(1983) J. Biol. Chem. 258, 2327-2333.
Renard, A., & Verly, W. G. (1980) FEBS Lett. 114, 98-102.
Schendel, P. F., & Robins, P. E. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 6017-6020.
Siegel, L. M., & Monty, K. J. (1966) Biochim. Biophys. Acta 112, 346-362.
Singer, B. (1975) Prog. Nucleic Acid Res. Mol. Biol. 15, 219-284.

Isolation and Characterization of Rat Pancreatic Elastase†

Corey Largman

ABSTRACT: Proelastase has been purified to homogeneity from rat pancreatic tissue by a combination of CM-Sephadex and immobilized protease inhibitor affinity resins. Trypsin activation yields an elastolytic enzyme that possesses a specificity toward small hydrophobic residues in synthetic amide substrates, similar to those of porcine elastase 1 and canine elastase. However, the rat enzyme also rapidly hydrolyzes a substrate containing tyrosine in the P_1 position. N-Terminal sequence analysis reveals that rat proelastase has an identical activation peptide with that of porcine proelastase 1 and has two conservative amino acid sequence differences from the activation peptide of canine proelastase. The sequence data established that rat proelastase corresponds to the elastase 1 mRNA clone isolated by MacDonald et al. [MacDonald, R. J., Swift, G. H., Quinto, C., Swain, W., Pictet, R. L., Nikovits, W., & Rutter, W. J. (1982) *Biochemistry 21*, 1453]. The sequence and substrate data obtained for rat and canine elastases suggest that there is a family of pancreatic elastases with properties similar to those of the classically described porcine elastase 1.

The pancreatic serine proteases are related by extensive sequence homology, maintenance of crucial active site catalytic residues, and highly conserved three-dimensional structure. X-ray crystal structure studies have provided rational explanations for the substrate specificities of trypsin (Stroud et al., 1971) and chymotrypsin (Mathews et al., 1967). In contrast, there is no general agreement concerning the peptide-bond specificity of pancreatic elastases. Much of this ambiguity results from the fact that elastases are operationally defined on the basis of their capacity to hydrolyze elastin (Shotten, 1970). In recent years, a number of pancreatic and nonpancreatic serine proteases with elastolytic activity have been reported that preferentially hydrolyze low molecular weight substrates containing different P_1[1] residues (Shotton, 1970; Gertler et al., 1977; Largman et al., 1976; Zimmerman & Ashe, 1977).

Essentially all of the early studies on elastases were focused on porcine pancreatic elastase 1 (Shotton, 1970). The few studies on the frequency of peptide bonds cleaved by porcine elastase 1 indicate a broad specificity for isoleucine, valine, and alanine (Naughton & Sanger, 1961, Powers et al., 1977). Nevertheless, the desire for a rapid and simple chemical assay based on synthetic substrates (Gertler & Hofman, 1969; Atlas & Berger, 1972; Bieth & Wermuth, 1973; Kasafirek et al., 1976) has resulted in the development of a literature that has emphasized the specificity of porcine pancreatic elastase 1 for hydrolysis of substrates with alanine in the P_1 position. The X-ray crystal structure of porcine elastase 1 has been interpreted in support of this specificity by suggesting that a partially obstructed binding pocket exists in this enzyme compared to chymotrypsin (Shotton & Watson, 1970). However, Mallory & Travis (1975) isolated an alanine-specific protease from human pancreas tissue that lacks elastolytic activity. Furthermore, a second elastase isolated from porcine pancreatic tissue and a similar enzyme isolated from human pancreas (Gertler et al., 1977; Largman et al., 1976) degrade elastin but possess specificities for more bulky amino acids in the P_1 position of synthetic substrates (Gertler et al., 1977; Del Mar et al., 1980). These elastases have chymotrypsin-like activation peptides (Lamy et al., 1977; Largman et al., 1980a) but, unlike chymotrypsin, are capable of rapid elastolysis. Finally, the finding that a serine protease from leukocytes rapidly hydrolyzes elastin but possesses a specificity for valine in the P_1 position (Zimmerman & Ashe, 1977) further obscures the relationship between the apparent configuration of the substrate binding site of an enzyme and its capacity to effect elastolysis.

MacDonald et al. (1982) have recently reported the nucleotide sequences of two messenger RNAs from rat pancreas, which the authors suggest correspond to the two types of pancreatic elastase described in other species. These authors have relied on comparisons of the inferred amino acid sequences with those of known pancreatic proteases and the existing substrate and X-ray structure data to make assignments for the proteins corresponding to these two messenger RNAs as elastolytic enzymes. At present, however, neither protein has been expressed from the cloned messenger RNA or compared with proteins isolated from rat pancreas tissue. The authors note that the isolation of the cDNA clones corresponding to the nucleotide sequence for pancreatic elastase(s) presents opportunities for the engineering and production of altered enzymes by specific mutagenesis, which would facilitate further understanding of the catalytic mechanism and determinants required for serine protease activity. It appears that such experiments would be especially useful in gaining insight

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[1] The nomenclature introduced by Schechter & Berger (1967) is used to describe the positions of amino acids in a substrate. Amino acid residues and terminal acyl substituents are numbered P_1, P_2, P_3, etc. in the N-terminal direction from the scissle bond. The corresponding subsites of the enzyme's active site are numbered S_1, S_2, S_3, etc. in an analogous fashion.

concerning the factors that influence the capacity of an enzyme to effect elastolysis.

Prior to embarking on experiments to modify cDNA clones, it is necessary to demonstrate the existence and properties of the corresponding proteins in rat pancreas tissue. We have previously briefly described the isolation of a proelastase from rat pancreas for use in studies on clearance of proelastases from the bloodstream (Largman et al., 1980b). In the present study, the isolation and purification of rat pancreatic proelastase is described. The P_1 specificity of rat elastase toward synthetic substrates and an elastin derivative are compared to those of other elastases. Finally, amino acid sequence analysis has been performed to demonstrate that the rat proelastase isolated corresponds to one of the mRNA sequences reported by MacDonald et al. (1982).

Materials and Methods

Materials. Frozen rat pancreas tissue was obtained from Pel Freeze Biologicals, Rogers, AR, in lots of 25–50 pancreata per package. Porcine enterokinase was purified from semi-purified material supplied by Miles Labs, Elkhardt, IN, as previously described (Brodrick et al., 1978b). RBB-elastin[2] (Rinderknecht et al., 1968) and RBB-hide (Rinderknecht et al., 1970) were prepared as described. Tos-Lys-CH_2Cl, Tos-Arg-OMe, lima bean trypsin inhibitor, and turkey egg white inhibitor were obtained from Sigma Chemical Co., St. Louis, MO. LTI–Sepharose (Brodrick et al., 1978a) and TEI-Sepharose (Largman et al., 1980c) were prepared as previously described. Bovine pancreatic trypsin inhibitor was a gift from Bayer-AG, Darmstadt, Federal Republic of Germany. Suc-Ala-Ala-Pro-Phe-pNA and Suc-Ala-Ala-Pro-Abu-pNA were prepared in a previous study (Del Mar et al., 1980). Bovine trypsin and α-chymotrypsin were obtained from Worthington Biochemical Corp.

Assay of Elastolytic Activity. RBB-elastin assays were performed in a final volume of 2 mL containing 10 mg of substrate suspended in 50 mM Tris-HCl–0.025% Triton X-100, pH 8.8. Assay tubes were incubated at 37 °C for 2 h with intermittent vortexing, placed on ice, and centrifuged at 4 °C for 10 min in a Sorval HS-4 rotor at 3000 rpm, and the absorbance of the supernatant was read at 595 nm. In order to assay for proelastase in column fractions, duplicate 500-μL aliquots were taken. One aliquot was activated by incubation at 37 °C for 1 h with 200 μL of 0.4 M Tris-HCl–0.15 M $CaCl_2$, pH 8.3, containing 40 μg of bovine trypsin. Tos-Lys-CH_2Cl (0.05 mg/mL) was added to the unactivated aliquots to prevent zymogen activation during the assay.

Assay of Amidase and Esterase Activities. Column fractions activated with trypsin as described above were assayed for chymotryptic activity with Suc-Ala-Ala-Pro-Phe-pNA and for amidase activity with Suc-Ala-Ala-Pro-Abu-pNA as previously described (Del Mar et al., 1979, 1980). Tryptic activity toward Tos-Arg-OMe was assayed following activation of column fractions with human enterokinase in 0.4 M Tris–0.15 M $CaCl_2$ (pH 8.0) as previously described (Brodrick et al., 1978b).

Assay of General Protease Activity. Nonspecific protease activity was measured with RBB-hide as previously described (Rinderknecht et al., 1970) in assay mixtures containing 5 mg/mL substrate with a total volume of 2 mL. After incubation, assay mixtures were centrifuged at 3000 rpm for 10 min, and the absorbance of the supernatant at 595 nm was recorded.

Kinetic Studies. Hydrolysis of *p*-nitroanilide substrates was determined at 410 nm as previously described (Del Mar et al., 1980) in a Gilford Model 251 spectrophotometer with an automatic curvette positioner. All assays were performed in 0.2 M Tris–HCl (pH 8.0). For K_m studies, elastase was employed at a final concentration of 0.2, 0.4, or 0.6 μg/mL. Substrate concentrations covered a minimal range of 0.1–3 times K_m for each substrate measured. The data were first plotted according to the method of Lineweaver & Burk (1934) to check for linearity of the reciprocal plot and to allow for rejection of obviously erroneous data points. The parameters K_m and V_{max} were then estimated by an interactive least-squares fit to the Michaelis–Menton equation by the use of the computer program described by Cleland (1967). A unit of esterase activity is defined as the hydrolysis of 1 μmol of substrate/min at 25 °C.

Amino Acid Composition. The amino acid composition was derived from analysis of protein samples that had been hydrolyzed in 6 N HCl at 110 °C for 24 h. The values for serine and threonine are uncorrected. Cysteine was identified as cysteic acid following hydrolysis of a protein sample subjected to performic acid oxidation (Hirs, 1967).

N-Terminal Sequence Analysis. An aliquot of proelastase (80 nmol) was reduced with dithiothreitol in 8 M urea (containing 10 mM benzamidine to reduce proteolysis by trace contamination by trypsin) and alkylated with iodo[^{14}C]acetic acid according to the procedure described by Crestfield et al. (1963). A $NaDodSO_4$ gel of the reduced and alkylated material showed a single band with an apparent molecular weight of 29 500. Automatic amino acid sequence analysis was performed in a Beckman spinning-cup sequenator with a dilute (0.1 M) QUADROL program (Beckman no. 03 1281) with 2 mg of polybrene added prior to sequencing. Phenylthiohydantoins were identified by high-pressure liquid chromatography, thin-layer chromatography, and gas–liquid chromatography. An aliquot of each phase was counted for ([^{14}C]carboxymethyl)cysteine. A second aliquot of proelastase (40 nmol) was subjected to performic acid oxidation (Hirs, 1967) prior to automatic sequence analysis.

Isoelectric Focusing. Isoelectric focusing was performed in an LKB flat-plate apparatus. A polyacrylamide gel with pH 7–11 ampholines was employed to set up a pH gradient of approximately 7.7–10.3. The values reported are the means of three determinations.

Results

Five separate partial purifications of rat proelastase were performed during the development of procedures to isolate this protein. The entire procedure given below was carried out twice. In all cases, only a single peak of Suc-Ala-Ala-Pro-Abu-pNA activity was observed.

Purification of Rat Proelastase. All purification steps were carried out at 4 °C. All buffers contained 1 mM benzamidine to reduce activation of proelastase by trypsin.

(*1*) *Preparation of Acetone Powder.* Acetone powders of lots of approximately 25 pancreata were prepared as previously described (Largman et al., 1976). Powders were stored at −76 °C until used.

(*2*) *Preparation of Initial Extract.* Acetone powder (6–14 g) was extracted with 20:1 by weight of 50 mM NaOAc (pH 4.5) in a Virtis homogenizer. The extract was stirred for 1

[2] Abbreviations: RBB-elastin, Remazol brilliant blue stained elastin; RBB-hide, Remazol brilliant blue stained hide powder; Suc, succinoyl (3-carboxypropionyl); pNA, *p*-nitroanilide; Abu, α-aminobutyric acid; Tos-Lys-CH_2Cl, N^{α}-tosyl-L-lysine chloromethyl ketone; Tos-Arg-OMe, *p*-toluenesulfonyl-L-arginine methyl ester; LTI–Sepharose, turkey egg white inhibitor bound to Sepharose; PTI, bovine pancreatic trypsin inhibitor; Tris, tris(hydroxymethyl)aminomethane; $NaDodSO_4$, sodium dodecyl sulfate; Mes, 4-morpholineethanesulfonic acid.

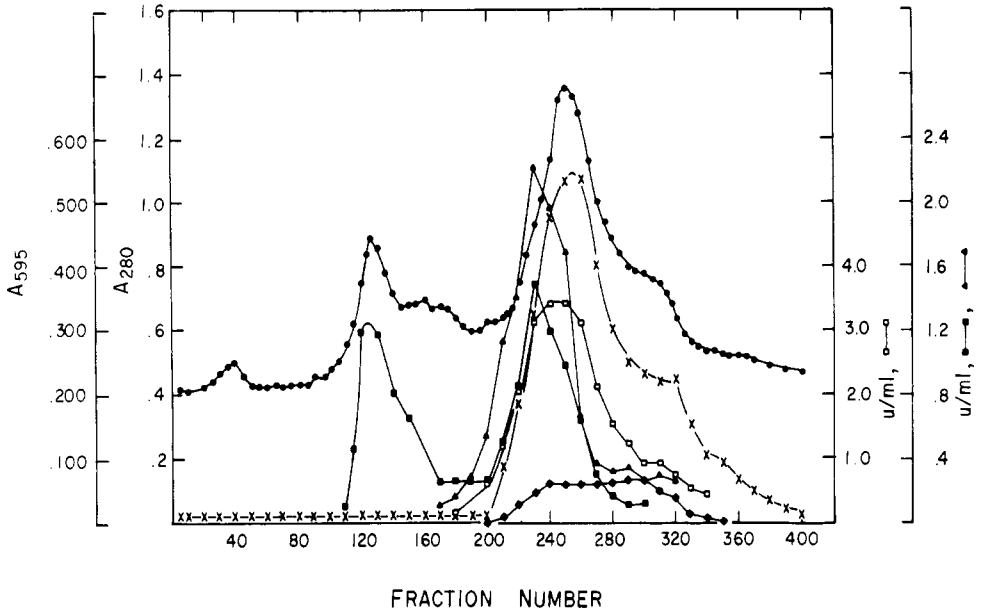

FIGURE 1: Initial fractionation of pancreas extract on CM-Sephadex: (×) elastolytic activity following tryptic activation of column fractions; (♦) elastolytic activity prior to activation; (□) activity toward Suc-Ala-Ala-Pro-Abu-pNA, (▲) activity toward Suc-Ala-Ala-Pro-Phe-pNA; (■) activity toward Tos-Arg-OMe; (●) A_{280}. Other than the initial elastolytic activity, all assays were performed on activated fractions and reflect a combination of initial enzyme and potential zymogen activity in each fraction. The A_{280} values are high due to a base-line absorbance of approximately 0.4 OD from the benzamidine in the buffers. Fractions 210–270 were pooled for proelastase activity. Chromatography conditions are described under Materials and Methods.

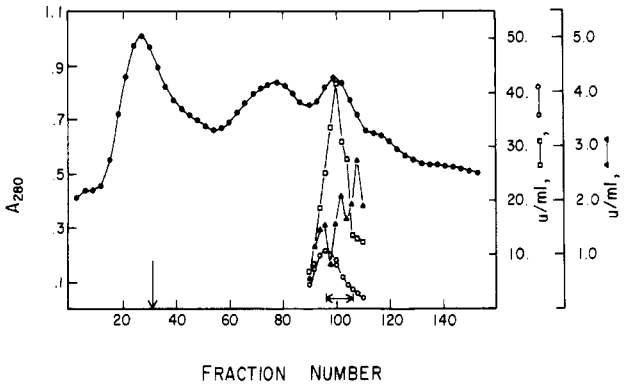

FIGURE 2: CM-Sephadex chromatography at pH 6.5: (□) activity toward Suc-Ala-Ala-Pro-Abu-pNA following trypsin activation; (○) activity toward Suc-Ala-Ala-Pro-Abu-pNA prior to activation; (▲) chymotryptic activity toward Suc-Ala-Ala-Pro-Phe-pNA following activation with trypsin; (●) A_{280}. Chromatography conditions are described under Materials and Methods.

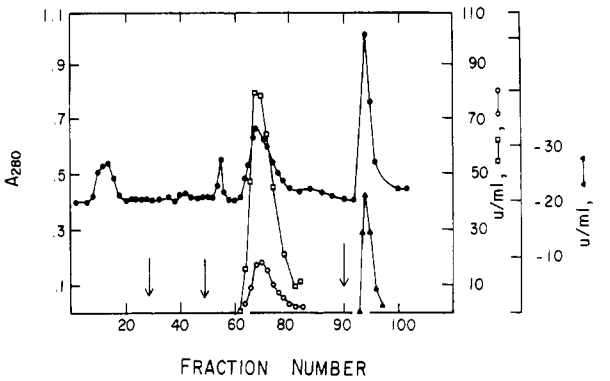

FIGURE 3: Chromatography of proelastase on LTI–Sepharose: (□) activity toward Suc-Ala-Ala-Pro-Abu-pNA following trypsin activation of column fractions; (○) activity toward Suc-Ala-Ala-Pro-Abu-pNA without trypsin activation; (▲) activity toward Suc-Ala-Ala-Pro-Phe-pNA following tryptic activation; (●) A_{280}. Chromatography conditions are described under Materials and Methods.

h and then centrifuged for 15 min at 15 000 rpm in a Sorval centrifuge equipped with an SS-34 rotor. Fractional precipitation of the resulting supernatant was accomplished by adjusting the extract to 40% of saturation by adding 22.9 g/100 mL of ammonium sulfate. The resulting suspension was stirred 1 h and centrifuged at 10 000 rpm for 15 min. The resulting pellet was redissolved in 50 mM NaOAc, containing 0.05 mg/mL PTI (pH 4.5), and dialyzed against the same buffer without inhibitor.

(*3*) *CM-Sephadex Chromatography at pH 4.5.* The dialyzed $(NH_4)_2SO_4$ fraction was applied to a 4 × 20 cm column of CM-Sephadex in 50 mM NaOAc (pH 4.5) (Figure 1). The column was washed with starting buffer, 50 mM NaOAc (pH 5.3), and eluted with a gradient of 0–0.35 M NaCl in 50 mM NaOAc (pH 5.3). In early experiments, formic acid was placed in fraction tubes to lower the pH of the fractions in order to reduce tryptic activation of proelastase. However, proelastase appeared to precipitate under the conditions employed, and low recovery was observed. The column fractions containing activatable elastolytic activity were pooled and concentrated in an Amicon concentration cell with a PM-10 membrane. Aliquots of PTI (1 mg) were added at the start and finish of the concentration step. The resulting concentrate was dialyzed against 50 mM sodium phosphate (pH 4.4).

(*4*) *CM-Sephadex Chromatography at pH 6.5.* Following removal of most of the trypsin and trypsinogen from the proelastase by chromatography at pH 4.5, rechromatography on CM-Sephadex at pH 6.5 was employed to further purify the proelastase. The dialyzed pool from the first CM-Sephadex column was adjusted to pH 6.5 with 1 M NaOH and applied to a 4 × 15 cm column of CM-Sephadex C-50 equilibrated with 50 mM sodium phosphate (pH 6.5) (Figure 2). The column was washed with starting buffer, and the proelastase was eluted with a gradient of 750 mL of 0.05–0.2 M sodium phosphate (pH 6.5). Fractions containing proelastase was pooled, concentrated by ultrafiltration, and dialyzed against 10 mM Mes (pH 4.0).

(*5*) *Chromatography on LTI–Sepharose.* The dialyzed pool from the second CM-Sephadex column was adjusted to 20 mM $CaCl_2$ and pH 6.7 and applied to a 2 × 7 cm column of

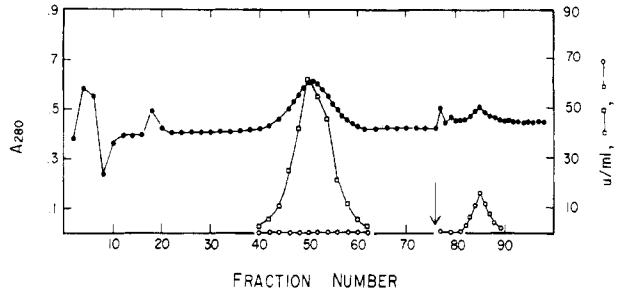

FIGURE 4: Resolution of proelastase from elastase on TEI–Sepharose: (□) activity toward Suc-Ala-Ala-Pro-Abu-pNA following trypsin activation; (○) activity toward Suc-Ala-Ala-Pro-Abu-pNA without trypsin activation; (●) A_{280}. Fractions 44–56 were pooled for proelastase. Chromatographic conditions are given under Materials and Methods. The arrow indicates elution with 0.1 M sodium formate (pH 2.1).

Table I: Purification of Rat Pancreatic Proelastase

step	vol (mL)	total act.	% active enzyme	proelastase yield (g)
initial extract	330	336	0.5	100
40% $(NH_4)_2SO_4$ pellet	45	362.3	0.31	108
first dialyzate	55	356.0	6.8	98.7
CM-Sephadex (pH 4.5)	290	133.4	17.4	32.8
CM-Sephadex (pH 6.5)	35	59.5	12.0	15.5
LTI-Sepharose	9.1	29.8	21.7	7.0
TEI-Sepharose	7.0	15.0	<0.2	4.5

LTI–Sepharose in 10 mM Mes–20 mM $CaCl_2$ (pH 6.7) (Figure 3). The column was washed with the following series of buffers: equilibration buffer, 0.1 M sodium formate (pH 5.0), 0.1 M sodium formate (pH 3.5), and 0.1 M sodium formate (pH 2.1). The fractions containing proelastase were pooled, concentrated in the diaflow apparatus, and dialyzed against 10 mM Mes (pH 4.0).

(*6*) *Chromatography on TEI–Sepharose.* The dialyzed pool from LTI–Sepharose chromatography was applied to a 1.2 × 4 cm column of TEI–Sepharose equilibrated in 10 mM Mes (pH 6.7) (Figure 4). The column was washed sequentially with equilibration buffer, 20 mM NaOAc (pH 5.0), and a gradient of 0–0.3 M NaCl in 20 mM NaOAc (pH 5.0). Fractions containing proelastase was pooled and concentrated in the diaflow apparatus. A summary of the purification of rat pancreatic proelastase is presented in Table I. The yield of proelastase from 15 g of pancreas powder is approximately 5 mg of pure protein.

Characterization of Rat Pancreatic Proelastase. The purified material from TEI–Sepharose chromatography was a single band on $NaDodSO_4$ gel electrophoresis and migrated as a single band on polyacrylamide gel isoelectric focusing with an isoelectric point of 9.60 ± 0.26. Samples of porcine elastase, human proelastase 2, and bovine chymotrypsinogen focused at pH values of 9.76 ± 0.25, 9.37 ± 0.21, and 9.26 ± 0.21, respectively. The purified proelastase had less than 0.2% active esterase activity compared to the maximum activity achieved following activation of the zymogen. Antisera raised against proelastase in rabbits showed a single line of identity in Ouchterlony double diffusion with the zymogen or active enzyme.

Enzymatic Studies. (*1*) *Elastin Degradation.* In order to obtain elastase for enzymatic studies, an aliquot of purified proelastase was diluted 1:1 with 0.4 M Tris-HCl–0.15 M $CaCl_2$ (pH 8.3) and incubated at 37 °C with 1:100 by weight of bovine trypsin. Esterase activity toward Suc-Ala-Ala-Pro-Abu-pNA was followed until maximal activity was obtained (45 min), and the resulting solution was adjusted to 1 mM Tos-Lys-CH_2Cl and incubated at 37 °C until there was no detectable trypsin activity (1 h). The solution was dialyzed against 1 mM HCl and stored at −76 °C until use.

Table II: Relative Elastolytic and Proteolytic Activities of Mammalian Pancreatic Elastases

enzyme	relative sp act. toward RBB-elastin[a]	relative sp act. toward RBB-hide powder[a]
porcine elastase	100	100
rat elastase	67.1	33.2
human elastase 2[b]	16.6	31.8
canine elastase[c]	199	164
baboon elastase[d]	37.9	50.4
bovine trypsin	0.3	274.0
bovine α-chymotrypsin	1.5	126.6

[a] Relative specific activity vs. porcine pancreatic elastase. [b] Largman et al., 1976. [c] Geokas et al., 1980. [d] C. Largman and J. W. Brodrick, unpublished observations.

The relative rates of hydrolysis of RBB-elastin by rat, porcine, human, canine, and baboon pancreatic elastases are reported in Table II. For comparison, the relative rates for hydrolysis of a general protease substrate (RBB-hide) are also reported. As shown in the table, rat pancreatic elastase rapidly hydrolyzes both elastin and hide powder at rates comparable to those of the other elastases. Table II also shows the virtual absence of hydrolysis of RBB-elastin by trypsin and chymotrypsin, demonstrating that the rat enzyme isolated is a true elastolytic enzyme.

(*2*) *Substrate Specificity.* The kinetic parameters k_{cat} and K_m were determined for the hydrolysis by rat pancreatic elastase of a series of synthetic peptides substrates of the form Suc-Ala-Ala-Pro-amino acid-pNA. As shown in Table III, rat elastase rapidly hydrolyzes several of the substrates tested. The rat enzyme appears to preferentially hydrolyze substrates with alanine, tyrosine, or α-aminobutyric acid in the P_1 position. For comparison, selected kinetic constants for human, porcine, and canine elastases are also presented in Table III. Since the rapid hydrolysis of the tyrosine substrate was not anticipated, the data for this substrate were obtained in three separate determinations. Furthermore, the same solution of substrate was employed in the studies on porcine and canine elastases reported in Table III.

(*3*) *Structural Studies.* The amino acid composition for rat pancreatic proelastase is shown in Table IV. For comparison, the amino acid composition calculated from the presumed amino acid sequence of rat proelastase as deduced from the mRNA sequence reported by MacDonald et al. (1982) is also presented. The data obtained from hydrolysis of the protein is in close agreement with the composition derived from the nucleotide sequence. The molecular weight of proelastase determined by $NaDodSO_4$ gel electrophoresis with the procedure of Weber & Osborn (1969) was 26 500, in agreement with the value of 27 092 calculated from the gene sequence data.

The sequence of the N-terminal 29 amino acid residues of rat pancreatic proelastase is given in Table V. The mRNA nucleotide sequence and the deduced amino acid sequence for rat preproelastase 1 reported by MacDonald et al. (1982) are also shown. The amino acids deduced from the mRNA corresponding to residues −26 to −11 represent the signal peptide (Blobel & Sabatini, 1971), which is presumably removed by proteolysis during cellular processing of the protein. On the first sequenator run of reduced and alkylated proelastase, a major sequence (80%) starting at residue 3 (Asp)

Table III: Kinetic Parameters for Hydrolysis of Succinoyl Tetrapeptide *p*-Nitroanilides by Elastases

substrate	rat elastase k_{cat} (s^{-1})	rat elastase K_m (mM)	rat elastase k_{cat}/K_m (s^{-1} M^{-1})	porcine elastase 1 k_{cat} (s^{-1})	porcine elastase 1 K_m (mM)	porcine elastase 1 k_{cat}/K_m (s^{-1} M^{-1})
Suc-Ala-Ala-Pro-Met-pNA	0.12	1.42	83			
Suc-Ala-Ala-Pro-Ile-pNA	0.20	0.91	220			
Suc-Ala-Ala-Pro-Val-pNA	0.70	1.21	580			
Suc-Ala-Ala-Pro-Phe-pNA	1.45	1.12	1 290			
Suc-Ala-Ala-Pro-Leu-pNA	2.99	1.47	2 030	4.9	0.49	10 000[a]
Suc-Ala-Ala-Pro-Ala-pNA	4.89	0.18	27 200	54.0	0.19	290 000[a]
Suc-Ala-Ala-Pro-Tyr-pNA	4.28	0.21	20 400	0.03	1.64	15.7
Suc-Ala-Ala-Pro-Abu-pNA	3.59	0.10	35 300	10.2	0.03	351 000
Suc-Ala-Ala-Ala-pNA	1.59	1.09	1 460	15.4	3.11	4 950

substrate	canine elastase k_{cat} (s^{-1})	canine elastase K_m (mM)	canine elastase k_{cat}/K_m (s^{-1} M^{-1})	human elastase 2 k_{cat} (s^{-1})	human elastase 2 K_m (mM)	human elastase 2 k_{cat}/K_m (s^{-1} M^{-1})
Suc-Ala-Ala-Pro-Tyr-pNA	0.05	0.06	700	2.8	1.0	2 700
Suc-Ala-Ala-Pro-Val-pNA	8.72	2.31	3 800	0.01	3.5	3[a]
Suc-Ala-Ala-Pro-Leu-pNA	23.5	0.71	33 000	5.1	1.4	3 600[a]
Suc-Ala-Ala-Pro-Ala-pNA	45.8	0.23	199 000	0.34	2.4	140
Suc-Ala-Ala-Pro-Abu-pNA	24.9	0.05	461 000	1.4	2.4	600[a]

[a] Del Mar et al., 1980.

Table IV: Amino Acid Composition of Rat Pancreatic Elastase[a]

amino acid	residues calculated from mRNA sequence[b]	residues determined by amino acid analysis
Asp	25	26.4
Thr	20	17.2
Ser	28	26.9
Glu	19	21.2
Pro	8	9.3
Gly	24	27.8
Ala	15	15.9
Val	26	24.9
Cys	8	5.7
Met	6	3.5
Ile	9	7.4
Leu	16	15.1
Tyr	12	10.0
Phe	4	4.5
Try	7	
His	6	6.1
Lys	4	3.9
Arg	11	12.8

[a] Calculated M_r 27 092. [b] MacDonald et al., 1982.

Table V: N-Terminal Amino Acid Sequence of Rat Pancreatic Elastase

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                                   -26                        -20

Determined Sequence:
Deduced Sequence:a         met leu arg phe leu val phe ala ser leu val leu tyr gly
Nucleotide Sequence:a      AUG CUG CGC UUC CUG GUG UUC GCU UCC CUG GUC CUG UAU GGA

        -10                                            +1

        |(thr-gln)asp phe pro glu thr asn ala arg || val val gly gly ala glu ala
his ser | thr gln asp phe pro glu thr asn ala arg || val val gly gly ala glu ala
CAC AGU↓ ACC CAG GAC UUU CCG GAA ACU AAU GCC CGC ⇓ GUG GUU GGA GGG GCU GAA GCC

        +10                                 +19
arg arg asn ser (-) pro ser(gln)ile ser leu(gln). . .
arg arg asn ser trp pro ser gln ile ser leu gln . . .
CGG CGG AAC UCC UGG CCA UCU CAG AUU UCC CUC CAG . . .
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[a] The reported nucleotide sequence for messenger RNA and the corresponding inferred amino acid sequence for rat preproelastase 1 from MacDonald et al. (1982).

and a minor sequence (20%) starting at residue 1 (Thr) were detected. When a second sample of performic acid oxidized rat proelastase was analyzed in the sequenator, the sequence starting with aspartic acid was present at a 2:1 ratio over the sequence beginning with threonine. No other sequences were detected in either sequenator run, suggesting a relatively clean mixture of these two forms of proelastase. The N-terminal 29 amino acid residues are identical with those predicted by the mRNA sequence. In addition, as shown in Table VI, the activation peptide sequence is identical with that reported for porcine pancreatic proelastase 1 (Lamy et al., 1977). For comparison, a sample of canine pancreatic proelastase (Geokas et al., 1980) was subjected to automatic Edman sequence analysis. As shown in Table VI, the sequence of the activation peptide is extremely similar to those of rat elastase and porcine elastase 1. It should be noted that this latter sequence determination also revealed a minor sequence starting at the aspartic acid at residue 3 (approximately 15%).

Discussion

The purification of pancreatic zymogens is plagued by the problem of activation of proenzymes by trypsin during the purification. A related problem is the potential for partial proteolysis of the desired proenzyme during purification. In the present study, these problems were minimized by performing the initial tissue extraction and column fractionation steps at acidic pH in which proteolytic activity due to pancreatic enzymes is minimal, by performing subsequent steps at as low a pH as feasible, and by adding exogenous inhibitors throughout the purification procedure. As shown in Table I, the major loss of yield came from the very restrictive pooling of proelastase in the first CM-Sephadex column, due to the desire to avoid including too much trypsinogen/trypsin with resultant potential for continuing zymogen activation throughout the purification. In preliminary experiments, this first CM-Sephadex column was run at pH 6.0 to permit better resolution of proelastase from trypsinogen, as had been reported in a brief description of the purification of rat pancreatic endopeptidases (Genell et al., 1977). However, as noted by the previous authors, under these conditions, the zymogens were rapidly activated.

We have previously described the use of TEI–Sepharose to isolate human pancreatic proelastase (Largman et al., 1979) and the use of LTI–Sepharose to purify human pancreatic cationic trypsinogen (Brodrick et al., 1978a,b). Although the respective active proteases bind more tightly to these resins, it is apparent that the zymogens exhibit significant affinity to these immobilized inhibitors, due perhaps to the partial formation of an incipient active site in the zymogen (Largman

Table VI: Comparison of N-Terminal Sequences of Pancreatic Proelastase[a]

	-10													+1		
Rat Proelastase 1	(thr	gln)	asp	phe	pro	glu	thr	asn	ala	arg	val	val	gly	gly		
Porcine Proelastase 1[b]	thr	glx	asx	phe	pro	glx	thr	asx	ala	arg	val	val	gly	gly		
Canine Proelastase	thr	glu	asn	val	pro	glu	thr	asn	ala	arg	val	val	gly	gly		
	-10										+1					
Human Proelastase 2[c]	cys	gly	asp	pro	thr	tyr	pro	pro	tyr	val	-	-	-	thr	arg	val	val	gly	gly
Porcine Proelastase 2[b]	cys	gly	val	pro	ala	ile	lys	pro	ala	leu	asx	phe	asx	glx	arg	ile	val	asx	gly
Porcine chymotrypsinogen[d]	cys	gly	val	pro	ala	ile	pro	pro	val	leu	ser	gly	leu	ser	arg	ile	val	asn	gly
Rat Proelastase 2[e]	cys	gly	val	pro	thr	tyr	glu	-	val	gln	his	asp	val	ser	arg	val	val	gly	gly

[a] The sequences are shown with inserted spaces to maximize homology for comparison. [b] Lamy et al., 1977. [c] Largman et al., 1980a. [d] Hartley & Shotton, 1971. [e] Proposed amino acid sequence derived from mRNA sequence reported by MacDonald et al. (1982).

et al., 1979; Bode et al., 1978). In the present study, these resins were extremely useful for fractionating proelastase both from contaminating proteins and especially from the active enzyme (Figure 4). Since the active enzyme differs from the zymogen by a single 10-residue peptide, these proteins have extremely similar properties on ion-exchange and size-fractionation columns.

As seen in Figure 1, a single peak of elastolytic activity is detected following fractionation of the initial pancreas tissue extract and tryptic activation of column fractions. This finding is consistent with the results of Genell et al. (1977), who reported a single peak of elastolytic activity following fractionation of rat pancreatic juice. Initial screening experiments indicated that one of the substrates previously synthesized for studies on human pancreatic elastase 2 (Del Mar et al., 1980), Suc-Ala-Ala-Pro-Abu-pNA, was a particularly good substrate for rat elastase. As seen in Table III, this substrate is 2 orders of magnitude better than the commercially available substrate Suc-Ala-Ala-Ala-pNA, as judged by the criteria of the kinetic parameter k_{cat}/K_m. The use of this substrate during the purification of rat pancreatic elastase greatly decreased the time necessary for column assays while permitting a more quantitative assessment of yields and percentage of active enzyme vs. zymogen at each step.

Porcine pancreatic elastase 1 (Thompson & Blout, 1973), human pancreatic elastase 2 (Del Mar et al., 1980), and bovine chymotrypsin (Segal, 1972) show significant rate enhancements for hydrolysis of substrates with proline in the P_2 position. Furthermore, substantial rate enhancements have been observed for pancreatic elastase hydrolysis of substrates containing a negatively charged group in the P_5 position (Kasafirek et al., 1976; Del Mar et al., 1980). In the present study, a series of compounds of the form Suc-Ala-Ala-Pro-amino acid-pNA were employed to characterize the P_1 specificity for rat pancreatic elastase. As seen in Table III, the rat enzyme has specificity for alanine and α-aminobutyric acid in the P_1 position. This specificity is similar to those of porcine elastase 1 and canine elastase (Table III). However, the rat enzyme also rapidly hydrolyzes a substrate containing tyrosine in the P_1 position, which is a very poor substrate for porcine elastase 1 or canine elastase, while being a moderately good substrate for human elastase 2. Although human elastase 2 and porcine elastase 2 hydrolyze substrates containing bulky residues in the P_1 position, it is unclear why a tyrosine substrate is rapidly hydrolyzed by rat elastase while the phenylalanine- and leucine-containing substrates are poor substrates. These results suggest that the rat enzyme has a less restricted binding pocket than other alanine-specific elastases.

As shown in Table III, the K_m values for the hydrolysis of the best amide substrates are quite similar (0.02–0.2 mM) for the rat, porcine, and canine elastases and are 10-fold higher for human elastase 2. In contrast, the k_{cat} values for these substrates are 10-fold lower for the rat and human enzymes compared to those for the porcine and canine elastases. These data suggest that the rat elastase binds substrates as efficiently as the porcine and canine elastases but that the inherent catalytic efficiency is somewhat lower for the rat enzyme. The finding that rat elastase hydrolyzes a derivatized elastase substrate and a high molecular weight protein substrate at rates similar to porcine elastase 1 (Table II) indicates that the relative activities of the enzymes on peptide substrates are only an approximate reflection of activity toward high molecular weight substrates.

The structural data in Table V demonstrate that the mRNA sequence reported for elastase 1 by McDonald et al. (1982)

represents the elastolytic enzyme isolated from rat pancreas. The apparent isolation of a mixture of proelastase with the N-terminal Thr-Gln sequence predicted by these authors and a major form of the protein missing these two residues is difficult to explain in terms of known proteolytic degradation patterns. Thus none of the specific pancreatic endopeptidases would be expected to cleave a Gln–Asp bond. Furthermore, the cleavage of this bond by endopeptidases or pancreatic aminopeptidase (Uriel & Avrameas, 1965) would be anticipated to generate significant contaminating sequences and N-terminal heterogeneity, which were not observed. As shown in Table VI, the activation peptide of rat pancreatic elastase 1 is homologous with porcine elastase 1 and canine elastase. These enzymes are characterized by activation peptides that are removed from the enzyme by limited tryptic hydrolysis at arginine. This is distinct from the elastases that appear to be related to the chymotrypsinogen family, in which the activation peptide remains attached to the enzyme via a disulfide bond between the N-terminal cysteine residue and an internal cysteine in the protein (Hartley & Shotton, 1971).

The properties that confer elastolytic activity on these proteases remain unclear. Gertler et al. (1977) have reported that the two types of porcine pancreatic elastase degrade elastin by preferentially hydrolyzing peptide bonds that reflect their respective specificities. Gertler (1971a,b) has provided substantial evidence that elastolysis is dependent on the cationic character of an elastase and that there is an electrostatic interaction between the enzyme and elastin. This requirement is supported by the isolation of an anionic serine endopeptidase from human pancreas that has alanine specificity but that lacks significant elastolytic activity[3] (Mallory & Travis, 1975; Largman et al., 1976). A similar enzyme has been isolated from porcine pancreas (Kobayashi et al., 1981). Chymotrypsins, although possessing basic isoelectric points and similar peptide-bond specificities to some elastases, do not effect elastolysis (Table II). Finally, MacDonald et al. (1982) have reported a second mRNA sequence that they suggest may correspond to an elastolytic enzyme on the basis of a relatively high sequence identity with porcine pancreatic elastase 1. These authors note that the putative rat elastase 2 would have a chymotrypsin-like activation peptide, and they suggest that its enzymatic specificity would be more like that of chymotrypsin. The fact that a second peak of elastolytic activity was not observed in the current study may imply that the enzyme denoted rat elastase 2 by MacDonald et al. (1982) does not possess significant elastolytic activity or that it is not extracted efficiently under the conditions employed.

Prior to the present study, porcine pancreatic elastase 1 was the only well-described elastase from mammalian pancreas tissue that possessed specificity for small hydrophobic amino acids. The results of the present study demonstrate that both rat pancreatic elastase 1 and canine elastase are closely related to porcine elastase 1. The data in Tables III and VI taken together with additional kinetic data for porcine elastase 2 (Gertler et al., 1977) and human elastase 2 (Del Mar et al., 1980) suggest that there are two distinct families of pancreatic elastases. One family, represented by rat elastase 1, porcine elastase 1, and canine elastase, is characterized by a released activation peptide, a general specificity for small amino acid side chains in the P_1 site of low molecular weight substrates, and very basic isoelectric points. The second family, represented by porcine elastase 2 and human elastase 2, is characterized by a chymotrypsin-like activation peptide, a specificity for bulky hydrophobic amino acids at the P_1 specificity site of substrates, and a basic isoelectric point. In light of the existing literature, which generalizes porcine elastase 1 as the quintessential elastase, the author proposes that the enzymes of the family containing porcine elastase 1 be referred to as pancreatic elastases while the enzymes of the second family be referred to as chymotrypsin-like elastolytic proteases.

[3] The protease referred to as human pancreatic elastase 1 by Largman et al. (1976) has been shown in additional experiments to be capable of effecting significant elastolysis only on preparations of elastin that had been subjected to very harsh conditions. When less damaged preparations of elastin are employed, this protease demonstrates very low levels of elastolytic activity (C. Largman, and J. W. Brodrick, unpublished observations).

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Registry No. Suc-Ala-Ala-Pro-Met-pNA, 72682-73-6; Suc-Ala-Ala-Pro-Ile-pNA, 72682-77-0; Suc-Ala-Ala-Pro-Val-pNA, 72682-76-9; Suc-Ala-Ala-Pro-Phe-pNA, 70967-97-4; Suc-Ala-Ala-Pro-Leu-pNA, 70968-04-6; Suc-Ala-Ala-Pro-Ala-pNA, 72682-69-0; Suc-Ala-Ala-Pro-Tyr-pNA, 72682-75-8; Suc-Ala-Ala-Pro-Abu-pNA, 72682-70-3; Suc-Ala-Ala-Ala-pNA, 52299-14-6; elastase, 9004-06-2; proelastase, 9023-43-2.

References

Atlas, D., & Berger, A. (1972) *Biochemistry 11*, 4719.

Bieth, J., & Wermuth, C. G. (1973) *Biochem. Biophys. Res. Commun. 53*, 383.

Blobel, G., & Sabatini, D. (1971) *Biomembranes 2*, 193.

Bode, W., Schwager, P., & Huber, R. (1978) *J. Mol. Biol. 118*, 99.

Brodrick, J. W., Largman, C., Johnson, J. H., & Geokas, M. C. (1978a) *J. Biol. Chem. 253*, 2732.

Brodrick, J. W., Largman, C., Hsiang, M., Johnson, J. H., & Geokas, M. C. (1978b) *J. Biol. Chem. 253*, 2737.

Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol. 11*, 350.

Crestfield, A. M., Moore, S., & Stein, W. H. (1963) *J. Biol. Chem. 238*, 622.

Del Mar, E. G., Largman, C., Brodrick, J. W., & Geokas, M. C. (1979) *Anal. Biochem. 99*, 316.

Del Mar, E. G., Largman, C., Brodrick, J. W., Fassett, M., & Geokas, M. C. (1980) *Biochemistry 19*, 468.

Genell, S., Gustafsson, B. E., & Ohlsson, K. (1977) *Scand. J. Gastroenterol. 12*, 811.

Geokas, M. C., Largman, C., Brodrick, J. W., & Fassett, M. (1980) *Am. J. Physiol. 238*, G238.

Gertler, A. (1971a) *Eur. J. Biochem. 23*, 36.

Gertler, A. (1971b) *Eur. J. Biochem. 20*, 541.

Gertler, A., & Hofman, T. (1970) *Can. J. Biochem. 48*, 384.

Gertler, A., Weiss, Y., & Burstein, Y. (1977) *Biochemistry 16*, 2709.

Hartley, B. S., & Shotton, D. M. (1971) *Enzymes, 3rd Ed. 3*, 323–373.

Hirs, C. H. W. (1967) *Methods Enzymol. 11*, 197.

Kasafirek, L., Fric, P., Slaby, J., & Malis, F. (1976) *Eur. J. Biochem. 69*, 1.

Kobayashi, R., Kobayashi, Y., & Hirs, C. H. W. (1981) *J. Biol. Chem. 256*, 2460.

Lamy, F., Gibson, D., Ledoux, M., & Moreux, J. C. (1977) in *Elastin and Elastic Tissues* (Sandberg, L., Gray, W. R., & Franyblau, C., Eds.) pp 165, Plenum Press, New York.

Largman, C., Brodrick, J. W., & Geokas, M. C. (1976) *Biochemistry 15*, 2491.
Largman, C., Brodrick, J. W., Geokas, M. C., Sischo, W. M., & Johnson, J. H. (1979) *J. Biol. Chem. 254*, 8516.
Largman, C., Brodrick, J. W., & Geokas, M. C. (1980a) *Biochim. Biophys. Acta 623*, 208.
Largman, C., Ray, S. B., Brodrick, J. W., & Geokas, M. C. (1980b) *Am. J. Physiol. 239*, G504.
Largman, C., Brodrick, J. W., Geokas, M. C., Johnson, J. H., & Fassett, M. (1980c) *Am. J. Physiol. 238*, G177.
Lineweaver, H., & Burk, D. (1934) *J. Am. Chem. Soc. 56*, 658.
MacDonald, R. J., Swift, G. H., Quinto, C., Swain, W., Pictet, R. L., Nikovits, W., & Rutter, W. J. (1982) *Biochemistry 21*, 1453.
Mallory, P. H., & Travis, J. (1975) *Biochemistry 14*, 722.
Mathews, B. W., Sigler, P. B., Henderson, R., & Blow, D. M. (1967) *Nature (London) 214*, 652.
Naughton, M. A., & Sanger, F. (1961) *Biochem. J. 78*, 156.
Powers, J. C., Gupton, B. F., Harley, A. D., Nishino, N., & Whitley, R. J. (1977) *Biochim. Biophys. Acta 485*, 156.
Rinderknecht, H., Geokas, M. C., Silverman, P., Lillard, Y., & Haverback, B. J. (1968) *Clin. Chim. Acta 19*, 327.
Rinderknecht, H., Silverman, P., Geokas, M. C., & Haverback, B. J. (1970) *Clin. Chim. Acta 28*, 239.
Schechter, I., & Berger, A. (1967) *Biochem. Biophys. Res. Commun. 27*, 157.
Segal, D. M. (1972) *Biochemistry 11*, 349.
Shotton, D. M. (1970) *Methods Enzymol. 19*, 113.
Shotton, D. M., & Watson, H. C. (1970) *Nature (London) 225*, 811.
Stroud, R. M., Ray, K. M., & Dickerson, R. E. (1971) *Cold Spring Harbor Symp. Quant. Biol. 36*, 125.
Thompson, R. C., & Blout, E. R. (1973) *Biochemistry 12*, 51.
Uriel, J., & Avrameas, S. (1965) *Biochemistry 4*, 1740.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.
Zimmerman, M., & Ashe, B. M. (1977) *Biochim. Biophys. Acta 480*, 241.

Proteolytic Specificity and Cobalt Exchange of Hemorrhagic Toxin *e*, a Zinc Protease Isolated from the Venom of the Western Diamondback Rattlesnake (*Crotalus atrox*)†

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ABSTRACT: Hemorrhagic toxin *e*, a zinc protease causing hemorrhage, was isolated from the venom of the western diamondback rattlesnake (*Crotalus atrox*) by a previously published procedure [Bjarnason, J. B., & Tu, A. T. (1978) *Biochemistry 17*, 3395–3404]. The proteolytic specificity of hemorrhagic toxin *e* was investigated by using the oxidized A and B chains of bovine insulin. The enzyme cleaves the Ala_{14}–Leu_{15} bond of insulin B chain very rapidly and the Ser_9–His_{10} and Asn_3–Gln_4 of the same chain more slowly. The enzyme cleaves Tyr_{14}–Gln_{15} of insulin A chain rapidly and Ala_6–Ser_9 of the same chain more slowly. The cleavage of insulin A chain by hemorrhagic toxin *e* was inhibited by ethylenediaminetetraacetic acid but not by aprotinin. Hemorrhagic toxin *e* containing cobalt(II) instead of zinc cleaved the A and B chains of insulin at the same sites and with similar rapidity as the native toxin. The cobalt-containing toxin was prepared by direct exchange with dialysis and contained approximately 1 mol of cobalt per mol of toxin but was devoid of zinc. No structural changes were observed accompanying the metal exchange by using circular dichroism (CD) and ultraviolet spectroscopy as structural probes, whereas considerable structural changes had occurred upon simple removal of zinc from the toxin, i.e., formation of the apoenzyme. The absorption spectrum of the cobalt hemorrhagic toxin *e* in the visible region had a maximum at 505 nm (170 cm^{-1} M^{-1}), and the CD spectrum in the visible region had a minimum at 480 nm (−3200 deg cm^2 $dmol^{-1}$) indicative of a distorted tetrahedral complex with oxygen and nitrogen ligands. Cobalt hemorrhagic toxin *e* was both hemorrhagic and proteolytic to a similar extent as the native toxin.

Five hemorrhagic toxins from the venom of *Crotalus atrox* have been characterized as metalloproteases containing zinc in the native form (Bjarnason & Tu, 1978). Proteolytic activities of each of the hemorrhagic toxins on the general protease substrates dimethylcasein and dimethylhemoglobin were measured by reacting 2,4,6-trinitrobenzenesulfonic acid (TNBS)[1] with the newly generated amino groups. It was furthermore demonstrated that the hemorrhagic activity of hemorrhagic toxin *e*, one of the five hemorrhagic toxins, correlates with its proteolytic activity and with its zinc content.

Although there has been considerable confusion as to the proteolytic activities of the hemorrhagic toxins from the venoms of Japanese snakes, probably due to the use of the casein–trichloroacetic acid precipitation method as a criteria of proteolytic activity, some of them have been demonstrated to act on isolated basement membrane, releasing protein and

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; CD, circular dichroism; TNBS, 2,4,6-trinitrobenzenesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet; HPLC, high-performance liquid chromatography; $NaDodSO_4$, sodium dodecyl sulfate; HT-*e*, hemorrhagic toxin *e*; TIU, trypsin inhibitor units.

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