The Protein Composition of Human Pancreatic Juice*

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A large body of information has been gained during the past decade concerning the enzymatic composition of pancreatic juice. Keller, Cohen, and Neurath (1) reported in 1958 on the proteins contained in bovine pancreatic juice, relating their observations to enzymes previously isolated from bovine pancreatic tissue. Greene, Hirs, and Palade (2) extended these observations to include amylase and lipase. Subsequently, members of Desnuelle's group have analyzed pancreatic juice from dog (3), pig (3), and rat (4, 5), and an excellent review of this work has been written by Marchis-Mouren (5). In the present report we will present our observations concerning the enzymes, zymogens, and inhibitors contained in human pancreatic juice.

EXPERIMENTAL PROCEDURE

**Human Pancreatic Juice**—Freshly collected human pancreatic juice was made available to us by Dr. Thomas T. White and Dr. Patricia J. Keller and Barbara J. Allan

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Ronald Elmslie of the University of Washington, Department of Surgery. After collection at room temperature the juice was treated with 1 mM DFP, lyophilized, and stored as a dry powder at -20°. Prior to use, the powder was restored to its initial protein concentration by the addition of distilled water.

Lyophilized powder containing approximately 33 g of protein was accumulated from one individual, Patient M, over a period of 5 weeks. This material was used for the major part of our experimentation. Additional samples of human pancreatic juice were obtained from a second individual, Patient T. This material was stored at -20° without DFP treatment and lyophilization.

**Enzymes**—Crystalline bovine pancreatic trypsin inhibitor and the crystalline bovine pancreatic enzymes, trypsin (EC 3.4.4.4), α-chymotrypsin (EC 3.4.4.5), carboxypeptidase A (EC 3.4.2.1), and DNase I (deoxyribonucleotide-oligonucleotide-hydrolase, EC 3.1.4.5) were obtained from Worthington. Crystalline bovine RNAse (polynucleotide:oligonucleotide-transferase (cyclizing), EC 2.7.7.1) was obtained from Armour and Company. The porcine pancreatic enzymes, elastase (pancreatopeptidase E, EC 3.4.4.7) (crystallized twice), lipase (glycerol ester hydrolase, EC 3.1.1.3), grade PL III, and carboxypeptidase B (EC 3.4.2.2), grade COB, were also obtained from Worthington. Human salivary amylase (α-1,4-glucan 4-gluconohydrolase, EC 3.2.1.1), crystallized twice, was a gift of Dr. Edmond Fischer of the Department of Biochemistry, University of Washington.

**Substrates**—The substrates purchased from Mann Research Laboratories were BAE, ATE, and elastin. Hippuryl-L-phenylalanyl acid (sodium salt) and naphthol-AS-nonanoate were purchased from Cycle Chemical Corporation, Los Angeles. Worthington supplied DNA (highly polymerized) and yeast RNA. The yeast RNA for the RNAase agar slide assay (described below) was a product of Nutritional Biochemicals. Hippuryl-L-arginine was purchased from Calbiochem; olive oil, highly refined, low acidity, was a product of Sigma; and Noredux starch, soluble, was obtained from Ganes Chemical Works, Inc., New York. The hydrolyzed starch used for the amyrase starch slide technique was supplied by the Connaught Medical Research Laboratories, Toronto, Canada.

**Methods of Assay**—Amylase was assayed as described by Fischer and Stein (6) with the exception that the temperature was increased to 30°, and the NaCl concentration was increased to 0.01 M to maintain maximal activity at this temperature (7).

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2 The abbreviations used are: DFP, diisopropyl phosphorofluoridate; BAE, benzoyl-L-arginine ethyl ester; ATE, N-acetyl-L-tyrosine ethyl ester; PTI, pancreatic trypsin inhibitor.

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For the lipase the amount of free acid released from olive oil was determined by the method of Marchis-Mouren, Sarda, and Deuuelle (8) the reaction being followed with a Radiometer pH-stat, type TTT1e. Null point titrimetric methods were also utilized to follow the hydrolytic activities of trypsin, chymotrypsin, and carboxypeptidase A toward BAE (9), ATE (9), and hippuryl-L-phenylalactic acid (10), respectively.

Alternatively, the hydrolyses of BAE and ATE were determined spectrophotometrically by the method of Schwert and Takenaka (11). For the spectrophotometric assays potassium phosphate buffer was used at pH 8.0 for BAE and at pH 7.0 for ATE. All phosphate buffers were prepared from the monopotassium salt.

Carboxypeptidase B activity was also measured by a spectrophotometric assay (12). A Hitachi Perkin-Elmer spectrophotometer, model 130, was used for all optical measurements. RNase was determined by the method described by Kalnitsky et al. (13).

Elastase was assayed by a modification of the method of Grant and Robbins (14), as follows. Covered 25-ml Erlenmeyer flasks containing 20 mg of elastin and 4.5 ml of 0.1 M Tris buffer, pH 8.8, were incubated at 37° with vigorous shaking (100 strokes per min). The reaction was started with 0.5 ml of enzyme in buffer. The usual assay time was 30 min, but with human pancreatic juice incubation periods up to 90 min were necessary. The reaction was stopped by the addition of 2.0 ml of 0.7 M phosphate buffer, pH 6.0, at 0°. The flask contents were centrifuged in chilled 12-ml centrifuge tubes for 45 min at 710 × g in the International Centrifuge, model EKD. The absorbance at 280 μA corrected for the appropriate enzyme and substrate blanks, was a measure of the digested elastin. Hog pancreatic elastase was used as a standard enzyme. A sigmoid curve was observed, as had been observed by Hall and Czerkawski (15) and by Naughton and Sanger (16) with other elastase assay systems. After an initial lag there is a pseudolinear portion, and this region was used to estimate the elastase activity in human pancreatic juice.

Protein Concentration—The absorbance at 280 μA was used to determine protein concentrations. For whole juice, a provisional factor of 16 was used as the optical density at 280 μA in 1-cm cells of 1% protein solution. For some of the more purified fractions of human pancreatic juice, factors previously used for the corresponding bovine enzymes were applied (17).

Polyacrylamide Disc Electrophoresis For routine analyses the Neidle and Waelsch modification (17) of the method of Riesfeld, et al. (13) was used. Prior to use the DEAE-cellulose was washed with 1 M K2HPO4 until no more yellow was extracted. The usual procedures (27) were followed for pouring and packing the columns, with the exception that gradually increasing pressures were used to pack the columns. Final packing was done with 5 p.s.i. of nitrogen. With type 40 DEAE-cellulose, final flow rates of 16 ml per hour were obtained.

Chromatographic Techniques Human pancreatic juice was separated into cationic and anionic fractions by the procedure described by Keller, Cohen, and Neurath (1) with the following minor modifications. The initial dialysis step was replaced by salt exchange on a column of Sephadex G-25, and chromatography on columns of DEAE-cellulose was carried out at pH 6.5 as well as at pH 8.0.

RESULTS

Enzymes of Whole Pancreatic Juice

We have analyzed whole reconstituted pancreatic juice for the enzymes commonly found in the pancreatic juice of other species. Amylase, lipase, RNase, and DNase are detectable in whole, UVF-treated pancreatic juice without prior activation. Other

The RNA solution, pH 7.2, was mixed with the agar (about 5%) when the concentrated agar solution had cooled to 41°. The slides were stored in a humid atmosphere at 4°.
enzymatic activities are found after prior treatment with trypsin, namely, elastase, carboxypeptidases A and B, chymotrypsin, and trypsin. Collagenase is absent, and lysozyme was not detected.

Polyacrylamide gel analyses have been used for comparison of samples of human pancreatic juice. Because our early studies indicated that some zymogen activation occurred during electrophoresis in anionic gel systems, we have utilized, with few exceptions, cationic gel systems. Typical patterns obtained with the cationic system are shown in Fig. 1. These samples, obtained from two different individuals, show a marked similarity in pattern. Freshly stained gels contain 2 or 3 zones in Region A, 5 zones in Region B, 4 zones in Region C, and several weakly staining zones in Region D, for a total of 15 cationic components under the conditions of the run. Although the patterns are qualitatively similar, the staining intensity of certain bands varies in the two individuals despite the application of equivalent protein loads.

**Pancreatic Trypsin Inhibitor**

In preliminary experiments, we noted that the zymogens of human pancreatic juice showed a marked resistance to activation by bovine trypsin. This behavior is due to the presence of a pancreatic trypsin inhibitor which suppresses the activity of trypsin added to the pancreatic juice. As shown in Fig. 2, 1 ml of unfractionated pancreatic juice, containing 4 mg of protein, inhibits about 0.08 mg of crystalline bovine trypsin. In a comparable experiment, 4 mg of pH 6.5 cationic proteins inhibit about 0.25 mg of added trypsin. There is thus a 3-fold enrichment of the inhibitor in the basic protein fraction.

Several observations suggest that the molecular size of the inhibitor is small. Although it is not retarded by Sephadex G-25, it is lost during dialysis and is soluble in 2.5% trichloroacetic acid at 80°C, a characteristic shared with several other pancreatic trypsin inhibitors.

The solubility of human pancreatic trypsin inhibitor in 2.5% trichloroacetic acid has been used as a means of purification. The procedure developed by Grossman (30) and by Kals and Grossman (31) for purification of trypsin inhibitors from the pancreatic juice of the rat and dog was followed. Fig. 3 presents the polyacrylamide gel pattern obtained with purified preparations of the inhibitor (Fig. 3c). Two major components are visible; two minor components are present, but are barely visible, even in freshly stained gels. Zones corresponding to the two major components are seen in patterns of whole human pancreatic juice (Fig. 3a) and the pH 6.5 cationic proteins (Fig. 3b).

For comparison we have examined preparations of the bovine trypsin inhibitor crystallized by Kunitz with the same polyacrylamide system. The two pancreatic inhibitors (Fig. 3c and d) are electrophoretically distinct, with the bovine inhibitor exhibiting a faster migration rate. No zones corresponding to the bovine inhibitor are visible in the human juice.

Some kinetic properties of the purified human inhibitor have been compared with those of the bovine inhibitor (Kunitz). When amounts of bovine PTI and human inhibitor that were equivalent in their trypsin inhibitory power were added to solutions containing γ-chymotrypsin, the bovine inhibitor gave complete inhibition of the chymotryptic activity against ATE, while the human inhibitor gave none.

Human PTI preparations have also been tested for temporary inhibition of trypsin. Samples were mixed with bovine trypsin...
in 0.01 M calcium chloride-0.04 M borate buffer, pH 8.0. Aliquots were removed from the control and experimental samples immediately after mixing and again after 22 hours of incubation at 25°C, and assayed by the spectrophotometric procedure. The results are given in Table I. Immediately after mixing with inhibitor, the activity of trypsin falls to approximately 25% of the control rate. However, after 22 hours at 25°C, the activity of the inhibited samples is restored to approximately 75% of the control rate.

**Separation into Cationic and Anionic Components**

In preliminary experiments we noted that pH 6.5 was superior to pH 8.0 for our purposes. A typical experiment was carried out as follows. Approximately 100 mg of lyophilized pancreatic juice protein was taken up in 20 ml of water containing DFP (1 mM). Nineteen milliliters were applied to a column (4.5 × 25 cm) of Sephadex G-25 that had been equilibrated with 0.005 M potassium phosphate buffer, pH 6.5. Fractions of 4.9 ml were collected at a flow rate of 75 ml per hour. After a hold-up volume of 115 ml had passed through the column, the proteins emerged in a volume of 65 ml in 95% yield. The fractions were combined, lyophilized, and stored at -20°C.

The powder was dissolved in 10 ml of cold distilled water, 1 mM in DFP, and stirred for 30 min at 0°C. This solution (9 ml, 85 mg) was applied to a column (1.8 × 63 cm) of DEAE-cellulose which had been equilibrated with 0.005 M potassium phosphate buffer, pH 6.5, at 4°C. The cationic proteins were collected at a flow rate of 16 ml per hour in 4.5-ml fractions by washing the column with 0.005 M potassium phosphate buffer, 0.1 mM in DFP; the anionic proteins were eluted in one step with 0.4 M potassium phosphate (KH₂PO₄-KOH) buffer, pH 6.5, containing 0.1 mM DFP. A typical diagram of the batch separation of cationic and anionic proteins is shown in Fig. 4.

**Cationic Proteins**—Cationic proteins account for 20 to 25% of the total protein of human pancreatic juice. The lyophilized powder containing the cationic proteins was dissolved in 0.005 M potassium phosphate buffer, pH 6.5, or in distilled water, and analyzed for enzymatic activity. Significant levels of amylase, lipase, RNase, and PTI were present. The activities toward ATE, hippuryl-L-arginine, and elastin were at very low levels, even though sufficient trypsin had been added to overcome the inhibition by pancreatic trypsin inhibitor. Activity toward BAE was not significant after correction for the added trypsin.

**Analyze the cationic proteins by the cationic polyacrylamide gel system reveals four prominently stained areas (Fig. 5).  With the specific staining techniques described under "Experimental Procedures," we found that the cationic proteins were stained for amylase, lipase, RNase, and PTI.**

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**Table I**

<table>
<thead>
<tr>
<th>Activity after 2 min</th>
<th>Activity after 22 hours</th>
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<td>Specific activity</td>
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<tr>
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<td>51</td>
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<tr>
<td>Patient T ..............</td>
<td>9</td>
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<tr>
<td>Patient M ...............</td>
<td>15</td>
</tr>
</tbody>
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**Fig. 3. Polyacrylamide disc electrophoresis of fractions containing human pancreatic trypsin inhibitor and bovine PTI. From left to right: whole human juice (454 µg), pH 6.5 cationic proteins (240 µg), human PTI, and bovine PTI (25 µg). The running conditions are the same as those given in the legend to Fig. 1.**
mental Procedure,” three of these areas have been shown to contain amylase, lipase, and RNase as indicated. The fastest running zones have been associated with the trypsin inhibitor as described above. When the cationic proteins are examined by the anionic polyacrylamide gel system of Davis a minimum of three zones with amylase activity can be resolved.

**Anionic Proteins**—Fig. 6 presents the anionic peak of Fig. 4 in finer detail. Protein concentrations were followed by the absorbance at 260 nm in a 1-cm cell, and the individual fractions were analyzed for endoproteolytic activity against ATE and BAE. From the elution pattern it is evident that the protein showing activity against ATE is eluted earlier than the two peaks showing activity against BAE. During chromatography some activation occurred in the anionic fractions, even in the presence of DFP. Presumably, this takes place after separation of the cationic proteins containing the pancreatic trypsin inhibitor.

Anionic proteins have also been resolved by gradient elution and the fractions obtained have been analyzed for carboxypeptidase A, carboxypeptidase B, and chymotrypsin. In this instance, the activities of carboxypeptidases A and B appeared before the main chymotryptic fraction as determined from the relative specific activities against hippuryl-L-arginine, hippuryl-L-phenyllactic acid, and ATE. We have tentatively concluded that the order of emergence of the anionic components from DEAE-cellulose columns equilibrated with potassium phosphate buffer at pH 6.5 is as follows: procarboxypeptidase B, carboxypeptidase A, chymotrypsinogen, and anionic “trypsins” 1 and 2.

**Other Enzymes**—Two enzymes, DNase and elastase, which are found in unfractionated pancreatic juice have not yet been located in either the anionic or cationic protein fractions. Experiments are in progress to determine their chromatographic behavior. DNase activity was detected in whole pancreatic juice by the
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FIG. 8. The elastase activity of human pancreatic juice with and without added trypsin. The change in optical density at 280 m/ per ml of supernatant solution (see “Experimental Procedure”) is plotted against the amount of human pancreatic juice protein, in milligrams per ml of assay mixture. O, pancreatic juice without added trypsin; , pancreatic juice activated with added trypsin (0.48 mg per ml). The 90-min assay was used.

polyacrylamide disc technique described by Boyd and Mitchell (24). This system uses the standard anionic gel system, with the exception that DNA is incorporated into the gels. When amounts of human pancreatic juice containing 5 μg of protein are applied to a polyacrylamide gel, two DNase zones are seen (Fig. 7). With higher amounts of sample (240 μg) as many as four zones are visible, two near the top of the running gel and two in the central region.

The elastase activity of human pancreatic juice was measured before and after trypsinic activation. Trypsin (final concentration, 0.48 mg per ml) was added to pancreatic juice containing approximately 4.7 mg of protein and incubated at 25° for 15 min. Samples with and without added trypsin were assayed as described under “Experimental Procedure.” Elastase activity was present after trypsinic activation, but no elastin digestion was shown by nonactivated juice or by trypsin alone (Fig. 8).

Discussion

Human pancreatic juice has not been characterized previously by the chromatographic and enzymatic techniques used in the present study, but it has been reported to contain amylase (32, 33), lipase (33–36), cholesterol esterase (36), phosphatase (36), and phosphatase (33), as well as chymotrypsinogen, trypsinogen (32, 37), and a trypsin inhibitor (37). Extracts of human pancreas have been reported to contain, in addition, ribonuclease (38) and elastase (39). Several enzymes have been isolated in purified form from extracts of human pancreas, notably crystalline amylase by Fischer, Duckert, and Bernfeld (40), two forms of ribonuclease by Uchita et al. (38), and trypsin by Buck, Bier, and Nord (41). In our investigation we have found that the juice contains procarboxypeptidases A and B, DNase, RNase, and proelastase, in addition to those enzymes already cited.

Pancreatic juices from a number of mammalian species, namely, steer (1), rat (4), and pig and dog (3), have been analyzed chromatographically on DEAE cellulose at pH 8.0, and each has presented a characteristic chromatographic profile. Marchis-Mouren (5) has reviewed the differences in the proportions and chromatographic behavior of the respective enzymatic components of the different species. In the absence of equivalent data concerning the pancreatic juice of higher species we can only compare the pancreatic juice of man with those of steer, rat, pig, and dog. The comparison reveals that man and rat have characteristics in common. In both species, amylase, lipase, and RNase are found in the cationic fraction, whereas trypsinic and chymotryptic activities are found in anionic fractions. Moreover, the ability to hydrolyze BAE appears in two anionic peaks in both species. Without further investigation it cannot be said whether the two human enzymes hydrolyzing BAE represent different activation products of a single zymogen or discrete enzymatic entities. Croston (42) has observed two anionic endopeptidases with trypsin-like properties in extracts of the pancreas of the Chinook salmon (Oncorkhyus tshawytscha), and I’rhal and Neurath (43) have reported an anionic trypsinogen in the pancreas of the Pacific spiny dogfish (Squalus acanthias). It is thus increasingly apparent that the pancreatic endopeptidases, trypsin and chymotrypsin, exist in different molecular forms in different species.

Although proelastase was shown to be present in unfractonated juice, we were unable to detect elastolytic activity in either the cationic or anionic subfractions of the juice. This apparent discrepancy may have resulted from instability of the proenzyme to lyophilization, as has been reported for porcine proelastase (44).

Four DNase zones were observed on anionic polyacrylamide gels of whole human pancreatic juice developed by the technique of Boyd and Mitchell (24). Crystalline preparations of bovine pancreatic DNase also contained four or more anionic components when analyzed at high concentrations by this method (24). However, the pH of the running gel during electrophoresis by this system is above 9.0, and we have observed that thezymogens of human pancreatic juice frequently become activated under these conditions. Thus the multiple forms of DNase detected in human juice might reflect altered forms of the same enzyme rather than unique enzymes. Further experimentation would be necessary to specify the number and charge properties of DNases in human pancreatic juice. In contrast, proteolysis does not seem to be a contributory factor to the isoamylases observed, and these are being studied further.

Two kinds of trypsin inhibitor have been isolated in crystalline form from bovine pancreatic tissue, the Kunitz inhibitor (28) and the Kazal inhibitor (29). Of the two, the Kunitz inhibitor is the better characterized: it is a basic protein, isoelectric near pH 10.5, with a molecular weight of 6513 (45) and a known amino acid sequence (46–48). Less well characterized is the crystalline Kazal inhibitor, which contains three components, two of which are known to be active and to have isoelectric points below 6.0 (29).

Laskowski and Wu (49) compared the kinetic behavior of the two kinds of bovine pancreatic trypsin inhibitors. The Kunitz inhibitor was found to inhibit α-chymotrypsin as well as trypsin, and to be resistant to trypsin digestion. In contrast, the Kazal inhibitor does not inhibit α-chymotrypsin, and is susceptible to trypsin digestion. The susceptibility of the latter to trypsin digestion results in a slow reversal of inhibition of trypsin, and...
because of this the Kazal inhibitor has been designated a "temporary" inhibitor by Laskowski and Wu (49).

Trypsin inhibitors have been isolated from the pancreatic juice of rat (30), dog (31), and steer (50). In general, these have resembled the Kazal inhibitor in biological activity and, where tested, in charge properties. The inhibitor from human juice porcine" inhibitor by Laskowski and Wu (49).


behave differently when tested with purified preparations of the human pancreatic juice. This is not unlikely, in view of the differences in charge properties of the bovine and human endopeptidases.

Human pancreatic juice can be collected only out of surgical necessity and thus never from a fully healthy individual. This shows the inhibitory properties typical of the Kazal inhibitor, whether tested with bovine trypsin and human chymotrypsin, the human inhibitor may fail to inhibit bovine chymotrypsin, as behrave differently when tested with pure preparations of the corresponding human enzymes. This is not unlikely, in view of the differences in charge properties of the bovine and human endopeptidases.

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