Two-Dimensional Gel Analysis of Soluble Proteins

CHARACTERIZATION OF GUINEA PIG EXOCRINE PANCREATIC PROTEINS

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SUMMARY

A two-dimensional gel technique using slab gel isoelectric focusing in the first dimension and sodium dodecyl sulfate gradient gel electrophoresis in the second dimension has been developed for the separation of soluble proteins larger than 10,000 daltons. The technique is sensitive to 0.6 μg of protein and recovery of radiolabeled proteins averages 90%. Analysis of secretory protein from the guinea pig exocrine pancreas shows the presence of 19 distinct high molecular weight proteins. Each of these proteins has been characterized by isoelectric point, molecular weight, and proportionate mass. Thirteen of the 19 proteins have been identified by actual or potential enzymatic activity, accounting for 96% of the protein mass resolved by the two-dimensional gel.

Exocrine pancreatic proteins have been studied in the past by a number of laboratories (1–7, 10–16) using classical methods for separation of proteins. In most of these studies exocrine proteins were obtained by sulfuric acid extraction of tissue or by direct cannulation of the pancreatic duct, temporarily stored at -20°C after deionization and lyophilization, and analyzed by column chromatographic methods. While these studies have yielded important information about proteins in general and exocrine pancreatic proteins in particular, this approach has several disadvantages for further investigation of pancreatic proteins at the cell biological level: (a) secretory proteins are extracted from tissue and stored under nonphysiological conditions, (b) large amounts of starting material are required, (c) separation of proteins on columns is frequently incomplete, (d) proteins eluted from columns are recovered in relatively dilute solutions requiring subsequent concentration procedures, and (e) the time and degree of manipulation necessary to obtain adequate separation of exocrine proteins is burdensome.

Recently, exocrine pancreatic proteins of the guinea pig have been fractionated by Tartakoff et al. (17) using column isoelectric focusing and sodium dodecyl sulfate gel electrophoresis. Secretory proteins used in these fractionation procedures were obtained under physiological conditions using in vivo pancreatic lobules as described by Scheele and Palade (18). Eight proteins (anylase, ribonuclease, trypsinogen, and chymotrypsinogen) were then determined on Na dodecyl-SO₄ gels. Na dodecyl-SO₄ gel electrophoresis revealed two additional proteins not identified on focusing columns and the relative Kₑ values for these proteins were then determined on Na dodecyl-SO₄ gels. Na dodecyl-SO₄ gel electrophoresis was used to obtain complete separation of all secretory proteins, a two-dimensional gel technique was developed to be used on mixtures of soluble proteins. This technique involves slab gel isoelectric focusing in 5% polyacrylamide in the first dimension and sodium dodecyl sulfate gel electrophoresis in gradient (10 to 20%) polyacrylamide in the second dimension. In the first dimension proteins are separated by charge; in the second dimension, by molecular weight.

EXPERIMENTAL PROCEDURE

Materials

Acrylamide, N,N’-methylenebisacrylamide, and N,N,N’,N’-tetramethylethlenediamine were purchased from Eastman Kodak, Rochester, N. Y. Ribonavin, ammonium persulfate, lauryl sulfate (sodium dodecyl sulfate), trisomy base, glycine, Triton X-114, bromphenol blue, iodoacetamide, benzoylalanine-methyl ester, yeast (cornula) RNA grade V, and tripalmitin were obtained from Sigma Chemical Co. Porcine α-anaylase (AA), bovine DNase I (D), bovine chymotrypsinogen A (WGC GFOA), bovine trypsinogen (TG 11D), bovine RNase A (ROGR), and lyophilized trypsin (TRL 1GA, 180 μg/mg) were obtained from Worthington Biochemical Corp., Freehold, N. J. Coomassie brilliant blue, sucrose, ultrapure, carbodol diisopropylfluorophosphate, hippuryl-arginine, hippuryl-phenylalanine, benzoyl-tyrosine-ethyl ester, and tosyl-arginine-methyl ester were from Schwarz/Mann. Ampholines (pH 3.5 to 10, pH 9 to 11, pH 5 to 7) were from LKB. I%, amino-acids, reconstituted from an algal protein hydrolysate, 1 mCi/ml, Catalog No. 2133-11, were purchased from Schwarz/Mann Radiochemicals. Bovine plasma albumin (crystallized) was obtained from Armour Pharmaceutical Co. Benzamidine hydrochloride (HCl) was from Aldrich Chemical Co. Hydrogen peroxide was from Fisher. Xylene was from Mallinckrodt, Whatman No. 3MM chromatography paper and glass fiber paper, GF/A (2.4 cm), were from W. and R. Balston Inc., England. Dithiothreitol was from W. and R. Balston Inc., England. Dithiothreitol was from W. and R. Balston Inc., England.

1 The abbreviations used are: Na dodecyl-SO₄, sodium dodecyl sulfate; bisacrylamide, N,N’-methylenebisacrylamide; TEMED, N,N,N’,N’-tetramethylethlenediamine; carbodol, carbanil chloride.
Nutritional Biochemical Co. Sephadex G-25 was from Pharmacia Fine Chemicals, Inc. (11-13)Trihalmitan was from Applied Sciences Laboratories. Soluble starch powder was from Amend Drug and Chemical Co. Porcine enterokinase was a kind gift from Drs. Suzanne Maroux and Jacque Baratti, Centre National de la Recherche Scientifique, Marseille, France.

Methods

Extraction of Secretory Protein

Secretory proteins are extracted by physiological methods. Pancreatic lobules are prepared from male albino guinea pigs (Rockefeller University colony), weighing 450 to 500 g, by the method of Scheele and Palade (18). Carbamylcholine (10⁻⁴ m) is used to stimulate the discharge of proteins from pancreatic lobules to the incubation medium (Kreb-Ringer bicarbonate + glucose + amino acids) as described by Scheele and Palade (18). Three-hour incubation of lobules from one pancreas gland in 10 ml of incubation medium results in a solution of secretory protein approximately 2 to 3 mg/ml in concentration. The medium then is centrifuged at 105,000 × g for 30 min at 3° to remove small pieces of connective tissue, and the supernatant is stored in 1.5-ml Eppendorf plastic tubes. Porcine enterokinase (Becton-Dickinson) is added following rapid freezing in liquid nitrogen. Samples can be stored at -70° with the protein sample in a 10 ml diisopropylfluorophosphate (19) and 1 mM benzamidine-HCl (20). No attempt is made to deionize or lyophilize samples. In addition, the secreted proteins can be radiolabeled by the addition of an algali hydrolyzate mixture of 15 NaC-amino acids to the incubation medium (5 pCi/ml) in the absence of cold amino acids (17). Three-hour incubation of these samples can be stored with or without the protease inhibitors, 1 mM benzamidine-HCl, 10 mM diisopropylfluorophosphate (pH 3.5 to 10), 0.25 ml of Ampholine (pH 9 to 11), 0.10 ml of Am- pholine (pH 3 to 6), 0.06 ml of Amorphine (pH 5 to 7), 6 ml of 60% sucrose, and water to 30-ml total volume. Sucrose is added for two reasons: (a) to increase the density of the focusing slab gel which facilitates the application of the focusing strip to the second dimension gel and (b) to increase the viscosity of the focusing medium which inhibits drift in the pH gradient toward the cathode due to the movement of the Polyacrylamide gel by diffusion. 

By the addition of 9 drops of a saturated solution of riboflavin and 0.075 ml of TEMED, and 60 ml of water, the gel mixture is immediately delivered into the gel container (positioned vertically) by gravity through 0.040 internal diameter Solveflex plastic tubing (Technic Corp., Tarrytown, N. Y.). Slow delivery prevents formation of unwanted air bubbles in the focusing medium. Polya crylamide gel polymerization, assisted by the presence of a fluorescent light set 2 inches from the gel, occurs in 60 min at 24 or 3°. After polymerization the gel is placed in the horizontal position with the plastic plate on top and this plate is carefully removed (using thin spatulas to pry between the side plastic shims and the top plastic plate). This somewhat difficult step, successful approximately 90% of the time, is facilitated by the hydrophobic surface of the plastic plate and by the removal of the plate as early after polymerization as possible. Following removal of the plastic plate, the three plastic shims and the outside sealing acrylamide are removed, leaving only the focusing gel adhered to the underlying glass plate. At this point the gel can be used immediately or set on the 8° cooling plate and used 1 to 2 hours later.

Samples of protein are applied to the focusing slab gel by one of two methods. In the first method, the sample of protein is applied to a square of glass chromatography GF/A and the paper square is placed on the surface of the gel. Glass fiber paper was used since preliminary trials with Whatman 3MM chromatography paper showed significant adherence of the protein to the paper. Since isoelectric focusing is an equilibrium procedure, the protein sample can be applied to any part of the surface of the gel although as shown below (cf. Figs. 6 and 7) it may be advantageous if the samples are separated by 0.5-cm intervals can be applied to the surface of a slab gel 12.5 cm wide. In the second method, the sample is added to the focusing gel and directly polymerized into the slab gel, either within a narrow segment of the gel or spread diffusely throughout the gel. The slowly polymerizing gel is facilitated by the hydrophobic surface of the glass plate. Samples can be stored with or without the protease inhibitors, 1 mM benzamidine-HCl, 10 mM diisopropylfluorophosphate (pH 3.5 to 10), 0.25 ml of Ampholine (pH 9 to 11), 0.10 ml of Am- pholine (pH 3 to 6), 0.06 ml of Amorphine (pH 5 to 7), 6 ml of 60% sucrose, and water to 30-ml total volume. Sucrose is added for two reasons: (a) to increase the density of the focusing slab gel which allows for concentration of protein from dilute solutions (24 out of 30 ml of gel solution is water), and is preferred for the determination of isoelectric points since banding of proteins will only occur at these equilibrium points. While both methods of sample application are successful, each has certain advantages. Absorption of the sample to glass fiber paper allows simultaneous comparison of different samples in isoelectric focusing, is convenient for use with small sample volumes (10 to 50), and shows banding of proteins during movement toward isoelectric points (similar to banding during electrorophoresis). Direct polymerization of the sample into the gel matrix ensures 100% recovery in the focusing gel, is ideal for preparative procedures, allows for concentration of protein from dilute solutions (24 out of 30 ml of gel solution is water), and is preferred for the determination of isoelectric points since banding of proteins will only occur at these equilibrium points.

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stained, and destained while the rest of the gel is covered with Saran wrap and stored frozen at -20°C. Later the gel is thawed at 3°C (15 min) and the segment with the desired protein is removed. All cutting of gels is done by hand using a straight edge razor blade and an underlying piece of graph paper for guidance.

Sodium Dodecyl Sulfate Gradient Gel Electrophoresis (Second Dimension)—Na dodecyl-SO₄ gel electrophoresis is done according to the method of Maizel (21) with the following modifications: (a) the running gel is made as a polyacrylamide gradient, 10 to 20% from top to bottom; (b) the proteins to be separated are loaded into the gel by using a heavy solution (40 ml of acrylamide/bisacrylamide; 12 ml of 2 M Tris, pH 8.8; 25 ml of TEMED; 6 ml of 60% sucrose; 0.6 ml of 10% Na dodecyl-SO₄) and a light solution (20 ml acrylamide/bisacrylamide; 12 ml of 2 M Tris, pH 8.8; 25 ml of TEMED; 0.6 ml of 10% Na dodecyl-SO₄, and water up to 60 ml), initiating polymerization by the separate addition of 150 µl of 10% Na dodecyl-SO₄, and water up to 50 ml, plus 0.2 ml of 10% ammonium persulfate to each and introduction of these solutions into the gel container (sitting vertical) through a relatively large horizontal interface at the gel edges. Polymerization takes 60 min. Once the running gel is polymerized, indicated by the formation of a third interface at the top of the gel (air/isobutyl alcohol, isobutyl alcohol/water, and water/acylamide), the stacking solution (12 ml of acrylamide/bisacrylamide (30%/0.8%); 6 ml of 0.5 M Tris, pH 6.8, 25 µl of TEMED; 0.5 ml of 10% Na dodecyl-SO₄; water up to 50 ml, plus 0.2 ml of 10% ammonium persulfate) is poured in, the "slot" forms (molds which project down into the focusing gel arc: (a) removal of the "slot" forms, (b) removal of the bottom form (assisted by the lubricating effect of several drops of water), and (c) sealing the two glass plates with gel to the gel apparatus by using a wide strip of parafilm, acrylamide sealing solution are added to the transferred strip and the adjacent empty slots in 5- to 50-µl (usually 25-µl) quantities. Molecular weight standards used are porcine amylase and bovine plasma albumin, DNaec, chymotryptsinogen, trypsinogen, and ßNaec from Worthington Biochemical Corp.

Radioautography of Radiolabeled Proteins

Gels containing ³⁵S-radiolabeled proteins are covered with Saran wrap and dried down on Whatman No. 3MM paper under vacuum. Dried gels are then exposed to Dupont chronex II x-ray film in the dark for 1 to 3 weeks, following which the film is developed.

Quantitation of Radiolabeled Proteins

Gel bands or gel spots containing radiolabeled proteins are removed by razor blade or Bard Parker dissection blade, transferred to counting vials and dissolved within 2 days in 1 ml of 30% H₂O₂ at 60°C. Ten milliliters of scintillation solution (25% Triton X-114, 75% xylene, v/v, and 8% 2-(4'-t-butylphenyl)-5-(4'-bi-phenyl-1,3,4-oxadiazole (22)) are added and "thi via is vigorously shaken, set at room temperature for 1 day in order to discharge chemical illuminance, and then counted in a Beckman model 350 liquid scintillation counter. In the case of proteins labeled with ³¹C, the degree of quenching was uniform regardless of the size of the gel piece and the efficiency of counting was 89%.

Elution of Secretory Proteins from Isoelectric Focusing Gel Fractions

Gel segments were homogenized in a 5-ml solution of 0.1 M Tris (pH 8.0)/0.1 M KCl/0.02 M CaCl₂/20 µl/ml of bovine plasma albumin with 10 strokes of a Teflon homogenizer (0.004 to 0.006 mmol/g gel). Gel segments were then centrifuged at 6,000 rpm for 10 min. Supernatant volumes were typically 2.5 to 3.0 ml and when measured by enzymatic analysis, efficiency of elution was 35 to 55%.

Identification of Pancreatic Proteins by Enzyme Activities

All measurements were made under conditions giving a linear relationship between measured activity and protein concentration. Procedures used for the activation of the guinea pig zymogens trypsinogen, chymotrypsinogen, and the procarboxypeptidase A and B, and the assay of the guinea pig enzymes amylase, ribonuclease, trypsin, chymotrypsin, and the carboxypeptidases A and B are summarized by Sechele and Paleode (18).

Lipase—The method of Kaplan (23) was used with [1-³¹C]tripalmitin (1.5 × 10⁶ dpm/ml) as substrate. Prior to use, contaminating labeled free fatty acids were removed from substrate by extracting three times with 0.1 N NaOH in ethylene glycol. Reactions were carried out at 37°C for 20 min and activity is expressed as counts per min of free fatty acids extracted into alkaline solvent. The background activity was usually 200 cpm.

Procolastase—Activation was initiated by the addition of 10 µl of trypsin (10 µg) and carried out at 3 or 30°C for 5 min to 24 hours. Preliminary studies on guinea pig pancreatic secretion showed that, (a) without added trypsin there was no activation of the zymogen, (b) with trypsin activation occurred immediately at 3 or 30°C and remained stable for at least 24 hours, and (c) under the...
RESULTS

Slab Gel Isoelectric Focusing (First Dimension)—Separation of pancreatic exocrine proteins was studied using isoelectric focusing in polyacrylamide slab gels. Fig. 1 shows the banding pattern of secretory protein polymerized directly into the acid portion of the gel and focused for 6 hours. There are 12 prominent bands and several faint bands. The gel is bisected since a 1-cm strip was removed and run in second dimension (not shown). The pH gradient profile is displayed immediately below and aligned to demonstrate the case with which isoelectric points are determined. The bands are labeled as subsequently identified by enzymatic assay.

The focusing procedure results in a small degree of gel distortion usually at the acidic end of the gel (cf. Figs. 1 and 4). Distortions in this area are greater at the sides than in the center, and when present are evidenced by rippling in the gel surface which is easily seen at the end of the run. Distortions are minimized when focusing current is maintained below 5 ma.

Sodium Dodecyl Sulfate Gradient Gel Electrophoresis (Second Dimension)—Separation of pancreatic exocrine proteins was studied using Na dodecyl-SO4 gel electrophoresis in a 10 to 20% polyacrylamide gradient. The two-dimensional gel technique described in this paper requires that the proteins be run in their nonreduced state to preserve enzymatic activity during the focusing procedure. However the banding pattern of nonreduced pancreatic proteins in Na dodecyl-SO4 gradient gel electrophoresis is considerably different from that of reduced and alkylated proteins (Fig. 2), indicating that these proteins are relatively abundant in disulfide bonds. While there is better separation (12 versus 10 bands) and better spread of proteins in the nonreduced state, log molecular weight correlates better with RF values of reduced and alkylated proteins (Fig. 2a) than with the RF values of nonreduced proteins (Fig. 2b). For example, in the nonreduced state, trypsinogen migrates more rapidly than expected and bovine plasma albumin does so to the extent that its relative mobility with that of lipase 1 is reversed. However, except for trypsinogen the RF values of nonreduced pancreatic proteins can be used as a rough guide to molecular weight values (cf. Table 1).

Two-dimensional Gel Analysis—The possibility of combining isoelectric focusing and Na dodecyl-SO4 gradient gel electrophoresis to achieve further separation of pancreatic proteins was studied. Transfer of focusing strips into appropriate slots in the electrophoresis gel was facilitated by immersion of strips in 1% Na dodecyl-SO4. It was not necessary to remove ampholytes by prolonged soaking since they are small, less than 1000 daltons, and are electrophoresed ahead of proteins. The mobility of strongly basic ampholytes is retarded but still sufficient not to interfere with proteins larger than 10,000 to 12,000 daltons. Although it was sufficient to place the strip in the appropriate slot without polymerization, polymerization did retard lateral diffusion of proteins passing across the interface formed by the strip and the Na dodecyl-SO4 stacking gel. The presence of an acrylamide gradient in the Na dodecyl-SO4 running gel was critical, allowing for continuous stacking of proteins into concentrated spots. A small amount of lateral diffusion occurred during electrophoresis, particularly when the load was large (cf. Fig. 4).

Fig. 3 shows a two-dimensional gel of radiolabeled guinea pig pancreatic proteins, unreduced, accompanied by the banding patterns of pancreatic secretion and protein standards (porcine amylase, and bovine DNase, chymotrypsinogen, trypsinogen, trypsin, and RNase) in one-dimension Na dodecyl-SO4 gradient gel electrophoresis. Below the Coomassie blue staining pattern is the radioautogram developed after 19 days' exposure. Proteins are run in their unreduced state. A companion focusing strip (fixed, stained, and destained) is shown immediately above for reference. Ampholytes have run off the gel except for strongly basic ones, which stain heavily at the bottom of the gel. No Coomassie blue bands remain in the focusing strip. Twenty protein spots are stained with Coomassie blue, each conveniently separated from the others. All but one (spot 19) appear in the radioautogram as radiolabeled proteins and are therefore true secretory proteins. Spot 19 is bovine plasma albumin which was added to prevent adherence of secretory proteins to glass or
Reduced and Alkylated Nonreduced

Bovine plasma albumin
Lipase I
Amylase
Lipase
Procarboxypeptidases A B B
Proelastase
Chymotrypsinogen
Trypsinogen
Ribonuclease

FIG. 2. Sodium dodecyl sulfate electrophoresis in a polyacrylamide gradient gel of guinea pig pancreatic exocrine proteins: a, reduced (10 mM dithiothreitol) and alkylated (20 mM iodoacetamide); and b, nonreduced. Polyacrylamide gradient, 10 to 20%. Running time, 16 hours. Protein load, 50 pg each. Bands are labeled as subsequently identified by two-dimensional gel analysis. 2a, semilog plot of molecular weight versus $R_F$ value for reduced and alkylated exocrine proteins. Standards, similarly reduced and alkylated, are: bovine plasma albumin, $\triangle$; porcine amylase, $\nabla$; bovine chymotrypsinogen, A; bovine trypsinogen, $\Box$; bovine RNase A, $\diamondsuit$; bovine RNase I, $\blacklozenge$; semilog plot of molecular weight versus $R_F$ value for nonreduced exocrine proteins. Standards, similarly nonreduced, are the same as 2a.

plastic surfaces (18). Its isoelectric point is 4.8, its apparent molecular weight is 68,000, and it frequently appears in heterogeneous form. Focusing, run for 2 hours at 500 to 1,000 volts in this case, was not complete as evidenced by the trails of protein associated with spots 3, 5, 6, 7, 16, 17, and 18, those having moved furthest from the glass fiber paper positioned at the acidic end of the focusing gel (see arrow). However, incomplete focusing here offers two advantages: the trails indicate molecular weight isolines, and spots 3, 5, and 7 (subsequently identified as RNase, trypsinogen, and chymotrypsinogen) which, under complete focusing conditions have the same isoelectric point (pH 8.7), migrate as separate entities to that point. Fig. 4 shows a two-dimensional gel obtained following complete focusing (6 hours) in
FIG. 4. Two-dimensional gel analysis of guinea pig pancreatic exocrine proteins with companion isoelectric focusing strip. Secretory protein (60 μg) absorbed to glass fiber paper (1 x 1 cm) and applied to acid end of isoelectric focusing slab gel (arrow). Focusing complete, time 6 hours (maximum volts, 500; maximum ma, 5). Focusing strip soaked in 1% Na dodecyl-SO₄-0.05 M Tris-HCl, pH 6.8, for 0.5 min, then applied to Na dodecyl-SO₄ gradient gel (polyacrylamide, 10 to 20%) by polymerization with 3% acrylamide. Na dodecyl-SO₄ gel running time, 16 hours. Second dimension spots, fixed with 10% acetic acid-30% methanol and stained with Coomassie blue, 0.06%, are numbered from 2 to 20.

the first dimension and no protein trails are seen. Protein 19 (bovine plasma albumin) appears less apparent and more heterogeneous since, in this case, 20 μg/ml instead of 200 μg/ml were added to the incubation medium.

Separation of proteins in two dimensions reveals instances where proteins have similar or identical RF values in either single dimension analysis. For instance, in single dimension Na dodecyl-SO₄ gel electrophoresis (Fig. 3), this occurs among the following groups of proteins: (a) proteins 1, 2, 3, and 4, (b) proteins 7 and 12, (c) proteins 8, 9, 10, and 11, and (d) proteins 13, 14, and 15. In single dimension slab gel isoelectric focusing (Fig. 3), this occurs among: (a) proteins 12, 14, 19, and 20, (b) proteins 11 and 15, (c) proteins 2 and 8, and (d) proteins 5, 17, and 18.

Enzymatic Identification of Proteins—Enzymatic activity or potential activity of secretory proteins was determined by direct assay of fractions derived from focusing gels (see “Methods”). Fig. 5 shows three enzymatic activities and five potential activities (after activation) found in 15 fractions derived from a gel loaded with 2 mg of secretory protein and focused 6 hours. The center reference strip (fixed, stained, destained, and dried) is placed at the top. No protease activity was found in any of the fractions prior to activation of zymogens. Each zymogen was activated separately under conditions shown to be optimal for that guinea pig zymogen (cf. Ref. 18 for trypsinogen, chymotrypsinogen, and procarboxypeptidases A and B and “Methods” for proelastase).

Enzyme activities allowed specific identification of protein bands in isoelectric focusing gels and where focusing bands contained a single protein, identities could be translated to two-
we were used to provide a means of quantitation. Labeled amino acids were removed by filtration through Sephadex G-25. Electrophoresis of radiolabeled proteins on Na dodecyl-SO₄ gradient gels (one dimension) showed recovery of 88.5% (85% in one case and 92% in another) of labeled protein, indicating that 10 to 12% of proteins are lost either by migration off the Na dodecyl-SO₄ gel due to their low molecular weight, or during fixation and staining. Fixation and staining does not cause serious loss of proteins since recovery in gels neither fixed nor stained was no better than those fixed and stained. Two-dimensional gels, regardless of method of sample application and fixation technique (10% acetic acid/50% methanol or 10% sulfosalicylic acid/5% trichloroacetic acid) showed recovery of 80% of radiolabeled protein, comparing favorably to 88% found in one-dimensional Na dodecyl-SO₄ gels. Furthermore, radiolabeled proteins appeared only in Coomassie blue stained spots since recovery from gels assayed for stained spots was equal to that of gels comprehensively sliced and counted. Manipulations required in the transfer of proteins from the first to the second dimension may result in the additional 8% loss. When the sample was directly polymerized into the gel matrix, 5% of the radiolabeled proteins remained in the focusing strip. When glass fiber paper was used for sample application, 2.5% of the radioactivity remained in the paper square and 3% remained in the focusing strip. These amounts of radioactivity probably represent labeled amino acids incorporated into molecules, possibly peptidoglycans (17), unable to migrate out of 5% polyacrylamide due to size or inability to bind sufficient Na dodecyl-SO₄. There was no selective loss of proteins on glass fiber paper since secretory protein profiles obtained during recovery experiments were the same using either method of sample application.

**Modifications in Isoelectric Points Due to Sample Loading**—When the sample of secretory protein was polymerized throughout the gel and focused for 10 hours to insure complete migration of proteins to their isoelectric points, several proteins, amylase, chymotrypsinogen 2, trypsinogen, and ribonuclease, showed two isoelectric points. One point corresponded to the pI1 reported in the paper while the other corresponded to a higher pI, similar for each of the four proteins (pI = 9.4). The two isoelectric points of these proteins are easily visualized in the two-dimensional gel pattern derived from this study (Fig. 6). Each pair of spots (18-18', 17-17', 7-7', 5-5', 3/4-3/4') is connected by a narrow band of stained protein. No differences are seen in the migration of neutral or acidic proteins. Since there is no change in molecular weight, the occurrence of two isoelectric points could not be due to dimerization caused by oxidation of sulfhydryl groups and formation of intermolecular disulfide bridges or to significant proteolytic degradation. However, since autoactivation of trypsinogen and activation of chymotrypsinogen involve the removal of only a few amino acid residues, this possibility was seriously considered. Trypsin and chymotrypsin activity were determined under these conditions on isoelectric focusing gel fractions both before and after activation with enterokinase and trypsin, respectively. There was no activity of either protease prior to activation. Following activation, large amounts of trypsin and chymotrypsin activity appeared at the two isoelectric points of each of the zymogens. In addition, in several other experiments, modifications in isoelectric points for these proteins is due neither to activation nor denaturation of proteins.

**Conversion of Proteins from One Isoelectric Point to the Other**

Dimensional spots. Where focusing bands contained more than one protein, additional evidence was required to distinguish identities of spots in two-dimensional gels. Thus, the heavy band in focusing fraction 13 is identified by enzymatic activity as a mixture of trypsinogen, chymotrypsinogen 2, and RNase. Evidence derived from considerations of molecular weight and comigration with protein standards (Fig. 3) indicates that spot 7 is chymotrypsinogen 2, spot 5 is trypsinogen, and spot 3 or 4 is RNase. The band in focusing fraction 12 is identified as amylase and on second dimension it results in the appearance of two spots, 17 and 18, which comigrate with the two bands obtained from porcine amylase. The band at the interface of focusing fractions 11 and 12 is identified as lipase 2 and corresponds to spot 16 on the two-dimensional gel. The faint band in focusing fraction 11 is unidentified and corresponds to spot 2 in the two-dimensional gel. The faint band in focusing fraction 10 is unidentified and corresponds to spot 9 on the two-dimensional gel. There are two bands in focusing fraction 9, identified as procarboxypeptidase B and proelastase. Molecular weight considerations identify spot 15 in the two-dimensional gel as the former and spot 11 as the latter. The faint band in focusing fraction 8 remains unidentified and corresponds to spot 1 in the two-dimensional gel. The bands appearing in focusing fractions 4, 5, and 6 are slightly distorted and must be considered as a group. By enzymatic activity they are identified as chymotrypsinogen 1, procarboxypeptidase A, and lipase 1. Molecular weight and isoelectric point considerations (Fig. 3 and Ref. 17) identify spot 12 as chymotrypsinogen 13 and spots 13 and 14 as two forms of procarboxypeptidase A. By elimination, spot 20 is identified as lipase 1. Finally, the band in focusing fraction 14 is unidentified and corresponds to spot 6 in two-dimensional gel.

Further studies were done to isolate and identify protein 20, presumed to be lipase 1 above. Secretory protein was applied to the basic end of a focusing gel and incompletely focused 3.5 hours. Protein 20 being larger in molecular weight migrated more slowly towards its isoelectric point and was intercepted alone under these conditions. Assays of fractions derived from this focusing gel revealed lipase activity coincident with the intercepted band. The complete isolation of this protein and its identification as protein 20 was demonstrated by two-dimensional gel analysis.

The secretory protein mixture used here to identify protein bands and spots by enzymatic activity did not contain protein 10 on two-dimensional analysis. Since it was likely that this protein along with protein 11 might be a form of proelastase, further studies were done on the secretory protein mixture containing both proteins, 10 and 11. The band pattern of this secretion is identical to that seen in Figs. 1 and 4. Each of the bands, representing proteins 10 and 11, was individually cut out and the rest of the isoelectric focusing gel was fractionated. There was no hydrolysis of benzoyl-alanine-methyl ester in any of the fractions prior to activation with trypsin. Following the addition of trypsin (10 μg/300 μl of elution buffer) at 3° there was immediate elastase activity in each of the fractions corresponding to the two proteins of interest (9.1 and 8.1 × 10⁻³ units, respectively). Proteins 10 and 11 also had a very small degree of chymotrypsin activity, 4.8 and 9.2 × 10⁻³ units, respectively. Accordingly, spots 10 and 11 on the two-dimensional gel are identified as two forms of proelastase.

**Recovery of Radiolabeled Proteins in Two-dimensional Gel Analysis**—Using the two-dimensional gel analysis described, recovery of proteins applied to focusing gels by both direct polymerization into the gel and absorption onto glass fiber paper was studied. Radiolabeled proteins (5 × 10⁶ cpm/mg of protein)
FIG. 6 (left). Two-dimensional gel analysis of guinea pig pancreatic exocrine proteins. Sample, 2 mg of protein, was polymerized throughout isoelectric focusing slab gel. Focusing time, 10 hours. Isoelectric focusing gel was fixed in 5% trichloroacetic acid/5% sulfosalicylic acid/20% methanol, stained with 0.0125% Coomassie blue, and destained with 30% methanol/10% acetic acid. Fixed, stained, and destained isoelectric focusing strip was applied to Na dodecyl-SO4 gradient gel after a 1-hour soak in 1% Na dodecyl-SO4/0.05 M Tris-HCl, pH 6.8. Na dodecyl-SO4 gel running time, 16 hours. Second dimension spots were fixed, stained, and destained as described under "Experimental Procedure." Artist’s rendition of the isoelectric focusing strip is shown above the two-dimensional gel.

FIG. 7 (right). Two-dimensional gel analysis of guinea pig pancreatic exocrine proteins. 7b shows the two-dimensional pattern resulting from sample loading (75 μg of protein) at the basic end of the isoelectric focusing gel. 7c shows the two-dimensional pattern resulting from sample loading (75 μg of protein) at the acidic end of the same isoelectric focusing slab gel. Corresponding isoelectric focusing strips are shown at the top of each two-dimensional pattern. 7a shows a side-by-side comparison of the isoelectric focusing strips, b and c, taken from the same slab gel. Isoelectric focusing strips were fixed, stained, and destained as described under “Experimental Procedure.”

These results indicate that modifications in isoelectric points are not due to aggregates of proteins (intra- or interspecies) at one isoelectric point and movement of the aggregate to the other isoelectric point during the focusing procedure.

The basic pattern was converted to the acidic pattern when the basic protein band (pH 9.4) was removed from the focusing slab gel, the polyacrylamide homogenized, and the protein eluted into 100 mM Tris-HCl buffer, pH 6.8, polymerized into the acidic end of an isoelectric focusing gel, and refocused. Each of the four proteins, amylase, chymotrypsinogen 2, trypsinogen and ribonuclease, migrated to the isoelectric points seen in the acidic pattern. This study suggests that modifications in isoelectric points are not due to chemical modifications involving covalent bonds.

Characteristics of Guinea Pig Pancreatic Exocrine Proteins—The 20 spots separated by the two-dimensional gel analysis and their physical characteristics (molecular weight, isoelectric points, enzymatic function, and proportionate mass) are summarized in Table I. Molecular weights were estimated from proteins run in Na dodecyl-SO4 gradient gels under two conditions, unreduced and reduced and alkylated. As discussed previously, the latter gives more accurate estimates. Isoelectric points were reproducible from gel to gel, never varying more than 0.2 pH units. Proportionate mass was determined by the per cent...
distribution of radioactivity among individual secretory proteins of pancreatic lobules continuously labeled with a mixture of 15 \(^{14}\)C-amino-acids and discharged into physiological medium during 3 hours of carbachol (10\(^{-5}\) M) stimulation.

**DISCUSSION**

Two-dimensional gel analysis for the separation of complex mixtures of proteins dates back to 1964. Early studies (26, 27) showed increased resolution in the separation of plasma proteins using two-dimensional gel electrophoresis. Two studies were published showing the potential usefulness of tube gel isoelectric focusing (pH 3 to 10) in the second dimension and slab gel electrophoresis (pH 8.9) in the second dimension for the separation of plasma proteins (28) and potato proteins (29). In 1970, Kalt- (30) however, insoluble proteins have been studied necessitating the presence of urea in both gel dimensions.

The procedure presented here was developed for separation of soluble proteins, such as those found in pancreatic secretion. It consists of slab gel isoelectric focusing in the first dimension and Na dodecyl-\(\text{SO}_4\) gradient gel electrophoresis in the second dimension. The absence of urea in the first dimension has two advantages: (a) proteins are separated according to native charge, and (b) enzymatic identification can be made of separated proteins. Insertion of gradient acrylamide (10 to 20\%) in the second dimension allows for better resolution of separated proteins. The procedure has been shown to be effective in the complete separation of all high molecular weight exocrine pancreatic proteins (larger than 10,000); sensitive to 0.6 \(\mu\)g of protein/spot (some proteins to 0.3 \(\mu\)g) rapid, requiring 24 hours, and to yield good recovery (\(\approx\) 80\%) of radio labeled proteins.

No attempt has been made to include proteins smaller than 10,000 molecular weight in the two-dimensional gel analysis since separation of these proteins in Na dodecyl-\(\text{SO}_4\) gels is not proportional to log molecular weight and since the presence of ampholytes interferes with the staining of these proteins in the two-dimensional gel. Consequently, the secretory trypsin inhibitor (39), colipase (40), and possibly other unidentified proteins less than 10,000 are lost in the two-dimensional analysis of secretory proteins. Insertion of gradient acrylamide (10 to 20\%) in the second dimension allows for better resolution of separated proteins.

### Table I

**Characteristics of guinea pig exocrine pancreatic proteins**

<table>
<thead>
<tr>
<th>Spot number</th>
<th>Enzyme or zymogen</th>
<th>Mass proportion</th>
<th>Molecular weight</th>
<th>Isoelectric point</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>0.2</td>
<td>14,000</td>
<td>6.4</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>0.3</td>
<td>14,000</td>
<td>7.8</td>
</tr>
<tr>
<td>3</td>
<td>Ribonuclease</td>
<td>0.7</td>
<td>14,000</td>
<td>8.7</td>
</tr>
<tr>
<td>4</td>
<td>Trypsinogen</td>
<td>0.4</td>
<td>14,600</td>
<td>8.8</td>
</tr>
<tr>
<td>5</td>
<td>Trypsinogen</td>
<td>33.0</td>
<td>18,750</td>
<td>8.7</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>2.0</td>
<td>21,800</td>
<td>&gt;9.3</td>
</tr>
<tr>
<td>7</td>
<td>Trypsinogen</td>
<td>16.4</td>
<td>35,000</td>
<td>8.7</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>0.7</td>
<td>28,200</td>
<td>7.8</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>27,500</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Proelastase 2</td>
<td>8.0</td>
<td>28,700</td>
<td>7.5</td>
</tr>
<tr>
<td>11</td>
<td>Proelastase 1</td>
<td>28,200</td>
<td>6.9</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Chymotrypsinogen 1</td>
<td>1.7</td>
<td>25,000</td>
<td>4.8</td>
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<tr>
<td>13</td>
<td>Procarboxypeptidase A1</td>
<td>3.5</td>
<td>45,100</td>
<td>4.6</td>
</tr>
<tr>
<td>14</td>
<td>Procarboxypeptidase A2</td>
<td>8.8</td>
<td>45,300</td>
<td>4.6</td>
</tr>
<tr>
<td>15</td>
<td>Procarboxypeptidase B</td>
<td>8.8</td>
<td>46,000</td>
<td>4.6</td>
</tr>
<tr>
<td>16</td>
<td>Lipase 2</td>
<td>3.4</td>
<td>49,900</td>
<td>8.1</td>
</tr>
<tr>
<td>17</td>
<td>Amylase</td>
<td>3.6</td>
<td>51,000</td>
<td>8.4</td>
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<tr>
<td>18</td>
<td>Amylase</td>
<td>54,000</td>
<td>53,000</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Lipase 1</td>
<td>8.5</td>
<td>64,700</td>
<td>5.0</td>
</tr>
</tbody>
</table>

\(a\) Per cent distribution of radioactivity among individual secretory proteins of pancreatic lobules continuously labeled with a mixture of 15 \(^{14}\)C-amino-acids and discharged into physiological medium during 3 hours of carbachol (10\(^{-5}\) M) stimulation.

\(b\) Estimated from semilog plot of molecular weight versus \(R_F\) values for nonreduced proteins (Fig. 2b).

\(c\) Estimated from semilog plot of molecular weight versus \(R_F\) value for reduced and alkylated proteins (Fig. 2a).

\(d\) Average of three determinations for each protein.
Differences in isoelectric points have been found for a number of secretory proteins (amylase, chymotrypsinogen 2, trypsinogen, ribonuclease, proelastases 1 and 2, and procarboxypeptidase A) depending on whether these proteins are loaded at the acidic or basic end of the isoelectric focusing gel. The isoelectric points of these proteins resulting from sample loading at the acidic end of the gel are the same reported in this paper. The isoelectric points resulting from sample loading at the basic end of the gel are similar to those reported by Tartakoff et al. (17) using 72-hour column isoelectric focusing. Studies reported in this paper indicate that modifications in the isoelectric points of these proteins are not due to the following possibilities: (a) oxidation of sulphydryl groups with formation of intermolecular disulfide bonds, (b) proteolytic degradation, (c) autoactivation of trypsinogen or activation of chymotrypsinogen 2, (d) formation of intermolecular aggregates at one isoelectric point and movement of aggregates to a second isoelectric point during the focusing procedure, or (e) chemical modification of proteins involving covalent bonds. It would appear from these studies that modifications in isoelectric points which occur when secretory proteins are loaded at the basic end of the gel (pH 9.4 or higher) result from changes in configuration of protein molecules related to extreme pH. Changes in configuration would result in different charge expressions of these molecules and therefore different isoelectric points. Differences in charge expression might occur solely as a result of isoelectric points which occur when secretory proteins are loaded at pH 9.4 or higher. Since the focusing pattern resulting from sample loading at the acidic end is the same as that loaded near neutrality and since under physiological conditions these proteins are not exposed to pH 9.4 or higher, the isoelectric points reported here would seem to be those related to the native molecules.

The development of this technique using secretory protein from the guinea pig exocrine pancreas has allowed the majority of these proteins to be characterized by isoelectric point, apparent molecular weight, and proportionate mass (using radiolabeling techniques). Within the range of adequate separation of proteins by Na dodecyl-SO₄ gel electrophoresis (proteins larger than 10,000 daltons) 19 protein spots have been found. Thirteen of these proteins have been identified by enzymatic or potential enzymatic activity, accounting for 96% of the high molecular weight protein mass. Multiple molecular forms have been detected for many of the zymogens and enzymes. Primarily on the basis of charge separation two forms have been found for chymotrypsinogen, proelastase, and procarboxypeptidase A. On the basis of size separation two forms of amylase have been found. These forms are detected only when a polyacrylamide gradient is present in the Na dodecyl-SO₄ gel. The small difference in molecular weight, estimated to be 1,000, may be due to differing amounts of attached carbohydrate. On the basis of both charge and size separation, two forms of lipase have been found. A comparison of amino acid analysis data from both molecules will be necessary to determine if the relatively large difference in apparent size (approximately 17,000) is due to amino acid or carbohydrate content or both. One form of procarboxypeptidase A cofocuses (isolectric point = 4.8) with chymotrypsinogen 1 and may exist in aggregate form similar to that seen between bovine procarboxypeptidase A and chymotrypsinogen C (42). No attempts were made to measure DNase or phospholipase activity in this study. Two forms of phospholipase, consisting of 123 and 130 amino acid residues, have been isolated from porcine pancreas (43). It is likely that spots 1 and 2 in the two-dimensional pattern of guinea pig pancreatic proteins are phospholipases.

Since 1958 exocrine pancreatic proteins have been studied in detail in six mammalian species: ox, pig, human, dog, rat, and guinea pig (1-17). Using the two-dimensional gel analysis presented here, the high molecular weight exocrine pancreatic proteins of the guinea pig have been described in greater detail than has been possible for those of the other species using column chromatographic and immunoelectrophoretic methods. The two-dimensional gel technique is effective in the complete separation of high molecular weight exocrine proteins of the pancreas, which is not the case with the other fractionation methods. It is rapid, inexpensive, and relatively easy to perform. It should prove useful not only in the further study of exocrine pancreatic proteins but in the study of other soluble proteins as well.

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