ANNA WILIMOWSKA-PELC, ANTONI POLANOWSKI, MARIA K. KOŁACZKOWSKA, MACIEJ WIECZOREK and TADEUSZ WILUSZ

 ASPARTYL PROTEINASE FROM CUCUMBER (CUCUMIS SATIVUS) SEEDS. PREPARATION AND CHARACTERISTICS* 

Institute of Biochemistry, Wroclaw University, Tamka 2, 50-137 Wroclaw, Poland

Aspartyl proteinase (EC 3.4.23) from cucumber seeds was purified by ammonium sulphate fractionation, chromatography on immobilized pepstatin and gel filtration on Sephacryl S-200. The preparation obtained, homogeneous on polyacrylamide-gel electrophoresis in acidic and alkaline media, has a molecular mass of 42 000, pI of 5.2, and shows the highest activity with denatured haemoglobin at pH 3.2. The proteinase is stable in slightly alkaline medium, whereas it is inactivated in acidic medium, especially in the presence of NaCl. The enzyme activity is affected neither by the inhibitors of serine proteinases, sulphydryl-proteinases and metalloproteinases, nor by divalent metal ions, whereas the enzyme is inactivated by the inhibitors of aspartyl proteinases: 1,2,3-epoxy(p-nitrophenoxy)propane, diazoacetyl-DL-norleucine and pepstatin.

Aspartyl proteinases — carboxyl (acid) proteinases (EC 3.4.23) — have free carboxyl groups of aspartic acid in the active centre. The enzymes are specifically inhibited by EPNP¹, DAN and pepstatin (Barrett, 1977). Purification of proteinases from this group, especially those of plant origin, is connected with numerous difficulties. Carboxyl proteinases of animal origin were obtained in highly purified form with the use of immobilized protein substrates (Turk et al., 1977) or pepstatin (Kregar et al., 1977). Fungal

---

* This work was supported by the Ministry of Science, Education and Technology within the program R.1.9.

¹ Abbreviations used: DAN, diazoacetyl-DL-norleucine methyl ester; DFP, diisopropylfluorophosphatase; pCMPSA, p-chloromercuribenzenesulphonic acid; DTT, dithiothreitol; EPNP, 1,2,3-epoxy(p-nitrophenoxy)-propane; PMSF, phenylmethylsulphonyl fluoride; STI, soybean trypsin inhibitor; TNBS, trinitrobenzene sulphonic acid; Hb, haemoglobin; BAPNA, α-N-benzoyl-DL-arginine-p-nitroanilide·HCl.
carboxyl proteinases were successfully purified with the use of immobilized antibiotics: gramicidin S and bacitracin (Rudenskaya et al., 1980). However, the corresponding enzymes from higher plants have not so far been more closely characterized. Low level of activity of these enzymes in plant material, especially in dormant seeds, and the use of traditional, non-specific methods made impossible preparation of these enzymes in homogeneous form.

The aim of the present work was to apply biospecific sorbents in purification of aspartyl proteinases from cucumber seeds, and to determine some properties of the isolated enzyme.

MATERIALS AND METHODS

Seeds of cucumber, variety Wisconsin, were obtained from the Production Department of Garden Seeds in Wroclaw (Poland).

Protein was determined by the method of Lowry et al. (1951) and by the microbiuret method according to Gooa (1953).

The content of sugar was measured by the phenol method according to Dubois et al. (1956).

Determination of proteolytic activity. One millilitre of acid-denatured 4% haemoglobin in 0.2 m-acetate/HCl buffer, pH 3.2, was incubated for 30 min at 36°C with 1 ml of the enzyme solution. The reaction was stopped by adding 3 ml of 5% trichloroacetic acid. The sediment was centrifuged and in the supernatant absorption at 280 nm was measured. One unit of proteolytic activity was defined as that amount of the enzyme which, under the conditions applied, gave an increase in absorption A_{280} = 0.1. For determination of substrate susceptibility, haemoglobin was replaced alternatively with serum albumin, methylated serum albumin, egg albumin, edestin, or globulin from cucumber seeds; the activity was determined by measuring both absorption at 280 nm after deproteinization as above, and monitoring the increase in free amino groups by the method of Snyder & Sobociński (1975).

Activation of trypsinogen. Bovine trypsinogen, 0.15 mg, was incubated with 0.15 mg of the enzyme in 1 ml of 0.1 m-acetate/HCl buffer, pH 3.2. After 30 min incubation at 36°C, trypptic activity was determined with BAPNA according to Erlanger et al. (1961).

Milk-clotting activity. The enzyme (0.1 ml, 190 μg) was incubated according to Yu et al. (1969) with 10% solution of dried milk powder in 0.01 m-CaCl₂ at 35°C.

Pepstatin was immobilized on AH-Sepharose 4B under conditions described by Linde & Persliden (1978).

Electrophoresis was run in 15% polyacrylamide gel at pH 4.5 according to Reisfeld et al. (1962) and at pH 8.3 according to Davis (1964). Gels were stained for protein with Coomassie Brilliant Blue R-250.

Isoelectric focusing was performed according to Karlsson et al. (1973) at
pH gradient from 3.5 to 9.0 in a 3 mm layer of polyacrylamide gel containing 12.5%, of sucrose.

**Molecular mass** of the enzyme was determined by gel filtration on a Sephadex G-75 column (1.5 x 90 cm) equilibrated with 0.05 M-phosphate buffer, pH 7.3, containing 0.1 M-NaCl. The following protein standards were used: cytochrome c (mol. mass 12 400), chymotrypsinogen A (25 700), β-lactoglobulin (36 800), egg albumin (45 000) and serum albumin (66 000). The proteins at a concentration of 1 mg/ml, were applied separately in 1 ml, and were eluted at a rate of 10 ml/h.

**Influence of effectors.** Divalent metal ions: Ca, Cu, Mn (chlorides), Mg, Zn, Hg (acetates) and Fe (sulphate), at 2 mM concentration, the -SH-group reagents (pCMPSA, iodoacetamide, iodoacetic acid, DTT), synthetic inhibitors of serine proteases (PMSF, DFP) and EDTA at 1 mM concentration were preincubated with the enzyme (5 μg) for 30 min at 36 C in 0.1 M-Na-acetate/HCl buffer, pH 3.2, or 0.02 M-phosphate buffer, pH 7.2. Natural trypsin inhibitors from the seeds of soybean (STI), lima bean and cucumber, and from bovine pancreas (Kunitz) were preincubated with the enzyme for 30 min at 36 C, at a weight ratio of 20:1, in 0.1 M-Na-acetate/HCl buffer, pH 3.2. After preincubation, the residual proteolytic activity was determined.

**The effect of diazoacetyl-DL-norleucine** on the enzyme activity was determined according to Gripon (1976). To 0.6 ml of the enzyme solution (87.5 μg/ml) 0.1 ml of 0.01 M-Cu²⁺, 0.1 ml of 0.01 M-DAN and 2 ml of 0.25 M-acetate buffer, pH 5.4, were added (final concentrations of the reagents were 1 mM, 1 mM and 50 mM, respectively). The mixture was incubated at 36 C. In the control sample, the enzyme was incubated with copper ions without DAN. At the time intervals indicated in legends to Tables and Figures, 50 μl samples were withdrawn for determination of proteolytic activity. The effect of 1,2,3-epoxy(-p-nitrophenoxo)propane was determined as described by Gripon (1976). EPNP in substantial, 3.3 mg, was added to 1 ml of the enzyme solution (0.14 mg protein) in 0.1 M-acetate buffer, pH 4.5. The sample was incubated at 28 C with gentle shaking. At the time intervals indicated for the particular experiments, 50 μl samples were withdrawn for determination of proteolytic activity.

**The effect of pepstatin.** The enzyme, 4.2 μg, was preincubated for 10 min at 36 C in 1 ml of 0.1 M-Na-acetate/HCl buffer, pH 3.2, with increasing amounts of pepstatin, then 1 ml of substrate was added, and proteolytic activity was determined.

**The effect of pH, temperature and NaCl.** The enzyme (50 μg/ml) in 0.02 M-acetate buffer of pH 3.6, 5.0 and 0.02 M-Tris/HCl buffer of pH 8.0 were heated for 30 min at 20 - 60 C, then cooled in ice, and 100 μl samples were taken for determination of the proteolytic activity. The effect of pH and NaCl was studied with the enzyme (70 μg/ml) stored at 5 C for 6 days at pH 2.2, 4.8 (0.02 M-phosphate — 0.01 M-citric acid) and 8.4 (0.05 M-Tris/HCl). In one of the two series studied, the samples contained 0.5 M-NaCl. At
two-day intervals samples of 50 μl were taken for determination of proteolytic activity.

Reagents. Ovoalbumin, crystalline bovine serum albumin, methylated bovine serum albumin, hemp edestin (2× cryst.), bovine haemoglobin, cytochrome c from horse heart, β-lactoglobulin from cow milk, chymotrypsinogen A (6× cryst.), bovine trypsinogen (1× cryst.), trypsin inhibitors from lima bean and soybean. pepstatin A, DAN, EPNP, PMSF, pCMPSA. DFP. DTT, were products of Sigma Chemicals (St. Louis, Mo., U.S.A.), Sepharose 4B, Sephacryl S-200 were from Pharmacia Fine Chemicals (Uppsala, Sweden), Coomassie Brilliant Blue R-250 and reagents for polyacrylamide-gel electrophoresis from Fluka A. G. (Buchs S. G., Switzerland), ampholine from LKB (Bromma, Sweden); trypsin inhibitors from bovine pancreas (Kunitz) and from cucumber seeds were prepared according to Wilusz et al. (1973, 1981), and cucumber globulin, according to Hara et al. (1976). Skimmed milk powder was from the Dairy Cooperative in Lubawa (Poland). All other reagents, of analytical grade, were supplied by POCh (Gliwice, Poland).

RESULTS AND DISCUSSION

Isolation and purification of proteinase from cucumber seeds. Ground cucumber seeds, 1 kg, were extracted with 5 litres of 0.1 M-acetate buffer, pH 4.5, for 1 h at room temperature with constant stirring. The insoluble material was centrifuged off (2000 g, 30 min) and the supernatant was left overnight in a refrigerator. The precipitated sediment was centrifuged off, and from the supernatant proteins were salted out at 0.8 (NH₄)₂SO₄ saturation. The precipitate was dissolved in water, and to the protein solution, acidified with 1 M-CH₃COOH to pH 3.6, NaCl was added at the concentration of 0.5 M. The mixture was left overnight in a refrigerator, the sediment precipitated was centrifuged off, and the supernatant was applied to the column (1.7×4.0 cm) filled with immobilized pepstatin equilibrated with 0.1 M-acetate buffer, pH 3.6. The non-adsorbed material was eluted with the same buffer. The enzyme was eluted from the column with 0.1 M-Tris/HCl buffer, pH 8.3, containing 0.5 M-NaCl. Under these conditions, about 30% of total enzyme activity was adsorbed on immobilized pepstatin, therefore the non-adsorbed material was subjected twice more to the same chromatographic procedure. In the material pooled from three successive runs over 50% of the activity was recovered, with a 400-fold increase in specific activity. The enzyme solution was dialysed 1 h against water, then concentrated to a volume of about 3 ml in a stream of cold air, and applied to a Sephacryl S-200 column (1.0×80.0 cm) equilibrated with 0.05 M-Tris/HCl buffer, pH 8.3. On chromatography, the material applied was resolved into two fractions (Fig. 1A), the first of which contained all the proteolytic activity. The enzyme preparation obtained by the above procedure (13.6 mg from 1 kg of seeds) was homo-
Fig. 1. Chromatography on Sephacryl S-200 of the cucumber seed proteinase purified on immobilized pepstatin (A), and polyacrylamide-gel electrophoresis of the preparation obtained (B). (A) Fractions of 3.5 ml were collected at the rate of 20 ml/h; ●, A_280; ×, proteolytic activity. (B) 1, 15', gels, pH 8.3 (45 μg); 2, 15', gel, pH 4.5 (45 μg).

Table 1

Purification of the proteinase from cucumber seed

For preparation of the enzyme, 1 kg of seeds was used. The activity of the enzyme in the extract is not presented due to the lack of proportionality. Protein in the extract was determined by the methods of Goo (1953), and at further steps of the procedure according to Lowry et al. (1951).

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Activity (units)</th>
<th>Spec. act. (units/mg)</th>
<th>Yield (%)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extract</td>
<td>4020.0</td>
<td>40800.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Precipitation with (NH_4)_2SO_4</td>
<td>205.0</td>
<td>17630.0</td>
<td>37023</td>
<td>2.1</td>
<td>100.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Pepstatin-Sepharose 4B</td>
<td>110.0</td>
<td>24.8</td>
<td>21025</td>
<td>847.8</td>
<td>56.8</td>
<td>403.7</td>
</tr>
<tr>
<td>Sephacryl S-200</td>
<td>19.5</td>
<td>13.6</td>
<td>15762</td>
<td>1159.0</td>
<td>42.6</td>
<td>551.9</td>
</tr>
</tbody>
</table>

geneous on polyacrylamide-gel electrophoresis at pH 8.3 and 4.5 (Fig. 1B). The successive steps of the proteinase purification are presented in Table 1.

Properties of cucumber proteinase. Molecular mass of the enzyme, estimated by molecular sieving, is 42 000 (Fig. 2). The isoelectric point, determined by isoelectric focusing, is 5.2. The enzymatic preparation contains 2% of sugars.

The maximum activity of the enzyme with 2% haemoglobin in 0.1 M-acetate buffer, is at pH 3.2 (Fig. 3A). Changes in ionic strength of the buffer over the range 0.05 - 0.25 M do not affect the activity at pH 3.2.

Thermal stability of the enzyme at pH 3.6, 5.0 and 8.0 is presented in Fig. 3B. On 30-min heating at temperatures ranging from 20 to 60°C, the enzyme is the most stable at pH 5.0.

The proteinase kept for 6 days at 5°C in slightly alkaline medium (pH 8.0) retains over 80% of its activity. Stability of the enzyme decreases
Fig. 2. Determination of molecular mass of the proteinase from cucumber seed by chromatography on Sephadex G-75 column. 1. Cytochrome c; 2. chymotrypsinogen A; 3. β-lactoglobulin; 4. ovoalbumin; 5. bovine serum albumin; P, cucumber seed proteinase.

Fig. 3. Effect of pH (A), temperature (B) and NaCl (C) on the proteinase activity. (A) The enzyme (7 μg) was incubated for 30 min at 36 °C with 2% haemoglobin, at various pH values: ●, 0.1 M-Na-acetate/HCl buffer; ○, 0.1 M-Na-acetate/acetic acid buffer. (B) The enzyme was incubated at: ●, pH 3.6; ○, pH 5.0 and □, pH 8.0; for details see Methods. (C) The enzyme was stored at 5 °C at: □, pH 2.2; △, pH 5.0 or ○, pH 8.0, either without NaCl added (continuous line) or in the presence of 0.5 M-NaCl (broken line).

with decreasing pH value. At pH 5.0 and 8.0 it is not significantly affected by 0.5 M-NaCl, whereas at pH 2.2 NaCl distinctly lowers the enzyme stability (Fig. 3C).

Influence of effectors. The enzyme activity is practically unaffected by the -SH-group reagents (pCMPSA, iodoacetamide, iodoacetate), divalent metal ions (Ca, Mg, Zn, Cu, Mn, Fe, Hg), synthetic inhibitors of serine proteinases (PMSF, DFP) or protein inhibitors of serine proteinases (STI, Kunitz inhibitor from bovine pancreas, inhibitors from lima bean and cucumbers). DTT
preincubated with the enzyme at pH 7.2 reduced the activity by half both in
the presence and absence of EDTA, whereas at pH 3.2 even a slight
increase in enzymatic activity was observed.

The rate of inactivation of the proteinase by 800-fold molar excess of
DAN and Cu²⁺ is presented in Fig. 4A. Incubation for 25 min at pH 5.6
at 36°C resulted in almost complete inactivation of the enzyme. Cu²⁺ applied
at the same concentration but in the absence of DAN, had no effect.

Similarly, the enzyme was inactivated on incubation at 28°C and at pH
4.5 in the presence of EPNP (Fig. 4B).

Pepstatin is a strong inhibitor of the proteinase. From the inactivation
curve (Fig. 4C) it appears that 1 mole of the enzyme is inhibited by about

Fig. 4. Effect of diazoacetyl-DL-norleucine (A), 1,2,3-epoxy(p-nitrophenoxy)propane (B) and
pepstatin, (C) on the enzyme activity. (A) ●, Activity in the presence of DAN and Cu²⁺;
○, activity in the presence of Cu²⁺, without DAN. (B) ●, Activity in the presence of
EPNP; ○, control, without EPNP. (C) The enzyme concentration in the incubation mixture
was 5 × 10⁻⁸ M.

Fig. 5. Susceptibility of various proteins to digestion by the cucumber seed proteinase.
The activity was determined by measuring the increase both in absorption at 280 nm
(outlined columns) and free amino groups with TNBS (hatched columns) in samples de-
proteinized with trichloroacetic acid. The activity with acid-denatured protein was taken as 100.
0.75 mole of pepstatin. This may indicate that the enzyme preparation contains about 25% of inactive protein — assuming that pepstatin reacts with the proteinase at an equimolar ratio.

_Susceptibility of various proteins to digestion by the proteinase._ The enzyme hydrolyses both animal proteins (denatured and non-denatured haemoglobin, methylated and non-methylated serum albumin, egg albumin) and plant proteins (edestin from hemp seed, globulin from cucumbers). From the data presented in Fig. 5 it appears that haemoglobin is the best substrate. Determination of trichloroacetic acid-soluble products of all remaining substrates (except egg albumin) with TNBS turned out to be several-fold more sensitive than the monitoring of absorption at 280 nm. Endogenous globulin from cucumbers was degraded to the trichloroacetic acid-soluble products to a lesser extent than haemoglobin.

The proteinase from cucumber seeds, like many fungal proteinases (Matsubara & Feder, 1971) is able to coagulate milk casein: 190 μg of the enzyme coagulates 5 ml of 10% reconstituted skim milk within 1 min at 35°C. However, unlike some fungal proteinases, it is unable to activate trypsinogen.

The properties of the enzyme presented in this paper: optimum pH in acidic medium, and inactivation by EPNP, DAN and pepstatin, inhibitors of aspartyl proteinases. indicate that the enzyme isolated from cucumber seeds is an aspartyl proteinase (EC 3.4.23).

REFERENCES


PROTEINAZA ASPARTYLOWA Z NASION OGÓRKÓW (CUCUMIS SATIVUS). PREPARACJA I CHARAKTERYSTYKA

Streszczenie

Proteinazę aspartylową z nasion ogórków (Cucumis sativus) oczyszczono poprzez frakcjonowanie siarczanem amonu, chromatografię na immobilizowanej pepstatynie oraz filtrację żelową na Sephacryl S-200. Otrzymany preparat homoenny w elektroforezie w celu polisakryloamidowym w środowisku kwaśnym i zasadowym, posiada masę cząsteczkową 42 000, pI 5.2 i wykazuje najwyższą aktywność w pH 3.2 wobec zdenaturowanej hemoglobiny. Proteinaza jest stabilna w środowisku lekko zasadowym, natomiast w środowisku kwaśnym ulega inaktywacji, szczególnie w obecności NaCl. Inhibitory proteaz serynowych, sulphydrylowych i metaloproteinaz, jak również jony metali dwuwartościowych nie wpływają na aktywność enzymu. Natomiast inhibitory proteinaz aspartylowych: 1,2,3-epoksy(p-nitrofenoksyl)propan, dwuacetylo-DL-borneolina i pepstatyna inaktywują enzym.

Received 30 June, 1982.