ISOLATION OF KISTOMIN MILK COAGULANT FROM *CALLOSELASMA RHODOSTOMA* VENOM

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ABSTRAK

Renet adalah enzim yang digunakan dalam proses penggumpalan susu untuk pembuatan keju. Enzim renet dihasilkan daripada perut binatang yang sedang menyusu terutamanya anak lembu. Namun, disebabkan peningkatan permintaan keju yang semakin meningkat bersama-sama dengan penyelidikan berterusan untuk produk keju yang inovatif telah menggalakkkan para penyelidik untuk meneroka sumber dan alternatif enzim yang baru untuk proses koagulasi susu. Sumber protease baru daripada haiwan, tumbuhan, microb dan kulat telah diterokai. Bisa ular adalah antara salah satu sumber yang kaya dengan protease enzim dan sumber ini perlu diterokai. Objektif kajian kali ini adalah untuk menyaring sebelas bisa ular daripada pelbagai spesis untuk aktiviti penggumpalan susu, mengasingkan protease enzim daripada spesis bisa ular yang mempunyai keupayaan untuk aktiviti penggumpalan susu menggunakan teknik kromatografi, menentukan parameter optimum enzim dan membandingkan dengan aktiviti enzim protease komersial. Hasilnya menunjukkan, bisa ular daripada *Calloselasma rhodostoma* memberikan masa penggumpalan susu yang paling cepat iaitu 2.83±0.17 minit dan telah dipilih untuk penulenan selanjutnya. Enzim koagulasi susu telah ditulenkan dengan menggunakan kromatografi turus pertukaran ion *HiTrap SP FF* dan telah dipisahkan lagi dengan menggunakan turus ruang pengasingan saiz *HiPrep 26/60 Sephacryl S 200 HR*. Selepas proses penulenan, aktiviti enzim terhadap penggumpalan susu telah meningkat sebanyak 4.41±0.06 kali ganda dan mempamerkan pemulihan aktiviti sebanyak 54.34 %. Analisis SDS-PAGE menunjukkan jalur tunggal dengan jisim molekul kira-kira 26 kDa. Aktiviti protease yang telah ditulenkan telah direncat sepenuhnya oleh EDTA dan 1,10 phenanthroline, mendedahkan bahawa protease ini adalah daripada keluarga protin metalloprotease SVMP P-1 Kistomin dan telah disahkan oleh analisis Spektrometri Jisim. Aktiviti spesifik kistomin adalah 62.20 (U/mg). Aktiviti koagulasi susu (MCA) dibawah keadaan optimum adalah 810.44±42.45 (SU/mL) dan aktiviti proteolisis (PA) kistomin adalah 1.39±0.01 (U/mL), memberikan nilai nisbah yang tinggi untuk MCA/PA iaitu 585.05. Aktiviti koagulasi kistomin pada susu yang tertinggi pada kepekatan enzim 0.76 mg/mL, 8 % (w/v) kepekatan kalsium klorida, suhu 48 °C dan stabil pada julat pH yang luas pada puncak aktiviti pH 6.5. Penambahan Ba^{2+}, Mn^{2+} dan Ca^{2+} ion sebagai kofaktor telah meningkatkan aktiviti kistomin dengan ketara, namun aktiviti kistomin terencat dengan penambahan Hg^{2+}, Pb^{2+} dan Fe^{2+} ion. Nilai K_m kistomin pada kasein ialah 1.153±0.08 mg/mL. Nilai K_m yang rendah oleh kistomin terhadap kasein menunjukkan sifat afiniti tinggi kistomin terhadap kasein. Kistomin menunjukkan belahan terhadap kappa kasein dengan tinggi dan menunjukkan belahan yang rendah terhadap beta casein. Dari kajian ini, dapat disimpulkan bahawa kistomin berpotensi untuk menjadi koagulan dalam industri tenusu. Namun disebabkan sumber protease dari bisa ular yang dianggap bahaya oleh kebanyakan pendapat, kajian yang berterusan terhadap keselamatan pengunaannya adalah disyorkan.

ABSTRACT

Rennet is an enzyme used in the milk clotting process for cheese production. Rennet is produced in the stomach of ruminant mammal especially calf. However, increasing demand for cheese with ongoing research for innovative cheese products has encouraged the researchers to explore the other new sources of milk clotting protease. The new sources of milk clotting protease include animal, plant, microbes and fungi have been investigated. Snake venom contains a rich and good source of protease that needs to be explored as well for its potential in milk clotting. The objectives of this study were to screen eleven different species of snake venoms, to isolate the protease enzyme from the most potential venom capable to coagulate milk using chromatography techniques, to determine the enzymes milk clotting parameters and compare with a commercial milk clotting protease. The results revealed that a crude venom from *Calloselasma rhodostoma* showed the fastest clotting time that is 2.83±0.17 minutes and was chosen for further purification process. The milk clotting enzyme was purified by HiTrap SP FF ion-exchange chromatography and further separated using HiPrep 26/60 Sephacryl S 200 HR size exclusion column. It was purified 4.41±0.06 fold and exhibited recovery activity of 54.34 %. SDS-PAGE analysis indicated a single band with a molecular mass of approximately 26 kDa. The purified protease was completely inhibited by EDTA and 1,10 phenanthroline revealing to be a metalloprotease SVMPs P-I Kistomin confirmed by Mass Spectrometry analysis. The specific activity of kistomin is 62.20 (U/mg). The milk clotting activity (MCA) of kistomin under optimum conditions, was 810.44±42.45 (SU/mL) and the PA of kistomin was 1.39±0.01 (U/mL), resulted in high ratio of MCA/PA value of 585.05. The clotting activity of kistomin on milk was the highest at 0.76 mg/mL kistomin concentration, 8 % (w/v) of calcium chloride concentration, temperature of 48 °C and stable over wide range of pH 5-7 with the peak of pH 6.5. The addition of Ba^{2+}, Mn^{2+} and Ca^{2+} ions as cofactor significantly increased the enzyme activity but inhibited by Hg^{2+}, Pb^{2+} and Fe^{2+} ions. The K_m value of kistomin on casein is 1.153±0.08 mg/mL. The low K_m value of kistomin on casein showed it has high affinity to casein. Kistomin promoted extensive cleavage of kappa casein and low level of beta casein hydrolysis. From this preliminary study, it can be concluded that kistomin has potential to be a coagulant in the dairy industry. However, due to the source of the protease being from a snake and always been considered lethal by people in general, a thorough safety investigation warranted before been utilised in any industry.

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LIST OF SYMBOLS

α	Alpha
β	Beta
κ	Kappa
K_m	Michaelis-Menten
V_{max}	Maximum velocity
S	Substrate concentration
V	Velocity
°C	Degree celcius
w/v	Weight solute per volume solution
v/v	Volume solute per volume solution
kDa	Kilo Dalton
µL	Microlitre
mM	Millimolar
MPa	Megapascal pressure unit
pI	Isoelectric point
M	Molar
rpm	Rotation per minute
R*f*	Relative migration distance
SU	Sohxlet Unit
mg	Milligram

LIST OF ABBREVIATIONS

PLA_2	Phospholipase A_2
MCE	Milk clotting enzyme
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
MCA	Milk clotting activity
PA	Proteolytic activity
TLC	Thrombin-like enzyme
SVMP	Snake venom metalloproteinases
MCP	Milk clotting properties
LAB	Lactic acid bacteria
BCA	Bicinchoninic acid assay
rpm	Rotation per minute
CV	Column volume
MWCO	Molecular weight cut-off
UV	Ultraviolet
2DE	Two-dimensional gel elctrophoresis
sp.	species
PMSF	Phenylmethylsulfonyl fluoride
EDTA	Ethylenediaminetetraacetic acid
MW	Molecular weight
HCl	Hydrochloric acid

CHAPTER 1

INTRODUCTION

1.1 Introduction

A worldwide shortage of rennet for cheese production has existed for a few decades. Since the 18th century, the cheesemakers have used other organic materials to coagulate milk when they were short on rennet. Increased sales from fast food segment, growth by region and customer-driven in nutritional cheese, the global cheese market to exceed 100 billion dollars by 2019 (Hal Conick, 2016). In addition, the increased demand for cheese coagulant especially from natural sources led to a growing necessity for rennet substitutes which promote a search for new sources of protease with coagulant properties. Currently, the researchers are trying to find the solution for rennet scarcities by exploring the new sources and increase the new coagulant effectiveness using genetic engineering technology. In this issue, the original rennet extracted from calf has been genetically engineered (chymosin) to fulfil the demand of dairy industries that rapidly growing every year. As the amino acid composition and kinetic properties of chymosin and pepsin have been extensively studied. There is more or less agreement of opinion in favour of chymosin as the enzyme in cheese making; presently three companies that producing calf chymosin through recombinant DNA technology and the cloned chymosin preparations produced by different microorganisms have been tested for cheese manufacture in various country (Garg & Johri, 1994).

Abundance of proteolytic enzymes can induce milk clotting and these proteases result not only from its ability to clot the milk but also from the relationship between milk clotting ability and the general proteolysis which enzyme may produce (Tavares et al., 1997). Milk clotting can be facilitated by inducing micelle aggregation; by enzymatic action (cheese) or also by adding acid (form fermented milk) (Hallen, 2008). In addition, previous studies have found a similarity between the clotting of blood and milk. Jolles and Henschen (1982) compared the clotting of blood and milk and found the common

features of the blood and milk clotting process. Snake venom are renowned for its ability to coagulate blood.

There are about 600 species of venomous snakes out of total 3000 snake species that exist on earth. The venomous species are categorized into major families i.e. the Elapidae, Viperidae, Crotalidae, Hydrophidae and Colubidae. The venomous snakes are described as poisonous which immobilize their prey via their fangs by injecting the venom (modified saliva) that contains toxins into their prey tissues. Aside from hunting, immobilizing and digesting their prey, they also used their venoms for self-defence and will bite people who startle, provoke and threaten them. Snake venoms are synthesized and stored in the venomous gland which is secretion of a venomous snake (Goswami et al. 2014). Venom consists of different proteins, peptides and enzymes which generally not dangerous when ingested by mouth. The complex mixture of enzymatic and toxic protein in snake venoms which includes phospholipase A_2 (PLA_2s), myotoxins, hemorrhagic metalloproteinases. In addition, proteolytic enzymes, coagulant components, cardiotoxins, cytotoxins and neurotoxins also present in the venom (Kini, 2003). The proteins and peptides fill up to 90 to 95 percent of the dry weight of venom. If ingested in liquid or crystal form after drying through mouth, the snake venom is harmless and it is only toxic if contact with blood (Vyas et al., 2013).

Despite venom being lethal as a whole in a mixture of protein, however once isolated its entities shown to be useful for mankind. Human can share benefits from snake venom for it have been proven snake venom contributed in many fields of pharmacology, toxicology and clinical field. There are three families of snakes (Colubridae, Elapidae and Viperidae) which show medical significance found in Malaysia. Majority of snakes found in Malaysia are non-venomous and possess no threat to humans. Researchers had studied the benefit of the snake venom especially in pharmacology and clinical field. It has been proved that snake venom contained a defibrinating agent, anti-cancer properties (Zhou et al. 2000), HIV treatment, anti-hypersensitive activities, treatment for Alzheimer (Lacoste et al., 2011), skin diseases (eczema, scleroderma, psoriasis) etc.

Nowadays, potential products based on snake venom has been produced and commercialized. Captopril has been long known for its service of saving millions of humans lives through its anti-hypersensitive activities. This innovation not only results in advance of potential human therapeutic agents, yet the discovery of captopril which

creates a new class of anti-hypersensitive drug (McCleary & Kini, 2013). Malayan pit viper contributes to widely held snake envenomation drugs. The prominent thrombin like enzyme from *C. rhodostoma* known as Arvin or Ancrod has been useful as defibrinating agent clinically. It had been trialled as a clinical anticoagulant (a common brand called Viprinex).

C. rhodostoma locally known as Malayan pit viper has been chosen as one of the venoms for causing clotting effects of milk. The screening for clotting property of *C. rhodostoma* snake venom shows that this snake venom has the potential to clot milk. It has been reported that enzyme present in snake venom hydrolyse proteins and membrane component which lead to blood clotting. *C. rhodostoma* crude venom has a dual effect on blood clotting; at particularly low concentration, it is procoagulant while at higher dosage it acts as an anticoagulant. The fibrinogen clotting enzymes found in snake venom sample are the most frequent type of blood clotting activator, they are well known among members of the family Viperidae (Viperinae and Crotalinae).

1.2 Problem statement

Since early times, dairy products such as yogurt and cheese have been produced by the addition of stomach extract from the new-born ruminants. The exact contributor of the conversion of milk to coagulated milk was uncertain for some time. However due to advance technology, it was identified as rennet or more precisely known as chymosin, a protease capable to cleave specifically the milk constituent e.g. kappa casein from soluble to curd (a non-soluble form of micelle). Since then, rennet has been commercialized as an industrialized enzyme to produce coagulated dairy products. Though within cottage industries the use of calf and other animal's (even the controversial pig's rennet) stomach extracts still been used. However, currently genetically engineered rennet is being chosen due to the huge magnitude of the dairy industry.

To be competitive over the lucrative commercial enzyme market i.e. increases demand in fast food market etc, manufacturers have been genetically modifying rennet to enhance its capacity to be more productive or/and achieve desired trait or stability. Nevertheless, recent possibility to modify the gene of the rennet has been limited or exhausted to a point researcher resorting to search in various microbes or other animal sources for a protease with milk clotting capabilities. Such has been the extend for the

latter sources that other than the commonest ones, some have been very peculiar rennet substitutes including codfish, dogfish, cat, camel, buffalo, lamb, kid, swine, chicken, seal, red crab and etc.

It is certain that the quest of a more productive protease within the rich animal biodiversity does not end as long as there is a market for milk coagulant. Hence, with the knowledge of snake venom that contains multitude of useful protein for the benefit of mankind (especially in medical and pharmacological field), similarly an effort has been carried to investigate its potential in deriving a protease in this study. Certain viper venom been proven to be haemolysin, which is capable to coagulate blood very well. Such correlation may as well lead to milk clotting and as of now none reported with such potentials. *C. rhodostoma* (Malayan Pit Viper) in our preliminary investigations managed to coagulate milk and therefore sets the catalysis for snake venoms to be studied for its milk clotting activities.

1.3 Objectives

1. To screen different species of eleven venoms obtained from the most abundant snakes in Malaysia, for milk clotting activities.

2. To isolate the relevant protease enzyme from the most potential venom capable to coagulate milk using chromatography techniques.

3. To characterize enzymes milk clotting parameters and compare with a commercial milk clotting protease.

1.4 Scope of research

In this project, the milk clotting ability of different species of eleven snake venoms was investigated. The eleven snake venoms are *Calloselasma rhodostoma, Trimeresurus purpureomaculatus, Trimeresurus wagleri, Trimeresurus sumatranus, Naja sputatrix, Bungarus candidus, Bungarus fasciatus, Naja kaouthia, Naja nivea, Ophiophagus hannah and Trimeresurus hageni*. The venoms were examined into their protein content using Bradford protein determination method. The milk clotting end point was determined by observing the clot formation after addition of crude venom and a drop of samples was observed under light microscope. The venom that have the most rapid

clotting activity was selected for further fractionation and purification using ion exchange chromatography and size exclusion chromatography.

The proteases from the most promising venom were isolated to single entity using bioassay guided purification procedures involving ion exchange chromatography initially, followed by size exclusion chromatography. In ion exchange chromatography, the protein fraction was eluted with linear gradient using 1.0M NaCl in 50mM ammonium acetate (pH 6). Then were further eluted using size exclusion chromatography using 50mM ammonium acetate (pH 7). The collected fractions in every chromatography steps were screened for the clotting activity. Sequentially, the purity of active protease or/and fraction obtained from each chromatography steps were analysed using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) until the purified protease was obtained. The pure protease was identified using LCMS/MS.

Next, after the protease identity was known, two assays of protease activity were conducted which are Milk Clotting Activity (MCA) and Proteolytic Activity (PA). Milk Clotting Activity (MCA) of isolated protease was determined using skim milk as a substrate and casein as a substrate for Proteolytic Activity (PA) of isolated protease. The optimum parameters of the protease such as optimum temperature, pH, $CaCl_2$ concentration and isolated enzyme concentration for milk clotting were determined. In addition, the effect of protease inhibitors and metal ions was evaluated. The kinetic parameters of isolated protease such as K_m and V_{max} values were determined using proteolytic activity assay which was calculated using GraphPad Prism 7 software.

1.5 Significance of research

It is already well known that snake venom is proven to be contributed in many fields such are clinical, toxicology and pharmacology field. It is expected many other applications of snake venom is yet to be discovered. As the preliminary studies of snake venom were found able to coagulate milk, therefore produces a novel discovery linking venom protein for a newer application which is in the dairy industry. This work not only investigated a newer application for snake venom but also provided potentially a more productive catalyst for the food industry. The current study expected to tap into the less ventured animal source instead i.e. snakes for useful protease. As the demand for cheese has increased, it may generate income to Malaysia if able to isolate the protease and

investigated for its efficacy and safety bettering existing commercial coagulants. However, prior to being able to reach commercial standard, the protease been isolated needed to be studied comprehensively on its capability and safety. Alternatively, instead of sourcing from snake venom the protease can be genetically engineered in microbes. This is so as much safety stigma exist on the use of snake venom for biotechnological purposes and on its limited yield from snakes. This study similar to the many preliminary study on protein in snake venom intends to introduce to biotechnologist the usefulness of protease that eventually can be studied in stages until commercialization.

CHAPTER 2

LITERATURE REVIEW

2.1 Milk clotting properties

2.1.1 Protein composition in milk

Milk is made up of 3-4 % total protein and its protein consists of all the 9 essential amino acids required by humans. Milk proteins are synthesized in the mammary gland, but 60 % of the amino acids used to build the proteins are obtained from the cow's diet. Total milk protein content and amino acid composition varies with cow breed and individual animal genetics. There are 2 major categories of milk protein that are broadly defined by their chemical composition and physical properties. The casein family contains phosphorus and will coagulate at pH 4.6. In cow's milk, approximately 80 % of milk protein is casein and the remaining 20 % is serum, or whey protein. Like the other major milk components, each whey protein has its own characteristic composition and variations.

Whey proteins do not contain phosphorus, by definition, but do contain a large amount of sulfur-containing amino acids. These form disulfide bonds within the protein causing the chain to form a compact spherical shape. The disulfide bonds can be broken, leading to loss of compact structure, a process called denaturing. Denaturation is an advantage in yogurt production because it increases the amount of water that the proteins can bind, which improves the texture of yogurt. This principle is also used to create specialized whey protein ingredients with unique functional properties for use in foods. One example is the use of whey proteins to bind water in meat and sausage products (Hui and Chandan, 2006).

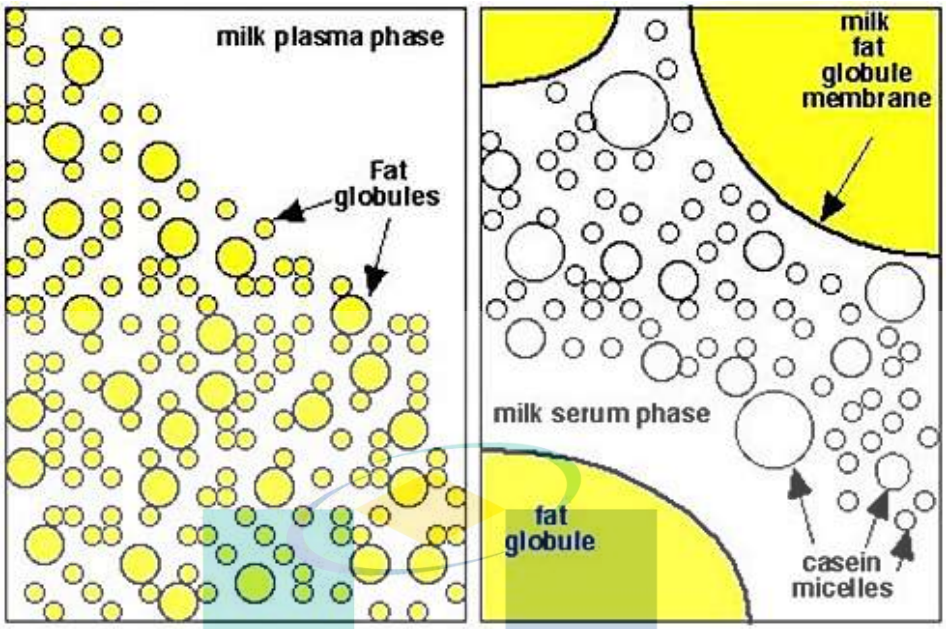


Figure 2.1 Illustration of emulsion milk fat globules (left) in a partially stable emulsion in plasma phase (skim milk) and casein micelles in a colloidal suspension in the serum phase of milk (whey).

Source: Hurley (2014)

2.1.2 Milk clotting

Milk clotting is the clotting process from milk (liquid) into curd (gel-like) formation. Milk clotting is always the first step in cheese making process. Herbert et al. (1999) stated that clotting of milk will be the primary step in most of the dairy products such as cheese and yogurt for their texture development. Since the milk clotting properties significantly affect the yield and quality of dairy products, they are getting more and more attention in recent years (Tabayehnejad et al., 2012). Milk clotting can be caused by adding rennet enzymes or altering the acidity of milk to the isoelectric point of casein, which is approximately pH 4.6 (Kindstedt, 2011).

Other than cheese making industry, milk clotting is essential for tofu making. The clotting process of soy milk is an important step in tofu making industry (Li et al., 2011). It was reported that tofu is a traditional food that is widely consumed in East Asian countries due to its rich nutrition as well as its health benefits. There are approximately 90 % of proteins in soy milk are glycinin and β-conglycinin. These major storage proteins are involved in clotting step of soy milk into tofu.

2.1.2.1 Enzyme-induced milk clotting

Enzyme-induced clotting of milk is done by the rennet enzymes. According to Hyslop (2003), this milk clotting process using enzymes can be generally divided to three steps: activation of milk clotting particles (casein micelles), aggregation of enzyme-modified casein micelles as shown in Figure 2.2 and lastly is the modification for the properties and structure of the coagulum. During the first step, the rennet enzymes play role in shaving off the segments on the micelle surfaces and thus exposing the micelle cores.

The exposed micelle cores are hydrophobic in nature and aggregate to form gel-like curd. During the aggregation of casein micelles, concentration of calcium ions plays an important role. Calcium ions reduce the electrostatic resistance of micelles by neutralizing the surface charge on casein micelles (Landfeld et al., 2002), hence promoting the casein micelles aggregation. Besides, it was reported that addition of calcium ions reduced the milk clotting time (Sandra et al., 2012).

The rennet obtained from calf contains more than 90 % of chymosin which is responsible for the milk clotting of milk to curd in dairy products industry (Kumar et al., 2010). Chymosin is a protease secreted in the stomach of young ruminants like calves and lambs. With the increase demands of rennet enzymes, other possible alternative sources of rennet such as the sources from plants, animals and microorganisms are studied and exploited. The advanced technology of recombinant DNA permits the cloning of chymosin using microorganisms (Hyslop, 2003). In instances, the protein of interest is scarce. Additionally, genetic engineering can be employed to enhance the protein stability and modulate the activity (Kamionka, 2011).

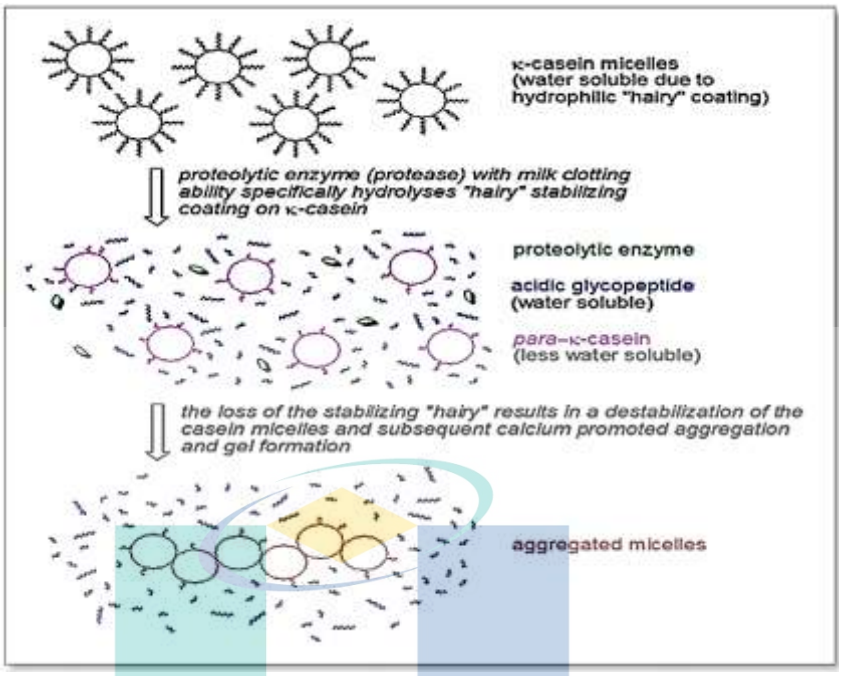


Figure 2.2 Activation of casein micelles using enzymes and aggregation of enzyme modified casein micelles.

Source: Lersch (2014)

2.1.2.2 Acid-induced milk clotting

Shaker et al. (2000) mentioned that addition of acid on milk clotting removes the calcium bonds between casein micelles and thus destabilizes the micelles and followed by forming curd due to aggregation. Acid-induced milk clotting is usually done by acidify the milk to pH below 4.6 by using hydrochloric acid (HCl). The addition of acid will reduce the negative charge repulsion between the casein micelles which will then lead to the aggregation. In nature, the negative charge of casein micelles prevents the micelles aggregation. The acidification results in the physicochemical properties change of casein micelles, including salvation and dissociation of caseins at the pH range of 5.5 to 5.0 (Phadungath, 2005). Based on the studies conducted, the caseins are liberated into the serum phase and thus the aggregation of caseins happen when the isoelectric point which is around pH 4.6 is reached.

2.2 New sources of rennet

In the last decade, there has been an increased demand for cheese consumption and production due to the population explosion (Tajalsir et al., 2014). According to new report from Transparency Market Research, the global cheese market to hit 118 billion

dollars by 2019 which driven by consumer driven in nutritional cheese (Natural product insider, 2014). This combined with the reduced quantity of natural calf rennet, elevated price of calf rennet (Ahmed et al., 2010), restricted use of animal rennet for certain religion (e.g. Islam, Hinduism and Judaism), diets (vegetarianism), safety (bovine spongiform encephalopathy) and activist against genetically engineered foods (Badgujar and Mahajan, 2014). All these reasons have demanded the search for the new enzyme that can be used as a rennet substitute. Consequently, a scientist has been focused on the discovering of milk-clotting enzymes from other sources such as animal, plant and microbial.

2.2.1 Animal

Besides bovine chymosin, camel chymosin is aspartic peptidases that are industrially used in cheese production and both cleave the Phe_{105}-Met_{106} bond of the milk protein kappa casein. Camel chymosin has 85 % sequence identity with bovine chymosin. Despite that, the camel chymosin shows 70 % higher milk clotting activity than that of bovine chymosin towards bovine milk. This is because both bovine and camel chymosins possess local positively charged patches on their surface that play the role in interaction with negatively charged C-terminal of κ-casein, however, the camel chymosin contains two additional positives patches that favours the interaction with the substrate. Variation in the surface charges and the greater malleability both in domain movements and substrate binding improved the electrostatic interactions thus contribute to the better milk clotting activity of camel chymosin towards bovine milk (Langholm Jensen et al., 2013).

Traditionally, the rabbit rennet has been used in the manufacturing of highly preferred cheese in the south-east region of Anatolia. Due to its superior taste and properties in storage period such as low bitterness and softening, this type of cheese is demanded and consumed admiringly by locals. The latest research by Alihanoglu et al. (2018) on *Orycyolagus cuniculus* (rabbit) rennet shows that the rabbit rennet has a comparable cheese-making performance with camel chymosin and could be a good alternative for calf chymosin. The other coagulant extracted from an animal that has been investigated are tuna fish (Tavares et al., 1997), turkey (Mekhaneq et al., 2018) and pepsin enzyme from bovine, porcine (Fox, 1969) and chicken (Gordin & Rosenthal, 1978).

2.2.2 Plant sources

The search for substitutes of milk clotting enzyme from plant has escalated as the use of traditional animal rennet is limited for dietary (vegetarianism) or religious (Judaism, Hinduism and Islam) reasons and the extraction of calf rennet involve several steps which making the enzyme supply expensive and laborious. Since ancient times, the vegetables extracts have been used as coagulant in cheese making (Puglisi et al., 2014), although their mode of action is relatively unknown (Bey et al., 2018). Over time, the cheeses made with plant coagulant spreads to many regions to the world mainly in west African, Mediterranean and Southern European countries (Roseiro et al., 2003). The application of plants in cheese industries still limited because they produce cheeses that has extremely bitter tastes and poor textual characteristics (Puglisi et al., 2014). This is due to high level of proteolytic activity of plant proteases, which inducing the production of short peptides that effect both the flavour and texture of the cheese (Oner & Akar, 1993). Moreover, most of these plants has high toxin content which constrains their use in food industry (Silva & Malcata, 2005). However, the extracts of *Cynara cardunculus* represent the exception as the extracted enzyme mixture is commonly utilized in Spain and Portugal for the production of soft-bodied cheeses from ovine milk (Roseiro et al., 2003), as the excessive bitterness in cheese can be avoided by better quantity control in cheese making (Galán et al., 2012).

Proteolytic enzymes that possessed milk clotting activity extracted from plants, such as *Lactuca sativa* (Lo Piero et al., 2002), *Streblus asper* (Tripathi et al., 2011), *Solanum elaeagnifolium* (Gutierrez-Mendez et al., 2012), Kiwi (Grozdanovic et al., 2013; Puglisi et al., 2014), *Moringa oleifera* (Pontual et al., 2012; Tajalsir et al., 2014), globe artichoke (Llorente et al., 2004), sunflower and albizzia seeds (Egito et al., 2007). Many of proteolytic enzyme that have been studied for milk clotting activity belong to acidic protease group (e.g. chymosin). These also include the commercially used microbial milk-clotting aspartic protease from *Mucor pusillus*, *Cryphonectria parasitica* and *Mucor miehei* (Grozdanovic et al., 2013). Aspartic protease with pH optimum of 5.0 and possessed a milk clotting activity have also been isolated from globe artichoke (Llorente et al., 2004). Puglisi et al. (2013) reported the application of kiwi fruit aqueous extract in the production of mozzarella cheese. The extract exhibits high levels of milk clotting activity and this probably due to the presence of enzyme actinidin in the mixture with

other proteolytic enzymes. In addition, no flavour defect was detected in fresh cheese made with kiwi extract.

2.2.3 Microbial and fungi

The worldwide reduced supply of calf rennet and increased of cheese consumption and production has stimulated the research for milk clotting enzyme especially from microbial and fungal sources as rennin substitutes. The attempts to use microbial and fungus milk clotting enzyme has been made by some researchers (Kumar et al., 2005; Silva et al., 2014). *Penicillium citrinum* was studied for their milk clotting activity and was cultured using different types of media cultures (Abdel-Fattah et al., 1972). In 1999, the production of extracellular milk-clotting enzymes by six fungal cultures included was investigated. The lists of the fungal strains used included *Aspergillus oryzae* NRRL 3486, *A. oryzae* NRRL 3483, *A. oryzae* NRRL 3488, *Tricoderma viride NRRL 24631, Penicllium oxalicum* and *Aspergillus terrus.* Static culture of eight days old *P. oxalicum* showed the most promising fungus as rennin producer which achieved the highest yield of milk clotting enzyme using sucrose (5 %) as carbon source and a mixture of yeast extract and peptone as nitrogen source (Hashem, 1999).

Protease from fungal have been widely investigated because of the enzymes that they secrete, in most cases extracellularly. The extracellular enzymes facilitate the process of extraction and recovery (Silva et al., 2014). In addition, it is cost effective, has fast production, the ease with which the enzymes can be modified, and mycelium can be easily removed by filtration (Souza et al., 2015). The other milk clotting enzyme from microbial and fungus that have been focused on for use as rennin substitutes includes *Aspergillus versicolor* (Abdel-Fattah & Saleh, 1979), *Nocardiopsis sp.* (Cavalcanti et al., 2004; Cavalcanti et al., 2005), *Penicillium expansum* (Mabrouk et al., 1976), *Basidiomycetes* (Kawai & Mukai, 1970), *Absidia* cylindrospora (Ismail et at., 1987), fungal strain of *Mucor miehei* ATCC 3420 (Ayhan et al., 2001) and *Bacillus subtilis natto* (Shieh et al., 2009; Wu et al., 2013). However, only milk clotting enzymes produced by strains of *Rhizomucor pusillus var. Lindt, Rhizomucor miehei, Enthothia parasitica* and *A. oryzae* are widely used (Thakur & Karanth, 1990; Crawford, 1985; Birkkjaer & Joehnk, 1985). The latest research is the milk clotting enzyme extracted from *Hercium*

erinaceum which can be used as an effective coagulant in cheesemaking (Sato et al., 2018).

Other than protease produces by bacteria and fungi, the cheese production with lactic acid bacteria (LAB) is effective, cheap and has a safe history application. LAB in the form of defined or undefined starter culture, produce the lactic acid and cause an acidification of milk, with the consequent decrease in pH value, thus affecting several aspects of the cheese manufacturing process and ultimately cheese composition and quality (Briggiler-Marcó et al., 2007; Kongo, 2013). *Lactococcus lactis* is a microbe classified as lactic acid bacteria because it ferments lactose to lactic acid. It has two subspecies which are *cremoris* and *lactis.* Both sub-species are essential in manufacture varieties of cheese and fermented milk products. The other LAB includes *Streptococcus thermophilus*, *Lactobacillus* and *Leuconostoc spp.* (Mahony et al. 2016).

2.3 Halal issue of dairy coagulant

The last two decade has seen the emergence of halal industry as one of the fastest growing sectors in Malaysia and by opening international restaurants in the 1970s, Malaysia has entered the global food market (Lever, 2013). The word halal means permissible, allowed and lawful and the reverse is haram (non-halal). In Islamic jurisprudence, a fatwa is the opinion of a scholar based on understanding of Islam, when there are some doubts whether a practice is permissible (halal) or prohibited (haram) in Islam. The Fatwa Committee in 1990 on cheese ruled that cheese as food ingredient must be produced using enzyme which is obtained from halal sources either from plants, fungi or slaughtered animals according to Islamic law, is halal (Asa & Azmi, 2018). Genetically modified organism in food and drink processed using DNA of halal sources or animal slaughtered according to Islamic law, is halal. However, the use of DNA from porcine sources in the production of food should not be allowed because there is no urgency in using DNA derived from porcine sources as there are plenty of other halal ingredients that can be used to replace the usage of such DNA (Asa & Azmi, 2018). In this research, the protein or enzyme that has the clotting activity on milk was isolated from snake venom. There is no specific fatwa on the use of venom protein or enzyme as rennet substitutes however, the Quran and Sunnah have already outlined the basic guidelines behind the permissibility and impermissibility of consumption of food items. Most reptiles including snake are considered as haram to consume. There is a fatwa on using

the venom or non-halal material as a medicine. It is permissible to take medicine made from non-halal materials under the supervision of experts whenever the pure one or the permissible sources are unavailable (Halib et al., 2017; Muhamad Rusdi et al., 2018). This view is confirmed taken into consideration the opinion of Shaf'i and Hanafi scholars (Halib et al., 2017). Based on the fatwa that has been discussed the use of isolated protein in snake venom as clotting agent in dairy industry is haram because there is no urgency and halal cheese coagulant sources such as rennet of animal origin which are slaughtered and enzyme extracted from plant, fungus and bacteria are available.

2.4 Similarities between blood and milk clotting

The final stage of blood clotting is the conversion of the soluble fibrinogen to the insoluble fibrin-clot which is induced by thrombin (Jollès & Henschen, 1982). This conversion occurs by the hydrolysis of the protease thrombin of two peptide bonds which are located in the Aα and Bβ chains of fibrinogen molecule (Kaye & Jollès, 1978). Meanwhile, for milk clotting, it was stated that κ-casein (casein fraction which acts as the substrate of chymosin) is the main protagonist in primary phase of milk clotting.

Kaye & Jollès (1978) suggested that there is possibility of similarities between the primary and secondary structures of fibrinogen and κ-casein molecules. It was also suggested that there is certain degree of homology may present between the Bβ and γ chains of human fibrinogen with cow κ-casein. According to Rutherfurd & Gill (2000), there are similarities of sequence between fibrinogen γ-chain and κ-casein. In addition, the structural and functional similarities between fibrinogen γ-chain C-terminal dodecapeptide and peptides from 106-116 region of κ-casein were mentioned.

The clotting of blood and milk process possesses common feature on the action of thrombin on fibrinogen and the action of chymosin on κ-casein. It was suggested that they are having similar mechanism for these enzymatic cleavages. Both processes are said to involve limited proteolysis where both use protease enzyme to hydrolyse specific linkages. It was stated that thrombin cleaves two R-G bonds to produce fibrin and fibrinopeptides while para-k-casein and glycomacropeptides were formed by cleaving a single unique F-M bond by chymosin (Rutherfurd & Gill, 2000).

2.5 Composition of venom

Meier and Stocker (1991) concluded that snake venoms are unique mixtures with their properties in biochemical and pharmacological. Most of the bioactive proteins and polypeptides (enzymatic and non-enzymatic) in snake venoms are responsible for their pharmacological properties (Mukherjee et al., 2013). These enzymatic and non-enzymatic proteins may be coagulant, anticoagulant, fibrinolytic or possess other effects in nature.

Other than bioactive molecules such as phospholipase A_2, metalloproteinases, peptidases, proteinases and L-amino acid oxidases, snake venoms also contain some inorganic cations like sodium, potassium and calcium as well as carbohydrates, lipids and free amino acids. Their compositions vary from venom to venom and it may be due to the difference or variation in age, origin, diet of the snakes. For example, Cobras typically possess neurotoxic venom while vipers' venoms mostly consist of hemotoxins (Clemetson & Clemetson, 2005).

Additionally, Kashani et al. (2012) said that snake venoms underwent evolution and became more specifically in facilitating snakes' hunting, immobilizing and digesting the prey animals. It was believed that snakes venoms had a function just to lubricate their food before undergoing evolution. According to Izidoro et al. (2014), as the time passed, snake venoms developed into more specialized mixture to help in their hunting and immobilization of prey.

With their complex composition, snake venom possesses various potential to be developed in different fields especially in medical field for improving the quality of environment and human life.

Table 2.1 Selected constituents found in snake venoms

Snake type	Venom composition	References
C. rhodostoma	C-type lectin, L-amino acid oxidase, metalloproteinase; P-I, P-II, P-III venom serine protease; Phospholipase A_2, Cysteine-rich secretory protein, Phospholipase B, Neurotrophin, Nerve growth factor, 5'Nucleotidase, Phosphodiesterase, Kinesin-like protein, Flavin monoamine oxidase, Aminopeptidase, glutaminyl-peptide cyclotransferase, ankyrin	Tang et al. (2016); Kunalan et al. (2018)
T. purpureomaculatus	5'-nucleotidase, protease, arginine ester hydrolase, arginine amidase, phospholipase A, acetylcholinesterase and alkaline phospomonoesterase	Tan & Tan (1988)
T. wagleri	Hyaluronidase, L-amino acid oxidase, phospholipase A, 5'-nucleotidase, protease, alkaline phosphomonoesterase, arginine ester hydrolaxe, phosphodiesterase, hemotoxin	Tan & Tan (1989); Reid et al. (1963)
T. sumatranus	L-Amino acid oxidase, Hyaluronidase, Phosphomonoesterase, 5'-Nucleotidase, Arginine ester hydrolase, Phosphodiesterase, Phospholipase A	Tan & Tan (1989)
N. sputatrix	Phospholipase A_2 postsynaptic neurotoxins and polypeptide cardiotoxins	Tan & Tan (1988); Tan (2004)
B. candidus	Low protease, 5'- nucleotidase, phosphodiesterase and alkaline phosphomonoesterase activities; moderately high in acid oxidase activity and high in phospholipase A, acetylcholinesterase and hyaluronidase activities; two polypeptide toxins, three finger toxins, L-amino acid oxidase and Kunitz-type inhibitors	Tan et al. (1989); Rusmili et al. (2014)

Table 2.1 Continued

Snake type	Venom composition	References
B. fasciatus	Neutrotoxins (presynaptic (beta) and postsynaptics (alpha) neurotoxins). serine proteases, L-amino acid oxidases, cholinesterases, metalloproteinases, PLA2s, 3 finger toxins, cardiotoxins, membrane toxins, neurotoxins and cathelecidin	Kruck and Logan (1982); Rusmili et al. (2014); Xu et al. (2007); Wang et al. (2008); Gomes et al. (2017); Wei et al. (2009)
N. kaouthia	Three-finger toxins, Kunitz-type serine protease inhibitor, natriuretic peptide, neprilysin, snake venom C-type lectin/lectin-like protein, phosphodiesterase, aminopeptidase, serine protease, waprin, phospholipase B, 5′nucleotidase, dipeptidylpeptidase-IV, clotting factor, acetylcholinesterase, hyaluronidase and insulin-like growth factor, neurotoxins (LNTXs, long neurotoxins; SNTXs, short neurotoxins), cytotoxins and phospholipases A_2, adenosine monophosphatase, adenosine triphosphatase, acetylcholinesterase, myotoxin	Tan & Tan (1988); Tan et al. (2017); Tan et al. (2015); Mukherjee & Maity (2002).
N. nivea	L-amino acid oxidase, hyaluronidase, alkaline phosphomonoesterase, protease, phosphodiesterase, acetylcholinesterase, phospholipase A, 5'-nucleotidase, neurotoxin, myotoxin, basic phospholipase A_2, L-amino acid oxidase and low molecular weight membrane active polypeptides	Tan & Tan (1988); Mukherjee & Maity (2002); Earl & Excell (1972)
O. hannah	L-amino acid oxidase, phospholipase A_2, arginine esterase, acetylcholine esterase, phosphodiesterase, three finger toxins, cysteine-rich secretory proteins, cobra venom factor, muscarinic toxin, hypothetical proteins, low cysteine protein, proteases, vespryn toxin, Kunitz, growth factor activators and others (clotting factor, endonuclease, 5'-nucleotidase)	Guo et al. (2007); Danpaiboon et al. (2014)

2.5.1 Proteins of venoms

Proteins are responsible for 90 % of dry weight in the complex mixture of snake venoms (Stábeli et al., 2012). Many of them had been purified from crude snake venoms and further characterized. Other than proteins and peptides, snake venoms also contain some inorganic cations such as sodium, magnesium, potassium, calcium as well as zinc, iron, cobalt and manganese (Vyas et al., 2013). Additionally, some of the venoms also contain carbohydrates, lipids, amines and free amino acids.

2.4.1.1 Thrombin-like enzymes

Thrombin-like enzymes (TLCs) are serine proteases which can be found from viper snake venoms as well as some elapid venoms. Serine proteases have dual effects both fibrinogenolytic and fibrinolytic activities. Only the enzyme with fibrinogenolytic activity are regarded as 'thrombin like' proteases if they exhibit fibrinogen clotting activity. Serine proteases are enzymes that cleave peptide bonds in proteins, in which serine serves as the nucleophilic amino acid at the (enzyme's) active site. They are named as thrombin-like enzymes as they are like thrombin, acting on fibrinogen. It forms mammalian α-thrombin (prothrombin in activated form; blood clotting factor II) serve as a, multipurpose serine protease. Extensive research on function and structure of it revealed that thrombin plays much crucial part in hemostasis and thrombosis in mammals. By limit proteolysis, serine protease convert fibrinogen (factor I) to fibrin clot and can be regarded as fibrinogenase enzyme. These enzymes induce coagulopathy by cleaving fibrinopeptide released by thrombin and produce fibrin clot. The fibrin clots produced by TLCs from snake venoms are easier degradable and less stable than that of thrombin. Pradniwat and Rojnuckarin (2014) stated that these enzymes isolated from snake venoms have the potential to be developed as therapeutic agents for thrombic disorders.

2.4.1.2 Metalloproteinases

A metalloproteinase is a protease enzyme where the water molecule that is used for hydrolysis is complexed to a metal ion in catalytic centre of the enzyme and the metal ions itself hold in the position by several amino acid residues. SVMPs are classified into P-I to P-III according to their domain organization which are P-I SVMPs contain only a

metalloproteinase (M) domain which is the simplest class of this enzyme. P-II SVMPs contain a metalloproteinase domain followed by disintegrin (D) domain and P-III contain M, disintegrin-like (D) and cysteine-rich (C) domains (Takeda et al., 2012).

Snake Venom Metalloproteinases (SVMPs) are proteases enzymes that can be found in snake venoms from the members of Colubridae family, Viperidea family and small amounts in Elapidea family (Markland & Swenson, 2013). They can be found in large number from Viperidae venom. Metalloproteinases are responsible for the lethal toxicity in these venoms which they can produce variable tissue and cellular pathology in bite victims. The majority of metalloproteinase induce profuse hemorrhage, blood clotting, and the inactivation/activation of complement proteins (Gasanov et al., 2014). Markland and Swenson, (2013) stated that metalloproteinases from snake venoms possess fibrinogenolytic activity and they act as prothrombin activators, inhibits platelet aggregation and inactivates blood serine proteinase inhibitors. With their fibrinogenolytic activity, they are developed in the clinical use for reducing fibrinogen in plasma. Similar to serine proteases, metalloproteinases come with fibrinogenolytic activity applicable for clinical management that reduces fibrinogen value in plasma or dissolves coagulated plasma (thrombolysis) (Matsui et al., 2000).

Kistomin is one of the metalloproteinases which extracted from *C. rhodostoma* venom. It has been shown that kistomin inhibit von Willebrand factor (vWF)-induced platelet aggregation which is the binding of vWF to platelet glycoprotein (GP) Ib-IX-V mediates platelet activation in the early stage of thrombus formation. The analysis shows that kistomin specifically inhibited vWF-induces platelet aggregation through binding and cleavage of platelet GPIbalpha and vWF (Hsu et al., 2007). In addition, it also inhibits collagen-, convulxin- and ristocetin-induced platelet aggregation which has potential to being developed as an antithrombotic agent (Hsu et al., 2008).

Table 2.2 Snake venom metalloproteinase kistomin description.

Feature Key	Description
Name	Snake venom metalloproteinase (*C. rhodostoma*)
Metal binding	At position 333, 337 and 343 (Zinc)
Amino acid sequence	MIEVLLVTIC LAAFPYQGSS IILESGNVND YEVVYPRKIT ALSEGAAQQK YEDTMQYEFK VNGEPVVLHL EKNKELFAKD YSETHYSPDG TRITTYPSVE DHCYYQGRIH NDADSTASIS TCNGLKGHFK FHGERYFIEP LKLPGSEAHA VYKYENIEKE DETPKMCGVI QKWKSDELIK KPFRLNLTPQ QQESPQAKVY LVIVADKSMV DKHNGNIKKI EEQGHQMVNT MNECYRPMGI IIIMAGIECW TTNDFFEVKS SAKETLYSFA KWRVEDLSKR KPHNDAQFLT NKDFDGNTVG LAFVGGICNE KYCAGVVQDH TKVPLLMAIT MGHEIGHNLG MEHDEANCKC KACVMAPEVN NNPTKKFSDC SRNYYQKFLK DRKPECLFKK PLRTDTVS VSGNEPLEVI TMDDFYA
Amino acid length	417 amino acid
Subunit	Monomer
Subcellular location	Secreted
Tissue specificity	Expressed by the venom gland
Mass (Da)	47446

Source: Hsu et al. (2008) & Huang et al. (1992)

2.4.1.3 Disintegrins

Disintegrins from viper venoms are the family of small proteins that function as inhibitors of both platelet aggregation and integrin-dependent cell adhesion. Disintegrins are derived from the processed precursors, metalloproteases in snake venoms (Selistre-de-Araujo et al., 2010). The proteins are named as disintegrins as they are involved as integrin-binding proteins. Integrins are cell receptors involved in cell–cell and cell–extracellular matrix interactions, serving as the final common pathway leading to aggregation via formation of platelet–platelet bridges, which are essential in thrombosis and haemostasis. The role of disintegrin in preventing blood clotting renders it of medical interest, particularly about its use as an anti-coagulant. For the growth of tumour cells, endothelial cell migration is required in order to provide essential blood supply to the cells. Hence, the disintegrins from snake venoms are believed to be useful in studying the blocking of tumour cells growth. The current knowledge of this protein is useful to be further studied on anti-inflammatory therapy and cancer treatment of patients (Sajevic et al., 2011).

2.4.1.4 C-type lectin-like proteins

C-type lectin-like proteins (CLPs) are one of the classified components of snake venoms which activate or inhibit coagulant factors and platelets and thus affect haemostasis (Clemetson & Clemetson, 2005). Many of them have been characterized and studied for the development involving blood clotting and platelet activation. In snake venom, lectins are classified into true C-type lectins (CTLs) and snaclecs (also known as C-type lectin-like proteins or CLPs) that lack the carbohydrate-binding loop present in true C-type lectins and consequently do not bind sugars (Drickamer, 1999). Lectins bind carbohydrate groups through a combination of hydrogen bonding, van der Waals interactions and hydrophobic interactions. It was also discussed that these CLPs can be obtained from *Agkistrodon acurus* venoms (Paulchamy, 2010).

2.4.1.5 Phospholipases A_2

Phospholipases enzymes are classified based on their action on phospholipid molecules. For instance, phospholipases A_2 hydrolyzes bond at the 2-acyl group, releases the second carbon group of glycerol (Stábeli et al., 2012). Phospholipases A_2 can be obtained from snake venoms of Elapidae and Viperidae families. These enzymes in snake

venoms may induce myotoxic and neurotoxic effects after envenomation. Marcussi et al. 2007 mentioned that the inhibitors of enzymatic and pharmacological effects induced by phospholipases A_2 from snake venoms provide models for conducting more studies on biotechnological and therapeutic use potential.

2.4.1.6 Angiotension-converting enzyme inhibitors

Angiotension-converting enzyme (ACE) inhibitors were developed from a Brazilian pit viper, which is *Bothrops jararaca* (Bomback et al., 2007). This was known for the clinical therapy for hypertension, heart failure and kidney diseases. Angiotensin-converting enzyme (ACE) inhibitors help relax blood vessels. ACE inhibitors prevent an enzyme in the body from producing angiotensin II, a substance that narrows blood vessels and releases hormones that can raise blood pressure. This narrowing can cause high blood pressure and force heart to work harder. ACE inhibitors can help reduce blood pressure in people who have high blood pressure. ACE inhibitors can also help prevent a heart attack or stroke. ACE inhibitors are prescribed to prevent or reverse the hypertrophy of the heart and vessel walls (Sweitzer, 2003).

2.4.1.7 Others

There are many other proteins in snake venoms have been isolated and studied by previous researchers. For instance, Hydrophidae and Elapidae venoms generally contain neurotoxins (Clemetson & Clemetson, 2005). Neurotoxins like taipoxin, muscarinic toxin, α-neurotoxin, cholinesterase and others work by attacking the nervous system of the prey. Dendrotoxins is another type of neurotoxins that has been isolated from the African mamba, *Dendroaspis sp.* (Koh et al., 2006). It was found to potentiate acetylcholine release and thus lead to excessive muscular activity and trembling of the prey. According to Ranawaka et al. (2013), the neurotoxic properties of venoms like antimicrobial activity, binding to specific cells, inhibition of apoptosis and anti-inflammatory revealed their potential to be studied for treating infections, cancer and various neurological disorders.

Besides, the other proteins found in snake venoms include amino acid oxidases, peptide bradykinin potentiators, myotoxins, hyluronidase, dehydrogenase, cardiotoxin, growth factors, nucleotidases and many others. Izidoro et al. (2014) stated that L-amino acid oxidase was detected from venom of *Vipera aspis* and studied. Doley and Kini (2009)

discussed that this responsible for the yellow colour of venom. It can induce changes on platelet function and thus causes effects on plasma clotting disorders. This protein showed antimicrobial and antiparasitic activity which can help in the development of therapeutic agents. Other than those mentioned, complex mixture of snake venoms contains many other proteins which are varies in different types of snakes.

2.6 Benefit and medical importance of snake venom

The dangerous effects of snake venom on humans is well known, but there are also many benefits that could be obtained from snake venom. Most of the bioactive proteins and polypeptides (enzymatic and non-enzymatic) in snake venoms are responsible for their pharmacological properties (Mukherjee, et al., 2013).

2.6.1 Anti-cancer properties

Snake venoms often act only on certain types of cells, and this specificity has led to an important research into treatments for cancer. Typical chemotherapy drugs cause many undesirable side effects because they don't discriminate between cancerous and healthy cells in the body. Some research that is currently under way is experimenting with using snake venom to destroy only those blood vessels that carry nutrients specifically to the tumour cells by starving it to death (Liu et al., 2014). The blockage of both tumour and endothelial cell migration and invasion is an interesting approach for the treatment of cancer patients. The key receptors involved in cell migration are the integrins, which connect the cells to the extracellular matrix of the tumour microenvironment. Integrin blocking usually results in inhibition of cell migration and tumour angiogenesis (Selistre-de-Araujo et al., 2010). Disintegrin is made from lower molecular weight, comes varies configuration, strength and specificity. It is found in snake venom isolated commonly from viperid snake venom contain integrin, an agent responsible for development of therapeutics to cure malignancy. Element integrins important in cell linkage, cell movement, tissue organization, cell growth, hemostasis and inflammatory responses, therefore further study could be done in configuring drugs for cancer treatment (Koh & Kini, 2012).

2.6.2 Defibrinating agent

Snake venom has potency to be beneficial in clinical research. Its venom has been valued understanding developments of blood clotting and usage as anticoagulant (Daltry et al., 1996). The prominent thrombin like enzyme from *C. rhodostoma* known as Arvin or Ancrod has been useful as defibrinating agent clinically. It is being trialled as clinical anticoagulant (common brand called Viprinex). Metabolism of action includes haemostatic dysfunction. This is done basically by metalloproteases and serine proteases which cleave or / and inhibit fibrinogen (Ali et al., 2013).

2.6.3 Anti-HIV activity

A cure for Human Immunodificiency Virus (HIV) is still an ongoing intense research that had met only a few successes throughout the years. Ande et al. (2006) stated in his research L-amino acid oxidases (LAAOs) present in snake venom of crotalids, elapids and viperids shows anti-HIV activity which could induce cell death.

HIV virus entry into cells mediated by binding of envelope glycoprotein–gp120, since there is similar homology of sequence HIV-1 gp 120 and conserved 30-40 amino acid residues of snake venom neurotoxins. Therefore, individually both compete for same receptor binding site and can be used against HIV. Moreover, phospholipase A_2 (PLA_2) in venom protects blood leukocytes from other replication from macrophages. Others include metalloprotease inhibitors which inhibit formation of new viruses by blocking protease enzyme (Meenakshisundaram et al., 2009).

2.6.4 Alzheimer

Bothrops jararaca venom has pharmacologically active peptide fraction of bradykinin-potentiating factor. This facilitates proteolytic enzyme inhibition which inactivates bradykinin and catalyses conversion of angiotensin-1 into angiotensin-2 (Ferreira et al., 1970). Research found that Bradykinin is induced in the brain of patients suffering from Alzheimer's disease (Lacoste et al., 2011).

2.6.5 Multiple sclerosis treatment

Multiple sclerosis (MS) is an autoimmune disease which occurs due to chronic inflammatory demyelination of the central nervous system. Until now, a specific

treatment or medication have not been found for this disease due to its complexity and heterogeneity. It was found that fibrinogen depleting agents such as dendrotoxin I complement depleting feature plays an important role in therapeutic measure in multiple sclerosis. From the previous research done by Mirshafiey (2007) venom from *C. rhodostoma* which contains ancrod can stimulate (defibrinogenation) to cure this disease.

2.6.6 Treatment for Leishmaniasis (parasitic disease)

Leishmaniasis is a parasitosis infection caused by species of protozoa *Leishmania* spread by female sandfly. Snake venom having a complex mixture of proteins possess various physiologic effects and induce systemic alterations; for example, systemic bleeding coagulopathy and many more. Brazil found *Bothrops moojeni* contains a wide range of proteases. *B. moojeni* found to kill *Leishmania in vitro*. Enzyme myotoxic phospholipase A_2, known as MjTX-2 display antiparasitic effect against *Leishmania* (Castilhos et al., 2011).

2.6.7 Anti-microbial

Antimicrobial properties of the snake venom have been investigated. Phua et al. (2012) conducted a study by screening the king cobra venom using three strains of *Staphylococcus aureus*, three other species of gram-positive bacteria and 6 gram-negative bacteria and the results showed that the venom was active against all the 12 bacteria tested and was most effective against *Staphylococcus spp. (S. aureus* and *S. epidermidis*). It shows the L-amino acid oxidase enzyme present in King Cobra has distinctive property. In another study conducted in 11 species of snakes common in Malaysia. The value of snake venom in therapeutic field was recognized. Toxin venom from *C. rhodostoma* and *O. hannah* could produce bacterial inhibition zone with maximum value of 12 mm, while other snakes only managed to inhibit up to 10 mm. Confirmation has been done using minimum inhibitory value, which proven their studies. Further studies on crude venom action against Gram-positive bacteria found to give more significant tested on Viperidae species. Crude venoms from Viperidae species results in major inhibition regions between 6.6-12.5 mm, with the Malayan Pit Viper showed largest inhibition ranging from 10.2-12.5 mm stated by Perumal et al. (2007).

2.7 Biotechnology benefit and potential product developed from snake venom

Vyas et al. (2013) stated that snake venoms are produced in the glands throughout the life of snakes. Snakes can replenish their venom glands periodically. Therefore, the benefits of snake venoms should be further studied and maximized. It was shown that some isolated and characterized proteins from snake venoms are useful in the field of medical, pharmacology and toxicology. With biotechnology techniques, the bioactive components from snake venoms are discovered and studied to develop new useful products and technologies. Studies have been carried out especially in medical applications:

- **Captopril**

It is an anti-hypertensive drug derived from *C. rhodostoma* venoms (Lathan & Staggers, 2006; Earl et al., 2012). Captopril has long been used to save millions of humans lives through its anti-hypertensive activity by inhibiting the Angiotension Converting enzyme (Dhananjaya, 2013). This medication is a new class of anti-hypertensive drug which can be used to treat hypertension and heart failure (Koh et al., 2006).

Figure 2.3 Captopril is an angiotensin-converting enzyme inhibitor for hypertension and congestive heart failure treatment.
Source: CSC Pharmaceuticals (2019)

- **Tirobifan (Aggrastat)**

It is extracted from *Echis carinatus* venoms and acts as a specific antagonist for platelet aggregation (Cook et al., 1999). It is used for heart diseases/ coagulopathies for treatment of angina (Dhananjaya, 2013).

Figure 2.4 Tirobifan (Aggrastat) new bolus vial format which has been approved by FDA in 2016.

Sources: Food and Drug Administration (2016)

Other than the uses of snake venom in medical applications and drug development, snake venoms have also been studied for diagnostic use. Some of the components in snake venom have been found to be useful in the diagnosis of haemostatic disorders (Mukherjee et al., 2013). For example, the toxins in Russell's viper venom was used to assay blood clotting factors, platelet factor and some clotting proteins like fibrinogen and prothrombin.

Furthermore, snake venoms had been studied to produce skincare products. With their properties of paralyzing effects, snake venoms were mimicked and synthesized to be used in producing skincare products. For instance, the synthetic version of the Temple Viper venom was used to prevent the wrinkles formation or reducing aging process by stopping the muscle contraction and relaxing muscles. There are several skincare products made using this synthetic snake venom available in the market: Planet Skincare anti-aging moisturiser, INKANAT face cream, DermaFi snake venom peptide cream and others.

On more odd uses of snake venom is on reports of rare and unusual addictions among drug users on snake and scorpion venom and wasp stings to get high (Pradhan et al. 1990; Malhotra et al. 2007; Katshu et al. 2011; Senthilkumaran et al. 2013; Umate et al, 2015; Krishnamurthy & Braganza, 2013; "Addicted To,"2001). These reports suggested some addicts have resorted to use snake venom as recreational drug and unperturbed by its lethality by letting the snake to bite over the tip of their tongue for the craving associated with happiness, grandiosity, and excessive sleepiness.

2.8 *Calloselasma rhodostoma* (viper)

Malayan Pit Viper (*C. rhodostoma*) which was previously known as *Agkistrodon rhodostoma*, is a distant cousin of the North American Copperheads. *C. rhodostoma* is a monotypic genus with only one species, *C. rhodostoma*. They have a variable background coloration from brown to grey with the typical copperhead style triangular bands on the sides of the body. They also have a stripe extending from their eye to their chin, which is scalloped on the bottom.

This snake species is endemic to southern Vietnam, Cambodia, Thailand, northwest of Peninsular Malaysia and Java Indonesia. Because of its wide distribution and present in many protected areas, this species is listed as Least Concern of threatened species. The habitat of Malayan Pit Viper mostly in lowland and sub-montane forest and plantation where it is associated with both dense undergrowth and rocky areas (Das, 2015). With an average length of 70-80 cm and rarely more than 1 meter. Its nature is lying motionless on the ground and only explosively strike if endangered. By having dark brown scales patterning from the head to tail, it camouflages itself among leaf litter and weeds in rubber and palm estates (Bruserud et al., 2013).

These snakes have some unusual characteristics that visually distinguish them from their North American relatives. First, they have smooth scales. This is a feature unique to the genera Calloselasma and Azemiops; every other viper and pit-viper in the world has keeled scales. It is the only Asian pit viper with large scales on the crown. Second, they are one of the few pit-vipers that lay eggs rather than give live birth. *Deinagkistrodon acutus*, another Agkistrodon relative, is also an egg-layer (Daltry et al., 1996). These snake are so dangerous because it is a bad tempered and not consistent with their behaviour. It is known in Malaysia as 'Ular Kapak Bodoh: the 'stupid viper'. It earned the reputation of being "bodoh" (translated from Malay as stupid) because the snake usually remains at the site of biting and hence usually killed easily.

Figure 2.5 *C. rhodostoma* snake
Source: Das et al. (2015)

The principal characteristics of systemic Malayan pit viper venom poisoning is systemic bleeding characterized by defibrination and thrombocytopenia (Reid et al., 1963). In addition, throughout its large distribution range, it is also the leading cause of venomous snakebite, many victims suffer permanent debilitating injuries and there is small percentage of bites cases are fatal (Daltry et al., 1996).

In humans, the venom of *C. rhodostoma* has been shown to have primarily pro-coagulant effects at low concentrations, in that it converts fibrinogen to fibrin, and then precipitates the fibrin out of the blood, leaving the rest of the blood uncoagulable. However, at higher concentrations, the venom has shown to have anticoagulant effects with a progressively shortened clotting process. It is also notable that venom composition seems to be determined by natural selection for different prey in different areas. This is due to inherited variation, which in turn is due to natural selection, as opposed to direct induction due to different animals being eaten by an individual (Daltry et al., 1996).

2.9 Protein assay

Protein concentration quantitation assay is an integral part of any laboratory workflow that involving protein extraction, purification, labelling and analysis. Protein quantification are important to determine protein concentration during each step of protein isolation technique, determined enzyme activity and before submitting the protein samples for electrophoresis (Goldring, 2012). In addition, it also essential step before

submitting the protein samples for immunochemical test. In pharmaceutical industry, protein quantification is important to understand the total protein content in the sample or in formulated product and to generate the accurate data. Furthermore, accurate protein quantification is important for protein analysis and quality control which means that the protein yield can be determine and it can measure the success of the experiments and compare it with others. For example, when analysing the kinetic under different parameters/conditions or testing and developing the new drugs, the small changes in protein concentration can dramatically alter the results.

In purification process, the quantity of protein is an important metric for calculating yields or the mass balance and determining the potency/target activity of the target protein (Noble & Bailey, 2009). The selection of method used to determine protein or peptide concentration in solution depend on many factors and majority of methods require a soluble analyte such as proteins, peptides, post-translationally modified protein (glycosylated), or chemically modified protein (PEGylated) (Noble & Bailey, 2009). According to Olsen & Markwell (2007), the primary determinants of the appropriate assay are protein and buffer composition.

The most difficult aspects of assaying protein concentration are the selection of an assay compatible with the sample since the assays available detect specific properties of protein. For example, a protein with high in cysteine would produce an artificially high result using the BCA assay, but would likely to produce better results with Bradford or Lowry assay (Brady & Macnaughtan, 2015). The choice among available protein assays usually based upon the compatibility of the method of the samples to be assayed and required the least pre-treatment of the samples containing substances that interferes with the assay (Wrolstad et al., 2005). As for buffer composition, the assays procedure must be compatible with the buffer system of the protein sample since some of buffer components interfere with chromophore production. For example, the BCA assay is compatible with detergent sodium dodecylsulfate (SDS) but does not tolerate reducing agents such as dithiothreitol (DTT) (Brown et al., 1989).

The other criteria that need to be considered when selecting an assay includes sample volume, sample recovery, protein aggregation, chemical modification (Noble & Bailey, 2009), high- throughput adaptations, selection of microtiter plate and plate reader (Olsen & Markwell, 2007). There are three traditional assays of total protein quantitation

such as measurement of Ultraviolet absorbance at 280nm, Bradford Assay and Bicinchoninic acid (BCA); and the alternative methods like Lowry assay or convenient kit of novel assay developed by commercial suppliers (Johnson, 2012). The other methods include western blot, enzyme-linked immunosorbent assay (ELISA) and mass spectrometry which for individual protein quantification methods.

2.9.1 Bradford protein assay

Bradford protein assay also known as Coomassie Blue protein assay was first described by Bradford (1976). This assay includes various preparations of dye Coomassie Brilliant Blue G-250 used to quantify the protein concentration. This assay is popular among researchers because it is sensitive, inexpensive, rapid and simple (Olson, 2007). The protein concentration in the sample is determined by the amount of dye in a blue ionic form measured using a spectrophotometer at absorbance of 595 nm.

The basic mechanism of Bradford assay is based on the direct binding of Coomassie brilliant blue G-250 dye at acidic pH to arginine, phenylalanine, tyrosine, tryptophan and histidine residues (de Moreno et al., 1986; Olson, 2007) and hydrophobic interactions (Fountoulakis et al., 1992) which is the negatively charged Coomassie brilliant blue dye binds to the positively charged proteins. The assay primarily responds to arginine residues eight times as much as other listed residues; therefore, it is necessary to use an arginine-rich standard. The free dye in the solution has a maximum absorbance at 470 nm whereas the anionic Coomassie brilliant blue G-250 dye produced a maximum absorbance at 595nm when binds to these protein residues. Therefore, upon binding protein, due to the stabilization of the anionic form of Coomassie brilliant blue dye, a metachromatic shift from 465 to 595 nm is observed.

The advantage of Bradford assay is it is compatible with most reducing agents used to stabilize protein in the solution during pre-treatment process which are not compatible with Lowry assay and BCA assay. In addition, in the presence of contaminating peptides, this assay can measure the concentration of high molecular weight (MW) proteins (larger than 3 kDa) because the Coomassie brilliant blue dye does not bind with low molecular weight (MW) proteins (Kruger, 1994; Olson & Markwell, 2007). Cheng et al. (2016) described that Bradford assay-based method is a method that

allows for rapid protein quantification even when the solution contains interfering substances.

The disadvantages of Bradford assay are its incompatibility with most detergents which usually used to solubilize membrane proteins (Johnson, 2012). In addition, most researchers use Bovine Serum Albumin (BSA) as protein standard for Bradford method since it is easily available and inexpensive, but it may lead to under-estimating the protein concentrations of samples since BSA exhibits a strong dye response. This is not a problem if only relative concentrations of samples are needed. However, depending on the protein sample, the lysozyme, immunoglobulin G (IgG) or other protein standard are better choices if a precise quantification of protein concentration is necessary (Kruger, 2009).

Figure 2.6 Bradford protein assay.
Source: Eiro forum (2019)

2.9.2 Lowry protein assay

Lowry assay was proposed by Oliver H. Lowry in 1951 (Lowry et al., 1951). This assay has a detection limit 10-1000 µg/mL and based on two chemical reactions. Firstly, the Biuret reaction involves the reduction of copper ions (Cu^{2+} to Cu^{+}) under alkaline solutions which forms a complex with peptide bonds. This followed by the enhancement stage which is the reduction of Folin Ciocalteu reagent (phosphomolybdate and phosphotungstate) by the copper peptide bond complex, which causes a colour change of the solution into blue with maximum absorbance at 750 nm detectable with a spectrophotometer (Olson & Markwell, 2007), but it can be measured at any wavelength between 650 nm and 750 nm. The colour development of this assay is not only due to

reduced copper-amide bond complex but also to tyrosine, tryptophan and lesser extend to cysteine, histidine and cystine residues (Noble & Bailey, 2009; Wu et al., 1978; Peterson, 1977).

The advantages of Lowry assay are its precision and high sensitivity however, the procedures are time consuming and the assay are incompatible with reducing agents and detergents which commonly used in buffer for protein preparation such as carbohydrates, Tricine Tris, EDTA and glycerol. Nowadays, the Lowry assay has been modified to increase the dynamic range, reduce its sensitivity to interfering agents and increase the speed that resulting the stability of the colour formation (Noble & Bailey, 2009; Peterson, 1979).

Figure 2.7 Lowry assay.
Source: Pudidotdk (2014)

2.10 Fractionation and purification of snake venom proteins

Snake venom consists of various pharmacologically active proteins and polypeptides. These include the toxins like hemotoxins, neurotoxins and myotoxins which will bring different affects to the prey and mankind. In order to neutralize the envenomation effects and prevent the further damages by venoms, study on active molecules in snake venoms were required to develop antivenom. Furthermore, there are a large number of proteins in snake venoms were selected as a source for biotechnology applications (Koh et al., 2006). Recent years, due to the rich sources of bioactive

components present in snake venoms, they are getting more and more attention to be studied in medical and therapeutic applications. According to Byoki and Mirakabadi (2013), over the years, a number of toxins have been isolated and characterized from various snake venoms for development of therapeutic agents. Therefore, the fractionation, isolation and purification are the important procedures for the study and research on the proteins in snake venoms.

There are various techniques had been used by researchers for fractionation and purification of snake venom proteins, including size exclusion chromatography, metal ion-affinity chromatography, ion exchange chromatography and others. After the purification processes, determination of protein purity is a basic procedure in protein chemistry (Mohan, 1992). Mohan (1992) stated that a protein can be said as pure when it yields a single band after electrophoresis on sodium dodecyl sulphate polyacrylamide (SDS-PAGE) gel and also elute as a single symmetrical absorbance peak from chromatography techniques like gel filtration, ion exchange and High-Performance Liquid Chromatography (HPLC). The purity determination of proteins from snake venom is very important for their applications in biotechnology, medical and therapeutic use. This will especially influence the safety of biopharmaceutical products (Rhodes & Laue, 2009).

Due to the scarcity of venom, upon purification, further biotechnology development of obtaining the end product may require genetic engineering technology. Recombinant DNA technology offers engineering tools to produce the desired protein molecules in large quantities on lab and industry scale (Kamionka, 2011). Viprinex manufactured by Abbott is an example of snake venom proteins produced using genetic engineering. Ancrod, an enzyme from pit viper venom with defibrinogenating properties was made as Viprinex (Yeo & Sharma, 2013). Viprinex was involved in the clinical trials of stroke treatment investigation.

2.11 Chromatography techniques in the purification of proteins

Purification of proteins involves a series of processes aimed to isolate a specific protein from a crude mixture. Chromatography techniques are the common and widely used methods to separate and purify individual protein. Gel filtration, ion exchange, hydrophobic interaction and affinity chromatography are some chromatography techniques available for proteins purification (GE Healthcare Life Sciences, 2010). In order to isolate a specific protein, the physical and chemical properties of the individual protein to be purified are utilized in different chromatographic separation. There is no single or simple step to purify all kinds of proteins. Purification to obtain a pure protein cannot be achieved in a single step of chromatography.

2.11.1 Ion exchange chromatography

Ion exchange chromatography is the technique for the separation of molecules according to their overall charge. Ion exchange chromatography can be divided into anion exchange chromatography and cation exchange chromatography. The separation of molecules is based on the affinity of analytes to the ion exchanger on the matrix. Ion exchange separation involves the adsorption of charged molecules to the matrix with immobilized ion groups which have an opposite charge to the charged molecules and followed by the release of charged molecules.

The media used in this chromatography is the matrix with immobilized charge groups. For cation exchange chromatography, cation exchanger (negatively charged) will be attached on the matrix and bind to the positively charged molecules from the samples. On the other hand, carboxymethyl-cellulose (CM-cellulose) is one of examples of the cation exchanger. According to Ramirez-Avila et al. (2004), cation exchange chromatography with a MONO S column was performed to purify phospholipase A_2 from *Bothrops asper* venom. The other ion exchangers available include as sulfopropyl (SP) and methyl sulfonate (S) as cation exchanger as well as quaternary ammonium (Q) and diethylaminopropyl (ANX) as anion exchanger (GE Healthcare Life Sciences, 2010).

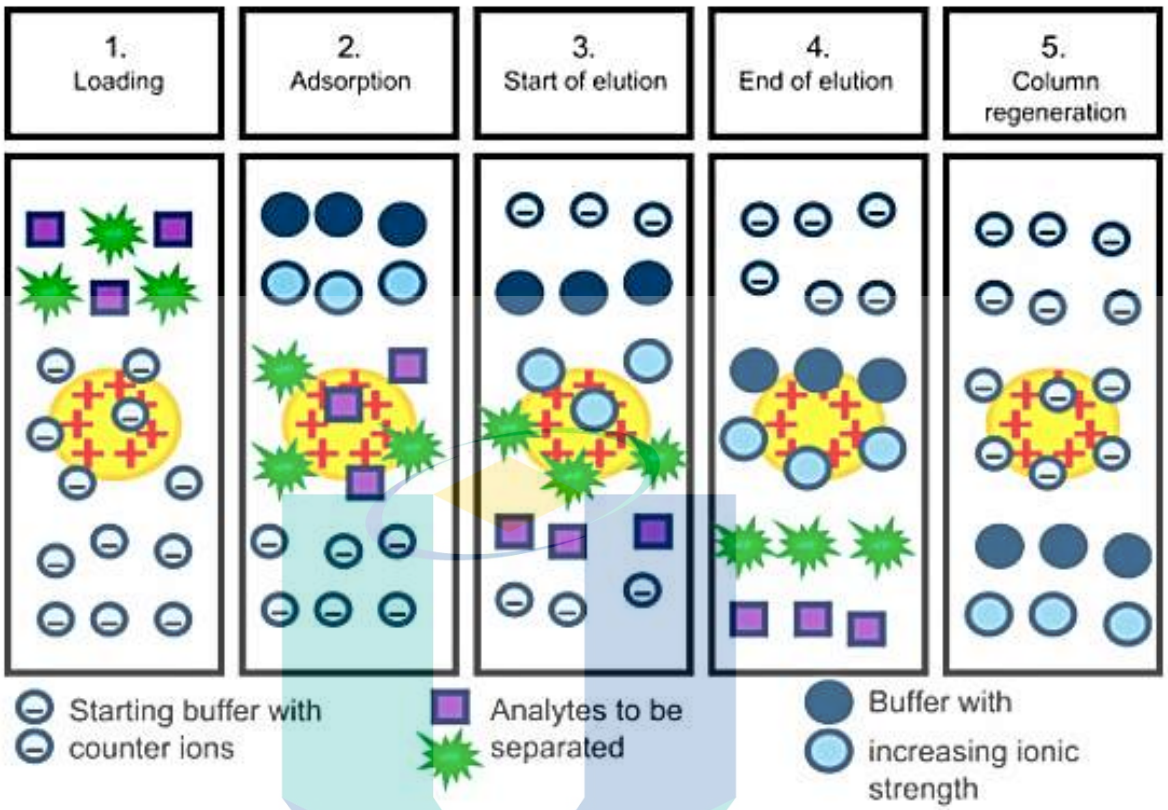


Figure 2.8 Ion exchange chromatography principle.

Source: Smoluch et al. (2016)

The overall number and types of amino acid side chain groups present in proteins affect the charge of the proteins, which thus influences the fractionation of proteins using ion exchange chromatography. The isoelectric point of protein is also important in this chromatography. The protein will not bind to the media while it is at its isoelectric point. When the protein is below its isoelectric point, it has a positive charge that will bind to negatively charged beads while above its isoelectric point, it has a negative charge that will bind to positively charged beads (Widmann et al., 2010).

2.11.2 Size exclusion chromatography

Size exclusion chromatography is also known as gel filtration chromatography. This technique separates the molecules like proteins, enzymes, polysaccharides and nucleic acids according to their size difference as they pass through the packed medium in the column (GE Healthcare Life Sciences, 2014). Medium of chromatography which consists of porous matrix is packed into a column to form a packed bed to perform the separation of molecules. There is a variety of matrix available for size exclusion

chromatography with different selectivity and molecular weight range such as Sepharcryl, Superose, Sephadex and Superdex.

The separation using size exclusion chromatography is achieved by the exclusion difference of the samples from the pores of packing materials when the samples pass through the porous materials. Larger molecules are excluded from the bead while the smaller one will be retained in the bead and be eluted later. The resolution of this chromatography may be affected by the molecular weight range of media used, height of column, quality of column packing and some other experimental related factors like volume of sample and flow rate.

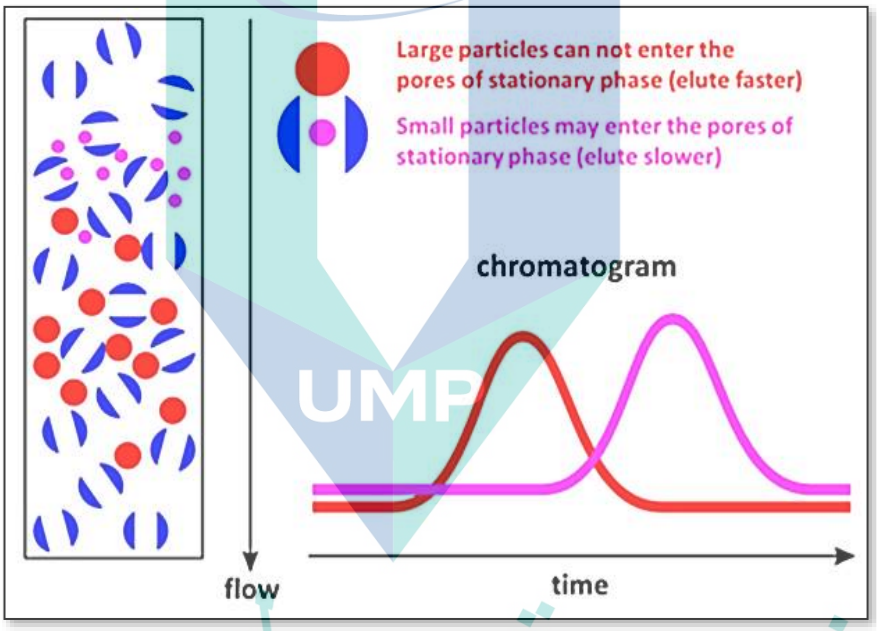


Figure 2.9 Size exclusion chromatography principle.
Source: Pronalyse (2019)

Size exclusion chromatography is one of the common techniques used for the fractionation and purification of snake venom proteins. According to Kashani et al. (2012), the partial fractionation of Iranian Vipers venoms was performed using gel filtration chromatography with Sephadex G-100 column. With this, two separated fractions were found to show the antiplatelet activity. Besides, for the purification and characterization of anticoagulant factor from *Echis carinatus* venom, gel filtration on Sephadex G-75 was performed to obtain the fractions for anticoagulant factor (Byoki & Mirakabadi, 2013).

2.11.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Weber and Osborn (1969) discussed that the separation of proteins by SDS-PAGE is dependent on the molecular weight of their polypeptide chains. The separation is achieved based on the differential migration rates of proteins with different molecular weight through a gel matrix when an electrical field is applied.

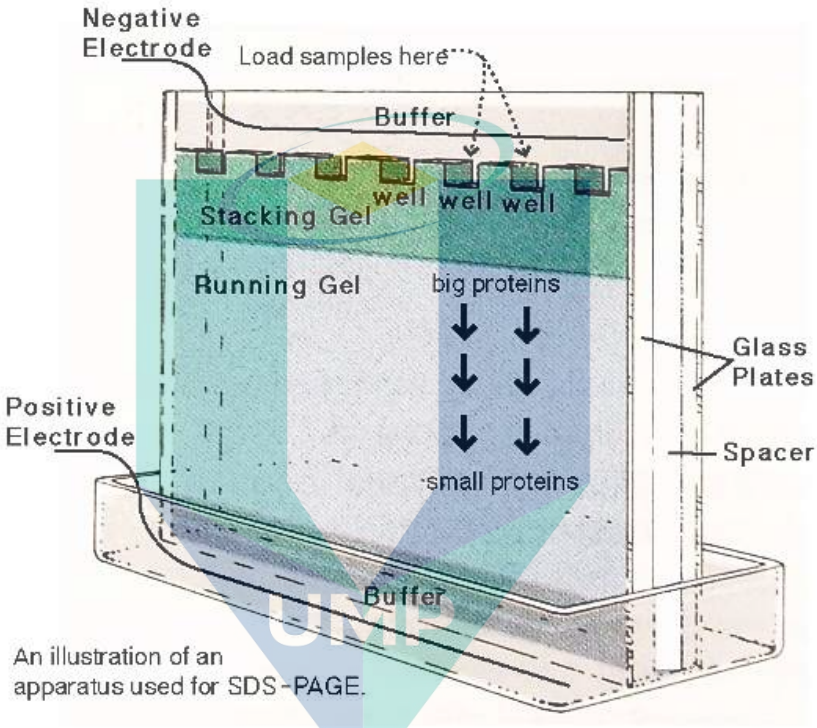


Figure 2.10 Apparatus set up for SDS-PAGE as well as the travel of proteins with different molecular weight.
Source: Pronalyse (2019)

Sodium dodecyl sulphate (SDS) is important to reduce the proteins to linear molecules for the separation of proteins based on their molecular weight only. SDS acts as anionic detergent and works with reducing agent like dithiothreitol (DTT) to disrupt the tertiary structure of protein molecules and forming linear molecules. Besides, SDS masks the proteins with a uniform negative charge to make them to be separated according only to the molecular weight when electrical field is applied.

Polyacrylamide gel matrix is used in SDS-PAGE. The concentration of acrylamide for making gel can be altered to produce the gel with different pore size. SDS-PAGE involves the use of separating gel and stacking gel. When electric field is applied, the proteins (negatively charged) will move towards the positive electrode depending on

their molecular weight. Hence, smaller molecules travel across the gel further than that of larger molecules.

2.12 Factors affecting milk clotting

The factors affecting milk clotting, an important process in dairy products industry have been determined and studied. While determining the factors, clotting time and curd firmness are used as indicators to evaluate the clotting properties. The factors or parameters include temperature, pH or acidity and calcium contents. Cecchinato et al. (2012) suggested that casein content, acidity, pH and fat content influence clotting properties. Among these, acidity and casein content may be the factors which can contribute on improving the milk clotting. Additionally, it was found that a lower pH with a higher temperature may give a better clotting process by having shorter clotting time (Toffain V et al., 2012). Calcium ions content also influence milk clotting by promoting aggregation of casein micelles and thus shorten the clotting time (Landfeld et al., 2002; Sandara et al., 2012).

2.12.1 pH

In milk, all the negatively charged micelles bounce off each other due to all having a negative charge. Caseins are often called “polar” due to all these charges. Adding acid, in effect, is like adding positive charge and the goal is to neutralize the negative charge that is surrounding the casein micelles. The addition of acid neutralizes the micelle surfaces, and this allows them to bump into each other and stick. Adding acid lowers the pH and a lower pH increases enzyme activity and neutralizes charge repulsion between micelles (Nájera et al., 2003). Therefore, both primary and secondary stages of clotting proceed more quickly at a lower pH. The optimum hydrogen-ion concentration for rennet activity has been reported to lie in the zone between pH 5.1 and 5.3. The reaction of the milk affects the rapidity of clotting and the character of the curd formed. Ordinarily when the reaction of the milk is alkaline, clotting does not occur.

2.12.2 Temperature

The optimum clotting temperature of calf rennet is about 40 ℃ to 42 ℃. Clotting is less rapid below this temperature and no clotting occurs above 60 ℃. The clot is softer at low temperatures and tougher and stringy at high temperatures. Optimum temperature

is the temperature at which clotting takes place most rapidly for a definite concentration of rennin and milk (Sharma et al., 1993).

2.12.3 Calcium chloride concentration

In addition to chymosin, cations are necessary to bring about clotting of milk. Due to casein and calcium being so closely involved in milk, the cation calcium is important in bringing about clotting. Calcium is not required for the primary stage but is essential in the aggregation of the casein micelles. At low levels of calcium, the primary phase goes to completion. Subsequently, instantaneous clotting can be induced by adding sufficient calcium chloride. If milk is diluted with sufficient water, clotting is both delayed and incomplete, the clot being soft. If calcium chloride is added to the water, diluted milk clotting properties are restored, which suggests that the concentration of calcium ions is more important than that of the casein ions (Nájera et al., 2003).

2.13 Criteria of good coagulant in dairy industry (MCA/PA)

Clotting of milk is the product of the action of protease that destabilize casein micelles, which are particles present in fresh milk dispersed in a continuous phase comprising whey proteins, lactose, water and salt (De Kruif, 1999). Summer et al. (2002) stated concentration and nature of clotting enzyme is one of the several factors which have the influenced-on Milk Clotting Properties (MCP) other than milk temperature, protein (casein and its fraction), titratable acidity (TA), somatic cell count, phosphorus and calcium content.

Dairy industry characterizes chymosin enzymes using two properties which are Milk clotting activity (MCA) and Proteolytic activity (PA). The Soxhlet unit has been widely used for the characterization of the milk clotting activity (rennet strength) that was developed and sold by Franz Soxhlet, the first commercial rennet with a standardized enzymatic activity. The Soxhlet method is easy and still used by small dairy industry. Although this method has been modified over time and new kind of unit is used today (Jacob et al., 2011). As the method is regarded as a mere indicative determination of rennet strength because the rennet strength depends on the quality of milk and pH, and not related to any reference standard of rennet. Therefore, the International Dairy Federation (1997 ISO/IDF) introduced methods 199 for sheep and goats rennet, 157 for cattle rennet and 179 for microbial rennet (Kozelková et al., 2013).Milk clotting activity

(MCA) is expressed in International Milk-Clotting Unit (IMCU) which was determined by a standard method of (International Dairy Federation, 2007) that describes the ability of the rennet to aggregate milk by cleaving the Phe_{105}-Met_{106} the nearby bond of κ-casein. The second property is proteolytic activity (PA) of the coagulant, which explained by Kappeler et al. (2006) as the ability of the coagulant to cleave any bond in casein.

The criteria of good coagulant are depending on the relationship between these criteria, which is the ratio of milk clotting activity/ proteolytic activity that captures the essential quality of a milk clotting enzyme. The high ratio of milk clotting activity/ proteolytic activity is considered a good coagulant so the higher the value, the better the coagulant (Nasr et al., 2016; Langholm Jensen et al., 2013). Good quality cheese production is related to high milk clotting activity and specificity of the enzyme to digest on κ-casein at amino acid Phe_{105}-Met_{106} (Elagamy, 2000). Chymosin is an aspartic proteinase which specifically breaking the bonds between amino acids present in the casein structure. The coagulant with low specificity may destroy peptide bonds and other milk proteins which may leads to loss of yield, excessive softening, formation of off-flavours, and other functional defects. In addition, excessive proteolysis during storage limits the shelf life of the cheese and results in formation of bitter peptides (Alihanoglu et al., 2018). Therefore, the clotting agents with high specificity at the Phe_{105}-Met_{106} peptide bond and limited proteolytic activity are highly desirable by dairy industry.

2.14 Enzyme kinetics

Enzymes kinetics is the study of the chemical reactions that are catalysed by enzymes. Enzymes are the protein catalyst that speed up the rate of chemical reaction without being used up in the process. They can achieve their effect by temporarily binding to the substrate and lowering the activation energy needed to convent the substrate into product. The rate of enzyme is influenced by several factors such as the concentration of the substrate molecules, temperature, the presence of inhibitors and pH. The rates of enzyme-catalysed reactions are described mathematically using the Michaelis-Menten Kinetics equation. This mathematical description of enzyme action was developed by Leonor Michaelis and Maud Menten in 1913.

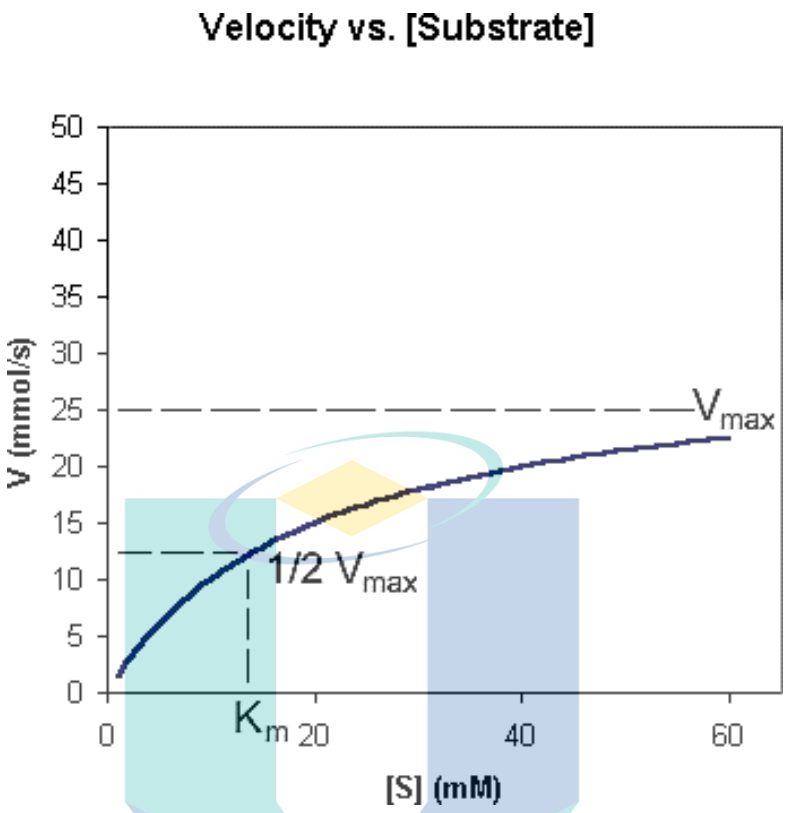


Figure 2.11 Enzyme kinetics

Source: Lubert Stryer (1995)

Two enzyme kinetics constant which is K_m and V_{max} play an important role, both to understand the enzyme activity on a macroscale and to understand the effects of different types of enzyme inhibitors. As the enzymes bind to their substrate and transform them into products. A plot of the initial reaction velocity (v) equals to $(V_{max}\ [A])/(K_m + [A])$ as described by the Michaelis-Menten equation where V_{max} is the maximum velocity, [A] is the substrate concentration and K_m is the Michaelis constant, or the substrate concentration at half maximum velocity (Robert, 2015).

The K_m and V_{max} are determined using initial velocity measurement obtained at varying substrate concentrations where all conditions such as pH, ionic strength, temperature and enzyme concentration are kept constant (Robert, 2007). In details, Maximum velocity (V_{max}) is the point when there are enough substrate molecules to completely saturate the enzyme active sites, as increasing the substrate concentration indefinitely does not increase the rate of an enzyme catalysed reaction beyond certain point. V_{max} reflects how fast the enzyme can catalyse the reaction. The Michaelis constant (K_m) is describes as the substrate concentration at which half the enzyme's active sites

are occupied by substrate. a low K_m means only a small amount of substrate is needed to saturate the enzyme which indicating a high affinity for substrate and vice versa.

Historically, the enzyme kinetic constants were determined graphically using linear plots and secondary plots but nowadays, kinetic constant of an enzymes are determined by compute using various software programs such as GraphPad Prism or SigmaPlot. This software determined the K_m and V_{max} values in the fashion that are used to draw the Lineweaver-Burk lines of the 'best fit' for illustrative purpose in journal articles. In the laboratory, plotting the substrate concentration versus velocity, and 1/[A] versus 1/v, provide an indication of trends and may indicate spurious experimental points (Robert, 2015). There was considerable discussion concerning which graphical procedures gave the most accurate results, with general agreement that Lineweaver-Burk plots were among the least accurate owing to the magnification of errors corresponding to low substrate concentrations (large 1/[S] values), and these values have a disproportionate weighting when linear regressions are performed (Robert, 2007).

2.15 Inhibition of enzyme

Enzyme inhibition means decreasing in the enzyme activity by the enzyme inhibitor. The enzyme inhibitor is the substance that abolished the rate of enzyme reaction. The enzyme inhibition is classified into competitive inhibition and non-competitive inhibition.

Competitive inhibition is a type of inhibition where there is structural similarity between the inhibitor and substrate. The substrate and the inhibitor compete to bind to the active site of the enzyme. This inhibition is reversible, and it can be relieved by increasing the concentration of substrate. Competitive inhibition increases K_m but does not affect V_{max}. The examples of competitive inhibition are the enzymatic process of enzyme succinate dehydrogenase which the substrate (succinic acid) and the inhibitor (Malonic acid) has some structural similarity.

Noncompetitive inhibition is a type of inhibition where the inhibitor is not structurally similar to the substrate. The inhibitor does not bind to the catalytic site as the substrate, but it binds to another site. It also binds to an enzyme or enzyme substrate complex. When it binds it can change the shape of active site and prevent the substrate from binding to the active site of the enzyme.

Uncompetitive inhibition is irreversible and cannot be relieved by increasing substrate concentration. Non-competitive inhibition decreases V_{max} but does not affect K_m value. In addition, non-competitive inhibition may be caused by inhibition of sulphahydryl group, inhibition of cofactors and inhibition of specific ion activator.

CHAPTER 3

METHODOLOGY

3.1 Introduction

A few procedures were developed to discover the potential of protease from snake venom for milk clotting activity. The research design is shown in Figure 3.1.

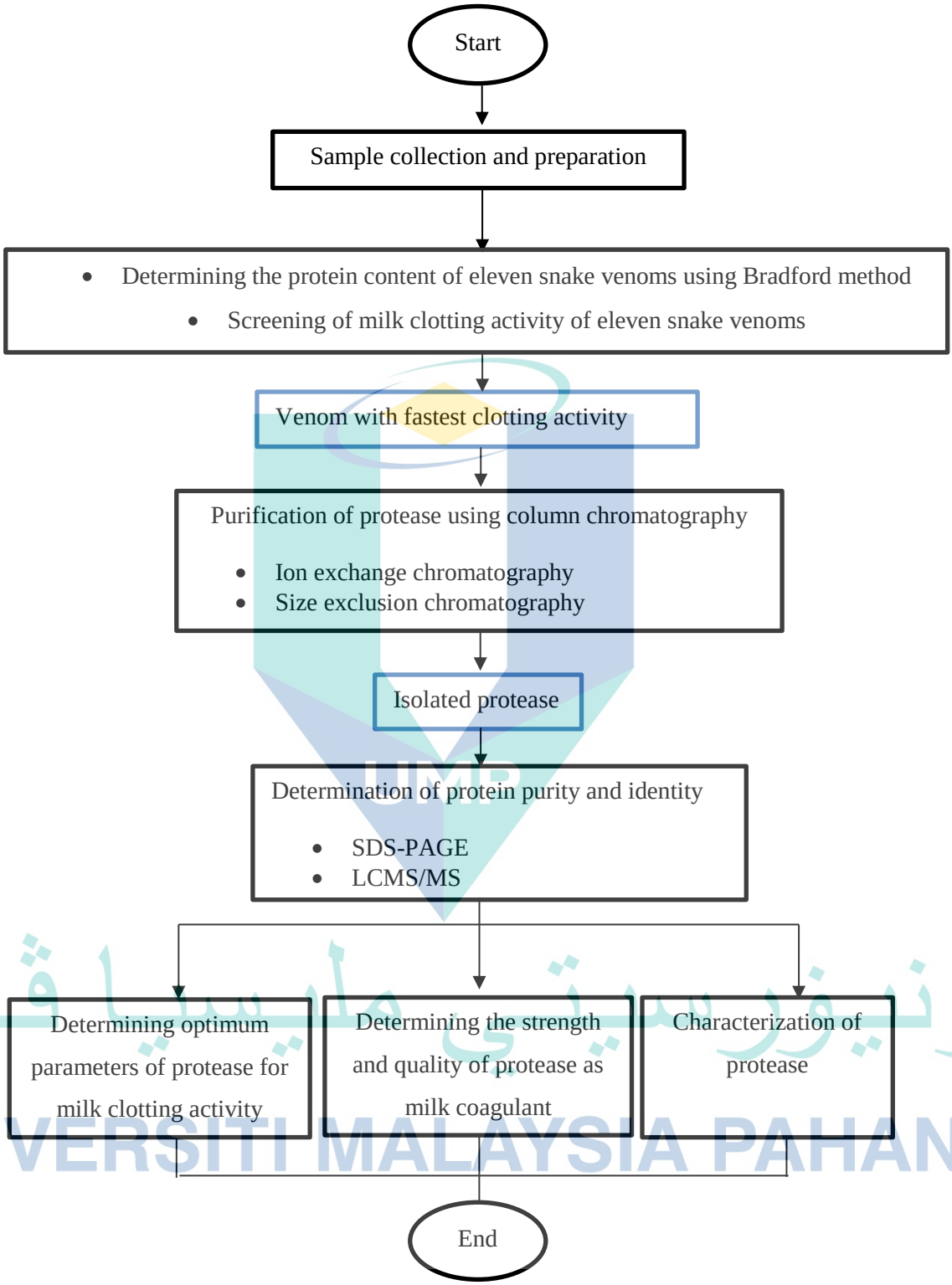


Figure 3.1 The overview of research design for the isolation of milk clotting protease from snake venom.

3.2 Sample collection and list of chemicals

Snake venoms from various species of viper origin were obtained from the usual source i.e. Snake and Reptile Farm, Sungai Batu Pahat, Perlis. The *N. nivea* venom which endemic to Southern Africa was a personal collection donated by Prof. Dr. Stephen Ambu from International Medical University, Kuala Lumpur. Venoms were milked from selected snakes by a trained snake handler and collected in a sterile universal container, the venoms were freeze-dried (lyophilized) and stored at -20 ^{o}C until further use. Rennet from *Mucor miehei* (fungi) was purchased from Sigma Aldrich, USA.

As for the fresh milk samples, cow milk (Good Day Fresh Milk) was purchased from Giant Supermarket Malaysia, goat milk and soy milk were purchased from local vendors and was freeze dried into powder, while the lyophilized camel milk was purchased from Products Pvt. Ltd. through Amazon.com. All the milk samples and coagulants were stored at -20 ℃.

Table 3.1 List of venom samples

Species	*Dry weight (mg)
C. rhodostoma	2380
N. sputatrix	430
T. wagleri	345
B. candidus	258
N. kaouthia	75
O. hannah	233
N. nivea	594
B. fasciatus	215
T. hageni	86
T. sumatranus	459
T. purpureomaculatus	20

*After freeze-drying

Table 3.2 List of chemicals

Procedure	Chemical	Company
Bradford protein determination	Bovine serum albumin (BSA) Coomassie Blue G-250	Thermo Fisher Scientific, USA
	85 % Phosphoric acid (≥85 %) Absolute ethanol (≥99.8 %)	Sigma Aldrich, USA
Protein purification	Ammonium Acetate (≥98 %) Hydrochloric acid (HCl) (36.5 – 38 %) Sodium Hydroxide (NaOH) (≥97 %) Sodium Chloride (NaCl) (≥99.0 %) Ethanol (≥99.8 %)	Thermo Fisher Scientific, USA
SDS-PAGE	Low Protein molecular weight marker	Takara, Japan
	Acrylamide (≥99 %) Tetramethylethylenediamine (~ 99 %) Bromophenol blue	GE Healthcare, USA
	Bis-acrylamide Ammonium persulphate (APS) (≥99.99 %) Dithiothreitol (DTT) (≥97 %)	Bio-Rad Laboratories Inc., USA
	Glycine (≥99 %) Sodium Dodecyl Sulfate (SDS) (≥99.0 %) Tris base (≥99.9 %) Glycerol (≥99.5 %)	Merck, Germany
Milk clotting activity	BD DifcoTM skim milk	Thermo Fisher Scientific, USA
	Calcium Chloride ($CaCl_2$) (≥99.9 %) Monobasic sodium phosphate (≥99.0 %) Dibasic sodium phosphate (≥99.0 %)	Sigma Aldrich, USA
Enzyme characterization	κ-casein (≥ 70%) β-Casein (≥ 99%) α-casein (≥ 70%) Phenylmethanesulfonyl fluoride (≥98.5 %) Pepstatin A (≥90 %) Ethylenediamine tetraacetic acid (≥99 %) Iodeacetamide (≥99 %) 1,10 phenanthroline (≥99 %) Copper sulfate ($CuSO_4$) (≥99.99 %) Magnesium chloride ($MgCl_2$) (≥98 %) Magnesium sulfate ($MgSO_4$) (≥99.5 %) Iron (II) sulfate ($FeSO_4$) (≥99 %) Lead nitrate ($Pb(NO_3)_2$) (≥99 %) Mercury chloride ($HgCl_2$) (≥99.5 %) Barium chloride ($BaCl_2$) (≥99 %) Manganese sulfate ($MnSO_4$) (≥99 %)	Sigma Aldrich, USA

Table 3.2 Continued

Procedure	Chemical	Company
Proteolytic activity	Casein powder	Nacalai Tesque, Japan
	Potassium phosphate dibasic, (≥99.0 %) Trichloroacetic acid (≥99.0 %) Folin & Ciolcaltea's Phenol Reagent (2N) Sodium Carbonate (≥99.5 %) L-tyrosine (≥98 %)	Sigma Aldrich, USA

Experiments were performed in triplicate and repeated three times (n=3) under the same conditions to estimate the variability and to increase the accuracy of the results. This is absolute replication which increase the sample size (n) and contributes to testing an experimental hypothesis (Lazic et al., 2018).

3.3 Bradford protein determination

Total protein in various samples were determined using protein determination technique as stated by Bradford et al. (1976), with an initial stock of 2 mg/mL of bovine serum albumin (BSA), 0.1 mg/mL stock solution was prepared. Bradford reagent was prepared by dissolving 100 mg of Coomassie Blue G-250 in 50 mL of 95 % ethanol and 100 mL of 85 % (w/v) phosphoric acid. The mixture was topped up to 1000 mL with deionized water and filtered using Whatman No. 1 filter paper before use.

BSA content from 0 to 10 µg was calculated and diluted with distilled water from 0.1 mg/mL stock solution. The venoms and the rennet were prepared by dissolving 1 mg each sample with 1 mL of distilled water. The samples were then diluted using serial dilution. A total of 1 mL of Bradford reagent was added to 100 µL of samples (venoms and rennet) and BSA solution compromising six standard solutions to obtain a standard curve. The mixtures were incubated in dark at room temperature for 20 minutes. The absorbance reading of protein concentration was recorded at 595 nm by using a spectrophotometer. The calibration curve of BSA proteins was constructed and the equation was generated from using MS Excel software. The protein concentration of samples was calculated by substituted the absorbance value of samples in the BSA protein calibration curve equation.

Table 3.3 Preparation of standard curve using bovine serum albumin (BSA) with different concentration.

Concentration (µg)	BSA stock solution (µL)	Distilled water (µL)
0	0	100
2	20	80
4	40	60
6	60	40
8	80	20
10	100	0

3.4 Determining milk clotting activity of various venoms

The commencement of clotting was determined by observing for visible clot formation as described by Farah & Bachmann (1987), with few modifications using three visible parameters i.e. change in viscosity, colour changes and white spot formation (separation between curd and whey) and a drop of the sample was observed under light microscope. The clotting time was defined as the time required for the first appearance of graininess in the moving film of milk on the surface of the glass wall.

3.5 Determining milk clotting activity of *C. rhodostoma* and *Mucor miehei* by different types of milk

The clotting activity of *C. rhodostoma* venom was tested using four different milk i.e. cow, goat, camel and soy milk. 1 mL of 10 % (w/v) of each freeze-dried milk samples were dissolved using phosphate buffer pH 7.0 containing 10 mM $CaCl_2$ except for the soy milk ($CaCl_2$ is a clotting agent for soy milk). The milk samples were pre-incubated in water bath at 45.5 ˚C (Vejayan et al., 2017). Then, 0.65 % (w/v) of *C. rhodostoma* venom and 0.32 % (w/v) *Mucor miehei* rennet were added into each milk samples and milk clotting time was measured according to the condition stated above in 3.4.

3.6 Purification of protease from *C. rhodostoma* venom

C. rhodostoma venom is an extracellular protein produced and released by venom glands in a solubilized form, therefore not requiring preliminary steps such as extraction, precipitation and ultracentrifugation. *C. rhodostoma* venom was fractionated and its protease was isolated to homogeneity using various chromatography techniques sequentially. The standard protein purification techniques included Ion Exchange Chromatography technique and Size Exclusion Chromatography.

3.6.1 Ion exchange chromatography

Fifty-five milligrams of freeze-dried venom was diluted using 2 mL of start buffer (50 mM ammonium acetate buffer, pH 6) and was filtered using using 0.45 µm syringe filter to remove any particle impurities present in the sample. The sample then was injected into a strong cation, 5 mL HiTrap SP FF (GE Healthcare Life Sciences) pre-packed with SulfopropylSepharose Fast Flow ion exchange chromatography column connected to the ÄKTAexplorer through 2 mL of sample injection loop.

The column was equilibrated with 50 mM of ammonium acetate buffer pH 6 (Start Buffer) and the protein fraction was eluted with linear gradient using 1.0 M NaCl in 50 mM ammonium acetate, pH 6.0 (Elution Buffer) at flow rate 5.0 mL/min. The parameters programmed to a method template as follows: Sample injection: 2 mL; Equilibration volume: 6 Column Volume (CV); Wash out unbound protein: 5 CV; Elution volume: 15 CV; Target concentration of Elution Buffer: 100 %; Clean after elution: 5 CV; Re-equilibration volume: 5 CV; Eluate fraction size: 5 ml; Column pressure limit: 0.3 mPa; Wavelength ($^{UV}\lambda$): 280 nm.

The fractions collected from ion exchange column were desalted and concentrated using Vivaspin 20 centrifugal concentrator with 3000 MWCO (GE Healthcare, USA). Protein fractions were pipetted into the concentrator and was centrifuged up to 8000 rpm for 3 hours at 20 °C. Fractions were screened for clotting abilities using the method described in 3.4.

3.6.2 Size exclusion chromatography

The active fraction from ion exchange chromatography was further separated using HiPrep 26/60 Sephacryl S 200 HR size exclusion column (CV:318.56 mL; Pressure: 0.15 mPa; Wavelenght ($^{UV}\lambda$): 280 nm). A total of 2 mL of the active fraction from ion exchange chromatography was injected through 2 mL sample injection loop. The column was equilibrated with 1 CV of 50 mM ammonium acetate buffer, pH 7.0 and the protein fractions was eluted with 1 CV with the same buffer at flow rate 1 mL/min. Fractions with size of 6 mL were collected and concentrated using centrifugal concentrator. The concentrated fraction then was screened for the clotting activity and the purity of the active fraction was assessed by SDS-PAGE.

3.7 Determination of protein purity by SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the purity of fraction samples. First, a gel caster was installed. The gel casting included two glass plates, casting frame and casting stand. 12 % separating gel and 5 % stacking gel were prepared by mixing all the ingredients shown in Table 3.3. Freshly made 10 % ammonium persulphate (APS) solution and tetramethylethylenediamine (TEMED) were added to the gel solution before loading to the gel caster. The prepared separating gel solution is then pipetted into the gap between the glass plates and leave about 2 cm below the bottom of the comb for the stacking gel. The separating gel is polymerized in 30 minutes. Then, the stacking gel solution was pipetted on top of the polymerized separating gel and the comb was inserted straight on down without trapping air under the comb.

The 4X sample buffer was prepared by mixing 2.5 mL of 1 M Tris-HCl (pH 6.8), 1 g SDS, 0.8 mL of 0.1 % bromophenol blue and 4 mL glycerol then bring the sample buffer solution up to 10 mL. 50 mg of Dithiothreitol (DTT) was added into 1 mL of prepared sample buffer. To prevent waste of reagent and sample, only 100 µL of sample buffer was then added to 300 µL sample solution before denaturing them at 95 ℃ to 5 - 10 minutes. A total of 5 µL of low protein molecular weight marker was loaded into the first lane and 10 µL of the prepared samples into the other wells. The gel was run with 150 Volts for 75 minutes or until dye almost reaches the bottom of the gel.

Table 3.4 The preparation of separating and stacking gel for SDS-PAGE

Content	12 % Separating gel	5 % Stacking gel
Deionized water	3.30 mL	6.80 mL
30 % Acrylamide/ Bis acrylamide solution	4.00 mL	1.70 mL
1.5 M Tris-HCl, pH 8.8	2.50 mL	-
0.5 M Tris-HCl, pH 6.8	-	1.25 mL
10 % SDS	100 µL	100 µL
10 % APS	100 µL	100 µL
TEMED	15 µL	10 µL

After the run, the gel was removed and rinsed with distilled water. The gel is stained with warm Coomassie blue staining solution for 10 minutes to allow the visualization of the bands. Then the gel was destained with 10 % acetic acid solution on a shaker overnight. The molecular weight of unknown protein was calculated from the equation obtained from the linear plotted graph of the logarithm of the molecular weight of an SDS-denatured polypeptide and its relative migration distance (*Rf*).

3.8 Mass spectrometry analysis

The proteins bands from SDS-PAGE gel was excised and the sample was sent for identification using Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) by Proteomics International, Australia. The protein sample was digested by trypsin and the peptides was extracted. Peptides were analyzed by electrospray ionization mass spectrometry using the Shimadzu Prominence nano HPLC system (Shidmadzu, Japan) coupled to a 5600 TripleTOF mass spectrometer (Sciex). Tryptic peptides were loaded onto an Agilent Zorbax 300SB-C18, 3.5 µm (Agilent Technologies, USA) and separated with a linear gradient of water/acetonitrile/0.1 % formic acid (v/v).

3.9 Evaluating milk clotting activity (enzyme assay)

3.9.1 Milk clotting activity (MCA)

The milk clotting activity of the coagulant was determined using the method of (Arima et al., 1970; Majumder et al., 2015) with few modifications. A 10 % (w/v) of BD DifcoTM skim milk (Thermo Fisher Scientific, USA) containing 10 mM of $CaCl_2$ as a substrate. A total of 1 mL of substrate was added to the 0.5 mL enzyme solution at 35 °C. The milk clotting time was measured according to the condition stated in section of 3.3. Milk clotting activity was expressed in Soxhlet Unit (SU/mL). One SU was defined as the enzyme amount which clots 1 mL of substrate within 40 minutes at 35 °C. The MCA for coagulant was calculated by using Equation (3.1).

$$MCA = (\mathrm{M} \times 35 \times 2400)/(\mathrm{E} \times \mathrm{t} \times \mathrm{T}) \quad (3.1)$$

Where,

M = volume of substrate (mL) t = clotting time (s)

E = volume of enzyme (mL) T = reaction temperature (°C)

3.9.2 Proteolytic activity (PA)

The proteolytic activity was measured by using 1 % (w/v) casein. The casein solution was prepared by mixing 10 mg/mL of casein powder in the 50 mM potassium phosphate buffer (pH 7.5). The solution was stirred gently, and the solution temperature was gradually increased to 80-85 ˚C for 10 minutes (it is very important not to boil the solution) until a homogenous solution is achieved.

Three vials were prepared with 2.5 mL of 1 % (w/v) casein in each vial. The casein solution was equilibrated in a water bath at 37 ˚C for 10 minutes. The enzymes were added to the two of the test sample vials as shown in Table 3.4. The solution was swirled and incubated at 35 ˚C for 30 minutes. After incubation, 2.5 mL of 440 mM trichloroacetic acid (TCA) was added to stop the reaction. Then the appropriate volume of enzyme solution was added to each vial, even in the blank so that the final volume of enzyme solution in each vial is 1 mL. The solution was incubated again at 35 ˚C for 30 minutes and was centrifuged at 12000 g for 10 minutes. A total of 5 mL of 500 mM sodium carbonate was added to 2 mL supernatant and 1 mL of Folin and Ciocalteu's phenol

reagent was added immediately afterward. The absorbance of the solution was measured at 660 nm.

Table 3.5 The procedure of proteolytic activity for both kistomin and rennet.

	Blank	**Kistomin**	**Rennet**
Casein (mL)	2.5	2.5	2.5
Enzyme (mL)	0	0.1	1
Incubate 35 °C for 30 minutes (Reaction time)			
440 mM trichloroacetic acid (mL)	2.5	2.5	2.5
Enzyme added after reaction (mL)	1	0.9	0
Incubate 35 ˚C for 30 minutes, centrifuge 12000 g for 10 minutes			
Test Filtrate (mL)	-	2	2
Blank filtrate (mL)	2	-	-
500 mM sodium carbonate (mL)	5	5	5
Folin and Ciocalteu's phenol reagent (mL)	1	1	1

Standard calibration curve of tyrosine standard was prepared using 1.1 mM L-tyrosine standard stock solution. This solution was prepared by dissolving 0.2 mg/mL L-tyrosine powder in purified water. The solution was heated gently (it is important not to boil the solution) until dissolved. The solution was cooled to room temperature.

Six vials were prepared, and 1.1 mM tyrosine standard stock solution was added with the following volumes: 0.05, 0.1, 0.2, 0.4 and 0.5 mL. Then, the appropriated volume of deionized water was added to bring the volume to 2 mL. A total of 5 mL of 500 mM sodium carbonate and 1 mL of Folin reagent was added immediately. The absorbance of the solution was measured at 660 nm using UV-Vis spectrophotometer.

Table 3.6 The preparation of Standard calibration curve of tyrosine standard using 1.1 mM L-tyrosine standard stock solution.

Volume of standard solution, 1.1 mM L-tyrosine stock (mL)	Volume of deionized water (mL)	Volume of Sodium carbonate (mL)	Volume of Folin reagent (mL)	L-tyrosine concentration (µMoles)
0 (Blank)	2.00	5	1	0.000
0.05	1.95	5	1	0.055
0.10	1.90	5	1	0.111
0.20	1.80	5	1	0.221
0.40	1.60	5	1	0.442
0.50	1.50	5	1	0.553

The calibration curve of L-tyrosine concentration (µMoles) versus Absorbance (660 nm) was constructed and the equation was generated using MS Excel software. The enzyme activity and specific activity for both kistomin and rennet was calculated by using Equation (3.2) and (3.3)

$$\text{Activity } \left(\frac{\text{Unit}}{\text{mL}}\right) = \frac{\text{tyrosine equivalent released (µMoles)} \times \text{Total in assay (mL)}}{\text{Time of assay (min)} \times \text{protease (ml)} \times \text{volume used in calometric determintion (ml)}} \quad (3.2)$$

$$\text{Specific activity } \left(\frac{\text{Unit}}{\text{mg}}\right) = \frac{\text{Activity } \left(\frac{\text{Unit}}{\text{mL}}\right)}{\text{concentration of protein used } \left(\frac{\text{mg}}{\text{mL}}\right)} \quad (3.3)$$

3.10 Determining optimum parameters of isolated enzyme

In this experiment, a total of 1 ml of 10 % (w/v) skim milk containing 10 mM $CaCl_2$ was used as a substrate except for determination of $CaCl_2$ concentration in method (d). The parameters of temperature and pH maintained to be constant (except for the investigated parameter) at 35 °C and pH 6.5.

a) Optimum concentration of coagulant:

Isolated protease from venom was serially diluted based on their protein content range from 0.01-1.28 mg/mL. A total of 1 mL of substrate was then introduced to 0.5 mL

of different concentrations of protease at 35 °C, pH 6.5. The time taken for the milk to coagulate was recorded. The protein concentration was increased until the clotting rate become constant and the graph of Milk Clotting Activity (MCA) versus concentration was plotted.

b) Optimum pH:

pH stability of protease was investigated at pH 5 - 9. Acetate buffer (pH 5), phosphate buffer (pH 6 to pH 8) and glycine-NaOH buffer (pH 9) was prepared by mixed the buffer solution with the acid or base as shown in Appendix A1. Each 0.5 mL of sample solution consisting of protease and an appropriate buffer with varying pH of 5 to pH 9 was mixed with 1 mL of substrate and swirled for 10 seconds at 35 °C. The time taken for the milk to coagulate was recorded. The graph of Milk Clotting Activity (MCA) versus pH was plotted.

c) Optimum temperature:

The substrate (pH 6.5) with protease was incubated in water bath with varying temperature settings i.e. 20 °C to 80 °C. The time taken for the milk to coagulate was recorded. The graph of Milk Clotting Activity (MCA) versus temperature (°C) was plotted.

d) Optimum Calcium ion concentration:

Various concentrations of $CaCl_2$ ranging from 2 to 14 % (w/v) was prepared. Appropriated mass of calcium chloride was dissolved in 1 mL of 10 % (w/v) skim milk at 35 °C, pH 6.5. 0.5 mL of the isolated protease was mixed with the skim milk and swirled for 10 seconds. The time taken for the milk to coagulate was recorded. The graph of Milk Clotting Activity (MCA) versus calcium chloride concentration (w/v) was plotted.

3.11 The clotting activity of protease to the other types of milk

The clotting activity of protease on cow milk and goat milk was carried out using 1 mL of 10 % (w/v) of freeze dried of cow and goat milk containing 10 mM of $CaCl_2$ as substrates. The samples were pre-incubated at 48°C in water bath for 5 minutes. Then,

100 µL of protease was added to the substrates and the milk clotting time was recorded and were calculated by using equation 3.1.

3.12 Kinetic activity of kistomin

The kinetic activity of protease was determined at 48 °C in a substrate range of 0.25-30 mg/mL. 2 % (w/v) of casein dissolved in 50 mM potassium phosphate buffer (pH 6.5) was used as a substrate. 1 mL of substrate was incubated with 100 µL (0.015 mg) protease. The reaction was stopped by adding 900 µL of trichloroacetic acid (TCA) and the solution was incubated for 30 minutes. After incubation, the solution was centrifuged at 12000 g for 10 minutes. One mililiter of supernatant was mixed with 900 µL of 500 mM sodium carbonate and 100 µL of Folin reagent. The absorbance of the solution was measured at 660 nm. The kinetic parameters (K_m and V_{max}) of kistomin was calculated by Michaelis Menten equation with nonlinear regression using GraphPad Prism 7 software.

3.13 Substrate specificity of kistomin

Substrate specificity of protease was determined using κ-casein, β-casein and α-casein in a substrate range of 0.05-5.0 mg/mL (2.63-263.16 mM), 0.05-5.0 mg/mL (2.08-208.33 mM) and 0.05-8.0 mg/mL (2.17-347.83 mM), respectively. The substrates were dissolved in 50 mM potassium phosphate buffer (pH 6.5) and was serially diluted. The reaction was initiated by adding 100 µL (0.015 mg) of kistomin and was incubated for 10 minutes at 48 °C.

The reaction was stopped by adding 900 µL of trichloroacetic acid (TCA) and the solution was incubated for 30 minutes. After incubation, the solution was centrifuged at 12000 g for 10 minutes. One mililiter of supernatant was mixed with 900 µL of 500 mM sodium carbonate and 100 µL of Folin reagent. The absorbance of the solution was measured at 660 nm. The substrate specificity of kistomin toward substrates were compared using the K_m value calculated by Michaelis Menten equation with nonlinear regression using GraphPad Prism 7 software.

3.14 The effects of metal ions and protease inhibitor on Kistomin activity

The effects of various metal ions and protease inhibitor were determined using 2 % (w/v) of casein dissolved in 50mM potassium phosphate buffer (pH 6.5) as a substrate. 100 µL of 20 mM/mL of various metal ions ($CuSO_4$, $MgCl_2$, $MgSO_4$, $FeSO_4$, $Pb(NO_3)_2$, $HgCl_2$, $BaCl_2$, $MnSO_4$ and $CaCl_2$) and protease inhibitor: 10 mM PMSF, 1 mM Pepstatin A, 10 mM EDTA, 5 mM Iodeacetamide and 10 mM 1,10 phenanthroline was pre-incubated with 100 µL (0.015 mg) protease for 10 minutes in 48 °C. One milliliter of substrate was then added to each tube containing the mixture of the enzyme and metal ions/inhibitor.

The solution was incubated in the water bath for 30 minutes at 48 °C and the reaction was stopped by adding 900 µL of trichloroacetic acid (TCA). The solution was incubated for 10 minutes. After incubation, the solution was centrifuged at 12000 g for 10 minutes. One mililiter of supernatant was mixed with 900 µL of 500 mM sodium carbonate and 100 µL of Folin reagent. The absorbance of the solution was measured at 660 nm. The residual activities were determined and compared to the control which prepared by preincubation the enzyme with buffer and corresponds to 100 % activity.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

The objective of this study was to discover the potential of *C. rhodostoma* venom in milk clotting activity. Thus, the study on the milk clotting activity (MCA) and parameters optimization of the protein was performed to analyse the potential and maximize the enzymatic activity; after the isolation and the identification of the protein responsible for the clotting activity. Proteolytic activity, enzyme inhibition and kinetic parameters were also performed for the protein characterization.

4.2 Determining protein content and milk clotting activity of various venoms.

Snake venom mainly composed of a complex mixture of proteins. The amounts and the types of proteins present in various snake venom also different (Anthony, 1996). The protein contents of various snake venoms were determined by using Bradford protein assay. Bradford protein assay works by the conversion of red to blue form of dye (Coomassie Brilliant Blue G-250) upon binding of dye to the proteins present in the samples (Bradford, 1976). Therefore, the absorbance measured which indicates the amount of complex formed was used to determine the concentration of proteins in solutions.

In this study, the protein content of the eleven snake venoms was between 30 and 80 %. Generally, snake venoms mainly comprise of protein which makes 70-90 % of the dry weight of snake venom (Tu, 1977; Vyas et al., 2013). Different species have different types of venoms and even the composition of protein within the single species vary depending on age, climate, geographical location, its habitat, diet and etc (Aragon and Gubensek, 1981; Alape-Girón et al., 2008; Goswami et al., 2014; Vyas et al., 2013).

Table 4.1 Milk clotting time of various snake venoms.

No.	Sample	Protein content (µg) Mean ± SD	Percentage of protein (%)	Clotting time (minutes) mean ± SD
1.	*C. rhodostoma* (viper)	778.89±0.05	77.8	2.83±0.17
2.	*N. sputatrix* (cobra)	268.40±0.02	26.8	3.33±0.59
3.	*T. wagleri* (viper)	574.36±0.03	57.4	3.67±0.97
4.	*B. candidus* (krait)	478.63±0.03	47.9	12.00±0.88
5.	*N. kaouthia* (cobra)	384.91±0.00	38.5	15.03±0.33
6.	*O. hannah* (cobra)	287.18±0.03	28.7	30.00±0.32
7.	*N. nivea* (cobra)	752.14±0.03	75.2	47.00±0.81
8.	*B. fasciatus* (krait)	678.63±0.02	67.9	>60*
9.	*T. hageni* (viper)	628.59±0.02	62.9	>60*
10	*T. sumatranus* (viper)	428.21±0.00	42.8	>60*
11.	*T. purpureomaculatus* (viper)	547.01±0.03	54.7	>60*

Data presented as standard deviation (SD) with repeats of n=3. *denotes those samples unable to show convincing clotting immediately.

Aragon and Gubensek (1981) found that the crude venoms of *Bothrops asper* originating from Pacific and Atlantic zones remarkably differ in their specific activities and protein component due to taxonomic differentiation between *B. asper* snakes from two zones. In addition, analysis of pooled venoms of neonate samples with those mature snakes from Caribbean and Pacific regions revealed a prominent ontogenetic change in both geographical populations. The changes appear to be a secretion of PLA_2 molecules in adults is different than in the neonates and shift from a PIII-SVMP rich to a PI-SVMP rich venom. Venom composition change results in increasing venom complexity due to the requirement for the venom to immobilize prey and initiate digestion change with the age (Alape-Giron et al., 2008). Mackessy et al. (2006) has conducted a study of protein content in four growth categories (neonates, juveniles, adults and large adults) of *Boiga irregularis* snake and found that the percentage of the protein content in this snake is increasing from 47.5 to 90.2 % as the snake aged.

Milk clotting activity is essential in the dairy industry as the properties of milk clotting activity greatly influence cheese and yogurt making. Screening for clotting property was performed to detect the milk clotting properties of the snake venoms. There are few methods that have been proposed to monitor the milk clotting activity. For instance, the visual observation method by Berridge (1952), which state that the clotting time is determined as the time of appearance of flocks of renneted standard milk substrate on the wall of a test tube of flocks of milk. In addition, Farah & Bachman (1987) defined the clotting time as the time required for the first appearance of graininess in the moving film of milk on the surface of the glass wall. The Berridge (1952) method is the current standard method of IDF Standard 157:2007/ISO 11815 for bovine rennet and measures milk-clotting activity.

However, the IDF Standard 157:2007 method is subjective because it depends on operator's skill to consistently identify milk flocculation (Tabayehnejad et al., 2012). Besides, Formagraph had been used to study the milk clotting time. According to Cecchinato (2012), the milk clotting measured using Formagraph works by monitoring the movement of pendulums which are immersed in oscillating samples of clotting milk. Jacob et al. (2011) stated that rotational viscometer was used to determine the milk clotting time. It involves the measurement of viscosity of milk using rotating spindle viscometers. However, most of the proposed methods are not applicable in this research due to the quantity of snake venom available. Hence, an effort to miniaturise the whole screening activity was required, and the visual method was selected described in Section of 3.3.

The screening method shows *C. rhodostoma* have rapid milk clotting activity averaging at 2.83±0.17 minutes in comparison to other ten venom samples. Two viper species, which are *C. rhodostoma* and *T. wagleri* have rapid clotting while the other five elapid snake species shows the clotting time less than an hour. Generally, venoms of Viperidae contain more haemotoxin and metalloproteinase and possess very strong proteolytic activity while Elapid venoms contain neurotoxins and cardiotoxin and have very weak proteolytic activity (Farid et al., 1989; Li et al., 2004). The previous study by Vejayan et al. (2014) shows that the 2DE profiles of Viperidae and Elapidae show clear distinction which viperidae venoms are typically observed in high molecular mass regions and at neutral pI while Elapidae venoms observed in lower molecular mass ranges and

concentrated around the basic pI. *C. rhodostoma* possess moderate hemorrhage activities (Tan & Ponnudurai, 1996) and has clotting enzyme activities of 133 NIH unit/mg compared to other venoms (Tan, 2004). *C. rhodostoma* venom exhibits strong thrombin-like enzyme. Ancrod, the major form of thrombin like-enzymes in *C. rhodostoma* venom which acts as a coagulant in-vitro accounts approximately 7 % of venom dry weight (Esnouf and Tunnah, 1967). In addition, ancrod is a serine proteinase synthesized as a pre-zymogen. As well as reptilian fibrinogenases, ancrod exhibit a high degree of sequence similarity to this mammalian serine proteinase (Burkhart et al., 1992; Tan, 2004). Hence highlighting the contribution of enzymes within *C. rhodostoma* in cleaving protein of milk into curd and whey.

Meanwhile, the elapid snake from this study also possess clotting activity but the time taken for clotting is longer than viper species. Elapid species mainly comprised of neurotoxins (Vejayan et al., 2014) and have weak proteolytic activity (Farid et al., 1989). In addition, elapid venoms contain various active basic polypeptides (non-enzymatic) which constitute 25-60 % of the venom dry weight and have fewer enzymes than viper venoms (Tan, 2004). However, *N. sputatrix*, (Javan splitting cobra) also gave rapid endpoint clotting, 3.33±0.59 minutes i.e. second to *C. rhodostoma* venom. *N. sputatrix* venom may have evolved from a specialization of prey ingestion rather than prey capture since the venom can cause disruption of the cornea and produces intense pains when in contacts with eye (Ismail et al., 1993; Young et al., 2004). Moreover, Tan (2004) stated that, 5 % of *N. sputatrix* venom dry weight consists of high molecular weight proteins and enzymes (> 30000 MW); and the enzyme composition included phosphodiesterase, 5'-nucleotidase, protease, L-amino acid oxidase, hyaluronidase, alkaline phosphomonoesterase and acetylcholinesterase (Tan & Tan, 1987).

In this study, *O. hannah* venom also exhibited milk clotting activity. *O. hannah* or King Cobra has higher enzyme content than venoms of other cobras. The biological active elements that have been characterized are neurotoxin, hemorrhagic and non-hemorrhagic proteases, L-amino acid oxidase, PLA_2 enzymes and alkaline phosphomonoesterases (Tan, 2004). This venom contains five proteases which likely hemorrhagic protease which has molecular weight around 70000 Dalton (Yamakawa and Omori-Satoh, 1988). Furthermore, the previous study by Vejayan et al. (2014) found the protein map of *O. hannah* display dissimilarities in relation to the other two elapids (*N.*

kaouthia and *B. fasciatus*). In 2-DE proteome venom profile, protein spots of *O. hannah* venom were displayed at the higher molecular mass region similar to the *C. rhodostoma*.

4.3 Determining milk clotting activity of *C. rhodostoma* venom towards four types of milk

C. rhodostoma venom exhibited good milk clotting activity and was screened for its ability to coagulate different types of milk. Table 4.2 shows four types of milk were used in this experiment which are cow, goat, camel and soy milk to investigate the action of *C. rhodostoma* venom and *M. miehei* rennet. In previous study by Vejayan et al. (2017), *C. rhodostoma* venom has clotting activity on cow's milk, as the other types of milks also highly been consumed, the clotting activity of *C. rhodostoma* venom on other types of milks were also been tested in this study. The control is the milk sample without the addition of *C. rhodostoma* venom and *M. miehei* rennet. Protein content of the various milk was determined ensured to be similar protein concentration for all. The concentration of *C. rhodostoma* venom and rennet used in this experiment were 0.65 % (w/v) and 0.32 % (w/v), respectively, with other parameters include temperature at 45.5 °C and at pH 7 (Vejayan et al., 2017). Additionally, 10 mM of calcium chloride ($CaCl_2$) was added to the milk samples as a clotting enhancer except for soy milk. Calcium chloride is calcium salt under "nigari-type" or chloride type coagulant which has been used as a coagulant for production of soybean curd. In addition, according to previous study by Li Tay et al. (2006), $CaCl_2$ has the strongest clotting power based on the formation of precipitates, states of curds and turbidity measurement on soy proteins defeating other salt coagulant (magnesium chloride, calcium sulphate and magnesium sulphate). In short, calcium ion served as cofactor for protease to cleave milk protein to curd. However, for soymilk it served as the coagulant itself. Therefore, requiring omission of calcium chloride for soymilk screening.

According to Table 4.2, the venom shows a faster clotting time to cow milk compared to rennet which are 58.33±2.89 s and 88.33±2.89 s, respectively. *C. rhodostoma* venom contains a protease enzyme that has high catalytic activity towards the casein protein compared to the protease enzyme in *M. miehei* rennet, which is chymosin. *C. rhodostoma* clotting time is much faster under optimum conditions compared to rennet.

Table 4.2 The clotting activity of *C. rhodostoma* venom and *M. miehei* rennet on cow, goat, camel and soy milk.

Sample	Control	Cow	Goat	Camel	Soy
Venom	No clotting	Coagulate 58.33±2.89 sec	Coagulate 51.67±2.89 sec	Coagulate >3 hours	*No clotting
Centrifugation at 10 000 rpm					
Amount of curd	-	0.146±0.010 g	0.099±0.005 g	0.046±0.007 g	-
Rennet	No clotting	Coagulate 88.33±2.89 sec	Coagulate 36.67±2.89 sec	Coagulate >3 hours	No clotting
Centrifugation at 10 000 rpm for 1 minute					
Amount of curd	-	0.129±0.004 g	0.094±0.004 g	0.042±0.004 g	-

*without $CaCl_2$; n=3

The time taken for both venom and rennet to coagulate goat milk is faster than the time taken for both coagulants to coagulate cow milk. Goat milk differ from cow milk in having a higher digestibility of protein and fat. It has smaller fat globules and more short and medium chain fatty acids (MCT). This is because there is less total casein in the goat milk content compared to the cow milk (Park, 2004). In addition, the difference in chemical composition in cow and goat milk related to the individual casein micelle structures. Goat milk has slightly smaller micelle structure than cow milk and results in slower reaction rate of cow milk with the protease enzyme compared to goat milk. Due to the smaller micelles present in goat milk, the κ-casein protein on the outside of

individual micelles is broken down at a faster rate compared to cow milk, thus takes longer to rupture the micelle structures of cow milk. The uniqueness of renneting kinetic of goat milk has also been characterized as having a shorter clotting time and greater hardening rate (Selvaggi & Tufarelli, 2012).

The clotting time for camel milk was more than 3 hours. The clotting time for camel is longer due to the molecular difference in camel milk proteins compared to other milk proteins such as various fractions of the casein, the distribution and size of the casein micelles, sites of the potential cleavage and many more (Boudjenah-Haroun et al., 2012). The clotting time varies with the micelle size and reaches an optimum in the medium and small size micelles. Camel milks have a bigger micelle structure, and this affects the availability of κ-casein where the content of κ-casein decreases with increasing micelle size (Ekstrand et al., 1980).

There is no milk clotting occur for soy milk with the addition of venom or rennet. This is because soy milk does not contain casein (Rozenfeld et al., 2002) and casein can only be found on mammalian milk. The protease enzyme in the venom and rennet only act on casein protein especially on κ-casein (for rennet), a type of protein which can be found in all mammalian milks. Soy milk now gaining importance as alternative to dairy milk especially for those customers experiencing lactose intolerance, cow milk allergy, calorie concern and prevalence of hypercholesterolemia (Sethi et al., 2016).

The samples were centrifuged at 10 000 rpm in order to separate the curd and the whey completely. It was found that the amount of curd obtained from cow milk for *C. rhodostoma* venom and *M. miehei* rennet higher than the amount of curd obtained from goat milk for *C. rhodostoma* venom and *M. miehei* rennet. Cow milk forms more curd compared to goat milk due to their higher composition of αs1-casein which is a structural component of casein micelle. Goat milk contains lower level of αs1-casein which is around 4 to 26 % compared to cow milk which is around 36 to 40 % (Clark & Sherbon, 2000). This is due to αs1-casein proteins that precipitate on contact with calcium forming the aggregates and are responsible for the amount of coagulated curd yielded (Clark & Sherbon, 2000).

As for camel milk, the amount of curd obtained due to *C. rhodostoma* venom is and *M. miehei* rennet was less than the mass amount obtained from both cow and goat

milk. This is due to more αs1-casein and β-casein proteins present in each cow and goat milk micelle molecule compared to camel milk (Boudjenah-Haroun et al., 2012). The yield of curd hence dependent on the clotting time of the various milk.

4.4 Purification of protease from *C. rhodostoma* venom

C. rhosdostoma venom was chosen for further purification process to isolate the protein responsible for milk clotting activity. Milk clotting enzyme produced by *C. rhodostoma* venom was fractioned using two standard purification techniques which were Ion Exchange chromatography and followed by Size Exclusion chromatography.

4.4.1 Ion exchange chromatography

The crude venom of *C. rhodostoma* was initially fractionated using ion exchange chromatography technique. The venom sample was injected into a strong cation, HiTrap SP FF column attached to AKTA Explorer and was equilibrated with 50 mM of ammonium acetate buffer (pH 6). The separations of proteins were observed at 280 nm because protein absorbs UV light at this wavelength. The crude venom was separated into four peaks on the HiTrap SP FF column eluted with linear gradient of 1.0 M NaCl in 50 mM ammonium acetate buffer (pH 6). Linear gradient is an ideal method when starting with unknown protein. Figure 4.1 shows the cation exchange chromatography of 25 mg *C. rhodostoma* crude venom and a total of 4 peaks were eluted out. The first fraction was an unbound fraction where the proteins does not bind to the negatively charged resin and was eluted out. The unbound fraction contains unwanted proteins (negatively charged molecules) and other impurities. A salt gradient was used to separate the protein of interest from other bound proteins and the protein was eluted in an order depending on their net surface charge. The second and third peak was eluted at a lower ionic strength whereas the fourth peak was eluted at high salt concentration. The protein of interest has positive net charge based on the chromatogram profile and has isoelectric point (pl) higher than 6.

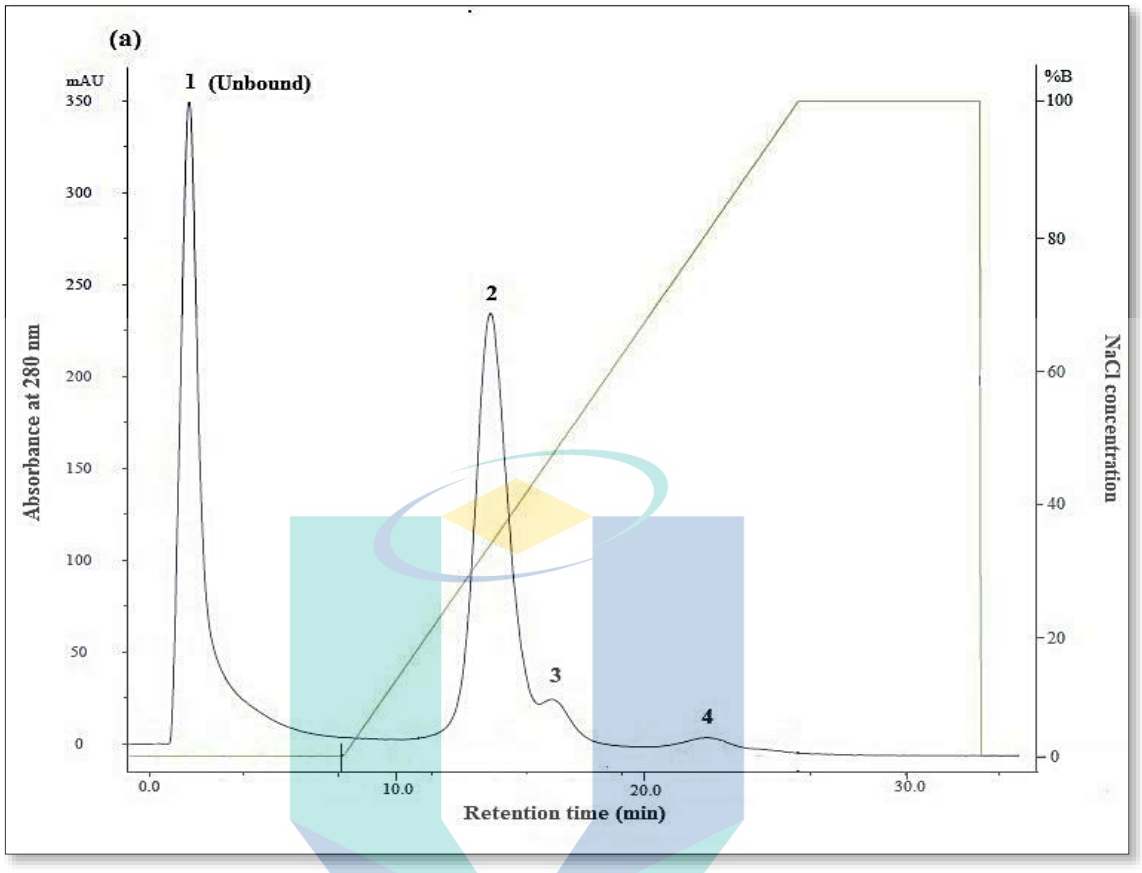


Figure 4.1 HiTrap SP FF cation exchange chromatography of 25 mg *C. rhodostoma* crude venom. A total of 4 peaks were eluted out and the desalting done for all cumulated fractions. Upon screening the fractions for clotting activity, only peak 2 observed with activity and will be further separate using size exclusion chromatography.

Proteins content of the *C. rhodostoma* venom evenly composed of basic and acidic protein, in which the venoms proteins were shown to be evenly dispersed across the pI on the proteomic profiles of 2DE (Vejayan et al., 2014). In addition, at least 96 distinct proteins comprised of 29 basic and 67 acidic proteins in 11 families were identified from the venom (Tang et al., 2016). The four fractions were screened for their clotting activities and only one, peak 2 which eluted at retention time of 14.55 minutes, exerted a milk clotting activity. Peak 2 was eluted at 26 % to 37 % of salt concentration which shows that the protein of interest is weak positively charged protein. This fraction was desalted and concentrated using Vivaspin 20 centrifugal concentrator. This fraction was then subjected to size exclusion chromatography on a Sephacryl S 200 HR.

4.4.2 Size exclusion chromatography

The active fraction from ion exchange chromatography was further purified using size exclusion chromatography. The fraction from ion exchange chromatography was injected to HiPrep 26/60 Sephacryl S-200 HR pre-packed column. The column was equilibrated with 50 mM of ammonium acetate buffer (pH 7) and the elution was carried out using the same buffer with 2 mL sample injection at flow rate of 1 mL/min. Figure 4.2 shows the chromatogram of size exclusion chromatography of active fraction from peak 2 from ion exchange chromatography were fractionated into six peaks in one column volume. Table 4.3 shows the screening results where the milk clotting activity were tested on each of the fractions and only the highest peak which is peak 5b at retention time 199.09 minutes exhibited milk clotting activity. The peak 5b has small molecular weight since it eluted at the end of the fractionation. The proteins which have molecular weight bigger than the range 5-250 kDa cannot enter the Sephacryl S-200 HR beads. In size exclusion chromatography, the larger molecules elute first, and the smaller molecules elute last.

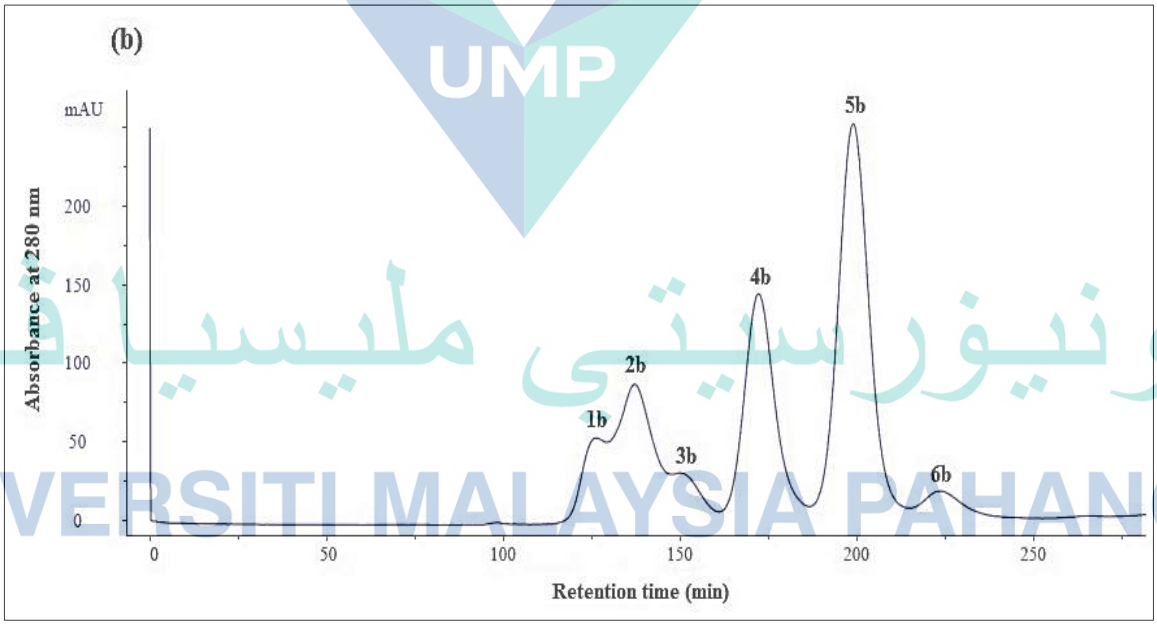


Figure 4.2 Size exclusion of active peak 2a (25 mg) separated into peaks of 1b - 6b. Only peak 5b shows clotting activity on milk during screening process.

Table 4.3 The screening result of active peak 2a of size exclusion chromatography.

Peak	Clotting activity
1b	No
2b	No
3b	No
Pooled peak (1b, 2b, 3b)	No
4b	No
5b	Yes
6b	No

The results of activity and protein assays from a protein purification steps of kistomin starting from first sample which is crude venom to the last step of purification are summarized in purification Table 4.4. Such a table important to evaluate the effectiveness of the purification steps (Burgess, 2009). In addition, the development of the purification procedures should be considered on the stability of protein, the ability of step to remove contaminant and the ability to maintain the biological activity of the protein of interest.

Table 4.4 shows the purification scheme of milk clotting enzyme from *C. rhodostoma*. The desired purification scheme should consider both purification fold and yield (Berg et al., 2002). Yield is a parameter to measure the protein/enzyme activity retained or based on the recovery of the activity after each purification step as a percentage of the activity in the crude extract. The amount of enzyme activity in the initial extract is taken to be 100 %. Purification fold is a parameter to measure the increase in purity and is obtained by dividing the specific activity which calculated after each purification step by the specific activity of the initial crude extract. A high yield with low purification fold leaves many contaminants which proteins other than protein of interest in the fraction and complicates the experiment interpretation. A high purification fold and poor yield leave little protein that can be used for experiment (Berg et al., 2002).

Table 4.4 Summary of the purification of protease from *C. rhodostoma* venom, n=3.

Step	Volume (mL)	Total Protein (mg)	Activity (SU/mL) ± SD	Total Activity (SU) ± SD	Specific Activity (SU/mg) ± SD	Yield (%)	Fold ± SD
Crude	20	10.00	10.39±0.16	207.8±3.27	20.78±0.32	100	-
HiTrap SP FF	20	3.46	6.03±0.03	121.54±0.51	34.84±0.15	58.48	1.68±0.02
Sephacryl S 200 HR	24	1.22	4.66±0.02	112.92±0.37	91.74±0.30	54.34	4.41±0.06

In every purification step, the fractions were collected, screened for their clotting activity and calculated the protein content of active fractions via protein assay. The first purification step which is the ion exchange chromatography showed a yield of 58.48 % and purification fold of approximately 1.68. After the selected fraction from ion exchange chromatography was desalted and concentrated using Vivaspin 20 centrifugal concentrator, the fraction passed through size exclusion column, the protein purification fold increased to 4.41 and the yield was 54.34 %. The overall performance of the proposed method has high protein recovery/yield and good purification fold. The high recovery value was achieved using Sephacryl as size exclusion media and centrifugal concentrator. Centrifugal concentrator was a better tool to remove the salt content in the sample fractions after the ion exchange chromatography since it can remove 99 % of the initial salt content after 3 cycles of centrifugation. In addition, it is non-denaturing method, reduced sample loss, better protein recovery (90-95 %), no sample dilution and time saving when compared to the conventional method. The conventional method of freeze-drying followed by desalting with Sephadex G-25 column was time consuming and possible to lose some of the protein during the process.

In this experiment, two chromatography techniques were chosen to isolated enzyme responsible for milk clotting which are ion exchange chromatography and size exclusion chromatography. The purification of metalloproteases is commonly performed using two or three chromatography steps with a predominance of size exclusion and ion exchange chromatography (Menaldo et al., 2015). However, a single purification process using a single chromatography step was described for purification of metalloproteinase P-I Neuwiedase from *Bothrops neuwiedi* (Rodrigues et al., 2000). Some studies also

showed the isolation of snake venom metalloprotease using three chromatography steps of combining ion exchange, size exclusion and affinity chromatographies to isolate BpirMP from *B.pirajai* and BmooMP-α from *B. moojeni* (Bernardes et al., 2008; Bernardes et al., 2013). Although purification method of kistomin proposed by Huang et al. 1992 widely used 3 types of columns i.e. CM-Sephadex column, gel filtration on Sephadex G-75 and Sephacryl S-200, however the new method described in the present study was simple and can reduce the loss of sample during fractionations since this method only needed two steps to isolate the kistomin to purity.

4.5 Protein identification and characterization

Protein identification and characterization is one of the important steps in protein study. Protein identification includes estimation of protein molecular weight, assessment of protein purity and protein identification by mass spectrometry. In this study, three methods were applied to identify and characterize the isolated protein; SDS-PAGE for estimation of protein molecular weight and assessment of protein purity, inhibition study for identification of their catalytic site architecture and mass spectrometry for protein identification.

4.5.1 Determining the protein purity by SDS-PAGE

To determine the success of protein purification scheme, each step of procedure was monitored by determining the specific activity of protein and performed the SDS-PAGE. The protein profile of *C. rhodostoma* freeze dried venom was analysed using 12 % SDS-PAGE separating gel under reducing condition shows the proteins of the venom is in between 15 kDa to 150 kDa based on low molecular weight marker which shown in Figure 4.3 Lane 2. Two particularly condensed bands can be observed in two sites which is in between 14-20 kDa and the most condensed band region is in between 20-30 kDa.

Venom of *C. rhodostoma* consists of mainly snake venom metalloproteinases (SVMP) which is 41.17 % of total venom proteins, within which the kistomin (P-I) is 20.4 % and rhodostoxin (P-II) which is 19.8%. This followed by C-type lectins (snaclec) which covered 26.3 % of venom total protein, snake venom serine protease (14.9 %), L-amino acid oxidase (7.0 %), phospholipase A_2 (4.4 %), cysteine-rich secretory protein (2.5 %) and five minor toxins totaling 2.6 % which are nerve growth factor, phospholipase B, 5' nucleotidase, neurotrophin and phosphodiesterase (Tang et al., 2016). The

condensed sites between 14-20 kDa suspected to be C-type lectins (snaclec) which covered 26.6 % of total protein content in *C. rhodostoma* venom with the molecular weight range from 14 kDa to 20 kDa (Tang et al. 2016). In addition, Vejayan et al. 2014 conducted a comparative analysis of the venom proteome of *C. rhodostoma* and the study found that rhodocytin subunit beta (snaclec) and rhodocetin alpha subunit (snaclec) has molecular weight of 14 kDa and 15 kDa, respectively. The other protein suspected between 14-20 kDa were thrombin-like enzyme, disintegrin, cysteine-rich secretory protein and L-amino acid oxidase (Tang et al., 2016).

The most condensed region of protein band which is between 20-30 kDa were suspected to be SVMP protein family P-I kistomin which account for 20.4 % of total venom protein. The other protein in this region were ancrod-like protein, venombin A (Vejayan et al., 2014) and kinesin-like protein (Tang el al., 2016). 2 % of the total venom proteins in *C. rhodostoma* (Tang et al., 2016). The protein band between 30-60 kDa were protein from SVMP family Class II (P-II), ancrod-like protein 2 (36, 40 and 50 kDa different level of glycosylation), thrombin-like enzyme contortrixobin and L-amino acid oxidase (Vejayan et al., 2014; Tang et al., 2016).

The protein band between 66 to 100 kDa were suspected to be protein from SVMP family Class III (P-III), phosphodiesterase 1, snake venom 5'-nucleotidase, thrombin-like enzyme ancrod and L-amino acid oxidase (Tang et al., 2016). This is because the SVMPs protein family that is abundant in *C. rhodostoma* venom were subdivided into three classes which are P-I, P-II and P-III and the characteristics that distinguished the different classes was their molecular weight and domain structure. P-I is the small SVMPs that have molecular weight of 20-30 kDa, the medium size SVMPs (P-II) have molecular weight of 30-60 kDa and P-III that is the large SVNPS have molecular weight of 60-100 kDa (Markland & Swenson, 2013).

After the purification steps of crude *C. rhodostoma* venom, the active fraction from size exclusion chromatography was subjected for protein identification and characterization. In this study, three steps applied to identify the isolated protein that possessed milk clotting activity. The first step to evaluate the purity and molecular weight of the protein by running the active fraction rom size exclusion chromatography to SDS-PAGE for estimation of protein molecular weight and assessment of protein purity.

The active fraction from size exclusion was subjected to SDS-PAGE to evaluate the purity of the protein by visualizing the protein with Comassive blue staining. Figure 4.3 Lane 3 showed the purified protease yielded a single band suggesting that this protein is monomer and its molecular mass was approximately 26 kDA according to the calculation from standard curve obtained from protein molecular weight standard (APPENDIX B1).

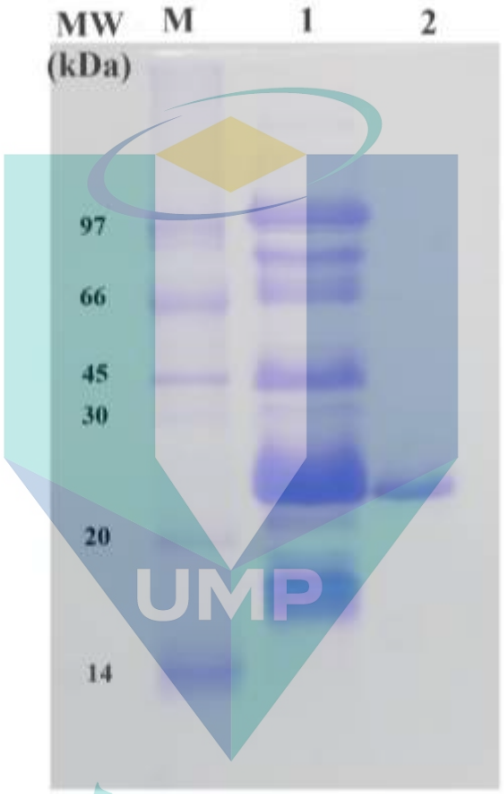


Figure 4.3 Visualization of protein band of *C. rhodostoma* crude venom and HiPrep 26/60 Sephacryl S 200 HR size exclusion chromatography peak 5 on SDS-PAGE gel. Lane 1: Low Molecular Weight Calibration Kit (GE Healthcare Biosciences, USA) molecular marker, Lane 2: Crude venom of *C. rhodostoma*, Lane 3: HiPrep 26/60 Sephacryl S 200 HR size exclusion chromatography peak 5.

Two proteins from *C. rhodostoma* venom which have the molecular weight around 26 kDa are SVMPs protein family P-I Kistomin and thrombin-like enzyme ancrod. Both proteins are expressed by a venom gland and has monomer subunit structure. As kistomin belong to small SVMP family, with molecular weight between 20-30 kDa. Purified kistomin from *C. rhodostoma* showing a single band about 25 kDa on SDS-PAGE (Hsu et al., 2008). Huang et al. (1992) isolated purified fibrinogenase kistomin using three column chromatography steps and revealed that kistomin was a single peptide-chain with a molecular mass of about 21800 Dalton. According to Barrett et al.

(2012), the calculated molecular weight of kistomin is 28142 Dalton and gel-filtration chromatography of kistomin yielded a molecular weight of 30 kDa. In 2014, Vejayan et al. (2014), conducted comparative analysis of the venom proteome of four important Malaysian snake species including *C. rhodostoma* venom. The protein spots from *C. rhodostoma* venom was extracted and analysed with MALDI-TOF MS, and the identification of the proteins isolated from 2-DE gels was performed by scanning the monoisotopic masses with two separate Internet search programs which were MASCOT and ExPASY. The result found that the molecular weight of metalloproteinase kistomin is 25846 Dalton. The molecular weight for ancrod from the database is 25213 Dalton and 29314 Dalton for ancrod-like protein (MASCOT) and 26571 Dalton for Ancrod Venombin A (ExPASY) from SDS-PAGE.

SDS-PAGE is effective method for assessment of protein purity of purification scheme, estimation of molecular weight of the protein of interest and sometimes identification of protein. However, since there are two proteins with similar molecular weight (ancrod and kistomin) in *C. rhodostoma* venom, the isolated protein was subjected to inhibition study and Mass Spectrometry analysis for conclusive protein identification.

4.5.2 The effect of protease inhibitor and metal ions to purified protease

Protease grouped into four classes based on their catalytic action included as aspartic, cysteine, metallo and serine proteases. Protease inhibitors are molecules that block the activity of proteases. Protease inhibitor typically function on classes of proteases with similar mechanism of action and commercially available to use experimentally during protein purification. In order to determine the nature of protein of interest, the effect of different protease inhibitors on proteolytic activity of protein were tested. In addition, this step is usually done to determine the identity of isolated unknown protein (Merheb-Dini et al., 2009; El-Bendary et al., 2007; Egito et al., 2007; Li et al., 2012).

Table 4.5 shows the effect of protease inhibitors on protease to determine the classes of purified protease from peak 5. The protease from peak 5 were incubated with five different protease inhibitor and the residual activities of the protease were determined and compared with the control which corresponds to 100 % of activity. The purified protease was completely inhibited in the presences of EDTA and 1,10-phenantroline and

the maintenance of activity in the present of iodeacitamide (cysteine protease inhibitor), pepstatin A (aspartic protease inhibitor) and PMSF (serine inhibitor) inhibitors. Ancrod is a serine protease and as the purified protease still retained its activity in the addition of PMSF a serine inhibitor, the purified protein is not an Ancrod. It was concluded that this purified protein is metalloprotease protein of SVMP P-I Kistomin. Kistomin is a metalloprotease and as expected both EDTA and 1,10-phenantroline inhibited protein in peak 5 (Huang et al., 1992; Hsu et al., 2007). The pre-treatment of EDTA inhibited the fibrinogenolytic activity of protein in peak 5 suggesting that it is kistomin metalloproteinase (Huang et al., 1992). EDTA is a strong chelator of metal ions (Hazra et al., 2012). EDTA and 1, 10- phenanthroline inhibit metalloprotease activity by lowering the concentration of metal ion and removed it from the active site of the enzyme.

Table 4.5 Effect of different protease inhibitors on the activity of protease, n=3.

Protease Inhibitor	**Activity (%) ±SD**
Control (no inhibitor)	100
PMSF (serine inhibitor)	64.99±2.77
EDTA (Metalloproteinase inhibitor)	0
Iodeacitamide (Cysteine protease inhibitor)	81.43±3.48
Pepstatin A (Aspartic protease inhibitor)	88.58±6.39
1, 10-phenantroline (Metalloproteinase inhibitor)	0

The most important rennet substitutes include enzymes of microbial origin, genetically modified recombinant proteases and plant proteases. Different from other milk clotting protein which mostly from aspartic proteinase, this milk clotting enzyme is from metalloproteinase. All commercial clotting enzymes are aspartic proteases that specially cleave the Phe_{105}-Met_{106} of kappa casein (Jacob et al., 2010), with the exception of the metalloprotease from *Termitomyces clypeatus* MTCC 5091 (Majumder et al., 2015) and *Paenibacillus spp.* BD3226 (Hang et al., 2016), which both have high milk clotting activity.

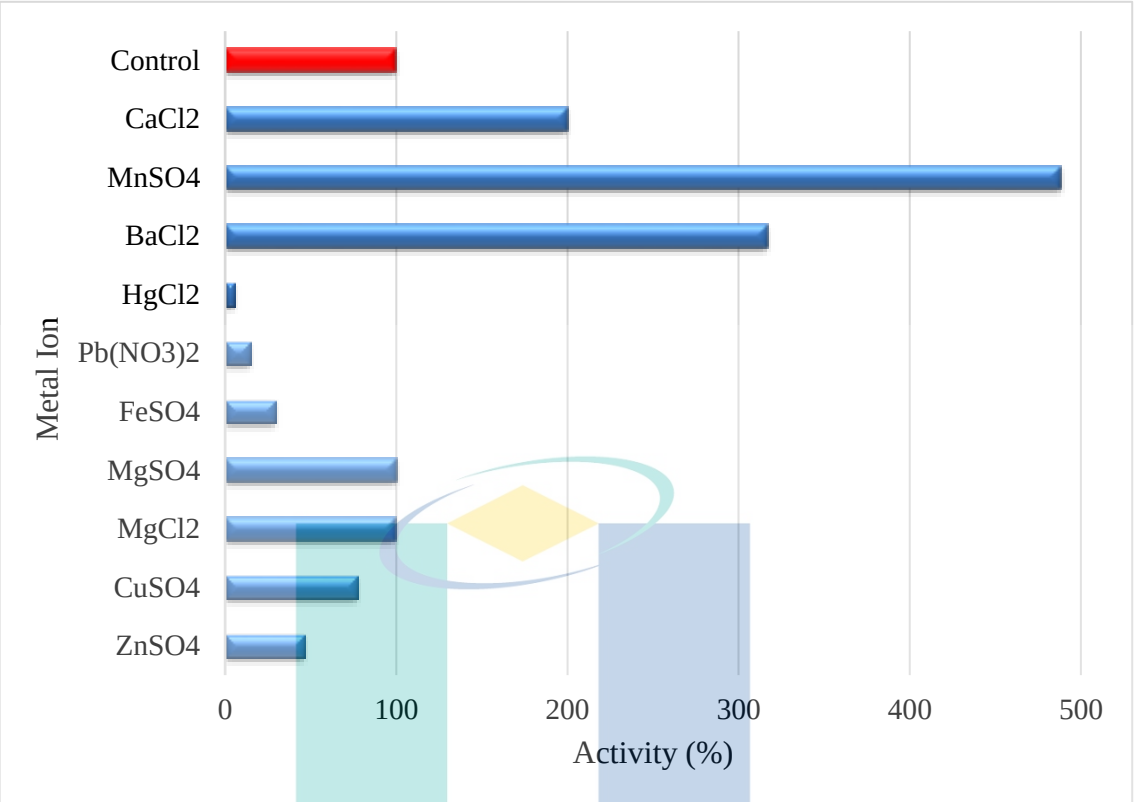


Figure 4.4 The effects of various metal ions on milk clotting activity of the kistomin.

Figure 4.4 shows the effects of metal ions on milk clotting activity of the kistomin enzyme. As shown in Figure 4.4, the addition of Mn^{2+}, Ba^{2+} and Ca^{2+} significantly increased the enzyme activity. However, kistomin was inhibited by Hg^{2+}, $Pb^{2+}$and Fe^{2+} ions. The effect of ions over protease stability because of interaction between them, with the formation of complexes such as ions used as cofactors, substrates and co-substrates (Merheb-Dini et al., 2009). The effects of ions on enzyme activity may be caused by the competitive and noncompetitive inhibition of the enzyme. According to Zhao (2005), halophilic enzyme requires high salt concentration to keep the activity and high stability. Therefore, some of the metal ions studied exhibited an activating effect while some exhibited inhibiting effect which caused by interaction between substrate, enzyme and salts (Merheb-Dini et al., 2009). Kistomin is metalloprotease enzyme and this class of proteases is characterized by its requirement of divalent metal ions for its catalytic activity (Rao et al., 1998). Metalloprotease usually associated with Zn^{2+}, but other metals have been found at active site of metalloprotease, such as Mn^{2+}, Ca^{2+}, Mg^{2+}, Cu^{2+} and Co^{2+} (Holmquist & Vallee, 2006; Hase & Finkelstein, 1993; Asoodeh & Mohammadian Musaabadi, 2012; Graham et al., 2005). Pb^{2+} and Hg^{2+} was found to be toxic when present in high amount. A similar result was found where the metalloprotease isolated from

Oudemansiella radicata was inhibited by Hg^{2+}, Pb^{2+} and Fe^{2+} (Geng et al., 2017). In addition, Hg^{2+} also has been reported to inhibit keratinolytic metalloprotease from *Microbacterium* sp. (Thys & Brandelli, 2006)

4.5.3 Mass spectrometry analysis

Mass spectrometry has been an established analytical technique for many years and had permitted the development of peptide mass fingerprinting. The development was assisted by effectively dispersing proteins and other macromolecules into gas phase. The method is called *matrix-assisted laser desorption-ionization* (MALDI) and *electrospray spectrometry*. In this technique, the protein ions are generated and accelerated through an electrical field. The smallest protein ion traveling fastest and arriving at the detector first as they travel through the flight tube. Thus, the *time of flight* (TOF) in the electrical field is a measure of the mass or mass/charge ratio. The single protein band from SDS-PAGE or 2DE spot is extracted and cleaved specifically by trypsin. The masses of protein fragments are then determined with mass spectrometry and the peptide masses was matched against the fingerprint found in the databases of proteins that have been "electronically cleaved" by a computer simulating the same fragmentation technique used for the experimental sample (Berg et al., 2002).

Table 4.6 Protein Identification Summary on the SDS PAGE Gel Band of Peak 5b.

Search parameters	**Results**
Database/version: MSPnr100/Mascot 2.4.1	protein hit and rank: 1
Variable modification: oxidation (M)	protein accession: P0CB14
Taxonomy filter: Serpentes (June 2017; 121,572 sequences)	Protein description: sp|P0CB14|VM1K_CALRH Snake venom metalloproteinase kistomin n=1 Tax_Id=8717 [*Calloselasma rhodostoma*]
Enzyme: Trypsin	Protein score: 658
Maximum Missed Cleavages: 1	emPAI: 1.49
Peptide & fragment mass tolerances: ±0.2Da	peptides matched: 30 (18 nonduplicate, 12 duplicate)
Mass values: Monoisotopic	Peptides (non-duplicate only): 107, 140, 142, 153, 211, 575, 576, 702, 705, 796, 889, 917
Instrument type: 5600 TripleTOF mass spectrometer [AB Sciex], ESI-QUADTOF	Peptides found significant and top ranking: 9

The Data in Table 4.6 indicated the protein hit ranked number one due to the highest score attained. The protein identified as snake venom metalloproteinase (SVMP) kistomin belonging to the snake of *C. rhodostoma*. Evidently, in this result no other protein belonging to the database of *C. rhodostoma* identified within the hit list. Based on protein identification and characterization results from SDS-PAGE, inhibition study and mass spectrometry, it was concluded that this purified protein is metalloprotease protein which is SVMP protein family P-I Kistomin with molecular weight 26 kDa.

4.6 Factors affecting kistomin activity

There are several factors affecting the enzymatic reaction of kistomin in milk clotting. In milk clotting activity for new coagulant, the reaction parameters involved are enzyme concentration, temperature, pH and $CaCl_2$ concentration.

4.6.1 Enzyme concentration

The enzyme concentration is one of the important factors in milk clotting activity. The effect of enzyme concentration on milk clotting activity was investigated using 10 % (w/v) skim milk as a substrate. For enzyme concentration study, the reaction conditions for other parameters were as follows: 35 °C, pH 6.5, 10 mM $CaCl_2$ concentration.

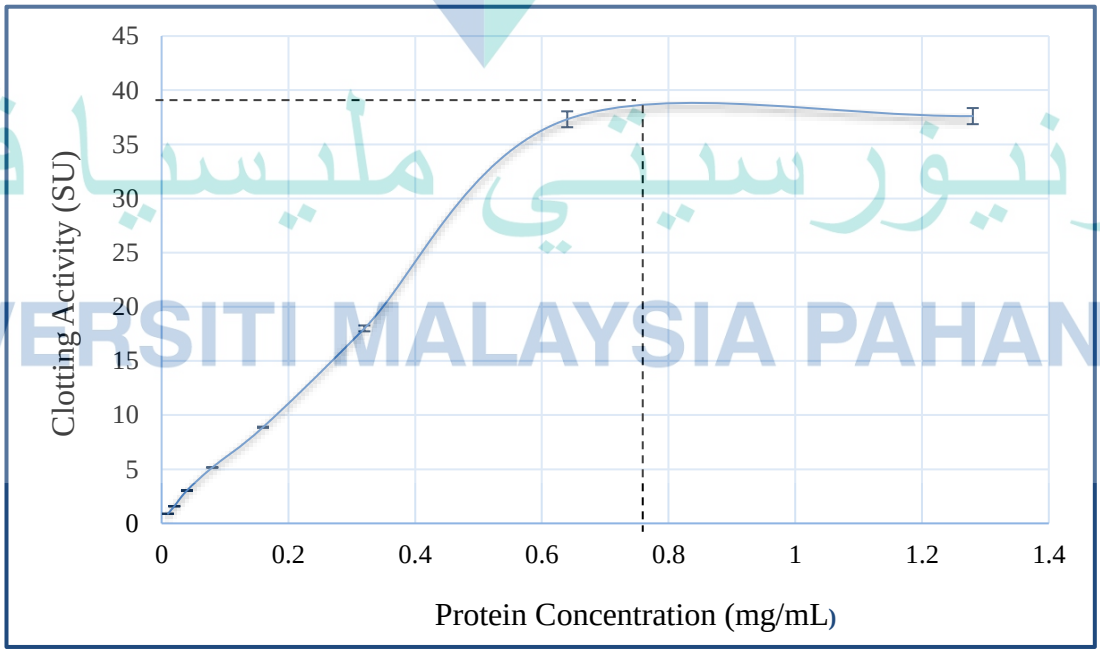


Figure 4.5 Graph of milk clotting activity using kistomin isolated from *C. rhodostoma* venom in increasing concentration.

Figure 4.5 shows the milk clotting activity against the kistomin concentration. The highest milk clotting activity achieved by kistomin concentration is with 0.76 mg/mL. For the kistomin concentration from 0 to 0.76 mg/mL, the rate of milk clotting activity is proportional to the increase of kistomin concentration. The clotting activity increase when enzyme concentration increased because of higher level of proteolysis of kappa casein (Najera et al., 2003).

Naturally, there is an abundance of kistomin in the more concentrated substrate. The rate of an enzyme catalyst reaction is directly dependent on the enzyme concentration, as the concentration of enzyme is significantly lower than the concentration of substrate. If there is substrate available to bound with the enzyme, the increasing enzyme concentration will speed up the reaction rate.

The addition of kistomin beyond 0.76 mg/mL did not increase the rate of clotting activity because the enzyme saturation has been reached and all the active site of the enzymes were fully occupied by a substrate. Once all the substrate is bound, the reaction will no longer speed up since there will be none of the enzyme available. The reaction can only increase the rate if more enzyme been added.

4.6.2 Temperature

Temperature is one of essential parameter in enzymatic reaction which is important to preserve the structure of enzyme and substrate. The milk clotting is strongly dependent on the temperature of the reaction (Najera et al., 2003). The effect of temperature of kistomin on the milk clotting activity was studied by using seven different temperatures (20, 30, 40, 50, 60, 70 and 80 °C). The reaction condition for other parameters was conducted as follows: 0.76 mg/mL kistomin, pH 6.5, 10 mM $CaCl_2$ concentration. At low temperature, there is no milk clotting activity detected as the number of successful collisions between the substrate and the enzyme is reduced because of their molecular movement is decreases.

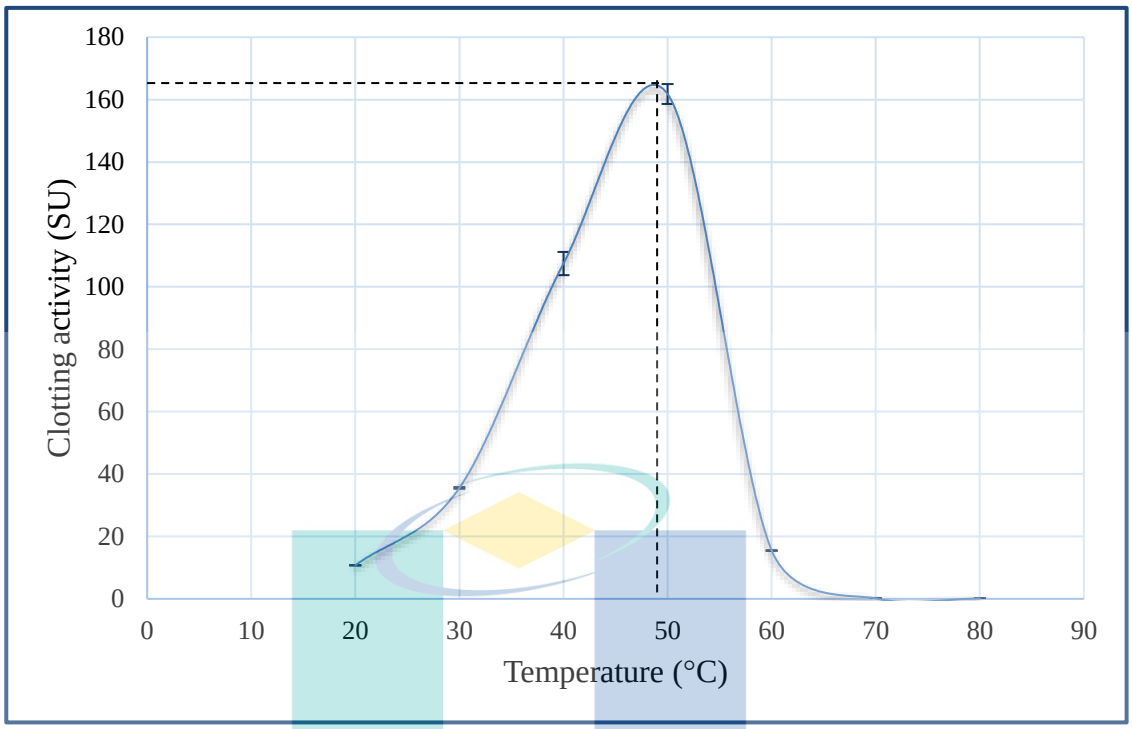


Figure 4.6 The rate of clotting activity at different temperatures.

Figure 4.6 shows the effects of temperature on milk clotting activity and indicates that the milk clotting activity improves by increasing the reaction temperature from 30 °C to 50 °C. The optimum temperature for kistomin is 48 °C which gives the highest milk clotting activity 165 (SU/mL). Landfeld et al. (2002) states that, the increasing milk clotting activity was achieved as the clotting time become shorter with the increase in temperature until the optimum temperature reached. This is because the enzymes and the substrates gain kinetic energy which resulted in frequent collision between substrate and enzyme molecules and have a higher affinity to bind to each other as the reaction temperature increases.

At 60 °C, the enzyme activity dropped steeply and the milk clotting activity of kistomin was completely inactivated at temperature 70 °C and above. This is due to the protein denaturation which involves a change in the protein structure with the loss of activity. The heat denaturation and loss of biological activity have been linked to the breakup of protein 2-D spanning water network which is due to the increased of breakage of hydrogen bond due to increasing temperature (Koizumi et al., 2007), which acts restrictively on protein vibrational dynamics (Broychenko et al., 2005). In addition, the protein structures become looser and allowing them to take up more water as the protein denature with the water-exposed surface increasing up to 50 % (Groot & Bakker, 2016).

Other researches show the optimum temperature for metalloproteinase enzyme between 37 °C to 55 °C as shown in the studies of Mazzi et al. (2004) with the hemorrhagic metalloprotease isolated from *Bothrops jararacussu* snake venom found to have optimum temperature at 37 °C, Majumder et al. (2015) which found high milk clotting enzyme purified from *Termitomyces clypeatus* and Wu et al. (2009) with novel fibrinolytic protease from *Fusarium sp.*, both having optimum temperature at 45 °C and metalloprotease isolated from *Serratia sp*. having optimum temperature in the range of 50 °C to 55 °C (Salarizadeh et al., 2014), which agrees with the results obtained from this research that optimum temperature for metalloprotease enzymes vary between 37 °C to 55 °C. Furthermore, only exception was metalloproteinase extracted from thermophilic fungus *Thermoascus aurantiacus* which was 75 °C (Merheb-Dini et al., 2009).

The ability of the milk clotting enzyme to act at elevated temperatures is one of the great biotechnological finding. This is importance because it is allowing the process to occur at high temperatures which reduced the risk of contamination by mesophilic microorganisms (Merheb-Dini et al., 2009; Gomes et al., 2007) that able to grow at moderate temperature between 20 °C to 45 °C and with optimum growth temperature between 30 °C to 39 °C (Schiraldi & De Rosa, 2016). It also favours substrate and product solubility, increase reaction rates for reducing viscosity and for increasing the diffusion coefficients of substrates (Merheb-Dini et al., 2009; Gomez et al., 2007). The commercial calf rennet shows the optimum temperature between 39 °C to 45.5 °C (Mamo & Balasubramaniam, 2018; Vejayan et al., 2017; Najera et al., 2003) and the optimum temperature are varied depended on the substrate.

The new alternative clotting enzyme in cheese production which is recombinant lamb chymosin (RLC) was evaluated and compared with the recombinant calf chymosin (RCC), cow rennet (CR) and microbial coagulant (MC) and the results showed that the optimum temperature of RLC at 40 °C, for both CR and RCC at 45 °C and for MC at 60 °C (Rogelj et al., 2001). This show that kistomin from *C. rhodostoma* has a potential as a coagulant in the cheese industry as it has a broad range of thermal stability and has high optimum temperature. Additionally, if required in the future, a recombinant kistomin variant can be generated to the desired trait.

4.6.3 pH stability

The pH stability of kistomin enzyme was studied at pH 5 to 9. Due to the acid induced clotting occur at pH below 4.6, the buffer below pH 5 was not executed. The experiment was carried out at 35 °C. Among the pH-dependent properties of protein, the activity and stability of the protein are two of the particular interest. The pH-dependence of activity and stability is typically bell-shaped curve with a single or several maxima and the maximum (maxima) is termed pH-optimum, which serves as a convenient metric for pH-dependent properties of protein which can be used to characterize protein adaptation to cellular and subcellular pH (Talley & Alexov, 2010). Based in Figure 4.7, the maximum milk clotting activity was detected at pH 6.5 (33.03 SU/mL), followed by pH 6 (31.44 SU/mL), pH 7 (30.19 SU/mL), pH 5.5 (30.00 SU/mL) and finally pH 5 (28.80 SU/mL). The optimum temperature of kistomin was at pH 6.5 (33.03 SU/mL). At pH 7.5, kistomin activity was decreased dramatically.

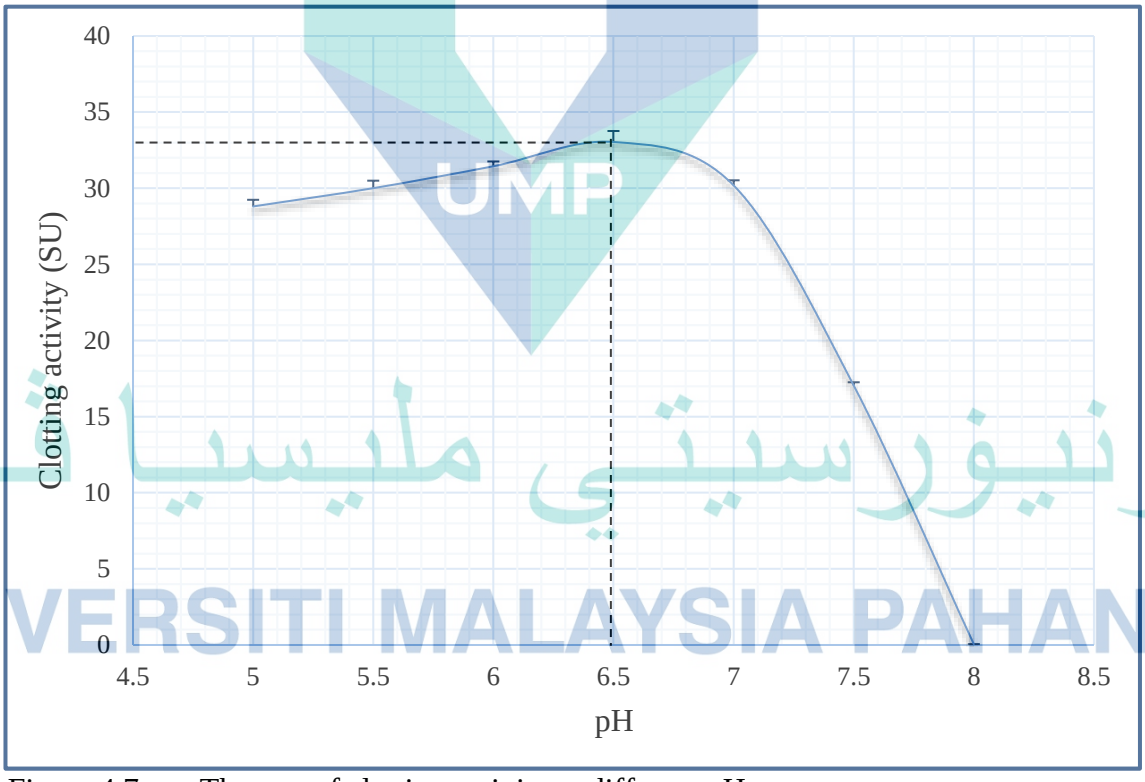


Figure 4.7 The rate of clotting activity at different pH.

The amino acid found in proteins have acidic or basic side chains and the pH of the environment influences the conformations of the protein molecule and the interactions between these charged side chains. The enzyme shows the maximum activity at the optimum pH. At the most favorable pH value, the enzyme activity is most active and has

the highest enzyme activity. The kistomin shows to be favorable in acidic environment as it stable at pH 5 to pH 6.9. The amino acid sequence of kistomin were computed using ProtParam tool online software and the results shows that the total number of negatively charged residues (Aspartic acid and Glutamic acid) is higher than the total number of positively charged residues (Arginine and Lysine) (Gasteiger et al., 2005). This protein also stable at pH 7 which maintained 71 % of its activity.

There many enzyme stabiles at neutral pH. For example, the metalloprotease isolated from *Eupenicillium javanicum* which has optimum pH at pH 6 (acidic condition) but also maintained a good activity at neutral pH (Hamin Neto et al., 2017) and the protease isolated from *Thermophilic Archaeon* which has maximal activity at pH 8 to pH 9 possessed a good activity at pH 7 (Jia et al., 2015). However, the activity of kistomin is completely loss at extreme basic condition which is due to the protein unfolding and denaturation of an enzyme (Piper & Fenton, 1965).

The remarkable activity with pH lower than pH 8 reveals that highly acidic nature of kistomin which makes it suitable applications in acidic environments especially in cheese production industry. In comparison to the chymosin (industrial rennets), according to (Reid at al., 1997), the chymosin cleaves the κ-casein at pH 4.6 and Van Hooydonk et al. (1984) stated that the optimum pH for proteolysis of κ-casein by chymosin is at pH 5.5. Shalabi & Fox (1982) also states that optimum pH for rennet is at 5.1-5.3 in order to coagulate milk. In dairy industry, the acidic condition is favourable because as the pH increases (alkaline condition), the rate of clotting was found to steadily decrease. In addition, it also has been reported that lowering the pH can increase in the curd firming rate (Daviau et al., 2000). Ong et al. (2012) investigated the effect of milk pH (pH 6.1-6.7) at renneting on composition, microstructure and texture of cheddar cheese. The gel renneted at pH 6.1 showed a dense protein network and this structure become more compact after cooking and forming an irregular and coarse matrix with lower porosity. The yield in dry matter of cheese renneted at pH 6.1 or pH 6.3 was 11-13 % higher than the cheese renneted at pH 6.7.

4.6.4 $CaCl_2$ concentration

Calcium chloride is one of the important factors in milk clotting. The addition of calcium chloride was reported to affect the milk clotting as it reduces the milk clotting

time (Sandra et al., 2012). Figure 4.8 shows the rate of milk clotting activity of kistomin with the addition of calcium chloride. The rate of clotting activity is gradually increase as the concentration calcium chloride increase until it reached optimum concentration which is 8 % (w/v) concentration of calcium chloride. According to Landfeld et al. (2002), the calcium chloride added to adjusts the calcium ions present in milk. It was stated that calcium chloride speeds up the milk clotting as calcium ions reduced the electrostatic resistance of micelles by neutralizing the surface charge on casein micelles.

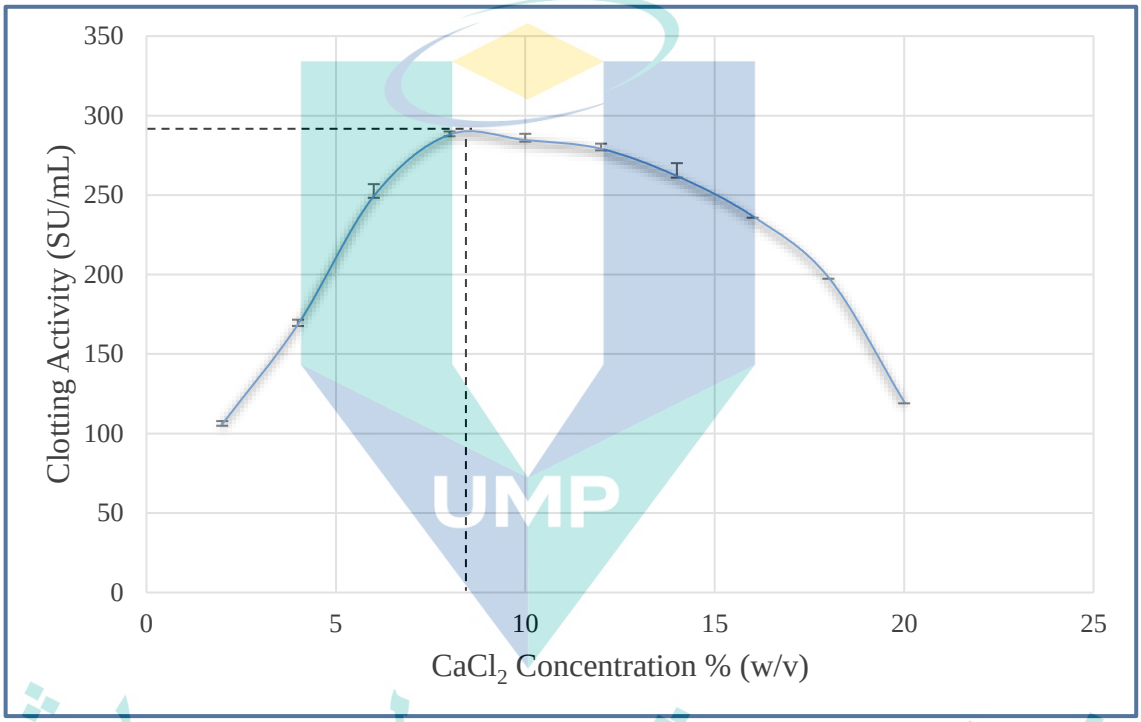


Figure 4.8 The rate of clotting activity with the increase of calcium chloride concentrations.

The calcium ions also form bridges between micelles of para casein indicating that, the more there are calcium ions (0 – 20 mM) in milk, the more there will be linkage. This promotes the aggregation of casein micelles and leads to faster milk clotting. In addition, presence of calcium chloride also reduces the pH of milk thus resulting in an increased protein aggregation rate (Najera et al., 2003). The rate of clotting activity was maintained more than 98 % of clotting activity at calcium chloride concentration 8 % to 12 % (w/v). However, addition of calcium chloride more than 12 % (w/v), the rate of milk clotting activity was gradually decrease. This is due to the at high concentration of calcium chloride, Ca^{2+} ions present inhibits the milk clotting network (Najera et al., 2003;

Patel & Reuter, 1986). Therefore, 10 mM $CaCl_2$ concentration was used as a constant variable to help speed up the clotting (Ekstrand et al., 1980).

4.7 Evaluating milk clotting activity of kistomin

Milk clotting activity of kistomin was evaluated using two properties which are Milk Clotting Activity (MCA) and Proteolytic Activity (PA). The milk clotting activity of kistomin were compared with rennet which is the commercial coagulant and other alternatives coagulant.

4.7.1 Milk clotting activity (MCA) for kistomin

The Milk Clotting Activity (MCA) was expressed in Soxhlet Unit (SU). One Soxhlet unit (SU) of milk clotting activity was defined as the amount of enzyme required to coagulate 1 mL of substrate within 40 minutes at 35 °C (El et al., 2017; Food, 2015). MCA is the ability of the rennet to aggregate milk by cleaving the amino acid of Phe_{105}-Met_{106} of κ-casein. The high value of MCA indicating that the enzyme has a good potential as a coagulant in dairy industry. Second property is proteolytic activity (PA) which is the ability of the coagulant to cleave any bond of casein (Kappeler et al., 2016). Evaluating an enzymatic activity in terms of MCA and PA is a crucial step in the selection of an appropriate substitute of rennet (Ben Amira et al., 2017). A high value of milk clotting activity/ proteolytic activity (MCA/PA) reflects an excellent end product of cheese with desirable firmness with no bitter flavours. The high ratio of MCA/PA is considered a good coagulant so the higher the value, the better the coagulant (Nasr et al., 2016; Langholm Jensen et al., 2013).

Table 4.7 The value of MCA, PA and MCA/PA ratio of samples.

Samples	MCA (SU/mL)	PA (U/mL)	MCA/PA
Kistomin *	810.44±42.45	1.39 ±0.01	583.05
Mucor miehei *rennet***	121.08 ± 0.62	0.03 ± 0.00	4036.0

*Optimum conditions of kistomin: 48 °C, pH 6.5, 8 % (w/v) $CaCl_2$, 0.76 mg/mL protein concentration.
**Conditions for rennet: 35 °C, pH 6.5, 10 % (w/v) $CaCl_2$.

Table 4.7 shows the value of MCA of kistomin and *M. meihei* rennet. The MCA for kistomin in optimum condition and rennet are 810.44±42.45 (SU/mL) and 121.08 ± 0.62 (SU/mL), respectively. The MCA of kistomin toward milk higher than commercial rennet. This shows that in optimum condition, kistomin possessed a high milk clotting activity and specificity to digest on kappa casein. Apart from milk MCA, MCA/PA ratio is very important criteria to evaluate the enzyme as rennet substitutes. The milk clotting enzyme (MCE) with high PA would excessively hydrolyse caseins, which led to loss of yield, texture and flavour defects (Hang et al., 2016; Alihanoglu et al., 2018). The determination and definition of PA methods of milk clotting are varied which can complicate the comparison among different studies (Vishwanatha et al., 2010). Thus, MCA/PA of different milk-clotting enzyme should be accessed under same conditions and similar to those used in the cheese-making. One of the assays that is usually employed to measure the proteolysis of protease is the released of amino acid (e.g. tyrosine) in whey. This assay is efficient to reflects the proteolytic activity of coagulant (Hang et al., 2016; Narwal et al., 2016).

In this study, proteolytic activity (PA) of *M. miehei* rennet is 0.03 ± 0.00 (U/ml) which is the lowest PA value in this study and followed by kistomin which is 1.39 ±0.01 (U/ml). MCA/PA ratio of the *M. miehei* rennet is the highest which is 4036.0, followed by kistomin which is 583.05. Higher MCA/PA ratio is desirable for milk clotting enzyme and its generally indicates the restricted degradation of casein substrate (Sato et al., 2004). However, kistomin has high PA compared to rennet. The specific activity of kistomin on casein in this study is 62.20 U/mg. According to Barrett et al. (2012), kistomin exhibits a strong proteolytic activity of 61 U/mg when casein used as substrate. Although kistomin has higher MCA value to *M. miehei* rennet, due to the high PA value, the MCA/PA ratio of kistomin has decreases abruptly. The major drawback of the protease extracted from most plant sources has the excessive proteolytic nature and low MCA/PA ratios which limited their use in cheese manufacturing such as ginger, cucumisin or hieronymain protease (Shah et al., 2014; Ben Amira et al., 2017).

In comparison Hang et al. (2016) has isolated the high milk clotting activity expressed by the newly Isolated *Paenibacillus spp*. and compared the milk clotting activity with three types of commercial rennet which are calf rennet, recombinant chymosin (Chy-MAX) and *R. miehei* (Marzyme 150MG) coagulant. The result shows

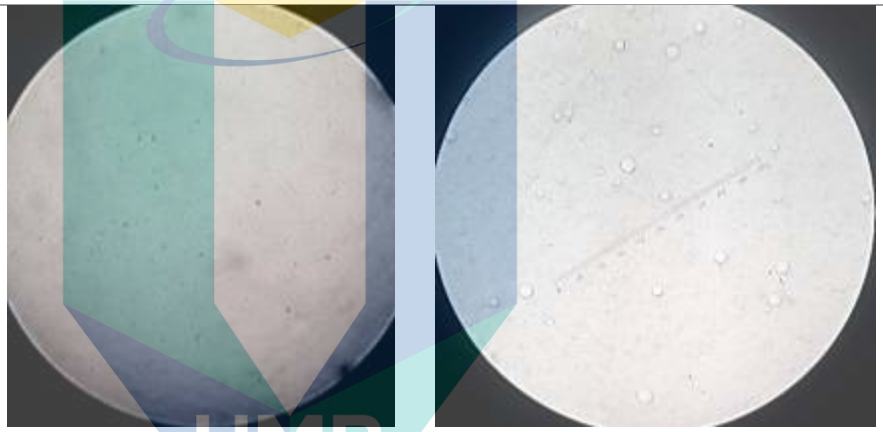
that the MCA/PA ratios of chymosin, rennet, *R. miehei* coagulant and *Paenibacillus spp*. protease were 200, 133.33, 44.44 and 33.33, respectively, and summarized that the MCA/PA ratio of *Paenibacillus spp*. protease was approximately in the same level of *R. miehei* and has potential as a rennet substitute. Some studies have also revealed that high MCA value of a plant coagulant as compared with chymosin. Ahmed et al. (2009) partially purified milk-clotting enzyme from *Solanum dubium* Fresen seeds and concluded that the extract of *S. dubium* is an appropriate rennet substitute, as *S. dubium* (880 U/mL) MCA value exceeds the calf rennet (249.6 U/mL) and Mucor rennet (551 U/mL) significantly. The study shows that the MCA/PA ratios of rennet, Mucor rennet, *S. dubium* enzyme and Papain were 4992, 4650, 2490 and 367, respectively.

The high value of PA of kistomin can be overcome using genetic engineering which is the recent advances in dairy industry. The fermentation produces chymosin (FPC) was the first processing aid for food processing produced with recombinant DNA technology which has been registered by U.S Food and Drug Administration and the other parts of the world also show increasing acceptance (Jacob et al., 2010). The FPC from animal other than calf is lamb prochymosin, using *E. coli* as a host and resulted the clotting and proteolytic activity of protease similar to calf chymosin (Rogelj et al., 2001). Vallejo et al. (2008), has studies the PFC from water buffalo chymosin using *Pichis pastoris* as a host and produced a protease which has higher affinity to kappa casein compared to conventional buffalo chymosin. Kistomin though been with lower MCA/PA value but improved once optimum conditions utilized. The measured value of 593 for kistomin found higher than 367 for papain. Papain been suggested not useful as a coagulant due to the bitterness of the curd obtained (Lemieux & Simard, 1991).

4.8 Comparison of kistomin action toward cow and goat milk

The kistomin that responsible for the milk milk clotting was tested on cow and goat milk. The result shows that the kistomin shows significant clotting activity for cow milk and goat milk which are 16.67 ± 2.89 s and 13.33 ± 2.89 s, respectively. The clotting activity of crude venom from *C. rhodostoma* and kistomin isolated from *C. rhodostoma* shows the same results on the cow's and goat's milk. However, as founded earlier, the goat milk shows a faster clotting time.

Table 4.8 Comparison of the clotting activity of metalloproteinase kistomin on cow and goat milk at optimum parameters (n=3)

Milk	Cow	Goat
Clotting time	16.67 ± 2.89 sec	13.33 ± 2.89 sec
Mass of curd	0.256 ± 0.012 g	0.133 ± 0.007 g
Texture of curd	Firm	Fragile
Before clotting (100X magnification)	 (A1)	(B1)
After clotting (100X magnification)	 (A2)	(B2)

*The following were the parameter for kistomin on cow and goat milk: pH 6.5, 48 °C, 8 % $CaCl_2$ concentration.

The amount of curd obtained for cow milk formed was higher amount (0.256 ± 0.012 g) compared to goat milk (0.133 ± 0.007 g). This is because goat milk has 2 % of curd as compared with 10 % curd in cow milk. This difference in curd tension is attributed to the low levels of αs1-casein in goat milk, compared to cow milk. This is the reason the goat milk coagulates faster than the cow milk. This is also a reason goat's milk is considered more easily digestible than cow milk. A softer casein curd could be expected to result in more rapid digestion of milk proteins, and this was confirmed in vitro by Jasińska (1995).

Figure 4.8 (A2) and (B2) showed the milk clotting time of kistomin for both cow and goat milk under optimum conditions for temperature, pH as well as concentrations of enzyme and $CaCl_2$. The images viewed under microscopic magnification clearly showed the formation of gaps represented by whey after milk coagulated. The observation before coagulation showed milk without any aggregation of casein micelles however subsequently obvious colloidal structure of aggregation occurred.

The texture of the curd formed from cow and goat milk was also analysed and it was found that cow milk forms a considerably firm curd compared to goat milk which forms a slightly fragile curd. This shows goat milk has poorer cheese making ability compared to cow milk. Microscopic observations were also made for the cow and goat milk before and after clotting. According to the images obtained, cow and goat milk have almost similar microscopic structures before clotting as can be seen in (A1) and (B1). However, after kistomin was added, the cow milk shows a better clotting and formation of more curd which can be seen as many dark spots in (A2). The goat milk on the other hand, slightly lighter and lesser spots which can be seen in (B2). This is because goat milk has lower amount of alpha-casein compared to cow milk.

4.9 Kinetic activity of kistomin

The kinetic parameters of kistomin and rennet were determined by varying the initial concentration of casein as the substrate. The kinetics parameters such as maximum reaction velocity (V_{max}) and Michaelis constant (K_m) were calculated by the Michaelis-Menten equation using GraphPad Prism 7. The V_{max} and K_m values are listed in Table 4.9.

Table 4.9 Kinetic parameters of kistomin

Kinetic parameters	Kistomin
K_m (mg/mL)	1.153 ± 0.08
V_{max} (µmol/min)	3.37×10^{-2}± 0.00

V_{max} or maximum velocity is defined as the maximum rate of an where the substrate completely saturated the enzyme active site. Table 4.9 revealed that the V_{max} values of kistomin was 0.0337± 0.00 µmol/min. K_m or Michaelis constant is defined as the substrate concentration at which half the enzyme's active sites are occupied by substrate. Table 4.9 shows that the K_m values of kistomin was 1.153 ± 0.08 mg/mL. This result shows that kistomin enzyme has high affinity towards casein substrate. Meaning that the kistomin requires low concentration of substrate/casein to achieve maximum catalytic efficiency. This might also happen because protease acts with low specificity and the casein molecule contains more cleavage sites (Merheb-Dini., 2009).

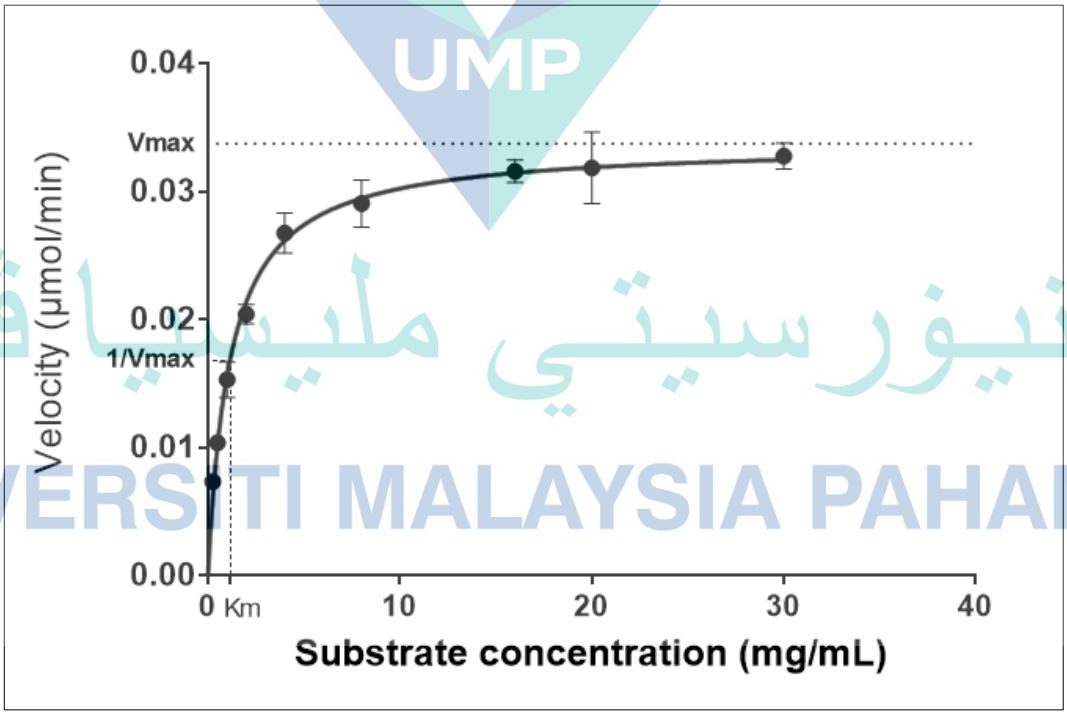


Figure 4.9 Michaelis-Menten curve of the effect of substrate concentration on the reaction velocity of kistomin.

4.10 Substrate specificity of kistomin

To further determine whether kistomin is suitable for dairy industry, the proteolytic activity and the substrate specificity were investigated. The substrate specificity of kistomin was investigated using α-casein, β-casein and κ-casein.

Table 4.10 compared the V_{max} and the K_m of α-casein, β-casein and κ-casein by kistomin isolated from *C. rhodotoma* venom. The results from Table 4.10 shows that the V_{max} and K_m value of κ-casein is the lowest which are 0.0275 ± 0.0019 µmol/min and 27.15 ± 5.277 mM, respectively. These followed by the V_{max} and K_m values of α-casein which have the V_{max} and K_m value at 0.0406 ± 0.0013 µmol/min 38.10 ± 2.090 mM, and β-casein at 0.0719 ± 0.0065 µmol/min 114.5 ± 16.67 mM. The lowest K_m value and V_{max} value indicates the kistomin has high affinity towards the substrate thus showed the high specificity towards the substrates. The kistomin has high specificity towards κ-casein as its digested kappa casein substrate faster than other substrates. This is a good parameter since the milk clotting protease that exclusively cleaves the peptide bond in Phe_{105}-Met_{106} of κ-casein is considered to be the most efficient protease for cheese making industry (Silva & Malcata, 2005; Yegin et al., 2011). The use of calf rennet substitutes for cheese making such as *Mucor miehei*, porcine pepsin A, porcine pepsin C and *Endothia parasitica* protease is common in several countries. The specificity studies of these enzyme towards bovine κ-casein shows all these enzymes cleaved the Phe_{105}-Met_{106} bond except for *Endothia parasitica* which only cleaved the Ser_{104}-Phe_{105} bond. However, this difference in cleavage does not seem to affect the clotting (Drohse & Foltmann, 1989: Egito et al., 2007).

Table 4.10 The substrate specificity study of kistomin

Substrate	Vmax (µmol/min)	Km (mM)
κ-casein	0.0275 ± 0.0019	27.15 ± 5.277
α-casein	0.0406 ± 0.0013	38.10 ± 2.090
β-casein	0.0719 ± 0.0065	114.5 ± 16.67

The major enzyme of calf rennet, chymosin has been extensively used in the production of cheese to produce a stable curd with good flavour due to its high specificity for κ-casein (Rao et al., 1998). The specificity of kistomin for casein was similar to chymosin which extensively cleaved κ-casein and promoted very slight hydrolysis of β-casein. The specificity of kistomin on κ-casein followed by α-casein. Kistomin enzyme does not has a high specificity towards β-casein because it has low affinity towards β-casein. This can avoid the bitterness development of cheese which is considered as defect in cheese. It has been reported that the hydrolysis of casein especially β-casein generates hydrophobic peptides which when accumulating is responsible for bitterness of dairy products (Savijoki et al., 2006). Both kinetic and specificity studies of kistomin proved that it has potential as milk coagulant. The higher the affinity towards β-casein leads to the product of hydrophobic peptides, hence becoming bitter cheese. In this instance, it was found kistomin having lower affinity towards β-casein based on higher K_m.

CHAPTER 5

CONCLUSION

5.1 Conclusion

The potential of kistomin from C. *rhodostoma* venom as one of the alternatives in milk clotting industry has been evaluated. Kistomin managed to be isolated using only two steps of chromatography techniques. These steps successfully produced 4.41±0.06 fold of protein and exhibited high protein recovery which is 54.34 %, in comparisons to other methods that required more than two chromatography steps which produced lower protein fold and recovery. The MCE kistomin has been found belong to metalloprotease SVMPs P-I which have the molecular mass of approximately 26 kDa in SDS-PAGE analysis and was completely inhibited by EDTA and 1,10 phenanthroline.

At the optimum conditions which are pH 6.5, 48 °C, 0.76 mg/mL protein concentration and 8 % (w/v) of calcium chloride concentration, the MCA/PA ratio calculated as 585.05 in comparison to rennet of 4036.0. It is not possible to ascertain whether this MCA/PA ratio obtained for kistomin is suitable as a dairy industry protease as there is no sufficient data available on the lower cut-off value for this ratio.

The acidic optimum pH of kistomin is favourable in dairy industry since the acidic condition can increase the cheese quality in the terms cheese firmness. In addition, kistomin has relatively high MCA in its optimum conditions which is higher than any other milk clotting alternatives and this will make a kistomin a good coagulant for dairy industry. The high PA possessed by kistomin towards casein can be overcome by using genetic engineering technology which can be used as a tool to produce the PA of protease with lower or similar to calf chymosin.

Lastly, kistomin has high affinity to casein and especially active on κ-casein. The results on kinetics activity shows that kistomin has high affinity to casein. The kistomin highly active towards κ-casein and less active to β-casein indicative of dairy product

without bitter taste. From this study, it can be concluded that kistomin has potential to be a coagulant in dairy industry.

5.2 Future recommendation

As demonstrated in this study, the kistomin has potential to be a coagulant in dairy industry and further investigation should be performed for improvement.

The problem of yield and using venom of snakes. The source of kistomin is hard to obtain due to the scarcity and the needs of expertise to extract the venom from the *C. rhodostoma*. This problem can be solved by using genetic engineering. The expression of kistomin gene in the microbe to determine whether can obtain the kistomin without snake. Therefore, yield to be adequate for production.

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APPENDICES

APPENDIX A

MATERIAL AND METHODS

Buffer preparation

1. Citrate-phosphate buffer

   Solution A: 0.2M of citric acid

   Solution B: 0.1M of dibasic sodium phosphate

Mix solution A and solution B in the proportions indicated and adjust the final volume to 100 ml with distilled water.

pH	Solution A (mL)	Solution B (mL)
5.0	24.3	25.7
5.2	23.3	26.7
5.4	22.2	27.8
5.6	21.0	29.0
5.8	19.7	30.3
6.0	17.9	32.1

2. Sodium-phosphate buffer

   Solution A: 0.2M monobasic sodium phosphate

   Solution B: 0.2M dibasic sodium phosphate

Mix solution A and solution B in the proportions indicated and adjust the final volume to 200 mL with distilled water.

pH	Solution A (mL)	Solution B (mL)
6.0	87.7	12.3
6.2	81.5	18.5
6.4	73.5	26.5
6.6	62.5	37.5
6.8	51.0	49.0
7.0	39.0	61.0
7.2	28.0	72.0
7.4	19.0	81.0
7.6	13.0	87.0
7.8	8.5	91.5
8.0	5.3	94.7

3. Glycine – NaOH buffer

Solution A: 0.2M of glucine

Solution B: 0.2M of sodium hydroxide

Mix 25 mL of the solution A with solution B in the proportions indicated and adjusted the final volume to 200 mL with distilled water

pH	Solution B (mL)
8.6	4.0
8.8	6.0
9.0	8.8
9.2	12
9.4	16.8
9.6	22.4
9.8	27.2
10.0	32.0

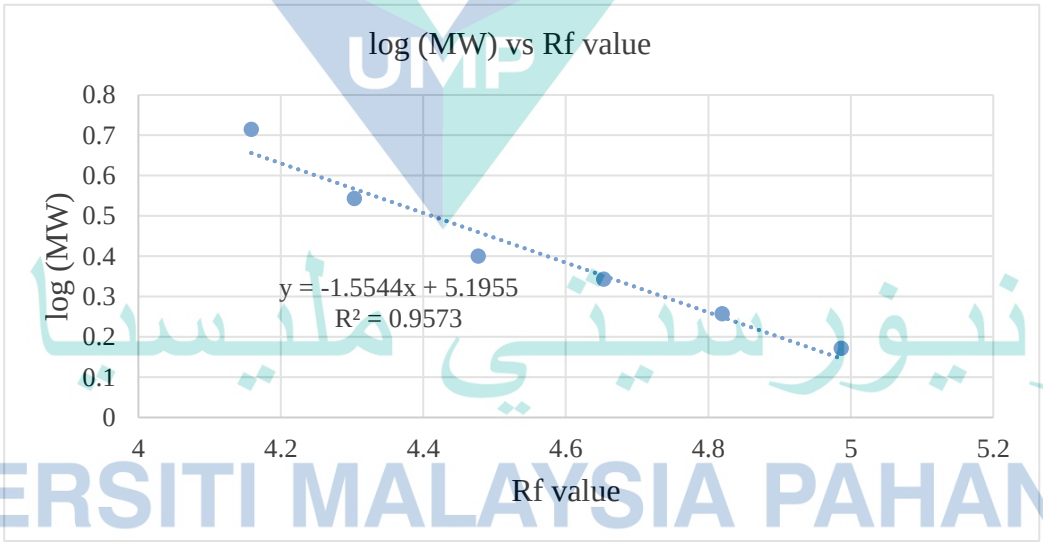
APPENDIX B

EXPERIMENTAL DATA

Appendix B1: Calculation of isolated protein molecular weight

$$\text{Relative migration distance (Rf)} = \frac{\text{migration distance of the protein}}{\text{migration distance of the dye front}}$$

Marker band	Molecular weight (MW)	Distance (cm)	log (MW)	Rf value
1	97000	1.2	4.9868	0.1714
2	66000	1.8	4.8195	0.2571
3	45000	2.4	4.6532	0.3429
4	30000	2.8	4.4771	0.4000
5	20100	3.8	4.3032	0.5429
6	14400	5.0	4.1584	0.7143



Rf value of single band = 3.5/7.0 = 0.5

y = -1.5544x + 5.1955

y = -1.5544(0.5) + 5.1955

y = 4.4183

log MW = 4.4183

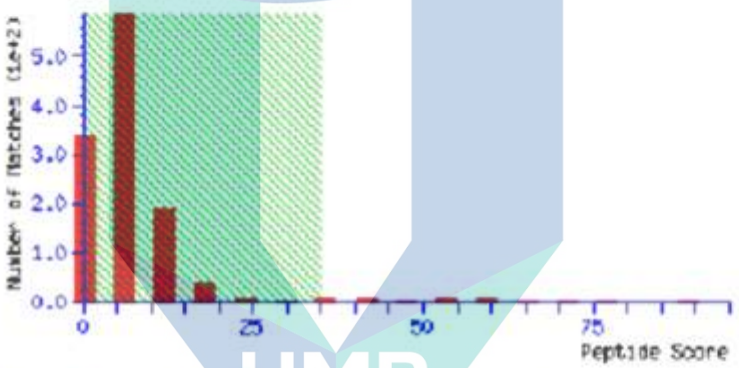
MW = 26 119 Dalton

Appendix B2: MASCOT search results for protein identification

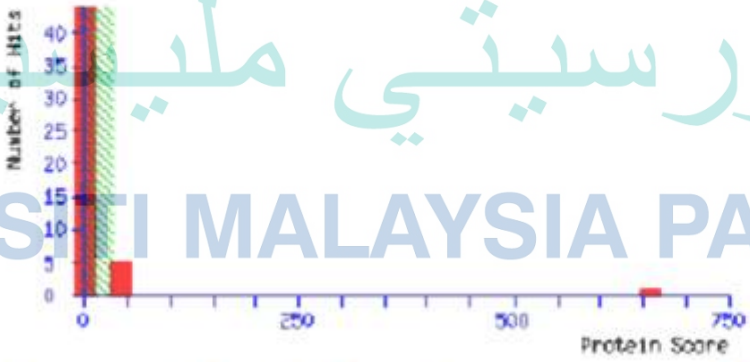
Search parameters

Type of search	: MS/MS Ion Search
Enzyme	: Trypsin
Variable modifications	: Oxidation (M)
Mass values	: Monoisotopic
Protein mass	: Unrestricted
Peptide mass tolerance	: ± 0.2 Da
Fragment mass tolerance	: ± 0.2 Da
Max missed cleavages	: 1
Instrument type	: ESI-QUAD-TOF
Number of queries	: 1,919

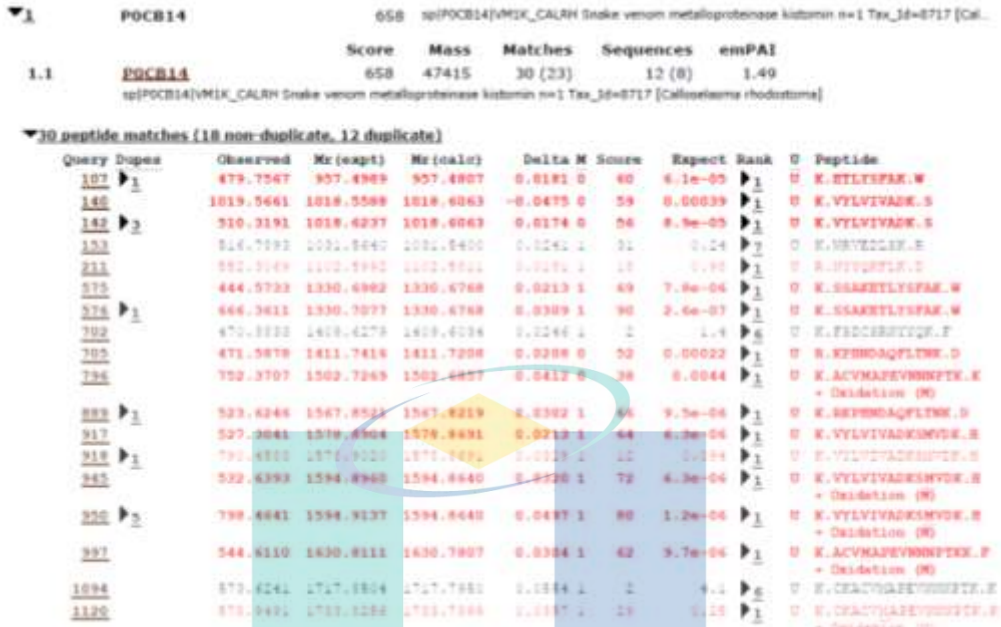
Score distribution



Peptide score distribution. Ions score is $-10\log(P)$, where P is the probability that the observed match is a random event. Individual ions scores > 35 indicate **identity** or **extensive homology** ($p<0.05$).



[Deprecated] Score distribution for family members in the first 50 proteins. Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein families.



1 P0CB14 658 sp|P0CB14|VM1K_CALRH Snake venom metalloproteinase kistomin n=1 Tax_Id=8717 [Cal...

		Score	Mass	Matches	Sequences	emPAI
1.1	P0CB14	658	47415	30 (23)	12 (8)	1.49

sp|P0CB14|VM1K_CALRH Snake venom metalloproteinase kistomin n=1 Tax_Id=8717 [Calloselasma rhodostoma]

30 peptide matches (18 non-duplicate, 12 duplicate)

Query	Dupes	Observed	Mr(expt)	Mr(calc)	Delta	M	Score	Expect	Rank	U	Peptide
107	1	479.7567	957.4989	957.4807	0.0181	0	40	6.1e-05	1	U	K.ETLTSFAK.W
140		1019.5661	1018.5588	1018.6063	-0.0475	0	59	0.00039	1	U	K.VYLVIVADK.S
142	3	510.3191	1018.6237	1018.6063	0.0174	0	56	8.9e-05	1	U	K.VYLVIVADK.S
153		516.7093	1031.8040	1031.8400	-0.0361	1	31	0.24	7	U	K.VNYEGLSK.E
211		552.3149	1102.5952	1102.5511	0.0441	1	19	0.93	1	U	R.FYYQETLK.D
575		444.5732	1330.6982	1330.6768	0.0213	1	69	7.6e-06	1	U	K.SSAKETLYSFAK.W
576	1	666.3611	1330.7077	1330.6768	0.0309	1	90	2.6e-07	1	U	K.SSAKETLYSFAK.W
702		470.3333	1408.4279	1408.6036	-0.1756	1	1	1.4	6	U	K.FSDCSEYYQK.F
705		471.5878	1411.7415	1411.7208	0.0208	0	52	0.00022	1	U	R.KFHMDAQFLTMK.D
796		752.3707	1502.7269	1502.6857	0.0412	0	38	0.0044	1	U	K.ACVMAPEVMMKFTK.K + Oxidation (M)
889	1	523.6246	1567.8519	1567.8219	0.0302	1	66	9.5e-06	1	U	K.KFHMDAQFLTMK.D
917		527.3041	1579.8904	1579.8631	0.0273	1	64	6.3e-06	1	U	K.VYLVIVADKSMVDK.S
918	1	790.4800	1579.9020	1579.8631	0.0389	1	12	1.8e+04	1	U	K.VYLVIVADKSMVDK.S
945		522.6393	1594.8960	1594.8640	0.0320	1	72	6.3e-06	1	U	K.VYLVIVADKSMVDK.S + Oxidation (M)
950	5	798.4041	1594.9137	1594.8640	0.0497	1	80	1.2e-06	1	U	K.VYLVIVADKSMVDK.S + Oxidation (M)
997		544.6110	1630.8111	1630.7807	0.0304	1	62	9.7e-06	1	U	K.ACVMAPEVMMKFTKK.F + Oxidation (M)
1094		873.4241	1747.8804	1747.7981	0.0824	1	1	4.1	6	U	K.CKACYQAPEYMMKFTK.K
1120		875.9401	1751.8584	1751.7956	0.0627	1	29	0.15	1	U	K.CKACYQAPEYMMKFTK.K + Oxidation (M)

Appendix B3: Results for kinetic activity of kistomin (K_m and V_{max}).

Table B3.1: Michaelis-Menten data of kistomin

	Velocity (micromoles/min)
Michaelis-Menten	
Best-fit values	
Vmax	0.03373
Km	1.153
Std. Error	
Vmax	0.0004991
Km	0.07765
95 % CI (profile likelihood)	
Vmax	0.0327 to 0.03478
Km	1 to 1.327
Goodness of Fit	
Degrees of Freedom	25
R square	0.9798
Absolute Sum of Squares	4.728e-005
Sy.x	0.001375
Replicates test for lack of fit	
SD replicates	0.001431
SD lack of fit	0.00122
Discrepancy (F)	0.7269
P value	0.6517
Evidence of inadequate model?	No
Constraints	
Km	Km > 0
Number of points	
# of X values	27
# Y values analyzed	27

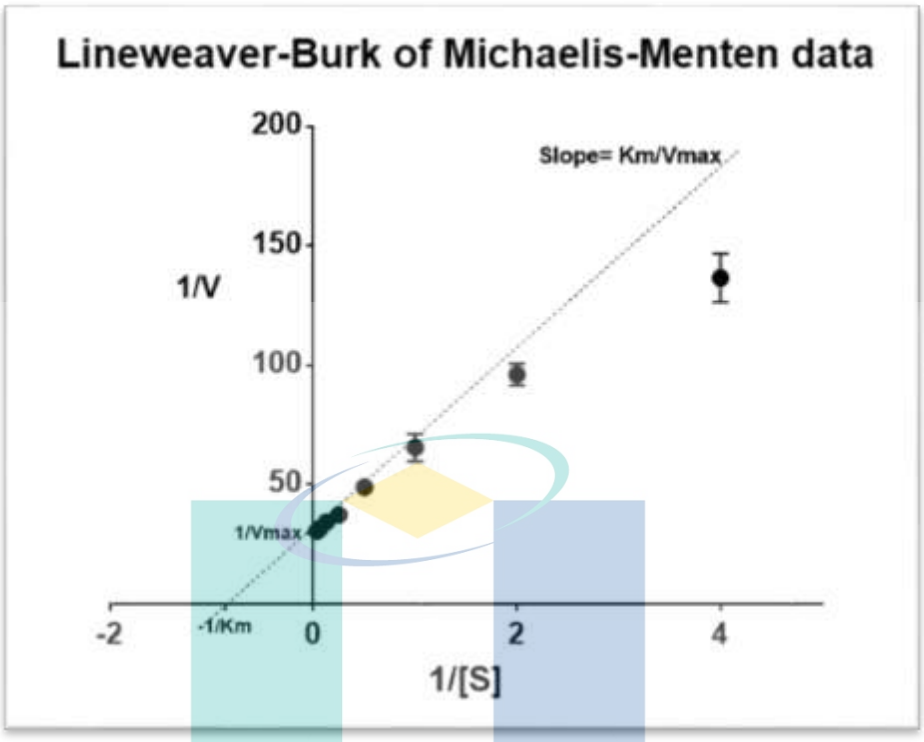


Figure B3.1: Lineweaver-Burk plot of initial velocity of reaction on casein by kistomin

Appendix B4: Substrate specificity of kistomin towards alpha-casein, beta-casein and kappa-casein.

Table B4.1: Michaelis-Menten data of kistomin towards alpha-casein

	Enzyme Activity
Michaelis-Menten	
Best-fit values	
Vmax	0.04062
Km	38.1
Std. Error	
Vmax	0.001341
Km	2.901
95 % CI (profile likelihood)	
Vmax	0.03796 to 0.04375
Km	32.45 to 45.05
Goodness of Fit	
Degrees of Freedom	19
R square	0.9925
Absolute Sum of Squares	1.421e-005
Sy.x	0.0008647
Replicates test for lack of fit	
SD replicates	0.0006434
SD lack of fit	0.001297
Discrepancy (F)	4.064
P value	0.0173
Constraints	
Km	Km > 0
Number of points	
# of X values	21
# Y values analyzed	21

Table B4.2: Michaelis-Menten data of kistomin towards beta-casein

	Enzyme Activity
Michaelis-Menten Best-fit values	
Vmax	0.07197
Km	114.5
Std. Error	
Vmax	0.006537
Km	16.07
95% CI (profile likelihood)	
Vmax	0.06083 to 0.08938
Km	87.42 to 157.7
Goodness of Fit	
Degrees of Freedom	19
R square	0.993
Absolute Sum of Squares	1.76e-005
Sy.x	0.0009625
Replicates test for lack of fit	
SD replicates	0.001084
SD lack of fit	0.0004819
Discrepancy (F)	0.1978
P value	0.9581
Constraints	
Km	Km > 0
Number of points	
# of X values	21
# Y values analyzed	21

Table B4.3: Michaelis-Menten data of kistomin towards kappa-casein

	Velocity (micromoles/min)
Michaelis-Menten	
Best-fit values	
Vmax	0.02753
Km	27.15
Std. Error	
Vmax	0.001927
Km	5.277
95% CI (profile likelihood)	
Vmax	0.02413 to 0.03198
Km	18.7 to 40.23
Goodness of Fit	
Degrees of Freedom	19
R square	0.9407
Absolute Sum of Squares	7.7e-005
Sy.x	0.002013
Replicates test for lack of fit	
SD replicates	0.001851
SD lack of fit	0.00241
Discrepancy (F)	1.696
P value	0.2002
Constraints	
Km	Km > 0
Number of points	
# of X values	21
# Y values analyzed	21

APPENDIX C

PUBLICATION AND AWARD

Specific to this study:

1. Vejayan, J., Zulkifli, A. A., Syed, M., Saufi, N. M., Ibrahim, H., & Ambu, S. (2017). Uncovering a protease in snake venom capable to coagulate milk to curd. *International Journal of Advanced Biotechnology and Research*, *8*(4), 409-423.

2. Silver Medal Award at Creation, Innovation, Technology & Research Exposition (CITREX) 2017, Kompleks Sukan Universiti Malaysia Pahang.

Other award and publication achieved during the candidature:

3. Vejayan, J., Mohamed, A. N., Zulkifli, A. A., Yahya, Y. A. C., Munir, N., & Yusoff, M. M. (2018). Marker to authenticate Eurycoma longifolia (Tongkat Ali) containing aphrodisiac herbal products. *Current science*, *115*(5), 886-894.

4. Co-author of article 'Tongkat Ali: Pusaka bumi dipersada dunia'- Publication for Wadah Magazine of UMP (May, 2017)