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Comparison of cysteine proteases of four laticiferous plants and characterization of *Euphorbia nivulia* Buch.-Ham. latex glycosylated cysteine peptidase

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Investigations have been carried out on proteolytic activities of four laticiferous plants, viz. *Calotropis procera* (Ait.) R. Br. (*Ruie*), *Carica papaya* Linn. (*Papaya*), *Euphorbia nivulia* Buch.-Ham. (*Sabar*) and *Ficus carica* Linn. (*Anjir*) using Hammerstein grade casein and skimmed milk powder. Ratio of milk clotting to proteolytic activity was highest in the latex sample of *C. papaya* followed by *F. carica*, *E. nivulia* and *C. procera*. The crude enzyme of *Sabar*, *Ruie*, *Papaya* and *Anjir* latices exhibit high proteolytic activity with in a *p*H range from 6.0-7.8, 6.5-8.0, 6.0-7.5 and 6.0-7.0, while maximum milk clotting activity with in a *p*H range from 6.0-6.5, 6.0-7.0, 6.0-6.5 and 6.5-7.0, respectively. The optimum temperature of proteolytic enzymes of plant latices were recorded over the temperature range 10-80°C at *p*H 7.0. Whereas, the optimum temperature range of milk clotting protease(s) for milk coagulation was found in between 45-55°C. Proteolytic activity of all latices was inhibited by cysteine protease inhibitors. Thus, cysteine protease is a unique property of all plant latices. This article describes comparative accounts of proteolytic activities of four plants and characterization of glycosylated cysteine protease of *E. nivulia* latex.

Keywords: Cysteine protease, *Euphorbia nivulia*, Latex, Milk clotting, Proteases, Proteolytic activity

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Introduction

Proteolytic enzymes are multifunctional enzymes that have many physiological functions in plants and animals including germination, senescence, apoptosis, complement activation, inflammation process, etc. They also have commercial importance in food, leather and textile industry. Commercially they are extremely important as more than 60% of the total enzyme market is made up of proteases[1]. In mammalian physiology, proteases play an important role in the pathophysiology of several diseases such as asthma, cancer, AIDS and wound healing process. These findings have created interest in the study of proteases. Besides the mammalian proteases, several extracellular fungal and plant proteases have also been characterized[1]. Proteolytic enzymes from plant sources have received special attention because they are active over wide ranges of temperatures and *p*H, as well as in the presence of surfactants, organic solvents and denaturing agents. This stability enables their use in processes that restrict the use of conventional enzymes for industrial applications[2]. We have already reported the proteolytic or caseinolytic, milk clotting and gelatinolytic activity in twenty one plant of seven different laticiferous families out of them, four plants, viz. *Carica papaya* Linn. (*Papaya*), *Calotropis procera* (Ait.) R. Br. (*Ruie*), *Euphorbia nivulia* Buch.-Ham. (*Sabar*) and *Ficus carica* Linn. (*Anjir*) have high potential[3]. Hence, an attempt has been made here to furnish detailed studies on hydrolytic enzyme profile of these plants.

During investigation on proteolytic enzymes of these promising plants, we have come across a lot of work, appeared in literature: (i) *Carica papaya* exhibits excellent cysteine protease enzyme, papain has been crystallized[4]. (ii) Two novel cysteine proteases of the latex of *Calotropis procera*, viz. procerain are well documented[5] and very recently, procerain B is well characterized[6]. (iii) Recently, an important milk clotting protease (with respect to its use in production of popular and traditional milk product i.e. cheese) of fig tree latex i.e. *F. carica* is well documented[7]. Very recently, couple of two group

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researchers published same report at two different places i.e. India and Germany on proteolytic activity of *E. nivulia* latex along with twenty one plants[3] and sixty four members of laticiferous plants[8]. *E. nivulia* is a wild, thorny, xerophytic, succulent plant, found in boundaries of the agricultural field and also in dry barren areas[9]. The secretion of milky juice (latex) is characteristic property of this plant. Phytochemical studies have led to the isolation of ingol diterpenes (3-acetyl-8-methoxyl-7-angolyl-12-hydroxylingol; 3, 12-diacetyl-7-hydroxy-8-methoxylingol; 3,12-diacetyl-7-angolyl-8-hydroxylingol; 3, 12-diacetyl-8-benzoylingol and 3, 12-diacetyl-7-benzoyl-8-nicotinylingol)[10] along with three macrocyclic ingol diterpenes derivatives (3, 7, 12-triacetyl-8-benzoylingol; 3, 12-diacetyl-7-angeloyl-8-methoxyingol and 7-angeloyl-12-acetyl-8-methoxyingol)[11]. A pharmacological activity study reported the efficacy of *E. nivulia* as an antioxidant and immunomodulator *in vitro* and demonstrated that the latex is cytotoxic, anti-inflammatory, wound healing, haemostatic and possesses antiproliferative activity[12]. However, the proteins and other biochemical constituents of the latex have not been investigated in detail. In view of such medicinal importance we conducted a search for biochemical constituents in the plant latex. During the course of screening for biochemical constituents, a substantial amount of proteolytic activity was observed in the latex of this plant. This manuscript describes the identification and purification, as well as the biochemical characterization, of a cysteine like protease isolated from the latex of *E. nivulia*.

Materials and Methods

Chemicals

All chemicals were of the highest purity, analytical HPLC grade purchased from Sigma Chemicals, USA; Himedia Laboratories, Mumbai; SRL Chemicals, Bangalore; Qualigen Fine Chemicals, Mumbai; Merck Chemicals, India; Bangalore Genie, India. Skimmed milk powder was thankfully received from Central Dairy, Aarey Milk Colony, Mumbai, India. Sequencing grade Hybond-PVDF membrane (Amershyam) was gifted by Department of Biochemistry, TMC, Advanced centre for Treatment, Research and Education in Cancer (ACTREC), Navi Mumbai, India.

Plant material and latex collection

The plant latex samples of *Carica papaya, Calotropis procera, Euphorbia nivulia* and *Ficus carica* were collected early in the morning by superficial incisions of stem, fruit or trunk of healthy plant and allowing the milky latex to drain in clean glass vials separately, brought to the laboratory and kept in refrigerator (till the experiment started)[9].

Preparation of crude enzyme

All operations were carried out at 0-5°C. Latex was homogenized in a homogenizer under chilled condition and filtered through four folds of muslein cloth. Filtrate latex sample was centrifuged at 15,000 rpm for 45 min at 4°C. The resulting supernatant of latex enzyme called "crude enzyme" or "centrifugal fraction", which was used for further investigation of protease enzyme assay[9].

Protease assay

Proteolytic activity was determined by the colorimetric assay using 1% casein (Hammarsten type) as a substrate as described by Khan *et al*[13]. The protease activity was expressed as amount of enzyme required to produce peptide equivalent to μg of tyrosine/min/mg protein at 37°C and protein content was measured according to Lowry's method[14] using Bovine serum albumin as the standard protein.

Substrate preferences assay

The substrate preferences were determined as proteolytic activity of crude enzyme according to method described by Patil[15] using casein, bovine serum albumin, keratin, gelatin, haemoglobin and egg albumin. Assay was made at 37°C in 0.01 M phosphate buffer (*p*H 7.0). A 2.0 ml aliquot of each substrate (2% w/v) was prepared in 0.01 M phosphate buffer (*p*H 7.0). Each substrate individually was treated with crude enzyme. The resulting reaction mixture was incubated at 37°C for 1 hour. The reaction was terminated by the addition of 3 ml of ice chilled 5% trichloroacetic acid and maintained for 30 min at room temperature, followed by filtration through Whatman filter paper number 01. Then the residual protease enzyme activity was calculated as per Khan *et al* method[13].

Milk clotting activity

The enzyme was assayed as described by Greenberg method[16] with some modification. The enzyme source (0.2 ml crude enzyme) was added to 2 ml of substrate solution (12% skim milk powder in 0.01M $CaCl_2$). The time necessary for the formation of milk clot was measured and its validity was confirmed by using pointed curve needle. Milk

clotting activity is expressed in terms of Soxhlet unit[17].

Effect of *p*H on enzyme activity

The effect of *p*H on proteolytic activity of crude enzyme was measured with casein as substrate (*p*H range 4.5-9.5) using 0.01 mM sodium salts of buffer. The buffers used were: acetate buffer (*p*H 4.5-5.5), phosphate buffer (*p*H 5.5-7.5) and carbonate buffer (*p*H 7.5-9.5). Due to precipitation of casein at *p*H 4.5, haemoglobin was used as substrate. Also, effect of *p*H on milk clotting activity was studied by the method similar to that of followed by Greenberg[7] with some modification. Twelve per cent solution of skimmed milk powder i.e. substrate was adjusted to various *p*H with the help of appropriate buffers (*p*H between 3 and 8). Milk clotting activity of crude enzyme with these substrates having different *p*H values was determined. At each *p*H, a control assay was run without enzyme and treated as blank.

Effect of temperature on enzyme activity

The effect of temperature on enzyme activity was studied by using casein. The crude enzyme was incubated at the desired temperature, in the range of 10-80°C, for 15 min in sodium phosphate buffer (*p*H 7) and an aliquot was used for the activity measurement at the respective temperature. Additionally, the milk clotting activity of crude enzyme was determined within the temperature range between 20 and 80°C. 0.2 ml aliquot of enzyme fraction was treated with the preincubated skimmed milk substrate at various temperatures for milk clotting enzyme assay. The time required for clotting of milk was noted and residual milk clotting activity was calculated according standard assay procedure as mentioned above. Suitable controls without the addition of enzyme were run simultaneously.

Effect of inhibitors on proteolytic activity

Inhibition of the hydrolysis of casein by crude enzyme was investigated using 5 mM PMSF (Phenylmethanesulfonyl fluoride), 0.01 mM pepstatin A, 5 mM 1, 10 phenathroline, 5 mM EDTA (Ethylene diammine tetra acetic acid), 5 mM IAA (Iodoacetic acid) and 5 mM $HgCl_2$. The enzyme preparation was incubated with inhibitors individually at room temperature for 60 minutes. The residual proteolytic activity against casein was determined by the standard assay procedure[13]. Controls were prepared by preincubating the enzyme fraction with the appropriate solvent used to dissolve the inhibitors. A control assay of the enzyme activity was done without inhibitors and the resulting activity was taken as 100%.

Purification of glycosylated cysteine protease of *Euphorbia nivulia* latex

Solvent precipitation

For the initial purification step, the crude enzyme was precipitated with ice chilled acetone (20-40%) at 4°C. The resulting precipitate was separated by centrifugation, washed with acetone and dissolved in 0.01 M ice chilled phosphate buffer (*p*H 6.0). This preparation, was named ‘‘partially purified preparation’’ of enzyme.

Chromatographic procedures

The partially purified preparation was applied (3 ml) on to a column (1.8 × 30 cm) of DEAE-Cellulose pre-equilibrated with 0.01 M phosphate buffer at *p*H 6.0. Elution of protein was carried out by batch wise addition of 40 ml portions of increasing molarities (0.0-0.5 M) of NaCl in 0.01 M phosphate buffer (*p*H 6.0). Effluents were collected in 5.0 ml fractions at a flow rate of 20 ml/h. The absorbance at 280 nm as well as the proteolytic activity in all fractions was tested. The fractions showing a peak activity were pooled and dialyzed using dialysis tubing with a cutoff of 12000-14000 Da against three 2-L changes of 0.01M phosphate buffer (*p*H 6.0) over 16 hours. The dialysate was centrifuged at 10000 rpm for 20 min at 4°C to remove any insoluble material and was immediately subjected to rechromatography on DEAE cellulose column of same dimension and finally eluted with a 0.2-0.3 M NaCl linear gradient in the same buffer.

Characterization of cysteine protease

Thermal stability

The thermal behaviour of the partially purified enzyme was evaluated by measuring the residual proteolytic activity at 37°C (*p*H 6.7) for 2 min after incubation of samples for 20, 40, 60, 80 and 100 min at 40-70°C.

Electrophoresis (SDS-PAGE)

Chromatographically active fraction of protease enzyme was analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with Tris-glycine buffer (*p*H 8.2) in 12% polyacrylamide gel according to Laemmli[18]. Detection was done by the Coomassie Brilliant Blue R-250 staining method

and molecular weight was estimated by comparing relative mobility of marker proteins, namely Bovine serum albumin (66 kDa), Ovalbumin (43 kDa), Carbonic anhydrase (29 kDa) and Lysozyme (14.3 kDa) (GeNei, Bangalore Genei, India). Substrate Polyacrylamide gel electrophoresis (Substrate-PAGE) was performed to detect the band with protease activity on a 4% stacking gel and 0.2% casein incorporated into 10% separating gel as per the method described by Heussen and Dowdle[19].

Carbohydrate content

Carbohydrate content of chromatographically active fraction of protease enzyme was determined by phenol sulphuric acid method using glucose as the standard sugar[20].

Glycoprotein detection

The PAS method of Kapitany and Zebrowski[21] was adopted in order to detect glycoproteins electroblotted onto a PVDF membrane. After electrotransfer, the PVDF membrane was washed in 50 mL of 12% TCA for 5 min. All the further treatments of the membrane were performed in the dark at 4°C. The membrane was treated by 50 ml of 1% (w/v) periodic acid for 15 minutes. Three washings with 75 ml of 15% acetic acid were performed for 5 min each. A volume of 50 ml of Schiff's reagent was then added for 30 min and the membrane was washed 6-8 times with 200 ml of 7.5% acetic acid for more than 60 minutes.

Results and Discussion

The total protein concentration of the crude enzymes of *E. nivulia*, *F. carica*, *C. procera* and *C. papaya* were found to be 6.10, 15.3, 3.23 and 19.2 mg/g of milky latex, respectively (Table 1). The order of potentiality with respect to proteolytic activity (PA) and milk clotting activity (MCA) of most promising (selected) plants are: *E. nivulia* > *C. papaya* > *C. procera* > *F. carica* (Table 1). The ratio of milk clotting to proteolytic activity was recorded as 107.36, 134.30, 77.51 and 147.23 for crude enzymes of *E. nivulia*, *F. carica*, *C. procera* and *C. papaya*, respectively. The ratio of milk clotting to proteolytic activities (MCA/PA) of enzyme is an important standard for replacement of calf rennet. Therefore, it must be considered MCA/PA ratio as searching calf rennet replacer[22]. Thus the higher MCA/PA ratio enzyme which has higher milk clotting activity and lower proteolytic activity is suitable for cheese making[23]. Therefore, this ratio is a key index for deciding the suitability of role of protease in traditional cheese production[24].

Crude enzyme of *E. nivulia* latex exhibited the highest substrate specificity with 2% casein at 37°C and *p*H 7.0, compared to 69.28% proteolytic activity with keratin, 52.68% activity with egg albumin, 50.00% activity with gelatin, 32.75% activity with haemoglobin and 21.68% activity with bovine serum albumin. Protease of *C. papaya* latex exhibited the highest substrate specificity with 2% casein (Fig. 1), compared to 72.34, 51.56, 58.34, 30.43 and 20.52% proteolytic activity of keratin, egg albumin, gelatin, haemoglobin and bovine serum albumin respectively. Casein was found to be most useful substrate whereas; bovine serum albumin was relatively poor substrate for proteolytic activity of protease enzyme of latices of four plants. The order of substrate preference for protease of *C. procera* is casein >

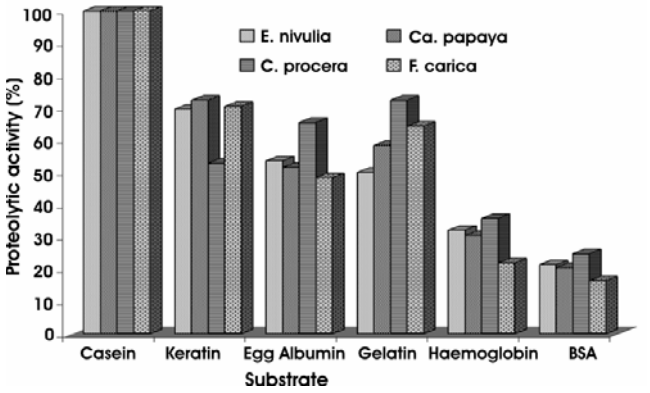

Fig. 1—Substrate specificity of the proteolytic activity of crude enzymes of selected laticiferous plants.

Table 1—Proteolytic activity of latex enzyme of some laticiferous plants

S. No.	Crude Enzyme	Total proteins (mg)	Milk clotting activity (MCA)		Proteolytic activity (PA)		MCA/PA
			TU*	SA**	TU*	SA**	
1	*Euphorbia nivulia*	6.10 ± 0.14	465.5 ± 0.37	76.38 ± 0.12	4.34 ± 0.03	0.72 ± 0.01	107.36 ± 0.68
2	*Ficus carica*	15.3 ± 0.33	180.2 ± 0.12	11.75 ± 0.04	1.34 ± 0.05	0.09 ± 0.06	134.30 ± 4.56
3	*Calotropis procera*	3.23 ± 0.05	141.0 ± 0.12	43.63 ± 0.10	1.83 ± 0.02	0.56 ± 0.08	77.51 ± 0.74
4	*Carica papaya*	19.2 ± 0.45	360.2 ± 0.12	18.69 ± 0.12	2.44 ± 0.04	0.13 ± 0.03	147.23 ± 2.25

Values are expressed as mean ± S.D., n=6 set in each group; *TU: Total Unit; **SA: Specific activity (Units/mg protein)

gelatin > egg albumin > keratin > haemoglobin > bovine serum albumin and for *F. carica* is casein > keratin > gelatin > egg albumin > hemoglobin > bovine serum albumin (Fig. 1).

The protease of *E. nivulia* (*Sabar*), *C. procera* (*Ruie*), *C. papaya* (*Papaya*) and *F. carica* (*Anjir*) latices exhibit high proteolytic activity with in a *p*H range from 6.0 to 7.8, 6.5 to 8.0, 6.0 to 7.5 and 6.0 to 7.0, while optimum activity was observed around *p*H 6.6, 7.0, 6.5 and 6.6, respectively (Fig. 2). A similar type of *p*H profile of *Papaya* latex protease (papain) was reported by Roy *et al*[25]. The resultant optimal *p*H range of *Anjir* latex protease was confirmed the reports published by Devaraj *et al*[26]. The optimal *p*H range of *Ruie* latex protease (6.5 to 8.0) is closely related with the earlier work on optimal *p*H range of Procerain and Procerain B of *C. procera*[5,6].

The hydrolytic enzyme of *Sabar*, *Papaya*, *Ruie* and *Anjir* latices exhibits high milk clotting activity with in a *p*H range from 6.0-6.5, 6.0-7.0, 6.0-6.5 and 6.5-7.0, while optimum milk clotting activity was observed around *p*H 6.3, 6.6, 6.3 and 6.8, respectively (Fig. 3). The optimum *p*H of milk clotting enzyme of *Ruie* latex was 6.3, our result is good in agreement with the earlier observations of milk clotting enzyme with optimum *p*H is 6.4 of Sodom apple i.e. *C. procera*[27].

The optimum temperature of proteolytic enzymes of *Sabar*, *Papaya*, *Ruie* and *Anjir* latices were measured by incubating with the substrate (Hammerstein grade casein) over the temperature range 10-80°C at *p*H 7.0 (Fig. 4). The protease activity (i) increased progressively as a function of temperature, (ii) was optimum at 45°C (*Sabar*), 60°C (*Papaya*), 55°C (*Ruie*) and 50°C (*Anjir*), (iii) on either side of the optimum temperature, the enzyme activity of protease enzymes of latices decreases (Fig. 4). Whereas, the optimum temperature range of milk clotting protease(s) for milk coagulation of investigated plants (*Sabar*, *Papaya*, *Ruie* and Anjir) was found in between 45-55°C. Earlier reports indicated, 55-60°C and 40-60°C is the optimal range of temperature for cysteine proteases i.e. Procerain[5] and Procerain B[6] of *Calotropis procera* latex. The optimum temperature of protease of *Anjir* latex (50°C) is closely similar with the optimal temperature range (45-55°C) of cysteine protease i.e. ficin of this latex[28]. Our result of *papaya* latex protease is justified with the earlier observation reported in the characterization of endolytic cysteine protease of *papaya* latex[25].

In order to identify the classes of protease enzymes of plant latices (*Sabar*, *Papaya*, *Ruie* and *Anjir*), the effect of different protease inhibitors have been

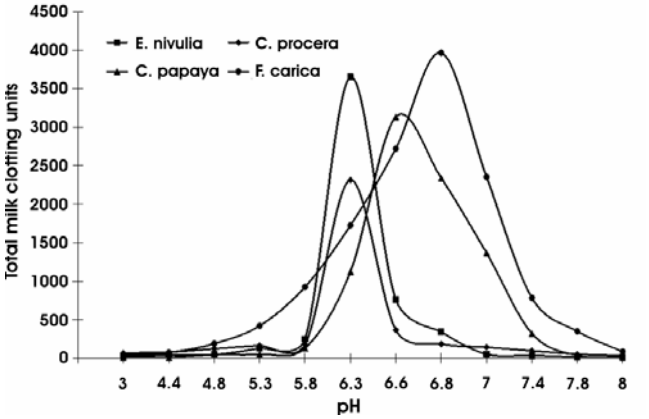

Fig.3—Effect of *p*H on milk clotting activity of latex proteases of some laticiferous plants.

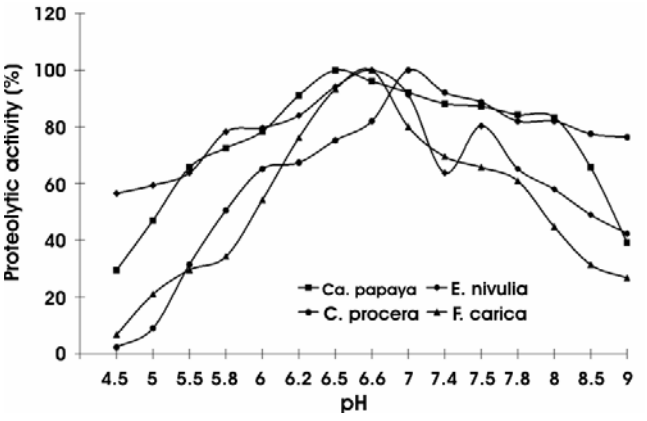

Fig. 2—Effect of *p*H on the proteolytic activity of latex proteases of some laticiferous plants.

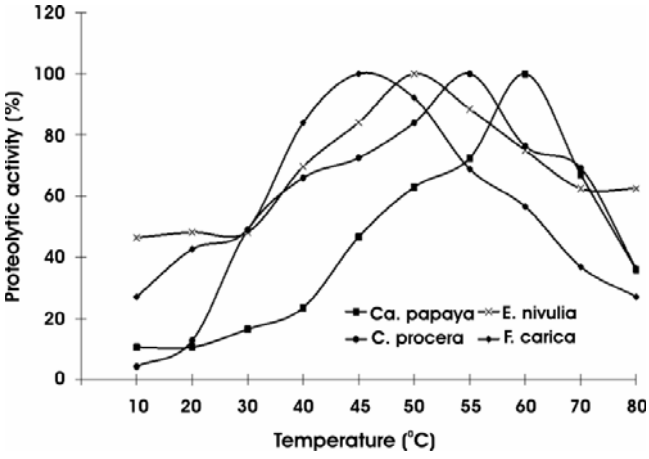

Fig. 4—Effect of temperature on the proteolytic activity of latex proteases of some laticiferous plants.

evaluated. Table 2 shows the residual activity of the protease enzymes of latices after its inhibition with the following class-specific inhibitors mercuric chloride and iodoacetic acid (inhibitors of cysteine proteases), PMSF (inhibitor of serine proteases), EDTA and phenanthroline (inhibitors of metalloproteases) and pepstatin A (inhibitor of aspartic proteases) using casein substrate. Maximum inhibition (89 to 97% inhibition) of proteolytic activity of plant latices containing enzyme occurs in presence of mercuric chloride and iodoacetic acid. Enzymes did not show significant inhibition in the presence of PMSF and Pepstatin-A. Metalloprotease inhibitors like Phenanthroline and EDTA showed no significant effect on the activity of latex enzymes ruling out the possibly of the protease enzyme of plant latices being a metalloprotein. These results confirm that the enzymes present in the latices of *Sabar*, *Papaya*, *Ruie* and *Anjir* belongs to cysteine protease family. Very recently, similar results of enzyme inhibition profile of cysteine protease of *Triticum aestivum* Linn.[29] and *Curcuma longa* Linn.[30] by iodoacetic acid are reported. Our work is very well close with the inhibition profile of Procerain B, cysteine protease of *C. procera* latex[6].

A summary of purification procedure and yield of enzyme profile of *E. nivulia* latex is presented in Table 3. The enzyme extracted with 20-40% acetone precipitation shows notable activity and hence this fraction of enzyme was subjected for chromatographic separation of protease. The unbound material to column as well as buffer wash of the column did not show any enzyme activity. The bound proteins were eluted from the column with increasing concentration of NaCl while the column elution profile resolved in to three protein peaks as shown in Fig. 5. Fractions of all the peaks were assayed for proteolytic activity. The magnitude of activity of the fractions (25-29) of peak II is higher relative to the pools from peak I and III. The dialysate of pooled fractions of peak II was used for further purification through rechromatographed on DEAE-cellulose column. The elution profile (Fig. 6) constitutes a major symmetrical peak followed by small peak

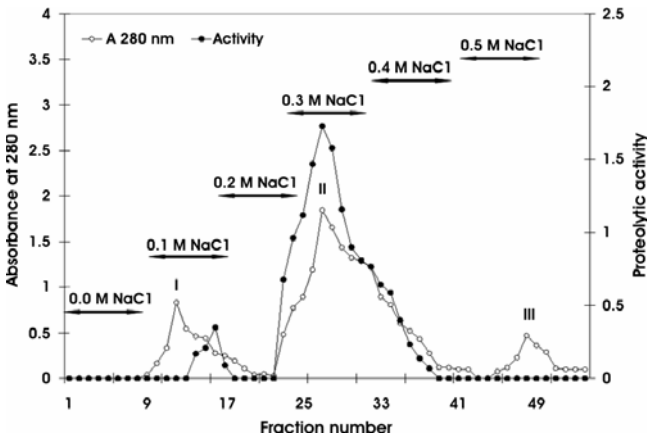

Fig. 5—Chromatography of acetone precipitate fraction of enzyme on a DEAE-cellulose column.

Table 2—Effect of inhibitors on proteolytic activity of latex enzyme of some laticiferous plants

Type of Inhibitor	Inhibitors [a]	Residual activity (%)			
		*Ruie**	*Papaya**	*Sabar**	*Anjir**
	Control [b] (W/O inhibitor)	100	100	100	100
Cysteine Protease	Iodoacetic acid[+]	8.67	8.11	15.46	10.34
	Mercuric Chloride[+]	4.37	2.67	9.48	8.61
Serine Protease	PMSF[+]	86.26	100	76.05	93.16
Metalloprotease	EDTA[+]	100	91.56	76.63	100
	Phenanthroline[+]	100	89.36	87.92	98.22
Aspartic Protease	Pepstatin A[++]	98.34	94.34	72.49	69.45

[a]Enzyme was incubated with inhibitors at 37°C for 60 min and residual activity was measured using casein as substrate; [b]The enzyme activity towards without (W/O) inhibitor was taken as 100%; [+] Concentration of inhibitors: 5 mM; [++] Concentration of inhibitor: 0.1 mM; *Botanical name of plant- Ruie: *Calotropis procera*; *Papaya*: *Carica papaya*; Sabar: *Euphorbia nivulia*; Anjir: *Ficus carica*.

Table 3—Purification scheme of cysteine protease of *Euphorbia nivulia* latex

S. No.	Step	Total protein (mg)	Total activity (Units/min)	Specific activity (Units/mg)	Yield (%)	Purification fold
01	Crude Enzyme	12.40	12.648	1.02	100	1.00
02	Acetone Precipitation	5.80	7.670	1.32	60.64	1.29
03	DEAE-Cellulose	1.405	3.183	2.26	25.16	2.22
04	Rechromatographed on DEAE-Cellulose	0.54	1.710	3.18	13.58	3.12

(fraction 31-36). The active fractions (11-15) of the former peak were pooled. SDS-PAGE electrophoresis of this pooled fractions shows two protein bands and their molecular masses are 52.96 and 43.42 kDa, out of these, 43.42 kDa protein showed notable proteolytic activity, which is confirmed by the corresponding zymogram i.e. substrate-PAGE. Zymogram shows a single band of proteolysis of casein present in separating gel (Fig. 7). These results, demonstrate the presence of two proteins in pooled fractions of symmetrical peak of rechromatography column. Interestingly, the smaller protein is proteolytically active and other protein is proteolytically inactive protein. The purified active form of protein is named as nivulian

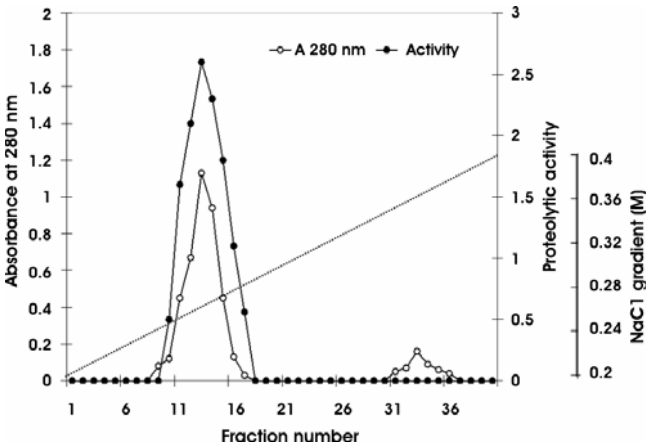

Fig. 6—Rechromatography of dialyzed fraction of enzyme on DEAE-cellulose column.

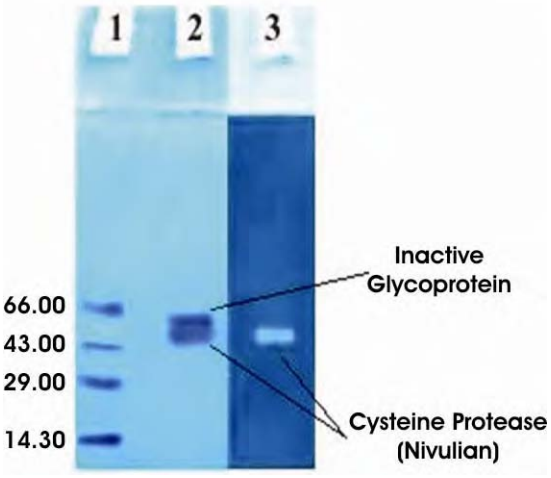

Fig. 7—Electrophoretic behaviour of purified protease enzyme of *Euphorbia nivulia* latex. SDS-PAGE and Substrate-PAGE (Zymogram) of purified enzyme fraction. Lane-1, molecular weight markers; Lane-2, Purified enzyme fraction of rechromatography; Lane-3, Zymogram of purified protease.

(43.42 kDa) according to protease nomenclature and previous suggestions[31].

The molecular architecture of isolated protease enzyme comprises of about 4.5% carbohydrate reveals that the enzyme is a glycoprotein. Further its evidence for glycosylation was obtained by SDS–PAGE and blotting to a PVDF membrane followed by subsequent carbohydrate staining. As shown in Fig. 8, the protein bands stained with Schiff's reagent indicate that a carbohydrate moiety is attached to the purified enzyme. The result demonstrated that, both proteins (52.96 and 43.42 kDa) i.e. inactive protein and active cysteine protease (Nivulian) are glycoproteins. Further, it was confirmed from zymography that only low molecular weight 43.42 kDa proteins is a protease (Fig. 8). The result demonstrated that, isolated protease enzyme is a type of glycoprotein. Similar results have been reported in some plant cysteine proteases like calotropin F-I and F-II[32], ginger protease GP-I and GP-II(Ref. 33) and pergularain eI[34] are glycoproteins. The glycocarbohydrate moiety may be part of the functional architecture of the purified enzyme, and might be responsible for its thermal stability. Very recently, similar results of thermostability of plant protease named dubiumin of *Solanum dubium* Fresen[35] and sterblin of *Sterblus asper* Lour.[36] is appeared in literature due to presence of glycocarbohydrate moiety. This protease showed a remarkable thermal stability. Proteolytic activity remains practically unchanged after 1.67 h at 40 and 50°C and the enzyme activity is notably high even after 1 h at 60°C (78.43%), but it was inactivated by heating uniformly for 60 min at 70°C (Fig. 9). Earlier studies reported similar thermal profile of cysteine endopeptidase, asclepain f(Ref. 37).

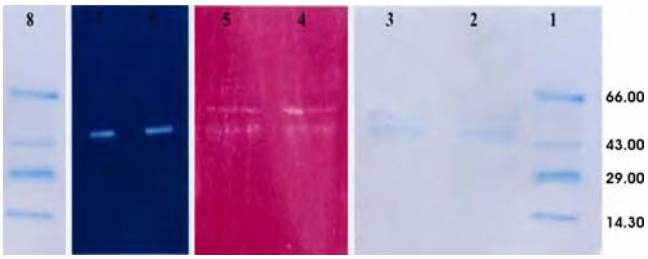

Fig. 8—Electroblotting of purified protease enzyme of *Euphorbia nivulia* latex. The electroblotting, glycoprotein detection and zymography of purified pooled enzyme fraction. Lane-1 and 8, molecular weight markers; Lane-2 and 3, Purified enzyme fraction of rechromatography; Lane-4 and 5, PAS staining of glycoprotein detection; Lane-6 and 7, Zymogram of purified protease.

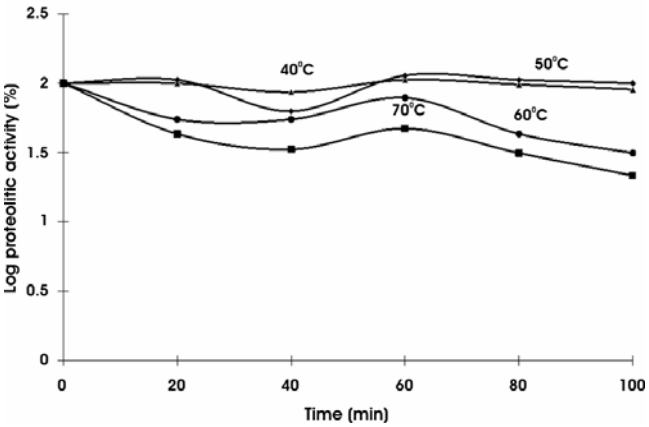

Fig. 9—Thermal stability of purified protease enzyme of *Euphorbia nivulia* latex.

Conclusion

Comparative studies on hydrolytic enzyme profile is described and it indicate that, the latex of *E. nivulia* is a promising candidate for proteolytic and milk clotting activity. A new cysteine protease is reported here for the first time from the latex of *E. nivulia*, using simple purification procedure. Easy availability of *E. nivulia* latex, with simple and economic purification process of cysteine protease, provides great possibility for its large scale preparation. This protease is also a potential endopeptidase with many interesting properties and may be subjected to many useful applications in biotechnological industries.

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