INACTIVATION OF SOME PANCREATIC AND LEUCOCYTE ELASTASES BY PEPTIDE CHLOROMETHYL KETONES AND ALKYL ISOCYANATES

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1. Introduction

Several authors recently demonstrated that polymorphonuclear leucocytes from various mammalian species contain elastase-like proteases of rather broad specificity see [1–3]. The enzymes from human leucocytes are supposed to be involved in pathological processes such as acute arthritis, pulmonary emphysema and various inflammatory and immunological reactions [4–8]. Synthetic inactivators of these enzymes might have some medical applications apart from being used in studies of biological functions and catalytic mechanism of elastases. Since leucocyte elastase-like proteases contain serine and histidine in the active centre [2,3] site-specific inactivators may be directed towards these two amino acid residues.

Powers and Tuhy [9] synthesized a series of alanine chloromethyl ketones that bind irreversibly to the histidine residue in the active centre of pancreatic [9,10] and human granulocyte [12,13] elastases. Independently, Brown and Wold [14,15] introduced alkyl isocyanates as active site-specific reagents for studying the structure of substrate-binding pocket of serine proteases. They observed that butyl isocyanate preferentially inactivates pancreatic elastase [15] while we confirmed this for two horse leucocyte elastases [2].

Now we report here comparative studies on the kinetics of inactivation of two highly purified pancreatic elastases and four leucocyte proteases by some tri-alanyl chloromethyl ketones and alkyl isocyanates. Our results indicate that all these enzymes, despite similar substrate specificity, can be clearly differentiated owing to variable sensitivity to the inactivators employed. Moreover, butyl-oxycarbonyl-L-trialanine chloromethyl ketone (Boc-Ala_3CH_2Cl) appears to be a much better inhibitor against all examined enzymes than its acetyl analogue.

2. Materials and methods

Pancreatic elastase I (pancreatopeptidase E, EC 3.4.21.11) and pancreatic elastase II were isolated from porcine pancreas by the procedure of Ardelt [16] leading to homogenous freeze-dried preparations.

Horse leucocyte proteases 1, 2A and 2B were purified as described by Dubin et al. [1] and stored frozen until used. Proteases 2A and 2B represented homogenous preparations in SDS-polyacrylamide gel electrophoresis but protease 1 was approximately 50% pure as determined by a gel densitometry scan, and suitable correction was applied for calculations of the enzyme concentration in the incubation mixture. Homogenous preparation of dog leucocyte protease was prepared as described by Ardelt et al. [3] and stored at 20°C. Some molecular and catalytic parameters of the employed enzymes are compared in table 1.

Activity of all enzymes in the course of inactivation

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Table 1
Some molecular and catalytic parameters of the pancreatic and leucocyte elastase employed

Enzyme	Molecular weight	Isoelectric point	K_M Ac-Ala$_3$-OMe (mM)	Reference
Porcine pancreatic elastase I	25 900[a]	above 11	1.02	[17,16]
Porcine pancreatic elastase II	25 000[b]	10.7	n.d.	[18]
Horse leucocyte proteinase 1	38 000[c]	5.3	1.60	[1,2]
Horse leucocyte proteinase 2A	24 500[c]	8.8	5.55	[1,2]
Horse leucocyte proteinase 2B	20 500[c]	above 10	0.98	[1,2]
Dog leucocyte proteinase	23 500[c]	n.d.	3.33	[3]

[a] Determined from sequence studies.
[b] Determined by sedimentation/diffusion studies.
[c] Determined by sodium dodecyl sulphale-polyacrylamide gel electrophoresis
n.d. – not determined.

studies was determined with N-acetyl-tri-L-alanine methyl ester (Ac-Ala$_3$-OMe) from Cyclo Chemical Co. This substrate is regarded as highly specific for elastase [19]. Hydrolysis of Ac-Ala$_3$-OMe was measured colorimetrically [20] at pH 7.4.

Derivatives of tri-alanine chloromethyl ketones were synthesized by Dr K. Bańkowski (Institute of Fundamental Problems of Chemistry, Warsaw University). *N*-acetyl-tri-L-alanine chloromethyl ketone (Ac-Ala$_3$-CMK) and Boc-Ala$_3$-CMK had melting points of 220–222°C (decomp.) and 198–202°C (decomp.), respectively.

The inactivators were dissolved in methanol and diluted ten times with 0.1 M phosphate buffer, pH 6.5. For inactivation studies, enzyme and inactivator solutions were rapidly combined (the final enzyme concentrations were 4–8 μM) and incubated at 30°C. At suitable intervals aliquots of the mixture were transferred to the substrate solution (final conc. of Ac-Ala$_3$-OMe 5 mM, in 0.1 M phosphate buffer, pH 7.4) and further incubated at 30°C for 10 min. The initial rate of substrate hydrolysis was determined from 5–6 samples taken at 2 min intervals.

Butyl isocyanate (BuNC) and octyl isocyanate (OcNC) were synthesized by Dr Z. Moskal (Jagiellonian University, Kraków). Stock solutions and dilutions of isocyanates were made in anhydrous acetone immediately before use. The actual concentration of isocyanates was determined with benzylamine [15]. For inactivation studies, the enzymes were incubated (5 min, 30°C) with a suitable amount of each isocyanate (final enzyme conc. 1–3 μM, in 0.1 M phosphate buffer, pH 7.4, 5% v/v acetone) and residual Ac-Ala$_3$-OMe-activity was determined.

3. Results and discussion

Fig.1 shows the kinetics of inactivation of elastase I by Boc-Ala$_3$CH$_2$Cl, tested at two different concen-

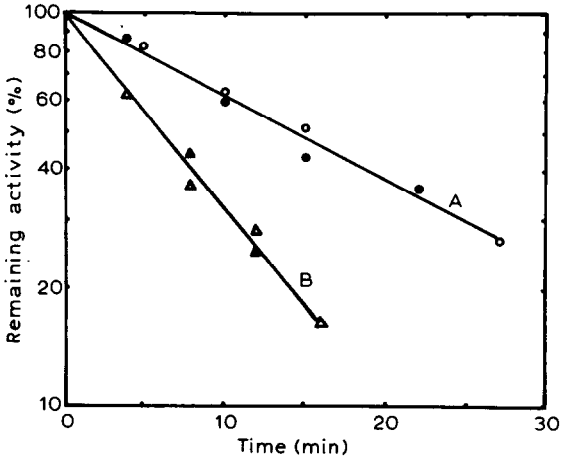

Fig.1. Kinetics of inactivation of pancreatic elastase I by Boc-Ala$_3$ CH$_2$ Cl. Enzyme concentration in the incubation mixture was 8 μM at pH 6.5 and 30°C. At suitable intervals, aliquots of this mixture were tested for residual Ac-Ala$_3$-OMe activity at pH 7.4. (A) 5-fold molar excess of the inhibitor; (B) 10-fold molar excess of the inhibitor. Open or full symbols represent the results obtained in separate runs.

trations. It appears that during the period employed, the reaction follows the first-order kinetics and the results obtained in independent runs are quite reproducible. From the slope of the curve, the inactivation rate constant (k) could be calculated using the equation $k = 0.693/T_{1/2}$ (where $T_{1/2}$ is the apparent half-life in min). Knowing the initial concentration of the inactivator, k [I] ratio could be further computed. This remained constant within the limits of experimental error over the employed range of the inactivator concentrations. From the data shown in fig.1 the following k [I] values were calculated: A = 976 M^{-1} min^{-1}; B = 1117 M^{-1} min^{-1}. The same procedure was employed for other enzymes and for Ac-Ala_3CH_2Cl. The reaction between horse leucocyte proteases (1 and 2A) and Ac-Ala_3CH_2Cl deviated from the first-order kinetics. Therefore in these cases we determined the initial inactivation rate from the first part of the plot (up to 10 min). It must be admitted that our procedure is less accurate than that of Powers and Tuhy [9–11] who employed the equation: log $V/V_o = -k_{obsd} \cdot t$ and least-squares computer program. However, our method is very simple and seems to be valuable for approximate comparison of relative effectiveness of structurally related inactivators as well as various enzymes of similar catalytic mechanism. On the other hand, numerical values of our parameter (k/[I]) are not directly comparable to those of k_{obsd}/[I] used by other authors [9–11].

Table 2 shows the mean k/[I] values determined for two inactivators and six elastolytic enzymes. Considerable differences between examined proteases are clearly visible. They might be associated with differences in the structure of binding sites of the enzymes. It is interesting that two horse leucocyte proteases 1 and 2A, showing the lowest isoelectric points, are most resistant to both inhibitors. In all cases, however, Boc-Ala_3CH_2Cl was several times more efficient than its acetyl analogue and probably was as effective as acetyl tetra-alanine chloromethyl ketone [11]. Since it is easily synthesized and water soluble it may replace other elastase-directed peptide chloromethyl ketones. We observed that Boc-Ala_3CH_2Cl does not affect trypsin and chymotrypsin so it can be employed as a rather simple and effective inhibitor of various elastolytic enzymes.

Previous comparative studies on inactivation of various proteases by alkyl isocyanates [2,15] depended on measurements of residual enzymic activity after incubation with 50-fold molar excess of an inhibitor. We found now that more accurate data can be obtained after graphical determination of isocyanate concentration essential for 50% inhibition of the enzyme activity (fig.2). The results of such determinations for all six enzymes are shown in table 3. All examined proteases are easily inactivated by BuNC but differ considerably in a resistance to OcNC. Horse leucocyte proteases and pancreatic elastase I require

Table 2
Inactivation of some pancreatic and leucocyte elastases by the two derivatives of tri-alanyl-chloromethyl ketone

Enzyme	Boc-Ala_3CH_2Cl		Ac-Ala_3CH_2Cl	
	k/[I] (M^{-1} min^{-1})	[I]/[E]	k/[I] (M^{-1} min^{-1})	[I]/[E]
Porcine pancreatic elastase I	1046	5– 10	202	20–100
Porcine pancreatic elastase II	65	5–100	13	50–100
Horse leucocyte proteinase 1	25	50–100	6	100–300
Horse leucocyte proteinase 2A	30	50–100	3	100–300
Horse leucocyte proteinase 2B	356	5– 50	140	20– 40
Dog leucocyte proteinase	224	10– 25	97	20– 50

Concentration of enzymes in the incubation mixture with the inhibitor was 4–8 μM at pH 6.5 and 30°C. [I]/[E] indicate molar excess of the inhibitor in respect to the enzyme when the inactivation rate constant (k) was determined with Ac-Ala_3-OMe as a substrate. Values k/[I] were calculated as the mean values of 2–4 determinations with at least 2 different concentrations of the inhibitor (cf. fig.1).

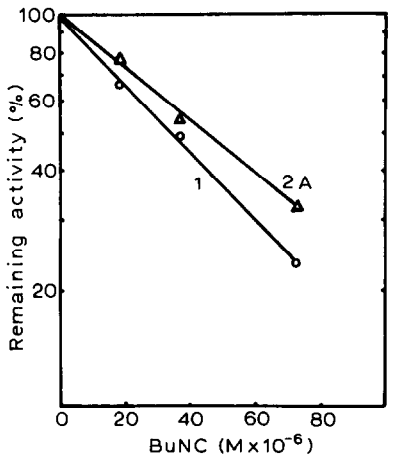

Fig.2. Inactivation of horse leucocyte proteases 1 and 2A by various concentrations of butyl isocyanate (BuNC). Concentration of protease 1 in the incubation mixture with the inhibitor was 2.1 μM, protease 2A, 1.3 μM, both at pH 7.4 and 30°C. After 5 min the substrate solution was added (final conc. 6 mM Ac-Ala_3-OMe) and residual enzymic activity was determined.

5 to 20 times higher concentrations of OcNC for 50% inhibition than pancreatic elastase II or dog leucocyte protease. In this respect the latter two enzymes resemble chymotrypsin. This is again in agreement with their substrate specificity, since they also attack synthetic substrates of chymotrypsin.

In conclusion, peptide chloromethyl ketones and alkyl isocyanates can be successfully employed for further differentiation of serine-histidine proteases showing elastase-like specificity. All the enzymes tested were most effectively inhibited by Boc-Ala_3-CH_2Cl and BuNC.

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Table 3
Inactivation of some pancreatic and leucocyte elastases by butyl isocyanate (BuNC) and octyl isocyanate (OcNC)

Enzyme	BuNC ($\times 10^{-6}$ M)	OcNC ($\times 10^{-6}$ M)	BuNC:OcNC
Porcine pancreatic elastase I	39	530	0.073
Porcine pancreatic elastase II	26.5	48	0.55
Horse leucocyte proteinase 1	39	1728	0.022
Horse leucocyte proteinase 2A	31.5	1549	0.020
Horse leucocyte proteinase 2B	26	1258	0.021
Dog leucocyte proteinase	39.5	190	0.210

Concentration of enzymes in the incubation mixture was 1–3 μM at pH 7.4, 30°C. The results are given as the initial molar concentration of BuNC or OcNC required for 50% inhibition of enzymic activity with Ac-Ala_3OMe as a substrate.

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