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A Stable Serine Protease, Wrightin, from the Latex of the Plant *Wrightia tinctoria* (Roxb.) R. Br.: Purification and Biochemical Properties

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Today proteases have become an integral part of the food and feed industry, and plant latex could be a potential source of novel proteases with unique substrate specificities and biochemical properties. A new protease named "wrightin" is purified from the latex of the plant *Wrightia tinctoria* (Family Apocynaceae) by cation-exchange chromatography. The enzyme is a monomer having a molecular mass of 57.9 kDa (MALDI-TOF), an isoelectric point of 6.0, and an extinction coefficient ($\epsilon^{1\%}_{280}$) of 36.4. Optimum activity is achieved at a pH of 7.5–10 and a temperature of 70 °C. Wrightin hydrolyzes denatured natural substrates such as casein, azoalbumin, and hemoglobin with high specific activity; for example, the K_m value is 50 μM for casein as substrate. Wrightin showed weak amidolytic activity toward L-Ala-Ala-*p*-nitroanilide but completely failed to hydrolyze *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), a preferred substrate for trypsin-like enzymes. Complete inhibition of enzyme activity by serine protease inhibitors such as PMSF and DFP indicates that the enzyme belongs to the serine protease class. The enzyme was not inhibited by SBTI and resists autodigestion. Wrightin is remarkably thermostable, retaining complete activity at 70 °C after 60 min of incubation and 74% of activity after 30 min of incubation at 80°. Besides, the enzyme is very stable over a broad range of pH from 5.0 to 11.5 and remains active in the presence of various denaturants, surfactants, organic solvents, and metal ions. Thus, wrightin might be a potential candidate for various applications in the food and biotechnological industries, especially in operations requiring high temperatures.

KEYWORDS: Plant protease; serine protease; wrightin; glycosylated; *Wrightia tinctoria*

INTRODUCTION

Proteases are one of the industrially most important enzymes and account for nearly 60% of total worldwide enzyme market. Proteases are extensively employed in various industries such as food, detergent, pharmaceutical, and leather. In the food industry the hydrolytic property of proteases is exploited for degradation of the turbidity complex resulting from protein in fruit juices and alcoholic liquors, the improvement of quality of protein-rich foods, soy protein hydrolysis, gelatin hydrolysis, casein and whey protein hydrolysis, meat protein recovery, and meat tenderization (*1*). There are many operations in the food industry that require the performance of proteolytic enzymes at high temperatures (65–85 °C) such as hydrolysis of proteins at high temperature, enzymatic production of aspartame and other peptides, baking, and brewing. Thermostability is desirable because the efficiency of enzymes is markedly improved at higher temperatures. Also, holding time for specific processes can be shortened, thereby minimizing undesirable chemical reactions. This is particularly valuable in food processing, where nutrients may be lost during processing at high temperatures. In addition, conducting thermal processes at higher temperatures minimizes microbial contamination (*2*).

Proteolytic enzymes or proteases are a class of proteins ubiquitously found in all organisms from microbes to higher organisms; they act as catalysts and perform diverse vital functions. In plants, proteases are involved in all aspects of the life cycle ranging from the mobilization of storage proteins during seed germination to the initiation of cell death and senescence programs (*3*). Different parts of plants are valuable reservoirs of biomolecules, and proteases from plant latex represent an important segment of them. Members of several plant families such as Euphorbiaceae, Apocynaceae, Moraceae, and Caricaceae exude latex on wounding, which constitutes organic and inorganic compounds, waxy materials, and enzymes mainly proteases. These extracellular proteases play a defensive role for plants against herbivores, insects, and pests (*4, 5*). Besides, plant latex could be a potential source of proteases due to easy purification methods, low levels of interfering substances during purification, and good yield of proteases.

Plant-derived proteases find promising use in food and biotechnological industries due to broad substrate specificity, high stability in extreme conditions, good solubility, and activity over a wide range of pH and temperature. Thiol proteases from plant latex such as papain, bromelain, ficin, and calotropins are

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utilized widely in several processes in the food and dairy industries (*6, 7*). However, a limitation in the use of thiol proteases is that their proteolytic activity is readily suppressed by air oxidation and metal ions. Therefore, these proteases require mild reducing and chelating agents for activation and proper activity and, thus, are not so economical and handy. In contrast, serine proteases from plant sources are both stable and active under harsh conditions (at temperatures of 70–80 °C and high pH), in the presence of surfactants or oxidizing agents; thus, they are more useful and economical for industrial applications. Therefore, the search for new potential plant serine proteases continues.

Although there is high diversity of serine proteases in the plant kingdom, as revealed by cDNA cloning experiments performed on *L. esculantum* (tomato), *L. longiflorum* (lily), and *A. thaliana* (*8*), to date most of the data relating to substrate and cleavage specificities, kinetic parameters, and other enzymatic properties are limited to cucumisin (EC 3.4.21.25), the first plant subtilase from the fruit of *C. melo*, and cucumisin-like proteases isolated from fruits of other cucurbits (*9–11*). Therefore, the present study aimed to purify a novel thermostable serine protease from the latex of the plant *Wrightia tinctoria* (Roxb.) R. Br. and investigate its physicochemical and kinetic properties. *W. tinctoria* belongs to the Apocynaceae family and is a small deciduous tree distributed in all parts of India. *W. tinctoria* is a medicinally important plant, and its extract shows antibacterial, antidandruff, and antipsoriasis activities (*12–14*). It exudes copious amounts of latex upon wounding of the leaf or stem throughout the year, and there is no seasonal change in the protein levels or composition of the latex. Thus, latex can be harvested at any time of the year for protease purification. The purified enzyme was named wrightin according to the nomenclature of proteases.

MATERIALS AND METHODS

Bovine serum albumin (BSA), hen egg white lysozyme, azoalbumin, hemoglobin, DTNB (5,5′-dithiobis[2-nitrobenzoic acid]), DTT (dithiothreitol), DFP (diisopropyl fluorophosphate), GuHCl (guanidine hydrochloride), urea, *o*-phenanthroline, EDTA (ethylenediaminetetraacetic acid), EGTA (ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid), SBTI (soyabean trypsin inhibitor), $HgCl_2$ (mercuric chloride), PCMB (*p*-chloromercuric benzoate), β-ME (β-mercaptoethanol), PMSF (phenylmethanesulfonylfluoride), acrylamide, *N*,*N*-methylene bisacrylamide, E-64 {L-*trans*-epoxysuccinylleucylamide(4-guanidino)butane-*N*-[*N*-(L-3-*trans*carboxyirane-2-carbonyl)-L-leucyl]agimatine}, Coomassie brilliant blue G-250, all synthetic substrates, triethanolamine, agarose, *o*-phenylenediamine, and other standard proteins were obtained from Sigma Chemical Co. (St. Louis, MO). Coomassie brilliant blue G 250 was from Eastman Kodak. Ampholine carrier ampholytes were from LKB. Acetonitrile, methanol, ethanol, butanol and dioxan were of high-performance liquid chromatography grade. All chemicals were of highest purity.

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

Step 1. Removal of Gum. Latex was collected by making a superficial incision on young stems of *W. tinctoria* into falcon tubes containing chilled 0.01 M acetate buffer, pH 4.5, and subsequently stored at −20 °C for 24 h. Frozen latex was thawed to room temperature and centrifuged at 17000*g* for 20 min to remove any insoluble debris. The resulting clear supernatant called crude latex was used in the next step.

Step 2. Cation-Exchange Chromatography on SP-Sepharose. Chromatography was performed at room temperature. Supernatant obtained in the above step was subjected to cation-exchange chromatography on a SP-Sepharose fast flow column pre-equilibrated with 0.01 M acetate buffer, pH 4.5. The column was washed with the same buffer until no protein was detected in the eluant. The bound proteins were eluted with a linear gradient of 0–0.8 M NaCl in the same buffer at a flow rate of 5 mL/min, and fractions of 4 mL were collected. The absorbances at 280 nm as well as caseinolytic activity of the proteins in all the fractions were determined. Bound proteins were eluted in a single peak with caseinolytic activity through out the peak, and all of the fractions were heterogeneous on SDS-PAGE. Therefore, all of the fractions in the peak were pooled, and such a pool was used for further purification. In subsequent purifications, crude latex was subjected to a SP-Sepharose column and bound proteins were eluted directly with 0.8 M NaCl and pooled.

Step 3. Cation-Exchange Chromatography on CM-Sepharose. The pool of fractions after SP-Sepharose were dialyzed extensively against the 0.01 M acetate buffer, pH 4.9, and subjected to cation-exchange chromatography on a CM-Sepharose fast flow column, which was pre-equilibrated with 10 mM acetate buffer, pH 4.9. The column was washed with the same buffer until no protein was detected in eluant, and the bound proteins were eluted with a linear salt gradient of 0–0.5 M NaCl at a flow rate of 3 mL/min. Fractions of 3 mL were collected, and each fraction was assayed for protein content as well as protease activity.

Protein Concentration. Protein concentration at different stages of purification was determined by absorbance at 280 nm as well as according to the method of Bradford (*15*) using BSA as a standard.

Protease Assay. The hydrolyzing activity of the protease was determined using denatured natural substrates such as casein, hemoglobin, and azoalbumin (chromogenic substrate) (*16*). Enzyme at a concentration of 15 μg in total volume of 0.5 mL of 0.05 M Tris-HCl buffer, pH 7.5, was incubated at 37 °C for 10 min. An equal volume of 1% casein solution (w/v) prepared at the same pH was added to the enzyme solution, making the final volume 1 mL, and the reaction mixture was incubated further for 30 min at 37 °C. The reaction was stopped by an addition of 0.5 mL of 10% TCA and allowed to stand for 10 min at room temperature. Soluble peptides were separated by centrifugation for 10 min using a tabletop centrifuge. The absorbance of the TCA-soluble peptides in the supernatant was measured at 280 nm. In the case of azoalbumin (0.6%, w/v) as 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 absorbance at 440 nm. With hemoglobin (1% w/v) as a substrate, activity is taken in the same manner as of casein. A control assay, without the enzyme, was done and used as 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 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.

Assay for Amidolytic Activity toward Synthetic Substrates. The enzymatic hydrolysis of different synthetic substrates peptidyl-*p*NA (peptidyl *p*-nitroanilide) by the purified protease wrightin was studied by a spectrophotometric method (*16*). Substrates used were *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), L-Ala-Ala-*p*-nitroanilide, *N*-succinyl-Phe-*p*-nitroanilide, L-Glu-*p*-nitroanilide, L-Ala-*p*-nitroanilide, and L-Leu-*p*-nitroanilde. In every case, a stock of 1–12 mM solution of synthetic substrate was prepared by dissolving the required amount of substrate in a minimum volume of DMSO and made up to the final volume with 0.05 M Tris-HCl buffer, pH 7.5. The reaction mixture contained approximately 15 μg of enzyme in 0.5 mL of Tris-HCl buffer, pH 7.5, and 0.5 mL of peptidyl *p*-NA. After 60 min of incubation at 37 °C, the reaction was terminated by the addition of 0.2 mL of 30% acetic acid and the liberated *p*-nitroanilide was determined by absorbance measurements at 410 nm using the extinction coefficient of 8800 M^{-1} cm^{-1} for *p*-nitroanilide as a measure of hydrolysis (*20*).

Electrophoresis and Gelatin Zymography. Homogeneity and intactness of the enzyme during all stages of purification were assessed by SDS-PAGE (*17*). For molecular mass determination, purified enzyme is subjected to electrophoresis on 12.5% SDS-PAGE under both reducing and nonreducing conditions. The protein bands on the gel were visualized by staining with 0.2% Coomassie brilliant blue R-250. Molecular mass markers used were phosphorylase *b* (97.40 kDa), BSA (66.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), soybean trypsin inhibitor (20.1 kDa), and chicken egg white lysozyme

(14.3 kDa). The molecular weight of the enzyme was extrapolated from the plot of log molecular weight versus electrophoretic mobility of molecular weight markers.

Proteases that degrade gelatin (denatured collagen) can be detected directly in gels. Gelatin is incorporated prior to polymerization and remains immobile during electrophoresis. Protease activity is indicated by unstained (i.e., proteolytically degraded) regions against a stained background, in contrast to conventional gels. To confirm the proteolytic activity of purified protease in gel, gelatin zymography was performed. Gelatin solution (1.5% w/v) was copolymerized with a 12.5% resolving gel. Protein sample was prepared in nonreducing sample buffer and heated to 37 °C before loading on the gel. After electrophoresis, the gel was washed for about 1 h with two changes of 2.5% Triton X-100 to remove SDS and placed in zymogram development solution (50 mM Tris buffer, pH 8.0) for 15 h at 37 °C. Gel was stained with 0.2% Coomassie brilliant blue R-250 and destained until a clear region against a dark background appeared (negative staining).

Mass Determination by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-MS). The purity and molecular weight of the purified protein were also checked by mass spectrometry. Enzyme at a concentration of 7 mM was mixed with saturated sinapinic acid solution in 0.1% trifluoroacetic acid in water/acetonitrile (2:1) mixture. One microliter was spotted onto the sample plate and allowed to dry. Data were collected on a Shimadzu Axima CFR-*plus* MALDI-TOF mass spectrometry in linear mode. MALDI-TOF standard BSA was used for calibration of the instrument prior to data collection.

Isoelectric Focusing. The isoelectric point of the purified enzyme was determined by isoelectric focusing in tube gels as described for ervatamin B (*18*) with some modifications. Electrophoretic runs were carried out with ampholine, in the pH range of 5.0–8.0. A 5% polyacrylamide gel containing 2% desired ampholine was cast in tube gels. Anodic and cathodic chamber buffers were 0.1 M phosphoric acid and 0.1 M sodium hydroxide, respectively. The gels were subjected to a prerun at a constant current of 1 mA per rod for 2 h to develop the pH gradient. Protein sample (100 μg) containing 10% (v/v) ampholine and 25% glycerol was loaded on each gel and electrophoresed at a constant current of 2 mA per rod for 4 h. After the run, tube gels were fixed in fixing solution (30 mL methanol, 70 mL of distilled water, 3.45 g of sulfosalicylic acid, and 11.5 g of trichloroacetic acid in 100 mL of distilled water) for 1 h. The gels were stained with 0.1% (w/v) Coomassie brilliant blue R-250 dissolved in 100 mL of staining solution (25% ethanol, 8% acetic acid in distilled water). Gels were destained with repeated changes of destaining solution (25% ethanol, 8% acetic acid in distilled water) until a clear background was obtained.

Carbohydrate Content. Carbohydrate moieties in glycoprotein 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 serine proteases as glycoproteins and also the fact that wrightin is a secreted protein, determination of the carbohydrate content of wrightin became essential. The carbohydrate content, if any, of the protease was determined using the phenol−sulfuric acid method (*19*). A solution of enzyme in water in the concentration range of 1–10 μg per 10 μL was taken in a microtiter plate. To each well 25 μL of 4% aqueous phenol was added. After 5 min 200 μL of H_2SO_4 was added, and the increase in absorbance was measured at 492 nm. A standard plot was generated with known concentrations of galactose (absorbance at 492 nm vs concentration), and the unknown values of the sample were extrapolated.

Effect of Various Inhibitors on Activity of Wrightin. The effect of increasing concentration of various protease inhibitors on the proteolytic activity of the purified protease was examined to identify the mechanistic class of enzyme. The inhibitors used in the present study were specific to cysteine proteases (IAA, E-64, mercuric chloride NEM, PCMB), serine proteases (PMSF, DIFP, SBTI), and metalloproteases (EDTA, EGTA and *O*-phenanthroline). A control assay of the enzyme activity was done without inhibitors, and the resulting activity was taken as 100%. The enzyme was incubated with an inhibitor, at a given concentration, for 30 min at room temperature, and an aliquot was used for the activity measurement. The activity assay was done using azoalbumin as a substrate as described earlier.

pH and Temperature Optima. The proteolytic activity of wrightin was examined in the pH range of 1.0–11.5 to determine the optimum pH. The buffers used were 0.05 M KCl−HCl (pH 1.0–1.5), 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). Because of the insolubility of azoalbumin below pH 4.0, enzyme assays at lower pH were carried out using hemoglobin as substrate. Substrate solution of azoalbumin (0.6% w/v) or hemoglobin (1% w/v) was prepared in the respective pH buffers, and activity was taken at the same pH as per the method described earlier at 37 °C. For temperature optimum activity of wrightin was assayed in the temperature range of 10–95 °C. Prior to assay both protease and substrate (azoalbumin) solution were incubated at the desired temperature for 15 min, and activity measurements were done at the same temperature.

Stability. Stability of an enzyme dictates its utility and commercial applicability. The stability of wrightin was assessed over a wide range of pH and temperature and in the presence of chemical denaturants. Wrightin was incubated for 24 h in given buffers in the pH range of 0.5–12.0 and assayed for residual activity. The different buffers used were already mentioned in an earlier section. Similarly, enzyme was incubated at various concentrations of the denaturants urea and GuHCl for 24 h and assayed for residual activity, whereas enzyme was incubated at different temperatures in the range of 10–95 °C for various periods of time and an aliquot was utilized to determine the residual activity. Although the enzyme was incubated over a range of temperatures, the activity measurements were carried out at pH 7.5 and 37 °C. In all of the above cases the residual activities of pretreated samples were measured by the standard protease assay described earlier.

The stability of enzyme activity was also analyzed in the presence of organic solvents, SDS, oxidizing agent (H_2O_2), bleaching agent (sodium perchlorate), and metal ions. In these studies, enzyme was incubated in 0.5 mL of 0.05 M phosphate buffer, pH 7.5, containing different concentrations of a given additive. Such a pretreated enzyme sample is added to 0.5 mL of substrate (casein) solution prepared in the same buffer, and the residual activity was determined. Enzyme assays were done as described earlier. Effective concentration of the organic solvent in the solvent composition did not alter the solubility of casein, which is confirmed by light scattering measurements in control experiments.

Effect of Substrate Concentration on Enzyme Kinetics. The effect of increasing substrate concentration on the velocity of the enzyme-catalyzed reaction was studied using casein as substrate at pH 7.5 at 37 °C. In each case, 15 μg of wrightin was used, and the concentration of casein was in the range of 1–150 μM. A Lineweaver–Burk plot was plotted, and the value of the Michaelis–Menten constant (K_m) was calculated. Proteolytic assays were performed as described previously.

Autodigestion. Proteases, in general, are prone to autodigestion, and the extent of autolysis depends on enzyme concentration, pH, time of incubation, temperature, and type of activator, if any. In the present study different concentrations of wrightin in the range of 0.05–0.45 mg/mL were incubated with 0.05 M Tris-HCl, pH 7.5, at room temperature for 48 h. An aliquot of enzyme was used to determine the protease activity with casein as substrate. The activity of the enzyme after the first 10 min was taken as 100% for the calculation of the residual activities.

Extinction Coefficient. The extinction coefficient of wrightin was determined using dry weight as well as spectrophotometric methods as described for procerain (*21*). The average value of the extinction coefficients obtained from the two methods was used for all other practical purposes.

Tyrosine and Tryptophan Contents. Total numbers of tryptophan and tyrosine residues in purified protein were determined according to the method of Goodwin and Morton (*22*), where absorbance spectra of the purified enzyme in 0.1 M NaOH were recorded between 300 and 220 nm using a Beckman DU 640B spectrophotometer. Under similar conditions, tyrosine and tryptophan contents of BSA and lysozyme were also determined to validate the current measurements.

Free and Total Sulfhydryl Content. Estimation of free and total cysteine residues of wrightin were carried out using Ellman's method

(*23*). For the free sulfhydryl group measurement, the purified enzyme was activated with 0.01 M β-mercaptoethanol in 0.05 M Tris-HCl, pH 8.0, for 15 min and then dialyzed against 0.1 M acetic acid. Similarly, for the measurement of total sulfhydryl content, the enzyme was reduced in the presence of 6 M GuHCl (*24*). The free cysteine and total sulfhydryl contents were measured by the DTNB reaction (*25*), where the liberated TNB anion after reaction of sulfhydryl group with DTNB was monitored spectrophotometrically at 412 nm. The molar extinction coefficient of TNB anion at 412 nm is 14150 M^{-1} cm^{-1}. Under similar conditions, free and total sulfhydryl contents of BSA and lysozyme were carried out to validate the current measurements.

Polyclonal Antibodies and Immunoasays. Antibodies to the purified enzyme were raised in a male albino rabbit (weight about 1.0 kg). The enzyme (200 μg) was emulsified with Freund's complete adjuvant in 0.01 M sodium acetate buffer, pH 5.5, and the emulsion was injected subcutaneously at multiple sites. After 1 week, a booster dose of 350 μg of protein was given as emulsion with Freund's incomplete adjuvant. Two similar doses were administered at intervals of 7 and 15 days as emulsions with Freund's incomplete adjuvant. At 7 days after the last booster dose, the rabbit was bled through its marginal ear vein. Blood was allowed to clot initially for 1 h at room temperature and later for 12 h at 4 °C. Supernatant was collected as antisera, by centrifuging at 5000 rpm, and stored at −20 °C until further use. Preimmune serum was collected prior to the immunization of the rabbit and used as a control for immunoassays. The presence of antibodies was confirmed by Ouchterlony's double-immunodiffusion assay (*26*). One percent agarose in phosphate buffer saline containing 0.02% sodium azide was solidified in a Petri dish, and appropriate holes were punched into it. Wrightin, trypsin, and protienase K (each 40 μg) were loaded into the peripheral wells, and 100 μL of the antiserum was added in the central well. The plate was left at room temperature for 24 h in a moist environment. A control assay was carried out with preimmune serum.

RESULTS AND DISCUSSION

Purification of Wrightin. A novel serine protease from the latex of *W. tinctoria* (family Apocynaceae) was purified to homogeneity by a simple procedure involving cation-exchange chromatography. Crude latex (devoid of gum and any insoluble matter) was applied to a SP-Sepharose Fast Flow column. In initial experiments the bound proteins were eluted with a linear gradient of 0–0.8 M NaCl, which resulted as a single peak having caseinolytic activity throughout the peak (data not shown), but the fractions were heterogeneous when submitted to SDS-PAGE; therefore, all of the active fractions were pooled and dialyzed extensively with several changes of 0.01 M acetate buffer, pH 4.9. In all subsequent preparations of the protein, the SP-Sepharose column was eluted with 0.8 M NaCl directly. The protein pool from SP-Sepharose was rechromatographed on a CM-Sepharose Fast Flow column, and the bound proteins were eluted using a linear gradient of 0–0.5 M NaCl. The resulting elution profile showed two resolved peaks, A and B (**Figure 1**). Most of the fractions of the two peaks exhibited some caseinolytic activity, whereas most of the activity loaded to the column appeared in peak B. Proteolytic activity observed in the separated peaks suggests the existence of multiple proteases in the latex of *W. tinctoria*. Such multiple proteases in latex are not an uncommon feature as evidenced by the presence of multiple proteases in the latex of *Euphorbia tirucalli*, *Euphorbia lacteal* (*27*) *Eravatamia caronaria* (*18*), and others. All of the fractions of the two peaks were assessed for homogeneity by SDS-PAGE. Fractions 147−168 of the ascending limb of peak B are homogeneous with higher magnitude of the activity (**Figure 1**). The active fractions from CM-Sepharose other than the homogeneous ones (147–168) when subjected to SDS-PAGE result in multiple bands, which are distinctly different in molecular size from the fractions, which are

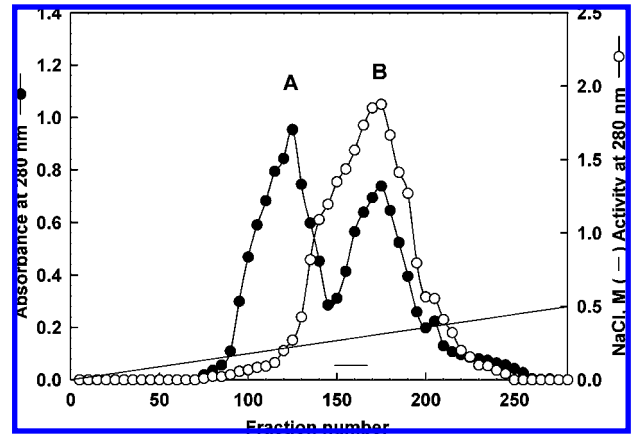

Figure 1. Elution of wrightin on cation-exchange chromatography on CM-Sepharose. Protein pool from SP-Sepharose after dialysis was applied to a CM-Sepharose column pre-equilibrated with 0.01 M acetate buffer, pH 4.9. The bound proteins were eluted with a linear gradient of 0–0.5 M NaCl. The fractions of 3 mL volume were collected at a flow rate of 3 mL/min and assayed for protein content (●) and activity (○) using casein as a substrate. The fractions from 147 to 168 were pooled and are indicated by a horizontal line.

Table 1. Purification of Wrightin from Latex of *W. tinctoria*

step	total protein (mg)	total activity (units)[a]	specific activity (units/mg)	% recovery
1. crude extract	341.5	6660	19.5	100
2. SP-Sepharose	276.4	5640	20.4	84.6
3. CM-Sepharose	24	753	31.3	11.3

[a] Definition of 1 unit: the amount of enzyme under the assay conditions described giving rise to an increase of 0.01 unit absorbance at 280 nm per minute of digestion. Casein was used as substrate.

homogeneous between 147 and 168. Preliminary experiments such as pH and temperature optima and kinetics showed some differences between the pools, confirming that the crude latex contains multiple proteases with distinct properties. Therefore, only the pure fractions from CM-Sepharose were chosen for further studies. The pool of fractions was concentrated by an Amicon membrane concentrator using a membrane with a molecular weight cutoff of 10 kDa followed by dialysis against 0.05 M phosphate buffer, pH 7.5, and stored at 4 °C for further use. Typically, the yield of purified protein is 11 ± 1% and specific activity is 31 ± 0.5 units/mg. The low yield of protease activity might be due to the presence of multiple proteases in the latex. The purification results of a typical batch are summarized in **Table 1**. The purification has been carried out many times. The purification protocol is highly reproducible, and the yields as well as specific activity of the enzyme preparation were consistent. It is worth mentioning that application of crude latex after removal of gum (step 1), directly to CM-Sepharose at pH 4.9 or 4.5 bypassing chromatography on SP-Sepharose (step 2), resulted in a single peak, without any resolution, where all of the fractions are active but heterogeneous. However, no loss of activity relative to the total activity loaded to the column is observed. Therefore, step 2 is necessary. It appears that some unknown constituents present in the latex either might have been removed in the unbound while loading on SP-Sepharose, leaving only proteins bound to the column, or remained tightly bound to the column even in the presence of 0.8 M NaCl. To the best of our knowledge, this is the first report on the identification and purification of a serine

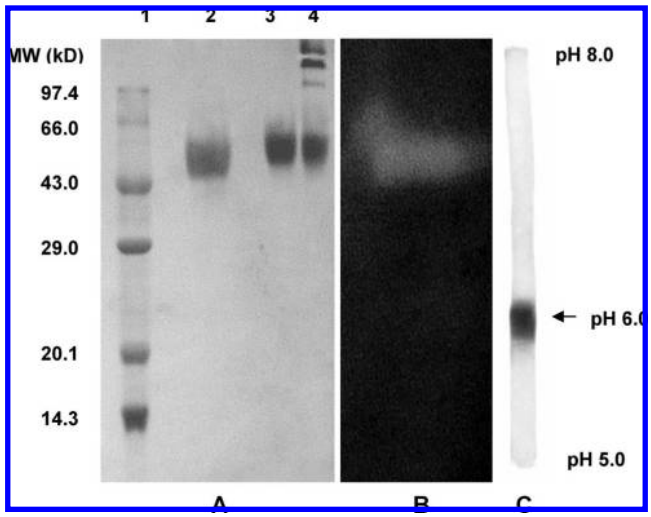

Figure 2. Electrophoretic analysis of purified protease: (**A**) SDS-PAGE (lane 1, protein markers; lanes 2 and 3, wrightin under reduced and nonreduced conditions, respectively; lane 4, crude latex after removal of gum); (**B**) zymogram (in gel activity) of serine protease wrightin (clear region shows the hydrolysis of gelatin by enzyme); (**C**) isoelectric focusing (electrophoresis was performed using 5% polyacrylamide disk gels with ampholine carrier ampholytes, pH 5–8; 100 μg of samples was loaded and electrophoresed for 3 h at a constant voltage of 300 V).

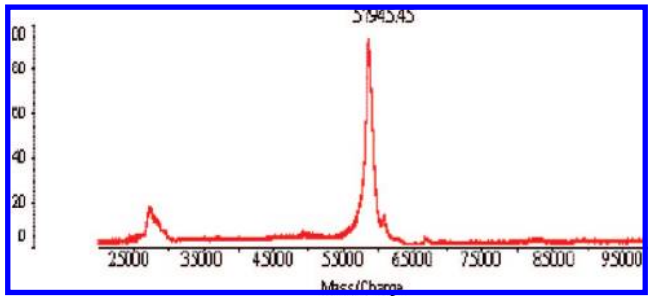

Figure 3. Mass spectrometry of wrightin. MALDI-TOF standard BSA was used for calibration of the instrument, and data were collected in linear mode.

protease from the genus *Wrightin* of the Apocynaceae family. Hence, it is important to study the biochemical properties of wrightin and compare them with those of other plant serine proteases.

Homogeneity and Physical Properties of Purified Protease. Wrightin appeared as a single band on SDS-PAGE under reducing and nonreducing conditions with an estimated molecular mass of 57 kDa (**Figure 2**). Mass spectrometric analysis of the protein confirms the homogeneity as well as monomeric nature of the enzyme with a single peak of molecular mass of 57.9 kDa (**Figure 3**). The average value of extinction coefficient of the enzyme was 36.4 by dry weight and spectroscopic methods. Similarly, purified protein appeared as a single band on isoelectric focusing with an approximate isoelectric point of pH 6.0 (**Figure 2**). Molecular masses of known plant serine proteases are in the range of 19−110 kDa, the majority of proteases falling in the range of 60−80 kDa (*28*) with some exceptions such as MRP (proteinase I) and RSIP from *Zea mays* L. (*29, 30*), which were reported to have molecular masses of 59 and 54 kDa, respectively. Most of the serine proteases derived from plant sources have isoelectric points in the range of 4.0–7.0 and rarely have highly basic p*I* values, as reported for endoprotease from melon fruit (*31*). The molecular mass and isoelectric point of wrightin were similar to those of other known plant

Table 2. Specific Activity of Wrightin with Natural and Synthetic Substrates

substrate	specific activity[a]
casein	31.3 ± 0.5
azoalbumin	20.8 ± 0.5
hemoglobin	22.4 ± 0.5
L-Ala-*p*-nitroanilide	2.1 ± 0.5
LAla-Ala-*p*-nitroanilide	0.8 ± 0.5
LLeu-*p*-nitroanilide	no activity
N-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA)	no activity
N-succinyl-Phe-*p*-nitroanilide, L-Glu-*p*-nitroanilide	no activity

[a] Specific activity is defined as the number of enzyme units per milligram of protein with natural substrates. Specific activity is defined as the number of moles of *p*-nitroaniline liberated per minute of synthetic substrate digestion per mole of enzyme.

serine proteases. The proteolytic nature of wrightin was also confirmed by gelatin zymography, where a digested gelatin appeared as a white band corresponding to the position of the protein in the gel (**Figure 2**). A comparison of physicochemical properties of wrightin with other plant serine proteases is summarized in **Table 3**.

Carbohydrate Content. Wrightin is a glycoprotein having a detectable amount of carbohydrate in its molecular structure. The amount of carbohydrate content was about 8% as estimated by the phenol−sulfuric acid method. Furthermore, on staining with Schiff's reagent after SDS-PAGE, the wrightin band appeared with a magenta color (data not shown), which confirms the glycosylation of wrightin. Glycosylation to varying degrees was seen in serine proteases purified from the Euphorbiaceae family (*27*) as well as in cucumisin (*32*) and taraxalisin (*33*). However, the biological role of such glycans has not been well documented yet. In general, glycan chains played a role in protecting the proteins from degradation and were as well responsible for the thermal stability and solubility of the glycoproteins. Glycans influence the conformation, stability, and biological activity of the glycoprotein as well.

Effect of Inhibitors on the Activity of Wrightin. Inhibition of a protease by inhibitors specific for different class of proteases is a useful tool to establish the mechanistic class of purified unknown protease (*28*). The effect of various inhibitors on the activity of wrightin is summarized in **Table 4**. The activity of the enzyme was effectively blocked, by almost >93%, by serine protease inhibitors such as DFP and PMSF. At the same time, inhibitors of metalloprotease (EDTA and *o*-phenanthroline) and cysteine protease (iodoacetic acid, E-64, PCMB) failed to inhibit the proteolytic activity of wrightin. However, $HgCl_2$ inhibited the enzyme activity to some extent (about 20%). Such inhibition was reported for cucumisin (*32*). The observed inhibition profile places wrightin in the class of serine proteases. The absence of inhibition by soybean trypsin inhibitor (SBTI), which is abundantly present in many protein-rich foods, rules out the possibility that the protease reported here was a trypsin-like protease. Thus, wrightin can be a potential enzyme in the food industry, where it may be effectively used in the processing of protein-rich food.

pH and Temperature Optima. The hydrolyzing activity of wrightin as a function of pH is shown in **Figure 4A**. Wrightin exhibited full activity over a broad range of pH from neutral to alkaline, pH 7.5−10.0, with a slow decrease afterward. The protein retained 75% of the activity even at pH 11.5 and 73% of activity at pH 5.0 as well. The protein was inactive below pH 2.0, and such loss of activity of the protein at lower pH could be due to incorrect protonation state of amino acid side chain in the active site of enzyme or altering protonation state of substrate, hence resulting in improper binding of the substrate

Table 3. Comparison of Physicochemical Properties of Wrightin with Other Plant Serine Proteases

		optima		stability			
plant source	mol mass (kDa)	pH	temp (°C)	pH	temp (°C)	glycoprotein	p*I*
W. tinctoria (wrightin)	57.9	7.5–10	70	5.5–11.5	75–80	yes	6.0
Z. mays[a] (RSIP)	59	6.5–7.0	nd	5–9	nd	nd	4.5
T. kirrilowi[b]	50	10.0	70	4–12.5	50	yes	nd
T. officinale[c]	67	8.0	40	6–9	40	yes	4.5
C. melo[d] (cucumisin)	54	7.1	70	4–11	50	yes	nd
M. pomifera[e]	65	8.5	58	7.0–9.0	nd	yes	nd
C. trigonus[f]	67	11	65	4–10	65	nd	nd
E. milli[g] (Milin)	51.4	8	60	5–12	65	yes	7.2
B. hispida[h]	67	10.0	60	7–11	60	nd	nd
P. hindsii[i]	82.0	6.5–10.5	30	nd	nd	nd	nd

[a] Reference *30*. nd, not determined. [b] Reference *37*. [c] Reference *33*. [d] Reference *32*. [e] Reference *38*. [f] Reference *9*. [g] Reference *39*. [h] Reference *11*. [i] Reference *40*.

Table 4. Effect of Inhibitors on the Activity of Wrightin

inhibitor type	inhibitor (I)	[I][a]	% residual activity
serine protease	PMSF	100 μM	5.0
	DFP	20 μM	6.7
	SBTI	1 mM	99
cysteine protease	E-64	100 μM	96
	iodoacetic acid	100 μM	98
	$HgCl_2$	100 μM	80
	PCMB	100 μM	100
	NEM	100 μM	100
metalloprotease	EDTA	5 mM	100
	o-phenanthroline	5 mM	99

[a] Minimum amount of inhibitor required for maximum inhibition.

to enzyme. The wide range of optimum pH of wrightin is similar to other serine proteases that have, in general, optimum pH values of 7.0–11.0 with the exceptions of hordolisin and SEP-1 from barley (optimum pH of 6.0 and 6.5, respectively), as well as Ara12 from *A. thaliana* and protease C1 from soybean (which acts best in the pH range 3.5–6.5) (*28*). However, the pH optimum of wrightin is different from that of proteases of the Euphorbiaceae family, which is pH 6.0–8.0 (*27*).

The protease activity of wrightin was also monitored at different temperatures in the range of 10–95 °C and is shown in **Figure 4B**. The enzyme activity increased linearly from 20 to 70 °C with a rapid decrease of activity afterward and was negligible by 90 °C. Therefore, the optimum temperature for the activity of wrightin is around 70 °C. Such a high temperature optimum is not common as most of the plant serine proteases are reported to act best in the range of 30–60 °C (**Table 3**).

pH and Temperature Stability. The remarkable feature of wrightin is its stability with respect to pH as well as high temperature. Wrightin is stable with full activity in a broad range of pH from 5.5 to 11.5 (**Figure 4A**) when exposed to a given pH for 24 h. It also showed a considerable amount of activity even at low pH of 3.0. The thermal stability of wrightin was examined by measuring the residual activity after the enzyme had been subjected to different temperatures for various times. The protease exhibited residual activities of 95% and 74% at 80 °C when the time of exposure of the enzyme was 15 and 30 min, respectively (data not shown). The protease retained full activity up to a temperature of 70 °C followed by a decrease of activity to 50% at 80 °C and complete loss by 90 °C when the time of exposure to a given temperature was increased to 60 min (**Figure 4B**). These results show that wrightin is a thermostable protease, and its heat stability is higher than that of other known plant serine proteases when compared as in

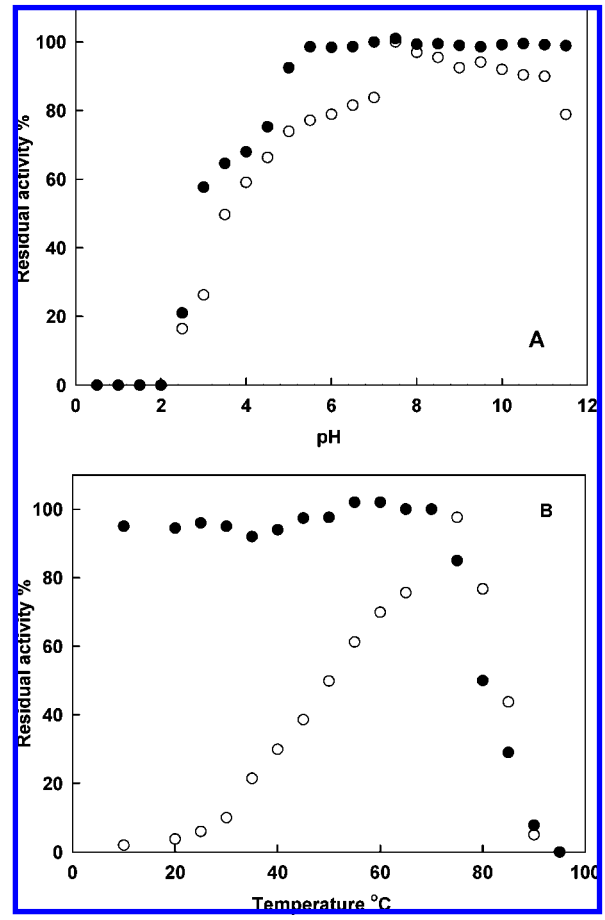

Figure 4. (**A**) Effects of pH on activity (○) and stability (●) of wrightin. Fifteen micrograms of enzyme was used for all activity measurements. For pH optima enzyme activity was determined at various pH values in the range of 0.5–12.0. Enzyme was equilibrated at given pH, and activity was taken at the same pH using substrate prepared in the corresponding buffer. For pH stability enzyme was incubated for 24 h at various pH values and activity was taken at pH 7.5 with azoalbumin as substrate. In both cases the specific activity of 20.8 ± 0.5 units/mg determined at pH 7.5 and 37 °C is taken as 100%. (**B**) Effect of temperature on activity (○) and stability (●) of wrightin. Temperature optimum was determined by incubating the enzyme at different temperatures from 10 to 95 °C for 15 min and taking activity at the same temperature. The specific activity of 68.33 ± 0.5 units/mg determined at pH 7.5 and 70 °C is taken as 100%. For temperature stability measurements, enzyme was incubated for 60 min at different temperatures and activity was measured at 37 °C at pH 7.5. The specific activity of 20.8 ± 0.5 units/mg determined at pH 7.5 and 37 °C is taken as 100%.

Table 5. Stability of Wrightin under Various Conditions

condition	stability	% residual activity
pH	5.5–11.5	100
temperature	75–80 °C	100
urea	8 M	100
methanol	50%	100
isopropanol	50%	86
acetonotrile	50%	64
dioxan	50%	57
ethanol	50%	53
butanol	50%	20
SDS	0.1%	86.7
H_2O_2	5%	100
sodium perchlorate	4%	100
metal ions (Ca^{2+}, Mg^{2+} and K^{+})	10 mM	100
GuHCl	1.5	100

Table 3. Besides, wrightin was also stable with 100% activity in 8 M urea and 1.5 M GuHCl when incubated for 24 h (**Table 5**). Thus, wrightin may be a potential enzyme in processes of the food industry, which require elevated pH or temperatures.

Effect of Organic Solvents, Metal Ions, SDS, and Oxidizing and Bleaching Agents. The stability of wrightin was examined as a function of increasing percentage of different organic solvents (**Figure 5**). At 50% concentration of organic solvents wrightin retained full activity in methanol and isopropanol and half of the activity in ethanol, dioxan, and acetonitrile, whereas the activity was reduced to one-third in butanol. Hence, the protein is most stable in methanol and least in butanol. Besides, the enzyme retained full activity in the presence of 0.1% SDS (detergent), 5% H_2O_2 (oxidizing agent), and 4% sodium perchlorate (bleaching agent). Metal ions such as K^{+}, Ca^{2+}, and Mg^{2+} did not affect the activity of wrightin up to 10 mM concentration. Thus, wrightin could be advantageous to microbial proteases, which require metal ions, mainly Ca^{2+}, as activator for proteolytic activity (*34, 35*). The stability of wrightin under different conditions is presented in **Table 5**. The retention of considerable activity by wrightin under adverse conditions as described above may be useful to explore the possibilities of utilization in biotechnology, the food industry, and others.

Effect of Substrate Concentration on the Reaction Velocity. The effect of increasing substrate concentration on reaction velocity of the enzyme obeyed the Michaelis–Menten kinetics when denatured casein was used as substrate as shown **Figure 6A**. At higher concentration of the substrate, enzyme activity was found to reach saturation, resulting in a typical hyperbolic curve. The value of K_m as estimated from the Lineweaver–Burk plot (**Figure 6B**) is 50 ± 0.5 μM.

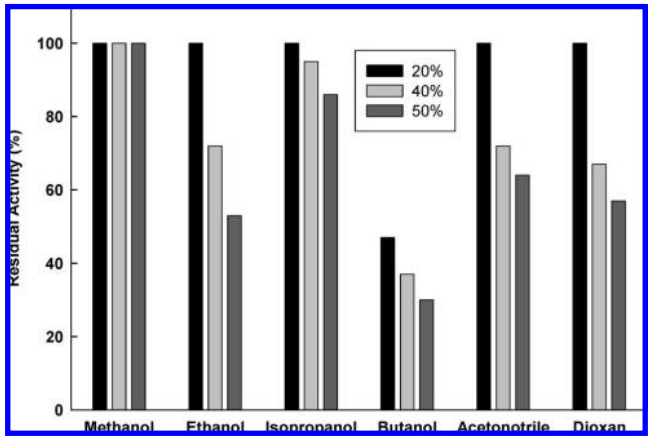

Figure 5. Effect of organic solvents on the activity of wrightin. The enzyme was incubated at given percentage of each organic solvent for 30 min at room temperature, and the residual activity was measured with casein as substrate. The specific activity of 31.3 ± 0.5 units/mg determined at pH 7.5 and 37 °C in the absence of organic solvent was taken as 100%.

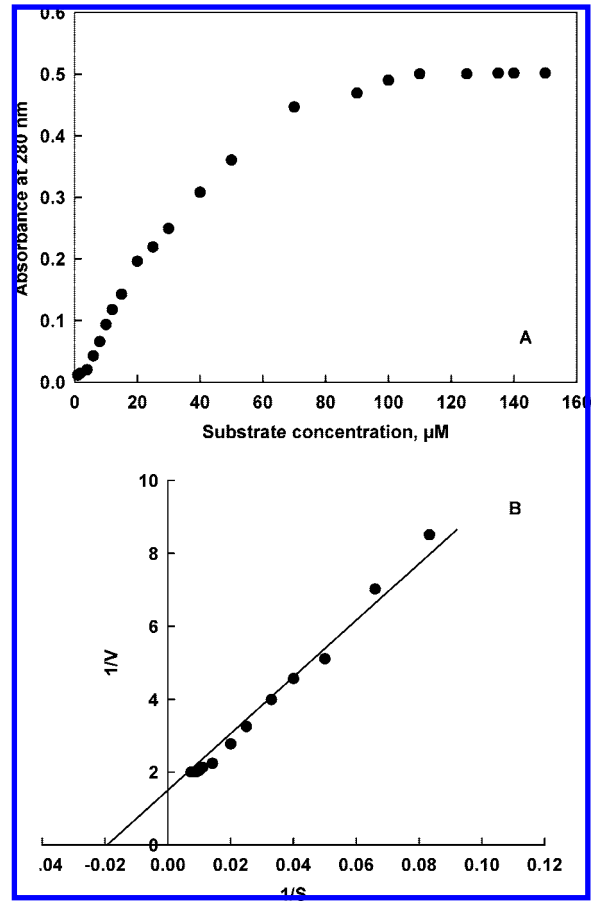

Figure 6. Effect of substrate concentration on reaction velocity of wrightin. (**A**) Effect of substrate concentration on the activity of wrightin. At the higher substrate concentrations the activity of wrightin attains saturation in accordance with the Michaelis–Menten equation. Different concentrations of substrate in the range from 0.0 to 50 μM and 15 μg of enzyme were used in the measurements. Casein was used as a substrate. (**B**) Lineweaver–Burk plot. K_m was calculated according to the Michaelis–Menten equation. The K_m value of wrightin was found to be 50 ± 0.5 μM.

Substrate Specificity. The activity of wrightin was examined against denatured natural substrates as well as chromogenic synthetic substrates. The specific activity of wrightin against natural and synthetic substrates is summarized in **Table 2**. Wrightin hydrolyzes denatured natural substrates such as casein, hemoglobin, and azoalbumin with significant activity. Wrightin showed weak amidolytic activity toward synthetic substrates such as L-Ala-*p*-nitroanilide and L-Ala-Ala-*p*-nitroanilide. The chain length of the substrate as well as amino acids at positions P_1 and P_2 may have strong influence in the catalytic action of the enzyme. The lower activity of wrightin toward L-Ala-Ala-pNA compared to L-Ala-pNA might be due to a preference for alanine at the P_1 position by the enzyme, not at the P_2 position. Wrightin failed to hydrolyze other synthetic substrates such as L-Leu-*p*-nitroanilide, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), *N*-succinyl-Phe-*p*-nitroanilide, and L-Glu-*p*-nitroanilide used in this study. Besides, lack of hydrolysis of BAPNA, a synthetic substrate for trypsin, by wrightin makes it distinct from trypsin-like enzymes (*36*).

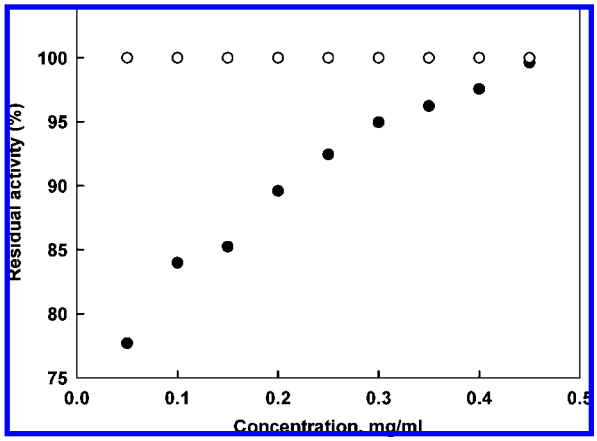

Figure 7. Autodigestion of wrightin as a function of protein concentration. Wrightin at different concentrations in the range of 0.05−0.5 mg/mL was incubated at room temperature for 48 h. An aliquot was used to determine residual activity (●). The activity of enzyme (○) after 10 min of incubation of the protein at every concentration was taken as 100%.

Autodigestion. Generally protease undergoes autolysis, which is dependent on protein concentration, temperature, time, and activators, if any. Therefore, it is important to check the conditions for the storage of the enzyme without any loss of proteolytic activity. Autocatalyis, if any, of wrightin was monitored as a function of increasing protein concentration in the range of 0.05–0.45 mg/mL at room temperature under neutral conditions (**Figure 7**). The magnitude of loss of activity decreases with increase in enzyme concentration from 0.05 to 0.45 mg/mL, and no loss of activity was seen at higher concentrations. The enzyme retains >70% of activity even at a very low protein concentration of 0.05 mg/mL. Most proteases undergo autodigestion at low protein concentrations, for example, cysteine proteases such as procerain (*21*). Wrightin is less susceptible to autodigestion, which in turn indicates its high stability. In our experience, the enzyme is stable for 6 months at 4 °C without any loss in activity.

Specific Amino Acid Residues. The total sulfhydryl content of the wrightin was found to be 9 (measured value = 9.15 ± 0.01) with one free (measured value = 1.09 ± 0.01) cysteine and the other eight forming four disulfide bonds. The tryptophan and tyrosine contents of the protein were 20 (mesured value = 20.16 ± 0.1) and 75 (measured value = 75.2 ± 0.5), respectively. The total cysteine content of wrightin is comparable to that of serine proteases such as Euphorbains y3 (total cysteine = 10) and Euphorbains y1 (total cysteine = 9) (*27*).

Antigenic Properties. Polyclonal antibodies against wrightin were raised in the male albino rabbit. The presence of antibodies in the anti-rabbit serum was checked by Ouchterlony's double-immunodiffusion method. The precipitin line became distinctly visible by approximately 24 h. One merging precipitin band devoid of spur was observed (**Figure 8**) between antiwrightin serum and wrightin. This band not only indicates the purity of wrightin but also that the antibodies are specific to the enzyme. At the same time other serine proteases such as trypsin and proteinase K did not cross-react with antiwrightin serum, revealing the antigenic distinction of wrightin from the latter two serine proteases. Control experiments with preimmune serum did not show any cross-reactivity (data not shown).

Easy availability of the latex and the simple purification procedure reported in this paper make large-scale production of wrightin possible and economical. The stability of the enzyme at higher temperatures and over a broad range of pH also make it a potential enzyme for industrial applications at high temperatures in the food industry. Wrightin could also be successfully employed in protein-rich foods containing soybean trypsin inhibitors. Besides, other properties of the enzyme such as its low susceptibility to autodigestion and its ability to withstand the harsh conditions of organic solvents and various chemical denaturants may allow various other applications to be explored. Moreover, wrightin can also be used as a model system to understand thermostability or thermoactivity, which would be useful for protein engineering.

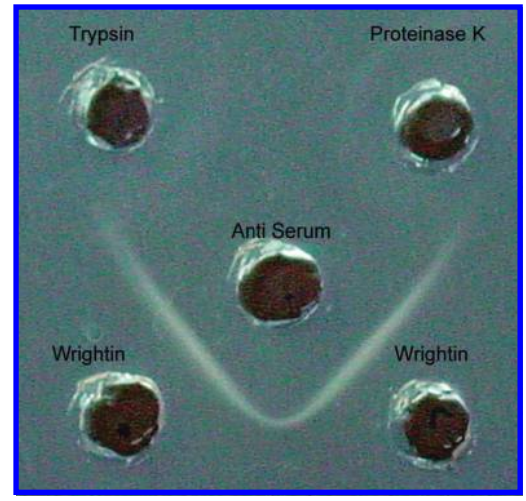

Figure 8. Ouchterlony's double immunodiffusion. Assay was carried out in (1%) agarose in phosphate-buffered saline containing 0.02% sodium azide. Anti-wrightin serum (100 μL) was added in the central well, and 40 μg of wrightin was added in the lower two peripheral wells. Forty micrograms of proteinase K and 40 μg of trypsin were added in the remaining upper wells. One merging precipitin band between antisera and wrightin appeared in 24 h.

ABBREVIATIONS USED

SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TEMED, *N*,*N*,*N*,*N*-tetramethylethylenediamine; UV, ultraviolet; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; PCMB, *p*-chloromercuribenzoate; NEM, *N*-ethylmaleimide; GuHCl, guanidine hydrochloride; DTNB, 5,5μ-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(α-aminoethyl ether)tetraacetic acid; SBTI, soybean trypsin inhibitor; PMSF, phenylmethanesulfonyl fluoride; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; BAPA, NR-benzoylarginine *p*-nitroanilide.

SAFETY

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

LITERATURE CITED

(1) Sumantha, A.; Larroche, C.; Pandey, A. Microbiology and industrial biotechnology of food-grade proteases: a perspective. *Food Technol Biotechnol.* **2006**, *44* (2), 211–220.

(2) Zamost, B. L.; Nielsen, H. K.; Starnes, R. L. Thermostable enzymes for industrial applications. *J. Ind. Microbiol. Bioechnol.* **1991**, *8* (2), 71–82.

(3) Schaller, A. A cut above the rest: the regulatory function of plant proteases. *Planta* **2004**, *220*, 183–187.

(4) Konno, K.; Hirayama, C.; Nakamura, M.; Tateishi, K.; Tamura, Y.; Hattori, M.; Kohno, K. Papain protects papaya trees from herbivorous insects: role of cysteine proteases in latex. *Plant J.* **2004**, *37*, 370–378.
(5) Rajesh, R.; Nataraju, A.; Gowda, C. D.; Frey, B. M.; Frey, F. J.; Vishwanath, B. S. Purification and characterization of a 34-kDa, heat stable glycoprotein from *Synadenium grantii* latex: action on human fibrinogen and fibrin clot. *Biochimie* **2006**, *88* (10), 1313–1322.
(6) Caffini, N. O.; Lopez, L. M. I.; Natalucci, C. L.; Priolo, N. S. Proteases of higher plants. General features, physiological roles and applications. *Acta Farm. Bonaerense* **1988**, *7*, 195–213.
(7) Wang, J.; Zhao, M.; Zhao, Q.; Jiang, Y. Antioxidant properties of papain hydrolysates of wheat gluten in different oxidation systems. *Food Chem.* **2007**, *101* (4), 1658–1663.
(8) Popovic, T.; Puizdar, V.; Brzin, J. A novel subtilase from common bean leaves. *FEBS Lett.* **2002**, *530* (1–3), 163–168.
(9) Asif-Ullah, M.; Kim, K.; Yu, Y. G. Purification and characterization of a serine protease from *Cucumis trigonus* Roxburghi. *Phytochemistry* **2006**, *67*, 870–875.
(10) Uchikoba, T.; Yonezawa, H.; Kaneda, M. Cleavage specificity of cucumisin, a plant serine protease. *J. Biochem. (Tokyo)* **1995**, *117* (5), 1126–1130.
(11) Uchikoba, T.; Yonezawa, H.; Kaneda, M. Cucumisin like protease from the sarcocarp of *Benincasa hispida* var. Ryukyu. *Phytochemistry* **1998**, *49* (8), 2215–2219.
(12) Dang, R.; Sabitha, J. S.; Shivanand, B. G. Anti-microbial activity of herbs used in psoriasis. *Pharma Rev.* **2005**, August, 71–76.
(13) Krishnamoorthy, J. R.; Ranganathan, S.; Shankar, S. G.; Ranjith, M. S. Dano: a herbal solution for dandruff. *Afr. J. Biotechnol.* **2006**, *5* (10), 960–962.
(14) Mitra, S. K.; Seshadri, S. J.; Venkataranganna, M. V.; Gopumadhvan, S. Reversal of parakeratosis, a feature of psoriasis by *Wrightia tinctoria* (in emulsion) histological evaluation based on mouse tail test. *Ind. J. Dermatol.* **1998**, *43* (3), 102–104.
(15) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.
(16) Arnon, R. Papain. *Methods Enzymol.* **1970**, *19*, 226–244.
(17) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.
(18) Kundu, S.; Sundd, M.; Jagannadham, M. V. Purification and characterization of a stable cysteine protease ervatamin B, with two disulfide bridges, from the latex of *Ervatamia coronaria*. *J Agric. Food Chem.* **2000**, *48*, 171–179.
(19) Hounsell, E. F.; Davies, M. J.; Smith, K. D. Chemical methods of analysis of glycoprotein. In *The Protein Protocols Handbook*; Walker, J. M., Ed.; Humana Press: Totowa, NJ, 1997; pp 633–634.
(20) Erlanger, B. F.; Kokowsky, N.; Cohen, N. The preparation and properties of two new chromogenic substrates of trypsin. *Arch. Biochem. Biophys.* **1961**, *95*, 271–278.
(21) Dubey, V. K.; Jagannadham, M. V. Procerain, a stable cysteine protease from the latex of *Calotropis procera*. *Phytochemistry* **2003**, *62*, 1057–1071.
(22) Goodwin, T. W.; Morton, R. A. The spectrophotometric determination of tyrosine and tryptophan in proteins. *Biochem. J.* **1946**, *40* (5–6), 628–632.
(23) Ellman, L. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **1959**, *82*, 70–77.
(24) Riddles, P. W.; Blakeley, R. L.; Zerner, B. Reassessment of Ellman's reagent. *Methods Enzymol.* **1983**, *91*, 49–60.
(25) Creighton, T. E. Disulfide bonds between protein residues. In *Protein Structures Practical Approach*; Creighton, T. E., Ed.; IRL Press: Oxford, U.K., 1989; pp 155–160.
(26) Ouchterlony, O.; Nilsson, L. A. Immunodiffusion and immunoelectrophoresis. In *Handbook of Experimental Immunology*; Weir, D. M., Herzerberg, L. A., Blackwell, C., Herzerberg, L. A., Eds.; Blackwell: Oxford, U.K., 1986; Vol. 1, pp 32.1–32.50.
(27) Lynn, K. R.; Clevette-Radford, N. A. Proteases of euphorbiaceae. *Phytochemistry* **1988**, *27* (1), 45–50.
(28) Antao, C. M.; Malcata, F. X. Plant serine proteases: biochemical, physiological and molecular features. *Plant Physiol. Biochem.* **2005**, *43* (7), 637–650.
(29) Goodfellow, V. J.; Solomonson, L. P.; Oaks, A. Characterization of a maize root proteinase. *Plant Physiol. Biochem.* **1993**, *101*, 415–419.
(30) James, F.; Brouquisse, R.; Suire, C.; Pradet, A.; Raymond, P. Purification and biochemical characterization of a vacuolar serine endopeptidase induced by glucose starvation in maize roots. *Biochem. J.* **1996**, *320*, 283–292.
(31) Noda, K.; Koyanagi, M.; Kamyia, H. C. Purification and characterization of an endoprotease from melon fruit. *J. Food Sci.* **1994**, *59* (3), 585–587.
(32) Kaneda, M.; Tominaga, N. Isolation and characterization of a proteinase from the sarcocarp of melon fruit. *J. Biochem. (Tokyo)* **1975**, *78*, 1287–1296.
(33) Bogacheva, A. M.; Rudenskaya, G. N.; Preusser, A.; Tchikileva, I. O.; Dunaevsky, Y. E.; Golovkin, B. N.; Stepanov, V. M. A new subtilisin-like proteinase from roots of the dandelion *Taraxacum officinale* Webb S. L. *Biochemistry (Moscow)* **1999**, *64*, 1030–1037.
(34) Adinarayana, K.; Ellaiah, P.; Prasad, D. S. Purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. *AAPS PharmSciTech.* **2003**, *4* (4), E56.
(35) Zamost, B. L.; Nielsen, H. K.; Starnes, R. L. Thermostable enzymes for industrial applications. *J. Ind. Microbiol. Biotechnol.* **1991**, *8* (2), 71–82.
(36) Souza, A. A. G.; Amaral, I. P. G.; Santo, A. R. E.; Carvalho, L. B., Jr.; Bezerra, R. S. Trypsin-like enzyme from intestine and pyloric caeca of spotted goatfish (*Pseudupeneus maculatus*). *Food Chem.* **2007**, *100* (4), 1429–1434.
(37) Uchikoba, T.; Horita, H.; Kaneda, M. Proteases of the sarcocarp of yellow snake-gourd. *Phytochemistry* **1990**, *29* (6), 1879–1881.
(38) Rudenskaya, G. N.; Bogdanova, E. A.; Revina, L. P.; Golovkin, B. N.; Stepanov, V. M. Macluralisin − a serine proteinase from fruits of *Maclura pomifera* (Raf.) Schneid. *Planta* **1995**, *196*, 174–179.
(39) Yadav, S. C.; Pande, M.; Jagannadham, M. V. Highly stable glycosylated serine protease from the medicinal plant *Euphorbia milii*. *Phytochemistry* **2006**, *67* (14), 1414–1426.
(40) Arima, K.; Uchikoba, T.; Yonezawa, H.; Shimada, M.; Kaneda, M. Isolation and characterization of a serine protease from the sprouts of *Pleioblastus hindsii* Nakai. *Phytochemistry* **2000**, *54*, 559–565.

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