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Research Article

Uncovering a Protease in Snake Venom Capable To Coagulate Milk to Curd

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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 eleven venoms mostly predominantly found in tropical region singled out one with the most rapid coagulating timei.e by *Calloselasmarhodostoma* (CR). Optimization of CR venom related to several parameters provided venom concentration, 0.07 (w/v%); pH,7.0; temperature, 45.5^0C while that of rennet were determined to be 0.04±0.02 (w/v%); pH,7.0; temperature, 45.5^0C, 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 kistominwithin 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.

Keywords: Coagulation; protease; dairy; snake; protein; curd

[I] INTRODUCTION

The thought of snake venom being only lethal to mankind is outdated and no longer a current perception for at least those working in the field of toxin. Due to many evidences been brought to attention showing venom being beneficial for mankind strengthened this fact further. Human can share benefits from snake venom for it have been proven snake venom contributed in many field of pharmacology, toxicology and clinical field. Researchers had studied the function and benefit of the snake venom in pharmacology and clinical field especially for its anti-cancer properties[1-12], as defibrinating agent [13-14], antihypertensive activities [15-16] and related

cure for cardiovascular or systemic problems[14, 17], HIV treatment[18-20], treatment for Alzheimer and other degenerative diseases[21], anti-microbial[22-27], tools in diagnostics[28-31] and so on.

To highlight just one research area in detail, in cancer therapy, Vyas et al.,(2013), isolated active constituents of certain snake venom capable to bind specifically to tumor cells, thus affects the migration and its cell proliferation [2]. It is proven that, cytotoxic effects of snake venom show potential in fighting against tumor cells. These components included cardiotoxin-3 (CTX-3) [32]and integrin [33-34]. Cytotoxins found in *Calloselasmarhodostoma* (formerly known as *Agkristodonrhodostoma*) snake venom revealed to have anti-cancer property by killing at least half percentage of the (CTC_{50}) tumor cells studied [35].

In reality, all these only concluded that venom as a mixture no doubt being lethal, however once isolated to partial or singular entity been a very powerful remedy. Observing and imitating nature has been an excellent window into innovations in science and technology and here as well that conception been well captured to improve on another area of life i.e. in food industry. 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 [36]. Proteases from animal, plant and microbial sources has been investigated as rennet substitute and milk clotting enzymes. Different sources of proteases differ in their physical and catalytic properties with some of them have capacity to coagulate milk, but most of them are not suitable for food industry because of their hydrolytic action end in lower yields, fat lost from the curd and undesirable changes in texture and flavor during cheese aging [37]. Researchers have been encouraged to find other sources and alternatives of coagulant due to the difficulties in finding a suitable coagulant in food industries. Milk coagulation can be facilitated by inducing micelle aggregation; by enzymatic action (cheese) or also by adding acid (form fermented milk) [38]. 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 [39]. 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 [40-45]. Hence, addition of chymosin (rennet) to milk causes coagulation.

It has been reported that enzyme present in snake venom especially those in vipers hydrolyze proteins and membrane component which lead to blood clotting [46-47]. Nevertheless, no strong indications support homology between coagulation of blood and milk other than that studied long time ago by Jolles&Henschen[48]. They compared the clotting of blood and milk and found the common features of both process. Evidently there are structural homologies between κ-casein and N-terminal part of fibrinogen y-chain. In addition, it has been also suggested similarity in cleavage mechanisms between the action of thrombin (blood) on fibrinogen and that of chymosin (EC 3.4.23.4) on the κ-casein fraction of milk. Both of these proteases are known to hydrolyze specific linkages resulting in development of soluble peptides and insoluble polymerized fractions. The specificity found to be dependent on amino acid sequence surrounding thrombin or chymosin sensitive peptide links (7-9, 13-16) determine in part of susceptibility of thrombin/chymosin hydrolysis [49-50]. Currently, unfortunately no other studies found to actually correlate these two activities ever since then. Nevertheless, these authors' suggestions of linking coagulation of milk to that of blood clothing process were more than enough for us to

hypothesize and eventually experiment on the possibility of snake venom (a highly capable blood coagulant) to also coagulate milk. Viper venom such as *C. rhodostoma*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 [47]. Freitas et al. [51], stated that, the venom has no effect on platelet activity at low concentration however, at high concentration its effects are confounded in by formation of weak fibrin mesh. The fibrinogen clotting enzymes found in snake venom sample are the most frequent type of blood coagulation activator, they are well known among members of the family Viperidae (Viperinae and Crotalinae). Based on purification efforts, Malayan pit viper contains, ancrod which is the fibrinogen clotting enzyme responsible to target on alpha chain [52-54].

[II] METHOD

2.1.Sample collection and preparation

A total of ten snake venom samples from various species in Malaysia were collected from Perlis Snakes Farm, State of Perlis, Malaysia. Venoms were milked into sterile universal containers and the container stored in an ice-filled insulated box to prevent venom deprivation. Samples were kept in freezer immediately upon arrival at laboratory followed by freeze-drying into powder form. The lyophilized powder was then stored at -20 □ freezer until further usage. Additionally, *Najanivea* (endemic to Southern Africa) was a personal collection of one researcher of this study.Rennet from *Mucormiehei*was purchased from Sigma-Adrich. BD Difco^{TM} skim milk was purchased from Thermo Fisher Scientific, USA.

2.2. Determining Protein Content

Protein content in samples were determined by following the modified procedures of Bradford [55],with an initial stock of 2 mg/ml of bovine serum albumin (BSA) purchased from Thermo Scientific, USA to be used to derive the standard curve.

2.3. Determining Coagulation Time of Various Snake Venom Samples

A total of 0.04% (w/v) of venom was weighed and dissolved in 1.5 ml of 5% (w/v) freshly prepared skim milk with temperature and pH maintained constant at 25^0C and pH 7.0, respectively. The amount of $CaCl_2$ was provided in excess at concentration of 0.8% (w/v). Immediately after the venom was added into milk solution, the end point of coagulation was determined by observing for visible clot formation as described by Farah & Bachmann [56], (with few modifications). Briefly, this was done using three visible parameters i.e. change in viscosity, colour changes and white spot formation (separation between curd and whey). Consequently, upon observing this changes a final confirmation of coagulation done by observing a drop of the fluid under magnification of a light microscope. The coagulation time was defined as the time required for the first appearance of graininess in the moving film of milk on the surface of the glass wall [56].

2.4.Determining Optimum Parameters of *C. rhodostoma* and *M. meihei* rennet

The parameters determined were optimum protein concentration of samples, temperature and pH. In determining the optimum protein concentration, serial dilution of CR venom and*M. meihei*rennet were prepared ranging from 0.0045 % - 0.2845 % (w/v) of different protein concentration of rennet and snake venom in 1.5 mL of skim milk sample with other parameters maintained constant at 25^0C temperature and pH 7.0. The time taken for coagulation was recorded.

Next, the protein concentration of 0.07% (w/v) of CR venomand 0.04% (w/v) *M. meihei*rennet were used for the determination of the optimum temperature andpH.For the latter, 0.1M Potassium

Phosphate buffer was prepared ranging from pH 6.0 to 8.0, while incubation in water bath at temperature ranges between 30□ to 60□ used for the other parameters. Each of the coagulant samples was dissolved in 1.5 mL of skim milkcontaining $CaCl_2$ of 0.8% (w/v). The parameters of temperature and pH maintained to be constant (except for the investigated parameter) at 25^0C and pH 7.0. Time taken to record coagulation time is as described earlier.

2.5. Microscopy Techniques

2.5.1. Optical Microscope

An observation was carried out by observing a drop of milk (uncoagulated and undergoing coagulation) in a light microscope (Nikon Eclipse E100, USA) fitted with digital imager (DinoEye Eyepiece Camera Software).

2.5.2. Scanning Electron Microscope (SEM)

The freeze-dried specimens (curd formed due to CR and rennet, and milk alone) were sent for SEM (Fei Quanta 450, Thermo Fisher Scientific, USA) analysis at Central Laboratory, Universiti Malaysia Pahang, Malaysia.

2.6. Milk Coagulation Guided Isolation and Identification of Active Protein

2.6.1.Ion Exchange Chromatography

The HiTrap SP FF (GE Healthcare Life Sciences) pre-packed with SulfopropylSepharose 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.

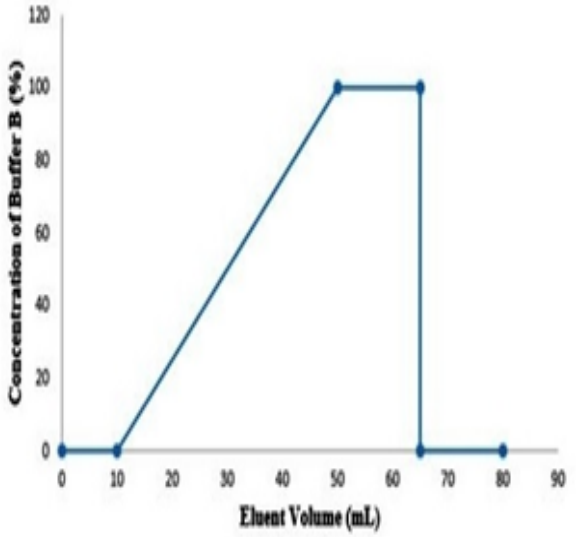

Fig: 1. Concentration of Buffer B (%) versus Eluent Volume (mL).

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.

2.6.2. 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 1ml/min. The fractions of 6 ml were collected and the peak was monitored by recording the absorbance at 280nm.

2.6.3. SDS PAGE

SDS PAGE was performed to determine the purity of fractions with positive results. A 12% separating gel and 5% stacking gel were prepared and ran on 150 Volts until the dye reached the

bottom of the gel. The gel was then stained with warm Coomassie blue staining solution (PhastGel^{TM} Blue R, GE Healthcare Life Sciences, USA) to allow the visualization of bands. The staining solution was discarded and de-stained with intermittent changes of 10% acetic acid solution until a clear visualization of bands on a shaker.

2.6.4. Mass Spectrometry Analysis

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

[III] RESULTS

Table 1. Milk coagulation time of various snake venoms.

Sample	**Protein content (µg) Mean ± SE**	**Percentage of protein (%)**	**Coagulation time (minutes) mean ± SE**
Calloselasmarhodostoma (viper)	778.89±0.03	77.8	2.83±0.10
Najasputatrix (cobra)	268.40±0.01	26.8	3.33±0.34
Trimeresuruswagleri(viper)	574.36±0.01	57.4	3.67±0.56
Bungaruscandidus (krait)	478.63±0.02	47.9	12.00±0.51
Najakaouthia (cobra)	384.91±0.00	38.5	15.03±0.19
Ophiophagushannah(cobra)	287.18±0.02	28.7	30.00±0.19
Najanivea (cobra)	752.14±0.02	75.2	47.00±0.47
Bungarusfasciatus (krait)	678.63±0.01	67.9	・60*
Trimeresurushageni(viper)	628.59±0.01	62.9	・60*
Trimeresurussumatranus(viper)	428.21±0.00	42.8	・60*
Trimeresuruspurpureomac-ulatus (viper)	547.01±0.02	54.7	・60*

Data presented as standard error (SE) with repeats of n=3. The end point was recorded when discrete particles (whitish liquid) were discernible. *denotes those samples unable to show convincing coagulation immediately.

3.1. Coagulation Time of Various Snake Venom Samples

The milk coagulation time for various snake venoms are as shown in Table 1. CR found with the most rapid coagulating activity of 2.83±0.10 minutes (also incidentally with the highest protein content of 77.8%), and *Najasputatrix*(26.8% protein content) and*Trimeresuruswagleri*(57.4% protein content) followed next with coagulating time to be less than four minutes. Hence, no direct correlations can be made between coagulation time and protein content.

3.2. Optimum Parameters of CR and rennet

In determining the optimum coagulating parameters for CR and rennet, an initial investigation to find out the optimum amount required for the coagulating activity was carried out. As shown in Fig 2 (graph A & B) the amount of 0.07% and 0.04% (w/v) of CR and rennet respectively determined as the optimum protein content required for each of the coagulant.

These amounts were next utilized in the determination of other parameters and results been summarized in

Table 2. The optimum values for each parameter (pH and temperature) were obtained based on graphs (C to F) shown in Fig 2.

The graphs of A-B provided similar patterns for the rate of coagulation whereby observed to be reaching a plateauing effect. While the graphs of C-F, a pattern of non-symmetrical bell shape with an obvious peak.

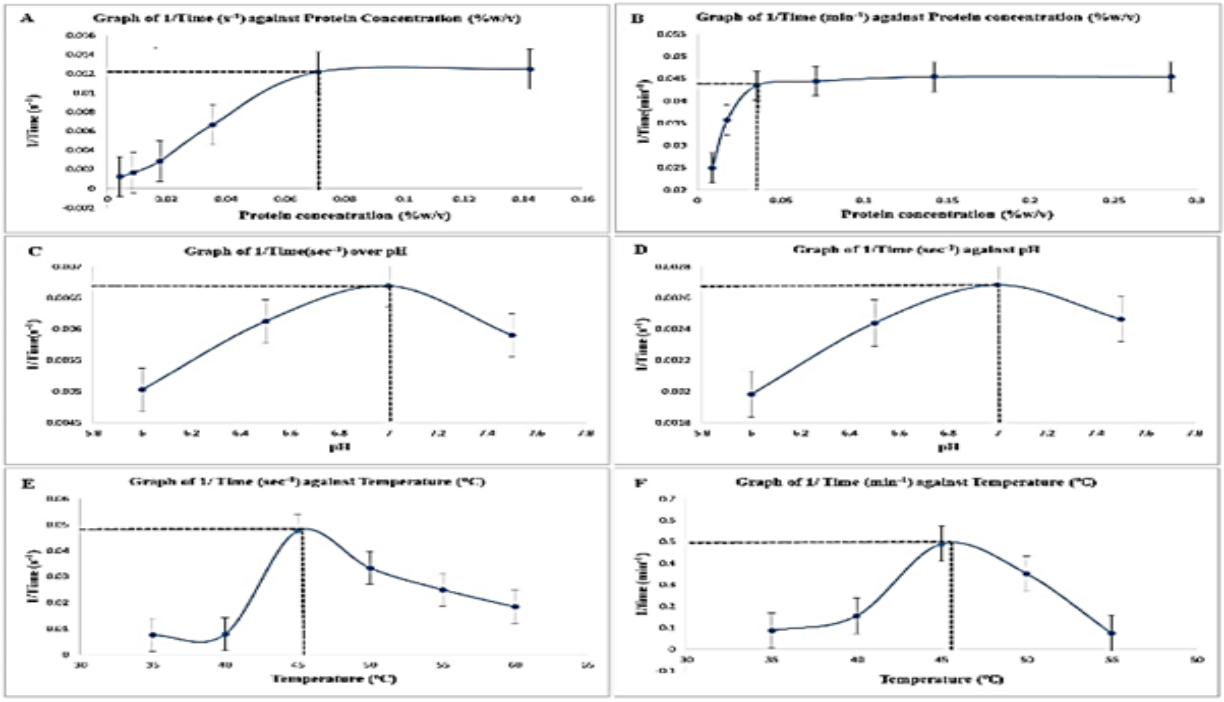

Fig: 2. Determining optimum parameters byA: increasing CR protein concentration (w/v%); B: increasing rennet protein concentration (w/v%); C: varying pH environment for CR; D: varying pH environment for rennet; E: varying temperature for CR; F: varying temperature for rennet.

As shown in Table 2, the optimum pH and temperature determined for both rennet and CRwere pH 7.0 and 45.4^{o}C, respectively. Though both coagulants had similar optimum conditions (i.e. pH 7.0 and temperature of 45.5^{o}C), the coagulation time differed markedly as CR was found better in its coagulating rate once tested with their optimum conditions as shown in Table 2.

Table 2. Summary of optimum conditions for *C. rhodostoma* venom and rennet.

Parameter	CR		Rennet	
	Optimum condition	Coagulation time (minute)	Optimum condition	Coagulation time (minute)
Protein concentration (w/v%)	0.07	1.36±0.01	0.04	23.00±0.58
pH	7.0	2.50±0.03	7.0	6.20±0.12
Temperature	45.5 °C	0.34±0.03	45.5 °C	2.00±0.25
#Comparison of CR and rennet	0.07; 7.0; 45.5^{o}C	0.41±0.02	0.04; 7.0; 45.5^{o}C	4.23±0.05

#The coagulation time of CR venom and rennet under their respective optimum conditions.
All data presented as ±SE with repeats of n= 3.

3.3. Microscopic View of Samples

The visuals (Fig 3) of pre- and post-coagulations showed (X-Z) images of milk before and after coagulation by CR and rennet viewed in three different observations i.e. after centrifugation, under light microscope and SEM. Evidently, milk appeared soluble throughout even after centrifugation while in both CR and rennet treated milk a clear curd (precipitated at the bottom) and whey (solution) being separated signifying coagulation.

The curds formed (moldy looking) due to these two coagulants were easily differentiated from milk under a low magnification using a light microscope.

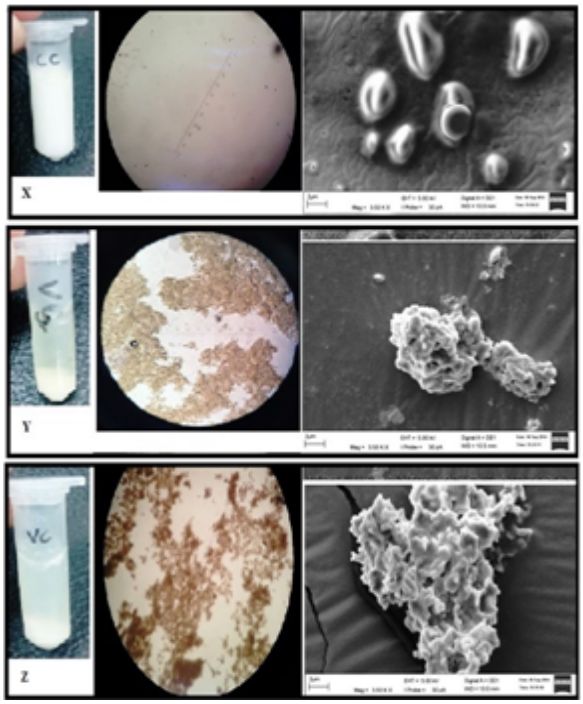

Fig: 3.pre- and post-coagulations of milk: Inset of X boxed area showing a tube containing 1 ml of uncoagulated milk (control), structure of milk at low magnification (100X) viewed with light microscopy (center) and SEM image of freeze dried milk (far right) at 3.50KX. Inset of Y boxed area showing a tube of milk with clear visible separation of curd and whey after coagulation by CR and centrifuged at 10,000 rpm for 2 minutes, structure of CR coagulated milk at low magnification (100X) viewed with light microscopy (center) and SEM image of freeze dried curd (far right) at 3.50KX. Inset of Z boxed area showing a tube of milk with clear visible separation of curd and whey after coagulation by rennet and centrifuged at 10,000 rpm for 2 minutes, structure of rennet coagulated milk at low magnification (100X) viewed with light microscopy (center) and SEM image of freeze dried curd (far right) at 3.50KX.

The images of freeze-dried milk, viewed under SEM showed individual structures of casein micelles. It has round, spherical like shape and distributed evenly throughout the milk sample. The image of caseinate curd due to CR showed strong shadowing effects due to surface roughness by the development of strands of gel formed due to coagulation. The particles surface showed clump of uneven surface forming compact structure. Caseinate curd of rennet showed uneven, irregular clumps with much larger hollow space which appeared as if to be large globular particles emerging from their interior and forming a thread of network.

3.4. Identification of the Coagulant within *C. rhodostoma*

As shown in Fig 4(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(b) with significant coagulation to be present in peak 5b only. The coagulation activity is as shown in Fig. 5.

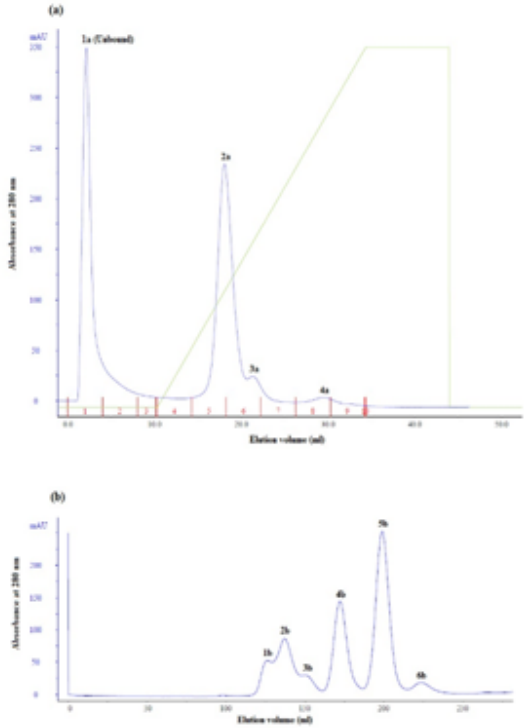

Fig: 4.Chromatograms of protein separations.(a) HiTrap SP FF cation exchange chromatography of 10mg CR crude venom and (b) Size exclusion of active peak 2a (25mg) separated into peaks of 1b - 6b.

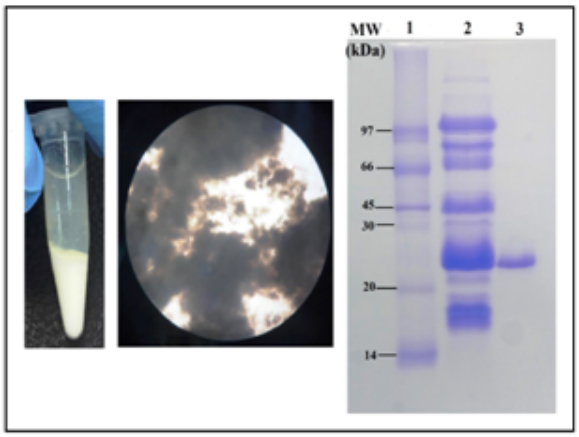

Fig: 5.Coagulation by fraction in peak 5b.Inset of boxed area showing a tube of milk with clear visible separation of curd and whey after coagulation by fraction in peak 5b once centrifuged at 10,000 rpm for 2 minutes and structure of coagulated milk at low magnification (100X) viewed with light microscopy (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 3. 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 [Calloselasmarhodostoma]
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 3 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 *Calloselasmarhodostoma*. 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 asingle 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 *Calloselasmarhodostoma* identified kistomin as an intense two dimensional electrophoresis (2-DE) spot between molecular weight of 20-30kDa.

Table 4. 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 showed results of0.03% (w/v) kistomintreated to milk in the absence and the presence of 50mM ethylenediaminetetraacetic acid (EDTA). Only milk treated with CR and kistominprovided 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 kistominto be the protease acting as the coagulant within the CR venom.

[IV] DISCUSSION

Generally, venoms of Viperidae known to contain more haemotoxin and metalloproteinase exerting strong proteolytic activity while Elapid venoms contain neurotoxins and cardiotoxin hence with weaker proteolytic activity [58-59]. The

comparison of proteome in four snake venoms belonging to Viperidae and Elapidae by Vejayan et al. [57], showed clear distinction with Viperidae venoms prominent with more of higher molecular weight spots in 2-DE compared to the Elapidae venoms studied with mostly lower molecular weight 2-DE spots (presumably belonging to neurotoxins). Apart from the two viper species i.e. *Calloselasmarhodostoma*and*Trimeresuruswagleri* shown with rapid milk coagulations while the other three vipers *Trimeresurushageni, Trimeresurussumatranus and Trimeresuruspurpureomaculatus*provided unconvincing activity of more than an hour. Similarly, no direct correlation can be made between protein content of venom and coagulating activity. In this study, the protein content of the eleven snake venoms was between 30 and 80%. Generally, snake venoms mainly comprise of protein which make 70-90% of the dry weight of snake venom [2, 60]. Different species have different types of venoms and even the composition of protein within the single species vary depending on age, climate, geographical location, its habitat, diet and etc[61-63].Mackessy et al. [64] has conducted a study of protein content in four growth categories (neonates, juveniles, adults and large adults) of *Boigairregularis*snake and found that the percentage of the protein content in this snake is increasing from 47.5 to 90.2% as the snake aged.

Interestingly, *Najasputatrix*(spitting cobra) venom found with high milk coagulating activity even though with the lowest protein content. *Najasputatrix* (also known as Javan spitting cobra) venom may have evolved from a specialization of prey ingestion rather than prey capture, since the venom can cause disruption of the cornea and produces intense pains when in contacts with eye [65].Moreover, it has been found that, 5% of *N. sputatrix* venom dry weight consists of high molecular weight proteins and enzymes (> 30000 MW); and the enzymes composition include phosphodiesterase, 5'-nucleotidase, protease, L-amino acid oxidase, hyaluronidase, alkaline phosphomonoesterase and acetylcholinesterase [66-67].

Meanwhile, elapid snake (with the exception of *Najasputatrix*) from this study also possess coagulation activity but the time taken for coagulation is much longer than viper species. Elapid species mainly comprised of neurotoxins [57] and have weak proteolytic activity [58]. In addition, elapid venoms contain various active basic polypeptides (non-enzymatic) which constitute 25-60% of the venom dry weight and have fewer enzymes than viper venoms [66]. *Najakaouthia* venom consists of enzymes mostly being high molecular and composition differing from *Najasputatrix* venom. The former has higher neurotoxin content than *Najasputatrix*[66]. In this study, *Ophiophagushannah*venom also exhibited milk coagulation activity. *Ophiophagushannah*or also known as King Cobra has relatively higher enzyme content than venoms of most cobras. The biological active elements that have been characterized are neurotoxin, hemorrhagic and non-hemorrhagic proteases, L-amino acid oxidase, phospholipase A_2 enzymes and alkaline phosphomonoesterases[66]. This venom contains five proteases which is likely hemorrhagic protease types having molecular weight around 70000daltons[68]. Furthermore, the previous study by Vejayan et al. [57], found the protein map of *Ophiophagushannah* displayed dissimilarities in relation to the other two elapids (*Najakaouthia* and *Bungarusfasciatus*). In the 2-DE proteome profile of the venoms, protein spots of *Ophiophagushannah* venom were displayed at the higher molecular mass region similar to the *C. rhodostoma*.

In this study, *C. rhodostoma*venom sample exhibited excellent milk clotting activity and was selected for optimization of parameters together with commercialized *Mucormieheir*ennet. Apart from the protein concentration, all other parameters i.e. pH and temperature were found similar as optimum conditions of both samples. Calcium chloride was added in excess to the skim milk as a firming agent. Zerrin[69] claimed

accumulation of calcium escalates rate of firming of rennet-induced milk gel (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 bridges which speed up the clotting as well. Interestingly, distinctive bell shaped curves with maximum peaks denoting optimum pH and temperature commonly obtained for any enzyme. Upon performing coagulating activity using these ideal conditions for each sample, it was determined *C. rhodostoma*venom was superior to rennet. The rennet used is a recombinant fungal, *Mucormiehei,*derived source. It comprises of proteins with higher level of purity compared to the crude venomincidentally explains the use of almost double the amount of *C. rhodostoma*venom for the optimum coagulating activity.

Three visual observations clearly separated the coagulated (by CR and rennet) and uncoagulated outcomes of milk. Such indicators relevant as the evaluation method for coagulation required to be customized for this study due to the low amount of venom material available from the snakes. Though a number of milk coagulation method available none found suitable to be used in evaluating the milk coagulating activity of snake venoms such as ultrasonic[70-72], heated thermistor [73], formagraph [74-75], rheological assay [76-77], NIR light backscatter [77-78],mid-Infrared reflectance spectroscopy [79] and diffuse reflectance [80].Based on the SEM images of freeze dried milk sample, the micelles are markedly found to be separated from each other and found to contain in groups. According to Mimouni et al. [81], the surface of the particles was principally smooth; any roughness on its surface is due to the consequence of freeze-dried technique done.

Isolation of the coagulant present within CR involvedstep wise assay guided purification by initial ion exchange separation followed by size exclusion chromatography and consequent gel band *de novo* peptide mass spectrometry analysis to finally identifySVMP kistomin as the highest scored match. The approach involved mass spectra data of the unknown peptide within protein in peak 5b submitted and found the most appropriate match with known peptide sequence of CR protein database. The results of the protein identified was in contrast to the earlier expected protease i.e. ancrod, being the major form of thrombin like-enzymes in *C. rhodostoma* venom. Ancrod is a reptilian fibrinogenases with a high degree of sequence similarity to those of mammalian serine proteinase [66, 82]. While SMVP kistomin is also a fibrinogenase capable of cleaving (protease activity) specifically the glycoprotein platelets to impair their function to cause hemostasis. This zinc containing enzyme (as such known to be inhibited by EDTA chelating agent) having 25kDa molecular weight belongs to the *C. rhodostoma* venom metaloproteinase (M12B) family capable to selectively cleave human platelet glycoprotein Ib[83-85]. Hence, the function of SMVP kistomin in coagulating milk can be explained due to its protease activity. In general, as it is similar to chymosin function, likely SMVP kistomin too specifically cleaves κ-casein fraction of milk resulting in development of insoluble fractions which polymerizes[86-87]. The recent studies had found that metalloproteinases isolated from *Paenibacillusspp*. [88-89]and *Termitomycesclypeatus*[90] have the ability to induce milk clotting. The specificity of the coagulant in cleaving casein within its amino acid sequence is crucial as other viper venom studied (expected to contain protease as well) unable to coagulate milk as comparable to *C. rhodostoma*.

[V] 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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