FURTHER CHARACTERISTICS OF CATHEPSIN D INHIBITOR FROM POTATOES

H. KEILOVÁ and V. TOMÁŠEK

Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6

Received December 5th, 1975

The inhibitor from potatoes, which inhibits the activity of cathepsin D, a tissue proteinase of the carboxyl type, simultaneously inhibits also trypsin and chymotrypsin. By contrast, it is without any effect on hog and chicken pepsin, rennin, or cathepsin E. Its cathepsin D- and trypsin-inhibiting activity is retained no matter whether the inhibitor has been purified by affinity chromatography on trypsin-Sepharose, by dissociation of the trypsin-inhibitor complex, or of the cathepsin D-inhibitor complex. The character of this potato inhibitor thus seems to be that of a polyvalent inhibitor which interacts both with cathepsin D and trypsin. The inhibition of both enzymes is reversible and competitive. The approximate K_i-value of cathepsin D inhibition is $3{\cdot}8 \cdot 10^{-7}$M and of trypsin inhibition $8{\cdot}6 \cdot 10^{-9}$M.

We have reported recently on the isolation of a cathepsin D inhibitor (PDI) from potatoes (*Solanum tuberosum*)[1]. We have observed that the inhibitor is stable over the pH-range 2–10·5. It can be heated 30 min at 60°C without any loss of activity and is resistant to pepsin hydrolysis. Its molecular weight determined in gel filtration experiments is 27000. So far the anticathepsin activity of the inhibitor has always been paralleled by antitryptic activity. We made therefore an effort in this study to separate the cathepsin D inhibitor from the trypsin inhibitor. Since PDI is the first naturally occurring cathepsin D inhibitor ever isolated, another aim of this study was to provide more information on the interaction of PDI with the enzyme.

EXPERIMENTAL

Material

Cathepsin D (EC 3.4.23.5) was prepared from bovine spleen in this Laboratory[2]. Hog pepsin (EC 3.4.23.1) was a commercial product of Worthington Biochemical Corporation, Freehold, N. J., U.S.A. Chicken pepsin (EC 3.4.23.1) was prepared from chicken forestomachs in this Laboratory[3]. Bone marrow cathepsin E (EC 3.4.23.5) was prepared by the method of Lapresle[4] in this Laboratory. Rennin (EC 3.4.23.4) was a commercial product of Lachema, Prague. Trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1), 3times crystallized preparations, and bovine hemoglobin were purchased from Léčiva, Prague. N^{α}-Benzoyl-D,L-arginine-*p*-nitroanilide (BAPN) was synthetized in this Institute. Acetyltyrosine methyl ester was from Schuchart, Munich, FRG.

The cathepsin D inhibitor from potatoes (PDI) was isolated in this Laboratory by the method described in our preceding paper[1]. Sephadex G-75 and Sepharose 4B were from Pharmacia Fine Chemicals, Uppsala, Sweden.

Methods

Proteolytic activity determination. The activity of cathepsin D was assayed with 2% hemoglobin as substrate at pH 3·5; the method has been described earlier[1]. A mixture containing 50 μl of cathepsin D solution (15—20 mU), 200 μl of Mc Ilvain buffer at pH 3·5, and 1 ml of substrate solution was incubated 20 min at 40°C. The hydrolysis was discontinued by the addition of 2 ml of 5% trichloroacetic acid, the precipitate was filtered off, and the absorbance of the filtrate was measured at 280 nm. The proteolytic activity of rennin was established in a similar manner. Two % hemoglobin solution at pH 2·5 and a McIlvain buffer at the same pH-value were used in the cathepsin E assay and 2% hemoglobin and 0·01M-HCl in the pepsin assay. Tryptic activity was determined in terms of cleavage of N^{α}-benzoyl-D,L-arginine-*p*-nitranilide (BAPN). A mixture of 100 μl of trypsin (2 μg), 1 ml of 0·1M Tris-HCl buffer containing 1 mM-$CaCl_2$, and 50 μl of substrate solution (40 mg in 1 ml of dimethylformamide) was incubated 20 min at 40°C. The cleavage was discontinued by the addition of 100 μl of glacial acetic acid. *p*-Nitroaniline released was quantitated spectrophotometrically at 405 nm. Chymotrypsin activity was measured in terms of cleavage of 1 mM acetyltyrosine methyl ester according to Schwert and Takenaka[5]; the decrease of absorbance at 237 nm was determined in 0·05M phosphate buffer at pH 7·0 and 25°C.

The inhibition of enzymic activity was effected as described in our preceding paper[1]. The enzyme solution and the inhibitor solution were preincubated 10 min at ambient temperature in a buffer whose pH was the same as the pH-optimum of the corresponding enzyme. McIlvain buffer at pH 3·5 was used for cathepsin D and rennin and the same buffer at pH 2·5 for cathepsin E. Trypsin was preincubated in 0·1M Tris-HCl buffer at pH 8·0 containing 1 mM-$CaCl_2$ and chymotrypsin in 0·05M phosphate buffer at pH 7·0. Following this preincubation the residual activity of the corresponding enzyme was determined as described and the inhibition degree was expressed in % of control treated in the absence of inhibitor.

Trypsin–Sepharose 4B. Ten ml of the filtered-off slurry of CNBr-activated[6] Sepharose was rapidly washed with 0·1M phosphate buffer at pH 6·9 containing 0·5M-NaCl. The gel was filtered off and suspended in 10 ml of the same buffer containing 100 mg of trypsin. The suspension was gently stirred 24 h at 4°C. Sepharose was then washed in turns with 0·1M phosphate buffer containing 1M-NaCl, pH 7·8 and with 0·1M acetate buffer containing 1M-NaCl, pH 4·5 until the supernatant was free of A_{280nm} absorbance and tryptic activity.

Complex of PDI *and cathepsin* D. Cathepsin D (5·0 mg) was dissolved in 500 μl of water. PDI (24·9 mg) was dissolved in 1·5 ml of McIlvain buffer (diluted 1 : 2), 0·1M in KCl, pH 3·5. Both solutions were mixed, the mixture adjusted to pH 3·5 and allowed to stand 5 min at ambient temperature (molar cathepsin D to PDI ratio 1 : 8·2). A slight turbidity was removed by centrifugation and the clear supernatant was subjected to gel filtration through a Sephadex G-50 column equilibrated with dilute (1 : 2) McIlvain buffer, 0·1M in KCl, pH 3·5. Cathepsin D activity and inhibition of trypsin were determined in individual fractions. Fractions containing the complex were pooled and lyophilized.

Complex of PDI *and trypsin.* Trypsin (15 mg) was dissolved in 500 μl of cold water. PDI (19 mg) was dissolved in 1·5 ml of 0·01M Tris-HCl buffer (1 mM-$CaCl_2$ + 0·1M-KCl) at pH 8·0. Both solutions were mixed, the pH of the mixture was adjusted to 8·0 by 1M Tris, and the mixture allowed to stand 10 min at ambient temperature (molar enzyme to inhibitor ratio 1 : 1·1). A slight

opalescence was removed by centrifugation. The clear supernatant was subjected to gel chromatography on a Sephadex G-50 column in 0·01M Tris-HCl buffer (1 mM-$CaCl_2$ + 0·1M-KCl), pH 8·0. Trypsin activity and inhibition of cathepsin D activity were determined in individual fractions. Fractions containing the complex were pooled and lyophilized.

Gel chromatography of PDI, cathepsin D, complex of PDI and cathepsin D, and of complex of PDI and trypsin, as well as the dissociation of these complexes were carried out on a column of Sephadex G-50 (2·3 × 69·1 cm, V_t = 287 ml, V_0 = 91·15 ml) equipped with a three-way stopcock, in the ascending arrangement; a 2 ml all-glass hypodermic syringe was used for the application of the sample. The flow rate (16 ml/h) was maintained by a peristaltic pump (LKB-Producter Bromma, Sweden); 3·0-ml fractions were collected. The chromatography was carried out at 5°C.

RESULTS

Affinity Chromatography on Trypsin-Sepharose 4B

It is possible to purify PDI by this method because PDI does not change its activity toward cathepsin D even after it has been incubated 4 h with trypsin at a weight ratio of PDI to trypsin equal 1 : 5.

The material used in these experiments had been purified first by chromatography on DEAE-Sephadex[1], *i.e.* most of the trypsin inhibitors had been removed and only the one always accompanying cathepsin D inhibitor was present. Affinity chromatography was carried out batchwise: 9 ml of thick, centrifuged-off trypsin-Sepharose slurry was suspended in 4·5 ml of 0·1M Tris-HCl buffer, pH 7·6, containing 0·5M-NaCl. PDI (10 mg) was dissolved in 4·5 ml of the same buffer and added to Sepharose with gentle stirring. Ten minutes later, when the supernatant was free of both antitryptic and anticathepsin activity, Sepharose was poured to a column; the latter was washed with 0·1M Tris-HCl buffer, pH 7·6, containing 0·5M-NaCl. The inhibitor attached was displaced from trypsin-Sepharose by a McIlvain buffer, pH 3·8, containing 0·5M-NaCl. Both fractions, *i.e.* fraction I not adsorbed to Sepharose and fraction II, which had been eluted by the acid buffer, were desalted on a Bio Gel P-2 column equilibrated with 0·02M-NH_4HCO_3 and lyophilized. The two lyophilisates were dissolved in water and their anticathepsin and antitrypsin activity was determined. Fraction I showed traces of antitryptic activity only whereas inhibition of both trypsin and cathepsin D was detected in fraction II. This fraction was homogeneous when subjected to disc electrophoresis at pH 4·3; two very weak zones in addition to the main zone were observed when fraction II was examined by electrophoresis at pH 8·3. The specific activity of the inhibitor increased by 15–20% in terms of inhibition of both cathepsin D and trypsin.

Complex of PDI *and Cathepsin* D *and* PDI *and Trypsin*

The profile of gel filtration of the free inhibitor is shown in Fig. 1*a* (V_e = 168 ml) and of free cathepsin D in Fig. 1*b* (V_e = 138 ml). The chromatography of a mixture

of cathepsin D and PDI at pH 3·5, *i.e.* at the pH-optimum of activity of cathepsin D, is shown in Fig. 1*c*. Fractions of peak *I*, where we suppose the presence of the cathepsin D–PDI complex, showed cathepsin D activity at pH 3·5 (curve *2*). Since the elution volume of free cathepsin D is higher (Fig. 1*b*) we explain the presence of cathepsin D in this peak by partial spotaneous dissociation of the cathepsin D–PDI complex during storage of the material. When these fractions are exposed to pH 8·0 at which the cathepsin D–PDI complex dissociates, they show antitryptic activity (curve *3*). So far we are lacking data explaining why the inhibition of tryptic activity by the cathepsin D–PDI complex appears in repeated experiments always in two peaks of which one coincides with the maximum of cathepsin D activity (curve *2*) and the other one with the maximum absorbance of peak *I*. This problem will be dealt with in our future studies. Fractions of peak *II*, whose elution volume corresponds to that of the free inhibitor, inhibit both cathepsin D and trypsin. Clearly they contain an excess of the unreacted inhibitor.

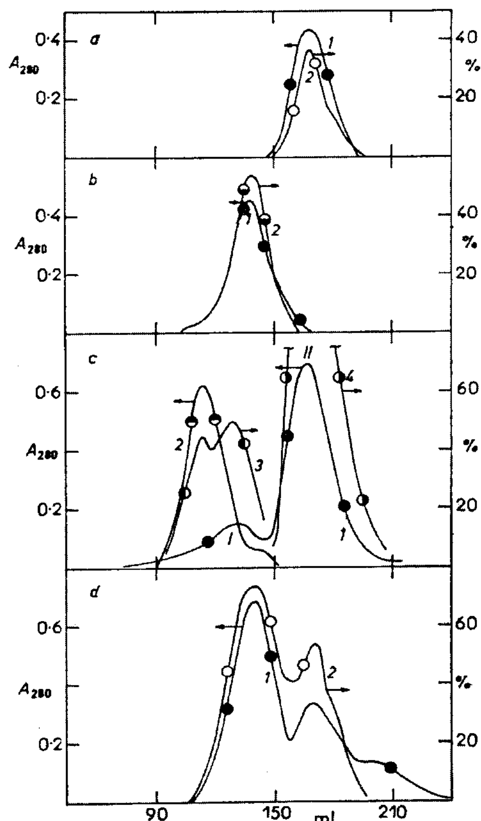

FIG. 1

Gel Chromatography of PDI, Cathepsin D, Cathepsin D–PDI Complex, and Trypsin–PDI Complex

See Experimental for details. A_{280} absorbance at 280 nm, % inhibition of enzymatic activity; *a* PDI (15·1 mg), *1* absorbance, *2* inhibition of cathepsin D (500 μl of each fraction); *b* cathepsin D (16·2 mg), *1* absorbance, *2* enzymatic activity (50 μl); *c* cathepsin D–PDI complex, *1* absorbance, *2* activity of cathepsin D (250 μl), *3* inhibition of trypsin (50 μl); *d* trypsin–PDI complex, *1* absorbance, *2* inhibition of cathepsin D (100 μl).

The gel chromatography profile of a mixture of trypsin and PDI at pH 8·0, *i.e.* at the pH-optimum of trypsin activity is shown in Fig. 1*d*.

Fractions of the first peak whose elution volume corresponds to the trypsin–PDI complex, show inhibition of cathepsin D activity when exposed to pH 3·5 where this complex dissociates. The elution volume of the second peak corresponds to the elution volume of the unreacted inhibitor. Fractions of this peak inhibit both cathepsin D and trypsin.

The results of both cross experiments shown in Fig. 1*c* and 1*d* complement each other.

If cathepsin D has been used for complex formation, the inhibitor released from such a complex shows also antitryptic activity which therefore must be inherent in the PDI molecule. Since the inhibitor forms a complex also with trypsin and the inhibitor liberated after dissociation of this complex inhibits cathepsin D, we conclude from our observations that both inhibitory activities, *i.e.* anti-cathepsin D and anti-trypsin are inseparable and represent an intrinsic characteristic of the PDI molecule.

Specificity of PDI

We have examined the effect of PDI on other proteinases of the carboxyl type: on hog and chicken pepsin, rennin, and cathepsin E. None of these enzymes is inhibited by the cathepsin D inhibitor from potatoes. Neither is inhibited cathepsin B_1, an SH-proteinase. Of proteinases of the serine type, chymotrypsin in addition to trypsin is also inhibited by PDI.

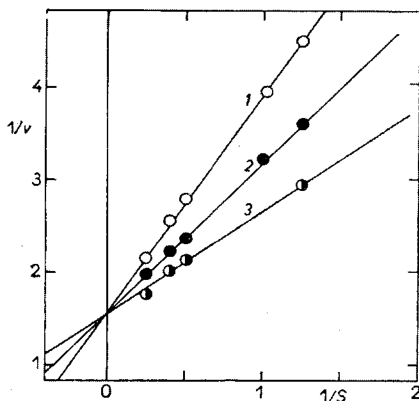

FIG. 2

Hydrolysis of Various Concentrations of Bovine Hemoglobin by Constant Amounts of Cathepsin D (22 mU) in Presence of PDI

1 25 μg of PDI, *2* 50 μg of PDI, *3* control in the absence of PDI, S substrate (0·8—4% hemoglobin), *v* initial hydrolysis rate in absorbance units at 280 nm. Time of hydrolysis 20 min at 40°C.

Character of Inhibition

The profile of the plot of hydrolysis rate *versus* cathepsin D and trypsin concentration in the presence of varying quantity of PDI indicates inhibition of the reversible type.

The plot of reaction rate *versus* substrate concentration at constant quantity of cathepsin D and in the presence of 25 or 50 μg of the inhibitor or its absence (Fig. 2) shows that the inhibition is competitive. Likewise, the inhibition of trypsin

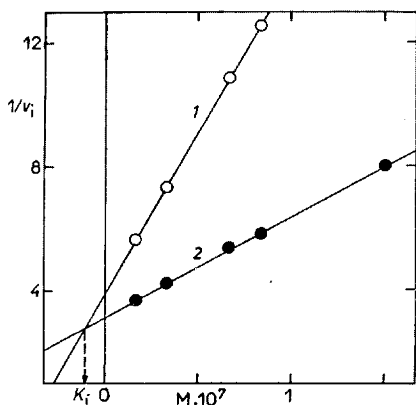

FIG. 3

Cleavage of Different Amounts of N^{α}-benzoyl-D,L-arginine-*p*-nitroanilide as Substrate by Constant Amount (2 μg) of Trypsin in Presence of Different PDI Concentrations

1 1 mg of BAPN, *2* 2 mg of BAPN, M molar concentration of PDI, *v* initial hydrolysis rate in absorbance units at 405 nm.

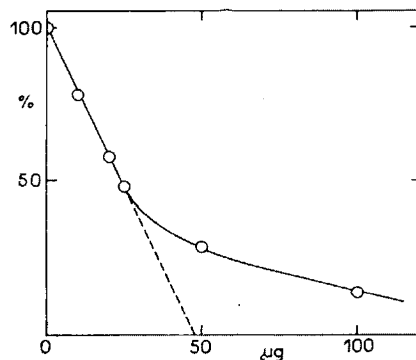

FIG. 4

Reaction of Cathepsin D with PDI

The reaction mixture (1·3 ml) containing 50 μg of cathepsin D in 100 μl of H_2O, 100 μl of McIlvain buffer at pH 3·5, PDI in 100 μl of water, and 1 ml of 2% hemoglobin solution at pH 3·5, was incubated 20 min at 40°C. % degree of hydrolysis in μg of PDI.

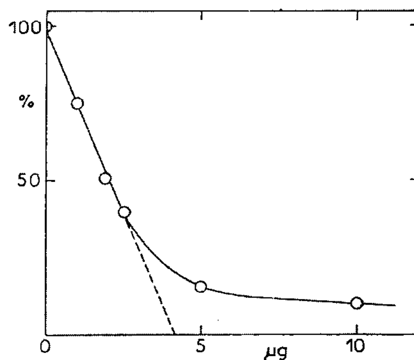

FIG. 5

Reaction of Trypsin with PDI

The reaction mixture containing 1 μg of trypsin in 100 μl of H_2O, PDI in 100 μl of H_2O, 1 ml of 0·1M Tris-HCl buffer, pH 8·0 and 1 mM in $CaCl_2$, and 50 μl of BAPN solution was incubated 20 min at 40°C. % degree of hydrolysis, corresponding to the quantity of free enzyme, in μg of PDI.

is of competitive character and the K_i-value read from the graph (Fig. 3) is $1{\cdot}01 \,.\, 10^{-8}$mM.

The approximate dissociation constants of the complex were determined by the method of Green and Work[7] on the assumption that both cathepsin D and trypsin form a complex with PDI at a 1 : 1 ratio. The values given in Fig. 4 and 5 were used in the calculations. The dissociation constant of the cathepsin D–PDI complex determined by this method was $3{\cdot}8 \,.\, 10^{-7}$M and of the trypsin–PDI complex $8{\cdot}6 \,.\, 10^{-9}$M.

DISCUSSION

It has been known that the tissue proteinases of the carboxyl type, cathepsin D and cathepsin E, share a number of enzyme characteristics in common with pepsin[3,4]. We have observed on the other hand that the behavior of these proteinases toward naturally occurring inhibitors widely differs. The pepsin inhibitor from *Ascaris lumbricoides* inhibits cathepsin E yet not cathepsin D (ref.[8]). The cathepsin D inhibitor from potatoes does not inhibit either pepsin or cathepsin E. This leads us to conclude that the part of the enzyme molecule which acts as the binding site for these inhibitors is similar in pepsin and cathepsin E whereas a completely different part of the molecule of cathepsin D is involved in the binding of these inhibitors. It deserves interest to extend the studies on the specificity of these inhibitors to include other carboxyl proteinases, mainly proteinases of the bacterial origin which have already been classified to a certain degree with respect to their binding sites[9].

The majority of naturally occurring inhibitors are capable of interaction with more than one enzyme. The binding site on the inhibitor molecule is either identical for various enzymes or the molecule bears separate binding sites for the individual enzymes. In the latter case the binding sites may more or less overlap; these inhibitors have been named multiheaded inhibitors. One inhibitor can inhibit several enzymes which may be of different origin yet mostly of the same reaction type, *e.g.* various enzymes belonging to serine proteinases. Therefore when we repeatedly observed that our inhibitor of cathepsin D, *i.e.* of a proteinase with pH-optimum of activity at 3·8, also inhibits trypsin, a proteinase of the serine type with pH-optimum of activity at 8, we suspected that our inhibitor is inhomogeneous rather than polyvalent. The results of this study, however, clearly show that we are dealing with one inhibitor only which inhibits the carboxyl proteinase cathepsin D at acid pH and the serine proteinases trypsin and chymotrypsin at pH 8. This is evidenced by the fact that the inhibitor which has been obtained by affinity chromatography on trypsin Sepharose also inhibits cathepsin D. We have observed in our preceding study that the inhibitor preparation obtained by affinity chromatography on Sepharose with cathepsin D attached inhibits not only cathepsin D but also trypsin[1]. Even more convincing are the results of experiments in which the inhibitor has been obtained by dissociation

of the inhibitor–enzyme complex. The inhibitor liberated from the cathepsin D–inhibitor complex inhibits trypsin. The inhibitor obtained by dissociation of the trypsin–-PDI complex inhibits cathepsin D.

In our opinion the results described above can be interpreted only by assuming that the cathepsin D inhibitor from potatoes has a polyvalent character and also inhibits the serine proteinases trypsin and chymotrypsin.

REFERENCES

1. Keilová H., Tomášek V.: This Journal *41*, 489 (1976).
2. Keilová H. in the book: *Tissue Proteinases* (A. J. Barrett, T. J. Dingle, Eds), p. 45. North-Holland, Amsterdam 1971.
3. Keilová H., Kostka V.: This Journal *40*, 574 (1975).
4. Lapresle C. in the book: *Tissue Proteinases* (A. J. Barrett, T. J. Dingle, Eds), p. 135. North-Holland, Amsterdam 1971.
5. Schwert G. W., Takenaka Y.: Biochim. Biophys. Acta *26*, 570 (1956).
6. Porath J., Aspberg K., Drevin H., Axén R.: J. Chromatogr. *86*, 53 (1973).
7. Green N. M. and Work E.: Biochem. J. *54*, 347 (1953).
8. Keilová H., Tomášek V.: Biochim. Biophys. Acta *284*, 461 (1972).
9. Nakatani H., Hiromi K., Satoi S., Oda K., Murao S., Ischishima E.: Biochim. Biophys. Acta *391*, 415 (1975).

Translated by V. Kostka.