Human Pancreatic Enzymes: Purification and Characterization of a Nonelastolytic Enzyme, Protease E, Resembling Elastase†

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ABSTRACT: An enzyme with proteolytic activity has been isolated from activated extracts of human pancreatic tissue. The purification procedure included salt fractionation followed by ion-exchange chromatography on SE-Sephadex C-25 and on DEAE-Sephadex A-50. The homogeneity of this enzyme, designated protease E, was demonstrated by disc electrophoresis and by sedimentation equilibrium centrifugation studies. The homogeneous enzyme shows the ability to hydrolyze many of the conventional synthetic substrates used for the identification of elastase activity; however, it demonstrates no significant elastolytic activity. A comparison of human protease E with porcine elastase reveals a high degree of similarity between the two proteases with respect to inhibition by active-site directed peptide chloromethyl ketones, stability, decreased susceptibility to naturally occurring proteinase inhibitors, and specificity for synthetic substrates as well as several other physical properties. The major difference between human protease E and porcine elastase, other than the lack of elastolytic activity by human protease E, seems to be in the ionic character and the amino acid composition of these two proteins. Porcine elastase is a cationic enzyme, while human protease E appears to be anionic in nature. These dissimilarities concerning elastolytic activity and ionic character appear to be directly related.

Elastase, by definition, is a proteolytic enzyme which has the ability to digest elastin, as well as other protein substrates. This elastin-digesting capability sets it apart from other pancreatic endopeptidases in that it is the only enzyme with such a function. Since the initial report by Balo and Banga (1950) concerning the elastolytic activity of porcine pancreatic tissue, a wealth of information has been obtained on porcine elastase (Mandl, 1962; Shotton, 1970; Hartley and Shotton, 1971).

Conflicting reports as to whether an elastolytic enzyme is secreted from the human pancreas have been made over the past several years (Hall *et al.*, 1952; Lamy and Tauber, 1963; Trowbridge and Moon, 1969). However, Trowbridge and Moon (1972) reported that a purified preparation of human pancreatic elastase had been obtained by salt fractionation of homogenized pancreatic tissue followed by adsorption of the enzyme on powdered human elastin. Detailed characterization of this molecule, however, was not accomplished. Clemente *et al.* (1972), employing immunological techniques and synthetic substrates, observed the presence of two protein components with elastase-like esterase activity in activated human pancreatic juice.

Feinstein *et al.* (1974) have recently reported the partial purification and characterization of two human pancreatic enzymes which demonstrate $Ac(Ala)_3OMe$[1] esterase activity. They have termed these two enzymes "elastases."

In our laboratory, using activated extracts of human pancreatic tissue, we have also been able to detect two enzyme components which hydrolyze $Ac(Ala)_3OMe$. One of these components, however, has no appreciable elastolytic activity. It does nevertheless have many striking properties in common with porcine pancreatic elastase. The purification and characterization of this enzyme, which we have called human protease E, are reported in this communication.

Experimental Section

Materials

Human pancreas were obtained quick frozen at autopsy from St. Joseph's Hospital, Marshfield, Wis., and Athens General Hospital, Athens, Ga. Pipes and elastin-orcein were purchased from Calbiochem. Cyclo Chemical Company provided CAP and TBAP. Powdered bovine neck ligament elastin, Congo Red-elastin, Tos-Lys-CH_2Cl, and Tos-Phe-CH_2Cl were products of Sigma Chemical Company. Chicken ovomucoid, soybean trypsin inhibitor, lima bean trypsin inhibitor, Kunitz bovine pancreatic trypsin inhibitor, and chromatographically purified porcine elastase were obtained from Worthington Biochemical Company. iPr_2FP was a product of Aldrich Chemical Company. DEAE-Sephadex A-50 as well as SE-Sephadex C-25 were from Pharmacia Fine Chemicals. $Ac(Ala)_3OMe$ was obtained from Miles Research Laboratories. Hippuryl-L-arginine and hippuryl-L-phenylalanine were purchased from Mann Research Laboratories. Bz-Leu-OEt was a gift of Dr. J. Folk,

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[1] Abbreviations used are: iPr_2FP, diisopropyl phosphorofluoridate; Tos-Lys-CH_2Cl, 1-chloro-3-tosylamido-7-aminoheptanone; Tos-Phe-CH_2Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) monosodium monohydrate; BzArgOEt, *N*-benzoyl-L-arginine ethyl ester; BzLeuOEt, benzoyl-L-leucine ethyl ester; TosArgOMe, toluenesulfonyl-L-arginine methyl ester; $Suc(Ala)_3NA$, succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide; $Ac(Ala)_3OMe$, acetyl-L-alanyl-L-alanyl-L-alanine methyl ester; Z-Ala-NP, carboxybenzoyl-L-alanine *p*-nitrophenyl ester; *t*-Boc-Ala-NP, *tert*-butyloxycarbonyl-L-alanine *p*-nitrophenyl ester; AcTyrOEt, acetyl-L-tyrosine ethyl ester.

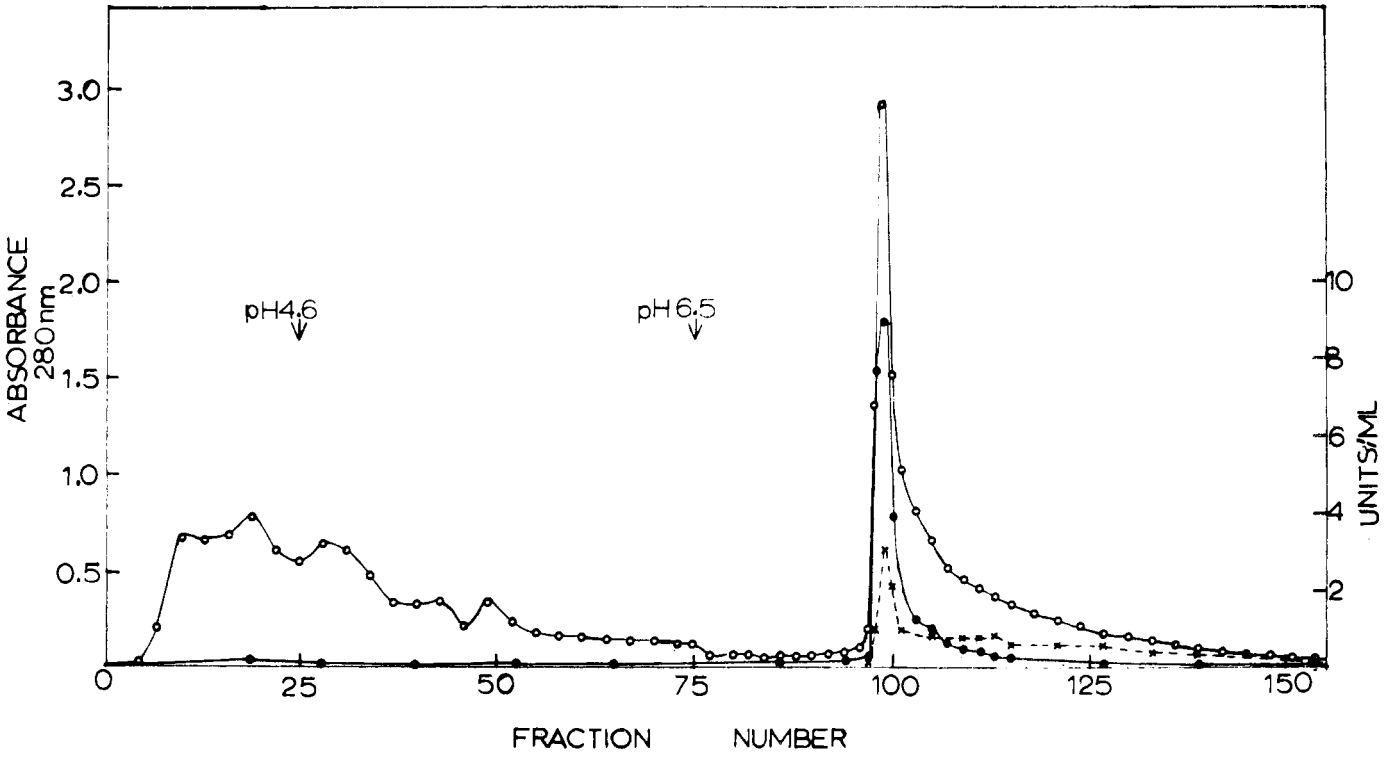

FIGURE 1: SE-Sephadex C-25 chromatography of activated acetone powder extracts of human pancreatic tissue. The column was equilibrated with 0.005 M Pipes-HCl (pH 6.5) containing 0.025 M $CaCl_2$. The column was alternately washed with 0.005 M acetic acid–0.025 M $CaCl_2$ (pH 4.6) and 0.005 M Pipes-HCl–0.025 M $CaCl_2$ (pH 6.5) as indicated. Column dimensions, 1.7 × 28 cm; flow rate, 20 ml/hr; fraction size, 5 ml. Curves are designated as follows: optical density at 280 nm (O—O), left ordinate, activity against BzArgOEt, (●—●), right ordinate; activity against Z-Ala-NP (x- - - -x), right ordinate.

National Institutes of Health, Bethesda, Md., and Suc-$(Ala)_3$-NA was a gift of Dr. J. Bieth, Civil Hospital, Strasbourg, France. Porcine pancreatic secretory trypsin inhibitor was a gift of Dr. L. J. Greene, Brookhaven National Laboratory, Upton, N.Y. The Bowman–Birk soybean trypsin inhibitor was donated by Dr. I. E. Liener, University of Minnesota, St. Paul, Minn. The five active-site specific inhibitors of porcine elastase were generously provided by Dr. J. C. Powers, Georgia Institute of Technology, Atlanta, Ga. Homogeneous α-1-antitrypsin was prepared in our laboratory (Travis and Pannell, 1973). All other reagents were of analytical grade obtained from various commercial sources.

Methods

Enzyme Assays. Elastase esterolytic activity was measured spectrophotometrically at room temperature using Z-Ala-NP or *t*-Boc-Ala-NP in 0.05 M Pipes (pH 6.5) (Visser and Blout, 1972). One unit of activity was defined as an absorbance change of one optical density unit per minute at 347.5 nm. Specific activity was calculated as units of esterase activity per milligram of protein. Ac$(Ala)_3$OMe and Suc-$(Ala)_3$-NA were assayed by the method of Bieth and Meyers (1973) and Bieth *et al.* (1974), respectively. Protein concentration was determined spectrophotometrically by the method of Warburg and Christian (1942). For purified preparations of enzyme a specific extinction coefficient of 24.5 (1% solution, 280 nm), as determined in this paper, was utilized. Proteolytic activity was measured by the casein hydrolysis method of Kunitz as described by Laskowski (1955). Elastin digestion assays were routinely performed by the method of Gertler and Birk (1970).

Inhibition Studies. Inhibition experiments using naturally occurring proteinase inhibitors were carried out by mixing various quantities of inhibitor with enzyme in 0.05 M Tris-HCl (pH 8.0) containing 0.05 M $CaCl_2$. After incubation for 45 min at 25°, the mixtures were assayed for proteolytic activity as described above. The enzyme concentration used was 10^{-6} M.

Inhibition by peptide chloromethyl ketones was determined spectrophotometrically with *t*-Boc-Ala-NP. The appropriate inhibitor was dissolved in 0.1 M Pipes (pH 6.5) containing 10% (v/v) methanol prior to incubation with enzyme at 25°. Aliquots of the incubation mixture were removed at regular intervals for assay. The concentrations of inhibitor and enzyme were 10^{-4} and 10^{-5} M, respectively.

Polyacrylamide Electrophoresis. Acid and alkaline disc electrophoresis were carried out by the procedure of Brewer and Ashworth (1969). The gels were 7.5% acrylamide with a running pH of 2.3 and 8.3, respectively.

Sodium Dodecyl Sulfate Gel Electrophoresis. Protein solutions were electrophoresed in 10% acrylamide gels after incubation in sodium dodecyl sulfate solutions as described by Weber and Osborn (1969). The molecular weight of human protease E was determined using proteins of known molecular weights as standards.

Gel Chromatography. The estimation of the molecular weight of human protease E was performed by chromatography on Sephadex G-100 as described by Whitaker (1963). Standard proteins of known molecular weight were used for comparisons.

Analytical Ultracentrifugation and Amino Acid Analysis. Experiments to determine amino acid composition and analytical ultracentrifuge studies to determine sedimentation coefficient, molecular weight, and extinction coefficient of the protease preparation were carried out as previously described (Mallory and Travis, 1973). For ultracen-

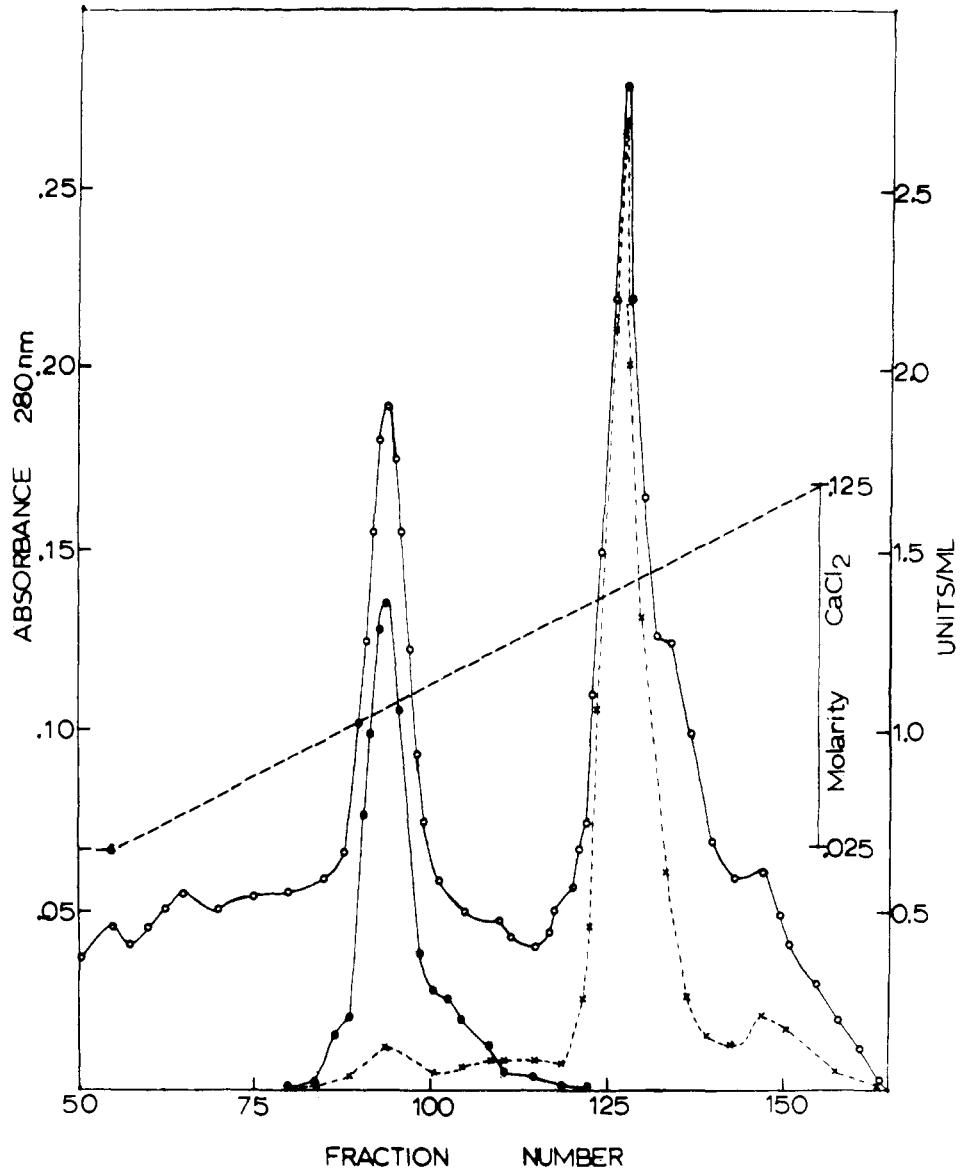

FIGURE 2: DEAE-Sephadex A-50 chromatography of active fractions from SE-Sephadex C-25 column chromatography. The column was equilibrated with 0.005 M Pipes-HCl (pH 6.5), containing 0.025 M $CaCl_2$, and eluted with a linear gradient to 0.125 M $CaCl_2$ as indicated. Column dimensions, 1.7 × 26 cm; flow rate, 20 ml/hr; fraction size, 5 ml. Curves are designated as follows: optical density at 280 nm (O—O), left ordinate; activity against BzArgOEt (●—●), right ordinate; activity against Z-Ala-NP (x- - - -x), right ordinate.

trifugation experiments requiring high protein concentrations, iPr_2FP human protease E was utilized (see Stability).

Results

Purification of Human Protease E. In initial attempts to isolate the anionic trypsin component in activated human pancreatic extracts (Mallory and Travis, 1973), it became apparent that the anionic fraction containing the trypsin activity was also composed of a second enzyme possessing strong proteolytic activity. This protease was insensitive to both Tos-Lys-CH_2Cl and Tos-Phe-CH_2Cl but was rapidly inhibited by iPr_2FP (see Inhibition Studies). Because this second protease had strong elastase esterase activity, it was felt that the purification and characterization of the molecule would be of significant interest.

Unless otherwise stated, all operations were performed at 4°, and aqueous solutions were prepared in double distilled water.

INITIAL PROCEDURE. Human protease E purification followed the experimental conditions for the isolation of human anionic trypsin (Mallory and Travis, 1973). Briefly, 40 g of acetone powder, representing 1000 g of pancreatic tissue, was extracted in 0.01 M HCl (pH 2.6), salt fractionated between 0.2 and 0.8 with solid ammonium sulfate, and dialyzed at alkaline pH against 0.05 M Tris-HCl (pH 8.0) containing 0.05 M $CaCl_2$, to remove salt and initiate activation of zymogens. The details of this procedure have been reported previously (Coan *et al.*, 1971).

SE-SEPHADEX *C*-25 CHROMATOGRAPHY. The activated material obtained after dialysis was adjusted to pH 4.6 with 0.1 M acetic acid, diluted with water to an ionic strength equivalent to that of a buffer consisting of 0.005 M Pipes–0.025 M $CaCl_2$ (pH 6.5) (buffer A), and applied to a column of SE-Sephadex C-25 equilibrated in the same buffer. The column was then washed with 0.005 M acetate buffer (pH 4.5) containing 0.025 M $CaCl_2$, until the A_{280} was less than 0.020. When the column was developed by addition of buffer A, a single protein peak was eluted containing approximately 30–40% of the applied CAP esterase activity (Figure 1) as well as trypsin esterase activity due to anionic

Table I: Purification of Protease E from Human Pancreas.[a]

Fractionation Step	Total Protein (mg)	Total Activity (units × 10^{-2})	Recovery (%)	Specific Activity (units/mg)	Purification	Elastolytic Activity[c]
1. Crude extract	4000	200[b]	100	0.05	1.0	
2. 0.2–0.8 salt fraction	1360	186[b]	93	0.14	2.8	0.83
3. SE-Sephadex C-25 column	81	150[b]	81	1.85	37.0	7.80
4. DEAE-Sephadex A-50 column	3[d]	78	39	26.00	520.0	0.01

[a] Purification based on human protease E esterase activity toward Z-Ala-NP, assayed as described under Methods. [b] Corrected for nonspecific esterase activity due to trypsin and chymotrypsin. [c] Elastolytic activity measured on elastin Congo Red as described under Methods. The activity of pure porcine elastase was taken as 100, after 1-hr digest. [d] Milligrams of protein determined using $E_{280\ nm}(1\%)$ 24.5.

trypsin. The significant loss of esterase units was found to be primarily due to the removal of large amounts of cationic trypsin and chymotrypsin, both of which show considerable Z-Ala-NP esterase activity (Visser and Blout, 1972; Janoff, 1969).

DEAE-SEPHADEX A-50 CHROMATOGRAPHY. The peak tubes of the SE-Sephadex chromatography were pooled and applied to a DEAE-Sephadex A-50 column equilibrated against buffer A. After the passage of inactive unretarded protein, human protease E was eluted by initiation of a linear gradient from 0.025 M $CaCl_2$ to 0.125 M $CaCl_2$, both in 0.005 M Pipes buffer (pH 6.5). As shown in Figure 2, human protease E eluted separately from anionic trypsin at about 0.1 M $CaCl_2$ concentration. The purification scheme is shown in Table I. Because of the instability of anionic trypsin, any delay following SE-Sephadex chromatography resulted in substantially lower amounts of this enzyme whereas the recovery of human protease E was not noticeably affected.

Criteria of Homogeneity and Molecular Weight Studies. Human protease E routinely eluted from DEAE-Sephadex A-50 with constant specific activity. Rechromatography of peak fractions on DEAE-Sephadex resulted in a single peak with constant specific activity identical with the applied material. Additional evidence of the homogeneity of this preparation was obtained by subjecting it to gel filtration on Sephadex G-75. The preparation eluted as a single symmetrical peak with a constant specific activity comparable to that of the applied enzyme.

Analytical disc electrophoresis of human protease E in 7.5% gels at pH 2.3 and 8.3 showed only a single band with no minor contaminants, again indicative of homogeneity (Figure 3).

Sedimentation velocity experiments were performed on iPr_2FP enzyme in 0.005 M Pipes–0.05 M $CaCl_2$ (pH 6.5) at protein concentrations of 10 and 7.5 mg/ml; both resulted in singly symmetrical Schlieren peaks and a sedimentation constant ($s_{20,w}$) of 3.19 S was calculated from these data.

Boundary depletion sedimentation equilibrium experiments were used to calculate the molecular weight of iPr_2FP human protease E. A protein concentration of 0.19 mg/ml in 0.005 M Pipes–0.025 M $CaCl_2$ (pH 6.5) was used with a rotor speed of 40,000 rpm. The molecular weight calculated from these data was 31,059, using a partial specific volume of 0.722 calculated from the amino acid composition. An extinction coefficient ($E_{1\ cm}(1\%)$) of 24.5 was computed from ultracentrifuge studies using interference optics (Babul and Stellwagen, 1969). The extinction coefficient experiments were conducted in 0.005 M Pipes–0.025 M $CaCl_2$ (pH 6.5) using iPr_2FP enzyme concentrations of 1.7 and 2.7 mg/ml.

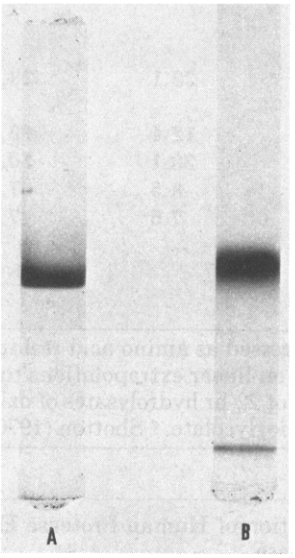

FIGURE 3: Polyacrylamide disc electrophoresis of human protease E (80 μg). Patterns were stained with 1% Amido Schwarz in 7.5% acetic acid: (A) pH 2.3, 7.5% gel, direction of migration is from anode (top) to cathode (bottom); (B) pH 8.3, 7.5%, direction of migration is from cathode (top) to anode (bottom).

Sodium dodecyl sulfate gel electrophoresis of iPr_2FP human protease E was performed after incubation in sodium dodecyl sulfate solution in order to determine both homogeneity and molecular weight. A single component was detected and a molecular weight of 30,500 was estimated using proteins of known molecular weights as standards. The molecular weight of human protease E as estimated by gel filtration chromatography on Sephadex G-100 was 30,000.

Amino Acid Composition. The amino acid composition of human protease E is presented in Table II. These data were calculated from analysis after 22-, 48-, and 72-hr hydrolysis of 1-mg samples of enzyme. The number of residues of each amino acid present was based on an assumed value of 20 residues of alanine per molecule of protein. A molecular weight of 30,792 was calculated from these data. The amino acid compositions of porcine elastase (Shotton, 1970) and the human "elastase 1" isolated by Feinstein *et al.* (1974) are also presented in Table II for comparison.

Stability. Human protease E was quite stable in dilute solutions of protein concentrations less than 0.4 mg/ml within a pH range of 4.0–8.0. No loss of activity could be

Table II: Amino Acid Composition[a] of Human Protease E and Other Related Proteases.

	Human Protease E					Other Related Proteases	
	Time of Hydrolysis						
Amino Acid	22 hr	48 hr	72 hr	Value Taken	Nearest Integer	Porcine Elastase[e]	Human "Elastase 1"[f]
Lysine	8.2	9.0	11.1	9.4	9	3	8
Histidine	6.7	8.1	7.4	7.4	7	6	6
Arginine	10.9	9.6	10.0	10.0	10	12	10
Aspartic acid	28.5	27.4	29.4	28.4	28	24	25
Threonine	16.1	15.4	14.9	16.4[b]	16	19	14
Serine	23.9	23.8	23.6	24.2[b]	24	22	16
Glutamic acid	24.2	25.1	24.1	24.4	24	19	21
Proline	18.3	15.2	15.8	17.4	17	7	16
Glycine	31.3	35.9	38.4	35.2	35	25	26
Alanine				20.0	20	17	16
Half-Cystine				15.3[c]	15	8	16
Valine	22.1	22.9	24.9	25.3[b]	25	27	24
Methionine				0.9[c]	1	2	0
Isoleucine	12.4	12.9	13.3	13.7[b]	14	10	10
Leucine	20.1	20.6	20.9	20.5	21	18	17
Tyrosine	8.5	7.6	7.3	7.8	8	11	7
Phenylalanine	7.6	7.1	6.4	7.1	7	3	6
Tryptophan				9.0[d]	9	7	n.d.
				No. of residues	290	240	238
				Mol wt	30,792	25,900	25,050

[a] Data are expressed as amino acid residues/molecule, assuming 20 alanines/molecule of protein. [b] Threonine and serine values are based on linear extrapolations to zero time, and valine and isoleucine values are based on linear extrapolation to 96 hr. [c] Averages of 22-hr hydrolysates of oxidized samples. [d] Tryptophan determined by average of 22-hr hydrolysates in the presence of 4% thioglycolate. [e] Shotton (1970). [f] Feinstein *et al.* (1974).

Table III: Inhibition of Human Protease E[a] with Active-Site Specific Peptide Chloromethyl Ketone Inhibitors of Porcine Pancreatic Elastase.

					Relative Reactivity[c]	
Inhibitor	pH	$[I] \times 10^4$	$10^3 K_{obsd}$[b] (sec^{-1})	$K_{obsd}/[I]$ (M^{-1} sec^{-1})	Human Protease E $K_{obsd}/[I]$ (rel)	Porcine[d] Elastase $K_{obsd}/[I]$ (rel)
Ac-Ala-Ala-$AlaCH_2Cl$	6.5	10	0.7	7	1.0	1.0
Z-Gly-Leu-$AlaCH_2Cl$	6.5	10	7.5	75	10.7	1.6
Ac-Ala-Ala-Ala-$AlaCH_2Cl$	6.5	10	20.4	204	34.6	18
Ac-Ala-Ala-Pro-$AlaCH_2Cl$	6.5	10	61	610	88	71
Ac-Ala-Ala-Phe-$AlaCH_2Cl$	6.5	10	13.8	138	19.7	16

[a] Human protease E concentration: 10^{-5} M, all enzyme assays were performed using *t*-Boc-Ala-NP as described under Methods. Kinetic constants calculated by the method of Powers and Tuhy (1973). [b] Averages of at least four runs. Controls containing no inhibitor exhibited no significant decrease in esterolytic activity. [c] Relative reactivity assuming Ac-Ala-Ala-$AlaCH_2Cl$ is equal to 1.0. These values for relative comparison only (see Discussion). [d] These constants were taken from Powers and Tuhy (1973) and were calculated by Powers and Tuhy (1973) for pH 6.5 from original inhibition studies at pH 5.0.

detected after 3 months, even at 24°. Below pH 4.0, however, the enzyme gradually lost activity, presumably due to irreversible denaturation. At protein concentrations above 0.4 mg/ml, rapid autolysis occurred; therefore, for experiments requiring high protein concentrations, iPr_2FP enzyme was employed.

The pH optimum for protease E was determined on casein substrates using the following buffers: 0.1 M sodium citrate (pH 2.0–5.5), 0.1 M Pipes–HCl (pH 6.0–7.7), 0.1 M Tris-HCl (pH 7.2–8.0), and 0.1 M sodium carbonate (pH 7.7–10.5). The pH optimum for casein hydrolysis was between pH 7.7 and pH 9.5.

Effect of Inhibitors on Human Protease E. SYNTHETIC INHIBITORS. In order to determine the degree of similarity between porcine elastase and human protease E, several synthetic active-site directed reagents designed specifically to inhibit porcine elastase were tested (Powers and Tuhy, 1973). The data were processed using a least-squares computer program and the results are listed in Table III. All of the compounds tested were found to be strong inhibitors of human protease E at pH 6.5.

NATURALLY OCCURRING INHIBITORS. Human prote-

Table IV: Inhibition Spectrum of Human Protease E[a] (Expressed as % Proteolytic Activity Remaining).

Inhibitor	Molar Ratio Inhibitor : Enzyme 1:1	5:1	10:1
Soybean trypsin	83	64	59
Lima bean trypsin	96	91	85
Kunitz bovine pancreatic trypsin	60	55	39
Chicken ovomucoid	100	96	89
Porcine pancreatic secretory trypsin	95	91	85
Bowman–Birk (soybean)	96	94	86
Human serum α_1-antitrypsin	0		

[a] Assays for inhibition of proteolytic activity were performed at pH 8.0 as described under Methods.

Table V: Relative Proteolytic Activity[a] of Several Human and Porcine Pancreatic Proteases.

Protein Substrate	Relative Activity: Porcine Elastase	Porcine Trypsin	Human Protease E	Human Trypsin
Casein	100		98	108
Undyed elastin[b]	100	0.50	1.50	0.70
Elastin–Orcein[b]	100	0.28	0.66	0.04
Elastin Congo Red[b]	100	0.10	0.17	0.06

[a] Porcine elastase has been assigned a value of 100. [b] Assay procedure as described by Shotton (1970). Values obtained from 8-hr incubation of protease with substrate.

ase E was also tested against a wide range of naturally occurring inhibitors including soybean trypsin inhibitor, lima bean trypsin inhibitor, Kunitz bovine pancreatic trypsin inhibitor, chicken ovomucoid, porcine Kazal inhibitor, Bowman–Birk (soybean) trypsin inhibitor, and α-1-antitrypsin. With the notable exception of α-1-antitrypsin, all the naturally occurring inhibitors tested showed a surprising lack of inhibition even at inhibitor–enzyme molar ratios of 10:1. These results are summarized in Table IV. Although detailed results have not been published, porcine elastase has reportedly little if any susceptibility to chicken ovomucoid (Mandl, 1962), pancreatic trypsin inhibitor (Mandl, 1962), or the Bowman–Birk soybean inhibitor (Gertler and Birk, 1970). Human serum at 1:100 dilutions, however, has been found to inhibit porcine elastase from 50 to 90%, while human protease E (0.1 mg/ml) is totally inhibited by a 1:100 dilution of human serum. In particular, human protease E and porcine pancreatic elastase have both been shown to be subject to complete inhibition by α-1-antitrypsin even at 1:1 molar ratios of inhibitor to enzyme.

Substrate Specificity and Kinetic Parameters. Human protease E was tested against three synthetic substrates which are commonly used to measure elastase activity. The human enzyme was found to have strong activity toward Z-Ala-NP and *t*-Boc-Ala-NP. The specific activity of human protease E toward the former was 25.8 as compared to 26.9 for porcine elastase. The K_m value for the latter substrate using human protease E was determined from eight different substrate concentrations (ranging from $2.0K_m$ to $0.1K_m$)) to be 1.66×10^{-4} M. The K_m value for this substrate using porcine elastase was reported by Visser and Blout (1972) to be 3.0×10^{-4} M. $Ac(Ala)_3OMe$, a highly specific substrate for porcine elastase (Gertler and Hofmann, 1970), was also hydrolyzed by human protease E but at only 25% the rate of that for porcine elastase. Human protease E was also tested against $Suc(Ala)_3NA$, another highly specific substrate for porcine elastase (Bieth *et al.*, 1974). Although the substrate was hydrolyzed, the rate was only 7.5% of that of porcine elastase. This was, however, 500 times greater than the rate of substrate hydrolysis by porcine chymotrypsin and 1000 times greater than hydrolysis by porcine trypsin (Bieth *et al.*, 1974).

Human protease E showed no detectable activity toward BzArgOEt or TosArgOMe, both synthetic substrates for trypsin or toward AcTyrOEt or BzLeuOEt which are used to detect human and bovine chymotrypsin and bovine chymotrypsin C activity, respectively. In addition, no carboxypeptidase A or B activity could be measured using hippuryl-L-phenylalanine and hippuryl-L-arginine as substrates.

Strong proteolytic activity against casein and hemoglobin was observed with human protease E representing 98% of that shown by porcine elastase when equal milligram quantities of both proteases were tested on these same substrates.

Assays for Elastolytic Activity. Using the procedure as described by Shotton (1970), Congo Red-elastin, orcein-elastin, and powdered bovine elastin were used to assay for elastolytic activity. Although a strong elastolytic activity was detected in the initial steps of the purification procedure, the protein which we have isolated has no significant elastolytic activity. In addition to the above procedures, the elastin-plate assay as described by Sbarra *et al.* (1960) also yielded negative results. The adsorption of human protease E on elastin was measured by the method of Gertler (1971). Using an assay mixture containing 0.125 mg of human protease E in 4.0 ml of 0.1 M borate buffer (pH 8.8), we found that less than 1% of the CAP esterase activity of human protease E was adsorbed on 20 mg of elastin. In identical experiments with porcine elastase, 96% of the Z-Ala-NP esterase activity was adsorbed on elastin and complete digestion occurred within 2 hr.

In order to ascertain the absolute elastolytic activity of human protease E as related to other pancreatic proteases, equal microgram quantities of porcine elastase, porcine trypsin, human protease E, and human trypsin were tested against a variety of elastin substrates. The results of this experiment are shown in Table V. Although human protease E does demonstrate trace activity against the elastin substrates tested, this activity would appear to be insufficient to justify calling this enzyme an elastase.

Discussion

Previous communications regarding the properties of human pancreatic proteolytic enzymes have suggested that there are two elastases present in activated extracts of human pancreatic tissue and in activated human pancreatic juice (Feinstein *et al.*, 1974; Clemente *et al.*, 1972). In our laboratory, however, we have been able to detect only one protein component which possesses elastolytic activity. Yet, we have resolved two proteases which show strong elastase-like esterase activity. The more anionic of these components we have called human protease E. Although this enzyme does have many properties in common with the well-charac-

Table VI: Comparative Properties of Human Protease E and Porcine Elastase.

Property	Human Protease E	Porcine Elastase[a]
$s_{20,w}$	3.19 S	2.60 S
Molecular weight		
(a) Sed. equilibrium	31,059	25,000
(b) Amino acid analysis	30,792	25,900
Extinction coefficient (E_{1cm} (1%))	24.5	23.6
pH optimum	7.7–9.5	8.8
K_m (*l*-Boc-Ala-NP)	1.66×10^{-4} M	1.6×10^{-4} M
Stability		
(a) Below pH 4.0	Slowly inactivated	Slowly inactivated
(b) Between pH 4.5 and 8.0	Stable	Stable
(c) Above pH 8.5	Autolysis occurs	Autolysis occurs

[a] Hartley and Shotton (1971).

terized porcine elastase, the ability of this protease to digest native insoluble elastin is almost undetectable.

The protease which we have isolated, besides having a unique specificity for the synthetic substrates of porcine elastase, is also inhibited by peptide chloromethyl ketones which have been demonstrated to be specific for porcine elastase (Powers and Tuhy, 1973). It is not inactivated by Tos-Lys-CH_2Cl or Tos-Phe-CH_2Cl, potent inhibitors of trypsin and chymotrypsin, respectively, but is, presumably, a serine protease since it is completely inhibited by iPr_2FP in 25 min when present at an inhibitor-enzyme molar ratio of 10:1. Furthermore, preliminary experiments using insulin A chain indicate that human protease E possesses a fairly broad specificity which is not unlike that of the porcine enzyme. In an earlier report from this laboratory, Gates and Travis (1973) demonstrated that human protease E (referred to in their publication as human pancreatic neutral protease) hydrolyzed the Leu-Ala and Gly-Ser linkages of an active site peptide isolated from shrimp trypsin.

It is interesting that the principal differences between human protease E and porcine elastase are in overall ionic characters and amino acid composition. The human enzyme is somewhat larger in size and the additional peptide sequence may be responsible for both dissimilarities. Of principal importance to elastolytic activity is the fact that whereas human protease E is an anionic protein, porcine elastase with an isoelectric point of 9.6 (Shotton, 1970) is a strongly cationic molecule. Gertler (1971) has shown that for a protease to digest elastin it must satisfy two requirements. First, the enzyme must be basic enough to be adsorbed on elastin. Second, it must have a side-chain specificity directed toward nonpolar, nonaromatic amino acids which form the bulk of the amino acids of elastin. Although human protease E appears to have the appropriate specificity, it is obvious that, because of its more anionic character, this protein would be adsorbed poorly, if at all, on elastin. Thus, it is not surprising that human protease E demonstrates no significant elastolytic activity. This type of phenomenon has been well documented through several studies employing the alkaline protease from *Aspergillus sojae* (Gertler and Hayashi, 1971; Gertler, 1971).

Human protease E and porcine elastase have a number of properties in common other than substrate specificity and inhibition by specific active-site directed reagents. For example, (a) both are quite stable at neutral or slightly alkaline pH but are unstable below pH 4.0, (b) neither enzyme shows strong susceptibility to naturally occurring proteinase inhibitors but each is totally inhibited by human serum α-1-antitrypsin, (c) both proteins are characterized by unusually high extinction coefficients as well as similar amino acid compositions, (d) both enzymes demonstrate approximately equivalent proteolytic activity when tested against protein substrates other than elastin. A summary of the properties of human protease E and porcine elastase is given in Table VI.

Powers and Tuhy (1972, 1973) were primarily responsible for the development of active-site directed chloromethyl ketone inhibitors specific for porcine pancreatic elastase as well as elastases from other sources. The functional similarity of human protease E and porcine elastase is perhaps best reflected through the relative reactivities of these two enzymes with a number of these specific inhibitors (Table III). This comparison is best facilitated through the $K_{obsd}/[I](rel)$ values listed in Table III. Although no absolute comparison can be established, due to variations in technique and the distortion of $K_{obsd}/[I]$ from nonlinear concentration effects (Powers and Tuhy, 1973), the relative reactivities approximated by $K_{obsd}/[I](rel)$ illustrate several fundamental similarities. All of the inhibitors tested showed strong inhibition of both enzymes. The relative order of effectiveness demonstrated by these inhibitors is identical for both human protease E and porcine elastase, with the tetrapeptide derivatives being better inhibitors than the tripeptide derivatives. The subtle differences observed in the $K_{obsd}/[I](rel)$ values, especially concerning the peptide derivative containing glycine and leucine, confirm the previous specificity studies involving human protease E (Gates and Travis, 1973) which indicates a possible preference for the hydrolysis of peptide bonds involving these two amino acids.

Clemente *et al.* (1972) have reported two "proelastases" after immunoelectrophoresis of human pancreatic juice as assayed by $Ac(Ala)_3OMe$. One of these "proelastases" ("proelastase 1") is an anionic molecule which migrates slightly ahead of anionic trypsin toward the anode. Judging from the elution pattern of human protease E from DEAE-Sephadex (Figure 2), the "proelastase 1" detected by Clemente almost certainly corresponds to the elastase-like enzyme we have isolated.

Feinstein *et al.* (1974), in a recent publication concerning human pancreatic proteases, described the purification and partial characterization of two trypsins and two chymotrypsins previously purified and characterized in this laboratory (Travis and Roberts, 1969; Coan *et al.*, 1971; Coan and Travis, 1972; and Mallory and Travis, 1973). In addition to these, Feinstein and coworkers reported two enzymes which they termed "elastases" by virtue of their esterolytic activity. The authenticity of these "elastases," however, is questionable since true elastolytic activity was not verified by crucial elastin-digestion experiments. Using the procedure of Feinstein *et al.* (1974), we have found that human protease E demonstrates an elution behavior from SE-Sephadex C-25 which is identical with that which Feinstein *et al.* (1974) have reported for their "elastase 1." Obvious consistencies in amino acid compositions confirm the above results and also indicate that the "elastase 1" reported by

Feinstein *et al.* (1974) corresponds to the nonelastolytic human protease E which we have described here. The elastolytic component which we have detected elutes from SE-Sephadex C-25 (using the procedure of Feinstein *et al.*, 1974) following the elution of human protease E and possesses both chymotrypsin-like and elastase-like esterase activity. This enzyme undoubtedly corresponds to the "elastase 2" observed by Feinstein *et al.* (1974) and to the cationic "proelastase 2" that Clemente *et al.* (1972) identified in human pancreatic juice. Both of these previous reports also suggest that this component possesses definite chymotrypsin-like esterase activity in addition to its strong elastase-like esterase activity.

Trowbridge and Moon (1972) reported purification of a human pancreatic elastase by its adsorption on powdered human aortic-elastin. Gertler (1971) demonstrated that the adsorption of porcine elastase and other basic proteins on elastin is nonspecific and electrostatic in nature. Consequently, the elastolytic fraction which Trowbridge and Moon (1972) have obtained undoubtedly contains the more cationic "elastase 2" reported by Clemente *et al.* (1972) and Feinstein *et al.* (1974). Investigations in our laboratory confirm the results of Trowbridge and Moon (1972) in that the elastolytic component which we have observed appears to be a cationic molecule. In the purification procedure reported here, it elutes from SE-Sephadex C-25 with human protease E and anionic trypsin (Figure 1), however, it passes unretarded through DEAE-Sephadex A-50 at pH 6.5. Subsequent investigations have revealed the cationic nature of this elastolytic component in that it can be further purified by gradient elution from DEAE-Sephadex A-50 only above pH 8.0. In addition it demonstrates a strong nonspecific adsorption on elastin followed by rapid digestion of this insoluble protein substrate. This true human pancreatic elastase is currently under investigation in our laboratory.

In the aforementioned investigation by Feinstein *et al.* (1974) of several proteases originally isolated and characterized in this laboratory (Travis and Roberts, 1969; Coan *et al.*, 1971; Coan and Travis, 1972; and Mallory and Travis, 1973), Feinstein and coworkers indicated that the molecular weights (and thus the amino acid compositions) of these pancreatic proteases were distinctly lower than the values originally reported by our laboratory (Feinstein *et al.*, 1974). These conflicting reports prompted a thorough investigation into the molecular weight of human protease E ("elastase 1"). The molecular weight of this elastase-like protease was estimated to be (a) 31,059 as determined by high-speed sedimentation equilibrium experiments using interference optics, (b) 30,817 as a result of low-speed sedimentation equilibrium experiments employing absorption optics, (c) 30,500 based on sodium dodecyl sulfate gel electrophoresis, and (d) 30,000 according to gel chromatography on Sephadex G-100. These independently determined molecular weight values are remarkably consistent and presumably indicate a high degree of reliability and accuracy in the molecular weight which we have obtained for this protease. In contrast to these results, however, the molecular weight reported by Feinstein *et al.* (1974) for "elastase 1" (human protease E) was 24,100. This value is similar to the molecular weights calculated for the other pancreatic proteases by Feinstein *et al.* (1974) in the respect that it is considerably lower than the molecular weight values obtained in this laboratory. The apparent reason for the differences between laboratories concerning molecular weights appears to arise from the low rotor speed used by Feinstein *et al.* (1974) for molecular weight determination. This speed does not appear to be sufficient for equilibrium conditions to be established (Teller, 1973). Therefore, the linear ln c *vs.* r^2 plots which Feinstein *et al.* (1974) have obtained do not indicate true homogeneity. Thus, the sedimentation equilibrium experiments which represent the only reliable criterion for molecular weight estimation employed by Feinstein and coworkers seem to be in error.

Our results indicating only slight inhibition of human protease E by lima bean trypsin inhibitor also may seem to contradict those of Feinstein *et al.* (1974) who used lima bean trypsin inhibitor-Sepharose to isolate proteases from extracts of human pancreatic tissue. However, under their conditions, it is possible that ionic interactions between the elastase esterase enzymes and the lima bean trypsin inhibitor may have been responsible for the binding of these proteins rather than true affinity.

Although human protease E lacks the ability to digest the structural protein elastin, its biological significance as a digestive enzyme appears scarcely altered. The fairly broad specificity demonstrated by human protease E apparently complements the specificities of human trypsin and human chymotrypsin. This, in addition to its decreased susceptibility to naturally occurring inhibitors, indicates that human protease E plays a prominent role in digestive functions.

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Functional Properties of Chemically Modified Hemocyanin. Fixation of Hemocyanin in the Low and the High Oxygen Affinity State by Reaction with a Bifunctional Imido Ester†

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ABSTRACT: Hemocyanin of *Helix pomatia* is a respiratory protein with a molecular weight of 9×10^6; it contains 180 oxygen binding sites. The reaction of hemocyanin with the bifunctional reagent dimethyl suberimido ester, which reacts with amino groups,has been studied. Up to 75% of the amino groups can be modified without inactivation of oxygen binding sites or dissociation of the protein. It appears that hemocyanin can be fixed in a state with low oxygen affinity by modification of the deoxy protein, and in a state with high oxygen affinity by modification of the oxy protein. Using conditions under which native hemocyanin binds oxygen cooperatively (Hill coefficient 2.9), modification of deoxy- and oxyhemocyanin yields derivatives with different oxygen affinities (P_{50} = 10 and 2.2 mm, respectively). Both the deoxy and oxy derivatives show strongly reduced cooperativity (Hill coefficients 1.4 and 1.1, respectively). Modification of oxy- and deoxyhemocyanin subunits (molecular weight one-tenth of the native protein), which bind oxygen noncooperatively, results in derivatives with oxygen binding properties identical with those of unmodified subunits. Parallel experiments have been carried out with a unifunctional reagent, methyl acetoimido ester. Modification of partially oxygenated hemocyanin under conditions at which the protein binds oxygen cooperatively yields derivatives with reduced cooperativity (Hill coefficients 1.1–1.2) and an oxygen affinity depending on the oxygen saturation at which modification had been carried out. The results are consistent with a simple two-state model for the cooperativity of oxygen binding by these giant hemocyanin molecules.

Hemocyanin is a very large respiratory protein which occurs freely dissolved in the hemolymph of the Roman snail, *Helix pomatia*. Its molecular weight is 9×10^6, and it contains about 180 oxygen binding sites. The protein can be dissociated into ten equal subunits (Konings *et al.*, 1969b), each containing a small number of very large polypeptide chains (Brouwer and Kuiper, 1973). Under the right conditions, oxygen binding is cooperative. Evidence has been presented that cooperativity may be interpreted as an oxygen-linked change from a state with low oxygen affinity, predominant at low oxygen saturation, to a state with high oxygen affinity at high oxygen saturation (Er-el *et al.*, 1972; Van Driel, 1973; Van Driel *et al.*, 1974).

The present paper reports the use of a bifunctional, cross-linking agent, dimethyl suberimido ester,[1] introduced by Davies and Stark (1970). This reacts with amino groups, forming amidine groups (Wold, 1967). Extensively modified hemocyanin retains its ability to combine reversibly with oxygen.

The aim of this study was to investigate oxygen linked conformational changes by cross-linking the protein at various degrees of oxygen saturation, and subsequent analysis of the oxygen binding properties of the hemocyanin derivatives obtained.

Materials and Methods

Helix pomatia α-hemocyanin was isolated, stored, and regenerated as described previously (Konings *et al.*, 1969a; Van Driel, 1973).

Protein concentration was determined routinely by measuring the absorbance at 278 nm in 0.1 M borate buffer (pH 9.3) (Heirwegh *et al.*, 1961). Under these conditions, unmodified hemocyanin dissociates into subunits. However,

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[1] Abbreviations used are: DSI, dimethyl suberimido ester hydrochloride; MAI, methyl acetoimido ester hydrochloride; DSI-deoxy-Hc, the product of reaction of DSI with deoxyhemocyanin (regardless of the oxygenation state of the product); likewise, DSI-oxy-Hc, MAI-deoxy-Hc, and MAI-oxy-Hc.