

Evaluation of caseinolytic, gelatinolytic and milk-clotting activities from germinated seeds of green gram (*Vigna radiata*), chick pea (*Cicer arietinum*), fenugreek (*Trigonellafoenum-graecum*) and moth bean (*Vigna aconitifolia*)

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Abstract

This work depicts the caseinolytic, gelatinolytic and milk-clotting activities from germinated seeds of green gram (*Vigna radiata*), chick pea (*Cicer arietinum*), fenugreek (*Trigonellafoenum-graecum*) and moth bean (*Vigna aconitifolia*). Proportional evaluations of these activities were done with rhizomes of ginger (*Zingiber officinale*) and turmeric (*Curcuma longa*). Caseinolytic activities of the crude proteins were assessed qualitatively by casein agar diffusion method. The clear distinct zone of milk casein hydrolysis was observed on the casein agar plates of all samples. Beside caseinolytic activity these samples were also able to hydrolyze gelatin assessed by Dot blot assay. The crude extracts mildly coagulated Na-caseinate solution within varied time periods. On quantitative estimation, the milk clotting activity in term of Soxhlet units (SU) was calculated and found to be highest for the turmeric, fenugreek and chick pea. Solvent precipitation with acetone revealed increased SU in turmeric and moth bean whereas decrease in SU observed in other samples. Comparatively high ratios of SU to proteolytic activity of turmeric and fenugreek advocates milk clotting potential. These ratios significantly increased upon solvent precipitations in these samples. Electrophoretic characterizations by Native and SDS PAGE showed varied migration of proteins on the gels. About 2 to 3 moderately migrating strong proteases were observed in all samples assessed by non denaturing SDS-PAGE zymography. Detailed biochemical investigations of these promising proteases may provide alternative, nutritive and cost effective source of milk clotting for cheese making.

Keywords

Proteases
Milk clotting enzyme
Na-caseinate
Dot blot assay
Zymography

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Introduction

A dairy product, cheese has occupying important portion in human diet worldwide. Casein accounts nearly 80% of proteins in cheese (Lucey *et al.*, 2004). In milk about 95% of this casein is found as casein micelles due to aggregation of colloidal structures (Farrell *et al.*, 2006). The clotting of milk by enzymatic methods is elementary step in the production of cheese. Milk-clotting proteases act on the Phe-Met bond of the soluble portion of the caseins called kappa-casein, thus creating an unstable micellar state that results in coagulation of milk (Law, 1999). Chymosin, a predominant aspartic protease is present in the natural calf rennet and has been used for milk-clotting (Jacob *et al.*, 2011; Rodriguez, 2013). It acts on Phe105–Met106 peptide bond of the kappa-casein. The falloff in calf rennet supply; the steady increase in world cheese production and increase in disease associated with calf have led to an increased mandate for new rennet substitutes (Lopes *et al.*, 1998). In addition religious concerns (e.g.,

Islam and Judaism) and diet (vegetarianism) have stimulated researchers to search for alternative milk-clotting proteases (Jacob *et al.*, 2011). Previously, milk clotting proteases have been isolated and studied from microbes (Ahmed *et al.*, 2010). Additionally genetically engineered microorganisms are also demonstrated to have milk clotting proteases (Rodriguez, 2013).

Increasing interest has been directed toward the milk-clotting enzymes extracted from plants as it believed to have application like target cheese production. This will improve nutritional input of those populations on whom limitations of the use of animal rennet (Silva and Malcata, 2005). Further plant proteases have received special attention because of their wide substrate specificity, well property of being active over a wide range of temperature and pH, as well as distinctive active site chemistry and safety of its usage (Uhlig, 1998; Schaller, 2004). Earlier plant extracts as a milk clotting protease has been mostly used by several investigators (Roseiro *et al.*, 2003). In recent times many plant proteases employed for

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cheese production include papain, bromelain, ficin, oryzasin, cucumisin, sodom apple protease and lettuce protease (Uchikaba and Kaneda, 1996; Lo Piero *et al.*, 2002). One restraint in the use of crude plant proteases in milk clotting is that it provides bitter test to the cheese. But this curb can be curtailed using purified enzymes with optimum extraction concentration (Yousif *et al.*, 1996). A number of studies revealed the involvement of plant serine and cysteine like proteases in milk clotting, but paucity of reports on purifications and characterizations such proteases from plants. Successful use of plant proteases in milk clotting will lead to improve the nutritional quality of cheese, fulfil growing demand and increase the acceptability from the vegetarian population.

The activities of proteases in plants dramatically increase upon germination of seeds and earlier could have been utilized in preparation of soy sauces, meat tenderization, leather processing, detergent industry, hydrolysis of gelatin, soy protein, casein and whey proteins (Wang and Wang, 2004). Their potential in cheese making industry can also be evaluated in detail, if the appropriate source and efficient procedure of extraction, purification and characterization is developed.

To accomplishment the above said goal, as initial efforts here we are reporting extraction and partial characterization of milk clotting proteases from the germinating seeds of green gram (*Vigna radiata*), Chick pea (*Cicer arietinum*), Fenugreek (*Trigonella foenum-graecum*), and Moth bean (*Vigna aconitifolia*) and compared with milk clotting activity of rhizomes of (turmeric (*Curcuma longa*), ginger (*Zingiber officinale*). Unambiguous objectives of this study were a) Isolation of crude proteolytic extract b) Qualitative estimation of milk-clotting activity, c) Quantative estimation of milk-clotting activity and d) Electrophoretic characterization of milk-clotting proteases.

Materials and Methods

Experimental Materials

Seeds of green gram (*Vigna radiata*), Chick pea (*Cicer arietinum*), Fenugreek (*Trigonella foenum-graecum*), and Moth bean (*Vigna aconitifolia*) and rhizomes of (turmeric (*Curcuma longa*), ginger (*Zingiber officinale*) were obtained from local market of Aurangabad (MS), India. The Cow milk (Govind milk, Yamakanmardi, Belgaum, Karnataka, India) obtained from a local dairy of Aurangabad (MS), India. Mini electrophoresis system was purchased from Genei, India. Trypsin (bovine pancreas,

E.C.3.4.21.4) was purchased from Loba Chemie. Casein (Hammarsten bovine), acrylamide, bisacrylamide, tetramethylethylenediamine (TEMED) and ammonium persulfate, were obtained from Sisco Research Laboratories (SRL), Mumbai. All supplementary chemicals used were of the available utmost purity.

Extractions of the crude proteolytic/milk clotting enzymes from germinated seeds

In this study partially germinated seeds were analyzed for proteolytic/milk clotting activity. For convenience partial germination is defined as the period from imbibition of the dry seed through radicle advent. The seeds (green gram, fenugreek, chick pea and moth bean) approximately 100 grams were washed separately and then soaked in distilled water at room temperature (27°C) for overnight. These germinated seed samples and rhizomes of turmeric and ginger were homogenized in 0.1M phosphate buffer (pH 6.5) containing 10 mM CaCl₂ (Calcium chloride) in chilled mortal pestle separately. Homogenization was carried out at 4°C. The resulting homogenate were centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant fractions were used as crude source of proteolytic/milk clotting activity.

Sodium caseinate preparation

Whole casein was obtained from cow milk (250 ml) via isoelectric precipitation following acidification to pH 4.6 with diluted acetic acid with distilled water (1:5 v/v). The mixture of caseins and whey was warmed to 37°C. The caseins were recovered by filtration through a clean cheese cloth, and washed several times with distilled water. The casein was then suspended in distilled water (to the initial volume), and pH was adjusted to 7.0 with 1 mM NaOH (Sodium hydroxide). This Na-caseinate solution was used as substrate (milk casein) of milk clotting proteases in entire study.

Casein hydrolysis detection by agar diffusion method

Preliminary test was performed to detect caseinolytic activity from partially germinated crude extracts. About 2 grams of agar was dissolved in 50 ml of distilled water and heated in oven at 100°C. When temp decreased to 50°C, 1 ml Na-caseinate solution was added and mixed well. Approximately 20 ml solution poured in each petri plate and allowed it to solidify. After solidification circular wells were made with the help of well borer and 200 µl crude extracts were applied onto it. Plate was incubated at 37°C for 3-4 hours and flooded with CBBR-250 (Coomassie brilliant blue R-250) or 10% TCA (Trichloro acetic

acid) checked for the zone of casein hydrolysis.

Gelatin hydrolysis detection by dot-blot assay/ Spot test

Dot-Blot assay method for detection of proteases on x-ray film developed by Pichare and Kachole (1994) was used to detect gelatinolytic activity from crude extracts. The principle of this technique is that the x-ray film is coated with gelatin and as we placed a drop of protease on the film, it hydrolyzes gelatin and forms clear transparent spot against a dark background, hence proteases present in the sample are detected. The extracted crude samples (10 µl) were spotted onto a strip of x-ray film and visually monitored gelatin hydrolysis on different time interval at room temperature. The water was used as negative control and trypsin as positive control in the experiment.

Acetone precipitation of crude extracts

The solvent precipitation was used for partial purification of proteases. Crude protein samples usually contain substances that affect the study. Acetone precipitation is a brilliant method for precipitating most soluble proteins. Crude enzyme was precipitated with chilled acetone (1:4 v/v) for 2 hours at -20°C. The resulting extract is centrifuged at 10,000 rpm for 10 min at 4°C. After centrifugation, protein pellets were collected and dissolved in appropriate volume of buffer or distilled water.

Milk clotting assay

The crude extracts were assessed for milk-clotting activity as described by Arima *et al.* (1970) with slight modification. A 2 ml Na-caseinate solution containing 0.1M phosphate buffer, pH 6.5, 10 mM CaCl₂ was used as substrate. The crude and partially purified proteases (1ml) were then added in Na-caseinate solution separately. The clotting point was determined by periodic manual rotation of the test tube, at very short time intervals at 37°C. The clotting time was recorded when discrete particles were visible. The milk clotting activity (MCA) was expressed in terms of Soxhlet unit (SU). The SU were calculated using the following equation:

Soxhlet unit (SU) = $M \times 35 \times 2400 / E \times t \times T$, Where M is the volume of substrate (mL), E is the enzyme concentration (mg), t is the clotting time (sec) and T is the reaction temperature.

Assay of proteolytic activity

For quantitative estimation, caseinolytic activity of crude and partially purified protease solution

was determined by measuring the release of TCA soluble peptides at 37°C for 30 min. The assay was performed by incubating 0.9 mL of substrate with 0.1 mL of crude and partially purified protease solution, and the mixture was incubated at 37°C for 30 min, followed by the addition of an equal volume of 10% TCA to stop the reaction. A blank was prepared by adding TCA to the casein solution before addition of enzyme. After 20 min standing at room temperature, the precipitated proteins were removed by centrifugation at 12,000 rpm for 10 min. The TCA soluble peptides content in the supernatant (0.5 ml) was determined by adding 3 ml of 0.5M Na₂CO₃ (sodium carbonate) and incubating for 20 min in dark. To this 0.5 ml of diluted FCR (Folin–Ciocalteu reagent) was added and the optical density (OD) was read at 660 nm. Ratio of the milk clotting activity to proteolytic activity is expressed as a milk clotting unit (SU) per the OD₆₆₀'s obtained in the assay (Shieh *et al.*, 2009). Protein was estimated according to method of Lowry *et al.* (1951) using BSA (Bovine Serum Albumin) as the standard.

Native polyacrylamide gel electrophoresis

For native protein profiles, polyacrylamide gel electrophoresis under “non-denaturing” condition was carried out in discontinuous buffer system according to the method of Davis (1964). The 12% gel was prepared and about 20-40 µg of protein was loaded in the wells of gel. A constant current (20 mA) was supplied to the gel until the tracking dye BPB (Bromophenol blue) reached the bottom of the gel. After the electrophoretic run, proteins were visualized using CBBR-250.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis ((SDS-PAGE)

For denatured protein profiles, polyacrylamide gel electrophoresis under “denaturing” condition was carried out in discontinuous buffer system according to the method of Laemmli (1970). SDS–PAGE was done using 12% acrylamide separating (resolving) gel and 4% acrylamide stacking gel containing 0.1% SDS.

Zymographical analysis of proteases

Protease activities of crude protein extracts were detected by zymography. The 12% non-reducing SDS gels containing 0.1% casein were prepared and about 20-40 µg of protein was loaded in the wells of gel. The gel was run at 20 mA till the tracking dye BPB reached the bottom of the gel and soaked in 2.5% Triton X-100 to displace the SDS. Gel were then incubated in incubation buffer (0.1M phosphate

Table 1. Protein, MCA and Proteolytic activity from crude extracts of selected samples

Samples	Protein content (mg/gram)	Milk Clotting Time (in minutes)	MCA ^a (SU/ml)	Proteolytic activity (OD 660 nm)	Ratio (SU units/OD 660 nm)
Turmeric	59.25 ± 3.2 ^b	16 ± 2 ^b	28.73	0.13	221
Chick Pea	46.50 ± 4.3	21 ± 3	27.89	0.28	99.60
Fenugreek	54.00 ± 2.1	18 ± 2	28.08	0.165	170.80
Green Gram	67.50 ± 3.4	20 ± 3	20.18	0.33	61.15
Moth Bean	60.00 ± 5.0	20 ± 2	22.70	0.42	54.04
Ginger	82.50 ± 5.5	20 ± 1	16.51	0.30	55.03

^aThe milk clotting activity (MCA) was expressed in terms of Soxhlet unit (SU). The SU were calculated using the following equation: $M \times 35 \times 2400 / E \times t \times T$. Where M is the volume of substrate (mL), E is the enzyme concentration (mg), t is the clotting time (sec) and T is the reaction temperature.

^bAverage of three readings. The results are presented as the mean ± standard deviation (SD); n = 3.

buffer, pH 6.5) for overnight at 37°C and later stained with CBBR-250. The unstained region of the gel reveals the caseinolytic activity of the enzyme.

Results and Discussion

Industrial applicability of widespread occurring proteases is increasing nowadays. As far food industries where large amount of cheese produced to meet demand increasing population, these protease is prime factor which is utilized for milk clotting. Seeds of leguminous plant may have played a major role in the search of milk clotting proteases owing to the great protein content (Muntz, 1996; Shah *et al.*, 2013). This protein content augmented during the process of germination due to increased expression of proteolytic enzymes (Mostafa and Rahma, 1987). In this study we tried to explored milk clotting potential of crude proteases expressed in partially germinated seeds of green gram, chick pea, fenugreek and moth bean and rhizomes of turmeric and ginger. Earlier milk clotting proteases were isolated and characterized from seeds of *Ficus glabra*, *Oriza sativa*, *Cucumis melo* ssp, *Albizia lebbeck*, *Helianthus annuus* and *Solanum dubium* (Asakura *et al.*, 1997; Lo Piero *et al.*, 2002; Egito *et al.*, 2007; Ahmed *et al.*, 2010).

Estimation of total protein content

For quantitative estimation of crude protein, Lowry method (1951) was employed. The highest amount of protein was present in green gram (67.5 ± 3.4 mg/gram of sample) whereas moth bean, fenugreek and chick pea has 60 ± 5.0 mg, 54 ± 2.1 mg and 46.5 ± 4.3 mg of protein per gram of sample respectively (Table 1). Total protein present in rhizomes of turmeric and ginger was found to be 59.25 ± 3.2 mg and 82.5 ± 5.5 mg

per gram of sample respectively. Upon germination, elevated protein content may be attributed to the action of proteolytic enzymes on storage proteins and peptides and amino acids produced may persist in the storage tissue or translocate to the developing parts of the plant (Callis, 1995). The substances like salts and lipids commonly found in crude extracts and may interfere in the study (Garcia-Rodriguez *et al.*, 2003). To isolate soluble proteins, we employed acetone precipitation method. For routine work it is efficient with high pellet stability, solubility and high yield of concentrated proteins (Nejadi *et al.*, 2014). In agreement with above finding, we recovered comparable amount of soluble protein from crude extracts as depicted in Table 2.

Qualitative detection of proteolytic activity

In qualitative analysis all crude protein extracts of germinating seeds exhibits caseinolytic activity confirmed by casein agar diffusion method (Figure 1 A and B). Protein of acetone precipitates corresponding to all samples have also showed significant proteolytic activity by agar diffusion method (data not shown). The caseinolytic activity was detected by the presence of clear zone of hydrolysis of casein on agar plate. The clear distinct zone of casein hydrolysis was observed after the addition of CBBR-250 dye reagent and 10% TCA. These results show that the crude extracts from germinated seeds of green gram, fenugreek, chick pea and moth bean contained a good quantity of proteases. Rhizomes of turmeric and ginger produced hydrolysis zone as well and could be related to the amount of protease present in samples.

Alongside caseinolytic activity these samples were also able to hydrolyze gelatin when spotted on gelatin coated X-ray film (Figure 1 C) as assessed

Table 2. Protein, MCA and Proteolytic activity from acetone precipitate of selected samples

Samples	Protein content (mg/ml)	Milk Clotting Time (in minutes)	MCA ^a (SU/ml)	Proteolytic activity ^b (OD 660 nm)	Ratio (SU units/OD 660 nm)
Turmeric	6.0 ± 0. 2 ^b	22 ± 2 ^b	34.39	0.04	859.75
Chick Pea	7.7 ± 1.0	24 ± 1	24.57	0.43	57.13
Fenugreek	8.5 ± 0.7	21 ± 2	25.43	0.14	181.64
Green Gram	11.2 ± 0.9	23 ± 1	17.62	0.42	41.95
Moth Bean	5.7 ± 1.3	25 ± 2	31.86	0.58	54.93
Ginger	13.6 ± 0.8	25 ± 1	13.35	0.36	37.08

^aThe milk clotting activity (MCA) was expressed in terms of Soxhlet unit (SU). The SU were calculated using the following equation: Soxhlet unit (SU) = $M \times 35 \times 2400 / E \times t \times T$. Where M is the volume of substrate (mL), E is the enzyme concentration (mg), t is the clotting time (sec) and T is the reaction temperature.

^bAverage of three readings. The results are presented as the mean ± standard deviation (SD); n = 3.

by Dot blot assay (Pichare and Kachole, 1994). The gelatinolytic activity was assessed at various time intervals and complete hydrolysis of gelatin coated on X-ray film was observed at 25 minutes in each sample visually detected by the presence of clear zone of hydrolysis. These activities are accompanied with trypsin and H₂O as positive and negative control respectively. The dot blot assay is simple, sensitive rapid, less expensive and used to screen large number of samples for proteases and protease inhibitors (Padul *et al.*, 2012; Shaikh *et al.*, 2014).

Quantification of milk clotting / proteolytic activity

Milk clotting is the major step in the development of texture and flavor of cheese and depends on proteolytic degradation of milk casein (Yousif *et al.*, 1996). The germinating seeds were chosen as source because it has high percentage of proteolytic activity which may contribute in degradation of Na-caseinate. The MCA in term of SU was the highest for the turmeric, fenugreek and chick pea (Table 1). Moderate MCA was observed in moth bean and green gram. Interestingly less amount of SU units were detected in ginger which is well known milk coagulant (Nafi *et al.*, 2013). This noticeable activity advocates the potentials of turmeric, chick pea, fenugreek, green gram and moth bean crude protein extracts as milk coagulants.

The crude extract of selected samples mildly coagulated Na-caseinate solution within varied time periods as represented in Table 1 and Figure 1 with strongest activity found in turmeric as it taken lowest time to forming first clot in solution (16 ± 2 minutes) at 37°C. Although chick peas have highest SU, it took relatively longer time period for milk coagulation to start (21 ± 3 minutes) (Figure 2). Upon acetone

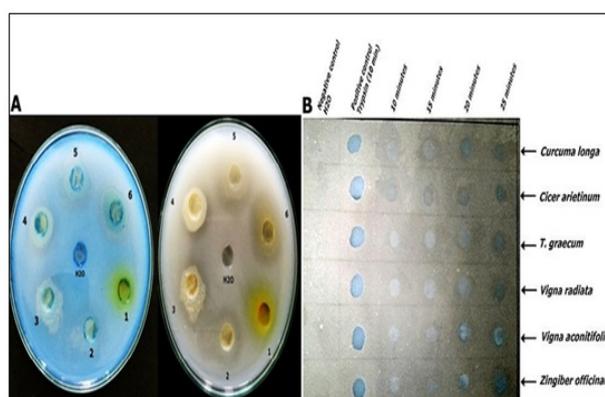


Figure 1. Proteolytic activity of crude extract of selected samples A) Proteolytic transparent zone was developed in casein incorporated agar plate flooded with CBBR-250 dye reagent or 10% TCA. 1) Turmeric (*Curcuma longa*); 2) Chick pea (*Cicer arietinum*); 3) Fenugreek (*Trigonella foenum-graecum*); 4) Green gram (*Vigna radiata*); 5) Moth bean (*Vigna aconitifolia*) and 6) Ginger (*Zingiber officinale*). Middle well with H₂O represents negative control. B) Dot-blot assay to detect protease activity (gelatin degradation). The proteolytic degradation was studied at various time intervals with water as negative control and trypsin as positive control. Detailed methodology is mentioned in materials and methods section.

precipitation increased SU in turmeric and moth bean were observed. Whereas reduced yield of SU were observed in chick pea, fenugreek, green gram and ginger (Table 2). Quantitative analysis produced high ratio of MCA to proteolytic activity from crude extracts of all samples with comparatively high in turmeric (221) and fenugreek (170.80) (Table 1). This ratio was found to increase after acetone precipitation of crude extracts of turmeric (859.75) and fenugreek (181.64) suggesting partial purification of enzymes (Table 2). Remarkably the ration declined in other all

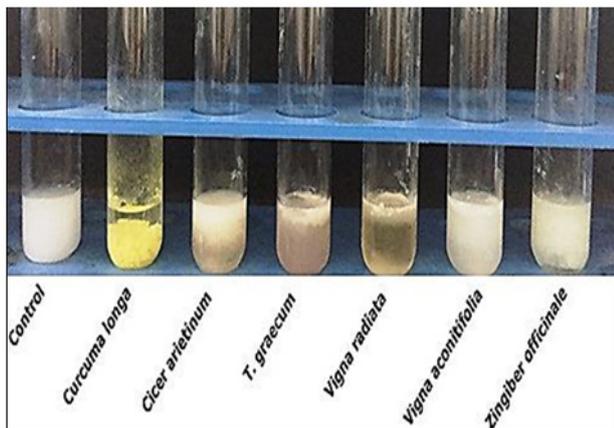


Figure 2. Milk-clotting activity of crude protein extract of selected samples.

samples suggesting loss of enzyme activity.

Electrophoretic characterization

For the electrophoretic characterizations, initially crude protein extract derived from selected samples was analyzed by 10% native PAGE (Figure 3 A). Profile of the native proteins present in crude extracts (40 μ l) showed varied migration pattern on the gel. In crude extract of turmeric (*Curcuma longa*), 3 to 4 fast migrating protein bands were observed (Lane I). The crude extracts of chick pea, fenugreek, green gram, and moth bean have maximum no. of protein bands with modest migration pattern on the gel (Lane II, III, IV and V). The protein profiling of ginger was varied among all samples with moderated to fast moving proteins on the gel ((Lane VI).

Electrophoretic patterns of proteins present in crude extracts (40 μ l) were also revealed by 12% SDS-PAGE (Figure 3 B). Protein distribution patterns in crude seed extract by SDS-PAGE demonstrated bands of varied migration on the denatured gel. The protein profiles were completely different from that native condition as assessed by Native PAGE. Most of the protein bands present on Native PAGE, were absent in the SDS-PAGE and dissimilar migration patterns of these proteins were observed due to denaturation. Effectiveness of SDS-PAGE zymography for detection and characterization of proteases was well recognized (Choi *et al.*, 2001; Shinde *et al.*, 2012). Zymography is a two-stage technique involving protein separation by electrophoresis followed by in situ detection of proteolytic activity. Profile of the proteases activity of crude protein extracts of selected samples using non-denaturing 12% SDS-PAGE containing 0.1% casein is shown in Figure 3 C. On a casein-containing zymogram, a clear area of gelatin hydrolysis was formed, whereas the undigested gelatin stained blue. The zone of gelatin clearing corresponded to

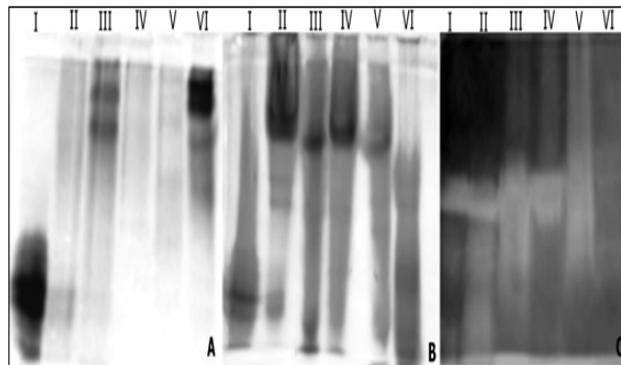


Figure 3. Electrophoretic characterization of crude protein extracts of selected samples. A) Native PAGE was performed on 10% gel B) None reducing SDS-PAGE was performed on 12% gel C) None reducing SDS-PAGE casein zymography analysis. Detailed methodology is mentioned in materials and methods section. Lane I: Turmeric (*Curcuma longa*); Lane II: Chick pea (*Cicer arietinum*); Lane III: Fenugreek (*Trigonella foenum-graecum*); Lane IV: Green gram (*Vigna radiata*); Lane V: Moth bean (*Vigna aconitifolia*) and Lane VI: Ginger (*Zingiber officinale*).

a migration distance of proteases. Among all crude samples, turmeric and chick have 3 to 4 fast migrating protease bands whereas crude samples of fenugreek, green gram and moth bean showed 2 to 3 moderately migrating strong protease bands (Lane III, IV and V). In ginger (*Zingiber officinale*) very light protease banding pattern was observed (Three bands) (Lane V). Detailed biochemical and molecular analysis of these promising proteases will provide alternative, nutrition rich and cost effective source of milk clotting for chees making industry.

Conclusion

In conclusion, milk coagulants are necessary for the production of cheese and plant proteases have been isolated from several plant sources. In this study we reveal the presence of milk clotting proteases in crude extracts of partially germinated seeds of green gram, chick pea, fenugreek and moth bean. Quantative estimations by solution assays and qualitative characterization by gel electrophoresis unveil potent caseinolytic, gelatinolytic and milk-clotting activities. The milk clotting potential found to be equivalent or even more than milk clotting potential of crude extracts of rhizomes of turmeric and ginger, a well-known milk coagulant. The detailed biochemical analysis and purifications to homogeneity may provide unconventional, nutritive and cost effective source of milk clotting for cheese making industry, is future prospective of this study.

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