INTERACTION OF CLOSTRIPAIN WITH NATURAL TRYPSIN INHIBITORS AND ITS AFFINITY LABELING BY N^{α}-*p*-NITROBENZYLOXYCARBONYL ARGININE CHLORMETHYL KETONE

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Received 17 May 1976

1. Introduction

Clostripain (EC 3.4.4.20) is a protease from the culture filtrate of *Clostridium histolyticum*, with a highly limited specificity directed at the carboxyl bond of arginyl residues in proteins and in synthetic substrates [1]. According to the specificity clostripain is close to trypsin-like enzymes, on the other hand its catalytic site is that of a SH-protease.

Recently we have developed new types of affinity chromatography which allow us to obtain highly active clostripain on a preparative scale [2]. The availability of the pure enzyme prompted us to study its inhibition by natural trypsin inhibitors and by a synthetic inhibitor suitable for the affinity labeling of its active site.

The experimental results show that the basic pancreatic trypsin inhibitor (Kunitz) is without effect on clostripain whereas the soy bean inhibitor shows a competitive type of inhibition. The synthetic affinity labeling reagent *p*-NO_2-ZACK was prepared for the first time in a pure and stable form by a substantial modification of a synthesis described previously [3]. It is highly active as an irreversible inhibitor of both clostripain and trypsin. The active site of clostripain is protected against this substitution by the competitive inhibitor benzamidine.

Abbreviations: *p*-NO_2-ZACK, N^{α}-*p*-nitrobenzyloxycarbonyl arginine chlormethyl ketone; TACK, N^{α}-tosylarginine chlormethyl ketone; TLCK, N^{α}-tosyllysine chloromethyl ketone; BAE, α-*N*-benzoylarginine ethyl ester; DTT, dithiothreitol.

2. Materials and methods

2.1. *Enzymes and peptidic trypsin inhibitors*

Clostripain was prepared from crude *Clostridium* collagenase (Institut Pasteur Production) by a combination of molecular sieve, ion exchange and affinity chromatography [2]. Specific activity of the lyophilized enzyme under conditions described below was 4.5 μkat (270 units) per mg. β-Trypsin prepared from the commercial trypsin (Worthington) in the laboratory contained 76% of the active site according to titration with *p*-nitrophenyl *p'*-guanidinobenzoate [4]. Trypsin inhibitor from ovomucoid (egg white) type 11-0 was purchased from Sigma, soy bean trypsin inhibitor (5 X cristallized) from National Biochem. Corp., basic pancreatic trypsin inhibitor was a gift from Institut Choay.

2.2. p-NO_2-ZACK

This compound was obtained by a substantial modification of the procedure described previously [3].

α-(*p*-Nitrobenzyloxycarbonyl)arginine (706 mg) prepared according to Gish and Carpenter [5] was suspended in anhydrous tetrahydrofuran (25 ml), chilled in an ice-salt bath and to this was added under stirring phosphorous pentachloride (840 mg) in several portions. The mixture was stirred for 120 min during which the temperature was allowed to rise to 0°C. After addition of anhydrous ether (100 ml) the precipitate was filtered off, washed with ether and dried in vacuo over sodium hydroxide pellets at 4°C.

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The yield of resulting α-(*p*-nitrobenzyloxycarbonyl)-arginyl chloride hydrochloride was 700 mg (86%), m.p. = 120°C, infrared spectrum (KBr) = 1785 cm^{-1}.

To the product dissolved in anhydrous tetrahydrofuran (15 ml) was added at 0°C an excess of ethereal solution of diazomethane, until a yellow color persisted. After 60 min of stirring 80 ml of anhydrous ether was added. The light yellow precipitate was filtered off, washed several times with anhydrous ether and dried in vacuo. The yield was 500 mg (77%) of the diazoderivative. To 380 mg (1 mmol) of this product was added N HCl in acetic acid (15.2 ml) at room temperature. After 60 min the evolution of nitrogen ceased. The solvent was evaporated in vacuo at 35°C, the last traces being removed on an oil pump.

The resulting crude product was purified consecutively by two procedures: after a passage through a column of silica gel (Kiesel gel, methanol–chloroform, 9:1 by vol) the product was taken to dryness, dissolved in water and freed from salts by passage through a column of Biogel P_2 (400 mesh). Lyophilisation of the eluate gave 150 mg of the desired *p*-NO_2-ZACK.

M.p. = 98–105°C, 38% yield. Infrared spectrum (KBr) = 1739, 1700, 1675, 1520 cm^{-1} (lit. [3]: 1735, 1695, 1675, 1520 cm^{-1}). Anal. calcd. for $C_{15}H_{21}O_5N_5Cl_2$, H_2O: C, 40.90; H, 5.22; N, 15.90; Cl, 15.90; found: C, 40.70; H, 6.01; N, 15.36; Cl, 14.27. Thin-layer chromatography (Kieselgel GE 254, Merck; 1-butanol–acetic acid–water 18:2:5, by vol) followed by Sakaguchi detection revealed a single spot.

2.3. *Enzyme assay*

The standard assay of clostripain employed the spectrophotometric determination of the initial rate of hydrolysis of α-*N*-benzoyl arginine ethyl ester (BAE) [6] as described by Mitchell and Harrington [7] in a volume of 3 ml, containing 2.5×10^{-4} M BAE, 2.5 mM DTT, and 50 mM Tris-HCl buffer (pH 7.4) made 50 mM in $CaCl_2$. Initial rates were determined with a Zeiss PQM-2 recording spectrophotometer at 25°C. Specific activity is expressed in μkat mg^{-1} of protein (micromoles of BAE hydrolyzed sec^{-1} mg^{-1}). A unit of activity according to [7] corresponds to 16.67 nkat. Molar concentrations of clostripain were calculated using a molecular weight of 50 000 [7]; a molar absorptivity difference of 1150 M^{-1} cm^{-1} at 253 nm was used for the substrate hydrolysis.

The standard assay of trypsin was performed spectrophotometrically [6] with BAE as substrate.

2.4. *Inhibition of clostripain by peptidic trypsin inhibitors*

Clostripain was activated at a concentration of 2×10^{-5} M in 50 mM Tris-HCl buffer, pH 7.4, containing 2.5 mM DTT and 50 mM $CaCl_2$ for 2 h at room temperature. 1 or 2 μl of this solution were used in the standard spectrophotometric assay.

Peptidic trypsin inhibitors were dissolved in the same buffer. Equal volumes of enzyme and inhibitor solutions were mixed and incubated for 8 min at room temperature; 2 to 10 μl of the mixtures were withdrawn afterwards for the assay. The final concentrations of inhibitors in the mixture were 4×10^{-5} M and 2×10^{-5} M for the soy bean inhibitor, 2×10^{-5} M for the ovomucoid inhibitor and 7.7×10^{-5} M for the basic pancreatic trypsin inhibitor, respectively; the final concentration of the enzyme was 1×10^{-5} M.

2.5. *Inhibition of clostripain and of trypsin by p-NO_2-ZACK*

Clostripain was activated prior to the assay in the buffer as described in 2.4. Trypsin was dissolved in a buffer of the same composition without DTT. The stock solution of the inhibitor was 2.4×10^{-3} M in buffer of the same composition as used to dissolve the enzymes. After mixing and incubation of the enzyme and inhibitor solution for 2 and 10 min at room temperature the residual activity of the inhibited enzyme was examined. Incubation of a solution containing only the enzyme (1×10^{-5} M) was run in parallel with the inactivation experiment to determine the uninhibited enzymatic activity level.

In the experiments aiming at the protective effect of benzamidine, the solutions of the enzyme were mixed with a solution of benzamidine in the same buffer to give the final concentration of benzamidine 4×10^{-4} M; after an incubation for 8 min different amounts of *p*-NO_2-ZACK were added, and the resulting solution was incubated another 8 min prior to activity assays. The substrate solutions were made 4×10^{-4} M in benzamidine.

3. Results and discussion

3.1. *Interaction of clostripain with natural trypsin inhibitors*

Studies on the mechanism of interaction between trypsin and natural peptidic trypsin inhibitors have shown, that in some inhibitors the binding is effected through a lysine residue, in others through an arginine residue; this fits with the general specificity of trypsin towards synthetic substrates and protein chains. Two of the inhibitors which we have examined in the present study, soy bean inhibitor and ovomucoid inhibitor, are of the arginine-type [8], on the other hand, the binding of the basic pancreatic inhibitor to trypsin is effected through a lysine residue [9].

Clostripain preferentially cleaves arginine bonds in proteins but the results show that its binding to different trypsin inhibitors does not follow an analogous pattern to that of trypsin.

In fig.1 is shown a series of initial rate determinations at different concentrations of BAE while maintaining the inhibitors at a fixed concentration. The plots are compatible with competitive inhibition in the case of soy bean trypsin inhibitor, whereas the basic pancreatic trypsin inhibitor is without any effect on clostripain, and the ovomucoid inhibitor influenced the rate very little under the experimental conditions. The apparent Michaelis constant K_M app for the clostripain-catalyzed hydrolysis of BAE was 0.174 mM. The K_i values calculated from the slope of

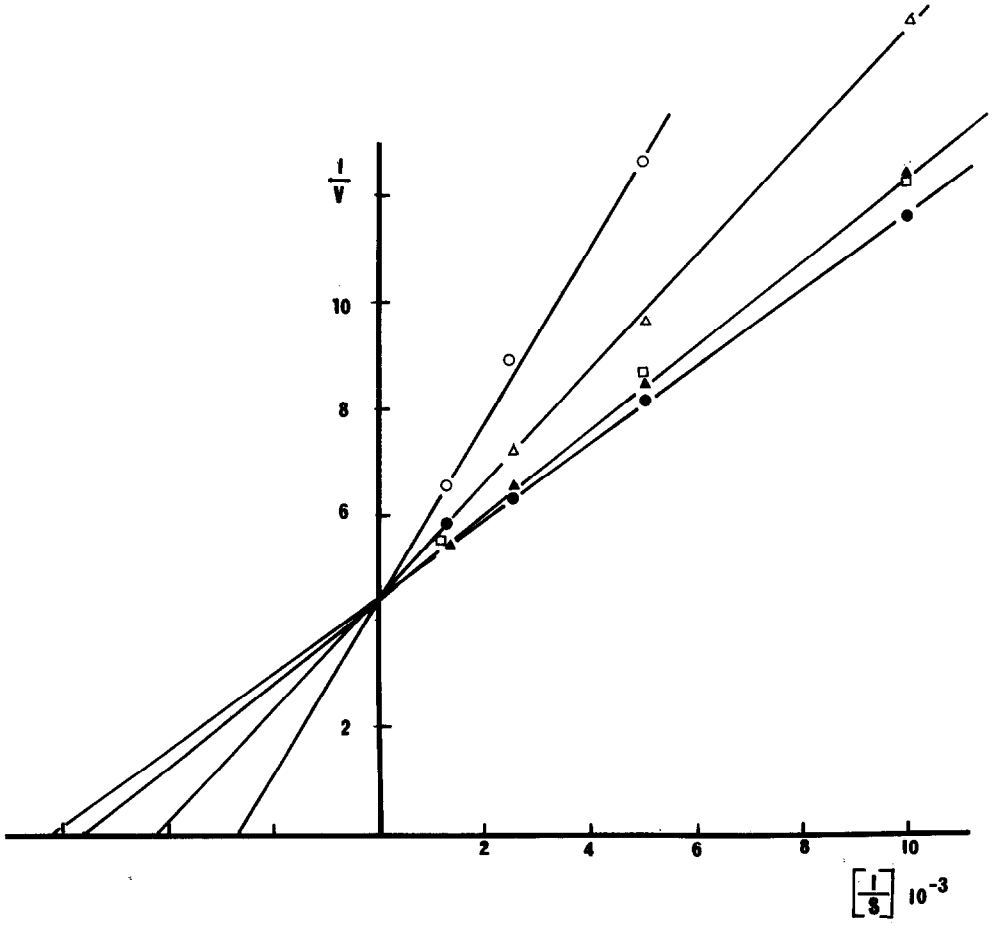

Fig.1. Lineweaver-Burk plot of clostripain catalyzed hydrolysis of BAE (□——□) enzyme conc. 10^{-5} M, and in presence of soy bean inhibitor, 2×10^{-5}, (△——△); 4.10^{-5} M, (○——○); pancreatic basic inhibitor, 7.7×10^{-5} (▲——▲); and ovomucoid inhibitor 2×10^{-5} M, (●——●). For experimental details see the text.

the plots are for soy bean trypsin inhibitor 8.8×10^{-5} (at inhibitor concentration 4×10^{-5} M) and 8.7×10^{-5} (at inhibitor concentration 2×10^{-5} M).

The inhibition of clostripain by soy bean trypsin inhibitor and the lack of inhibition by basic pancreatic trypsin inhibitor fit with the 'arginine' and 'lysine' classification of trypsin inhibitors; the results with ovomucoid, however, does not fit at all.

Clostripain preferentially cleaves arginine substrates; nevertheless, it also cleaves lysine substrates at a reduced rate. The basic pancreatic inhibitor binds to trypsin with a remarkably high $K_i = 10^{-11}$. A competitive inhibition of clostripain, even with a K_i six orders of magnitude lower, could be demonstrated. The results with ovomucoid and basic pancreatic inhibitor seem therefore to indicate that an analogy between the fixation sites of trypsin and clostripain can hardly be drawn. As regards soy bean inhibitor, we still have no evidence which indicates that the surface which combines with clostripain is the same as in the case of trypsin.

Nevertheless, the finding of the inhibition of clostripain by soy bean trypsin inhibitor had a practical consequence: affinity chromatography for the purification of clostripain [2].

3.2. *Reaction of p-NO_2-ZACK with clostripain and β-trypsin*

Shaw and Glover [3] made the first attempt to synthesize an arginine chlormethyl ketone derivative for affinity labeling of the active sites of trypsin-like enzymes. Although the synthesis yielded a mixture containing mainly an inactive cyclic product and only about 2% of the active chlormethyl ketone (p-NO_2-ZACK), they could show that this agent was an extremely rapid inactivator of trypsin. Three years later, Yoshida et al. [10,11] synthesized N^{α}-tosyl arginine chlormethyl ketone (TACK) and they found that it inactives trypsin 3.4 times more rapidly than the corresponding lysine derivative, but it was apparently less efficient that the p-NO_2-ZACK proposed by Shaw and Glover.

The synthesis described in the present study followed by a two-step chromatographic purification gives the p-nitrobenzyloxycarbonyl arginine chlormethyl ketone (p-NO_2-ZACK) in a homogeneous state and in a good yield.

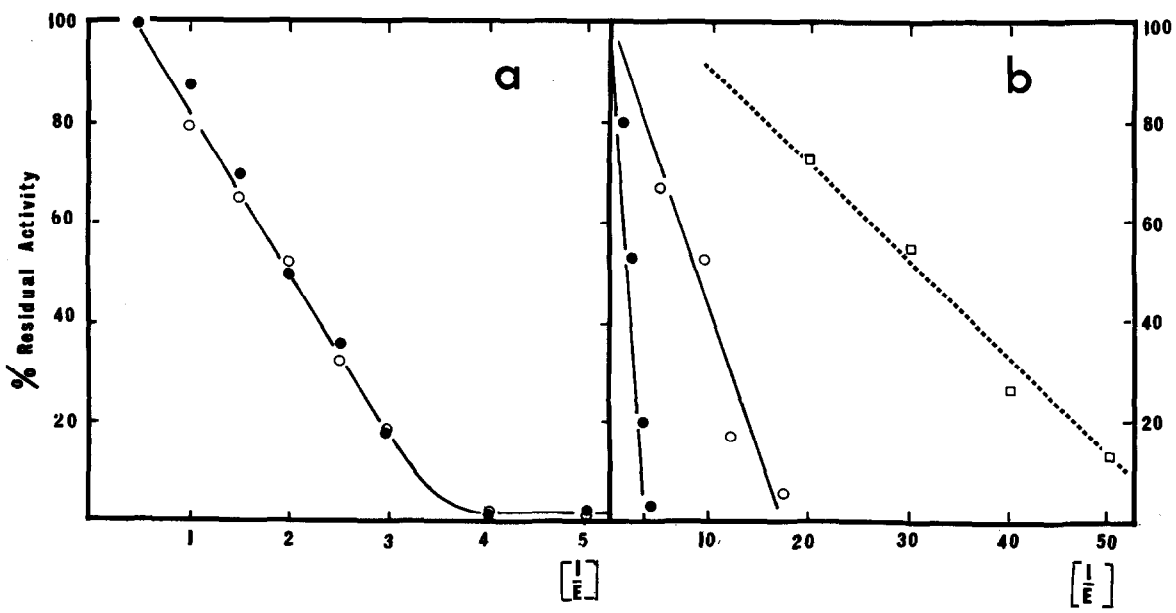

Fig.2. Effect of p-NO_2-ZACK on the enzymatic activity of clostripain and of β-trypsin under different inhibitor-enzyme ratios. (a) Residual activity of clostripain (initial conc. 10^{-5} M) after 2 min (●——●) and 10 min (○——○) of reaction at room temperature. (b) Comparison of residual activity of clostripain (●——●) and β-trypsin, 10^{-5} M, (○——○) after 10 min of reaction. (□——□) trypsin inhibition by crude p-NO_2-ZACK as deduced from fig.1 of [3].

The availability of this reagent gave us the opportunity to study its reactivity with β-trypsin and with highly active clostripain. Porter et al. [12] have described the inactivation of clostripain by α-*N*-tosyllysine chlormethyl ketone (TLCK). Their enzyme preparation incorporated approximately 4 mol of TLCK per 50 000 g of protein instead of expected 1:1 molar ratio. Because they found by independent means that the enzyme was homogeneous, they supposed that a large fraction to the enzyme was still in an inactive state.

Fig.2 summarizes results of our inactivation experiments. Clostripain is irreversibly inactivated by *p*-NO_2-ZACK within few minutes. It was difficult to follow the initial rate of the reaction; the assays after 2 and 10 min gave the same results (fig.2a). As a consequence the 10 min reaction time was used to compare the excess of reagent needed for the complete inactivation of clostripain and β-trypsin (fig.2b).

As expected, pure *p*-NO_2-ZACK inhibits trypsin much more readily than the crude agent studied previously [3]. The reactivity between *p*-NO_2-ZACK and clostripain is even more pronounced. A four molar excess of the reagent removes the activity in less than 2 min at room temperature.

Evidence that the reaction is oriented to the active site of clostripain is given by the same experiment in which we used benzamidine. Porter et al. [12] have found that benzamidine which inhibits trypsin is also an efficient competitive inhibitor of clostripain with a K_i of 4.29×10^{-4} M. Fig.3 shows that benzamidine is effective in protecting the active site of clostripain from reaction with *p*-NO_2ZACK.

At present we have no evidence as to which residue in clostripain is substituted by the action of the arginine chlormethyl ketone. Porter et al. [12] suppose that TLCK reacts with a free SH group in an analogous way as in papain, but no direct experimental evidence was given which showed that only one inhibitor residue was incorporated per molecule of enzyme and that this residue was a cystein. Although we used another reagent than Porter et al. [12] and a highly active enzyme, the curve in fig.2a indicates also an incorporation of 3–4 mol of *p*-NO_2-ZACK per 50 000 g of protein. This phenomenon together with the problem of which amino acid residue was labeled by the reagent can be solved only by a more detailed structural study of the enzyme.

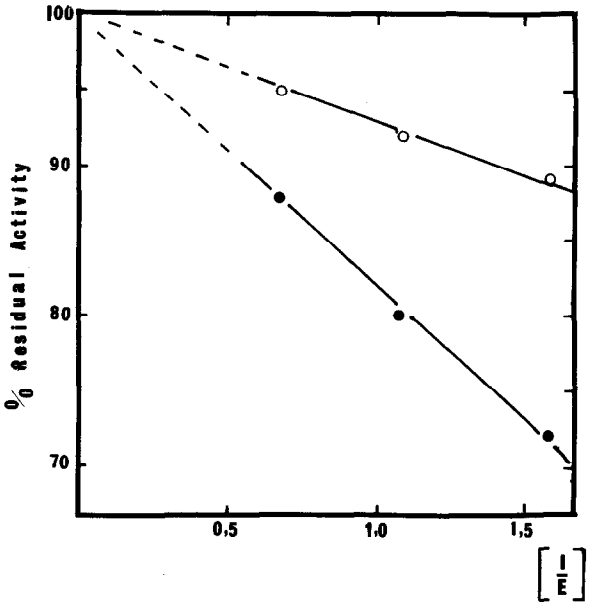

Fig.3. Protection of clostripain from inactivation by *p*-NO_2-ZACK by benzamidine. Residual activity of clostripain (initial conc. 10^{-5} M) after 8 min of reaction with excess of *p*-NO_2-ZACK at room temperature (●——●) and in presence of 4×10^{-4} M benzamidine (○——○).

Acknowledgement

We are grateful for an excellent technical assistance to Mrs M. Pasquet.

References

[1] Mitchell, W. M. and Harrington, W. F. (1971) in: The enzymes, 3rd edn., (P. Boyer, ed.) Vol.3, pp. 699–719. Academic Press, New York, London.
[2] Emöd, I. and Keil, B. FEBS Lett. submitted for publication.
[3] Shaw, E. and Glover, G. (1970) Arch. Biochem. Biophys. 139, 298–305.
[4] Chase, T. Jr. and Shaw, E. (1967) Biochem. Biophys. Res. Commun. 29, 508–514.
[5] Gish, D. T. and Carpenter, F. H. (1953) J. Amer. Chem. Soc. 75, 5872–5877.
[6] Schwert, G. W. and Takenaka, Y. (1955) Biochim. Biophys. Acta 16, 570–575.
[7] Mitchell, W. M. and Harrington, W. F. (1968) J. Biol. Chem. 243, 4683–4692.

[8] Laskowski, M. and Sealock, R. W. (1971) in: The Enzymes, 3rd edn (P. Boyer ed.) Vol.3, pp. 375–473. Academic Press, New York, London.
[9] Chauvet, J. and Acher, R. (1967) J. Biol. Chem. 242, 4274–4275.
[10] Yoshida, N., Sasaki, A. and Inouye, K. (1973) Biochim. Biophys. Acta 321, 615–623.
[11] Inouye, K., Sasaki, A. and Yoshida, N. (1974) Bull. Chem. Soc. Japan 47, 202–203.
[12] Porter, W. H., Cunningham, L. W. and Mitchell, W. M. (1971) J. Biol. Chem. 246, 7675–7682.