

Substrate and inhibitor studies on proteinase 3

Chih-Min Kam^a, John E. Kerrigan^a, Koert M. Dolman^b, Roel Goldschmeding^b,
Albert E.G.Kr. Von dem Borne^b and James C. Powers^a

^a*School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA 30332, USA* and ^b*Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Laboratory for Experimental and Clinical Immunology and Department of Hematology, Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands*

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Various amino acid and peptide thioesters were tested as substrates for human proteinase 3 and the best substrate is Boc-Ala-Ala-Nva-SBzl with a k_{cat}/K_m value of $1.0 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$. Boc-Ala-Ala-AA-SBzl (AA = Val, Ala, or Met) are also good substrates with k_{cat}/K_m values of $(1-4) \times 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. Substituted isocoumarins are potent inhibitors of proteinase 3 and the best inhibitors are 7-amino-4-chloro-3-(2-bromoethoxy)isocoumarin and 3,4-dichloroisocoumarin (DCI) with $k_{obs}/[I]$ values of 4700 and 2600 $\text{M}^{-1} \cdot \text{s}^{-1}$, respectively. Substituted isocoumarins, peptide phosphonates and chloromethyl ketones inhibited proteinase 3 less potently than human neutrophil elastase (HNE) by 1–2 orders of magnitude.

Peptide chloromethyl ketone; Peptide phosphonate; Proteinase 3; Substituted isocoumarin; Peptide thioester

1. INTRODUCTION

Proteinase 3 is a serine protease in the azurophilic granules of polymorphonuclear leukocytes (PMN) and is distinct from human neutrophil elastase (HNE) and cathepsin G [1]. Recently, proteinase 3 has been cloned [2] and found to be identical to the antimicrobial protein p29b/AGP7 [3–5] and to myeloblastin, a serine protease derived from HL-60 leukemia cells [6,7]. Proteinase 3 is at least as prominent in neutrophil azurophilic granules as HNE [2]. The active sites of both enzymes are very similar [5]. Proteinase 3 degrades elastin [8], induces emphysema when administered to hamsters by intra-tracheal instillation [1] and serves as an antigen recognized by Wegener's Granulomatosis autoantibodies [8–11]. Proteinase 3 has also been found to degrade a variety of other matrix proteins [12]. We report here the reactivity of proteinase 3 with various peptide thioester substrates to further elucidate the substrate reactivity of proteinase 3, and the development of inhibitors which

may be useful for elucidating the function of proteinase 3 in vitro and in vivo.

2. EXPERIMENTAL

Percoll was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Triton X-100 was purchased from Sigma Chemical Co., St. Louis, MO. Adenosine triphosphate (ATP) was obtained from Boehringer, Mannheim, Germany. ^{125}I -Na was obtained from the Radiochemical Centre, Amersham, UK. Matrex Gel orange A was purchased from Amicon Div., Danvers, MA. Bio-Rex 70 was obtained from Bio-Rad Laboratories, Richmond, CA. XAR5 film was purchased from Eastman Kodak Co., Rochester, NY. Purified human neutrophil elastase (HNE) was obtained from Elastin Products, Pacific, MO and from the research laboratory of Dr. James Travis, University of Georgia. EDTA was purchased from Aldrich Chemie, Brussels, Belgium. Sheep anti-serum against cathepsin G was obtained from ICN Biochemicals, Lisle, IL. HEPES was obtained from Research Organics Inc., Cleveland, OH. 4,4'-Diithiodipyridine was purchased from Aldrich Chemical Co., Milwaukee, WI. All the peptide thioesters were prepared as previously described [13,14]. Substituted isocoumarins [15–17], peptide phosphonates [18], and peptide chloromethyl ketones [19] were prepared previously.

2.1. Preparation of proteinase 3

Blood from healthy donors was anticoagulated with trisodium citrate. Mononuclear cells and platelets were removed by centrifugation over isotonic Percoll with a specific gravity of 1.076 g/ml (1,000 $\times g$, 20 min, 20°C). Erythrocytes were subsequently lysed with NH_4Cl (155 mM), KHCO_3 (10 mM), EDTA (10 μM) at 4°C. Thereafter, the neutrophils were washed twice with PBS/0.1% (v/v) BSA and finally resuspended in medium that consisted of 100 mM KCl, 3 mM NaCl, 1 mM ATP, 3.5 mM MgCl_2 , 10 mM PIPES, pH 7.3. More than 95% of the cells were neutrophils.

Purified neutrophils, 75×10^6 in 300 ml of medium were nitrogen cavitated [20]. Nuclei and unbroken cells were pelleted by centrifugation at 500 $\times g$ for 10 min at 4°C. The supernatant was collected and centrifuged for 30 min at 35,000 $\times g$ at 4°C. The pellet containing the mixed granules fractions was resuspended in 0.08 M citrate phosphate

Correspondence address: J.C. Powers, School of Chemistry and Biochemistry, Georgia Institute of Technology, Atlanta, GA 30332, USA. Fax: (1) (404) 894 7452.

Abbreviations: Abu, α -aminobutanoic acid; Boc, *t*-butoxycarbonyl; 3-BrEtOIC, 3-(2-bromoethoxy)isocoumarin; DCI, 3,4-dichloroisocoumarin; DFP, diisopropyl fluorophosphate; 3-EtOIC, 3-ethoxyisocoumarin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; HNE, human neutrophil elastase; MeO, methoxy; Nva, Norvaline; PIPES, piperazine-*N,N*-bis(2-ethanesulfonic acid); PMSF, phenylmethanesulfonyl fluoride; 3-PrOIC, 3-propoxyisocoumarin; pNA, *p*-nitroanilide; PPE, porcine pancreatic elastase; ONp, *p*-nitrophenylester; SBzl, thiobenzyl ester.

buffer pH 3.0/0.1% (v/v) Triton X-100 and sonicated at 45 kHz for three periods of 10 s each. Thereafter, soluble and insoluble materials were separated by centrifugation at $220,000 \times g$ for 1 h at 4°C and the supernatant was stored at -70°C .

Proteinase 3 was purified from crude granule extract by dye-ligand affinity chromatography over Matrex Gel orange A followed by ion-exchange chromatography over Bio-Rex 70, as described previously [1]. Proteinase 3 was detected by hydrolysis of α -naphthyl acetate and by ELISA using a MAb 12.8 against proteinase 3. The fractions containing proteinase 3 were pooled and concentrated by ultrafiltration using an Amicon unit (filtration cut off 10,000 Da) and dialyzed against PBS. Contamination with HNE and cathepsin G in the proteinase 3 preparation was excluded by ELISA's [9], using MAb NP 57 (anti-NE) [21] and sheep anti-serum against cathepsin G. Purity was further proven by the absence of contaminants in SDS-PAGE analysis with subsequent silver staining and autoradiography after radiolabeling (Fig. 1). Protein concentration was measured by the Bradford technique, using a BSA standard. A total of 1.5 mg of proteinase 3 was purified from approximately 150 mg of crude granule extract. Aliquots of 500 $\mu\text{g/ml}$ were stored at -70°C .

2.2. Substrate kinetics

The enzymatic hydrolysis of peptide thioester substrates catalyzed by human proteinase 3 was measured in 0.1 M HEPES, 0.5 M NaCl, pH 7.5 buffer containing 9% Me_2SO and at 25°C in the presence of 4,4'-dithiodipyridine [22]. All stock solutions of substrate were prepared in Me_2SO and stored at -20°C . The initial rates were measured at 324 nm ($\epsilon_{324} = 19800 \text{ M}^{-1} \cdot \text{cm}^{-1}$) using a Beckman 35 spectrophotometer when a 10–25 μl of an enzyme stock solution was

added to a cuvette containing 2.0 ml of buffer, 150 μl of 4,4'-dithiodipyridine (5 mM) and 25 μl of substrate. The same volume of substrate and 4,4'-dithiodipyridine was added to the reference cell in order to compensate for the background hydrolysis rate of the substrates. Initial rates were measured in duplicate for each substrate concentration and were averaged in each case. The kinetic constants k_{cat} , K_m and k_{cat}/K_m were obtained from Lineweaver-Burke plots which had correlation coefficients greater than 0.99. For Z-Ala-SBzl, the plot went through the origin and only k_{cat}/K_m could be determined.

2.3. Enzyme inactivation – incubation method

An aliquot of inhibitor (25 μl) in Me_2SO was added to 0.25 ml of a buffered enzyme solution (0.13 μM) containing albumin (10–25 μl of 0.1 g/ml) to initiate the inactivation. Aliquots (50 μl) were withdrawn at various intervals and the residual enzymatic activity was measured as described above using Suc-Ala-Ala-Nva-SBzl (88 μM) as the substrate. The inhibitor concentrations are shown in Table II. Pseudo first-order inactivation rate constants (k_{obs}) were obtained from plots of $\ln v/v_0$ vs. time, and the correlation coefficients were greater than 0.98.

3. RESULTS AND DISCUSSION

3.1. Substrate kinetics

The substrate activity of proteinase 3 has been studied previously with oxidized insulin chains, and chromogenic and fluorogenic substrates [12]. The cleavage of insulin chains by proteinase 3 suggests that this enzyme preferentially attacks peptide bonds having small aliphatic amino acids such as Ala, Val, Leu and Ser at the P_1 and P_1' sites. The hydrolysis of synthetic peptides also indicates proteinase 3 prefers small aliphatic residues at the P_1 site and Pro at the P_2 site. Proteinase 3 did not hydrolyze HNE substrates such as Suc-Ala-Ala-*p*NA, and Suc-Ala-Ala-Val-*p*NA, but hydrolyzed Boc-Val-ONp, Boc-Ala-ONp, MeO-Suc-Ala-Ala-Pro-Val-*p*NA, and MeO-Suc-Ala-Ile-Pro-Met-*p*NA. We have tested amino acid and peptide thioesters including Boc-Ala-Ala-AA-SBzl (AA = Ala, Abu, Gly, Arg, Asp, Asn, Met, Met(O), Pro, Nva, Val, Ile, Leu, Nle, Phe, and Ser) as substrates of proteinase 3. The best substrates are tripeptide thioesters with Nva in the P_1 site. Z-Ala-SBzl, Boc-Ala-Ala-Ala-SBzl, Suc-Ala-Ala-Met-SBzl, Boc-Ala-Ala-Met-SBzl, Boc-Ala-Ala-Abu-SBzl, Boc-Ala-Ala-Val-SBzl, and MeO-Suc-Ala-Ala-Pro-Val-SBzl are also good substrates. All other thioesters show little activity. The kinetic constants k_{cat} , K_m and k_{cat}/K_m of several better substrates for proteinase 3 and HNE are shown in Table 1. The kinetic constants of these thioester substrates for HNE have been reported previously [13] and are shown here for comparison with proteinase 3. The best substrate is Boc-Ala-Ala-Nva-SBzl with k_{cat}/K_m value of $1.0 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ which is ca. 9-fold lower than the value of HNE. Suc-Ala-Ala-Nva-SBzl is the only substrate which has a higher k_{cat}/K_m value for proteinase 3 than for HNE. Substitution of Boc with Suc in Boc-Ala-Ala-Met-SBzl and Boc-Ala-Ala-Nva-SBzl slightly reduced the k_{cat}/K_m values for proteinase 3 by 1.5- to 2.1-fold, however this substitution resulted in a 50-fold reduction

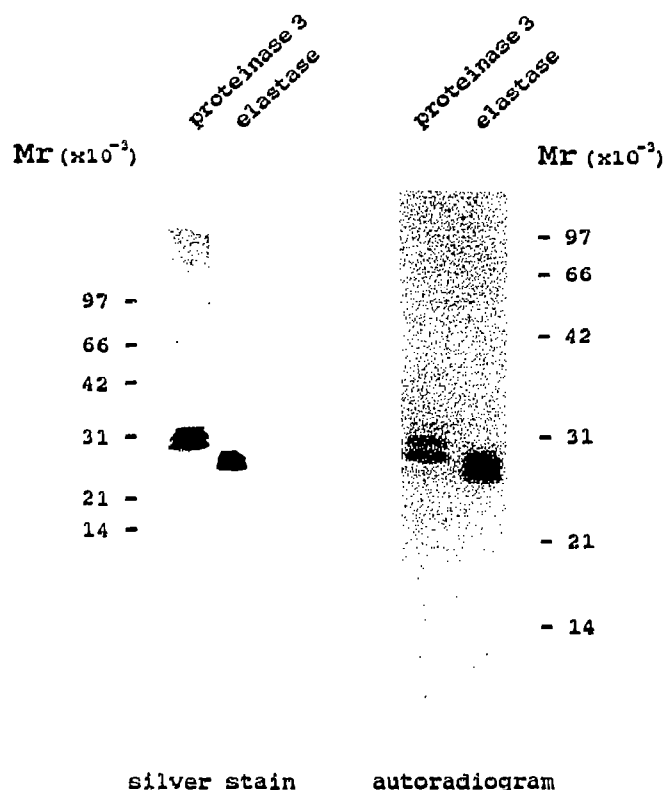


Fig. 1. SDS-PAGE analysis of proteinase 3 and HNE. Purified proteins (200 ng per lane) were applied on a 10–15% gradient gel under non-reducing conditions and stained with silver after separation. ^{125}I -labeled proteins (10 ng per lane) were applied on a 12% gel under non-reducing conditions and visualized by autoradiography after separation. Proteinase 3 appeared to be pure and exhibited an electrophoretic mobility different from HNE.

Table II

Inhibition constants of proteinase 3 and HNE by substituted isocoumarins, peptide phosphonates and peptide chloromethyl ketones^a

Inhibitors	Proteinase 3		HNE	
	[I] (μM)	$k_{\text{obs}}/[\text{I}]$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)	[I] (μM)	$k_{\text{obs}}/[\text{I}]$ ($\text{M}^{-1} \cdot \text{s}^{-1}$)
3,4-dichloroisocoumarin	3.6	2,600	1.1	8,900 ^b
7-NH ₂ -4-Cl-3-EtOIC	40.7	560	4	9,400 ^c
7-NH ₂ -4-Cl-3-PrOIC	38	740	2.6	54,000
7-NH ₂ -4-Cl-3-BrEtOIC	7.3	4,700	0.07	200,000
7-CH ₃ CONH-4-Cl-3-PrOIC	7.7	2,200	1.0	>190,000
7-PhNHCONH-4-Cl-3-PrOIC	7.7	1,700	1.2	140,000
7-S-(Naphthyl)(CH ₃)CHNHCONH-4-Cl-3-BrEtOIC	6.8	2,100	0.44	310,000
		310		
7-R-(Naphthyl)(CH ₃)CHNHCONH-4-Cl-3-BrEtOIC	6.2	1,800	0.64	170,000
		330		
7-S-PhNHCOOCH(CH ₃)CONH-4-Cl-3-BrEtOIC	7.4	2,000	0.8	140,000
		300		
MeO-Suc-Ala-Ala-Ala-Nva ^P (OPh) ₂	427	3.3		
MeO-Suc-Ala-Ala-Ala-Val ^P (OPh) ₂	426	30	5	1,500 ^d
MeO-Suc-Ala-Ala-Pro-Nva ^P (OPh) ₂	427	21	95	380 ^d
MeO-Suc-Ala-Ala-Pro-Val ^P (OPh) ₂	78	150	4.9	7,100 ^d
Boc-Val-Pro-Val ^P (OPh) ₂	323	46	4.5	27,000 ^d
MeO-Suc-Ala-Ala-Pro-Val-CH ₂ Cl	400	5.9		1,600
Boc-Ala-Ala-Pro-Val-CH ₂ Cl	416	1.4		
Z-Gly-Leu-Ala-CH ₂ Cl	400	14% ^e		20 ^f
Ac-Ala-Ala-Ala-CH ₂ Cl	395	11% ^e		2.8 ^f
Ac-Ala-Ala-Pro-Ala-CH ₂ Cl	400	6% ^e		44 ^f

^a Inhibition constants were measured in 0.1 M HEPES, 0.5 M NaCl, pH 7.5 buffer, 9% Me₂SO, 0.3 mg of albumin and at 25°C. Suc-Ala-Ala-Nva-SBzl (88 μM) was used as the substrate.^b Data obtained from reference [15].^c Data obtained from reference [16].^d Data obtained from reference [18].^e Percentage of inhibition was obtained after 10 min of incubation.^f Data obtained from reference [27].

of 7-amino-4-chloro-3-propoxyisocoumarin generally improves the inhibitory potency toward HNE by 3- to 4-fold, whereas the same substitution in 7-amino-3-bromoethoxy-4-chloroisocoumarin did not change the inhibitory potency significantly. These 7-acylamino groups are directed toward the S' subsites [26] which indicates that proteinase 3 and HNE must have significant difference in this region of the active site. It has been shown that Phe-41 which is part of the S₁' and S₃' subsites in HNE remains unchanged in proteinase 3 [12,23]. However, in this region there are two insertions in proteinase 3 when compared with HNE, a two amino acid insertion between Gly-38 and Gly-39, and a single amino acid insertion between Gly-39 and His-40. These insertions may account for the reactivity differences between proteinase 3 and HNE toward acylaminoisocoumarins. The inhibition reaction of 7-substituted-3-bromoethoxy-4-chloro-isocoumarin towards proteinase 3 was biphasic and the rate constants were based on the 40–50% inhibition observed during the first 1–2 min incubation.

Peptide phosphonates are good inhibitors of HNE [18], but did not inhibit proteinase 3 very well. The best inhibitor is MeO-Suc-Ala-Ala-Pro-Val^P(OPh)₂ ($k_{\text{obs}}/[\text{I}]$

= 27,000 $\text{M}^{-1} \cdot \text{s}^{-1}$) which inhibits proteinase 3 ca. 50-fold less potently than HNE. The best HNE inhibitor Boc-Val-Pro-Val^P(OPh)₂ inhibits proteinase 3 with $k_{\text{obs}}/[\text{I}]$ of 50 $\text{M}^{-1} \cdot \text{s}^{-1}$. As previously described, all the peptide chloromethyl ketones which we tested also inhibited proteinase 3 poorly. Roe et al. [12] found no inhibition of proteinase 3 by MeO-Suc-Ala-Ala-Pro-Ala-CH₂Cl.

In summary, we have found that peptide thioesters are good substrates of proteinase 3 and substituted isocoumarins inhibit this enzyme quite potently. Comparison of the activity of proteinase 3 and HNE indicates that there are significant differences in the extended substrate binding sites (S₄ subsite is more polar, S₁ and S' subsites are more restrictive in proteinase 3) and the catalytic residues (proteinase 3 has lower reactivity toward most substrates and inhibitors). Thioester substrates and isocoumarin inhibitors should be useful for future studies on the functional role of proteinase 3.

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