AJHS.
Asian Journal of Home Science
Volume **8** | Issue 2 | December, 2013 | 456-460

Research Paper

Assessment of milk clotting activities of plant latex

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Received: 20.04.2013; **Revised:** 24.08.2013; **Accepted:** 18.09.2013

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ABSTRACT : A number of proteolytic enzymes are widely employed in food industries for cheese manufacture. The coagulant which is widely used in cheese production or manufacturing of cheese is animal rennet, which contains chymosin, an asparatic protease which is responsible for milk clotting. Thus, there's a need for the identification of a milk clotting enzyme from other sources that can meet the industrial demand of rennet. In this context, the present investigation was carried out in order to extract a milk clotting enzyme from different plant sources which can be utilized in cheese production. Three plants, *Carica papaya*, *Euphorbia splendens* and *Musa paradasica* belonging to the families Annonaceace, Euphorbiaceae and Musaceae were examined for the presence of proteinases in the present study. The proteinase activity was estimated by determining the milk clotting property of crude latex by identifying specific activity through their partial purification. The results indicated that *Carica papaya* plant latex had the highest milk clotting activity (2006.20 ± 3.77 U/ml) than *Musa paradasica* (187.19 ± 4.44 U/ml) and minimum in *Europhorbia splendens* (105.20 ± 4.15 U/ml) (p =0.01). SDS-PAGE analysis of the plant latex exhibiting maximum milk clotting activity displayed 5 bands ranging from 14.3 KD to 97.4 KD.

KEY WORDS : Milk clotting enzyme, SDS-PAGE, Plant latex, Carica papaya, Musa paradasica, Euphorbia splendens

HOW TO CITE THIS PAPER : Upasana, Chakravarty, Archana, Chaturvedi, Adya and Tripathi, Y.B. (2013). Assessment of milk clotting activities of plant latex. *Asian J. Home Sci.*, **8** (2): 456-460.

The transformation of raw milk to cheese is very important for developing countries which are widely achieved through natural calf rennet (rennin or chymosin) extracted from the calf's abomasums *i.e.* from fourth stomach before weaning. Rennet is a natural complex of enzyme produced in any mammalian stomach to digest the mother's milk as well as used in the production of cheese. Rennet contains a proteolytic enzyme (protease) that coagulates the milk causing it to separate into casein and whey protein. It is well known that coagulation of milk is a combination of two phases: initial enzymatic hydrolysis reaction and subsequent enzyme dependent protein aggregation reaction (Hooydonk and Walstras, 1987 ; Najera *et al.*, 2003) Although primary proteolysis is an essential phase in the development of proper cheese texture, secondary proteolysis is of great importance to assure the proper breakdown of curd protein in order to avoid formation of low viscosity and high bitterness that is related to cheese flavour (Visser,1993 ; Silva and Malcata, 2000). The coagulation occurs due to change in physio-chemical status of casein as a result of cleavage of peptide bond (between phenylalanine and methionine) in k-casein fraction. The reduced supply of rennin has led to search for coagulant substitutes like vegetable rennet, microbial rennet and genetically engineered rennet. As the proper coagulation is done by the enzymatic activity, the task of the researcher is to find the alternative source of enzyme for cleaving the casein that would result in taste as well as texture similar to natural calf rennet. A number of proteolytic enzymes from various sources have been used such as bovine, porcine, chicken pepsin, fish chymotrypsin as well as proteases from fungi and transgenic microorganism (Lopez *et al.*, 1998). However, the use of these coagulants give rise to unwanted final products and also causes ethnic (been against genetically modified foods), religious (Hinduism and Islam), diet (vegetarianism) and public health problem (bird flu, bovine spongiform encephalopathy, H_1N_1 virus and microbial toxins) (Roseiro *et al.*, 2003). Due to this reason, much research

interest has been aroused towards discovering the milk clotting enzymes from vegetable or plant sources which could satisfactorily replace calf's rennet in cheese making process or production. Applications of plant coagulants allow target cheese production and hence contribute to improve the nutritional input of those populations on whom restrictions are improbable by the use of animal rennets (Silva and Malcata, 2005). Plant coagulants show many biochemical features similar to rennin (Chymosin). Both rennin or chymosin (EC 3.4.23.4) and plant coagulants cleave the Phel 05-Metl 06 peptide bond of k-casein but plant coagulants are more proteolytic and have broader specificity on α S_1 and β-caseins than rennin (Chymosin) (Macedo, 1993; Esteves *et al*., 2003).

Several plant preparations are known for cheese making. Species from *Cynara* genus are used successfully as source of milk clotting enzymes in the lberian Peninsula and Latin America. Tropical plants such as: *Carica papaya*, *Ananas comocus*, *Ficus glabra*, *Calotropis procera* and *Latuca sativa* have also milk clotting enzymes *i.e.* papain, bromelain, ficin, Sodom apple protease and lettuce protease, respectively (Uchikoba and Kaneda, 1996; Asakura *et al*., 1997; Lo Piero *et al*., 2002). Unfortunately, most of these coagulants have been found to be unsuitable in cheese making, because, they produce bitter cheese (Ahmed *et al*., 2009) as well as due to ecological variations or bio-diversity.

Therefore, the present study was accomplished to explore three plant sources, *Carica papaya* (Annonaceace), *Euphorbia splendens* (Euphorbiaceace) and *Musa paradasica* (Musaceace) for the extraction of milk clotting enzymes that can be utilized for various food applications such as cheese production. Further, partial characterization of these enzymes was also attempted.

■ RESEARCH METHODS

Plant materials :

The plant materials used in the study were *Carica papaya, Euphorbia splendens* and *Musa paradasica*. Triplicate samples of plant latex were collected from Jodhpur colony, BHU campus, Varanasi, and Department of Horticulture, Institute of Agricultural Sciences, BHU, Varanasi U.P., India. Bovine serum albumin (BSA), acrylamide and bisacrylamide were purchased from Aldrich – Sigma while TEMED and SDS were purchased from Merck. All other chemicals were commercially available.

Collection of latex :

Latex samples were collected early in the morning from these selected plants from fruits or by nippling the leaves near the stem or by incision of the trunk or branches and allowing the milk to drain in separating tubes maintained at 4°C on ice. Tubes were brought to the laboratory and kept at 4°C till the experiment commenced.

Milk clotting activity :

The coagulant activities were determined according to the method of Berridge (1952) and as modified by Collin *et al.* (1977) with slight modifications. The milk clotting activity was analysed by using Nestle every day milk powder as a substrate which was locally available in the market. The milk powder was dissolved in 10 mM of $CaCl_2$ at pH6.5 to a final concentration of 0.12 kilogram per litre. Enzyme sample was added in the proportion drop-wise by titrimetric method. The coagulation time was determined by manual rotation of test tube at a short time. The end point was noted by the appearance of milk clot. The unit of milk clotting activity was defined as the amount of plant extract or latex that coagulates 1mL of milk at room temperature. Milk coagulating activity was calculated by following equation : where,

$$\mathbf{MCA} = \frac{\mathbf{2400xV}}{\mathbf{vxt}}$$

where,

MCA = Milk clotting activity
V = Volume of milk in ml.
T = clotting time in second
V = volume of plant latex or extract in ml.

Protein estimation :

The estimation of protein was carried out by following Lowry *et al*. (1951) method and the protein absorbance was read at 660 nm using Bovine serum albumin (BSA) as the standard.

Electrophoresis :

After protein estimation, protein samples (40µg) were denatured in Laemmli sample buffer with 5 per cent mercaptoethanol and a gel electrophoresis was performed and denatured protein sample (40 µg) was loaded to each well in 4 per cent (v/v) stacking gel and 8 per cent (v/v) separating or resolving gel in sodium dodecyl sulphate–polyacrylamide gel for electrophoresis (SDS-PAGE) (Laemmli, 1970). The gel was stained with silver staining and the molecular weight of milk clotting protease of *Carica papaya* was estimated using the protein standards (GeNei™): Myosin, rabbit muscle (205.0kD) phosphorylase b (97.4 kD), bovine serum albumin (66.0 kD), ovalbumin (43.0 kD), carbonic anhydrase (29.0 kD), soyabean trypsin inhibitor (20.1 kD), lysozyme (14.3 kD), aprotonin (6.5kD) and insulin (α and β chains) is of (3.0 kD).

■ RESEARCH FINDINGS AND DISCUSSION

The experimental findings obtained from the present

study have been discussed in following heads:

Milk clotting activity from selected plant sources :

The aim of this work was to assess the milk clotting activity from three selected plant sources *i.e. Carica papaya*, *Euphorbia splendens* and *Musa paradasica*. The milk clotting activity, protein content, specific activity and total activity of the three plant extracts are presented in Table 1. It is well known to all of us that all proteolytic enzymes clot milk but important is to determine, which plant latex or extract is more suitable for cheese making that could be used for commercial applications. Results showed that *Carica papaya* plant latex had the maximum milk clotting activity as well as protein content than *Musa paradasica* and minimum in *Euphorbia splendens* (p =0.01). Similar higher milk clotting activities were shown from the extracts of *Carica papaya* leaves (Dahot *et al*., 1990) and unripe fruit latex (Badgujar and Mahajan, 2009). The specific activity of *Carica papaya* plant latex (220.59±3.55 U/mg) (p =0.01) can be compared with crude enzyme extract of *Solanum elaegaifolium*, *Solanum dubium* and *Asclepias curassavica* L. which was 15.63 MCU/mg, 128.44 U/mg and 1.0586 U/mg, respectively. The total activity of *Carica papaya* plant latex (801.40±6.47 U) (p =0.01) can also be compared with crude enzyme of *Solanum dubium* and *Asclepias curassavica* L. which was 1650.5 U and 1.480 U, respectively. These results showed that crude latex of *Carica papaya* with further purification could be a potent source of milk clotting activity. Further, the milk clotting activity was also assessed by altering the concentration of crude plant latex or extract of *Carica papaya*, *Musa paradasica* and *Euphorbia splendens*. It was observed that the milk clotting activity of crude plant latex also decreases on dilution of crude latex by the addition of water.

where,

$$\text{Milk clotting activity (U/ml)} = \frac{\text{v x 2400}}{\text{v x t}}$$

V = volume of milk in ml

t = clotting time in seconds

v = volume of enzymatic extract in ml.

Electrophoresis of *Carica papaya* :

SDS-PAGE analysis of *Carica papaya* showed 5 distinct bands of different intensities ranging from 14.3 KD to 97.4 KD (Fig. 1). In addition, some light intensity protein bands were also found. Some of the bands observed in *Carica papaya* had similar molecular weights as reported for asparatic proteinases such as chymosin which is of 33kD.These results were in agreement with the study of Nitsawang *et al.* (2006) where they also detected five bands while purifying the papain enzyme from *Carica papaya*. Our results were also comparable with the religiosin a milk clotting serine protease from the latex of *Ficus reliogiosa* (Kumari *et al.*, 2010). The electropherogram profile showed one single band of religiosin under reducing and non-reducing condition of molecular weight of 43 KD which is also present in crude latex of *Carica papaya* in the present study. These results can be compared with the study of *Jacartia corumbensis* O. Krutze a new vegetable source for milk clotting enzymes reported by Duarte *et al.* in 2009 which reported a protein band with molecular weight of approximately 33 KD. Other works showed the presence of approximately 23.5 KD of protein band in the latex of *Asclepias currassavica* by Liggieri *et al.*, 2009 and supports the present findings. These results suggest that crude enzyme solution of *Carica papaya* may be an interesting source of milk clotting enzyme which may further be useful in cheese manufacturing.

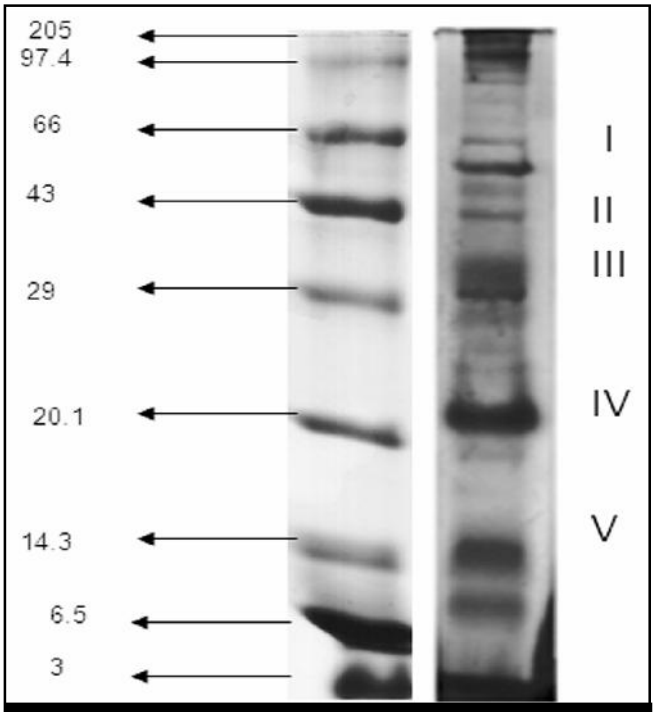

Fig. 1 : SDS-Page electropherogram of *Carica papaya* plant extract

Table 1: Milk clotting activities of selected plant latex (given in means ± S.D. (n=5)

Name of plant	Volume of latex used (ml)	Time (sec)	Volume of milk (ml)	Milk clotting activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	Total activity (U)
Carica Papaya	0.4	3± 0.79 [c]	1.0	2006.20 ± 3.77[a]	9.24 ± 0.19 [a]	220.59 ± 3.55 [a]	801.40± 6.47 [a]
Musa Paradasica	1.1	12 ± 0.79[b]	1 .0	187.19 ± 4.44[b]	0.84 ± 0.02 [b]	210.08± 3.68 [b]	394.60 ± 4.56 [b]
Euphorbia Splendens	1.2	20 ± 0.79[a]	1 .0	105.20 ± 4.15[c]	0.49 ± 0.04 [c]	187.23 ± 4.65 [c]	120.20 ± 3.49 [c]

a-c: Values in a column are significantly different at p? 0.01

Conclusion :

In the present study, three plants namely, *Carica papaya*, *Euphorbia splendens* and *Musa paradasica* were used for study. The results of this study showed that *Carica papaya* plant latex had maximum milk clotting activity as well as protein content than *Musa paradasica* and minimum in *Euphorbia splendens*. Electrophoresis assessment by SDS-PAGE was only done for *Carica papaya* which showed the highest milk clotting activity. Through SDS-PAGE it was shown that 5 bands of different intensities were seen between 14.3 KD to 97 KD along with some light bands with molecular weight marker and some of the bands were approximately similar to chymosin molecular weight.

However, it is not clear from the study that the coagulation of milk by latex as a source of milk clotting enzyme is due to single action of enzyme or may be due to the synergistic action of several enzymes. The proteinases used for the study belongs to which group *i.e.* cysteine, serine, asparatic, metalloprotein or threonine proteinases is not clear from this study. Further studies on characterization and properties of purified enzymes and the mechanism of milk clotting activity will clear the above point and if required with certain modification can be later used in cheese production.

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