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A High Cysteine Containing Thiol Proteinase from the Latex of *Ervatamia heyneana*: Purification and Comparison with Ervatamin B and C from *Ervatamia coronaria*

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A cysteine protease, with a high cysteine content and a high degree of amino terminal sequence homology with ervatamins B and C, has been purified from the latex of *Ervatamia heyneana* (Family Apocynaceae). The enzyme designated as heynein (M_r = 23 kDa) has a comparatively high cysteine content (11), high isoelectric point (10.8), and high stability against pH (2.5−11.5), temperature (63 °C, 15 min), strong denaturants, and organic solvents. The enzyme has high specific activities for natural substrates such as casein and azoalbumin. The pH and temperature optima are pH 8.0−8.5 and 52 ± 2 °C, respectively. Hydrolysis of synthetic substrates and digestion of bovine serum albumin confirm a distinct specificity of heynein as compared to ervatamins and papain. Also, heynein has distinct immunogenicity as monitored by enzyme-linked immunosorbent assay and Ouchterlony's double immunodiffusion. Strong enzyme activation by reducing agents such as β-mercaptoethanol, dithiothreitol, and strong enzyme inhibition by thiol proteinase inhibitors such as E-64 and iodoacetic acid have evidenced heynein to be a cysteine protease. High stability, specific activity, and easy purification may make heynein a potential protease for food and biotechnology applications.

KEYWORDS: Azoalbumin; cysteine protease; ELISA; food industry; sequence homology; substrate specificity

INTRODUCTION

A number of intracellular and extracellular processes of higher plants are accomplished by virtue of their proteases (*1*), and such processes include seed germination, proteolytic activation of proenzymes, scavenging of defective metabolites, and senescence (*2*). All plants primarily use cysteine endopeptidases for protein degradation, whereas most of the microorganisms utilize serine endopeptidases (*3*). It is noted that expression of germination cysteine proteases and senescence cysteine proteases is enhanced dramatically during germination of seeds and senescence of plant organs (*4*, *5*).

The majority of plant proteinases isolated so far belong to the cysteine protease class, and only a few are of aspartic and serine proteases. Plant proteases have been purified from various parts of the plants such as fruit, seeds, stems, and latices (*6*). Plant latices have yielded a number of industrially important endopeptidases such as calotropins (*7*), papain (*8*), and ficain (*9*). The precise biological role of plant latex proteinases still remains speculative, but by virtue of their broad substrate specificity, they have been found to protect ripening fruits against plant pathogens especially fungi and insects (*10*). In addition to their biological roles, plant proteases have also been exploited commercially for ages in food, leather, and textile industries (*11*). Proteolytic fractions from fig latex have found use in unmasking antigens in serology (*12*) and digestion of gastrointestinal nematodes (*13*). According to estimates, more than half of the total commercial industrial enzymes used are proteases (*14*). In food industries, proteases are indispensable for processes such as tenderization of meat, brewing, cheese elaboration, and bread manufacturing (*15*). The quest for new potential plant proteases still continues to make the industrial applications cost effective.

In this study, isolation and biochemical characterization of a new cysteine protease from the latex of a medicinal plant *Ervatamia heyneana* (Family Apocynaceae) are reported. Because of a high degree of amino terminal sequence homology, properties of the enzyme have been compared with those of ervatamin B and ervatamin C, the two cysteine proteases reported from the latex of another medicinal plant of the same genus, *Ervatamia coronaria* (*16*, *17*). *E. heyneana* has a wide range of important medicinal applications including anticancerous activities of its root, leaf, and stem extracts in addition to its antiinflammatory effect over wounds and yielding a number of medicinally valuable alkaloids (*18*, *19*). In view of the occurrence of highly stable cysteine proteases in the latex of *E. coronaria* (*16*), the latex from *E. heyneana* was also screened for its biochemical constituents. Preliminary studies indicated the presence of a protease in the letex, which has been

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purified to homogeneity and established to be a cysteine protease. The enzyme was named as heynein as per the nomenclature of cysteine proteases. It exhibits distinct substrate specificity and other physicochemical properties as compared to ervatamins B and C, although it conserves a high degree of amino terminal sequence homology with them.

MATERIALS AND METHODS

Safety. The preparation of sodium tetrathionate, which is a highly exothermic reaction, was carried out at 4 °C. 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.

Materials. Fresh latex was collected from the apexes of young stems of plant *E. heyneana* in 0.01 M sodium acetate buffer, pH 4.5, containing 0.005 M sodium tetrathionate. CM Sepharose was purchased from Pharmacia Biotech (Sweden). Ampholine was from LKB (Sweden). Hen egg white lysozyme, bovine serum albumin (BSA), ribonuclease A, azoalbumin, azocasein, hemoglobin, DTNB, DTT, GuHCl, urea, EDTA, *o*-phenanthroline, EGTA, leupeptin, PCMB, SBTI, NEM, PMSF, β-mercaptoethanol (β-ME), acrylamide, *N*,*N*-methylene bis acrylamide, Coomassie brilliant blue R-250, l-*trans*-epoxysuccinyl-leucylamide(4-guanidino)butane-*N*-[*N*-(L-3-*trans*-carboxyirane-2-carbonyl)-L-leucyl]agimatine (E-64), all synthetic amides, and triethanolamine were procured from Sigma Chemical Co. (U.S.A.). Coomassie brilliant blue G-250 was purchased from Eastman Kodak. HEPES buffer and dimethyl sulfoxide were obtained from Spectrochem. Pvt. Ltd. (India). All other chemicals were of the highest purity available commercially. Hemoglobin was denatured with urea before the activity assay (*20*). Sodium tetrathionate ($Na_2S_4O_6 \cdot 2H_2O$) was synthesized by the method of Gilman (*21*).

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

Step 1: Removal of Gum. Fresh latex of *E. heyneana* was collected from young stems in 500 mL of 0.01 M sodium acetate buffer, pH 4.5, containing 0.005 M sodium tetrathionate. The latex was frozen at −20 °C for 24 h. Subsequently, the latex was thawed to room temperature and centrifuged at 24 000*g* for 15 min to remove insoluble gum and cell debris. The clear supernatant was dialyzed against 0.01 M sodium acetate buffer, pH 4.5, and centrifuged as above. The clear supernatant thus obtained after centrifugation was used in the next step. During the entire purification, 0.005 M sodium tetrathionate was maintained in all of the solutions.

Step 2: Ammonium Sulfate Precipitation. The latex was subjected to 80% ammonium sulfate saturation to precipitate all of the soluble proteins. For this, the clear supernatant from the previous step was taken to 80% ammonium sulfate saturation at 4 °C and precipitation of the proteins was allowed to proceed for 15 h at the same temperature. The resulting precipitate was collected by centrifugation at 24 000*g* for 15 min at 4 °C. The precipitate was dissolved in 20 mL of 0.01 M sodium acetate buffers, pH 4.5, and dialyzed against 1 L of the same buffer for 24 h at 4 °C with three changes of the buffer. Any insoluble content in the dialysis bag was removed by centrifugation as above.

Step 3: Cation Exchange Chromatography. Protein solution from step 2 was loaded at a flow rate of 6 mL/min on a CM Sepharose fast flow ion exchange column that was preequilibrated with 0.01 M sodium acetate buffer, pH 4.5, containing 0.005 M sodium tetrathionate. The column was thoroughly washed with the same buffer, and the protein was eluted with a linear salt gradient of 0−1 M sodium chloride. Fractions of 5 mL were collected at a flow rate of 6 mL/min, and the protein content in the fractions was monitored by absorbance at 280 nm. Proteolytic activity of every fraction was assessed using casein as substrate. Homogeneity of the fractions was also checked on sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) under nonreducing conditions. (*22*). Fractions with maximum activity and homogeneity were pooled and concentrated by 80% ammonium sulfate precipitation followed by dialysis against 0.01 M sodium phosphate buffer, pH 7.0. The clear protein solution was stored at 4 °C for further use. The purified protein contained 0.005 M sodium-tetrathionate; therefore, a reducing agent was necessary to activate the protein before activity measurements.

Protein Concentration. Protein concentration was measured spectrophotometrically (absorbance at 280 nm) as well as by the Bradford assay (*23*). BSA was used as a standard to generate a calibration curve for the Bradford reagent.

Assay for Proteinase Activity. The proteolytic activity of the enzyme was monitored using denatured natural substrates such as casein, hemoglobin, azoalbumin, and azocasein following the method of Arnon (*22*). Before the assay, the tetrathionate-inactivated enzyme (5−10 μg) was activated at 37 °C with 0.05 M β-ME in 0.05 M tris HCl buffer, pH 8.0, containing 0.02 M EDTA and the remaining procedure for enzyme activity was followed as described by Dubey and Jagannadham (*24*). One unit of enzyme activity was defined as the amount of enzyme that gave rise to an increase of one unit of absorbency at 280 nm per minute of casein digestion.

Assay for Amidolytic Activity toward Synthetic Substrates. The enzymatic hydrolysis of different synthetic peptidyl-*p*NA (peptidyl *p*-nitroanilide) substrates by the purified protease was studied by spectrophotometry (*22*). The different substrates used for studies were BAPA, l-Ala-Ala-*p*-nitroanilide, *N*-succinyl-Phe-*p*-nitroanilide, L-Glu-*p*-nitroanilide, L-Ala-*p*-nitroanilide, *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide, and L-Leu-*p*-nitroanilde. An extinction coefficient of 8800 M^{-1} cm^{-1} for *p*-nitroanilide was used for calculation of activity (*25*).

Study of Substrate Specificity. Studies on the activity of enzyme over natural and synthetic substrates were substantiated with comparative substrate specificity studies of a few selective cysteine proteases. Equal amounts (1 μg) of activated samples of papain, ervatamin B, ervatamin C, ervatamin A, procerain, and heynein were incubated with 50 μg of BSA at 37 °C for 5 min. The resulting fragments were separated on a Tricine-SDS−PAGE following the method of Schagger and Jagow (*26*).

Dependence of Enzyme Activity on pH and Temperature. The effect of pH in the range of 0.5−11.5 and temperature in the range of 10−80 °C on the activity of heynein was investigated. Because of insolubility of azoalbumin below pH 4.0, enzyme assays at lower pH were carried out using haemoglobin as substrates (*20*). The buffers used for different pH were 0.05 M KCl−HCl (pH 0.5−1.5), 0.05 M glycine−HCl (pH 2.0−3.5), 0.05 M sodium acetate (pH 4.0−5.5), 0.05 M sodium phosphate (pH 6.0−7.5), 0.05 M tris-HCl (pH 8.0−10.0), and 0.05 M sodium carbonate (pH 10.5−11.5). For temperature dependence of enzyme activity, enzyme as well as substrate solutions were equilibrated at different temperatures for 15 min and assays were done as described (*24*). At each temperature, a control assay was done without enzyme and used as a blank.

Effect of Various Compounds on the Activity. The effect of various thiol specific activators and protease inhibitors on the activity of heynein was studied. Among activators, the effect of β-ME, DTT, L-cysteine, and reduced glutathione was investigated. Samples of enzyme (5 μg) were incubated for 15 min at 37 °C in the presence of a specific activator. The proteolytic activity was measured as a function of activator concentration in the range of 0−50 mM. Inhibition of the enzyme activity of heynein by thiol specific as well as nonspecific inhibitors was also studied. The tetrathionate-inactivated enzyme was activated by 0.05 M β-ME at 37 °C in 0.05 M tris-HCl buffer, pH 8.0, containing 0.002 M EDTA for 15 min. The activator was removed subsequently by dialysis for 24 h against 500 mL of 0.1 M acetic acid at 4 °C with three changes of the dialysate. Before the assays, 1 μM of enzyme was incubated in the presence of an inhibitor (1−100 μM) in 0.05 M tris-HCl buffer, pH 8.0, for 20 min at 37 °C. Subsequently, the enzyme assays were performed with azoalbumin as substrate. Various protease inhibitors used were EDTA, EGTA, E-64, iodoacetic acid (IAA), DFP, leupeptin, mercuric chloride, NEM, *o*-phenanthroline, PCMB, PMSF, SBTI, and sodium tetrathionate. In each case, a control assay of the enzyme was done without inhibitor and the resulting activity was taken as 100% for calculation of residual activity.

Effect of Substrate Concentration on Reaction Velocity. The effect of increasing concentrations of synthetic substrate BAPA on the enzyme activity was studied at the optimum pH. For assays, 15 μg of the enzyme was incubated in the presence of increasing concentration of BAPA (0−7 mM) in a total volume of 1 mL and the activity was

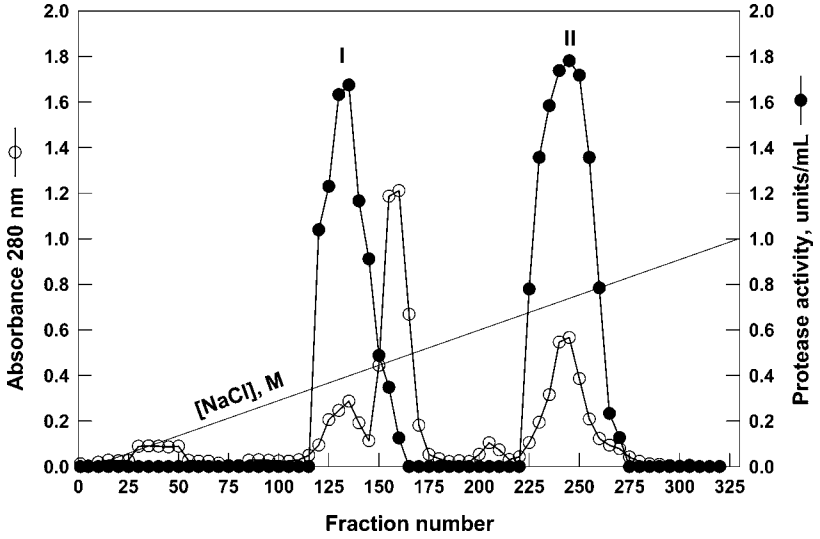

Figure 1. Elution of heynein from cation exchange column. Proteins were loaded on a CM Sepharose column preequilibrated with 10 mM sodium acetate buffer, pH 4.5. The unbound proteins were washed out with the equilibration buffer, and the column was eluted with a linear salt gradient of 0–1 M sodium chloride (—) at the same pH. Fractions of 5 mL were collected and assayed for protein content (○) and proteolytic activity (●).

assayed as described in the Materials and Methods. The values of Michaelis−Menten constant (K_m) for BAPA were calculated from a Lineweaver−Burk plot.

Electrophoresis. Assessment of homogeneity of the enzyme, at different stages of purification as well as molecular mass determination of the purified enzyme, was carried out using 15% SDS−PAGE by the method of Laemmli (*27*, *28*).

Determination of Isoelectric Point (pI). The pI of the purified enzyme was determined by isoelectric focusing in a tube gel as described for procerain by Dubey and Jagannadham (*24*). Ampholines in the range of pH 9−11 were used to generate the pH gradient. Protein bands were stained using 0.04% (w/v) Coommassie G-250 dye dissolved in 6% perchloric acid (v/v) (*30*).

Determination of Extinction Coefficient. The extinction coefficient of heynein was determined by dry weight (*31*) and spectrophotometer methods (*32*). The values of extinction coefficients obtained from the two methods were averaged to a resultant and used for all practical purposes.

Estimation of Tryptophan and Tyrosine Content. The total number of tryptophan and tyrosine residues in the protein were estimated by spectrophotometry (*33*). To validate the measurements, similar contents of papain, ribonuclease A, and chicken egg white lysozyme were also done.

Measurement of Free and Total Sulfhydryl Content. Determinations for free and total sulfhydryl contents of the enzyme were carried out following the method of Ellman (*34*) using DTNB. For free cysteine measurements, the enzyme was activated 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. Excess reducing agent DTT or β-ME in the reaction mixture was removed by extensive dialysis against 0.1 M acetic acid (*35*). The number of cysteine residues was calculated using an extinction coefficient of 14 150 M^{-1} cm^{-1} at 412 nm for NTB anion (*36*). To validate the results, similar estimations for papain, ribonuclease A, and lysozyme were carried out simultaneously.

Measurement of 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. In view of the occurrence of a few plant cysteine proteases such as calotropins FI, FII (*7*), and bromelain (*37*) as glycoproteins and also because heynein is a secreted protein, determination of glycosylation of heynein, if any, became essential. The carbohydrate content in the purified protein was estimated using the phenol sulfuric acid method (*38*). Galactose was used to generate a calibration curve.

Autodigestion. Because cysteine proteases have been reported to undergo autolysis in the presence of activators (*16*, *28*), autodigestion of heynein in the presence of an activator was followed as a function of enzyme concentration. The activity of the enzyme after 15 min of activation was taken as 100% for calculation of residual activity.

Stability. Because the stability of an enzyme dictates its applicability, the effect of extreme pH (0.5−11.5), temperature (10−80 °C), denaturants, and typical organic solvents on the proteolytic activity of the enzyme was studied. The enzyme was incubated under specified conditions of pH, chemical denaturant, or organic solvent for 24 h, and residual activity was assessed as described (*24*).

Amino Terminal Sequence Analysis. The amino terminal sequence of heynein was determined to study its homology and evolutionary relationship with other cysteine proteases of its class. This enzyme sample was freeze-dried on a Virtis lyophilizer and sequenced on an Applied Biosystems 477A protein sequencer by the method of Matsudaria (*39*).

Polyclonal Antibodies. Immunological studies have been used to distinguish proteases from the same source as well as similar sources (*40*). Polyclonal antibodies were raised against heynein in a male albino rabbit to study its immunological homology with ervatamin B, ervatamin C, and other cysteine proteases. The antibodies were raised as described by Dubey and Jagannadham (*24*). All sera were stored at −20 °C.

Immunoassays. Because heynein has a high degree of amino terminal sequence homology with ervatamin B and ervatamin C, immunoassays by Ouchterlony's double immunodiffusion (*41*) and indirect ELISA (*42*) were performed to check the antigenic similarity of heynein, if any, with ervatamins B and C. Heynein, ervatamin B, ervatamin C, and papain (40 μg each) were loaded into the peripheral wells, and 100 μL of the antiserum was added in the central well. A control was carried out with preimmune serum. Furthermore, antigenic similarities between heynein, ervatamin B, ervatamin C, and papain were also studied using indirect ELISA as described by earlier (*24*).

RESULTS

Purification. The proteins in the crude latex, after gum removal and 80% ammonium sulfate precipitation, were resolved into three protein peaks in cation exchange chromatography over CM Sepharose (**Figure 1**). No proteolytic activity was detected in unbound as well as the buffer wash of the column. In the elution profile, two peaks with proteolytic active were obtained (peaks I and II). Peak I fractions were heterogeneous, whereas those in the descending shoulder of peak II were found to be homogeneous on SDS−PAGE. No detectable differences were seen between the proteases of the two peaks in preliminary biochemical studies; therefore, the homogeneous

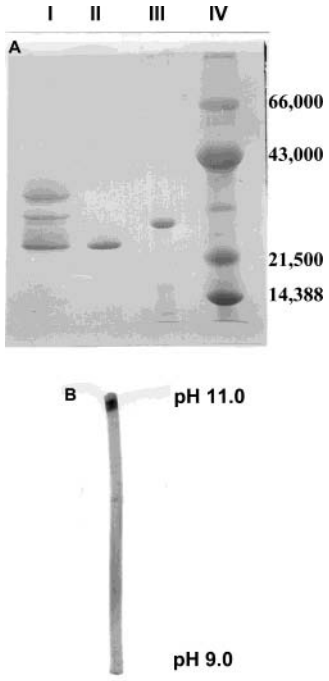

Figure 2. Assessment of the homogeneity of heynein. (**A**) Heynein under nonreduced (lane II) and reduced (lane III) conditions was electrophoresed on a 15% SDS–PAGE to assess its homogeneity. Lane I contained the starting material (crude latex) under nonreducing conditions. Standard proteins (reducing condition) BSA (66 000), ovalbumin (43 000), SBTI (21 500), and chicken egg white lysozyme (14 388) were used as protein molecular mass markers (lane IV). (**B**) To check homogeneity of heynein as well as to determine its pI, ampholines in the pH range of 9–11 were used to form a pH gradient in tube gel. One hundred micrograms of protein was loaded, and its pI was determined from its mobility in the tube gel.

fractions were pooled and concentrated by 80% ammonium sulfate precipitation. The specific activity of purified enzyme was found to be 8.2 unit/mg using casein as a substrate while the specific activity of crude was 4.2 units/mg in the same conditions. The total enzyme unit recovery of heynein amounted to about 29%. The pure enzyme was named heynein as per the nomenclature of cysteine proteinases. The enzyme was stored in its sodium tetrathionate blocked state at 4 °C and activated prior to activity measurements.

Homogeneity of the Enzyme. The purified enzyme showed a single band on SDS−PAGE under reducing as well as nonreducing conditions (**Figure 2A**). Similarly, a single protein band was observed on isoelectric focusing with a pI of 10.8 (**Figure 2B**). Besides, heynein eluted as a single symmetrical protein peak on reverse phase high-performance liquid chromatography (HPLC) on a C-18 column (data not shown).

Physical Properties of the Enzyme. The approximate molecular mass (M_r) of the purified enzyme was determined to be 23 kDa on SDS−PAGE (**Figure 2A**) utilizing the plot of relative mobility of marker proteins vs molecular mass (data not shown). There was no detectable carbohydrate component in heynein as observed in ervatamins, the cysteine proteases from the same genus. The extinction coefficient of the enzyme obtained by dry weight and spectrophotometric methods was averaged to a value of 23.2 and used for all practical applications.

Substrate Specificity of the Enzyme. The enzyme shows broad substrate specificity and hydrolyzes denatured natural substrates such as casein, azocasein, azoalbumin, and hemoglobin with high specific activities (**Table 1**). Under similar

Table 1. Comparative Specific Activities of Heynein, Ervatamin B,[a] and Ervatamin C[b]

substrate	specific activity		
	heynein	ervatamin B	ervatamin C
casein[c]	8.2 ± 0.3	9.0 ± 0.5	11.0 ± 0.5
azocasein[c]	6.6 ± 0.3	5.5 ± 0.5	5.0 ± 0.5
azoalbumin[c]	6.2 ± 0.3	7.5 ± 0.5	6.5 ± 0.5
BAPA[d]	0.5 ± 0.1		
N-succinyl-Ala-Ala-Ala-*p*-nitroanilide[d]	4.7 ± 0.1	12.5 ± 1	

[a] Ref *17*. [b] Ref *16*. [c] Specific activity is defined as the number of enzyme units per mg of protein. [d] Specific activity is defined as the number of moles of *p*-nitroaniline liberated per minute of substrate digestion per mole of enzyme; blank entry, no amidolysis.

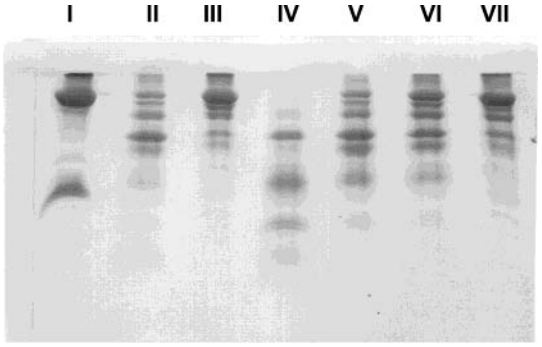

Figure 3. Comparative substrate specificity studies using digestion of BSA. Equal amounts of BSA (50 μg) were digested with ervatamin B, ervatamin C, ervatamin A, papain, procerain, and heynein. In each case, an equal amount of enzyme (5 μg) was used, and the substrate digestion was allowed to proceed for 5 min. The resulting fragments were electrophoresed on a Tricine SDS–PAGE as discussed in the Materials and Methods. Lane I, BSA and hen egg white lysozyme as markers; lane II, digestion by ervatamin B; lane III, digestion by ervatamin C; lane IV, digestion by ervatamin A; lane V, digestion by heynein; lane VI, digestion by papain; and lane VII, digestion by procerain.

conditions, specific activities of ervatamins B and C are also shown in the table. The enzyme also showed amidolytic activity on synthetic substrates BAPA, an ideal substrate for papain (*22*) and *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide. Heynein resembles ervatamin B in hydrolyzing *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide. It can be noted that ervatamins B and C both fail to hydrolyze BAPA. Thus, these three enzymes have distinct substrate specificities although they have been purified from two species of the same genus. Proteolysis of BSA by heynein, ervatamin B, ervatamin C, ervatamin A, procerain, and papain yielded different length fragments on Tricine-SDS−PAGE (**Figure 3**) confirming the distinct substrate specificity of heynein.

Dependence of Enzyme Activity on pH and Temperature. Heynein retains its proteolytic activity over a broad range of pH and temperature. The enzyme effectively catalyzes its natural substrates at pH 5.5−10.5 with an optimum activity at pH 8.0−8.5 (**Figure 4A**). The half-maximal proteolytic activity of heynein was observed at pH 6.0 and 10.5. Similarly, substrate hydrolysis can be accomplished by heynein at temperatures in the range of 25−63 °C with a maximal activity at 50−55 °C (**Figure 4B**). The enzyme shows its half-maximal activities at 36 and 62 °C.

Enzyme Activation and Inhibition Studies. The enzyme was strongly activated in the presence of reducing agents such as β-ME, DTT, L-cysteine, and reduced glutathione (data not

Table 2. Effect[a] of Various Protease Inhibitors on the Activity of Heynein, Ervatamin B,[b] and Ervatamin C[c]

		heynein		ervatamin B		ervatamin C	
inhibitor class	inhibitor	[I]	residual activity (%)	[I]	residual activity (%)	[I]	residual activity (%)
cysteine protease	IAA	50 μM	5				
	iodoacetamide			40 μM	9.6	40 μM	9.3
	E-64	10 μM	2				
	$HgCl_2$	50 μM	4	2 μM	5.7	6 μM	10.5
	NEM	50 μM	10	50 μM	29.8	20 μM	16.5
	sodium-tetrathionate	1 mM	10	40 μM	17.4	20 μM	15.7
	PCMB	50 μM	7	8 μM	8.9	6 μM	16.8
Ser/Cys	leupeptin	25 μM	2	8 μM	13.1	10 μM	25
serine protease	DFP	100 μM	95				
	PMSF	50 μM	90	40 μM	95.7	40 μM	70
	SBTI	1 mM	100	100 μg/mL	100	100 μg/mL	95
metallo protease	EDTA	5 mM	100	1 mM	100	1 mM	100
	EGTA	5 mM	100	1 mM	100	1 mM	100
	o-phenan-throline	1 mM	100	1 mM	100	1 mM	100

[a] Enzyme assays were performed with casein as substrate as described in the Materials and Methods. In all inhibition studies, 1 mM enzyme was used. [b] Ref *17*; μM. [c] Ref *16*; blank entry, data unavailable; [I], inhibitor concentration.

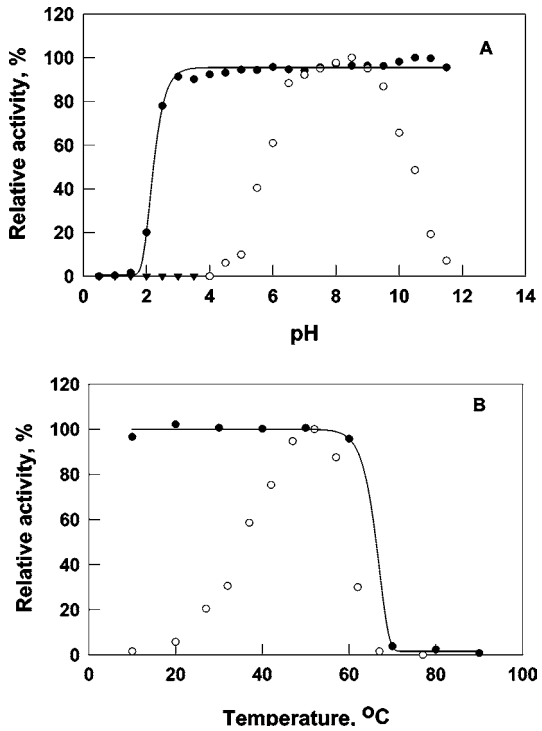

Figure 4. Effect of pH and temperature on the activity and stability of heynein. (**A**) Effect of pH on the activity of the enzyme was studied by carrying out the activity assays at different pH values in the range of 0.5–11.5. The enzyme was equilibrated at a particular pH, and assays were carried out at the same pH. Enzyme assays at and above pH 4.0 were done with azoalbumin (○) as substrate, whereas, below pH 4.0, denatured hemoglobin was used as a substrate (▼). For the evaluation of the stability of heynein (●) at different pH values, the enzyme was incubated at a given pH for 24 h and its activity was assayed at pH 8.0 as discussed in the Materials and Methods. (**B**) For determining the temperature optimum of heynein (○), the enzyme was equilibrated at a particular temperature for 15 min followed by its activity assay at the same temperature using azoalbumin. For the temperature stability (●) of heynein, the enzyme sample was equilibrated for 15 min at different temperatures and an aliquot was assayed for activity at 37 °C.

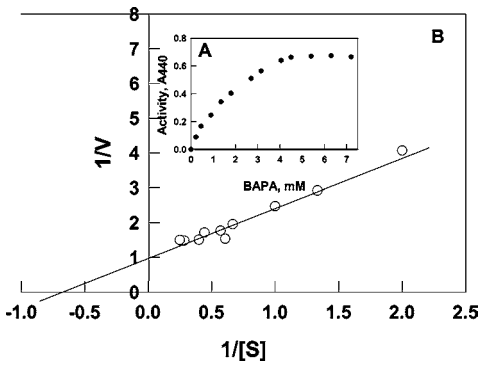

Figure 5. Effect of synthetic substrate concentration on the activity of heynein. (**A**) Enzyme activity was assayed with increasing concentration of BAPA. The enzyme activity reached saturation at higher concentrations of substrate (●). (**B**) Double reciprocal plot between 1/[S] and 1/V was used to determine the K_m of the enzyme over this synthetic substrate (○).

shown). Overall, an average 12−15 mM concentration of activator is needed for maximum activation. Whereas the amount of DTT required for the maximum activity of the enzyme was very low (3−5 mM), a higher concentration of DTT resulted in some inhibition. The inhibitory effect of DTT over heynein is similar to that seen in the case of ervatamins. The mechanism of inhibition at higher DTT concentrations is not fully understood. It may be due to either perturbation of functional structure by disulfide reduction or irreversible complexation of DTT with active site free cysteine residue of the enzyme thereby blocking the catalytic site of the enzyme. Enzyme inhibition studies using different classes of protease inhibitors concluded that heynein is a cysteine protease. Comparative data of inhibition studies on heynein and ervatamins (**Table 2**) show that these enzymes were strongly inhibited by thiol proteinase inhibitors such as E-64, IAA, PCMB, NEM, mercuric chloride, and sodium tetrathionate. Leupeptin, an inhibitor for cysteine/serine proteases, also inhibited the activity effectively. The serine protease inhibitors such as PMSF, SBTI, DFP, and metallo protease inhibitors such as EDTA, EGTA, and *o*-phenanathroline had no significant inhibitory effect over heynein. Such activation of heynein by reducing agent and activation by cysteine specific activators confirms that the enzyme belongs to cysteine protease.

Effect of Substrate Concentration. The enzyme obeyed Michaelis−Menten kinetics with denatured natural substrates (data not shown) as well as synthetic substrates (**Figure 5A**). The nature of kinetics with respect to both of the substrates is

Table 3. Salient Features of Heynein as Compared to Ervatamin B and Ervatamin C

enzyme	pI	M_r (kDa)	Tyr	Trp	total Cys	$\epsilon_{280}^{1\%}$	carbohydrate (%)
heynein	10.8	23	16	6	11	23.2	0.00
ervatamin B[a]	9.35	26	10	7	5	20.5	0.00
ervataminC[b]	9.54	25.5	16	7	7	24.6	0.00

[a] Ref *17*. [b] Ref *16*.

typically hyperbolic, and at higher concentrations of the substrate, the enzyme activity was found to undergo saturation. The values of K_m, as obtained from a Lineweaver−Burk plot, was 1.3 mM for BAPA (**Figure 5B**).

Specific Amino Acid Residues. The number of free cysteine residues in the protein was one (measured value, 1.14), whereas the total number of cysteine residues in the protein was 11 (measured value, 11.12), of which 10 cysteine residues formed five disulfide linkages. The total numbers of tryptophan and tyrosine residues in the enzyme were estimated to be six and 16, respectively (**Table 3**).

Autodigestion. At a fixed concentration of an activator, autodigestion of heynein was found to be enzyme concentration-dependent. Similar to ervatamins B and C, heynein exhibited slow autolytic degradation at its lower protein concentrations but autodigestion was less at higher protein concentrations (*16*, *17*). Typical results after 20 h of incubation shows that no significant loss in activity was observed at 1.0 mg/mL and above, while a maximum reduction in activity was at the lowest concentration of enzyme used (0.1 mg/mL) and only 20% residual activity was observed after 20 h. It is noted that a control enzyme sample, incubated at 37 °C without any activator under similar conditions, retained its complete activity. In addition, heynein at different concentrations (0.1−0.75 mg/mL), after 20 h of incubation in the presence of activator, was subjected to SDS−PAGE (data not shown). The gel patterns showed many fragments with molecular mass lower to the native enzyme at lower concentrations of the protein and the intensity of the band at the native position increases with an increase in protein concentration suggesting the protein is less susceptible to autodigestion at higher concentrations. This observation confirms the lesser extent of autodigestion at higher protein concentration, which is evident from the residual activity plot vs protein concentration.

Stability. Heynein exhibited remarkable stability under various denaturing conditions. It retained its activity over broad range of pH, 2.5−11.5 (**Figure 4A**), and temperature up to 63 °C (**Figure 4B**). The enzyme retained its full activity even in the presence of 8 M urea and 3.5 M GuHCl. The enzyme exhibited remarkable stability in the presence of organic solvents methanol, acetonitrile, and 2-propanol as compared with ervatamins B and C (**Table 4**).

N-Terminal Sequence. The amino terminal sequence of the first 15 amino acid residues of heynein has been compared (**Table 5**) to a few selected plant cysteine proteinases such as ervatamin B (*17*), ervatamin C (*16*), papain (*43*), asclepain (*44*), and bromelain (*45*). The N-terminal sequence of heynein has remarkable homology with that of ervatamins B and C and other cysteine proteases.

Antigenic Properties. Polyclonal antibodies were successfully raised in the male albino rabbit. The presence of antibodies in the serum was confirmed by precipitin bands obtained in Ouchterlony's double immunodiffusion (**Figure 6A**) and also by typical color development in indirect ELISA (**Figure 6B**).

Table 4. Stability of Heynein Relative to Ervatamin B[a] and Ervatamin C[b]

denaturant	stability: heynein	ervatamin B	ervatamin C
pH	2.5–11.5	3.0–10.5	2.0–12.0
urea (M)	8	8	8
GuHCl, pH 7.0 (M)	3.5	2.5	4
acetonitrile (%)	50	40	40
methanol (%)	65	50	50
2-propanol (%)	80		
temperature (°C)	63	62	70

[a] Ref *17*. [b] Ref *16*. Enzyme was incubated in the presence of a denaturant for 24 h prior to activity measurements where time of incubation at a given temperature is 15 min.

Table 5. Amino Terminal Sequence of Heynein as Compared to Other Cysteine Proteases[a]

enzyme	amino terminal sequence (first 15 residues)														
heynein	**L**	**P**	**E**	Q	I	**D**	X	**R**	X	X	**G**	**A**	**V**	N	P
ervatamin B[b]	**L**	**P**	S	F	V	**D**	**W**	X	S	K	**G**	**A**	**V**	N	S
ervatamin C[c]	**L**	**P**	**E**	Q	I	**D**	**W**	**R**	K	X	**G**	**A**	**V**	T	P
papain[d]	I	**P**	**E**	Y	V	**D**	**W**	**R**	Q	K	**G**	**A**	**V**	T	P
asclepain[e]	**L**	**P**	N	S	I	**D**	**W**	**R**	Q	K	N	V	**V**	F	P
bromelain[f]	V	**P**	Q	S	I	**D**	**W**	**R**	N	Y	G	**A**	**V**	T	S
morrenain oII[g]	**L**	**P**	D	S	V	**D**	**W**	**R**	K	K	N	L	**V**	F	P
morrenain bII[g]	**L**	**P**	**D**	S	V	**D**	**W**	**R**	K	K	N	L	**V**	F	P

[a] Bold letters represent the conserved amino acid residues. [b] Ref *17*. [c] Ref *16*. [d] Ref *43*. [e] Ref *44*. [f] Ref *45*. [g] Ref *57*.

There was no cross-reaction of antiheynein serum with other cysteine proteases such as ervatamin B, ervatamin C, and papain.

DISCUSSION

A cysteine protease has been purified from the latex of a valuable medicinal plant *E. heyneana* by a simple procedure using cation exchange chromatography on CM Sepharose. The protease named as heynein exhibited distinct physicochemical properties from other reported cysteine proteases including ervatamin B and ervatamin C, two proteases isolated already from *E. coronaria*, another species of the same genus. A single protein band on SDS−PAGE, isoelectric focusing, and a single symmetrical peak on reverse phase HPLC are indicative of the purity of the enzyme. Such a high degree of purity can be exploited to carry out further biophysical studies to understand the structure−function relationship of the protein molecule.

Like ervatamin B and ervatamin C, heynein showed broad substrate specificity. The enzyme hydrolyzed natural substrates casein, azoalbumin, azocasein, and also synthetic amides such as BAPA and *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide. Cysteine proteases have been supposed to play an important role in a variety of physiological processes of plants by virtue of their broad substrate specificities (*46*). Such contribution from heynein is therefore very much probable. The precise reason for the broad substrate specificity of cysteine proteases is not known, but it has been speculated to depend on specific amino acid residues in their substrate-binding site (*47*). Because of its specificities for a broad range of substrates, heynein can also be an important defense warrior against pathogens of the plant in vivo.

Heynein, like ervatamin B, hydrolyzes *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide, but unlike ervatamins B and C, it also hydrolyzes BAPA, which is an ideal substrate for papain (*22*). Besides, comparative BSA digestion studies with selected

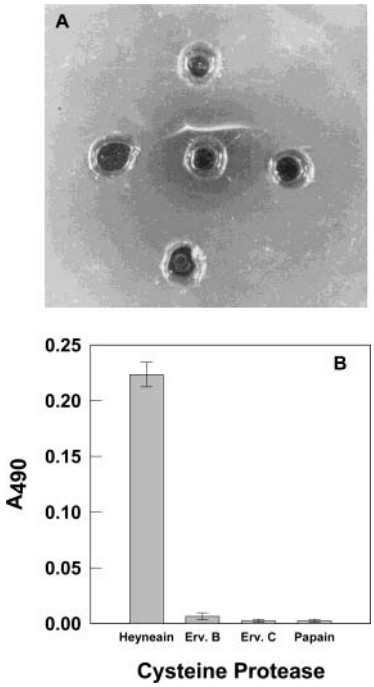

Figure 6. Immunoassay to check cross reactivity of anti-heynein with other related proteases, (**A**) Ouchterlong's double immunodiffusion: Agarose (1%) in phosphate buffered saline containing 0.02% sodium azide was solidified in a petri plate and appropriate holes were punched into it. Anti serum against heynein (100 μL) was added in the central well. Cysteine proteases (40 μg each) were added in to the peripheral wells. Heynein, ervatamin B, ervatamin C and papain were loaded in upper, right, bottom and left wells respectively. (**B**) Cross-reactivity of anti-heynein serum was assessed by a typical indirect-ELISA experiment. Antigens were used at a concentration of 1 μg/mL and antisera to heynein was diluted 1:200 times was added at 100 μL/well. The goat anti-rabbit IgG linked to horseradish peroxidase was diluted 10000 times in PBS-T. H_2O_2 was used as a substrate and o-phenylenediamine as a color indicator. The color development was measured at 490 nm. Background contribution has been deduced.

cysteine proteases using equal amounts of substrate and enzyme incubated for the same time show different molecular mass fragments on Tricine-SDS−PAGE. Therefore, the substrate specificity of heynein is quite different from these cysteine proteases.

The estimated molecular mass of heynein (23 kDa) lies well in the range of 20−35 kDa reported for most of the cysteine proteases (*46*, *48*). The enzyme is a monomer and consists of a single polypeptide chain as seen from its mobility on SDS−PAGE under reducing and nonreducing conditions.

Most of the cysteine proteases reported from plant sources are basic with a few exceptions such as actinidain, isolated from the latex of *Actinidia chinensis*, which is an acidic protease (*49*). Heynein (pI 10.8) is also basic in character, but it is exceptionally more basic than the related cysteine proteases, ervatamin B (pI 9.35) and ervatamin C (pI 9.53), indicating thereby the presence of more basic amino acids in its primary sequence. The pH specificity curve for heynein is found to be broad, symmetrical, and bell-shaped as seen in the case of ervatamin B (*17*), ervatamin C (*16*), ananain (*50*), caricain (*51*), and asclepain (*52*). Such a broad pH specificity of heynein may make it a highly applicable enzyme in various food and biotechnology industries for application under different conditions of pH.

Enzyme activation and inhibition studies revealed similar trends to those seen in the case of ervatamins B and C and other cysteine proteases. The presence of an activator was essential for the enzyme to exhibit its full activity. Thiol specific reducing agents such as β-ME, L-cysteine, reduced glutathione, and DTT activated the enzyme strongly. Such activation of the enzyme may be because of the removal of half-cysteine residues from the enzyme with concomitant liberation of a free thiol group on the catalytic site of the enzyme, which is a must for activity (*53*). The activity of heynein was inhibited in the presence of inhibitors specific for cysteine proteases such as E-64, IAA, PCMB, NEM, and sodiumtetrathionate. Serine and acid protease inhibitors did not inhibit the activity of the enzyme; therefore, the enzyme has been assigned to the class of cysteine proteases. Lack of inhibition of the activity by proteinous inhibitors such as SBTI, which are abundant in protein rich foods such as soybean, makes the enzyme a potential protease for food industry (*54*).

The enzyme is stabile against pH, temperature, denaturants, and organic solvents as it retains full activity over a broad range of pH values (2.5−11.5) and temperatures (4−63 °C). The enzyme activity remains unaffected even after prolonged exposure to 8 M urea and up to 3.5 M GuHCl. Heynein shows more stability than ervatamin B and other related cysteine proteases except ervatamin C. The broad stability of the enzyme against pH, temperature, and denaturants may make it a valuable tool for food and biotechnology industries for applications at extremes of pH, temperature, and under denaturing conditions.

The most striking property of heynein is its total cysteine content. It has an exceptionally higher number of cysteine residues (11) than ervatamin B (5) and ervatamin C (7). Heynein has only one free cysteine residue indicating the presence of five disulfide bonds. Such a high number of disulfide bonds asserts attention from the point of view of their contribution to the overall structural properties of the enzyme. Protein folding studies on cysteine proteases have yielded a number of valuable information in particular as well as in general (*55*). In this light, biophysical studies on heynein may lead to important conclusions.

The amino terminal sequence of heynein has a high degree of homology with that of ervatamin B and ervatamin C, and also, there is a considerable homology with other members of the cysteine protease class. Because proteins resemble each other in amino acid sequence only if they have a common ancestor (*56*), the conservation of amino terminal sequence may be due to divergent evolution of these enzymes from an ancestral precursor. Besides, the amino terminals of two proteases morrenain bII and morrenain oII from *Morrenia brachystephana* and *Morrenia odorata*, respectively, also bear almost full sequence homology (*57*), and also, the N-terminal sequence of fruit bromelain is identical to that of stem bromelain (*58*); therefore, a high degree of amino terminal sequence homology with ervatamins B and C is not surprising. Like ervatamins B and C and other cysteine proteases of the papain superfamily, a proline residue is conserved at the second position of the amino terminal of heynein, which probably prevents its unwanted proteolysis (*59*) in vivo. The marked amino terminal homology of heynein with ervatamins B and C suggests that their source plants *E. heyneana* and *E. coronaria* might have a recent divergence in their evolution.

As seen from Ouchterlony's double diffusion and ELISA, the antiheynein serum from immunized rabbit failed to cross-react with other cysteine proteases thereby establishing their antigenic diversity. Therefore, despite having close similarity in their amino terminal sequences, heynein and ervatamins are antigenically different.

Because cysteine proteases find a number of applications in industries such as meat tenderization, food processing, and dehairing of hide (*60*), by virtue of its high activity, high stability, easy purification, and bulk availability of the latex, heynein seems to be a potential peptidase for industrial applications.

ABBREVIATIONS USED

BAPA, Nα-benzoylarginine *p*-nitroanilide; DFP, diisopropylfluorophosphate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediamminetetraacetic acid; EGTA, ethylene glycol bis(β-amino ethyl ether)-*N*,*N*,*N*,*N*-tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; GuHCl, guanidine hydrochloride; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; NEM, *N*-ethyl-maleimide; PCMB, *p*-chloromercurybenzoate; PMSF, phenyl-methanesulfonyl fluoride; SBTI, soybean trypsin inhibitor; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TEMED, *N*,*N*,*N*,*N*-tetramethylethylenediamines.

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