Trypsin Inhibitor from Bovine Pancreatic Juice*

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SUMMARY

A trypsin inhibitor has been isolated in 54% yield from bovine pancreatic juice by gel filtration on Sephadex G-75 at pH 8.1 and by elution chromatography on DEAE-cellulose at pH 9.0. It appears to be homogeneous by equilibrium chromatography, equilibrium sedimentation ultracentrifugation, and amino acid analysis, and on the basis of the stoichiometry of its interaction with trypsin. The polypeptide inhibitor has a molecular weight of 6155 and has the following amino acid composition: Asp, Thr, Ser, Glu, Pro, Gly, Ala, Cys, Val, Met, Ile, Leu, Tyr, Lys, and Arg.

The inhibitor is secreted in the pancreatic juice in the free form (not in complex with trypsin) and it prevents the trypsin-catalyzed activation of the proteolytic zymogens. The amount of inhibitor is equivalent to 1% of the total potential trypsin in pancreatic juice.

Although two trypsin inhibitors have been isolated from acid extracts of the gland, only one (Kazal type) is present in the secretion. This suggests that the inhibitors are segregated at the subcellular level in the pancreatic acinar cells.

The acinar cell of the exocrine pancreas elaborates the proteolytic enzymes as inactive precursors (zymogens) and segregates the secretory protein to prevent autodigestion. The secretory protein is kept separate from the cytoplasm immediately after its synthesis and for the remainder of the secretory cycle by intracellular membrane systems, i.e. the endoplasmic reticulum (during intracellular transport), the Golgi complex (during concentration), the zymogen granule membrane (during storage), and the cell membrane (after extrusion) (1, 2). The effectiveness of this interposition device depends primarily on the impermeability of the membranes to the secretory proteins and on the resistance of the membranes to lipase activity. Trypsin plays the central role in the zymogen mechanism because the activation process consists of one or more trypsin-catalyzed partial proteolysis reactions (3). It is clear that the stability of the entire zymogen system depends on limiting the concentration of trypsin to a minimum since trypsinogen represents more than 20% of the bovine secretory protein (4-6) and trypsinogen activation is autocatalytic.

Trypsin inhibitors have been isolated from acid extracts of pancreas (7, 8), and inhibitor activity has been demonstrated in pancreatic juice (9, 10). Kunitz and Northrop (7) isolated a basic trypsin inhibitor from acid extracts of bovine pancreas as a crystalline 1:1 molar complex with trypsin. The interaction of this inhibitor with trypsin has been studied extensively as an example of specific protein-protein interaction (cf. 11-14). The complete amino acid sequence of the basic inhibitor has been determined (15-20) and it has recently been shown to be identical with the kallikrein inactivators from bovine lung (21) and bovine parotid gland (22, 23). Kazal, Spicer, and Brahinsky (8) isolated a mixture of three anionic trypsin inhibitors from acid-alcohol extracts of 8 tons of bovine pancreas. This preparation was readily distinguishable from the basic inhibitor of Kunitz by differences in isoelectric point and inhibitor properties. However, detailed chemical investigations were not pursued because of the limiting amounts available.

We have isolated and characterized a trypsin inhibitor from bovine pancreatic juice of the anionic type described by Kazal et al. (8).

EXPERIMENTAL PROCEDURE

Collection of Pancreatic Juice

Bovine pancreatic juice was collected by direct cannulation of the main pancreatic duct (24). Cannulation does not completely deprive the animal of the secretion because the cow has a functional accessory duct system (25). The daily secretion, 4 to 8 liters of pancreatic juice containing 0.5 to 2% protein, was collected into bottles at 4°. Four 5-ml aliquots were frozen immediately for the later determination of enzyme and zymogen concentrations, and the remainder was treated with DFP (0.1 m isopropyl alcohol, 10 ml per liter of juice) for 1 hour with continuous stirring, and was then lyophilized. The animal was maintained on a normal ration and was permitted water ad libitum. Electrolyte loss was compensated by the addition of 30 g each of NaCl and NaHCO3 to the feed.

Determination of Enzyme Activity

Trypsin—The rate of hydrolysis of p-toluenesulfonyl-L-arginine methyl ester was determined at pH 7.8 and 25° in a pH-

1 The abbreviations used are: DFP, diisopropyl phosphofluoridate; TAME, p-toluenesulfonyl-L-arginine methyl ester; ATE, acetyl-L-tyrosine ethyl ester.

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were added to 1.0-ml aliquots of each fraction and the tubes were stored at 4°. The solutions were assayed for ATE and TAME activity at 0, 24, and 48 hours after addition of trypsin.

**Preparation of Material for Amino Acid Analyses**—The column effluents containing inhibitor were lyophilized and the concentrated solutions were dialyzed in 23/22 NoJax Visking cellulose casings at 4° for 42 hours against two changes of 0.1 M KCl, and for an additional 52 hours against four changes of deionized water (27). The material retained was lyophilized and stored at -20°. Samples of 1 to 1.5 mg of protein were hydrolyzed in an evacuated, sealed tube with 1 ml of twice distilled, constant boiling HCl. The tubes were held at 110 ± 0.2° for 22 and 52 hours.

Amino acid analysis of the hydrolysates was performed according to the method of Spackman, Stein, and Moore (28). The long column was run for 30 min after the elution position of phenylalanine to permit observation of glucosamine. The cystic acid content of a sample oxidized by the method of Moore was determined on a 22-hour hydrolysate (29). The ammonia content was determined by correcting the observed value for traces of ammonia in acid and buffers as well as for serine and threonine decomposition. The correction amounted to less than 15% of the observed value.

**Ultraviolet Absorption Spectra**—A Zeiss PMQ-II spectrophotometer was used for absorbance measurements. Absorbance indices, A°T (1% solution), were selected from the literature: bovine pancreatic juice, 18.0 (3); trypsin, 14.4 (30); ribonuclease A, 6.98 (31); basic trypsin inhibitor, 11.98 (15); Kazal's trypsin inhibitor, 6.5 (8); bovine pancreatic juice trypsin inhibitor, 5.89 (this study). Absorption spectra were determined with a Cary recording spectrophotometer, model 14, at 24°. Acetyltirosinamide from Cycle Chemical Company (Lot R 4581) (Dumas nitrogen; found, 12.41% (theory, 12.60%); εTyrosine) was recrystallized from ethanol before use (32). Protein concentrations for spectral studies were determined from the 22-hour acid hydrolysates by amino acid analysis in the usual manner.

**Disc Electrophoresis**—Electrophoresis was carried out in 15% acrylamide by the method of Reisefeld, Lewis, and Williams (33) at pH 8.0 as described by Davis (24) at 4 mA per tube for 120 min at 4°. After fixing and staining with Amido Schwarz in 7% acetic acid excess stain was removed by diffusion. The gels were photographed by transmitted light and the negative was examined by densitometry. The data were corrected for the film response (Kodak Super XX) to calibrated variable light intensities.

**Equilibrium Ultracentrifugation**—The Spinco model E analytical ultracentrifuge was equipped with a scanning photoelectric absorption system and Rayleigh interference optics. The analytical runs were made in a three-channel Rexelite aluminum-epoxy cell of 3-mm column height at 30,000 rpm at 22°. Solutions containing 0.085, 0.0425, and 0.017% protein were examined at pH 4.28 (0.01 M CaCl₂, 0.0158 M KCl, 0.005 M citric acid-NaOH) and at pH 7.81 (0.01 M CaCl₂, 0.026 M KCl, and 0.005 M Tris-HCl). Weight average molecular weights were obtained from plots of the logarithm of observed absorbance as a function of the square of the radius. The interference optics were used to obtain z-average molecular weights by a modification of Method II of Van Holde and Baldwin (35).
Preparation of Inhibitor

Gel Filtration on G-75—Sephadex G-75, bead form, was swollen in 50% acetic acid and the fines were removed by decantation. Lyophilized pancreatic juice was dissolved in distilled water containing $10^{-4} \text{M} \text{DFP}$ to a final concentration of 1.5 to 2% protein. The solution, 600 to 800 ml, was applied to a Sephadex G-75 column, 7.6 x 175 cm, previously equilibrated with 0.5 m KCl, 0.01 m Tris-hydrochloride, and $10^{-4} \text{M} \text{DFP}$ at pH 8.1. A flow rate of 4.8 cm hr$^{-1}$ was reduced to 2.6 cm hr$^{-1}$ in 2% protein. The solution, 600 to 800 ml, was applied to a water containing $10^{-4} \text{M} \text{DFP}$ solution. Lyophilized pancreatic juice was dissolved in distilled water containing 10% acetic acid and the fines were removed by decantation. Lyophilized and submitted again to gel filtration for a second time on the same column (Fig. 1, bottom). Effluent tubes with more than 12 specific activity units were pooled, lyophilized, and combined with Fraction 1. This combined pool is denoted "low molecular weight fraction."

Chromatography on DEAE-cellulose—DEAE-cellulose was treated with m KCl followed by 0.1 N KOH-0.25 KCl until the filtrate was colorless. After washing with 0.025 m KCl and 0.02 m Tris-hydrochloride, the resin was equilibrated by suspending the basic form in 5 volumes of 0.025 m KCl and 0.020 m Tris-hydrochloride, pH 9.0, and correcting the pH to 9.0. A column, 7.6 x 40 cm, was poured (36) and was washed with 0.025 m potassium chloride, 0.01 m Tris-hydrochloride, and 0.1 m Tris-hydrochloride, the resin was equilibrated by suspension in 0.5 m Tris-HCl, 1 X $10^{-4}$ m diisopropyl phosphofluoridate at pH 8.1, 4°C. Sample, 30 $A_{280} \text{ml} \times 900 \text{ml}$. $\bullet \longrightarrow$, absorbance at 280 nm; $\circ \longrightarrow \circ$, trypsin inhibition (TAME hydrolysis). Bottom, gel filtration of effluent corresponding to Region 2 (top) derived from 25 preparative columns. Conditions are the same as indicated for the top. Sample, 20 $A_{280} \text{ml} \times 700 \text{ml}$. Note change of scale for comparison with the top.

RESULTS

Isolation of Trypsin Inhibitors

Low Molecular Weight Fraction Obtained by Gel Filtration on Sephadex G-75—A typical elution profile of lyophilized bovine pancreatic juice on Sephadex G-75 is presented at the top of Fig. 1. The inhibitor activity $\bullet$ is retarded relative to most of the secretory protein (○). The recovery of inhibitor activity was $100 \pm 5\%$.

When DFP was omitted from the eluting buffers, the recovery of inhibitor activity remained high but there was evidence for the activation of the zymogens of carboxypeptidase A, carboxypeptidase B, chymotrypsin, and trypsin after the fractions had been collected.

The fast side of the inhibitor activity peak, Region 2, was submitted to gel filtration for a second time on the same column (Fig. 1, bottom). It had the same retention volume as the inhibitor activity in Region 1.

The designation low molecular weight fraction has been arbitrarily applied to a pool of material derived from several preparative columns corresponding to Region 1 (Fig. 1, top) and tubes 97 to 112 (Fig. 1, bottom). The protein composition of the low molecular weight fraction was studied by means of quantitative enzyme activation and assay after analytical scale gel filtration on G-75 (cf. Fig. 2). The retention volumes of the enzyme activities were the same as on the preparative columns but the resolution was improved. Tryptsinogen was eluted in the same position as chymotrypsin.

Activation by trypsin was required for ATE and TAME activity even when CaCl$_2$ was added immediately after collection of the fractions. There was no increase in enzyme activity after acidification. This procedure would dissociate enzyme inhibitor complexes of the Kunitz or Kazal type if present (11).

The recovery of protein, ribonuclease, and inhibitor activity was quantitative. Of the total protein in the fraction, 15.7% could be accounted for in the following manner: 14% ribonuclease, 0.5% chymotrypsinogen A equivalents, and 0.16% trypsinogen. The weight assignments are based on activity determinations and the following specific activities: trypsin, 410 units per mg; chymotrypsin A, 600 units per mg;
ribonuclease A, 3 units per mg; trypsin inhibitor, 1600 units per mg.

**Elution Chromatography on DEAE-cellulose**—The elution diagram presented in Fig. 3B is typical of those obtained on both a preparative and analytical scale. After the sample was applied to the column, the chromatogram was developed by “elution analysis” (37), i.e., eluent composition was held fixed before, during, and after the development of the first activity peak. The inhibitor activity was quantitatively recovered in two regions. The major fraction, tubes 20 to 30, accounting for 86% of the activity (○), was eluted after 5 column volumes of buffer had passed through the column. The elution position varied between 5 and 7 column volumes depending on the particular lot of DEAE-cellulose.

A second peak of inhibitor activity and “other proteins” (△) were eluted when buffer containing 0.025 M KCl and 0.020 M Tris-hydrochloride, pH 9.0, was applied to the column. When 1.0 M KCl was applied, protein without inhibitor activity was eluted. The A of Fig. 3A refer to effluent conductivity. When the second peak was rechromatographed under similar conditions, 60% of the inhibitor activity was eluted in the same position (tubes 80 to 85), but 40% was eluted in the position corresponding to the major peak (tubes 20 to 30).

When the effluent corresponding to tubes 20 to 30 in Fig. 3B (specific activity, 2000) was rechromatographed, inhibitor activity was recovered in a single peak of constant high specific activity, 2780.3

**Amino Acid Composition**

The amino acid composition of the pancreatic juice trypsin inhibitor is presented in Table I. Phenylalanine, histidine, tryptophan, and glucosamine were not detected in any acid hydrolysate. The low sensitivity of the analytical data for tryptophan is due to the small sample size and the acid lability of this amino acid. The absence of tryptophan in the sample

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3 Inhibitor with a specific activity of 2750 has been prepared in the pH 9.0 system. The amino acid compositions of inhibitor prepared at pH 6.5 and pH 9.0 were identical.
has been confirmed by spectrophotometric procedures (cf. "Ultraviolet Spectra" below). The integral molar ratios of the constitutive amino acids and the inability to detect phenylalanine, histidine, and tryptophan may be taken as strong evidence for the homogeneity of the polypeptide.

The amino acid composition of the basic trypsin inhibitor is also presented in Table I. Although both bovine pancreatic trypsin inhibitors have high cystine content and are devoid of histidine and tryptophan, there is little other similarity in their amino acid composition.

**Ultraviolet Spectra**

A time-dependent change in the ultraviolet absorption spectrum was observed when pancreatic juice trypsin inhibitor was dissolved in 0.1 N NaOH (Fig. 6). Vestigial "acid" peaks of tyrosine at 278.0 and 284.7 nm were apparent even after 3 min. The 294.5 nm phenolate ion peak increased with time and reached a maximum value between 46 and 70 hours. There was no evidence for the 281 or 288 nm peaks present in the alkaline spectrum of tryptophan (cf. Fig. 7). A 6-min incubation with 8 M urea in 0.1 N NaOH removed most of the time dependence from the tyrosine ionization.

The shoulder between 320 and 330 nm formed during alkaline treatment was present in the 70-hour sample after neutralization to pH 6.9. The chromophore responsible for this shoulder is unknown but it may be related to the peak in this region observed after the tyrosinase-catalyzed oxidation of peptides and proteins (38-40).

Tyrosine and tryptophan were estimated by the spectrophotometric method of Goodwin and Morton (41) on a sample after 70-hour exposure to 0.1 N NaOH. The absorbances at 280.0 and

![Fig. 5. Chromatography of pancreatic juice trypsin inhibitor on DEAE cellulose at pH 6.5. The column was 1.8 X 71 cm in 0.001 M potassium phosphate at 4°C. Sample, 3.2 A_280 radiation X 6.1 ml.](image)

![Fig. 6. The change in absorption spectra of 0.99 X 10^-4 M pancreatic juice trypsin inhibitor with time in 0.1 N NaOH. The 6-min urea treatment was in 8 M urea. All other curves are in the absence of urea.](image)
WAVELENGTH, Å

Fig. 7. Absorption spectra in 0.1 N NaOH. Absorption curves: —, pancreatic juice trypsin inhibitor, $0.99 \times 10^{-4}$ M, 46 hours; --, acetyl-L-tyrosinamide, $2.07 \times 10^{-4}$ M.

294.4 $\mu$m were corrected for scattering by extrapolation of the absorbance between 370 and 450 $\mu$m. The tyrosine and tryptophan content was found to be 2.5 and 0.093 residue per molecule, respectively, in good agreement with amino acid analysis (cf. Table I).

Since the inhibitor contains tyrosine as the only chromophore absorbing to any appreciable extent above 250 Å, we have compared its spectrum to those of N-acetyl-L-tyrosine amide (Figs. 7 and 8). With the exception of peptide bond end absorption, the spectrum of the inhibitor was similar to that of the model compound.

The molar absorbance indices, $e_{280}$, were 3630 and 1200 at 280 $\mu$m for inhibitor and acetyltyrosinamide, respectively. The value at the maxima was 4090 at 276.1 $\mu$m and 1400 at 274.4 $\mu$m for these compounds. The ratio of absorbance at 280 $\mu$m compared to 260 $\mu$m was 1.42 for the inhibitor.

The observed molar absorbivity, $e_{280}$, 3800 (corrected for scattering), may be compared to that calculated by adding the contribution of the chromophores: 2(acetyl-L-tyrosinamide), $2 \times 1400 (\lambda_{max}) = 2800$, plus 3(cystine), $3 \times 150 (280 \mu m) = 450$ (42). The difference of 550 ($\Delta e$ at $\lambda_{max}$) between the observed value, 3800, and the calculated value, 3250, may be attributed to the increased absorbance of "buried" tyrosine residues. When the absorption of native ribonuclease is compared with that of a total peptic digest in the same manner a difference of 600 (for three buried tyrosines) is obtained (49). Similarly, a value of 200 to 250 per tyrosine residue may be calculated from the data of Laskowski (44) and Warrington and Laskowski (45).

**Acrylamide Gel Electrophoresis**

Fig. 9 shows the result obtained when 140 μg of the pancreatic juice trypsin inhibitor were subjected to gel electrophoresis in 15% acrylamide gel. More than 95% of the material migrated as a single component.

**Molecular Weight Determination**

Weight average ($M_w$) and z-average ($M_z$) molecular weights have been calculated from absorption and interference measurements, respectively. Determinations were made over a 5-fold range of protein concentration. As shown in Fig. 10, all of the log concentration versus $r^2$ plots conform to a straight line as expected for a homogeneous material. Molecular weights were obtained from the slope of the line with the calculated partial specific volume of 0.719. The values of $M_w$ and $M_z$ (each is the result of averaging results for three protein concentrations) were 6020 and 6220 at pH 4.28 and 5920 and 6270 at pH 7.81. The grand average of all the $M_w$ and $M_z$ values is 6100 and the standard deviation, $\pm 260$, is well within the $\pm 8\%$ error estimated for these experiments. The polypeptide could not be distinguished from a homogeneous monomer under the experimental conditions employed.

**Trypsin Inhibitor Activity**

Pancreatic Juice—The direct determination of trypsin inhibitor activity in bovine pancreatic juice is complicated by the presence of trypsinoget. The trypsin activity of the mixture first decreases and then rises sharply (Fig. 11, 0). The increase in activity is due presumably to the autocatalytic conversion of trypsinoget to trypsin. Inhibitor activity was stable for 1 to 2 hours when the partially purified low molecular weight fraction (Fig. 11, △) or pancreatic juice trypsin inhibitor was assayed. The time-dependent liberation of TAMe activity did not interfere with the quantitative determination of inhibitor in pancreatic juice activity provided that the 1- and 2-min time points were taken (Fig. 12). No inhibition of chymotrypsin activity could be demonstrated "temporary" inhibition.

4 Under these conditions, 15 to 30 hours would be required to demonstrate "temporary" inhibition.
Pancreatic Juice Trypsin Inhibitor

There is a linear relationship between the extent of trypsin inhibition and concentration of inhibitor until approximately 95% of the trypsin activity has been abolished (Fig. 13).

Extrapolation of the linear portion of the dose response curve yields $7.0 \times 10^{-7}$ M for inhibitor required to inactivate $0.90 \times 10^{-4}$ M "active" trypsin. Similarly, the highest specific activity observed for column effluents, 2,780, corresponds to an inhibitor to trypsin molar ratio of 0.97 when 23,800 and 6,155 are taken as the molecular weights of trypsin and inhibitor, respectively. These results are in good agreement with the 1:1 molar ratio usually observed for polypeptide trypsin inhibitors (13, 15, 46).

A residual activity of 3 to 4% persists even when the molar ratio of inhibitor to trypsin is 2.0. When this ratio is equal to 7, the residual activity is 2%. Experiments are currently under way to distinguish between two possible explanations for residual activity when the molar ratio of inhibitor to trypsin is greater than 1.0. (a) The inhibitor-trypsin complex has activity equiva-
respect to trypsinogen was achieved and the fraction contained no detectable activity toward TAMe or ATE. The initial fraction presented by Finkenstadt and Laskowski (49), gel filtration on G-75 in the presence of DFP at the pH of pancreatic juice commercial preparations and the recent evidence for peptide bond cleavage during trypsin-soybean trypsin inhibitor complex formation by molecular size also permitted the isolation of a new component of the secretion rather than the Kazal inhibitor (54); and (e) temporary inhibition (52). Grossman (9) identified the inhibitor from rat pancreatic juice as a Kazal type on the basis of criteria d and e.

The inhibitor concentration in pancreatic juice is 0.6 mg per g of protein. This corresponds to $10^{-4}$ m in an average pancreatic juice that contains 10 mg per ml of protein. It is present as a free monomer and does not interact with other proteins in the mixture under the conditions of gel filtration examined. The role of the inhibitor in preventing the trypsin-mediated activation of zymogens was demonstrated by the activation of the proteases after the inhibitor was removed during gel filtration in the absence of DFP.

The secretion of inhibitor was correlated with the protein content of the juice and not with the secretion of water. This confirms the observations of Kalser and Grossman (47) on canine pancreatic juice and supports their conclusion that the inhibitor is a constituent of the protein secretion that is synthesized and secreted by the pancreatic cells. It is reasonable to assume that the Kazal-type inhibitor is present in zymogen granules, i.e., the intracellular storage sites for the pancreatic secretion (4, 6), and one may anticipate that there is segregation of the two types of trypsin inhibitors at the subcellular level.

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Inhibitor activity in tubes 131 and 132 is probably an artifact due to eluting the column with 1 M KCl. 

Note—Kazal-type trypsin inhibitors have recently been isolated from human, dog, pig, and cow pancreatic juice (55, 56). An acidic trypsin inhibitor with an amino acid composition similar to the one reported in this paper has been isolated from bovine pancreas by Burk, Cerwinsky, and Grinnan (Abstracts of the American Chemical Society Meeting, New York, September 1966, No. 272).
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