Trypsin Inhibitor from Human Pancreas and Pancreatic Juice*

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SUMMARY

Human pancreatic secretory trypsin inhibitor has been isolated in 55% yield from human pancreatic juice and in 45% yield from human post-mortem pancreata by gel filtration on Sephadex G-75 in the presence of 10⁻¹⁴M diisopropyl phosphofluoridate and by ion exchange chromatography on DEAE-cellulose and SP-Sephadex. The polypeptide inhibitor has a molecular weight of 6242 and contains 56 amino acid residues per molecule: Asp, Asn, Thr, Ser, Glu, Gln, Pro, Gly, Ala, Cys, Val, His, Leu, Tyr, Phe, Lys, and Arg, and does not contain glucosamine or galactosamine. The inhibitor was isolated as five chromatographic forms with identical amino acid content, specific activity for trypsin inhibition and molecular weight on sodium dodecyl sulfate gel electrophoresis, amino acid analysis, and on the basis of molecular weight on sodium dodecyl sulfate gel electrophoresis, amino acid analysis, and on the basis of molecular weight on sodium dodecyl sulfate gel electrophoresis. All forms of the inhibitor are homogeneous by acrylamide gel electrophoresis, amino acid analysis, and on the basis of the stoichiometry of their interaction with trypsin. All of the three forms subjected to Edman degradation had Asx-Ser as their NH₂-terminal residues. The multiple chromatographic forms could be distinguished on the basis of chromatographic behavior on ion exchange resins, by acrylamide gel electrophoresis and, for the three major forms, on the basis of amide content and susceptibility to enzymatic digestion. It is concluded that the multiple chromatographic forms differ in amide content.

The inhibitor is secreted in the pancreatic juice in the free form (not combined with trypsin) in an amount equivalent to 0.1 to 0.8% of the total protein in pancreatic juice. The inhibitor is of the "Kazal" type and similar to the pancreatic secretory trypsin inhibitors isolated from pancreatic juice of other species. The human inhibitor is the only pancreatic secretory trypsin inhibitor studied which effectively inhibits human cationic trypsin.

The study of the interaction of polypeptide trypsin inhibitors with trypsin and trypsin-like enzymes affords the opportunity to examine specific protein-protein interactions (1) of physiological and perhaps clinical significance (2-4). Although detailed structural (2), kinetic (5, 6) and crystallographic (9, 7) data are available for trypsin inhibitors from bovine and porcine pancreases, the inhibitor from human pancreas has not yet been characterized. In view of the demonstration that bovine and porcine pancreatic secretory trypsin inhibitors do not effectively inhibit human cationic trypsin (8) and the probable role of the pancreatic secretory trypsin inhibitor in preventing autodigestion of the pancreas (9, 10), it is important to characterize the human pancreatic secretory trypsin inhibitor (PSTI)-trypsin system. In a preliminary report we have described the isolation of one form of pancreatic trypsin inhibitor from human post-mortem tissue (11). Here we extend this study to the isolation of inhibitor from human pancreatic juice and characterize the inhibitor from both sources.

EXPERIMENTAL PROCEDURES

Materials

Human pancreatic juice was collected by catheterization of the pancreatic duct after related surgical procedures (9, 12-14). Although some of the pancreatic juice was from patients having pancreatitis, the pancreatic juice documented in the elution diagrams presented here was from a patient without pancreatic disease. Post-mortem pancreatic tissue was obtained from patients who did not have diagnosed pancreatic disease. Human cationic pancreatic trypsin (15, 16) was supplied by Dr. James Travis, University of Georgia, Athens, Ga. Other sources of materials not given in the text have been described previously (17-19).

Methods

Analytical Procedures: Amino Acid Analysis—Samples containing 10 to 30 nmoles of inhibitor were hydrolyzed in evacuated sealed tubes with 1 ml of twice distilled constant boiling HCl for 22 to 66 hours at 110°. Amino acid analysis of hydrolysates was performed by the method of Spackman et al. (20) on an automatic instrument with provisions for multiple sample application (21). Cysteine and cystine were determined as cysteic acid after oxidation with performic acid and acid hydrolysis (22). The glutamine and asparagine content of the multiple chromatographic forms of the inhibitor was determined by amino acid analysis (23) of enzymatic hydrolysates of S-2-aminoethylated inhibitor. Inhibitor (0.2 to 2 μmoles) was reduced with 30 molar excess of diithothreitol, reacted with ethylenimine in a pa-stat and isolated by gel filtration on Sephadex G-25 (19). S-2-Aminoethylated-inhibitor (36 nmoles) was incubated with 10 μg each of trypsin, chymotrypsin, pronase, carboxypeptidase B, and 125 μg of aminopeptidase M (Henley & Co., New York, Lot E:L-37-71) in 0.5 ml of 0.02 M sodium phosphate buffer, pH 7.5 for 24 hours at 21°.

Edman Degradation—A subtractive procedure using the method of Gray (24) was used on 15-nmole samples of S-2-aminoethylated-inhibitor (25).

Polyacrylamide Gel Electrophoresis—Electrophoresis was carried out on the inhibitor in a 7% polyacrylamide gel, in the presence of 3% sodium dodecyl sulfate, according to the method of Davis (26). Electrophoresis was carried...
out in a vertical slab apparatus using the Tris-glycine buffer, pH 8.3, and 20% acrylamide in the presence and absence of 10% SDS (20). The β-alanine-acetic acid, pH 4.5 buffer system of Heidt et al. (27) was also run in 20% acrylamide gels. Inhibitor, 2 to 5 μg, was detected by staining at 40° for 1 hour with Coomassie blue, 0.25% in methanol-acetic-water (5:1:4). Gels were destained by diffusion.

**Analytical Ultracentrifugation**—Sedimentation-equilibrium experiments were performed in a Spinco model E analytical ultracentrifuge, by the high speed equilibrium technique of Yphantis (29) and Roark and Yphantis (30). A glass-sandwiched polarizing filter placed above the usual Kodak 77A filter, and oriented to polarize the light in the radiation direction, reduced the effect of cell window distortions. The centrifuge cell was a 12-mm multi-channel, external-loading cell with 5 mm column height (31). "Blank" corrections were performed according to the protocol of Ansevin et al. (31). Experiments were performed at 20° and 56,000 rpm in 0.1 M KCl 0.01 M Tris, pH 7.8. Kodak type II G photo graphic plates were read with an automated Nikon microcomparator. Fringe measurements were analyzed by computational methods described by Roark and Yphantis (30). Partial specific volume of 0.71 was calculated from the amino acid composition.

**Trypsin Inhibitor and Enzyme Assays**—The activity of trypsin was measured in a pH-stat at pH 8.2, 25°, using 0.01 M TAme, 0.1 M KCl, 0.005 M Tris-HCl, 0.020 M CaCl₂. One unit of inhibitor activity is the amount of inhibition that caused a reduction of Tamelase by 1 µmole per min. Inhibitor specific activity is defined as inhibitor units per mg enzyme. Each value was determined in column effluents with bovine trypsin. Details of the assay are given in Reference 17. Chymotrypsin activity was also measured by the pH-stat method with 0.01 M ATFe, 0.01 M KCl, 0.005 M Tris-HCl, 0.002 M CaCl₂ (17). Carboxypeptidase A activity was measured by the spectrophotometric procedure of Folk and Schirmer (32) by monitoring the increase in absorbance of 254 nm at 25° of the substrate solution 0.001 M hippuryl-L-phenylalanine in 0.025 M Tris-HCl, pH 7.5, containing 0.5 M NaCl. Carboxypeptidase B activity was measured in a similar manner with 0.001 M HA in 0.025 M Tris-HCl buffer, pH 7.6, containing 0.1 M NaCl. Leucine amino peptide activity was assayed with 0.005 leucine-β-naphthylamide in 0.05 M Tris-HCl, 0.1 M NaCl by incubation at 37° for 3 h. Enzyme activity is reported as the increase of absorbance at 550 nm after diazonitration of the product of the reaction, β-naphthylamide (33).

**Activation of Zymogen**—No trypsin, chymotrypsin, carboxypeptidase A or B activity was detected in Sephadex G-75 column effluent before activation with trypsin. Aliquots of the column effluent were incubated with an equal volume of bovine trypsin, 0.08 mg per ml in 0.1 M KCl, 0.02 M CaCl₂, 0.05 M Tris-HCl buffer, pH 7.8. Chymotrypsin and trypsin activity were determined after incubation at room temperature for 10 and 240 min, respectively. Separate activation mixtures were held at 35° for 30 min before assay of carboxypeptidase A and B activity. The presence of 10⁻³ M diisopropylphosphofluoridate in the Sephadex G-75 effluent did not prevent the tryptic activation of the zymogens under these conditions, but did preclude their quantitative estimation.

**Preparation of Inhibitors**

Individual samples of pancreatic juice and tissue were initially screened for trypsin inhibitor activity to ensure that no trypsin activation had occurred. This precaution was taken so that the inhibitor could be separated from trypsinogen by gel filtration and isolated in the "free" form. The tissue was subjected to a preliminary fractionation by ammonium sulfate precipitation and this enriched inhibitor fraction was then processed in the same manner as pancreatic juice. The procedure is similar to that described for the isolation of trypsin inhibitor from bovine (17) and porcine pancreatic juice (18). After purification on the basis of size by gel filtration on Sephadex G-75 and Sephadex G-25 the inhibitor was separated into multiple chromatographic forms by gradient elution chromatography on DEAE-cellulose and SP-Sephadex.

**Extraction of Inhibitor from Human Pancreas**—Minced tissue in 100 g portions was suspended in 5 volumes (w/v) of chilled 10⁻⁴ M diisopropyl phosphofluoridate and homogenized in a Waring Blender for 2 min at maximum speed. The suspension was adjusted to pH 4.5 with 6 N HClO₄ and centrifuged at 13,000 × g for 45 min at 5° in a Sorvall GSA rotor. This procedure was repeated on the sediment and the combined supernatants were brought to 70% saturation by the slow addition of 472 g per liter of ammonium sulfate. The suspension was centrifuged for 45 min at 12,000 × g, 0° and the supernatant discarded. The precipitate was resuspended in 10⁻⁴ M diisopropyl phosphofluoridate and stored at - 15°. Each preparation was assayed for inhibitor activity before being combined with others for gel filtration on Sephadex G-75.

**Gel Filtration**—Two Sephadex G-75 columns (7.6 × 180 cm) containing inhibitor in series, were prepared as described in Greene et al. (18) and in the legend to Fig. 1. Lyophilized pancreatic juice suspended in 200 to 400 ml of 10⁻¹⁴ M diisopropyl phosphofluoridate to a final protein concentration of 1 to 2%, was held at 4° for 2 hours before gel filtration. Effluent with an inhibitor specific activity greater than 12 was combined and lyophilized. This fraction, defined "low molecular weight fraction" was dissolved in 400 ml of 10⁻¹⁴ M diisopropyl phosphofluoridate and desalted on a Sephadex G-25 column (7.6 × 72 cm) developed at 4° with 0.05 M ammonium bicarbonate buffer, pH 8.1, containing 10⁻⁴ M diisopropyl phosphofluoridate. Fractions with an inhibitor specific activity greater than 20 were pooled and lyophilized. Since the inhibitor activity was partially included in the Sephadex G-25 resin from further purification, the preparation was then submitted to gel filtration on two columns (1.8 × 160 cm) of Sephadex G-25 (superfine) connected in series and developed with 0.05 M ammonium bicarbonate buffer at 4°. Effluent with inhibitor specific activity greater than 90 was combined.

The procedures used for gel filtration of inhibitor prepared by ammonium sulfate fractionation of tissue were essentially the same as used for pancreatic juice, with the exception that the G-75 column was 7.6 × 180 cm in length and the load solution was 600 to 750 ml.

**Chromatography on DEAE-cellulose**—Columns of DEAE-cellulose (Whitman DE32) (1.8 × 45 cm) were prepared and developed as described in Greene et al. (18) and in Fig. 2. At fraction 66 (4° and 6000 g) a linear gradient prepared from 2 liters each of 0.0285 M Tris-HCl buffer, pH 0.0, and 0.0285 M Tris-HCl buffer, pH 0.9, containing 0.2 M potassium chloride was applied to the column. Fractions containing inhibitor activity were combined, lyophilized and desalted by gel filtration on Sephadex G-25 (superfine) (1.8 × 72 cm), developed with 0.05 M ammonium bicarbonate buffer, pH 8.1, at 4°.

**Chromatography on SP-Sephadex**—SP-Sephadex C-25 (40 to 120 μm) was treated with 0.5 M acetic acid overnight and fine particles were removed by settling several times in distilled water. The resin was equilibrated with 0.1 M ammonium acetate, pH 4.5. (0.1 M in acetic acid) by decadation before the column was prepared. Lyophilized desalted material from the DEAE-cellulose column was dissolved in 2 to 5 ml of 0.05 M ammonium acetate buffer, pH 4.5, and applied to a column (0.9 × 70 cm). The column was developed at 4° with a linear gradient prepared from 400 ml each of 0.1 M ammonium acetate buffer, pH 4.5, and 0.1 M ammonium acetate buffer, pH 7.0.

**RESULTS**

**Trypsin Inhibitor Content of Pancreatic Tissue and Juice**

The average amount of trypsin inhibitor activity found in unactivated human pancreatic tissue was equivalent to 3 mg/100 g wet weight tissue. Most of the samples were within the range 1 to 6 mg/100 g and the maximum inhibitor content detected in a single gland was 30 mg/100 g. Approximately 40% of the 10 kg of human pancreata, examined in 50 to 100 g portions,
was activated as indicated by the presence of trypsin activity. These values for the inhibitor content of human pancreas were approximate and probably underestimated because of the variability of collection, storage, and secretory state of post mortem tissue.

The average amount of trypsin inhibitor activity in human pancreatic juice was equivalent to 0.3 mg/100 mg of protein with a range of 0.1 to 0.6 mg of trypsin inhibitor/100 mg of protein. These values for the inhibitor content of pancreatic juice like those given for tissue are approximate and probably underestimates. The pancreatic juice documented in the elution diagrams presented here contained 0.35 mg of trypsin inhibitor/100 mg of protein.

Isolation of Trypsin Inhibitor

**Gel Filtration**—Fig. 1 is a representative elution diagram of human pancreatic juice obtained by gel filtration on Sephadex G-75. The inhibitor activity (○) was retarded relative to most of the secretory protein (●). Effluent corresponding to Peak 1, indicated by the solid bar, was combined and is denoted low molecular weight fraction. The elution positions for leucine aminopeptidase activity (1) and the zymogen, procarboxypeptidase A (2), procarboxypeptidase B (3), chymotrypsinogen (ATEase) (4) and trypsinogen (5) are also indicated in the diagram. Amylase activity was eluted after the trypsin inhibitor in effluent corresponding to fractions 240 to 270. In five preparative experiments of the type illustrated in Fig. 1, more than 87% of the trypsin inhibitor activity present in the juice was isolated as part of the low molecular weight fraction. On the basis of an inhibitor specific activity of 49 for the low molecular weight fraction, a 16-fold purification was achieved and the inhibitor activity had been quantitatively separated from trypsinogen during filtration on Sephadex G-75. During desalting and gel filtration on Sephadex G-25 columns (cf. Methods) an additional 4-fold purification was achieved with essentially quantitative recovery of inhibitor activity.

The low molecular weight fraction from the 70% ammonium sulfate precipitate derived from tissue was prepared by procedures similar to those used for pancreatic juice. The G-75 elution diagram is given in Fig. 1 of Greene and Pabols (11). In contrast to the high recovery of trypsin inhibitor from pancreatic juice, an average of 70% of the inhibitor activity was recovered from the G-75 column and part of the trypsinogen was activated in the collection tubes after gel filtration.

**Chromatography on DEAE-cellulose**—The gradient elution chromatography on DEAE-cellulose of the low molecular weight fraction from human pancreatic juice is given in Fig. 2 (top). The inhibitor activity (○) was separated into two chro-

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**Fig. 1.** Gel filtration of human pancreatic juice on Sephadex G-75. The column (7.6 × 360 cm) was equilibrated and developed at 4° with 0.5 M potassium chloride, 0.01 M Tris-HCl buffer, pH 8.1, containing 10⁻⁴ M disopropyl phosphorofluoridate. Sample, 34 A₁₀₀° × 190 ml; ●, absorbance at 280 nm; ○, trypsin inhibition (TAME hydrolysis). The fractions containing trypsin inhibitor activity indicated by the bar were combined. Elution profiles are given for: 1, leucine aminopeptidase (— — —); 2, procarboxypeptidase A (— — —); 3, procarboxypeptidase B (— — —); 4, chymotrypsinogen (— — —), and 5, trypsinogen (— — —). See Methods for zymogen activation and enzyme assay conditions.

**Fig. 2 (left).** Chromatography of human pancreatic low molecular weight fraction on DEAE-cellulose. The column (1.8 × 15 cm) was equilibrated and developed with 0.028 M Tris-HCl buffer, pH 9.0, at 4°. At Tube 66 (indicated by the arrow) a linear gradient prepared from 2 liters each of equilibrating buffer and 0.028 M Tris-HCl buffer, 0.2 M potassium chloride was applied to the column. Top, pancreatic juice low molecular weight fraction, 4.3 A₁₀₀° × 30 ml. Bottom, pancreatic tissue low molecular weight fraction 2.4 A₁₀₀° × 35 ml. – — —, absorbance at 280 nm; ○ – ○, trypsin inhibition (TAME hydrolysis).

**Fig. 3 (center).** Chromatography of human pancreatic trypsin inhibitor activity A and B (Fig. 2, top) on SP-Sephadex. The column (0.9 × 70 cm) was equilibrated with 0.1 M ammonium acetate buffer, pH 4.5. After sample application the column was developed at 4° with a linear gradient prepared from 400 ml each of equilibrating buffer and 0.1 M ammonium acetate buffer, pH 7.0. The pH values given in the figure indicate the pH of effluent for the center tube of each peak. Top, Form A, 2.7 A₁₀₀° × 2 ml. Bottom, Form B, 5.1 A₁₀₀° × 4 ml. ● — ●, absorbance at 280 nm; ○ – ○, trypsin inhibition (TAME hydrolysis).

**Fig. 4 (right).** Comparison of SP-Sephadex chromatography elution profiles of the multiple chromatographic forms of human pancreatic trypsin inhibitor isolated from pancreatic juice and tissue. The column was operated as described in the legend to Fig. 3. Forms A and B of inhibitor were prepared by chromatography on DEAE-cellulose (cf. Fig. 2). ○ – ○, trypsin inhibitor derived from tissue; ● — ●, trypsin inhibitor derived from pancreatic juice; — — —, effluent pH.
matographic forms, A and B. A similar elution pattern was obtained from pancreatic tissue (Fig. 2, bottom). Forms A and B from both sources were eluted with the same elution conductivity, and present in similar proportions. The recovery of trypsin inhibitor activity was 85 ± 5% from both sources. At this stage of the purification the specific activity of inhibitor isolated from pancreatic juice approached 70 to 90% of the maximum specific activity achieved after Sephacryl S-200 chromatography. The inhibitor specific activity of material derived from pancreatic tissue was approximately 30 to 50% of the maximum achieved after Sephacryl S-200 chromatography.

**Chromatography on Sephacryl S-200—**Forms A and B of the pancreatic juice inhibitor prepared by DEAE-cellulose chromatography (Fig. 2) were individually rechromatographed on Sephacryl S-200 and eluted by a pH gradient as shown in Fig. 3. The A Form was separated into three components: A1, A2, and A3, which were eluted at pH 4.70, pH 4.75, and pH 4.92, respectively. The B form was resolved into two components, B1 and B2, eluted at pH 4.70 and pH 4.75. The inhibitor specific activity for all of the forms was 1500 to 1800. The calculated specific activity based on a 1:1 molar complex with trypsin is 1790. The recovery of inhibitor activity was 75 ± 5% for six Sephacryl S-200 columns.

The SP-Sephadex chromatography of the A and B forms of inhibitor prepared by DEAE-cellulose chromatography from pancreatic tissue (Fig. 4) is compared with inhibitor isolated from pancreatic juice (Fig. 5). In addition to the Forms A1, A2, A3, B1, and B2 present in juice, tissue contained a small amount of A1 and A2, eluted at pH 4.95 and pH 5.02, respectively, of inhibitor recovered from the column. Sixty-two per cent of tissue inhibitor activity was recovered from the SP-Sephadex columns in these experiments. The inhibitor specific activity for each of the multiple chromatographic forms of inhibitor isolated from pancreatic tissue was the same as inhibitor isolated from pancreatic juice.

The chromatograms presented in Figs. 2 and 4 demonstrate that both pancreatic juice and tissue contain the same major multiple chromatographic forms of trypsin inhibitor. The inhibitor specific activity for all forms is, within the error of the experiments, in good agreement with the calculated specific activity based on a 1:1 molar complex of inhibitor with bovine trypsin (17, 18). A somewhat unexpected chromatographic result is that Form A resolved from Form B by DEAE-cellulose chromatography, contains inhibitor (i.e. A1 and A2) which is eluted from Sephacryl S-200 at the same pH as B1 and B2 prepared from Form B. We have not been able to distinguish between forms A1 and B1 and Forms A2 and B2 by the analytical methods given below.

**Acrylamide Gel Electrophoresis—**The multiple chromatographic forms of human inhibitor were subjected to acrylamide gel electrophoresis at pH 8.3 and pH 4.5 in an attempt to differentiate the multiple forms and assess homogeneity. Fig. 5 gives the result of electrophoresis at pH 8.3. The multiple forms behaved as two classes: A1, A2, B1, and B2 migrated with the same mobility which was faster than forms A3, A4, and A5. Bovine (17) and two porcine pancreatic secretory trypsin inhibitors (18) were run for comparison. The porcine inhibitors I and II migrated toward the cathode.

At pH 4.5 (cf. Fig. 6) Forms A1, A2, and A4 are separated but Forms A1, A2, B1, and B2 are still not resolved. All forms of human PST1 migrated less rapidly than the homologous inhibitors of bovine and porcine origin. The Coomassie blue staining pattern indicates that the five forms of the human inhibitor are essentially homogeneous when examined by acrylamide gel electrophoresis at pH 4.5 and pH 8.3.

An SDS-acrylamide gel electrophoretogram of the five forms of inhibitor after reduction and denaturation is given in Fig. 7. A single component was observed for all forms of the human inhibitor. This corresponded in mobility and hence molecular weight to the homologous bovine (34) and porcine (25, 35) inhibitors which are single polypeptide chains with molecular weights of approximately 6000. Porcine insulin gave only one band under these conditions, probably corresponding to the β chain having a molecular weight near 3400. The presence of material of approximately 6000 molecular weight as well as the absence of detectable lower molecular weight peptides in SDSPAGEs for each of the multiple chromatographic forms of the human inhibitor, indicates that internal peptide bond cleavage (5, 36, cf. Laszowsk and Scalock (1) for a recent review) cannot be used to explain the existence of the multiple chromatographic forms of the inhibitor.

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**Fig. 5 (left).** Polyacrylamide gel electrophoresis of pancreatic secretory trypsin inhibitors in Tris-glycine buffer, pH 8.3. Electrophoretic migration was from top (cathode) to bottom (anode). The gel was run for 14 hours at 100 volts. B1 and B2, human inhibitor (cf. Fig. 4, bottom); A1 through A5, human inhibitor (cf. Fig. 4, top); cow, bovine inhibitor (17); P-I and P-II, porcine inhibitors I and II (18) which migrated toward the cathode.

**Fig. 6 (center).** Polyacrylamide gel electrophoresis of pancreatic secretory trypsin inhibitors in β-alanine-acetic acid buffer, pH 4.5. Electrophoretic migration was from top (anode) to bottom (cathode). The gel was run for 20 hr at 140 volts. The samples are identified in the legend to Fig. 5.

**Fig. 7 (right).** Polyacrylamide gel electrophoresis of pancreatic secretory trypsin inhibitors in Tris-glycine buffer, pH 8.3, containing 10% sodium dodecyl sulfate. Electrophoretic migration was from top (cathode) to bottom (anode). The samples were treated with β-mercaptoethanol and SDS at 105° for 2 min before electrophoresis. The gel was run for 13 hours at 60 volts. I; porcine insulin. The remaining samples are identified in the legend to Fig. 5.
Amino Acid Composition of Human Pancreatic Secretory Trypsin Inhibitors

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<th>Calculated value</th>
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<th>B2</th>
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<th>A2</th>
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<td></td>
<td>22 hrs 44 hrs 66 hrs</td>
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<tr>
<td>Step 2</td>
<td>Asx Ser</td>
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</table>

a Average of two determinations; deviation from average was ±3% or less.
b Average of 22-, 44-, and 66-hour hydrolysates unless otherwise indicated.
c Value obtained by extrapolation to intercept at zero time.
d Twenty-two-hour hydrolysate of performic acid oxidized protein.
e The histidine, methionine, and tryptophan content was 0.02 residue per molecule or less.
f Determined by subtractive Edman degradation of S-2-aminoethylated inhibitor.

Amino Acid Composition—The amino acid compositions of the five chromatographic forms of the human inhibitor isolated from pancreatic juice are presented in Table I. Data for 22-, 44-, and 66-hour hydrolysates are given for B1, and the results obtained for 22-hour hydrolysates are given for the remaining forms. Glucosamine and galactosamine were not detected in acid hydrolysates under conditions where 0.05 mole per mole of peptide would have been detected. This indicates that the inhibitors are not glycopeptides. The analytical data for all the amino acids in the five forms are in good agreement with integral molar ratios which, in combination with the absence of methionine, histidine, tryptophan, and hexosamines, may be taken as strong evidence for the homogeneity of these polypeptides. All five forms of the inhibitor prepared from juice and tissue have the same amino acid composition. The minimum chemical molecular weight calculated on the basis of 56 amino acid residues per molecule is 6242.

The NH₂-terminal sequence of the major forms of the inhibitor, B1, B2, A1, determined by subtractive Edman degradation of S-2-aminoethylated inhibitors is also given in Table I. The NH₂-terminal sequence Asx-Ser was common to the three chromatographic forms.

Amide Content of Multiple Chromatographic Forms—The amide content of the A2, B1, and B2 forms of the inhibitor was determined in order to examine the possibility that differences in amide content may be responsible for the multiple chromatographic forms. Enzymatic hydrolysis was employed rather than the chemical total amide method because it provides information on the amides of both aspartic and glutamic acids. The inhibitor forms were converted to the S-2-aminoethylated derivatives in order to achieve total enzymatic hydrolysis (19, 25). The data presented in Table II show that total enzymatic hydrolysis was achieved with recoveries of 88 to 100%. The amino acids not given in the table were within ±5% of the values found for acid hydrolysates. Tyrosine values have been included in Table II to supplement the data from acid hydrolysates which indicated approximately 2.5 moles per mole of peptide (cf. Table I).

The glutamic acid and glutamine values are 4 and 2 moles per mole of peptide, respectively, for all three forms, accounting for the 6 glutamic acid residues recovered in the acid hydrolysates. Form A1 contains 3 aspartic and 5 asparagine residues per molecule accounting for all 8 aspartic acid residues in the acid hydrolysates. Although 3 moles per mole of peptide of aspartic acid were recovered from the enzymatic hydrolysates of Form B1, 1 less residue each of asparagine, glycine, and S-2-
TABLE II
Enzymatic hydrolysis of S-2-aminoethylated human pancreatic secretory trypsin inhibitors

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Integer value</th>
<th>A₂</th>
<th>B₁</th>
<th>B₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamic acid</td>
<td>6</td>
<td>3.82</td>
<td>4.00</td>
<td>4.00</td>
</tr>
<tr>
<td>Glutamine</td>
<td>8</td>
<td>2.13</td>
<td>1.96</td>
<td>2.00</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>8</td>
<td>2.88</td>
<td>3.00</td>
<td>3.60</td>
</tr>
<tr>
<td>Asparagine</td>
<td>5</td>
<td>5.00</td>
<td>4.17</td>
<td>4.01</td>
</tr>
<tr>
<td>Glycine</td>
<td>6</td>
<td>4.65</td>
<td>3.93</td>
<td>4.48</td>
</tr>
<tr>
<td>S-2-aminoethylicysteine</td>
<td>6</td>
<td>5.88</td>
<td>4.85</td>
<td>6.00</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3</td>
<td>3.02</td>
<td>3.05</td>
<td>2.98</td>
</tr>
</tbody>
</table>

Yield (moles amino acid/mole peptide)

<table>
<thead>
<tr>
<th>Form</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₂</td>
<td>91%</td>
</tr>
<tr>
<td>B₁</td>
<td>88%</td>
</tr>
<tr>
<td>B₂</td>
<td>100%</td>
</tr>
</tbody>
</table>

aAminoethylcysteine was recovered. Form B₂ yielded 0.6 residue of aspartic acid more than either Form A₁ or B₁, and like B₁, contained 1 residue loss of asparagine than A₂. The glycine and S-2-aminoethylycysteine content of B₂ was similar to that found for Form A₂.

These data show that the three chromatographic forms of human PSTI differ in amide, free carboxylic acid content, and have different susceptibility to enzymatic hydrolysis with respect to the release of aspartic acid, glycine, and S-2-aminoethylcysteine. The data suggest the possibility of deamidation at Asx-Gly sequences involving imide formation followed by hydrolysis to α and β aspartic acid peptide bonds (37). This is supported by the existence of the following sequences in homologous inhibitors: Asp²-Gly⁴ in bovine and porcine PSTI and Asn⁴-Gly⁴-Cys⁸ in bovine inhibitor. During the sequence determination of these inhibitors peptides containing these sequences were isolated as multiple chromatographic forms and some were not susceptible to enzymatic hydrolysis (19, 25, 38). However, in contrast to the results presented here for human inhibitors B₁ and B₂, it should be noted that it was possible to obtain complete enzymatic digestion of the S-2-aminoethylated derivatives of the bovine (19) and porcine (25) inhibitors as was obtained here for Form A₂.

Ultracentrifugation—The B₁ form of the human PSTI behaved as a homogeneous, ideal solute when examined by equilibrium ultracentrifugation. The Z-average molecular weight from experiments conducted at three loading concentrations, 0, 0.5 mg per ml; 0, 1.0 mg per ml, and 0, 2.0 mg per ml. The values of local concentration are given on the abscissa. The ordinate gives the corresponding Z-average molecular weight.

Fig. 8. Z-average molecular weight of human pancreatic secretory trypsin inhibitor B₁ determined by equilibrium ultracentrifugation at 60,000 rpm. The initial loading concentrations were: 0, 0.5 mg per ml; 0, 1.0 mg per ml, and 0, 2.0 mg per ml. The values of local concentration are given on the abscissa. The ordinate gives the corresponding Z-average molecular weight.

Inhibition of Trypsin and Chymotrypsin

The stoichiometry of the interaction between inhibitor and bovine trypsin has been estimated by using the specific activity of trypsin and the molecular weights of trypsin and the inhibitor (17). On this basis a 1:1 molar complex is equivalent to an inhibitor specific activity of 1790. The specific activity of the inhibitor in the SP-Sephadex column effluents was 1700 ± 100.

No significant differences in specific activity were detected among the chromatographic forms of human PSTI react with bovine trypsin on a 1:1 molar basis and may be considered homogeneous by this criterion. This result is in cell (cf. Fig. 8). The measured molecular weight was 6300 ± 200. This value is in good agreement with the minimum chemical molecular weight 6242 calculated from the amino acid composition and results of SDS-gel electrophoresis.

Ultraviolet Spectra—The ultraviolet absorption spectra of inhibitors B₁ and B₂ are identical and consistent with the absence of tryptophan in the molecules. The molar absorptivity, ε₁cm³ (10 mg per ml) is 8.4 for both forms of inhibitor. The spectra were essentially the same in shape as that previously reported for bovine PSTI (cf. reference 17) (Fig. 8) with differences due to the fact that human inhibitor contains 3 moles of tyrosine per mole of peptide rather than 2 for the bovine.
agreement with the 1:1 molar ratios observed for the interaction of bovine trypsin with bovine (17) and porcine (18) pancreatic secretory trypsin inhibitors and other polypeptide trypsin inhibitors (cf. Laskowski and Sealock (1)).

The data given in Fig. 9 show that B1 effectively inhibits human cationic trypsin (X), bovine trypsin (O), and porcine trypsin (●). There is a linear relationship between the extent of trypsin inhibition and the concentration of inhibitor until 90 to 95% of the TAME esterase activity has been abolished. The titration curves show the "residual" trypsin activity of 3 to 5% when inhibitor is in excess, a phenomenon observed previously for bovine and porcine secretory trypsin inhibitors (17, 18, 39). The differences in the slopes of the titration curves cannot be explained at this time. These differences may be due to heterogeneity in the trypsin preparations which are reflected when TAME hydrolysis used here rather than an active center titration is employed to measure the amount of active trypsin (1), or they may be characteristic of the inhibitor-trypsin interaction.

The specific activity for the inhibitor with bovine trypsin calculated from the data given in Fig. 9 is lower than that observed during the preparation of the inhibitor. This is due to loss of activity during lyophilization. It has been shown that cationic human trypsin is not effectively inhibited by bovine or porcine pancreatic secretory trypsin inhibitors (8, 11, 16). The data presented in Fig. 9 demonstrate that the human inhibitor, in contrast to the homologous bovine and porcine inhibitors, is capable of effectively inhibiting cationic human trypsin.

Human pancreatic trypsin inhibitor did not effectively inhibit the hydrolysis of ATEe by bovine chymotrypsin. When incubated with 20-fold molar excess of human PSTI, the chymotrypsin activity was inhibited 35%.

**DISCUSSION**

The trypsin inhibitor activity present in human pancreas and pancreatic juice has been studied by several groups of investigators (28, 40-44). The inhibitor activity has been identified as a Kazal-type inhibitor on the basis of its inability to inhibit chymotrypsin and kallikrein (28, 41) and because it exhibits temporary inhibition (28, 43). The inhibitor in whole pancreatic juice (44) or after extraction from juice by trichloroacetic acid treatment (28) behaved as two electrophoretic components. Although partially purified inhibitor preparations have been obtained by several groups, none of these preparations were sufficiently homogeneous for detailed chemical, enzymatic, or structural studies of the kind carried out on pancreatic secretory trypsin inhibitors from other species.

In this paper we document the isolation of the trypsin inhibitor from the pancreatic juice of one individual, and in a separate preparation, from a pool of post mortem pancreata of approximately 40 individuals. The inhibitor was isolated in the free form (not complexed with trypsin) by gel filtration on Sephadex G-75 and purified by ion exchange chromatography on DEAE-cellulose and SP-Sephadex using procedures developed for the isolation of trypsin inhibitors from bovine (17) and porcine (18) pancreatic juice.

The inhibitor was isolated as five different chromatographic forms which were the same from both pancreatic juice and tissue. These multiple forms had identical amino acid compositions after acid hydrolysis and specific activity with respect to trypsin. The minimum molecular weight for each form determined by amino acid analysis, SDS-gel electrophoresis and the stoichiometry of interaction with trypsin agreed well with the value 6300 ± 200 determined for B1 by ultracentrifugation. Forms B2, B3, and B4 had Asx-Ser as the NH2-terminal residues. The major differences demonstrable between the multiple forms were in amide content and susceptibility to enzymatic digestion associated with an Asx-Gly sequence. The available evidence suggests that amide differences and probably the presence of β-aspartyl peptide bonds derived from cyclic imides of aspartic acid account for the multiple chromatographic forms observed for human PSTI. Definite structures, however, have not been assigned to the individual forms. Many examples of Asx-Gly rearrangements have been described in the peptide literature and recently Bornstein (37) has presented evidence that cyclic imides of aspartic acid exists in intact collagen. Multiple chromatographic forms of bovine PSTI were observed during isolation from pancreatic juice (17) and tissue (19). M. Laskowski Sr. (reported in Reference 5) has isolated a form of bovine PSTI from tissue differing in one full charge unit from the form of bovine PSTI isolated and sequenced in our laboratory. Laskowski and Sealock (5) have reported that these two forms of bovine PSTI could not be distinguished on the basis of kinetic or thermodynamic measurements of peptide bond hydrolysis of the reactive site. Other explanations for the existence of multiple chromatographic forms of proteins which have been documented for pancreatic secretory proteases are carbohydrate heterogeneity (45-48) and allotypic variation (49). The first can be ruled out because of the absence of hexosamines in human PSTI. The second could be explored only by amino acid sequence determination of the individual forms which is not possible at this time because of the limited amounts of material available.

The availability of human PSTI as a homogeneous polypeptide will now permit the amino acid sequence determination of the inhibitor. This will provide information for examining the structural basis of the species differences observed in the pancreatic secretory trypsin inhibitor-trypsin system. Knowledge of the properties of human PSTI and its use as the basis of a radioimmunoassay should prove useful in the study of human pancreatitis.

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**REFERENCES**
