The Chemistry of Connective Tissues

5. THE ELASTASE ACTIVITY OF PROTEOLYTIC ENZYMES*

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The dissolution of elastin by pancreatic extract was first demonstrated by Balo & Banga (1950), but since that time there have been many conflicting views about the mechanism of elastolysis. Hall, Reed & Tunbridge (1952) attributed a mucolytic function to pancreatic elastase, Lansing, Rosenthal, Alex & Dempsey (1952) suggested that the action might be lipolytic, Pepler & Brandt (1954) considered that sulphatase action might be involved and Partridge & Davis (1955) concluded that the dissolution of the fibrous protein is brought about by the rupture of peptide links.

Hall *et al.* (1952), from histological and chemical studies, suggested that the fundamental structural element of elastic tissue consists of two chemically distinct components, and that the fibres behaved as though composed of bundles of inner fibrils embedded in a mucoprotein matrix or sheath. During dissolution by pancreatic elastase there was a release of polysaccharide and sulphate, which led to the suggestion that elastase was not a proteolytic enzyme but rather a mucase. Banga & Balo (1953) supported the mucolytic view when they observed the release of reducing substances during elastase action and concluded that elastin was a glucoprotein containing 10–12% of carbohydrate resembling glucosamine, but this figure was subsequently reduced to below 2% (Banga, 1953). Hall (1955) traced the removal of collagen, polysaccharide and sulphate from elastic tissue during the progress of various purification procedures, including the action of hot dilute acetic acid, alkali and urea, and concluded that these methods of freeing so-called elastin from associated proteins are either inadequate or so drastic as to commence the degradation of the fundamental unit of elastic tissue itself. Hall & Gardiner (1955) advanced evidence that pancreatic elastase consists of two enzymes which act on two separate components of elastic-tissue preparations. Banga & Balo (1956), recognizing the heterogeneity of the pancreatic-elastase preparations then in use, suggested the limitation of the name elastase to the enzyme which has proteolytic activity. They reported the presence in purified preparations of pancreatic elastase of an enzyme which they called elastomucoproteinase, which released mucoprotein from elastic tissue. Hall (1957) showed that pancreatic-elastase preparations subjected to electrophoresis revealed the presence of two enzymes, one of which was inactive except in the presence of the other. He suggested that one of these enzymes is involved in the hydrolysis of the mucoprotein sheath and the other in the proteolytic hydrolysis of the central fibril.

Enzymes other than pancreatic elastase have been reported to have elastolytic activity. Thus Yokota (1957) demonstrated that a commercial preparation of α-amylase dissolved elastin, and Pepler & Brandt (1954) claimed that a preparation of bacterial chondrosulphatase was also active. Lansing *et al.* (1952) believed that elastin could be classified as a lipoprotein and observed the release of lipid during digestion with pancreatic elastase. This view was supported by Labella (1957), who suggested that lipid was essential for maintaining the integrity of elastin and that elastolysis involved the release of lipid.

Other groups of workers believe that elastolysis is fundamentally a proteolytic process. Partridge & Davis (1955) pointed out that in elastic tissue the elastin fibres are intimately associated with collagen and mucopolysaccharide, but demonstrated that by a purification procedure based on repeated autoclaving with water at neutral pH, alternated with fine grinding, the basic fibres of elastic tissue could be separated in a form which maintained their original morphological structure and staining properties. Prepared in this way the elastin fibres contained only traces of carbohydrate and ester sulphate, and fractionation of the material after rendering it soluble by partial hydrolysis with dilute oxalic acid showed that it was homogeneous in amino acid composition. Lewis, Williams & Brink (1956) reported that crystalline pancreatic elastase hydrolysed other proteins in addition to elastin. The presence of the enzyme in the pancreatic juice of animals led to the suggestion that elastase should be regarded as a digestive proteinase. About the same time the significant observation was made that certain plant proteolytic enzymes will dissolve elastin. Thus Miyada & Tappel (1956) demonstrated the activity of ficin

* Part 4: Partridge & Davis (1958).

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and papain and Wang, Weir, Birkner & Ginger (1958) that of bromelin. Miyada & Tappel (1956) also reported the activity of a commercial proteolytic preparation of fungal origin, Rhozyme P-11. Lewis & Thiele (1957) showed by chromatography that their crystalline pancreatic elastase was heterogeneous and that, of the five components separated, only one component possessed elastase activity. Grant & Robbins (1957), using pancreatic elastase which had been partially purified by adsorption on to elastin, showed that it had strong proteolytic activity on proteins other than elastin. Naughton & Sanger (1958) extended the purification of pancreatic elastase by Lewis *et al.* (1956) to chromatographic fractionation and obtained on elution a well-defined peak which possessed all the elastase activity. When tested against the B chain of insulin it exhibited a wide range of peptide-bond specificity. The presence of two elastases in the pancreas has been reported by Dvonch & Alburn (1959), by the use of electrophoretic fractionation. Both these fractions had elastolytic and proteolytic activities.

It is clear that much of the confusion which exists in current views of the nature of elastin and the mechanism of elastolysis will be resolved only by a more detailed knowledge of the molecular architecture of the protein, and the final objective of the present work is to utilize elastolytic enzymes as tools for its stepwise degradation. However, to place such structural studies on an unequivocal basis it was considered necessary first to undertake a preliminary survey of the various enzyme preparations which have been reported as displaying elastolytic activity, with a preparation of elastin of established purity as substrate.

EXPERIMENTAL

Materials

Elastin. Extraction from bovine ligamentum nuchae was carried out as described by Partridge, Davis & Adair (1955). The carbohydrate content measured against a glucose standard was not more than 0·2% with the orcinol reagent (Sørensen & Haugaard, 1933) and 0·25% with anthrone (Yemm & Willis, 1954). The uronic acid content estimated by the carbazole method of Dische (1947) gave not more than 0·3% (as galacturonic acid). A colour corresponding to 0·14% of glucosamine was obtained by the Elson & Morgan (1933) reaction. The ash content was 0·14%.

Enzyme preparations. Pancreatin was obtained from Armour and Co. and Greeff and Co.; crude preparations of ficin were from Mann Research Laboratories, Merck and Co. and Nutritional Biochemical Corp.; papain was from British Drug Houses Ltd. and Harrington Bros. Ltd.; bromelin was from L. Light and Co. Ltd.; crystalline trypsin was from Armour and Co.; cereal α-amylases were from E. Gurr Ltd. and L. Light and Co. Ltd.; testicular hyaluronidase was from Benger Laboratories Ltd.; Rhozyme P-11 was from Rohm and Haas Co.; crystalline hog pancreatic and salivary α-amylases were generously given by Dr W. J. Whelan and bacterial chondroitinase was given by Dr A. G. Lloyd. Intracellular enzymes were extracted from kidney and spleen of a freshly killed ox by autolyses in acidic solution, followed by ammonium sulphate fractionation according to the instructions of De La Haba, Cammarata & Fruton (1955).

Carboxymethylcellulose. This was prepared as described by Petersen & Sober (1956).

Methods

Assay of elastase activity. The method was based on that of Naughton & Sanger (1958). Elastin (20 mg.) was weighed into a 10 ml. centrifuge tube and Congo red added (2 ml. of 0·02% solution in 0·1 M-sodium carbonate–HCl buffer, pH 8·8). No excess of dye remained in the supernatant after shaking at room temperature for 2–3 min. The enzyme solution was adjusted to the same buffer concentration and added to the dyed elastin suspension. More 0·1 M-sodium carbonate–HCl buffer (pH 8·8) was then added to give a final incubation volume of 5 ml. When cysteine was required 0·5 ml. of 0·25 M-cysteine solution (pH 8·8) was added, giving a final concentration of 25 mM. During incubation at 37·5° the elastin was kept in suspension by occasional shaking and the amount dissolved after 30 min. was measured by centrifuging and estimation of the colour in the supernatant with a Spekker colorimeter and filter no. 604.

In some experiments activity was also determined by the modification of Lewis *et al.* (1956) of the gravimetric method of Banga (1952).

A unit of elastase activity (e.u.) was defined as that amount of enzyme which dissolved 10 mg. of elastin in 0·1 M-sodium carbonate–HCl buffer, with or without cysteine (25 mM), when incubated at 37·5° for 30 min. The specific elastase activity (specific e.a.) was the number of units/mg. of enzyme.

Assay of proteolytic activity. This was based on the viscosity-decrease method of Whitaker (1957). A stock solution of gelatin was prepared by dissolving gelatin (2·5 g.) in water (50 ml.) with stirring and heating at 80°. After cooling, an equal volume of 0·2 M-phosphate buffer, pH 7·5, was added and mixed. To the enzyme solution (1 ml.) was added 0·1 M-phosphate buffer, pH 7·5 (1 ml.), and the mixture was brought to 37·5°. Gelatin solution (8 ml.) at 37·5° was added, the components were rapidly mixed and 5 ml. was transferred to an Ostwald viscometer (with a flow rate for water of 12·5 sec.) placed in a constant-temperature water bath at 37·5°. The flow rate was measured immediately and at regular intervals up to 10 min. When cysteine (25 mM) was required the enzyme solution was added to cysteine (0·25 M) in phosphate buffer, pH 7·5 (1 ml.). A blank was performed in the absence of enzyme solution.

A unit of proteolytic activity (p.u.) was defined as that amount of enzyme which decreased the specific viscosity of 100 mg. of gelatin to one-half when incubated in 0·1 M-phosphate buffer, pH 7·5, with or without cysteine (25 mM), after 10 min. at 37·5°.

Fractionation of ficin with ammonium sulphate. Ficin (20 g.) was stirred into 220 ml. of 0·1 M-acetate, pH 5·5, containing 20 mM-cysteine, for 30 min., the mixture was

centrifuged and the supernatant decanted. The residue was re-extracted with the acetate–cysteine mixture and centrifuged. The washings were combined with the supernatant and solid ammonium sulphate was added with stirring until the extract was brought to 30% saturation. The precipitate was removed by centrifuging and to the supernatant was added more ammonium sulphate until the saturation reached 40%. The precipitates obtained at 30, 40, 50 and 100% saturation were dissolved in salt solution and dialysed against frequent changes of distilled water containing 10 mM-cysteine. The euglobulin precipitates obtained during dialysis were collected by centrifuging, giving soluble and euglobulin fractions at each percentage of ammonium sulphate saturation.

Chromatographic fractionation of the 0–40% *ammonium sulphate-soluble fraction from crude ficin.* The column (23 cm. × 0·8 cm.) was packed with carboxymethylcellulose (Petersen & Sober, 1956) and equilibrated with 25 mM-sodium acetate buffer, pH 4·8, containing 5 mM-cysteine and 1 mM-ethylenediaminetetra-acetic acid. The same buffer was used to apply the ficin fraction (30 mg.) and to fill the mixing chamber (175 ml.) used for gradient elution. The column was developed by passing M-sodium acetate buffer, pH 4·8, into the mixing chamber at 2 ml./hr. and the effluent was collected in 4 ml. fractions. The protein content of each fraction was estimated by measuring E at 280 mμ.

Zone electrophoresis. The method used was essentially that of Svensson, Hagdahl & Lerner (1957), in which a vertical column is stabilized by a sucrose-density gradient. The column was 42 cm. high × 3·2 cm. diameter; buffer, 30 mM-sodium barbitone, pH 8·67; the sucrose concentration varied from 8% (w/v) at the point of injection of the sample to 30% (w/v) at the outlet. The solution of ficin (52 mg. in 10 ml. of sucrose–buffer of the appropriate density) was injected into the column and migration allowed to proceed in the downward direction overnight at room temperature (6·7 v/cm.; 20 mA). Fractions (10 ml.) were then collected by discharge from the outlet tube at the bottom of the column and were estimated for protein by measuring the extinction with the Folin–Ciocalteu reagent (Lowry, Rosebrough, Farr & Randall, 1951).

RESULTS

The measurement of elastase activity is a matter of some difficulty owing to the complexity of the kinetics of the reaction. Banga (1952) defined a unit of elastase activity as the amount of enzyme which dissolved 5% of a specified quantity of elastin in 30 min. under defined conditions, and difficulties due to the lag phase of the reaction were avoided by adjusting the dilution of the enzyme until the assay tube contained a quantity close to one unit. In our hands this scheme appeared to give reproducible results when assaying a single sample of enzyme, but when enzymes from different sources are to be assayed there is an obvious danger of error if the preparation of elastin should contain a small amount of foreign protein as impurity. Accordingly for the purposes of the present work the elastase unit was redefined as the amount of enzyme which dissolved 50% of a 20 mg. portion of elastin in 30 min. under the conditions specified.

Assay of proteolytic enzymes. The elastolytic and proteolytic activities of crude commercial preparations of various enzymes which are known to hydrolyse proteins are summarized in Table 1. The report by Miyada & Tappel (1956) that Rhozyme P-11 hydrolysed elastin was not confirmed, despite the proteolytic activity exhibited. However, in agreement with these workers and Wang *et al.* (1958), it was shown that ficin, papain and bromelin possess strong elastolytic and proteolytic activities. These plant enzymes require activation by a reducing agent, e.g. cysteine, and in its absence there was a marked decrease in activity towards elastin and gelatin. The two commercial pancreatin preparations tested were very much less active than the plant enzyme both in elastolytic and proteolytic effect. It is to be noticed that the ratio of elastase to proteolytic activity in all the preparations used did not vary over a wide range. In contrast with these results, the preparations of crystalline trypsin, chymotrypsin and pepsin were either completely inactive or showed a slight dissolution of elastin after several days.

Assay of mucolytic enzymes. Pepler & Brandt (1954) claimed that a sample of chondrosul-

Table 1. *Elastolytic and proteolytic activities of proteolytic enzymes*

The definition and method of estimation of specific e.a. and specific proteolytic activity (p.a.) are given under Methods. Cysteine (0·025 M) was present in incubation mixtures with ficin, papain and bromelin.

Enzyme	Commercial source	Specific e.a.	Specific p.a.	Specific e.a./ specific p.a.
Ficin	Merck and Co.	0·186	87·0	0·00214
	Mann Research Laboratories	0·218	87·6	0·00249
	Nutritional Biochemicals Corp.	0·143	83·3	0·00172
Papain	British Drug Houses Ltd.	0·50	77·0	0·00649
	Harrington Bros. Ltd.	0·50	84·0	0·00596
Bromelin	L. Light and Co. Ltd.	0·21	44·0	0·00477
Pancreatin	Armour and Co.	0·007	1·25	0·00560
	Greeff and Co.	0·004	0·85	0·00471
Rhozyme P-11	Rohm and Haas Co.	0	12·0	—

phatase from *Proteus vulgaris* dissolved elastic fibres. In the present assays a preparation of dried cells from *P. vulgaris* (National Collection of Type Cultures, no. 4636), which contained both chondrosulphatase and chondroitinase, was tested against purified elastin but was found to be inactive. A sample of pure chondroitinase from *P. vulgaris* (Dodgson & Lloyd, 1957) showed no proteolytic activity, and also failed to digest elastin. In view of the report by Yokota (1957) that a commercial preparation of α-amylase possessed elastolytic activity, an assay was made of a number of samples of α-amylase extracted from different sources. Two commercial cereal preparations and crystalline salivary α-amylases showed no activity towards elastin or gelatin. However, a crystalline preparation of hog pancreatic α-amylase showed weak elastolytic activity but this disappeared when the enzyme was recrystallized. A purified sample of testicular hyaluronidase (Benger Laboratories Ltd.) had no elastolytic or proteolytic activity.

Kathepsins. The presence in mammalian tissues of intracellular proteolytic enzymes known collectively as kathepsins has been known for many years. By the method of De La Haba *et al.* (1955) for extraction of these enzymes from fresh kidney and spleen, fractions were obtained which possessed proteolytic activity when assayed in the presence of reducing agents but did not digest elastin either in the presence or the absence of cysteine.

Fractionation and purification of ficin. The commercial preparations are described as the products of drying fig latex, and the question arises whether the elastolytic activity observed is a property of the proteolytic enzyme which forms a large part of the product or whether it is due to the presence of a separate specific elastase. Accordingly, one of the preparations (Merck and Co.) was extensively fractionated first by ammonium sulphate precipitation and then by zone electrophoresis. A highly purified product was then isolated by chromatographic fractionation with a column of carboxymethylcellulose. Table 2 shows the results of fractionation with ammonium sulphate, and it will be observed that the distribution of elastase activity follows closely that of proteolytic activity as measured by following the decrease of viscosity of gelatin. Most of the active enzyme is precipitated at 50% saturation with ammonium sulphate or below, and activity is associated with the pseudoglobulin fraction which is soluble in water after dialysis.

From the data in Table 2 the elastolytic and proteolytic activity appeared to be concentrated in the 30 and 40% ammonium sulphate-soluble

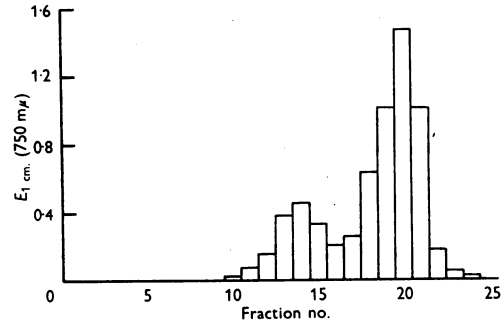

Fig. 1. Zone-electrophoresis pattern given by 52 mg. of ficin (water-soluble fraction precipitated at 40% ammonium sulphate saturation) with a sucrose-density-gradient column. Protein concentrations of fractions were estimated from E at 750 mμ after addition of Folin–Ciocalteu reagent.

Table 2. *Fractionation of ficin with ammonium sulphate*

All estimations were carried out in the presence of cysteine (25 mM). The definitions of p.u. (proteolytic unit), specific p.a. (specific proteolytic activity), e.u. (elastase unit) and specific e.a. are given under Methods.

Fraction	Dry wt. (g.)	Total p.u. ($\times 10^{-3}$)	Specific p.a.	Total e.u.	Specific e.a.	Specific e.a./ specific p.a.
Commercial ficin	20·000	1820	91	3720	0·186	0·00205
Insoluble in acetate	0·245	0	0	0	0	0
30% satn. $(NH_4)_2SO_4$ ppt.						
Soluble in water	1·500	667	445	1480	0·99	0·0022
Euglobin	0·694	136	196	417	0·61	0·0031
40% satn. $(NH_4)_2SO_4$ ppt.						
Soluble in water	0·316	240	760	277	0·88	0·0012
Euglobin	0·087	13	154	35	0·41	0·0027
50% satn. $(NH_4)_2SO_4$ ppt.						
Soluble in water	2·120	1074	506	930	0·44	0·0087
Euglobin	0·980	85	87	202	0·21	0·0024
100% satn. $(NH_4)_2SO_4$ ppt.						
Soluble in water	1·570	194	123	175	0·11	0·0089
Euglobin	0·144	11	74	35	0·24	0·0033

fractions and these were combined for further fractionation by zone electrophoresis with a sucrose-density-gradient column. The resultant curve, showing two protein peaks, is given in Fig. 1. Fractions 14 and 20 were tested for elastolytic and proteolytic activities. All the activity was associated with the slower-moving fraction 20, the ratio of elastolytic to proteolytic activity being 0·00163 compared with 0·00256 for the starting material. The peak represented by fraction 14 possessed no elastolytic or proteolytic activity.

The same ammonium sulphate fraction was further fractionated by gradient elution with a carboxymethylcellulose column. The buffer system contained cysteine and ethylenediaminetetra-acetic acid and the elution was conducted by increasing the concentration of the sodium acetate buffer, the pH being maintained at 4·8 throughout. The resulting curve of protein concentration is shown in Fig. 2. Fractions 3, 14, 18, 20, 28, 38 and 48 were dialysed against distilled water containing 5 mM-cysteine and 1 mM-ethylenediaminetetra-acetic acid and then tested for elastolytic and proteolytic activities. Fractions 3 and 48 were devoid of both activities, 28 and 38 showed slight activity but fractions 14 and 20 were both highly active. In both fractions the ratio of elastolytic to proteolytic activity was the same.

Pancreatic elastase. Although pancreatic elastase is not regarded as an SH-dependent enzyme (Naughton & Sanger, 1958) it was observed that the addition of cysteine (25 mM) to the incubation mixture increased the activity of crude preparations of the enzyme two- or three-fold. The possibility that pancreatin contains a second, SH-active elastase was tested by fractionating a preparation of crude pancreatin and comparing the properties of the fractions. An extract of commercial pancreatin in sodium acetate buffer was fractionated with ammonium sulphate according to the directions of Lewis *et al.* (1956). The fractionation results are shown in Table 3. The most active material was the water-insoluble (euglobulin) fraction obtained after dialysis of the fraction

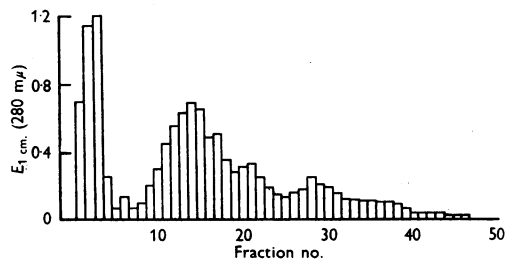

Fig. 2. Ultraviolet-absorption diagram from 30 mg. of ficin (water-soluble fraction precipitated at 40% ammonium sulphate saturation) obtained after elution with a concentration gradient of acetate buffer, pH 4·8, with a carboxymethylcellulose column.

Table 3. *Fractionation of pancreatin with ammonium sulphate*

For explanation of abbreviations see Table 2.

Fraction	Dry wt. (g.)	Total p.u. ($\times 10^{-3}$)	Specific p.a.	Total e.u.	Specific e.a.	Specific e.a./ specific p.a.
Starting material	100	120·0	1·20	690	0·007	0·0059
Insoluble in acetate	4·08	15·2	3·72	123	0·030	0·0081
45% satn. $(NH_4)_2SO_4$ ppt.						
Soluble in water	1·04	25·6	24·60	213	0·204	0·0083
Euglobin	0·10	3·2	32·40	200	2·000	0·0618
100% satn. $(NH_4)_2SO_4$ ppt.	0·43	4·2	9·80	5	0·120	0·0122

Table 4. *Effect of activators and inhibitors on purified pancreatic elastase*

For explanation of abbreviations see Table 2.

Molarity of carbonate buffer (pH 8·8)	Addition	Specific e.a.	Effect
0·1	None	2	—
0·1	Cysteine (25 mM)	5	Activates × 2·5
0·1	Di*iso*propyl phosphorofluoridate (0·1 mM)	0	Complete inhibition
0·1	H_2O_2 (30 mM)	4	Activates × 2·0
0·1	Iodoacetic acid (20 mM)	2	No effect
0·05	None	7	Activates × 3·5
0·05	Cysteine (25 mM)	7	Activates × 3·5; cysteine has no effect
0·1	NaCl (0·1M)	0·25	Inhibition
0·1	NaCl (0·1M) + cysteine (25 mM)	0·60	Cysteine reduces inhibition

precipitated at 45% ammonium sulphate concentration. The specific activity of this fraction was 286 times that of the starting material. The activity of this highly enriched fraction was increased by the presence of cysteine in the same ratio (1:2·5) as was observed with crude pancreatin and the other less active fractions. It was therefore concluded that cysteine activation is a property of pancreatic elastase itself and is not due to the presence of a second elastolytic enzyme. As would be expected from the known presence of several proteolytic enzymes in crude pancreatin, the ratio of elastolytic to proteolytic activity of the fractions varied over a wide range.

The effect of activators and inhibitors on the euglobulin fraction is reported in Table 4. Iodoacetic acid (20 mM), an inhibitor of SH-dependent enzymes, had no effect and hydrogen peroxide behaved like cysteine in producing marked activation. The complete inhibition by di*iso*propyl phosphorofluoridate at 0·1 mM concentration reported by Naughton & Sanger (1958) was confirmed. This inhibitor has no effect on SH-dependent enzymes and the complete loss of activity in its presence tells strongly against the possibility of the existence of a separate SH-elastase in pancreatin. All the elastase assays described above were performed in the presence of 0·1 M-carbonate buffer, but it was found that if the buffer concentration was reduced to 50 mM there was a 3·5-fold increase in activity. At this lower ionic strength cysteine was found to have no effect on activity, thus confirming the report of Lewis *et al.* (1956), who used a low buffer concentration in their assays and observed no increase in activity with added cysteine. The addition of sodium chloride (0·1 N) to an incubation mixture containing 0·1 M-carbonate caused marked inhibition of elastase activity. The inhibition, however, was decreased in the presence of cysteine.

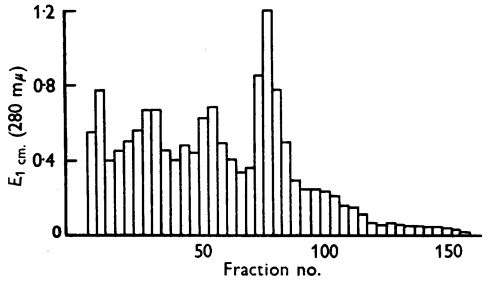

Fig. 3. Ultraviolet-absorption diagram from 25 mg. of pancreatin (euglobulin fraction precipitated at 45% ammonium sulphate saturation) obtained after elution with a concentration gradient of acetate buffer, pH 4·8, with a carboxymethylcellulose column.

Further purification of the euglobulin fraction of pancreatic elastase was obtained by fractionation on a carboxymethylcellulose column (Naughton & Sanger, 1958), gradient elution with acetate buffer, pH 4·8, being used. Fig. 3 shows the presence of five protein peaks, nearly all of the activity being associated with the slowest moving peak (fraction 78). The specific elastase activity of the material from this peak was 5·0, showing a purification factor of 714, based on the activity of crude pancreatin. The specific proteolytic activity of the preparation was 34·4.

DISCUSSION

In carrying out this survey of elastolytic enzymes it was considered of importance that the substrate used should be one of established purity. By this it is meant that the preparation of elastin should represent the major structural element of elastic tissue, and that it should be substantially free from all contaminating material, arising from other components of connective tissue, that can be removed by physical means. We consider that the preparation of elastin described by Partridge, Davis & Adair (1955) satisfied these requirements in that: (i) the method of isolation avoids the use of hydrolytic reagents such as alkalis and acids; (ii) the fibrous preparation has the morphological structure and staining reactions of the parent tissue; (iii) it is substantially free from collagen and carbohydrate; (iv) on fractionation after partial hydrolysis the fractions have substantially the same amino acid analysis. Elastin prepared in this way may be regarded as a fibrous protein in a relatively high state of purity, but, as pointed out by Partridge & Davis (1955), only 95% of the protein dry weight could be accounted for by amino acid analysis and it is possible that nitrogenous or non-nitrogenous residues other than amino acids may be involved in its structure.

On digestion with pancreatic elastase Banga (1951), using the formol-titration and van Slyke methods of estimation, failed to detect release of α-amino groups. However, Partridge & Davis (1955), using the more sensitive fluorodinitrobenzene technique of Sanger (1945), observed release of amino groups during elastolysis and concluded that the mechanism was essentially a proteolytic one. Purified pancreatic-elastase preparations have a wide range of proteolytic activity and hydrolyse heat-treated collagen (Banga, 1953), haemoglobin (Patridge & Davis, 1955), fibrin, albumin, casein and soya-bean protein (Lewis *et al.* 1956). Esterase activity has also been demonstrated towards acetyl-L-tyrosine ethyl ester (Grant & Robbins, 1957) and rennet activity (Lewis *et al.* 1956).

The present work confirms the finding of Miyada & Tappel (1956) and Wang *et al.* (1958) that certain proteolytic enzymes of vegetable origin have a high elastolytic activity, but no elastolytic or proteolytic activity was detected in any of the mucolytic enzymes tested. A sample of α-amylase prepared from pancreas extract did indeed dissolve elastin, but this activity disappeared when the enzyme was recrystallized and it was concluded that the activity was the result of contamination with pancreatic elastase. In this connexion it should be mentioned that the presence of an unidentified proteolytic enzyme in crystalline preparations of pancreatic α-amylase has already been reported by Bernfeld (1951).

In all enzyme preparations tested elastolytic activity was always associated with proteolytic activity. The ratio of these activities was about the same in crude preparations of bromelin, ficin and papain. With ficin the crude commercial product was fractionated and purified, and a fraction was obtained by chromatography on a carboxymethylcellulose column in which the specific activity was sevenfold that of the starting material. All the major active fractions and the finally purified product had about the same ratio of elastolytic to proteolytic activity and it is therefore concluded that the elastase of fig latex is identical with the enzyme responsible for the larger part of the proteolytic activity.

The purification of pancreatic elastase was based on the procedure of Naughton & Sanger (1958). During the course of the assays the observation was made that under some conditions of pH and ionic strength the enzyme was markedly activated by the presence of reducing agents such as cysteine, suggesting the presence of an SH-enzyme. However SH-inhibitors such as iodoacetic acid had no effect, and hydrogen peroxide, instead of decreasing the activity of the enzyme, appeared to enhance it somewhat. In agreement with Naughton & Sanger it was found that di*iso*propyl phosphorofluoridate inhibited the elastase activity and thus there appears little doubt that the active centre of pancreatic elastase is similar to that of trypsin and chymotrypsin (cf. Dixon, Neurath & Pechere, 1958). The enhanced activity due to cysteine and hydrogen peroxide may then be due to the rupture of an –SS– bond elsewhere in the molecule. It is considered possible that the active –SS– bond may constitute an intermolecular link, and in this case the presence of cysteine or hydrogen peroxide would reduce the number of dimers present. A decrease in salt concentration from 0·1 M to 50 mM results in activation of the same order and this may also be due to a similar disturbance of an association equilibrium. This indication of association phenomena could account in part for the complex kinetics of elastase action observed by many workers, but in considering this question the possibility of the presence of an inactive zymogen (Grant & Robbins, 1955) should not be forgotten.

The results of the survey as a whole show that whereas all non-proteolytic enzymes so far tested are inactive towards elastin several well-characterized proteolytic enzymes have a high activity. The supposition that there is a specific non-proteolytic 'elastase' which dissolves the fibrous protein and thus renders it susceptible to proteolytic hydrolysis by a second enzyme is scarcely tenable in view of the failure of the fractionation experiments with ficin and pancreatin to disclose such a system. Since the specificity of proteolytic enzymes as a whole seems to be associated with the rupture of specific peptide links, it may well be asked why some proteolytic enzymes are active as elastases and others only slightly active or not at all. It has already been pointed out (Newman, 1949; Partridge & Davis, 1955) that elastin has an unusual amino acid composition, being very high in residues with hydrocarbon side chains, particularly valine, and very low in acidic and basic amino acids. There is also evidence that the fibrous protein has a cross-linked structure (Partridge, Davis & Adair, 1955). It is possible that in order to split a sufficient number of peptide links to disrupt the net-like structure of the protein an enzyme displaying a wide peptide-bond specificity is essential. Enzyme preparations such as crystalline trypsin may fail because they attack a too small proportion of the links. It is also probable that in some cases a proteolytic enzyme may show little activity because of steric factors concerning its size, shape and position of active centre; thus both the activity of pancreatic elastase, and its adsorption by elastin, is greatly enhanced if the elastin is pretreated with boiling dilute sodium hydroxide (Grant & Robbins, 1957). This treatment would be expected to open up the structure of the network by the rupture of a proportion of the links.

SUMMARY

1. A number of proteolytic and mucolytic enzymes have been assayed for elastolytic activity with a preparation of elastin of established purity as substrate. This was prepared from bovine ligamentum nuchae; it contained less than 0·3% of carbohydrate and was free from collagen.

2. In addition to pancreatic elastase, three proteolytic enzymes, papain, ficin and bromelin, were found to have a high activity towards elastin. Crystalline trypsin, chymotrypsin, pepsin, Rhozyme and preparations of kathepsin from kidney and spleen were found to be either totally inactive or very slow.

3. Preparations of bacterial chondrosulphatase, purified chondroitinase and testicular hyaluronidase showed no activity.

4. Preparations of α-amylase from saliva and cereal seeds were inactive but a crystalline sample of pancreatic α-amylase showed activity. This disappeared on further recrystallization, and it is concluded that the elastolytic activity of α-amylase reported in the literature is due to contamination with pancreatic elastase.

5. Various commercial preparations of ficin all showed the same ratio of elastolytic to proteolytic activity as measured by viscosity reduction of gelatin. This same ratio was maintained throughout fractionation and purification of commercial dried fig latex. It was concluded that 'fig-latex elastase' is identical with the major proteolytic enzyme in preparations of 'ficin'.

6. The results of the survey confirm the view that elastolysis is the result of proteolytic activity. The failure of certain well-characterized proteolytic enzymes such as trypsin and chymotrypsin to dissolve elastin is attributed either to their failure to penetrate the cross-linked structure of elastin or to the very restricted peptide-bond specificity exhibited by these enzymes.

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The Formation of Phenolic Substances in *Eucalyptus gigantea* and *Eucalyptus sieberiana*

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It has been pointed out (Hillis, 1956; Hathway, 1958) that resins and kinos produced in the cambial region contain components which are absent from heartwood of the species which produced them. For these and other reasons, it was suggested that the phenolic substances in bark and wood were produced elsewhere. It was further suggested that, in some instances, the leucoanthocyanins were the precursors of heartwood extractives (see also Bate-Smith & Swain, 1953; King & White, 1957; Roux, 1958; Roux & Evelyn, 1958). Recent information obtained with the aid of a staining technique (Hillis, 1958) indicated that the leucoanthocyanins in some eucalypts were synthesized *in situ*. A