ENZYME IN VENOM USEFUL IN COAGULATING MILK TO CURD FOR FOOD INDUSTRY

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(ENZIM DALAM BISA ULAR DALAM PENGUBAHAN SUSU KEPADA DADIH BAGI INDUSTRI PEMAKANAN)

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ABSTRACT

Snake venom been studied for its lethality and various benefits for mankind. The latter been studied a plenty of recent but none related to coagulation of milk to curd. The coagulation time of milk by samples were done using visible parameters i.e. change in viscosity, colour changes, white spot formation (separation between curd and whey) and finally observing a drop of coagulating fluid under magnification of a light microscope. Optimum parameters determined included concentration of coagulants, temperature and pH. Microscopic viewing included observing after centrifugation, under light microscope and SEM. Screening selected venoms mostly predominantly found in tropical region singled out one with the most rapid coagulating time i.e by Calloselasma rhodostoma (CR). Optimization of CR venom related to several parameters provided venom concentration, 0.07 (w/v%); pH, 7.0; temperature, 45.5^{0}C while that of rennet were determined to be 0.04±0.02 (w/v%); pH,7.0; temperature, 45.5^{0}C, respectively. Under these ideal conditions for both coagulants, comparison of their milk coagulation time found CR superior i.e. 0.41±0.02 min compared to 4.23±0.05 min for rennet. Milk coagulating assay guided fractionation of CR venom by using HiTrap SP FF and consecutively followed by HiPrep 26/60 Sephacryl S200 HR pre-packed columns led to a single band on coomassie stained SDS-PAGE gel. Next by LCMS analysis on the SDS PAGE band identified the presence of metalloproteinase kistomin within the venom. EDTA inactivated the venom presumably chelating zinc hence suggesting further towards identifying kistomin as the likely protease within this venom with milk-clotting activity. Snake venom been potentially identified for yet another application for the benefit of mankind. In this investigation Malayan Pit Viper's protease can play major role in dairy industry if studied further.

ABSTRAK

Bisa ular telah dikaji untuk pelbagai faedah bagi manusia. Dalam kajian terkini merupakan keupayaan bias membeku susu. Masa pembekuan susu mengikut sampel telah dilakukan dengan menggunakan parameter yang jelas dilihat iaitu perubahan kelikatan, perubahan warna, pembentukan titik putih (pemisahan antara dadih dan larutan) dan akhirnya memerhatikan setitik cairan pembekuan di bawah pembesaran mikroskop cahaya. Parameter optimum ditentukan termasuk kepekatan koagulan yang diguna, suhu dan pH. Penglihatan melalui mikroskop termasuk juga pemerhatian selepas pemendapan, di bawah mikroskop cahaya dan SEM. Kebanyakan bisa dipilih adalah dari negara beriklim tropika dan yang paling menonjol bagi kegiatan koagulasi susu adalah *Calloselasma rhodostoma* (CR). Pengoptimuman bisa CR yang berkaitan dengan beberapa parameter memberikan hasil kajian seperti berikut, 0.07 (w / v%); pH, 7.0; suhu, 45.5^{0}C manakala bagi rennet ditentukan sebagai 0.04 ± 0.02 (w / v%); pH, 7.0; suhu, 45.5^{0}C, masing-masing. Di bawah keadaan yang ideal bagi kedua-dua koagulan ini, perbandingan masa pembekuan susu dilakukan dan didapati CR lebih unggul i.e 0.41 ± 0.02 min berbanding dengan 4.23 ± 0.05 min untuk rennet. Dalam penulenan yang dilakukan pada bisa CR dengan menggunakan HiTrap SP FF dan diikuti oleh HiPrep 26/60 Sephacryl S200 HR membawa kepada satu "band" pada SDS-PAGE gel. Seterusnya oleh analisis LCMS pada

“band” SDS PAGE tersebut mengenal pasti kehadiran metalloproteinase kistomin dalam CR. EDTA tidak mengaktifkan CR yang berkemungkinan mengusikkan kandungan zink dan seterusnya menggangu aktiviti CR secara keseluruahn. Ini mencadangkan ke arah mengenal pasti kistomin sebagai protease yang berkemungkinan besar bertanggungjawap dalam aktiviti pembekuan susu dalam CR. Dengan demikian bisa ular telah dikenal pasti sebagai satu lagi aplikasi bagi faedah manusia. Dalam penyiasatan ini, protease ular Kapak Bodoh boleh memainkan peranan penting dalam industri tenusu jika dikaji lebih lanjut.

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CHAPTER 1

INRODUCTION

1.1 RESEARCH BACKGROUND

There are about 600 species of venomous snakes out of 3000 snake species that exist on earth. They are categorized into four major families including the Families of 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-defense and will bite people who startle, provoke and threaten them. Snake venoms are synthesized and stored in the venomous gland which are secretion of venomous snake (Goswami et al. 2014). Particularly, the bites caused by Elapidae (kraits and cobras) families and Viperidae (pit vipers) families are dangerous (Kasturiratne, 2008). Venom consist of various 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 A2 (PLA2s), myotoxins, hemorrhagic metalloproteinases. In addition, proteolytic enzymes, coagulant components, cardiotoxins, cytotoxins and neurotoxins also present in the venom (Leon et al., 2013; Kini, 2003). The proteins and peptides fills 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 only toxic if contacted with blood (Vyas et al., 2013). The protein composition in venom varied depend on the many factors. Different species have different types of venoms and even the composition of protein within the different species and even 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

Human can share benefits from snake venom for it have been proven snake venom contributed in many field 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 defibrinating agent, anti-cancer properties, HIV treatment, anti-hypersensitive activities, treatment for Alzheimer and etc.

Cancer treatment is one of the major challenge to the medical world. Component in the snake venom are frequently studied by scientist for its anti-cancer properties. According to Zhou et al. (2000), Contortrostatin, a disintegrin from copperhead snake venoms can disrupt cancer cell adhesion and invasion. Disintegrin is made from lower molecular weight, comes varies configuration, strength and specificity which works by binding to integrin which plays role in cell migration. Affecting of cell migration thus results in blocking of tumor cells growth (Koh and Kini et al., 2011).

Other than that, Malayan pit viper contributes to widely held snake envenomation drugs. *Calloselasma rhodostoma* gives venom consists of enzymes responsible for hemostasis. This crude venom has dual special effects on blood coagulation: the enzyme similar to thrombin which could cause clotting action *in vitro*, whereas, defibrination (anticoagulant) reaction placed *in vivo*. The thrombin-like enzyme has high coagulant value and plays role in conversion of fibrinogen to fibrin (Pornmuttakun and Ratanabanangkoon et al., 2013). Thrombin- like Enzyme from *Calloselasma rhodostema* venom has been valued understanding developments of blood coagulation 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.

In addition, medical application of snake venom that has also been studied are Multiple Sclerosis (MS) therapy using ancrod which can be found from *Calloselasma rhodostoma* venom that capable to stimulate (defibrinogenation) to cure this disease (Mirshafiey, 2007). Finding for effective cure for Human Immunodeficiency Virus (HIV) is still an ongoing process that had met few successes. 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 A2 (PLA2) in venom protects blood leukocytes from other replication from macrophages. Others, include metaloprotease inhibitors which inhibit formation of new viruses by blocking protease enzyme (Meenakshisundaram et al., 2009). L-amino acid oxidases (LAAOs) present in snake venom of crotalids, elapids and viperids shows anti-HIV activity, which could induce cell death. The other studies of the therapeutic application of snake venom are treatment for antibacterial using element of L-Amino acid oxidases (LAAO) found in snake venom which effective in inducing antibacterial property against *Escherichia* (Phua et al., 2012); 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) and Leishmaniasis parasitic disease studies using Enzyme mytoxic phospholipase A2 , known as MjTX-2 which display antiparasitic effect against *Leishmania* (Castilhos et al., 2011).

Nowadays, the potential products based on snake venom has been produced and commercialized. Captopril has been long known for its service of saving millions of human lives through its anti-hypersensitive activities. This innovation not only results in advance of potential human therapeutic agents, yet discovery of captopril which creates new class of anti-hypersensitive drug (McCleary & Kini., 2013). Furthermore, Snake venom was recently found to be effective in treating skin diseases such as dermatomyositis, eczema, scleroderma, and psoriasis. The pure snake venom also contains nerve growth which speeds up the healing developments of damaged skin (Sun et al., 2003). Furthermore, research found that Bradykinin is induced in the brain of patients suffering from Alzheimer's disease (Lacoste et al., 2011).This facilitates proteolytic enzyme inhibition which inactivates bradykinin and catalyses conversion of angiotensin-1 into angiotensin-2 (Ferreira et al., 1970).

Abundance of proteolytic enzymes can induce milk coagulation and these proteases results 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 coagulation can be facilitated by inducing micelle aggregation; by enzymatic action

(cheese) or also by adding acid (form fermented milk) (Hallen, 2008). Milk is the emulsion of fat globules and suspension of casein micelles in water. Casein micelles are hydrophobic with their natural tendency to aggregate. Coagulation is achieved by adding specific proteolytic enzyme usually rennet to the milk when casein micelles stick together. κ-casein is a substrate of chymosin, is the primary agent for milk clotting and proteinases which productively coagulate milk could recognize sequence from His 98 to Lys111, but exhibit specificity for Phe105- Met106 bond of κ-casein of the casein micelle (Smith et al.,1991). Addition of chymosin (rennet) to milk cause coagulation. Chymosin recognize the sequence from His at lag phase, the enzyme hydrolyses κ-casein used in the stabilization of casein micelles (Blecker et al., 2012).

Previous studies has found similarity between coagulation of blood and milk. Jolles and Henschen (1982) compared the between the clotting of blood and milk and found the common features of the blood and milk clotting process. The structural homologies between κ- casein and N-terminal part of fibrinogen y-chain. In addition, there is similarities between the action of thrombin on fibrinogen and that of chymosin on the κ- casein fraction of milk, that these enzymic cleavage are proceed through similar mechanisms. There is definite degree of homology among the structure of κ-casein and human fibrinogen (Nicholas et al., 1978).

C. rhodostoma locally known as Malayan pit viper is chosen to study on the importance of snake venom in causing coagulating effects of milk. The screening for coagulation property of *C. rhodostoma* snake venom shows that this snake venom has the potential to clot milk in food industry. It has been reported that enzyme present in snake venom hydrolyze proteins and membrane component which lead to blood clotting. *C. rhodostoma* crude venom has dual effect on blood clotting; at particularly low concentration it is procoagulant while at higher dosage it acts as anticoagulant, the venom is an activator of Factor X, which speed up conversion of prothrombin to thrombin, results in formation of normal fibrin. This clotting activity observed at low concentrations estimated due to Factor X activator predominant, while at high concentration defibrinogenating mechanism is predominant (Dambisya et al., 1994). The fibrinogen clotting enzymes found in snake venom sample are the most frequent type of blood coagulation activator, they are best well known among members of the family Viperidae (Viperinae and Crotalinae).

1.2 PROBLEM STATEMENT

The field of enzyme technology in utilizing enzyme commercially is a lucrative business. The manufacturers of commercial enzymes are in constant search for catalytically productive and stabile enzyme. The latter refers to the ability of the enzyme to be mined from its original state or genetically manipulated to withstand in desired condition e.g. to modify enzyme optimum condition from low acidity or high alkaline to neutral condition to save expenditure of the industry using protease.

Generally, the approach to mine for useful enzyme relied on all sources such as microbial, animal and plants. Many of such useful enzymes been found for not only industry but also other fields such as healthcare and even for military purposes. Next to enhance the enzyme capabilities, newer innovations been ventured and this included genetic engineering, unnatural substrate or environment, immobilization and also artificial enzymes. Consequently, the use of genetic engineering may overcome the issue of obtaining from kilogram to ton metric quantity of the enzyme for industry needs. Snake venom been studied extensively for its potentials in healthcare and commercial and even some products and drugs are already out in the market.

Additionally, some snakes such as Malayan Pit Viper and Malayan Krait are found indigenous and carry the name of Malaysia. Furthermore, snake venom contains rich and good source of protease e.g with much studies on its blood coagulation. All these only encourage the need to investigate useful protease from this less ventured source of biodiversity.

1.3 SCOPE OF RESEARCH

Snake venoms have attracted attention to be studied and developed in medical, biotechnological, toxicology and other applications due to having rich sources of bioactive components especially inorganic and organic components predominantly constituted by peptides and proteins (Menaldo et al., 2012). There are many of the proteins have been discovered, isolated and were tested for their activity. This provide knowledge and tools on further studies for further biotechnological applications. In most instances fractionation and

purification methods on snake venoms are required to isolate the complex mixture of snake venoms into single entity of protein.

In this project, the coagulating ability of at least ten snake venoms will be investigated. The venom that have the most rapid coagulating activity was selected for further fractionation and purification using ion exchange and size exclusion chromatographies. Ion exchange chromatography separates molecules according to protein overall charge whereas size exclusion chromatography separates protein molecules based on the molecular size and shape.

The proteases in selected snake venom with promising coagulating property on milk are isolated to single entity using bioassay guided purification procedures involving ion exchange chromatography initially followed by size exclusion chromatography using ion exchange chromatography and the fractions will be test for the activity. The fraction from ion exchange chromatography that has the coagulating. The fractions obtain from the chromatography techniques are collect and screen for milk coagulation activities. Sequentially, in order to analyze the purity of proteases, Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is carried out after each chromatography techniques. The pure proteases is sent to Proteomics International for identification using LCMS/MS. Next, once the protease identity known, the optimum parameters of the protease such as optimum temperature, pH, $CaCl_2$ concentration and isolated enzyme concentration for milk coagulation to be determined. Additionally, the kinetic parameters of isolated protease and rennet will be determined to compare coagulation activity of both coagulant based on Km, Kcat and Kcat/Km values.

1.4 OBJECTIVES

1. To screen selected venoms obtained from the most abundant snakes in Malaysia, for milk coagulating activities.
2. To isolate the relevant protease enzymes from the most potential venom capable to coagulate milk.

3. To determine the enzymes coagulating parameters i.e. optimum concentration, optimum pH, optimum temperature, dependency on calcium ions and enzyme kinetics.
4. To compare the efficiency of the isolated enzyme with commonly available commercial milk coagulating proteases.

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. The other applications of snake venom are yet to be discovered by the researchers. As the preliminary studies of snake venom is able to coagulate milk, it will produce new novel discovery linking venom protein for a newer application which is in dairy industry. In addition, it will provide new alternative coagulant in dairy industry upon isolating protein of interest to purity. This work not only investigates a newer application for snake venom but also providing potentially a more productive catalyst for the food industry. Due to the prospecting of enzyme from the common sources i.e. plants and microbes have been exhausted. The current way expected to tap into the less ventured animal source instead i.e. snakes for useful protease. As the demand of 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.

CHAPTER 2

LITERATURE REVIEW

2.1 DESCRIPTION OF SNAKES FOR STUDY

There are approximately 400 venomous snakes among around 2600 different species of snakes in the world. In Malaysia, there are approximately 40 species of poisonous snakes, including those found on the land and in the sea (Halim et al., 2012). These venomous snakes can be further categorized into families of Viperidae, Elapidae and Colubridae (Underwood, 1979):

- Viperidae family is a common family of venomous snakes which include vipers, pit vipers, rattlesnakes and copperheads. Most of the Viperidae family members contain hemostatically active components which cause strong local swelling, necrosis and disturbs the blood clotting system (Clemetson et al., 2005).
- Elapidae family includes cobras, sea snakes, coral snakes, kraits and the King cobras. They are highly venomous and contain neurotoxins which cause disable muscle contraction and even paralysis when injected (Clemetson et al., 2005).
- Colubridae family consists of non-venomous and venomous snakes. They are mainly not harmful to humans while there is only some of the venomous snakes of Colubridae family like twig snake may cause local and systemic damages when injected.

2.1.1 *Calloselasma rhodostoma* (viper)

Malayan Pit Viper (*Calloselasma rhodostoma*) which was previously known as *Agkistrodon rhodostoma*, is a distant cousin of the North American Copperheads. Calloselasma 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, Combodia, 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 submontane forest and plantation where it is associated with both dense undergrowth and rocky areas (Das, 2010). With an average length of 70-80 centimeter and rarely more than 1 meter. Its nature are 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 'stupid' because the snake usually remains at the site of biting and hence usually killed. 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 *Calloselasma 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 incoagulable. However, at higher concentrations, the venom has shown to have anticoagulant effects with a progressively shortened coagulation 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).

2.1.2 *Trimeresurus purpureomaculatus* (viper)

Trimeresurus purpureomaculatus commonly known as mangrove pit viper or shore pit viper by the locals. This is one of the medically important poisonous land snake found in Malaysia (Tan, 2010). There are about 30 species of the genus Trimeresurus. *T. purpureomaculatus* falls under those without spines on the hemipenis (Tan & Tan, 1988). This snake venom was found abundance with enzyme for example 5'-nucleotidase, protease, arginine ester hydrolase, arginine amidase, phospholipase A, acetylcholinesterase and alkaline phospomonoesterase activities. Nget-Hong & Gnanajothy (1992) also emphasized on the absence of L-Amino acid oxidase and hyaluronidase in its venom content. In their studies, it is proven *T. purpureomaculatus* exhibit hemorrhagic activity, an edema-inducing effect, anticoagulant effect and thrombin like activity. The thrombin-like activity was not comparable to *Calloselasma rhodostoma*. Based on Denson (1969), *C. rhodostoma* has 8 fold higher coagulant effects compared to *T. purpureomaculatus* venom. The action of *T. purpureomaculatus* venom has coagulation activity at increased venom concentration, whereas anticoagulant effect in a lower venom concentration. Studies shown, only few significant research have been done based on this venom, the most prominent outcome is the present of both procoagulant and anticoagulant principles in it.

2.1.3 *Trimeresurus wagleri* (viper)

Trimeresurus wagleri is also commonly known as speckled pit viper or Wagler's pit viper. Brattstrom (1964) stated that *Trimeresurus wagleri* has many morphological characteristics that distinguish it from other species of Trimeresurus and therefore it can be put under the subgenus Tropidolaemus. *Tropidolaemus wagleri* is a venomous pitviper species native to Southeast Asia. They have a large triangular-shaped head, with a relatively thin body. They are almost entirely arboreal, and the tail is prehensile to aid in climbing (Tan & Tan, 1989).

They are found in a wide variety of colours and patterns, often referred to as "phases". In the past, some researchers classified the different phases as subspecies. The phases vary greatly from having a black or brown coloration as a base, with orange and yellow banding to others having a light green as the base colour, with yellow or orange banding, and many

variations. Nocturnal and arboreal, they appear quite sluggish as they remain motionless for long periods of time waiting for prey to pass by. When prey does pass by, or if disturbed, they can strike quickly. It contains hemotoxic toxins which is used to attack its prey. Their primary diet consists of rodents, birds, and lizards. The snake is abundant in lowland, primary forest and secondary forest. It is often seen climbing in low bushes. *T. Wagleri* is regarded as one of the most primitive crotaline species and its bite may cause severe pain and local inflammation (Reid, et al., 1963).

Tan & Tan (1989) stated that *Trimeresurus wagleri* exhibited the usual set of enzymes contain in pit viper venoms which are hyaluronidase, Lamino acid oxidase, phospholipase A, 5'-nucleotidase, protease, alkaline phosphomonoesterase, arginine ester hydrolaxe and phosphodiesterase, but it has unusually high content alkaline phosphomonoesterase, whereas the contents of arginine ester hydrolase and protease were very low. In addition, the lethal toxicity of t. wagleri venom is relatively high among Trimeresurus venoms which is LD_{50} 0.7-0.9µg/g.

2.1.4 *Naja sputatrix* (cobra)

The species found throughout most of Peninsular Malaysia and known as Malayan cobra. The snake is brown or black usually with some white marks on the throat. The average length 1-1.25 m, maximum about 1.5 m. This species of cobra has the habit of spraying or `spitting' venom. The Javan spitting cobra is found mostly in tropical forests and wet forest, but the species adapts incredibly well to a wide variety of habitats across its range on the islands, including more arid regions, dry woodlands, and cultivated hill country (Batchelor, 1958).

Naja sputatrix is very defensive and readily spits out venom when it feels threatened. It is a terrestrial snake that's nocturnal in nature. It preys predominantly on small mammals such as small rats and mice, but it will also feed on frogs, other snakes and lizards. The most significant constituents of the venom include high-molecular-weight proteins and enzymes, phospholipase A_2 enzymes, postsynaptic neurotoxins and polypeptide cardiotoxins. The high molecular weight protein and enzymes more than 30000 which approximately 5% of the venom dry weight. Like all cobra species, this species' venom also consists of postsynaptic

neurotoxins which account 4.5% of venom dry weight (Tan, 2004). However, the main components of its venom are cardiotoxins with cytotoxic activity which account 60% of venom dry weight (Tan, 2004). The venom was also found to exhibit an in vitro anticoagulant activity much stronger than most common cobra (genus Naja) venoms. The anticoagulants, phospholipase A_2 enzymes make up 15% of the venom dry weight (Tan & Tan, 1988).

A spitting cobra can launch its venom a distance of 6 to 8 feet (1.8 to 2.4 meters) at lightning speed. Just as rattlesnakes only rattle when they sense danger, spitting cobras only spit in order to defend themselves. This self-defense mechanism is a deep-rooted instinct. *N. sputatrix* often contains a combination of neurotoxins and cytotoxins which are substances that can damage nerve tissue and shut down individual cells. On human skin, *N. sputatrix* venom is not harmful, but if it gets in the eyes, inside the nostrils or into a cut in the skin, it can cause serious damage (Reid, et al., 1963). This snake is nocturnal and spends most of its time on the ground. It is a common prey for the Komodo dragon, the largest monitor lizard.

2.1.5 *Ophiophagus Hannah* (cobra)

Ophiophagus Hannah or popularly known as king cobra is the world's largest venomous snake, which could reach up-to 5 meter length. It is found mostly in Southeast Asia and South China. People regard it as one of deadliest snake having abundance venom yield. Around the world only 35 cases of cobra bites reported, about one third end up in fatality. Successful survivors were mostly saved by monospecific Thai Red Cross Society king cobra antivenin stated by Gold & Pyle (1998). Qualitative records on the enzymatic actions including (L-amino acid oxidase, phospholipase A2, protease, arginine esterase, acetylcholine esterase, phosphodiesterase and 5'-nucleotidase).

Action of cytotoxicity of L-amino acid oxidase was seen in stomach malignancy, murine melanoma, fibrosarcoma, colorectal tumor and Chinese hamster ovary cell lines. This protein successfully decreased level of cell proliferation by 74% when checked using thymidine uptake test. Generally, proteins with hemorrhagic effect are frequently found in snake venoms from viperadae and crotalidae families, while neurotoxic peptides were widely dispersed among elapid snakes. Pathogenic diseases such as venom-prompted pathogenesis, hemorrhage edema, hypotension, hypovolemia, inflammation and cell death contributed by

snake venom metalloproteinase domain. This protein commonly found in component from viperid venoms, however there have been studies showed its presence in elapid snake venoms indicated by Guo et al. (2007).

2.2 COMPOSITION OF SNAKE VENOM

Meier and Stocker (1991) concluded that snake venoms are unique mixtures with their properties in biochemical and also pharmacological. Most of the bioactive proteins and polypeptides (enzymatic and non-enzymatic) in snake venoms are responsible for their pharmacological properties (Mukherjee et al., 2011). 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 et al., 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.

2.2.1 PROTEINS FROM SNAKE 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.

2.2.1.1 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 coagulation 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. They have been found to have roles in coagulation and digestion as well as in the pathophysiology of neurodegenerative disorders such as Alzheimer's and Parkinson's induced dementia. 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.

In addition, Ancrod is one of the important TLCs which can be found from *C. rhodostoma* venoms. It is a proteolytic enzyme which has been used as defibrinogenating

agent in clinical trials. Anticoagulant effects can be produced by defibrinogenating the blood. Purified ancrod functions for the treatment of acute ischemic stroke due to this properties (Lathan & Staggers, 1996). Ancrod has long been viewed as a potential treatment for acute ischemic stroke. It possesses a serine protease that cleaves fibrinopeptide A (FPA) from fibrinogen. This fibrinogenolytic effect underlies ancrod's potential in clinical benefit, which would be based upon limited clot propagation, reduced plasma viscosity, improved microcirculatory flow, and activation of endogenous fibrinolysis (Dempfle et al., 2000).

2.2.1.2 Metalloproteinases

A metalloproteinase is a protease enzyme whose catalytic activity need a metal to work properly. Metalloproteinases 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 are able to produce variable tissue and cellular pathology in bite victims. The majority of metalloproteinase induce profuse hemorrhage, blood coagulation, and the inactivation/activation of complement proteins (Rael et al., 1993). Metalloproteinases responsible for the local and systemic hemorrhage, hemostatic system disturbances and local tissues damage when venom is injected. Markland and Swenson, (2013) stated that metalloproteinases from snake venoms possess fibrinogenolytic activity and they acts 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 comes with fibrinogenolytic activity applicable for clinical management that reduces fibrinogen value in plasma or dissolves coagulated plasma (thrombolysis) (Matsui et al., 2000).

Jararhagin is one of the metalloproteinases obtained and isolated from *Bothrops jararaca* venom. Based on the studies, jararhagin enhanced fibrinolysis and interfered with platelet functions (Moura-da-Silva & Baldo, 2012) and stated that, the applications on biotechnological and therapeutic use of jararhagin should be further studied.

2.2.1.3 Disintegrins

Disintegrins from viper venomms 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 coagulation renders it of medical interest, particularly with regard to 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).

2.2.1.4 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 et al., 2005). Many of them have been characterized and studied for the development involving blood coagulation 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).

Besides, convulxin and crotacetin are some of the CLPs isolated and studied from snake venoms. (Rádis-Baptista et al., 2005) stated that convulxin from South American rattlesnakes, Crotalus durissus has been isolated and studied. They possess coagulant, anticoagulant and antagonist of platelet activation properties. Their properties acting on

platelets and plasma provide tool for exploring facet of platelet functions. C-type lectin-like proteins possess various biological activities, including anticoagulant- and platelet-modulating activities, and, in contrast to C-type lectins, they have no lectin activity. Common C-type lectins are non-enzymatic proteins that bind carbohydrate in a calcium-dependent manner (Morita, 2005).

2.2.1.5 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.

2.2.1.6 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. The drop of blood pressure due to the toxic effects of snake venoms revealed the potential of Angiotension-converting enzyme inhibitors. ACEs control the blood pressure through the conversion of angiotension I to angiotension II, which acts as the blood pressure regulator. Angiotensin II is a hormone that circulates in the blood and has many effects on the cardiovascular system; its main role is to constrict blood vessels. This constriction can cause high blood pressure and increase the work required for the heart to pump blood into the body's main arteries. This causes a problem for the heart muscle if it has been weakened by a heart attack or heart failure.

Angiotensin-converting enzyme (ACE) inhibitors help relax blood vessels. ACE inhibitors prevent an enzyme in your body from producing angiotensin II, a substance in your

body that narrows your blood vessels and releases hormones that can raise your blood pressure. This narrowing can cause high blood pressure and force your 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. With this potential in therapeutic use, Captopril, an anti-hypertensive drug was developed and designed in order to treat hypertension and heart failure. In addition to the role it plays in constricting arteries and raising blood pressure, angiotensin II is also "growth-promoting" in a negative way, in that it causes increases in size or thickness of several cardiovascular structures. High levels of circulating angiotensin II lead to thickening of the heart, a condition known as hypertrophy. Hypertrophy of the heart has long been recognized as a marker for high risk of death caused by heart disease. In the presence of high levels of angiotensin II, the walls of blood vessels also become thicker and stiffer, in addition to constricting, and this is thought to predispose to cholesterol deposits and blockages in the arteries, which can lead to heart attacks and strokes. ACE inhibitors are prescribed to prevent or reverse the hypertrophy of the heart and vessel walls (Sweitzer, 2003).

2.2.1.7 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 et al., 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 is able to 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. Myotoxin (myonecrotic toxin) that affects muscular system can be found in rattlesnakes and some pit vipers venoms. Other than the mentioned proteins, complex mixture of snake venoms contain many other proteins which are varies in different types of snakes. The proteins present in venoms have their own uses for defence uses and also possess valuable potential that can be further studied for other applications.

2.3 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). An example of a well-known disease that could be controlled by snake venom is excessive bleeding. A blood-clotting protein in Taipan venom has been found to stop excessive bleeding during surgery or after major trauma. Another example of a well-known disease that could potentially be cured by snake venom is stroke. Components of Malayan Pit Viper venom has shown potential for breaking blood clots. In its pure form, the venom causes prey to die of massive hemorrhaging by preventing blood coagulation but among humans, it is used to treat patients who suffer from blood clots.

Cobra venom, on the other hand, has the potential to slow down the degeneration of joints as in Rheumatoid Arthritis. It also reportedly has anti- depressive activity. At Recepto pharm, researchers are also currently doing research on Herpes, HIV/AIDS and Multiple Sclerosis using cobra venom and its constituents. Enzymes from cobra venom also may be instrumental to finding cures for neurological diseases such as Parkinson's disease and Alzheimer's disease. Cobra venom cytotoxins, for example, may also be utilized as anti-cancer agents since they are efficient at destroying certain types of cancer cells including

leukemia. Snake venom cytotoxins are highly basic amphipathic proteins and they constitute as much as 40–70% of cobra venom (Alsarraj, 1997).

In addition, snake venom also consists of many type of toxins and each of this toxin could be used as a cure for various diseases.Neurotoxins target the central nervous system and they stop the muscles from working. They work by inhibiting or completely blocking nerve activity, so they are interesting research targets for diseases, such as epilepsy, in which there is too much electrical brain activity, for the treatment of pain or for helping drug addicts trying to escape their dependency. According to (Ranawaka, et al., 2013), the neurotoxic properties of venoms like antimicrobial activity, binding to specific cells, inhibition of apoptosis and anti-inflammatory also revealed their potential to be studied for treating infections, cancer and various neurological disorders.

Another type of toxin is the Hemotoxin and one example of a therapeutic use of Hemotoxin is in the circulatory system. These toxin has anti- clotting activity and prevents the clotting of blood. The protein 'Ancrod' which is present in hemotoxins prevent clotting compounds from functioning correctly, which causes uncontrollable bleeding. Ancrod seems able to dissolve the blood clots that cause stroke for as long as 6 hours after stroke symptoms start. Ancrod can also prevent new blood clots from forming (Kini, 2011).

Cytotoxin is another toxin which is present in the snake venom and has many therapeutic uses. Some physiological effects of cytotoxin include cytotoxity, inhibition of platelet aggregation, cardiac arrest and hemolysis. Cytotoxicity refers to a substance or process which results in cell damage or cell death. Cytotoxins exhibit various uses such as in cancer treatment. Cytotoxic effects of snake venom have potential to degrade/destroy tumor cells. These may treat breast cancer, ovarian cancer or other type of cancers (Liu et al., 2014).

2.3.1 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 tumor cells by starving it to death (Liu et al., 2014). In order for a tumor to grow and metastasize, both tumor and endothelial cells must migrate and invade surrounding tissues. Once in the blood vessels, tumor cells must adhere to the endothelium and escape to a new site, two processes that depend on their invasive abilities. Therefore, the blockage of both tumor 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 tumor microenvironment. Integrin blocking usually results in inhibition of cell migration and tumor 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).

According to Jamunaa et al. (2012), *Ophiophagus hannah* and *C. rhodostoma* venoms were the most cytotoxic venoms. Their cytotoxic properties are further studied for the development in cancer treatment. The disintegrins proteins derived from this species venom also revealed the potential in development of anti-inflammatory therapy and cancer treatment (Selistre-deAraujo et al., 2010).

Additionally, snake venom extracted from Walterinessia aegyptia alone or in combination with silica nano- particles can decrease the proliferation of human breast carcinoma cell line. The snake venom toxin from Vipera lebentina turnica also can induce apoptotic cell death of ovarian cancer cells. An enzyme derived from copperhead venom could also be used for treatment of breast cancer (Jain & Kumar, 2012).

2.3.2 Defibrinating agent

Snake venom has potency to be beneficial in clinical research. Its venom has been valued understanding developments of blood coagulation 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).

This glycoprotein have molecular weight ranging from 29 000 to 35 000 Da. Since it displays action as defibrinating agent, researches have more focused on study of this enzyme on venom pro-coagulant or anti-coagulant. Research done by using ancrod before the formation of induced thrombus in dog, shows positive results in preventing thrombosis and vessel remained unobstructed (Vyas et al., 2013).

2.3.3 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 can could induce cell death. A study done in India on the helpfulness of venom transmitted to a patient encountered with drug resistant human immunodeficiency virus (HIV) infection, who was also undergoing anti-retroviral therapy shows positive response after administering snake venom with great increase in CD4 count in and lower count in viral load.

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 A2 (PLA2) in venom protects blood leukocytes from other replication from macrophages. Others, include metaloprotease inhibitors which inhibit formation of new viruses by blocking protease enzyme (Meenakshisundaram et al., 2009).

2.3.4 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).

2.3.5 Multiple sclerosis treatment

Multiple sclerosis (MS) is an autoimmune disease which occurs due to chronic inflammatory demyelination of 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's depleting feature plays an important role in therapeutic measure in multiple sclerosis. From the previous research done by Mirshafiey (2007) venom from *Calloselasma rhodostoma* which contains ancrod can stimulate (defibrinogenation) to cure this disease.

2.3.6 Treatment for Leishmaniasis (Parasitic Disease)

Human parasitic infections include malaria, Chagas' disease, leishmaniasis, filariasis is a sleeping sickness and schistosomiasis contribute to destruction impact on human health. Leishmaniasis is a parasitosis infection caused by species of protozoa *Leishmania* spread by female sandfly. Snake venom having complex mixture of proteins having various physiologic effects and induce systemic alterations; for example, systemic bleeding coagulopathy and many more. Brazil found *Bothrops moojeni* contains wide range of proteases. *Bothrops moojeni* found to kill *Leishmania in vitro*. Enzyme mytoxic phospholipase A2, known as MjTX-2 display antiparasitic effect against *Leishmania* (Castilhos et al., 2011).

2.3.7 Anti-microbial

Antimicrobial properties of the snake venom have been investigated. The element of L-Amino acid oxidases (LAAO) found in snake venom effective in inducing antibacterial

property. In a test by using six gram bacteria, it shows inhibition zones produced around the tested area in hole-plate method. King cobra venom when tested its effectiveness, shows most efficient antibacterial property against *Escherichia coli* and less efficient against *Pseudomonas aeruginosa*. Phua et al. (2012) conducted a studies by screening the king cobra venom using three strains of *Staphylococcus aureus*, three other species of gram-positive bacteria and six gram-negative bacteria and the results shows 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 LAAO 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 *Calloselasma rhodostoma* and *Ophiophagus 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).

2.4 BIOTECHNOLOGY BENEFIT /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:

- Ancord (Viperinex)

  It is a thrombin-like enzyme isolated from *C. rhodostoma* venoms which was discovered by H. Alistair Reid in 1960 (Lathan & Staggers, 1996). It is useful in

clinical trials of treating acute ischemic stroke and heart diseases. Viprinex manufactured by Abbott is an example of snake venom product produced using genetic engineering. Formerly Arvin, Viprinex is an orphan drug from Malayan pit viper venom, used to treat patients with acute ischemic stroke to anticoagulate heparin-intolerant patients undergoing coronary artery bypass graft. Ancrod, an enzyme from pit viper venom with defibrinogenating properties was made as Viprinex. Viprinex was involved in the clinical trials of stroke treatment investigation. However, it failed those trials and the product was banned and not commercialised (Yeo and Sharma, 2013).

- Angiotension-converting enzyme inhibitors (Captopril)

  It is an anti-hypertensive drug derived from *C. rhodostoma* venoms (Lathan & Staggers, 2006). Captopril has long been used to save millions of human lives through its anti-hypertensive activites. It is an example of therapeutics derived from snake venoms (Earl, et al., 2012). These medications could block angiotension converting enzyme (ACE) which cleaves and converts angiontension-I into angiontension-II. Angiotension I has vasodilation effects while angiontension-II has vasoconstriction effects. When the ACE is blocked, vasoconstriction cannot take place and this is very much needed for people with hypertension. This medication is a new class of anti-hypertensive drug which can be used to treat hypertension and heart failure (Koh, et al., 2006).
- Phospholipases A_2

  It can be extracted from Elapidea and Viperidae family members. They possess anti-tumor and anti-microbial activity. Koh et al. (2006) stated that the study on this enzyme provides potential more specific pharmacological tools for treatment of neural trauma such as Alzheimer's disease and Parkinson's disease.
- Tirobifan (Aggrastat)

  It is extracted from *Echis carinatus* venoms and and acts as a specific antagonist for platelet aggregation (Cook et al., 1999). It is useful in inhibiting platelet aggregation and angina treatment.
- C-type lectin-like proteins

It can be found from *Agkistrodon acutus* and acts as an antagonist in platelet aggregation (Paulchamy, 2010).

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., 2011). For example, the toxins in Russell's viper venom was used to assay blood clotting factors, platelet factor and some coagulation proteins like fibrinogen and prothrombin. Besides, disintegrin from snake venom which prevents platelet aggregation are used to study the platelet-platelet and platelet-endothelium interactions. According to Mukherjee et al., (2011), the researchers discussed that Protein C activator from *Agkistrodonc. Contortrix* venom was found to be useful in activating Protein C for assay.

Furthermore, snake venoms had been studied for the production of 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 a number of skincare products made using this synthetic snake venom available in the market: Planet Skincare anti-aging moisturiser, INKANAT face cream, Auralux snake venom peptide cream and others.

2.5 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.

2.5.1 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.

2.5.2 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 anion exchange chromatography, anion exchanger (positively charged) will be attached on the matrix and bind to the negatively charged molecules from the samples. Figure 2.8 shows the illustration of protein molecules separation using anion exchange column. Diethtlaminoethyl (DEAE) Sepharose column is one of the example of anion exchanger used in chromatography techniques to purify snake venom (Babaie et al., 2013).

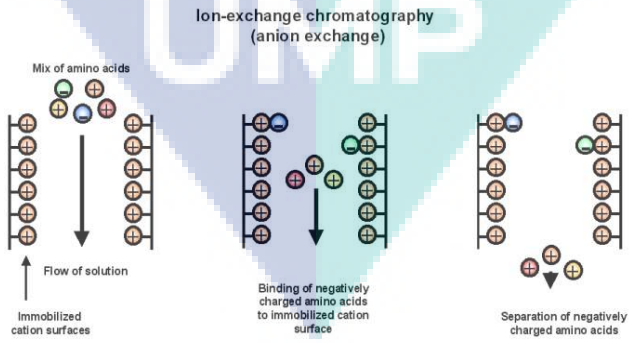

Figure 2.8. Separation of protein molecules in an anion exchange chromatography column. Source retrieved from http://chemwiki.ucdavis.edu/Core/Analytical_Chemistry/Instrumental_Analysis/Chromatography/Liquid_Chromatography/Ion_Exchange_Chromatography

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 quaternary ammonium (Q) and diethylaminopropyl (ANX) as anion exchanger as well as sulfopropyl (SP) and methyl sulfonate (S) as cation exchanger (GE Healthcare Life Sciences, 2010)

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 cation exchanger. While for the one above the pH of its isoelectric point, the protein is negatively charged and will bind to anion exchanger.

2.5.3 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 as shown in Figure 2.7.

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.

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).

2.5.4 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.

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. Figure 2.9 shows the illustration of the apparatus set up for SDS-PAGE as well as the travel of proteins with different molecular weight from negative to positive electrode.

2.6 MILK COAGULATION PROPERTIES

2.6.1 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).

2.6.2 Milk Coagulation

Milk coagulation is the clotting process from milk (liquid) into curd (gel-like) formation. Milk coagulation is always the first step in cheese making process. Herbert et al. (1999) stated that coagulation 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 coagulation 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 coagulation 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 coagulation is essential for tofu making. The coagulation process of soymilk 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 coagulation step of soy milk into tofu.

2.6.2.1 Enzyme-induced Milk Coagulation

Enzyme-induced coagulation of milk is done by the rennet enzymes. According to Hyslop (2003), this milk coagulation 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 2.10 and lastly will be the modification for the properties and structure of the coagulum. During the first step, the rennet enzymes play role in shave 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 coagulation time (Sandra et al., 2012).

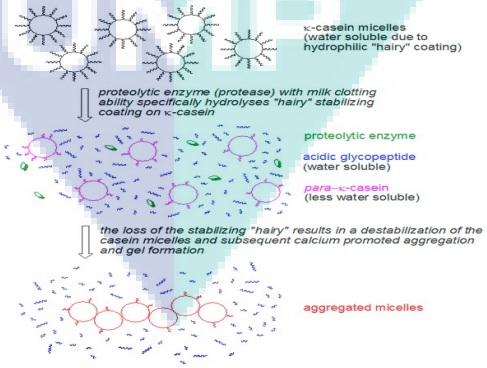

Figure 2.10. Activation of casein micelles using enzymes and aggregation of enzyme modified casein micelles.

Source retrieved from http://blog.khymos.org/wp-content/2014/02/casein.png

The rennet obtained from calf contains more than 90 % of chymosin which is responsible for the milk coagulation 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).

2.6.2.2 Acid-induced Milk Coagulation

Shaker et al. (2000) mentioned that addition of acid on milk coagulation removes the calcium bonds between casein micelles and thus destabilizes the micelles and followed by forming curd due to aggregation. Acid-induced milk coagulation is usually done by acidify the milk to pH below 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.

2.6.2.3 Factors Affecting Milk Coagulation

The factors affecting milk coagulation, an important process in dairy products industry have been determined and studied. While determining the factors, coagulation time and curd firmness are used as indicators to evaluate the coagulation 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 coagulation properties. Among these, acidity and casein content may be the factors which can contribute

on improving the milk coagulation. Additionally, it was found that a lower pH with a higher temperature may give a better coagulation process by having shorter coagulation time (Toffain V et al., 2012). Calcium ions content also influence milk coagulation by promoting aggregation of casein micelles and thus shorten the coagulation time (Landfeld et al., 2002; Sandara et al., 2012).

2.7 POTENTIAL SIMILARITIES BETWEEN BLOOD AND MILK COAGULATION

The final stage of blood coagulation 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 coagulation.

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 cleavage. 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).

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Sample collection

Snake Venoms Snake venoms from various species of viper origin will be obtained from the usual source i.e. Snake and Reptile Farm, Sungai Batu Pahat, Perlis. Venoms will be milked from selected viper snakes by a trained snake handler and collected in a sterile universal container, which will be freeze-dried (lyophilized) and stored at -80^{o}C until further use. Rennet from Mucor miehei (fungi) will be purchased from Sigma-Aldrich.

3.2 Determining milk coagulating end point time

The commencement of coagulation will be determined using three visible parameters i.e viscosity, colour changes, and development of white spots. Upon these initial coagulating indications, immediate observations under light microscope done to confirm the end point time with certainty.

3.3 Bradford Protein Determination

Total protein in various venoms as well as in milk and curdles form will be determined using protein determination technique as stated by Bradford et al. (1976).

3.4 Evaluating Milk Coagulating Activity in Various Venoms

All freeze-dried viper venoms will be individually assessed for coagulating activity as describe in (ii).

3.5 Purification of Protease from Selected Venom

The proteases in venom will be fractionated and eventually isolated to homogeneity using various chromatography techniques sequentially. The standard protein purification techniques include Size Exclusion Chromatography and Ion Exchange Chromatography technique, fractions will be screened for coagulating abilities using method described in (ii). Chromatography techniques such as appropriate size exclusion gel and ion exchange chromatography will be utilized. The purity assessed

by SDS-PAGE and two dimensional electrophoresis. The bands and spots from them will be cleaved to determine the identity of the protease in the venom using LCMS-MS.

3.5.1 Ion Exchange Chromatography

The HiTrap SP FF (GE Healthcare Life Sciences) pre-packed with Sulfopropyl Sepharose Fast Flow, a strong cation connected to the ÄKTAexplorer (Amersham Biosciences, Sweden). A 50mM ammonium acetate at pH 6.0 and 1.0M NaCl in 50mM ammonium acetate at pH 6.0 were the start buffer and elution buffer (Buffer B). The parameters programmed to a method template as follows:

Flow rate: 1.0 mL/min; Fraction size: 4.0 mL; Sample injection: 0.5 mL; Equilibration volume: 15 mL; Wash out unbound: 10 mL; Elution volume: 30 mL; Re-equilibration volume: 15 mL and the linear gradient elution as shown in Fig 1.

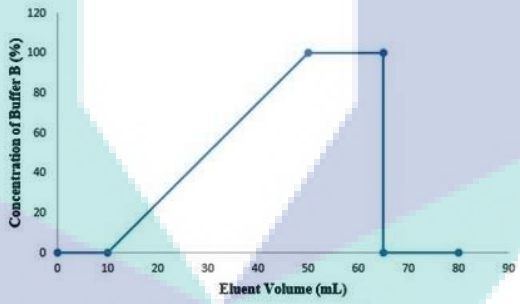

Fig 3.1: Concentration of Buffer B (%) versus Eluent Volume (mL).

The fractions were collected, freeze dried and consequently desalted using HiTrap Desalting (GE Healthcare Life Sciences, USA), a pre-packed Sephadex G-25 Superfine column. The column was connected to the ÄKTAexplorer (Amersham Biosciences, Sweden) and utilized a standard template program for desalting contained within it.

3.5.2 Size Exclusion Chromatography

The active fraction eluted from ion exchange chromatography column (HiTrap SP FF) was applied onto HiPrep 26/60 Sephacryl S 200 HR pre-packed column (GE Healthcare Life Sciences, USA). The column was connected to the ÄKTAexplorer (Amersham Biosciences, Sweden). The column was equilibrated with 50mM of ammonium acetate buffer of pH 7 and the elution was carried out using the same buffer with 2 ml sample injection at flow rate of 1 ml/min. The fractions of 6 ml were collected and the peak was monitored by recording the absorbance at 280nm.

3.6 Determining Optimum Parameters of Isolated Coagulants

a) Optimum concentration of coagulant:

Both purified proteases from venom and commercial rennet will be serially diluted based on their protein content. A total of 1ml of 10% (w/v) skim milk will be measured and added to the basic require Calcium chloride (0.83mg). The 1 ml milk will then introduce to 0.5 mL of different rennet and snake venom protease and swirled for 10 seconds. Timer set to record duration of time taken until there was signs of coagulation based on end point determination as state in method (ii).

b) Optimum Calcium ion concentration:

To determine the optimum concentration of calcium chloride required various concentrations of Calcium chloride preparations will be dissolved in 1ml milk and followed by mixing with 0.5 ml of the coagulants and swirled for 10 seconds. Timer set to record duration of time taken until there was signs of coagulation based on end point determination as stated in method (ii).

c) Optimum pH:

Each 0.5 mL of sample solution consisting of the individual coagulants and an appropriate buffer with varying pH of 5 - 7 will be mixed with 1 mL of fresh milk and swirled for 10 seconds. Timer set to record duration of time taken until there was signs of coagulation based on end point determination as stated in method (ii).

d) Optimum temperature:

Milk with respective coagulants will be incubated in water bath with varying temperature settings i.e 30 ℃, 35 ℃, 40 ℃, 45 ℃, 50 ℃, 55 ℃, and 60 Timer set to record duration of time taken until there was signs of coagulation based on end point determination as stated in method (ii).

e) Determining Kinetic Parameters

Kinetic parameters such as Km, Kcat and Kcat/Km will be determined for the isolated protease as well as commercial rennet. Kinetic parameters can be determined graphically by measuring velocity of enzyme-catalyzed at different concentration of substrate (Casein) i.e graph of Vo vs [substrate]. Km = Michaelis-Menten constant; Kcat = the turnover number; Kcat/Kcat = the catalytic efficiency of the enzyme.

3.7 Comparing Coagulation Capabilities of Isolated Protease with Commercial Rennet

Determining Coagulating end point time: Once the optimum parameters of the isolated protease determined for their respective parameters stated above they will be utilized to determine coagulating end point time as described in method (ii) above. The commercial rennet of Mucor meihei also with its optimum parameters will be assessed for its coagulating time. All protease will be ranked based on their coagulating end point time. Determining kinetic parameters of commercial rennet and comparing with kinetic parameters of isolated protease e.g. lower Km signify to be better coagulant.

3.8 Scanning Electron Microscopy (Visualizations of Coagulation)

The freeze-dried specimens (curds formed due to coagulants of selected venoms, purified proteases, commercial rennet and milk) will be sent for SEM analysis at University Malaysia Pahang, Central Laboratory.

CHAPTER 4

RESULTS AND DISCUSSION

This section included with selected results to be reported. The investigation into snake venoms identified one most capable venom for the purpose of coagulating milk. Hence it allowed a thorough study on the venom for its capabilities which included optimum parameters, visible and microscopic visualizations, comparison on its milk coagulation to a known protease, bioassay purification and identification of its active protease etc.

4.1 BRADFORD PROTEIN DETERMINATION

4.1.1 Bovine Serum Albumin Standard (BSA) Standard Curve

Table 4.1: Absorbance readings of different concentration BSA solution

Bovine Serum Albumin (BSA) concentration (µg)	Absorbance (A)
0	0
0.2	0.084
0.4	0.180
0.6	0.259
0.8	0.365
1.0	0.397

The concentration of *Calloselasma rhodostoma*, *Ophiophagus hannah Trimeresurus purpureomaculatus*, and *Trimeresurus purpureomaculatus* were determined using Bradford protein assay. The protein calibration standard curve is shown in Figure 4.1. The BSA concentrations used is as show in Table 4.1

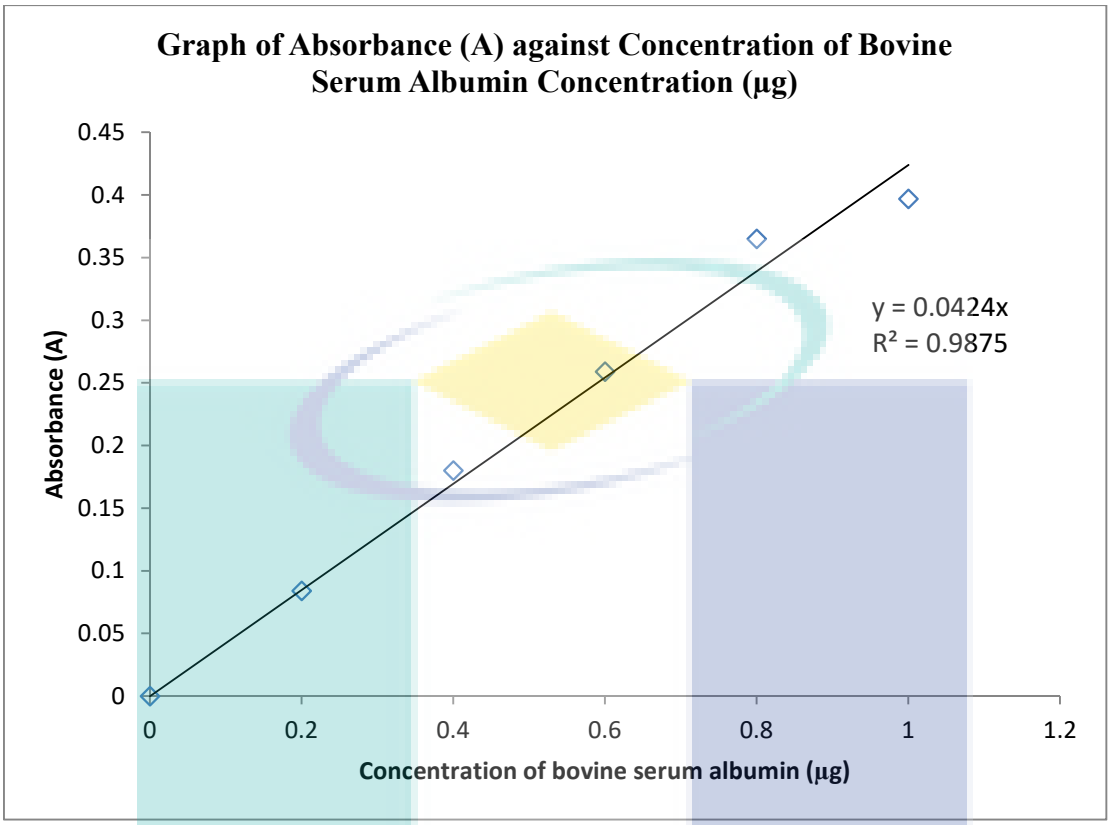

Figure 4.1: Protein calibration standard curve for Bradford protein assay

Figure 4.1 shows the protein calibration standard curve for Bradford protein assay. Bradford reagent measuring 1 mL was added to 100 µl of BSA (0-100 µg/mL). The homogeneous mixture was placed in dark for 20 minutes before measuring absorbance at 595 nm.

4.1.2 Determination of Protein Concentration

Table 4.2: Snake venom samples with its respective protein content (µg)

Venom sample	Protein content (µg)
Calloselasma rhodostoma	570.65
Trimeresurus purpureomaculatus	347.30
Ophiophagus hannah	268.41
Trimeresurus wagleri	109.45

The first step of this experiment would be to determine the quantity of proteins present in the venom and rennet. According to Bradford et al. (1976), this can be achieved by using Coomassie Brilliant Blue G-250 which exists in two different forms of color, red and blue. This dye will react with the protein to form a complex which has a very high molar absorptivity. This binding process makes the Bradford testing to be highly sensitive to the amount of protein present in the mixture. Moreover, it is a very rapid process and the protein-dye complex will remain stable up to one hour, allowing for a stable and fairly accurate reading.

Rennet protein content is 44000 µg. Its highest protein content is due to its high level of purity since it is based on recombinant fungal technology. The amount of protein determined from *Calloselasma rhodostoma* is 570.65 µg per 1 mg of the sample crude venom, which is equivalent to 57% (w/w). It clearly shows *C. rhodostoma* gives the highest protein content among the four venom samples studied as stated in Table 4.2.

4.2 SCREENING FOR MILK COAGULATION FOR SNAKE VENOM SAMPLES

Individual snake venoms were weighed and dissolved in 1 mL of distilled water to yield 1600 µg of protein present in that solution. Then, 0.5 mL of mixture was used to determine the time taken for 1 mL of milk to coagulate. The time taken was recorded as shown in Table 4.3.

Table 4.3: Time required for all four venoms to exhibit coagulation activity

Sample(s)	Time of coagulation (hours)
Negative control	no coagulation
Calloselasma rhodostoma	3.125
Ophiophagus hannah	>24
Trimeresurus purpureomaculatus.	>24
Trimeresurus wagleri	>24

>24 = Milk coagulation beyond 24hr

Screening for coagulation property was performed to detect the milk coagulating properties of the snake venom. The screening method proved *Calloselasma rhodostoma* to have rapid milk coagulation activity averaging at 3.125 hours in comparison to other 3 venom samples which require more than 24 hours to coagulate the milk. Hence, it can be observed that *C. rhodostoma* is proven to induce coagulation in a shorter duration of time compared to the other three venoms tested

Milk consists of complex components containing variety of fluorescent molecules (aromatic amino acids, nucleic acid and riboflavin). Some researchers studied on the use of amino acids (such as tryptophan) or vitamin A as an indicator to monitor milk coagulation.

For effective milk coagulation study, sophisticated apparatus such as turbiscan instrument will be helpful. Its operating procedure is based on measurements of concentrated emulsion and suspension instability. It can detect aggregation phenomena on concentrated thick media. Besides that, Bittante et al. (2011) studied milk coagulation by using Formagraph. It measures based on movement of small loop pendulums immersed in linearly oscillating samples of coagulating milk. Gel development in the moving milk solution studied by the minute forces registered. These techniques were used in large scale of measurements. Zerrin (2013) also stated on the usage of Nametre viscometer to evaluate on-line measurements of coagulation time and gel firmness. It is not applicable to use this technique in this experiment since snake venom samples obtained in minute quantity and require great risk of difficulties to milk the snake in order to extract the venom.

Initially, when coagulation activity have been spotted, it was hard to obtain an actual time of coagulation (end point) since determination was done visually. Absorbance readings at 500 nm were taken before and once the solution centrifuged at 13.4 rpm for 2 minutes. Absorbance readings were taken once there are signs of coagulation. Comparison was done between initial readings and after (at time of coagulation) to check the changes in absorbance from the initial value. Centrifugation is used to separate suspended materials in a liquid medium. The centrifugal force generated to the rotation rate of the rotor (in rpm) may denature the enzymes found in the sample. Since the proceeding experiments i.e: determining parameters of milk coagulations are continues process requiring the presence of milk and venom. Therefore, this method was found not effective and replaced with method described in section 3.2.

4.3 PARAMETER DETERMINATION

4.3.1 Determination of Optimum Concentration to Effectively Coagulate Milk

4.3.1.1 Determination of Milk Coagulation using Different Concentration of Rennet

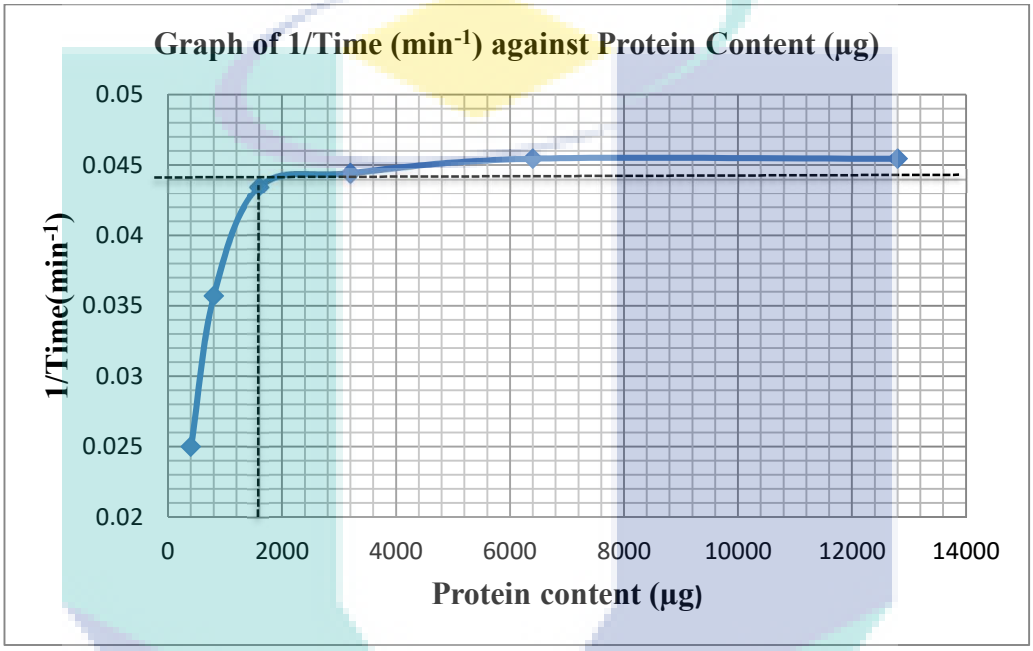

Figure 4.2: Graph for determination of milk coagulation by using different concentration of rennet.

Figure 4.2 shows 1600 µg of rennet protein content is the saturation point for effective coagulation activity with an average time of coagulation of 23 minutes. An increase in concentration beyond this point does not increase the rate of coagulation.

4.3.1.2 Determination of Milk Coagulation using Different Concentration of *Calloselasma rhodostoma*

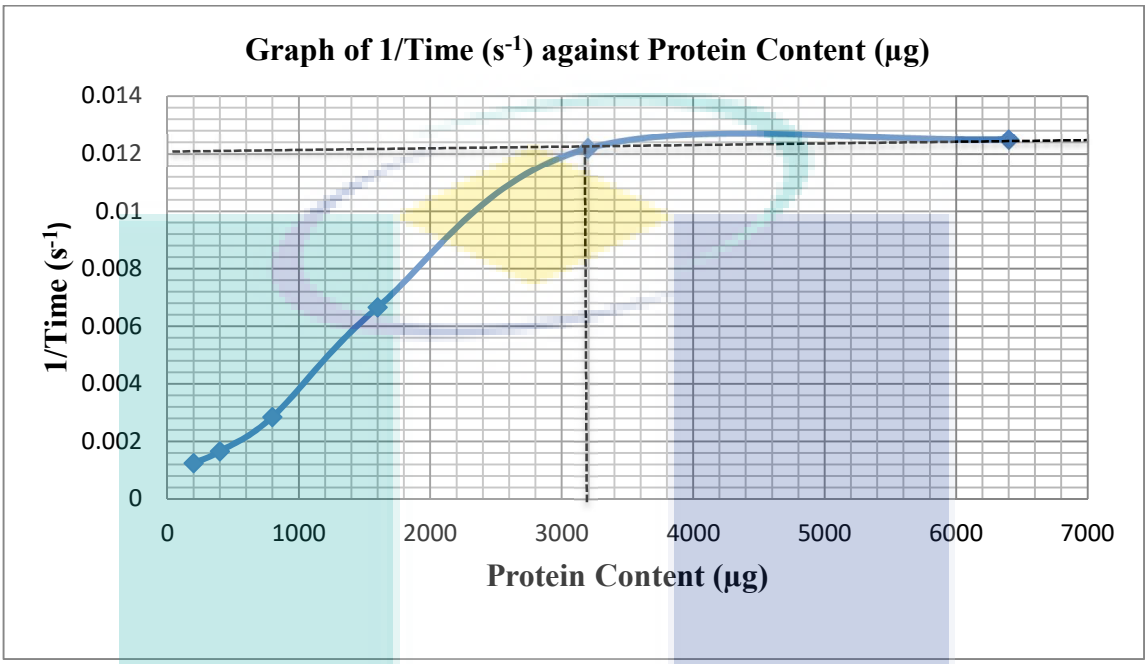

Figure 4.3: Graph for determination of milk coagulation by using different concentration of *C. rhodostoma.*

Figure 4.3 shows that the curve reaches plateau at 3200 µg protein content with an estimated time of milk coagulation at 82 seconds. The graph pattern shows the rate reaches constant value at this point. Beyond this value, further addition on protein content does not increase the rate of coagulation.

Protein concentration determination was done to determine optimum concentration for both samples to coagulate milk effectively. *Mucor miehei* rennet concentration graph shows proteins saturation occurs at 1600 µg, while for *C. rhodostoma*, higher amount of 3200 µg is needed. Any addition of protein content for both samples does not yield any significant increase in rate of milk coagulation. The rennet used is a recombinant fungal derivative source. It comprises of proteins with higher level of purity compared to venom of *C.*

rhodostoma therefore less amount of protein content is sufficient to cause coagulation compared to crude *C. rhodostoma* venom used.

4.3.2 Determination of Concentration of Calcium Chloride ($CaCl_2$)

4.3.2.1 Determination of Ideal Calcium Chloride ($CaCl_2$) Concentration for Rennet

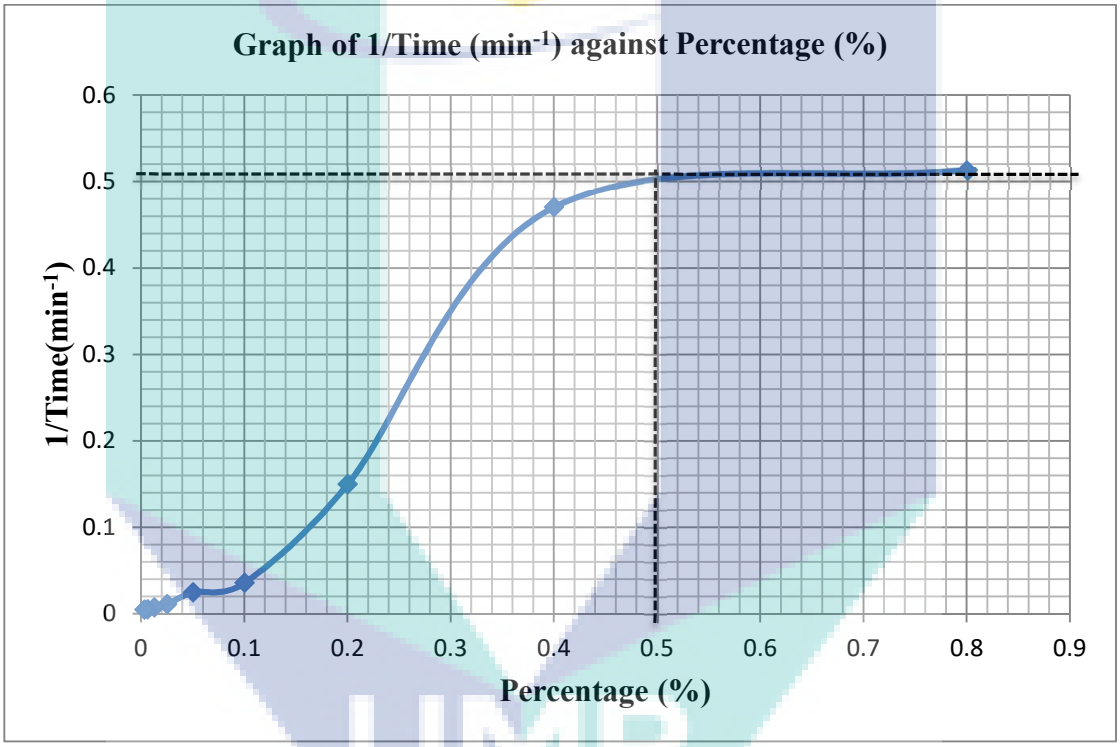

Figure 4.4: Determination of optimum calcium chloride ($CaCl_2$) concentration with 1600 µg rennet protein content.

Figure 4.4 shows a perfect sigmoid curve. The curve reaches plateau at concentration of 0.5% with an average timing of 2 minutes. This is the optimum concentration. Further addition of calcium ion concentration does not increase the rate of coagulation.

4.3.2.2 Determination of Calcium Chloride ($CaCl_2$) Concentration for *C. rhodostoma*

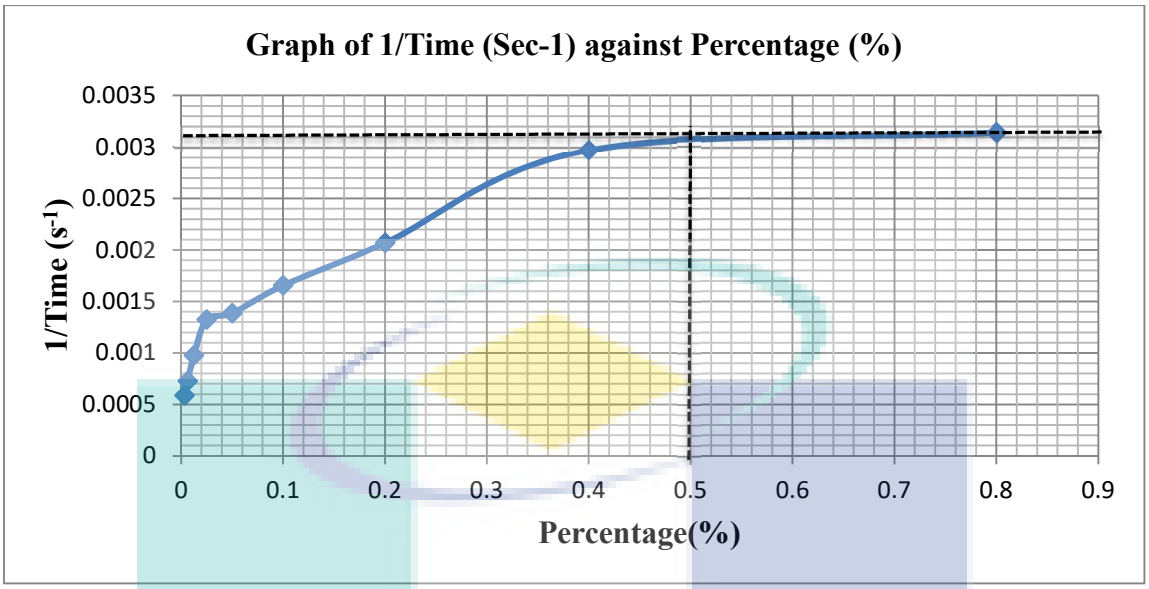

Figure 4.5: Determination of optimum calcium chloride ($CaCl_2$) concentration for 3200 µg of *Calloselasma rhodostoma.*

Figure 4.5 describes a sigmoid curve reaching a constant value at concentration of 0.5% calcium chloride. The effective optimum coagulation time estimated is 322 seconds. Addition of calcium ion concentration beyond this optimum concentration does not increase the rate of coagulation.

The study on calcium chloride concentration required for milk coagulation using rennet and *C. rhodostoma* shows 0.5% (w/v) was needed to produce maximum coagulation activity. Any increase in concentration of calcium chloride does not change the overall activity. According to Landfeld et al. (2002), calcium chloride is added to milk for an adjustment of the content of calcium ions. The rate of coagulation is directly proportional to the amount of calcium chloride added up to a constant value reached which in this case at the concentration of 0.5%. Processed milk sample will likely lose its nutrients especially calcium ion content during homogenization and pasteurization process, therefore it is crucial to add calcium chloride to rebalance its content in milk. Zerrin (2013) claimed accumulation of calcium escalates rate of firming of rennet-induced milk gels (curd) and firmness of gel (curd). Calcium chloride causes charge neutralization of negatively charged groups found on

casein micelle surface and possible development of calcium bridge which speed up the clotting as well. Kugelmass et al. (1937) defined addition of calcium ion (Ca^{2+}) not needed at pH 4.7 and below, due to formation of casein chloride instead of calcium caseinate.

4.3.3 Determination of Optimum pH to Effectively Coagulate Milk

4.3.3.1 Determination of Optimum pH for 1600 µg of Rennet by 0.1M Phosphate Buffer

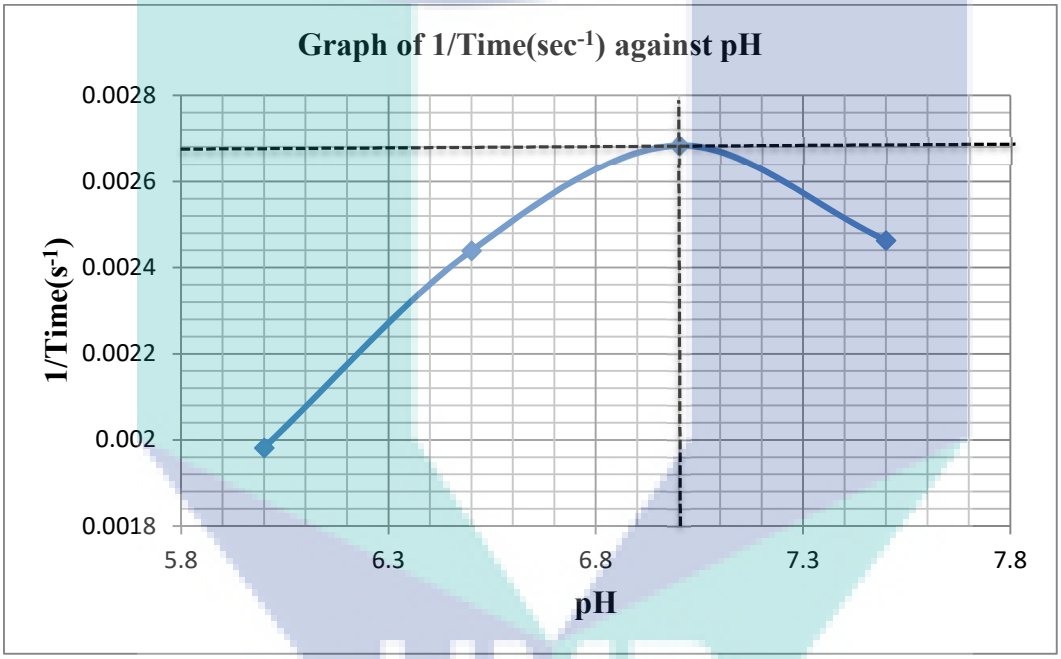

Figure 4.6: Determination of optimum pH using 1600 µg of rennet by 0.1M phosphate buffer.

Figure 4.6 shows a bell curve having its peak at optimum pH 7 with estimated time for milk coagulation around 370 seconds. Below pH 5, acid induced coagulation occurred, from pH 6 the coagulation rate increases gradually until pH 7, then gradual decrease till pH 7.5. Beyond pH 8, milk coagulation does not occur.

4.3.3.2 Optimum pH Determination using 3200 µg of *Calloselasma Rhodostoma* by 0.1M Phosphate Buffer

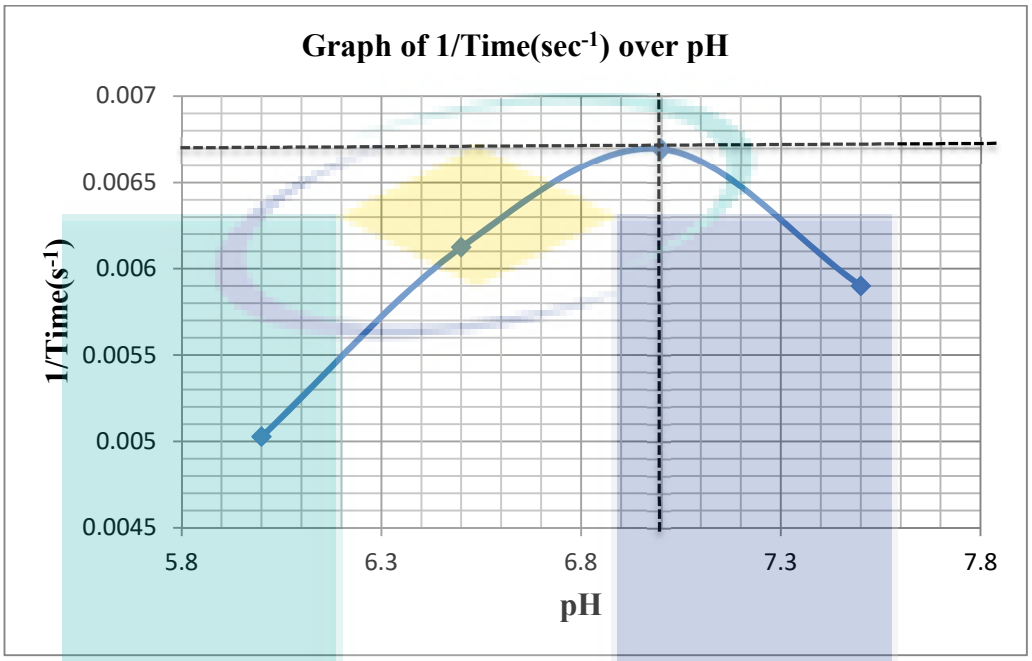

Figure 4.7: Optimum pH determination using 3200 µg of *Calloselasma rhodostoma* by 0.1M phosphate buffer.

Figure 4.7 shows a bell curve having peak at optimum pH 7 with estimated coagulation time of 151 seconds. This shows *C. rhodostoma* requires an optimum pH of 7 for effective coagulation activity. Below pH 5, acid induced coagulation occurred, from pH 6 the coagulation rate increases gradually until pH 7, then gradual decrease till pH 7.5. Beyond pH 8, milk coagulation does not occur.

Landfeld et al. (2002) indicated optimum pH of milk should be in the range of 6.2-6.5. Therefore, milk coagulation generally occurs in slightly acidic pH environment in contrast to alkali, which inhibits clotting of milk. From Figure 4.6, increasing clotting activity can be observed in rennet from pH 6.0 to pH 7.0; the peak at pH 7.0 gives an average clotting rate of 370 sec^{-1}. The same bell shape curve can be observed in Figure 4.7 for *C. rhodostoma*. Clotting rate rose gradually from pH 6.0 to pH 7.0 until the best clotting time of 151 sec^{-1} attained at pH 7.0. Kugelmass et al. (1937) elaborated high buffer action of milk at about pH

6.0 masks any change in pH during rennet clotting. The study also confirmed that coagulation occurs at wider pH range for clotting. The optimum zone is about pH 6.0 which falls on the range for complete transformation of casein to paracasein. In the process, clot formation is believed to occur in the form of a soft gel in consistency near to pH 7.0 and get firmer and thicker eventually. Despite this, coagulation below pH 5.3 is identified as coagulation due to acid precipitation and causes rennet activity to cease. Based on the findings of researchers, the rennet clotting activity is optimum at about pH 6.2 and yields practically 100% clot. Shalabi et al. (1982), hypothesized rennet coagulation sensitive to pH change from pH 6.5~7.0.

4.3.4 Determination of Optimum Temperature for Rennet and *C. rhodostoma*

4.3.4.1 Temperature Determination by using 1600µg of Rennet Protein Content

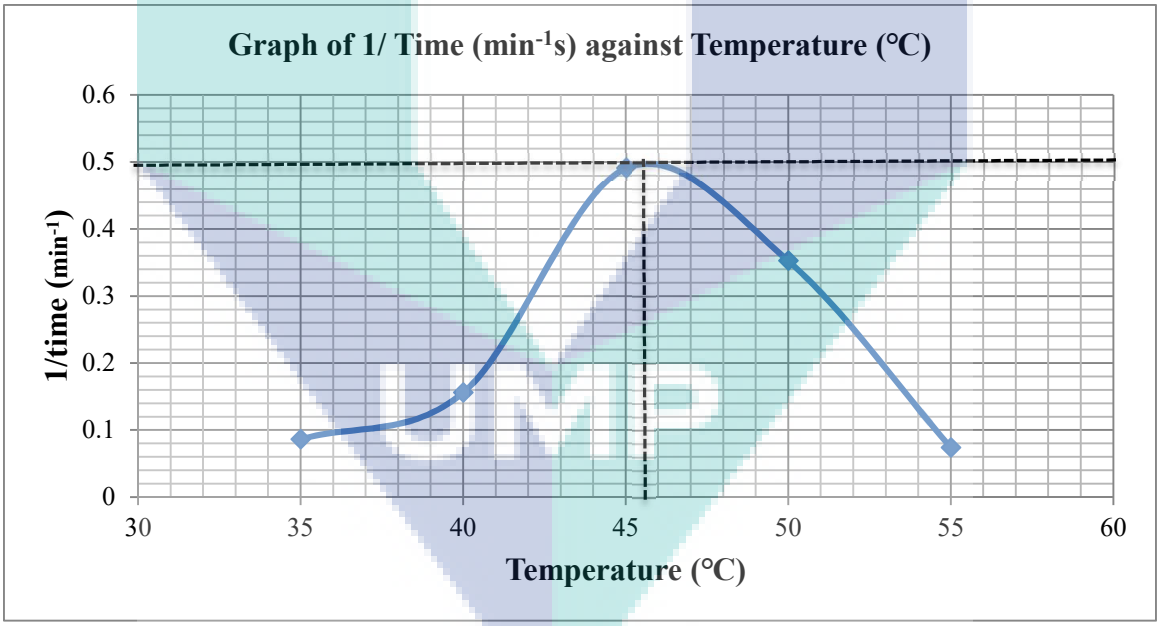

Figure 4.8: Determination of optimum temperature for rennet milk coagulation activity.

Figure 4.8 shows a bell curve reaching its peak at optimum temperature at 45.5 ℃ at estimated time of 2 minutes. At 35 ℃, the coagulation process begins and increases exponentially from 40 ℃ to 45 ℃. Beyond 55 ℃, there is rate of milk coagulation deceases.

4.3.4.2 Optimum Temperature Determination by using 3200µg of *Calloselasma rhodostoma* Protein Content

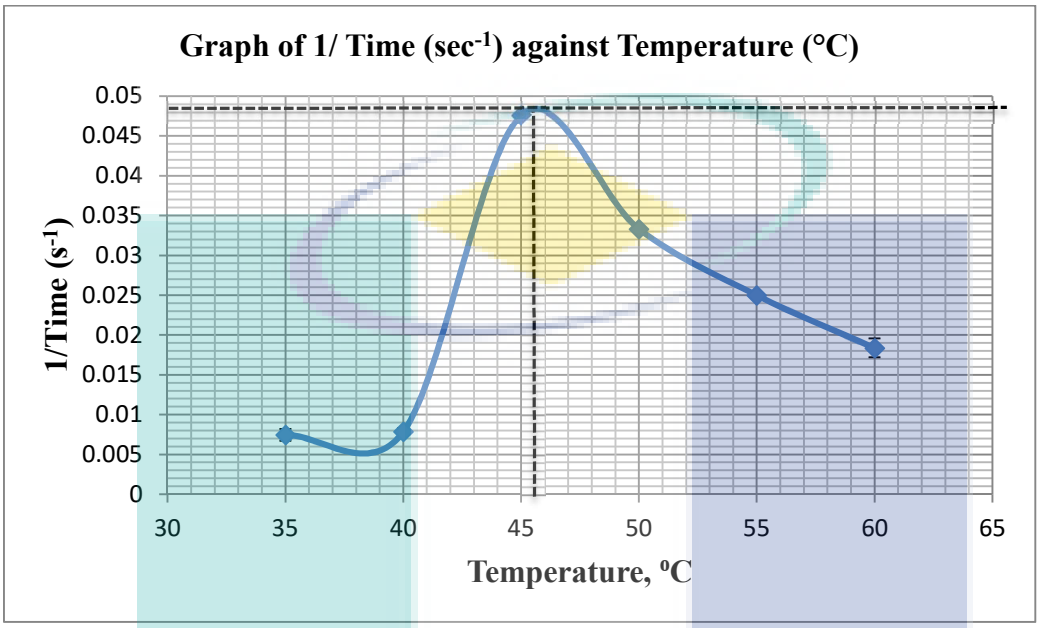

Figure 4.9: Determination of optimum temperature for *C. rhodostoma* milk coagulation activity.

Figure 4.9 shows a bell curve reaching its peak at optimum temperature of 45.5 ℃ in 20 seconds. Coagulation begins at 35 ℃, increase very little at 40 ℃, and shoots up to 46℃. Beyond this temperature, the coagulation activity decreases periodically.

Thermostability plays a vital role in determination for chymosin functionality. From figure 4.8 and 4.9, it shows both rennet and *C. rhodostoma* effectively coagulate milk at 45.5 ℃. At this optimum temperature, the enzyme responsible for coagulation worked at its full capacity. At this temperature, rennet shows coagulation rate of 0.5 min^{-1} while *C. rhodostoma* shows coagulation rate of 0.048 min^{-1}. Increase in temperature after 45.5 ℃ cause the reaction activity to drop. It is well known that heat treatment higher than 55 ℃ will allow the denaturation of whey protein and chymosin. Denatured whey protein and chymosin present on surface of casein micelles prevents aggregation of rennet-altered micelles, thus results in longer duration required for rennet coagulation, indicated by Blecker et al. (2012). Landfeld

et al. (2002) stated that time of coagulation will reduce with increasing temperature, with the optimum temperature achieved at 45.5 ℃. Subsequent increase of reaction temperature will reduce the rate of reaction.

4.4 SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE) RESULT

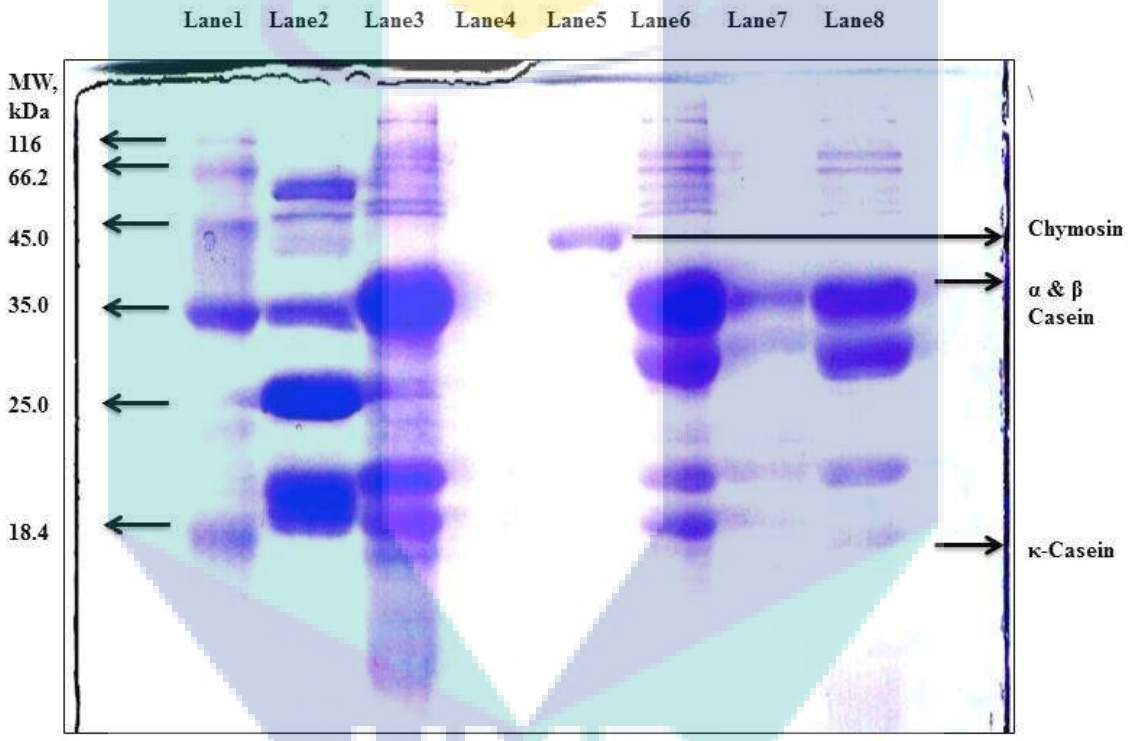

Figure 4.10: SDS-PAGE gel; lane 1 (molecular marker, with proteins from 18.4 to 116.0 kDa), lane 2 (C.R), lane 3 (C.R curd), lane 4 (C.R whey), lane 5 (rennet), lane 6 (rennet curd), lane 7 (rennet whey), lane 8 (milk).

The key aspects based on SDS-PAGE results indicated several significant comparable predictions based on molecular size. Casein proteins are milk substrate present in milk, in which κ-casein was cleaved in order for coagulation to occur. Component κ-casein has molecular weight of 19,037 Da and 19,006 Da stated by Farrell Jr et al. (2004) while, Jollès et al. (1982) and Hurley et al. (2010) confirmed it was 20 kDa. In lane 8, there is presence of

κ-casein, however the amount is minute as shown in the thin band produced in the gel. This is possibly due to processed milk solution loaded which comprises of less concentration of casein molecules. Similar band is also found in C.R curd and rennet curd proving the presence of κ-casein micelles in the curd. Hydrophilic κ-casein has been cleaved and aggregated which then responsible for formation of the lattice curd structure.

Other casein molecules found in milk sample would be α-Casein and β-Casein. Formation of similar bands at 35kDa, from lane 2, lane 3, lane 6, lane 7 and lane 8 indicate the presence of casein molecules in these lanes suggested by Zanabria et al. (2013), Nikkhah et al. (2011) and Veith et al. (2004).

The SDS-PAGE result indicates that on lane 5 shows single band formation at 45kDa. It is predicted to be chymosin found in rennet. Parallel, but less intense band was formed on lane 2 indicating the is presence of coagulating agent in *C. rhodostoma*. Molecular weight of *Mucor miehei* rennet is from 39 kDa to 42kDa; as stated by Smith et al. (1991) and Sternberg et al. (1971). It is a monomeric enzyme comprise of two domains. In addition, Rao et al (1998) stated chymosin molecular weight ranging from 30 to 40kDa while active rennin is 30.7 kDa. Rickert et al. (1973) claimed studies on determination of *Mucor miehei* rennet molecular weight are widely found. He even identified the molecular weight of rennet to be 41.8kDa. Nevertheless, *C. rhodostoma* molecular weight determination has not been studied. The band formed in the SDS-PAGE has less intensity in *C. rhodostoma* compared to single band in rennet since *Mucor miehei* rennet used was in pure form, meanwhile crude mixture of *C. rhodostoma* sample is used. Crude venom also contains other significance proteins which might masks the presents of this specific protein.

Whey proteins have low molecular weight. It may not be completely fixed and could diffuse from the gel during staining. The relative size of whey proteins β-lactoglobulin determined was 18kDa, while α-lactalbumin had relative size of 14kDa as indicated by Hurley et al. (2010). Theoretically, bands supposedly do not form on lane 4 and lane 7. The presence of bands on lane 7 might be due to cross-over contaminations from lane 6. Hurley et al. (2010) wrote that the major whey proteins in milk are β-lactoglobulin and α-

lactalbumin, the former is responsible for synthesis of lactose and in milk synthesis while the function of the latter is unknown.

Molecular weight determination by SDS-PAGE is an effective method. Unknown protein's molecular weight (in this case at 45kDa in lane 2) can be obtained by extracting the unknown protein band and run analysis via mass spectrometry. Mass spectrometry gives precise reading for molecular weight as well as identity of the protein. It has higher degree of accuracy, due to individual amino acid of a protein is analyzed.

Overall, certain bands formed were unclear at certain molecular weight. Researchers' highlighted, staining method such as coomasie blue does not effectively detect very less amount of protein. Silver nitrate staining should be practiced since it is highly sensitive and will detect even nanograms of protein contents.

4.5 MORPHOLOGY IDENTIFICATION OF SAMPLE STRUCTURE

4.5.1 Scanning Electron Microscope (SEM) of Freeze Dried Milk

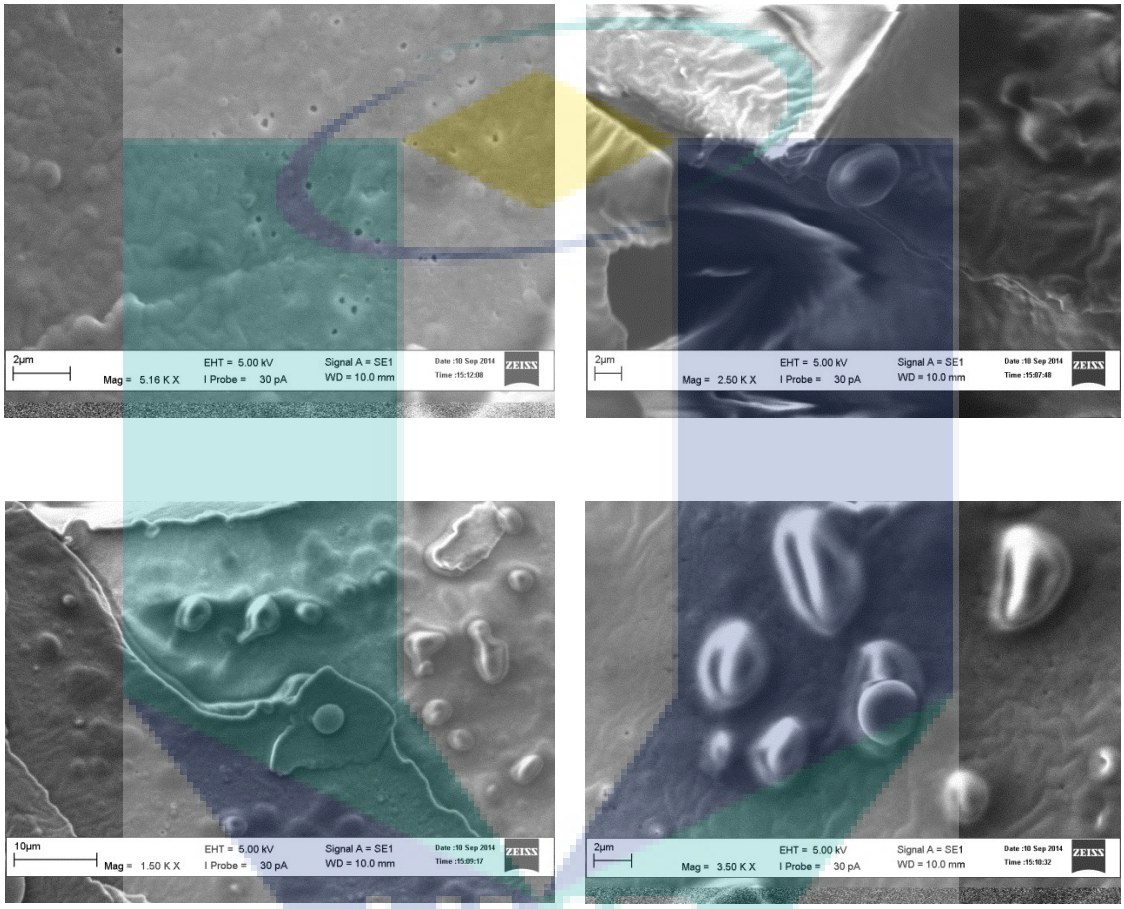

Figure 4.11: SEM image of freeze dried fresh milk

The images were taken focused with 1.5 kX, 2.5 kX, 3.5 kX, and 5.16 kX magnification. These images of freeze-dried milk, viewed under SEM shows individual structures of casein micelles. It has round, spherical like shape and distributed evenly throughout the milk sample. Researchers also confirmed on formation of small vacuoles on the milk particles. The micelles are markedly found to be separated from each other and found to contain in groups (Kalab et al. 1973). According to Mimouni et al. (2010), the

surface of the particles was principally smooth; any roughness on its surface is due to the consequence of freeze-dried technique done.

4.5.2 Scanning Electron Microscope (SEM) of Caseinate Curd from *Calloselasma Rhodostoma*

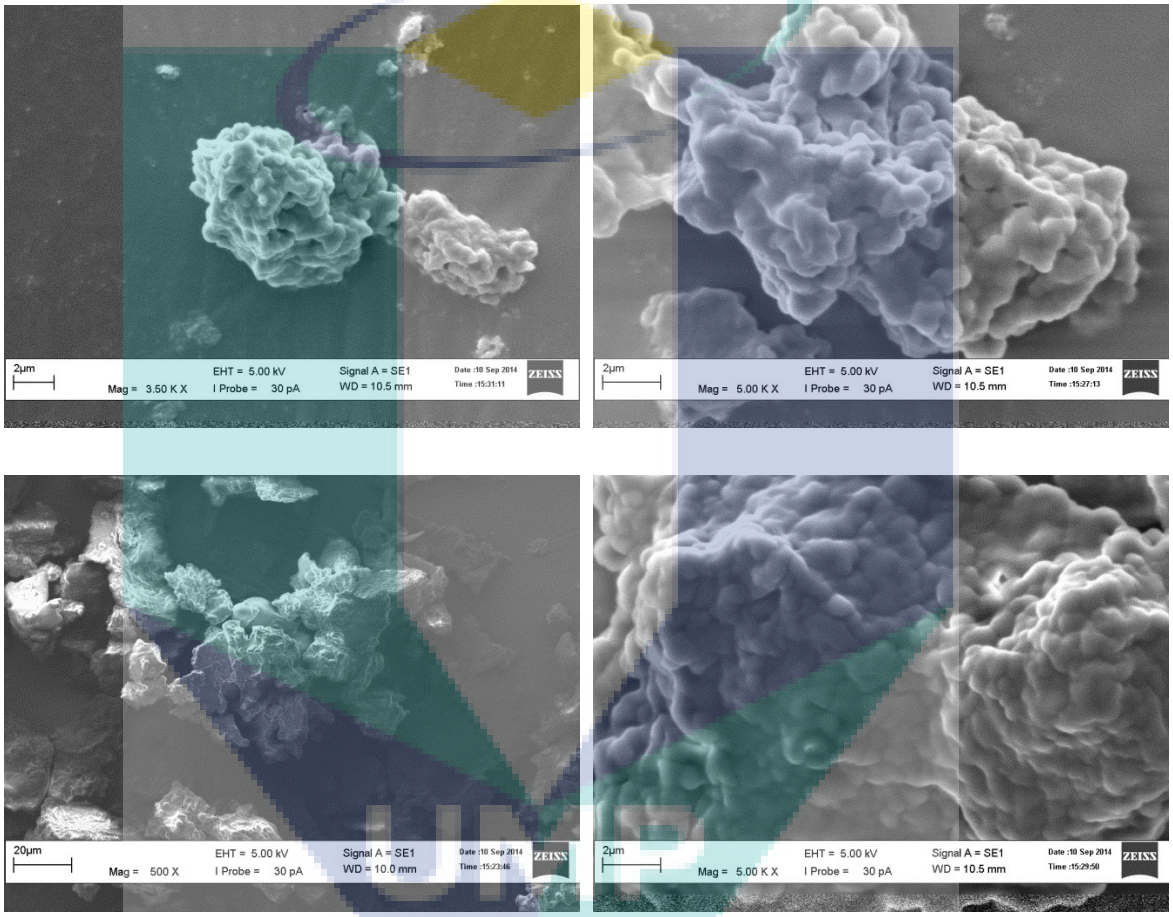

Figure 4.12: SEM image of caseinate curd of *Calloselasma rhodostoma* contrast image showing different edge resolutions in the same direction

The detector image shows strong shadowing effects due to surface roughness by the development of strands of gel formed due to coagulation. The particles surface shows clump of uneven surface of particles forming compact structure. The structure is predicted to be casein micelles attached together after joined with calcium ions. This structure captured when

compared with rennet shows small size distribution of cavities with thicker thread of protein stated by Lee et al. (1981). The images were captured with magnification of 500 X, 3.50 kX, and 5.00 kX. The distance measured for each individual structure is 20µm.

4.5.3 Scanning Electron Microscope (SEM) of Caseinate of *Mucor miehei* Rennet

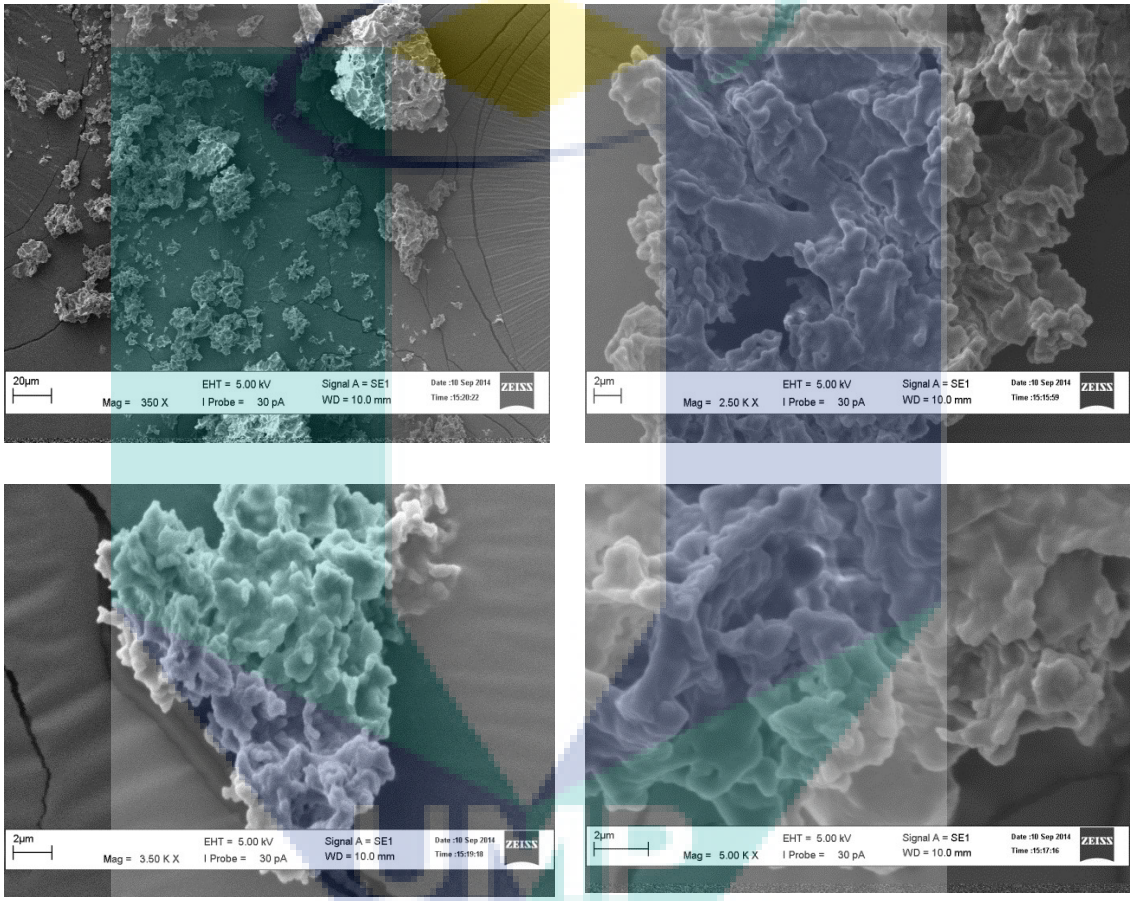

Figure 4.13: SEM image of caseinate curd from *Mucor miehei* rennet contrast image showing different edge resolutions in the same direction

The detector image shows coagulated curd in globular shape particles which appear as if large globular particles are emerging from their interior and forming a thread of network. They have a compact surface, similar with images of curd from *C. rhodostoma* (Kalab et al., 1973). However, rennet shows much coarser porous network microstructure compared to *C.*

rhodostoma. Chemicals such as calcium chloride increased firmness of the gel formed besides help casein micelles to fuse, explained by Lee et al. (1981). Images were captured at 2.50 kX, 3.50 kX, and 5.00 kX magnification. It shows uneven, irregular clumps with much larger hollow space compared to curd coagulated using *C. rhodostoma*.

Scanning electron microscopy (SEM) proposed fast and simple preparation of specimens with high depth of focus which may produce image with three-dimensional impression. In addition, it offers an outstanding tool in the research of gel structures as identified by Goldstein et al. (1981).

According to Parameswaran et al. (2011), SEM analysis results in molecules exposed to high energy electron beam which results in structural and surface modifications in which will confer a morphological character of the sample. Gold-palladium alloy for sputter coating on sample was sprayed and coated. For analysis, electron beam is generated at the specimen surface. Moisture found on the samples will disrupt movement of electrons, since the specimen is in a continually changing state of surface potential. It does not to give a fixed image identified by Newbury et al. (1975).

Scanning electron microscope (SEM) utilizes a focused electron probe to extract structural and chemical data point-by-point from parts of concern in the sample. SEM has high spatial resolution which makes it to characterize a large range of specimens at the nanometer to micrometer length scales Mendis et al. (2001).

The three samples; freeze dried milk, freeze dried curd from *C. rhodostoma* and rennet are stored at -20 ℃. Conventionally, it is widely known that freeze drying causes shrinkage of variable degrees. Cryofixation techniques aimed to replace freeze dying in most of the research studies done.

4.6 PRESENCE OF COAGULANT IN *CALLOSELASMA RHODOSTOMA* AND RENNET

Venoms from snakes have been recognized to contain anticoagulant and coagulant features. One of it, isolated from *Calloselasma rhodostoma* is ancrod used for anticoagulant and coagulation activity. Ancrod is a glycosylated enzyme with molecular weight of 38 kDa.

There is also presents of coagulant in this venom based on the overall studies and research done in this project. Generally, the DNA sequences for the polypeptides present in *C. rhodostoma* venoms have never been isolated and their amino acid sequences have never been determined. It was predicted, there is presence of protease enzyme which is responsible to acquire coagulant property particularly in this venom. This enzyme is recognized to show similar enzymatic action in comparison with aspartyl protease (EC 3.4.23) found in rennet stated by Silveira et al. (2005) and Smith et al. (1991).

4.6.1 Identification of the Coagulant within *C. rhodostoma*

As shown in Fig 4.14(a), a total of 4 peaks were eluted out and the desalting done for all cumulated fractions. Upon screening the freeze dried fractions for coagulating activity only peak 2a observed with activity. Consequent, stepwise size exclusion chromatography of protein mixture within the relevant peak 2a provided profile as shown in Fig 4.14(b) with significant coagulation to be present in peak 5b only. The coagulation activity is as shown in Fig. 4.15.

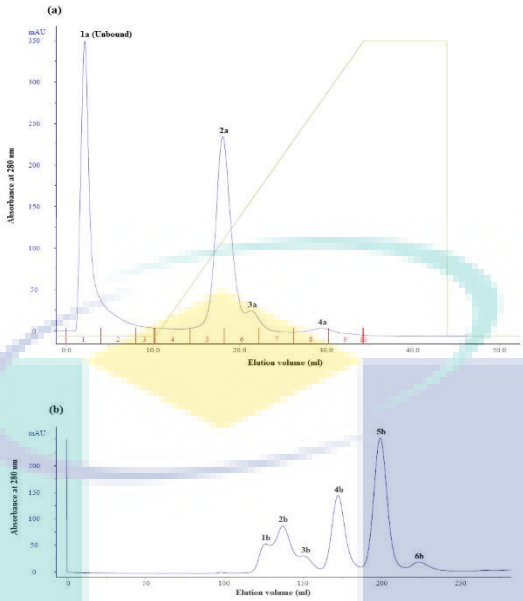

Fig: 4.14 Chromatograms of protein separations. (a) HiTrap SP FF cation exchange chromatography of 10mg CR crude venom and (b) Size exclusion of active peak 2a (25mg) separated into peaks of 1b - 6b.

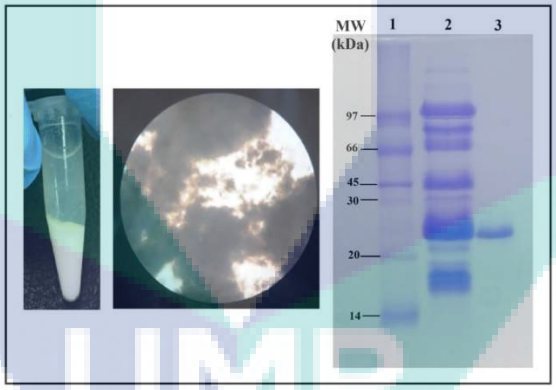

Fig: 4.15. Coagulation by fraction in peak 5b. Inset of boxed area showing a tube of milk with clear visible separation of curd and whey after coagulation by fraction in peak 5b once centrifuged at 10,000 rpm for 2 minutes and structure of coagulated milk at low magnification (100X) viewed with light microscopy (center). On the far right, SDS-PAGE gel; Lane 1: Low Molecular Weight Calibration Kit (GE Healthcare Biosciences, USA) molecular marker, Lane 2: Crude venom of CR, Lane 3: HiPrep 26/60 Sephacryl S 200 HR size exclusion chromatography obtained peak 5b.

Table 4.4: 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 non-duplicate, 12 duplicate)
Mass values: Monoisotopic	Peptides (non-duplicate only): 107, 140, 142, 153, 211, 575, 576, 702, 705, 796, 889, 917, 918, 945, 950, 997, 1094, 1120
Instrument type: 5600 TripleTOF mass spectrometer [AB Sciex], ESI-QUAD-TOF	Peptides found significant and top ranking: 9

Data in Table 4.4 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 Calloselasma rhodostoma. Evidently, no other protein belonging to the database of CR identified within the hit list. Hence, the kistomin enzyme was purified from C. rhodostoma as a single band with molecular weight found between 20-30kDa on SDS-PAGE (Fig 5). Similarly, Vejayan et al. [57], in their attempt to map the proteome of Calloselasma rhodostoma identified kistomin as an intense two dimensional electrophoresis (2-DE) spot between molecular weight of 20-30kDa.

Table 4.5: Coagulation activity in presence of a chelating agent.

Samples	0.07% (w/v) CR only	0.07% (w/v) CR in 50mM EDTA	Kistomin only	Kistomin in 50mM EDTA
Coagulation of 5 % (w/v) milk	+	--	+	--

+, coagulated within a minute; --, no coagulation

Table 4.5 showed results of 0.03% (w/v) kistomin treated to milk in the absence and the presence of 50mM ethylenediaminetetraacetic acid (EDTA). Only milk treated with CR and kistomin provided a tube with clear separation between whey and curd after centrifugation at 10,000 rpm for 2 minutes (figure not provided). Kistomin been reported to be deactivated in the presence of EDTA (a chelating agent). Evidently in this experiment no coagulation observed once the EDTA been included in tubes containing either CR or the isolated kistomin alone. Hence, indicating kistomin to be the protease acting as the coagulant within the CR venom.

CHAPTER 5

CONCLUSION

This study identified the potentials of snake venom in coagulating milk as whole and more importantly the ability of one outstanding venom related to this activity. A comparison with commercial milk coagulant indicated potentials of the Malayan Pit Viper venom and consequently identified the protease within this venom to be metalloproteinase of kistomin. Though yet again doubts may arise in considering venom protease as a likely coagulant for the dairy industry due to its low abundance from snake however it is expected with the eventual genetic engineering of it's gene into a suitable microbe may overcome this challenge. This work merely proposes a protease originating from snake venom uniquely capable to coagulate milk. Whether it is able to be utilized commercially is another challenge altogether.

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-Note: only selected citations been included here-