Purification and Characterization of Porcine Elastase II and Investigation of Its Elastolytic Specificity†

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ABSTRACT: A new porcine pancreatic serine protease, elastase II (Ardelt, W. (1974), *Biochim. Biophys. Acta 341*, 318–326), was further purified by removing the accompanying proteases on a turkey ovomucoid–Sepharose column. The purified enzyme was found to be homogeneous by ultracentrifugal sedimentation analysis ($s_{20,w}$ = 3.16), by electrophoresis on cellulose acetate and polyacrylamide gels at different pHs, by sodium dodecyl sulfate gel electrophoresis, and by electrofocusing in polyacrylamide gels. Elastase II is composed of about 250 amino acids, with a molecular weight of 26 500, and an NH_2-terminal amino acid sequence which strongly resembles the B chain of bovine chymotrypsin B. Preliminary experiments indicated that it may be composed of two chains held together by a disulfide bond in which the NH_2-terminal half-cystine participates. Elastase II has no activity towards specific substrates of porcine elastase I (EC 3.4.21.11) but exhibits characteristic chymotrypsin specificity. It has an extended binding site but like in other chymotrypsins the subsite S_1 plays a dominant role in the binding. Similar to elastase I, elastase II solubilizes elastin by hydrolyzing about 6% of the peptide bonds. However, while elastase I hydrolyzes Ala-Ala and Ala-Gly peptide bonds, elastase II hydrolyzes the bonds formed between leucine, phenylalanine, or tyrosine with glycine or alanine. It seems, therefore, that the specificity of elastase II is completely different from that of elastase I. Elastase II also differs from porcine chymotrypsin A, B, and C, but strongly resembles a previously described proelastase B, which upon activation hydrolyzes elastin and exhibits chymotrypsin specificity.

A few years ago, we proposed that an elastolytic enzyme could be defined as an alkaline protease which adsorbs on elastin and exhibits specificity toward amino acids with short aliphatic side chains, the predominating species in elastin (Gertler, 1971a). This specificity is demonstrated by the ability of elastolytic enzyme to hydrolyze the esteric or amide bond adjacent to C-terminal alanine in substrates such as Ac-Ala_3-OMe[1] (Gertler and Hofmann, 1970), Ac-Ala_3-Nan (Feinstein et al., 1973), or succinyl-Ala_3-Nan (Bieth and Wermuth, 1973). Since then, it has been shown that other serine proteases which have wide specificity and ability to hydrolyze specific synthetic chymotrypsin substrates also solubilize elastin (Gertler and Trop, 1971; Gertler, 1971b). On the other hand, it was found that anionic serine proteinases of microbial (Gertler and Hayashi, 1971) or vertebrate (Mallory and Travis 1975) origin which were capable of hydrolyzing Ac-Ala_3-OMe were devoid of any elastolytic activity. We also demonstrated that elastolytic enzymes do not necessarily show structural similarities and that a pancreatic serine protease from the African lungfish, which is structurally related to chymotrypsins, hydrolyzes both Ac-Ala_3-OMe and elastin (de Haen and Gertler, 1974).

More recently, a new type of an elastolytic enzyme from porcine pancreas, elastase II, was described (Ardelt, 1974, 1975). This elastase exhibited very high activity toward Ac-Tyr-OEt, thus raising the question of the mode of action and specificity of elastolysis performed by this enzyme. The present study was devoted to further purifying and characterizing this enzyme and to investigating its elastolytic specificity.

Experimental Section

Materials

Pure porcine elastase I was prepared according to Shotton (1970), turkey ovomucoid was prepared according to Feeney et al. (1967), and DFP-treated carboxypeptidase A and elastin were purchased from Worthington Biochemical Corp.

Acetyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide (Ac-Ala_3-Nan) was a product of Miles-Yeda, Rehovot, Israel. Acetyl-L-tyrosine ethyl ester (Ac-Tyr-OEt), benzoyl-L-tyrosine ethyl ester (Bz-Tyr-OEt), tosyl-L-arginine methyl ester (Tos-Arg-OMe), acetyl-L-tyrosine *p*-nitroanilide (Ac-Tyr-Nan) and *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride were obtained from Sigma Chemical Co. *p*-Nitrophenyl *p'*-(ω-dimethylsulfonioacetamido)benzoate bromide, a specific active-site titrant of chymotrypsin (Wang and Shaw, 1972) was a gift of Dr. E. Shaw.

The inhibitors, all of them derivatives of phenylalanine chloromethyl ketone, namely: acetyl-L-alanyl-L-phenylalanine chloromethyl ketone (Ac-Ala-Phe-CK), acetyl-L-leucyl-L-phenylalanine chloromethyl ketone (Ac-Leu-Phe-CK), acetyl-L-alanylglycyl-L-phenylalanine chloromethyl ketone (Ac-Ala-Gly-Phe-CK), *N-tert*-butoxycarbonyl-L-alanylglycyl-L-phenylalanine chloromethyl ketone (Boc-Ala-Gly-Phe-CK), and *N-tert*-butoxycarbonylglycyl-L-leucyl-L-phenylalanine chloromethyl ketone (Boc-Gly-Leu-Phe-CK), were a gift from Dr. K. Kurachi. Their syntheses and characterizations have been described previously (Segal et al., 1971; Kurachi et al., 1973). Tosyl-L-phenylalanine chloromethyl ketone (Tos-Phe-CK) was purchased from Cyclo Chemical

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[1] Abbreviations used are: Ac, *N*-acetyl; Bz, N-benzoyl, Tos, *N*-*p*-toluenesulfonyl; OMe, methyl ester; OEt, ethyl ester; Nan, *p*-nitroanilide; DFP, diisopropyl fluorophosphate; Pth, phenylthiohydantoin; CK, chloromethyl ketone; Boc, *N-tert*-butoxycarbonyl; DEAE, diethylaminoethyl; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

Co. and Sepharose 6B was purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden.

Methods

Assay of Enzymatic Activities. Esterolytic activities were determined titrimetrically, the assay being carried out at 30 °C in a Radiometer Titrator in 0.1 M KCl–0.005 M Tris–0.05 M $CaCl_2$ buffer, pH 8.0, by using Tos-Arg-OMe (0.01 M) and Ac-Tyr-OEt (0.01 M).

The spectrophotometric assay of elastase II, using Bz-Tyr-OEt (Walsh and Wilcox, 1970) or Ac-Tyr-Nan (Bundy, 1963) was carried out at 30 °C. K_m and k_{cat} values for Ac-Tyr-OEt, Bz-Tyr-OEt, and Ac-Tyr-Nan were calculated from Lineweaver–Burk plots obtained with substrate concentrations of 0.5–10, 0.1–0.5, and 0.07–0.5 mM, respectively. The elastase I like activity was estimated spectrophotometrically at 30 °C using Ac-Ala_3-Nan (0.5 mM) as described by Feinstein et al. (1973). All spectrophotometric assays were carried out in a Gilford 2400 spectrophotometer.

The elastolytic activity was determined by a modification (Gertler, 1971b) of the direct spectrophotometric method (Ardelt et al., 1970), and the activity was expressed as ΔA_{276} min^{-1} (mg of enzyme)$^{-1}$ in the reaction mixture.

Affinity Chromatography on the Turkey Ovomucoid–Sepharose Column. Insolubilized turkey ovomucoid was prepared according to March et al. (1974). Eighty milligrams of a lyophilized fraction of elastase II obtained from chromatography of DEAE-Sephadex (Ardelt 1974) was dissolved in 16 mL of 0.1 M triethanolamine hydrochloride, 0.02 M $CaCl_2$ (pH 8.1), and applied to a turkey ovomucoid–Sepharose column (2 × 10 cm) previously equilibrated with the same buffer. The elution was performed with the same buffer at the rate of 80 mL/h. The "breakthrough" fraction was pooled, dialyzed, and lyophilized. This fraction, which was used for further studies, was designated as pure elastase II. The fraction which was adsorbed on the column was eluted from it with 0.3 M KCl adjusted to pH 2 (with HCl). All steps were performed at 4 °C.

Electrophoresis on cellulose acetate membranes was performed in a Beckman Microzone Electrophoresis System Model R-100 in 0.08 M collidine acetate buffer (pH 7.0), or 0.1 M pyridine acetate buffer (pH 4.5, the molarity is that of the cationic component). Samples (0.25–0.75 μL) of protein solution were applied to the membrane and the electrophoresis was performed for 30 min, at 400 V. The membranes were stained with Ponceau-S as described in the Beckman Method Manual (1967).

Electrophoresis in Polyacrylamide Gels. Samples of 100 μg of freeze-dried elastase II were subjected to polyacrylamide gel electrophoresis (Reisfeld et al., 1962) in 0.35 M β-alanine acetate buffer (pH 4.5) for 90 min with a current of 3.5 mA/tube (0.5 × 7 cm). The gels were then stained with Amido Black 10. The gels were prerun for 60 min prior to protein application.

Electrofocusing in polyacrylamide gels was performed in tubes (0.5 × 12 cm) in the pH range 3–10 with a final concentration of polyacrylamide of 4.6% (Wrigley, 1968). The protein (50–100 μg) was applied in 10% (w/v) sucrose solution, and electrofocusing was carried on for 6 h at 350 V with an initial current of 1 mA/tube. The protein was fixed with 12% (w/v) trichloroacetic acid and stained with 1% Coomasie Blue in 10% trichloroacetic acid. The gels were destained with water–methanol–acetic acid (13:5:2, v/v). At least one gel per run was electrofocused without protein. The pH gradient in these gels was checked by measuring the pH of slices of the gel soaked in water.

Sodium Dodecyl Sulfate Gel Electrophoresis. Protein samples were denatured by adding them to a boiling solution of 1% sodium dodecyl sulfate in 0.01 M sodium phosphate buffer (pH 7.0) containing 1% β-mercaptoethanol. After 10 min at that temperature, the samples were cooled and applied to gels following the general procedure of Weber et al. (1972). Molecular weights were calculated using chicken ovoinhibitor (mol wt 48 000), bovine pepsin (mol wt 35 000), Kunitz soybean trypsin inhibitor (mol wt 21 000) and chicken egg-white lysozyme (mol wt 14 400) as standards.

Ultracentrifugal Analysis. Determination of the Sedimentation Coefficient. A sedimentation-velocity analysis was carried out in a Spinco Model E ultracentrifuge at 56 000 rpm, 20 °C, with 0.44% protein in 0.1 M NaCl–0.01 M sodium acetate buffer, pH 5.0. Measurements of diffusion were performed in the same ultracentrifuge using 0.44% of enzyme in the same buffer at 4800 rpm.

Determination of the Molecular Weight. The high-speed equilibrium method of Yphantis (1964) was used. The ultracentrifuge was operated at 15 000 rpm at 20 °C. The concentration of elastase II was 4.4 mg/mL in 0.1 M NaCl–0.01 M sodium acetate buffer, pH 5.0. The samples were run in a four-place cell, after overnight dialysis against the same buffer.

Amino acid analysis was performed with the LKB 3201 Automatic Amino Acid Analyzer. The freeze-dried protein (about 3 mg) dissolved in about 3 mL of 5.7 M HCl was hydrolyzed in 0.5-mL batches under vacuum for 24, 48, 72, and 96 h at 110 °C. Norleucine was added as standard. For determination of half-cystine as cysteic acid, 20 μL of dimethyl sulfoxide was added before hydrolysis (Wold, 1969).

Tryptophan was determined after 24-h hydrolysis in the presence of 4% thioglycolic acid (Matsubara and Sasaki, 1969).

Active-site titration was performed with a Gilford 2400 spectrophotometer using either *p*-nitrophenyl *p*-guanidinobenzoate (Chase and Shaw, 1970) or *p*-nitrophenyl *p'*-(ω-dimethylsulfonioacetamido)benzoate bromide (Wang and Shaw, 1972).

Determination of free sulphydryl groups was carried out by spectrophotometric titration with *p*-mercuribenzoate according to Benesh and Benesh (1962).

Stability Studies. Solutions of elastase II (0.25 mg/mL) in various buffers with or without $CaCl_2$ (0.05 M) were incubated at 4 and 25 °C for 10 days. The following buffers were used: 0.05 M glycine hydrochloride (pH 3), 0.05 M sodium acetate (pH 4–6), 0.05 M Tris-HCl (pH 7–9), 0.05 M glycine-NaOH (pH 10). At different times, aliquots were withdrawn from the incubated samples and assayed for their ability to hydrolyze Ac-Tyr-OEt.

Inhibition of Elastase II by Peptide Chloromethyl Ketones. The reaction mixtures were prepared by adding 0.02 mL of the respective inhibitor dissolved in dioxane to 0.4 mL of enzyme (0.5 mg/mL) in 0.05 M Tris-HCl buffer (pH 8.0) and kept at 25 °C. At various intervals, aliquots were removed from the inhibition mixture and assayed immediately with Ac-Tyr-OEt for residual enzymatic activity. Pseudo-first-order rate constants of inhibition (k_{obsd}) were calculated from semilogarithmic plots. The kinetic parameters, namely, the acylation constant k_2 and the dissociation constant of the enzyme–inhibitor complex K_I, were calculated by plotting $1/k_{obsd}$ as a function of the reciprocal of inhibitor concentration, [I] (Kurachi et al., 1973). It should be noted that under these

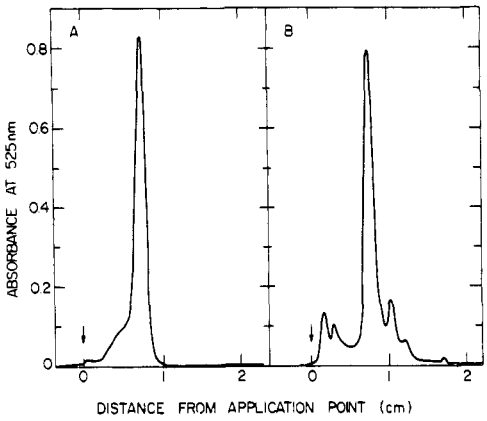

FIGURE 1: Electrophoresis of elastase II before (B) and after (A) purification by turkey ovomucoid-Sepharose column, on cellulose acetate membranes. The electrophoresis was performed at room temperature in 0.08 M collidine acetate buffer (pH 7.0), for 30 min, at the potential of 400 V (cathode on the right). Samples (0.75 μL) containing approximately 15 μg of protein were applied.

conditions $k_{obsd}/[I] = k_2/K_I$ and, therefore, in the cases where separate evaluation of the constants was impossible, a second-order rate constant ($k_{obsd}/[I]$) was calculated.

Sequence analyses were performed with a Beckman sequencer Model 890C according to a modification (Hermodson et al., 1972) of the method of Edman and Begg (1967). Phenylthiohydantoin amino acids were identified and determined quantitatively by gas-liquid chromatography or by amino acid analysis after hydrolysis with hydroiodic acid (47%) for 18 h at 130 °C in evacuated sealed tubes. The sequencer chemicals were products of Beckman and Pierce.

Solubilization of Elastin with Elastase I and Elastase II. An elastin suspension (80 mL) 10 mg/mL in 6 mM NaCl was incubated at 30 °C with 2.5 mg of elastase I or 1.7 mg of elastase II at pH 8.7, using a Radiometer pH meter Model 26, equipped with an autotitrator 11 and 0.65 M NaOH as a titrant. The extent of solubilization was estimated by measuring the absorption at 276 nm in centrifuged aliquots of the digest. When 50% of the elastin was solubilized ($A_{276} = 2.1$), half of the digest was removed; the unsolubilized elastin was spun down and the clear supernatant was lyophilized. The remaining portion of the digest was incubated until full solubilization was achieved and was then lyophilized. Since the amount of lysine in mature elastin is negligible, the extent of hydrolysis was determined by measuring free amino groups with ninhydrin (Rosen, 1957) using leucine as standard.

Digestion of Solubilized Elastin with Carboxypeptidase A. The reaction mixture (2.3 mL) was composed of 4 mg of elastin digested by elastase I or elastase II dissolved in 0.2 M *N*-ethylmorpholine-acetate buffer (pH 8.5), 1 μmol of norleucine, and 170 μg of carboxypeptidase A (prepared according to method 1, Ambler, 1967).

The digestion was carried out at 25 ° C. Aliquots of 0.2 mL were withdrawn after various incubation periods, mixed with 3.8 mL of 0.2 M sodium citrate buffer (pH 2.2) to stop the reaction, and frozen. Samples of 0.5 or 1.0 mL were analyzed for amino acid content. No free amino acids were found prior to digestion with carboxypeptidase A or in the enzyme itself.

Results

Further Purification and Characterization of Elastase II. Following Ardelt (1974), elastase II was partially purified by ion-exchange chromatography on a DEAE-Sephadex column

TABLE I: Enzymatic Activities of Elastase II Before and After Purification by Turkey Ovomucoid-Sepharose Column.

	Ac-Tyr-OEt (μmol min^{-1} mg^{-1})	Tos-Arg-OMe (μmol min^{-1} mg^{-1})	Ac-Ala$_3$-Nan (μmol min^{-1} mg^{-1})	Elastin (ΔA_{276} min^{-1} mg^{-1})
DEAE-Sephadex eluate	905	13.7	0.330	0.20
Turkey ovomucoid-Sepharose breakthrough fraction	995	1.3	0.003	0.07
Elastase I	ND[a]	ND[a]	8.280	0.35

[a] ND, not determined.

in the presence of 3 M urea. As he reported, this preparation exhibited a major and a minor diffusible protein band in disc electrophoresis at pH 4.3. Electrophoresis on cellulose acetate at neutral pH (Figure 1B), however, revealed one major and four minor protein bands. The preparation also exhibited small but detectable enzymatic activities on specific substrates of porcine elastase I and bovine trypsin (Table I). We have shown that such enzymatic activities could be inhibited by turkey ovomucoid, which inhibits bovine trypsin and chymotrypsin, as well as porcine elastase I (Gertler and Feinstein, 1971). Since we found that this inhibitor did not inhibit elastase II, a Sepharose–turkey ovomucoid affinity column was therefore utilized to remove those minor contaminants. Over 80% of protein applied to the column was recovered in the "breakthrough" fraction, which we shall refer to as pure elastase II.

As can be seen in Table I, affinity chromatography removed traces of elastase I like and trypsin like activities, while the activity on AcTyrOEt was slightly elevated. The preparation also lacked any activity on substrates of carboxypeptidase A and B, such as hippurylphenylalanine and hippurylarginine. The elastolytic activity of elastase II, as estimated by ability to solubilize elastin, was only 20% as compared to pure elastase I and 35% as compared to the fraction eluted from DEAE-Sephadex column. It seems therefore that removal of traces of elastase I and trypsin by the Sepharose–turkey ovomucoid column results in drastic decrease in the elastolytic activity. This is most likely due to their synergistic effect on elastolysis that was described previously (Gertler and Birk, 1970; Ledoux and Lamy, 1975). This conclusion is supported by preliminary experiments in which we found that about 65% of the elastolytic activity of the DEAE-Sephadex eluate could be inhibited by turkey ovomucoid. We suppose that the relatively higher elastolytic activity reported by Ardelt (1974) resulted most likely from contamination by small amounts of elastase I.

Figure 1A represents the electrophoretic picture of elastase II after chromatography on a Sepharose–turkey ovomucoid column. As can be seen, most of the contaminating material was removed, confirming the results indicated by trypsin and elastase I activities (Table I). A single protein band was also obtained by electrophoresis on cellulose acetate at pH 4.0, on polyacrylamide at pH 4.5, and by sodium dodecyl sulfate gel electrophoresis in the presence of β-mercaptoethanol.

Electrofocusing (pH 3–10) also resulted in a single band situated on top of the gel (pH 8.5). This merely indicated the absence of acidic proteins, as the more basic proteins, if any, would have been eluted from the gel.

Amino Acid Composition. Amino acid composition of el-

TABLE II: Amino Acid Composition of Porcine Elastase II Compared to that of Porcine Proelastase B and Porcine Chymotrypsins A, B, and C.

Amino acid	Residues/ molecule[a]	Nearest integral	Residues per molecule				
			Elastase II[b]	Proelastase B[c]	Chymotrypsin A[d]	Chymotrypsin B[d]	Chymotrypsin C[d]
Lys	4.9 ± 0.5	5	4	5.5	11.4	5.7	7.2
His	4.2 ± 0.1	4	5	5.0	2.3	2.8	6.1
Arg	6.4 ± 0.4	6	6	8.2	5.6	7.7	8.9
Asp	22.6 ± 0.3	23	19	24.2	21.0	20.0	25.2
Thr[e]	18.4	18	17	17.8	19.8	17.4	16.7
Ser[e]	28.3	28	26	25.9	23.9	28.3	22.5
Glu	21.3 ± 0.7	21	19	20.8	16.6	14.9	26.4
Pro	12.5	12	19	15.0	15.8	13.7	13.5
Gly	23.4 ± 0.8	23	23	25.0	21.8	22.0	26.6
Ala	19.0	19	19	19.0	21.9	21.9	14.9
1/2-cystine[f]	11.2	12	10	10.0	10.5	9.6	10.3
Val[g]	24.7 ± 0.8	25	20	22.8	24.9	24.6	23.0
Met	1.9 ± 0.2	2	3	2.7	1.9	2.0	1.0
Ile	12.7 ± 0.2	13	11	10.5	10.6	11.2	13.8
Leu	20.8 ± 0.5	21	19	20.7	19.4	16.6	21.7
Tyr	6.1 ± 0.4	6	6	6.7	4.8	4.2	6.3
Phe	4.3 ± 0.2	4	4	5.1	5.9	7.9	4.2
Trp[h]	10.4	10	8	—	8.4	12.7	12.1

[a] The number of residues per molecule was the average (±SD) from four analyses after 24, 48, 72, and 96 h of hydrolysis assuming 19 alanines/mol. [b] Ardelt (1975). [c] Uram and Lamy (1969), recalculated assuming 19 alanines/mol. [d] Wilcox (1970). [e] Extrapolated to zero time of hydrolysis. [f] Measured as cysteic acid after oxidation. [g] After hydrolysis of 72 and 96 h. [h] After hydrolysis in the presence of 4% thioglycollic acid.

astase II was compared to that reported by Ardelt (1975), to porcine chymotrypsins A, B, and C, and to porcine proelastase B (Uram and Lamy 1969). This is presented in Table II. The compositions of both ours and Ardelt's preparations are almost identical, with some differences appearing in the proline, methionine, and tryptophan residues. On the other hand, elastase II differs appreciably from porcine chymotrypsins A, B, and C, but shows striking similarity to porcine proelastase B. Since no free SH groups could be detected by titration with *p*-mercuribenzoate, it was assumed that all 12 half-cystine residues in elastase II participate in disulfide bridging.

Molecular Weight. Sedimentation analysis showed one symmetrical peak with $s_{20,w} = 3.16$, thus indicating size homogenity, and the $D_{20,w}$ was calculated as 11.65×10^{-7} cm^2 s^{-1}. Both values were obtained at 0.44% protein solution. The molecular weight was calculated from sedimentation equilibrium using the short-column method of Yphantis (1964), using the partial specific volume, $\bar{v} = 0.730$, as calculated from amino acid composition. A molecular weight of 26 500 ± 260 (av ± SD) was calcuated from four runs at 15 000 rpm. An almost identical value, namely, 26 750, was obtained from sodium dodecyl sulfate-polyacrylamide gel electrophoresis, while the value of 26 812 was calculated from the amino acid composition assuming 19 alanine residues per mol of protein.

Molar Absorption Coefficient. The molar absorption coefficient at 280 nm, calculated from the amino acid composition, assuming 19 alanine residues per mole and using norleucine as an internal standard, was found to be 7.24×10^4 mol^{-1} and $A_{280}^{0.1\%} = 2.74$. This is a rather high value which probably results from the high tryptophan content.

Active-Site Titration. Elastase II can be titrated with the trypsin active-site titrant, *p*-nitrophenyl *p*-guanidinobenzoate or with a chymotrypsin active-site titrant, *p*-nitrophenyl *p'*-(ω-dimethylsulfonioacetamido)benzoate bromide. Both titrations gave identical results, namely 0.96 mol of active site per mol of enzyme. Since activity on a specific trypsin substrate Tos-Arg-OEt was negligible, it can be assumed that the titration reflects action of elastase II only.

N-Terminal Sequence. The NH_2-terminal sequence of the first ten amino acids was found to be Ile-Val-Asx-Gly-Glu-Asx-Ala-Val-Pro-Gly, a sequence strongly resembling that of the B chain of chymotrypsins. It should be noted, however, that considerable amounts of alanine and glycine were found, respectively, in cycles one and two after hydrolysis of the Pth-amino acids and Pth-Gly was found in the second cycle. Since no Pth-Ala could be detected in the first cycle by gas-liquid chromatography, it seems that its appearance after hydrolysis resulted from the destruction of Pth-Cys (Smithies et al., 1971). We would like to suggest, therefore, that elastase II, like other chymotrypsins, is most probably composed of two chains: the B chain whose NH_2-terminal sequence is presented above and a small A chain which was rapidly washed out from the sequencer cup. Experiments to evaluate this assumption are in progress. It should be noted that the NH_2-terminal sequence of almost all known chymotrypsinogens starts with Cys-Gly (de Haen et al., 1975). As mentioned before, the sodium dodecyl sulfate electrophoresis of reduced elastase II showed only one protein band; however, a small peptide composed of a few amino acids could be washed out during the staining or destaining processes.

Ardelt (1975) reported leucine as the NH_2-terminal amino acid of elastase II (by using the dansyl chloride method). That erroneous identification resulted most likely from the poor separation of Pth-leucine from Pth-isoleucine by thin-layer chromatography.

Determination of Kinetic Parameters with Synthetic Substrates. Elastase II was found to be highly active on all synthetic substrates of chymotrypsins. The kinetic parameters are presented in Table III. Parameters for the ester substrates resemble the values reported for various chymotrypsins (Cunningham, 1965), except for the significantly higher k_{cat} value for hydrolysis of Ac-Tyr-Nan than that reported for bovine α-chymotrypsin (Bundy, 1963).

TABLE III: Kinetic Parameters of Hydrolysis of Synthetic Substrates by Elastase II and Chymotrypsin A.

Enzyme	Substrate Assayed: Ac-Tyr-OEt $k_{cat}(s^{-1})$	Ac-Tyr-OEt K_m(mM)	Bz-Tyr-OEt[a] $k_{cat}(s^{-1})$	Bz-Tyr-OEt[a] K_m(mM)	Ac-Tyr-Nan[b] $k_{cat}(s^{-1})$	Ac-Tyr-Nan[b] K_m(mM)
Elastase II[c]	374	0.59	20.4	0.81	5.10	2.42
Chymotrypsin A[d]	195	0.70	43.0	2.6	0.30	1.13

[a] In 30% methanol. [b] In 5% dimethylformamide. [c] At pH 8.0, 30 °C, this investigation. [d] At pH 7.8, 25 °C, from Cunningham (1965) for Ac-Tyr-OEt and Bz-Tyr-OEt and from Bundy (1963) for Ac-Tyr-Nan.

TABLE IV: Inhibition of Elastase II by Peptide Chloromethyl Ketones.

	Concn range (mM)	k_2 (min^{-1})	K_I (mM)	k_2/K_I (min^{-1} M^{-1})
Boc-Gly-Leu-Phe-CK	0.01–0.10	0.11	0.37	300
Boc-Ala-Gly-Phe-CK	0.05–0.50	0.39	0.15	2600
Ac-Ala-Gly-Phe-CK	0.01–0.50	0.50	0.16	3120
Ac-Leu-Phe-CK	0.04–0.50	0.03	0.54	55
Ac-Ala-Phe-CK[a]	0.09–0.45			70
Tos-Phe-CK[a]	0.50			<1.4

[a] The experimental data did not allow separate estimation of k_2 and K_I.

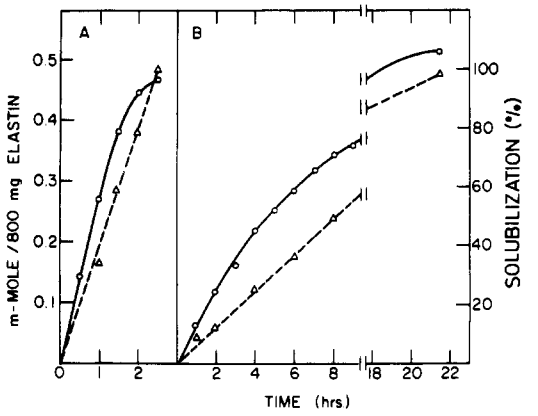

FIGURE 2: Digestion of elastin by elastase I or elastase II. Elastin (800 mg) in 6 mM NaCl (80 mL), pH 8.7, 30 °C was digested by 2.5 mg of elastase I (A) or 1.7 mg of elastase II (B). The digestion was followed by OH^- uptake (O—O) or by measuring the absorption at 276 nm in the centrifuged aliquots of the digest. (Δ- - -Δ).

Inhibition of Elastase II by Peptide Chloromethyl Ketones. Although the number of inhibitors assayed is limited, the results presented in Table IV give preliminary characterization of the binding site of porcine elastase II.

In general extension of the inhibitor peptide chain resulted in slightly better binding and higher acylation constant, thus increasing the total reaction rate.

Stability Studies. Elastase II is a surprisingly stable enzyme. No loss of activity was observed during 10 days, in the pH range of 3–10 at 4 °C or at room temperature, in the presence or the absence of Ca^{2+}.

Solubilization of Elastin by Elastase II or Elastase I. The kinetics of elastolysis by either elastase II or elastase I is presented in Figure 2. As shown, full solubilization of elastin was achieved by both enzymes. The solubilization rate was measured from both the OH^- uptake and the absorbance of the soluble products at 276 nm. Although elastase II solubilizes elastin more slowly than elastase I, the total OH^- uptake required to reach 100% solubilization was almost identical for both. The number of peptide bonds cleaved in each hydrolysate was determined by measuring the concentration of released free amino groups with ninhydrin. It was found that the total solubilization of elastin by elastase I and elastase II was achieved by corresponding hydrolysis of 7.6 and 6.1% of total peptide bonds. Similar values, namely, 7.4 and 5.1%, were obtained in the soluble fraction obtained after solubilization of 50%, merely indicating that the elastolysis was essentially homogeneous. The slightly higher value obtained after full solubilization by elastase II (6.1 vs. 5.1%) may indicate some further degradation of the soluble product that took place after solubilization.

Identification of the Cleaved Peptide Bonds of Elastin Digested with Elastase I and Elastase II. The new NH_2-terminal residues in digested elastin were determined by automatic Edman degradation. Table V presents the amino acids found in the first cycle of degradation. The results are expressed in moles of new residues per mole of cleaved bond, and the latter value was calculated from the data presented above. No corrections were made for losses during the degradation process or during hydrolysis of the Pth-amino acids and this may account for the fact that the total sum of residues is less than 100%. An additional two cycles of degradation were performed in each case. However, since digested elastin contains many hydrolyzed bonds, and small peptides are rapidly washed out from the cup of the sequencer, interpretation of the additional results was impossible. As shown, each enzyme exhibited similar results after either 50 or 100% solubilization. However, while in elastase I solubilized elastin the new NH_2-terminal amino acids consisted of approximately equal amounts of alanine and glycine, in elastase II digested elastin the main NH_2-terminal amino acid was glycine.

Results of the digestion of 100% solubilized elastin by carboxypeptidase A are presented in Figure 3. A remarkable difference was observed in the two digests. In elastin digested by elastase I alanine appears to be the main if not the sole new C-terminal amino acid, while in elastase II digested elastin three new C-terminal residues, namely, leucine, phenylalanine, and tyrosine, were found. Although the total amount of released alanine (Figure 3B) is greater than that of tyrosine or phenylalanine, the kinetics of the release indicate that alanine is not the C-terminal residue. Similar results were also observed with digestion of fractions obtained after 50% solubilization. It may therefore be concluded that elastase I hydrolyzes mainly Ala-Ala and Ala-Gly bonds, while those hydrolyzed by elastase II are most likely Leu-Gly, Phe-Gly, Tyr-Gly, Leu-Ala, Phe-Ala and Tyr-Ala.

Discussion

A main object of this investigation was to clarify whether elastase II, a new porcine pancreatic serine protease described

TABLE V: The NH_2-Terminal Amino Acids in Elastin Solubilized by Elastase I or Elastase II.

Enzyme	Solubilization (%)	NH_2-Terminal residues (mol/mol of hydrolyzed bond)					
		Pro	Gly	Ala	Val	Ile	Leu
Elastase I	50	0.02	0.25	0.25	0.02	0.02	0.02
	100	0.05	0.32	0.26	0.06	0.02	0.06
Elastase II	50	0.03	0.62	0.13	0.01	0.00	0.02
	100	0.02	0.41	0.14	0.01	0.00	0.02

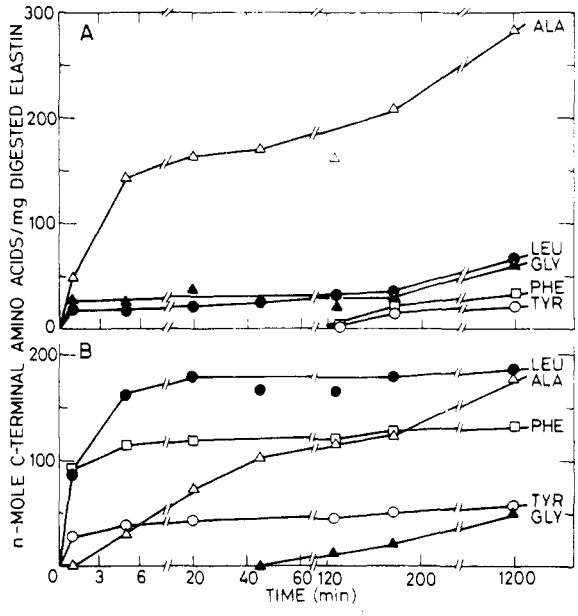

FIGURE 3: Digestion of solubilized elastin with carboxypeptidase A. Four milligrams of solubilized elastin (2.3 mL) in 0.2 M *N*-ethylmorpholine-acetate buffer (pH 8.5) was digested by 0.17 mg of carboxypeptidase A at 25 °C. Aliquots were withdrawn after various incubation periods and analyzed for amino acid content. (A) Elastin digested by elastase I; (B) elastin digested by elastase II.

recently (Ardelt 1974, 1975), is truly an elastolytic enzyme and whether it differs from other previously described porcine serine proteases, like elastase I or chymotrypsin A, B, or C. It was therefore necessary to ensure that elastase II preparations were uncontaminated by other proteases and particularly by elastase I, whose elastolytic activity is strongly enhanced in the presence of other basic proteases like trypsin or chymotrypsin (Gertler and Birk, 1970). Such purification was achieved by passing partially purified enzyme through a turkey ovomucoid–Sepharose column, which removed the contaminating elastase I and trypsin. The uniqueness of elastase II was then clearly demonstrated by its particular specificity toward synthetic substrates, its amino acid composition, its NH_2-terminal sequence, and its electrophoretic mobility.

Since the specificity of elastase II resembles that of chymotrypsins and since no data about elastolytic activities of the latter enzymes have been reported, our conclusion that elastase II differs also from the known porcine chymotrypsins was derived mainly from its amino acid composition, NH_2-terminal sequence, and electrophoretic data. Analysis of its amino acid composition (Table II) showed distinct differences in the ratio of Lys/His, Lys/Arg, and Tyr/Phe, as compared with chymotrypsins A and B, and in the ratio of Gly/Ala, as compared with chymotrypsin C. Our conclusion was further substantiated by the fact that chymotrypsins B and C are anionic proteins, while elastase II is a cationic protein and that the NH_2-terminal residue of chymotrypsin C is valine. On the other hand, the amino acid composition of elastase II is almost identical to that of proelastase B (Uram and Lamy 1969), thus suggesting that the latter is the zymogen of elastase II. The fact that activation of proelastase B results in a high Ac-Tyr-OEt-ase activity (Uram and Lamy 1969; Ledoux and Lamy 1975) makes this suggestion even more acceptable.

Kinetic studies using different substrates and peptide chloromethyl ketones gave preliminary characterization of the binding site of elastase II. Adopting the notation introduced by Schechter and Berger (1967), it can be concluded that elastase II like chymotrypsin A_α (Segal et al., 1971, Kurachi et al., 1973) has an extended binding site, although the enzyme subsite S_1 plays probably the dominant role in binding. Elastase II like chymotrypsin hydrolyzed rapidly the substrates having P_1 only but its inhibition by TosPheCK was negligible, most likely due to some interference by the tosyl group. The requirements of subsite S_2 are not completely clear. In tripeptides, replacement of a large hydrophobic residue, like leucine at P_2 by glycine, improved both the binding and the acylation constants, contrary to the results reported for chymotrypsin A_α. However, in dipeptides a similar replacement by alanine had almost no effect on the second-order rate constant of the inhibition. The importance of S_3 is clearly demonstrated by the fact that tripeptides bind better and in a more productive way than the related dipeptides.

The finding that elastin could be completely solubilized by elastase II (with the maximal contamination of elastase II by elastase I not exceeding 0.1%) indicates clearly that elastase II is a true elastolytic enzyme. Moreover, the investigation of the products of elastolysis revealed that solubilization by elastase II results from cleavage of peptide bonds completely different from those split by elastase I. Elastase II cleaved peptide bonds adjacent to amino acids with aromatic or large hydrophobic side chains, like phenylalanine, tyrosine and leucine. This distinct specificity of elastase II was further demonstrated by the fact that it hydrolyzed additional synthetic chymotrypsin substrates, and its active site could be titrated by either trypsin or chymotrypsin active-site titrants which do not react with elastase I.

It should be noted that all known elastolytic enzymes that hydrolyze elastin at alkaline pH also hydrolyze specific elastase substrates like Ac-Ala_3-OEt or Ac-Ala_3-Nan. The present study is to our best knowledge the first demonstration that a basic serine protease with a characteristic chymotrypsin specificity can lack this activity but is still able to solubilize elastin. Analysis of the model of elastin based on its partial sequence (Gray et al., 1973, Gallop and Paz, 1975) reveals that the peptide bond hydrolyzed by elastase II, namely, Leu-Gly, Tyr-Gly, Phe-Gly, and Phe-Ala, and the peptide bonds hydrolyzed by elastase I, namely, Ala-Ala and Ala-Gly, are found in the α-helical cross-link regions. On the other hand, the regions between cross links that form "oiled coil" (Gray et al., 1973), whose conformation controls the protein extensibility,

are composed mainly of glycine, proline, and valine. In view of this model, it can be assumed that both elastase I and elastase II disrupt the highly cross-linked structure of elastin by hydrolyzing the peptide bonds in the α-helical parts that separate the extensible regions. Therefore, the mutual synergistic effect on the rate of elastolysis, which was also observed by Ledoux and Lamy (1975), is fully understood by this assumption. It remains to be answered, however, why elastin is not hydrolyzed by bovine α-chymotrypsin, which shares the specificity of elastase II. So far, no clear answer can be given; however, the fact that leucine forms over 50% of the bonds hydrolyzed in elastin by elastase II suggests that the lack of activity may be due to the low ability of α-chymotrypsin to hydrolyze bonds adjacent to leucine (Cunningham, 1965). It was clearly demonstrated that α-chymotrypsin enhances the rate of elastolysis of elastin by elastase I by up to 200% (Gertler and Birk, 1970), but only after some bonds were initially hydrolyzed by elastase I did α-chymotrypsin show this effect. Preliminary experiments performed in our laboratory have shown that the elastolytic activity of elastase II was also enhanced by α-chymotrypsin.

The partial structural and specificity data presented in this paper allow us to make at least a preliminary evaluation of the evolutionary relationship of elastase II to other pancreatic serine proteases. Comparison of the NH_2-terminal fragments suggests that elastase II is closely related to the chymotrypsins and particularly to chymotrypsin B (Dayhoff, 1972), whose NH_2-terminal sequence of 10 amino acids is identical to that of elastase II. Comparison with porcine elastase I (Dayhoff, 1972) or African lungfish elastase B (de Haen and Gertler, 1974), on the other hand, reveals a much less similarity with only three out of the ten amino acids being identical. It seems therefore that elastase II is a new, undescribed chymotrypsin, structurally and kinetically similar to other chymotrypsins and which most likely acquired its ability to solubilize elastin, as a result of some minor changes in its binding site. We hope that the elucidation of its full primary structure will provide us with a more accurate explanation of the elastolytic activity.

Until the present investigation, two types of vertebrate elastases have been described: porcine elastase I and African lungfish elastase B, which are structurally related and have similar specificity, and African lungfish elastase A, which while sharing similar specificity is structurally related to chymotrypsins (de Haen and Gertler, 1974). Another anionic serine protease which hydrolyzes synthetic substrates of elastase I but is devoid of elastolytic activity was recently isolated from the human pancreas (Mallory and Travis, 1975). The data presented in this paper increase the heterogeneity of the elastases even more, since elastase II lacks the ability to hydrolyze the specific synthetic substrates of elastase I and is structurally related to chymotrypsins. It is obvious, therefore, that classification of elastases (or pancreatopeptidases E) as a separate family of serine proteases is not acceptable any more. Consideration should be given as to whether these enzymes should be classified according to specificity, ability to solubilize elastin, or structural similarity.

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pH, Isotope, and Substituent Effects on the Interconversion of Aromatic Substrates Catalyzed by Hydroxybutyrimidylated Liver Alcohol Dehydrogenase†

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ABSTRACT: The mechanism of hydrogen transfer catalyzed by horse liver alcohol dehydrogenase (EC 1.1.1.1) with amidinated amino groups was studied with steady-state kinetics. Hydroxybutyrimidylation of the enzyme increases the maximum velocities of the enzymatic reactions and the rates of dissociation of the enzyme-coenzyme complexes. Results of product inhibition studies with the modified and native enzymes acting on benzyl alcohol and benzaldehyde are consistent with an ordered mechanism. Primary deuterium isotope effects obtained for oxidation of pentadeuterioethanol (V_H/V_D = 3.1) and [1,1-D_2]benzyl alcohol (V_H/V_D = 3.6) and for reduction of a series of para-substituted benzaldehydes (V_H/V_D = 2.4) indicate that the turnover numbers reflect the rates of hydrogen transfer with hydroxybutyrimidylated enzyme. Isotope effects were not observed for native enzyme. The magnitudes and signs of the ρ values obtained for oxidation of para-substituted benzyl alcohols ($\rho^+ = -0.2$) and for reduction of a series of para-substituted benzaldehydes ($\rho^+ = 1.1$) catalyzed by hydroxybutyrimidylated enzyme suggest that interconversion of central complexes occurs via concerted hydride and proton transfer in which only a small amount of charge develops in the transition state. The rate of oxidation of benzyl alcohol depends upon a group with a pK of 8.4, which must be unprotonated for maximum activity but which allows partial activity in its protonated form. The rate of reduction of benzaldehyde is essentially independent of pH over the range from pH 6.0 to 9.9. The maximum velocity for benzaldehyde reduction catalyzed by native enzyme required protonation of a group with a pK of 8.0 but did not show an isotope effect, indicating that steps in the mechanism other than hydrogen transfer are rate limiting. The pH effects for both enzymes can be explained by a coherent model that is consistent with the structure of the enzyme as determined by x-ray crystallography. This model postulates that a water (or hydroxide) molecule coordinated to the zinc ion acts as a proton donor (or acceptor) and that the state of protonation of His-51 modulates the rate of transfer of hydrogen in the central complexes.

The work of Klinman (1972, 1975, 1976) on the mechanism of the interconversion of para-substituted benzyl alcohols and benzaldehydes catalyzed by yeast alcohol dehydrogenase (EC 1.1.1.1) has led to the conclusion that little or no charge develops on C-1 of the substrate during hydrogen transfer. Mechanisms involving either concerted general acid-base catalysis of a hydride transfer or a protonated radical intermediate and hydrogen atom transfer are consistent with the results. Similar studies with horse liver alcohol dehydrogenase have been more difficult and have led to differing conclusions. The main difficulty is that the chemical conversion of substrate to product catalyzed by the native liver enzyme cannot be observed using steady-state techniques, because transfer of hydrogen is not rate limiting for turnover. The rate-limiting step for the oxidation of ethanol or benzyl alcohol is dissociation of the enzyme-NADH complex (Dalziel, 1963; Wratten and Cleland, 1965), and for the reduction of benzaldehyde release of benzyl alcohol is limiting (McFarland and Bernhard, 1972). More recently, transient kinetic techniques have been used to study interconversion of ternary complexes of native enzyme. Large primary deuterium isotope effects were obtained, but the ρ values obtained from Hammett plots do not appear to be compatible with each other. Jacobs et al. (1974) found a small positive ρ value for reduction of some benzaldehydes, which led them to suggest that there was a highly positively charged transition state; from this result, a large negative ρ value for the oxidation of the benzyl alcohols is predicted. But Hardman et al. (1974) found a small negative ρ value for the alcohols and suggested that a hydride ion and a proton are transferred synchronously.

We now report effects of modification of amino groups of liver alcohol dehydrogenase on rate-limiting steps in reactions of various substrates and the applicability of modified enzymes for steady-state studies on the mechanism of hydrogen transfer. A deuterium isotope effect of 4.8 on steady-state turnover had been obtained previously for the oxidation of ethanol catalyzed by picolinimidylated enzyme (Plapp et al., 1973). Extending this approach to para-substituted benzyl alcohols and benzaldehydes, we have found that substituent effects with the liver enzyme are similar to those found for the yeast enzyme. Furthermore, we have integrated our results on the effects of pH

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