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RELATIONSHIP BETWEEN DIGESTIVE ENZYMES, PROTEINS AND ANTI-NUTRITIVE FACTORS IN MONOGASTRIC DIGESTION

By:

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'To my beloved mother, whom I couldn't have done this without and to the memory of my beloved father that passed away during my PhD.'

ABSTRACT

Monogastrics' lack of some digestive enzymes is compensated by using exogenous enzymes in the animal feed. Those interact with feed ingredients causing enzyme inhibition. Two known inhibiting substances are phytic acid and polyphenolics.

Phytase is an important exogenous enzyme that is breaking down phytic acid. The effect of sorghum polyphenolic-rich extracts on the 6-phytase activity was investigated using an ITC by calculating its relative activity. Results show inhibition of the phytase, from all three extracts. For a better understanding of this assay, a real-time mass spectroscopic analysis was performed, that showed the pattern in which the phytase is hydrolysing phytic acid.

Consecutively, the effect of phytic acid on the activity of three proteases exogenous and endogenous chymotrypsin and endogenous trypsin was studied. A colorimetric assay using casein as a substrate was used. The results showed different levels of inhibition of all three enzymes by phytic acid.

Finally, the interaction between the exogenous chymotrypsin and the phytase was studied by using the ITC enzyme assay and an SDS-Page gel analysis. ITC results showed partial inhibition of the phytase activity, while SDS-Page results showed complete inhibition.

Literature supports the results of this study which can have a negative nutritional and economic effect. This information could be used to create a conceptual model, that would be a useful tool to understand the fundamental interactions of enzymes, substrates and anti-nutritive factors in order to improve monogastrics' nutrition.

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CHAPTER 1 'GENERAL INTRODUCTION'

1.1 SUMMARY

With a higher demand for animal products every year, the need for more efficient animal feed is clear. Monogastric animal feed mainly consists of grains, protein meals and oils from plant and animal source and feed additives. Pigs and poultry rely on digestive enzymes for their digestion and it is a common practice, for exogenous enzymes to be used in animal feed.

Exogenous enzymes are important for animal nutrition, because monogastrics do not have the capacity to utilise all the nutrients from the feed. Hence exogenous enzymes aid to their nutrition. Two of the most commonly used and important enzymes are proteases and phytases. Protease aids to the digestion while phytase breaks down phytic acid that is both a source of phosphorus and also has anti-nutritive properties. Other than phytic acid, there are other anti-nutritive factors found in grains, like polyphenolics. Both phytic acid and polyphenolics are found in high concentrations in grains like sorghum. They both have the tendency to bind to positively charged molecules, making them unavailable to digest. Although research has been done in the field regarding enzyme efficiency and interaction of the anti-nutritive factors with feed components, there is not a model that explains all the interactions that take place in the digestive tract of the monogastric animals during the digestion, nor the specific exogenous enzymes used for this project have been intensively studied before.

The objective of this project was to produce a map that would describe the interactions between endogenous and exogenous enzymes, feed grains and anti-nutritive factors, like phytic acid and polyphenolics. This would be achieved using

Isothermal titration Calorimetry (ITC), a technique that has been used before to investigate the binding between molecules and the kinetics of enzyme reactions.

1.2 INTRODUCTION

Through the past few decades with a growing human population, the livestock sector has shown a major growth too. As a result, there has been a massive expansion in meat, milk and egg production. Pig and chicken meat demand has the highest growth per annum. The research in the field of livestock production focuses on the improvement of the reproduction, nutrition and growth rates of livestock animals. With an increasing production of poultry and pork and with animal feed being a major part of the cost in animal production and the global enzyme feed market to be around US$1bn per annum (Cowieson & Roos, 2016, Opio et al., 2011), there is a need for more efficient nutrition in monogastrics, to minimise costs.

Because of the high demand in animal products, and the need for higher efficiency on the livestock production, enzymes used as animal feed additives are in the spotlight of research. Exogenous enzymes work as catalysts and helping the digestion of different components of the feed like proteins, carbohydrates, lipids and anti-nutritional factors (dos Passos and Kim, 2014). Proteases, phytase, lipases and carbohydrases are commonly used as feed additives in the diet of monogastrics (Cowieson & Roos, 2016).

So far, the use of digestive enzyme in the animal feed industry has not received the required attention from the general scientific community. Although there has

been an extensive study of the effect of enzyme additives on the performance of farmed pigs and poultry, the interactions of the enzymes with the other ingredients of the feed or the secretions of the animal itself have not been fully understood. Since there is still an uncertainty regarding the models of action of the exogenous enzymes it is clear that there is a need for further investigation of their action (Cowieson and Roos, 2016).

Phytase and proteases have been widely used in the past few decades and both have a very important role in the digestion of monogastric animals. Monogastrics produce endogenous proteases that aid to the protein digestion, but not in the required levels for the digestion of the whole protein included in their feed. Therefore, exogenous proteases need to be added to the feed, so that there is a higher level of protein is digested. Proteins are very important for the animal nutrition at all stages, but especially at the early stages of growth (Cowieson & Adeola, 2005). On the contrary, phytase is not secreted at all by the organism, so it is only present as an animal feed additive. Its role is to break down phytic acid, which is the natural way of storing phosphorus in the plants. Animals require phosphorus in their diets as it is an essential inorganic component for their growth. With the price of phosphorus being high and an uncertainty in its availability in the future it is beneficial to allow the animals utilize this phosphorus in phytic acid. The role of phytase is to break down the phytic acid and therefore release the phosphorus included in it. The efficiency of the commercial phytases at the moment is not the maximum possible and therefore phosphorus still goes to waste since it is excreted by the animals in the form of phytic acid (Truong et al., 2014; Mohebbifar et al., 2015). Phytic acid not only is indigestible from

monogastrics, but it is also a reactive, polyanionic molecule. Phytic acid has the capacity to interact with proteins in grains and negatively charged molecules of phytic acid have the ability to form binary complexes with proteins that have a positive charge (O'Dell et al., 1972; Kahindi et al., 2014; Selle *et al.*, 2012). This results in the blockage of the digestion of other nutrients that it gets attached to. That is why phytic acid is considered as an anti-nutritional factor. Another anti-nutritional factor is the polyphenolics. Polyphenolics are tannins that also have the ability to attach to other nutrients and in that way blocking their digestion. Polyphenolics and phytic acid are present in most grains and legumes used as ingredients of the diet of monogastric animals (Frazier *et al.*, 2010; Selle *et al.*, 2010; Przywitowski *et al.*, 2016).

Both phytic acid and polyphenolics have been in the centre of research because of their anti-nutritional properties. Despite that, the interactions between endogenous and exogenous enzymes, grain proteins and phytic acid and polyphenolics has not been studied before. Therefore, there is not a model proposing the interactions that occur in the stomach of monogastric animals (Woyengo et al., 2015; Ficco et al., 2009).

Isothermal Titration Calorimetry (ITC) is a widely used biophysical technique and it can be used both for studying the binding between different substances as well as the kinetics of enzymes (Ghai, Falconer and Collins, 2012).

The objective of this project is to study these interactions and produce a model that will explain them. This will be accomplished by the use of Isothermal Titration Calorimetry (ITC), since it has been used before for understanding both enzyme kinetics (Freyer and Lewis, 2008) and the binding of phytic acid and tannins

(McRae, Falconer and Kennedy, 2010; Bye and Falconer, 2013). To produce this model, the interactions two exogenous enzymes, a phytase (HiPhos) and a protease (ProAct) both produced and provided by DSM for this project, will be investigated. The complexes formed in the stomach of monogastric animals and the efficiency of these enzymes in the presence or absence of other components will provide the information needed for the creation of the digestions model.

1.3 LITERATURE REVIEW

1.3.1 NUTRITION IN MONOGASTRIC ANIMALS

The animal body is mainly consisted of water, organic and inorganic components. The organic components are divided into three main categories, carbohydrates, fats and nitrogenous substances. Regarding the inorganic components, the two main present components in the animal body are calcium and phosphorus.

Like every other organism, animals are in a constant need of food intake to maintain all their body functions. Animal feed needs to fulfil the intake needs of the animal in (i) carbohydrates and (ii) fats for energy as well (iii) protein as a source of amino acids (Liamadis (Part 1), 2000; Vasilopoulou 1984; Lloyd, 1978; Schmidt-Nielsen, 1997).

i. Carbohydrates are the main source of energy (75%) for the livestock and they are divided into two main categories, sugars and non-sugars. Monosaccharides and oligosaccharides are sugars, while polysaccharides and complex carbohydrates are non-sugars.

ii. Fats are the second source of energy for farm animals and the fact that they contain a lot of energy in a concentrated form makes them an important part of the feed. Fats are especially valuable for diets of broiler chickens.

iii. Protein for animal feed includes all the amino acids, peptides, proteins, protein derivatives and the non-protein nitrogenous substances are all falling under the category of nitrogenous substances. They are very important for the animal growth and muscle gain.

1.3.2 DIGESTION IN MONOGASTRICS

According to Liamadis (Part 1), (2000) the digestion of big molecules like proteins, fats and carbohydrates is accomplished by the combination of three functions:

- Mechanical digestion: Chewing the feed in the mouth and the muscular contraction in the stomach both aid to the better digestion of the macromolecules.
- Chemical digestion: Enzymes, secreted by the digestive glands and HCl chemically aid to the digestion of the feed.
- Microbial digestion: Enzymes produced by microorganisms that live in the digestive tract of the animal and are not produced by the digestive glands. In ruminants these microorganisms exist in the rumen while in monogastric animals they are mainly found in the caecum and in the colon.

1.3.3 DIGESTIVE SYSTEMS IN MONOGASTRICS

Livestock have important differences in the anatomy and the physiology of their digestive systems. These differences are profound especially between ruminants and non-ruminants. Cows, sheep and goats are ruminants, while pigs, rabbits, horses and poultry are non-ruminants. There are four main types of digestive systems:

1. Simple stomach digestive system (pig)
2. Herbivore non-ruminant digestive system (horse)
3. Ruminant digestive system (cow)

4. Poultry digestive system (chicken)

1.3.4 DIGESTION IN PIGS

The digestive system of the pig has is very characteristic, since it is consisted of a single stomach that is not divided, like it is in ruminants. Pigs also have a caecum very small in volume. First the feed enters the mouth of the animal, where it gets a mechanical digestion while it gets chewed and mixed with the saliva. Amylase is present in the saliva as well as water, mucine and inorganic salts. Lysozyme is also present in the saliva of the pig. As the feed moves to the stomach the digestion gets mainly chemical. The presence of the feed in the stomach triggers the secretion of the gastric juice. It mainly consists of HCl with a pH between 1-3 is secreted into the stomach along with pepsinogen that is the precursor of pepsin and is transformed into pepsin by the presence of HCl. All the products of the digestion then move from the stomach to the duodenum. The intestinal digestion takes place by the secretion pancreatic juice, which consists of enzymes like trypsin, chymotrypsin, carboxypeptidase, pancreatic lipase and pancreatic amylase. In that way the intestine digestion is aiding to the breakdown of proteins, fats and starch. In the small intestine the digestion is promoted by the presence of different enzymes like enterokinase, a few peptidases, carbohydrases like maltase, sucrase and lactase, lipase, nucleotidase and prolinase. The final part of the digestion takes place in the colon. Everything that has not been absorbed by the small intestine is being digested by enzymes that have been transferred to the colon along with the feed or by the microorganisms that live in the colon. The

product of the digestion in the colon is then excreted (Demertzi 1977, Vasilopoulou (Part A), 1984, Liamadis (Part 2), 2000).

1.3.5 DIGESTION IN POULTRY (CHICKEN)

The poultry digestive system has four main characteristics that differentiate it from the rest livestock digestive systems. From top to bottom, it has a beak instead of lips and cheeks and it lacks teeth. Furthermore, the stomach consists of two completely different parts: the glandular stomach, which is a tight tube with thick walls and the muscular stomach, which is spherical, with thick walls and contains small pebbles that are obtained by the bird. Another distinctive characteristic of the poultry digestive system is that there are two caecums forming a "Y" shape at the bit where the small intestine and the colon are joined. Finally, the colon is very short in length and it ends to the vent from which both urine and faeces are excreted. Except these anatomical differences, there are also physiological differences in the poultry digestive system (Demertzi 1977, Vasilopoulou (Part A), 1984, Liamadis (Part 1), 2000).

The digestion begins in the mouth, where the feed stays for a very short amount of time. Poultry have a very complex system of salivary glands that are tubular, and the saliva secreted has an average pH of 6.75. Then the feed moves to the gizzard where it gets wetted both by the saliva and the glandular mucus secretions. Despite the fact that there is no enzyme secretion in the gizzard, the saliva-deriving amylase continues acting on the starch and there is also a bacterial fermentation that aids to the feed digestion. In the glandular stomach the feed just gets mixed with the gastric juice and then is forwarded to the muscular stomach,

where with the presence of the small pebbles it gets crushed. The gastric juice action takes place in the duodenum, which does not have any glands. Amylase, lipase and proteinase are secreted by the pancreas, while sucrase and amylase are present in the intestinal juice. The two caecums are useful for the absorption of the nutrients. Finally, everything left moves to the colon and is excreted through the vent (Wood 1974 Bondi 1987, Demertzi 1977, Vasilopoulou (Part A), 1984, Liamadis (Part 1), 2000).

1.3.6 DIGESTIVE/ENDOGENOUS ENZYMES

Enzymes are responsible for the production of all the organic compounds both in animals and plants. They are organic catalysts, produced by living cells thus are usually called biocatalysts. A catalyst is every substance that affects the speed of a chemical reaction, without being present in the final products and being unchanged after the completion of the reaction. Enzymes are a group of proteins that has a very important biological role in the chemical digestion of feed, since all the biochemical reactions that are aiding to the different functions of the living organisms are called metabolism and these reactions cannot take place without the presence of the enzymes. The substances that the enzymes catalyse are called substrates (Jennings 1965).

The digestive enzymes are secreted by the digestive glands. Their role is to break down the nutrients by hydrolysis and their optimum pH is usually between 6-8. An exception to that are pepsin and chymosin that their function better in an acidic environment (Demertzi, 1977; Vasilopoulou (Part B), 1984; Liamadis (Part 2), 2000; Rook, 1983).

There are three main groups of digestive enzymes:

1) Carbohydrases: are enzymes that break down carbohydrates. Amylase, maltase, lactase and sucrase are respectively hydrolysing starch, maltose, lactose and sucrose.

2) Proteases (or Proteinases): are hydrolysing peptide bonds. Peptide bonds are used by amino acids to connect to each other. Proteases are divided into two categories: a. Endopeptidases that are acting on bonds in central areas of the proteins and b. exopeptidases that act on bonds at the ends of the peptide chain. Pepsin, chymosin, trypsin and chymotrypsin are all endopeptidases, while carboxypeptidase, aminopeptidases and dipeptidases are exopeptidases.

3) Esterases: are breaking down oils and fats, by hydrolysing the ester bonds that they have. Lipases like pancreatic lipase and intestinal lipase are esterases.

All the known enzymes so far are complex, high molecular weight proteins. The active centre of the enzyme is responsible for its catalytic action and it is consisted of some parts of the polypeptide chain that is folded in a certain way. If their folding changes, it is highly likely that the enzyme will lose its function even if the amino acids are still in the right order on the chain. Many enzymes need a prosthetic group that usually is a inorganic, non-protein -component that is used as the active centre of the enzyme. These prosthetic groups are called coenzymes or cofactors (Bondi 1987, Vasilopoulou (Part B), 1984, Liamadis (Part 2), 2000).

1.3.7 MONOGASTRIC ANIMAL FEED

The ingredients of the feed prepared for monogastrics are generally classified into grains, protein meals, oils and fats, feed additives, minerals, and other raw materials like tubers and roots.

1.3.7.1 GRAINS

In the general category of grains both cereal grains and their by-products are included. The aim of the grains is to provide the animal with the required amount of energy. The most commonly used grains worldwide are corn and wheat. Corn is the dominant grain in US, South America and Asia, while wheat is the most dominant in Europe, Canada, Russia and Australia. Following corn and wheat sorghum is a widely used grain in Australia due to its availability and it is replacing wheat during the summer season. On the contrary, Barley and rye are the most commonly used grains in Scandinavia.

1.3.7.2 PROTEIN MEALS

Protein is provided from both vegetable and animal sources, such as oilseed meals, legumes and abattoir and fish processing by-products.

1.3.7.3 VEGETABLE PROTEIN SOURCES

By-products of oilseed crops like soybean, canola, sunflower, palm kernel, copra, peanut and sesame seed are mostly used as vegetable protein sources. They are used for up to the 25% of the diet and their use varies in different countries. In Australia the most commonly used vegetable protein sources are soybean and canola meals (Yilmaz, Brandolini and Hidalgo, 2015). A lot of the oilseeds and

legumes used as animal feed ingredients contain anti-nutritive factors (Liamadis (Part 1), 2000).

1.3.7.4 ANIMAL PROTEIN SOURCES

The most commonly used animal protein sources used in pig and poultry diets are meat, bone, fish, blood and feather meal. There are some challenges associated with the use of animal protein sources mainly due to concerns regarding food safety since the prion disease bovine spongiform encephalopathy (BSE – mad cow disease) and a variant Creutzfeldt-Jakob disease in humans (Liamadis (Part 2), 2000).

1.3.7.5 OILS AND FATS

Oils and fats are regularly used in monogastric feed to fulfil the energy requirements of the animal since they are highly concentrated energy sources. A lot of important vitamins like A, D, E and K are fat soluble and on top of that essential fatty acids and linoleic acid are important for the diet. A variety of oils and fats, from both animal (lard, fish oil or tallow) and vegetable origin are used (soy, canola/rapeseed, sunflower, palm or cottonseed oil) (Liamadis (Part 2), 2000).

1.3.7.6 MINERALS AND VITAMINS

Minerals and vitamins are essential for a normal growth and development of animals. They have vital roles in body processes such as enzyme activation. Minerals like calcium, phosphorus, potassium and sodium are required in big quantities, while other minerals, like manganese, cobalt, iodine, zinc, iron, selenium, molybdenum and copper are only required in really small quantities.

Vitamins, both fat (A, D, E, K) and water soluble (biotin, choline, folic acid, niacin, riboflavin, thiamine, pyridoxine, pantothenic acid and B12) need to be present in the feed (Liamadis (Part 2), 2000).

1.3.8 FEED ENZYMES

Specific proteins that are catalysing a chemical reaction are called enzymes. Despite the fact that endogenous enzymes are produced by the animals and their gut microflora they are not produced in sufficient quantities and therefore there is a need for exogenous enzymes to be added in the animal feed. Exogenous enzymes are being used as feed additives for the past 20-25 years because of their ability to improve the nutrients utilization. It all started in the 1980s with β-glucanases produced and used in Scandinavia, it then shifted to the production and use of xylanases in the 1990s in the UK and Northern Europe and at the moment the use of different enzymes like amylase, protease, phytase and carbohydrase is a global phenomenon in the livestock sector (Walsh, Power and Headon, 1994; Cowieson and Roos, 2016).

The use of enzymes in animal feed has become so popular because of their ability to reduce the feed cost since the need for protein/amino acid or phosphorous sources is lower with the enzymes effect. With the demand for meat growing rapidly, the production of poultry and pork has been increasing and this has resulted for the global enzyme feed market to be around US$1bn per annum (Cowieson & Roos, 2016; Cowieson & Adeola, 2005).

Exogenous enzymes are beneficial for the animal since they not only improve the efficiency of the utilization of feed ingredients like proteins, amino acids, lipids and starch but they also allow the use of a variety of feed components that would not be used without the presence of the enzymes. Furthermore, the use of enzymes allows the use of ingredients with variations in quality since by improving the digestion the animal gets all the nutrients it needs. Finally enzymes aid to the gut health since they promote the proliferation of the favourable bacterial species and therefore they have a positive effect of the overall health of the animal (Wenk, 2000; Cowieson and Adeola, 2005; Cowieson and Roos, 2016).

Since animals obtain all the nutrients they require from the feed through digestion, by improving this process results to a better nutrition. Enzymes can improve the digestion up to 5% that translates into approximately 5% reduction in the cost of the feed.

As shown in Figure 1.1, the higher the level of nutrients in the feed, the higher cost. In order to minimise the cost of the feed, the effect of the margin should be considered. The maximum margin is not achieved by reducing feed cost (red circle), but at the point where the animal is fed to ensure margin is in the maximum margin zone. This is accomplished by maintaining or increasing the nutrients in the diet and having them utilised by the animal at the maximum possible rate (green circle).

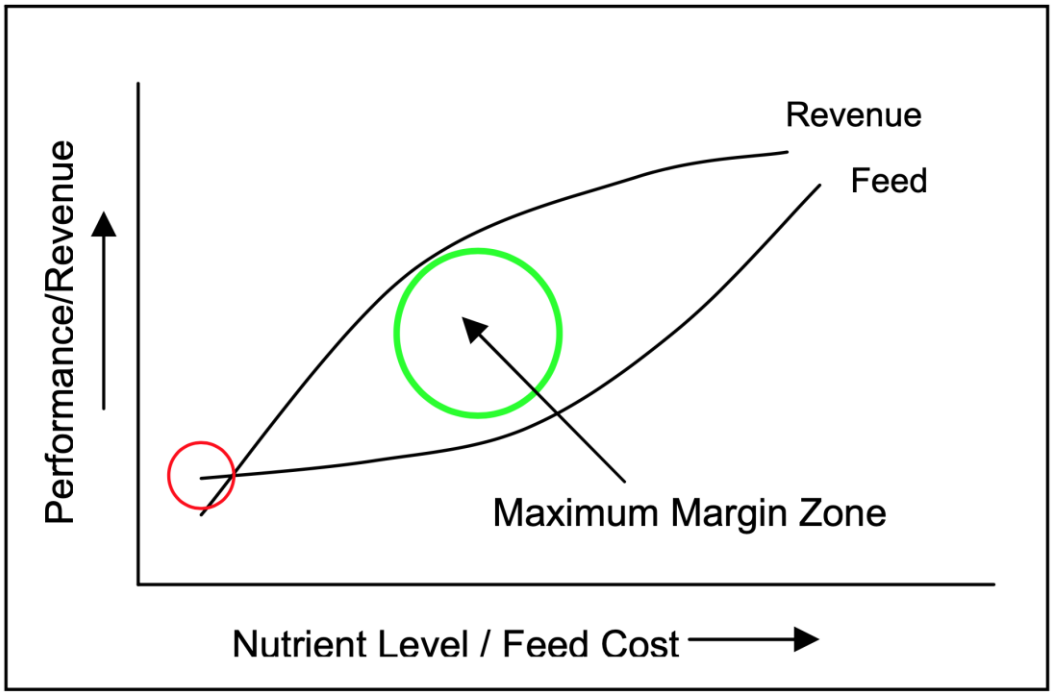


Figure 1.1: Diagram representing the relationship between the nutrient level and feed cost and performance and revenue. (Taken from Waller, 2007).

Protein, energy and phosphorus are the three most expensive components of animal feed and the exogenous enzymes produced today aim to their better digestibility. Carbohydrases and amylase aid to the better use of energy, proteases enhance the digestibility of proteins, while phytases release phosphorus by breaking down the phytic acid that is present in grains (Cowieson & Adeola, 2005; Cowieson & Roos, 2016).

1.3.8.1 PROTEASES

This group of enzymes contains all the peptidases, proteinases and proteolytic enzymes. Proteases are enzymes that degrade proteins by hydrolysing peptide bonds. The purpose of proteases used as animal feed additives is an increased protein hydrolysis and an improved nitrogen utilization, which could lead to a

lower protein content in the diets of the non-ruminants (Swiatkiewicz *et al.*, 2016; van der Aar, Molist and van der Klis, 2016). The use of exogenous protease as an animal feed additive was first mentioned by Lewis et al. 1955 and Baker et al. 1956. Protease was first used in pig diets in 1957 when it was added into a mixture of pre-digested feed, but there was no improvement on the growth performance of the pigs (Cunningham and Brisson, 1957). Today proteases are used at all stages of pig production, from nursery until finishing pigs (Guggenbuhl et al. 2012, Wang et al. 2011, Mc Alpine et al., 2012). Protease has been used as a part of commercial enzyme admixture for the past two decades but just in the last 5-10 years as mono-component enzyme (Oxenboll et al., 2011; Cowieson & Adeola, 2005; Cowieson & Roos, 2016).

Monogastric animals produce their own endogenous digestive proteases (pepsin, trypsin, chymotrypsin and carboxypeptidases) that aid to the digestion of feed proteins. Yet these enzymes are not enough since a part of the protein is still with the faeces. The need for an exogenous protease that improves the protein utilization is profound (Parsons et al., 1997, Lemme et al., 2001). Although proteases are already being used as feed additives there still are gaps in the literature regarding their function and interactions with other components of the feed like phytate, polyphenolics and other enzymes (Cowieson & Adeola, 2005). Further information on proteases and their ways of action are shown in Chapters 3.2 and 4.2.

1.3.8.2 PHYTASE

Phytase has the ability to hydrolyse phytate (or phytic acid) that is present in most plants and is the reason why the majority of plant phosphate is not available to

the animals through digestion. Phytic acid is also well known for its anti-nutritional properties as a result of binding to other nutrients like minerals and proteins. Phytic acid (myo-inositol 1, 2, 3, 4, 5, 6-hexakis phosphate) is the storage form of P in cereal grains and oil seeds (Cheryan, 1980). Monogastrics do not secrete phytase themselves and therefore they cannot utilise the phosphorus available in phytic acid. By the use of phytase as a feed additive they can overcome this issue.

Although phytase has been commercially used for the past few decades there is still scope for further research in the field in order to move to a completely "phytate-free nutrition" (Selle & Ravindran (Part A), 2007; Cowieson et al., 2015). Not only phytase breaks down phytic acid and releases phosphorus, but recent research has shown that it has other physiological effects in animals, including the retention of amino acids, trace minerals, calcium and energy as well as the enhancement of the performance of the animals (Cowieson and Roos, 2016). However, these functions of phytase are not fully understood and there is a need for further research in the field of the 'extra-phosphoric' effects of phytase. Finally, there is a profound need for further research in the interactions of phytase with other enzymes as well as polyphenolics. More information on the phytases is shown in Chapter 2.2.

1.3.9 ANTI-NUTRITIONAL FACTORS IN ANIMAL FEED

Phytic acid and polyphenolics are both known for their anti-nutritional properties. Sorghum is a grain that has been in the centre of research since it contains both phytic acid and polyphenolics (Elzubeir and Jubarah, 1993). According to Selle

(2010), phytic acid and polyphenolics have a lot of anti-nutritive properties in common (Woyengo, Beltranena and Zijlstra, 2015).

Phytic acid is the way of plants storing phosphorus and has a clear anti-nutritional effect (Selle *et al.*, 2010; Truong *et al.*, 2014). Its anti-nutritional properties come from its high binding abilities through electrostatic mechanisms. This results in phytic acid binding to proteins and other nutrients and therefore reducing their solubility and making them indigestible (Selle and Ravindran, 2007b; Bye *et al.*, 2012; Cowieson and Roos, 2016).

Tannins are a subgroup of polyphenolics and they are well known for their anti-nutritional and even toxic properties. Their natural aim is to deter ingestion to animals and insects. They interact with nutrients like starch and therefore they influence the nutritional properties of different feed ingredients. In that way polyphenolics reduce the starch hydrolysis in grains like maize, barley and wheat (Tomasik and Schilling, 1998; Barros, Awika, and Rooney, 2012; Kandil, Vasanthan and Bressler, 2012; Zhu, 2015). Their ability to bind on protein substrates results in affecting the activity of digestive enzymes (Supagro, 2007).

Since there is not in-depth knowledge of the pathways through which phytic acid and polyphenolics interact with enzymes and nutrients, further research is required in the field. The anti-nutritive effect of phytic acid and polyphenolic compounds is described in more detail at Chapter 2.2.

1.3.10 ISOTHERMAL TITRATION CALORIMETRY

Isothermal titration calorimetry (ITC) is a technique used for the past 25 years used to investigate the biophysical reactions occurring between molecular complexes. The use of ITC started as a tool for studying binding interactions. It has been widely used for binding studies using carbohydrates, nucleic acids and synthetic molecules. Now it is commonly used not only for the characterization of the thermodynamics occurring in biopolymer binding interactions but for enzyme kinetics as well. Despite the fact that ITC is mostly used for researching simple one-site binding interactions and enzyme kinetics, there are more applications of ITC, like protein folding , where ITC can be used as an analytical technique (Falconer, 2016).

ITC is a very sensitive technique and it can measure changes in heat from 0.1μcal, making the measurement of large binding constants possible ($K > 10^{8}$-$10^{9}M^{-1}$). Its sensitivity also allows the determination of the Micahelis-Menten constant ranging from K_m=10^{-2}-10^{3}μM and the catalysis constant in between k_{cat}=0.05-500sec^{-1} (Atkins and De Paula, 2006; Freyer and Lewis, 2008; Transtrum, Hansen and Quinn, 2015).

ITC is widely used for studying the intermolecular binding interactions (Falconer & Collins, 2011; Ghai et al., 2012) and that has also been applied on interactions between tannin and proteins (Frazier et al., 2006; Prigent et al., 2009; Pascal *et al.*, 2007; Frazier *et al.*, 2010; McRae, Falconer and Kennedy, 2010).

Since the aim of this project is to study the interactions between enzymes, grain proteins, phytic acid and tannins, it is clear that ITC is the appropriate technique to be used for these studies.

1.3.10.1 ITC THEORY AND OPERATION

There are three different ways a calorimeter can function, by measuring (a) the temperature change, (b) the power compensation (ITC) and (c) the heat conduction (Eatough et al., 1985). In an isothermal titration calorimeter, the measurement cell is kept in a constant temperature by the instrument using either a cooler or a heater. During a chemical reaction that takes place in the measurement cell, any heat input is perceived by the instrument and therefore the power applied to the cell by the control heater is reduced so that its temperature remains constant. The raw signal in an ITC is the power applied to the control heater in order to keep the temperature in the measurement cell constant as a function of time. The heat change can be calculated by the integral of the heat power over the time. In Figure 1 a typical isothermal titration calorimeter is shown (Atkins and De Paula, 2006; Freyer and Lewis, 2008).

1.3.10.2 ITC FOR ENZYME KINETICS

The need for a better understanding of the catalytic activity of enzymes is profound. Enzyme kinetics are being used both for the verification and establishment of the mechanisms in catalytic reactions and biochemical pathways, as well as for drug discovery and development. Analysis of the enzyme kinetics by the use of ITC is rather recent (Todd and Gomez, 2001) although this use of ITC was proposed a lot earlier (Spink and Wadsö, 1976; Morin and Freire, 1991; Williams and Toone, 1993). Using ITC for the determination of the kinetic behaviour of enzymes allows the measurement of a complete set of kinetic parameters for an enzyme-catalysed reaction in a single experiment (Freyer and Lewis, 2008; Ghai, Falconer and Collins, 2012; Falconer, 2016).

After the establishment of ITC as a tool for the determination of the catalytic activity of enzymes by Todd and Gomez in 2001, it has been increasingly used within the last 15 years. The kinetics of trypsin-catalysed hydrolysis have been a major part of the research done in the field (Aguirre *et al.*, 2015; Maximova and Trylska, 2015). The kinetics of other enzymes like xylanase (Baumann *et al.*, 2011), glucanases (Murphy *et al.*, 2012; Rohatgi, Gudmundsson and Rolfsson, 2015) and many more have been successfully measured since 2001 using ITC (Cheleski *et al.*, 2011; Ertan *et al.*, 2012; Mertens, 2013; Siddiqui *et al.*, 2014).

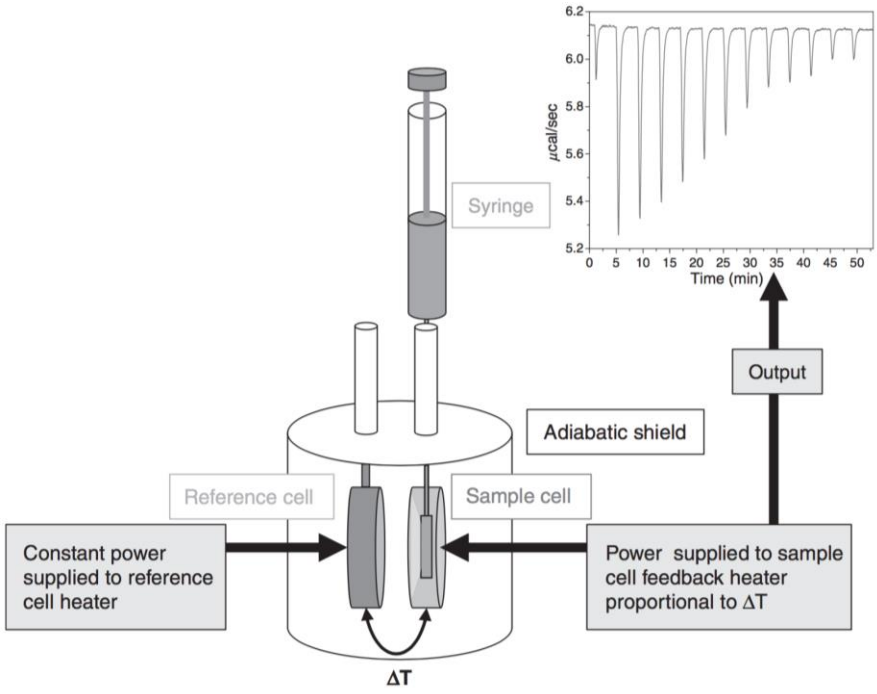


Figure 1.2: Diagram representing the way ITC works. When heat change is identified in the reference cells, power is used to compensate that change. This power is applied by the instrument to bring the temperature of the sample cell to the same temperature that the reference cell has. This signal is recorded and exported as a thermogram. Taken from: Freyer and Lewis, 2008.

1.3.10.3 KINETIC ITC EXPERIMENTS

The typical experiment for the enzyme kinetics involves enzymes, substrate and any other reactants that are involved in the catalysis. ITC is the optimal instrument of the analysis of enzyme kinetics since the signal produced is a direct measurement of the rate of the reaction. Enzyme catalysis reactions have an enthalpy in the range of -10 to -100 kcal/mol with reaction rates from 10 to 100 pmol/sec allowing the instrument to produce accurate measurements (Freyer and Lewis, 2008; Transtrum, Hansen and Quinn, 2015). For the best use of ITC for enzyme kinetics the following rules should be followed:

- Use of reasonable concentrations for both the enzyme and substrate
- The concentration of the enzyme should be enough to convert the substrate into product in a reasonable amount of time.
- The concentration of the enzyme should vary from 1nM to 10μM while the concentration of the substrate should be between 1μM and 10mM.

Typical ITC enzyme activity assays measure the Michaelis-Menten constants; ΔH, Km and Vmax values and are always performed in simple, single-step reactions.

The output of ITC consists of peaks that are a measure of the power that is used to compensate for the temperature difference between the reference and measurement cell. This is a result of all processes taking place in the measurement cell caused by the injection and mainly describe a binding event between the substances of the cell and the syringe (Buurma and Haq, 2007).

ITC is based on the simple principle of thermodynamics, which stated that when there is contact between two or more molecules, that results in production of heat or heat loss. This varies according to the type of binding, that takes place and it

can be exothermic or endothermic, respectively. Both endothermic and exothermic are identifiable and the direction of the peaks on the thermogram corresponds to the loss or production of energy respectively.

For single binding site interactions providing that the c-value is between 1-1000, information such as the stoichiometry, disassociation constant (KD), change in free energy (ΔG) and change in enthalpy (ΔH) are information deriving from the data exported as an output form ITC. Using this information, the change in entropy (ΔS) can bet then calculated. On the other hand, when it comes to data deriving from multiple binding site experiments, the same calculations need to be conducted for the different binding events. For example, if there are two binding events, the ΔG, ΔH and ΔS values will be calculated for both events. Finally, when quantifying high affinity binding interactions, assuming that the receptor would be titrated into a mixture of competing ligands with both high and moderate affinity, a biphasic isotherm would be generated and would be used to measure the disassociation constants (KD) as well as the binding enthalpies (ΔH) for both ligands. Finally,

1.4 AIMS AND OBJECTIVES

As mentioned above, there is a clear problem with animal feed, mainly caused by the presence of antinutritive factors, that are causing poor utilisation of protein, probably due to enzyme inhibition. Enzymatic reactions and the effect of anti-nutritive factors have been studied before, but not with a calorimetric assay, like ITC.

The aim of this work is to provide information regarding the interactions between phytic acid, polyphenolics, phytase and proteases. This would be achieved by using ITC, a powerful tool that would provide valuable information that could be used to help build a map based on the schematic shown in Fig. 1.2.

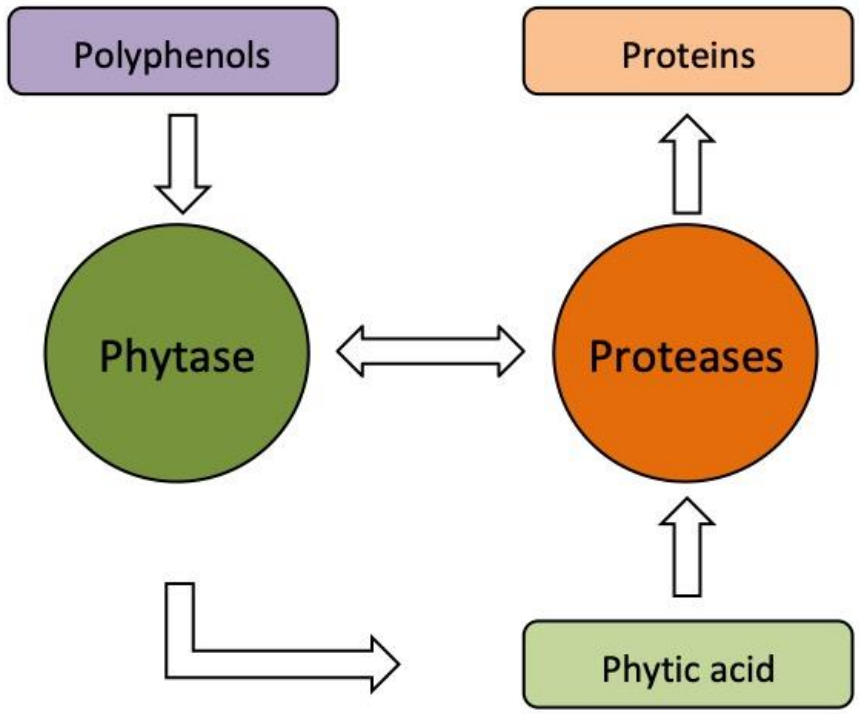


Figure 1.3: Map of interactions between phytic acid, phytase, protein, proteases and polyphenolic compounds, to be used as a model for nutrition.

1.5 HYPOTHESES

I. Sorghum polyphenolics have an inhibitory effect on phytase activity

Polyphenolics are known to be powerful anti-nutritive factors due to their ability to bind to proteins and other positively charged molecules. This is the hypothesis of Chapter 2 of this thesis, where polyphenolics will be extracted from sorghum and their effect on the activity of an exogenously produced, commercially available phytase will be estimated.

II. Phytic acid has an inhibitory effect on the activity of endogenous and exogenous proteases

Phytic acid has a high negative charge and this promotes its binding to positively charged molecules, like metal ions and proteins. It is known to have an inhibitory effect on digestive enzymes. This is the hypothesis of Chapter 3, where the effect of phytic acid on an exogenously produced, commercially available protease will be estimated. At the same time, its effect on two endogenously produced proteases will also be estimated, to provide a better understanding of the dynamics between the enzymes.

III. Phytase is degraded by proteases

Enzyme-enzyme interactions are common in nature, especially in animal's nutrition. Most exogenously produced enzymes are designed to be resistant to degradation by other enzymes. This hypothesis is tested in experiments described in chapter 4 of this thesis. It is the final piece of the puzzle that will provide all the necessary information need to create a model with the interactions between phytase, proteases, proteins, phytic acid and polyphenolics.

CHAPTER 2 'THE EFFECT OF SORGHUM POLYPHENOLS ON THE ACTIVITY OF A 6-PHYTASE'

2.1 SUMMARY

Phytic acid is a known anti-nutritive factor, present in most grains. It is a highly negatively charged molecule and its anti-nutritive effect in monogastrics digestion was established a few decades ago. To counter this effect, phytases have been used as feed additives. Phytases are enzymes that hydrolyse phytic acid, by cleaving the phosphate groups from the inositol ring and therefore reducing its charge. These enzymes are still not working as efficiently, due to other inhibitory substances present in the feed, that interact with the enzymes and stop then from catalysing the digestive reactions. Potential inhibitors of the phytases could be polyphenolics compounds that are also known to have anti-nutritive effects and are present in many grains. ITC was the technique chosen to study the interactions between polyphenolics compounds and phytase.

ITC is a calorimetric technique that has been used to study enzymatic reactions in the past, by measuring the ΔH, Km and Vmax values. This was achieved before in simple enzymatic reactions. When studying the phytase-phytic acid reactions, it was obvious that it was a complex/multiple stage reaction, and the thermogram obtained from ITC for this reaction were complex, with peaks overlapping each other and therefore unable to be used for standard enzymatic kinetics. Despite that, the plethora of information that can be gathered from these thermograms provided enough information for estimating the rate of the reaction and therefore the relative activity of the enzyme. Using that information, different concentrations of three polyphenolics-rich extracts obtained from three different sorghum varieties were used to estimate their effect on the activity of a commercial phytase that is used as an additive. All three had different inhibitory effects on the phytase.

2.2 INTRODUCTION

Phytic acid, or phytate, or myo-inositol hexakisphosphate is a phosphate ester of inositol. Phytic acid consists of six phosphate groups attached on the cyclic 6C myo-inositol (Pallauf and Rimbach, 1997) and it is the natural way of plants storing phosphorus. Thus, it can be found in plant tissue, legumes, cereals and grains (Loewus and Loewus, 1980).

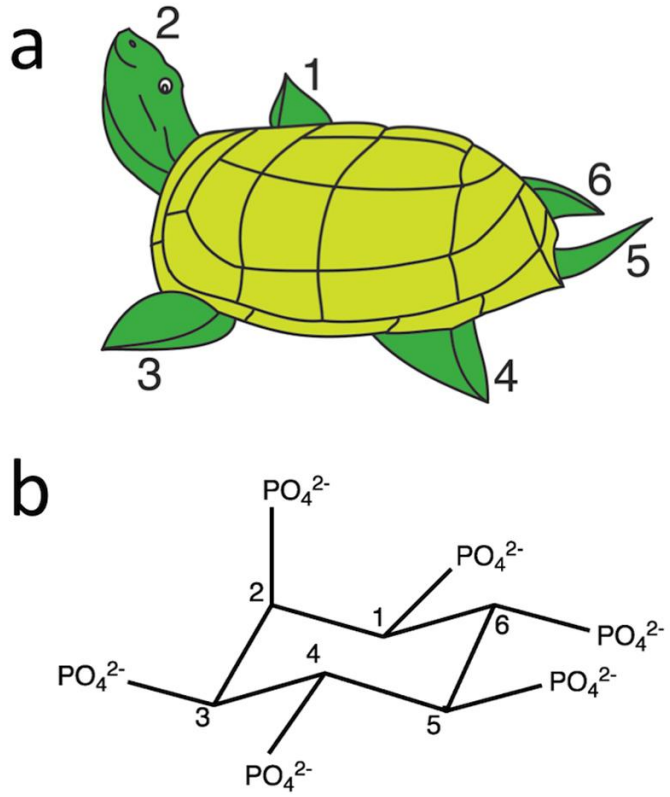


Figure 2.1: (a) Agranoff's interpretation of IP6 as a turtle. (b) The molecule of phytate numbered according to Agranoff's nomenclature. In both (a) and (b) the 2-phosphate axial is pointing upwards. Taken from Ariza et al., 2013.

Phytic acid's molecular composition is $C_6H_{18}O_{24}P_6$ and it contains 54 bonds; 36 are non-H bonds, 12 are rotatable bonds and the final 6 are double bonds. It is consisted of a six-membered ring and 6 phosphate groups with 12 hydroxyl groups (Figure 2.1). Due to the six phosphate groups, phytic acid is negatively ionised, which results in a strong binding affinity. This is leading to interactions with positively charged molecules, such as metals and minerals (iron, zinc, calcium), or even proteins. In that way it inhibits the absorption of these nutrients and for that, it is considered to be an anti-nutritive factor (Zhu *et al.*, 1990; Barker *et al.*, 2009).

To address this and to improve digestibility of nutrients in the diets of monogastric, several studies have been conducted to understand how to get over this issue and proved that exogenously produced phytases can aid and improve the nutrient digestibility. This is achieved by the phytase cleaving the phosphate groups of the inositol ring and thus reducing its negative charge.

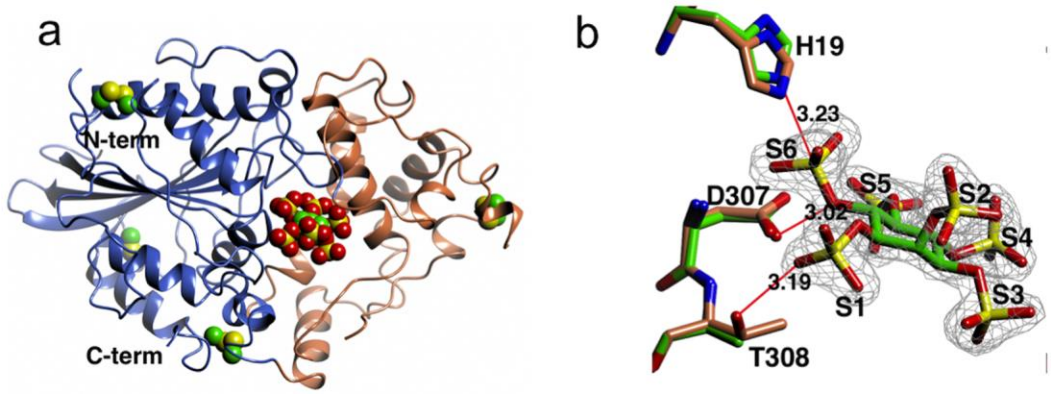


Figure 2.10: (a) Ribbon representation of the *Hafnia alvei* phytase fold. The α domain is shown in coral, while the α/β domain is shown in blue. (b) Mono view of the binding of myo-inositol- hexakissulphate to the active site of *Hafnia alvei* phytase, with the density shown at the 1s level for the myo-inositol-hexakissulphate. Taken from Ariza et al., 2013.

Therefore, phytases are essential for the nutrition of monogastric animals as phytase is the only way to catalyse the hydrolysis of phytic acid. Phytases can be found in the animal's mucus and in the gut microflora to a very limited extend, as well as in plants and microbial organisms. Since the mucosal phytase in monogastric animals is not capable of hydrolysing the whole amount of phytic acid present in the animal feed, exogenous phytase is supplemented to the feed**.** In this way not only nutrients and minerals are absorbed, but also the organic phosphorus present in the phytic acid becomes available to the animal when cleaved (Humer, Schwarz and Schedle, 2015).

As mentioned above phytic acid consists of six phosphate groups attached on an inositol ring. Therefore, phytic acid is carrying a large amount of organic phosphorus in its molecule. Phosphorus is essential for all living matter, including plants, animals, and bacteria and it has no substitute in food production. Approximately 90% of phosphate rock is used for food production: 82% for

fertilizers, 5% for animal feed, and 2–3% for food additives. On the other hand, the size of remaining fossil phosphate resources is uncertain and certainly finite. Phosphorus security is emerging as one of the twenty-first century's greatest global sustainability challenges. Therefore, fossil P resources may become depleted by continuous mining and despite calls for phosphorous conservation, fossil P is being mined at an increasing rate. It is essential to improve the phosphorous efficiency for agricultural use and also to recycle P to contribute to the reduction of demand for fossil P resources (Cordell, Drangert and White, 2009; Cordell and White, 2014).

The use of phytase to de-sequester phosphate from phytic acid in animal feed to increase P efficiency in animal husbandry of poultry and swine has been a common practice in the last decade. This has resulted in a reduction in P excretion by 25–50% dependent on type of animal, species and activity of added phytase (Reijnders, 2014). In that way phytases, reduce both environmental impact and feed costs. The amount of plant phosphorus available to the animal and the liveweight gain and feed conversion are greatly increased as well as, far less inorganic phosphorus needs to be added to the diet.

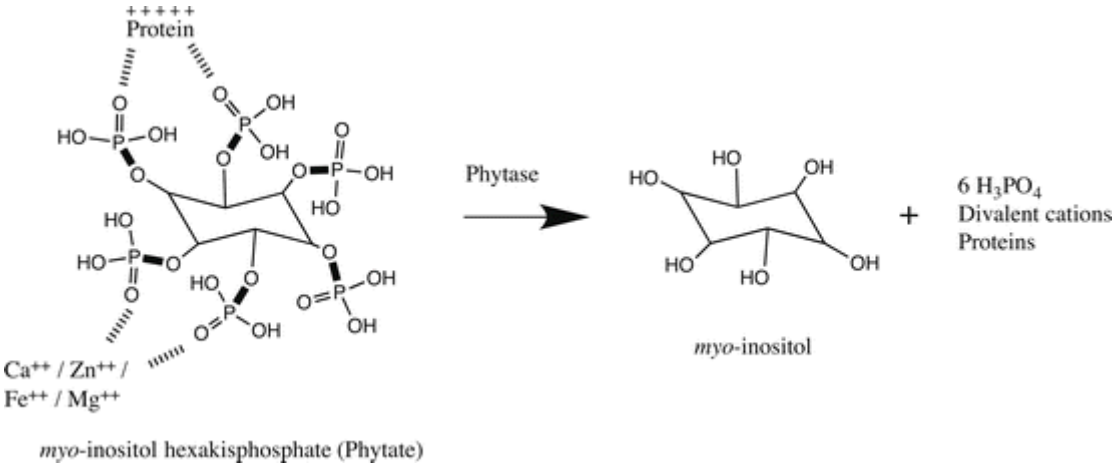


Figure 2.19: Degradation of phytic acid by phytase taken from A V Shivange & U Schwaneberg, 2017.

As shown in Figure 2.3, phytases are divided into two main categories, 3-phytases and 6-phytases; they are named after the site that the hydrolysis starts from (Selle and Ravindran, 2007b). The use of microbial phytase as an additive to monogastrics nutrition was first observed by Nelson *et al.* (1971) and since then, the efficiency of the phytases used as a supplement for monogastric nutrition has increased and as has their stability (Humer, Schwarz and Schedle, 2015).

Although monogastrics' diets are supplemented with high doses of phytases, there is still an amount of organic phosphorus being excreted by the animal (Cowieson et al., 2006). And since phytase is provided in an excess amount to the animal, the reason why this is happening is probably due to inhibition of the enzyme by some other anti-nutritive factors present in grains.

A grain well known for containing a high percentage of polyphenols, which are also regarded as anti-nutritive factors is sorghum (Figure 2.4). Sorghum (Sorghum bicolor) is commonly produced in Africa, Asia and Australia, due to its ability to grow under extreme climates (Dicko *et al.*, 2006). Therefore, it is being used both

for human and animal diets as it is a good source of protein, vitamins, energy and minerals. It is not a very expensive ingredient that is produced with low effort and in conditions that most other grains would not grow. Therefore, it is a great substitute for more expensive grains like wheat, maize and barley, which are commonly used for the diets of monogastrics worldwide.

All sorghum varieties contain polyphenols, and most contain flavonoids, which are a subgroup of polyphenols. Tannin content should not be related to the pericarp colour, as it is not the ultimate indicator. All sorghum types have different tannin content, which is influenced by the presence of B_1 and B_2 genes (Boren and Waniska, 1992, Bohin *et al.*, 2012).

There are a lot of studies discussing the polyphenolic profile in different sorghum varieties and Liberty, a white sorghum variety that was used for the experimental part of this chapter, was found to have a simpler polyphenol profile compared to five coloured grain genotypes, red and black.

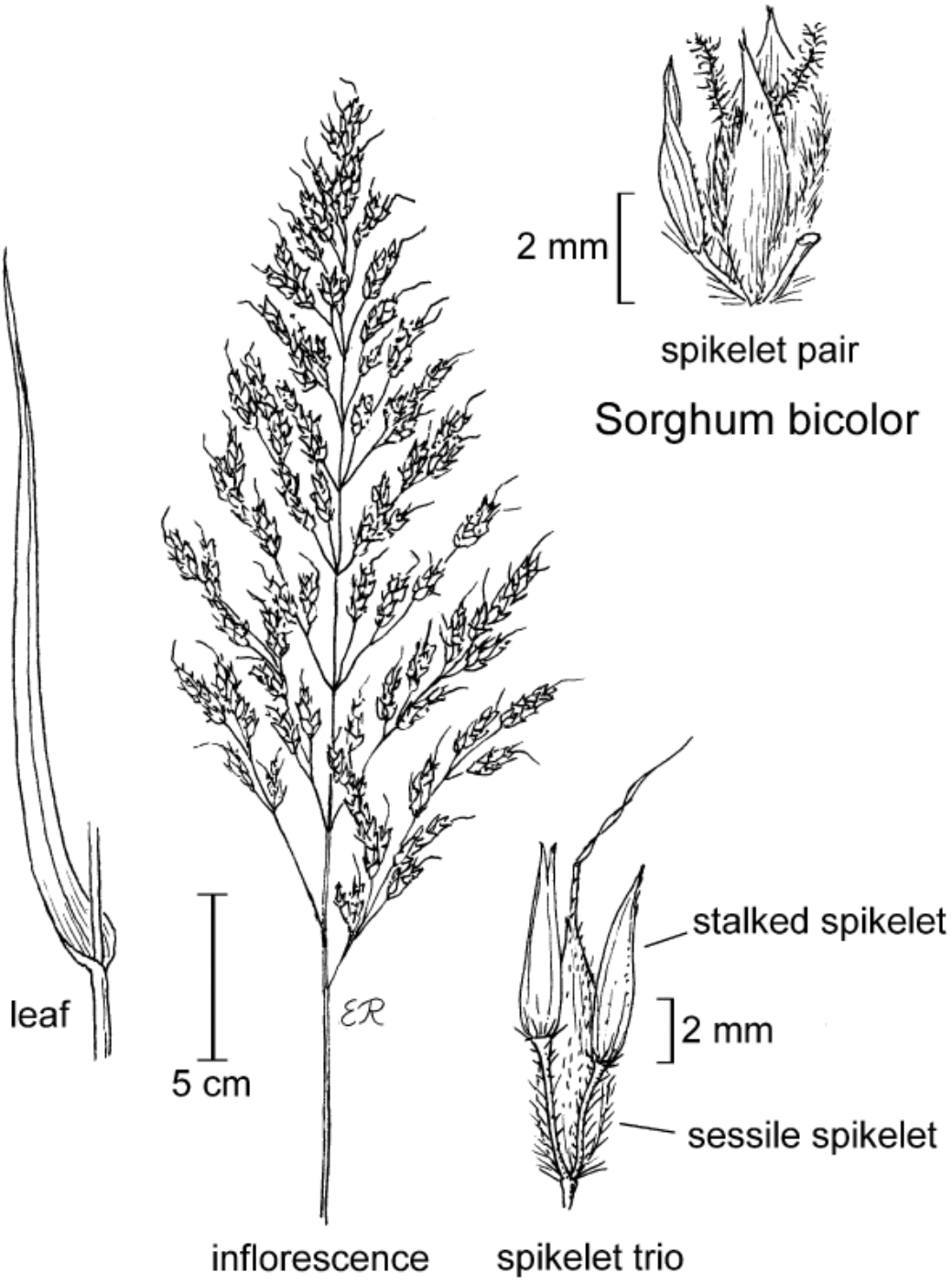


Figure 2.27: Sorghum Bicolor taken from: de Wet 1978 Amer J Bot 65:477—484.

Sorghum can comprise up to 100% of the grains' percentage in a chicken's diet but usually it is used in combination with other grains. For pig diets it usually makes up 20% of the diet. As shown in Table 1, in a typical diet of a sow, there are roughly 350g of sorghum daily. Although sorghum can be toxic to animals, birds are known to be resistant to the sorghum tannins and therefore they can consume it in large amounts. Despite the fact that they are not toxic for the birds that does not mean that there are no other implications on their nutrition due to the high level of polyphenols present in sorghum.

Table 2.6: Typical sow diet, focusing on sorghum

Typical daily sow diet	
Diet mass	2.5kg
Grains	70% of diet
Sorghum	20% of grains
Mass of sorghum	350g

Sorghum phenolic acids are divided into two main categories, hydroxybenzoic and hydroxycinnamic acids. The main difference is that hydroxybenzoic acids derive from benzoic acid, while hydroxycinnamic acids have a C6-C3 structure. Hydroxybenzoic acids include gallic, vanillic and syringic acids, while hydroxycinnamic acids include coumaric, ferulic and sinapic acids (Dykes and Rooney, 2006).

Polyphenols are present in a plethora of plants, fruit and grains. They are found in high concentrations in green tea, red grape skin, cocoa and most grains. They are divided into three main categories; phenolic acids, flavonoids and tannins. Catechin and epicatechin are two of the most common and well-known phenols in nature (Figure 2.5), but the list of phenolic compounds is really long as more complex phenolics are different combinations of smaller compounds. Flavonoids are the most abundant polyphenols in nature, consisting of at least two aromatic rings, connected to one or more hydroxyl groups and a bridge of carbon. Therefore, catechin and epicatechin are considered as the building blocks of tannins. Other common anti-nutritive factors are polyphenolic biomolecules, such as tannins. Sorghum is known to contain significant amounts of polyphenols such as phenolic acids, procyanidins and anthocyanidins (Massel *et al.*, 2016). Some more complex polyphenolic compounds like the ones mentioned above are shown in Figure 2.6.

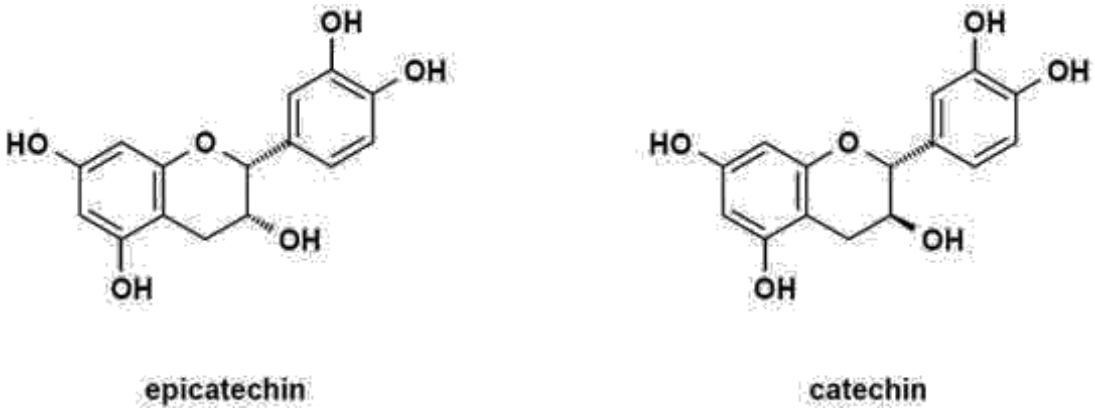
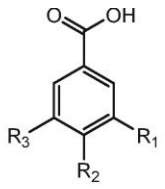
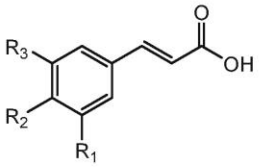


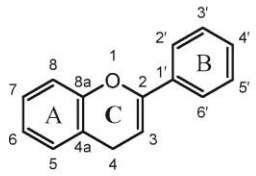
Figure 2.32: Structure of Epicatechin and catechin, adapted from Ramalho et al., 2014.



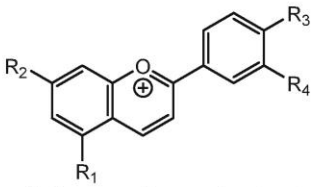
Major **benzoic acids** in sorghum;
Gallic acid, $R_1 = R_2 = R_3 = OH$
Protocatechuic acid, $R_1 = R_2 = OH$, $R_3 = H$
Vanillic acid, $R_1 = OCH_3$, $R_2 = OH$, $R_3 = H$
p-Hydroxybenzoic acid, $R_1 = R_3 = H$, $R_2 = OH$



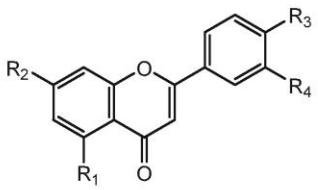
Major **cinnamic acids** in sorghum;
Ferulic acid, $R_1 = OCH_3$, $R_2 = OH$, $R_3 = H$
Caffeic acid, $R_1 = R_2 = OH$, $R_3 = H$
Sinapinic acid, $R_1 = R_3 = OCH_3$, $R_2 = OH$
p-Coumaric acid, $R_1 = R_3 = H$, $R_2 = OH$



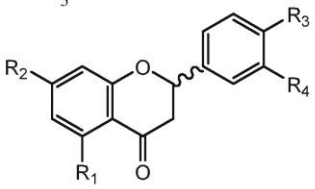
Basic flavonoid structure



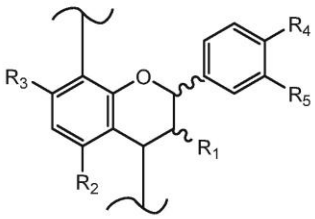
Sorghum **3-deoxyanthocyanin** structure;
Apigeninidin derivatives, $R_3 = OH$, $R_4 = H$
Luteolinidin derivatives, $R_3 = R_4 = OH$
R_1 & R_2 are usually OH, O-glycosides, and/or OCH_3



Sorghum **flavone** structure;
Apigenin derivatives, $R_3 = OH$, $R_4 = H$
Luteolin derivatives, $R_3 = R_4 = OH$
R_1 & R_2 are usually OH, O-glycosides, and/or OCH_3



Sorghum **flavanone** structure;
Naringenin derivatives,$R_3 = OH$, $R_4 = H$
Eriodictyol derivatives, $R_3 = R_4 = OH$
R_1 & R_2 are usually OH and/or O-glycoside



Sorghum **proanthocyanidin** unit structure;
Proapigeninidin , $R_1 = R_5 = H$, $R_4 = OH$
Proluteolinidin, $R_1 = H$, $R_4 = R_5 = OH$
Procyanidin, $R_1 = R_4 = R_5 = OH$
R_1 & R_2 are usually OH, but can rarely have OCH_3

Figure 2.41: Structure of the major phenolic acids and flavonoids in sorghum taken from Girard & Awika, 2018.

Polyphenols are plant secondary metabolites and one of their main purposes is to defend plants against predators. Polyphenols are known to have an anti-nutritional effect by inhibiting digestive enzymes and protein absorption (Hagerman and Butler, 1981). This is accomplished by linking with

macronutrients and thus creating indigestible complexes or by chelating metal ions and reducing their bioavailability (Dicko, 2005). Their natural aim is to deter ingestion to animals and insects; thus, they have anti-nutritional and even toxic properties in animals' diets. They interact with nutrients like starch and they influence the nutritional properties of different feed ingredients. This is caused by their ability to bind on protein substrates and results in affecting the activity of digestive enzymes. Therefore, they can potentially interfere with phytase activity.

Although the anti-nutritive effect of polyphenols is profound, not all the interactions causing this effect are known. For the purpose of this project, Isothermal Titration Calorimetry (ITC) was used to estimate the effect of the polyphenol-rich extracts on the phytic acid-phytase reaction.

Enzymatic reactions were studied by calorimetric techniques since the 1950s, but ITC started being used for this reason in 1990s. Enzymes like yeast oxydases and hexokinases, chitinases and pyruvate kinase were the first to be studied using ITC (Bianconi, 2007). Since Todd and Gomez, (2001) explained the potential of ITC to study enzymatic reactions and explained how that is done, ITC is constantly being used for such purposes. ITC is a technique that has been commonly used for the investigation of binding interactions between molecules and for the analysis of enzymatic kinetics. Despite its power as an analytical tool ITC has primarily been used for the analysis of simple/one stage reactions due to the complexity of the data generated from multiple-step reactions. It is a very sensitive technique and it can measure changes in heat from 0.1μcal (Falconer, 2016). Enzymatic reactions are not always simple. In order for ITC to be used for the estimation of the kinetics in complex enzymatic reactions, the ΔH, Km and Vmax values, which will be

different for every step of the reaction, need to be calculated. In such cases, due to overlap of the peaks, the relative activity of the enzyme can be calculated instead. This can be achieved by comparing the time needed to complete a percentage of the reaction, to the time needed to complete a percentage of the reaction in the presence of another substance, or at a different temperature, pH, presence of salts and any other parameter that is of interest

The objective of this study was first to manage using ITC for the study of the kinetics of the reaction between the 6-phytase and phytic acid, which is novel, as ITC has not been used to study the kinetics of complex enzymatic reactions before. Furthermore, the second objective was to use this technique in order to determine the effect of sorghum polyphenols on the activity of a 6-phytase and also to show that ITC can be used to interpret one-stage enzymatic reactions as well as complex enzymatic reactions. This will provide information for a better understanding of the reactions between phytic acid, phytase, protein, proteases and polyphenols. As shown in Figure 2.7, this is one of the steps towards the mapping of these reactions in order to create a model that could be useful for the better nutrition of monogastrics.

The hypothesis of this Chapter is that sorghum polyphenolics have an inhibitory effect on phytase activity, and that ITC can be used to study complex enzymatic reactions.

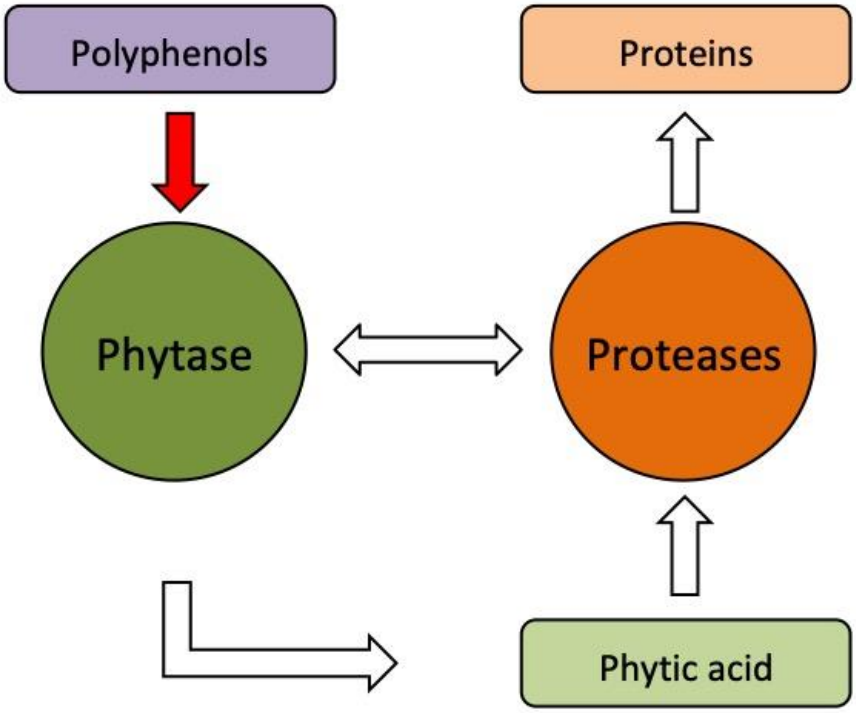


Figure 2.49: Map of interactions between phytic acid, phytase, protein, proteases and polyphenolic compounds. As shown with the red arrow, in this chapter the focus will be on the interactions of polyphenolic compounds with phytase.

2.3 MATERIALS AND METHODS

2.3.1 CHEMICALS AND MATERIALS

- Phytic acid sodium salt hydrate, rice extract, sodium chloride and sodium acetate were sourced from Sigma Aldrich, Gillingham, UK.
- 6-phytase (the enzyme originating from Citrobacter braakii) in solution, was sourced from DSM, Kaiseraugst, Switzerland.
- N- Hexane, For residue analysis, ≥99.0%, Honeywell Riedel-de Haën™, Bucharest, Romania.
- Acetone, HPLC Plus, for HPLC, GC, and residue analysis, ≥99.9%, Sigma Aldrich, Gillingham, UK.
- Hydrochloric acid, Reag. Ph Eur, Reag. USP, Sigma Aldrich, Gillingham, UK.
- Ethanol, BioUltra, for molecular biology, ≥99.8%, (absolute alcohol, without additive, A15 o1), Sigma Aldrich, Gillingham, UK.
- (+)-Catechin, Sigma Aldrich, Gillingham, UK.
- Na2CO3, Sigma Aldrich, Gillingham, UK.

2.3.2 EXTRACTION OF POLYPHENOLS

Three different sorghum varieties were used for this study, one white (Liberty) and two red (MR-Buster and Cracka) sorghum varieties. 20g of each sorghum variety were soaked into MilliQ water over night. The husk was isolated after crushing the seeds and air dried overnight. The husks were then defatted for 5 hours using a soxhlet and 150ml of n-hexane. The defatted sorghum husks were air-dried overnight and then the polyphenols were extracted by using 70% v/v aqueous acetone, while vigorously mixing in an orbital mixer for 30 minutes at

200rpm. Finally, the supernatant was filtered through glass microfiber filters under pressure. The acetone was evaporated from the solution using a rotary evaporator under pressure. The final solutions were frozen and then freeze dried, leaving a powder-form substance, which was used for the experiments below.

2.3.3 Analysis of Polyphenolics

The polyphenol extracts were assessed by FTIR (IRAffinity-1S by Shimadzu) analysis, using an automated linear subtraction across the spectrum of the blank. A resolution of 1cm-1, taking 32 scans in each measurement and scanning wavelengths from 400 to 4000nm. Before the sample scans, an empty background scan was performed, and baseline was corrected accordingly. For the FTIR analysis a sufficient amount of powdered polyphenolic rich extracts and catechin as a standard (enough to cover the surface of the measurement space) were added on the measurement space and the analysis was performed. The data was normalised based on the highest observed peak to remove any abnormalities. A UV-vis analysis (UV: GENESYS 10S UV-Vis spectrophotometer by Thermo Scientific) was performed by using samples of the different polyphenolic rich extracts and catechin as a standard. Wavelength (for single measurement or scans), used quartz cuvette and sample volume according to cuvette's standards (~1 mL).

2.3.4 Total Phenolic Content Assay

The total phenolic content assay was performed according to Ainsworth and

Gillespie (2007). 100 µl of each sample (in 95% methanol), standard (in 95% methanol) or 95% (vol/vol) methanol blank were added in duplicates in the 2 mL microtubes. Samples and standard were in concentrations of 1µM. 200 µl 10% (vol/vol) Folin Ciocalteau reagent were added and then all samples were vortexed thoroughly. Then, 800 µl of 700 mM Na_2CO_3 were added into each tube and they were then incubated at room temperature for 2 h. Finally, 200 µL of each (sample, standard or blank) were transferred into a clear 96-well Microplate or 1 mL to a cuvette and read the absorbance of each well at 765 nm.

Using concentrations from 0 up to 0.2 nmol of Gallic acid equivalents and plotting them against their absorbance, a standard curve was created. Using this and the known absorbance, the concentration of each sample in gallic acid equivalents was back calculated.

2.3.5 ITC

A 1 µM 6-phytase, originating from *Citrobacter braakii* with a minimum phytase activity of 10,000 FYT/g, stock was made by appropriately diluting the enzyme solution provided by DSM into 5% ethanol.

Respectively, a 20 Mm phytic acid stock was prepared by dissolving the appropriate mass of phytic acid into 5% ethanol. These stocks were used to estimate the optimal concentrations of the substrate and enzyme that would be used for the ITC enzyme activity assay.

2.3.5.1 ITC OPTIMIZATION

Prior to analysis the samples were all degased for 20 minutes to eliminate the possibility of air bubbles that would alternate the data. ITC analysis was conducted on an iTC200 (MicroCal, Northampton, MA, USA). 50 µl of the 20 mM stock of Phytic acid was loaded into the syringe and titrated into the sample cell, which contained 300 µl of 0.0625 µM of 6-phytase in a sequence of 4 injections in 2, 5, 5 and 5 µl respectively. Experiments were conducted at room temperature (20°C) and 285 rpm.

2.3.5.2 ITC ENZYME ACTIVITY ASSAY

To estimate the enzymatic activity of the phytase in the presence of different concentrations of the polyphenol-rich extracts of the three sorghum varieties, the inverse of the time for 90% completion of the phytase catalysed reaction to occur was used as activity in this assay. Since the ITC assay was unable to calculate the Michaelis-Menten constants, by estimating the 90% time point of the completion of the reaction made it possible to estimate the relative activity of the enzyme in the presence of the polyphenolic-rich extracts.

The time for the completion of the reaction without the presence of polyphenolics was estimated, which was then used to compare the effect of the polyphenolics on the reaction. Inhibition of the reaction was identified by a slower reaction. The longer it took for the reaction to complete at its 90%, the higher the inhibition.

The 6-phytase was incubated with the sorghum polyphenol-rich extract for 25 minutes at room temperature prior to the titration. The enzyme assay used a TA Instruments NanoITC; injecting 5 µL of 20 mM phytate into 300 µL of 0.125 µM 6-

phytase in 5% ethanol. Note, the ethanol is present to solubilise the polyphenol-rich extract and does not affect the assay.

2.3.6 Anion Exchange Chromatography

A Metrisep A supp 4 column (polyvinyl alcohol with quaternary ammonium groups – medium ion exchange capacity, pH 3-12) – 250/4.0mm was used on a Ion chromatograph 883 Basic IC plus (Metrohm, Runcorn, UK). UV–Vis spectra were recorded on a UV–Vis HELIOS α spectrophotometer (Unicam, Cambridge, UK) in 1-cm quartz cell.

Detection was suppressed ion conductivity. Column temperature was set at 25 °C. The injection volume was 20 µL, whereas total flow rate was 1 mL min^{-1}. Samples of 20mm phytic acid and 0.0625µM 6-phytase were prepared in stock. 3ml of 1M HCl was used to stop the reaction.

2.3.7 Mass spectrometry

Phytate (Phytic acid sodium salt hydrate from rice) made up to a concentration of 20 mM with UHP water. 6-phytase was sourced from DSM Nutritional Products Ltd (Basel, Switzerland) and was diluted to a working concentration of 0.0625 µM with UHP water.

MS analysis was performed on a MALDI Synapt G2-Si mass spectrometer (Waters, USA) with an ESI source. MS analysis was performed in negative ion mode and was tuned to the phytate ion (m/z 658.8). Spectra were produced for both phytate and phytase individually, as well as for the phytate-phytase reaction. For the

individual spectra, solutions were injected at a flow rate of 20 µL min^{-1}, with the source temperature set at 350°C and capillary voltage set to -2.4 kV. Cone gas (nitrogen) flow rate was 10 L h^{-1}, with desolvation gas flow rate at 700 L h^{-1}. Spectra were acquired as an average of 174 scans with a scan time of 1 second, over a mass range of 50-800 Da.

For the phytate-phytase reaction, the system was first purged with 0.0625 µM phytase for a period of 20 minutes at a flow rate of 20 µL min-1. The syringe containing 15 mL of 0.0625 µM phytase was then connected to an SP100i microprocessor-controlled syringe pump (World Precision Instruments, Hertfordshire, UK) set to an infusion flow rate of 10 µL min-1 with refill intervals every 20 minutes. To begin the reaction, 0.25 mL of a 20 mM phytate solution was pipetted into the phytase solution and was manually agitated for 5 seconds at room temperature. Simultaneously, the syringe pump was set to fill and begin infusion to the mass spectrometer. Source temperature was set to 350°C, with a capillary voltage of -2.4 kV. Cone gas, and desolvation gas flow rates were set to 10 L h^{-1} 1 and 700 L h^{-1} respectively. A total of 2490 scans were performed with a scan time of 1 second over a mass range of 50-800 Da.

2.3.8 SOFTWARE

Spectra were analysed using MassLynx™ V4.1 (Waters, USA). For analysis of the phytate-phytase reaction, total spectral counts were collected for peaks present at the m/z values of expected reaction products. Total counts for each inositol phosphate and their sodium adducts were summed to provide a total count value for each inositol phosphate at each scan interval. The reaction figure was

produced in Microsoft Excel (Microsoft Corporation, USA) by plotting total counts of each expected inositol phosphate (plus their respective sodium adducts) over time.

2.3.9 MATHEMATICAL CALCULATIONS

All calculations for the correlation of the findings of this chapter with the diet of monogastrics were done by estimating the percentages of the different feed components from principle, based on literature.

2.4 RESULTS AND DISCUSSION

2.4.1 POLYPHENOL EXTRACTION AND ANALYSIS

Three different varieties of sorghum were used for their extracts. Two red (MR-Buster & Cracka) and one white (Liberty) sorghum varieties were used for these experiments. The extracts from Liberty and Cracka were very similar in final mass of extract, as shown in Table 2.2, but both Liberty and Cracka extracts were almost half in size compared to MR-Buster's extract.

Table 2.12: Polyphenol-rich extract masses for all three sorghum varieties.

	Liberty	Cracka	MR-Buster
Initial mass	20g	20g	20g
Mass after de-branning	5.70g	7.12g	4.66g
Mass of extract	0.0292g	0.0334g	0.0504g
Mass of extract per gram of sorghum	1.46mg	1.67mg	2.52mg
Mass of polyphenols in a typical sow diet per day	0.511g	0.585g	0.882g

That could be due to the genetic differences of the three varieties, as well as to the method of extraction, as using the technique described in the Materials and Methods, only polyphenols with a smaller molecular weight would be extracted.

To have a better understanding of each extract, a total phenolic content (TPC), as well as UV-Vis and a FTIR analysis were performed on all samples. The TPC assay was performed using gallic acid as a standard. Using different concentrations of gallic acid against their absorbance at 765nm, a standard curve was created. Using this standard curve and knowing the absorbances of each sample diluted in liquid form, the concentrations of all samples in gallic acid equivalents were estimated. To back calculate the sample concentrations, the equation of the trendline of the standard curve (Figure 2.8) was used:

$$y = 8.81x \qquad (R^2 = 0.99253)$$

Gallic acid (or 3,4,5-trihydroxybenzoic acid) is a type of phenolic acid, commonly used for the determination of the phenolic content in samples. Its chemical formula is $C_6H_2(OH)_3COOH$ and it is usually found in plant products. With a molecular weight of 170.12 g/mol, is considered to be a small phenolic compound.

As shown in Figure 2.9, Cracka's extract had a significantly higher concentration of gallic acid equivalents compared to the other two extracts. The second higher was MR-Buster and Liberty had the lowest concentration of gallic acid equivalents, out of all three.

This result agrees with the literature, as the extracts from the two red sorghum varieties had higher concentration of gallic acid equivalent, compared to the white sorghum variety.

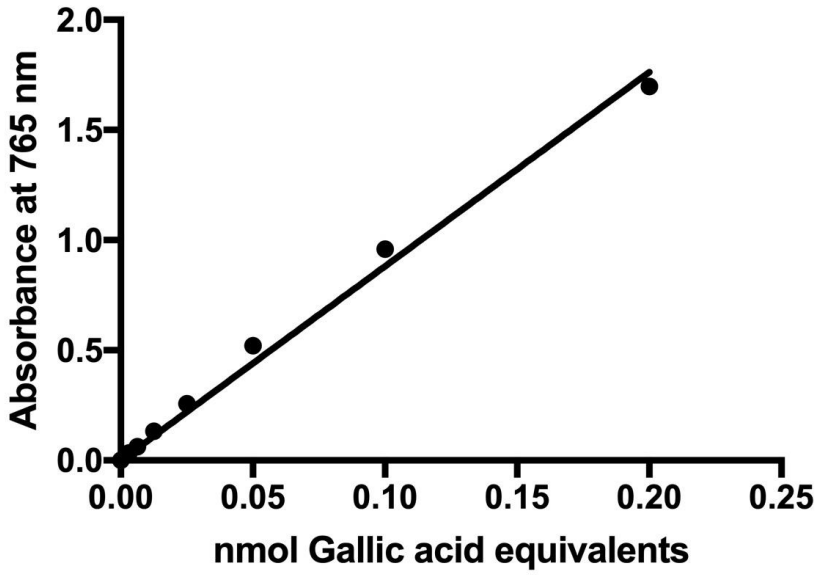


Figure 2.59: Standard curve of the absorbance at 765nm for different concentrations of gallic acid (0-0.2nmol).

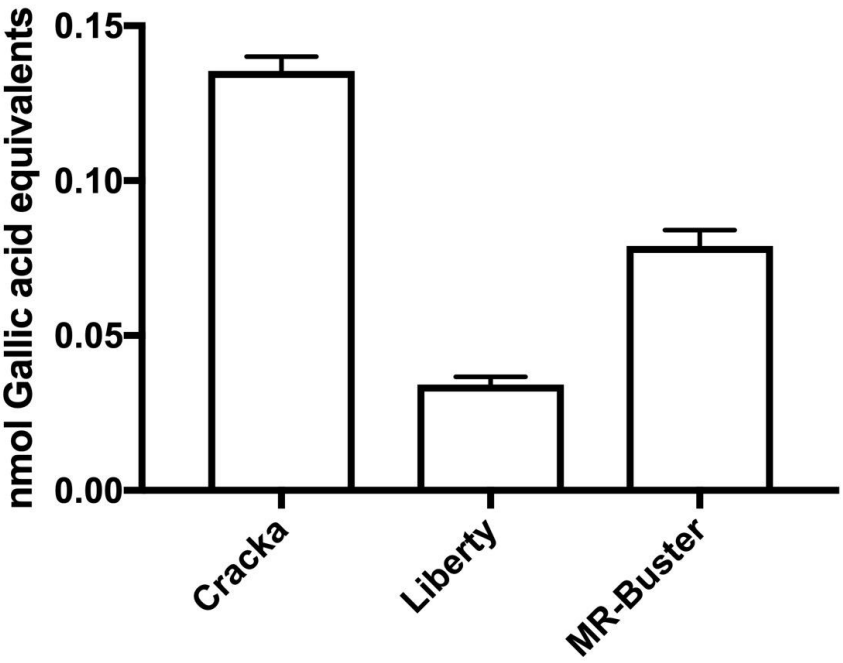


Figure 2.67: Total phenolic content of all three sorghum varieties in nmol of mg/ml gallic acid equivalent.

Table 2.21: Polyphenol-rich extract concentrations in nmol of gallic acid equivalents and their standard deviations for all three sorghum varieties.

Sorghum Variety	Concentration nmol (mg/ml gallic acid equivalent)	Standard Deviation
Liberty	0.034	0.002
Cracka	0.135	0.004
MR-Buster	0.078	0.005

Following the TPC assay, a UV-Vis analysis was performed on all three extracts and on Catechin, which was used as a standard. As shown in Figure 2.10, the

extracts from the red sorghum varieties have a very similar UV spectrum, which was expected. Their spectra consist of two wide peaks at around 290 and 490nm, as well as two smaller but steep peaks at around 310 and 365nm for MR-Buster and Cracka extracts respectively. On the other hand, the white sorghum extract has a different spectrum, with three distinct peaks, at around 210, 240 and 310 nm, which are not very distinct as they are overlapping. Finally, the catechin spectrum shows two peaks, one at around 220 and the other around 280nm. The initial peak of catechin is present in all three extracts and that

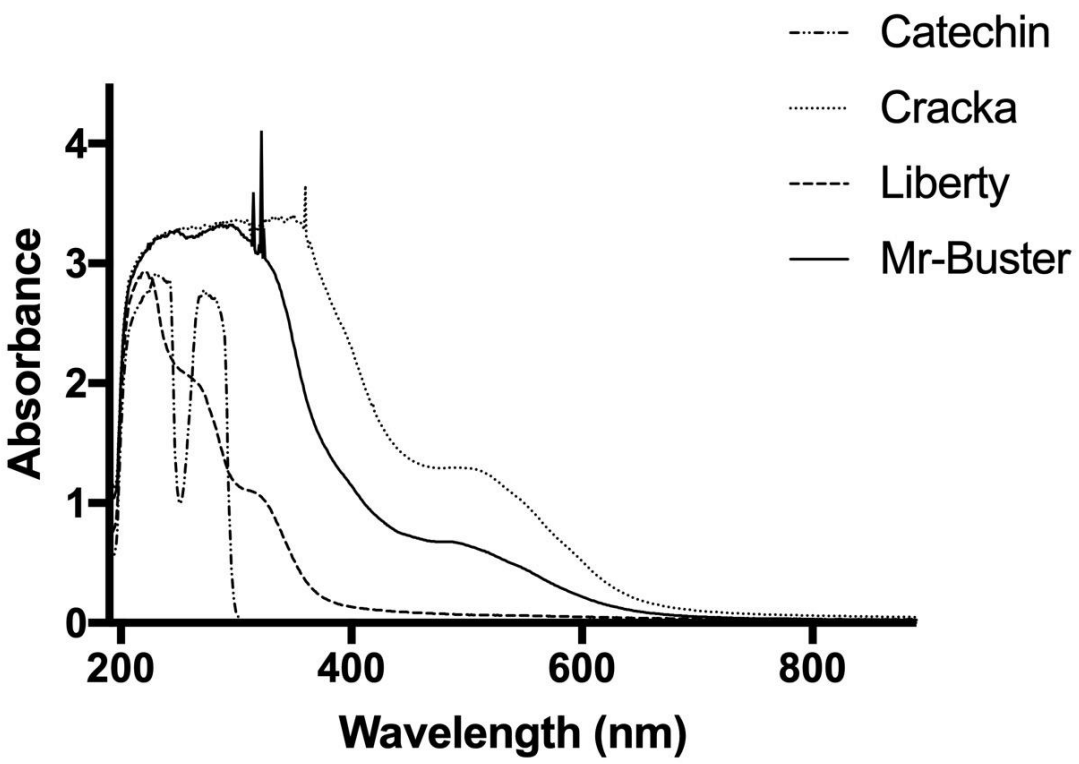


Figure 2.76: The UV-vis data comparing the polyphenol-rich extract data from the three different sorghum varieties (Cracka, Liberty and MR-Buster), to catechin (chosen as a standard).

Based on data published by Anouar *et al.* (2012), most polyphenol compounds, such as flavones, flavonols, anthocyanidins and many more appear in the UV-Vis spectrum between 249-546nm, which is the area where the three sorghum extract samples have most of their peaks. As shown in Table 2.4, which is modified from a table found in the work done by Anouar *et al.* (2012), the difference between the two red sorghum varieties and the white sorghum, depend on the type of polyphenol compounds that are present in each.

In the two red sorghum varieties, there are peaks even until 600nm, while the white sorghum peaks end at around 400nm. The interpretation of this data, using the information found in Table 2.4, is that there are no anthocyanidins in the white sorghum, while the presence of anthocyanidins in the red sorghum varieties is the one creating the peaks shown in Figure 2.10. Furthermore, catechin is expected to be present at 287nm that is validated in Figure 2.10.

Table 2.30: Experimental nm values for different polyphenol compounds (modified from Hassane Anouara et al., 2012).

Polyphenol compounds	nm
Flavones (i.e. chrysin, apigenin, luteolin)	313-349
Flavonols (i.e. galangin, kaempferol, quercetin)	359-370
Flavonones (i.e. eriodictyol, hesperetin, naringenin)	289-290
Flavan-3-ols (i.e. catechin)	287
Anthocyanidins (i.e. pelargonidin, cyanidin, delphinidin)	520-546
Isoflavones (i.e. daidzein, genistein, isoluteolin)	249-261
Chalcones (i.e. 4',4-diOHchalcone, isoliquiritigenin)	348-370

Comparing the UV-Vis data from Figure 2.10, to data shown in research done by Ramos-Tejada *et al.* (2002), it is clear that the spectra obtained by the analysis of catechin is accurate, as it is identical to the one, they have produced. Furthermore, Ramos-Tejada *et al.* (2002) also performed an FTIR analysis on the catechin and the same peaks shown in Figure 2.11 are present in their data.

On top of the UV-Vis analysis, an FTIR analysis of the three sorghum extracts as well as catechin was performed. As shown in Figure 2.11, all three extracts follow a similar, but not identical pattern, while catechin has a different pattern, but yet has some similarities with the three extracts. Both UV-Vis and FTIR analysis were performed in a more qualitative rather than quantitative way, to get a rough understanding of the extract quality.

Table 2.35: FTIR vibrational bands from 6 green tea varieties (modified from Senthilkumar et al., 2018)

Wave number (cm^{-1})	Vibration band/group	Chemical compound
3270 ~ 3320	O–H stretch, H–Bonded	Phenols, alcohols
2946	C–H stretch (asym.) O–H stretch	Alkanes Carboxylic acid
2833	C–H stretch (sym.)	Alkanes
1629 ~ 1663	C=O stretch (carbonyls)	Flavonoids Polyphenols, catechins
1449	C–C stretch (in ring)	Aromatics
1239	C–N stretch	Aliphatic amines
1113	C–O stretch	Alcohols, esters, carboxylic acids
1014 ~ 1019	C–O stretch	Alcohols, esters, carboxylic acids

Comparing the peaks present in the FTIR data obtained from the analysis of the three sorghum extracts and the catechin standard, to published data, it is obvious that the peaks around 3300-3400 cm^{-1} show the presence of phenols and/or alcohols. Furthermore, the peaks at around 1600 cm^{-1} are related to polyphenols and flavonoids as well as catechins. Such peaks are present in all four samples, all three sorghum extracts and the catechin standard. These comparisons are made with the data shown in Table 2.5, published by Senthilkumar *et al.*, (2018), where they analyse the phenolic content of six green tea extracts.

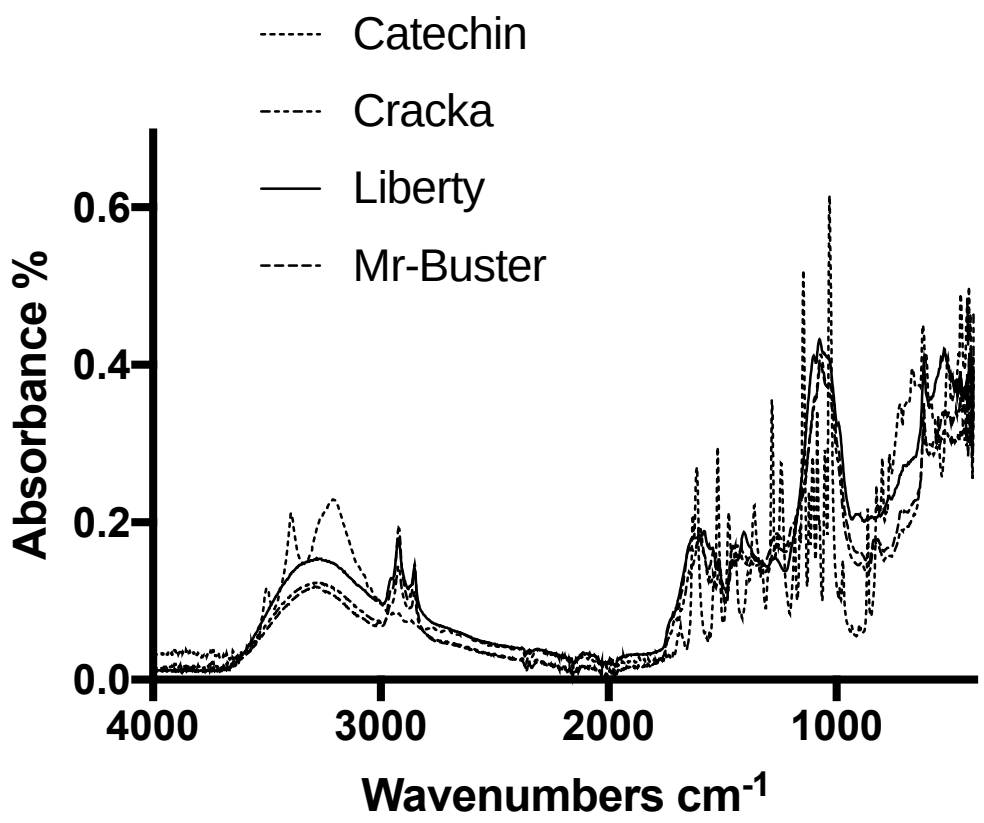


Figure 2.85: FTIR data comparing the polyphenol-rich extract data from the three different sorghum varieties (Cracka, Liberty and MR-Buster), to catechin (chosen as a standard).

Although the source of the phenolic compounds is different, sorghum and green tea, since the structure of the compounds is very similar, so should be their FTIR vibrational bands.

While that was the correlation to green tea polyphenolics bands, an FTIR Spectrum for *Allium sativum*, created by Trifunschi *et al.* (2015), also allows the comparison of the bands shown in Figure 2.11, to their findings. Peaks at 3339cm-1 and 2979cm-1 are specific for OH group and for the CH2 symmetric frequency. Those peaks are both present in the FTIR data from the sorghum extracts as well as the analysis of the *Allium sativum*. Furthermore, peaks around 1384cm-1

correspond to the OH phenolic group, again present in both Figure 2.11 and the analysis performed by Trifunschi *et al.* (2015).

Finally, comparing the data in Figure 2.11, to the data shown in the research by Harwansh *et al.*, (2016), it is clear that the catechin standard data is identical in both pieces of work.

2.4.2 ITC ENZYMATIC ACTIVITY ASSAY

2.4.2.1 OPTIMIZATION

Initially a four-injection experiment was performed to estimate the reproducibility of the technique. As shown in Figure 2.12, each of the three last injections has an almost identical shape to the rest, with a small decrease on the steep peak, due to less energy originating from dilution (heat of dilution), as the inositol phosphates (with 0-6 phosphate groups) concentration in the cell becomes higher after each injection.

This preliminary experiment also showed the expected complexity of the reaction between phytic acid and 6-phytase, which was also mentioned by Ariza et al. (2013). To have a better understanding of the reaction of the 6-phytase that is being used for these experiments, anion exchange chromatography that is described in Ariza *et al.* (2013)'s paper was replicated with but with no success.

As a final resort, I had the idea of performing a real time mass spectrometric analysis of the enzymatic reaction, using the same concentrations, but on a larger scale (See below: Section 2.4.2.2).

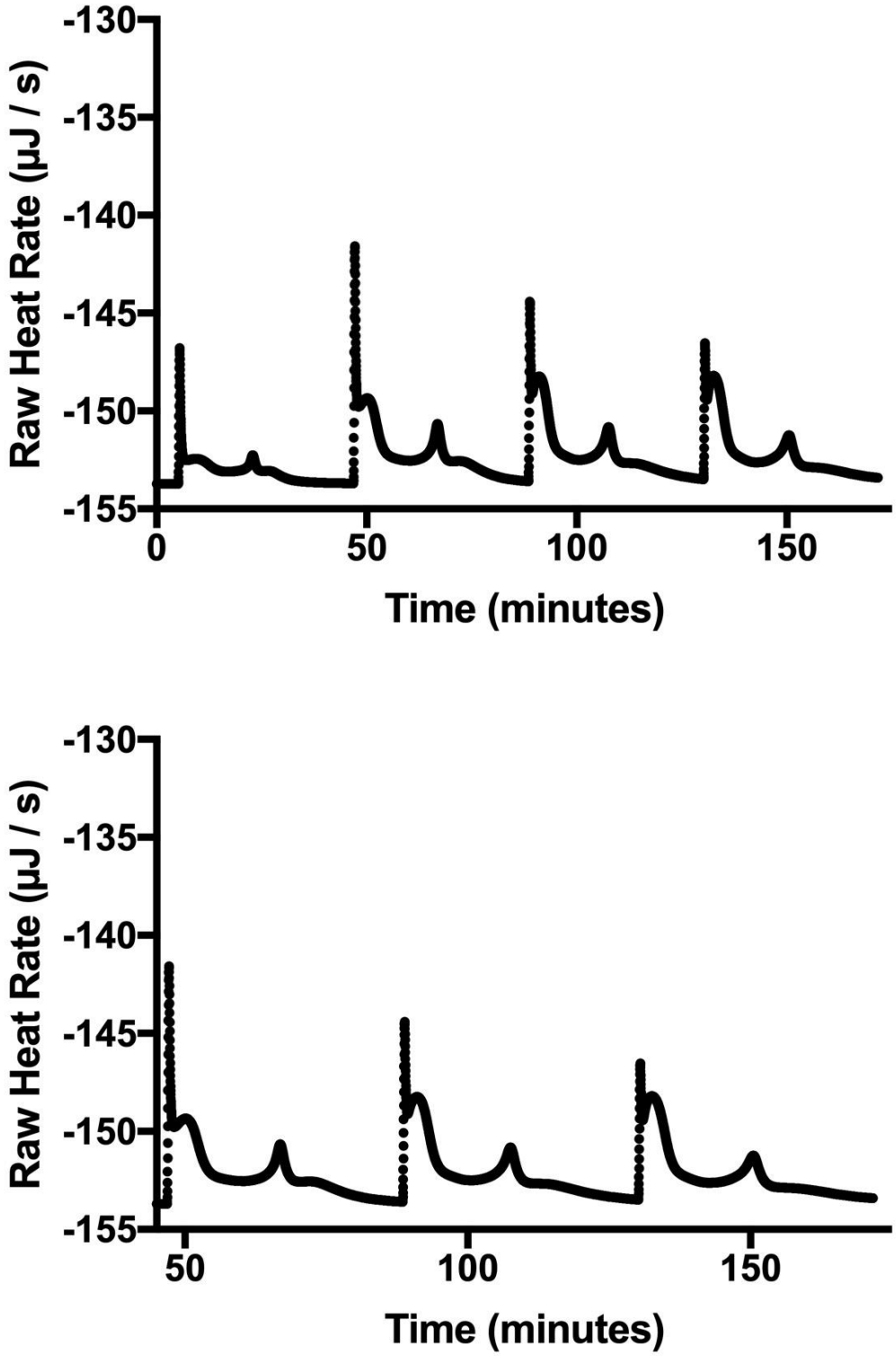
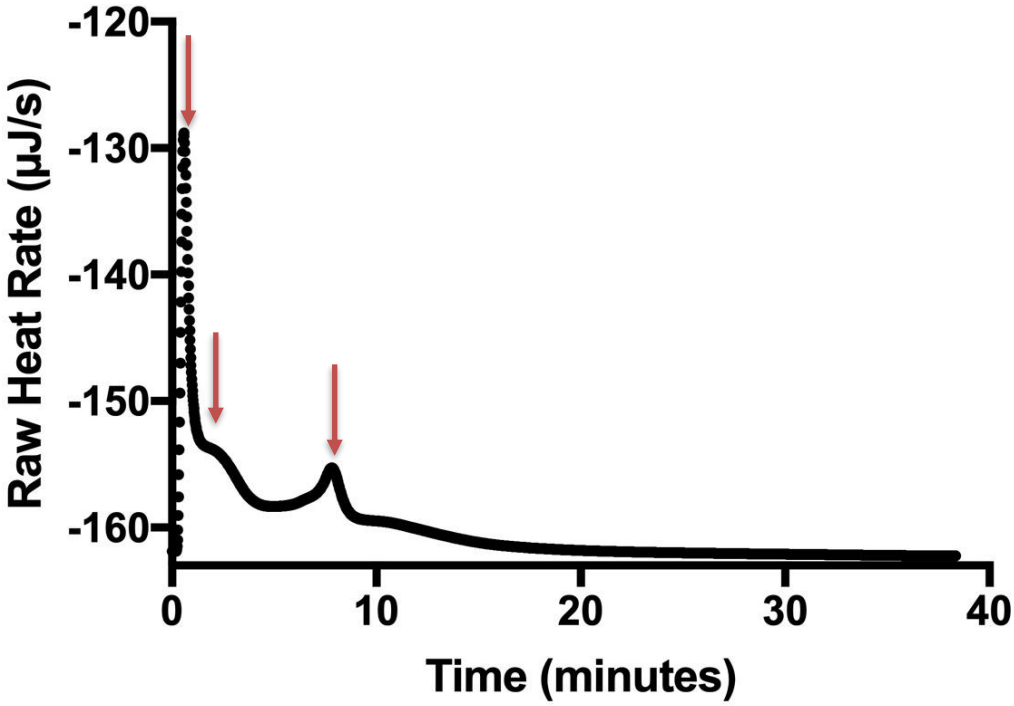


Figure 2.12: Injection of 5 µL of 20mm phytic acid into 0.0625 µM of 6-phytase. (A). Initial 4 injection experiment, with long intervals between injections to estimate the reproducibility of the reaction and (B) the same reaction, focusing on the latter 3 peaks of interest.

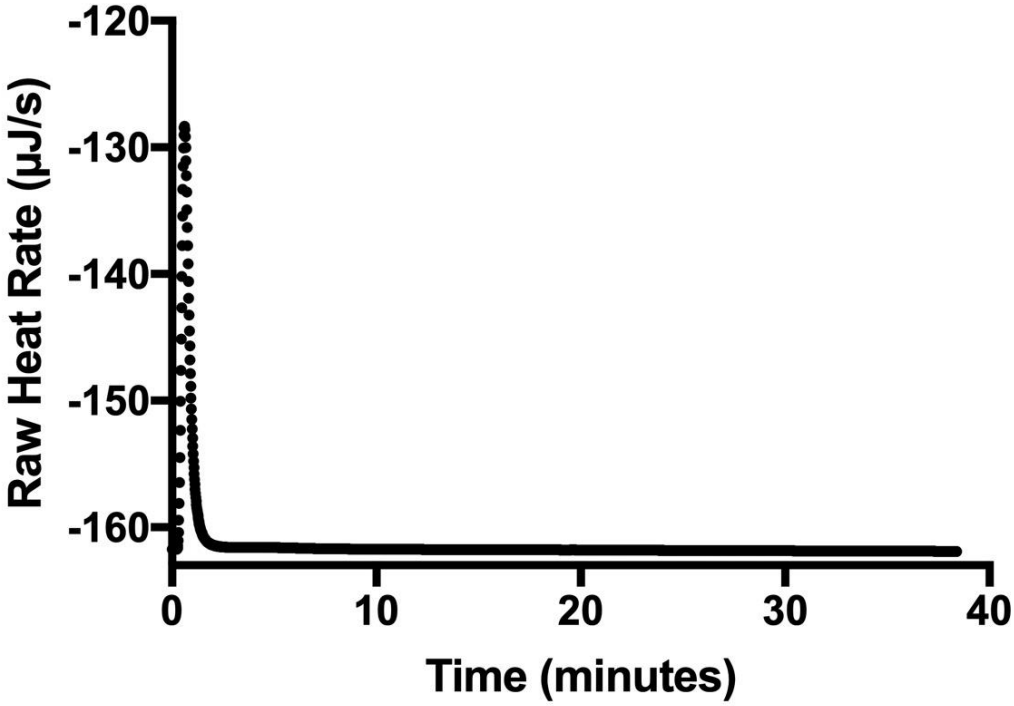
The phytase thermogram is different to a standard thermogram generated by an enzyme that catalyses a single reaction. A single, one-stage reaction, starts with maximum reaction rate and is followed by a decrease on the reaction rate as the substrate is being catalysed, following Michaelis–Menten kinetics. There are at least three peaks shown in the thermogram of the reaction, one of which is the heat of dilution. Therefore, there are at least two well-separated stages of this reaction, since the second peak appears 20 minutes after the initiation of the reaction. This is probably due to a bottleneck in the reaction pathway flowed by a step with rapid kinetics.

Since the shape of the peaks is uniform, different concentrations of phytase and phytic acid were used to find the optimal concentration for this assay. After consideration, 0.125μM of phytase and 20mm of Phytic acid were selected to be the concentrations used for the further experiment, as the thermograms obtained at that concentration were providing the most information as the peaks were most obvious. Figure 2.13A shows the reaction of phytase with phytic acid, as well as the injection of phytic acid in a cell containing 5% ethanol, which will show the heat of dilution (Figure 2.13(B)). In order to analyse the enzymatic activity of the phytase in the presence of different concentrations of all three polyphenol-rich extracts, the 90% of the completion of each reaction was estimated for all reactions, with and without the presence of the polyphenol-rich extracts.

For every calculation, the heat of dilution was deducted from the reaction's energy, and then the time for the 90% of the completion of the reaction was calculated.



(A)



(B)

Figure 2.13: (A) Injection of 5 µL of 20mm phytic acid into 0.125 µM of 6-phytase (red arrows indicating the three peaks) and **(B)** injection of 5 µL of 20mm phytic acid into MilliQ water (blank).

2.4.2.2 Ion Exchange Chromatography and Mass Spectrometry Analysis

As shown in Figure 2.14, different concentrations of phytate were easily identified by HPIC, which suggests that the products of the phytate-phytase reaction (IP0-IP6) could also be identified. Doing this for each time point of the reaction would increase the precision of the de-convolution of the ITC data and help identify all the different stages of the reaction.

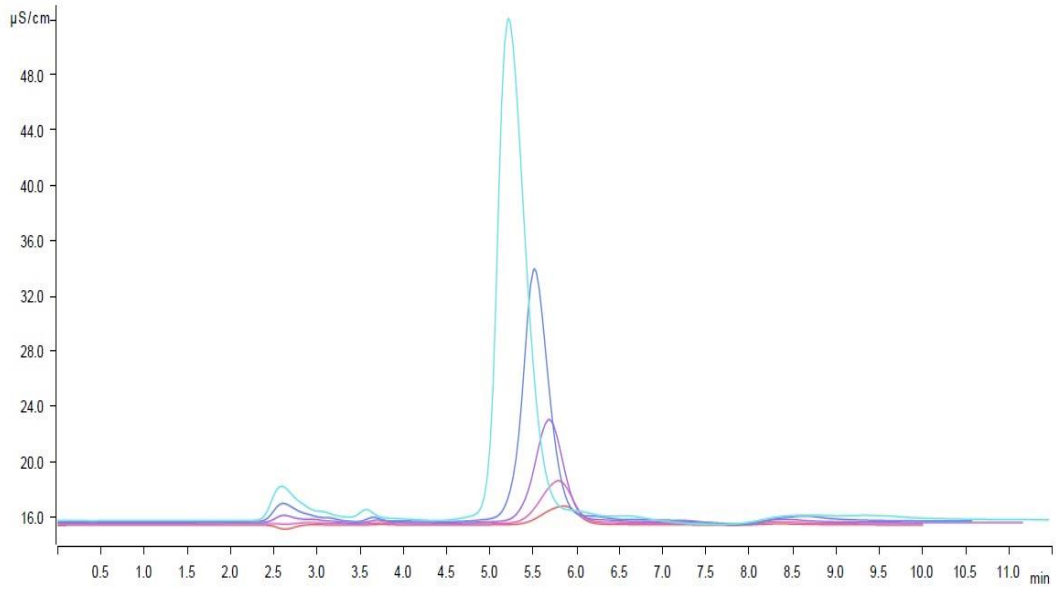


Figure 2.87: The profile of five 1:1 dilution of phytate (100-6.25ppm) using HPIC. 100ppm (light blue), 50ppm (dark blue), 25ppm (purple), 12.5ppm (red) and 6.25ppm (pink).

Although the peaks for the different concentrations of phytate where very distinct (Figure 2.14), when the reaction was taking place and 3ml of 1N HCl where added to the mixture to stop the reaction at different time points, HCl produced a huge peak that covered every other peak present.

After low success of ion chromatography, tried to change some parameters, but still the problem was not resolved. Initially, changed reaction terminator to heat, instead of HCl. No difference in peaks between 0, 10 and 20 minutes observed. Then, changed eluent from medium to low and used a higher molarity of Na_2CO_3, but still there was no difference in the resolution.

Unfortunately, due to the data quality of the datasets the de-convolution of the Ion Exchange chromatography was not possible. Therefore, the following experiment of real time mass spectrometric analysis of the phytase phytic acid was carried out.

As shown in Figure 2.15, the enzymatic reaction of phytic acid and 6-phytase follows a specific pattern. The degradation of IP6 to IP5 is almost instant. The initial higher amount of IP5 compared to the amount IP6 present in the solution, is probably due to a 1,3-hydride type substitution, originating from a spontaneous ionisation within the cone voltage. From minute 2, IP4 increases and when that starts to decrease, IP3 starts rising. At 5 minute there is a mixture of IP5, IP4 and IP3, all competing for binding to the catalytic site. IP2 and IP1 start taking off after IP is almost completely degraded, but the data for that part of the graph is not very clear as it contains noise, due to the optimisation of the technique for the larger, rather than the smaller sized inositol molecules.

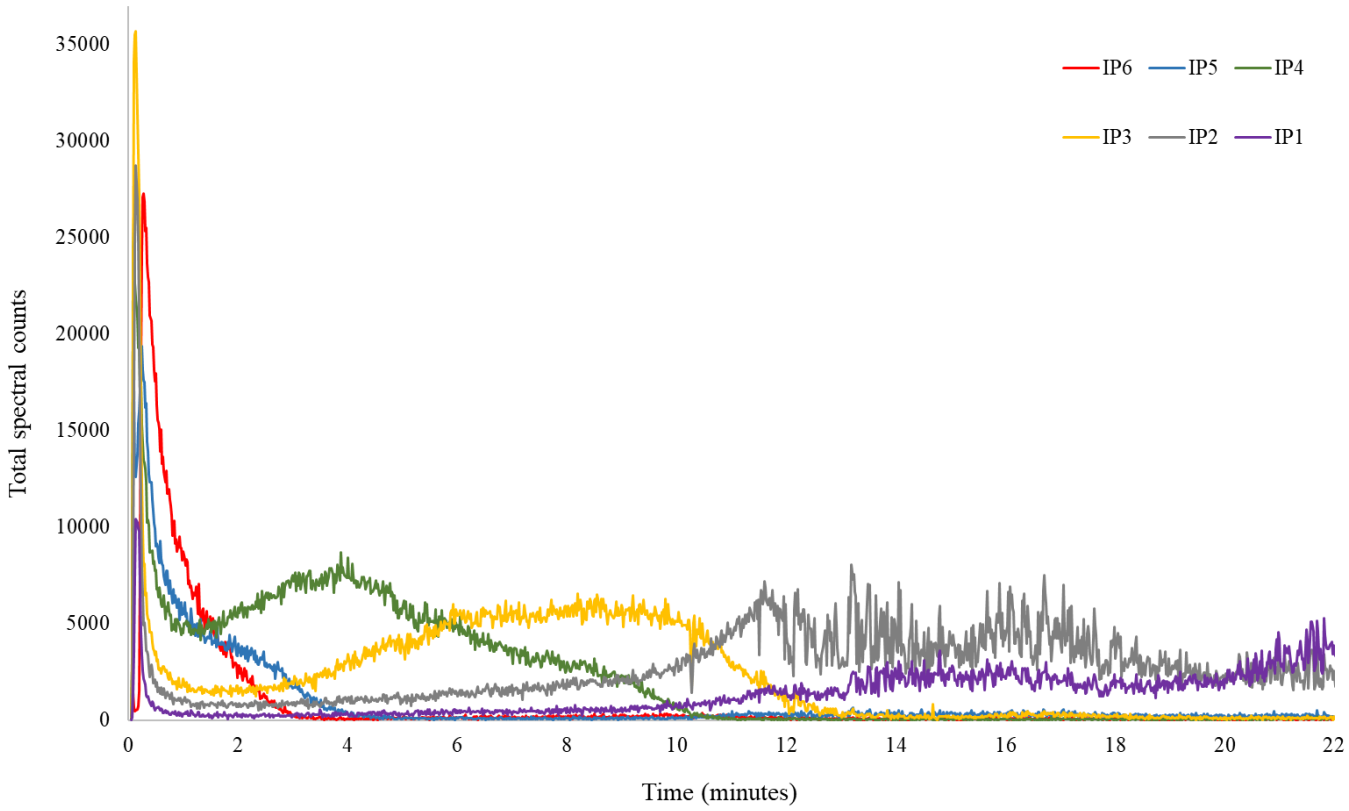


Figure 2.96: Mass spectroscopy analysis over 0-20 minutes showing m/z ratios of expected inositol phosphate (IP) molecules for a single addition of 5 µL 20mM phytate into 0.0625µM phytase. The figure is the sum of intensities for up to two sodium adducts (eg. IP6 + IP6Na + IP6Na2). Red line – IP6, Blue line – IP5, Green line – IP4, Yellow line – IP3, Grey line – IP2 and Purple line – IP1.

Further information and analysis of this data will be found on Niall Bradshaw's thesis, as he will continue this piece of work. For the purpose of this thesis, this mini experiment proves that the phytase has a specific pattern of degrading phytic acid, as expected.

2.4.2.3 INTERPRETATION OF THE PHYTASE-PHYTIC ACID THERMOGRAMS

To study the kinetics of the phytic acid digestion by the 6-phytase, using ITC, a 20 mM sodium phytate was placed into the syringe and 32.6 FYT/mL of phytase into the sample cell (One (FYT) is the amount of enzyme which releases 1 mmol of inorganic phosphate/minute from a 0.0051 M Na-phytate solution, at pH 5.5 and 37 ˚C.). To do so, an initial 2 μL injection was performed, followed by a 5 μL injection. The initial injection of 2 μL is always disregarded because it contains errors, as it is recommended by the AT Instruments, the manufacturers of NanoITC. The signal caused by the thermal power was measured (Figure 2.13A) and as shown in Figure 2.13B, the initial spike is identified as the heat of dilution caused by the injection of phytic acid into the cell containing water. Following that initial peak, there is another peak, which reached its maximum peak at around 2 minutes and then slowly declined. This second peak is associated with the degradation of phytic acid by the 6-phytase. Unexpectedly, at around 20 minutes after the second peak, there was a smaller peak between 40-50 minutes into the reaction.

This complex thermogram obtained by the reaction of phytic acid and 6-phytase is quite unlike the standard enzymatic thermogram. The fact that the thermogram is not consisted of a maximum reaction rate, followed by a decline in the reaction rate, caused by the shortage of the substrate, which is what is happening in simple enzymatic reactions, is not allowing the standard Michaelis-Menten enzymatic kinetic analysis (Olsen, 2006).

The presence of three peaks, instead of one, makes this reaction differ from the rest. Simple reactions are the ones that have been studied in the past using ITC, as

they are easily interpreted. A reaction like this is complex, as it has different stages, some of which are obvious, and some are not. It is known that the phytase is breaking down phytic acid with different patterns and it goes from one phosphate group to the other. That explains the different peaks shown in the thermogram. Despite that, there are 6 phosphate groups, so it would be expected that there should be 6 peaks present in the thermogram, since the reaction is reaching completion. That is not shown in the thermogram, as some of the different stages of the reaction are done close to each other and they are piling together into bigger peaks. The best way to interpret this reaction, regarding the second peak in heat generated at around 20 minutes, is that there is a bottleneck in the pattern of this reaction pathway followed by a final step during which there is a rapid change in the reaction's kinetics. A closer look into this reaction and specifically into the way that phytase is functioning would provide a better understanding of this complex thermogram.

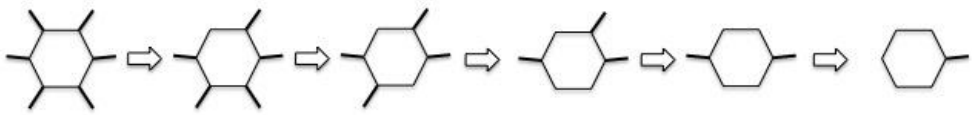
Most phytases belong into the bigger group of histidine phosphatases. The way that they function is that they are breaking the bond between the inositol ring and the phosphate group. This is a two-step process, as the histidine in the active site of the phytase causes a nucleophilic attack on the phosphorous atom in the scissile phosphate creating a phospho-histidine intermediate (Vincent and Kimura, 1992; Wo *et al.*, 1992).

The way in which the phytase is binding on the phytate has been previously studied using an Escherichia coli phytase, which is very similar to the one used in these experiments. Using X-ray crystallography, it was understood that the His 17 required for the formation of the phospho-histidine intermediate was mutated to

an alanine. The phytate in the binding site did not fully occupy the binding pocket (Lim *et al.*, 2000). The scissile phosphate is guided to its position in the catalytic site by Arg 16, Arg 30 in the conserved RHGXRXP sequence at the active site, along with conserved Arg 92, His 303 and Asp 304. His 17 has an electron pair that undergoes nucleophilic attack on the scissile phosphate forming a covalently linked phospho-histidine intermediate. In that way the remainder of the phytate is released from the binding site and the phospho-histidine intermediate is hydrolysed in order the phosphate to be separated from the histidine (Harbertson *et al.*, 2014). The phosphate groups are cleaved from the inositol ring in a specific way, depending on the phytase each time. Each phytase has a preferential way in which it cleaves the phosphate groups, but there are always secondary paths followed too (Ariza *et al.*, 2013; Sun *et al.*, 2017). As shown in Figure 2.15, an analysis of the reaction between *Citrobacter braakii* 6-phytase and sodium phytate done using mass spectroscopy revealed that the sequence of phytate degradation from phytate (IP6) down to myo-inositol monophosphate (IP1).

During the degradation of phytate, the 6-phytase firstly works on the IP6 molecule in order to remove the first phosphate group from it. The association constant for this first step of the reaction is Ka6.1. This association is dominated by the electrostatic attraction that takes place between the positive charge of the active site of the phytase and the strong negative charge on the phytic acid which is due to the phosphate groups that are attached to the inositol ring. When this reaction initiates, the enzyme is attached to the active site of the IP6 and then the intermediate phospho-histidine is created and then the myo-inositol pentakisphosphate (IP5) is released. When the IP5 is released back to the solution, the phosphate molecule is free and has dissociated from the phytase with a Ka6.2

association constant. The combination of these two association constants would give the combined association constant k_6, which is considering both parts of the reaction to change IP6 to IP5.



IP6 IP5 IP4 IP3 IP2 IP1

$$\text{Phytase} + \text{IP6} \overset{K_{a6.1}}{\Leftrightarrow} \text{Phytase:IP6} \overset{k_6}{\Rightarrow} \text{Phytase:Pi} + \text{IP5} \overset{K_{a6.2}}{\Leftrightarrow} \text{Phytase} + \text{IP5} + \text{Pi} \overset{K_{a5.1}}{\Leftrightarrow} \text{Phytase:IP5} + \text{Pi} \overset{k_5}{\Rightarrow}$$

$$\text{Phytase:Pi} + \text{IP4} + \text{Pi} \overset{K_{a5.2}}{\Leftrightarrow} \text{Phytase} + \text{IP4} + 2\text{Pi} \overset{K_{a4.1}}{\Leftrightarrow} \text{Phytase:IP4} + 2\text{Pi} \overset{k_4}{\Rightarrow} \text{Phytase:Pi} + \text{IP3} + 2\text{Pi} \overset{K_{a4.2}}{\Leftrightarrow}$$

$$\text{Phytase} + \text{IP3} + 3\text{Pi} \overset{K_{a3.1}}{\Leftrightarrow} \text{Phytase:IP3} + 3\text{Pi} \overset{k_3}{\Rightarrow} \text{Phytase:Pi} + \text{IP2} + 3\text{Pi} \overset{K_{a3.2}}{\Leftrightarrow} \text{Phytase} + \text{IP2} + 4\text{Pi} \overset{K_{a2.1}}{\Leftrightarrow}$$

$$\text{Phytase:IP2} + 4\text{Pi} \overset{k_2}{\Rightarrow} \text{Phytase:Pi} + \text{IP1} + 4\text{Pi} \overset{K_{a2.2}}{\Leftrightarrow} \text{Phytase} + \text{IP} + 5\text{Pi}$$

Figure 2.104: The sequence of association, cleavage and disassociation shown for each step of the reaction between phytase and phytic acid.

In the same way, the association constants are calculated until IP6 is left with only one phosphate group attached to it (IP1). This procedure is shown in Figure 2.16 and the association constants are shown for each step of the enzymatic reaction. Since the negative charge of the phytic acid is due to the phosphate groups attached to it, for every step of the reaction, there is one less phosphate group attached on the inositol ring and therefore, the degradation products' association constants are reduced for every step of the reaction.

$$K_{a6.1} > K_{a5.1} > K_{a4.1} > K_{a3.1} > K_{a2.1}$$

As the degradation of the phytic acid by the phytase is a complex, multiple stage reaction, there are times during the reaction, when there are multiple products of the reaction present in the solution. As this is also indicated by the data obtained by the mass spectrometry experiment (Figure 2.16), at around minute 5, there is definitely IP5, IP4 and IP3 present all at the same time. During that time, all three products are competing for binding to the catalytic site of the enzyme, but because the negative charge on the IP5 molecule is higher than the one on IP4 and respectively that has a higher negative charge compared to IP3, the reaction is also following that sequence. Therefore, by the time that most of the IP5 is degraded, there will be a plethora of IP4 in the solution, but at the same time IP4 should have already started being degraded by the phytase and therefore IP3 should also be present in the mixture. When the whole amount of IP6 is degraded to IP1, the reaction probably stops, as due to the fact that the association constant of IP1 is much lower than the previous by-products of this reaction (IP5, IP4, IP3 and IP2), there is a high probability that it is so weak that it does not allow it to displace the phosphate that is attached to the phytase and therefore initiate its degradation.

As explained above, the complexity of this reaction is not allowing the standard Michaelis-Menten kinetics analysis. To overcome this limitation and use the useful data obtained by this experiment, to study the enzymatic kinetics, the decision was to use an innovative way of analysing this data. To do so, the complexity of this reaction was not considered, and simply the time point of a percentage of

completion of this reaction was chosen (90% in this case). Then the rate of the reaction for the time needed for the 90% of its completion was calculated.

$$Rate \sim 1/t_{90\%}$$

This method is providing enough information for the relative estimation of the increase or decrease of the enzymatic activity. In that way, the activity of the enzyme can be estimated under different conditions. Either that is the presence of another organic substance, a change in temperature, a change in pH, or even different concentrations salts present in the solution, the effect of all these conditions on the enzymatic activity can be estimated.

This is a novel way of interpreting the enzymatic kinetics of complex/multiple stage reactions using ITC, when unable to make exact estimations of the ΔH, Km and Vmax values. Estimating the relative activity of the enzyme using ITC and comparing it for different conditions can be used as a valid and accurate technique for measuring the kinetics of such reactions. Furthermore, if and when these results are backed up by another technique, such as Ion Exchange chromatography, or Mass spectroscopy, ITC can be one of the most powerful techniques for this type of analyses.

2.4.2.4. INHIBITION OF PHYTASE BY SORGHUM POLYPHENOLS

On this occasion, the effect of polyphenolics compounds, extracted from sorghum, on the phytase activity was estimated using the method described above. The way this method was used for this specific experiment is described in greater detail

below. This method was applied to the reaction between phytase and phytic acid in the presence of the three different polyphenol-rich extracts in different concentrations. Figure 2.18 shows the thermograms obtained for the reaction of phytase and phytic acid, in the presence of different concentrations of Liberty polyphenol-rich extracts, as well as the blank. The thermograms show the heat change after the subtraction of the blank. After deducting the blank from each thermogram, the time point for the completion of the 90% of the reaction was calculated. Figure 2.17 shows how the relative activity is calculated.

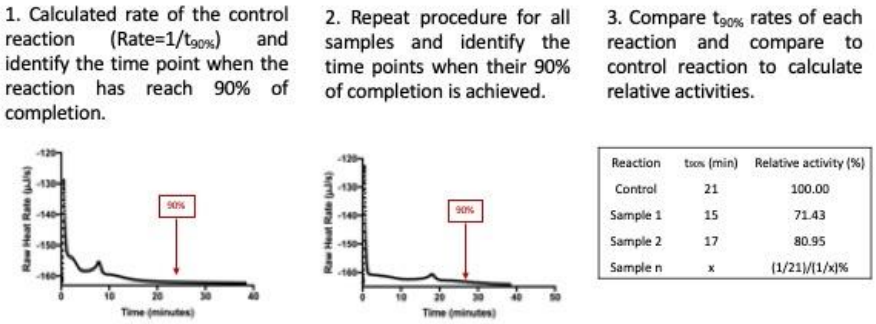


Figure 2.108: Indicating how the relative activity is calculated.

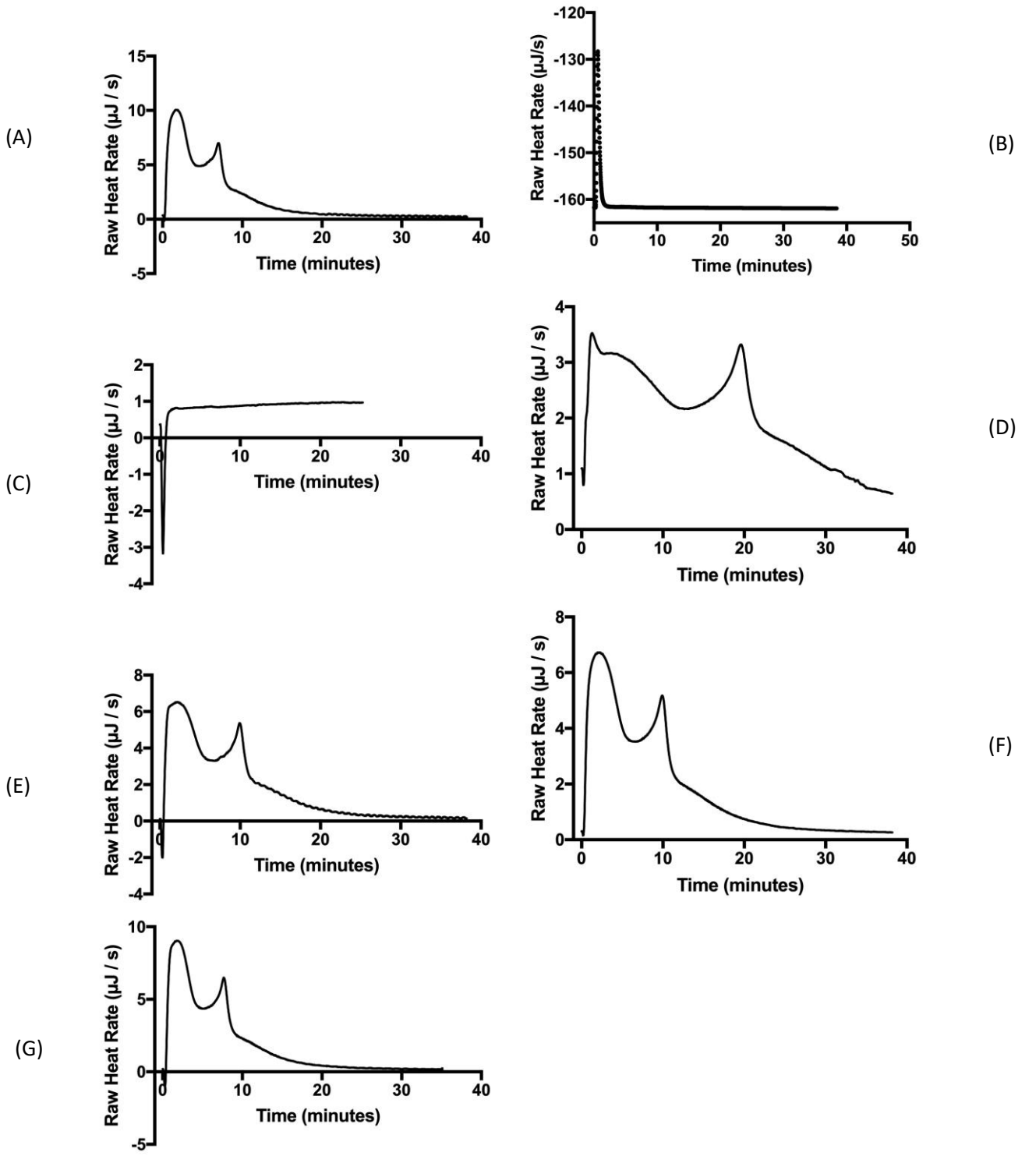


Figure 2.118: The raw heat rate of (A) phytic acid-phytase reaction, (B) phytic acid into 5% ethanol, phytic acid-phytase reaction with (C)0.109, (D)0.054, (E)0.028, (F)0.014 and (G)0.007 mg/ml polyphenol-rich extract from Liberty sorghum.

The same procedure was performed for each different extract. Each experiment was conducted in triplicates and the results for each sorghum variety are shown below in Figures 2.19, 2.20 and 2.21. Finally, the comparison of the different effect of each extract on the enzymatic reaction is shown in Figure 2.22.

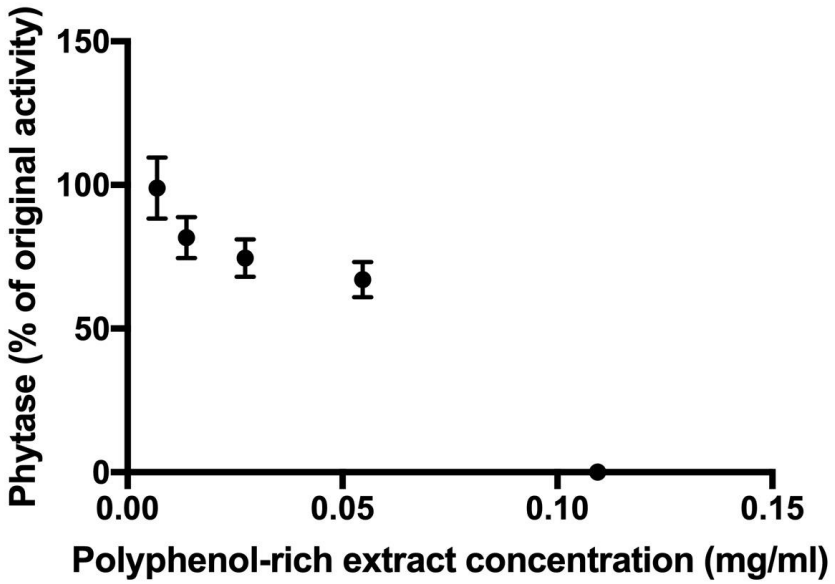


Figure 2.122: The relative reduction in phytase activity as measured by ITC in the presence of acetone extracts from the sorghum varieties Liberty.

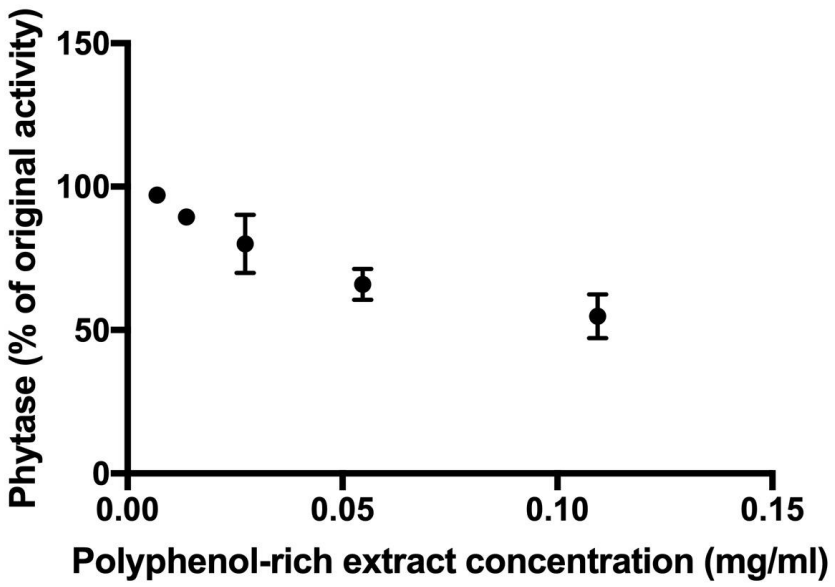


Figure 2.123: The relative reduction in phytase activity as measured by ITC in the presence of acetone extracts from the sorghum varieties MR-Buster.

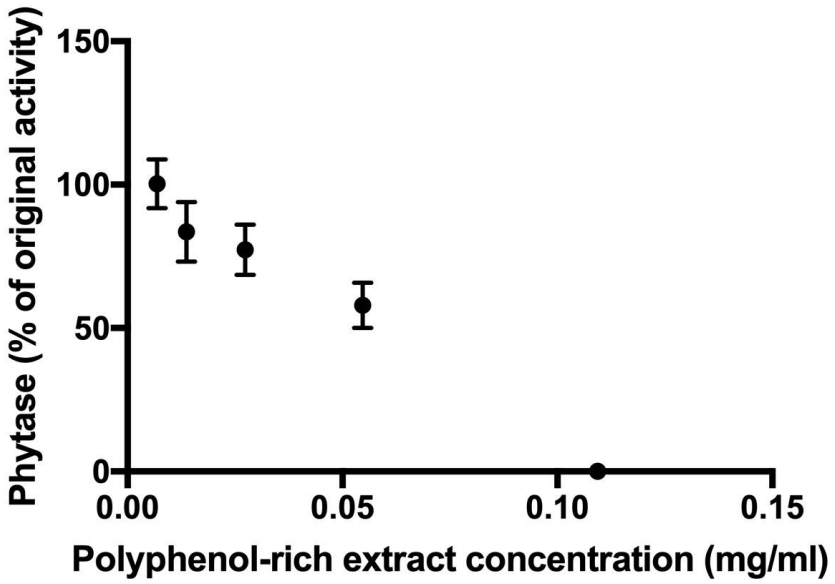


Figure 2.132: The relative reduction in phytase activity as measured by ITC in the presence of acetone extracts from the sorghum varieties Cracka.

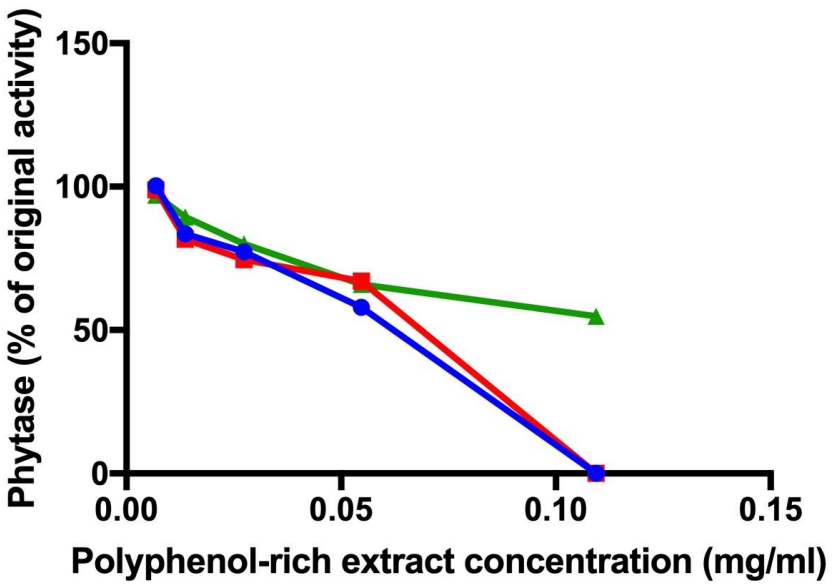


Figure 2.133: The relative reduction in phytase activity as measured by ITC in the presence of acetone extracts from the sorghum varieties (Blue) Liberty, (Red) Cracka and (Green) MR-Buster.

Extracts from both Liberty and Cracka sorghum at the concentration of 0.109mg/ml had a completely inhibitory effect on the phytase – phytic acid reaction, while the extract from MR-Buster sorghum inhibited the reaction to 45.3% compared to the control. Although this is a very clean environment with only a handful of substances that can react with each other, polyphenols have a great effect on the enzymatic activity as expected.

Despite the fact that Liberty is a white sorghum, which has a lower number of flavonoids, hence the lack of pigmentation, the effect of its extract on the phytase activity is as great as the extract from Cracka, a red sorghum, with a higher percentage of flavonoids. This essentially could mean that smaller polyphenolic compounds are probably also inhibiting the enzymatic activity, as much as flavonoids do. Despite that, inhibition is usually related to the specific phenolic

compounds in extracts rather than total quantity, as inhibition is not necessarily related to size.

To understand better what are the specific substances that are inhibiting the phytase, an analysis to identify the composition of each extract should be performed, in order to allow us to identify the phenolic compounds with the greatest inhibitory effect on the reaction.

According to previous studies, the phenolic profile of the white-grained sorghum genotype hybrid line Liberty was much simpler compared to red-grained sorghum genotypes (Wu et al., 2017). Liberty had the lowest amount of flavonoids and caffeic acid compared to other red and black sorghum varieties, while there were no 3-Deoxyanthocyanidins or flavones (apigenin. taxifolin and naringenin) found. At the same time, phenolic and flavonoid fractions, were found in percentages up to 50%, which indicates that total phenolic contents may be underestimating its phenolic content (Wu, 2017). MR-Buster compared to 3 other sorghum varieties (two white and one red), was found to have the highest concentration of total phenolic compounds (4.13 g CE (Catechin)/100 g). Further HPLC analysis showed that catechin, rutin, hesperidin, fisetin, quercetin and naringenin were all present in the MR-Buster extract (Plessis, 2014).

Table 2.39: Percentage of inhibition of different concentrations of the polyphenol-rich extract from three sorghum varieties on the activity of phytase.

	Polyphenol-rich extract Concentration (mg/ml)				
	0.007	0.014	0.027	0.055	0.109
Liberty	0.0%	16.4%	22.7%	52.1%	100%
Cracka	1.2%	18.4%	25.5%	32.9%	100%
MR-Buster	3.0%	10.5%	20%	34.1%	45.3%

2.4.3. APPLICATION OF RESULTS IN MONOGASTRIC ANIMAL NUTRITION

The amount of sorghum present daily in a sow's diet as shown in Table 2.1 is roughly 350 g. Using the extraction information in Table 2.2, the amount of polyphenol-rich extract present in a sow's daily diet would be 0.511 g for Liberty, 0.585 g for Cracka and 0.882 g for MR-Buster. In a typical sow's diet, phytase is being added at the ratio of 50000 FTU/kg (One FTU is the quantity of enzyme that can release 1 μmol of inorganic phosphorus/minute from 5 mM sodium phytate at pH 5.5 and 37 ◦C). In that way, 125000 FTU of phytate would be used for the 2.5 kg of feed. That would translate in 60mg of phytase per kg of feed and therefore 0.15 g in 2.5 kg of feed.

Having an analogy of 0.15 g of phytase and 350 g of polyphenols and in 2.5 kg of feed, translates into having the following ratio of phytase and polyphenols:

$$\frac{phytase}{polyphenols} = \frac{0.15\ g}{(extraction\ contant)\ g}$$

Since the phytase concentration used for the experiments is 0.000203 g per ml, and the polyphenol concentration varies from 0.000109 to 0.000013 g per ml. The calculated ratios of phytase against the different concentrations of polyphenols are shown in Table 2.7.

Table 2.40: Phytase against polyphenols ratios for all sorghum varieties in sow diet (20% sorghum).

Sorghum Variety	Liberty	Cracka	MR-Buster
Grams of phenol per kg sorghum	1.46	1.67	2.52
Phytase / Polyphenols Ratio	0.29	0.26	0.17

Looking at Table 2.8, it is obvious that the highest concentration of the polyphenol-rich extract used (0.109 mg/ml) for the experiments has a ratio of phytase against polyphenols 15-26 times higher than the ratios in the typical sow's diet.

In a chicken's diet instead, assuming that 100% of its diet consisted of sorghum, the ratios of phytase against polyphenols are much lower than the concentrations used for the enzymatic activity experiments. The different ratios of phytase and polyphenols, for all three sorghum varieties are shown in Table 2.9.

As shown, the bigger the ratio, the less the effect, as the number of polyphenols is lower. Therefore, the effect of the polyphenol-rich extract on phytase should at least partially inhibit the reaction. Despite the fact that there is complete inhibition of the phytase – phytic acid reaction for some concentrations of polyphenols, that is probably not the case in reality, as there are many more

substances present in an animals diet that can and will interfere with the somewhat 'clean' system that was used for the above experiments.

Table 2.49: Phytase against polyphenols ratios for all sorghum varieties in chicken diet (100% sorghum).

Sorghum variety	Polyphenols (g/kg)	Phytase / polyphenols Ratio
Liberty	1.46	0.041
Cracka	1.67	0.035
MR-Buster	2.52	0.023

Table 2.57: Ratio of phytase against polyphenols for the different polyphenol-rich extract concentrations used of the ITC enzyme activity assay.

Polyphenol-rich extract Concentration (mg/ml)	Ratio of phytase / polyphenols
0.1090	1.86
0.0545	3.72
0.0272	7.44
0.0136	14.89
0.0068	29.79

Previous studies investigating the interactions between phenols and phytase have shown similar results. According to Goel and Sharma (1979), phloroglucinol, which is a phenol, was inhibiting the activity of plant phytases. The effect varied from source to source, ranging from 1.5-100% inhibition. The inhibition of pumpkin seed phytase and the barley phytases were mostly inhibited by phloroglucinol, while the effect was higher as the concentration increased. Similar to the results of this chapter, the highest concentration of phloroglucinol had a full inhibition on the pumpkin seed phytase.

According to Quesada et al. (1996), phenolic-rich extracts containing proanthocyanidins and condensed tannins from cocoa, pears and lentils were able to inhibit trypsin's activity, but remarkably the lentil and cocoa extracts had a 10 times higher inhibition compared to the pear extract, while the lentil extract had a 100% inhibition on the trypsin activity. Similar to the results of this chapter, different extracts have different effects on the enzymatic activity because of the different types of polyphenols in the extracts.

Those two studies certainly can support the results of this study, but there is a lack of studies that have investigated the impact of polyphenols on phytase, while there are a few studies on the effect of other phytase inhibitors. The effect of other substances on phytase has been studied further. Liu et al. (1998) discovered that phytase was inhibited when glucose was present in high concentrations and the aeration was poor. At the same study they revealed that phytase was inhibited by EDTA, Zn^{+2}, Ba^{+2}, Cu^{+2}, Cd^{2+}, Fe^{2+} and Al^{+3}. Finally, Greiner et al. (1997) found that fluoride, phosphate, molybdate and vanadate had a great inhibitory effect on the phytase activity.

2.5 CONCLUSION

Isothermal Titration Calorimetry can be used as an alternative analytical method to the traditional colorimetric assays for phytase activity (Heinonen and Lahti, 1981; Engelen *et al.*, 1994). In this occasion, using ITC to study the activity of phytase under different conditions proved to be challenging as the reaction between phytase and phytic acid is a complex, multiple stage reaction. So far, the ITC enzyme kinetics assay was widely used to study the kinetics of simple, one stage reactions by calculating the ΔH, Km and Vmax values. In this occasion, because of the way that phytase catalyses phytic acid (multiple cleavage sub-reactions, IP6 to IP5, IP5 to IP4, etc.), this reaction is unable to be analysed using the standard Michaelis-Menten kinetics and needs an alternative approach to using this data.

Using ITC to study the complex kinetics of this reaction is an innovative way of utilising the useful and meaningful data obtained by the ITC analysis. Using this method for studying the enzymatic kinetics for complex, multiple stage reactions, makes it obvious that taking the association constants and the rate constants under consideration, provide great support in the understanding and interpretation of the data of complex reactions, like to phytase-phytic acid degradation.

Using this novel analytic method, the activity of the phytase was able to be estimated under different conditions, which were the different sorghum polyphenolics-rich extracts. The effect of all three sorghum extracts on the activity of the 6-phytase when reacting with phytic acid was assessed by ITC. The effect of the polyphenol-rich extracts is profound on the phytase activity and this finding

can be used for a better understanding of the nutrition of monogastrics and potential alterations in their diet, to accomplish a better nutrition.

Although ITC has been used before for the analysis of enzymatic kinetics it was mainly for one-step reaction and by estimating the Km and Vmax values. The reaction of 6-phytase and phytic acid is a multiple stage reaction and thus these values cannot be estimated. Instead, the rate of the reaction was estimated and the relative activity of the 6-phytase was estimated with time as the main value of interest. This is a new perspective of how to use ITC for multiple stage enzymatic reactions that widens the area where ITC can be used.

The hypothesis of this chapter that Sorghum polyphenolics have an inhibitory effect on phytase activity was confirmed.

CHAPTER 3 'THE EFFECT OF PHYTIC ACID ON THE ACTIVITY OF PROTEASES'

3.1 SUMMARY

Proteases are vital for the digestion of monogastrics, as protein is the most important nutrient in their feed. They also play a very important role in the economic value of the feed, as protein is also the most expensive nutrient in the feed. The protein is almost never fully digested and there is a percentage of it is excreted by the faeces although exogenously produced proteases are added to the feed.

The reason behind that is that the proteases are not functioning as efficiently as they should, due to environmental inhibitors. This chapter investigates the role of phytic acid as a potential protease inhibitor. The enzymes tested for the inhibitory effect of the phytic acid are an exogenous chymotrypsin, commercially available and commonly used as an animal feed additive, an endogenous trypsin and an endogenous chymotrypsin.

Initially, the ITC enzyme activity assay was used to estimate inhibitory effects, but that was not possible due to the limitations of the technique when using the reactants chosen for this chapter. Therefore, the inhibitory effect of phytic acid on the proteases was estimated using Sigma's non-specific protease activity assay, which is a colorimetric assay using casein as a substrate.

There was a significant effect on the enzymatic activity of all three enzymes at high concentrations of phytic acid and although in lower concentrations the effect was much lower, there is still an inhibitory effect. Since the system used is quite clean and there are not many other reactants that can also interfere with the enzyme, the results show the minimal effect possible, as there are many more anti-nutritive factors that can also inhibit the enzymes.

3.2 INTRODUCTION

Proteases are enzymes essential for the digestion of most organisms, but especially for monogastric as they do not rely on microorganisms for the digestion of the nutrients present in their feed, as ruminants do. Exogenous enzymes added to the diets of pigs and poultry increase the protein utilisation and the dietary energy (Murugesan, Romero and Persia, 2014). Proteases are endogenously produced and/or secreted in their digestive tract to aid digestion (Glitsø *et al.*, 2012).

Pepsin, trypsin and chymotrypsin are the most widely known proteases. Trypsin and chymotrypsin are secreted by the pancreas and belong to the family of serine proteases. The serine proteases are a family of enzymes that identifies and cuts certain peptide bonds in proteins. This activity is defined by a set of amino acids, one of which is serine, which is present on the active site of the enzyme. On the other hand, pepsinogen, the precursor of pepsin, is produced by the chief cells of the stomach. When pepsinogen is released into the stomach, it is mixed with hydrochloric acid and instantly converted to the active enzyme pepsin. Pepsin functions best at low pH (pH 1.5–2.5), while it is no longer effective at neutral or basic pH (pH > 7).

Although already present in the digestive tract, proteases (mainly chymotrypsin) are also exogenously produced and added in the animal feed to enhance digestion of proteins. Protein is the most important and most expensive ingredient of the animal feed and the endogenous amounts of proteases are not enough to digest the whole amount of present in the feed. Therefore, a large amount of protein is excreted with the faeces. The addition of exogenously produced proteases to the

animal feed definitely improves the digestion of proteins, but still there is an amount of protein loss. The bio-efficacy of the exogenous proteases is influenced by a number of factors. *In vivo*, the effect of proteases is highly affected by the presence of other digestive enzymes, as their mechanisms and sub-sequent effects seem to not be independent (Romero and Plumstead, 2013).

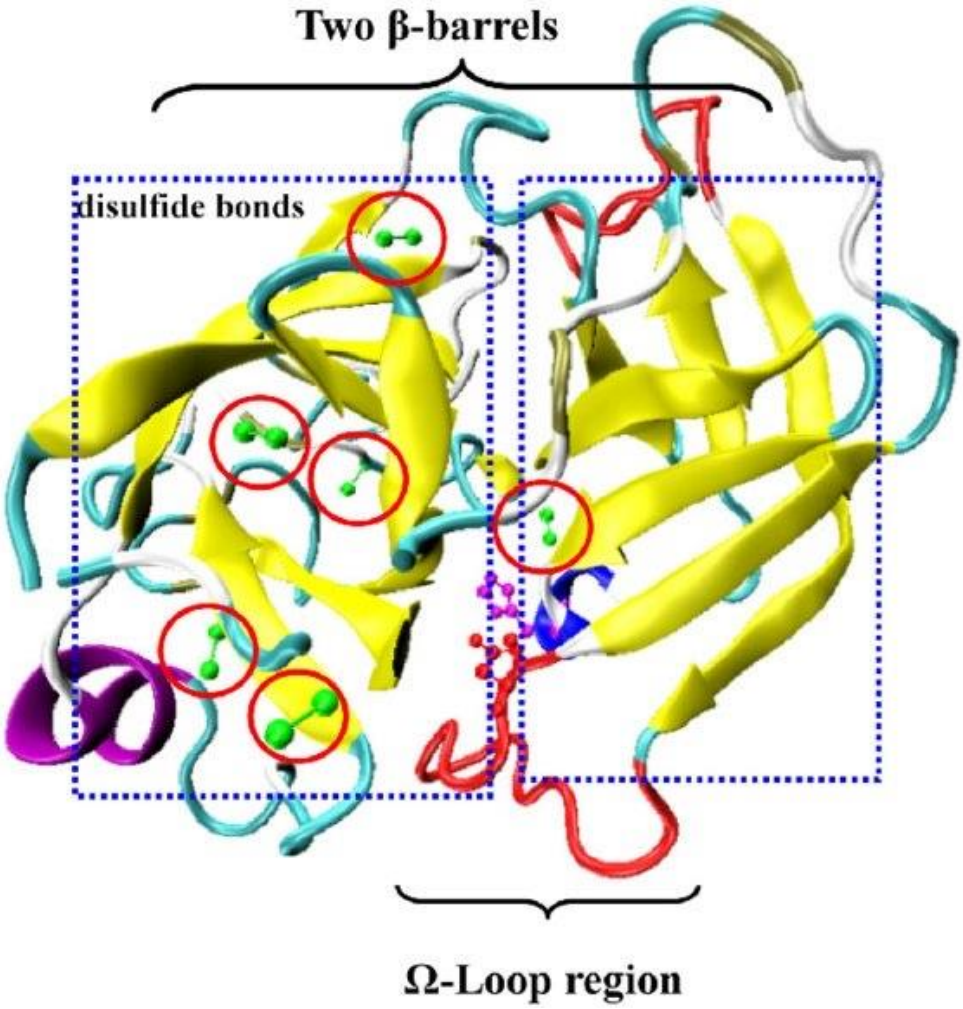


Figure 3.7: The crystal structure of a porcine trypsin shown using the Visual Molecular Dynamics software, obtained from the Protein Data Bank file of trypsin, identifying the two β-barrels and the Ω-loop region (Taken from Guo et al., 2016)

Trypsin consists of 13 beta-strands, six of which create the beta-barrel structure and there are four regions of alpha helix, as well as six disulphide bridges (Figure

3.1) (Guo *et al.*, 2016). Chymotrypsin on the other hand starts as chymotrypsinogen, which is inactive and gets activated by getting cleaved by trypsin. After that, the enzyme is active and consists of 3 polypeptide chains that are bound together by two inter-chain disulphide bonds and three intra-chain disulphide bonds too. Generally, it consists of two beta barrels with α-helices and β-strands. Chymotrypsin, a serine protease, has a unique active site structure of Aspartate-Histidine-Serine. Serine proteases belong to the category of endopeptidases, which break non-terminal peptide bonds. Chymotrypsin is a digestive enzyme secreted by the pancreas, but the mechanism of chymotrypsin is what makes it interesting, as it cleaves the peptide bonds by attacking the unreactive carbonyl group with a powerful nucleophile, the serine 195 residue that is located in the enzyme's active site. Following that, it briefly becomes covalently bound to the substrate and it forms an enzyme-substrate intermediate (Gupta *et al.*, 2010).

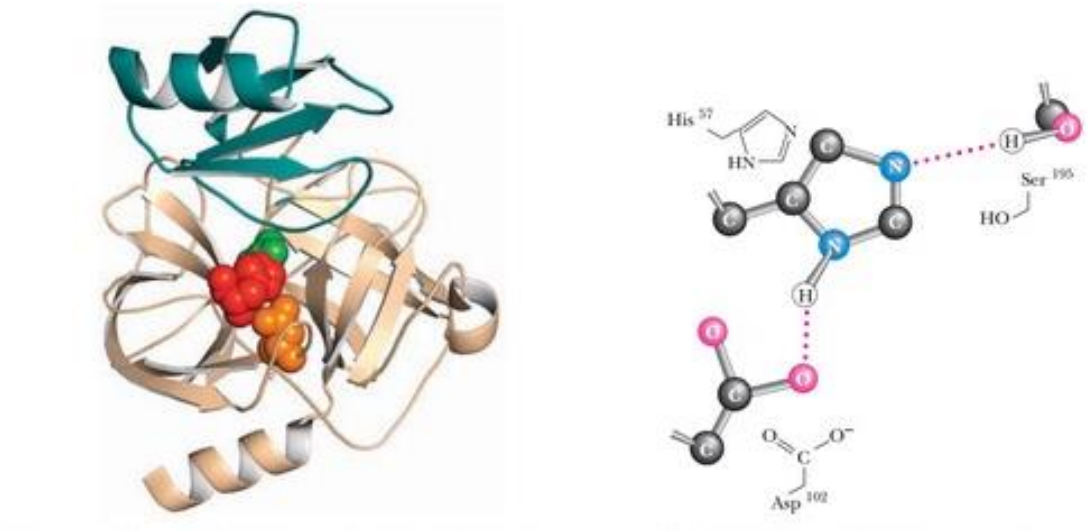


Figure 3.16: (Left) a ribbon representation of chymotrypsin's structure when it has a protein attached to its active site. (Right) Aspartate-Histidine-Serine, the catalytic triad of chymotrypsin. (Taken from Garrett & Grisham, Biochemistry, 4th edition)

As mentioned before, on top of the endogenously produced protease, it is a common practice, exogenously produced proteases to be added to the feed to improve protein digestibility. A serine protease from the strain *Nocardiopsis* was identified to have a good acid stability and activity when compared to different commercial protease containing products (Glitsø, 2012). Based on these findings the Nocardiopsis protease was selected to be the best protease to survive the passage through the acidic conditions of the stomach and function in the small intestine. This protein shows the double β-barrel architecture characteristic of most chymotrypsin family members. In contrast with most of the chymotrypsin family members, it has a unique small β-hairpin that connects the N- and C-terminal β-barrels. Finally, this protein consists of another larger β-hairpin in the C-terminal domain and is an integral site for Pro region binding and has a role in

the folding catalysis (Kelch *et al.*, 2007). This chymotrypsin is being used for the purposes of this chapter. This is an innovative protease that increases protein digestion across a range of feed ingredients, with an ultimate purpose of reducing the feed costs. Animal feed protein ingredients had a market size of USD 160.95 Billion in 2019, while the Value Projection for 2026 is USD 245.06 Billion (Ahuja & Singh, 2020). While increasing the protein digestibility, at the same time it is improving the protein utilization by the animal and is helping the animal utilise protein sources of lower quality. It is designed in a way that it complements the endogenously produced proteases. It has a high heat tolerance, as it is stable in a range of temperatures and thus is easier to be used as a feed additive.

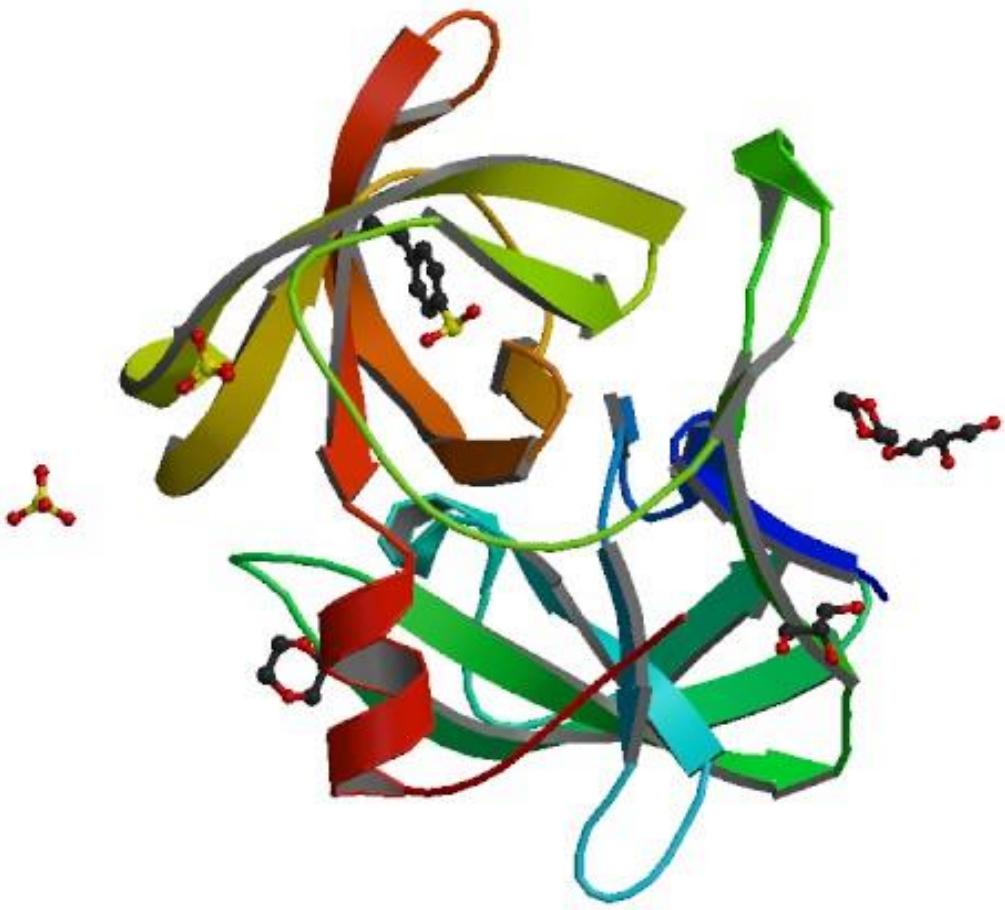


Figure 3.25: Crystal Structure of *Nocardiopsis* Protease. (Taken from: https://www.rcsb.org/structure/2OUA)

Anti-nutritive factors, such as phytic acid and polyphenols alongside with non-starch polysaccharides and lectins inhibit the proteases (Murugesan, Romero and Persia, 2014). As discussed in chapter 2, the effect of the sorghum polyphenol-rich extract on the phytase activity is profound. Therefore, less phytic acid is being eliminated for the feed and hence there is a higher amount to interact with other digestive enzymes such as proteases.

The stomach works like a bioreactor and there is a plethora of substances, biological or not, interacting with each other. Those interactions can cause inhibition, prohibit, or even be necessary to initiate reactions. The ultimate goal of digestion is to simplify nutrients, by breaking them down into smaller, easier to digest molecules and therefore promote nutrition. In this very complicated matrix of enzymes, proteins and other substances, the importance of protein digestion and its bioavailability is of high importance.

Therefore, identifying potential inhibitors of protease reactions is extremely significant. As, in order to be able to resolve this problem, an extended understanding of what is actually causing it, is really important.

Proteins are positively charged molecules and therefore they are prone to interactions with other negatively charged molecules. Such interactions could potentially decrease the efficiency of the proteolytic enzymes to hydrolyse the proteins that are not a part of a more complex system (protein + negatively charged molecule). Phytic acid, as mentioned in Chapter 2, consists of an inositol ring that has a phosphate group attached on each carbon molecule. These phosphate groups give a high negative charge to the phytic acid's molecules, which then are likely to interact with other positively charged molecules. This is one of the most important characteristics of phytic acid and the one that makes it be considered as an anti-nutritive factor.

A very common protein used for the estimation of protease activity in vitro is bovine serum albumin (BSA), which is a serum albumin protein isolated from cows. BSA is used in areas such as immunology, biochemistry and biotechnology. Some of the assays that use BSA are ELISA, immunoblots and

immunocytochemistry. It is also used as a nutrient in cell and microbial culture as well as a stabilizer for restriction enzymes during digestion of DNA and other processes. BSA is commonly used for all these processes mainly because it does not affect the functions of other proteins or enzymes (Karonen *et al.*, 2015).

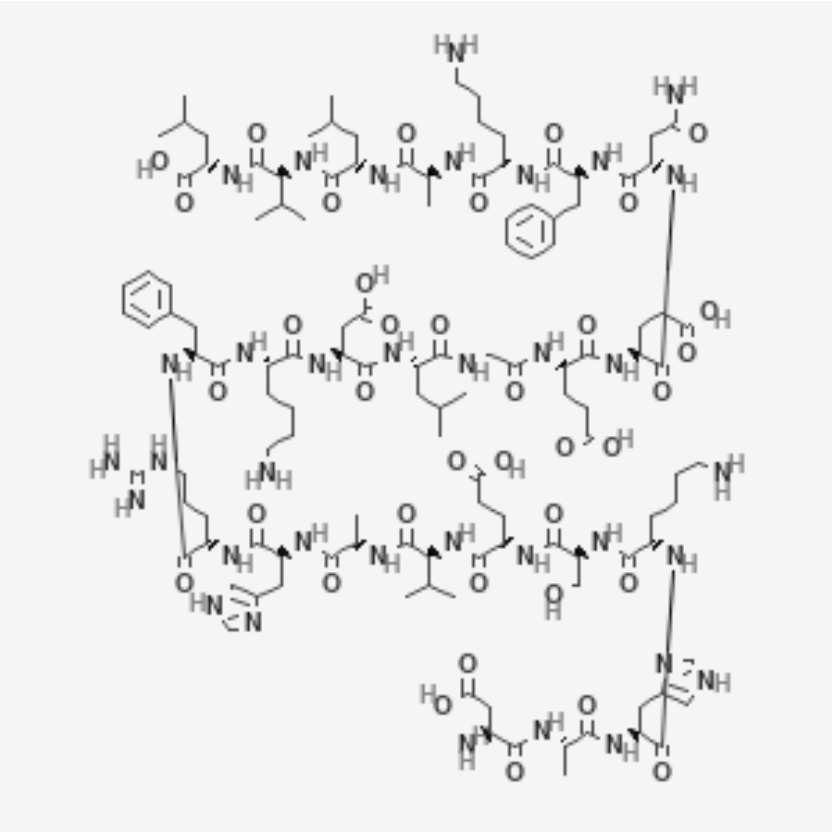


Figure 3.34: Structure of serum albumin (taken from PubChem).

Another enzyme widely used in laboratory experiments and especially in protease activity assays is casein. Casein is one of the major milk proteins and it is found in milk in percentages usually up to 3%. The white colour of the milk is due to the casein micelles present in it. Those micelles are usually stable, but when interacting with acids and/or salts they can easily be disturbed. Casein is easily

isolated and when hydrolysed produces l-tyrosine which can be easily measured. Due to these reasons, it is a protein commonly used in the assays mentioned above.

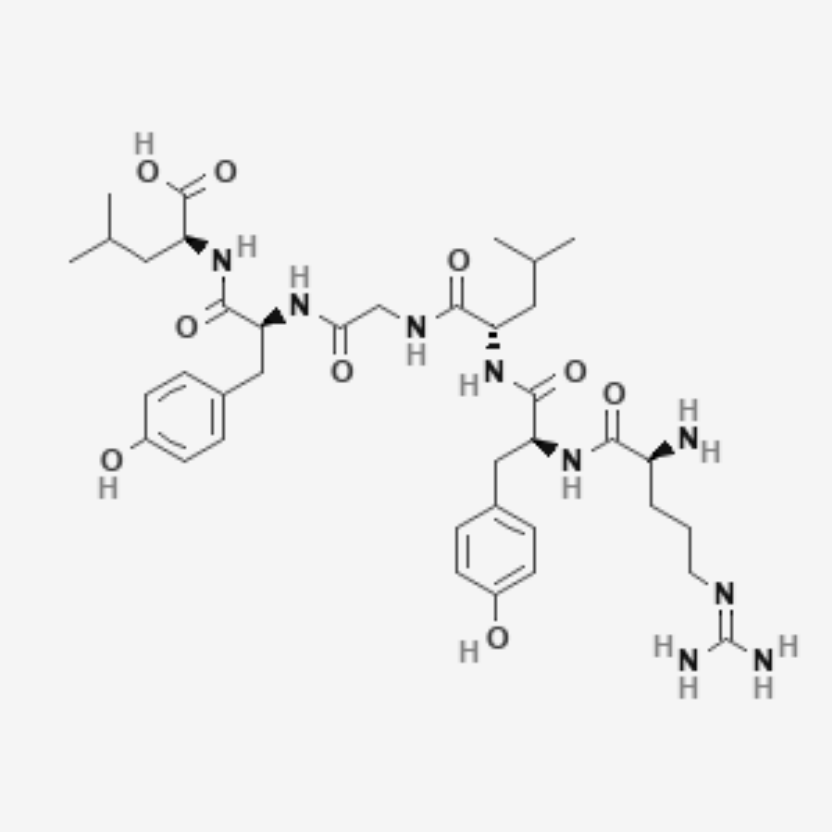


Figure 3.43: Structure of casein (taken from PubChem).

Inhibition of the activity of trypsin, chymotrypsin and amylase by phytate has been identified in the past (Sharma, Goel and Irshad, 1978; Singh and Krikorian, 1982; Deshpande, 1984; Caldwell, 2005). At the same time, when pepsin is trying to digest protein substrate that is bound to phytate, the digestion is not as effective as when the substrate is a just a protein (Knuckles *et al.*, 1989). In pig and poultry diets the anti-nutritional properties of phytate have an economic impact where it

reduces the yield of animal meat from grain-based feed (Selle and Ravindran, 2007a; Selle, Cowieson and Ravindran, 2009).

Though the effect of anti-nutritive factors on the activity of proteases has been researched in the past, it has never been done for the specific exogenously produced protease, neither has it been tried to be estimated before using ITC.

In a previous study of casein degradation by trypsin, using a single injection of the casein in the ITC cell containing the enzyme, resulted in an irregular thermogram. This is indicative of multiple cleavage reactions, which would then translate into different enzyme kinetics and even different ΔH values (Maximova and Trylska, 2015). As enzymes identify their binding site and they start hydrolysing the substrate, having a substrate (like casein) with more than one binding sites for a specific enzyme, would be a problematic situation, as it would be hard to analyse such data.

To try and estimate the inhibition caused to proteases by the presence of phytic acid, the ITC enzyme activity assay that was used in Chapter 2, for the estimation of the inhibitory effect of polyphenol-rich substances to the 6-phytase was believed that it could also be used to estimate the inhibitory effect of phytic acid on proteases.

Though, to avoid the complexity of the multiple binding sites of casein, BSA was chosen as a substrate. This also proved to be a problematic situation, as the heat of dilution produced when the protein was injected into the cell greatly exceeded the heat produced/absorbed by the enzymatic reaction. Coming to this realisation, different assays that estimate enzymatic activity were considered.

Initially, an SDS-Page gel analysis was performed to make sure the enzyme was still active. Following that, Sigma's kit with trypsin as standard using casein as a substrate was used and showed that the protease used for the ITC enzyme activity assay was still hydrolysing the substrate and therefore the limitation came from the assay. Therefore, Sigma's non-specific protease activity assay using casein as a substrate was chosen to be the assay used for this chapter. The effect of phytic acid on the activity of three different proteases was tested. An endogenous porcine trypsin and chymotrypsin as well as an exogenous chymotrypsin were used. A colorimetric assay was used to estimate phytic acid's effect on them, which works at a neutral pH, therefore the effect on pepsin's activity could not be estimated with this assay as pepsin functions only in low, acidic pH (1-3).

A typical sow diet, as mentioned in Chapter 2, is roughly 2.5kg daily, 70% of which is consisted from grains. With a roughly a 0.20% phytate in cereals like maize, oats, rye, wheat and barley and with a 20% content of sorghum, with a phytic acid assuming that these are the only grains used in this diet, there are roughly 3.5g of phytate present in the daily feed intake.

With a phytic acid concentration of 15.43mg/g, there would be 5.40g of phytic acid in 350g of sorghum. At the same time for the rest of the grains there is an amount of 2.80g of phytic acid. Hence, in total, there are 8.2g of phytic acid in 2.5kg of the feed.

The exogenous chymotrypsin liquid formulation has a target activity of 75,000 PROT/g (One PROT is the amount of enzyme that releases 1 mmol of p-nitroaniline from 1 mmol of Suc-Ala-Ala-Pro-Phe-pNA per minute at pH 9.0 and 37 °C.). The liquid formulation of the exogenous chymotrypsin is directly sprayed

onto the feed to obtain an enzyme activity of 15,000 PROT per kg of feed. Since the daily feed of sow is 2.5kg per day, 37,500 PROT need to be added to it, which translates to 0.5g or the exogenous protease's liquid formulation.

Therefore, there is a ratio of 0.5g of exogenous protease solution to 8.2g of phytic acid. According to the calculations that were carried out to estimate the amount of the enzyme in its liquid solution (7.7% w/w), which would be 0.0385g of the exogenous chymotrypsin. This would give the following ratio of enzyme and phytic acid in a typical sow feed intake:

$$\frac{0.0385g\ of\ exogenous\ chymotrypsin}{8.2g\ of\ phytic\ acid} = 0.0047$$

For broiler chickens, assuming that they consume 100g of feed per day and the feed has a high phytate content (13.1g/kg), the ratio of enzyme to phytic acid content in the feed is as described below:

$$\frac{0.00154g\ of\ exogenous\ chymotrypsin}{1.31g\ of\ phytic\ acid} = 0.00117 = \sim 0.0012$$

As shown in the above calculations, there is much more phytic acid present in the broiler chicken feed, and therefore the ration is almost four times lower, compared to that of a typical sow's diet. The same calculations can also be

performed for weaning pigs, laying hens and other categories, but the chosen two (sow and broiler) are the most commonly used ones.

In this chapter, the understanding of the effect of phytic acid on the reaction between protein and endogenous and exogenous proteases will provide another vital piece of information, to aid to the mapping and understanding of the interactions shown in Figure 3.6.

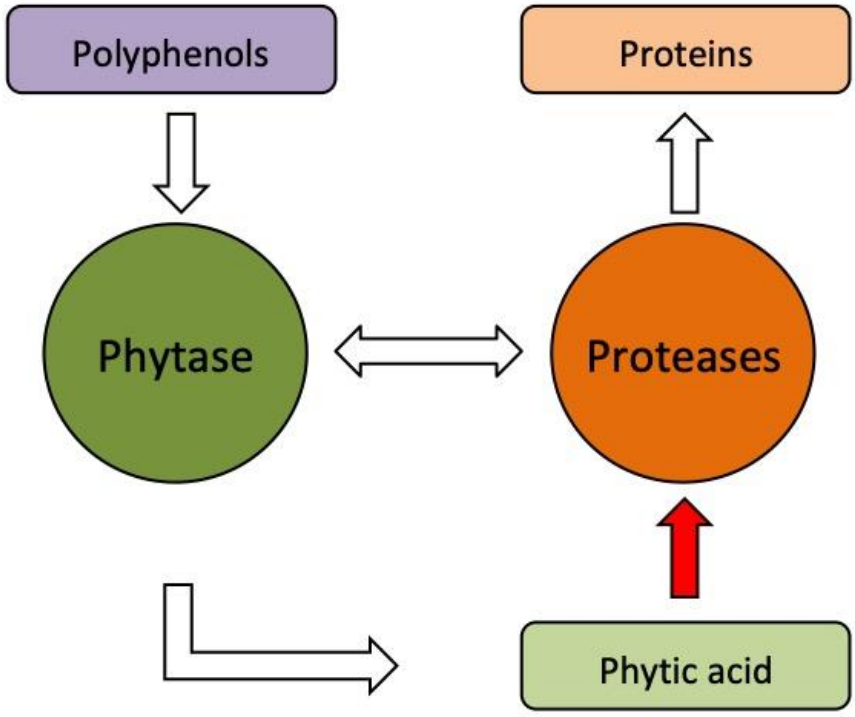


Figure 3.52: Map of interactions between phytic acid, phytase, protein, proteases and polyphenolic compounds. As shown with the red arrow, in this chapter the focus will be on the interactions of phytic acid with proteases.

The hypothesis of this chapter is that phytic acid has an inhibitory effect on the activity of endogenous and exogenous proteases.

3.3 MATERIALS AND METHODS

3.3.1 CHEMICALS AND MATERIALS

- Phytic acid sodium salt hydrate, rice extract, sodium chloride and sodium acetate were sourced from Sigma Aldrich, Gillingham, UK.
- Serine chymotrypsin was sourced from DSM, Kaiseraugst, Switzerland.
- A-Chymotrypsin from bovine pancreas, Sigma Aldrich, Gillingham, UK.
- Trypsin from porcine pancreas, Sigma Aldrich, Gillingham, UK.
- Pepsin from porcine gastric mucus, Sigma Aldrich, Gillingham, UK.
- Potassium Phosphate, Dibasic, Trihydrate Sigma-Aldrich, Gillingham, UK.
- Casein Sigma-Aldrich, Gillingham, UK.
- Trichloroacetic Acid Sigma-Aldrich, Gillingham, UK.
- Folin & Ciocalteu's Phenol Reagent Sigma-Aldrich, Gillingham, UK.
- Sodium Carbonate, Anhydrous Sigma-Aldrich, Gillingham, UK.
- Sodium Acetate, Trihydrate Sigma-Aldrich, Gillingham, UK.
- Calcium Acetate Sigma-Aldrich, Gillingham, UK.
- L-Tyrosine, Free Base Sigma-Aldrich, Gillingham, UK.
- Bovine serum albumin, Sigma-Aldrich, Gillingham, UK.
- Pierce™ Colorimetric Protease Assay Kit, Thermo Scientific,
- Whatman® Puradisc syringe filters 0.45 µm, PES, sterile, Little Chalfont, UK.
- Falcon™ 15mL Conical Centrifuge Tubes, Waltham, Massachusetts, USA.
- Jenway Quartz Semi-micro Cuvette, Staffordshire, UK.

- PageBlue Protein Staining Solution, Thermo Scientific, Waltham, Massachusetts, USA.
- MES SDS Running Buffer (20X), Thermo Scientific, Waltham, Massachusetts, USA.
- NuPAGE™ 4-12% Bis-Tris Protein Gels, 1.5 mm, 10-well, Thermo Scientific, Waltham, Massachusetts, USA.
- NuPAGE LDS Sample Buffer (4X), Thermo Scientific, Waltham, Massachusetts, USA.
- Novex XCell SureLock Mini-Cell, Thermo Scientific, Waltham, Massachusetts, USA.
- LABOSI PowerPro 300, Fisher Scientific, Hampton, New Hampshire, USA.
- Novex™ Sharp Pre-stained Protein Standard, Thermo Scientific, Waltham, Massachusetts, USA.
- BioSpectrum 410 Imaging system, 230V~50Hz, UVP, LLC Upland, CA. Ultra-Violert Products Ltd., Cambridge, UK.
- Eppendorf Thermomixer C, Eppendorf, UK Limited, Stevenage, UK.

3.3.2 ITC PROTEASE ACTIVITY ASSAY

A 1 μM of serine protease from *Bacillus licheniformis* containing a minimum protease activity of 75,000 protease activity units/g, stock was made by appropriately diluting the enzyme solution provided by DSM into MilliQ water. Respectively, a 1 Mm BSA stock was prepared by dissolving the appropriate mass of BSA into MilliQ water. These stocks were used to estimate the optimal concentrations of the substrate and enzyme that would be used for the ITC enzyme activity assay.

Prior to analysis the samples were all degassed for 20 minutes to eliminate the possibility of air bubbles that would alter/affect the data. ITC analysis was conducted on an iTC200 (MicroCal, Northampton, MA, USA). 50 µl of the 1 Mm BSA stock was loaded into the syringe and titrated into the sample cell, which contained 300 µl of 1 µM the serine protease in a sequence of 4 injections in 2, 5, 5 and 5 µl respectively. Experiments were conducted at room temperature (20°C) and 285 rpm.

The behaviour of the ITC Protease activity assay proved to be very different to the ITC phytase assay, see Figure 3.7. We believe the heat of dilution was overpowering the heat difference caused by the amino acids and peptides created when BSA was digested by the Serine Chymotrypsin. The controls were not able to explain the phenomenon but as you can see in Figure 3.7 the heat of dilution decreases with each addition as the concentration of amino acids and peptides increases. It was decided that this ITC assay was never going to be rugged enough for the research that was planned. A recently published work that measured trypsin activity using ITC added polyethylene glycol to the buffer presumably to eliminate this effect (Aguirre et al., 2015), which is something we didn't want to do, as this would add another substance in the matrix which could affect the reaction in many ways and impact the results. As an alternative, the Pierce™ Colorimetric Protease Assay Kit was assessed as a replacement for the ITC enzyme activity assay (see Figure 3.9). Despite the effectiveness of this kit, there were still limitations, both financially but also experimentally. Due to the volume of the samples, instead of the Pierce™ Colorimetric Protease Assay Kit, Sigma's non-specific Protease Activity Assay was the best option for these experiments and

should be reliable as long as the correct controls were included in the experiment design.

3.3.3. SDS-PAGE GELS

3.3.3.1 SAMPLE PREPARATION

Samples of protease, BSA and protease with BSA were used for this experiment by dissolving the appropriate amount of BSA and enzyme stock into MilliQ water. After 30 min of incubation at 30 degrees Celsius, 40µL of each sample was loaded into the gel and then followed the general procedure of running an SDS-Page gel (Carter et al., 2013).

All samples were heated to 70 degrees Celsius for 10 minutes, while the ladder was only heated at 90 degrees Celsius for 2 minutes.

3.3.3.2 GENERAL PROCEDURE

Once the gel is placed into the sure-lock mini-cell, the gel comb is removed, and each well is rinsed with some of the MES buffer (containing 2-(N-morpholino)ethanesulfonic acid) that is going to be used to run the gel. When all wells are rinsed, 40µL of each sample and 5µL of the ladder are added to the wells. Then MES buffer is used to fill the tank, until the liquid in the inner compartment of the gel assembly is covering the top of the wells and is over halfway full up the gel? in the outer part.

Following that, the gel runs for 35-45 minutes at 200V. Once the dye front has run all the way to the bottom of the gel, the gel is washed three times with MilliQ water (30 minutes) in an orbital mixer and is then stained with 30ml of PageBlue Protein Staining Solution for an hour, while gently shaken in an orbital mixer. After the

completion of the staining procedure, the gel is rinsed with MilliQ water twice and then rinsed three times, 10 minutes each, with 100-200ml of MilliQ water, while shaken in the orbital mixer. Each time the supernatant is discarded and fresh MilliQ water is used. Once the gel was washed, it was read using the BioSpectrum 410 Imaging system.

3.3.4 PIERCE PROTEASE ASSAY KIT

A control blank was created for each unknown and standard sample. The blank consisted of the Assay Buffer and protease (unknown or standard) but no Succinylated Casein Solution, so there is no reaction. When assayed in parallel to the samples, these blanks control for colour development caused by amines in the protease sample, including those cause by self-cleavage. 100µL of Succinylated Casein Solution were added to one set of micro plate wells and 100µL Assay Buffer were added to a duplicate set of wells to serve as blanks. Following that, 50µL of each unknown or standard sample (containing no casein) were added to both Succinylated Casein wells and corresponding blank wells. The plate was then incubated at room temperature for 20 minutes and at the end of that time, 50µL TNBSA Working Solution was added to each well. The plate was then incubated again at room temperature for another 20 minutes and following that the absorbance of each well in the plate was read using a plate reader set to 450nm.

The absorbance at 450nm for each well was calculated by deducting the absorbance for the blank for each sample cell at 450nm. The ΔA450 generated is the absorbance generated by the proteolytic activity of the exogenous serine chymotrypsin hydrolyzing the casein. Using the ΔA450 for the standards, the

standard curve was plotted (Figure 3.9). Using the equation from the trendline shown below the relative activity of the unknown samples was estimated.

3.3.5 SIGMA'S NON-SPECIFIC PROTEASE ACTIVITY ASSAY - CASEIN AS A SUBSTRATE

3.3.5.1 ASSAY VALIDATION

Precision:

The Precision for this assay was estimated by performing an experiment to estimate its repeatability. This was achieved by performing the exact experiment under the same conditions over three days. The repeatability expresses the precision under of the assay under the same operating conditions and over a short interval of time.

To estimate the precision of the assay, 7 samples of the same concentration (1 ml of the 1 μM enzyme solution) were analysed under the same conditions, repeatedly in over the period of three days. This resulted in having 21 replicates of the same reaction.

Linearity:

The optimal concentration of each enzyme that is being used for this assay was estimated by performing a linearity validation for each enzyme. The linearity of each enzyme was estimated by using a range of concentrations. To identify the optimal concentration for each, the concentration at which the curve is the most linear was chosen.

Sensitivity:

The sensitivity of this assay was estimated by understanding what its ability was to detect the enzymatic reaction at very low concentrations of the enzyme used. Using the results of the linearity validation, the limit of detection was estimated and used for understanding the sensitivity of the assay by performing the same experiment ten times to measure the standard deviation of those replicates.

3.3.5.2. ANALYSIS

All procedures were performed following, Sigma's non-specific protease activity assay (Cupp-Enyard and Aldrich, 2008). After preparing all the different reagents stated at the materials and methods section, the 50 mM Potassium Phosphate Buffer was adjusted to pH 7.5 with 1M HCl. This solution is placed at 37°C prior to use. A 0.65% weight/volume casein solution was prepared using the 50 mM potassium phosphate buffer. Gradually increased the solution temperature with gentle stirring to 80-85 °C for about 10 minutes until homogenous. The pH is adjusted again if necessary, with NaOH and/or HCl (Cupp-Enyard and Aldrich, 2008).

An enzyme diluent solution, which consists of 10 mM Sodium Acetate Buffer with 5mM Calcium Acetate, pH 7.5, was used to dilute enzyme solutions and kept at 37°C. Finally, a 1.1 mM L-tyrosine Standard stock solution was prepared using purified water and heated gently until homogeneous. For each enzyme that was tested, 4 vials were used. One for a blank and three replicates of the actual sample. 5mls of the 0.65% casein solution were pipetted into each vial and they were placed in a water bath at 37°C for about 5 minutes. 1ml of the enzyme solution containing different concentrations of phytic acid was placed into the three

sample vials, but not the blank. Mixed by swirling and incubate for 37°C for exactly ten minutes. After this 10-minute incubation, 5 mls of the TCA reagent were added into each tube to stop the reaction (including the blank vials). Then, 1 ml of the enzyme solution was added to the blank vials. This is done to account for the absorbance value of the enzyme itself and to make sure that the final volume in each tube is equal. All vials were then incubated at 37°C for 30 minutes. A phytate control was also tested without having a difference to the blank, therefore it was not used. During the incubation, the tyrosine standard dilutions were prepared. In six vials, 1.1 mM tyrosine standard stock was added in the following volumes in mls: 0.05, 0.10, 0.20, 0.40, and 0.50. No tyrosine standard was added to the blank. Following that, the appropriate volume of purified water was added to each of the standards to bring the volume up to 2 mls (Cupp-Enyard and Aldrich, 2008).

After the 30-minute incubation, each of the test solutions and the blanks was filtered using a 0.45 um polyethersulfone syringe filter. 2 mls of the filtrate of each sample was added into a new vial. To all of the vials 5mls of sodium carbonate were added, followed by the addition of 1 ml of Folin's reagent immediately afterwards. The vials were mixed by swirling and incubated at 37°C for 30 minutes. 2mls of these solutions were filtered using a 0.45 um polyethersulfone syringe filter into suitable cuvettes and their absorbance was measured using a spectrophotometer using a wavelength of 660nm. The light path is set to 1cm and the absorbance values for the standards, standard blank, the different test samples, and test blank were recorded. Once all of the data was collected, the standard curve was created (Figure 3.11, 3.15 & 3.17). To generate the curve, difference in absorbance between the standard and standard blank must be

calculated. This is the absorbance value attributable to the amount of tyrosine in the standard solutions. After this simple calculation, the standard curve was created using a graphing program to plot the change in absorbance of the standards on the Y-axis, versus the amount in micromoles for each of our 5 standards on the X-axis (Cupp-Enyard and Aldrich, 2008).

After data points have been entered, generate a line of best fit and corresponding slope equation. The change in absorbance in the samples was estimated by calculating the difference between the sample absorbance and the absorbance of the blank. Using the slope equation for the absorbance value and solving it gives us the micromoles of tyrosine liberated during this particular proteolytic reaction (Cupp-Enyard and Aldrich, 2008).

To get the activity of enzyme in units per/ml, the following calculation was performed:

$$(\mu mole\ tyrosine\ equivalents\ released) * (A)\ Units/ml\ Enzyme$$
$$= (C) * (B) * (D)$$

A= Total volume (ml) of assay

B= Time of assay (min) as per the Unit definition

C= Volume of Enzyme (ml) of enzyme used

D= Volume (ml) used in Colorimetric Determination

The number of micromoles tyrosine equivalents released, that was obtained from the slope equation was multiplied by the total volume of the assay in mls (11mls).

This value was then divided by three other quantities: the time of the assay, which we ran for 10 minutes, the volume of enzyme used in the assay, which was 1ml and the volume of millilitres used in colorimetric detection (2 mls). The micromoles of tyrosine divided by time in minutes, gives the measurement of protease activity called "units". We can cancel out the units for volume measurement in the numerator and denominator, leaving a measurement of enzyme activity in terms of units/ml (Cupp-Enyard and Aldrich, 2008).

To determine the activity in a solid protease sample diluted in enzyme diluent, we divide our activity in units/ml by the concentration of solid used in this assay originally in mg/ml., leaving us with activity in terms of units/mg (Cupp-Enyard and Aldrich, 2008).

$Unit$ / mL enzyme * Units / mg of enzyme = mg solid / ml enzyme

To estimate the effect of phytic acid for each enzyme, except a range of different concentrations of phytic acid, five concentrations were analysed in five replicates, to estimate the variation in each experiment (Cupp-Enyard and Aldrich, 2008).

3.4 RESULTS AND DISCUSSION

3.4.1 ITC ENZYME ACTIVITY ASSAY

Initially tried to estimate the effect of phytic acid on proteases, using the ITC enzymatic activity assay that was used in Chapter 2 to estimate the effect of polyphenols on the activity of phytase.

To do so, a four-injection experiment of BSA being titrated into the ITC cell containing the exogenous serine chymotrypsin was performed (Figure 3.7). Unfortunately, due to the nature of the interaction between the BSA and the serine chymotrypsin, in combination with the limitations of this assay, it was not successful.

As shown in Figure 3.7, the heat identified is an exothermic reaction. This was due to the fact that the assay is identifying changes in temperature, but there is a heat of dilution produced when BSA is being titrated into the cell and is overpowering the heat change caused by the reaction itself. The heat produced by this dilution is higher to the amount of heat produced by the actual hydrolysis of the BSA itself, the results show no interaction, which is an inaccuracy as the protein-protease interaction was shown to perfectly work using a different assay below.

3.4.2 SDS PAGE ANALYSIS

In order to verify that this was the reason why the ITC assay was not effective and that the serine chymotrypsin was still active and able to hydrolyse proteins, an SDS Page gel was run, containing samples of BSA, chymotrypsin and a solution that contained both and was incubated at 30 degrees Celsius for 30 minutes. As shown

in Figure 3.8, the protease completely hydrolysed the BSA, which proves that the enzyme is still active and able to hydrolyse proteins.

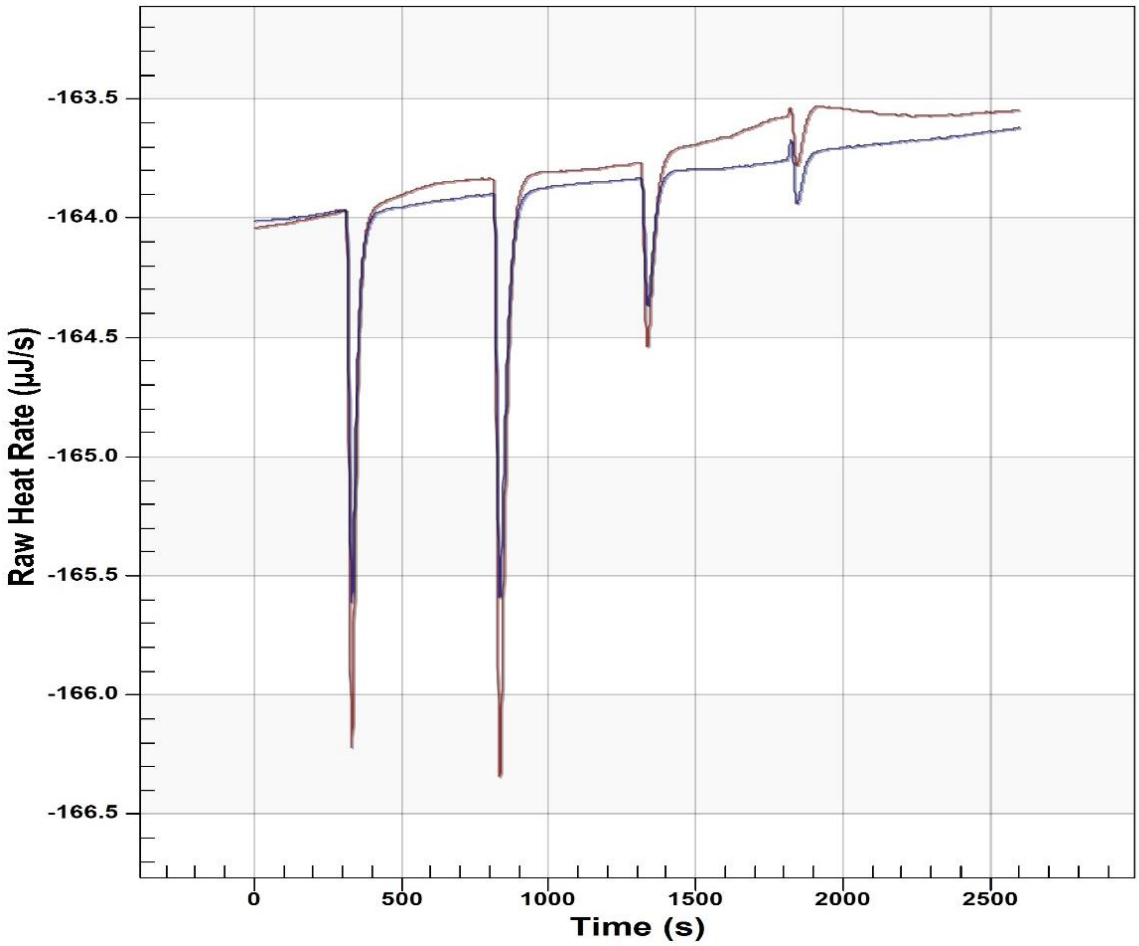


Figure 3.61: Thermogram obtained by the ITC enzyme activity assay. Two replicates of the 4-injection experiment with 2, 5, 5 and 5µL injections respectively. 1mM BSA titrated into 1µM of the exogenous chymotrypsin under the same conditions. (Note the baseline drift of the brown line is indicative of a small air bubble in the cell and is an artefact.)

As shown in Figure 3.8, the first well contains the protein size ladder. The contents of the other wells are described in 3.4.1 and are as follows: exogenous chymotrypsin (well 2); the syringe solution (1mM BSA) used for the ITC experiment (well 3); the solution from ITC cell (well 4); 1mM BSA (well 5) and

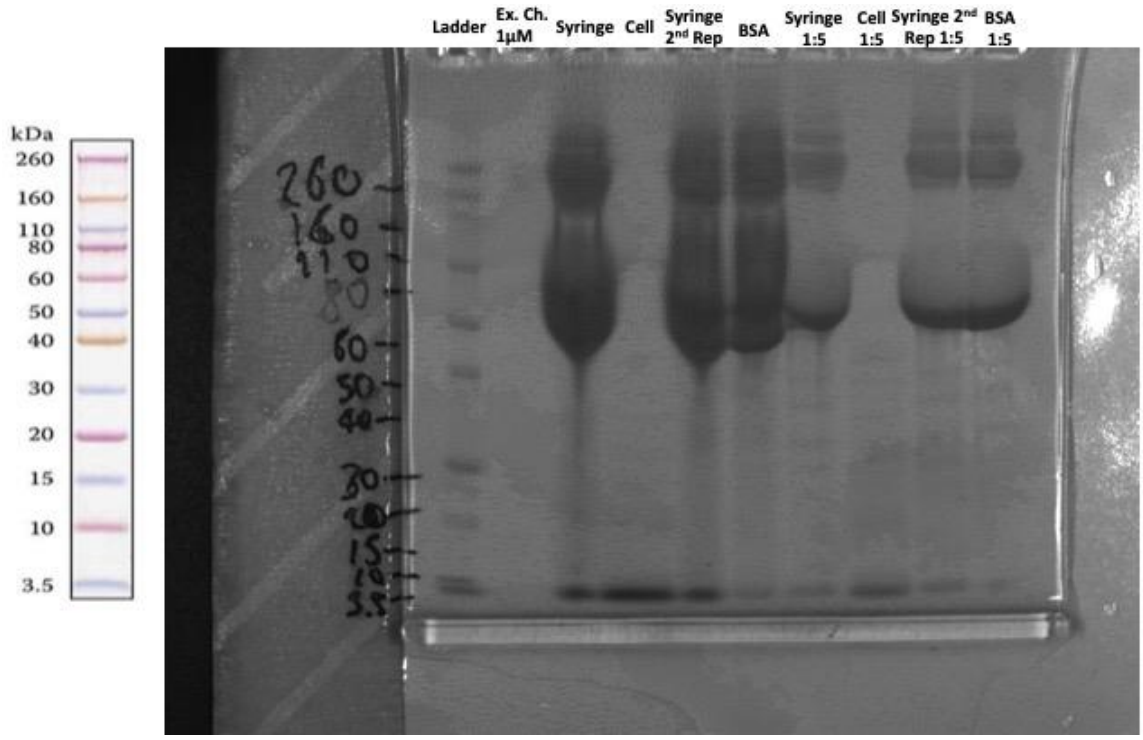


Figure 3.70: SDS gel electrophoresis of ladder, exogenous chymotrypsin, syringe contents, cell contents, replicate of syringe contents, 1mM BSA and 1:5 dilutions of the syringe contents, cell contents, replicate of syringe contents, 1mM BSA.

1:5 dilutions of the contents of the wells 3,4,5 and 6 are in wells 7, 8, 9 and 10 respectively. The exogenous chymotrypsin is not showing any bands, due to the very low concentration used (1μM). As the syringe contained 1mM BSA, it was expected that wells 3 and 5 would be identical to well number 6, and 7 and 9, identical to well number 10. Wells 6-10 that contain the 1:5 dilutions of the samples are much clearer and give a better understanding of the contents of the wells. The most important wells though, are wells 4 and 8, which contain the samples obtained from the cell of the ITC.

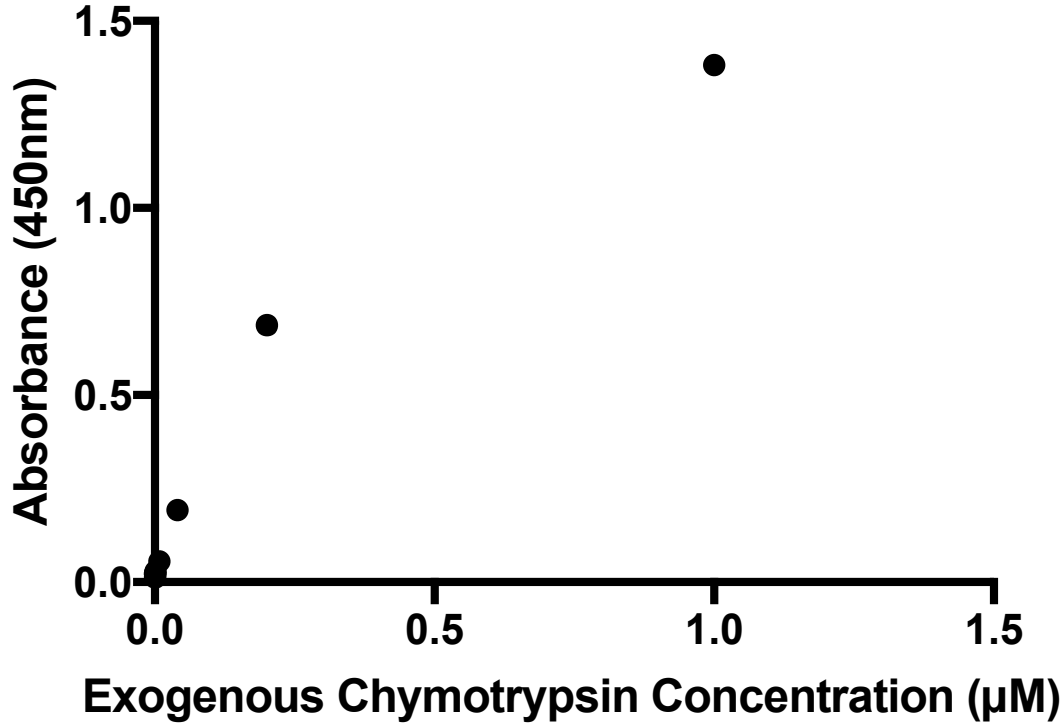
Since there are no BSA bands for cells 4 and 8, but only a band at the very bottom of the gel, it means that the whole BSA was hydrolyzed by the chymotrypsin.

Therefore, it is certain that the reaction is happening as expected and that the reason why the ITC enzyme activity assay was unable to provide the necessary information regarding this reaction, is due to the limitations of the technique, when it comes to reactions that involve more than one endothermic or exothermic reactions.

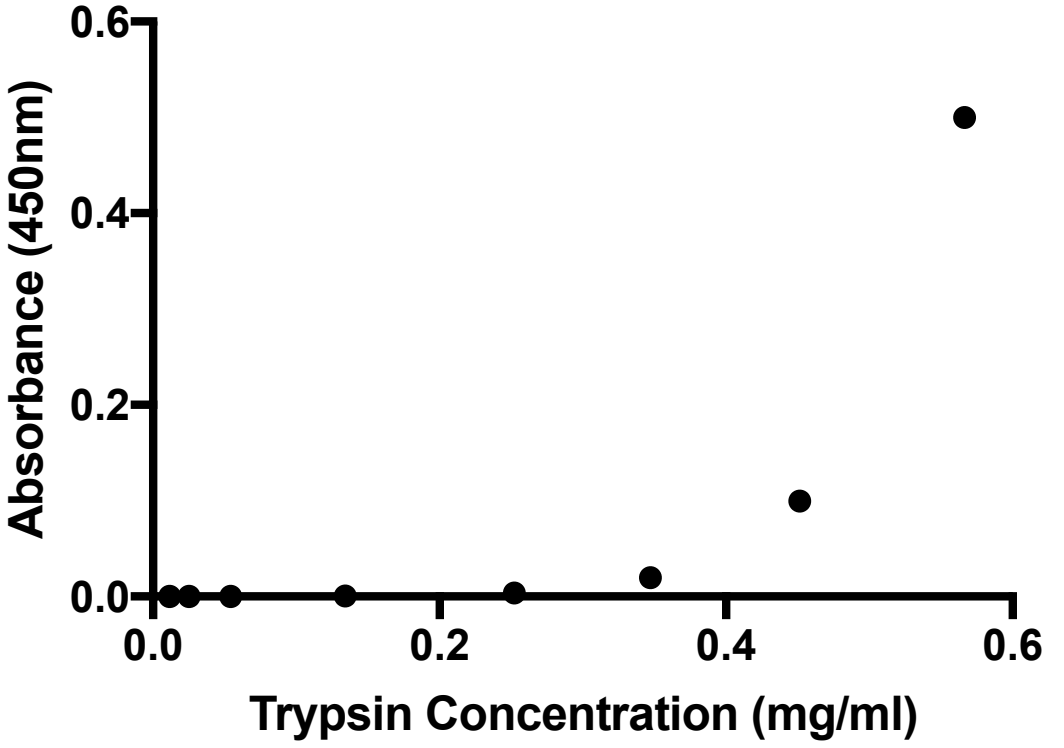
3.4.3 PIERCE PROTEASE ASSAY KIT

Although the SDS-Page gel analysis shows that the chymotrypsin is still active and able to break down BSA, since the ITC enzyme activity assay cannot be used for the estimation of the effect of the phytic acid on the activity of the proteases, an alternative had to be considered. To do so, the Pierce Protease Assay Kit was used to confirm that both the protease was active, able to hydrolyse casein and that all in all, this would be an accurate assay that could be used for the needs of this experiment.

Pierce protease Assay Kit uses trypsin as a standard and casein as a substrate. Therefore, it provides all the necessary information in order to proceed in the final colorimetric assay that was used for this experiment, which is Sigma's non-specific protease activity assay – casein as a substrate. The Pierce protease Assay Kit proves the efficiency of the protease to cleave casein and it can be measured by a colorimetric assay. As shown in Figure 3.9, trypsin in concentrations as low as 0.0000064 mg/ml. The units in Figure 3.9 differ because of the format of the stocks used.



(a)



(b)

Figure 3.78: Standard curves created using the absorbance at 450nm against the enzyme concentration, using the Pierce™ Colorimetric Protease Assay Kit. (a) Trypsin standard in the concentration range 0.0000064-0.5mg/ml and (b) exogenous chymotrypsin in concentrations 0.0000128-1µM.

3.4.4 PROTEASE ACTIVITY ASSAY

3.4.4.1 VALIDATION

The Precision of the Sigma's non-specific protease activity assay was estimated by performing the same experiment 21 times in total, 7 times a day for 3 consecutive days. After calculating the mean of the 21 replicates and calculating the standard deviation, the coefficient of variation was also calculated. The coefficient of variation was calculated by the following equation.

$$Coefficient\ of\ Variation\ =\ (Mean\ *\ Standard\ Deviation)\ *\ 100$$

The Mean of those replicates was found to be 13.77 Units/mg, with a Standard Deviation of 0.27 and a Coefficient of Variation equal to 1.97%.

Following that, an experiment for linearity of the exogenous chymotrypsin was performed (Figure 3.10), in order to estimate the sensitivity of the assay.

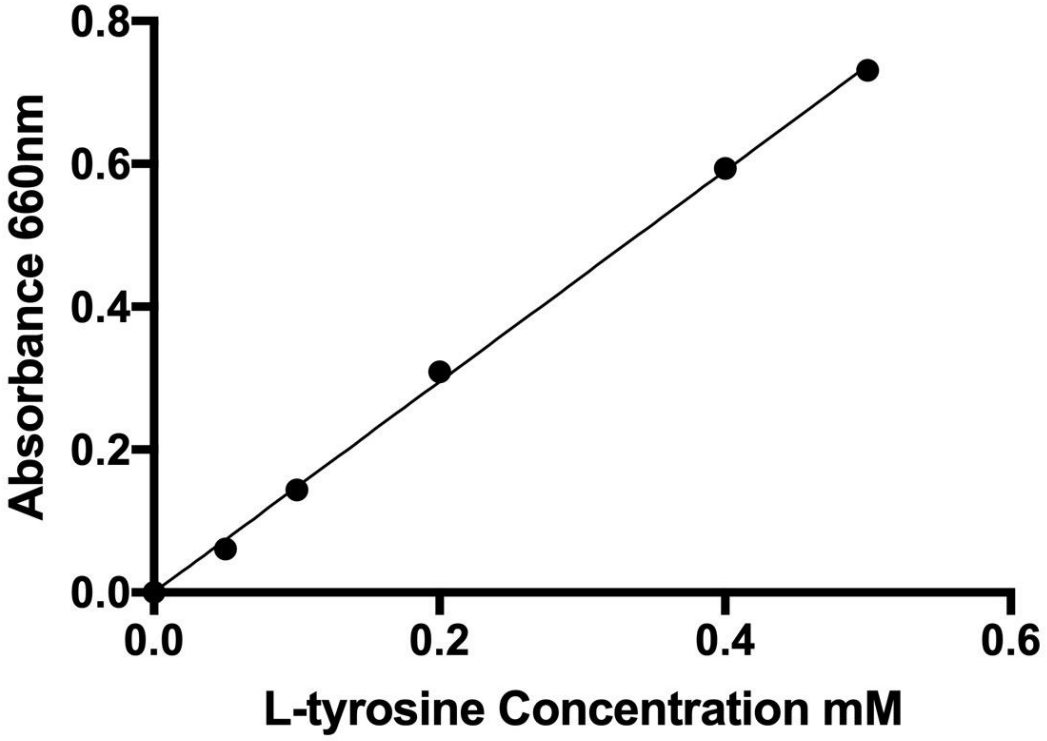


Figure 3.10: Standard curve of L-tyrosine against Absorbance at 660nm, created for the calculations of the enzymatic activity of the exogenous chymotrypsin.

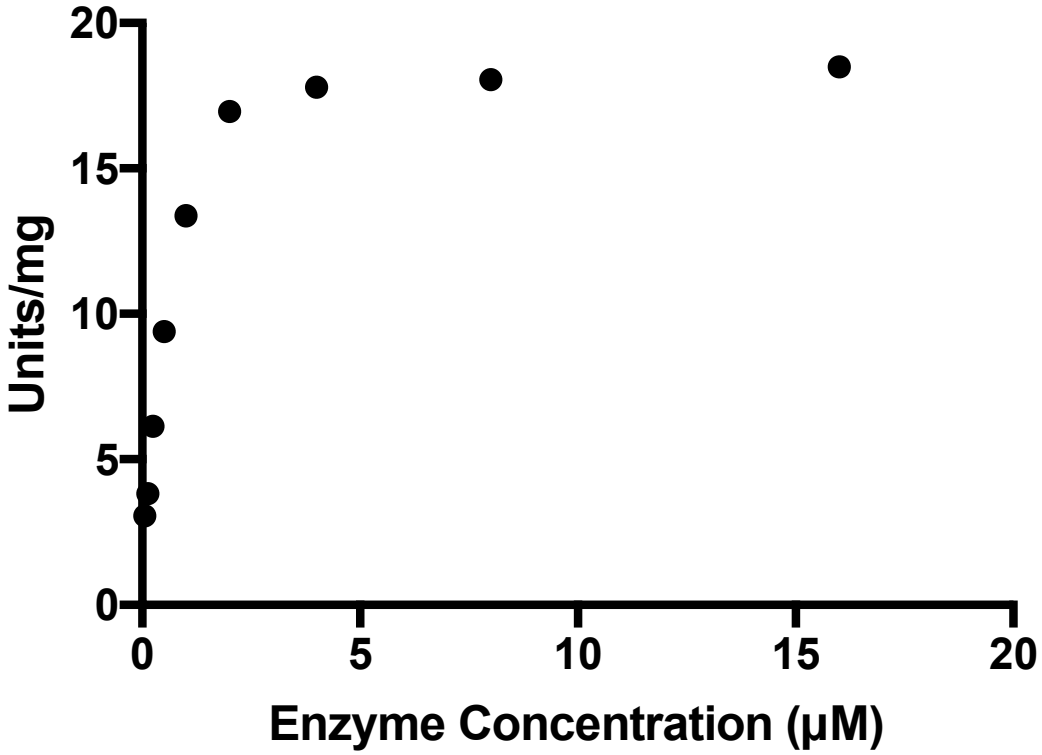


Figure 3.88: Linearity curve of different concentrations of the exogenous chymotrypsin against the enzymatic activity in units/mg for each.

The lowest concentration of detectable enzyme was identified to be 0.05µM and for this concentration, 10 replicates were performed for this concentration. The results showed a mean of 2.53 Units/mg, with two times the Standard Deviation equal to 0.05.

3.4.4.2. ANALYSIS

Exogenous chymotrypsin

After performing the standard curve experiment, while conducting the experiments exogenous chymotrypsin experiments, the slope for the Figure 3.11, which was used for the calculations of the enzyme activity of the following experiments, is:

$$x = 1.4086y$$

To identify the optimal concentration for the exogenous chymotrypsin, a linearity curve was created (Figure 3.12).

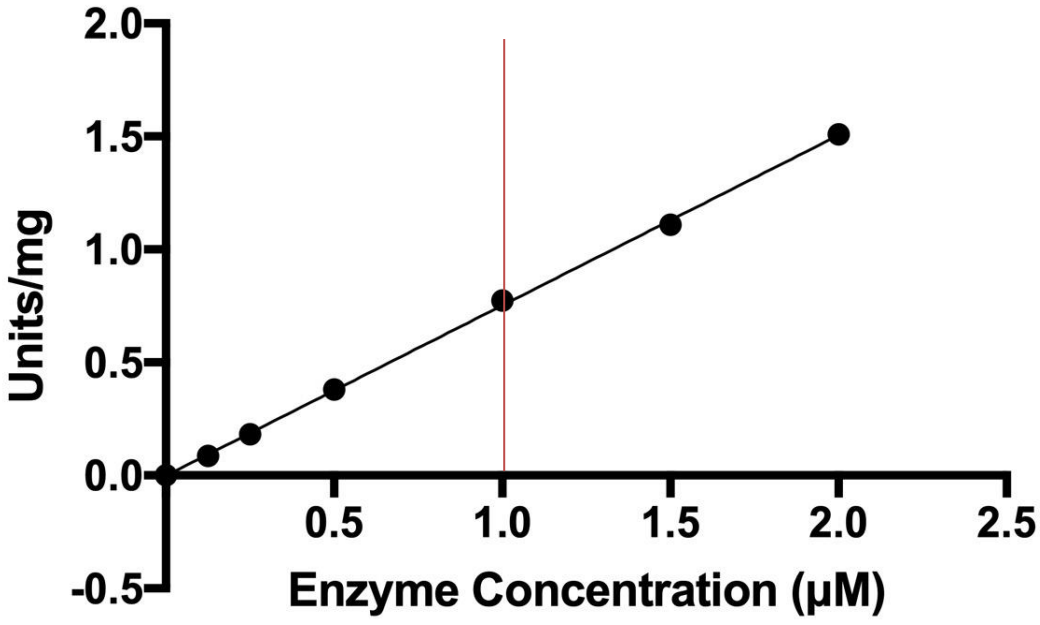


Figure 3.97: Linearity curve of different concentrations of the exogenous chymotrypsin against the enzymatic activity.

After performing the same experiment with different concentrations of the exogenous enzyme and creating a standard curve, the 1μM concentration was selected as the best option for conducting the phytic acid experiments, as it was at the middle of the linear line of concentrations (Figure 3.12).

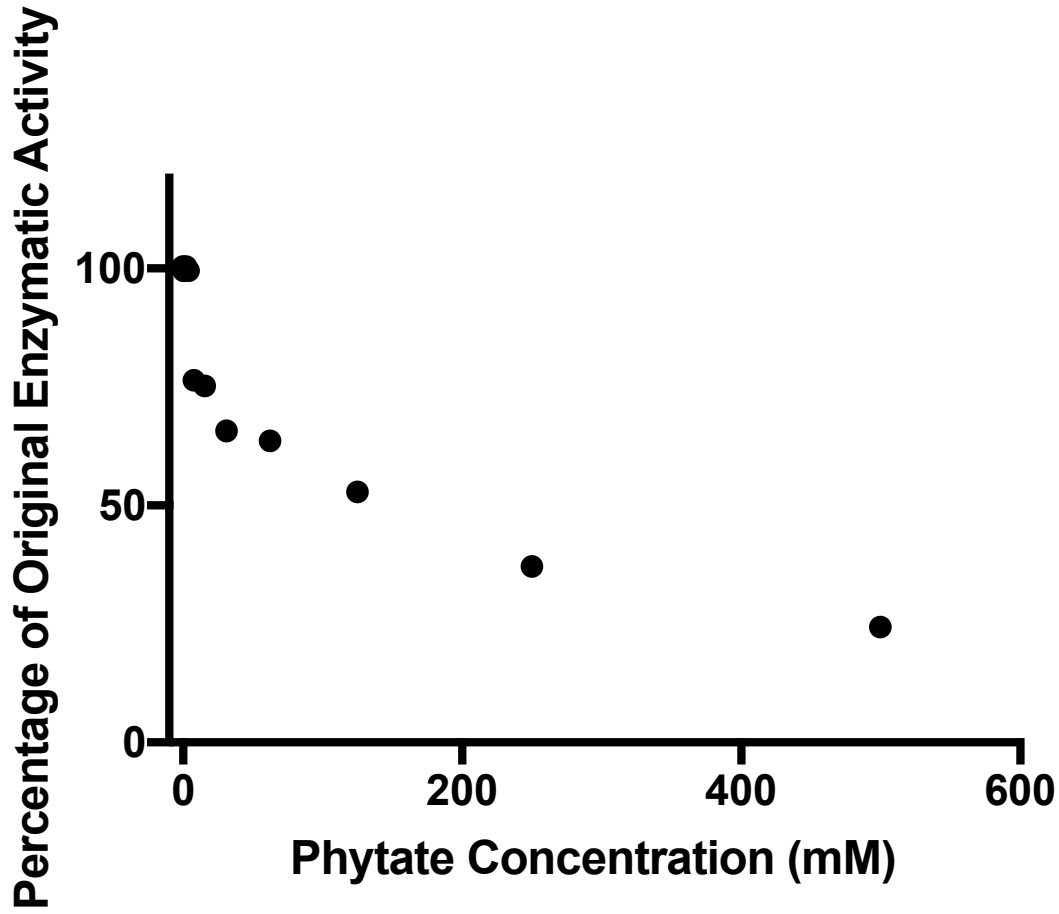


Figure 3.106: The effect of a range of phytic acid concentrations (0-500mM) on the enzymatic activity of the exogenous chymotrypsin as a percentage of the original enzymatic activity. All samples done in duplicate.

As shown in Figure 3.13, there is a small inhibitory effect caused by the phytic acid on the exogenous chymotrypsin, while the effect is greater as the phytic acid concentration is increasing.

Following that, an experiment for just five of the above concentrations was performed in quintuplicate, to estimate the standard deviation for these. As shown in Figure 3.14, the standard deviation for those is really low.

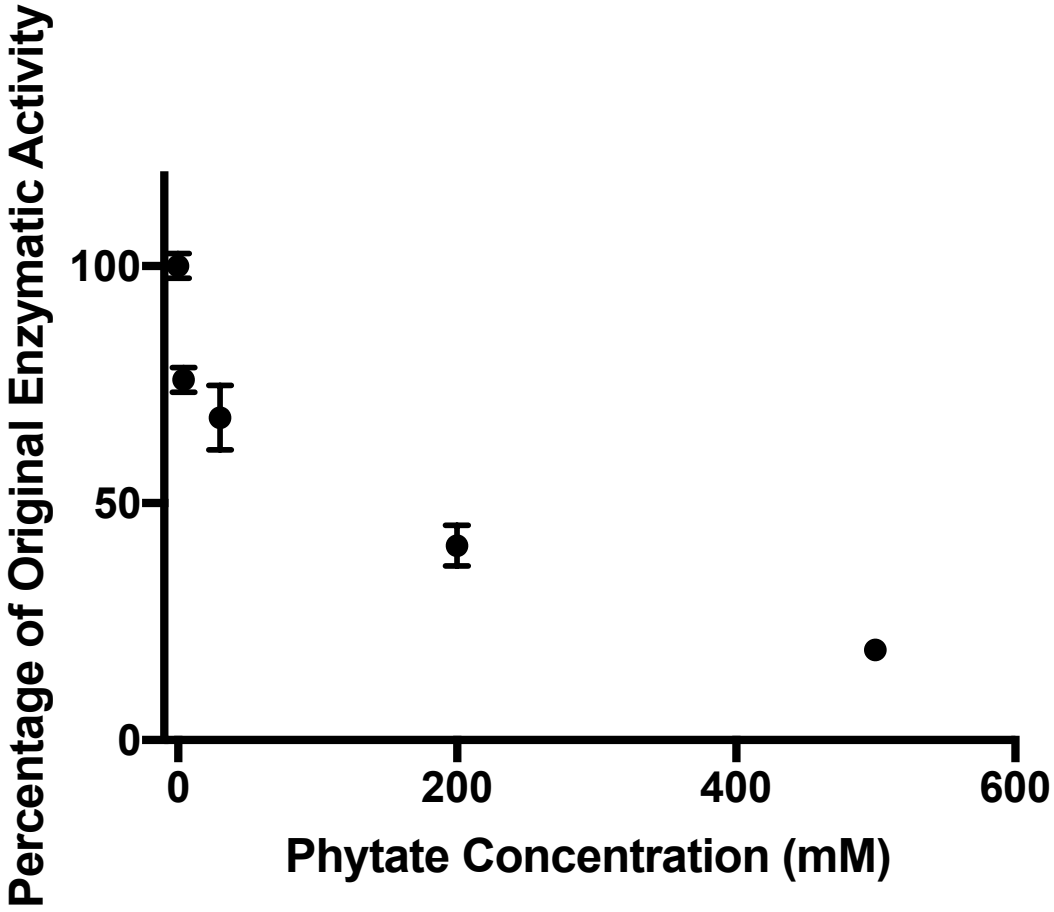


Figure 3.115: The effect of five phytic acid concentrations (0,4, 30, 200 & 500mM) on the enzymatic activity of the exogenous chymotrypsin as a percentage of the original enzymatic activity. All done in five replicates, error bars show STDEV.

Endogenous Trypsin

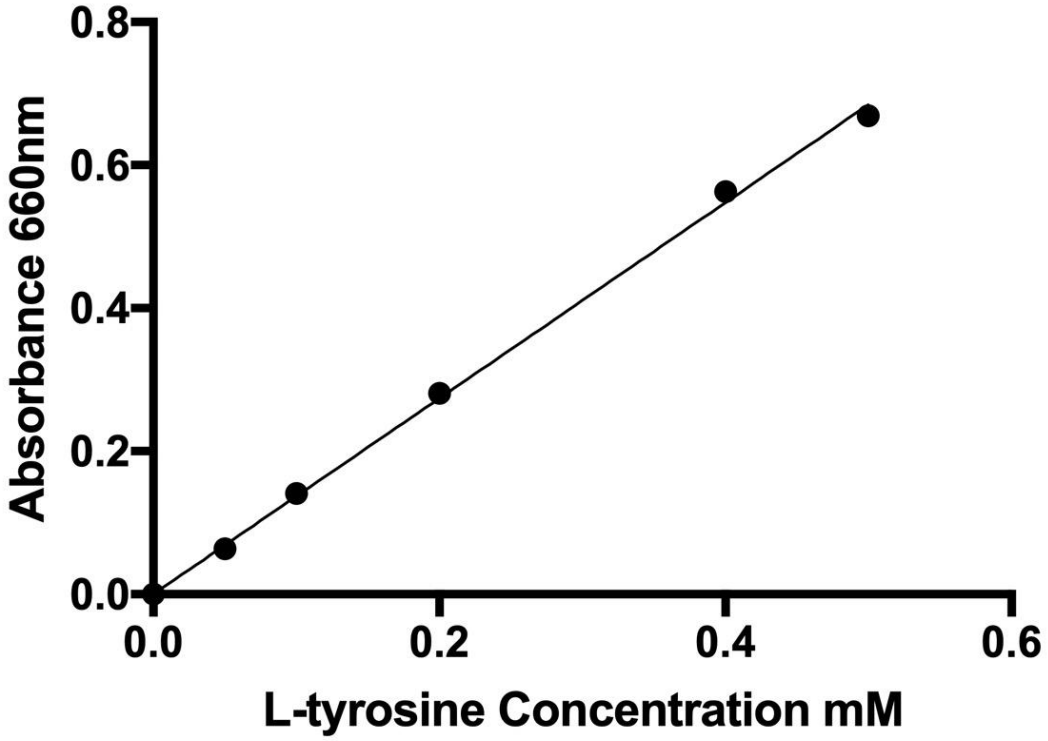


Figure 3.124: Standard curve of L-tyrosine against Absorbance at 660nm, created for the calculations of the enzymatic activity of the endogenous trypsin.

After performing the standard curve experiment, while conducting the experiments exogenous chymotrypsin experiments, the slope for the Figure 3.15, which was used for the calculations of the enzyme activity of the following experiments, is:

$$x = 1.4754y$$

To identify the optimal concentration for the exogenous chymotrypsin, a linearity curve was created (Figure 3.16).

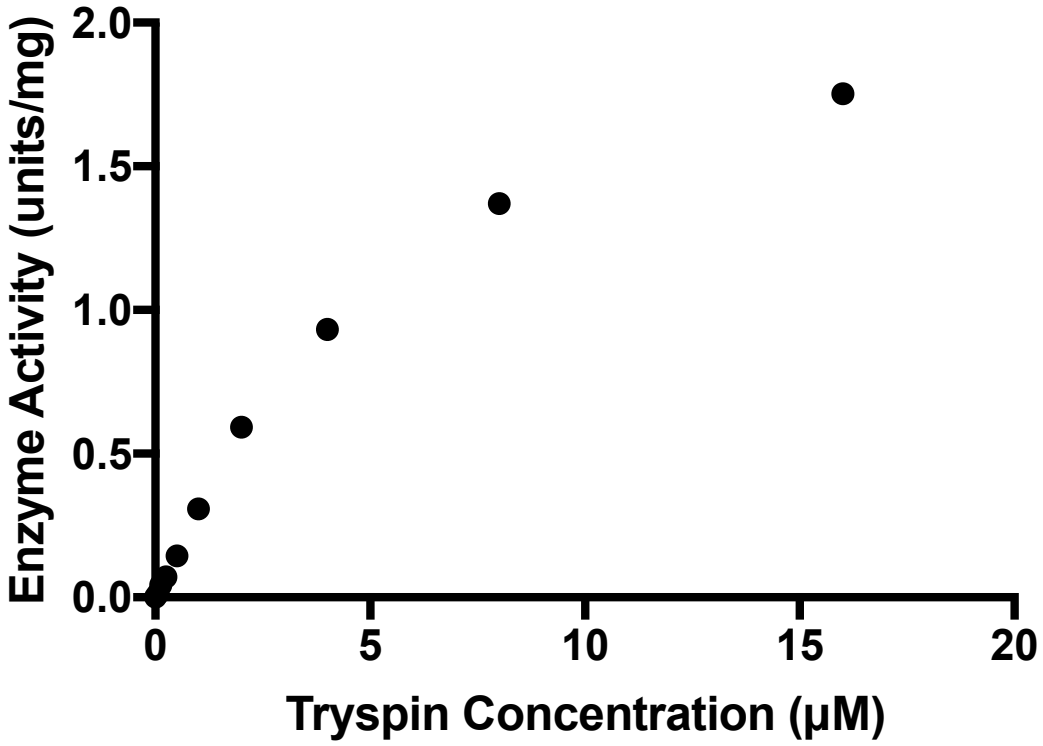


Figure 3.133: Linearity curve of different concentrations of the endogenous trypsin against the enzymatic activity.

Since higher concentrations of the enzyme create a non-linear curve (Figure 3.16), the same linearity experiment was performed for lower concentrations (0-1μM).

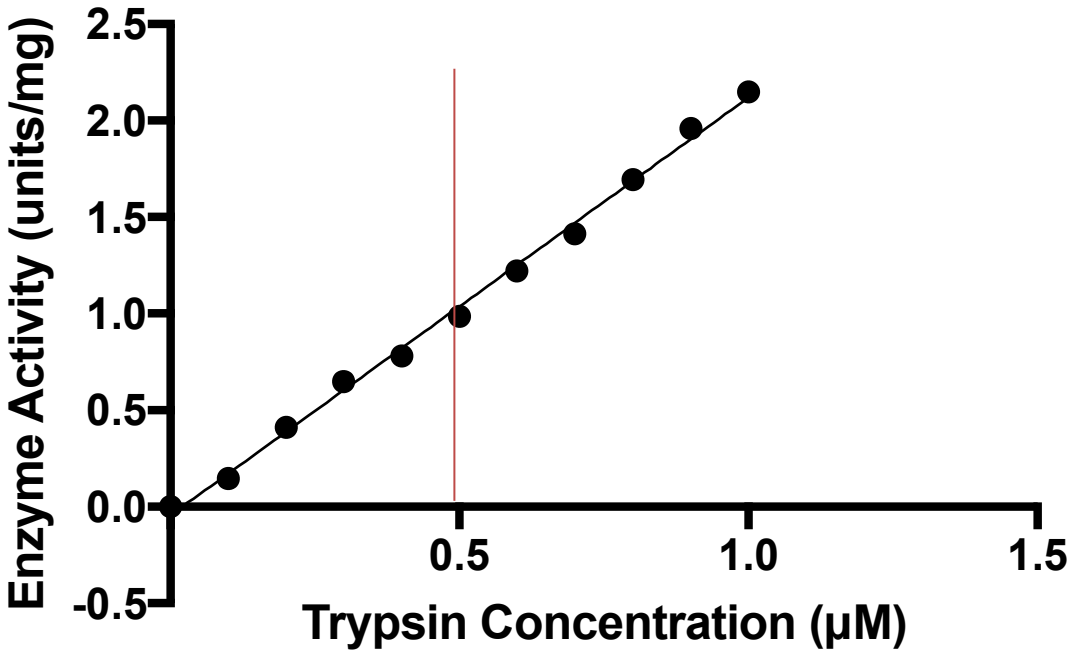


Figure 3.142: Linearity curve of different concentrations of the endogenous trypsin against the enzymatic activity on a lower scale to achieve a linear curve.

After performing the same experiment with different concentrations of the exogenous enzyme and creating a standard curve, the 0.5µM concentration was selected as the best option for conducting the phytic acid experiments, as it was in the middle of the linear concentration range (Figure 3.17).

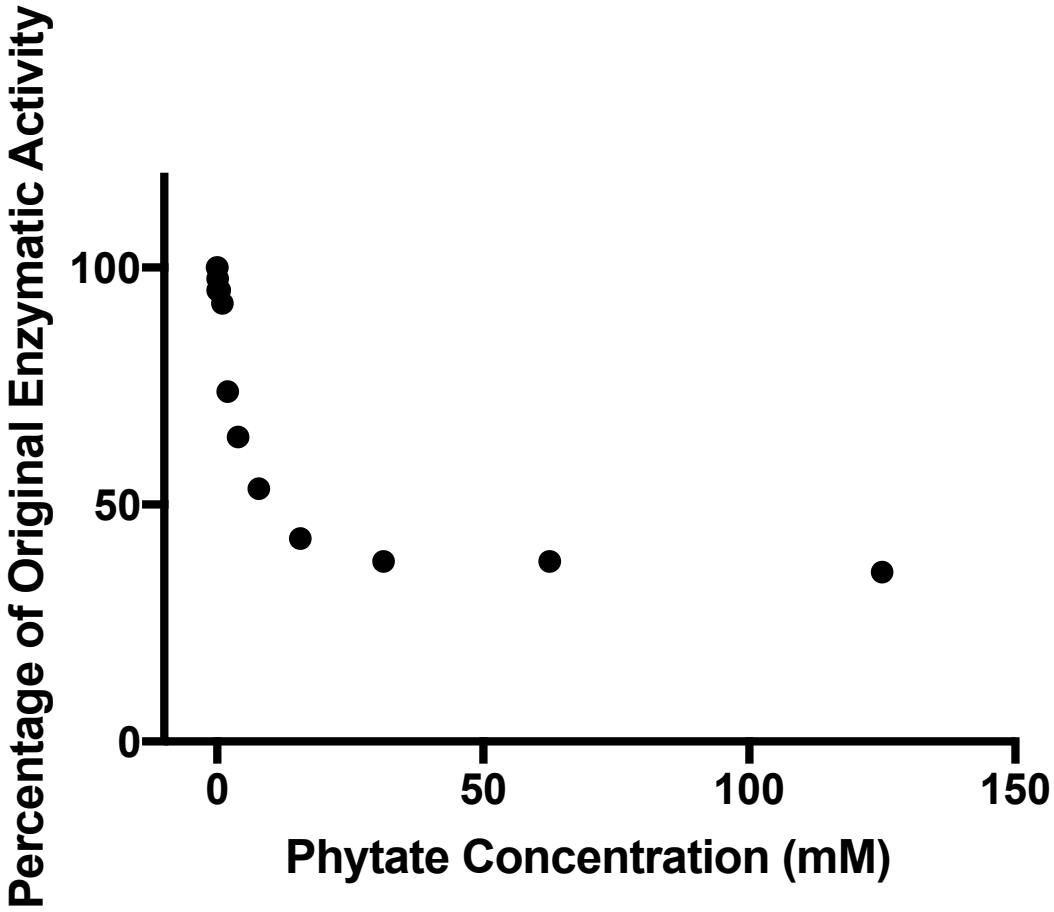


Figure 3. 151: The effect of an array of phytic acid concentrations (0-125mM) on the enzymatic activity of the endogenous trypsin as a percentage of the original enzymatic activity. All samples done in duplicates.

As shown in Figure 3.18, there is a small inhibitory effect caused by the phytic acid on the exogenous chymotrypsin, while the effect is greater as the phytic acid concentration is increasing.

Following that, an experiment for just five of the above concentrations was performed in quintuplicate, to estimate the standard deviation for these. As shown in Figure 3.19, the standard deviation for those is really low.

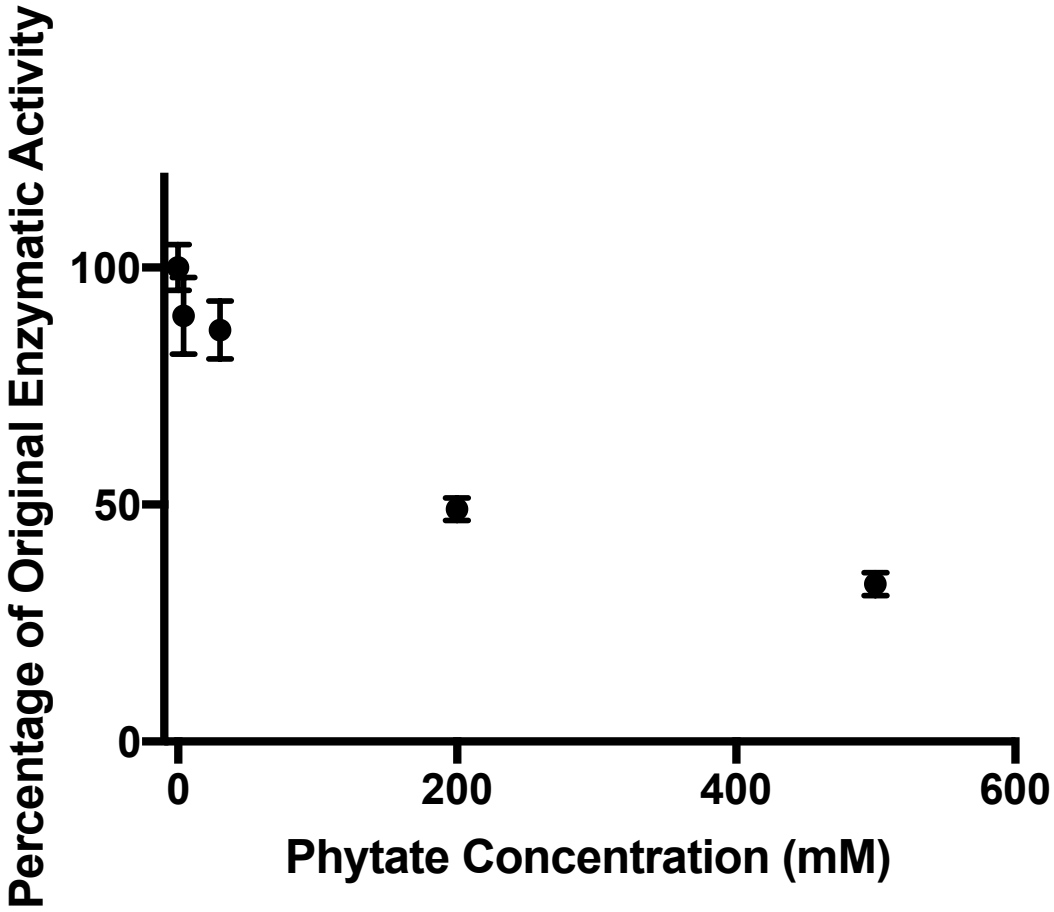


Figure 3.160: The effect of five of phytic acid concentrations (0, 4, 30, 200 & 500mM) on the enzymatic activity of the endogenous trypsin as a percentage of the original enzymatic activity. All done in five replicates, error bars show STDEV.

Endogenous Chymotrypsin

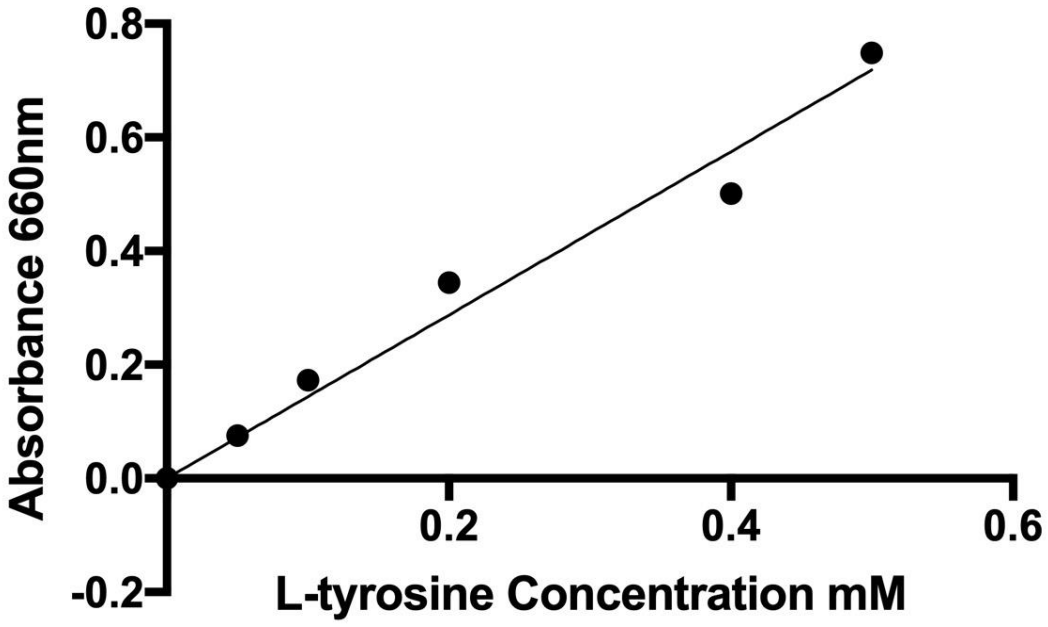


Figure 3.169: Standard curve of L-tyrosine against Absorbance at 660nm, created for the calculations of the enzymatic activity of the endogenous chymotrypsin.

After performing the standard curve experiment, while conducting the experiments exogenous chymotrypsin experiments, the slope for the Figure 3.20, which was used for the calculations of the enzyme activity of the following experiments, is:

$$x = 1.2383y$$

To identify the optimal concentration for the exogenous chymotrypsin, a linearity curve was created (Figure 3.21).

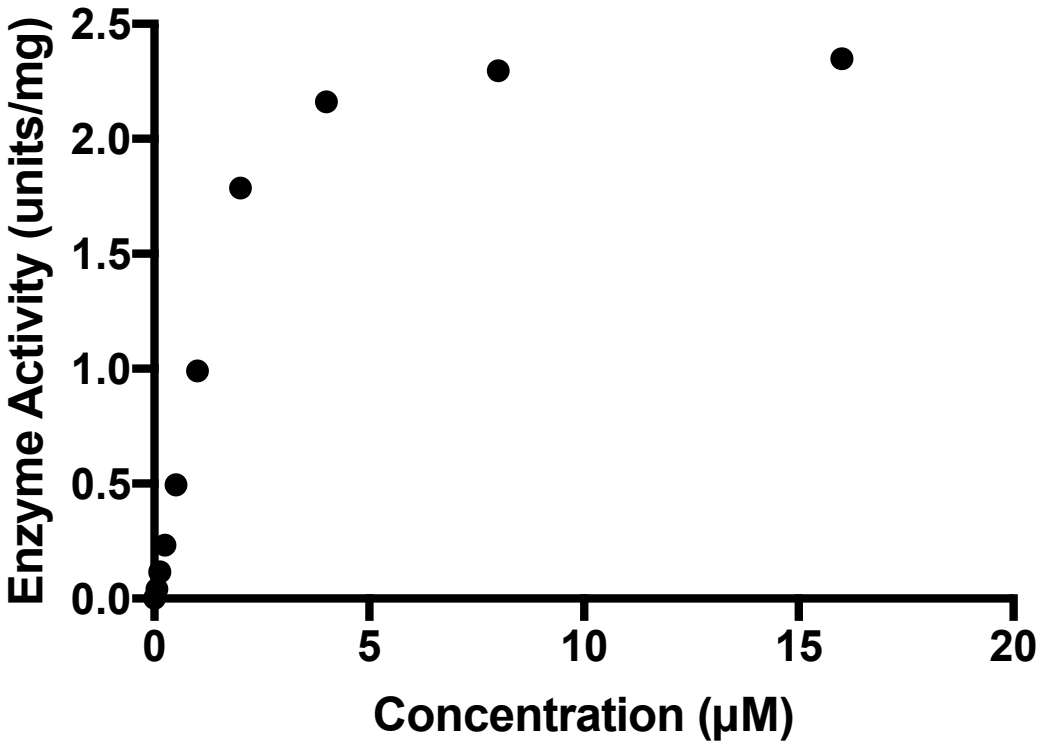


Figure 3.178: Linearity curve of different concentrations of the endogenous chymotrypsin against the enzymatic activity.

Since higher concentrations of the enzyme create a non-linear curve (Figure 3.21), the same linearity experiment was performed for lower concentrations (0-2 μM).

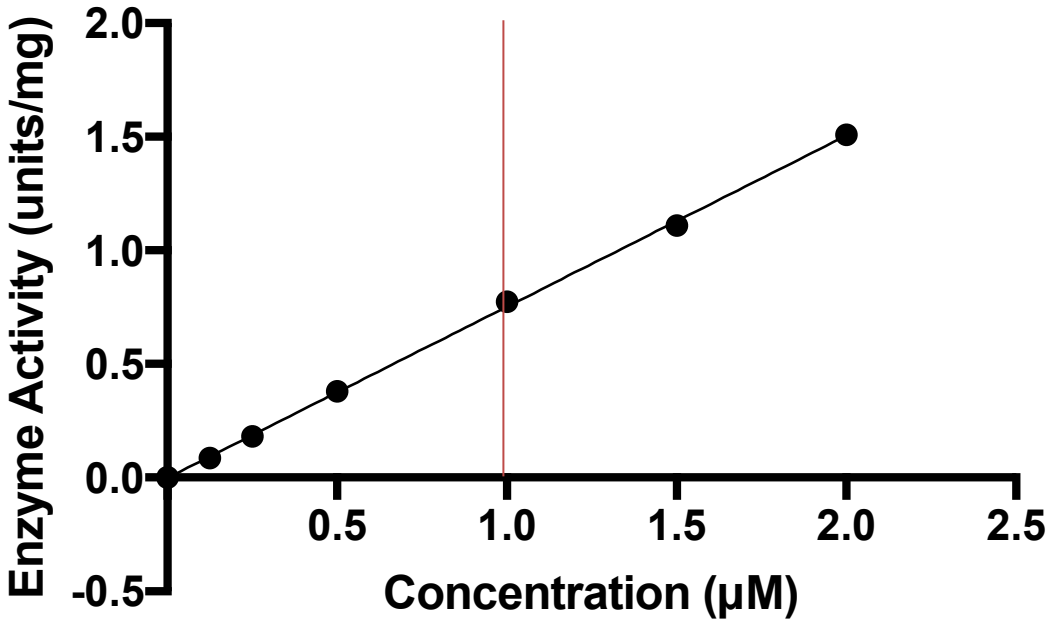


Figure 3.187: Linearity curve of different concentrations of the endogenous chymotrypsin against the enzymatic activity on a lower scale to achieve a linear curve.

After performing the same experiment with different concentrations of the exogenous enzyme and creating a standard curve, the 1µM concentration was selected as the best option for conducting the phytic acid experiments, as it was at the middle of the linear line of concentrations (Figure 3.22).

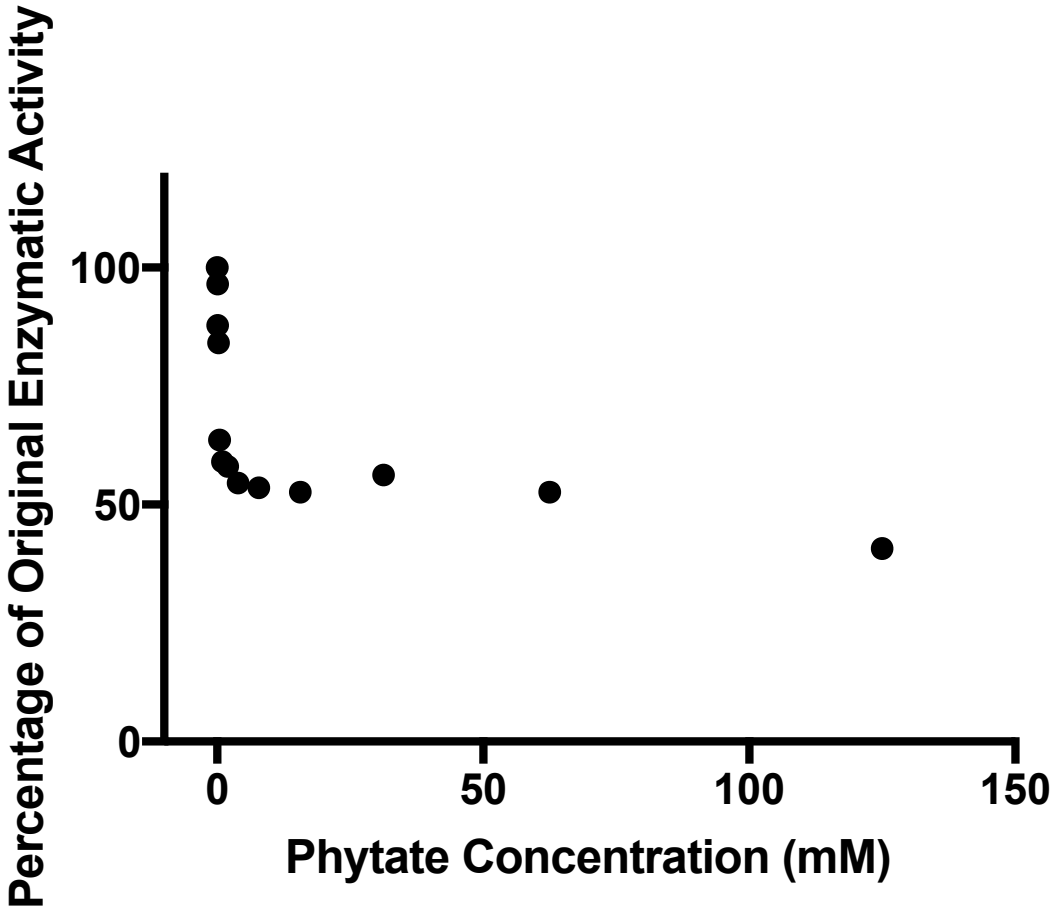


Figure 3.196: The effect of an array of phytic acid concentrations (0-125mM) on the enzymatic activity of the endogenous chymotrypsin as a percentage of the original enzymatic activity. All samples done in duplicate.

As shown in Figure 3.23, there is an inhibitory effect caused by the phytic acid on the exogenous chymotrypsin, while the effect is greater as the phytic acid concentration is increasing. In comparison to the exogenous chymotrypsin, there was a much greater than effect observed for the endogenous chymotrypsin.

Following that, an experiment for just five of the above concentrations was performed in quintuplicate, to estimate the standard deviation for these. As shown in Figure 3.24, the standard deviation for those is really low.

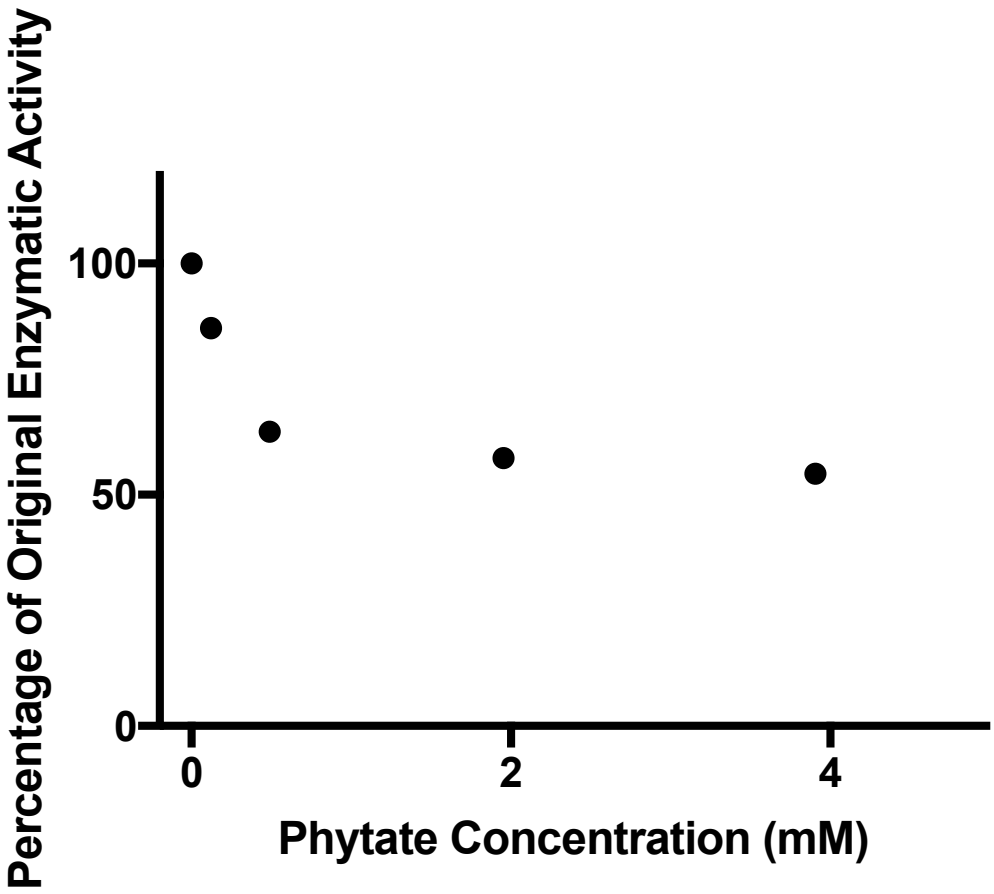


Figure 3.204: The effect of five of phytic acid concentrations (0,0.12, 0.48, 1.95 & 3.90mM) on the enzymatic activity of the endogenous chymotrypsin as a percentage of the original enzymatic activity. All done in five replicates, error bars show STDEV.

3.4.5 GENERAL DISCUSSION

Summarizing the effect of the phytic acid for all three different enzymes, as shown in Table 3.1, it is obvious that in high concentrations there is a great inhibitory effect in all three, with the exogenous chymotrypsin being the least affected by the presence of the phytic acid.

Table 3.6: Percentage of inhibition of different protease from different phytic acid concentrations.

	Phytic Acid Concentration (μM)				
	0.48	1.9	3.9	7.8	15.62
Exogenous Chymotrypsin	3.75%	13.30%	18.65%	29.34%	30.49%
Endogenous Trypsin	4.77%	26.20%	35.72%	46.67%	42.85%
Endogenous Chymotrypsin	26.33%	42.00%	45.48%	46.48%	47.40%

The effect of phytic acid on all three proteases is profound. According to the ratios of exogenous chymotrypsin to phytic acid in sow and broiler diets, there would be no drop on the enzymatic activity in the sow diet, but the drop in the enzymatic

activity (exogenous chymotrypsin) would be 7.5% for broiler chicken diet equivalent.

This is only indicative of the actual effect of the phytic acid on the exogenous chymotrypsin, as the system used is very clean and there are not many other substances/anti-nutritive factors that could also have an effect on the protease activity, like polyphenolics.

Furthermore, the exogenous chymotrypsin is added in great excess to the feed, in order to avoid any such interactions. In contrast to that, the endogenously produced proteases not only are secreted in much lower concentrations by the animal itself, but also since they are not engineered to be as robust and efficient as possible the phytic acid is affecting their activity greatly.

Assuming that the ratio of enzyme and phytic acid is the same for the endogenously produced trypsin, the decrease in trypsin would be around 5% in the sow diet and 7.5% in the broiler diet, which is the same as the exogenous chymotrypsin. While at the same time, the activity of the endogenously produced chymotrypsin would be decreased by 16% in the sow diet and almost 41% in the broiler diet equivalent.

That would have a detrimental effect in the digestion and nutrition of the animals, even in a clean system as this. Therefore, the addition of the exogenously produced chymotrypsin to their diet is necessary, as without its presence, much less protein would be digested and that would not only slow down the growth of the animals, but it would also have a great economic effect to the production, since as

mentioned above, protein is the most expensive and most important nutrient for the animals growth.

In higher concentrations of phytic acid (between 2-8mM), the inhibition of the exogenous chymotrypsin ranges from 13-29%. For the same phytic acid concentrations, the endogenous trypsin is inhibited by 26-46%, while the effect of the phytic acid on the endogenous chymotrypsin's activity is the highest with an inhibition between 42-47%.

These phytic acid concentrations are only 2-8 times higher than the concentration in the broiler chicken. Therefore, if the diet consisted of grains and meals that have a higher phytic acid concentration, the proteases' activity would drop incrementally and that would have all the negative effects that were mentioned above.

In the work published by Khan & Ghosh (2013), who investigated the effect of phytic acid on fish proteases, the results showed a 2 -61.4% inhibition of the protease extracted from three fish species cause by the phytic acid. There was a documented inhibition of proteases even at low phytic acid concentrations. Those results align with the inhibition of the three enzymes by phytic acid as shown in Table 3.1, as the inhibition shown there was from 3.75-47.4%. In another study by Singh & Krikorian (1982), investigated the effect of phytic acid on trypsin, demonstrating inhibition between 2.7-45.9%. Similar to the results of this Chapter, Singh & Krikorian's (1982) work showed a highest inhibition of 45.9%, which was achieved when the enzyme was preincubated with 0.1M of phytate at 37°C.

Deshpande and Damodaran (1989), work on the effect of phytate on solubility, activity and conformation of trypsin and chymotrypsin, revealed that trypsin was inhibited by 2.6% at ratios higher than 10.40:1 of phytic acid:trypsin in acidic pH (3), and reached a 24.3% inhibition for a ration of 52:1. On the other hand, they demostrated that chymotrypsin was less affected as it was inhibited from a ration 14:1 of phytic acid:chymotrypsin at pH 3 only by 1.2% and reached an inhibition of 7.9% at a ratio of 28:1.

Similarly, when Vaintraub & Bulmaga (1991) investigated the effect of phytic acid on the in vitro activity of proteinases, showed that the degree of the enzyme inhibition increases rapidly as the phytate concentration increases and inhibition of up to 90% was observed, while once again the preincubated samples showed a higher inhibition.

The studies discussed above support the results shown in this chapter as phytic acid is certainly binds to proteins and does not allow the proteinases to hydrolyse the substrates.

3.5 CONCLUSIONS

The ITC enzyme activity assay can be used to analyse simple and complex enzymatic reactions, but it can face severe limitations when there is a presence of another energy source or energy intake. In this occasion, the heat of dilution of BSA was so high, that it was covering any heat change coming from the enzymatic reaction and therefore making it impossible to further analyse it.

Despite that, the efficiency of the enzyme was proved by the SDS-Page gel analysis and the Pierce protease activity colorimetric assay kit was used to make sure that such technique would be able to provide good quality data for the interactions of phytic acid and proteases. Since the protease activity kit gave good quality data, sigma's non-specific protease activity assay with casein as a substrate, was used to investigate the potential inhibition of the proteases by the phytic acid.

Different concentrations of phytic acid were used to estimate the effect of it on the enzymatic activity of an exogenously produced chymotrypsin, two endogenously produced proteases, a trypsin and a chymotrypsin. As expected, due to its high negative charge, phytic acid inhibited all three proteases and therefore it is correctly considered to be an anti-nutritive factor. The inhibitory effect of phytic acid on the proteases' activity was profound even in low concentrations, while as the concentration of phytic acid increased, so did its inhibitory effect. Furthermore, it is worth pointing out that the inhibition does not seem to go much higher than 50%, which shows that the enzyme is well engineered to be able to function in the presence of ANFs, but not as efficiently.

The inhibitory effect on the exogenously produced chymotrypsin was lower compared to that on the endogenous proteases, which was also expected, since

this enzyme is engineered to work as efficiently as possible. Therefore, the need for an exogenous protease is profound, as especially in diets with high percentages of phytic acid, the endogenous enzymes would not be able to process all the protein that is provided to the animal, the nutrition would slow down and big amounts of protein would not be utilised and excreted by the animal through its faeces, which would have a high economic impact on the animal farming.

The hypothesis of this Chapter, that phytic acid has an inhibitory effect on the activity of endogenous and exogenous proteases was confirmed.

CHAPTER 4 'INTERACTIONS BETWEEN PHYTASE AND PROTEASES'

4.1 SUMMARY

Enzyme-enzyme interactions have been identified in the past. Those could either prohibit or inhibit the enzymatic reaction each time, depending on the occasion. During the digestion in monogastrics, there is a plethora of enzymes, endogenous that are essential to nutrition and exogenous, which are all necessary for the catalysis of reactions and all aim to the better nutrition of the animal. Despite the fact that the proteases are used to enhance the nutrition, there is a high probability of the proteases hydrolysing other enzymes as long as they carry their binding site. If so, the hydrolysis would be inevitable and that could initiate a chain reaction of multiple problems, with a not so great effect on their digestion.

Hydrolysis of amylases and carbohydrases by a protease has been documented before. In this chapter, the effect of a serine chymotrypsin on the activity of a 6-phytase was estimated initially by using the ITC enzyme activity assay, where different concentrations of chymotrypsin were incubated together with the 6-phytase and then the activity of the 6-phytase was estimated and compared to the original activity without the presence of the chymotrypsin. For a better understanding of those interactions, an SDS-Page gel analysis was performed on the interaction between the two enzymes.

The ITC enzyme activity assay showed a roughly 30% inhibition of the phytase by the presence of the chymotrypsin, while there was a small (10%) positive effect on the phytase activity in the lowest concentrations of chymotrypsin. On the other hand, there was complete hydrolysis of the phytase in the SDS gel analysis. In both scenarios, the hydrolysis of the phytase is profound and is something that needs to be considered.

4.2 INTRODUCTION

The importance of phosphorus as a nutrient for all living organisms is profound. Therefore, the ability of monogastrics to utilize the organic phosphorus present in their feed is essential, as due to the physiology of their nutrition, they are not able to do so naturally. This is thus achieved, by the usage of the exogenously produced phytase to their feed. This is a common practice for the chicken and pig farmers in the past few decades.

Alongside with the phytase though, a variety of other exogenously produced enzymes is added to the feed, such as proteases for enhanced protein digestibility, amylases to make the most out of the starch present in the feed, lipases to release as much energy as possible from the fats and oils present in the feed and a lot more. Except those, there is a plethora of digestive enzymes produced from the animal itself. Those are not identical for pigs and chicken, but they both produce pepsin, trypsin and chymotrypsin, as well as lipase, amylase and carbohydrases.

Proteases have the ability to break down enzymes, but when referring to proteases, a big group of enzymes is considered. There is not just one protease enzyme that breaks down all proteins. Proteases, like every other enzyme, are really specific and they only break down molecules that they can bind to, when those fit the enzyme's binding site. This can be a problem in occasions where a protease breaks down other enzymes that are essential for the catalysis of different reaction and the two enzymes are able to interact and react with each other. An example where this is happening and creating issues, is the catalysis of amylases by proteases, when the amylases are trying to saccharify corn and produce bioethanol, but they are hydrolysed by the proteases at the same time,

because they carry on them the binding site that the proteases are looking for (Pervez et al., 2014). A similar situation could be taking place in the monogastrics' digestion and the proteases to be hydrolysing the phytase. Since the phytase inhibition is allowing an excess of phytic acid, which would then inhibit the proteases' activities. By understanding if there are any interactions between the two enzymes, would provide a big amount of information on the general mapping of the digestion for monogastrics.

As described in Chapter 2, the effect of the polyphenols on the activity of the phytase is profound. Furthermore, the presence of phytic acid during the proteolysis of trypsin, chymotrypsin and the serine chymotrypsin, is inhibiting their activities. Therefore, because of the higher the polyphenols levels of polyphenols in the feed, the inhibition of phytase is also higher. This is leading to a higher concentration of phytic acid present in the stomach, as there is not enough phytase to hydrolyse it. The presence of the phytic acid in the feed is then causing inhibition of the proteases and thus less protein is being digested.

This can have dramatic effects on the animal nutrition, and since protein is the most important and most expensive ingredient of the animal feed, can also have a great economic impact on the farmer.

This makes the digestion a very complex system, as there are a lot of parameters inhibiting and prohibiting the hydrolysis of most substances. As shown in Chapter 2 for instance, polyphenols are inhibiting the activity of phytase. The same way different polyphenols affect the phytase activity differently; in the same way phytate is affecting the activity of different proteases in various ways. It is clear that the matrix that we are dealing with is very complex and all the variants can

cause a different reaction that will have a final result to the nutrient absorption. It is thus very important to study as many of these interactions as possible in order to identify all potential inhibitors and try to eliminate them so that the nutrition is as efficient as possible.

Proteases break down protein to amino acids. Enzymes are biologically proteins too, so the chances are that they could easily be hydrolysed by a protease. Since the possibilities of enzyme-enzyme interactions are definite, the risk of phytases being hydrolysed is a real issue that needs to be dealt with. In the past years there are a few reports on the production of a protease-resistant phytase (Chantasartrasamee *et al.*, 2005; Singh and Satyanarayana, 2006; Schramm *et al.*, 2017).

According to Sapna and Singh (2013), phytase is a vital animal feed supplement, and it is crucial that it is resistant to the trypsin and pepsin action as these proteases are present in animal digestive tracts. The phytase's activity was not greatly affected when incubated with trypsin and pepsin, but after an over 60-minute treatment with trypsin, there was a 30-35% reduction of the enzymatic activity, while pepsin had absolutely no effect on phytase activity. Since the difference between trypsin and chymotrypsin is that they select different amino acids for hydrolysis, chymotrypsin could also have an effect on the phytase activity, but this needs to be investigated.

Enzyme inhibitors can reduce the rate of an enzyme catalysing a reaction by interfering with the enzyme in some way. This can be either a temporary inhibition, or a permanent one. Competitive enzyme inhibitors have similar shape to the substrate and the enzyme creates a formation with them instead of

formatting enzyme-substrate complexes (Bjelaković, 2002). This is usually a temporary effect, and it is affected by the concentration of the inhibitor and substrate, as they are the competitors for the active site. On the other hand, non-competitive enzyme inhibitors work by not allowing the substrate to react with the enzyme and create the product of the reaction. There are many non-competitive enzyme inhibitors and most of them cause a non-reversible/permanent inhibition (Najapfour, 2007).

Despite it is stated that there are not significant interactions between the chymotrypsin and phytase in the publication 'Safety and efficacy of Ronozyme® ProAct (serine protease) for use as feed additive for chickens for fattening' (2009), it still is unclear if there are any positive or negative interactions between the enzymes, as that conclusion was only achieved by using both enzymes in animal trials. That could have masked any interactions that could be taking place between phytase and chymotrypsin, as the presence of other substances, as temperature, pH and the presence of salts can also have hidden the interactions (Niu et al., 2020, Zhao et al. (2010)). Furthermore, if one or both enzymes were used in big excess that could have also been a reason why no interactions are found. Finally, the composition of the feed can differentiate massively between different farms. It can also change throughout the different seasons depending on the availability of grains. Finally feed composition can vary based on the area where the farm is based, as different grains grow in different climates and their costs differentiate accordingly. All these parameters can affect the availability and cost of the grains used for the diet of the animals.

The objective of this chapter is to identify any interactions between the 6-phytase and the chymotrypsin, in a cleaner, more regulated environment. Those interactions could have either a positive or a negative effect on the phytase's activity, or there could also be no effect whatsoever. Such an analysis would provide more in-depth information on any interactions. In that way, there would be a full understanding the relationship between the two enzymes, which then can be used to have a more holistic mapping of interactions between phytase, proteases, polyphenols and phytic acid. As shown in Figure 4.1, the interactions between the proteases and phytase would be the missing part, to complete the mapping of this whole system.

The hypothesis of this Chapter is that phytase is being degraded by proteases.

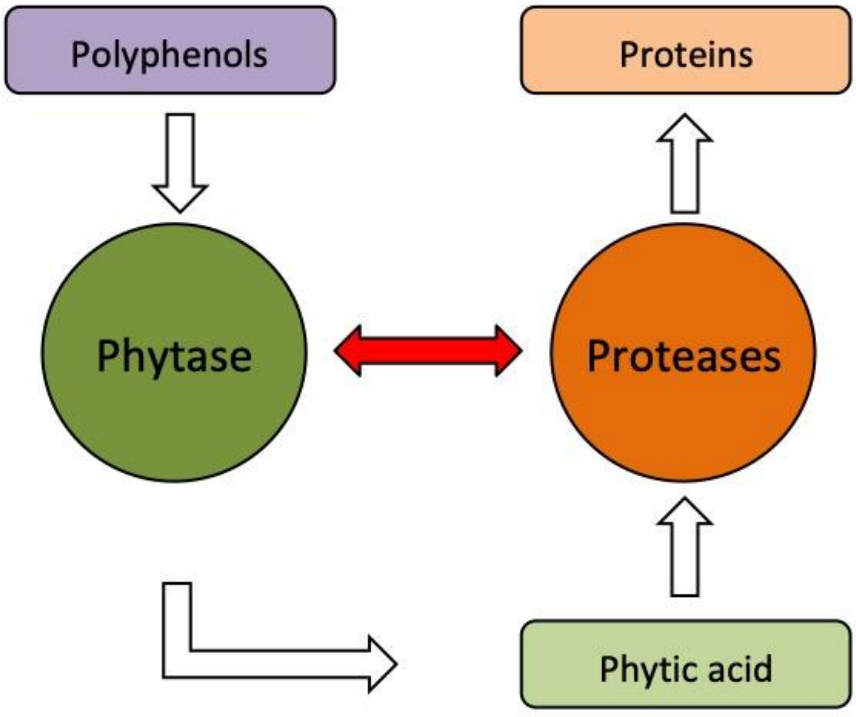


Figure 4.2: Map of interactions between phytic acid, phytase, protein, proteases and polyphenolic compounds. As shown with the red arrow, in this chapter the focus will be on the interactions of proteases and phytase.

4.3 MATERIALS AND METHODS

4.3.1 CHEMICALS AND MATERIALS

- Phytic acid sodium salt hydrate, rice extract, Sodium chloride and Sodium acetate were sourced from Sigma Aldrich, Gillingham, UK.
- 6-phytase was sourced from DSM, Kaiseraugst, Switzerland.
- Serine protease (chymotrypsin) was sourced from DSM, Kaiseraugst, Switzerland.
- PageBlue Protein Staining Solution, Thermo Scientific, Waltham, Massachusetts, USA.
- MES SDS Running Buffer (20X), Thermo Scientific, Waltham, Massachusetts, USA.
- Bolt™ 12% Bis-Tris Plus 1.0 mmX 12 well, Thermo Scientific, Waltham, Massachusetts, USA.
- NuPAGE LDS Sample Buffer (4X), Thermo Scientific, Waltham, Massachusetts, USA.
- Novex XCell SureLock Mini-Cell, Thermo Scientific, Waltham, Massachusetts, USA.
- LABOSI PowerPro 300, Fisher Scientific, Hampton, New Hampshire, USA.
- LI-COR Biosciences Chameleon Duo Pre-Stained Protein Ladder, Thermo Scientific, Waltham, Massachusetts, USA.
- BioSpectrum 410 Imaging system, 230V~50Hz, UVP, LLC Upland, CA. Ultra-Violert Products Ltd., Cambridge, UK.
- Eppendorf Thermomixer C, Eppendorf, UK Limited, Stevenage, UK.

4.3.2 ITC

Initially, the experimental idea was to use the ITC enzyme activity assay that was used in chapter 2, to estimate the effect of different concentrations of the exogenous chymotrypsin on the phytase activity. To do so, the following steps were followed.

4.3.2.1 ITC SAMPLE PREPARATION

A 1 μM 6-phytase, originating from *Citrobacter braakii* with a minimum phytase activity of 10,000 FYT/g, and a 1 μM serine chymotrypsin, from *Bacillus licheniformis* containing a minimum protease activity of 75,000 protease activity units/g, stocks were prepared by appropriately diluting the enzyme solution provided by DSM into MilliQ water. Respectively, a 20 Mm phytic acid stock was prepared by dissolving the appropriate mass of phytic acid into MilliQ water.

Except for the single enzyme samples, two mixed enzyme solutions were prepared. The first contained phytase and chymotrypsin in a 1:1 ratio, while the second one also had both enzymes in a 1:8 ratio respectively. Both solutions were incubated at 30 °C for 30 minutes, before they were used for the ITC experiment.

4.3.2.2 ITC ENZYME ACTIVITY ASSAY

To estimate the enzymatic activity of the 6-phytase in the presence of the serine chymotrypsin, different concentrations of the chymotrypsin were incubated with the 6-phytase prior to placing it into the sample cell. Following that, each enzyme solution was placed into the sample cell and phytic acid was injected in it to estimate the effect of the chymotrypsin on the enzymatic activity of the 6-phytase.

To analyse this data, the exact same method that was used in chapter 2 to estimate the effect of polyphenol-rich extracts on the activity of the phytase was used here. To estimate the relative activity of the phytase, the inverse of the time for 90% completion of the phytase catalysed reaction to occur was used as activity in this assay. That was achieved by estimating the rate of the reaction and then measuring the time needed for the completion of 90% of the reaction. Then by using this as the positive control, the effect of different substances on the enzymatic activity was estimated by comparing their reaction completion time to the standard. The enzyme assay used a TA Instruments NanoITC; injecting 5 μL of 20 mM phytate into 300 μL of 0.125 μM 6-phytase. All reactions were performed in duplicates and the mean was calculated.

4.3.3 SDS-PAGE

Following the ITC analysis, an SDS-page gel analysis was performed to verify those results. To do so, the phytase was incubated with the chymotrypsin and then run into a gel to estimate the percentage of degradation. To do so, the following steps were followed.

4.3.3.1 SDS-PAGE GEL ANALYSIS

20μL of each sample, 7.6μL of the sample buffer and 3.0μL of reducing agent were added together. The mixture was heated to 90°C for 5 min and following that 10μL was loaded in each well of the gel. Electrophoresis was performed in the running buffer (1×) at 200V voltage for 45 minutes. Protein standard was loaded on the gel as molecular weight marker. After electrophoresis, the gel was stained using

PageBlue Protein Staining Solution and then de-stained with water, to remove any leftovers of the dye and it was scanned at a BioSpectrum 410 Imaging system. The images of the gels were then used for further analysis.

In each of the three replicates of the gels, concentrations from 0-5mg/ml of the 6-phytase were loaded into the first six wells. Chymotrypsin was loaded in well number 7 at a concentration of 5mg/ml. in well number 8 was the ladder and in wells 9 and 11 was the solution containing both phytase and chymotrypsin in a ratio 1:1, while in cells 10 and 12 were loaded with the solution containing both phytase and chymotrypsin in a ratio of 8:1 respectively.

4.3.3.2 SDS-PAGE GEL DATA ANALYSIS

To quantify the phytase levels in the gels, a standard curve was created using different concentrations of the phytase and analysed them using the ImageJ software, which allows quantification of bands according to their intensity.

Using the equation of the standard curve, would allow quantification of the samples that contained both the phytase and the chymotrypsin, to estimate if there is a degradation of the phytase. This analysis was based on the work published by Carter *et al.*, (2013).

4.4 RESULTS AND DISCUSSION

4.4.1 ITC ENZYME ACTIVITY ASSAY FOR 6-PHYTASE IN THE PRESENCE OF SERINE CHYMOTRYPSIN

After a 30-minute incubation of the 6-phytase with different concentrations of the serine chymotrypsin, the activity of the 6-phytase was estimated using the ITC enzyme activity assay that was also used in Chapter 2. The reaction between phytic acid and phytase (Figure 4.2(a)) without the presence of any protease was used as a negative control.

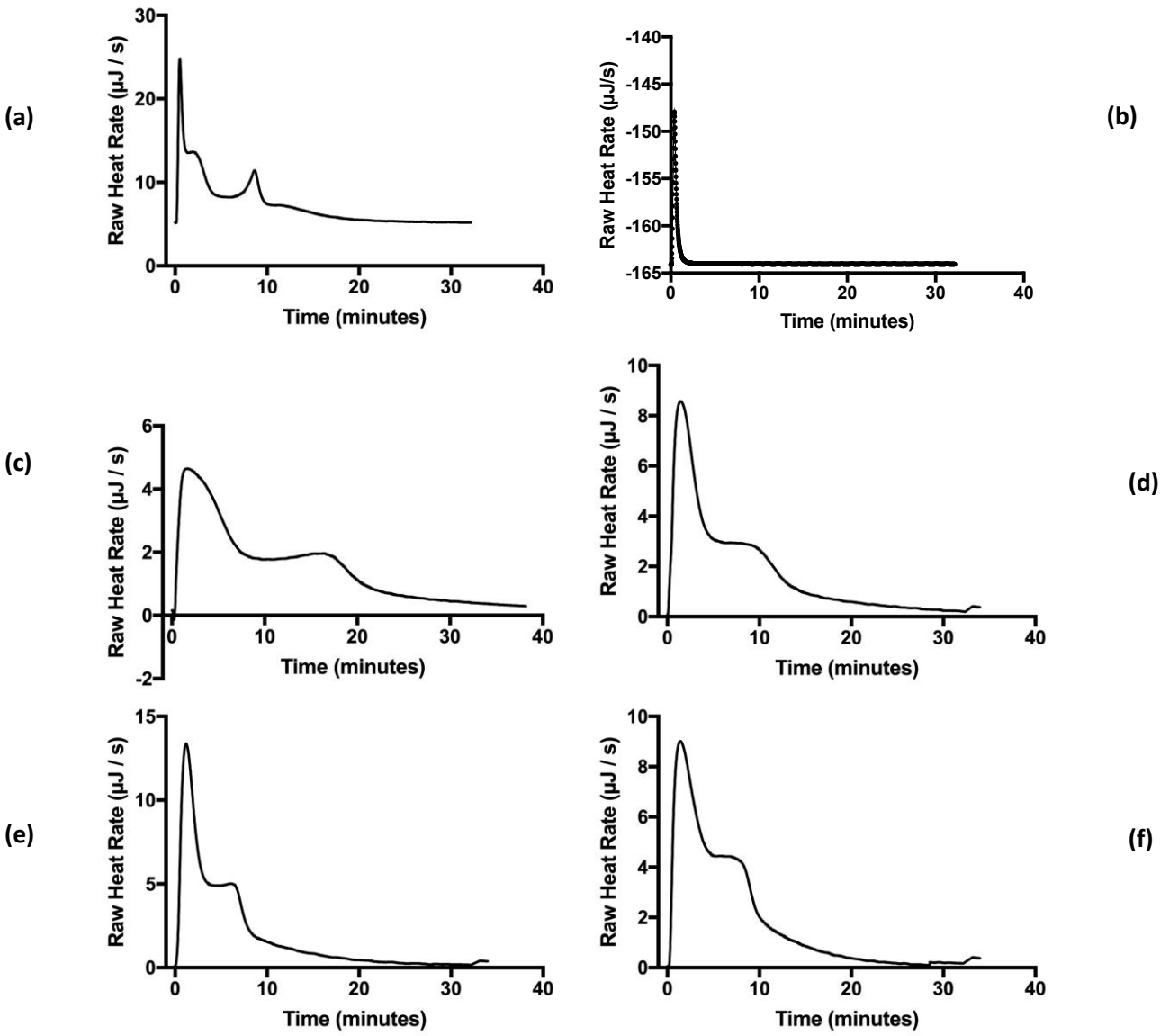


Figure 4.6: The raw heat rate of (a) the phytic acid - phytase reaction, (b) phytic acid injected into MilliQ water (Blank), the phytic acid - phytase reaction in the presence of (c) 1, (d) 0.5, (e) 0.25 and (f) 0.125 mg / ml chymotrypsin.

The raw heat rate of the reaction between the phytase and the phytic acid under different conditions is shown in Figure 4.2. Figure 4.2a shows the reaction between the enzyme and the phytic acid, without the presence of chymotrypsin. Figure 4.2b is the blank, which is phytic acid titrated into MilliQ water. Figures

4.2c to Figure 4.2f represent the reaction between phytase and phytic acid in the presence of different concentration of chymotrypsin, 1-0.125mg/ml respectively.

The analysis of this data was carried out by first deducting the blank from each reaction. Following that, the activity of the 6-phytase without the presence of the chymotrypsin was estimated as a function of time (90% completion of the reaction).

As shown in Figure 4.3, the relative activity of the phytase is slightly increasing in low concentrations of chymotrypsin (0.125 and 0.25 μM), by 8.5% to 14.5% respectively. Despite that slight increase, for higher concentrations (0.5 and 1 μM), the relative activity of the phytase is decreasing by 11.5% and 29% respectively.

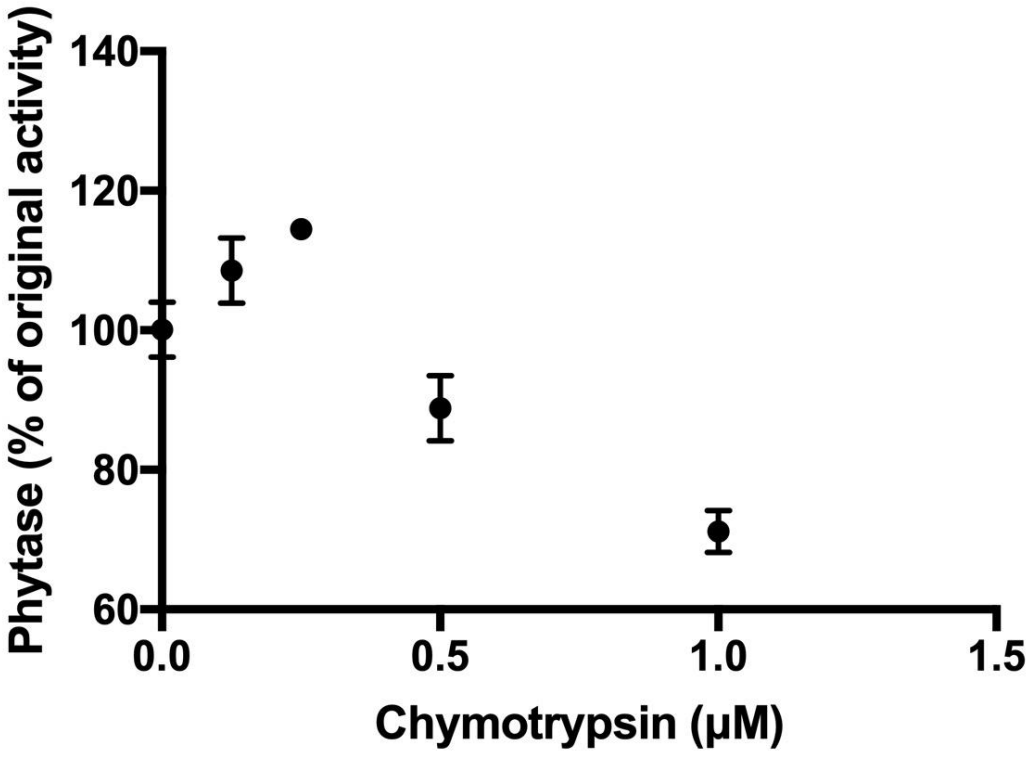


Figure 4.19: The effect of the presence of different concentrations of chymotrypsin on the relative activity of 6-phytase, using the ITC enzyme activity assay.

As the presence of proteases (endogenous and exogenous) is quite high during digestion in monogastrics, the higher concentrations of the chymotrypsin are the most important out of Figure 4.3. This is because those are more likely to represent the actual ratio of phytase and protease in the digestive tract. Since the phytase activity is decreased and the only substances present in the solution are the phytase, the phytic acid and the chymotrypsin, it is very likely, if not definite that the phytase is being hydrolysed by the chymotrypsin, which was the hypothesis on which this chapter was based on.

On the other hand, the increase on the activity of the phytase in the lower concentrations is something that is also interesting scientifically and it would be worth exploring further.

To confirm the ITC enzyme activity assay results, an SDS-Page gel analysis was done for the same reaction.

4.4.2 SDS-PAGE GEL ANALYSIS FOR THE INTERACTION BETWEEN THE 6-PHYTASE AND THE SERINE CHYMOTRYPSIN

Using the method for quantification of the protein bands as described by Carter et al., 2013, a standard curve was created, using different concentrations of the 6-phytase (Figure 4.4). The reaction samples of phytase and chymotrypsin were incubated for 30 minutes at 30 degrees Celsius and then injected into the gel wells for analysis. Except the reaction and the standard curve samples, chymotrypsin was also injected into a cell as a standard. Two ratios of phytase and chymotrypsin

were used, 1:1 and 1:8 Figures 4.5a, 4.5b & 4.5c; show the triplicates of the SDS-Page gels that were used for the calculations below.

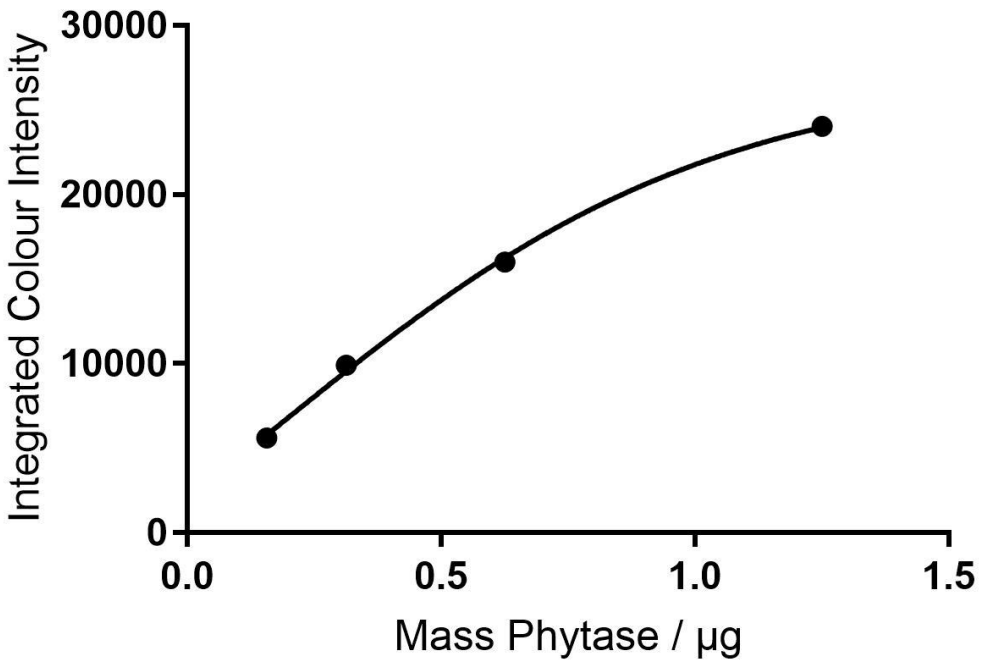
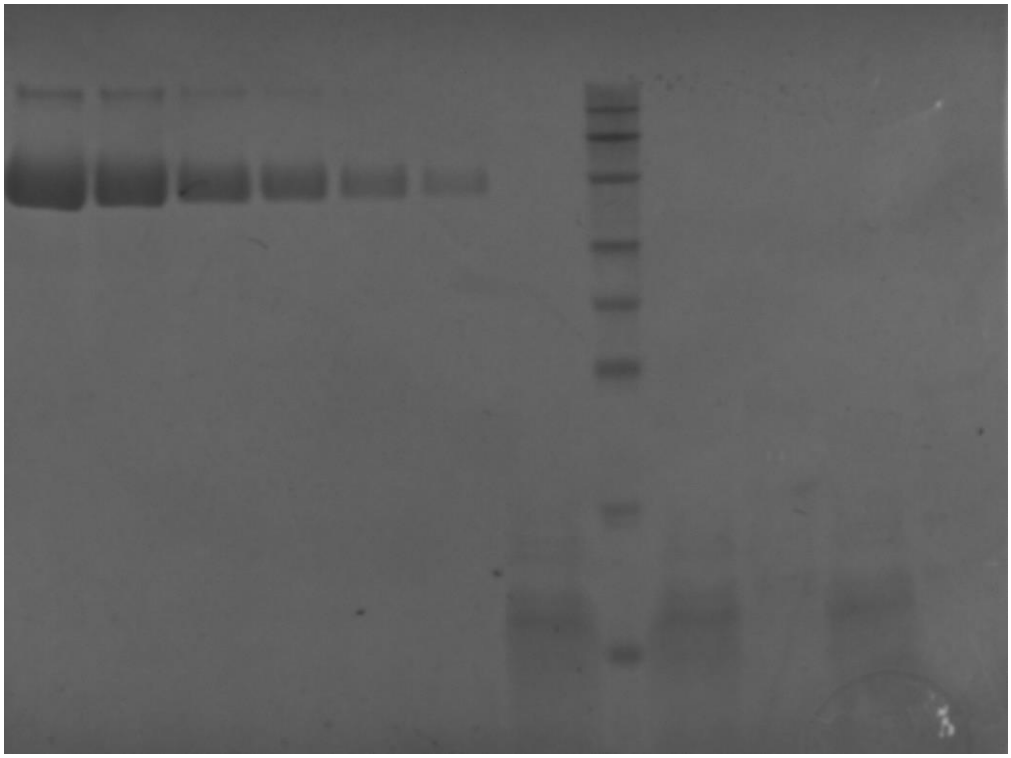
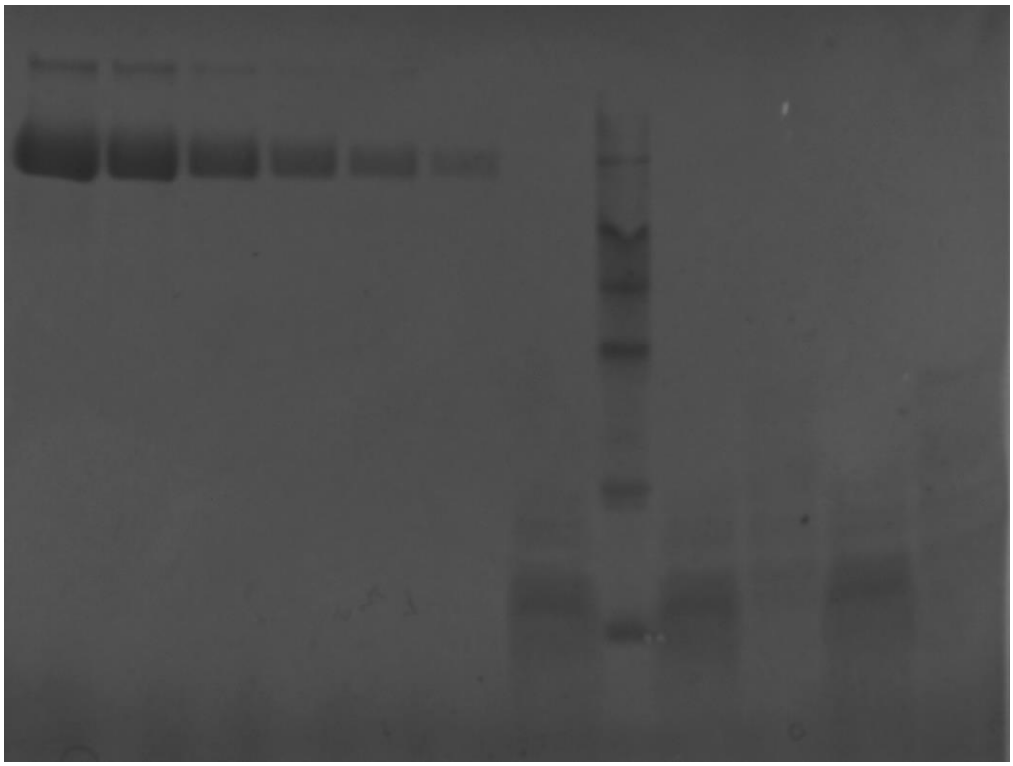


Figure 4.27: Standard curve of different concentrations of 6-phytase (0-5 mg/ml), against the integrated colour intensity, estimated using the ImageJ software.



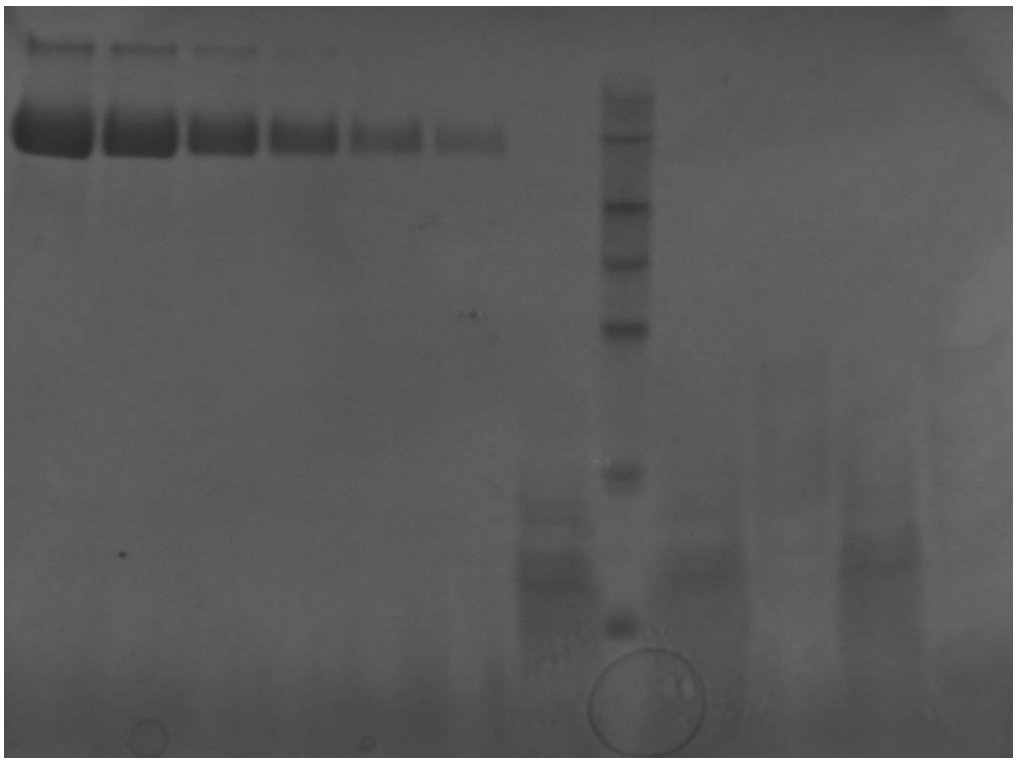
4.5A

Well: 1 2 3 4 5 6 7 8 9 10 11 12



4.5B

Well: 1 2 3 4 5 6 7 8 9 10 11 12



4.5C

Figure 4.35 A, B & C: Triplicates of the SDS gel electrophoresis of 6-phytase in concentrations between 0-5 mg/ml, chymotrypsin, ladder and samples of the interaction between the 6-phytase and the chymotrypsin in 1:1 and 1:8 ratio.

Using the ImageJ software, the masses for the phytase were quantified and a standard curve (Figure 4.4) was created. Using the curve's equation stated below and the integrated photo intensity of the bands, the concentration of the phytase in the reaction samples was back calculated. The equation coming from the standard curve is as follows:

$\boldsymbol{y = 8856.3ln(x) + 21123,}$ with $\mathbf{R^2 = 0.9819}$

Using this equation, the mass of phytase per μg was back calculated both for 1:1 and 8:1 ratio. As shown in Figure 4.5 Wells 9-12, the whole amount of phytase was

hydrolyzed by the chymotrypsin in both 1:1 and 1:8 ratio of phytase to chymotrypsin respectively. This is interesting, as it is not identical, but similar to the ITC enzyme activity assay result., which shows a partial inhibition of the enzyme, but not a complete degradation of the enzyme. In the case of the complete degradation of the phytase by the chymotrypsin, the reaction between phytase and phytic acid would not have taken place at all and the thermogram would be identical to Figure 4.2(b) which represents the titration of phytic acid in the cell containing only MilliQ water.

As shown in Table 4.1, all replicates or the reaction for both ratios had a complete digestion of the 6-phytase by the chymotrypsin.

Table 4.6: Percentage of the 6-phytase hydrolysed by the chymotrypsin in 1:1 and 8:1 ratio in all SDS-Page gel replicates, after ImageJ analysis.

Sample	**6-Phytase:Chymotrypsin ratio**	
	1:1	**8:1**
Gel 3A Replicate 1	100%	100%
Gel 3A Replicate 2	100%	100%
Gel 3B Replicate 1	100%	100%
Gel 3B Replicate 2	100%	100%
Gel 3C Replicate 1	100%	100%
Gel 3C Replicate 2	100%	100%

Despite the differences in the results between the two assays, the hydrolysis of the phytase by the chymotrypsin was expected as it has been stated multiple times in the literature that proteases tend to hydrolyse other enzymes when they can identify their binding site on the other enzyme, as enzymes are also proteins.

This indicates that the exogenous chymotrypsin is cleaving the phytase and that it should be further investigated, but at the same time provides information on the differences of the two types of analysis used for this experiment.

The ITC data indicates that there is cleaving taking place as the different concentrations of chymotrypsin slow down the activity of the phytase, but yet the phytase is still active. On the other hand, the SDS-page gel analysis shows complete degradation of the phytase by the chymotrypsin. This could be due to the different limitations of each technique. Which could alter the results accordingly.

In a study by Singh (2013) phytase that was treated with trypsin resulted in 27% and 38% reduction of the phytase activity after 60 and 120 minutes, respectively, while there was no significant effect on phytase activity when treated with pepsin. It is worth mentioning that this phytase that is protease-resistant by *Aspergillus oryzae* and yet it still was inhibited in the presence of trypsin. When Zhao et al. (2010) tried to engineer a protease-resistant phytase from *Penicillium* sp., they measured its resistance to proteases. The results showed that the phytase was sensitive to the trypsin digestion and a percentage as high as 80% of its activity was lost after a 2h incubation with trypsin ratio of 0.001 (w/w). Hence confirming the possibility of phytase degradation by trypsin. Furthermore, they used SDS-PAGE to investigate this interaction and results revealed that it was digested

completely. Having similar results with trypsin and using the same technique (SDS-PAGE) supports the results reported in this chapter.

Similarly, Kim et al. (2003) investigated the effect of proteases on the activity of a *Citrobacter braakii* phytase, similar to the phytase used for the experiments of this thesis. Kim et al. incubated their phytase with different proteases including pepsin, trypsin, elastase, pancreatin, and papain for 2 h at 37 Degrees Celsius. Results showed that the phytase activity was not significantly changed with either pepsin or trypsin, but papain, elastase and pancreatin reduced the phytase activity by 15, 20 and 30% respectively compared to the original activity. Finally, when Niu et al. (2020) worked on *Yersinia* phytases to improve pepsin and trypsin resistance, they discovered that the activity of two out of four phytases had a drop on their activity to 88.7−90.6% after 2 h of trypsin treatment, while the other two lost 40−46% activity. Niu et al.'s results were as expected given that the first two phytases were engineered to have a higher protease resistance. Comparing the effect of the trypsin to the latter two phytases, to the results of this chapter, the inhibition is of a similar level (~45%), supporting the results shown above.

4.5 CONCLUSIONS

The difference between the two assays is the way the samples were incubated at 30 degrees Celsius. Prior to the ITC assay, the chymotrypsin and the phytase were incubated in the ITC itself, while prior to the SDS-Page gel analysis, the samples were incubated in water bath at 30^{o}C for 30 minutes. The two different types of incubation, water bath vs. ITC could potentially have a diverse effect, as the water bath's temperature could have a deviation from the 30 degrees Celsius, shown on the thermometer. The difference in temperature could easily affect the speed and the efficiency of the chymotrypsin's reaction rate.

In any case, both the ITC enzyme activity assay and the SDS-Page gel analysis, show that there is an interaction between the two enzymes, which is a result that both DSM and the users of these enzymes, should be aware of and manage the ratios of the two enzymes accordingly, to avoid the complete inhibition of the phytase by the chymotrypsin, as this could trigger other problems, such as the inhibition of proteases by the excess of phytic acid present in the digestive tract.

To conclude, there is a definite inhibition of the phytase activity, caused by the presence of the exogenous chymotrypsin. Therefore, it is apparent that there is an interaction taking place between the phytase and the chymotrypsin and although according to the ITC assay there is a partial inhibition of the reaction, while the SDS-PAGE analysis showed full hydrolysis of the phytase.

The hypothesis of this Chapter, that phytase is being degraded by proteases was confirmed.

CHAPTER 5 'GENERAL DISCUSSION'

5.1. GENERAL CHEMISTRY

The inhibitory effect of anti-nutritive factors such as phytic acid and polyphenolic compounds on various enzymes' activity is already established. Phytic acid is known to inhibit trypsin (Selle *et al.*, 2000), as well as other enzymes like a-amylase (Khan and Ghosh, 2013) or lipase (Onyango, Asem and Adeola, 2009) and to bind to proteins, making them unavailable to digestion (Van Der Poel, 1990). Polyphenolics and tannins also have a similar effect on protein digestion according to Van Der Poel, 1990. Furthermore, berry polyphenolics have been found to inhibit proteases (McDougall, Gordon and Stewart, 2005).

The chemistry behind the inhibition caused both by the phytic acid and the polyphenols is well known. Due to their high negative charge, they have a tendency to bind positively charged molecules and, in that way, making them unavailable to the enzymes.

These interactions not only affect the animal nutrition, but at the same time have a negative environmental impact, as there is phosphorus pollution taking places in water deposits, due to the high amount of phytic acid being excreted by the animal and goes into the wastewater (see Appendix A2). This could be avoided with a better utilisation of the phytate available in the grains used in the animal feed.

The novelty in this study is, estimating their effect on commercially available, exogenously produced enzymes (phytase and chymotrypsin), while at the same time using polyphenols extracted from sorghum, a grain known for its high concentration in polyphenolics and tannins that is commonly part of monogastrics' feed composition, especially in Australia and South East Asia.

Goel and Sharma (1979) showed that phloroglucinol was inhibiting the activity of plant phytases and Quesada et al. (1996) found that cocoa, pear and lentil phenolic-rich extracts, containing proanthocyanidins and condensed tannins, were also inhibiting the activity. These two studies support the results of Chapter 2. Other studies showed that phytases can be inhibited by other substances like glucose, EDTA, Zn^{+2}, Ba^{+2}, Cu^{+2}, Cd^{2+}, Fe^{2+} and Al^{+3}) according to Liu et al. (1998). A remarkable point is that there is certainly a lack of studies on the effect of polyphenolic compounds on phytase activity. There is certainly a need for more publications in this field as there is a plethora of compounds that could have an effect on phytase and should be identified.

Khan & Ghosh (2013), showed inhibition on three proteases caused by phytic acid in similar levels to the data shown in this study. Similar results were also published by Singh & Krikorian (1982), demonstrating inhibition of trypsin by phytic acid, as well as Deshpande and Damodaran (1989), who also revealed trypsin inhibition by phytic acid. Finally, Vaintraub & Bulmaga (1991) showed inhibition of proteinases by phytic acid supporting the results of Chapter 3.

Singh (2013) published a study showing the inhibition of trypsin by phytase and Zhao et al. (2010) published a similar study with similar results, but also successfully engineered a phytase to be more protease-resistant and still demonstrated an inhibition. Kim et al. (2003), also showed a phytase inhibition by a variety of proteases, as did Niu et al. (2020) who studied the effect of trypsin on four phytases, that were all inhibited at different levels. All these studies support the results of Chapter 4, while some even used SDS-PAGE to investigate the interactions between phytase and proteases.

5.1.1 MAPPING THE PHYTASE, PROTEASE AND ANTI-NUTRITIVE FACTORS SYSTEM

All three hypotheses of this thesis are shown in Figure 5.1, polyphenolic compounds have an inhibitory effect on the phytase/phytic acid reaction. Furthermore, phytic acid is decreasing the activity of endogenous and exogenous proteases. Finally, there is an interaction between the phytase and the proteases, which means that this is also causing an inhibitory effect on the phytase activity.

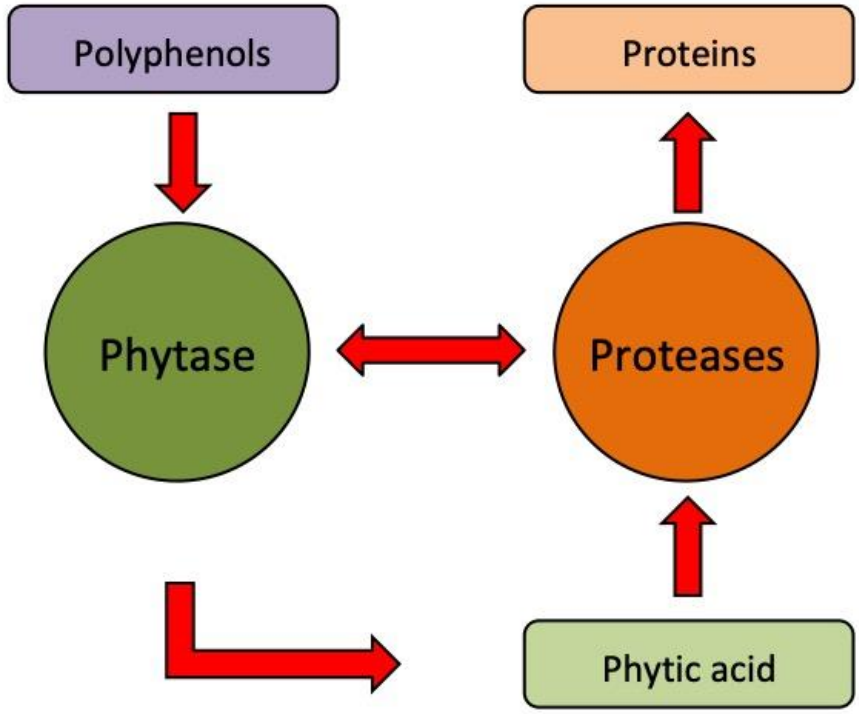


Figure 5.7: Map of interactions between phytic acid, phytase, protein, proteases and polyphenolic compounds.

Putting that into context, the more polyphenolics, the less active the phytase. This then translates into higher concentrations of phytic acid in the stomach of the animal, which is then itself causing an inhibitory effect on the proteases' activity.

At the same time, some proteases might be degrading phytase instead of proteins, which would increase the phytic acid ratio in the feed and cause extra inhibition to the proteases.

5.1.2 COMPARISON OF DIFFERENT ENZYMATIC ACTIVITY TECHNIQUES

Although the hypotheses for chapter 2 and chapter 3 were similar and theoretically could be investigated using the same technique, that was not possible. Due to limitations of the ITC enzyme activity assay and the nature of the biologics used for each experiment, a colorimetric assay had to be used for the understanding of the inhibitory effect of phytic acid on proteases.

Not only a different assay had to be used for the proteases, but also due to the nature of the data, even the ITC enzyme activity assay was not used in the conventional way. As shown in the literature, this assay is commonly used for investigating the enzymatic kinetics in simple enzymatic reactions, by estimating the ΔH, Km and Vmax values.

This was not possible for the estimation of the activity of phytase, as its reaction with phytic acid is a complex, multiple-stage reaction that creates a complex thermogram, which does not allow the conventional enzymatic kinetics calculations. To overcome that issue, a different approach was adopted to interpret the data. Instead of calculating the ΔH, Km and Vmax values, the relative activity of the enzyme was estimated. That was achieved by estimating the rate of the reaction and then measuring the time needed for the completion of 90% of the reaction. Then by using this as the positive control, the effect of different

substances on the enzymatic activity was estimated by comparing their reaction completion time to the standard. With that information the activity of the enzyme under different conditions could be calculated, relatively, based on the time needed for the completion of the 90% of the reaction in standard conditions.

5.2. GUT COMPLEXITY ON ENZYME INTERACTIONS

5.2.1. ANTI-NUTRITIVE FACTORS

Other than the polyphenolic compounds and phytic acid, other anti-nutritive factors are the substances like protease inhibitors, haemagglutinin, glucosinolates, cyanogen, saponins, phytoestrogens, gossypol, antivitamins, amylase, invertase, cholinesterase and arginase inhibitors, dihydroxyphenylalanine, mimosine and cyclopropenoic acids (Tadele Yilkal., 2015). Out of those, the impact of protease inhibitors is of great importance and can be directly related to the results as they can affect the proteases' enzymatic activity. They are usually found in legume seeds like soybean, kidney beans and mung bean. They are separated into two main categories; Kunitz inhibitors, which inhibit trypsin and Bowman-birk inhibitors that inhibit both trypsin and chymotrypsin. Their inhibitory effect can be inactivated by heat treatment. Haemagglutinins are present in soybean and other legumes and they have a toxic effect on animals. Saponins have a great effect in the monogastrics diet as they can have a severe impact on monogastrics (Tadele Yilkal., 2015). They can cause a slow-down on the growth rate, since they cause a reduction in feed intake. Especially in poultry, as they are more susceptible than pigs, a low amount of saponins in the feed (0.4- 0.5%) can cause major feed consumption, egg production and body weight loss in birds.

Cyanogenic glycosides that are naturally present in legumes can be cleaved by the action of the β-glucosidase enzyme producing cyanide, which is a toxicant. Other anti-nutritional compounds in legumes include α-amylase inhibitors, saponins, allergens, and toxic amino acids that have been known to exhibit antivitamin and

anti-hormonal activity (Dalgetty & Baik, 2003). Oxalic acid is mainly affecting monogastrics in a way very similar to the phytic acid. The response to oxalate poisoning is dependent on the species of animal and the species of the plant. When released, the oxalic acid binds with nutrients, making them unavailable to the body. When present in large concentrations and regularly, can cause severe irritation to the gastrointestinal tract mucosal lining. Oxalic acid can create strong chemical and chelated bonds with various minerals, such as Calcium, Magnesium, Sodium, and Potassium, making them unavailable to the animal. This chemical combination results in the formation of oxalate salts, as they are usually insoluble.

5.2.2 THE EFFECT OF PH, TEMPERATURE, SALTS AND WATER

The presence of specific metallic ions along with food content can inhibit or enhance amylase activity, and therefore the rate of digestion. Each enzyme has an optimal temperature of functioning, but there is also a broad temperature range of each enzyme activity. In other temperatures except the optimal, the enzymatic activity is decreasing. The same applies to the pH, as each enzyme has an optimal pH of functioning, but there is also a broad pH range at which each enzyme can work. In other pH except the functioning range, the enzymatic activity is completely inhibited.

Water is one of the most important nutrients for monogastrics. Water can affect the body temperature, the transportation of nutrients around the body. It can also help with the removal of toxins and help with filtration, as well as aid the general digestion. Water also affects the relative concentrations of nutrients and makes

them dynamic. Dynamism of concentrations for both enzymes and substrates is important as it has a direct effect on nutrition.

5.2.3 A MODEL THAT OUTLINES THE INTERACTIONS BETWEEN FEED INGREDIENTS

As shown in Figure 5.2, there are other factors inhibiting the enzymatic reactions, such as pH, salts, water and temperature, as well other anti-nutritive factors as mentioned above.

This model can be used for the better understanding of interactions between feed ingredients, like enzymes, substrates and anti-nutritive factors. For example, the exact same model could be used for the effect of cyanogen, saponins or phytoestrogens on those enzymes.

If necessary, this model could then become even more complex, by taking into account the effect of changes in pH, temperature, salts and water. By adding all those other parameters in this model, which is a clean system, it would provide much more information that could help optimise the feed to a higher extend.

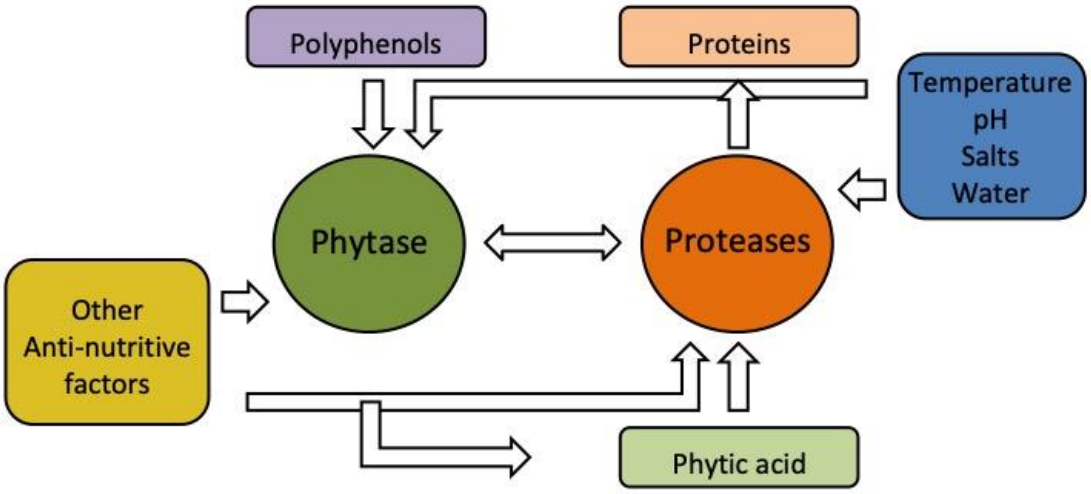


Figure 5.15: Map of interactions between phytic acid, phytase, protein, proteases, polyphenolic compounds and other potential enzymatic inhibitors, like other anti-nutritive factors or temperature, pH, salts and water.

This is a conceptual model that can be used as a tool based on the fundamental interactions of enzymes, substrates and anti-nutritive factors. Despite being a clean system, by understanding the interactions between its components, could provide a plethora of information that could help design a better, more sustainable and efficient animal feed. In that way the animal nutrition would improve, and the cost of the animal feed would decrease, two positive outcomes of great importance for livestock farming.

5.3 IMPLICATIONS OF INTERACTIONS IN NUTRITION

As described above, the interactions between the different components of animal feed can have a negative impact on the animal nutrition, the cost of the animal feed and therefore the animal products, as well as the environment. To avoid these, the animal feed should be designed differently in order to achieve a better animal feed composition that would have a less negative impact.

5.3.1 HOW CAN THESE INTERACTIONS AFFECT THE FEED COMPOSITION

Being aware of the negative effect of anti-nutritive factors present in grains, farmers tend to use those in as low percentages as possible in the animal feed. At the same time, there are treatments that can aid to a less negative effect in nutrition.

Post-harvest processing methods, or the addition of specific amino-acids, vitamins and minerals definitely can have a positive effect. Another technique used to decrease the negative impact of anti-nutritive factors is germination of grains. Germination is commonly used for legumes and while it is reducing the antinutrients present in the grain, at the same time is improving the bioavailability and digestibility of their nutrients. Furthermore, a better selection of varieties and types of feed components that are the least impactful on the animal nutrition is also a common practise (Tadele Yilkal., 2015). The use of white sorghum more frequently than red and black sorghum is a prime example of this selection process. It is known that white sorghum has less condensed tannins compared to red and black sorghum and therefore it is used more frequently and in higher s, as it has a less negative effect.

5.3.2 HOW TO DESIGN A BETTER ANIMAL FEED

Other than what has been mentioned above, taking into consideration of the interactions between the different substances present in the feed can allow the design of a more efficient feed composition. This could be achieved by doing something small, such as milling and soaking of the grains, fermentation, germination, grinding or heat treatment, prior to using them in the feed (Barekatain *et al.*, 2015).

For example, as a general practice for all sorghum varieties, by separating the husk from the endosperm would instantly help remove a part of polyphenols and would make sorghum instantly a better grain for fed. Despite the cost of such practice, I believe that the outcome would be more beneficial compare to the economic loss, as a better protein utilisation would compensate for this loss, plus more phosphorus would be available for the animal, while using sorghum which is still a relatively not costly grain (Soni *et al.*, 2016).

In legumes there are anti-nutritional factors such as goitrogens that interact with iodine; this effect can be prevented in soybeans by heating. Another anti-nutritive factor found in legumes the mineral chelating agents like as phytic and oxalic acid. Those could be avoided by removing the husk of the grains or by the addition of phytase in the feed to support the degradation of phytic acid.

5.4 CONCLUSIONS LIMITATIONS AND FUTURE WORK

Limitations that should be taken under consideration and be addressed are as mentioned below:

- ITC being unable to investigate the kinetic constants for the both enzymes. This was overcome by measuring the relative activity of the phytase, but a completely different technique had to be used to study the proteases' reactions. Using a different substrate could potentially allow ITC to study the activity of the proteases as well.

- Mass Spectroscopy was used to verify/ help us understand the reaction of phytase/phytic acid. The same could be done for the different proteases in order to get some secondary data for their reactions.

- The effect of the polyphenolic compounds could also be studied fully using Mass Spectroscopy, which would probably provide further data.

- The unknown consistency of the polyphenolic-rich extracts. This could be overcome by Mass Spectroscopic analysis of the extracts or by any other technique that could provide all the necessary information.

- The effect of pH, temperature and the presence of salts is something that should also be investigated as it would make the model to be created much more realistic and closer to the reality.

- Further research needs to be done in the reaction between the exogenous chymotrypsin and the phytase to clarify the exact interaction that happens

between the two. Mass spectroscopy could be used for that. The interaction of phytase with all proteases should also be studied to complete the map.

Using the techniques above, in combination with investigating other grain extracts and/or other digestive enzymes, can provide valuable data, that in combination with the data in this study and some programming, could create a model to simulate the interactions that could potentially take place during the monogastrics' nutrition in order to optimise their feeding.

This research could be further developed using the techniques already used in this study by:

- Investigate the effect of polyphenolics on the proteases' activities using the Sigma's non-specific protease activity assay.
- Using Mass Spectroscopy to identify the specific polyphenolics present in each sorghum extract.
- Using the identified polyphenolics, run substance-specific experiments to find the most inhibiting polyphenolics.

The results of this thesis show that sorghum polyphenolics have a negative effect on the phytase's activity. At the same time, phytic acid was found to inhibit the proteases' activity and finally an interaction between the phytase and the proteases was established. Combining all that information, a model that provides valuable information on the interactions between those substances was created.

To be able to make the most out of this model being used as a tool, some further needs to be conducted. Initially a better understanding of the interactions

between the phytase and the exogenous chymotrypsin would be some really useful information. Furthermore, similar studies with the other endogenous proteases would also provide some meaningful and important data, which would help us make more accurate estimations on the levels of phytase that need to be added to the feed, so that there is always enough to hydrolyse as much phytic acid as possible.

In regard to polyphenolics compounds and how they affect the phytase's activity, a thorough analysis of all three extracts would give meaningful information on which are the specific compounds that are causing the highest inhibition on the enzymes. Then separating those and running the same assay would confirm which these substances are. By knowing them, a better selection of which sorghum is used for feeds could be done in order to select varieties with less of the inhibiting compounds and therefore promote a better nutrition to the animal.

The information of that model would also provide information on combinations of different feed ingredients that should be avoided or preferred. Also knowing the possible interactions would indicate all the treatments that could be applied to each ingredient to minimise the negative effect on the animal nutrition.

Finally, all hypotheses of this thesis were confirmed, and the model created could be used for other anti-nutritive factors, while there is a freedom to increase or decrease its complexity based on the needs of each project.

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APPENDIX

A1 ABSTRACT FOR ORAL PRESENTATION AT THE INTERNATIONAL SOCIETY OF ENZYMOLOGY ABSTRACT

Enzymatic kinetics analysis of phytase using Isothermal Titration Calorimetry: Using ITC for complex/multiple stage reactions.

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Isothermal titration calorimetry (ITC) is a technique that has been commonly used in for the investigation of binding interactions between molecules and for the analysis of enzymatic kinetics. Despite its power as an analytical tool ITC has primarily been used for the analysis of simple/one stage reactions due to the complexity of the data generated from multiple-step reactions. The reaction between phytase and phytic acid is known to be a complex reaction due to the stoichiometry of the phytic acid molecule. In this work, ITC was used to study the action of phytase when cleaving phosphate groups from phytic acid sodium salt hydrate. The thermogram obtained from this enzymatic reaction is complex, with at least three clear peaks produced. This was expected since phytases have a preferential mechanism of degrading phytic acid. To deconvolute the data obtained by ITC, a 'real-time' mass spec analysis of the same reaction was performed. By quantifying the peaks produced in the mass spectra by the lower inositol phosphates throughout the reaction, the pattern of phytic acid dephosphorylation was revealed. The phytase was found to rapidly cleave the first two phosphate groups of phytic acid, after which the rate of dephosphorylation slows to eventually produce a final product of the singly phosphorylated inositol monophosphate. By combining the ITC data with the MS data, the thermogram obtained by ITC can be deconvoluted and each step of the reaction can be identified since both datasets are against time. Thus, the kinetics of phytase can be calculated for the different steps of the reaction.

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A2 ABSTRACT FOR POSTER PRESENTATION AT THE 3RD EUROPEAN SUSTAINABLE PHOSPHORUS CONFERENCE (ESPC3)

INHIBITION OF PHYTASE ACTIVITY BY POLYPHENOL-RICH EXTRACTS FROM SORGHUM, IN RELATION TO PHOSPHORUS SUSTAINABILITY

Polyphenols are present in a variety of grains used for the nutrition of monogastric animals and together with phytic acid are considered as anti-nutritive factors. The anti-nutritive effect of phytic acid is tackled by using phytase in the animal feed, but there is no solution for the polyphenols' effect. For this project, polyphenols were extracted from different sorghum varieties and the polyphenol-rich extracts were used to estimate their effect on the activity of a commercially used phytase. The results proved a negative impact of the polyphenol-rich extract on the phytase activity. By slowing down the enzymatic reaction, less phosphates are cleaved off the phytic acid, which has a series of negative effects. Initially, with less phosphorous released, there is an addition of inorganic phosphorous in the feed to meet the nutritional requirements of the animal. Having in mind that the world's phosphorus banks are being drained, this aids to the phosphorus unsustainability. Finally, the inefficiency of the phytase leads to higher levels of phosphorus present in the animal's excreta that needs to be eliminated since it is then released in the wastewater stream and ends up in rivers.

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A3 FIGURES SHOWING THE DIFFERENT STAGES OF THE ITC ENZYME ACTIVITY ASSAY FOR LIBERTY, CRACKA AND MR-BUSTER SORGHUM VARIETIES

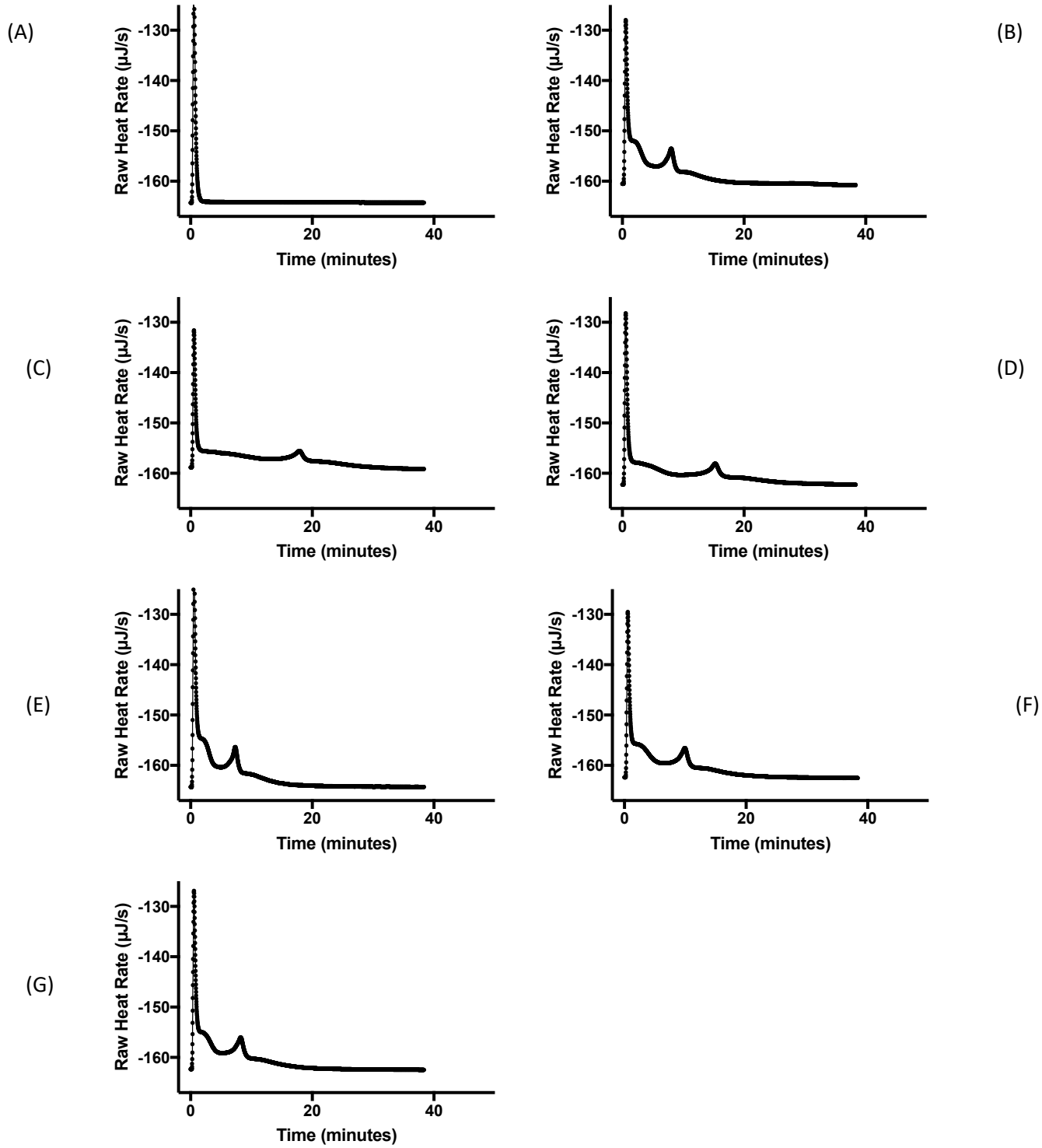

Figure A.1: The raw heat rate of (A) phytic acid-phytase reaction, (B) phytic acid into 5% ethanol, phytic acid-phytase reaction with (C)0.109, (D)0.054, (E)0.028, (F)0.014 and (G)0.007 mg/ml polyphenol-rich extract from MR-Buster sorghum.

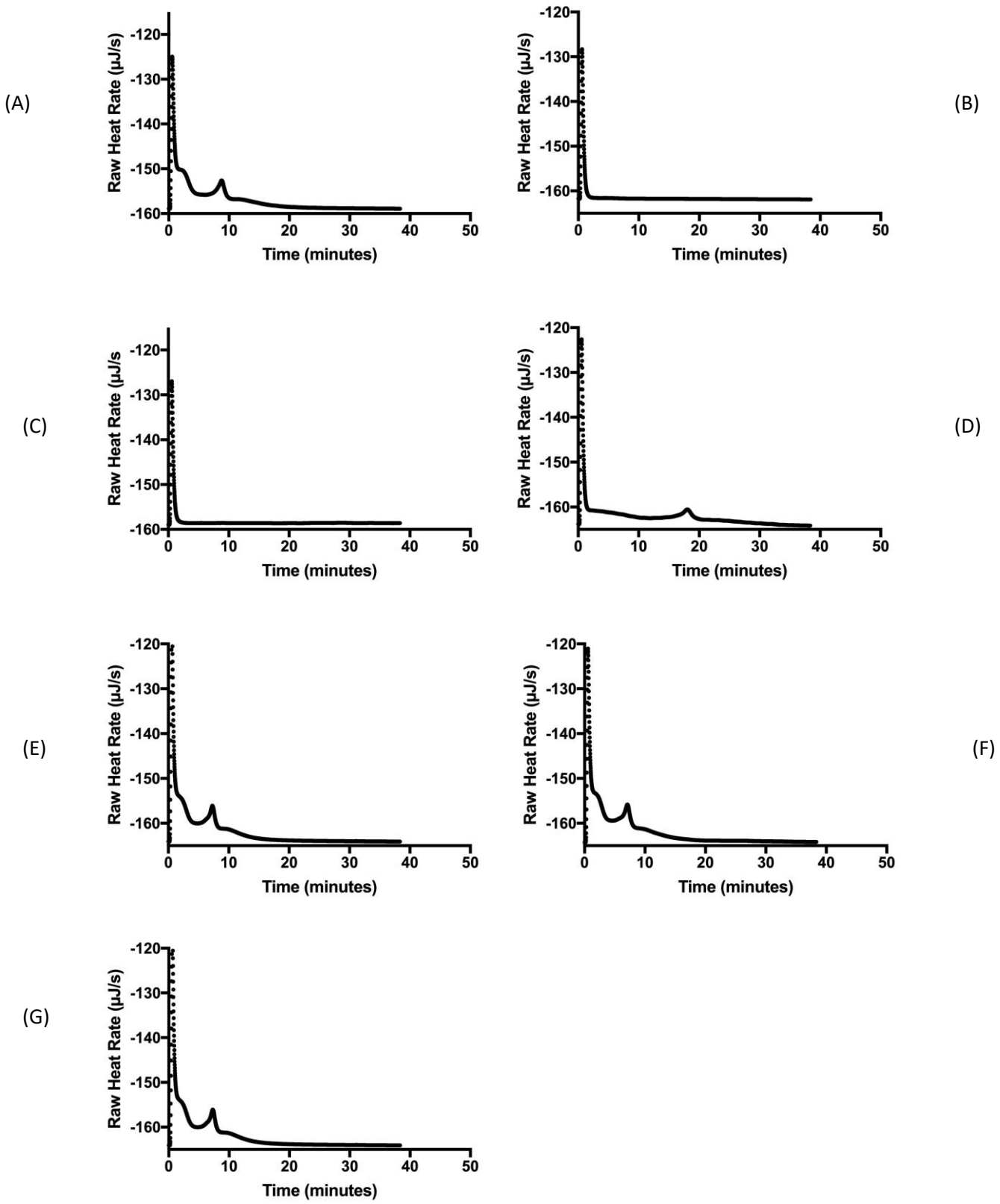

Figure A.2: The raw heat rate of (A) phytic acid-phytase reaction, (B) phytic acid into 5% ethanol, phytic acid-phytase reaction with (C)0.109, (D)0.054, (E)0.028, (F)0.014 and (G)0.007 mg/ml polyphenol-rich extract from Liberty sorghum.

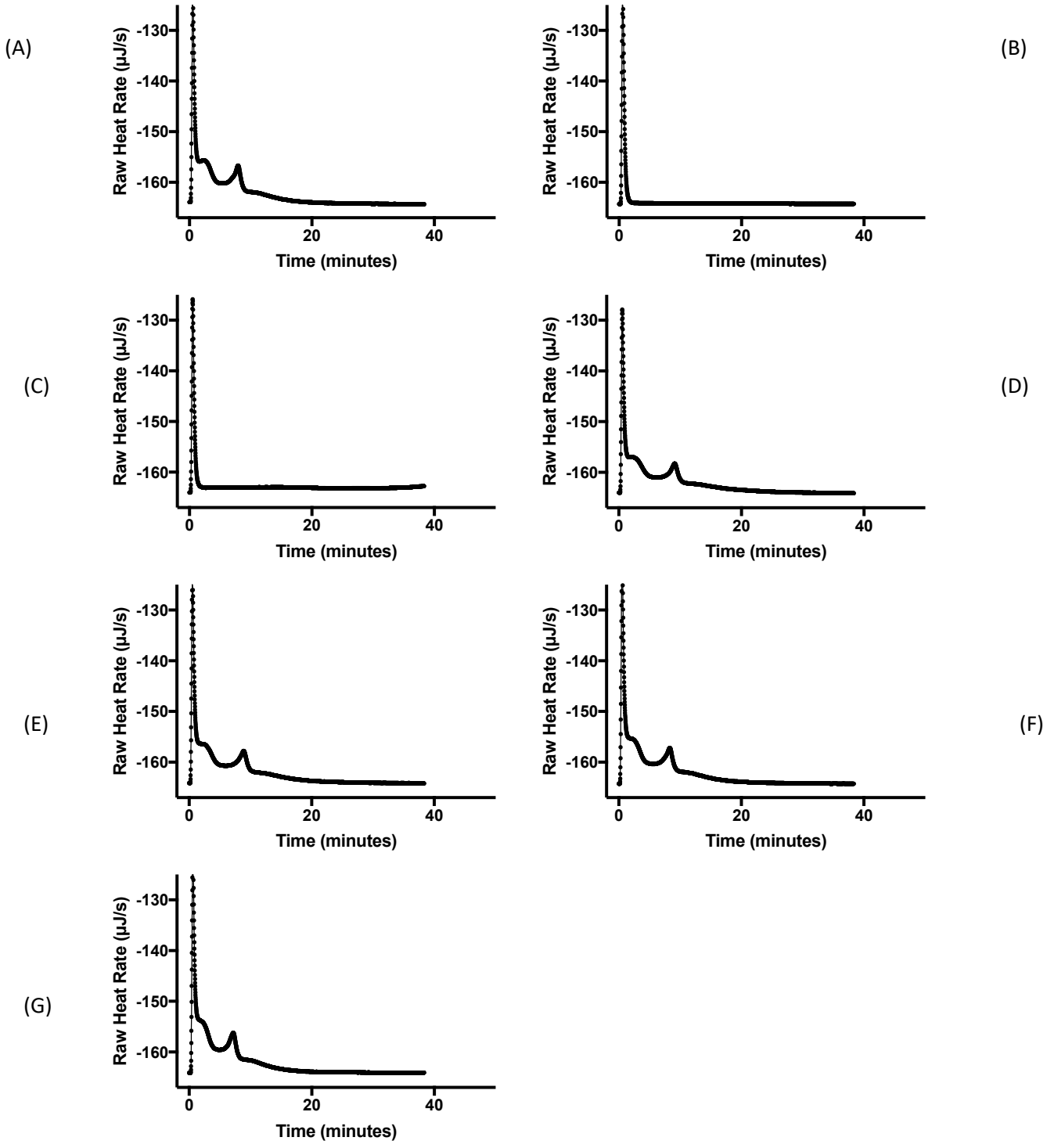

Figure A.3: The raw heat rate of (A) phytic acid-phytase reaction, (B) phytic acid into 5% ethanol, phytic acid-phytase reaction with (C)0.109, (D)0.054, (E)0.028, (F)0.014 and (G)0.007 mg/ml polyphenol-rich extract from Cracka sorghum.

A4 TABLES FOR DETAILED INHIBITION OF PROTEASES USING THE COLORIMETRIC ASSAY (CHAPTER 3)

Table A.7: The effect of different phytic acid concentrations on the activity of the exogenous chymotrypsin.

Phytic Acid Concentration (μM)	Exogenous Chymotrypsin Activity (Percentage of the original activity)
0	100.00
0.06	100.00
0.12	100.41
0.24	100.00
0.48	99.58
0.97	100.00
1.95	93.80
3.90	88.01
7.81	76.44
15.62	75.20
31.25	65.70
62.5	63.63
125	52.89
250	37.19
500	24.38

Table A.16: The effect of different phytic acid concentrations on the activity of the trypsin.

Phytic Acid Concentration (μM)	Exogenous Chymotrypsin Activity (Percentage of the original activity)
0.00	100.00
0.06	97.61
0.12	97.61
0.24	95.23
0.48	95.23
0.97	92.47
1.95	73.80
3.90	64.28
7.81	53.33
15.62	42.85
31.25	38.09
62.50	38.09
125	35.71

Table A.24: The effect of different phytic acid concentrations on the activity of the endogenous chymotrypsin.

Phytic Acid Concentration (μM)	Endogenous Chymotrypsin Activity (Percentage of the original activity)
0.00	100.00
0.06	96.52
0.12	87.83
0.24	84.17
0.48	63.67
0.97	59.01
1.95	58.00
3.90	54.52
7.81	53.52
15.62	52.60
31.25	56.26
62.50	52.60
125.00	40.71