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ISOLATION AND PARTIAL CHARACTERIZATION OF AN ELASTASE-ASSOCIATED ACIDIC ENDOPEPTIDASE AND ITS INTERACTION WITH ELASTASE AT LOW IONIC STRENGTH

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SUMMARY

An acidic endopeptidase has been isolated from dried preparations of swine pancreas by column chromatography on DEAE cellulose. The yield of acidic endopeptidase from 100 g of pancreas powder was 210 mg. The product was found to be electrophoretically homogeneous and produced a single peak in the analytical ultracentrifuge. The sedimentation coefficient ($s^0_{20,w}$), diffusion coefficient ($D_{20,w}$), apparent molecular weight, electrophoretic mobility, $E^{1\%}_{1cm}$ at 280 mμ and pH optimum were respectively, 3.13, $8.7 \cdot 10^{-7}$ cm^2·sec^{-1}, 35 300, $5.9 \cdot 10^{-5}$ cm^2·V^{-1}·sec^{-1}, 14.6 and 10.7. The enzyme was inhibited by diisopropyl phosphorofluoridate, by swine serum and to a lesser extent by human serum. It was not "activated" by $1 \cdot 10^{-3}$ M $FeCl_2$, $MnCl_2$, $MgCl_2$, $CaCl_2$ or $CoCl_2$ or by $1 \cdot 10^{-3}$ M $ZnSO_4$; however, the activity of the enzyme was increased by $1 \cdot 10^{-3}$ M cysteine. The similarity of the acidic endopeptidase to proteolytic enzymes described by other investigators is discussed.

Gradient elution ion-exchange chromatography of water insoluble elastase euglobulin produces two major water soluble components, elastase and the acidic endopeptidase. When distilled water solutions of the purified enzymes were mixed complex formation occurred (precipitation) between the basic protein elastase and the acidic endopeptidase. The formation of the euglobulin when acid extracts of swine pancreas are dialyzed against distilled water is thus explained. Complex formation was maximal at low ionic strength when the enzymes were oppositely charged and was minimal below the isoelectric point of the acidic endopeptidase ($\sim$ pH 4.1) or above the isoelectric point of elastase (pH 9.5). The reaction was essentially abolished at I 0.011. The equivalence point for the reaction was 2.8 mg of elastase : 1 mg of acidic endopeptidase. One mole of acidic endopeptidase is capable of binding approximately four moles of elastase. Although trypsin, another basic protein, was precipitated by the acidic endopeptidase the data suggest a preferential reaction of the latter enzyme with elastase.

INTRODUCTION

Since the discovery of the enzyme elastase (Pancreatopeptidase E (EC 3.4.4.7)) by BALO AND BANGA[1] several methods describing procedures for the isolation of the enzyme from swine pancreas have been published. Of those employing column chromatography, the starting material has been either the euglobulin[2,3] or crystalline elastase[4]. Both of the latter preparations are insoluble in water and the elastase produced, *via* ion exchange chromatography, from either preparation is soluble in water. In addition, gradient elution ion exchange chromatography of these preparations produces an acidic endopeptidase which is also soluble in water. It was reasoned, therefore, that the water insolubility of and, therefore, the formation of elastase euglobulin might be due to the interaction of the basic protein elastase and the acidic endopeptidase. When water solutions of elastase and the acidic endopeptidase were mixed, a euglobulin precipitate was immediately formed showing that the latter premise was indeed correct. Further, the virtual absence of trypsin, which like elastase is a basic protein, in purified elastase preparations has been somewhat of a paradox since the initial stages of the procedure for the extraction of both enzymes from swine pancreas are similar[5]. More importantly, trypsin, like elastase, reacts with the acidic endopeptidase at low ionic strength to produce an insoluble euglobulin.

The present report deals with the isolation and partial characterization of the acidic endopeptidase, the reaction of elastase and the acidic endopeptidase in detail and explains the absence of trypsin in purified elastase preparations.

A preliminary account of a portion of this work has been published[6].

MATERIALS AND METHODS

Isolation and partial characterization of the acidic endopeptidase

Pancreatic elastase euglobulin was prepared from 100 g of Trypsin 1-300 (Nutritional Biochemicals), a dried preparation of swine pancreas, according to the method of LEWIS *et al.*[7] with the exception that dialysis was carried out with two successive 16 l volumes of distilled water rather than running tap water.

The euglobulin precipitate was washed four times in cold distilled water, dissolved in 100 ml of 0.07 M carbonate buffer (molar ratio $NaHCO_3$:Na_2CO_3 = 15, in 0.04 M NaCl), pH 8.9, and dialyzed overnight against 16 l of distilled water. This procedure was repeated twice more.

Column chromatography

DEAE cellulose (Reeve Angel Co., Clifton, New Jersey; A23, 0.9/milliequivalent per g) was prepared, adjusted to pH 8.9, washed with starting buffer (carbonate) and poured into 2.1 cm × 80 cm columns as previously described[8,9]. One liter of 0.07 M carbonate buffer (as above), pH 8.9, was passed through the column prior to the addition of 407 mg of elastase euglobulin (dissolved in 200 ml carbonate buffer). It is necessary that the euglobulin solution added to the column be water-clear. Failure to completely dissolve the euglobulin will result in the appearance of elastase in the acidic endopeptidase peak. The chromatograms were developed with a two-chambered gradient (Varigrad; 2 × 400 ml) with 1.07 M buffer (1 M NaCl–0.07 M carbonate) in chamber 2 and 0.07 M carbonate in chamber 1.

The fractions comprising the acidic endopeptidase peak were pooled, dialyzed against 16 l of 0.07 M carbonate buffer and subjected to rechromatography on DEAE cellulose under the above conditions. The peak from the second DEAE run was also subjected to chromatography using a three-chambered gradient system (3 × 400 ml) in which chambers 1, 2, and 3 contained respectively 0.07 M carbonate buffer, 0.17 M NaCl-carbonate (0.1 NaCl — 0.07 M carbonate) and 1.07 M NaCl-carbonate (as above). A single symmetrical peak was produced in both instances. All final products (pooled peaks) were dialyzed against two 16 l volumes of distilled water and lyophilized.

Zone electrophoresis in agar gel

These experiments were carried out as previously described for elastase[10]. Microslides as well as 5 cm × 25 cm photographic plates were used. A 0.2 cm × 4 cm sample slit, which accomodated a 0.1 ml sample (1 mg acidic endopeptidase), was used in conjunction with the photographic plates and a 3 mm sample well for microslides (5 μl samples).

The electrophoretic mobility of the acidic endopeptidase was determined, relative to albumin, from hemoglobin-impregnated agar on microslides as previously described[10]. Immunoelectrophoresis (used in the determination of electrophoretic mobility) was carried out according to the method of SCHEIDEGGER[11].

Ultracentrifugation

The Beckman Model E ultracentrifuge was used to determine the dependence of the sedimentation coefficient ($s_{20,w}$) on concentration and, therefore, the value of the sedimentation coefficient at infinite dilution. Purified acidic endopeptidase was used at concentrations of 1.76, 3.52, and 7.03 mg/ml.

An apparent diffusion coefficient was determined from sedimentation boundary curves as suggested by FUJITA[12] using the equation:

$$(A/H_{max})^2 = 4\pi Dt \qquad (1)$$

where A is the area under the sedimentation boundary curve, H_{max} the height of the maximum ordinate of the same curve, D the diffusion coefficient and t is the time in seconds. The slope of a plot $(A/H_{max})^2$ *vs.* $4\pi t$ is D, the diffusion coefficient[12].

Areas were determined by planimetry (average of three separate measurements), on a 10 × tracing (Model 6 Nikon comparator) of the sedimentation boundary curves* representing frames 7-10 (96–144 minutes). Areas from the tracings were converted to the areas in the cell using the equation:

$$A_{cell.}\ (cm^2) = \frac{A_{planimeter.}\ (cm^2)}{F^2 m_c} \qquad (2)$$

where F is the magnification of the enlarger (10×) and m_c the magnification of the camera lens.

Values obtained for the sedimentation and diffusion coefficients were converted to standard conditions ($s_{20,w}$ and $D_{20,w}$) as described by SCHACHMAN[13].

* The concentration of the acidic endopeptidase was 7.03 mg/ml. The boundaries in frames 7-10 (16 min per frame) were sufficiently removed from the meniscus to permit the superimposition of a solvent baseline. The baseline determination was made following the protein experiment.

Determination of apparent molecular weight

An apparent molecular weight (mol. wt.$_{app}$) was calculated from the well known Svedberg equation:

$$\text{mol. wt.}_{app} = \frac{RTs}{D(1-\bar{v})} \quad (3)$$

using the value for the diffusion coefficient (D) as determined above and the sedimentation coefficient computed from the same run. The partial specific volume, $\bar{v}$, was assumed to be 0.75 ml/g.

The mol. wt. of the acidic endopeptidase was also determined as previously described[8], on Sephadex G-200[14] (Pharmacia) at pH 8.3, I 0.1. A 2.1 cm × 50 cm column was employed. The column was calibrated with 1 ml of human whole serum, which provided the elution positions (V) of α_2-macroglobulin (α_2M; mol. wt. 820 000), γG-globulin (mol. wt. 160 000) and albumin (mol. wt. 69 000). The void volume (V_0) of the column was taken to be the elution position of α_2M. A third point on the plot of V/V_0 *vs.* log mol. wt. was provided by the elution position of elastase (mol. wt. 25 000)[7]. A third and final run was carried out to determine the elution position and subsequently the apparent molecular weight of the purified acidic endopeptidase.

Absorption spectrum

The absorbance of a solution of the acidic endopeptidase (1 mg/ml) in 0.1 acetic acid and Miller Golder buffer[15], pH 10, I 0.1 + 1% NaCl was determined between 230 and 630 mμ. The extinction coefficient, $E^{1\%}_{1cm}$, was determined from the absorbance value obtained at 280 mμ.

pH optimum

The buffers of MILLER AND GOLDER[15], I 0.1, were used. Five ml volumes of 2% hemoglobin (Hb; Nutritional Biochemicals) substrate powder in 0.1 M NaCl were adjusted to the appropriate pH with 0.1 M HCl or 0.1 M NaOH and subsequently diluted to 10 ml with buffer of the same pH. The final hemoglobin concentration was 1%.

The reaction mixture consisted of the following: 2 ml of 1% hemoglobin at the appropriate pH, 0.1 ml of acidic endopeptidase (1 mg/ml in 0.1 M NaCl) and buffer, of the proper pH, to a total volume of 4 ml. The reaction was allowed to proceed for 30 min at 37°, and was stopped with 6 ml of 2.5% trichloroacetic acid. After standing for 1.5 h at room temperature, the reaction mixtures were centrifuged. The absorbance, at 280 mμ, of the supernatants was determined and, after blank correction, was plotted as a function of pH.

An identical procedure was employed for 0.025 M diethylbarbiturate buffers.

Effect of heavy metals and cysteine

The activity of the acidic endopeptidase on hemoglobin was tested in the presence of the following compounds: $FeCl_2$, $MnCl_2$, $CaCl_2$, $CoCl_2$, $ZnSO_4$ and cysteine. The concentration of all compounds tested was $1 \cdot 10^{-3}$ M. The reaction mixture contained the following: 95 μg of acidic endopeptidase, 2 ml of 2% hemoglobin in 0.027 M diethylbarbiturate buffer, pH 8.6, $4 \cdot 10^{-3}$ mmoles of test compound, and diethylbarbiturate buffer to a total volume of 4 ml. The remainder of the procedure was carried out as described above under "pH optimum".

Inhibition by serum and diisopropylphosphorofluoridate (DFP)

Since elastase, trypsin and chymotrypsin are inhibited by proteins in serum

and the fact that all of the above enzymes are DFP sensitive, it was desirable to ascertain if purified acidic endopeptidase shared these properties.

Human or swine serum* was mixed with an equal volume of acidic endopeptidase solution (1 mg/ml) and the proteolytic activity of 0.1 ml of the mixture determined as described under "pH optimum". The pH was 8.6. The percentage inhibition was calculated from absorbance values (280 mμ) of the supernatants (with and without serum) after correction for the blanks.

Inhibition of the acidic endopeptidase by DFP (Aldrich Chemical Co.) was carried out as above with 100 μg of acidic endopeptidase and 200 millimoles of DFP (in 2-propanol) in a total volume of 4 ml.

Proteolytic activity of the acidic endopeptidase. Peptide mapping of digests of the A and B chains of oxidized insulin

For purposes of comparing the activity of the acidic endopeptidase with data published for other endopeptidases, peptide mapping of digests of the A and B chains of oxidized insulin was carried out according to KATZ *et al.*[16]. The reaction mixture(s) contained the following: 12 mg of A and B chain (Mann Research) in 5 ml of 0.1 M NH_4HCO_3, pH 8.0; 0.05 ml of acidic endopeptidase (1.0 mg/ml) was added at the beginning of the experiment and 0.05 ml after 1.5 h. The total incubation time was 3 h at 37°. The digest was lyophilized, rehydrated with 1.2 ml of distilled water and 0.2 ml subjected to peptide mapping on Whatman 3 MM filter paper[16].

Comparative reaction rates

Comparative reaction rates for the hydrolysis of the A chain of oxidized insulin (Mann Research) and denatured hemoglobin (Nutritional Biochemicals) were determined for the acidic endopeptidase and α-chymotrypsin (Worthington, 2 × crystallized). The consumption of 0.1 M NaOH was followed in the pH-Stat (Radiometer). The reaction mixtures contained either 6 mg of A chain or 25 mg of hemoglobin in 2 ml of 0.1 M KCl 0.001 M $CaCl_2$ and 0.04 mg (in 40 μl) of enzyme. The reaction was carried out at pH 8.0 and 37° under an atmosphere of CO_2-free nitrogen. The rate of hydrolysis was determined from the slope(s) of the line produced during the first 3 to 5 min of the reaction.

The activity of the acidic endopeptidase on *N*-benzoyl-L-arginine ethyl ester and *N*-acetyl-L-tyrosine ethyl ester (Worthington) was determined according to SCHWERT AND TAKENAKA[17]. Fifty μg of acidic endopeptidase was used in the determination. Trypsin and chymotrypsin were used at concentrations of 5 μg per run.

Interaction of elastase and the acidic endopeptidase

The acidic endopeptidase and elastase were prepared as described above. Trypsin (5 × crystallized) was a product of the Armour Chemical Co. and was kindly supplied by Dr. W. R. Thomas. It was further purified by a single passage through DEAE cellulose under the conditions used above for elastase.

Elastase activity was determined on orcein-dyed elastin (Worthington) using a modification of the method of SACHAR *et al.*[18]. Tryptic activity was determined using *N*-benzoyl-L-arginine ethyl ester as described by SCHWERT AND TAKENAKA[17]. Protein was determined using the method of LOWRY *et al.*[19].

Equivalence point

This parameter was determined by reacting a constant amount of elastase with

* Dialyzed overnight against 200 volumes of 0.027 M diethylbarbiturate buffer, pH 8.6.

increasing amounts of acidic endopeptidase. The reaction mixture contained the following: 0.2 ml of elastase (0.95 mg/ml in distilled water), graded amounts of acidic endopeptidase (0.115 mg/ml in distilled water) up to 1.0 ml and distilled water to a total volume of 1.2 ml. The final pH was 6.9. The ionic strength was zero (or approaching zero) since distilled water was used. The reaction does not occur at high ionic strength; hence, in this and subsequent experiments the ionic strength was kept as low as possible in order to obtain maximal precipitation. Following 30 min incubation at 0°, the precipitates were removed by centrifugation at 3000 rev. per min for 20 min. The supernatants were used for elastase activity and protein determinations.

The reaction mixtures for the determination of elastase activity[18] contained the following: 0.2 ml of elastase–acidic endopeptidase supernatant, 10 mg of orcein elastin, and 1.8 ml of 0.07 M carbonate buffer, pH 8.9 (molar ratio $NaHCO_3$:Na_2CO_3 15, in 0.04 M NaCl). The reaction was allowed to proceed for 3 h at 37° at which time the tubes were plunged into an ice bath followed by centrifugation (2°) at 3000 rev. per min for 30 min. The absorbance at 590 mμ of the supernatants was determined and, after blank correction, was plotted as a function of increasing amounts of acidic endopeptidase.

The precipitates were pooled, washed 5 times in cold distilled water, dissolved in 0.027 M diethylbarbiturate buffer, pH 8.6, and subjected to zone electrophoresis in agar gel. Following electrophoresis, the gel layers were overlaid with elastin particles in agar for demonstrating elastase activity or impregnated with hemoglobin to demonstrate proteolytic activity. These methods have previously been described in detail[10].

Effect of ionic strength

These reaction mixtures contained the following: 0.1 ml of elastase (2.43 mg/ml in distilled water), 0.1 ml of acidic endopeptidase (2.54 mg/ml in distilled water), an appropriate aliquot of 1 M NaCl and distilled water to a total volume of 1.0 ml. The reaction mixtures were incubated at 0° for 30 min and centrifuged as above. The supernatants (0.2 ml aliquots) were analyzed for elastase activity, as above, and the enzyme activity plotted as a function of ionic strength.

Effect of pH

One mg samples of acidic endopeptidase were dissolved in 2.0 ml of 0.001 M NaOH or 0.001 M HCl. Each sample was adjusted to the appropriate pH with 0.01 M NaOH or 0.01 M HCl and dialyzed overnight (4°) against 1 liter of 0.001 M NaOH or 0.001 M HCl or mixtures of the same to give an ionic strength of approximately 0.001 and a pH between 3.0 and 10.5. Samples between pH 7.0 and 10.5 were dialyzed under an atmosphere of nitrogen to prevent CO_2 absorption.

Following dialysis, a protein determination[19] was made on the contents of each dialysis bag. One ml aliquots of each pair (acidic endopeptidase and elastase of a given pH) were mixed, incubated at 0° for 30 min and centrifuged. The protein content of the supernatants was determined. This data was used in the construction of a histogram showing the quantity of protein precipitated as a function of pH.

RESULTS

Isolation and partial characterization of the acidic endopeptidase

DEAE cellulose chromatography of "euglobulin precipitate"

Fig. 1A illustrates the results obtained when the "euglobulin precipitate" was

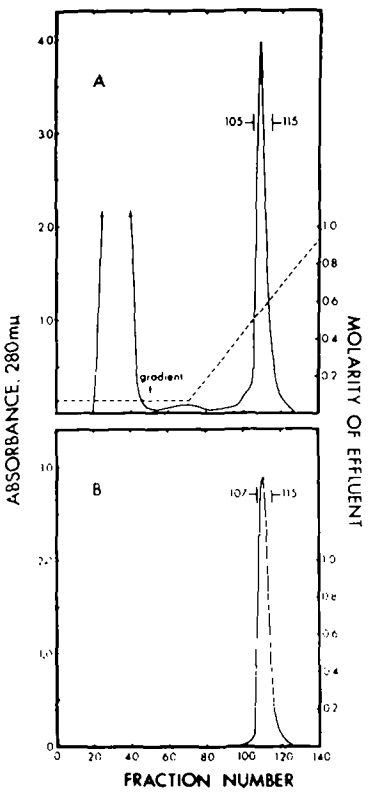


Fig. 1. Chromatographic separation of the acidic endopeptidase on 2.1 cm × 80 cm columns of DEAE cellulose. (A) 407 mg (200 ml) of "euglobulin precipitate." Large breakthrough peak is elastase. Large second peak eluted after addition of gradient is the acidic endopeptidase. Fractions pooled are indicated at upper portion of peak. (B) Rechromatography of peak from (A); fractions pooled are indicated at top of peak. Experimental conditions were identical. The pH throughout was 8.9 (0.07 M carbonate); Gradient, 2 × 400 ml, was begun in both cases at fraction 51 (arrows); flow rate, 60 ml/h, fraction volume 10 ml. The temperature was 4°. See text for details.

subjected to chromatography on DEAE cellulose at pH 8.9. Two major peak areas are apparent. The first peak off the column (breakthrough) is elastase. The broadness of the peak is due to the large volume of sample added. The second major peak is the acidic endopeptidase. The fractions comprising the symmetrical portion of the acidic endopeptidase peak were pooled, dialyzed against starting buffer, and rechromatographed on DEAE cellulose under the same conditions. These results appear in Fig. 1B. It can be seen that a single symmetrical peak was produced. The fractions comprising this peak were pooled, as indicated, dialyzed against distilled water and lyophilized.

The yield of the acidic endopeptidase from Fig. 1B was 109 mg and the calculated yield of the enzyme from 100 g of Trypsin 1-300 was 210 mg.

Zone electrophoresis in agar gel

The "euglobulin precipitate" which was used above as a source of the acidic endopeptidase is used also in the purification of elastase. Three examples of potential

sources of the acidic endopeptidase and elastase are shown in the hemoglobin impregnated electrophoretograms presented in Fig. 2. Fig. 2A represents the "euglobulin precipitate" which had been dialyzed against water only one time. Fig. 2B presents the results obtained with euglobulin used in the chromatographic separation of the acidic endopeptidase (Fig. 1). Fig. 2C is representative of crystalline "elastase" (Worthington). With the exception of the euglobulin sample represented by the electrophoretogram in Fig. 2B, the other preparations are probably unsuitable for the separation of both elastase and the acidic endopeptidase on a single DEAE column, *i.e.* both crystalline "elastase" and 1 × precipitated euglobulin are multi-component rather than two-component mixtures.

Figs. 2D and 2E are electrophoretograms of purified acidic endopeptidase, one

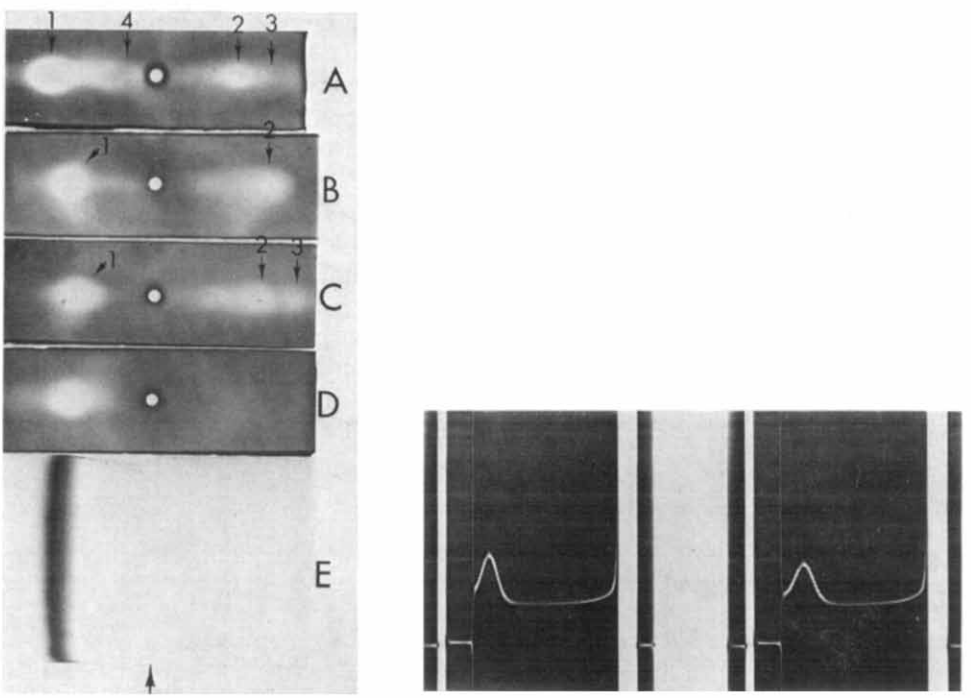


Fig. 2. Zone electrophoretic separation of crude and purified preparations of acidic endopeptidase. (A) "Euglobulin precipitate," 17.4 mg/ml; one precipitation against distilled water; 1, acidic endopeptidase; 2, elastase; 3, trypsin?; 4, chymotrypsin? One percent agar in diethylbarbiturate buffer, pH 8.6, I 0.027; field intensity 9.3 V/cm, 1 h; sample volume 5 μl. Following electrophoresis the agar was immersed in 0.2% hemoglobin (in the above buffer) for 1 h at 37° followed by incubation for an additional 2 h, fixing and staining (Amido black 10B). Zones of clearing are indicative of proteolytic activity. The anode is at the left. (B) "Euglobulin precipitate," 1.0 mg/ml, prepared as outlined in METHODS. Note absence of enzymes other than the acidic endopeptidase and elastase. Other conditions and designations as in (A). (C) Crystalline "elastase." Worthington, 1.8 mg/ml. Other conditions and designations as in (A). (D) Purified acidic endopeptidase, 1 mg/ml. Other conditions and designations as in (A). (E) Agar gel electrophoretograms of purified acidic endopeptidase, 10 mg/ml. Following electrophoresis the agar slide (5 cm × 25 cm) was immersed in acetic acid saturated picric acid, (25:75, v/v), for 30 min followed by drying under filter paper and staining (Amido black); 9.3 V/cm for 1.5 h.

Fig. 3. Ultracentrifuge patterns of purified acidic endopeptidase. Protein concentration 7.05 mg/ml in Miller–Golder buffer, pH 10, I 0.1, + 1% NaCl. First exposure 34 min after reaching 59 780 rev. per min., exposure interval 16 minutes; diaphragm angle 60°.

impregnated with hemoglobin (Fig. 2D) and one which has been immersed in saturated picric acid-20% acetic acid, (75:25, by vol.)[20] and stained with Amido Black 10B. Both electrophoretograms attest to the absence of either extraneous proteolytic activity (Fig. 2D) or contaminating protein (Fig. 2E).

The electrophoretic mobility of the acidic endopeptidase relative to human serum albumin was found to be $-5.9 \cdot 10^{-5}$ $cm^2 \cdot V^{-1} \cdot sec^{-1}$ or exactly the equivalent of human serum albumin.

Ultracentrifugal analysis

These runs were undertaken first to supply additional data on the homogeneity of the purified acidic endopeptidase and secondly to ascertain the effect of concentration upon the sedimentation coefficient.

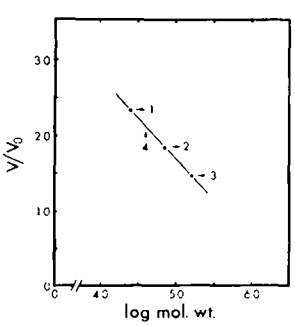
Fig. 3 presents the Schlieren pattern obtained when the acidic endopeptidase was subjected to analysis in the analytical ultracentrifuge at a concentration of 7.03 mg/ml. Only one symmetrical peak was produced.

When the $s_{20,w}$ was plotted as a function of concentration, a horizontal line was produced indicating that the $s_{20,w}$ is not dependent upon concentration. The infinite dilution value ($s^0_{20,w}$), as well as the values of all the experimental points on the plot was $3.13 \cdot 10^{-13}$ sec.

Since the sedimentation coefficient was not dependent upon concentration in this buffer system, it was possible to utilize FUJITA's method[12], *i.e.* equation (1), to determine the diffusion coefficient. The value obtained was $D_{20,w}$ $8.7 \cdot 10^{-7}$ $cm^2 \cdot sec^{-1}$.

Apparent molecular weight

Fig. 4 presents the elution positions of three reference proteins as well as the elution position of the purified acidic endopeptidase from a column of Sephadex G-200. The V/V_0 value for the acidic endopeptidase (Fig. 4, No. 4, arrow) was 2.13 which corresponded to an apparent molecular weight of 39 000.



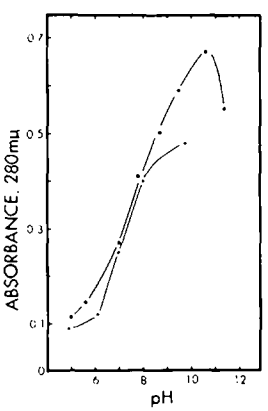


Fig. 4. Determination of the apparent mol. wt. of the acidic endopeptidase by gel filtration on Sephadex G-200. Column dimensions 2.1 cm × 50 cm; flow rate, 20 ml/h; fraction volume, 5 ml, phosphate buffer, pH 8.3, I 0.1, V, elution volume; V_0, void volume. 1, elastase; 2, human albumin; 3, human γG-globulin; 4, acidic endopeptidase. See text for further details.

Fig. 5. Proteolytic activity of the acidic endopeptidase as a function of pH. Substrate, 2 ml of 1% hemoglobin; enzyme concentration 0.1 mg; total volume of reaction mixture, 4 ml. Incubated 30 min at 37°. (●), Miller–Golder buffers, I 0.1; (▲), diethylbarbiturate buffers, I 0.025. See text for details.

The molecular weight calculated from the sedimentation-diffusion data (equations (1) and (3)) was found to be 35 300 which is in good agreement with the value obtained by gel filtration.

pH optimum

Fig. 5 depicts the results obtained when the activity of the acidic endopeptidase is plotted as a function of pH in two different buffer systems. The pH of optimal enzyme activity was approx. 10.7.

"Activation" or apparent inhibition by heavy metals and cysteine

Table I presents the results obtained when various salts of heavy metals and

TABLE I

EFFECT OF HEAVY METALS AND CYSTEINE ON THE PROTEOLYTIC ACTIVITY OF ACIDIC ENDOPEPTIDASE

Molar concentration of all compounds tested are $1 \cdot 10^{-3}$; enzyme concn. in all cases was 95 μg; substrate, 2 ml of 2% hemoglobin in 0.027 M diethylbarbiturate buffer, pH 8.6; total volume of reaction mixture, 4 ml; incubated at 37° for 30 min. See text for details.

Compound tested	*Enzyme activity**
Enzyme alone**	1.00
$FeCl_2$	0.86
$MnCl_2$	0.78
$MgCl_2$	0.98
$CaCl_2$	0.98
$CoCl_2$	0.56
$ZnSO_4$	0.42
Cysteine	1.18

* (A_{280} with test compound)/(A_{280} enzyme alone).
** Enzyme alone gave absorbance at 280 mμ (A_{280}) = 0.55.

cysteine, at concentrations of $1 \cdot 10^{-3}$ M, were tested for their ability to "activate" or diminish the activity of the enzyme. The data indicated that the acidic endopeptidase is apparently not a metal-associated enzyme. Indeed, the activity in the presence of all cations tested was less than with the enzyme alone. Cysteine, however, increased the activity of the enzyme.

Inhibition by serum and diisopropyl phosphorofluoridate

The proteolytic activity of the enzyme was inhibited 37% by human serum and 80% by swine serum. This result is no doubt due to species differences since the source of the acidic endopeptidase was swine pancreas.

The activity of the enzyme was found to be completely inhibited by DFP and is in this respect similar to elastase, trypsin and chymotrypsin.

Absorption spectrum

A typical curve with a single peak between 230 and 300 mμ was obtained when the absorbance of a solution of the acidic endopeptidase was plotted as a function of wavelength between 230 and 630 mμ. The extinction coefficient, $E^{1\%}_{1cm}$, at 280 mμ was 14.6 in 0.1 M acetic acid and 16.6 in Miller Golder buffer, pH 10, *I* 0.1, *plus* 1% NaCl.

Table II summarizes the physicochemical properties of the purified acidic endopeptidase.

TABLE II

SOME PHYSICAL AND CHEMICAL PROPERTIES OF ACIDIC ENDOPEPTIDASE

Constant or property	*Value*
Sedimentation coefficient, $s^{\circ}_{20,w}$	$3.13 \cdot 10^{-13}$ sec
Apparent diffusion coefficient, $D_{20,w}$	$8.7 \cdot 10^{-7}$ cm^2·sec^{-1}
Apparent molecular weight:	
gel filtration	39 900
sedimentation-diffusion	35 300
Extinction coefficient ($E^{1\%}_{1cm}$, 280 mμ) in:	
Miller–Golder buffer, ionic strength 0.1 ; 1% NaCl	16.6
0.1 M acetic acid	14.6
Electrophoretic mobility	$5.9 \cdot 10^{-5}$ cm^2·V^{-1}·sec^{-1}
pH optimum	10.7
Inhibited by:	
human serum	+
swine serum	-
DFP	+
Solubility in water	+
Elastase activity	-

Proteolytic and esterolytic activity of the acidic endopeptidase

The peptide maps of acidic endopeptidase digests of the A and B chains of oxidized insulin are presented in Fig. 6. The results suggest a rather broad specificity for the enzyme and further substantiate its proteolytic nature.

When the proteolytic activity of the acidic endopeptidase was compared to that of α-chymotrypsin, the results embodied in Table III were obtained. It is seen that the rate of hydrolysis of the A chain of oxidized insulin is greater for the acidic endopeptidase than for α-chymotrypsin. The converse was true for denatured hemoglobin. The activity of the enzyme on acetyl-L-tyrosine ethyl ester, a substrate specific for chymotrypsin, was approximately 13-fold less than α-chymotrypsin. This result could reflect a slight contamination of the acidic endopeptidase preparation with chymotrypsin. The acidic endopeptidase did not hydrolyze benzoyl-L-arginine ethyl ester.

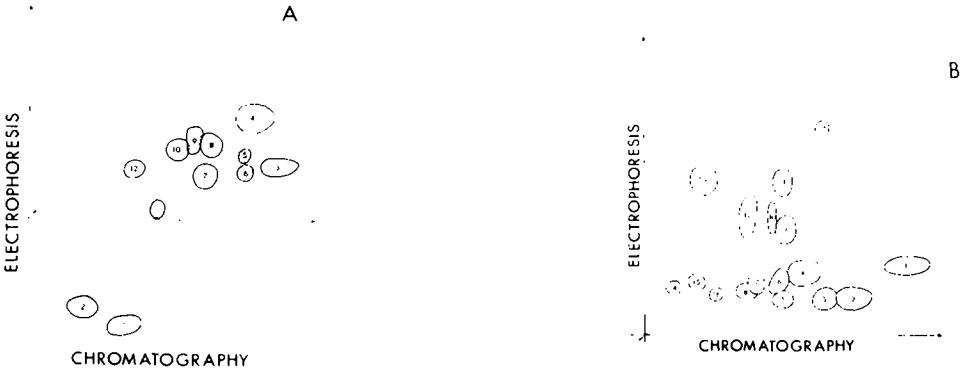


Fig. 6. Peptide maps of acidic endopeptidase digests of the chains of oxidized insulin. (A) A chain; (B) B chain. Substrate to enzyme ratio = 120; approximately the equivalent of 2 mg of initial substrate was placed on the chromatographic-electrophoretic origin (arrows).

TABLE III

COMPARATIVE PROTEOLYTIC ACTIVITIES OF THE ACIDIC ENDOPEPTIDASE AND α-CHYMOTRYPSIN ON VARIOUS SUBSTRATES

Substrate	*α-Chymotrypsin*	*Acidic endopeptidase*
A chain of oxidized insulin	5.24*	7.19*
Hemoglobin	12.40*	9.75*
N-acetyl-L-tyrosine ethyl ester	19.40**	1.45**

* μmoles NaOH consumed per min per mg enzyme.
** Absorbance change (237 mμ) per minute per mg enzyme.

Interaction of elastase and the acidic endopeptidase

When water solutions of the acidic endopeptidase and elastase were mixed a euglobulin precipiate was immediately formed. When the elastase–acidic endopeptidase precipitate was dissolved in buffer and subjected to zone electrophoresis in agar gel, the results shown in Fig. 7 were obtained. This experiment conclusively demonstrates that the precipitate is composed of the two initial reactants (Fig. 7A), elastase and the acidic endopeptidase. In addition, it is apparent that the acidic endopeptidase is devoid of elastase activity (Fig. 7B).

In an effort to quantitate the reaction, a constant amount of elastase was reacted with increasing amounts of acidic endopeptidase. The precipitate was removed by centrifugation and residual elastase activity of the supernatants was determined.

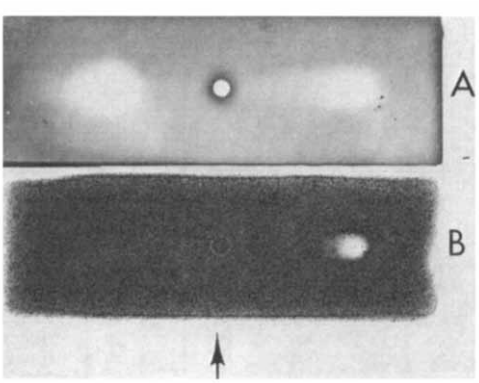


Fig. 7. Direct demonstration of elastase and proteolytic activities in agar gel electrophoretograms of dissolved precipitate (0.027 M diethylbarbiturate buffer) from elastase-acidic endopeptidase reaction mixtures. (A) Impregnated with 0.2% hemoglobin; incubated for 3 h at 37°. (B) Overlaid with 0.5% elastin particles in agar; incubated at 37° for 4 h. Note resolution of precipitate into two proteolytic components in (A) and absence of elastase activity at the position of the acidic endopeptidase in (B). One percent agar in diethylbarbiturate buffer, pH 8.6, *I* 0.027; sample volume, 5 μl; protein concentration, 0.54 mg/ml; 9.3 V/cm for 1 h; arrow indicates electrophoretic origin; the anode is at the left.

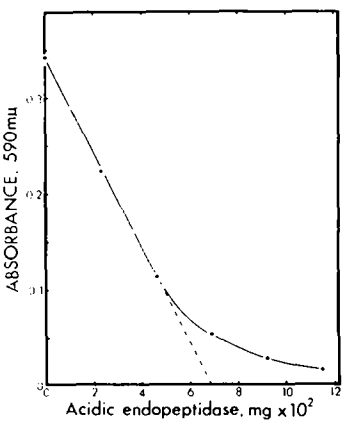


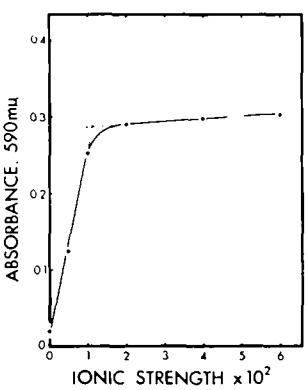
Fig. 8. Elastase activity of supernatant fractions of elastase-acidic endopeptidase reaction mixtures as a function of increasing amounts of acidic endopeptidase. Equivalence point is designated by dashed line from stoichiometric portion of the plot. Please see text for details.

The results of a typical experiment are presented in Fig. 8. It is apparent that a stoichiometric reaction has occurred. The equivalence point for the reaction was 2.8 mg elastase : 1 mg of acidic endopeptidase. Two additional experiments produced values respectively of 2.76 and 2.96. Also, it will be noted that the line produced in Fig. 8 deviates from linearity in the area of the equivalence point. This behavior is probably due to a slight dissociation of the elastase-acidic endopeptidase complex. Efforts to demonstrate soluble complexes in the analytical ultracentrifuge by performing the reaction in an excess of acidic endopeptidase were not successful. Protein determinations on precipitate-free supernatants of elastase-acidic endopeptidase reaction mixtures produced curves which matched the slope of Fig. 8.

The stoichiometry of the reaction (Fig. 8) can conveniently be worked out by consideration of the mol. wt. of the acidic endopeptidase, calculated from the equivalence point (2.8 mg elastase : 1 mg acidic endopeptidase) using 25 000 for the molecular weight of elastase[6]. The mol. wt. calculated for the acidic endopeptidase on the above basis would be 8929 which as the results above have shown is too low by a factor of about four. The stoichiometry of the reaction, therefore, would be 3.95 moles of elastase bound per mole of acidic endopeptidase. ((2.8/25 000)/(1/35 300) or 35 300/8 929.) The "valence" of acidic endopeptidase for elastase would be approximately four.

Since the reaction between elastase and the acidic endopeptidase occurred at low ionic strength it was of interest to determine the ionic strength at which the reaction would be abolished. These results appear in Fig. 9. There is a gradual decrease in the reaction as the ionic strength increases. The reaction is essentially abolished at the intersection of the extrapolated lines in Fig. 9. This ionic strength was 0.011.

The electrophoretograms in Fig. 7 showed that the elastase-acidic endopeptidase precipitate produced at low ionic strength could be resolved into two reactants, one with a negative charge density (acidic endopeptidase) and one with a positive charge



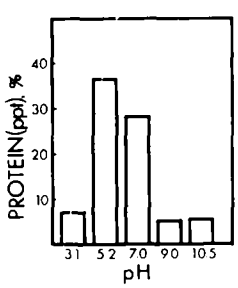


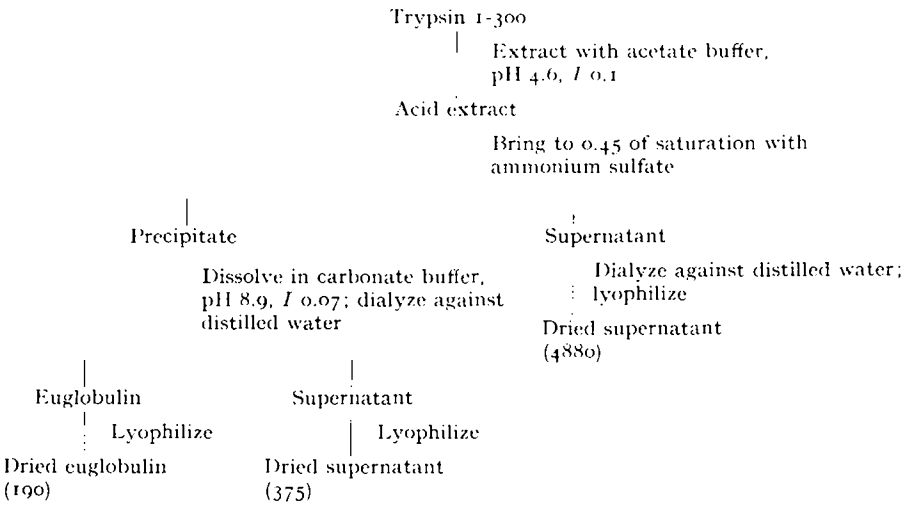
Fig. 9. Interaction of elastase and the acidic endopeptidase as a function of ionic strength. The ordinate (A_{590} mμ) represents the elastase activity of the supernatant fractions of elastase-acidic endopeptidase reaction mixtures following removal of precipitate by centrifugation. The intersection of the dashed lines was found to be I 0.011. Precipitation was minimal above this ionic strength. Please see text for details.

Fig. 10. Effect of pH on the mutual precipitation of elastase and the acidic endopeptidase. Ordinate represents the protein precipitated in elastase acidic endopeptidase reaction mixtures of I 0.001. Please see text for details.

TABLE IV

STEPWISE DEMONSTRATION OF TRYPTIC ACTIVITY IN THE PREPARATION OF ELASTASE EUGLOBULIN FROM DRIED SWINE PANCREAS

Numbers in parentheses refer to the specific activity (units/mg protein) of trypsin. A unit of tryptic activity is defined as the change in absorbance (237 mμ) of 0.001/min at 25°.



density (elastase). It was likely, therefore, that the reaction was electrostatic and was a function of pH. The results presented in Fig. 10 indicate that the reaction was indeed pH dependent, therefore, a function of charge. At a pH below the isoelectric point of acidic endopeptidase (approx. 4)*, both reactants carry the same charge and the reaction is minimal. The same held true for the reaction above the isoelectric point of elastase (9.5; see ref. 7). Where the proteins were oppositely charged (at intermediate pH's), however, the reaction was maximal (Fig. 10).

Trypsin like elastase reacts with the acidic endopeptidase at low ionic strength to produce an insoluble complex (euglobulin). It was of interest, therefore, to examine the initial stages of the extraction procedure for a quantitative evaluation of the specific activity of the products when reacted with BAE. Table IV presents the results of this study. The results show that a total of 5445 units of tryptic activity could be accounted for. 89.6% of the tryptic activity was associated with the ammonium sulfate supernatant, 3.5% with the euglobulin precipitate and 6.9% with the euglobulin supernatant. Further, it is seen (Table IV) that in the presence of elastase only 33.6% of the available trypsin reacted with the acidic endopeptidase. Two additional precipitations of the euglobulin against distilled water produced a product which was negative for tryptic activity.

DISCUSSION

The heterogeneity of crystalline "elastase" and elastase euglobulin preparations

* The enzyme precipitates at pH 4.1, I 0.001.

has been known for many years. Although it has been shown that these preparations contain proteolytic activity in addition to elastase[21,22], the primary focus of attention has been on the elastase moiety to the exclusion of the other enzyme(s) present.

The results of the present study have shown (Fig. 2) that neither pure elastase nor pure acidic endopeptidase can be prepared from once-precipitated euglobulin (Fig. 2A) or crystalline "elastase" (Fig. 2C). These results suggest that the source of pancreas powder and also the method (or care) used in the preparation of the euglobulin or crystalline "elastase" will greatly influence the outcome of the purification procedure. For example, the crystalline "elastase" described by LEWIS *et al.*[23] would probably be suitable for the simultaneous isolation of elastase and the acidic endopeptidase, *i.e.* on a single DEAE column.

The protein designated as "IV" in moving boundary electrophoretograms of crystalline "elastase" by LEWIS *et al.*[23] is probably the same as the acidic endopeptidase isolated here. These authors, however, did not ascribe any enzymatic properties to their protein "IV" rather it was referred to primarily as a "contaminant" or "impurity" found in crystalline "elastase" preparations. The electrophoretic mobility of their protein "IV" was $5.3 \cdot 10^{-5}$ $cm^2 \cdot V^{-1} \cdot sec^{-1}$ (pH 10) as compared to the value of $5.9 \cdot 10^{-5}$ $cm^2 \cdot V^{-1} \cdot sec^{-1}$ found here for the acidic endopeptidase. This similarity plus the zone electrophoretic data presented in Fig. 2 strongly suggest that these components are the same enzyme. The only data which speak against sameness is the finding of LEWIS *et al.*[23] that protein "IV" was insoluble in water. The acidic endopeptidase to the contrary is quite water soluble.

It is also possible that the acidic endopeptidase isolated here is similar or the same as the "esteroproteolytic enzyme" described by GJESSING AND HARTNETT[24] and the enzyme called "chymotrypsin C" by FOLK AND SCHIRMER[25]. All three enzymes are acidic in nature and are inhibited by DFP. The acidic endopeptidase and chymotrypsin C are water soluble while the esteroproteolytic enzyme is not. Both the esteroproteolytic enzyme and chymotrypsin C exhibit an appreciable activity toward acetyl-L-tyrosine ethyl ester whereas the acidic endopeptidase does not (Table III). The esteroproteolytic enzyme showed a strong hydrolytic activity toward poly-L-glutamic acid and this activity was shared by chymotrypsin C. When the acidic endopeptidase was tested on this substrate (mol. wt. 175 000; Mann), in the pH Stat, under conditions used by GJESSING AND HARTNETT[24] (pH 5.3), gel formation occurred causing an appreciable uptake of base. The value obtained for the "specific activity" approximated the constant obtained for the esteroproteolytic enzyme and chymotrypsin C. The same result was obtained with or without enzyme. The results were negative also when acidic endopeptidase "digests" (pH 8.0) of poly-L-glutamic acid were subjected to paper chromatography on Whatman 3 MM filter paper using butanol acetic acid water (4:1:5, by vol.). Other discrepancies which exist between chymotrypsin C and the acidic endopeptidase are their molecular weights (mol. wt. chymotrypsin C 23 800, ref. 25) and the dependency of the sedimentation coefficient of the former enzyme upon concentration. The molecular weight of the esteroproteolytic enzyme reported by MCCONNELL AND GJESSING[26] was 32 800. This value is more in line with the value of 35 300 obtained for the acidic endopeptidase (Table II). The peptide maps of digests of the A and B chains of oxidized insulin produced by chymotrypsin C[27] and the acidic endopeptidase were found to be surprisingly similar (Fig. 6). In addition, paper chromatographic separation of digests (as above) of the peptides,

Leu Val and Leu-Tyr (Nutritional Biochemicals) showed that the acidic endopeptidase was capable of catalyzing the hydrolysis of these bonds. These results are in accord with those obtained for chymotrypsin C[27]. In view of the discrepancies between the three enzymes outlined above it is possible that the association-dissocation of the acidic endopeptidase with elastase during the purification procedure* could have resulted in a partial alteration in its specificity. In addition it must be emphasized that the methods used in the isolation of the enzymes are drastically different. The method described here is based on the ability of the acidic endopeptidase to interact, at low ionic strength, with elastase to produce a two-component euglobulin. Obviously, this is or can be considered to be a crystallization step. FOLK AND SHIRMER[25] when comparing chymotrypsin C with the esteroproteolytic enzyme of GJESSING AND HARTNETT[24] concluded that the latter enzymes might be "different molecular forms of the same enzyme". This appears to be a reasonable appraisal and one which appears to fit the present case.

Homogeneity of the acidic endopeptidase preparation has been demonstrated by at least two criteria, zone electrophoresis in agar gel using two different methods of detection (Fig. 2D and 2E) and the production of a single peak in the analytical ultracentrifuge (Fig. 3). The results with acetyl-L-tyrosine ethyl ester, a synthetic substrate "specific" for chymotrypsin, however, indicated that the acidic endopeptidase preparation could contain as high as 7.5% of chymotrypsin (Table III). The chromatogram in Fig. 1A shows a small shoulder (fraction no. 100) on the leading edge of the acidic endopeptidase peak which is absent in the rechromatographed preparation shown in Fig. 1B. Chromatography of 2 times crystallized swine chymotrypsin under the same conditions (Fig. 1) produced a major peak whose maximum 280 mμ absorbance occurred at fraction 96. It is possible, therefore, that the small shoulder observed on the leading edge of the acidic endopeptidase peak in Fig. 1A is indeed chymotrypsin but it is considered to be most unlikely that this "contamination" would be, if present, of the order of 7.5%. This premise is strengthened and indeed probably eliminated altogether by the absence of proteolytic activity in the α_2-globulin position in Fig. 2D, *i.e.* in the characteristic position of swine chymotrypsin. In addition, no zone is present at this position in Fig. 2E.

Many years have passed since LEWIS *et al.*[7] described the first truly preparative method for the separation of elastase from Trypsin 1-300. The resulting euglobulin or crystalline "elastase" were both insoluble in water and until the present writing, little if anything was known about the chemistry of the euglobulin.

It is apparent from the results presented here (Figs. 7-10) that the formation of elastase euglobulin is the result of the low ionic strength association of the basic protein elastase and the acidic endopeptidase. Since the reaction was maximal when the enzymes were oppositely charged (Fig. 10) and became minimal as the ionic strength increased (Fig. 9), the type of bonding is no doubt electrostatic. Other protein pairs have been shown to exhibit similar properties[28,29].

The equivalence point for the interaction of elastase and the acidic endopeptidase gave approx. 4 moles of elastase per mole of acidic endopeptidase. LEWIS *et al.*[7] found that crystalline "elastase" contained 80% elastase and 20% "contami-

* It is assumed that upon being dissolved in buffer, the euglobulin is dissociated into the elastase and acidic endopeptidase moieties. The possibility that this may not be entirely the case is discussed below.

nant" or in the light of the present work, 20% acidic endopeptidase. In a later paper by LEWIS *et al.*[23] the percentage of acidic endopeptidase ("contaminant") was reported to be as high as 50% in some preparations. The molar ratio for 80:20 and 50:50 mixtures would be respectively about 5:1 and 1.5:1 using 25 000 for the mol. wt. of the elastase[7], and 35 300 (Table II) for the mol. wt. of the acidic endopeptidase. Crystalline elastase is less soluble in buffers than the euglobulin. This greater insolubility of the elastase-acidic endopeptidase complex in crystalline "elastase" could be due to conformational changes brought about by the crystallization from concentrated ammonium sulfate solutions. Under any circumstances, the solubility of elastase and acidic endopeptidase produced from crystalline "elastase" (Worthington), and the "euglobulin" compound formed when water solutions of the enzyme were mixed was no different from the result obtained when the enzymes were derived from the euglobulin.

The physiological implication(s) of the interaction of elastase and the acidic endopeptidase are presently somewhat obscure but may be important. Perhaps a weak association exists even at physiological ionic strength(s). Although soluble complexes could not be demonstrated in the analytical ultracentrifuge direct evidence in favor of such complexes is embodied in Table IV. These results showed that only 34% of the available trypsin in the euglobulin precipitate reacted with the acidic endopeptidase. Such an apparently preferential reaction with elastase could be taken to mean that soluble complexes exist. Other evidence for soluble complexes is the fact that when the euglobulin is subjected to electrophoresis in agar gel, an elongated zone, reminescent of γG-globulin, is produced. The fastest migrating portion of this zone possesses a mobility of the order of $2.8 \cdot 10^{-5}$ cm$^2 \cdot$volt$^{-1} \cdot$sec^{-1} as opposed to $0.9 \cdot 10^{-5}$ cm$^2 \cdot$volt$^{-1} \cdot$sec^{-1} for pure elastase[10].

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