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Proteolytic Enzymes of Some Laticiferous Plants Belonging to Khandesh Region of Maharashtra, India

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ABSTRACT

To identify the potent source of protease that can be used as ideal milk clotting enzyme(s) in handmade dairy products of latex obtained from twenty one plant species belonging to Northern (Khandesh) region of Maharashtra, India, was analyzed for caseinolytic activity, milk clotting activity, gelatinolytic activity and peroxidase activity. Highest caseinolytic activity was noted in latex of *Pedilanthus tithymaloides* (L.) Poit., followed by *Ficus carica* L., *Calotropis gigantea* (L.) R. Br. and *Carica papaya* L. Moderate caseinolytic activity was seen in *Euphorbia milii* Desmoul., *Ficus hispida* L.f. and *Calotropis procera* (Ait.) R. Br., and remaining plants possess less activity. Milk clotting activity was found highest in stem latex of *Euphorbia nivulia* L., followed by *Carica papaya* L., *Ficus carica* L., *Calotropis procera* (Ait.) R. Br., and *Tabernaemontana divaricata* (L.) R. Br. Six plants showed moderate milk clotting activity, however, such activity was not noticed in remaining six plants. Highest gelatinolytic activity was found in the latex of twelve plants, lowest in four plants and remaining plants were devoid of such activity. The result of present study reveals some promising identification of few laticiferous plant species for their possible proteolytic activity. A comparative account on enzymes viz., caseinolytic activity, milk clotting activity, gelatinolytic activity and peroxidase activity are discussed in the present research.

Keywords: Proteolytic activity, Milk coagulation, Gelatination, Laticiferous plants, *Euphorbia nivulia*

INTRODUCTION

Protease refers to a group of enzymes whose catalytic function is to hydrolyze peptide bond of proteins i.e. proteases conduct proteolysis and they begin protein catabolism. Proteases are also called as proteolytic enzyme or proteinases, belong to hydrolase class of enzyme and subclass is 3.4. Proteinases constitute a large family and divided as endopeptidases and exopeptidase on the basis of cleavage site at which they breakdown peptide chain. Endopeptidases are categorized according to the reactive groups at the active site involved in catalysis viz., serine proteinase, cysteine proteinase, aspartic proteinase and metalloproteinase (Hartley, 1960 and Barrett, 1994) and threonine protease is the most recently discovered enzyme (Seemuller et al., 1995). Proteases are also classified on the basis of origin such as animal protease, plant protease and microbial protease (Neurath, 1989). It may be divided as extracellular or intracellular proteinase on the basis of their action. The extracellular proteinase includes pepsin (active in acid medium), trypsin and chymotrypsin (more functional in neutral or slightly alkaline medium) and some microbial protease. Animal origin proteases belong to intracellular proteinase, called as cathepsins, found in every kind of tissue and organs (powerful in acidic medium). The extracellular proteinases also include plant origin proteolytic enzymes including papain extracted

from *Carica papaya* (Awoyinka and Shokunbi, 2005), ficin from *Ficus* species (Pant and Srivastava, 1966), bromelin (from *Ananas comosus*), most fungal and bacterial protease. The present paper is focused on experimental studies performed on enzymatic assay of plant latex, also include the experimental procedures on protease assay, which refers to a group of enzymes regarding caseinolytic activity (Casein digestion method), milk clotting activity (Milk coagulation), gelatinolytic activity (Gelatin digestion method) and peroxidase activity.

Materials and methods

Plant material

All laticiferous plants were collected in rainy season from Khandesh region of Maharashtra and the corresponding voucher specimen were deposited in the Department of Zoology, Moolji Jaitha College, Jalgaon 425 001, Maharashtra, India (*Alstonia scholaris* R. Br. LAT 81, *Calotropis gigantea* (L.) R.Br. LAT 82, *Calotropis procera* (Ait.) R.Br. LAT 83, *Carica papaya* L. LAT 84, *Euphorbia hirta* L. LAT 85, *Euphorbia milii* Desmoul. LAT 100, *Euphorbia nivulia* Buch.-Ham. LAT 87, *Euphorbia prunifolia* Jacq. LAT 101, *Ficus carica* L. LAT 90, *Ficus hispida* L.f. LAT 91, *Ficus racemosa* L. LAT 92, *Ficus religiosa* L. LAT 93, *Ipomoea carnea* Jacq. LAT 95, *Manilkara zapota* (L.) P. van Royen LAT 97, *Pedilanthus tithymaloides* (L.) Poit LAT 102, *Plumeria rubra* L. LAT 103, *Plumeria rubra* L. forma *acuminata* (Ait.) Santapau and Irani ex Shah LAT 104, *Synadenium grantii* Hook. F. LAT 105, *Tabernaemontana citrifolia* L. LAT 98, *Tabernaemontana divaricata* (L.) R. Br. LAT 99 and *Thevetia peruviana* (Pers.) K. Shum.

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LAT 106)

Collection of Latex

Latex samples were collected early in the morning from each plant by nipping the leaves near the stem or by incision of the trunk and branches of the plant and allowing the milk to drain in clean glass tube separately, brought to the laboratory and kept in refrigerator (till the experiment starts). Latex was homogenized in a homogenizer under chilled condition and filtered through four folds of muslein cloth. Filtrate latex sample was used for determining various enzyme activities.

Chemicals

Casein (Hammerstein grade) was purchased from Sisco Research Laboratories, Mumbai. Skimmed milk powder was obtained from Jalgaon Jilha Sahakari Dudh Utpadak Sangh Marayadit, Jalgaon, Maharashtra. Bovine albumin fraction V (powder) was purchased from Sigma Chemical Company, Mumbai. All other chemicals used are AR Grade purchased either from Qualigen fine Chemicals or E. Merck, India.

Caseinolytic activity

The hydrolyzing activity of the protease was determined by casein using the I.P. method (IP, 1985) with some alteration. Accurately weighed 0.5 g of latex sample as a source of enzyme diluted up to 10 ml by 0.01 M phosphate buffer pH 6.4. Fifteen ml of phosphate cysteine EDTA buffer pH 6.0 and 8 ml of 2 % casein solution was poured in two separate flasks. Both flasks were incubated at 60°C for 5 min. In one flask, 2 ml solution containing enzyme sample was added. To the second flask, 2 ml of the previously boiled and cooled enzyme sample was added. Both the flasks were incubated at 60°C for 30 min, gently cooled at room temperature. Then 5 ml neutral formaldehyde and 1 - 2 drops of phenolphthalein indicator was added. Both solutions were titrated with standard 0.1 N sodium hydroxide to a definite pink colour. The caseinolytic activity was expressed as a number of ml of 0.1 N sodium hydroxide solution required to neutralize the reaction mixture.

Milk clotting activity

Milk clotting activity was analyzed by using low heat skim milk powder as a substrate (Silva and Malcata, 2005). Low heat skim milk powder was dissolved in 10 mM calcium chloride at pH 6.5 to a final concentration of 0.12 Kg per lit. Enzyme sample was added in the proportion of 0.1 ml per ml of the milk. The coagulation point was determined by manual rotating of test tube periodically at short time intervals and sharp visual clot formation in milk was checked and its validity confirmed by using pointed curve needle. Milk clotting activity was defined as the amount of enzyme of plant latex that coagulates 1 ml milk at 50°C in one minute.

Gelatinolytic activity

Gelatinolytic activity was expressed by using gelatin as a substrate (Yamaguchi, 1982). Mixture of preincubated (40°C) 2 ml of 9% gelatin solution and 1 ml combination of enzyme sample of plant latex and 0.05 M phosphate buffer pH 5.9 (1:1) was kept at 40°C for 15 min and reaction mixture was cooled in ice bath (4°C) for 10 min. Then prevention of gelatination was compared with control for which distilled water was used instead of latex. Gelatinolytic activity was measured in terms of index such as gelatin was liquefied completely, con-

siderably, slightly and not liquefied.

Peroxidase activity

The contact with air and storage of plant latex undergoes oxidation so we decided to analyze peroxidase activity and it was determined using Guaiacol as substrate, according to Baaziz (Baaziz, 1989). The assay mixture consist of 3 ml of 12 mM sodium phosphate buffer (pH 6.4) and 0.05ml of 20mM hydrogen donor i.e. 2-methoxy phenol (Guaiacol). To this added 0.1 ml of enzyme (plant latex) and 0.05 ml of 1mM Hydrogen peroxide. Total volume of reaction mixture was 3.2 ml. Peroxides activity was detected by the formation of oxidised form of guaiacol with brown in colour. Peroxidase activity was measured in terms of degree such as Guaiacol was dark brown, considerably, slightly and not brown i.e. colorless.

Protein estimation

Estimation of protein was carried out by colorimetrically at 660 nm using Bovine serum albumin as the standard protein (Lowry et al., 1951).

Biostatistical analysis

Specific activity was analyzed in all latex samples of laticiferous plants for caseinolytic activity and milk clotting activity. The scale was selected for drawing figure in order to accommodate the data precisely and it was normalized for graphical presentation by converting values obtained in the scale of zero to one (Gurumani, 2005).

Results and discussion

Complete details of identified laticiferous plants with botanical name, family, vernacular name, habitat, nature and part used as a source of latex is summarized in *Table 1*. Out of twenty one laticiferous plants, leaves of ten, fruits of six, stem bark of three and pair of whole plants were used for obtaining latex. These belong to the wide group of laticiferous families viz., *Apocynaceae*, *Asclepiadaceae*, *Caricaceae*, *Euphorbiaceae*, *Moraceae*, *Convolvulaceae* and *Sapotaceae*. The result in *Table 1* and *2* summarizes all latex have caseinolytic activity and milk clotting activity as seen in 15 latex samples. Gelatinolytic activity and peroxidase activity was demonstrated in 16 and 13 plant latex respectively. Rich source of caseinolytic activity, milk clotting activity, gelatinolytic activity and peroxidase activity were found in 9, 6, 11 and 7 laticiferous plants respectively. Highest ratio of milk clotting activity to caseinolytic activity was found in the latex of *Euphorbia nivulia* followed by *Carica papaya*, *Calotropis procera* and *Tabernaemontana divaricata* i.e.50.60, 22.37, 12.59 and 10.36 respectively. The grades described in *Table 2* are designed on the basis earlier report of gelatinolytic activity (Yamaguchi et al., 1982) and for peroxidase activity (Rani et al., 2004). We adopted such grades in the present investigation. Leafy latex of *C. gigantea* and *C. procera* showed highest caseinolytic activity and this finding supports results reported by Dahot et al., 1990 and and Srivastava, 1966, their finding are confirmed by this study. The unripe fruit latex of papaya showed greater caseinolytic activity, milk clotting activity and gelatinolytic activity. Our results are good in agreement of the earlier claim made by Awoyinka and Shokunbi, 2005 and Frankel et al., 1937. Additionally peroxidase activity was also detected in some plant latex. Evidently reciprocal relationship would be established towards caseinolytic activity and peroxidase activity. Largest quantity of pro-

Table 1: Details of Laticiferous plants with their caseinolytic and milk clotting activity

Sr. No.	Botanical Name	Vernacular name and Part used*	Habitat# and Nature ^s	Protein (mg/g latex)	Caseinolytic activity (U/g latex)	Milk clotting activity (U/g latex)
01	<i>Alstonia scholaris</i> R. Br.	Satveen, LF	T, WD	4.40 ± 0.16	10.2 ± 0.09	---
02	<i>Calotropis gigantea</i> (L.) R.Br.	Ruchkin, LF	S, WD	3.10 ± 0.08	16.2 ± 0.20	5.53 ± 0.26
03	<i>Calotropis procera</i> (Ait.) R.Br.	Rui, LF	S, WD	3.23 ± 0.05	11.2 ± 0.12	141.0 ± 0.12
04	<i>Carica papaya</i> L.	Papai, FT	S, CU	19.2 ± 0.45	16.1 ± 0.12	360.2 ± 0.12
05	<i>Euphorbia hirta</i> L.	Dudhi, WP	H, WE	3.96 ± 0.17	13.3 ± 0.12	5.60 ± 0.22
06	<i>Euphorbia milii</i> Desmoul.	Christ Plant, LF	H, OR	5.33 ± 0.17	14.8 ± 0.24	60.1 ± 0.25
07	<i>Euphorbia nivulia</i> Buch.-Ham.	Sabar, SB	S, WD	6.10 ± 0.14	9.20 ± 0.08	465.5 ± 0.37
08	<i>Euphorbia prunifolia</i> Jacq.	Dudhi, WP	H, WE	7.70 ± 0.22	9.20 ± 0.08	50.1 ± 0.16
09	<i>Ficus carica</i> L.	Anjir, FT	T, WD	15.3 ± 0.33	28.1 ± 0.17	180.2 ± 0.12
10	<i>Ficus hispida</i> L.f.	Bhui-umbar, FT	T, WD	31.2 ± 0.12	13.5 ± 0.12	---
11	<i>Ficus racemosa</i> L.	Umbar, FT	T, WD	15.0 ± 0.05	19.1 ± 0.20	---
12	<i>Ficus religiosa</i> L.	Pimpal, LF	T, WD	5.30 ± 0.08	12.1 ± 0.20	10.4 ± 0.46
13	<i>Ipomoea carnea</i> Jacq.	Besharam, LF	S, WD	3.73 ± 0.20	13.1 ± 0.08	5.67 ± 0.20
14	<i>Manilkara zapota</i> (L.) P. van Royen	Chiku, FT	T, CU	9.43 ± 0.05	5.53 ± 0.27	---
15	<i>Pedilanthus tithymaloides</i> (L.) Poit	Vilayati sher, SB	H, WD	13.2 ± 0.24	52.4 ± 0.05	29.9 ± 0.20
16	<i>Plumeria rubra</i> L.	Lal chafa, LF	T, OR	9.10 ± 0.16	12.2 ± 0.23	---
17	<i>Plumeria rubra</i> L. forma <i>acuminata</i> (Ait.) Santapau and Irani ex Shah	Pandhara chafa, LF	T, OR	11.8 ± 0.37	3.07 ± 0.17	---
18	<i>Synadenium grantii</i> Hook. F.	Irhonda, SB	S, OR	3.53 ± 0.12	9.77 ± 0.40	39.9 ± 0.33
19	<i>Tabernaemontana citrifolia</i> L.	Chandani, LF	S, OR	2.23 ± 0.12	12.9 ± 0.20	55.0 ± 0.04
20	<i>Tabernaemontana divaricata</i> (L.) R. Br.	Chandani, LF	S, OR	7.26 ± 0.29	11.2 ± 0.12	116.0 ± 0.25
21	<i>Thevetia peruviana</i> (Pers.) K. Shum.	Piwali khaner, FT	S, WD	6.23 ± 0.09	26.2 ± 0.28	5.46 ± 0.33

*Part used: LF: Leaf, FT: Fruit, WP: Whole plant, SB: Stem bark; #Habitat: T: Tree, S: Shrub, H: Herb, ^sNature: WD: Wild, CU: Cultivable, WE: Weed, OR: Ornamental; Data represented in average values ± SD of n=6 experiments.

Table 2: Peroxidase and Gelatinolytic activity of Laticiferous plants

Sr. No.	Botanical Name	Peroxidase activity	Gelatinolytic activity
01	<i>Alstonia scholaris</i> R. Br.	+++	---
02	<i>Calotropis gigantea</i> (L.) R.Br.	+++	+++
03	<i>Calotropis procera</i> (Ait.) R.Br.	+++	+++
04	<i>Carica papaya</i> L.	---	+++
05	<i>Euphorbia hirta</i> L.	++	+++
06	<i>Euphorbia milii</i> Desmoul.	---	++
07	<i>Euphorbia nivulia</i> Buch.-Ham.	++	+++
08	<i>Euphorbia prunifolia</i> Jacq.	---	+++
09	<i>Ficus carica</i> L.	---	+++
10	<i>Ficus hispida</i> L.f.	+++	++
11	<i>Ficus racemosa</i> L.	+	---
12	<i>Ficus religiosa</i> L.	++	+++
13	<i>Ipomoea carnea</i> Jacq.	+++	+++
14	<i>Manilkara zapota</i> (L.) P. van Royen	---	---
15	<i>Pedilanthus tithymaloides</i> (L.) Poit	++	+++
16	<i>Plumeria rubra</i> L.	+++	---
17	<i>Plumeria rubra</i> L. forma <i>acuminata</i> (Ait.) Santapau and Irani ex Shah	---	---
18	<i>Synadenium grantii</i> Hook. F.	+	++
19	<i>Tabernaemontana citrifolia</i> L.	---	+++
20	<i>Tabernaemontana divaricata</i> (L.) R. Br.	---	+++
21	<i>Thevetia peruviana</i> (Pers.) K. Shum.	---	+

Grades: +++ Higher enzymatic activity; ++ Moderate enzymatic activity; + Less enzymatic activity; --- Absence of enzyme activity

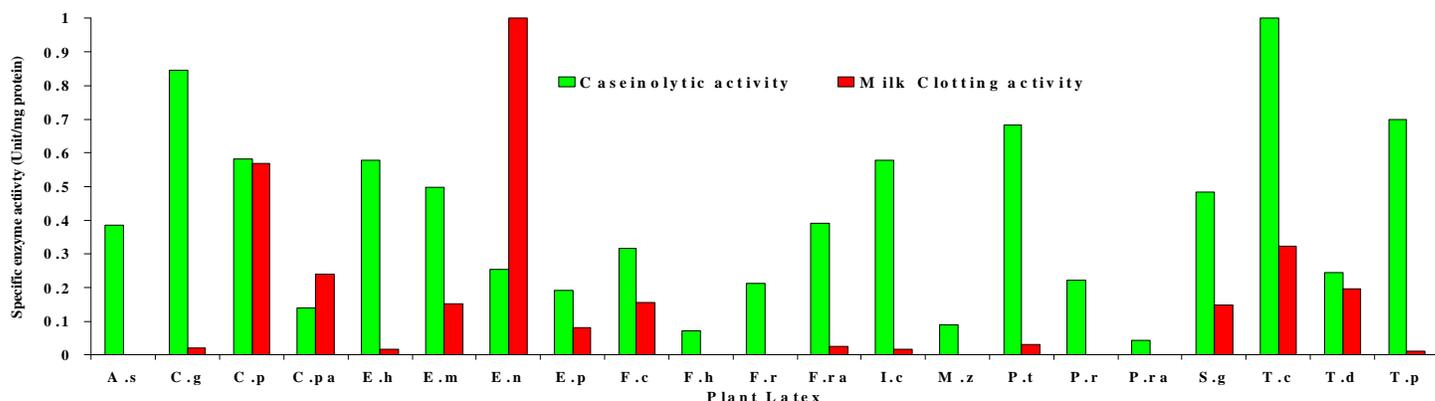


Figure 1 Specific Activity of Laticiferous Plants
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proteolytic activity warrants us further to analyze them using diverse group of substrates. Proteases of plant latex undertaken for present study belongs serine proteinase, cysteine proteinase, aspartic proteinase, metalloproteinase or threonine protease is not clear from this study. This assures to extend this by chemical analysis and electrophoretic pattern. This is very important from enzymological view point with respect to characterization, enzyme catalysis, kinetic study of enzyme etc. We think the list of laticiferous plants, presented in this study is useful to researchers and in industry regarding food, leather and detergent.

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