ISOLATION AND CHARACTERIZATION OF ENZYME PROTEASE FROM PUMPKIN AND ITS AFFINITY TO DIFFERENT PROTEIN SUBSTRATES

By

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A project report submitted to the Department of Chemical Science,

Faculty of Science,

Universiti Tunku Abdul Rahman

In partial fulfillment of the requirement for the degree of

Bachelor of Science (HONS) Chemistry

September 2016

ABSTRACT

ISOLATION AND CHARACTERIZATION OF ENZYME PROTEASE FROM PUMPKIN AND ITS AFFINITY TO DIFFERENT PROTEIN SUBSTRATES

TAN SUE JIN

Proteolytic enzymes are multifunctional enzymes that contribute many physiological functions in living organisms such as germination, inflammation process, complement activation etc. and also have been widely used in food, leather and detergent industry. They are commercially dedicated over 60% of the global total enzyme market. Proteolytic enzyme can be isolated from plants, animals, bacteria and fungi. Proteolytic enzyme from the plant sources has receive immense attention due to their broad substrate specificity and their activeness in wide range of pH, temperature and in the presence of organic compound as well as other additives. This project is aimed to determine the optimal pH and temperature of pumpkin proteases enzyme activity that can be applied during the manufacturing processes that involve this enzyme. In addition, the determination of substrate affinity is also an objective in this project. The resulting substrate that shows the best affinity towards the pumpkin proteases can be used in further researches and studies regarding pumpkin proteases.

ABSTRAK

PENGASINGAN DAN PENCIRIAN ENZIM PROTEASE DARIPADA LABU DAN PERTALIANNYA DENGAN SUBSTRAT YANG BERBEZA

TAN SUE JIN

Enzim proteolitik merupakan enzim yang mempunyai pelbagai fungsi psikologi terhadap organisma hidup seperti percambahan, proses keradangan, pengaktifan pelengkap dan lain-lain lagi. Enzim proteolitik juga banyak menyumbang dalam industri makanan, kulit dan bahan pencuci. Ia telah memberi penyumbangan yang melebihi daripada 60% dalam pasaran enzim globalisasi. Selain itu, enzim proteolitik boleh diperoleh daripada tumbuhan, haiwan, bakteria, dan kulat. Pengasingan enzim proteolitik daripada tumbuhan telah menerima perhatian yang tegang kerana ia mempunyai kekhususan substrat dan keaktifannya dalam pH dan suhu yang luas, serta dalam keadaan kompaun organik dan bahan tambahan yang lain. Tujuan projek ini adalah untuk mengetahui dan menentukan pH dan suhu optimum dalam aktiviti enzim protease daripada labu. Maklumat ini akan menyumbang kegunaan yang boleh diaplikasikan dalam industri pembuatan. Selain itu, pertalian enzim protease ini dengan pelbagai jenis substrat akan diketahui dalam projek ini. Substrat yang menunjuk pertalian yang paling tegang dengan enzim protease labu boleh dipilih dan digunakan dalam penyelidikan and kajian tentang enzim protease labu pada masa depan.

ACKNOWLEDGEMENT

First of all, I wish to express my sincere gratitude to my project supervisor, Asst. Prof. Dr Hnin Pwint Aung for her continuous assisting throughout my project. I am glad and appreciate her patience, encouragement and numerous interesting and useful knowledge given by her. I am thankful that she was always willing to discuss and help me to solve and overcome any problems crisis situations that I have met throughout this project. I am able to complete my project successfully thank to her guidance and support.

Besides, I wish to grant my thankfulness to the lab officers who have provided enormous assistance during my bench work in the laboratory. Beside preparing and providing chemicals and glassware, I have also gained a lot of useful knowledge and opinion regarding to my project from them. I am appreciated that they are able to maintain the instruments in good conditions and willing to teach me the way to use the instruments.

Last but not least, I would like to thank my friends and family for giving me chances to learn what I am interested in and encouragement to me whenever I am in problem and feel helpless. I am really appreciated for their love, concern and support towards me throughout the tough time.

DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

_____

TAN SUE JIN

DATE:

APPROVAL SHEET

This project report entitle "**ISOLATION AND CHARACTERIZATION OF ENZYME PROTEASE FROM PUMPKIN AND ITS AFFINITY TO DIFFERENT PROTEIN SUBSTRATES**" was prepared by TAN SUE JIN and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) Chemistry at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

It is hereby certified that TAN SUE JIN (ID No.: 13ADB00713) has completed this thesis entitled "ISOLATION AND CHARACTERIZATION OF ENZYME PROTEASE FROM PUMPKIN AND ITS AFFINITY TO DIFFERENT PROTEIN SUBSTRATES" under the supervision of Dr. Hnin Pwint Aung from the Department of Chemical Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my thesis in pdf format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

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TAN SUE JIN

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LIST OF ABBREVIATIONS

Ca^{2+}	Calcium (II) ion
Co^{2+}	Cobalt (II) ion
Cu^{2+}	Copper (II) ion
Da	Dalton
Fe^{2+}	Iron (II) ion
HCl	Hydrochloric acid
Hg^{2+}	Mercury (II) ion
K	Kilo
M	Molar
Mg	Milligram
Mg^{2+}	Magnesium (II) ion
Min	Minute
mL	Milliliter
Mn^{2+}	Manganese (II) ion
mol	mol
Ni^{2+}	Nickel (II) ion
nm	Nanometer
Pb^{2+}	Lead (II) ion
rpm	Revolutions per minute
Zn^{2+}	Zinc (II) ion

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CHAPTER 1

INTRODUCTION

1.1 Proteins

Protein is a large molecule that is built up by many monomeric units called amino acids. The amino acids are linked up by peptide bond, forming a polypeptide chain.

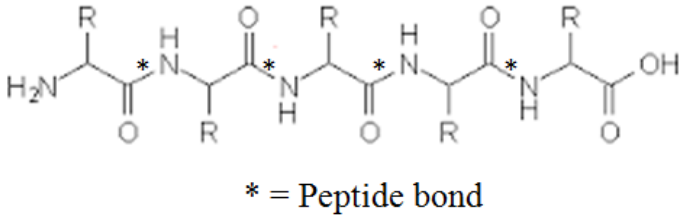

Figure 1.1: The primary structure of polypeptide chain.

Amino acids are considered as organic compounds which contain carbon-hydrogen bonding. In addition, amino acids are also consists of nitrogen, oxygen, and sometimes sulphur. The basic structure of a free amino acid is found to include a carbon atom, a hydrogen atom, an amino group ($-NH_2$), a carboxyl group (-COOH), and a side chain which is always represented by R-group. The side

chain is different for each type of free amino acid. Generally, amino acids are found to have α configuration, where the amino group, carboxyl group, hydrogen atom and the R-group are attached to the carbon atom. The carbon atom is known as α-carbon and the amino acid with α-carbon is known as α-amino acid.

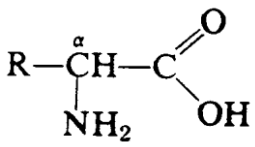

Figure 1.2: Basic structure of a free amino acid

1.2 Enzyme

All enzymes are protein (Sumner, 1926). Enzymes are the proteins that are produced by living organisms to regulate their biochemical processes. Enzymes can be also known as catalysts that speed up biochemical processes without itself being consumed (Mantsala and Niemi, 2009). Enzyme can be classified into six major types according to the reaction types catalyzed. The six major types of enzyme are tabulated in Table 1.1 (Webb, 1992).

Table 1.1: Classification of enzymes.

Class	Type of chemical reaction catalyzed	Example
Hydrolase	Hydrolysis reaction	Digestive enzymes
Isomerase	Rearrangement of atoms within a molecule	Fumarases
Ligase	Joining two molecules by forming a new bond.	Citric acid synthetases
Lyase	Nonhydrolytic removal of a groups	Decarboxylases, Aldolases
Oxidoreductase	Oxidation or reduction reactions which hydrogen or oxygen atoms are gained or lost.	Dehydrogenase, Cytochrome oxidase
Transferase	Transfer of function group	Transaminase

1.2.1 Digestive enzyme

Digestive enzymes are one of an example of hydrolase enzymes that are produced by living organisms to break down polymeric macromolecules, also known as substrate, into their monomeric units. Digestive enzymes can be classified into three major groups: proteases, amylase and lipase according to their specific target substrates as listed in Table 1.2 (Whitecomb and Lowe, 2007).

Table 1.2: Classification of digestive enzymes

Digestive enzyme	Substrate	Monomeric units	Example
Protease	Protein	Amino acids	Pepsin, Bromelain
Amylase	Starch	Glucose	Maltase
Lipase	Lipids	Fatty acid and glycerol	Gastric lipase

1.3 Protease and Proteolytic Activity

Protease is a digestive enzyme that breaks down protein into amino acids. The breaking down of peptide bonds involves addition of water molecules, which is known as hydrolysis reaction. The hydrolysis reaction of protease is known as proteolysis (Berg *et al.*, 2002).

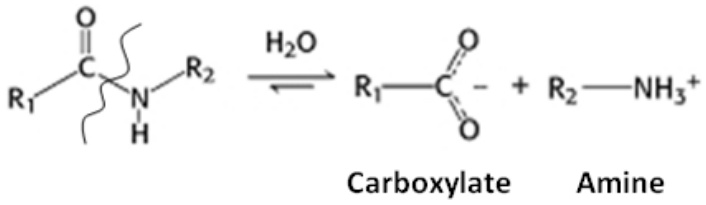

Figure 1.3: Proteolysis of peptide bond.

1.3.1 Classification of proteases

Proteases can be further classified according to three main factors, which are catalytic residues, optimum pH, and cleavage sites on peptide bonds (Akpinar and Penner, 2002).

1.3.1.1 Classification based on catalytic residues

This classification is done according to the nucleophilicity of the amino acid residue, which is also the protease active site involved in the proteolysis reaction. Proteases are classified under four catalytic types, which are serine proteases, cysteine proteases, metalloproteases and aspartic proteases as listed in Table 1.3 (Rawlings and Barette, 1993).

Table 1.3: Classification of proteases based on amino acid residue.

Proteases	Amino acid residue involved
Serine	Serine alcohol
Cysteine	Cysteine thiol
Metallo-	Metal, usually zinc
Aspartic	Aspartate carboxylic acid

1.3.1.2 Classification based on optimum pH

Different types of proteases will perform optimal proteolytic activity at certain pH. They can be classified into acid proteases, neutral proteases and alkali proteases, as shown in Table 1.4 (Rao, 1998).

Table 1.4: Classification of protease based on optimum pH.

Proteases	Optimum pH
Acid	2.0 – 6.0
Neutral	~ 7.0
Alkaline	≥ 8.0

1.3.1.3 Classification based on cleavage site on peptide bond

Polypeptide chain consists of an amino group terminal and a carboxyl group terminal. They are known as N terminus and C terminus respectively. The cleaving of protease on the peptide bonds can take place at any site on the chain. Based on the cleavage site, proteases can be classified into 2 major types, which are endo- and exopeptidases. Endopeptidases hydrolyse internal peptide bonding, whereas exopeptidases hydrolyse the external peptide bonding. Exopeptidases can be further grouped into 2 types, which are aminopeptidases and carboxypeptidases.

The exopeptidases is recognized as aminopeptidases when the hydrolysis occurs at N terminus, while if the hydrolysis occurs at C terminus, it is recognized as carboxypeptidases (Akpinar and Penner, 2002).

1.4 Industrial Application of Protease

Proteases execute large contributions to industrial application nowadays. It shows a great importance in the overall enzyme industrial, by having a great sales account for over 60% of the total worldwide sales of the biochemical products (Godfrey and West, 1995). It has been widely used due to its naturally existence within living organisms causing it to be easily available. Proteolytic properties of proteases are the major actions used in the industrial application, mainly to degrade various natural substances (Kirk, 2002). The two major industries that involve the application proteases are food industries and detergent industries. The application of proteases in various industries is tabulated in Table 1.5, (Kirk, 2002; Rao, 1998).

Table 1.5: The industrial applications of proteases.

Industry	Examples	Actions
Detergent	Laundry detergent	Break down protein stains on clothes, such as food and blood.
Food	Meat tenderization	Break down protein collagen that hold meat together, making the meat soften.
	Baking	Reduces baking time and increases loafing volume by hydrolysis of gluten chains.
	Cheesemaking	Hydrolysis of specific peptide bond to generate para-κ-casein and macropeptides
Leather	Dehairing, tanning	Hydrolysis of noncollagenous constituents of the skin and for removal of nonfibrillar proteins such as albumins and globulins
Pharmaceutical	Therapeutic agents	Wound debridement by breaking down dead or damaged tissues on wound. (Klasen, 2000)

1.5 Problem Statements

- The presence of proteases in pumpkin is unknown.
- The characterization of proteases from pumpkin has not been studied.

1.6 Objective

- To extract enzyme proteases from pumpkin flesh.
- To determine the presence of proteolytic enzyme in pumpkin.
- To determine the optimum temperature and pH for the protease enzyme activity.
- To identify the affinity of pumpkin enzyme protease to different protein substrate.

CHAPTER 2

LITERATURE REVIEW

2.1 The Presence of Enzymes Proteases in Plants and Fruits

Enzyme proteases have been reported to be found in a variety of fruits and plants. Enzyme proteases were found in dicotyledonous plants such as papaya, fig, euphorbia and milkweed and monocotyledonous plants such as pineapple and cereals (Greenberg, 1955). In addition, a numbers of enzymes proteases were also been isolated from latex, fruits and seeds. (Boller, 1986). Examples of enzyme proteases that can be found in certain plants and fruits sources were listed in Table 2.1.

Table 2.1: List of enzyme proteases existed in certain plants and fruits.

Types of plants and fruits	Proteases
Melon	Cucumisin
Papaya	Papain
Fig	Ficin
Kiwifruit	Actinidin
Pineapple	Bromelain

2.2 Detection of Enzyme Proteases

Screening methods have been widely used for the detection of proteases in sample extracts. Generally, there is a few types of protein substrate agar plates have been used in detection of proteases, such as skimmed milk agar (Wery *et al*., 2003), casein agar (Frazier and Rupp, 1928), gelatin agar (Whaley *et al*., 1982), fibrin agar (Safarik *et al*., 1983), and elastin agar (Rust *et al*., 1994).

In screening method for proteases, protein substrate agar plates in petri dishes with punched holes were prepared and the sample extracts will be loaded into the holes. The changes in the appearance around the holes will be observed. Casein and skimmed milk agar are the most used type of agar for the screening test for proteases. Both of these substrate agars are white in color which mostly contributed by casein. The hydrolysis of casein to smaller fragments will cause the substrates to lose their white color to colorless. The presence of protease in the sample extract is indicated by the clear zones around the holes, with a visible appearance (Adinarayana *et al*., 2003).

2.3 Extraction and Purification of Enzymes

Proteins were enriched within plant cells. The cell membranes must be disrupted to release and extract the proteins within it. The homogenizer can be a simple and effective way to disrupt the cell membranes by Berg *et al*., (2002).

Proteins can be purified according to different characteristics such as solubility, size, charge and specific binding affinity. Purification of proteins involves a few steps according to these characteristics until completely purified. The yields, specific enzyme activity and protein concentration were determined and recorded at each step of purification. Different type of purification techniques were introduced as listed in Table 2.2 (Berg *et al*., 2002).

Table 2.2: Protein purification techniques

Purification steps	**Dependant characteristics**
Ammonium sulphate precipitation (Salting out)	Solubility
Dialysis	Size
Gel-Filtration Chromatography	Size
Ion-Exchange Chromatography	Net charge
Affinity Chromatography	Specific binding affinity
High-Pressure Liquid Chromatography	Hydrophobicity

Berg *et al*., (2002) reported that, the purification fold increases by steps of purifications by using a fictitious protein, as shown in Table 2.3.

Table 2.3: Summary of purification of fictitious protein

Purification steps	**Specific activity, (units/mg)**	**Yield (%)**	**Purification fold**
Homogenization	10	100	1
Salt fractionation	30	92	3
Ion-exchange chromatography	90	77	9
Molecular exclusion chromatography	1,100	50	110
Affinity chromatography	30,000	35	3,000

The percentage yields indicate the resulting amount of components left within the sample extract after each stages of purification. When going through each stage of purification, the amount of the unwanted substances removed from the sample extract increases, causing the percentage yield to decrease. As purification goes on, the amount of proteins of interest in the sample extracts increases which indicate the purity level of the sample extract increases. The purity level is expressed as purification fold.

2.4 Factors that Affect the Enzyme Activities

There were four main factors being reported to have effect on enzyme activities. The four factors were pH, temperature, metal ions and inhibitors.

Modilal *et al*. (2011) have reported that proteases isolated from cauliflower and elephant yam performed maximum activity on casein substrate at pH 4.5, 55 ℃ and pH 5, 50 ℃ respectively. They suggested that both proteases isolated from cauliflower and elephant yam are acidic proteases according to their optimum pH. The researchers have used ethylenediaminetetraacetic acid (EDTA) and dimethyl sulphonium bromide (DMSB) inhibitors to determine their effects on the enzyme activity. They have found that the overall proteolytic activity decreases when the inhibitors are added into the reaction. EDTA was found to be a weaker inhibitor than DMSB. Besides pH, temperature and inhibitors, they have also studied the effect of metal ions on the proteolytic activity. Mn^{2+}, Ca^{2+} and Mg^{2+} were added to the enzymatic reactions separately and an enhancement of the activities occurred for every metal ions. They suggested that metal ions have a capability to protect the enzymes from denaturation and their active conformation were maintained by the metal ions as well.

Besides, Asker *et al*. (2013) have isolated and purified enzyme protease from *Bacillus megaterium* and studied their characterization. The enzyme assays were done by using gelatin as substrate. The enzyme protease from *Bacillus megaterium* exhibited maximum activity at pH 7.5 and 50 ℃ in the effect of pH and temperature experiments. Ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) and phenylmethylsulfonyl fluoride (PMSF) were added into the enzyme activity to study the effect of inhibitors on the reactions. The type of proteases can be identified by using different type of inhibitors. Each inhibitor can exhibit inhibition of certain proteases by interfering the proteases' active site. They found that both EDTA and EGTA show no significant affect on the enzyme activity when low concentration of the inhibitors was added. The enzyme activity decreases by 20% when higher concentration of EDTA was added, while a low concentration of PMSF completely inhibited the enzyme activity. However, they were unable to conclude that the enzyme protease from *Bacillus megaterium* is a metalloproteases even though addition of EDTA causes the decreasing in enzyme activity. This is due to EDTA is not a good indicator of metalloproteases because most enzymes contain calcium for their activity and EDTA is a strong calcium chelator. In addition, EGTA which is also a metalloproteases inhibitor did not decrease the enzyme activity of the enzyme protease from *Bacillus megaterium*, indicating that the enzyme protease is not metalloproteases. They suggested that enzyme protease from *Bacillus megaterium* is a type of serine protease since PMSF is a standard serine protease inhibitor. PMSF inhibits the serine protease by blocking their active site through sulfonating the essential serine residue. In

addition, metal ions Fe^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Mn^{2+} and Zn^{2+} were used to study their effect on the proteolytic activities. They found that Mn^{2+}, Zn^{2+} and Fe^{2+} show losing in activity after the addition, while Mg^{2+}, Ca^{2+}, Cu^{2+} show significant increases in the proteolytic activity.

Jinka *et al.* (2009) in their study on purification and characterization of cysteine protease from germinating cotyledons of horse gram have found that the cysteine protease they extracted is an acidic proteases which performed their optimum activities on azocasein as substrate at pH 5.5; haemoglobin at pH 4.0 and gelatin and BSA at pH 5.2. In addition, the optimum temperature of the cysteine proteolytic activity towards azocasein as substrate is 40 ℃, whereas the optimum temperatures for the rest substrates were not shown. They have also used Mn^{2+}, Zn^{2+}, Hg^{2+}, Cu^{2+}, Ni^{2+}, $Co^{2+}$and Pb^{2+} to study their effect on the enzyme activity. They have found that the proteolytic activities were inhibited by low concentration (0.5 mM) of Zn^{2+}, Hg^{2+} and Cu^{2+}, and high concentration (1.0 mM) of Ni^{2+}, $Co^{2+}$and Pb^{2+}. However, the Mn^{2+} at high concentration (>1.0 mM) enhanced the proteolytic activity. In addition, they have employed various types of inhibitors specific to each class of proteases to determine the amino acid residue that contribute to the active site of the enzyme. The inhibitors used are listed in Table 2.4.

Table 2.4: The various types of inhibitors specific to each protease class used.

Protease class	Inhibitor
Serine	Diisopropyl fluorophosphate (DIPF)
	Soybean trypsin inhibitor (STI)
	Eserine
	Phenylmethylsulfonyl fluoride (PMSF)
	Aprotinin
Cysteine	p-chloromercuribenzoate (pCMB)
	Iodoacetamide
	N-Ethylmaleimide (NEM)
	Diethylpyrocarbonate (DEPC)
	Dithionitrobenzoic acid (DTNB)
Aspartic	Pepstatin
Metallo-	1,10-phenanthrolone
	Ethylenediaminetetraacetic acid (EDTA）

They have found that the activities of cysteine protease from germinating cotyledons of horse gram were not inhibited by serine, aspartic and metalloproteases inhibitors, but were inhibited by the cysteine protease inhibitors especially pCMB and DEPC which have completely inhibited the activities. They suggested that the protease from germinating cotyledons of horse gram is a type of cysteine protease.

2.5 Enzyme Kinetic Studies

Substrate affinity is the relationship between the targeted enzyme and the specific substrates. It is about how well the substrate can bind onto the active site of the enzyme. It can affect the rate of reaction of the enzyme on different concentration of certain substrate. The rate of reaction increases with the substrate concentration, with the enzyme concentration being constant. The increasing rate indicates that the rate of encounter and binding of the substrates onto the enzymes. The substrate affinity can be expressed as the Michaelis-Menten constant, K_m value. The rate of reaction will reach the theoretical maximum when a relatively high substrate concentration is reached. This is due to the active sites on the enzymes are already almost being occupied, causing the limitation for the substrates to continue to bind onto the active sites. This can be expressed as V_{max} value. The substrate affinity determination can be done by comparing the K_m value and the V_{max}/K_m value. A lowest K_m value and a highest V_{max}/K_m value of a studied substrate would be more preferable, indicating that the substrate is performing the best with the targeted enzyme. (Michaelis and Menten, 1913; Scott and Williams, 2012)

Enzyme kinetic study was done to determine the enzyme protease affinity to different protein substrate by Corzo *et al*. (2011). Isolation and characterization of enzyme bromelain from pineapple fruit have been carried out, with five types of different substrates used: azoalbumin, azocasein, casein, sodium caseinate and

haemolgobin. For the enzyme kinetic study experiment, activity assay was done by varying the substrate concentration, for each of every five substrates at their optimum pH and temperature as listed in Table 2.5.

Table 2.5: The optimum pH and temperature for the proteolytic activity of bromelain on different substrates.

Substrates	Optimum pH	Optimum temperature (℃)
Azoalbumin	7.5	55
Azocasein	6.5	55
Casein	7.7	59
Sodium caseinate	6.5	59
Haemoglobin	2.9	37

The Michaelis-Menten K_m constant, maximum reaction velocity V_{max} and the reaction specificity V_{max}/K_m were determined by plotting the activity data using Lineweaver-Burk method with Sigma Plot 11 program. Azoalbumin and azocasein were found to have lowest K_m value, and highest V_{max}/K_m value. Both of these substrates were highly recommended for the determination of fruit bromelain activity at optimal conditions.

2.6 Determination of Purity of Protein Extract after Purification

Errors of screening the wrong enzyme can always happen; hence the enzyme identity must be confirmed to prevent this problem from happening. The enzyme is identified based on the molecular weight of the targeted enzyme. Besides, contaminants might still existed in the enzyme extract even after a few steps of purification. Those remaining contaminants have the possibility to interfere with the enzyme activity assay (Scott and Williams, 2012). Therefore, both enzyme identity and enzyme purity should be determined for every enzyme characterization study to provide a more precise and persuasive result and conclusion. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) has been suggested as a good technique to determine the enzyme identity and enzyme purity. This method normally carried out along with the usage of Coomassie blue staining or silver staining. The molecular weights of proteins presence in the sample extract are expressed as bands on the SDS gels. The presence of number of bands can help to identify the enzyme purity. SDS-PAGE exhibits fast and simple analysis, hence this is a famous technique used in the researches on characterization of enzymes (Scott and Williams, 2012).

Jackson et al. (1981) used SDS-PAGE to check the purity the enzyme nitrite reductase isolated from *Eschmchza col.* The enzyme extract subjected the SDS-PAGE shows migration as a single band on the SDS gel, after purified with 50%

ammonium sulphate precipitation, DEAE-Cellulose chromatography and DEAE-Sephadex chromatography. The purification fold determined was 121 times to the crude extract. The single band observed from the SDS gel and the high purification fold shows that only the targeted enzyme was isolated with high purity. The purification fold determined would have been decreased if the single protein band shown in the SDS gel is not the targeted enzyme.

CHAPTER 3

METHODOLOGY

3.1 Materials

3.1.1 Pumpkin fruit sample

The pumpkin fruit used is purchased from morning market located in Kampar, Perak.

3.1.2 Ready-to-use chemicals

The ready-to-use chemicals and reagents used and the supplement sources were listed in Table 3.1.

Table 3.1: List of ready-to-use chemicals and reagents used and their supplement sources.

Chemicals and reagents	Supplier
Acetic acid	Qrec
Acrylamide-Bis	Bio Basic Canada Inc.
Agar	Merck
Ammonium persulphate (APS)	Merck
Ammonium sulphate	John Kellin Chemicals
Bovine serum albumin (BSA)	Merck
Bradford reagent	Nacalai tesque
Bromelain	Merck
Bromophenol blue	HIMEDIA Laboratories
Coomassie Brilliant Blue R-250	Fisher Scientific
Dialysis tubing with molecular weight cut-off 12-14 kDa	Fisher Scientific
Disodium hydrogen phosphate	Bio Basic Canada Inc.
Ethanol	GENE Chemicals
Glycerol	Qrec
Glycine	Fisher Scientific
Haemoglobin	Sigma Aldrich
Hydrochloric acid	Qrec
L-Tyrosine	Bio Basic Canada Inc.
Methanol	Fisher Scientific
Phosphoric acid	Fisher Scientific
Skimmed milk powder	Merck
Sodium caseinate	Sigma Aldrich
Sodium dihydrogen phosphate	R&M Chemicals
Sodium dodecyl sulphate (SDS)	Bendosen
Sodium hydroxide	R&M Chemicals
Tetramethylethylenediamine (TEMED)	Merck
Trichloroacetic acid	Sigma Aldrich
Tris-base	R&M Chemicals
β-merchaptoethanol	Sigma Aldrich

3.1.3 Instruments

The model of the instruments used and their manufacturer were listed in Table 3.2.

Table 3.2: List of instruments, model and the manufacturers

Instruments	Model	Manufacturer
Centrifuge machine	CT15RT Versatile Refrigerated Centrifuge	Techcomp
Centrifuge machine	centrifuge-5702	Eppendoff
Electrophoresis cell	OLCVS10	NYX TECHNIK
Juice blender	-	Panasonic
pH meter	FE20	Mettler Tolepo
UV-Vis spectrophotometer	UV-mini-1240	Shimadzu
Vortex mixer	VM-300	Gemmy Industrial Corp
Water bath	WNE 29	Memmert

3.2 Preparation of Chemicals and Reagents

3.2.1 Preparation of 0.1 M sodium hydroxide

Sodium hydroxide pellets (4 g) were dissolved in 1 L deionized water in 1 L volumetric flask. The solution was homogenized using magnetic stirrer and stirring bar.

3.2.2 Preparation of 0.1 M phosphoric acid

Eighty five percent of phosphoric acid (6.8 mL) was diluted with 1 L deionized water in 1 L volumetric flask. The solution was mixed using magnetic stirrer and stirring bar.

3.2.3 Preparation of 0.1 M sodium phosphate buffer

Sodium phosphate buffers at different pH (pH 4-9) were prepared by dissolving sodium dihydrogen phosphate and disodium hydrogen phosphate in deionized water. The solution was homogenized using magnetic stirrer and stirring bar. The amounts of sodium dihydrogen phosphate and disodium hydrogen phosphate used to prepare each pH is listed in Table 3.3. The pH of the buffers were adjusted with 0.1 M sodium hydroxide and 0.1 M phosphoric acid by using pH meter.

Table 3.3: The amounts of sodium dihydrogen phosphate and disodium hydrogen phosphate used to prepare 100 mL buffer at each pH.

pH	Disodium hydrogen phosphate (g)	Sodium dihydrogen phosphate (g)
4	0.0036	1.3781
5	0.0360	1.3614
6	0.3218	1.2143
7	1.5466	0.5836
8	2.4970	0.0942
9	2.6605	0.0100

3.2.4 Preparation of 10% trichloroacetic acid

Trichloroacetic acid (10 g) was dissolved in 100 mL deionized water. The solution was homogenized using magnetic stirrer and stirring bar.

3.2.5 Preparation of 0.5 mg/mL enzyme bromelain

Commercial bromelain (5 mg) was dissolved in 10 mL deionized water. The solution was homogenized by using vortex mixer.

3.2.6 Preparation of 70% ethanol

Ninety five percent of ethanol (74 mL) was added to 100 mL deionized water. The solution was mixed using magnetic stirrer and stirring bar.

3.2.7 Preparation of 0.2 mg/mL L-tyrosine stock solution

L-tytosine (5 mg) was dissolved in 25 mL deionized water in 25 mL volumetric flask. The solution was homogenized using magnetic stirrer and stirring bar.

3.2.8 Preparation of 2 mg/mL bovine serum albumin (BSA) stock solution

BSA (50 mg) was dissolved in 25 mL deionized water in 25 mL volumetric flask. The solution was mixed using magnetic stirrer and stirring bar.

3.2.9 Preparation of 20 mg/mL enzyme bromelain

Commercial bromelain (200 mg) was dissolved in 10 mL deionized water. The solution was homogenized by using vortex mixer.

3.2.10 Preparation of 2% agar solution

Agar powder (2 g) was dissolved in 100 mL deionized water. The solution was homogenized using magnetic stirrer and stirring bar.

3.2.11 Preparation of 10% skimmed milk solution

Skimmed milk powder (10 g) was dissolved in 100 mL deionized water. The solution was mixed using magnetic stirrer and stirring bar.

3.2.12 Preparation of 2% sodium caseinate solution

Sodium caseinate (0.5 g) was dissolved in 25 mL sodium phosphate buffer at certain pH (pH 4-9). The solution was homogenized using magnetic stirrer and stirring bar.

3.2.13 Preparation of 1% haemoglobin solution

Haemoglobin (0.25 g) was dissolved in 25 mL sodium phosphate buffer at certain pH (pH 4-9). The solution was mixed using magnetic stirrer and stirring bar.

3.3 Screening Test for Enzyme Proteases Detection from Pumpkin

3.3.1 Extraction of proteases from pumpkin flesh

Peeled pumpkin flesh without seeds (76 g) was blended thoroughly with 76 mL pre-chilled (4 ℃) 0.1 M sodium phosphate buffer at pH 7. The pumpkin juice was then filtered through cheesecloth to remove suspension. The pumpkin juice was centrifuged at 10000 rpm at 4 ℃ for 20 minutes to remove impurities. The

supernatant from the resulting pumpkin juice was then collected at stored in 4 ℃ for further use. The sediments were discarded.

Figure 3.1: The resulting solutions after centrifugation of the blended and filtrated pumpkin crude extract.

3.3.2 Preparation of skimmed milk agar plate

The petri dish was placed under UV lamp 15 for 15 minutes. The UV lamina used was sterilized with 70% ethanol beforehand. The freshly prepared 2% agar solution and 10% skimmed milk solution were mixed together. The solution was homogenized by using magnetic stirrer and stirring bar. 20 mL of the solution was then transferred to a petri dish and autoclaved. The agar plate was left for 1 hour for solidification. Once the agar plate solidified, it was placed under UV lamp for another 5 minutes to sterile it. The agar plate was sealed with parafilms and stored at 4 ℃ for further use.

3.3.3 Screening test on skimmed milk agar plate

Three holes were punched on the skimmed milk agar plate by using the non-pointy end of a 1 mL micropipette tip. The distance between the three holes was approximately equal to each other, in a triangle shape. The three holes on the agar plate were filled separately with 20 mg/mL enzyme bromelain, deionized water and pumpkin crude extract, with 80 μL of each sample in each hole. The enzyme bromelain was acted as reference enzyme, whereas the deionized water acted as blank. The agar plate was incubated at 37 ℃ for 24 hours. The clear ring zone around each hole can be observed afterward.

3.4 Partial Purification of Pumpkin Crude Extract

Ammonium sulphate precipitation method was used to partial purify the pumpkin crude extract. 50 mL of the pumpkin crude extract was preceded to partial purification by adding ammonium sulphate salt. The partial purification was done up to 95% of saturation based on the ammonium sulphate precipitation table attached in Appendix A. Ammonium sulphate (33 g) was added slowly into the pumpkin crude extract with constant stirring using magnetic stirrer and stirring bar at 4 ℃. The addition of the ammonium sulphate was carried out for 3 hours. The pumpkin extract was then left to stir for overnight at 4 ℃ to assure completely dissolving of the ammonium sulphate. After that, the extract was centrifuged at 10000 rpm at 4 ℃ for 20 minutes.

Figure 3.2: The resulting protein pellet after centrifugation of pumpkin crude extract after ammonium sulphate precipitation.

The sediment pellet was collected and the supernatant was discarded. The pellet was redissolved in 15 mL pre-chilled (4 ℃) 0.1 M sodium phosphate buffer at pH 7. The solution was filled into dialysis tubing and tied tightly. The solution was then dialyzed in 1 L pre-chilled 0.1 M sodium phosphate buffer (pH 7) at 4 ℃ with constant stirring using magnetic stirrer and stirring bar. During the dialysis, the dialysate was replaced with new sodium phosphate buffer every 4 hours and the dialysis was left to stand for overnight for a better removal of ammonium sulphate salt from the pumpkin partial purified extract. The total volume of sodium phosphate buffer used to dialyze the extract is 3 L. The partial purified pumpkin

extract was then collected and centrifuges at 10000 rpm at 4 ℃ for 20 minutes to remove suspension. The supernatant was collected and stored at 4 ℃ for further use. The sediment was discarded.

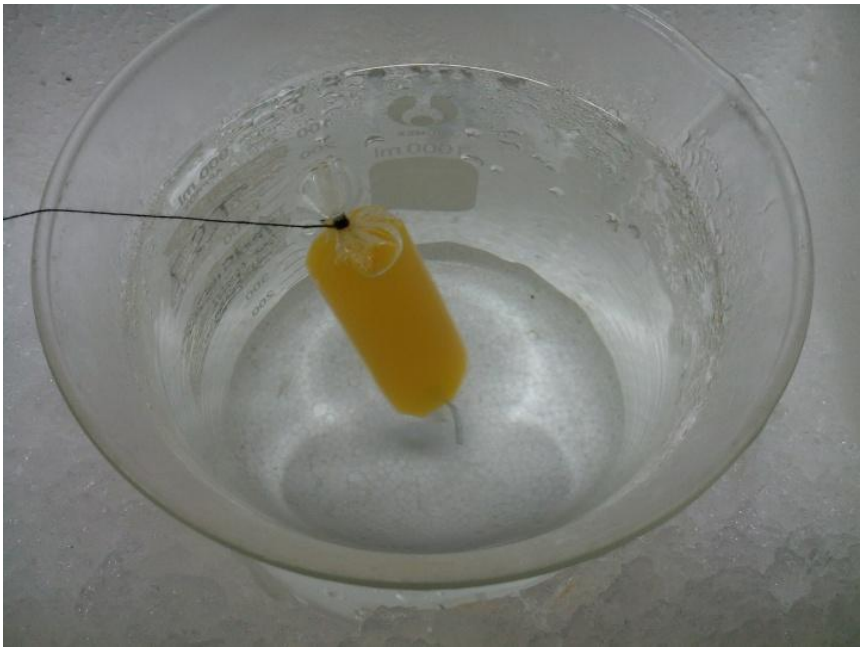

Figure 3.3: Dialysis of pumpkin extract in 1 L of sodium phosphate buffer.

3.5 Preparation of Standard Calibration Curves

3.5.1 Bovine serum albumin (BSA) calibration curves

Bradford assay is used for the determination of protein concentration. In this assay, a series of BSA standard solutions at different concentrations were prepared from the 2 mg/mL BSA stock solution prepared (refer section 3.2.8). The BSA stock solution was diluted to different concentrations of standard solutions (0.1 - 1.2 mg/mL) using deionized water as shown in Table 3.4.

Table 3.4: The volumes of BSA stock solution required in the standards preparation.

Concentration (mg/mL)	Volume of BSA stock solution added (µL)	Volume of deionized water added (µL)
0.0	0	1000
0.1	50	950
0.2	100	900
0.4	200	800
0.6	300	700
0.8	400	600
1.0	500	500
1.2	600	400

The absorbance of each standard solution was measured by using Bradford assay, with UV-Vis spectrophotometer. Bradford assay is done by adding 1 mL of Bradford reagent to 20 μL of each standard solution, and homogenized by using vortex mixer. The samples were incubated in dark conditions for 10 minutes and the absorbance was read at 595 nm. The blank used for the analysis is 20 μL deionized water instead of samples, then add 1 mL of Bradford reagent.

3.5.2 Tyrosine calibration curves

A tyrosine standard calibration curve is constructed to determine the amount of tyrosine released after the proteolytic activity. A series of tyrosine standard solutions at different concentrations (5 - 200 μg/mL) were prepared from the 0.2 mg/mL L-tyrosine stock solution (refer section 3.2.7) with deionized water. The volumes of L-tyrosine stock solution used to prepare different concentrations of standard solutions were listed in Table 3.5.

Table 3.5: The volumes of L-tyrosine stock solution required in the standards preparation.

Concentration (µg/mL)	Volume of BSA stock solution added (µL)	Volume of deionized water added (µL)
0	0	1000
5	25	975
10	50	950
20	100	900
30	150	850
40	200	800
50	250	750
75	375	625
100	500	500
125	625	375
150	750	250
175	875	125
200	1000	0

The absorbance of the standard solutions was measured at 280 nm by using UV-Vis spectrophotometer. Deionized water was used as blank.

3.6 Enzyme Activity Assay

A total of three enzymes and two substrates were used to undergo the enzyme activity assay. The three enzymes used were 0.5 mg/mL bromelain as reference, pumpkin crude extract and pumpkin partial purified extract, while the two substrates used were 2% sodium caseinate and 1% haemoglobin. Table 3.6 shows the overview of the enzyme activity assay to be done by using different enzymes and substrates.

Table 3.6: The overview of enzyme activity assay with different enzymes and substrates.

Enzyme	Substrate
Bromelain (reference)	Sodium caseinate
	Haemoglobin
Pumpkin crude extract	Sodium caseinate
	Haemoglobin
Pumpkin partial extract	Sodium caseinate
	Haemoglobin

The enzyme concentration was determined using Bradford assay as described in section 3.5.1 and the BSA standard calibration curve.

The solutions involved in the enzyme activity were all prepared freshly before use. 2 mL of buffered substrates at pH 7 were added to 10 mL test tubes and incubated in water bath at 37 ℃ for 5 minutes. 0.2 mL of enzyme was added into each test tube and homogenized by using vortex mixer. Once the enzyme is added, the enzyme activity started and the timer was started. The reaction was left to stand for 1 hour in the water bath for 37 ℃. After 1 hour, the enzyme activity was stopped immediately by adding 2 mL of 10% trichloroacetic acid and stand at room temperature for 15 minutes. The solutions were then centrifuged at 4400 rpm at room temperature for 20 minutes. The supernatant was collected to measure the absorbance at 280 nm by using UV-Vis spectrophotometer to determine the

amount of tyrosine released after the proteolytic activity. The concentration of the tyrosine was then calculated using the tyrosine calibration curve.

3.6.1 Optimization of enzyme activity by the effect of pH and temperature

The optimum pH was determined by using buffered substrates in pH range of 4-9. The incubation temperature was fixed at 37 ℃ throughout the determination of optimum pH. In addition, the optimum temperature was determined by using buffered substrates at the optimum pH determined. The incubation of the activity reaction was done in water bath with different temperature (30 – 70 ℃).

3.7 Determination the Relative Abundance of Major Proteins in Pumpkin after Partial Purification using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the relative abundance of the pumpkin proteins after partial purification.

3.7.1 Preparation of chemicals to be used in SDS-PAGE

3.7.1.1 Preparation of 6 M hydrochloric acid

Thirty seven percent of hydrochloric acid (508.5 mL) was diluted with 1 L deionized water in 1 L volumetric flask. The solution was mixed by using magnetic stirrer and stirring bar.

3.7.1.2 Preparation of Tris-HCl buffer

Tris-HCl buffers were prepared at different concentrations and pH. The amounts of Tris-Base required to prepare Tris-HCl buffer at certain concentration and pH were listed in Table 3.7.

Table 3.7: Conditions of Tris-HCl buffers required

Concentration (M)	Weight of Tris-Base required (g)	Desired pH
0.5	6.055	6.8
1.0	12.11	6.8
1.5	18.165	8.8

The required amount of Tris-Base was dissolved in 80 mL deionized water. The pH value of the solution was adjusted by using 6 M hydrochloric acid to the desired pH. The solution was topped up to the mark with deionized water in 100 mL volumetric flask. The solution was homogenized using magnetic stirrer and stirring bar. The solution was stored in 4 ℃ for further use.

3.7.1.3 Preparation of 10% and 20% sodium dodecyl sulphate (SDS)

SDS (1 g) was dissolved in 10 mL deionized water to prepare 10% SDS. 2 g of SDS was dissolved in 10 mL deionized water to prepare 20% SDS. The solutions were homogenized by using vortex mixer.

3.7.1.4 Preparation of 10% ammonium persulphate

Ammonium persulphate (0.1 g) was dissolved in 1 mL deionized water. The solution was homogenized by using vortex mixer.

3.7.1.5 Preparation of SDS-PAGE sample loading buffer

One molar of Tris-HCl buffer at pH 6.8 (2.4 mL), 3 mL of 20% SDS, 3 mL 100% glycerol, 1.6 mL of β-Mercaptoethanol and 0.006 g of Bromophenol Blue were added into a 10 mL volumetric flask, and topped up to the mark. The solution was homogenized by inverting the volumetric flask repeatedly. The solution was stored at 4 ℃ for further use.

3.7.1.6 Preparation of 10 x SDS-PAGE electrophoresis buffer

Tris-Base (30.3 g), 144 g of glycine and 10 g SDS were dissolved in deionized water in 1 L volumetric flask. The solution was homogenized by inverting the volumetric flask repeatedly. The pH of the solution was adjusted to pH 8.3 by using 6 M hydrochloric acid. The solution was stored at room temperature for further use. The solution was diluted 10 times before every usage.

3.7.1.7 Preparation of Coomassie Blue staining solution

Coomassie Brillant Blue R-250 (0.25 g) and 25 mL glacial acetic acid were added into deionized water in 250 mL volumetric flask. The solution was homogenized by inverting the volumetric flask repeatedly.

3.7.1.8 Preparation of destaining solution

Methanol (40 mL) and 10 mL of acetic acid was added into a 100 mL volumetric flask. The solution was topped up to the mark with deionized water. The solution was homogenized by inverting the volumetric flask repeatedly.

3.7.2 Preparation of loading samples

SDS-PAGE sample loading buffer (20 μL) was added to 10 μL of pumpkin crude extract and pumpkin partial purified extract separately. Triplicate solutions were prepared. The solutions were homogenized by using vortex mixer. The solutions were then let to boil in boiling water bath for 30 minutes. After 30 minutes, the solutions were centrifuged for 1 minute by using micro-centrifuge machine.

3.7.3 Preparation of 4% stacking gel

The stacking gel required in the SDS-PAGE was prepared according to the chemicals listed in Table 3.8. All of the chemicals were prepared freshly before usage. The solution was homogenized by using vortex mixer.

Table 3.8: The chemicals needed in 5 mL stacking gel preparation

Chemicals	Volume (mL)
Deionized water	2.975
0.5 M Tris-HCl at pH 6.8	1.250
10% SDS	0.050
Acrylamide-Bis	0.670
10% Ammonium persulphate (APS)	0.050
Tetramethylethylenediamine (TEMED)	0.005

Remarks: The stacking gel solution will start to solidify after APS and TEMED are added, hence both of them must be added last.

3.7.4 Preparation of 12% separating gel

The separating gel required in the SDS-PAGE was prepared according to the chemicals listed in Table 3.9. All of the chemicals were prepared freshly before usage. The solution was homogenized by using vortex mixer.

Table 3.9: The chemicals needed in 10 mL stacking gel preparation

Chemicals	Volume
Deionized water	3.2 mL
1.5 M Tris-HCl at pH 8.8	2.6 mL
10% SDS	0.10 μL
Acrylamide-Bis	4.0 mL
10% Ammonium persulphate (APS)	100 μL
Tetramethylethylenediamine (TEMED)	10 μL

Remarks: The separating gel solution will start to solidify after APS and TEMED are added, hence both of them must be added last.

3.7.5 Setting up the plates on the electrophoresis cell rack

There were two plates used for the casting of the gel, which are short plate and spacer plate. Both of the plates were cleaned thoroughly before setting up. The short plate was placed facing inward the electrophoresis cell while the spacer plate was placed facing outward. Distilled water was first filled into the space between both plates to check whether leakage happened. The water was then poured out and left to dry.

3.7.6 Casting of the gel

The separating gel and stacking gel were prepared only after the plates and cell were well-prepared. 5 mL of 12% separating gel was inserted into the space between the plates first. Distilled water was then added to the upper part of the separating gel to ensure the separating gel is evenly distributed on the plates. The solution was left to stand for approximately 30 minutes for solidification. The remaining separating gel prepared left in the test tube can be used as indicator for the solidification. After 30 minutes, the distilled water was poured away and 2 mL of stacking gel was added to the upper part of the solidified separating gel. A comb used to create sample holding wells was inserted onto the stacking gel solution. The plates were left aside for approximately 15 minutes for solidification. The comb was removed carefully after the stacking gel solidified. The 10 times diluted SDS-PAGE electrophoresis buffer was added into the cell, covering the plates to prevent the gel from drying.

3.7.7 SDS-PAGE process

20 μL of each samples was loaded into the wells generated by the comb separately. The loading process must be careful so that the sample will not overflow into the neighbor wells. The cell was let to stand for 5 minutes until the entire samples sink to the bottom of the well. After that, the electrophoresis cell was connected to a power supply to create electric field across the electrophoresis buffer. The current and voltage supplied to the cell was 20 mA and 100 V respectively, for the first 1 hour. This is to let the samples to move across the stacking gel. After 1 hour, higher current and voltage supplies were fixed at 30 mA and 140 V to let the samples to move across the more concentrated and longer separating gel. The whole separating process took around 5 hours to complete. The gel was removed carefully from the cell and plates.

3.7.8 Staining process

The gel was immersed in the Coomassie Blue staining solution with continuous agitation until obvious bands appeared on the gel.

3.7.9 Destaining process

The gel was washed carefully with distilled water for a few times, and then immersed in destaining solution prepared. The destaining solution was replaced with new solution once it is intense with the blue color staining solution. The destaining process was left for overnight.

3.8 Enzyme Kinetic Studies

The purpose of performing enzyme kinetic studies is to identify the substrate affinity. For enzyme kinetic studies, the enzyme activity was determined at the optimum pH and temperature for each enzyme and its respective substrate pairings as shown in Table 3.6. Besides, a series of substrate solutions at different concentrations between the range of 0 mg/mL to 30 mg/mL were prepared. The enzyme activities on different concentration of substrates were determined as described in section 3.6.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Determination of Presence of Enzyme Protease in Pumpkin using Screening Test

The screening test was conducted to determine the presence of enzyme proteases in pumpkin. During the screening test, the distilled water was used as blank, while commercial enzyme bromelain was used as reference. Milk contains of high amount of casein which is favorably to be hydrolyzed by enzyme proteases. Besides, it also contains a high component of lipids. The enzyme subjected to hydrolyze the lipid is lipase. It is uncertain that whether the lipase is presence in the pumpkin extract, hence skimmed milk agar plate was used. Skimmed milk is the milk with all cream, which is the milk fats being removed. Hence, the test will not be interfered by the lipase enzymatic reaction. Clear zone formation will occur at the surrounding of the holes punched on the agar plate that was used to fill the extract sample if there is any proteolytic reaction happened (Adinarayana et al., 2003). The reaction can be indicated based on the commercially pure bromelain used as reference. The result of the screening test is illustrated in Figure 4.1.

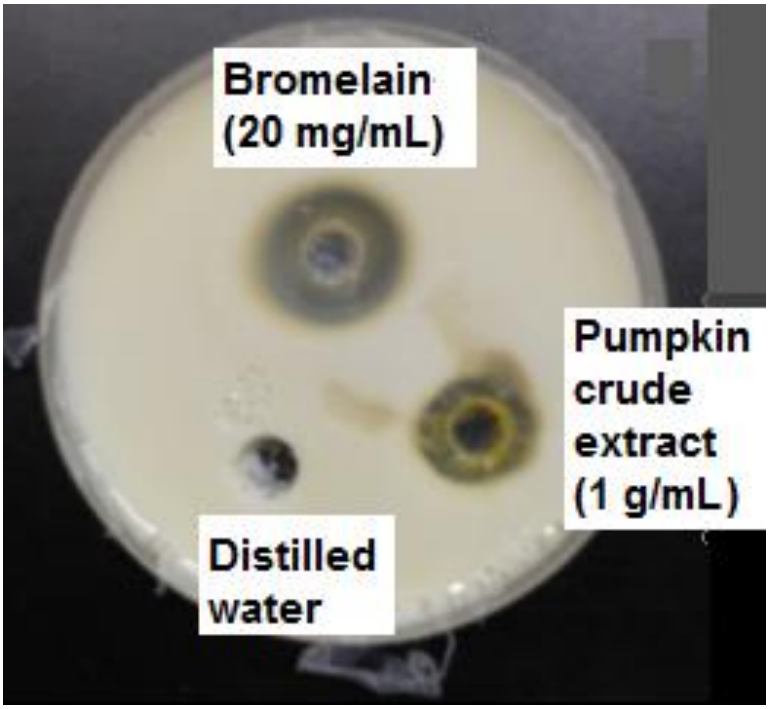

Figure 4.1: The skimmed milk agar plate with after incubation for 24 hours.

The screening test shows a positive result with clear zone formed on both reference bromelain and the pumpkin crude extract. This indicates that enzyme proteases that digested the skimmed milk are present in the pumpkin crude extract.

4.2 Calibration Curves

4.2.1 Bovine serum albumin (BSA) standard calibration curve for determination of protein concentrations

Bradford assay used BSA as a standard compound. BSA is used due to its stability, low cost and easily available and it is lack of interference within biological reactions. BSA standard calibration curve is a common and famous method used for calculating protein concentration of an unknown sample. A series of known protein standard solutions at different concentrations are prepared to build up this standard calibration curve.

Bradford assay is a rapid and sensitive colorimetric protein assay used to estimate protein concentration in a sample. Bradford assay is based on the binding of the dye Coomassie Brilliant Blue G-250 or R-250, which is also known as Bradford reagent to the protein to be assayed. The free cationic Coomassie Brilliant Blue is in red or green form that absorbance maxima at 470 and 650 nm respectively. When the dye binds onto the protein, it is deprotonated by donating free electron to the protein, causing disruption of the protein structure which leads to the exposure of the hydrophobic part of the protein. The dye then forms strong noncovalent bonding with the protein via electrostatic interaction with amino groups or sulfonic groups and also van der waals' forces. Both of the ionic and

hydrophobic interactions stabilized the deprotonated dye, leading to changing color from red to blue form that has absorbance maxima at 595 nm (Bradford, 1976; Georgiou *et al*., 2008). The more concentrated the extract sample, the higher the intensity of blue color, the higher the absorbance readings. The BSA standard calibration curve constructed is shown in Figure 4.3.

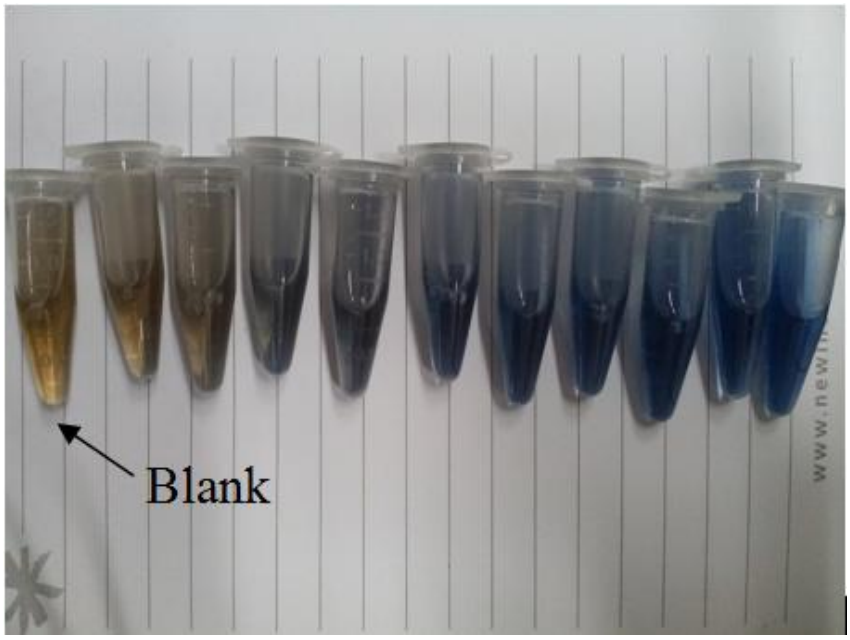

Figure 4.2: The trend of increasing intensity of blue color when concentration of BSA increases from left to right.

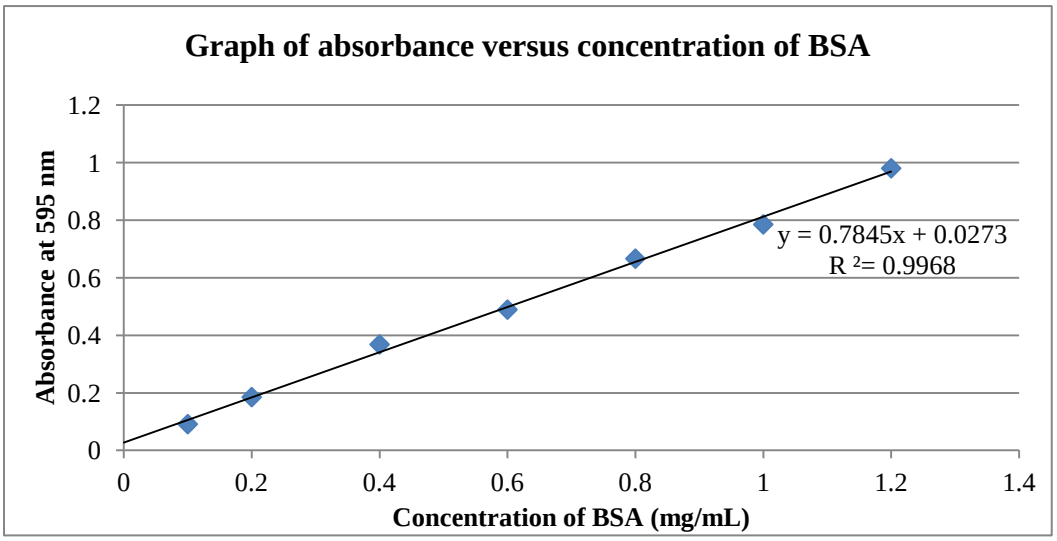

Figure 4.3: The standard calibration curve of BSA.

4.2.2 Tyrosine standard calibration curve for the determination of release of tyrosine residue in protein fragments.

Tyrosine is one of the reported twenty amino acids. It is used as representative among the amino acids products to be analyzed from the proteolytic reaction due to its existence of benzene ring. The aromatic ring gives the hydrophobic properties to tyrosine, causing them to have no preference towards aqueous environment and buried inside the protein core during folding (Betts and Russell, 2003). The tyrosine will be exposed to the exterior environment after the proteolytic activity on the protein molecule, hence can be detected. The conjugated aromatic ring causes tyrosine to be able to absorb light at UV region, at maximum

wavelength of 280 nm. The higher absorbance indicated the higher amount of tyrosine.

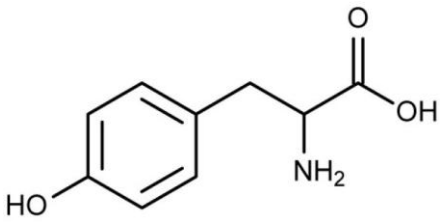

Figure 4.4: Structure of tyrosine.

The tyrosine standard calibration curve constructed is shown in Figure 4.5.

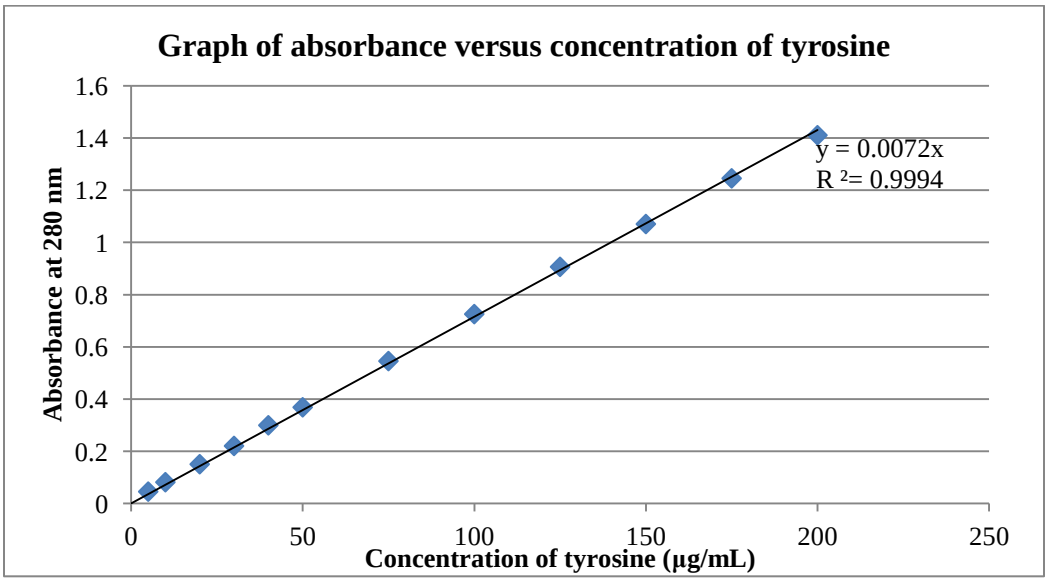

Figure 4.5: Standard calibration curve of tyrosine.

4.3 Determination of Optimal Conditions for Proteolytic Activity

Enzyme activities are highly dependent on pH and temperature (Home and Peck, 1993). The active site of the enzymes must have the right conformations for the protein substrate to bind onto it. pH and temperature alteration are the common ways used to enhance the desirably of the enzymatic reaction. Generally, enzyme works well within a range of temperature specific to the organism. The reaction rates basically increase with increasing in temperature until a limit. At the limit temperature, the enzyme and protein will be denatured resulting from the breaking down of the weak ionic and hydrogen bonding that stabilize the three-dimensional structure of the enzyme active site, hence losing its secondary and tertiary structure (Daniel *et al*., 2010).

In addition, pH can affect the enzymatic activities by varying the ionic state of amino acid residues within the enzyme and substrate. The changes in ionic state will influence the efficiency of the substrate binding affinity onto the enzyme active site. The effect of pH can be reversible within a narrow range of pH around the optimum pH of certain enzymatic activity, but the protein structure will be denatured permanently at the extreme pH region, either acidic or alkaline, due to the critical distortion of the protein structure (Home and Peck, 1993).

4.3.1 Relative enzyme activity

Enzyme activity is a measure of quantity of active enzyme present in an extract sample. It is always expressed in Unit/mol. In this project, non-specific protease activity assay was conducted with casein sodium salt as one of the substrates. In the non-specific protease activity assay, one unit indicates the amount of enzyme that liberated 1 μg of tyrosine in 1 minute of hydrolysis reaction at 37 ℃ (Cupp-Enyard, 2008). Besides, when the enzyme activity is conducted with haemoglobin as substrate, one unit is defined as the amount of enzyme that produces trichloroacetic acid-soluble product that is detectable at 280 nm in 1 minute at pH 2 and 37 ℃.

The negatively charged trichloroacetate ions in trichloroacetic acid can interfere the electrostatic interaction between the protein molecules and destabilized it, causing partially unfolding of the protein structure. The hydrophobic region of the partially disordered protein is therefore being exposed to the solvent environment, causing the protein precipitation due to the hydrophobic interaction. It is found that the protein precipitation by trichloroacetic acid is less effective on the completely unfolded protein (Rajalingam *et al.,* 2009). Hence, the detectable tyrosine will not be precipitated out from the activity assay solution. The uncleaved sodium caseinate and haemoglobin are in white and reddish brown color respectively. When trichloroacetic acid is added during the enzyme activity assay, the colored

unreacted protein will be precipitated, leaving the cleaved colorless protein solution with a signal that is detectable at UV region at 280 nm. The relative enzyme activity of the enzyme proteases was determined by using Equation (1).

Relative enzyme activity, Unit/mL

$$= \frac{\text{μmol of tyrosine liberated x Total volume of assay (mL)}}{\text{Time taken for the assay (min) x Volume of enzyme used (mL)}} \quad \text{……..…..Equation (1)}$$

The amount of tyrosine liberated is calculated from the tyrosine standard calibration curve with the absorbance reading obtained by using the linear regression line equation y = 0.0072x as shown in Figure 4.5. The amount of tyrosine shown in the calibration curve is expressed in μg/mL, it is then converted to μmol/mL (calculation shown in Appendix B) for the calculation of enzyme activity. The complete relative enzyme activities calculated for enzyme bromelain, pumpkin crude extract and pumpkin partial purified extract on sodium caseinate and haemoglobin substrates at their optimal conditions is shown in Appendix B.

4.3.2 Specific enzyme activity

Specific activity is a measure of active enzyme capability at a specific substrate concentration. It is usually expressed in Unit/mg, using equation (2):

$$\text{Specific activity, Unit/mg} = \frac{\text{Relative enzyme activity } (\frac{\text{Unit}}{\text{mL}})}{\text{Enzyme concentration } (\frac{\text{mg}}{\text{mL}})} \quad \text{.....Equation (2)}$$

The enzyme concentration is calculated from the BSA standard calibration curve with the absorbance readings by using the linear regression line equation y = 0.7845x + 0.0273 as shown in Figure 4.3. The complete specific enzyme activities calculated for bromelain as reference enzyme protease, pumpkin crude extract and pumpkin partial purified extract on sodium caseinate and haemoglobin substrates at their optimal conditions is shown in Appendix B. The optimal conditions expressed in specific enzyme activity for each enzyme and substrate pairings are shown in Figure 4.6 until Figure 4.17 and Table 4.1.

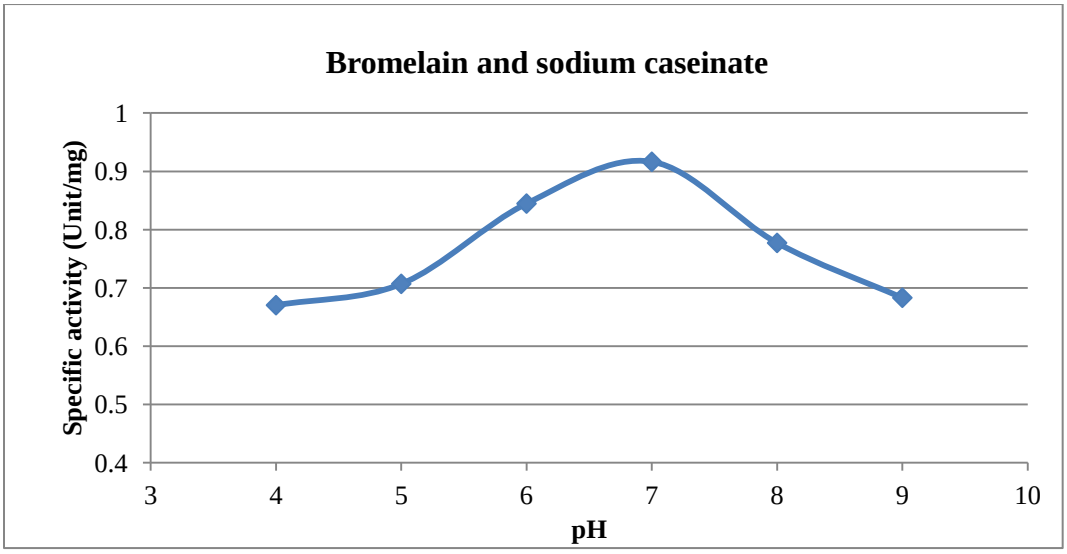

Figure 4.6: Effect of pH on proteolytic activity between enzyme bromelain and sodium caseinate substrate at 37 ℃.

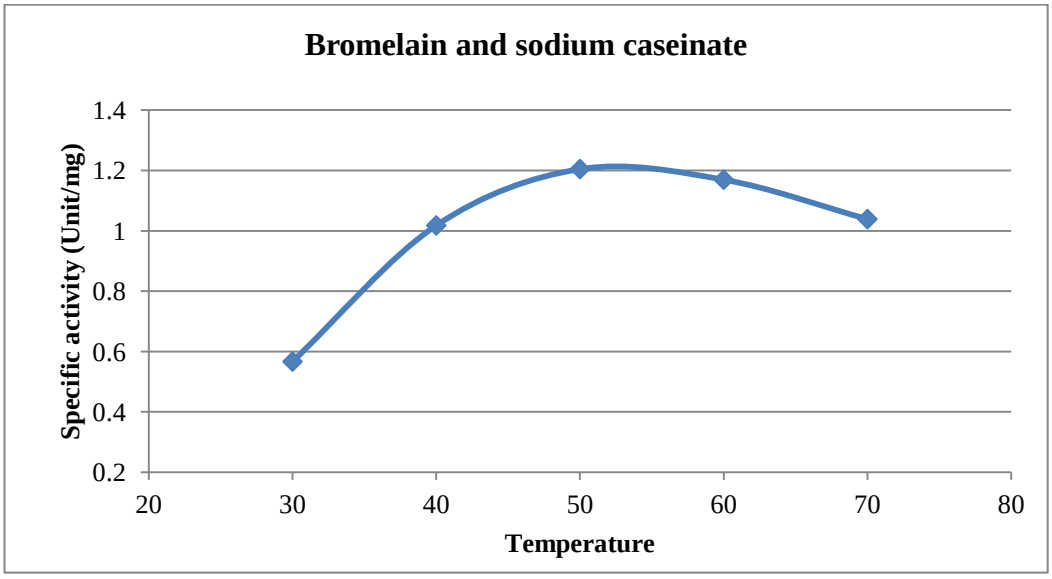

Figure 4.7: Effect of temperature on proteolytic activity between enzyme bromelain and sodium caseinate substrate at pH 7.

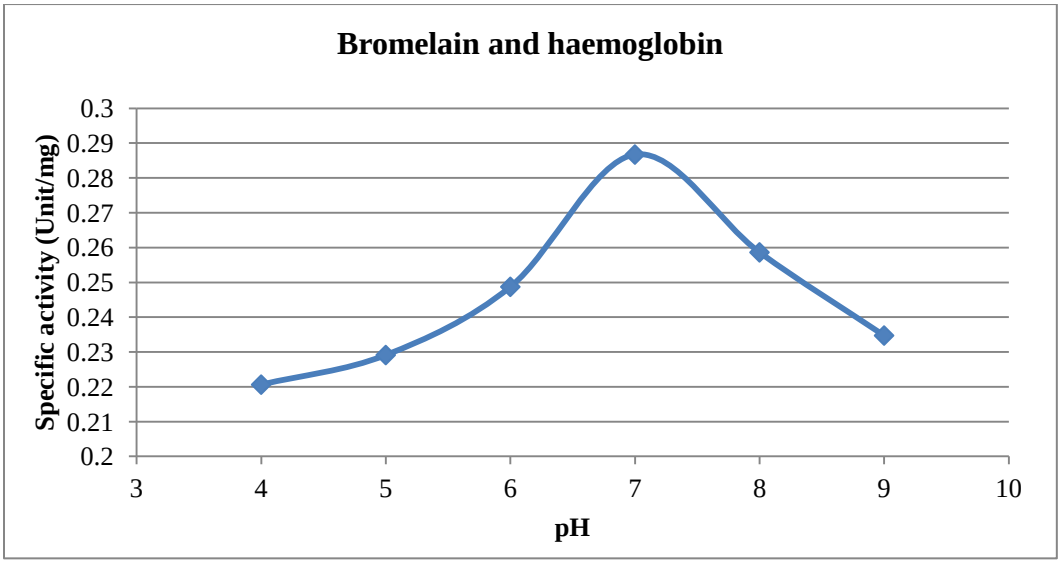

Figure 4.8: Effect of pH on proteolytic activity between enzyme bromelain and haemoglobin substrate at 37 ℃.

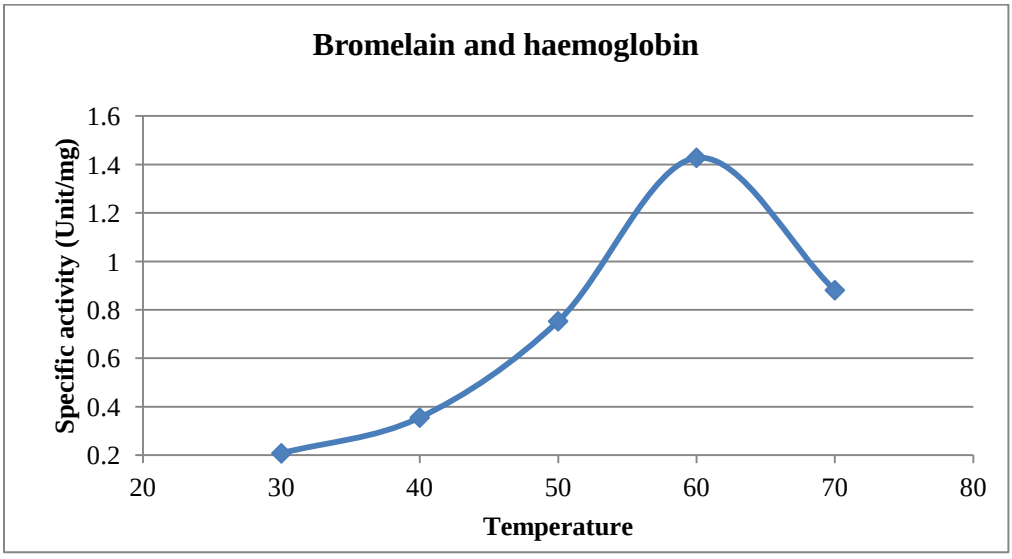

Figure 4.9: Effect of temperature on proteolytic activity between enzyme bromelain and haemoglobin substrate at pH 7.

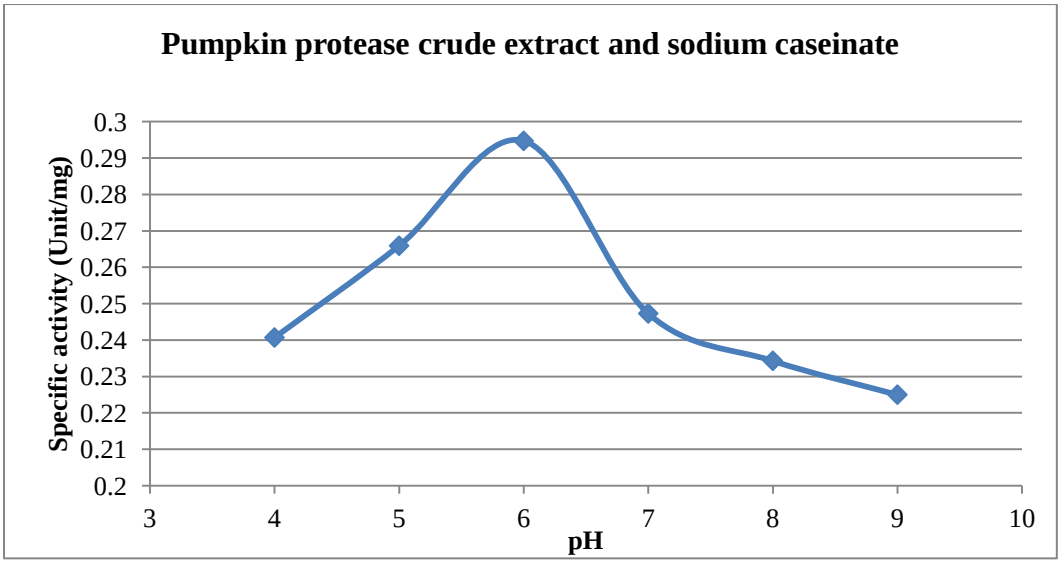

Figure 4.10: Effect of pH on proteolytic activity between enzyme proteases from pumpkin crude extract sodium caseinate substrate at 37 ℃.

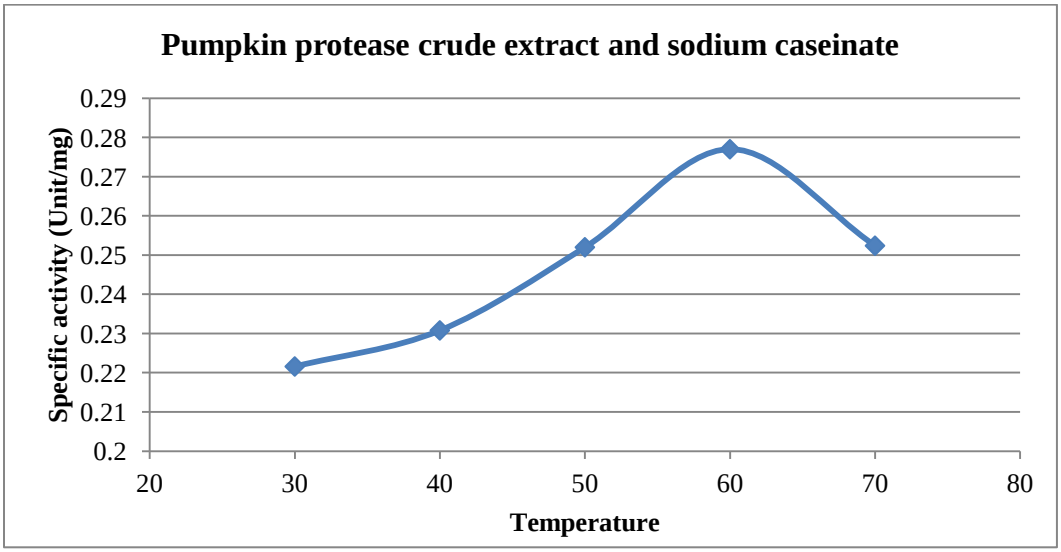

Figure 4.11: Effect of temperature on proteolytic activity between enzyme proteases from pumpkin crude extract sodium caseinate substrate at pH 6.

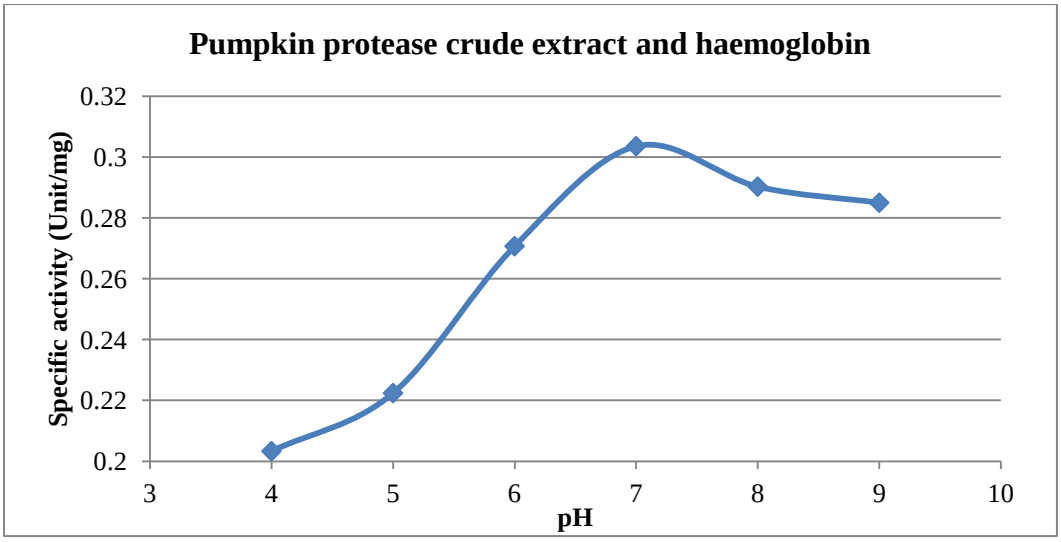

Figure 4.12: Effect of pH on proteolytic activity between enzyme proteases from pumpkin crude extract haemoglobin substrate at 37 ℃.

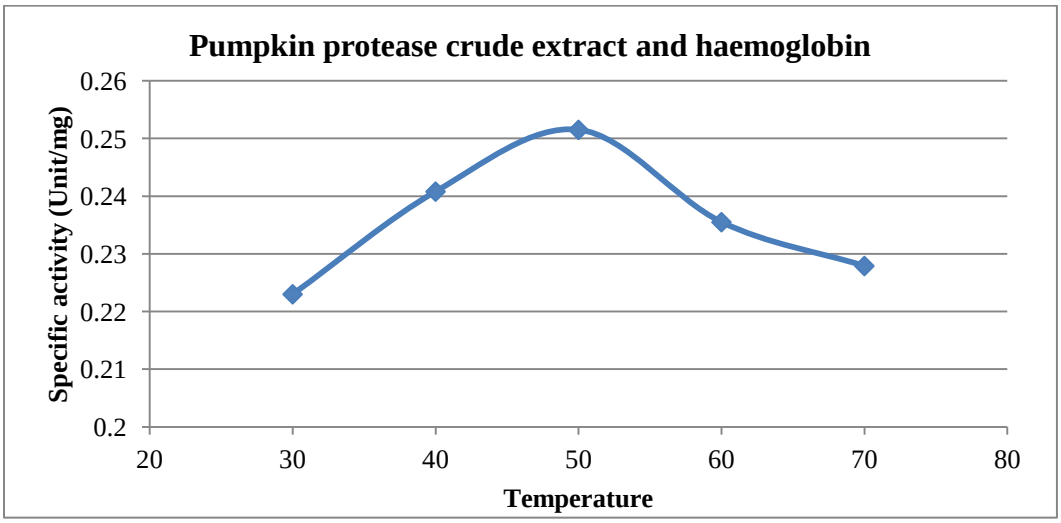

Figure 4.13: Effect of temperature on proteolytic activity between enzyme proteases from pumpkin crude extract haemoglobin substrate at pH 7.

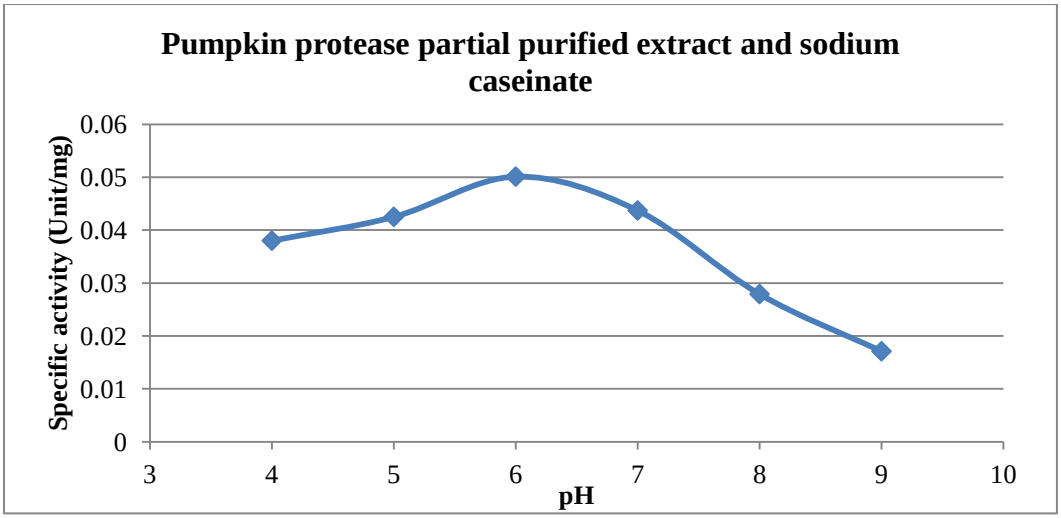

Figure 4.14: Effect of pH on proteolytic activity between enzyme proteases from pumpkin partial purified extract sodium caseinate substrate at 37 ℃.

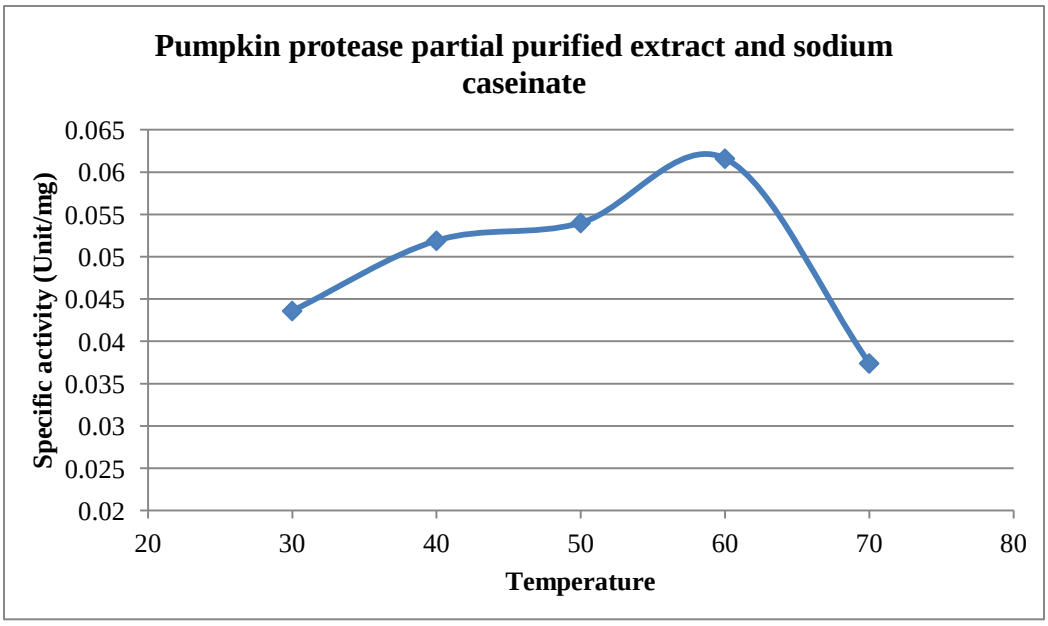

Figure 4.15: Effect of temperature on proteolytic activity between enzyme proteases from pumpkin partial purified extract sodium caseinate substrate at pH 6.

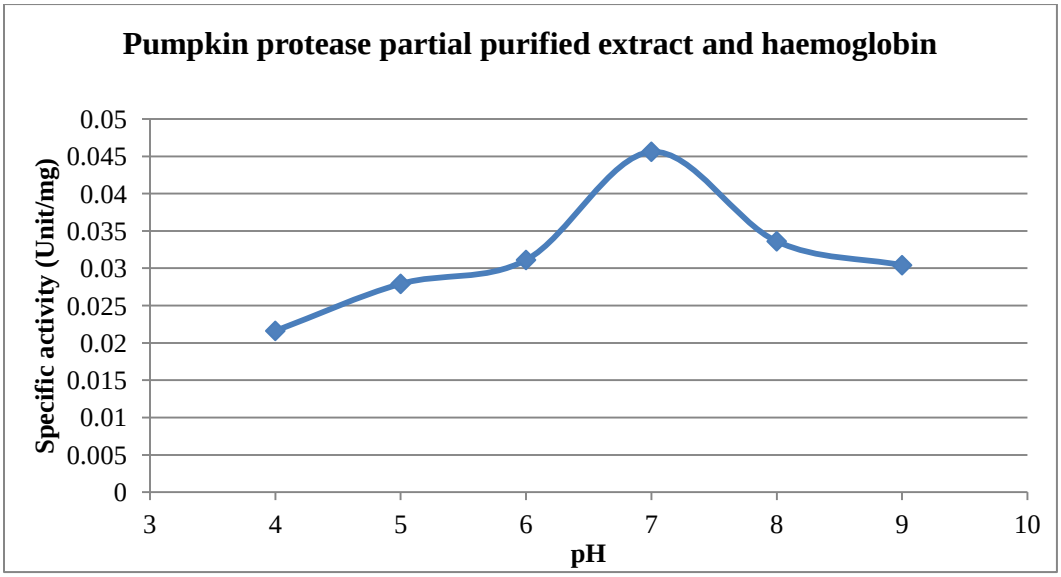

Figure 4.16: Effect of pH on proteolytic activity between enzyme proteases from pumpkin partial purified extract haemoglobin substrate at 37 ℃.

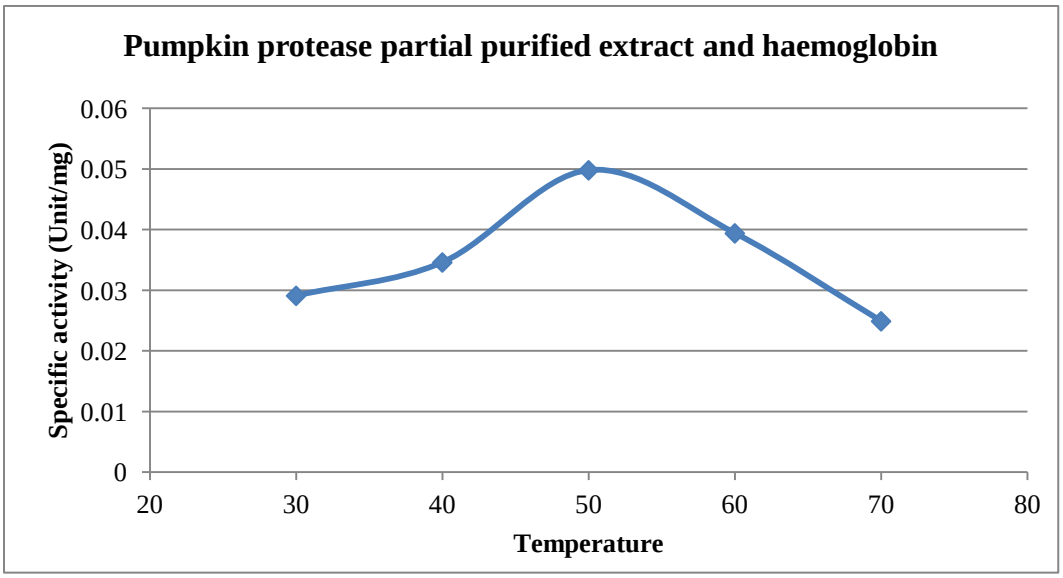

Figure 4.17: Effect of temperature on proteolytic activity between enzyme proteases from pumpkin partial purified extract haemoglobin substrate at pH 7.

Table 4.1: Optimal conditions of proteolytic activity between different enzyme and substrate pairings.

Protease and Substrate	Optimum pH	Optimum temperature (℃)	Specific activity (Unit/mg)	Remarks
Bromelain and sodium caseinate	7	50	1.2043	**Refer to Figure 4.6 and Figure 4.7.**
Bromelain and haemoglobin	7	60	1.4278	**Refer to Figure 4.8 and Figure 4.9**
Pumpkin crude extract and sodium caseinate	6	60	0.2770	**Refer to Figure 4.10 and Figure 4.11**
Pumpkin crude extract and haemoglobin	7	50	0.2515	**Refer to Figure 4.12 and Figure 4.13**
Pumpkin partial purified extract and sodium caseinate	6	60	0.0616	**Refer to Figure 4.14 and Figure 4.15**
Pumpkin partial purified extract and haemoglobin	7	50	0.0498	**Refer to Figure 4.16 and Figure 4.17**

Different enzymes may have different optimum pH and temperature on different substrates (Home and Peck, 1993). Based on the data tabulated in Table 4.1, it was found that the enzyme activities are dependent on substrates type. The same type of enzyme shows different optimal conditions on different types of substrates. Bromelain performs optimal activity on sodium caseinate at pH 7 and 50 ℃; and pH 7 and 60 ℃ towards haemoglobin as substrate. Both pumpkin crude and partial purified extract shows their highest activities at pH 6 and 60 ℃ and at pH 7 and

50 ℃ on sodium caseinate and haemoglobin substrates respectively. It was observed that the pumpkin protease partial purified extract show enzyme activity approximately 5 times lower than pumpkin crude extract on both sodium caseinate and haemoglobin substrates. Corzo *et al.* (2012) in their study on the enzyme bromelain activity on different protein substrates found that the optimal conditions on sodium caseinate and haemoglobin substrates are pH 6.5 and 59 ℃, and pH 2.9 and 37 ℃ respectively.

4.4 Partial Purification of Pumpkin Crude Enzyme Protease

The pumpkin crude enzyme protease is partial purified by using ammonium sulphate precipitation method. It is a method that involves the precipitation of proteins from the sample extract by the ammonium sulphate salts. It is the most common used method as the salt is easily available and the steps are simple and the ammonium sulphate salts show the highest effectiveness in Hofmeister series (Yang, 2009). The Hofmeister series is a classification of ions according to their ability to precipitate out the proteins. The Hofmeister series for cations is shown as $F^- \approx SO_4^{2-} > HPO_4^{2-} >$ acetate $> Cl^- > NO_3^- > Br^- > ClO_3^- > I^- > ClO_4^- > SCN^-$, while the Hofmeister series for anions is shown as $NH_4^+ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+} >$ guanidinium (Zhang and Cremer, 2006). When ammonium sulphate salt is added into the protein extracts, the solubility of proteins increase with the concentration of ammonium sulphate salts added. The proteins are surrounded by

the hydrated counterions, which prevents the aggregation of the proteins in the solution. This is known as salting in effect. When the concentration of salts added reaches a limit, the salts withdraws the hydrated water molecules from the proteins, and thus leads to aggregation and precipitation of protein molecules due to the hydrophobic effect between each other. This phenomenon is known as salting out.

The ammonium sulphate salts within the crude extract was then removed by conducting dialysis against 0.1 M sodium phosphate buffer at pH 7. During dialysis, the crude extract is filled in a dialysis tubing which is a selectively permeable membrane, allowing the small ion molecules such as ammonium sulphate salt, and unwanted small molecules such as free amino acids, sugars and natural products to flow into the dialysate through the membrane through diffusion against concentration gradient. The dialysis tubing with molecular weight cut-off (MWCO) of 12-14 kDa was used in this project. Molecular weight cut-off is the lowest molecular weight of solute that can be retained by the permeable membrane. The peptide molecules with size lower than 12 kDa will diffuse through the membrane into the dialysate. The enzyme activities will be interfered by the presence of ammonium sulphate salt if dialysis is not conducted.

4.5 Purification Fold

The proteolytic activities of pumpkin proteases were done with both crude and partial purified extracts to determine the purification fold of the enzyme in order to determine any interference during the purification steps. It can be calculated by using the Equation (3):

Purification fold

$$= \frac{\text{Specific enzyme activity of partial purified enzyme protease}}{\text{Specific enzyme activity of crude enzyme protease}} \quad \text{.....Equation (3)}$$

Determination of purification fold can help to know the purity level fold of the proteins and the specific enzyme activities fold. The purification fold calculated is tabulated in Table 4.2.

Table 4.2: The partial purification fold calculated for each enzyme and substrate pairings.

Extract	Volume (mL)	Protein concentration (mg/mL)	Total protein concentration (mg)	Relative activity (U/mL)	Total relative activity (U)	Specific activity (U/mg)	Partial purification fold
Pumpkin crude extract and sodium caseinate	50	0.3196	15.98	0.0885	4.425	0.2770	1
Pumpkin partial purified extract and sodium caseinate	20	0.2031	4.062	0.0125	0.25	0.0616	0.2224
Pumpkin crude extract and haemoglobin	50	0.3163	15.815	0.0795	3.975	0.2515	1
Pumpkin partial purified extract and haemoglobin	20	0.2031	4.062	0.0101	0.202	0.0498	0.1980

Based on the Table 4.2, it is shown that the purification fold for the pumpkin protease extract on both substrates decreases with folding of only 0.2224 and 0.1980 on sodium caseinate and haemoglobin respectively. Josephine *et al.* (2012), Devi *et al*. (2014) and Berg *et al*. (2002) in their studies on the proteases from plants and fungi have suggested that the purification fold would increases after every steps of purification, including ammonium sulphate precipitation.

The assumption made for this result is that the ineffective removal of ammonium sulphate salts from the partial purified extract during dialysis. The diffusion involves the net movement of molecules from a higher molecule concentration area to lower concentration until equilibrium is reached. The difference in diffusion pattern between the protease extract and dialysate leads the diffusion of molecules to happen. The concentration of sodium phosphate buffer used to dissolve the centrifuged pellet after the ammonium sulphate precipitation is 0.1 M, which is the same as the concentration of the sodium phosphate buffer used to dialyze against. This can cause the differential diffusion pattern between the dialysis tubing and the dialysate to be similar, leading to very slow diffusion of the ion molecules through the permeable membrane; therefore it might need a longer time. During the experiment, the dialysis process was forced end before the completion of removal of all ammonium sulphate salts, causing the binding of the salts onto the protein molecules and inactivates it.

In addition, the extraction of proteins from the natural sources such as plants, fruits, animals and fungi not only release the targeted enzymes but also endogenous proteases. Endogenous proteases can degrade the other enzymes during the extraction and purification. They will affect the enzyme of interest activity by degrading them (Thermo Scientific, 2011). In this project, proteolytic enzymes are extracted from the pumpkin fruits. There is a possibility to have more than one type of proteolytic enzymes being extracted, including the endogenous proteases. Therefore, degradation of the targeted enzyme might happen during purification. This can be a reason that caused the decrement of enzyme activities. In order to avoid this problem, proteases inhibitors or stabilizers can be added in the extraction buffer and the resulting enzymatic activities with and without the inhibitors and stabilizers will be observed. There is also possibility that the proteases lose it enzymatic activities during the purification stage and upon storage. Stabilizer can help to prolong the enzyme activity lifetime upon storage. The protease inhibitors such as ethylenediaminetetraacetic acid (EDTA) can inhibit the enzymes other than the targeted enzyme, while the stabilizer such as EDTA, ascorbic acid and cysteine can stabilize the targeted proteases and enhance their proteolytic activities (Nafi *et al.*,2012). Another assumption will be explained in the following section 4.6.

4.6 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is commonly used to estimate the relative molecular mass of protein, the relative abundance of major proteins in a sample, and the distribution of proteins among fractions. Electrophoresis is a phenomenon where the charged molecules move in response to an electric field applied. This can be used to separate protein effectively. SDS which is a strong anion reducing agent is used to denature the protein macromolecules. The SDS will bind onto the protein macromolecules and break down its structure into long, rod-like shape conformations. The negatively charged of SDS give the nature of negative charge to the bound proteins. When an electric field is applied across the electrophoresis chamber, the proteins are forced to migrate through the gel from cathode to anode. (Hames, 1998). Generally, the SDS-bound proteins would migrate in the gel according to their sizes, enabling the determination of molecular weight of the proteins. The molecular weight of the proteins will be expressed as bands on the resulting SDS-PAGE gel. In this project, SDS-PAGE is done to determine the relative abundance of major protein within the pumpkin proteases. The resulting SDS-PAGE gel is shown in Figure 4.18.

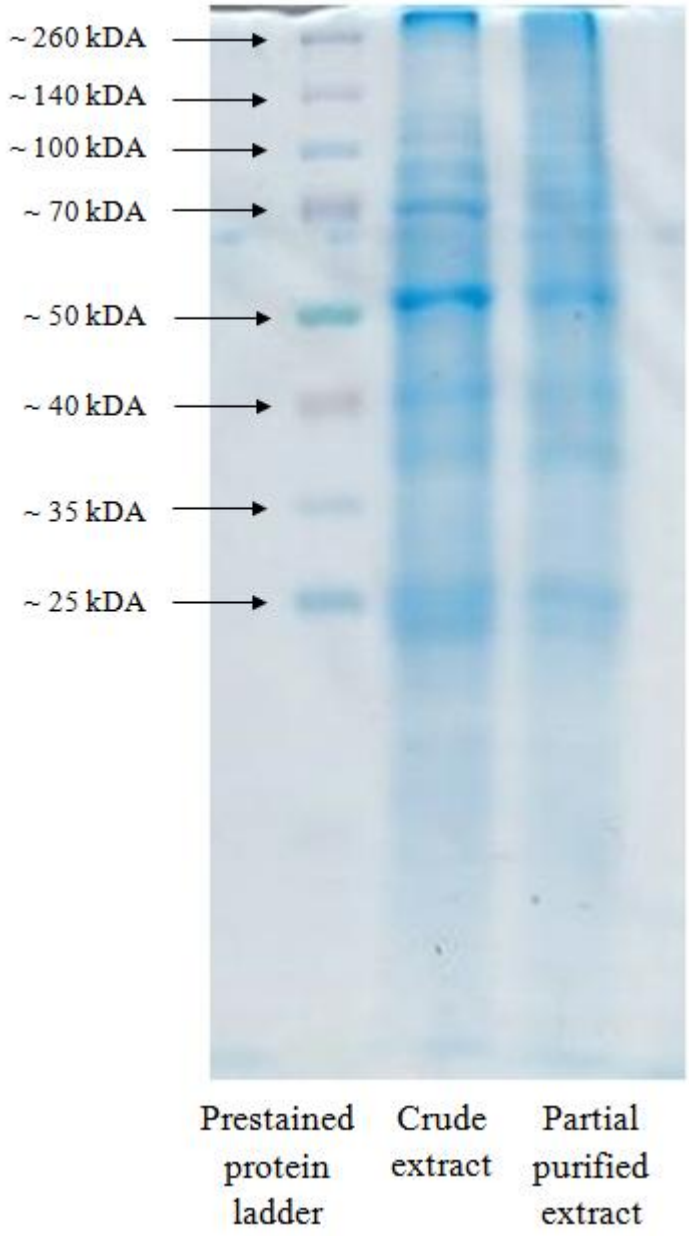

Figure 4.18: The resulting SDS-PAGE gel

Based on the Figure 4.18, it is found that there is losing of important bands, either completely disappearing of the band or decreases in intensity of blue color of the bands. It is known that the intensity of Coomassie Blue would increases with the

concentration of proteins. The missing of bands might be caused by the losing of that specific protein during partial purification, since the partial purification was only done up to 95% saturation. The lost protein might be failed to be precipitated out and being left in the remaining 5% of crude extract. Assumption is made that the losing of the proteins in the SDS-PAGE analysis might be the main targeted enzymes that provide most of the proteolytic activity. It may be due to the presence of endogenous protease or the protease is unstable during purification stage as explained in section 4.5.

4.7 Proteases Enzyme Kinetic Studies

Enzyme kinetic study can be used to examine the mechanism of the enzyme kinetic activities or to determine the substrate affinity towards the specific enzymes. The Michaelis-Menten method is the simplest and effective way for enzyme kinetic study. The Michaelis-Menten equation shown below is used to determine the K_m and V_{max} values which are important as the results of enzyme kinetic studies.

$$v = \frac{V_{max}[S]}{K_M + [S]} \quad \text{.....Equation (4)}$$

where v is reaction rate (absorbance change), V_{max} is maximum reaction rate, [S] represents substrate concentration and K_m represents the Michaelis-Menten constant. A standard plot of velocity against substrate concentration is normally

constructed as a way for the enzyme kinetic determination as shown in Figure 4.19**.**

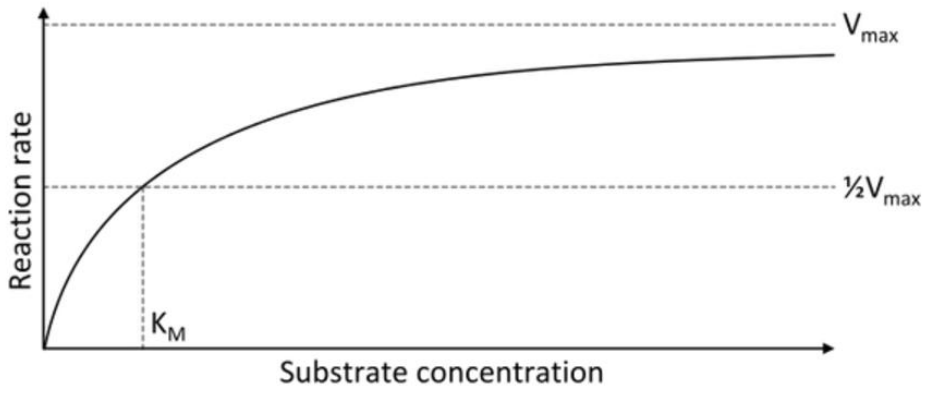

Figure 4.19: The standard plot of Michaelis-Menten curve.

The Michaelis-Menten constant (K_m) indicates the substrate concentration required to reach half of the V_{max}. It is suggested that the substrate with lower K_m value is having better affinity to the enzyme, which it can bind to enzyme active site and react in a short time and achieve ½ V_{max}. (Berg *et al.*, 2002).

In the project, the affinity of enzyme to different protein substrates is studied at optimal conditions as listed in Table 4.1. The substrates used are sodium caseinate and haemoglobin at different substrate concentration. Commercial bromelain from pineapple fruit is used as reference enzyme protease. The K_m value and V_{max} value are calculated with Michaelis-Menten equation using Sigma Plot 12.5 Software. The calculated K_m and V_{max} value for each protease and substrate pairings are shown is Figure 4.20 to Figure 4.23, and Table 4.3.

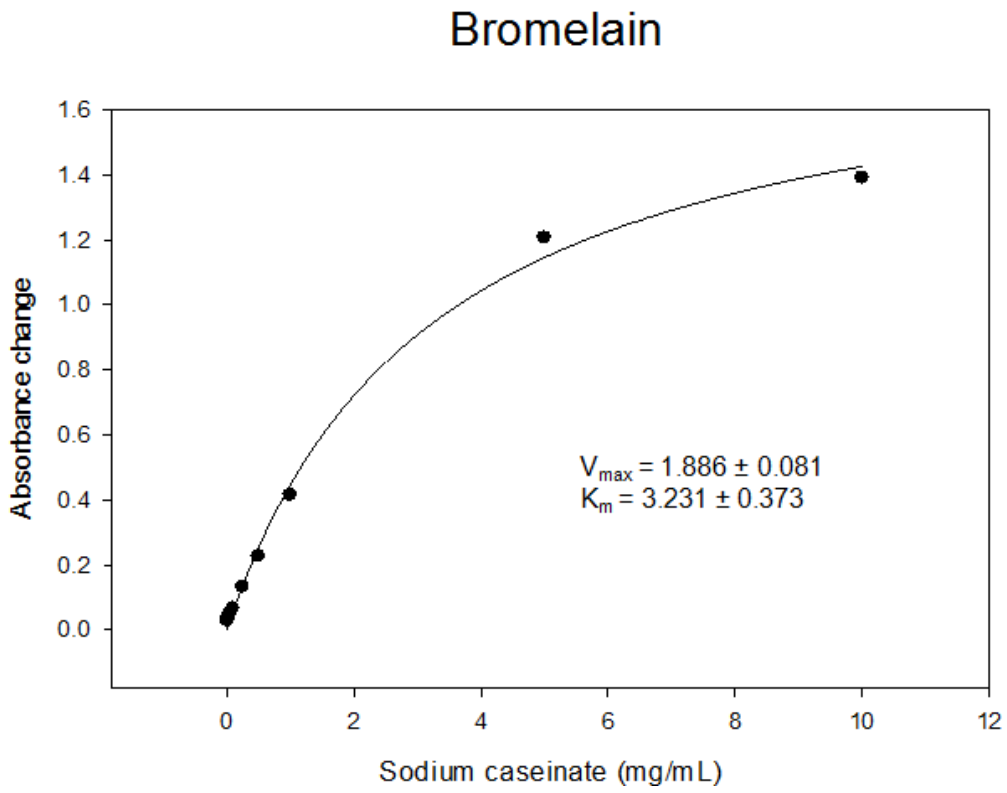

Figure 4.20: The Michaelis-Menten plot for enzyme bromelain acitivity on sodium caseintate pH 7 and 50 ℃.

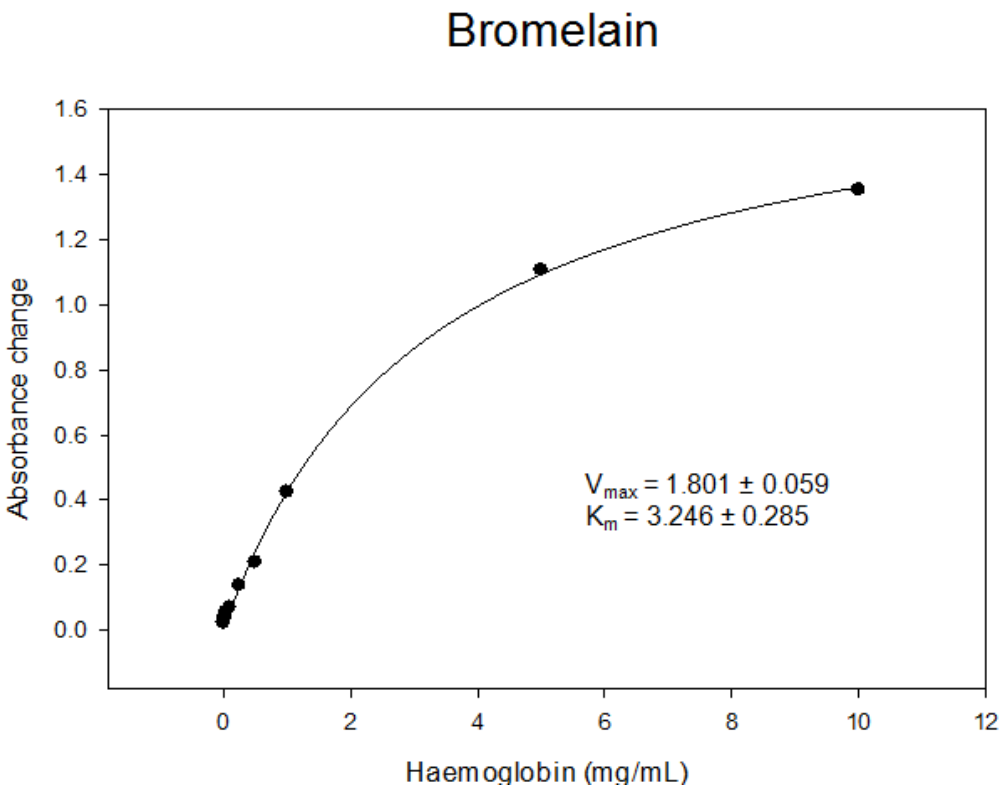

Figure 4.21: The Michaelis-Menten plot for enzyme bromelain acitivity on haemoglobin at pH 7 and 60 ℃.

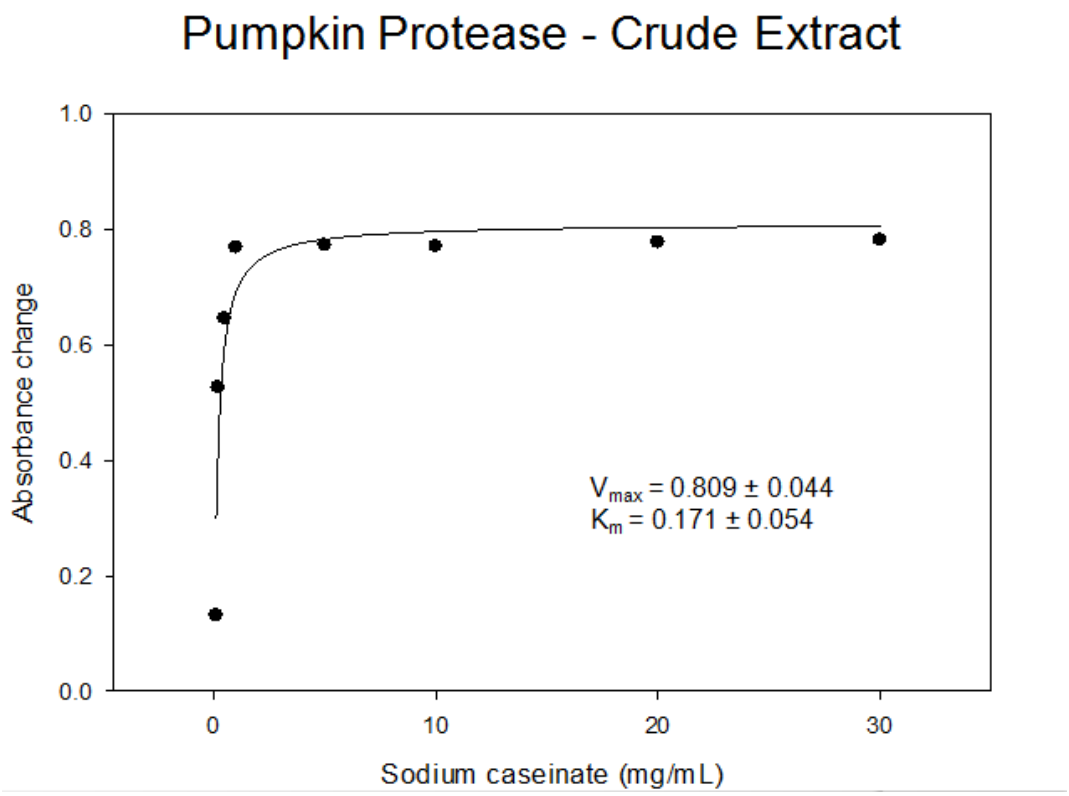

Figure 4.22: The Michaelis-Menten plot for pumpkin protease (crude extract) activity on sodium caseintate at pH 6 and 60 ℃.

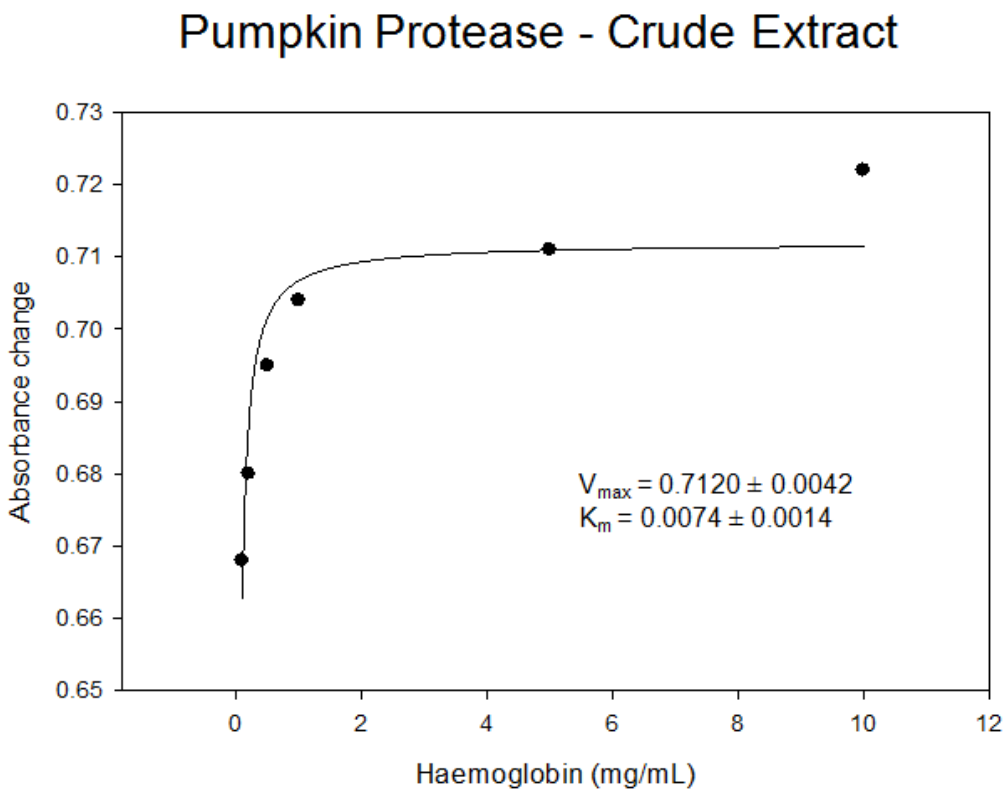

Figure 4.23: The Michaelis-Menten plot for pumpkin protease (crude extract) activity on haemolgobin at pH 7 and 50 ℃.

Table 4.3: The K_m calculated for each enzyme and substrate pairings.

Enzyme and substrate	**K_m value (mg/mL)**
Bromelain and sodium caseinate	K_m = 3.231 ±0.373
Bromelain and haemoglobin	K_m = 3.246 ±0.285
Pumpkin protease crude extract and sodium caseinate	K_m = 0.171 ±0.054
Pumpkin protease crude extract and haemoglobin	K_m = 0.0074 ±0.0014

The pumpkin protease partial purified extract was unable to perform kinetic studies. It may be due to their very low enzymatic activities as shown in section 4.3. Based on the Table 4.3, the sodium caseinate is having similar K_m value with haemoglobin towards enzyme bromelain, which indicates that both of them are having similar affinity to the enzyme bromelain. In addition, the haemoglobin is having lower K_m value than sodium caseinate towards pumpkin protease, which indicates that the haemoglobin is more compatible with pumpkin protease compared to sodium caseinate. Haemoglobin is hence recommended for the determination of pumpkin protease enzyme activity at optimal conditions in further studies. A similar studies is reported by Corzo *et al*,(2012) on their research on the enzyme bromelain characterization, they have found that azocasein and azoalbumin are more suitable as a substrate for enzyme bromelain as they are having the lowest K_m value among casein, sodium caseinate and haemoglobin. They have recommended that azoalbumin and azocasein to be the most suitable substrate for standardization and characterization of enzyme bromelain in future studies.

CHAPTER 5

CONCLUSION

5.1 Conclusion

In this project, the enzyme proteases have been extracted and partial purified using ammonium sulphate precipitation method. The presence of proteolytic enzyme in the pumpkin protease extract has been proven by conducting screening test of the enzyme on the skimmed milk agar plate. The pumpkin protease crude and partial purified extracts were used to study the effect of pH and temperature on their proteolytic activities using sodium caseinate and haemoglobin as substrates.

Both of the pumpkin protease crude and partial purified extracts show tally optimum pH and temperature to each other, which is pH 6 and 60 ℃ on sodium caseinate substrate and pH 7 and 50 ℃ on haemoglobin as substrate. The relative abundance of proteins within the pumpkin proteases extract after partial purification is determined using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE result shows that there are missing bands, indicating there might be protein loses during the purification step.

The enzyme activity is found to decrease after the partial purification which is expressed as purification fold of 0.2224 and 0.1980 towards sodium casienate and haemoglobin as substrates respectively. In addition, the pumpkin protease is found to have higher affinity to haemoglobin substrate than sodium caseinate in the enzyme kinetic studies. Haemoglobin is suggested to be used in the future researches and studies regarding the pumpkin enzyme proteases.

5.2 Further Studies

The partial purification of the pumpkin protease crude extract is only one fraction which is up to 95%. It recovers almost all proteins contained within the pumpkin, causing the targeted proteases to be unidentified. Hence, it is suggested that fractional partial purification at different saturation level can be done in future studies. Besides, further purification after partial purification can be conducted using a variety of chromatography to obtain the highest purity and enzyme activities. The molecular weight and characteristic of the main targeted enzyme within pumpkin can be discovered by using SDS-PAGE analysis.

Since proteases provide a very wide contribution in industrial application, the stability of proteases under various conditions is a key concern. In order to improve the protein yield, stability and proteolytic activity, protease stabilizers

such as cysteine, ethylenediaminetetraacetic acid (EDTA), and ascorbic acid can be added during the enzyme activity. Besides, the presence of endogenous enzymes must be considered also. It can be inhibited by adding proteases inhibitors such as EDTA, phenylmethylsulfonyl fluoride (PMSF), and aprotinin.

Furthermore, the pumpkin protease can be classified by adding various types of inhibitors specific to different protease classes into the enzyme activity assay. The effect of metal ions on the enzyme activities are suggested to be studied in future researches as well.

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APPENDIX

APPENDIX A

Table A.1: Ammonium sulphate precipitation table.

Initial concentration of ammonium sulfate	Solid ammonium sulfate (grams) to be added to 1 liter of solution																				
0	106	134	164	194	226	258	291	326	361	398	436	476	516	559	603	650	697				
5	79	108	137	166	197	229	262	296	331	368	405	444	484	526	570	615	662				
10	53	81	109	139	169	200	233	266	301	337	374	412	452	493	536	581	627				
15	26	54	82	111	141	172	204	237	271	306	343	381	420	460	503	547	592				
20	0	27	55	83	113	143	175	207	241	276	312	349	387	427	469	512	557				
25		0	27	56	84	115	146	179	211	245	280	317	355	395	436	478	522				
30			0	28	56	86	117	148	181	214	249	285	323	362	402	445	488				
35				0	28	57	87	118	151	184	218	254	291	329	369	410	453				
40					0	29	58	89	120	153	187	222	258	296	335	376	418				
45						0	29	59	90	123	156	190	226	263	302	342	383				
50							0	30	60	92	125	159	194	230	268	308	348				
55								0	30	61	93	127	161	197	235	273	313				
60									0	31	62	95	129	164	201	239	279				
65										0	31	63	97	132	168	205	244				
70											0	32	65	99	134	171	209				
75												0	32	66	101	137	174				
80													0	33	67	103	139				
85														0	34	68	105				
90															0	34	70				
95																0	35				
100																	0				

Adapted from "Data for Biochemical Research" (R.M.C. Dawson, D.C. Elliott, and K.M. Jones, eds.), 2nd Ed. Oxford Univ. Press, London, 1969.

APPENDIX B

Equation to convert of tyrosine in μg/mL to μmol/mL is shown as:

Concentration of tyrosine released (μmol/mL)

$$= \frac{\text{Concentration of tyrosine released } (\frac{\mu g}{mL})}{\text{Molecular weight of tyrosine}}$$

$$= \frac{\text{Concentration of tyrosine released } (\frac{\mu g}{mL})}{181.16 \frac{g}{mol}} \quad \text{.....Equation (5)}$$

Table B.1: Specific activity of enzyme bromelain on sodium caseinate at 37 ℃.

pH	Absorbance	Concentration of tyrosine released (μg/mL)	Concentration of tyrosine released (μmol/mL)	Relative activity (U/mL)	Specific activity (U/mg)
4	0.477	66.2500	0.3657	0.0670	0.6703
5	0.503	69.8611	0.3856	0.0707	0.7069
6	0.601	83.4722	0.4608	0.0845	0.8446
7	0.652	90.5556	0.4999	0.0916	0.9163
8	0.553	76.8056	0.4240	0.0777	0.7771
9	0.486	67.5000	0.3726	0.0683	0.6830

Remarks: Concentration of enzyme bromelain is 0.1 mg/mL.

Table B.2: Specific activity of enzyme bromelain on sodium caseinate at pH 7.

Tempera-ture	Absorb-ance	Concentration of tyrosine released (µg/mL)	Concentration of tyrosine released (µmol/mL)	Relative activity (U/mL)	Specific activity (U/mg)
30	0.403	55.9722	0.3090	0.0566	0.5663
40	0.724	100.5556	0.5551	0.1017	1.0174
50	0.857	119.0278	0.6570	0.1204	1.2043
60	0.832	115.5556	0.6379	0.1169	1.1692
70	0.739	102.6389	0.5666	0.1039	1.0385

Remarks: Concentration of enzyme bromelain is 0.1 mg/mL.

Table B.3: Specific activity of enzyme bromelain on haemoglobin at 37 ℃.

pH	Absorbance	Concentration of tyrosine released (µg/mL)	Concentration of tyrosine released (µmol/mL)	Relative activity (U/mL)	Specific activity (U/mg)
4	0.157	21.8056	0.1204	0.0221	0.2206
5	0.163	22.6389	0.1250	0.0229	0.2291
6	0.177	24.5833	0.1357	0.0249	0.2487
7	0.204	28.3333	0.1564	0.0287	0.2867
8	0.184	25.5556	0.1411	0.0259	0.2586
9	0.167	23.1944	0.1280	0.0235	0.2347

Remarks: Concentration of enzyme bromelain is 0.1 mg/mL.

Table B.4: Specific activity of enzyme bromelain and haemoglobin at pH 7.

Tempera-ture	Absorb-ance	Concentration of tyrosine released (µg/mL)	Concentration of tyrosine released (µmol/mL)	Relative activity (U/mL)	Specific activity (U/mg)
30	0.148	20.5556	0.1135	0.0208	0.2080
40	0.253	35.1389	0.1940	0.0356	0.3555
50	0.536	74.4444	0.4109	0.0753	0.7532
60	1.016	141.1111	0.7789	0.1428	1.4278
70	0.627	87.0833	0.4807	0.0881	0.8811

Remarks: Concentration of enzyme bromelain is 0.1 mg/mL.

Table B.5: Specific activity of pumpkin protease crude extract on sodium caseinate at 37 ℃.

pH	Absorbance	Concentration of tyrosine released (µg/mL)	Concentration of tyrosine released (µmol/mL)	Relative activity (U/mL)	Specific activity (U/mg)
4	0.517	71.8056	0.3964	0.0728	0.2407
5	0.572	79.4444	0.4385	0.0804	0.2659
6	0.634	88.0556	0.4861	0.0891	0.2947
7	0.532	73.8889	0.4079	0.0748	0.2473
8	0.504	70.0000	0.3864	0.0708	0.2343
9	0.484	67.2222	0.3711	0.0680	0.2250

Remarks: Concentration of pumpkin protease crude extract is 0.3023 mg/mL.

Table B.6: Specific activity of pumpkin protease crude extract on sodium caseinate at pH 6.

Tempera-ture	Absorb-ance	Concentration of tyrosine released (µg/mL)	Concentration of tyrosine released (µmol/mL)	Relative activity (U/mL)	Specific activity (U/mg)
30	0.504	70.0000	0.3864	0.0708	0.2216
40	0.525	72.9167	0.4025	0.0738	0.2308
50	0.573	79.5833	0.4393	0.0805	0.2520
60	0.630	87.5000	0.4830	0.0885	0.2770
70	0.574	79.7222	0.4401	0.0807	0.2524

Remarks: Concentration of pumpkin protease crude extract is 0.3196 mg/mL.

Table B.7: Specific activity of pumpkin protease crude extract on haemoglobin at 37 ℃.

pH	Absorbance	Concentration of tyrosine released (µg/mL)	Concentration of tyrosine released (µmol/mL)	Relative activity (U/mL)	Specific activity (U/mg)
4	0.383	53.1944	0.2936	0.0538	0.2033
5	0.419	58.1944	0.3212	0.0589	0.2224
6	0.510	70.8333	0.3910	0.0717	0.2707
7	0.572	79.4444	0.4385	0.0804	0.3036
8	0.547	75.9722	0.4194	0.0769	0.2903
9	0.537	74.5833	0.4117	0.0755	0.2850

Remarks: Concentration of pumpkin protease crude extract is 0.2648 mg/mL.

Table B.8: Specific activity of pumpkin protease crude extract on haemoglobin at pH 7.

Tempera-ture	Absorb-ance	Concentration of tyrosine released (µg/mL)	Concentration of tyrosine released (µmol/mL)	Relative activity (U/mL)	Specific activity (U/mg)
30	0.502	69.7222	0.3849	0.0705	0.2230
40	0.542	75.2778	0.4155	0.0762	0.2408
50	0.566	78.6111	0.4339	0.0795	0.2515
60	0.530	73.6111	0.4063	0.0745	0.2355
70	0.513	71.2500	0.3933	0.0721	0.2279

Remarks: Concentration of pumpkin protease crude extract is 0.3163 mg/mL.

Table B.9: Specific activity of pumpkin protease partial purified extract on sodium caseinate at 37 ℃.

pH	Absorbance	Concentration of tyrosine released (µg/mL)	Concentration of tyrosine released (µmol/mL)	Relative activity (U/mL)	Specific activity (U/mg)
4	0.060	8.3333	0.0460	0.0084	0.0380
5	0.067	9.3056	0.0514	0.0094	0.0425
6	0.079	10.9722	0.0606	0.0111	0.0501
7	0.069	9.5833	0.0529	0.0097	0.0437
8	0.044	6.1111	0.0337	0.0062	0.0279
9	0.027	3.7500	0.0207	0.0038	0.0171

Remarks: Concentration of pumpkin protease partial purified extract is 0.2217 mg/mL.

Table B.10: Specific activity of pumpkin protease partial purified extract on sodium caseinate at pH 6.

Tempera-ture	Absorb-ance	Concentration of tyrosine released (µg/mL)	Concentration of tyrosine released (µmol/mL)	Relative activity (U/mL)	Specific activity (U/mg)
30	0.063	8.7500	0.0483	0.0089	0.0436
40	0.075	10.4167	0.0575	0.0105	0.0519
50	0.078	10.8333	0.0598	0.0110	0.0540
60	0.089	12.3611	0.0682	0.0125	0.0616
70	0.054	7.5000	0.0414	0.0076	0.0374

Remarks: Concentration of pumpkin protease partial purified extract is 0.2031 mg/mL.

Table B.11: Specific activity of pumpkin protease partial purified extract on haemoglobin at 37 ℃.

pH	Absorbance	Concentration of tyrosine released (µg/mL)	Concentration of tyrosine released (µmol/mL)	Relative activity (U/mL)	Specific activity (U/mg)
4	0.034	4.7222	0.0261	0.0048	0.0216
5	0.044	6.1111	0.0337	0.0062	0.0279
6	0.049	6.8056	0.0376	0.0069	0.0311
7	0.072	10.0000	0.0552	0.0101	0.0456
8	0.053	7.3611	0.0406	0.0074	0.0336
9	0.048	6.6667	0.0368	0.0067	0.0304

Remarks: Concentration of pumpkin protease partial purified extract is 0.2217 mg/mL.

Table B.12: Specific activity of pumpkin protease partial purified extract on haemoglobin at pH 7.

Tempera-ture	Absorb-ance	Concentration of tyrosine released (µg/mL)	Concentration of tyrosine released (µmol/mL)	Relative activity (U/mL)	Specific activity (U/mg)
30	0.042	5.8333	0.0322	0.0059	0.0291
40	0.050	6.9444	0.0383	0.0070	0.0346
50	0.072	10.0000	0.0552	0.0101	0.0498
60	0.057	7.9167	0.0437	0.0080	0.0394
70	0.036	5.0000	0.0276	0.0051	0.0249

Remarks: Concentration of pumpkin protease partial purified extract is 0.2031 mg/mL.