Chapter 4

Further Enzyme Studies

4.1. Introduction

Enzymes are a group of proteins possessing catalytic ability. The mechanism of an enzyme-catalysed reaction can be expressed as below:

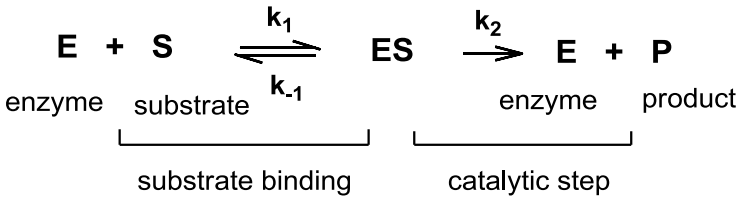

Figure 4-1 Single-substrate mechanism for an enzyme reaction (Michaelis-Menten kinetic model)

Enzyme kinetics is the study of the chemical reactions that are catalyzed by enzymes, with a focus on their reaction rates. The study of the kinetics of an enzyme reveal the catalytic mechanism of the enzyme, its role in metabolism, how its activity is controlled, and how a drug or a poison might inhibit the enzyme (Stauffer, 1989; Bisswanger, 2008).

4.1.1. General Principles

Like other catalysts, enzymes do not alter the position of equilibrium between substrates and products. Unlike un-catalyzed chemical reactions, enzyme-catalyzed reactions display saturation kinetics. For a given enzyme concentration and for relatively low substrate concentrations, the reaction rate increases linearly with substrate concentration; the enzyme molecules are largely free to catalyze the reaction, and increasing substrate concentration means an increasing rate at which the enzyme and substrate molecules encounter one another (Tipton, 2002). However, at relatively high substrate concentrations, the reaction rate asymptotically approaches the theoretical maximum; the enzyme active sites are almost all occupied and the reaction rate is determined by the intrinsic turnover rate of the enzyme (Tipton, 2002). The substrate concentration midway between these two limiting cases is denoted by **Km**. The relationship between enzyme velocity and substrate concentration is shown in figure 4-2.

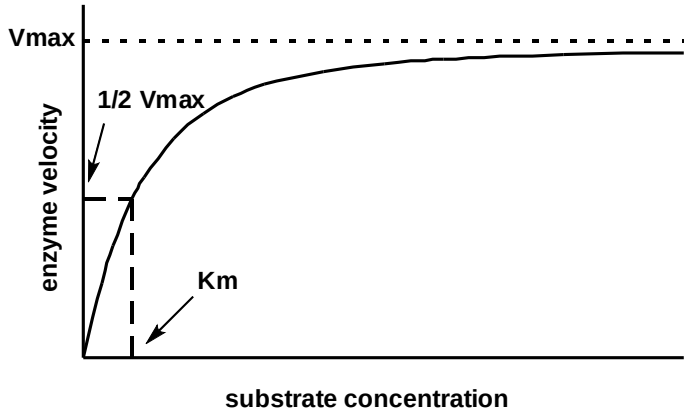

Figure 4-2 Saturation curve for an enzyme showing the relationship between substrate concentration and reaction rate

The two most important kinetic properties of an enzyme are how quickly the enzyme becomes saturated with a particular substrate, and the maximum rate it can achieve (Bisswanger, 2008).

4.1.2. Enzyme Assay

Enzyme assays are laboratory procedures that measure the rate of enzyme reactions. Because enzymes are not consumed by the reactions they catalyze, enzyme assays usually follow changes in the concentration of either substrates or products to measure the rate of reaction (Bisswanger, 2004; Schnell *et al.*, 2006).

A typical progress curve for an enzyme assay is shown in figure 4-3. The enzyme produces product at a linear initial rate at the start of the reaction. Later in the progress curve, the rate slows down as substrate is used up or products accumulate. So the curve starts to level off. Eventually the concentration of product reaches a plateau and doesn't change with time (Tipton, 2002). The slope in the initial rate period is the initial rate of reaction V (or V_0), which is also called steady state because the concentration of enzyme-substrate complex doesn't change. However, the concentration of product accumulates, so the system is not truly at steady state until, much later, the concentration of product doesn't change any further. The length of the initial rate period depends on the assay conditions and can range from milliseconds to

hours (Tipton, 2002). The Michaelis-Menten equation can describe how the slope varies with the concentration of substrate.

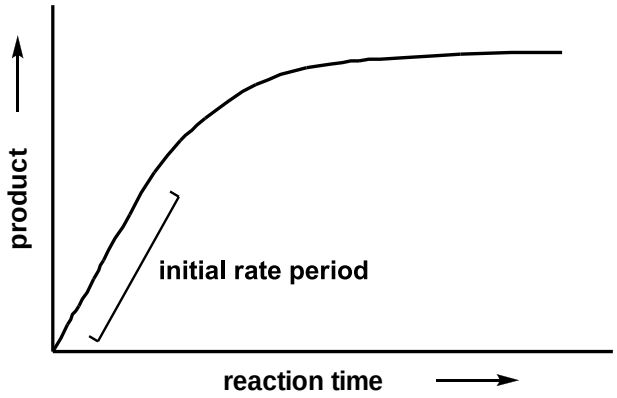

Figure 4-3 Progress curve for an enzyme reaction

Most enzyme kinetic studies concentrate on the initial, linear part of enzyme reactions. However, it is also possible to measure the complete reaction curve and fit the data to a non-linear rate equation (Bisswanger, 2004). This way of measuring enzyme reactions is called progress-curve analysis, used as an alternative to rapid kinetics when the initial rate is too fast to measure accurately (Copeland, 2000).

Rather than fit the enzyme progress curve, most analyses of enzyme kinetics fit the initial velocity of the enzyme reaction as a function of substrate concentration. The velocity of the enzyme reaction is the slope of the linear phase, expressed as amount of product formed per time (Bisswanger, 2008). If the initial transient phase is very short, the product formed at a single time can be measured, and define the velocity to be the concentration divided by the time interval (Schnell *et al.*, 2006).

4.1.2.1. Types of Assay

Enzyme assays measure either the consumption of substrate or production of product over time (Tipton, 2002). A large number of different methods of measuring the concentrations of substrates and products exist and many enzymes can be assayed in several different ways.

- Initial rate experiments

When an enzyme is mixed with a large excess of the substrate, the enzyme-substrate intermediate builds up in a fast initial transient phase. Then the reaction achieves steady-state kinetics in which enzyme-substrate intermediates remain approximately constant over time and the reaction rate changes relatively slowly (Tipton, 2002). Rates are measured for a short period after the attainment of the quasi-steady state, typically by monitoring the accumulation of product with time. Because the measurements are carried out for a very short period and because of the large excess of substrate, the assumption that free substrate is approximately equal to the initial substrate can be made (Schnell *et al.,* 2006). The initial rate experiment is the simplest to perform and analyze, being relatively free from complications such as back-reaction and enzyme degradation. It is therefore by far the most commonly used type of experiment in enzyme kinetics (Xie & Lu, 1999; Bisswanger, 2004).

- Progress curve experiments

In these experiments, the kinetic parameters are determined from expressions for the species concentrations as a function of time. The concentration of the substrate or product is recorded after the initial fast transient change and for a sufficiently long period to allow the reaction to approach equilibrium (Tipton, 2002). Progress curve experiments were widely used in the early period of research into enzyme kinetics while they are less commonly used now.

- Transient kinetics experiments

In these experiments, reaction behaviour is tracked during the initial fast transient phase as the intermediate reaches the steady-state kinetics period. These experiments are more difficult to perform than either of the above two classes because they require rapid mixing and observation techniques (Tipton, 2002).

- Relaxation experiments

In these experiments, an equilibrium mixture of enzyme, substrate and product is perturbed, for instance by a temperature, pressure or pH jump, and the return to equilibrium is monitored. The analysis of these experiments requires consideration of the fully reversible reaction. Moreover, relaxation experiments are relatively

insensitive to mechanistic details and are thus not typically used for mechanism identification, although they can be under appropriate conditions (Tipton, 2002; Bisswanger, 2004).

4.1.2.2. Assay Methods

Enzyme assays can be split into two groups according to the sampling method: continuous assays, where the assay gives a continuous reading of activity, and discontinuous assays, where samples are taken, the reaction stopped and then the concentration of substrates or products determined (Tipton, 2002).

Continuous assays are most convenient, with one assay giving the rate of reaction with no further work necessary (Tipton, 2002). There are many different types of continuous assays which include spectrophotometeric assays, direct versus coupled assays, fluorometric assays, colorimetric assays, chemiluminescent assay, light scattering assays, etc.

Discontinuous assays are when samples are taken from an enzyme reaction at intervals and the amount of product producted or substrate consumed is measured. Discontinuous assays include radiometric assays and chromatographic assays (Tipton, 2002).

4.1.3. Michaelis-Menton Kinetics

A single-substrate enzyme reaction is shown in figure 4-1. If enzyme velocity is measured at many different concentrations of substrate, the graph generally looks like figure 4-2. Enzyme velocity as a function of substrate concentration (denoted as [S]), often follows the Michaelis-Menten equation (Bisswanger, 2008):

$$\text{Velocity} = V = \frac{V_{max}[S]}{[S] + K_M}$$

Vmax is the limiting velocity as substrate concentrations get very large. Vmax (and V) are expressed in units of product formed per unit of time. If the molar concentration of enzyme is known, the observed velocity can be divided by the concentration of enzyme sites in the assay, and Vmax be expressed as moles of product formed per

second per mole of enzyme sites (Bisswanger, 2008). This is the *turnover* number, the number of molecules of substrate converted to product by one enzyme site per second.

Km is expressed in units of concentration, usually in molar units. Km is the concentration of substrate that leads to half-maximal velocity. Under certain reaction conditions, Km is a constant which can be used to show the affinity of the enzyme binding with substrate (Browne *et al.*, 1987). The lower the Km is, the stronger binding ability the enzyme has. Generally speaking, Km values range from 10^{-6}-10^{-2} M (Tipton, 2002; Bisswanger, 2008).

Double reciprocal the previous equation and simplifying:

$$\frac{1}{V} = \frac{[S] + K_M}{Vmax\,[S]} = \frac{[S]}{Vmax[S]} + \frac{K_M}{Vmax[S]} = \frac{1}{Vmax} + \frac{K_M}{Vmax} * \frac{1}{[S]}$$

So the most common way of generating this data is with a series of experiments at constant enzyme concentration and different substrate concentration (Keleti, 1986; Duggleby, 1995; Schnell *et al.*, 2006).

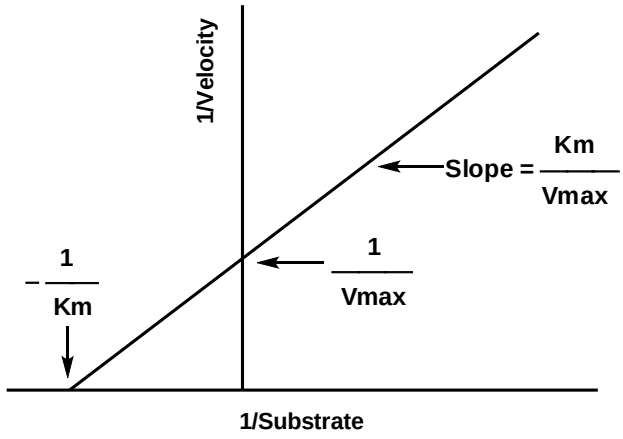

Figure 4-4 Displaying enzyme kinetic data on a Lineweaver-Burk plot

4.1.4. Other Enzyme Studies

A comprehensive study of a particular enzyme includes (Stauffer, 1989):

- Identifying the catalytic activity of interest. Develop an assay to quantify the activity. At this stage, simplicity and speed are more important for an assay than sophistication and accuracy. For example, the hydrolysis of p-

nitrophenyl-acetate in a rack full of test tubes may be very useful for testing fractions from a chromatographic separation of esterase, although the accuracy may be no better than ± 20%.

- Purifying the enzyme. This may be only partial isolation, not the achievement of complete purity. However, it should be carried to the point at which there is only one enzyme present (not two or more with similar activities) and endogenous inhibitors or activators are removed or identified.
- Characterizing the enzymatic activity. This determines the kinetics of the enzyme, and more care must be taken in choosing the assay methods which will give appropriate experimental data. In investigating different properties (e.g., the pH-dependence of activity and the rate of heat-denaturation under various conditions), different substrates and assay conditions may be needed. Other characterizing parameters might be specificity (rate of reaction with different substrates) and the effect of inhibitors and activators.
- Determining the enzyme's chemical and physical properties. Once a pure (single molecular species) material is obtained, there are a number of factors to be found: composition (amino acids, sugars, metal ions, and any other prosthetic groups), molecular weight (quaternary structure, if any), amino acid sequence, secondary and tertiary structure. Of practical importance is the stability of the enzyme against denaturation under conditions which might be found in use.
- Integrating the protein characteristics and enzymatic nature of the molecule. The amino acid residues which make up the active site are identified by chemical and kinetic studies. By crystallographic studies the orientation of the substrate at the active site is determined. If the source of the enzyme is amenable to gene engineering, it may be possible to tailor the amino acids around the active site to alter activity or specificity in ways which are technologically desirable.

Undoubtedly, different people are interested in different points. Although the aim of this study is looking for a suitable enzyme (source) to develop a novel food(s) which contain flavonoid glycoside or aglycone used for the study of flavonoid absorption,

further enzyme studies could be carried out if possible as described in subsequent sections.

4.1.4.1 Inhibitors

Almost all enzymes will combine with some chemical species which reduce their catalytic efficiency. These materials are termed inhibitors (Stauffer, 1989). Activators are molecules that also bind to enzymes but increase their activity. According to their reacting mechanism (their effects on Km and Vmax), the inhibitors can be divided into *irreversible* inhibitors (i.e., the inhibitor permanently inactivates the enzyme) and *reversible* inhibitors (i.e., removal of the inhibitor restores enzyme activity) which including competitive inhibition and non-competitive inhibition and mixed-type inhibitions (Copeland, 2000). The type of inhibitions can be distinguished by kinetic study.

Inhibitor studies are another important part of enzyme study especially when used on natural substrates. For most cases, the substance possessing similar structure to the product will be considered. So some monosaccharides especially hexoses, need to be tested like glucose, lactose, fructose, etc. (Dale *et al.*, 1985; Yan & Lin, 1997; Barbagallo *et al.*, 2007). The inhibition of inhibitors on enzyme activity can be determined by adding various concentrations of inhibitor into reaction mixtures with p-NP-Glc as substrate (Bols *et al.*, 2007).

The reversible enzyme inhibitors generally do not undergo chemical reactions when bound to the enzyme and can be easily removed by dilution or dialysis. The different type of effects results from the inhibitor binding to the enzyme E, to the enzyme–substrate complex ES, or to both, as shown in the figure 4-5.

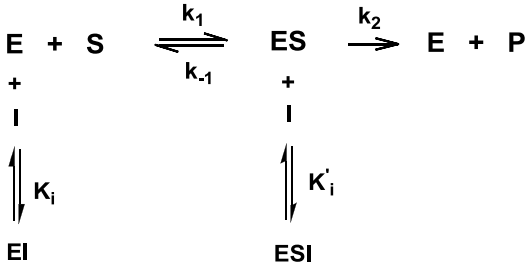

Figure 4-5 Kinetics scheme for reversible enzyme inhibitors

The particular type of an inhibitor can be discerned by studying the enzyme kinetics as a function of the inhibitor concentration (Copeland, 2000). The four types of inhibition produce Lineweaver-Burk plots that vary in distinctive ways with inhibitor concentration. Figure 4-6 shows the Lineweaver-Burk plots of different types of reversible enzyme inhibitors (Bisswanger, 2004). For brevity, two symbols are used:

$$\alpha = 1 + [I]/ K_i \quad \text{and} \quad \alpha = 1 + [I]/ K'_i$$

where K_i and K'_i are the dissociation constants for binding to the enzyme and to the enzyme-substrate complex, respectively. In the presence of the reversible inhibitor, the enzyme's apparent Km and Vmax become (α/α') Km and ($1/\alpha'$) Vmax, respectively, as shown in table 4-1 for common cases.

Table 4-1 Inhibitors' effects on kinetic parameters

Type of inhibition			Km apparent	Vmax apparent
competitive	K_i only	$\alpha' = 1$	Km α	Vmax
uncompetitive	K_i' only	$\alpha = 1$	Km / α'	Vmax / α'
non-competitive	$K_i = K_i'$	$\alpha = \alpha'$	Km	Vmax / α'
mixed	$K_i \neq K_i'$	$\alpha \neq \alpha'$	Km α/ α'	Vmax / α'

Enzyme inhibitors can inactivate enzymes irreversibly, usually by covalently modifying active site residues. These reactions, which are also called suicide substrates, follow exponential decay functions and are usually saturable.

4.1.4.2. SDS-PAGE

The enzyme β-D-glucosidase from different sources is reported to consist of 2 subunits but of different molecular weights (Esen, 1993). SDS-PAGE (Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis) is a technique widely used to separate proteins and determine their molecular weight according to their electrophoretic mobility (Yan *et al.*, 1998; Hu *et al.*, 2007; Yu *et al.*, 2007).

4.1.4.3. Determine the Active Site Concentration

Enzyme quantification methods vary widely in complexity and reliability. Methods that assume an enzyme is homogeneous and fully active (gravimetric or total protein

assays) are far less reliable than those measuring the concentration of functional enzyme (active titration and substrate turnover rate assays). Of those methods that measure functional enzyme, active site titrations tend to be more reliable than activity assay methods (Czjzek *et al.*, 2000; Martin, 2002).

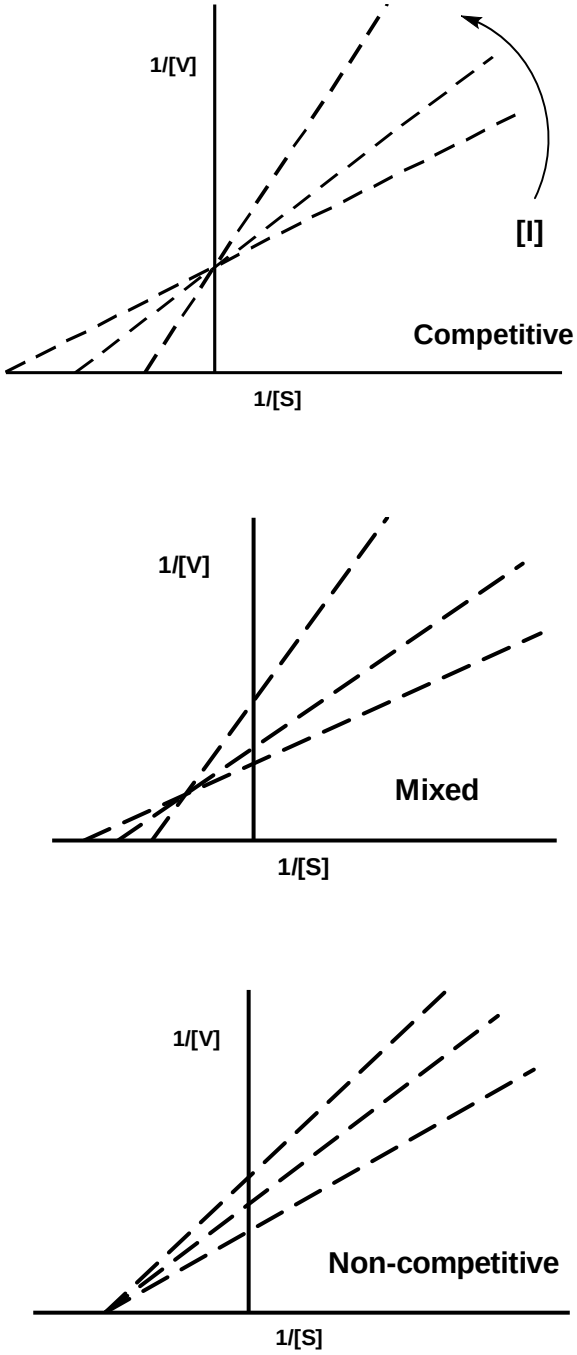

Figure 4-6 Lineweaver-Burk plots of different types of reversible enzyme inhibitors. The arrow shows the effect of increasing concentrations of inhibitor.

In contrast to activity methods, titrations do not require primary standards of 100% pure and active enzyme and are less likely to be affected by small variations in assay conditions (i.e. pH, ionic strength, temperature). However, despite the attractiveness of active site titration, there are a large number of well-known enzymes for which no titration protocols exist because of the challenge to design titration methods. Fortunately, a number of excellent titration protocols have been developed for a diverse collection of catalysts (Martin, 2002).

4.1.5. Factors to Control in Enzyme Assays

4.1.5.1. Salt Concentration

Most enzymes cannot tolerate extremely high salt concentrations. The ions interfere with the relatively weak ionic bonds of proteins. Typical enzymes are active in salt concentrations of 1-500mM. As usual there are exceptions such as enzymes from the halophilic (salt loving) algae and bacteria (Bisswanger, 2004).

4.1.5.2. Effects of Temperature

All enzymes work within a range of temperature specific to the organism. Increases in temperature generally lead to increases in reaction rates. But there is a limit to the increase because higher temperatures lead to a sharp decrease in reaction rates due to the denaturing (alteration) of protein structure resulting from the breakdown of the weak ionic and hydrogen bonding that stabilize the three dimensional structure of the enzyme (Daniel *et al*., 2009). The optimum temperature for human enzymes is usually (but not always) between 35ºC and 40ºC. Enzymes from *Thermophilic archaea* found in the hot springs are stable up to 100ºC (Cowan, 1997; Daniel *et al*., 2009).

4.1.5.3. Effects of pH

Most enzymes are sensitive to pH and have specific ranges of activity. All have an optimum pH. The pH can stop enzyme activity by denaturing the three dimensional shape of the enzyme by breaking ionic, and hydrogen bonds (Tipton, 2002). Most

enzymes function between a pH of 6 and 8; however pepsin in the stomach works best at a pH of 2 and trypsin at a pH of 8 (Copeland, 2000; Bisswanger, 2004).

4.1.5.4. Substrate Saturation

Increasing the substrate concentration increases the rate of reaction (enzyme activity). However, enzyme saturation limits reaction rates. An enzyme is saturated when the active sites of all the molecules are occupied most of the time. At the saturation point, the reaction will not speed up, no matter how much additional substrate is added (Tipton, 2002). The graph of the reaction rate will plateau (see figure 4-2).

4.1.5.5. Level of Crowding

Large amounts of macromolecules in a solution will alter the rates and equilibrium constants of enzyme reactions, through an effect called macromolecular crowding (Minton, 2001).

4.1.6. Expression of Enzyme Activity

In order to express the activity of an enzyme in absolute terms it is necessary to ensure that the assay procedure used is measuring the true initial velocity and that is proportional to the enzyme concentration. Under these conditions the ratio (velocity/enzyme concentration) will be a constant that can be used to express the activity of an enzyme quantitatively (NC-IUB, 1979). This can be valuable for comparing data obtained with the same enzyme from different laboratories, assessing the effects of physiological or pharmacological challenges on cells or tissues, monitoring the extent of purification of enzymes and comparing the activities of different enzymes, or of the same enzyme from different sources or with different substrates (Tipton, 2002).

4.1.6.1. Units and Specific Activity

The activity of an enzyme may be expressed in any convenient units, such as absorbance change per time per mg enzyme protein, but it is preferable to have a more

standardized unit in order to facilitate comparisons. The most commonly used quantity is the *Unit*, sometimes referred to as the International Unit or Enzyme Unit. One Unit of enzyme activity is defined as that catalyzing the conversion of 1μmol substrate (or the formation of 1μmol product) in 1min (Bisswanger, 2004). The specific activity of an enzyme preparation is the number of Units per mg protein (NC-IUB, 1979).

If the relative molecular mass of an enzyme is known it is possible to express the activity as the *molecular activity*, defined as the number of Units per μmol of enzyme; in other words, the number of mol product formed, or substrate used, per mol enzyme per min (Martin, 2002). This may not correspond to the number of mol substrate converted per enzyme active-site per min since an enzyme molecule may contain more than one active site. If the number of active sites per mol is known the activity may be expressed as the *catalytic centre activity*, which corresponds to mol substrate used, or product formed, per min per catalytic centre (active site).

4.1.6.2. The Katal

Although the Unit of enzyme activity, and the quantities derived from it, have been proven to be most useful, the Nomenclature Commission of the International Union of Biochemistry has recommended the use of the katal (abbreviated to kat) as an alternative, which is also called the International System of Units (SI Units) (NC-IUB, 1979).

One katal corresponds to the conversion of 1mol of substrate per second. Thus it is an inconveniently large quantity unit (NC-IUB, 1979). The relationships between katals and Units are:

$$1 \text{ kat} = 60 \text{ mol/min} = 6 \times 10^7 \text{ Units}$$

$$1 \text{ Unit} = 1 \text{ μmol/min} = 16.67 \text{ nano-katal}$$

4.2. Aims of Chapter

The aims of this chapter are:

- To investigate the properties of β-glycosidase extracted from apple seeds
- To optimize reaction conditions for β-glycosidase from apple seeds in order to hydrolyse isoflavone glycosides
- To determine the kinetic parameters of β-glycosidase from apple seeds in order to compare with its activity with other sources (especially with almond)

4.3. Materials and Methods

4.3.1. Chemicals

The following protein standard agents were obtained from Sigma-Aldrich plc, Poole, Dorset, UK:

- Bovine serum albumin (CAS number: 9048-46-8)
- Coomassie Brilliant Blue G-250 (MW: 854; CAS number: 6104-58-1)
- Sodium chloride (NaCl; MW:58.5) analytical grade

The following protein agents were obtained from BDH Chemicals Ltd, UK:

- Coomassie Brilliant Blue G-250 (MW: 854; CAS number: 6104-58-1)

4.3.2. Preparation of Protein Reagents

- Coomassie Brilliant Blue (CBB) G-250: 100mg CBB G-250 was dissolved in 50ml 95% ethanol, to this solution 100ml 85% (w/v) phosphoric acid was added, and finally diluted to 1000ml. Final concentrations were 0.01% (w/v) CBB G-250 in 4.7% (w/v) ethanol, and 8.5% (w/v) phosphoric acid.

- Bovine serum albumin (BSA): 100mg BSA was dissolved either in 0.15M NaCl or water to reach final volume of 100ml. The concentration of protein was 1000μg/ml.

4.3.3. Optimization

Optimization experiments were designed to find out optimum conditions for the enzyme reaction catalyzed by apple seed extracts. On the basis of previous experiments, an orthogonal experiment which contains three factors and three levels was designed for optimization of the reaction conditions using the orthogonal table $L_9(3^4)$. The hydrolysis percentage of flavonoid glycosides were used as results. Both range R_Σ and Variance were used to determine the significance of the factors tested. The consequential optimum reaction conditions were used for enzyme assay later.

4.3.4. Stability of Crude Enzyme Extracts

The enzyme activity of apple seeds was monitored every week for a period of 12 weeks from the day of extraction. Monitoring was carried out through the determination of the percentage of hydrolysis of daidzin and genistin (see 3.3.5.2) at pH 5.5 and 50°C for one hour.

4.3.5. Protein Determination

The concentrations of protein contained in the crude enzyme extracts were determined by the CBB-BSA binding method, which is normally called the Bradford Method (Bradford, 1976).

Protein assay (standard method): protein solution containing 10 to 100μg protein in a volume up to 0.10ml was pipetted into 5ml micro-centrifuge tubes and the volume was adjusted to 0.10ml with appropriate buffer. 5.0ml of protein reagent (CBB G-250) was added to the tubes and the contents mixed by vortexing. The absorbance at 595 nm was measured after 2min and before 1 hr in 1.5ml cuvettes against a reagent blank prepared from 0.1ml of the appropriate buffer and 5.0ml of protein reagent. The

weight of protein was plotted against the corresponding absorbance resulting in a standard curve used to determine the protein in unknown samples.

Micro-protein assay: protein solution containing 1 to 10μg protein in a volume up to 0.10ml was pipetted into 5ml micro-centrifuge tubes and then the appropriate amount of buffer was added to give a final volume of 0.10ml. Then 5.0ml of protein reagent (CBB G-250) was added to the test tubes and the contents mixed as in the standard method. Absorbance at 595nm was measured as in the standard method.

Since the Bradford method can be affected by many factors, experiments were carried out under different conditions for the standard curve and sample determination. 'BDH' indicates that the CBB G-250 used was the product of BDH rather than from Sigma.

4.3.6. Enzyme Assay

4.3.6.1. p-NP Standard Curves

Since a higher concentration of p-NP-Glc was used and greater amounts of p-NP were liberated, another standard curve for p-NP at high-concentration was made, in which the p-NP concentration was double the normal one.

4.3.6.2. Progress Curve

The progress curve of an enzyme reaction can display the relationship of the product concentration against reaction time. Theoretically, the reaction time for the Vmax and Km assay should be in the period at which the enzyme velocity is constant (in the initial period in figure 4-3 where the graph of product concentration vs. time should be linear).

To make a progress curve, the experiment was carried out in one test tube and analysed at particular time intervals.

The reaction was described in 3.3.5.1 by using p-NP-Glc as substrate. 0.70ml buffer (0.1M, pH5.5), 0.20ml p-NP-Glc solution (0.05M, same pH value as buffer), and 0.10ml enzyme extracts (apple seed extracts or almond extracts) were added into a 2ml micro-centrifuge tube and incubated under optimum temperature (65°C for apple seeds and 50°C for almond) . After certain times (every 2-3mins for the first 10mins, then every 5mins until 30mins, then every 10mins until 60mins and longer time intervals until finish), 0.02ml of the reaction mixture was taken and added into a cuvette, then 0.98ml buffer (0.1M, pH 5.5) and 1.0ml Na_2CO_3 buffer (0.1M, pH 10.5) added, and absorbance measured on a spectrophotometer at a wavelength of 400nm.

4.3.6.3. The Linearity of Velocity against Enzyme Concentration

The linearity of velocity against enzyme concentration was tested by adding 0.70ml buffer (0.1M, pH5.5), 0.20ml p-NP-Glc solution (0.05M, pH5.5), and 0.10ml crude enzyme extracts or their series dilutions (in buffer, immediately before the experiment), into a 2ml micro-centrifuge tube and incubated at the enzyme's optimum temperature (65°C for apple seed extracts and 50°C for whole almond extracts) for 10mins. Then a certain amount of reaction mixture was removed and put in a cuvette, a corresponding volume of buffer (0.1M, pH5.5) and 1.0ml Na_2CO_3 buffer (0.1M, pH 10.5) added to make the final volume of 2.0ml, and then absorbance measured at 400nm. The amount of p-NP liberated was calculated according to the standard curve obtained in 4.3.6.1. Finally the velocity against the enzyme concentration was plotted.

4.3.6.4. Determination of Km and Vmax

In order to determine the Km and Vmax, a series of experiments at constant enzyme concentration and different substrate concentration were carried out and then expressed by Lineweaver-Burk plot.

Immediately before the experiment, the enzyme extracts were diluted by buffer (0.1M, pH5.5). Apple seed extracts (1.0ml) were diluted to 5.0ml, whole almond extracts (1.0ml) were diluted to 10.0ml.

0.10ml, 0.20ml, 0.30ml, 0.40ml, 0.50ml, 0.60ml, 0.80ml of 0.05M p-NPG were pipetted into 2ml micro-centrifuge tubes, a corresponding amount of buffer (0.1M, pH 5.5) added to reach 0.90ml, then 0.10ml dilution of crude enzyme extracts added (apple seeds or almond), incubated at optimum temperature as finally determined (65°C for apple seed and 50°C for almond) for 5mins.

0.10ml of reaction mixtures for apple seed extracts and 0.20ml of reaction mixtures for whole almond extracts respectively, were taken from reaction tubes and put into a cuvette, 0.90ml or 0.8ml of buffer (0.1M, pH5.5) and 1.0ml Na_2CO_3 buffer (0.1M, pH10.5) added, then absorbance was read at a wavelength of 400nm.

The amount of p-NP liberated was found from the standard curve of p-NP and then 1/[S] and 1/V were calculated.

4.4. Results

4.4.1. p-NP Standard Curves

Standard curves at pH 5.5 for p-NP in 0.1mM (figure 4-7) and 0.2mM (figure 4-8) were obtained by the method described in 3.3.3. Both of them had a very good R^2, showing very good linearity.

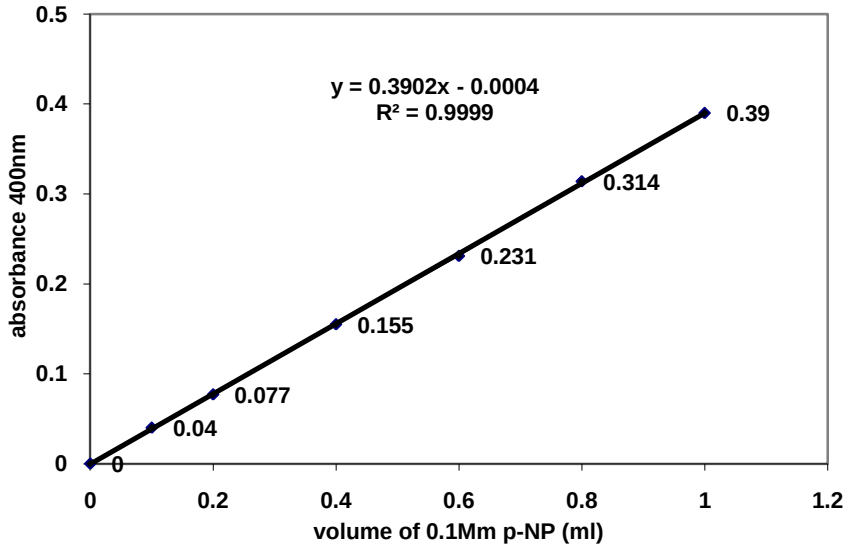

Figure 4-7 Standard curve for p-NP at pH 5.5

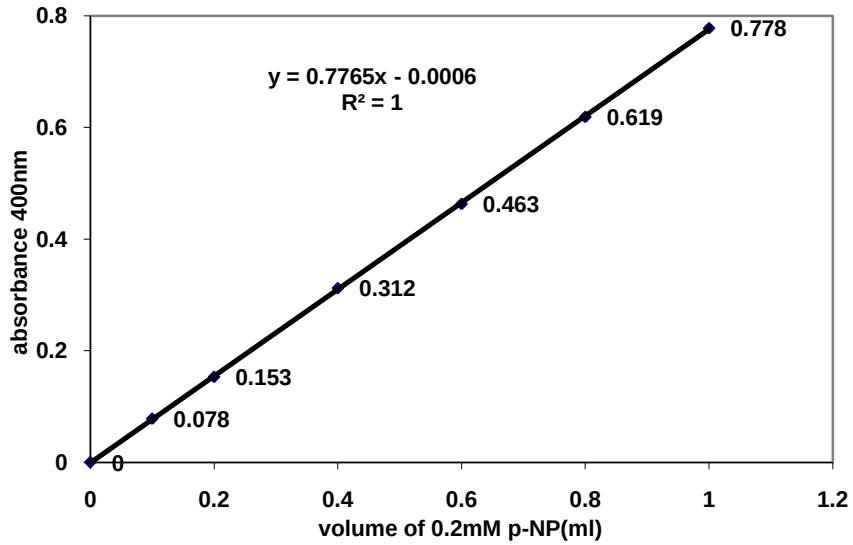

Figure 4-8 Standard curve for p-NP (high concentration) at pH 5.5

4.4.2. Stability of Apple Seed Extracts

The stability of apple seed extracts was tested by using daidzin and genistin as substrates, with product measured after extraction by HPLC (Merck-Hitachi Lachrom L-series) weekly. The enzyme reaction conditions were pH 5.5, incubated at 50°C for 1.0hr.

The results are shown in table 4-2 and figure 4-9, from the average of triplicate experiments. It was found that storage at 4°C was sufficient to maintain the stability of the enzyme since it was stable under these conditions for at least 12 weeks. It was noted that the hydrolysis ability of the enzyme increased with storage time, eventually reaching the highest level at 4 weeks after extraction.

Table 4-2 Stability tests of the enzyme extracted from apple seeds

Days of storage at 4°C	Percentage hydrolysis of daidzin					Percentage hydrolysis of genistin				
	1	2	3	M	SD	1	2	3	M	SD
2	73.6	77.8	78.0	76.4	2.5	37.6	39.3	39.1	38.7	0.91
7	77.2	75.3	77.0	76.5	1.1	38.9	41.3	40.7	40.3	1.3
14	76.9	75.4	77.5	76.6	1.1	45.3	47.3	46.3	46.3	0.98
20	93.6	96.3	95.1	95.0	1.4	50.8	53.4	53.9	52.7	1.7
30	100	100	100	100	0	74.8	76.4	75.3	75.5	0.78
41	100	100	100	100	0	74.9	73.6	75.8	74.8	1.2
50	100	100	100	100	0	76.2	74.4	76.7	75.8	1.3
55	98.1	100	99.1	99.1	0.97	68.1	70.3	69.0	69.2	1.1
63	100	100	100	100	0	70.9	69.5	69.2	69.9	0.93
70	100	100	100	100	0	73.4	71.6	72.9	72.6	0.95
77	99.7	100	98.6	99.4	0.76	70.3	72.2	71.9	71.5	1.0
83	100	100	100	100	0	74.7	73.1	72.2	73.3	1.3

1, 2, 3: triplicate determinations; M: mean; SD: standard deviation;

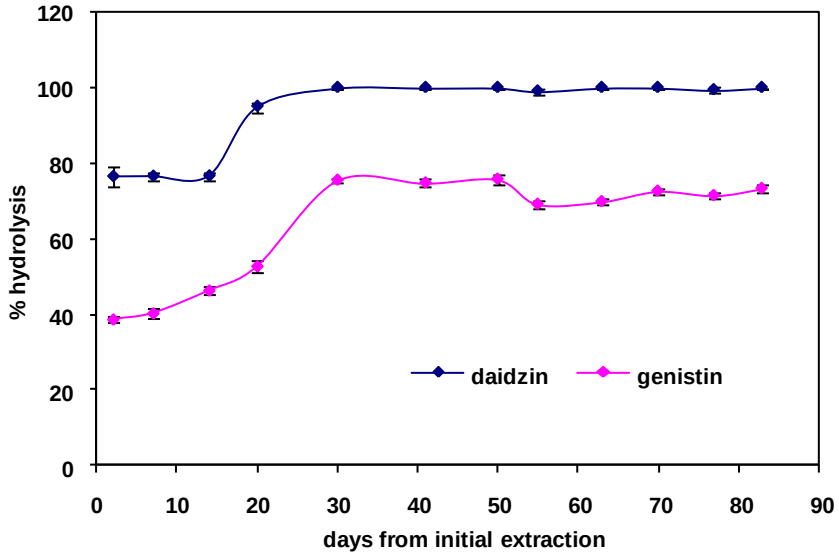

Figure 4-9 Stability of enzyme extracted from apple seeds

4.4.3. Optimum Reaction Conditions

4.4.3.1. Optimization

As with the almond extracts in previous experiments, an orthogonal experiment was designed for optimization of the reaction conditions for apple seed extracts. The experimental points are shown in the factor-level table 4-3 and the experiments were carried out according to the $L_9(3^4)$. Only the percentage hydrolysis of genistin was used since daidzin was easily hydrolysed completely at the tested concentration.

Table 4-3 Factor-level table for apple seed extracts optimization

Factor	pH Value	Temperature	Reaction Time
Level 1	4.5	50 ˚C	1.0 hr
Level 2	5.5	60 ˚C	1.5 hr
Level 3	6.5	70 ˚C	2.0 hr

From the range (R_Σ) calculated in table 4-4, it is seen that incubation temperature was the most critical factor. Both pH value and reaction time were less important since they were at the same level as the error given by the blank (column 4). The initially determined optimum conditions were given by 70°C, pH 5.5, for 2.0hr.

Table 4-4 Design of orthogonal experiment, results and statistics ($L_9(3^4)$) (apple seed extracts).

No. of experiment	Column 1 (pH)	Column 2 (temperature)	Column 3 (time)	Column 4 (error)	Result (hydrolysis%)
1	1 (4.5)	1 (50°C)	1 (1.0hr)	1	45.18
2	1 (4.5)	2 (60°C)	2 (1.5hr)	2	71.92
3	1 (4.5)	3 (70°C)	3 (2.0hr)	3	96.32
4	2 (5.5)	1 (50°C)	3 (2.0hr)	2	78.33
5	2 (5.5)	2 (60°C)	1 (1.0hr)	3	77.50
6	2 (5.5)	3 (70°C)	2 (1.5hr)	1	87.94
7	3 (6.5)	1 (50°C)	2 (1.5hr)	3	48.62
8	3 (6.5)	2 (60°C)	3 (2.0hr)	1	70.90
9	3 (6.5)	3 (70°C)	1 (1.0hr)	2	85.60
Σ_1 (level 1)	213.42	172.13	208.28	204.02	Σ = 662.31
Σ_2 (level 2)	243.77*	220.32	208.48	235.85	
Σ_3 (level 3)	205.12	269.86*	245.55*	222.44	
$R_\Sigma=\Sigma_{max}-\Sigma_{min}$	38.65	97.73	37.27	31.83	

Σ_1: the sum of all level 1 results; Σ_2: the sum of all level 2 results; Σ_3: the sum of all level 3 results, Σ: the sum of all results.

Variance analysis was then carried out since it could quantify the effect of the different factors. The result is shown in table 4-5. The significance of these considered factors was:

Temperature * > Time of Hydrolysis ≈ pH Value > Error

(*: the effect of the factor is significant)

The conclusion from the variance test was similar to that from table 4-4 with the significance order of pH value and reaction time reversed. This is rarely observed, which is also an advantage of using the variance analysis.

The effect of temperature reached significance level because its F value was bigger than $F_\alpha(f_1, f_e)$ ($\alpha = 0.10$). The effects of other two factors considered, pH value and reaction time, were much smaller. Their F values were 1.62 and 1.80 respectively, which meant that neither of them had a significant contribution towards the total variance and it was unnecessary to consider these factors apart from the convenience. Hence the optimal reaction conditions could be ensured at 70°C, pH 5.5, and 1.0hr.

Table 4-5 Result of variance analysis (apple seed extracts).

Source of Variance	Sum of Variance	Freedom	Variance	F Value	$F_{\alpha}(2,2)$
pH Value	275.98	2	137.99	1.62	$F_{0.05}(2,2)$ =19.0 $F_{0.10}(2,2)$ = 9.0 $F_{0.25}(2,2)$ = 3.0
Temperature	1591.96	2	795.98	9.35*	
Reaction Time	307.03	2	153.52	1.80	
Error	170.25	2	85.13		
Total	2345.22	8			

The sum of variance is calculated from the Σ values in table 4-4. The formula: sum of variance (factor)=$1/3(\Sigma_1^2+\Sigma_2^2+\Sigma_3^2)-1/9(\Sigma)^2$; sum of variance (total)=(sum of result^2)$-1/9(\Sigma)^2$.

4.4.3.2. Effect of Temperature on Apple Seed Extracts

Since the incubation temperature was the most important factor, and the optimum conditions concluded from orthogonal experiments was at the extreme experimental point (at the edge of the experimental area), another experiment was carried out to deeply test the effect of temperature and the results are shown in table 4-6 and figure 4-10. Since the hydrolysis percentage of genistin reached a stable situation around 60-75°C (plateau period), double the volume of genistin was used in order to try and differentiate any differences. The result at 70°C was better than at 60°C as 4.4.3.1., but 65°C was better than at 70°C.

Table 4-6 Effect of temperature on apple seed extracts

Temp	% hydrolysis of initial 75µl genistin					% hydrolysis of initial 150µl genistin				
(°C)	1	2	3	M	SD	1	2	3	M	SD
40	57.7	59.3	58.0	58.4	0.83	46.6	48.4	48.8	48.0	1.2
50	73.4	75.9	75.1	74.8	1.3	62.3	64.3	62.6	63.0	1.1
55	83.3	85.4	83.8	84.2	1.1	66.9	65.2	67.3	66.5	1.1
60	95.9	97.2	94.4	95.9	1.5	72.3	70.8	72.7	71.9	0.98
65	96.7	98.3	97.1	97.4	0.86	80.3	82.3	81.1	81.2	0.99
70	96.9	95.5	97.0	96.4	0.85	73.4	75.6	74.1	74.4	1.2
75	93.0	94.1	92.0	93.1	1.1	68.6	66.4	67.0	67.4	1.2
80	66.7	68.2	68.7	67.8	1.1	32.2	34.4	33.2	33.4	1.2

1, 2, 3: triplicate determinations; M: mean; SD: standard deviation

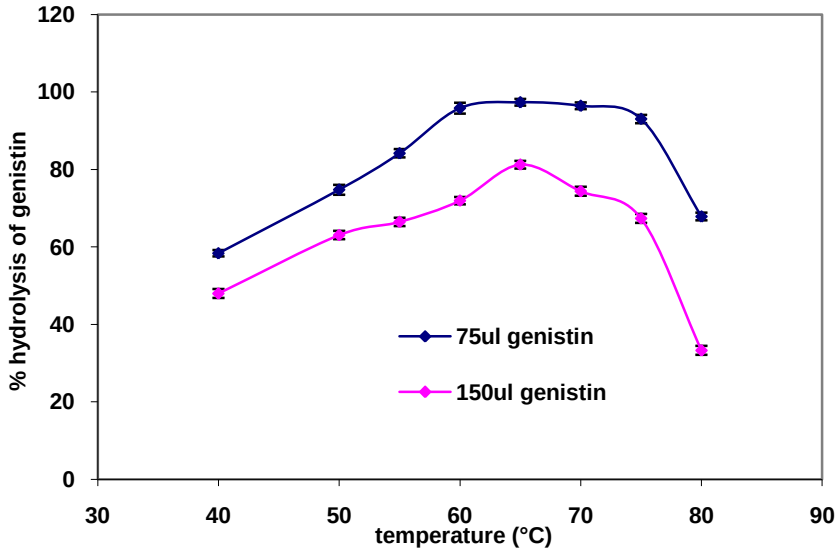

Figure 4-10 Effect of temperature on activity of apple seed extracts

4.4.3.3. Confirmation of the Optimum Conditions

An experiment was carried out to confirm the results of optimization by comparing the percentage hydrolysis of flavonoid glycosides under different enzyme reaction conditions. These were: (a) hydrolysis by almond extracts under the previously determined optimum conditions, (b) by apple seed extracts under the previously determined almond extracts' optimum conditions (i.e. apple seed tested conditions), and (c) by apple seed extracts under apple seed extracts' optimum conditions.

The results (table 4-7 and figure 4-11) show that the optimization enhanced the reaction efficiency significantly since the percentage hydrolysis of flavonoid glycosides increased noticeably. The optimum reaction conditions for the β-D-glucosidase extracted from apple seeds were finally confirmed as 65°C, pH 5.5, 1.0 hr.

Table 4-7 Confirmation of optimum conditions

% hydrolysis		almond-optimal conditions	apple seed-test conditions	apple seed-new conditions
Daidzin	1	98.5	77.6	100
	2	100	77.2	100
	3	98.9	74.9	100
	M	99.1	76.6	100
	SD	0.78	1.5	0
Genistin	1	66.9	54.7	100
	2	65.5	53.2	100
	3	69.1	50.2	100
	M	66.5	52.7	100
	SD	2.3	2.4	0
Quercetin -4'- glucoside	1	100	85.8	100
	2	100	87.8	100
	3	100	86.4	100
	M	100	86.7	100
	SD	0	1.1	0

1, 2, 3: triplicate determinations; M: mean; SD: standard deviation

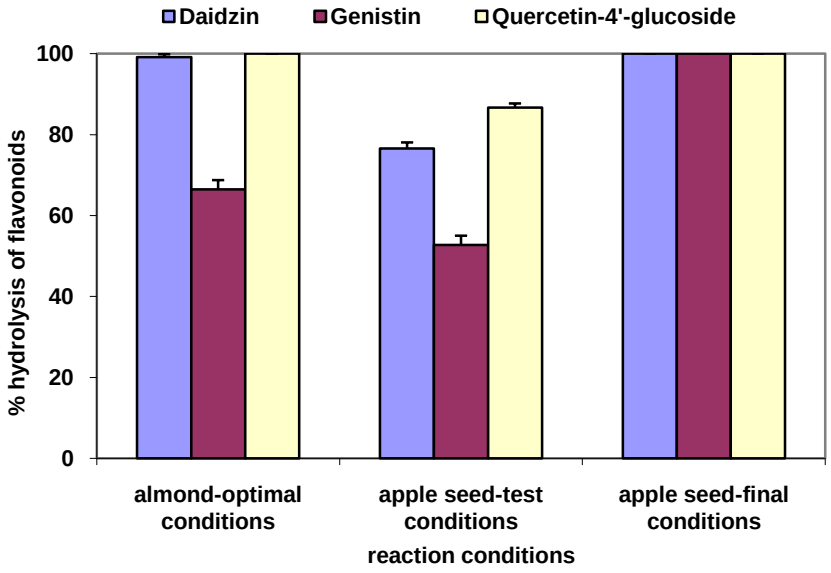

Figure 4-11 Optimum reaction conditions for almond and apple seed extracts

4.4.4. Content of Protein

Experiments were carried out at different conditions for the standard curve and for sample determination since the Bradford method can be affected by many factors (Bradford, 1976; Friedenauer & Berlet, 1989). The Bradford method is generally regarded as the best of the broad-spectrum protein methods being less susceptible to interference, in general, than other procedures (Kamizake *et al*., 2003). This method has been widely used to determine protein in food and enzyme samples by others (Hsieh & Graham, 2001; Barbagallo *et al*., 2007; Yu *et al*., 2007). The data is summarized in table 4-8 and the results are shown in table 4-9. BDH indicates that the CBB G-250 used was the product of BDH rather than from Sigma (see highlighted columns in table 4-8).

From table 4-8, it can be seen that:

- There was a significant difference between using low-concentration BSA (100μg/ml) and high-concentration BSA (1000μg/ml) and the absorbance readings of these 2 standard curves were not proportional. If the standard curve and sample were tested in the same way at the same time, the results did not show any significant difference.
- There was a significant difference between using CBB from Sigma and CBB from BDH. If the standard curve and samples were tested in the same way at the same time, the results did not show any significant difference.
- There was not any significant difference in results between using the BSA in water and BSA in 0.15M sodium chloride, so the data obtained from these two conditions could be put together in calculating protein concentration in the apple seed extracts and almond extracts in order to reduce the error. So, the data for standard curve y=3.0032x+0.0113 and y=3.0749x+0.0101 was put together to create a new standard curve y= 3.0391x+0.0107 which was used in determinations (figure 4-12).

Table 4-8 Summary of protein determination of apple seed extracts

Method	1mg/ml BSA in H_2O		1mg/ml BSA in NaCl		1mg/ml BSA in H_2O/BDH		1mg/ml BSA in NaCl/BDH		100ug/ml BSA in H_2O		100ug/ml BSA in NaCl	
	BSA(ml)	A595nm ±SD (n=3)	BSA(ml)	A595nm±SD (n=3)	BSA(ml)	A595nm ±SD (n=3)	BSA(ml)	A595nm ±SD (n=3)	BSA(ml)	A595nm ±SD (n=3)	BSA(ml)	A595nm ±SD (n=3)
	0	0	0	0	0	0	0	0	0	0	0	0
	0.01	0.033±0.002	0.01	0.039±0.002	0.01	0.240±0.003	0.01	0.241±0.002	0.1	0.024±0.001	0.1	0.109±0.002
	0.02	0.082±0.002	0.02	0.082±0.002	0.02	0.382±0.003	0.02	0.398±0.002	0.2	0.085±0.002	0.2	0.209±0.003
	0.04	0.143±0.005	0.04	0.146±0.002	0.04	0.593±0.006	0.03	0.517±0.005	0.4	0.275±0.004	0.4	0.348±0.003
	0.06	0.205±0.003	0.06	0.181±0.004	0.06	0.798±0.008	0.04	0.622±0.006	0.6	0.440±0.005	0.6	0.500±0.005
	0.08	0.241±0.010	0.08	0.262±0.010	0.08	1.020±0.007	0.05	0.841±0.008	0.8	0.557±0.006	0.8	0.612±0.009
	0.10	0.306±0.006	0.10	0.314±0.004	0.10	1.202±0.009	0.06	0.946±0.006	1.0	0.656±0.008	1.0	0.688±0.009
							0.08	1.140±0.006				
Equation	y=3.0032x+0.0113		y=3.0749x+0.0101		y=11.454x+0.0977		y=14.078x+0.0778		y=0.7082x-0.0227		y=0.6897x+0.0468	
R^2	0.9899		0.9923		0.9851		0.9832		0.9884		0.9814	
A (0.1ml sample)	0.226		0.228		0.924		0.616*		0.481		0.563	
	0.236		0.241		0.910		0.605*		0.492		0.555	
	0.231		0.230		0.920		0.603*		0.482		0.568	
Average	0.231		0.233		0.918		0.608		0.485		0.562	
SD	0.005		0.007		0.008		0.007		0.006		0.007	
conc. of protein (mg/ml)	0.715		0.709		0.721		0.763**		0.711		0.748	
	0.748		0.751		0.709		0.750**		0.730		0.737	
	0.732		0.715		0.718		0.746**		0.716		0.756	
average	0.732		0.725		0.716		0.753		0.719		0.747	
SD	0.017		0.023		0.007		0.009		0.010		0.010	

* absorbances were obtained with 0.05ml apple seed extracts and ** were converted to 0.1ml apple seed extracts

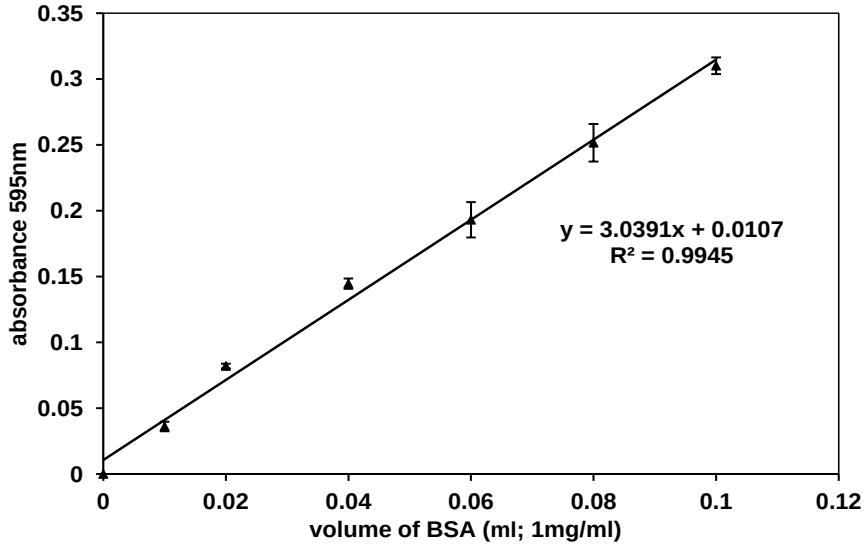

Figure 4-12 Standard curve for protein determination by the Bradford method (for further details, see text).

Table 4-9 Results of protein determination

Replicate number	Apple seed extracts (0.1ml)		Almond extracts (0.05ml)	
	A 595nm	Protein (mg/ml)	A 595nm	Protein (mg/ml)
1	0.226	0.708	0.210	1.31
2	0.236	0.741	0.201	1.25
3	0.231	0.725	0.204	1.27
4	0.228	0.715	0.197	1.23
5	0.241	0.758	0.202	1.26
6	0.230	0.722	0.207	1.29
M	0.232	**0.728**	0.204	**1.27**
SD	0.0056	0.019	0.0046	0.031

A: absorbance; M: mean; SD: standard deviation

Using equation y = 3.0391x + 0.0107, the protein content of tested crude enzyme extracts were calculated to be 0.728mg/ml in apple seed extracts and 1.27mg/ml in whole almond extracts.

4.4.5. Progress Curves

In order to make progress curves and determine the reaction time for Km and Vmax measurement, the product concentrations against reaction time were plotted using p-NP-Glc as substrate, and the results are shown in figures 4-13 and 4-14.

Because the enzyme catalyzed reaction rate would be constant at reaction times of less than 10mins, 5mins as reaction time was applied in determination of Km and Vmax.

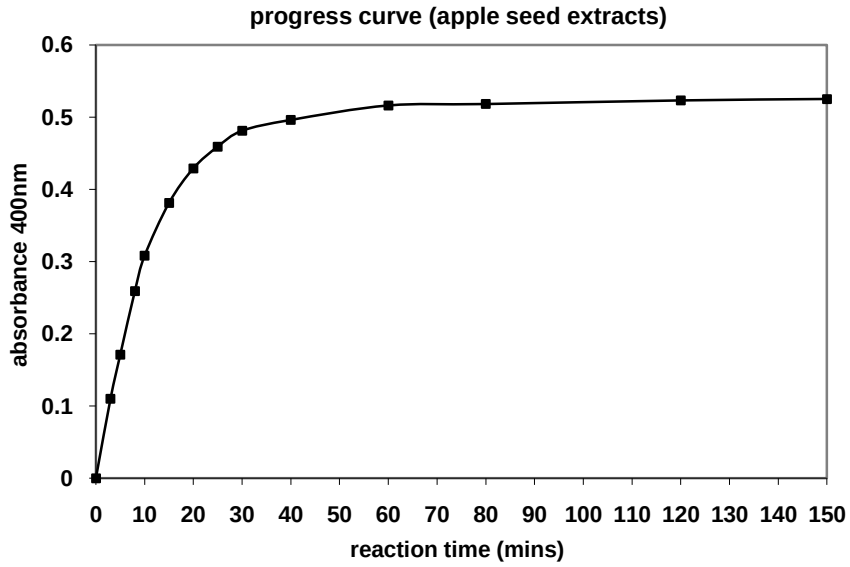

Figure 4-13 Product concentration against time (apple seed extracts)

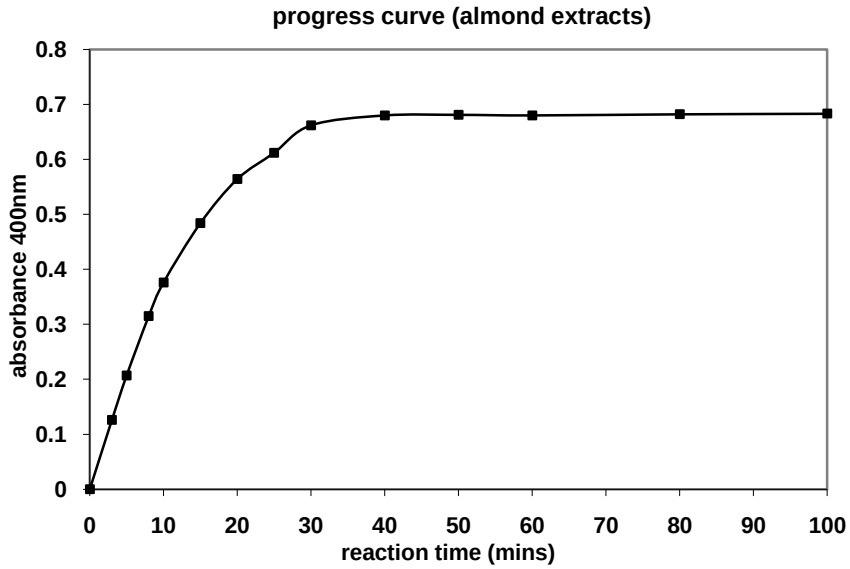

Figure 4-14 Product concentration against time (almond extracts)

4.4.6. The Linearity of Velocity against Enzyme Concentration

Experiments were carried out to test the linearity of velocity against enzyme concentration. (Table 4-10 and figure 4-15 show the results for apple seed extracts, table 4-11 and figure 4-16 show the results for whole almond extracts). The averages of triplicate results with standard deviation show the linearity to be very good.

Table 4-10 Relationship between velocity and enzyme concentration (apple seed extracts)

Enzyme (ml of original extract)	Incubation volume (ml)	A 400nm	V (μmol)	V (μmol/min)	SD
0.004	0.2	0.179	0.2299	0.02299	0.0011
0.01	0.2	0.356	0.4567	0.04567	0.0032
0.02	0.1	0.393	1.008	0.1008	0.0093
0.04	0.05	0.350	1.796	0.1796	0.010
0.1	0.02	0.341	4.375	0.4375	0.022

Substrate was 0.20ml p-NP-G (0.05M); reaction time was 10mins;
SD: standard deviation

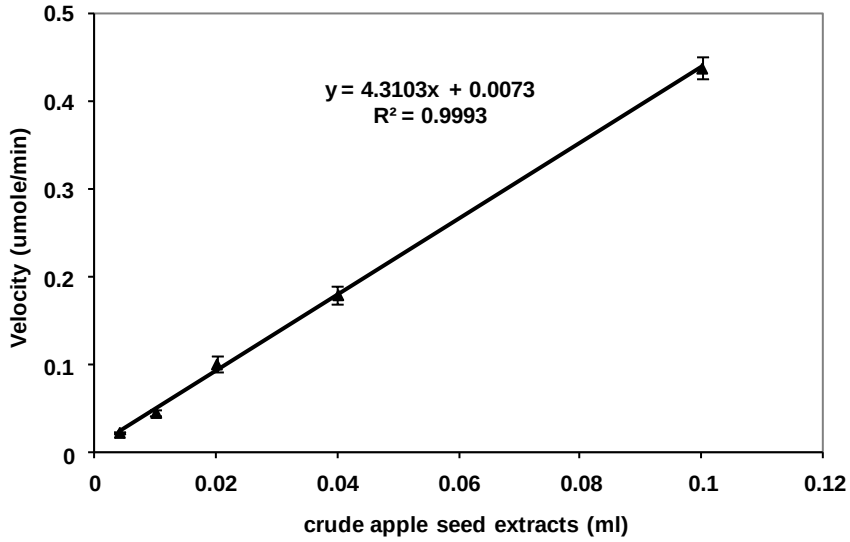

Figure 4-15 Velocity against enzyme concentration (apple seed extracts)

Table 4-11 Relationship between velocity and enzyme concentration (whole almond extracts)

Enzyme (ml of original extract)	Incubation volume (ml)	A 400nm	V (μmol)	V (μmol/min)	SD
0.005	0.2	0.582	0.7503	0.07503	0.0054
0.01	0.1	0.516	1.331	0.1331	0.0098
0.02	0.02	0.168	2.158	0.2158	0.018
0.05	0.02	0.384	4.926	0.4926	0.031
0.1	0.02	0.715	9.216	0.9216	0.035

Substrate was 0.20ml p-NP-G (0.05M); reaction time was 10mins;
SD: standard deviation

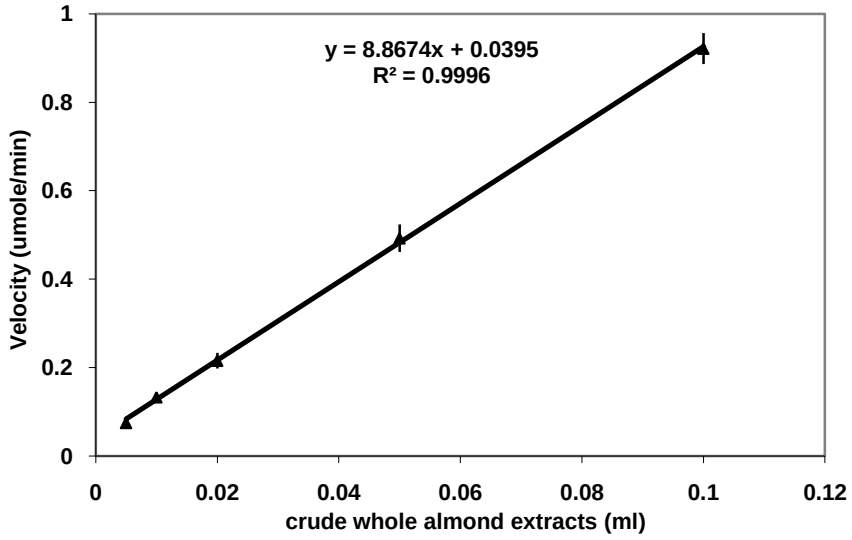

Figure 4-16 Velocity against enzyme concentration (whole almond extracts)

4.4.7. Km and Vmax

The determination of Km and Vmax was repeated 8 times for apple seed extracts, 3 times for almond extracts. The data is summarized in table 4-12 and table 4-13 respectively. By plotting 1/V against 1/[S], the Lineweaver-Burk plot was obtained, the results are shown in table 4-14.

Table 4-12 Data for Vmax and Km determination (apple seed extracts). Columns contain data from replicate determinations at the conditions described.

0.05M p-NPG (ml)	0.10	0.20	0.30	0.40	0.50	0.60	0.80
substrate (mM)	5	10	15	20	25	30	40
1/[S] (mM^{-1})	0.200	0.100	0.067	0.050	0.040	0.033	0.025
Absorbance 400nm	0.208	0.268	0.306	0.341	0.359	0.368	0.380
1/V (min/µmol)	9.362	7.269	6.367	5.7147	5.428	5.296	5.129
Absorbance 400nm	0.211	0.273	0.303	0.328	0.348	0.368	0.377
1/V (min/µmol)	9.229	7.136	6.430	5.941	5.600	5.296	5.170
Absorbance 400nm	0.199	0.256	0.293	0.319	0.337	0.337	0.363
1/V (min/µmol)	9.784	7.609	6.650	6.108	5.782	5.782	5.369
Absorbance 400nm	0.216	0.295	0.340	0.369	0.386	0.401	0.387
1/V (min/µmol)	9.016	6.605	5.731	5.2815	5.049	4.860	5.036
Absorbance 400nm	0.224	0.298	0.331	0.365	0.396	0.408	0.419
1/V (min/µmol)	8.694	6.538	5.887	5.339	4.922	4.777	4.652
Absorbance 400nm	0.220	0.305	0.334	0.359	0.373	0.386	0.410
1/V (min/µmol)	8.852	6.388	5.834	5.428	5.225	5.049	4.754

0.075M p-NPG (ml)	0.10	0.20	0.30	0.40	0.60	0.80
substrate (mM)	7.5	15	22.5	30	45	60
1/[S] (mM^{-1})	0.133	0.067	0.044	0.033	0.022	0.017
Absorbance 400nm	0.262	0.325	0.364	0.392	0.413	0.400
1/V (min/µmol)	7.435	5.996	5.354	4.972	4.719	4.641
Absorbance 400nm	0.250	0.305	0.342	0.376	0.396	0.399
1/V (min/µmol)	7.792	6.388	5.698	5.183	4.922	4.885

Table 4-13 Data for Vmax and Km determination (whole almond extracts). Columns contain data from triplicate determinations at the conditions described.

0.05M p-NPG (ml)	0.10	0.20	0.30	0.40	0.60	0.80
substrate (mM)	5	10	15	20	30	40
1/[S] (Mm^{-1})	0.200	0.100	0.067	0.050	0.033	0.025
Absorbance 400nm	0.203	0.252	0.276	0.287	0.306	0.318
1/V (min/µmol)	9.592	7.730	7.059	6.788	6.367	6.128
Absorbance 400nm	0.184	0.226	0.247	0.256	0.284	0.281
1/V (min/µmol)	10.58	8.617	7.886	7.609	6.860	6.933
Absorbance 400nm	0.220	0.268	0.297	0.302	0.326	0.345
1/V (min/µmol)	8.852	7.269	6.560	6.452	5.977	5.649

As shown in table 4-14, the kinetic parameters of tested enzyme extracts were:

- apple seed extracts (n = 8): Km 5.48 ± 0.34 mM; Vmax 15.60 ± 0.95 U/mg protein; Vmax/Km 2.85;
- almond extracts (n = 3): Km 3.32 ± 0.067 mM; Vmax 13.54 ± 1.2 U/mg protein; Vmax/Km 4.08

Table 4-14 Summary of Vmax and Km determinations

		Formation (y = a + bx)	R^2	a	b	1/a	Vmax (U/ml)	Vmax (U/mg prot.)	Km (mM) = b/a	Vmax/Km
apple seed extracts	1	y = 24.679x + 4.551	0.9904	4.551	24.679	0.2197	10.987	15.091	5.423	2.783
	2	y = 23.169x + 4.6957	0.9909	4.6957	23.169	0.2130	10.648	14.626	4.934	2.964
	3	y = 25.039x + 4.8843	0.9926	4.8843	25.039	0.2047	10.237	14.062	5.126	2.743
	4	y = 24.462x + 4.2285	0.9945	4.2285	24.462	0.2365	11.824	16.242	5.785	2.808
	5	y = 25.762x + 4.4516	0.9851	4.4516	25.762	0.2246	11.232	15.428	5.787	2.666
	6	y = 24.145x + 4.1635	0.9923	4.1635	24.145	0.2402	12.009	16.496	5.799	2.845
	7	y = 23.317x + 4.1155	0.9921	4.1155	23.317	0.2430	12.149	16.688	5.666	2.946
	8	y = 22.789x + 4.2564	0.9968	4.2563	22.789	0.2349	11.747	16.136	5.354	3.014
		Average					**11.35**	**15.60**	**5.48**	**2.85**
		Standard Deviation					0.70	0.95	0.34	0.12
almond extracts	1	y = 19.457x + 5.737	0.9975	5.737	19.457	0.1743	17.431	13.725	3.391	4.047
	2	y = 21.232x + 6.4001	0.9894	6.4001	21.232	0.1562	15.625	12.303	3.317	3.709
	3	y = 17.601x + 5.3997	0.9875	5.3997	17.601	0.1852	18.520	14.582	3.260	4.474
		Average					**17.19**	**13.54**	**3.32**	**4.08**
		Standard Deviation					1.5	1.2	0.067	0.39

Vmax (U/ml) = 1/a * 50; 1U = the amount of enzyme releasing 1 μmol of p-nitrophenol/min under assay conditions

4.5. Discussion

4.5.1. The Optimum Conditions for Enzyme Activity

The idea of an optimum rate of an enzyme reaction is misleading, as the rate observed at any temperature is the product of two rates, the reaction rate and the denaturation rate (Stauffer, 1989; Bisswanger, 2004). An assay measuring activity for one second would give high activity at high temperatures, while an assay measuring product formation over an hour would give low activity at these temperatures (Daniel *et al.*, 2009).

In this case, the optimum pH value, temperature and reaction time were tested as a whole by an orthogonal table arrangement and calculated by variance analysis, so the result is reliable not only because the orthogonal design is more representative of the total experimental range, but also because error can be displayed and compared with other tested factors. As shown in table 4-4, the most important factor was temperature, which was different to almond (see chapter 3). The others factors were less important as their variance contributed at the level of error. This also meant all the important factors were tested and the real optimum conditions were found. The conditions were confirmed by observing the increased percentage hydrolysis of tested flavonoid glycosides (see table 4-7 and figure 4-11).

On the other hand, the reaction times selected in table 4-3 were from 1.0 hr to 2.0 hr. As can be seen in the enzyme progress curves (figure 4-13 and figure 4-14), the absorbance (representing product concentration) has already reached the highest stable level by 60mins, so there should not be much difference beyond this period (1.0hr-2.0hr).

4.5.2. Enzyme Stability

From the apple seed extracts stability experiment, it can be seen that the activity increases on storage at 4°C, reaching the highest point after 4 weeks, and then remains

steady. This may be due to the particular enzyme source, the molecular state of the enzyme released by extraction, or the loss of endogenous inhibitor activity (Shewale 1982; van den Tweel *et al.*, 1993), in contrast to the state of enzyme extracted from almond (see chapter 3).

4.5.3. Protein Determination

The Bradford method is a fast and sensitive method for determining protein content especially for large numbers of samples. However, the linearity of the standard curve is a limitation (Peterson, 1977; Splittgerber & Sohl, 1989). Another weak point is that the reaction time of the protein-dye binding is an important factor influencing the determination (see figure 4-17), although it was reported that the dye binding process is virtually complete in approximately 2min with relatively good colour stability for 1hr (Bradford, 1976). The standard curve and the sample must be prepared at the same time, making sure that the time interval between adding CBB G-250 to every tube to reading the absorbance is the same, and that experiments are finished in 20mins.

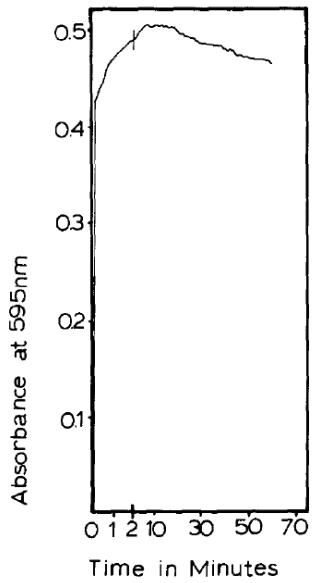

Figure 4-17 Protein-dye complex formation rate and colour stability (Bradford, 1976)

Whether using 0.15M sodium chloride or not for making the BSA solution did not look to be critical, especially for short time storage. However, using different company's products seriously influenced the absorbance values (see table 4-8). Using

a commercially-available Bradford Protein Kit, which contains protein standard and protein agents that have been matched and ready for use, could be a good choice to minimise variation.

4.5.4. Enzyme Kinetic Parameters

4.5.4.1. The Real Progress Curve

Enzymes are catalysts which reduce the required activation energy so these reactions proceed at rates that are useful especially in living systems which might otherwise occur at extremely slow rates.

In most cases, an enzyme converts one chemical (the *substrate*), into another (the *product*). A real graph of product concentration vs. time follows three phases as shown in the following graph (Bisswanger, 2008).

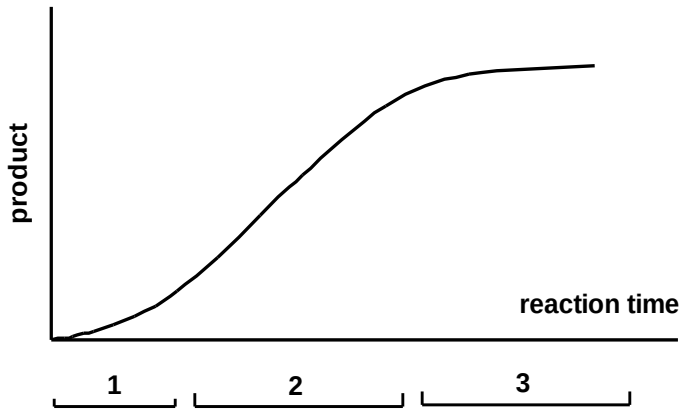

Figure 4-18 Real progress curve for an enzyme reaction

At very early time points, the phase 1, the rate of product accumulation increases over time. But special techniques are needed to study the early kinetics of enzyme action, since this transient phase usually lasts less than a second (the figure greatly exaggerates the first phase) (Tipton, 2002). For an extended period of time, phase 2, the product concentration increases linearly with time, which is the so-called "initial period".

Although figure 4-18 is the real situation of a progress curve, most enzyme studies consider data collected only in the second phase. The terminology describing these phases can be confusing (Bisswanger, 2008). The second phase is often called the "initial rate", ignoring the short transient phase that precedes it. It is also called "steady state".

4.5.4.2. The Linearity of V against [E]

One of the prerequisites of the enzyme assay is that V_0, the initial velocity, is proportional to the enzyme concentration. To test this, an experiment was carried out and the results showed that the linearity was very good with the R^2 reaching 0.999.

The linearity of velocity against enzyme concentration is an important prerequisite of enzyme assays. As enzymes are catalysts, the initial velocity of the reaction would be expected to be proportional to the concentration of the enzyme. In the progress of the enzyme reaction, the period of linearity may be prolonged by decreasing the enzyme concentration, slowing down the rate of product formation, and increasing the sensitivity of the assay method, if necessary (Tipton, 2002). This is indeed the case for most enzyme-catalysed reactions, where the graph of initial velocity against enzyme concentration should be a straight line passing through the origin (zero activity at zero enzyme concentration). However, in some cases this simple relationship does not appear to hold and it is thus important to check for linearity in all studies. The graph of initial velocity against enzyme concentration can show either upward or downward curvature, and is caused by different factors.

In this case, the linearity of velocity against enzyme concentration is very good but the graph of initial velocity against enzyme concentration did not pass through the origin (zero point) – there was absorbance at zero enzyme concentration, i.e. there was a blank rate. The cause resulting in such behaviour was quite simple, because the artificial substrate used was p-NP-Glc which is relatively unstable in aqueous solution and steadily hydrolysed to liberate p-nitrophenol (Tipton, 2002). This blank rate due to non-enzyme reaction should be corrected by being subtracted from the rate given in

the presence of enzyme. In this case, simply subtract the absorbance reading, i.e. subtract the reagent control from the sample reading.

4.5.4.3. Evaluation of Enzyme Kinetic Analyses

Standard analyses of enzyme kinetics usually assume (Bisswanger, 2008):

- The production of product is linear with time during the time interval used.
- The concentration of substrate greatly exceeds the concentration of enzyme. This means that the free concentration of substrate is very close to the concentration added, and that substrate concentration is constant throughout the assay.
- A single enzyme forms the product.
- There is negligible spontaneous creation of product without enzyme.
- Non co-operativity. Binding of substrate to one enzyme binding site doesn't influence the affinity or activity of an adjacent site.
- Neither substrate nor product acts as an allosteric modulator to alter the enzyme velocity.

As some of the assumptions are not always true, there are questions to ask when evaluating the results of enzyme kinetic experiments:

- Is the production of product linear with time?

The concentration of product must be checked at several times to test this assumption.

In this case, this is not a problem. Figure 4-13 & 4-14 show the progress curves of the hydrolysis reaction catalysed by apple seed extracts and almond extracts respectively. The rate was absolutely constant for the first 10mins, so the production of product was linear in the chosen reaction time of 5mins.

- Was only a small fraction of the substrate converted to product?

The analysis assumes that the free concentration of substrate is almost identical to the concentration added during the time course of the assay. This assumption can

be tested by comparing the lowest concentration of substrate used in the assay with the concentration of product created at that concentration.

In this case, the lowest amount of substrate used was 5μmol. At this concentration, the average corresponding amount of product created was 0.547μmol by apple seed extracts and 0.520μmol by almond extracts. The percentages of substrate consumption were 10.94% and 10.40% respectively.

It has often been assumed that restricting measurements of reaction rates to a period in which less than 10-20% of the total substrate consumption has occurred will provide a true measure of the initial rate (Tipton, 2002). So this assumption was fulfilled very well because in general, the higher concentration will cause the lower percentage of substrate consumption.

In this case, one way to determine the amount of enzyme used is simple: hydrolysing 0.10ml p-NP-G (0.05M) with different enzyme dilutions, and then using 0.10ml reaction mixture to measure the absorbance (as described in 4.5.4.2). According to the p-NP standard curve equation y = 0.3902x – 0.0004, the corresponding readings for 10-20% of substrate consumption were 0.1947-0.3898. As a result, 0.02ml original extract solution of apple seed extracts was chosen (see table 4-10, where it was shown that at 10μmol substrate, 0.10ml of apple seed extracts could cause 43.75% of substrate consumption while 0.02ml caused 10.08 % only), and for almond extracts, it was decided 0.01ml was to be used (for almond extracts, using 0.02ml at 5μmol substrate amount could create 30.58% substrate consumption with an absorbance reading of 0.593).

- Is a high enough concentration of substrate being used?

The ratio of the highest substrate concentration used divided by the best-fit value of Km (both in the same concentration units) should be calculated. Ideally, the highest concentration should be at least 10 times the Km.

In this case, the highest concentration of p-NP-Glc used for apple seed extracts was 60mM, which is more than 10 times the Km=5.48mM; the highest

concentration of p-NP-Glc used for almond extracts was 40mM, which is more than 10 times the Km=3.32mM. 60mM was the limit under this experimental condition since 0.075M p-NP-Glc is nearly at saturation.

- Are the standard errors too large? Are the confidence intervals too wide?

The standard deviation of the Vmax should be divided by the Vmax, and the SD of the Km divided by the Km. If either ratio is much larger than about 20%, the reasons should be understood.

In this case, all of the ratios were less than 10%.

- Is product produced in the absence of enzyme?

The analysis assumes that all product formation is due to the enzyme. If some product is produced spontaneously, alternative adjustment or correction is required.

It is not uncommon to observe an apparent rate of reaction in the absence of one of the components of the complete assay mixture, especially the enzyme. It is important to understand the causes of such blank rates in order to make appropriate corrections. It is possible that a blank rate will only occur with certain components of an incomplete assay mixture and thus it is necessary to test for such rates using different combinations of the system, for example, by omitting the enzyme and each of the substrates in turn. Some of the more common causes of blank rates include: settling of particles; precipitation; contamination of one of the components of the assay mixture; and non-enzymatic reaction (Tipton, 2002).

In this case, para-nitrophenyl-β-D-glucopyranoside (p-NP-Glc) was used as artificial substrate of the enzyme β-D-glycosidases. Similar to many other p-nitrophenol esters, p-NP-Glc is relatively unstable in aqueous solution and steadily hydrolysed to liberate p-nitrophenol (Tipton, 2002). The blank rate due to non-enzyme reaction should be corrected for by subtracting it from the rate given in the presence of enzyme. In this case, spectrophotometric assay was applied which means the correction can be most conveniently be done by using a double-

beam spectrophotometer that automatically records the difference between the absorbance of the sample and that of the blank. So, in every determination, reagent control was applied in order to subtract the blank absorbance. This also means that the conversions observed are related to enzyme activity alone.

- Was a time point chosen at which enzyme velocity was constant?

Product formation was measured at several time points straddling the time used for the assay. The graph of product concentration vs. time should be linear.

See figures 4-13 & 4-14. The enzyme velocities were constant at the first 10mins, so 5mins was chosen as the time point for the determination.

- Is there any evidence of co-operativity?

The standard analysis assumes no co-operativity. This means that binding of substrate to one binding site does not alter binding of substrate to another binding pocket. Since many enzymes are multimeric, this assumption is often not true.

In this case, the graph of V vs. [S] bent slightly toward the x-axis (showed in figures 4-19 and 4-20), which is the correct situation, so there was no evidence of co-operativity.

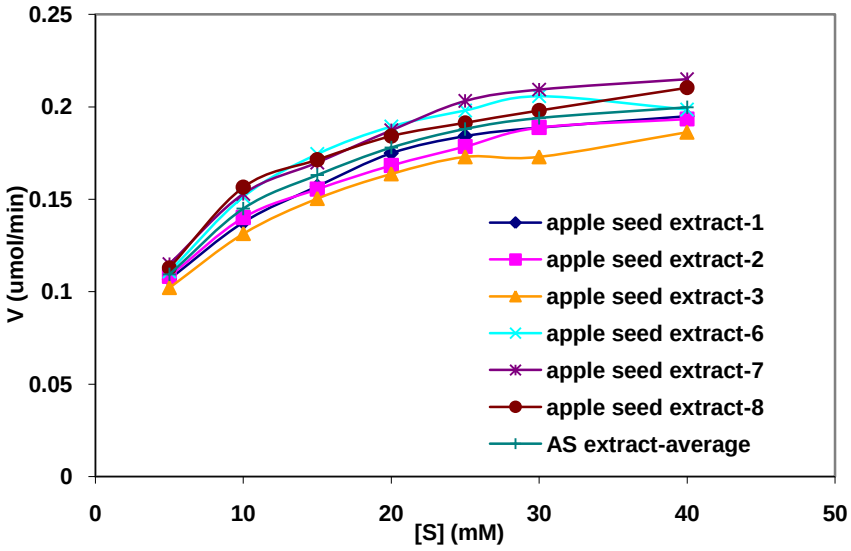

Figure 4-19 Graph of V vs. [S] to test co-operativity (apple seed extracts, data converted from table 4-12)

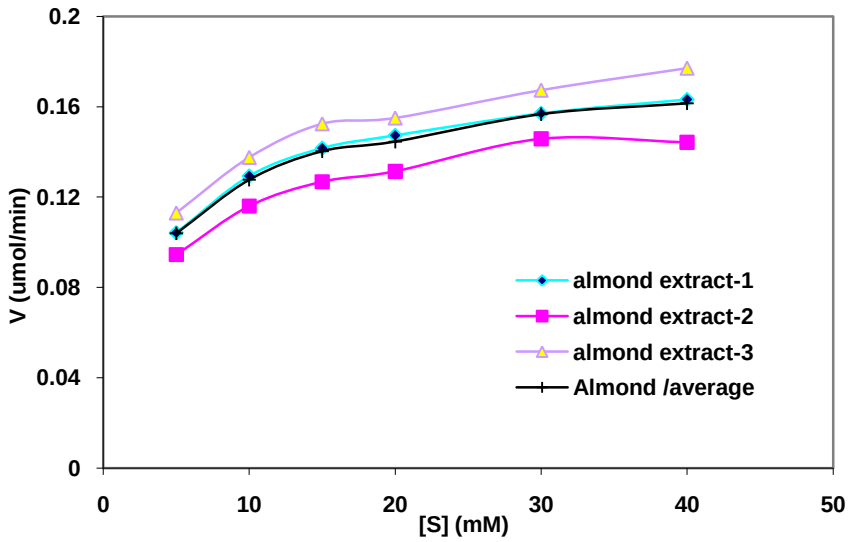

Figure 4-20 Graph of V vs. [S] to test co-operativity (almond extracts, data converted from table 4-13)

In summary, all of the assumptions were fulfilled well and the enzyme assay was successful.

4.5.4.4. The Michaelis-Menten Kinetic Model

There is an initial bimolecular reaction between the enzyme E and substrate S to form the enzyme-substrate complex ES. Although the enzymatic mechanism for the unimolecular reaction $ES \xrightarrow{k_{cat}} E + P$ can be quite complex, there is typically one rate-determining enzymatic step that allows this reaction to be modelled as a single catalytic step with an apparent unimolecular rate constant k_{cat}. If the reaction path proceeds over one or several intermediates, k_{cat} will be a function of several elementary rate constants, whereas in the simplest case of an single elementary reaction (e.g. no intermediates) it will be identical to the elementary unimolecular rate constant k_2 (see figure 4-1) (Xie & Lu, 1999). The apparent unimolecular rate constant k_{cat} is also called *turnover* number and denotes the maximum number of enzymatic reactions catalyzed per second.

The Michaelis–Menten equation describes how the (initial) reaction rate V_0 depends on the position of the substrate-binding equilibrium and the rate constant k_2 (Tipton, 2002).

$$V_0 = \frac{Vmax\,[S]}{[S] + K_M}$$ (Michaelis-Menten equation)

with the constants:

$$K_M \overset{def}{=} \frac{k_2 + k_{-1}}{k_1} \approx K_D \quad \text{and} \quad Vmax \overset{def}{=} k_{cat}\,[E]_{total}$$

So the k_{cat} can be calculated if the $[E]_{total}$ is known. It is also be used to estimate the enzyme velocity and express the enzyme efficiency, which means how many substrate molecules can be converted into product by each enzyme molecule per time (preferably per second) (Bisswanger, 2004). Generally k_{cat} ranges from $1\text{-}10^4$ s^{-1} and the bigger the better.

In this case, the k_{cat} of apple seed extracts and almond extracts were $(1.30\times10^{-2})\times W$ s^{-1} and $(2.26\times10^{-2})\times W$ s^{-1} respectively. If the relative molecular weight (W) of the enzyme is known, it is possible to express the [E] as the *molecular activity*, then the k_{cat} can be calculated.

This Michaelis-Menten equation is the basis for most single-substrate enzyme kinetics. Two crucial assumptions underlie this equation (apart from the general assumption about the mechanism only involving no intermediate or product inhibition, and there is no allostericity or co-operativity) (Copeland, 2000). The first assumption is the so called quasi-steady-state assumption (or pseudo-steady-state hypothesis), namely that the concentration of the substrate-bound enzyme (and hence also the unbound enzyme) changes much more slowly than those of the product and substrate and thus the change over time of the complex can be set to zero ($d[ES]/dt = 0$). The second assumption is that the total enzyme concentration does not change over time, thus $[E]_{total} = [E] + [ES] = \text{constant}$ (Tipton, 2002).

The Michaelis constant Km is experimentally defined as the concentration at which the rate of the enzyme reaction is half Vmax, which is the smaller the better. If the rate-determining enzymatic step is slow compared to substrate dissociation ($k_2 << k_{-1}$),

the Michaelis constant Km is roughly the dissociation constant K_D of the ES complex (Bisswanger, 2004).

If [S] is small compared to K_M then the term [S]/(Km+[S]) ≈ [S]/Km and also very little ES complex is formed, thus $[E]_0 \approx [E]$. Therefore, the rate of product formation is:

$$V_0 \approx \frac{k_{cat}}{K_M}[E][S] \qquad \text{if} \quad [S] << K_M$$

Thus the product formation rate depends on the enzyme concentration as well as the substrate's concentration, the equation resembles a bimolecular reaction with a corresponding pseudo-second order rate constant k_2/K_M (Tipton, 2002). This constant is a measure of catalytic efficiency. The most efficient enzymes reach a k_2/K_M in the range of 10^8-10^{10} $M^{-1}\cdot s^{-1}$. These enzymes are so efficient they effectively catalyze a reaction each time they encounter a substrate molecule and have thus reached an upper theoretical limit for efficiency (diffusion limit); these enzymes have often been termed *perfect enzymes* (Bisswanger, 2004).

In this case, for apple seed extracts, $k_2/Km = k_{cat}/Km = Vmax/([E]\ Km) = 2.37W\ M^{-1}s^{-1}$; for almond extracts, $k_2/Km = 6.81W\ M^{-1}\ s^{-1}$. If the relative molecular weight (W) of the enzyme is known, it is possible to express the [E] as the *molecular activity*, then the k_2/Km can be compared. It looks as if the almond extracts were better than apple seed extracts according to this parameter.

Since sometimes the molecular weight (W) of the enzyme is not known, frequently Vmax/Km is used to estimate the enzyme efficiency replacing k_2/Km which is also the bigger the better (Tipton, 2002). In this case the Vmax/Km were 2.85 and 4.08 respectively, which also showed that the almond extracts were better than apple seed extracts although there was a low enzyme concentration in the apple seed extracts.

4.5.4.5. Linear Plots of the Michaelis-Menten Equation

The plot of V versus [S] is not linear; although initially linear at low [S], it bends over to saturate at high [S]. Before the modern era of nonlinear curve-fitting on computers, this nonlinearity could make it difficult to estimate K_m and V_{max} accurately. Therefore,

several researchers developed linearizations of the Michaelis-Menten equation, such as the Lineweaver–Burk plot, the Eadie-Hofstee diagram and the Hanes-Woolf plot (Hanes, 1932; Hofstee, 1959; Atkins & Nimmo, 1975).

The Lineweaver-Burk plot or double reciprocal plot is a common way of illustrating kinetic data. However, naturally no experimental values can be taken at negative 1/[S]; the lower limiting value 1/[S] = 0 (the y-intercept) corresponds to an infinite substrate concentration, where $1/V=1/V_{max}$; thus, the x-intercept is an extrapolation of the experimental data taken at positive concentrations. More generally, the Lineweaver-Burk plot skews the importance of measurements taken at low substrate concentrations and, thus, can yield inaccurate estimates of V_{max} and K_m (Counotte & Prins, 1979; Duggleby, 1995). Another linear plotting method is the Eadie-Hofstee plot. In this case, V is plotted against V/[S] (Hofstee, 1959). In the third common linear representation, the Hanes-Woolf plot, [S]/V, is plotted against [S] (Hanes, 1932). All of these linear representations can be useful for visualizing data. However, the best way to determine kinetic parameters should be using computer software, such as Prism, which is readily available that allows for more accurate determination by nonlinear regression methods.

4.6. Conclusions

- Apple seeds are a good source of the enzyme β-D-glycosidase. The optimum reaction conditions were observed at 65°C and pH 5.5.
- The enzyme from apple seeds was stable for at least 12 weeks at 4°C. Activity increased during the first 4 weeks of storage.
- The content of protein was 0.728 ± 0.019 mg/ml in the apple seed extracts, 1.27 ± 0.031 mg/ml in the whole almond extracts.
- The kinetic parameters of the enzyme were:
  apple seed extracts (n=8): Km 5.48 ± 0.34 mM; Vmax 15.60 ± 0.95 U/mg protein
  almond extracts (n=3): Km 3.32 ± 0.067 mM; Vmax 13.54 ± 1.2 U/mg protein
- The enzyme from apple seeds has properties similar to that from almond (which is commercially available). The enzyme from apple seeds might be a more economically attractive option.