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Original Article

Report on Biotechnological Applications of Proteolytic Enzymes from Lattices of Euphorbian Plants

Raghunath Mahajan[1*] Gunjan Chaudhari[2], Manoj Chopadaa[1]

Abstract

Proteases catalyze the hydrolysis of peptide bonds in proteins. They are widely distributed in nature, nearly in all plants, animals and microorganisms. Plant latex is a rich source of proteases. Latex production is unique property of Euphorbian plant, which could be a potential source of proteolytic enzymes. The highest proteolytic activity was observed in the latex of *Pedilanthus tithymaloides* Linn., which is followed by lattices of *Euphorbia tirucalli* Linn., *Euphorbia nivulia Buch.-Ham*., and *Euphorbia nerifolia* Linn. Therefore, *P. tithymaloides*, *E. tirucalli*, *E. nivulia*, and *E. nerifolia* lattices were selected as potential sources of proteases. These proteases were effectively used to remove hair from goat skin indicating its potential in leather processing industry. These proteolytic enzymes showed potential environmental waste management applications such as degradation of chicken feather waste. Additionally, the crude enzymes of the selected plants exhibited potent gelatinolytic activity almost correlated to release of silver from waste X–ray film. They also showed remarkable destaining property, indicating their importance in detergent industry. These enzymes have remarkable silk degumming property demonstrating their use in textile industries.

Keywords: Plant Protease, Latex, Euphorbiaceae, Biotechnological Applications

1. Department of Zoology, Moolji Jaitha College, Jalgaon, India
2. Department of Biochemistry, Moolji Jaitha College, Jalgaon, India

*** Corresponding Author**
Raghunath T Mahajan
Department of Zoology, Moolji Jaitha College, Jalgaon , India
E-mail: rt_mahajan@rediffmail.com

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Introduction

Proteases refer to a group of enzymes whose catalytic function is hydrolysing peptide bonds of proteins. They are also called proteolytic enzymes or proteinases. Proteases form a large group of enzymes belonging to the class of hydrolases. They are ubiquitous in nature and perform an important role with respect to their applications in both physiological and commercial fields. The mostly used industrial enzymes today are proteases, carbohydrate-hydrolyzing enzymes, and ester cleavage fat hydrolyzing enzymes. The specific applications of such technical enzymes are in major areas of food processing, beverage production, animal nutrition, leather, paper and pulp, textiles, detergents, etc. Proteolytic enzymes account for nearly 60% of the industrial enzyme market and are widely used in food industry iccluding cheese ripening, meat tendering, production of protein hydrolysate, and bread making [1] and with the advent of new frontiers in biotechnology, the spectrum of amylase and protease application has been expanded into many new fields, such as clinical, medicinal, and analytical chemistry [2].

The characteristic property of each protease through irreversible cleavage is crucial for functional activation of proteins that are involved in blood coagulation, fibrinolysis, complement activation, and protein digestion in biological system; therefore, they have become the focus of wide range of industrial and medical applications [3]. Enzymes, especially proteases, have become an important and indispensable part of the processes used in many applications by the modern food and feed industries to produce a large and diversified range of products for human and animal consumption. Enzyme technology has evolved to become an integral part of the food industry [4]. Proteases have potential applications in food, dairy, detergent, leather, alcoholic beverages, brewing, meat, pharmaceutical, and photographic industries [5, 6].

Although proteases, used in industry have been obtained mostly from microorganisms, the unique substrate specificity of plant proteases is absent in microbial or mammalian systems. Due to the nature of enzyme isolation and purification, many commercial enzyme preparations usually contain some unwanted side activities. However, plant based enzymes tend to be naturally free of unwanted side enzyme activities due to their unique specificity [7]. This factor makes the plant resources a valuable enzyme source serving increasingly significant roles in our processing systems. For instance, industrial utilization of cysteine protease family has received increasing interest due to a better understanding of their role in numerous important physiological and developmental processes. These natural processes are mimicked *in vitro* and explored for industrial application [8]. Even though there is a vast information on plant protease, majority of these proteases have been subjected for isolation to homogeneity and characterized. Some of them are Millin (*Euphorbia milli* Des Moul.), Papain (*Carica papaya* Lynn), Euphorbain (*Euphorbia lathyris* Willd), Curcain (*Jatropa curcas* Lynn), Calatropin (*Calatropis gigantean* Robert brown), and Pedillanthin (*Pedilanthus tithymaloides*). However, data for the comparative evaluation of biotechnological and industrial applications of Euphorbiaceae family is the prevailing re-

search gap and this necessitates the study. The aim of the present study is to compare proteases of some members of Euphorbiaceae family for their industrial applications such as bioprocessing of used X-ray film, dehairing, destaining, and silk degumming.

Materials and Methods

The lattices of latex bearing medicinal plants of Euphorbiaceae family mainly *Euphorbia tirucalli*, *Euphorbia nivulia*, *Euphorbia nerifolia* and *Pedilanthus tithymaloides* were collected early in the morning by superficial incisions of stem, or trunk of healthy plant and allowing the milky latex to drain in clean glass vials separately, brought to the laboratory and store at 4°C. All the chemicals used in the study were analytical grade from Fisher Scientific Company and Hammerstein casein from Hi Media, Mumbai, India.

Preparation of crude enzyme

All operations were carried out at 0-5ºC. The plant part e.g. leaf, stem, fruit and latex was homogenized in homogenizer under chilled condition and filtered successively through four folds of muslin cloth and Whatman filter paper No. 1. Filtrate called "Crude enzyme", was used for further investigation of Proteolytic activity.

Protein Estimation

Protein concentration in the enzyme extract was determined using Folin Ciocalteu reagent as per the procedure of Lowry *et al*., 1951 [9], Crystalline Bovine Serum Albumin used as standard protein for preparation of standard curve. The different aliquots of protein standard allowed reacting with Folin phenol reagent. The absorption of the developed blue color was measured at 660 nm using a spectrophotometer.

Protease Assay

The protease activity was assayed as described by Badgujar and Mahajan 2011 [10]. One ml of crude enzyme was incubated with 2 ml of substrate (1 % casein) in presence of 0.01 M phosphate buffer (pH 7.0) for 60 minute at 37°C. The residual protein precipitated by adding 5% ice chilled trichloroacetic acid. The precipitates were allowed to settle for 30 minutes at room temperature. The content of tube was filtered through Whatman filter paper no. 1. After filtration, 1 ml aliquot of the diluted filtrate was mixed with 2.5 ml 15% sodium carbonate. Then 0.5 ml of diluted Folin Ciocaltenu reagent was added and the contents were mixed. The produced blue colour was read by a spectrophotometrically (Shimadzu, Japan) at 650 nm exactly after 15 minutes. The protease activity was expressed as µg of tyrosine equivalents liberated/minutes/mg protein under standard conditions.

Blood stain removal

A clean piece of cloth was soaked in blood and allowed to dry the blood cloth. The cloth was cut to equal sizes and they were incubated with the crude protease (5 U) at 45-50ºC for different incubation time. After incubation, each piece was rinsed with water for 2 min and then dried. The same procedure was done for the control, except incubation with the enzyme solution.

Application of crude enzyme (5 U mg-1 of protein) in 0.02 M phosphate buffer pH 7.4 as a detergent additive was studied on white cotton cloth pieces (1.5" × 1.5") stained with blood sample [11]. The stained cloth pieces were taken in separate trays. The following groups were set up:

I. Tray with 50 ml of 0.02M phosphate buffer pH 7.4 and blood stained cloth
II. Tray with 50 ml of crude enzyme in 0.02M phosphate buffer pH 7.4 and blood stained cloth
III. Tray with 50 ml of detergent (7 mg/ml) and blood stained cloth
IV. Tray with 50 ml of detergent (7 mg/ml) and crude enzyme and blood stained cloth

These trays were incubated at 30ºC for 25 min. The cloth pieces were taken out from each set at regular intervals of 5 min, rinsed with water, dried and visually examined. The untreated cloth pieces stained with blood were taken as control. Additionally, after washing, dried cotton pieces were subjected for cutting. The resulting little pieces of individual destained cotton cloth pieces were suspended in a saline at 30°C and centrifuged at 10000 rpm for 20 min. The Progress of destaining of blood stain was monitored by measuring the absorbance of resulting supernatant at 420 nm. The destaining ability of crude protease was confirmed in triplicate experiment.

Dehairing studies

We adopted the method described by Badgujar and Mahajan, 2013, for evaluation of dehairing activity [12]. The fresh fleshed goat skin was washed with a commercial detergent and cut into 2 × 2 cm pieces. Eight to twelve grams of the skin (usually two to three pieces) was processed in a flask with crude protease enzyme and 0.01 M phosphate buffer (control) in a proportion of 5.0 mL of liquid enzyme per g of skin. At the end of the process, the skin pieces were gently scraped with fingers to remove loose hairs. This procedure was necessary because rubbing in this laboratory-scale process was not as vigorous as in industrial drums. The skin depilation started with 5.0 U/ mg of protein and it was completed during 18 h at pH 7.0 and temperature 30°C.

Silver release from X ray film

The used x-ray film degradation assay was performed according to standard method [13]. The used x-ray films were washed with distilled water and wiped with cotton impregnated with ethanol. The films were dried in an oven at 50ºC for 30 min. Ten grams of X-ray film (cut into 2 × 2 cm^2 pieces) was then incubated with 100 ml of diluted protease of lattices of four selected plants in 0.02M phosphate buffer pH 7.4 (such that the film is completely submerged in the enzyme) at 30°C with continuous shaking until the gelatin-silver layer was stripped completely. The obtained slurry was washed 2-3 times and filtered to remove the gelatin from the slurry and then dried. The dried slurry was smelted in the presence of Na_2CO_3 and hard coked at 900°C in a furnace. The silver settles and the waste burn out.

Feather degradation

Chicken feather degradation assay using crude enzyme of latex of four selected plants was carried out using standard method [14]. Feathers were collected from chicken shop and then washed properly by tap water and allowed to dry

and cut into small pieces. Conical flask was taken and 100 mg of feathers where added to each flask along with 100 ml crude enzyme diluted in Phosphate buffer (0.1 M with pH7.4). Papain was taken as standard and four plants crude enzymes were taken as test samples and allowed to incubate for 24 hours on rotary shaker at 100 rpm. After that, all samples were filtered and allowed to dry and then weighed. The amount of degraded feathers was measured on the basis of decreased weight of the samples.

Silk degumming

The role of crude enzyme of latex of four selected plants in order to remove sericin from the silk fibers was studied according to the method described by Nakpathom *et al*., 2009 [15]. The different concentration of crude protease of lattices of four selected plants were added to 0.1 mg of silk fiber for degumming at room temperature for 24 h. Degummed silks were then washed with hot and cold distilled water and finally air dried. The percent weight loss of the silk fibers after degumming process was calculated from following equation:

$$\%\, Weight\ loss = \frac{W_0 - W_t}{W_0} \times 100$$

Where, W0 and Wt are the weight of the silk fiber samples before and after degumming, respectively.

Results and Discussion

Proteolytic activity

In our laboratory, we screened twenty plants belonging to Euphorbiaceae family for their proteolytic activity using casein as a substrate. Among all selected laticiferous plants, latex of *P. tithymaloides* has the highest caseinolytic activity followed by *E. tirucalli*, *E. nivulia* and *E. nerifolia* 9.0, 6.42, 4.56 and 4.38 U/mg of protein, respectively. The remaining plants also showed proteolytic activity, however, it was comparatively smal (Data published in proceedings ECUBE-2016) [22].

Destaining efficacy

Investigations have been carried out on destaining efficacy of crude enzymes of four different laticiferous plants of *Euphorbiaceae* family viz. *E. tirucalli*, *E. nivulia*, *E. nerifolia*, *P. tithymaloides*. The highest destaining efficacy was observed by crude enzyme of *E. nivulia* followed by *E. nerifolia*, *E. tirucalli* and the crude enzyme of *P. tithymaloides* showed lesser destaining performance (Figure 1). These destaining profiles made clear the idea about removal of blood stains with minimum use of commercial detergent within 25 min [12]. Our results of washing performance of crude protease of lattices of four selected plants are in good accordance with the earlier observations reported in washing performance of protease of Bacillus subtilis [16], *Calotropis gigantea* (L.) latex [17], *Bacillus sp*. JB-99 [18] and *Euphorbia nivulia* Buch-Ham. [12].

Dehairing activity

Enzymatic dehairing process is getting importance as an alternative to chemical methodology in leather processing. This process is significant in reduction of toxicity in addition to improvement of leather quality [19].

Higher dehairing activity observed in the case of standard papain enzyme in comparison to the selected Euphorbiaceae members. The crude enzyme of *E. nerifolia* showed considerable dehairing activity followed by *E. tirucalli*, *E. nivulia* and then *P. tithymaloides*. In the case of negative control, no hair removal was observed as shown in Figure 2. Similar results are noticed by Rao *et al*., with thermostable protease of *Bacillus circulans* [20]. Our results are in accordance with the findings of dehairing property of cysteine proteases of *Euphorbia nivulia* latex in rat skin [12].

Silver release

The treatment of plant lattices with used X- ray film result in hydrolysis of gelatin layer and thus colour of film changes from black to blue (Figure 3). The release of silver particles from X- ray film was determined by titration. Among the selected plant lattices, crude enzyme of *P. tithymaloides* latex exhibited highest silver release followed by *E. tirucalli*, *E. nivulia* and *E. nerifolia* (Figure 4).

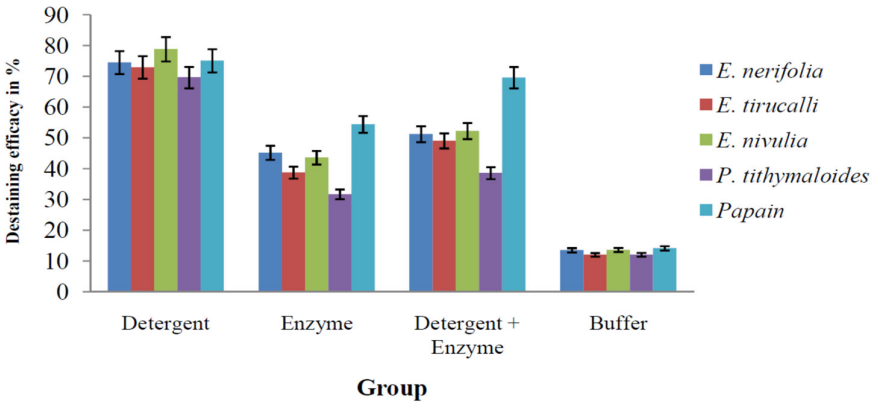

Figure 1. Destaining efficacy of crude enzymes of laticiferrous plants.

In this study Papain was used as standard and exhibited silver release about 54.00 mg/10 g of X-ray film. Phosphate buffer saline was used as negative control and did not release silver. Our findings are in accordance with the work carried out by Shankar et al., 2010 using alkaline protease from *Conidiobolus coronatus* protease [13].

Figure 2. Dehairing activity of latex of some members of Euphorbiaceae family.

Feather Degradation

The degradation of feathers with keratinolytic enzymes is the best eco-friendly approach in poultry waste management. Crude enzyme of *E. nerifolia* shows the highest activity followed by *E. tirucalli*, E. nivulia and *P. tithymaloides* but not higher than Industrial Papain (Table 1) which is in accordance with the results of keratinolytic activity, it is observed the highest in *E. nerifolia* and the lowest in *P. tithymaloides*. Similar results are noticed by Kumar et al., with bacterial isolate *Bacillus altitudinis* GVC [21].

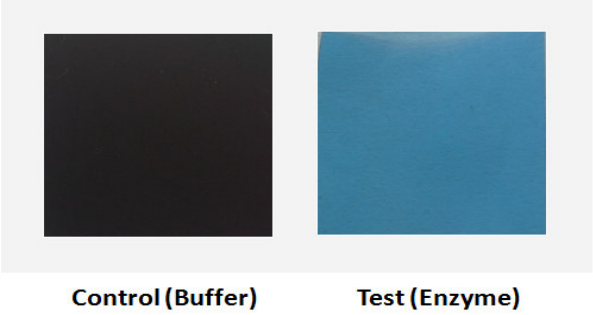

Figure 3. Gelatin hydrolysis of waste X-ray films by protease of laticiferrrous plants.

Silk Degumming

The highest degumming avtivity of raw silk fiber was observed using crude enzyme of *E. nerifolia* latex, followed by 10% papain, *E. nivulia*, *E. tirucalli*, alkali, 5% papain and *P. tithymaloides* (Table 2). For the direct dye staining test, raw silk was stained dark red color as a result of a large quantity of sericin present in the fibers. Degummed silk sample which appeared pale pink, indicate that there was a small amount of sericin remaining. Moreover, dull and stiff handle of raw silk fibers disappeared and the degummed silk became shinier and softer.

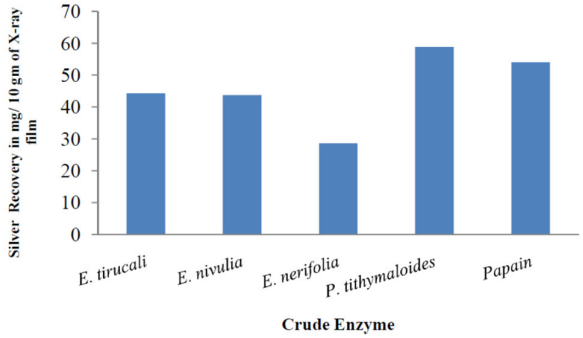

Figure 4. Release of silver by crude enzymes of laticiferrous plants.

Table 1. Feather Degradation Potential of Plant Crude Enzymes.

No.	Plant Name	Feather degradation (%)
1	Buffer	-
2	*E. tirucalli*	5 ±0.34
3	*E. nivulia*	4.2 ±2.91
4	*E. nerifolia*	15.06 ±4.46
5	*P. tithymaloides*	3.53 ±0.30
6	Papain	24.43 ±5.75

Table 2. Silk Degumming Potential of Plant protease.

No.	Degumming method	Weight loss (%)
1	Alkaline bath	9.3 ±0.02
2	5% papain	7.55 ±0.36
3	10% papain	13.75 ± 0.14
4	*E. tirucalli*	10.95 ± 0.72
5	*E. nivulia*	12.3 ± 0.29
6	*E. nerifolia*	15.1 ± 0.43
7	*P. tithymaloides*	3.8 ± 0.09
8	Control	ND

Conclusion

In conclusion, characterization and environmental friendly potential application of protease of Euphorbian plant latex were studied for the first time. Studies indicated its utility for blood stain removal and dehairing properties. This study also demonstrates that these proteolytic enzymes have the potential of being applied for reusing of silver from used x-ray films in an eco-friendly manner. This work also showed that the proteases of selected Euphorbian plants could be used in detergent, leather processing, and textile industries.

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References

1. Poldermans B., Proteolytic Enzymes. *In: Proteolytic Enzymes in Industry: Production and Applications,* W. Gerhartz (Ed.), VCH Publishers, Weinheim, Germany (1990) pp. 108–123.
2. Negi S., Banerjee R., Optimization of Amylase and Protease Production from *Aspergillus awamori* in Single Bioreactor Through EVOP Factorial Design Technique. *Food Technol Biotechnol*, 2006, Vol. 44 (2), pp. 257–261.
3. Dayanand C. D., Evaluation of comparative total Proteolytic activity in plant lattices. *Int J Life Sci Biotech Pharm Res,* 2013, Vol. 2(1), pp. 47-55.
4. Demir, N., Bahçeci, K. S., Acar, J., The effect of processing method on the characteristics of carrot juice. *J of Food Quality*, 2007, Vol. 30(5), pp. 813-822.
5. Mahajan, R. T., Badgujar, S. B., Biological aspects of proteolytic enzymes: A Review. *J Pharm Res*, 2010, Vol. 3(9), pp. 2048-2068.
6. Mahajan, R. T., Chaudhari, G. M., Plant latex as vegetable source for milk clotting enzymes and their use in cheese preparation, *Int J Adv Res*, 2014, Vol. 2(5), pp. 1173-1181.
7. Gary D. J., Weiping J., Characterization of cathepsin L secreted by Sf21 insect cells, *Arch Biochem Biophys*, 2005, Vol. 444(1), pp. 7-14.
8. Bassa O. Y., Isolation, Partial Purification and Characterization of a Protease from Ginger *Zingiberaceae officinale* Roscoe, A research project, Department of Biochemistry, Ahmadu Bello University Zaria, Nigeria. July 2011.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J., Protein measurement with the Folin phenol reagent. *J biol Chem*, 1951, Vol. 193(1), pp. 265-275.
10. Badgujar S. B. Mahajan R. T., Proteolytic enzymes of some latex bearing plants belonging to Khandesh region of Maharashtra. Ph D. Thesis, North Maharashtra University, Jalgaon 2011, pp 108-109.
11. Vishalakshi, N., Lingappa, K., Amena, S., Prabhakar, M., Dayanand, A., Production of alkaline protease from *Streptomyces gulbargensis* and its application in removal of blood stains. *Indian J Biotechnol*, 2009, Vol. 8(3), pp. 280-285.
12. Badgujar, S. B., Mahajan, R. T., Characterization of thermo- and detergent stable antigenic glycosylated cysteine protease of *Euphorbia nivulia* Buch.-Ham. and evaluation of its ecofriendly applications. *Sci World J*, 2013.
13. Shankar, S., More, S. V., Laxman, R. S., Recovery of silver from waste X-ray film by alkaline protease from *Conidiobolus coronatus*. *Kathmandu Univ J Sci Eng Techno*, 2010, Vol. 6(1), pp. 60-69.
14. Tiwary E., Gupta, R., Rapid Conversion of Chicken Feather to Feather Meal Using Dimeric Keratinase from *Bacillus licheniformis* ER-15. *J Bioprocess Biotech*, 2012, Vol. 2, pp.123-128.
15. Nakpathom, M., Somboon, B., Narumol, N., Papain enzymatic degumming of Thai *Bombyx mori* silk fibers. *J Microsc Soci Thailand*, 2009, Vol. 23(1), pp. 142-146.
16. Kumar, M. D. J., Rameetha, R., Lincy, L., Priyadarshini, S., Sandhiyachittybabu, Kalaichelvan, P.T., Destaining and dehairing capability of partially purified *Bacillus subtilis* protease from optimized fermentation medium. *Asian J Exp Biol Sci*, 2012, Vol. 3, pp. 613- 620.
17. Bindhu, O. S., Singh, M. K. Hemostatic, milk clotting and blood stain removal potential of cysteine proteases from *Calotropis gigantea* (L.) R. Br. Latex. *Pharmacognosy magazine*, 2014, Vol. 10, pp. 350-355.
18. Shivasharana, C. T., Naik, G. R. Ecofriendly applications of thermostable alkaline protease produced from a *Bacillus sp.* JB-99 under Solid State Fermentation. *Int J Environ Sci*, 2012, Vol. 3, pp. 956-964.
19. Sivasubramanian, S., Manohar, B. M., Rajaram, A., Puvanakrishnan, R., Ecofriendly lime and sulfide free enzymatic dehairing of skins and hides using a bacterial alkaline protease. *Chemosphere*, 2008, Vol. 70(6), pp.1015-1024.
20. Rao, C. S., Sathish, T., Ravichandra, P., Prakasham, R. S., Characterization of thermo-and detergent stable serine protease from isolated *Bacillus circulans* and evaluation of eco-friendly applications. *Process Biochem*, 2009, Vol. 44(3), pp. 262-268.
21. Kumar, E. V., Srijana, M., Chaitanya, K., Reddy, Y.H.K., Reddy, G., Biodegradation of poultry feathers by a novel bacterial isolate *Bacillus altitudinis* GVC 11. *Indian J Biotechnol*, 2011, Vol.10 (4), pp. 502-507.
22. Mahajan R. T., Chaudhari G. M. and Chopada M. Z., Comparative proteolytic activity of some members of Euphorbiaceae family. *Proceedings ECUBE-2016*.