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Purification and characterization of a gelatinolytic serine protease from the seeds of ash gourd *Benincasa hispida* (Thunb.) Cogn.

Niloy Das[#], Sanhita Maity, Jishnu Chakraborty, Sampa Pal, Subrata Sardar & Umesh Chandra Halder*
Organic Chemistry Section, Department of Chemistry, Jadavpur University, Jadavpur, Kolkata-700 032, West Bengal, India

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In Ayurveda, *Benincasa hispida* (Thunb.) Cogn. (Ash gourd) was recommended for management of diabetes, peptic ulcer, and other diseases. This plant is rich in proteolytic enzymes and proteases have wide application in food and laundry industry. Therefore, the search for new potential plant proteases continues. A soluble gelatinolytic plant serine protease (AG2) had been purified from the seeds of *Benincasa hispida*. The molecular mass of the monomer was estimated to be about 11 kDa by SDS-PAGE and 11211.1 Da by MALDI-TOF. The protease activity was strongly inhibited by PMSF only but not at all by soyabean trypsin inhibitor and resists autodigestion. Thus AG2 belongs to subtilisin family. The optimum pH and temperature are 10.0 and 30°C respectively. This protease was quite stable in presence of a cationic surfactant, an oxidizing agent and in basic pH medium. The protease AG2 can hydrolyze casein, azoalbumin and TAME but it was inert towards BAPNA. The kinetic parameters K_m and V_{max} were 0.117 ± 0.00067 mM and 470.592 ± 0.631 unit mg^{-1} min^{-1} respectively using casein as substrate. The CD spectrum showed it as a typical α/β class of protease. The N-terminal sequence of first 17 amino acid residues (MQQFFNEPSSLLIVVVR) is unique in nature.

Keywords: Ash Gourd (AG), *Benincasa hispida*, Gelatinolytic, Plant serine protease, Subtilisin-like

Proteases are one of the important groups of enzymes widely present in plant and animal kingdom. Apart from their physiological roles as endopeptidase and exopeptidase, they are extremely used in various industries such as food, pharmaceutical, detergent, paper, and leather[1]. Cysteine proteases such as papain, bromelain, and ficin are utilized in food and dairy industries and also have medicinal applications. Plant serine proteases are involved in many physiological processes such as protein degradation, signal transduction, microsporogenesis, and hypersensitive response[2] and in fibrinolytic activity[3]. Again plant serine proteases are stable and active under harsh conditions, thus they are more useful and economical for industrial applications. The fruits of some cucurbitaceous plants contain a significant amount of serine protease activity. A serine protease, isolated from the fruit of melon, *Cucumis melo* L. var. prince called cucumisin (EC 3.4.21.25) and had been well characterized[4,5]. This serine protease has the milk-clotting activity like papain. Subsequently, more cucumisin-like proteases had been isolated and characterized from other plants such as, *Euphorbia supine*, *Cucumis trigonus*, and *Ficus religiosa*[6-8]. However little information was available about the enzymatic characteristics of plant subtilisin-like serine proteases[9,10] except the cucumisin-like enzymes. Some subtilisin-like serine proteases had been isolated from the latex of different plants[11,12]. *Benincasa hispida* (Ash Gourd), belonging to the family Cucurbitaceae, is a vegetable used widely in India and other semi-tropical countries. In Ayurveda, ash gourd was recommended for management of peptic ulcer, diabetics, epilepsy and other nervous disorders[13]. Cucumisin-like proteases of molecular weight 67 kDa had been isolated from the sarcocarp of *Benincasa hispida*[14]. In the course of our studies with the serine proteases from the plants of the Cucurbitaceae family; we had isolated and characterized a gelatinolytic subtilisin-like serine protease, AG2, from the seeds of *Benincasa hispida* (Ash gourd). In this paper purification, physicochemical characterization and the N-terminal amino acid sequence of such gelatinolytic subtilisin-like serine protease, AG2, from the Cucurbitaceae family has been described.

Material and Methods

Materials and chemicals

Ash gourd (*Benincasa hispida*) seeds were purchased from the local market and used to isolate

*Correspondence:
Phone: +91 33 2414 6223 (Mob); Fax: +91 33 2413 7902
E-mail: uhalder2002@yahoo.com
[#]Present Address: Department of Chemistry, Durgapur Government College, Jawaharlal Nehru Avenue, Paschim Bardhaman, Durgapur- 713 214,West Bengal, India

the subtilisin-like serine proteases. All the protease inhibitors, substrates (casein and azoalbumin), bovine trypsin (twice crystallized), p-tosyl-L-arginine methyl ester (TAME), N_{α}-benzoyl-DL-arginine-p-nitroanilide (BAPNA), dialysis tubing, PMSF, iodoacetamide, β-mercaptoethanol, SDS, DTT, STI, Coomassie brilliant blue R-250 and acrylamide were obtained from Sigma Chemical Co.. SP-Sephadex C-50 and DEAE-cellulose were Pharmacia products. AR grade sodium dihydrogen phosphate, sodium hydrogen phosphate, acetic acid, methanol, SDS, and TCA were purchased from Sisco Research Laboratory, India. All other chemicals were of the highest purity available. The sample solutions were prepared in double distilled water and filtered through 0.45 μM filters.

Purification of the protease AG2

Fresh mature ash gourd seeds were collected from the local market and 200 g dry seeds were crushed. It was soaked overnight in 700 mL 0.03 M phosphate buffer, pH-7.0 containing 1% NaCl at 4°C. Then the seeds were homogenized in a blender. The homogenate was filtered through a cotton cloth and then it was centrifuged at 5000 rpm for 15 min. The supernatant was collected for ammonium sulphate precipitation. Solid ammonium sulphate was added slowly to the extract with constant stirring till 90% saturation was obtained. The extract was allowed to stand for overnight at 4°C. The protein precipitation was obtained at 10000 rpm for 10 min. The pellet was collected and dialyzed for 24 h at 4°C against 0.04 M sodium phosphate buffer, pH 7.5 with several changes. Then 20 mL of dialyzed supernatant was subjected to anion-exchange chromatography and loaded on a DEAE-cellulose packed column (2.6 × 30 cm) pre-equilibrated with 0.04 M sodium phosphate buffer, pH 7.5. Proteins were collected using an automatic fraction collector (Eyelet, DC-1000) maintaining the flow rate at 20 mL h^{-1}. The absorbance of protein fractions was measured at 280 nm using a Hitachi UV-Vis spectrophotometer (Hitachi U-2000). The chromatogram showed the appearance of a single broad peak in the unabsorbed part. Proteolytic activity in each fraction was measured using casein as a substrate. The active fractions were pooled and lyophilized to reduce the volume. Then it was dialyzed against 0.02 M sodium phosphate buffer, at pH 7.0 for 10 h. The dialyzed sample was loaded on an SP-Sephadex C-50 column (2 × 45 cm) pre-equilibrated with the 0.02 M sodium phosphate buffer, pH 7.0. Protein fractions were eluted from the column by passing the same buffer maintaining the flow rate at 16 mL h^{-1}. The protein fractions under the major peak (AG2) having proteolytic activities were pooled and lyophilized to reduce the volume. It was then dialyzed against distilled water with several changes. The purified protease (AG2) was dried by lypohilization and stored at −20°C for further work. The protein concentration was determined using bovine serum albumin (BSA) as a standard.

SDS-PAGE and zymography study

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the molecular mass and also to check the homogeneity and intactness of AG2 in the preparation. Gel electrophoresis was carried out in 20% separating gel containing 0.1% SDS[15]. The electrophoresis was performed at pH 8.3 for 2 h under both reducing and non-reducing conditions. The gel was stained for proteins for 5 h with Coomassie brilliant blue R-250. It was destained by washing several times with a mixture of methanol/acetic acid/water (50/10/40, v/v/v), (destaining solution). The electrophoresis was done with the protein markers having a molecular weight ranging from 10 kDa to 130 kDa (Prestained Protein ladder, Fermentus page ruler™, # SM0671). The electrophoretic pattern was photographed. The molecular mass of the protease AG2 was extrapolated from the plot of log molecular weight *vs.* electrophoretic motilities of the standard protein markers[16]. Proteases those degrade gelatin (denatured collagen) can be directly identified in the gel. Thus to confirm the gelatinolytic activity of the purified AG2, gelatin zymography was performed. The 0.5 mL gelatin solution (12 mg in 1 mL 0.02 M phosphate buffers, pH 7.02) was co-polymerized with 20% acrylamide solution (resolving gel) and PAGE-gelatin zymography was carried out in absence of SDS[17]. The protease solution was loaded on the gel and electrophoresis was carried out at 20°C, after completion of electrophoresis the gel was washed by 2.5% Triton X 100 with several changes for 1 h. Finally, the gel was washed thoroughly with water and incubated in 10% 0.05 M Tris-HCl, pH 7.6 for 24 h. Then the staining-destaining procedure was followed in the usual way.

Mass spectrometry

The purity and molecular weight of the purified protease were determined by mass spectrometry in a Bruker Daltonics Auto flex II TOF/TOF mass

spectrometer. About 3.8 mg of DHAP matrix was dissolved in 250 µL of 3:1 absolute ethanol and 10 mM diammonium hydrogen citrate solution. The protein sample was prepared in 10 mM NH_4HCO_3 solution at pH 8.0. To the 6 µL of a protein sample, 6 µL of matrix and 1 µL of 10%TFA were added. 1-2 µL of this mixture was loaded on the MALDI target plate and air dried. External calibration standard prot-mix from Bruker was used to calibrate the instrument daily. Mass spectra were acquired in the linear mode in the m/z range of 6000-25000. For each spectrum, 200 laser shots were summed and three spectra were averaged for each sample.

Carbohydrate content

The presence of carbohydrate content of the protease was checked by the phenol-sulfuric method[18]. A stock solution of D-Glucose (1 mg mL^{-1}) was prepared. To the 1 mL aqueous solution containing 20-60 µg of D-glucose, 1 mL of 5% (v/v) phenol was added and mixed well. Then 5 mL H_2SO_4 (96%, v/v) was added with thorough mixing. Standards and blank solutions were prepared simultaneously. The solutions were allowed to stand for 30 min when a yellow-orange color developed. The absorbance of these solutions was measured at 490 nm. A standard curve for D-Glucose was prepared using these absorbance values. The carbohydrate content of the purified protease AG2 having the protein concentration 1 mg mL^{-1} was calculated using this standard curve.

Protease activity assay

Caseinolytic assay

Proteolytic activity of the purified enzyme AG2 was measured using denatured natural substrates like casein[19]. A stock solution of the casein was prepared by dissolving 500 mg casein in 50 mL of 0.1 M Tris-HCl buffer, pH 8.1. The assay was carried out by adding 100 µL of purified protease to the 1 mL of 1% casein solution. The reaction mixture was incubated at 30°C for 20 min and the reaction was stopped by the addition of 3 mL 10% (w/v) trichloroacetic acid (TCA). After 10 min the precipitate was removed by centrifugation at 10000 rpm for 5 min. The absorbance of the filtrate was measured at 280 nm. One unit of enzyme activity was defined as the amount of enzyme, under given assay conditions which give rise to an increase of 0.001 unit of absorbance at 280 nm min^{-1} of digestion. The number of units of activity mg^{-1} of protein was taken as the specific activity of the protease.

Azoalbumin assay

A stock solution of the azoalbumin (1%) was prepared in distilled water. Now the mixture of Tris-HCl buffer, pH ~8.1, and azoalbumin and protease solution was incubated for 1 h at 30°C. The reaction was terminated by the addition of 0.5 mL of 5% TCA. After 10 min, it was centrifuged at 10000 rpm for 5 min. Now 0.5 mL of the supernatant after TCA precipitation was mixed with an equal volume of 0.5 M NaOH and incubated for 15 min. The development of color was measured spectro-photometrically by measuring the absorbance at 440 nm[20,21]. Each assay was done in triplicate.

Esterase activity

The esterase activity of purified protease was measured spectrophotometrically at pH ~8.2 using N-α-p-tosyl-L-arginine methyl ester (TAME) as a substrate[22]. The hydrolysis of the substrate was studied by measuring the absorbance at 247 nm using 0.05 M of Tris-HCl buffer (pH ~8.2) and 0.01 M TAME. One protease unit was expressed as one micromole of TAME hydrolyzed min^{-1} of reaction.

Amidase activity

The amidase activity of purified protease was measured spectrophotometrically at pH ~8.2 using N-α-p-benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPNA) as a substrate with the help of Hitachi spectrophotometer[23]. The hydrolysis of the substrate was studied by measuring the absorbance liberated p-nitro aniline at 410 nm using 0.05 M Tris-HCl buffer, pH ~8.2, 1 mM BAPNA solution and 30% (v/v) glacial acetic acid.

Kinetic parameters

For the protease activity, Kinetic parameters V_{max} and K_m values were determined by using casein as a substrate according to Lineweaver-Burk's method[24]. The 30 µL of that protease solution having concentration 3 mg mL^{-1} was added to the varying amount of 1% casein solution. The total volume of the solution was adjusted to 1 mL by adding 0.1 M Tris-HCl buffer, pH ~8.1. These reaction mixtures were incubated for 30 min at 30°C. The reaction was stopped by the addition of 1.2 mL 10% (w/v) trichloroacetic acid (TCA). The velocity of the reaction was determined spectrophotometrically at 280 nm and it was defined as an increase of 0.001 unit of absorbance at 280 nm min^{-1} of digestion by the one milligram of protease under given assay conditions[25].

Effect of various inhibitors on the activity of AG2

Effect of metal ions

The effect of various metal ions on the enzymatic activity of the protease was determined by incubating the solution of the purified protease with different metal salt solutions[26]. The 10 mM stock solution of different metal ions (Ca^{2+}, Mg^{2+}, Ba^{+2}, Cu^{+2}, Co^{2+}, Fe^{+3} and Zn^{2+}) were prepared. The concentration of protein solution was kept at 1 mg/mL. To the 100 µL protein solution, an equal volume of metal salt solution was added and incubated for 30 min at 30°C. Each solution containing the protease and metal ions were assayed with casein acting as a substrate. A control assay for the measuring of protease activity was done without the metal ions and the resulting activity was taken as 100%.

Effect of inhibitors, surfactants, and oxidizing agents

The influence of different kinds of protease inhibitors like EDTA, iodoacetamide, and phenylmethylsulfonylfluoride (PMSF) on protease activity was tested by incubating the compounds with protease AG2 in the assay buffer 0.1 M Tris-HCl buffer, pH 8.1 for 1 h at 30°C prior to the addition of the substrate. Enzyme activities were then measured by using casein as a substrate. The influences of different surfactants like SDS, Tween 80, Triton X-100 and an oxidizing agent (H_2O_2) on enzyme activity were measured following the above method.

pH and temperature optima

The pH and temperature affect the activity of a protease. Therefore the activity of the protease (AG2) was measured by incubating the protease at different pH and temperatures. For the pH range 6-7, NaH_2PO_4-Na_2HPO_4 buffer and for pH ranges 8.0-11.0 glycine-NaOH buffer solutions were used and the residual activities were measured by caseinolytic assay method.

Similarly, the effect of temperature on the activity of the protease AG2 was measured by incubating the enzyme solution in the temperature controlled water bath in the temperature range 20-80°C for 15 min and then the residual activities of the enzyme were measured by caseinolytic assay method at the same temperature.

Autodigestion

Generally, proteases are susceptible to autodigestion and the extent of this autodigestion depends on factors like pH, temperature, enzyme concentration, incubation time and activator/inhibitor if any. In our study, the extent of autodigestion of the protease AG2 was monitored at 37°C after incubating the protease in the concentration range 0.05-1.0 mg mL^{-1} in 50 mM Tris-HCl buffer, pH 8.0 for 24 h. An aliquot of the protease was used for the determination of the residual protease activity using casein as a substrate. The activity of the protease after first 15 min was taken to be 100% for the calculation of the residual activity.

Fluorescence study

Tryptophan fluorescence study with native protease AG-2 was carried out with a Shimadzu spectrofluorophotometer (RF-540) at 25°C using a quartz cuvette of 1 cm path length. The slits for excitation and the emission were set at 2/1 for the protease. The concentration of the protease solution used for this study was 0.3 mg/mL. The excitation was done at 295 nm and emission wavelength was fixed in the wavelength range 300-400 nm. The microenvironment of the tryptophan residue of AG-2 was investigated by using different neutral (acrylamide) and ionic quenchers (copper sulphate and potassium iodide) with Stern-Volmer equation[27].

$F_0/F = 1 + K_{SV}[Q]$; Where F_0 and F are the fluorescence intensities in the absence or presence of quencher, respectively, K_{SV} is the quenching rate constant and [Q] is the concentration of quencher.

Acrylamide solution (2 µL) from a stock of 0.5 M solution was added gradually to 2.6 mL AG-2 (1 mg/mL) solution and the fluorescence intensities were measured. Similar studies were done with potassium iodide (stock 0.5 M) and copper sulphate (stock 0.03 M) solution.

Circular dichroism study

The circular dichroism (CD) spectra of native AG2 were recorded on a Jasco-815 CD spectropolarimeter (Jasco, Tokyo, Japan). The secondary structures of the protein were determined using far-UV CD spectra in the region 200-250 nm at 30°C in a 10 mm path-length quartz cuvette with a protein concentration of 0.2 mg mL^{-1}. Typical instrumental parameters were bandwidth: 1.0 mm and scan speed 20 nm/min. Each spectrum was an average of three consecutive scans. The secondary structural contents (α-helix and β-sheets) of the protein were calculated using CDNN 2.1 software.

Tryptic digestion of protease AG2 for MS analysis and de-novo sequencing

To the 20 µL of purified protease solutions, 6µL 100 mM DTT and 34 µL double distilled H_2O were

added in order to reduce the disulfide bonds. The mixture was incubated at 56°C for 45 min and then allowed to cool to room temperature. Then they were alkylated by incubation with 55 mM iodoacetamide for 30 min at room temperature in dark. A working solution of trypsin was prepared using sequencing grade trypsin from Promega (USA). To these alkylated samples, trypsin solution (concentration 0.1 µg/µL) was added at a 1:50 ratio (trypsin: protein) and gently flick to mix. The samples were placed on a 37°C water-bath and continue to incubate for 17-18 h. The reaction was stopped by adding 5 µL 10% TFA in the digested solution. From this solution, 5 µL of the sample was taken and 5 µL of CHCA (α-cyano-4-hydroxycinnamic acids) was added to it and 1-2 µL was applied on the MALDI target plate and air dried. MALDI MS studies were carried out by Bruker Daltonics Autoflex II TOF/TOF mass spectrometer, at Bose Institute, Calcutta, India. External calibration standard prot-mix from Bruker was used to calibrate the instrument daily. Mass spectra were acquired in the linear mode in the m/z range of 4000. For each spectrum, 200 laser shots were summed and three spectra were averaged for each sample. MASCOT search tool (URL http://www.matrixscience.com) was used for identification of tryptic maps. MS/MS fragmentation study was performed with some selected peptides to determine the partial amino acid sequence of AG-2. MS/MS was achieved by 1 or 2 kV collision induced dissociation (CID) in positive ion mode. The raw data was accessible in BioTools 3.0. The RapiDeNovo module in BioTools was used for *de-novo* sequencing, resulting in sequence tags. In combination with the peptide mass, these were then used to automatically create peptide sequence proposals.

N-Terminal amino acid sequencing

The sequence of first 17 amino acid residues from the N-terminal end of the protease AG2 was determined by Edman's automated degradation with a Shimadzu PPSQ-31A/33A Protein Sequencer analyzing the PTH-amino acid in isocratic mode with RP HPLC. The N-terminal sequence of AG2 was compared with other plant serine proteases using BLAST search.

Results and Discussion

Purification of the protease

The purification procedure involved extraction, ammonium sulphate precipitation, anion-exchange chromatography on DEAE-cellulose and finally on SP-Sephadex C-50 column. Results of the purification showing the recovery, a fold of purification and the specific activity at each step starting from 200 g of ash gourd seeds were shown in Table 1. The chromatographic profile of DEAE-cellulose column was shown in Fig. 1A. The fraction coming out from SP-Sephadex C-50 column was resolved into two peaks (Fig. 1B). Both the peak showed the proteolytic activity. The second peak having higher proteolytic activity and greater protein content is designated as AG2. The protein content in the isolated protease

Table 1 — Purification of ash gourd seed serine protease

Stage	Total Protein (mg)	Total activity (U)	Specific Activity (U/mg)	Purification Fold factor	Recovery %
Crude extract from $(NH_4)_2SO_4$ precipitation	2938	7345	2.5	1	100
DEAE- Cellulose	640	2480	3.875	1.55	34
SP-Sephadex C-50	60	1398	23.3	9.32	19

Table 2 — Comparison of some physicochemical properties of AG2 with other plant subtilisin-like proteases

Plant source	MW (kDa) of monomer	Optima		Stability		Glycoprotein
		pH	Temp (°C)	pH	Temp (°C)	
AG2 from seed of *B. hispida*	11.2	10	30	7-11	70	No
W. tinctoria[a]	57.9	7.5-10	70	5.5-11.5	75-80	Yes
E. milli (milin)[b]	31.5	8.0	60	nd	nd	Yes
T. officinale[c]	67	8.0	40	6-9	40	Yes
C. melo[d](cucumisin)	54	7.1	70	4-11	50	Yes
C. trigonus[e]	67	11.0	65	4-10	65	nd
B. hispida[f]	67	10.0	60	7-11	70	nd

[nd = not determined; a=11; b= 29; c=37; d= 5; e= 7; f= 14]

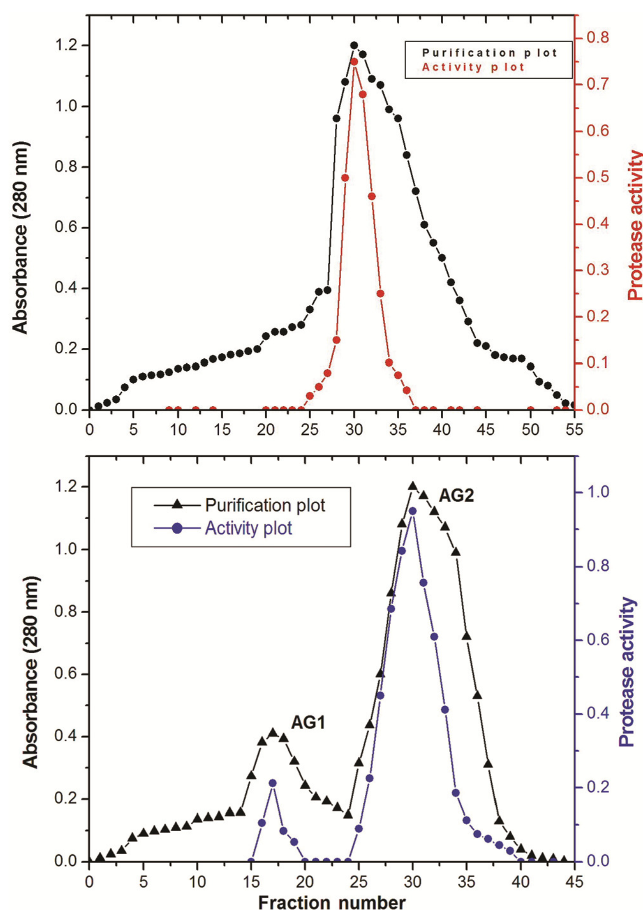

Fig. 1— (A) Elution profiles of ammonium sulphate extract of ash gourd seeds on a DEAE-cellulose (2.6 × 30 cm) column. The unbound protein was eluted with 0.04 M sodium phosphate buffer, pH 7.5 at a flow rate 20 mL/h. Absorbance at 280 nm for each fraction was shown by black circles and protease activity was assayed with casein (red circles) as a substrate. Fractions with protease activity were collected at the flow rate of 20 mL/h, (B) Elution profiles of ash gourd serine protease from SP-Sephadex C-50 column (2 × 45 cm) pre-equilibrated with 0.02M sodium phosphate buffer, pH-7.0. The flow rate for elution was 16 mL/h. Absorbance at 280 nm for each fraction was shown by black triangles and protease activity was assayed with casein (blue circles) as a substrate. Fractions under second peak (AG2) with high protease activity were collected

(AG2) from ash gourd seed extract is high (Table 1) and it is comparable to that of cucumisin, a subtilisin-like serine protease from melon fruit[5]. The test tubes under these peaks were collected separately and lyophilized. The content of the purified protease (AG2) was estimated according to the method of Lowry[28]. In brief, the procedure used for the purification of protease AG2 is very simple and cost effective[29].

Homogeneity and zymography study of purified protease

The purified protease AG2 appeared as a single subunit of the mass of about 11 kDa in 20% SDS-PAGE both in presence and absence of 2-mercaptoethanol (Fig. 2A), this confirms the homogeneity in preparation.

Zymography is an efficient technique to study the protease activity in polyacrylamide gel. Gelatin zymography was performed with the native protease AG2. The protease activity was visualized as clear (unstained) areas of digested gelatin against a blue background following Coomassie blue staining. The gelatin zymographic pattern of AG2 was shown in Fig. 2B.

Mass spectrometry

Mass spectrometric analysis of the protein AG2 by MALDI-TOF experiment confirmed the homogeneity as well as the mass of the single monomeric unit up to m/z ratio 16000 Da. Fig. 2C shows the appearance of a single peak having the exact mass 11211.1 Da. The result showed good agreement with that obtained by SDS-PAGE. The reported molecular masses of the plant serine proteases were in the range of 20-120 kDa and those from Cucurbitaceae family were in the region of 50-80 kDa. Since all the serine proteases reported so far are monomeric except a few[30], AG2 isolated from ash gourd would be a low molecular weight protease.

Carbohydrate content

Attempts were made with the protease AG2 to determine the neutral sugar content by Dubio's method[18] and also amino sugar content by Spiro's method[31]. No neutral and amino sugars were found in the protease AG2, indicating that protease was not a glycoprotein like Cucumisin and Cucumisin-like other serine proteases isolated from Cucurbitaceae family.

Substrate specificity

The specific activity values of protease AG2 in the presence casein, azoalbumin and TAME were 274.4 U/mg, 68 U/mg and 23.3 U/mg but it had no amidase activity hence it is specific for the substrates[32]. The K_m and V_{max} values calculated using casein as substrate were 0.117 ±.00067 mM and 470.592 ± 0.631 units/mg/min respectively. The protease AG2 thus showed greater substrate affinity than the plant serine protease, Wrightin.

Protease activity in presence of various inhibitors

Protease specific inhibitors were examined to establish the mechanistic class of purified protease AG2. Results showed that EDTA, IAA had no effect on its activity. The phenylmethylsulphonyl fluoride (PMSF), a general inhibitor of a serine protease,

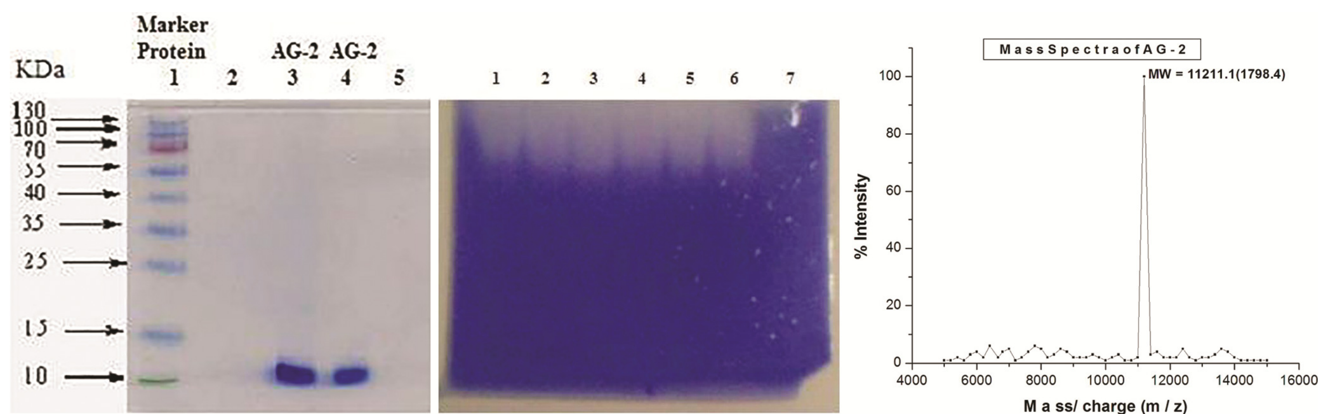

Fig. 2 — (A) Assessment of homogeneity and molecular weight of purified protease (AG2) from *Benincasa hispida* by SDS-PAGE: Lane-1: M_r Marker proteins, Lane-3: 15 µg of purified AG2 in presence of 2-MCE, Lane 4: 15 µg of purified AG2 in absence of 2-MCE, Lane 2 and 5: blank solution (sample loading buffer only); (B) 20% Gelatin zymography study with the purified protease AG2. Lane 1, 2, 3, 4, 5 and 6: purified protease AG2; Lane 7: blank solution (sample loading buffer only) and then subjected to native PAGE analysis. The gel was incubated in 10% 0.05 M Tris-HCl, pH-7.6 buffer for 24 h; (C) MALDI- mass spectra of the purified protease AG2 carried out in a Bruker Daltonics Autoflex II TOF/TOF mass spectrometer. External calibration standard prot-mix from Bruker was used to calibrate the instrument daily. Matrix used was α-cyano-4-hydroxycinnamic acid and data were collected in a linear mode

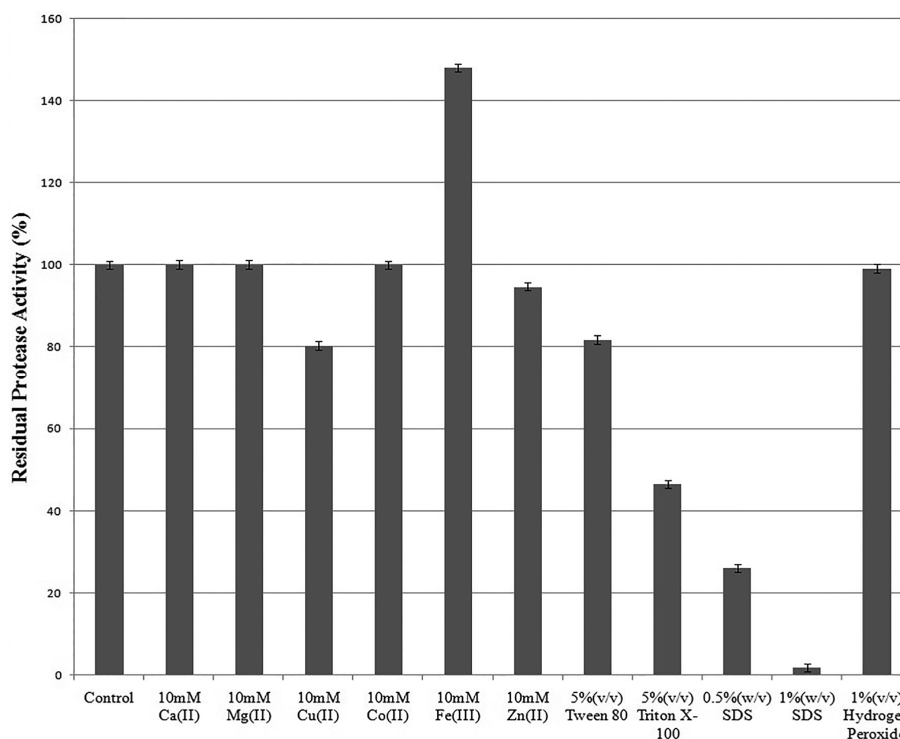

Fig. 3 — The bar diagram represents the residual activity of the protease AG-2 after incubation with different metal ions like Ca^{2+}, Mg^{2+}, Ba^{+2}, Cu^{+2}, Co^{2+}, Fe^{+3} and Zn^{2+}, surfactants like Tween 80, Triton X-100 and SDS and oxidizing agent H_2O_2. [Protease activity using casein as a substrate in absence of metal ions, surfactant and oxidizing agent was considered as a control]

strongly inhibited its protease activity but no inhibition with soyabean trypsin inhibitor (STI) which is abundantly present in different protein-rich foods. These results ruled out the possibility of the existence of trypsin-like character of the AG2. The inhibition profile classified the AG2 as a member of the subtilisin-like class of serine protease.

Protease activity in presence of metal ions, surfactants, and an oxidizing agent

Main group metal ions like Ca^{2+} and Mg^{2+} ions had no influence on the protease activity of AG2. But

transition metal ions like Cu^{2+} (residual enzymatic activity ~80%), Zn^{+2} (residual enzymatic activity ~94%) slightly inhibited the enzymatic activity[33] whereas about 48% increased of activity was observed in presence of Fe^{3+}. The enzyme was highly stable in presence of non-ionic surfactant Tween 80 (residual enzymatic activity ~82%) and moderately stable in Triton X-100 (residual enzymatic activity ~47%) but the enzyme was highly stable in presence of an oxidizing agent, H_2O_2. It retained 99% of its protease activity in 1% (v/v) H_2O_2 after 1 h incubation. On the other hand, the protease activity of AG2 was strongly inhibited in presence of 1% (w/v) SDS (anionic surfactant) while it retained ~30% enzyme activities in 0.5% (w/v) SDS solution after 1 h of incubation (Fig. 3). The inertness in presence of the oxidizing agent and high stability in presence of non-ionic surfactant the protease appears to be a potential candidate for industrial use[34].

pH and temperature optima

The protease activity of AG2 at pH range from 7 to 11 was measured spectrophotometrically using casein as a substrate. The protease activity gradually increased up to pH 10.0 and then decreased at higher pH showing optimum pH at 10.0. It retained 75% of its initial activity at pH 11.0. The protease showed very low activity in the pH range 4 to 6. Thus the purified protease was more stable in the alkaline pH. The protease activity of the AG2 was monitored in the temperature range 20°C to 80°C. The protease was active over a wide range of temperature (20-60°C), showing its optimum activity at temperature 30°C. The activity rapidly decreased as the incubation temperature increased higher than 60°C. It retains ~30% of initial activity at 70°C. Its optimum pH and temperature are comparable to those of subtilisin-like other plant serine proteases (Table 2).

Autodigestion

Generally, proteases are susceptible to autodigestion causing great difficulty in their storage or their industrial applications. Autodigestion depends on protein concentration, pH, temperature and time.

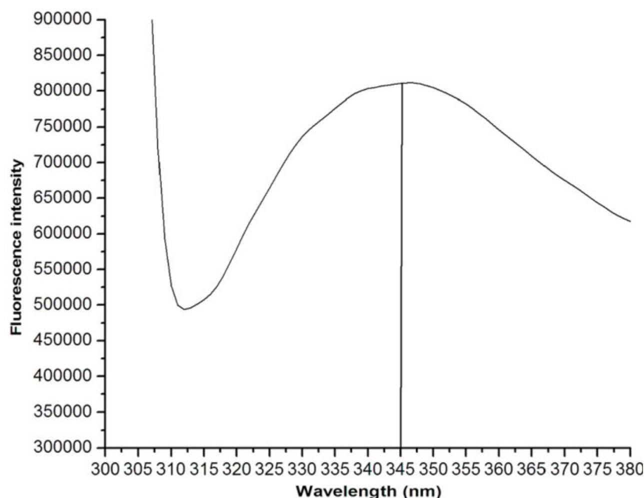

Fig. 4—Fluorescence emission spectra of the native protease AG-2. Protein solution was prepared in water (1 mg/mL) and the spectrum was recorded within the range of 300-380 nm at room temperature. The excitation wavelength was 295 nm

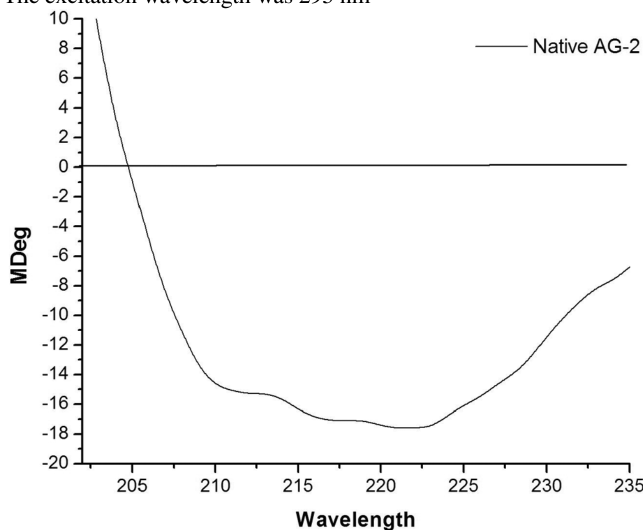

Fig. 5 — Far UV-CD spectral pattern of the protease AG2 in the native condition in water at 30°C keeping the protein concentration 0.2 mg/mL. [All the data points were an average of three consecutive scans and correspondingly blank corrected]

Table 3 — Comparison of the N-Terminal sequence of AG2 with other known plant serine proteases

Protease	Amino Terminal Sequence (first 17 residues)																
AG2	M	Q	Q	F	F	N	E	P	S	S	L	**L**	I	**V**	V	**V**	**R**
Cucumisin	T	T	R	S	W	D	F	L	G	F	P	**L**	T	**V**	P	R	**R**
White gourd	T	T	R	S	W	D	F	L	N	F	P	Q	N	I	Q	**V**	V
Tomato P69A	T	T	H	T	S	S	F	L	G	L	Q	Q	N	M	G	**V**	M
Lily LiM9	T	T	H	T	P	D	Y	L	G	I	Q	T	V	W	G	**V**	W
Alnus AG 12	T	T	H	T	P	R	F	L	S	L	N	P	T	G	G	L	W
SP NSP82	T	P	R	T	P	Q	F	L	G	L	A	E	S	V	F	L	V

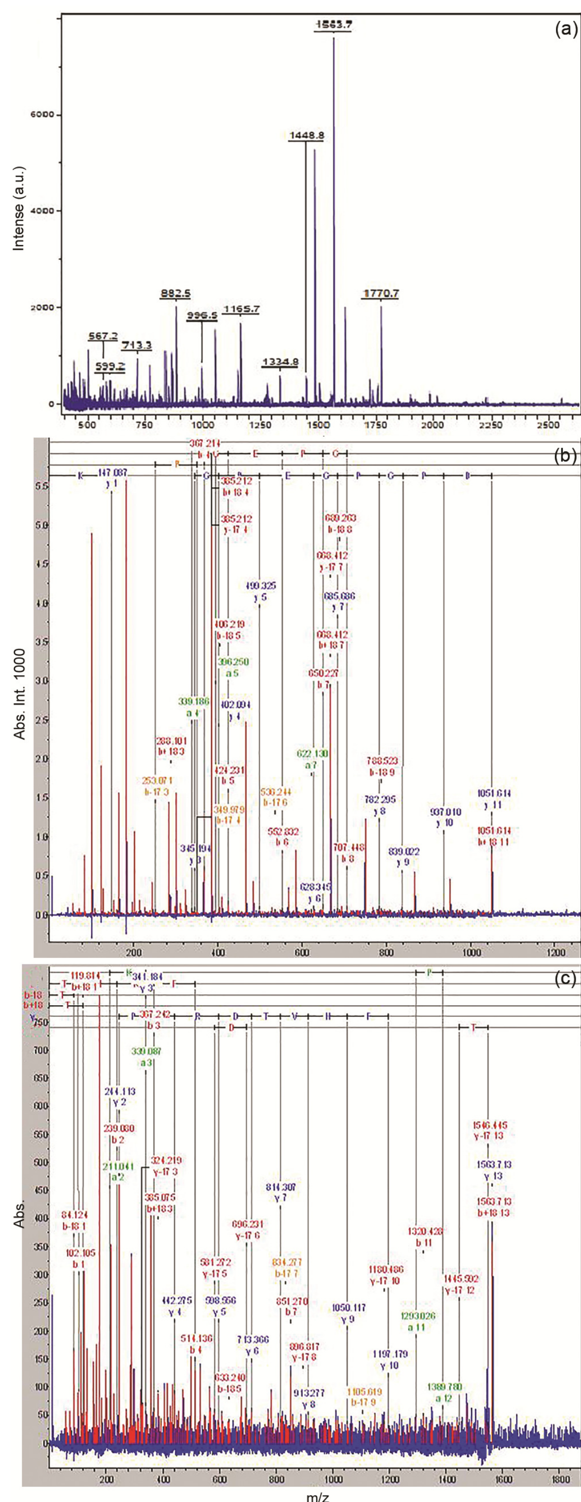

Fig. 6—(A) MALDI-TOF mass spectral analysis of tryptic digested fragments of protease AG2. The α-cyano-4-hydroxycinnamic acid (CHCA) was used as a matrix; (B) The amino acid sequence of the peptide 1051.614 Da was derived by *de-novo* sequencing technique by using BioTool 3.0 software; and (C) The amino acid sequence of the peptide 1563.697 Da was derived by *de-novo* sequencing technique by using BioTool 3.0 software

In our study of autodigestion very small activity has been lost in lower concentrations (the protease retains more than 90% of activity at 0.05 mg mL^{-1} concentration) of the protease. During our work, we have noticed that the protease AG2 is stable at 4°C and does not lose activity even after one year.

Fluorescence study

Fluorescence study of native protease AG-2 showed normal emission spectra of tryptophan with an emission maximum at 345 nm after excitation at 295 nm. The result indicated the partial exposure of tryptophan moiety toward solvent (water) molecule or the influence of the microenvironment around excited fluorophore of the protease shifted the emission maxima towards the longer wavelength (Fig. 4). The linear Stern-Volmer plots were obtained for both the neutral (acrylamide) and an ionic quencher (copper sulphate and potassium iodide) indicating accessibility the fluorophore of AG-2 to both the neutral and ionic quencher. The quenching rate constant (K_{SV}) values calculated for the acrylamide, copper sulphate and potassium iodide were 16.43 M^{-1} sec^{-1}, 7846.36 M^{-1} sec^{-1} and 8.88 M^{-1} sec^{-1} respectively. The results indicated that the microenvironment of the tryptophan was strongly affected by the cationic quencher.

Circular dichroism study of native protease AG2

The circular dichroism spectrum of the purified protease AG2 was recorded in water at 30°C in far-UV (200-250 nm) region. The far-UV spectrum showed negative troughs at 210 nm and 222 nm along with a small minimum at 215 nm which indicated the presence of both the α-helix and β-sheet structures in the protease (Fig. 5). The secondary structural contents as estimated from the CD spectra with CDNN 2.1 software were 28.4 % α-helix, 19.6 β-sheet structure and 17.8 % β-turn. Thus the protease AG2 was a type of α/β class of proteins[35]. The native protease showed well integrity in its secondary and tertiary structure.

Mass spectral analysis and partial amino acid sequences of the trypsin-digested fragments

The mass spectral pattern of the different trypsin-digested fragments of the proteases AG2 was shown in Fig. 6A. MASCOT search tool (URL http://www.matrixscience.com) was used for identification of protein mass fingerprint (PMF) analysis. No matches were found with other plant serine proteases. Protein identification and

differentiation by PMF has been adopted as an effective tool to differentiate the proteases with very similar physicochemical and functional properties[36]. Hence to find out the partial amino acid sequence of the protease AG2, the *de-novo* sequencing technique using BioTool 3.0 software was applied to the MS/MS spectrum of the peptides 1051.614 Da and 1563.697 Da. The partial amino acid sequences of the protease AG2 were the followings- DPGPGEPGVVK and THKFHVTDRTTPK (Fig. 6B & C). With these sequences, BLAST searches (blastp) were performed but no matches were found with other plant serine proteases. Hence, the results indicate that the partial amino acid sequence of the AG2, belonging to a Cucurbitaceae family, is unique in nature. No putative conserved domains have been detected using these sequences.

N-terminal sequence

The sequence of 17 amino acid residues from the N-terminus (MQQFFNEPSSLLIVVVR) of AG2 was determined and compared with other plant serine proteases (Table 3). Very little sequence homology is observed with other serine proteases like alnus ag12, lily LIM9, tomato P69 A, SP NSP 82, Arabidopsis ARA12 while greater homology (nearly 20%) is observed with cucumisin protease[10]. AG2 contains Met (M) residue at the N-terminal while it contains Val (V) at the position 14, common in most serine proteases including AG2.

Conclusion

This is the first report of the purification and partial characterization of a low molecular weight subtilisin-like serine protease AG2 from the seeds of *Benincasa hispida* of Cucurbitaceae family. The raw material (plant seeds) is easily available and its protein content is very high. The purification procedure is very simple and economic. All these things may be useful for the large-scale production of the protease for its industrial application. As the protease is stable in presence of a non-ionic surfactant, an oxidizing agent and as well as in the wide range of temperature and high pH, it (AG2) may be a potent candidate for detergent formulation. Knowledge of N-terminal amino acid sequence and partial sequence of AG2 will help to understand the structure-function relationship and finding genetic approaches to generate mutant protease with improved physicochemical properties.

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