ISOLATION AND SOME PROPERTIES OF CATHEPSIN D INHIBITOR FROM POTATOES

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An inhibitor of cathepsin D was isolated from potato juice by ammonium sulfate precipitation, gel filtration on Sephadex G-50, chromatography on DEAE-Sephadex A-25, SE-Sephadex C-25, and affinity chromatography on Sepharose 4B with covalently bound cathepsin D. The purification of the inhibitor was 80 − 90 fold. The inhibitor is stable in the pH-range 2 − 10·5. The activity of the inhibitor remains unaltered after 30 min heating at 60°C yet is partly destroyed after 1 min heating at 85°C. The inhibitor is stable toward pepsin digestion. Its molecular weight determined in gel filtration experiments on Sephadex G-75 is 27000. The degree of inhibition was proportional to quantity of inhibitor added up to 50% inhibition.

Still more attention has been focused on naturally occurring inhibitors of proteolytic enzymes which may serve as models in studies on specific interactions between two macromolecules and which are also of practical importance from the therapeutical viewpoint.

Very little has been known about the naturally occurring inhibitors of proteinases of the carboxyl type. Umezawa and coworkers[1] have isolated pepstatin, an inhibitor from *Streptomyces*; this peptide of molecular weight 700 inhibits pepsin, gastricsin, rennin, cathepsin D and E (ref.[2,3]). Another inhibitor of pepsin was isolated from the body walls of the roundworm *Ascaris lumbricoides*[4] in 1970; this inhibitor inhibits besides pepsin also cathepsin E, it does not inhibit, however, cathepsin D and rennin[5]. Werle and coworkers[6] showed in 1959 that a potato extract inhibits among others also the proteolytic activity of a homogenate of bovine spleen at pH 3·5. By using precipitation with ammonium sulfate to 0·3 saturation they were able to enrich partly the inhibitory activity of the material; these authors did not, however, continue these investigations. The proteolytic activity of the spleen homogenate at pH 3·5 can be ascribed predominantly to cathepsin D and potatoes must therefore contain an inhibitor of cathepsin D. Since no naturally occurring high molecular weight inhibitor of the tissue proteinase cathepsin D has been reported so far, we have made an attempt to purify this inhibitor and to determine some of its basic characteristics.

EXPERIMENTAL

Potatoes (*Solanum tuberosum*) were of common commercial origin. Cathepsin D(3.4.23.5) was prepared from bovine spleen in this Laboratory[7]. Pepsin (3.4.23.1) was a commercial product of Worthington Biochemical Corporation, Freehold, N.J., U.S.A. Ribonuclease was from Koch-Light, England. Trypsin (3.4.21.4), human serum albumin, bovine hemoglobin and chymotrypsinogen were purchased from Léčiva, Prague. Nα-Benzoyl-D,L-arginine-*p*-nitroanilide (BAPN) was prepared in this Institute. Sephadex G-50, G-75, DEAE-Sephadex A-25, SE-Sephadex C-25, and Sepharose 4B were from Pharmacia Fine Chemicals, Sweden. Bio-Gel P-2 was from Bio Rad Laboratories, U.S.A.

Methods

Assay of enzymatic activities. 1) *Proteolytic activity of cathepsin D.* A solution of cathepsin D (50 μl, 15—20 mU), 200 μl of 0·1M acetate buffer at pH 3·5, and 1 ml of 2% hemoglobin solution denaturated and adjusted to pH 3·5 by HCl were incubated 20 min at 40°C. The digestion was discontinued by the addition of 2 ml of 5% trichloroacetic acid. The reaction mixture was filtered and the A_{280nm} of the filtrate was determined. One unit of enzyme activity was defined as that amount of enzyme which increased the absorbance (A_{280nm}) by 1·0 in 1 min. The specific activity is expressed in the number of enzymatic units in the protein solution of A_{280nm} 1·0. Milliunits (mU) were used in most cases.

2) *Inhibition of cathepsin D.* A solution of cathepsin D (50 μl, 15—20 mU) was preincubated at room temperature with 100 μl of inhibitor solution and 100 μl of 0·1M acetate buffer at pH 3·5. The proteolytic activity of uninhibited cathepsin D was determined as described above. One unit of inhibitory activity was defined as that amount of inhibitor which inhibits one cathepsin D unit by 50%. The specific activity denotes the number of inhibitor units in solution of $A_{280nm} =$ $= 1·0$. The values are given in milliunits.

3) *Enzymatic activity of trypsin.* Trypsin (2 μg in 100 μl of H_2O), 1 ml of 0·1M-Tris-HCl buffer at pH 8·0, and 50 μl of substrate (40 mg of BAPN in 1 ml of dimethylformamide) were incubated 20 min at 40°C. The digestion was discontinued by the addition of 100 μl of glacial acetic acid. The quantity of *p*-nitroaniline liberated was determined spectrophotometrically at 405 nm.

4) *Inhibition of trypsin.* A trypsin solution (100 μl, 2 μg) and 1 ml of 0·1M Tris-HCl buffer at pH 8·0 were preincubated with 100 μl of the inhibitor solution 5 min at room temperature before the addition of the substrate. The residual enzymatic activity was determined as described above.

Preparation of Sepharose 4B *with covalently bound cathepsin* D. Sepharose 4B (10 ml) was activated by cyanogen bromide[8], washed repeatedly, and suspended in 0·1M phosphate buffer, pH 6·8 (at 4°C). The buffer was sucked off and Sepharose was suspended in 10 ml of the same buffer which contained 100 mg of cathepsin D. The suspension was gently stirred 24 h at 4°C. Unbound cathepsin D was removed by washing the suspension alternately with dilute acetic acid at pH 4·0 containing 1M-NaCl and with 0·1M phosphate buffer at pH 7·8 containing 1M-NaCl until the eluate showed no absorbance at 280 nm or cathepsin D activity. One ml of the cathepsin D-Sepharose wet slurry contained 300 mU of cathepsin D as judged by the hydrolysis of the hemoglobin substrate.

Disc electrophoresis was carried out in 7·5% polyacrylamide gel and Tris-glycine buffer at pH 8·3 or in β-alanine-acetic acid buffer at pH 4.

Molecular weight determinations were effected by gel chromatography on Sephadex G-75 in 0·1M-KCl whose pH had been adjusted to 4. Human serum albumin (mol. wt. 68000), chymotrypsinogen (mol. wt. 25000), and ribonuclease (mol.wt. 13700) served as elution volume standards. The protein (15 mg in 3 ml of H_2O) was applied to a column of 2·3 × 69 cm (V_t = 287 ml). The flow rate was 16 ml/h and 3 ml fractions were collected.

Purification of potato cathepsin D *inhibitor* (PDI). Purified potatoes (1 kg) were homogenized with 1800 ml of 0·1M acetate buffer, pH 4, containing 1% of n-butyl alcohol. The suspension was stirred overnight at 4°C. Large particles were removed by filtration through double gauze and the extract was centrifuged at 5000 G (20 min). To the filtrate solid ammonium sulfate was added to give 30% saturation. After standing overnight the precipitate was collected by centrifugation, dissolved in water (40—50 ml), and chromatographed on Sephadex G-50.

Gel filtration on Sephadex G-50 *fine* was carried out in two piston columns connected in series (each column 3 × 85 cm) in the upward flow arrangement. Dilute acetic acid, pH 4·0, containing 1% of n-butyl alcohol was used as eluant. The flow rate was 40 ml/h and 13·3 ml fractions were collected. Fractions containing PDI were pooled and lyophilized (sample A).

Chromatography on DEAE-*Sephadex* A-25. The ion exchanger was equilibrated with 0·01M Tris-HCl buffer at pH 7·6. Sample A (100 mg) was dissolved in the same buffer and centrifuged. The supernatant was applied to a 1·6 × 10 cm column. The flow rate was 12 m/h. The initial buffer was 0·01M-Tris-HCl at pH 7·6. As soon as the absorbance of the effluent dropped to zero, the column was eluted by a linear gradient of increasing concentration of NaCl in the same buffer (0—0·3M-NaCl). The fraction containing cathepsin D-inhibiting activity was desalted and lyophilized (sample B).

Chromatography on SE-*Sephadex* C-25. SE-Sephadex was equilibrated with 0·01M acetate buffer at pH 5·0, and sample B was dissolved in the same buffer. After application of the sample, the column was eluted first by the same buffer and subsequently by a linearly increasing concentration of NaCl in the same buffer. Fractions containing PDI were pooled, desalted, and lyophilized.

Affinity chromatography on Sepharose 4B with covalently bound cathepsin D as affinant. Preparation A or B (20—50 mg), dissolved in dilute acetic acid, pH 4, containing 0·5M-NaCl was applied to the cathepsin D-Sepharose column (0·9 × 15·7 cm). Unbound material was washed off with the same solution and PDI bound was displaced from the complex by 0·1M phosphate buffer, pH 7·6, containing 0·5M-NaCl. Fractions containing PDI were pooled, desalted and lyophilized.

The samples were desalted on a column of Bio-Gel P-2 equilibrated with dilute acetic acid. The ratio of the column volume to the sample volume was 10 : 1.

Determination of pH-*stability* of PDI. PDI (10 mg) was dissolved in 2 ml of a Britton-Robinson buffers; the pH of the buffers increased by one pH-unit in the pH-range 2—10·5. The sample solutions in the buffers were set aside for 18 h at room temperature. The inhibitory activity on cathepsin D and trypsin was then tested by standard procedures.

Thermal stability of PDI. A solution of 10 mg of PDI in 1 ml of 0·1M acetate buffer at pH 4 was heated in a water bath at 60° and 85°C for 1, 5, 10, and 30 min. The sample withdrawn was cooled down rapidly in an ice bath and its inhibitory activity on cathepsin D and trypsin was determined.

Action of pepsin on PDI. Pepsin (200 μg) was added to 20 mg of PDI dissolved in 1 ml of dilute acetic acid at pH 3·0. After 4 h incubation at 40°C pepsin was irreversibly inactivated by in-

creasing the pH of the solution to 8·5 by 2M-Tris-HCl buffer, pH 9, and the inhibitory activity of PDI on cathepsin D and trypsin was determined. In a control experiment, PDI was incubated under identical conditions yet in the absence of pepsin.

RESULTS AND DISCUSSION

Potatoes represent a rich source of inhibitors of various proteinases. Potato inhibitors have been reported of trypsin and chymotrypsin[9,10], kallikrein[11], carboxypeptidase B (ref.[12]), and of some bacterial proteinases[13]. Some of these inhibitors have been obtained in homogeneous state and their amino acid composition is known[14,15]. The partial amino acid sequence of one potato chymotrypsin inhibitor has been reported[16]. In spite of the fact that many inhibitors have been isolated from potatoes, none of these inhibitors is known to inhibit proteinases of the carboxyl type.

Purification of PDI

The inhibitor can be extracted from the homogenate both by acidic and alkaline[17] solutions. Acid extracts are less colored; we used therefore 0·1M acetate buffer at

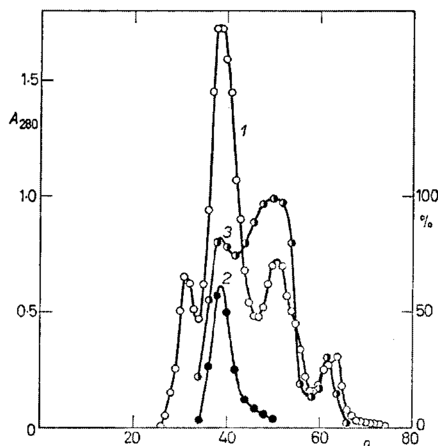

FIG. 1

Chromatography of Material Precipitated by Ammonium Sulfate on Sephadex G-50 fine

1 Absorbance at 280 nm, *2* inhibition of proteolytic activity of cathepsin D in %, *3* inhibition of enzymatic activity of trypsin in %, *n* tube number. See text for details.

FIG. 2

Disc Electrophoresis in Polyacrylamide Gel at pH 8·3 (*a* — *d*) and pH 4 (*e*)

a Preparation after gel filtration on Sephadex G-50, *b* after chromatography on DEAE-Sephadex A-25, *c* fraction I after chromatography on SE-Sephadex C-25, *d* after affinity chromatography (pH 8·3), *e* after affinity chromatography (pH 4).

pH 4·0 for extraction. When the extract is treated with ammonium sulfate PDI is salted out at 10–30% saturation. A typical course of gel chromatography of the material obtained after ammonium sulfate fractionation is shown in Fig.1. PDI emerges in the main protein peak together with the trypsin inhibitors. This fraction is com-

TABLE I

Partial Purification of PDI

The averaged result of three experiments is given.

Treatment	Spec. act. mU PDI/A_{280}	Purification factor	Activity recovery %
Initial extract	24	1	100
Precipitate with $(NH_4)_2SO_4$[a]	87	3·6	85
Sephadex G-50 fine	193	8·1	74
DEAE-Sephadex A-25	900	37·5	60
SE-Sephadex C-25 peak I	2 000	83·5	29
peak II	1 400	58·5	16
Affinity chromatography[b]	1 060	44	—

[a] 30% saturation, [b] on Sepharose 4B with covalently bound cathepsin D. The sample after gel filtration on Sephadex G-50 was subjected to affinity chromatography.

TABLE II

Thermal Stability of PDI

The values are % of inhibition.

Time min	Cathepsin D	Trypsin	Cathepsin D	Trypsin
	60°C		85°C	
1	45	75	14	57
5	40	75	13	50
10	40	74	10	45
30	45	71	5	24

plex as shown by disc electrophoresis (Fig. 2*a*). When this material was chromatographed on DEAE-Sephadex A-25 at pH 7·6, PDI was not adsorbed to the ion exchanger and emerged when the column was washed with the equilibrating buffer, whereas most proteins, among their number also certain trypsin inhibitors were adsorbed and could be displaced by increased concentration of NaCl. The disc electrophoresis pattern at pH 8·3 of the material from the first peak not adsorbed shows one main zone at the origin and three minor zones located closely under the major zone (Fig. 2*b*). The quantity of this fraction obtained from 1 kg of potatoes was 60 mg.

When PDI is subjected to additional chromatography on SE-Sephadex C-25 at pH 5·0, it is displaced by a linear gradient in the range 0–0·1M-NaCl. PDI is resolved into two fractions (I and II). As can be seen in Fig. 3, both fractions inhibit also the enzymatic activity of trypsin. In this manner, a 85-fold purification on the average of PDI was obtained, as shown in Table I. However, not even this material was homogeneous on disc electrophoresis (Fig. 2*c*).

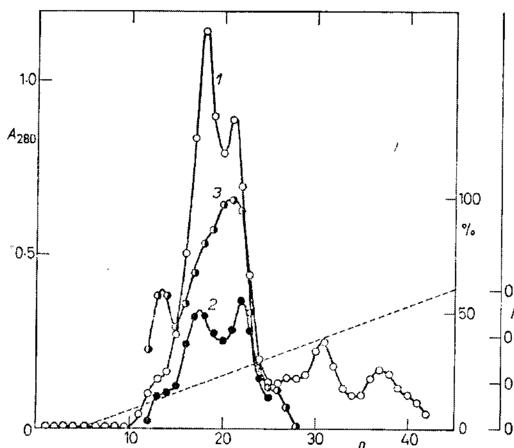

FIG. 3

Chromatography on SE-Sephadex C-25

PDI (30 mg, sample B) was applied onto a 0·9 × 11 cm column. Flow rate 7 ml/h. Initial buffer 0·01M Na-acetate, pH 5. Elution by a linear gradient of increasing concentration of NaCl (0–0·3M-NaCl, 100 + 100 ml).

1 Absorbance at 280 nm, *2* inhibition of proteolytic activity of cathepsin D in %, *3* inhibition of tryptic activity in %, *n* tube number, ------ molarity of NaCl (0–0·3M).

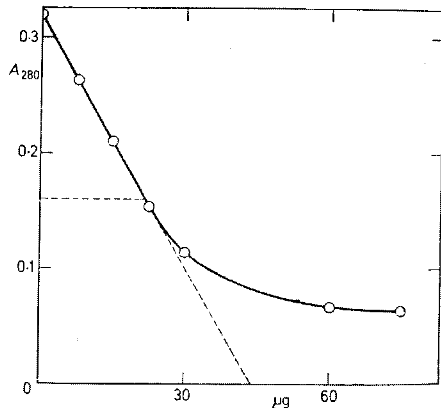

FIG. 4

Dependence of Inhibition of Proteolytic Activity of Cathepsin D on Amount of PDI

Abscissa amount of PDI in μg, ordinate residual activity of cathepsin D in measured values of A_{280nm}. The quantity of cathepsin D taken for the activity tests was 16 mU; the hydrolysis of 2% hemoglobin at pH 3·5 was allowed to proceed 20 min at 40°C.

Since the cathepsin D-inhibiting activity of PDI of all the fractions obtained was paralleled by trypsin-inhibiting activity, we made an effort to retain PDI specifically on Sepharose with covalently bound cathepsin D. We tested the activity of cathepsin D bound to Sepharose 4B and also the pH-optimum of proteolysis. We found that this optimum lies between pH 3 and 3·5 when assayed with hemoglobin as substrate and that it does not essentially differ from the pH-optimum of free cathepsin D. This finding of ours significantly differs from the data reported by Kazakova and coworkers[18]. According to the latter authors rat liver cathepsin D bound to Enzacryl Polyacetal or Sepharose 4B shows a shift of pH-optimum from the acidic range to pH 7 when assayed with hemoglobin as substrate. By contrast, Turk and coworkers[19], similarly to us, did not observe any shift of the pH-optimum of cathepsin D bound to Sepharose 4B. Immobilized cathepsin D retained its pH-optimum at pH 3·3. These differences can be explained *a*) by the different sources of the two cathepsins (rat liver and bovine spleen) and *b*) possibly also by the different conditions under which the binding of the enzyme to the solid support was effected.

After affinity chromatography of sample A (chromatographed on Sephadex G-50) on cathepsin D-Sepharose the specific activity of PDI increased approximately 6-times (Table I). This operation is quick, it does not yield, however, a homogeneous preparation either. The preparation showed antitryptic activity and on disc electrophoresis at pH 8·3 (Fig. 2*d*) a pattern similar to that of the preparation chromatographed on DEAE-Sephadex; one sharp zone only was found at pH 4 (Fig. 2*e*).

Some Properties of PDI

As noted above, all preparations of PDI showed also antitryptic activity. We ascribe this fact to the unhomogeneity of the preparation rather than to the polyvalent character of the inhibitor; therefore when studying the properties of PDI we examined also the presence of antitryptic activity in an effort to use the possible differences between the two inhibitors for the isolation of the cathepsin D inhibitor.

Thermal stability. When PDI is heated at 60°C for 30 min, the anticathepsin and antitrypsin activity of PDI remain unaltered. When heated at 85°C, the cathepsin D inhibitor looses its activity already after 1 min and is completely inactive after 30 min. The antitrypsin activity also decreases under these conditions yet the decrease is not so sharp as in the preceding case (Table II).

pH-*Stability.* PDI is stable in the pH range 2–10·5. Both the cathepsin D-inhibiting activity and the antitryptic activity remain unaltered.

Dependence of inhibition on period of preincubation of cathepsin D with PDI. In an effort to determine how fast a complex is formed of cathepsin D and its inhibitor we preincubated both components at room temperature for 1, 5, 10, 30, and 60 min. Having determined the residual activity of cathepsin D we found that the inhibition

is the same in all cases and that the interaction of the enzyme with the inhibitor is most probably instantaneous.

The dependence of the degree of inhibition of catepsin D on the amount of PDI is shown in Fig. 4. The profile of the curve shows that the dependence is linear for amounts of PDI which inhibit the enzymatic activity of cathepsin D by less than 50%. Therefore the inhibitory potency of different PDI preparations was calculated for inhibition lower than 50%. The affinity of PDI for cathepsin D seems to be considerably weaker than that of *e.g.* the highly potent pancreatic, soy-bean and potato trypsin inhibitors, *etc.* From preliminary experiments the K_i-value is of the order of 10^{-7}M.

The molecular weight of PDI was determined by gel filtration on Sephadex G-75. The elution volume indicates a molecular weight of approximately 27000.

Action of pepsin on PDI. It has been observed that the potato chymotrypsin inhibitor(I) is cleaved by pepsin at pH 2 (ref.[20]). We assumed that antitrypsin activity could be removed from our preparation in this manner. It was necessary to determine first whether PDI interacts with pepsin or not. A fact deserving interest is that PDI does not inhibit the activity of porcine pepsin. It was therefore possible to subject PDI to peptic hydrolysis. We found that PDI is entirely resistant to pepsin and neither its anticathepsin nor its antitrypsin activity are altered by the action of pepsin.

The data presented here permit us to compare further two related proteinases, cathepsin D and pepsin. Both enzymes fall into the group of carboxyl proteinases, are inhibited by pepstatin, diazomethylketo compounds in the presence of Cu^{2+}-ions, and have a similar specificity when assayed with the B-chain of oxidized insulin as substrate[7]. On the other hand pepsin cleaves a number of simple peptides whereas cathepsin D prefers larger peptides[21]. This seems to indicate some differences in the conformation of their binding sites. Cathepsin D and pepsin markedly differ in their behavior toward certain naturally occurring inhibitors. We have found earlier that the pepsin inhibitor from the body walls of the roundworm *Ascaris lumbricoides* does not inhibit the proteolytic activity of cathepsin D (ref.[5]). On the other hand, our experiments described in this paper have shown that, by contrast, PDI does inhibit cathepsin D and does not inhibit pepsin. This very interesting fact provides evidence showing that the part of the molecule of cathepsin D and pepsin which bears the binding site for these inhibitors, is different in structure.

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REFERENCES

1. Umezawa H., Aoyagi T., Morishima H., Matsuzaki M., Hamada M., Takeuchi T.: J. Antibiot. *23*, 259 (1970).
2. Aoyagi T., Kunimoto S., Morishima H., Takeuchi T., Umezawa H.: J. Antibiot. *24*, 687 (1971).
3. Barrett A. J., Dingle J. T.: Biochem. J. *127*, 439 (1972).
4. Peanasky R. J., Abu-Erreish Ghaleb M., in the book: *Proceeding of the International Research Conference on Proteinase Inhibitors* (H. Fritz, H. Tschesche, Eds), p. 289. Walter de Gruyter, Berlin 1971.
5. Keilová H., Tomášek V.: Biochim. Biophys. Acta *284*, 461 (1972).
6. Werle E., Appel W., Happ E.: Z. Vit. Hormon Fermentforsch. *10*, 127 (1959).
7. Keilová H. in the book: *Tissue Proteinases* (A. J. Barrett, T. J. Dingle, Eds), p. 45. North-Holland, Amsterdam 1971.
8. Porath J., Axén R., Ernback S.: Nature *215*, 1491 (1967).
9. Balls A. K., Ryan C. A.: J. Biol. Chem. *238*, 2976 (1963).
10. Hochstrasser K., Werle E., Siegelmann R., Schwarz S.: Hoppe-Seyler's Z. Physiol. Chem. *350*, 897 (1969).
11. Hojima Y., Moriya H., Moriwaki Ch.: J. Biochem. *69*, 1019 (1971).
12. Rancour J. M., Ryan C. A., Arch. Biochem. Biophys. *125*, 380 (1968).
13. R. Vogel, I. Trautschold, E. Werle in the book: *Natural Proteinase Inhibitors,* p. 36. Academic Press, London 1968.
14. Hojima Y., Moriwaki Ch., Moriya H.: J. Biochem. *73*, 923 (1973).
15. Iwasaki T., Kiyohara T., Yoshikawa M.: J. Biochem. *75*, 843 (1974).
16. Richardson M.: Biochem. J. *137*, 101 (1974).
17. Mansfeld V.: Personal communication.
18. Kazakova O. V., Orekhovich N. V.: Int. J. Peptide Protein Res. *7*, 23 (1975).
19. Turk V., Urh I., Kregar I., Gubenšek F., Smith R.: Abstracts II International Symposium on Intracellular Protein Catabolism, p. 56. Ljubljana, Yugoslavia 1975.
20. Ryan C. A.: Biochemistry *5*, 1592 (1966).
21. Keilová H., Bláha K., Keil B.: Eur. J. Biochem. *4*, 442 (1968).

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