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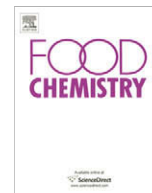

Crinumin, a chymotrypsin-like but glycosylated serine protease from *Crinum asiaticum*: Purification and physicochemical characterisation

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ABSTRACT

Plant latex could be a potential source of novel proteases usable in the food and feed industries because of broad substrate specificity with high stability in extreme conditions. Crinumin, a glycosylated serine protease with chymotrypsin-like activity was purified from the latex of *Crinum asiaticum* using cation-exchange column chromatography. Crinumin shows activity over a wide range of pH (4.5–11.5 and optimum at 8.5), temperature (75 °C and optimum at 70 °C) and is also functional against chaotrophs, organic solvents, and detergents, even after prolonged exposure. The molecular mass (67.7 kDa), extinction coefficient (17.7), isoelectric point (6.9), and numbers of tryptophan (13), tyrosine (24) and cysteine (15 with 7 disulphide bridges) residues were estimated. K_m of the enzyme was 31.7 μM with casein and 5×10^4 μM with *N*-succinyl-L-phenylalanine-*p*-nitroanilide. Easy availability of the aqueous latex, simple purification procedure, high yield (33%), stability and activity in adverse conditions makes it applicable for the pharmaceutical and food industries.

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1. Introduction

Proteases are one of the leading groups of industrial enzymes, with significant application in the food industry. In the food industry proteases are applied for degradation of the turbidity complex resulting from protein in fruit juices and alcoholic liquors, the improvement of quality of protein-rich foods, soy protein hydrolysis, gelatin hydrolysis, casein and whey protein hydrolysis, meat protein recovery, and meat tenderisation (Tomar, Kumar, & Jagannadham, 2008). They are also used in brewing, cheese elaboration, and bread manufacturing (Pande, Dubey, Yadav, & Jagannadham, 2006). Proteolytic enzymes from plant sources are better suited for pharmaceutical as well as food industries, as they are active over a wide range of temperature and pH, and possess broad substrate specificity and high stability in extreme conditions (Patel, Singh, & Jagannadham, 2007). Therefore, valuable and potential plant proteases are always in demand.

Thiol proteases from plant latex, such as bromelain, calotropins, ficin and papain, are commonly used in several processes in the food and dairy industries, but their proteolytic activity is inhibited by air oxidation or metal ions. Thus, these proteases require reducing and chelating agents for their activity, which restricts their commercial application. In contrast, different serine proteases, including plant proteases, do not need such co-factors (Tomar et al., 2008).

In human health, serine proteases are helpful in non-specific digestion as well as highly regulated functions, like embryonic development, immune response and blood coagulation. Insufficient or excess protease activity can promote significant pathologies, like cancer, inflammation, haemophilia, heart attack, stroke and pancreatitis, and also parasitic infection (Cera, 2008). Type II trans-membrane serine protease may provide a remedy for specific diseases in which neurohypophysial hormone participates, in particular, diabetes or diabetic retinopathy (Yamanouchi, 2003).

Crinum asiaticum of the family Amaryllidaceae has a wide range application, in traditional as well as modern medicine. *C. asiaticum* is native to tropical south-eastern Asia, and is also a popular landscape plant in Florida, the Gulf Coast, California and other warm areas (Scheper, 1998). The herb is useful to treat inflamed joints and sprains (ICBSI, 1998–2000). The leaves of the plant are used to induce vomiting (Fennel & Staden, 2001) and for the treatment of haemorrhoids, contusions, sprains, fractures, (Nguyen & Doan, 1989), earache and other inflammations (ICBSI, 1998–2000). The

Abbreviations: BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DTNB, 5,5í-dithiobis(2-nitrobenzoicacid); IAA, iodoacetic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(Raminoethyl ether) tetraacetic acid; GuHCl, guanidine hydrochloride; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulphate; TCA, trichloroacetic acid; DTT, di-thiothreitol; β-ME, β-mercaptoethanol; TEMED, *N*,*N*,*N*,*N*-tetramethylethylenediamine.

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bulb shows cytotoxicity against tumour cells. Its root has activity against HIV-1 virus (Min, Gao, Nakamura, Kim, & Hattori, 2001). The seed is useful in kidney diseases (Oudhia, 2001, 2002, 2003). The latex of the plant shows inhibition of calprotectin-induced cytotoxicity (Satoru, Masaaki, Mikio, & Yamazaki, 1998), anti-inflammatory activity and also may contain compound(s) with anti-histaminic properties (Samud, Asmawi, Sharma, & Yusof, 1999). In Ayurveda, the plant is useful in the treatment of biliousness, vomiting, urinary discharges and tumours, and also in diseases of the vagina, abdomen and blood. According to the Unani system of medicine, it is used in the treatment of bronchitis, chest, lung, and spleen diseases, gonorrhoea, night-blindness, urinary concretions, lumbago, anuria and toothache (Oudhia, 2001, 2002, 2003).

Phytochemical research has identified 170 different compounds (more than 150 different alkaloids) in this genus, some of which have analgesic, antitumour, and antiviral activity (Fennel & Staden, 2001; Neergheen, Bahorun, Jen, & Aruoma, 2007). The bulb of the plant also contains alkaloids, notably criasiaticidine (Min et al., 2001), lycorine and crinamine (Nguyen & Doan, 1989). However, the proteins and other biochemical constituents of the plant have not been investigated in detail. To understand the potential applications of the aqueous latex, efforts were focused on identifying and purifying the protein constituents, if any. While screening different parts of the plant for biological activities, the aqueous latex was found to possess a considerable amount of proteolytic activity. In this paper, the purification and physiochemical and immunological characterisation of a serine protease from the aqueous latex of *C. asiaticum* are reported.

2. Materials and methods

2.1. General

The latex was collected by incisions on the midrib of leaf from the plant *C. asiaticum* found abundantly on the Banaras Hindu University campus (Varanasi, India). CM-Sepharose was purchased from Pharmacia. Molecular weight marker was purchased from Bangalore Genie. Coomassie Brilliant Blue R-250, all synthetic substrates, bovine serum albumin (BSA), lysozyme, agarose, azocasein, azoalbumin, haemoglobin, 5,5í-dithiobis(2-nitrobenzoicacid) (DTNB), iodoacetic acid (IAA), $HgCl_2$, dithiothreitol (DTT), guanidine hydrochloride (GuHCl), urea, glycerol, *o*-phenanthroline, ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis(Raminoethyl ether) tetraacetic acid (EGTA), soybean trypsin inhibitor (SBTI), β-mercaptoethanol (β-ME), *N*,*N*,*N*,*N*-tetramethylethylenediamine (TEMED), phenylmethanesulfonyl fluoride (PMSF), acrylamide, *N*,*N*-methylenebisacrylamide, *o*-phenylenediamine, and other standard proteins were obtained from Sigma Chemical Co. (St. Louis, MO). Coomassie Brilliant Blue G-250 was from Eastman Kodak. Ampholine carrier ampholytes were from LKB. All other chemicals were commercially available and of high purity.

2.2. Precaution

The handling of acrylamide is done with safety gloves because it is a potent neurotoxin and carcinogen. Phenol and trichloroacetic acid (TCA) are highly corrosive to skin.

2.3. Protein purification

All purification steps were carried out at 4 °C unless stated otherwise. Latex was collected in 0.01 M acetate buffer, pH 5.0, and frozen at −20 °C for 36 h. The latex was thawed to room temperature and centrifuged at 15000*g* for 30 min to remove any insoluble material and gum. The supernatant was filtered and the clear supernatant thus obtained, which was referred to as crude latex, was subjected to cation-exchange chromatography (CM-Sepharose pre-equilibrated with 0.01 M sodium acetate buffer, pH 5.0). The column was washed with the same buffer until no protein was detected in the eluate. The bound proteins from the column were eluted with a linear salt gradient of 0–1.0 M NaCl in the same buffer. Fractions of 4.0 ml in volume were collected at a flow rate of 4.0 ml/min. Protein content (by absorbance at 280 nm) and caseinolytic activity of all the fractions were measured. Protein concentration was determined by absorbance at 280 nm, as well as by the Bradford method (Bradford, 1976) with BSA as a standard. Similarly, homogeneity of the protein in the fraction was judged by SDS–PAGE. The fractions with maximum activity and high homogeneity were pooled and concentrated by membrane filtration, using an Amicon concentrator with YM10 membrane. The clear protein solution was stored at 4 °C for further use.

2.4. Assay for protease activity

The hydrolysing activity of the protease was monitored using denatured natural substrates like casein, haemoglobin, and azoalbumin as described (Sarath, Motte, & Wagner, 1989). An aliquot of enzyme was incubated in a final volume of 500 μl of 50 mM Tris–HCl buffer (pH 7.5) at 37 °C. After 15 min, 0.5 ml of 1% (w/v) casein solution in the same buffer were added to the reaction mixture and the reaction was allowed to proceed at 37 °C. After 90 min the reaction was terminated by addition of 0.5 ml of 10% TCA and allowed to stand for 10 min at room temperature. The precipitate was removed by centrifugation and TCA-soluble peptides in the supernatant were estimated by absorbance at 280 nm. In the case of azoalbumin or haemoglobin as a substrate, the supernatant after TCA precipitation was mixed with an equal volume of 0.5 M NaOH and incubated for 10 min. The development of colour was measured at 440 nm. A control assay was done without any enzyme in the reaction mixture and was used as a reference. One unit of the enzyme activity was defined as the amount of enzyme, under given assay conditions that gave rise to an increase of 1 U of absorbance at 280 or 440 nm/min of digestion. The specific activity is the number of units of activity per milligram of protein.

2.5. Electrophoresis

Homogeneity of the enzyme was checked by SDS–PAGE (Laemmli, 1970). On SDS–PAGE, samples of crude latex, active enzyme and inactive enzyme (PMSF-treated) along with molecular weight markers were applied. The gel was stained with Coomassie Brilliant Blue R-250. The molecular weight of the purified enzyme was estimated from the plot of log (molecular weight) *vs.* electrophoretic mobility of the proteins.

2.6. Isoelectric point

Isoelectric point of the purified enzyme was determined by isoelectric focusing in tube gels as described for procerain (Yadav, Pande, & Jagannadham, 2006). Ampholine in the pH range of 5.0–8.0 was used to generate the pH gradient. Anodic and cathodic chamber buffers were 0.1 M phosphoric acid and 0.1 M NaOH, respectively. Protein bands were stained with 0.04% (w/v) Coomassie Brilliant Blue G-250 dissolved in 6% perchloric acid.

2.7. Carbohydrate content

For carbohydrate content, various amounts of the enzyme were taken in a final volume of 50 μl in different wells of a microtitre plate and 25 μl of 4% aqueous phenol were added to each well.

After 5 min, 200 µl of concentrated sulphuric acid were added to each well and the increase in absorbance was measured at 492 nm using the Emax Precision microtitre plate reader (Molecular Devices, Sunnyvale, CA). Carbohydrate content of the enzyme was extrapolated from the curve generated under similar conditions, using galactose as standard.

2.8. Extinction coefficient

Dry weight, as well as spectrophotometric (Aitken & Learmoth, 1997), methods were used to determine the extinction coefficient of the enzyme. In the dry weight method, several solutions of the enzyme were prepared by serial dilution and the absorbance of each sample at 280 nm was recorded. These samples were dried thoroughly in an oven and the dry weight of each sample was determined using an analytical balance. The extinction coefficient was calculated using Beer–Lambert's law. In spectrophotometric method, the formula:

$$\varepsilon^{1\%}_{280\ \mathrm{nm}} = 10(5690n_w + 1280n_y + 120n_c)/M$$

where n_w, n_y, n_c are the number of tryptophan, tyrosine, and cysteine residues in the protein, and M is the molecular mass of the protein) was used to determine the extinction coefficient of the protein.

2.9. Tryptophan and tyrosine content

Total tyrosine and tryptophan contents of the enzyme were measured by the method of Goodwin and Morton (1946). Absorbances at 280 and 294.4 nm were obtained from the absorbance spectra of the purified enzyme in 0.1 M NaOH recorded between 300 and 220 nm. By the use of these values the total tyrosine and tryptophan content in the protein were calculated using the formula:

$$w = (A_{280} - x\varepsilon y)/(\varepsilon w - \varepsilon y)$$

where w is the estimated tryptophan content in moles per litre; A_{280} is the absorbance at 280 nm from the protein spectra, εw and εy are molar extinction coefficients of tryptophan and tyrosine in 0.1 M NaOH at 280 nm (εw = 5225 and εy = 1576), respectively, and x is total tyrosine and tryptophan content in the protein, calculated using $\varepsilon_{294.4}$ = 2375.

2.10. Measurement of free and total cysteine content

The free and total cysteine residues of the protein were determined by the DTNB method (Ellman, 1959). The enzyme was incubated at 37 °C for 30 min in the presence of 0.05 M β-ME in 0.05 M Tris–HCl buffer pH 8.0 and dialysed overnight against 0.1 M acetic acid. An aliquot of enzyme (50 µl) was taken in 700 µl of 0.1 M Tris–HCl buffer (pH 7.3) and allowed to stand for 10 min. Subsequently, 50 µl of 5 mM DTNB solution were added and the liberated TNB anion was monitored by the measurement of absorbance at 412 nm. The numbers of free cysteine residues were calculated using an extinction coefficient for DTNB of 14,150 $M^{-1}\ cm^{-1}$ at 412 nm (Creighton, 1989). Similarly, to determine the total sulfhydryl content, the enzyme was reduced in the presence of 6 M GuHCl with 0.05 M DTT in 0.05 M Tris–HCl buffer pH 8.0 and dialysed against 0.1 M acetic acid, to remove the excess DTT from the reaction mixture (Riddles, Blakeley, & Zerner, 1983). By comparison of the number of free and total cysteine molecules, the numbers of disulphide bonds in the protein were deduced (Goodlett et al., 2000). To validate the current measurements, similar contents of papain, ribonuclease, BSA, and lysozyme were determined under similar conditions.

2.11. Amidolytic activity with synthetic substrate

The amidolytic activity of the enzyme was studied using different synthetic peptidyl-*p*NA (peptidyl *p*-nitroanilide) substrates. In each case, a stock solution of the synthetic substrate was prepared by dissolving the required amount of substrate in a minimum volume of DMSO and making up to the required volume with 0.01 M Tris–HCl buffer (pH 8.0). The reaction mixture contained 20 µg of enzyme and 100 µl of the synthetic substrate in the same buffer. After 30 min of incubation at 37 °C, the reaction was terminated by addition of 0.2 ml of 30% acetic acid. The liberated *p*-nitroanilide was monitored by absorbance measurement at 410 nm, using an extinction coefficient of 8800 $M^{-1}\ cm^{-1}$ for *p*-nitroanilide as a measure of hydrolysis (Erlanger, Kokowsky, & Cohen, 1961).

2.12. pH and temperature optima

The activity of the purified enzyme is measured as a function of pH and temperature, to determine the pH and temperature optima for activity. For pH optimum determination, substrates as well as reaction mixture were prepared at desired pH, and activity was measured as already described. The buffers used were: 0.05 M KCl–HCl (pH 1.0–1.5); 0.05 M glycine–HCl (pH 2.0–3.5); 0.05 M Na acetate (pH 4.0–5.5); 0.05 M Na phosphate (pH 6.0–7.5); 0.05 M Tris–HCl (pH 8.0–10.0) and 0.05 M sodium carbonate (pH 10.5–12.0). Due to insolubility of casein below pH 4.0, haemoglobin was used as substrate.

Similarly, the effect of temperature on the activity was also studied, to determine the temperature optimum of the enzyme. Casein was used as substrate. The enzyme was incubated at the desired temperature, in the range of 20–90 °C, for 15 min in 0.05 M Tris–HCl buffer, pH 8.0 and an aliquot was used for the activity measurement at the same temperature. At each temperature, a control assay was carried out without enzyme.

2.13. Effect of inhibitors on the protease activity

The effects of different classes of protease inhibitors were also monitored to classify the protease. Inhibitors used were iodoacetic acid (IAA), PMSF, EDTA, EGTA, *o*-phenanthroline, mercuric chloride, DTT and SBTI. In this study, the enzyme (10 µg) was incubated with each inhibitor at varying concentrations for 60 min under experimental conditions and an aliquot was used for the activity measurement. The assay was similar as described above. To calculate the change, a control assay was done without inhibitor.

2.14. Effect of substrate concentration on the reaction velocity

The effect of substrate concentration on the reaction velocity of the enzyme hydrolysis was studied using natural as well as synthetic substrates. The enzyme (10 µg) and the substrate casein (in a range of 0.01–0.25 mM) or *N*-succinyl-L-phenylalanine-*p*-nitroanilide (over a range of 5–250 mM) were used for the activity measurements. In each case, a Lineweaver–Burk plot was plotted and the Michaelis–Menten constant (K_m), V_{max} and K_{cat} were calculated.

2.15. Peptide mass fingerprinting

For peptide mass fingerprinting, the target protein band was excised from the gel and subjected to in-gel trypsin digestion. Mass spectrometry was carried out by the Department of Molecular and Cellular Neurobiology of Free University, Amsterdam on a MALDI/TOF-TOF mass spectrometer. The obtained peak lists were submitted to the Matrix Sciences (http://www.matrixscience.com) programme for protein identification. An additional match of the

mascot peptide mass fingerprint of the protease with other existing protease sequences (http://www.ncbi.nlm.nih.gov/) was also performed.

3. Results and discussion

3.1. Purification

A protease was purified from the latex of *C. asiaticum* using cation-exchange chromatography. The proteins bound to the column resulted in an elution profile consisting of two peaks with most of the activity appearing in peak II (Fig. 1). Protein in most of the fractions of second peak was homogeneous on SDS–PAGE. These homogeneous fractions were pooled and concentrated for further use. The specific activity of the enzyme was 79.9 ± 0.05 U/mg of protein. The total recovery of the activity was 33.32% with 1.94-fold purification. The purification protocol was highly reproducible within experimental error. Such single step purification with high yield enables the possibility of industrial application. The protease was named as crinumin, according to protease nomenclature.

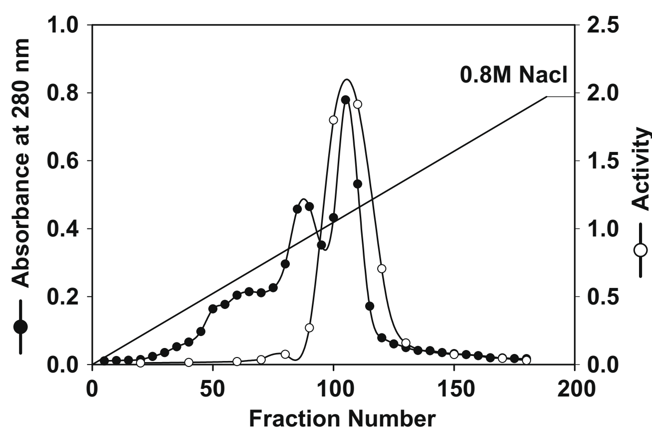

Fig. 1. Elution profile of crinumin on cation-exchange chromatography. All fractions were assayed for activity (○) and for protein content (●).

3.2. Physical properties

Purified enzyme, under active and inactive conditions, appeared as a single band in SDS–PAGE, with an estimated molecular mass of 67 kDa (Fig. 2a), in the range 60–80 kDa, similar to the majority of serine proteases (Antao & Malcata, 2005). The wide-smeared bands of crinumin in electrophoresis may be due to intense differential glycosylation of the protein (Lara et al., 2004). A single band was also observed on isoelectric focusing with a p*I* of pH 6.9 (Fig. 2c), confirming the purity of the enzyme; the protease belongs to the class of neutral proteases. The extinction coefficient of the enzyme was averaged to a value of 17.7 by dry weight (determined value 18.06) and spectrophotometric (determined value 17.28) methods and used for all other practical applications. The isoelectric point of crinumin along with other physiological and biochemical properties were compared with those of other plant serine proteases and presented in Table 1.

3.3. Proteolytic property and inhibition

Zymography also showed a clear region against a stained background, corresponding to the crinumin position (Fig. 2b), confirming the protease nature of the purified protein. Most of the activity of the enzyme was inhibited by PMSF up to 95% while other inhibitors, such as EDTA, *o*-phenanthroline, SBTI, DTT, IAA and mercuric chloride failed to inhibit the protease activity (Table 2). Inhibition of the enzyme activity with PMSF suggests the protein belongs to the serine proteases class. Known serine protease inhibitors like SBTI did not cause any inhibition of proteolytic activity of crinumin. SBTI inhibits the activity of bacterial or animal serine proteases but does not inhibit the activity of plant serine proteases, such as cucumisin, bamboo sprout proteases, etc. (Arima, Uchikoba, Yonezawa, Shimada, & Kaneda, 2000; Uchikoba, Yonezawa, & Kaneda, 1995). Lack of inhibition of the activity by proteinaceous inhibitors, such as SBTI, which is abundant in protein-rich foods like soybean, makes the current enzyme a potential protease in the food industry. No metal is involved in the proteolytic activity of crinumin, as the activity was not inhibited by *o*-phenanthroline, EDTA or EGTA, which are specific for metalloproteases.

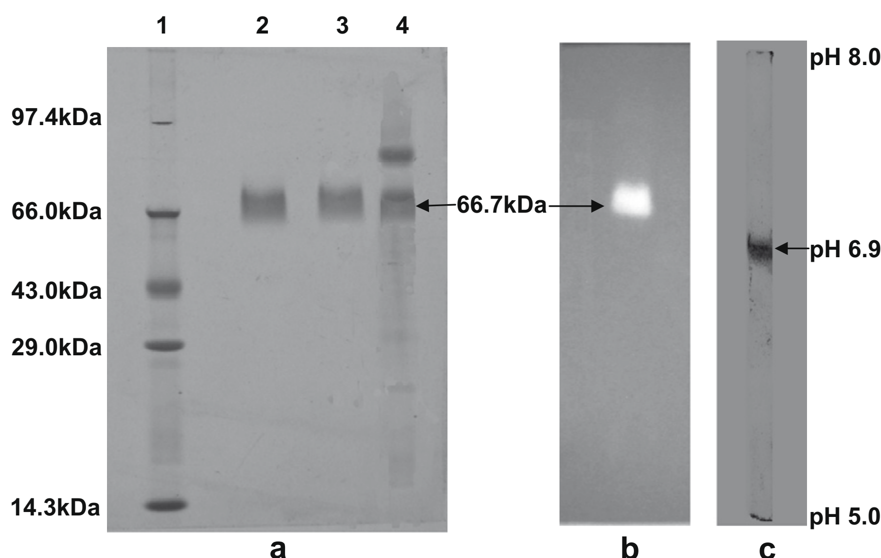

Fig. 2. Electrophoretic analysis of purified protease (a) SDS–PAGE of proteins includes 10 μg of molecular weight marker (lane 1), 15 μg of inactive (incubated with PMSF) protease (lane 2), 15 μg of active pure protease (lane 3), and 50 μg of crude latex (lane 4). (b) Zymogram of 15 μg of pure protease, showing proteolytic activity of the protein. (c) Isoelectric focusing of crinumin.

Table 1
Physicochemical properties of crinumin in comparison with other plant serine proteases.

Enzyme	Mol. mass (kDa)	$\varepsilon^{1\%}_{280\ nm}$	p*I*	K_m (μM/min)	Glyco-sylation (%)	pH optima	pH stability	Temp optima (°C)	Temp stability (°C)	Tyr	Trp	Free Cys	Total Cys
Crinumin	67	17.7	6.9	31.7	13	8.5	4.5–11.5	70	75	27	14	1	15
Milin[a]	51.4	29	7.2	33.3	7–8	8.0	5.5–12.0	60	65	14	23	2	14
Cryptolepain[b]	50.5	26.4	6.0	101	6–7	8–10	2.5–11.5	65–70	80	41	15	0	08
Carnein[c]	80.2	37.1	5.6	310	No	6.5	3–10	65	70	76	35	3	07
Wrightin[d]	57.9	36.4	6.0	50	8	7.5–10	5.5–11.5	70	75	75	20	1	09
Ara12[e]	76.1	N	N	N	N	5.0	N	80	90	N	N	N	N
Euphorbains l_C[a]	70	N	8.0	N	Yes	8.3	N	N	N	27	13		20
Protease[d]	67.0	N	N	N	N	11	4–10	70	70	N	N	N	N
Protease[a]	50	N	N	N	N	9.2	4.5–9.5	70	65	N	N	N	N
Cucumisin[a]	67	N	N	N	N	10.5	4–12.5	70	N	N	N	N	N

Name of amino acids are in 3-letter abbreviations. N in the table represents data not reported.
[a] Yadav et al. (2006).
[b] Pande et al. (2006).
[c] Patel et al. (2007).
[d] Tomar et al. (2008).
[e] Hamilton et al. (2003).

Table 2
Activity of crinumin under various conditions.

Conditions		Concentration[a]	% Residual activity
Protease inhibitor	IAA	1 mm	97
	Mercuric chloride	1 mm	99
	EDTA	1 mM	99
	o-Phenanthroline	1 mM	95
	PMSF	1 mM	5
	SBTI	1 mM	95
Chaotrophs	GuHCl	3.0 M	98
	Urea	6.0 M	99
Organic solvents	Methanol	50%	98
	Ethanol	40%	92
	Isopropanol	90%	87
	Butanol	40%	87
	Acetonitrile	70%	95
	DMSO	50%	97
	Dioxane	90%	98
Detergents	SDS	0.05%	88
	Triton X-100	2%	98
	Exalin	0.3%	89

[a] Minimum concentration for activity more than 80%.

Furthermore, the features of crinumin are special, as most proteases seen in plant latex are thiol proteases (Uchikoba et al., 1995).

3.4. Carbohydrate content

The molecular structure of crinumin contains at least 13% of carbohydrate moieties. Carbohydrate moieties play a crucial role in protein stabilisation, protection from degradation, control of protein solubility, and transport inside the cell (Yadav et al., 2006).

3.5. Effect of temperature and pH on enzyme activity

The enzyme retains full activity in the temperature range of 30–80 °C with optimum activity at 70 °C (Fig. 3a). Such a high-temperature optimum is seen with a limited number of serine proteases (Table 1). Most plant serine proteases show optimum activity in the temperature range of 30–60 °C (Pande et al., 2006).

Similarly, the enzyme is highly active in the range of pH 3.0–11.5, with a maximal activity at pH 8.5 (Fig. 3b). This pH optimum of crinumin places the enzyme closer to subtilisin-like serine proteases, as most of the plant subtilases have pH optima in the alkaline region (Hamilton, Simpson, Hyman, Ndimba, & Slabas, 2003).

3.6. Stability

The enzyme retains activity at temperatures up to 75 °C and is stable over the range of pH 4.5–11.5 as shown in Fig. 3a and b. Also the protein is reasonably active up to pH 3.0 with an activity of more than 64%. Further, the enzyme was also stable against chaotrophs (GuHCl and urea), organic solvents (DMSO, acetonitrile, methanol, ethanol, isopropanol, and butanol), and detergents (Triton X-100, SDS, and Exalin). The enzyme exhibited more than 80% activity in the presence of higher concentrations of chaotrophs, organic solvents, and detergents (Table 2). The stability of this enzyme under various extreme conditions indicates its potential use in various food, biotechnology and pharmaceutical applica-

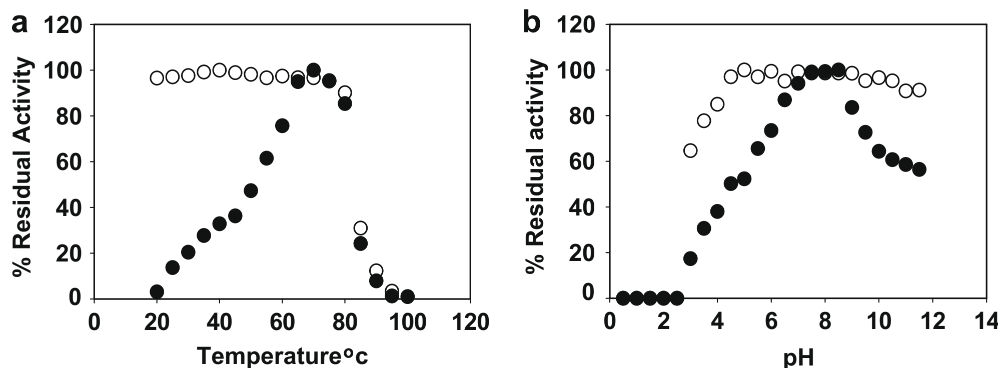

Fig. 3. Effects of temperature (a) and pH (b) on stability (○) and activity (●) of crinumin.

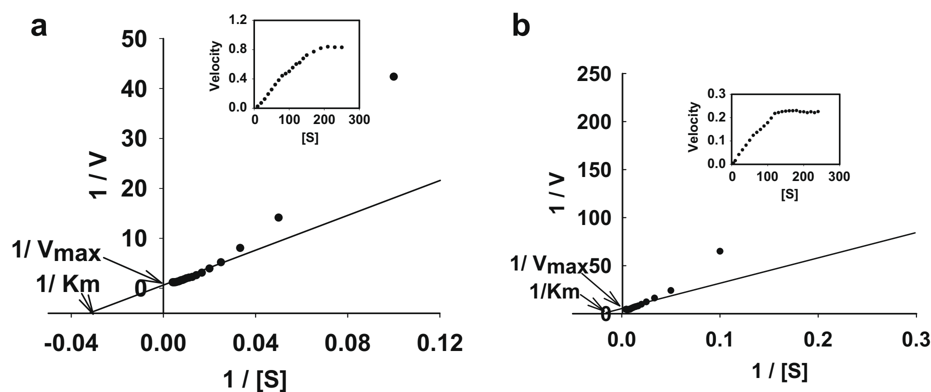

Fig. 4. Effect of substrate concentration on reaction velocity of crinumin (a) casein (0.01–0.25 mM) and (b) *N*-succinyl-L-phenylalanine-*p*-nitroanilide (5–250 mM).

tions as well as an excellent system for biophysical studies, to elucidate the structure–function relationship of serine proteases.

3.7. Enzyme kinetics

The nature of the kinetics is typically hyperbolic with natural substrates (casein) as well as synthetic substrates (*N*-succinyl-L-phenylalanine-*p*-nitroanilide) and at higher concentrations of the substrate the enzyme activity attains saturation (inset of Fig. 4a and b). The apparent K_m, V_{max} and K_{cat} values obtained from the Lineweaver–Burk plot were 31.7 μM, 0.03 μM/min and 0.15 with casein as substrate (Fig. 4a) and 5×10^4 μM, 0.316 μM/min and 0.73 with *N*-succinyl-L-phenylalanine-*p*-nitroanilide as substrate (Fig. 4b). In the case where the substrate is a native protein with multiple cleavage sites per mole of protein, results deviate at lower protein concentrations, suggesting that the Michaelis–Menten enzyme kinetic model may not be appropriate for plant proteases (Patel et al., 2007). However, such deviation is not seen with other proteases studied in our laboratory. Therefore, this kind of deviation may be protease-dependent.

3.8. Substrate specificity

The denatured natural substrates, such as casein, azocasein, azoalbumin and haemoglobin were hydrolysed by crinumin with very high specific activity. The enzyme also showed amidolytic activity against synthetic substrates, *N*-succinyl-L-Phe-*p*-nitroanilide and α-leucine-*p*-nitroanilide but failed to hydrolyse l-γ-glutamyl-*p*-nitroanilide, l-Ala-*p*-nitroanilide, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPA), *p*-nitrophenyl acetate and Ala-Ala-Ala-*p*-nitroanilide. Therefore, crinumin cleaves peptides at the carboxyl side of amino acids containing phenyl rings and also hydrolyses other amide bonds, particularly those with l-donated carboxyls. This was proved by the amidolytic activity against various synthetic substrates, which indicate that crinumin is a protease with a cleaving site similar to chymotrypsin (Uchikoba et al., 1995).

3.9. Specific amino acid residues

The tryptophan and tyrosine contents in the protein were 14 (measured value, 14.4) and 27 (measured value, 27.5), respectively. Total cysteine content was 15 (measured value, 15.7) with one free cysteine (measured value 0.58), resulting in seven disulphide bridges. Specific amino acid residues is the striking feature of crinumin, which place it close to euphorbain l_c, milin and other serine proteases of the euphorbiaceae family (Table 1).

3.10. Mass fingerprint analysis

Out of 15 peptide fragments of this protease, the Mascot peptide fingerprint of only one peptide hit (Score >60, http://www.matrixscience.com) with subtilase family, putative of *Oryza sativa* (gi|62733786 Score: 78), Os10g0524600 of *O. sativa* (gi|115483032 Score: 78) and a predicted protein of *Physcomitrella patens* subsp. Patens (gi|168024416 Score: 73). An additional match of the mascot peptide mass fingerprint of the protease with the other existing protein sequence in the NCBI database was also performed. One subtilisin-like protease precursor of *Zea mays* (gi|195614714) also matches with score 78 (http://www.matrixscience.com/cgi/master_results.pl?file=../data/20090507/FtiofiTwR.dat). These data suggested that crinumin may be a novel protease of the subtilase family.

4. Conclusion

This is the first report about the presence of proteolytic activity in the latex of the medicinally important plant *C. asiaticum*. During the course of the work a glycosylated serine protease was purified and also its physiochemical characterisation was performed. Physiochemical character suggests its closeness to subtilisin-like protease with a cleaving site similar to chymotrypsin. Easy and economic purification with high yield, stability in different stream conditions and active over a broad range of pH, temperature and variety of substrates makes this enzyme crucial for biotechnology, food and textile industries and also an excellent model system to study the structure–function of plant serine proteases, as well as protein folding. The role of the plant latex and serine proteases in inflammation, indicate that crinumin can play an important role in its treatment. The ability of the enzyme to function, even with inhibitor having proteinaceous nature (abundantly found in protein-rich food), make it more special and useful than other serine proteases being used in the food industry.

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