A Low Molecular Weight Alkaline Serine Protease from the Seeds of *Nelumbo nucifera*: Purification and Characterization

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Summary: A low molecular weight alkaline serine protease from the seeds of *Nelumbo nucifera was* purified to electrophoretic homogeneity. Three steps of purification consisting of ammonium sulfate precipitation, gel filtration and ion exchange chromatography techniques were employed to achieve purified alkaline serine protease. Its molecular mass was estimated by SDS-PAGE to be 24 kDa. The optimum pH of the enzyme was 8.0 using 1 % casein as a substrate. The enzyme exhibited maximum activity at 40 °C. The K_m and V_{max} of the enzyme were found to be 2.8 x 10^{-4}M and 200 μM/ min. respectively. The purified enzyme was strongly inhibited by PMSF, indicating the presence of serine residue at the active site and was weakly inhibited by EDTA. The enzyme activity was enhanced in the presence of Ca^{++} and weakly inhibited by Cu^{++}. Na^{+} showed no effect on the enzyme activity while slight changes in the enzyme activity were observed in the presence of K^{+} and Mg^{++}.

Introduction

Proteolytic enzymes are essential for the survival of all kinds of organisms and are encoded by approx. 2 % of all genes [1]. Proteases are hydrolytic enzymes capable of specifically cleaving the peptide bond in the protein. They play key roles in various physiological processes such as digestion, coagulation, protein turnover and degradation of defective and exogenous proteins. In plants, significant progress has been made in understanding the mechanisms by which short-lived proteins are targeted and then degraded during their normal cellular turnover [2].

Although many proteases have been isolated from plant latex, fruits and seeds [3], little is known about the endopeptidases from underground parts of the plant. Some reported plant proteases are as follows: prolyl endopeptidases from carrot root [4], a peptidase from sweet potato tuberous root, protease of potato tuber [5-9] and cysteine proteases from ginger rhizome [10].

Most plant proteases have been classified as cysteine proteases, serine protease or rarely, as aspartic proteases [11]. Serine proteases are a large class of proteolytic enzymes that have a reactive serine residue, as a part of catalytic triad, in the active center to hydrolyze the peptide bonds. A serine protease from *Cucumis melo var.* has been well purified and characterized [12-13]. Recently, cucumisin-like proteases have been found in the fruits of the cucurbitaceae [14]. It is interesting to note that the primary structures of proteases in the cucumisin family are homologous to those of proprotein processing proteases, such as Kex2 from yeast and human furin [15].

In this paper for the first time ever, we describe the purification and characterization of low molecular weight serine protease from the seeds of *Nelumbo nucifera.*

Results and Discussion

Enzyme Purification

For the enzyme purification, the ammonium sulfate precipitation, gel filtration and ion exchange chromatographic steps were applied. The enzyme activity units and specific activity were not exhibited in the form of purification table since the crude extract and ammonium sulfate precipitation did not exhibit enzyme activity. A crude extract prepared from fresh dry seeds of *Nelumbo nucifera* was precipitated with ammonium sulfate to remove the impurities other than proteins. After salt precipitation, the resuspended pellet was first applied to a Sephadex G-50 column. The separation profile of

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the enzyme, using casein as substrate, from a Sephadex G-50 column is shown in Fig. 1. The enzyme active fractions obtained from the first peak of gel filtration column were collected. These collected enzyme active fractions were further purified by DEAE cellulose column. The elution profile, using casein as substrate, from DEAE cellulose is shown in Fig. 2.

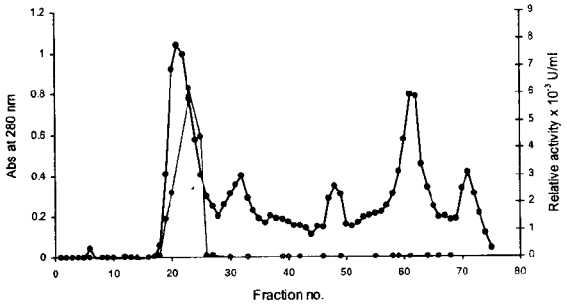

Fig. 1. Separation profile of alkaline serine protease from the seeds extract of *Nelumbo nucifera* after ammoniumsulfate precipitation on Sephadex G-50 column (2.5 x 54 cm) eluted with Tris HCl buffer pH 7.4. The flow rate was 12 ml/hour. 3 ml fractions were collected and measured for protease activity (●·····●) and protein (A_{280}, ●——●).

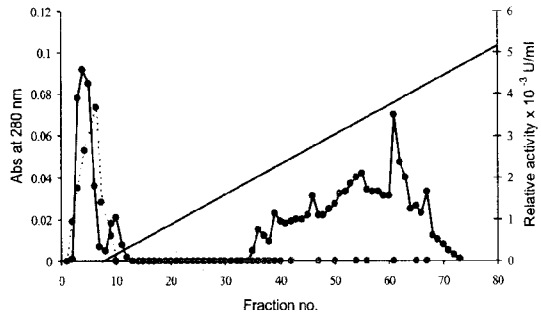

Fig 2. Elution profile of the first peak obtained from Sephadex G-50 on DEAE Cellulose column (2.5 x 24 cm). Elution was performed by Tris HCl buffer pH 7.4 with 0 - 0.25 M NaCl at flow rate of 24 ml/hour. Fractions of 6 ml were collected and were measured for protease activity by Anson Method (●------●), Protein at 280 nm, (●——●) and NaCl gradient (0-0.25 M), (———).

The enzyme active fractions eluted as unbound were collected and analyzed by SDS-PAGE. The purified enzyme showed a single band in reducing the condition on SDS-PAGE. The single band analyzed by SDS-PAGE is shown in Fig. 3, indicating that the enzyme has no subunits. The molecular mass of the purified protease was estimated to be 24 kDa. Zymogram staining activity also revealed one clear zone of proteolytic activity against the blue background (Fig. 4). The molecular mass of the purified enzyme was observed to be smaller than the serine protease already reported from other plants [16] and greater than some other reported plant serine proteases [17].

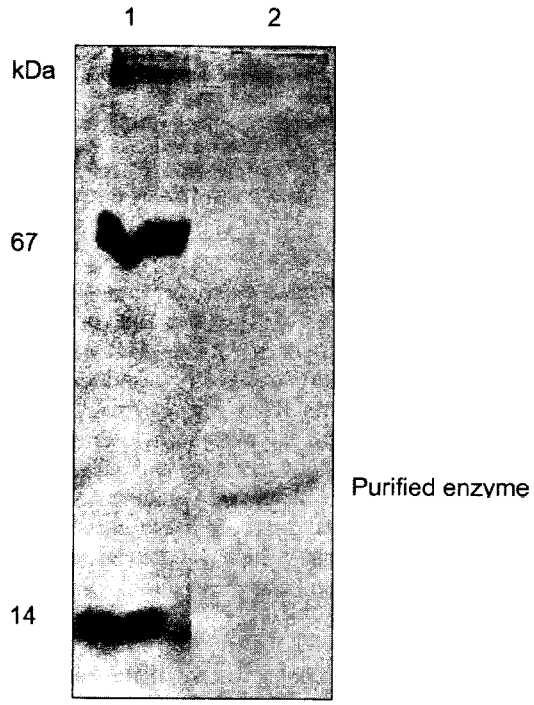

Fig. 3. 10 % SDS-PAGE of the purified *N. nucifera* alkaline serine protease, Lane 1: BSA, 67 kDa and Lysozyme, 14 kDa (standards), Lane 2: purified enzyme.

Characterization

The optimum pH for the hydrolysis of casein was found to be pH 8.0 (Fig. 5). The maximal activity of *Nelumbo nucifera* protease was found at 40 °C (Fig. 6). The activity of *Nelumbo nucifera* protease toward different substrates was examined (Table-1). The best substrate for purified enzyme was casein. The enzyme also hydrolyzed hemoglobin, bovine serum albumin (BSA) and gelatin, but to a

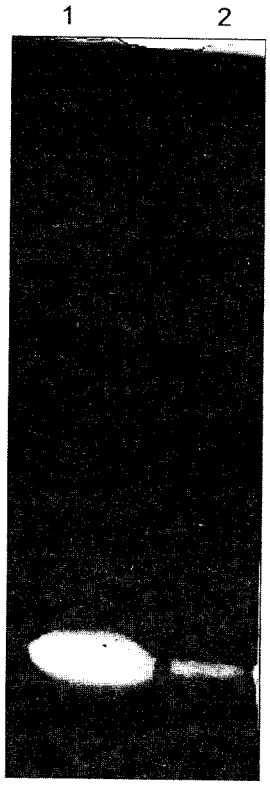

Fig. 4: Activity gel electrophoresis (10 % SDS-PAGE) of purified. *N. nucifera* alkaline serine protease, Lane 1. purified protease (5 μg), Lane 2. purified protease (1 μg).

Table-1: Substrate specificity of *N. nucifera* alkaline serine protease.

Substrate	Concentration (%)	nm	Relative activity (%)
Casein	0.5	280	100
Hemoglobin	0.5	280	84
BSA	0.5	280	76
Gelatin	0.5	280	87

Table-2: Effects of metals ions and compounds on enzyme activity

Metal Ions/compounds	Conc.(mM)	Relative activity (%)
None		100
NaCl	2	100
KCl	2	105
$CaCl_2$	2	120
$MgCl_2$	2	108
$CuSO_4$	2	92
PMSF	2	20
EDTA	0.5	88

lesser extent than casein. The K_m and V_{max} of the enzyme were determined to be 2.8 x 10^{-4}M and 200 μM/ min. respectively. The effects of various metal ions and compounds as possible activators and

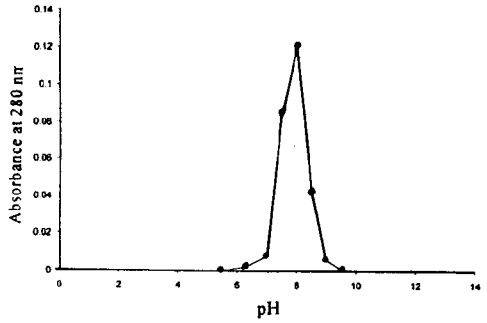

Fig. 5. Optimum pH of purified *N.nucifera* alkaline serine protease (The absorbance recorded were pH 5.5: 0, pH 6.5: 0.002, pH 7: 0.008, pH 7.5: 0.084, pH 8: 0.121, pH 8.5: 0.042, pH 9:0.006, pH 9.5: 0.001 as shown in the figure).

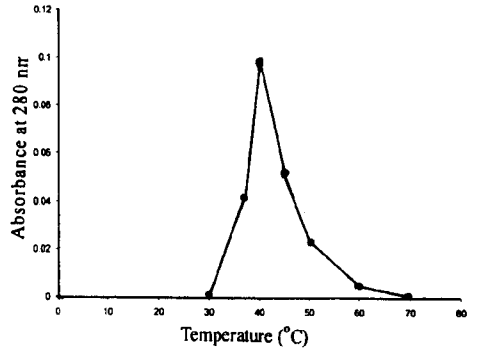

Fig. 6. Optimum temperature of purified *N. nucifera* alkaline serine protease (The absorbance recorded were 30 °C: 0.0012, 37 °C: 0.0455, 40 °C: 0.108, 45 °C: 0.045, 50 °C: 0.0265, 60 °C: 0.0075, 70 °C: 0.001 as shown in graph).

inhibitors on the enzyme activity using casein as substrate are shown in Table 2. The enzyme was strongly inhibited by PMSF but weak inhibition was observed in the presence of EDTA and Cu^{++}. The slight increase in the enzyme activity was observed in the presence of Ca^{++}, Mg^{++} and K^{+}. These results indicated that enzyme is a serine protease having serine residue in the active site of the enzyme.

Experimental

Materials

Phenylmethanesulphonylfluoride (PMSF) was purchased from Sigma. Sephadex G-50 was obtained from Pharmacia, while DEAE cellulose was obtained

from Sigma. Commercial *Nelumbo nucifera* dry seeds were purchased from local market.

Extraction

The dry seeds of *Nelumbo nucifera* were ground and soaked in 10 mM Tris HCI buffer, at pH 7.4 containing sodium azide (1 %), stirred for 10 hours and kept at 4 °C overnight. The seed's extract was filtered and centrifuged at 15000 x g for 15 min. The pellet was discarded while the supernatant was subjected to protein purification steps.

Protein Concentration

Protein concentration was determined at each step by dye binding method of Bradford [18], using bovine serum albumin as standard.

Enzyme Assay

At each step of activity directed purification, the caseinolytic activity of the purified enzyme was determined using casein as a substrate, according to Anson's method [19]. The reaction mixture consisting of 150 μl of enzyme solution, 100 μl of 1 % casein (w/ v) in 10 mM Tris HCI buffer at pH 8.0 and 1 ml of 50 mM Tris HCI buffer at pH 8.0 was incubated at 37 °C for 30 min. The reaction was stopped by the addition of 200 μl 40 % TCA, kept on ice for 30 min and then centrifuged at 14000 x g for 10 min. The control was prepared in the same quantity but the enzyme was added to the mixture of substrate and TCA. The TCA soluble fractions were measured at 280 nm. The protease activity was expressed as the difference of absorbance at 280 nm between the control sample and the test sample. One unit activity was defined as the activity giving 0.001 A_{280} unit of change per min under these conditions.

Enzyme Purification

Step l. Ammonium Sulfate Precipitation

Solid ammonium sulfate was added gradually to the clear supernatant to 67 % saturation. The saturated solution was kept at -20 °C for 30 min and then the resulting precipitates were collected by centrifugation at 15000 x g for 15 min. The precipitates were resuspended in 10 mM Tris HCI buffer pH 7.4.

Step 2. Gel Filtration on Sephadex G-50

The resuspended ammonium sulfate precipitates were applied to Sephadex G-50 column (2.5 x 54 cm) already equilibrated with 10 mM Tris HCI buffer at pH 7.4. The flow rate was maintained at 12 ml /hour and fractions of 3 ml/ 15 min were collected and then monitored at 280 nm. The protease activity of some fractions was measured by the above described method. The enzyme active fractions were collected and then applied to ion exchange column.

Step 3. DEAE Cellulose Chromatography

The enzyme active peak from Sephadex G-50 was placed in DEAE-cellulose column (2.5 x 24 cm) previously equilibrated with 10 mM Tris HCI pH 7.4. The column was washed with linear gradient 0-0.25 M NaCI in the same buffer. The flow rate was 24 ml/hour and 6 ml fractions were collected and monitored at 280 nm. The enzyme activity was found in an unbound peak.

Electrophoresis

SDS-PAGE

The SDS-PAGE was conducted by the method of Laemmli [20], on slab gel using 10 % (w/v) polyacrylamide. The molecular mass of the purified protease was estimated using standard proteins. The protein bands were visualized by Coomassie Brilliant Blue R-250.

Activity Detection by Zymogram Staining

For the detection of enzyme activity by Zymogram staining, the purified enzyme was electrophoresed in 10 % Zymogram-resolving gel containing SDS (0.1 %) and gelatin (0.3 %) as substrate. In order to remove SDS, the gel was washed in Tritron X-100 (2.5 % w/v) three times for 20 min. After washing, the gel was incubated in 50 mM Tris-HCI buffer at pH 8.0 containing 0.1M NaCI and 1mM $CaCl_2$ for 8 hours at 37 °C and then stained with Coomassie Brilliant Blue R-250. The activity band was observed as a clear colorless area depleted of gelatin in the gel against the blue back-ground.

Characterization of Protease

Effect of pH and Temperature on Enzyme Activity

The optimal pH for activity of enzyme was determined for casein. The enzyme was preincubated in a series of buffers spanning the range 5.5-9.5 for a period of 15 min. After this period, the substrate was added as described previously. The buffers used were acetate pH (5.5-6.5), phosphate pH (7.0-7.5) and Tris pH (8.0-9.5). For the determination of optimum

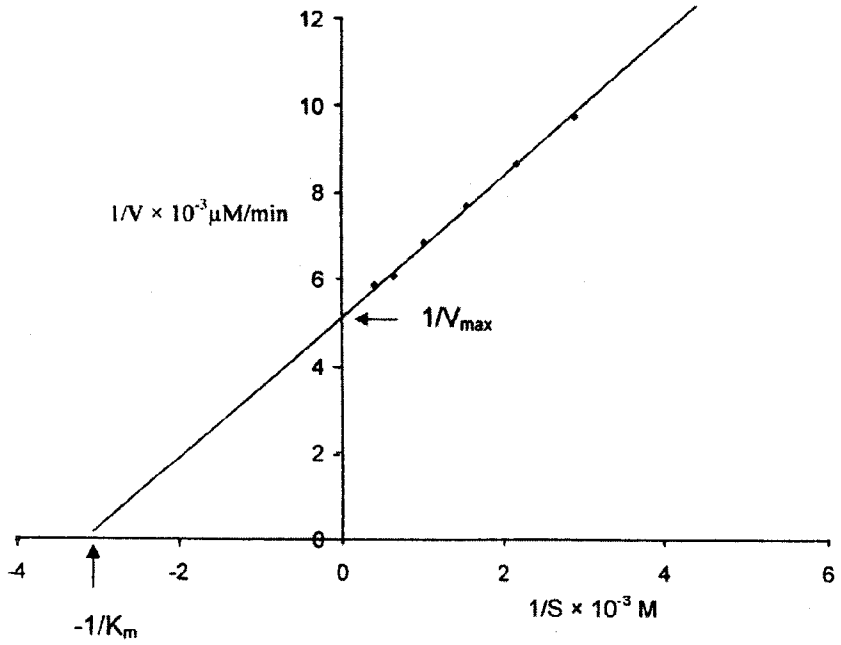

Fig. 7. Lineaweaver-Burk curve for Michaelis-Menten constant (K_m) and V_{max} determination of *Ne/umbo nucifera* alkaline serine protease. The K_m and V_{max} were found to be 2.8 x 10^{-4}M and 200 μM/min respectively from the curve.

temperature, the enzyme activity was assayed at different temperatures from 30 to 70 °C in 10 mM Tris HCl pH 8.0 (Fig. 6).

Effect of Metals and Compounds on Enzyme Activity

The effect of metal ions and compounds as possible activators and inhibitors on enzyme activity was determined. The enzyme was incubated with each compound at different concentrations for 20 min at 37 °C, followed by the measurement of residual activity under the standard assay conditions (Table-2).

K_m Determination and Substrate Specificity

The K_m value for purified enzyme was also determined using increasing substrate concentrations and plotted 1/V against 1/[S]. The enzyme hydrolyzing activity toward the different substrates for purified enzyme was also examined (Fig. 7).

References

1. A. J. Barrett, N. D. Rawling and E. A. O'Brien, *J. Struc. Bio.l,* **134**, 95 (2001).
2. R. D. Vierstra, *Annual Review of Plant Physiology and Plant Molecular Biology,* **44**, 385 (1993).
3. T. Boller, Plant proteolytic enzymes: Roles of proteolytic enzymes in interactions of plants with other organisms, *CRC Press,* FL, **1**, 67 (1986).
4. T. Yoshimoto, A. M. A. Sattar, W Hirose and D. Tsuru, *Biochem.Biophys. Acta,* **916**, 29 (1987).
5. Y. H. Lin and H. Y. Chan, *Bot. Bull. Acad. Sin,* **33**, 253 (1992).
6. Y. H. Un and H.Y. Chan, *Bot. Bull. Acad. Sin,* **33**, 263 (1992).
7. K. Santarius and H. D. Belitz, *Planta,* **141**, 145 (1978).
8. N. Kitamura and Y. Maruyama, *Agric. Biol. Chem.,* **53**,381 (1986).
9. N. Kitamura, A. Okitani and Y. Maruyama, *Agric. Biol. Chem.,* **53**, 1159 (1989).
10. K. Ohtsuki, K. Taguchi, K. Sato and M. Kawabata, *Biochem. Biophys. Acta,* **1243**, 181 (1995).
11. M. Kaneda, N. Tominaga, *Journal of Biological Chemistry,* **78**, 1287 (1975).
12. H. Yamagata, T. Masuzawa, Y. Nagaoka, T. Ohnishi and T. Iwasaki, *Journal of Biological Chemistry,* **269**, 32725 (1994).

13. T. Uchikoba, H. Yonezawa and M. Kaneda, *Journal of Biochemistry,* **117**, 1126 (1995).
14. M. Kaneda and T. Uchikoba, *Phytochemistry,* **33**, 583 (1994).
15. M. Nishikata, *Journal of Biological Chemistry,* **95**, 1169 (1984).
16. K. R. Lynn and N. A. Clevette-Radford, *Phytochemistry,* **24**, 925 (1985).
17. B. Kowalsaka-Loth, T. Burdzynski, K. Toczko and I. Chmielewska, *Acta. Biochem., Pol,* **23**, 369 (1976).
18. M. Bradford, *Anal. Biochem.,* **72**, 248 (1976).
19. M. L. Anson, *J. Gen. Physiol.,* **22**, 79 (1938).
20. U. K. Laemmli, *Nature,* **227**, 680 (1970).