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Porcine Elastase. I. The Presence of Tyrosinate-Splitting Enzymes as Impurities in Elastase Preparations*

Thomas H. Marshall,† John R. Whitaker,‡ and Myron L. Bender§

ABSTRACT: The presence in crystalline elastase preparations of several components with high specific activity against *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate has been demonstrated by both chromatographic and kinetic methods. The amounts of these components are variable from one preparation of crystalline elastase to another and the amounts are not related to the activity of the preparation against casein or elastin–orcein. Crystalline elastase which has been subjected subsequently to preparative electrophoresis has lower, but still measurable, activity against *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate than does the initial crystalline enzyme. These minor components of elastase can be essentially removed by chromatography of crystalline elastase on CM-cellulose or DEAE-Sephadex A-50 at pH 8.0–8.8. The relationship between elastase and the enzymatic impurities is best demonstrated by chromatography of crude elastoproteinase on DEAE-Sephadex A-50 at pH 8.8. Three components with high tyrosinate activity are found to be chromatographically distinct from the single component containing the elastase activity.

Pancreatic elastase (EC 3.4.4.7; Commission on Enzymes, 1961) has long been recognized to present a formidable purification problem. Crystalline elastase has been fractionated into various components of elastolytic, proteolytic, and esterolytic activity (Lewis and Thiele, 1957; Dvonch and Alburn, 1959; Lamy *et al.*, 1961; Avrameas and Uriel, 1965; Ling and Anwar, 1966). The complexity of elastase has been inferred from selective inhibition studies (Walford and Kickhöfen, 1962; Sólyom and Tolnay, 1965) and from the bifunctional nature of the action of elastase on elastin (Hall, 1953). The *in vivo* effect of elastase has been attributed to the presence of its "impurities" (Sólyom *et al.*, 1964). Elastomucase and elastoproteinase have been separated and shown to act synergistically during elastolysis (Loeven, 1963).

Highly purified elastase, which was determined by titration against diethyl *p*-nitrophenyl phosphate to be 80% active, contained enzymatic impurities which reacted only with *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate (Bender *et al.*, 1966). (We shall designate these impurities as tyrosinate enzymes.) The tyrosinate enzymes were a maximum of 7% of the total amount of protein present. They reacted some 20 times more slowly than elastase with the titrating agent diethyl *p*-nitrophenyl phosphate. The tyrosinate enzymes do not interfere with either the titration assay or a kinetic study of elastase-catalyzed ester hydrolysis (Bender and Marshall, 1968).

In the research reported here, the relationships between elastase and the tyrosinate enzymes and among the elastolytic, proteolytic, and tyrosinate activities were investigated by the further purification of both crystalline elastase and of elastoproteinase.

Materials and Methods

Substrates. The nitrophenyl esters used in this research have been characterized previously (Bender *et al.*, 1966). Casein (Hammarsten quality) was from Nutritional Biochemical Corp. Elastin–orcein was prepared by the method of Mandl (1962). Stock solutions of synthetic substrates were prepared in acetonitrile (Eastman Spectrograde) which had been distilled from P_2O_5 and finally from K_2CO_3.

* From the Department of Chemistry, Northwestern University, Evanston, Illinois 60201, and the Department of Food Science and Technology, University of California, Davis, California 95616. *Received August 25, 1969.* Abstracted in part from the Ph.D. thesis of Thomas H. Marshall, Northwestern University, 1967.

† Present address: Department of Chemistry, Northern Illinois University, DeKalb, Ill. 60115.

‡ Department of Food Science and Technology, University of California, Davis, Calif.

§ Department of Chemistry, Northwestern University.

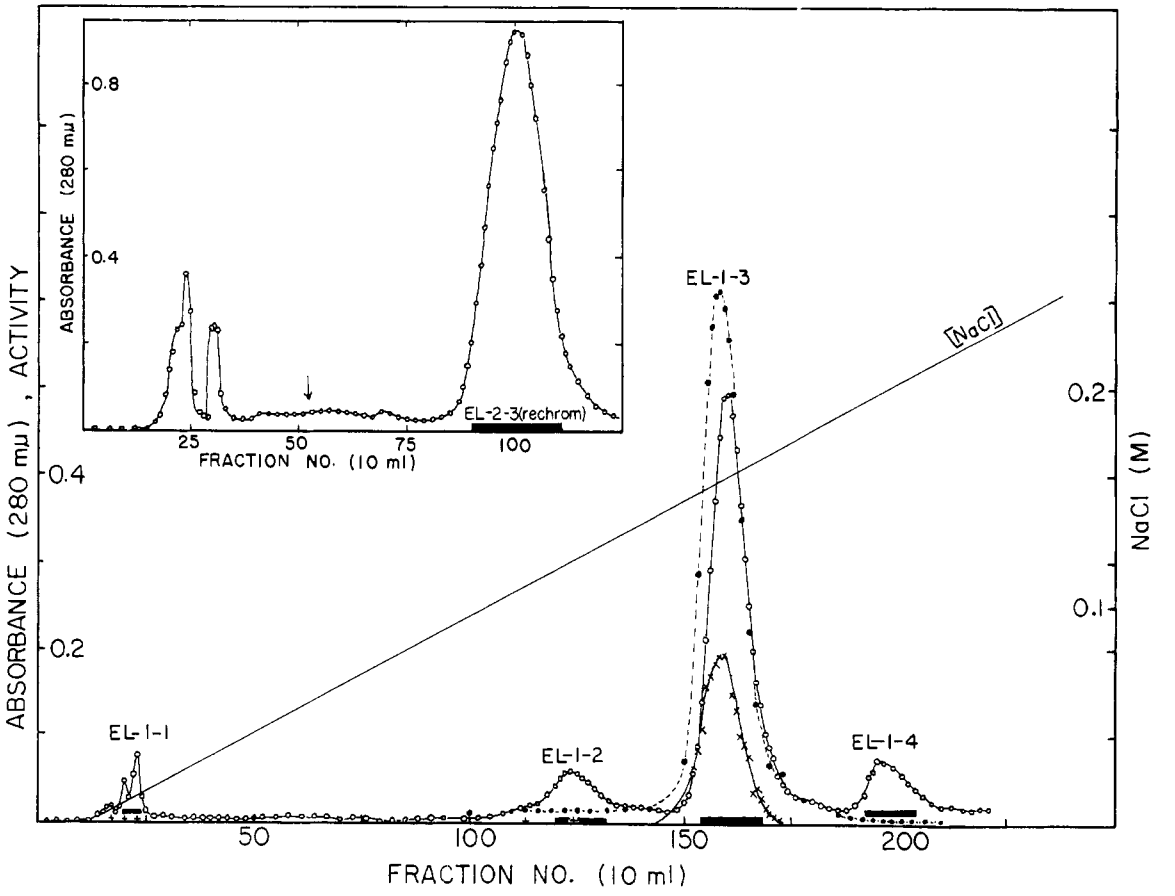

FIGURE 1: Chromatography of crystalline elastase on CM-cellulose at pH 4.6. Chromatography was performed as described in the text. (○) Absorbance of collected fractions at 280 mμ, (●) activity against casein, and (X) and (+) activity on elastin–orcein at 20-min and 2-hr incubation times, respectively. Insert: rechromatography of elastase prepared as in Figure 1. EL-2-3[1] was dialyzed, lyophilized, and redissolved in 0.05 M ammonium acetate buffer (pH 4.6), containing 0.11 M NaCl and applied to a CM-cellulose column equilibrated against this same solution. At tube 52 the elution fluid was changed to 0.05 M ammonium acetate buffer (pH 4.6), containing 0.14 M NaCl. Fractions 90–111 were pooled, dialyzed, and lyophilized to give preparation EL-2-3 (rechromatographed).

Enzymes. Several preparations of crystalline elastase and of crystalline elastase which had been further purified by electrophoresis (Lewis *et al.*, 1956) were obtained from Worthington Biochemical Corp. The authors also made several preparations of crystalline elastase by the method of Lewis *et al.* (1956) using porcine pancreas powder from Nutritional Biochemical Corp. (lot no. 5057).

Buffer solutions were made from reagent grade chemicals as described by Long (1961) to give a specific pH at an ionic strength of 0.05 with no added salt unless otherwise stated. Water was distilled in a Corning AG-2 still after passing through an Amberlite MB 3 resin and a cation-exchange resin.

Kinetic Measurements. The kinetics of nitrophenyl ester hydrolysis were followed spectrophotometrically at 347.5 or 400 mμ with a Cary 14 recording spectrophotometer. The spectral properties of the compounds have been reported previously (Bender and Marshall, 1968). Casein hydrolysis was determined on 0.5% casein at pH 7.6 and 35° (Kunitz, 1947) and the activity is expressed as change in absorbance at 280 mμ/min per ml of enzyme solution (or per mg of protein). Activity against *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate is expressed either as a first-order rate constant or as an initial rate under the following conditions: 5.91×10^{-5} M substrate, 0.01 M Tris-HCl (pH 8.00, 25°), and acetonitrile concentration as stated. The initial rate is expressed as moles of substrate hydrolyzed per liter per second per milliliter of enzyme solution (or per milligram of protein).

Enzyme Concentration. The concentration of true elastase was determined by titration with diethyl *p*-nitrophenyl phosphate (Bender *et al.*, 1966). Protein concentration was determined by the biuret method (Layne, 1957) using crystalline bovine serum albumin as a standard or by absorbance at 280 mμ.

Chromatography. Crystalline elastase was prepared by the authors from porcine pancreas powder according to the method of Lewis *et al.* (1956). After crystallization it was dissolved at pH 10.4 and treated with DEAE-Sephadex A-50 (Smillie and Hartley, 1964), dialyzed, lyophilized, and chromatographed under either of two sets of conditions. Chromatography at pH 4.60 was done on CM-cellulose (Naughton and Sanger, 1961). A column (2.1 × 45 cm) was equilibrated with 0.05 M ammonium acetate buffer (pH 4.60) and developed with a linear NaCl gradient. Rechromatography of the major component (EL-1-3, Figure 1) was performed on a CM-cellulose column (2.1 × 45 cm) equilibrated with 0.05 M ammonium acetate buffer (pH 4.60), containing 0.11 M NaCl. After elution of three minor components which had no measurable activity on either casein, elastin, or *p*-nitrophenyl

TABLE I: Activity of Various Preparations of Elastase on Casein and on *p*-Nitrophenyl *N*-Benzyloxycarbonyl-L-tyrosinate.[a]

Preparation	Casein[b] (Δ/sec per mg of Protein 1) (× 10^2)	*p*-Nitrophenyl *N*-Benzyloxycarbonyl-L-tyrosinate[c] (moles/l. of Substrate Hydrolyzed per sec per mg of Protein) (× 10^7)
Elastase-1[d]	2.40	55.6
Elastase-1–3[e]	2.17	2.69
Elastase-2[d]	2.58	128
Elastase-2–3[e]	2.58	2.73
Elastase-2–3 (rechromatographed)[f]	2.89	2.86
Elastase-2–2[e]	2.22	18.4
Elastase-2–4[e]	0.0	5.37
Elastase-3[g]	3.81	670
Elastase-7–1 (tube 12)[h]	2.12	14.3
Elastase-7–2 (tube 86)[h]	2.86	0.444
Elastase-7–2 (lyophilized)[h]	2.19	0.331
Fraction 50 of Figures 3 and 4	7.70	4840

[a] Preparations designated elastase-2–2, elastase-2–4, elastase-7–1, and fraction 50 of Figures 3 and 4 had no activity on elastin–orecin; all other preparations had this activity. [b] Activity on 0.5% casein at pH 7.60 and 35°. Initial rate on 5.91×10^{-5} M *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate at pH 8.00 (0.02 M Tris-HCl) and 25° in presence of 12.2% acetonitrile. [d] Crystalline elastase prepared from porcine pancreas powder by the authors. Crystalline elastase prepared as in footnote *d*, then chromatographed on CM-cellulose as described in Figure 1 and text. Elastase-2 refers to a second preparation of crystalline elastase which gave identical results upon chromatography under the same conditions as elastase-1. The designated fractions were pooled, dialyzed, and lyophilized. [f] Elastase-2–3 was rechromatographed on CM-cellulose as shown in Figure 1 (insert). [g] Elastase prepared and chromatographed by the procedure of Loeven (1963). The results are shown in Figures 3 and 4 where this preparation represents the pooling of tubes 21–27, which were then dialyzed and lyophilized. [h] Crystalline elastase, prepared as described in footnote *d*, was chromatographed on CM-cellulose at pH 8.0 (initially equilibrated against 0.02 M Tris-HCl). The results are shown in Figure 2.

N-benzyloxycarbonyl-L-tyrosinate, the major component, with activity against all three substrates, was eluted with 0.05 M ammonium acetate buffer (pH 4.60), containing 0.14 M NaCl. Chromatography at pH 8.00 was done on a CM-cellulose column (2.1 × 50 cm) initially equilibrated against 0.02 M Tris-HCl buffer. A linear NaCl gradient was used for eluting the material (Figure 2).

Crude elastoproteinase was prepared and chromatographed by the procedure described by Loeven (1963). Chromatography was performed on a DEAE-Sephadex A-50 column

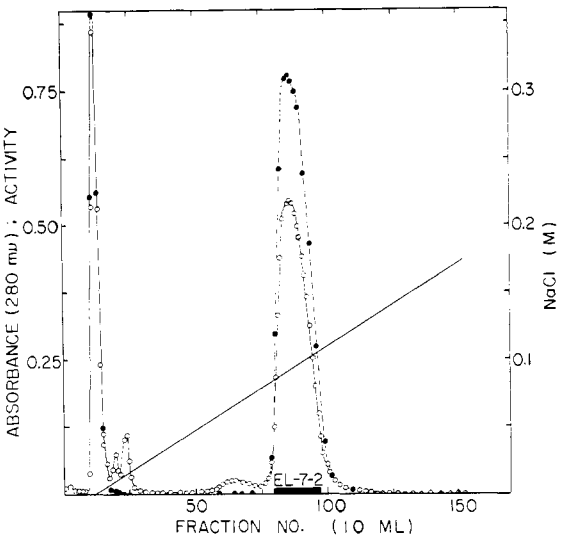

FIGURE 2: Chromatography of crystallized elastase on CM-cellulose at pH 8.0. Chromatography was performed as described in the text. (○) Absorbance at 280 mμ and (●) activity against casein. The major peak was pooled, dialyzed, and lyophilized to give EL-7–2.

(2.1 × 45 cm) equilibrated against 0.0654 M carbonate (pH 8.80). Protein was eluted from the column by increasing the ionic strength stepwise by adding NaCl to the buffer as indicated in Figure 3.

Crystalline elastase preparation no. 5704A (Worthington Biochemical Corp.) was chromatographed on DEAE-Sephadex A-50 at pH 8.8 according to the procedure of Loeven (1963).

Results

Effect of Calcium Ions. Several samples of Worthington crystalline elastase became insoluble in water and most buffers after being stored in the refrigerator for several weeks. Calcium ions were found to be a specific solubilizing agent. Dialysis of the precipitated protein against 0.1 M calcium acetate (pH 4.7), caused it to redissolve. Pipetting the enzyme solution containing calcium ions into citrate buffer caused a slow precipitation of protein. This may be due to chelation of calcium ions by citrate. A specific effect of calcium ions has been reported (Hörmann and Fujii, 1962) and the inhibitory effects of citrate and EDTA have been explained by calcium ion chelation (Hall, 1964). In contrast to crystalline elastase, electrophoretically purified enzyme is quite soluble in a variety of buffer solutions. This agrees with the report of Lewis *et al.* (1956) that electrophoresis of crystalline elastase removes an insoluble protein.

Results of Chromatography. The results of chromatography of crystalline elastase on CM-cellulose are shown in Figures 1 and 2. The fractions were assayed for proteolytic and elastolytic activity. Chromatography of crystalline porcine elastase on CM-cellulose at pH 4.6 separated the material into five components. The first two minor components, EL-1–1, which came off the column near the beginning, had no activity on any of the substrates tested. The remaining three components all had activity on *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate but none had anywhere near the activity

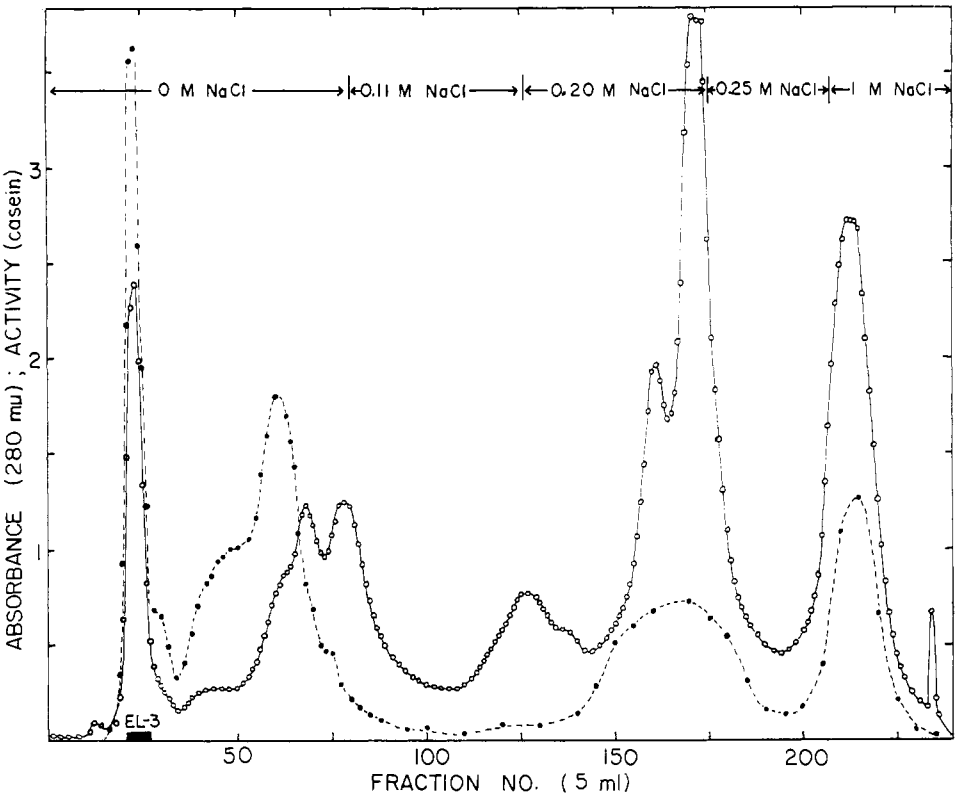

FIGURE 3: Chromatography of elastoproteinase on DEAE-Sephadex A-50 at pH 8.8. Chromatography was performed as described in the text. (○) Absorbance at 280 mμ and (●) activity on casein. The column was eluted with stepwise increases in NaCl concentration in 0.0654 M carbonate as indicated.

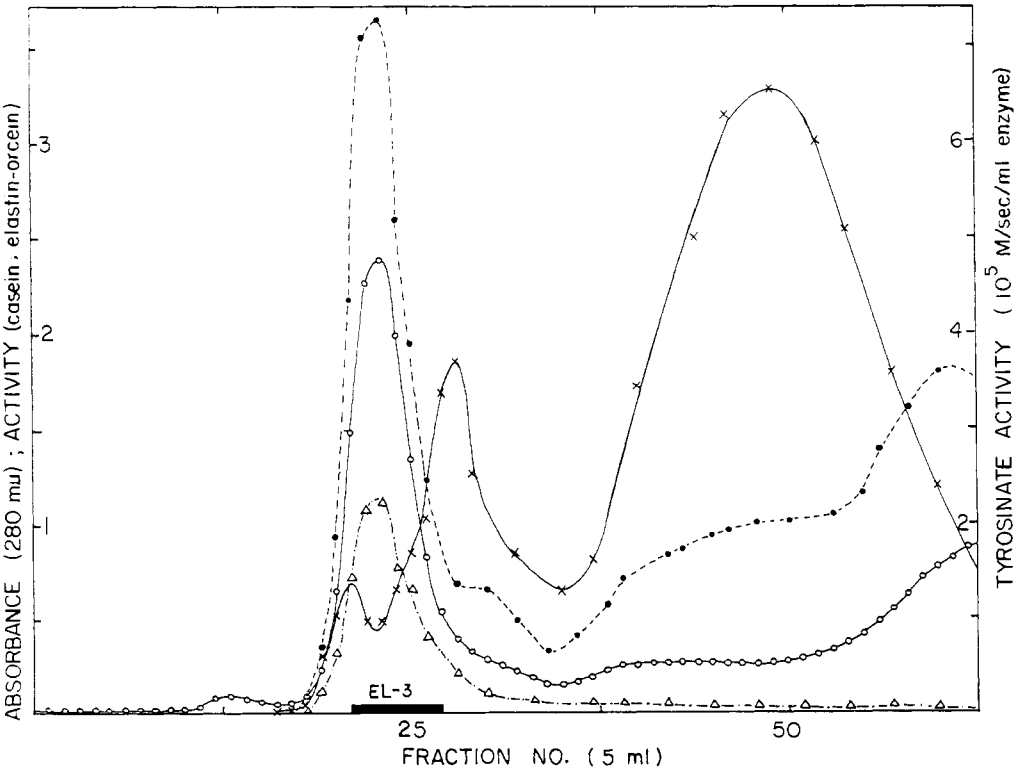

FIGURE 4: Chromatography of elastoproteinase (same data as Figure 3). (○) Absorbance at 280 mμ, (●) activity on casein, (Δ) activity on elastin–orcein, and (X) initial rate of hydrolysis of *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate at pH 8.0 in 12% acetonitrile. Tubes 21–27 were pooled, dialyzed, and lyophilized to produce EI-3 (Table I).

TABLE II: Comparison of the Activities of Worthington Crystalline (A) and Electrophoretically Purified (B) Elastase.[a]

p-Nitrophenyl Ester	Rel Act. (*A*/*B*)
N-Acetyl-L-leucinate	42[b]
N-Acetylglycinate	68[b]
N-Acetyl-L-tryptophanate	129[b]
Trimethylacetate	0.7[c]

[a] Comparison is made on the basis of a standard rate assay. Crystalline elastase (lot 5678) and electrophoretically purified elastase (lot 6505) were used. [b] Assay conditions: pH 5.15, sodium acetate buffer. [c] Assay conditions: pH 8.7, Tris-HCl buffer.

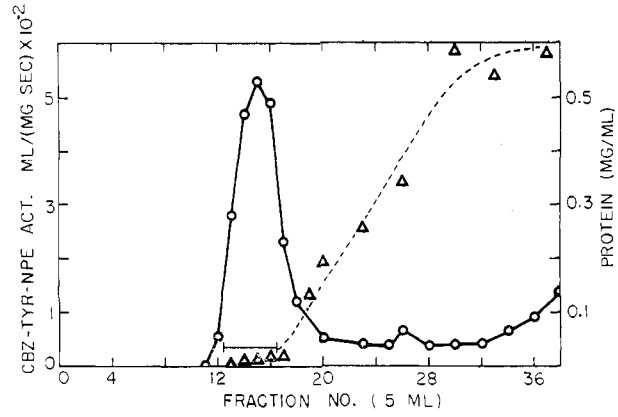

FIGURE 5: Elution pattern obtained from crystalline elastase (Worthington 5704A) by chromatography on DEAE-Sephadex A-50 at pH 8.8 (Loeven, 1963). (O) Protein concentration and (Δ) *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate activity.

of the crystalline material before chromatography (Table I). Components EL-1-2 and EL-1-3 had about the same specific activity on casein but only EL-1-3 (the major component) had activity on elastin-orcein. Rechromatography of component EL-2-3[1] on CM-cellulose (Figure 1 insert) did not change the ratio of elastin, casein, and *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate activities.

Chromatography of crystalline elastase on CM-cellulose at pH 8.0 gave two major components (Figure 2 for elastase preparation 7). Both components had approximately the same specific activity on casein (Table I). Component one had no activity on elastin-orcein and a rather high activity on *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate. Component two had high activity on elastin-orcein and quite low activity on *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate.

Figure 3 shows the results of chromatography of crude elastoproteinase on DEAE-Sephadex A-50. The results for the first 65 fractions are shown in Figure 4 on a larger scale. The fractions were assayed for activity against casein, elastin-orcein, and *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate. The first protein peak (designated EL-3) corresponds to elastoproteinase and the double peak of protein (fractions 65–90) should be considered elastomucase (Loeven, 1963). There are a minimum of five separable components in crude elastoproteinase with activity against casein. The first of these components (fractions 20–25) contains all of the activity against elastin-orcein (Figure 4). As shown in Figure 4 there are three components with activity against *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate. The first two components with activity against this substrate overlap the component with activity on elastin-orcein but are chromatographically distinct from it. The majority of the tyrosinate activity is in the component with a peak of activity at fraction 49. In terms of total protein and activity on casein this component represents a very minor part of crude elastoproteinase. The three tyrosinate enzymes are chromatographically distinct from elastoproteinase and elastomucase and should be considered associated with the "second fraction" of Loeven (1963).

[1] The designation EL-2 refers to a second preparation of crystalline elastase chromatographed under the same conditions and with the same results as EL-1.

Figure 5 shows the results of chromatographing Worthington crystalline elastase on DEAE-Sephadex A-50 at pH 8.8. This produced elastase of the highest purity (highest diethyl *p*-nitrophenyl phosphate titer and the lowest tyrosinate activity) obtained in this research. From Figure 5 it is possible to estimate the maximum amount of tyrosinate enzymes at 2.1% of the total protein present in the main protein peak which titrated to be 85% active elastase against diethyl *p*-nitrophenyl phosphate.

Kinetic Evidence for Heterogeneity of Crystalline Elastase. Table I shows the results of assaying the various factions obtained from the chromatography described in Figures 1–4 for activity against casein and against *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate. With the exception of the preparations designated elastase-2-2, elastase-2-4, elastase-7-1, and fraction 50 of Figures 3 and 4, all preparations had activity against elastin-orcein. There is a remarkable constancy of activity against casein except for elastase-2-4 which had no activity against casein. However, the activity against *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate ranged from 4840 to 0.331 M sec^{-1} mg^{-1}, a variation of some 14,600-fold. Among those preparations which involved a crystallization of elastase the tyrosinate activity ranged from 128 to 2.69 M sec^{-1} mg^{-1} while the activities against casein and against elastin-orcein were constant.

Commercially obtained crystalline elastase is richer than commercial electrophoretically purified elastase in an impurity having amino acid esterase activity. This activity, which is distinct from that of the tyrosinate enzymes, is removed by electrophoresis; this removal slightly enriches the *p*-nitrophenyl trimethylacetate (elastase) activity. These results are given in Table II where representative crystalline and electrophoretically purified enzymes are compared using four substrates.

Table III shows that different preparations of electrophoretically purified elastase contain varying amounts of tyrosinate enzymes. The maximal amount of tyrosinate impurity was 200-fold above that of what we believe to be pure elastase (5704A (81–79)). In contrast, Ling and Anwar (1966) found electrophoretically purified elastase to be essentially free of contamination. The activities reported in

TABLE III: *p*-Nitrophenyl *N*-Benzyloxycarbonyl-L-tyrosinate Activity of Several Purified Elastases.

Electrophoretically Purified Elastase	% Elastase Act.[a]	*N*-CBZ-Tyr-$C_6H_5NO_2$ Act.[b] $\times 10^3$ (ml/mg sec)
5691/923	75	625
6505		338
6506	50	96
6507A	51	458
6507B	62	490
Chromatographed		
5704A (81–79)[c]	85	3.2
7–2[d]	67	11

[a] Activity is recorded as a per cent of a theoretical activity based on protein concentration (A_{280} = 0.54 mg/ml) and an equivalent weight of 25,000. The actual sample activity was determined either by titration with diethyl *p*-nitrophenyl phosphate (Bender *et al.*, 1966) or by a standard rate assay related to such a titration. [b] First-order rate constant for hydrolysis of 1.5×10^{-5} M substrate, pH 7.8, phosphate, 25°, divided by the protein concentration. [c] DEAE-Sephadex A-50, pH 8.8 (see Figure 5). [d] CM-cellulose, pH 8.0 (see Figure 2).

Table III can be related to those in Table I using enzyme 7–2 which appears in each.

Two elastases prepared in this research (7–2 of Tables I and III and 5704A (81–79) of Table III) contained less tyrosinate activity than Worthington crystalline enzyme and Worthington enzyme prepared by crystallization followed by electrophoresis. The pH at which chromatography is performed appears to be of crucial importance since both purifications were carried out at pH 8.0 or above, one using DEAE-Sephadex A-50 (pH 8.8) and the other CM-cellulose (pH 8.80). On the other hand, inferior separation of the elastase and tyrosinate activities was obtained with the latter resin at pH 4.6. Electrophoretically purified elastase contains significantly less tyrosinate activity than crystalline enzyme. Crystalline elastase is richer in enzymatic impurities in addition to the tyrosinate enzymes.

Discussion

The results reported here leave little doubt that preparations of crystalline elastase contain several minor enzymatic impurities. As shown by chromatography on CM-cellulose at pH 4.6 and at 8.0 there are components in crystalline elastase which have activity on casein and on *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate but which have no demonstrable activity on elastin–orcein. These components can be partially removed by repeated chromatography or by preparative electrophoresis of the crystallized material. Chromatography at pH 8.0–8.8 appears to be more effective in this purification than chromatography at pH 4.6. The best preparation of elastase obtained in this research involved the chromatography at pH 8.8 of a preparation from Worthington Biochemical Corp. which had been crystallized and subsequently subjected to electrophoresis (preparation 5704A).

The relationship among the components which possess activity against casein, elastin–orcein, and *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate is best demonstrated by the chromatography of crude elastoproteinase on DEAE-Sephadex A-50 at pH 8.8 (Figures 3 and 4). Chromatography under these conditions shows the presence of three components with tyrosinate activity which are chromatographically distinct from the single component with elastase activity.

The heterogeneity of crystalline elastase has been noted before. Lewis *et al.* (1956) reported that crystalline elastase contains two electrophoretically distinct components in the ratio of approximately 4:1. After removal of the minor component by preparative electrophoresis the preparation was found to be homogeneous by electrophoresis at six different pH values, by ultracentrifugation, and by diffusion. However, it is doubtful if they would have detected traces of components with tyrosinate activity by these procedures. Naughton and Sanger (1961) reported that their preparations of crystalline elastase still possessed activity against *N*-acetyl-L-tyrosine ethyl ester equivalent to 1–2% of the specific activity of beef α-chymotrypsin.

Several workers have reported the presence of two components in crystalline elastase with activity against elastin (Hall, 1957; Czerkawski and Bingle, 1963; Bingle and Czerkawski, 1963; Avrameas and Uriel, 1965). Avrameas and Uriel (1965) found these two elastolytic components to be distinct on the basis of electrophoretic mobility, antigenic properties, specific activity on elastin–congo red, and activation with cysteine. We found no evidence for two components with activity against elastin. However, the presence of a second elastase in our preparation cannot be excluded since elastase 1 of Avrameas and Uriel represents 4.7% of the total protein with elastase activity and has a specific activity of only 7.1% of that of elastase 2. In addition, we did not use cysteine in our reaction mixture which is an activator of elastase-1 (Avrameas and Uriel, 1965).

The presence in porcine pancreas of five components with activity against *N*-acetyl-L-tyrosine ethyl ester has been demonstrated by immunoelectrophoretic and chromatographic techniques (Uriel and Avrameas, 1965; Avrameas and Uriel, 1965). They identified two of these components as chymotrypsins 1 and 2. They designated the third component as elastase-1 because it had a low specific activity on elastin–congo red. It is interesting that this component had the highest specific activity against *N*-acetyl-L-tyrosine ethyl ester of all the components which they isolated from porcine pancreas. The other two components, pancreatic proteases I and II, had a small activity on poly-L-glutamic acid as well as native egg albumin. Avrameas and Uriel (1965) have documented well the distinct nature of these components. It is not possible due to the different methods used, to indicate the exact relationship among tyrosinate enzymes I, II, and III reported in this research and pancreatic proteases I and II and elastase-1 of Avrameas and Uriel or even to decide if any of the enzymes are the same.

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Porcine Elastase. II. Properties of the Tyrosinate-Splitting Enzymes and the Specificity of Elastase*

Thomas H. Marshall,† John R. Whitaker,‡ and Myron L. Bender§

ABSTRACT: Three enzymes catalyzing the hydrolysis of *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate were partially purified from elastoproteinase by chromatography on DEAE-Sephadex A-50 at pH 8.8. The chromatographed enzymes showed complex kinetics including activation by acetate ion, acetonitrile, and reaction products. When the tyrosinate-splitting enzymes were assayed without having been separated from elastase, they exhibited normal kinetics. The tyrosinate enzymes were distinguished from elastase by (1) high activity against *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate, (2) greater sensitivity to inhibition by soybean trypsin inhibitor, and (3) slower rates of reaction with diethyl *p*-nitrophenyl phosphate. The tyrosinate enzymes appear to be different from all the other known esterases of pancreas. The specificity of the tyrosinate enzymes may have possible significance in relation to the transitory nature of the dityrosine cross-linkage in fetal elastin.

The specificity of elastase was measured against eight substrates and compared with that of α-chymotrypsin and hydroxide ion.

Porcine pancreas has been shown to contain three enzymes with high specific activity against *p*-nitrophenyl *N*-benzyloxycarbonyl-L-tyrosinate (Marshall *et al.*, 1969). Preparations of crystalline elastase, including those prepared in these laboratories and those obtained commercially, contained variable amounts of these enzymes. These tyrosinate enzymes are distinct from elastase which has very little if any activity against this substrate. The tyrosinate-splitting enzymes can be partially removed from elastase by electrophoretic purification of crystalline elastase or by repeated chromatography on CM-cellulose or on DEAE-Sephadex A-50, particularly at alkaline pH. In this paper we report some properties of the tyrosinate enzymes which may be used to distinguish them from elastase. We also report on the specificity of elastase as measured against eight substrates and as compared with the specificity of α-chymotrypsin and hydroxide ion. The comparative specificities of elastase and α-chymotrypsin are of interest since the X-ray studies now in progress (Shotton *et al.*, 1968) should reveal in detail the differences in their three dimensional structures.

* From the Department of Chemistry, Northwestern University, Evanston, Illinois 60201, and the Department of Food Science and Technology, University of California, Davis, California 95616. *Received August 25, 1969.* Abstracted in part from the Ph.D. thesis of Thomas H. Marshall, Northwestern University, 1967.

† Present address: Department of Chemistry, Northern Illinois University, DeKalb, Ill. 60115.

‡ Department of Food Science and Technology, University of California, Davis, Calif. 95616.

§ Department of Chemistry, Northwestern University, Evanston, Ill. 60201.