Systematic Fractionation of Swine Pancreatic Hydrolases. II. Fractionation of Enzymes Insoluble in Ammonium Sulfate Solution at 0.40 Saturation*

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ABSTRACT: A systematic fractionation of the swine pancreatic enzymes insoluble in ammonium sulfate solutions at 0.40 saturation has been carried out. The progressive resolution and purification of the enzymes on Sephadex ion exchangers have been followed by immunochemical tests and identification reactions in gel-diffusion media. Two enzymes with elastolytic properties, designated as elastases 1 and 2, have been isolated, as well as the pancreatic protease 1, an enzyme showing hydrolytic activity against *N*-acetyl-L-tyrosine ethyl ester, native egg albumin, and poly-L-glutamic acid. The carboxypeptidases A and B have also been obtained in immunologically pure form. The five isolated enzymes are immunochemically distinct.

Aqueous extracts from swine activated pancreas were separated by salting out with ammonium sulfate in two fractions designated P 0.40 and S 0.40–0.75. One fraction (P 0.40) contained enzymes precipitating in ammonium sulfate solution up to 0.40 saturation; the second fraction was salted out between 0.40 and 0.75 saturation. The systematic fractionation by ion-exchange chromatography of the fraction S 0.40–0.75 was reported in the preceeding paper (Uriel and Avrameas, 1965). In the present work, a similar fractionation of the P 0.40 fraction has been carried out. The gel-diffusion techniques described in the foregoing paper were employed to follow the progressive resolution and purification of the enzymes present in the fractions and to study the antigenic relationships among them.

Material and Methods

The preparation of the P 0.40 fraction from "Pancreatine" and of the rabbit antiserum against pancreatic extract have been described in article I of this series (Uriel and Avrameas, 1965). Furthermore, the same ion exchangers were utilized, and the techniques for electrophoresis, immunoelectrophoresis, and gel diffusion, as well as the identification reactions on antigen-antibody precipitates, are also described in the preceding paper.

Synthetic Substrates. Carbonaphthoxy-DL-phenylalanine and hippuryl-L-arginine were purchased from Mann Research Laboratories (New York, N.Y.), and poly-L-glutamic acid from Yeda (Israel).

Natural Substrates. Elastine was obtained from Sigma Chemical Co., St. Louis, Mo.

Quantitative Assays for Enzymes. Chymotryptic activity was measured by a continuous-titration method (Kaufman *et al.*, 1949) using ATEE[1] as substrate. Hydrolytic activity against poly-L-glutamic acid was determined by a similar method (Gjessing and Hartnett, 1962). Elastolytic activity was determined by the elastine–Congo red method (Naughton and Sanger, 1961) with minor modifications. A spectrophotometric method (Folk *et al.*, 1960) was used to estimate the hydrolytic activity of carboxypeptidase B.

Carboxypeptidase A activity was measured using a spectrophotometric technique similar to that employed for leucine aminopeptidase analysis (Uriel and Avrameas, 1965). The rate of formation of free 2-naphthol from carbonaphthoxy-DL-phenylalanine (Ravin and Seligman, 1951) was followed at 328 mμ in a Beckman Model DU spectrophotometer.

Protein Assays. Protein content was determined by a modified biuret method (Uriel, 1961).

Fractionation and Chromatography of Fraction P 0.40. Fraction P 0.40 (2 g) was suspended in 20 ml of 0.01 M potassium phosphate buffer, *p*H 6.5, and dialyzed for 14 hours at 4° against 10 liters of the same buffer. The suspension was centrifuged for 30 minutes at 4° and 17,000 *g*. The pellet fraction PP 0.40 was collected and stored at −20°. The supernatant, fraction SP 0.40, was placed on a column of DEAE-Sephadex A-50 medium, equilibrated with 0.01 M phosphate buffer at *p*H 6.5. A stepwise gradient of phosphate buffer at *p*H 6.5 and of increasing molarity from 0.01

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[1] Abbreviation used in this work: ATEE: *N*-acetyl-L-tyrosine ethyl ester.

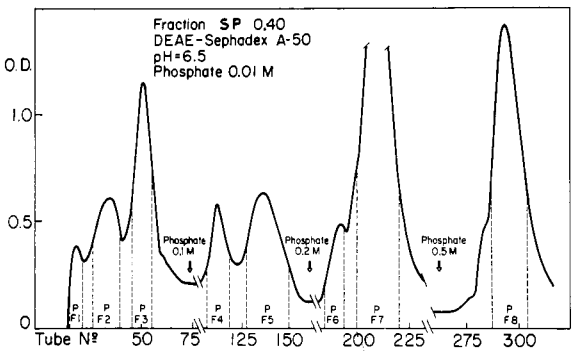

FIGURE 1: Elution diagram of the SP 0.40 fraction. In each peak, the two vertical interrupted lines delineate the eluents combined and designated as fractions PF1, PF2 . . . PF8. Arrows are positioned at the points where the different buffers were applied to the column.

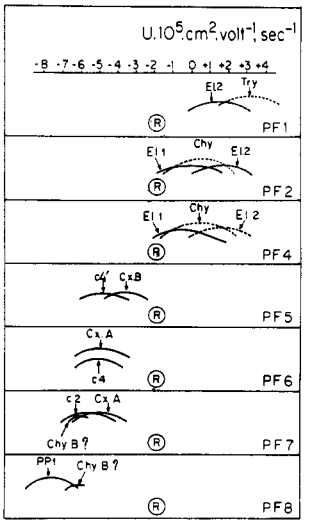

FIGURE 2: Immunoelectrophoretic diagram of the PF fractions obtained from the chromatographic resolution of the SP 0.40 fraction. The antigenic constituents were revealed with a rabbit serum antiswine whole pancreatic extract. Minor constituents in any given fraction are represented as interrupted arcs. Abbreviations: E1.1 and E1.2 (elastases 1 and 2), Chy. (chymotrypsin), Try (trypsin), Cx.A and Cx.B (carboxypeptidases A and B), Chy.B (chymotrypsin B), PP1 (pancreatic protease 1), c2, c4′ (antigenic constituents 2 and 4′), R (starting reservoir).

to 0.5 M was applied to the column giving a flow rate of 3–4 ml/minute at 15°. Separated fractions of 11 ml each were collected. The elution diagram, represented in Figure 1, was obtained by measuring the optical density of the eluates at 280 mμ. The eluates were mixed as indicated on the diagram and designated as fractions PF1, PF2, . . . PF8.

For analytical purposes samples of the PF fractions were saturated with ammonium sulfate and centrifuged. The precipitates were collected in demineralized water,[2] dialyzed against water, and lyophilized. The fractions were subjected to electrophoretic and immunoelectrophoretic analyses in agarose. Identification reactions were then carried out in the gels in order to demonstrate the enzymes and other constituents present. The immunoelectrophoretic patterns of the PF fractions are represented in Figure 2.

No further purification of fractions PF1, PF2, PF3, and PF6 was attempted. The first two fractions contained small amounts of trypsin and chymotrypsin. The isolation of these enzymes was carried out from the S 0.40–0.75 fraction (Uriel and Avrameas, 1965). No protein material was recovered from fraction PF3. The PF6 fraction contained a small amount of a carboxypeptidase A enzyme, immunochemically identical to the carboxypeptidase A present in the PF7 fraction but having a slower electrophoretic mobility (see Figure 2).

Chromatography of Fraction PF4 and PP 0.40: Isolation of Elastases 1 and 2. Solid ammonium sulfate was added progressively to the PF4 fraction up to 0.60 saturation. The suspension was allowed to stand for 30 minutes at room temperature, and centrifuged.[3] The precipitate was collected in 10 ml water and dialyzed for 14 hours against two 10-liter volumes of water, and for 6 hours against 10 liters 0.01 M phosphate buffer, *p*H 6.5. After dialysis the bag contents were centrifuged and the supernatant solution was stored at −20° until used. This fraction was thawed at 4° and chromatographed at this temperature in a column of CM-Sephadex (40 × 2 cm) equilibrated with 0.01 M phosphate buffer, *p*H 6.5. A discontinuous gradient elution was performed with phosphate buffer starting at 0.01 M, *p*H 6.5, and followed by 0.05 M at the same *p*H. The elution rate was 75 ml/hour and fractions of 4.5 ml each were collected. The elution diagram is represented in Figure 3. Fractions eluting with 0.05 M phosphate buffer were mixed as indicated in the figure. These were then precipitated with ammonium sulfate at 0.60 saturation and centrifuged. The precipitate, collected in 3–4 ml water, was dialyzed for 16 hours against three 10-liter volumes of water. The precipitate formed during the dialysis was discarded after centrifugation and the supernatant (elastase 1) was stored at −20°.

Fraction PP 0.40 was suspended in 15 ml of 0.1 M phosphate buffer at *p*H 6.5. The suspension was gently stirred for 15 minutes and then centrifuged for 15 minutes at 17,000 *g* and 4°. The precipitate was discarded and the supernatant was chromatographed at 4° in a column of CM-Sephadex (32 × 2 cm)

[2] Unless otherwise specified demineralized water was used throughout this procedure. Dialyses were performed in a cold room at 4°.

[3] Unless otherwise specified, the ammonium sulfate-precipitated fractions were centrifuged for 30 minutes at 4° and 17,000 *g* whereas the dialyzed fractions were centrifuged for 15 minutes at 4° and 17,000 *g*.

TABLE I: Quantitative Data on the Hydrolysis of Elastin–Congo Red.[a]

Fraction	Total Protein (mg)[b]	Total Units[c]	Specific Activity[c]
Aqueous extracts[d]	13,608	2,585,520	190
S 0.40–0.75	1,525	106,750	70
SP 0.40	2,352	437,472	186
PP 0.40	749	749,000	1000
PF4	57.7	2,365	41
Elastase 1 (from PF4 fraction)	2.41	279	106
Elastase 2 (from PP 0.40 fraction)	49.06	73,010	1490

[a] The elastase-containing fraction was diluted in 0.05 M Tris buffer, *p*H 8.8. One ml of the solution was added to 10 mg of elastin–Congo red suspended in 3 ml of the same buffer. Incubation time was 120 minutes at 37°. Readings of solubilized Congo red were made at 500 mμ in a Beckman Model DU spectrophotometer. [b] Data based on 100 g Pancreatine powder. [c] One unit is defined as the quantity of elastase which solubilizes 1 mg of elastin after 120 minutes incubation at 37°. Specific activity is expressed in units per mg of protein. [d] Prepared as previously described (Uriel and Avrameas, 1964).

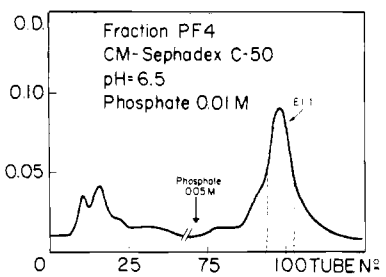

FIGURE 3: Elution diagram of the fraction PF4 showing the isolation of elastase 1 (E1.1). Vertical interrupted lines delineate the eluates combined. Arrows are positioned at the points where the different buffers were applied to the column.

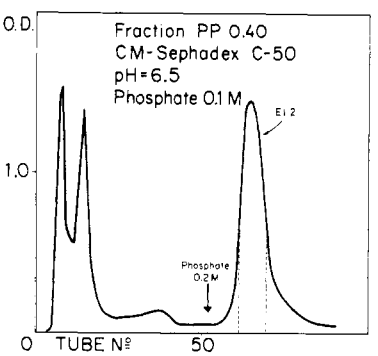

FIGURE 4: Elution diagram of fraction PP 0.40 showing the isolation of elastase 2 (E1.2). Vertical interrupted lines delineate the eluates combined. Arrows are positioned at the points where the different buffers were applied to the column.

equilibrated with 0.1 M phosphate buffer, *p*H 6.5. The column was washed with this buffer to eliminate the unwanted proteins and then 0.2 M phosphate buffer was passed at a flow rate of 2 ml/min. The resulting eluates of 10 ml each were combined (see Figure 4) and treated according to the procedure utilized for fraction PF 4. After dialysis, the uncentrifuged suspension (elastase 2) was stored at −20°. The preparations of elastase 1 and 2 were subjected to immunoelectrophoresis and double diffusion in gel media. Each preparation appeared to contain only one antigen (Figure 5). The two antigens were immunologically distinct. In addition, using the Osserman (1960) technique, a reaction of immunological identity has been observed between our elastase 2 and a commercial preparation of elastase (Sigma, St. Louis, Mo.). Data on elastolytic activity of these preparations as well as those of the intermediate fractions from the pancreatic extracts are recorded in Table I.

It can be seen in Table II that the two elastases show significant differences in physicochemical and catalytic properties. It is worth noting that the ATEE-hydrolyzing activity of elastase 1 is about sixty times greater than that of the elastase 2. The elastolytic activity of the elastase 2 is, on the other hand, almost thirteen times greater than that of the elastase 1. In addition, the activity of the elastase 1 against elastin–Congo red is increased by 45% in the presence of cysteine while the activity of elastase 2 remains unchanged under the same conditions. The catalytic properties against ATEE and elastin–Congo red of both enzymes are completely inhibited by DFP at a final concentration of 10^{-3} M. Neither elastase 1 nor elastase 2 hydrolyzes poly-L-glutamic acid.

Chromatography of Fraction PF5: Isolation of Carboxypeptidase B. The PF5 fraction was precipitated with ammonium sulfate under the same conditions as described for fraction PF4. The precipitate was col-

TABLE II: Some Physicochemical and Catalytic Properties of Elastases 1 and 2.

	Elastase 1	Elastase 2
Electrophoretic mobility (U)	−0.60[a]	+1.48[a]
Solubility in water	Very soluble	Almost insoluble
Elastolytic activity on elastine–Congo red	112	1490
Elastolytic activity (cysteine added)[b]	160	1490
Esterase activity on ATEE	230	3.70
Chromatography on CM-Sephadex, 0.01 M phosphate buffer, *p*H 6.5	Elution with 0.05 M phosphate buffer	Elution with 0.2 M phosphate buffer
Chromatography on SE-Sephadex, 0.01 M phosphate buffer, *p*H 5.5	Elution with 0.07 M phosphate buffer	Elution with 0.15 M phosphate buffer

[a] U × 10^5, v^{-1} sec^{-1} in agarose buffered with 0.025 M Veronal, *p*H 8.2. [b] Same experimental conditions as those described in Table I, except that the incubation mixture contains cysteine at 2.5 × 10^{-2} M final concentration.

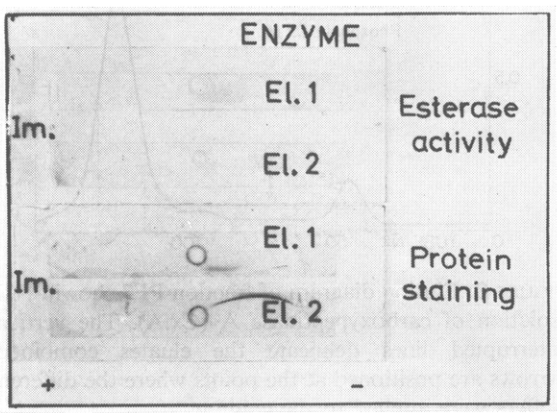

FIGURE 5: Immunoelectrophoretic analysis of elastases 1 and 2 revealed with a rabbit serum antiswine pancreatic extract (Im.). Note that elastase 2 lacks esterase activity against ATEE.

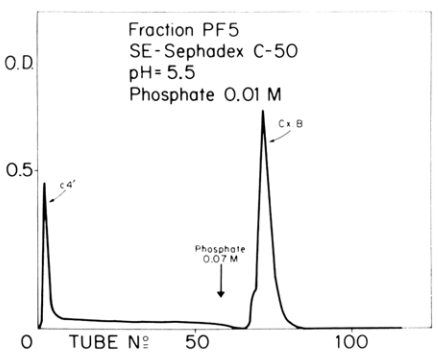

FIGURE 6: Elution diagram of fraction PF5 showing the isolation of carboxypeptidase B (Cx.B). The vertical interrupted lines delineate the eluates combined. Arrows are positioned at the points where the different buffers were applied to the column.

lected in 10 ml water and dialyzed for 14 hours against two 10-liter volumes of water, followed by a 6-hour dialysis against 10 liters of 0.01 M phosphate buffer, *p*H 5.5. After centrifugation, the supernatant was stored at −20° until used. The thawed fraction was placed on a refrigerated column (+4°) of SE-Sephadex (30 × 2 cm) equilibrated with 0.01 M phosphate buffer, *p*H 5.5. Starting with this buffer, a stepwise gradient was applied to the column at an elution rate of 120 ml/hour. Fractions of 8.5 ml each were eluted with 0.07 M phosphate buffer and mixed as indicated in Figure 6. The purification procedure was carried out as described above for fraction PF4. The final supernatant which contained carboxypeptidase B was stored at −20°.

The immunochemical tests revealed only one antigen in this preparation. This antigen was shown by identification reactions in gel-diffusion media to possess hydrolytic activity against hippuryl-L-arginine and hippuryl-L-lysine.

Data on the catalytic activity of this preparation as well as those of intermediate fractions from the aqueous extracts are given in Table III. The value of 14,600 units/mg of protein for the specific activity of the isolated carboxypeptidase B is slightly lower than the value previously obtained (Folk *et al.*, 1960).

More than 50% of the total carboxypeptidase B activity remained in the S 0.40–0.75 fraction (see Table III). However, the carboxypeptidase B isolation from this source was abandoned, since when fraction S 0.40–0.75 was chromatographed on DEAE-Sephadex carboxypeptidase B and PP2 enzyme were eluted in a single fraction (Uriel and Avrameas, 1965). No further attempts were made to purify carboxypeptidase B from this fraction as subsequent subfractionation was accompanied by a rapid and progressive degradation and loss of enzymatic activity. The immunoelectro-

TABLE III: Quantitative Data on the Hydrolysis of Hippuryl-L-arginine.

Fraction	Total Proteins[a] (mg)	Total Units[b]	Specific Activity[b]
Water extracts[c]	13,068	7,076,160	520
S 0.40–0.75	1,525	3,766,750	2,470
SP 0.40	2,352	2,929,240	1,245
Carboxypeptidase B (from PF5 fraction)	24.14	352,364	14,600

[a] Data based on 100 g of pancreatine powder. [b] Units of activity are defined as per cent hydrolysis per minute of a solution of hippuryl-L-arginine at a concentration of 0.001 M (Folk *et al.*, 1960). Specific activity is expressed in units per mg of protein. [c] Prepared as described previously (Uriel and Avrameas, 1964).

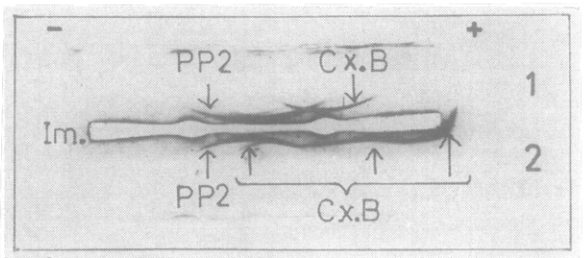

FIGURE 7: Immunoelectrophoretic analysis of two fractions SF5 revealed with a rabbit serum antiswine whole pancreatic extract (Im.). (1) The fraction SF5 was analyzed immediately after its chromatographic elution. (2) The fraction SF5 was dialyzed (12 hours; 4°) against neutral demineralized water. See details in the text.

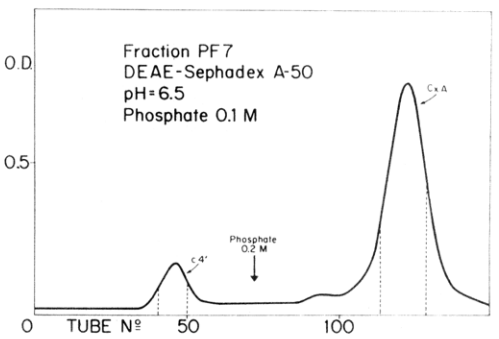

FIGURE 8: Elution diagram of fraction PF7 showing the isolation of carboxypeptidase A (Cx.A). The vertical interrupted lines delineate the eluates combined. Arrows are positioned at the points where the different buffers were applied to the column.

phoretic pattern of the enzymes changed in the manner shown in Figure 7. The modified forms of the enzyme still reacted with the homologous antibody to carboxypeptidase B, but they had lost some of their catalytic properties, as witnessed by subsequent enzymatic tests in gel.

Chromatography of Fraction PF7: Isolation of Carboxypeptidase A. The PF7 fraction was precipitated with ammonium sulfate at 0.60 saturaton. After centrifugation, the precipitate was collected in 10 ml water and dialyzed for 14 hours against two 10-liter volumes of water and for 6 hours against 10 liters of 0.1 M phosphate buffer, *p*H 6.5. After centrifugation, the supernatant was stored at −20° until use. After thawing at 4° the fraction was chromatographed at this temperature on a column of DEAE-Sephadex (40 × 2 cm) equilibrated with 0.1 M phosphate buffer, *p*H 6.5. The chromatogram (Figure 8) was developed by a stepwise gradient with aliquots of 0.1 and 0.2 M phosphate buffer at *p*H 6.5. Eluates were collected in 10-ml fractions at an elution rate of 60 ml/hour. The fractions obtained with 0.2 M phosphate were mixed as indicated in Figure 8. The combined fraction was precipitated with ammonium sulfate at 0.60 saturation and centrifuged. The precipitate was collected in 3–4 ml water and dialyzed for 16 hours against three 10-liter portions of water. A fine suspension formed to which was added solid LiCl up to 0.1 M concentration. The suspension was adjusted to *p*H 7.5 with 0.1 M NaOH, gently stirred at room temperature for 15 minutes, and centrifuged at 4° during 15 minutes at 12,000 *g*. The supernatant containing carboxypeptidase A was stored at −20°.

Only one antigen was revealed in this preparation by immunological tests. The enzyme-antibody complex showed hydrolytic activity against carbonaphthoxy-DL-phenylalanine and carbobenzoxyglycyl-L-phenylalanine. Activity data using the former substrate are recorded in Table IV for the isolated enzyme as well as for the more crude preparations.

The isolated enzymes hydrolyze poly-L-glutamic acid at a rate of 0.30 μmole of NaOH per minute per mg of protein (25°, *p*H 5.3). The hydrolysis was not inhibited by DFP up to a final concentration of 10^{-3} M.

Chromatography of Fraction PF8: Isolation of Pancreatic Protease 1 (PP1). The PF8 fraction was precipi-

TABLE IV: Quantitative Data on the Hydrolysis of Carbonaphthoxy-DL-phenylalanine.[a]

Fraction	Total Proteins (mg)[b]	Total Units[c]	Specific Activity[c]
Water extracts[d]	13,608	1,088	0.08
S 0.40–0.75	1,525	152	0.10
SP 0.40	2,352	681	0.29
PF6	254	196	0.77
Carboxypeptidase A	113	101	0.90

[a] The carboxypeptidase A fraction was diluted in 0.05 M Tris buffer, *p*H 7.65. One ml of the solution was added to 4 ml of 3.125×10^{-3} M carbonaphthoxy-DL-phenylalanine. Readings were made at 328 mμ in a Beckman Model DU spectrophotometer provided with a temperature-controlled cell regulated at 37°. [b] Data based on 100 g of Pancreatine powder. [c] One unit is defined as the amount which liberates 1 μmole of β-naphthol in 1 minute. Specific activity is expressed in units per mg of protein. [d] Prepared as previously described (Uriel and Avrameas, 1964).

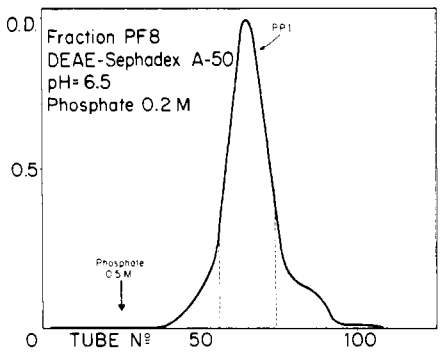

FIGURE 9: Elution diagram of fraction PF8 showing the isolation of pancreatic protease 1 (PP1). The vertical interrupted lines delineate the eluates combined. Arrows are positioned at the points where the different buffers were applied to the column.

tated with ammonium sulfate at 0.60 saturation and centrifuged. The precipitate was collected in 10 ml of water and dialyzed for 14 hours against two 10-liter portions of water and for 6 hours against 10 liters of 0.2 M phosphate buffer, *p*H 6.5. After centrifugation, the supernatant was stored at −20° until use.

The fraction was subsequently chromatographed at 4° on a column of DEAE-Sephadex (25 × 2 cm) equilibrated with 0.2 M phosphate buffer, *p*H 6.5. A stepwise gradient, starting with the same buffer, was applied for elution at a rate of 138 ml/hr. Fractions of 7 ml each were eluted with 0.5 M phosphate buffer, combined as indicated in Figure 9 and precipitated with ammonium sulfate at 0.80 saturation. The isolation of this fraction was continued according to procedure utilized for the isolation of PP1 from the SF8 fraction (Uriel and Avrameas, 1965). Immunological tests in agarose with this preparaton showed it to be identical with the PP1 enzyme isolated from SF8 fraction, and also with another preparation which has been designated as pancreatic protease 1 and obtained by a different method (Uriel and Avrameas, 1963).

Discussion

Concerning the enzymes isolated from the P 0.40 fraction, the following remarks are of interest: An enzyme possessing elastolytic properties has been isolated from swine pancreatic extracts (Lewis *et al.*, 1956; Hall, 1957; Naughton and Sanger, 1961). The preparation obtained by us from similar extracts and designated elastase 2 can be considered as identical with the elastase described by these authors. Thus both enzymes are euglobulin in nature and possess very similar chromatographic properties (Naughton and Sanger, 1961; Baumstark *et al.*, 1963).

Naughton and Sanger first observed that purified pancreatic elastase possesses an esterase activity against ATEE which represented 1–2% of that of beef α-chymotrypsin. The ATEE-hydrolyzing activity of the elastase 2 preparation isolated by us was 1.3% that of a commercial preparation of bovine α-chymotrypsin.

The existence of a second elastolytic enzyme in swine pancreas has been suggested (Hall, 1957; Czerkawski and Bingle, 1963; Bingle and Czerkawski, 1963). After starch electrophoresis of a crude elastase preparation, Dvonch and Alburn (1959) noted the presence of four electrophoretic constituents. Eluted from the gel, two of these showed elastolytic properties, but no further studies were attempted. On the other hand, we have reported in an earlier paper (Uriel and Avrameas, 1964) the existence of two zones of elastolytic activity on swine pancreatic extracts submitted to electrophoresis and immunoelectrophoresis in agarose.

It has been reported (Thomas and Partridge, 1960) that the elastolytic activity of a crude elastase preparation was increased upon the addition of cysteine. These authors suggested that this activation by cysteine was an effect on a single pancreatic elastase rather than the demonstration of the presence of a second distinct elastolytic enzyme. However, according to our results only the elastase 1 is potentiated by cysteine. This elastase 1 appears to be a distinct enzyme as judged by its antigenic, chromatographic, and catalytic properties. It is worth noting that the specific activity of elastase 1 against ATEE is the highest among all the isolated enzymes from swine pancreas. This is the sole catalytic property which elastase 1 has in common with the four other ATEE-hydrolyzing enzymes (chymotrypsins 1 and 2, PP1 and PP2) isolated from the S 0.40–0.75 fraction. Thus it was shown that

elastase 1 has no activity against poly-L-glutamic acid or native egg albumin, substrates which are hydrolyzed by the other four enzymes.

The isolation of carboxypeptidase A from swine pancreas was described (Folk, 1963; Folk and Schirmer, 1963) during the time when this work was in progress. These authors reported the existence of three electrophoretically distinct forms of the enzyme and succeeded in isolating two of them, which they designated as carboxypeptidase A_1 and A_2. These two forms probably correspond to the carboxypeptidase which we have found in PF6 and PF7 fractions. Both are immunologically identical and differ only in their electrophoretic and chromatographic properties.

These findings, together with the recent observation of multiple molecular forms of beef pancreatic carboxypeptidase A (Bargetzi *et al.*, 1963; Cox *et al.*, 1964) and the isolation of two molecular forms of swine chymotrypsins (Uriel and Avrameas, 1965), suggest that molecular multiplicity of single enzymatic species is probably not an unusual characteristic of pancreatic hydrolases.

It has been reported (Green and Stahmann, 1952) that carboxypeptidase A from beef pancreas hydrolyzes poly-L-glutamic acid, and it was suggested that other enzymes able to hydrolyze this substrate should be present in pancreatic extracts. Our present studies confirm this point. In addition to swine carboxypeptidase A, other enzymes (chymotrypsins 1 and 2, and pancreatic proteases 1 and 2) from the same source possess a hydrolyzing activity against poly-L-glutamic acid.

In the present work, swine pancreatic powder from the autolyzed organ was used as starting material. When different lots of the commercial pancreatic powder Pancreatine were studied by immunochemical tests, the same total number of enzymic constituents was found, provided that their concentrations in the starting extracts were adequate. Alternatively, we have prepared from fresh swine pancreas an acetone powder containing zymogens almost exclusively (Henry, 1961). The aqueous extract of this powder was activated with small amounts of trypsin and analyzed. The immunochemical results obtained were identical qualitatively to those results obtained with aqueous extracts of the commercial preparation. Other commercial swine pancreatic powders were analyzed and found also to possess similar enzymic composition. However, some variations in the quantitative distribution of individual constituents were observed with different preparations. Since no qualitative differences were found between the various preparations of pancreas tested, a single large supply of Pancreatine was used in these studies.

The demonstration by immunochemical methods of a multiplicity of proteins possessing an ATEE-hydrolyzing activity raises the question of whether this multiplicity represents an artifact resulting from partial degradation of the native enzymes, particularly during the autolytic activation of the starting material. However, the following observations support the view that these ATEE-hydrolyzing enzymes are normal constituents of the swine pancreas: (a) The constancy of their presence, as distinct antigens, in all the aqueous extracts of swine pancreatic powders which were analyzed. (b) If partial degradation occurs, it may be detectable by means of immunochemical and immunological tests. Such an observation was made in this study in the case of carboxypeptidase B. The degradation of the enzyme, resulting in reduction of enzymatic activity, produced a marked change in the immunological property of this constituent. This type of immunological modifications was not seen either before, during, or after the isolation of the five ATEE-hydrolyzing enzymes. (c) The significant differences observed in catalytic activities of the two molecular forms of chymotrypsin and the three other isolated enzymes (pancreatic proteases 1 and 2, elastase 1) using poly-L-glutamic acid as substrate provided evidence for the presence of four distinct proteolytic enzymes in swine pancreas. (d) Experiments in progress in this laboratory indicate that at least three immunochemically distinct precursors possessing potential hydrolytic activity against ATEE are present in swine pancreas. (e) The existence in swine pancreas of more than one precursor with ATEE-hydrolyzing potential has been suggested (Desnuelle and Rovery, 1961).

The final yields of the different enzymes isolated in these studies were of necessity lower than those normally reported: since the starting material was a mixture of enzymes in their active state and since we wished to identify the total number of enzymes present, it was anticipated that some denaturation and/or loss of activity by extensive degradation would occur.

A number of hydrolases were identified but not isolated because of their low concentration (deoxyribonuclease, constituents c1 and c4) or instability (lipase, α-amylase, constituents c2) under the conditions of fractionation employed. As deduced from the immunoelectrophoretic analysis and from the activity data, the distribution of the different hydrolases between the two major fractions (S 0.40–0.75 and P 0.40) of swine pancreas extracts varied greatly, and different degrees of saturation with ammonium sulfate did not result in appreciably better distributions. With the exception of the pancreatic protease 1, all the enzymes were isolated from one of the two salted-out fractions. The final choice of a fraction to be used for further purification of a given enzyme was based on preliminary chromatographic treatments and systematic control of the resolved fractions by immunodiffusion methods.

The degree of purity of each isolated enzyme was determined by immunological tests. In order to establish its catalytic properties, each preparation was systematically checked by identification reactions in gel media against all the substrates used. According to the information gained in this fashion, activity data were obtained on the substrates which were actively hydrolyzed by each of the enzymes.

In summary, in these studies the following enzymes have been isolated as immunochemically pure antigens: trypsin, chymotrypsins 1 and 2, pancreatic proteases 1 and 2, elastases 1 and 2, carboxypeptidases A and B,

ribonuclease, and leucine aminopeptidase. In addition to the systematic fractionation of swine pancreas extracts, another purpose of the present work was to check the usefulness of an immunochemical approach, both as an analytical tool and as a source of information on the antigenic structure of pancreatic hydrolases.

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References

Bargetzi, J. P., Sampath Kumar, K. S., Cox, D. J., Walsh, K. A., and Neurath, H. (1963), *Biochemistry 2*, 1463.

Baumstark, J. S., Bardawil, W. A., Sbara, A. H., and Hayes, N. (1963), *Biochim. Biophys. Acta 77*, 676.

Bingle, J. P., and Czerkawski, J. W. (1963), *Biochem. J. 87*, 34 P.

Cox, D. J., Bovard, F. C., Bargetzi, S. M., Walsh, K. A., and Neurath, H. (1964), *Biochemistry 3*, 44.

Czerkawski, J. W., and Bingle, J. P. (1963), *Biochem. J. 87*, 33.

Desnuelle, P,, and Rovery, M. (1961), *Advan. Protein Chem. 16*, 139.

Dvonch, W., and Alburn, H. E. (1959), *Arch. Biochem. Biophys. 79*, 146.

Folk, J. E. (1963), *J. Biol. Chem. 238*, 3895.

Folk, J. E., Piez, K. A., Caroll, W. R., and Gladner, J. A. (1960), *J. Biol. Chem. 235*, 2272.

Folk, J. E., and Schirmer, E. W. (1963), *J. Biol. Chem. 238*, 3884.

Gjessing, E. C., and Hartnett, J. C. (1962), *J. Biol. Chem. 237*, 2201.

Green, M., and Stahmann, M. A. (1952), *J. Biol. Chem. 197*, 771.

Hall, D. A. (1957), *Arch. Biochem. Biophys. 67*, 366.

Henry, H. J. (1961), *Biochem. Prepn. 8*, 102.

Kaufman, S., Neurath, H., and Schwert, G. W. (1949), *J. Biol. Chem. 177*, 793.

Lewis, J. U., Williams, D. E., and Brink, H. G. (1956), *J. Biol. Chem. 222*, 705.

Naughton, M. A., and Sanger, P. (1961), *Biochem. J. 78*, 156.

Osserman, E. (1960), *J. Immunol. 84*, 93.

Ravin, H. A., and Seligman, A. M. (1951), *J. Biol. Chem. 190*, 217.

Thomas, J., and Partridge, S. M. (1960), *Biochem. J. 74*, 600.

Uriel, J. (1961), *Biol. Med. (Paris) 50*, 23.

Uriel, J., and Avrameas, S. (1963), *Compt. Rend. 257*, 3247.

Uriel, J., and Avrameas, S. (1964), *Ann. Inst. Pasteur 106*, 396.

Uriel, J., and Avrameas, S. (1965), *Biochemistry 4*, 1740 (this issue; preceding paper).