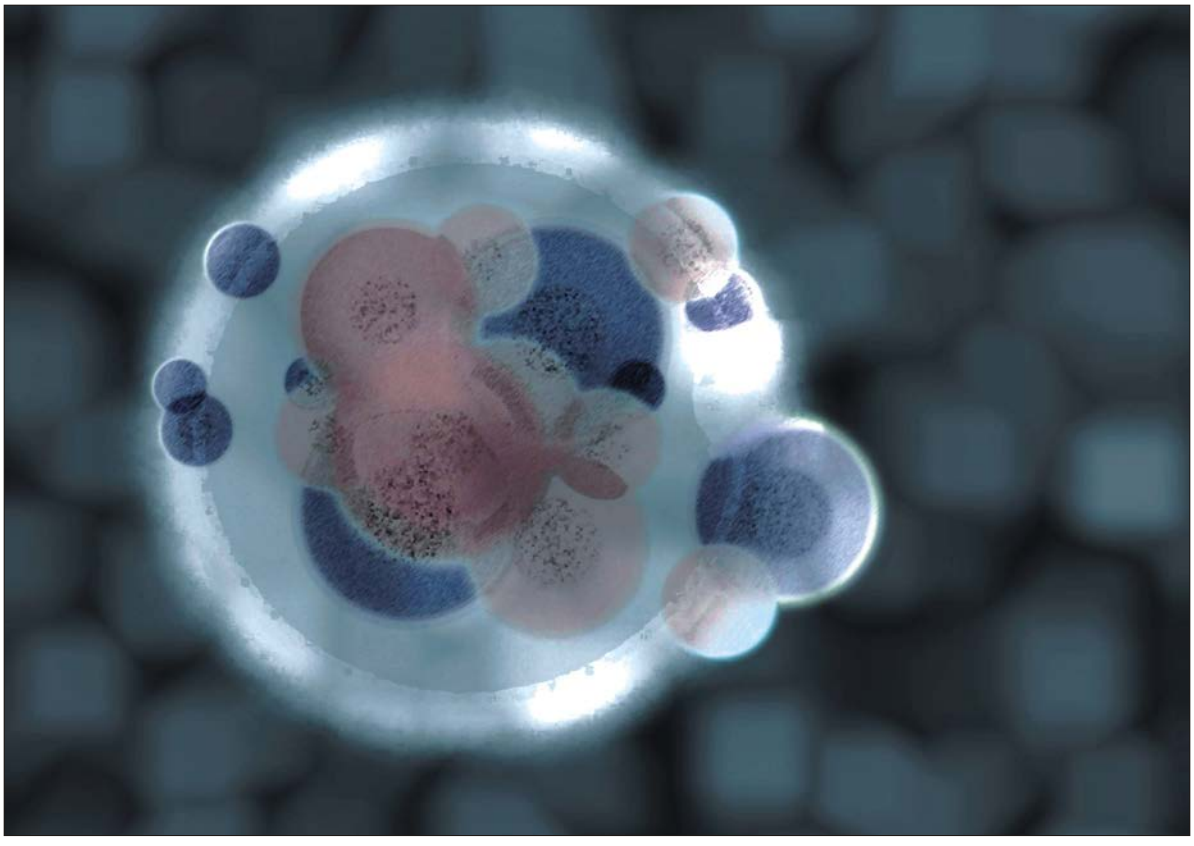

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Research Article
Enzymic Milk Clotting Activities of Metalloproteinase Kistomin

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Abstract

Background and Objectives: The dairy industry is in constant search for newer milk clotting coagulants to substitute rennet due to its growing demands and controversies such as; its origin from young slaughtered animals. Kistomin (EC:3.4.24) is a protease long identified on its role to cleave platelet and fibrinogen. In this study, kistomin discovered to function as a milk coagulant and investigated for its milk clotting activities. **Materials and Methods:** Kistomin investigated by measuring its Milk Clotting Activity (MCA) and Proteolytic Activity (PA), optimum conditions of pH, temperature, concentrations of enzyme and calcium chloride, exposures to various chelating agents and cofactor ions. **Results:** The MCA of kistomin was 810.44 (SU mL^{-1}) and the PA was 1.39 (U mL^{-1}), resulted in a ratio of MCA/PA value of 583. The coagulating activity of kistomin on milk was the highest at 0.76 mg mL^{-1} enzyme concentration, 8% (w/v) of $CaCl_2$ concentration, the temperature of 48°C and stable over a wide range of pH 5-7 with activity peaking at pH6.5. The protease completely inhibited by EDTA and 1,10 phenanthroline verifying to be a metalloprotease. The addition of Ba^{2+}, Mn^{2+} and Ca^{2+} significantly increased the enzyme activity but inhibited by Hg^{2+}, Pb^{2+} and Fe^{2+} ions. Kistomin promoted extensive hydrolysis of κ-casein and low level of β-casein cleavage. **Conclusion:** This concluded that kistomin can be used as a milk clotting candidate for the dairy industry.

Key words: Milk coagulation, dairy product, curd, protease, hydrolysis, casein cleavage

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Milk is made up of 3-4% total protein with approximately 80% of milk protein is casein and will coagulate or clot at isoelectric pH of casein at pH4.6 or driven by protease hydrolysis to form curd (gel-like) and whey (liquid)[1]. Since, the milk coagulation properties significantly affect the yield and quality of dairy products such as cheese or yogurt, they are getting more and more attention in recent years[2]. Enzyme milk clotting can be generally divided into three steps: Activation of milk clotting particles (casein micelles), aggregation of enzyme-modified casein micelles and lastly is the modification for the properties and structure of the coagulum[3]. During the aggregation of casein micelles, the concentration of calcium ions reduces the electrostatic resistance of micelles by neutralizing the surface charge on casein micelles, hence promoting the casein micelles aggregation[4].

The rennet obtained from calf contains more than 90% of chymosin which is responsible for the milk coagulation of milk to curd in the dairy products industry[5]. Chymosin is a protease secreted in the stomach of young ruminants like calves and kids raising ethical awareness in the slaughtering of such young animals. The advanced technology of recombinant DNA permits the cloning of chymosin using microorganisms[3]. Additionally, genetic engineering can be employed to enhance protein stability and modulate the activity[6]. Genetically modified organism or living modified organism experiencing their challenges suspected to cause allergic reactions prompting Genetically Modified (GM) labeling of such products. In the last decade, there has been an increased demand for cheese consumption and production due to the population explosion[7]. With the increased demands of rennet enzymes, restricted use of animal rennet for certain religions (e.g., porcine rennet for Islam), diets (vegetarianism), safety (bovine spongiform encephalopathy) and activist against genetically engineered foods, other possible alternative sources of rennet are studied and exploited[8]. Kistomin being a homogenous protease allows the opportunity to be studied on its ability to coagulate milk and as an alternative source for rennet.

The final stage of blood coagulation is the conversion of the soluble fibrinogen to the insoluble fibrin-clot[9]. This conversion occurs by the hydrolysis of the protease thrombin of fibrinogen molecule[10]. Meanwhile, for milk clotting, it was stated that κ-casein (casein fraction which acts as the substrate of chymosin) is the main protagonist in the primary phase of milk coagulation. According to Rutherfurd and Gill[11], there are similarities of sequence between fibrinogen γ-chain and κ-casein. The clotting of the blood and milk process possesses a common feature on the action of thrombin on fibrinogen and the action of chymosin on κ-casein. It was suggested that they are having a similar mechanism for these enzymatic cleavages. Both processes are said to involve limited proteolysis where both use protease enzymes to hydrolyze specific linkages[11]. Kistomin cleaves alpha- and gamma-chains of fibrinogen leaving the beta-chain unaffected[12]. It is one of the metalloproteinases extracted from *Calloselasma rhodostoma* venom. A metalloproteinase is a protease enzyme where the water molecule that is used for hydrolysis is complexed to a metal ion in the catalytic center of the enzyme and the metal ions itself hold in the position by several amino acid residues[13]. Kistomin is a 47446 Da molecular weight monomer of 417 amino acids with zinc bound at positions of 333, 337 and 343[12,14]. Kistomin being a protease needs to be explored as well for its potential in milk clotting. In this study, Kistomin is known to cleave specific blood protein hence rationalized able to also cleave protein specifically casein in milk. Kistomin was investigated for its important features to coagulate milk to become curd and whey.

MATERIALS AND METHODS

Study area: This study conducted between Sept, 2017 and Aug, 2019.

Isolation of kistomin using stepwise chromatography techniques: The purification of kistomin to homogeneity done as previously described in Vejayan *et al.*[15]. Briefly, freeze-dried *C. rhodostoma* venom (purchased from Snake and Reptile Farm, Sungai Batu Pahat, Perlis, Malaysia) fractionated to its active peak using a 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 50 mM ammonium acetate at pH 6.0 and 1.0 M NaCl in 50 mM ammonium acetate at pH 6.0 were the start buffer and elution buffer, respectively. The active fraction from the ion exchange chromatography collected, freeze-dried and consequently desalted by using HiTrap Desalting (GE Healthcare Life Sciences, USA), a prepacked Sephadex G-25 Superfine column. Next, the active fraction eluted from the ion exchange chromatography column was applied onto HiPrep 26/60 Sephacryl S 200 HR pre-packed column (GE Healthcare Life Sciences, USA). The column was equilibrated with 50 mM of ammonium acetate buffer of pH 7 and the elution was carried out using the same buffer with 2 mL sample injection at a flow rate of 1 mL min^{-1}

to yield kistomin known peak (denoted as peak 5b)[15]. The protein content of the kistomin estimated by using the dye-binding technique of Bradford[16] with Bovine Serum Albumin (BSA) at 2.0 mg mL^{-1} concentration, purchased from Thermo Fisher Scientific, USA.

Milk Clotting Activity (MCA): The milk clotting activity of the kistomin determined by using the method of Arima *et al.*[17] and Majumder *et al.*[18] with few modifications. A 10% (w/v) of BD Difco skim milk (Thermo Fisher Scientific, USA) containing 10 mM of $CaCl_2$ as a substrate. A total of 1 mL of milk substrate was added to the 0.5 mL enzyme solution at 35°C. The milk clotting time was measured according to 3 visible parameters, i.e., viscosity, color changes and the development of white spots[19,20]. Milk clotting activity was expressed in Soxhlet Unit (SU mL^{-1}). One SU was defined as the enzyme amount which clots 1 mL of the substrate within 40 min at 35°C. The MCA for coagulant was calculated by using the equation:

$$MCA = \frac{M \times 35 \times 2400}{E \times t \times T}$$

Where:

M = Volume of substrate (mL)
t = Clotting time (s)
E = Volume of enzyme (mL)
T = Reaction temperature (°C)

Proteolytic Activity (PA): The proteolytic activity was measured by using 2.5 mL of 1% (w/v) casein (Nacalai Tesque, Japan) in 50 mM potassium phosphate buffer (pH 7.5). The casein solution was equilibrated in a water bath at 37°C for 10 min. A total of 0.1 mL kistomin and a blank (without any coagulant) added to the casein preparations and incubated at 35°C for 30 min. After incubation, 2.5 mL of 440 mM trichloroacetic acid (Sigma Aldrich, USA) was added to stop the reaction. Then the appropriate volume of enzyme solution was added, also in the blank so that the final volume of enzyme solution in each is 1 mL. The solution was incubated again at 35°C for 30 min and was centrifuged at 12000 g for 10 min. A total of 5 mL of 500 mM sodium carbonate (Sigma Aldrich, USA) was added to 2 mL supernatant and 1 mL of Folin and Ciocalteu's phenol reagent (Sigma Aldrich, USA) was added immediately afterward. The absorbance of the solution was measured at 660 nm.

The standard calibration curve of L-tyrosine (Sigma Aldrich, USA) was prepared between range 0.0-0.60 µMoles. In each 2 mL concentration of tyrosine, a total of 5 mL of 500 mM sodium carbonate and 1 mL of Folin reagent added immediately. The absorbance of the solution was measured at 660 nm by using a UV-V is spectrophotometer.

The calibration curve of L-tyrosine concentration (µMoles) versus Absorbance (660 nm) was constructed and the equation was generated using MS Excel software. The enzyme activity for kistomin calculated by using this equation[19]:

$$\text{Activity}\left(\frac{\text{units}}{\text{mL}}\right) = \frac{\text{Tyrosine equivalent released (µMoles)} \times \text{Total in assay (mL)}}{\text{Time of assay (min)} \times \text{protease (mL)} \times \text{volume used in calometric determination (mL)}}$$

Determining optimum parameters of kistomin coagulant: A total of 1 mL of 10% (w/v) skim milk (BD Difco, Thermo Fisher Scientific, USA) containing 10 mM $CaCl_2$ (Sigma Aldrich, USA) was used as the milk substrate (except during the determination of optimum $CaCl_2$ concentration). The temperature and pH maintained to be constant at 35°C and pH 6.5 (except during the determination of optimum temperature and pH).

Enzyme concentration: Kistomin was serially diluted in the range of 0.01-1.28 mg mL^{-1} protein content. A total of 1 mL of milk substrate was then introduced to 0.5 mL of different concentrations of the coagulant at 35°C, pH 6.5. The time taken for the milk to coagulate was recorded based on three visible parameters, i.e., viscosity, color changes and the development of white spots[19,20]. The protein concentration was increased until the coagulation rate become constant and the graph of MCA versus concentration was plotted.

pH: The pH stability of kistomin investigated at pH 5-9. Acetate buffer (pH 5), phosphate buffer (pH 6-pH 8) and glycine-NaOH buffer (pH 9) used. Each 0.5 mL of sample solution consisting of coagulant and an appropriate buffer with varying pH of 5- pH 9 was mixed with 1 mL of milk substrate and swirled for 10 sec at 35°C. The time taken for the milk to coagulate was recorded. The graph of MCA versus pH was plotted.

Temperature: The milk substrate (pH 6.5) with kistomin was incubated in a water bath with varying temperature settings i.e., 20-80°C. The time taken for the milk to coagulate was recorded. The graph of MCA versus temperature (°C) was plotted.

Calcium ion concentration: Various concentrations of $CaCl_2$ ranging from 2-14% (w/v) dissolved in 1 mL of 10% (w/v) skim milk at 35°C and pH 6.5. A volume of 0.5 mL of the coagulant

was mixed with the skim milk and swirled for 10 sec. The time taken for the milk to coagulate was recorded. The graph of MCA vs. $CaCl_2$ concentration (w/v) was plotted.

Substrate specificity of kistomin: Substrate specificity of coagulant was determined by using a substrate range of 0.05-5.0 mg mL^{-1} for κ-casein and β-casein while 0.05-8.0 mg mL^{-1} of α-casein (all purchased from Sigma Aldrich, USA). The substrates were dissolved in 50 mM potassium phosphate buffer (pH 6.5) and were serially diluted. The reaction was initiated by adding 100 µL (0.015 mg) of kistomin and was incubated for 10 min at 48°C.

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

Effects of protease inhibitor and metal ions on kistomin activity: All materials for this experiment sourced from Sigma Aldrich, USA. The effects of various metal ions and protease inhibitors were determined by using 2% (w/v) of casein dissolved in 50 mM potassium phosphate buffer (pH 6.5) as a substrate. A total of 100 µL of 20 mM of various metal ions ($CuSO_4$, $MgCl_2$, $MgSO_4$, $FeSO_4$, $Pb(NO_3)_2$, $HgCl_2$, $BaCl_2$, $MnSO_4$ and $CaCl_2$) and protease inhibitor: 10 mM PMSF, 1 mM Pepstatin A, 10 mM EDTA, 5 mM Iodoacetamide and 10 mM 1,10 phenanthroline was pre-incubated with 100 µL (0.015 mg) protease for 10 min in 48°C. One milliliter of the substrate was then added to each tube containing the mixture of the enzyme and metal ions/inhibitor.

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

RESULTS AND DISCUSSION

The values determined for MCA, PA and ratio of MCA/PA of kistomin were 810.44±42.45 SU mL^{-1}, 1.39±0.01 U mL^{-1} and 583.05, respectively. In general term Milk Coagulating Activity (MCA) is the ability of the rennet to aggregate milk by cleaving the amino acid of Phe_{105}-Met_{106} of κ-casein[21]. The high value of MCA indicating that the enzyme has good potential as a coagulant in the dairy industry. The second property is Proteolytic Activity (PA) which is the ability of the coagulant to cleave any bond of casein[22]. A high value of Milk Clotting Activity/Proteolytic Activity (MCA/PA) reflects an excellent end product of cheese with desirable firmness with no bitter flavors. The high ratio of MCA/PA is considered a good coagulant so the higher the value, the better the coagulant and its generally indicates the restricted degradation of casein substrate[23-25]. The major drawback of the protease extracted from most plant sources has the excessive proteolytic nature and low MCA/PA ratios which limited their use in cheese manufacturing such as ginger, curcumin or hieronyma in protease[26,27]. The measured value of 583 for kistomin found higher than 367 for papain. Papain has been suggested not useful as a coagulant due to the bitterness of the curd obtained[28]. Although the MCA/PA ratio calculated for kistomin better than papain nevertheless not possible to ascertain whether this MCA/PA ratio obtained is suitable as a dairy industry protease as there is no sufficient data available on the lower cut-off value for the ratio.

As shown in Fig. 1 the several factors affecting the enzymatic reaction of kistomin in milk clotting. The profiles of the graphs were as expected of an enzyme reacting on a substrate. Initially, to determine the optimum enzyme concentration the temperature and pH maintained constant to 35°C and pH7, respectively while $CaCl_2$ given more than 10 mM. The clotting activity was expressed in Soxhlet Unit (SU). One Soxhlet Unit (SU) of milk clotting activity was defined as[29] the amount of enzyme required to coagulate 1 mL of the substrate within 40 min at 35°C. The clotting activity increase when enzyme concentration increased because of a higher level of proteolysis of kappa casein[19]. The optimum concentration of 0.76 mg mL^{-1} determined based on Fig. 1a as the highest milk clotting activity achieved by kistomin. Consequently, all other optimum conditions for kistomin with the highest milk clotting activity determined to be 48°C for temperature (Fig. 1b), pH 6.5 (Fig. 1c) and $CaCl_2$ concentration of 8% w/v (Fig. 1d).

Figure 2a,b showed the milk clotting time of kistomin under optimum conditions for temperature, pH as well as concentrations of enzyme and $CaCl_2$. The images viewed

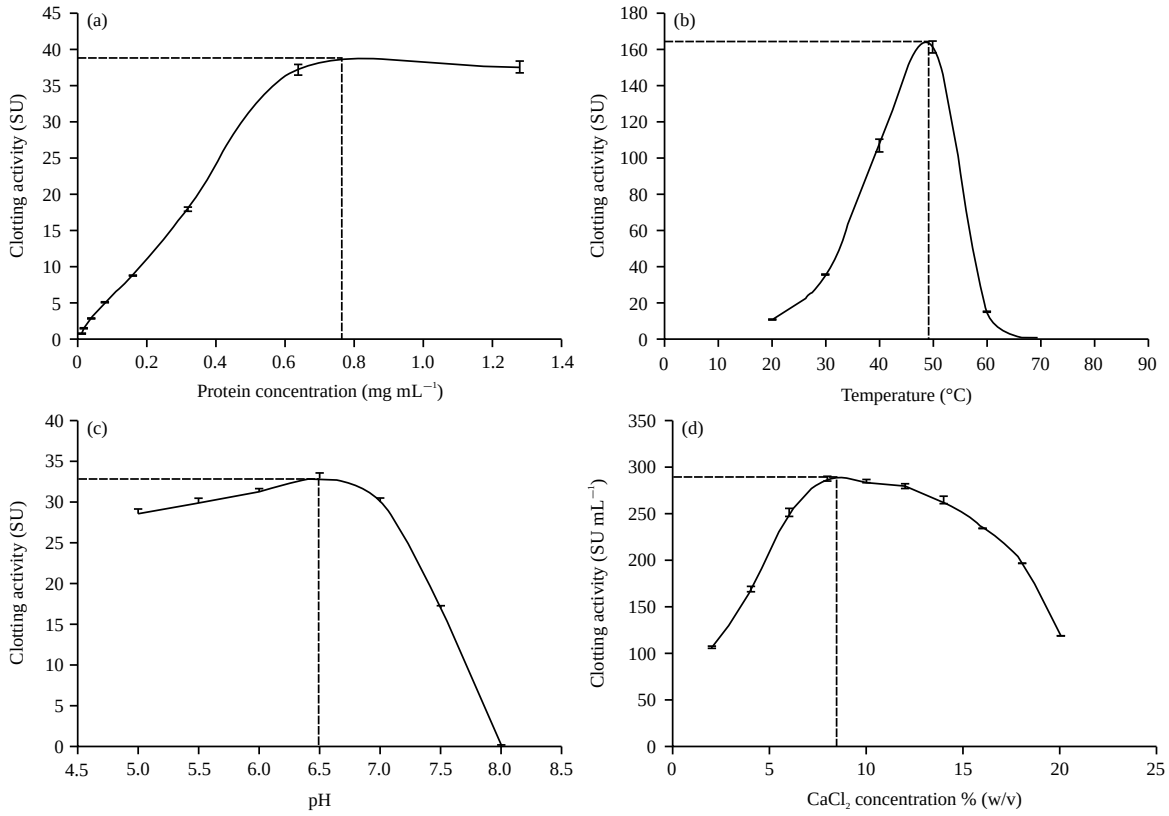

Fig. 1(a-d): Graph of (a) Milk clotting activities versus enzyme concentration, (b) Temperature, (c) pH, (d) $CaCl_2$ concentration
Values are the Mean±Standard Deviation, n = 4

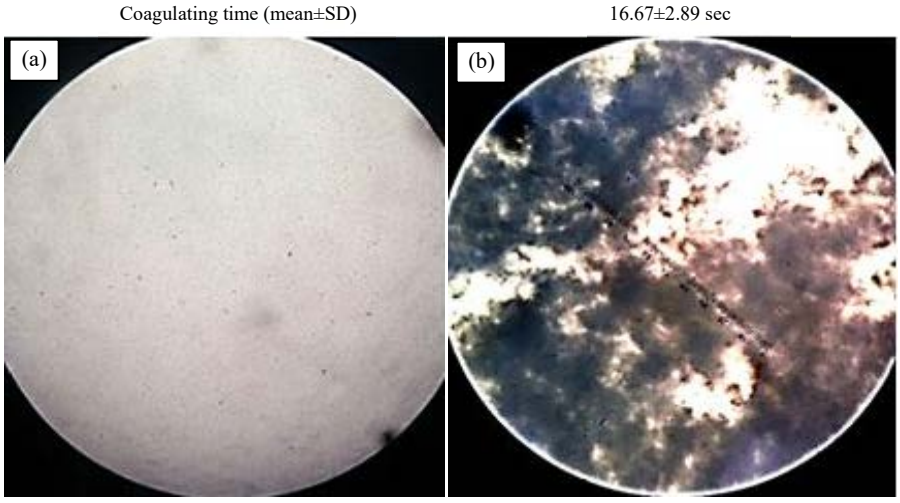

Fig. 2(a-b): Coagulating activity of kistomin on milk at optimum parameters (a) Before Coagulation (b) After coagulation
The coagulation time determined (n = 4) at optimum conditions of kistomin: Enzyme concentration of 0.76 mg mL^{-1}, 48°C for temperature, pH 6.5 and $CaCl_2$ concentration of 8% (w/v). Observation under light microscope at low magnification (100X) of; (a) Control, bovine skim milk and (b) Coagulated by kistomin

under microscopic magnification clearly showed the formation of gaps represented by whey after milk coagulated. The observation before coagulation showed milk without any aggregation of casein micelles however subsequently obvious colloidal structure of aggregation occurred.

The optimum temperature[30] for metalloproteinase enzyme stated between 37-55°C. The optimum temperature for kistomin reduced the risk of contamination by mesophilic microorganisms that able to grow at a moderate temperature between 20-45°C and with optimum growth temperature between 30-39°C. The elevation of temperature additionally favors substrate and product solubility, increases reaction rates for reducing viscosity and for increasing the diffusion coefficients of substrates[31]. Higher than room temperature is favored for the milk coagulant in the dairy industry. The commercial calf rennet shows the optimum temperature between 39-45.5°C[15,19,32]. Genetically engineered calf chymosin for cheese production found with an optimum temperature of 45°C[33]. This shows that kistomin has potential as a coagulant in the cheese industry as it has a broad range of thermal stability and has a high optimum temperature. Additionally, if required in the future, a recombinant kistomin variant can be generated to lower the optimum temperature in reducing the cost of cheese production.

The kistomin is shown to be favorable in the acidic environment as it stable at pH 5 to pH 6.9 with an optimum pH at 6.5. The amino acid sequence of kistomin was computed by using ProtParam tool-ExPASy online software and the results showed that the total number of negatively charged residues (Aspartic acid and Glutamic acid) is higher than the total number of positively charged residues (Arginine and Lysine)[34]. This protein also moderately stable at pH 7 which maintained 71% of its activity. In comparison to the chymosin (industrial rennet) found to cleave κ-casein of milk at the optimum pH between 5.1-5.5[35]. In industry additional cost required to be considered to adjust pH to be highly acidic or basic.

The addition of calcium chloride was reported to affect the milk coagulation as it reduces the milk coagulation time[36]. Calcium chloride speeds up the milk coagulation as calcium ions reduced the electrostatic resistance of micelles by neutralizing the surface charge on casein micelles[4]. The calcium ions also form bridges between micelles of para casein indicating that the more there are calcium ions in milk, the more there will be linkage[19]. This promotes the aggregation of casein micelles and leads to faster milk coagulation. However, the addition of calcium chloride beyond what is required leads to the rate of milk clotting activity to gradually decrease. This is due to the high concentration of calcium chloride, Ca^{2+} ions present inhibits the milk clotting network[19]. It is therefore necessary in providing the optimum or even slightly lower concentration of $CaCl_2$ rather than resorting to excess for milk clotting activity.

Table 1: Kistomin specificity toward various casein substrates

Substrate	V_{max} (μmoL min^{-1})	K_m (mM)
κ-casein	0.0275±0.0019	27.15±5.277
α-casein	0.0406±0.0013	38.10±2.090
β-casein	0.0719±0.0065	114.50±16.67

Values are the Mean±Standard deviation, n = 4

Table 2: Effect of different protease inhibitors on the activity of kistomin

Protease inhibitor	Activity (%)±SD
Control (no inhibitor)	100
PMSF (serine inhibitor)	64.99±2.77
EDTA (metalloproteinase inhibitor)	0
Iodoacetamide (cysteine protease inhibitor)	81.43±3.48
Pepstatin A (aspartic protease inhibitor)	88.58±6.39
1, 10-phenanthroline (metalloproteinase inhibitor)	0

Values are the Mean±Standard Deviation, n = 4

Table 1 compared the V_{max} and the K_m of α-casein, β-casein and κ-casein by kistomin. The results showed the V_{max} and K_m value of kappa-casein is the lowest, 0.0275±0.0019 μmoL min^{-1} and 27.15±5.277 mM, respectively. This indicated kistomin has a high affinity towards kappa casein and potentially preferred cleaving it to curd and whey fastest. The major enzyme of calf rennet, chymosin has been extensively used in the production of cheese to produce a stable curd with good flavor due to its high specificity for κ-casein[37]. The specificity of kistomin for casein was similar to chymosin which extensively cleaved κ-casein and promoted very slight hydrolysis of β-casein. The specificity of kistomin on κ-casein followed by α-casein. This can avoid the bitterness development of cheese which is considered as a defect in cheese. It has been reported that the hydrolysis of casein especially β-casein generates hydrophobic peptides which when accumulating is responsible for the bitterness of dairy products. The higher the affinity towards β-casein leads to the product of hydrophobic peptides hence becoming bitter cheese[38]. In this instance, it was found kistomin having a lower affinity towards β-casein based on higher K_m.

Table 2 showed the effect of protease inhibitors on kistomin. Kistomin was incubated with five different protease inhibitor and the residual activities of the protease were determined and compared with the control which corresponds to 100% of activity. Kistomin was completely inhibited in the presence of EDTA and 1,10-phenanthroline and the maintenance of activity in the presence of iodoacetamide (cysteine protease inhibitor), pepstatin A (aspartic protease inhibitor) and PMSF (serine inhibitor) inhibitors. Kistomin is a metalloprotease and as expected both EDTA and 1,10-phenanthroline inhibited it similar to previous observations[14,39]. EDTA and 1, 10-phenanthroline inhibit metalloprotease activity by lowering the concentration of metal ion and removed it from the active site of the enzyme[40].

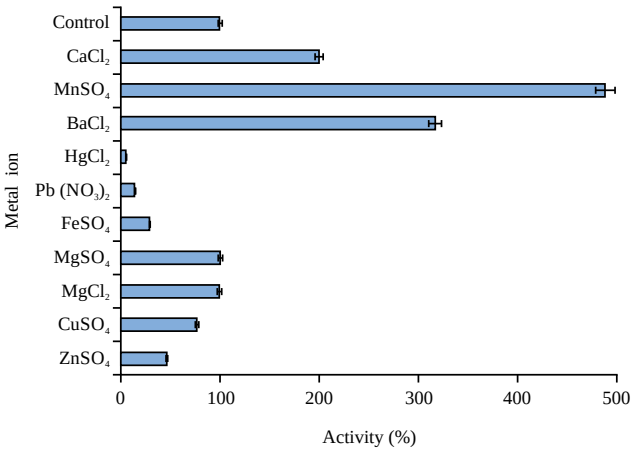

Fig. 3: Effects of various metal ions on milk clotting activity of kistomin
Values are the Mean±Standard Deviation, n = 4

Figure 3 showed the effects of metal ions on milk clotting activity of the kistomin enzyme. The addition of Mn^{2+}, Ba^{2+} and Ca^{2+} (in descending orders) significantly increased the enzyme activity. However, kistomin was inhibited by Hg^{2+}, Pb^{2+} and Fe^{2+} ions. The effect of ions over protease stability because of interaction between them, with the formation of complexes such as ions used as cofactors, substrates and co-substrates[31]. Kistomin is a metalloprotease enzyme and this class of proteases is characterized by its requirement of divalent metal ions for its catalytic activity[37]. Metalloprotease usually associated with Zn^{2+}, but other metals have been found at the active site of metalloproteases[41-43], such as; Mn^{2+}, Ca^{2+}, Mg^{2+}, Cu^{2+} and Co^{2+}. Pb^{2+} and Hg^{2+} were found to be toxic when present in high amount. A similar result was found where the metalloprotease isolated from *Oudemansiella radicata* was inhibited[44] by Hg^{2+}, Pb^{2+} and Fe^{2+}. Also, Hg^{2+} also has been reported to inhibit keratinolytic metalloprotease from *Microbacterium* sp.[45].

CONCLUSION

Kistomin is shown as a capable milk coagulant with MCA/PA ratio better than papain and having optimum conditions of 0.76 mg mL^{-1} enzyme concentration, 48°C temperature, pH6.5 and 8% $CaCl_2$ concentration. Its affinity at least towards β-casein likely producing cheese with less bitter taste. Kistomin completely inhibited by EDTA and 1,10 phenanthroline verifying it as a metalloproteinase and required most the metal ion of Mn^{2+} as a cofactor.

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SIGNIFICANCE STATEMENT

This study discovered the kistomin function of coagulating milk to curd and whey. The study determined and revealed the protease MCA/PA ratio, rapid coagulation time, specificity towards kappa casein rather than beta-casein, its preferred cofactor and optimum conditions. Overall this work introduces a potential protease in its natural and homogenous state capable to coagulate milk to curd with important features highlighted. Its potential capabilities can be improved further by other researchers by genetic engineering to become an alternative coagulant to chymosin.

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