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ARTICLE

Benghalensin, a Highly Stable Serine Protease from the Latex of Medicinal Plant *Ficus benghalensis*

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A serine protease was purified to homogeneity from the latex of medicinal plant *Ficus benghalensis* by a single step procedure using anion exchange chromatography. The enzyme, named benghalensin, has a molecular mass of 47 kDa (MALDI-TOF and SDS−PAGE). The purified protein is a glycoprotein, and the enzymatic activity is solely inhibited by PMSF and chymostatin, indicating that the enzyme belongs to the serine protease class. The isoelectric point of the enzyme is pH 4.4 with optimum pH and temperature of pH 8.0 and 55 °C respectively. The extinction coefficient ($\varepsilon^{1\%}_{280}$) of the enzyme is 29.25, and the molecular structure consists of 17 tryptophan, 31 tyrosine and 09 cysteine residues. Peptide mass fingerprinting and *de novo* sequencing of tryptic-digested fragments of the protein did not find any putative conserved domains in BLAST analysis. The enzyme is stable and retains full activity over a broad range of pH and temperature or prolonged storage at 4 °C. Simple purification, high yield and stability enable exploration of the protein for structure−function relationship studies as well as other applications.

KEYWORDS: Benghalensin; *Ficus benghalensis*; latex; medicinal plant; serine protease

INTRODUCTION

Proteases in the plants play a significant role in various cellular and extracellular processes such as fruit development and ripening and participate in the degradation of storage proteins in germinal seeds (*1*−*3*) as well as in the activation and degradation of proteins (*4*). The precise biological role of the proteases in the latex of plants is still a debate, but they are suggested to be involved in protecting the plant against predator attacks (*5*, *6*). Proteases are the one of the most important enzymes in industries and occupy 60% of the total worldwide enzyme market. Proteases are also extensively used in food, leather, detergent and pharmaceutical industries. Many operations in food industry are carried out at high temperature such as hydrolysis of proteins at high temperature, enzymatic production of aspartame and other peptides, baking and brewing (*7*).

However, in some cases aqueous or organic solvent extracts of plants are known to possess several activities. Aqueous extracts of several wild plants are shown to protect deoxyribose and DNA from degradation suggesting their utility in some practical applications of those plants, viz., as ingredients in the formulation of nutraceutical beverages and/or foods (*8*). Similarly, extracts of *Centaurea calcitrapa* degrade caseins from milk from different species suggesting that such plant extracts can be used as an alternative to commercial animal rennets especially in the manufacture of caprine and ovine milk cheeses (*9*). The fruit extract of *Opuntia ficus-indica* is apparently a good substitute for animal rennet, as it exhibits both the clotting and caseinolytic activities (*10*).

However, the plant extracts or latex may constitute several components like organic compounds, proteins etc. with distinct features in terms of function and structure. In this direction a lot of work is being done in exploring the identification and characterization of biochemical constituents of the latex of some medicinally important plants in our laboratory (*11*−*16*). The latex of the plants constitutes several proteins with distinct characteristics. In most of the cases, the proteins present in the plant latex are cysteine proteases, e.g., ervatamins from *Ervatamia coronaria*, and several proteases from papaya latex with distinct characteristics. Likewise, the lattices of *Ficus glabrata* and *Ficus carica* contain a number of cysteine proteases, which are chromatographically and electrophoretically distinct (*17*).

However, the utility of cysteine proteases is limited, as they require oxidizing and chelating agents for their function. Therefore, the search for other proteases from plant lattices is still of importance to explore their therapeutic use as well as other applications in biotechnology. The majority of the proteases from plant latex are thiol proteases while the presence of other classes of proteases is also known. Serine proteases were once thought to be rare in plants; in recent years, however, several of those enzymes have been isolated (and duly purified) from various plant species, in which they occur in distinct parts, ranging from the seeds to the latex and the fruits (*18*−*20*).

Serine proteases are one of the largest groups of proteolytic enzymes involved in numerous regulatory processes. The role of serine proteases in various cellular mechanisms like microsporogenesis, symbiosis, hypersensitive response, signal transduction, differentiation and senescence has been reviewed by Antao and Malcata (*21*). Despite being the largest class of proteases in plant, the function and regulatory role of serine proteases is poorly understood. Plant serine proteases are stable and active under harsh conditions like high temperature and high pH and also in

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the presence of surfactant and oxidizing agents as compared to cysteine proteases. Therefore, serine proteases may be more useful and economical for industrial applications.

Ficus benghalensis (Moraceae, Mulberry family) is commonly known as Banyan tree or Indian Bargad and grows up to 30 m in height in tropical and subtropical areas of Asia. *F. benghalensis* contains a high amount of good quality natural rubber (*22*). Besides, various parts of the plant are widely used in traditional systems of medicine. An aqueous decoction of the fresh aerial roots of the Indian Banyan is also used by ayurvedic medical practitioners to boost the immune system in various diseases (*23*). The decoction is also used in the treatment of diarrhea, dysentery and piles, tooth disorders, rheumatism, skin disorders like sores, to boost immune system, and as a hypoglycemic. Various extracts of *Ficus benghalensis* were screened for antiallergic and antistress potential activities in asthma by milk induced leucocytosis and eosinophilia (*24*). Stem latex is used to treat heel cracks, and young twigs are used as toothbrushes (*25*). In view of various applications of different parts of the plant, it is important to look into biochemical constituents of the latex and their characteristics, which may provide a rationale for such applications, if any. In the course of screening the latex for various activities, a substantial amount of proteolytic activity is observed. This manuscript describes the identification, purification and biochemical characterization of a serine protease from the latex of *F. benghalensis*.

MATERIALS AND METHODS

Acetonitrile, acrylamide, ammonium bicarbonate, ampholine carrier ampholites, azocasein, azoalbumin, bovine serum albumin, BSA, ribonuclease A, casein, chymostatin, Coomassie brilliant blue, DEAE−Sepharose Fast Flow, DTNB, DTT, EDTA, EGTA, glycerol, GuHCl, hemoglobin, hen egg white lysozyme, $HgCl_2$, IAA, *N*, *N*-methylene bis-acrylamide, *o*-phenanthroline, PMSF, SBTI, TFA, urea, β-mercaptoethanol, Sigma Chemical Co, USA. All other chemicals were of the highest purity commercially available.

Purification of Enzyme. All purification steps were carried out at 4 °C unless stated otherwise.

Step 1. Gum Removal. Fresh latex was collected from the stem by making longitudinal incisions in to 0.01 M Tris-HCl buffer, pH 8.0 and frozen at −20 °C for more than 48 h. Subsequently, the latex was thawed to room temperature and centrifuged at 24000 × *g* for 20 min to remove gum and other debris. The resulting clear supernatant was termed as crude latex and used in the next step.

Step 2. Anion Exchange Chromatography. The crude latex was subjected to anion exchange chromatography on DEAE−Sepharose fast flow in a column pre−equilibrated with 0.01 M Tris buffer, pH 8.0. The column was washed thoroughly with the same buffer until no protein or activity was seen in the eluate. Bound proteins were eluted with a linear salt gradient from 0 to 1.0 M NaCl. All the fractions were monitored by absorbance at 280 nm for protein content and assayed for enzymatic activity with casein as substrate. Intactness and homogeneity of the enzyme in all the fractions were also assessed by SDS−PAGE.

Protein Concentration. Protein concentration was measured by absorbance at 280 nm as well as by Bradford's assay (*26*) using BSA as standard.

Assay for Proteolytic Activity. The proteolytic activity of the proteins during purification was monitored using natural substrates casein and hemoglobin (*27*). For the activity measurement, 10 μg of enzyme in 0.5 mL of 0.05 M Tris-HCl buffer, pH 8.0 was added to 0.05 mL of 1% substrate in the same buffer and the reaction was allowed to proceed for 30 min at 37 °C. The reaction was terminated by addition of 0.5 mL of 10% TCA and kept for 10 min. The resultant precipitate was removed by centrifugation, and TCA soluble peptides in the supernatant were measured by absorbance at 280 nm. A control assay, without enzyme in the reaction mixture, was used as blank. One unit of enzyme activity is defined as the amount of enzyme that gave rise to an increase of one unit of absorbency at 280 nm per min of substrate digestion. The specific activity is the number of units of activity per milligram of protein.

Electrophoresis. Homogeneity, intactness and molecular mass (M_r) of the purified protein were assessed by SDS−PAGE under reducing and nonreducing conditions as described by Laemmli (*28*). Proteins in the gel were stained by coomassie R-250.

Zymography. Zymography was performed to visualize the proteolytic activity of the purified protein in the gel, using the same protocol as in the case of wrightin from the latex of *Wrightia tinctoria* (*20*).

Isoelectric Focusing. The isoelectric point (p*I*) of benghalensin was determined by isoelectric focusing on polyacrylamide disc gels as described (*11*). Electrophoretic runs were carried out with ampholine carrier ampholytes in the pH range 4−6 at 300 V for 2 h using 0.1 M NaOH as catholyte and 0.1 M orthophosphoric acid as anolyte. The enzyme in the gel was visualized by staining with coomassie G-250 (*29*).

Carbohydrate Content. Carbohydrate moieties in glycoproteins have been known to impart protein stabilization, protection from degradation, control of protein solubility and transport inside the cells. Some plant serine proteases were reported as glycoprotein such as milin (*39*) and wrightin (*20*). As the protein benghalensin is a secreted protein, it is essential to determine glycosylation, if any. The carbohydrate content in the purified protein was estimated using the phenol sulfuric acid method (*30*). Galactose was used to generate a calibration curve.

Glycostaining. Glycostaining indicates the presence of carbohydrate moiety in benghalensin. Schiff's reagent was used for glycoprotein staining using the reported protocol (*31*).

pH and Temperature Optima. The activity of an enzyme is affected by pH and temperature and in turn dictates the utility of an enzyme for industrial processes. Therefore, the activity of benghalensin was measured at different pH and temperature. The enzyme assay was carried out at 37 °C as described earlier. Casein could not be used as substrate below pH 4.0 due to insolubility, thus hemoglobin was used as substrate at lower pH (*32*). A control assay at the same pH without enzyme in the reaction mixture was used as blank. The effect of temperature on the activity of purified enzyme was also investigated. Enzyme was incubated for fifteen minutes at the desired temperature, and the activity was measured at the same temperature.

Stability. The stability of an enzyme dictates its applicability therefore; the effect of pH (1.0−12.0) and temperature (20−80 °C) as well in the presence of different concentrations of denaturants and organic solvents on the proteolytic activity of benghalensin was examined. The enzyme was incubated under specified conditions of pH, chemical denaturant or organic solvent for 24 h. In the case of temperature the sample was incubated for 15 min before activity measurement. The residual activity was assessed as described above.

Effect of Various Inhibitors on the Activity. The effect of different inhibitors on the activity of purified enzyme was studied to classify the protein. The effect of various protease inhibitors (PMSF, chymostatin, IAA, $HgCl_2$, EDTA, EGTA, *o*-phenanthroline) on hydrolyzing activity of benghalensin was monitored. Ten micrograms of enzyme was incubated with increasing concentration of specific inhibitor (0−50 mM) in 0.05 M Tris-HCl buffer pH 8.0 for 30 min at 37 °C and assayed. A control assay was performed without inhibitor, and the activity was considered as 100%.

Effect of Substrate Concentration on Reaction Velocity. The effect of increasing substrate concentration on the reaction velocity of the enzyme hydrolysis was studied using natural substrates at pH 8.0 and 37 °C. In the case of natural substrate, ten micrograms of the enzyme was used, and the concentration of the substrate, casein, was in the range of 20−410 μM. Assays were performed as already described under Assay for Proteolytic Activity. A blank was used at the specific substrate concentrations without the enzyme. A Lineweaver−Burk plot was plotted and the value of the Michaelis−Menten constant (K_m) was calculated.

Tryptophan and Tyrosine Contents. The total number of tryptophan and tyrosine residues in the benghalensin was determined by the method of Goodwin and Morton (*33*). To validate the measurements, similar contents of papain, ribonuclease and lysozyme were also carried out.

Free and Total Cysteine Content. Free and total cysteine residues of milin were determined using the DTNB method of Ellman (*34*) using

DTNB. For free cysteine content determination, the enzyme was reduced with 0.05 M β-ME, while for the estimation of total number of cysteine residues, the enzyme was denatured in 6 M GuHCl and reduced with 0.05 M DTT. After the reduction the excess reducing agents were removed by dialysis with 0.1 M acetic acid. After dialysis an aliquot of the dialyzed enzyme was added to DTNB solution. The liberated TNB anion after reaction of sulfhydryl group with DTNB was monitored spectrophotometrically by the measurement of absorbance at 412 nm. The numbers of disulfide bonds, in the protein, were deduced by comparison of the number of free and total cysteine residues. To validate the measurements, similar contents of papain, ribonuclease A, and lysozyme were also determined.

Extinction Coefficient. The estimation of extinction coefficient of benghalensin was determined by spectrophotometric method (*35*).

Autolysis. Proteases are prone to autolysis. Autolysis depends upon concentration of enzyme, pH, temperature, and any type of activator, if any. Extent of autolysis of the benghalensin was monitored at 37 °C as a function of enzyme concentration. The enzyme at different concentrations in the range of 0.01−1.0 mg/mL was incubated in 50 mM Tris-HCl, pH 8.0 at 37 °C. An aliquot of enzyme, at different time intervals, was used to determine the residual activity with casein as substrate. Reaction mixture without enzyme was used as a blank. Activity of the enzyme after the first 2 h was taken to be 100% for calculating the residual activity.

Tryptic Digestion for MS Analysis and *de novo* Sequencing by MALDI-TOF/TOF. Trypic digestion of benghalensin was done using the reported protocol (*36*). A tryptic digested sample of benghalensin was mixed with matrix and spotted on the polished stainless steel MALDI target plate for MALDI analysis. *de novo* sequences of peptides were determined by using the reported protocol (*37*).

RESULTS AND DISCUSSION

Purification. A new serine protease was purified from the latex of *Ficus benghalensis* to homogeneity using anion exchange chromatography. No proteolytic activity is seen in the column unbound or buffer wash. The protein elution profile resolved into four peaks (**Figure 1**), and the total activity loaded is seen in the second peak. Fractions of peak II with higher activity as well as homogeneity were pooled (70−140). The yield of the purified protein is 80 ± 5% with specific activity 62.4 ± 5 U/mg. The purified protein was named as benghalensin according to the nomenclature of proteases. The purification results of a typical batch are summarized in **Table 1**. The purification protocol is highly reproducible in terms of yield as well as specific activity within experimental errors. Proteolytic activity observed in only one peak suggests the existence of only one protease in the latex of *F. benghalensis*. Such single protease is an uncommon feature as plant latex consists of several proteases, e.g., *Elaeophorbia drupifera* (*38*), *Euphorbia milli* (*39*, *40*) and others. To the best of our knowledge, this is the first report of identification and purification of a serine protease from the genus *Ficus* of the Moraceae family. The simple purification of benghalensin, together with easy availability of a large quantity of latex, makes large-scale production of enzyme possible and thereby enables exploration of industrial as well as biotechnological applications.

Homogeneity and Physical Properties. Homogeneity of benghalensin was assessed on SDS−PAGE under nonreducing and reducing conditions (inset of **Figure 2**). The molecular mass (M_r) deduced from the plot between log of molecular weight vs relative

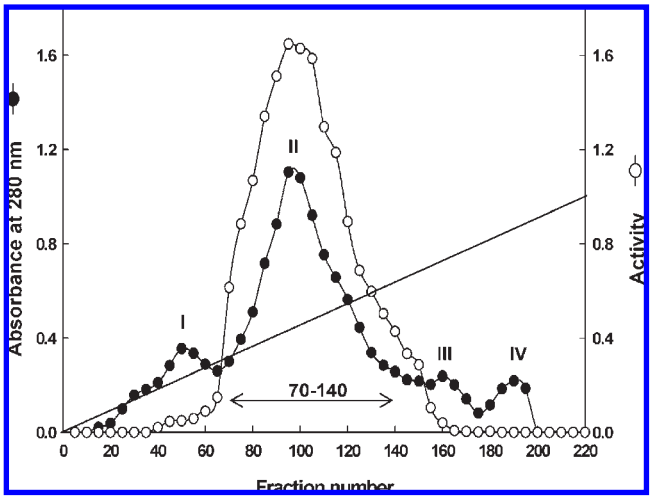

Figure 1. Elution profile of crude latex on anion exchange chromatography. DEAE−Sepharose column was pre-equilibrated with 10 mM Tris buffer pH 8.0. The bound proteins were eluted with a linear salt gradient of 0−1.0 M NaCl at the same pH. Fractions of 4 mL volume at a flow rate of 4 mL/min were collected and assayed for protein concentration (●) and proteolytic activity (○). The fractions of pool II (70−140) were pooled as indicated by a horizontal arrow.

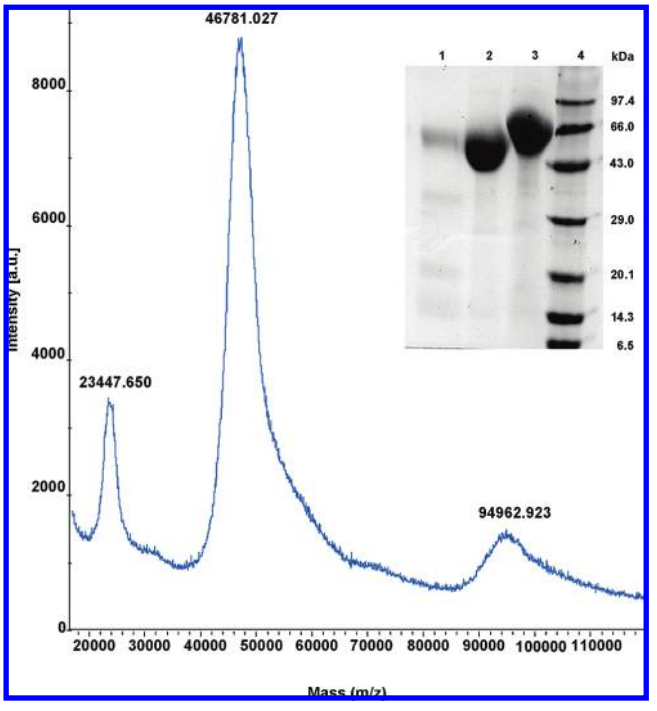

Figure 2. Assessment of homogeneity and molecular weight of benghalensin by SDS−PAGE and mass spectrometry. Gel electrophorized lanes 1−4 represent crude latex, benghalensin (30 μg) under nonreducing and reducing conditions and molecular weight marker respectively as shown in the inset. Benghalensin (10 ng) was used for MALDI-TOF analysis. BSA was used as a standard for calibration of the instrument, and mass resolution was obtained in the linear modes.

Table 1. Purification of Benghalensin from the Latex of *Ficus benghalensis*

step	total protein (mg)	total act. (units[a])	sp act. (units/mg)	recovery (%)	purification factor (fold)
crude latex	150.0	3750	37.5	100.0	1.0
DEAE-Sepharose	72.0	3000	62.5	80	1.6

[a] One unit of enzyme activity is defined as the amount of enzyme under the assay conditions described and gives rise to an increase of one unit of absorbance at 280 nm per min of digestion.

Table 2. Characteristics of Some Plant Serine Proteases

protein	plant source	M_r (kDa)	pH optima	temp optima (°C)	isoelectric point (p*I*)	glycosylation
benghalensin	*F. benghalensis*	47	8.0	50	4.4	yes
serine protease[a]	*Heliantus annas*	25	7.8	55	ND[b]	ND
serine protease[c]	*Cucurbita ficifolia*	60	9.2	55	ND	ND
cryptolepain[d]	*C. buchanani*	50.5	8.0−10.5	70−75	6.0	yes
wrightin[e]	*W. tinctoria*	57.9	7.5−10	70	6.0	yes
protease A and B[f]	*T. kirrilowi*	50	10−11	35	ND	yes
cucumisin[g]	*C. melo*	54	7.1	70	ND	yes
hordolisin[h]	*H. vulgare*	74	6.0	60	6.9	ND
taraxilisin[i]	*T. officinale*	67	8.0	40	4.5	ND
indicain[j]	*Morus indica*	134.3	8.5	80	4.8	no

[a] Reference *43*. [b] ND represents no data available. [c] Reference *44*. [d] Reference *18*. [e] Reference *20*. [f] Reference *43*. [g] Reference *45*. [h] Reference *46*. [i] Reference *4*. [j] Reference *42*.

mobility was 47 and 66 kDa under nonreducing and reducing conditions respectively. The purified protein showed a single peak with molecular mass of 47 kDa by MALDI-TOF analysis (**Figure 2**). Most of the time, the protein bands in SDS−PAGE were diffused under reducing and nonreducing conditions. Such diffusion of bands may be due to the glycosylation of the protein. However, the gel scanning of the bands gave a single symmetrical peak, confirming the homogeneity of the purified protein (data not shown). Further, the difference in the mobility of the protein, with and without reducing agent, in gel electrophoresis shed light on the contribution of the disulfide bonds on the protein structure as well as hydrodynamic nature of the protein. The biochemical properties of benghalensin are compared with those of some of the other known proteases in **Table 2**. Molecular masses of known plant serine proteases are in the range of 19−110 kDa while the majority of them lie between 60 and 80 kDa. The molecular mass of benghalensin (47 kDa) falls below the average range, which may be of some significance. The proteolytic nature of the protease was confirmed by gelatin zymography, where digested gelatin band appeared at a similar position of the protein on SDS−PAGE (**Figure 3A**). The single band on isoelectric focusing (IEF) with an isoelectric point of pH 4.4 indicates that the protein is an acidic protein (**Figure 3C**). The p*I* value of other known serine proteases generally lies between pH 4.0 and pH 8.0 (**Table 2**). Overall the single band on SDS−PAGE and IEF as well as MALDI-TOF analysis demonstrates the high purity of the enzyme identified and characterized.

Carbohydrate Content. Benghalensin contains about 10−12% carbohydrate content in the molecular structure. Further, the presence of carbohydrate moiety is confirmed by staining of the protein with Schiff's reagent, which appeared as magenta color as described in **Figure 3B**. Many plant proteases are glycoproteins though the biological advantage of such glycosylation is still not clear. Deglycosylation of benghalensin with TFMS treatment results in loss of activity, and no band is seen in zymogram (data not shown), leading to the conclusion that glycosylation is essential for the proteolytic activity of the protein. Carbohydrate moieties in glycoproteins played a role in protecting the protein from degradation, enhance thermal stability as well as solubility and enable transport inside the cell (*39*).

pH and Temperature Optima. The hydrolyzing activity of benghalensin was monitored at different temperatures in the range of 20−90 °C and pH from 1.0 to 12.0. The temperature and pH optima for the activity of benghalensin are 50 °C and pH 8.0 respectively (**Figures 4A** and **4B**).

Stability. Benghalensin retains more than 80% of the activity in the range of temperature 20−80 °C and pH 5.5−10 (**Figures 4A** and **4B**). Similarly, the protein also is stable in the presence of denaturants and organic solvents. The stability of the enzyme under different conditions is summarized in **Table 3**. The enzyme

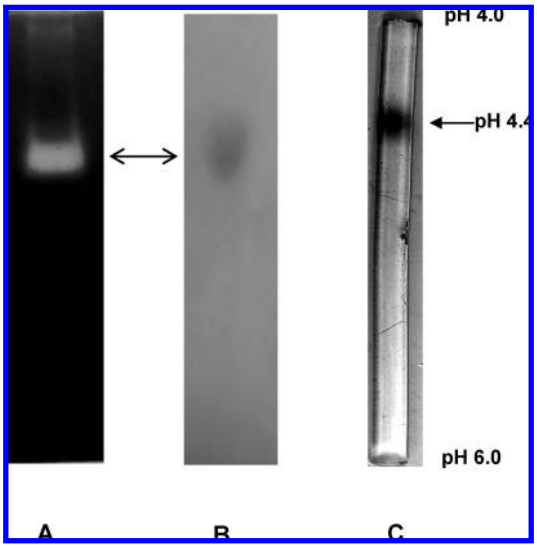

Figure 3. Zymogram, glycostaining and isoelectric focusing of benghalensin. (**A**) Zymogram (in gel activity) of benghalensin in the clear region (indicated by arrow) showed the hydrolysis of casein by the enzyme. (**B**) Glycostaining by Schiff's base. The magenta color (indicated by arrow) confirms the presence of carbohydrate content with protein. (**C**) Isoelectric focusing was performed on polyacrylamide disc gel with ampholine ampholyte, pH 4.0−6.0 at constant current. Isoelectric point of purified protease is indicated by arrow.

is fairly stable at high temperatures and broad range of pH, but low concentration of denaturant or organic solvent affects the activity considerably. Such contrary observation of stability may be a distinct feature of this enzyme. The high stability of the enzyme against pH and temperature along with the low stability under other conditions facilitates exploration of the possibility of utilization of the enzyme in industrial and biotechnological applications.

Effect of Inhibitors. The effect of inhibitors on the activity of the enzyme is shown in **Figure 5**. The enzyme activity was inhibited considerably by the PMSF and chymostatin as compared to other protease inhibitors. Such inhibition of the enzyme activity by PMSF and chymostatin indicates that the enzyme belongs to the class of serine proteases. SBTI, which is present in protein rich foods like soybeans, fails to inhibit the enzyme thus paving the way for its industrial usage. Generally, SBTI inhibits the protease activity of animal and bacterial serine proteases but fails to do so in the case of plant proteases such as cucumisin, miliin, indicain etc. (*39*, *41*, *42*). Inhibitors of cysteine protease ($HgCl_2$, IAA, DTT) and metalloprotease (EDTA, EGTA, *o*-phenanthroline) did not affect the enzymatic activity of the protein.

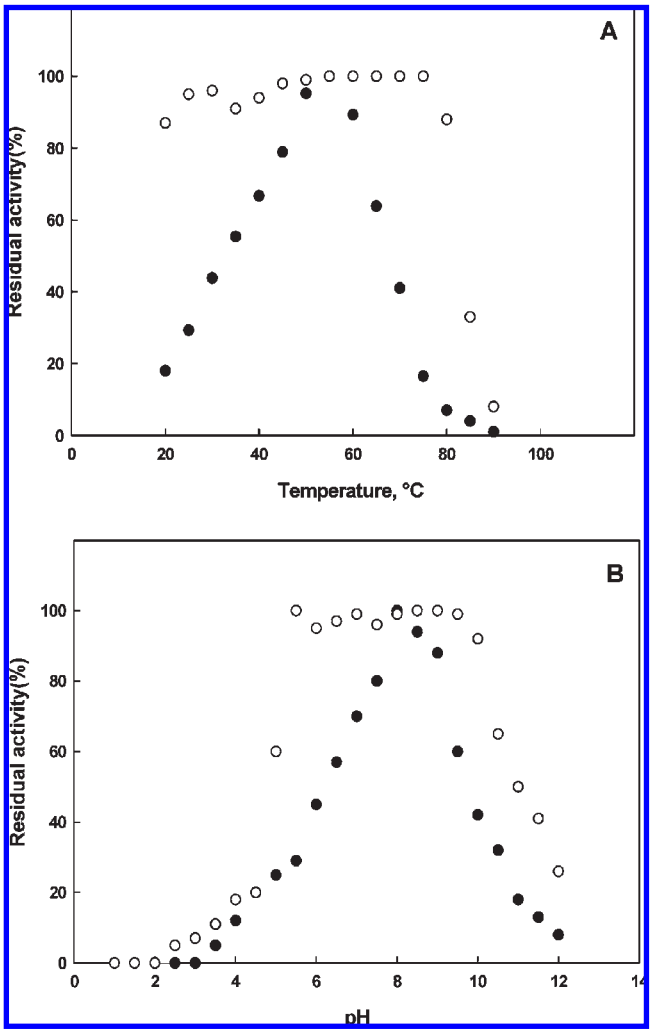

Figure 4. Effect of temperature (**A**) and pH (**B**) on the activity (●) and stability (○) of benghalensin. For temperature optima, 10 μg of benghalensin was used for activity assay at the required temperature. For temperature stability, the enzyme was incubated at the required temperature for 15 min and the activity was measured at 37 °C and pH 8.0. Similarly to see the effect of pH on activity, 10 μg of benghalensin was taken at required pH and activity was measured using substrate at the corresponding buffer. For pH stability, enzyme was incubated overnight at the required pH, and the activity was measured at 37 °C and pH 8.0.

Table 3. Stability of Benghalensin under Various Conditions

condition	concn	residual act. (%)
urea	6.5 M	50
GdnHCl	2.5 M	80
acetonitrile	20%	55
methanol	35%	60
DMSO	40%	50
pH	5.5−9.5	100
temp	45−75 °C	100

Effect of Substrate Concentration. The enzyme follows the typical Michelis−Menten equation with increasing concentration of casein as a substrate (**Figure 6A**). The nature of the curve was hyperbolic, and at higher concentrations of substrate, enzymatic activity attains saturation. The value of K_m from the Lineweaver−Burk plot (**Figure 6B**) was 0.33 mM with casein as a substrate.

Specific Amino Acid Residues and Extinction Coefficient. The tryptophan and tyrosine contents of the protein were 17 (measured value 16.86 ± 0.04) and 31 (measured value 31.08 ± 0.04) respectively. The total cysteine content was 09 (measured value 08.79) with 03 free cysteine (measured value 03.21) and six cysteine forming three disulfide bridges. The extinction coefficient of benghalensin, measured by spectrophotometric method, was 29.25.

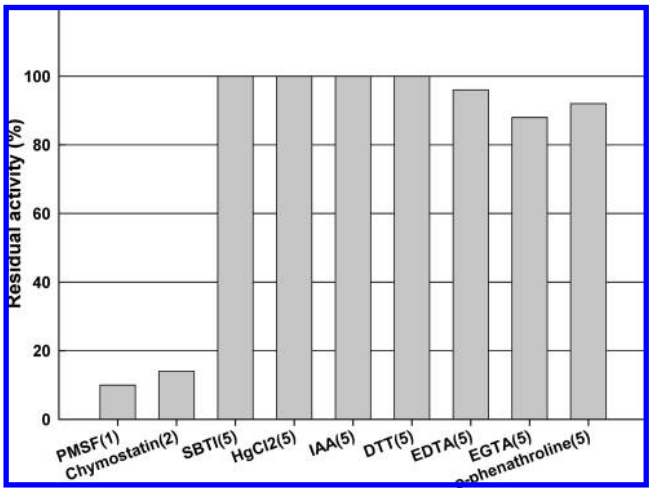

Figure 5. Effect of inhibitors on the caseinolytic activity of benghalensin. The enzyme was incubated in the presence of specified concentration of inhibitor (shown in parentheses, mM), with assay buffer for 30 min and assayed. The activity of the enzyme without inhibitor was considered as 100%.

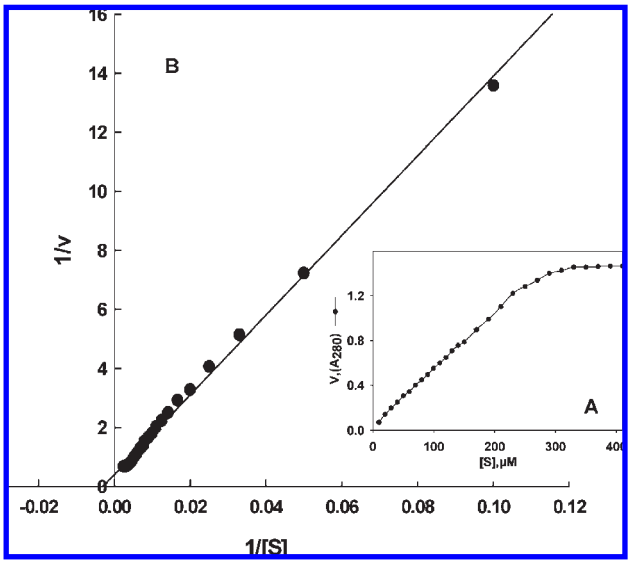

Figure 6. (**A**) Effect of substrate concentration on reaction velocity (●) of benghalensin. Ten micrograms of enzyme in 0.5 mL of 0.5 M Tris-HCl, pH 8.0, was added to 0.5 mL of casein in a concentration range of 1−410 μM. The activity was determined as described in the text. (**B**) Lineweaver−Burk plot. K_m was calculated according to Michaelis−Menten equation.

Autolysis. Generally, proteases are prone to autolysis, which becomes a hindrance in the utilization of the same. Under neutral conditions benghalensin undergoes autolysis. Loss of activity of benghalensin, in the concentration range 0.01−1.0 mg/mL, after 72 h of incubation at room temperature is shown in **Figure 7**. The magnitude of loss of activity decreases with increase in the enzyme concentration from 0.01−0.20 mg/mL, and further increase in loss of activity was not observed. Benghalensin

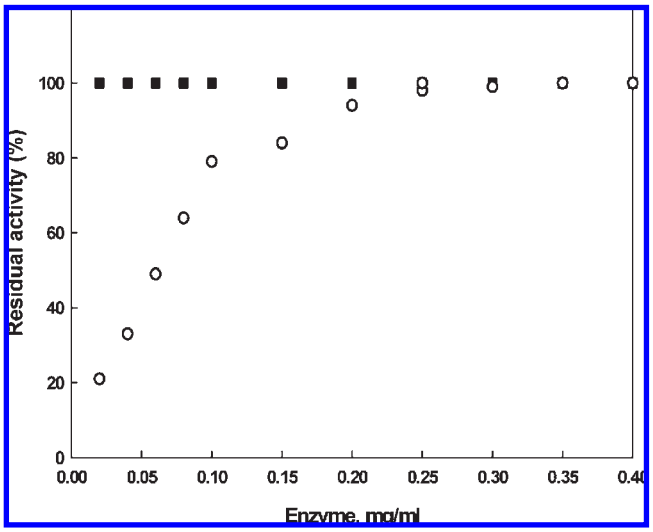

Figure 7. Autolysis of benghalensin as a function of protein concentration. The enzyme of different concentrations (0.02−0.40 mg/mL) was used. After 72 h of incubation at room temperature an aliquot of enzyme (10 μg) was used for residual proteolytic activity measurement under optimal conditions. The activity of enzyme after 30 min was considered as 100% activity.

Table 4. *de novo* Sequences of Some Tryptic Peptides of Benghalensin

m/z PMF	sequences	score
842.5255	—[a]	—
1186.5575	SSESMMKAKR	57
1218.5460	—	—
1255.6848	—	—
1329.7214	—	—
1619.7783	SSLWVTLDDEWLR	72

[a] (—) No sequence and score obtained because of low intensity of signals and weak MS/MS spectra.

retains more than 80% activity even at very low concentration, indicating its possible utilization in food, textile and biotechnological industries. In our experience, the enzyme is stable for four months at 4 °C, under neutral conditions, without loss in activity.

Protein Mass Fingerprint (PMF) and *de novo* Sequence of Trypsin-Digested Peptides of Benghalensin. Attempts were made to identify the protein by peptide mass finger printing. For this, the protein was enzymatically digested by trypsin and resolved into a number of peptides. The mass of the peptides was determined and searched against relevant databases in NCBI. No significant hits were obtained based on peptide masses. Subsequently, the different peptide fragments of benghalensin generated and with good resolution were subjected to *de novo* sequencing by MALDI-TOF/TOF. The TOF/TOF spectra of representative peptides and their *de novo* sequence are listed in **Table 4**. Due to the low intensity and weak MS/MS spectra we could get the *de novo* sequencing of only two peptides. The low signal intensity may be because of improper ionization and fragmentation of the peptides. These sequences when submitted to BLAST search in NCBI database with different variables show the uniqueness of the protein sequence. No putative conserved domains have been detected using these sequences as search query. However, good scores were obtained with similarities to esterase and ATP dependent protease. BLAST analysis proves that the purified protease is a serine protease as esterases also have the same catalytic triad as serine proteases.

ABBREVIATIONS

BLAST, Basic Local Alignment Search Tool; BSA, bovine serum albumin; CBB, Coomassie Brilliant Blue; DEAE, diethylaminoethyl; DFP, diisopropylfluorophosphate; DMSO, dimethyl sulfoxide; DTNB, 5,5μ-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(α-aminoethyl ether)tetraacetic acid; GuHCl, guanidine hydrochloride; $HgCl_2$, mercuric chloride; IAA, iodoacetic acid; MALDI-TOF, matrix assisted laser desorption/ionization time-of-flight; PAGE, polyacrylamide gel electrophoresis; NCBI, National Center for Biotechnology Information; PMSF, phenylmethanesulfonyl fluoride; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TFA, trifluoroacetate; TFMS, trifluoromethanesulfonic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

SAFETY

Acrylamide is a potent neurotoxin and carcinogen, and it was handled with safety gloves. The handling of phenol and TCA was done carefully because of their highly corrosive nature to skin. All other experiments were carried out with the utmost precaution and care.

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LITERATURE CITED

(1) Brady, C. J. Fruit ripening. *Annu. Rev. Plant Physiol.* **1985**, *38*, 155–178.

(2) Kembhavi, A. A.; Buttle, D. J.; Knight, C. G.; Barrett, A. J. The two cysteine endopeptidases of legume seeds: purification and characterization by use of specific fluorometric assays. *Arch. Biochem. Biophys.* **1993**, *303*, 208–213.

(3) Taylor, R. M.; Cuming, A. C. Purification of an endoproteinase that digests the wheat 'Em' protein in vitro, and determination of its cleavage sites. *FEBS Lett.* **1993**, *27*, 76–80.

(4) Rudenskaya, G. N.; Bogacheva, A. M.; Preusser, A.; Kuznetsova, A. V.; Dunaevsky, Ya. E.; Golovkin, B. N.; et al. Taraxalisin−a serine proteinase from dandelion Taraxacum officinale Webb s.l. *FEBS Lett.* **1998**, *437*, 237–240.

(5) Boller, T. Roles of proteolytic enzymes in interactions of plants with other organisms. In *Plant Proteolytic Enzymes*; Dalling, M. J., Ed.; CRC Press: 1986; pp 67−96.

(6) Smit, H. F.; Woerdenbag, H. J.; Singh, R. H.; Meulenbeld, G. J.; Labadie, R. P.; Zwaving, J. H. Ayurvedic herbal drugs with possible cytostatic activity. *J. Ethnopharmacol.* **1995**, *47*, 75–84.

(7) Zamost, B. L.; Nielsen, H. K.; Starnes, R. L. Thermostable enzymes for industrial applications. *J. Ind. Microbiol. Biotechnol.* **1991**, *8*, 71–82.

(8) Tavaria, F. K.; Sousa, M. J.; Domingos, A.; Malcata, F. X.; Bordelius, P.; Clemente, A.; Paris, M. S. Degradation of caseins from milk of different species by extracts of *Centaurea calcitrapa*. *J. Agric. Food Chem.* **1997**, *45*, 3760–3765.

(9) Pintado, A. I.; Macedo, A. C.; Teixeira, G.; Paris, M. S.; Clemente, A.; Malcata, F. X. Caseinolytic activity of fruit extract from Opuntia ficus-indica on bovine, caprine, and ovine sodium caseinates. *Biotechnol. Prog.* **2001**, *17*, 643–646.

(10) Giao, M. S.; Gonzalez-Sanjose, M. L.; Muniz, P.; Rivero-Perez, M. D.; Kosinska, M.; Pintado, M. E.; Malcata, F. X. Protection of deoxyribose and DNA from degradation by using aqueous extracts of several wild plants. *J. Sci. Food Agric.* **2008**, *88*, 633–640.

(11) Kundu, S.; Sundd, M.; Jagannadham, M. V. Purification and characterization of a stable cysteine protease ervatamin B, with

two disulfide bridges, from the latex of *Ervatamia coronaria*. *J. Agric. Food Chem.* **2000**, *48*, 171–179.
(12) Nallamsetty, S.; Kundu, S.; Jagannadham, M. V. Purification and biochemical characterization of a highly active cysteine protease Ervatamin A from the latex of *Ervatamia coronaria*. *J. Protein Chem.* **2003**, *22*, 1–13.
(13) Patel, B. K.; Jagannadham, M. V. A high cysteine containing thiol proteinase from the latex of *Ervatamia heyneana*: purification and comparison with ervatamin B and C from *Ervatamia coronaria*. *J. Agric. Food Chem.* **2003**, *51*, 6326–6334.
(14) Patel, A. K.; Singh, V. K.; Moir, A. J.; Jagannadham, M. V. Biochemical and spectroscopic characterization of morning glory peroxidase from an invasive and hallucinogenic plant weed *Ipomoea carnea*. *J. Agric. Food Chem.* **2008**, *56*, 9236–9245.
(15) Glazer, A. N.; Smith, E. L. Papain and other plant sulfhydryl proteolytic enzymes. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press:New York,1971; Vol. 3, pp 501−546.
(16) Robinson, G. W. Isolation and characterization of papaya peptidase A from commercial chymopapain. *Biochemistry* **1975**, *14*, 3695–3700.
(17) Sgarbieri, V. C.; Gupte, S. M.; Kramer, D. E.; Whitaker, J. R. Ficus enzymes I. Separation of the proteolytic enzymes of *Ficus carica* and *Ficus glabrata* latices. *J. Biol. Chem.* **1964**, *239*, 2170–2177.
(18) Pande, M.; Dubey, V. K.; Yadav, S. C.; Jagannadham, M. V. A novel serine protease cryptolepain from *Cryptolepis buchanani*: purification and biochemical characterization. *J. Agric. Food Chem.* **2006**, *54*, 10141–10150.
(19) Patel, A. K.; Singh, V. K.; Jagannadham, M. V. Carnein, a serine protease from noxious plant weed *Ipomoea carnea* (morning glory). *J. Agric. Food Chem.* **2007**, *55*, 5809–5818.
(20) Tomar, R.; Kumar, R.; Jagannadham, M. V. A stable serine protease, wrightin, from the latex of the plant Wrightia tinctoria (Roxb.) R. Br.: purification and biochemical properties. *J. Agric. Food Chem.* **2008**, *56*, 1479–1487.
(21) Antao, C. M.; Malcata, F. X. Plant serine proteases: biochemical, physiological and molecular features. *Plant Physiol. Biochem.* **2005**, *43*, 637–650.
(22) Kang, H.; Kim, Y. S.; Chung, G. C. Characterization of natural rubber biosynthesisin *Ficus benghalensis*. *Plant Physiol. Biochem.* **2000**, *38*, 979–987.
(23) Khan, T.; Tatke, P.; Gabhe, S. Y. Immunological studies on the aerial roots of the Indian banyan. *Indian J. Pharm. Sci.* **2008**, *70*, 287–291.
(24) Aswar, M.; Aswar, U.; Wagh, A.; Watkar, B.; Vyas, M.; Gujar, K. N. Antimicrobial activity of ficus benghalensis. *Pharmacologyonline* **2008**, *2*, 715–725.
(25) Muthu, C.; Ayyanar, M.; Raja, N.; Ignacimuthu, S. Medicinal plants used by traditional healers in Kancheepuram District of Tamil Nadu, India. *J. Ethnobiol. Ethnomed.* **2006**, *2*, 43.
(26) Bradford, M. M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein−dye−binding. *Anal. Biochem.* **1976**, *72*, 248–254.
(27) Arnon, R. Papain. *Methods Enzymol.* **1970**, *19*, 226–244.
(28) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.
(29) Merril, C. R. Gel−stain techniques. *Methods Enzymol.* **1990**, *182*, 477–488.
(30) Hounsell, E. F.; Davies, M. J.; Smith, K. D. Chemical methods of analysis of glycoprotein. In *The Protein Protocols Handbook*; Walker, J. M., Ed.; Humana Press: Totowa, NJ, 1997; pp 633− 634.
(31) Zacharius, R. M.; Morrison, T. E. Z. H.; Woodlock, J. J. Glycoprotein staining following electrophoresis on acrylamide gels. *Anal. Biochem.* **1969**, *30*, 148–152.
(32) Sarath, G.; da la Motte, R. S.; Wagner, F. W. Protease assay methods. In *Proteolytic Enzymes−a Practical Approach*; Beynon, R. J., Bond, J. S., Ed.; IRL Press: Oxford, 1989; pp 25−55.
(33) Goodwin, W.; Morton, R. A. The spectrophotometric determination of tyrosine and tryptophan in proteins. *Biochem. J.* **1946**, *40*, 628–632.
(34) Ellman, L. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **1959**, *82*, 70–77.
(35) Aitken, A.; Learmonth, M. Protein determination by UV absorption. In *The Protein Protocol Handbook*; Walker, J. M., Ed.; Humana Press: Totowa, NJ, 1997; pp 3−6.
(36) Shevchenko, A.; Wilm, M.; Vorm, O.; Mann, M. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal. Chem.* **1996**, *68*, 850–858.
(37) Yadav, S. C.; Jagannadham, M. V.; Kundu, S.; Jagannadham, M. V. A kinetically stable plant subtilase with unique peptide mass fingerprints and dimerization properties. *Biophys. Chem.* **2009**, *139*, 13–23.
(38) Lynn, K. R.; Clevette Radford, N. A. Two proteases from the latex of *Elaeophorbia drupifera*. *Phytochemistry* **1985**, *24*, 2843–2845.
(39) Yadav, S. C.; Pande, M.; Jagannadham, M. V. Highly stable glycosylated serine protease from the medicinal plant *Euphorbia milii*. *Phytochemistry* **2006**, *67*, 1414–1426.
(40) Moro, L. P.; Murakami, M. T.; Cabral, H.; Vidotto, A.; Tajara, E. H.; Arni, R. K.; et al. Purification, Biochemical and functional characterization of miliin, a new thiol dependent serine protease isolated from the latex of *Euphorbia milii*. *Protein Pept. Lett* **2008**, *15*, 724–730.
(41) Uchikoba, T.; Yonezawa, H.; Kaneda, M. Cleavage specificity of cucumisin, a plant serine protease. *J. Biochem.* **1995**, *117*, 1126–1130.
(42) Singh, V.; Patel, A. K.; Moir, A. J.; Jagannadham, M. V. Indicain, a dimeric serine protease from *Morus indica* cv. K2. *Phytochemistry* **2008**, *69*, 2110–2119.
(43) Uchikoba, T.; Horita, H.; Kaneda, M. Proteases of the sarcocarp of yellow snake-guard. *Phytochemistry* **1990**, *29*, 1879–1881.
(44) Curroto, E.; Gonzalez, G.; O'Reilly, S.; Tapia, G. Isolation and partial characterization of a protease from *Cucurbita ficifolia*. *FEBS Lett.* **1989**, *243*, 363–365.
(45) Yamagata, H.; Ueno, S.; Iwasaki, T. Isolation and characterization of a possible native cucumisin from developing melon fruits and its limited autolysis to cucumisin. *Agric. Biol. Chem.* **1989**, *53*, 1009–1017.
(46) Terp, N.; Thomsen, K. K.; Svendsen, I.; Davy, A.; Simpson, D. J. Purification and characterization of hordolisin, a subtilisin−like serine endoprotease from barley. *J. Plant. Physiol.* **2000**, *156*, 468–476.

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