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# Partial properties of an aspartic protease in bitter gourd (*Momordica charantia* L.) fruit and its activation by heating

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**Abstract**

Bitter gourd (BG fruit) is usually heated in hot water to reduce bitterness and improve flavour before being served. Protein extract from BG was analyzed for protease activity by gelatin-gel electrophoresis. The study showed that the proteolytic activity in BG flesh was enhanced by heat-treatment at temperatures ranging from 50 °C to 75 °C. An aspartic protease (AP) was characterized by gel electrophoresis. The optimal AP activity was at pH 7; the pI of the AP was demonstrated to be 4.8; the protein molecular weight of the BG–AP was estimated to be 60 KD by SDS–PAGE. The AP was implicated in the proteolysis of the photosynthetic enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase.

The AP was further purified and submitted for analysis of peptide mass fingerprint (PMF). The Mascot peptide mass fingerprint of the AP protein hit no existing protein (score > 60), and it proved to be a novel AP.


*Keywords:* Bitter gourd; Aspartic protease; Heat-treatment; Activation; SDS–gelatin–PAGE

## 1. Introduction

Protein degradation in plants has been linked to different developmental stages, such as germination, differentiation and morphogenesis, senescence, and programmed cell death. In certain circumstances, proteolysis is also associated with various stresses (Palma et al., 2002). Thus, the role of proteases in plant development is essential.

Protein degradation in plants is a complex process involving a multitude of proteolytic pathways that can be catalyzed by different classes of proteases. Aspartic proteinases (APs; EC 3.4.23) have been found in seeds, tubers, flowers and petals of many species (Simoes & Faro, 2004). Plant APs are specifically inhibited by pepstatin and have two aspartic acid residues responsible for the catalytic activity. Most plant APs so far identified are synthesized as preproenzymes and are subsequently converted to mature enzymes (Dunn, 2002; Rawlings & Barrett, 1995), and show preferential specificity for cleavage at peptide bonds between hydrophobic amino-acid residues. Information is very limited on the biological function of APs in plants (Voigt, Biehl, Heinrichs, & Voigt, 1997). It has been suggested that an AP could be involved in the digestion of insects in Nepenthes (An, Fukusaki, & Kobayashi, 2002), in the degradation of plant proteins in response to pathogens (Xia et al., 2004), and during development processes and senescence (Chen & Foolad, 1997).

Proteolysis during plant senescence is well documented, but little is known about the proteases involved in the response to processing of vegetables and fruit. Heat-treatments (thermotherapy) have been used for over a century to increase storage life and improve the flavour

*Abbreviations*: AP, aspartic protease; BG, bitter gourd; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfatepolyacrylamide; RA, relative activity.

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of a number of fruits. However, serious injury to the host may occur if temperatures are too high or the treatment period too long. There are many hypotheses to explain heat injury. Most of the concepts and experimental evidence involve protein denaturation, disruption of protein synthesis and loss of membrane integrity (Paull & Chen, 2000).

Protein denaturation at lethal temperatures is regarded as non-reversible, while lower temperatures can lead to a reversible inactivation (Bernstam, 1978). However, the temperatures and exposure times involved in protein denaturation *in vitro* are considerably above those used in post-harvest treatments and may not apply to the same protein *in vivo*. Less than lethal temperatures and duration can lead to short-term reversible disruption of transcription and translation steps in protein synthesis.

Bitter gourd (*Momordica charantia* L.) (BG) is a cucurbit, native to Asia, and now widely cultivated in tropical areas, including parts of the Amazon Basin, Africa, Asia, the Caribbean, and South America for the immature fruits, and sometimes for the tender leafy shoots or the ripe fruits. The immature fruit, called bitter melon, bitter gourd or balsam pear, harvested at developmental stages up to seed hardening, is sliced for use as a vegetable. Bitter gourd fruits, used as an alternative therapy for diabetes for a long time, and recently proposed as an antiviral and antineoplastic agent (Basch, Gabardi, & Ulbricht, 2003), should attract great interest because of their nutritional and medicinal properties. To reduce bitterness and improve flavour, BG fruit is usually heated in hot water and then served for meals. Heat-treatment can greatly inactivate the enzymes that may reduce the quality of BG fruit; however, it can also activate enzymes, such as proteases. Little is known about the BG protease and how the BG protease may be influenced by high temperatures.

We have used a previously developed analysis system (Jiang, Lers, Lomaniec, & Aharoni, 1999) to detect whether or not proteases are affected in BG fruit during heat-treatment and to determine any related biochemical changes, particularly during proteolysis in the fruit. Our study showed that an aspartic protease in bitter gourd was activated by the heating treatment.

## 2. Materials and methods

### 2.1. Plant materials and treatments

Green bitter gourd (*Momordica charantia* L. cv. Wanningqinglv) was obtained from a local market in Beijing. The fruits used in the experiment were selected for uniformity and freedom from defects.

The flesh of bitter gourd (BG)was cut into slices (about 1 cm wide) along the longitudinal axis after removing the core tissue. After being incubated at 50 °C, 55 °C, 60 °C, 65 °C, 70 °C, 75 °C, 80 °C or 85 °C in water for 5 min or the durations as defined in the text, soluble proteins were extracted from the BG tissues immediately after the treatments or incubation, as indicated in the Results section.

### 2.2. Extraction and measurements

Ten gram of the BG flesh were ground in 10 ml of 50 mM Tris–HCl buffer, pH 7.5. The homogenate was centrifuged at 12,000*g* for 20 min at 4 °C and the resulting supernatants were used for the assays (Jiang et al., 1999).

Chlorophyll contents of in the BG samples were measured according to the method of Jiang, Sheng, Zhou, Zhang, and Liu (2002).

### 2.3. Proteolytic activity

Protease activities in the extract were assayed essentially as described by Jiang et al. (1999), with some modifications. Regular SDS–PAGE (Laemmli, 1970) was performed with a mini-gel apparatus (gel size: 60 × 70 × 1 mm; 10 lanes) except that the 7.5% resolving gel contained 0.1% (w/v) gelatin. Protein samples (20 μl each lane) were mixed with an equal volume of a non-reducing sample buffer [0.1 M Tris–HCl, pH 6.8; 2% (w/v) SDS, 10% (v/v) glycerol, 0.01% bromophenol blue] and incubated at 37 °C for 10 min before loading on the gel. Following electrophoresis at 4 °C, the gel was washed in a renaturing buffer (2% Triton X-100, 50 mM Tris–HCl, pH 7.0) for 20 min. Protease activities were investigated by incubating the gel in 50 mM Tris–HCl, pH 7.5, for 7 h at 37 °C, which is the optimal temperature for protease catalyzing in the gel according to our primary experiment. After staining the gel with Coomassie blue (0.05% Coomassie blue, 10% acetic acid, 30% ethanol) and destaining with a solution of 10% acetic acid and 10% ethanol, white bands, representing the site of protease activity, were visualized.

### 2.4. Determination of optimal pH

For determination of optimal pH, multiple protein samples of BG were fractionated on gelatin-gels as described above. Following electrophoresis and renaturation, single-lane gel slices were incubated at 37 °C for 4 h in different Tris–HCl buffers ranging in their pH from 4 to 11. Staining was carried out as described above.

### 2.5. Protease inhibitor assay

Effects of various protease inhibitors on the BG protease were tested to determine the mechanistic class of the protease. Following electrophoresis and renaturation, gel slices containing the BG protease were incubated in buffer (50 mM Tris–HCl, pH 7.5) with the defined inhibitor at 37 °C for 7 h. Inhibitors included pepstatin A (10 mM, Sigma), EDTA (4 mM), PMSF (2 mM, Sigma), E-64 (10 mM, Sigma), and Leupeptin (100 μM, Sigma).

### 2.6. Assay of effect of heat-treatment on the sample

Effect of pre-treatment temperature on the samples was examined by incubating the extract with the sample buffer

at 37, 45, 50, 55 °C for 10 min before loading on the gel. The electrophoresis and assay of protease activity were carried as described above.

### 2.7. Isoelectric focusing electrophoresis

An apparatus for IEF (model DYCZ37B, Beijing Liuyi Scientific Equipment Ltd, China) was used for running the gels, as specified by the manufacturer, with ampholyte (pH range 4–9). When the electrofocussing was finished, the gel was sliced, from anode to cathode, into pieces about 0.5 cm wide. The pH and protease activity in each of the fractions were determined.

### 2.8. Analysis of effect of the protease on Rubisco

The protein extract from spinach leaves was fractionated on SDS–PAGE as described above and the protein of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) was electric-eluted out. Then the extracted Rubisco was incubated with the partial purified BG protease (as described below) for 0, 0.5, 1, 1.5 or 2 h at 45 °C, respectively, and was analyzed by regular SDS–PAGE.

### 2.9. Partially purifying the protease

Our previous study showed that activity of the BG protease could be remarkably enhanced by heating the BG tissue. Therefore, the heat-treated BG (70 °C, for 5 min) was used for the extraction. About 10 kg of the heated BG with 300 ml of Tris–HCl buffer (1.5 M, pH7.5) were homogenized in a juice-extractor. The homogenate was filtered through cheesecloth and centrifuged at 12,000*g* for 20 min at 4 °C; the supernate was frozen at −50 °C, and loyphilized in a vacuum-lyophilizer (LGJ-10, Beijing). The concentrated extract (about 1 mg protein/ml) was treated with ammonium sulfate to 30% (w/v), and incubated for 4 h at 4 °C, the precipitate was collected after the mixtures were centrifuged at 12,000*g* for 10 min. The same procedure was repeated by adding 40, 50, 60, 70 or 80% ammonium sulfate (AS) to the supernatant, respectively. The precipitated fractions were dialyzed against Tris–HCl buffer (0.02 M, pH 7.5). The BG protease was enriched in the fraction of the 40% AS precipitate; thereafter, this fraction was used for the subsequent protease-assay.

### 2.10. Protein microsequencing and peptide mass fingerprinting

The partially purified BG protease (1 mg of total protein) was separated by SDS–PAGE and electro-eluted to a PVDF (polyvinylidene difluoride) membrane, visualized with Coomassie Blue, and submitted to a commercial institution for N-terminal sequence assay by Edman degradation with an Applied Biosystems sequencer (PROCISE491, ABI, USA).

For peptide mass fingerprinting, isolation of the protein was performed on 2DE electrophoresis (Bjellqvist et al., 1993). The target protein spots were excised from the gel, directly, and subjected to in-gel trypsin digestion (Shevchenko, Wilm, Vorm, & Mann, 1996). Mass spectrometry was carried out by the Proteome Centre at the University of China Medical on an AB Voyager MALDI/TOF mass spectrometer.

The obtained peak lists were submitted to the Matrix Sciences (http://www.matrixscience.com) programme for protein identification.

Additional match of the mascot peptide mass fingerprint of the BG protease with the other existing protease sequence in http://www.ncbi.nlm.nih.gov/ was also performed as in http://www.expasy.org/tools/findpept.html.

## 3. Results and discussion

### 3.1. Conditions for assay of the protease activity in bitter gourd

To identify proteolytic activities in the bitter gourd (BG), protein extract was resolved on SDS–PAGE containing gelatin substrate. Following renaturation, incubation period and protein staining, the proteolytic activity was visualized as clear bands in which the substrate was hydrolyzed. After the electrophoresis, protease activity was clearly observed in the gel.

To make sure that the proteins in the extract could be well separated with high protease activity, pre-tests were carried to find the optimal conditions for the protease catalyzing in the gel. The protease extract with the sample buffer was pre-treated at 37 °C, 45 °C, 50 °C or 55 °C for 10 min before being loaded for electrophoresis. Then activity of the protease in the gel was analyzed as described above. The protease activity was maximal in the sample pre-treated at 45 °C for 10 min (Fig. 1).

The optimal pH for assay of the BG protease activity in the gel was showed at pH 7 (Fig. 2).

### 3.2. Biochemical parameter of the BG protease

To determine pI of the BG protease, the protein extract of BG was separated in an IEF gel (Fig. 3a), then the gel

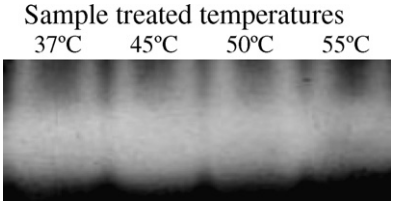


Fig. 1. Effect of pre-treatment temperature of the samples before the electrophoresis. The extracts from the 70 °C-heated BG were incubated with the sample buffer at the defined temperatures for 10 min before loading onto the gelatin-gel. After electrophoresis, the gel was incubated with reaction buffer (pH 7.5) at 37 °C for 7 h. Then, the gel slices were stained for protease activity.

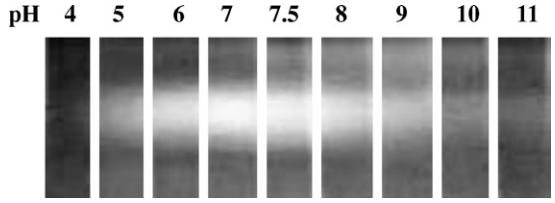


Fig. 2. Effect of pH of the reaction buffer on the protease activity. Gelatin-gel slices containing resolved protein extracted from 70 °C-heated BG were incubated with reaction buffer at the defined pH at 37 °C for 7 h. Then, the gel slices were stained for protease activity.

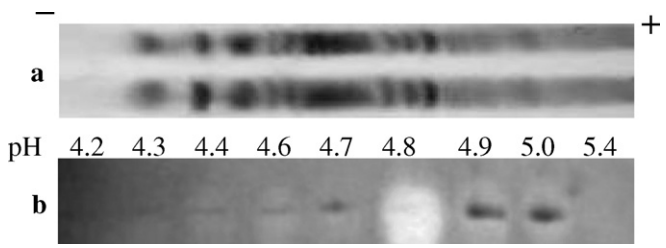


Fig. 3. Analysis of isoelectronic point of BG protease. The protein extracted from 70 °C-heated BG was electro-focussed; then the gel was sliced from anode to cathode into pieces about 0.5 cm wide. The pH and protease activity in each of the fractions were determined.

was sliced and assayed for protease activity. The pI of the BG protease was demonstrated to be 4.8 (Fig. 3b).

As shown in Fig. 4, the proteinase activity of BG was inhibited by pepstatin of the aspartic proteinase (AP) inhibitor, whereas it did not seem to be affected by the inhibitors cysteine, serine, or metal-proteinase. This result suggests that the proteinase of BG is an aspartic protease.

The proteolytic activity in the BG flesh was enhanced by heating BG fruit at temperatures ranging between 50 °C and 75 °C. The protease activity in the BG flesh was greatly reduced by the heating-treatment at 80 °C for 5 min, and was completely inhibited by the heating-treatment at 85 °C for 5 min (Fig. 5). Fig. 6 further shows that in the BG slices incubated at 45 °C, 50 °C, 55 °C, 60 °C, 65 °C or 70 °C for various times, activities of the protease also

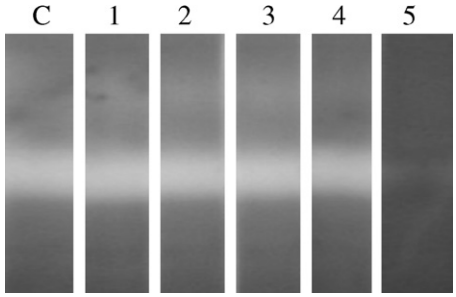


Fig. 4. Effects of various protease inhibitors on the BG protease. The performance is referred to in Materials and methods. Inhibitors: (1) metalloproteases (4 mM EDTA), (2) serine (2 mM PMSF), (3) serine or cysteine (100 μM leupeptin), (4) cysteine (10 mM E-64), and (5) aspartic proteases (10 mM pepstatin A). C-lane is the control without any inhibitor of protease.

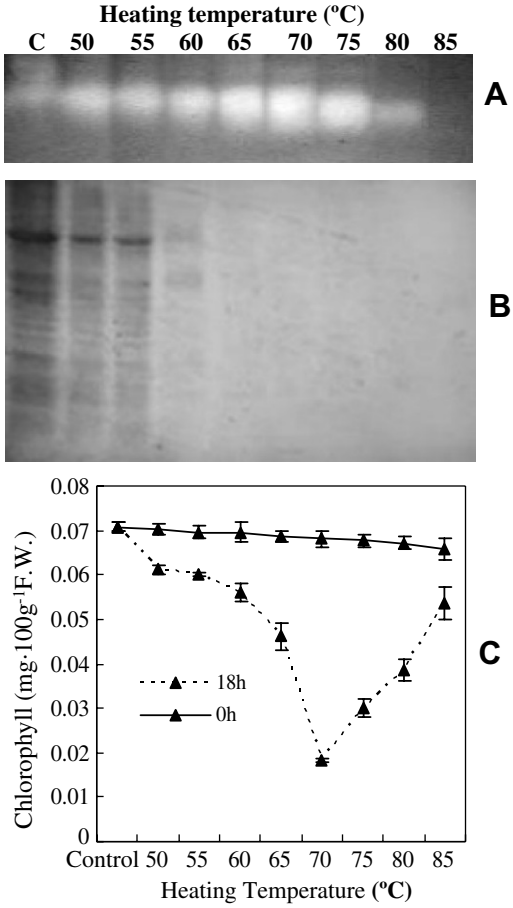


Fig. 5. Effects of heat-treatment on protease, soluble proteins and chlorophyll content in the BG flesh. Protein extractions were performed immediately after the BG flesh was incubated at 50 °C, 55 °C, 60 °C, 65 °C, 70 °C, 75 °C, 80 °C and 85 °C in water for 5 min. Then, the extracts were gel-assayed for protease activity (A) or resolved in SDS–PAGE (B). To evaluate the effect of heat-treatment on chlorophyll degradation (C), chlorophyll contents in the heated BG slices were determined immediately after the heat-treatment or after storage for 18 h at 25 °C. RA = relative activity (%).

increased with the time of heating. The relative activity (RA) of the protease increased almost 3 times in the sample heated at 45 °C for 150 min, and 50 °C and 55 °C for 90 min; the RA increased 4 times in the sample heated at 60 °C for 60 min or at 65 °C for 40 min. However, the RA only increased 2 times in the sample heated at 70 °C for 5 min, and increased even less in the sample heated at 70 °C for more than 5 min. Activation of protease by heat-treatment was also observed in leaves of lettuce, spinach, cole and celery (data not shown here).

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, in $C_3$ plants) represents most of the protein of the leaf tissue. After the protein extract from spinach leaves was incubated with a protease fraction partially purified from BG, as shown in Fig. 7, the large subunit (LSU) of spinach Rubisco was gradually degraded during the time of incubation, and was almost undetectable after being incubated at 45 °C for 2 h.

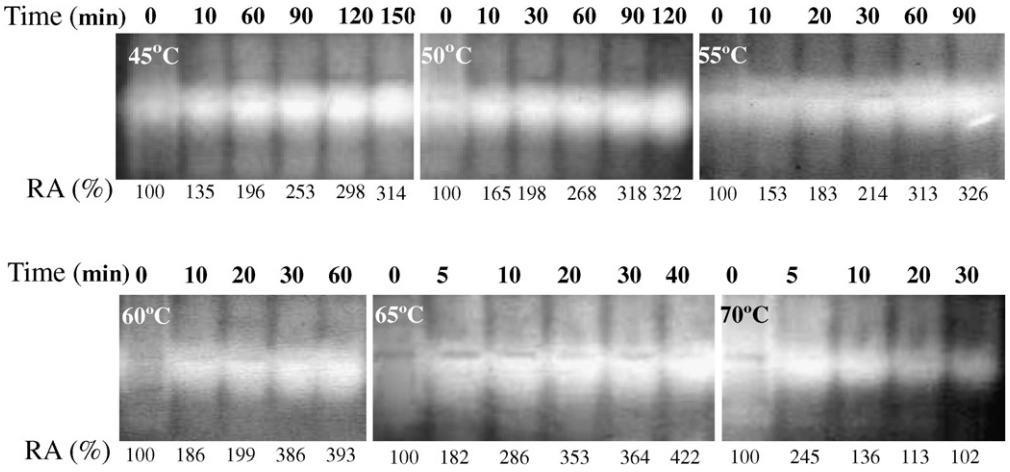


Fig. 6. Effect of heat temperature and duration on activity of BG protease. Protein extractions were performed immediately after the BG flesh was incubated at 45 °C, 50 °C, 55 °C, 60 °C, 65 °C and 70 °C for the defined durations. Then, the extracts were gel-assayed for protease activity. The numerical values under each of the bands are the relative activities estimated with software for gel-analysis (Scion Image, 4.0.2).

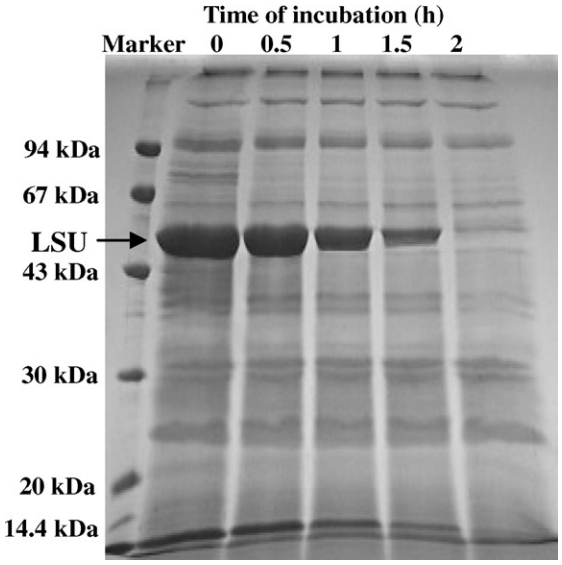


Fig. 7. Effect of the BG protease on protein degradation of spinach leaf. The protein of Rubisco extracted from spinach leaves with the partially purified-protease of the BG flesh was incubated for 0, 0.5, 1, 1.5 or 2 h at 45 °C; thereafter, it was fractionated on SDS–PAGE and stained with Coomassie blue. LSU indicates the large subunit of the Rubisco.

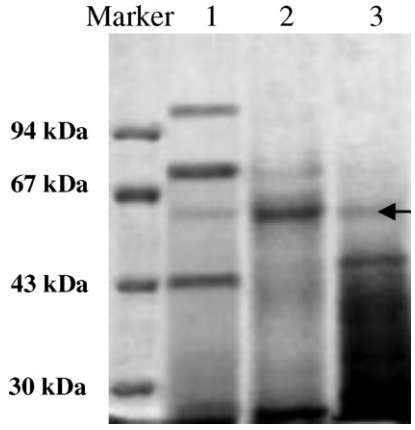


Fig. 8. Analysis of the isolated BG protease by SDS–PAGE. The fraction enriched in the BG protease (about 150 μg protein loaded on one gel) was resolved by SDS–PAGE as for the assay of protease activity except without gelatin in the gel. After electrophoresis, the gel was horizontally sliced into 1 mm wide slices (parallel with the bottom), and then analyzed for protease activity in each of the slices. Proteins in the slice enriched with the protease, and the up/low adjacent slices were electro-eluted out and resolved on regular SDS–PAGE. Then, the gel was stained with Coomassie blue. Lane 1: proteins from the up-slice of the protease locus; lane 2: proteins from the gel slices of the protease locus; lane 3: proteins from the low-slice of the protease locus.

### 3.3. Protein isolation of the BG protease

The soluble protein extract of BG was vacuum-lyophilized and then precipitated with 40% ammonium sulfate. Thereafter, the fraction containing the protease was further resolved by SDS–PAGE. Proteins in the slice of the protease-locus and the up/low adjacent slices were electro-eluted out and re-analyzed by SDS–PAGE. As shown in Fig. 8, a 60 kDa band was supposed to be the putative protein of the BG protease and was isolated and eluted out for the further assays. A few protein spots in fraction of the isolated protease were observed on the 2D-PAGE, as shown in Fig. 9. The major protein spot was analyzed further by matrix-assisted laser deionization time-of-flight (MALDI-TOF) mass spectrometry (Fig. 10). The Mascot peptide mass fingerprint of this protein hit no existing protein (score > 60, http://www.matrixscience.com), and closest sequence similarity to aspartic-type endopeptidase/pepsin A (*Arabidopsis thaliana*, NP_172655) (score, 31), suggested that protease may be a novel aspartic protease. Additional match of the mascot peptide mass fingerprint of the BG protease with the other existing aspartic protease sequence in http://www.ncbi.nlm.nih.gov/ was also performed (http://www.expasy.org/tools/findpept.html). The highest sequence similarity (Δmass, −0.005 daltons) was found in Zea mays nucellin-like aspartic protease

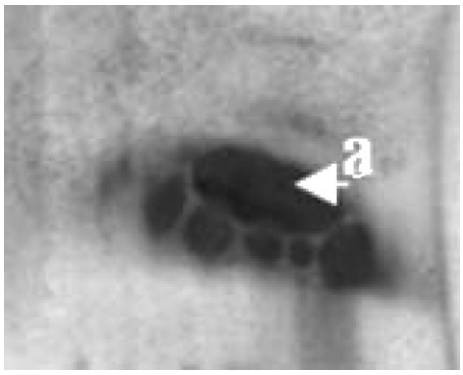


Fig. 9. Assay of the isolated BG protease by two-dimensional electrophoresis The 2D-PAGE was performed as described in the Materials and method and the Results. The major protein spot (a) was excised for the MS assay.

(AAM76716) which is tissue-specifically and-developmentally expressed in kernel tissue of Zea mays.

The 60 kDa protease in BG was inhibited by the specific aspartic proteinase (AP) inhibitor, pepstatin A, unequivocally showing that it belongs to the family of APs. Our result further showed that the AP activity in BG was activated by heat-treatment. Induction of AP gene expression has also been detected in tomato leaves by wounding or treatments with systemin and methyl jasmonate (Schaller & Ryan, 1996). AP activity is induced in potato tubers/leaves in the defence responses of potato plants against pathogens and/or insect herbivores (Guevara et al., 2005; Guevara, Oliva, Huarte, & Daleo, 2002). The BG AP exhibited its maximal activity at pH 7, which is different from the AP in other plants with maximal activity at acidic pH (<5.0) (Wang & Ng, 2001).

The mechanisms involved in the protease-activation by heating have so far remained unclear. Because the protease can be activated by a heat-treatment in only a few minutes (such as at 70 °C for 5 min), it might be unlikely due to *de novo* synthesis of the protease in BG tissues. One hypothesis is that heat-treatment may trigger activation of proenzymes of the protease. Indeed, most plant APs identified so far are synthesized as proenzymes and undergo several proteolytic cleavages to produce the mature form of the enzyme. Zymogen conversion generally occurs by limited proteolysis and removal of the "activation segment", involving accessory molecules that trigger activation or proteolysis, being autocatalytic as is described for the gastric APs (Khan & James, 1998).

Within the family of plant APs only few enzymes have been functionally characterized. For the great majority of plant APs, no definitive role has been assigned. The biological functions of APs are still hypothetical. Our results showed BG protease is implicated in the proteolysis of the proteins, particularly the photosynthetic enzyme, ribulose-1,5-bisphosphate carboxylase (Fig. 7). Similar results have also been observed in citrus leaves (Garciamartinez & Moreno, 1986) and senescence alfalfa leaves (Nieri, Canino, Versace, & Alpi, 1998). In addition, the degradations of chlorophyll were closely correlated with the protease activity in the bitter gourd treated at different temperatures (Fig. 5C). Bhalerao et al. (2003), studied gene expression during autumn leaf senescence in aspen trees and found, among others, two genes encoding similar APs that were upregulated in autumn leaves. According to their data, APs may play an important role during chloroplast degradation.

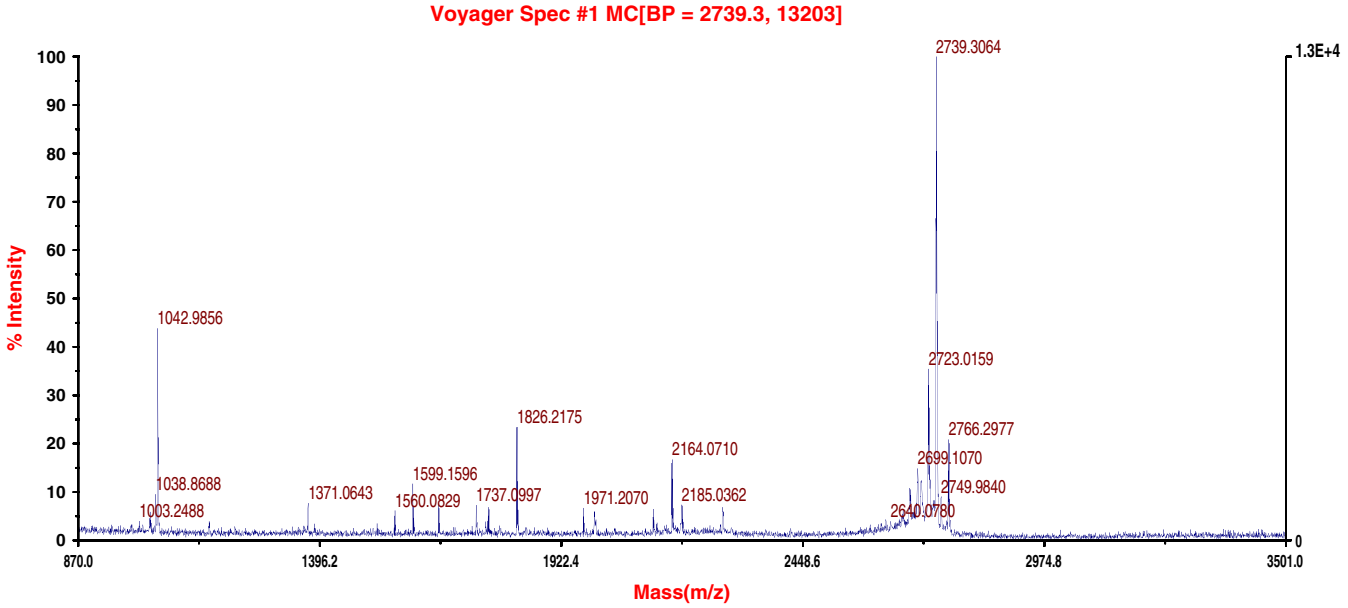


Fig. 10. Assay of peptide mass fingerprinting of the BG protease by MS-chromatogram.

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