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THE ENZYMATIC DIGESTION OF ELASTIN AT ACIDIC PH

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Summary

The enzymatic degradation of insoluble elastin has been studied at several pH values using purified pepsin and cathepsin D, and neutrophil extracts. Pepsin degraded elastin throughout the pH range of 1.2—4.0 with the optimum pH below 2.0. Molecular sieve chromatography and gel electrophoresis indicated that a spectrum of molecular weight degradation products was produced. The degradation by pepsin was inhibited by sodium dodecyl sulfate (SDS), NaCl and pepstatin. Cathepsin D which, like pepsin, degrades hemoglobin at acid pH and is inhibited by pepstatin, had no activity against insoluble elastin in the pH range of 3.2—7.2. Extracts of neutrophils degraded elastin above pH 4.0. The pH profile of elastin degradation by neutrophil extracts generally followed that of purified human leukocyte elastase. Our results suggest that during alimentation or pulmonary aspiration of gastric contents, extracellular elastin may be digested by gastric juice at acid pH. Inflammatory cells would not appear to be capable of contributing to such actions until local pH approaches neutrality. Cathepsin D, a major constituent of inflammatory cells, does not digest all types of connective tissue proteins.

Introduction

The incidence of pulmonary emphysema is increased in individuals deficient in alpha-1-antiprotease [1]. Intratracheal instillation of proteases with elastolytic activity, moreover, produces lesions which resemble emphysema morpho-

Abbreviations: Suc-(Ala)$_3$-NA, succinylalanylalanylalanyl-*p*-nitroanilide.

logically and physiologically [2,3]. These observations suggest that emphysema may result in part from a relative excess of proteases over antiproteases in the lung interstitium [4]. Since the pH of the extracellular matrix is within the neutral range, most studies of possible proteolytic mechanisms for emphysema have centered on enzymes optimally active at neutral pH. During pulmonary aspiration of gastric contents, however, lung extracellular pH is temporarily lowered, so that enzymes accessible to and capable of digesting lung structural proteins at acid pH are afforded an opportunity to act. Two candidates for such actions are pepsin and cathepsin D. Pepsin, a normal constituent of gastric juice, is active against both collagen [5] and elastin [6—8], but the activity of pepsin against elastin has received only cursory attention. Cathepsin D is a constituent of neutrophil [9] and alveolar macrophage [10] lysosomes and would, therefore, be released during pulmonary inflammation, such as that which accompanies acid aspiration [11]. Cathepsin D levels, moreover, are increased in the neutrophils of cigarette smokers [10]. Although much of the previously presumed collagenolytic activity of cathepsin D has been assigned to other cathepsins [12], the enzyme has limited activity against collagen [13]. Its activity against elastin has not been characterized.

Although pepsin and cathepsin D could conceivably damage lung connective tissue during acid aspiration, it is not known whether these enzymes could continue to act after extracellular pH is corrected, or, in the case of cathepsin D, if its release is not accompanied by lowered extracellular pH. To further identify possible roles for pepsin and cathepsin D in the catabolism of exogenous and perhaps endogenous elastin, we characterized the activities of these enzymes against insoluble elastin, denatured hemoglobin, and Suc-$(Ala)_3$-NA and compared them with the activities of neutrophil extracts and human leukocyte elastase (EC 3.4.21.11).

Experimental

Materials

Porcine stomach pepsin (twice crystallized) (EC 3.4.23.1), bovine *ligamentum nuchae* elastin and hemoglobin were products of Worthington Biochemical Corp. (Millipore Corp.). Cathepsin D (EC 3.4.23.5) (specific activity = 15.1 units/mg protein, where 1 unit produces an increase of 1 A_{280nm} per min/ml of trichloroacetic acid-soluble hemoglobin at 37°C) from bovine spleen, and pepstatin were products of Sigma Chemical Co. Suc-$(Ala)_3$-NA was a product of Bachem, Inc. NaB^3H_4 (specific activity = 240—270 Ci/mmol) and [^{14}C]methylmethemoglobin (specific activity = 15.1 μCi/μg) were products of New England Nuclear. For use in our studies, the NaB^3H_4 was diluted with unlabeled material to a specific activity of 5 mCi/mmol [14]. The ^{3}H-labelled elastin substrate was prepared using previously described methods [14—16]. Briefly, *ligamentum nuchae* elastin was treated with 0.1 M NaOH at 90°C for 50 min, washed several times with distilled water, then lyophilized. 1 g lyophilized elastin was suspended in 50 ml of 0.1 mM EDTA, pH 9.0, and reduced with 25 mCi NaB^3H_4 (specific activity = 5 mCi/mmol) at room temperature for 2 h. The tritiated elastin was then washed on a Buchner funnel with distilled water to remove unincorporated tritium, washed by centrifugation, then suspended in

800 ml of 0.1 M Na_2CO_3, pH 8.9, containing 0.05% NaN_3. The preparation used in this study contained approx. $1.33 \cdot 10^5$ dpm ^3H/mg elastin.

Enzyme assays

For assay of elastolysis, 0.3 ml of the elastin substrate was transferred to a 1.5 ml polyethylene centrifuge tube and the insoluble elastin was pelleted at 15 600 × *g* for 5 min. The supernatant was aspirated, and the soluble components of the assay added. These included 0.2 M sodium formate, pH 3.2/10 mM $CaCl_2$/0.05% NaN_3/enzyme in a total volume of 0.3 ml. Incubation was carried out for 16 h at 37°C in a Dubnoff shaking incubator. Reaction mixtures were then centrifuged for 10 min at 15 600 × *g* and 0.2 ml of the supernatant was aspirated and counted in 5 ml of Hydromix or Instabray (Yorktown Research) in a Beckman LS-230 liquid scintillation spectrometer with efficiencies of 32 and 30%, respectively.

For studies with inhibitors, the inhibitor was added after all other reaction components except the enzyme, which was added last. For study of the pH profile, 1 M salt or acid solutions were prepared and mixed to achieve a desired buffer pH and then diluted in the assay mixture to a final concentration of 0.2 M. For pH values in the range 1.4—2.9, 1 M H_3PO_4 and 1 M NaH_2PO_4 were used; in the range of 2.6—4.4, 1 M HCOOH and 1 M HCOONa were used; for higher pH values mixtures of 1 M HCOONa and 1 M Na_2HPO_4 were used. At pH 8.5, 1 M Tris-HCl was used.

The assay using hemoglobin as substrate contained, in a total volume of 0.1 ml: 0.15 M sodium formate, pH 3.2/0.1 mg [^{14}C]methylmethemoglobin (approx. 45 000 dpm)/enzyme. After 16 h at 37°C, 0.1 ml 10% trichloroacetic acid was added. The reaction was chilled, then centrifuged for 10 min at 1000 × *g*. One-tenth ml of the supernatant was counted in 5 ml Hydromix.

For assay with Suc-$(Ala)_3$-NA, enzyme was incubated with 1.25 mM substrate and either 0.1 M phosphate or formate buffer for 44 h at 37°C [17].

Column chromatography

For analysis of the peptide products of pepsin digestion, 100 mg unlabelled elastin were digested with 4 mg pepsin in 0.2 M phosphate buffer, pH 1.48/10 mM $CaCl_2$, for 16 h at 37°C. Insoluble material was removed by centrifugation. The pepsin was inactivated by adjusting to pH 7.6 with 10 M NaOH, and the preparation was stored frozen. It was later chromatographed on a 2.6 × 53 cm calibrated column of Sephadex G-100. Fractions were pooled, concentrated, and then analyzed by SDS-polyacrylamide gel electrophoresis [18].

Preparation of neutrophil extracts

Venous blood was collected in acid/citrate/dextrose solution, 30 ml blood/6 ml solution and centrifuged at 300 × *g* for 20 min. The plasma layer was removed and centrifuged at 1000 × *g* to pellet platelets. The platelet-poor supernatant was then added back to the original cell pellet. The reconstituted blood was then diluted with an equal volume of normal saline and layered over Ficoll-Hypaque (Sigma Chemical Co.) in a ratio of 7.5 ml diluted blood to 4 ml Ficoll-Hypaque. This suspension was then centrifuged at 400 × *g* for 40 min, following which the layer containing mononuclear cells was then removed

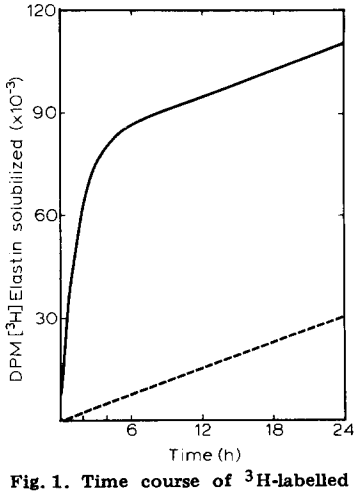
[19]. The remaining cell pellets were pooled, diluted with 6% Dextran (M_r = 500 000) (Sigma Chemical Co.), gently agitated, and allowed to sediment at unit gravity. The neutrophil-rich supernatant layer was aspirated as soon as red blood cells settled. Neutrophils were collected by centrifugation at 300 × *g* for 10 min. Cells were washed first in red cell lysing buffer [20] and then twice in divalent cation-free phosphate buffered saline, pH 7.2, containing 5.6 mM glucose. Cells were counted, assessed for viability (by exclusion of Trypan blue dye), and differentially enumerated in smears stained with buffered differential Wright's stain (DIFF-QUIK®, CAMCO). Cell suspensions prepared in this manner contained more than 95% neutrophils. After adjustment of cell counts, neutrophils were lysed in 0.1% Triton X-100 (Sigma Chemical Co.) in normal saline and homogenized in a Teflon-plexiglas homogenizer (Bellco Glass, Inc.). Insoluble material was removed by centrifugation at 1000 × *g* for 10 min. Protein was estimated by the Lowry technique [21] using bovine serum albumin as the standard.

Purity of enzymes

Analysis of the enzymes used by SDS-polyacrylamide gel electrophoresis [18] showed single bands of appropriate molecular weight for pepsin and human leukocyte elastase, while cathepsin D showed the expected band at 42 000 plus the 28 000 degradation product as discussed by others [22].

Results

The reaction of 100 μg pepsin with 500 μg elastin was nearly complete within 6 h, while 3 μg pepsin degraded elastin in an approximately linear fashion for 24 h (Fig. 1). The dose response to pepsin is shown in Fig. 2. There



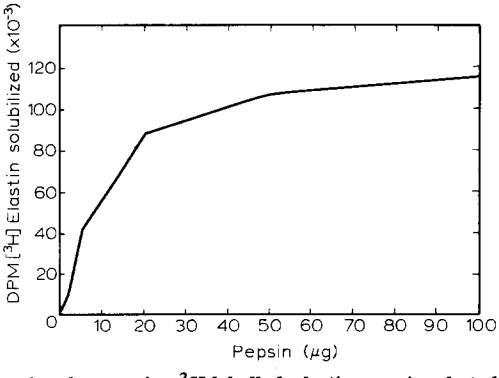


Fig. 1. Time course of ^{3}H-labelled elastin solubilization by pepsin. ^{3}H-labelled elastin was incubated with either 100 μg pepsin (solid line) or 3 μg pepsin (dashed line) at pH 1.48 and 37°C. Samples were taken at 1, 2, 4, 6 and 24 h.

Fig. 2. ^{3}H-labelled elastin solubilization as a function of pepsin. A constant amount (500 μg) of ^{3}H-labelled elastin was incubated with increasing amounts of pepsin (0—100 μg) at pH 1.48 for 16 h at 37°C.

TABLE I

PEPSIN SOLUBILIZATION OF ELASTIN AND METHEMOGLOBIN

For elastin, the complete system contained 0.2 M phosphate, pH 1.48/10 mM $CaCl_2$/500 μg ^{3}H-labelled elastin/3 μg pepsin. After 16 h at 37°C, 15 200 dpm of insoluble ^{3}H-labelled elastin, (approx. 114 μg) were solubilized. For methemoglobin, the complete system contained 0.25 M phosphate, pH 1.48/100 μg (22 000 cpm) [^{14}C]methylmethemoglobin/0.1 μg pepsin. The substrate was completely solubilized in 16 h at 37°C.

System	Relative activity (%)	
	Elastin	Methemoglobin
Complete	100	100
—$CaCl_2$	116	—
—$CaCl_2$, +26 mM EDTA	82	—
+1 M NaCl	26	—
+0.03% SDS	27	11
+0.29 M ethanol	106	95
+729 pmol pepstatin	1	<1
+73 pmol pepstatin	34	3

was a linear response to at least 20 μg pepsin. 10 μg pepsin (287 pmol) solubilized 41 000 dpm ^{3}H-labelled elastin, or approx. 308 μg. If tropoelastin (M_r = 70 000) [23] is considered to be the building block of elastin, then an amount of insoluble elastin equivalent to 15 pmol tropoelastin was solubilized per pmol pepsin in 16 h at 37°C.

Pepsin degraded elastin over a wide pH range with the optimum pH occurring below pH 2 (Fig. 3). The effect of various components and potential inhibitors and activators on the reaction is shown in Table I. $CaCl_2$ was not

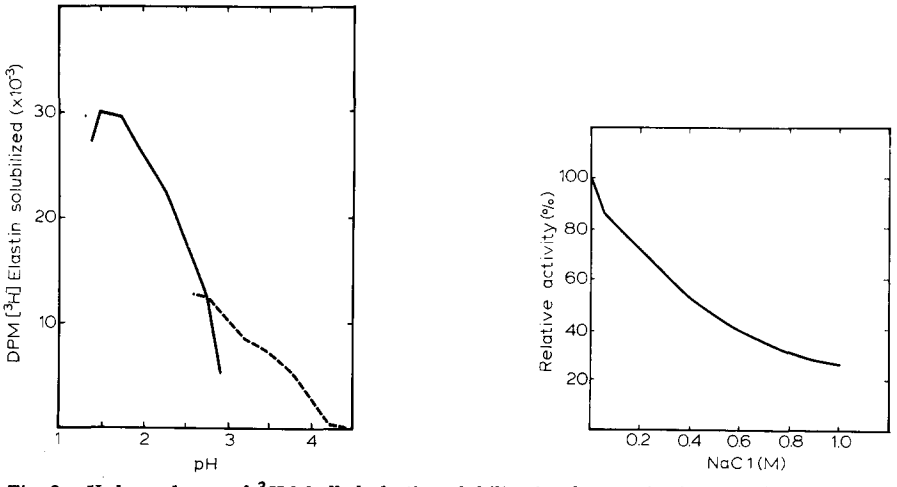


Fig. 3. pH dependence of ^{3}H-labelled elastin solubilization by pepsin. Stock buffers (1 M) were prepared as described in Methods. They were diluted to 0.2 M final concentration in the assay. Pepsin (3 μg) was incubated with ^{3}H-labelled elastin in the various buffers for 16 h at 37°C. The solid line is for phosphate buffers and the dashed line for formate buffers.

Fig. 4. NaCl inhibition of ^{3}H-labelled elastin solubilization by pepsin. ^{3}H-labelled elastin was incubated with 3 μg pepsin in 0.2 M phosphate, pH 1.48, and increasing amounts of NaCl for 16 h at 37°C.

TABLE II

ACTIVITY OF PEPSIN AND CATHEPSIN D AGAINST SUC-(ALA)$_3$-NA

The 1.25 ml reaction contained 1.25 mM Suc-(Ala)$_3$-NA and 0.1 M Tris, pH 8.1/0.1 M phosphate buffer, pH 1.48, or 0.1 M formate buffer, pH 3.2. Incubation was for 44 h at 37°C.

Enzyme (μg)	pH	ΔA_{405mm}/μg protein
Pancreatic elastase (5)	8.1	75
Porcine pepsin (100)	8.1	0.002
Porcine pepsin (100)	1.5	0
Spleen cathepsin D (16)	8.1	0.001
Spleen cathepsin D (16)	3.2	0

required, and ethylenediaminetetraacetic acid did not appreciably inhibit the enzyme. Ethanol, which inhibits the reaction of pepsin with other substrates [24], did not inhibit elastolysis by pepsin. SDS, which enhances the reaction of pancreatic elastase with elastin at slightly alkaline pH [25,26], was inhibitory at acidic pH. NaCl, which inhibits elastolysis by pancreatic but not leukocyte elastase at 0.15 M [27], inhibited elastolysis by pepsin at and above 0.05 M (Fig. 4). Pepstatin, a standard inhibitor of pepsin, inhibited elastolysis at nearly one molecule of pepstatin per molecule of pepsin (Table I). Pepsin had no activity against Suc-(Ala)$_3$-NA (Table II).

The products of the reaction whereby pepsin digested elastin were characterized by chromatography on Sephadex G-100 (Fig. 5). A continuous spectrum of products was produced with the peak molecular weight near that of bovine serum albumin (M_r = 68 000). When pools of various molecular weight

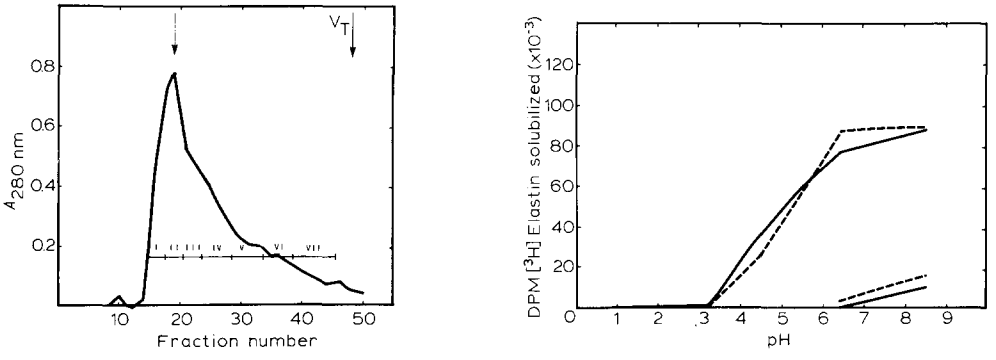


Fig. 5. Sephadex G-100 chromatography of the peptide products of elastin solubilization by pepsin. Unlabelled base-treated elastin (100 mg) was digested with 4 mg pepsin at pH 1.48 for 16 h at 37°C. After centrifugation and neutralization, the soluble materials was chromatographed on Sephadex G-100, as described in Methods and 5 ml fractions were collected. The groups of fractions indicated by Roman numerals were pooled and analyzed by SDS-polyacrylamide gel electrophoresis. The arrows at fractions 19 and 48 denote the elution positions of bovine serum albumin (M_r = 68 000) and ^3H_2O (VT = total bed volume), respectively. Lactate dehydrogenase (M_r = 140 000) eluted at fraction 16 and myoglobin (M_r = 17 000) at fraction 30.

Fig. 6. pH dependence of ^{3}H-labelled elastin solubilization by neutrophil extracts and human leukocyte elastase. ^{3}H-labelled elastin was digested at the indicated pH values with either 36 μg neutrophil extract (dashed line) or 5 μg purified leukocyte elastase (solid line) for 16 h at 37°C. At the higher pH values the lower lines are values obtained with either 3.6 μg neutrophil extract or 0.5 μg purified leukocyte elastase.

ranges were made as shown in Fig. 5 and subjected to SDS-polyacrylamide gel electrophoresis the only discrete band was pepsin. The elastin degradation products appeared as a continuous area of stain with molecular weight ranges paralleling their elution volumes from the Sephadex column (data not shown).

Cathepsin D neither cleaved Suc-$(Ala)_3$-NA (Table II) nor solubilized ^3H-labelled elastin. In the pH range 3.2—7.2, less than 1% of the elastin substrate was solubilized after 64 h by 0.4 U cathepsin D, both in the presence and in the absence of SDS [25,26]. When neutrophil extracts from $5 \cdot 10^5$ cells were tested in the pH range 1.48—8.5, less than 1% of the ^3H-labelled elastin substrate was digested at pH 1.48 and 3.2. Above pH 4, the elastolytic activity paralleled that of leukocyte elastase (Fig. 6).

Discussion

In this report, the degradation of elastin by pepsin has been further characterized. Pepsin appears to be the only known protease capable of solubilizing elastin at low pH. Since digestion of elastin has been implicated in the pathogenesis of pulmonary emphysema, availability of gastric juice to lung parenchyma due to pulmonary aspiration of gastric content [11] is a potential contributing mechanism to the development of this disease.

SDS, at a level optimal for the enhancement of elastin digestion, inhibits pepsin-catalyzed elastin degradation (Table I), in contrast to its enhancing effect with pancreatic [25,26] and macrophage elastase [28]. The binding of pancreatic elastase to elastin is postulated to occur through attraction of negatively charged carboxyl groups on elastin to positively charged groups on elastase [29]. Binding of SDS to elastin induces an increase in negative charge and a change in conformation. At low pH, elastin would be more positively charged. If the binding of pepsin is facilitated by the presence of positive charges on elastin, SDS, since it does not become protonated, would be expected to lower the net positive charge and decrease the interaction. This is consistent with a mechanism for pepsin action in which a negatively charged carboxyl group in pepsin is postulated to react with a protonated amide on the protein substrate [30]. Sodium chloride (Fig. 4) would also be expected to interfere with the electrostatic interaction of enzyme and substrate [29].

Chromatographic analysis of the peptides produced by pepsin digestion (Fig. 5) yielded a variety of degradation products skewed toward molecular weights around 68 000. Both ethanol-KOH [31] and oxalic acid [32] digests of elastin contained peptides of varied molecular weight. With time, the average molecular weight of the peptides produced by ethanolic-KOH decreased [31]. Although reports of the molecular size distribution of the products following digestion of elastin by other enzymes are not available, both pancreatic and leukocyte elastase produce a variety of soluble peptides which can be distinguished by paper electrophoresis [33].

Although cathepsin D has similarities to pepsin including inhibition by pepstatin [34] and activity against collagen [12], it was unable to solubilize elastin, either at low pH, where it maximally digests hemoglobin, or near neutrality, where it is still active against some substrates [34]. Lysates of neutrophils

were unable to digest insoluble elastin at low pH, indicating that elastolytic acid proteases were not present (Fig. 6).

The large number of tyrosine and phenylalanine residues in elastin [23] probably serve as sites of attack by pepsin. The lack of activity of pepsin against the alanyl-nitroanilide bond of Suc-$(Ala)_3$-NA is consistent with the inability of pepsin to hydrolyze amide bonds other than peptide bonds [35]. This inability has also been seen with the macrophage metalloprotease which digests insoluble elastin at neutral pH [28]. These observations, along with the finding that Suc-$(Ala)_3$-NA can be hydrolyzed by enzymes which do not digest elastin [36,37], reemphasize the fact that Suc-$(Ala)_3$-NA can only be used to assay elastases of the serine protease type.

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