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Aspartic proteinase from the seeds of figleaf gourd (*Cucurbita ficifolia*)*

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Aspartic proteinases (EC 3.4.23) of animal origin represent a family of enzyme with well-defined physiological functions. They are involved in many biological processes such as digestion (pepsin), lysosomal protein degradation [1], generation of biologically active peptides (cathepsin D) [2], and hormone maturation (renin) [3], moreover these enzymes may serve as a prognostic factor in human neoplastic diseases [4]. However, in contrast to the extensively studied aspartic proteinases from animal tissues, relatively little is known about these enzymes from plant material. Although plant aspartic proteinases have been purified from several sources [5–7], the detailed amino-acid sequence has been elucidated only for the enzyme separated from barley grain [8, 9].

In this communication we present a method of purification of aspartic proteinase from the seeds of figleaf gourd *(Cucurbita ficifolia)*, and describe some properties of the enzyme, its N-terminal amino-acid sequence and the mechanism of inactivation of the endogenous squash trypsin inhibitor (CMTI I) by the purified enzyme.

To purify the enzyme, ground seeds were extracted with 2 volumes (w/v) of 0.05 M acetate buffer, pH 4.7. After 1 h of constant stirring the mixture was centrifugated at 2000 r.p.m. for 30 min and to the supernatant ammonium sulfate was added to 0.9 saturation. The precipitate was collected by centrifugation and suspended in water. After 24 h dialysis against distilled water the solution was mixed with activated charcoal (2 g/100 ml) and left for 15 min at room temperature with constant stirring. The clarified solution was acidified to pH 3.6 and the proteinase was adsorbed batch-wise onto a pepstatin-AH Sepharose [10] at 5°C. The non-adsorbed material was washed out with 0.1 M acetate buffer, pH 3.6, followed by 0.05 M Tris/HCl buffer, pH 6.8. The enzyme was eluted with 0.3 M carbonate buffer, pH 8.6. The proteinase was finally purified by ion-exchange chromatography on Mono Q column equilibrated with 0.02 M diethanolamine/HCl buffer, pH 8.4. From 3 kg of seeds, 5.5 mg of the enzyme preparation was obtained.

The proteinase appeared to be homogenous on 7% polyacrylamide gel electrophoresis at pH 8.3 under nondenaturating conditions. However, two protein bands of 30 kDa and 11 kDa were detected on 10%–20% SDS-PAGE [11]. The enzyme showed the highest activity against acid-denatured hemoglobin at pH 3.6.

Using oxidized B-chain of porcine insulin as a substrate it was established that the proteinase hydrolysed the peptide bonds involving amino groups of hydrophobic amino-acid residues.

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Fig. 1. The cleavage site of the native squash trypsin inhibitor CMTI I by C. ficifolia aspartic proteinase.

C. ficifolia					S	D	I	V	A	L	K	N	Y	M	D	A	Q	Y	Y	G	E	I	G	I	G	T	P	P
Cathepsin D				G	P	I	P	E	V	L	K	N	Y	M	D	A	Q	Y	Y	G	E	I	G	I	G	T	P	P
Barley	S	E	E	E	G	D	I	V	A	L	K	N	Y	M	N	A	Q	Y	F	G	E	I	G	I	G	T	P	P

Fig. 2. Alignment of N-terminal amino-acid sequence of purified proteinase from C. ficifolia seeds with those of porcine cathepsin D and barley-grain aspartic proteinase.

The group-specific inhibitors of aspartic proteinases: pepstatin, 1,2 -epoxy-3-(*p*-nitrophenoxy)-propane, and diazoacetyl-DL-norleucine methyl ester in the presence of Cu^{2+}, effectively inhibited the enzyme.

The cleavage site of the native endogenous trypsin inhibitor (CMTI I), isolated from *C. ficifolia* seeds [12], by the purified proteinase is presented in Fig. 1. After 30 h incubation at 30°C of the inhibitor with the enzyme immobilized on Sepharose 4B, the antitrypsin activity of the inhibitor decreased significantly. The proteolysis products of the inhibitor were resolved upon chromatography on SP-Sephadex C25 into two fractions of which only the first one was active. In both fractions the amino-acid composition of the proteins was the same. However, the inactive protein contained, in addition to arginine, a new N-terminal amino-acid residue, namely methionine. The presence of a new N-terminal residue in the inactive protein fraction proves that the proteinase, like pepsin [13], selectively hydrolyzed in CMTI I only one peptide bond, i.e. Leu7—Met8, inducing inactivation of the inhibitor.

The N-terminus of the amino-acid sequence of the 30 kDa band shows a strong homology of the proteinase to porcine cathepsin D and aspartic proteinase from barley grains (Fig. 2).

Our results indicate that the seeds of *C. ficifolia* contain a proteolytic enzyme which belongs to the aspartic proteinase family (EC 3.4.23) and its N-terminal amino-acid sequence resembles that of porcine cathepsin D and barley seed proteinase. The enzyme may be involved in selective inactivation of the endogenous trypsin inhibitor CMTI I.

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