The Purification of the Proteolytic Component of Elastase

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Of the many hypotheses advanced to explain the onset of atherosclerosis, one (Baló & Banga, 1949) implicates the pancreatic enzyme elastase and its inhibitor (Baló & Banga, 1949; Graham, 1958). Since these first claims, interest in elastase has not diminished, although the simplicity of the original concept has been considerably impaired by the realization that not only is the level of the circulating inhibitor lowered in old age, but the elastase content of the pancreas is also decreased (Baló & Banga, 1953). Many studies of the interaction between the protein elastin, or the intact elastic fibre and the enzyme have been reported (Balo & Banga, 1949; Lansing, Rosenthal, Alex & Dempsey, 1952; Hall, Reed & Tunbridge, 1952, 1955).

A number of methods have been described for the preparation of elastase (Baló & Banga, 1950; Banga, 1952; Bagdy & Banga, 1958; Hall & Gardiner, 1955; Hall, 1957; Grant & Robbins, 1957; Lewis, Williams & Brink, 1956). Most of these appear to have certain failings. In all the earlier papers it was realized that the enzyme was not necessarily pure and Bagdy & Banga (1958) record that their preparation is not electrophoretically pure. Lewis *et al.* (1956) claimed electrophoretic homogeneity for a preparation obtained from their 'crystalline' material, but in the authors' Laboratories this also had a polydisperse appearance after paper electrophoresis.

Hall (1953, 1957) showed that at least two active fractions, E_2 and E_1 , were present in crude preparations, and it became clear that two steps were necessary in any preparative procedures, namely: the separation of the active material from the other components of crude pancreas extract and the division of the active material into its two separate entities. Electrophoretic separation proved adequate for both these steps but the yields so obtained were insufficient for kinetic studies on E_2 (Czerkawski, 1958) or studies on E_1 *in vivo* (Saxl, 1957, and unpublished work, 1959); Hall, 1958 and unpublished work, 1959). The present paper reports a method for the bulk preparation of the fraction E_2 in an electrophoretically pure state.

EXPERIMENTAL

Materials

Most of the reagents used were of A.R. grade but a good technical grade of aluminium sulphate proved adequate for

the preparation of the gel, provided that the number of molecules of water of crystallization was known. Dialyses were carried out in Visking cellophan tubing (2 in. diam.) against approx. 3 vol. of resin-demineralized water at 5°.

Pancreas powder. As in previous studies from these Laboratories (Hall, 1955; Hall & Gardiner, 1955; Hall, 1957), a commercial hog-pancreas powder was employed as starting material in preference to fresh pancreas, since this enabled more constant recoveries of activity to be achieved. The source of the powder (Viokase) was Viobin Inc., Monticello, Ill., U.S.A.

Elastin. Ox ligamentum nuchae powder passing a 40 but retained by a 60 mesh/in. sieve was employed throughout. Ground substance and collagen were removed as reported previously (Hall, 1955) by autoclaving with 2% (v/v) acetic acid.

Alumina gel. Aluminium hydroxide in the form of the C-γ gel was prepared by a modification of the method of Willstätter & Kraut (1923). The gel was formed by the addition of the following two reagents: *A*, 90 g. of $(\text{NH}_4)_2\text{SO}_4$ was dissolved in water, 83.1 ml. of aq. NH_3 soln. (sp.gr. 0.88) was added and the whole diluted to 1950 ml.; *B*, 142.7 g. of $\text{Al}_2(\text{SO}_4)_3 \cdot 16\text{H}_2\text{O}$ was dissolved in a little water and the solution diluted to 300 ml. Reagent *A* was maintained at 60° and *B* was added slowly with stirring. After further stirring at 65° for 15 min. the suspension was poured into 18 l. of tap water (soft, calcium and magnesium salts 50 p.p.m.), at room temperature, containing 50 ml. of aq. 2N- NH_3 soln. The gel was allowed to settle overnight and after the supernatant had been removed by decantation, was washed twice with aq. 0.05N- NH_3 soln. Great care was taken to ensure that the gel never became acid, during either its preparation or its subsequent use.

Measurement of protein concentrations in enzyme preparations

Of the numerous methods available, u.v.-absorption at 280 mμ was standardized as the most suitable method, being least dependent on the composition of the protein but having a sensitivity suitable for the measurement of microgram quantities of enzyme.

Elastolysis. The extent of elastolysis in terms of the material passing into solution was measured by means of an interferometer (Claesson, 1946) and expressed as percentage of protein dissolved, on the original air-dried weight of substrate (moisture content of air-dried material 14.7%).

The vessel containing the reaction mixture replaces the adsorption column in the apparatus as originally designed by Claesson (1946). It is thus mounted on the top of the cuvette which lies in one of the light paths of the Rayleigh interferometer, but is separated from it by a filter disk. At frequent intervals throughout the course of the reaction, small samples can be drawn into the cuvette by suction, the increase in refractive index can be measured and then

the samples are returned to the reaction vessel. The refractive-index increments of soluble elastin, NaCl and a soluble polysaccharide do not vary by more than 5%, and since the non-protein content of elastin is less than 5% the error in using a value of 0.018 for the increment is less than 0.5%. Hence the method estimates the total amount of solid passing into solution and replaces laborious gravimetric estimations.

Definition of an elastase unit. The elastase unit (e.u.) is defined as that amount of elastase which dissolves 1 mg. of ox ligament (initial concentration, 5 mg. of solid/ml.) in 3 hr. at 37°, the buffer used being borate, pH 8.7, *I* 0.364. The amount of enzyme is chosen so that not more than 60% and not less than 20% of the initial substrate is hydrolysed, since under these conditions the activity is on the essentially linear central portion of a sigmoid curve. The definition differs considerably from those of other workers (see Discussion).

Electrophoresis. The progress of the purification was followed throughout by high-voltage paper electrophoresis. The apparatus was of the 'sandwich' type, in which the paper (Whatman no. 3 MM) was clamped tightly between the upper and lower surfaces of two Perspex cooling chambers. In every case standard conditions of 16 v/cm., giving a 60 ma current across a paper (55 cm. × 12 cm.) in veronal buffer, pH 8.6, *I* 0.15, were used. Protein constituents were dyed, after fixing by heating at 140° for 60 min., by immersion for 10 min. in a hot (65°) 0.05% solution of Solvay Purple in aq. 0.5% H₂SO₄. Surplus dye was rinsed off with warm water.

Method of purification

Pancreas powder (200 g.) was shaken gently for 15 min. with 1 l. of acetate buffer, pH 4.7, *I* 0.1, and allowed to stand at 5° for 5 hr. This was then centrifuged at 5° at 1890 *g* and the residue extracted for a further 16 hr. with 1 l. of the same buffer, under identical conditions. The supernatants were pooled and the residues discarded. The supernatant, which was a suspension of very fine particles, was centrifuged again at high speed (22 700 *g*) at 5° when a clear golden-yellow liquid was obtained.

First dialysis. The acid extract was dialysed at 5° against 3 l. of water for 3 days, the water being changed at least twice a day. After this time interval, a copious precipitate of euglobulin was formed, and this was removed by centrifuging at 5° at 1890 *g* for 15 min., followed by decantation. The euglobulin was kept at 5° while the supernatant was subjected to further dialysis under the same conditions as those described above. This was repeated daily until precipitation was complete, usually after 2 days of further

dialysis. The final supernatant and the precipitates could be freeze-dried at this stage to give preparations AES and AEI (Fig. 1), their differences in properties (Table 1) indicating that the majority of the proteolytic enzyme was segregated in the euglobulin fraction (AEI). The euglobulins were pooled and suspended in 200 ml. of cold water, the suspension was centrifuged and the supernatant decanted. The wet solid was then resuspended in 500 ml. of water and finely powdered NH₄HCO₃ added with stirring until the whole protein was dissolved. At this stage the solution could be freeze-dried and kept indefinitely at 5°

Fig. 1. Electrophoresis diagrams of fractions of elastase obtained at various stages during the purification of the proteolytic component E₂. Electrophoresis was carried out as described in the text. S, Stationary material adsorbed at point of application; E₁, so-called 'mucolytic fraction' (Hall, 1957); E₂, proteolytic fraction; E₂', fraction preventing synergistic effects apparent when E₁ and E₂ are tested together; A₁–A₄, acidic fractions having no effect on elastolysis; B₁–B₃, basic fractions having no effect on elastolysis.

Table 1. *Elastolytic activity of fractions*

All the values listed were calculated on the dry weight of each sample. Values of the fifth column were determined by the method described in the text.

Fraction	Water content (%)	Nitrogen content (%)	Yield (g.)	Specific activity (e.u./mg.)	10 ³ × Total no. of e.u.
Pancreatic powder	8.9	10.7	182.0	8.5	1560
AES	3.7	11.1	27.0	6.2	168
AEI	13.0	14.7	7.1	86.5	612
SI	12.3	7.5	0.91	22.8	22
E ₂ S	11.0	9.4	1.51	16.3	25
E ₂ I	14.5	14.5	0.40	368.0	145

until required for further purification, or alternatively the solution could be used as such for the next step of the purification procedure.

Adsorption. The solution of euglobulin was made slightly alkaline (pH 7.5-8.0) with aq. 2N-NH₃ soln. and mixed with the C-γ gel. The total volume was brought to 2 l. with water and the suspension shaken gently for 15 min., after which it was allowed to stand at 5° overnight. The suspension was centrifuged and decanted, and the residue of C-γ gel, together with adsorbed elastase, was washed with 200 ml. of cold water. The supernatant was dialysed against tap water for 3 days and freeze-dried to give a fraction S1.

Extraction and second dialysis. The gel was shaken gently with 1.25 l. of 0.2M-Na₂HPO₄ solution, containing enough HCl to give pH 8.0-8.5, and allowed to stand at 5° for 45 hr. with intermittent stirring. The suspension was then centrifuged and the residue washed with 0.75 l. of the same acidified phosphate solution. The supernatant and washings were dialysed under the same conditions as those used in the preparation of the euglobulin fraction from the original acid extract. A precipitate appeared after 2 days, and precipitation was complete after 5 days. The supernatant was freeze-dried to give fraction E₂S and the solids were pooled and washed with a little cold water. The solid was dissolved in 1 l. of M-NH₄HCO₃ soln. and filtered before freeze-drying to give fraction E₂I.

RESULTS

Electrophoretic examination of intermediate stages in the purification. As can be seen from Fig. 1 the crude acid extract contains at least nine bands: three acid components, a stationary band S, the important components E₁ and E₂ and three strongly basic components B₁, B₂ and B₃. There is also a band E₂', which has practically the same mobility as E₂ and which stains to a grey shade with Solvay Purple as compared with the E₂ which stains purple with the dye. This compound is only separable from E₂ in buffers of relatively high ionic strength such as are employed here. The material in band E₁ appears to be very soluble and becomes visible only after prolonged heating before staining. Components of this band appear to have a synergistic effect on the activity of E₂, which is the only electrophoretic component showing elastolytic activity when examined alone.

The first dialysis gives the fractions AES and AEI. The former retains all A₂ and little A₁, most of the basic components B and nearly all E₁ and E₂'. The fraction AEI is still rich in the elastolytically inactive component A₃; it contains a large amount of E₂ and a relatively large amount of insoluble material. The fraction S1 contains a little E₁ and E₂', and very little of the unadsorbed E₂ component. The second dialysis confines the small amount of acidic material to fraction E₂S to give a fraction E₂I, which consists solely of E₂ with a small amount of insoluble material. By centrifuging this can be removed from E₂ solutions in

buffer or sodium chloride without any loss of activity.

Isoelectric point. Several electrophoresis runs were made in veronal buffers, I 0-15, having the pH indicated in Table 2. Otherwise, standard conditions were used and, to correct for electroendosmosis, glucose was subjected to electrophoresis simultaneously. It will be seen from the figures in Table 2 that after making the necessary corrections the isoelectric point appears to be near pH 8.5.

Effect of pH on activity. Hall & Gardiner (1955) obtained curves relating activity to pH with a relatively impure enzyme, and showed that for this material the curve has two maxima, at pH 7.8 and 8.7, the upper of which was ascribed to E₂ (Hall, 1957). With the purified enzyme E₂I, only one maximum, at pH 8.45, is obtained.

Specific activity. It will be seen from Table 1 that, within the limits of experimental error, the purification produced after the first two steps is nearly tenfold, whereas the complete procedure gives a material which is about 40 times as active as the starting material; the question of the validity of these figures is considered in the Discussion section.

Variation of extent of elastolysis with substrate concentration. Table 3 shows a typical set of results illustrating the variation of extent of elastolysis with the initial amount of substrate at different time intervals. It will be seen that the percentages dissolved in corresponding time intervals approach a constant value when the substrate content

Table 2. *Determination of the isoelectric point of elastase by paper electrophoresis*

Conditions used were 60 mA, 16V/cm., 1½ hr. with veronal buffers, I 0-15.

pH	Distance moved by E ₂ (cm.)	Distance moved by glucose (cm.)	Net distance moved by E ₂ (cm.)
7.6	+3.5	+2.6	+0.9
8.6	+2.2	+2.3	-0.1
9.6	+1.4	+2.5	-1.1
11.0	+0.5	+2.5	-2.0

Table 3. *Variation of elastolytic activity with substrate/enzyme ratio*

Enzyme concentration was constant and equal to 54 μg./ml.

Starting amount of substrate (mg./10 ml.)	Percentage of elastin dissolved		
	Time 1 hr.	Time 2 hr.	Time 3 hr.
10	36	84	100
20	28	70	94
30	24	62	88
40	22	57	83
60	20	56	80

reaches about 6 mg./ml. It was also found that the curve relating activity to enzyme concentration is linear only for relatively low enzyme concentration (not higher than 4.0 $\mu\text{g.}$ of $\text{E}_2\text{I./ml.}$) (Figs. 2, 3.)

Ultraviolet absorption of $\text{E}_2\text{I.}$ Absorption spectrum of $\text{E}_2\text{I.}$ dissolved in 0.1 % sodium chloride and

in Clark and Lubs 0.5 M-borate buffer, pH 8.7, was determined. In each there was a definite maximum at a wavelength of 275 $\mu\text{m.}$ Extinction was proportional to concentrations up to 500 $\mu\text{g./ml.}$, giving an extinction coefficient of 13.

DISCUSSION

The progress of the elastolytic reaction, measurement of which is an essential for the assessment of any purification of elastase, has been estimated by a variety of methods. These include qualitative methods based on histological sections and electron-microscope studies (Baló & Banga, 1949; Saxl, 1958), gravimetric methods (Baló & Banga, 1950), biuret estimation of the protein released into solution (Hall & Gardiner, 1955; Hall, 1955) and the measurement of dye released into solution after the action of the enzyme on a previously dyed elastin preparation (Sachar, Winter, Sicher & Frankel, 1955; Roberts & Samuel, 1957).

Of these the histological method evaluates the enzyme activity under conditions as near physiological as possible, but the results can be no more than semiquantitative. The determination of protein release measures only one aspect of the whole effect of the enzyme whereas the solubilization of the dyed tissue may represent a reaction far removed from the normal physiological conditions. In a molecule with so few polar groups as elastin, sites of dye attachment may well be intimately related to those at which the enzyme becomes attached. Findlay (1954) states that staining with resorcinol fuchsin inhibits elastase attack on whole tissue but not on powdered material, and with the latter tissue which has been pre-stained it is conceivable that the points of attack are quite dissimilar to those in fresh tissue.

The gravimetric method measures solubilization of all the components of the tissue, and the interferometric method described here permits this effect to be measured continuously throughout a reaction without the tedious manipulations of the gravimetric method.

One of the difficulties which has arisen over the past few years about the comparison of the results for elastase activity provided by a variety of workers has been the differences in the criteria on which they have based these measurements. Four or five definitions of a unit for elastase have been proposed. Baló & Banga (1950) first defined an elastase unit as that amount of elastase which dissolves 1 mg. of elastin in 0.5 hr. This unit has been used widely but, as will be seen from the following discussion, it is very artificial and rather unsatisfactory. Such a unit should be examined critically in the light of the latest developments reported here and by other workers. The

Fig. 2. Activity (expressed in terms of increased refractive index of supernatant)-time curves for various concentrations of E_2 : A, 11.4; B, 7.6; C, 3.8; D, 1.9 $\mu\text{g./ml.}$ In each, 5 mg. of solid elastin/ml. was used.

Fig. 3. Relationship between activity (expressed as increases in refractive index of the supernatant) and concentration of enzyme, for four different periods of incubation: A, 1; B, 2; C, 3; D, 4 hr. In each, incubation was carried out at 37° with an initial substrate concentration of 5 mg./ml.

elastolysis-time curve is sigmoid, which makes it difficult to identify the percentage elastolysis after a given time as being identical with the classically defined 'initial reaction velocity'. There is experimental evidence that the solubilizing action of elastase is accompanied by a certain amount of purely hydrolytic action, in which the large soluble protein fragments are split into smaller polypeptides (Partridge & Davis, 1955). This implies that the function of the E_2 component is dual in nature and that at any one time only a part of the available enzyme is concerned in solubilization, which is the measurable effect. It follows therefore that the magnitude of the elastase unit will vary with the substrate/enzyme ratio (cf. Table 3). It can be shown that the number of elastase units in a given preparation increases considerably with the starting amount of elastin, provided that enzyme concentration remains constant (Fig. 4).

Many workers have used buffer solutions of relatively low ionic strength. This enables a higher specific activity to be recorded for the enzyme, since activity varies inversely with salt concentration (Lewis *et al.* 1956) and consequently the results of purification procedures can appear very attractive. It was decided that rather than sacrifice reproducibility, it would be better to use strongly buffering solutions such as borate ($I\ 0.36$), with its unavoidable decrease in activity, especially in a system such as this which is accompanied by the liberation of free acid.

Further, the time interval of 0.5 hr. is too short because to get a measurable effect with relatively inactive fractions of crude tissue extracts it is necessary to use large amounts of protein material. Lewis *et al.* (1956) in their paper on the preparation

of elastase report that the incubation period had to be extended to 2 hr. when certain preparations were estimated, thus invalidating direct comparisons such as they make between various stages in the purification.

A further complication in the definition of an elastase unit is the observation of Hall (1957) that the component E_1 , although elastolytically inactive, increases the activity of E_2 by nearly 50%. This means that the specific activity of many of the early fractions is higher than would be expected for a given amount of E_2 and in the extreme case a fraction consisting mostly of E_1 with little E_2 would show a specific activity, completely out of keeping with its E_2 content.

Although the primary object of this method of preparation is to obtain the elastolytic component E_2 , at the same time it throws some light on the possible means of isolation of component E_1 , which apart from its synergistic action on E_2 has been shown (Saxl, 1957, 1958) to be of importance in the solubilization of the mucopolysaccharide portion of elastic fibres.

It can be seen from the investigation of the enzyme-substrate reaction that the best conditions under which to define an elastase unit would be such that enzyme concentration is relatively small and the initial amount of substrate is as large as possible. The ensuing difficulties of measurement under such conditions and the presence of a large excess of reaction products would necessitate limiting the reaction to about one-fifth of its complete course. There is every indication from the shape of the curve (Fig. 2) that the reaction is not of the same type from beginning to end, so that the first fifth of its course may not be a true representation of the elastolytic reaction as a whole, and it is safer until such time as the processes concerned are fully characterized to make measurements near the region of 50% elastolysis.

It can be seen from the foregoing that it is very difficult to evolve a really satisfactory elastase unit. Work is now in progress and preliminary reports have been made (Czerkawski, 1958), which it is hoped will lead to the elucidation of the kinetics of the whole elastin-elastase system; this would then provide a sound basis for definition of the elastase unit. However, until such a time it is thought that the unit defined in the Experimental section utilizes the best possible conditions, is convenient and is capable of reproduction.

It must be stressed that for good reproducibility one must adhere to the stated conditions, especially for initial amount of substrate. Fig. 3 shows the increase in the number of elastase units contained in a given sample of elastase with increasing initial substrate concentration. Since the actual amount of enzyme used is constant the real value of the

Fig. 4. Relationship between the activity of a given elastase fraction (here E_2) and the amount of substrate initially available for reaction. Each point represents results obtained for a system containing $5.4\ \mu\text{g.}$ of $E_2/\text{ml.}$ and incubated for 3 hr. at 37° .

elastase unit becomes smaller with increasing elastin concentration.

An important point emerges from the successful purification of the proteolytic component of elastase. Partridge & Davis (1955) have shown that soluble elastin prepared by the oxalic acid extraction of solid elastin can be fractionated into two proteins, only one species, the α -elastin, producing coacervation on warming at low pH. This property of coacervation was rapidly destroyed by a crude elastase preparation such as was used by these authors.

We have found that great amounts of this material can be produced during the middle third of the reaction between solid elastin and purified elastase. When α -elastin obtained in this fashion is subjected to the same enzyme the power of coacervation disappears fairly rapidly, but a crude elastase having much lower elastolytic activity removes the power to coacervate very rapidly indeed.

One could infer from Partridge's work that during enzymic solubilization of elastin, only β -elastin is produced and production of α -material is only inherent in oxalic acid extraction. This would appear not to be so, and the results demonstrate the danger of employing impure enzymes until adequate proof is available that the contaminating proteins play no part in the main reaction. In this context it is of interest that Saxl (1958) has observed the formation of globular degradation products of elastase attack only after the use of highly purified enzyme.

SUMMARY

1. A practical unit of elastase is defined.
2. A method of purification, based on acid extraction, fractional dialysis, adsorption and extraction, is described.
3. Electrophoretic examination of each fraction is reported, together with an estimation of the isoelectric point of the final fraction E₂I. The iso-

electric point of elastase appears to be at pH 8.5, which virtually coincides with the single peak of the pH-activity curve.

4. The mode of variation of specific activity of elastase with initial amount of substrate and enzyme is reported. Most suitable conditions for the definition of elastase unit are discussed. Difficulties due to the complex nature of the elastase system are indicated.

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