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An unusual thermostable aspartic protease from the latex of *Ficus racemosa* (L.)

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Abstract

The most extensively studied ficins have been isolated from the latex of *Ficus glabrata* and *Ficus carica.* However the proteases (ficins) from other species are less known. The purification and characterization of a protease from the latex of *Ficus racemosa* is reported. The enzyme purified to homogeneity is a single polypeptide chain of molecular weight of 44,500 ± 500 Da as determined by MALDI-TOF. The enzyme exhibited a broad spectrum of pH optima between pH 4.5–6.5 and showed maximum activity at 60 ± 0.5 °C. The enzyme activity was completely inhibited by pepstatin-A indicating that the purified enzyme is an aspartic protease. Far-UV circular dichroic spectra revealed that the purified enzyme contains predominantly β-structures. The purified protease is thermostable. The apparent T_m, (mid point of thermal inactivation) was found to be 70 ± 0.5 °C. Thermal inactivation was found to follow first order kinetics at pH 5.5. Activation energy (E_a) was found to be 44.0 ± 0.3 kcal mol^{-1}. The activation enthalpy (ΔH^*), free energy change (ΔG^*) and entropy (ΔS^*) were estimated to be 43 ± 4 kcal mol^{-1}, −26 ± 3 kcal mol^{-1} and 204 ± 10 cal mol^{-1} K^{-1}, respectively. Its enzymatic specificity studied using oxidized B chain of insulin indicates that the protease preferably hydrolyzed peptide bonds C-terminal to glutamate, leucine and phenylalanine (at P_1 position). The broad specificity, pH optima and elevated thermal stability indicate the protease is distinct from other known ficins and would find applications in many sectors for its unique properties.

Keywords: *Ficus racemosa*; *Moraceae*; Aspartic protease; Plant proteases; Endopeptidases; Ficin

1. Introduction

Proteolytic enzymes are multifunctional class of enzymes that have many physiological functions ranging from generalized protein digestion to more specific regulated processes such as the activation of zymogens, blood coagulation, complement activation, inflammation process and liberation of physiological peptides from the precursor proteins. They are ubiquitous in nature (Neurath, 1984). Proteolytic enzymes constitute nearly half of the commercially utilized enzymes; frequently used in food processing, tenderization of meat, brewing, cheese elaboration, bread manufacturing, leather and textile industry. Many proteolytic enzymes have been identified and studied from the latex of several plant families such as *Caricaceae, Moraceae, Asclepiadaceae, Apocynaceae* and *Euphorbiaceae* (Caffini et al., 1988). Most of the plant derived proteases have been classified as cysteine proteases and more rarely belong to aspartic proteases (Boller, 1986). Proteolytic enzymes from the plant sources have received special attention because of their property of being active over a wide range of temperature and pH (Uhlig, 1998). The quest for valuable proteases with distinct specificity is always a continuous challenge for varied industrial applications.

The name ficin has been used to describe the endoproteolytic enzyme activity in tree latex of the genus *Ficus* (Jones and Glazer, 1970). This genus contains more than 1800 named species making them one of the largest genuses in the *Moraceae* family. The ficins isolated from the latex of different *Ficus* trees possess different characteristic properties. The most extensively studied ficins (EC 3.4.22.3) are

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the cysteine proteases found in the latex of *Ficus glabrata* and *Ficus carica*. These cysteine proteases are known to exist in a number of charged forms (Englund et al., 1968; Kramer and Whitaker, 1964). Similar proteases have been isolated and studied from *Ficus anthelmintica* latex (Marini-Bettolo et al., 1963). These proteases differ from each other chromatographically, compositionally and quantitatively with an essential cysteine at the active site (Liener and Friedenson, 1970). Ficin-E isolated from *Ficus elastica* is a serine-centered protease, indicating for the first time that the latex of trees of the genus *Ficus* contains other classes of proteases (Lynn and Clevette-Radford, 1986).

The aim of this work is to identify new and unique plant proteases from *Ficus racemosa* that can be used in food and pharma industry. In the present investigation we report the purification and biochemical characteristics of an endoprotease from the latex of *F. racemosa*. It is shown that this protease is an aspartic protease hitherto not known in the genus *Ficus* with novel cleavage specificities that would find applications both in food and pharma industry. This aspartic protease shares a number of common characteristics with several other plant aspartic proteases with respect to pH optimum, molecular weight, inhibition by pepstatin, thermostability and its secondary structure and stability.

2. Results and discussion

2.1. Purification of protease from the latex

F. racemosa is a moderate sized to large tree found in all parts of India in moist locality. Gelatin-embedded PAGE of the crude extracts of *F. racemosa* latex used specifically to detect the proteases indicated the presence of a single protease (Fig. 1a, inset). The presence of a single isoform in the *F. racemosa* latex is distinct as compared to the presence of several isoforms in the latex of other *Ficus* trees. The lattices of *F. glabrata* and *F. carica* are known to contain a number of ficins (Liener and Friedenson, 1970). This single protease isoform was purified to as per the criterion of MS (mass spectrometry) homogeneity from the latex by conventional techniques that included salt precipitation, molecular size exclusion and anion exchange chromatography as described in the methods.

The latex was clarified by centrifugation at 10,000*g* for 60 min. The clarified latex was saturated to 80% ammonium sulfate at 4 °C. This step afforded the removal of several non-protein components including the polyphenols that absorb at 280 nm. The total activity recovered was 95%. This first step served in the separation and concentration of protein. Size exclusion chromatography of the concentrate by HPLC on BioSep-SEC-S2000 column was used as the second step of purification. The proteins resolved into three major fractions (Fig. 1a). Fraction two that exhibited maximum activity was pooled separately and concentrated by using a centrifugal device

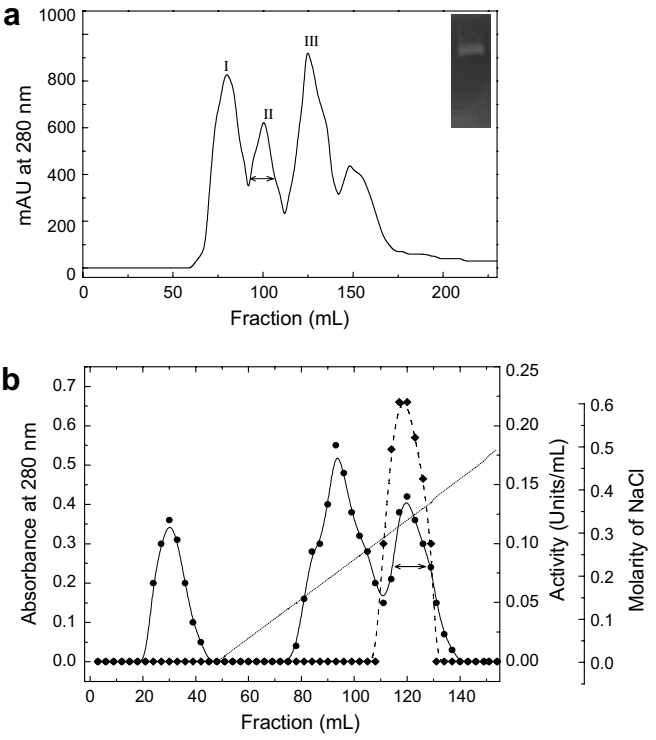

Fig. 1. (a) Size exclusion chromatography profile of the latex proteins on BioSep-SEC-S2000. The column was pre-equilibrated with 0.05 M Tris–HCl buffer pH 7.0. A flow rate of 5 mL/min was used and fractions were detected at 280 nm. The active fractions were pooled as shown. Inset: gelatin-embedded PAGE of crude extracts of *Ficus racemosa* latex showing the protease activity. (b) DEAE-Sephadex A-50 anion exchange chromatography profile of protein (–●–) and activity (--◆--). The column was washed with 0.05 M sodium acetate buffer pH 5.5 and protease eluted using a linear gradient of 0–0.5 M NaCl in the same buffer. The active fractions were pooled as shown.

(10,000 Da cutoff). This step afforded a 3.9-fold increase in the specific activity. A final step of anion exchange chromatography on DEAE-Sephadex A-50 was employed, to obtain the enzyme in a homogenous form. Several contaminating proteins were removed in the unbound fraction. A linear NaCl gradient (0–0.5 M) was used to elute the bound protease (Fig. 1b). The fractions showing a high specific activity were recovered and pooled as indicated. The pooled fraction was dialyzed extensively (5×1 L of 50 mM sodium acetate buffer pH 5.5). The results of the purification have been summarized in Table 1. The specific activity of the purified protease was 0.64 U/mg with a recovery of ~20% and existed as a single form with no isoenzymes. Among the several active components of commercial latex of *F. glabrata*, one major ficin was purified by CM-cellulose chromatography after reversible inhibition by sodium tetrathionate (Englund et al., 1968). Metrione et al. (1967) found evidence for three isoenzymes in a salt fractionated ficin. In contrast Sgarbieri et al. (1964) reported the separation of nine proteolytic components when salt fractionated *F. glabrata* latex was subjected to CM-cellulose chromatography.

Table 1
Purification of the protease from the latex of *Ficus racemosa*[a]

Steps	Total activity (Units)	Total protein (mg)	Specific activity (U/mg)	Purification (Fold)	Recovery (%)
Clarified latex	13.50	150	0.09	–	100
80% $(NH_4)_2SO_4$ precipitation	12.83	143	0.09	1	95
Size exclusion chromatography (BioSep-SEC-S2000)	6.07	17.3	0.35	3.9	45
DEAE-Sephadex A-50 chromatography	2.60	4.06	0.64	7.1	19.3

[a] These are the results of a typical purification from 50 mL of the clarified latex of *Ficus racemosa* and are reproducible.

2.2. Homogeneity and molecular mass

The homogeneity of the purified enzyme was evaluated by Native-PAGE and gelatin-embedded PAGE. The purified enzyme was homogeneous, migrating as a single species in native-PAGE (Fig. 2a, Lane 1). Gelatin-embedded PAGE used specifically to detect proteases also shows a single activity band (Fig. 2a, Lane 2). The single protein band visualized is an indicative of a single isoform. The apparent molecular mass of the purified enzyme estimated by SDS–PAGE is 43,500 ± 500 Da (Fig. 2b Inset) and by analytical gel filtration on a BioSep-SEC-S2000 column was 44,000 ± 1500 Da, indicated that the enzyme is a single polypeptide chain protein. The molecular weight also determined by MALDI-TOF was found to be 44,500 ± 500 Da (Fig. 2b). These results put together indicate that the purified protease is homogenous. The molecular mass of this protease differs from the other reported ficins. The molecular weight of the major ficin of *F. glabrata* determined by ultracentrifugation was 25,500 ± 750 Da (Englund et al., 1968). These results taken together showed that the purified protease of *F. racemosa* is distinct from other known ficins with respect to its existence as a single isoform and molecular weight.

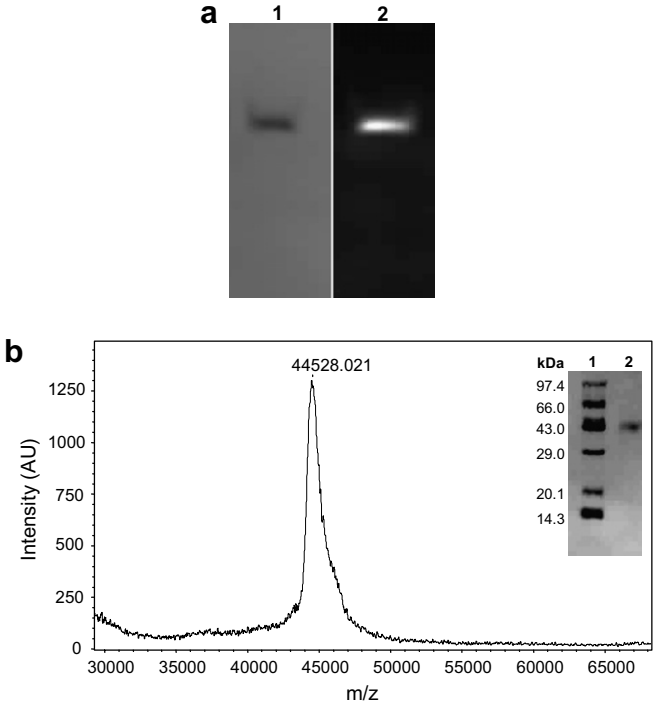

Fig. 2. (a) Lane 1: Native-PAGE (10% T, 2.7% C) of the purified protease at pH 8.8. Lane 2: Gelatin-embedded PAGE for detecting protease activity stained with Coomassie brilliant blue and (b) MALDI-TOF spectrum of the purified protease. Inset: SDS–PAGE (12% T, 2.7% C) of the purified protease. Lane 1: M_r markers, Lane 2: purified protease.

2.3. Effect of various inhibitors on the protease activity

Relative activity of the purified protease in presence of various inhibitors was carried out to establish the nature of the protease and its characteristics (Table 2). The inhibitors evaluated include serine protease inhibitors (DFP and PMSF), cysteine protease inhibitors (PCMB, iodoacetamide and sodium tetrathionate), metalloprotease inhibitor (EDTA) and the aspartic protease inhibitor (pepstatin-A). Pepstatin, a high binding inhibitor specific for aspartic protease, inhibits the enzyme at relatively low concentrations. At pepstatin-A concentration as low as 80 μM, the inhibition of azocasein digestion is complete. Cynarase A, an aspartic protease from artichoke showed a very low K_i for pepstatin of 30 nM (Sidarch et al., 2005). This Inhibition of the protease by pepstatin-A was further evaluated using oxidized insulin B chain as the substrate. The results show that in the presence of pepstatin-A, the peptide was not digested (Fig. 3a). These results further evidence that the protease is an aspartic protease. The proteolytic activity of this enzyme was not inhibited by PCMB, sodium tetrathionate and iodoacetamide ruling out that this protease is a thiol protease, as most proteases seen in the latex are thiol proteases in general. The ficin isolated from the latex of *F. glabrata* and *F. carica,* as well as papain from latex of *Carica papaya* are all thiol proteases (Englund et al., 1968; Kortt et al., 1974). Different from these, a serine protease

Table 2
Effect of inhibitors on the activity of the protease

Inhibitor	Concentration (mM)	Residual activity (%)
PCMB	1.0	98
Iodoacetamide	1.0	97
Sodium tetrathionate	1.0	99
DFP	1.0	97
PMSF	1.0	98
EDTA	5.0	99
Pepstatin-A	0.08	0

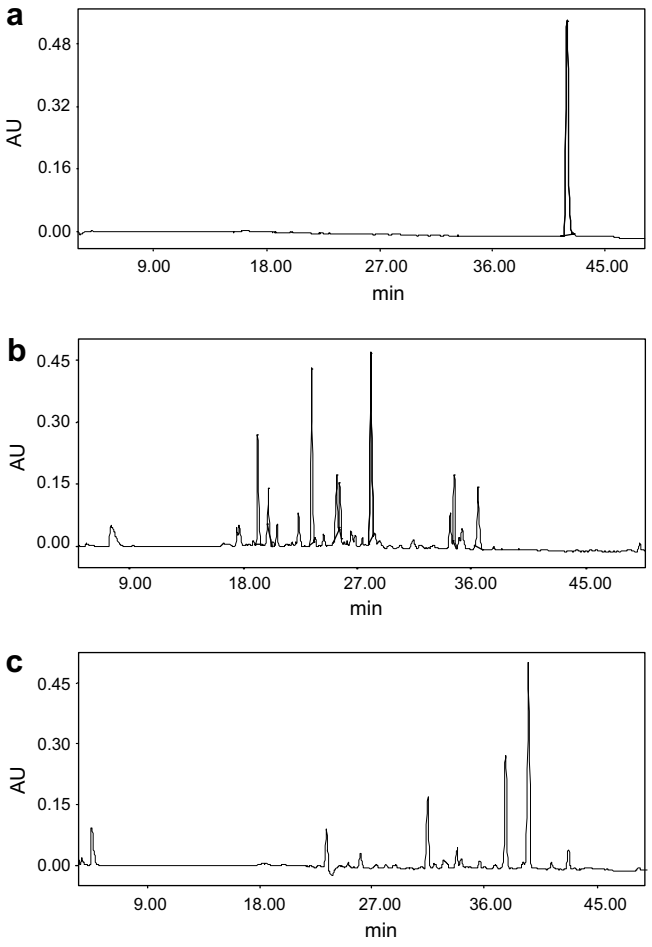

Fig. 3. (a) RP-HPLC profile showing the Pepstatin-A inhibition of the insulin B chain digestion by the protease of *Ficus racemosa*. (b) RP-HPLC profile of the *Ficus racemosa* protease cleaved peptides of oxidized B chain of insulin. (c) Cleavage pattern of B chain of insulin hydrolyzed by ficin from the latex of *Ficus carica*. The peptides were resolved using a Symetry Shield C-18 column (4.6 × 150 mm). The solvents used were 0.1% TFA and 70% acetonitrile containing 0.05% TFA. The peptides were detected at 230 nm. These peptides were subjected to amino-terminal sequencing to deduce the cleavage specificity.

has been reported in the latex of *Euphorbia milii* (Yadav et al., 2006).

2.4. Effect of pH and stability

The isolated protease had a pH activity profile as shown in Fig. 4a. The maximum activity of the purified enzyme with azocasein was observed between pH 4.5–6.5. The enzyme is relatively active at low pH exhibiting 80% of its activity at pH 4.0. However beyond pH 6.5 there is a sharp decline in the activity. Half the maximal activity is observed at pH 7.0. Varying the ionic strength from 0.1 to 0.6 M of the acetate buffer at pH 5.5 had no effect on the activity of the enzyme. Further increase in the ionic strength led to decrease in the activity. The enzyme also showed high stability over a wide pH range when incubated at 25 ± 2 °C for 120 min. The residual activity was 95–80%

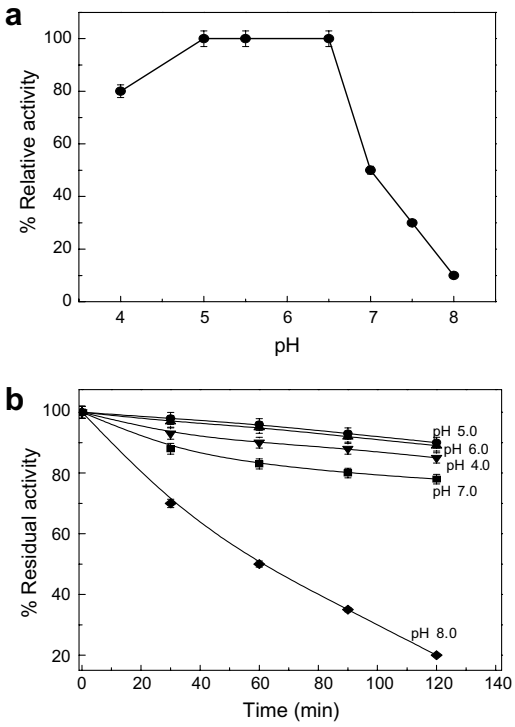

Fig. 4. (a) Effect of pH on the activity of the purified protease and (b) pH stability. Experimental conditions and buffer used are as specified in methods.

when incubated at pH 4.0–7.0. However, the activity decreased rapidly when incubated at pH 8.0 (Fig. 4b).

2.5. Thermal stability

The optimum temperature for the proteolysis of azocasein was found to be 60 ± 0.5 °C. Even at higher temperatures (70 °C) the enzyme exhibited 50% of its original activity indicating the thermal stability of the enzyme. The effect of temperature on the proteolytic activity was determined after incubating the enzyme for a reference time of 15 min (Fig. 5a). The enzyme was incubated over a temperature range of 40–85 °C and the residual activities measured at 60 °C. The enzyme was stable up to 60 °C with an apparent T_m of 70 ± 0.5 °C. The semi-logarithmic plots of residual activity versus incubation time at different temperatures are characterized by straight lines of $r > 0.98$. Therefore the inactivation process can be attributed to a single exponential decay. Further the plots also indicate the thermal inactivation follows first order kinetics. The time at which loss of activity reached 50% was taken as the experimental half-life for the enzyme, and the rate constants were calculated (Fig. 5b). The Arrhenius plot for irreversible denaturation is linear. The thermodynamic parameters were determined from rate constants obtained at different temperatures. From the slope of Arrhenius plot, the activation energy (E_a) for inactivation is 44.0 ± 0.3 kcal mol^{-1}. The activation enthalpy (ΔH^*), free energy change (ΔG^*) and entropy (ΔS^*) were estimated to

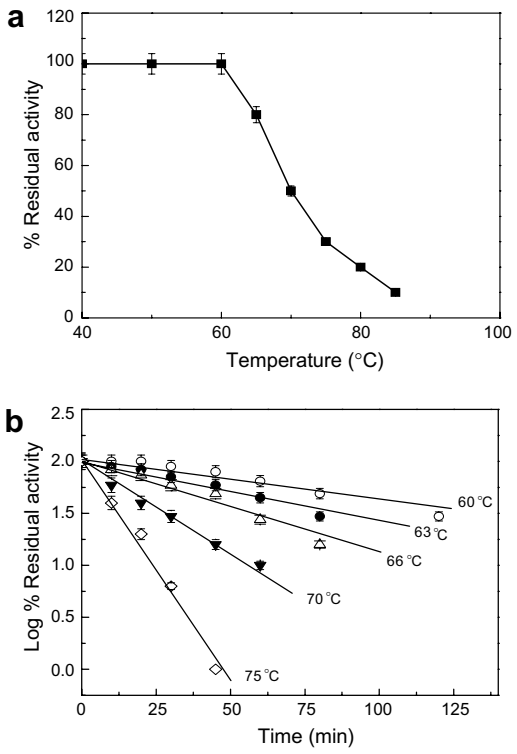

Fig. 5. (a) Thermal stability of the protease at different temperatures. Enzyme samples in 0.05 M acetate buffer pH 5.5 were incubated at the test temperatures for a reference time of 15 min, cooled rapidly and assayed for the remaining activity at 60 °C. (b) Thermal inactivation profile of the protease. Enzyme was incubated at different test temperatures. Aliquots were drawn at different time intervals and cooled to 4 °C and their residual activities were measured under standard assay conditions.

be 43 ± 4 kcal mol^{-1}, -26 ± 3 kcal mol^{-1} and 204 ± 10 cal mol^{-1} K^{-1}, respectively.

2.6. Cleavage specificity

An investigation of specificity of the purified enzyme was carried out by determining the sites of the cleavage catalyzed by the enzyme using oxidized B chain of insulin. The cleaved peptides were resolved by RP-HPLC as shown in Fig. 3b and their amino acid sequence was determined by automated Edman degradation on an Applied Biosystems 447A protein sequencer. Analysis of the P_1–P_1' bond specificity of the protease indicated that the P_1 residue was either Phe, Leu or Glu and the preferred P_1' residue was Tyr (P_1–P_1' notations according to Schechter and Berger, 1967). The RP-HPLC peptide profile of B chain of insulin digested with ficin of *F. carica* (Fig. 3c) is different, clearly indicating that the enzyme isolated from *F. racemosa* is different from that of *F. carica*. Englund et al. (1968) showed that ficin obtained from the latex of *F. glabrata* preferably hydrolyzed the peptide bonds following an aromatic residue more efficiently compared to others. A protease of musk melon fruit also showed broad specificity towards Leu, Phe, Glu, Ala, Val, Gly, Pro at P_1 position (Kaneda et al., 1997), but the preferential cleavage sites, as in the present study were hydrophobic and acidic amino acid residues at the P_1 position. Like the majority of the other aspartic proteases, the purified enzyme cleaves peptide bonds between residues with hydrophobic side chains (Fruton, 1976).

2.7. Effect of the protease on food proteins

In order to study the possible food applications of the protease of *F. racemosa* the activity of the protease using different food proteins as substrates was studied. The highest activity was observed with casein (Table 3). Casein digestion constitutes the main process in the first phase of milk clotting in cheese making industry. The purified *F. racemosa* aspartic protease preferentially cleaves carboxy-terminus of Phe. It is well established that the first step of milk clotting starts with the cleavage of κ-casein at the Phe^{105}–Met^{106} bond (Sousa et al., 2001). Taking together the cleavage specificity of the purified protease and its ability to digest casein is suggestive of a milk clotting property. Nevertheless this will require a more detailed study. The purified enzyme also showed similar but lower activity towards arachin and glycinin digestion. Several endogenous seed storage proteins have been tested *in vitro* as substrates for plant aspartic proteases (Belozersky et al., 1989). The aspartic protease of *F. racemosa* hydrolyses arachin and glycinin, seed storage proteins of peanut and soy, respectively. Therefore it may not be unreasonable to assume that the *F. racemosa* latex aspartic protease is physiologically associated with protein degradation for mobilization of nitrogen to the developing fruit. Runeberg-Roos and Saarma (1998) showed that the aspartic protease is localized at the developing tracheary elements of barley root tip cells.

2.8. Amino-terminal sequence analysis

The purified enzyme showed the following sequence from the amino-terminus, NH_2-EQELEQAGGYLA... This sequence differed from the amino-terminal sequence NH_2-LPEVDWAXFGAV..., determined for a commercially available ficin from *F. carica*. A BLAST search of the determined sequence did not show any matches with other plant proteases. Therefore the first 12 amino acid residues of the protease from *F. racemosa* were aligned and compared with amino-terminus sequences of other plant aspartic proteases (Table 4) like cardosin A, cardosin B (*C. cardunculus* L.), phytepsin (*H. vulgare*), At-Asp1 (*A. thaliana*), chlapsin (*Chlamydomonas reinhardtii*), cyprosin

Table 3
Hydrolyzing activity of the protease against different substrates

Substrate	Activity (Units/mg)
Casein	178 ± 5.0
Albumin	169 ± 5.0
Gelatin	133 ± 6.0
Arachin	152 ± 6.0
Glycinin	135 ± 5.0

B (*C. cardunculus*) and Vigna AP (*V. unguiculata*). A similar amino-terminal Glutamate is observed in phytepsin. Val and Leu corresponding to Val^{7} and Leu^{9} are the first conserved amino acids in plant aspartic proteases (Domingos et al., 2000). Leu^{11} of *F. racemosa* protease corresponding to Leu^{9} of phytepsin is conserved. The amino-terminal sequences of plant aspartic proteases show heterogeneity and this could be the reason for the absence of any matches with BLAST.

2.9. Spectral studies

The far-UV CD spectrum for the protease was determined over the range of 190–260 nm in acetate buffer pH 5.5 (0.05 M). The enzyme contains about 13% α-helix, 63% beta structures and aperiodic structures of 24%. The overall secondary structure of the purified aspartic protease of *F. racemosa* shows mainly β-structures with very little α-helix. The three dimensional structure of cardosin A of *Cynara cardunculus* L. (PDB code: 1B5F) (Frazao et al., 1999) and prophytepsin of barley (PDB code: 1QDM) (Kervinen et al., 1999) show essentially β-structures and very little α-helix. The intrinsic fluorescence emission spectrum of purified protease was determined in acetate buffer pH 5.5 (0.05 M). Tryptophans within hydrophobic milieus have been shown to display emission maxima at 330 nm or less, whereas tryptophans localized in more hydrophilic surroundings have an emission peak around 350 nm (Eftink and Ghiron, 1981). The purified enzyme exhibited an emission maximum at 338 ± 0.2 nm, indicating that tryptophan residues were partially exposed to hydrophilic microenvironments.

3. Experimental

3.1. Materials and chemicals

DEAE-Sephadex A-50 was purchased from Pharmacia, Sweden. Ficin (*F. carica*), insulin B-chain, trifluroacetic acid (TFA), pepstatin-A, 4-chloromercurobenzoate (PCMB), iodoacetamide, phenyl-methanesulfonylfluoride (PMSF), EDTA, sodium tetrathionate, Di-isopropylfluorophosphate (DFP), polyvinyledine difluoride membrane (PVDF), casein, azocasein, gelatin, BSA, acrylamide, bis-acrylamide, SDS, 2-mercaptoethanol, ammonium persulfate, N,N,N',N'-tetramethylethylenediamine (TEMED) were purchased from Sigma Chemical Co., St., Louis., Mo., USA. All other chemicals used were of analytical/HPLC grade obtained from Merck.

3.2. Collection of latex

Immature green fruits from *F. racemosa* trees growing locally were collected. The fruits were freed of suckers and ants. Latex was collected by incising the stalk of the green fruit from the main branch. The collected latex was clarified by centrifugation at 10,000*g* at 4 °C for 60 min to remove the gum and other debris. The clarified extract was stored at −20 °C until further use.

3.3. Protein determination

Protein concentration was determined by the dye binding method of Bradford (1976). Bovine serum albumin (BSA) was used as the standard protein.

3.4. Protease assay

Protease activity was assayed using azocasein as the substrate according to the reported method (Bendicho et al., 2002). The assay mixture consisting of 1 mL of 0.5% azocasein (w/v) in 0.05 M sodium acetate buffer pH 5.5 and 10–100 μg of enzyme was incubated at 60 ± 2 °C for 30 min. The reaction was stopped by addition of ice cold TCA (10% w/v) and centrifuged at 10,000*g*. In the control enzyme was inactivated by TCA prior to incubation with substrates. The supernatant (0.5 mL) was mixed with an equal volume of 0.5 M NaOH and incubated for 15 min. The absorbance of the developed color was measured at 440 nm. All the assays were corrected for non enzyme hydrolysis using buffer in place of enzyme. One unit of enzyme activity is defined as the amount of enzyme that increases the absorbance by 1.0 per minute under the assay conditions.

Proteolytic activity was also examined against natural food proteins that are commonly hydrolyzed or used in protease assay. The different substrates used were: casein, albumin, gelatin, arachin (isolated according to Monteiro and Prakash, 1994) and glycinin (isolated as described by Tanh and Shibasaki, 1976). Substrate concentration of

Table 4
Comparison of N-terminal amino acid sequence of *Ficus racemosa* protease with other aspartic proteases[a]

Enzyme	Amino-terminal sequence											
Ficus racemosa protease	E	Q	E	L	E	Q	A	G	G	Y	L	A
Phytepsin (*H. vulgare*)	E	E	E	G	D	I	V	A	L	K	N	Y
Vigna AP (*V. unguiculata*)	G	T	E	T	D	I	V	A	L	K	N	Y
At-Asp 1 (*A. thaliana*)	G	G	D	A	D	V	V	V	L	K	N	Y
Cardosin A (*C. cardunculus* L.)	D	S	G	G	A	V	V	A	L	T	N	D
Cardosin B (*C. cardunculus* L.)	D	S	G	G	G	I	V	A	L	T	N	D
Chlapsin (*C. reinhardtii*)	S	S	D	Q	G	Q	V	T	L	K	N	G
Cyprosin B (*C. cardunculus*)	D	S	D	G	E	L	I	A	L	K	N	Y

[a] Domingos et al. (2000).

1% (w/v) was prepared in acetate buffer pH 6.0. One milliliter of purified enzyme (100 μg) was added to 1 mL substrate. The reaction mixture was incubated at 60 °C for 30 min. The reaction was stopped by the addition of 2 mL of 10% TCA and incubated for 10 min at room temperature and centrifuged. The absorbance of soluble peptides in the supernatant was measured at 280 nm. In case of blank substrate was added after the enzyme was first inactivated by TCA. One unit of activity is defined as the amount of enzyme that increases the absorbance by 0.001 per min under given assay conditions.

3.5. Purification of protease from the latex

To the clarified extract, solid ammonium sulphate (51.6 g/100 mL) was added to obtain 80% saturation at 4 °C. The precipitated protein was removed by centrifugation at 10,000*g* for 30 min. The precipitate thus obtained was redissolved in 0.05 M Tris–HCl buffer pH 7.0. This protein fraction was loaded onto a BioSep-SEC-S2000 column (600 × 21.2 mm, Phenomenex) pre-equilibrated in the same buffer at a flow rate 5 mL/min at room temperature (25 ± 2 °C). The protease active fractions were pooled (Fig. 1a) and concentrated using the 10,000 Da cutoff Amicon Ultra centrifugal filter devices. The retentate was loaded onto a DEAE-Sephadex A-50 column (20 × 1.5 cm) pre-equilibrated with 0.05 M Tris-HCl buffer pH 7.0 at a flow rate of 25 mL/h at 4 °C. The unbound fraction did not show any protease activity. Subsequently elution of bound protein, using a linear gradient of 0–0.5 M NaCl in the same buffer afforded the protease fraction with a specific activity of 0.64 U/mg. This pooled fraction (Fig. 1b) was used in all further studies.

3.6. Polyacrylamide gel electrophoresis (PAGE)

Non-denaturing PAGE (10% T, 2.7% C) was performed at pH 8.8 according to the procedure of Laemmli (1970). SDS–PAGE (12% T, 2.7% C) was performed after denaturing the proteins with SDS and β-mercaptoethanol.

3.7. Gelatin-embedded PAGE for protease activity

Gelatin–PAGE (Felicioli et al., 1997) was performed by including gelatin (0.5% w/v final concentration) to the polyacrylamide (10% T, 2.7% C) gel as described above. Following electrophoresis at pH 8.8 the gel was washed three times in distilled water and then incubated at 37 °C in 0.05 M sodium acetate buffer pH 5.5 for 30 min. After incubation the gel was stained with Coomassie brilliant blue. The presence of protease is detected as clear white band against a dark blue background due to the hydrolysis of gelatin.

3.8. Molecular weight determination

The apparent molecular mass of the native enzyme was determined using a BioSep-SEC-S2000 (300 × 7.8 mm, Phenomenex) analytical column, in a Waters HPLC system. The eluant used was 0.05 M sodium phosphate buffer, pH 7.4 at a flow rate of 1 mL/min. The proteins were detected at 230 and 280 nm. The column was calibrated using bovine serum albumin (66.3 kDa), ovalbumin (43.5 kDa), carbonic anhydrase (29 kDa), ribonuclease (13.7 kDa), cytochrome c (12.4 kDa) and aprotinin (6.5 kDa). The molecular weight of the purified protease was also determined by SDS-PAGE (as described earlier) and MALDI-TOF. Matrix Assisted Laser Desorption Ionization Mass Spectroscopy (MALDI-MS) analysis were performed on a Bruker Daltonics Ultraflex MALDI TOF/TOF system (Bruker-Daltonics, Bremen, Germany) in the reflective positive ion mode. The instrument was calibrated with the masses ranging between 10,000-100,000 Da. The purified enzyme was dialyzed extensively against water. The samples were prepared by mixing equal volumes of protein and matrix. Matrix was saturated α-cyano-4-hydroxycinnamic acid (Sigma–Aldrich Chemie GmbH) prepared separately in CH_3CN/H_2O/TFA (80:20:0.1). The samples were then dried at 25 °C under atmospheric pressure and loaded on to probe slide. Data were collected between the molecular weight 30,000 and 65,000 Da.

3.9. Amino-terminal sequence

The purified protease was transferred following SDS–PAGE to polyvinylidene difluoride (PVDF) membrane in 10 mM CAPS-10% methanol buffer by electroblotting at 0.8 A/cm^2 at constant current for 1.5 h and stained with Coomassie brilliant blue R-250. The band corresponding to the protease was excised and loaded directly to the gas phase sequencer (Applied Biosystems 447A) for automated Edman degradation. β-lactoglobulin (Applied Biosystems) was used as the standard to calibrate the instrument.

3.10. Effect of pH and stability studies

The protease activity as a function of pH was determined using azocasein as the substrate. The buffers used were acetate (0.05 M) 4.0–6.0 and sodium phosphate (0.05 M) pH 6.5–8.0. A plot of the relative activity versus pH was employed to obtain the pH optimum. To test the pH stability, the enzyme (0.5 mg/mL) in different specified buffers was incubated for different time intervals. At the end of the incubation period, aliquots of incubated enzyme were assayed at pH 5.5 against azocasein as described in assay method. The effect of ionic strength on the protease activity was studied by assaying the enzyme using sodium acetate buffer pH 5.5 of molarities ranging from 0.1 to 1.0 M.

3.11. Thermal stability

Kinetics of thermal inactivation of the purified protease was studied using a constant temperature bath, at the

desired temperatures. Aliquots of enzyme removed at periodic intervals, were subjected to assay, after cooling to 4 °C. Residual activity was measured as a percentage of initial activity. From the semi-logarithmic plot of residual activity as a function of time the inactivation rate constants (k_r) were calculated. The apparent half-life values were estimated and energy of inactivation (E_a) calculated from the slope of the Arrhenius plot. Thermodynamic parameters were calculated according to the equations:

$$\Delta H^* = E_a - RT \quad (1)$$

$$\Delta G^\circ = -RT \ \ln(k_r)h/K_B T \quad (2)$$

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T \quad (3)$$

where h is the Plank constant, K_B the Boltzmann constant, R is the gas constant and k_r is the rate constant.

3.12. Determination of the protease group specificity

The residue involved in catalytic activity of the purified protease was identified by using group specific chemical inhibitors of protease. The enzyme was pre-incubated with different inhibitors specific for all classes of proteases in pH 5.5 buffer for 30 min at 37 °C. The residual activity was determined using azocasein as previously indicated. The assay performed without any of the inhibitors served as the reference initial activity.

3.13. Determination of the protease cleavage specificity

The cleavage specificity of the enzyme was investigated by digesting the oxidized B chain of bovine insulin and determining amino acid sequences of the peptides produced. The assay was carried out according to the method described earlier (Kim et al., 1995). The digestion was carried out in acetate buffer pH 5.5 (0.05 M) for 1 h at 60 °C. The ratio of purified enzyme to substrate was 2% (w/w). The reaction was terminated by addition of TFA and clarified by centrifugation. The clear supernatant which contained the digested insulin peptides were fractionated directly by RP-HPLC using a C18 Symmetry Shield column (4.6 × 150 mm, 5 μm) on a Waters HPLC system equipped with a 1525 binary pump and Waters 2996 photodiode array detector set at 230 nm. The solvent used were 0.1% TFA (A) and 70% acetonitrile containing 0.05% TFA (B). A linear gradient traversing from 0% to 70% B in 60 min at a flow rate of 0.7 mL/min was employed. The peptide fractions detected at 230 nm were collected. The peptides were identified by their sequence following Edman degradation in an automated gas phase protein sequencer as described earlier.

3.14. Fluorescence studies

Fluorescence measurements were recorded on a Shimadzu RF-5000 spectrofluorometer using a 10 mm path length quartz cell at 25 °C. Excitation and emission slit widths were set at 5 nm and 5 nm, respectively. The measurements were carried out in a 0.05 M acetate buffer pH 5.5. The protein was excited at 280 nm and emission was recorded between 300 and 400 nm. Appropriate blanks were used for baseline correction of fluorescence intensity.

3.15. Conformational analysis

Far UV-Circular dichroic spectral measurements of the purified protease were carried out using an automatic recording Jasco J-810 spectropolarimeter fitted with a xenon lamp and calibrated with +d-10-camphor sulphonic acid, using a protein concentration of 0.335 mg/mL for far-UV regions. Dry nitrogen was purged continuously before and during the experiment. The measurements were made at 25 °C. The enzyme solution was prepared in acetate buffer pH 5.5 (0.05 M). Far-UV measurements were recorded in the range of 190–260 nm using a 1mm path length cell. The scan speed was 10 nm/min using a bandwidth of 1 nm, the spectra were taken as an average of three scans. The results were expressed as the mean residual ellipticity $[\theta]_{MRW}$ obtained from the relation $[\theta] = 100 \times \theta_{obs}/l \times c$ where θ_{obs} is the observed ellipticity in degrees. The $[\theta]_{MRW}$ was calculated using a value of 115 for mean residue mass of the protease, c is the concentration in grams/liter and l is the length of the light path in cm. The values obtained were normalized by subtracting the base line recorded for the buffer under similar conditions. The analysis of the data for the secondary structure elements was done according to computer program of Yang et al. (1986).

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