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Purification and Partial Characterization of a Protease from Tomato (*Lycopersicon esculentum* Mill)

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Abstract

A protease from tomato flesh was purified to homogeneity by $(NH_4)_2SO_4$ precipitation followed by Sephadex G-75 and DEAE-cellulose column chromatography. Molecular weight of the enzyme was estimated to be 81-79 KD by gel filtration and SDS-PAGE respectively. The enzyme was found to be a single polypeptide chain as revealed by SDS-PAGE under either reducing or non-reducing conditions. Optimum activity was observed at pH 7.0 and 45°C with a k_m value of 0.48 per cent determined by using casein as substrate. The enzyme appears to be a serine protease being inhibited greatly by DFP and PMSF and to a lesser extent by heavy metals such as Pb^{2+} and Fe^{2+}.

Introduction

The ubiquous nature of proteolysis in biological system provides a means for cells to change their protein content during development and adaptation to altered environmental conditions. Significant progress have been achieved in understanding how short-lived proteins in plants are targeted and then degraded during their normal cellular turnover. Also in response to pathogenic attack, plants assemble a number of biochemical weapons to antagonize the growth of the invading pathogen as well as to develop a resistance toward subsequent pathogenic attack. This general response is accompanied by the accumulation of a characteristic set of proteins referred to as pathogenesis related (PR) proteins (Bol *et al.*, 1990). A number of protease and PR proteins have been reported in tomato leaves (Schaller and Ryan, 1996, Drake *et al.*, 1996, and Pautat *et al.*, 1993).

Tomato is a very popular seasonal vegetables almost all over the world. It is one of the most widely cultivated vegetables in Bangladesh. Several hybrid varieties of tomato have recently been developed here. Their productivity, quality, and degree of resistance to pathogens have also been studied (Hoque *et al.*, 1981; Rahman and Hoque, 1986). One of the varieties, Manik, has been selected to be superior over other varieties in terms of these parameters (Hoque *et al.*, 1981). We have reported the variety to possess highest protease activity among the newly developed varieties at green stage (Karim *et al.*, 1995). The results led us to assume that the high proteolytic activity in this variety might have some correlations with its high resistance against pathogens. This assumption motivated us further to investigate the characteristic properties of the enzyme. Although reports on serine protease in plants are not abundant, the euphorbains and hevains spp. largely have serine protease (Lynn *et al.*, 1988). The protease have so far been reported in tomato leaves and fruits are mostly aspartic and cysteine protease (Schaffer and Fischer, 1990, Schaller and Ryan, 1994; Tornero *et al.*, 1996). But Michaud *et al.* (1993) reported the presence of a serine protease in tomato fruit. In our present study we report the purification and characterization of a protease that seems to be a serine protease.

Materials and Methods

Materials: Sephadex G-75 and DEAE-Cellulose were purchased from Pharmacia Biotech Inc. Diisopropylfluorophosphate (DFP) was from ICN and phenylmethyl sulphonylfluoride (PMSF) was from Wako Pure Chemical Industries Ltd. Casein (Hammarsten) was purchased from E. Merck, Darmstadts. All other chemicals were purchased from sigma.

Plant materials: The tomato of Manik variety at green stage were collected from Agricultural Research Substation Shampur, Rajshahi, Bangladesh.

Protein determination: Protein was determined by the method of Lowry et al. (1951) using BSA as standard or by measurement of absorbance at 280 nm.

Enzyme assay: The enzyme was routinely assayed following the method as described by Hashinaga et al. (1983) using casein as substrate. One unit of enzyme activity is defined as the amount producing 1 micromole tyrosine per minute at 45°C. A_{280} of the enzyme digest in the TCA supernatant was read and referred to a standard curve for tyrosine.

Purification of the enzyme: Unless mentioned otherwise, all the operations were performed at 4°C. The tomato flesh of manik variety at green stage was cut into pieces and crushed into paste using a homogenizer, with addition of 0.5 per cent PVP (w/w), at a low medium speed. The suspension was filtered through double layer of cheese cloth and the filtrate was clarified futher by centrifugation at 8,000 g for 15 minutes. The clear supernatant was then

saturated to 75 per cent by adding solid ammonium sulfate with slow stirring. The precipitate collected by centrifugation at 12,000 g for 20 minutes was dissolved in a minimum volume of 5 mM phosphate buffer saline (PBS), pH 7.6 and dialysed against the same buffer for overnight with four changes of the buffer. After centrifugation the clear supernatant was used as crude enzyme and stored at -20°C if not used immediately.

The crude enzyme extract was loaded onto a Sephadex G-75 colunm previously equilibrated with 5 mM PBS, pH 7.6 and the proteins were eluted from the column with the same buffer. The fractions (40-60) showing the protease activity were pooled and dialysed against 10 mM Tris-HCl buffer, pH 8.4 for overnight with four changes of buffer. After centrifugation the supernatant was applied onto a DEAE-Cellulose column, previously equilibrated with Tris-HCl buffer, pH 8.4 and the proteins were recovered from the column by stepwise elution with the same buffer containing different concentrations of NaCl. Protease eluted in the fractions 19-38 were pooled and tested for homogeneity.

Electrophoresis. Polyacrylamide disc gel electrophoresis was conducted at room temperature, pH 8.4 on 7.5 per cent gel as described by Ornstein (1964), and the enzyme was stained with amido black.

Determination of molecular weight. Molecular weight of the purified native enzyme was determined by gel filtration on Sephadex G-75 column (0.9 X 90 cm) as described by Andrews (1965). The marker proteins used were Lysozyme (14.6 KD), Egg Albumin (45 KD), BSA (66 KD), β-Galactosidase (116 KD) and β-Amylase (200 KD). The molecular weight was also determined by SDS-PAGE according to the method of Laemmli (1970) using the marker proteins, Myosine (205 KD), β-Galactosidase (116 KD), BSA (66 KD), Carbonic anhydrase (29 KD), β-Lactalbumin (18 KD), and Aprotinin (6.5 KD).

Results

Manik variety was chosen as the source for isolation of protease as it possessed the highest protease activity. Using casein as substrate to monitor protease activity routinely during purification, a protease, referred here as tomato flesh protease (TFP), was purified as summerized in Table 1. The specific activity increased only over 1.33 fold following 75 per cent ammonium sulfate precipitation whereas gel filtration on sephadex G-75 column effected a further 31 fold increase in specific activity (Fig. 1). A final purification of 88 fold was obtained following ion- exchange chromatography on DEAE-cellulose column (Fig. 2). The recovery of protease activity was low (34%) since over 99 per cent of the extracted protein was removed during the purification steps due to denaturation of the enzyme during purification and /or during storage at -20°C.

The protease activity eluted in DEAE-cellulose column appeared to be homogeneous by the criterion of single protein band on PAGE at pH 8.4. SDS-PAGE in the presence of MSH and in its absence resulted in a singl band of the same mobility under dissociating and as well a non-dissociating conditions showing that the protea consisted of a single polypeptide chain. References to th calibration lines constructed from the relative mobilities o the Mr marker proteins run in parallel with TFP on SDS PAGE gave Mr of ca 79 KD (Fig. 3) whereas it showed M

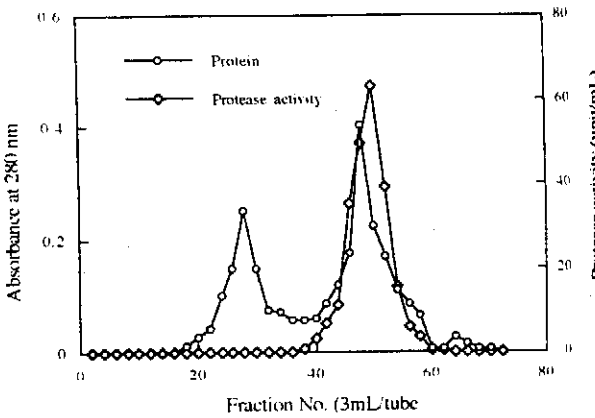

Fig. 1: Gel filtration profile of 75 per cent $(NH_4)_2S$ saturated fraction of crude enzyme on Sephad G-75 column (1.8 x 120 cm) pre-equilibrated wi phosphate buffer saline pH 7.8. Protein was elut with the same buffer at a flow rate of 25mL/mi

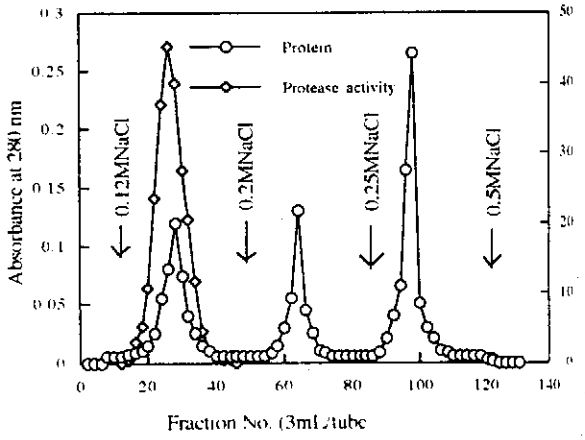

Fig. 2: Stepwise elution profile of TFP on DEAE-Cellul column. The active fractions (40-60) were appl to a column (0.9x90 cm) pre-equilibrated with T HCl buffer pH 8.4 and the activity was eluted w the same buffer containing different concentrat of NaCl.

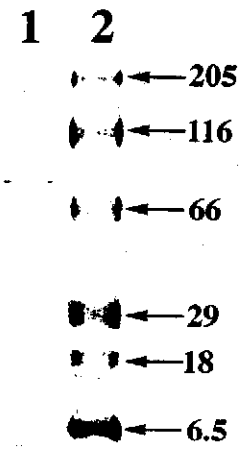

Fig. 3: SDS-PAGE for the determination of molecular weight of purified TFP. Lane 1 is for the pure enzyme and lane 2 for molecular marker proteins. Molecular weight was expressed in killo dalton (KD).

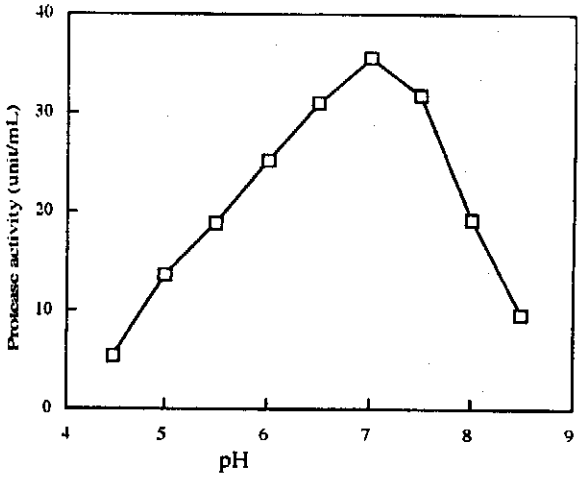

Fig. 4: Effect of pH on the activity of the purified tomato flesh protease.

of ca 81 KD on gel filtration. This discrepancy in Mr might fall within the error of SDS-PAGE technique. Thus we can predict an avarage Mr of 79-81 KD. TFP is active over a pH range of 6 to 8 and maximum activity was observed at pH 7 (Fig. 4). Below pH 6 and above pH 8 the activity of TFP decreased sharply, and very little activity was observed at pH 4.5 and at pH 9.

The effect of temperature on the activity of TFP is shown in Fig. 5. As observed, the activity of the protease increased gradually up to 30°C, then increased rapidly reaching the peak at 45°C. A gradual decrease in activity started with further increase in temperature; and very little activity was observed at or above 90°C.

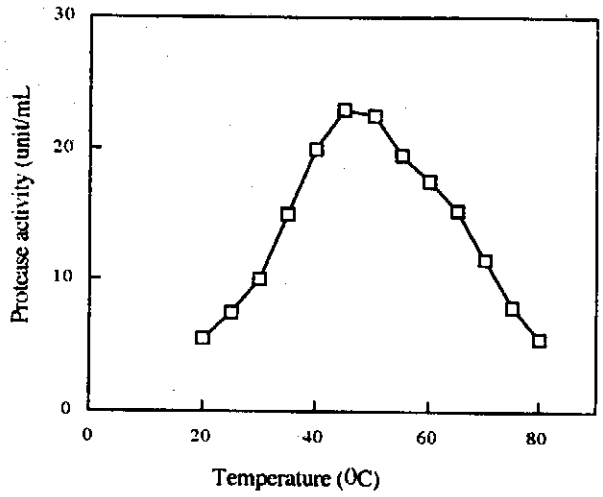

Fig. 5: Effect of temperature on the activity of the purified tomato flesh protease.

Effect of several mono- and divalent metal ions on TFP activity were examined at 1 and 10 mM concentration of each metal ion (Table 2). None of the metal ions tested had any significant activating effect on the enzyme. However, Pb^{2+} and Ag^{+} were around 50 per cent inhibitory at 1 mM concentration while Pb^{2+} was totally inhibitory at 10 mM, the later being 65 per cent. Fe^{2+} being only 25 per cent inhibitory at 1mM concentration, it can completely inhibit the enzyme at 10 mM. Sn^{2+}, Mn^{2+}, Ca^{2+}, and Cu^{2+} were also partially inhibitory at 10 mM concentration.

Effect of various organic compounds and some inhibitors on the activity of the TFP at 1 mM concentration has also been investigated (Table 3). Thiol activating agents such as MSH and Cysteine had no activating effect on the enzyme. Iodoacetamide, a thiol blocking agent had also no inhibitory effect on the activity of the enzyme. The metal chelating agent, EDTA, had no inhibitory effect on the activity of the enzyme.

The purified TFP was strongly inhibited by a typical serine protease inhibitor, DFP (Table 3). However, trypsin inhibitor from soybean had little effect on the enzyme activity. Tyrosine also had no significant activation effect on the enzyme. But, PMSF, another serine protease inhibitor showed significant inhibitory effect on the enzyme.

Though the substrate specificity of TFP was not thoroughly investigated through the study due to unavailability of different peptide substrates, we just tested the K_m value of the enzyme against the routinely used substrate casein and found to be 0.48 per cent.

Table 1: Purification of tomato flesh protease

Purification steps	Total protein (mg)	Total activity (units)*	Specific activity (unit/mg)	Yield (%)	Purification fold
Crude extract	570.0	1094.4	1.92	100.0	1.00
75% $(NH_4)_2SO_4$ ppt.	420.0	1071.0	2.55	97.8	1.33
Sephadex G-75	6.52	528.0	80.98	48.2	42.10
DEAE-Cellulose	2.2	372.0	169.0	34.0	88.00

* One unit of enzyme activity is defined as the amount of protein releasing 1 μmole of tyrosine per minute at 45°C.

Table 2: Effect of metal ions on the activity of purified tomato flesh protease

Metal ions	Residual activity (%)	
	1 mM	10 mM
Zn^{2+}	82	80
Mg^{2+}	97	96
Cu^{2+}	88	68
Ag^{+}	59	35
Ca^{2+}	84	60
Sn^{2+}	86	45
Fe^{2+}	75	0
Pb^{2+}	52	0
Mn^{2+}	82	54

Enzyme activity in the absence of any metal ion was taken as 100.

Table 3: Effect of inhibitor and effectors on the activity of purified tomato flesh protease.

Inhibitor/effector	Residual acitivity (%)
Iodoacetamide	99
Diisopropyl flurophosphate	0
Trypsin inhibitor from soy bean	75
L-Cysteine	102
L-Tyrosine	99
2-mercaptoethanol	107
EDTA	97
Phenylmethylsulfonyl fluoride	25
L-Aspertic acid	101

Enzyme activity in the absence of any added organic substances was taken as 100.

Discussion

Proteolytic activity in tomato fruit and leaves are not new. But to the best of our knowledge we report for the first time the purification and characterization of a serine protease. Michaud *et al.* (1993) just reported the presence of such enzyme in tomato fruit extract but did not report its purification. Although the protease in tomato leaves are reported to have Mr ranging from 50 KD to 69 KD (Schaller and Ryan, 1994; Tornero *et al.*, 1996), the TFP appears to have the Mr higher than that of the tomato leaf protease. Molecur weight of TFP closely resembles to a serine protease namely artocarpin from jackfruit latex (Renuka and Virupaksha, 1990).

A broad pH range is most likely observed in case of serine proteases in plants like asparagus, calotropis and soybean (Guo *et al.*, 1998, Abraham and Joshi, 1979; Yamaguchi *et al.*, 1982). Tomato proteases in our report also showed a broad pH-activity profile indicating the similarity with other serine proteases.

The temperature-activity profile of TFP though covers a broad range, the significant activity enhancement region is sharp and lies between 35 to 50°C. This temperature optimumm shows consistency with that reported for green asparagus and miut (Yamaguchi *et al.*, 1982).

In order to identify the mechanistic properties of TFP, we investigated the effect of some important organic compounds and inhibitors on its activity. Since thiol activating agents such as MSH and Cysteine had no activating effect on the enzyme, it rules out the possibility of the enzyme to be a cysteine protease. Iodoacetamide, a thiol blocking agent also had no inhibitory effect on the enzyme that points to the same conclusion. Lack of inhibition in the presence of the metal chelating agent, EDTA, and lack of activation by added metal ions does not support any possibility of TFP to be a metalloprotease.

The purified TFP was strongly inhibited by a typical serine protease inhibitor, DFP indicating the possibility that the enzyme is a serine protease. However, trypsin inhibitor from soybean had little effect on the enzyme activity. Tyrosine also had no significant activation effect on the enzyme. But PMSF, another serine protease inhibitor showed significant inhibitory effect on the enzyme. Therefore, it is suggested that TFP should belong to the mechanistic class of serine protease.

The K_m value of the enzyme for casein as substrate was high (0.48%) showing that casein is not a good substrate for the enzyme. It may show good activity against some synthetic substrates. Takahashi and Kikuchi (1993) reported a milk clotting proteolytic enzyme that showed K_m value of 0.27 per cent for casein.

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