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SERINE PROTEINASE FROM *CUCURBITA FICIFOLIA* SEEDS *

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A new serine proteinase was isolated from *Cucurbita ficifolia* seeds by the purification procedure, which includes: extraction, salting out with ammonium sulphate, chromatography on CM-cellulose. Sephacryl S-300 gel filtration and h.p.l.c. on DEAE-2SW TSK column. The enzyme was homogeneous both in native and SDS PAGE. Three independent methods showed its molecular mass to be approximately 77 kDa. The enzyme was inhibited by specific serine proteinase organic inhibitors, and was active in the presence of inhibitors specific for other proteinase classes. Surprisingly, squash proteinase exhibited a very high and broad pH optimum with a maximum at 10.7. It hydrolysed many different peptide bonds in B-chain of insulin and was able to cleave four bonds in endogenous serine proteinase inhibitor (CMTI).

Animal proteolytic enzyme are known to play a crucial role in biological processes such as digestion, zymogen activation, hormone maturation, blood coagulation, fibrinolysis, predestination, protein remodelling [1]. However, relatively little is known about the function of proteinases in plants and many researches have focused their attention on the role of proteinases in the mobilization of storage proteins.

The major subject of our previous studies were the smallest protein inhibitors of serine proteinases isolated from the seeds of *Cucurbitaceae* plants [2, 3, 4] but so far we were unable to find any endogenous serine proteinase inhibited by these inhibitors [5]. However, we have isolated the aspartic proteinase [6, 7], which inactivates squash inhibitors by limited proteolysis (to be published).

In this communication we describe properties of serine proteinase isolated from *Cucurbita ficifolia* seeds which is able to split several peptide bonds in the squash inhibitor.

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MATERIALS AND METHODS

The seeds of *C. ficifolia* were obtained from the Garden Seeds Company, Wrocław. The reagents and methods used were essentially the same as used previously [3]. Routinely, the activity of proteinase was determined at pH 8.6 using casein as a substrate [8]. The oligopeptide substrate was from Sigma Co. The *N*-terminal amino acids were determined using dansyl-method and were identified by thin-layer chromatography [9].

RESULTS AND DISCUSSION

Proteinase isolation. Typically, proteins from 100 g of the figleaf gourd seeds were extracted with 50 mM Na-acetate, pH 4.6, salted out with ammonium sulphate (0.3 saturation) and then applied on CM-cellulose column (0.02 M Na-phosphate, pH 6.0). The active fractions eluted at 0.1 M NaCl were precipitated with ammonium sulphate and subjected to chromatography on Sephacryl S-300 column, equilibrated with 0.1 M acetic acid. Finally, the enzyme was purified by h.p.l.c. (TSK DEAE-2SW column, isocratic elution with 0.1 M Na-phosphate, pH 7.6). Typically 8 mg of the enzyme was obtained from 100 g portion of seeds and 20% yield with the purification factor of 350. A major difficulty during purification was due to proteinase instability. However, the enzyme was stable in ammonium sulphate solution (0.3 saturation) at 5°C.

Enzyme properties. The apparent molecular mass of the enzyme, as determined by gel filtration and SDS PAGE, was about 77 kDa. The maximum activity of enzyme was at pH 10,7 both with casein and Suc-Ala-Ala-Pro--Leu-*p*-nitroanilide as substrates. The enzyme was inhibited by diisopropyl fluorophosphate and phenylmethanesulphonyl fluoride but not by EDTA, iodoacetamide, dithiothreitol, 2-mercaptoethanol or pepstatin. This unequivocally indicates that the isolated proteinase is a serine enzyme. None of the examined protein inhibitors of serine proteinases (Kunitz and Bowman-Birk inhibitors from soybean, Kunitz and Kazal inhibitors from bovine pancreas, turkey and chicken ovomucoid) including the endogenous serine proteinase inhibitor (CMTI I) is able to inhibit the isolated proteinase.

Proteinase specificity. The potential sites of proteolysis were determined using the oxidized B-chain of insulin as a substrate. Incubation of B-chain with a catalytic amount of the enzyme at pH 8.6, produced 14 small peptides which could be separated by reverse phase h.p.l.c. (Ultrapack Column Lichrosorb RP 10 μm, linear gradient 0-50% of acetonitrile in 0.1% trifluoroacetic acid). *N*-Terminal amino acid analysis and amino acid composition of these peptides made possible to determine unequivocally localization of the hydrolyzed peptide bonds. As can be seen from Fig. 1, the enzyme exhibits very broad specificity and there is no preference for specific recognition of any amino acid

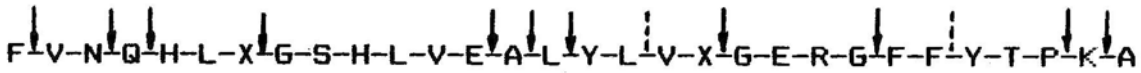

Fig. 1. Peptide bonds in oxidized B-chain of insulin which are susceptible to proteolytic attact by *Cucurbita ficifolia* serine proteinase. For details of procedure see Materials and Methods. Peptide bonds hydrolyzed after 20 ↓ and 50 ¦ minutes of incubation with 0.01 mol % of proteinase at pH 7.8 are indicated; x, cysteic acid residue

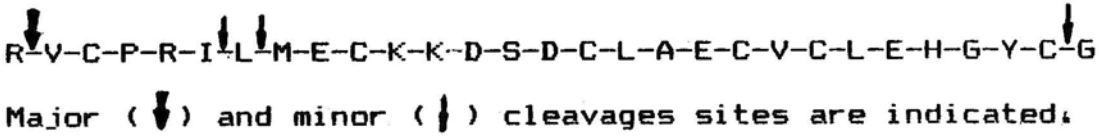

Fig. 2. Proteolysis of native *Cucurbita maxima* trypsin inhibitor by endogenous serine proteinase. Major ↓ and minor ↓ cleavages sites are indicated

residues. Incubation of the native proteinase inhibitor (CMTI I) from *C. ficifolia* seeds with the proteinase increased inhibitor mobility in gel electrophoresis at pH 8.6. This resulted from cutting off the *N*-terminal arginine residue from the inhibitor molecule. This form still linearly inhibits bovine trypsin as it was found in the routine test using Bz-Arg-*p*-nitroanilide as a substrate. We also detected cleavage of three additional peptide bonds (Ile^6-Leu^7, Leu^7-Met^8) and cleaves Cys^{28}-Gly^{29} but with a very low yield (Fig. 2) It is known that specific cleavage of the Leu^7-Met^8 peptide bond by pepsin and squash seeds aspartic proteinase completely inactivates the inhibitor.

The chromogenic chymotrypsin substrate Suc-$(Ala)_2$-Pro-Leu-*p*--nitroanilide was found to be a good substrate of the enzyme.

The enzyme from *C. ficifolia* seeds resembles in some properties the serine proteinase isolated from fruit juice of *C. ficifolia* [10] and *Cucumis melo* [11] but it significantly differs in molecular mass and amino-acid composition from those two proteinases.

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