Potential biological properties of peptides derived from oilseed proteins

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Han, R., Maycock, J., Murray, B.S., & Bösch, C. Antioxidant, anti-diabetic properties and potential cytotoxicity of commercial protein hydrolysates produced using pepsin and alcalase. *The 6th Annual Food Science and Nutrition PhD conference* – University of Leeds, Leeds, UK, November 21st, 2019.

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Details of the contributions

Ruixian Han: development of study designs, in silico methodology, experimental methodologies, data analysis, publication draft, final version

Joanne Maycock: provided partial supervision, feedback on designs of different experiments and methodologies, contributing to proofreading and editing

Alan Javier Hernández-Álvarez: supervision, feedback on experimental design, data visualization, contributing to proofreading and editing

Brent S. Murray: feedback on designs of different experiments and methodologies, data visualization, contributing to proofreading and editing

Christine Bösch: provided supervision and feedback on experimental designs, data visualization, contributing to proofreading and editing

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Abstract

Bioactive peptides are short amino acid chains liberated from parent proteins that can exert a wide range of activities, including antioxidant, anti-hypertensive, anti-diabetic and anti-inflammatory properties, which are beyond the nutritive value of the protein. Compared with peptides derived from animal proteins, such as egg, milk and fish, the peptides from plant proteins are much less explored. Therefore, the aim of this project was to investigate the biological properties of plant protein peptides using a range of methodologies, including *in silico* prediction of bioactive properties, *in vitro* testing of *in silico* predictions and the application of peptides to cell cultures.

Five oilseed proteins, flaxseed, rapeseed, sunflower, sesame and soybean were selected as peptide sources as well as two well-documented dairy proteins, whey and casein. Three hydrolysis conditions were applied to liberate peptides from these proteins, which were alcalase (pH 8.0) and pepsin (pH 1.3 and pH 2.1), respectively. Based on the frequency indices and potency indices of angiotensin converting enzyme (ACE) and dipeptidyl peptidase-IV (DPP-IV) inhibitory peptides, the *in silico* analysis predicted oilseed proteins as good sources of ACE inhibitory peptides, but weaker sources of DPP-IV inhibitory peptides. The average scores of the oilseed proteins aligned via the PeptideRanker indicated that oilseed proteins were potentially significant sources of bioactive peptides. When analyzed via Pepsite2, 65 novel peptides exerting ACE and/or DPP-IV inhibitory properties were predicted, based on the binding sites of peptides in ACE and DPP-IV.

The protein hydrolysates, together with their low molecular weight fractions (M_w < 3kDa), separated via ultrafiltration, were tested in several *in vitro* bioassays. These tests demonstrated that, apart from DPP-IV inhibitory activity, the M_w < 3 kDa fraction showed stronger bioactivity. Limited correlation was found between the *in silico* predictions and the *in vitro* findings. This discrepancy was thought to be largely caused by incomplete proteolysis and the lacking cover of protein sequence information in the available protein databases. Nevertheless, oilseed proteins, especially soybean, were confirmed as adequate sources in comparison with whey and casein for liberating ACE and α-glucosidase inhibitory peptides.

Further, anti-inflammatory properties of oilseed peptides were explored using cell culture models. The anti-inflammatory activities of protein hydrolysates and the three different M_w fractions (M_w > 10 kDa, 3-10 kDa and

< 3 kDa) were initially screened via RAW Blue^{TM} cells. The fractions with M_w < 3kDa of pepsin (pH 1.3)-treated protein hydrolysates were then selected to confirm their anti-inflammatory activities in RAW 264.7 macrophages. Results showed that rapeseed had the greatest potency to attenuate LPS-induced inflammation via downregulation of pro-inflammatory markers such as IL-6, IL-1β, iNOS and COX-2 and upregulation of anti-inflammatory IL-10, effects which were linked to the modulation of transcription factor NF-κB and supported by evidence for pre-receptor binding to LPS albeit these effects were much weaker as compared to whey and casein.

Taken together, the data indicate that peptides from oilseed proteins, especially rapeseed and soybean, demonstrate biological properties with a potential significance to beneficially affect chronic disease. The current findings should therefore encourage further research into the development of functional foods as well as nutraceutical and pharmaceutical applications.

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Chapter 1
General Introduction

1.1 Overall background of this research

Bioactive peptides are short amino acid sequences that can be released from parental proteins through enzymatic hydrolysis. Once released, these peptides can exert various biological activities, including antioxidant, antimicrobial, mineral binding, immunomodulation and cholesterol lowering, which are beyond their basic nutritional values (supplements of amino acids) (Li-Chan, 2015; Daliri et al., 2017). So far, bioactive peptides have been studied extensively for their potential benefits on human health. *In vitro* assays have been widely employed in measuring the biological activities. Simultaneously, various techniques have been used to produce, purify and identify peptides with robust biological activities. Compared with bioactive peptides derived from animal resources, such as fish (Cipolari et al., 2020), milk (Nielsen et al., 2017) and egg (Liu et al., 2018), peptides derived from plant protein, apart from soybean (Chatterjee et al., 2018), have so far received less research attention. In addition, there is limited information available as yet on some biological activities such as inhibition of inflammatory response. Importantly, studies describing peptide-mediated effects on modulation of cellular pathways are largely missing.

For this project, five oilseed proteins, flaxseed, rapeseed, sunflower, sesame and soybean, were selected and biological properties with health relevance determined after enzymatic hydrolysis and ultrafiltration. The selected properties were antioxidant, anti-hypertensive, anti-diabetic and anti-inflammatory capabilities. Whey and casein were used as reference proteins for comparison. In order to achieve the research goal, several methodologies were used: *in silico* prediction of bioactive properties, *in vitro* preparation of protein hydrolysates and their ultrafiltration into different fractions, and several different bioassays to confirm the *in silico* findings and cell culture-based anti-inflammatory assays.

1.1.1 Research Aim

Systematic investigation of the bioactive properties of oilseed protein derived peptides via *in silico* and *in vitro* methodologies to address the role of oilseed protein as appropriate sources of bioactive peptides.

1.1.2 Research Objectives

Designing the bioinformatic approaches to predict the bioactive properties of peptides, obtained via hydrolysing oilseed and milk proteins by alcalase (subtilisin), pepsin (pH 1.3) or pepsin (pH > 2); The bioactive properties to be predicted include angiotensin converting enzyme (ACE) inhibition and dipeptidyl peptidase IV (DPP-IV) inhibition

Producing bioactive peptides from oilseed and dairy protein isolates/concentrates via *in vitro* hydrolysis with alcalase (pH 8.0) and pepsin (pH 1.3 and 2.1); Separating hydrolysed proteins into three different molecular weight (M_w >10 kDa, 3 - 10 kDa and < 3 kDa) fractions via ultrafiltration

Analysing and comparing the antioxidant, anti-hypertensive and anti-diabetic capability of oilseed and dairy protein hydrolysates, together with their low M_w fractions (M_w < 3 kDa); validating the *in silico* predictions

Screening protein hydrolysates and different M_w fractions for anti-inflammatory properties using a reporter cell model and exploring with a subset of samples potential mechanisms of anti-inflammatory response

1.1.3 Research Hypothesis

Oilseed proteins can be sources of bioactive peptides, comparable to casein and whey proteins.

Biological activities of bioactive peptides can be predicted using *in silico* tools, which will be confirmed through experimental validation.

1.2 From protein to peptides

1.2.1 Proteins

Proteins are well-known macromolecules composed of amino acids linked together via peptide bonds, there are 20 naturally occurring amino acids. Each protein has a specific amino acid sequence, structure (shape) and

size (length, molecular weight), which markedly affects its biophysical and biochemical behaviour. According to their solubility, proteins can be simply classified as albumin, globulin, glutelin, prolamin, histone, protamine and scleroprotein, respectively (Sun, 2013).

Protein is also an essential macronutrient in the human body and plays a critical role in a wide range of biological processes, including muscle protein synthesis, cell growth and differentiation, synthesis of enzymes and transport proteins as well as signalling molecules as part of immune function. In addition, protein also can be considered as a source of energy, for 1 gram of protein equals to 4 kcal of energy. Whilst proteins can be synthesized endogenously, some of the amino acids, i.e. essential amino acids, need to be obtained through the diet. In order to suit the requirements of the human body, the British Nutrition Foundation recommends a daily intake of 0.6 g/per kg protein for an adult (the reference nutrient intake is 0.75 g/per kg).

The protein content in different food sources can vary significantly. Animal sources, including meat, fish and milk, are abundant in protein, whilst beans, lentils, cereals and potatoes are documented as the major dietary sources of plant proteins. According to the amino acid profiles, proteins originating from animal sources tend to present more promising biological and nutritional values compared with plant protein. The patterns of amino acids in animal proteins are similar to the requirements of humans, but very different from the ones in plant proteins (Young and Pellett, 1994). Plant proteins, apart from soybean and potato, cannot provide the complete range or required amino acids. They may lack one or more of the essential amino acids that cannot be synthesized by the human metabolism, such as lysine (grains and wheat), methionine (beans, seeds and nuts) or tryptophan (corn). The strategy to overcome this drawback is to combine different (plant) proteins in a certain ratio to complement amino acid profiles and therefore overcome limitations in individual amino acid profiles (Woolf et al., 2011).

Despite a lower content of essential amino acids, plant proteins have drawn increasing research attention due to their health and environment related advantages. Consumption of plant-based diet is closely associated with health benefits including cholesterol regulation (Jenkins et al., 2008) and blood glucose management (Trapp et al., 2010). In addition, plant proteins are more sustainable than animal proteins. Since they require less usage of land and freshwater, and do not emit greenhouse gases in the process (Harwatt, 2019). Therefore, plant proteins are regarded as a promising choice for

meeting the increasing demand for protein resulted from the global population explosion.

1.2.2 Enzymes – hydrolysing protein to peptides

Enzymes can efficiently promote the rate of chemical reactions via significantly decreasing their activation energies. In that way, substrates are relatively rapidly converted to different products. The catalysis action of enzymes is highly regulated via their specificity, which is determined via their unique structure and amino acid sequences. Single enzyme only works on one or on a set of reactions with the same basic mechanism. The active site of the enzyme binds with the substrate to form an 'enzyme-substrate' complex. Internal distortion of this complex brings about the breakage and/or formation of bonds, usually in a number of stages, until the product is released. Then the enzyme reverts back to its original state before binding of the substrate.

Since the active site structure is an important factor affecting the enzyme activity, any structural disruption, induced via heat or exposure to chemical denaturant, will cause the enzyme to lose its activity. Therefore, enzyme activity is affected via a range of physio-chemical factors, including pH, temperature, salt content, etc. With regard to external factors, the catalytic activity is determined by the enzyme and substrate concentration. Increasing the concentration of substrate will largely promote the rate of catalysis, because the free active site of enzyme can now bind to substrate. After that, the rate of activity will reach the highest value, which means even though more substrate is added, the rate will not increase any further, as all sites are occupied (Eed, 2012; Grahame et al., 2015).

1.2.3 Peptides

As protein fragments, peptides are short sequences of amino acids. Some peptides, such as bradykinin and oxytocin, are natural metabolites, whilst most peptides are liberated from the precursor proteins during hydrolysis. In the body, enzymatic hydrolysis is the major mechanism generating peptides from proteins. Due to the specificity of enzymes and the inaccessibility of some peptide bonds, some peptides can resist complete proteinase hydrolysis to the free amino acids.

Like proteins, the unique amino acid sequences of peptides determine their functional and bioactive properties. Compared with proteins, moderately

large peptides (polypeptides, over 30 residues) usually present better functional properties, including solubility, foaming, emulsifying and surface activity (Aluko and McIntosh, 2001; Guan et al., 2007; Klost and Drusch, 2019; Karami and Akbari-Adergani, 2019). Low M_w peptides (generally comprising 2 - 30 amino acid residues) generally exert more potent biological activities. In this project, anti-antioxidant, anti-hypertensive, anti-diabetic and anti-inflammatory properties of oilseed protein hydrolysates and their low M_w fractions (M_w < 3 kDa) were investigated and compared with diary proteins. The abovementioned bioactive properties of plant protein derived peptides are reviewed in **Chapter 2**.

1.3 Rationale behind selecting of oilseeds as sources of bioactive peptides, compared with dairy proteins (whey and casein).

1.3.1 Dairy proteins

The health benefits and high nutritional value of milk have been frequently reported in different types of studies. Milk is a complete food, providing abundant nutrients, including lactose, protein, lipid and mineral salts. Currently, milk proteins, whey and casein, have been claimed as good sources of bioactive peptides to modulate the developments of several chronic non-communicable diseases, including obesity, hypertension, diabetes and cardiovascular diseases (Korhonen, 2009; Nagpal et al., 2011).

Milk protein derived peptides, released via protease hydrolysis, have been confirmed under *in silico*, *in vitro* and *in vivo* conditions to exert numerous biological activities, including opioid-like, antioxidant, antihypertensive, anti-diabetic, immunomodulatory, antimicrobial activities (Korhonen, 2009; Nongonierma and FitzGerald, 2015; Brandelli et al., 2015)

Based on these key findings of research, it has been increasingly acknowledged that milk proteins are excellent sources of peptides with remarkable biological activities. Meanwhile, several commercial peptide products, such as BioZate (hydrolysed whey proteins) and PeptoPro (hydrolysed casein protein) have been launched, with claimed functions of blood pressure reduction and muscle recovery, respectively (Korhonen and Pihlanto, 2006).

1.3.2 Oilseed proteins

Currently, soybean is the predominant oilseed crop worldwide, the yield reached 362.05 million metric tons in 2020/2021, followed by rapeseed (68.87 million metric tons) and sunflower (49.46 million metric tons). Oilseeds are widely utilized as the raw material for producing edible vegetable oils, whilst the defatted meal, the by-product of oil extraction, is only used as fertilizer and animal feeds. Due to their appreciable level of protein, these defatted meals have gained more research interest for their potential application in nutritional and biological areas.

With the aid of the extraction technology, the proteins in oilseeds can be efficiently recovered, which brings the opportunity to apply these oilseed proteins in a broad research area. In this project, three leading types of oilseed proteins: soybean, rapeseed and sunflower, together with flaxseed and sesame were selected as the samples to investigate for their potential of releasing bioactive peptides. Whey and casein derived peptides were used as comparisons.

1.4 *In silico* prediction

In vitro techniques have been widely applied to the study of bioactive peptides, starting from enzymatic hydrolysis generating peptides from parental proteins, then various bioassays used to explore and confirm their bioactive properties, finally using chromatography to identify the peptide sequences. Although these *in vitro* experiments make great contributions to the field of peptide research, they are always expensive and time-consuming. Therefore, affordable and rapid bioinformatic approaches, based on mathematics, computer science and biological principles, have been developed for predicting and evaluating peptides released from the given protein sequences.

As shown in **Figure 1.1**, databases, including NCBI (https://www.ncbi.nlm.nih.gov/), UniProt KB (https://www.uniprot.org/) and BIOPEP (https://www.uwm.edu.pl/), contain the information for the amino acid sequences of proteins. With the given sequences, *in silico* hydrolysis tools, available from BIOPEP ((https://www.uwm.edu.pl/) and/or PeptideCutter (https://web.expasy.org/peptide_cutter/), can predict the peptide profiles liberated from precursor proteins based on the specificity of protease. Afterwards, peptides in the profiles are screened according to the record of

peptides sequences in the database. With regard to 'unrecorded' peptides, PeptideRanker (http://distilldeep.ucd.ie/PeptideRanker/) may be used to align the score to each peptide, in which '0' means the least likelihood of being active, whilst '1' refers to the most promising peptides. In addition, 'Pepsite 2' (http://pepsite2.russelllab.org/) or molecular docking can be used to predict the interaction between peptides and proteins can be predicted via, thus, is able to predict the binding sites of enzymes (e.g. ACE and DPP-IV) to evaluate their potential of being inhibitors. Furthermore, the potential toxicities of all the peptides are analysed via the ToxinPred (http://crdd.osdd.net/raghava/toxinpred/) and the allergenicity can be predicted via AlgPred 2.0 (https://webs.iiitd.edu.in/raghava/algpred2/) (Tu et al., 2018).

Although bioinformatic approaches bring great benefits in potentially discovering peptides, there are several drawbacks. The structure of the protein will limit the accessibility of protease, so that the predicted peptides may not be released by *in vitro* digestion. In addition, the predicted activities depend on the accuracy and completeness of information recorded in databases, which will be undoubtable to affect the accuracy of the prediction. **Chapters 3 and 4** will discuss the limitations of *in silico* predictions and the correlation between *in silico* and *in vitro* data in detail.

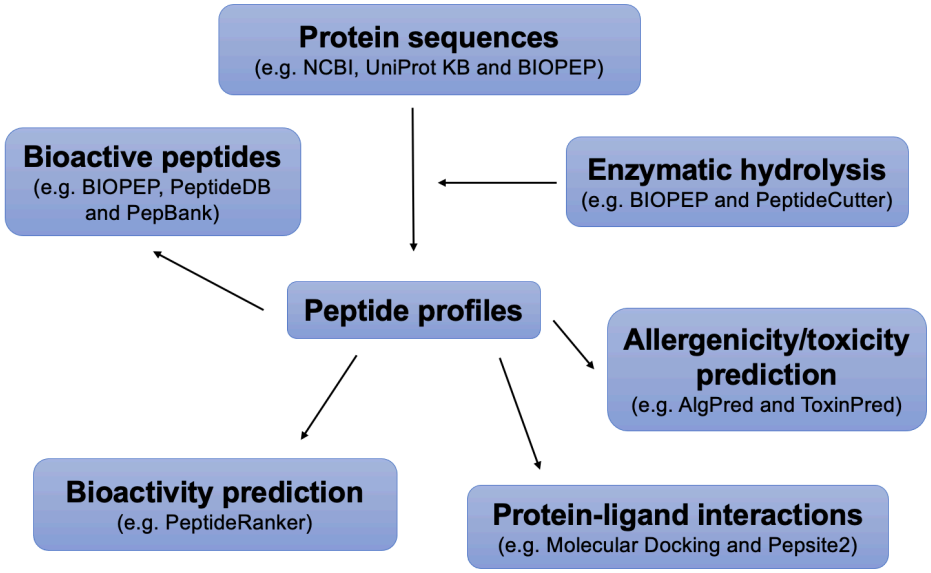

Figure 1.1 Bioinformatic analysis for predicting and studying the bioactive peptides liberated from the proteins

1.5 Rationale behind the selection of *in vitro* techniques

In order to evaluate the potential of five selected oilseed proteins as sources of bioactive peptides, a range of analytical assays and experimental instruments were employed in this research project. The antioxidant, anti-hypertensive and anti-diabetic properties of oilseed protein hydrolysates and their ultrafiltration fractions were measured and subsequently compared with the ones of whey and casein proteins. The background theory and methodology of these assays are discussed below.

1.5.1 The alkaline protein extraction

There are many conventional methods for recovering plant proteins from defatted oilseed meals, including water, salt, alkaline and organic solution-based extraction. The efficiency of recycling protein is largely dependent on the nature of protein sources. Alkaline extraction was selected for extracting the protein in this project, in which defatted oilseed meal is dissolved in alkali-based solution. The higher pH of the solution increases the protein solubility due to the ionization of amino acids and breakdown of the disulphide bonds. The dissolved protein can then be precipitated at its isoelectric point. Compared with the other methods, alkaline extraction tends to produce higher protein yield, and also efficiently removes anti-nutritional factors, such as trypsin inhibitors, tannins and phytic acid (Jyothi and Sumathi, 1995; Gao et al., 2020).

1.5.2 Protein separation using gel electrophoresis

The molecular weight distribution of protein concentrate/isolates in this project was measured via sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). This technique is based on the negatively charged proteins moving towards the opposite charge electrode in the electric field. Sodium dodecyl sulphate (SDS) is a detergent and denaturant that unfolds the protein and binds to it in a ratio proportional to the protein M_w. The presence of a reducing reagent, such as dithiothreitol, breaks any disulfide bond and further ensures that the protein is fully unfolded so that the amount of negative charge due to SDS binding reflects the full M_w and so smaller proteins move faster in the same electric field in the acrylamide or agarose gel to which the proteins are applied.

After separated proteins are stained, e.g., via Coomassie Blue, and comparison with protein with knowing M_w allows estimation of the M_w and identification of unknown bands. For example, the M_w of albumin of sunflower is between 10 -18 kDa, while the acidic and basic subunits (polypeptides) of helianthinin (11S) have the M_w in the 32 – 44 kDa and 21 – 27 kDa ranges, respectively (Žilić et al., 2010). In addition, Achouri et al. (2012) summarized the M_w of sesame albumin (13 kDa), globulin (45 – 50 kDa) and subunits of 11S globulin (acidic subunit 30 - 35 kDa, basic subunit 20 - 25 kDa). Based on the findings of previous literature, the names of proteins in sunflower and sesame, together with five other protein isolates/concentrates can be identified from the bands shown in the gel. An example is given in the **Figure 1.2.**

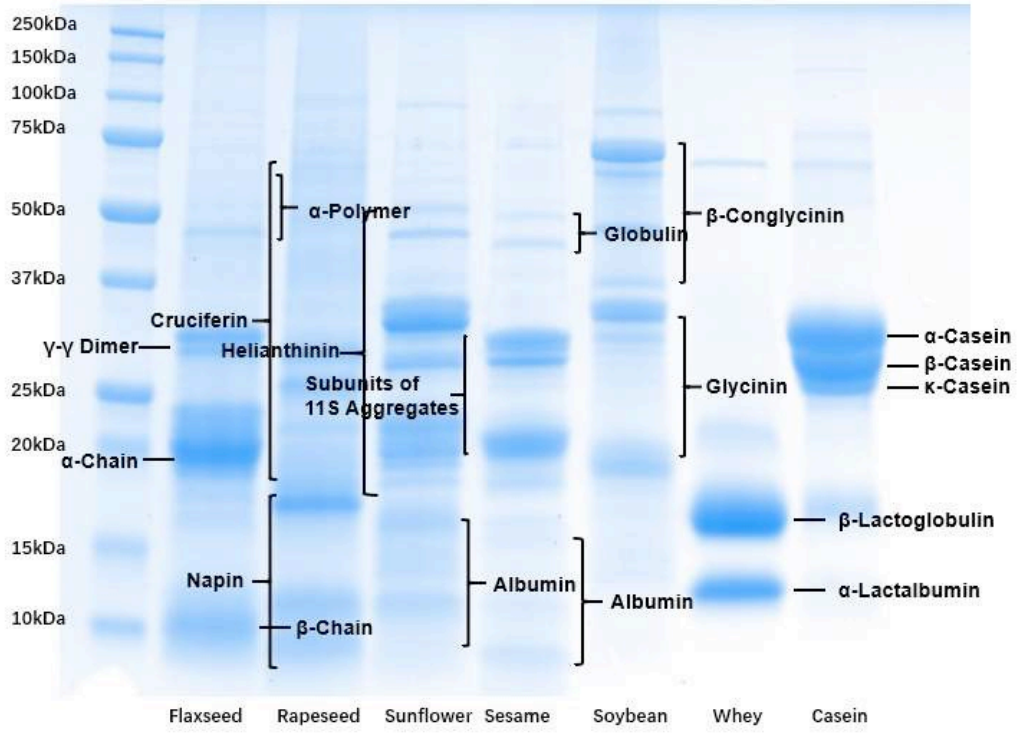

Figure 1.2 Electrophoretic profile of oilseed and dairy protein concentrates and isolates. The names of identified bands are labelled according to Nandish et al. (2018), Akbari and Wu (2015), Žilić et al. (2010), Achouri et al. (2012), Barać et al. (2011), Garcia-Campayo et al. (2018) and Turpeinen et al. (2016). (Molecular weight)

1.5.3 Preparation of protein hydrolysates

Enzymatic protein hydrolysis was conducted to liberate peptide fragments from the oilseed and dairy proteins. The specificity of the different

proteases determines the disruption of specific peptides bonds. In addition, the structural characteristics of protein limited the bio-accessibility of the protease (Adler-Nissen, 1986). Therefore, protease treatment usually results in partial hydrolysis, which means the resultant products are mixtures of different sized peptides, rather than free amino acid residues.

In this thesis, all the protein samples were hydrolysed via pepsin and alcalase, separately. Pepsin is a key physiological protease found in gastric digestions, while alcalase is a serine endopeptidase produced via *Bacillus licheniformis*. Pepsin preferentially cleaves peptide linkages involving phenylalanine, tyrosine, tryptophan and leucine. While, alcalase presents a broader specificity with a preference for phenylalanine, tryptophan, tyrosine, glutamic acid, methionine, leucine, alanine, serine and lysine residues. Both proteases have wide differences in specificities, which is expected to lead to large dissimilarity in peptide profiles after treating the same protein samples. Applying different protein treatments will give a wider comparison between oilseeds and dairy proteins as sources of bioactive peptides.

Following proteolysis, the extent of hydrolysis can be quantified by measuring the free amine content, for example via 2,4,6-trinitrobenzene sulfonic acid (TNBS) assay. Amine groups rapidly react with TNBS to form an orange-coloured derivative (**Figure 1.3**). L-leucine can be used as a standard and therefore calculate the L-leucine equivalent amine group concentration (Fields, 1972).

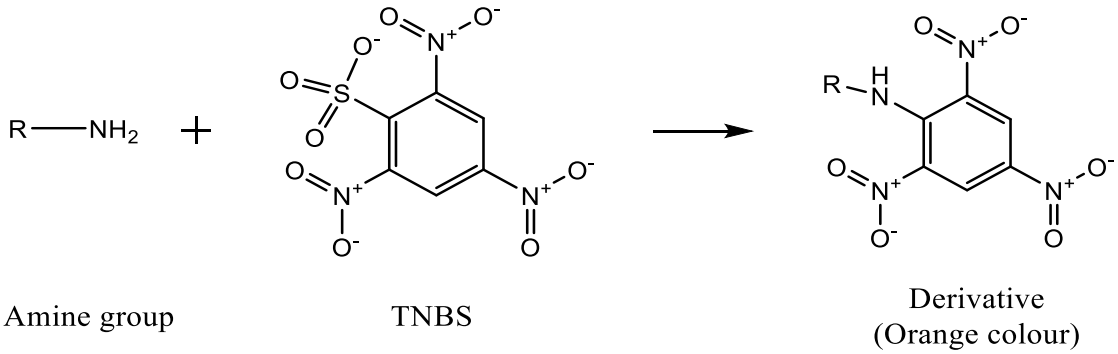

Figure 1.3 Basic chemical mechanism for TNBS assay

1.5.4 Ultrafiltration

Ultrafiltration, a membrane filtration process, is capable to separate the particles according to their pore sizes. As shown in **Figure 1.4**, driven via the low transmembrane pressure, only the molecular with size smaller than molecular size cut-off (MWCO) can cross the semipermeable membrane, whilst the larger ones are retained in solvents (Strathmann et al., 2011). Consequently, the low M_w molecules are concentrated and separated from the previous solution. Previous research claimed that low M_w peptides (e.g. M_w < 3 kDa, < 1 kDa) tend to present more promising biological activity, compared with the larger peptides (He et al., 2013; Olagunju et al., 2018). Therefore, ultrafiltration is applied to enrich peptides with a specific range of M_w, and thereby allows separation of protein hydrolysates into different fractions.

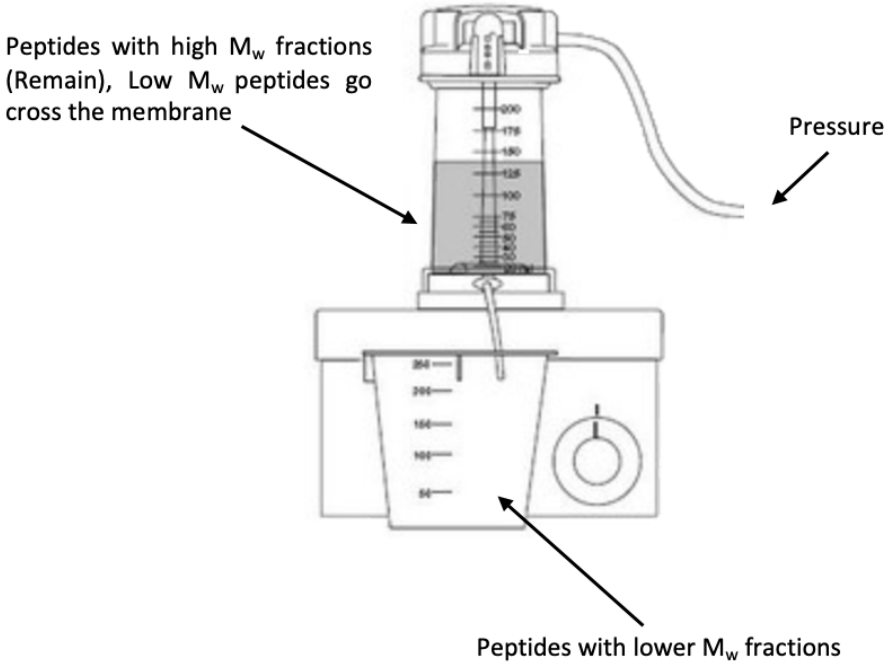

Figure 1.4 Illustration of an ultrafiltration device (Amicon@ stirred cells) (figure taken from Life Science Research - Merck Millipore).

1.5.5 Antioxidant assay

A great variety of *in vitro* chemical assays have been employed to measure the antioxidant capability of protein hydrolysates and peptides. These assays are based on hydrogen atom transfer (HAT) or single electron

transfer (SET) mechanisms. (Huang et al., 2005; Samaranayaka and Li-Chan, 2011; Gulcin, 2020). However, no standardized methods are available yet to test the overall antioxidant capability. Therefore, more than one method is recommended to characterize the antioxidant capability of samples.

Trolox equivalent antioxidant assay (TEAC) and ferric reducing antioxidant power assay (FRAP) were selected to investigate the antioxidant properties of oilseeds and dairy peptides, respectively. Both are spectrophotometric assays for quantifying the reducing capability of samples. TEAC assay measures the scavenging of ABTS radicals (water-soluble blue chromophores) of samples, while FRAP assay is based on the reduction of Fe^{3+} to Fe^{2+} (Re et al., 1999; Benzie and Strain, 1996). Trolox was used as the standard in both assays and the antioxidant capability was expressed as mM Trolox equivalent/ per mg protein hydrolysates. The results of assays will be compared for better understanding and evaluating the antioxidant properties of oilseed peptides (compared with dairy peptides).

1.5.6 Enzyme inhibitory assay

Physiologically, the overexpression and hyperactivation of angiotensin converting enzyme (ACE) and dipeptidyl-peptidase IV (DPP-IV) are related to the development of hypertension and type 2 diabetes, respectively. ACE is a carboxypeptidase that can generate angiotensin II (vasoconstrictor) from angiotensin I via cleaving dipeptide (HL) from C-terminus (Caldwell et al., 1976). In addition, it can degrade bradykinin, a potent vasodilator. On the other hand, DPP-IV can quickly degrade and cleave glucose dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), both are closely associated with insulin synthesis and secretion (Lambeir et al., 2003). With the action of α-amylase and α-glucosidase, carbohydrates ingested will be rapidly degraded to free glucose units, leading to increased blood sugar level (Telagari and Hullatti, 2015). Therefore, the effects of protein hydrolysates on direct inhibition of these four enzymes was investigated in this project. All the enzyme inhibitory assays are colorimetric assays, as the absorbance of the hydrolysed product is dose dependent. With a given substrate (summarised in **Table 1.1**), the hydrolysis rate (activity) of an enzyme can be measured via the changes in absorbance at a particular wavelength. Afterwards, the inhibitory effect of peptides on enzyme activity can be easily calculated according to **Equation (1.1)**. Importantly, a positive inhibitor of the enzyme should also be used in order to validate the results.

$$Enzyme\ inhibition\ (\%) = \frac{\Delta Abs\ of\ \ negative\ cotorol - \Delta Abs\ of\ samples}{\Delta Abs\ of\ nagetive\ control} x\ 100 \qquad \textbf{(1.1)}$$

Table 1.1 List of enzyme inhibitory assays involved in this project.

Enzyme	**Substrate**	**Positive inhibitors**	**Principle of assay**	**References**
Angiotensin-converting enzyme	N-[3-(2-Furyl) acryloyl]-Phe-Gly-Gly (FAPGG)	Captopril	ACE hydrolyses FAPGG to FAP and GG. Absorbance of FAP recorded at 340 nm	(Murray et al., 2004)
Dipeptidyl peptidase IV	Gly-Pro-p-Nitroanilide	Diprotin A	*Para* nitroaniline is released from substate via DPP-IV, which leads to an increase in absorbance at 405 nm	(Gu et al., 2015)
α-amylase	Starch	Acarbose	After hydrolysis, the produced maltose will react with DNS solution to generate a product with brown colour (Absrobance measured at 540 nm)	(Luo et al., 2019)
α-glucosidase	p-nitrophenyl glucopyranoside	Acarbose	p-nitrophenol liberated from p-nitrophenyl glucopyranoside present a yellow colour can be measured at 405 nm	(Zhang et al., 2020)

1.6 Cell culture experiments: the role of oilseed peptides in modulating LPS-induced inflammation

Cell culture refers to maintaining the growth and differentiation of cells in a cultured medium under artificial simulated environments. It is an important tool to investigate the anti-inflammatory properties of the peptides. As described later in **Chapter 5**, RAW Blue^{TM} and RAW 264.7 macrophage cells were employed. Various methodologies, including NO measurement, sandwich ELISA (IL-6 and TNF-α), qPCR (β-action, interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α), IL-1β, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), nuclear factor κ B subunit 1 (NF-κB1), p65, nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor, α (IκBα), toll-like receptor 4 (TLR4) and cluster of differentiation 14 (CD14)) were used to explore the potential roles of oilseeds in suppressing Lipopolysaccharide(LPS)-induced inflammation. The basis of these methods is described below.

1.6.1 Cell lines and inflammatory stimulators

Macrophages are important immune cells that produce a range of markers upon inflammatory stimulus such as cytokines and chemokines (Oishi and Manabe, 2018). RAW 264.7 cells, a macrophage-like cell line, was used to investigate the anti-inflammatory effects of the protein hydrolysates and peptides. Considering the large number of samples (84 in total), RAW Blue^{TM} cell, a nuclear factor-kappa B (NF-κB)-secreted alkaline phosphatase (SEAP) reporter cell line generated from the RAW 264.7 macrophages, was utilized to screen the inhibitory effects of all protein hydrolysates on the activation of NF-κB and activator protein 1 (AP-1) pathway. After being transfected with pNF-κB-SEAP-neomycin phosphotransferase (NPT) plasmid, RAW-Blue^{TM} cell is permitted to express SEAP reporter gene in response to the activity of NF-κB. Therefore, the expression of SEAP was correlated with NF-κB activation (Moon et al., 2001). Different from the ones generated from intracellular reporters, the activity of enzyme, encoded via SEAP gene, is easier to quantify because it is secreted into the cell supernatant. SEAP enzyme activity is directly proportional to their protein and gene expression level, which can be used to quickly determine NF-κB activity without the need for cumbersome assay (Berger et al., 1988). In this project, the secreted embryonic alkaline phosphatase (SEAP) in cultured medium was

quantified via QUANTI-Blue™ solution in an absorbance-based measurement.

Lipopolysaccharide (LPS), an endotoxin secreted via gram-negative bacteria, was utilized to induce inflammation response in both cell lines. It is a stimulus to evoke the multiple downstream intracellular signalling cascades, including NF-κB and MAPK releasing a wide range of cytokines and mediators (Park et al., 2011; Wang et al., 2012)

1.6.2 Measurement of cytotoxicity

Before loading cells with peptides, protein hydrolysates or other compounds, it is necessary to test the effect of the sample on cell viability and proliferation. Neutral Red uptake assay is a common method to measure the effect of samples on the cell viability. Only viable cells can take up the eurhodin dye, Neutral Red, then the dye is transported to lysosomes which are thus stained. After destaining, absorbance measurement of the released dye can directly reflect the number of viable cells and the cytotoxicity of samples (Repetto et al., 2008).

1.6.3 Nitric oxide measurement

Nitric oxide (NO) is considered as a signalling molecule and pro-inflammatory mediator during the pathogenesis of inflammation in endothelial cells (Sharma et al., 2007). After the activation of NF-κB pathway, excessive amount of NO, synthesized from L-arginine, is produced via the catalysis by iNOS (Aktan, 2004). Therefore, quantification of NO synthesized in RAW 264.7 macrophages before/after LPS with/without protein hydrolysates co-treatment is a strategy to evaluate the potential of the samples in suppressing the inflammatory process.

Griess assay was used in this project to measure the nitrite ion levels in cell supernatant. It is a colorimetric assay based on nitrite reacting with sulfanilamide, then N-(1-naphthyl) ethylenediamine to form a pink-red colour component of high absorbance at 540nm (**Figure 1.5).** Sodium nitrite ($NaNO_2$) was used as the standard to calculate NO levels (after reconversion of nitrate/nitrite to NO) (Berkels et al., 2004).

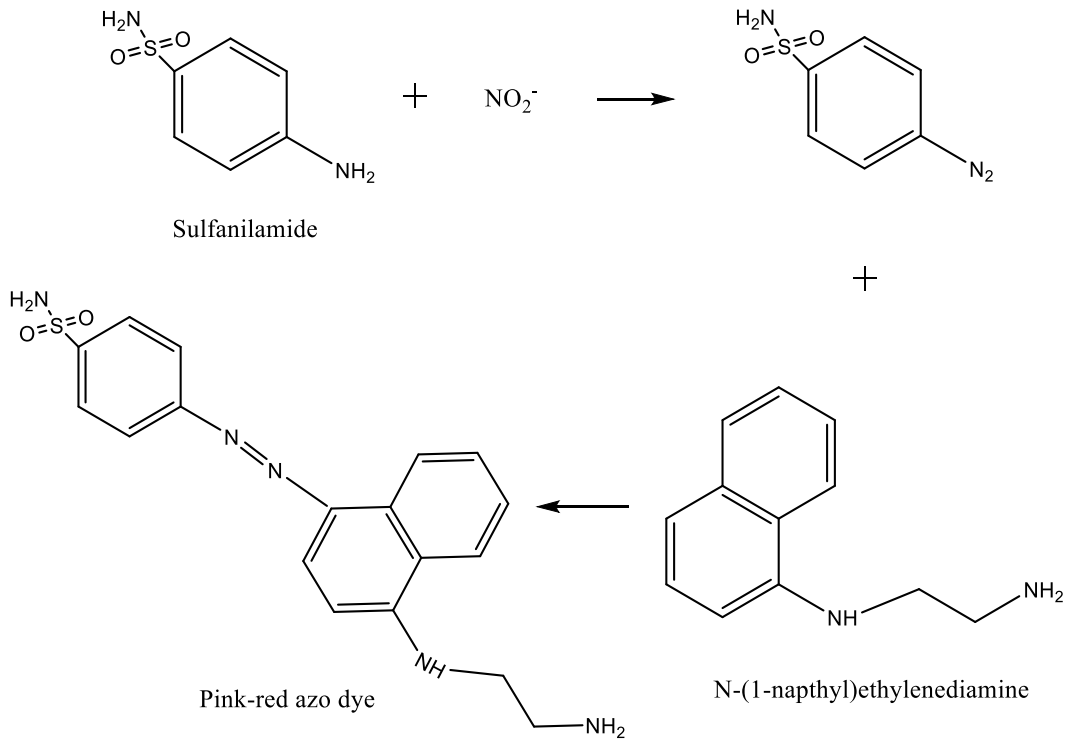

Figure 1.5 Two step chemical reaction of the Griess test.

1.6.4 Sandwich enzyme-linked immunosorbent assay (ELISA)

ELISA is a biochemical solid-phase technique used to quantify antigens and/or antibodies. There are two major types of ELISA assay, the first one is the competitive ELISA, which based on the conjugation of antigen and enzyme or antibody and enzyme (Voller et al., 1978). In our project, the second type, 'sandwich' ELISA assay which was applied for the detection of IL-6 and TNF-α in cell supernatants.

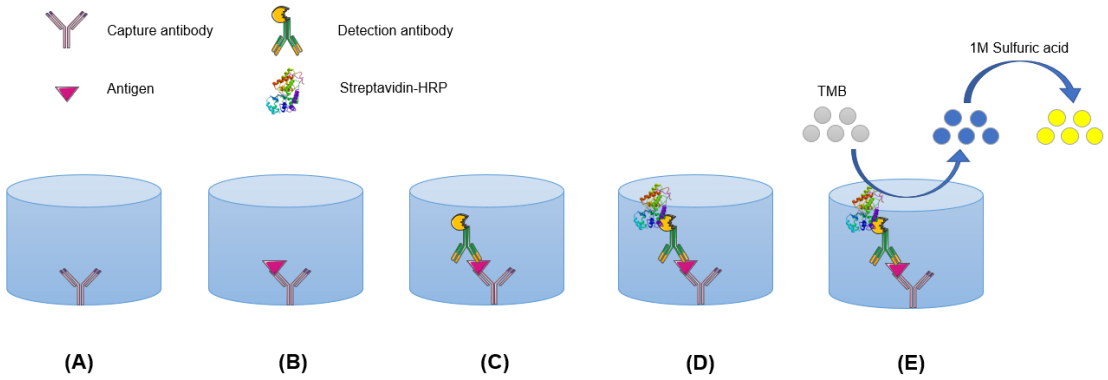

Figure 1.6 Five stages of sandwich enzyme-linked immunosorbent assay

As shown in **Figure 1.6**, 'sandwich' ELISA contains two types of antibodies (the capture and detection antibodies). The capture antibody was firstly immobilized onto the well plate via noncovalent interactions **(A)**. Then supernatant, being tested, containing the antigen of interest is then applied. After an incubation and washing step, the specific antigen will bind to the capture antibody **(B)**. Subsequently, the detection antibody is added followed by further incubation and washing **(C)**. The detection antibody is conjugated to biotin, which can bind to streptavidin-HRP **(D)**. HRP, a horseradish peroxidase, can oxidise colourless 3,3′,5,5′-Tetramethylbenzidine (TMB) to form blue coloured intermediate products. Finally, the oxidative reaction is stopped via sulfuric acid addition that turns the colour of the product to yellow **(E)**. (Frey et al., 2000) The intensity of this colour is directly proportional to the concentration of the original antigens of interest (e.g., IL-6 and TNF-α).

1.6.5 Quantitative real time polymerase chain reaction (PCR)

Quantitative real time polymerase chain reaction (RT-PCR) was utilized to quantify the gene expression in this project. PCR is a thermocycle technique, which can rapidly generate millions of identical copies of the targeted DNA sequences (Nolan et al., 2006). RT-PCR is the combination of PCR and fluorimetry. The number of specific DNA is obtained based on the fluorescence intensity. Therefore, the changes in gene expression in each cycle can be monitored and quantified in real time. In this experiment, SYBR@ Green is used as the fluorescent dye, which binds the double stranded of DNA (Arikawa et al., 2008).

In response to LPS stimulation, the gene expression of anti- / pro -inflammatory cytokines, meditators and transcript factors in cells (e.g., RAW 264.7 macrophages) was dramatically changed (Guha and Mackman, 2001). The anti-inflammatory capabilities of peptides/protein hydrolysates can be directly evaluated via their regulation of LPS induced changes in mRNA levels of inflammation-related genes. Therefore, quantification of mRNA levels of IL-6, TNF-α, IL-1β, IL-10, iNOS, COX-2, NF-κB1, p65, IκBα, TLR4 and CD14 before and after LPS treatment and the mRNA of these primers that is up- or down- regulated via peptide-based protein hydrolysates was performed using RT-PCR.

1.7 Outline of the thesis

This project, investigating nutritionally relevant biological properties of bioactive peptides derived from plant proteins, has the following chapter outline:

Chapter 1 provides a general introduction to the topic of bioactive peptides including methods used in this thesis.

Chapter 2 reviews the currently available literature on antioxidant, anti-hypertensive, anti-diabetic and anti-inflammatory activity of plant protein derived peptides, investigated via *in silico*, *in vitro* and/or *in vivo* methodologies. This includes a critical analysis on the potential for some plant proteins as peptide sources and identifies gaps in the research field.

Chapter 3 presents two integrated bioinformatic approaches to predict the ACE and DPP-IV inhibitory peptides liberated from oilseed and dairy protein sequences. The results demonstrate the potential of oilseed protein as comparable bioactive peptide sources, especially with regards to ACE inhibition in comparison with dairy proteins. In addition, the limitations of *in silico* studies are also discussed. This chapter has been published in *Food Research International* (**Reference:** Han, R., Maycock, J., Murray, B.S. and Boesch, C., 2019. Identification of angiotensin converting enzyme and dipeptidyl peptidase-IV inhibitory peptides derived from oilseed proteins using two integrated bioinformatic approaches. *Food Research International*, **115**, pp.283-291.)

Chapter 4 'translates' the *in silico* predictions (**Chapter 3**) into *in vitro* data, using a range of bio-assays to test the predictions. Only a weak correlation between *in silico* predictions and *in vitro* data was detected. However, the *in vitro* data also support the hypothesis that oilseed proteins, particularly soybean and rapeseed, can be appropriate sources of bioactive peptides, in comparison to whey and casein. This chapter has been published in *Current Research in Food Science*. (**Reference:** Han, R., Álvarez, A.J.H., Maycock, J., Murray, B.S. and Boesch, C., 2021. Comparison of alcalase-and pepsin-treated oilseed protein hydrolysates–Experimental validation of predicted antioxidant, antihypertensive and antidiabetic properties. *Current Research in Food Science*, **4**, pp.141-149.)

Chapter 5 further explores and compares the anti-inflammatory effects of oilseed and dairy protein hydrolysates in RAW Blue^{TM} and RAW 264.7 macrophages. Potential mechanisms of peptide-rich protein hydrolysates interacting with LPS-induced inflammatory signalling is also investigated.

Among all the tested samples, low M_w (M_w < 3 kDa) fractions of pepsin (pH 1.3)-treated rapeseed proteins presented the greatest anti-inflammatory activities.

Chapter 6 provides an overall discussion and synthesis of all the main findings as well as giving recommendations for further research.

References

Achouri, A., Nail, V. and Boye, J.I. 2012. Sesame protein isolate: Fractionation, secondary structure and functional properties. *Food research international.* **46**(1), pp.360-369.

Adler-Nissen, J. 1986. *Enzymic hydrolysis of food proteins.* Elsevier applied science publishers.

Akbari, A. and Wu, J. 2015. An integrated method of isolating napin and cruciferin from defatted canola meal. *LWT-Food Science and Technology.* **64**(1), pp.308-315.

Aktan, F. 2004. iNOS-mediated nitric oxide production and its regulation. *Life sciences.* **75**(6), pp.639-653.

Aluko, R.E. and McIntosh, T. 2001. Polypeptide profile and functional properties of defatted meals and protein isolates of canola seeds. *Journal of the Science of Food and Agriculture.* **81**(4), pp.391-396.

Arikawa, E., Sun, Y., Wang, J., Zhou, Q., Ning, B., Dial, S.L., Guo, L. and Yang, J. 2008. Cross-platform comparison of SYBR® Green real-time PCR with TaqMan PCR, microarrays and other gene expression measurement technologies evaluated in the MicroArray Quality Control (MAQC) study. *BMC genomics.* **9**(1), pp.1-12.

Barać, M., Pešić, M., Žilić, S., Srebrić, M. and Drinić, S. 2011. Identification of soluble protein fractions and their subunits in soybeans with black and yellow kernel coat. In: *46th Croatian and 6th International Symposium on Agriculture, Opatija, Croatia, 14-18 February 2011. Proceedings*: University of Zagreb Faculty of Agriculture, pp.681-685.

Benzie, I.F. and Strain, J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analytical biochemistry.* **239**(1), pp.70-76.

Berger, J., Hauber, J., Hauber, R., Geiger, R. and Cullen, B.R. 1988. Secreted placental alkaline phosphatase: a powerful new quantitative indicator of gene expression in eukaryotic cells. *Gene.* **66**(1), pp.1-10.

Berkels, R., Purol-Schnabel, S. and Roesen, R. 2004. Measurement of nitric oxide by reconversion of nitrate/nitrite to NO. *Nitric Oxide Protocols.* Springer, pp.1-8.

Brandelli, A., Daroit, D.J. and Corrêa, A.P.F. 2015. Whey as a source of peptides with remarkable biological activities. *Food Research International.* **73**, pp.149-161.

Caldwell, P., Seegal, B.C., Hsu, K.C., Das, M. and Soffer, R.L. 1976. Angiotensin-converting enzyme: vascular endothelial localization. *Science.* **191**(4231), pp.1050-1051.

Chatterjee, C., Gleddie, S. and Xiao, C.-W. 2018. Soybean bioactive peptides and their functional properties. *Nutrients.* **10**(9), p1211.

Cipolari, O.C., de Oliveira Neto, X.A. and Conceição, K. 2020. Fish bioactive peptides: A systematic review focused on sting and skin. *Aquaculture.* **515**, p734598.

Daliri, E.B.-M., Lee, B.H. and Oh, D.H. 2017. Current trends and perspectives of bioactive peptides. *Critical reviews in food science and nutrition.* pp.1-12.

Eed, J. 2012. Factors affecting enzyme activity. *Essai.* **10**(1), p19.

Fields, R. 1972. [38] The rapid determination of amino groups with TNBS. *Methods in enzymology.* Elsevier, pp.464-468.

Frey, A., Meckelein, B., Externest, D. and Schmidt, M.A. 2000. A stable and highly sensitive 3, 3′, 5, 5′-tetramethylbenzidine-based substrate reagent for enzyme-linked immunosorbent assays. *Journal of immunological methods.* **233**(1-2), pp.47-56.

Gao, Z., Shen, P., Lan, Y., Cui, L., Ohm, J.-B., Chen, B. and Rao, J. 2020. Effect of alkaline extraction pH on structure properties, solubility, and beany flavor of yellow pea protein isolate. *Food Research International.* **131**, p109045.

Garcia-Campayo, V., Han, S., Vercauteren, R. and Franck, A. 2018. Digestion of food ingredients and food using an in vitro model integrating intestinal mucosal enzymes. *Food and Nutrition Sciences.* **9**(06), p711.

Grahame, D., Bryksa, B. and Yada, R. 2015. Factors affecting enzyme activity. *Improving and tailoring enzymes for food quality and functionality.* Elsevier, pp.11-55.

Gu, L., Liao, L., Hu, H., Bligh, S.A., Wang, C., Chou, G. and Wang, Z. 2015. A thin-layer chromatography-bioautographic method for detecting dipeptidyl peptidase IV inhibitors in plants. *Journal of Chromatography A.* **1411**, pp.116-122.

Guan, X., Yao, H., Chen, Z., Shan, L. and Zhang, M. 2007. Some functional properties of oat bran protein concentrate modified by trypsin. *Food Chemistry.* **101**(1), pp.163-170.

Guha, M. and Mackman, N. 2001. LPS induction of gene expression in human monocytes. *Cellular signalling.* **13**(2), pp.85-94.

Gulcin, İ. 2020. Antioxidants and antioxidant methods: An updated overview. *Archives of toxicology.* **94**(3), pp.651-715.

Harwatt, H. 2019. Including animal to plant protein shifts in climate change mitigation policy: a proposed three-step strategy. *Climate Policy.* **19**(5), pp.533-541.

He, R., Girgih, A.T., Malomo, S.A., Ju, X. and Aluko, R.E. 2013. Antioxidant activities of enzymatic rapeseed protein hydrolysates and the membrane ultrafiltration fractions. *Journal of Functional Foods.* **5**(1), pp.219-227.

Huang, D., Ou, B. and Prior, R.L. 2005. The chemistry behind antioxidant capacity assays. *Journal of agricultural and food chemistry.* **53**(6), pp.1841-1856.

Jenkins, D., Kendall, C., Faulkner, D., Kemp, T., Marchie, A., Nguyen, T., Wong, J., De Souza, R., Emam, A. and Vidgen, E. 2008. Long-term effects of a plant-based dietary portfolio of cholesterol-lowering foods on blood pressure. *European journal of clinical nutrition.* **62**(6), pp.781-788.

Jyothi, V. and Sumathi, S. 1995. Effect of alkali treatments on the nutritive value of common bean (Phaseolus vulgaris). *Plant Foods for Human Nutrition.* **48**(3), pp.193-200.

Karami, Z. and Akbari-Adergani, B. 2019. Bioactive food derived peptides: A review on correlation between structure of bioactive peptides and their functional properties. *Journal of food science and technology.* **56**(2), pp.535-547.

Klost, M. and Drusch, S. 2019. Functionalisation of pea protein by tryptic hydrolysis–Characterisation of interfacial and functional properties. *Food Hydrocolloids.* **86**, pp.134-140.

Korhonen, H. 2009. Milk-derived bioactive peptides: From science to applications. *Journal of functional foods.* **1**(2), pp.177-187.

Korhonen, H. and Pihlanto, A. 2006. Bioactive peptides: production and functionality. *International dairy journal.* **16**(9), pp.945-960.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *nature.* **227**(5259), pp.680-685.

Lambeir, A.-M., Durinx, C., Scharpé, S. and De Meester, I. 2003. Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. *Critical reviews in clinical laboratory sciences.* **40**(3), pp.209-294.

Li-Chan, E.C. 2015. Bioactive peptides and protein hydrolysates: research trends and challenges for application as nutraceuticals and functional food ingredients. *Current Opinion in Food Science.* **1**, pp.28-37.

Liu, Y.-F., Oey, I., Bremer, P., Carne, A. and Silcock, P. 2018. Bioactive peptides derived from egg proteins: A review. *Critical reviews in food science and nutrition.* **58**(15), pp.2508-2530.

Luo, K., Kim, N.-g., You, S.-M. and Kim, Y.-R. 2019. Colorimetric determination of the activity of starch-debranching enzyme via modified tollens' reaction. *Nanomaterials.* **9**(9), p1291.

Moon, K.-Y., Hahn, B.-S., Lee, J. and Kim, Y.S. 2001. A cell-based assay system for monitoring NF-κB activity in human HaCat transfectant cells. *Analytical biochemistry.* **292**(1), pp.17-21.

Murray, B., Walsh, D. and FitzGerald, R. 2004. Modification of the furanacryloyl-L-phenylalanylglycylglycine assay for determination of angiotensin-I-converting enzyme inhibitory activity. *Journal of Biochemical and Biophysical Methods.* **59**(2), pp.127-137.

Nagpal, R., Behare, P., Rana, R., Kumar, A., Kumar, M., Arora, S., Morotta, F., Jain, S. and Yadav, H. 2011. Bioactive peptides derived from milk proteins and their health beneficial potentials: an update. *Food & function.* **2**(1), pp.18-27.

Nandish, S.K.M., Kengaiah, J., Ramachandraiah, C., Shivaiah, A., Girish, K.S., Kemparaju, K. and Sannaningaiah, D. 2018. Anticoagulant, antiplatelet and fibrin clot hydrolyzing activities of flax seed buffer extract. *Pharmacognosy Magazine.* **14**(55), p175.

Nielsen, S.D., Beverly, R.L., Qu, Y. and Dallas, D.C. 2017. Milk bioactive peptide database: A comprehensive database of milk protein-derived bioactive peptides and novel visualization. *Food chemistry.* **232**, pp.673-682.

Nolan, T., Hands, R.E. and Bustin, S.A. 2006. Quantification of mRNA using real-time RT-PCR. *Nature protocols.* **1**(3), pp.1559-1582.

Nongonierma, A.B. and FitzGerald, R.J. 2015. The scientific evidence for the role of milk protein-derived bioactive peptides in humans: A Review. *Journal of Functional Foods.* **17**, pp.640-656.

Oishi, Y. and Manabe, I. 2018. Macrophages in inflammation, repair and regeneration. *International immunology.* **30**(11), pp.511-528.

Olagunju, A.I., Omoba, O.S., Enujiugha, V.N., Alashi, A.M. and Aluko, R.E. 2018. Pigeon pea enzymatic protein hydrolysates and ultrafiltration peptide fractions as potential sources of antioxidant peptides: An in vitro study. *LWT.* **97**, pp.269-278.

Park, H.Y., Han, M.H., Park, C., Jin, C.-Y., Kim, G.-Y., Choi, I.-W., Kim, N.D., Nam, T.-J., Kwon, T.K. and Choi, Y.H. 2011. Anti-inflammatory effects of fucoidan through inhibition of NF-κB, MAPK and Akt activation in lipopolysaccharide-induced BV2 microglia cells. *Food and Chemical Toxicology.* **49**(8), pp.1745-1752.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free radical biology and medicine.* **26**(9-10), pp.1231-1237.

Repetto, G., Del Peso, A. and Zurita, J.L. 2008. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nature protocols.* **3**(7), p1125.

Samaranayaka, A.G. and Li-Chan, E.C. 2011. Food-derived peptidic antioxidants: A review of their production, assessment, and potential applications. *Journal of functional foods.* **3**(4), pp.229-254.

Sharma, J., Al-Omran, A. and Parvathy, S. 2007. Role of nitric oxide in inflammatory diseases. *Inflammopharmacology.* **15**(6), pp.252-259.

Strathmann, H., Giorno, L. and Drioli, E. 2011. *Introduction to membrane science and technology.* Wiley-VCH Weinheim.

Sun, Z. 2013. Foundations for the Study of Structure and Function of Proteins. *Basics of Bioinformatics.* Springer, pp.303-336.

Telagari, M. and Hullatti, K. 2015. In-vitro α-amylase and α-glucosidase inhibitory activity of Adiantum caudatum Linn. and Celosia argentea Linn. extracts and fractions. *Indian journal of pharmacology.* **47**(4), p425.

Trapp, C., Barnard, N. and Katcher, H. 2010. A plant-based diet for type 2 diabetes. *The Diabetes Educator.* **36**(1), pp.33-48.

Tu, M., Cheng, S., Lu, W. and Du, M. 2018. Advancement and prospects of bioinformatics analysis for studying bioactive peptides from food-derived protein: Sequence, structure, and functions. *TrAC Trends in Analytical Chemistry.* **105**, pp.7-17.

Turpeinen, A., Kautiainen, H., Tikkanen, M.-L., Sibakov, T., Tossavainen, O. and Myllyluoma, E. 2016. Mild protein hydrolysation of lactose-free milk further reduces milk-related gastrointestinal symptoms. *Journal of Dairy Research.* **83**(2), pp.256-260.

Voller, A., Bartlett, A. and Bidwell, D. 1978. Enzyme immunoassays with special reference to ELISA techniques. *Journal of clinical pathology.* **31**(6), pp.507-520.

Wang, Z., Jiang, W., Zhang, Z., Qian, M. and Du, B. 2012. Nitidine chloride inhibits LPS-induced inflammatory cytokines production via MAPK and NF-kappaB pathway in RAW 264.7 cells. *Journal of ethnopharmacology.* **144**(1), pp.145-150.

Woolf, P.J., Fu, L.L. and Basu, A. 2011. vProtein: identifying optimal amino acid complements from plant-based foods. *PloS one.* **6**(4), pe18836.

Young, V.R. and Pellett, P.L. 1994. Plant proteins in relation to human protein and amino acid nutrition. *The American journal of clinical nutrition.* **59**(5), pp.1203S-1212S.

Zhang, X., Li, G., Wu, D., Yu, Y., Hu, N., Wang, H., Li, X. and Wu, Y. 2020. Emerging strategies for the activity assay and inhibitor screening of alpha-glucosidase. *Food & function.* **11**(1), pp.66-82.

Žilić, S., Barać, M., Pešić, M., Crevar, M., Stanojević, S., Nišavić, A., Saratlić, G. and Tolimir, M. 2010. Characterization of sunflower seed and kernel proteins. *Helia.* **33**(52), pp.103-114.

Chapter 2
Plant proteins as sources of bioactive peptides: preparation, identification, and biological activities

Abstract

The applications of plant proteins as sources of bioactive peptides have fuelled great interest over the past twenty years. A great number of bioactive peptides have been generated and identified from various species of plant proteins. Among such literature, enzymatic hydrolysis is described as the major approach to liberate peptides.

This chapter revisits and discusses the status for the investigation of plant protein derived bioactive peptides, with a focus on antioxidant, anti-hypertensive, anti-diabetic and anti-inflammatory beneficial effects, irrespective of utilizing *in silico*, *in vitro* or *in vivo* models. Many studies have employed a similar workflow in research of bioactive peptides for the preparation and analysis of biological activities. Simultaneously, the *in silico* tools bring the opportunity for rapid analysis of peptide profiles from given protein, whilst seldom papers 'translate' these bioinformatic predictions into *in vitro* and *in vivo* data.

Based on the current understanding, the crucial role of plant proteins as sources of bioactive peptides has been clearly highlighted. However, more accurate comparisons of releasing bioactive peptides from plant and animal sources under exactly the same conditions are required. In addition, there is limited information available as yet on the bioavailability of peptides, as well as pre-clinical and clinical human trials. These current hurdles make it challenging to commercialize plant peptides as dietary health-promoting functional food or supplements.

2.1 Introduction

Dietary protein is a crucial nutrient for supplying energy and essential amino acids to humans. Protein can be hydrolysed into peptides, which are described as 'intermediate products' of protein breakdown to free amino acid

residues. Some of them present promising health benefits beyond their basic nutrition levels, and thereby defined as ‘bioactive peptides’. These peptides are documented as potential health-promoting agents in preventing or controlling the progress of metabolic diseases, and possibly supporting traditional therapeutic treatments without apparent toxic or adverse effects (Rochlani et al., 2017; Mada et al., 2019).

Bioactive peptides are specific protein fragments which are inactive when contained within the sequence of the parental protein. Once released, these peptides, exert diverse biological activities (summarized in **Figure 2.1**), including but not limited to antioxidant (Wen et al., 2020), anti-hypertensive (Garcia-Vaquero et al., 2019), anti-diabetic (Wang, J. et al., 2020) and anti-inflammatory (Hu et al., 2020) properties, determined by their amino acid content and sequence. Among them, most bioactive peptides are of 2 to 20 amino acids in length. Compared with chemical hydrolysis, autolysis and microbial fermentation, enzymatic hydrolysis is the major and most widely used technique to obtain peptides from precursor proteins. Numerous commercial digestive enzymes (including, pepsin, trypsin, chymotrypsin and pancreatin) (Brodkorb et al., 2019) and proteases (such as, alcalase, papain, flavourzyme and neutrase) (Ma et al., 2019; Xiao et al., 2020; Zheng et al., 2019a) have been applied in peptide research. Protein hydrolysates are increasingly recognized as value-added ingredients of functional foods and dietary supplements for nutraceutical, pharmaceutical and cosmeceutical applications.

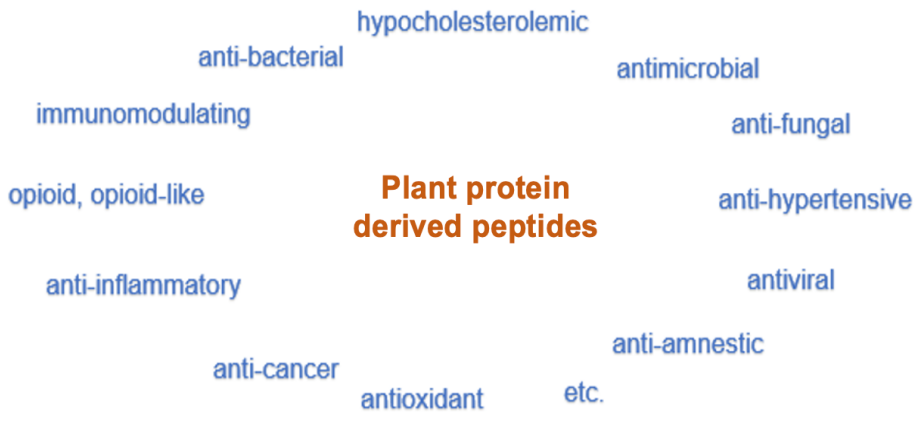

Figure 2.1 Biological activities of plant protein derived peptides

To date, a large proportion of the research has been devoted to animal-based protein hydrolysates and peptides, mostly egg (Liu, Y.F. et al.,

2018), milk (Mohanty et al., 2016) and fish (Halim et al., 2016), as well as their production from precursor proteins, purification and identification of these peptides. Their biological importance and health benefits after oral administration also have been highlighted in different types of research. Compared with animal-based proteins, the same yield of plant-based proteins is more sustainable (requires less land, water and no fossil fuels to produce and gives lower greenhouse gas emissions) plus cholesterol-free and low in saturated fatty acid products (González et al., 2011). In addition, plant-based foods are more likely to meet the increasing requirement of protein caused by the global population growth (Henchion et al., 2017). Furthermore, mixing plant proteins of various origins can overcome the deficiency in certain amino acids (e.g. lysine) (Chiesa and Gnansounou, 2011). For examples, pulse proteins (lack methionine, but rich in ysin) blended with cereal proteins (rich in methionine, but lack in lysin) offer a promising completed source of essential amino acids (Boye et al., 2010; Boukid, 2021). Therefore, it is highly desirable to employ plant proteins as the ingredients for generating bioactive peptides, as a substitution for animal proteins. Despite the importance of plant proteins, less thorough research of plant-based protein hydrolysates and peptides has been undertaken with respect to management of non-communicable diseases such as hypertension, diabetes and cardiovascular diseases.

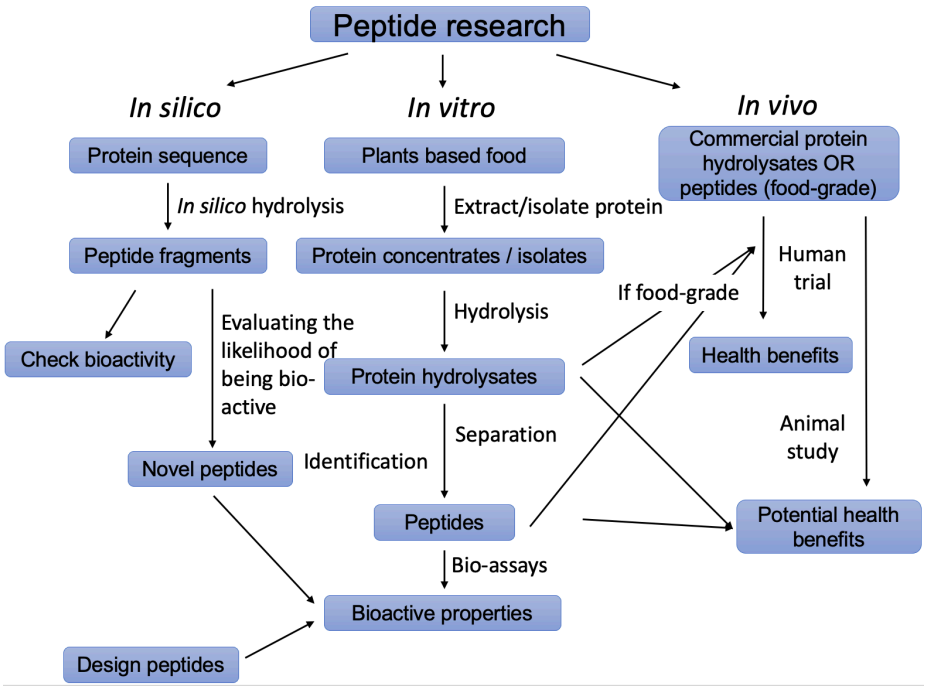

Figure 2.2 General flowchart for the analysis of plant protein derived peptides via *in silico*, *in vitro* and *in vivo* approaches.

This chapter provides a detailed overview of the issues relevant to the production of plant protein hydrolysates and peptides, as well as a comprehensive review of current research status of important bioactive properties derived from plants from *in silico*, *in vitro* and *in vivo* measurements. See **Figure 2.2** for a general flowchart of peptide research. Furthermore, the research gaps of plant-protein derived peptides and the direction of future research are addressed and discussed.

2.2 Preparation of plant-derived protein hydrolysates and peptides

2.2.1 Isolating proteins from the food samples

The application of protein extraction technologies can efficiently improve the protein content of plant-based food matrices via removal of carbohydrates, fat, fibre, among other compounds. The most common extraction method is alkaline extraction, based on the changes of protein dispersibility (solubility) at different pH (Deleu et al., 2019). This procedure always starts with solubilizing the crushed/ground plant-food matrix in an alkaline solution (typically at pH 9), combined with stirring for a given time (e.g. 2-4 hours). Afterwards, the solubilized plant food matrices are centrifuged and then the pH of collected supernatant adjusted near the isoelectric point (e.g. pH 4.0 – 5.0, dependent on the type of plant protein) via addition of acid (e.g. 1M HCl) to precipitate the protein. Finally, the precipitated protein concentrates/isolates are separated via a second centrifugation and dried for further uses via lyophilization or spray-drying. Slight variances in parameters, such as the pH of dissolution and precipitation, type and concentration of alkaline and acid solution, as well as centrifugation settings (temperature, speed, etc.) in extraction procedures are reported in the literature.

Apart from alkaline extraction, some studies utilize salt extraction-dialysis or micellization precipitation to isolate proteins (Stone et al., 2015; Gerzhova et al., 2016; Mohan and Mellem, 2020). Furthermore, as first mentioned in the Osborne scheme, specific protein fractions can be obtained with sequential extraction (water, salt, alkaline and alcohol solution), theoretically into protein fractions of albumin, globulin, glutelin and prolamin, respectively (Osborne, 1924; Chang et al., 2014). For example, Chin et al. (2019) used the Osborne method to isolate four protein fractions from blue

lupin for investigating nutritional composition and the ACE inhibitory properties of their alcalase and flavourzyme hydrolysed products.

2.2.2 Releasing bioactive peptide through enzymatic hydrolysis

Enzymatic hydrolysis is the most common strategy for releasing peptides, as it requires mild reaction conditions without changing or damaging nutritional and chemical features. In addition, this approach requires less reaction time, is easily controlled and gives reproducible peptide profiles. One or more proteases (multiple proteases are expected to generate higher yields of peptides rather than single systems) are used, including pepsin, trypsin, α-chymotrypsin, alcalase, flavourzyme, neutrase, protamex, bromelain, papain and many others, to release peptides, usually under optimum hydrolysis conditions. Some studies have applied a sequential scheme of pepsin and pancreatin (or a mixture of trypsin and α-chymotrypsin) to mimic the gastrointestinal digestion of protein (Brodkorb et al., 2019). This can also be used to evaluate the stability of the peptides themselves under gastrointestinal conditions (Vilcacundo et al., 2017; Hu et al., 2020; Yang et al., 2020; Sánchez-Velázquez et al., 2021). The hydrolytic procedure can be terminated via pH adjustment, heating or by addition of protease inhibitors. The type of protease used is a critical factor that determines the released peptides, since the cleavage of specific peptide bonds depends on the enzyme specificity. These enzyme specificities have been summarized and recorded in Peptidecutter service (https://web.expasy.org/peptide_cutter/peptidecutter_enzymes.html), an *in silico* tool used to mimic the enzyme hydrolysis. Disparate specificity among proteases largely affects the peptide profiles, and equally, same proteases may exhibit diverse catalysis capabilities in different plant proteins. Therefore, some studies have screened the most suitable enzymes prior peptides production for further research (Ren et al., 2016; Saisavoey et al., 2018; Zheng et al., 2019a; Famuwagun et al., 2020).

The structural complexity of proteins in the food matrix may limit the enzyme-substrate interaction, which consequently, leads to poor accessibility of protease and a low hydrolysis rate. Several researchers pre-treat proteins prior to the hydrolysis process to expose better the hydrolysis sites (peptide bonds) of protein. Heating, high pressure or ultrasound treatments are three major pre-treatment processes used. Heating denatures proteins and can expose inner hydrophobic residues (Grigera and McCarthy, 2010). High pressure treatment also induces protein unfolding, exposing more cleavage

sites (Meersman et al., 2006). Ultrasound plays a less significant role in modifying the structure of proteins, but potentially leads to better protein dispersion (Jiang, L. et al., 2014).

Subsequent centrifugation of the hydrolysed material removes undigested protein or high Mw insoluble peptides. The extent of hydrolysis and lengths of peptides produced is not only influenced by the parental protein and types of protease, but also with the reaction parameters enzyme/substrate ratio, temperature, pH, buffer medium and digestion time, among others. Adler-Nissen (1979) developed and defined the term 'degree of hydrolysis' (DH), which directly refers to the percentage of peptide bonds in protein structure cleaved. DH remains a key factor to specify when producing protein hydrolysates, generally, limited enzymatic hydrolysis (e.g. DH < 10%) improves the functional properties (such as water absorption, oil/fat absorption, emulsifying and foaming capability) of the proteins, whilst much higher DH is usually required for generating high proportions of bioactive peptides.

2.2.3 Peptide fractionation, purification and identification

Enzymatic hydrolysis produces a complex mixture of peptides/ polypeptides with a wide range of M_w. Considering that the peptides may exert numerous bioactive properties, a purification process is needed to exclude undesirable peptides and to concentrate the ones with the desired activity. Generally, ultrafiltration is the initial step to fractionate the hydrolysates on the basis of M_w range, e.g. <1, 1-3, 3-5, 5-10, >10 kDa (Chan-Zapata et al., 2019). Thereafter, various chromatographic purification techniques, including gel filtration chromatographic (GFC), reversed-phase-high-performance liquid chromatographic (RP-HPLC), ion exchange chromatography (IEC) and affinity chromatography, are used to enrich and recover targeted peptides. Following chromatography, tandem-mass spectrometry (LC-MS/MS) or matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry (MALDI-TOF-MS) are used to confirm the amino acid sequences of the peptides, in order to explain their bioactive properties determined via *in vitro* and/or *in vivo* assays.

For examples, Zheng et al. (2019a) created a general workflow for discovering and identifying ACE inhibitory and antioxidant peptides from coconut cake albumin hydrolysates using ultrafiltration and chromatographic techniques. The authors started with ultrafiltration with two molecular (Mw 3

and 5 kDa) weight cut-off membranes to separate crude protein hydrolysates into 3 fractions and selected the most active fraction (Mw < 3kDa) after testing ACE inhibitory and hydroxyl radical scavenging activity. Afterwards, Sephadex G-25 GFC (column Φ 2.6 cm x 60 cm) and sequential RP-HPLC (C_{18} columns, Φ 9.4 mm x 250 mm and Φ 4.6 mm x 250 mm) were applied to elute the most active fraction. Finally, three novel peptides (KQAYPYV, KIIIYN and KILIYG) were identified via LC-MS/MS, and their antioxidant (hydroxyl radical scavenging, ion chelating and superoxide radical scavenging capability) and anti-hypertensive (ACE inhibition) properties confirmed via *in vitro* bio-assays. As a result of the latest ultrafiltration and chromatographic technologies, peptides can successfully be identified and these are derived from a wide range of plant species, including watermelon (Wen et al., 2020), sesame (Lu et al., 2019), quinoa (Vilcacundo et al., 2017) and lychee seed (Saisavoey et al., 2018).

2.3 Characteristics of bioactive properties of peptides

2.3.1 *In silico* prediction

Bioinformatic (*in silico*) techniques are gaining importance as a novel approach for studying the activity of biological materials, although experimental techniques are still the predominant and essential test. Nevertheless, the *in silico* approach has immense potential due to its speed, and when combined with *in vitro* and *in vivo* techniques to validate predictions, is producing new channels of bioactive research. *In silico* methods can predict the peptide profiles released, effectiveness of enzyme inhibitors and other aspects, summarized i**n Appendix A - Table A1**.

In silico methodologies involve a wide range of *in silico* tools and different databases and software. Typically, starting with *in silico* hydrolysis tools (e.g. BIOPEP and PeptideCutter) for predicting the peptide profiles released from a given protein sequence. Protein sequences can be obtained from NCBI, UniProtKB, SwissProt and Protein Data Bank, or identified experimentally. Predicted peptide profiles can be rapidly analysed to find the relevant bioactive fragment, after matching with peptide databases (e.g. BIOPEP, PeptideDB and Pepbank). With regard to novel peptides, PeptideRanker can predict the likelihood of peptides being bioactive via a ranking score. Based on this information, researchers can rapidly select the most suitable proteases for releasing specific peptides. In addition, the

allergenicity and toxicity of peptide fragments can also be predicted via AlgPred and ToxinPred tools, respectively. Compared with the classic experimental approach for discovering bioactive peptides, the bioinformatic approach can rapidly predict all the peptide fragments from a given protein based on the specificity of proteases, in principle. In reality less peptide bonds will be cut *in vitro* hydrolysis due to the inaccessibility of all peptide bonds to the protease. Therefore, it is not guaranteed that the predicted peptides will match those produced *in vitro*. Li et al. (2020) identified 420 peptides from zein treated with trypsin-chymotrypsin via nano-LC-MS/MS, of which only 27.6% (116) were predicted via *in silico* analysis.

As previously discussed, protein hydrolysates and even their more-purified chromatographic fractions usually contain large numbers of peptides which makes it challenging to assess the overall bioactivity due to presence of a wide number of bioactives. Some studies use *in silico* tools to narrow the number of hydrolysis experiments and conditions and then use *in vitro* experiments to validate the biological activity of the predicted peptides. Zheng, Y. et al. (2020) identified 17 peptides from a chromatographic fraction derived from brown seaweed (sargassum maclurei) after pepsin and papain hydrolysis. With the aid of *in silico* analysis, RWDISQPY was finally selected as a candidate and proved to exert a promising ACE inhibitory property (IC_{50} 72.24 μM). Their group also used the similar *in silico* methodology to isolate SSYYPEK (ACE inhibition IC_{50} 91.82 μM) derived from naked oat globulin (Zheng, Yajun et al., 2020) and RGQVIYVL (ACE inhibition IC_{50} 38.16 μM) from quinoa bran albumin (Zheng et al., 2019b). However, Marseglia et al. (2019) reported two tri-peptides, VPI and SPV, selected from 19 peptides derived from cocoa protein, which presented weak ACE inhibition and cannot explain the high activity of the whole protein digestates (after simulated gastrointestinal digestion). Clearly further studies are required to narrow the gap between *in silico* predictions and *in vitro* experiments.

Molecular docking approaches play an important role in predicting the affinity of peptides for target enzymes and thereby can screen peptides for their enzyme inhibitory properties. For example, Zhao et al. (2019) applied molecular docking analysis to predict the binding site of 7 tri-peptides to ACE, and finally selected EGW, DMG and DTW, because of the low docking energy needed. Afterwards, these peptides were validated in terms of their ACE inhibitory properties (IC_{50} value 3.95 ± 0.11mM). In addition, Mirzaei et al. (2019) used molecular docking to predict YGKHVAVHAR as a non-competitive inhibitor, which has been proved via *in vitro* experiments.

Also *in vitro* inhibition models of VPW and IPR for DPP-IV are aligned with molecular docking analysis. Quantitative structure activity relationship (QSAR) analysis was also utilized for predicting the bioactive properties of peptides and designing novel peptides, based on mathematically quantifying the regularity of similar structure or amino acid contributing to high biological activity (Iwaniak et al., 2015). Currently, this technique has been used only rarely for discovering and designing peptides with ACE inhibition (Udenigwe and Mohan, 2014), renin inhibition (Udenigwe et al., 2012), and antioxidant(Li, Y.-W. et al., 2011) peptides.

Despite bioinformatic techniques being widely applied for investigating the plant protein derived peptides, some limitations remain: 1) The parent proteins are often complex mixtures and not all the sequences may be known; 2) Screening of the peptides for activity depends on the peptides recorded in databases and for some peptides information is lacking; 3) Incomplete knowledge of peptides released by virtue of enzymatic hydrolysis; 4) Low accuracy in predicting *in vivo* data, due to complex metabolism; 5) Lack of standard protocols for validating *in silico* predictions.

2.3.2 Biological activities of plant protein derived peptides

2.3.2.1 Antioxidant activity

Reactive oxygen species (ROS) and free radicals are generated via physiological oxidative reactions, including respiration and host defence mechanisms (Brieger et al., 2012). Additionally, a high-fat diet, air pollutants, smoking, drugs and irradiation may also be sources of oxidants (Żukowski et al., 2018). In living organisms, the endogenous defence system secretes enzymatic and non-enzymatic antioxidants to arrest ROS, modulate oxidative stress and avoid ROS damaging living cells. Immoderate amounts of ROS and free radicals cannot be neutralized and this results in oxidation of lipids, DNA and proteins, leading to tissue injury and several chronic diseases, such as diabetes, stroke and atherosclerosis (Jakus, 2000; Devasagayam et al., 2004). Oral administration of food-grade antioxidants may well inhibit oxidative reactions for health maintenance. The effectiveness of natural antioxidants may lower than synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BTA), but their easier absorption, reduced side effects and permissible high intake means their importance for health should not be ignored. Like other well-known dietary antioxidants, including polyphenols (Pulido et al., 2000), vitamin C(Padayatty

et al., 2003), bioactive peptides (Lorenzo et al., 2018) are seen as good candidates to modify ROS and free radicals.

Many studies have attempted to evaluate the antioxidative effect of plant-derived peptides and protein hydrolysates, although there is a lack of specific standardised assays to accurately measure the overall antioxidant capability, due to the diversity and complexity of oxidative processes in biological systems. *In vitro* chemical assays based on electron transfer, including 2,2-diphenyl-1-picrylhydrazyl radical-scavenging capacity (DPPH) and Trolox Equivalent Antioxidant Capacity (TEAC), hydrogen atom transfer, such as, Total Radical Trapping Antioxidant Parameter (TRAP) and the Oxygen Radical Absorbance Capacity (ORAC) and metal ion chelating, are widely used for screening promising antioxidant candidates (Wen et al., 2020). Ideally, more than one assay should be applied to evaluate overall effectiveness in the different antioxidant mechanisms, summarized in **Appendix A – Table A2**. Even so, it is usually hard to compare results across different studies. Apart from varying DH, minor variations such as the characteristics of the starting materials such as protein content and the presence of other antioxidant compounds, such as polyphenols, may have an effect.

Despite these complications, most studies conclude that there is a positive correlation between high antioxidant capability and high DH, i.e., low Mw fractions (typically Mw < 1 to 3 kDa). A wide range of plant sources have been tested, including foxtail millet (PFLF, IALLIPF) (Ji et al., 2020), watermelon seed (RDPEER, KELEEK, DAAGRLQE) (Wen et al., 2020), chickpea (LTEIIP) (Famuwagun et al., 2020), corn (LAYPQ, YGPQ, PPY, LSPY) (Liu et al., 2020), mungbean (WGN, AW, RGWYE) (Xia et al., 2020), sesame (RDRHQKIG, TDRHQKLR, MNDRVNQGE) (Lu et al., 2019) and zein (PF, LPF) (Tang and Zhuang, 2014). All protein hydrolysates were confirmed as sources of antioxidant compounds.

Apart from these *in vitro* chemical assays, antioxidant peptides were also investigated for their cytoprotective properties in cell lines. Wen et al. (2020) reported RDPEER, identified from alcalase-treated watermelon seeds, exerted cytoprotective effects (100 μmol/L) in a H_2O_2-damaged HepG2 cell line via increasing the activity of superoxide dismutase (SOD) (48.08%), glutathione peroxidase (GSH-Px) (37.47%) and catalase (CAT) (55.84%), and decreased 15.85% malondialdehyde (MDA) levels, compared with damaged group. Xiao et al. (2020) claimed 1 mg/mL of alcalase-treated rice bran protein significantly promoted cellular antioxidant activity (31.76%) in same

cell line. Protein hydrolysates (0.5 mg/mL) obtained from wheat gluten significantly increased the glutathione level in human peripheral blood mononuclear cells (Cruz-Chamorro et al., 2020). The cytoprotective effect of antioxidant plant peptides was also studied in other cellular models, such as Caco-2 (Torres-Fuentes et al., 2015), Raw 264.7 macrophage (Wen et al., 2018), HT-29 (Wongaem et al., 2020) cell lines. However, the correlation between the results of these cell culture experiments and their effects in human subjects is still unclear and needs further investigation.

2.3.2.2 Anti-hypertensive activity

Hypertension has become a global health issue. WHO (2020) claimed 1.13 billion people have been diagnosed as having hypertension. This noncommunicable disease is closely associated with increased blood pressure. In the human body, renin-angiotensin system (RAS) is the most crucial regulator of body fluids and blood pressure. This pathway starts with active renin hydrolysis angiotensinogen to a decapeptide angiotensin I, through cleaving the Leu. Then, with the interaction of angiotensin converting-(I) enzyme, angiotensin I is conversed to angiotensin II (cleaving His-Leu), a vasoconstrictor. ACE is also involved in kinin-kallikrein system and degrades bradykinin, a potent vasodilator, to three inactive fragments. Therefore, great interest has been attached to plant-derived peptides exerting renin and/or ACE inhibitory properties for their potential in modulating hypertension (**Figure 2.3**).

Various plant-based proteins, such as oilseed (Wang, J. et al., 2020), cereal (Zhang, P. et al., 2020), microalgae (Aiello et al., 2019), legume (Ciau‐Solís et al., 2018) and fruit (Vásquez-Villanueva et al., 2015), have been hydrolysed and the generated peptides and protein hydrolysates have been shown to exert ACE inhibitory properties (shown in **Appendix A - Table A3**). In the work of Nuchprapha et al. (2020) physiological digestive enzymes (pepsin-pancreatin) were applied to hydrolyse Longan seeds and ACE inhibitory protein hydrolysates with IC_{50} value 1.74 ± 0.006 mg/ mL were obtained. This finding suggests gastrointestinal digestion may significantly improve the ACE inhibitory activity of certain plant proteins. Also, Ciau‐Solís et al. (2018) claimed that protein hydrolysates with M_w < 3kDa obtained via pepsin-pancreatin exert even stronger ACE inhibitory activity (60.15%) than those generated via Alcalase-flavourzyme (16.91%). Other proteases also produced some promising ACE inhibitory peptides. In a very early study, the peptide (Pro-Ser-Gly-Gln-Tyr-Tyr) from corn gluten, generated by pescalase,

showed ACE inhibitory properties *in vitro* (IC_{50} 0.1Mm) and also proved to antagonize the response against angiotensin I in a rat model (at 30mg / kg body weight) (Suh et al., 1999). Karami et al. (2019) reported a much lower IC_{50} value (0.093 mM) for the ACE inhibitory properties of SGGSYADELVSTAK, identified from proteinase K treated wheat germ. With regard to short chain peptides, VNP and VWP, are considered promising ACE inhibitors from alcalase-treated rice protein, with IC_{50} value 6.4 and 4.5 μM (Chen, J. et al., 2013).

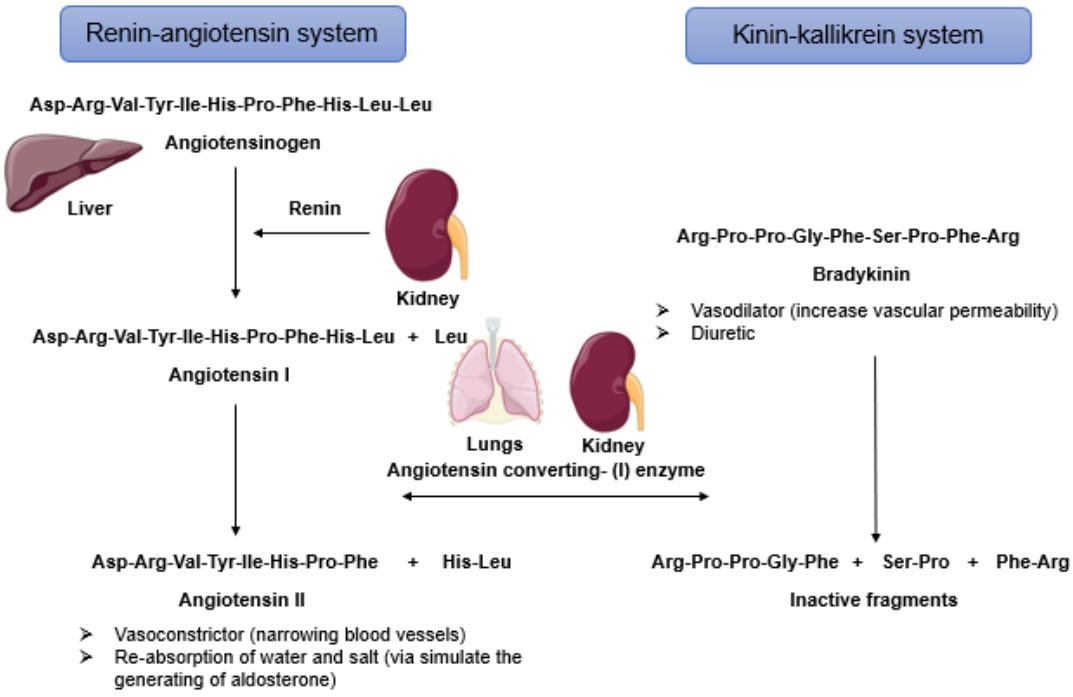

Figure 2.3 The mechanism of Renin and Angiotensin (I)- converting enzyme in increasing blood pressure.

Besides ACE inhibitory activity, renin inhibitors have less frequently been identified. Amaranth-derived peptides have been reported as good sources of renin inhibitors (SFNLPILR, FNLPILR, AFEDGFEWVSFK, with IC_{50} values from 0.41- 2.50μM) (Nardo et al., 2020). In addition, LY, TF, RALP from alcalase-treated rapeseed protein exert dual inhibition of renin and ACE, which may result in a robust anti-hypertensive effect in the human body. It may be that combinations of peptides with different inhibitory mechanisms may exert synergistic effects, but this has not been widely investigated yet.

The anti-hypertensive activity of LVPPHA, derived from *Radix Astragali*, has been investigated in spontaneous hypertensive rats (SHR), and

significantly decreased systolic blood pressure (SBP) at a dosage of 10 μM/kg body weight (BW) at 2 hours after oral administration (Wu, J.-S. et al., 2020). Likewise, hazelnut peptide (YLVR) reduced SBP at dosages of 10 to 50 mg/kg BW (Liu, C. et al., 2018).

Furthermore, Zheng et al. (2019b) claimed quinoa peptide (RGQVIYVL) exerted a lowering effect in SBP and diastolic blood pressure (DBP) in SHRs, although there was no significant dose-dependent relationship. On the other hand, the same group reported RWDISQPY derived from *Sargassum maclurei* presented dose-dependent lowering effect in DBP and SBP in SHRs (Zheng, Y. et al., 2020).

2.3.2.3 Anti-diabetic activity

As shown in **Figure 2.4** α-amylase, α-glucosidase and DPP-IV are responsible for an increase in blood glucose levels. Salivary and pancreatic α-amylase are digestive endoenzymes that break down dietary starch or other long-chain polysaccharides into absorbable low molecular weight oligo- and di- saccharides (e.g. maltose). Then α-glucosidase, in intestinal brush border, further hydrolyses these oligo- or di- saccharides into monosaccharides (e.g. glucose, fructose), which are then released into the blood stream. Simultaneously, active glucose dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), both incretin hormones, promote the synthesis and secretion of insulin to lower blood glucose levels. However, DPP-IV is widely recognized for its rapid degradation and cleavage of GIP and GLP-1, resulting in an insensitive insulin response and loss of incretin secretion function, hence inducing abnormal blood glucose levels. Therefore, as leading enzymes of increasing blood glucose level in human body, their inhibition is closely linked with modifying the blood glucose and potential anti-diabetic properties.

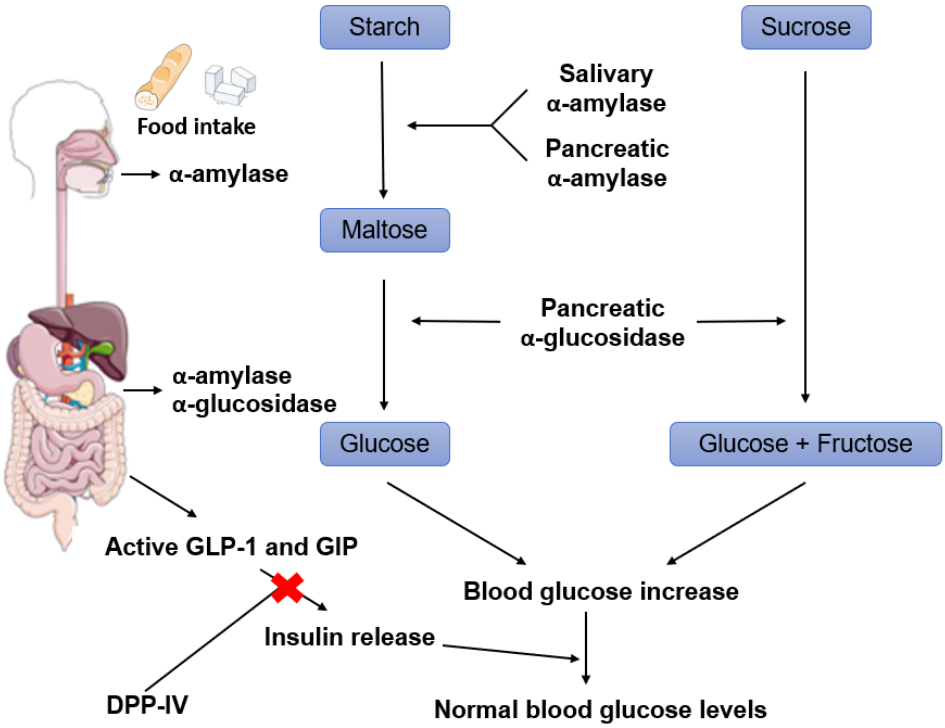

Figure 2.4 The role of DPP-IV, α-amylase and α-glucosidase in increasing the blood glucose after food (starch or sucrose-based) intake.

Several studies have identified peptides inhibiting α-amylase and/or α-glucosidase to delay the digestion of carbohydrates (**Appendix A - Table A4**). For example, Admassu et al. (2018) claimed pepsin-treated red seaweed exerted promising α-amylase inhibition (50.34%) at 1.86 mg/mL and then identified two peptides, Gly-Gly-Ser-Lys and Glu-Leu-Ser, with IC_{50} values of 2.58 ± 0.08 mM and 2.62 ± 0.05 mM, respectively. In addition, three long chain peptides derived from cumin seed proteins also can be considered as α-amylase inhibitors, with IC_{50} value ranges from 1.58 – 5.6 mg/mL (Siow et al., 2017). With regard to α-glucosidase inhibition, alcalase-treated rice bran albumin exerted 47.9 ± 2.6% inhibition per mg protein with Mw < 3 kDa. Meanwhile, Ren et al. (2016) isolated two novel hemp peptides, Leu-Arg and Pro-Leu-Met-Leu-Pro, against α-glucosidase with IC_{50} values 0.027 ± 0.002 and 0.032 ± 0.003 mg/mL, respectively. Soybean protein was also considered as a source of α-glucosidase inhibitors and released two peptides Gly-Ser-Arg and Glu-Ala-Lys after trypsin treatment, with IC_{50} values of 20.4μM and 520.4μM, respectively (Jiang, M. et al., 2018). Furthermore, dual inhibition of α-amylase and α-glucosidase were reported in chymotrypsin treated yellow field pea (α-amylase inhibition: 30.52 ± 0.01% at 225 μg/mL with Mw 1-3 kDa; α-glucosidase inhibition: 53.35 ± 2.78% at 20 mg/mL with Mw < 1kDa)

(Awosika and Aluko, 2019). Same findings were also reported in a peptide (LPLLR) derived from alcalase-treated walnut protein, exerting 50.12% and 39.08% of α-glucosidase and α-amylase inhibition, respectively, at 2 mM (Wang, J. et al., 2020).

Protein hydrolysates and peptides from different plant proteins, such as rice (Hatanaka et al., 2015), rapeseed (Xu, F. et al., 2019), quinoa (Nongonierma et al., 2015), macroalga (Harnedy et al., 2015), brewers spent grain (Connolly et al., 2017), common carp (Zhang, C. et al., 2020) and oat (Wang, F. et al., 2015) have been reported to be sources of the DPP-IV inhibitors. Among them, IIAP derived from Corolase PP-treated macroalga protein exerted the lowest IC_{50} value (43.40 ± 1.40 μM). Importantly, common bean cultivars, including black, pinto, red, navy, great northern and Negro 8025 are also considered as good hypoglycaemic agents, exerting considerable inhibition of DPP-IV, α-amylase and α-glucosidase (Valencia-Mejía et al., 2019). Likewise, pepsin-pancreatin co-treated quinoa protein hydrolysates with Mw < 5 kDa (comprises IQAEGGLT, DKDYPK, GEHGSDGNV, etc.) exerted considerable inhibitory properties of DPP-IV, α-amylase and α-glucosidase (Vilcacundo et al., 2017). Also legumes, such as Black, pinto, red, navy, great northern bean were proved as promising hypoglycaemic agents, as their protein hydrolysates inhibited all three enzymes as well (Mojica et al., 2015; Mojica and De Mejía, 2016).

2.3.2.4 Anti-inflammatory activity

Inflammation is a kind of complex and innate defensive immune response that is activated by injury, infection or other harmful stimuli. Acute inflammation is essential for defeating such infections and for aiding tissue repair, whilst uncontrolled and prolonged inflammation is closely linked with the pathogenesis of several chronic diseases, including metabolic disorders, atherosclerosis and cancers (Hotamisligil, 2006).

To access the anti-inflammatory potential of plant-derived protein hydrolysates and peptides, cell lines are used in particular macrophage cell lines (RAW 264.7), and also EA.hy926 cell, Caco-2, HT-29, MIN-6, vascular smooth muscle,THP-1 and RBL-2H3 cells. Lipopolysaccharides (LPS), are endotoxins secreted via gram-negative bacteria, and are widely used as a stimuli to evoke the multiple downstream intracellular signalling cascades of inflammation, including nuclear factor -kappa b (NF-κB) and mitogen-activating protein kinases (MAPK) in various cell types for studying

inflammatory responses (Park et al., 2011; Wang, Z. et al., 2012). Toll-like receptor 4 is currently implicated as a receptor for LPS-induced low-grade inflammatory response in macrophages (Pålsson‐McDermott and O'Neill, 2004). Activation of TLR4 was shown to generate abundant inflammatory effects and meanwhile induced expression of cytokines, including interleukin-6 (IL-6), tumour necrosis factor (TNF-α) and pro-inflammatory markers, such as nitric oxide (NO) and prostaglandins (PGs) (Tornatore et al., 2012) (See **Figure 2.5**).

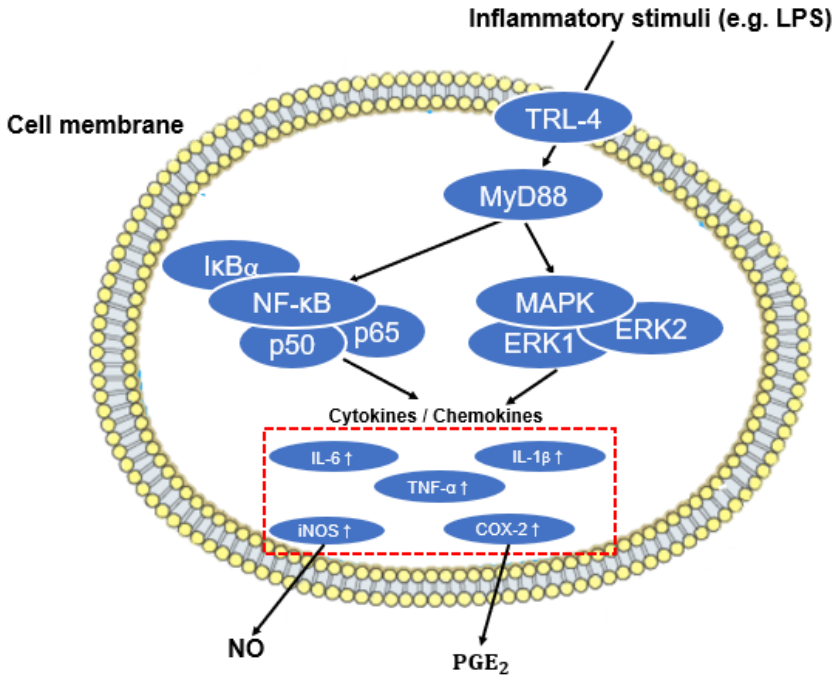

Figure 2.5 Potential mechanisms of LPS induced the activation of NF-κB and MAPK pathway.

A recent study reported that two peptides, PDLF and IALLIPF, isolated from alcalase-treated foxtail millet protein dampened NO, TNF-α, IL-6 and IL-1β in LPS-simulated RAW 264.7 macrophage cells. This study also found that both peptides suppressed the translocation of NF-κB (lowering the level of phosphorylated p-IκB and p65 submit) and MAPK pathways (supressing JNK, p38 and Erk1/2) (Ji et al., 2020). Hu et al. (2020) also used foxtail millet protein as material to generate two longer chain peptides, EDDQMDPMAK and QNWDPCEAWEPCF, that suppressed the lowering NO, TNF-α (42.29 and 44.07%, respectively) and IL-6 (56.59 and 43.45%, respectively) under same type of cell model. Likewise, rapeseed, hazelnut, lychee seed and many

others have also been shown to be sources of anti-inflammatory peptides (summarized in **Appendix A – Table A5**).

With regard to other cell lines, zein hydrolysates and three isolated peptides (PPYLSP, IIGGAL, FLPPVTSMG) are reported to exert anti-inflammatory properties in TNF-α-induced EA.hy925 cells via down-regulating of TNFR1 and supressing phosphorylation of p65 (Liang et al., 2020). LSW, derived from soy protein and previously reported as an anti-hypertensive peptide, suppressed the generation of COX-2 and decreased the phosphorylation of Src, ERK1/2 and P50 in Ang II-induced vascular smooth muscle cells (Lin et al., 2017).

It is important to note that the type of cell line may play a role in assessing the effectiveness of the anti-inflammatory properties of peptides. For example, extruded amaranth hydrolysates (after simulated gastrointestinal digestion) reduced secretion of TNF-α (33.5 and 36.5%), PGE_2 (31.4 and 15.4%) and COX-2 (67.6% and 38.1%), also reduced phosphorylation of IKK-α (88.2 and 52.5%), IκB-α (66.2 and 86.1%) and p65 NF-κB (70.7 and 64.2%) in RAW 264.7 and THP-1 cells (Montoya‐Rodríguez et al., 2014). Also, Chen, Y. et al. (2019) claimed peptide fractions from navy bean and light red kidney bean milk exerted the stronger inhibition in IL-8 secretion in Caco-2 cells than in HT-29 cells, due to di/tripeptides transporters (PepT1). Therefore, although these studies proposed that these peptides present anti-inflammatory properties via down-regulation of the pathways, suppression of the phosphorylation of gene expression, and inhibition of inflammatory cytokines, more studies are required to understand the mechanisms and the peptide transporters on the regulation of the inflammatory response.

2.3.3 *In vivo* evidence on bioactive effects of peptides

Despite current available studies offering plenty of *in silico* and *in vitro* scientific evidence to support the bioactive potential of plant-derived peptides, most studies do not take into consideration that peptides will probably be further broken down by digestive enzymes in the human gastrointestinal tract (GIT) after oral administration. A recent review also showed that some bioactive peptides may actually modulate the digestive enzyme activity, regulating the nutrient and mineral absorption or even exerting the benefit related to gut and intestinal health (Xu, Q. et al., 2019). Since the health benefits of peptides is influenced not only by the initial bioactive properties,

but also by the bioavailability and bio-accessibility, it is crucial that more human trials need to be conducted for confirming the bioactive properties.

Peptides liberated from plant proteins, such as chickpea (Torres-Fuentes et al., 2015), common bean (Mojica et al., 2015), Quinoa (Vilcacundo et al., 2017), longan seeds (Nuchprapha et al., 2020), foxtail millet (Hu et al., 2020) and oat (Sánchez-Velázquez et al., 2021), via simulated gastrointestinal digestion are always believed to be stable in GIT. Advances in *in vitro* digestion models (such as INFOGEST (Brodkorb et al., 2019)) are improving our understanding of the changes of peptide profiles and the capability of their bioactive properties. Intestinal uptake of peptides can be also evaluated in cell culture, typically in Caco-2 cell monolayers. Several studies have used animal protein to investigate the *in vivo* bioactive properties of peptides. However, to date, few clinical trials have been performed in human subjects. A recent study by Rein et al. (2019) explored the anti-inflammatory properties of rice protein hydrolysates. 20 grams of peptide powder contributed to a significant decrease in IP-10 (12 h and 24 h) and nitrogen oxidant (1 h to 8 h) levels in the blood serum. In addition, Claessens et al. (2009) found that the area under the curve (AUC) of glucose, when pea, soy and gluten hydrolysates (0.2 g per kg of body weight) were applied, was lower than with the control (0.2 g maltodextrin), whilst the AUC of insulin and glucagon increased. Apart from these acute response, Li, H. et al. (2011) conducted a three-week randomized double-blind placebo-controlled crossover trial, and found 3 g/day of pea protein hydrolysates resulted a significant average reduction of SBP (5 to 6 mmHg) in 7 healthy volunteers (3 males and 4 females) after intake for 2 or 3 weeks, with a maximum reduction of 10 mmHg. Furthermore, in a nine-week crossover human intervention trial (146 participants), 4 g per day of corn peptides resulted in increasing activity of superoxide dismutase (SOD) (7.83 ± 0.73 U/mL) and glutathione peroxidase (CPx) (24.97±2.68 U/mL) in the serum , along with decreased levels of malondialdehyde (MDA) (-0.41 ± 0.05 nmol/mL) and TNF-α (-25.56 ± 2.22 pg/mL) (Wu, Y. et al., 2014). The limited number of human studies and the discrepancies between *in vitro* and *in vivo* results means that much more work needs to be done in this area to substantiate health claims.

2.4 Concluding remarks and future trends

Plant proteins are well known as alternative sources of bioactive peptides and as such are actively under extensive investigation as functional

foods. With the proliferation of *in vitro* experiments (including cell culture) and animal models, evidence supporting their application in food areas as potential health promoting agents in general seems accumulating. However, much more work is required to: 1) isolate and concentrate protein isolates from a wide variety of raw plant matrices; 2) investigate pre-treatments and processing technologies for protein extraction; 3) investigate the effects of different enzymatic hydrolysis conditions; 4) access purification and identification of the active peptides; 5) conduct *in vitro* bioassay or animal models to test and confirm the bioactive properties. In general, it would appear that there is hope that plant peptides as functional ingredients may be able to replace those based on animal proteins. However, a key requirement is better quantification of the peptides of targeted biological activity, no matter what the source protein. This would allow better comparison of the efficacy of plant and animal proteins.

In silico (bioinformatic) tools potentially provide an excellent replacement of costly and time-consuming proteomics and peptidomics. In principle one can rapidly screen the peptide profiles liberated in precursor proteins with any known proteases, assuming the mechanisms of their bioactive functions are known (e.g., via molecular docking analysis) as well as predicting other characteristics such as toxicity, allergenicity and bitterness and even designing new peptides (QSAR). At present, however, the accuracy of the predictions is limited by no standard protocols to link *in silico* predictions with *in vitro* or in *vivo* data.

Although it is well known that some short chain peptides can be transported across the gut epithelial membrane and reach the ‘target’ tissues in an active form, resistance to digestive enzymes during GIT is a prerequisite challenging for bioactive peptides. Therefore, selecting peptides in advance or searching methods to extend its fate such as encapsulation before being delivered to digestive systems is necessary and further investigation in this area is needed. In addition, the release of, or transformation into, active peptides as a result of digestive enzymes on proteins and peptides in the GIT also needs to be considered.

Most previous research has used *in vitro* methods and in fewer cases animal models, with a notable lack of human trials. Some protein hydrolysates have been shown to exert biological activity but the active components have not been identified. Variations in the physiological dosage of peptides, their stability, bioavailability and metabolic breakdown broadens the gap between the conclusions of *in vitro* and *in vivo* experiments. To date,

few preclinical studies on peptides have been carried out and safety of these products also needs to be considered.

References

Adler-Nissen, J. 1979. Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *J Agric Food Chem.* **27**(6), pp.1256-1262.

Admassu, H., Gasmalla, M.A., Yang, R. and Zhao, W. 2018. Identification of bioactive peptides with α-amylase inhibitory potential from enzymatic protein hydrolysates of red seaweed (Porphyra spp). *Journal of agricultural and food chemistry.* **66**(19), pp.4872-4882.

Aiello, G., Li, Y., Boschin, G., Bollati, C., Arnoldi, A. and Lammi, C. 2019. Chemical and biological characterization of spirulina protein hydrolysates: Focus on ACE and DPP-IV activities modulation. *Journal of Functional Foods.* **63**, p103592.

Awosika, T.O. and Aluko, R.E. 2019. Inhibition of the in vitro activities of α - amylase, α - glucosidase and pancreatic lipase by yellow field pea (Pisum sativum L.) protein hydrolysates. *International Journal of Food Science & Technology.* **54**(6), pp.2021-2034.

Boukid, F. 2021. *Cereal-based Foodstuffs: The Backbone of Mediterranean Cuisine.* Springer Nature.

Boye, J., Zare, F. and Pletch, A. 2010. Pulse proteins: Processing, characterization, functional properties and applications in food and feed. *Food research international.* **43**(2), pp.414-431.

Brieger, K., Schiavone, S., Miller, F. and Krause, K.-H. 2012. Reactive oxygen species: from health to disease. *Swiss medical weekly.* **142**, pw13659.

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R. and Carrière, F. 2019. INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature protocols.* **14**(4), pp.991-1014.

Chan-Zapata, I., Arana-Argáez, V.E., Torres-Romero, J.C. and Segura-Campos, M.R. 2019. Anti-inflammatory effects of the protein hydrolysate and peptide fractions isolated from Salvia hispanica L. seeds. *Food and Agricultural Immunology.* **30**(1), pp.786-803.

Chang, S.K., Ismail, A., Yanagita, T., Esa, N.M. and Baharuldin, M.T.H. 2014. Biochemical characterisation of the soluble proteins, protein isolates and hydrolysates from oil palm (Elaeis guineensis) kernel. *Food Bioscience.* **7**, pp.1-10.

Chen, J., Liu, S., Ye, R., Cai, G., Ji, B. and Wu, Y. 2013. Angiotensin-I converting enzyme (ACE) inhibitory tripeptides from rice protein hydrolysate: Purification and characterization. *Journal of Functional Foods.* **5**(4), pp.1684-1692.

Chen, Y., Zhang, H., Liu, R., Mats, L., Zhu, H., Pauls, K.P., Deng, Z. and Tsao, R. 2019. Antioxidant and anti-inflammatory polyphenols and peptides of common bean (Phaseolus vulga L.) milk and yogurt in Caco-2 and HT-29 cell models. *Journal of Functional Foods.* **53**, pp.125-135.

Chiesa, S. and Gnansounou, E. 2011. Protein extraction from biomass in a bioethanol refinery–Possible dietary applications: Use as animal feed and potential extension to human consumption. *Bioresource Technology.* **102**(2), pp.427-436.

Chin, Y.Y., Chew, L.Y., Toh, G.T., Salampessy, J., Azlan, A. and Ismail, A. 2019. Nutritional composition and angiotensin converting enzyme inhibitory activity of blue lupin (Lupinus angustifolius). *Food Bioscience.* **31**, p100401.

Ciau‐Solís, N.A., Acevedo‐Fernández, J.J. and Betancur‐Ancona, D. 2018. In vitro renin‐angiotensin system inhibition and in vivo antihypertensive activity of peptide fractions from lima bean (Phaseolus lunatus L.). *Journal of the Science of Food and Agriculture.* **98**(2), pp.781-786.

Claessens, M., Calame, W., Siemensma, A.D., van Baak, M.A. and Saris, W.H. 2009. The effect of different protein hydrolysate/carbohydrate mixtures on postprandial glucagon and insulin responses in healthy subjects. *European journal of clinical nutrition.* **63**(1), pp.48-56.

Connolly, A., O'Keeffe, M.B., Nongonierma, A.B., Piggott, C.O. and FitzGerald, R.J. 2017. Isolation of peptides from a novel brewers spent grain protein isolate with potential to modulate glycaemic response. *International Journal of Food Science & Technology.* **52**(1), pp.146-153.

Cruz-Chamorro, I., Álvarez-Sánchez, N., Santos-Sánchez, G., Pedroche, J., Fernández-Pachón, M.-S., Millán, F., Millán-Linares, M.C., Lardone, P.J., Bejarano, I. and Guerrero, J.M. 2020. Immunomodulatory and Antioxidant Properties of Wheat Gluten Protein Hydrolysates in Human Peripheral Blood Mononuclear Cells. *Nutrients.* **12**(6), p1673.

Deleu, L.J., Lambrecht, M.A., Van de Vondel, J. and Delcour, J.A. 2019. The impact of alkaline conditions on storage proteins of cereals and pseudo-cereals. *Current Opinion in Food Science.* **25**, pp.98-103.

Devasagayam, T., Tilak, J., Boloor, K., Sane, K.S., Ghaskadbi, S.S. and Lele, R. 2004. Free radicals and antioxidants in human health: current status and future prospects. *Japi.* **52**(794804), p4.

Famuwagun, A.A., Alashi, A.M., Gbadamosi, O.S., Taiwo, K.A., Oyedele, D., Adebooye, O.C. and Aluko, R.E. 2020. Antioxidant and enzymes inhibitory properties of Amaranth leaf protein hydrolyzates and ultrafiltration peptide fractions. *Journal of Food Biochemistry.* pe13396.

Garcia-Vaquero, M., Mora, L. and Hayes, M. 2019. In Vitro and In Silico Approaches to Generating and Identifying Angiotensin-Converting Enzyme I Inhibitory Peptides from Green Macroalga Ulva lactuca. *Mar Drugs.* **17**(4), p204.

Gerzhova, A., Mondor, M., Benali, M. and Aider, M. 2016. Study of total dry matter and protein extraction from canola meal as affected by the pH, salt addition and use of zeta-potential/turbidimetry analysis to optimize the extraction conditions. *Food chemistry.* **201**, pp.243-252.

González, A.D., Frostell, B. and Carlsson-Kanyama, A. 2011. Protein efficiency per unit energy and per unit greenhouse gas emissions: potential contribution of diet choices to climate change mitigation. *Food policy.* **36**(5), pp.562-570.

Grigera, J.R. and McCarthy, A.N. 2010. The behavior of the hydrophobic effect under pressure and protein denaturation. *Biophysical journal.* **98**(8), pp.1626-1631.

Halim, N., Yusof, H. and Sarbon, N. 2016. Functional and bioactive properties of fish protein hydolysates and peptides: A comprehensive review. *Trends in Food Science & Technology.* **51**, pp.24-33.

Harnedy, P.A., O'Keeffe, M.B. and FitzGerald, R.J. 2015. Purification and identification of dipeptidyl peptidase (DPP) IV inhibitory peptides from the macroalga Palmaria palmata. *Food Chemistry.* **172**, pp.400-406.

Hatanaka, T., Uraji, M., Fujita, A. and Kawakami, K. 2015. Anti-oxidation activities of rice-derived peptides and their inhibitory effects on dipeptidylpeptidase-IV. *International Journal of Peptide Research and Therapeutics.* **21**(4), pp.479-485.

Henchion, M., Hayes, M., Mullen, A.M., Fenelon, M. and Tiwari, B. 2017. Future protein supply and demand: strategies and factors influencing a sustainable equilibrium. *Foods.* **6**(7), p53.

Hotamisligil, G.S. 2006. Inflammation and metabolic disorders. *Nature.* **444**(7121), pp.860-867.

Hu, S., Yuan, J., Gao, J., Wu, Y., Meng, X., Tong, P. and Chen, H. 2020. Antioxidant and anti-inflammatory potential of peptides derived from the in vitro gastrointestinal digestion of germinated and heat-treated foxtail millet (Setaria italica) proteins. *Journal of Agricultural and Food Chemistry.*

Iwaniak, A., Minkiewicz, P., Darewicz, M., Protasiewicz, M. and Mogut, D. 2015. Chemometrics and cheminformatics in the analysis of biologically active peptides from food sources. *Journal of Functional Foods.* **16**, pp.334-351.

Jakus, V. 2000. The role of free radicals, oxidative stress and antioxidant systems in diabetic vascular disease. *Bratislavske lekarske listy.* **101**(10), pp.541-551.

Ji, Z., Mao, J., Chen, S. and Mao, J. 2020. Antioxidant and anti-inflammatory activity of peptides from foxtail millet (Setaria italica) prolamins in HaCaT cells and RAW264. 7 murine macrophages. *Food Bioscience.* p100636.

Jiang, L., Wang, J., Li, Y., Wang, Z., Liang, J., Wang, R., Chen, Y., Ma, W., Qi, B. and Zhang, M. 2014. Effects of ultrasound on the structure and physical properties of black bean protein isolates. *Food Research International.* **62**, pp.595-601.

Jiang, M., Yan, H., He, R. and Ma, Y. 2018. Purification and a molecular docking study of α-glucosidase-inhibitory peptides from a soybean protein hydrolysate with ultrasonic pretreatment. *European Food Research and Technology.* **244**(11), pp.1995-2005.

Karami, Z., Peighambardoust, S.H., Hesari, J., Akbari-Adergani, B. and Andreu, D. 2019. Antioxidant, anticancer and ACE-inhibitory activities of bioactive peptides from wheat germ protein hydrolysates. *Food Bioscience.* **32**, p100450.

Li, H., Prairie, N., Udenigwe, C.C., Adebiyi, A.P., Tappia, P.S., Aukema, H.M., Jones, P.J. and Aluko, R.E. 2011. Blood pressure lowering effect of a pea protein hydrolysate in hypertensive rats and humans. *Journal of agricultural and food chemistry.* **59**(18), pp.9854-9860.

Li, M., Zheng, H., Lin, M., Zhu, W. and Zhang, J. 2020. Characterization of the protein and peptide of excipient zein by the multi-enzyme digestion coupled with nano-LC-MS/MS. *Food Chem.* **321**, p126712.

Li, Y.-W., Li, B., He, J. and Qian, P. 2011. Quantitative structure–activity relationship study of antioxidative peptide by using different sets of amino acids descriptors. *Journal of Molecular Structure.* **998**(1-3), pp.53-61.

Liang, Q., Chalamaiah, M., Liao, W., Ren, X., Ma, H. and Wu, J. 2020. Zein hydrolysate and its peptides exert anti-inflammatory activity on endothelial cells by preventing TNF-α-induced NF-κB activation. *Journal of Functional Foods.* **64**, p103598.

Lin, Q., Liao, W., Bai, J., Wu, W. and Wu, J. 2017. Soy protein-derived ACE-inhibitory peptide LSW (Leu-Ser-Trp) shows anti-inflammatory activity on vascular smooth muscle cells. *Journal of Functional Foods.* **34**, pp.248-253.

Liu, C., Fang, L., Min, W., Liu, J. and Li, H. 2018. Exploration of the molecular interactions between angiotensin-I-converting enzyme (ACE) and the inhibitory peptides derived from hazelnut (Corylus heterophylla Fisch.). *Food chemistry.* **245**, pp.471-480.

Liu, W.-Y., Fang, L., Feng, X.-W., Li, G.-M. and Gu, R.-Z. 2020. In vitro antioxidant and angiotensin I-converting enzyme inhibitory properties of peptides derived from corn gluten meal. *European Food Research and Technology.* pp.1-11.

Liu, Y.F., Oey, I., Bremer, P., Carne, A. and Silcock, P. 2018. Bioactive peptides derived from egg proteins: A review. *Crit Rev Food Sci Nutr.* **58**(15), pp.2508-2530.

Lorenzo, J.M., Munekata, P.E., Gómez, B., Barba, F.J., Mora, L., Pérez-Santaescolástica, C. and Toldrá, F. 2018. Bioactive peptides as natural antioxidants in food products–A review. *Trends in Food Science & Technology.* **79**, pp.136-147.

Lu, X., Zhang, L., Sun, Q., Song, G. and Huang, J. 2019. Extraction, identification and structure-activity relationship of antioxidant peptides from sesame (Sesamum indicum L.) protein hydrolysate. *Food Res Int.* **116**, pp.707-716.

Ma, F.-F., Wang, H., Wei, C.-K., Thakur, K., Wei, Z.-J. and Jiang, L. 2019. Three novel ACE inhibitory peptides isolated from Ginkgo biloba seeds: Purification, inhibitory kinetic and mechanism. *Frontiers in pharmacology.* **9**, p1579.

Mada, S.B., Ugwu, C.P. and Abarshi, M.M. 2019. Health Promoting Effects of Food-Derived Bioactive Peptides: A Review. *International Journal of Peptide Research and Therapeutics.* pp.1-18.

Marseglia, A., Dellafiora, L., Prandi, B., Lolli, V., Sforza, S., Cozzini, P., Tedeschi, T., Galaverna, G. and Caligiani, A. 2019. Simulated gastrointestinal digestion of cocoa: Detection of resistant peptides and in silico/in vitro prediction of their ace inhibitory activity. *Nutrients.* **11**(5), p985.

Meersman, F., Dobson, C.M. and Heremans, K. 2006. Protein unfolding, amyloid fibril formation and configurational energy landscapes under high pressure conditions. *Chemical Society Reviews.* **35**(10), pp.908-917.

Mirzaei, M., Mirdamadi, S., Safavi, M. and Hadizadeh, M. 2019. In vitro and in silico studies of novel synthetic ACE-inhibitory peptides derived from Saccharomyces cerevisiae protein hydrolysate. *Bioorg Chem.* **87**, pp.647-654.

Mohan, N. and Mellem, J.J. 2020. Functional properties of the protein isolates of hyacinth bean [Lablab purpureus (L.) Sweet]: An effect of the used procedures. *LWT.* p109572.

Mohanty, D., Mohapatra, S., Misra, S. and Sahu, P. 2016. Milk derived bioactive peptides and their impact on human health–A review. *Saudi journal of biological sciences.* **23**(5), pp.577-583.

Mojica, L., Chen, K. and de Mejía, E.G. 2015. Impact of commercial precooking of common bean (Phaseolus vulgaris) on the generation of peptides, after pepsin‐pancreatin hydrolysis, capable to inhibit dipeptidyl peptidase‐IV. *Journal of food science.* **80**(1), pp.H188-H198.

Mojica, L. and De Mejía, E.G. 2016. Optimization of enzymatic production of anti-diabetic peptides from black bean (Phaseolus vulgaris L.) proteins, their characterization and biological potential. *Food & function.* **7**(2), pp.713-727.

Montoya‐Rodríguez, A., de Mejía, E.G., Dia, V.P., Reyes‐Moreno, C. and Milán‐Carrillo, J. 2014. Extrusion improved the anti‐inflammatory effect of amaranth (A maranthus hypochondriacus) hydrolysates in LPS‐induced human THP‐1 macrophage‐like and mouse RAW 264.7 macrophages by preventing activation of NF‐κB signaling. *Molecular nutrition & food research.* **58**(5), pp.1028-1041.

Nardo, A.E., Añón, M.C. and Quiroga, A.V. 2020. Identification of renin inhibitors peptides from amaranth proteins by docking protocols. *Journal of Functional Foods.* **64**, p103683.

Nongonierma, A.B., Le Maux, S., Dubrulle, C., Barre, C. and FitzGerald, R.J. 2015. Quinoa (Chenopodium quinoa Willd.) protein hydrolysates with in vitro dipeptidyl peptidase IV (DPP-IV) inhibitory and antioxidant properties. *Journal of Cereal Science.* **65**, pp.112-118.

Nuchprapha, A., Paisansak, S., Sangtanoo, P., Srimongkol, P., Saisavoey, T., Reamtong, O., Choowongkomon, K. and Karnchanatat, A. 2020. Two novel ACE inhibitory peptides isolated from longan seeds: purification, inhibitory kinetics and mechanisms. *RSC Advances.* **10**(22), pp.12711-12720.

Osborne, T.B. 1924. *The vegetable proteins.* Longmans, Green and Company.

Padayatty, S.J., Katz, A., Wang, Y., Eck, P., Kwon, O., Lee, J.-H., Chen, S., Corpe, C., Dutta, A. and Dutta, S.K. 2003. Vitamin C as an antioxidant: evaluation of its role in disease prevention. *Journal of the American college of Nutrition.* **22**(1), pp.18-35.

Pålsson‐McDermott, E.M. and O'Neill, L.A. 2004. Signal transduction by the lipopolysaccharide receptor, Toll‐like receptor‐4. *Immunology.* **113**(2), pp.153-162.

Park, H.Y., Han, M.H., Park, C., Jin, C.-Y., Kim, G.-Y., Choi, I.-W., Kim, N.D., Nam, T.-J., Kwon, T.K. and Choi, Y.H. 2011. Anti-inflammatory effects of fucoidan through inhibition of NF-κB, MAPK and Akt activation in

lipopolysaccharide-induced BV2 microglia cells. *Food and Chemical Toxicology.* **49**(8), pp.1745-1752.

Pulido, R., Bravo, L. and Saura-Calixto, F. 2000. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *Journal of agricultural and food chemistry.* **48**(8), pp.3396-3402.

Rein, D., Ternes, P., Demin, R., Gierke, J., Helgason, T. and Schön, C. 2019. Artificial intelligence identified peptides modulate inflammation in healthy adults. *Food & function.* **10**(9), pp.6030-6041.

Ren, Y., Liang, K., Jin, Y., Zhang, M., Chen, Y., Wu, H. and Lai, F. 2016. Identification and characterization of two novel α-glucosidase inhibitory oligopeptides from hemp (Cannabis sativa L.) seed protein. *Journal of functional foods.* **26**, pp.439-450.

Rochlani, Y., Pothineni, N.V., Kovelamudi, S. and Mehta, J.L. 2017. Metabolic syndrome: pathophysiology, management, and modulation by natural compounds. *Therapeutic advances in cardiovascular disease.* **11**(8), pp.215-225.

Saisavoey, T., Sangtanoo, P., Reamtong, O. and Karnchanatat, A. 2018. Anti-inflammatory effects of Lychee (Litchi chinensis Sonn.) seed peptide hydrolysate on RAW 264.7 macrophage cells. *Food Biotechnology.* **32**(2), pp.79-94.

Sánchez-Velázquez, O.A., Cuevas-Rodríguez, E.O., Mondor, M., Ribéreau, S., Arcand, Y., Mackie, A. and Hernández-Álvarez, A.J. 2021. Impact of in vitro gastrointestinal digestion on peptide profile and bioactivity of cooked and non-cooked oat protein concentrates. *Current Research in Food Science.* **4**, pp.93-104.

Siow, H.-L., Tye, G.-J. and Gan, C.-Y. 2017. Pre-clinical evidence for the efficacy and safety of α-amylase inhibitory peptides from cumin (Cuminum cyminum) seed. *Journal of Functional Foods.* **35**, pp.216-223.

Stone, A.K., Karalash, A., Tyler, R.T., Warkentin, T.D. and Nickerson, M.T. 2015. Functional attributes of pea protein isolates prepared using different extraction methods and cultivars. *Food research international.* **76**, pp.31-38.

Suh, H.J., Whang, J. and Lee, H. 1999. A peptide from corn gluten hydrolysate that is inhibitory toward angiotensin I converting enzyme. *Biotechnology Letters.* **21**(12), pp.1055-1058.

Tang, N. and Zhuang, H. 2014. Evaluation of antioxidant activities of zein protein fractions. *Journal of food science.* **79**(11), pp.C2174-C2184.

Tornatore, L., Thotakura, A.K., Bennett, J., Moretti, M. and Franzoso, G. 2012. The nuclear factor kappa B signaling pathway: integrating metabolism with inflammation. *Trends Cell Biol.* **22**(11), pp.557-566.

Torres-Fuentes, C., del Mar Contreras, M., Recio, I., Alaiz, M. and Vioque, J. 2015. Identification and characterization of antioxidant peptides from chickpea protein hydrolysates. *Food Chemistry.* **180**, pp.194-202.

Udenigwe, C.C., Li, H. and Aluko, R.E. 2012. Quantitative structure–activity relationship modeling of renin-inhibiting dipeptides. *Amino Acids.* **42**(4), pp.1379-1386.

Udenigwe, C.C. and Mohan, A. 2014. Mechanisms of food protein-derived antihypertensive peptides other than ACE inhibition. *Journal of Functional Foods.* **8**, pp.45-52.

Valencia-Mejía, E., Batista, K.A., Fernández, J.J.A. and Fernandes, K.F. 2019. Antihyperglycemic and hypoglycemic activity of naturally occurring peptides and protein hydrolysates from easy-to-cook and hard-to-cook beans (Phaseolus vulgaris L.). *Food Research International.* **121**, pp.238-246.

Vásquez-Villanueva, R., Marina, M.L. and García, M.C. 2015. Revalorization of a peach (Prunus persica (L.) Batsch) byproduct: Extraction and characterization of ACE-inhibitory peptides from peach stones. *Journal of Functional Foods.* **18**, pp.137-146.

Vilcacundo, R., Martínez-Villaluenga, C. and Hernández-Ledesma, B. 2017. Release of dipeptidyl peptidase IV, α-amylase and α-glucosidase inhibitory peptides from quinoa (Chenopodium quinoa Willd.) during in vitro simulated gastrointestinal digestion. *Journal of Functional Foods.* **35**, pp.531-539.

Wang, F., Yu, G., Zhang, Y., Zhang, B. and Fan, J. 2015. Dipeptidyl peptidase IV inhibitory peptides derived from oat (avena sativa L.), buckwheat (fagopyrum esculentum), and highland barley (hordeum vulgare trifurcatum (L.) trofim) proteins. *Journal of agricultural and food chemistry.* **63**(43), pp.9543-9549.

Wang, J., Wu, T., Fang, L., Liu, C., Liu, X., Li, H., Shi, J., Li, M. and Min, W. 2020. Anti-diabetic effect by walnut (Juglans mandshurica Maxim.)-derived peptide LPLLR through inhibiting α-glucosidase and α-amylase, and alleviating insulin resistance of hepatic HepG2 cells. *Journal of Functional Foods.* **69**, p103944.

Wang, Z., Jiang, W., Zhang, Z., Qian, M. and Du, B. 2012. Nitidine chloride inhibits LPS-induced inflammatory cytokines production via MAPK and NF-kappaB pathway in RAW 264.7 cells. *J Ethnopharmacol.* **144**(1), pp.145-150.

Wen, C., Zhang, J., Feng, Y., Duan, Y., Ma, H. and Zhang, H. 2020. Purification and identification of novel antioxidant peptides from watermelon seed protein hydrolysates and their cytoprotective effects on H2O2-induced oxidative stress. *Food Chemistry.* p127059.

Wen, C., Zhang, J., Zhou, J., Duan, Y., Zhang, H. and Ma, H. 2018. Effects of slit divergent ultrasound and enzymatic treatment on the structure and antioxidant activity of arrowhead protein. *Ultrasonics sonochemistry.* **49**, pp.294-302.

Wongaem, A., Reamtong, O., Srimongkol, P., Sangtanoo, P., Saisavoey, T. and Karnchanatat, A. 2020. Antioxidant properties of peptides obtained from the split gill mushroom (Schizophyllum commune). *Journal of Food Science and Technology.* pp.1-12.

Wu, J.-S., Li, J.-M., Lo, H.-Y., Hsiang, C.-Y. and Ho, T.-Y. 2020. Anti-hypertensive and angiotensin-converting enzyme inhibitory effects of Radix Astragali and its bioactive peptide AM-1. *Journal of Ethnopharmacology.* p112724.

Wu, Y., Pan, X., Zhang, S., Wang, W., Cai, M., Li, Y., Yang, F. and Guo, H. 2014. Protective effect of corn peptides against alcoholic liver injury in men with chronic alcohol consumption: a randomized double-blind placebo-controlled study. *Lipids in health and disease.* **13**(1), p192.

Xia, J.a., Song, H., Huang, K., Li, S. and Guan, X. 2020. Purification and characterization of antioxidant peptides from enzymatic hydrolysate of mungbean protein. *Journal of Food Science.*

Xiao, J., Li, Y., Chen, B., Gong, S., Li, Z., Ou, Y., Ou, Z., Kang, A., Jia, Y. and Yang, A. 2020. Enzymatic preparation and antioxidative activity of hydrolysate from Rice bran protein. *Journal of Food Measurement and Characterization.* pp.1-12.

Xu, F., Yao, Y., Xu, X., Wang, M., Pan, M., Ji, S., Wu, J., Jiang, D., Ju, X. and Wang, L. 2019. Identification and Quantification of DPP-IV-Inhibitory Peptides from Hydrolyzed-Rapeseed-Protein-Derived Napin with Analysis of the Interactions between Key Residues and Protein Domains. *Journal of agricultural and food chemistry.* **67**(13), pp.3679-3690.

Xu, Q., Hong, H., Wu, J. and Yan, X. 2019. Bioavailability of bioactive peptides derived from food proteins across the intestinal epithelial membrane: A review. *Trends in food science & technology.* **86**, pp.399-411.

Yang, X., Wang, L., Zhang, F. and Ma, H. 2020. Effects of multi-mode S-type ultrasound pretreatment on the preparation of ACE inhibitory peptide from rice protein. *Food Chemistry.* p127216.

Zhang, C., Gao, S., Zhang, C., Zhang, Y., Liu, H., Hong, H. and Luo, Y. 2020. Evaluating in vitro dipeptidyl peptidase IV inhibition by peptides from common carp (Cyprinus carpio) roe in cell culture models. *European Food Research and Technology.* **246**(1), pp.179-191.

Zhang, P., Chang, C., Liu, H., Li, B., Yan, Q. and Jiang, Z. 2020. Identification of novel angiotensin I-converting enzyme (ACE) inhibitory peptides from wheat gluten hydrolysate by the protease of Pseudomonas aeruginosa. *Journal of Functional Foods.* **65**, p103751.

Zhao, W., Xue, S., Yu, Z., Ding, L., Li, J. and Liu, J. 2019. Novel ACE inhibitors derived from soybean proteins using in silico and in vitro studies. *Journal of food biochemistry.* **43**(9), pe12975.

Zheng, Y., Li, Y. and Li, G. 2019a. ACE-inhibitory and antioxidant peptides from coconut cake albumin hydrolysates: purification, identification and synthesis. *RSC advances.* **9**(11), pp.5925-5936.

Zheng, Y., Wang, X., Zhuang, Y., Li, Y., Shi, P., Tian, H., Li, X. and Chen, X. 2020. Isolation of novel ACE‐inhibitory peptide from naked oat globulin hydrolysates in silico approach: Molecular docking, in vivo antihypertension and effects on renin and intracellular endothelin‐1. *Journal of Food Science.* **85**(4), pp.1328-1337.

Zheng, Y., Wang, X., Zhuang, Y., Li, Y., Tian, H., Shi, P. and Li, G. 2019b. Isolation of Novel ACE-Inhibitory and Antioxidant Peptides from Quinoa Bran Albumin Assisted with an In Silico Approach: Characterization, In Vivo Antihypertension, and Molecular Docking. *Molecules.* **24**(24), p4562.

Zheng, Y., Zhang, Y. and San, S. 2020. Efficacy of a Novel ACE-Inhibitory Peptide from Sargassum Maclurei in Hypertension and Reduction of Intracellular Endothelin-1. *Nutrients.* **12**(3), p653.

Żukowski, P., Maciejczyk, M. and Waszkiel, D. 2018. Sources of free radicals and oxidative stress in the oral cavity. *Archives of Oral Biology.* **92**, pp.8-17.

Chapter 3
Identification of Angiotensin Converting Enzyme and Dipeptidyl Peptidase-IV inhibitory peptides derived from oilseed proteins using two integrated bioinformatic approaches

Abstract

Angiotensin-converting enzyme (ACE) and dipeptidyl peptidase-IV (DPP-IV) play critical roles in the development of hypertension and type 2 diabetes, respectively. Inhibiting the ACE and DPP-IV activity using peptides has become part of new therapeutic strategies for supporting medicinal treatment of both diseases. In this study, oilseed proteins, including soybean, flaxseed, rapeseed, sunflower and sesame are evaluated for the possibility of generating ACE and DPP-IV inhibitory peptides using different integrated bioinformatic approaches (UniProt knowledgebase, ProtParam, BLAST, BIOPEP, PeptideRanker, Pepsite2 and ToxinPred), and three bovine proteins (β-lactoglobulin, β-casein and κ-casein) as comparisons. Compared with bovine proteins, the potency indices of ACE and DPP-IV inhibitory peptides, calculated using the BIOPEP database, suggest that oilseed proteins may be considered as good precursors of ACE inhibitory peptides but generate a relative lower yield of DPP-IV inhibitory peptides following subtilisin, pepsin (pH=1.3) or pepsin (pH>2) hydrolysis. Average scores aligned using PeptideRanker confirmed oilseeds proteins as significant potential sources of bioactive peptides: over 105 peptides scored over 0.8. Pepsite2 predicted that these peptides would largely bind via Gln281, His353, Lys511, His513, Tyr520 and Tyr523 of ACE to give the inhibition, while Trp629 is the predominant binding site of peptides in reducing DPP-IV activity. All peptides were capable of inhibiting ACE and DPP-IV whilst 65 of these 105 peptides are not currently recorded in BIOPEP database. In conclusion, our *in silico* study demonstrates that oilseed proteins could be considered as good precursors of ACE and DPP-IV inhibitory peptides as well as so far unexplored peptides that potentially have roles in ACE and DPP-IV inhibition and beyond.

3.1 Introduction

Defatted oilseeds, such as flaxseed, rapeseed, sunflower and sesame, are by-products from the food industry and currently used as livestock feed or waste. In the last two decades these under-utilised materials have gained growing interest, due to their high protein content, which in turn makes them a potential low-cost source of bioactive peptides. Peptides derived from some oilseed proteins have demonstrated a wide range of bioactive properties including: antioxidative (He et al., 2013), mineral chelating (Megías et al., 2008), anti-inflammatory (Udenigwe et al., 2009) and cholesterol lowering (Cho et al., 2007) activities. These activities have been linked to beneficial health outcomes and peptides could be applied as value-added components in functional foods, nutraceuticals and pharmaceuticals (Korhonen and Pihlanto, 2003; Hartmann and Meisel, 2007; Udenigwe and Aluko, 2012; Li-Chan, 2015).

Among several health benefits of bioactive peptides, inhibition of Angiotensin-Converting Enzyme (ACE) and Dipeptidyl Peptidase-IV (DPP-IV) have been well documented, as well as their synergistic effects in combination with synthetic drugs (Marczak et al., 2003; Guang and Phillips, 2009; Nongonierma and FitzGerald, 2015; Patil et al., 2015). In the human body, ACE, is associated with elevated blood pressure, by cleaving a dipeptide (HL) from the decapeptide angiotensin I to form a potent vasoconstrictor, angiotensin II. Further, ACE inhibits and degrades bradykinin, a potent vasodilator (Bénéteau-Burnat and Baudin, 1991). DPP-IV is a metabolic serine peptidase which is widely distributed in almost all human tissues, causing the degradation and inactivation of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), both are incretin hormones and responsible for stimulating the secretion of insulin (Lambeir et al., 2003). Therefore, inhibiting ACE and DPP-IV activities have become two major therapeutic targets for the management of hypertension and type 2 diabetes mellitus, directly leading to significant reduction in blood pressure and blood glucose levels (Kieffer et al., 1995; Hansson et al., 1999), respectively.

Proteomic approaches are widely applied for exploring and evaluating the biological activities of bioactive peptides and are traditionally comprised of several steps: 1) manually quantifying the protein content; 2) extracting the protein isolate; 3) hydrolysing protein with the aid of one or more proteases to release the bioactive peptides; 4) fractionating and purifying the protein hydrolysates; 5) identifying the amino acid sequences of peptides; 6)

synthesizing the identified peptides and confirming the bioactive properties (Carrasco-Castilla et al., 2012; Sánchez-Rivera et al., 2014; Dupont, 2017). These methods are time-consuming and expensive, and yield low amounts of targeted peptides, which limits their further appraisal with respect to *in vivo* studies.

Complementary to traditional proteomic approaches, database-aided bioinformatic evaluations (*in silico*) are suggested as a potential cost-effective tool to screen and theoretically predict the potency of specific protein sequences as precursors for ACE and DPP-IV inhibitors. The release of peptide fractions can be predicted through simulation of enzymatic hydrolysis of identified protein sequences based on protease cleavage specificities, which allow evaluation of the hydrolysis capability of enzymes and gastrointestinal digestive tolerance of the peptides. Such screening delivers information on the potential production of potent bioactive peptides and can highlight novel bioactive peptides for further chemical or recombinant DNA synthesis (Udenigwe, 2014). Peptide sequences with ACE and DPP-IV inhibiting activities have already been extensively explored and identified in the literature, as well being included in appropriate databases; this therefore greatly improves the accuracy and reliability of *in silico* screening for these particular peptide activities. In addition, a number of *in silico* studies have already been published with the purpose of predicting other biological activities of peptides derived from food material including milk (Vukic et al., 2017), deer skin (Jin et al., 2015), rice (Pooja et al., 2017), crude barley (Gangopadhyay et al., 2016), green algae Caulerpa (Agirbasli and Cavas, 2017) and cumin (Siow and Gan, 2016). These studies suggest that integrated bioinformatic evaluations are effective in predicting the peptides released from the parent proteins. However, to the best of our knowledge, the comparison of two *in silico* predicting approaches for potency of precursor proteins generating ACE and DPP-IV inhibitors is missing. Thus, the aims of this study are: (a) to screen peptides released from precursor proteins using BIOPEP and (b) to rank the peptides using PeptideRanker, together with predicting the binding sites of promising peptides to ACE and DPP-IV using Pepsite2.

3.2 Methods

3.2.1 Protein sequences

In this study, ten storage proteins from five oilseeds sources, flaxseed (*Linum usitatissimum* Q8LPD4), rapeseed (*Brassica napus* P17333, P11090), sunflower (*Helianthus annuus* P19084, P15461), sesame (*Sesamum indicum* Q9XHP1, Q9XHP0) and soybean (*Glycine max* P04347, P11827, P13916), were selected and assessed, together with three bovine proteins (*BOS Taurus* P02754, P02666, P02666) for comparison, to investigate their potency as precursors of ACE and DPP-IV inhibitory peptides. An overview of the integrated bioinformatic approach is presented in **Figure 3.1**.

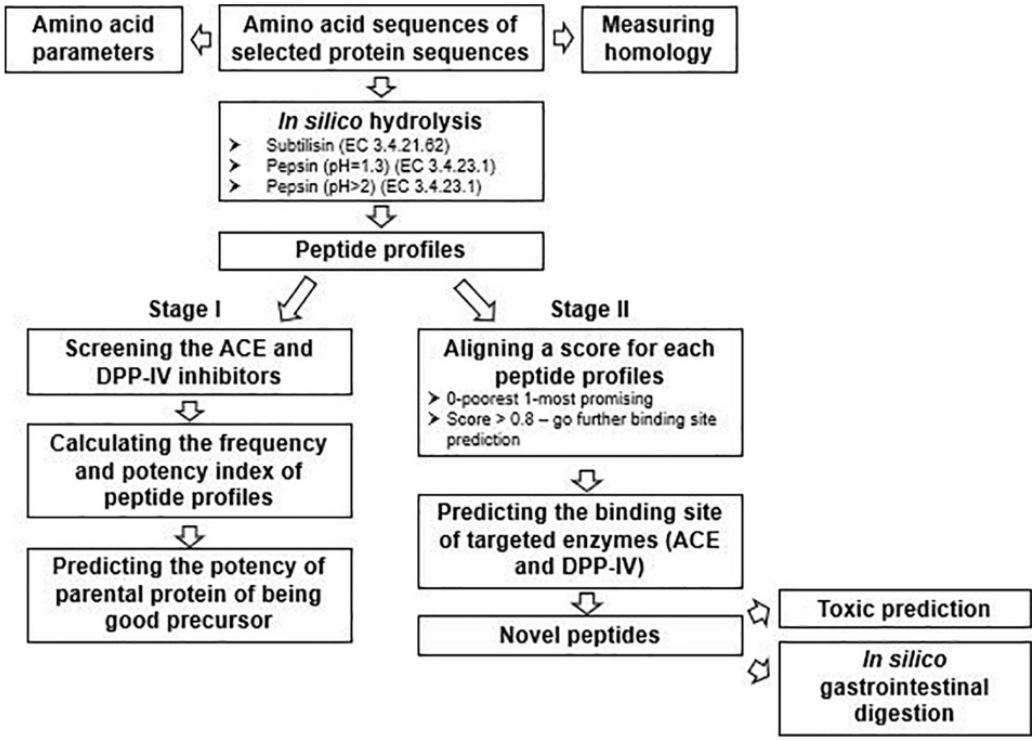

Figure 3.1 Bioinformatic methodology applied for screening and predicting ACE and DPP-IV inhibitory peptides from oilseed and bovine proteins.

All sequence information was retrieved from UniProt Knowledgebase (http://www.uniprot.org/) (**Table 3.1**). The specific oilseed crops chosen were selected based on the protein levels in their seeds as well as the availability of their amino acid sequences in databases. The bovine caseins β-casein and κ-casein, plus the principal bovine whey protein β-lactoglobulin, are all well-documented as abundant resources of ACE and DPP-IV inhibitory peptides (Maruyama and Suzuki, 1982; Pihlanto-Leppälä et al., 2000;

Nongonierma and FitzGerald, 2013; Silveira et al., 2013). ProtParam (https://web.expasy.org/protparam/) was used to count the amino acid percentage in the selected proteins (Gasteiger et al., 2005). In addition, BLAST server (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to evaluate significant similarities (homologies) of the 13 protein sequences (Papadopoulos and Agarwala, 2007).

3.2.2 *In silico* hydrolysis

The peptide bond cleavage was simulated according to the specificity of the enzymes subtilisin (EC 3.4.21.62), pepsin (pH = 1.3) (EC 3.4.23.1) and pepsin (pH > 2) (EC 3.4.23.1) using the BIOPEP 'Enzyme(s) action' tool. The peptide profiles generated were identified based on the information recorded in BIOPEP database (currently 3669 peptides). Two parameters, frequency index (A) and potency index (B) for generating ACE and DPP-IV inhibitory peptides were calculated using **Equation 3.1** and **Equation 3.2**, respectively (Minkiewicz et al., 2008).

$$\boldsymbol{A} = \frac{\boldsymbol{a}}{\boldsymbol{N}} \qquad \textbf{(3.1)}$$

A: the frequency index of targeted peptides released from precursor proteins

a: the number of targeted peptides

N: the total number of amino acid residues

$$\textbf{B} = \frac{\sum_{i=1}^{K} \frac{a_i}{IC_{50}}}{N} \qquad \textbf{(3.2)}$$

B: the potency index of targeted biological activity

a_i: the number of repetitions of peptides with same amino acid residues released from precursor proteins

IC_{50}: the concentration of bioactive peptides needed to inhibit half-maximal enzyme activity

K: the number of different peptides with targeted biological activities

N: the total number of amino acid residues

Table 3.1 Overview on oilseed and bovine protein sequences used for bioinformatic analyses

Protein	UniProtKB Code	Length	References
Flaxseed			
Linin	-	-	(Truksa et al., 2003; Chung et al., 2005)
Conlinin	Q8LPD4	169	
Rapeseed			
Napin	P17333	180	(Ericson et al., 1986; Gueguen et al., 1990)
Cruciferin	P11090	488	
Sunflower			
11S globulin seed storage protein	P19084	493	(Allen, R.D. et al., 1985; Allen, R. et al., 1987)
2S seed storage protein	P15461	295	
Sesame			
2S seed protein protein	Q9XHP1	148	(Tai et al., 1999; Orruno and Morgan, 2007)
11S globulin seed storage protein	Q9XHP0	459	
Soybean			
Glycinin	P04347	516	(Meinke et al., 1981; Fujiwara et al., 1992)
β-conglycinin, α' chain	P11827	639	
β-conglycinin, α chain	P13916	605	
Bovine			
β-lactoglobulin	P02754	178	(Madureira et al., 2007; Dalgleish, 2011)
β-casein	P02666	224	
κ-casein	P02668	190	

3.2.3 The potential of peptide profiles exerting biological activities

All the peptide profiles generated via *in silico* digestion were evaluated for their likelihood of being bioactive using PeptideRanker. This tool assigns a score to each peptide, within the range 0 (poorest activity) to 1 (most promising activity). The peptides with score > 0.8 were described as 'promising bioactive peptides' and subsequently subjected to toxicity prediction using ToxinPred (http://crdd.osdd.net/raghava/toxinpred/) and further binding site prediction (Mooney et al., 2012; Gupta et al., 2013). In addition, an average score of all the predicted peptides generated from oilseed and bovine proteins was calculated via **Equation 3.3** to assess overall protein promise of releasing bioactive peptides.

$$\boldsymbol{Average\ score} = \frac{\sum_{i=1}^{k} \boldsymbol{b_i}}{\boldsymbol{N}} \qquad \textbf{(3.3)}$$

b_i: the score of peptides

k: the number of peptides released from precursor protein

N: the total number of amino acid residues

3.2.4 Predicting ACE and DPP-IV binding sites within the generated peptides

The interactions between the peptides and the targeted enzyme were predicted using Pepsite2 (http://pepsite2.russelllab.org/) (Trabuco et al., 2012). The three-dimensional structures of human DPP-IV (PDB code: 1NU6) and ACE (PDB code: 1O8A) were obtained from Protein Data Bank (PDB) (https://www.rcsb.org/). Colour scales were applied in this study to evaluate the predictions, which are 1) Red colour refers to highly significant; 2) Yellow colour means moderately significant; 3) White colour is considered as no significance (Trabuco et al., 2012). For each peptide, only the prediction with the lowest p-value (red colour) was selected. In addition, sequences comprising more than ten amino acid residues were ignored (the maximum length accepted by this database).

3.2.5 Potential gastrointestinal digestive tolerance of peptides

The bioavailability of peptides *in vivo* is also determined by their survival during digestion. Peptide profiles with a score over 0.8 (aligned by PeptideRanker) were evaluated for their tolerance against the cleavage of

pepsin (pH > 2.0, EC 3.4.23.1), trypsin (EC 3.4.21.4) and chymotrypsin (3.4.21.2), using the 'Enzyme(s) action' tool obtained from BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/en/biopep).

3.3 Results and Discussion

3.3.1 Amino acid compositions and homology of the oilseed proteins

All sequences underwent homology analysis using BLAST with the method 'compositional matrix adjustment'. The distributions of alignment scores among selected proteins conducted in pairs are shown in **Table 3.2**. Out of 78 pairs, 7 gave high scores (> 200), meaning that these pairs show similar molecular features and therefore the peptides derived from them might also be expected to have similar sequences and biological activities (Pooja et al., 2017). **Table 3.3** summarises the details, including the identities, positives, gaps, and bit scores, of protein sequences with alignment scores over 200. 76% identities and 83% positives were found between the α-chain and α'-chain of β-conglycinin, both from soybean. In addition, 11S globulin (sunflower), cruciferin (rapeseed), 11S globulin (sesame) and glycinin (soybean) displayed similarity. This finding agrees with the previous research on related proteins - for example, Chang and Alli (2012) suggested there are approximately 30% similar amino acid residues between legumin (chickpea) and 12S globulin (oat).

Table 3.2 Distribution of alignment scores for 13 proteins sequences. (**BLAST**)

Alignment scores	< 40	40 – 50	50 - 80	80 - 200	> 200
Number of groups	65	4	2	0	7

High value of alignment score indicates high homology

Quantitative structure-activity relationship (QSAR) studies, e.g., Lafarga et al. (2014) have shown how 7 amino acid residues - Gly, Ile, Leu, Phe, Pro, Trp and Tyr, are present at high frequencies in ACE inhibitory peptides. In addition, Ala, Gly, Pro and Tyr play key roles in the composition of DPP-IV inhibitory peptides (Lacroix and Li-Chan, 2012; Jin et al., 2015). Therefore,

protein sequences containing high concentrations of the above amino acid residues are expected to be promising sources of ACE or DPP-IV inhibitory peptides. The number and percentage of specific amino acid residues were counted via ProtParam (**Table 3.4**). The α chain and α' chain of β-conglycinin (soybean) demonstrated similar percentages of these specific amino acids. In addition, cruciferin (rapeseed) has a similar amino acid composition to 11S globulin storage protein (sunflower) and 11S Globulin Seed storage protein (sesame), but differences could be found in their Ala, Ile and Pro contents when compared with glycinin (soybean). Other sequences showed major differences when compared with each other.

Table 3.3 Summary of protein sequences with alignment scores over 200. (**BLAST**)

	Identities	Positives	Gaps	Scores
P19084 vs P11090	189/494(38%)	265/494(53%)	87/494(17%)	304
P11090 vs Q9XHP0	150/442(34%)	251/442(56%)	35/442(7%)	277
P11090 vs P04347	167/520(32%)	258/520(49%)	116/520(22%)	254
P19084 vs Q9XHP0	161/471(34%)	257/471(54%)	49/471(10%)	275
Q9XHP0 vs P11090	146/442(33%)	246/442(55%)	35/442(7%)	271
P04347 vs Q9XHP0	149/495(30%)	249/495(50%)	83/495(16%)	252
P13916 vs P11827	495/649(76%)	540/649(83%)	54/649(8%)	900

P19084: Sunflower, 11S Globulin seed storage protein; **P11090**: Rapeseed, Cruciferin; **Q9XHP0**: Sesame, 11S Globulin seed protein; **P04347**: Soybean, Glycinin; **P13916**: Soybean, β-conglycinin, α'-chain; **P11827**: Soybean, β-conglycinin, α-chain; **Identities**: same amino acid residues in same position; **Positives**: amino acid residues with the similar molecular characteristics in same position; **Gaps**: Spaces for deletions and insertions; **Scores**: reflects homology.

Obviously, the variations in amino acid content and sequence between oilseed and milk proteins contributes to the differences in bioactive properties of peptides generated.

Table 3.4 Number and percentage of amino acid residues frequently discovered in ACE and DPP-IV inhibitory peptides present in oilseed and bovine proteins (**ProtParam**)

Protein	Ala	Gly	Ile	Leu	Phe	Pro	Trp	Tyr
Flaxseed								
2S Conlinin	10	19	8	9	7	2	3	3
%	5.90%	11.20%	4.70%	5.30%	4.10%	1.20%	1.80%	1.80%
Rapeseed								
Napin	12	9	6	15	9	15	2	3
%	6.70%	5.00%	3.30%	8.30%	5.00%	8.30%	1.10%	1.70%
Cruciferin	33	47	22	45	22	25	5	10
%	6.80%	9.60%	4.50%	9.20%	4.50%	5.10%	1.00%	2.00%
Sunflower								
11S Globulin Seed Storage Protein	38	35	24	37	25	22	8	5
%	7.70%	7.10%	4.90%	7.50%	5.10%	4.50%	1.60%	1.00%
2S Seed Storage Protein	14	17	16	15	10	13	0	1
%	4.70%	5.80%	5.40%	5.10%	3.40%	4.40%	0%	0.30%
Sesame								
2S Seed Storage Protein	12	6	2	7	6	3	2	4

%	8.10%	4.10%	1.40%	4.70%	4.10%	2.00%	1.40%	2.70%
11S Globulin Seed Storage Protein	34	33	20	35	18	19	4	12
%	7.40%	7.20%	4.40%	7.60%	3.90%	4.10%	0.90%	2.60%
Soybean								
Glycinin	20	41	17	41	18	38	4	15
%	3.90%	7.90%	3.30%	7.90%	3.50%	7.40%	0.80%	2.90%
β-conglycinin, α' chain	28	31	29	49	32	35	3	15
%	4.40%	4.90%	4.50%	7.70%	5.00%	5.50%	0.50%	2.30%
β-conglycinin, αchain	28	26	31	54	30	40	2	15
%	4.60%	4.30%	5.10%	8.90%	5.00%	6.60%	0.30%	2.50%
Bovine								
β-lactoglobulin	19	5	10	27	4	8	2	4
%	10.70%	2.80%	5.60%	15.20%	2.20%	4.50%	1.10%	2.20%
β-casein	9	5	11	27	9	35	1	4
%	4.00%	2.20%	4.90%	12.10%	4.00%	15.60%	0.40%	1.80%
κ-casein	16	3	13	13	7	21	1	9
%	8.40%	1.60%	6.80%	6.80%	3.70%	11.10%	0.50%	4.70%

3.3.2 Stage I: Screening the ACE and DPP-IV inhibitory peptides

Oilseed and bovine protein sequences were analysed using BIOPEP database. Meanwhile, the bacterial protease subtilisin and the human gastric enzyme pepsin, were selected as enzymes for protein hydrolysis. Udenigwe (2016) suggested that pepsin exerts a narrower specificity when the pH of the medium is below 2. Therefore, two pH conditions, pH =1.3 and pH > 2, were selected for pepsin hydrolysis. Only peptides with short amino acid sequences, such as di- and tri-peptides, contribute to the major part of peptide bioactivity (Iwaniak and Dziuba, 2009), exerting antithrombotic, antiamnestic, antioxidative, hypotensive and ubiquitin-mediated proteolysis (**Supplementary Table 1S**). However, here only ACE and DPP-IV inhibitory activity were investigated.

Table 3.5 presents the frequency index of ACE and DPP-IV inhibitory peptides generated from 13 protein sequences. Apart from pepsin (pH = 1.3)-treated napin (rapeseed), pepsin (pH = 1.3)-treated 2S storage protein (sunflower), pepsin (pH = 1.3)-treated 2S storage protein (sesame) and pepsin (pH > 2)-treated napin (rapeseed), the frequency index of ACE inhibitory peptide was lower than that of DPP-IV inhibitory peptides generated from the same sequences. In addition, pepsin (pH > 2) gave the highest frequency index among the three enzymes, followed by subtilisin, with pepsin at pH 1.3 being lowest. Among all the pepsin (pH > 2)-treated oilseed proteins, napin, derived from rapeseed (A 0.0883), exerted the highest frequency index of ACE inhibition, only κ-casein being higher (A 0.0947) when compared with the three milk proteins. Regarding the frequency index of DPP-IV inhibition, cruciferin (rapeseed) gave the highest value (A 0.1127) amongst the oilseed proteins, but this was lower than for all 3 milk proteins (A between 0.1180 and 0.1518).

High predicted frequency values of ACE and DPP-IV inhibition do not directly translate from the precursor protein to a good source of ACE and DPP-IV inhibitors. The value of IC_{50} of each active peptide should be used to adjust the frequency to get the potency index (μM^{-1}).

Table 3.5 Frequency indices of ACE and DPP-IV inhibitory peptides generated *in silico* from oilseed and bovine proteins using enzymatic hydrolysis with subtilisin and pepsin (pH 1.3 and pH>2) (**BIOPEP)**

	Subtilisin		**Pepsin (pH 1.3)**		**Pepsin (pH > 2)**	
	ACE inhibitor	DPP-IV inhibitor	ACE inhibitor	DPP-IV inhibitor	ACE inhibitor	DPP-IV inhibitor
Flaxseed						
2S Conlinin	0.0296	0.0355	n/a	n/a	0.0533	0.1006
Rapeseed						
Napin	0.0389	0.0444	0.0111	0.0056	0.0883	0.0883
Curciferin	0.0246	0.0574	0.0164	0.0184	0.084	0.1127
Sunflower						
2S Seed Storage Protein	0.0102	0.0271	0.0068	0.0068	0.0644	0.0949
11S Globulin Seed Storage Protein	0.0243	0.0446	0.0101	0.0142	0.0649	0.0852
Sesame						
2S Seed Storage Protein	n/a	0.0405	0.0068	n/a	0.0743	0.0946
11S Globulin Seed Storage Protein	0.0194	0.0367	0.0043	0.013	0.0475	0.0907
Soybean						
Glycinin	0.031	0.0581	0.0078	0.0116	0.0523	0.0969

β-conglycinin, α' chain	0.0203	0.0423	0.0063	0.0125	0.0673	0.1095
β-conglycinin, α chain	0.0198	0.0347	0.0099	0.0165	0.0793	0.1124
Bovine						
β-lactoglobulin	0.0169	0.0337	0.0056	0.0225	0.0562	0.118
β-casein	0.0268	0.0938	0.0268	0.0357	0.067	0.1518
κ-casein	0.0158	0.0789	0.0053	0.0211	0.0947	0.1421

Table 3.6. Potency indices (x 10^{-3} μM^{-1}) of ACE and DPP-IV inhibitory peptides generated from oilseeds and bovine proteins **(BIOPEP)**

	Subtilisin		**Pepsin (pH 1.3)**		**Pepsin (pH > 2)**	
	ACE inhibitor	DPP-IV inhibitor	ACE inhibitor	DPP-IV inhibitor	ACE inhibitor	DPP-IV inhibitor
Flaxseed						
2S Conlinin	1.4197	0.0060	n/a	n/a	1.9097	0.0375
Rapeseed						
Napin	3.0663	0.0009	0.0445	n/a	6.2213	0.2050
Curciferin	3.9421	0.1985	0.2079	0.0031	3.8966	0.2328
Sunflower						

2S Seed Storage Protein	0.4810	0.0497	0.1125	0.0038	1.0453	0.0876
11S Globulin Seed Storage Protein	1.5792	0.0274	0.2115	n/a	1.6763	0.0688
Sesame						
2S Seed Storage Protein	n/a	0.1826	0.9543	n/a	0.873	0.2536
11S Globulin Seed Storage Protein	0.9832	0.0153	0.0940	0.0050	1.6192	0.0818
Soybean						
Glycinin	2.9164	0.1214	0.3297	0.0015	3.5738	0.1779
β-conglycinin, α' chain	0.4772	0.0653	0.0549	n/a	1.5270	0.1141
β-conglycinin, α chain	0.2859	0.0453	0.2303	0.0013	1.6910	0.0831
Bovine						
β-lactoglobulin	1.4124	0.1646	0.0431	0.0887	1.6405	0.3243
β-casein	0.7556	0.2810	0.6728	0.0410	1.0948	0.3079
κ-casein	0.6648	0.2997	0.0404	0.0060	4.6858	0.2614

Pepsin (pH > 2)-treated napin showed the highest potency index (B 6.22135 x 10^{-3} μM^{-1}) of ACE inhibitor amongst all the proteins investigated (**Table 3.6**). With regards to DPP-IV inhibition, pepsin (pH > 2)- treated milk proteins gave more promising values than oilseed proteins: 3.2434 x 10^{-4} μM^{-1} (β-lactoglobulin), 3.0789 x 10^{-4} μM^{-1} (β-casein) and 2.6140 x 10^{-4} μM^{-1} (κ-casein), whilst the most promising amongst the oilseed proteins was pepsin (pH > 2)-treated napin (B 0.00023281μM^{-1}). Thus, bovine milk proteins might be a more promising source of DPP-IV inhibitors than oilseed proteins in general.

In comparison to animal peptide data, plant protein-derived peptide sequence availability is limited which may have an impact on the outcome of prediction analysis and therefore contribute to underestimation of frequency and potency indices of plant proteins. To be able to predict unrecorded ACE and DPP-IV inhibitor candidate peptides potentially obtainable from oilseed and milk proteins, the PeptideRanker application was used together with Pepsite2 (Stage II - see **Figure 3.1** and below).

The frequency and potency indices among all proteins vary notably, even though sequences possessing significant similarity (aligned score > 200 via BLAST). Lafarga et al. (2014) also highlighted that the peptides derived from one 'parent' protein might not always be generated from highly similar proteins.

3.3.3 Stage II: Predicting ACE and DPP-IV inhibitory peptides

Entire peptide profiles from *in silico* hydrolysis are provided with scores using PeptideRanker (**Supplementary Table 2S**). A threshold of 0.8 was set in order to reduce the number of false positives Mooney et al. (2012) and the resulting numbers of peptides for each source are shown in **Figure 3.2**. Unlike the results of Udenigwe et al. (2013), the numbers of bioactive peptides did not always appear to be strongly dependent on the type of enzyme, or correlate with the frequency index calculated using the BIOPEP database. In most cases, pepsin (pH > 2) gave the highest number bioactive peptides, except for colinin (flaxseed) and glycinin (soybean). Subtilisin treatment predicts the same number of bioactive peptides in 2S storage protein (sunflower) as in the α-chain and α'-chain of β-conglycinin (soybean) and β-lactoglobulin (bovine). For the other proteins, pepsin (pH = 1.3) gave the lowest number of bioactive peptides, correlating with the trends in the frequency index of the proteins with different enzymes. In addition, the highest

numbers of bioactive peptides were predicted from oilseed proteins compared to milk proteins. However, the total numbers of peptide fragments are remarkably different for each protein sequence. Therefore, the average PeptideRanker score for all the sequences was calculated (see **Table 3.7**). Pepsin (pH > 2) gave the highest average score, pepsin (pH = 1.3) the lowest. This tendency is the same as the influence of enzyme on frequency Index of ACE and DPP-IV inhibitor peptides. For the pepsin (pH>2)-treated proteins, colinin exerted the highest average score (0.2678), while the lowest was for κ-casein (0.1972). Thus, the oilseed proteins (0.2103 – 0.2678) might have equal or even better release of bioactive peptides compared to β-lactoglobulin (0.2406), β-casein (0.2260) or κ-casein (0.1972).

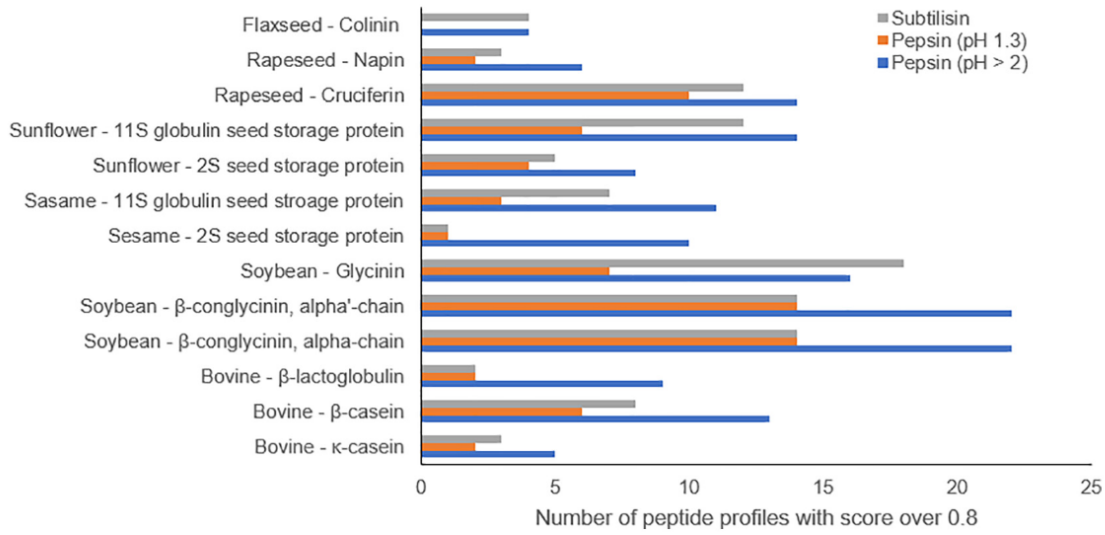

Figure 3.2 Peptide profiles generated from in silico hydrolysis of thirteen proteins which demonstrated scores over 0.8

Peptides with a score > 0.80 via PeptideRanker suggest high bioactive possibilities. However, their biological activity still needs be explored via Pepsite2. Remembering that this tool ignores peptides with > 10 amino acid residues, 89 oilseed peptides and 16 milk protein peptides were finally investigated (**Supplementary Table 3S**). (In addition, 10 peptides were removed because their sequences were identical in the oilseed- and milk-derived systems). Studies investigating the binding models of ACE and DPP-IV inhibitors are available but the binding sites for different inhibitors are not always the same (**Table 3.8**). Nevertheless, the important amino acids in ACE binding are summarized as Glu162, Gln281, His353, Ala354, His383, Glu384, His387, Glu411, Lys511, Phe512, His513, Val518, Tyr520 and

Tyr523 in the study by Ngoh and Gan (2017) whilst the predominant amino acids of DPP-IV binding have been described as Arg125, Glu205, Glu206, Val207, Ser209, Phe357, Arg358, Tyr547, Gly(Trp)629, Ser630, Tyr631, Gly633, Val656, Trp659, Tyr662, Tyr666, Asp708, Asn710, Val711and His740. These are slightly different from the ones summarized by Mudgil et al. (2018), who do not mention Arg356, Glu403, Val404 and Tyr585 and who modelled DPP-IV (PDB code: 4A5S) forming complexes with the inhibitor, whereas the one used in our study is human DPP-IV (PDB Code: 1NU6).

Table 3.7 Average scores of fragments released from oilseeds and bovine proteins (**PeptideRanker**)

Protein sequences	Average score		
	Subtilisin	Pepsin (pH 1.3)	Pepsin (pH > 2)
Flaxseed - Colinin	0.0936	0.0498	0.2678
Rapeseed - Napin	0.1628	0.0731	0.2476
Rapeseed - Cruciferin	0.1095	0.0622	0.2553
Sunflower - 2S seed storage protein	0.0520	0.0393	0.2053
Sunflower - 11S globulin seed storage protein G3	0.0946	0.0582	0.2538
Sesame - 2S seed storage protein	0.0771	0.0391	0.2673
Sesame - 11S globulin seed storage protein	0.0902	0.0480	0.2381
Soybean - Glycinin	0.0981	0.0515	0.2299
Soybean -β-conglycinin, α'-chain	0.0940	0.0650	0.2103
Soybean -β-conglycinin, α-chain	0.1015	0.0748	0.2137
Bovine -β-lactoglobulin	0.1068	0.0699	0.2406
Bovine -β-casein	0.1236	0.0701	0.2260
Bovine -κ-casein	0.0975	0.0581	0.1972

Table 3.9 enumerates the 105 peptides binding to the amino acids presented in **Table 3.8**. Gln281, His353, Lys511, His513, Tyr520 and Tyr523 are major binding sites of these peptides predicting high ACE inhibiting activity, whilst only W629 is frequently bound by these peptides to exert the DPP-IV inhibition. PF, TF, VF, SF, PSF, MKF, KF, IPF, IF, HF, CF, NF and PM

are considered as promising ACE inhibitory peptides, while MW, AW, WF, AF, MKF, KF, QCAW, HWL, WA, IPF, MAPF, WM, IF, ACQCL, PQNIPPL and VYPF could be considered as promising DPP-IV inhibitory peptides, due to all their predicted binding sites being at the critical amino acid in ACE and DPP-IV. In addition, ACF shows the highest p-value (0.05557) for predicting ACE binding sites. Regarding DPP-IV, the highest p-value is 0.06617, coming from the dipeptide IF. This means all the candidates could be considered to interact with both ACE and DPP-IV. Comparison of the sequences recorded in the BIOPEP databases revealed that out of these, 105 peptides are unrecorded in this database (**Supplementary Table 4S**). The toxicity of the peptides was analysed using ToxinPred as suggested by Gupta et al. (2013) but no toxic properties were predicted.

The stability of peptides in the gastrointestinal (GI) tract (gastric phase + intestinal phase) determines their availability and in vivo efficacy (You et al., 2010). In order to predict whether the GI tract could be a barrier for effectivity of oral administration of peptides, we used *in silico* GI digestion (mainly, pepsin (pH > 2), trypsin and chymotrypsin) applied to the peptides with high PeptideRanker scores. Out of the 105 peptides, only 21 were predicted as stable on exposure to these enzymes during digestion (PG, VCPF, PF, PL, VF, SF, PSF, SPF, CL, VPF, IPF, IF, CG, CY, SPM, CF, PF, CM, PM, VPPF and IPPL). In this case, the most promising peptides exerted a low oral bioavailability, which is similar to the finding of Udenigwe and Fogliano (2017) which is indicating that peptides may need to be protected by appropriate encapsulation techniques as recently suggested (Mohan et al., 2015).

Table 3.8 Summary of important active sites or binding sites in ACE and DPP-IV *(Homo sapiens*)

Important sites	References
Angiotensin-converting enzyme	
Binding sites of lisinopril in ACE: Glu384, Val518, Glu162, Lys511 and Tyr520	(Natesh et al., 2003)
	(Priyanto et al., 2015)
Active sites: Glu384, Ala354, Glu162, His353,	
Active sites of ACE binding by Lisinopril and Enalaprilat: Glu162, His353, Ala354, Glu384, His387, Glu411, His383, Tyr523, Tyr520, Lys511	(Wang et al., 2011)
Important binding sites of two natural ACE inhibitory peptides: Gln281, Tyr520, Lys511, Tyr523, His353, Ala354, His513, His353, Ala354, Phe512, Glu384	(Masuyer et al., 2012)
Dipeptidyl-peptidase IV	
Active sites: Ser630, Asp708, His740, Gly629, Gly633, Tyr631, Glu205 and Glu206	(Lambeir et al., 2003)

Important binding sites of commercial DPP-IV inhibitors: Ser630, Tyr666, Tyr547 (vildapliptin and saxagliptin); Tyr547 and Trp629 (alogliptin and linagliptin); Asn710 (sitagliptin and teneligliptin); Glu205 and Glu206 play a key role in DPP-IV inhibiting activities for all the DPP-IV inhibitor	(Nabeno et al., 2013)
Active sites: Val207, Ser209, Phe357, Arg358	
Binding sites of saxagliptin in DPP-IV: Val711, Val656, Tyr662, Tyr666, Trp659, Tyr547, Asn710, Glu205, Glu206, Tyr 547and Arg125	(Metzler et al., 2008)
DPP-IV inhibitors binding sites: Ser630, Glu205, Glu206, Arg125, Phe357, Tyr 547, Arg125, Trp629	(Berger et al., 2018)
Active sites: Ser630, His740, Asp708; Tyr547; Tyr666; Tyr662; Val711; Val656; Trp659; Arg125; Asn710; Glu205; Glu206 and Arg358	(Engel et al., 2003)

Table 3.9 The frequency of 105 peptides binding to the amino acids detained in **Table 3.8** (**Pepsite2**)

Binding sites in ACE	Number of peptides	Binding sites in DPP-IV	Number of peptides
E(Glu)162	0	R(Arg)125	0
Q(Gln)281	103	E(Glu)205	3
H(His)353	102	E(Glu)206	5
A(Ala)354	0	V(Val)207	0
H(His)383	57	S(Ser)209	0
E(Glu)384	36	F(Phe)357	12
H(His)387	27	R(Arg)358	0
E(Glu)411	54	Y(Tyr)547	33
K(Lys)511	86	W(Trp)629	93
F(Phe)512	0	S(Ser)630	47
H(His)513	102	Y(Tyr)631	9
V(Val)518	0	G(Gly)633	0
Y(Tyr)520	104	V(Val)656	2
Y(Tyr)523	102	W(Trp)659	0
		Y(Tyr)662	7
		Y(Tyr)666	25
		D(Asp)708	0
		N(Asn)710	2
		V(Val)711	2
		H(His)740	5

3.3.4 Limitations

The predicted generation of peptides *in silico* is based on the specificity of enzymes and the primary structure of precursor proteins. However, accessibility of individual peptide bonds through an enzyme will be influenced by different factors such as the tertiary structures of the protein and the presence of enzyme inhibitors will affect the hydrolysis capability of the enzyme (Nishinari et al., 2014; Agyei et al., 2018). In addition, frequency and potency indices of protein sequences are calculated basing on the current knowledge recorded in BIOPEP databases. Therefore, with more peptide sequences added to databases, changes in the frequency and potency indices will undoubtedly occur (BIOPEP database). Then, the bioinformatic tools involved in this study only consider the amino acid sequences of peptides to predict the toxicity (ToxinPred); to align the score (PeptideRanker) and to predict the binding site of ACE and DPP-IV (Pepsite 2). Furthermore, the binding of promising peptides at the pertinent site of the targeted enzyme is not necessarily correlated with their inhibiting activity, due to the fact that the interaction may not be associated with the targeted activity (Pepsite 2) (Li-Chan, 2015). Finally, extracted or commercial protein isolates contain different protein sequences, only considering that the storage proteins will also lead to a bias when targeted biological activities predicted.

3.4 Conclusion

Our results, based on selected amino acid sequences from different protein sources hydrolyzed *in silico* using subtilisin and pepsin, indicate that oilseed proteins may be good sources for bioactive peptides, in particular for ACE inhibitory peptides, compared to bovine milk proteins. Further studies are highly warranted to validate the predictions, in particular to confirm the presence and activity of peptides that are currently not described and to establish their overall relevance for enzyme inhibition and beneficial health properties *in vitro* and *in vivo*.

References

Agirbasli, Z. and Cavas, L. 2017. In silico evaluation of bioactive peptides from the green algae Caulerpa. *Journal of Applied Phycology.* **29**(3), pp.1635-1646.

Agyei, D., Tsopmo, A. and Udenigwe, C.C. 2018. Bioinformatics and peptidomics approaches to the discovery and analysis of food-derived bioactive peptides. *Analytical and bioanalytical chemistry.* pp.1-10.

Allen, R., Cohen, E., Haar, R.V., Adams, C., Ma, D., Nessler, C. and Thomas, T. 1987. Sequence and expression of a gene encoding an albumin storage protein in sunflower. *Molecular and General Genetics MGG.* **210**(2), pp.211-218.

Allen, R.D., Nessler, C.L. and Thomas, T.L. 1985. Developmental expression of sunflower 11S storage protein genes. *Plant molecular biology.* **5**(3), pp.165-173.

Bénéteau-Burnat, B. and Baudin, B. 1991. Angiotensin-converting enzyme: clinical applications and laboratory investigations on serum and other biological fluids. *Critical reviews in clinical laboratory sciences.* **28**(5-6), pp.337-356.

Berger, J.P., SinhaRoy, R., Pocai, A., Kelly, T.M., Scapin, G., Gao, Y.D., Pryor, K.A.D., Wu, J.K., Eiermann, G.J. and Xu, S.S. 2018. A comparative study of the binding properties, dipeptidyl peptidase‐4 (DPP‐4) inhibitory activity and glucose‐lowering efficacy of the DPP‐4 inhibitors alogliptin, linagliptin, saxagliptin, sitagliptin and vildagliptin in mice. *Endocrinology, Diabetes & Metabolism.* **1**(1), pe00002.

Carrasco-Castilla, J., Hernández-Álvarez, A.J., Jiménez-Martínez, C., Gutiérrez-López, G.F. and Dávila-Ortiz, G. 2012. Use of proteomics and peptidomics methods in food bioactive peptide science and engineering. *Food Engineering Reviews.* **4**(4), pp.224-243.

Chang, Y.-W. and Alli, I. 2012. In silico assessment: Suggested homology of chickpea (Cicer arietinum L.) legumin and prediction of ACE-inhibitory peptides from chickpea proteins using BLAST and BIOPEP analyses. *Food Research International.* **49**(1), pp.477-486.

Cho, S.-J., Juillerat, M.A. and Lee, C.-H. 2007. Cholesterol lowering mechanism of soybean protein hydrolysate. *Journal of agricultural and food chemistry.* **55**(26), pp.10599-10604.

Chung, M., Lei, B. and Li-Chan, E. 2005. Isolation and structural characterization of the major protein fraction from NorMan flaxseed (Linum usitatissimum L.). *Food chemistry.* **90**(1-2), pp.271-279.

Dalgleish, D.G. 2011. On the structural models of bovine casein micelles—review and possible improvements. *Soft matter.* **7**(6), pp.2265-2272.

Dupont, D. 2017. Peptidomic as a tool for assessing protein digestion. *Current Opinion in Food Science.* **16**, pp.53-58.

Engel, M., Hoffmann, T., Wagner, L., Wermann, M., Heiser, U., Kiefersauer, R., Huber, R., Bode, W., Demuth, H.-U. and Brandstetter, H. 2003. The crystal structure of dipeptidyl peptidase IV (CD26) reveals its functional regulation and enzymatic mechanism. *Proceedings of the National Academy of Sciences.* **100**(9), pp.5063-5068.

Ericson, M., Rödin, J., Lenman, M., Glimelius, K., Josefsson, L.-G. and Rask, L. 1986. Structure of the rapeseed 1.7 S storage protein, napin, and its precursor. *Journal of Biological Chemistry.* **261**(31), pp.14576-14581.

Fujiwara, T., Hirai, M.Y., Chino, M., Komeda, Y. and Naito, S. 1992. Effects of sulfur nutrition on expression of the soybean seed storage protein genes in transgenic petunia. *Plant Physiology.* **99**(1), pp.263-268.

Gangopadhyay, N., Wynne, K., O'Connor, P., Gallagher, E., Brunton, N.P., Rai, D.K. and Hayes, M. 2016. In silico and in vitro analyses of the angiotensin-I converting enzyme inhibitory activity of hydrolysates generated from crude barley (Hordeum vulgare) protein concentrates. *Food chemistry.* **203**, pp.367-374.

Gasteiger, E., Hoogland, C., Gattiker, A., Wilkins, M.R., Appel, R.D. and Bairoch, A. 2005. Protein identification and analysis tools on the ExPASy server. *The proteomics protocols handbook.* Springer, pp.571-607.

Guang, C. and Phillips, R.D. 2009. Plant food-derived angiotensin I converting enzyme inhibitory peptides. *Journal of Agricultural and Food Chemistry.* **57**(12), pp.5113-5120.

Gueguen, J., Bollecker, S., Schwenke, K.D. and Raab, B. 1990. Effect of succinylation on some physicochemical and functional properties of the 12S storage protein from rapeseed (Brassica napus L.). *Journal of agricultural and food chemistry.* **38**(1), pp.61-69.

Gupta, S., Kapoor, P., Chaudhary, K., Gautam, A., Kumar, R., Raghava, G.P. and Consortium, O.S.D.D. 2013. In silico approach for predicting toxicity of peptides and proteins. *PLoS One.* **8**(9), pe73957.

Hansson, L., Lindholm, L.H., Niskanen, L., Lanke, J., Hedner, T., Niklason, A., Luomanmäki, K., Dahlöf, B., de Faire, U. and Mörlin, C. 1999. Effect of angiotensin-converting-enzyme inhibition compared with conventional therapy on cardiovascular morbidity and mortality in hypertension: the Captopril Prevention Project (CAPPP) randomised trial. *The Lancet.* **353**(9153), pp.611-616.

Hartmann, R. and Meisel, H. 2007. Food-derived peptides with biological activity: from research to food applications. *Current opinion in biotechnology.* **18**(2), pp.163-169.

He, R., Girgih, A.T., Malomo, S.A., Ju, X. and Aluko, R.E. 2013. Antioxidant activities of enzymatic rapeseed protein hydrolysates and the membrane ultrafiltration fractions. *Journal of Functional Foods.* **5**(1), pp.219-227.

Iwaniak, A. and Dziuba, J. 2009. Animal and plant proteins as precursors of peptides with ACE Inhibitory Activity—An in silico strategy of protein evaluation. *Food Technology and Biotechnology.* **31**(4), p441.

Jin, Y., Yan, J., Yu, Y. and Qi, Y. 2015. Screening and identification of DPP-IV inhibitory peptides from deer skin hydrolysates by an integrated approach of LC–MS/MS and in silico analysis. *Journal of Functional Foods.* **18**, pp.344-357.

Kieffer, T.J., McIntosh, C. and Pederson, R.A. 1995. Degradation of glucose-dependent insulinotropic polypeptide and truncated glucagon-like peptide 1 in vitro and in vivo by dipeptidyl peptidase IV. *Endocrinology.* **136**(8), pp.3585-3596.

Korhonen, H. and Pihlanto, A. 2003. Food-derived bioactive peptides-opportunities for designing future foods. *Current pharmaceutical design.* **9**(16), pp.1297-1308.

Lacroix, I.M. and Li-Chan, E.C. 2012. Evaluation of the potential of dietary proteins as precursors of dipeptidyl peptidase (DPP)-IV inhibitors by an in silico approach. *Journal of Functional Foods.* **4**(2), pp.403-422.

Lafarga, T., O'Connor, P. and Hayes, M. 2014. Identification of novel dipeptidyl peptidase-IV and angiotensin-I-converting enzyme inhibitory peptides from meat proteins using in silico analysis. *Peptides.* **59**, pp.53-62.

Lambeir, A.-M., Durinx, C., Scharpé, S. and De Meester, I. 2003. Dipeptidyl-peptidase IV from bench to bedside: an update on structural properties, functions, and clinical aspects of the enzyme DPP IV. *Critical reviews in clinical laboratory sciences.* **40**(3), pp.209-294.

Li-Chan, E.C. 2015. Bioactive peptides and protein hydrolysates: research trends and challenges for application as nutraceuticals and functional food ingredients. *Current Opinion in Food Science.* **1**, pp.28-37.

Madureira, A.R., Pereira, C.I., Gomes, A.M., Pintado, M.E. and Malcata, F.X. 2007. Bovine whey proteins–overview on their main biological properties. *Food Research International.* **40**(10), pp.1197-1211.

Marczak, E.D., Usui, H., Fujita, H., Yang, Y., Yokoo, M., Lipkowski, A.W. and Yoshikawa, M. 2003. New antihypertensive peptides isolated from rapeseed. *Peptides.* **24**(6), pp.791-798.

Maruyama, S. and Suzuki, H. 1982. A peptide inhibitor of angiotensin I converting enzyme in the tryptic hydrolysate of casein. *Agricultural and Biological Chemistry.* **46**(5), pp.1393-1394.

Masuyer, G., Schwager, S.L., Sturrock, E.D., Isaac, R.E. and Acharya, K.R. 2012. Molecular recognition and regulation of human angiotensin-I converting enzyme (ACE) activity by natural inhibitory peptides. *Scientific reports.* **2**, p717.

Megías, C., Pedroche, J., Yust, M.M., Girón-Calle, J., Alaiz, M., Millán, F. and Vioque, J. 2008. Production of copper-chelating peptides after hydrolysis of sunflower proteins with pepsin and pancreatin. *LWT-Food Science and Technology.* **41**(10), pp.1973-1977.

Meinke, D., Chen, J. and Beachy, R. 1981. Expression of storage-protein genes during soybean seed development. *Planta.* **153**(2), pp.130-139.

Metzler, W.J., Yanchunas, J., Weigelt, C., Kish, K., Klei, H.E., Xie, D., Zhang, Y., Corbett, M., Tamura, J.K. and He, B. 2008. Involvement of DPP‐IV catalytic residues in enzyme‐saxagliptin complex formation. *Protein Science.* **17**(2), pp.240-250.

Minkiewicz, P., Dziuba, J., Iwaniak, A., Dziuba, M. and Darewicz, M. 2008. BIOPEP database and other programs for processing bioactive peptide sequences. *Journal of AOAC International.* **91**(4), pp.965-980.

Mohan, A., Rajendran, S.R., He, Q.S., Bazinet, L. and Udenigwe, C.C. 2015. Encapsulation of food protein hydrolysates and peptides: a review. *RSC Advances.* **5**(97), pp.79270-79278.

Mooney, C., Haslam, N.J., Pollastri, G. and Shields, D.C. 2012. Towards the improved discovery and design of functional peptides: common features of diverse classes permit generalized prediction of bioactivity. *PloS one.* **7**(10), pe45012.

Mudgil, P., Kamal, H., Yuen, G.C. and Maqsood, S. 2018. Characterization and identification of novel antidiabetic and anti-obesity peptides from camel milk protein hydrolysates. *Food chemistry.* **259**, pp.46-54.

Nabeno, M., Akahoshi, F., Kishida, H., Miyaguchi, I., Tanaka, Y., Ishii, S. and Kadowaki, T. 2013. A comparative study of the binding modes of recently launched dipeptidyl peptidase IV inhibitors in the active site. *Biochemical and biophysical research communications.* **434**(2), pp.191-196.

Natesh, R., Schwager, S.L., Sturrock, E.D. and Acharya, K.R. 2003. Crystal structure of the human angiotensin-converting enzyme–lisinopril complex. *Nature.* **421**(6922), p551.

Ngoh, Y.-Y. and Gan, C.-Y. 2017. Identification of Pinto bean peptides with inhibitory effects on α-amylase and angiotensin converting enzyme (ACE) activities using an integrated bioinformatics-assisted approach. *Food Chemistry.*

Nishinari, K., Fang, Y., Guo, S. and Phillips, G. 2014. Soy proteins: A review on composition, aggregation and emulsification. *Food hydrocolloids.* **39**, pp.301-318.

Nongonierma, A.B. and FitzGerald, R.J. 2013. Inhibition of dipeptidyl peptidase IV (DPP-IV) by proline containing casein-derived peptides. *Journal of functional foods.* **5**(4), pp.1909-1917.

Nongonierma, A.B. and FitzGerald, R.J. 2015. Investigation of the potential of hemp, pea, rice and soy protein hydrolysates as a source of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides. *Food Digestion: Research and Current Opinion.* **6**(1-3), pp.19-29.

Orruno, E. and Morgan, M. 2007. Purification and characterisation of the 7S globulin storage protein from sesame (Sesamum indicum L.). *Food Chemistry.* **100**(3), pp.926-934.

Papadopoulos, J.S. and Agarwala, R. 2007. COBALT: constraint-based alignment tool for multiple protein sequences. *Bioinformatics.* **23**(9), pp.1073-1079.

Patil, P., Mandal, S., Tomar, S.K. and Anand, S. 2015. Food protein-derived bioactive peptides in management of type 2 diabetes. *European journal of nutrition.* **54**(6), pp.863-880.

Pihlanto-Leppälä, A., Koskinen, P., Piilola, K., Tupasela, T. and KORHONEN, H. 2000. Angiotensin I-converting enzyme inhibitory properties of whey protein digests: concentration and characterization of active peptides. *Journal of Dairy Research.* **67**(1), pp.53-64.

Pooja, K., Rani, S. and Prakash, B. 2017. In silico approaches towards the exploration of rice bran proteins-derived angiotensin-I-converting enzyme inhibitory peptides. *International Journal of Food Properties.* pp.1-14.

Priyanto, A.D., Doerksen, R.J., Chang, C.-I., Sung, W.-C., Widjanarko, S.B., Kusnadi, J., Lin, Y.-C., Wang, T.-C. and Hsu, J.-L. 2015. Screening, discovery, and characterization of angiotensin-I converting enzyme inhibitory peptides derived from proteolytic hydrolysate of bitter melon seed proteins. *Journal of proteomics.* **128**, pp.424-435.

Sánchez-Rivera, L., Martínez-Maqueda, D., Cruz-Huerta, E., Miralles, B. and Recio, I. 2014. Peptidomics for discovery, bioavailability and monitoring of dairy bioactive peptides. *Food research international.* **63**, pp.170-181.

Silveira, S.T., Martínez-Maqueda, D., Recio, I. and Hernández-Ledesma, B. 2013. Dipeptidyl peptidase-IV inhibitory peptides generated by tryptic hydrolysis of a whey protein concentrate rich in β-lactoglobulin. *Food chemistry.* **141**(2), pp.1072-1077.

Siow, H.-L. and Gan, C.-Y. 2016. Extraction, identification, and structure–activity relationship of antioxidative and α-amylase inhibitory peptides from cumin seeds (Cuminum cyminum). *Journal of Functional Foods.* **22**, pp.1-12.

Tai, S.S., Wu, L.S., Chen, E.C. and Tzen, J.T. 1999. Molecular cloning of 11S globulin and 2S albumin, the two major seed storage proteins in sesame. *Journal of Agricultural and Food Chemistry.* **47**(12), pp.4932-4938.

Trabuco, L.G., Lise, S., Petsalaki, E. and Russell, R.B. 2012. PepSite: prediction of peptide-binding sites from protein surfaces. *Nucleic acids research.* **40**(W1), pp.W423-W427.

Truksa, M., MacKenzie, S.L. and Qiu, X. 2003. Molecular analysis of flax 2S storage protein conlinin and seed specific activity of its promoter. *Plant Physiology and Biochemistry.* **41**(2), pp.141-147.

Udenigwe, C.C. 2014. Bioinformatics approaches, prospects and challenges of food bioactive peptide research. *Trends in Food Science & Technology.* **36**(2), pp.137-143.

Udenigwe, C.C. 2016. Towards rice bran protein utilization: In silico insight on the role of oryzacystatins in biologically-active peptide production. *Food chemistry.* **191**, pp.135-138.

Udenigwe, C.C. and Aluko, R.E. 2012. Food protein‐derived bioactive peptides: production, processing, and potential health benefits. *Journal of Food Science.* **77**(1).

Udenigwe, C.C., Gong, M. and Wu, S. 2013. In silico analysis of the large and small subunits of cereal RuBisCO as precursors of cryptic bioactive peptides. *Process Biochemistry.* **48**(11), pp.1794-1799.

Udenigwe, C.C., Lu, Y.-L., Han, C.-H., Hou, W.-C. and Aluko, R.E. 2009. Flaxseed protein-derived peptide fractions: Antioxidant properties and inhibition of lipopolysaccharide-induced nitric oxide production in murine macrophages. *Food Chemistry.* **116**(1), pp.277-284.

Vukic, V.R., Vukic, D.V., Milanovic, S.D., Ilicic, M.D., Kanuric, K.G. and Johnson, M.S. 2017. In silico identification of milk antihypertensive di-and tripeptides involved in angiotensin I–converting enzyme inhibitory activity. *Nutrition Research.* **46**, pp.22-30.

Wang, X., Wu, S., Xu, D., Xie, D. and Guo, H. 2011. Inhibitor and substrate binding by angiotensin-converting enzyme: quantum mechanical/molecular mechanical molecular dynamics studies. *Journal of chemical information and modeling.* **51**(5), pp.1074-1082.

Chapter 4
Comparison of alcalase- and pepsin-treated oilseed protein hydrolysates – experimental validation of predicted antioxidant, antihypertensive and antidiabetic properties

Abstract

There is emerging evidence on the importance of food-derived bioactive peptides to promote human health. Compared with animal derived proteins, plant proteins, in particular, oilseed proteins, are considered as affordable and sustainable sources of bioactive peptides. Based on our previous bioinformatic analysis, five oilseed proteins (flaxseed, rapeseed, sunflower, sesame and soybean) were enzymatically hydrolysed using alcalase and pepsin (pH 1.3 and pH 2.1). Further, low molecular weight (M_w < 3 kDa) fractions were generated using ultrafiltration. The protein hydrolysates and their low M_w fractions were evaluated for their *in vitro* antioxidant, antihypertensive and antidiabetic capabilities, in comparison with two dairy proteins (whey and casein). Apart from dipeptidyl-peptidase IV inhibition, significantly stronger bioactivities were detected for the low M_w fractions. In partial agreement with *in silico* predictions, most oilseed hydrolysates exerted comparable angiotensin-converting enzyme inhibitory capability to dairy proteins, whilst whey protein was the most promising source of dipeptidyl-peptidase IV inhibitors. Apart from alcalase-treated soybean, dairy proteins were more efficient in releasing antioxidant peptides as compared to oilseed proteins. On the other hand, soybean protein hydrolysates showed the highest α-glucosidase inhibitory activity amongst all protein sources. Overall, there was limited correlation between *in silico* predictions and *in vitro* experimental results. Nevertheless, our results indicate that oilseed proteins have potential as bioactive peptide sources, and they might therefore be suitable replacers for dairy proteins as well as good sources for development of functional foods.

4.1 Introduction

A growing amount of research is focused on developing strategies to valorise food waste and exploit its potential usage for different purposes, including nutrition and health related applications. Proteins from defatted oilseed meal, remainders of oil pressing industries, are extracted from sources such as flaxseed, rapeseed, sunflower, sesame and soybean, and have shown to be promising sources of bioactive peptides with *in vitro* antioxidant (Alashi et al., 2014), antihypertensive (He, Rong et al., 2013), and antidiabetic (Nongonierma and FitzGerald, 2015) properties. Bioactive peptides, defined as peptide fragments of 2-20 amino acid residues in length, are considered to have potential to complement synthetic drugs and become part of new therapeutic strategies against diseases such as hypertension, type 2 diabetes and cardiovascular disease (Patil et al., 2015; Nasri, 2017). There may be some drawbacks, in that plant proteins can be difficult to digest and also some peptides may lead to off flavours, but these negative aspects may depend greatly on the pre-processing/treatment of the protein samples, leading to loss or degradation of unwanted components.

Peptides can be generated from the parental proteins via chemical and enzymatic hydrolysis. Enzymatic methods are preferentially adopted for releasing peptides from precursor proteins because of the specificity of proteases and the mild hydrolysis conditions required that are unlikely to reduce the protein quality and its biological value (Panyam and Kilara, 1996; Tavano, 2013). Nevertheless, protease hydrolysates will contain a wide range of peptides of varying molecular weight (M_w) and sequences (Sarmadi and Ismail, 2010).

Amongst bioactive peptides, one of the most frequently reported bioactivity refers to antioxidant properties, which can occur via a range of mechanisms, including chelating metal ions, scavenging free radicals and exhibiting reducing power (Elias et al., 2008; Zambrowicz et al., 2015). In addition, angiotensin converting enzyme (ACE) and dipeptidyl-peptidase IV (DPP-IV) inhibitory peptides are well documented as antihypertensive and antidiabetic agents, respectively (Megías et al., 2004; Nongonierma et al., 2017). ACE is a carboxypeptidase, which cleaves a dipeptide (HL) from the C-terminus of angiotensin I, generating angiotensin II, a vasoconstrictor. Meanwhile this enzyme inhibits and degrades bradykinin, a potent vasodilator (Bénéteau-Burnat and Baudin, 1991). DPP-IV is an enzyme widely recognized for its rapid degradation and cleaving of glucose dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1), both

incretin hormones being associated with insulin synthesis and secretion (Juillerat-Jeanneret, 2013). Furthermore, bioactive peptides have been reported to suppress postprandial blood glucose via inhibiting α-amylase and α-glucosidase enzymes and potentially attenuate glucose absorption (do Evangelho et al., 2017; Vilcacundo et al., 2017). Both carbohydrase enzymes are critically involved in hydrolysing dietary starch and other long-chain carbohydrates into absorbable monosaccharides (Tundis et al., 2010).

In order to rapidly screen the possible bioactive peptide profiles of proteins, *in silico* approaches have been developed to replace expensive and time-consuming laboratory analyses (FitzGerald et al., 2020). *In silico* analysis is also able to evaluate bioactive potency and thereby allowing comparison with other protein sources, such as bovine derived whey and casein, both of which are considered excellent sources of bioactive peptides (Abd El-Salam and El-Shibiny, 2017; Sultan et al., 2018). Although *in silico* prediction may be fast and cost-effective, it is limited by the lack of representative amino acid sequence information in some cases and missing experimental data on the specific enzyme inhibition of all possible peptides, plus a lack of knowledge of activity in real protein mixtures.

In our previous bioinformatics analysis, we screened peptide profiles of a range of proteins generated by *in silico* hydrolysis via subtilisin (alcalase) and pepsin. These results suggested that several oilseed proteins, including napin, cruciferin and glycinin, could generate promising bioactive peptides, especially with ACE inhibitory activity, as compared to dairy proteins (Han et al., 2019). Based on these *in silico* results, it was hypothesized that rapeseed and soybean protein hydrolysates could exert comparable biological activities to those derived from dairy proteins. A key aim of the present study was to validate the *in silico* predictions for ACE and DPP-IV inhibitory activities of oilseed protein hydrolysates through *in vitro* measurements, something that is rarely done. In particular, we evaluated the impact of low M_w (M_w < 3 kDa) peptide fractions *versus* the whole hydrolysates on bioactive properties. In addition, antioxidant and α-glucosidase inhibitory activities were investigated and compared with *in silico* predictions. It should be emphasized that it was important to use recognized methodology, i.e., we did not aim to develop new analytical tools, although we did optimize the protocols of the enzyme inhibitory assays (ACE, DPP-IV, alpha-amylase and alpha-glucosidases), as explained in what follows.

4.2 Materials and Methods

4.2.1 Materials and reagents

Pepsin from porcine gastric mucosa, alcalase from *Bacillus licheniformis*, 2,4,6-trinitrobenzenesulfonic acid solution (TNBS), L-leucine, (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium peroxodisulfate, sodium acetate-trihydrate, iron (III)-chloride-hexahydrate-solution, 2,4,6-tri(2-pyridyl)-s-triazine, human angiotensin converting enzyme expressed in HEK 293 cells, N-[3-(2-Furyl)acryloyl]-Phe-Gly-Gly (FAPGG), captopril, α-amylase from *Aspergillus Oryzae*, starch, α-glucosidase from *Saccharomyces cerevisiae*, *p*-nitrophenyl-α-D-glucopyranoside, human dipeptidyl peptidase IV expressed in baculovirus infected Sf9 cells and diprotin A were purchased from Sigma-Aldrich (Dorset, UK). Acarbose and Gly-Pro *p*-nitroanilide hydrochloride (Gly-Pro-pNA) were obtained from LKT labs (Minnesota, USA) and Cambridge Bioscience (Cambridge, UK), respectively. Oilseeds and dairy proteins were food grade commercial products purchased from local supermarkets (Leeds, UK).

4.2.2 Preparation of oilseed protein isolates

Defatted oilseed meals were prepared using Soxhlet extraction. Briefly, ground oilseeds were mixed with hexane 1:10 (w/v) and the defatted residues recovered after 24 h. Residual samples were resuspended in distilled water to a final concentration of 100 mg/mL and the pH adjusted to 9.5 with 1 M NaOH. After stirring for 4 h, the mixture was centrifuged at 3,500 x g for 20 min and the protein fraction was recovered from the supernatant after adjusting the pH to 4.5 with 1 M HCl. Following a second centrifugation, the protein fraction was lyophilized and the protein content of samples was determined using the Kjeldahl method. Different multiplicators were applied for individual proteins (**Appendix C – Table C1**).

4.2.3 Preparation of oilseed and dairy protein hydrolysates and fractions

For pepsin hydrolysis, each protein isolate was suspended in 0.034 M NaCl solution with a final protein concentration of 50 mg/mL, adjusted to pH 1.3 and 2.1, respectively. Pepsin was added to the protein solution in a 1:25

E/P ratio (dry weight of sample x protein content) (w/w) and incubated for 6 h at 37℃. For alcalase hydrolysis, protein samples were mixed with 0.1 M phosphate-buffered saline, pH 8, with an E/P ratio of 1.5:25 (w/w) and incubated 6 h at 60°C. All enzymatic hydrolysis samples were inactivated by placing them in boiling water for 10 min and centrifuged after which the pH of the supernatant was adjusted to 7.0. The degree of hydrolysis (DH) was determined via the trinitro-benzene sulfonic acid (TNBS) method using L-leucine as standard (Adler-Nissen, 1979). In addition, the predicted DH of protein samples was calculated based on the percentage of peptide bonds cut using *in silico* hydrolysis in the whole protein sequence. Low M_w fractions of protein hydrolysates were prepared through ultrafiltration using 3 kDa molecular weight cut-off membranes (Ultracel® regenerated cellulose, 76 mm diameter). Subsequently, samples were lyophilized and stored at -20°C for further measurements.

4.2.4 ACE and DPP-IV inhibitory activity assay

Inhibition of ACE activity was determined according to Vermeirssen et al. (2002) with minor modifications. Briefly, 20 µL of sample (1.5 mg/mL) was added to 100 µL of 1 mM FAPGG and preincubated for 10 min at 37°C. Both sample and substrate were dissolved in 50 mM Tris buffer (pH 8.3 with 0.3 M NaCl). The reaction was then initiated by adding 20 µL of ACE (50 mU/mL in Tris base buffer). The absorbance was recorded over 10 min at 340 nm in 30 sec intervals using a Tecan Spark10M plate reader. Captopril was used as positive control (IC_{50} = 2.9 ± 0.2 nM). The DPP-IV inhibition assay was performed according to Nongonierma and FitzGerald (2013) with modifications. Briefly, 25 µL of sample (prepared as 1.5 mg/mL), was added to 25 µL 10 mM Gly-Pro-pNA and pre-incubated for 10 min at 37°C. The reaction was initiated by adding 50 µL of DPP-IV enzyme (500 U/mL). The absorbance was measured at 405 nm over 30 min in 2 min intervals. Diprotin A was used as positive control (IC_{50} = 134.5 ± 3.6 µM). Inhibition of ACE and DPP-IV were expressed as per cent of non-inhibited control.

4.2.5 Antioxidant activity assays

Antioxidant activity of samples was determined using ABTS radical scavenging (TEAC) and Ferric-Reducing Power Assay (FRAP) assays. ABTS radical stock solution was prepared using 14 mM ABTS stock solution and 4.9 mM potassium peroxodisulfate and then incubated for 24 h in the dark.

The ABTS radical working solution was obtained through diluting the stock solution to reach an initial absorbance of 0.700 ± 0.020 at 734 nm. ABTS radical scavenging activity was tested though adding 10 µL of sample (1 mg/mL, dissolved in distilled water) to 300 µL ABTS radical working solution. The absorbance was taken after 6 min at 734 nm. The FRAP reagent was prepared by mixing 300 mM Acetate buffer (pH 3.6), 5 mM TPTZ solution and $FeCl_3$ in the ratio of 10:1:1 (v/v/v). Reducing capability was measured via mixing 10 µL sample (1 mg/mL, dissolved in 5% DMSO) with 300 µL FRAP reagent. The absorbance was recorded at 594 nm after incubation at 37°C for 15 min. Trolox was applied as standard compound in both assays, and antioxidant capability of samples was expressed as mM Trolox equivalents (TE)/g.

4.2.6 α-glucosidase inhibitory activity assay

Inhibitory properties of peptides towards α-glucosidase activity were determined in a microplate based assay according to Zhang et al. (2017) with some modifications. Briefly, 100 µL of protein hydrolysate (20 mg/mL) was added to 50 µL of 0.5 U/mL α-glucosidase solution (dissolved in 0.1 M PBS, pH 7.0), and pre-incubated at 37°C for 10 min. Then, 50 µL of 2.5 mM pNPG substrate was added to start the reaction. The absorbance was recorded at 405 nm over 10 min. Acarbose was used as positive control for α-glucosidase inhibitory (IC_{50} = 1.12 ± 0.03 mM) assay. Results are expressed in percent of non-inhibited control.

4.2.7 Statistical analysis

Detailed results for ACE, DPP-IV, α-glucosidase inhibitory capability together with antioxidant capability of all the protein hydrolysates and their low M_w fractions are presented in **Appendix C – Figure C1, C2 and C3**, respectively. These single values were then scaled relative to value of alcalase-hydrolysed whey protein. In addition, predicted values were averaged to give a single value for each protein source. Both, experimental results and predicted values are shown in **Figure 4.1 and 4.2**.

Statistical analysis was performed using the Student's t-test and two-way analysis of variance (ANOVA) with post hoc analysis (95% confidence interval), depending on the number of groups to compare. Significant differences were considered at p-value < 0.05. Experiments were conducted in triplicate and data were expressed as mean ± standard deviation

(SD). The IC_{50} value, defined as the compound concentration inhibiting 50% enzyme activity, was calculated using GraphPad Prism 7.0.

4.3 Results and discussion

4.3.1 Protein content and degree of hydrolysis of protein hydrolysates

The protein contents of oilseed and dairy protein concentrates and isolates varied, ranging from 46.4 ± 1.2 to 92.4 ± 1.0%, as determined via the Kjeldahl method (**Table 4.1**). To take this into account when subsequently assessing the activity of the peptide mixtures, the same concentration of protein was used from each source when conducting protease hydrolysis. The hydrolysis time was 6 h; increased DH is not expected if the catalysis time is extended further (do Evangelho et al., 2017; Kimatu et al., 2017).

As with our previous bioinformatics analysis, alcalase (pH 8) and pepsin (pH 1.3, pH 2.1) enzymes were utilized to release peptides from the protein samples (Han et al., 2019). Alcalase, a serine S8 endoproteinase family member, has a broad protease specificity with preference for large uncharged residues in P1 position (Adamson and Reynolds, 1996). Pepsin cleavage is more specific at pH 1.3 as compared to pH ≥ 2, with a preference to cleave hydrophobic and aromatic residues in the P1 and P1' position (Inouye and Fruton, 1967). Therefore, *in silico* prediction is for DH activity to be in the order of pepsin (pH 2.1) > alcalase > pepsin (pH 1.3), yet the TNBS results indicated the highest DH following alcalase hydrolysis amongst all proteins (**Table 4.1**). In addition, except for sesame and casein, increasing the pH from 1.3 to 2.1 significantly raised the DH ($p < 0.05$), however, the value of DH is generally lower than expected. The TNBS assay measures the N-terminal amino groups of proteins, leading to differences in DH calculated based on one or more given protein sequences (Adler-Nissen, 1979). On the other hand, proteolysis is not only ruled by enzyme specificity, but also amino acid profiles, tertiary structure of proteins, minor variations in hydrolysis conditions and sources of protease (Panyam and Kilara, 1996; Tavano, 2013). For example, the folded calyx structure of β-lactoglobulin was reported to be resistant to pepsin digestion, which might explain the much lower DH of whey protein (9.2 ± 1.5%) in comparison to calculated DH for β-lactoglobulin (17.5%) (Reddy et al., 1988).

Alcalase exerted the lowest effect with soybean protein (DH 12.4±0.7%) (**Table 4.1**). It appeared more efficient in liberating peptides from whey (DH 25.3 ± 1.4%) and casein (DH 27.0 ± 2.0%). Pepsin exerted the most efficient hydrolysis with flaxseed protein, especially at pH 2.1 (DH 16.5±0.4%, $p < 0.5$), while the DH of the other oilseed protein hydrolysates (ranging from 8.4 ± 0.9% to 13.3 ± 1.6%) was comparable with that of the dairy proteins (ranging from 7.6 ± 1.7% to 12.8 ± 2.0%). Alcalase and pepsin hydrolysis of whey protein resulted in DH 25.1 ± 1.4% and 10.5 ± 1.8%, which is within the range reported by Zheng et al. (2008) and Pena-Ramos and Xiong (2001), respectively. Overall, *in vitro* alcalase and pepsin (pH 1.3) treatments resulted in similar DH values to the *in silico* predictions, whilst DH of pepsin (pH 2.1) hydrolysis seemed highly over-estimated in the predictions.

Ultrafiltration methodology was sequentially applied to fractionate the hydrolysed samples to enrich smaller peptides in the M_w < 3 kDa fractions, which represent the major part of bioactive peptides. Both hydrolysates and < 3 kDa fraction were analysed for biological activities.

4.3.2 *In vitro* ACE and DPP-IV inhibitory activity of oilseed and dairy protein hydrolysates

4.3.2.1 ACE inhibitory activity

Figure 4.1 (A,B,C) presents a comparison of ACE-inhibitory activity of the seven protein hydrolysates and their low M_w fractions (M_w < 3 kDa) treated using the three enzyme conditions referred to above, i.e., alcalase, pepsin (pH 1.3) and pepsin (pH 2.1) at the same concentration (1.5 mg/mL). All values have been scaled relative to that of alcalase-treated whey protein (46.0 ± 4.5%), in order to ease comparison and accommodate the fact that absolute values are subject to variations in protein source and enzyme conditions, as discussed above. Relative values are also advantageous for easier identification of plant proteins that are 'superior' to dairy (or other animal-based) proteins. Among alcalase-treated protein samples, the highest ACE inhibitory activity was detected in casein protein hydrolysates (**Figure 4.1A**) followed by rapeseed, sunflower and whey protein, which all exerted similar inhibitory capabilities ($p > 0.05$). Soybean presented the second lowest activity, only slightly higher than flaxseed ($p < 0.05$). With regard to pepsin (pH 1.3) hydrolysis, flaxseed, sesame and sunflower exerted similarly high ACE inhibitory properties, stronger than the dairy proteins **(Figure 4.1B**). Apart from soybean, no significant change was found on increasing the pH from 1.3

to 2.1 with pepsin (**Figure 4.1C**). The inhibitory value of pepsin (pH 2.1)-treated soybean protein was lowered to a similar level for the casein, but much more promising than whey protein hydrolysates. Apart from pepsin (pH 1.3)-treated sesame and pepsin (pH 2.1)-treated sunflower protein, the activity of all protein hydrolysates was higher for the low M_w (< 3 kDa) fractions. Similar findings have been reported for tilapia (Raghavan and Kristinsson, 2009) and cowpea (Segura Campos et al., 2010) protein hydrolysates.

Unlike protein hydrolysates, the low M_w fraction of alcalase-treated flaxseed protein exerted a similar activity to rapeseed, sesame soybean and whey protein hydrolysates. Alcalase-treated casein protein still exerted the most promising inhibition amongst all the low M_w samples, but only slightly higher than that of sunflower protein hydrolysates. For pepsin (pH 1.3), casein produced similar activity to flaxseed and soybean protein samples, whilst low M_w peptides derived from oilseed exerted a comparable or even higher ACE inhibition compared to those from whey. After the pH of pepsin hydrolysis increased to 2.1, the activity of soybean decreased and consequently the inhibitory capability was significantly lower than casein protein samples, whilst low Mw peptides derived from rapeseed protein hydrolysates were now similar in activity with those from casein. Taken together, oilseed proteins should be recommended as potential sources of ACE inhibitors compared to dairy protein, especially whey.

In contradiction to our results, Michelke et al. (2017) demonstrated highest ACE inhibition for whey peptide mixtures, compared with soybean and rice. However, their results were based on tryptophan- and tyrosine-containing dipeptides only, whereas our samples contain a mixture of peptides, and were therefore not limited to dipeptide bioactivity. Interestingly, three ACE inhibitory dipeptides derived from whey protein, IW, WL and VY, were also found in soybean and rice, supporting that plant proteins could be comparable sources for ACE inhibitory peptides (Michelke et al., 2017).

Table 4.1 Protein contents of protein samples and degree of hydrolysis predicted *in silico* and measured *in vitro.*

Protein	Protein content (%)	Degree of hydrolysis (%)					
		Subtilisin (Alcalase)		Pepsin (pH 1.3)		Pepsin (pH >2)	
		Predicted	*In vitro*	Predicted	*In vitro*	Predicted	*In vitro*
Flaxseed	59.6 ± 3.9		23.6 ± 0.5^{F}		13.1 ± 0.8^{C}		16.5 ± 0.4^{E}
Colinin		22.0		8.9		73.8	
Rapeseed	70.3 ± 1.5		19.5 ± 0.4^{E}		9.8 ± 1.2^{B}		12.2 ± 1.2^{CD}
Napin		25.1		13.4		70.4	
Cruciferin		29.4		13.8		71.0	
Sunflower	46.4 ± 1.2		18.8 ± 0.9^{E}		9.33 ± 0.97^{B}		13.3 ± 1.6^{BD}
11S globulin seed storage protein		26.0		12.6		71.3	
2S seed storage protein		16.0		8.5		69.0	
Sesame	92.4 ± 1.0		21.7 ± 1.7^{D}		8.5 ± 0.3^{AB}		8.4 ± 0.8^{A}
2S seed storage protein		22.4		8.8		67.3	
11S globulin seed storage protein		27.5		11.6		67.5	
Soybean	75.8 ± 0.3		12.4 ± 0.7^{C}		8.7 ± 0.5^{AB}		10.7 ± 0.8^{C}
Glycinin		28.5		11.5		67.2	
Beta-conglycinin, alpha'-chain		26.0		12.7		70.1	
Beta-conglycinin, alpha-chain		26.8		13.9		70.2	
Whey	90.0 ± 3.4		25.3 ± 1.4^{B}		9.2 ± 1.5^{AB}		12.8 ± 2.0^{BD}
Beta-lactoglobulin		28.2		17.5		76.3	
Casein	75.8±0.3		27.8 ± 2.0^{A}		7.6 ± 1.7^{A}		8.2 ± 2.0^{A}
Beta-casein		33.6		16.1		65.5	
Kappa-casein		29.1		10.6		65.1	

Degree of hydrolysis is expressed as mean with SD of triplicate measurements. Different superscript letters within a column indicate significant differences

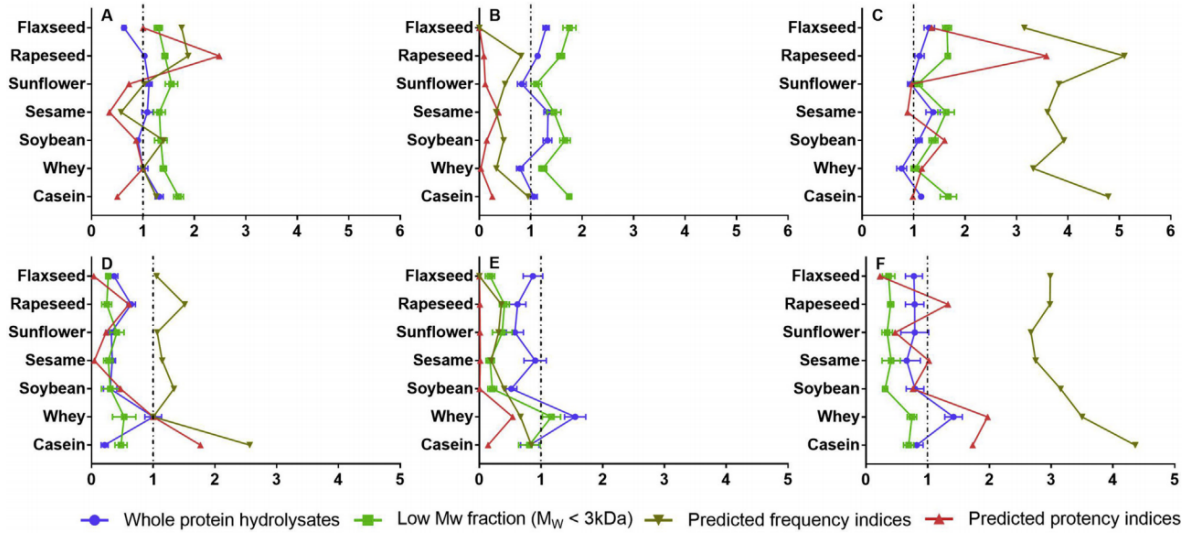

Figure 4.1 Angiotensin converting enzyme (A,B,C) and dipeptidyl peptidase-IV (D,E,F) inhibitory capability of protein hydrolysates and their low M_w fractions obtained using alcalase (A,D), pepsin (pH 1.3) (B,E) and pepsin (pH 2.1) (C,F) hydrolysis, respectively, determined using *in vitro* enzyme assays. Predicted frequency indices and potency indices (mean) are presented to compare with experimental data (mean ± SD).

4.3.2.2 DPP-IV inhibitory activity

Similar to ACE activity, DPP-IV inhibitory activity values, determined at 10 mg/mL, were scaled relative to that of alcalase-treated whey protein (51.3±6.4%) as summarized in **Figure 4.1 (D,E,F)**. **Figure 4.1D** shows that whey protein exhibited the strongest DPP-IV inhibition amongst all alcalase-treated protein samples, followed by rapeseed protein. The other five protein samples exerted similar DPP-IV inhibitory activity ($p > 0.05$) (**Figure 4.1E**). Similar to ACE inhibition, increasing the pH of pepsin hydrolysis from 1.3 to 2.1 did not increase DPP-IV inhibitory capability. Pepsin-treated whey exerted the most promising DPP-IV inhibitory properties. Pepsin (pH 1.3)-treated soybean showed lower inhibition compared to flaxseed, sesame and casein, but all five oilseed protein samples and casein demonstrate similar inhibitory capabilities after pepsin (pH 2.1) reaction (**Figure 4.1F**)

Turning to the low M_w fractions, apart from alcalase-treated casein, the M_w < 3 kDa peptides did not show higher DPP-IV inhibition than the whole hydrolysates, which is opposite to ACE inhibition. Lacroix and Li-Chan (2012) obtained similar results, reporting inhibition of four fractions to be 63% (<

1 kDa), 83% (1-3 kDa), 82% (3-10 kDa) and 78% (> 10 kDa), respectively. This is in contrast to the work of Konrad et al. (2014), who demonstrated greatest DPP-IV inhibitory properties in peptide fractions below 3 kDa, obtained from whey protein hydrolysates using serine protease, and emphasized the highest activity to be in the range of 3-10 kDa after further purification. Alcalase-treated whey protein hydrolysates showed relatively lower DPP-IV inhibition, similar to sunflower, sesame, soybean and casein. With regard to pepsin hydrolysates, whey exerted the highest inhibition at pH 1.3, showing similar inhibition to that for casein at pH 2.1. Flaxseed and sesame were significantly lower than rapeseed ($p < 0.05$), in contrast to the whole hydrolysates, although, all five protein samples showed similar activity with pepsin at pH 2.1 which is similar to the trends with the whole hydrolysates. Based on these results, dairy proteins seem to be a better source of DPP-IV, compared with oilseeds. In addition, unlike ACE inhibition, whey exerted the highest potential of releasing DPP-IV inhibitory peptides amongst all the proteins. The important active sites and binding sites in ACE and DPP-IV are not identical, which has an impact on the different requirements in terms of amino acid residues, peptide lengths and conformation, which can at least partially explain the different behaviour of whey protein released ACE and DPP-IV inhibitors (Lacroix et al., 2016).

4.3.3 Other biological activities

4.3.3.1 Antioxidant activity

Despite antioxidant properties of peptides having been widely reported, no specific assay has been developed that quantifies their overall antioxidative potential and summarizes the differing mechanisms of antioxidant and radical scavenging activity (Samaranayaka and Li-Chan, 2011). Therefore, in line with most other work, the present study utilized two established methods to determine antioxidant activity, the TEAC and FRAP assays, which evaluate radical scavenging and metal reducing capability, respectively.

Figure 4.2 (A,B,C) shows the results of TEAC in protein hydrolysates and their low Mw fractions. For ease of comparison, and in line with other assays, the value for alcalase-treated whey protein hydrolysate, with a TEAC value of 5.48 ± 0.12 mM TE/g, was used to normalize the results. Note that a lower value (1.16 ± 0.05mM TE/g) was reported by Mann et al. (2015), which might be the result of a lower DH (19.12%). As clearly seen in **Figure 4.2A**,

soybean hydrolysates were the strongest antioxidants amongst all alcalase-treated proteins, but still significantly weaker than whey and casein protein hydrolysates ($p < 0.05$). An increase of ABTS radical scavenging capability was detected amongst most proteins when the pH value increased to 2.1, soybean and sesame protein being the exceptions. In addition, **Figure 4.2 (A,B,C)** clearly showed that, in each case, the low M_w fractions showed stronger ABTS radical scavenging activity compared with the whole protein hydrolysates. Foh et al. (2010) and Phongthai et al. (2018) also claimed that low M_w fractions tended to show better capability of trapping the ABTS radical. Low M_w fractions of dairy protein were still the most promising sources of antioxidants, along with their corresponding whole protein hydrolysates. With regards to oilseed proteins, the only difference with the low M_w is that the alcalase-treated rapeseed exerted similar capability to soybean. In summary, in alignment with other literature, the TEAC results reflected the superior antioxidant activity of whey and casein compared to oilseed proteins.

The results of FRAP assay, a frequently applied method to determine antioxidant capability based on electron transfer mechanisms, are displayed in **Figure 4.2 (D,E,F).** Alcalase-treated whey protein only exerted 0.53 ± 0.03 mM TE/g antioxidant capability (used to scale all the other values, as previously), only 9.6% of the value measured via the TEAC assay. Overall, the TEAC and FRAP results seemed weakly correlated ($r^2 = 0.4436$), as also shown elsewhere (Dong et al., 2013; Choonpicharn et al., 2016). The reducing power of soybean was the highest amongst all proteins treated with alcalase, while casein exerted the second highest antioxidant activity ($p < 0.05$) **(Figure 4.2D**). In addition, whey protein also presented mild antioxidant capability after alcalase treatment, stronger than flaxseed and sesame protein hydrolysates. With regards to pepsin (pH 1.3), rapeseed protein hydrolysates exerted comparable antioxidant capability as the whey and soybean protein samples, being more promising than casein and the other oilseed samples. The only decrease of reducing power after raising the pH of pepsin hydrolysis from pH 1.3 to 2.1 was found for the rapeseed and sesame protein ($p < 0.05$). Both dairy proteins exerted stronger reducing power than oilseeds after pepsin (pH 2.1) hydrolysis. Meanwhile, rapeseed, together with soybean samples, showed the strongest antioxidant activity amongst oilseed proteins.

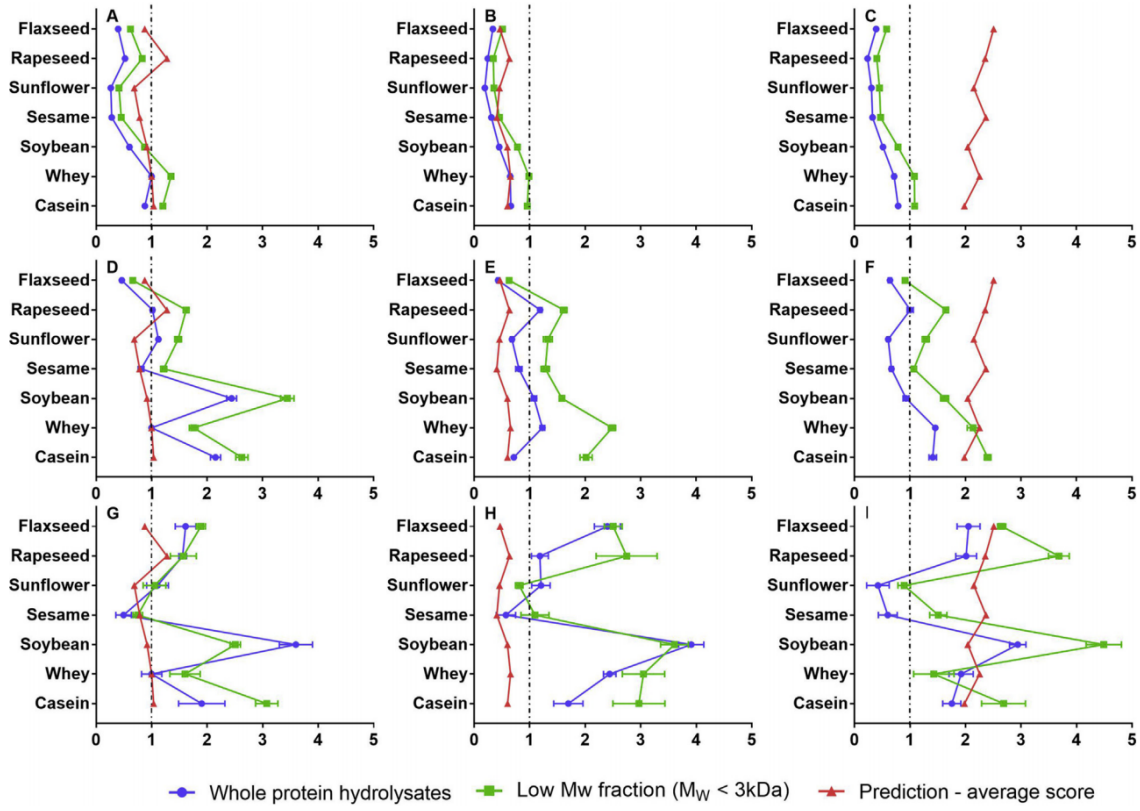

Figure 4.2 Antioxidant properties determined using TEAC (A,B,C) and FRAP (D,E,F) assays in protein hydrolysates and their low Mw fractions obtained using alcalase (A,D,G), pepsin (pH 1.3) (B,E,H) and pepsin (pH 2.1) (C,F,I) hydrolysis, respectively, together with α-glucosidase inhibitory properties (G,H,I) measured using *in vitro* enzyme assay. *In silico* prediction (aligned using PeptideRanker) (mean) was also presented in order to allow comparison with *in vitro* data (mean ± SD).

Comparing the behaviour of whole protein hydrolysates as described above with the low M_w fractions, similarly to the TEAC assay, all M_w < 3 kDa fractions exerted stronger reducing power. In contrast, Arise et al. (2016) reported that only fractions with Mw 5-10 kDa from Bambara groundnut protein hydrolysates had promising reducing power, compared to fractions with M_w < 5 kDa, whereas, He, R. et al. (2013) showed only fractions with M_w < 1 kDa exerted measurable reducing power from rapeseed protein hydrolysates. Ajibola et al. (2011) suggested only fractions with lower M_w from African yam bean seed protein hydrolysates were directly linked to stronger reducing activity. As with the whole protein hydrolysate, M_w < 3 kDa soybean fractions from alcalase-treatment exerted the highest reducing power capability amongst all the protein hydrolysates, 31% and 96% higher than the values for casein and whey protein, respectively. On the other hand, both

dairy proteins exerted stronger reducing power capability than soybean and the other oilseed proteins after pepsin (pH 1.3) hydrolysis. Reducing power decreased in sesame and whey protein samples after adjusting pepsin pH to 2.1. The differences amongst pepsin (pH 2.1)-treated low M_w fractions were similar with the whole protein hydrolysates. Overall, alcalase-treated soybean protein hydrolysates should be considered as good potential sources of antioxidants, compared with dairy proteins. In addition, whole pepsin (pH 1.3)-treated rapeseed hydrolysates presented noticeably high antioxidant capability, whilst, its low M_w fraction presented weak reducing power compared to dairy proteins.

4.3.3.2 α-glucosidase inhibitory activity

Inhibition of α-amylase and α-glucosidase activities are considered as antidiabetic properties. Our samples displayed only very low α-amylase inhibitory activity, in protein hydrolysates as well as their fractions up to 100 mg/mL (raw data not shown). Admassu et al. (2018) found that pepsin-treated red seaweed protein hydrolysate exerted 50.3% α-amylase inhibitory activity (1.86 mg/mL) and Ngoh and Gan (2016) reported fractions with Mw < 3 kDa from protamex treated pinto bean tended to reduce 62.1% of α-amylase activities.

Further investigation was carried out to compare the potential of oilseed and dairy protein releasing α-glucosidase inhibitory peptides at a concentration of 20 mg/mL. **Figure 4.2 (G,H,I)** illustrated the α-glucosidase inhibitory capability relative to alcalase-treated whey protein hydrolysates (15.2 ± 2.8%) at 20 mg/mL. The highest α-glucoside inhibition was found in soybean protein hydrolysates for all three enzyme treatments. Alcalase-treated flaxseed and rapeseed proteins exerted similar α-glucosidase inhibitory capability, compared to casein, but stronger than for the whey protein samples. Using pepsin at pH 1.3, whey protein gave the second strongest inhibition alongside the flaxseed protein hydrolysates. A significant increase of inhibition was detected in rapeseed after raising the pH from 1.3 to 2.1 and consequently it then had similar inhibitory capability to the flaxseed and dairy protein samples. In addition, the sunflower and whey protein hydrolysates showed a slight decrease in inhibitory capability.

After ultrafiltration, the only decrease of inhibitory capability for the low M_w fractions was found in alcalase-treated soybean protein, which was then weaker than for casein. The low M_w fractions of soybean protein hydrolysates

still exerted a noticeable inhibitory capability, the second strongest inhibitor. With regard to pepsin (pH 1.2)-treated hydrolysates, no significant difference was detected in the soybean sample before and after ultrafiltration, but it was still considered as a promising α-glucosidase inhibitor, similar to the whey protein samples. Uraipong and Zhao (2016) reported low M_w (< 3 kDa) fractions from rice bran protein hydrolysates exerted promising α-glucosidase inhibition, while Awosika and Aluko (2019) recommended that high M_w (M_w 3 to 5 kDa and 5 to10 kDa) fractions of yellow field pea protein hydrolysates could exhibit high α-glucosidase inhibition.

A similar tendency of α-glucosidase inhibition was found in the low M_w fractions, compared to the whole protein hydrolysates, apart from soybean and rapeseed. Low M_w fractions of alcalase-treated soybean exerted weaker inhibition than casein samples. Meanwhile, rapeseed samples identified as the second promising inhibitor amongst pepsin-treated protein samples. Taken together, soybean protein could therefore be considered as a promising source of α-glucosidase inhibitors, whilst dairy proteins, together with flaxseed and rapeseed, could also regarded as alternative sources, especially their low M_w (< 3 kDa) fractions.

4.3.4 Comparison with *in silico* predictions

A positive correlation between *in silico* prediction and *in vitro* analysis has been reported in several studies and consequently *in silico* has been suggested as a novel and fast screening tool to predict the potential of a protein as a source of targeted bioactive peptides, after hydrolysis by proteases (Gangopadhyay et al., 2016; Hsieh et al., 2016; Wang et al., 2017). However, our work suggested that, frequency indices, potency indices and average scores of fragments of protein only partly agreed with *in vitro* experiments. In particular, the DH, ACE inhibitory capability, DPP-IV inhibitory capability and antioxidant of pepsin (pH 2.1)-hydrolysed proteins seem to be over-evaluated by the *in silico* analysis. These disagreements are probably mostly due to incomplete *in vitro* protein hydrolysis and poor representation of the complete range of polypeptides presented in the various sources.

The potential great advantage of *in silico* methods is that they provide a rapid and affordable strategy for predicting and investigating the peptide profiles in proteins. In this approach, peptides released from the precursor proteins are more idealistic, since breakdown of peptide bonds is assumed to occur at very specific cutting sites of the polypeptide chain. However, in real

hydrolysis a range of factors including: solution conditions, characteristics of enzyme(s) and substrate(s), protease bio-accessibility (surface activity), presence of protease inhibitors, interactions with other compounds present in the complex food matrix, among others, could lead to incomplete hydrolysis (Amit et al., 2018). therefore, the predicted DH of the protein sequences were higher than those measured via the *in vitro* TNBS assay, with one exception. This outlier was for flaxseed protein (alcalase and pepsin pH 1.3 treated). Possibly this is explained by the lack of fully sequenced proteins from this source, such as linin - the major storage protein (58–66%) and not available in protein databases, and conlinin 2S (20–42%) the protein used for the *in silico* analysis. In addition, this protein may be more sensitive to protein hydrolysis, thus improving the overall DH obtained experimentally (Rabetafika et al., 2011). Of course using the same protease conditions *in vitro* with different proteins from the same plant source can produce different DH, affecting the peptidic profiles of protein hydrolysates (Cheison et al., 2010). Thus some predicted peptides with bioactive properties may not be obtained via *in vitro* hydrolysis due to the disagreement of predicted DH (Chatterjee et al., 2015). The DH of pepsin (pH 2.1) hydrolysed proteins is significantly lower than those predicted by *in silico* analysis, which undoubtedly is the main explanation for the over-estimation of their potential ACE inhibitory capability, DPP-IV inhibitory capability and antioxidant capacity.

Additionally, only a few representative protein sequences were selected for *in silico* analysis, according to their presence in the intact protein sources (Cheung et al., 2009; Gangopadhyay et al., 2016). The plant storage proteins that have their sequences recorded in Uniport database were chosen because these proteins represent a very large proportion of the edible proteins consumed (Shewry et al., 1995; Shewry and Halford, 2002). However, there are other proteins present in these sources that might also release peptides with significant bioactivity.

Also, an absence of standard protocols for enzyme and substrate (protein) preparations, protein hydrolysis and bioassays of bioactive peptides may also complicate the evaluation of the relationship between *in silico* prediction and *in vitro* experiments (Nongonierma and FitzGerald, 2017). Despite this limited correlation, soybean protein was confirmed as a good source for bioactive peptides, especially ACE and α-glucosidase inhibitory peptides. Rapeseed protein is also a good source of α-glucosidase inhibitors, and a notable oilseed protein for releasing antioxidant peptides. However, the moderate levels of ACE inhibition measured experimentally for rapeseed

peptides disagreed significantly with the highest predicted frequency and potency indices amongst oilseeds and dairy proteins. This disagreement could be due to the potential interaction of other compounds present in the rapeseed protein hydrolysates (phenolics, carbohydrates, phytates and glucosinolates) that might be interacting or competing with the ACE active site or forming complexes with peptides, thus reducing the ACE inhibition compared to the predicted potency indices (Mansour et al., 1993; Ruan et al., 2021)

Overall, the protein sources with a higher value of frequency index, potency index and/or average scores were supposed to be more likely to release peptides with comparable or more promising bioactive capabilities, in comparison with other sources. The findings are in accordance with the work of Hsieh et al. (2016), who observed a positive correlation between *in silico* and *in vitro* analysis based on this tendency. However, after classifying oilseed and dairy protein sources according to their strength of bioactive capabilities in a descending order, a limited correlation between *in silico* prediction and *in vitro* experiments was detected.

In silico tools are, of course, solely based on the protein sequences available in the databases used. They are therefore most reliable and helpful for screening the properties of pure protein samples. The complex structure of biomacromolecules, the interactions between them and with other relevant food components (such as polyphenols) and the food matrix in general, plus the fact that peptides might be produced that are highly bioactive but as yet are not recognized as such, will clearly have a negative impact on the accuracy of *in silico* predictions. Finally, tools such as PeptideRanker are designed for predicting the potential of a peptide to being bioactive, but this is not limited to any specific biological activity, plus these predictions are based purely on structural chemical features (Mooney et al., 2012). It is possible that the high scoring fragments may play roles in biological activities other than those under scrutiny here. In consequence, complete agreement between any experimental assessment and *in silico* analysis is unlikely, unless all the protein sequences are available and their proportions in a protein isolate/concentrate have been clearly identified beforehand, together with the intrinsic and extrinsic factors mentioned above being taken into account.

4.4 Conclusion

In the present study, oilseed and dairy proteins have been demonstrated as good sources of bioactive peptides. Dairy proteins are more promising in releasing antioxidant and DPP-IV inhibitory peptides, while oilseed proteins could be considered as comparable sources of ACE and α-glucosidase inhibitory peptides, especially soybean. Apart from DPP-IV inhibition, ultrafiltration is an approach to enrich targeted bioactive peptides. *In silico* analysis predicted rapeseed and soybean as comparable sources to dairy protein and this was partly born out in the *in vitro* experimental results. However, the relative bioactive capability of oilseeds and dairy proteins predicted by *in silico* and *in vitro* analysis largely disagreed. This disagreement may be largely due to incomplete representation of the full range of protein sequences in the protein isolates/concentrates and/or incomplete enzyme hydrolysis. Nevertheless, this current study provides direct *in vitro* evidence to support the view of replacing dairy proteins with affordable and sustainable oilseed proteins as a source of functional foods, without any apparent drawbacks. Future studies should address the corroboration of the *in vitro* release of peptides and their bioactive properties predicted via *in silico* analysis. In addition, it will be necessary to compare *in vitro* with *in vivo* digestion studies, plus acute or chronic human studies to confirm the predicted health benefits of the peptides released, and its potential to reach the selected target. In addition, the antagonistic and/or synergistic role of polyphenols on the bioactivity of these peptides also needs clarifying.

References

Abd El-Salam, M.H. and El-Shibiny, S. 2017. Preparation, properties, and uses of enzymatic milk protein hydrolysates. *Crit Rev Food Sci Nutr.* **57**(6), pp.1119-1132.

Adamson, N.J. and Reynolds, E.C. 1996. Characterization of casein phosphopeptides prepared using alcalase: Determination of enzyme specificity. *Enzyme and microbial technology.* **19**(3), pp.202-207.

Adler-Nissen, J. 1979. Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *J Agric Food Chem.* **27**(6), pp.1256-1262.

Admassu, H., Gasmalla, M.A., Yang, R. and Zhao, W. 2018. Identification of bioactive peptides with α-amylase inhibitory potential from enzymatic protein hydrolysates of red seaweed (Porphyra spp). *Journal of agricultural and food chemistry.* **66**(19), pp.4872-4882.

Ajibola, C.F., Fashakin, J.B., Fagbemi, T.N. and Aluko, R.E. 2011. Effect of peptide size on antioxidant properties of African yam bean seed (Sphenostylis stenocarpa) protein hydrolysate fractions. *Int J Mol Sci.* **12**(10), pp.6685-6702.

Alashi, A.M., Blanchard, C.L., Mailer, R.J., Agboola, S.O., Mawson, A.J., He, R., Girgih, A. and Aluko, R.E. 2014. Antioxidant properties of Australian canola meal protein hydrolysates. *Food Chem.* **146**, pp.500-506.

Amit, K., Nakachew, M., Yilkal, B. and Mukesh, Y. 2018. A review of factors affecting enzymatic hydrolysis of pretreated lignocellulosic biomass. *Research Journal of Chemistry and Environment.* **22**(7), pp.62-67.

Arise, A.K., Alashi, A.M., Nwachukwu, I.D., Ijabadeniyi, O.A., Aluko, R.E. and Amonsou, E.O. 2016. Antioxidant activities of bambara groundnut (Vigna subterranea) protein hydrolysates and their membrane ultrafiltration fractions. *Food & function.* **7**(5), pp.2431-2437.

Awosika, T.O. and Aluko, R.E. 2019. Inhibition of the in vitro activities of α - amylase, α - glucosidase and pancreatic lipase by yellow field pea (Pisum sativum L.) protein hydrolysates. *International Journal of Food Science & Technology.* **54**(6), pp.2021-2034.

Bénéteau-Burnat, B. and Baudin, B. 1991. Angiotensin-converting enzyme: clinical applications and laboratory investigations on serum and other biological fluids. *Critical reviews in clinical laboratory sciences.* **28**(5-6), pp.337-356.

Chatterjee, A., Kanawjia, S., Khetra, Y. and Saini, P. 2015. Discordance between in silico & in vitro analyses of ACE inhibitory & antioxidative peptides from mixed milk tryptic whey protein hydrolysate. *Journal of food science and technology.* **52**(9), pp.5621-5630.

Cheison, S.C., Schmitt, M., Leeb, E., Letzel, T. and Kulozik, U. 2010. Influence of temperature and degree of hydrolysis on the peptide composition of trypsin hydrolysates of β-lactoglobulin: Analysis by LC–ESI-TOF/MS. *Food chemistry.* **121**(2), pp.457-467.

Cheung, I.W., Nakayama, S., Hsu, M.N., Samaranayaka, A.G. and Li-Chan, E.C. 2009. Angiotensin-I converting enzyme inhibitory activity of hydrolysates from oat (Avena sativa) proteins by in silico and in vitro analyses. *Journal of agricultural and food chemistry.* **57**(19), pp.9234-9242.

Choonpicharn, S., Tateing, S., Jaturasitha, S., Rakariyatham, N., Suree, N. and Niamsup, H. 2016. Identification of bioactive peptide from Oreochromis niloticus skin gelatin. *J Food Sci Technol.* **53**(2), pp.1222-1229.

do Evangelho, J.A., Vanier, N.L., Pinto, V.Z., De Berrios, J.J., Dias, A.R.G. and da Rosa Zavareze, E. 2017. Black bean (Phaseolus vulgaris L.) protein hydrolysates: Physicochemical and functional properties. *Food chemistry.* **214**, pp.460-467.

Dong, S.-Y., Zhao, Y.-H., Xu, D.-X., Liu, Z.-Y. and Zeng, M.-Y. 2013. Assessing the antioxidant activity of the ultrafiltration fractions from

silver carp protein hydrolysate by different antioxidant methods. *Journal of aquatic food product technology.* **22**(6), pp.573-583.

Elias, R.J., Kellerby, S.S. and Decker, E.A. 2008. Antioxidant activity of proteins and peptides. *Crit Rev Food Sci Nutr.* **48**(5), pp.430-441.

FitzGerald, R.J., Cermeño, M., Khalesi, M., Kleekayai, T. and Amigo-Benavent, M. 2020. Application of in silico approaches for the generation of milk protein-derived bioactive peptides. *Journal of Functional Foods.* **64**, p103636.

Foh, M.B.K., Qixing, J., Amadou, I. and Xia, W. 2010. Influence of ultrafiltration on antioxidant activity of tilapia (Oreochromis niloticus) protein hydrolysate. *Advance Journal of Food Science and Technology.* **2**(5), pp.227-235.

Gangopadhyay, N., Wynne, K., O'Connor, P., Gallagher, E., Brunton, N.P., Rai, D.K. and Hayes, M. 2016. In silico and in vitro analyses of the angiotensin-I converting enzyme inhibitory activity of hydrolysates generated from crude barley (Hordeum vulgare) protein concentrates. *Food Chem.* **203**, pp.367-374.

Han, R., Maycock, J., Murray, B.S. and Boesch, C. 2019. Identification of angiotensin converting enzyme and dipeptidyl peptidase-IV inhibitory peptides derived from oilseed proteins using two integrated bioinformatic approaches. *Food Research International.* **115**, pp.283-291.

He, R., Alashi, A., Malomo, S.A., Girgih, A.T., Chao, D., Ju, X. and Aluko, R.E. 2013. Antihypertensive and free radical scavenging properties of enzymatic rapeseed protein hydrolysates. *Food Chem.* **141**(1), pp.153-159.

He, R., Malomo, S.A., Alashi, A., Girgih, A.T., Ju, X. and Aluko, R.E. 2013. Purification and hypotensive activity of rapeseed protein-derived renin and angiotensin converting enzyme inhibitory peptides. *Journal of Functional Foods.* **5**(2), pp.781-789.

Hsieh, C.H., Wang, T.Y., Hung, C.C., Jao, C.L., Hsieh, Y.L., Wu, S.X. and Hsu, K.C. 2016. In silico, in vitro and in vivo analyses of dipeptidyl peptidase IV inhibitory activity and the antidiabetic effect of sodium caseinate hydrolysate. *Food Funct.* **7**(2), pp.1122-1128.

Inouye, K. and Fruton, J.S. 1967. Studies on the specificity of pepsin. *Biochemistry.* **6**(6), pp.1765-1777.

Juillerat-Jeanneret, L. 2013. Dipeptidyl peptidase IV and its inhibitors: therapeutics for type 2 diabetes and what else? *Journal of medicinal chemistry.* **57**(6), pp.2197-2212.

Kimatu, B.M., Zhao, L., Biao, Y., Ma, G., Yang, W., Pei, F. and Hu, Q. 2017. Antioxidant potential of edible mushroom (Agaricus bisporus) protein hydrolysates and their ultrafiltration fractions. *Food chemistry.* **230**, pp.58-67.

Konrad, B., Anna, D., Marek, S., Marta, P., Aleksandra, Z. and Józefa, C. 2014. The evaluation of dipeptidyl peptidase (DPP)-IV, α-glucosidase

and angiotensin converting enzyme (ACE) inhibitory activities of whey proteins hydrolyzed with serine protease isolated from Asian pumpkin (Cucurbita ficifolia). *International journal of peptide research and therapeutics.* **20**(4), pp.483-491.

Lacroix, I.M. and Li-Chan, E.C. 2012. Dipeptidyl peptidase-IV inhibitory activity of dairy protein hydrolysates. *International Dairy Journal.* **25**(2), pp.97-102.

Lacroix, I.M., Meng, G., Cheung, I.W. and Li-Chan, E.C. 2016. Do whey protein-derived peptides have dual dipeptidyl-peptidase IV and angiotensin I-converting enzyme inhibitory activities? *Journal of functional foods.* **21**, pp.87-96.

Mann, B., Kumari, A., Kumar, R., Sharma, R., Prajapati, K., Mahboob, S. and Athira, S. 2015. Antioxidant activity of whey protein hydrolysates in milk beverage system. *J Food Sci Technol.* **52**(6), pp.3235-3241.

Mansour, E., Dworschák, E., Lugasi, A., Gaál, Ö., Barna, É. and Gergely, A. 1993. Effect of processing on the antinutritive factors and nutritive value of rapeseed products. *Food chemistry.* **47**(3), pp.247-252.

Megías, C., Yust, M.d.M., Pedroche, J., Lquari, H., Girón-Calle, J., Alaiz, M., Millán, F. and Vioque, J. 2004. Purification of an ACE inhibitory peptide after hydrolysis of sunflower (Helianthus annuus L.) protein isolates. *Journal of Agricultural and Food Chemistry.* **52**(7), pp.1928-1932.

Michelke, L., Deussen, A., Dieterich, P. and Martin, M. 2017. Effects of bioactive peptides encrypted in whey-, soy-and rice protein on local and systemic angiotensin-converting enzyme activity. *Journal of Functional Foods.* **28**, pp.299-305.

Mooney, C., Haslam, N.J., Pollastri, G. and Shields, D.C. 2012. Towards the improved discovery and design of functional peptides: common features of diverse classes permit generalized prediction of bioactivity. *PloS one.* **7**(10), pe45012.

Nasri, M. 2017. Protein hydrolysates and biopeptides: Production, biological activities, and applications in foods and health benefits. A review. *Advances in food and nutrition research.* Elsevier, pp.109-159.

Ngoh, Y.-Y. and Gan, C.-Y. 2016. Enzyme-assisted extraction and identification of antioxidative and α-amylase inhibitory peptides from Pinto beans (Phaseolus vulgaris cv. Pinto). *Food chemistry.* **190**, pp.331-337.

Nongonierma, A.B. and FitzGerald, R.J. 2013. Inhibition of dipeptidyl peptidase IV (DPP-IV) by proline containing casein-derived peptides. *Journal of functional foods.* **5**(4), pp.1909-1917.

Nongonierma, A.B. and FitzGerald, R.J. 2015. Investigation of the potential of hemp, pea, rice and soy protein hydrolysates as a source of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides. *Food Digestion: Research and Current Opinion.* **6**(1-3), pp.19-29.

Nongonierma, A.B. and FitzGerald, R.J. 2017. Strategies for the discovery and identification of food protein-derived biologically active peptides. *Trends in Food Science & Technology.* **69**, pp.289-305.

Nongonierma, A.B., Lalmahomed, M., Paolella, S. and FitzGerald, R.J. 2017. Milk protein isolate (MPI) as a source of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides. *Food Chemistry.* **231**, pp.202-211.

Panyam, D. and Kilara, A. 1996. Enhancing the functionality of food proteins by enzymatic modification. *Trends in food science & technology.* **7**(4), pp.120-125.

Patil, P., Mandal, S., Tomar, S.K. and Anand, S. 2015. Food protein-derived bioactive peptides in management of type 2 diabetes. *Eur J Nutr.* **54**(6), pp.863-880.

Pena-Ramos, E.A. and Xiong, Y.L. 2001. Antioxidative activity of whey protein hydrolysates in a liposomal system. *J Dairy Sci.* **84**(12), pp.2577-2583.

Phongthai, S., D'Amico, S., Schoenlechner, R., Homthawornchoo, W. and Rawdkuen, S. 2018. Fractionation and antioxidant properties of rice bran protein hydrolysates stimulated by in vitro gastrointestinal digestion. *Food Chem.* **240**, pp.156-164.

Rabetafika, H.N., Van Remoortel, V., Danthine, S., Paquot, M. and Blecker, C. 2011. Flaxseed proteins: food uses and health benefits. *International journal of food science & technology.* **46**(2), pp.221-228.

Raghavan, S. and Kristinsson, H.G. 2009. ACE-inhibitory activity of tilapia protein hydrolysates. *Food chemistry.* **117**(4), pp.582-588.

Reddy, I.M., Kella, N.K. and Kinsella, J.E. 1988. Structural and conformational basis of the resistance of. beta.-lactoglobulin to peptic and chymotryptic digestion. *Journal of Agricultural and Food Chemistry.* **36**(4), pp.737-741.

Ruan, S., Xiong, J., Li, Y., Huang, S., Wang, X. and Ma, H. 2021. Improvement in enzymolysis efficiency and bioavailability of rapeseed meal protein concentrate by sequential dual frequency ultrasound pretreatment. *Process Biochemistry.*

Samaranayaka, A.G. and Li-Chan, E.C. 2011. Food-derived peptidic antioxidants: A review of their production, assessment, and potential applications. *Journal of functional foods.* **3**(4), pp.229-254.

Sarmadi, B.H. and Ismail, A. 2010. Antioxidative peptides from food proteins: a review. *Peptides.* **31**(10), pp.1949-1956.

Segura Campos, M.R., Chel Guerrero, L.A. and Betancur Ancona, D.A. 2010. Angiotensin‐I converting enzyme inhibitory and antioxidant activities of peptide fractions extracted by ultrafiltration of cowpea Vigna unguiculata hydrolysates. *Journal of the Science of Food and Agriculture.* **90**(14), pp.2512-2518.

Shewry, P.R. and Halford, N.G. 2002. Cereal seed storage proteins: structures, properties and role in grain utilization. *Journal of experimental botany.* **53**(370), pp.947-958.

Shewry, P.R., Napier, J.A. and Tatham, A.S. 1995. Seed storage proteins: structures and biosynthesis. *The plant cell.* **7**(7), p945.

Sultan, S., Huma, N., Butt, M.S., Aleem, M. and Abbas, M. 2018. Therapeutic potential of dairy bioactive peptides: A contemporary perspective. *Crit Rev Food Sci Nutr.* **58**(1), pp.105-115.

Tavano, O.L. 2013. Protein hydrolysis using proteases: An important tool for food biotechnology. *Journal of Molecular Catalysis B: Enzymatic.* **90**, pp.1-11.

Tundis, R., Loizzo, M. and Menichini, F. 2010. Natural products as α-amylase and α-glucosidase inhibitors and their hypoglycaemic potential in the treatment of diabetes: an update. *Mini reviews in medicinal chemistry.* **10**(4), pp.315-331.

Uraipong, C. and Zhao, J. 2016. Identification and functional characterisation of bioactive peptides in rice bran albumin hydrolysates. *International Journal of Food Science & Technology.* **51**(10), pp.2201-2208.

Vermeirssen, V., Van Camp, J. and Verstraete, W. 2002. Optimisation and validation of an angiotensin-converting enzyme inhibition assay for the screening of bioactive peptides. *J Biochem Biophys Methods.* **51**(1), pp.75-87.

Vilcacundo, R., Martínez-Villaluenga, C. and Hernández-Ledesma, B. 2017. Release of dipeptidyl peptidase IV, α-amylase and α-glucosidase inhibitory peptides from quinoa (Chenopodium quinoa Willd.) during in vitro simulated gastrointestinal digestion. *Journal of Functional Foods.* **35**, pp.531-539.

Wang, T.Y., Hsieh, C.H., Hung, C.C., Jao, C.L., Lin, P.Y., Hsieh, Y.L. and Hsu, K.C. 2017. A study to evaluate the potential of an in silico approach for predicting dipeptidyl peptidase-IV inhibitory activity in vitro of protein hydrolysates. *Food Chem.* **234**, pp.431-438.

Zambrowicz, A., Eckert, E., Pokora, M., Bobak, Ł., Dąbrowska, A., Szołtysik, M., Trziszka, T. and Chrzanowska, J. 2015. Antioxidant and antidiabetic activities of peptides isolated from a hydrolysate of an egg-yolk protein by-product prepared with a proteinase from Asian pumpkin (Cucurbita ficifolia). *RSC Advances.* **5**(14), pp.10460-10467.

Zhang, A.J., Rimando, A.M., Mizuno, C.S. and Mathews, S.T. 2017. α-Glucosidase inhibitory effect of resveratrol and piceatannol. *The Journal of nutritional biochemistry.* **47**, pp.86-93.

Zheng, H., Shen, X., Bu, G. and Luo, Y. 2008. Effects of pH, temperature and enzyme-to-substrate ratio on the antigenicity of whey protein hydrolysates prepared by Alcalase. *International dairy journal.* **18**(10-11), pp.1028-1033.

Chapter 5
Differential effects of oilseed protein hydrolysates to attenuate inflammation in murine macrophages

Abstract

Proteins from underutilized defatted oilseed meals have been observed as good sources of bioactive peptides.

This study was conducted to compare the anti-inflammatory activities of five oilseed proteins (flaxseed, rapeseed, sunflower, sesame and soybean) treated via alcalase, pepsin (pH 1.3 and pH 2.1) hydrolysis, respectively. Two dairy proteins (whey and casein) were used as comparisons. The effects of protein hydrolysates and their three different molecular weight fractions (M_w > 10 kDa, 3-10 kDa, < 3 kDa) on suppressing the activation of NF-κB pathway were screened via RAW-Blue^{TM} cells. Fractions with M_w < 3kDa of pepsin (pH 1.3)-treated protein hydrolysates were subsequently selected to validate their anti-inflammatory properties in RAW 264.7 macrophages.

Rapeseed fractions showed the greatest potency to attenuate inflammation on a cellular level, via efficiently down-regulating the expression of IL-6 (49.1%), IL-1β (58.6%), iNOS (41.9%) and COX-2 (58.7%) and up-regulating the IL-10 (47.2%) mRNA level at 2000 μg/mL.

Rapeseed, sesame and casein demonstrated marked repression of NF-κB pathway, via down-regulating NF-κB1, p65 and/or IκBα mRNA levels. In addition, rapeseed, sesame and soybean reduced the expression of TLR4 and/or CD14 associated with attenuated LPS recognition. Furthermore, more efficiently scavenging LPS were detected in rapeseed fractions, contributing to its more promising anti-inflammatory activity

The current study demonstrates the differing capacity of plant hydrolysates to interact with inflammatory signalling, indicating the need for further studies to identify active peptides and confirm their potential to attenuate inflammation.

5.1 Introduction

Oilseed crops are widely utilized in agricultural industry mainly due to their high oil content. Oilseeds, such as rapeseed and soybean, are of

significant economic importance as they are not only profitable in yielding edible vegetable oils with low cholesterol content, but also being utilized as biofuel. Thus, they contributes significantly to the incomes of farmers (Jaeger and Siegel, 2008; Adeleke and Babalola, 2020). The remainder of oil industries, defatted oilseed cake, contains approximately 20-25% protein (Moure et al., 2006) and was previously utilized either as organic fertilizer or cheap livestock feed. At present, there is an increasing interest in plant proteins, including oilseed proteins, for their functional properties and potential health benefits, some of which are linked to bioactive peptides. Bioactive peptides are increasingly recognized as an important group of bioactive compounds with a range of properties, such as antioxidant (He et al., 2013), anti-hypertensive (Aondona et al., 2021), anti-diabetic (Nongonierma and FitzGerald, 2015) and anti-inflammatory (He et al., 2019) activities. They can be released from parental proteins upon hydrolysis or fermentation and usually comprise 2 to 20 amino acid residues.

Inflammation is a complex and innate defensive immune response that involves various immune-system cells (such as macrophages) and can be activated against injury, infection or other harmful stimuli. Acute inflammation is essential for defeating damage of infection and tissue repair, whilst uncontrolled prolonged inflammation is closely linked to the pathogenesis of several chronic diseases, including metabolic disorders, atherosclerosis and cancers (Hotamisligil, 2006). Lipopolysaccharide (LPS), an endotoxin secreted via gram-negative bacteria, is widely used as a stimulus to evoke the multiple downstream intracellular signalling cascades, including nuclear factor-kappa b (NF-κB) and mitogen-activating protein kinases (MAPKs), in various cell types for studying inflammatory response. Dysregulation of NF-κB and MAPKs activation generates abundant inflammatory effects and meanwhile promotes the expression of various pro-inflammatory cytokines, including interleukin-6 (IL-6), interleukin-1β, tumour necrosis factor (TNF-α), proinflammatory mediators, such as nitric oxide (NO) and prostaglandins (PGs) as well as inducible nitric oxide synthases (iNOS) and cyclooxygenase-2 (COX-2) (Tornatore et al., 2012). Therefore, targeting macrophages and their products after activation can be a crucial and effective strategy for characterizing natural anti-inflammatory agents.

Anti-inflammatory activities of oilseed derived peptide and protein hydrolysates have been reported in several studies. According to Udenigwe et al. (2009), low M_w fractions of pepsin-, ficin- or papain- treated flaxseed protein hydrolysates, which significantly reduced the generation of nitric oxide

in RAW 264.7 macrophages with IC_{50} values of 0.250, 0.504 and 0.215 mg protein/mL, respectively. Using the same cell line, He et al. (2019) identified the three rapeseed peptides, LY, RALP and GHS, as markedly suppressing the secretion of nitric oxide, and leading to reduce the expression of pro-inflammatory cytokines and mediators, including iNOS, IL-6 and TNF-alpha. They further confirmed the anti-inflammatory properties of LY, RALP and GHS *in vivo* using a spontaneously hypertensive rat (SHR) model. In addition, four novel sunflower derived peptides (YFVP, SGRDP, MVWGP and TGSYTEGWS) were confirmed to blunt the activation of NF-κB signalling in human monocytic leukemia THP-1 cells (Velliquette et al., 2020). Compared with these abovementioned oilseeds, soybean was widely reported as a noticeable source of anti-inflammatory peptides, such as Lunasin and LSW (Lin, Q. et al., 2017; González-Montoya et al., 2018; Hao et al., 2020). Nevertheless, there is still lack of the details about the molecular mechanisms underlying the anti-inflammatory activities of peptides derived from sesame, therefore, needed to be explored.

In view of the heterogeneity of enzymatic hydrolysis conditions applied to different protein samples, our study aimed to directly compare the anti-inflammatory properties of alcalase- or pepsin- treated oilseed protein hydrolysates (flaxseed, rapeseed, sunflower, sesame and soybean), against two dairy protein hydrolysates (whey and casein). The potency of protein hydrolysates and their three different M_w fractions (M_w > 10, 3-10 and <3 kDa) to attenuate NF-κB signalling were determined via RAW-Blue^{TM} reporter cells. Wild-type RAW264.7 macrophages were used to confirm the effects of selected fractions of pepsin (pH 1.3)-treated protein hydrolysates on the expression of pro- and anti- inflammatory cytokines, mediators and transcription factors, In addition, this study investigated the potential of peptide-containing samples binding and immobilizing bacterial LPS to modulate inflammation.

5.2 Materials and methods

5.2.1 Materials and reagents

Pepsin from porcine gastric mucosa, alcalase from *Bacillus licheniformis*, Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4, and from *Escherichia coli* O111:B4 FITC conjugate, neutral red, casein and o-Phenylenediamine (OPD)). Dulbecco's modified Eagle's medium (DMEM),

foetal bovine serum, 100U/mL penicillin and 100 µg/mL streptomycin were purchased from Sigma (Dorset, UK). Primers used in RT-PCR were obtained from Invitrogen™ (Inchinnan, UK). RAW-Blue™ cell line (mouse macrophage reporter cell line) and QUANTI-Blue™ reagent were purchased from InvivoGen (Toulouse, France). Trisure and SensiFAST™ SYBR® Hi-ROX Kit were purchased from Bioline (Nottingham, UK) and iScript reagent kit was from Bio-Rad (Watford, UK). RAW 264.7 macrophages were purchased from the European Collection of Authenticated Cell (Salisbury, UK) Commercial food grade oilseeds and dairy proteins were collected from local supermarket (Leeds, UK).

5.2.2 Preparation of protein hydrolysates and their ultrafiltered fractions

Sample preparation and enzyme hydrolysis procedures were performed as recently described (Han et al., 2021). Briefly, oilseed and dairy proteins were hydrolysed at 60^{o} C for 6 hours with alcalase enzyme using an Enzyme/Protein (E/P) ratio of 1.5:25 at pH 8. For pepsin hydrolysates, proteins were digested at 37^{o} C for 6 hours using pepsin with a 1:25 E/P ratio at pH 1.3 or 2.1, respectively. Three different M_w (M_w > 10, 3-10 and < 3 kDa) fractions were prepared for each sample through ultrafiltration using 3 and 10 kDa M_w cut-off membranes.

5.2.3 Cell culture and treatments

RAW-BLUE™ cells and RAW 264.7 macrophages were both cultivated in high glucose Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were incubated at 37°C in a humidified atmosphere (5% CO_2).

5.2.4 Measurement of cell viability

The cytotoxicity of protein hydrolysates in RAW-Blue™ and RAW 264.7 macrophages was determined using Neutral Red assay according to Repetto et al. (2008) with slight modifications. Briefly, RAW-BLUE™ and RAW 264.7 cells were seeded into 24-well plates at a density of 200,000 cells per well. Upon reading 70 – 80% confluence, protein hydrolysates or low Mw fractions were diluted 1:10 from a stock solution in PBS into the medium for

final concentrations of 250, 1000 and 2000 µg/mL. After incubation for 24 hours, the medium was removed and replaced by medium containing neutral red (40 µg/mL). After 3 hours of incubation at 37°C, the neutral red containing medium was removed, the cells washed with PBS and bleach solution (50% ethanol, 49% deionized H_2O and 1% glacial acid) added to dissolve the accumulated neutral red dye. The absorbance was recorded at 540 nm using a Spark10M plate reader (Tecan, Männedorf, Switzerland) against bleach solution as blank and calculated in per cent of untreated control cells (medium only).

5.2.5 NF-κB reporter assay

RAW-BlueTM cells are a commercially available NF-κB reporter cell line derived from murine macrophages, with secreted embryonic alkaline phosphatase (SEAP) reporter construct chromosomally integrated. Activation of inflammatory signalling via NF-κB and AP-1 leads to increased secretion of SEAP into the cell culture medium. RAW-Blue cells, 70-80% confluent, were incubated with protein hydrolysates and their different Mw fractions (250 µg/mL and 1000 µg/mL) for 1 h and then stimulated with LPS (100 ng/mL). After 24 h, 50 µL of supernatant was mixed with 150 µL of QUANTI-BlueTM substrate and incubated for 1 h at 37ºC. SEAP activity, indicating the magnitude of transcriptional activation, was measured via absorbance at 620 nm using Spark10M plate reader. Absorbance of non-stimulated control samples was subtracted from absorbance values of stimulated samples. The results were expressed as inhibition in per cent of the sample treated with LPS only.

5.2.6 Real-time quantitative PCR (qPCR) analysis

The mRNA expression levels of IL-6, TNF-α, IL-1β, iNOS, COX-2, NF-κB1, p65, IκBα, TLR4, CD14 were determined using quantitative RT-PCR. To this end, RAW 264.7 macrophages were stimulated with 100 ng/mL LPS in the absence or presence of protein hydrolysates samples (2000 µg/mL) for 6 hours. The total RNA was isolated using Trisure reagent according to manufacturer's instructions, and the quality and quantity of RNA were determined using Nanoquant plate (Spark plate reader). For cDNA synthesis, 0.5 µg of RNA was reserve transcribed using iScript reagent kit (BIO-Rad) according to manufacturer's instructions. Amplification of target gene mRNA was performed using SensiFast SybrGreen reagent on a StepOne cycler

(ABI) using recommended settings. Primers were designed using NCBI tools, the sequence information is shown in **Table 5.1**. The β-actin gene was used as housekeeping gene to normalize the target gene expression. The relative gene expression was calculated basing on $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

5.2.7 LPS-binding assay

The LPS-binding capacity in peptide-containing samples was determined via immunoassay according to Martínez-Sernández et al. (2016) with slight modifications. Briefly, wells of a 96-well plate were coated with 200 µL of casein hydrolysate solutions (protein concentration 12.5 µg/mL) covered with adhesive film and incubated at 4°C overnight. The following day, 60 µL of LPS O111:B4-FITC (15 µg/mL) was mixed with the same volume of each of the seven low Mw fractions of pepsin (pH 1.3)-treated protein hydrolysates (0 to 256 µg/well) and incubated for 1 h at room temperature. The contents of the wells in the 96-well plate were aspirated and the wells washed three times with PBS using plate washer (Tecan). To each well 100 µL of the preincubated solution was transferred, and subsequently incubated at room temperature for 30 min. Afterwards, the plate was washed five times with 200 µL of PBS-T (0.05% Tween 20) per well. Then 100 µL of 1/4000 diluted sheep anti-FITC: HRP was added and incubated for a further 30 mins at room temperature at 750 rpm on a rocking platform. Following a washing step, OPD solution (100 µL) was added to each well and incubated for 20 mins in the dark. After that the reaction was stopped via adding 25 µL of 3N H2SO4. Finally, the absorbance of per well was read at 492 nm.

5.2.8 Statistical analysis

Data were analysed via student's t-test, depending on group number, multiple t-test and two-way analysis of variance (ANOVA) with post hoc analysis using GraphPad Prism 9.0. Significant differences were considered when p-value < 0.05. All experiments were performed in triplicate and data were expressed as mean ± standard error of the mean (SEM).

Table 5.1 Mouse primer pairs used for RT-PCR.

Gene	Forward sequence (5'-3')	Reverse sequence (5' -3')
β-actin	CCTCTATGCCAACACAGTGC	CCTGCTTGCTGATCCACATC
IL-6	AGTTGCCTTCTTGGGACTGA	CAGAATTGCCATTGCACAAC
TNF-α	GTGCCTATGTCTCAGCCTCT	AGTTGGTTGTCTTTGAGATCCA
IL-1β	CAGGCAGGCAGTATCACTCA	CAGGCAGGCAGTATCACTCA
IL-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
iNOS	GCAGCCTGTGAGACCTTTG	GCATTGGAAGTGAAGCGTTTC
COX-2	TTCAACACACTCCTATCACTGGC	AGAAGCGTTTGCGGTACTCAT
NF-κB1	ATGGCAGACGATGATCCCTAC	TCTTCACAGTGGTATTTCTGGTG
p65	AGGCTTCTGGGCCTTATGTG	TGCTTCTCTCGCCAGGAATAC
IκB-α	TGAAGGACGAGGAGTACGAGC	TTCGTGGATGATTGCCAAGTG
TLR 4	AGGCAGCAGGTGGAATTGT	GGTCCAAGTTGCCGTTTCTT
CD14	CTCTGTCCTTAAAGCGGCTTAC	GTTGCGGAGGTTCAAGATGTT

5.3 Results and Discussion

5.3.1 Inhibition of SEAP secretion via peptide fractions

NF-κB is an inducible transcription factor which plays a pivotal role in regulating the cellular inflammatory response. The activation of NF-κB pathway directly promotes the expression of pro-inflammatory target genes, including cytokines and chemokines (Liu et al., 2017). Interruption of NF-κB signalling has been demonstrated by many anti-inflammatory agents and is being applied as part of therapeutic approaches to modulate inflammation and its progression (Liu et al., 2017). In this study, the potency of protein hydrolysates and their three different M_w (M_w > 10, 3 - 10 and < 3 kDa) fractions to modulate the activation of NF-κB signalling were evaluated. RAW Blue™ reporter cells were used as a macrophage-based screening model responsive to (anti-)inflammatory triggers which can be quantified according to secreted alkaline phosphatase (SEAP) levels.

As shown in **Table 5.2**, the inhibition of SEAP secretion of oilseed and dairy peptides were tested at 250 and 1000 μg/mL. At both concentrations, no significant reduction of cell viability was found by any of the samples (data not

shown). In general, inflammation inhibitory effects were stronger at higher concentrations, apart from pepsin (pH 1.3)-treated sunflower and alcalase-treated whey protein hydrolysates, alcalase-treated rapeseed (M_w > 10 kDa) and whey (M_w < 3 kDa); pepsin (pH 1.3)-treated flaxseed (whole); pepsin (pH 2.1)-treated rapeseed (M_w > 10 kDa), sesame (M_w > 10 kDa) and casein (M_w > 10 kDa). The M_w > 10 kDa fractions presented the overall lowest effectiveness in modulating SEAP secretion, on the other hand, the low M_w fraction (< 3 kDa) tended to exhibit the strongest effects on attenuating NF-κB signalling. These findings were correlated with Vo et al. (2013) and (Sangtanoo et al., 2020), who claimed the low M_w (M_w < 3kDa) fractions obtained from edible microalgae (*Spirulina maxima*) protein hydrolysates and peanut worm protein hydrolysates, exerting the most promising anti-inflammatory potential, compared with other fractions (e.g. M_w 3-10 kDa or > 10 kDa), respectively. In contrast, Sandoval-Sicairos et al. (2021) claimed higher M_w (> 10 and 3-10 kDa) fractions from amaranth protein hydrolysates tended to exert more promising anti-inflammatory responses, compared with the fraction with M_w < 3 kDa. In addition, Kim et al. (2016) confirmed M_w > 5 kDa fractions of blue mussel (*Mytilus edulis*) hydrolysates inhibited NO generation after LPS stimulation by 92.4%, which was the highest value compared to all fractions in their study. Both findings were aligned with some of our results, which are alcalase-treated sesame (84.5%) and pepsin (pH 2.1)-treated rapeseed (61.5%) fractions with M_w 3-10 kDa, showing the highest inhibitory potency among other fractions, together with whole protein hydrolysates.

Utilization of RAW-Blue™ cells to evaluate the anti-inflammatory potential of bio-compounds has also been reported via Li, L.-H. et al. (2017). In their work, GW-A2, a peptide with sequence of GAKYAKIIYNYLKKIANALW, dose-dependently suppressed the activation of NF-κB transcriptional activities in the reporter cell line, which was directly linked to downregulation of pro-inflammatory markers iNOS, COX-2, TNF-α, IL-6 and NO in LPS-activated RAW 264.7 macrophage cells. Similarity, Xie et al. (2012). reported four flavonoids, including velutin, luteolin, apigenin and chrysoeriol, were identified from the pulp of açaí fruit, effectively inhibited SEAP secretion, with IC_{50} values ranging from 2.0 – 20.0 μM, findings that were confirmed in RAW 264.7 macrophages, through reduced TNF-α and IL-6 secreted in cultured medium, blocking of IκB degradation, or inhibition of p38 and JNK phosphorylation.

In general, Low M_w (M_w < 3kDa) fractions exert the most promising anti-inflammatory potential compared with the other two fractions (M_w > 10 kDa and 3 – 10 kDa). Therefore, low M_w < 3 kDa fraction of pepsin (pH 1.3)-treated rapeseed, sesame, soybean and whey fractions were selected to validate their anti-inflammatory properties in wild type macrophages since they inhibited around 90% SEAP activity in NF-kB reporter cells. Meanwhile, flaxseed (17.3%), sunflower (32.3%) and casein (73.8%) under the same treatment were used as comparisons.

5.3.2 Effect of protein hydrolysates on cell viability in RAW 264.7 macrophages

Prior to the evaluation of anti-inflammatory activity in RAW 264.7 macrophages, the potential cytotoxicity of low M_w fraction of pepsin (pH 1.3)-treated protein hydrolysates was examined via Neutral Red assay. As shown in **Figure 5.1**, none of the tested protein hydrolysates tested exerted any negative effects on the viability of RAW 264.7 macrophages. In contrast, flaxseed, rapeseed, sunflower, sesame and casein samples showed enhanced cell viability values at 2000 µg/mL, in the range of 9.4 – 25.6% above the medium control. These above control values in % cell viabilities may indicate an increase in cell proliferation that has also been observed by others. For example, He et al. (2019) reported that three rapeseed derived peptides, LY, RALP and GHS, increased cell viability by 13.4%, 20.8% and 24.1% respectively. Further, Zhao et al. (2016) demonstrated that velvet antler protein hydrolysates increased cell viability by 24.3% and 31.7% (at 200 and 500 µg/mL). The enhancement of proliferation and survival (phagocytic activities) of macrophages indicated the positive immunostimulatory effects, since macrophages are the target cells of the immune system (Li, E.W. and Mine, 2004; Girón-Calle et al., 2010).

Table 5.2. Anti-inflammatory properties (% inhibition) of oilseed and diary protein hydrolysates and different M_w fractions as determined using SEAP reporter assay in LPS (100ng/mL) stimulated RAW-Blue™ cells. Data are expressed as mean with SEM of three independent experiments performed in duplicate. Different capital or small letters within the same column and row indicate significant differences, respectively ($p<0.05$). * means significant differences compared to 250 and 1000 µg/mL ($p<0.05$).

	Condition	Whole		>10kDa		3-10kDa		<3kDa	
Conc.		250 µg/mL	1000 µg/mL	250 µg/mL	1000 µg/mL	250 µg/ml	1000 µg/ml	250 µg/ml	1000 µg/ml
Flaxseed	Alcalase	7.2 ± 0.5^{Ab}	$16.3\pm1.5^{ABCDb*}$	n/a	n/a	15.2 ± 0.2^{BCc}	$13.4\pm0.2^{Abb*}$	33.6 ± 3.2^{EFGHa}	$81.5\pm1.8^{IJKa*}$
	Pepsin pH 1.3	18.3 ± 2.1^{Bb}	23.8 ± 1.9^{DEa}	6.1 ± 0.4^{ABCa}	n/a	4.7 ± 1.0^{Aa}	$17.1\pm1.1^{Aba*}$	n/a	$17.3\pm2.5^{Aba*}$
	Pepsin pH 2.1	6.8 ± 0.9^{Aa}	$22.3\pm0.9^{CDEc*}$	0.6 ± 0.1^{Ab}	$7.3\pm1.3^{Aba*}$	n/a	n/a	3.0 ± 0.3^{Aa}	$11.1\pm0.4^{Ab*}$
Rapeseed	Alcalase	n/a	$10.5\pm0.4^{Aba*}$	1.3 ± 0.1^{ABa}	1.4 ± 0.1^{Ab}	n/a	$8.1\pm1.4^{A*}$	n/a	$19.6\pm2.9^{ABCa*}$
	Pepsin pH 1.3	13.1 ± 1.8^{ABb}	$31.3\pm1.9^{EFb*}$	10.1 ± 1.1^{ABCDb}	$24.8\pm0.8^{CDc*}$	n/a	$43.8\pm2.6^{EFb*}$	48.3 ± 2.4^{IJa}	$86.2\pm2.0^{JKa*}$
	Pepsin pH 2.1	n/a	$8.6\pm2.2^{Ac*}$	14.4 ± 2.7^{DEa}	15.0 ± 4.3^{BCc}	40.6 ± 4.3^{Fb}	$61.5\pm4.5^{GHb*}$	18.9 ± 1.5^{BCDa}	$44.9\pm2.3^{DEFa*}$
Sunflower	Alcalase	n/a	$21.9\pm4.9^{BCDEb*}$	n/a	$21.1\pm2.5^{CDb*}$	n/a	n/a	30.4 ± 2.6^{CDEFGa}	$68.6\pm4.3^{GHIa*}$
	Pepsin pH 1.3	8.1 ± 2.3^{Ab}	$37.6\pm2.2^{Fab*}$	23.5 ± 1.7^{EFa}	$49.7\pm3.8^{Fb*}$	n/a	$29.2\pm4.1^{CDa*}$	n/a	$32.3\pm5.9^{CDEa*}$
	Pepsin pH 2.1	n/a	$51.8\pm1.6^{Gd*}$	3.7 ± 0.7^{ABCb}	$10.9\pm0.6^{ABCc*}$	5.5 ± 0.3^{Ab}	$16.3\pm0.5^{ABb*}$	17.2 ± 1.9^{BCa}	$38.6\pm2.7^{DEa*}$
Sesame	Alcalase	n/a	$26.6\pm1.5^{DEFc*}$	n/a	$39.0\pm2.3^{Ea*}$	44.2 ± 2.8^{Fb}	$84.5\pm2.7^{Ib*}$	21.3 ± 0.9^{BCDEa}	$46.8\pm2.1^{EFa*}$
	Pepsin pH 1.3	17.6 ± 3.5^{Bc}	20.6 ± 4.2^{DEFc}	n/a	$39.1\pm1.6^{Eb*}$	28.4 ± 2.3^{DEb}	$42.5\pm1.8^{EFb*}$	38.2 ± 2.4^{GHIa}	$88.7\pm1.6^{Ka*}$

	Pepsin pH 2.1	n/a	10.4±2.4^{Abd*}	29.1±4.1^{Fb}	29.6±3.1^{DEc}	31.2±2.0^{Eb}	71.5±4.4^{HIb*}	52.5±1.9^{Ja}	96.1±1.8^{Ka*}
Soybean	Alcalase	n/a	7.1±1.9^{Ac*}	n/a	n/a	n/a	18.2±1.3^{ABCb*}	25.5±3.8^{BCDEFa}	82.0±5.7^{IJKa*}
	Pepsin pH 1.3	n/a	n/a	n/a	n/a	7.1±0.1^{ABb}	19.2±0.5^{BCb*}	26.5±1.9^{BCDEFGa}	93.0±2.1^{Ka*}
	Pepsin pH 2.1	8.1±0.9^{Ab}	20.8±0.7^{BCDEc*}	n/a	8.4±1.5^{Abb*}	n/a	9.3±1.3^{ABb*}	19.3±2.4^{BCDa}	72.9±1.4^{HIJa*}
Whey	Alcalase	8.5±1.1^{Ab}	25.8±1.2^{DEcd*}	10.5±2.2^{BCDb}	20.5±1.4^{CDcd*}	10.0±0.2^{ABb}	15.5±0.3^{ABbd*}	20.0±4.8^{BCDa}	31.2±4.2^{BCDac}
	Pepsin pH 1.3	9.1±0.7^{Ab}	30.7±0.9^{EFb*}	n/a	9.2±1.0^{ABc*}	n/a	35.8±1.1^{DEb*}	37.3±2.0^{FGHIa}	90.2±3.0^{Ka*}
	Pepsin pH 2.1	n/a	51.3±2.0^{Ga*}	n/a	n/a	12.4±1.6^{ABa}	36.7±2.3^{DEb*}	16.4±2.0^{Ba}	62.0±1.6^{Ga*}
Casein	Alcalase	n/a	11.7±2.1^{ABCb*}	n/a	n/a	9.3±0.5^{ABb}	16.5±1.1^{ABb*}	26.9±1.5^{BCDEFGa}	55.5±1.5^{FGa*}
	Pepsin pH 1.3^{AB}	12.8±0.7^{ABb}	23.5±2.0^{DEc*}	1.8±0.3^{ABCa}	n/a	15.2±1.1^{BCb}	34.9±1.7^{DEb*}	30.5±1.7^{DEFGa}	73.8±1.0^{HIJa*}
	Pepsin pH 2.1^{AB}	10.8±1.6^{ABc}	17.3±1.4^{ABCDd*}	8.0±2.0^{ABCDc}	9.2±0.5^{ABc}	22.0±0.3^{CDb}	52.8±1.0^{FGb*}	45.4±4.0^{HIJa}	89.2±2.9^{Ka*}

Based on the cell toxicity results, peptide-containing factions at all three concentrations (250, 1000 and 2000 μg/mL) did not exert cytotoxicity. 2000 μg/mL was selected as the concentration of protein hydrolysates for further experiments.

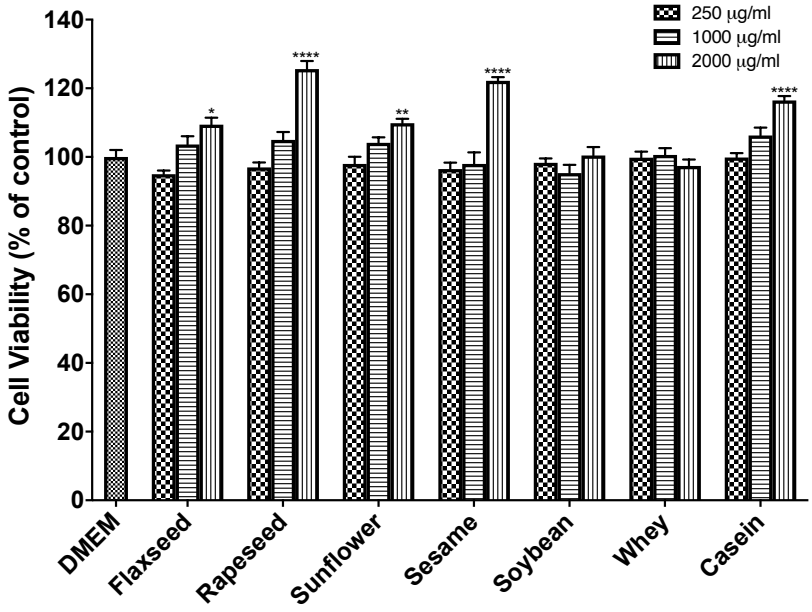

Figure 5.1 Effects of low M_w fraction of pepsin (pH 1.3)-treated protein hydrolysates on cell viability in RAW 264.7 macrophages. Data are mean with SEM of three independent experiments performed in duplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ compared with the value of cell viability of RAW 264.7 macrophages treated with medium only.

5.3.3 Protein hydrolysates modulate the expression of cytokines and mediators

In the NF-κB reporter assays, low M_w ($M_w < 3$ kDa) fractions of pepsin (pH 1.3)-treated rapeseed, sesame and soybean protein hydrolysates presented the potential of suppressing activation of NF-κB, together with whey and casein. Attenuating the NF-κB pathway is associated with the regulating transcriptional induction of anti-/pro-inflammatory cytokines and mediators. Based on these facts, IL-6, TNF-α, IL-1β and IL-10 were chosen as the presentative cytokines to evaluate the outcome of protein hydrolysates attenuating NF-κB activation. Therefore, all low M_w fraction of pepsin (pH 1.3)-treated protein hydrolysates were evaluated for their inhibition on the mRNA levels of cytokines in LPS-stimulated RAW 264.7 macrophages at 2000 μg/mL. As shown in **Figure 5.2a and 5.2b**, mRNA levels of IL-6 and TNF-α were significantly increased after LPS stimulation comparing with

negative control. However, only rapeseed significantly reduced IL-6 mRNA levels by 37.5%. Whilst no peptides supressed the inflammation via downregulating the TNF-α expression. Ren et al. (2018) also claimed LDAPGHR (at 50μM) peptides derived from hazelnut decreased IL-6 mRNA levels by 66.1%. Meanwhile, two other cytokines, IL-1β (pro-inflammatory) and IL-10 (anti-inflammatory), were evaluated during the inflammatory progress. Our results showed that rapeseed, sesame and casein significantly inhibited the expression of IL-1β by 67.7%, 51.8% and 50.1% respectively (**Figure 5.2c**). Apart from soybean and whey, all samples showed up-regulation of IL-10 mRNA levels at 2000 μg/mL in the range of 44.5% to 57.3% (**Figure 5.2d**), compared with positive control. Coincidentally, cell viability of these five samples was also significantly increased.

In addition to cytokines, the inducible form of nitric oxide (iNOS) and cyclooxygenase-2 (COX-2) are two major inflammatory mediators regulated via NF-κB in inflammatory progress. iNOS converts NO from arginine and COX-2 synthesizes prostaglandin E2 using arachidonic acids (Aktan, 2004; Nasry et al., 2018). Large quantities of produced NO and PGE2 are directly associated with inflammatory diseases, including injuries and rheumatoid arthritis. iNOS and COX-2 are undetectable in RAW 264.7 macrophages, whilst they remarkably promote the gene expression in response to LPS stimulation. Therefore, the roles of peptides in regulating iNOS and COX-2 gene expression were analysed in order to evaluate their anti-inflammatory potential. As shown in **Figure 5.2e and 5.2f**, only rapeseed efficiently reduced 47.2% of iNOS expression after 6 hours LPS stimulation. Rapeseed was also associated with the modulation of COX-2 transcriptional level (decrease 44.5%), to a similar level as casein (56.5%). Taken together, our results suggest that rapeseed was the most promising sources of anti-inflammatory peptides, since it efficiently down-regulated the IL-6, IL-1β, iNOS and COX-2 and up-regulated IL-10 transcriptional activities. In addition, apart from soybean, the other protein hydrolysates, especially sesame and casein, tended to modulate one or more cytokines, indicating their potential to lower inflammation.

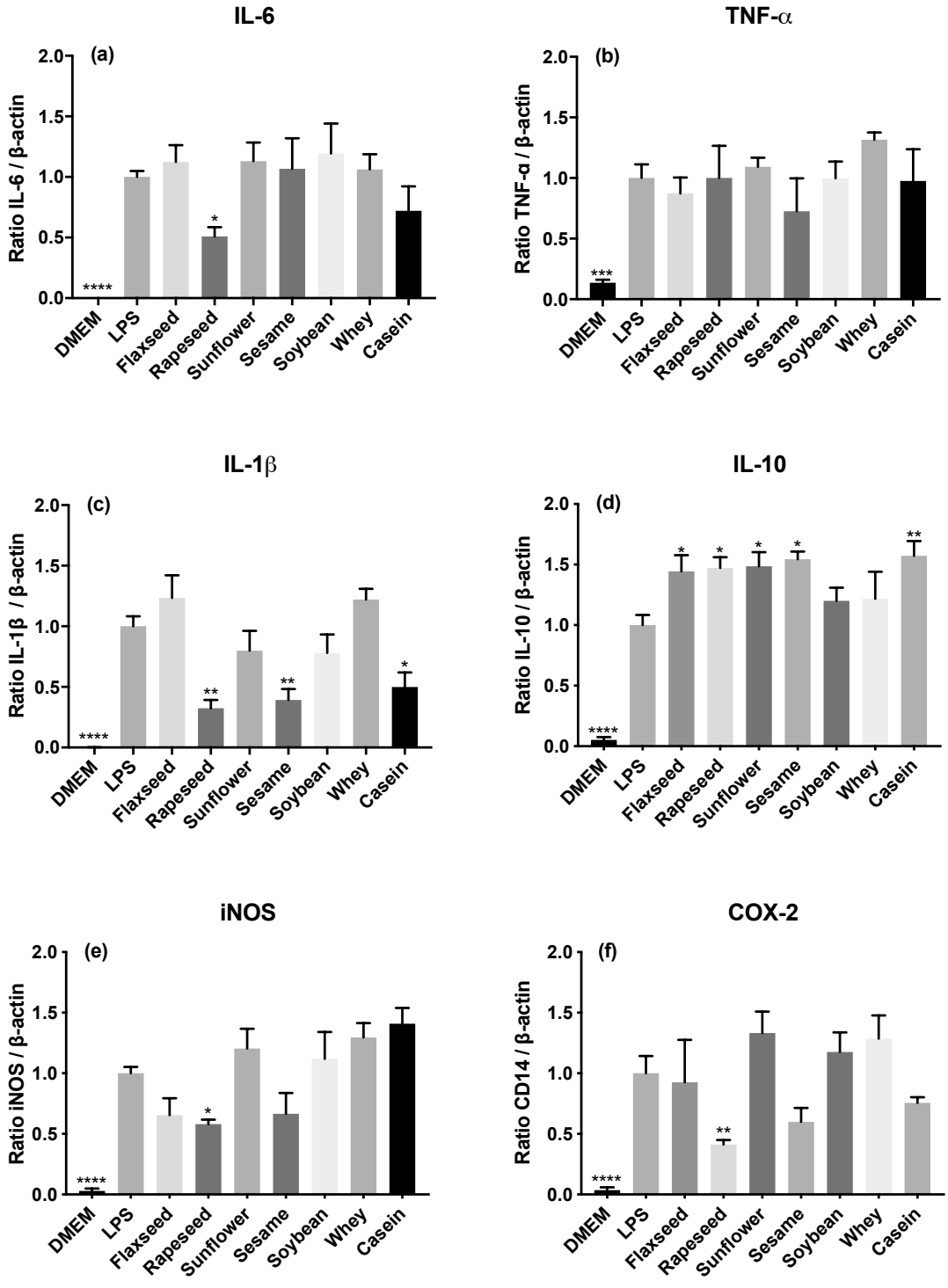

Figure 5.2 Effects of low M_w fractions (2000 µg/mL) on mRNA levels of pro-inflammatory cytokines, a) IL-6, b) TNF-α and c) IL-1β, anti-inflammatory cytokine d) IL-10, and pro-inflammatory mediators e) iNOS and f) COX-2 in LPS (0.1 µg/mL)-stimulated RAW 264.7 macrophages. Data are mean with SEM of three independent experiments performed in duplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ compared with the positive control (LPS-stimulated RAW 264.7 macrophages)

5.3.4 Attenuation of NF-κB activation in LPS-stimulated RAW 264.7 macrophages

To validate the changes of NF-κB in RAW 264.7 macrophages, the mRNA levels of NF-κB1, p65 and IκBα were also analysed via RT-PCR. NF-κB1 (p50) and p65 are the two most common subunits of NF-κB/Rel proteins, which act as transcriptional regulators and so affect inflammatory-related gene expression. After 6-hour LPS stimulation, the mRNA levels of p50 and p65 were significantly up-regulated in RAW 264.7 macrophages. As shown in **Figure 5.3a and 5.3b**, sesame significantly reduced 50.7% expression of NF-κB1, while rapeseed (59.1%) and casein (47.8%) down-regulated the expression of p65. IκBα, functioning as an inhibitor of NF-κB transcription, keeps NF-κB in place in the cytoplasm and thus prevents them from activation and binding to DNA (Jacobs and Harrison, 1998). The LPS-induced increase in IκBα showed a strong decrease (73.9%) upon co-incubation with rapeseed Meanwhile, casein also downregulated 58.1% of IκBα expression.

TLR 4 is the specific endogenous receptor to recognize LPS. Activation of TLR4 leads to inflammatory cytokines be synthesized via the TLR4/NF-κB pathway. In this study, the gene expression of TLR 4 was assessed in RAW 264.7 macrophages. As shown in **Figure 5.3d**, TLR 4 mRNA levels were detectable in macrophages, whilst no significant increase was shown after LPS-stimulation for 6 hours. Matsuguchi et al. (2000) also claimed no significant elevation of TLR4 gene expression after 2 or 8 hours LPS stimulation in macrophages. Rapeseed and soybean evidently decreased the expression of TLR 4 by 43.0% and 41.8% at 2000 μg/mL, respectively. Regarding to CD14, the other crucial LPS receptor is on the cell membrane. Unlike TLR4, a 2-fold increase of CD14 expression was found after LPS stimulation, compared to the negative control (**Figure 5.3e**). Rapeseed and sesame exerted similar suppression of CD14 expression, which were 49.5% and 54.4%, respectively.

Since all tested genes above are targets of NF-κB, any decrease in the mRNA level of these genes directly leads to the reduction of pro-inflammatory mediators and the proteins involved in NF-κB signalling. Taken together, the possible suppression of activation of NF-κB pathway of rapeseed, sesame and casein were proved in RAW 264.7 macrophages, whilst soybean and whey were unable to suppress the NF-κB related gene expression. Rapeseed, sesame and soybean may also delay the LPS recognized via reducing the expression of TLR4 and CD14.

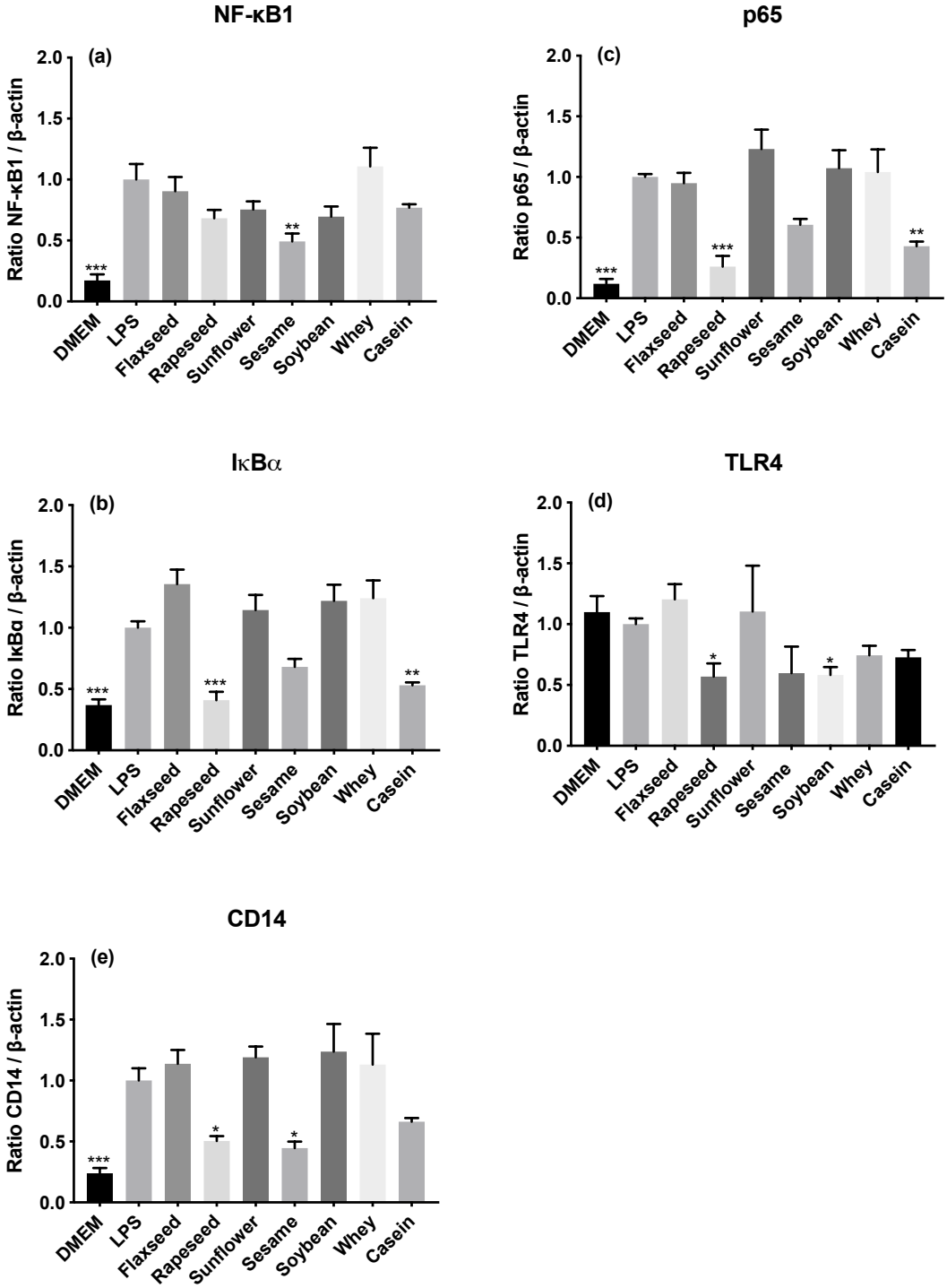

Figure 5.3 Effect of low M_w fraction of pepsin (pH 1.3)-treated protein hydrolysates on the expression of a) NF-**k**B1, b) p65, c) IκBa, d) TLR 4 and e) CD14 in LPS-stimulated raw 264.7 macrophages. Data are shown as mean with SEM of three independent experiments preformed in duplicate. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 compared with the positive control (LPS-stimulated RAW 264.7 macrophages)

5.3.5 LPS binding capacity of peptides

Apart from suppressing the activation of the NF-κB pathway, protein/peptide-LPS binding may also contribute to the modulation of inflammation via avoiding the recognition of LPS receptors in cell surface. In order to determine the affinity of peptides to LPS, all the low M_w fractions of pepsin (pH 1.3)-treated protein hydrolysates were evaluated for their scavenging capabilities of LPS in solution. Polymyxin B sulfate salt, an antibiotic with the function of binding to Lipid A, located in the outer leaflet of LPS membrane, which efficiently treated the inflammation and other gram-negative bacterial induced infections, it was used as positive control (Morrison and Jacobs, 1976; Domingues et al., 2012; Steimle et al., 2016).

As shown in **Figure 5.4a**, the peptides exerted a dose-dependent increase of LPS binding capability (from 0.004 to 256 μg peptides/per well). At 0.004 and 0.016 μg/ per well, casein demonstrated the highest binding capability, which scavenged 14.4 and 20.7% of LPS, respectively. However, when the concentration of peptides/protein reached 0.063 μg/ per well, polymyxin B sulfate salt showed the most robust binding capability (33.5% shown at **Figure 5.4b**), 8.9% higher than casein. The inhibition of Polymyxin B sulfate salt increased to 72.7% at 1 μg/ per well, while casein and soybean presented similar affinity of LPS, 28.7% and 22.7%, respectively (**Figure 5.4c**). At the highest concentration tested (256 μg/ per well, shown in **Figure 5.4d**), 83.9% of LPS bound with casein fractions. Among oilseed proteins, rapeseed and soybean scavenged 42.9% and 52.4% LPS, respectively (without significant difference). While whey was more promising to bind LPS (reduced 65% LPS content), compared with oilseeds, de Haas et al. (1999) also reported peptide fragments from Serum Amyloid P binding to LPS, with IC_{50} value of 0.06 μM to 4.41 μM. In addition, CM4, an anti-bacterial peptide with the sequence RWKIFKKIEKVGQNIRDGIVKAGPAVAVVGQAATI, was shown to almost completely suppress LPS binding at 5 -10 μM and significantly attenuated LPS-induced TNF-α, IL-6 and NO secretion in RAW 264.7 macrophages (Lin, Q.-P. et al., 2008).

The worth-mentioning LPS binding capabilities of rapeseed and soybean may directly reduce the LPS recognized via its receptors to the cell surface, whilst whey and casein failed to have any effects on the TLR4 and CD14 mRNA levels. Meanwhile, BSA also presented marked LPS binding activity, which increased to 70% at 4.0 μg/per well, and superior compared to all the tested oilseed and dairy protein hydrolysates. However, no evidence suggested that un-hydrolysed BSA can act as an anti-inflammatory agent.

This might be because the moiety of LPS that most contributes to the pathophysiological effects of LPS is the hydrophobic lipid A, located in the membrane-anchoring region. It is possible that BSA and peptides may bind to the other parts of LPS and fail to block lipid A recognized via the receptors. Therefore, these peptides cannot efficiently block the LPS biological activity and avoid triggering the cascade that results in the NF-κB activation. Rosenfeld et al. (2006) also claimed direct interaction of peptides with LPS in solution did not directly block the LPS biological activity. Only peptides with function of LPS-detoxifying (neutralization) could dissociate of LPS aggregating to the receptors.

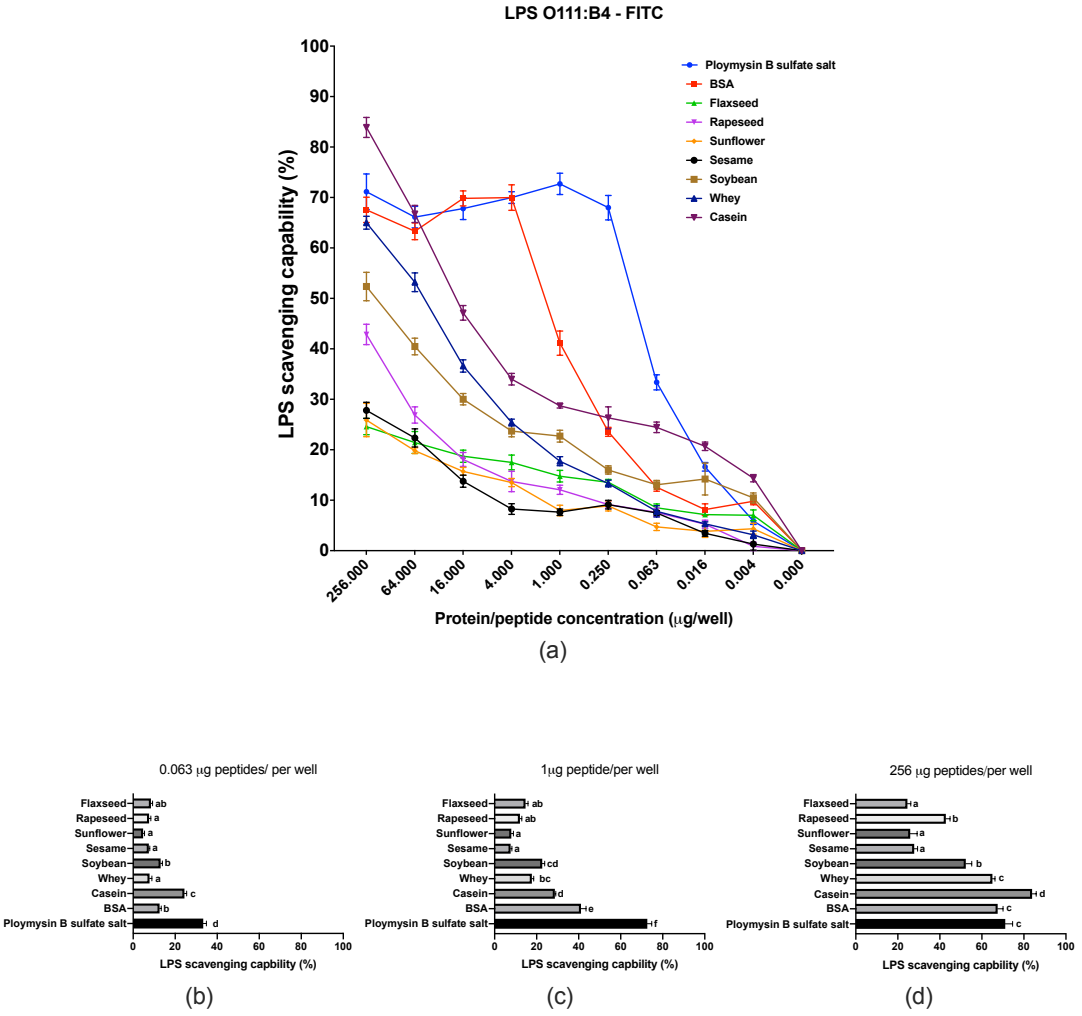

Figure 5.4 Concentration-response curves of low M_w (M_w < 3kDa) fractions of pepsin (pH 1.3)-treated protein hydrolysates interact with LPS-FITC. Data are shown as mean with SEM of three independent experiments performed in duplicate. Different letters indicate significant differences at p-value < 0.05.

5.3.6 Anti-inflammatory mechanisms of protein hydrolysates

Based on our experimental and literature findings, an overview of mechanisms involved in peptide molecular actions is proposed (Shown in **Figure 5.5**). The LPS-induced macrophage activation starts with LPS interacting with LPS-binding protein (LPS), which will accelerate the binding of CD14 (primary receptor of LPS) due to high affinity. The complex formed (LPS-LBP-CD14) will thereby become attached to the toll-like receptor-4 (TLR-4) to induce intracellular signalling. Meanwhile, the degradation of IκBα indued via LPS lead to the subunits of NF-κB are translocated into the nucleus. After nuclear translocation, NF-κB subunits promote the gene expression of pro-inflammatory cytokines and mediators (Liu et al., 2017).

Our study has demonstrated the potential mechanisms of protein hydrolysates/ peptides to attenuate LPS-induced inflammation. LPS can be scavenged via peptides and consequently the rapid recognition of LPS via the receptors is thus limited. In addition, the knockdown of TLR4 and CD14 (rapeseed and soybean) can further suppress the LPS-activated signalling. Meanwhile, the downregulation of NF- κB1 (p50) (sesame) and p65 (rapeseed and casein) reflected the NF- κB activation is efficiently attenuated. Regulation of IκBα (rapeseed and casein) expression indicates the phosphorylation of IκBα is inhibited. Consequently, the pro-inflammatory cytokines, including IL-6 (rapeseed) and IL-1β (rapeseed, sesame and casein), are reduced and anti-inflammatory cytokines, such as IL-10 (flaxseed, rapeseed, sunflower, sesame and casein), will be secreted. Meanwhile, mediators, such as, iNOS (rapeseed) and COX-2 (rapeseed) will be inhibited, which are expected to result in the reduction of NO and PGE2 in cultured medium.

However, apart from NF-κB pathway, MAPK signalling can also be activated via LPS stimulation. It mainly consists of three subtypes, which are p38, JNK and ERK. Each subtype plays a significant role in producing inflammatory cytokines and mediators. For example, the activation of p38 contributes to TNF-α generation, which may explain why rapeseed, sesame and casein suppressed the activation of NF-κB, but failed to reduce the TNF-α mRNA levels. In addition, JNK and ERK are also essential for the expression of iNOS and COX-2, respectively (Paul et al., 1999; Uto et al., 2005). LDAPGHR, a hazelnut derived peptide, was proved to suppress the MAPK signalling at 50 μM (Ren et al., 2018). In addition, Gao et al. (2021) reported a fraction from pepsin-treated sturgeon muscle efficiently decreased ERK1/2

levels by 25.5%, JNK by 29.7% and 46.29% of p38 phosphorylation at 0.5mg/mL.

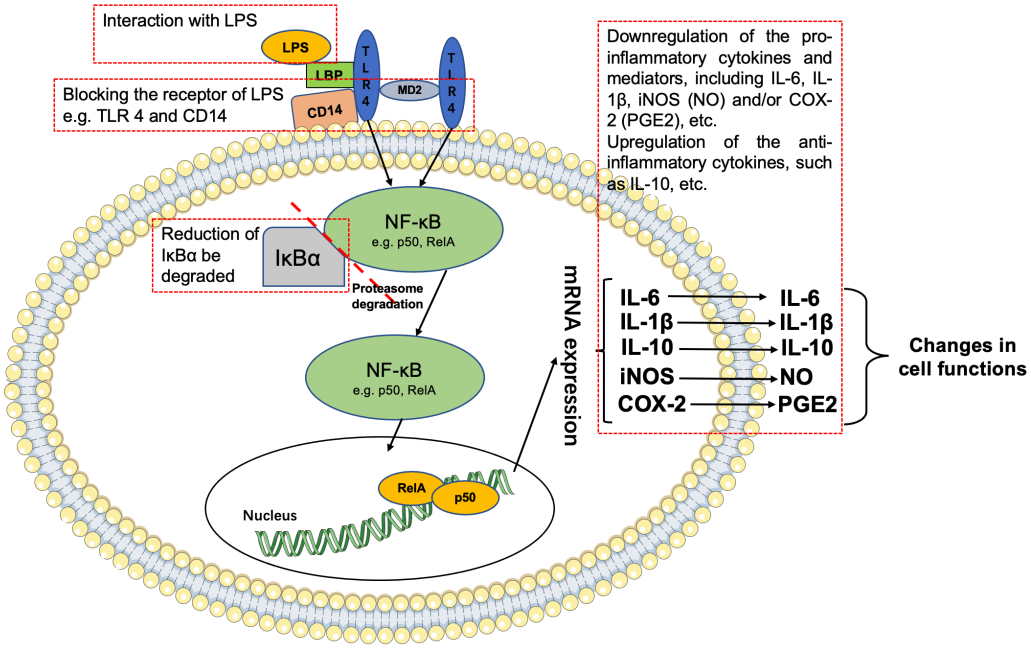

Figure 5.5 The potential anti-inflammatory mechanisms of hydrolysates in LPS-induced RAW 264.7 macrophages.

5.4 Conclusion

Our study shows that the major part of hydrolysates derived from oilseed protein effectively inhibited the SEAP secretion in RAW-Blue^{TM} reporter cells. Except the ones from flaxseed and sunflower, low M_w (M_w < 3kDa) fractions from pepsin (pH 1.3)-treated protein hydrolysates presented the most promising anti-inflammatory potential in RAW-Blue^{TM} reporter cells with inhibition ranging from 73.8% to 93%. The rapeseed fraction was considered as the most promising source of anti-inflammatory peptides since it efficiently downregulated the expression of pro-inflammatory cytokines and mediators in RAW 264.7 macrophages. Attenuation of NF-κB signalling was also confirmed for sesame and casein fractions. In addition, noticeable LPS binding capability were detected in rapeseed and soybean at 256 μg/well, but these were much lower compared with whey and casein. Further research efforts should be directed towards the characterization and purification of bioactive target fractions from these sources in order to understand the structure-function mechanisms of active peptides. These data will support the

development of formulations where peptide fractions could be incorporated and subsequently evaluated for *in vivo* efficacy to lower inflammatory activity in animals or humans.

References

Adeleke, B.S. and Babalola, O.O. 2020. Oilseed crop sunflower (Helianthus annuus) as a source of food: Nutritional and health benefits. *Food Science & Nutrition.* **8**(9), pp.4666-4684.

Aktan, F. 2004. iNOS-mediated nitric oxide production and its regulation. *Life sciences.* **75**(6), pp.639-653.

Aondona, M.M., Ikya, J.K., Ukeyima, M.T., Gborigo, T.w.J., Aluko, R.E. and Girgih, A.T. 2021. In vitro antioxidant and antihypertensive properties of sesame seed enzymatic protein hydrolysate and ultrafiltration peptide fractions. *Journal of Food Biochemistry.* **45**(1), pe13587.

de Haas, C.J., van der Zee, R., Benaissa-Trouw, B., van Kessel, K.P., Verhoef, J. and van Strijp, J.A. 1999. Lipopolysaccharide (LPS)-binding synthetic peptides derived from serum amyloid P component neutralize LPS. *Infection and immunity.* **67**(6), pp.2790-2796.

Domingues, M.M., Inácio, R.G., Raimundo, J.M., Martins, M., Castanho, M.A. and Santos, N.C. 2012. Biophysical characterization of polymyxin B interaction with LPS aggregates and membrane model systems. *Peptide Science.* **98**(4), pp.338-344.

Gao, R., Shu, W., Shen, Y., Sun, Q., Jin, W., Li, D., Li, Y. and Yuan, L. 2021. Peptide fraction from Sturgeon muscle by pepsin hydrolysis exerts anti-inflammatory effects in LPS-stimulated RAW264. 7 macrophages via MAPK and NF-κB pathways. *Food Science and Human Wellness.* **10**(1), pp.103-111.

Girón-Calle, J., Alaiz, M. and Vioque, J. 2010. Effect of chickpea protein hydrolysates on cell proliferation and in vitro bioavailability. *Food Research International.* **43**(5), pp.1365-1370.

González-Montoya, M., Hernández-Ledesma, B., Silván, J.M., Mora-Escobedo, R. and Martínez-Villaluenga, C. 2018. Peptides derived from in vitro gastrointestinal digestion of germinated soybean proteins inhibit human colon cancer cells proliferation and inflammation. *Food Chemistry.* **242**, pp.75-82.

Han, R., Álvarez, A.J.H., Maycock, J., Murray, B.S. and Boesch, C. 2021. Comparison of alcalase-and pepsin-treated oilseed protein hydrolysates–Experimental validation of predicted antioxidant, antihypertensive and antidiabetic properties. *Current research in food science.* **4**, pp.141-149.

Hao, Y., Fan, X., Guo, H., Yao, Y., Ren, G., Lv, X. and Yang, X. 2020. Overexpression of the bioactive lunasin peptide in soybean and evaluation of its anti-inflammatory and anti-cancer activities in vitro. *Journal of bioscience and bioengineering.* **129**(4), pp.395-404.

He, R., Girgih, A.T., Malomo, S.A., Ju, X. and Aluko, R.E. 2013. Antioxidant activities of enzymatic rapeseed protein hydrolysates and the

membrane ultrafiltration fractions. *Journal of Functional Foods.* **5**(1), pp.219-227.

He, R., Wang, Y., Yang, Y., Wang, Z., Ju, X. and Yuan, J. 2019. Rapeseed protein-derived ACE inhibitory peptides LY, RALP and GHS show antioxidant and anti-inflammatory effects on spontaneously hypertensive rats. *Journal of Functional Foods.* **55**, pp.211-219.

Hotamisligil, G.S. 2006. Inflammation and metabolic disorders. *Nature.* **444**(7121), pp.860-867.

Jacobs, M.D. and Harrison, S.C. 1998. Structure of an IκBα/NF-κB complex. *Cell.* **95**(6), pp.749-758.

Jaeger, W.K. and Siegel, R. 2008. Economics of oilseed crops and their biodiesel potential in Oregon's Willamette Valley.

Kim, Y.-S., Ahn, C.-B. and Je, J.-Y. 2016. Anti-inflammatory action of high molecular weight Mytilus edulis hydrolysates fraction in LPS-induced RAW264. 7 macrophage via NF-κB and MAPK pathways. *Food chemistry.* **202**, pp.9-14.

Li, E.W. and Mine, Y. 2004. Immunoenhancing effects of bovine glycomacropeptide and its derivatives on the proliferative response and phagocytic activities of human macrophagelike cells, U937. *Journal of agricultural and food chemistry.* **52**(9), pp.2704-2708.

Li, L.-H., Ju, T.-C., Hsieh, C.-Y., Dong, W.-C., Chen, W.-T., Hua, K.-F. and Chen, W.-J. 2017. A synthetic cationic antimicrobial peptide inhibits inflammatory response and the NLRP3 inflammasome by neutralizing LPS and ATP. *PLoS One.* **12**(7), pe0182057.

Lin, Q., Liao, W., Bai, J., Wu, W. and Wu, J. 2017. Soy protein-derived ACE-inhibitory peptide LSW (Leu-Ser-Trp) shows anti-inflammatory activity on vascular smooth muscle cells. *Journal of Functional Foods.* **34**, pp.248-253.

Lin, Q.-P., Zhou, L.-F., Li, N.-N., Chen, Y.-Q., Li, B.-C., Cai, Y.-F. and Zhang, S.-Q. 2008. Lipopolysaccharide neutralization by the antibacterial peptide CM4. *European journal of pharmacology.* **596**(1-3), pp.160-165.

Liu, T., Zhang, L., Joo, D. and Sun, S.-C. 2017. NF-κB signaling in inflammation. *Signal transduction and targeted therapy.* **2**(1), pp.1-9.

Livak, K.J. and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *methods.* **25**(4), pp.402-408.

Martínez-Sernández, V., Orbegozo-Medina, R.A., Romarís, F., Paniagua, E. and Ubeira, F.M. 2016. Usefulness of ELISA methods for assessing LPS interactions with proteins and peptides. *PLoS One.* **11**(6), pe0156530.

Matsuguchi, T., Musikacharoen, T., Ogawa, T. and Yoshikai, Y. 2000. Gene expressions of Toll-like receptor 2, but not Toll-like receptor 4, is induced by LPS and inflammatory cytokines in mouse macrophages. *The Journal of Immunology.* **165**(10), pp.5767-5772.

Morrison, D.C. and Jacobs, D.M. 1976. Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. *Immunochemistry.* **13**(10), pp.813-818.

Moure, A., Sineiro, J., Domínguez, H. and Parajó, J.C. 2006. Functionality of oilseed protein products: a review. *Food research international.* **39**(9), pp.945-963.

Nasry, W.H.S., Rodriguez-Lecompte, J.C. and Martin, C.K. 2018. Role of COX-2/PGE2 mediated inflammation in oral squamous cell carcinoma. *Cancers.* **10**(10), p348.

Nongonierma, A.B. and FitzGerald, R.J. 2015. Investigation of the potential of hemp, pea, rice and soy protein hydrolysates as a source of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides. *Food Digestion: Research and Current Opinion.* **6**(1-3), pp.19-29.

Paul, A., Cuenda, A., Bryant, C.E., Murray, J., Chilvers, E.R., Cohen, P., Gould, G.W. and Plevin, R. 1999. Involvement of mitogen-activated protein kinase homologues in the regulation of lipopolysaccharide-mediated induction of cyclo-oxygenase-2 but not nitric oxide synthase in RAW 264.7 macrophages. *Cellular signalling.* **11**(7), pp.491-497.

Ren, D., Wang, P., Liu, C., Wang, J., Liu, X., Liu, J. and Min, W. 2018. Hazelnut protein-derived peptide LDAPGHR shows anti-inflammatory activity on LPS-induced RAW264. 7 macrophage. *Journal of Functional Foods.* **46**, pp.449-455.

Repetto, G., Del Peso, A. and Zurita, J.L. 2008. Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nature protocols.* **3**(7), p1125.

Rosenfeld, Y., Papo, N. and Shai, Y. 2006. Endotoxin (lipopolysaccharide) neutralization by innate immunity host-defense peptides: peptide properties and plausible modes of action. *Journal of Biological Chemistry.* **281**(3), pp.1636-1643.

Sandoval-Sicairos, E.S., Milán-Noris, A.K., Luna-Vital, D.A., Milán-Carrillo, J. and Montoya-Rodríguez, A. 2021. Anti-inflammatory and antioxidant effects of peptides released from germinated amaranth during in vitro simulated gastrointestinal digestion. *Food Chemistry.* **343**, p128394.

Sangtanoo, P., Srimongkol, P., Saisavoey, T., Reamtong, O. and Karnchanatat, A. 2020. Anti-inflammatory action of two novel peptides derived from peanut worms (Sipunculus nudus) in lipopolysaccharide-induced RAW264. 7 macrophages. *Food & function.* **11**(1), pp.552-560.

Steimle, A., Autenrieth, I.B. and Frick, J.-S. 2016. Structure and function: Lipid A modifications in commensals and pathogens. *International Journal of Medical Microbiology.* **306**(5), pp.290-301.

Tornatore, L., Thotakura, A.K., Bennett, J., Moretti, M. and Franzoso, G. 2012. The nuclear factor kappa B signaling pathway: integrating metabolism with inflammation. *Trends in cell biology.* **22**(11), pp.557-566.

Udenigwe, C.C., Lu, Y.-L., Han, C.-H., Hou, W.-C. and Aluko, R.E. 2009. Flaxseed protein-derived peptide fractions: Antioxidant properties and inhibition of lipopolysaccharide-induced nitric oxide production in murine macrophages. *Food Chemistry.* **116**(1), pp.277-284.

Uto, T., Fujii, M. and Hou, D.-X. 2005. 6-(Methylsulfinyl) hexyl isothiocyanate suppresses inducible nitric oxide synthase expression through the inhibition of Janus kinase 2-mediated JNK pathway in lipopolysaccharide-activated murine macrophages. *Biochemical pharmacology.* **70**(8), pp.1211-1221.

Velliquette, R.A., Fast, D.J., Maly, E.R., Alashi, A.M. and Aluko, R.E. 2020. Enzymatically derived sunflower protein hydrolysate and peptides

inhibit NFκB and promote monocyte differentiation to a dendritic cell phenotype. *Food Chemistry.* p126563.

Vo, T.-S., Ryu, B. and Kim, S.-K. 2013. Purification of novel anti-inflammatory peptides from enzymatic hydrolysate of the edible microalgal Spirulina maxima. *Journal of Functional Foods.* **5**(3), pp.1336-1346.

Xie, C., Kang, J., Li, Z., Schauss, A.G., Badger, T.M., Nagarajan, S., Wu, T. and Wu, X. 2012. The açaí flavonoid velutin is a potent anti-inflammatory agent: blockade of LPS-mediated TNF-α and IL-6 production through inhibiting NF-κB activation and MAPK pathway. *The Journal of nutritional biochemistry.* **23**(9), pp.1184-1191.

Zhao, L., Wang, X., Zhang, X.-L. and Xie, Q.-F. 2016. Purification and identification of anti-inflammatory peptides derived from simulated gastrointestinal digests of velvet antler protein (Cervus elaphus Linnaeus). *journal of food and drug analysis.* **24**(2), pp.376-384.

Chapter 6
General discussion

The utilization of food-derived bioactive peptides as ingredients of functional foods has been highlighted as a promising strategy to beneficially impact chronic disease development (Chakrabarti et al., 2018; Singh et al., 2021). Although both, animal and plant proteins, are documented as sources of bioactive peptides, the research in this area has been focussing on peptides derived from animal sources, e.g., milk proteins. Therefore, knowledge on plant protein derived peptides is still incomplete and lacks systematic comparisons of biological capabilities of animal- and that of plant-protein hydrolysates and peptides. So far, there is no clear message on the equivalence of plant proteins to replace animal protein from the perspective of bioactive peptide release. In particular, milk proteins have been well-documented as good sources of bioactive peptides (FitzGerald and Meisel, 2003; Nongonierma and FitzGerald, 2015; Brandelli et al., 2015) with several epidemiological studies that have proven their health benefits in human studies (Xu et al., 2008; Sartorius et al., 2019). Therefore, milk protein may serve as a suitable reference in the evaluation of plant protein as sources of bioactive peptides, which was applied in the present study.

Currently, different types of research have been conducted to explore and identify peptides from plant proteins (Reviewed in **Chapter 2**). Among them, proteins from oilseeds, covering flaxseed, rapeseed, sunflower, sesame and soybean, showed potential to be used as sources of bioactive peptides, such as antioxidant (He et al., 2013), anti-hypertensive (Wang et al., 2020) and anti-diabetic (Lammi et al., 2016) peptides. Therefore, in this project, oilseed proteins were selected as an example category of plant proteins, to be compared with milk proteins (whey and casein). Additionally, the anti-inflammatory activity of oilseed peptides was explored, which is less documented in the available literature.

In view of above, the potential of oilseeds to release ACE and DPP-IV inhibitory peptides under separate *in silico* alcalase and pepsin (pH 1.3 and pH > 2) hydrolysis was predicted via the potency indices, calculated with the BIOPEP tool database (**Chapter 3**). ACE and DPP-IV play a key role as a strategy for prevention and treatment of hypertension and type 2 diabetes, respectively, and have indeed been shown to have strong presence in many hydrolysed samples. On the other hand, PeptideRanker was used to align

scores. The values of scores for each peptide directly reflect the likelihood of them being active. Novel ACE and DPP-IV inhibitory peptides were subsequently predicted according to the high score (score > 0.8) obtained via PeptideRanker and interaction between these peptides and enzymes (ACE and DPP-IV) were predicted via Pepsite2. Since *in silico* prediction cannot be directly linked with *in vitro* data, a range of in *vitro* bioassays were conducted to confirm the biological activity of protein hydrolysates and their low M_w fractions (M_w < 3kDa) (**Chapter 4**): *in vitro* ACE and DPP-IV inhibitory capabilities of oilseed peptides were compared with their *in silico* potency indices. Besides, *in vitro* TEAC radical scavenging capability, ferric reducing power and α-glucosidase inhibitory capability of tested samples were compared with the *in silico* data, which is the average scores (aligned via PeptideRanker) of peptide profiles in given protein sequences. The last part of the study was to explore the effect of oilseed peptides in modulating LPS-induced inflammation in macrophages, cellular models of inflammation (**Chapter 5**). The anti-inflammatory activity of protein hydrolysates and their three different M_w fractions (M_w > 10 kDa, 3 – 10 kDa and < 3kDa) were screened in RAW-Blue^{TM} cells via the inhibition of SEAP stimulated in cultured medium. Accordingly, low M_w fractions of pepsin (pH 1.3)-treated protein hydrolysates were selected to confirm their anti-inflammatory activities via modulating the gene expression of anti- and pro-inflammatory cytokines and mediators. Moreover, the mechanisms behind the anti-inflammatory activities of oilseed peptides, including modulating relevant transcription factors and LPS binding capability were studied. For all the *in silico* and *in vitro* analysis, dairy proteins (milk, whey and casein) were used as comparisons to evaluate the role of oilseeds in liberating promising bioactive peptides.

6.1 Concluding outcomes of the comparison between oilseed and dairy proteins in releasing bioactive peptides

6.1.1 Degree of hydrolysis

In silico hydrolysis, the specificity of protease determines the peptide profiles liberated from the given protein sequences, thus, the amino acid composition is the only factor to affecting the *in silico* DH. According to **Table 4.1**, Colinin (Flaxseed, 22.0% and 8.9%), 2S storage protein (Sunflower, 16.0% and 8.5%) and 2S storage protein (Sesame, 22.4% and 8.8%) presented lower value of DH after subtilisin and pepsin (pH 1.3) hydrolysis, compared with the DH outcomes of three milk proteins, ranges from 28.2 -

33.6% and 10.6 - 17.5%, respectively. In contrast, the remaining oilseed samples demonstrated a similar DH compared to diary proteins. Importantly, the M_w of almost all the peptide profiles was below 3 kDa.

Regarding *in vitro* experimental hydrolysis, it is much more complex than the *in silico* prediction. When compared with animal-derived protein hydrolysis, applying the same conditions, plant proteins are harder to be hydrolysed/digested, which is due to several factors. The structure of plant proteins overall contains less α-helixes and more β-sheets. Also, plant proteins show more conjugation and worse solubility (more hydrophobic amino acids), as well as interacting with fibre, carbohydrates, phenolics and some others. In addition, plant proteins contain antinutrients such as protease inhibitors, which will also have a negative effect on protein digestibility and bioavailability (Duodu et al., 2003; Hwang et al., 1977; Ismail et al., 2020). The DH of whey and casein is much higher than that of oilseed proteins after alcalase treatment (**Table 4.1**). However, after pepsin (pH 1.3 and pH 2.1) treatment, more peptides were released from flaxseed, compared with diary proteins. Given the long hydrolysis time that was applied in the present study, the prolonged enzymatic activity might have resulted in increased peptide cleavage in the inner structures of plant proteins.

To conclude, plant proteins are harder to be hydrolysed via alcalase, but present the same or even higher digestibility with pepsin treatment. The proteins present in both oilseed and dairy protein isolates/concentrates could not be hydrolysed completely via proteolysis, because the fractions with M_w > 10 kDa and 3 - 10 kDa were separated via ultrafiltration. The existence of peptides with such M_w were not predicted by the pervious bioinformatic analysis. Furthermore, only a few peptide sequences predicted via i*n silico* prediction were identified via LC-MS/MS.

6.1.2 Biological capabilities

After *in silico* hydrolysis, peptide profiles released from parent protein sequences were matched to the peptides recorded in BIOPEP databases to calculate their potency indices (μM^{-1}) of ACE and DPP-IV inhibitory peptides. As shown in **Table 6.1**, compared with the highest values of ACE inhibitory potency indices (μM^{-1}) among oilseed and dairy proteins under the same protease treatment, some oilseeds are able to release more promising ACE inhibitory peptides. To the contrary, dairy protein hydrolysates were predicted to present stronger DPP-IV inhibitory capabilities. Since the highest value of

average score among oilseed protein sequences is higher than the ones calculated from dairy proteins, it is possible that oilseed protein sequences can release peptides with noticeable biological activities. Therefore, *in silico* prediction supported the hypothesis that oilseed proteins, such as rapeseed, sesame and soybean, can be potential sources of high-quality bioactive peptides, especially for ACE inhibitory peptides.

The next step was to validate the predicted outcomes using a wide range of *in vitro* bioassays. *In vitro* ACE inhibitory assays reflect that there is no significant difference between the strongest inhibition found in oilseeds and dairy protein hydrolysates (with M_w < 3kDa). Correlated with *in silico* findings, casein proved to exert promising DPP-IV inhibition, stronger than the ones of oilseed protein hydrolysates. Regarding antioxidant capabilities, only alcalase-treated soybean presented a robust ferric reducing power, higher than the ones of dairy proteins. Peptides derived from dairy protein contributed to the higher ABTS scavenging capability and ferric reducing power, which is opposite to the average score aligned via the PeptideRanker. Nevertheless, oilseed peptides, such as soybean peptides, presented effective α-glucosidase inhibition after enzymatic hydrolysis, compared to the ones from casein and whey.

After alcalase hydrolysis, flaxseed peptides contributed to more promising anti-inflammatory potential. Meanwhile, some oilseeds showed comparable inhibition in SEAP secretion in cultured medium (LPS -stimulated RAW-Blue^{TM} cells) with dairy proteins under the pepsin treatment. Low M_w fractions of pepsin (pH 1.3)-treated samples on RAW 264.7 macrophages confirmed their anti-inflammatory capability, rapeseed and sesame exerted the ability to modulate mRNA levels of several inflammatory related cytokines and/or mediators and NF-κB signalling, which is comparable to the ones of casein.

Taken together, *in silico* prediction strongly suggested oilseed proteins as potential sources of bioactive peptides, especially ACE inhibitory peptides. Whilst *in vitro* data did not support this finding, it suggested that oilseeds (particularly soybean) may release more promising α-glucosidase inhibitory peptides. Regarding ACE inhibitory and anti-inflammatory effects, only a few oilseeds (e.g., rapeseed) exerted comparable capability with dairy protein hydrolysates.

6.2 Limitations

The protein content and purity of the different sources used differs among them, from flours rich in protein to protein isolates and concentrates, ranging from 46.4 ± 1.2% to 92.4 ± 1.0%. It is almost impossible to match all the compounds in the starting material except for variety of proteins. Also, the alkaline extraction progress tends to recycle more types of proteins from defatted oilseed cakes than expected. Consequently, the bioactive properties and capability of extracted protein isolate/concentrates become less predictable after protease treatments. Nevertheless, from the proteins extracted by alkaline extraction, novel bioactive peptides may also be released.

In vitro bioassays test the “biological” activities of protein hydrolysates in controlled conditions followed by a given reaction, rather than the complex conditions linked to living organisms. Even though the major conditions of the physiological process in human body are carefully simulated, there are still many details in the complex human systems that cannot be accounted for in this relatively simplified *in vitro* assays.

Table 6.1 List of most promising oilseed and dairy peptides/protein hydrolysates.

		Alcalase	**Pepsin (pH 1.3)**	**Pepsin (pH > 2)**
Potency indices (x 10^{-3} μM^{-1}) of ACE inhibitory peptides	Oilseed	3.9421 (curciferin, rapeseed)	0.9543 (2S storage protein, sesame)	6.2213 (napin, rapeseed)
	Dairy	1.4124 (β-lactoglobulin, whey)	0.6728 (β-casein, casein)	4.6858 (κ-casein, casein)
Potency indices (x 10^{-4} μM^{-1}) of DPP-IV inhibitory peptides	Oilseed	1.985 (curciferin, rapeseed)	0.050 (11S globulin, sesame)	2.536 (2S storage protein, sesame)
	Dairy	2.997 (κ-casein, casein)	0.887 (β-lactoglobulin, whey)	3.248 (β-lactoglobulin, whey)
Average score	Oilseed	0.1628 (napin, rapeseed)	0.0748 (β-conglycinin, α-chain, soybean)	0.2673 (2S storage protein, sesame)
	Dairy	0.1236 (β-casein, casein)	0.0701 (β-casein, casein)	0.2046 (β-lactoglobulin, whey)
Inhibition (%) of ACE activity	Oilseed	71.52 ± 5.56 (< 3 kDa, sunflower)	76.61 ± 4.62 (< 3 kDa, soybean)	76.53 ± 0.97 (< 3 kDa, rapeseed)
	Dairy	77.85 ± 4.37 (< 3 kDa, casein)	80.35 ± 1.51 (< 3 kDa, casein)	77.08 ± 7.26 (< 3 kDa, casein)
Inhibition (%) of DPP-IV activity	Oilseed	28.22 ± 2.94 (whole, rapeseed)	39.71 ± 7.95 (whole, sesame)	34.89 ± 6.28 (whole, soybean)

		Alcalase	Pepsin (pH 1.3)	Pepsin (pH > 2)
	Dairy	43.87 ± 5.97* (whole, whey)	68.12 ± 7.53* (whole, whey)	62.27 ± 6.24* (whole, whey)
ABTS radical scavenging capability (TE mM/g)	Oilseed	4.55 ± 0.10 (< 3 kDa, rapeseed)	4.27 ± 0.15 (< 3 kDa, soybean)	4.30 ± 0.17 (< 3 kDa, soybean)
	Dairy	7.40 ± 0.27* (< 3 kDa, whey)	5.41 ± 0.18* (< 3 kDa, whey)	5.94 ± 0.11* (< 3kDa, whey)
Ferric reducing power (TE mM/g)	Oilseed	1.83 ± 0.06 (< 3 kDa, soybean)	0.86 ± 0.03 (< 3 kDa, rapeseed)	0.87 ± 0.02 (< 3 kDa, rapeseed)
	Dairy	1.39 ± 0.06* (< 3 kDa, casein)	1.32 ± 0.03* (< 3 kDa, whey)	1.27 ± 0.03* (< 3 kDa, casein)
Inhibition (%) of α-glucosidase	Oilseed	54.58 ± 4.53 (whole, soybean)	59.30 ± 3.44 (whole, soybean)	68.11 ± 2.78 (< 3 kDa, soybean)
	Dairy	46.61 ± 2.97* (< 3 kDa, casein)	46.26 ± 2.76* (< 3 kDa, whey)	40.73 ± 2.81* (< 3kDa, casein)
Inhibition (%) of SEAP secretion	Oilseed	81.5 ± 1.8 (< 3 kDa, flaxseed)	88.7 ± 1.6 (< 3 kDa, sesame)	96.1 ± 1.8 (< 3 kDa, sesame)
	Dairy	55.5 ± 1.5* (< 3 kDa, casein)	90.2 ± 3.0 (< 3 kDa, whey)	89.2 ± 2.9 (< 3 kDa, casein)

* Indicates significant differences compared with oilseed and dairy protein hydrolysates ($p < 0.05$)

6.3 Conclusion

To conclude, the antioxidant, anti-diabetic, anti-hypertensive and/or anti-inflammatory activities of oilseed protein hydrolysates, together with their different M_w (M_w > 10 kDa, 3 - 10 kDa, < 3 kDa) fractions were evaluated in this project. *In silico* analysis indicated that oilseed proteins maybe good sources of bioactive peptides, especially for ACE inhibitory activity. Despite limited correlations, *in vitro* tests confirmed *in silico* findings that, oilseeds, especially soybean, could be considered as a comparable source of peptides with ACE and α-glucosidase inhibitory potential. In addition, a M_w fraction < 3 kDa of pepsin (pH 1.3)-treated rapeseed protein hydrolysates efficiently attenuated LPS-induced inflammation in RAW-Blue^{TM} and RAW 264.7 macrophages. Therefore, oilseed proteins, in particular rapeseed and soybean, are affordable, sustainable and environmental-friendly. Even though, for soybean, there has been some controversy regarding its environmental impact (Fearnside, 2001). Rapeseed and soybean proteins are promising sources of bioactive peptides with potential health benefits. Thus, although their correlation is overall somewhat limited, both *in silico* and *in vitro* findings support the idea that oilseeds can be used as sources of bioactive ingredients for development of functional, nutraceutical and pharmaceutical applications.

6.4 Future perspectives

Despite promising *in vitro* experimental findings, there is still a long way to transfer these plant-based peptides/protein hydrolysates into edible commercial products. In order to achieve this, several research aspects are suggested for future work.

- To fully identify all the peptides generated through the enzymatic hydrolysis procedures, plus further purification and fractionation in order to clearly identify and enrich the targeted bioactive peptides.

- To investigate the bioavailability of the peptides i.e. the changes in the peptide profiles achieved under more realistic digestion conditions such as application of in vitro digestion models.

- To test peptide profiles resulting from application of other proteases, such as trypsin, papain and flavourzyme and to explore other biological activities related to hypocholesterolaemic, anti-obesity, antibacterial, anticancer and antithrombotic properties.

- To investigate in vivo efficacy of plant protein peptide interventions in animals and/or humans, which would validate the in silico and in vitro findings.

- To develop hydrolysate/peptide containing formulations and food products and to explore sensory properties of these to determine suitability for human consumption, as texture and flavour changes, e.g. through production of umami flavour are impacting food quality.

- To ensure food safety status for peptide-based ingredients/products and establish safe consumption levels of these.

- To optimize experimental hydrolysis protocols to maximize yield and minimize use of enzymes in order to reduce the cost and to translate and upscale these technologies to industrial scale level.

References

Brandelli, A., Daroit, D.J. and Corrêa, A.P.F. 2015. Whey as a source of peptides with remarkable biological activities. *Food Research International.* **73**, pp.149-161.

Chakrabarti, S., Guha, S. and Majumder, K. 2018. Food-derived bioactive peptides in human health: Challenges and opportunities. *Nutrients.* **10**(11), p1738.

Duodu, K., Taylor, J., Belton, P. and Hamaker, B. 2003. Factors affecting sorghum protein digestibility. *Journal of cereal science.* **38**(2), pp.117-131.

Fearnside, P.M. 2001. Soybean cultivation as a threat to the environment in Brazil. *Environmental Conservation.* pp.23-38.

FitzGerald, R. and Meisel, H. 2003. Milk protein hydrolysates and bioactive peptides. *Advanced dairy chemistry—1 Proteins.* Springer, pp.675-698.

He, R., Girgih, A.T., Malomo, S.A., Ju, X. and Aluko, R.E. 2013. Antioxidant activities of enzymatic rapeseed protein hydrolysates and the membrane ultrafiltration fractions. *Journal of Functional Foods.* **5**(1), pp.219-227.

Hwang, D.L.-R., Lin, K.-T.D., Yang, W.-K. and Foard, D.E. 1977. Purification, partial characterization, and immunological relationships of multiple low molecular weight protease inhibitors of soybean. *Biochimica et Biophysica Acta (BBA)-Protein Structure.* **495**(2), pp.369-382.

Ismail, B.P., Senaratne-Lenagala, L., Stube, A. and Brackenridge, A. 2020. Protein demand: review of plant and animal proteins used in alternative protein product development and production. *Animal Frontiers.* **10**(4), pp.53-63.

Lammi, C., Zanoni, C., Arnoldi, A. and Vistoli, G. 2016. Peptides derived from soy and lupin protein as dipeptidyl-peptidase IV inhibitors: in vitro biochemical screening and in silico molecular modeling study. *Journal of agricultural and food chemistry.* **64**(51), pp.9601-9606.

Nongonierma, A.B. and FitzGerald, R.J. 2015. The scientific evidence for the role of milk protein-derived bioactive peptides in humans: A Review. *Journal of Functional Foods.* **17**, pp.640-656.

Sartorius, T., Weidner, A., Dharsono, T., Boulier, A., Wilhelm, M. and Schön, C. 2019. Postprandial effects of a proprietary milk protein hydrolysate containing bioactive peptides in prediabetic subjects. *Nutrients.* **11**(7), p1700.

Singh, B.P., Aluko, R.E., Hati, S. and Solanki, D. 2021. Bioactive peptides in the management of lifestyle-related diseases: Current trends and future perspectives. *Critical Reviews in Food Science and Nutrition.* pp.1-14.

Wang, R., Lu, X., Sun, Q., Gao, J., Ma, L. and Huang, J. 2020. Novel ACE inhibitory peptides derived from simulated gastrointestinal digestion in vitro of sesame (Sesamum indicum L.) protein and molecular docking study. *International journal of molecular sciences.* **21**(3), p1059.

Xu, J.-Y., Qin, L.-Q., Wang, P.-Y., Li, W. and Chang, C. 2008. Effect of milk tripeptides on blood pressure: a meta-analysis of randomized controlled trials. *Nutrition.* **24**(10), pp.933-940.

Appendix A
Supporting information for Chapter 2

Table A.1 *In silico* prediction of bioactive peptides derived from plant proteins

Sample	*In silico* prediction	Peptide(s)	Bioactive properties	*In vitro* / *in vivo*	Comments	References
Zein (Excipient)	Digestion and Function	Trypsin/chymotrypsin (5120 peptides), Trypsin/thermolysin (1151 peptides) and Trypsin/LysC (130 peptides)	ACE inhibitory, DPP-IV inhibitory, antioxidant, etc.	420 peptides derived from 71 protein were identified via LC-MS/Ms analysis, from which 116 peptides were predicted	*In vitro* validation of peptides released predicted via *in silico* digestion	(Li, Menglin et al., 2020)
Microalgae (*Isochrysis Zhanjiangensis*)	Molecular docking	FEIHCC	ACE inhibitory	Combined *in vitro* and molecular docking to examine the ACE inhibitory capability of peptides and explore the inhibitory mechanism	*In silico* analysis and *in vitro* experiments for mechanism analysis	(Chen, J. et al., 2020)
Pea	Evaluvation of peptide activity	14 peptides, in which, 3 peptides (YSSPIHIW, ADLYNPR, HYDSEAILF) have a score > 0.5 aligned via Peptideranker	Antioxidant	No validation	Using Peptideranker to select most promising peptides to contribute to antioxidant activity	(Ding et al., 2020)

Sample	*In silico* prediction	Peptide(s)	Bioactive properties	*In vitro* / *in vivo*	Comments	References
					in pea protein hydrolysates	
Naked Oat (globulin)	Screening peptides and molecular docking	SSYYPRK	ACE inhibitory, Renin inhibitory and endothelin-1 suppressing capability	Validating the SSYYPRK present in protein hydrolysates, confirming the bioactive properties of peptides	*In silico* analysis assisted to select SSYYPPK peptide from 14 peptides, and predicted its anti-hypertensive mechanism, being the same as captopril	(Zheng et al., 2020a)
Red Alga (*Grateloupia asiatica*)	Digestion and Screening ACE inhibitory peptides	190 potential ACE inhibitory peptides can be derived from chloroplast proteins, which 21 peptides can be derived via in silico thermolysin digestion	ACE inhibitory	Authors claimed *in vitro* ACE activity correlated with *in silico* prediction	Authors did not clearly explain the correlation between *in silico* and *in vitro* analysis (	(Sumikawa et al., 2020)
Brown seaweed (*Sargassum maclurei*)	Screening peptides and molecular docking	RWDISQDY	ACE inhibitory	Validating the ACE inhibitory properties of RWDISQDY,showing this peptide can reduce intercellular Endthelin-1.	*In silico* analysis assisted to select the RWDISQDY from 17 peptides, and predicted the anti-hypertensive mechanism of	(Zheng et al., 2020b)

Sample	*In silico* prediction	Peptide(s)	Bioactive properties	*In vitro* / *in vivo*	Comments	References
					RWDISQDY which binds to active sites S1 and S2 of ACE	
Quinoa	Digestion, ACE and DPP-IV peptides screening, ranking predicted peptides	MAF, NMF, HPG and MCG	ACE inhibitory, DPP-IV inhibitory	ACE and DPP-IV inhibitory properties of four peptides were confirmed via *in vitro* bioassays	*In silico* analysis predicted four peptides which exerted ACE and DPP-IV inhibitory activity, validated via *in vitro*	(Guo et al., 2020)
Quinoa Bran	Screening ACE and Antioxidant peptides from 12 peptides	RGQVIYVL (ACE inhibitory and Antioxidant) ASPKPSSA (Antioxidant) QFLLAGR (Antioxidant)	ACE inhibitory, Antioxidant	*In vitro/ in vivo* experiments confirmed ACE and antioxidant properties of predicted bioactive peptides, as well as the competitive mechanism of RGQVIYVL	*In silico* analysis assisted to select peptides obtained via LC-MS/MS, in addition, *in vitro* bioassay confirmed the inhibitory mode predicted using molecular docking	(Zheng et al., 2019b)
Flaxseed	Hydrolysis, screening antioxidant	In total, 253 antioxidant peptides screened from 23 mature flaxseed storage proteins	Antioxidant	None	Antioxidant *In silico* peptides screening in flaxseed, without	(Ji, D. et al., 2019)

Sample	*In silico* prediction	Peptide(s)	Bioactive properties	*In vitro* / *in vivo*	Comments	References
	peptides, predicting toxicity and allergenicity				validation	
Soybean	Hydrolysis, screening tripeptides, ranking peptides, predicting toxicity and solubility, molecular docking	161 tripeptides screened, from which 7 peptides HDW, EGW, DMG, LPR, CIT, DTW and MDY were used for molecular docking. EGW, DMG and DTW were synthesized for ACE inhibitory assessment and docking	ACE inhibition	DMG proved to exert promising ACE inhibitory activity	*In silico* analysis assisted to select 3 peptides from 161 tripeptides using several *in silico* tools	(Zhao et al., 2019)
Sea grass (*Halophila stipulacea*)	Screening bioactive peptides	All peptide profiles released from a protein sequence (H6TAS9)	Antioxidant, ACE, DPP-IV, hypolipidemic, etc	None	*In silico* screening of bioactive peptides, without validation	(Kandemir-Cavas et al., 2019)
Saccharomyces cerevisiae	Physicochemical characteristics and molecular docking	YGKHVAVHAR (YHR-10), CKPVAVPA (GA-8), PAR (PAR-3), GKPVAVPA (GA-8)	ACE inhibitory	All five peptides exerted *in vitro* ACE inhibitory activity. *In vitro* analysis showed that YR-10, YHR-10 and GHA-8 are non-competitive inhibitors. Molecular docking analysis predicted that YHR-10 exerted strongest ACE inhibitory activity, linked to h-bonding.	*In silico* analysis aided to predict the ACE inhibitory properties and inhibitory mechanisms of synthesized peptides	(Mirzaei et al., 2019)
Tuber storage proteins (potato,	Digestion, screening peptide	387 bioactive peptides predicted	DPP-IV, ACE, antioxidant,	none	Screening bioactive peptides	(Ibrahim et al., 2019)

Sample	*In silico* prediction	Peptide(s)	Bioactive properties	*In vitro* / *in vivo*	Comments	References
sweet potato, yam and taro)	profiles, ranking peptides, toxicity prediction		antimicrobial, etc		using *in silico* tools	
Chia Seed (*Salvia hispanica L.*)	Physicochemical properties Molecular docking	TAQEPTIRF, PGLTIGDTIPNL, LSLPNYHPNPRL, LIVSPLAGRL, IVSPLAGRL	ACE inhibition	none	Predicted the mechanism of peptides to inhibit ACE by interaction with its catalytic site	(San Pablo-Osorio et al., 2019)
Cocoa	Screening ACE inhibitory peptides Molecular docking	19 peptides were predicted as ACE inhibitory peptides. Two tripeptides (VPI & SPV) were valeted *in vitro*	ACE inhibition	VPI and SPV were very weak inhibitors	Predicted ACE inhibitory peptides, while poor *in vitro* ACE inhibition was found.	(Marseglia et al., 2019)
Microalgae (*Chlorella sorokiniana*)	Hydrolysis, screening bioactive peptides	Peptide profiles released from 4 identified protein sequences, using 15 different enzymes	Antioxidant, ACE and DPP-IV	None	Use of *in silico* tools to predict peptide profiles from identified proteins, to rapid selection the most promising sequences	(Tejano et al., 2019)
Green Macroalga (*Ulva*	Digestion, screening bioactivity	16 di-/tri- bioactive peptides were found after GI enzymatic simulation.	Mainly ACE and DPP-IV	None	Papain hydrolysis was investigated	(Mirzaei et al., 2019)

Sample	*In silico* prediction	Peptide(s)	Bioactive properties	*In vitro* / *in vivo*	Comments	References
lactuca)	after simulation as well as toxicity and allergenicity. Ranking the peptides	70 novel peptides were predicted, from which probably 42 peptides were non-allergenic. Two novel peptides GPPPPSP and GTF showed over 0.8, no peptide exerted toxicity	inhibitory properties		by *in vitro* experiments and *in silico* tools to predict the stability of peptides after digestion, as well as the toxicity and allergenicity. Selecting peptides with high scores, but without proper validation	
Red Alga (*Palmaria sp.)*	Compared two protein sequences (plastid proteins in *Palmaria* sp. and *proximate AA* in P.palmata) in structure , amino acid composition and ACE inhibitory peptides.	A total of 742 peptides from *p.palmata* while 751 peptides from *palmaria* sp. The number of ACE inhibitory peptides was similar	ACE	None	Used different *in silico* tools to compare the difference among two given amino acid sequences from 2 proteins.	(Kumagai et al., 2019)
Oilseed	Comparison of similarity and amino	Peptides released from 15 given protein sequences. 105 novel	ACE and DPP-IV	None	Systematic *in silico* comparison of	(Han et al., 2019)

Sample	*In silico* prediction	Peptide(s)	Bioactive properties	*In vitro* / *in vivo*	Comments	References
	acid composition of selected protein sequences. Pepsin and subtilisin *in silico* hydrolysis, screening bioactive peptides, ACE and DPP-IV peptides prediction. Stability of peptides under GI digestion	peptides were found, only 21 peptides showed stability to GI digestion.			oilseeds and bovine proteins as sources of ACE and DPP-IV inhibitory peptides	
Ribosomal Hazelnut	*In silico* digestion and hydrolysis, screening bioactive peptides (e.g. ACE and DPP-IV), peptides ranking	Peptide profiles released from 23 ribosomal hazelnut proteins using 6 different proteases	Major ACE and DPP-IV inhibitory properties	None	*In silico* evaluation of hazelnut as source of bioactive peptides after hydrolysis with proteases	(Gülseren, 2018)
Bambara bean	Similarity of each protein sequences, *in silico* hydrolysis and ACE, antioxidant and DPP-IV inhibitors	Predict the similarity of storage proteins and bioactive properties of 5 LC-EST-TOF-MS fractions	Antioxidant, ACE and DPP-IV	None	*In silico* prediction and *in vitro* assessment of peptides released from thermolysis hydrolysis	(Mune et al., 2018)

Sample	*In silico* prediction	Peptide(s)	Bioactive properties	*In vitro* / *in vivo*	Comments	References
	screening				contributing to antioxidant, ACE and DPP-IV inhibitory activity.	
Chlorella vulgaris	*In silico* digestion, screening of ACE inhibitory peptides (di- or tripeptides), molecular docking	10 tri-peptides were selected from the peptide profiles, due to high ACEI inhibition potential. TTW and VHW were selected as novel stronger ACE inhbitors	ACE inhibitory	The ACE inhibition of targeted peptides was assessed *in vitro* and *in vivo*. Whilst the predicted inhibitory mechanism through active site binding via hydrogen bonds was not validated	*In silico* analysis to mimic the hydrolysis and selection of promising ACE candidates, by means of *in vitro* assays to validate ACE inhibition and selection of two strong inhibitors. Molecular docking analysis showed TTW and VHW interact with ACE by active bonding, but *in vitro* results showed that VHW is more stable.	(Xie et al., 2018)
Wheat	Screening opioid tri-	10 opioid peptides were identified	Opioid	*In vitro* data validated the opioid	*In silico* analysis	(Garg et al.,

Sample	*In silico* prediction	Peptide(s)	Bioactive properties	*In vitro* / *in vivo*	Comments	References
	and oligo peptides with Y (tyrosine) and P (proline), bioactivity prediction.	within sequences. YPG, YYPG and YIPP were selected to undergo *in vitro* experiments, due to high scores via peptide ranker		activity of YPG, YYPG and YIPP	assisted to select three potential promising peptides and proved *in vitro* activity	2018)
Rice bran	*In silico* homology and of selected protein sequence. Screening bioactive peptides (especially DPP-IV), toxicity and allergenicity prediction	Peptide profiles produced via *in silico* hydrolysis (pepsin, ficain, papain and bromelain)	Mainly focus on DPP-IV	None	Use of *in silico* tools to evaluate whether rice bran can be source of bioactive peptides, especially DPP-IV inhibitors.	(Pooja et al., 2017a)
Pigeon pea (*Cajanus cajan*)	Molecular docking	Val-Val-Ser-Leu-Ser-Ile-Pro-Arg	ACE inhibition	This peptide is a competitive inhibitor	Use of molecular docking analysis to study the interaction between VVSLSIPR and ACE and predicted their strong and stable interaction.	(Nawaz et al., 2017)
Oat (*Avena sativa*)	Hydrolysis, bioactivity	FFG, IFFFL, PFL, WWK, WCY, FPIL, CPA, FLLA, and FEPL	Renin, ACE and DPP-IV	IFFFL, WCY, PFL, FLLA AND WWK exerted renin inhibition. FFG, IFFL,	Used *in silico* analysis to narrow	(Bleakley et al., 2017)

Sample	*In silico* prediction	Peptide(s)	Bioactive properties	*In vitro* / *in vivo*	Comments	References
	prediction, ranking peptides			PFL, WWK, WCY, FLLA, FEPL exerted ACE inhibition. FPIL, CPA exerted DPP-IV inhibition	down the peptides for *in vitro* testing	
Chlorella vulgaris	Screening *chlorella vulgaris* proteins, digestion, screening DPP-IV inhibitory peptides, molecular docking	VPW, IPR, VPA, IPL	DPP-IV inhibition	Four peptides were proved to exert DPP-IV inhibitory activity, VPW and IPR were selected for further analysis due their low IC_{50}. *In vitro* inhibition mode is aligned with molecular docking analysis	In silico analysis helped to Peptide selection and prediction aided to explain the inhibitory mode and VPW higher activity than IPR	(Zhu, Q. et al., 2017)
Green algae *caulerpa*	Homology analysis, Hydrolysis, screening bioactive peptides, ranking peptides	Peptide profiles in protein and peptide release via *in silico* hydrolysis	ACE, DPP-IV, antioxidant, neuroprotective, etc	None	*In silico* evaluation whether green algae *caulerpa* can be a source of ACE inhibitory peptides	(Agirbasli and Cavas, 2017)
Black bean	Molecular docking	AKSPLF, ATNPLF, FEELN LSKSVL	Hypoglycaemic	*In vitro* and *in vivo* assay validated the hypoglycaemic properties of four peptides	Use of *in silico* analysis to screen most promising candidates for further *in vitro* and *in vivo* study	(Mojica et al., 2017a)
Rice bran	*In silico* homology analysis, screening	Peptide profiles in protein sequences and peptide release via *in silico*	Mainly ACE	None	*In silico* evaluation whether rice bran	(Pooja et al., 2017b)

Sample	*In silico* prediction	Peptide(s)	Bioactive properties	*In vitro* / *in vivo*	Comments	References
	bioactive peptides, screening ACE inhibitory peptides, sensory characteristics, toxicity and allergenicity predictions	hydrolysis			can be a source of ACE inhibitory peptides	
Cumin seed	Screening and ranking bioactive peptides, prediction of α-amylase binding site	WWQCHSFKLPDDGDLLCCW, CCCCSHSPLALNSAQEMCM, FFRSKLLSDGAAAAKGALLPQYW, RCMAFLLSDGAAAAQQLLPQYW, FTPDAPSAAPPGMALI, DPAQPNYPW TAVLVFRH	α-amylase inhibition	RCMAFLLSDGAAAAQQLLPQYW and DPAQPNYPW TAVLVFRH exerted noticeable α-amylase inhibitory activity	Use of *in silico* analysis to select promising candidates for α-amylase inhibition, and use of *in vitro* enzyme assay for validation	(Siow, H.-L. et al., 2017a)
Soy and lupin	Molecular docking	IAVPTGVA, YVVNPDNDEN, YVVNPDNNEN, LTFPGSAED, LILPKHSDAD, GQEQSHQDEGVIVR	DPP-IV	None	Molecular docking analysis to predict the interaction between peptides and DPP-IV via active site	(Lammi et al., 2016)
Cauliflower	Peptide ranking	AAGGFGGLR, AGQAAFGNMCR, FFAPYAPNFPFK,	ACE	GGPVPAPCCAGVSK, ILYDFCFLR and FFAPYAPNFPFK have strong	Use of *in silico* analysis to rapid	(Chiozzi et al., 2016)

Sample	*In silico* prediction	Peptide(s)	Bioactive properties	*In vitro* / *in vivo*	Comments	References
		GGPVPAPCCAGVSK, ILYDFCFLR, MLDFDFLCGR		ACE inhibitory activity	peptide selection	
Crude barley	Hydrolysis, ranking peptides	15 peptides from papain-treated barley protein hydrolysates were selected (low Mw)	ACE	15 peptides proved to exert ACE inhibition	*In silico* analysis to select promising proteases and peptides	(Gangopadhyay et al., 2016)
Basil Seed	Binding sites prediction	ACGNLPRMC, ACNLPRMC, AGCGCEAMFAGA	α-amylase inhibitory	None	Predicted the mechanisms of α-amylase peptides inhibition via prediction of ACE binding site	(Afifah and Gan, 2016)
Rice bran (oryzacystatins)	*In silico* hydrolysis	Peptide profiles released after hydrolysis, evaluating the possibility of peptides bioactivity	Antioxidant, ACE, DPP-IV, etc.	None	*In silico* analysis to evaluate oryzacystatins a sources of bioactive peptides, and selecting the most suitable protease to release target peptides	(Udenigwe, 2016)
Dietary	Screening DPP-IV inhibitors	68 peptides were selected	DPP-IV	none	Evaluate the possibility of protein to release	(Nongonierma and FitzGerald, 2014)

Sample	*In silico* prediction	Peptide(s)	Bioactive properties	*In vitro* / *in vivo*	Comments	References
					DPP-IV inhibitory peptides	
Pistachio	Molecular docking	ACKEP	ACE	None	ACKEP binds to seven active sites of ACE, showing inhibition mainly via Van der Waals interactions	(Li, P. et al., 2014)
Cereal (Rice, Barley, Wheat, Oat, Sorghum and corn)	Hydrolysis, screening bioactive peptides	Peptide profiles released via *in silico* hydrolysis	Including ACE, DPP-IV, Antioxidant and UbMP activating	None	Evaluation of cereal sources as bioactive peptides reservoirs	(Udenigwe et al., 2013)
Amaranth	Molecular docking	STHASGFFFFHPT, STNYFLISCLLFVLFNGCMGEG, GLTEVWDSNEQEF, TIEPHGLLLPSFTSAPELIYIEQ GNGITGMMIPGCPETYESGSQ QFQGGEDE	DPP-IV inhibitory	None	Predicted these four peptides binding to DPP-IV residue via hydrophobic interactions and hydrogen-bonds	(Velarde-Salcedo et al., 2013)
Chickpea	Homology, screening of ACE inhibitory peptides	Peptides profiles within the protein sequences, and peptide profiles released from *in silico* hydrolysis	ACE	None	Chickpea legumin is similar to oat 12S globulin 1 and rice glutelin precursor.	(Chang and Alli, 2012)

Sample	*In silico* prediction	Peptide(s)	Bioactive properties	*In vitro* / *in vivo*	Comments	References
					Use of *in silico* tools to evaluate chickpea as source of ACE inhibitors	
Wheat gliadin	*In silico* hydrolysis	Peptide profiles released via *in silico* hydrolysis	ACE	Hydrolysates obtained via *in vitro* experiments showed ACE inhibition, whilst *in silico* analysis predicted that some ACE peptides showed no correlation with *in vitro* results	Use of *in silico* analysis to select ficin, thermolysin and prolyl endopeptidase to hydrolyse wheat gliadin	(Thewissen et al., 2011)
Peanut (arachin)	Screening ACE peptides, molecular docking	ACE peptide profiles present in arachin, finding 2 potential peptides NAQRP and QRP	ACE	*In vitro* experiments validated arachin can be a source of ACE inhibitors, and proved QRP has stronger ACE inhibition than NAQRP	*In silico* analysis correlated with *in vitro* experiments. Arachin can be a potential source of ACE peptides. Molecular docking found QRP is more active as it binds to the ACE catalytic, and showed stronger ACE inhibition than	(Jimsheena and Gowda, 2011)

Sample	*In silico* prediction	Peptide(s)	Bioactive properties	*In vitro* / *in vivo*	Comments	References
					NAQRP	
Oat	*In silico* hydrolysis, screening of ACE inhibitory peptides, molecular docking	Peptide profiles released from *in silico* hydrolysis	ACE	*In vitro* study confirmed thermolysin can release ACE peptides from Oat proteins	Use of *in silico* tools to select promising proteases to release ACE peptides	(Cheung et al., 2009)
Amaranth	Ligand-protein docking	VIKP and ALEP were selected from 9 peptides	ACE	The ACE inhibitory properties of VIKP and ALEP were confirmed *in vitro*	*in silico* tools were promising for selection of peptides from Amaranth	(Vecchi and Añón, 2009)
Selected plant and animal	Screening bioactive peptides in six selected protein sequences	Peptide profiles present in protein sequences	Opioid, DPP-IV, antioxidant, ACE, etc.	None	Simple screening of bioactive peptides	(Iwaniak and Dziuba, 2009)
Pea and whey	*In silico* digestion, ACE peptides screening	Peptides profiles obtained via *in silico* gastrointestinal digestion	ACE	Positive correlation was found between *in vitro* experiments *and in silico* prediction	First study to use *in silico* analysis combined with *in vitro* assays to evaluate ACE inhibitory peptides released via digestion	(Vermeirssen et al., 2004)

Table A.2 Antioxidant properties of plant protein derived peptides

Sources	Enzymatic hydrolysis	Peptide/protein hydrolysates	Antioxidant Measurements	References
Faba bean seed (*Vicia faba*)	Pepsin, Trypsin, Alcalase (individually and sequentially)	Hydrolysates	ABTS scavenging capability DPPH scavenging capability	(Samaei et al., 2020)
Foxtail millet prolamin	Alcalase	Hydrolysates with Mw <1kDa PFLF, IALLIPF	Decreased ROS level in H_2O_2 – induced HaCaT cells	(Ji, Z. et al., 2020)
Watermelon Seed	Alcalase (Ultrasonic pre-treatment)	Hydrolysates Ultrafiltered fraction (<1kDa) Chromatographic fraction RDPEER, KELEEK, DAAGRLQE, LDDDGRL, GFGDDAPRA	DPPH radical scavenging capability ABTS radical scavenging capability Oxygen radical absorbance capability Inhibit ROS, MDA levels and increase the activity of SOD, GSH-Px and CAT activity in H_2O_2 – treated HepG2 cell	(Wen et al., 2020)
Wheat Gluten	Alcalase	Hydrolysates	ABTS scavenging capability Ferric reducing power Oxygen racial absorbance capability Copper reducing power Increase GHS levels in Human peripheral blood mononuclear cells	(Cruz-Chamorro et al., 2020)
Chickpea sprout	Trypsin, neutrase, alcalase and papain	Hydrolysates Chromatographic fraction LTEIIP	DPPH radical scavenging capability Hydroxyl radical scavenging capability	(Wali et al., 2020)
Amaranth leaf	Alcalase, Trypsin, Pepsin and Chymotrypsin	Hydrolysates Ultrafiltration fraction (<1, 1-3, 3-5, 5-10. >10	DPPH radical scavenging capability Superoxide radical scavenging	(Famuwagun et al., 2020)

Sources	Enzymatic hydrolysis	Peptide/protein hydrolysates	Antioxidant Measurements	References
		kDa)	capability Ferric reducing power Metal ion chelating Lipid peroxidation inhibition	
Rice bran	Papain, neutrase, pepsin, flavorase, trypsin, alcalase	Hydrolysates	DPPH radical scavenging capability ABTS radical scavenging capability Fe^{2+}chelating capability Cellular antioxidant capability in HepG2 cell	(Xiao et al., 2020)
Common bean	Flavourzyme, alcalase and neutrase (Combine)	Hydrolysates	DPPH radical scavenging capability Total antioxidant capability Reducing power	(Ohara et al., 2020)
Split gill mushroom	Alcalase	Hydrolysates Ultrafiltration fraction (<0.65, 0.65-3, 3-5, 5-10, >10 kDa) Chromatographic fraction	ABTS radical scavenging capability Cellular antioxidant capability in HT-29 cell	(Wongaem et al., 2020)
Corn gluten	Alcalase and Protex (Combine)	Hydrolysates (<1kDa) Chromatographic fraction LAYPQ, YGPQ, PPY, LSPY, LNSPY, AYLQQQ, AYPGPA, AYPQ, TYSGPK	ABTS radical scavenging capability Intracellular ROS scavenging capability	(Liu, W.-Y. et al., 2020)
Oat bran	Flavourzyme, papin or alcalase	Hydrolysates	Oxygen radical absorbance capability Hydroxyl radical scavenging capability Superoxide anion radical scavenging capability	(Walters et al., 2020)

Sources	Enzymatic hydrolysis	Peptide/protein hydrolysates	Antioxidant Measurements	References
Mungbean	Neutral protease	Hydrolysates Ultrafiltration fraction (<3, >3kDa) Chromatographic fraction WGN, AW, RGWYE, GVPFW	DPPH radical scavenging capability ABTS radical scavenging capability Hydroxyl radical scavenging capability Regulation MDA, CAT and GSH level in HepG2 cell	(Xia et al., 2020)
Garlic	Pepsin and trypsin (separately)	Hydrolysates	DPPH scavenging capability Ferric reducing power Lipid peroxidation inhibition Against H_2O_2-induced oxidative damage in L02 cells	(Gao, X. et al., 2020)
Wheat bran	Alcalase	Hydrolysates Ultrafiltration fraction (<1, 1-3, 3-5 and 5-10 kDa)	Oxygen radical absorbance capability	(Zou et al., 2020)
Cardamine violifolia	Alcalase	Hydrolysates Ultrafiltration fraction (<1, 1-3, 3-10, >10kDa) Chromatographic fraction GRVGSSSC, GRAGGSYM, CHPNFKLNGSGG, GTKSCKA, ASSNARDMI, TAGGCYIPI and KNCALQ	DPPH radical scavenging capability Hydroxyl radical scavenging capability ABTS radical scavenging capability Superoxide radical scavenging capability	(Zhu, S. et al., 2019)
Marine alga (*Gracilariopsis lemaneiformis*)	Pepsin, Trypsin, Papain, alpha-chymotrypsin and Alcalase	Hydrolysates Ultrafiltration fraction (<3, 3-10, >10kDa) Chromatographic fraction Glu-Leu-Trp-Lys-Thr-Phe	DPPH radical scavenging capability	(Zhang, X. et al., 2019)
Sorghum Kafirin	Alcalase	Hydrolysates Ultrafiltration fraction (<1, 1-3, 3-5, 5-10, >10kDa) Chromatographic fraction	DPPH radical scavenging capability ABTS radical scavenging capability Oxygen Radical scavenging capability	(Xu, S. et al., 2019)

Sources	Enzymatic hydrolysis	Peptide/protein hydrolysates	Antioxidant Measurements	References
			Ferric Ion Reducing power Metal chelating capability Lipid peroxidation inhibitory capability	
Peanut	Alcalase	Hydrolysates Ultrafiltration fraction (<3, >3kDa) Chromatographic fraction Thr-Pro-Ala, ile/Leu-Pro-Ser, Ser-Pro	Reducing power	(Xu, S. et al., 2019)
Sesame	Alcalase and trypsin (combined)	Hydrolysates Ultrafiltration fractions (<3, 3-5, 3-8, 8-10, >10) Chromatographic fractions RDRHQKIG, TDRHQKLR, MNDRVNQGE, RENIDKPSRA, SYPTECRMR, GGVPRSGEQEQQ and AGEQGFEYVTFR	DPPH radical scavenging capability ABTS radical scavenging capability	(Lu et al., 2019)
Moringa oleifera seed	Alcalase	Hydrolysates Ultrafiltration fraction (<1, 1-3, 3-5, 5-10kDa)	DPPH radical scavenging capability Ferric reducing power Metal ions chelating capability Hydroxyl radical scavenging capability	(Aderinola et al., 2019)
Arrowhead protein	Trypsin and alcalase (Ultrasonic pre-treatment)	Hydrolysates	DPPH radical scavenging capability ABTS radical scavenging capability Increasing the CAT and SOD activity in RAW 264.7 cells	(Wen et al., 2018)
Soy	Peptidases from latex of *Maclura pomifera* fruits	Hydrolysates Chromatographic fraction	ABTS radical scavenging capability Oxygen radical absorbance capability	(Jara et al., 2018)

Sources	Enzymatic hydrolysis	Peptide/protein hydrolysates	Antioxidant Measurements	References
Ficus Deltoidea	Trypsin	Hydrolysates Ultrafiltration fraction (<3, 3-10, 10-30 kDa) Chromatographic fraction	DPPH radical scavenging capability	(Abdullah et al., 2018)
Maize kernels	Alcalase	Hydrolysates	Oxygen radical absorbance capability	(Ortiz-Martinez et al., 2017)
Pinto Bean	Alcalase, Savinase (High pressure pre-treatment)	Hydrolysates Ultrafiltration fraction (<3kDa)	Oxygen radical absorbance capability Ferric reducing antioxidant capability ABTS radical scavenging capability Reducing power	(Garcia-Mora et al., 2016)
Ziziphus jujube seed	Alcalase, Papain and protease (separately)	Hydrolysates	ABTS radical scavenging capability DPPH radical scavenging capability Metal ions chelating capability Reducing power capability	(Kanbargi et al., 2016)
Walnut (*Juglans regia L.*)	Chymotrypsin, trypsin and proteinase K	Hydrolysates Ultrafiltration fraction (<3, 3-5, 5-10, >10 kDa)	ABTS radical scavenging capability ROS scavenging capability	(Jahanbani et al., 2016)
Black bean	Pepsin, alcalase	Hydrolysates	DPPH radical scavenging capability ABTS radical savenging capability	(EVANGELHO et al., 2016)
Almond (Iranian wild)	Pepsin, trypsin, chymotrypsin, alcalase, flavourzyme	Hydrolysates	ABTS radical scavenging capability Reducing power	(Mirzapour et al., 2016)
Rice bran	Trypsin	Hydrolysates Ultrafiltration fraction (<5 kDa)	Oxygen radical absorbance capability	(Wattanasiritham et al., 2016)
Corn	Alcalase, Flavourzyme, Alcalase + Flavourzyme and Flavourzyme + Alcalase	Hydrolysates Ultrafiltration fraction (<6 kDa) Chromatographic fraction	DPPH radical scavenging capability Fe^{2+} chelating capability Reducing power	(Jin et al., 2016)

Sources	Enzymatic hydrolysis	Peptide/protein hydrolysates	Antioxidant Measurements	References
		CSQAPLA, YPKLAPNE, YPQLLPNE	Hydroxyl radical scavenging capability Superoxide anion radical scavenging capability	
Flaxseed	Papain, trypsin, alcalase, pancreatin or Flavourzyme	Hydrolysates Chromatographic fraction	ABTS radical scavenging capability Ferric reducing power Fe^{2+} ion chelating capability	(Karamać et al., 2016)
Pea	Alcalase (None, High pressure or High temperature pre-treatment)	Hydrolysates	Oxygen radical absorbance capability DPPH radical scavenging capability Ferric reducing power Metal ion chelating activity Superoxidase radical scavenging activity Hydroxyl radical scavenging activity	(Girgih et al., 2015)
Chickpea	Pepsin and pancreatin (combined)	Hydrolysates Chromatographic fractions	Reducing power DPPH radical scavenging capability Cellular antioxidant capability in Caco-2 cell	(Torres-Fuentes et al., 2015)
Zein	Alkaline protease, papain, flavorzyme and trypsin	Hydrolysates Ultrafiltration fraction (<3, >3kDa) Chromatographic fraction Pro-Phe, Leu-Pro-Phe	DPPH radical scavenging capability ABTS radical scavenging capability Fe^{2+} ion chelating capability Cu^{2+} ion chelating capability Reducing power Lipid peroxidation inhibition capability	(Tang and Zhuang, 2014)
Palm kernel cake	Papain, Alcalase, Pepsin, Trypsin, Flavourzyme, Bromelain or	Hydrolysates Chromatographic fraction	DPPH radical scavenging capability Metal ions chelating capability	(Zarei et al., 2014)

Sources	Enzymatic hydrolysis	Peptide/protein hydrolysates	Antioxidant Measurements	References
	chymotrypsin	YLLLK, YGIKVGYAIP, GGIF, GIFE, WAFS, GVQEGAGHYALL, WAF, AWFS, LPWRPATNVF		
Red macroalgae (*Palmaria palmata*)	Alcalase, Corolase PP	Hydrolysates	Oxygen radical absorbance capability Ferric reducing power	(Harnedy et al., 2014)
Winged Bean	Papain	Hydrolysates Chromatographic fraction YPNQKV, FDIRA	DPPH radical scavenging capability Metal ions chelating capability	(Yea et al., 2014)
Rapeseed	Alcalase, Proteinase K, Pepsin + Pancreatin, Thermolysin or Flavourzyme	Protein hydrolysates Ultrafiltration (<1, 1-3, 3-5, 5-10kDa)	DPPH radical scavenging capability Superoxide radical scavenging capability Metal ions chelating capability Ferric reducing power Lipid peroxidation inhibition capability	(He et al., 2013a)
Parkia speciose seeds	Alcalase	Hydrolysates Ultrafiltration fraction (<10, 10-30, 30-50, >50kDa)	DPPH radical scavenging capability Ferric reducing power	(Siow, H.-L. and Gan, 2013)
Zizyphus jujube fruits	Alcalase, Trypsin (Separately and mixture)	Hydrolysates Ultrafiltration fraction (<3kDa) Chromatographic fraction VGQHTR, GWLK	ABTS radical scavenging capability DPPH radical scavenging capability Metal ions chelating capability Lipid peroxidation inhibitory capability Reducing power	(Memarpoor-Yazdi et al., 2013)
Sweet potato	Alcalase, proleather FG-F, ASI. 398, neutrase, papain and pepsin	Hydrolysates Ultrafiltration fraction (<3, 3-10, >10kDa)	Hydroxyl radical scavenging capability Fe^{2+} – chelating capability Protect DNA from oxidative damages	(Zhang, M. et al., 2012)

Sources	Enzymatic hydrolysis	Peptide/protein hydrolysates	Antioxidant Measurements	References
Canola	Alcalase, Flavourzyme, Alcalase-flavourzyme	Hydrolysates	DPPH radical scavenging capability Reducing power	(Cumby et al., 2008)
Soy, wheat gluten	Commercial	Hydrolysates Autofocusing fractions	DPPH radical scavenging capability Lipid peroxidation inhibitory capability	(Park et al., 2008)
Wheat gluten	Alcalase, Pancreatin, Pepsin, Protamex and Neutrase	Hydrolysates Ultrafiltration fraction (<3, 3-5, 5-10, >10 kDa)	Lipid peroxidation inhibitory capability DPPH radical scavenging capability Hydroxyl radical scavenging capability Superoxide radical scavenging capability	(Kong et al., 2008)
Soy	Flavourzyme	Hydrolysates	Ferric reducing power Hydroxyl radical scavenging capability ABTS radical scavenging capability Superoxide anion radical scavenging capability Beta-Carotene bleaching capability Reducing power	(Moure et al., 2006)

Table A.3 Anti-hypertensive properties of plant protein hydrolysates and peptides.

Sources	Type	Protease	Peptide/protein hydrolysates	Inhibitory properties	References
Rice (*Oryza sativa*)	ACE	Neutrase, simulated gastrointestinal digestion (pepsin + pancreatin) (Ultrasound pre-treatment)	Hydrolysates	Highest inhibition 56.33% using 20 kHz in mon-frequency ultrasound 63.39% using 20/40 kHz in	(Yang et al., 2020)

Sources	Type	Protease	Peptide/protein hydrolysates	Inhibitory properties	References
				dual-frequency ultrasound ACE inhibition of protein hydrolysate increased after Gastrointestinal simulated digestion	
Walnut	ACE	Neutrase	Hydrolysates Ultrafiltration fraction (<1, 1-8, 8 kDa-0.45μm, >0.45μM) Chromatographic fraction Three peptides (GVVPHN, EHSLDPLK, KTLLNFGPN)	Hydrolysate 42.65±0.76% inhibition Mw <1kDa 88.42±0.38% inhibition 75.66% inhibition (SEC fraction) 94.44% inhibition (HPLC fraction) IC_{50} value for three peptides are 27.3, 49.6 and 36.8μM/L, respectively	(Chen, Y. et al., 2020)
Radix astragali	ACE	-	LVPPHA	IC_{50} value 414.88±41.88μM Decrease 42 ± 2.65 mmHg at 10 μmol/ kg	(Wu, J.-S. et al., 2020)
Longan seeds	ACE	Pepsin and pancreatin	Hydrolysates Ultrafiltration fraction (<3, 3-5, 5-10, >10 kDa) Chromatographic fraction Two peptides: ETSGMKPTEL, ISSMGILVCL	Hydrolysates IC_{50} value 1.74 ± 0.006 mg/mL Mw<3kDa IC_{50} value 0.25 ± 0.004 mg/mL HPLC fraction F5 IC_{50} value 18.22 ± 0.029μg/mL	(Nuchprapha et al., 2020)

Sources	Type	Protease	Peptide/protein hydrolysates	Inhibitory properties	References
				IC_{50} value of two peptides 2.15 ± 0.016 mM and 3.88 ± 0.004 mM	
Sargassum maclurei	ACE inhibitor, Reduce the blood pressure in SHRs Inhibitor of intracellular endothelin-1	Pepsin - Papain	Hydrolysates Chromatographic fraction RWDISQPY	ACE inhibition of hydrolysates 43.67 ± 4.03% 61.79±5.33% are the highest inhibition among chromatographic fractions peptide IC_{50} value of peptide 210 μM Dose-dependent lowering effect in DBP and SBP of SHRs Suppression 16.64%-26.20% of ET-1 production	(Zheng et al., 2020b)
Sesame	ACE	*In vitro* simulated gastrointestinal digestion (pepsin, trypsin and α-chymotrypsin)	Hydrolysates Ultrafiltration fraction (<3, 3-5, 5, 5-10, 10-30, 30-50, 50-100, >100 kDa) Chromatographic fractions 11 peptides: GHIITVAR, IGGIGTVPVGR, HIGNILSL, FMPGVPGPIQR, PNYHPSPR, AFPAGAAHW, HIITLGR, LAGNPAGR, MPGVPGPIQR, AGALGDSVTVTR	Inhibition reached 81.21% after 10 h hydrolysis IC_{50} values for Mw< 3kDa is 2.720 ± 0.0003 μg/mL IC_{50} values for most promising HPLC fraction is 0.558±0.003 μg/mL GHIITVAR and ICCICTVPVGR have the lowest IC_{50} values 3.60±0.10 and 6.97±0.18 μM, respectively	(Wang, R. et al., 2020)

Sources	Type	Protease	Peptide/protein hydrolysates	Inhibitory properties	References
Wheat gluten	ACE	Alcalase, Protamex, PaproA, Alcalase-Protamex, Alclase-PaproA, Protamex-PaproA. Simulated gastrointestinal digestion (pepsin, tyrpsin)	Hydrolysates, Ultrafiltration (<1, 1-5, 5-10, >10 kDa) Chromatographic fraction 6 peptides: IHVTET, AGPCAPNP, APATPSFW, APAPRPPNAP, SAGGYIW and EEAIFLW	IC_{50} value of protein hydrolysates treated via alcalase and paproA is 0.21 ± 0.02 mg/mL IC_{50} value for most promising chromatographic fraction (0.03 mg/mL) SAGGYIW and APATPSFW are the two most promising ACE- inhibitory peptides, with IC_{50} values of 0.003 mg/mL and 0.036 mg/mL, respectively	(Zhang, P. et al., 2020)
Amaranth	Renin inhibitor	-	SFNLPILR, FNLPILR, AFEDGFEWVSFK	IC_{50} values are 2.50, 0.41 and 1.47μM, respectively	(Nardo et al., 2020)
Quinoa Bran Albumin	ACE inhibitor, decrease DBP and SBP in SHRs	Alcalase and trypsin	Hydrolysates Chromatographic fraction RGQVIYVL	Hydrolysates inhibition 62.38 ± 5.64% The highest inhibition of SEC fraction is 84.69%± 3.24% at 1 mg/mL Peptide IC_{50} value 38.16μM Significantly decrease DBP and SBP after oral administration 100-150 mg/kg body weight	(Zheng et al., 2019b)
Microalgae	ACE inhibitor	Trypsin and pepsin (Ultrasound	Hydrolysates	*In vitro* ACE inhibitory assay	(Aiello et al., 2019)

Sources	Type	Protease	Peptide/protein hydrolysates	Inhibitory properties	References
Spirulina	Cellular ACE inhibitory properties	pre-treatment)		IC_{50} values 0.1 ± 0.04 mg/ml (pepsin) and 0.28 ± 0.03 mg/ml (trypsin) cellular ACE inhibitory assay IC_{50} values 2.7 ± 0.3 mg/mL (pepsin) and 2.8 ± 0.9 mg/mL (Trypsin)	
Wheat germ	ACE inhibitor	Alcalase, pepsin or proteinase K	Hydrolysates Chromatographic fractions Seven peptides SGGSYADELVSTAK (proteinase K), MDATALHYENQK (proteinase K), KELPPSDADW (pepsin), SSDEEVREEKELDLSSNE (pepsin), TVGGAPAGRIVME (alcalase), VGGIDEVIAK (alcalase), CNPIPREPGQVPAY (alcalase)	0.093μM, 0.021μM, 0.245μM, 0.3μM, 0.48μM, 0.2μM and 0.254μM	(Karami et al., 2019)
Cashew	ACE inhibitor, Downregulate the ACE-AngII-AT1R	Alkaline protease, pepsin protease, neutral protease	Hydrolysates Ultrafiltration fraction Mw < 3.5 kDa Chromatographic fraction FETISFK	Alkaline hydrolysis 71.24±5.96%, IC_{50} value 401.36±1.33 μg/mL Ultrafiltration fraction 85.01±1.44%, IC_{50} 121.03 ± 2.67 μg/mL Most promising chromatographic fraction	(Shu et al., 2019)

Sources	Type	Protease	Peptide/protein hydrolysates	Inhibitory properties	References
				(majorly, FETISFK) 91.04 ± 0.31%, IC_{50} values 2.12 ± 0.067 µg/mL 0.8 g kg^{-1} ACE inhibitory peptides inhibit the mRNA levels of ACE and AT_1R in mice model	
Spent Grain prolamin	ACE inhibitor	Alcalase, Neutrase and Flavourzyme	Hydrolysates Ultrafiltration fraction Chromatographic fraction Six peptides: AVQ, NQL, YPQ, AYLQ, VLPVLS and VLPSLN	Highest inhibition Alcalase hydrolysates (79.05%) Mw <1 kDa has the lowest IC_{50} value 6.750 ± 0.37 µg/mL AVQ is the most promising ACE inhibitor with IC_{50} 181.0 ± 6.17 µM	(Wei et al., 2019)
Macroalga (*Ulva intestinalis*)	ACE inhibitor	Trypsin, pepsin, papain, alpha-chymotrypsin, Alcalase	Hydrolysates Ultrafiltration fraction Chromatographic fraction FGMPLDR, MELVLR	Trypsin hydrolysates has the greatest ACE inhibition (51.15 ± 3.78%), After optimum, the ACE inhibition reach to 64.07% Mw <3 kDa fractions has the lowest IC_{50} value 1.14±0.11 mg/ml Fraction C2-8 has the most promising ACE inhibition among all chromatographic fractions (62.35%)	(Sun, Siqi et al., 2019)

Sources	Type	Protease	Peptide/protein hydrolysates	Inhibitory properties	References
				Peptides IC_{50} 219.35μM and 236.85μM	
Coconut cake albumin	ACE inhibitor	Alcalase, flavourzyme, pepsin and trypsin (high pressure pre-treatment)	Hydrolysates Ultrafiltration fraction Chromatographic fraction Three peptides KAQYPYV, KIIIYN, KILIYG	The ACE inhibition of protein hydrolysates after treated via four enzymes is 48.34 ± 1.41% Fraction Mw < 3kDa exert 63.58 ± 5.14% at 1mg/mL Fraction A4e3 has the greatest ACE inhibitory properties among all chromatographic fractions (87.31 ± 1.89%) Peptides IC_{50} values 37.06μM, 58.72μM and 53.31μM respectively.	(Zheng et al., 2019a)
Ginkgo biloba seeds	ACE inhibitory peptides	Alcalase, flavourzyme, dispase and trypsin	Hydrolysates Ultrafiltration fraction (<1, 1-3, 3-5, 5-10kDa) Chromatographic fraction Three peptides: TNLDMY, RADFY, RVFDGAV	ACE inhibition (%) of alcalase-treated protein hydrolysates is 62.70% Mw < 1 kDA has the lowest IC_{50} value 0.224 mg/ml Fraction A3 is the most promising ACE inhibitor (74.96 % at 1mg/ml) Peptides IC_{50} values are 1.932, 1.35, and 1.006 mM, respectively	(Ma et al., 2019)

Sources	Type	Protease	Peptide/protein hydrolysates	Inhibitory properties	References
Cottonseed	ACE inhibitor	Papain	Hydrolysates Ultrafiltration fraction (<5, 5-10, 10-30, >30 kDa) Chromatographic fraction Pre-Pro-Ala-Ile-Gly-Met-Lys	ACE inhibition of protein hydrolysates 87.8 ± 0.23 % IC_{50} values of fraction with MW <5 kDa is 0.792 mg/mL FII-2 has the highest ACE inhibitory activity (91.4% at 100μg/mL) Peptide IC_{50} values of 46.7μg/mL	(Gao, D. et al., 2019)
Black soybean	ACE inhibitor	Alcalase (microwave pre-treatment)	Hydrolysates Ultrafiltration fraction (<3 kDa) Chromatographic fraction	Hydrolysates 70.37±0.57% Inhibition Mw <3 kDa exerts 72.38% of ACE inhibitory activity Fraction III has the highest ACE inhibitory activity (90.67%)	(Li, Meiqing et al., 2018)
Rhodophyta (*Gracilariopsis lamaneiformis*)	ACE inhibitor Reduce the blood pressure in SHR model	Trypsin	FQINMCILR, TGAPCR	IC_{50} values 9.64 ± 0.36 μM and 23.94 ± 0.82 μM In SHR model, FQINMCILR reduced 34 mmHg whilst TGAPCR decrease 28 mmHg	(Deng et al., 2018)
Microalgae (*Tetradesmus obliquus*)	ACE inhibitor	Alcalase	Hydrolysates Chromatographic fraction Two peptides	Fraction F9f2 is the most promising ACE inhibitor (inhibit 91.2%)	(Montone et al., 2018)

Sources	Type	Protease	Peptide/protein hydrolysates	Inhibitory properties	References
			GPDRPKFLDPF, WYGPDRPKFL	Peptides IC_{50} values are 5.73 and 0.82 μM/L, respectively.	
Hazelnut	ACE inhibitor Reduce blood pressure in SHR model	Alcalase	Hydrolysates Chromatographic fraction three peptides AVKVL, YLVR, TLVGR	Among fractions, fraction C2 exert 89.5 ± 3.23% inhibition (most promising) IC_{50} values of 73.06, 15.42, and 249.3 μM (50mg/kg BW) YLVR decrease 39.97 mmHg in SBP (8 hours)	(Liu, C. et al., 2018)
Walnut	ACE inhibitor	Pepsin	EPNGLLLPQY	IC_{50} value 233.178 μM.	(Wang, C. et al., 2018)
Lima bean	ACE inhibitor, Renin inhibitor, Anti-hypertensive activity	Alcalase-Flavourzyme (AF) Pepsin-pancreatin (PP)	Hydrolysates Ultrafiltration fraction (<1, >1, >3, >5, >10 kDa)	Highest ACE inhibition 60.15% (PP Mw > 3kDa) Highest Renin inhibition 31.73% (AF Mw > 3kDa) and 30.05% (PP Mw > 3kDa) highest reduction is 51% SBR and 64% DBP in Wistar rats	(Ciau-Solís et al., 2018)
Wild almond	ACE inhibitor	Alcalase, chymotrypsin, trypsin, pepsin, flavourzyme	Hydrolysates Ultrafiltration fraction (<3, 3-10, >10 kDa) Chromatographic fraction	All protein hydrolysates exert over 88.7% ACE inhibition, IC_{50} value 0.8 to 0.9 mg/mL Fraction Mw< 3kDa exert the highest ACE inhibitory activity (86.7%) (Alcalase)	(Mirzapour et al., 2017)

Sources	Type	Protease	Peptide/protein hydrolysates	Inhibitory properties	References
Amaranth	Chymase inhibitor Renin inhibitor	Alcalase	Hydrolysate Chromatographic fraction	IC_{50} value of renin is 0.6 mg/mL FV is the most promising Renin inhibitor (39%) among all the chromatographic fractions, equal to 67 ± 5% mg of peptide	(Quiroga et al., 2017)
Patatin	ACE inhibitor, Renin inhibitor	-	WG, PRY	ACE inhibition IC_{50} values 231.22 and 97.59μM Renin inhibition IC_{50} values 112.34 and 95.33 μM	(Fu et al., 2017)
Bambara groundnut	ACE inhibitor, Renin inhibitor	Alcalase, pepsin and trypsin	Hydrolysates Ultrafiltration fraction (<1, 1-3, 3-5, 5-10 kDa)	Highest ACE inhibition 93.9% inhibition (Alcalase Mw < 1 kDa) Highest Renin inhibition 59% inhibition (Alcalase hydrolysates)	(Arise et al., 2017)
Mucuna pruriens	ACE inhibitor	Pepsin, pancreatin	Hydrolysates Ultrafiltration fraction (<1, 1-3, 3-5, 5-10, >10 kDa) Chromatographic fraction	IC_{50} value for crude hydrolysate is 13.02μg/mL 0.0009 μg/mL (lowest IC_{50} value ultrafiltration fraction Mw <1kDa), whilst gel fraction has a IC_{50} value from 0.401 – 0.969 μg/mL And two HPLC fractions have	(Tuz and Campos, 2017)

Sources	Type	Protease	Peptide/protein hydrolysates	Inhibitory properties	References
				the IC_{50} 16.162 and 14.542 μg/mL, respectively.	
Rice bran	ACE inhibitor	Neutral protease, alkaline protease and trypsin	Hydrolysates Ultrafiltration fraction (<4, 3-6, >6 kDa) Chromatographic fraction Tyr-Ser-Lys	539 ± 12.78 μg/mL (protein hydrolysate) <4 kDa fraction has the lowest IC_{50} value 300±14.92μg/mL F2-a has the lowest IC_{50} values 30±3.4μg/mL Peptide IC_{50} 75.95 mM	(Wang, X. et al., 2017)
Enteromorpha clathrate	ACE inhibitor	Alcalase	Hydrolysates Chromatographic fraction Pro-Ala-Phe-Gly	IC_{50} value of protein hydrolysates 0.66 ± 0.026 mg/mL IC_{50} value of fraction with MW <10kDa is 0.21 ± 0.014 mg/mL Lowest IC_{50} value of most promising chromatographic fraction 0.014 ± 0.006 mg/mL IC_{50} value of 35.9 μM	(Pan et al., 2016)
Hemp	ACE inhibitor	AFP, HT, Pro-G, actinidin and zingibain	Hydrolysates	Highest inhibition 76.82 ± 0.06 % (HT, 2h, alkaline protein isolate)	(Teh et al., 2016)
Walnut	ACE inhibitor	Pepsin	Hydrolysates Ultrafiltration (<5, 5-10 and >10 kDa)	HydrolysateMw <5 kDa has the lowest IC_{50} value	(Wang, F.-J. et al., 2016)

Sources	Type	Protease	Peptide/protein hydrolysates	Inhibitory properties	References
			Chromatographic fraction YVPHWNL	IC_{50} of peptide is 0.173μM/mL	
Sweet sorghum grain	ACE inhibitor	Alcalase	Hydrolysates Ultrafiltration fraction (<1, 1-5, 5-10, >10 kDa) Chromatographic fraction Thr-Leu-Ser	Whole protein hydrolysates 20% -85% inhibition Fraction Mw <1 kDa has the highest ACE inhibition (IC_{50} 0.116 mg/mL) The most promising chromatographic fraction F1b-2b (IC_{50} value 31.6μg/mL) Peptide IC_{50}= 102.1 μM	(Wu, Q. et al., 2016)
Peach	ACE inhibitor	Alcalase, thermolysin, flavourzyme and protease P	Hydrolysates Ultrafiltration fraction (<3, 3-5, >5 kDa)	IC_{50}= 18.6 ± 0.7 μg/mL Thermolysin (Highest) Fraction with MW <3kDa has the lowest IC_{50} value (16.4 ±1.4μg/mL)	(Vásquez-Villanueva et al., 2015)
Date seed	ACE inhibitor	Alcalase, flazourzyme and thermolysin (Individually and sequentally)	Hydrolysates	Lowest IC_{50} value is 0.53 mg/ml (Alcalase + Thermolysin)	(Ambigaipalan et al., 2015)
Brewers' spent grain	ACE inhibitor	Alcalase, Corolase PP, Flavourzyme (Simulated gastrointestinal digestion)	Hydrolysates Ultrafiltration <3, <5, >5 kDa Chromatographic fraction	Alcalase-treated hydrolsyates has the lowest IC_{50} values 0.32 ± 0.02 mg/mL	(Connolly et al., 2015)

Sources	Type	Protease	Peptide/protein hydrolysates	Inhibitory properties	References
			Six peptides IVY, ILDL, VHSP, HHMP, GLQLPFSEE and ILLPGAQDGL	Fraction Mw <5kDa from alcalase treated (IC_{50} 0.33±0.08 mg/mL) and Flavourzyme (0.35±0.07 mg/mL) 91.9 ± 3.48% inhibition of ACE showed the highest inhibition among fractions Peptide Most promising peptides were IVY and ILDL, with IC_{50} values 80.4±11.9 and 96.4± 8.36 µM, respectively	
Lupin (*Lupinus angustifolius*) & *(Lupinus luteus)*	ACE inhibitor	Pepsin, trypsin, chymotrypsin, corolase PP	Hydrolysates Ultrafiltration (<3 kDa)	Pepsin-treated protein hydrolysate exert lowest IC_{50} value 197 ± 1.6 µg/ mL (Lupinus albus) Pepsin-treated protein hydrolysates exerts lowest IC_{50} value 185 ± 13.3 µg/ mL (*Lupinus angustifolius*) Chymotrypsin-treated protein hydrolysates exert lowest IC_{50} value 136±4.5 µg/ mL *(Lupinus luteus)*	(Boschin et al., 2014)

Sources	Type	Protease	Peptide/protein hydrolysates	Inhibitory properties	References
Rice (*Oryza sativa*)	ACE inhibitor	Alcalase	Hydrolysates Ultrafiltration fraction (<3, 3-10, >10 kDa) Chromatographic fraction VNP and VWP	Hydrolysates 0.460±0.003 mg/mL Fraction with Mw <3 kDa has the lowest IC_{50} value 0.280 ± 0.005 mg/mL Fraction 10-2 has the lowest IC_{50} value 0.0018±0.0002 mg/mL Peptides IC_{50} value of 6.4 and 4.5μM	(Chen, J. et al., 2013)
Rapeseed	Renin and ACE inhibition Reduce blood pressure in SHR model	Alcalase	Hydrolysates Chromatographic fraction LY, TF, RALP	Hydrolysates have 76.89 ± 0.93% ACE inhibition and 81.19 ± 0.82% Renin inhibition at 1mg/mL, respectively F5-FIII showed 70.05 ± 0.71% ACE inhibition, 44.50 ± 1.60% Renin inhibition. F5-FV has the highest Renin inhibition (53.60 ± 0.97%) Peptides ACE IC_{50} values are , LY 0.11mM, TF 0.81mM, RALP 0.65mM	(He et al., 2013b)

Sources	Type	Protease	Peptide/protein hydrolysates	Inhibitory properties	References
				Renin IC_{50} values, RALP 0.97 mM, LY 1.87 mM, TF 3.1 mM LY -26mmHg, RALP -16mmHg and TF -12 mmHg blood pressure in SHR model	
Sunflower	ACE inhibitor	Pepsin and pancreatin (sequentally)	Hydrolysates Chromatographic fraction FVNPQAGS	Up to 45% ACE inhibition Most promising fraction has lowest IC_{50} value 2.4 µg/mL IC_{50} of FVNPQAGS peptide is 6.9µM	(Megías et al., 2004)

Table A.4 Anti-diabetic properties of plant protein derived peptides and protein hydrolysates.

Protein source	Type of test	Peptide/protein hydrolysates	Inhibitory activity	References
Black Bean	DPP-IV inhibitor, α-amylase inhibitor, α-glucosidase inhibitor	Hydrolysates produced via proteinase K, pepsin, trypsin, papain, alcalase, flavourzyme, thermolysin EGLELLLLLLAG, ASKPLF, FEELN, TTGGKGGK, AKSPLF, WEVM	Highest inhibition, 96.7% (DPP-IV, alcalase, 1:20 E/S, 2h), 64.5% (alpha-amylase, flavourzyme, 1:20 E/S,2h), (75.3%-78.4%, papain, regardless of E/S and hydrolysis time). EGLELLLLLLAG, ASKPLF and FEELN (predicted promising DPP-IV inhibitor) TTGGKGGK (predicted promising α-glucosidase inhibitor) and AKSPLF and WEVM (α-amylase inhibitor)	(Mojica and De Mejía, 2016)

Protein source	Type of test	Peptide/protein hydrolysates	Inhibitory activity	References
Quinoa	DPP-IV inhibitor, α-amylase inhibitor, α-glucosidase inhibitor	Sequential hydrolysis by pepsin (120 min) and pancreatin (60 min, 120 min), followed by ultrafiltration. Fraction > 5kDa and < 5kDa HPLC fractions of protein hydrolysates after 120min pepsin and 60min pancreatin IQAEGGLT, DKDYPK, CHEGSDGNY	Gastric phase, only inhibit DPP-IV (IC_{50} 2.52±0.06mg protein/ML) After pancreatin hydrolysis (60 or 120min), the whole protein hydrolysates, together with Mw<5kDa factions inhibit DPP-IV, α-amylase, α-glucosidase. Whilst fraction with Mw>5kDa only exert DPP-IV inhibition. Three HLPC fractions exerted DPP-IV, α-amylase, α-glucosidase inhibitory activity. Only Fraction 3 showed a IC_{50} value of α-amylase (3 mg peptide/mL). IQAEFFLT show DPP-IV (17.05±0.06%) and α-glucosidase inhibitory activity (55.85±0.26%). DKDYPK exerts α-glucosidase inhibitory (22.16±0.61%) and GEHGSDGNV exerts α-amylase (6.86±0.16%) and α-glucosidase inhibitory properties (30.84±0.69%).	(Vilcacundo et al., 2017)
Common Beans (Black, pinto, red, navy, great northern)	DPP-IV inhibitor, α-amylase inhibitor, α-glucosidase inhibitor	Protein hydrolysates produced via pepsin and pancreatin (r aw or precooked)	DPP-IV inhibitory Navy bean has the strongest inhibition (IC_{50} 0.093 -0.095mg protein/mL). All samples exerted α-amylase inhibition, whilst pre-treated bean hydrolysates were weaker than raw. α-glucosidase inhibition, raw (60%-70%), precooked (40-60%)	(Mojica et al., 2015)
Hard to cook beans	DPP-IV inhibitor, α-amylase	Protein hydrolysates (alcalase or bromelain)	α-amylase (49.9±1.4%, pinto Durango-bromelain,	(Oseguera-Toledo

Protein source	Type of test	Peptide/protein hydrolysates	Inhibitory activity	References
(Negro, Pinto Durango)	inhibitor, α-glucosidase inhibitor, insulin secretion	followed by simulated gastrointestinal digestion. Ultrafiltration fractions <1, 1-3, 3-5, 5-10 and >10 kDa. LLSL, QQEG and NEGEAH	Mw <1kDa) α-glucosidase (76.4±0.5%, pinto Durango-alcalase) DPP-IV (55.3±1.6%, pinto Durango-alcalase) Mw<1 and 1-3kDa promote up to 57% insulin secretion	et al., 2015)
Yellow field pea	α-amylase inhibitor and α-glucosidase inhibitor	Alcalase, chymotrypsin, pepsin or trypsin treated protein hydrolysates, Ultrafiltration fractions, Mw <1, 1-3, 3-5, 5-10 kDa	Highest α-amylase inhibition 30.52±0.01%, chymotrypsin 1-3kDa fraction 225ug/ml α-glucosidase, up to 53.35±2.78% at 20mg/ml chymotrypsin <1 kDa	(Awosika and Aluko, 2019)
Palmaria palmata	DPP-IV inhibitor	Aqueous extract, Alkaline extract, Aqueous and Alkaline extract protein hydrolysates obtained via Alcalase, Flavourzyme or Corolase PP	Highest DPP-IV inhibition: IC_{50} 1.65±0.12 mg/ml Corolase PP treated Aqueous protein	(Harnedy and FitzGerald, 2013)
Walnut	α-glucosidase inhibitor, α-amylase inhibitor and alleviating insulin resistance	LPLLR	50.12% α-glucosidase and 39.08% α-amylase inhibition at 2000μM. Also 100 and 200 μM LPLLR can increase glycogen synthesis and glucose uptake in HepG2 cells	(Wang, J. et al., 2020)
Cowpea bean	DPP-IV inhibitor	Alcalase-treated germinated or no germinated cowpea bean protein hydrolysates	Most potent inhibitor is no-germinated cowpea, with 1 h alcalase hydrolysis (IC_{50} 0.58 mg SP/mL)	(de Souza Rocha et al., 2014)
Rapeseed (Napin)	DPP-IV inhibitor	Napin protein hydrolysates obtained via Alcalase (together with trypsin, pepsin, flavourzyme and papain) Ultrafiltration fractions Mw <1 kDa, 1-3 kDa and >3 kDa MPGPS, PAGPF, TMPGP, IPQVS, NIPQVS,	The most potential DPP-IV inhibitors were alcalase and pepsin-treated Napin proteins with Mw <1kDa. IC_{50} =0.68±0.09 mg/ml. IPQVS are the most promising DPP-IV inhibitors, with IC_{50} 52.16±5.91 μM	(Xu, F. et al., 2019)

Protein source	Type of test	Peptide/protein hydrolysates	Inhibitory activity	References
		KETMPGPS, HQEEPL, ELHQEEPL		
Bambara bean	DPP-IV inhibitor	Protein hydrolysates obtained via alcalase, trypsin and thermolysin	Alcalase-treated and thermolysin treated protein hydrolysates exerted the similar promising DPP-IV inhibition (IC_{50} 1.73mg/mL)	(Mune et al., 2018)
Quinoa	DPP-IV inhibitor	Protein hydrolysates obtained via papain or papain-like enzyme	IC_{50} 0.88 ±0.05 and 0.98±0.04 mg/mL respectively	(Nongonierma et al., 2015)
Soybean Lupin	DPP-IV inhibitor	Lup1 LTFPGSAED Soy1 IAVPTGVA	IC_{50} values 207.5 µM and 223.2 µM	(Lammi et al., 2018)
Common beans (Easy to cook and hard to cook beans)	α-amylase inhibitor, α-glucosidase inhibitor, antihyperglycemic activity decrease the glucose level after extension of glibenclamide	Non-hydrolysed or pepsin-pancreatin hydrolysed common beans. Ultrafiltration fractions Mw< 3kDa, 3-10 kDa, >10kDa	α-amylase, up to 89.1% inhibition α-glucosidase up to 89.2% inhibition Tested protein hydrolysates, especially low molecular fractions (<3kDa) showed antihyperglycemic activity and hypoglycaemic activity in animal models (male Wistar rats)	(Valencia-Mejía et al., 2019)
Wheat gluten	DPP-IV inhibitor	Nine wheat gluten protein hydrolysates (with Debitrase with E:S 0.5, 1.0 and 1.5% at 40,50,60 °C) Simulated gastrointestinal digestion of gluten and the most promising wheat gluten protein hydrolysates (E:S 1.5, 60°C)	IC_{50} value 0.24 ± 0.02 to 0.66±0.06 mg/ml Digestive hydrolysates: 0.40±0.03; 0.33±0.03 mg/ml Whilst the digestive proteins were 1.45±0.26 and >1.90 mg/ml Trp-Leu, Trp-Pro, Val-Pro-Leu most promising peptides	(Nongonierma et al., 2017)
Brown alga *Gamtae* (*Ecklonia*	α-amylase inhibitor, α-glucosidase inhibitor,	*Gamtae* enzymatic hydrolysates (Celluclast)	α-glucosidase: IC_{50} 0.62mg/mL α-amylase IC_{50} 0.59mg/mL	(Lee et al., 2012)

Protein source	Type of test	Peptide/protein hydrolysates	Inhibitory activity	References
cava)	postprandial hyperglycaemia		AUC Postprandial glucose Normal mice (6,142±101.3 to 4,099±131.3 mg min/dL^2) Normal mice (10,425±108.4 to 6,102±102.3 mg min/dL^2)	
Macroalga *Palmaria Palmata*	DPP-IV inhibitor	Corolase PP- treated protein hydrolysates HPLC fractions 10, 20, 25, 30, 40,60% ILAP, LLAP. MAGVDHI	IC_{50} value for hydrolysates 1.47±0.09 IC_{50} ranged from 0.33±0.03 to 0.54±0.03 for 25% -60% ACN HPLC fraction (mg/mL) IC_{50} value of peptides ranged from 43.40±1.40 to 159.27±13.67uM	(Harnedy et al., 2015)
Common bean (Mexican black and Brazilian Carioca bean)	DPP-IV inhibitor, alpha-glucosidase inhibitor	gastrointestinal digestion KTYGL, KKSSG, CPGNK, GGGLHK	DPP-IV inhibitor: Digests IC_{50} value from 0.14±0.01 to 0.33±0.03 mg DW/mL Peptides IC_{50} value from 0.02±0.01 to 0.87±0.02 mg DW/mL α-glucosidase inhibitors Digestates 46.90±7.10% to 50.10 ± 5.30 % Peptides 36.30±8.80% to 49.34±6.50 %	(Mojica et al., 2017b)
Kiwicha (*Amaranthus caudatus*)	DPP-IV inhibitor, α-amylase inhibitor	gastro and gastroduodenal digestion Ultrafiltration fraction >5kDa, <5kDa HPLC fraction F-1, F-2 from kiwicha protein hydrolysates (gastroduodenal digests 60 min)	DPP-IV inhibitor Digests IC_{50} values from 0.19±0.01 to 0.68±0.07 mg/ml F-1, F-2 IC_{50} values 0.38±0.04 & 0.18±0.01 mg/mL α-amylase inhibitor Digests IC_{50} values from 0.84±0.03 to 2.73±0.02	(Vilcacundo et al., 2019)

Protein source	Type of test	Peptide/protein hydrolysates	Inhibitory activity	References
			respectively F-1, F-2 0.42±0.03 & 1.17±0.05 mg/mL	
Red Seaweed	α-amylase inhibitor	Hydrolysates of pepsin, alcalase, neutrase or trypsin Ultrafiltration >10kDa 3-10kDa, <3kDa of pepsin-treated Sephadex G-15 fraction A-F HPLC fraction D 1-10 Gly-Gly-Ser-Lys Glu-Leu-Ser	Fraction D2 exerted 88.67±1.05% at 1mg/ mL IC_{50} value for 2.58±0.08mM (Gly-Gly-Ser-Lys) and 2.62±0.05 mM (Glu-Leu-Ser) respectively. Both peptides are non-competitive inhibitor	(Admassu et al., 2018)
Andean Lupin legume	DPP-IV inhibitor Glucose uptake enhancer Gluconeogenesis reducer	Hydrolysed gamma-conglutin fraction (pancreatin, pepsin, Mw <10 kDa)	Inhibit 100% DPP-IV activity at 5mg/mL Increase 6.5-fold glucose uptake, decrease 50% gluconeogenesis	(Muñoz et al., 2018)
Brewers spent grain	DPP-IV inhibitor	Protein hydrolysates of alcalase, corolase PP and flavourzyme Ultrafiltration fraction Mw > 5kDa, 3-5 kDa and <3kDa HPLC fraction I- VIII for alcalase-treated protein hydrolysates before and after simulated gastrointestinal digestion IL, LL, ILDL, ILLPGAQDGL	IC_{50} value for protein hydrolysates and ultrafiltration fractions are from 2.01±0.09 mg/mL to 4.12±0.29 mg/mL HPLC fractions of alcalase-treated brewers spent grain protein with/ without digestion up to 66.33±0.85 % at 3 mg/mL IL 171.2±15.1μM, LL 191.7±19.2μM, ILDL 1121.1±116.2μM, ILLPGAQDGL 145.5±10.7μM	(Connolly et al., 2017)
Palmaria palmata	DPP-IV inhibitor	Protein hydrolysates generated via Alcalase and Corolase PP	IC_{50} value 1.60 - 4.24 mg/mL	(Harnedy et al., 2014)
Common Carp (*Cyprinus carpio*) Roe	DPP-IV inhibitor	Protein hydrolysates generated via papain, neutrase, trypsin or pepsin. Ultrafiltration <3kDa, 3-5kDa, 5-10 kDa,	The highest inhibition of DPP-IV was found in papain-treated samples (58.60±2.06%) IC_{50} value for IPNVAVD is 777.35±5.50μM.	(Zhang, C. et al., 2020)

Protein source	Type of test	Peptide/protein hydrolysates	Inhibitory activity	References
		>10kDa Gel fraction SP1-SP3 HPLC SP1 F1-F10 IPNVAVD	Reduced 44.35% DPP-IV activity, increased GLP-1, and significantly improved Glucose uptake	
Porphyara dioica	DPP-IV inhibitor	Alcalase-flavourzyme protein hydrolysates HPLC fraction A – E, Subfraction 19 -22, YLVA	DPP-IV inhibition: Protein extract 30.81±5.1% Protein hydrolysates 60.27±4.6 Fraction up to 56.64±3.8% Subfraction 71.08±0.92% DPP-IV inhibition at 2.0 mg/ml IC_{50} value for YLVA is 439.5±34.4 μM	(Cermeño et al., 2019)
Moringa seed (*Moringa oleifera*)	α-amylase inhibitor	Protein hydrolysates generated via Trypsin, chymotrypsin, pepsin-trypsin	IC_{50} values up to 0.195 and 0.123 ug/μl for pepsin-trypsin treated fraction of protein hydrolysates	(Garza et al., 2017)
Rice bran albumin	α-glucosidase inhibitor	Protein hydrolysates obtained via Alcalase, Protamax, Flavourzyme or Neutrase Ultrafiltration fraction Mw < 3kDa, 3-10kDa, >10kDa	Highest α-glucosidase inhibition among whole protein hydrolysates: 43.1±2.1% Alcalase-treated rice bran albumin hydrolysates Fraction Mw < 3kDa, 3-10kDa, >10kDa from alcalase-treated protein hydrolysates inhibit 47.9±2.6%, 24.9±1.9% and 21.9±1.9% per mg protein	(Uraipong and Zhao, 2016)
Legume (*Phaseolus lunatus, Phaseolus vulgaris* and	α-glucosidase inhibitor, delay intestinal glucose absorption and antihyperglycemic agents	Protein hydrolysates obtained using Alcalase-Flavourzyme & pepsin-pancreatin Ultrafiltration fraction Mw < 1kDa	*In vitro* Inhibit 19.2% - 40% carbohydrate intestinal absorption IC_{50} value from 0.86 to 75mg/mL	(Nuñez-Aragón et al., 2019)

Protein source	Type of test	Peptide/protein hydrolysates	Inhibitory activity	References
mucuna pruriens)			*In vivo* Suppress postprandial hyperglycaemia ED_{50} 1.4 and 93 mg/kg	
Cumin	α-amylase inhibitor	FFRSKLLSDGAAAAKGALLPQYW (CSP3), RCMAFLLSDGAAAAQQLLPQYW (CSP4) DPAQPNYPWTAVLVFRH (CSP6)	IC_{50} value 5.6, 1.58 and 2.39 mg/ml	(Siow, H.-L. et al., 2017b)
Hemp	α-glucosidase inhibitor	Protein hydrolysates obtained via Flavourzyme, protamex, neutrase, trypsin, alcalase, with different E/S ratios Gel filtration fraction RP-HPLC fraction Leu-Arg, Pro-Leu-Met-Leu-Pro	Among protein hydrolysates, Alcalase-treated protein hydrolysates exerts the highest α-glucosidase inhibition (58.26±3.26%) IC_{50} value of the most promising Gel filtration fraction is 0.21±0.01mg/ml The most promising HPLC fraction has a IC_{50} value of 0.024±0.002 mg/ml IC_{50} value 0.027±0.002 0.032±0.003 mg/ml	(Ren, Y. et al., 2016)
Black bean	Modulation of glucose uptake via GLUT2 and SGLT1 transporters	Alcalase-treated protein hydrolysates	10mg/ml hydrolysates decrease 21.5% glucose absorption in caco-2 cell model. For hyperglycaemic rat model, lowest fasting glucose decreased to 150-200 mg/kg BW/day HPI	(Mojica et al., 2017a)
Soybean	α-glucosidase inhibitor, hypoglycemic agents	Trypsin-treated soybean protein hydrolysates. Ultrafiltration fraction MW<5kDa >5kDa Cation exchange column fraction I,II,III from Mw<5kDa Gel filtration 1-4 from faction III	IC_{50} value 1.93 mg/ml IC_{50} value 0.27mg/ml 3.31 mg/ml Lowest IC_{50} value among cation exchange column fraction 0.09mg/ml (III). Lowest IC_{50} value among gel filtration fraction 0.061mg/ml (2)	(Jiang et al., 2018)

Protein source	Type of test	Peptide/protein hydrolysates	Inhibitory activity	References
		RP-HPLC a-e from faction III-1 Gly-Ser-Arg and Glu-Ala-Lys	Lowest IC_{50} value among HPLC fraction is 0.049mg/ml (a) IC_{50} value for peptides are 20.4μM and 520.4μM Significantly decrease the fasting blood glucose level in mice	
Cumin	α-amylase inhibitor	Peptide mixture with Mw 2.0-28.5 kDa produced via Protamex and Novoenyme hydrolysis	22.67% amylase inhibition	(Siow, H.L. and Gan, 2017)
Oat Buckwheat and Highland Barley	DPP-IV inhibitor	Protein hydrolysates produced via alcalase, gastrointestinal or trypsin digestion LQAFEPLR	All protein hydrolysates exert DPP-IV inhibition. The lowest IC_{50} values are 0.13mg/ml alcalase-treated oat glutelin IC_{50} 0.35uM	(Wang, F. et al., 2015)

Table A.5 Anti-inflammatory properties of plant protein derived peptides

Sources	Enzymatic hydrolysis	Cell line	Peptide/ protein hydrolysates	Key results of anti-inflammatory properties	References
Foxtail millet	*In vitro* gastrointestinal digestion (pepsin + pancreatin)	RAW 264.7	Hydrolysates Mw < 3 kDa Chromatographic fraction (F4) EDDQMDPMAK, QNWDFCEAWEPCF	Hydrolysates Inhibit NO Fraction 4 NO from 24.33±0.56 to 17.84±0.60 μM IL-6 Slightly decreased TNF-α from 138.06±2.19 ng/mL to 110.57±4.17 ng/mL at 100μg/mL	(Hu et al., 2020)

Sources	Enzymatic hydrolysis	Cell line	Peptide/ protein hydrolysates	Key results of anti-inflammatory properties	References
				Peptide NO inhibition TNF-α 42.29% and 44.07% IL-6 56.59% AND 43.45%	
Foxtail millet	Alcalase	RAW 264.7	Hydrolysates Mw < 1kDa PDLF, IALLIPF	Dose-dependent decrease in NO, TNF-α, IL-6 and IL-1β p-IκB and p65 Exert anti-inflammatory property via NfκB and MAPK pathway (IALLIPF decrease NO from 15.1μM to 9μM)	(Ji, Z. et al., 2020)
Zein	Thermolysin	EA.hy926	Hydrolysates PPYLSP IIGGAL FLPPVTSMG	Reduced the expression of TNFR1 16.8-41.4% Significant decrease in IκBβ expression Restore phosphorylation of p65	(Liang et al., 2020)
Microalgae	Trypsin	RAW 264.7	Hydrolysates (Mw <3kDa)	Relative quantity at concentration of 7.5μg/ml iNOS 5.05 ± 1.10 (LPS 55.55±5.16) TNF-α 2.12 ± 1.01 (LPS 11.68±1.30) COX-2 17.62±7.27 (LPS 135±32.85) IL-6 9.25±0.58 (LPS 91.65±5.68)	(Suttisuwan et al., 2019)

Sources	Enzymatic hydrolysis	Cell line	Peptide/ protein hydrolysates	Key results of anti-inflammatory properties	References
Common bean milk and yogurt	Pepsin+ pancreatin	Caco-2 cells/ Caco-2, EA.hy926 cell co-culture/ C2BBe1 and EA.hy926 cell co-culture	Hydrolysate Mw <10 kDa Three peptides: s γ-E-S-(Me)C, γ-EL, and LLV	NBM, navy bean milk; NBY, navy bean yogurt; LKBM, light red kidney bean milk; LKBY, light red kidney bean yogurt. Inhibit/decrease as follows: IL-6 NBM, NBY, LKBM, LKBY IL-8 NBY, LKBM, LKBY TNF-α NBY, LKBM, LKBY IL-1β BNY, LKBM, LKBY IL-6 NBY, LKBM, LKBY IL-10 LKBM, LKBY (Caco-2) Caco-2 Cell/ EA,hy926 Cell NBM, NBY, LKBM, LKBY inhibit IL-8, ICAM-1, NCAM-1. IL-1β, IL-6 C2BBe1 cell/ EA.hy926 cell NBM, NBY, LKBM and LKBY inhibit expression of p-JNK p-IκB α Peptides All peptides inhibited IL-8	(Chen, Y. et al., 2019b)
Cereal	-	MIN-6 cell	Commercial soybean peptide (SP), mung	SP, CP, C2, WP reduced IL-6	(Sun, Suling et al.,

Sources	Enzymatic hydrolysis	Cell line	Peptide/ protein hydrolysates	Key results of anti-inflammatory properties	References
			bean peptide (MP), corn peptide (CP, C1 and C2), wheat peptide (WP, W1 and W2)	Levels	2019)
Rapeseed	-	RAW 264.7 cell	LY, GHS, RALP	iNOS (35.05%, 16.29% and 67.83%) NO (38.81%, 11.45% and 61.94%) at 1mol/mL IL-6 (25.55%, 16.42% and 12.93%) TNF-α (50.68%, 24.73% and 43.89%) at 1mM In SHR model (30mg/kg body weight, 5 week) Plasma IL-6 (41.02%, 16.62% and 29.8%) Plasma TNF-α (43.21%, 46.96% and 38.42%)	(He et al., 2019)
Common bean (milk and yogurt)	Pepsin and pancreatin	HT-29 and Caco-2 cell	Navy bean (NB) Light red kidney bean (LKB)	NBM NBY LKBM LKBY Apart from NMB 10-50 kDa, all samples inhibited IL-8 < 10kDa fractions more promising than 10-50kDa fraction (0.05 mg/ml) HT-29 NBY 0.04mg/ml, LKBM 0.2, 0.04	(Chen, Y. et al., 2019a)

Sources	Enzymatic hydrolysis	Cell line	Peptide/ protein hydrolysates	Key results of anti-inflammatory properties	References
				mg/mL, LKBY 0.04 mg/mL cannot inhibit IL-8 generation in HT-29 cells but all the samples inhibit IL-8 generation in Caco-2 cells Peptide fractions and phenolic fraction in protein hydrolysates inhibited IL-8 release in Caco 2 and HT-29 cells (at 0.2, 0.04 mg/mL)	
Millet grain	Gastrointestinal digestion (α-amylase, pepsin, pancreatin) (INFOGEST digestion)	Unknown	Albumin, Globulin 7S, Globulin 11S, Prolamin, Glutelin hydrolysates and Mw < 3kDa fraction	COX-1 globulin 11S and prolamin hydrolysates (65℃) IC_{50} value from 0.038 and 0.033mg/mL COX-2 globulin 7S hydrolysates (100℃) IC_{50} value 0.07mg/ml LOX IC_{50}= 0.15 mg/mL	(Jakubczyk et al., 2019)
Chia seeds *(Salvia hispanica L.)*	Pepsin and pancratin	Peritoneal murine macrophages	Protein hydrolysates Ultrafiltration (<1, 1-3, 3-5, 5-10, > 10kDa)	Fraction with Mw 1-3 kDa are most promising anti-inflammatory agents IL-1β 59% inhibition, IL-6 61.63% inhibition TNF-α 47.49% inhibition	(Chan-Zapata et al., 2019)
Hazelnut	-	RAW 264.7 macrophage	LDAPGHR	LDAPGHR (50μM) decrease 62.6% IL-1β and 61.6% IL-6	(Ren, D. et al., 2018)

Sources	Enzymatic hydrolysis	Cell line	Peptide/ protein hydrolysates	Key results of anti-inflammatory properties	References
				inhibition, but no TNF-α inhibition. 66.5% NO inhibition, 77.9% PGE 2 inhibition 65.0% and 65.6% iNOS and COX-2 mRNA expression 93.5% 93.5% inhibition of IκB-α 40.5% and 86.7% inhibition of p65 phosphorylation (nucleus)/(cytoplasm) Down-regulate JNK, ERK1/2 and p38 phosphorylation	
Zein	Alcalase, neutral protease, thermolysin (Gastric phase: pepsin Intestinal phase: pancreatin)	EA.hy926 Caco-2	Hydrolysates 11 peptides (IIGGAL, PPYLSP, FLPPVTSMG, LLPFNQ, LLPFN, TIFPQ, LPFNQ, FLPFN, FLPFNQ, LPPVTSMG, FLPPV)	Gastric phase Inhibits 53.8% VCAM-1 Intestinal phase inhibits 33.9% - 50.9% VCAM-1 (thermolysin hydrolysates are the most promising) After transport digestion, the digest inhibits 80.0% VCAM-1 and inhibit 54.9% ICAM-1 PPYLSP, IIGGAL and	(Liang et al., 2018)

Sources	Enzymatic hydrolysis	Cell line	Peptide/ protein hydrolysates	Key results of anti-inflammatory properties	References
				FLPPVTSMG inhibit 38.9 – 54% VCAM-1 and 28.6-36.5% inhibition at 0.2 mM The other eight peptides 35.1 -77.7% VCAM-1 4.7 – 42.6% at 0.3 mM	
Lychee seed	Alcalase, Flavourzyme, Neutrase	Raw 264.7 cell culture	Hydrolysates Ultrafiltration fractions (<0.65, 0.65-3, 3-5, 5-10, >10 kDa) Chromatographic fractions Four peptides: KVRTKLLPP, MKLCWQKSIHGS, XDVDRIDK, RPLVTHK	Neutrase hydrolysate has the strongest NO inhibition with IC_{50} 3.44± 0.82μg/mL. Fraction with Mw <0.65kDa exerted the most promising NO inhibition with IC_{50} 12.01±0.94μg/mL Gel chromatography, fraction G1 with lowest IC_{50} value 22.54±1.09μg/ml and also inhibit the gene expression of COX-2, IL-6, iNOS, and TNF-α PR-HPLC chromatography, fraction H3 was selected to identify the peptide sequences, with IC_{50} 2.81 ± 0.01μg/mL Peptides With IC_{50} values of inhibit NO are 10.90±0.44, 5.35±0.10, 2.81±0.01	(Saisavoey et al., 2018)

Sources	Enzymatic hydrolysis	Cell line	Peptide/ protein hydrolysates	Key results of anti-inflammatory properties	References
				and 4.42±0l.38 µg/ml, respectively.	
Soybean	-	Vascular smooth muscle cell	LSW	Down-regulate COX-2 (no inhibition in iNOS), AT1R, reduced the phosphorylation of AT1R and ERK1/2 Reduce the phosphorylation of p50, but not p65	(Lin et al., 2017)
Amaranth	Alcalase	Bacterial flagellin-activated Caco-2 CCL20: luc cells	Protein hydrolysates Chromatographic fraction Three peptides: SSEDIKE, IADEDPDEANDK and KPV	At 2.5 mg/ml, AH (DH 23%) and AH (AH30) significantly inhibit the activation of Caco-luc cell. Two fractions M1 and M2 exerts 81.13±1.82% and 98.91±0.47% inhibition at 1.112±0.82 mg/ml and 2.505±0.091 mg/ml concentration. Peptides SSEDIKE (more active) and KPV suppress the expression of CCL20	(Moronta et al., 2016)
Lupin (*Lupinus angustifolius L.*)	Alcalase	Human monocytic THP-1 cell, RAW 264.7 macrophages	Protein hydrolysates Ultrafiltration fraction (<10, >10 kDa) Chromatographic fraction GPETAFLR	Mw >10kDa fraction inhibit 41% and 32% of the gene expression of TNF and CCL2, while MW <10 kDa fraction inhibits 50% of both cytokines	(del Carmen Millán-Linares et al., 2015)

Sources	Enzymatic hydrolysis	Cell line	Peptide/ protein hydrolysates	Key results of anti-inflammatory properties	References
				HPLC fraction F3, F7 inhibit the expression of IL-6, CCR2, CCL2 and CCL18 Gel Fraction F3-5 downregulate the expression of TNF, CCR2 (-59.54%) and CCL2 (-74.45%) Peptide (100, 500μg/mL) Reduce 40% of TNF and IL-1β expression Reduce 50% CCL2 expression Only inhibit CCR2 at the concentration of 100 μg/ml Inhibit TNF (-42%) and IL-1β(-39%) cytokine production at 100 μg/mL (after 6 hours, but no effect after 48 hours),while increased the IL-10 production Inhibit the generation of NO	
Pinto bean	Alcalase, Savinase	CCD-18Co colon myofibroblasts	Hydrolysates with fraction (Mw < 3kDa)	Alcalase-treated protein hydrolysates exert the most promising inhibition of IL-6 (28% inhibition) (120 min hydrolysis) among all the samples	(Garcia-Mora et al., 2015)

Sources	Enzymatic hydrolysis	Cell line	Peptide/ protein hydrolysates	Key results of anti-inflammatory properties	References
Amaranth	Pepsin and pancreatin	THP-1 human cell RAW 264.7 macrophage	Hydrolysates (Unprocessed and extruded)	THP-1 human cells Inhibit the phosphorylation of IKK-α (58.7 and 52.5% respectively), IκB-α (74.1 AND 86.1%, respectively) Inhibit 71.9% of p65 expression (Unprocessed) Inhibit 67.2 and 64.2% of p50 and p65 (extruded) 17.1% reduce PGE2 production (unprocessed) 36.5% TNF-α and 15.4 % PGE2 (extruded) Reduced COX-2 expression by 25.4% and 38.1% RAW 264.7 mouse cells Inhibition phosphorylation of IKKα (85.4% and 88.2%) IκB (88.9% and 66.2%) reduced p50 by 66.5 and 72.4% and p65 84.5 and 70.7%	(Montoya-Rodríguez et al., 2014)

Sources	Enzymatic hydrolysis	Cell line	Peptide/ protein hydrolysates	Key results of anti-inflammatory properties	References
				Inhibit NO production 53.0% and 60.0% iNOS 71.4% and 59.0% Reduced 36.8% PGE2 production (Unprocessed) Inhibit TNF-α (33.5%) and PGE2 (31.4%) (extruded) reduction of 84.3% and 67.6% COX-2 expression	
Edible microalgal (*spirulina maxima*)	Trypsin and α-chymotrypsin	RBL-2H3 cell	Hydrolysates Ultrafiltration fraction (<3, 3-5, 5-10, >10 kDa) Chromatographic fraction LDAVNF, MMLDF	Decrease IL-8 production Fraction with Mw <3 kDa exert the strongest inhibition on histamine release and IL-8 production After HPLC and gel fraction chromatography FII-8 are found to be most promising against histamine release and IL-8 production Peptides Inhibit histamine release 34% and 39% at 200μM	(Vo et al., 2013)

Sources	Enzymatic hydrolysis	Cell line	Peptide/ protein hydrolysates	Key results of anti-inflammatory properties	References
				Reduced IL-8 level from 1624 pg/ml to 626 pg/ml and 532 pg/ml	
Lunasin	-	Raw 264.7 cells	Lunasin Four fragments (SKWQHQQDSCRKQLQGVNLTPC; DDDDDDDDD, EKHIMEKIQGRGDDDDDDDDD; EKHIMEKIQ)	Lunasin reduce 23% and 37% TNF-α and IL-6 level at 200 μM, (no inhibition found in four fragments) Lunasin and related peptides cannot inhibit NO generation	(Hernández-Ledesma et al., 2009)

Reference

Abdullah, F.I., Chua, L.S., Rahmat, Z., Soontorngun, N. and Somboon, P. 2018. Trypsin hydrolysed protein fractions as radical scavengers and anti-bacterial agents from Ficus deltoidea. *International Journal of Peptide Research and Therapeutics.* **24**(2), pp.279-290.

Aderinola, T.A., Fagbemi, T.N., Enujiugha, V.N., Alashi, A.M. and Aluko, R.E. 2019. In vitro antihypertensive and antioxidative properties of alcalase‐derived Moringa oleifera seed globulin hydrolysate and its membrane fractions. *Journal of Food Processing and Preservation.* **43**(2), pe13862.

Admassu, H., Gasmalla, M.A., Yang, R. and Zhao, W. 2018. Identification of bioactive peptides with α-amylase inhibitory potential from enzymatic protein hydrolysates of red seaweed (Porphyra spp). *Journal of agricultural and food chemistry.* **66**(19), pp.4872-4882.

Afifah, B.S.S.N.H. and Gan, C.-Y. 2016. Antioxidative and amylase inhibitor peptides from basil seeds. *International Journal of Peptide Research and Therapeutics.* **22**(1), pp.3-10.

Agirbasli, Z. and Cavas, L. 2017. In silico evaluation of bioactive peptides from the green algae Caulerpa. *Journal of Applied Phycology.* **29**(3), pp.1635-1646.

Aiello, G., Li, Y., Boschin, G., Bollati, C., Arnoldi, A. and Lammi, C. 2019. Chemical and biological characterization of spirulina protein hydrolysates: Focus on ACE and DPP-IV activities modulation. *Journal of Functional Foods.* **63**, p103592.

Ambigaipalan, P., Al-Khalifa, A.S. and Shahidi, F. 2015. Antioxidant and angiotensin I converting enzyme (ACE) inhibitory activities of date seed protein hydrolysates prepared using Alcalase, Flavourzyme and Thermolysin. *Journal of Functional Foods.* **18**, pp.1125-1137.

Arise, A.K., Alashi, A.M., Nwachukwu, I.D., Malomo, S.A., Aluko, R.E. and Amonsou, E.O. 2017. Inhibitory properties of bambara groundnut protein hydrolysate and peptide fractions against angiotensin‐converting enzymes, renin and free radicals. *Journal of the Science of Food and Agriculture.* **97**(9), pp.2834-2841.

Awosika, T.O. and Aluko, R.E. 2019. Inhibition of the in vitro activities of α‐amylase, α‐glucosidase and pancreatic lipase by yellow field pea (Pisum sativum L.) protein hydrolysates. *International Journal of Food Science & Technology.* **54**(6), pp.2021-2034.

Bleakley, S., Hayes, M., O'Shea, N., Gallagher, E. and Lafarga, T. 2017. Predicted release and analysis of novel ACE-I, renin, and DPP-IV inhibitory peptides from common oat (Avena sativa) protein hydrolysates using in silico analysis. *Foods.* **6**(12), p108.

Boschin, G., Scigliuolo, G.M., Resta, D. and Arnoldi, A. 2014. Optimization of the enzymatic hydrolysis of lupin (Lupinus) proteins for producing ACE-inhibitory peptides. *Journal of agricultural and food chemistry.* **62**(8), pp.1846-1851.

Cermeño, M., Stack, J., Tobin, P.R., O'Keeffe, M.B., Harnedy, P.A., Stengel, D.B. and FitzGerald, R.J. 2019. Peptide identification from a Porphyra dioica protein hydrolysate with antioxidant, angiotensin converting enzyme and dipeptidyl peptidase IV inhibitory activities. *Food & function.* **10**(6), pp.3421-3429.

Chan-Zapata, I., Arana-Argáez, V.E., Torres-Romero, J.C. and Segura-Campos, M.R. 2019. Anti-inflammatory effects of the protein hydrolysate and peptide fractions isolated from Salvia hispanica L. seeds. *Food and Agricultural Immunology.* **30**(1), pp.786-803.

Chang, Y.-W. and Alli, I. 2012. In silico assessment: Suggested homology of chickpea (Cicer arietinum L.) legumin and prediction of ACE-inhibitory peptides from chickpea proteins using BLAST and BIOPEP analyses. *Food research international.* **49**(1), pp.477-486.

Chen, J., Liu, S., Ye, R., Cai, G., Ji, B. and Wu, Y. 2013. Angiotensin-I converting enzyme (ACE) inhibitory tripeptides from rice protein hydrolysate: Purification and characterization. *Journal of Functional Foods.* **5**(4), pp.1684-1692.

Chen, J., Tan, L., Li, C., Zhou, C., Hong, P., Sun, S. and Qian, Z.-J. 2020. Mechanism Analysis of a Novel Angiotensin-I-Converting Enzyme Inhibitory Peptide from Isochrysis zhanjiangensis Microalgae for Suppressing Vascular Injury in Human Umbilical Vein Endothelial Cells. *Journal of Agricultural and Food Chemistry.* **68**(15), pp.4411-4423.

Chen, Y., Li, J., Dong, N., Zhang, Y., Lu, X., Hao, Y. and Qi, J. 2020. Separation and identification of ACE inhibitory peptides from defatted walnut meal. *European Food Research and Technology.* pp.1-10.

Chen, Y., Zhang, H., Liu, R., Mats, L., Zhu, H., Pauls, K.P., Deng, Z. and Tsao, R. 2019a. Antioxidant and anti-inflammatory polyphenols and peptides of common bean (Phaseolus vulga L.) milk and yogurt in Caco-2 and HT-29 cell models. *Journal of Functional Foods.* **53**, pp.125-135.

Chen, Y., Zhang, H., Mats, L., Liu, R., Deng, Z., Mine, Y. and Tsao, R. 2019b. Anti-inflammatory effect and cellular uptake mechanism of peptides from common bean (Phaseolus vulga L.) milk and yogurts in Caco-2 mono-and Caco-2/EA. hy926 co-culture models. *Journal of agricultural and food chemistry.* **67**(30), pp.8370-8381.

Cheung, I.W., Nakayama, S., Hsu, M.N., Samaranayaka, A.G. and Li-Chan, E.C. 2009. Angiotensin-I converting enzyme inhibitory activity of hydrolysates from oat (Avena sativa) proteins by in silico and in vitro analyses. *Journal of agricultural and food chemistry.* **57**(19), pp.9234-9242.

Chiozzi, R.Z., Capriotti, A.L., Cavaliere, C., La Barbera, G., Piovesana, S. and Laganà, A. 2016. Identification of three novel angiotensin-converting enzyme inhibitory peptides derived from cauliflower by-products by multidimensional liquid chromatography and bioinformatics. *Journal of Functional Foods.* **27**, pp.262-273.

Ciau‐Solís, N.A., Acevedo‐Fernández, J.J. and Betancur‐Ancona, D. 2018. In vitro renin–angiotensin system inhibition and in vivo antihypertensive activity of peptide fractions from lima bean (Phaseolus lunatus L.). *Journal of the Science of Food and Agriculture.* **98**(2), pp.781-786.

Connolly, A., O'Keeffe, M.B., Nongonierma, A.B., Piggott, C.O. and FitzGerald, R.J. 2017. Isolation of peptides from a novel brewers spent grain protein isolate with potential to modulate glycaemic response. *International Journal of Food Science & Technology.* **52**(1), pp.146-153.

Connolly, A., O'Keeffe, M.B., Piggott, C.O., Nongonierma, A.B. and FitzGerald, R.J. 2015. Generation and identification of angiotensin converting enzyme (ACE) inhibitory peptides from a brewers' spent grain protein isolate. *Food chemistry.* **176**, pp.64-71.

Cruz-Chamorro, I., Álvarez-Sánchez, N., Santos-Sánchez, G., Pedroche, J., Fernández-Pachón, M.-S., Millán, F., Millán-Linares, M.C., Lardone, P.J., Bejarano, I. and Guerrero, J.M. 2020. Immunomodulatory and Antioxidant Properties of Wheat Gluten Protein Hydrolysates in Human Peripheral Blood Mononuclear Cells. *Nutrients.* **12**(6), p1673.

Cumby, N., Zhong, Y., Naczk, M. and Shahidi, F. 2008. Antioxidant activity and water-holding capacity of canola protein hydrolysates. *Food chemistry.* **109**(1), pp.144-148.

de Souza Rocha, T., Hernandez, L.M.R., Chang, Y.K. and de Mejía, E.G. 2014. Impact of germination and enzymatic hydrolysis of cowpea bean (Vigna unguiculata) on the generation of peptides capable of inhibiting dipeptidyl peptidase IV. *Food research international.* **64**, pp.799-809.

del Carmen Millán-Linares, M., Millán, F., Pedroche, J. and del Mar Yust, M. 2015. GPETAFLR: A new anti-inflammatory peptide from Lupinus angustifolius L. protein hydrolysate. *Journal of Functional Foods.* **18**, pp.358-367.

Deng, Z., Liu, Y., Wang, J., Wu, S., Geng, L., Sui, Z. and Zhang, Q. 2018. Antihypertensive effects of two novel angiotensin I-converting enzyme (ACE) inhibitory peptides from Gracilariopsis lemaneiformis (Rhodophyta) in spontaneously hypertensive rats (SHRs). *Marine drugs.* **16**(9), p299.

Ding, J., Liang, R., Yang, Y., Sun, N. and Lin, S. 2020. Optimization of pea protein hydrolysate preparation and purification of antioxidant peptides based on an in silico analytical approach. *LWT.* **123**, p109126.

EVANGELHO, J.A.d., Berrios, J.d.J., Pinto, V.Z., Antunes, M.D., Vanier, N.L. and Zavareze, E.d.R. 2016. Antioxidant activity of black bean (Phaseolus vulgaris L.) protein hydrolysates. *Food Science and Technology.* **36**, pp.23-27.

Famuwagun, A.A., Alashi, A.M., Gbadamosi, O.S., Taiwo, K.A., Oyedele, D., Adebooye, O.C. and Aluko, R.E. 2020. Antioxidant and enzymes inhibitory properties of Amaranth leaf protein hydrolyzates and ultrafiltration peptide fractions. *Journal of Food Biochemistry.* pe13396.

Fu, Y., Alashi, A.M., Young, J.F., Therkildsen, M. and Aluko, R.E. 2017. Enzyme inhibition kinetics and molecular interactions of patatin peptides with angiotensin I-converting enzyme and renin. *International journal of biological macromolecules.* **101**, pp.207-213.

Gangopadhyay, N., Wynne, K., O'Connor, P., Gallagher, E., Brunton, N.P., Rai, D.K. and Hayes, M. 2016. In silico and in vitro analyses of the angiotensin-I converting enzyme inhibitory activity of hydrolysates generated from crude barley (Hordeum vulgare) protein concentrates. *Food chemistry.* **203**, pp.367-374.

Gao, D., Zhang, F., Ma, Z., Chen, S., Ding, G., Tian, X. and Feng, R. 2019. Isolation and identification of the angiotensin-I converting enzyme (ACE) inhibitory peptides derived from cottonseed protein: optimization of hydrolysis conditions. *International Journal of Food Properties.* **22**(1), pp.1296-1309.

Gao, X., Xue, Z., Ma, Q., Guo, Q., Xing, L., Santhanam, R.K., Zhang, M. and Chen, H. 2020. Antioxidant and antihypertensive effects of garlic protein and its hydrolysates and the related mechanism. *Journal of food biochemistry.* **44**(2), pe13126.

Garcia-Mora, P., Frias, J., Peñas, E., Zieliński, H., Giménez-Bastida, J.A., Wiczkowski, W., Zielińska, D. and Martínez-Villaluenga, C. 2015. Simultaneous release of peptides and phenolics with antioxidant, ACE-inhibitory and anti-inflammatory activities from pinto bean (Phaseolus vulgaris L. var. pinto) proteins by subtilisins. *Journal of Functional Foods.* **18**, pp.319-332.

Garcia-Mora, P., Penas, E., Frias, J., Zielinski, H., Wiczkowski, W., Zielinska, D. and Martinez-Villaluenga, C. 2016. High-pressure-assisted enzymatic release of peptides and phenolics increases angiotensin converting enzyme I inhibitory and antioxidant activities of pinto bean hydrolysates. *Journal of agricultural and food chemistry.* **64**(8), pp.1730-1740.

Garg, S., Apostolopoulos, V., Nurgali, K. and Mishra, V.K. 2018. Evaluation of in silico approach for prediction of presence of opioid peptides in wheat. *Journal of Functional Foods.* **41**, pp.34-40.

Garza, N.G.G., Koyoc, J.A.C., Castillo, J.A.T., Zambrano, E.A.G., Ancona, D.B., Guerrero, L.C. and García, S.R.S. 2017. Biofunctional properties of bioactive peptide fractions from protein isolates of moringa seed (Moringa oleifera). *Journal of food science and technology.* **54**(13), pp.4268-4276.

Girgih, A.T., Chao, D., Lin, L., He, R., Jung, S. and Aluko, R.E. 2015. Enzymatic protein hydrolysates from high pressure-pretreated isolated pea proteins have better antioxidant properties than similar hydrolysates produced from heat pretreatment. *Food chemistry.* **188**, pp.510-516.

Gülseren, İ. 2018. In silico methods to identify ACE and DPP-IV inhibitory activities of ribosomal hazelnut proteins. *Journal of Food Measurement and Characterization.* **12**(4), pp.2607-2614.

Guo, H., Richel, A., Hao, Y., Fan, X., Everaert, N., Yang, X. and Ren, G. 2020. Novel dipeptidyl peptidase‐IV and angiotensin‐I‐converting enzyme inhibitory peptides released from quinoa protein by in silico proteolysis. *Food Science & Nutrition.* **8**(3), pp.1415-1422.

Han, R., Maycock, J., Murray, B.S. and Boesch, C. 2019. Identification of angiotensin converting enzyme and dipeptidyl peptidase-IV inhibitory peptides derived from oilseed proteins using two integrated bioinformatic approaches. *Food Research International.* **115**, pp.283-291.

Harnedy, P.A. and FitzGerald, R.J. 2013. In vitro assessment of the cardioprotective, anti-diabetic and antioxidant potential of Palmaria palmata protein hydrolysates. *Journal of applied phycology.* **25**(6), pp.1793-1803.

Harnedy, P.A., O'Keeffe, M.B. and FitzGerald, R.J. 2015. Purification and identification of dipeptidyl peptidase (DPP) IV inhibitory peptides from the macroalga Palmaria palmata. *Food Chemistry.* **172**, pp.400-406.

Harnedy, P.A., Soler-Vila, A., Edwards, M.D. and FitzGerald, R.J. 2014. The effect of time and origin of harvest on the in vitro biological activity of

Palmaria palmata protein hydrolysates. *Food research international.* **62**, pp.746-752.

He, R., Girgih, A.T., Malomo, S.A., Ju, X. and Aluko, R.E. 2013a. Antioxidant activities of enzymatic rapeseed protein hydrolysates and the membrane ultrafiltration fractions. *Journal of Functional Foods.* **5**(1), pp.219-227.

He, R., Malomo, S.A., Alashi, A., Girgih, A.T., Ju, X. and Aluko, R.E. 2013b. Purification and hypotensive activity of rapeseed protein-derived renin and angiotensin converting enzyme inhibitory peptides. *Journal of Functional Foods.* **5**(2), pp.781-789.

He, R., Wang, Y., Yang, Y., Wang, Z., Ju, X. and Yuan, J. 2019. Rapeseed protein-derived ACE inhibitory peptides LY, RALP and GHS show antioxidant and anti-inflammatory effects on spontaneously hypertensive rats. *Journal of functional foods.* **55**, pp.211-219.

Hernández-Ledesma, B., Hsieh, C.-C. and Ben, O. 2009. Antioxidant and anti-inflammatory properties of cancer preventive peptide lunasin in RAW 264.7 macrophages. *Biochemical and Biophysical Research Communications.* **390**(3), pp.803-808.

Hu, S., Yuan, J., Gao, J., Wu, Y., Meng, X., Tong, P. and Chen, H. 2020. Antioxidant and anti-inflammatory potential of peptides derived from the in vitro gastrointestinal digestion of germinated and heat-treated foxtail millet (Setaria italica) proteins. *Journal of Agricultural and Food Chemistry.*

Ibrahim, M.A., Bester, M.J., Neitz, A.W. and Gaspar, A.R. 2019. Tuber storage proteins as potential precursors of bioactive peptides: An in silico analysis. *International Journal of Peptide Research and Therapeutics.* **25**(2), pp.437-446.

Iwaniak, A. and Dziuba, J. 2009. Analysis of domains in selected plant and animal food proteins-precursors of biologically active peptides-in silico approach. *Food science and technology international.* **15**(2), pp.179-191.

Jahanbani, R., Ghaffari, S.M., Salami, M., Vahdati, K., Sepehri, H., Sarvestani, N.N., Sheibani, N. and Moosavi-Movahedi, A.A. 2016. Antioxidant and anticancer activities of walnut (Juglans regia L.) protein hydrolysates using different proteases. *Plant foods for human nutrition.* **71**(4), pp.402-409.

Jakubczyk, A., Szymanowska, U., Karaś, M., Złotek, U. and Kowalczyk, D. 2019. Potential anti-inflammatory and lipase inhibitory peptides generated by in vitro gastrointestinal hydrolysis of heat treated millet grains. *CyTA-Journal of Food.* **17**(1), pp.324-333.

Jara, A.M.R., Liggieri, C.S. and Bruno, M.A. 2018. Preparation of soy protein hydrolysates with antioxidant activity by using peptidases from latex of Maclura pomifera fruits. *Food chemistry.* **264**, pp.326-333.

Ji, D., Udenigwe, C.C. and Agyei, D. 2019. Antioxidant peptides encrypted in flaxseed proteome: An in silico assessment. *Food Science and Human Wellness.* **8**(3), pp.306-314.

Ji, Z., Mao, J., Chen, S. and Mao, J. 2020. Antioxidant and anti-inflammatory activity of peptides from foxtail millet (Setaria italica) prolamins in HaCaT cells and RAW264. 7 murine macrophages. *Food Bioscience.* p100636.

Jiang, M., Yan, H., He, R. and Ma, Y. 2018. Purification and a molecular docking study of α-glucosidase-inhibitory peptides from a soybean protein hydrolysate with ultrasonic pretreatment. *European Food Research and Technology.* **244**(11), pp.1995-2005.

Jimsheena, V. and Gowda, L.R. 2011. Angiotensin I-converting enzyme (ACE) inhibitory peptides derived from arachin by simulated gastric digestion. *Food chemistry.* **125**(2), pp.561-569.

Jin, D.-x., Liu, X.-l., Zheng, X.-q., Wang, X.-j. and He, J.-f. 2016. Preparation of antioxidative corn protein hydrolysates, purification and evaluation of three novel corn antioxidant peptides. *Food Chemistry.* **204**, pp.427-436.

Kanbargi, K.D., Sonawane, S.K. and Arya, S.S. 2016. Functional and antioxidant activity of Ziziphus jujube seed protein hydrolysates. *Journal of food measurement and characterization.* **10**(2), pp.226-235.

Kandemir-Cavas, C., Pérez-Sanchez, H., Mert-Ozupek, N. and Cavas, L. 2019. In silico analysis of bioactive peptides in invasive sea grass Halophila stipulacea. *Cells.* **8**(6), p557.

Karamać, M., Kosińska-Cagnazzo, A. and Kulczyk, A. 2016. Use of different proteases to obtain flaxseed protein hydrolysates with antioxidant activity. *International journal of molecular sciences.* **17**(7), p1027.

Karami, Z., Peighambardoust, S.H., Hesari, J., Akbari-Adergani, B. and Andreu, D. 2019. Antioxidant, anticancer and ACE-inhibitory activities of bioactive peptides from wheat germ protein hydrolysates. *Food Bioscience.* **32**, p100450.

Kong, X., Zhou, H. and Hua, Y. 2008. Preparation and antioxidant activity of wheat gluten hydrolysates (WGHs) using ultrafiltration membranes. *Journal of the Science of Food and Agriculture.* **88**(5), pp.920-926.

Kumagai, Y., Miyabe, Y., Takeda, T., Adachi, K., Yasui, H. and Kishimura, H. 2019. In silico analysis of relationship between proteins from plastid genome of red alga Palmaria sp.(Japan) and angiotensin I converting enzyme inhibitory peptides. *Marine drugs.* **17**(3), p190.

Lammi, C., Bollati, C., Ferruzza, S., Ranaldi, G., Sambuy, Y. and Arnoldi, A. 2018. Soybean-and lupin-derived peptides inhibit DPP-IV activity on in situ human intestinal Caco-2 cells and ex vivo human serum. *Nutrients.* **10**(8), p1082.

Lammi, C., Zanoni, C., Arnoldi, A. and Vistoli, G. 2016. Peptides derived from soy and lupin protein as dipeptidyl-peptidase IV inhibitors: in vitro biochemical screening and in silico molecular modeling study. *Journal of agricultural and food chemistry.* **64**(51), pp.9601-9606.

Lee, S.-H., Park, M.-H., Han, J.-S., Jeong, Y., Kim, M. and Jeon, Y.-J. 2012. Bioactive compounds extracted from Gamtae (Ecklonia cava) by using enzymatic hydrolysis, a potent α-glucosidase and α-amylase inhibitor, alleviates postprandial hyperglycemia in diabetic mice. *Food Science and Biotechnology.* **21**(4), pp.1149-1155.

Li, M., Xia, S., Zhang, Y. and Li, X. 2018. Optimization of ACE inhibitory peptides from black soybean by microwave-assisted enzymatic method and study on its stability. *Lwt.* **98**, pp.358-365.

Li, M., Zheng, H., Lin, M., Zhu, W. and Zhang, J. 2020. Characterization of the protein and peptide of excipient zein by the multi-enzyme digestion coupled with nano-LC-MS/MS. *Food Chemistry.* p126712.

Li, P., Jia, J., Fang, M., Zhang, L., Guo, M., Xie, J., Xia, Y., Zhou, L. and Wei, D. 2014. In vitro and in vivo ACE inhibitory of pistachio hydrolysates and in silico mechanism of identified peptide binding with ACE. *Process Biochemistry.* **49**(5), pp.898-904.

Liang, Q., Chalamaiah, M., Liao, W., Ren, X., Ma, H. and Wu, J. 2020. Zein hydrolysate and its peptides exert anti-inflammatory activity on endothelial cells by preventing TNF-α-induced NF-κB activation. *Journal of Functional Foods.* **64**, p103598.

Liang, Q., Chalamaiah, M., Ren, X., Ma, H. and Wu, J. 2018. Identification of new anti-inflammatory peptides from zein hydrolysate after simulated gastrointestinal digestion and transport in caco-2 cells. *Journal of agricultural and food chemistry.* **66**(5), pp.1114-1120.

Lin, Q., Liao, W., Bai, J., Wu, W. and Wu, J. 2017. Soy protein-derived ACE-inhibitory peptide LSW (Leu-Ser-Trp) shows anti-inflammatory activity on vascular smooth muscle cells. *Journal of Functional Foods.* **34**, pp.248-253.

Liu, C., Fang, L., Min, W., Liu, J. and Li, H. 2018. Exploration of the molecular interactions between angiotensin-I-converting enzyme (ACE) and the inhibitory peptides derived from hazelnut (Corylus heterophylla Fisch.). *Food chemistry.* **245**, pp.471-480.

Liu, W.-Y., Fang, L., Feng, X.-W., Li, G.-M. and Gu, R.-Z. 2020. In vitro antioxidant and angiotensin I-converting enzyme inhibitory properties of peptides derived from corn gluten meal. *European Food Research and Technology.* pp.1-11.

Lu, X., Zhang, L., Sun, Q., Song, G. and Huang, J. 2019. Extraction, identification and structure-activity relationship of antioxidant peptides from sesame (Sesamum indicum L.) protein hydrolysate. *Food Res Int.* **116**, pp.707-716.

Ma, F.-F., Wang, H., Wei, C.-K., Thakur, K., Wei, Z.-J. and Jiang, L. 2019. Three novel ACE inhibitory peptides isolated from Ginkgo biloba seeds: Purification, inhibitory kinetic and mechanism. *Frontiers in pharmacology.* **9**, p1579.

Marseglia, A., Dellafiora, L., Prandi, B., Lolli, V., Sforza, S., Cozzini, P., Tedeschi, T., Galaverna, G. and Caligiani, A. 2019. Simulated gastrointestinal digestion of cocoa: Detection of resistant peptides and in silico/in vitro prediction of their ace inhibitory activity. *Nutrients.* **11**(5), p985.

Megías, C., Yust, M.d.M., Pedroche, J., Lquari, H., Girón-Calle, J., Alaiz, M., Millán, F. and Vioque, J. 2004. Purification of an ACE inhibitory peptide after hydrolysis of sunflower (Helianthus annuus L.) protein isolates. *Journal of Agricultural and Food Chemistry.* **52**(7), pp.1928-1932.

Memarpoor-Yazdi, M., Mahaki, H. and Zare-Zardini, H. 2013. Antioxidant activity of protein hydrolysates and purified peptides from Zizyphus jujuba fruits. *Journal of functional foods.* **5**(1), pp.62-70.

Mirzaei, M., Mirdamadi, S., Safavi, M. and Hadizadeh, M. 2019. In vitro and in silico studies of novel synthetic ACE-inhibitory peptides derived from Saccharomyces cerevisiae protein hydrolysate. *Bioorganic Chemistry.* **87**, pp.647-654.

Mirzapour, M., Rezaei, K. and Sentandreu, M.A. 2017. Identification of potent ACE inhibitory peptides from wild almond proteins. *Journal of food science.* **82**(10), pp.2421-2431.

Mirzapour, M., Rezaei, K., Sentandreu, M.A. and Moosavi‐Movahedi, A.A. 2016. In vitro antioxidant activities of hydrolysates obtained from Iranian wild almond (A mygdalus scoparia) protein by several enzymes. *International Journal of Food Science & Technology.* **51**(3), pp.609-616.

Mojica, L., Chen, K. and de Mejía, E.G. 2015. Impact of commercial precooking of common bean (Phaseolus vulgaris) on the generation of peptides, after pepsin–pancreatin hydrolysis, capable to inhibit dipeptidyl peptidase‐IV. *Journal of food science.* **80**(1), pp.H188-H198.

Mojica, L. and De Mejía, E.G. 2016. Optimization of enzymatic production of anti-diabetic peptides from black bean (Phaseolus vulgaris L.) proteins, their characterization and biological potential. *Food & function.* **7**(2), pp.713-727.

Mojica, L., de Mejia, E.G., Granados-Silvestre, M.Á. and Menjivar, M. 2017a. Evaluation of the hypoglycemic potential of a black bean hydrolyzed protein isolate and its pure peptides using in silico, in vitro and in vivo approaches. *Journal of Functional Foods.* **31**, pp.274-286.

Mojica, L., Luna‐Vital, D.A. and González de Mejía, E. 2017b. Characterization of peptides from common bean protein isolates and their potential to inhibit markers of type‐2 diabetes, hypertension and oxidative stress. *Journal of the Science of Food and Agriculture.* **97**(8), pp.2401-2410.

Montone, C.M., Capriotti, A.L., Cavaliere, C., La Barbera, G., Piovesana, S., Chiozzi, R.Z. and Laganà, A. 2018. Peptidomic strategy for purification and identification of potential ACE-inhibitory and antioxidant peptides in Tetradesmus obliquus microalgae. *Analytical and bioanalytical chemistry.* **410**(15), pp.3573-3586.

Montoya‐Rodríguez, A., de Mejía, E.G., Dia, V.P., Reyes‐Moreno, C. and Milán‐Carrillo, J. 2014. Extrusion improved the anti‐inflammatory effect of amaranth (A maranthus hypochondriacus) hydrolysates in LPS‐induced human THP‐1 macrophage‐like and mouse RAW 264.7 macrophages by preventing activation of NF‐κB signaling. *Molecular nutrition & food research.* **58**(5), pp.1028-1041.

Moronta, J., Smaldini, P.L., Docena, G.H. and Añón, M.C. 2016. Peptides of amaranth were targeted as containing sequences with potential anti-inflammatory properties. *Journal of Functional Foods.* **21**, pp.463-473.

Moure, A., Domínguez, H. and Parajó, J.C. 2006. Antioxidant properties of ultrafiltration-recovered soy protein fractions from industrial effluents and their hydrolysates. *Process Biochemistry.* **41**(2), pp.447-456.

Mune, M.A.M., Minka, S.R. and Henle, T. 2018. Investigation on antioxidant, angiotensin converting enzyme and dipeptidyl peptidase IV inhibitory activity of Bambara bean protein hydrolysates. *Food chemistry.* **250**, pp.162-169.

Muñoz, E.B., Luna-Vital, D.A., Fornasini, M., Baldeón, M.E. and de Mejia, E.G. 2018. Gamma-conglutin peptides from Andean lupin legume (Lupinus mutabilis Sweet) enhanced glucose uptake and reduced gluconeogenesis in vitro. *Journal of Functional Foods.* **45**, pp.339-347.

Nardo, A.E., Añón, M.C. and Quiroga, A.V. 2020. Identification of renin inhibitors peptides from amaranth proteins by docking protocols. *Journal of Functional Foods.* **64**, p103683.

Nawaz, K.A., David, S.M., Murugesh, E., Thandeeswaran, M., Kiran, K.G., Mahendran, R., Palaniswamy, M. and Angayarkanni, J. 2017. Identification and in silico characterization of a novel peptide inhibitor of angiotensin converting enzyme from pigeon pea (Cajanus cajan). *Phytomedicine.* **36**, pp.1-7.

Nongonierma, A.B. and FitzGerald, R.J. 2014. An in silico model to predict the potential of dietary proteins as sources of dipeptidyl peptidase IV (DPP-IV) inhibitory peptides. *Food chemistry.* **165**, pp.489-498.

Nongonierma, A.B., Hennemann, M., Paolella, S. and FitzGerald, R.J. 2017. Generation of wheat gluten hydrolysates with dipeptidyl peptidase IV (DPP-IV) inhibitory properties. *Food & function.* **8**(6), pp.2249-2257.

Nongonierma, A.B., Le Maux, S., Dubrulle, C., Barre, C. and FitzGerald, R.J. 2015. Quinoa (Chenopodium quinoa Willd.) protein hydrolysates with in vitro dipeptidyl peptidase IV (DPP-IV) inhibitory and antioxidant properties. *Journal of Cereal Science.* **65**, pp.112-118.

Nuchprapha, A., Paisansak, S., Sangtanoo, P., Srimongkol, P., Saisavoey, T., Reamtong, O., Choowongkomon, K. and Karnchanatat, A. 2020. Two novel ACE inhibitory peptides isolated from longan seeds: purification, inhibitory kinetics and mechanisms. *RSC Advances.* **10**(22), pp.12711-12720.

Nuñez‐Aragón, P.N., Segura‐Campos, M., Negrete‐León, E., Acevedo‐Fernández, J.J., Betancur‐Ancona, D., Chel‐Guerrero, L. and Castañeda‐Corral, G. 2019. Protein hydrolysates and ultrafiltered< 1 KDa fractions from Phaseolus lunatus, Phaseolus vulgaris and Mucuna pruriens exhibit antihyperglycemic activity, intestinal glucose absorption and α‐glucosidase inhibition with no acute toxicity in rodents. *Journal of the Science of Food and Agriculture.* **99**(2), pp.587-595.

Ohara, A., Cason, V.G., Nishide, T.G., Miranda de Matos, F. and de Castro, R.J.S. 2020. Improving the antioxidant and antidiabetic properties of common bean proteins by enzymatic hydrolysis using a blend of proteases. *Biocatalysis and Biotransformation.* pp.1-9.

Ortiz-Martinez, M., Otero-Pappatheodorou, J.T., Serna-Saldívar, S.O. and García-Lara, S. 2017. Antioxidant activity and characterization of protein fractions and hydrolysates from normal and quality protein maize kernels. *Journal of Cereal Science.* **76**, pp.85-91.

Oseguera-Toledo, M.E., de Mejia, E.G. and Amaya-Llano, S.L. 2015. Hard-to-cook bean (Phaseolus vulgaris L.) proteins hydrolyzed by alcalase and bromelain produced bioactive peptide fractions that inhibit targets of type-2 diabetes and oxidative stress. *Food Research International.* **76**, pp.839-851.

Pan, S., Wang, S., Jing, L. and Yao, D. 2016. Purification and characterisation of a novel angiotensin-I converting enzyme (ACE)-inhibitory peptide derived from the enzymatic hydrolysate of Enteromorpha clathrata protein. *Food Chemistry.* **211**, pp.423-430.

Park, E.Y., Morimae, M., Matsumura, Y., Nakamura, Y. and Sato, K. 2008. Antioxidant activity of some protein hydrolysates and their fractions

with different isoelectric points. *Journal of agricultural and food chemistry.* **56**(19), pp.9246-9251.

Pooja, K., Rani, S., Kanwate, B. and Pal, G.K. 2017a. Physico-chemical, sensory and toxicity characteristics of dipeptidyl peptidase-IV inhibitory peptides from rice bran-derived globulin using computational approaches. *International Journal of Peptide Research and Therapeutics.* **23**(4), pp.519-529.

Pooja, K., Rani, S. and Prakash, B. 2017b. In silico approaches towards the exploration of rice bran proteins-derived angiotensin-I-converting enzyme inhibitory peptides. *International Journal of Food Properties.* pp.1-14.

Quiroga, A.V., Aphalo, P., Nardo, A.E. and Añón, M.a.C. 2017. In Vitro Modulation of Renin–Angiotensin System Enzymes by Amaranth (Amaranthus hypochondriacus) Protein-Derived Peptides: Alternative Mechanisms Different from ACE Inhibition. *Journal of agricultural and food chemistry.* **65**(34), pp.7415-7423.

Ren, D., Wang, P., Liu, C., Wang, J., Liu, X., Liu, J. and Min, W. 2018. Hazelnut protein-derived peptide LDAPGHR shows anti-inflammatory activity on LPS-induced RAW264. 7 macrophage. *Journal of Functional Foods.* **46**, pp.449-455.

Ren, Y., Liang, K., Jin, Y., Zhang, M., Chen, Y., Wu, H. and Lai, F. 2016. Identification and characterization of two novel α-glucosidase inhibitory oligopeptides from hemp (Cannabis sativa L.) seed protein. *Journal of functional foods.* **26**, pp.439-450.

Saisavoey, T., Sangtanoo, P., Reamtong, O. and Karnchanatat, A. 2018. Anti-inflammatory effects of Lychee (Litchi chinensis Sonn.) seed peptide hydrolysate on RAW 264.7 macrophage cells. *Food Biotechnology.* **32**(2), pp.79-94.

Samaei, S.P., Ghorbani, M., Tagliazucchi, D., Martini, S., Gotti, R., Themelis, T., Tesini, F., Gianotti, A., Toschi, T.G. and Babini, E. 2020. Functional, nutritional, antioxidant, sensory properties and comparative peptidomic profile of faba bean (Vicia faba, L.) seed protein hydrolysates and fortified apple juice. *Food Chemistry.* p127120.

San Pablo‐Osorio, B., Mojica, L. and Urías‐Silvas, J.E. 2019. Chia Seed (Salvia hispanica L.) Pepsin Hydrolysates Inhibit Angiotensin‐Converting Enzyme by Interacting with its Catalytic Site. *Journal of food science.* **84**(5), pp.1170-1179.

Shu, Y., Cao, X.Y. and Chen, J. 2019. Preparation and antagonistic effect of ACE inhibitory peptide from cashew. *Journal of the Science of Food and Agriculture.* **99**(15), pp.6822-6832.

Siow, H.-L. and Gan, C.-Y. 2013. Extraction of antioxidative and antihypertensive bioactive peptides from Parkia speciosa seeds. *Food chemistry.* **141**(4), pp.3435-3442.

Siow, H.-L., Lim, T.S. and Gan, C.-Y. 2017a. Development of a workflow for screening and identification of α-amylase inhibitory peptides from food source using an integrated Bioinformatics-phage display approach: Case study–Cumin seed. *Food chemistry.* **214**, pp.67-76.

Siow, H.-L., Tye, G.-J. and Gan, C.-Y. 2017b. Pre-clinical evidence for the efficacy and safety of α-amylase inhibitory peptides from cumin (Cuminum cyminum) seed. *Journal of Functional Foods.* **35**, pp.216-223.

Siow, H.L. and Gan, C.Y. 2017. Optimization study in extracting anti‐oxidative and α‐amylase inhibitor peptides from Cumin seeds (Cuminum cyminum). *Journal of Food Biochemistry.* **41**(1), pe12280.

Sumikawa, K., Takei, K., Kumagai, Y., Shimizu, T., Yasui, H. and Kishimura, H. 2020. In Silico Analysis of ACE Inhibitory Peptides from Chloroplast Proteins of Red Alga Grateloupia asiatica. *Marine Biotechnology.* pp.1-12.

Sun, S., Xu, X., Sun, X., Zhang, X., Chen, X. and Xu, N. 2019. Preparation and identification of ACE inhibitory peptides from the marine macroalga ulva intestinalis. *Marine drugs.* **17**(3), p179.

Sun, S., Zhang, H., Shan, K., Sun, T., Lin, M., Jia, L. and Chen, Y.Q. 2019. Effect of Different Cereal Peptides on the Development of Type 1 Diabetes is Associated with Their Anti‐inflammatory Ability: In Vitro and In Vivo Studies. *Molecular nutrition & food research.* **63**(11), p1800987.

Suttisuwan, R., Phunpruch, S., Saisavoey, T., Sangtanoo, P., Thongchul, N. and Karnchanatat, A. 2019. Isolation and characterization of anti-inflammatory peptides derived from trypsin hydrolysis of microalgae protein (Synechococcus sp. VDW). *Food Biotechnology.* **33**(4), pp.303-324.

Tang, N. and Zhuang, H. 2014. Evaluation of antioxidant activities of zein protein fractions. *Journal of food science.* **79**(11), pp.C2174-C2184.

Teh, S.-S., Bekhit, A.E.-D.A., Carne, A. and Birch, J. 2016. Antioxidant and ACE-inhibitory activities of hemp (Cannabis sativa L.) protein hydrolysates produced by the proteases AFP, HT, Pro-G, actinidin and zingibain. *Food Chemistry.* **203**, pp.199-206.

Tejano, L.A., Peralta, J.P., Yap, E.E.S., Panjaitan, F.C.A. and Chang, Y.-W. 2019. Prediction of Bioactive Peptides from Chlorella sorokiniana Proteins Using Proteomic Techniques in Combination with Bioinformatics Analyses. *International journal of molecular sciences.* **20**(7), p1786.

Thewissen, B.G., Pauly, A., Celus, I., Brijs, K. and Delcour, J.A. 2011. Inhibition of angiotensin I-converting enzyme by wheat gliadin hydrolysates. *Food chemistry.* **127**(4), pp.1653-1658.

Torres-Fuentes, C., del Mar Contreras, M., Recio, I., Alaiz, M. and Vioque, J. 2015. Identification and characterization of antioxidant peptides from chickpea protein hydrolysates. *Food Chemistry.* **180**, pp.194-202.

Tuz, M.A.O. and Campos, M.R.S. 2017. Purification of Mucuna pruriens (L) peptide fractions and evaluation of their ACE inhibitory effect. *Biocatalysis and agricultural biotechnology.* **10**, pp.390-395.

Udenigwe, C.C. 2016. Towards rice bran protein utilization: In silico insight on the role of oryzacystatins in biologically-active peptide production. *Food chemistry.* **191**, pp.135-138.

Udenigwe, C.C., Gong, M. and Wu, S. 2013. In silico analysis of the large and small subunits of cereal RuBisCO as precursors of cryptic bioactive peptides. *Process Biochemistry.* **48**(11), pp.1794-1799.

Uraipong, C. and Zhao, J. 2016. Identification and functional characterisation of bioactive peptides in rice bran albumin hydrolysates. *International Journal of Food Science & Technology.* **51**(10), pp.2201-2208.

Valencia-Mejía, E., Batista, K.A., Fernández, J.J.A. and Fernandes, K.F. 2019. Antihyperglycemic and hypoglycemic activity of naturally

occurring peptides and protein hydrolysates from easy-to-cook and hard-to-cook beans (Phaseolus vulgaris L.). *Food Research International.* **121**, pp.238-246.

Vásquez-Villanueva, R., Marina, M.L. and García, M.C. 2015. Revalorization of a peach (Prunus persica (L.) Batsch) byproduct: Extraction and characterization of ACE-inhibitory peptides from peach stones. *Journal of Functional Foods.* **18**, pp.137-146.

Vecchi, B. and Añón, M. 2009. ACE inhibitory tetrapeptides from Amaranthus hypochondriacus 11S globulin. *Phytochemistry.* **70**(7), pp.864-870.

Velarde-Salcedo, A.J., Barrera-Pacheco, A., Lara-González, S., Montero-Morán, G.M., Díaz-Gois, A., de Mejia, E.G. and De La Rosa, A.P.B. 2013. In vitro inhibition of dipeptidyl peptidase IV by peptides derived from the hydrolysis of amaranth (Amaranthus hypochondriacus L.) proteins. *Food chemistry.* **136**(2), pp.758-764.

Vermeirssen, V., van der Bent, A., Van Camp, J., van Amerongen, A. and Verstraete, W. 2004. A quantitative in silico analysis calculates the angiotensin I converting enzyme (ACE) inhibitory activity in pea and whey protein digests. *Biochimie.* **86**(3), pp.231-239.

Vilcacundo, R., Martínez-Villaluenga, C. and Hernández-Ledesma, B. 2017. Release of dipeptidyl peptidase IV, α-amylase and α-glucosidase inhibitory peptides from quinoa (Chenopodium quinoa Willd.) during in vitro simulated gastrointestinal digestion. *Journal of Functional Foods.* **35**, pp.531-539.

Vilcacundo, R., Martínez‐Villaluenga, C., Miralles, B. and Hernández‐Ledesma, B. 2019. Release of multifunctional peptides from kiwicha (Amaranthus caudatus) protein under in vitro gastrointestinal digestion. *Journal of the Science of Food and Agriculture.* **99**(3), pp.1225-1232.

Vo, T.-S., Ryu, B. and Kim, S.-K. 2013. Purification of novel anti-inflammatory peptides from enzymatic hydrolysate of the edible microalgal Spirulina maxima. *Journal of Functional Foods.* **5**(3), pp.1336-1346.

Wali, A., Mijiti, Y., Yanhua, G., Yili, A., Aisa, H.A. and Kawuli, A. 2020. Isolation and Identification of a Novel Antioxidant Peptide from Chickpea (Cicer arietinum L.) Sprout Protein Hydrolysates. *INTERNATIONAL JOURNAL OF PEPTIDE RESEARCH AND THERAPEUTICS.*

Walters, M.E., Willmore, W.G. and Tsopmo, A. 2020. Antioxidant, Physicochemical, and Cellular Secretion of Glucagon-Like Peptide-1 Properties of Oat Bran Protein Hydrolysates. *Antioxidants.* **9**(6), p557.

Wang, C., Tu, M., Wu, D., Chen, H., Chen, C., Wang, Z. and Jiang, L. 2018. Identification of an ACE-Inhibitory peptide from walnut protein and its evaluation of the inhibitory mechanism. *International Journal of Molecular Sciences.* **19**(4), p1156.

Wang, F., Yu, G., Zhang, Y., Zhang, B. and Fan, J. 2015. Dipeptidyl peptidase IV inhibitory peptides derived from oat (avena sativa L.), buckwheat (fagopyrum esculentum), and highland barley (hordeum vulgare trifurcatum (L.) trofim) proteins. *Journal of agricultural and food chemistry.* **63**(43), pp.9543-9549.

Wang, F.-J., Yin, X.-Y., Regenstein, J.M. and Wang, J.-Z. 2016. Separation and purification of angiotensin-I-converting enzyme (ACE) inhibitory peptides from walnuts (Juglans regia L.) meal. *European Food Research and Technology.* **242**(6), pp.911-918.

Wang, J., Wu, T., Fang, L., Liu, C., Liu, X., Li, H., Shi, J., Li, M. and Min, W. 2020. Anti-diabetic effect by walnut (Juglans mandshurica Maxim.)-derived peptide LPLLR through inhibiting α-glucosidase and α-amylase, and alleviating insulin resistance of hepatic HepG2 cells. *Journal of Functional Foods.* **69**, p103944.

Wang, R., Lu, X., Sun, Q., Gao, J., Ma, L. and Huang, J. 2020. Novel ACE inhibitory peptides derived from simulated gastrointestinal digestion in vitro of sesame (Sesamum indicum L.) protein and molecular docking study. *International journal of molecular sciences.* **21**(3), p1059.

Wang, X., Chen, H., Fu, X., Li, S. and Wei, J. 2017. A novel antioxidant and ACE inhibitory peptide from rice bran protein: Biochemical characterization and molecular docking study. *LWT.* **75**, pp.93-99.

Wattanasiritham, L., Theerakulkait, C., Wickramasekara, S., Maier, C.S. and Stevens, J.F. 2016. Isolation and identification of antioxidant peptides from enzymatically hydrolyzed rice bran protein. *Food Chemistry.* **192**, pp.156-162.

Wei, D., Fan, W. and Xu, Y. 2019. In Vitro Production and Identification of Angiotensin Converting Enzyme (ACE) Inhibitory Peptides Derived from Distilled Spent Grain Prolamin Isolate. *Foods.* **8**(9), p390.

Wen, C., Zhang, J., Feng, Y., Duan, Y., Ma, H. and Zhang, H. 2020. Purification and identification of novel antioxidant peptides from watermelon seed protein hydrolysates and their cytoprotective effects on H2O2-induced oxidative stress. *Food Chemistry.* p127059.

Wen, C., Zhang, J., Zhou, J., Duan, Y., Zhang, H. and Ma, H. 2018. Effects of slit divergent ultrasound and enzymatic treatment on the structure and antioxidant activity of arrowhead protein. *Ultrasonics sonochemistry.* **49**, pp.294-302.

Wongaem, A., Reamtong, O., Srimongkol, P., Sangtanoo, P., Saisavoey, T. and Karnchanatat, A. 2020. Antioxidant properties of peptides obtained from the split gill mushroom (Schizophyllum commune). *Journal of Food Science and Technology.* pp.1-12.

Wu, J.-S., Li, J.-M., Lo, H.-Y., Hsiang, C.-Y. and Ho, T.-Y. 2020. Anti-hypertensive and angiotensin-converting enzyme inhibitory effects of Radix Astragali and its bioactive peptide AM-1. *Journal of Ethnopharmacology.* p112724.

Wu, Q., Du, J., Jia, J. and Kuang, C. 2016. Production of ACE inhibitory peptides from sweet sorghum grain protein using alcalase: Hydrolysis kinetic, purification and molecular docking study. *Food chemistry.* **199**, pp.140-149.

Xia, J.a., Song, H., Huang, K., Li, S. and Guan, X. 2020. Purification and characterization of antioxidant peptides from enzymatic hydrolysate of mungbean protein. *Journal of Food Science.*

Xiao, J., Li, Y., Chen, B., Gong, S., Li, Z., Ou, Y., Ou, Z., Kang, A., Jia, Y. and Yang, A. 2020. Enzymatic preparation and antioxidative activity of hydrolysate from Rice bran protein. *Journal of Food Measurement and Characterization.* pp.1-12.

Xie, J., Chen, X., Wu, J., Zhang, Y., Zhou, Y., Zhang, L., Tang, Y.-J. and Wei, D. 2018. Antihypertensive effects, molecular docking study, and isothermal titration calorimetry assay of angiotensin I-converting enzyme inhibitory peptides from Chlorella vulgaris. *Journal of agricultural and food chemistry.* **66**(6), pp.1359-1368.

Xu, F., Yao, Y., Xu, X., Wang, M., Pan, M., Ji, S., Wu, J., Jiang, D., Ju, X. and Wang, L. 2019. Identification and Quantification of DPP-IV-Inhibitory Peptides from Hydrolyzed-Rapeseed-Protein-Derived Napin with Analysis of the Interactions between Key Residues and Protein Domains. *Journal of agricultural and food chemistry.* **67**(13), pp.3679-3690.

Xu, S., Shen, Y. and Li, Y. 2019. Antioxidant activities of sorghum kafirin alcalase hydrolysates and membrane/gel filtrated fractions. *Antioxidants.* **8**(5), p131.

Yang, X., Wang, L., Zhang, F. and Ma, H. 2020. Effects of multi-mode S-type ultrasound pretreatment on the preparation of ACE inhibitory peptide from rice protein. *Food Chemistry.* p127216.

Yea, C.S., Ebrahimpour, A., Hamid, A.A., Bakar, J., Muhammad, K. and Saari, N. 2014. Winged bean [Psophorcarpus tetragonolobus (L.) DC] seeds as an underutilised plant source of bifunctional proteolysate and biopeptides. *Food & function.* **5**(5), pp.1007-1016.

Zarei, M., Ebrahimpour, A., Abdul-Hamid, A., Anwar, F., Bakar, F.A., Philip, R. and Saari, N. 2014. Identification and characterization of papain-generated antioxidant peptides from palm kernel cake proteins. *Food Research International.* **62**, pp.726-734.

Zhang, C., Gao, S., Zhang, C., Zhang, Y., Liu, H., Hong, H. and Luo, Y. 2020. Evaluating in vitro dipeptidyl peptidase IV inhibition by peptides from common carp (Cyprinus carpio) roe in cell culture models. *European Food Research and Technology.* **246**(1), pp.179-191.

Zhang, M., Mu, T.H. and Sun, M.J. 2012. Sweet potato protein hydrolysates: antioxidant activity and protective effects on oxidative DNA damage. *International journal of food science & technology.* **47**(11), pp.2304-2310.

Zhang, P., Chang, C., Liu, H., Li, B., Yan, Q. and Jiang, Z. 2020. Identification of novel angiotensin I-converting enzyme (ACE) inhibitory peptides from wheat gluten hydrolysate by the protease of Pseudomonas aeruginosa. *Journal of Functional Foods.* **65**, p103751.

Zhang, X., Cao, D., Sun, X., Sun, S. and Xu, N. 2019. Preparation and identification of antioxidant peptides from protein hydrolysate of marine alga Gracilariopsis lemaneiformis. *Journal of Applied Phycology.* **31**(4), pp.2585-2596.

Zhao, W., Xue, S., Yu, Z., Ding, L., Li, J. and Liu, J. 2019. Novel ACE inhibitors derived from soybean proteins using in silico and in vitro studies. *Journal of food biochemistry.* **43**(9), pe12975.

Zheng, Y., Li, Y. and Li, G. 2019a. ACE-inhibitory and antioxidant peptides from coconut cake albumin hydrolysates: purification, identification and synthesis. *RSC advances.* **9**(11), pp.5925-5936.

Zheng, Y., Wang, X., Zhuang, Y., Li, Y., Shi, P., Tian, H., Li, X. and Chen, X. 2020a. Isolation of novel ACE‐inhibitory peptide from naked oat globulin hydrolysates in silico approach: Molecular docking, in vivo antihypertension and effects on renin and intracellular endothelin‐1. *Journal of Food Science.* **85**(4), pp.1328-1337.

Zheng, Y., Wang, X., Zhuang, Y., Li, Y., Tian, H., Shi, P. and Li, G. 2019b. Isolation of Novel ACE-Inhibitory and Antioxidant Peptides from Quinoa Bran Albumin Assisted with an In Silico Approach:

Characterization, In Vivo Antihypertension, and Molecular Docking. *Molecules.* **24**(24), p4562.

Zheng, Y., Zhang, Y. and San, S. 2020b. Efficacy of a Novel ACE-Inhibitory Peptide from Sargassum maclurei in Hypertension and Reduction of Intracellular Endothelin-1. *Nutrients.* **12**(3), p653.

Zhu, Q., Chen, X., Wu, J., Zhou, Y., Qian, Y., Fang, M., Xie, J. and Wei, D. 2017. Dipeptidyl peptidase IV inhibitory peptides from Chlorella vulgaris: In silico gastrointestinal hydrolysis and molecular mechanism. *European Food Research and Technology.* **243**(10), pp.1739-1748.

Zhu, S., Du, C., Yu, T., Cong, X., Liu, Y., Chen, S. and Li, Y. 2019. Antioxidant Activity of Selenium‐Enriched Peptides from the Protein Hydrolysate of Cardamine violifolia. *Journal of Food Science.* **84**(12), pp.3504-3511.

Zou, Z., Wang, M., Wang, Z., Aluko, R.E. and He, R. 2020. Antihypertensive and antioxidant activities of enzymatic wheat bran protein hydrolysates. *Journal of Food Biochemistry.* **44**(1), pe13090.

Appendix B
Supporting information for Chapter 3

Supplementary data could be downloaded from

https://www.sciencedirect.com/science/article/pii/S0963996918309621?via%3Dihub#ec0005

Appendix C
Supporting information for Chapter 4

Table C.1 list of nitrogen to protein conversion factor

Protein	Conversion factor	Reference
Flaxseed	5.41	(Oomah and Mazza, 1998)
Rapeseed	5.6	(Gorrill et al., 1974)
Sunflower	5.36	(Freer and Dove, 1984)
Sesame	5.30	Other oilseeds and nuts (Mariotti et al., 2008)
Soybean	5.5	An average of soybean (Mariotti et al., 2008)
Whey	6.15	Purified milk proteins (Mariotti et al., 2008)
Casein	6.15	(Sosulski and Imafidon, 1990)

References

Freer, M. and Dove, H. 1984. Rumen degradation of protein in sunflower meal, rapeseed meal and lupin seed placed in nylon bags. *Animal Feed Science and Technology.* **11**(2), pp.87-101.

Gorrill, A., Walker, D. and Jones, J. 1974. Rapeseed protein sources and amino acid supplementation of diets for weanling rats. *Canadian Journal of Animal Science.* **54**(4), pp.659-667.

Mariotti, F., Tomé, D. and Mirand, P.P. 2008. Converting nitrogen into protein—beyond 6.25 and Jones' factors. *Critical reviews in food science and nutrition.* **48**(2), pp.177-184.

Oomah, B.D. and Mazza, G. 1998. Compositional changes during commercial processing of flaxseed. *Industrial Crops and Products.* **9**(1), pp.29-37.

Sosulski, F.W. and Imafidon, G.I. 1990. Amino acid composition and nitrogen-to-protein conversion factors for animal and plant foods. *Journal of Agricultural and Food Chemistry.* **38**(6), pp.1351-1356.

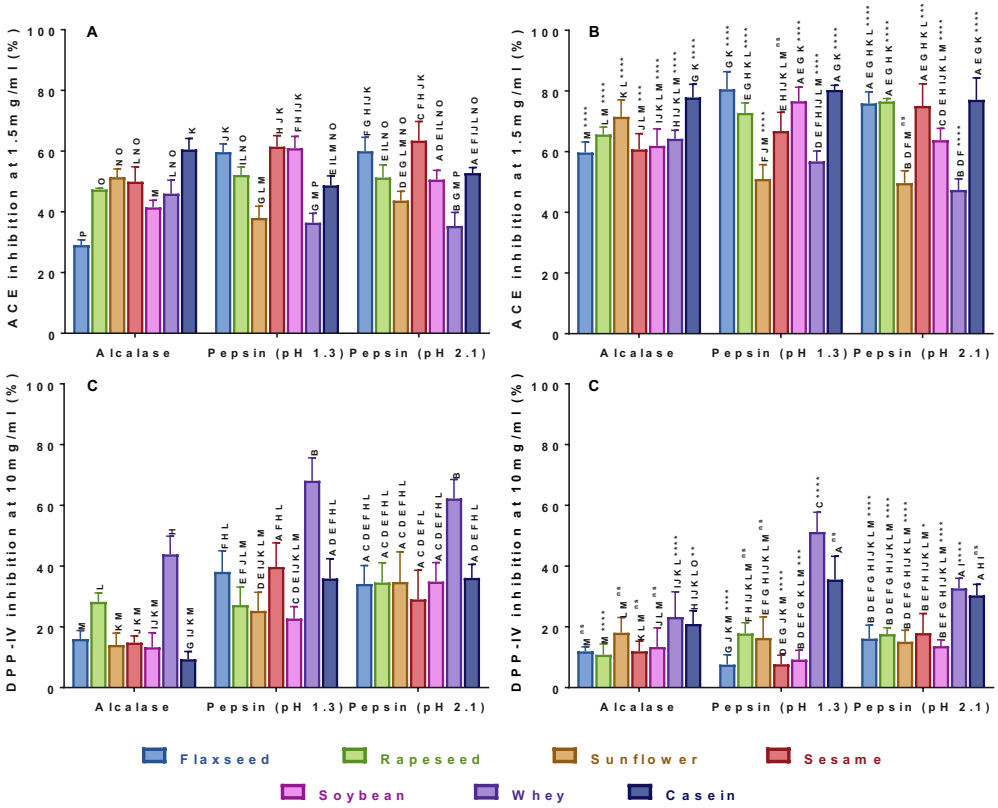

Figure C.1 Angiotensin converting enzyme (A,B) and dipeptidyl peptidase-IV (C,D) inhibitory capability of protein hydrolysates (A,C) and their low molecular weight (M_w <3 kDa) fraction (B,D) determined using *in vitro* enzyme inhibitory assay. Different capital letters indicate significant differences ($p<0.05$). * following capital letters represent significant differences between whole protein hydrolysates and their low M_w fractions. Error bars represent standard deviations.

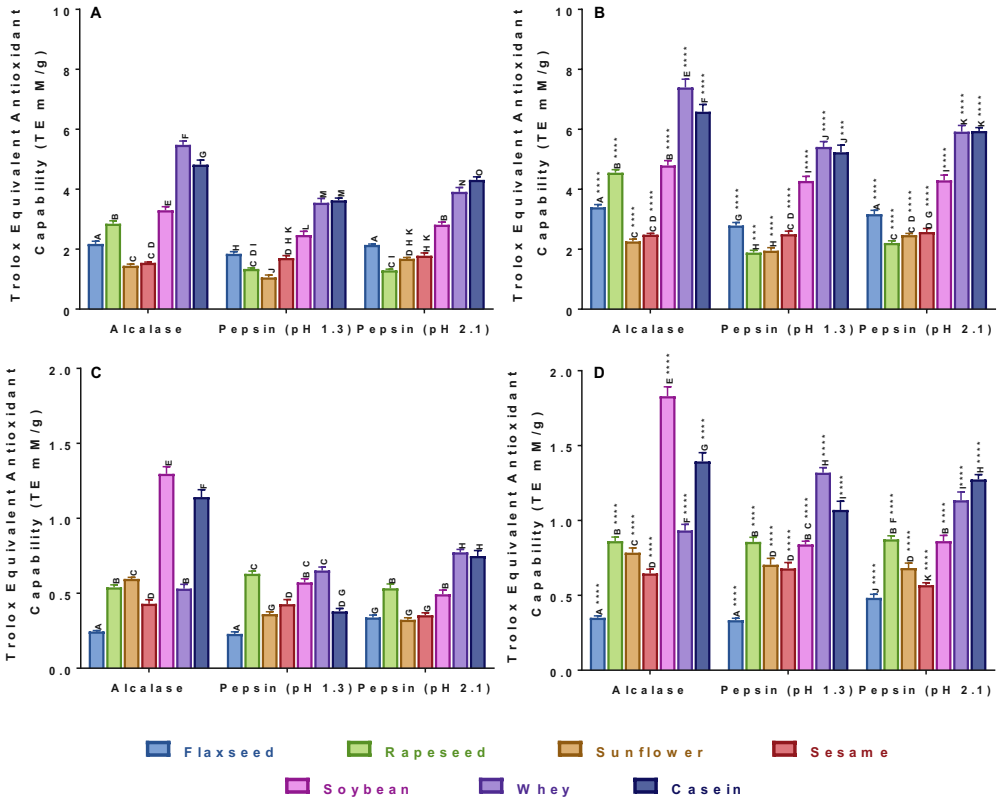

Figure C.2 Antioxidant activity of protein hydrolysates (A,C) and low molecular weight (M_w <3 kDa) fraction (B, D) determined using TEAC (A,B) assay and FRAP assay (C,D). Different capital letters indicate significant differences ($p<0.05$). * following capital letters represent significant differences between whole protein hydrolysates and their low M_w fractions. Error bars represent standard deviation.

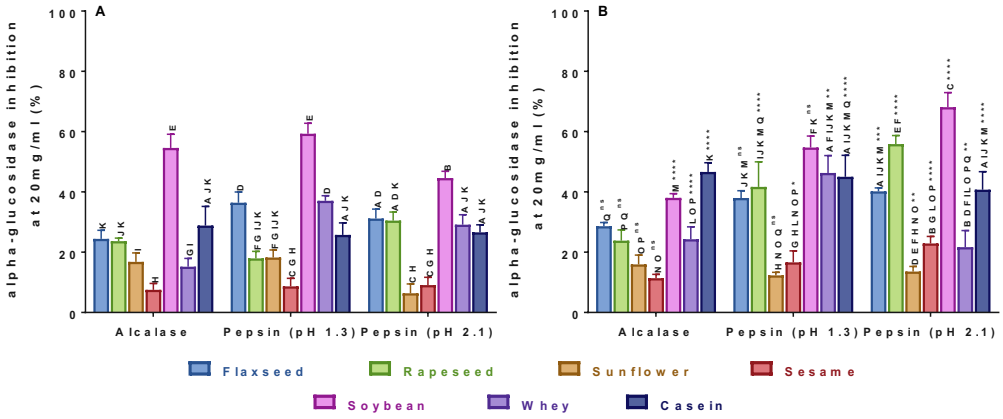

Figure C.3 α-glucosidase inhibitory activity of protein hydrolysates (A) and low molecular weight (M_w <3 kDa) fractions (B) determined using *in vitro* enzyme inhibitory assay. Different capital letters indicate significant differences ($p<0.05$). * following capital letters represent significant differences between whole protein hydrolysates and their low M_w fractions. Error bars represent standard deviation.