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 a 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 secretary protein to prevent autodigestion. The secretary 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 secretary 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 DFP¹ (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-toluenesulfonfyl-L-arginine methyl ester was determined at pH 7.8 and 25° in a pH-
stat (Radiator Model T114c, SHR-3e, ABU-1a, 0.25-ml piston). Four ml of a solution containing 0.01 M TAME, 0.1 M KCl, 0.02 M CaCl₂, 0.005 M Tris hydrochloride, and 1 ml of enzyme were used for each assay. The rate of hydrolysis was essentially linear with respect to time for at least the first 40% of the reaction; a linear relationship between initial rate and trypsin concentration was observed within the range of 2 to 20 μmoles per min. The standard deviation of replicate determinations was 1 and 2%. Activity was expressed as micromoles of TAME hydrolyzed per min.

In order to determine the amount of active enzyme in our trypsin preparations we have used the spectrophotometric benzoylarginine methyl ester assay and the specific activity reported by Kassell et al. (15) for chromatographically purified trypsin which combined stoichiometrically with homogeneous basic trypsin inhibitor. The specific activities of two trypsin preparations were determined and the percentage of active trypsin was calculated by dividing the observed specific activity by the value reported by Kassell et al. (51 units per mg). On this basis 100% active trypsin has a specific activity of 410 ± 34 units per mg in the titrimetric assay used in this study.

Trypsin Inhibitor—A stock trypsin solution (Worthington TRL 6257 or TRL 6261) containing approximately 1 mg per ml in 0.01 M KCl-HCl at pH 2.9, stored at 4° for 2 to 3 weeks, was used to prepare fresh trypsin daily. The standard trypsin solution contained approximately 10 units per ml in 0.1 M KCl, 0.015 M Tris, 0.020 M CaCl₂, and 0.005 M Tris hydrochloride at pH 7.8. No decrease of enzyme activity was observed when the trypsin standard was stored for 2 hours at 25° or 8 hours at 4°. The trypsin standard was incubated with the test solution (usually 1 ml of each) at 25° for 3 min and the activity remaining was determined on 1 ml of the incubation mixture. The difference in activity between the test solution and the diluted control is taken as trypsin inhibition. Inhibitor concentration was adjusted so as to inhibit 25 to 75% of the trypsin. The enzyme inhibitor complex was not dissociated by the large excess of substrate for at least the first 5 min of the assay. One inhibition unit is the amount of inhibition that caused the reduction of TAME hydrolysis by 1 μmole per min. Inhibitor specific activity is defined as inhibitor units per A₄₁₆₅⁰₆₅°.

Chymotrypsin—The rate of hydrolysis of 0.01 M acetyl-L-tyrosine ethyl ester in 0.1 M KCl, 0.020 M CaCl₂, and 0.005 M Tris hydrochloride containing 1% v/v acetonitrile was measured at pH 7.8 at 25° in a pH-stat as described previously for trypsin. A linear relationship existed between initial rate (first 20% of reaction) and chymotrypsin A concentration within the range of pH 7.8 at 25° in a pH-stat as described previously for trypsin. Activity was expressed as micromoles of TAMe hydrolyzed per min (initial rate).

Ribonuclease—The hydrolysis of cytidine 2',3' cyclic phosphate (Schwarz, Lot 6301) was measured at 256 μa by means of a Beckman DB spectrophotometer fitted with a logarithmic conversion circuit and potentiometric recorder. The reaction was measured at pH 7.0 at 25° by the procedure of Murdock et al. (26). One unit of activity is the change in absorbance at 256 μa per min (initial rate) with a 1-cm light path.

Activation of Trypsinogen and Chymotrypsinogen (cf. Fig. 2)—As soon as possible after fractions were collected, 100 μl of 0.5 M CaCl₂ were added to each even numbered tube. Ten microliters of a trypsin solution (1 mg per ml in 0.1 M KCl, pH 2.9) 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 23/32 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, A₁₆₅° (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°. Acetyltyrosinamide from Cycle Chemical Company (Lot R 4898) (Dumas nitrogen; found, 12.41% (theory, 12.6%); ε₄₅₃°, 1100 was used without further purification. Reagent grade urea (Mallineckrodt) 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 (34) 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 B analytical ultracentrifuge was equipped with a scanning photoelectric absorption system and Rayleigh interference optics. The analytical runs were made in a three-channel Revelite 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 90% 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 × 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 the presence of the protein sample. Effluent was collected in 60-ml fractions. Gel filtration and all subsequent manipulations were carried out at 2-4°C.

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The solution, 600 to 800 ml, was applied to a Sephadex G-75 column, 7.6 × 175 cm, previously equilibrated with 0.5 m KCl, 0.01 m Tris-hydrochloride, and $10^{-4} \text{m} \text{DFP}$ at

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Effluent fractions with an inhibitor specific activity in excess of 5 (Fig. 1, Region 1) were pooled and lyophilized. The effluent corresponding to Region 2 from 25 preparative columns (2.5 to 5 specific activity units) was lyophilized and submitted again to gel filtration (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 M 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 × 40 cm, was poured (36) and was washed with 0.0025 m

$\text{M}$

$\text{KCl}$ and 0.020 m Tris-HCl, 1 X $10^{-4}$ M $\text{Tris}$-$\text{HCl}$, 0.01 m $\text{Tris}$-$\text{HCl}$, and $10^{-4}$ m $\text{DFP}$ followed by 0.1

For acidification. This procedure would dissociate enzyme inhibitor complexes of the Kunitz or Kazal type if present (11).

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

The fast side of the inhibitor activity peak, Region 1, 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. Trypsinogen 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, 1% trypsin inhibitor, 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; KCl, 0.002 m Tris-hydrochloride, pH 9.0, until the conductivities of the influent and effluent were equal. Effluent pH was usually between 8.5 and 9.0. Buffer was protected from atmospheric carbon dioxide.

In order to equilibrate the low molecular weight fraction for DEAE-cellulose chromatography, 880 g (14,150 $A_{280}$, 121,000 inhibitor units) were suspended in 4 liters of $10^{-4}$ m DFP (0.1% isopropyl alcohol) and dialyzed in 28/32 Nojas Visking casings against distilled water. The material retained was lyophilized and resuspended in 550 ml of distilled water. The suspension was clarified by centrifugation at 13,200 × g for 30 min. The pH and conductivity of the combined supernatants were adjusted to those of equilibrating buffer with concentrated buffers.

Equilibrated sample, 615 ml ($8,550 A_{280}$, 105,000 inhibitor units), was applied to the DEAE-cellulose column, 7.6 × 40 cm. Elution was carried out with equilibrating buffer; flow rates of 540 to 600 ml per hr were achieved at 10 p.s.i. Fractions of 60 ml were collected. At tube 231 and at tube 278 the eluting solutions were changed to 0.025 m KCl, 0.020 m Tris-hydrochloride, pH 9.0, and 1.0 m KCl, respectively.

**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 ($\circ$). The recovery of inhibitor activity was 100 ± 5%. When DFP was omitted from the eluting buffers, the recovery of inhibitor activity remained high but there was evidence for the activation of thezymogens of carboxypeptidase A, carboxypeptidase B, chymotrypsin, and trypsin after the fractions had been collected.

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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, 2670 for 96% of the inhibitor activity (cf. Fig. 4). This preparation of bovine pancreatic juice trypsin inhibitor was used for further physical and chemical studies.

When it was chromatographed at pH 6.5 in 0.001 M potassium phosphate on DEAE-cellulose (Fig. 5), the activity was quantitatively recovered in a single peak of constant high specific activity, 2780.

**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

1 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 m\(\mu\) were apparent even after 3 min.

The 294.5 m\(\mu\) 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 m\(\mu\) 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 m\(\mu\) 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

Table I

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<th>Amino acid composition of bovine pancreatic trypsin inhibitors</th>
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<td>Pancreatic juice inhibitor* (Kazal type)</td>
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<td>22 hrs</td>
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<td>Aspartic acid</td>
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Molecular weight

6155

6513

* Average of two determinations; deviation from average was 1% or less. The values for aspartic acid, glutamic acid, glycine, alanine, valine, and leucine were used as the basis for the calculation of molar ratios of constituent amino acids.

* Kassell et al. (15).

* Average of 22- and 52-hour hydrolysates unless otherwise indicated.

* Fifty-two-hour hydrolysate.

* Value obtained by extrapolation to intercept at zero time.

* Cysteic acid determination on the oxidized protein yielded 6.06 moles.

* Estimated lower limit of detection.

* Determined on 22-hour acid hydrolysate corrected for destruction of \(\beta\)-hydroxyamino acids and ammonia content of solvents.

* The calculated molecular weight is based on 8 amide groups per molecule.
Fig. 7. Absorption spectra in 0.1 N NaOH. Absorption curves: ---, pancreatic juice trypsin inhibitor, $9.90 \times 10^{-4}$ M, 46 hours; ---, acetyl-L-tyrosinamide, $2.67 \times 10^{-4}$ M.

Fig. 8. Absorption spectra at pH 7.8. Absorption curves in 0.1 N KCl, 0.01 M Tris-HCl, pH 7.8. ---, pancreatic juice trypsin inhibitor, $1.32 \times 10^{-4}$ M; ---, acetyl-L-tyrosinamide, $3.50 \times 10^{-4}$ M.

294.4 m\(\mu\) were corrected for scattering by extrapolation of the absorbance between 370 and 450 m\(\mu\). 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 m\(\mu\), 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, \(\epsilon_{\text{cm}}\), were 3630 and 1200 at 280 m\(\mu\) for inhibitor and acetyltyrosinamide, respectively. The value at the maxima was 4090 at 276.1 m\(\mu\) and 1400 at 274.4 m\(\mu\) for these compounds. The ratio of absorbance at 280 m\(\mu\) compared to 260 m\(\mu\) was 1.42 for the inhibitor.

The observed molar absorbity, \(\epsilon_{\text{obs}}\), 3800 (corrected for scattering), may be compared to that calculated by adding the contribution of the chromophores: 2(2(acetyl-L-tyrosinamide), $2 \times 1400 (\lambda_{\text{max}}) = 2800$, plus 3(cystine), $3 \times 150 (280 \mu\text{m}) = 450$ (42). The difference of 550 (\(\Delta\epsilon\) at \(\lambda_{\text{max}}\)) 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 (43). Similarly, a value of 200 to 250 per tyrosine residue may be calculated from the data of Laskowski (44) and Warrington and Laskowski (45). who studied a system composed of ATE and 1.5% Schardinger's dextran. On the basis of this analysis and the time-dependent absorbance change in 0.1 N NaOH, it appears that both tyrosines are buried.

Acrylamide Gel Electrophoresis

Fig. 9 shows the result obtained when 140 \(\mu\)g 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 \(\langle M_w\rangle\) and z-average \(\langle M_z\rangle\) 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 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 trypsinogen. The trypsin activity of the mixture first decreases and then rises sharply (Fig. 11, O). The increase in activity is due presumably to the autocatalytic conversion of trypsinogen to trypsin. Inhibitor activity was stable for 1 to 2 hours when the partially purified low molecular weight fraction (Fig. 11, A) 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

4 Under these conditions, 15 to 30 hours would be required to demonstrate “temporary” inhibition.
be detected when 10 μg of α-chymotrypsin were incubated with 20 mg of bovine pancreatic juice protein.

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 $0.99 \times 10^{-7}$ M “active” trypsin. Similarly, the highest specific activity observed for column effluents, 2,750, corresponds to an inhibitor to trypsin molar ratio of 0.07 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-

![Graph](image)

**Fig. 10.** Molecular weight determination by equilibrium centrifugation. Pancreatic trypsin inhibitor at an initial concentration of 0.085%. The equilibrium distribution of protein was determined by the scanning absorption system after 18 hours at 30,000 rpm and 22°. The ordinate gives the logarithm of concentration (absorbance). On the abscissa are the values of the square of the distance (in centimeters) from the axis of rotation. The lines marked a and b refer to the top and bottom of the cell, respectively. $M_w = M_r = 6,230.$

![Graph](image)

**Fig. 11.** Time course of inhibition of a standard trypsin solution by: ○○, bovine pancreatic juice, $0.99 A_{280}^{2}$; and ▲▲, low molecular weight fraction, $0.24 A_{280}^{2}$. The incubation solution was 0.02 M CaCl$_2$, 0.1 M KCl, 0.015 M Tris-HCl, pH 7.8, at 25°.

![Graph](image)

**Fig. 12.** Inhibition of trypsin by bovine pancreatic juice. Conditions were the same as indicated for Fig. 11. The incubation period was 1 min.
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 structural homologues of pancreatic enzymes isolated from commercial insulin preparations. In view of the reports of alcohol extracts of whole pancreas obtained as by-products of trypsin inhibitor from pancreatic juice. The original procedures for the isolation of bovine trypsin inhibitors involved acid or acid-alcohol extracts of whole pancreas obtained as by-products of commercial preparations. In view of the reports of Plummer and Hirs (48) and Eaker, King, and Craig (31) of structural homologues of pancreatic enzymes isolated from commercial preparations and the recent evidence for peptide bond cleavage during trypsin-soybean trypsin inhibitor complex formation presented by Finkenstadt and Lackowski (40), gel filtration on G-75 in the presence of DFP at the pH of pancreatic juice was employed to separate free inhibitor from an 80-fold molar excess of trypsinogen. A 2000-fold purification of inhibitor with respect to trypsinogen was achieved and the fraction contained no detectable activity toward TAMe or ATE. The initial fraction by molecular size also permitted the isolation of a new class of low molecular weight polypeptides (mol wt, 4,000 to 14,000) not previously observed (4, 5). The fraction accounts for 3.5% of the D11m in bovine pancreatic juice and it probably represents authentic components of the secretion rather than partial proteolysis products. In addition to possible hydrolytic functions these polypeptides may be involved in the absorption and utilization of material by the intestine (50, 51).

When an authentic sample of Kazal’s inhibitor (examined 18 years after isolation) was chromatographed on DEAE-cellulose at pH 9.0 (Fig. 3C), three inhibitor activity peaks (O) were observed. Two of these components have the same chromatographic properties as the peaks of inhibitor activity present in pancreatic juice (cf. Fig. 3B). The number of activity peaks found is consistent with three components shown by free electrophoresis to be present in the Kazal inhibitor (8). The similarity between the Kazal inhibitor and the pancreatic juice inhibitor is further indicated by (a) residual activity (52), cf. Fig. 13; (b) time-dependent spectrophotometric changes of tyrosine spectrum in 0.1 N NaOH (53), cf. Fig. 8; (c) extinction coefficient and tyrosine content (11), cf. Table I; (d) inability to inhibit chymotrypsin (54); and (e) temporary inhibition (52). Grossman (9) identified the inhibitor from rat pancreatic juice as a Kazal type on the basis of criteria a 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 aminar 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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**REFERENCES**


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*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).
Trypsin Inhibitor from Bovine Pancreatic Juice
L. J. Greene, M. Rigbi, D. S. Fackre and With the technical assistance of J. R. Broich


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