

Proteolytic and Milk Clotting Activity of Fractionated Protein of *Lagenaria siceraria*

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ABSTRACT

The study was undertaken to evaluate milk clotting and proteolytic activity of protein fractions of seeds of *Lagenaria siceraria*. Protein fractions were isolated by the method of differential solubility, and their protein contents were estimated using Bio-Rad protein assay reagent and bovine serum albumin. The effects of pH and temperature on the milk-clotting and proteolytic activity were also evaluated. The isolated protein fractions showed highest milk clotting activity over a broad temperature range of 30-80 °C and pH range of 3-9. Based upon the observations, milk clotting enzymes present in different fraction of proteins of *Lagenaria siceraria* are promising candidates for application in industrial scale for production of cheese and might be a potential substitute for commercial animal rennet.

INTRODUCTION

Since a long time, the conventional milk clotting enzyme, calf rennet has been derived from the stomach of suckling calves and being used as coagulant for manufacturing of cheese worldwide. As the supply of calf rennet is going to be reduced by the manufacturers, the production of cheese is now under consideration for research in milk clotting enzymes from the alternative source. The alternative sources such as various animals, plants and microbial proteases have now been considered as milk coagulants (Shieh *et al.*, 2009). Due to high cost of calf rennet and its ethical and religious consideration associated with animals, prompted researchers to study on milk coagulants from natural sources. The milk coagulants from the plant source is of great interest since they are natural enzymes and can also be used for producing cheeses for lacto-vegetarian consumers (Bruno *et al.*, 2010). Proteolytic enzymes from plant sources play important role in production of cheese, protein hydrolyzates, and tenderization of meat and preparation of medicine for digestive and anti-inflammatory disorders (Huang *et al.*, 2011). There are so many rennet substitutes obtained from the plants such as fruits (e.g. *Actinidia chinensis* and *Cucumis melo*), latex (e.g., *Ficus carica* and *Calotropis procera*), flowers

(e.g., *Cynara cardunculus* and *Centaurea calcitrapa*), seeds (e.g., *Albizia lebeck* and *Solanum dubium*), leaves (e.g., *Lactuca sativa*) and roots (e.g., *Zingiber officinale*) have been investigated to meet the demand of conventional calf rennet (Gagaoua *et al.*, 2015). *Lagenaria siceraria* (*L. siceraria*) belonging to family Cucurbitaceae is known as bottle gourd and used throughout the India as common vegetable. Over a long time, it is used for treatment of various disorders such as immunosuppressant, diuretic, cardio-tonic, cardio-protective and nutritive agent. The fruits are also reported to have antihepatotoxic, antioxidant, hypolipidemic and triton-induced hyperlipidemic activities in rats. The fruit contains vitamin-B complex, choline, vitamin-C, β -carotene, Cucurbitacins, fibres and polyphenol. It also contains two sterols namely campesterol and sitosterol. Similarly the methanol extract was found to have flavones-C-glycosides. A ribosome inactivating protein (RIP), Lagenin isolated from the seeds of *L. siceraria* was claimed by Kumar *et al* for immunoprotective, antitumor, anti HIV and antiproliferative properties (Kumar *et al.*, 2012).

The fruits of *L. siceraria* was previously claim to have various amino acids such as leucines 0.8; phenylalanine 0.9; valine 0.3; tyrosine 0.4; alanine 0.5; threonine 0.2; glutamic acid 0.3; serine 0.6; aspartic acid 1.9; cystine 0.6; cysteine 0.3; arginine 0.4; and proline 0.3mg/g (kubde *et al.*, 2010). Studies on the vegetable proteins from the plant source is coming now in the lime light of research and more attention is paid due to increasing demand of cheap and affordable dietary protein .

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There is a growing interest in the utilization of plant proteins for the formulation of new food products. Seed flours also have appreciable functional properties that are suitable for innovative application in the food industry (Ogundele *et al.*, 2013). This leads to research on *L. siceraria* as the seeds are alternative source of dietary protein.

The seeds are edible and used in the preparation of local soups, fermented food product (ogiri), fried cake (robo) and pudding (igbalo or ugbaotiri) (Ogunbusola *et al.*, 2010). In view of the immense importance of the plant in the field of food industry it was thought worthwhile to investigate the proteolytic and milk clotting activity of fractionated protein of *L. siceraria*.

MATERIAL & METHODS

Isolation of protein

L. siceraria seed kernels are separated and dried at 50 °C. Then kernels were powdered using grinder and then subjected for de-fatting using n-hexane. Then the residue (10 mg) was mixed with 1 ml of distilled water at 4 °C for 1 h and centrifuged at 10000 g for 20 min. The supernatant containing albumins (L_{alb}) was collected, while the sediment was used for further extractions. It was rinsed with 2 ml of distilled water before homogenization, followed by centrifugation in the same condition as in the previous step to remove albumins (L_{alb}) completely. The residual pellet obtained passed through the similar series of steps using a mixture of Tris HCl 100 mmol in 0.5 NaCl at pH 8.1 to extract globulins (L_{glo}).

The pellet was then treated with 55 % isopropyl alcohol to separate prolamin (L_{pro}) and glutelin (L_{glu}) was separated by using 0.2 N acetic acid. Protein fractions were purified using acetone. The four protein fractions obtained were lyophilized and stored at -20 °C until further study (Teugwa *et al.*, 2013).

Determination of protein content and protein pattern analysis

The protein content was determined using a Bio-Rad protein assay reagent (Bio-Rad, USA) and bovine serum albumin (BSA) as described by Bradford (Bradford, 1976). Absorbance was measured at 595 nm after the mixture was allowed to stand for 5 min at room temperature.

Proteolytic activity

The proteolytic activity was determined at pH 6 using casein hydrolysis method described previously by Shieh *et al.* (Shieh *et al.*, 2009). The 5 ml of 1.2 % of casein solution in 0.05 mol phosphate buffer (pH 6) was added to 1ml of sample solution and incubated at 35 °C for 10 min. After incubation, 5 ml (0.44 mol) of tri-chloroacetic acid (TCA) was added to the mixture and filtered. 1 ml of the filtrate was then added to 2.5 ml of NaOH (0.28 N) solution and 1 ml phenol reagent. The optical density (OD) of the mixture was measured at 660 nm after incubation at 35 °C for 15 min. All the observations are carried out in triplicate.

Proteolytic activity = tyrosine equivalents released (μg) $\times 11 = 1 \times 10 \times 2$

Where,

11= total volume (ml) of assay

10= time of assay (min) as per unit definition

1= volume of enzyme (ml) of enzyme used

2= volume (ml) used in colorimetric determination

Milk clotting activity

The milk clotting activity of *L. siceraria* protein fraction was determined according to the method of Corrons *et al.* (Corrons *et al.*, 2012). The substrate containing 10 % skimmed milk in 0.01 mol CaCl_2 and its pH was adjusted to 6.5. The substrate (2.0 ml) was pre incubated at 37 °C for 5 min and then 0.2 ml of test sample was added.

The formation of curd was observed at 37 °C while test tube was rotated manually in different time intervals. The end point was recorded when discrete particles were fully separated. One milk clotting unit is defined as the amount of enzyme that clots 10 ml of the substrate within 40 min. All the observations are carried out in triplicate.

MCA (U/ml) = $2400 / \text{clotting time (s)} / \text{dilution factor}$

Effect of heating and pH on proteolytic and milk clotting activity

Proteolytic and milk clotting activities were determined at 30, 40, 50, 60, 70, 80 and 90 °C. To determine the effect of pH on the enzyme activities, protein was previously incubated in 50 mmol citrate- sodium phosphate buffer (pH 3.0 to 6.0, 24 h, 37 °C), 50 mmol tris-HCl (pH 6.0-8.0), 50 mmol glycine (pH 8.0 to 11.0, 24 h, 37 °C). The assays were performed as above.

RESULTS AND DISCUSSION

Milk clotting activity

In this experiment, all the test protein fractions were evaluated for their milk clotting activity. The milk clots obtained show stable consistency over the time period of 40 min and the exudates are clear and slightly yellow in colour. Milk clotting activity of $L_{(alb)}$, $L_{(glo)}$, $L_{(pro)}$ and $L_{(glu)}$ was found to be 26.2 ± 4.7 , 24 ± 3.9 , 35.9 ± 6.2 units/ml and 26.8 ± 2.5 respectively at 37 °C and pH 6.5. In this present study, $L_{(alb)}$, $L_{(glo)}$, $L_{(pro)}$ and $L_{(glu)}$ also registered specific activity of 7, 6.1, 18.89 and 5.32 units/mg respectively.

The results showed that $L_{(pro)}$ has highest milk clotting and specific activity in comparison to other test proteins. As shown in Fig. 5, the skimmed milk coagulations took place based on enzymatic activity of seed proteins. The milk clotting activity of isolated protein of *L. siceraria* was much higher than some plant extracts i.e. in agreement with previously reported article (Mazorra-Manzano *et al.*, 2013) such as *S. aculeastrum* (0.56 ± 0.01 U/ml), *S. aethiopicum* (2.62 ± 0.1 U/ml), *S. terminale* (1.33 ± 0.02).

Effect of heating, pH on Milk clotting activity

The effect of temperature and pH on milk clotting activity was shown in Fig 1 & 2. The isolated protein fraction is stable over a wide range of pH and retained all of its enzymatic activity. The previously incubated protein fractions of *L. siceraria* showed milk clotting activity at pH 3 and loss of activity at pH values higher than 10. L_(alb) and L_(glo) showed highest activity at pH 5, but L_(pro) and L_(glu) exhibited highest activity at pH 6 which is similar to milk clotting activity of culture of *Bacillus subtilis* (Shieh *et al.*, 2009).

The selection of protease for industrial processes is based on its high thermal stability and wide range of pH. It is well known that the reaction rate of enzymes increases with increasing temperature, but after a certain temperature, the rate of reaction decreases due to denaturation (Hashim *et al.*, 2011). In this study, L_(alb), L_(pro) and L_(glu) showed highest activity at 40° C, but the activity decreases with increase in temperature. But L_(glo) showed highest activity at 60° C which is similar to the activity reported by Ahmed *et al* (Ahmed *et al.*, 2009). It was found that, the milk clotting enzymes present in different fraction of proteins are promising candidates for application in industrial scale for production of cheese. Additionally, use of seed *L. siceraria* in human diet, being eaten raw or cooked is an indicative of safety for use in cheese production.

Proteolytic activity

The L_(alb), L_(glo), L_(pro) and L_(glu) showed caesinolytic activity 203.4 ± 0.9, 179.64 ± 1.3, 270.17 ± 2.7, 173.71 ± 0.7 units/ml and specific activity 54.38, 46.06, 142.19 and 34.53 units/mg respectively (Table-1). From the observed data it was found that L_(pro) has highest proteolytic and specific activity than the rest protein fractions. The caesinolytic activity of the protein fractions of *L. siceraria* was found more than the isolated proteins obtained from some other plant sources reported earlier (Monica *et al.*, 2008).

Effect of heating, pH on proteolytic activity

Proteolytic activity of L_(alb) and L_(glo) on the substrate, casein significantly enhanced at pH 4 but after pH 6 its activity gradually decreased. The proteolytic activity of L_(pro) and L_(glu) was found to be highest at pH 6, which was similar to *Euphorbia nivulia* (Badgujar *et al.*, 2012) and activity was lowered after pH 7. As shown in Fig. 3, these fractionated proteins were stable under a wide range of pH (3 to 11). L_(alb) and L_(pro) showed optimum activity at 40° C, which is in agreement with studies reported earlier by Thakur *et al* (Thakur *et al.*, 1990). Similarly, it was found that L_(glo) and L_(glu) exhibited highest activity at 70 °C, which correspond to proteolytic activity of the melon (Mazorra-Manzano *et al.*, 2013).

Table 1: Milk clotting activity and proteolytic activity of protein fractions of *L. siceraria*.

Protein fractions	Proteolytic activity(units/ml)	Milk clotting activity(units/ml)	Total protein (mg)	Specific activity for proteolytic activity (units/mg)	Specific activity for MCA (units/mg)
Albumin	203.4±0.9	26.2±4.7	3.74	54.38	7
Globulin	179.65±1.3	24.0±3.9	3.9	46.06	6.1
Prolamin	270.17±2.7	35.9±6.2	1.9	142.19	18.89
Glutelin	173.71±0.7	26.8±2.5	5.03	34.53	5.32

Values are expressed as mean ± SD

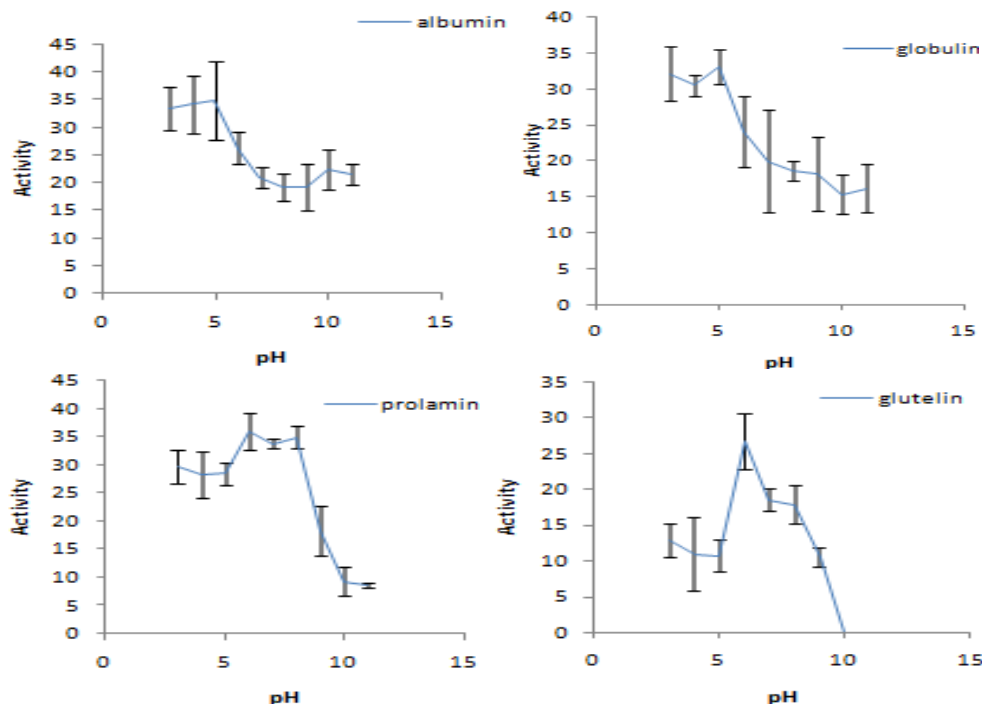


Fig. 1: Milk clotting activity of *L. siceraria* protein fraction of seed at different pH

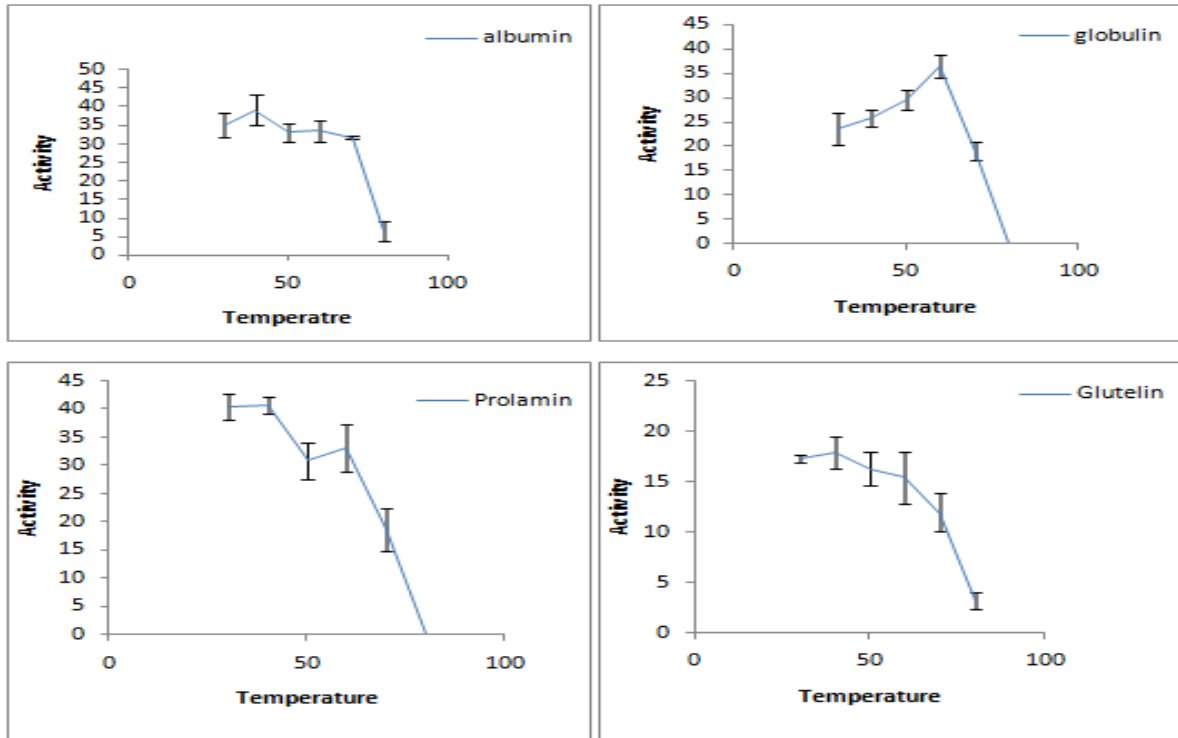


Fig. 2: Milk clotting activity of *L. siceraria* protein fraction of seed at different temperature.

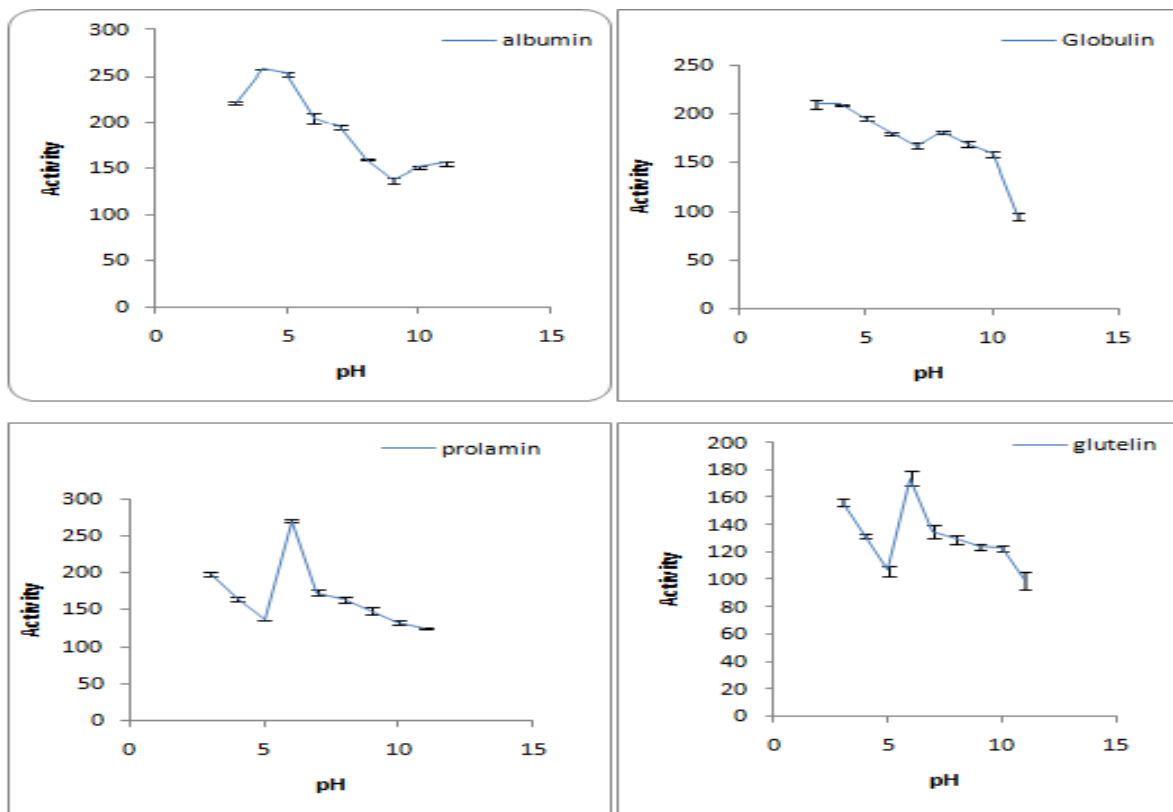


Fig. 3: Proteolytic activity of *L. siceraria* protein fraction of seed at different pH

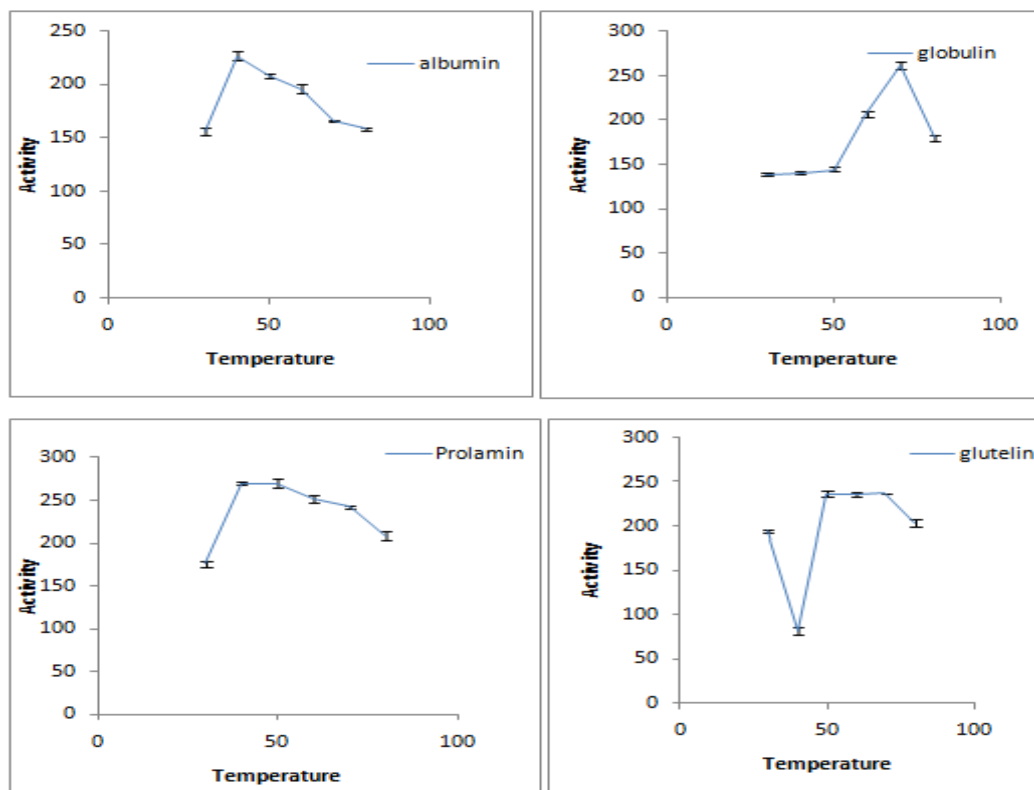


Fig. 4: Proteolytic activity of *L. siceraria* protein fraction of seed at different temperature.

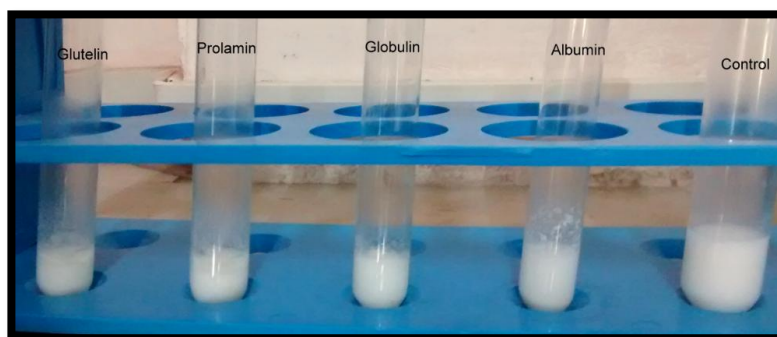


Fig. 5: Milk clotting activity of *L. siceraria*

CONCLUSIONS

In the present study, it is concluded that among the protein fractions of *L. siceraria* seed, prolamin might be a potentially suitable substitute for commercial animal rennet, being more active than other protein fractions and exhibiting both good milk clotting and caseinolytic activity required for cheese-ripening. As many plant rennets generate bitter peptides, experimental cheese-making needs to be carried out with *L. siceraria* to ensure that its seed protein can lead to cheese without bitterness.

REFERENCES

Ahmed I.A.M., Morishima I., Babiker E.E., Mori N. Characterisation of partially purified milk-clotting enzyme from *Solanum dubium* Fresen seeds. *Food Chem*, 2009; 116: 395–400.

Badgujar S.B. and Mahajan R.T. Comparison of cysteine proteases of four laticiferous plants and characterization of *Euphorbia nivulia* Buch.-Ham. Latex glycosylated cysteine peptidase. *Indian J Nat Prod Res*, 2012; 3: 152-160.

Bradford M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem*, 1976; 72: 248-254.

Bruno M.A., Lazza C.M., Errasti M.E., Lopez L.M.I., Caffini N.O., Pardo M.F. Milk clotting and proteolytic activity of an enzyme preparation from *Bromelia hieronymi* fruits. *LWT - Food Sci Technol*, 2010; 43: 695–701.

Corrons M.A., Bertucci J.I., Liggieri C.S., López L.M.I., Bruno M.A. Milk clotting activity and production of bioactive peptides from whey using *Maclura pomifera* proteases. *LWT - Food Sci Technol*, 2012; 47: 103-109.

Gagaoua M., Hoggas N., Hafid K. Three phase partitioning of zingibain, a milk-clotting enzyme from *Zingiber officinale* Roscoe rhizomes. *Int J Biol Macromol*, 2015; 73: 245–252.

Hashim M.M., Mingsheng D., Iqbal M.F., Xiaohong C. Ginger rhizome as a potential source of milk coagulating cysteine protease. *Phytochem*, 2011; 72: 458–464.

Huang X.W., Chen L.J., Luo Y.B., Guo H.Y., Ren F.Z. Purification, characterization, and milk coagulating properties of ginger proteases. *J Dairy Sci*, 2011; 94: 2259–2269.

Kumar A., Partap S., Sharma N.K., Jha K.K. Phytochemical, Ethnobotanical and Pharmacological Profile of *Lagenaria siceraria*: - A Review. *J Pharmacog Phytochem*, 2012; 1: 24-31.

Mazorra-Manzano M.A., Perea-Gutiérrez T.C., Lugo-Sánchez M.E., Ramirez-Suarez J.C., Torres-Llanez M.J., González-Córdova A.F., Vallejo-Cordoba B. Comparison of the milk-clotting properties of three plant extracts. *Food Chem*, 2013; 141: 1902–1907.

Meenal S., kubde S.S., Khadabadi I., Farooqui A., Deore S.L. *Lagenaria siceraria*: Phytochemistry, pharmacognosy and pharmacological studies. *Rep Opinion*, 2010; 2: 91-98.

Monica G., Parisi M.G., Moreno S., Fernandez G. Isolation and characterization of a dual function protein from *Allium sativum* bulbs which exhibits proteolytic and hemagglutinating activities. *Plant Physiol Biochem*, 2008; 46: 403-413.

Ogunbusola E.M., Fagbemi T.N., Osundahunsi O.F. Amino acid composition of *Lagenaria sicerari* seed flour and protein fractions. *J Food Sci Technol*, 2010; 47: 656–661.

Ogundele J.O., Oshodi A.A., Sanni T.A., Amoo I. A. Protein isolates of gourd melon seeds and their functional properties. *Am J Food Nutr*, 2013; 3: 176-181.

Shieh C., Thi L.P., Shih I. Milk-clotting enzymes produced by culture of *Bacillus subtilis* natto. *Biochem Eng J*, 2009; 43: 85–91.

Teugwa C.M., Boudjeko T., Tchinda B.T., Mejiato P.C., Zofou D. Anti-hyperglycaemic globulins from selected Cucurbitaceae seeds used as antidiabetic medicinal plants in Africa. *BMC Complement Altern Med*, 2013; 13: 63.

Thakur M.S., Karanth N.G. Nand K. Production of fungal rennet by *Mucor miehei* using solid state fermentation. *Appl Microbiol Biotechnol*, 1990; 32: 409–413.

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