Specific inhibition of human granulocyte elastase with peptide aldehydes

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The kinetic features of human granulocyte elastase, chymotrypsin, bovine pancreatic chymotrypsin, porcine pancreatic elastase and elastomucoproteinase were compared. Amino acyl ester substrates were assayed and K_m and k_{cat} values were defined. Aldehyde analogues of the *p*-nitroanilide substrates designed for granulocyte elastase as optimal for K_m appeared to be potent inhibitors. Suc-D-Phe-Pro-valinal (K_i = 40 μM) was found to inhibit granulocyte elastase competitively and specifically when measured with synthetic substrates, and the K_i was 3 μM with the natural protein substrate, elastin.

Peptide aldehyde *Enzyme inhibitor* *(Granulocyte)* *Proteinase* *Elastase* *Chymotrypsin* *Cathepsin G*

1. INTRODUCTION

Various neutral proteinases, such as elastase (EC 3.4.21.37), CG (chymotrypsin, EC 3.4.21.20) and collagenase can be found in the granules of polymorphonuclear leukocytes. When released into the circulation, they are inhibited by plasma proteinase inhibitors [1,2]. In the case of inhibitor deficiency states or when the inhibitors are consumed, granulocyte proteinases become uncontrolled and may induce disease states accompanied by tissue damage [3].

To inhibit granulocyte elastase and CG in vitro, peptide chloromethyl ketones [4,5], azapeptides [6], peptide boronic acids [7] and fermentation products, e.g. elasnin [8] and elastatinal [9,10], were successfully applied.

Abbreviations: EMP, elastomucoproteinase; GE, granulocyte elastase; CG, cathepsin G; PE, pancreatic elastase; PC, pancreatic chymotrypsin; PhCO-L-Tyr-OEt, *N*-α-benzoyl-L-tyrosine ethyl ester; Boc-L-Ala-ONp, *N*-*t*-butoxycarbonyl-L-alanine *p*-nitrophenyl ester; Z-, benzyloxycarbonyl-; Suc-, succinyl-; Boc-, *t*-butoxycarbonyl-; -NH-Np, *p*-nitroanilide; Ahx, 2-aminohexanoic acid

Our previous results obtained with peptide -NH-Np substrates [11] and the findings of Bajusz et al. [12] suggested that the aldehyde analogues of substrates having small Michaelis constants (K_m) may be good inhibitors of elastase.

To test the specificity of the inhibition, the effect of inhibitors was investigated on other enzymes as well. One of them was EMP, a usual contaminating enzyme of PE preparations [13]. This enzyme has both mucolytic and proteolytic activity and probably plays a role in the decomposition of glycoproteins [14].

2. MATERIALS AND METHODS

PhCO-L-Tyr-OEt, Boc-L-Ala-ONp and porcine PE (EC 3.4.21.11) were products of Sigma (St. Louis, MO), and bovine PC (EC 3.4.21.1) was obtained from Merck (Darmstadt).

Granulocyte proteinases were isolated from human granules. GE was purified according to Baugh and Travis [15], while CG was isolated as described by Feinstein and Janoff [4].

EMP was isolated from elastase crudum [14]. Elastin was prepared from human lung [3].

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The peptides were synthesized by the usual stepwise method [16]. The C-terminal amino acids (Ala, Ahx and Val) were reduced in the form of their Z-protected 2,5-dimethylpyrrazolide derivatives by $LiAlH_4$ [17], then the aldehyde function formed was protected as the semicarbazone. After decarbobenzoxylation the free amino aldehyde semicarbazones were acylated with Z-D-Phe-Pro and Z-Pro-D-Phe-Pro, respectively, by using the dicyclohexylcarbodiimide/*N*-hydroxybenzotriazole method [18]. The Z group of the peptides was changed for Suc-, and their aldehyde function liberated. The products were homogeneous by thin-layer chromatography detected with peptide and aldehyde reagents, and by elementary analysis. Details of the synthesis will be described elsewhere.

The effect of inhibitors was measured at 37°C with Boc-L-Ala-ONp and PhCO-L-Tyr-OEt substrates by monitoring spectrophotometrically the changes in absorption at 347.5 and 256 nm, respectively. The hydrolysis of Boc-L-Ala-ONp was examined in 50 mM phosphate buffer, pH 6.5, in 50–100 μM substrate concentration range, whereas that of PhCO-L-Tyr-OEt was investigated in 100 mM Tris-HCl buffer, pH 7.6, in the range 10–50 μM. Enzyme concentration was 10 nM. The concentration of inhibitors varied between 0.02 and 4 mM. When determining K_m and k_{cat}, the substrate concentration was changed between 1 and 100 mM and the enzyme concentration between 1 and 100 μM.

The inhibition of GE was also examined with Suc-D-Phe-Pro-valinal and human lung elastin substrate. The decomposition of elastin was measured by recording the changes in fluorescence intensity of fluorescamine reacted with the liberated N-terminus upon hydrolysis of peptide bonds of elastin by GE [19].

K_i inhibitory constant was calculated in the case of small molecular mass substrates according to Dixon, and by the method of Green and Work [20] using elastin substrate. The values of K_m and k_{cat} were determined according to the Lineweaver-Burk method.

3. RESULTS AND DISCUSSION

In our previous studies of granulocyte elastase 3 substrates of similar composition appeared to have the best Michaelis constant among the investigated tripeptide-NH-Np substrates. These substrates contained Boc-D-Phe-Pro- at the N-terminus and Ahx-, Val- or Ala-NH-Np at the specificity site; their K_m values were 4.2, 6.1, 8.1 μM, respectively [11]. Therefore 3 tripeptide aldehydes with the same sequence and 2 tetrapeptide aldehydes which are longer with a Pro- at the N-terminus were synthesized. To increase the solubility of substrates, the Boc- protective group was changed for Suc- at the N-terminus.

The effect of the inhibitors was examined with GE, and compared with CG, the pancreatic analogues of both enzymes, and with EMP, which has chymotrypsin-like specificity.

Assays on elastase were carried out with Boc-L-Ala-ONp, whereas PhCO-L-Tyr-OEt substrate was used for PC, CG and EMP. These substrates are widely used for the assay of these enzymes although the elastase-specific Boc-L-Ala-ONp is also hydrolyzed by CG, but at a 10-times lower rate than by elastase. PhCO-L-Tyr-OEt, on the other hand, is cleaved only by chymotrypsin-type proteinases. Table 1 shows the kinetic constants of the substrates and table 2 the inhibitory constants obtained in the presence of inhibitors. The inhibition appeared to be competitive in each case, as calculated by the Lineweaver-Burk method.

Table 1

Kinetic constants (K_m, mM; k_{cat}, s^{-1}) of granulocyte and pancreatic proteinases measured with ester substrates

Substrate	GE		PE		EMP		CG		PC	
	K_m	k_{cat}	K_m	k_{cat}	K_m	k_{cat}	K_m	k_{cat}	K_m	k_{cat}
Boc-L-Ala-ONp	0.20	2.36	0.45	2.07	4.30	2.10	0.21	0.13	1.25	0.90
PhCO-L-Tyr-OEt	–	–	–	–	0.34	1.59	0.26	20.00	0.18	48.30

Table 2

Inhibitory constants (K_i, mM) of granulocyte and pancreatic proteinases measured with peptide aldehydes

Inhibitor	GE	PE	EMP	CG	PC
Suc-D-Phe-Pro-alaninal	0.62	0.98	3.40	–	–
-2-aminohexanal	0.38	2.10	2.10	1.40	2.75
-valinal	0.04	1.40	3.50	–	–
Suc-Pro-D-Phe-Pro-2-aminohexanal	0.07	0.85	1.50	0.90	2.00
-valinal	0.04	0.52	–	–	–

GE and PE were assayed with Boc-L-Ala-ONp, CG, PC and EMP with PhCO-L-Tyr-OEt as substrate

3.1. *Elastases*

Elastases were inhibited by each of the examined inhibitors. The compound containing valinal at the C-terminus seemed to be the best for GE. An inhibitory constant of $K_i = 50\ \mu M$ was reported by Feinstein et al. [9] using elastatinal and Boc-L-Ala-ONp as inhibitor and substrate, respectively. With Suc-Ala-Ala-Ala-NH-Np substrate an almost 10-times more effective inhibition ($K_i = 6.2\ \mu M$) was observed [10]. 2-Aminohexanal was less effective in the tripeptide inhibitor form by one order of magnitude. PE is effectively inhibited with alaninal. The increase in chain length rendered the inhibition more effective. Similar observations were reported by Thompson [21] although his K_i values are far better than those described in this paper. This contradiction may be explained by the fact that different compounds and conditions were applied in the investigations. In addition, it was described that the increase of chain length enhanced both the productivity of enzymes with peptide-NH-Np substrates [9,22] as well as the inhibitory capacity of peptide chloromethyl ketones [5,23].

3.2. *PC and CG*

We have found that PC and CG are inhibited only with 2-aminohexanals, and the enzymes are able to cleave X-Y-Ahx-NH-Np substrates (unpublished). The inhibitory constants are of the millimolar order of magnitude and the increase in chain length brings about only a slight improvement, as in the case of chymostatin [9]. A more pronounced inhibition can be expected with various peptide-phenylalaninal derivatives which fit better into the binding pocket of PC and CG.

3.3. *EMP*

EMP, similarly to chymotrypsin, may be inhibited with peptide-2-aminohexanals as well as with alaninal and valinal, though to a small extent. Its inhibition with the examined compounds lies between that of PE and PC, as follows from the data obtained with the substrates (see table 1).

3.4. *GE*

Suc-D-Phe-Pro-valinal seemed to be specific for GE, since chymotrypsins were not inhibited, and it was also a 100-fold weaker inhibitor of PE. This was also examined with the high molecular mass substrate, human lung elastin. The inhibitory constant, $K_i = 3\ \mu M$, was one order of magnitude less than that obtained in the presence of the small molecular mass substrate.

We may conclude from the above data that peptide aldehydes may be efficient and specific inhibitors of GE and probably of other intracellular serine proteinases as well.

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