Isolation and Characterization of a Human Pancreas-Specific Protein

S. T. Nerenberg, R. Prasad, L. Pedersen, N. S. Biskup, and I. Faiferman

Homogenates of human pancreas in saline were centrifuged at 27,000 x g and the supernates were fractionated by preparative polyacrylamide gel electrophoresis. The gels were divided into sections and each section was injected into rabbits; after absorption with polymerized serum from apparently normal humans, the antiserum obtained by injecting one of the sections was tested against a variety of human tissue extracts but reacted only with saline extracts of human pancreas. The absorbed antiserum, polymerized and made insoluble with glutaraldehyde, was used to purify a pancreas-specific antigen by immunoaffinity batch technique. The purified antigen proved to be a protein with some carbohydrate content (180 mg/g by weight) and a molecular mass of about 2.25 x 10^5 daltons. The antigen is relatively thermostable, and precipitates in the range of 245.64–340.2 g/L saturated ammonium sulfate; its antigenic activity is not affected by incubation with ribonuclease or deoxyribonuclease, but is destroyed by incubation with trypsin or neuraminidase and by extraction with perchloric acid. Immunofluorescence studies show that the antigen is diffusely present in the cytoplasm of pancreatic acinar cells.

Additional Keyphrases: preparative electrophoresis • immunoaffinity chromatography • immunoassay

Immunological approaches to the etiology and particularly the diagnosis of pancreatic disease have received little attention. Early studies with hemagglutination and gel-diffusion techniques have demonstrated the presence of circulating autoantibodies to pancreas in patients with various pancreatic diseases (1); recent studies, with immunofluorescence staining procedures, have confirmed some of these observations (2). Although antiserum specific for human pancreas (3, 4) has been produced in rabbits and monkeys, data on the isolation and characterization of pancreas-specific antigens are scant; one pancreas-specific but not species-specific antigen has been detected and localized in the zymogen granules (6), and two pancreas-specific antigens recently detected in urine have been partially characterized (7).

Working on the hypothesis that "subclinical" pancreatitis could be detected by the determination in sera of pancreas-specific antigen(s) leached into the circulation as a consequence of the disease, we have isolated and characterized a human pancreas-specific protein and developed a rabbit antiserum specific to this protein for use in immunoassays.

Materials and Methods

Preparation of Pancreatic Extracts

Fat, vessels, and ducts were removed from fresh human pancreas obtained at autopsy within 12 h of death, then stored at −70 °C until extracted. All subsequent steps were carried out at 4 °C. The frozen tissue was thawed and minced, then suspended in two volumes of 9 g/L NaCl and homogenized in a Waring Blender at full speed for 1 min. We centrifuged the homogenate at 27,000 x g for 15 min and filtered the supernate through coarse filter paper. The filtrate was collected, divided into aliquots, and kept at −70 °C.

Preparation of Rabbit Antiserum to a Pancreatic Antigen

Saline extracts of human pancreas were dialyzed overnight against distilled water, then dialyzed against tris(hydroxymethyl)methylamine(Tris)–glycine buffer (pH 9.1, 0.04 mol/L) for two days. After centrifuging the dialyzed extract at 27,000 x g for 20 min, we collected the supernate and fractionated it by preparative polyacrylamide gel gradient electrophoresis (see below). The gels were divided into seven sections; each section was crushed, homogenized with Freund’s complete adjuvant (Difco Laboratories, Inc., Detroit, MI 48232), and injected intradermally into 25 to 30 sites on the backs of each of two rabbits (weight, 2 kg). To boost the titer of the developing antiserum, we filtered through a 45-μm (av pore size) Millipore filter 1 mL aliquots of human pancreatic saline extract containing 20 g of protein per liter and injected the filtrate intravenously into each of the rabbits three to four weeks later; the rabbits were bled five days later to prevent development of unwanted, extraneous antibodies. Sera were collected and absorbed with pooled normal human serum that had been polymerized and made insoluble with glutaraldehyde (8). After mixing 1 mL of rabbit antiserum with 1 g of polymer and incubating at 4 °C overnight on a shaker, we collected the supernates and tested them by double-gel diffusion and radial immunodiffusion against serum from healthy subjects and extracts of different human tissues.

Additional pancreas-specific antiserum was obtained by injecting rabbits with precipitin arcs, as described by Goudie et al. (9); the arcs were obtained by immunoelectrophoresis of saline extracts of human pancreas, followed by reaction with rabbit antiserum prepared as described above. The arcs were exhaustively washed with saline for one week to remove unreacted proteins, homogenized with equal amounts of complete Freund’s adjuvant, and injected intradermally in 25 to 30 sites in rabbits. Each rabbit was “boosted” by intravenous injection of material from two washed precipitin arcs homogenized in saline and bled three and five days later. The booster injections were repeated at weekly intervals for two additional weeks. Antisera were collected as previously described and routinely absorbed with polymerized normal human serum.

Purification of Pancreatic Antigen

Absorption with CM-Sephadex. We heated human pancreas saline extract to 60 °C, immediately cooled it to 4 °C, and centrifuged it at 27,000 x g for 20 min at 4 °C; the pellet was discarded. The supernate was dialyzed overnight against 0.05 mol/L sodium phosphate buffer (pH 6.0), centrifuged as above, and the resulting supernate filtered through a 0.45-μm av pore size Millipore filter. We mixed the filtrate with CM-Sephadex (C50-125 Sigma Chemical Co., St. Louis, MO 63178) that had been prewashed with 0.05 mol/L phosphate buffer, pH 6.0 (1 mL of filtrate to 2 g of CM-Sephadex), incubated the suspension for 1 h at room temperature, and filtered it with a Büchner funnel. The gel was thoroughly washed with 0.05 mol/L sodium phosphate buffer (pH 6.0), resus-

Department of Pathology, University of Illinois at the Medical Center, Chicago, IL 60612.

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Fig. 1. Fractionation of human pancreas extract by gel filtration on Sephadex.

The antigenic activity of each fraction was detected by radial immunodiffusion; + represents degree of activity. The y-axis is absorbance at 280 nm.

Pended in 0.05 mol/L sodium phosphate buffer containing 1 mol of sodium chloride per liter (also pH 6.0) for 1 h at room temperature, and filtered; the resulting filtrate was dialyzed against 0.05 mol/L sodium phosphate buffer (pH 7.4), concentrated under pressure in an Amicon filter unit (Model 402; Amicon Corporation, Lexington, MA 02173), and fractionated by gel chromatography.

Gel filtration through Sephadex G-200. Fractions were applied to a 75 x 2.5 cm (i.d.) Sephadex G-200 (Pharmacia, Uppsala, Sweden) column and eluted with 0.05 mol/L sodium phosphate buffer (pH 7.4) at a flow rate of 24 mL/h; 3-mL fractions were collected. Fractions containing antigenic activity, as determined by radial immunodiffusion, were pooled and subjected to preparative polyacrylamide gel electrophoresis.

Preparative polyacrylamide gel electrophoresis. Preparative electrophoresis was carried out on a 35 x 4.5 cm (i.d.) column of 50 g of polyacrylamide gel per liter of 0.04 mol/L glycine buffer (pH 9.2). We applied 5-mL samples containing 30 g of protein per liter to the gel and electrophoresed at 200 V and 50 mA for 18 h at 4°C. After staining a longitudinal slice of the gel with 0.01 mol/L Amido Black in 5 mL/L acetic acid to localize the protein bands, we eluted the proteins from the gel with phosphate-buffered saline and further fractionated the antigenically active protein band by immunoaffinity chromatography.

Samples containing 10 to 50 μg of purified pancreatic antigen were subjected to polyacrylamide disc gel electrophoresis in gels containing 75 g of polyacrylamide per liter of buffer (10). After electrophoresis, gels were stained for protein with Coomassie Blue.

Immunoaffinity chromatography, batch technique. Rabbit pancreas-specific antiserum was polymerized with glutaraldehyde at pH 5.5 to form an insoluble immunoabsorbent; this was then incubated at 4°C overnight with the antigenically active protein obtained by preparative polyacrylamide gel electrophoresis in the proportion of 1 g of polymer to 1 mL of eluted protein solution. We centrifuged the resulting suspension for 5 min at 6000 x g and 4°C; the pellet of immunoabsorbent was washed several times with 0.1 mol/L phosphate-buffered saline, pH 7.4. The antigen was eluted with 0.1 mol/L glycine HCl buffer, pH 2.8; the eluent was immediately brought to pH 7.2-7.4 with solid sodium bicarbonate and then dialyzed against 0.01 mol/L sodium phosphate buffer, pH 7.0.

Determination of Molecular Mass

Purified pancreatic antigen was filtered through a 100 x 0.9 cm (i.d.) column of Biogel A-1.5m (50-100 mesh; Bio-Rad Lab, Richmond, CA 94804) equilibrated with 0.1 mol/L sodium phosphate buffer (pH 7.4) containing 10 mL of 10 g/L bovine serum albumin solution. Highly purified preparations of 125I-labeled IgE (Pharmacia), follitropin (Amersham Corp., Arlington Heights, IL 60005), insulin (Med Vak, Inc., Columbia, MD 21045), carcinoembryonic antigen (Hoffman-LaRoche, Nutley, NJ 07110), and unlabeled blue dextran (Pharmacia) were used as reference markers. The proteins were loaded on the column and eluted with 0.1 mol/L phosphate buffer, pH 7.4, at a rate of 0.75 mL/min. We collected 0.5-mL fractions and determined the presence of proteins by measuring their radioactivity and their adsorption at 280 nm. We plotted the partition coefficient [Kav = (total bed volume - void volume)/(total bed volume - sample elution volume)] for the pancreatic antigen and the "marker proteins" vs molecular mass.

Immunoassays

We subjected the pancreatic antigen to double-gel diffusion (11), radial immunodiffusion (12), and immunoelectrophoresis (13) in agarose gels (10 g/L) in a 0.05 mol/L veronal buffer, pH 8.6.

Enzymatic Degradation

After human pancreatic antigen obtained by preparative electrophoresis had been incubated with enzymes at room temperature for 24 h, we tested for antigenic activity by radial immunodiffusion. Enzyme-treated and untreated preparations were analyzed simultaneously. The antigen (150 ng in phosphate-buffered saline) was treated with the following enzymes (all from Sigma): 100 units of Vibrio cholerae neuraminidase (EC 3.2.1.18) in 0.1 mol/L maleate buffer (pH 6.4) containing 20 mmol of CaCl2 per liter; 100 U of deoxyribonuclease I (EC 3.1.4.5) in 0.1 mol/L acetate buffer (pH 5.6) containing 5 mmol of MgSO4 per liter; 70 U of ribonuclease (EC 3.1.4.22) in 0.1 mol/L acetate buffer (pH 5.6); and 10 ng of trypsin (EC 3.4.21.4) in phosphate-buffered saline.

Immunofluorescent Staining

We froze fresh rat pancreas by sudden immersion into isopentane cooled in liquid nitrogen. Cryostate sections 6-μm thick were fixed in ethanol (950 mL/L) for 5 min at room
temperature, then rinsed for 10 min in phosphate-buffered saline. The sections were incubated with 50 μL of rabbit anti-serum for 45 min in a moist chamber at room temperature; then the slides were rinsed for 30 min in phosphate-buffered saline. Next, we added two drops of a mixture of fluorescein isothiocyanate-conjugated IgG fraction of goat-antirabbit IgG (Cappel Lab., Cochraville, PA 19330) and rhodamine (prepared by mixing 1 mL of 10-fold diluted fluoresceinated goat antirabbit antiserum and 10 μL of 10 g/L rhodamine to the sections) and continued the incubation in a dark, moist chamber at room temperature for 30 min. The sections were then rinsed with phosphate-buffered saline for 30 min and mounted with buffered glycerol. Normal rabbit serum was used as a negative control.

**Results**

**Purification of the Antigen**

Saline extracts of human pancreas were absorbed with CM-Sephadex; the absorbed proteins were eluted and further fractionated by gel filtration through Sephadex G-200. The antigenic activity was eluted in the void volume, indicating that the antigen possessed a high relative molecular mass (Mr) (Figure 1). This fraction was further separated by preparative polyacrylamide gel electrophoresis, which yielded six protein bands (Figure 2). The antigenic activity was located in proteins eluted from Band 3; these proteins were further fractionated by an immunoaffinity batch technique. The eluted protein was antigenically active, as determined by radial immunodiffusion.

We examined the purity of this antigen by polyacrylamide disc gel electrophoresis; only one band was detected after staining with Coomassie Blue for protein.

**Determination of Molecular Mass**

We determined the molecular mass of the purified pancreatic antigen by gel filtration through Biogel A-1.5 m. The antigen appeared as a single, symmetrical peak with a mass of $2.25 \times 10^6$ daltons, determined by comparison with reference proteins (Figure 3).

![Fig. 2. Fractionation by preparative polyacrylamide gel electrophoresis of antigenically active fraction of pancreatic extract obtained by gel chromatography. Arrows indicate direction of migration.](image)

![Fig. 3. Determination of the relative molecular mass of the antigen by filtration through Biogel A-1.5m.](image)
Properties of the Purified Antigen

The antigen contained 180 mg of carbohydrate per gram, as determined by the phenol–sulfuric acid method (14), and precipitated at between 245.64 and 340.2 g/L ammonium sulfate saturation. The antigenic activity was not affected by heating up to 60 °C for as long as 1 min or by storage for one year at −70 °C; moreover, it was not affected in the pH range of 2.8 to 8.5 but was destroyed at pH 2.5. The antigenic activity was destroyed by extraction with 0.25 mol/L perchloric acid or by incubation of the antigen with trypsin and neuraminidase. On the other hand, the antigenic activity was not affected by incubation with deoxyribonuclease or ribonuclease. When subjected to immunoelectrophoresis in agar, the antigen appeared in the β-globulin region (Figure 4).

Preparation of Pancreas-Specific Antiserum

Saline extracts of human pancreas fractionated by preparative polyacrylamide gel electrophoresis showed 14 protein bands when the gels were stained with Amido Black (Figure 5). Rabbit antiserum produced by injecting section 4 of the gel appeared specific for pancreas after its absorption with pooled polymerized normal human serum. Additional pancreas-specific antiserum was produced by injecting precipitin arcs as described by Goudie et al. (9). We tested the absorbed antiserum against saline extracts of various human tissues by radial immunodiffusion. A positive reaction was detected only with human pancreatic extracts, whereas extracts from colon, spleen, liver, kidney, lung, stomach, ovary, heart, uterus, adrenal, thyroid, gall bladder, testicles, prostate, parotid, submaxillary gland, and thymus showed no reaction. Extracts of adult human, fetal human, and adult baboon pancreas showed complete antigenic identity, whereas extracts of rat pancreas showed partial identity, as detected by double-gel diffusion. Extracts of rabbit, pig, guinea pig, and dog pancreas did not show any reaction with the rabbit antiserum; furthermore, we found no reactions between the rabbit antiserum and several pancreatic proteins (insulin, amylase, lipase, deoxyribonuclease, α-chymotrypsin, elastase, collagenase, carboxypeptidase A and B, leucine aminopeptidase, phospholipase A₂, carbonic anhydrase, and γ-glutamyltransferase), as determined by radial immunodiffusion.

Discussion

We produced antisera to pancreas-specific antigens by injecting rabbits with extracts of human pancreas. The rabbit antisera reacted with extracts from several tissues but was rendered specific for pancreas after a series of absorptions (3–5, 7). To eliminate the need for extensive absorptions and simplify the preparation of pancreas-specific antisera, we first fractionated saline extracts of human pancreas by preparative polyacrylamide gel electrophoresis and injected the different fractions into rabbits. Rabbits injected with fraction 4 (Figure 5) produced an antisem specific for pancreas after a single absorption with polymerized normal human serum. We also obtained specific antisem by injecting rabbits with precipitin arcs prepared by immunoelectrophoresis in agar of a saline extract of human pancreas reacted with rabbit pancreas-specific antisem. Once we had a large amount of pancreas-specific antisem available, we were able to develop a procedure for the purification of a human pancreas-specific antigen involving sequential steps of gel filtration, preparative gel electrophoresis, and immunoaffinity chromatography by a batch technique.

The antigenic activity was destroyed by incubation with trypsin or neuraminidase, indicating that the carbohydrate moiety is associated with, or is a part of, the antigenic site. The antigen appeared to be homogeneously distributed in the cytoplasm of rat pancreatic acinar cells, as detected by immunofluorescent staining (Figure 6); human pancreas showed a similar staining pattern, but the cell morphology was considerably altered by autolysis.

The existence of pancreas-specific antigens has been indicated in a number of reports (3–7, 15). Although these antigens have not been fully characterized, their physical characteristics seem to differ from those of the pancreatic antigens we have isolated.

Circulating antibodies to human pancreas in sera of patients with pancreatic disease have been detected by various procedures (1, 2). To determine whether the pancreas-specific antigen could be shed into the circulation as a consequence of a pathological process in that organ, we developed a radioimmunoassay for the quantitative determination of the pancreatic antigen in which we use 125I-labeled, purified antigen and rabbit pancreas-specific antisem. Studies of the amounts of the circulating antigen in sera of normal subjects and patients with clinically diagnosed pancreatitis is the subject of the following paper (this issue).

References

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