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

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ABSTRACT: A systematic fractionation of aqueous extracts from autolyzed swine pancreas has been carried out by salting-out and ion-exchange chromatographic methods. The extracts were first separated into two major fractions by precipitation with ammonium sulfate. This paper deals with the chromatographic resolution on Sephadex ion exchangers of the hydrolases soluble in ammonium sulfate solution up to 0.40 saturation. Immunochemical tests in gel-diffusion media were used to follow the progressive purification of the pancreatic constituents and to study their immunochemical relationships. No further purification of an enzyme preparation was carried out when the proportion of impurities detectable by immunochemical methods was estimated to be less than 1% of the total protein content. Four preparations capable of hydrolyzing acetyl-L-tyrosine ethyl ester and poly-L-glutamic acid have been isolated and designated as chymotrypsins 1 and 2 and pancreatic proteases 1 and 2. The chymotrypsins 1 and 2 are identical antigenically and probably represent molecular forms of the same enzyme. However, the pancreatic proteases 1 and 2 appear to be antigenically distinct preparations. The four preparations have been shown to differ in physicochemical, catalytic, and chromatographic properties. Trypsin and an enzyme with leucine aminopeptidase activity have been isolated. The immunochemical approach to the study of pancreatic enzymes with recently developed methods of analysis of antigen mixtures is useful, both as an analytical tool and as a source of information on the antigenic relationship among the enzymes.

Fractionation by chromatographic methods of beef and swine pancreatic juice have been published (Keller *et al.*, 1958; Marchis-Mouren *et al.*, 1961). In both studies the enzymes were resolved by a single passage on DEAE-cellulose followed by a single passage on a cationic exchanger for the nonadsorbed proteins. No further purification of these fractions was attempted.

Our previous studies (Uriel and Avrameas, 1964a) on aqueous extracts of autolyzed swine pancreas, using specific identification techniques applied after electrophoresis and immunoelectrophoresis in agarose, have revealed sixteen immunochemically distinct antigens. From among these, almost all of the known swine pancreatic hydrolases have been identified and located on immunoelectrophoretic diagrams. In addition, three new enzymes have been demonstrated, two of which were later isolated (Uriel and Avrameas, 1963).

The present work is a systematic fractionation of aqueous extracts from autolyzed swine pancreas by salting-out and ion-exchange chromatographic methods. Immunochemical tests in gel-diffusion media for the identification of the enzyme or enzymes present in the fractions were used to follow the progressive resolution and purification of each particular enzyme. Processes such as the inactivation of catalytic properties or the formation of degradation products can be detected by these methods. Simultaneously, a study of the immunochemical relationships among the enzymes separated from the extracts has been carried out.

The extracts were first separated into two major fractions by precipitation with ammonium sulfate. This report concerns the study of the fraction containing the hydrolases soluble in ammonium sulfate solutions at 0.40 saturation. The chromatographic resolution of the fraction containing the constituents insoluble at 0.40 saturation is reported in the second part of this work (Avrameas and Uriel, 1965).

Materials and Methods

Ion-Exchange Chromatography. The following ion exchangers purchased from Pharmacia (Uppsala, Sweden) were used: DEAE-Sephadex A-50 medium (lots 3303 and 1602; 3.5 ± 0.5 meq/g); CM-Sephadex C-50 medium (lot 160M, 4.7 meq/g); SE-Sephadex C-50 medium (lot 8694, 2.3 meq/g).

The cationic resins CM- and SE-Sephadex were regenerated according to the instructions given by the manufacturer. DEAE-Sephadex exchanger was re-

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† The material presented herein has been taken, in part, from a thesis submitted to the University of Paris in partial fulfillment of the requirement for the degree of Doctor of Sciences.

generated in the following manner. DEAE-Sephadex powder (100 g) was suspended in 10 liters of water and gently stirred for a few minutes. After standing for 1 hour, the supernatant was decanted and the whole operation was repeated. The hydrated powder was resuspended in water and allowed to stand for 4 days, during which time the aqueous supernatant was decanted and renewed twice daily. Five liters of 0.5 N HCl was added with stirring to the swelled exchanger and, after standing, the supernatant was removed and the exchanger was washed by decanting with four 10-liter volumes of demineralized water. Five liters of 0.5 N NaOH was added to the suspension which then was stirred for 15 minutes, filtered on a sintered-glass funnel, and washed with demineralized water under mild vacuum. Ten liters of the appropriate buffer was passed slowly through the exchanger after which the latter was transferred to a beaker containing the same buffer and allowed to stand under mild vacuum for a day. The buffer was decanted daily and renewed as many times as was necessary to obtain a clear supernatant. The procedure outlined here permits the removal of adsorbed CO_2 and the small-sized particles, thus allowing for a more efficient packing of the column as well as a faster flow rate.

Swine Pancreatic Powder. The starting material, "Pancreatine," was purchased from l'Industrie Biologique Francaise."[1] The Pancreatine powder is prepared from fresh pancreas glands, activated[2] by autolyzation under standardized conditions, then freed of lipids by successive ether extractions. A single lot of Pancreatine was used in the present work.

Rabbit Antisera versus Swine Pancreatic Extracts. The antisera were obtained by immunizing rabbits with a soluble pancreatic powder (Uriel and Avrameas, 1964a). This powder, containing 50% protein, was prepared by dialysis and lyophilization of an aqueous extract of Pancreatine. The immunization of each rabbit was performed as follows:

First day: intradermal injection of 30 mg of the soluble powder (SP) in 0.7 ml water and 0.3 ml complete Freund's adjuvant.[3]

10th, 11th and 12th days:[4] 40 mg of SP in 1 ml of 0.15 M NaCl.

19th, 20th, and 21st days: 60 mg of SP in 1 ml of 0.15 M NaCl.

29th, 30th, and 31st days: 120 mg of SP in 1 ml of 0.15 M NaCl.

Rabbits were bled on the 35th day.

Synthetic Substrates. The following substrates were purchased from Mann Research Laboratories (New York, N.Y.): acetyl-DL-phenylalanine 2-naphthyl ester (APNE),[5] acetyl-L-tyrosine ethyl ester (ATEE), benzoyl-DL-arginine-2-naphthylamide (BANA), *p*-toluenesulfonyl-L-arginine methyl ester, benzoyl-L-arginine ethyl ester (BAEE), carbonaphthoxy-DL-phenylalanine, hippuryl-L-arginine, L-leucyl-2-naphthylamide, and L-leucyl-4-methoxy-2-naphthylamide. 2′3′-*Cyclic*-cytidylic acid was purchased from Schwarz BioResearch (Orangeburg, N.Y.); and poly-L-glutamic acid from Yeda (Israel).

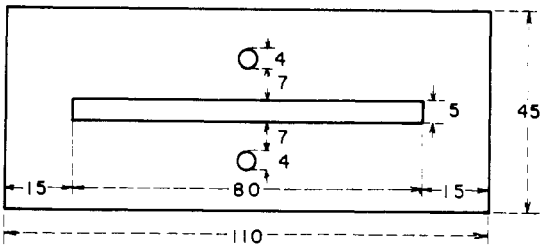

FIGURE 1: Diagrammatic representation of the plates used for immunoelectrophoresis showing the rectangular reservoir for the antiserum and the circular reservoirs for the antigen samples. Dimensions are in millimeters.

Natural Substrates. The following substrates were used: casein (Merck, Darmstadt, Germany); five-times-crystallized egg albumin (Nutritional Biochemicals Corp., Cleveland, Ohio), yeast RNA (Schwarz).

Electrophoresis and Immunoelectrophoresis in Agarose. Electrophoresis and immunoelectrophoresis have been carried out according to the method of Grabar and Williams (1953) as modified for agarose gels (Uriel *et al.*, 1963). Agarose gels were used at 0.8% in 0.025 M Na-Veronal buffer, *p*H 8.2. Some electrophoretic runs were carried out in gels prepared with 0.025 M Na-Veronal–Na acetate buffer, *p*H 6.4.

A schematic representation of the agarose plates used is shown in Figure 1. Samples of aqueous extracts, chromatographic fractions, or pure enzymes were dissolved or suspended in the buffer in dilutions ranging from 1 to 10 mg protein per ml prior to electrophoresis. The voltage gradient (approximately 8 v/cm) was applied for 60–90 minutes. All the electrophoretic runs were performed in a cold room at 5°. Identification reactions of enzymes were performed after electrophoresis and gel-immunodiffusion techniques in agarose.

The identification reactions of enzymes by their catalytic properties were carried out immediately after the electrophoretic run in the case of simple electrophoresis. When the immunodiffusion techniques were used, the catalytic properties of the enzyme-antibody precipitates served for the identification of the enzymes in gel-diffusion media.

[1] Gennevilliers, Seine (France).

[2] For explanation of "activated" see discussion in the following paper (Avrameas and Uriel, 1965).

[3] Difco Laboratories, Detroit, Mich.

[4] On the 10th, 19th and 29th days, the injections were administered intramuscularly, and the latter days by intravenous route.

[5] Abbreviations used in this work: APNE, *N*-acetyl-DL-phenylalanine-2-naphthyl ester; ATEE, *N*-acetyl-L-tyrosine ethyl ester; BANA, benzoyl-DL-arginine-2-naphthylamide; BAEE, benzoyl-L-arginine ethyl ester; LAP, leucine aminopeptidase.

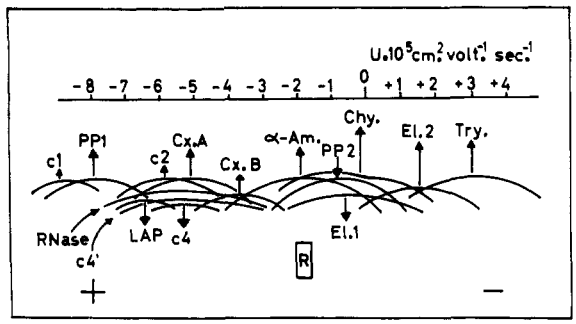

FIGURE 2: Immunoelectrophoretic diagram of swine pancreatic extracts. Individual enzymes were located by the appropriate reactions in gel media. Abbreviations: PP1 and PP2 (pancreatic proteases 1 and 2); Cx.A and Cx.B (carboxypeptidase A and B); RNase (ribonuclease); LAP (leucine aminopeptidase); α-Am (α-amylase); E1.1 and E1.2 (elastases 1 and 2); Chy. (chymotrypsin); Try. (trypsin); c1, c2, c4 (antigenic constituents c1, c2, and c4′); R (starting reservoir).

Techniques for the identification of enzymes with trypsin, chymotrypsin, carboxypeptidases A and B, and ribonuclease specificity, as well as those with elastolytic, proteolytic, and amylasic activities, have been described previously (Uriel, 1963; Uriel and Avrameas, 1964a,b).

Leucine aminopeptidase activity was identified by the following method. To 20 mg of L-leucyl-4-methoxy-2-naphthylamide in 3 ml dimethylformamide was added 7 ml of 0.05 M HCl–Na Veronal buffer, *p*H 8, containing 10 mg of diazo-blue B. The solution was prepared just before use, heated to 45°, and mixed with 10 ml of 1.4% melted agarose maintained at the same temperature in a water bath.

Enough agarose-substrate solution was poured over the plates to obtain a layer 1–2 mm thick. The plates were allowed to incubate at 37° for 2 hours, then washed for 4 hours in 2% acetic acid and dried. Leucine aminopeptidase activity is visualized as red-orange zones or arcs.

Quantitative Assays for Enzymes. Tryptic and chymotryptic activities were measured by a continuous titration method[6] at a constant *p*H of 7.9 using BAEE (Schwert *et al.*, 1948) for trypsin and ATEE (Kaufman *et al.*, 1949) for chymotrypsin and enzymes with chymotrypsinlike activity. Proteolytic activity against native egg albumin was measured by the same procedure. In all cases, the final volume of the reaction vessel was 10 ml and the temperature was 25°. Quantitative assays at *p*H 5.3 using poly-L-glutamic acid as substrate have been carried out according to a method previously described (Gjessing and Hartnett, 1962). The rate of hydrolysis was calculated from the initial slope of the curve (1 or 2 minutes of hydrolysis).

[6] A *p*H-stat (Radiometer, Copenhagen, Denmark) was used for the titration.

Leucine aminopeptidase activity was determined using L-leucyl-2-naphthylamide as substrate. The rate of formation of free 2-naphthylamine was measured at 340 mμ in a Beckman Model DU spectrophotometer (1-cm cells) at room temperature. This method avoids the coupling of the split 2-naphthylamine with a diazonium salt, thus eliminating a subsequent extraction of the azo dye formed (Ravin and Seligmann, 1951). Activity is expressed as μmoles of 2-naphthylamine liberated per minute.

Protein Assays. The concentration of proteins was determined with the biuret reagent according to a technique reported previously (Uriel, 1961).

Experimental Results

The Antigen-Antibody Reaction as a Criterion of Purity for the Isolated Enzymes. In gel-diffusion media, the sensitivity of the immunochemical reaction for a particular antigen depends mainly on the level of homologous precipitating antibodies present in the antiserum used. The antibody level may vary considerably with regard to one antigen or another in any given antiserum. To use the antigen-antibody reaction as a criterion of purity for enzyme preparations, isolated enzymes are necessary in order to detect the minimum quantity of an enzyme which still can be revealed as a precipitation line in gel. With the aid of the double-diffusion method (Ouchterlony, 1948), each of the purified enzyme preparations was tested in decreasing concentration against the antisera.

After development of the antigen-antibody complexes, the plates were washed in saline and stained in the usual manner. The sensitivity of the precipitation tests, as expressed by the minimum quantity of an antigen detectable, ranged from 0.01 to 0.02 mg proteins per ml, for all the constituents analyzed. Using this procedure as a criterion of purity, no further purification of an enzyme preparation was carried out when the proportion of impurities present was estimated by dilution to be less than 1% of the total protein content.

Immunoelectrophoretic Analysis of Swine Pancreatic Extracts. The schematic diagram shown in Figure 2 represents a reconstitution of the results with the swine pancreatic hydrolases from whole extracts as revealed by immunoelectrophoresis and identified with the aid of the different enzymatic reactions. Compared with the diagram given previously (Uriel and Avrameas, 1964a), two new constituents have been revealed, c4′ and the enzyme leucine aminopeptidase (LAP). Constituent c3 of the previous diagram has been identified as ribonuclease. Constituent c5 and the constituent previously identified as chymotrypsin have been shown to give an immunochemical cross-reaction.

The best results in immunoelectrophoresis were obtained at concentrations of about 10 mg protein per ml of pancreatic extracts (0.4 mg per sample in the starting reservoir). Some constituents such as c1, ribonuclease, leucine aminopeptidase, and trypsin re-

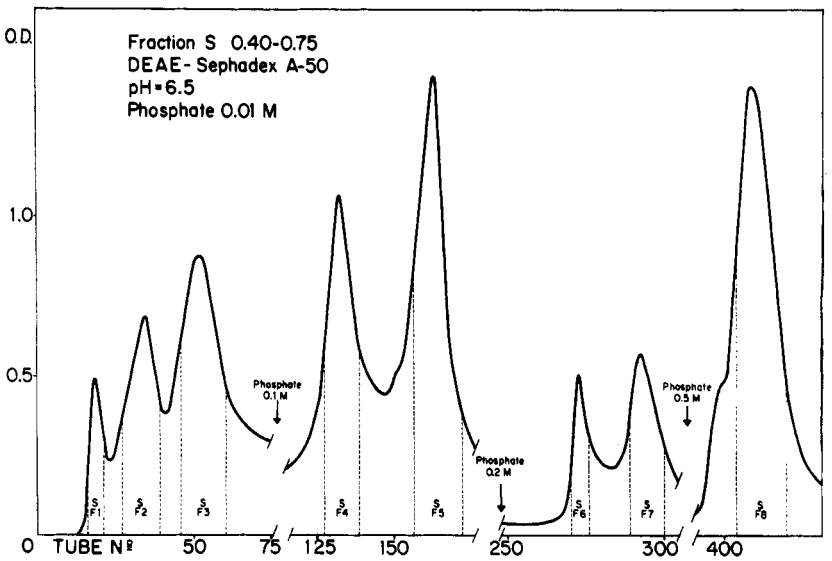

FIGURE 3: Elution diagram of the S 0.40–0.75 fraction. In each peak, the vertical interrupted lines delineate the various eluate fractions which were designated as SF1...SF8. Arrows are positioned at the points where the different buffers were applied to the column.

quired higher concentrations in order to be demonstrated.

Salting-out Fractionation of Swine Pancreatic Extracts. Pancreatine (100 g) was gently stirred in 1 liter of demineralized water[7] for 60 minutes at room temperature. The *p*H of the suspension was adjusted continuously to 5.5.

Subsequently, while the mixture was being stirred, ammonium sulfate was added to 0.40 saturation (240 g). The suspension was allowed to stand for 30 minutes and then was centrifuged for 45 minutes at 20° and 17,000 *g*. The supernatant (fraction S) was decanted and the precipitate (fraction P) was collected as described below. Ammonium sulfate up to 0.75 saturation (240 g/liter) was added to the supernatant and the *p*H was maintained at 5.5.

After standing for 30 minutes at room temperature, the suspension was centrifuged as before. The precipitate was collected, resuspended in 100 ml water, and dialyzed[8] for 16 hours against three 10-liter volumes of water. The solution was then lyophilized (fraction S 0.40–0.75).

The precipitate (fraction P) was suspended in 200 ml 0.15 M NaCl and stirred for 60 minutes at room temperature. The *p*H was continuously adjusted to 7.2. The suspension was centrifuged as before and the precipitate was discarded. Ammonium sulfate up to 0.45 saturation (280 g/liter) was added to the supernatant and the suspension was treated in the same way as was fraction S. The final lyophilization product has been designated fraction P 0.40.

[7] Unless otherwise specified, demineralized water was used throughout this procedure.

[8] All the dialyses were performed at 4°.

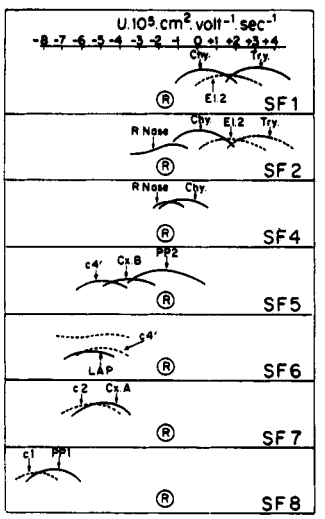

FIGURE 4: Immunoelectrophoretic diagrams of the SF fractions obtained from the chromatography of the initial S 0.40–0.75 fraction. The abbreviations are those used in Figure 2. Minor constituents in any given fraction are represented as interrupted arcs.

Chromatography of the Fraction S 0.40–0.75. Lyophilized fraction S 0.40–0.75 (2 g) was dissolved in 20 ml of 0.01 M potassium phosphate buffer, *p*H 6.5. The *p*H was readjusted to 6.5 and the solution was dialyzed overnight against 5 liters of the same buffer. The solution was applied to a column (40 × 3.5 cm) of DEAE-Sephadex A-50 (medium) equilibrated with 0.01 M phosphate buffer, *p*H 6.5.

The proteins were eluted with a stepwise gradient as

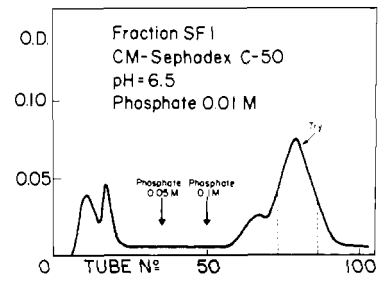

FIGURE 5: Elution diagram of fraction SF1 showing the isolation of trypsin (Try.). The vertical interrupted lines delineate the eluates combined. Arrows are positioned at the points where the different buffers were applied to the column.

shown in Figure 3. Separated eluents of a volume of 11 ml each were collected at a flow rate of 3–4 ml/minute. The chromatographic run was carried out at +15°. The elution diagram shown in Figure 3 was obtained by measuring the optical density of the separated fractions at 280 mμ (1-cm cells). The eluents were mixed as indicated on the diagram and designated as fractions SF1 through SF8.

Samples of the SF fractions were saturated with ammonium sulfate and centrifuged. The precipitates were resolubilized in water, dialyzed against water, and then lyophilized. The samples were submitted to immunoelectrophoretic analysis and the antigen-antibody precipitates which formed were subjected to the identification reactions. The immunoelectrophoresis patterns of the fractions are represented in Figure 4. The SF fractions were subjected to refractionation procedure except for SF3 and SF7, which contained little or no enzymatic activity.

Chromatography of Fraction SF1: Isolation of Trypsin. Fraction SF1 was adjusted to *p*H 3 with 1 N HCl, precipitated at room temperature by the addition of ammonium sulfate up to 0.80 saturation, and allowed to stand for 30 minutes. The suspension was then centrifuged[9] and the precipitate was dissolved in 10 ml 0.001 N HCl and dialyzed for 16 hours against two 10-liter volumes of 0.001 N HCl. The insoluble material formed during the dialysis was discarded after centrifugation and the clear supernatant was lyophilized. This product was dissolved in 5–7 ml of 0.01 M phosphate buffer, *p*H 6.5, and the *p*H was readjusted if necessary. The solution was placed in a column (30 × 2 cm) of CM-Sephadex equilibrated with the same buffer. As shown in Figure 5, a stepwise gradient was applied for elution at a rate of 60 ml/hour. The entire experiment was performed in a cold room at 4°. The effluents (6 ml each) obtained by elution with 0.1 M phosphate buffer were mixed as indicated in Figure 5.

[9] Unless otherwise specified, the ammonium sulfate–precipitated fractions were centrifuged for 30 minutes at 20° and 17,000 *g*, whereas the dialyzed fractions were centrifuged for 15 minutes at 4° and 17,000 *g*.

The combined fractions were immediately adjusted to *p*H 3 with 1 N HCl and submitted to the same treatment as the SF1 fraction. In gel-immunodiffusion tests, only one antigen was revealed in the purified fraction. The enzyme-antibody complex was shown to possess tryptic specificity against BANA and BAEE substrates. In addition, the complex appeared to possess very slight hydrolytic activity against ATEE but a strong activity against APNE. No other catalytic properties were observed in this complex. In Table I are recorded the activity data of fraction SF1 at each step of its purification from the pancreatic aqueous extracts.

TABLE I: Quantitative Data on the Hydrolysis of BAEE.[a]

Fraction	Total Proteins (mg)[b]	Total Units[c]	Specific Activity[c]
Aqueous extract[d]	13,608	20,956	1.54
S 0.40–0.75	1,525	1,830	1.20
P 0.40	3,534	883	0.25
SF1	18.9	378	20.00
Trypsin[e]	2	57	28.30
Novo[f]			27.60

[a] BAEE (0.001 M) in 0.1 M KCl (10 ml in the reaction vessel). Titration with 0.0223 M NaOH. Reaction at *p*H 7.9. [b] Data referred to 100 g of Pancreatine powder. [c] One unit is defined as the amount which hydrolyses 1 μeq of BAEE per minute. Specific activity is expressed in units per mg of protein. [d] Prepared according to Uriel and Avrameas (1964a). [e] Final step of purification. [f] A twice-crystallized commercial preparation of beef trypsin from Novo (Denmark).

Chromatography of Fraction SF2: Isolation of Chymotrypsins. Fraction SF2 was precipitated with ammonium sulfate at 0.80 saturation and centrifuged. The precipitate was dissolved in 10 ml of 10^{-4} N HCl, dialyzed against two 10-liter volumes of 10^{-4} N HCl for 16 hours, and centrifuged, and the supernatant was lyophilized. This product was dissolved in 5–7 ml of 0.01 M phosphate buffer, *p*H 6.5, the *p*H was readjusted, and the solution was placed on a column (30 × 2 cm) of CM-Sephadex equilibrated with the same buffer. A stepwise gradient of phosphate at *p*H 6.5 ranging from 0.01 to 0.2 M was applied, and the elution rate was 135 ml/hour. Fractions (9 ml) were collected according to the elution diagram shown in Figure 6. The eluents obtained with 0.05 M phosphate and with 0.1 M phosphate were combined as indicated. Each of these fractions, designated chymotrypsin 1 and chymotrypsin 2, were precipitated with ammonium sulfate, centrifuged, and dialyzed against 10^{-4} N HCl. After

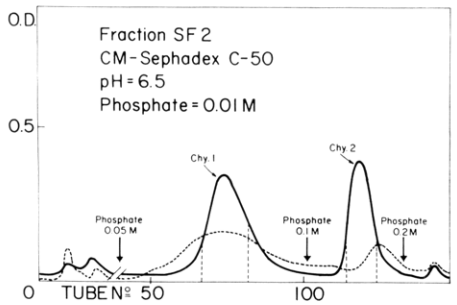

FIGURE 6: Elution diagram of fraction SF2 showing the isolation of chymotrypsins 1 and 2 (solid line). The broken line represents the elution diagram of an SF2 fraction allowed to stand at *p*H 3 before the chromatographic run.

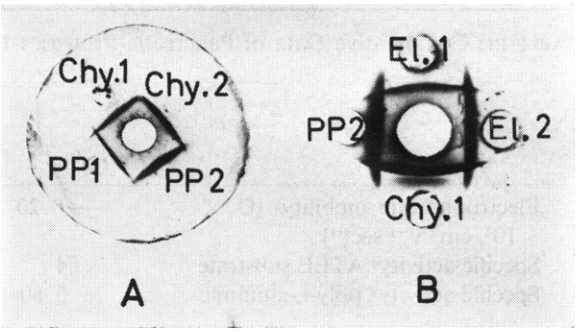

FIGURE 7: Double-diffusion plates demonstrating the lack of antigenic relationship between four and five isolated enzymes. The center well contains the antiserum. The lines of precipitation were stained with light green. The chymotrypsin 1 preparation contains an impurity which was subsequently identified as elastase 1. For explanation of the abbreviations, see Figure 2.

dialysis and centrifugation, the clear supernatants were lyophilized.

When the fraction SF2 was dialyzed against 10^{-3} N HCl instead of 10^{-4} N, changes in the chromatographic behavior of the two fractions were observed. The spreading of the chromatographic peaks when the SF2 fraction was dialyzed against 10^{-3} N HCl is illustrated in Figure 6.

The immunochemical analyses in gel-diffusion media revealed one single antigen in each of the Chy.1 or Chy.2 fractions. Both antigens appeared to possess chymotrypsin specificity with ATEE and APNE as substrates, and they were immunochemically identical in double-diffusion tests (Figure 7). The two preparations possess hydrolyzing ability against ATEE and native egg albumin near *p*H 8, and against poly-L-glutamic acid at *p*H 5.3. Catalytic activities of both enzymes were completely inhibited by DFP at a final concentration of 10^{-3} M, when assayed as described in Tables II and III. Chymotryptic activity of purified chymotrypsins 1 and 2 and pancreatic proteases 1 and 2 and of intermediate fractions is recorded in Table II. Comparative data of the four isolated enzymes are included in Table III.

Purification of Fraction SF4: Isolation of Ribonuclease. The SF4 fraction was precipitated with ammonium sulfate at 0.90 saturation and centrifuged. The precipitate, collected in 25 ml water, was treated by the phenol method (Rushizky *et al.*, 1963).

The lyophilized preparation was suspended in 1–2 ml of water, stirred for 10 minutes, and centrifuged. The supernatant was submitted to activity and purity tests for ribonuclease.

After simple electrophoresis of this preparation in agarose, a single zone of activity against RNA was detected. The electrophoretic mobility in agarose ranged between -1 and -2×10^{-5} cm^2 v^{-1} sec^{-1}. The immunoelectrophoretic analysis of this preparation revealed a single antigen, but no hydrolytic activity against 2′3′-cyclic-cytidylic acid was detected on the ribonuclease-antibody precipitate. It was impossible to demonstrate whether the absence of activity was due to

TABLE II: Quantitative Data on the Hydrolysis of ATEE.[a]

Fraction	Total Protein (mg)[b]	Total Units[c]	Specific Activity[c]
Aqueous extracts[d]	13,608	571,536	42
S 0.40–0.75	1,525	61,000	40
P 0.40	3,534	229,710	65
SF2	50.3	5,670	107
Chy.1 (from SF2)	16.5	1,891	115
Chy.2 (from SF2)	23.5	1,504	64
SF8	41	3,027	73.6
PP1 (from SF8)	37.1	2,854	77
SF5	112.5	7,087	63
PP2 (from SF5)	7.3	1,239	170

[a] ATEE (0.020 M) in 0.1 M KCl (10 ml in the reaction vessel). Titration with 0.079 M NaOH. Reaction at 25° and *p*H 7.9. [b] Data referred to 100 g of Pancreatine powder. [c] One unit is defined as the quantity which hydrolyzes 1 μmole of ATEE per minute. Specific activity is expressed in units per mg of protein. [d] Prepared as described by Uriel and Avrameas (1964a).

insensitivity of the reaction or to the inhibition of the active site by the antibody. A quantitative assay of the ribonuclease fraction on the *p*H-stat using RNA as substrate (Kalnitzky *et al.*, 1959) gave a specific activity of 0.17 μmole of NaOH consumed per minute per mg of protein (*p*H 7; + 25°). This is about one-tenth of the value reported for crystalline preparations of beef pancreatic ribonuclease.

Chromatography of Fraction SF5: Isolation of Pancreatic Protease 2 (PP2). The SF5 fraction was

TABLE III: Comparative Data of Pancreatic Proteases 1 and 2, Chymotrypsins 1 and 2, of Beef Chymotrypsin.

	Chy.2	Chy.1	PP2	PP1	Beef Chymotrypsin[a]
Electrophoretic mobility[b] [U × 10^5, cm^2 v^{-1} sec^{-1}]	−0.20	−1.0	−1.5	−8.0	
Specific activity[c] ATEE substrate	64	115	170	77	280
Specific activity[d] poly-L-glutamic substrate	5.80	6.08	0.10	1.27	None
Specific activity[e] native egg albumin substrate	1.28	1.29	0.72	1.21	0.52

[a] A three-times-crystallized commercial preparation of beef α-chymotrypsin from Worthington (Freehold, N.J.). [b] Calculated according to a method described previously (Grabar *et al.*, 1960). [c] For quantitative assay and units, see Table II. [d] Poly-L-glutamic acid (20 mg) in 5 ml of 0.1 M KCl (solution adjusted at *p*H 5.3). Titration with 0.079 M NaOH. Reaction at 25° and *p*H 5.3. One unit is defined as the amount which consumes 1 μmole of NaOH per minute. Specific activity is given in units per mg of protein. [e] Egg albumin (2.5%) in 0.1 M KCl (10 ml in the reaction vessel); *p*H adjusted to 8; titration with 0.079 M NaOH. Reaction at 25°. One unit is defined as the amount which consumes 1 μmole of NaOH per minute. Specific activity is given in units per mg of protein.

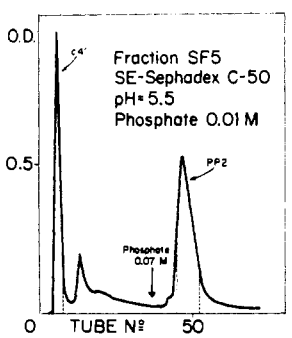

FIGURE 8: Elution diagram of fraction SF5 indicating the isolation of pancreatic protease 2 (PP2). The vertical interrupted lines delineate the eluates combined. Arrows are positioned at the points where the different buffers were applied to the column.

precipitated with ammonium sulfate at 0.80 saturation and centrifuged. The collected precipitate was dissolved in 10 ml water and dialyzed for 16 hours against 10 liters of water. This was followed by a second dialysis against two 5-liter portions of 0.01 M phosphate buffer, *p*H 5.5. After centrifugation, the supernatant was stored at −20° until use.

The fraction was thawed at room temperature and placed on a column (35 × 2 cm) of SE-Sephadex equilibrated with 0.01 M phosphate buffer, *p*H 5.5. A stepwise gradient of phosphate buffer at *p*H 5.5, ranging from 0.01 to 0.07 M, was applied, and the elution rate was 78 ml/hour. Fractions (10 ml) were collected at 4° according to the elution diagram shown in Figure 8.

Fractions eluting with 0.01 M phosphate were combined in the manner indicated on the diagram. The fraction appeared to contain a single antigen which was identified with constituent c4′ (see Figure 2) by the technique of Osserman (1960). None of the substrates employed in this work was hydrolyzed by this constituent.

With 0.07 M phosphate buffer, a chromatographically well-defined peak was eluted. The collected fractions, mixed as indicated in Figure 8, were precipitated with ammonium sulfate at 0.85 saturation, allowed to stand for 30 minutes at room temperature, and centrifuged. The precipitate was collected with 10 ml 10^{-4} N HCl and dialyzed at 4° against three 10-liter volumes of 10^{-4} N HCl. The precipitate formed during the dialysis was discarded after centrifugation and the supernatant containing the pancreatic protease 2 was lyophilized.

By electrophoresis, immunoelectrophoretic analysis, and identification reactions in gel-diffusion media, this preparation appeared to contain a single antigen which possesses hydrolytic properties against ATEE and native egg albumin. Its electrophoretic mobility was slightly higher than that of the same antigen contained in whole pancreatic extracts. The same observation was noted with certain other isolated enzymes.

The PP2-isolated enzyme was immunologically distinct from the other ATEE-hydrolyzing enzymes of swine pancreas (see Figure 7) but identical with an enzyme previously isolated by a different method from similar pancreatic extracts (Uriel and Avrameas, 1963).

The enzyme hydrolyzes ATEE and native egg albumin near *p*H 8 and poly-L-glutamic acid at *p*H 5.3. The hydrolysis of these three substrates was inhibited by DFP at 10^{-3} M final concentration when assayed as described in Tables II and III.

Activity data are recorded in Table II. Comparative data of this enzyme and the three other enzymes with chymotrypsinlike activity (Chy.1, Chy.2, and PP1) are included in Table III.

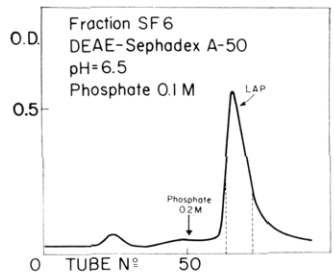

FIGURE 9: Elution diagram of fraction SF6 demonstrating the isolation of leucine aminopeptidase (LAP). The vertical interrupted lines delineate the eluates combined. Arrows are positioned at the points where the different buffers were applied to the column.

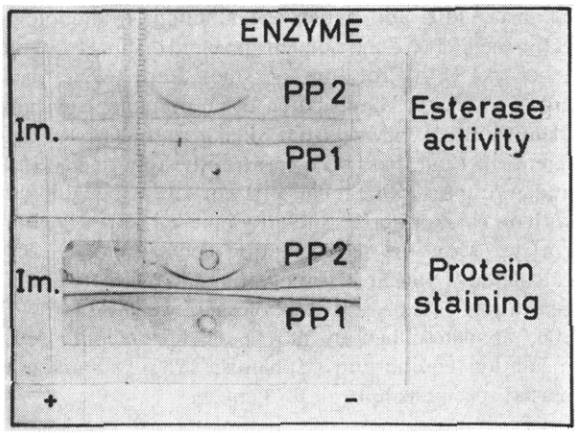

FIGURE 10: Immunoelectrophoretic analyses of the pancreatic proteases 1 and 2 (PP1 and PP2) demonstrating the purity of these preparations and the enzymatic activity associated with each of the precipitated arcs.

The SF5 fraction also contains an antigen with carboxypeptidase B activity. However, further attempts to purify the carboxypeptidase B from this fraction were discontinued because of a rapid degradation of the enzyme (see Isolation of Carboxypeptidase B in the following paper).

Chromatography of the SF6 Fraction: Isolation of Leucine Aminopeptidase. The SF6 fraction was precipitated with ammonium sulfate at 0.80 saturation and centrifuged. The precipitate was dissolved in 10 ml water and dialyzed for 16 hours against 10 liters of water and then twice against 5 liters of 0.1 M phosphate buffer, *p*H 6.5. After centrifugation, the supernatant was stored at −20° until use.

After thawing at room temperature, the fraction was placed on a column (25 × 2 cm) of DEAE-Sephadex equilibrated with 0.1 M phosphate buffer, *p*H 6.5. Approximately 275 ml of this buffer were passed through the column at an elution rate of 55 ml/hour. The eluted fractions were discarded. The elution was continued at the same rate with 250 ml of 0.2 M phosphate buffer, *p*H 6.5. The fraction mixed as indicated on the diagram of the Figure 9 was precipitated with ammonium sulfate at 0.85 saturation and the suspension was centrifuged. The precipitate was dissolved in 5–7 ml water and dialyzed three times against 10 liters of water. The dialysate was centrifuged and the supernatant was stored at −20°.

Only one antigen-antibody complex possessing hydrolytic activity against L-leucyl-4-methoxy-2-naphthylamide and L-leucyl-2-naphthylamide was revealed in this preparation. This complex was also active on the following substrates: L-alanyl-2-naphthylamide, L-leucyl-L-alanine, and DL-alanine-DL-leucine. In Table IV are recorded the activity data of fraction SF6 at each step of its purification from the pancreatic aqueous extract.

Purification of Fraction SF8: Isolation of PP1. The SF8 fraction was precipitated with ammonium sulfate at 0.80 saturation and centrifuged. The precipitate was suspended in 10 ml of water and dialyzed for 61 hours against three 10-liter volumes of water. To the dialysate, LiCl up to 0.15 M was added. The suspension was adjusted to *p*H 7.5, gently stirred for 5 minutes, and centrifuged. The supernatant was dialyzed for 40 hours at 4° against three 10-liter portions of Na acetate–acetic acid buffer, 0.005 M, *p*H 4.0–4.1. The suspension was centrifuged and the precipitate was dissolved in 3–4 ml, 0.1 M LiCl. The *p*H was adjusted to 6.5 and the clear solution stored at −20° (preparation designated as PP1 enzyme).

By immunoelectrophoretic analysis, a single antigen was revealed in this preparation (Figure 10). The identification reactions in gel-diffusion media have shown this antigen to possess hydrolytic activity

TABLE IV: Quantitative Data on the Hydrolysis of L-Leucyl-2-naphthylamide.[a]

Fraction	Total Protein (mg)[b]	Total Units[c]	Specific Activity[c]
Aqueous extracts[d]	13,608	178	0.013
S 0.40–0.75	1,525	38	0.025
SF 6	32.3	4.5	0.145
LAP	4.9	0.72	0.146

[a] The fraction containing leucine aminopeptidase was diluted in 0.05 M Tris buffer, *p*H 7.65. One ml of the solution was added to 4 ml of 10^{-3} M L-leucyl-2-naphthylamide in the same buffer. Readings were made at 340 mμ and 25° in a Beckman Model DU spectrophotometer. [b] Data refer to 100 g pancreatine powder. [c] One unit is defined as the amount which liberates 1 μmole of 2-naphthylamine per minute. Specific activity is expressed in units per mg protein. [d] Prepared as previously described (Uriel and Avrameas, 1964a).

against ATEE and native egg albumin. Immunologically, the PP1 enzyme isolated appeared distinct from the other ATEE-hydrolyzing enzymes from swine pancreatic extracts. The isolation of an immunochemically identical PP1 enzyme from similar extracts by a different method has been previously reported (Uriel and Avrameas, 1963). The PP1 enzyme is a euglobulin with an electrophoretic mobility of -7.5 to -8×10^{-5} $cm^2\ v^{-1}\ sec^{-1}$. Its sedimentation coefficient at $+20°$, calculated by linear extrapolation to zero protein concentration, is 3.03 and its molecular weight 24,000 ± 300, as determined by means of short-column sedimentation equilibrium (Yphantis, 1960), assuming a partial specific volume of 0.73 cm^3/g.

PP1 enzyme possesses hydrolytic activity against ATEE (Table II) and native egg albumin near *p*H 8 and on poly-L-glutamic acid at *p*H 5.3. At a final concentration of 10^{-3} M, DFP completely inhibited these activities. Incubation of the enzyme at *p*H 3 and 4° during 10 hours resulted in a loss of about 50% of the ATEE-hydrolyzing activity.

As shown in Table III, the four isolated enzymes with chymotrypsinlike activity have different electrophoretic mobilities. Those of Chy.1, Chy.2, and PP2 are slightly higher than the mobilities found for the same enzymes in the whole pancreatic extract (Uriel and Avrameas, 1964a).

The four enzymes hydrolyze ATEE, poly-L-glutamic acid, and native egg albumin. Using ATEE or native egg albumin as substrates, the differences in the specific activity of these enzymes are not apparently significant. On the contrary, the rate of hydrolysis of poly-L-glutamic acid by chymotrypsins 1 and 2 is 60-fold and 5-fold higher than that of PP2 and PP1, respectively. Under the same experimental conditions, bovine α-chymotrypsin has no hydrolytic activity against poly-L-glutamic acid.

Discussion

It has been reported (Inagami and Sturtevant, 1960) that beef trypsin possesses some hydrolytic activity against ATEE, a substrate considered to be specific for chymotrypsins. The swine trypsin isolated by us has been shown by immunochemical tests to possess a slight hydrolytic action against ATEE and in addition a strong activity against APNE, another substrate with a somewhat similar specificity. The observation that the specific complex, trypsin-antitrypsin, possesses chymotrypsinlike activity confirms the conclusion of Inagami and Sturtevant that this catalytic property is inherent in the trypsin molecule rather than due to an impurity in the trypsin preparations.

A zymogen from swine pancreas has been isolated (Rovery *et al.*, 1960). This zymogen, designated as chymotrypsinogen A, after activation by trypsin, gives rise to an enzyme with ATEE-hydrolyzing ability. However, the possibility that there exist in swine pancreas at least two enzymes splitting ATEE has been advanced by the same authors (Rovery *et al.*, 1960; Desnuelle and Rovery, 1961). Previous results on immunochemical analyses of swine pancreatic extracts (Uriel and Avrameas, 1964a) have shown the presence of at least five enzymatic constituents with ATEE-hydrolyzing activity. In the present work, four[10] of these constituents have been isolated and designated as chymotrypsins 1 and 2 and pancreatic proteases 1 and 2.

It is now known (Bettelheim and Neurath, 1955; Desnuelle and Rovery, 1961) that the activation of beef chymotrypsinogen A by trypsin involves the hydrolysis of a maximum of four peptide bonds leading to δ-chymotrypsin, or to the chymotrypsins of the α family. From an immunological point of view, it seems highly improbable that such a mechanism of activation would be accompanied by a significant loss in the immunological relationship between the zymogen and its active form(s). In fact, when α, β-, γ-, and δ-chymotrypsins were tested by Ouchterlony's technique (1948) against a rabbit anti-α-chymotrypsin serum, the four chymotrypsins showed immunologic identity (Uriel, 1963). Further assays with this antiserum have demonstrated an immunologic identity between these activated chymotrypsins and the zymogen preparation.[11]

If the activation mechanism of beef chymotrypsinogen is common to other pancreatic zymogens, it would be reasonable to conclude that the four enzymes isolated here which possess ATEE-hydrolyzing ability arose from three different precursors. As PP1 and PP2 are immunologically distinct enzymes, two different zymogens could give rise to them. However, the chymotrypsins 1 and 2 have been shown to be immunologically identical (Figure 7) and probably represent two molecular forms arising from a single precursor.

The designation of pancreatic proteases 1 and 2 and chymotrypsins 1 and 2 which we have proposed for the isolated chromatographic preparations can be considered as only provisional. Thus the PP2 enzyme, the chymotrypsins 1 and 2 described above, as well as the swine chymotrypsinogen A isolated by Rovery *et al.* (1960), possess the chromatographic and electrophoretic properties of cationic proteins.

As the physicochemical or chromatographic properties of the active form(s) derived from the chymotrypsinogen A have not been reported, their relationship to the enzymes studied here could not be established. On the basis of physicochemical, chromatographic, and catalytic properties, strong evidence has been presented to suggest that the pancreatic protease 1 and those enzymes designated as esterase 1 (Hofstee, 1952), pankrin (Grant and Robbins, 1956), and esterolytic protease (Gjessing and Hartnett, 1962) are one and the same hydrolytic enzyme. The four enzymes behave as euglobulins and are precipitated from low ionic strength solutions at about *p*H 4. The use of different experimental conditions for the isolation of these enzymes makes the comparative interpretation of their chromatographic behavior difficult. However, when

[10] The isolation of the fifth constituent, designated as elastase 1, is described in the second part of this work.

[11] Unpublished experiments.

the elution was performed with buffers of increasing molarity, as in the case of pankrin and PP1 (Avrameas and Uriel, 1965), both enzymes eluted at high ionic strength.

The four enzyme preparations show esterase activity against ATEE. The specific activities of PP1 and pankrin are, respectively, 20 and 12% of that of beef α-chymotrypsin, values considered quite similar. Both PP1 and the esterolytic protease possess hydrolytic ability against poly-L-glutamic acid. Neither esterase 1 nor pankrin has been assayed against this substrate.

When the S 0.40–0.75 fraction was chromatographed on DEAE-Sephadex, ribonuclease activity against RNA was detected in the eluates ranging between 0.01 and 0.2 M phosphate (SF1 to SF6 fractions). A similar finding has been reported (Marchis-Mouren *et al.*, 1961) for the chromatographic fractionation of swine pancreatic juice on DEAE-cellulose. By an identification reaction after electrophoresis (Uriel and Avrameas, 1961), we have revealed the presence of nucleic acid material in the chromatographic eluates of the S 0.40–0.75 fraction. Apparently, complexes between ribonuclease and nucleic acid constituents are formed. These complexes may likely explain the chromatographic heterogeneity of swine pancreas ribonuclease. Both ribonuclease activity and nucleic acid contaminants were absent in the purified enzymes obtained from the SF1, SF2, and SF6 fractions after a subsequent chromatographic run.

The isolation of a pancreatic aminopeptidase has not been reported previously. Its electrophoretic mobility in agarose and the broad substrate specificity which it exhibits are in good agreement with the data reported for the aminopeptidase isolated from swine kidney (Spackman *et al.*, 1955).

A general discussion on the systematic fractionation of swine pancreatic hydrolases that we have performed is given in the second paper of this series.

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