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Religiosin B, a milk-clotting serine protease from *Ficus religiosa*

Moni Kumari, Anurag Sharma, M.V. Jagannadham *

Molecular Biology Unit, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005, India

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ABSTRACT

A novel milk-clotting serine protease, named religiosin B, is purified from *Ficus religiosa*. The molecular mass of the protein is 63,000 with p*I* value of pH 7.6. The proteolytic activity of the enzyme is strongly inhibited by phenylmethanesulfonyl fluoride (PMSF) and chymostatin. Religiosin B acts optimally at pH 8.0–8.5 and temperature 55 °C. The molar absorption coefficient of the enzyme is 149,725 $M^{-1}cm^{-1}$ with 23 tryptophan, 15 tyrosine and 7cysteine residues per molecule of the enzyme. The enzyme shows broad substrate specificity with natural as well as synthetic substrates. Religiosin B is highly stable against denaturants and metal ions as well as over a wide range of pH and temperature. The *de novo* sequencing confirms the novelty of the enzyme. In addition to its high milk-clotting ability, it could be used in the cheese industry, as well as other food and biotechnological industries.

1. Introduction

Proteases constitute one of the most important groups of industrial enzymes, accounting for about 60% of the total enzyme market. Several useful proteases have been obtained from various sources such as plants, animals and microorganisms. Proteases have a large variety of applications in food industry. They have been routinely used for various purposes such as, cheese making, baking, meat tenderization and preparation of soya hydrolysates (Tomar, Kumar, & Jagannadham, 2008). The major application of proteases in dairy industry is in the manufacture of cheese. The milk coagulating enzymes fall into three main categories; animal rennet, microbial milk coagulants and genetically engineered chymosin. However, the increasing cheese production and consumption, the high price and reduced supply of rennets, as well as the associated ethical issues have led to the search for alternative or additional rennet substitutes produced either from plants or from genetically modified microorganisms (Ahmed, Morishima, Babiker, & Mori, 2009a, 2009b). Moreover, the use of plant as a source of proteases could be preferred over other sources in terms of availability of crude, easy purification processes and in isolation of natural coagulant. Also, the use of plant coagulant could be advantageous in improving the nutritional intake of vegetarian people worldwide (Tavaria, Sousa, & Malacata, 2001). In recent years, much research interest has been directed towards finding several new proteases from the plants and their milk coagulating activity has been explored.

Proteolytic enzymes from the plant sources have received special attention because of their broad substrate specificity, activity in a wide range of pH values, temperature, and presence of organic compounds as well as other additives. Many operations in food industry are carried out at high temperature such as, hydrolysis of proteins at high temperature, enzymatic production of aspartame and other peptides, baking and brewing (Sharma, Kumari, & Jagannadham, 2009). Therefore, the search for valuable proteases with distinct activity and specificity is always a continuous challenge for varied industrial applications.

Multiple proteases of the same family are quite often reported in latex bearing plants. However, the reason for such multiplicity of proteases has not been extensively highlighted. These multiple proteases, from the same source, show different behaviours in terms of stability, activity and specificity (Nallamsetty, Kundu, & Jagannadham, 2003). Such reports about multiplicity of proteases have prompted further screening of the latex of *Ficus religiosa.* During the process one more milk-clotting serine protease has been identified which is more active relative to other protease of the same source. The other protease namely religiosin, has been already characterised for its milk-clotting activity (Kumari, Sharma, & Jagannadham, 2010). Also, both the enzymes show distinct features from each other and from other well-known serine proteases in various terms. In this respect, it is essential to pursue studies on

Abbreviations: BSA, bovine serum albumin; CBB, coomassie brilliant blue; DEAE, diethylaminoethyl; DMSO, dimethyl sulfoxide; DTNB, 5,5-dithiobis (2-nitrobenzoicacid); DTT, di-thiothreitol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(raminoethyl ether) tetraacetic acid; GuHCl, guanidine hydrochloride; IAA, iodoacetic acid; IEF, iso electric focusing; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; SBTI, soybean trypsin inhibitor; SDS, sodium dodecyl sulphate; TCA, trichloroacetic acid; TEMED, *N,N,N,N*-tetramethylethylenediamine; β-ME, β-mercaptoethanol.

* Corresponding author. Tel.: +91 542 2367936; fax: +91 542 237568.
E-mail addresses: jvm@bhu.ac.in, monimbu@rediffmail.com (M.V. Jagannadham).

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these enzymes to get a better understanding of their features. This manuscript describes the identification, purification and biochemical properties of a new serine protease from the latex of *F. religiosa*.

2. Materials and methods

2.1. Materials

Acetonitrile, acrylamide, bovine serum albumin, casein, chymostatin, coomassie brilliant blue, DEAE–Sepharose fast flow, DTNB, DTT, EDTA, EGTA, glycerol, GuHCl, haemoglobin, hen egg white lysozyme, $HgCl_2$, IAA, *N*,*N*-methylene bis-acrylamide, *o*-phenanthroline, papain, PMSF, rennin, ribonuclease A, SBTI, TCA, 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.

2.2. Purification of enzyme

All the experiments of purification were carried out 4 °C unless stated otherwise.

2.2.1. Gum removal

Fresh latex was collected from the stem by making longitudinal incisions into 0.01 M acetate buffer, pH 4.5 and frozen at −20 °C for more than 48 h. Subsequently, the latex was thawed to room temperature and centrifuged at 24,000*g* for 30 min to remove gum and other debris. The resulting clear supernatant was termed as crude latex and used in the next step.

2.2.2. Cation exchange chromatography

The crude latex was subjected to cation exchange chromatography on SP-Sepharose fast flow, in a column pre-equilibrated with 0.01 M acetate buffer, pH 4.5. The column was washed thoroughly with the same buffer until no protein or activity was seen in the eluate. The bound proteins were eluted with a linear salt gradient from 0 to 1.0 M NaCl. All the fractions were monitored by absorbance, at 280 nm, for protein content and assayed for enzymatic activity with casein as substrate. Intactness and homogeneity of the enzyme in all the fractions were also assessed by SDS–PAGE.

2.3. Protein concentration

The protein concentration was measured by absorbance, at 280 nm, as well as by the method of Bradford using BSA as standard (Bradford, 1976).

2.4. Protease assay

The proteolytic activity of the enzyme during purification was monitored using natural substrates, casein and haemoglobin. For the assay, 10 μg of enzyme in 0.5 ml of 0.05 M Tris buffer, pH 8.0, was added to 0.05 ml of 1% (w/v) substrate in the same buffer and the reaction was allowed to proceed for 30 min, at 37 °C. The reaction was terminated by the addition of 0.5 ml of 10% (w/v) TCA and kept for 10 min. The resultant precipitate was removed by centrifugation and TCA soluble peptides in the supernatant were measured by absorbance at 280 nm. A control assay, without enzyme in the reaction mixture, was used as blank. One unit of enzyme activity is defined as the amount of enzyme that gave rise to an increase of one unit of absorbancy at 280 nm per min of substrate digestion. The specific activity is the number of units of activity per milligram of protein.

2.5. Electrophoresis and zymography

Homogeneity, intactness and molecular mass (M_r) of the purified enzyme were determined by SDS–PAGE under reducing and non-reducing conditions as described by Laemmli (1970). The gel was cast in 1 mm slab consisting of 15% separating gel (8 cm × 10 cm) and 5% stacking gel (2 cm × 10 cm). Electrophoresis was carried out at constant current of 20 mA, until the tracking dye (bromophenol blue) reached till the bottom of the gel (ca. 1 h running time). After electrophoresis, proteins in the gel were stained by coomassie R-250. Gelatin zymography was performed to confirm the proteolytic activity of religiosin B in the gel using the protocol of Tomar et al. (2008). After electrophoresis, protein in the gel was stained by coomassie G-250.

2.6. Isoelectric focusing

The isoelectric point (p*I*) of the purified enzyme was determined by isoelectric focusing on polyacrylamide disc gel, as described by Pande, Dubey, Yadav, & Jagannadham (2006). Electrophoretic runs were carried out with ampholine carrier ampholytes in the pH range 7–9, at 5 mA current for 2 h. A 5% polyacrylamide gel containing 2% desired ampholine was cast in tube gels. Anodic and cathodic chamber buffers were 0.1 M orthophosphoric acid and 0.1 M sodium hydroxide, respectively. The gels were subjected to a pre-run at a constant current of 1 mA per rod, for 2 h, to develop the pH gradient. Protein samples (100 μg) containing 10% (v/v) ampholine and 25% (v/v) glycerol were loaded on each gel and electrophoresed at a constant current of 2 mA per rod for 4 h. After the run, protein band was stained with 0.04% (w/v) coomassie G-250 dissolved in 6% perchloric acid.

2.7. pH and temperature optima

pH and temperature affects the activity of an enzyme and are of the prime importance when choosing an enzyme for industrial processes. The optimum protease activity of religiosin B was measured at different pH and temperature. The assays were carried at 37 °C, as described above. Below pH 4.0, casein could not be used as substrate due to insolubility. Therefore, haemoglobin was used as substrate below pH 4.0. A control assay at the same pH, without enzyme in reaction mixture, was used as blank. Effect of temperature on the activity of purified enzyme was also investigated and the activity assay was performed at different temperatures.

2.8. Stability

The stability of an enzyme dictates its applicability therefore; the effect of pH (1.0–12.0) and temperature (20–90 °C), as well as in the presence of different concentrations of denaturants, and organic solvents detergents and metal ions on the proteolytic activity of religiosin B was examined. The enzyme was incubated under specified condition of pH, denaturants, 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.

2.9. Effect of various inhibitors on the activity

Effect of different inhibitors on the activity of purified enzyme was studied to classify the protein. Effect of various protease inhibitors (PMSF, chymostatin, IAA, $HgCl_2$, EDTA, EGTA, *o*-phenanthroline) on hydrolysing activity of religiosin B were monitored. Ten micrograms of the enzyme was incubated, with increasing concentration of specific inhibitor (0–50 mM), in 0.05 M Tris–HCl buffer

pH 8.0, for 30 min, at 37 °C and assayed. A control assay was performed without inhibitor, and the activity was considered as 100%.

2.10. Assay for amidolytic activity towards synthetic substrates

Protease activity was determined by measuring *p*-nitroaniline liberation from the chromogenic synthetic peptide substrates, such as Nα-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. In every case, a stock of 20 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 buffer, pH 7.5. The reaction mixture contained approximately 15 μg of enzyme in 0.5 ml of Tris buffer, pH 7.5, and 0.5 ml of peptidyl *p*-NA. After 30 min of incubation at 37 °C, the reaction was terminated by addition of 0.2 ml of 30% (v/v) acetic acid and the liberated *p*-nitroaniline (ε = 8800 $M^{-1}cm^{-1}$ at 410 nm) was monitored by absorbance at 410 nm, against a reaction blank without enzyme. One unit of enzyme activity is defined as, the amount of enzyme that gives rise to an increase in one unit of absorbency at 410 nm per min substrate digestion, under standard assay conditions.

2.11. Kinetic parameters

The effect of increasing substrate concentration on the velocity of the enzyme-catalysed reaction was studied synthetic substrate at pH 8.0 and 37 °C. The concentration of synthetic substrates (L-Leu-*p*NA, L-γ-Glutamyl-*p*NA, L-Ala-*p*NA, L-Ala-Ala-*p*Na) were studied in the range of 0.02–40 mM. Kinetic constants of the purified enzymes were calculated from the product accumulation curves, with molar absorption coefficient for *p*-nitroaniline determined in the reaction buffer for different synthetic peptide substrates. The value of Michaelis constant, K_m was calculated by fitting the values into an excel plot. The value of the catalytic constant (k_{cat}) was obtained by dividing V_{max} by molar concentration of enzyme. The specificity constant was calculated by dividing k_{cat}/K_m.

2.12. Estimation of tryptophan and tyrosine content

Total numbers of the tryptophan and tyrosine residues in the enzyme molecule were measured as described by Sharma et al. (2009). An absorbance spectrum of the purified enzyme in 0.1 M NaOH was recorded, from 300 to 220 nm, and the absorbance values at 280 and 294.4 nm were deduced from the spectra. For calculations, the following formula was used:

$$w = (A_{280} - x.\varepsilon_y)/(\varepsilon_w - \varepsilon_y)$$

where A_{280} is the absorbance at 280 nm from the protein spectra; *w* is the molar concentration of tryptophan; ε_w and ε_y are the molar absorption coefficients of tryptophan and tyrosine in 0.1 M NaOH at 280 nm (ε_w = 5225 and ε_y = 1576), respectively. *x*, the total molar concentration of total tyrosine and tryptophan content, was calculated using $\varepsilon_{294.4}$ = 2375. The number of a particular amino acid residue per molecule of the protein, was calculated from the ratio of the molar concentrations of the amino acid residues, to that of the total protein. To validate the current estimation, papain, ribonuclease and lysozyme were used as standards.

2.13. Estimation of total and free cysteine content

The free and total cysteine residues of the enzyme were estimated by Sharma et al. (2009). 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 first denatured in 6 M GuHCl and then reduced with 0.05 M DTT. The excess reducing agents were removed by dialysis against 0.1 M acetic acid. An aliquot of the dialysed enzyme was added to DTNB solution and the liberated TNB anions were monitored by absorbance at 412 nm. The number of disulphide 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.

2.14. Estimation of molar absorption coefficient

The molar absorption coefficient of the enzyme was determined by spectrophotometric method as described by Pace, Vajdos, Fee, Grimsley, & Gray (1995). The molar absorption coefficient was determined by using the formula,

$$\varepsilon(280) = 5500n_w + 1490n_y + 125n_c$$

where n_w, n_y and n_c are the number of tryptophan, tyrosine, and cysteine residues in the protein, respectively; the molar absorption coefficients of tryptophan, tyrosine and cysteine are 5500, 1490, 125, respectively. The total numbers of tryptophan, tyrosine and cysteine residues in the protein were determined as described above.

2.15. Autolysis

Proteases are prone to autolysis. Autolysis depends upon concentration of enzyme, pH, temperature, and any type of activator, if any (Tomar et al., 2008). Extent of autolysis of the religiosin B was monitored at 37 °C. The enzyme at different concentrations, in the range of 0.01–1.0 mg/ml, was incubated in 50 mM Tris–HCl, pH 8.0 at 37 °C. An aliquot of enzyme was used for the determination of remaining proteolytic activity with casein as substrate. Reaction mixture without enzyme was used as a blank. The activity of the enzyme after the first 2 h was taken to be 100% for calculating the residual activity.

2.16. Milk-clotting activity

Milk-clotting activity was determined according to the methods described by Arima, Ya, & Iwasaki (1970) with a slight modification. The substrate (10% skim milk, w/v in 0.01 M $CaCl_2$) was prepared and the pH was adjusted to 6.0. The substrate (2.0 ml) was pre-incubated for 5 min, at 37 °C, and 0.2 ml of enzyme was added, and the curd formation was observed at 37 °C, while manually rotating the test tube from time to time. The end point was recorded when discrete particles were discernible. One milk-clotting unit is defined as the amount of enzyme that clots 10 ml of the substrate within 40 min.

$$\text{MCA(U/ml)} = (2400/\text{clotting time in sec}) \times \text{dilution factor}$$

2.17. Effect of $CaCl_2$ concentration on MCA of the enzyme

The effect of $CaCl_2$ on the milk-clotting activity of enzyme was determined. The substrate (1% casein, w/v) was equilibrated at different concentrations of $CaCl_2$ (0–0.8 M), for 5 min, at 30 °C. After that the enzyme (0.15 ml) was added and further incubated for 30 min at the same temperature. Casein hydrolysing activity was determined and expressed as a percentage of the activity measured in the absence of the salt.

2.18. de novo sequencing by MALDI TOF/TOF

Tryptic digestion of religiosin B was done using the protocol as described by Kumari et al. (2010), 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% (v/v) TFA and 50% (v/v) acetonitrile) and spotted on 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).

3. Results and discussion

3.1. Purification of enzyme

A novel serine protease is purified to homogeneity from the latex of *F. religiosa* using cation exchange chromatography. The protein elution profile resolves into four peaks (Fig. 1) and majority of the total proteolytic activity is observed only in peak II. The ascending limb of peak II (fractions 105–145) was homogeneous and resulted in a single band by SDS–PAGE. The homogenous fractions were pooled, dialysed and stored for further use. The enzyme is purified up to 2.5-fold with 34% yield and a specific activity of 69 U/mg. The purification protocol is highly reproducible in terms of purification fold, yield as well as specific activity. The purified protein was named as religiosin B according to the nomenclature of proteases. A simple purification procedure was developed in this study to obtain a very active and stable enzyme from the latex of *F. religiosa*.

3.2. Homogeneity and physico-chemical properties

Homogeneity and intactness of the protein are judged by SDS–PAGE under reducing and non-reducing conditions. A single band by SDS–PAGE suggests highly purified and monomeric nature of the enzyme. The relative molecular mass (M_r) of the purified enzyme is 63,000 by both MALDI–TOF and SDS–PAGE (Fig. 2A and B). The molecular mass of the enzyme is similar to that of the well-known plant serine protease cucumisin (Yamagata, Ueno, & Iwasaki, 1989), and with other cucumisin-like serine proteases (Asif-Ullah, Kim, & Yu, 2006; Uchikoba, Yonezawa, & Kaneda, 1998). The molecular mass of the purified enzyme was also similar to those of subtilisin-like endoproteases from the tomato plant (Messdaghi & Dietz, 2000). The proteolytic nature of religiosin B was also confirmed by gelatin zymography, where digested gelatine appeared as well-resolved band against a dark background, corresponding to the position of the protein in gel (Fig. 2C). Although the zymogram gel contained 0.1% SDS (w/v) and the sample was treated with 1% SDS (w/v), the enzyme still displayed activity, indicating that it was resistance to SDS denaturing. As recently reported, SDS resistance is a property often associated with heat-stable proteases of thermo-stable archaea and bacteria (Joo & Change, 2005). SDS resistance is the most striking property of the purified enzyme among all reported plant serine proteases, except for cucumisin (Yamagata et al., 1989). As shown in fig. 2D, the purified enzyme was apparently resolved as a single band on isoelectric focusing with an isoelectric point (p*I*) of pH 7.6 indicating that it is a weakly basic protein. Noda, Koyanagi, & Kamyia (1994) found similar results for plant serine protease from *Cucumis melo* L. with a basic isoelectric point (p*I*) of 9.5. In contrast, most of the recently isolated serine proteases from plants have isoelectric points in the range of pH 4.0–7.0 (Tomar et al., 2008). Religiosin B was found to contain no detectable carbohydrate moiety in the molecular architecture, as majority of the reported plant serine proteases are glycoproteins, as shown in table 1. Moreover, the previously reported serine protease from the same source by Kumari et al. (2010), is highly glycosylated with 12% of carbohydrate moiety in its protein architecture. The different physicochemical properties of the enzyme are compared with the other serine proteases as shown in table 2.

3.3. Effect of pH and temperature on the activity of religiosin B

The proteolytic activity of religiosin B was monitored at different pH values from 1.0 to 12.0 and temperatures in the range of

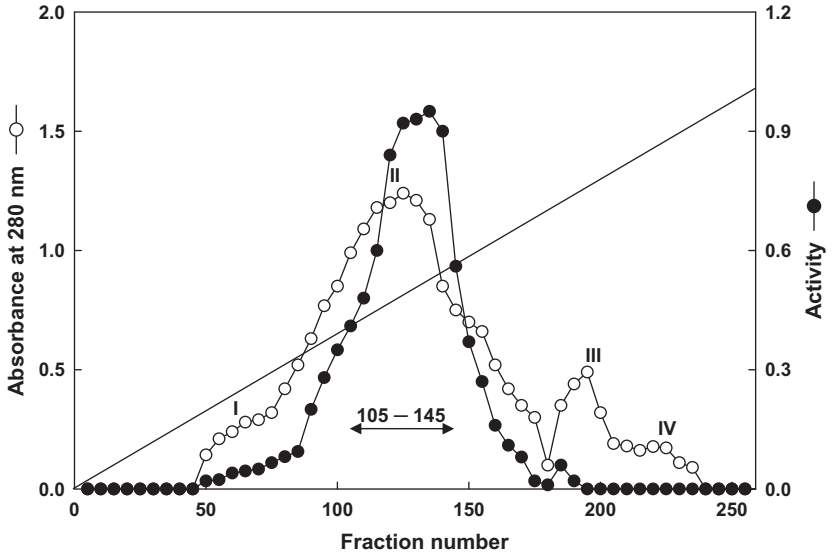

Fig. 1. Elution of the crude latex on cation exchange chromatography. SP-Sepharose fast flow, pre-equilibrated with 10 mM acetate buffer, pH 4.5. The bound proteins were eluted with a linear salt gradient of 0–1 M NaCl. Fractions of 4 ml, at a flow rate of 4 ml/min collected and assayed for the protein concentration (○) and the proteolytic activity (●). The fractions of ascending limb of pool II (105–145) were pooled, as indicated by a horizontal line.

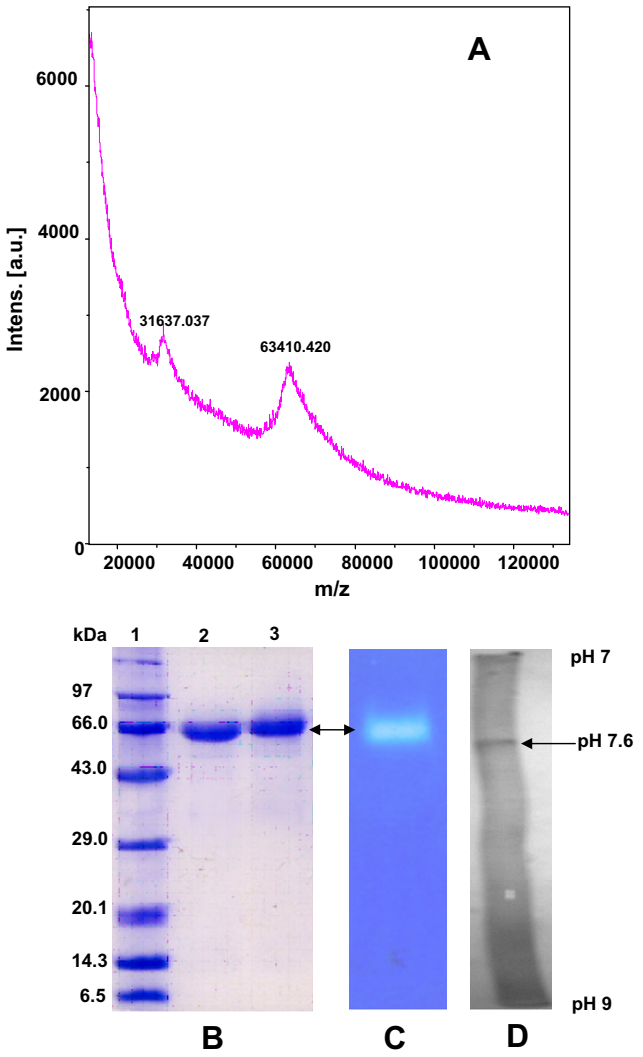

Fig. 2. Biochemical and physico-chemical properties of religiosin B. (A) MALDI–TOF analysis of religiosin B. (B) Assessment of homogeneity and relative molecular mass (M_r) of the enzyme by 15% SDS–PAGE. Gel electrophorized lanes 1–3 represent: marker, religiosin B (30 μg) under nonreducing and reducing conditions, respectively. (C) Zymogram (in-gel activity) of religiosin B. The unstained region in the gel (indicated by double head arrow) showed the hydrolysis of gelatin by the enzyme (D) Isoelectric focusing was performed by 5% polyacrylamide disc gel electrophoresis with ampholine carrier ampholyte, pH 7.0–9.0, at constant current of 5 mA. The isoelectric point of the purified protein is indicated by an arrow.

20–90 °C. Religiosin B acted optimally at pH 8 and 55 °C. Moreover, Religiosin B shows more than 80% of proteolytic activity in the pH and temperature range of pH 7.0–9.5 and 50–60 °C, respectively.

3.4. Effect of pH and temperature on the stability of religiosin B

The enzyme retains more than 80% of the activity from pH 5.5–11.0 and in the temperature range of 20–75 °C (Fig. 3A and B) and its stability is more comparable to other cucumisin-like serine proteases from *Cucumis trigonus* Roxburghi, *Cucumis melo* L. var. Prince and *Trichosantus kirrilowi* A. (Asif-Ullah et al., 2006; Uchikoba, Horita, & Kaneda, 1990; Yamagata et al., 1989). The thermostability of the enzyme was found to be 75 °C. The temperature profile of the purified enzyme was similar to those of subtilisin/cucumisin like plant serine proteases from *C. trigonus* Roxburghi, *C. melo* L. var. Prince, and *T. kirrilowi* A. (Asif-Ullah et al., 2006; Uchikoba et al., 1990; Yamagata et al., 1989).

3.5. Effect of denaturants and organic solvents on the activity of religiosin B

As shown in table 2, the purified enzyme exhibited remarkable stability under various conditions. Religiosin B retains full activity at higher concentrations of denaturants, up to 3.0 M GuHCl and 8 M urea. The enzyme also shows an increase in the proteolytic activity at lower concentrations of GuHCl up to 2.0 M. The increase in proteolytic activity of the enzyme is about twofold at 2.0–2.5 M GuHCl, which is not found when the enzyme is treated with urea. The enhanced activity might be correlated with high structural integrity of the active site of the enzyme in the presence of the denaturants, as reported for religiosin earlier by Kumari et al. (2010). The enzyme retains its complete activity in 50% (v/v) methanol, where as in 50%, v/v (ethanol, butanol, acetonitrile, dioxane and DMSO) the residual activity observed was more than 50%. Thus the enzyme is fairly stable at high temperatures, at a broad range of pH values, and at low concentrations of denaturant, where as organic solvent affects the activity of enzyme considerably. Such contrary observations of stability may be a distinct feature of this enzyme. The high stability of the enzyme against pH and temperature along with the high stability under mild denaturing conditions facilitates the possibility of utilisation of the enzyme in industrial and biotechnological applications.

3.6. Effect of inhibitors and metal ions on activity of religiosin B

Effect of inhibitors on the activity of the enzyme is shown in table 2. The proteolytic activity was inhibited considerably by the PMSF and chymostatin. Compared to other protease class inhibitors this indicates that the enzyme belongs to the class of serine proteases. However, the enzyme activity was inhibited by 20% from $HgCl_2$ (a cysteine protease inhibitor) and *o*-phenanthroline (a metalloprotease inhibitor), shows a distinct inhibition profile for religiosin B, with a cysteine residue near its active site and containing a metal in the protein architecture. Although, the complete structure of protein should be necessary for better understanding of the active site and catalysis of the enzyme. More inhibition studies can provide an insight into the nature of the enzyme, its cofactor requirement, and the nature of the active centre (Sedmak & Grossberg, 1977). It is noticeable that a proteinaceous inhibitor, such as soybean trypsin inhibitor (SBTI), which is present in protein rich foods like soybeans, fails to inhibit the enzyme up to 5 mM, thus paving the way for its industrial usage. Generally, SBTI inhibits the protease activity of animal and bacterial serine proteases, but fails to do so in the case of plant proteases (Sharma et al., 2009). Other inhibitors of cysteine protease ($HgCl_2$, IAA, DTT) and metalloprotease (EDTA, EGTA) did not affect the enzymatic activity significantly up to the concentration of 5 mM.

Metal ions, the monovalent cations (K^+, Rb^+, and Li^+), and the divalent cations (Ca^{2+}, Mg^{2+}) do not show any considerable inhibitory effect on the activity of religiosin B up to 10 mM, shown in table 2. However, the activity was inhibited by some heavy divalent cations such as Hg^{2+}, Co^{2+}, Zn^{2+}, and Cu^{2+}. The high stability of religiosin B, without perturbing the enzymatic activity by various agents, could make it beneficial to work under various conditions.

3.7. Substrate specificity

The enzyme hydrolyses denatured natural substrates such as casein and haemoglobin. Religiosin B also exhibits significant amidolytic activity against synthetic substrates such as, L-alanine-*p*-nitroanilide, L-alanine-alanine-*p*-nitroanilide, L-γ-glutamyl-*p*-nitroanilide and L-leucine-*p*-nitroanilide, while fails to hydrolyse *N*α-benzoyl-DL-arginine-*p*-nitroanilide, *N*-succinyl-L-phenylalanine-*p*-nitroanilide. The results indicated that the purified protease

Table 1
Comparison of biochemical and physico-chemical properties of religiosin B with other selected serine proteases.

Enzyme (Source)	Mol Mass (kDa)	Optimum		Stability		pI	Trp	Tyr	Cys	$\varepsilon^{1\%}_{280}$	Glycosylation(%)
		pH	Temp (°C)	pH	Temp (°C)						
Religiosin B (*F. religiosa*)	66	8–8.5	55	5.5–11	20–70	7.6	23	15	7	23.80	no
Dubiumin (*S. dubium*)[a]	66	11	70	3–12	60–70	9.3	NR	NR	NR	NR	yes
Religiosin (*F. religiosa*)[b]	43.3	8–8.5	60	5.5–10	20–65	3.8	16	26	11	29.47	12
Streblin (*S. asper*)[c]	64	9	65	3–12.5	15–85	9.2	3	7	7	5.3	9.7
Cucumisin (*C. melo*)[d]	54	7.1	70	4–11	50	NR	NR	NR	NR	NR	yes
Benghalensin (*F. benghalensis*)[e]	47	8	55	5.5–10	20–80	4.4	17	31	9	29.25	10–12
Wrightin (*W. tinctoria*)[f]	57.9	7.5–10	70	5.5–11.5	75–80	6	20	75	9	36.4	8

NR in the table represents data not reported.
[a] Ahmed et al. (2009a, 2009b).
[b] Kumari et al. (2010).
[c] Tripathi et al. (2011).
[d] Yagmata et al. (1989).
[e] Sharma et al. (2009).
[f] Tomar et al. (2008).

Table 2
Stability of religiosin B under different conditions.

Condition	Concentration	Residual activity (%)
pH (6.0–10.5)		>90
Temperature (20–70 °C)		>90
GuHCl	3 M	100.12 ± 0.14
Urea	8 M	100.02 ± 0.11
Methanol	50% (v/v)	100.04 ± 0.15
Ethanol	50% (v/v)	60.09 ± 0.18
Acetonitrile	50% (v/v)	64.03 ± 0.08
Butanol	50% (v/v)	57.24 ± 0.15
Dioxane	50% (v/v)	55.35 ± 0.22
DMSO	50% (v/v)	70.44 ± 0.12
PMSF	1 mM	8.55 ± 0.17
Chymostatin	2 mM	12.11 ± 0.15
SBTI	5 mM	95.02 ± 0.35
$HgCl_2$	5 mM	80.21 ± 0.06
IAA	5 mM	100.02 ± 0.35
DTT	5 mM	100.05 ± 0.24
EDTA	5 mM	100.20 ± 0.17
EGTA	5 mM	100.10 ± 0.12
o-phenanthroline	5 mM	80.09 ± 0.28
Metal ions (Na^+, K^+, Mg^{2+} and Ca^{2+})	10 mM	100.01 ± 0.01

Residual activities shown in the table as mean ± SD ($n = 3$).

preferred both hydrophilic and hydrophobic amino acid residues at the P1 position, whereas, the activity of enzyme over bulky aromatic groups at position P1 is not detectable. Thus, the specificity of religiosin B differs from that of cucumisin, a well known and characterised serine protease from the latex of *C. melo* by Arima, Yonezawa, Uchikoba, Shimada, & Kaneda (2000). The preference for the hydrophobic residue at the P1 position of this enzyme was comparable to that of chymotrypsin, and subtilisin. Moreover, aliphatic neutral residues (e.g., Ala) are preferred at the P2 site as seen also for the other proteases (Yamagata et al., 1989).

3.8. Kinetic parameters

The K_m values for the enzyme were estimated to be 0.04, 0.05, 1.32 and 0.93 mM against Ala-*p*NA, Ala-Ala-*p*NA, γ-glutamyl-*p*NA and Leu-*p*NA, respectively. The K_m value of the enzyme, with Ala at P1 position, was higher than with Leu and Glu at the same position. These results indicated that the enzyme preferred a small and non-polar residue at the P1 position to a charged residue at the same position. One of the notable aspects of serine proteases is their wide diversity of substrate specificities, coupled to a single catalytic mechanism (Ahmed et al., 2009a, 2009b). The kinetic parameters and specificity constant (k_{cat}/K_m) values of religiosin B with various synthetic substrates are shown in table 3.

3.9. Estimation of amino acid contents and molar absorption coefficient

The tryptophan and tyrosine contents of the protein are 23 (measured value 23.16 ± 0.03) and 15 (measured value 15.07 ± 0.04), respectively. The total cysteine content is found to be 7 (measured value 6.89 ± 0.11) with one free cysteine (measured value 1.22 ± 0.05) and six cysteine forming three disulphide bridges. The molar absorption coefficient of religiosin B, measured by spectrophotometric method is 149,725 $M^{-1}cm^{-1}$.

3.10. Autolysis

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. The loss of activity of religiosin B in the concentration range 0.01–1.0 mg/ml, under neutral conditions at room temperature was studied. An aliquot containing 10 μg of enzyme was used to measure the proteolytic activity after 24 h, 48 h and 72 h of incubation. The magnitude of loss of activity decreases with an increase in the enzyme concentration from 0.01–0.30 mg/ml and no loss of activity was observed at higher concentrations. Religiosin B retains more than 90% of activity, even at very low concentration up to 0.05 mg/ml, after 72 h of incubation. The result indicates that religiosin B shows high resistant to autolysis. The similar results have also been reported for other plant serine proteases like streblin and dubiumin (Tripathi, Tomar, & Jagannadham, 2011; Ahmed et al., 2009b). In contrast, many proteases studied undergo autolysis at low concentrations, such as benghalensin, wrightin, procerain and ervatamin A (Dubey & Jagannadham, 2003; Nallamsetty et al., 2003; Sharma et al., 2009; Tomar et al., 2008). The lower susceptibility to autodigestion at very low concentrations, in turn, indicates the possible use of religiosin B in food, textile, and other biotechnological industries. In our experience, the enzyme is stable for four months, at 4 °C, under neutral conditions, without loss in activity.

3.11. Effect of $CaCl_2$ on milk-clotting activity

Calcium has been described as an important substance in clot formation during milk clotting, which happens when its concentration is high enough (Anema, Lee, & Klostermeyer, 2005). Calcium helps coagulation by creating isoeletric conditions and by acting as a bridge between casein micelles. Milk-clotting activity of religiosin B was highest at 0.2–0.3 M of $CaCl_2$. It is known that calcium has important function on casein aggregation during the second

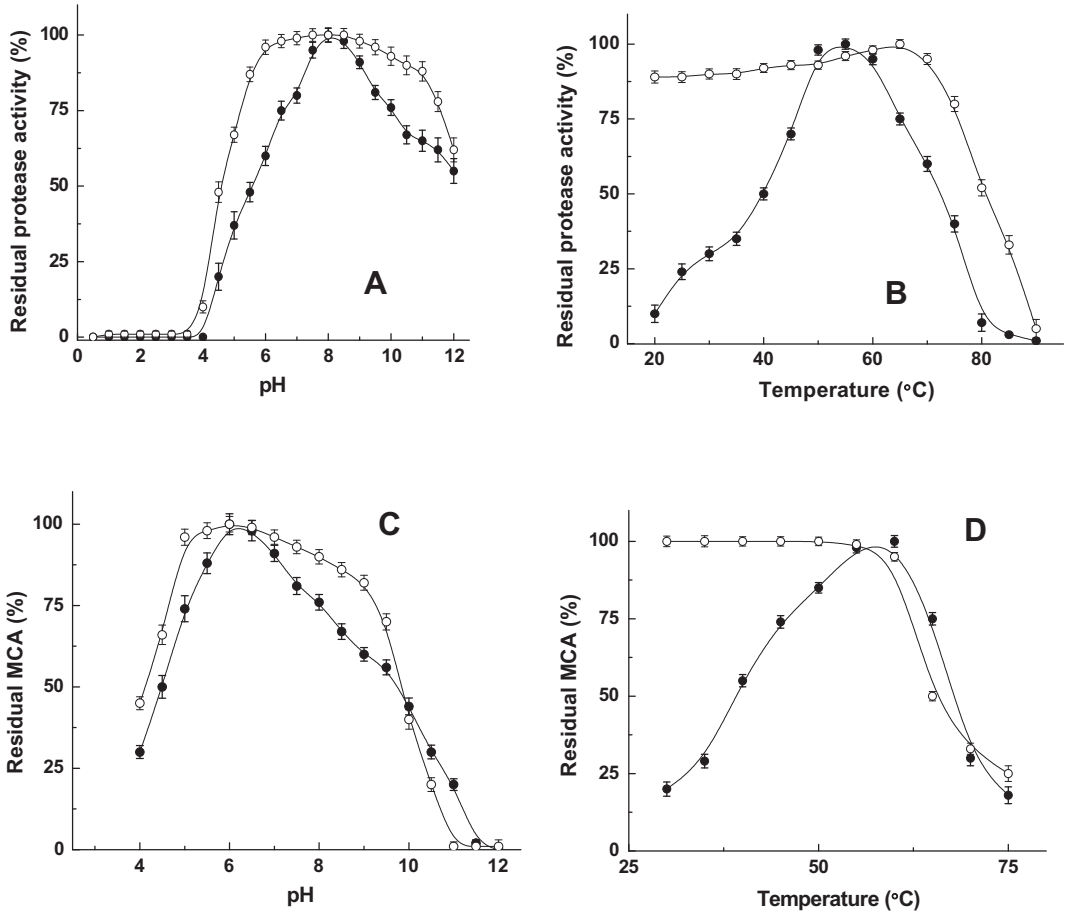

Fig. 3. Effect of pH (A) and temperature (B) on the proteolytic activity (●) and stability (○) of religiosin B. Likewise, effect of pH (C) and temperature (D) on the milk-clotting activity (●) and stability (○) of religiosin B. The assay protocols are described in the material and method section. Each value in all figures represented as mean ± SD (n = 3).

Table 3
Kinetic parameters of religiosin B with different synthetic substrates.

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
Leu-*p*NA	0.928 ± 0.005	64.81 ± 1.30	69.85 ± 3.24
Ala-*p*NA	0.038 ± 0.001	44.21 ± 2.42	1163.42 ± 34.66
Ala-Ala-*p*NA	0.052 ± 0.002	43.64 ± 1.89	837.76 ± 26.71
γ-Glu-*p*NA	1.320 ± 0.020	64.80 ± 2.10	49.05 ± 4.13

All values in the table are represented as mean ± SD (n = 3).

step (non-enzymatic) of milk clotting, caused by the neutralisation of casein micelles' negative residues (phosphoserine and carboxylic groups) by Ca^{2+} and calcium–phosphate complexes (Pires, Orellana, & Gatti, 1999) and so that an increase in its concentration leads to an increase in the coagulation rate (Arima et al., 1970; Kumar, Sharma, Saharan, & Singh, 2005). Milk-clotting activity of religiosin B decreased at concentrations higher than 0.3 M, probably due to the increase of ionic force or to the saturation of negative residues, of the micelles at increasing Ca^{2+} concentration in the medium. A similar behaviour was reported by Ahmed, Babikar, & Mori (2010) and Preetha and Boopathy (1997), where the purified proteases showed the maximum milk-clotting activity in the presence of 0.2 and 0.4 M of $CaCl_2$, respectively.

3.12. Milk coagulation

The enzyme coagulates skimmed milk and forms a white and firm curd. Moreover, the ratio of milk-clotting activity to proteolytic activity of religiosin B is determined to be 803.22 ± 23.67 U/OD 660 nm, comparable to those of (387 ± 12.67, 4989 ± 109.771, 367 ± 5.90, 3.6 ± 0.025, and 393 ± 6.12 U/OD 660 nm) religiosin, rennin, papain, trypain and ficin, respectively (Kumari et al. 2010). The ratio of milk-clotting activity to proteolytic activity is a useful indicator of the protease efficiency to be used as a coagulant for cheese making (Arima et al., 1970). The capacity of religiosin B to produce milk curds together with its high ratio of milk clotting to proteolytic activity, could make it useful as a new milk coagulants, although, more studies about quality of both milk curds and the cheese formed should be carried out in the future to confirm its usefulness in the dairy industry.

3.13. Effect of pH and temperature on milk-clotting activity of religiosin B

Highest milk-clotting activity of religiosin B was observed at pH 6.0 and temperature 55–60 °C (Fig. 3C and D). Similar behaviour of optimum pH was reported for the crude extract from *Mucor pusillus* var. Lindt (Arima et al., 1970) and the precipitated extract from *Penicillium citrinum* Abdel-Fattah, Mabrouk, & El-Hawwary, 1972), at pH 5.5 and pH 6.0, respectively. Calf rennet exhibits the same dependence towards pH, being weaker in alkaline conditions than in acidic conditions (Richardson, Nelson, Lubnow, & Schwarberg, 1967). The similar temperature optimum (60 °C) was observed in the case of the crude enzymatic extract from *Penicillium oxalicum* (Hashem, 1999), the precipitated extract from *P. citrinum* (Abdel-Fattah et al., 1972), and the purified protease from *Rhizopus oryzae* (Kumar et al., 2005).

The enzyme remains stable and shows more than 80% milk-clotting activity in the range of pH 5–9 and temperature from 30–60 °C. On both sides of the pH range, the stability decreases and at pH 10 the activity reduces to 40%. Similarly at 65 °C the stability of the enzyme reduces to 50% only. These observations confirm the high stability of the enzyme over a broad range of temperature and pH. In this regard, the isolated enzyme is unique, and therefore, might be suitable for uses in industry under alkaline conditions. These characteristics are important, because most enzymes are catalytically unstable at alkaline pH values, thus limiting their usefulness in the food industry especially as cheese-making coagulants (Lamas, Barros, Balcao, & Malcata, 2001). An exception to this general rule is represented by the aqueous extract and aspartic proteases from the flower of *Cynara cardunculus*, which have been employed successfully for the manufacture of traditional cheeses from ovine and caprine milk (Sousa & Malcata, 2002).The stability of proteins and enzymes is usually a factor that limits their usefulness in many applications.

3.14. de novo sequencing by MALDI TOF/TOF

Attempts were made to identify the protein by peptide mass finger printing. For this, religiosin B was digested enzymatically that resolved into a number of peptides. The masses of the peptides were determined and searched against relevant databases in NCBI. No significant hits were obtained based on peptide masses. Subsequently, the different peptide fragments of religiosin B generated above and with good resolution were subjected to de novo sequencing by MALDI–TOF/TOF. The TOF/TOF spectra of representative peptides and their *de novo* sequence were KLVMWSGMR, KGMGPWPGSAR, KFGGPVQLQW, DPFFEHPSADVR and CHSVVPR. Due to the low intensity and weak MS/MS spectra we could get the *de novo* sequencing of only five peptides. The low signal intensity may be because of improper ionisation and fragmentation of the peptides. These sequences when submitted to BLAST search in NCBI database, with different variables, show the uniqueness of the protein sequence. No putative conserved domains have been detected using these sequences as search query. However, good scores were obtained with similarities to cucumisin like serine proteases confirms that the purified protease is a new serine protease.

4. Conclusion

The present study describes the purification and characterisation of a novel serine protease, named religiosin B, from the latex of a valuable medicinal plant, *F. religiosa*. We have optimised a simple and economic purification procedure, combined with the availability of the plant latex, could possibly be used for large-scale production of the enzyme. The enzyme is stable in the presence of denaturants, organic solvents, and metal ions as well as over a wide range of temperature and pH; therefore this protease may turn out to be an efficient choice in food, pharmaceutical, and biotechnological industries. The enzyme is resistant to autolysis and can be stored at low temperature for long time, without loss of activity. Besides, high milk-clotting activity of the enzyme could pave the way for its use as a potential cheese making enzyme from a vegetal source in the cheese industry, as well as other food and biotechnological industries.

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