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Indicain, a dimeric serine protease from *Morus indica* cv. K2

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ABSTRACT

A high molecular mass serine protease has been purified to homogeneity from the latex of *Morus indica* cv. K2 by the combination of techniques of ammonium sulfate precipitation, hydrophobic interaction chromatography, and size-exclusion chromatography. The protein is a dimer with a molecular mass of 134.5 kDa and with two monomeric subunits of 67.2 kDa and 67.3 (MALDI-TOF), held by weak bonds susceptible to disruption on exposure to heat and very low pH. Isoelectric point of the enzyme is pH 4.8. The pH and temperature optima for caseinolytic activity were 8.5 and 80 °C, respectively. The extinction coefficient ($\varepsilon_{280}^{1\%}$) of the enzyme was estimated as 41.24 and the molecular structure consists of 52 tryptophan, 198 tyrosine and 42 cysteine residues. The enzyme activity was inhibited by phenylmethylsulfonylfluoride, chymostatin and mercuric chloride indicating the enzyme to be a serine protease. The enzyme is fairly stable and similar to subtilases in its stability toward pH, strong denaturants, temperature, and organic solvents. Polyclonal antibodies specific to enzyme and immunodiffusion studies reveal that the enzyme has unique antigenic determinants. The enzyme has activity towards broad range of substrates comparable to those of subtilisin like proteases. The N-terminal residues of indicain (T-T-N-S-W-D-F-I-G-F-P) exhibited considerable similarity to those of other known plant subtilases, especially with cucumisin, a well-characterized plant subtilase. This is the first report of purification and characterization of a subtilisin like dimeric serine protease from the latex of *M. indica* cv. K2. Owing to these unique properties the reported enzyme would find applications in food and pharma industry.

1. Introduction

Proteases play an important role in regulating the biological processes in plants, such as stress response, recognition of pathogen, induction of effective defense response, mobilization of storage protein during germination, initiation of cell death, and senescence (Schaller, 2004). Plant proteases also exhibit broad substrate specificity as well active in wide range of pH, temperature, and in presence of organic compounds as well as other additives. Therefore, plant protease may turn out to be an efficient choice in pharmaceutical, medicinal, food, and biotechnology industry (Kaneda and Tominaga, 1975).

Serine proteases are one of the largest groups of proteolytic enzymes, which catalyze the hydrolysis of specific peptide bonds in their substrates and such activity is dependant of a set of amino acids in the active site of the enzyme where one of them is serine. Besides, serine proteases also exhibit different type of activities including exopeptidase, endopeptidase, oligopeptidase and omega-peptidase (Rawling and Barret, 2004). There are over 40 serine protease families and grouped in 6 clans (Rawling et al., 2006). Structures of different clans are distinct suggesting at least four evolutionary foundations of serine proteases. However, the reaction mechanisms of several protease clans are similar (Barrett and Rawling, 1995). For example, Chymotrypsin, subtilisin and carboxypeptidase C clans have similar catalytic triad of serine, aspartate and histidine but their geometric order is different (Dodson and Wlodawer, 1998).

The subtilisin like proteases, generally known as subtilases, may be sub classified into two subfamilies as kexins and pyrolysins. Most mammalian subtilases belong to former subfamily whereas the majority of plant subtilases belong to the latter (Antão and

Abbreviations: BAPA, NR-benzoylarginine *p*-nitroanilide; BLAST, basic local alignment search tool; BSA, bovine serum albumin; DFP, diisopropylfluorophosphate; DMSO, dimethyl sulfoxide; DTNB, 5,5μ-dithiobis(2-nitrobenzoic acid); E-64, 3*S*)-3-(*N*-{(*S*)-1-[*N*-(4-guanidinobutyl)carbamoyl]3-ethylbutyl}carbamoyl)oxirane-2-carboxylic acid; EDTA, ethylene diammine tetra acetic acid; EGTA, ethylene glycol-bis(α-amino ethyl ether)tetraacetic acid; GuHCl, guanidine hydrochloride; GuSCN, guanidine isothiocynate; IAA, iodoacetic acid; MALDI-TOF, matrix assisted laser desorption/ionization time-of-flight; MES, 2-(*N*-morpholino)ethanesulfonic acid; NCBI, National Center for Biotechnology Information; NEM, *N*-ethyl maleimide; PAGE, polyacrylamide gel electrophoresis; PCMB, *p*-chloromercuribenzoate; PMSF, phenylmethanesulfonyl fluoride; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate.

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Malcata, 2005). Plant serine proteases, especially subtilases, are known to involve in many physiological and regulatory processes in the plants. However the role of subtilases in plants is not clear due to lack of information on the processing sites of the enzyme and substrates as well. Therefore, exploration for existence of valuable serine proteases as well as understanding the appropriate physiological role of such proteases in plants is still an open area of investigation.

Mulberry tree, a plant of the family of *Moraceae* and genus Morus, has been widely cultivated in countries all over the world including temperate to tropical areas; sea level to high altitudes; as well as humid tropics to semi arid lands (FAO Agricultural Services Bulletin, 1990). Different parts of the plant are used as herbal medicine for blood serum glucose reduction; cholesterol and lipids levels reduction; fighting arterial plaques and antiphlogistic; diuretic and expectorant effects (Andallu et al., 2001; Andallu and Varadacharyulu, 2003; Doi et al., 2000; Jang et al., 2002). Many of the medicinal applications have been proved by clinical studies and various compounds responsible for such therapeutic benefits, have been recognized (Asano et al., 2001; Cheon et al., 2000; Doi et al., 2001; Kim et al., 1999; Nomura, 1999). The screening of different parts of the plant for biochemical constituents, substantial amount of proteolytic activity is observed in the latex of the plant. This manuscript describes the identification, purification and biochemical as well as immunological characterization of a serine protease in the latex of *Morus indica* cv K2.

2. Results and discussion

2.1. Purification of the enzyme

A dimeric serine protease from the latex of *M. indica* cv. K2 was purifed to homogeneity by simple procedure using hydrophobic ineraction chromatography and gel filtration. The crude latex (devoid of gum and any insoluble material) was subjected to ammonium sulfate precipitation in 25 mM MES buffer pH 6.5 and applied to ether-toyopearl fast flow column pre-equilibrated in the same buffer. The unbound material to column as well as buffer wash of the column did not show any caseinolytic activity. The bound proteins were eluted from the column with decreasing concentration of ammonium sulphate while the column elution profile resolved in to three peaks as shown in Fig. 1a. Fractions of all the peaks were assayed for caseinolytic activity and subjected to SDS–PAGE to check the purity. The magnitude of activity as well as purity of the fractions of peak III is higher relative to the pools from peak II and I. Therefore, the pool of fractions of peak III was subjected to further purification. However, the pool of fractions of peak II is heterogeneous with a predominant band around 66 kDa molecular mass (data not shown). Preliminary examination of the proteins from the three peaks, such as temperature and pH optima, reaction kinetics, reveal that the proteins are different and eluted at different concentrations of ammonium sulphate in hydrophobic interaction chromatography. The pool of proteins from peak III was subjected to gel filtration on Sephacryl S-200 (Fig. 1b). The elution profile constitute of a major symmetrical peak followed by small broad peak. The active and homogenous fractions of the former peak were pooled, concentrated, dialyzed against 25 mM MES buffer, pH 6.5 for further use. Typically, the yield of purified protein is 21 ± 1% with specific activity 45 ± 0.5 U/mg. The purified protein is named as indicain according to protease nomenclature. The purification of indicain in a typical batch is summarized in Table 1. The purification protocol is highly reproducible and the yield of the enzyme as well as specific activity was consistent. Appearance of three peaks with activity suggests the presence of at least three different proteases in the latex of *M. indica*, authenticating the diversity of proteases in the plant latex.

2.2. Homogeneity and physical properties of indicain

Indicain migrates as single band on SDS–PAGE under reducing and non-reducing conditions as well and indicate that the purified protein is single entity as shown in lane 1 of Fig. 2a with a molecular mass of 134 kDa. Purified protein migrates as single band if protein sample is exposed to high temperature or very low pH (<2.0) in the absence of reducing agent (lane 2) with a molecular mass of 67 kDa. The mobility pattern of indicain on SDS–PAGE indicates that the protein is a dimer and susceptible weak bonds hold the monomers of the protein. Absence of monomerization in the presence of reducing agent rules out disulfide linking of the monomers. The proteolytic nature of indicain was also confirmed by casein zymography, where digested casein appeared as white band corresponding to the position of the protein in the gel (Fig. 2b). Similarly, the purified protein appeared as single band on isoelectric focusing with an approximate isoelectric point pH 4.8 (Fig 2c). There was no detectable carbohydrate moiety in the molecular structure of the purified protein. MALDI-TOF analysis also confirms the purity of the enzyme. The biochemical properties of indicain are compared to some of other well-known subtilases in Table 2.

2.3. Oligomeric state and molecular mass determination

MALDI-TOF of the purified protein appeared as two peaks of 67.195 and 67.303 kDa (Fig. 3) molecular mass. It is likely that during the process the subunits held by weak interactions dissociates resulting two monomers with differing molecular mass probably due to some post-translational modification. A molecular mass of 134 kDa was used for all the experimental calculations. The dimeric nature of indicain was also confirmed by gel filtration on Superdex S-200 where the native protein is eluted as single symmetric peak with an apparent molecular mass of 134 kDa. If the protein sample is exposed to high temperature or very low pH (<2.0) the enzyme elutes as single symmetric peak with an apparent molecular mass of 67 kDa (Fig. 4). This observation confirms the dimeric nature of the protein and the monomers are held by weak interactions, prone to disruption by heating or exposure to low pH. The monomeric form is inactive; dimerization is pre-requisite for activity suggesting either active site is located in the dimeric interface or complete catalytic site may be located in individual monomers but dimerization is still required for the activity may be the dimeric interface has some unique surface and large hydrophobic patches which is necessary for binding of the substrate. Indicain is a typical case of plant serine proteases with dimeric nature. However, occurrence of few dimeric serine proteases from non-plant sources like viruses is known (Darke et al., 1996; Shimba et al., 2004; Coelho et al., 2005). Further studies on indicain would be of immense importance in finding the precise physiological function of dimeric serine proteases.

2.4. Specific amino acid residues

The tryptophan and tyrosine contents of the protein were 52 (measured value 52.42 ± 0.05) and 198 (measured value 198.39 ± 0.05), respectively. The total sulfhydryl content of the protein was found to be 42 (measured value 42.18 ± 0.05) forming 21 disulfide bonds. Under the similar experimental conditions Ribonuclease, Papain, Proteinase K, and Lysozyme gave the reported values validating the current measurements. The extinction coefficient of indicain was 41.24 by spectrophotometric method.

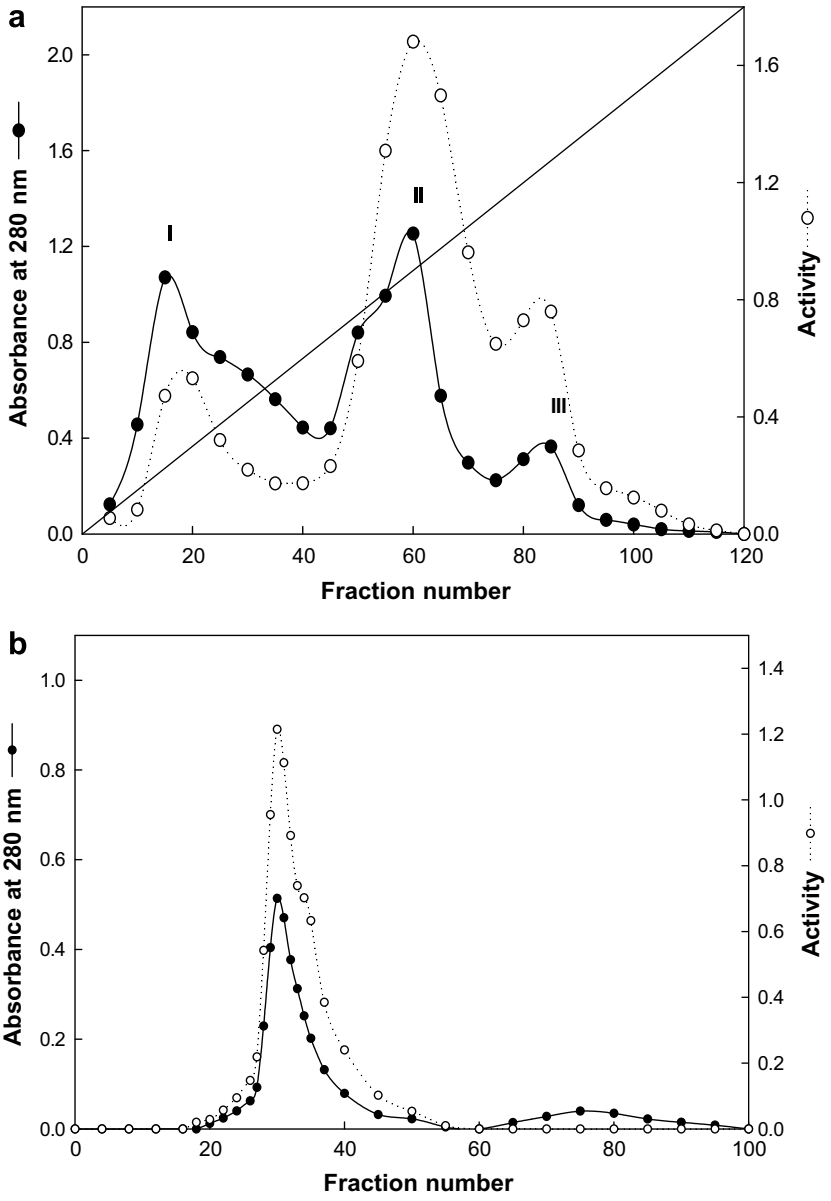

Fig. 1. Column chromatography of curde latex after removal of gum. (a) Hydrophobic interaction chromatography on Ether-toyopearl 650 S. Latex after removal of the gum and insoluble material was subjected to 80% ammonium sulphate precipitation. The precipitate was dissolved in 50 mM MES buffer pH 6.5 containing 1.5 M ammonium sulphate and loaded on Ether-toyopearl 650S. The bound proteins were eluted with decreasing concentration of ammonium sulphate. Fractions of 3 ml were collected. (b) Gel filtration on Sephacryl S-200 HR. Peak III fractions from the HIC elution profile were concentrated and loaded on Sephacryl S-200. All fractions of elution in the two chromatographies were assayed for activity (○) and protein content (●).

Table 1
Purification scheme for the protease indicain from the latex of *Morus Indica*

Steps	Total protein (mg)	Total activity[a] (units)	Specific activity (unit/mg)	Purification fold	Yield (%)
Crude extract	64.5	1430.61	22.18	1.00	100.0
Ammonium sulphate precipitation (80%)	47.0	1169.83	24.89	1.12	81.8
HIC ETP column	10.5	372.96	35.52	1.60	26.1
Gel filtration Sephacryl S-200	6.6	299.24	45.34	2.04	20.9

[a] One unit of enzyme activity is defined as the amount of enzyme that, under conditions described, gives rise to an increase of one unit of absorbance at 280 nm per 30 min of digestion.

2.5. pH and temperature optima

The hydrolyzing activity of indicain as a function of pH is shown Fig. 5a. It retains at least 50% of the proteolytic activity in the pH range of 5.5–12.0 with optimum activity at pH 8.0. The enzyme also retains >50% of the proteolytic activity in the temperature of 50–85 °C and the optimum activity is observed at 80 °C.

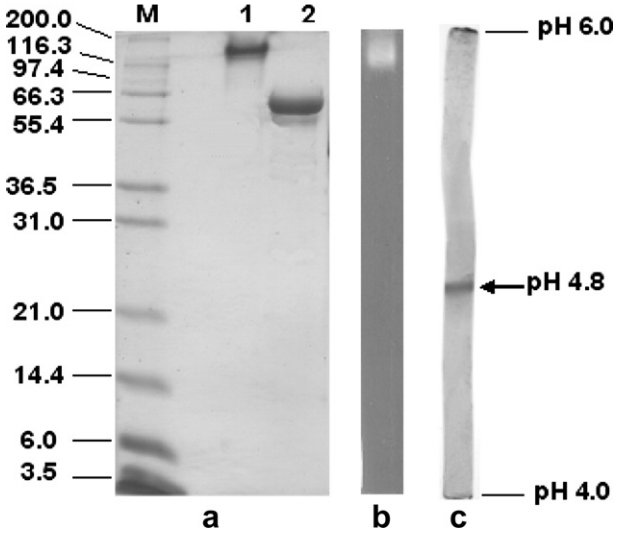

Fig. 2. Electrophoretic analysis of the purified protease. (a) SDS–PAGE: (Lane M) markers; Lane 1. indicain in reducing conditions. Lane 2. Purified protein was exposed to 90C for 15 min. (b) Zymogram (in gel activity) of indicain. Clear region showed the hydrolysis of casein by the enzyme. (c) Isoelectric focusing of indicain. Isoelectric focusing was performed using 5% polyacrylamide disc gels with ampholine ampholytes, pH 4.0–6.0. 100 μg of protein sample was loaded and electrophoressed for three hours at constant voltage of 300Volts.Isoelectric point of the purified protease is indicated by arrow.

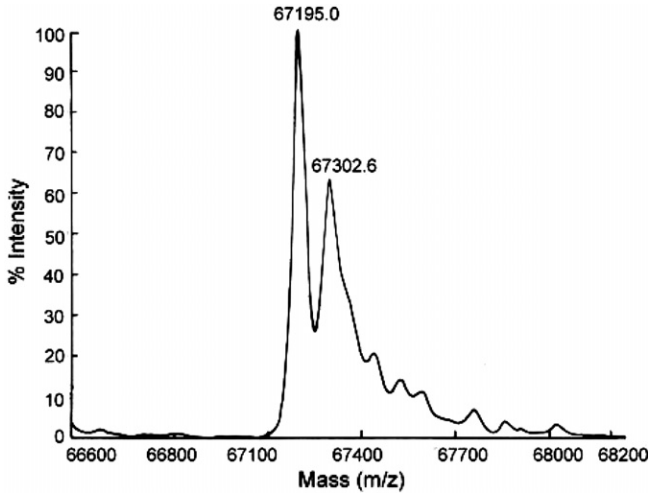

Fig. 3. Mass determination of indicain by MALDI-TOF MSES+. An external calibration was made using horse heart myoglobin. Data were processed using Mass Lynx software.

2.6. Stability

The stability of indicain as a function of pH and temperature is shown in Fig. 5b. The enzyme retains more than 80% of the activity in the range of pH 2.5–12.0. Similar amount of activity is seen in the temperature range of 25–90 °C. The stability of indicain in the presence of different additives commonly used in protein chemistry are also studied and summarized in Table 3. The enzyme is fairly stable and resembles subtilases in stability under adverse conditions like pH, strong denaturants, temperature, and organic solvents, which facilitate to explore the possibilities of utilization of the enzyme in industrial and biotechnological applications.

2.7. Effect of inhibitors

The effect of different inhibitors on the activity of the purified indicain was also studied. The minimum amount of inhibitor required for maximum inhibition is reported in Fig. 6. Inhibitors of cysteine proteases, metallopeptidases and trypsin like proteases do not affect the enzymatic activity. The enzyme retains only 1% activity with 1 mM PMSF and 0.2 mM chymostatin; it retains only 2% activity with $HgCl_2$. Chymostatin and PMSF inhibited indicain efficiently placing it in the class of serine proteases.

Table 2
Comparison of biochemical properties of indicain with other known plant serine proteases

Enzyme	Plant	Mol. mass (kDa)	pH optimum	Temp. optimum (°C)	Isoelectric point (pI)
Indicain	*Morus indica*	134.3	8.5	80	4.8
Protease[a]	*T. aestivum*	110.0	8–10	60	NR
Ara 12[b]	*A. thaliana*	76.1	5.0	80	NR
RSIP[c]	*Z. mays* L	59.0	6.0–6.5	NR	4.55
Cucumisin[d]	*C. melo* L	54.0	7.1	70	NR
Protease[e]	*C. cochinchinensis*	76.0	11.0	60	NR
Protease[f]	*P. hindsii*	82.0	6.5–10.5	30	NR
KLSP[g]	*P. vulgaris*	72.0	9.9	60	4.6
IBSP82[h]	*I. batatas*	82.0	7.9	40	NR
Artocarpin[i]	*A. heterophyllus*	79.5	8.0	60	6.3
Taraxilisin[j]	*T. officinale*	67.0	8.0	40	4.5
MCA[k]	*N. tobacum*	68.0	5–9	NR	5.8
Hordolisin[l]	*H.vulgare* L	74.0	6.0	60	6.9

NR in the table represents data not reported.
[a] Roberts et al. (2003).
[b] Hamilton et al. (2003).
[c] James et al. (1996).
[d] Yamagata et al. (1989).
[e] Uchikoba et al. (2000).
[f] Arima et al. (2000).
[g] Popovic et al. (2002).
[h] Chen et al. (2004).
[i] Prasad and Virupaksha. (1990).
[j] Rudenskaya et al. (1998).
[k] Messdaghi and Dietz. (2000).
[l] Terp et al. (2000).

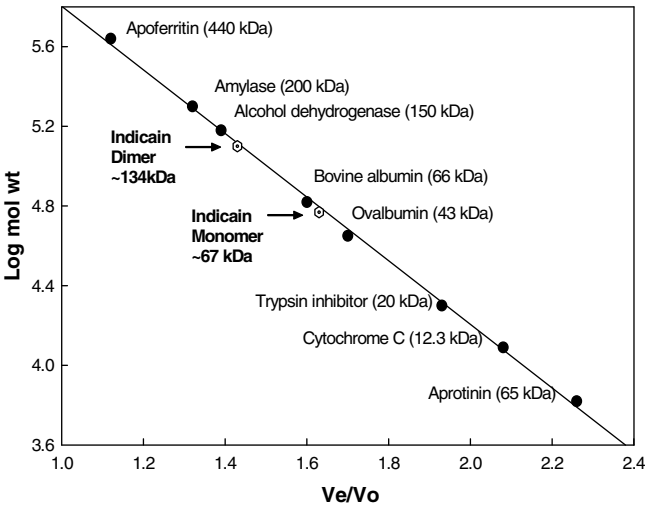

Fig. 4. Molecular mass assessment of dimeric and monomeric forms of indicain on gelfiltration. Indicain was loaded on on Superdex S-200 where the native protein is eluted as single symmetric peak with an apparent molecular mass of 134 kDa. If the protein sample is exposed to high temperature or very low pH (<2.0) the enzyme elutes as single symmetric peak with an apparent molecular mass 67 kDa. The dimeric and monomeric forms are indicated by arrow.

2.8. Substrate specificity

The enzyme hydrolyzes denatured natural substrates such as casein, and hemoglobin with high efficiency. The enzyme also shows amidolytic activity against synthetic substrates. The kinetic parameters of indicain with different substrates are listed in Table 4. Resembling other serine proteases, indicain showed amidolytic activity in addition to proteolytic activity. It hydrolyzes synthetic substrates such as Ala-Ala-*p*-nitroanilide, Alanine-*p*-nitroanilide, and Leucine-*p*-nitroanilide. Whereas the enzyme fails to hydrolyze BAPA, an ideal substrate for papain, ficin and other plant endopeptidases. This indicates that the enzyme acts mostly on the non-polar R group amino acids whereas its activity over polar and aromatic amino acid is not detectable.

2.9. Polyclonal antibodies and immunoassays

Polyclonal antibodies against indicain were raised in the male albino rabbit. The presence of antibodies in the anti rabbit serum was checked by Ouchterlony's double immunodiffusion method. Precipitin lines start appearing after 10–12 h of incubation at room temperature and are distinctly visible by about 24–30 h (Fig. 7a). The antibodies raised for indicain did not cross-react with morus 66 kDa protease (the pool of fractions of peak II of ETP column which is heterogeneous with a predominant band around 66 kDa molecular mass), carnein, a serine protease from noxious palnt weed Ipomoea carnea and crude cucumisin as shown in Fig. 7b. Similarly the polyclonal antibodies specific to indicain did not cross-react with the protein pool of fractions of Peak I of ETP column (data not shown). These observations confirm that the proteins present in the three active peaks seen on ETP column (Fig 1a) are distinct. The polyclonal antibodies raised for indicain would be of immense importance in detecting and as a ligand for various future studies.

2.10. N-terminal sequencing

The first 11 amino acid residues from N-terminal of indicain were determined and reported in Table 5 along with similar sequences of few other proteases. N-terminal sequence of indicain showed considerable similarity to the sequence of other plant subtilases when aligned using NCBI-BLAST and CLUSTALW. Highest similarity was with cucumisin and significant similarity with White gourd and *Medicago trunculata* proteases. Besides, the N-Terminal residues of indicain are also identical with other subtilisin like serine proteases. Therefore, indicain may be a member of subtilisin clan of proteases. Additional sequence analysis, catalytic site studies and structural determination may refine the classification of indicain.

3. Conclusion

This is the first report of purification and characterization of a subtilisin like dimeric serine protease from the latex of *M. indica*. The enzyme, indicain is fairly stable and resembles subtilases in stability under adverse conditions like pH, strong denaturants, temperature, and organic solvents, which facilitate to explore the possibilities of utilization of the enzyme in industrial and biotechnological applications.

4. Experimental

4.1. Materials

Sephacryl S-200 HR and Hi-Load Superdex 200 were purchased from Amersham Pharmacia. Ether-Toyopearl 650 S (TOSOH), BSA, Ribonuclease A, hen egg white lysozyme, azocasein, azoalbumin, hemoglobin, DTNB, DFP, IAA, Chymostatin, $HgCl_2$, E-64, GuHCl, GuSCN, glycerol, urea, *o*-phenanthroline, EDTA, EGTA, SBTI, NEM, β-mercaptoethanol, PMSF, Coomassie brilliant blue R-250, all synthetic substrates, Freund's complete and incomplete adjuvants, agarose were obtained from Sigma Chemical Co. (United States). Ampholine carrier ampholites were from LKB. All other chemicals were of the highest purity commercially available.

4.2. Purification of protein from the plant latex

4.2.1. Collection of latex, preliminary processing and ammonium sulphate precipitation

Latex was collected in 0.01 M Tris–HCl buffer pH 8.0 by superficial incisions on stems of *M. indica* cv K2 plants found abundantly in Banaras Hindu University campus, Varanasi, India and frozen at −20 °C for 24 h. The latex was thawed and centrifuged at 24,000g for 50 min to remove the gum and other insoluble material. The clear supernatant was subjected to 85% ammonium sulfate precipitation at 4 °C. After 24 h the resultant precipitate was recovered by centrifugation at 24,000g for 30 min at 4 °C.

4.2.2. Hydrophobic interaction chromatography

The precipitate obtained in the above step was resuspended in 25 mM MES buffer pH 6.5 containing 1.5 M ammonium sulphate and subjected to Hydrophobic Interaction Chromatography on an Ether-Toyopearl column (7 × 2 cm) pre-equilibrated with the same buffer. The column was washed with the same buffer and the proteins were eluted with decreasing concentration of ammonium sulphate in linear gradient of 1.5–0.0 M ammonium sulfate in the starting buffer at a flow rate of 3 ml/min. All the eluted fractions were assayed for protein content, extent of homogeneity and proteolytic activity by absorbance at 280 nm, SDS–PAGE and proteolytic activity measurement, respectively.

4.2.3. Gel filtration

Active fractions from the peak III of ETP column elution profile were pooled and concentrated using an Amicon membrane

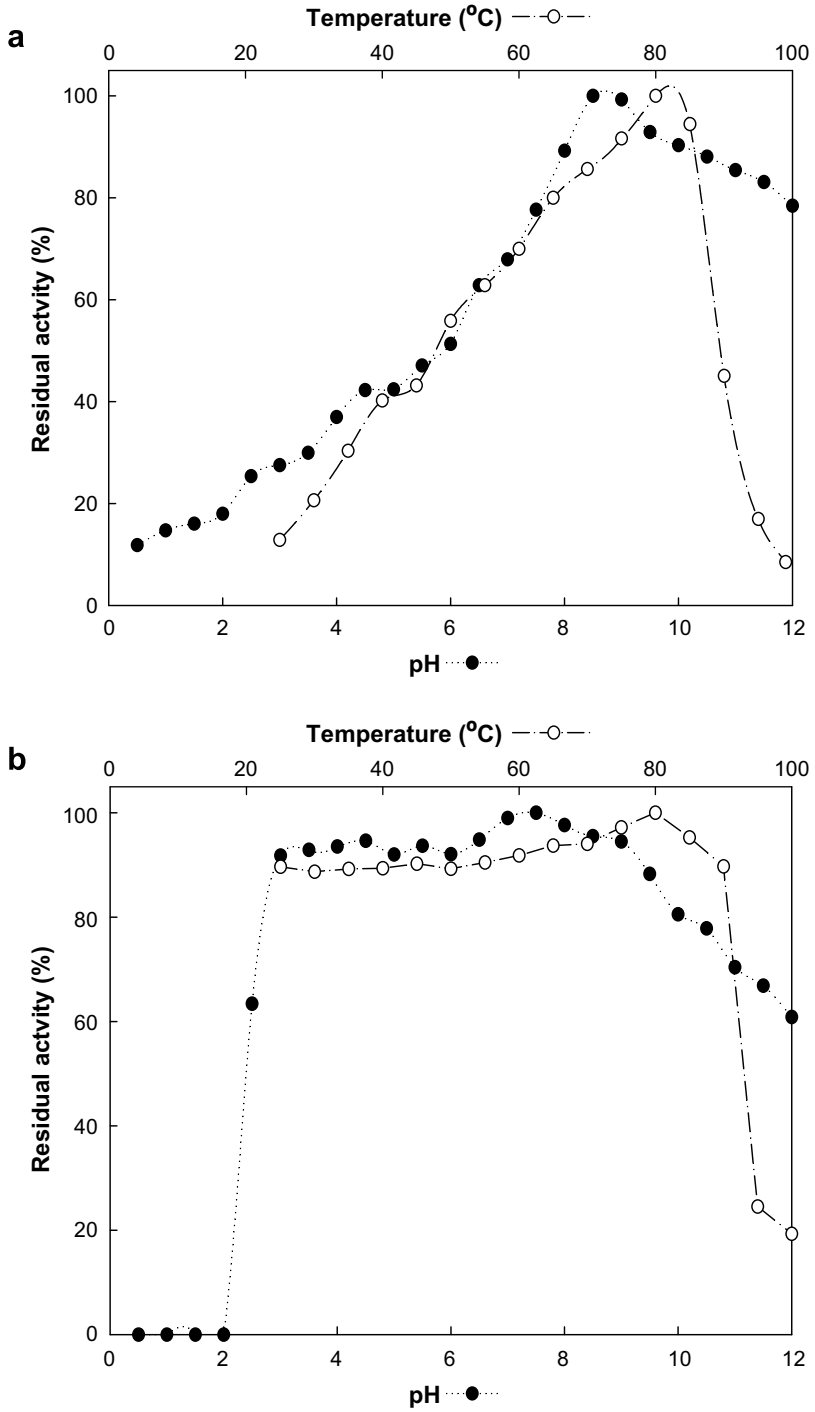

Fig. 5. Effect of temperature and pH on the activity of indicain. (a) Effect of temperature (●) and pH (○) on the activity of indicain. To determine the pH optima of the enzyme, the enzyme was incubated at different pH in the range of 0.5–12.0 and the activity was measured at the same pH. For temperature optima determination the enzyme sample was incubated at different temperatures for 15 min and the activity was measured at the same temperature. (b) Effect of temperature (●) and pH (○) on stability of indicain. The maximum activity was considered as 100% activity. To determine the pH stability the enzyme, the enzyme was incubated at different pH in the range of 0.5–12.0 for 24 h and the activity was measured at pH 8.0. For temperature stability determination the enzyme sample was incubated at different temperatures for 15 min and the activity was measured at 37 °C.

concentrator with 10 kDa cutoff. The resulting enzyme preparation was applied to gel filtration on Sehacryl S-200 HR (120 × 1 cm) preequilibrated with 25 mM MES buffer pH 6.5 containing 0.5 M NaCl and the column was eluted isocratically. All the fractions were analyzed as in the above step. The active and homogenous fractions were pooled, concentrated by ultrafiltration and stored at 4 °C for further experiments. The pure enzyme thus obtained was named as indicain.

Table 3
Stability of indicain under different conditions

Conditions	Concentration	Residual activity (%)
Urea	8.0 M	88
GuHCl	4.8 M	55
GuSCN	3.5 M	86
SDS	0.8%	62
DMSO	70%	58
Methanol	70%	53
Ethanol	60%	62
Isopropanol	55%	55
Butanol	25%	59
Acetonitrile	30%	84

Table 4
Kinetic parameter of indicain with different substrate

Substrates	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Casein	0.139 ± 0.002	92.9 ± 3.7	668.4 ± 33.4
Ala-Ala-pNA	6.570 ± 0.230	377.0 ± 22.0	57.5 ± 5.2
Leu-pNA	4.160 ± 0.090	258.6 ± 11.6	62.2 ± 4.2
Ala-pNA	2.860 ± 0.050	310.0 ± 15.2	108.4 ± 7.2

4.3. Protein concentration

Protein concentration was determined spectrophotometrically (absorbance at 280 nm) as well as by Bradford's method (Bradford, 1976) using BSA as standard.

4.4. Activity measurements

The hydrolyzing activity of the protease was monitored using denatured substrate casein and hemoglobin (Oteesen and Svendsen, 1970). In activity measurements, enzyme in 0.5 ml of 0.05 M Tris–HCl buffer pH 8.0 and incubated at 37 °C for thermal equilibration. After 15 min 0.5 ml of 1% (w/v) substrate was added and the reaction was allowed to proceed for 1 h at 37 °C. The reaction was terminated by an addition of 0.5 ml of 10% TCA and allowed to stand for 10 min. The resulting precipitate was removed by centrifugation at 10,000g for 10 min and the absorbance of TCA soluble peptides in the supernatant was measured at 280 nm. A control assay was done without any enzyme in the reaction mixture and used as reference. One unit of enzyme activity is defined as the amount of enzyme that, under conditions described, gives rise to an increase of one unit of absorbance at 280 nm per minute of digestion. The specific activity is the number of units of activity per milligram of protein.

The amidolytic activities of the enzyme were studied using BAPA, L-alanine alanine *p*-nitroanilide, succinyl phenylalanine *p*-nitroanilide, L-glutamyl *p*-nitroanilide, L-alanine *p*-nitroanilide, *N*-succinyl alanine alanine alanine *p*-nitroanilide and L-leucine *p*-nitroanilide by the method of Arnon (Arnon, 1970) with some modifications. The synthetic substrates (5–20 mM) were prepared by dissolving the required amount in minimum volume of DMSO and making up the final volume with 0.05 M Tris–HCl pH 8.0 buffers at 30 °C. The enzyme was incubated with the assay buffer and synthetic substrate at 37 °C for 30 min. The reaction was terminated by addition of acetic acid and the liberated *p*-nitroaniline was monitored spectrophotometrically by absorbance measurement at 410 nm against a blank sample containing no enzyme (Erlanger et al., 1961).

4.5. Electrophoresis and zymography

Electrophoresis was performed on 12% SDS–PAGE under non-reducing and reducing conditions (Laemmli, 1970). The proteins were stained with Coomassie brilliant blue R-250. Mark12 unstained from Invitrogen was used as molecular weight marker. For zymography, 1.2% casein was included in 12% polyacrylamide gel. Gel was run at 200 volts for 1 h and soaked in 2.5% Triton

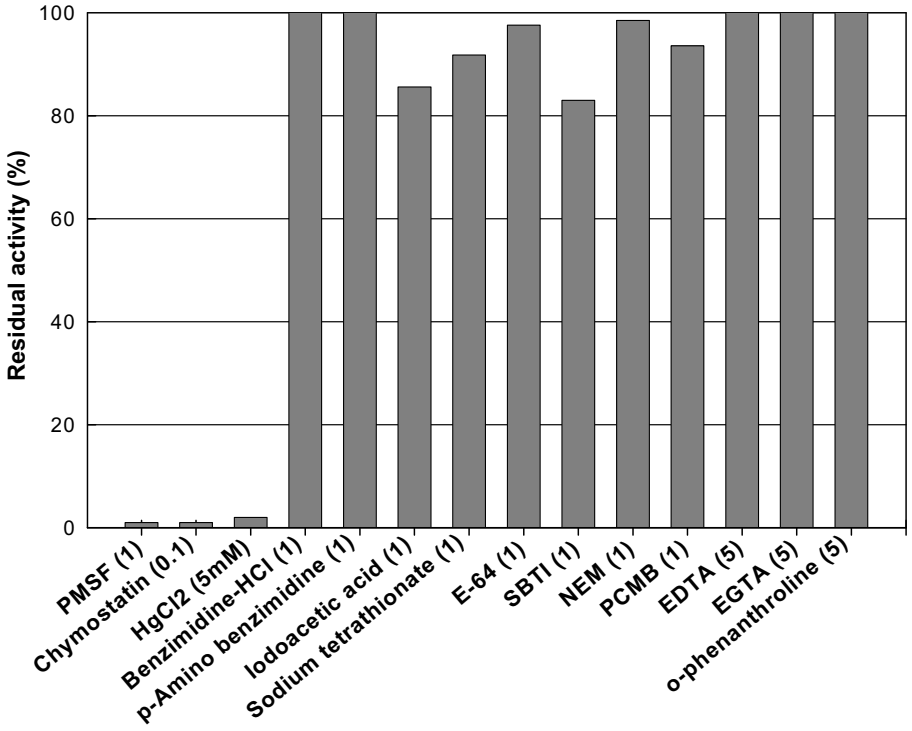

Fig. 6. Effect of inhibitors on the caseinolytic activity of indicain. The enzyme was pre-incubated in the assay buffer for 30 min with each compound at the specified concentration as indicated in the brackets and assayed for caseinolytic activity. The activity of the enzyme in the absence of any compound was taken as 100%.

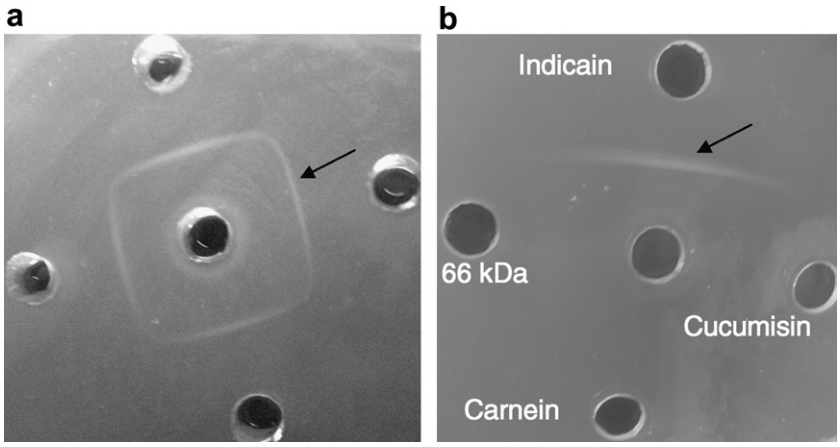

Fig. 7. Ouchterlony's double immunodiffusion. Anti-indicain serum was added in the central well and (a) indicain was added in the peripheral wells (b) indicain, Morus 66 kDa protease (peak II pool of elution profile of HIC column), carnein and crude cucumisin was added in the peripheral wells. The appearance of precipitin bands (indicated by arrow) was observed after 24 h of incubation.

Table 5
Comparison of N-terminal sequences of indicain with other known plant subtilases

protease	Amino terminal sequence (first 11 residues)											% Identity
Indicain	**T**	**T**	**N**	**S**	**W**	**D**	**F**	**I**	**G**	**F**	**P**	**100**
Cucumisin	**T**	**T**	R	**S**	**W**	**D**	**F**	L	**G**	**F**	**P**	**82**
C. metuliferous	**T**	**T**	R	**S**	**W**	**D**	**F**	L	**G**	**F**	**P**	**82**
White gourd	**T**	**T**	R	**S**	**W**	**D**	**F**	L	N	**F**	**P**	**73**
M.truncatula	**T**	**T**	R	**S**	**W**	**D**	**F**	**I**	**G**	V	H	**73**
Tomato P69B	**T**	**T**	R	**S**	P	T	**F**	L	**G**	L	E	**46**
Tomato P69A	**T**	**T**	H	T	S	S	**F**	L	**G**	L	Q	**36**
E. supina	**T**	**T**	R	T	P	N	**F**	L	**G**	L	V	**36**
ARA12	**T**	**T**	R	T	P	L	**F**	L	**G**	L	D	**36**
Lily LIM 9	**T**	**T**	H	T	P	**D**	Y	L	**G**	I	Q	**36**
Alnus AG12	**T**	**T**	H	T	P	R	**F**	L	S	L	N	**27**

X-100 for displacement of the SDS. Gels were incubated in reaction buffer (50 mM tris buffer, pH 8.0, 1 mM $CaCl_2$) for 15 h at 37 °C and later stained with Coomassie brilliant blue R-250. The unstained region of gel reveals the proteolytic activity of the proteases.

4.6. Mass spectrometry

Molecular mass of indicain was determined by mass spectrometry (MALDI-TOF MS ES+). Samples were dissolved at a concentration of 10 pmol/μl in 1:1 v/v 1% aqueous formic acid and methanol. Positive ionization was used for the sample analyses with an electro spray voltage of 1.0 kV. A sampling cone voltage of 40 V and MCP detector of 2700 V was adjusted. Nitrogen was employed as the API gas and data were acquired over the appropriate *m*/*z* range at a scan speed of 3.0 s in continuum mode. An external calibration was made using horse heart myoglobin (MW 16951.5 Da) and data were processed using the MassLynx suite of software programs supplied with the mass spectrometer.

4.7. Isoelectric focussing

The isoelectric point (pI) of purified indicain was determined by isoelectric focusing on polyacrylamide disc gels (0.6 × 10.0 cm) as described (Kundu et al., 2000). Electrophoretic runs were carried out with ampholine carrier ampholytes 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. Enzymes in IEF–PAGE gel were visualized by Coomassie G-250 staining.

4.8. Oligomeric state and molecular mass of the protein

The molecular mass and oligomeric state of indicain was determined using size exclusion chromatography. A column of Hi-Load Superdex-200 (1.6 × 60 cm) was equilibrated with 25 mM MES pH 8.0 containing 0.5 M NaCl. The column was calibrated with apoferritin (440 kDa), amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine albumin (66 kDa), ovalbumin (43 kDa), trypsin inhibitor (20 kDa), cytochrome C (12.3 kDa) and aprotinin

(6.5 kDa) obtained from Sigma Chemical Co., USA. The protein samples were applied and eluted at a flow rate of 1.0 ml/min isocratically. The molecular mass of the unknown sample was deduced from the plot of V_e /V_o versus log molecular mass of the known proteins.

4.9. pH and temperature optima

Dependence of the enzyme activity on pH was determined using following buffers in 0.05 M concentration: KCl–HCl (pH 0.5–1.5), sodium acetate (pH 2.5–5.5), sodium-potassium phosphate (pH 5.5–7.5), Tris–HCl (pH 7.5–9.0) and glycine/NaOH (pH 9–12). Casein/hemoglobin dissolved in corresponding buffer of required pH was used as substrate for the activity measurements of the enzyme. Denatured hemoglobin was used as substrate in the enzyme assays at low pH as casein is insoluble below pH 4.0 (Sarath et al., 1989). Activity measurements were carried as described above. Similarly the effect of temperature on the caseinolytic activity of the enzyme was carried out to determine the temperature optimum. Enzyme sample were incubated at different temperature in the range of 25–99 °C for 15 min and an aliquot was used for activity measurement at the same temperature.

4.10. Effect of different classes of inhibitors on the activity of indicain

The effect of increasing concentration of various protease inhibitors on hydrolysis of casein by the purified enzyme was monitored to identify the mechanistic class of the enzyme. The inhibitors used in the present study were specific to cysteine proteases (IAA, mercuric chloride, $HgCl_2$, E-64) serine proteases (DFP, PMSF, chymostatin, SBTI) metalloproteases (EGTA, EDTA, *o*-phenanthroline). In each case, 20 μg of enzyme was incubated in the presence of increasing concentration of the inhibitor in 0.05 M Tris–HCl buffer, pH 8.0, for 30 min at 37 °C and assayed for activity. A control assay of the enzyme activity was done without inhibitors and the resulting activity was taken as 100%.

4.11. Enzyme kinetics

The effect of increasing substrate concentration on the reaction rate of the enzyme-catalyzed reaction was studied using casein and synthetic substrates. A definite amount of enzyme was incubated in the assay buffer and substrate concentration was in the range of 0–0.5 mM. Activity measurements were done as described ealier. A Lineweaver–Burk plot was drawn and the value of Michaelis–Menten Constant (K_m) and V_{max} was calculated (Segel, 1976). The value of the catalytic constant (K_{cat}) was obtained by dividing V_{max} by amount of enzyme in mole. The specificity constant was calculated by dividing k_{cat}/K_m.

4.12. Estimation of tyrosine and tryptophan content

The tyrosine and tryptophan contents of the enzyme were measured spectrophotometrically using the method of Goodwin and Morton (Goodwin and Morton, 1946). The absorbance spectra of the enzyme in 0.1 M NaOH was recorded between 300 and 220 nm using a Beckman DU 640 B spectrophotometer and the absorbance values at 280 and 294.4 nm were deduced from the spectra. The standard formula given by Goodwin and Morton was used to estimate the tryptophan and tyrosine contents. To validate the current estimations tryptophan and tyrosine, contents of Papain, Ribonuclease, BSA and Lysozyme were determined in similar experimental conditions.

4.13. Estimation of free and total sulfhydryl content of indicain

The exposed and total cysteine residues of the enzyme were estimated by Ellman's method (Ellman, 1959). For exposed sulfhydryl group estimation; the purified enzyme was activated with 0.01 M β-mercaptoethanol in 0.05 M Tris–HCl buffer, pH 8.0, for 15 min, and then dialyzed against 0.1 M acetic acid at 4 °C for 24 h with frequent changes. For the estimation of total sulfhydryl content, the enzyme was reduced in the presence of 6 M GuHCl for 15 min at 37 °C and dialyzed against 0.1 M acetic acid (Creighton, 1989). To validate these estimations tryptophan, tyrosine, exposed and total sulfhydryl contents of Papain, Ribonuclease, BSA and lysozyme were determined in similar experimental conditions.

4.14. Extinction coefficient

The extinction coefficient of the enzyme was determined by spectrophotometric method (Aitken and Learmoth, 1997). Several solutions of the enzyme were prepared by serial dilutions and the concentration of the enzyme in each sample was determined by Bradford method. The absorbance of each sample at 280 nm was measured and extinction coefficient of the enzyme was calculated using Beer–Lambert's law. In spectrophotometric method, the extinction coefficient was determined using formula given by Aitken and Learmoth.

4.15. Stability

The ability of the purified enzyme to retain its activity under conditions of extreme pH, strong denaturants, temperatures, detergents and organic solvents was studied by exposing the enzyme to the respective conditions. 20 μg of enzyme was incubated at different pH in the range of pH 0.5–12.0, varying concentration of denaturants, detergents and organic solvents for 24 h. The enzyme after being exposed to different condition was incubated with Tris–HCl pH 8.0 and casein for 30 min at 37 °C and assayed.

4.16. Antigenic properties

Antibodies to the purified enzyme were raised in a male albino rabbit (1 kg body mass) as described (Kundu et al., 2000) and the presence of antibodies was confirmed by immunoassays. Pre-immune serum obtained from the rabbit before first injection of antigen and after the last booster dose was stored at −20 °C. The presence of antibodies was confirmed by Ouchterlony's double immunodiffusion (Ouchterlony and Nilsson, 1986). 1% agarose in phosphate-buffer saline containing 0.02% sodium azide was solidified in Petri dishes and appropriate holes were punched into it. Various antigens (40 μg) of crude cucumisin, carnein, a serine protease from noxious plant weed Ipomoea carnea (Patel et al., 2007), morus 66 kDa protease (the pool of fractions of peak II of ETP column which is heterogeneous with a predominant band around 66 kDa molecular mass) and the purified enzyme (indicain) in peripheral wells and 100 μl of antiserum were loaded in the central well and left at room temperature for 24 h.

4.17. N-terminal sequence

The protein sample for sequencing was electrophoresed according to the procedure given by Smith (Smith, 1997) and transferred as a blot onto a PVDF membrane. The N-terminal sequence was determined on an Applied Biosystem Procise Sequencer by Edman's automated degradation. The N terminal sequence of indicain was compared with other plant serine proteases using NCBI-BLAST and CLUSTAL W.

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References

Aitken, A., Learmoth, M., 1997. Protein determination by UV absorption. In: Walker, J.M. (Ed.), The Protein Protocol Handbook. Humana Press, Totowa, NJ, pp. 3–6.

Andallu, B., Suryakantham, V., Lakshmi, S.B., Reddy, G.K., 2001. Effect of mulberry (*Morus indica*) therapy on plasma erythrocyte membrane lipids in patients with type 2 diabetes. Clin. Chim. Acta 314, 47–53.

Andallu, B., Varadacharyulu, N.C., 2003. Antioxidant role of mulberry (*Morus indica* L cv. Anantha) leaves in streptozotocin-diabetic rats. Clin. Chim. Acta 338, 3–10.

Antão, C.M., Malcata, F.X., 2005. Plant serine proteases: biochemical, physiological and molecular features. Plant Physiol. Biochem. 43, 637–650.

Arima, K., Uchikoba, T., Yonezawa, H., Shimada, M., Kaneda, M., 2000. Isolation and characterization of a serine protease from the sprouts of *Pleioblastus hindsii* Nakai. Phytochemistry 54, 559–565.

Arnon, R., 1970. Papain. Methods Enzymol. 19, 226–244.

Asano, N., Yamashita, T., Yasuda, K., Ikeda, K., Kizu, H., Kameda, Y., Kato, A., Nash, R.J., Lee, H.S., Sun Ryu, K., 2001. Polyhydroxylated alkaloids isolated from mulberry trees (*Morus alba* L) and silkworms (*Bombix mori* L.). J. Agric. Food Chem. 49, 4208–4213.

Barrett, A.J., Rawling, N.D., 1995. Families and clans of serine peptidases. Arch. Biochem. Biophys. 318, 247–250.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Chen, T.E., Huang, D.J., Lin, Y.H., 2004. Isolation and characterization of a serine protease from the storage roots of sweet potato (*Ipomoea batatas* [L] Lam). Plant Sci. 166, 1019–1026.

Cheon, B.S., Kim, Y.H., Son, K.S., Chang, H.W., Kang, S.S., Kim, H.P., 2000. Effects of prenylated flavonoid and biflavonoids on lipolysaccharide-induced nitric oxide production from the mouse macrophage cell line RAW 264 7. Planta Med. 66, 596–600.

Coelho, M.G.P., Simone, S.G. De., Da Silva-Lopez, R.E., 2005. Characterization of an extracellular serine protease of Leishmania (Leishmania) amazonensis. Parasitology 131, 85–96.

Creighton, T.E., 1989. Disulphide bonds between cysteine residues. In: Creighton, T.E. (Ed.), Protein Structure – Practical Approach. IRL Press, Oxford, pp. 155–160.

Darke, P.L., Cole, J.L., Waxman, L., Hall, D.L., Sardana, M.K., Kuo, L.C., 1996. Active human cytomegalovirus protease is a dimer. J. Biol. Chem. 271, 7445–7449.

Dodson, G., Wlodawer, A., 1998. Catalytic triads and their relatives. Trends Biochem. Sci. 23, 347–352.

Doi, K., Kojima, T., Fujimoto, Y., 2000. Mulberry leaf extract inhibits the oxidative modification of rabbit and human low-density lipoprotein. Biol. Pharm. Bull. 23, 1066–1071.

Doi, K., Kojima, T., Makino, M., Kimura, Y., Fujimoto, Y., 2001. Studies on the constituents of the leaves of *Morus alba* L. Chem. Pharm. Bull. 49, 151–153.

Ellman, L., 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. 82, 70–77.

Erlanger, B.F., Kokowsky, N., Cohen, N., 1961. The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. 95, 271–278.

FAO Sericulture training manual, 1990. FAO Agricultural Services Bulletin 80, 117.

Goodwin, W., Morton, R.A., 1946. The spectrophotometric determination of tyrosine and tryptophan in proteins. Biochem. J. 40, 628–632.

Hamilton, J.M.U., Simpson, D.J., Hyman, S.C., Ndimba, B.G., Slabas, A.R., 2003. Ara12 subtilisin-like protease from *Arabidopsis thaliana*: purification, substrate specificity and tissue localization. Biochem. J. 370, 57–67.

James, F., Brouquisse, R., Suire, C., Pradet, A., Raymond, P., 1996. Purification and biochemical characterization of a vacuolar serine endopeptidase induced by glucose starvation in maize roots. Biochem. J. 320, 283–292.

Jang, M.H., Kim, H., Shin, M.C., Lim, B.V., Lee, T.H., Jung, S.B., Kim, C.J., Kim, E.H., 2002. Administration of folium Mori extract decreases nitric oxide synthase expression in the hypothalamus of rats. Jpn. J. Pharmacol. 90, 189–192.

Kaneda, M., Tominaga, N., 1975. Isolation and characterization of a proteinase from the sarcocarp of melon fruit. J. Biochem (Tokyo) 78, 1287–1296.

Kim, H.B., Choung, W.Y., Ryu, K.S., 1999. Sensory characteristics and blood glucose lowering effect of ice-cream containing mulberry leaf powder. Korean J. Seric. Sci., 129–134.

Kundu, S., Sundd, M., Jagannadham, M.V., 2000. Purification and characterization of a stable cysteine protease ervatamin B, with two disulfide bridges, from the Latex of *Ervatamia coronaria*. J. Agric. Food Chem. 48, 171–179.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 6850–6855.

Messdaghi, D., Dietz, K., 2000. Characterization of an extracellular chymostatin-sensitive serine protease preferentially expressed in young plant tissues. Biochim. Biophys. Acta 1480, 107–116.

Nomura, T., 1999. The chemistry and biosynthesis of isoprenilated flavonoids from Moraceous plants. Pure Appl. Chem. 71, 1115–1118.

Oteesen, M., Svendsen, I.B., 1970. The subtilisins. Methods Enzymol. 19, 199–215.

Ouchterlony, O., Nilsson, L.A., 1986. Immunodiffusion and immunoelectrophoresis. In: Weir, D.M., Herzerberg, L.A., Blackwell, C. (Eds.), The Hand book of Experimental Immunology, vol. 1. Blackwell, Oxford, pp. 321–325.

Patel, A.K., Singh, V.K., Jagannadham, M.V., 2007. Carnein, a serine protease from noxious plant weed *Ipomoea carnea* (Morning Glory). J. Agric. Food Chem. 55, 5809–5818.

Popovic, T., Puizdar, V., Brzin, J., 2002. A novel subtilase from common bean leaves. FEBS Lett. 530, 163–168.

Prasad, K.M.R., Virupaksha, T.K., 1990. Purification and characterization of a protease from jack fruit latex. Phytochemistry 29, 1763–1766.

Rawling, N.D., Barret, A.J., 2004. Families of serine peptidases. Methods Enzymol. 244, 19–61.

Rawling, N.D., Morton, F.R., Barrett, A.J., 2006. MEROPS: the peptidase database. Nucleic Acids Res. 34, D270–D272.

Roberts, I.N., Murray, P.F., Caputo, C.P., Passeron, S., Barneix, A.J., 2003. Purification and characterization of a subtilisin-like serine protease induced during the senescence of wheat leaves. Physiol. Plant 118, 483–490.

Rudenskaya, G.N., Bogacheva, A.M., Preusser, A., Kuznetsova, A.V., Dunaevsky, Y.E., Golovkin, B.N., Stepanov, V.M., 1998. Taraxalisin-a serine protease from dandelion *Taraxacum offýcinale* Webb s.l. FEBS Lett. 437, 237–240.

Sarath, G., Motte, R.S., Wagner, F.W., 1989. Protease assay methods. In: Beynon, R.J., Bond, J.S. (Eds.), Proteolytic Enzymes – A Practical Approach. IRL Press, Oxford, pp. 25–55.

Schaller, A., 2004. A cut above the rest: the regulatory function of plant proteases. Planta 220, 183–197.

Segel, I.H., 1976. Enzymes. In: Biochemical Calculations, second ed. Wiley and Sons, New York, pp. 208–323.

Shimba, N., Nomura, A.M., Marnett, A.B., Craik, C.S., 2004. Herpesvirus protease inhibition by dimer disruption. J. Virol. 78, 6657–6665.

Smith, B.J., 1997. Protein sequencing protocols. In: Smith, B.J. (Ed.), Methods in Molecular Biology, vol. 64. Humana Press Inc., Totowa, NJ, pp. 17–24.

Terp, N., Thomsen, K.K., Svendsen, I., Davy, A., Simpson, D.J., 2000. Purification and characterization of hordolisin, a subtilisin-like serine endoprotease from barley. J. Plant Physiol. 156, 468–476.

Uchikoba, T., Arima, K., Shimada, M., Yonezawa, H., Kaneda, M., 2000. Isolation and some properties of a serine protease from the fruits of *Cudrania cochinchinensis* (Lour) Kudo et. Masam. Biosci. Biotechnol. Biochem. 64, 623–624.

Yamagata, H., Ueno, S., Iwasaki, T., 1989. Isolation and characterization of a possible native cucumisin from developing melon fruits and its limited autolysis to cucumisin. Agric. Biol. Chem. 53, 1009–1017.