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IDENTIFICATION AND CHARACTERIZATION OF SERINE PROTEASES FROM *CUMINUM CYMINUM* (APIACEAE)

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Abstract

Cuminum cyminum L. of the family Apiaceae, commonly known as cumin or safaid zeera, has been popularly utilized as common spice around the world. Phytochemical analysis and pharmacological studies proved the presence of several biological active compounds with health beneficial effects. The present work aimed to identify the proteases in the aqueous extract of *Cuminum cyminum*. Total proteins were extracted and fractionated by size exclusion chromatography. Protein fractions were divided in two groups CSP I and CSP II on the basis of protein pattern and the presence of protease activities by SDS-PAGE and zymography, respectively. Additionally, both group showed optimum activity at pH range 5-6 and at 35ºC using casein as substrate. Significant inhibition of activity by phenylmethylsulfonyl fluoride, indicated that enzymes of both groups belonged to the family of plant serine proteases. These cumin proteases can be a significant addition in plant proteases family which is in continuous growth due to their wide range of application in industries like food, leather, detergent and pharmaceutical manufacturing, offering substitute to the harmful chemicals.

Key words: *Cuminum Cyminum*, Caseinolytic activity, Serine protease, Zymography, Plant proteases.

Abbreviations: CSPI, cumin serine protease type I; CSP II, cumin serine protease type II; DTT, Dithiotheritol; 2-ME, 2-mercaptoethanol; EDTA, Ethylendiamine tetracetic acid; PMSF, phenylmethanesulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Introduction

Proteases from plant source gained broad industrial applications with their wide pH and temperature stability along large substrate specificity. In the food industry, they are being utilized for diverse processes like brewing, tenderization of meat, elaboration of cheese and production of breads (David *et al*., 2022) Based on the catalytic activity, proteases are typically categorized in four major groups such as aspartic, cysteine, serine, and metallo proteases. Among these, the serine proteases found to be the most abundant class, present in plants.

S**erine proteases** (EC 3.4.21), an endopeptidase, catalyze hydrolysis of peptide bond with nucleophilic serine residue located in active site along with highly conserved histidine and aspartate residues. According to MEROPS database, serine proteases have been categorized into 52 different families with respect to their structural similarity and amino acid sequence homology (Rawlings & Barrett, 1999). In general, these enzymes have broad substrate specificity for proteins and specifically inhibited by phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP). They are stable at 40°C or above with optimum pH from alkaline to slightly acidic environments. These proteases are associated with numerous phases of plant physiology and development processes including protein synthesis and degradation, spore formation, signal transduction, symbiosis, senescence and hypersensitive response (Antao & Malcata, 2005).

Cuminum cyminum (cumin), a popular spice, rich in bioactive compounds, proved to exhibit wide range of beneficial effects on health (Sharif *et al*., 2018). Therapeutic potential in terms of anti-carcinogenic (Prakash & Gupta, 2014), antibacterial (Iacobellis *et al*., 2005; Gachkar, 2007; Wanner *et al*., 2010, Sharifi *et al*., 2021), antifungal (Hajlaoui *et al*., 2010; Romagnoli, *et al*., 2010, Tanapichatsakul *et al*., 2020), antioxidant (El-Ghorab *et al*., 2010; Satyanarayana, *et al*., 2004), antidiarrhoeal (Sahoo, *et al*., 2014) and antihyperglycemic effects (Kumar *et al*., 2009, Jagtap & Patil, 2010) have been documented from *Cuminum cyminum*. Despite these valuable properties, very little studies have been conducted for characterization of their biological active proteins.

Literature studies showed only few report about α and β amylase (Nomura, 1999) and pattern of seed storage proteins on SDS-PAGE (Masoumi *et al*., 2012). Previously, we have reported non-specific lipid transfer protein 1 (nsLTP1) that purified by classical protein chemistry techniques (Zaman & Abbasi, 2009) and more recently, for the first time shotgun proteomic approach has been utilized that allowed the identification of large number of proteins with their functional annotation (Zaman *et al*., 2018).

The present study described the pattern of two groups of serine protease, partially purified using size exclusion chromatography. They have been named as cumin serine protease type I (CSP I) and type II (CSP II) on the basis of molecular weights. Gel based proteomic approach has been applied to identify different type of proteases in slightly acidic extract of *Cuminum cyminum*, possessing potential role in plant development and pathogenesis.

Material and Methods

Protein extraction: 100 gram of *Cuminum cyminum* were fine grinded and soaked in sodium acetate solution (25 mM sodium acetate, 4 mM EDTA, 0.01% sodium azide and pH 5.5) for 24 hrs. Proteins were extracted out and concentrated with ammonium sulfate (60%) as described previously (Zaman & Abbasi, 2009). Protein precipitate

was collected by centrifugation at 10,000 Xg for 30 mins at 4ºC, stored for further analysis at -20ºC.

Size exclusion Chromatography: Re-suspended proteins in sodium acetate solution (25 mM sodium acetate, 4 mM EDTA, 0.01% sodium azide and pH 5.5) were fractionated by size exclusion chromatography. The Bio-Rad column with dimension of 2.5 **X** 65 cm was packed with Sephacryl S-100 (Pharmacia Biotech) and equilibrated with ammonium acetate solution (50 mM ammonium acetate, 100 mM NaCl, 2 mM EDTA, 0.01% sodium azide and pH 6.5). Fractions were collected at flow rate of 20 ml/hour by measured absorbance at 280 nm wavelength. Quantification of proteins was carried out by Bradford assay (Bradford, 1976) by utilizing known concentration of Bovine Serum Albumin as reference protein.

Electrophoresis and Zymography: Electrophoresis was done on SDS-PAGE (Laemmli, 1970). Crude proteins and fractions obtained from size exclusion chromatography were applied. Samples in Laemmli buffer were heated for 5 minutes at 100ºC under reducing condition and separated on 12% separating gels in Tris-glycine-SDS buffer for 60 min at 130V constant voltage. Staining with coomassie blue R-250 was followed by de-staining with mixture of acetic acid (10%) and methanol (30%) which assisted to visualize the bands on gels.

Protease activity in fractions was analyzed by zymography after optimized the method of Heussen and Dowdle (Heussen & Dowdle, 1980). Separating gel (10%) was polymerized along with 1% gelatin (molecular biology grade, MERCK) as substrate. Protein fractions (20ug) were re-suspended in Laemmli buffer under non reducing condition and loaded on gel without heat treatment. Trypsin was used as a reference enzyme. Electrophoresis was done at constant voltage of 160V for 60 min. Triton X-100 (2.5% for 20 min) was used to wash gels and protease activity was recovered after incubation in Tris-HCl solution (50 mM Tris-HCl, 100 mM NaCl and 10 mM $CaCl_2.2H_2O$, pH 7.6) for 16 hours at 37ºC. Enzyme activity was recovered as transparent bends against a blue background after stainig with coomassie blue R-250 followed by de-stained.

Biochemical characterization of proteases: Colorimetric method of Kunitz (Kunitz, 1947) was applied to characterize serine protease activity. Fractions of similar pattern on SDS-PAGE were pooled and incubated for 20 min at 37ºC with 1% casein (dissolved in solution of 50mM Tris-HCl, pH 7.6). The reaction was stopped with 40% TCA (trichloroacetic acid). Supernatant was collected after centrifugation (12,000 Xg for 20 min at 4ºC) and enzyme activity was calculated by monitoring the absorbance at 280 nm. Under the reaction condition, one unit of enzyme activity is the concentration of enzyme that increases the absorbance at 280 nm by 1.0. Trypsin was applied as reference enzyme and blank was prepared by addition of TCA before addition of the enzyme to the reaction mixture.

To investigate the optimum pH of enzyme activity, solutions with different pH (pH 4.5-9.0) were prepared such as Na-acetate (50 mM, pH 4.5-5.5), Phosphate (50 mM, pH 6.0-7.5) and Tris-HCl (50 mM, pH 8.0-9.0). Effect of pH on enzyme activity (100 l) was measured by incubating the pooled fractions in different pH solutions for 20 min at 37ºC along with 1% casein. Similarly pH stability was determine by pre-incubation in the various pH solution mentioned earlier (pH 4.5-9.0) for 30 min at 37ºC. Then residual activities were calculated after addition of casein and measured the absorbance of supernatant at 280 nm.

Temperature profile was determined in the range of 25-70ºC in solution of Tris-HCl (50 mM pH 6.0) for 30 min. Whereas thermo stability of pooled fractions was measured by pre-incubation in solution of Tris-HCl (50 mM pH 6.0) for 30 min at various temperatures followed by reaction with casein.

The effect of various substrates (Casein, bovine serum albumin, porcine hemoglobin and gelatin) on enzyme activity was analyzed for 20 min at 37ºC in solution of Tris-HCl (50 mM pH 6.0).

The stock solution of inhibitors such as Ethylendiamine tetracetic acid (EDTA), Dithiotheritol (DTT), Phenylmethylsulfonyl-floride (PMSF), 2-Mercaptoethanol, Urea and Sodium Dodecyl Sulfate (SDS) were prepared. The enzyme residual activity was measured after incubation with different concentrations of inhibitor in 50mM Tris-HCl pH 6.0 under the standard assay conditions. Effect of metal ions was also investigated (details of salt solution mentioned in supplementary Table S4).

Shotgun proteomic analysis: Total extracted crude protein (100 µg) was re-suspended in sample buffer (NuPAGE) with heating at 70 °C for 10 min and separated on gradient gel (4 -15% , NuPAGE Novex Bis-Tris Gels). Electrophoresis was done in MOPS-SDS buffer under reducing condition and at 200 V for 60 mins. Proteins bends were observed on gel by staining with colloidal Coomassie Brilliant Blue R-250 for overnight followed by de-staining with water. Separated proteins were in-gel digested by trypsin for overnight at 37ºC after reduction and alkylation steps. Peptides were extracted out with 5% formic acid (Schmidt & Urlaub, 2009) and analyzed by LTQ-Orbitrap Velos MS (Thermo-Scientific). Peptides were separated out online by nano-flow HPLC system (Agilent 1100 series, Agilent technologies, Boblingen, Germany) equipped with reverse phase C18 column (ReproSil-Pur C_{18}-AQ 3 µm resin, 150 X 0.075 mm, Dr. Maisch GmbH). Linear gradient of 3-36% acetonitrile was applied for 37 min then 95% acetonitrile for 8 min. The flow rate was adjusted to 250 nl/min.

For mass spectrometry, MS scan of 350-1600 *m/z* range was recorded in the Orbitrap mass analyzer with resolution of 30,000 (*m/z* 400). To record MS/MS scans, the fifteen most intense peaks (Top15) with charge state ≥ 2 were selected in the ion trap for CID fragmentation with normalized collision energy of 35%, activation time of 10 ms. The ion accumulation time for MS scans was 500 ms and for MS/MS scan was 100 ms. The ion selection threshold was 2000 counts. Identification of peptide/protein was done with MASCOT search engine (v.2.3, Matrix Science) by using Viridiplantae database from NCBI. Parameters selected for searches included monoisotopic mass with 20 ppm tolerance for parent ion mass and 0.8 Da

tolerance for fragment ion mass. Trypsin with maximum one or two allowed missed cleavage events was selected as proteolytic enzyme. In section of modification, oxidation of methionine as variable modification and carbamidomethylation of cysteine as fixed modification were selected. Further data validation was achieved with Scaffold v4.0.4 (Proteome Software, Portland, OR, USA) with a peptide threshold of 99% and protein probability threshold of 90%. A minimum of one unique peptide was required as selection criteria. The false discovery rate was 0.02% for peptides and 2.1% for proteins. At least two biological replicates were analyzed from each protein fraction.

Results and Discussion

Two groups of serine proteases have been purified from *Cuminum cyminum* under slightly acidic condition. Crude extract containing total proteins were fractionated by size exclusion chromatography (Fig. 1). Caseinolytic activity (Fig. 1) of fractions resulted in two peaks with good proteolytic activity, revealed the presence of two populations of proteases. The results of purification have been summarized in supplementary Table S1 and S2. Gelatin zymography (Fig. 2) of fractions showed two clear zones of activity against blue background confirmed the presence of two groups of proteases with different range of molecular weights (Fig. S2a and S2b). These results help to pool the fractions into two groups i.e. CSP I (cumin serine protease type I or high molecular weight serine proteases (Fig. S1a)) and CSP II (cumin serine protease type II or low molecular weight serine proteases (Fig. S1b)) for further biochemical characterization by Kunitz method (Kunitz, 1947). It was observed that CSP I showed optimum pH at 5.5 with casein as substrate (Fig. 3a). Whereas enzyme activity was decreased up to 40% at pH 7.0 and negligible activity observed at pH 8.0. The pH stability test revealed 60% residual activity between pH 5.0 and 6.5 after incubation in different pH solutions (pH 4.5-9.0) for 30 min at 37ºC (Fig. 3b). Similarly, the pH optimum of CSP II was about 6.0 at 37ºC (Fig. 4a) while the stability resembled to CSP I (Fig. 4b). Although plant serine proteases found to be active in the alkaline pH, however, hordolisin (Terp *et al*., 2000) and SEP-1 (Fontanini & Jones, 2002) from Barley reported to have pH optima around 6 and 6.5 respectively. Whereas Ara12 from *Arabidopsis thaliana*, (Hamilton, *et al*. 2003) RSIP from maize (James *et al*., 1996) and protease C1 from soybean (Qi *et al*., 1992) also showed optimum activities between 3.5-6.5 pH range respectively.

Both CSPI and CSPII were found to be heat labile, showed temperature optima at 35ºC (Fig. 3c and 4c) with increased initial activities at temperature up to 40ºC in a solution of pH 6.0 and negligible residual activities were observed at temperature above 40ºC (Fig. 3d and 4d).

The protease inhibition profile examination confirmed that both group of enzymes belonged to the family of plant serine protease as 76% enzyme activity of CSP I and 62% enzyme activity of CSP II was inhibited by 2mM PMSF (serine protease inhibitor). Whereas 2-mercaptoethanol (thiol proteases inhibitor) and EDTA (metalloproteases inhibitor) had no significant effect (supplementary Table S3) on enzyme activities of both groups. Substrate specificity behavior against various proteins was also similar; both exhibited the highest activity towards bovine serum albumin (BSA) and lowest activity towards gelatin (supplementary Table S5). Further metal ions sensitivity analysis showed that cations such as Ca, K and Mg were found to enhance proteases activity (supplementary Table S4).

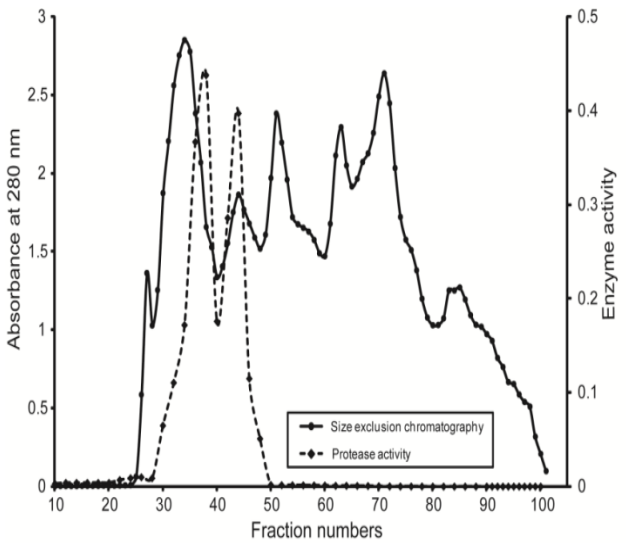

Fig. 1. Separation profile of total protein extracted from C*uminum cyminum* by size exclusion chromatography. Ammonium sulfate precipitate applied on Sephacryl S-100, 5 ml fraction was collected with the flow rate of 20ml/hr (solid line). The protease activity of fractions was measured at 37ºC for 20min with casein as substrate (dash line). Absorbance of fractions was recorded at 280nm.

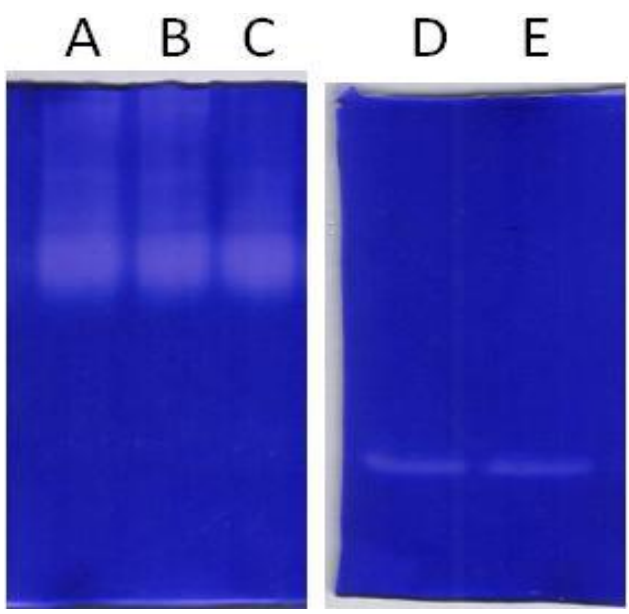

Fig. 2. Zymogram of fractions of C*uminum cyminum* separated by Sephacryl S-100. SDS-PAGE (10%) was prepared with Gelatin (1%).The lane A, B, C, represent CSPI, whereas D and E represent CSPII (fraction numbers 32, 34, 36, 42 and 44 respectively). Coomassie blue R-250 applied to stain gel.

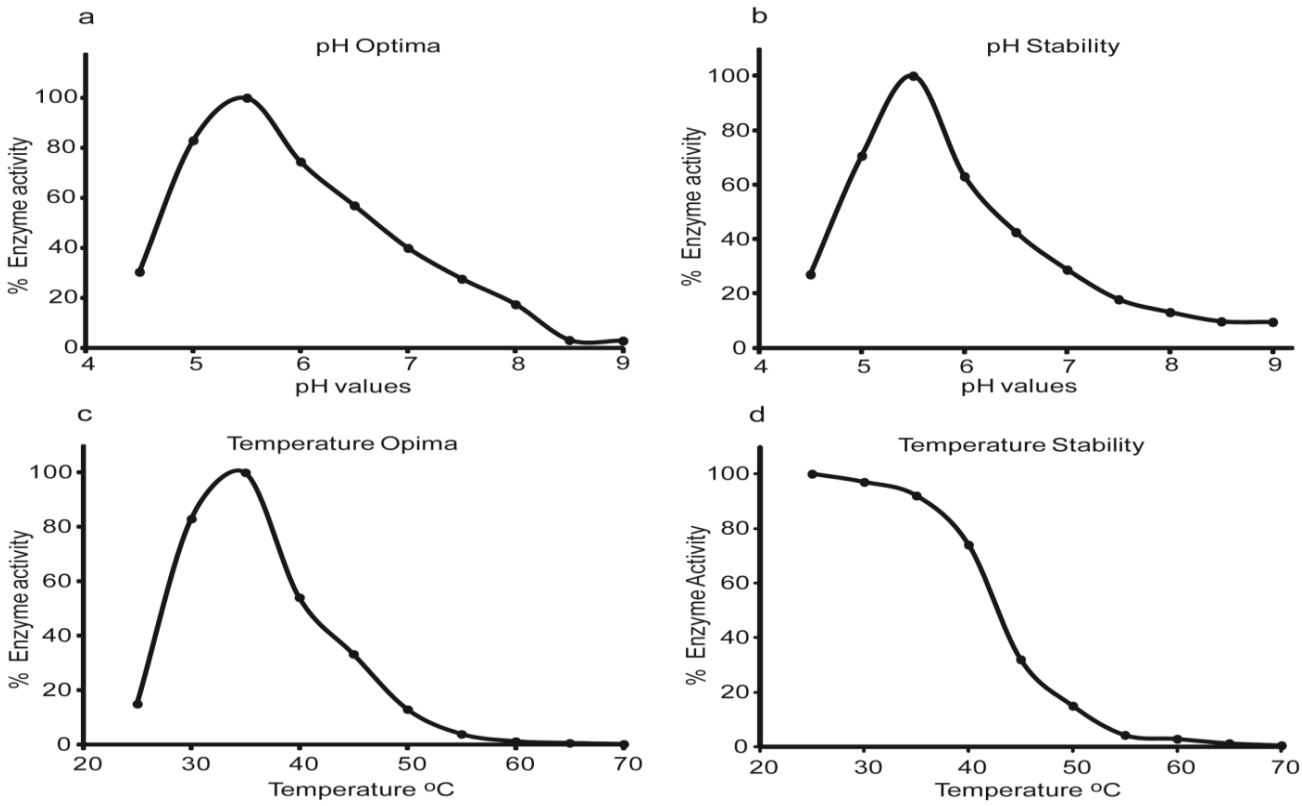

Fig. 3. Biochemical characterization of CSP1. Colorimetric method of Kunitz (Kunitz, 1947) was applied to characterize serine protease activity with 1% casein solution. Fractions of similar pattern on SDS-PAGE were pooled and incubated for 20 min at 37ºC. Graphical presentation shows the optimum pH (a) and pH stability (b) of CSPI, similarly optimum temperature (c) and thermal stability (d) of CSPI protease.

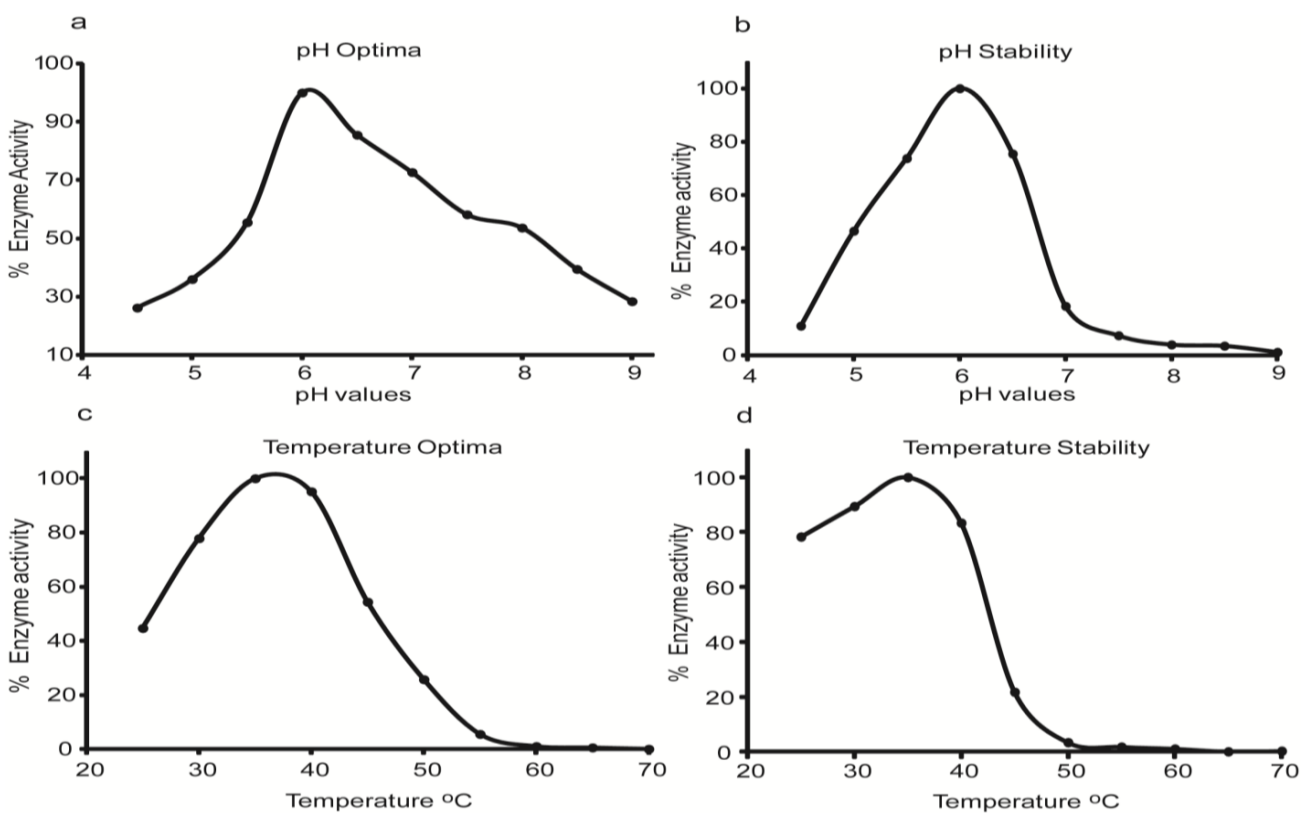

Fig. 4. Biochemical characterization of CSPII. Colorimetric method of Kunitz (Kunitz, 1947) was applied to characterize serine protease activity with 1% casein solution. Fractions of similar pattern on SDS-PAGE were pooled and incubated for 20 min at 37ºC. Graphical presentation shows the optimum pH (a) and pH stability (b) of CSPII, similarly optimum temperature (c) and thermal stability (d) of CSPII.

Table S1. Purification of CSPI from *Cuminum cyminum*.

Steps of purification	Total Protein (mg)	Volume (ml)	Specific activity (Unit/mg)	Total activity	Purification fold	% Yield
Crude protein	1506	850	9	13554	1	100
Ammonium sulfate precipitates	440	70	20.1	8844	2.2	65
Sephacryl S-100	134	52	32.1	4288	3.5	31.6

Table S2. Purification of CSP II from *Cuminum cyminum*.

Steps of purification	Total Protein (mg)	Volume (ml)	Specific activity (Unit/mg)	Total activity	Purification fold	% Yield
Crude protein	1506	850	9	13554	1	100
Ammonium sulfate precipitates	440	70	20.1	8844	2.2	65
Sephacryl S-100	72	16	29	2088	3.2	15.4

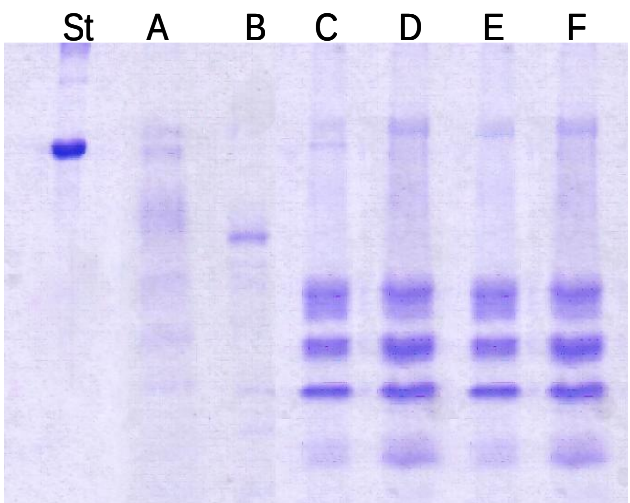

Fig. S1a. Electrophoresis on 12% SDS-PAGE for analysis of fractions of *Cuminum cyminum* separated and collected from Sephacryl S-100 chromatography. St. represent bovine serum albumin whereas lane A, B, C, D, E, and F correspond to fraction numbers 26, 27, 30, 32, 34 and 37, respectively.

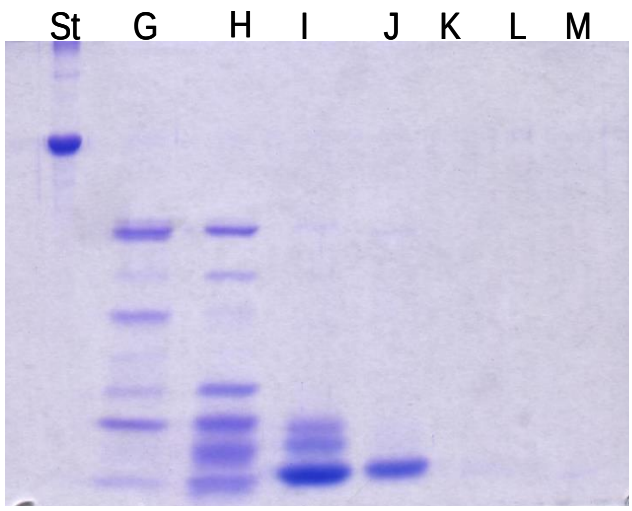

Fig. S1b. Electrophoresis on 12% SDS-PAGE for analysis of fractions of *Cuminum cyminum* separated and collected from Sephacryl S-100 chromatography. St. represent bovine serum albumin whereas lane G, H, I, J, K, L and M correspond to fraction numbers 41, 44, 51, 57, 63, 71 and 85, respectively.

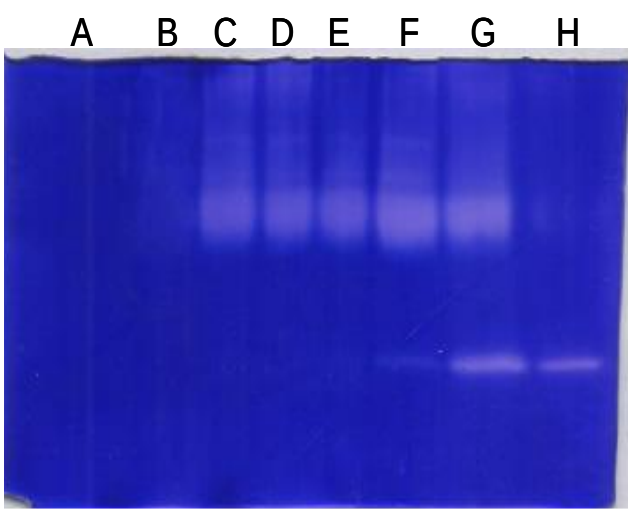

Fig. S2a. Zymogram of fractions of C*uminum cyminum* separated on Sephacryl S-100 chromatography. The lane A, B, C, D, E and F, G and H represent fraction numbers 26, 27, 30, 32, 34, 37, 41 and 44 respectively.

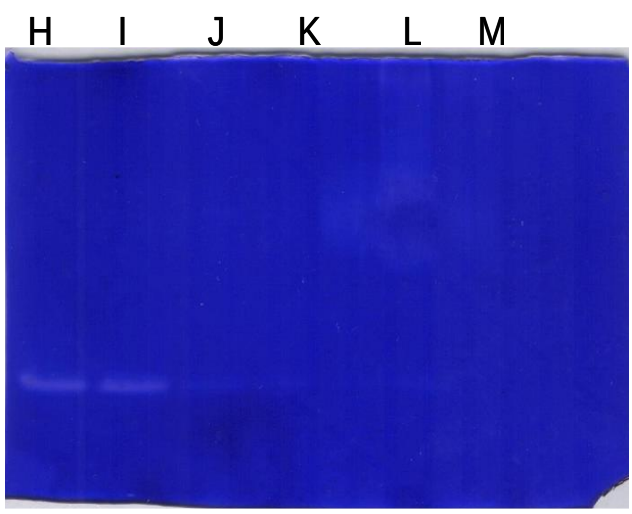

Fig. S2b. Zymogram of fractions of C*uminum cyminum* separated on Sephacryl S-100. The lane H, I, J, K, L and M represent fraction numbers 44, 51, 57,63, 71 and 85 respectively.

Table 1. Serine proteases identified from *Cuminum cyminum* by shotgun proteomic approach.

NCBI ID	UniProt ID	Identified Proteins	Homologous species	MW (KDa)
gi|194698772	B4FMX7	Serine carboxypeptidase	*Zea mays*	36
gi|225424228	F6HFI1	Serine protease	*Vitis vinifera*	49
gi|225457767	E0CQN9	Serine protease	*Vitis vinifera*	52
gi|224083898	B9H5R2	Serine protease	*Populus trichocarpa*	53
gi|45935141	Q6JJ33	putative serine carboxypeptidase	*Ipomoea trifida*	54
gi|224108896	B9NDJ5	Serine carboxypeptidase S28 protein	*Populus trichocarpa*	55
gi|226533357	B8A0Q3	Serine protease	*Zea mays*	57
gi|242050222	C5X8I6	Serine protease	*Sorghum bicolor*	57
gi|15237178	Q9FFB0	Serine carboxypeptidase-like 47	*Arabidopsis thaliana*	57
gi|115349976	Q06SH9	ATP-dependent Clp proteolytic subunit	*Stigeoclonium helveticum*	60
gi|115455505	Q94H95	serine protease Cucumisin-like	*Oryza sativa*	79
gi|255538108	B9R726	Xylem serine proteinase 1 precursor	*Ricinus communis*	81
gi|227053577	C3VDI0	Subtilisin-like serine protease	*Carica papaya*	83

The LC-MSMS data searched against viridiplantae database from NCBI using MASCOT search engine. NCBI ID- National Center for Biotechnology Information gene accession number, Uniprot ID- Uniprot gene accession number, MW -Molecular weight of protein in kDa.

Table S3. Effect of inhibitors on the Proteases activity.

Inhibitor	Concentration used	% Relative activity	
	(mM)	CSPI	CSPII
None		100	100
PMSF	0.5	37	32
	1	20	24
	2	15	11
EDTA	1	88	92
	2	85	88
	5	80	83
DTT	5	73	79
B-mercaptoethanol	1	79	71
	2	73	69
Urea	2	90	89
SDS	2	85	87

Effect of specific inhibitors on the activity of proteases purified from *cuminum cyminum*. (Concentrations mentioned are those used in the preincubation mixture. Activity of control without addition of inhibitor was taken as 100%).

Table S4. Effect of metal ions on the Proteases activity.

Metals	Concentration (mM)	% Relative activity	
		High molecular weight proteases	Low molecular weight proteases
CaCl2	2	116	124
NaCl	2	119	105
LiCl	2	89	59
CuSO4	2	35	6
KCl	2	105	115
Mg Cl2	2	122	117
ZnCl	2	65	13

Table S5. Substrate specificity of proteases purified from *cuminum cyminum.*

Substrate	Concentration	% Relative activity	
	(%)	CSPI	CSPII
Casein	1%	100	100
Gelatin	1%	24	32
Hemoglobin	1%	62	60
BSA	1%	184	159

Purification and characterization of proteins from plant such as spices is notoriously challenging. Beside the presence of relatively low amount of proteins, plant extracts are rich in proteases, polysaccharides, lipids, phenolic compounds and large number of secondary metabolites. Since these materials cause hindrance in protein separation and analysis, a gel based proteomic approach was designed for characterization of the serine proteases from *Cuminum cyminum*. This global proteomic screening approach by mass spectrometry has extended our knowledge and helped in further functional characterization. Total proteins have been extracted in slightly acidic buffer, separated out on NuPAGE gel. Peptides derived from in gel digestion of protein bends were analyzed by nano-ESI-LC-MS/MS and acquired data was searched against viridiplantae database from NCBI using MASCOT search engine. Further validation was done by scaffold. Using global proteomics approach, 13 serine proteases have been identified (Table 1) along other proteases.

Although the NCBI database of proteins is the major store of experimentally identified proteins, a large number of proteins present are still unnamed or hypothetical with uncharacterized function. For this reason, we manually validated these identified proteases using UniProt database which contained revised and manually annotated data. Due to lack of protein sequence information of *Cuminum cyminum*, all the proteins have been identified for homology with other plant species. Moreover, most of these proteins have been identified with only one unique peptide.

From the data it was found that all the identified serine proteases possessed molecular weight in the range of 83 kDa to 36 kDa. However, majority of serine proteases showed molecular weight range from 49-60 kDa (Fig. S1a), that matched to zymography result of CSP I (Fig. 2 and S1a). Very broad proteolytic band observed in the gelatin gel also confirmed the presence of more than one enzyme in this mass range. From the data we also found one serine protease with molecular weight 36 kDa (Fig. S1b), which could be matched to CSP II group. Zymogram also show and very thin clear band in this range (Fig. 2 and S1b).

Conclusion

Serine proteases from *Cuminum cyminum* have been partially purified. Attempts to purify these serine proteases using several methods and conditions did not succeed. The main reason being the low amount and the labile nature of these enzymes. Though CSP I and CSP II have different molecular weights, however, the enzymatic properties resembled closely. The biochemical properties of both enzymes suggested their placement in the serine protease family which was further confirmed by inhibition of protease activity by class specific inhibitor such as PMSF. Further in-gel shotgun proteomic approach applied and total 21 proteases have been detected, among these, 13 have been classified as serine proteases having different molecular weights.

Acknowledgments

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