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# Religiosin C, a cucumisin-like serine protease from *Ficus religiosa*

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ABSTRACT

A serine protease was purified to homogeneity from the latex of *Ficus religiosa*. The enzyme, named religiosin C is a monomer with molecular mass of 80 kDa. The enzymatic activity of the protein was inhibited by serine protease inhibitors. Isoelectric point of the enzyme is pH 4.6 with optimum pH and temperature of pH 6–8 and 45–50 °C, respectively. The specific extinction coefficient ($\varepsilon_{280}^{1\%}$) of the enzyme is 14.68 with 16 tryptophan, 20 tyrosine and 7 cysteine residues in its molecular structure. The enzyme shows broad substrate specificity and hydrolyzes both natural and synthetic substrates. The enzyme is highly stable over a broad range of pH and temperature as well as in the presence of high concentration of chemical denaturants, organic solvents and metal ions. The N-terminal residues of religiosin C exhibited considerable homology with cucumisin and other cucumisin/subtilisin-like serine proteases. The high milk-clotting ability of religiosin C supports its probable use in the food and other biotechnological industries.



## 1. Introduction

Proteolytic enzymes are ubiquitous in biological systems and play numerous cellular and extracellular processes. Serine proteases are one of the largest groups of proteolytic enzymes involved in several biological processes. In plants, they are widely distributed among different taxonomic groups and involved in physiological processes such as protein degradation and processing, microsporogenesis, symbiosis, hypersensitive response, signal transduction and differentiation, and senescence. Despite being their prevalence, the functions and regulatory roles of plant serine proteases are poorly understood, probably due to a lack of identification of their physiological substrates [1].

Serine proteases are grouped into six classes, and the second largest class being the subtilisins [2]. Nearly, all the members of the subtilisins are tripeptidylpeptidases or endopeptidases and best characterized in microorganisms. However, very little information is available about the features of the plant subtilisins, also referred as cucumisin-like proteases. Cucumisin from melon fruit (*Cucumis melo*) was the first plant serine protease characterized to date [3,4]. Subsequently, more cucumisin-like proteases were isolated from other plants, such as *Taraxacum officinale* Webb, *Euphorbia supine* and *Benincasa hispida* var. Ryukyu, and characterized for their broad substrate specificity and optimum temperature and pH [5].

Plant lattices are valuable reservoir of biomolecules including organic and inorganic compounds, waxy materials, and enzymes such as proteases. These extracellular proteases have been shown to play important defensive roles against herbivores, insects, and pests. Besides, plant latex could be a potential cost-effective natural source of enzymes in terms of easy purification methods, low levels of interfering substances during purification, and good yield. Moreover, the substantial uses of plant-derived proteases in food and biotechnological industries have been well documented. These proteases are found to be suitable under working conditions of different industries due to their broad substrate specificity, high stability in extreme conditions, good solubility, and activity over a wide range of pH and temperature. Some cysteine proteases such as papain, bromelain, ficin, and calotropins are utilized widely in several processes in the food and dairy industries. In addition, several plant serine proteases have been studied for their medicinal and industrial applications. Their enzymatic activity over a wide range of natural protein substrates is one of the factors which have made them suitable for commercial applications in industries. Cucumisin, a plant serine protease, could be an example of milk-clotting enzyme similar to papain, but also produced less bitter cheese than those formed by the cysteine proteases [6]. One of the most important applications of proteases in the

*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 sulfate; TCA, trichloroacetic acid; TEMED, N,N,N,N-tetramethylethylenediamine; β-ME, β-mercaptoethanol.

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food industry is the use of rennet in cheese formation. Numerous attempts have been made to find out a suitable alternative of calf rennet because of its limited supply, ethical issues and increasingly high prices. In this direction, attention has been drawn to enzymes from plant sources for the production of cheese and other food products. Recently, many proteases have been characterized from the different parts of the plants and their uses in food industries have been publicized. Milk-clotting enzymes have been found in almost all kinds of plant tissues. Therefore, the search for new potential plant proteases still continues in order to make them industrially applicable and cost effective [7].

Multiple proteases of the same family are quite often reported in latex bearing plants. However, the cause of such multiplicity of proteases has not been extensively highlighted. These multiple proteases from the same source show different behaviors in terms of stability, activity and specificity [8]. Such reports about the multiplicity of proteases have prompted further screening of the latex of *Ficus religiosa* and during the process one more milk-clotting serine protease has been identified which is more active than other proteases of the same source. The other proteases namely religiosin and religiosin B have already been characterized for their milk-clotting activity [9,10]. Also, all the three enzymes show distinct features from one other as well as from other well-known serine proteases in various terms. In this respect it is essential to pursue studies on 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, hemoglobin, 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 at 4 °C unless stated otherwise.

*Step 1. Gum removal*

Fresh latex was collected from the stem by making longitudinal incisions in to 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.

*Step 2. Ammonium sulfate precipitation*

The crude latex (devoid of gum and any insoluble material) was subjected to 70% ammonium sulfate precipitation. The clear supernatant after ammonium sulfate precipitation showed good amount of proteolytic activity and was used in the next step of purification.

*Step 3. Anion exchange chromatography*

The supernatant from previous step was subjected to anion exchange chromatography on DEAE-sepharose fast flow in a column pre-equilibrated with 0.01 M Tris buffer, pH 8.5. 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 0.6 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.

*Step 4. Gel-filtration chromatography*

Active fractions from previous column were subjected to gel filtration chromatography on superdex-200 pre-equilibrated with 0.01 M Tris buffer, pH 8.5 containing 0.2 M NaCl and the column was eluted isocratically. All the fractions were analyzed as in the above step. The active and homogenous fractions were pooled, dialysed and stored at 4 °C for further experiments.

### 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 [11].

### 2.4. Protease assay

The proteolytic activity of the enzyme during purification was monitored using natural substrates casein and hemoglobin. For the assay, 10 μg of enzyme in 0.5 ml of 0.05 M Tris–HCl buffer, pH 8.0 was added to 0.05 ml of 1% 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% 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 absorbency 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 15% SDS-PAGE under reducing and non-reducing conditions. After electrophoresis, proteins in the gel were stained by coomassie R-250. Gelatin zymography was performed to confirm the proteolytic activity of religiosin C in the gel using the protocol of Tomar et al. [7]. 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 Tomar et al. [7]. Electrophoretic run was carried out with ampholine carrier ampholytes in the pH range 4–6 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.

### 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 C 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 use as substrate due to its insolubility. Therefore, hemoglobin was used as substrate below pH 4.0. A control assay at 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 in the presence of different concentration of denaturants, and organic solvents detergents and metal on the proteolytic activity of religiosin C 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 hydrolyzing activity of religiosin C was monitored. Ten micrograms of 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–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 pNA. After 30 min of incubation at 37 °C, the reaction was terminated by 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 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-catalyzed reaction was studied synthetic substrate at pH 8.0 and 37 °C. The concentration of synthetic substrates (L-Leu-pNA, L-γ-glutamyl-pNA, L-Ala-pNA, L-Ala-Ala-pNa) was studied in the range of 0.001–40 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 in 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. [12]. 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:

$$w = \frac{A_{280} - x \cdot \varepsilon_y}{\varepsilon_w - \varepsilon_y}$$

where $A_{280}$ is the absorbance at 280 nm from the protein spectra; $w$ is the molar concentration of tryptophan; $\varepsilon_w$ and $\varepsilon_y$ are the molar extinction coefficients of tryptophan and tyrosine in 0.1 M NaOH at 280 nm ($\varepsilon_w$ = 5225 and $\varepsilon_y$ = 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. [12]. For the free cysteine content estimation, the enzyme was reduced with 0.01 M β-ME, whereas, for the 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 dialyzed enzyme was added to 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.

### 2.14. Estimation of specific extinction coefficient

The extinction coefficient of the enzyme was determined by spectrophotometric method as described by Tomar et al. [7]. The specific extinction coefficient was determined by using the formula,

$$\varepsilon_{280}^{1\%} = \frac{10(5690n_w + 1280n_y + 120n_c)}{M}$$

where $n_w$, $n_y$, and $n_c$ are the number of tryptophan, tyrosine, and cysteine residues in the protein, respectively; $M$ is the molecular mass of the protein; 5690, 1280, and 120 are the extinction coefficients of tryptophan, tyrosine and cysteine, 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. Extent of autolysis of the religiosin C 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. N-terminal sequencing

The protein sample for sequencing was electrophoresed according to the procedure given by Matsudaria [13] and transferred to PVDF membrane. The N-terminal sequence was determined an Applied Biosystems ABI 470 protein sequencer.

### 2.17. Milk-clotting activity

Milk-clotting activity was determined according to the methods described by Arima et al. [14] with a slight modification. The substrate (10% skim milk 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.

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

## 3. Results and discussion

### 3.1. Protein purification

A new serine protease was purified to homogeneity from the latex of *F. religiosa* by combination of procedure using ammonium sulfate precipitation, anion exchange and gel filtration chromatography. The crude latex (devoid of gum and any insoluble material) was subjected to 70% ammonium sulfate precipitation. The clear supernatant after ammonium sulfate precipitation showed good amount of proteolytic activity and was used in the next step of purification. The supernatant was applied to DEAE-sepharose fast flow column pre-equilibrated with 10 mM Tris buffer, pH 8.5. The bound proteins were eluted with a linear salt gradient of 0–0.6 M NaCl. The column elution profile resolved in to two peaks and denoted as peaks I and II as shown in Fig. 1A. Fractions of both the peaks were assayed for proteolytic activity and subjected to SDS-PAGE to check the purity.

The magnitude of activity as well as purity of the fractions of peak I was higher than the fractions of peak II. Therefore, the fractions of ascending limb of pool I (125–160) were pooled and subjected to further purification to gel filtration chromatography on Superdex-200. The elution profile constitutes of a major symmetrical peak followed by a small peak as shown in Fig. 1B. The active and homogenous fractions of the former peak were pooled, concentrated and dialyzed for further use. The purification fold of the purified protein is 2.49 with 11% yield and specific activity of 55 U/mg. The purified protein is named as religiosin C according to protease nomenclature.

The purification result of religiosin C is summarized in Table 1. The purification protocol is simple, highly reproducible with the consistent yield and specific activity of the enzyme.

### 3.2. Homogeneity and physical properties of purified protease

Religiosin C showed a single band when assayed by SDS-PAGE under both reducing and non-reducing conditions with estimated molecular mass of 80 kDa (Fig. 2A). The mobility pattern of the enzyme by SDS-PAGE indicates that the protein is a monomer and consists of single subunit of 80 kDa. The molecular mass of the purified enzyme is similar to those of cucumisin-like serine proteases from *Cucumis metuliferus*, and *Pleioblastus hindsii*, while different from the other proteases of the same source [9,10,15,16]. The molecular masses of plant serine proteases vary from 19 to 110 kDa where majority of proteases fall in the range of 60–80 kDa [1].

Gelatin zymography confirmed the proteolytic nature of the protein, where digested gelatine appeared as well-resolved white band against a dark background, corresponding to the position of the enzyme in gel (Fig. 2B). Although the zymogram gel contained 0.1% SDS and the sample was treated with 1% SDS, 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

**Table 1**
Purification of religiosin C.

| Steps | Total protein (mg) | Total activity[a] (units) | Specific activity (units/mg) | Purification fold | Yield (%) |
|---|---|---|---|---|---|
| Crude latex | 150 | 3599.97 | 23.99 | 1.00 | 100 |
| $(NH_4)_2SO_4$ supernatant (70%) | 30.22 | 900.43 | 29.79 | 1.24 | 25 |
| DEAE-sepharose | 15.1 | 605.40 | 40.09 | 1.67 | 16.8 |
| Superdex-200 | 12 | 400.01 | 55.01 | 2.29 | 11.1 |

[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.

bacteria [17]. SDS resistance is the most striking property of purified enzyme among all reported plant serine proteases except for cucumisin [18].

The isoelectric point (p*I*) of the purified enzyme was pH 4.6 which confirms the acidic nature of the protein (Fig. 2C). Most of the recently isolated serine proteases from plants have isoelectric points in the range of pH 4.0–7.0 [12]. However, serine protease from melon fruit was reported to have a highly basic isoelectric point [19].

The result of Schiff's staining confirms that there is no detectable carbohydrate moiety in the molecular architecture of the protein, as majority of the reported plant serine proteases are glycoproteins [1]. However, one of the previously reported serine proteases from the same source is highly glycosylated with 12% of carbohydrate moiety while the other has no detectable glycosylation in their protein architecture [9,10]. The different biochemical properties of the enzyme are compared with the other serine proteases as shown in Table 2. A single band obtained by SDS-PAGE and isoelectric focusing demonstrates the high purity of the enzyme.

### 3.3. Effect of pH on the activity and stability of religiosin C

The proteolytic activity of religiosin C was monitored at different pH from 1.0 to 12.0. Religiosin C acted optimally and shows 100% activity from pH 6 to 8 (Fig. 3A). Moreover, religiosin C shows more than 80% and 50% of proteolytic activity in the pH range of pH 5.5–8.5 and pH 4–10, respectively. The above observations suggest that religiosin C is active from acidic to basic pH range. The other cucumisin-like serine proteases show their optimal pH from acidic to basic ranges are MCA-protease and serine protease from *P. hindsii* [15,20]. However, most of the other plant serine proteases show their optimal activity in the alkaline range, pH 7–11 [1].

The stability of the enzyme dictates its usefulness in various applications. Religiosin C is stable under a wide range of pH 4–11, retains full activity within the same pH range when incubated for 1 and 24 h (Fig. 3A). Besides, the enzyme retains more than 80% of activity from pH 3.5 to 11.5. The pH stability of the enzyme is more comparable to other cucumisin-like serine proteases from *Cucumis trigonus* Roxburghi, *C. melo* L. var. Prince, and a milk-clotting enzyme from *Solanum dubium* [4,5,21]. However, only 30%

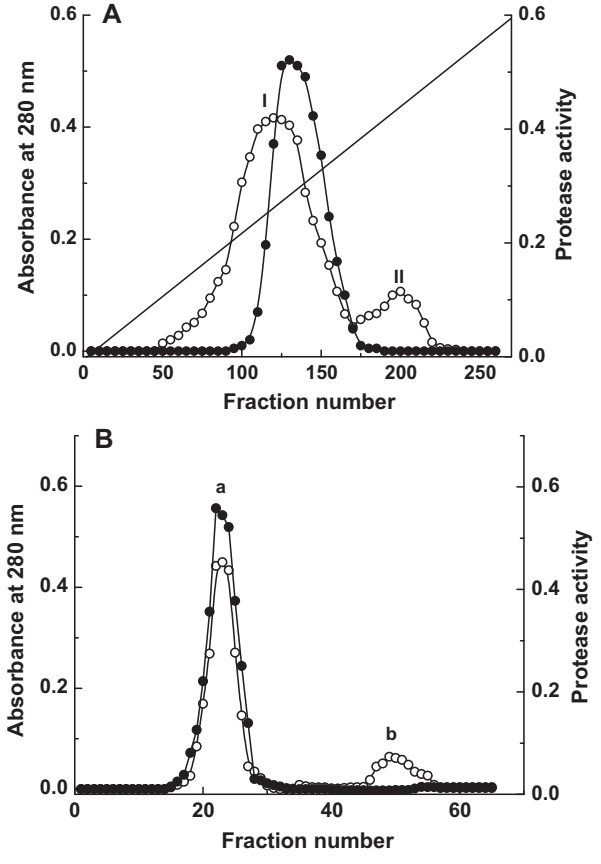


**Fig. 1.** Elution of the crude latex on cation exchange chromatography. (A) DEAE-sepharose fast flow, pre-equilibrated with 10 mM Tris buffer, pH 8.5. The bound proteins were eluted with a linear salt gradient of 0–0.6 M NaCl. Fractions of 3 ml volume at a flow rate of 3 ml/min were collected. The fractions of ascending limb of pool I (125–160) were pooled. (B) Superdex-200 column, pre-equilibrated with 0.01 M Tris buffer, pH 8.5 containing 0.2 M NaCl and the column was eluted isocratically. The fractions were assayed for the protein concentration (○) and the proteolytic activity (●).

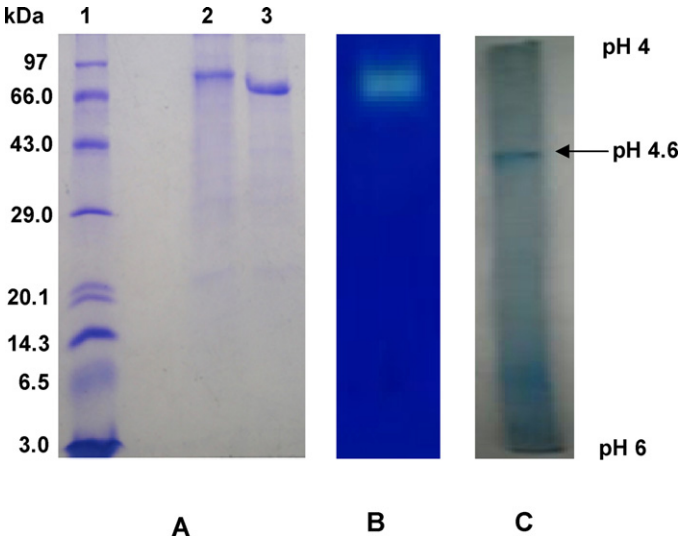


**Fig. 2.** Biochemical properties of religiosin C. (A) Assessment of homogeneity and molecular weight of the enzyme by 15% SDS-PAGE. Gel electrophoresed lanes 1–3 represent: marker, religiosin C (20 μg) under nonreducing and reducing conditions, respectively. (B) Zymogram (in-gel activity) of religiosin C. The unstained region in the gel (white colour band) showed the hydrolysis of gelatin by the enzyme. (C) Isoelectric focusing was performed by 5% polyacrylamide disc gel electrophoresis with ampholine carrier ampholyte, pH 4–6, at 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 C with other known serine proteases.

| Enzyme (source) | Mol. mass (kDa) | Optimum | | Stability | | pI | Trp | Tyr | Cys | $\varepsilon_{280}^{1\%}$ | Glycosylation (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | pH | Temp. (°C) | pH | Temp. (°C) | | | | | | |
| Religiosin C (*F. religiosa*) | 80 | 6–8 | 45–50 | 4–11.5 | 20–60 | 4.6 | 16 | 20 | 7 | 14.68 | No |
| Religiosin (*F. religiosa*) [9] | 43.3 | 8–8.5 | 60 | 5.5–10 | 20–65 | 3.8 | 16 | 26 | 11 | 29.47 | 12 |
| Religiosin B (*F. religiosa*) [10] | 63 | 8–8.5 | 55 | 5.5–11 | 20–70 | 7.6 | 23 | 15 | 7 | 23.8 | No |
| Dubiumin (*S. dubium*) [24] | 66 | 11 | 70 | 3–12 | 60–70 | 9.3 | NR | NR | NR | NR | Yes |
| Cryptolepain (*C. buchanani*) [27] | 50.5 | 8–10.5 | 70–75 | 6–12 | 10–75 | 6 | 15 | 41 | 8 | 26.4 | 6–7 |
| Cucumisin (*C. melo*) [18] | 54 | 7.1 | 70 | 4–11 | 50 | NR | NR | NR | NR | NR | Yes |
| Benghalensin (*F. benghalensis*) [12] | 47 | 8 | 55 | 5.5–10 | 20–80 | 4.4 | 17 | 31 | 9 | 29.25 | 10–12 |

NR in the table represents data not reported.

of the activity was retained when the enzyme was incubated at pH 2.5 and no activity was observed at or below pH 2.0, probably due to denaturation of the protein at highly acidic pH. The stability of religiosin C in regard to its pH is more comparable to other cucumisin-like serine proteases (Table 2). In this respect, the isolated enzyme is unique, and might therefore 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 [22]. 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 [23].

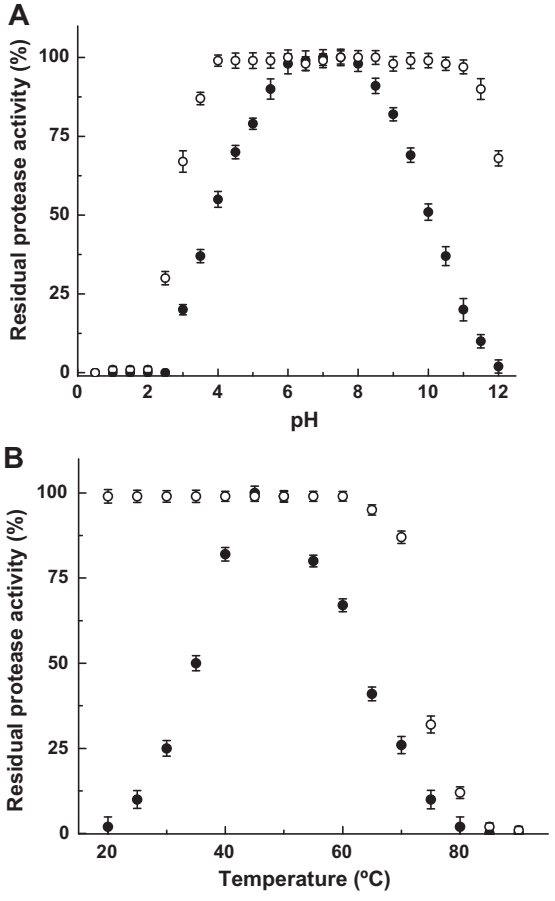


**Fig. 3.** Effect of pH (A) and temperature (B) on the proteolytic activity (●) and stability (○) of religiosin C. The assay protocols are described in Section 2. Each value in all figures represented as mean ± SD ($n = 3$).

### 3.4. Effect of temperature on the activity and stability of religiosin C

The proteolytic activity of religiosin C was monitored in the temperature range of 20–90 °C and the optimal activity was observed at 45–50 °C (Fig. 3B). More than 80% of the activity was observed from 40 to 55 °C. However, the activity decreases steadily as the temperature rose over 60 °C and no activity was observed at 80 °C. The optimum temperature for other plant serine proteases may vary from 30 to 80 °C but most of the plant serine proteases act optimally in the range of 20–50 °C [1].

The temperature stability of religiosin C was monitored in range of 20–90 °C and the enzyme showed 100% activity up to 60 °C (Fig. 3B). Moreover, 87% of activity was observed at 70 °C when incubated for 30 min which is significantly higher as compared to other subtilisin-like serine proteases [5]. The activity sharply reduced as the temperature increased higher than 70 °C probably due to thermal denaturation of the protein. The temperature profile of the purified enzyme was similar to those of other serine proteases from *C. trigonus* Roxburghi, *C. melo* L. var. Prince, and a milk-clotting enzyme from *S. dubium* [4,5,24].

### 3.5. Effect of denaturants, organic solvents and metal ions on the activity of religiosin C

The purified enzyme exhibited remarkable stability under various conditions. Religiosin C retains full activity at higher concentrations of denaturants, up to 4 M GuHCl and 8 M urea at pH 7.0. The retention of activity at such a high concentration of denaturants has also been observed in the case of religiosin and religiosin B. Moreover, the enzyme retains its complete activity in 50% methanol whereas in 50% of other organic solvents such as ethanol, butanol, acetonitrile, and dioxane, the residual activity observed was more than 60% (Table 3). Thus, the enzyme is fairly stable at high temperatures, broad range of pH, at high concentrations of denaturants as well as organic solvents. Such observation of stability may be a distinct feature of this enzyme. The high stability of religiosin C against

**Table 3**
Stability of religiosin C under different conditions.

| Condition | Concentration | Residual activity (%) |
|---|---|---|
| pH | pH 4.0–11.5 | >90 |
| Temperature | 20–60 °C | >90 |
| GuHCl | 4.0 M | 99.89 ± 0.16 |
| Urea | 8 M | 100.10 ± 0.21 |
| Methanol | 65% | 100.04 ± 0.45 |
| Ethanol | 50% | 75.09 ± 0.18 |
| Acetonitrile | 50% | 60.03 ± 0.58 |
| Butanol | 50% | 68.24 ± 0.45 |
| Dioxane | 50% | 65.35 ± 0.22 |
| Metal ions ($Na^+$, $K^+$, $Mg^{2+}$ and $Ca^{2+}$) | 10 mM | 100.01 ± 0.3 |

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

**Table 4**
Effect of inhibitors on the activity of religiosin C.

| Inhibitor | Concentration (mM) | Residual activity (%) |
|---|---|---|
| PMSF | 0.5 | 20.02 ± 0.21 |
| | 1 | 12.55 ± 0.11 |
| | 5 | 5.12 ± 0.34 |
| Chymostatin | 1 | 22.11 ± 0.15 |
| | 2 | 6.54 ± 0.31 |
| | 5 | 2.45 ± 0.11 |
| DIFP | 0.5 | 14.34 ± 0.39 |
| | 1 | 2.34 ± 0.17 |
| SBTI | 5 | 96.02 ± 0.35 |
| | 10 | 94.12 ± 0.41 |
| $HgCl_2$ | 5 | 99.21 ± 0.06 |
| | 10 | 98.97 ± 0.12 |
| IAA | 5 | 100.00 ± 0.35 |
| | 10 | 98.34 ± 0.31 |
| DTT | 5 | 99.05 ± 0.24 |
| | 10 | 97.54 ± 0.34 |
| EDTA | 5 | 100.10 ± 0.14 |
| | 10 | 98.35 ± 0.34 |
| EGTA | 5 | 100.10 ± 0.12 |
| | 10 | 99.76 ± 0.34 |
| o-Phenanthroline | 5 | 96.09 ± 0.28 |
| | 10 | 97.56 ± 0.38 |

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

pH and temperature along with the high stability under denaturing conditions facilitates the possibility of utilization of the enzyme to elucidate the structure–function relationship as well as in industrial and biotechnological applications. 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 C up to 10 mM, as shown in Table 3. The high stability of religiosin C without perturbing the enzymatic activity by various agents could make it beneficial to work under various conditions.

### 3.6. Effect of inhibitors and metal ions on activity of religiosin C

Inhibition studies can provide a first insight into the nature of the enzyme, its cofactor requirement, and the nature of the active centre [25]. Effect of inhibitors on the activity of the enzyme is represented in Table 4. The inhibitors of cysteine protease ($HgCl_2$, IAA, DTT) and metalloprotease (EDTA, EGTA) did not affect the enzymatic activity significantly up to the concentration of 10 mM. The strongest inhibition was observed with the inhibitors of serine protease such as PMSF, chymostatin, and DIFP. The above-mentioned inhibition profile classified the isolated protease as a member of the serine protease class. The inhibition profile of the purified enzyme is consistent with those reported for bamboo serine protease [15] and cucumisin [26]. Strong inhibition by PMSF was also reported for some plant serine proteases, such as cucumisin-like protease from latex of *Cryptolepis buchanani* [27] and subtilisin-like protease from *C. trigonus* Roxburghi [5].

It is noticeable that a proteinaceous inhibitor such as soybean trypsin inhibitor (SBTI), which is present in a typical protein-rich food such as soybean, did not inhibit activity of the purified enzyme. This property could, therefore, pave the way for the application of the purified enzyme in food industries. Generally, proteinaceous inhibitors known so far inhibit activity of either bacterial or animal serine proteases, but fail to do so in the case of plant serine proteases such as cucumisin, bamboo sprout proteases, benghalensin [12,15,22].

### 3.7. Substrate specificity

The enzyme hydrolyzes denatured natural substrates such as casein and hemoglobin. Religiosin C 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 hydrolyze Nα-benzoyl-DL-arginine-*p*-nitroanilide, N-succinyl-L-phenylalanine-*p*-nitroanilide. The results indicated that the purified protease preferred both hydrophilic and hydrophobic amino acid residues at the $P_1$ position, whereas, the activity of enzyme over bulky aromatic groups at position $P_1$ is not detectable. Thus, the specificity of religiosin C differs from that of cucumisin, a well known and characterized serine protease from the latex of *C. melo* [28]. The preference of hydrophobic residue at the $P_1$ position of this enzyme was comparable to that of chymotrypsin, and subtilisin. Moreover, aliphatic neutral residues (e.g., Ala) are preferred at the $P_2$ site as seen also for the other proteases [4].

**Table 5**
Kinetic parameters of religiosin C with different synthetic substrates.

| Substrate | $K_m$ (mM) | $K_{cat}$ ($s^{-1}$) | $K_{cat}/K_m$ ($mM^{-1}\ s^{-1}$) |
|---|---|---|---|
| Ala-pNA | 0.002 ± 0.001 | 32.13 ± 2.42 | 13,906.42 ± 414.66 |
| Ala-Ala-pNA | 0.004 ± 0.001 | 25.48 ± 3.89 | 6888.29 ± 120.97 |
| γ-Glu-pNA | 0.273 ± 0.03 | 207.80 ± 5.60 | 749.73 ± 43.13 |
| Leu-pNA | 0.093 ± 0.01 | 39.35 ± 1.30 | 422.19 ± 33.24 |

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

### 3.8. Kinetic parameters

The $K_m$ values for the enzyme were estimated to be 0.002, 0.004, 0.273 and 0.093 mM against Ala-pNA, Ala-Ala-pNA, γ-glutamyl-pNA and Leu-pNA, respectively. As the specificity constant ($K_{cat}/K_m$) values of religiosin C with various synthetic substrates are 13,906.42 ± 414.66, 6888.29 ± 120.97, 749.73 ± 43.13, 422.19 ± 33.24 $mM^{-1}\ s^{-1}$ against Ala-pNA, Ala-Ala-pNA, γ-glutamyl-pNA and Leu-pNA, respectively. The specific constant with Ala at $P_1$ 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 $P_1$ 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 [24]. The kinetic parameters and specificity constant ($K_{cat}/K_m$) values of religiosin C with various synthetic substrates are shown in Table 5.

### 3.9. Estimation of amino acid contents and specific extinction coefficient

The tryptophan and tyrosine contents of the protein are 16 (measured value 15.76 ± 0.03) and 20 (measured value 20.07 ± 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 disulfide bridges. The specific extinction coefficient of religiosin C measured by spectrophotometric method is 14.68.

### 3.10. Autolysis

Generally, proteases are prone to autolysis which becomes hindrance in the utilization of the same. The loss of activity of religiosin C in the concentration range 0.01–1.0 mg/ml, after 72 h of incubation at room temperature was studied. The magnitude of loss of activity decreases with increase in the enzyme concentration from 0.01 to 0.5 mg/ml and further increase in loss of activity was not observed. Religiosin C retains more than 90% activity even at very low concentration up to 0.03 mg/ml. This, in turn, indicates its high stability, and thus its possible application in food, textile, and biotechnological industries. In our experience, the enzyme is

**Table 6**
Comparison of N-terminal sequences of religiosin C with other serine proteases.

| Enzyme | N-terminal sequence (first 10 residues) | | | | | | | | | | % identity |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Religiosin C[a] | T | T | R | S | P | D | F | L | G | F | 100 |
| Cucumisin | T | T | R | S | W | D | F | L | G | F | 90 |
| Kiwano protease[b] | T | T | R | S | W | D | F | L | G | F | 90 |
| White gourd protease | T | T | R | S | W | D | F | L | N | F | 80 |
| Tomato P69B | T | T | R | S | P | T | F | L | G | L | 80 |
| Arabidopsis ARA12 | T | T | R | T | P | L | F | L | G | L | 70 |
| Lily LIM9 | T | T | H | T | P | D | Y | L | G | I | 60 |
| Tomato P69A | T | T | H | T | S | S | F | L | G | L | 50 |
| Alnus ag12 | T | T | H | T | P | R | F | L | S | L | 50 |

[a] This report.
[b] Ref. [16].
All other sequences are from Ref. [5].

stable for four months at 4 °C, under neutral conditions without loss in activity. Besides, religiosin C can also be stored for a longer time at neutral conditions and low temperatures with retention of full activity.

### 3.11. N-terminal sequencing

The sequence of 10 amino acids at the N-terminus of religiosin C was determined, and compared with other plant serine proteases (Table 6). The N-terminal sequence of religiosin C showed high similarity with other plant serine proteases. The highest similarity (90%) was with cucumisin and kiwano protease. The sequence similarity was 80% with white guard protease and tomato P69B. Besides, the N-terminal residues of religiosin C are also identical with other subtilisin/cucumisin like serine proteases. Therefore, religiosin C may be a member of subtilisin/cucumisin like serine proteases. Additional sequence analysis, catalytic site studies and structural determination may refine the classification of religiosin C.

### 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 C is determined to be $950.42 \pm 43.21$ U/OD 660 nm comparable to those of ($387 \pm 12.67$, $4989 \pm 109.771$, $367 \pm 5.90$, $3.6 \pm 0.025$, and $393 \pm 6.12$ U/OD 660 nm) religiosin, rennin, papain, trypsin and ficin, respectively [9]. 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 [14]. The capacity of religiosin C 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. However, the calf rennet used for cheese production is a relatively expensive enzyme due to its limited availability and ethical considerations associated with its use; therefore, the search for new enzymes from other sources still continues [29].

## 4. Conclusions

The present study describes purification and characterization of a new serine protease, named religiosin C from the latex of a medicinal plant, *F. religiosa*. The physico-chemical properties of the enzyme are similar to those of other known cucumisin/subtilisin-like proteases Further, religiosin C shows strong homology of N-terminal amino acid sequence with cucumisin as well as other cucumisin/subtilisin-like serine proteases. The enzyme is resistant to autolysis and can be stored at low temperature for long time without loss of activity.

Religiosin C shows striking physico-chemical differences with religiosin and religiosin B in terms of molecular mass, isoelectric point, extinction-coefficient, carbohydrate contents and mobility pattern in SDS-PAGE gel. Temperature optimum of religiosin C is slightly lower than the other two proteases from the same source. In addition, pH optimum of religiosin C is slightly towards the acidic region as compared to religiosin and religiosin B. The Michaelis constant of religiosin C is significantly lower than religiosin and religiosin B with Leu-pNA as a common substrate used in the study of all the three enzymes may dictate wide diversity in substrate specificity coupled to a single catalytic mechanism. Therefore, religiosins could be ideal model systems to study structure–function relationship. The stability of the enzyme in the presence of denaturants, organic solvents, and metal ions as well as over a wide range of temperature and pH is comparable to religiosin and religiosin B; therefore this protease may turn out to be an efficient choice in food, pharmaceutical, and biotechnological industries. Religiosin C shows highest milk-clotting activity out of the three proteases form the same source. Moreover, the detergent activity of religiosin is significantly higher than the other two proteases (data not shown). Therefore, the use of religiosin could be possible in detergent and food industry whereas religiosin B and C could be more ideal choices for food industry.

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