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Decolorization of Crude Latex by Activated Charcoal, Purification and Physico-Chemical Characterization of Religiosin, a Milk-Clotting Serine Protease from the Latex of *Ficus religiosa*

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The crude latex of *Ficus religiosa* is decolorized by activated charcoal. Decolorization follows the Freundlich and Langmuir equations. A serine protease, named religiosin, has been purified to homogeneity from the decolorized latex using anion exchange chromatography. Religiosin is a glycoprotein with a molecular mass of 43.4 kDa by MALDI-TOF. Religiosin is an acidic protein with a p*I* value of 3.8 and acts optimally at pH 8.0−8.5 and temperature 50 °C. The proteolytic activity of religiosin is strongly inhibited by PMSF and chymostatin indicating that the enzyme is a serine protease. The extinction coefficient ($\varepsilon^{1\%}_{280}$) of religiosin is 29.47 M^{-1} $cm^{-1}$with 16 tryptophan, 26 tyrosine, and 11 cysteine residues per molecule. The enzyme shows broad substrate specificity against natural as well as synthetic substrates with an apparent K_m of 0.066 mM and 6.25 mM using casein and Leu-pNA, respectively. MS/MS analysis confirms the novelty of the enzyme. Religiosin is highly stable against denaturants, metal ions, and detergents as well as over a wide range of pH and temperature. In addition, the enzyme exhibits milk-clotting as well as detergent activity.

KEYWORDS: Activated charcoal; *Ficus religiosa*; milk-clotting enzyme; religiosin; serine protease

INTRODUCTION

Plant latex is a complex emulsion of proteins, alkaloids, starches, sugars, oils, tannins, resins and gums etc. In most of the plants, the latex is white, but some have yellow, orange, or scarlet latex due to the presence of some organic compounds. Some melanin-like compounds present in the latex of the genus *Ficus* impart brown color to the latex. The melanin-like compounds bind to the proteins and prevent the adsorption of the proteins to the matrix of the ion exchanger, thus posing problems during the purification process. Besides, the retained color after purification hinders the precise absorbance measurements of pure protein. The removal of colored compounds by solvent partitioning, dialysis, and diafiltration is not always practical; these methods are often tedious, time-consuming, and expensive (*1*). Activated charcoal, the most widely used adsorbent for organic compounds, has many applications in isolation and purification of biomolecules from crude fermentation broths (*2*). In this view, a simple and inexpensive process for the decolorization of the crude latex of *Ficus religiosa* was optimized using activated charcoal.

Proteolytic enzymes regulate protein processing and intracellular protein levels by removing abnormal and damaged proteins from the cell as well as play a defensive role against herbivores, insects, and pests (*3*). In addition to their biological roles, proteases have also been exploited commercially in food, leather, textile, and detergent industries because of their broad substrate specificities and activities over a wide range of pH, temperature, and other denaturing conditions (*4*). High stability is generally considered as an economic advantage because of reduced enzyme turnover. In addition, stable enzymes permit the use of high process temperatures, which usually have a beneficial effect on reaction rate, reactant solubility, and the risk of microbial contaminations (*5*, *6*). In the food industry, proteases are also used for the coagulation of milk in the cheese industry. However, the calf rennet used for cheese production is a relatively expensive enzyme due to the limited availability and ethical considerations associated with its use; therefore, the search for new enzymes from other sources still continues to make it industrially applicable and cost-effective (*7*). In the detergent industry, proteases are used to improve the cleaning efficiency of laundry detergents. Nonenzymatic detergents are sometimes inefficient in removing protein-based stains because of their formulation for degrading mainly oil and grease. The addition of proteases leads to the distinctly better cleansing power of detergents through hydrolytic degradation of proteinaceous stains such as blood, egg-yolk, chocolate, etc.

Plant lattices yielded a number of industrially important proteases such as papain, bromelain, ficin, and calotropins (*8*, *9*). Recently, the feasibility of using chemically modified papain as a proteolytic enzyme component in detergents was explored and proved to be an inexpensive alternative to the microbial alkaline proteases used in the detergent industry (*10*).

Serine proteases are one of the largest groups of proteolytic enzymes involved in various physiological and regulatory processes. In recent years, several serine proteases have been purified

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and characterized from different parts of the plants including seeds, latex, and fruits (*11*). Unlike cysteine proteases, serine proteases do not require oxidizing and chelating agents for their function. Therefore, serine proteases could be more advantageous than cysteine proteases for industrial applications (*12*).

F. religiosa, commonly known as pipal, is a large glabrous evergreen tree that belongs to the family Moraceae. It is widely distributed in the tropical areas of the world. *F. religiosa* is a medicinal plant, and the different parts of the plant have been used for years in the preparation of traditional and ayurvedic medicines. The bark of *F. religiosa* shows antitumor and antimicrobial activities and helps in the treatment of asthma, diarrhea, gonorrhea, ulcers, scabies, hiccups, and vomiting (*13*). In view of the several medicinal applications of *F. religiosa*, exploration of biochemical constituents of the plant is worthwhile and was attempted in our laboratory. In the course of screening for biochemical constituents of the plant, high proteolytic activity was found in the latex; in view of this, a serine protease was purified and named as religiosin, following the nomenclature of proteases.

MATERIALS AND METHODS

Acetonitrile, acrylamide, activated charcoal, bovine serum albumin, BSA, casein, chymostatin, coomassie brilliant blue, DEAE−Sepharose fast flow, DTNB, DTT, EDTA, EGTA, glycerol, GuHCl, hemoglobin, hen egg white lysozyme, $HgCl_2$, IAA, *N*,*N*-methylene bis-acrylamide, *o*-phenanthroline, papain, PMSF, rennin, ribonuclease A, SBTI, TFA, trypsin, urea, β-mercaptoethanol, and all synthetic amides were purchased from Sigma Chemical Co., USA. Ampholine carrier ampholites were from LKB. All other chemicals were of the highest purity and commercially available.

Decolorization of Crude Latex by Activated Charcoal. All of the experiments of decolorization were carried out at 30 °C unless stated otherwise.

Step 1: Gum Removal. Fresh latex of *Ficus religiosa* was collected from stems in 0.01 M Tris-HCl buffer at pH 8.0 and frozen at −20 °C for 48 h. The frozen latex was thawed to room temperature and centrifuged at 24000 × *g* for 10 min to remove any insoluble debris. The resulting clear supernatant was termed crude latex and used for decolorization.

Step 2: Decolorization. One milliliter of the crude latex (c_o is equal to 1.32) was mixed with 20 mg of activated charcoal and kept on a shaker for 10 min, followed by centrifugation at 7200 × *g* for 10 min. The color intensity of the clear supernatant was measured by absorbance at 470 nm (*14*). The protein content and activity of the enzyme were also analyzed. The remaining color intensity after decolorization was calculated using the following formula:

$$\text{Remaining color intensity}(\%) = (c*/c_o) \times 100$$

where c_o and c^* are the absorbance of crude and decolorized latex, respectively. (c_o is the initial solute concentration before adsorption, and c^* is the equilibrium solute concentration after adsorption. Here, the solute is the color.)

Optimization of Conditions for Decolorization. Decolorization of crude latex was studied as a function of initial color intensity (c_o, 2.38 to 0.89), amount of charcoal (5 to 50 mg/mL), contact time (5 to 40 min), and temperature (20 to 50 °C). All of the experiments were carried out as described in the decolorization step.

Models. The Freundlich and Langmuir models were tested for decolorization to validate the adsorption by activated charcoal.

Freundlich Equation. The Freundlich equation, for the adsorption of solutes from dilute solution is:

$$c* = k[v(c_o - c*)]^n \quad (1)$$

where v is the latex volume to charcoal weight ratio; $[v(c_o - c^*)]$ is the apparent adsorption per unit weight of adsorbent; k and n are the constants. The value of n is the indicator of adsorption characteristics. A plot of ln c^* versus ln $[v(c_o - c^*)]$ is a straight line.

The Langmuir Equation. The equation for the adsorption isotherm is:

$$a = ABc*/1 + Ac* \quad (2)$$

where a is the amount of solute adsorbed per unit weight of adsorbent $[= v(c_o - c^*)]$ (from eq 1); A and B are the constants depending on the properties of the adsorbent and the solute being adsorbed. A plot of $1/c^*$ versus$1/a$ is a straight line.

Purification of the Enzyme. All of the experiments of purification were carried out at 4 °C unless stated otherwise. The decolorized crude latex obtained from the previous step was subjected to anion exchange chromatography on a DEAE-Sepharose fast flow column pre-equilibrated with 0.01 M Tris-HCl buffer at pH 8.0. The column was washed thoroughly with the same buffer until no protein was seen in the eluate. The bound proteins were eluted with a linear salt gradient of 0−0.6 M NaCl. The eluted fractions were monitored for protein content by absorbance at 280 nm and assayed for proteolytic activity with casein as a substrate. SDS−PAGE was used for the assessment of homogeneity and the intactness of all of the fractions.

Protein Concentration. Protein concentration was determined by absorbance at 280 nm as well as by Bradford's method (*15*).

Electrophoresis and Zymography. Homogeneity, intactness, and molecular mass (M_r) of the purified enzyme were determined by 15% SDS−PAGE under reducing and nonreducing conditions as described by Laemmli (*16*). After electrophoresis, proteins in the gel were stained by coomassie R-250. Zymography was performed to confirm the proteolytic activity of religiosin in the gel using the protocol of Tomar et al (*17*). After electrophoresis, protein in the gel was stained by coomassie G-250.

Activity Measurements. The proteolytic activity of religiosin was monitored using natural substrates casein and hemoglobin. Ten micrograms of the enzyme equilibrated in 0.5 mL of 0.05 M Tris-HCl buffer, pH 8.0, at 37 °C for 10 min, was added to 0.5 mL of 1% substrate in the same buffer, and the reaction was allowed to proceed at 37 °C for 30 min. The reaction was terminated by adding 0.5 mL of 10% TCA and kept for 10 min. The resultant precipitate was removed by centrifugation, and the TCA-soluble peptides were measured by absorbance at 280 nm. A control assay without enzyme in the reaction mixture was used as a blank. One unit of enzyme activity is defined as the amount of enzyme that gives rise to an increase in one unit of absorbency at 280 nm per min of casein digestion, under the standard assay conditions. The specific activity is the number of units of activity per milligram of protein.

Isoelectric Focusing. The isoelectric point (p*I*) of the purified enzyme was determined by isoelectric focusing on polyacrylamide disc gel as described by Kundu et al (*18*). The electrophoretic run was carried out with ampholine carrier ampholytes in the pH range 3−6, at 5 mA current for 2 h using 0.1 M NaOH as catholyte and 0.1 M orthophosphoric acid as anolyte. The protein band in the gel was visualized by coomassie G-250 staining.

Glycostaining and Carbohydrate Content. The glycostaining was performed to confirm the presence of the carbohydrate moiety of the protein molecule in the gel using Schiff's reagent. After SDS−PAGE, the gel was fixed in the mixture of 40% methanol and 20% acetic acid followed by reswelling in 7% acetic acid. Furthermore, the gel was kept in an oxidizing mixture of 1% periodic acid in 7% acetic acid in the dark for 1 h. Subsequently, the gel was washed in 7% acetic acid with several changes for 24 h. Finally, the gel was stained with Schiff's reagent at 4 °C in the dark for 1 h and differentiated by 1% sodium metabisulfite in 0.1 M HCl. The amount of carbohydrate content in the enzyme molecule was estimated by the phenol sulfuric acid method as described by Sharma et al (*12*).

Substrate Specificity. The amidolytic activities of the enzyme were studied using *N*α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), L-alanine-*p*-nitroanilide, L-alanine alanine-*p*-nitroanilide, L-leucine-*p*-nitroanilide, *N*-succinyl-L-phenylalanine-*p*-nitroanilide, and L-γ-glutamyl-*p*-nitroanilide by the method of Arnon (*19*) with some modifications. The synthetic substrates (5−20 mM) were prepared by dissolving the required amount in a minimum volume of DMSO and making up the final volume with 0.05 M Tris-HCl buffer, pH 8.0, at 30 °C. The enzyme was incubated with the assay buffer and synthetic substrate at 37 °C for 30 min. The reaction was terminated by the addition of 0.2 mL of 30% acetic acid, and the liberated *p*-nitroaniline ($\varepsilon = 8800\ M^{-1}\ cm^{-1}$ at 410 nm) was monitored by absorbance at 410 nm, against a reaction blank without the enzyme (*20*).

pH and Temperature Optima. The activity of religiosin was measured at different pH values and temperatures. The optimum pH of the enzyme was determined by measuring casein-hydrolyzing activity from pH 1.0 to 12.0. Hemoglobin was used as a substrate below pH 4.0 due to the insolubility of casein at lower pH value. A control assay at the same pH without enzyme in the reaction mixture was used as a blank. The effect of temperature on the activity of purified enzyme was also investigated. The enzyme was incubated at the desired temperature (20−90 °C) for 15 min, and the activity was measured at the same temperature value.

Stability. The stability of an enzyme dictates its applicability; therefore, the effect of pH (1.0−12.0), temperature (20−90 °C), as well as different concentrations of denaturants such as organic solutes, organic solvents, detergents, and metal ions on the proteolytic activity of religiosin was examined. The enzyme was incubated under the specified condition of pH, organic solutes, organic solvents, and metal ions for 24 h, whereas, in the case of temperature and detergents, the enzyme was incubated for 15 min and 6 h, respectively. The residual proteolytic activity was assayed as described above.

Effect of Inhibitors. In order to classify the enzyme, the effect of protease inhibitors on the activity of religiosin was studied. The inhibitors used were PMSF, chymostatin, SBTI, $HgCl_2$, IAA, leupeptin, EDTA, EGTA, and *o*-phenanthroline. The enzyme was incubated with increasing concentrations of a specific inhibitor (0−50 mM) at 37 °C for 30 min, and the residual proteolytic activity was assayed as described above. A control assay without inhibitor was performed, and the activity was considered as 100%.

Effect of Substrate Concentration. The effect of increasing substrate concentration on the velocity of the enzyme-catalyzed reaction was studied using natural as well as synthetic substrates at pH 8.0 and 37 °C. The concentrations of the natural substrate (casein) and the synthetic substrate (L-leucine-*p*-nitroanilide) were studied in the range of 20−450 μM and 5−20 mM, respectively. The enzyme activity was measured under the standard assay conditions as described above. A Lineweaver−Burk plot was drawn, and the value of the Michaelis constant, K_m, was calculated as described by Segel (*21*).

Estimation of Specific Amino Acid Contents and Extinction Coefficient. Total numbers of the tryptophan and tyrosine residues in religiosin were measured spectrophotometrically as described by Goodwin and Morton (*22*). The free and total cysteine residues of the enzyme were estimated by Ellman's method (*23*). For the free cysteine content estimation, the enzyme was reduced with 0.01 M β-ME, whereas for the of total cysteine content, estimation the enzyme was denatured in 6 M GuHCl and reduced with 0.05 M DTT. The excess reducing agents were removed by dialysis against 0.1 M acetic acid. An aliquot of the dialyzed enzyme was added to the DTNB solution, and the liberated TNB anions were monitored by absorbance at 412 nm. The number of disulfide bonds per molecule of the protein was calculated using the number of total and free cysteine residues in the molecule. To validate the current estimations, papain, ribonuclease, and lysozyme were used as standards. The extinction coefficient of the enzyme was determined by the spectrophotometric method as described by Aitken and Learmonth (*24*).

Peptide Mass Fingerprinting/Mass Spectrometric Sequencing. Tryptic digestion of religiosin was done using the protocol as described by Shevchenko et al (*25*) with minor modifications. After SDS−PAGE, gel pieces were excised, destained, washed, dehydrated in CH_3CN, and dried in a vacuum centrifuge. The gel pieces were cooled on ice and soaked in digestion buffer containing 50 mM NH_4HCO_3, 5 mM $CaCl_2$, and 12.5 ng/μL of trypsin (Promega, sequencing grade) overnight at 37 °C. The digested peptides were recovered from the gel by sonicating in a water bath for 10 min, and the process was repeated three times. The tryptic-digested sample was mixed with the matrix (saturated solution of α-cyano-4-hydroxy-cinnamic acid in 0.1% TFA and 50% acetonitrile) and spotted on a MALDI plate. The mixture was allowed to dry at room temperature and used for the MALDI analysis on an ultraflex MALDI-TOF/TOF mass spectrometer (Bruker Daltonics).

Milk-Clotting Activity and Proteinaceous Stain Removal Study. The milk-clotting activity of religiosin was determined according to the method described by Arima et al (*26*). One milk-clotting unit was defined as the amount of enzyme that coagulates 10 mL of the substrate in 40 min.

For the proteinaceous stain removal study, clean cotton cloth pieces (10 cm × 10 cm) were soiled with blood and chocolate and allowed to dry.

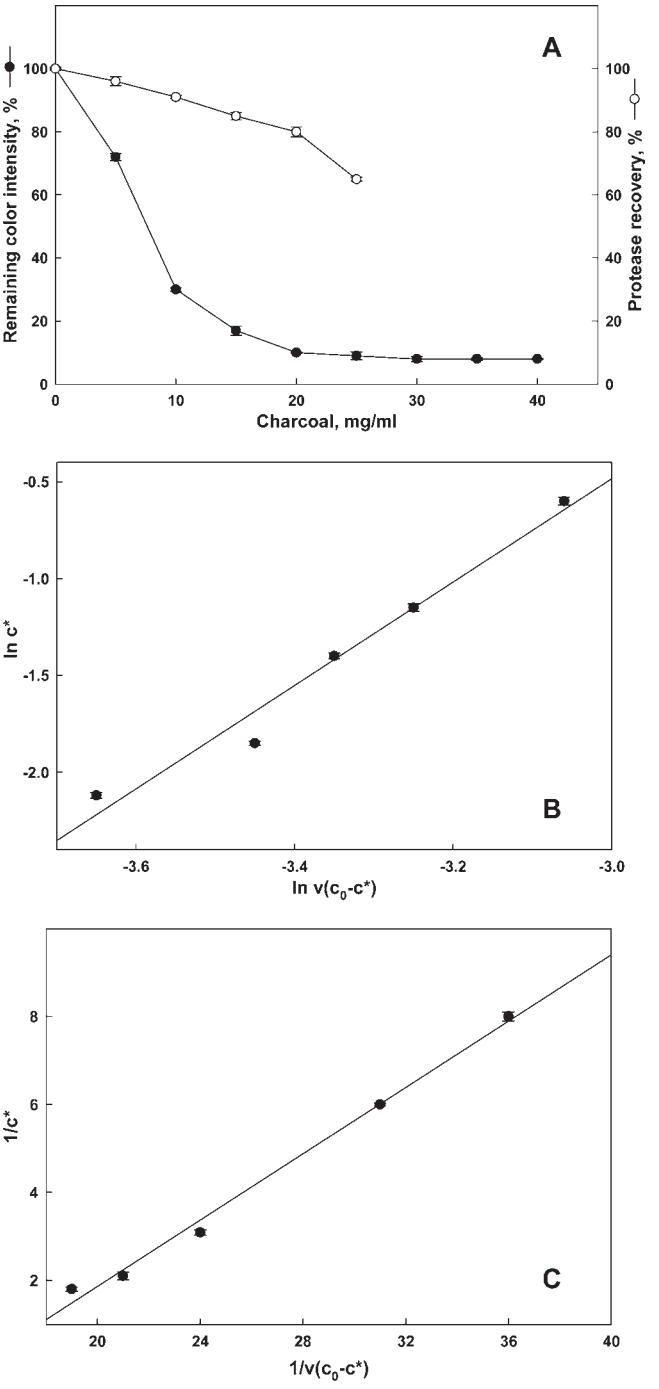

Figure 1. Decolorization of the crude latex. (**A**) Effect of the amount of activated charcoal on remaining color intensity (●) and protease recovery (○). (**B**) Fitting of the decolorization data to the Freundlich equation. (**C**) Fitting of the decolorization data to the Langmuir equation. Values in all of the figures are represented as the mean ± SD (n = 3).

The soiled cloth pieces were incubated with the 1% detergent and 0.02% enzyme solution for 5 h. Aliquots of 5 mL of washing liquid were taken out periodically, and the color intensity was measured by absorbance at 280 nm until the change in the color intensity leveled off. Stained-cloths of the same size soaked in 1% detergent without enzyme were used as the control. Commercial detergents Surf Excel (Hindustan Lever, India) and Ariel (Procter and Gamble, India) were used as references for this study.

RESULTS AND DISCUSSION

Decolorization of Crude Latex by Activated Charcoal. The rate of decolorization depends on the degree of adsorption, temperature,

Table 1. Purification of Religiosin from the Latex of *F. religiosa*

steps	total protein (mg)	total activity[a] (units)	specific activity (units/mg)	yield (%)	purification fold
crude latex	120	3000	25	100	1.00
decolorized latex	80	2406	30.07	80.2	1.20
DEAE- Sepharose	25	1440	57.6	48	2.3

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

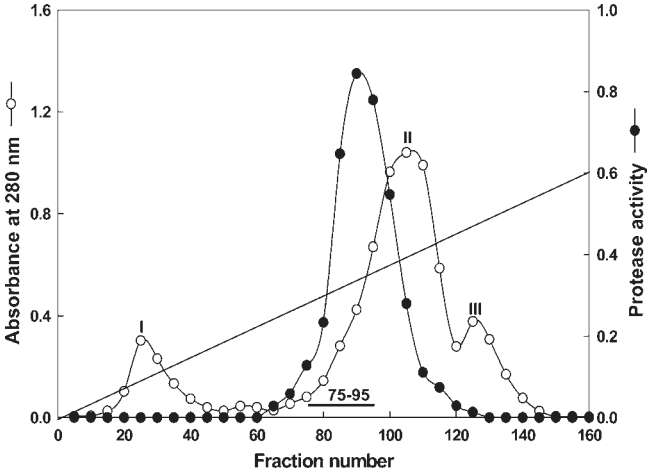

Figure 2. Elution of the crude latex on anion exchange chromatography. DEAE−Sepharose fast flow, pre-equilibrated with 10 mM Tris buffer, pH 8.0. The bound proteins were eluted with a linear salt gradient of 0−0.6 M NaCl. Fractions of 5 mL volume at a flow rate of 5 mL/min were collected and assayed for protein concentration (○) and proteolytic activity (●). The fractions of the ascending limb of pool II (75−95) were pooled as indicated by the horizontal line.

contact time, and amount of charcoal used, as shown in **Figure 1A**. The optimum amount of charcoal is found to be 20 mg/mL of the crude latex (c_o is equal to 1.32) that helps to achieve 90% decolorization and 80% of protease recovery. The optimum contact time and temperature for decolorization are found to be 10 min and 30 °C, respectively, without appreciable loss of the enzymatic activity. The rate of adsorption of colored solutes increases with increasing contact time as well as temperature and attains a constant value when equilibrium is established. Similar results were also observed in the case of yellow zein (*27*). In addition to decolorization, activated carbon helps in partial purification of religiosin indicated by increased specific activity and fold purification as presented in **Table 1**.

Models. From **Figure 1**, v, c^*, and v (c_o−c^*) were calculated. The linear plot of ln c^* versus ln [v(c_o−c^*)] testifies that the adsorption characteristics with the Freundlich constant n equal to 2.6 ± 0.012 validates the adsorption by activated charcoal as shown in **Figure 1B**. Also, the linear plot of $1/c^*$ versus $1/v$ (c_o−c^*) confirms the validity of the Langmuir model (**Figure 1C**).

Purification of the Enzyme. A new serine protease is purified to homogeneity from the decolorized latex of *Ficus religiosa* using anion exchange chromatography. The protein elution profile resolves into three peaks (**Figure 2**). The proteolytic activity is observed only in one peak (peak II). The ascending limb of peak II (fractions 75−95) is found to be homogeneous and resulted in a single band by SDS−PAGE. The pure enzyme is named as religiosin according to protease nomenclature. The enzyme is purified up to 2.3-fold with 48% yield and a specific activity of 57.6 U/mg. The purification protocol is simple and highly reproducible in terms of yield and specific activity of the enzyme within the experimental errors. Homogeneity and intactness of the protein are judged by SDS−PAGE under reducing and nonreducing conditions as shown in **Figure 3A**. The single band on SDS−PAGE suggests the monomeric nature of the enzyme. The results of purification are summarized in **Table 1**.

Physical Properties of Religiosin. The molecular mass (M_r) of the purified enzyme is 43 kDa by both SDS−PAGE and MALDI-TOF (**Figure 3A** and **B**). The molecular mass (M_r) of the majority of plant serine proteases lies in the range of 60−80 kDa, and the molecular mass of religiosin falls below the range (*11*). Gelatin zymography confirms the proteolytic nature of the enzyme in the gel (**Figure 3C**). Religiosin shows a single band in isoelectric focusing with an approximate isoelectric point (p*I*) of pH 3.8 (**Figure 3D**). Most of the plant serine proteases have isoelectric points in the range of pH 4.0−7.0 and rarely have highly acidic p*I* values (*11*). The p*I* value of religiosin is lower than those of the other plant proteases as compared in **Table 2**. Religiosin is a glycoprotein with 12% carbohydrate content. Furthermore, Schiff's staining confirms the presence of the carbohydrate moiety of the protein molecule in the gel (**Figure 3E**). The protein band seems diffused and wider in SDS−PAGE gel due to the intense differential glycosylation, a characteristic feature of glycoproteins. Similar observations were reported in the case of benghalensin (*12*). The deglycosylation of religiosin by TFMS results in the complete loss of proteolytic activity as no band appeared in the zymogram and reflects that the carbohydrate moiety may be the part of the functional architecture of the enzyme. The biochemical properties of religiosin are compared with those of the other known serine proteases in **Table 2**.

Substrate Specificity. The enzyme hydrolyzes denatured natural substrates such as casein and hemoglobin. Religiosin exhibits significant hydrolyzing activity against synthetic substrates such as L-alanine-*p*-nitroanilide, L-alanine−alanine-*p*-nitroanilide, and L-leucine-*p*-nitroanilide while failing to hydrolyze *N*α-benzoyl-DL-arginine-*p*-nitroanilide, *N*-succinyl-L-phenylalanine-*p*-nitroanilide, and L-γ-glutamyl-*p*-nitroanilide. Thus, religiosin shows broad substrate specificity, giving preferences to small and nonpolar amino acids at the P_1 position, whereas the activity of enzyme over polar and bulky hydrophobic groups at position P_1 is not detectable. Thus, the P_1 specificity of religiosin is somewhat similar to that of cucumisin and differs from that of subtilisin, trypsin, and α-chymotrypsin, confirming distinct substrate specificity for religiosin (*11*, *28*).

pH and Temperature Optima. Religiosin retains at least 50% of the proteolytic activity from pH 6.0−10.0 and in the temperature range of 35−65 °C. The pH and temperature optima for the activity of religiosin are pH 8.0−8.5 and 50 °C, respectively (**Figure 4A** and **B**). The pH and temperature optima of religiosin are compared with those of other serine proteases and shown in **Table 2**.

Stability. Religiosin retains more than 80% of the activity from pH 5.5−10.0 and in the temperature range of 20−65 °C (**Figure 4A** and **B**). Religiosin shows an increase in proteolytic activity at lower concentrations of GuHCl up to 2.0 M. The increase of proteolytic activity of the enzyme is about 2-fold at 2.0 M GuHCl, which is not found when the enzyme is treated with urea. Religiosin retains full activity at higher concentrations of

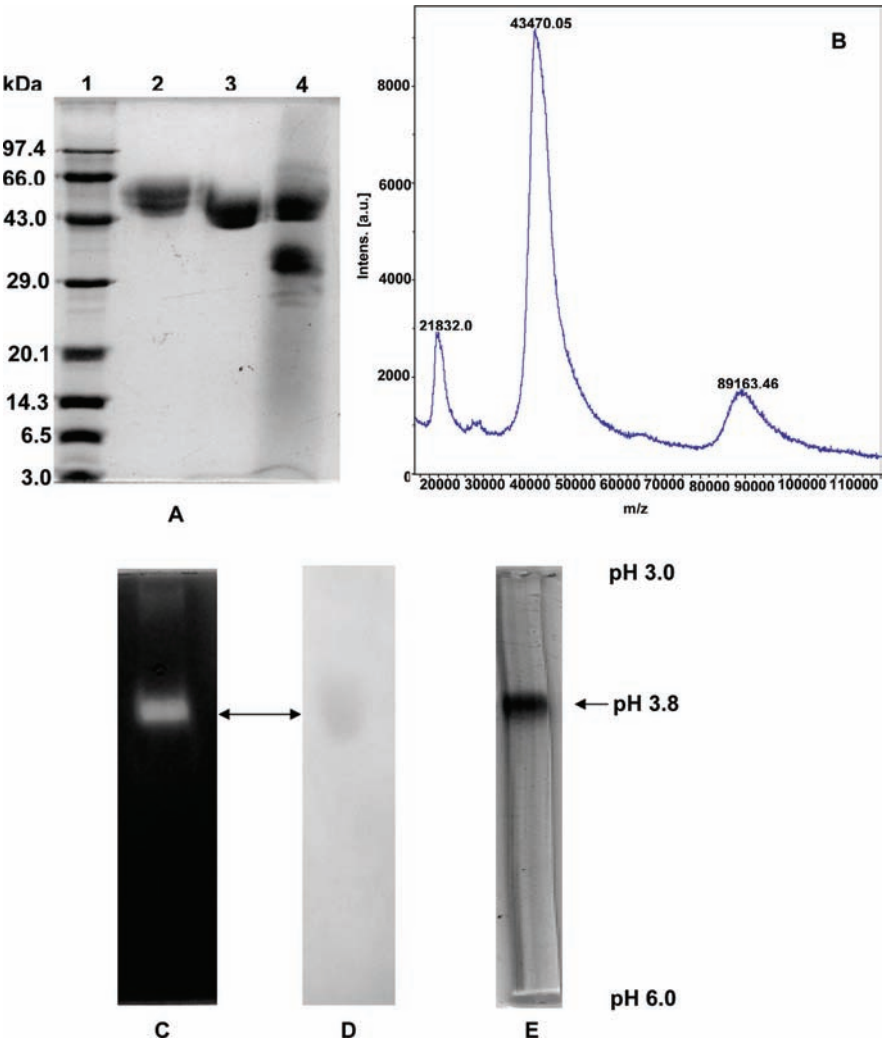

Figure 3. (**A**) Assessment of homogeneity and molecular weight of religiosin by 15% SDS−PAGE. Gel electrophorized lanes 1−4 represent the marker, religiosin (30 μg), under reducing and nonreducing conditions and the crude latex, respectively. (**B**) Religiosin (10 ng) was used for MALDI−TOF analysis. (**C**) Zymogram (in-gel activity) of religiosin. The clear region in the gel (indicated by the double head arrow) shows the hydrolysis of gelatin by the enzyme. (**D**) Glycostaining by Schiff's reagent. The magenta color (indicated by the double head arrow) confirms the presence of the carbohydrate moiety in the protein molecule. (**E**) Isoelectric focusing was performed by 5% polyacrylamide disk gel electrophoresis with the ampholine carrier ampholyte, pH 3.0−6.0, at a constant current of 5 mA. The isoelectric point of the purified protein is indicated by an arrow.

Table 2. Comparison of Biochemical Properties of Religiosin with Other Known Plant Serine Proteases

enzyme	plant	mol. mass (kDa)	pH optimum	temperature optimum (°C)	isoelecric point (p*I*)
religiosin	*F. religiosa*	43.3	8.0−8.5	50	3.8
benghalensin[a]	*F. benghalensis*	47	8.0	50	4.4
cucumisin[b]	*C. melo L*	54.0	7.1	70	NR[i]
RSIP[c]	*Z. mays L*	59.0	6.0−6.5	NR	4.55
taraxilisin[d]	*T. officinale*	67.0	8.0	40	4.5
cryptolepain[e]	*C. buchanani*	50.5	8.0−10.5	70−75	6.0
indicain[f]	*M. indica*	134.3	8.5	80	4.8
carnein[g]	*I. carnea*	80.24	6.5	65	5.6
KLSP[h]	*P. vulgaris*	72.0	9.9	60	4.6

[a] Ref *12*. [b] Ref *31*. [c] Ref *32*. [d] Ref *33*. [e] Ref *34*. [f] Ref *35*. [g] Ref *36*. [h] Ref *37*. [i] NR represents the data not reported.

denaturants, up to 3.5 M GuHCl and 7 M urea. The enhanced activity of religiosin might be due to the denaturant-induced alteration of tertiary structure and the conformational stability of the active site. By contrast, religiosin shows a considerable loss of proteolytic activity at low concentrations of organic solvents, which may be due to the profound effects of organic solvents on the structural integrity and catalytic activity of the enzyme as summarized in **Table 3**. Besides, religiosin can also be stored for a longer time at neutral conditions and low temperatures with the retention of full activity. Metal ions, such as Na^+, K^+, Mg^{2+}, and Ca^{2+}, do not show any considerable effect on the activity of religiosin up to 10 mM, as shown in the **Table 3**. Similar observations have been reported for other serine proteases, wrightin (*17*) and dubiumin (*29*). The high stability of religiosin, without perturbing the enzymatic activity by various agents, could make it possible to work under mild denaturing conditions.

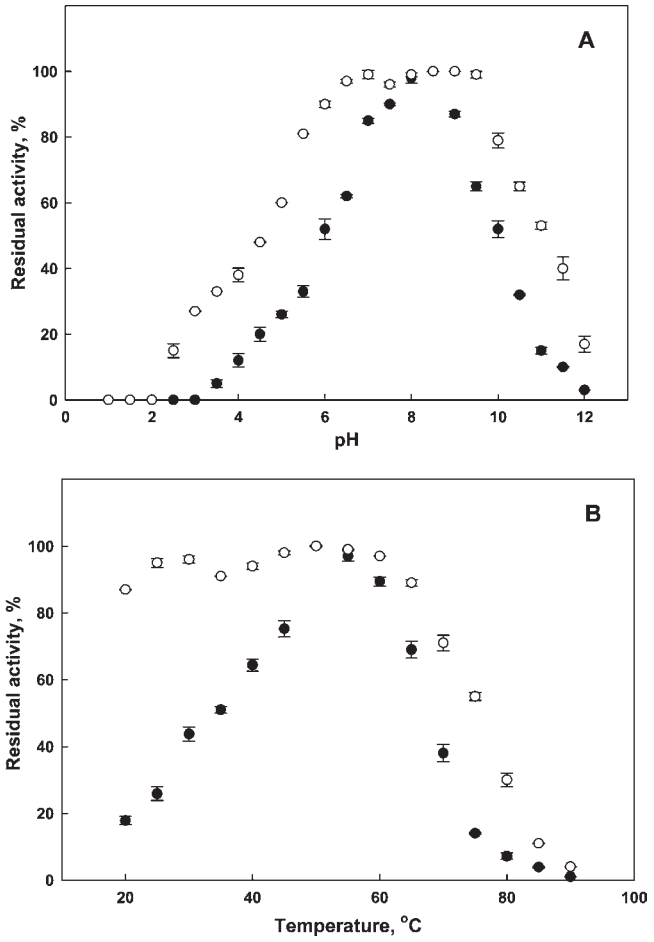

Figure 4. Effect of pH (**A**) and temperature (**B**) on the activity (●) and stability (○) of religiosin. To see the effect of pH on activity, 10 μg of religiosin was taken at required pH, and the activity was measured, using casein as substrate at the same pH. For pH stability, the enzyme was incubated overnight at the required pH, and the residual activity was measured at 37 °C and pH 8.0. Similarly, for temperature optima, 10 μg of religiosin was used for the activity assay at the required temperature. For temperature stability, the enzyme was incubated at the required temperature for 15 min, and the activity was measured at 37 °C and pH 8.0. Values in both figures are represented as the mean ± SD (n = 3).

Table 3. Stability of Religiosin under Different Conditions

condition	concentration	residual activity (%)[a]
pH	pH 6.5−9.5	98.57 ± 1.51
temperature	25−60 °C	96.5 ± 2.90
GuHCl	3.5 M	99.32 ± 0.64
urea	7 M	100.02 ± 0.34
methanol	30%	79.07 ± 0.05
acetonitrile	20%	80.03 ± 0.08
DMSO	50%	70.44 ± 0.12
metal ions (Na^+, K^+, Mg^{2+}, and Ca^{2+})	10 mM	100.01 ± 0.03
detergent (Surf-excel and Arial)	1%	100.02 ± 0.05

[a] Residual activities are shown as the mean ± SD (n = 3).

Effect of Inhibitors. PMSF and chymostatin inhibit more than 90% of the proteolytic activity of religiosin, while leupeptin, $HgCl_2$, IAA, *o*-phenanthroline, EDTA, and EGTA do not inhibit enzymatic activity (**Table 4**) and confirm that religiosin belongs to the serine protease class. However, soybean trypsin inhibitor (SBTI) does not alter the proteolytic activity of religiosin. Generally, SBTI effectively inhibits the activity of bacterial or animal serine proteases while failing to inhibit plant serine proteases such as benghalensin and milin (*12*, *30*).

Table 4. Effect of Inhibitors on the Activity of Religiosin

inhibitor	concentration (mM)	residual activity (%)[a]
PMSF	1	6.45 ± 0.17
chymostatin	2	10.21 ± 0.18
SBTI	5	100.02 ± 1.3
$HgCl_2$	5	100.01 ± 0.04
IAA	5	99.5 ± 0.35
leupeptin	5	100.05 ± 0.24
EDTA	5	98.20 ± 0.11
EGTA	5	96.21 ± 0.53
o-phenanthroline	5	99.09 ± 0.16

[a] Residual activities are shown as the mean ± SD (n = 3).

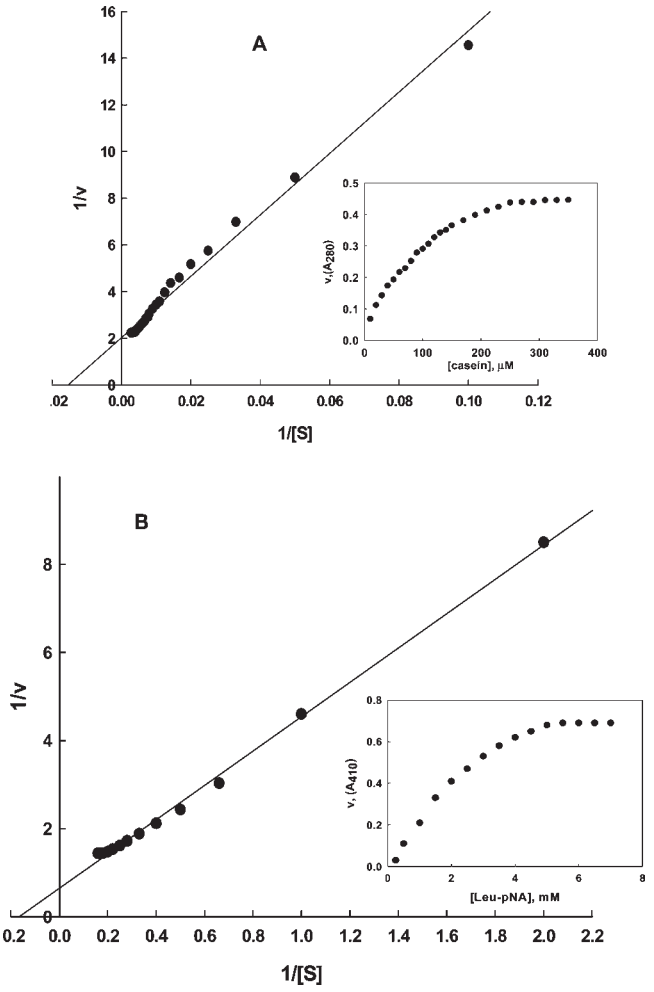

Figure 5. Effect of the substrate concentration on the reaction velocity (●) of religiosin with natural (**A**) and synthetic (**B**) substrates. (**A**) Lineweaver−Burk plot. The enzyme (10 μg) incubated in 0.5 mL of 0.5 M Tris−HCl buffer, pH 8.0, was added to 0.5 mL of casein in a concentration range of 20−410 μM. Inset: Michaelis−Menten curve. K_m was calculated according to the Lineweaver−Burk plot. (**B**) Lineweaver−Burk plot. The enzyme (10 μg) incubated in 0.5 mL of 0.5 M Tris−HCl buffer, pH 8.0, was added to 0.5 mL of Leu-pNA in a concentration range of 0.25−7.0 mM. The activity was determined under the standard assay conditions, as described in the text. Inset: Michaelis−Menten curve. K_m was calculated according to the Lineweaver−Burk plot.

Effect of Substrate Concentration. The enzyme obeys the Michaelis−Menten kinetics, and the nature of kinetics is typically hyperbolic with increasing substrate concentration, natural as

Table 5. Ratio of Milk-Clotting Activity to Proteolytic Activity of Religiosin and Other Proteases[a]

protease	milk-clotting activity (units/mL)	proteolytic activity (OD 660 nm)	ratio (units/OD 660 nm)
religiosin	211 ± 0.21	0.54 ± 0.02	387 ± 12.67
rennin	249 ± 0.500	0.05 ± 0.001	4989 ± 109.771
papain	216 ± 0.14	0.59 ± 0.01	367 ± 5.90
trypsin	1.6 ± 0.003	0.44 ± 0.002	3.6 ± 0.025
ficin	267 ± 0.15	0.68 ± 0.012	393 ± 6.12

[a] All data are shown as the mean ± SD (n = 3).

well as synthetic substrates (insets of **Figure 5A** and **B**). The value of K_m, obtained from the Lineweaver−Burk plot, is 0.066 ± 0.02 mM and 6.25 ± 0.10 mM, with casein and Leu-pNA as substrates, respectively (**Figure 5A** and **B**).

Specific Amino Acid Residues and Extinction Coefficients. The total cysteine residues in the protein are found to be 11 (measured value 11.34 ± 0.05), out of which 10 (measured value 9.89 ± 0.09) residues are involved in making five disulfide bridges, and one is free. The total numbers of tryptophan and tyrosine residues in the enzyme molecule are estimated to be 16 (measured value 16.42 ± 0.01) and 26 (measured value 26.09 ± 0.10), respectively. The extinction coefficient of the protein is 29.47 M^{-1} cm^{-1}.

Peptide Mass Fingerprinting Analysis. Attempts were made to characterize religiosin by peptide mass finger printing. Tryptic-digested religiosin resolved into a number of peptide fragments. The peptide masses with high relative intensities were selected for further mass spectrometry using peptide mass fingerprinting. Two major peptides with m/z of 1619.82 and 1186.5 were sequenced by mass spectrometry. The sequences obtained were submitted to BLAST analysis, and no significant hits were detected. However, a good score of 54 was obtained with sequence similarities to ATP dependent protease. This MS/MS result provides evidence that the purified protease is a new protein.

Milk-Clotting Activity and Proteinaceous Stain Removal Study. The enzyme coagulates skimmed milk and forms a white and firm curd. Moreover, the ratio of milk-clotting activity to proteolytic activity of religiosin is determined to be 390 ± 12.67 U/OD 660 nm and compared with those of other proteases and shown in **Table 5**. The capacity of religiosin to produce milk curds together with its high ratio of milk clotting to proteolytic activity could make it useful as a new milk coagulant, although more studies about the quality of both milk curds and the cheese formed should be carried out in the future to confirm its usefulness in the dairy industry.

The enzyme is highly stable in the presence of detergents shown in **Table 3**. The addition of religiosin to the nonenzymatic detergents helps in the removal of proteinaceous stains better than the detergent alone. Thus, religiosin might be a good choice for the detergent industry, though it includes a deeper understanding of the structure−function relationship of the enzyme as well as factors influencing cleaning performance.

ABBREVIATIONS

BSA, bovine serum albumin; DEAE, diethylaminoethyle; DFP, diisopropylfluorophosphate; DMSO, dimethyl sulfoxide; DTNB, 5,5-μ-dithiobis (2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylene diammine tetraacetic acid; EGTA, ethylene glycol-bis (α-amino ethyl ether) tetraacetic acid; GuHCl, guanidine hydrochloride; $HgCl_2$, mercuric chloride; IAA, iodoacetic acid; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; PAGE, polyacrylamide gel electrophoresis; PMSF, phenyl-methanesulfonyl fluoride; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; TFMS, trifluoromethanesulfonic acid; Tris-HCl, tris-(hydroxymethyl) aminomethane hydrochloride.

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.

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