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Acidic Bovine Pancreatic Trypsin Inhibitor. I. Purification and Physical Characterization*

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ABSTRACT: A polypeptide trypsin inhibitor has been isolated and purified from a 15% sodium chloride filtrate which is discarded during the commercial preparation of bovine insulin. The inhibitor was isolated by precipitation at 31% sodium chloride, reprecipitation with 0.6 saturated ammonium sulfate, and chromatography on CM-cellulose using pH 5.0 ammonium acetate buffers. The purified inhibitor behaved as a single component on acrylamide and starch gel electrophoresis. Column chromatography, gel electrophoresis, and amino acid content indicated that this inhibitor is more acidic and is distinctly different from the basic Kunitz pancreatic trypsin inhibitor.

The molecular weight of our inhibitor was found to be 6500 ± 300. Phenylalanine, tryptophan, and histidine were found to be absent. Our inhibitor is identical with an acidic pancreatic trypsin inhibitor isolated in much smaller quantities by Kazal *et al.* (Kazal, L. A., Spicer, D. S., and Brahinsky, R. A. (1948), *J. Am. Chem. Soc. 70*, 3034) from a similar side fraction, and later by Greene *et al.* (Green, L. J., Fackre, D. S., and Rigbi, M. (1966), *J. Biol. Chem. 241*, 5610) from pancreatic juice.

Kunitz and Northrup (1936) first crystallized a trypsin inhibitor from bovine pancreas. Kazal *et al.* (1948) isolated a crystalline trypsin inhibitor from a side fraction of the commercial bovine insulin process of Romans *et al.* (1940). Kazal's inhibitor differed from the Kunitz inhibitor in that it was found to be nondialyzable, and its ionic character was of a more acidic nature. The Kazal inhibitor was found to be electrophoretically heterogeneous. The crystalline inhibitor of Kazal was obtained in yields of 12–172 μg/kg of frozen pancreas. Haverback *et al.* (1960) and Keller and Allen (1967) found the Kazal-type inhibitor in human pancreatic juice. Fritz *et al.* (1966a–c) demonstrated the presence of a similar type of inhibitor in human, pig, cow, and dog pancreas and pancreatic juice. Grossman (1958) reported a Kazal-type inhibitor in rat pancreatic juice. Greene *et al.* (1966) found a trypsin inhibitor in bovine pancreatic juice and determined its amino acid content and physical characteristics. This paper presents a very short chromatographic procedure which gives an electrophoretically homogeneous pancreatic trypsin inhibitor in milligram quantities per kilogram of frozen bovine pancreas. The inhibitor isolated by us is identical with the inhibitor isolated by Kazal *et al.* (1948) from frozen pancreas and later by Greene *et al.* (1966) from pancreatic juice.

Materials and Methods

Assay. Trypsin esterase activity was assayed by the spectrophotometric method of Schwert and Takenaka (1955). The inhibitor was assayed by the method of Kassell *et al.* (1963) using the Determatubes TRY (Worthington Biochemical Corp.) as the substrate solution. Crystalline trypsin and crystalline KPTI[1] (Worthington Biochemical Corp.) were dried for 24 hr *in vacuo* over P_2O_5. Dried trypsin (8 μg) in the assay cuvet caused an increase in OD_{253} of 0.099/min. KPTI (1 μg) caused a decrease in the above ΔOD_{253} of 0.055/min. One unit of our inhibitor (APTI) activity is equivalent to the inhibitory activity of 1 mg of this standard KPTI. Specific activity is defined as units

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[1] Abbreviations used: KPTI, Kunitz pancreatic trypsin inhibitor; APTI, acidic pancreatic trypsin inhibitor; FDNB, fluorodinitrobenzene.

per milliliter of APTI divided by the OD_{280} of the inhibitor solution.

Column Chromatography. The chromatographic fractions were monitored at 280 mμ using a Beckman DU spectrophotometer. Flow rates were 15–20 ml/cm² per hr. CM-cellulose (Cellex-CM, Bio-Rad Laboratories) was regenerated by washing with 0.5 M NaOH–0.5 M NaCl for 30 min, followed by repeated water washes until the washes were at neutral pH. The resin was equilibrated with the appropriate buffer by titration with the acid form of the buffer and repeated buffer washing.

Starting Material. The starting material was the 15% NaCl filtrate from the commercial bovine insulin process. This filtrate was obtained by extracting frozen ground pancreas glands with acid–ethanol, filtering, removing the alcohol by vacuum distillation, precipitating the insulin from the resulting solution with 15% NaCl, and filtering (Maxwell and Hinkel, 1954). Frozen pancreas (102 kg) yields 100 l. of 15% NaCl filtrate.

Purification. All operations were performed at 5° unless otherwise noted.

STEP 1. The 15% NaCl filtrate was brought to 31% with solid NaCl (Kazal *et al.*, 1948) and stirred for 30 min. Hyflo Super-Cel was added to make a 1% suspension. The stirring was continued for another 15 min before filtering. The filter cake was extracted three times for 30 min with deionized water. The final volume of the combined extracts after filtering was one-tenth the volume of the 15% NaCl filtrate.

STEP 2. The water extract was brought to 0.6 saturated ammonium sulfate, and the resulting precipitate was allowed to settle overnight. Most of the supernatant fluid was decanted and the precipitate was collected by centrifuging at 10,000*g*. When small-scale preparations were made, the entire suspension was centrifuged. The collected precipitate was dissolved in 0.01 M ammonium acetate (pH 5.0) in a volume $^1/_{100}$ that of the 15% NaCl filtrate.

STEP 3. The solution was desalted at room temperature on a G-25C Sephadex (Pharmacia) column equilibrated with the solvent. The fractions were tested for sulfate ions with 1–2 drops of 1.0 N $BaCl_2$. The crude APTI was eluted with the front-running, salt-free, protein-rich peak. The fractions within this peak were pooled and lyophilized. The crude preparation was stored at 5°. The crude inhibitor was dissolved in 0.005 M ammonium acetate (pH 5.0) the volume being $^1/_{500}$ the starting material volume.

STEP 4. The solution of crude APTI was adsorbed onto a CM-cellulose column (height:diameter = 6:1) equilibrated with the solvent buffer at room temperature. Unadsorbed material was eluted through the column. Ammonium acetate (0.05 M) (pH 5.0) was passed through the column when the OD_{280} returned to a point near zero. The second peak following the buffer change contained the purified inhibitor. The fractions comprising this peak were pooled and lyophilized.

Starch Gel Electrophoresis. Hydrolyzed starch was purchased from Connaught Medical Research Laboratories. Starch gel electrophoresis was performed at pH 9.0 (Smithies, 1955, 1959) and 2.9. The pH 2.9 starch gel procedure was identical with the one described by Rasmussen *et al.* (1964), except that 0.16 M NaOH and 0.02 M Na_2 EDTA was used, and the pH was adjusted to 2.9 with 98–100% formic acid. A sample (1 mg) in 40 μl of buffer was placed in the sample slot. An 8-v/cm voltage gradient was applied for 16 hr at room temperature. Slicing, staining, and fixing of the gels were performed by the method of Edelman and Poulik (1961).

Disc Electrophoresis. pH 4.5 polyacrylamide gel electrophoresis was carried out by the method of Reisfeld *et al.* (1962) at 6 v/tube for 1 hr. Electrophoresis at pH 8.9 was performed according to Ornstein (1964) and Davis (1964).

N-Terminal Amino Acid Determination. Dinitrophenylamino acids were purchased from Mann Research Laboratories. The N-terminal amino acid analysis was done by the FDNB method of Sanger (1945, 1949), according to the procedure described by Fraenkel-Conrat *et al.* (1955). The thin layer chromatography system described by Brenner *et al.* (1961) was used to separate and identify the DNP-amino acids.

Amino Acid Analysis. Purified APTI was hydrolyzed for 21 hr at 110° with 6 N HCl in sealed-evacuated tubes (Moore and Stein, 1963), and the analysis was performed using a Spinco Model 120C amino acid analyzer (Hubbard, 1965).

Sulfhydryl group determinations were performed by the method of Ellman (1959). 5,5′-Dithiobis(2-nitrobenzoic acid) was purchased from Aldrich Chemicals.

Molecular weight measurements were made by sedimentation equilibrium techniques (Van Holde and Baldwin, 1958) and were carried out in a Beckman–Spinco Model E analytical ultracentrifuge with rotor temperatures of 18°. Equilibrium measurements were made on solutions of APTI at 2–7 mg/ml at rotor speeds from 20,410 to 35,600 rpm. These solutions had been dialyzed against either 0.03 M citrate buffer (pH 5.0) or 0.03 M phosphate buffer (pH 7.0). The partial specific volume of APTI was measured in an unbuffered water solvent at 30° and was found to be 0.71.

Results

Purification. As little as 10 l. and as much as 300 l. of starting material have been processed. The average yield of crude inhibitor was 22–24 units/kg of original pancreas. Yields as low as 15 units/kg and as high as 33 units/kg have been obtained. The crude inhibitor has been stored up to 3 weeks in solution without loss of activity prior to desalting on Sephadex G-25. Following desalting and subsequent lyophilization, the APTI has remained stable at 5° for several months.

Figure 1 shows the type of elution pattern obtained when crude APTI derived from 100 l. of 15% NaCl filtrate was purified using ammonium acetate (pH 5.0) for the CM-cellulose chromatography. The sym-

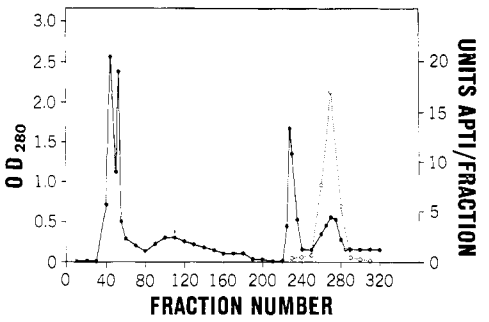

FIGURE 1: CM-cellulose chromatography of crude APTI with 0.005 M ammonium acetate (pH 5.0). Buffer concentration increased to 0.05 M at arrow. Column size 4.0 × 44 cm. Fractions (10 ml) were collected. (●) OD_{280}; (○) units of APTI per fraction.

metrical shape of the peak indicates a high degree of homogeneity. Smaller scale experiments exhibit similar patterns.

Table I summarizes the purification of crude APTI derived from 100 l. of starting material. Yields of purified APTI have usually been 9–12 units/kg of original pancreas. The highest yield obtained was 19 units/kg, the lowest 9 units/kg. The specific activity was 2.5–2.8.

Disc electrophoresis (pH 4.5 and 8.9) and starch gel electrophoresis (pH 9.0 and 2.9) reveal only one band present. A sedimentation velocity experiment revealed a single homogeneous peak with a $s_{20,w}$ = 1.08 S.

Following lyophilization, purified APTI has remained stable at 5° for at least several months. It also maintained stability for 1 month at 5° in 0–1.0 M sodium citrate (pH 5.0). After 2.5 months, these same solutions assayed at 75% of their original potency.

N-Terminal Amino Acid Determination. The DNP-trypsin inhibitor hydrolysate revealed only DNP-aspartic acid and ε-DNP-lysine. This indicates that aspartic acid is the N-terminal amino acid of APTI.

Amino Acid Analysis. The amino acid content of APTI is presented in Table II. The composition of the Kazal-type inhibitor and the basic KPTI also shown. APTI is devoid of phenylalanine, tryptophan, and histidine, as is the Kazal-type inhibitor. KPTI lacks only tryptophan and histidine. KPTI is similar to APTI in only two other aspects. The unusually high content of cysteine and proline is the same for each inhibitor. Both inhibitors contain only one methionine residue and six glycine residues. The remaining residues of each inhibitor are generally much different. APTI and the Kazal-type inhibitor have an acidic to basic amino acid ratio about three times that of KPTI. Sulfhydryl group determinations of APTI were negative, even when the inhibitor was incubated in 8 M urea. This indicates that the six cysteine residues most likely exist as three disulfide linkages.

Molecular Weight Determination. Shown in Figure 2 is a plot of log j *vs.* X^2 for the APTI in citrate buffer (pH 5.0) at 19°, 20,410 rpm, and an initial protein concentration of 7 mg/ml. The only curvature present is at the bottom of the cell, indicating very little heterogeneity in the system. All of the samples examined showed slight heterogeneity which was probably due to denatured and aggregated inhibitor. When higher rotor speeds were used, no lower molecular weight material nor any tendency for the inhibitor to undergo self-association or dissociation could be detected. The apparent weight-average molecular weight for APTI is 6500 ± 300. This amount of uncertainty in the molecular weight arises primarily from the partial specific volume term ($\bar{v}$) which was not measured under the same conditions as the sedimentation experiments. However, the agreement with the molecular weight calculated from the amino acid composition is good and would thus appear to confirm the value of 6155 determined from the amino acid analysis.

Discussion

We have isolated from bovine pancreas an anionic trypsin inhibitor in yields much higher than previously reported (Kazal *et al.*, 1948). The starting material is similar to that used by Kazal *et al.* in that it was a 15% NaCl filtrate derived from an acid–ethanol extract of bovine pancreas obtained in a commercial process for the purification of insulin. The much higher yields of inhibitor obtained in this investigation were made possible by avoiding the isolation of APTI as a complex with trypsin and the crystallization of APTI from trichloroacetic acid solutions. Kazal's crystalline inhibitor showed three distinct peaks during moving boundary electrophoresis. The APTI we have purified is

TABLE I: Purification of Crude APTI with Ammonium Acetate Buffers (pH 5.0) on CM-cellulose.

Purification Step	Units of Inhibitor	Units/kg of Pancreas	Sp Act.	% Yield
Step 3, crude APII	2240	18.6	0.076	100
Step 4, elution peak	1510	12.6	2.58	68
Purified APTI	958	9.5	2.79	51

TABLE II: Amino Acid Analysis of APTI.

Amino Acid	APTI[a] (residues/mole)		Integer	Kazal Type[b]	KPTI[c]
Lys	3.02	3.38[d]	3	3	4
Arg	3.02	2.87	3	3	6
Asp	7.12	6.89	7	7	5
Thr	3.86	3.78	4	4	3
Ser	1.95	2.76	2	2	1
Glu	7.18	6.89	7	7	3
Pro	3.98	3.97	4	4	4
Gly	5.05	5.18	5	5	6
Ala	1.15	1.50	1	1	6
Cys	5.39	5.70	6	6	6
Val	3.86	3.74	4	4	1
Met	0.89	0.94	1	1	1
Ile	2.83	2.74	3	3	2
Leu	3.64	3.87	4	4	2
Tyr	1.64	1.92	2	2	4
Phe			0	0	4
Total			56	56	58
Calculated molecular weight			6155	6155	6513

[a] Molar ratios calculated by using the values obtained for aspartic acid, glutamic acid, glycine, alanine, valine, and leucine. Integers obtained by averaging the molar ratios of the two analyses and rounding to nearest whole number. [b] Data obtained by Greene *et al.* (1966) from pancreatic juice inhibitor and Greene (1966) from the inhibitor isolated by Kazal *et al.* (1948). [c] Data obtained by Kassell *et al.* (1963). [d] Analysis by Dr. L. J. Greene of an identical sample of APTI.

homogeneous on the basis of disc electrophoresis, starch gel electrophoresis, and studies in the ultracentrifuge.

Fritz *et al.* (1966c) isolated a trypsin inhibitor of the Kazal type from bovine pancreas and pancreatic juice. Although bovine pancreas contains this inhibitor and KPTI, only the Kazal-type inhibitor is secreted in the pancreatic juice. Fritz *et al.* (1966c) separated their inhibitor from KPTI by complexing the latter with chymotrypsin or kallikrein. These enzymes did not complex with their Kazal-type inhibitor. This approach was not necessary in our procedure since KPTI was not found in the 15% NaCl filtrate used for the starting material for the purification of APTI.

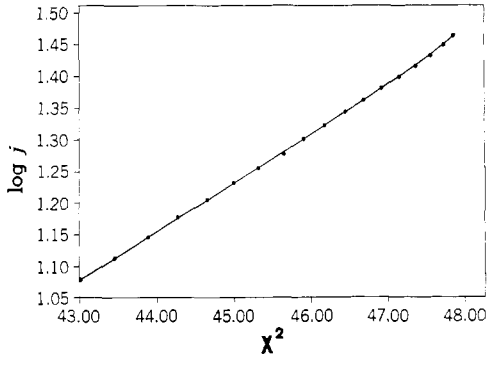

FIGURE 2: Sedimentation equilibrium experiment. See text for details. Molecular weight at meniscus = 6520. Molecular weight at cell bottom = 7880. Apparent weight-average molecular weight = 6500 ± 300.

Greene *et al.* (1966) purified an anionic trypsin inhibitor similar to the Kazal inhibitor from bovine pancreatic juice without the use of complex formation with trypsin and final crystallization. They separated the inhibitor from a large excess of trypsinogen by chromatography on Sephadex G-75; this was not necessary in our procedure since the pancreatic trypsinogen is not solubilized during the acid–ethanol extraction of pancreas. The low molecular weight fraction from the Sephadex G-75 column was further purified by column chromatography on DEAE-cellulose at pH 9.0. One major fraction was obtained, accounting for 86% of the total activity. Chromatography of Kazal's inhibitor using this same system yielded three active peaks, probably comparable to the three electrophoretically distinct fractions observed by Kazal *et al.*

The amino acid contents of APTI, Greene's inhibitor, and the Kazal inhibitor (Greene, 1966) are identical. APTI is also identical with the inhibitor isolated by Greene *et al.* (1966) on the basis of the following

criteria: (a) chromatographic behavior (L. J. Greene, personal communication); (b) molecular weight; (c) amino acid content; (d) inhibitory specificity; and (e) temporary inhibition of trypsin (Burck *et al.*, 1967).

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