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A Novel Serine Protease Cryptolepain from *Cryptolepis buchanani*: Purification and Biochemical Characterization

MONU PANDE,[†] VIKASH K. DUBEY,[‡] SUBHASH C. YADAV,[†] AND MEDICHERLA V. JAGANNADHAM*,[†]

Molecular Biology Unit, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005, India, and Department of Biotechnology, Indian Institute of Technology, Guwahati 781039, India

A novel protease is purified to homogeneity from the latex of a medicinally important plant *Cryptolepis buchanani* of family Apocynaceae (formerly Asclepiadaceae). The enzyme named cryptolepain has a molecular mass of 50.5 kDa. The isoelectric point and extinction coefficient ($\epsilon_{280nm}^{1\%}$) are 6.0 and 26.4, respectively. Cryptolepain contains 15 tryptophans, 41 tyrosines, and eight cysteine residues forming four disulfide bridges. The detectable carbohydrate moiety in the enzyme was found to be 6−7%. Cryptolepain hydrolyzes denatured natural substrates like casein, azocasein, and azoalbumin with high specific activity. The protease is exclusively inhibited by serine protease inhibitors phenylmethansulfonyl fluoride and diisopropyl fluorophosphate. Hydrolysis of azoalbumin by the cryptolepain is optimal in the pH range of 8−10 and temperatures of 65−75 °C. The enzyme shows high stability against pH (2.5−11.5), temperature (up to 80 °C), and chemical denaturants. The K_m value of the enzyme was found to be 10 μM with azocasein as the substrate. The N-terminal sequence of cryptolepain is unique and shows only little homology to other known serine proteases, which makes this enzyme an ideal candidate for our ongoing biochemical and structure−function investigations of proteases. Easy availability of the latex and simple purification procedures make the enzyme a good system for exploring the biophysical chemistry of serine proteases as well as applications in the food industry.

KEYWORDS: Asclepidaceae; Apocynaceae; serine proteases; plant endopeptidase; *Cryptolepis buchanani*; cryptolepain; anti-cryptolepain

INTRODUCTION

Most of the isolated plant proteases are cysteine and aspartic; occurrences of serine and metalloproteases are relatively rare (*1*). Several plant cysteine proteases were purified and characterized in our laboratory (*2*−*4*). Recently, we have focused our attention toward serine proteases to understand their comparative biochemical and biophysical properties. These studies may provide fundamental insights in to structure−function relationship and rationale behind the abundance of a particular class of protease (cysteine protease) among the plants. Although serine proteases are rare from the plant sources, in recent years, many serine proteases have been isolated and purified from various plant species in which they occur in distinct parts, ranging from the seeds to the latex and the fruits (*5*). However, very little information about the biochemical, biophysical, or structural properties of plant serine proteases is available. Most of the available data relating to plant serine proteases pertains to cucumisin-like proteases, a family of endopeptidases found in Cucurbitaceae (*6*−*9*). Furthermore, among plant sources, serine proteases are abundant in fruits and only few serine proteases are reported from the latex (*5*).

Proteolytic enzymes play a key role in plant physiology. They not only maintain the protein pool of the cell but also are involved in various intra- and extracellular processes like leaf senescence, breakdown of storage proteins in germinating seeds (*10*), development and ripening of fruits (*11*), regulatory mechanisms, etc. The tissues, which are metabolically more active, have abundant endopeptidases activity. The lattices of plants have yielded some valuable and commercially important endopeptidases, such as calotropins (*12*), papain (*13*), and ficin (*14*). The precise biological role of proteases in latex of plants is still a matter of debate, but they are suggested to be involved in the protection of plants against pathogens such as insects, fungi, etc. (*1*, *15*). The presence of bacteriolytic activity in lattices of *Carica papaya* (*16*), *Ficus glabarata* (*17*), and *Ervatamia coronaria* (*18*, *19*) confirms the fact that it acts in unison. The roles of serine proteases in microsporogenesis, symbiosis, hypersensitive response, signal transduction and differentiation, senescence, and protein degradation/processing are reviewed by Antao and Malcata (*5*).

Plant proteases have been well-known for ages for their industrial applications due to their broad substrate specificity

* To whom correspondence should be addressed. Tel: 91-542-2367936. Fax: 91-542-2367568. E-mail: jvm@bhu.ac.in or jaganmv@satyam.net.in.
[†] Banaras Hindu University.
[‡] Indian Institute of Technology.

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and their activity over a wide range of pH and temperature values. Quantitatively, more than half of the total commercially used industrial enzymes are proteases (*20*). In food industries, proteases are indispensable for processes such as tenderization of meat, brewing, cheese elaboration, and bread manufacturing (*21*). Cysteine proteases are abundant in plant systems, but they are readily inhibited by air oxidation or metal ions. Thus, the application of these proteases requires reducing agents and chelating agents, which restricts their commercial application, as they are not so economic and handy. So, the search for a new potential plant serine proteases to make industrial processes cost-effective is still in demand.

In the present study, isolation and biochemical characterization of a novel serine protease from the latex of a medicinally important plant *Cryptolepis buchanani* has been reported. *C. buchanani* belongs to the family Apocynaceae (formerly Asclepidaceae) and is commonly distributed throughout India, especially in hot, deciduous forests. It is worth mentioning that Asclepiadaceae is now included within the Apocynaceae family. It is a plant family whose members usually produce latex and includes five subfamilies: Rauvolfioideae, Apocynoideae, Periplocoideae, Secamonoideae, and Asclepiadoideae; the last three formerly belonged to the subfamily Asclepiadaceae. In turn, the Asclepiadoideae subfamily includes three tribes: Asclepiadeae, Ceropegieae, and Marsdenieae (*22*).

C. buchanani is a medicinally important plant, and various parts of the plant are used as antidiarrheal, antibacterial, antiulcerative, antiinflammatory, blood purifiers, and in curing rickets in children (*23*). The ethanolic extract of the plant has a potent immunostimulant activity and can be used as a therapeutic agent in immune-compromised patients (*24*). Preliminary studies on the latex of this valuable plant showed a high caseinolytic activity, indicative of the presence of protease(s). A serine protease was purified to homogeneity by ammonium sulfate fractionation and ion exchange chromatography. Following the nomenclature of proteases, the purified enzyme was named as cryptolepain. The similar nomenclature pattern has been used for naming other serine proteases; for example, a serine protease purified from the latex of dandelion root *Taraxacum officinale* is called taraxilisin (*25*), and artocarpin has been purified from *Artocarpus heterophyllus* (*26*).

MATERIALS AND METHODS

Materials. Superficial incisions on the *C. buchanani* yielded milklike latex. Fresh latex of the plant was collected. CM-Sepharose FF was purchased from Pharmacia. Bovine serum albumin (BSA), hen egg white lysozyme, azocasein, azoalbumin, hemoglobin, DTNB (5,5′-dithiobis-[2-nitrobenzoic acid]), DTT (dithiothretiol), DIFP (diisopropyl fluorophosphate), GuHCl (guanidine hydrochloride), urea, *o*-phenanthroline, EDTA (ethylene diamine tetraacetic acid), EGTA (ethylene glycol-bis(2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid), leupeptin, SBTI (soyabean trypsin inhibitor), $HgCl_2$ (mercuric chloride), PCMB (*p*-chloromercuric benzoate), NEM (*N*-ethylmaleimide), βME (β-mercaptoethanol), PMSF (phenylmethansulfonyl fluoride), acrylamide, *N*,*N*-methylene bisacrylamide, Coomassie brilliant blue R-250, E-64 {L-*trans*-epoxysuccinylleucylamide(4-guanidino)butane-*N*-[*N*-(L-3-*trans*-carboxyirane-2-carbonyl)-L-leucyl]agimatine}, hemoglobin, Coomassie brilliant blue R 250, all synthetic substrates, triethanolamine, Agarose, Tween-20, *o*-phenylenediamine, horseradish peroxidase conjugate, and other standard proteins were obtained from Sigma Chemical Co. (United States). Coomassie brilliant blue G250 was from Eastman Kodak. Trifluoric acetic acid was obtained from Applied Biosystems. Ampholine carrier ampholytes were from LKB. Acetonitrile was of high-performance liquid chromatography grade. Sodium tetrathionate ($Na_2S_4O_6 \cdot 2H_2O$) was synthesized by the method of Gilman et al. (*27*). Hemoglobin was denatured with urea before the assay as described (*28*). All other chemicals were of highest purity.

Purification. All purification steps were carried out in cold to minimize complications due to autodigestion of proteins, if any. Most of the purification was carried out at 4 °C unless stated otherwise.

Step 1. Removal of Gum. Latex was collected from the young stems of the plant in 0.01 M phosphate buffer, pH 7.0, containing 0.01 M sodium tetrathionate and was stored at −20 °C. Sodium tetrathionate reversibly blocked the cysteine proteases present in the latex of *C. buchanani* and prevented any complication due to autodigestion during the purification as the crude enzyme was catalytically active. Frozen latex was thawed to room temperature and centrifuged at 24000*g* for 10 min to remove any insoluble materials. The supernatant was used in the next step.

Step 2. Ammonium Sulfate Fractionation. The supernatant from the above step was brought to 50% saturation with solid ammonium sulfate and allowed to stand in cold for overnight. The resulting precipitate was removed by centrifugation at 20000*g* for 10 min and dissolved in 0.01 M acetate buffer, pH 4.5, and dialyzed against the same buffer. The supernatant was also dialyzed against 0.01 M acetate buffer, pH 4.5, for 24 h with frequent changes of buffer and loaded on sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). A major amount of protein with good activity was seen in the supernatant, which was used in the next step.

Step 3. Ion Exchange Chromatography on CM-Sepharose. Chromatography was performed at room temperature. The supernatant from the above step was loaded on a CM-Sepharose cation exchange column pre-equilibrated with 0.01 M acetate buffer, pH 4.5. The column was washed with the same buffer, and the bound proteins were eluted with a linear gradient of 0−0.6 M NaCl at a flow rate of 6 mL/min. Fractions of 4 mL were collected. The absorbance at 280 nm as well as the caseinolytic activity of the protein in all fractions was checked using casein as a substrate.

Protein Concentration. The protein concentration at different stages of purification was determined by absorbance at 280 nm as well as by the method of Bradford (*29*) using BSA as a standard.

Protease Activity. The hydrolyzing activity of the protease was determined using denatured natural substrates like casein, hemoglobin, azoalbumin, and azocasein using the method of Arnon (*30*). The enzyme solution (15 μg) was incubated in a final volume of 0.5 mL of 50 mM Tris-HCl buffer, pH 8.0, at 37 °C for 10 min. The casein solution (1%, w/v) prepared at the same pH was added to the enzyme solution making the final reaction volume to 1 mL, and the reaction mixture was incubated for 30 min at 37 °C. The reaction was stopped by adding 0.5 mL of 10% TCA and incubated further for 10 min at room temperature followed by centrifugation at 10000 rpm for 10 min. The absorbance of the soluble peptides in the supernatant was measured at 280 nm. In the case of azoalbumin or azocasein as the substrate, 0.5 mL of supernatant after TCA precipitation was mixed with an equal volume of 0.5 M NaOH and incubated for 15 min. The development of color was measured spectrophotometrically by taking the absorbance at 440 nm. A control assay, without the enzyme in the reaction mixture, was done and used as the blank in all spectrophotometric measurements. One unit of enzyme activity was defined as the amount of enzyme, under given assay conditions, that gives rise to an increase of 0.01 unit of absorbance at 280 nm or an increase of 0.001 unit of absorbance at 440 nm per minute of digestion. The number of units of activity per milligram of protein was taken as the specific activity of the enzyme.

Electrophoresis. Homogeneity and intactness of the enzyme, during purification as well as molecular mass determination of the purified enzyme, were determined by using SDS-PAGE (*31*). The purified enzyme was inactivated to avoid autolysis by treatment with DFP. The gels were stained with 0.1% Coomassie brilliant blue R-250. The molecular weight of the purified enzyme was extrapolated from the plot of log molecular weight vs electrophoretic mobility of markers. The gel was also stained with Schiff's reagent, which is specific for glycoproteins.

Mass Determination by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-MS). MALDI-TOF mass spectrometry was performed to confirm the purity of the protein as well as molecular weight determination. Mass spectrometry was performed by mixing a 7 mM concentration of the sample with saturated sinapinic acid solution in 0.1% trifluoroacetic acid in water/acetonitrile, 2:1. One

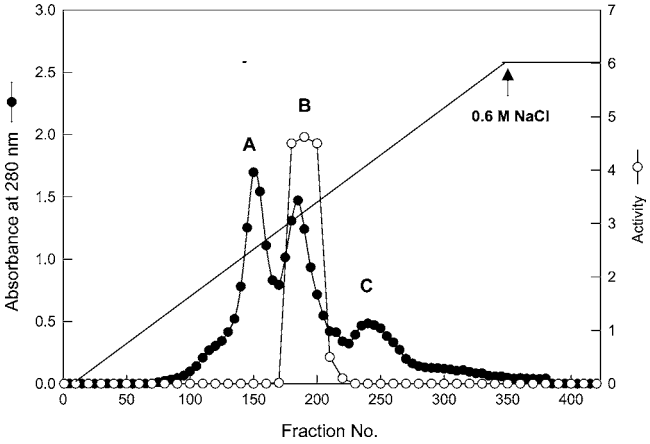

Figure 1. Elution profile of cryptolepain on the cation exchanger: The CM-Sepharose column was pre-equilibrated with 10 mM sodium acetate buffer, pH 4.5. The unbound proteins were washed out with the equilibration buffer, and the column was eluted with a linear salt gradient of 0.00–0.60 M NaCl at the same pH. Fractions of 4 mL were collected at a flow rate of 6 mL/min and assayed for protein content (●) and proteolytic activity (○).

Table 1. Purification of Cryptolepain from Latex of *C. buchanani*

steps	total protein (mg)	total activity (units[a])	specific activity (units/mg)	recovery (%)
1. crude latex	480	5568	11.6	100
2. ammonium sulfate supernatant (50%)	215	3060	14.2	55
3. CM-Sepharose	21	404.2	19.2	7.3

[a] Definition of 1 unit: 1 unit of enzyme activity is defined as the amount of enzyme under the assay conditions described and gives rise to an increase of 0.01 unit absorbance at 280 nm/min of digestion. Casein was used as the substrate.

microliter was spotted onto the sample plate. After spotting, the plate was allowed to dry and data were collected on a Simadzu Axima CFR-plus MALDI-TOF mass spectrometer in linear mode. MALDI-TOF standard BSA was used for calibration of the instrument.

Isoelectric Point. The isoelectric point of the purified enzyme was determined by isoelectric focusing in tube gels as described for procerain (*2*). Ampholines, in the pH range of 3.5−10.0, were used to generate the pH gradient. Protein bands were stained with 0.04% (w/v) Coomassie brilliant G-250 dissolved in 6% (w/v) perchloric acid (*32*).

Carbohydrate Content. Carbohydrate moieties attached to the protein surface were important to regulate the stability of the protein (*33*). The carbohydrate content of cryptolepain was determined by the phenol sulfuric acid method (*34*). The carbohydrate content of cryptolepain was determined by extrapolation from the calibration curve generated under similar conditions with galactose as the standard.

Extinction Coefficient. The extinction coefficient of cryptolepain was determined using dry weight (*35*) as well as spectrophotometric (*36*) methods. The average value of the extinction coefficients obtained from the two methods was used for all other practical purposes.

Tryptophan and Tyrosine Content. The total number of tryptophan and tyrosine residues in the purified protein was determined spectrophotometrically by the method of Goodwin and Morton (*37*), where the absorbance spectrum of the purified enzyme in 0.1 M NaOH was recorded. To validate the measurements, similar contents of papain, ribonuclease A, ervatamin C, and egg white lysozyme were also determined.

Measurement of Free and Total Sulfhydryl Content. Free and total cysteine residues of cryptolepain were determined out using DTNB method of Ellman (*38*) as described in great detail for procerain (*2*). The excess of DTT or β-ME in the reaction mixture was removed by extensive dialysis against 0.1 M acetic acid (*39*). The number of cysteine residues was calculated using an extinction coefficient of 14150 M^{-1} cm^{-1} at 412 nm for TNB anions (*40*). To validate the current measurements, similar contents of papain, ribonuclease A, and lysozyme were also determined.

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 a spectrophotometry method (*30*). Substrates used in this study were N-β-benzoyl-DL-arginine-*p*-nitroanilide (BAPA), L-Ala-Ala-*p*-nitroanilide, *N*-succinyl-Phe-*p*-nitroanilide, L-Glu-*p*-nitroanilide, L-Ala-*p*-nitroanilide, and L-Leu-*p*-nitroanilde. In each case, a stock of 1−12 mM solution of the synthetic substrate was prepared in DMSO and made up to the required volume with buffer. An extinction coefficient of 8800 M^{-1} cm^{-1} for *p*-nitroanilide was used in the calculation activity (*41*).

pH and Temperature Optima. The activity of the purified enzyme is measured as a function of varying pH to determine the pH optima of the enzyme. Ten micrograms of enzyme was used for activity measurement. The buffers used were as follows: 0.05 M KCl−HCl (pH 1.0−1.5), 0.05 M glycine−HCl (pH 2.0−3.5), 0.05 M Na−acetate (pH 4.0−5.5), 0.05 M Na−phosphate (pH 6.0−7.5), 0.05 M Tris−HCl (pH 8.0−10.0), and 0.05 M sodium carbonate (pH 10.5−12.0). The substrate solution of azoalbumin or hemoglobin was prepared in the respective buffers. Because of the insolubility of azoalbumin below pH 4.0, hemoglobin was used as the substrate for activity measurements at lower pH (*28*).

The effect of temperature (in the range of 10−80 °C) on the activity of cryptolepain was also studied using azoalbumin as the substrate. Prior to the assays, the substrate solution was also equilibrated at the corresponding temperature in the same buffer. At each temperature, a control assay was carried out without the enzyme and used as a blank.

Stability. As the stability of an enzyme dictates its applicability, the effect of pH (0.5−12), temperature (10−80 °C), denaturants (GuHCl and urea), and typical organic solvents such as methanol, acetonitrile, and dioxan on the proteolytic activity of cryptolepain was also studied. The enzyme was incubated under specified conditions of pH, chemical denaturant, or organic solvent for 24 h while the incubation time for temperature stability measurement was 15 min. The activity was measured as previously described.

Effect of Various Compounds on the Activity of Cryptolepain. The effect of protease inhibitors on the proteolytic activity of enzyme was studied. The inhibitors used were STT, IAA, PMSF, EDTA, EGTA, E-64, PCMB, *o*-phenanthroline, NEM, $HgCl_2$, DIFP, leupeptin, and SBTI. A control assay of the enzyme activity was done without inhibitors, and the resulting activity was taken as 100%. The enzyme was incubated with a specific inhibitor for 30 min at room temperature, and an aliquot was used for the activity measurement. The assay was done as described earlier.

Effect of Substrate Concentration on the Reaction Velocity. The effect of increasing substrate concentration on the reaction velocity of the enzyme hydrolysis was studied using azocasein as the substrate at pH 8.0 and 37 °C. Ten micrograms of the enzyme was used, and the concentration of azocasein was in the range of 1−150 μM. Assays were performed as already described under proteolytic activity measurements. A blank was used at the specific substrate concentrations without the enzyme. A Lineweaver−Burk plot was plotted, and the value of Michaelis−Menten constant (K_m) was calculated.

Autocatalysis. Proteases, in general, are prone to autodigestion, and the extent of autolysis depends on enzyme concentration, pH, and temperature. Cryptolepain at different concentrations in the range of 0.05−0.45 mg/mL in 0.05 M Tris−HCl, pH 8.0, was incubated at room temperature. An aliquot containing 5 μg of enzyme was used for the determination of protease activity after 24, 48, and 72 h. Casein was used as the substrate. The activity of the enzyme after the first 10 min of incubation was taken as 100% for the calculation of the residual activity.

Polyclonal Antibodies and Immunoassays. Polyclonal antibodies were raised against cryptolepain in a male albino rabbit (weight about 1.5 kg) to check its immunological properties. The antibodies were raised as described by Dubey and Jagannadham (*2*). All sera were stored at −20 °C. The Oüchterlony's double diffusion was performed as

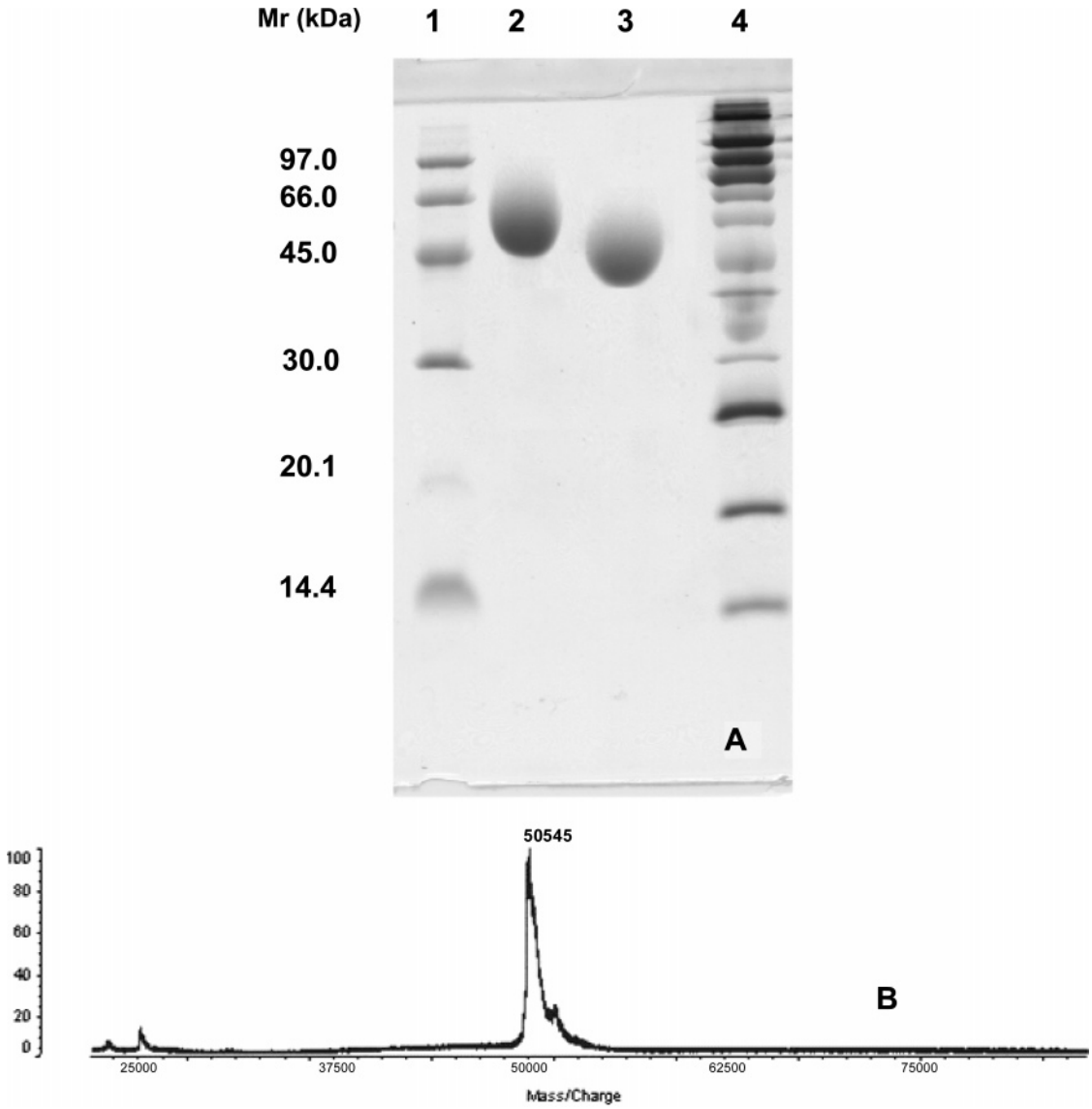

Figure 2. (**A**) SDS-PAGE of purified protease: Lanes 1–4 represent markers and cryptolepain under nonreduced and reduced conditions and crude latex, respectively. (**B**) Mass spectrometry of cryptolepain: The MALDI-TOF standard BSA was used for calibration of the instrument, and data were collected in linear mode.

described by Oüchterlony and Nilsson (*42*). Various antigens (40 μg) and 100 μL of antiserum were loaded in the wells and left at room temperature for 24−30 h. Control assays were performed with preimmune serum.

An indirect enzyme-linked immunosorbent assay (ELISA) (*43*) was also performed to check the cross-reactivity of various antigens with anti-cryptolepain serum. Wells of a microtiter plate were coated with 10 μg/μL of cryptolepain, trypsin, and proteinase K (50 μL per well) in sodium carbonate, pH 9.6, and incubated overnight at 4 °C. The color development was determined as described by Dubey and Jagannadham (*2*).

N-Terminal Amino Acid Sequencing. The N-terminal sequencing was performed with sample in a gel slice. Twenty micrograms of purified enzyme was loaded on SDS-PAGE and stained with 0.1% Coomassie Blue R-250 for the minimum time necessary to visualize the bands. Overnight destaining was performed with several changes to ensure adequate removal of SDS. The protein band was excised and used for N-terminal sequencing using an Applied Biosystems model 492 Precise Sequencer attached to a model 140C Micro-gradient System and a 610A Data Analysis System using the method of Matsudaira (*44*).

RESULTS AND DISCUSSION

Purification. The purification of cryptolepain from the latex of *C. buchanani* consisted of a two-step procedure. After the gum was removed, crude latex was subjected to 50% ammonium sulfate fractionation. The precipitate and supernatant both showed protease activity, but the precipitate was more heterogeneous as judged by SDS-PAGE. Besides, the specific activity of protease was found to be more in the supernatant fraction. Thus, the supernatant from the previous step was chosen for further purification on cation exchanger CM-Sepharose fast flow column after dialyzing it against 10 mM acetate buffer, 10 mm, pH 4.5. The bound proteins were eluted with a linear salt gradient of 0−0.6 M NaCl. The elution profile resolved into three peaks: A, B, and C (**Figure 1**). The majority of the total activity loaded to the column appeared in peak B. The fractions of the ascending limb of peak B were pure as judged by single band on SDS-PAGE. Such homogeneous fractions were pooled, concentrated, dialyzed against 50 mM phosphate buffer, pH 7.0, and stored at 4 °C for further use. The purification results were summarized in **Table 1**. The specific activity of the purified

Figure 3. Zymogram (gelatin gel PAGE) showing the protease activity of cryptolepain. Fifteen micrograms of enzyme was used for activity staining.

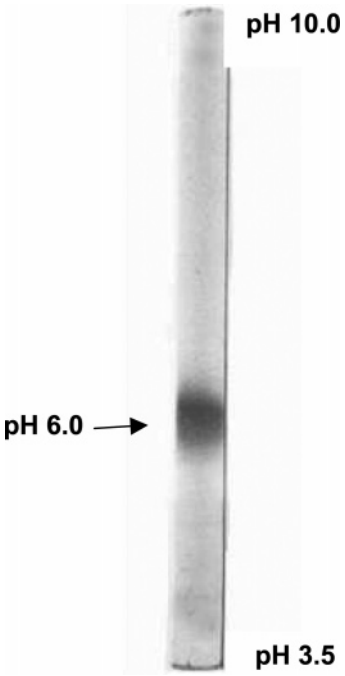

Figure 4. Isoelectric focusing of cryptolepain: Electrophoresis was performed using 5% polyacrylamide disc gels with ampholine carrier ampholytes, pH 3.5–10.0. A sample of 100 μg of protein containing 10% ampholine and 25% glycerol was loaded and electrophoresed for 3 h at a constant voltage of 300 V.

enzyme was 19.2 U/mg of protein as compared to the activity in the crude latex, which was 11.6 U/mg of protein with casein as substrate. The total recovery of the activity was 7.3%. The low recovery of the protease activity may be due to the presence of multiple proteases in the crude latex. It is also important to note that measurable activity that appeared in 50% ammonium sulfate precipitate might have affected the final yield. The zymogram of protease activity staining of the purified enzyme shows a clear zone of proteolytic activity against corresponding to cryptolepain position in the gel (**Figure 3**).

The abundance of latex together with the simple purification procedure of cryptolepain makes large-scale purification economical, which enables exploration of various industrial and biotechnological applications of the protein. Serine proteases are well-characterized in mammals and microorganisms, but they are somehow overlooked in plants. Till today and to the best of our knowledge, no serine proteases have been purified from the Apocynaceae family. So, it is important to study the biochemical properties of cryptolepain and compare them with the other members of the serine protease family.

Physical Properties. Cryptolepain showed a single band on SDS-PAGE under reducing and nonreducing conditions, which shows its purity (**Figure 2A**). However, the band positions were not the same. Under reducing conditions, because of breakage of disulfide bonds, the protein molecule will be more flexible, which facilitates an increase in mobility, whereas under nonreducing conditions, the molecule is compact resulting in a retardation in mobility. Furthermore, the homogeneity of the enzyme was also confirmed by mass spectrometry. The molecular mass of cryptolepain estimated by SDS-PAGE and mass spectrometry (**Figure 2B**) is 52.5 and 50.5 kDa, respectively. There is some discrepancy in the molecular mass of cryptolepain determined by these two methods, but as the mass spectrometric data are more accurate, the 50.5 kDa value is used for other biochemical calculations. The molecular masses of the plant serine proteases known at present vary from 19 to 110 kDa, but the majority are between 60 and 80 kDa (*5*). The molecular mass of cryptolepain falls well in the range of the molecular mass of plant serine proteases. The molecular mass of other well-studied plant serine proteases of the cucumisin family, falls in the range of 60−70 kDa (*45*−*50*). Cryptolepain showed a single band (**Figure 4**) on isoelectric focusing with an approximate isoelectric point (pI) of 6.0. Most of the plant serine proteases have acidic to near neutral isoelectric points (**Table 2**). Other serine proteases reported from euphorbia family also have isoelectric points in the same range (*51*). However, some of the serine proteases were also reported to have highly basic isoelectric points (*52*). The extinction coefficient ($\epsilon_{280\text{nm}}^{1\%}$) of purified enzyme as determined by dry weight and spectrometric methods was 26.4, and this value was used for all experimental purposes.

Carbohydrate Content. Cryptolepain is a glycoprotein having a detectable amount of carbohydrates in it. The majority of serine proteases reported from euphorbiaceae family also have sugar moieties in their molecular architecture. The amount of carbohydrate as estimated by the phenol sulfuric acid method in cryptolepain is 6−7%. The glycoprotein nature of cryptolepain is further confirmed by staining with Schiff's reagent after SDS-PAGE as described in the Materials and Methods. It shows a magenta color band (data not shown). The roles of sugar residues in proteases are not very well-defined, but in general, the carbohydrate residues of proteins have important roles in function, folding, and solubility. They also stabilize the proteins and protect them from degradation.

Specific Amino Acid Residues. The total cysteine content of cryptolepain is found to be eight with no free cysteine residues (measured value 0.03), thus forming four disulphide bridges. Serine proteases like euphorbian l, euphorbian y_1, and euphorbian y_3 have 7, 9, and 10 cysteine residues, respectively (*51*). The total number of tryptophan and tyrosine residues of cryptolepain is 15 and 41, respectively. These values are

Table 2. Physicochemical Properties of Cryptolepain in Comparison with Other Plant Serine Proteases[a]

protein	plant	mol mass (kDa)	pH optimum	temp optimum (°C)	isolectric point (pI)
cryptolepain	*C. buchanani*	50.5	8–10.5	70–75	6.0
Ara12	*A. thaliana*	76.1	5.0	80	NR
serine protease	*T. aestivum*	110.0	8–10	60	NR
serine protease	*C. melo*	26.0	NR	NR	9.5
protease D	*C. melo*	50.0	11	35	NR
RSPI	*Z. mays*	59.0	6.0–6.5	NR	4.5
cucumisin	*C. melo*	54.0	7.1	70	NR
serine protease	*C. cochinchinensis*	76.0	11	60	NR
serine protease	*P. hindsii*	80.0	6.5–10.5	30	NR
KLSP	*P. vulgaris*	72.0	9.9	60	4.6
serine protease	*C. trigonus*	67	11	70	NR

[a] NR in the table represents data not reported. *A. thaliana* (*55*), *T. aestivum* (*57*), *C. melo* (*52*), protease D *C. melo* (*58*), *Z. mays* (*59*), cucumisin *C. melo* (*57*), *C. cochinchinensis* (*60*), *P. hindsii* (*9*), *P. vulgaris* (*61*), and *C. trigonus* (*64*).

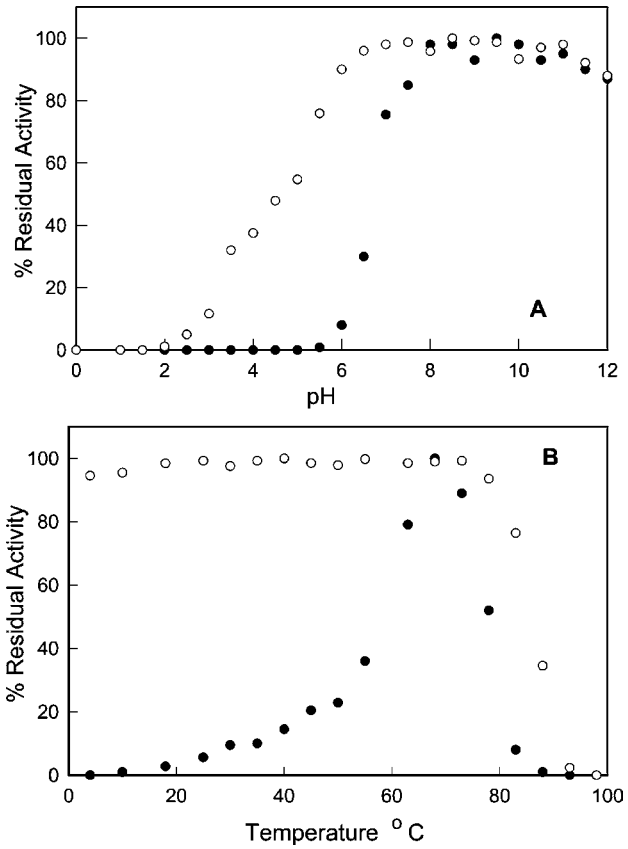

Figure 5. Effect of pH (**A**) and temperature (**B**) on activity (●) and stability (○) of cryptolepain. For pH optima, 10 μg of enzyme in 0.5 mL of buffer at the required pH was used for activity measurement using substrate prepared in the corresponding buffers. For pH stability measurements, 15 μg of enzyme was incubated overnight at the required pH. To see the effect of temperature on activity, 10 μg of cryptolepain was incubated at the required temperature for 15 min, 0.5 mL of substrate was added, and the activity was measured at the same temperature. For stability experiments (○), 15 μg of enzyme was incubated at the required temperature for 15 min, and the activity was measured at 37 °C and pH 8.0.

exceptionally high when compared to reported values for other serine proteases.

Substrate Specificity. Cryptolepain hydrolyzes natural denatured substrates like casein, hemoglobin, azoalbumin, and azocasein with remarkable activity. However, it does not show any significant activity for the synthetic substrates used in the current study like L-Ala-Ala-*p*-nitroanilide, L-Ala-*p*-nitroanilide, L-Leu-*p*-nitroanilide, N-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPA), *N*-succinyl-Phe-*p*-nitroanilide, and L-Glu-*p*-nitroanilide. For most plant serine proteases, like cucumisin, hydrolysis of these substrate is chain length-dependent and they cleave desirably at the C-terminal side of carboxyl amino acid residues such as glutamic acid and carboxymethylated cysteine and the N-terminal side of alanine (*53*). Thus, with these synthetic substrates, cryptolepain fails to exhibit any measurable activity.

Table 3. Stability of Cryptolepain under Various Conditions

conditions	stability	% residual activity
pH	6.0–11.0	100
urea	8.0 M	100
GuHCl	2.5 M	100
acetonitrile	55%	100
methanol	50%	100
dioxan	70%	100
temperature	75 °C	100

Table 4. Effect of Different Types of Inhibitors on the Activity of Cryptolepain

inhibitor type	name of inhibitor	[I]	residual activity (%)
serine protease	PMSF	100 μM	2.68
	SBTI	5 mM	97.7
	DIFP	50 μM	4.01
Ser/cysteine	leupeptin	25 μM	110.0
cysteine protease	iodoacetic acid	50 μM	98.9
	sodium tetrathionate	40 μM	92.0
	E-64	10 μM	97.3
	$HgCl_2$	100 μM	92.2
	NEM	5 μM	98.8
	PCMB	50 μM	105.0
metalloprotease	EDTA	5 mM	100.0
	EGTA	5 mM	97.4
	o-phenanthroline	1 mM	98.0

pH and Temperature Optimum. The optimal pH for the hydrolyzing activity of cryptolepain for azoalbumin is 8−12 (**Figure 5**A). Such a broad pH range specificity of cryptolepain may indicate its applicability in various food and biotechnology industries. Furthermore, the optimum pH of cryptolepain differs significantly with those proteases purified from euphorbia species, having pH optima between 6 and 8 (*51*). On the other hand, the value is close to cucumisin like serine protease of the cucurbitaceae family, which shows an optimum pH in the range of 8−10 (*45*, *54*). A sharp decrease in activity in the acidic region (**Figure 5A**) may be due to the formation of improper ionic forms of the enzyme active site or substrate or a

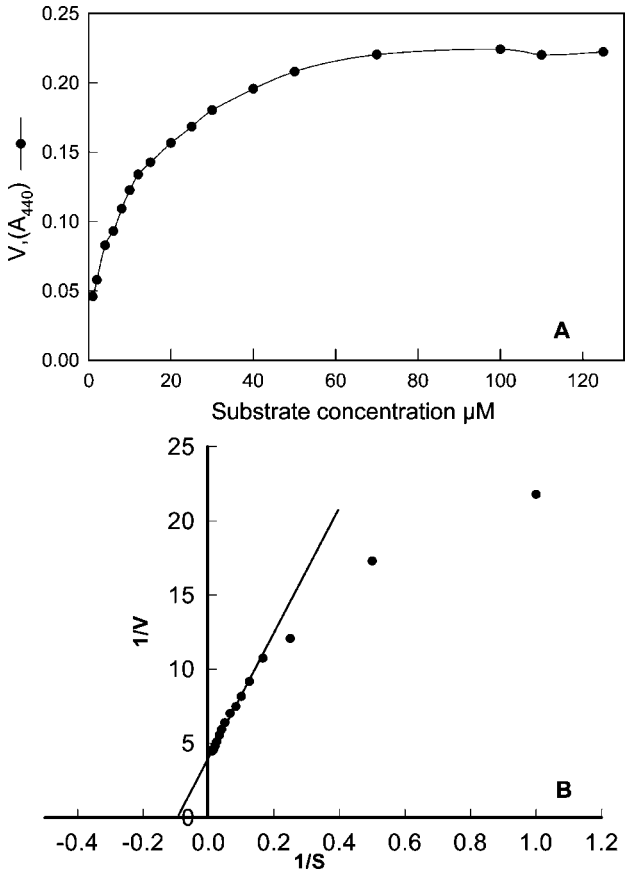

Figure 6. (**A**) Effect of substrate concentration on reaction velocity (●) of cryptolepain follows the Michaelis–Menten equation. Ten micrograms of the enzyme in 0.5 mL of 0.1 M Tris-HCl, pH 8.0, was added to 0.5 mL of azoalbumin in a concentration range of 1–150 μM. The activity was determined as described in the text. (**B**) Lineweaver–Burk plot. K_m was calculated according to the Michaelis–Menten equation.

combination of the two. Besides, the enzyme showed no detectable activity below 4.5.

The temperature optimum of cryptolepain is 70−75 °C (**Figure 5B**). There is a sudden decrease in the activity on either side of this optimal range. Such a high-temperature optimum is also reported in the case of serine proteases purified from *Arabidopsis thaliana* (*55*), *Cucumis trigonus* (*64*), and in cucumisin (*56*). However, most of the plant serine proteases show optimum activity in the range of 30−60 °C (**Table 2**). This unusually high-temperature optimum of cryptolepain makes the enzyme useful for those industries where high-temperature stability is required.

Stability. The stability of the enzyme under extreme conditions is a decisive factor for its usefulness as a potent industrial enzyme. Cryptolepain is remarkably stable under the conditions where most of the enzymes lose their activity. The protein retains full activity over a broad range of pH 6−12 (**Figure 5A**) as well as temperatures up to 75 °C for 15 min (**Figure 5B**). Retention of complete activity in 50% methanol, 55% acetonitrile, and 70% dioxan indicates a high stability of cryptolepain in organic solvents. The protease is stable up to 2.5 M GuHCl and retains maximum activity in 8 M urea. Such a high stability of cryptolepain under various harsh conditions makes it an excellent system for biophysical studies to elucidate the structure−function relationship. The stability of cryptolepain under different condition is presented in **Table 3**.

Effect of Inhibitors on Activity of Cryptolepain. Different inhibitors specific for different class of proteases are used to determine the class of the purified protease (**Table 4**). Maximum inhibition of activity of the enzyme occurs in the presence of DIFP and PMSF while PCMB, $HgCl_2$, IAA, NEM, E-64, *o*-phenthroline, EDTA, and EGTA have no effect on the activity. As the enzyme is inhibited exclusively by serine protease inhibitors, this indicates that the enzyme is serine protease. More than 95% inhibition occurs when the enzyme is incubated with 50 μM DIFP for 30 min at room temperature. The effective concentration of DIFP required for complete inhibition of cryptolepain is close to that required for cucumisin and other plant serine proteases (*54*). PMSF also inhibits the enzyme activity by 97% at 100 μM concentration. SBTI is a less

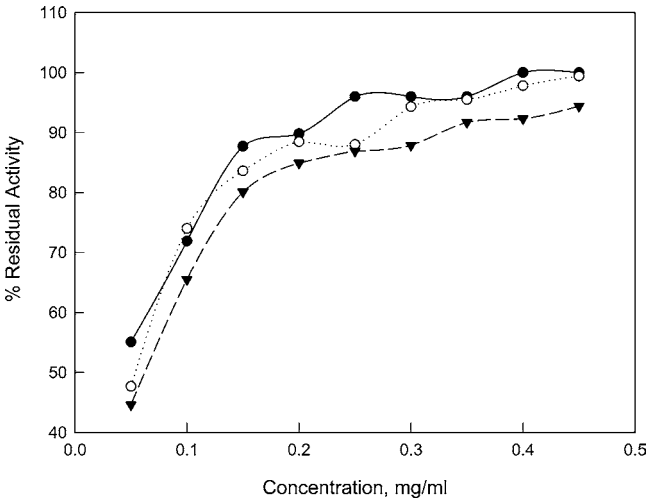

Figure 7. Autodigestion of cryptolepain, as a function of increasing protein concentration, was depicted. The enzyme at different protein concentrations (0.05–0.45 mg/mL) was incubated at room temperature for 24 (●), 48 (○), and 72 (▼) h, respectively. The residual proteolytic activity was measured under optimal conditions using 5 μg of enzyme as described in the Materials and Methods. The activity of the enzyme after 10 min of incubation was taken as 100% activity.

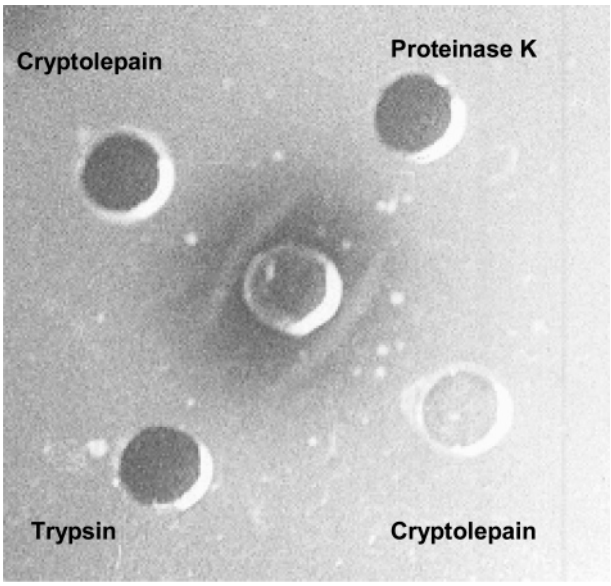

Figure 8. Ouchterlony's double immunodiffusion was carried out in (1%) agarose in phosphate-buffered saline containing 0.02% sodium azide. Anti-cryptolepain serum (100 μL) was added in a central well and 40 μg of cryptolepain was added in the right side lower and left side upper peripheral wells and 40 μg of proteinase K and trypsin were added in the remaining two right upper and left lower peripheral wells. The appearance of precipitin bands was observed after 24 h of incubation.

Table 5. Amino Terminal Sequence of Cryptolepain as Compared to Other Serine Proteases[a]

enzyme	amino terminal sequence (first 15 residues)														
cryptolepain	**M**	E	G	A	**S**	F	G	A	F	**L**	**S**	S	**T**	A	**R**
bamboo protease	T	T	R	T	P	S	F	L	R	**L**	**S**	A	V	G	**R**
pyrolysin	**M**	Y	N	S	T	W	V	I	N	A	L	Q	F	I	Q
cucumisn	T	T	R	S	W	D	F	L	G	F	P	L	**T**	V	P
tomato P69 A	T	T	H	T	**S**	S	F	L	G	**L**	Q	Q	N	M	G
tomato P69 B	T	T	R	S	P	T	F	L	G	**L**	E	G	R	E	S
arabdopsis ARA12	T	T	R	T	P	L	F	L	G	**L**	D	E	H	T	A
alnus ag12f	T	T	H	T	P	R	F	L	S	**L**	N	P	**T**	G	G

[a] The bold letters represent the similar amino acid residues. Bamboo protease (*9*), pyrolysin (*62*), cucumisin (*63*), tomato P69A (*46*), tomato P69B (*47*), Ara 12 (EMBL accession number X85974), and Alnus ag 12f (*48*).

effective inhibitor as complete inhibition was not achieved in the concentration range used (1−5 mM). This indicates that the enzyme is not a trypsinlike serine protease. However, structural determination of active site residues may provide more conclusive evidence in this direction. Lack of inhibition of the activity by proteinacesous inhibitors such as SBTI, which is abundant in protein-rich foods like soybean, makes the enzyme a potential protease for the food industry (*7*). Metalloprotease inhibitors like *o*-phenanthroline, EDTA, and EGTA showed no significant effect on the activity of cryptolepain ruling out the possibly of the protease being a metalloprotein. Likewise, cysteine protease inhibitors like iodoacetic acid, sodium tetrathionate, E-64, NEM, etc. also showed no significant effect on the proteolytic activity of purified enzyme.

Effect of Substrate Concentration on the Reaction Velocity. The effect of increasing substrate concentration on reaction velocity of cryptolepain follows the Michaelis−Menton equation (**Figure 6A**). The study was carried out using azocasein as the substrate. The kinetics shows a typical hyperbolic curve with increasing concentration of substrate and attaining saturation at higher concentrations. The value of K_m estimated from Lineweaver−Burk plot (**Figure 6B**) is 10 μM with azocasein as the substrate.

Autocatalysis. The autodigestion of cryptolepain is monitored in the protein concentration range of 0.05−0.45 mg/mL at pH 8.0. The reduction in the activity of enzyme after 24, 48, and 72 h of incubation is shown in **Figure 7**. In each case, the extent of loss of activity is inversely proportional to the concentration. The enzyme retains more than 50% of the activity after 24 h of incubation even at low concentrations (0.05 mg/mL) and the extent of loss of activity increases with the time of incubation from 24 to 48 h, while at higher concentrations, the enzyme is fully active. After 72 h of incubation, there is an overall decrease in the residual activity at every concentration in the chosen range. At higher concentrations, the enzyme is fully active after 24 h of incubation and maintains the trend up to 48 h of incubation with some decrease in residual activity after 72 h. Thus, cryptolepain resists autodigestion, which may make it a valuable enzyme in various food and biotechnology industries.

Polyclonal Antibodies and Immunoassays. Polyclonal antibodies specific to cryptolepain have been successfully raised in male albino rabbits indicating the immunogenicity of purified protein. These antibodies can be used to purify the enzyme and as an immunological probe for its conformational studies. The presence of polyclonal antibodies in immunized rabbit serum was confirmed by Oüchterlony's double immunodiffusion. Precipitin lines start appearing after about 10−12 h of incubation at 37 °C and are distinctly visible by 24−30 h. The precipitin band appeared devoid of any spur when anti-cryptolepain serum was loaded in a central well surrounded by cryptolepain in the left side upper and right side lower wells and proteinase K and trypsin in right upper and left lower wells, respectively (**Figure 8**). This reflects that antisera to cryptolepain did not cross-react with other serine proteases, indicating that antigenic determinants of cryptolepain are unique to it. This also confirms the specificity of the antibodies toward the protein. A control experiment was also performed with preimmune serum in the central well surrounded by cryptolepain antigen in peripheral wells where no precipitin line was observed (data not shown).

The antigenic properties were further investigated by indirect ELISA. The typical color development in indirect ELISA results from cryptolepain anti-cryptolepain complex formation, which further confirmed the presence of antibodies to cryptolepain in the serum of immunized rabbit (data not shown). As there was no perceivable (insignificant color development) cross-reaction of anti-cryptolepain serum with other serine proteases such as proteinase K and trypsin, this reveals that purified protein is antigentically different from them.

N-Terminal Sequence. The amino terminus sequence of cryptolepain has been compared to other known serine proteases (**Table 5**). Very little sequence similarity is seen to other serine proteases like cucumisin, tomato P69 A, tomato P69 B, and alnus ag12f while there is more homology of about 20% with bamboo protease (*9*). Most of serine proteases have Thr (T) residues at the first two places whereas cryptolepain has Met and Glu. However, leucine at position 10 is common among most serine proteases. The unique sequence of the enzyme may result different protein scaffold and folding. Thus, it is very important to pursue structure−function relationship studies of cryptolepain in detail.

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