Characterization of Human Exocrine Pancreatic Proteins by Two-Dimensional Isoelectric Focusing/Sodium Dodecyl Sulfate Gel Electrophoresis

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Exocrine proteins contained in human pancreatic juice were separated in two dimensions using isoelectric focusing and sodium dodecyl sulfate gel electrophoresis. Nineteen discrete proteins were found. Fifteen of these were identified by actual or potential enzyme activity and include three forms of trypsinogen, two forms each of procarboxypeptidase A, procarboxypeptidase B, proelastase, and colipase, and one form each for amylase, lipase, chymotrypsinogen, and prophospholipase A_2. Lipase and four unidentified proteins were found to contain carbohydrate by the periodic acid Schiff staining method. Each pancreatic protein was characterized by isoelectric point and molecular weight. Proteins were quantitated according to relative mass, as measured by the incorporation of a mixture of 15 ^3H-amino acids into secretory proteins contained in tissue slices, and according to the distribution of Coomassie blue R stain among proteins contained in pancreatic juice, as determined by two-dimensional gel scanning and computer analysis. The second form of pancreatic procarboxypeptidase B (IEP_n6.7) was present in only 4 of 10 subjects tested. Trypsinogens 1 and 3 were covalently labeled with $^{35}SO_4$. Trypsin derived from trypsinogen 2 showed no inhibition with soybean trypsin inhibitor or Trasylol.

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Studies on human exocrine pancreatic proteins have been difficult in the past because of the relative inaccessibility and lability of this gland. Despite the difficulties encountered, a number of major contributions have appeared which describe important aspects of the exocrine function of this tissue. Kern and Ferner have described the morphologic features of the human pancreas and have demonstrated that this tissue contains all of the important intracellular elements of the secretory pathway including the rough endoplasmic reticulum, Golgi complex, condensing vacuoles, and zymogen granules (1). Keller and Allan (2) and Allan et al. (3) have identified a number of actual and potential hydrolases in pancreatic juice. Figarella et al., working with pancreatic juice devoid of free proteolytic activity, have described a number of human pancreatic enzymes and zymogens isolated by column chromatography and have studied the biochemical and immunologic features of these proteins in detail (4-9). Greene et al. have purified the human pancreatic secretory trypsin inhibitor and described its physical characteristics and amino acid sequence (10,11).

Although each of the above studies has contributed to an understanding of the function of the human pancreas, the methodologies used could not be applied easily to the study of exocrine pancreatic proteins obtained from individual human subjects. Two methodologic advances made in the past decade now allow such studies to be carried out. The first of these methodologies is endoscopic retrograde cannulation of the pancreatic duct (12-14) which now allows access to pure juice contained in the pancreatic duct of human subjects. The second is two-dimensional isoelectric focusing/SDS gel electrophoresis, a procedure independently described in 1975 by Scheele (15) and O'Farrell (16) for the analysis of complex mixtures of proteins contained in microliter quantities of fluid. In the studies reported here, we combine these two methodologies and dem-

onstrate the feasibility of analysis of exocrine pancreatic proteins from individual human subjects. We also present, in detail, a thorough description of the exocrine proteins contained in normal human pancreatic juice.

Materials and Methods

Chemical Preparations

Acrylamide, *N,N'*-methylenebisacrylamide, and specially pure sodium lauryl sulfate were from BDH Chemicals (Poole, England). Ampholines were from LKB. Coomassie blue, types R and G, were from Sigma. Autoclaved, ^{14}C-labeled *E. coli* were a kind gift from Peter Elsbach of the New York University Medical Center (New York, N.Y.). Purified bovine enterokinase was a kind gift from Drs. Suzanne Maroux and Jacques Baratti (CNRS, Marseille, France); bovine trypsin (TRL3, 229 U/mg) was from Worthington Biochemicals (Freehold, N.J.). Ultrapure urea was from Schwarz/Mann Div., Becton, Dickinson & Co., Inc. (Orangeburg, N.Y.).

Biologic Samples

The majority of studies presented here were carried out on a single sample of pure pancreatic juice obtained by postoperative cannulation of the pancreatic duct (Dr. George Nardi, Massachusetts General Hospital and Harvard Medical School, Boston). Pancreatic secretion was collected in iced containers containing soybean trypsin inhibitor and stored at −80°C. Eight other samples of pancreatic juice were collected by endoscopic retrograde catheterization of the pancreas (ERCP), performed either by Dr. Jerome Waye of the Mount Sinai School of Medicine (New York) or Drs. Robert Kurtz and Charles Lightdale of Memorial Cancer Hospital (New York). These samples of pancreatic juice are considered to be normal.

Endoscopic retrograde catheterization of the pancreas samples were obtained after intravenous injection of 1 U/kg secretin (Boots Co., Nottingham, England), collected in 1.5-ml Eppendorf plastic vials containing 15 μl each of diisopropylfluorophosphate (100 mM), benzamidine (100 mM), Trasylol (10,000 KIU/ml)* and soybean trypsin inhibitor (2 mg/ml), and immediately stored at −80°C after rapid freezing in liquid N_2.

Human pancreatic tissue was obtained through the Downstate Medical Center Organ Transplant Program (Dr. Ismail Parsa, Director) from a cadaver kidney transplant donor perfused extracorporeally until removal of the desired organs. Tissue slices (0.5 × 7 × 9 mm) were prepared with a Stadie-Riggs tissue slicing blade (Arthur H. Thomas Co., Philadelphia, Pa.) at 4°C. Two tissue slices were incubated in 1.0 ml of Krebs-Ringer bicarbonate solution equilibrated with 95% O_2 and 5% CO_2 and containing the appropriate radioactive compound.

Two-Dimensional IEF/SDS Gel Electrophoresis

Separation of proteins in two dimensions using slab gel isoelectric focusing followed by gradient polyacrylamide gel electrophoresis in SDS was carried out as described by Scheele (15) with the following modifications: (a) The isoelectric focusing gel contained 1% Ampholine pH 5–8, and 0.33% Ampholine pH 3.5–10 (LKB) to achieve optimal separation of human exocrine pancreatic proteins; (b) 10 KIU/ml Trasylol, 2 μg/ml soybean trypsin inhibitor, and 0.1 mM diisopropylfluorophosphate were included in the isoelectric focusing gel to retard the autoactivation of trypsinogen, except in the studies where enzymes and zymogens separated in the isoelectric focusing gel were identified by their activities; (c) isoelectric focusing was carried out either in the absence of (15) or in the presence of (17) urea. In the procedure using urea, as described by Bieger and Scheele (17), isoelectric focusing gels were polymerized in the presence of ultrapure urea, added in crystalline form, at a concentration of 8 M. During the focusing procedure, gels were maintained in a humid environment to prevent precipitation of urea in the gel matrix. Samples of human pancreatic juice, which were applied to the focusing gel, contained 4% Ampholine, pH 3.5–10, and 4 M (added in crystalline form) urea. Added in this sequence Ampholine served as a scavenger for cyanate ions, which appear as an equilibrium product in solutions of urea (18). (d) Isoelectric focusing strips, cut 1 cm in width, were applied to the second dimension gels without an interposed bridge of polyacrylamide. Two-dimensional gels were stained with 0.06% Coomassie blue, type R, 50% methanol, and 10% acetic acid for 1 h and destained with 50% methanol and 10% acetic acid. Glycoproteins were identified in two-dimensional gels using the periodic acid Schiff staining procedure (19).

Measurements of Actual or Potential Enzyme Activities

Human pancreatic zymogens contained in pancreatic juice were diluted in 0.1 M KCl, 0.1 M Tris-HCl, pH 8.0, 0.02 M $CaCl_2$, and 100 μg/ml BSA (buffer A) to a concentration of 20–100 μg/ml and activated as follows. Trypsinogen was activated at 0°C for 60 min using purified bovine enterokinase at 1 μg/ml. Trypsin at 10 μg/ml was used to activate chymotrypsinogen (0°C, 60 min), procarboxypeptidase A (22°C, 60 min), procarboxypeptidase B (22°C, 60 min) and prophospholipase A_2 (0°C, 60 min). Proelastase contained in pancreatic juice diluted to a concentration of 50–200 μg/ml was activated at 22°C for 30 min with 20 μg/ml trypsin. Zymogens eluted from isoelectric focusing gel fractions into buffer A were activated under the same conditions except that concentrations of pancreatic proteins varied in these fractions from 5 to 50 μg/ml. Procedures for the assay of amylase, trypsin, chymotrypsin, and the carboxypeptidases A and B are summarized by Scheele and Palade (20). Elastase was measured

*Abbreviations used in this paper: IEF, isoelectric focusing; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; TCA, trichloroacetic acid; KIU, Kallikrein inhibitor units; M_r, apparent molecular weight; IEP, isoelectric point; IEP_n, isoelectric point in the absence of urea (native); IEP_u, isoelectric point in the presence of 8 M urea; and STI, soybean trypsin inhibitor.

according to the procedure of Bieger and Scheele (21) using soluble [α-^{3}H]elastin as substrate and measuring TCA soluble radioactivity after a 60-min incubation at 37°C. Lipase was measured using [^{14}C]triolein as substrate (22).

Potential phospholipase A_2 activity was determined on proteins eluted from second dimension gels which had been fixed in 50% methanol and 10% acetic acid and stained with Coomassie blue. Prophospholipase A_2 was stable in the presence of these solvents and after elution could be activated under conditions described above. Phospholipase A_2 activity was determined by the method of Franson et al. (23). The release of [^{14}C]oleate in mixtures containing 2.5×10^8 [^{14}C]oleate, autoclaved *E. coli* in 100 mM Tris-HCl, pH 8.0, and 20 mM $CaCl_2$ was determined after 15 min at 37°C. *E. coli* contain no triglyceride and incorporate [^{14}C]oleic acid exclusively into the 2 position of phospholipids. After extraction of lipids ^{14}C-labeled free fatty acids were separated from phospholipids by thin-layer chromatography. Coomassie blue interfered with neither the activation nor the assay of phospholipase A_2.

Characterization of Individual Exocrine Proteins

Secretory proteins separated by the two-dimensional gel procedure were characterized by the following procedures. Isoelectric points of individual proteins were determined from pH measurements made on focusing gels, either by using a surface pH electrode (Ingold Electrodes, Inc., Lexington, Mass.) or by regular pH measurement, at 8°C, of ampholytes eluted into distilled water from 0.5-cm gel fractions. Values could vary from 0.4 to 0.6 pH units between these methods. Isoelectric points reported here were calculated from pH measurements made on solutions of eluted ampholytes.

Molecular weights of individual proteins were determined by SDS-gel electrophoresis in a polyacrylamide gradient (12%–17%). Standard molecular weight markers were obtained from Bio-Rad Laboratories (Richmond, Calif.), and measurements were made on proteins which were reduced with 50 mM dithiothreitol and alkylated with 100 mM iodoacetamide. Under these conditions a quasilinear relationship is obtained between R_f and log M_r for proteins in the range of 10,000–100,000 daltons (15).

Relative mass of secretory proteins was determined by incorporation of a mixture of 15 ^{3}H-amino acids (50 μCi algal profile from Schwarz/Mann Radiochemicals) into two human pancreatic tissue slices incubated at 37°C in Krebs Ringer bicarbonate buffer gassed with 95% O_2. After 2 h of incubation in the presence of radioactive amino acids, pancreatic tissue was homogenized (20% wt/vol) in a solution containing 1% Triton X-100, 25 mM Tris-HCl, pH 9.0, 1.0 mM diisopropylfluorophosphate, 1 mM benzamidine, 100 KIU/ml Trasylol, and 20 μg/ml soybean trypsin inhibitor. Secretory proteins contained in tissue homogenates were separated by two-dimensional IEF/SDS gel electrophoresis and radioactivity contained in two-dimensional gel spots was analyzed as described previously (15). Under these conditions, ~95% of the TCA-insoluble radioactivity was incorporated into secretory proteins (24), and distribution of radioactivity among these proteins gave an adequate measure of relative mass.

Distributions of secretory proteins contained in samples of human pancreatic juice were determined by two-dimensional scanning of Coomassie blue (R) stained gels. Particular care was taken to minimize background staining in these gels. Scanning was performed on photographic reproductions of stained gels using an Optronics two-dimensional gel scanner, and optical density measurements were taken at 100 μm intervals (rasters). Measurements recorded on magnetic tape were analyzed on a PDP-11/70 Computer. Programs for the analysis of data were designed by Alan Ezer and Banvir Chaudhary of The Rockefeller University Computing Services and included: (a) selection of two-dimensional gel spots, (b) subtraction of background optical density values, and (c) determination of cumulative densities within and fractional densities among the selected two-dimensional spots. When background levels were low, all spots visualized by Coomassie blue staining were included in the computer analysis of two-dimensional gel scanning data.

Incorporation of $^{35}SO_4$ into pancreatic proteins was carried out on tissue slices (n = 2) incubated for 2 h in the presence of 1.2 mCi $^{35}SO_4$ (Amersham Corp.) and 1.0 ml Krebs-Ringer bicarbonate buffer as described above. $MgCl_2$ was substituted for $MgSO_4$ in the incubation medium. $^{35}SO_4$-labeled pancreatic proteins contained in tissue homogenates and separated by two-dimensional urea IEF/SDS-gel electrophoresis were analyzed for radioactivity both by scintillation spectroscopy (15) and fluorography (25).

Results

Two-Dimensional IEF/SDS Gel Electrophoresis of Exocrine Pancreatic Proteins and their Identification by Actual or Potential Enzyme Activity

Exocrine proteins contained in human pancreatic juice were separated in two-dimensions using the technique described by Scheele (15). Figure 1 shows the electrophoretic pattern obtained with a sample of pancreatic juice from a normal individual. In order to preserve the integrity of the secretory proteins it was necessary to add Trasylol and soybean trypsin inhibitor to the isoelectric focusing gel. In the absence of these inhibitors, trypsinogen 3 autoactivated during the focusing procedure, and the resulting trypsin activity activated and degraded those proteins which migrate with trypsinogen 3 including procarboxypeptidase B, lipase, and amylase. Fifteen pancreatic proteins are clearly separated in this figure, and they are labeled with abbreviations described in Table 2. Of the two trypsin inhibitors added, only soybean trypsin inhibitor appeared on the gel since Trasylol was not stained with Coomassie blue R. In addition, the two colipase molecules (unpublished observations) were not stained,

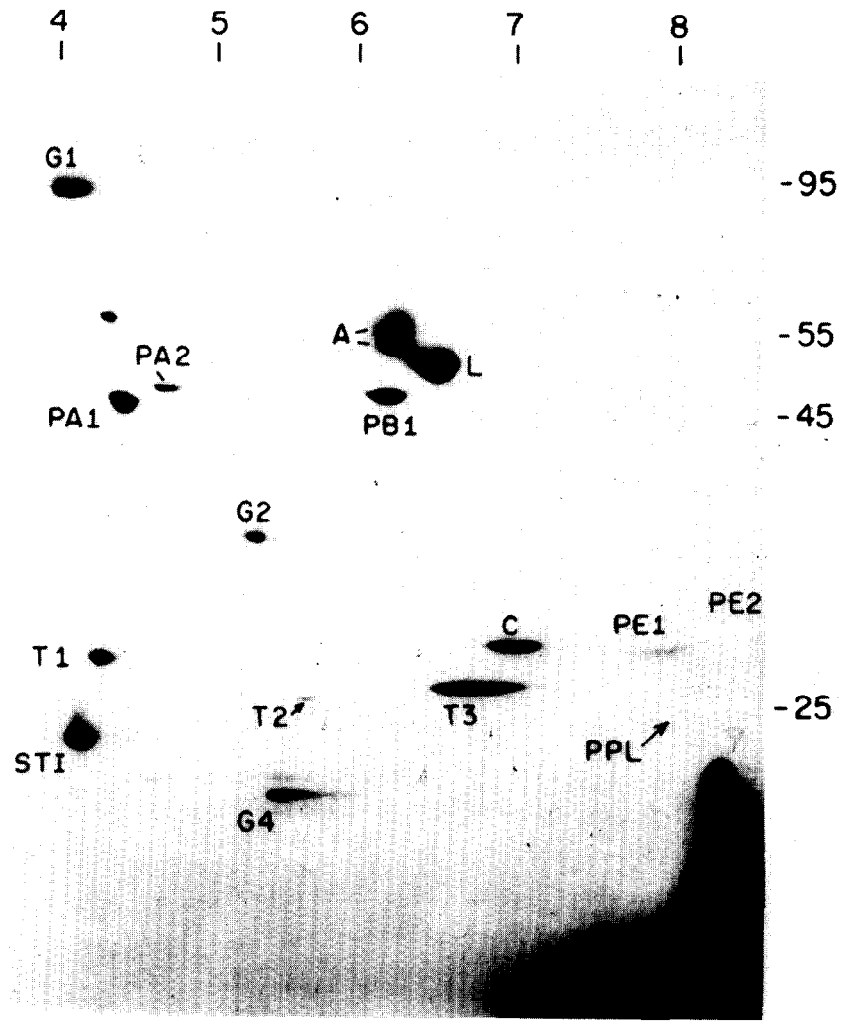

Figure 1. Two-dimensional isoelectric focusing/SDS-gel electrophoresis of human exocrine pancreatic proteins (200 μg) contained in human pancreatic juice. Proteins were separated by slab gel isoelectric focusing using the procedure described in Methods. The focusing strip (1 cm wide) was then cut from the slab gel, rinsed in a solution containing 1% SDS and 50 mM Tris-HCl, pH 6.8, and applied to a polyacrylamide slab gel containing SDS. Proteins were then separated in the second dimension by SDS-gel electrophoresis. Proteins separated in the two-dimensional gel were stained with Coomassie blue R as previously described (15). The *upper abscissa* gives the isoelectric points, and the *ordinate* gives the molecular weights $\times 10^{-3}$ of the separated proteins. Exocrine pancreatic proteins are labeled in abbreviated form according to their actual or potential enzyme activity. Abbreviations are described in Table 2. STI represents soybean trypsin inhibitor (IEP 4.6, M_r 21,000) which was added to both the isoelectric focusing gel and protein sample to inhibit autoactivation of human pancreatic trypsinogen. The dark stain in the *lower right corner* represents basic ampholytes which have not been cleared from the second dimension gel.

and prophospholipase A_2 was poorly stained using Coomassie blue R.

Exocrine pancreatic proteins were identified in the first dimension gel by their actual and potential enzyme activities. Isoelectric focusing slab gels were cut into 1.0-cm fractions and proteins in these fractions were eluted into 0.1 M KCl, 0.1 M Tris-HCl, pH 8.0, 0.02 M $CaCl_2$ containing 100 μg/ml BSA. Enzyme activities in these fractions were determined before and after activation, and the results are presented in Figure 2. Two forms of trypsinogen (labeled T1 and T3 in Figure 1) were found in this study, and the second form found in fractions 8–10 was autoactivated after elution overnight at 3°C. Later studies indicated a third form of trypsinogen which is labeled T2 in Figure 1. Single forms were observed for chymotrypsinogen, amylase, lipase, and procarboxypeptidase B. Procarboxypeptidase A, which was found in a single peak in this study, was later observed to exist in two discrete forms (17). Identifications of the proteins in the second-dimension gel were made according to the positions of actual and potential enzyme activities in the first-dimension gel and considerations of molecular weight based on commercially available pancreatic proteins, in the second-dimension gel.

The identity of amylase was confirmed by purification of the protein using shellfish glycogen (26) and analysis of the purified product by the two-dimensional gel procedure. Figure 3 shows the areas of interest in the two-dimensional gels used to analyze this procedure. This area of interest includes three

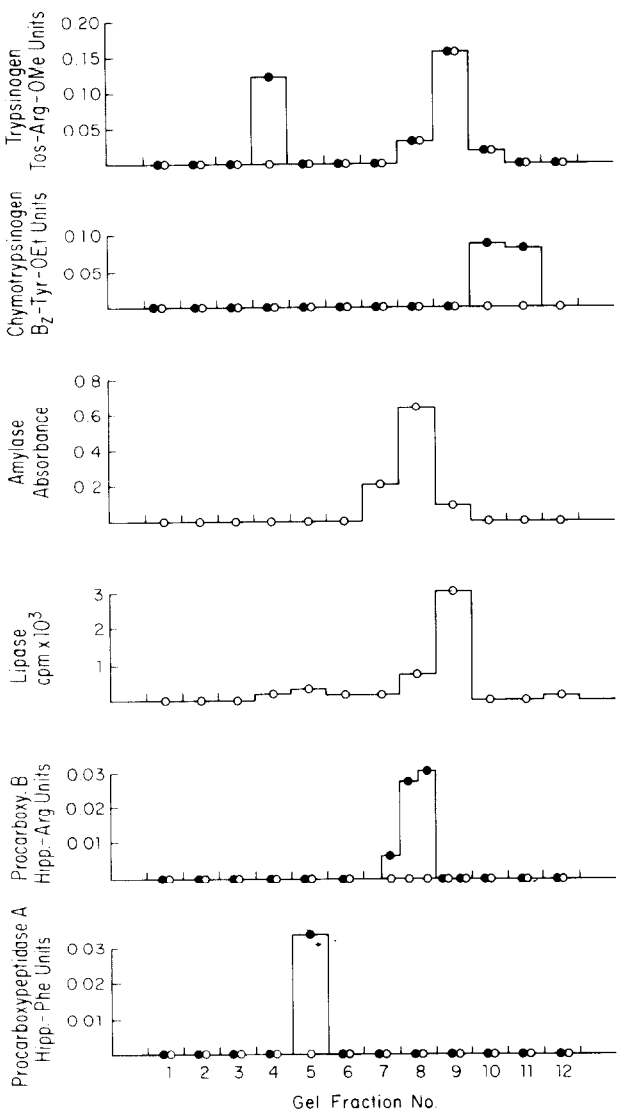

Figure 2. Histogram of actual and potential enzyme activities of exocrine pancreatic proteins eluted from isoelectric focusing gels. Pancreatic proteins contained in 1-cm focusing gel fractions were eluted into buffer containing 0.1 M KCl, 0.1 M Tris-HCl, pH 8.0, 0.02 M $CaCl_2$ and 100 μg/ml BSA at 3°C for 16 h. Zymogens were activated as described in Methods. Enzyme activities obtained before activation are given by the *open circles*. Activities obtained after activation are given by the *solid circles*. Substrates are as follows: Tos-Arg-OMe, tosyl arginine methyl ester; Bz-Tyr-OEe, benzoyl tyrosine ethyl ester; Hipp-Arg, Hippuryl arginine; and Hipp-Phe, hippuryl phenylalanine.

of the exocrine proteins shown in Figure 1, amylase (which appears as a vertical doublet), lipase, and procarboxypeptidase B. Figure 3*a* shows the two-dimensional gel analysis of pancreatic juice before the addition of shellfish glycogen. After addition of glycogen, pancreatic juice is separated into two fractions, a precipitate which contains the amylase, in complex with the glycogen (Figure 3*b*), and the supernate, which contains the residual proteins, in this case lipase and procarboxypeptidase B (Figure 3*c*). Figure 3*b* shows a third spot for amylase (*small arrow*) which could also be observed in pancreatic juice samples analyzed at higher protein concentrations. Two-dimensional gel scanning indicated that this second form of amylase represented approximately 5% of the amylase present.

Two-Dimensional Urea IEF/SDS Gel Electrophoresis of Exocrine Pancreatic Proteins

Because of the potential for autoactivation of trypsinogen 3 during the focusing procedure, we developed a modification of our two-dimensional gel procedure. This modification, which involves the ad-

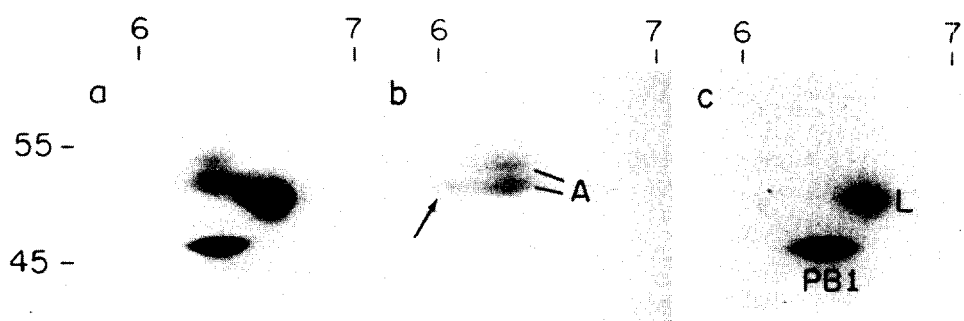

Figure 3. Purification of human pancreatic amylase by glycogen precipitation. Analysis by two-dimensional isoelectric focusing/SDS gel electrophoresis. Insets of two-dimensional gels showing the area of separation. amylase (*A*), lipase (*L*), and procarboxypeptidase B (*PB*) are shown. Figure 3*a* shows the proteins contained in pancreatic juice treated with 40% ethanol; Figure 3*b* shows the protein precipiated with shellfish glycogen; and Figure 3*c* shows the resulting supernate. The *small arrow* indicates a second, minor form of amylase detected in this study. Proteins are stained with Coomassie blue R.

dition of 8 M urea to the isoelectric focusing gel, was found to inhibit the autoactivation of this form of human trypsinogen (17). The pattern of two-dimensional gel spots (Figure 4) appears somewhat different because the isoelectric points of proteins focused in the presence of 8 M urea are different from those focused in the absence of urea (17). In addition, trypsinogen 3, which showed considerable charge heterogeneity when focused in the absence of urea, appeared to be homogeneous when focused in the presence of urea.

Exocrine pancreatic proteins separated by isoelectric focusing in the presence of 8 M urea were identified according to actual and potential enzyme activity after elution of proteins from 0.5 cm focusing gel fractions. We encountered no difficulty with enzyme measurements provided the urea concentration was diluted to less than 0.5 M during the elution procedure (17). Again, identification of proteins in the second-dimension gel was made by their position in the first-dimension gel and by considerations of molecular weight in the second-dimension gel.

Separation of Exocrine Proteins by Gradient Polyacrylamide Gel Electrophoresis in SDS: Identification of Proteins and Determination of Molecular Weights

Figure 5 shows the banding pattern, after reduction and alkylation, of proteins contained in human pancreatic juice. The positions of molecular weight markers are given to the left, and identities of proteins are given in abbreviated form to the right. Identifications were made in the following manner: Exocrine proteins were separated by isoelectric focusing, and proteins eluted from gel fractions as described in Methods were precipitated with 10% trichloroacetic acid. After centrifugation and removal of the supernatants, pellets were resuspended in the presence of 2% SDS, reduced and alkylated, and compared with pancreatic juice, similarly treated, using one-dimensional polyacrylamide gel electrophoresis in SDS. Since other studies had determined the identities of proteins contained in isoelectric focusing gel fractions, these identities could

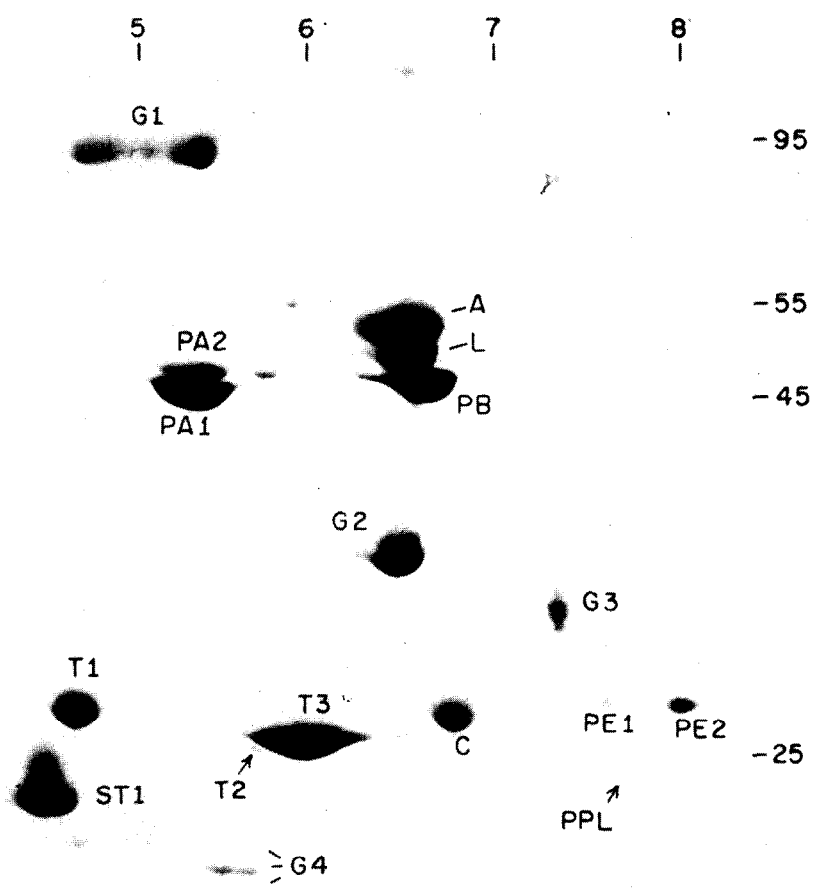

Figure 4. Two-dimensional *urea* isoelectric focusing/SDS gel electrophoresis of human exocrine pancreatic proteins. Proteins (200 μg) contained in pancreatic juice were separated in two dimensions as described in the legend to Figure 1 except that the isoelectric focusing slab gel contained 8 M urea and proteins were applied to the focusing gel in 4 M urea. Proteins were stained with Coomassie blue R. The *upper abscissa* gives the isoelectric points, and the *ordinate* gives the molecular weights × 10^{-3} of the separated proteins. Exocrine pancreatic proteins are labeled in abbreviated form as described in Table 2. Soybean trypsin inhibitor (STI) and basic ampholytes are observed as described in the legend to Figure 1.

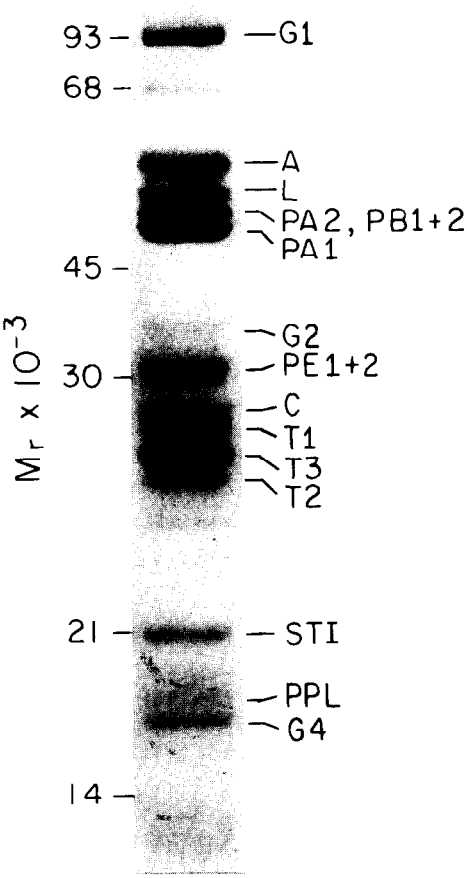

Figure 5. One-dimensional analysis of human exocrine pancreatic proteins using SDS-gel electrophoresis in a polyacrylamide gradient (12–17%). Proteins (50 μg) contained in pancreatic juice were carboxymethylated (see Methods) and prepared for SDS-gel electrophoresis as previously described (15). Coomassie blue R stained bands, individually identified as described in Results, are labeled in abbreviated form according to actual or potential enzyme activity on the right. The position of molecular weight marker proteins is presented on the left.

be assigned to bands appearing in one-dimensional gels.

Analysis of proteins in their carboxymethylated state allowed accurate assessment of their molecular weights using SDS-gel electrophoresis as shown previously for guinea pig pancreatic proteins (15). Separation of pancreatic proteins, in this form, additionally results in the complete separation of amylase and lipase.

Variation in the Number of Forms of Human Pancreatic Procarboxypeptidase B

The number of forms of enzymes and zymogens contained in human pancreatic juice and their two-dimensional migration on gels were generally the same among normal individual human subjects, with the exception of procarboxypeptidase B. Of 10 subjects tested, 6 contained only a single form of procarboxypeptidase B (IEP 6.2 and M_r 47,000) in their pancreatic juice, and 4 subjects contained two forms (the first as described above and the second with an IEP 6.7 and M_r 47,000). Figure 6*A* demonstrates pancreatic juice containing two forms of procarboxypeptidase B, and Figure 6*B* demonstrates pancreatic juice, obtained from a second individual, containing one form. Incorporation of 15 ^{3}H-amino acids into secretory proteins contained in tissue slices indicated that 4.4% of the radioactivity was incorporated into procarboxypeptidase B1, and 2.9% was incorporated into procarboxypeptidase B2. The distribution of potential enzyme activities among the two forms, separated and eluted from isoelectric focusing gels, was 61% and 39%, respectively. These findings indicate that the two forms of procarboxypeptidase B have similar specific activities.

Human Pancreatic Trypsinogens 1 and 3 Covalently Labeled with $^{35}SO_4$

Human pancreatic slices were labeled for 2 h with $^{35}SO_4$ under physiological conditions. Pancreatic tissue was then homogenized as described in Methods, and the proteins contained in tissue homogenates were separated by the two-dimensional gel procedure and analyzed by fluorography (25). Figure 7*A* shows the results of this analysis and indicates that the radioactivity appears in two spots, which coincide with trypsinogen 1 and trypsinogen 3. Other areas of the gel were devoid of radioactivity which indicates that the radioactive label was not metabolized to methionine or cysteine. Furthermore, the association of the radioactive label with a protein after two-dimensional IEF/SDS gel electrophoresis indicates that the association is mediated by a covalent bond. Quantitative analysis of radioactivity contained in two-dimensional gel spots by liquid scintillation spectrometry as previously described (15) indicated that greater than 95% of the radioactivity found in the two-dimensional gel appeared in the two trypsinogen spots. Sixty-seven percent appeared in trypsinogen 1, and 27 percent appeared in trypsinogen 3.

Figure 7*B* shows the lability of the sulfate linkage in these two trypsinogen forms. A 5-min treatment of tissue homogenate containing the $^{35}SO_4$-labeled proteins at 100°C and 0.1 N HCl (pH = 1.0) resulted in the removal of greater than 90% of the radioactive label from each of the proteins. This finding suggests that the sulfate moiety is covalently attached to a tyrosine residue. The radioactivity observed at the top of the gel most likely represents label attached to

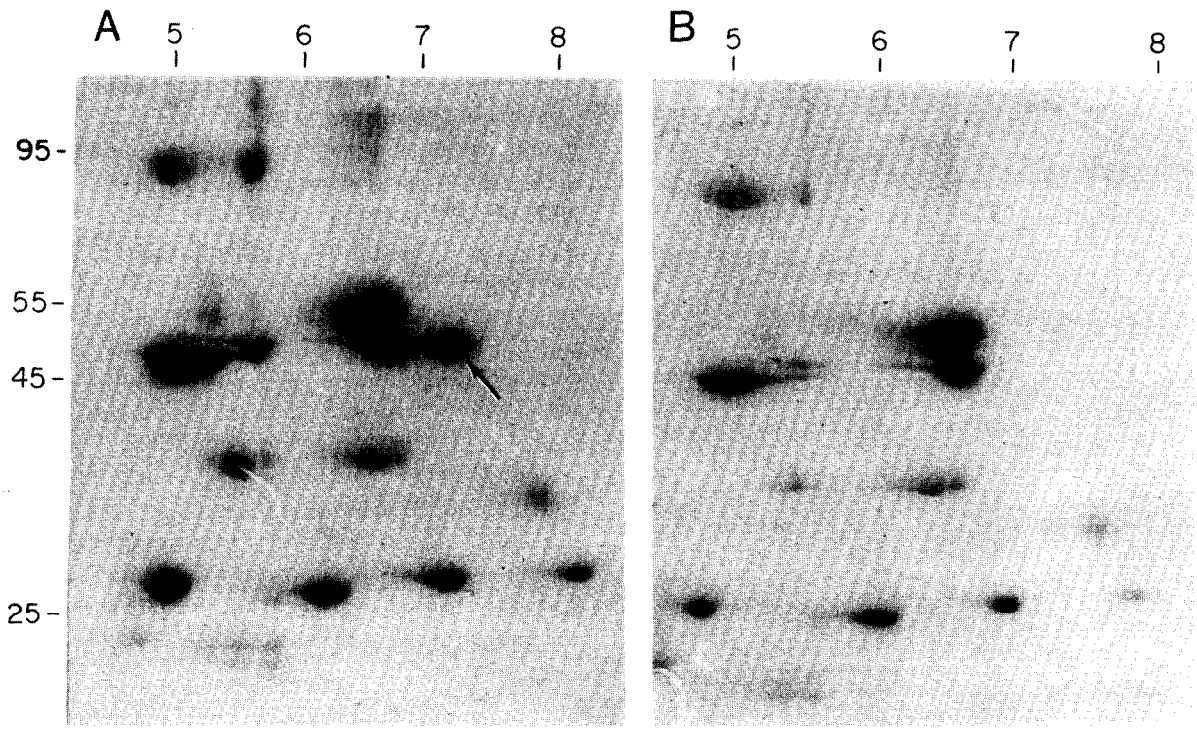

Figure 6. Variation in the number of forms of human pancreatic procarboxypeptidase B. Samples of pure pancreatic juice from two humans subjects were individually analyzed by two-dimensional urea isoelectric focusing/SDS gel electrophoresis. Figure 6*A* shows two forms of procarboxypeptidase B, and the second form, procarboxypeptidase B2, is indicated by the *arrow*. Figure 6*B* shows a single form of procarboxypeptidase B similar to that seen in Figure 4. Two forms of this zymogen were found in the pancreatic juice of 4 out of 10 human subjects tested.

proteoglycans. Most of this observed radioactivity appears at the interface of the stacking gel (5% polyacrylamide) and the running gel (12%–17% polyacrylamide), while some of the radioactivity appears to enter the running gel in the form of a heterogeneous smear.

Identification of a Third Form of Human Pancreatic Trypsinogen

Studies were carried out to determine if the small Coomassie blue stained spot observed between the two major trypsinogens in Figures 1 and 4 (see *arrow*) represented a third form of human pancreatic trypsinogen. Pancreatic proteins were separated using the two-dimensional gel technique and stained in second dimension gels with 0.03% Coomassie blue, type G, in the presence of 100 mM Tris-HCl, pH 9.0, and 5% glycerol. Chemical fixatives, including methanol and acetic acid, were omitted. After 12 h of staining, using this low concentration of Coomassie blue, areas of the gel which contained protein were stained more heavily than background areas. Stained spots were then excised, and proteins contained in these gel pieces were simultaneously eluted from the gel matrix and renatured in a solution containing 1% Triton X-100, 0.1 M KCl, 0.1 M Tris-HCl, pH 8.0, 0.02 M CaCl, and 100 μg/ml BSA for 16 h at 3°C. The two previously identified trypsinogens, forms 1 and 3, and the protein of interest were then activated using enterokinase as described in Methods. Coomassie blue, extracted into the elution buffer, did not interfere with either the activation of trypsinogen or the assay of its active product. Trypsin activity derived from the trypsinogen 1 spot amounted to 0.145 U, that derived from the trypsinogen 3 spot amounted to 0.190 U and that derived from the spot indicated by the *arrow* gave 0.029 U. Table 1 shows the effect of Trasylol and soybean

Table 1. Effect of Trypsin Inhibitors on the Percent Activities of Three Human Trypsins

		None	Trasylol, 0.4 U	Soybean, 0.4 U
Human trypsin 1	(0.30 U)	100	0	14
Human trypsin 2	(0.06 U)	100	99	100
Human trypsin 3	(0.34 U)	100	0	77
Bovine trypsin	(0.20 U)	100	0	0

Note: One trypsin inhibitor unit inhibits 1 μmol/min tosyl arginine methyl ester hydrolysis by bovine trypsin at 22°C.

Figure 7. Covalent linkage of $^{35}SO_4$ to human pancreatic trypsinogens 1 and 3. Human pancreatic tissue slices were incubated in vitro for 2 h with Krebs-Ringer bicarbonate buffer containing $^{35}SO_4$ as described in Methods. Tissue slices were then homogenized in 1% Triton X-100 and 25 mM Tris-HCl, pH 9.0. *A.* Proteins contained in tissue homogenates and separated by two-dimensional urea IEF/SDS gel electrophoresis were analyzed for radioactivity by fluorography of dried gels (25). Radioactivity found among proteins was coincident with Coomassie blue stained spots representing trypsinogens 1 and 3. Numbers on the *upper abscissa* and *right ordinate* are as described in the legend to Figure 1. *B.* Tissue homogenates containing $^{35}SO_4$-labeled proteins were adjusted to pH 1.0 with 0.1 N HCl and incubated at 100°C for 5 min. Proteins were then reduced with 50 mM dithiothreitol, alkylated with 100 mM iodoacetamide, separated by SDS-polyacrylamide gel electrophoresis, and analyzed by fluorography. Slot 1 shows the control; slot 2, the effect of the treatment.

trypsin inhibitor in concentrations sufficient to inhibit 0.4 U of bovine trypsin on each of the three forms of pancreatic trypsin. The acidic trypsin, form 1, was inhibited significantly by both Trasylol and soybean trypsin inhibitor. Trypsin 3 was inhibited to a considerably less extent by soybean trypsin inhibitor. Trypsin 2 showed no inhibition with either Trasylol or soybean trypsin inhibitor. These latter findings confirm the existence of a third form of human pancreatic trypsinogen, which is distinct from the two previously described.

Characterization of Human Exocrine Pancreatic Proteins

Table 2 summarizes a number of characteristics for each of the exocrine proteins studied. Proteins are listed generally by molecular weight, from high to low. For those proteins identified by actual or potential enzyme activity, the full descriptive name of the enzyme or zymogen is given along with the International Union of Biochemistry number. Four protein spots, which were positively stained by the periodic acid Schiff reaction, could not be identified according to the enzyme assays used in this study. They are designated arbitrarily as glycoproteins 1–4. Multiple forms of enzymes or zymogens are numbered consecutively from anode to cathode in accordance with the IUPAC-IUB Commission on the biochemical nomenclature of multiple forms of enzymes (27). Abbreviations for each of the exocrine pancreatic proteins are given and correspond to those used in Figures 1 and 4. Molecular weights are given for proteins in their carboxymethylated form, as described above. Isoelectric points are given both for the native pancreatic proteins (IEP_n) and for those denatured in 8 M urea (IEP_u). Pancreatic proteins in their native state cover a wider range of isoelectric points. In the presence of urea the isoelectric points of anionic and cationic proteins show a general tendency to shift toward neutrality (16). Proteins were quantitated, as described in Methods, according to both relative mass, as measured by the in-

Table 2. Characteristics of Human Exocrine Pancreatic Proteins

	I.U.B. Number	Abbrev.	M_r[a]	IEP_n[b]	IEP_u[c]	Mass proportion (%)[d]	Distribution of stain (%)[e]	PAS[f]
1. Glycoprotein 1		G1	93,000	3.9	5.0	3.1	7.5	+
2. α-Amylase	(EC 3.2.1.1)	A	54,800	6.3	6.4	5.3	18.3	–
3. Lipase	(EC 3.1.1.3)	L	50,500	6.5	6.6	0.7	3.0	+
4. (Pro)carboxypeptidase A1	(EC 3.4.17.1)	PA1	46,000	4.6	5.1	16.8	12.9	–
5. (Pro)carboxypeptidase A2	(EC 3.4.17.1)	PA2	47,000	4.7	5.5	8.1	1.2	–
6. (Pro)carboxypeptidase B1	(EC 3.4.17.2)	PB1	47,000	6.2	6.7	4.4	8.2	–
7. (Pro)carboxypeptidase B2	(EC 3.4.17.2)	PB2	47,000	6.7	6.8	2.9	—	–
8. Glycoprotein 2		G2	36,500	5.2	6.6	6.7	8.1	+
9. Glycoprotein 3[g]		G3	—	7.2	7.0	0.5	1.7	+
10. Trypsin(ogen) 1	(EC 3.4.21.4)	T1	28,000	4.4	4.9	23.1	9.3	–
11. Trypsin(ogen) 2	(EC 3.4.21.4)	T2	26,000	5.5	5.7	—	—	–
12. Trypsin(ogen) 3	(EC 3.4.21.4)	T3	26,700	6.4	6.2	16.0	18.1	–
13. Chymotrypsin(ogen)	(EC 3.4.21.1)	C	29,000	7.2	6.8	1.7	7.0	–
14. (Pro)elastase 1	(EC 3.4.21.11)	PE1	30,500	7.6	7.2	3.1	1.0	–
15. (Pro)elastase 2	(EC 3.4.21.11)	PE2	30,500	>7.9	8.0	1.2	1.3	–
16. Glycoprotein 4		G4	15,700	5.4	5.5	2.5	1.1	+
17. Colipase 1		CL1	—	7.2	7.3	—	—	–
18. Colipase 2		CL2	—	7.4	7.4	—	—	–
19 (Pro)phospholipase A_2	(EC 3.1.1.4)	PPL	17,500	7.5	7.9	—	—	–

[a] M_r = Molecular weight of proteins, reduced and alkylated, as determined by gradient polyacrylamide gel electrophoresis in SDS. [b] Isoelectric points of undenatured proteins (native). [c] Isoelectric points of proteins denatured in 8 M urea. [d] Mass proportion of secretory proteins were determined by incorporation of a mixture of 15 ^{3}H-amino acids into human pancreatic slices over 2 h and subsequent analysis of radioactive secretory proteins contained in tissue homogenates by two-dimensional IEF/SDS-gel electrophoresis. [e] Distribution of Coomassie blue R stain among secretory proteins contained in human pancreatic juice and separated by two-dimensional IEF/SDS-gel electrophoresis. [f] Periodic acid Schiff reactivity of two-dimensional gel spots. [g] Glycoprotein 3 which appeared as a discrete spot using the two-dimensional gel procedure with urea in the isoelectric focusing gel (Figure 4) was not observed using the two-dimensional procedure without urea in the isoelectric focusing gel (Figure 1).

Note: Enzymes and potential enzymes are numbered consecutively from anode to cathode in accordance with the IUPAC-IUB Commission on the biochemical nomenclature of multiple forms of enzymes. The four glycoproteins with unknown functions are numbered arbitrarily.

corporation of a mixture of 15 ^{3}H-amino acids into secretory proteins contained in tissue slices, and according to the distribution of Coomassie blue R stain among proteins contained in pancreatic juice, as determined by two-dimensional gel scanning and computer analysis.

Discussion

The two-dimensional gel procedure, using isoelectric focusing in the first dimension and SDS-gel electrophoresis in the second dimension, was developed by Scheele (15) for the analysis of complex mixtures of proteins, such as those secreted by the exocrine pancreas (28). Previous studies had indicated that guinea pig pancreatic proteins were stable during the two-dimensional gel procedure (15). Our initial experience with human pancreatic secretory proteins indicated that trypsinogen 3 (IEP = 6.4) autoactivated either during the isoelectric focusing procedure or during the transfer of the focusing strip to the second-dimension gel. Addition of soybean trypsin inhibitor and Trasylol* to the isoelectric focusing gel inhibited the autoactivation of trypsinogen 3 and allowed the successful analysis of pancreatic proteins in their native configuration as demonstrated in Figure 1. However, under these conditions the time of focusing was limited since each of the added inhibitors were themselves being focused during the procedure, soybean trypsin inhibitor to pH 4.6 and Trasylol to ~pH 10.0. Thus, if the focusing procedure was prolonged, the trypsin inhibitors were cleared from the field of the gel, and trypsinogen 3 was rapidly autoactivated. Activation of trypsinogen to trypsin resulted in both the activation and degradation of other proteins which migrated in close proximity with trypsinogen 3: procarboxypeptidase B, lipase, and amylase. Autoactivation of this type could be readily discerned using the two-dimensional gel technique by the disappearance of the proteins described above and the appearance of activation and degradation peptides in the second-dimension gel. Trypsinogen 1 remained relatively stable during the focusing procedure.

Addition of 8 M urea to the isoelectric focusing gel, in addition to the two trypsin inhibitors, com-

*See Figarella C, Negri GA, Guy O. Eur J Biochem 1975;53:457–63 for effect of Trasylol and STI on the two major forms of human trypsin.

pletely prevented the autoactivation of trypsinogen 3 (17). This modification provided, under routine use, a more reliable method for the analysis of human exocrine pancreatic proteins. Since 8 M urea significantly inhibited the activities of the two major forms of human trypsinogens (17), this modification of the two-dimensional gel procedure also allowed the analysis of pancreatic juice samples which were partially activated.

Analysis of human pancreatic juice by isoelectric focusing in the absence (Figure 1) or presence (Figure 4) of urea followed by SDS-gel electrophoresis gave similar results. Using Coomassie blue R stain, 17 distinct proteins were observed. Two forms of colipase which were not visualized by Coomassie blue R stain were identified and characterized in a separate study using Coomassie blue G. (unpublished observations). Multiple forms were observed for a number of enzymes and zymogens. Two major and one minor form of trypsinogen were observed. The occurrence of two forms of human pancreatic trypsinogen has been reported by a number of workers (2,4,29,30) and characterized in detail by Guy et al. (8). More recently Rinderknecht et al. have reported the presence of a third minor trypsinogen in human pancreatic juice which is similar to the minor form described here (31). Further discussion concerning this minor form of trypsinogen appears below. Based on amino acid composition data for trypsinogen 1 and trypsinogen 3 (8) and the differential effect of trypsin inhibitors on each of the three corresponding trypsins (cf. Table 1), the three forms of trypsinogen are believed to be derived from separate genes.

Two forms of each were observed for colipase, procarboxypeptidase A, proelastase, and in some cases carboxypeptidase B and amylase. Two forms of colipase have been isolated from human pancreatic tissue by Sternby and Borgström (32). Two forms each for human pancreatic carboxypeptidase A (33), carboxypeptidase B (34,35), and elastase (36) have been described in the literature. The two forms of proelastase, isolated and renatured from a two-dimensional gel, each possessed weak potential chymotrypsinlike activity using benzoyl tyrosine ethyl ester as substrate, a result consistent with the findings of Largman et al. (36) and De Caro et al. (6). The identity of amylase (IEP = 6.3) among the spots observed in the two-dimensional gel was confirmed by analysis of purified amylase, precipitated with shellfish glycogen. Using a variety of techniques, others have found multiple forms of human pancreatic amylase (37-39). However, it is important to distinguish physiologic forms from those appearing after incubation at 37°C or long-term storage at −20°C (39). While we have observed human pancreatic amylase to focus at pH 6.3 in polyacrylamide gels, other investigators have observed amylase to focus at pH 6.8 in glass columns (39). Discrepancies of this magnitude can be observed when isoelectric points determined in polyacrylamide gels and glass columns are compared.

Single forms were observed for prophospholipase A_2, chymotrypsinogen, and lipase. The isolation of one form of prophospholipase A_2 from human pancreatic juice has been reported (40). De Caro et al. have described the isolation of one major and one minor form of chymotrypsinogen from pooled pancreatic juice devoid of free proteolytic activity (6). The minor form represented 7% of the total potential chymotrypsin activity in the juice.

The finding of a single form of lipase, which was strongly stained with the periodic acid Schiff reaction, indicating the covalent attachment of carbohydrate, is in agreement with De Caro et al. who have isolated human pancreatic lipase and determined the composition of its carbohydrate content (7). Four Coomassie blue-stained spots in the two-dimensional gel which could not be identified with any of the enzymatic activities measured in this study were also strongly stained by the periodic acid Schiff reagent. Accordingly they were designated as glycoproteins 1-4. Although amylase was not stained by this procedure, limited binding of ^{125}I concanavalin A to this protein was observed. Each of these exocrine pancreatic proteins has been additionally characterized according to isoelectric point and molecular weight.

Isoelectric points are given for proteins separated both in the absence and the presence of urea. Investigation into the molecular weights of carboxymethylated secretory proteins indicated that amylase and lipase were completely separated by this one-dimensional procedure (cf. Figure 5). Separation of nonreduced, nonalkylated secretory proteins either by the one-dimensional gel or the two-dimensional procedure using 8 M urea in the focusing gel did not result in the complete separation of these two enzymes.

Two methods have been used to quantitate exocrine pancreatic proteins. The distribution of stain among two-dimensional gel spots significantly deviated from the measurements of relative mass using the radioactive amino acid incorporation technique. This finding was expected, since the binding of Coomassie blue dyes varies significantly with different proteins. Within limits, however, the distribution of Coomassie blue stain among two-dimensional gel spots can be used to quantitate proteins contained in human pancreatic juice, since, for any specific protein, dye binding is linearly related to the amount of protein.

During the course of these studies, three unique observations were made which relate to human exocrine pancreatic proteins. Patterns of Coomassie blue-stained proteins varied dramatically among individuals in regard to the number of forms of procarboxypeptidase B. While procarboxypeptidase B1 (IEP 6.2 and M_r 47,000) was found in the pancreatic juice of all human subjects, procarboxypeptidase B2 (IEP 6.7 and M_r 47,000) was found in only 4 of 10 subjects tested. The expression of this zymogen may represent a genetic polymorphism in humans.

Both major forms of human pancreatic trypsinogen were labeled with $^{35}SO_4$. The quantities of $^{35}SO_4$ incorporated into these two proteins were proportional to their mass as given in Table 1. Interestingly, trypsinogen 2 was not labeled with $^{35}SO_4$. Whether or not the covalent attachment of SO_4 to trypsinogens 1 and 3 has physiologic significance will require further study. Sulfate groups are known to be attached to certain types of proteoglycans through either a sulfate ester linkage with sugar residues or a sulfamide linkage with aminosugar residues (41). Sulfate groups are attached to fibrinogen (42) and polypeptide hormones, including CCK-pancreozymin and gastrin through a tyrosine-O-sulfate linkage (43). The attachment of SO_4 groups to these molecules is thought to occur in the Golgi complex (44,45). Brief acid hydrolysis of the two $^{35}SO_4$ human pancreatic trypsinogens resulted in the loss of greater than 90% of the attached sulfate. Since there is no evidence that carbohydrate is attached to these proteins, this finding suggests that these sulfate moieties are attached directly to tyrosine residues (46). Radioactive sulfate was also observed to attach to a class of large proteins with heterogeneous molecular weights, most likely representing proteoglycans similar to those described in the guinea pig pancreas by Tartakoff et al. (47) and Reggio and Palade (45).

Finally, a third form of human pancreatic trypsinogen, with an isoelectric point of 5.5 and mol wt of 26,300, has been described. This zymogen is of particular interest since its corresponding trypsin showed no inhibition with Trasylol or soybean trypsin inhibitor. As mentioned above, Rinderknecht et al. have identified a similar third and minor form of human pancreatic trypsinogen after nondenaturing polyacrylamide gel electrophoresis (31). In their study the corresponding form of trypsin showed no inhibition with human pancreatic secretory trypsin inhibitor as well as a number of commercially available trypsin inhibitors.

The present study has described, in detail, the exocrine proteins which appear in normal pancreatic juice. A comprehensive analysis of these proteins can be carried out on samples of pure human pancreatic juice, obtained by endoscopic retrograde cannulation of the pancreatic duct. This method should now allow a detailed study into the pathological changes and the pathophysiological mechanisms associated with pancreatic disease.

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