An unusual bovine pancreatic protein exhibiting pH-dependent globule–fibril transformation and unique amino acid sequence

(threads/helices/solubility/electron microscopy/primary structure)

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ABSTRACT An unusual hitherto unreported protein, extracted in acid from fresh bovine pancreas, has been purified and characterized biochemically. It precipitates in the neutral pH range in the form of uniform double-helical threads, each strand of which is smooth and of uniform diameter, about 7–8 nm. The threads dissolve to a nonviscous solution below pH 3.6 and above pH 9.4, and they reconstitute reversibly in the pH range in between. The monomer in acid has an apparent molecular weight of 17,800 and consists of two disulfide-linked nonidentical polypeptide chains of different lengths. It is rich in aromatic amino acids, particularly tryptophan. There is no significant content of carbohydrate, fatty acid, or bound phosphate. The amino acid sequences of the first NH₂-terminal 48 residues of the A chain and 35 residues of the B chain appear to be unique, differing from all other reported animal proteins, including those of the pancreas. Thus far, a function has not been found.

In the course of early studies on the structure of elastic fibers by electron microscopy utilizing trypsin digestion, the appearance of 12-nm-wide, uniformly helical threads in the "digestate" led to the erroneous conclusion that these coiled elements were structural components of elastin (1). Subsequent reexamination (2) led the author to conclude that the helical threads were either a conversion product of trypsinogen or a contaminant of the various commercial crystallized trypsin preparations, not observed earlier in the trypsin control for technical reasons. High-speed centrifugation of dissolved trypsin or trypsinogen alone at neutral pH produced a pellet consisting largely of straight, tightly wound, double-helical, 12-nm threads of variable length with a pitch of 47–58 nm and a right-handed screw axis. A variable proportion of these threads were uncoiled single filaments or parallel pairs. They dissolved at acid pH and rapidly reconstituted to helical threads on neutralization in a reversible manner (3). They were not a component of elastin as originally proposed (1) or of bacterial flagella (4).

Although there have been numerous studies of the composition of the pancreatic exocrine secretion from various mammals, including bovines (5–9), the protein described here has not been previously recognized.

We report here the partial molecular characterization of this unusual bovine pancreatic thread protein (PTP) with an as-yet-unrecognized function.

MATERIALS AND METHODS

Whole pancreases from freshly slaughtered calves were collected in cold 0.25 M sulfuric acid. Within 24 hr the tissue was homogenized in a blender in the cold and the acid extract was separated from the residue by filtration and sedimentation. It was then processed by sequential ammonium sulfate precipitation and re-solution in borate buffers as outlined by Kunitz and Northrup (10). At that stage in the described procedure where the third 70% saturated ammonium sulfate precipitate is obtained, the solution at pH 8 was allowed to stand at 4°C until a cloudy precipitate formed after 2–3 days. The gelled precipitate was removed by centrifugation, dissolved in 0.1 M HCl, centrifuged at 100,000 × g in the Spinco preparative ultracentrifuge to remove any undissolved material, and reprecipitated by neutralization to pH 7 for at least four cycles. The final precipitate was dialyzed free of salt and lyophilized from water to a pure white fluff.

Wet cakes of white gelatinous material forming spontaneously from the third 70% saturated ammonium sulfate precipitate during the large-scale commercial preparation of trypsin and normally discarded were kindly provided by J. D. Teller (Worthington). This material proved to be almost pure thread protein in the helical filamentous form. It behaved identically to the material purified from the pancreas as described above, thus making bovine PTP available in gram amounts.

pH Dependence of Thread Formation. Lyophilized threads were suspended in 2-mg aliquots in 2 ml of sodium citrate/phosphate buffers prepared in the pH range between 2.6 and 10.5 and the suspensions were allowed to stand overnight in the cold. They were centrifuged at 60,000 × g for 1 hr and the absorbance of the clear supernatant solutions was measured at 280 nm after appropriate dilution.

Electron Microscopy. Lyophilized purified PTP was suspended in water in opalescent dispersions or was reprecipitated from acid solution by neutralization and was deposited in drops on carbon-coated specimen grids. These were stained negatively with 4% aqueous sodium silicotungstate at pH 7.5 or shadowed at 7–10° angles with chromium. A JEOL 100B electron microscope was used.

Reduction and Radioalkylation. Five milligrams of lyophilized PTP was dissolved in 6 M guanidinium hydrochloride in 0.5 M Tris-HCl, pH 8.2/5 mM EDTA. Dithiothreitol was added to give 20 mM and after flushing with N₂ and incubation at 37°C for 90 min the PTP was alkylated at 4°C in the dark with iodo[¹³⁵]C]aceticamide. The reaction was stopped with 0.1 M acetic acid and the preparation was dialyzed against 0.01 M HCl.

Acrylamide Gel Electrophoresis. Protein heterogeneity, molecular weight, chain separation by reduction and alklylation, and the presence of associated neutral proteinase activity were evaluated by NaDodSO₄/polyacrylamide gel electrophoresis. Autoradiography of the gel patterns of

Abbreviations: PTP, pancreatic thread protein; Pth, phenylthiohydantoin.

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14C-labeled reduced and alkylated preparations was accomplished by the method of Laemmli (11). The relationship between the thread protein and neutral protease activity measured by quantitative radioactive gelatin assay (12), kindly performed by Yi-Shan Cheng of the Developmental Biology Laboratory, was assessed by electrophoresis in NaDodSO4/11% acrylamide gel in which 1.5% gelatin was incorporated, but plasminogen was omitted (13).

High-Pressure Liquid Chromatography. Reversed-phase HPLC was performed at ambient temperature, using a μBondapak C18 column (0.4 x 30 cm; Waters) and Corasil C18 guard column (Waters). Samples were applied while the column was equilibrated in 40% (vol/vol) acetonitrile/0.1% trifluoroacetic acid in water at a flow rate of 1 ml/min. Peptides were then eluted with a 15-min linear gradient of 40–75% acetonitrile and detected by absorbance at 280 nm. The UV detector effluent was directed to a Flow-One DR liquid scintillation spectrometer (Radiomatic Instruments & Chemical, Tampa, FL) for continuous monitoring of radioactivity or, alternatively, to a fraction collector for recovery of separated peptides.

Amino Acid Analysis. PTP was hydrolyzed in 3 M p-toluensulfonic acid plus 0.2% 3-(2-aminoethyl)indole (Eastman) in water to preserve tryptophan (14). Recoveries of individual amino acids were checked by using this hydrolysis protocol. Comparisons were also made with 6 M HCl hydrolysates. Analyses were performed in an automated Beckman updated 121B amino acid analyzer using the buffer system of Trelstad and Lawley (15).

Carbohydrate, Lipid, and Phosphate Analyses. Gas/liquid chromatographic analysis for sugars was accomplished with an Applied Biosystems model 470A gas/liquid analyzer on 0.5- to 10-nmol samples of intact PTP based upon a program reported previously for the spinning cap sequencer by Brauer et al. (19). The reaction program is similar to the Applied Biosystems MHTFA1 program except that phenylisothiocyanate delivery was replaced by o-phthalaldehyde. o-Phthalaldehyde (0.2 mg/ml) (Pierce) in n-butyl chloride containing dithiothreitol at 0.2 mg/ml was delivered from the S1 reagent reservoir. All of the sequencer programs, including the Polybrene wash cycle, did not include an S1 delivery except for the cycle at which blocking by o-phthalaldehyde was employed. The heptane ordinarily delivered from S1 in program MHTFA1 was mixed with ethyl acetate (1:2) in the S2 reagent reservoir. A 30-sec pause was inserted just after S1 delivery, followed by the usual argon drying and trimethylamine buffer (R2) delivery. The S2 wash in the o-phthalaldehyde cycle was extended to 600 sec. An additional S3 wash (n-butyl chloride) of 900 sec was used at the end of the cycle to reduce residual o-phthalaldehyde to a minimum. Following o-phthalaldehyde treatment a double coupling cycle similar to the Applied Biosystems MHTFLG program was used except that the delivery of trifluoracetic acid and associated flask functions prior to coupling was omitted (19). After this double coupling, the MHTFA1 program was used except that cleavage of the prolyl peptide bond with trifluoroacetic acid was extended between 1200 and 1800 sec. Phenylthiohydantoin (Pth) derivatives of the amino acids were identified and quantified by HPLC as previously reported (20).

A and B chains, reduced and alkylated with iodo[14C]acetamide and separated by HPLC were sequenced separately without o-phthalaldehyde derivatization.

RESULTS

Although the original thread preparations used for analyses were obtained from fresh pancreas, all the analytical work reported here was accomplished on protein isolated and purified by repeated solution and reprecipitation from wet cakes of precipitate from an intermediate stage of the Kunitz and Northrup enzyme isolation procedure (10), obtained from Worthington. These lyophilized precipitated thread preparations have been stable at -20°C and at refrigerator temperature over many years. PTP could not be obtained by the same fractionation technique from bovine kidney or liver.

Turbid suspensions of the lyophilized threads in distilled water or neutral buffer, when examined by phase microscopy, appeared in the form of flat, thin, irregular-shaped plaques, which when examined in polarized light were shown to consist of packed fibrous aggregates oriented in seemingly random directions. They exhibited positive birefringence in the axial directions as determined by color orientation with the quarter-wavelength plate in place.

Lyophilized threads were immediately and completely soluble in 0.01 M HCl and reprecipitated from solution either as a cloudy suspension or as a loose opalescent gel upon neutralization with NaOH or ammonium hydroxide. The pattern of pH-dependent solubility seemed to be relatively insensitive to the presence of various salts, including acetate, phosphate, bicarbonate, citrate, and borate. As shown in Fig. 1, there is a rapid fall in solubility above pH 3.6 with little or no protein remaining in solution above pH 4.2 until an abrupt increase occurred above pH 9.4. At pH 10 essentially all the protein had dissolved. As was the case at the lower pH range, return toward neutrality from high pH reprecipitated the protein in a reversible manner.

Electron microscopy of the opalescent suspensions produced on increasing the pH of an acid solution in the range between 4.2 and 9.4 revealed large numbers of helically coiled and single, smooth, unbranched threads of uniform diameter, without obvious periodic fine structure or frayed ends in negatively stained (Fig. 2) or metal-shadowed preparations (not shown). In some instances the coiled threads seemed to consist of more than two strands. No other ordered structures and very little amorphous material were seen in any of these preparations. The threads were similar to those

![Fig. 1](image-url)  
**FIG. 1.** pH dependence of bovine PTP solubility. Absorbance at 280 nm of the centrifuge-clarified supernatant of lyophilized purified PTP dispersed in citrate-phosphate buffer at 13 different pH values.
observed previously in pellets prepared by sedimentation from neutral solutions of commercial crystallized preparations of trypsin and trypsinojen (2).

Thread protein chromatographed on Sephadex G-75 in 0.01 M HCl or in 4 M guanidine hydrochloride and also on an HPLC reversed-phase column eluted with an acetonitrile/trifluoroacetic acid gradient resulted in single symmetrical peak elution patterns (data not shown). However, reduced and radioalkylated preparations were separated into two sharp peaks on HPLC (Fig. 3). UV absorption (Fig. 3A) and radioactivity (Fig. 3B) peaks correlated in position very closely.

NaDodSO₄/polyacrylamide gel electrophoresis performed on four different PTP preparations produced a major, relatively sharp band migrating in the 16- to 20-kDa range, depending on acrylamide gel concentration (12.5%, 14%, 15%, 17%). Five separate runs in 14% gels indicated an apparent molecular mass of 17.8 ± 0.6 kDa (mean ± SD). The major component was often associated with a slower, more diffuse, weakly staining band appearing in the reduced sample (Fig. 4, lane 3) but in this case not apparent in the unreduced preparation. Reduced and alkylated thread protein appeared as two new bands, one of about 14 kDa and the other, of nearly similar staining density, at the solvent front. Autoradiography indicated nearly equal labeling of the two bands (data not shown).

Because analyses for native proteinase revealed variable amounts of gelatinolytic activity associated with the PTP in three different preparations, a question of intrinsic or fortuitous association of proteolytic activity was explored by electrophoresis in NaDodSO₄/polyacrylamide gels impregnated with gelatin (Fig. 5). Proteolytic activity represented by a nonstaining band ran in the same position as trypsin (lane 1) and separate from the thread protein (lane 3). Several faint bands of protease activity are seen in the higher molecular mass range.

Amino acid analyses were performed on two different preparations of purified thread protein, five separate analyses on each (Table 1). Recovery of amino acids from the p-toluenesulfonic acid hydrolysates was nearly the same as for hydrolysates prepared with 6 M HCl except for the loss of tryptophan in the latter. One of the unusual features is the high tryptophan content, 8 residues per mol. Per mol of protein, less than 0.1 mol of carbohydrate, of fatty acids, and of bound phosphate was detected.

An initial sequence analysis of intact threads revealed two

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**Fig. 2.** Electron micrograph of dispersion of purified threads reconstituted from acid solution by neutralization. (Negatively stained with 4% aqueous sodium silicotungstate at pH 7.5; ×199,000.)

**Fig. 3.** HPLC of reduced and radioalkylated purified PTP. (A) Absorbance at 280 nm. (B) Radioactivity profile of same eluate.

**Fig. 4.** NaDodSO₄/polyacrylamide gel electrophoretic pattern of intact and reduced and alkylated PTP. Lane 1, molecular mass markers (kDa), unreduced. Lane 2, intact threads. Lane 3, reduced and alkylated threads. Lane 4, HPLC-isolated B chain identified by sequence analysis.
incorporating phoresis on Pth activity. Light bands reflect nonstaining region of gelatinolyis by migrating proteolytic activity.

Pth amino acids at most cycles in equimolar amounts. Only a single sequence was identified through cycles 35–48, the last residues identified with confidence. The results were consistent with the presence of two subunits of differing sequence. At certain cycles (e.g., 2, 11, 12, and 15) the yields of a single identified Pth amino acid were twice those expected, suggesting identical residues in both chains. Since proline residues were identified at positions 3 and 4 in this mixture, Edman degradation was repeated on the unfractonated PTP, using selective o-phthalaldehyde blockade (19) prior to cycle 4. The irreversible blocking of the primary amine released in one chain and the specific reversible blocking reaction of o-phthalaldehyde with the released proline residue in the other chain permitted sequencing solely in the latter. We were fortunate in that the two prolines at positions 3 and 4 were located one in each chain. Thus, a single sequence was observed in chain A beginning at position 4, extending 48 cycles. Pth amino acids were not identified at cycles 14 and 31. In a repeat degradation using o-phthalaldehyde treatment prior to cycle 3, the single sequence corresponding to chain B was identified for 35 cycles except for positions 6, 23, and 31. After complete reduction and alkylation with iodo[14C]acetic acid, Edman degradation on PTP was repeated. The results were analogous to the first experiment except that additional Pth S-carboxymethylcysteine residues were identified at cycles 3, 14, and 31 in the A chain and 6, 23, and 31 in the B chain, thus filling in the gaps. The yield of radioactivity at each cycle confirmed these identifications. These sequences are reported in Fig. 6.

The two fractions separated by HPLC were sequenced separately and the NH2-terminal residue of the A chain was identified as isoleucine and that of the B chain as serine. The first eight residues of each chain analyzed separately confirmed the identity and sequence of the two chains as determined by the selective o-phthalaldehyde derivatization method. NaDodSO4/polyacrylamide gel electrophoresis of the HPLC peaks identified the B chain with the fast-moving band at the solvent front, shown in Fig. 4, lane 4.

**DISCUSSION**

The uniform, helical, thread-like characteristic and globule–fibril transformation of this protein made it intriguing as a model for studying the labile polymorphism of certain structural proteins. Interest was maintained by the absence of any cross-reaction in the Ouchterlony diffusion system with any of the well-known pancreatic enzymes or other proteins, including insulin and glucagon, using a polyclonal antibody made against the purified pancreatic thread protein (unpublished data).

Waugh (21) had previously found that boiling insulin at acid pH produced morphologically very similar threads, although the conditions for polymerization proved to be quite different from those observed for this protein. The globule–fibril transition of actin had been described by Jakus and Hall (22), but again the required conditions were very different. It was subsequently reported that a wide range of "globular" proteins will make very similar thread-like structures; these include glucagon (23), tropomyosin (24), Bence-Jones protein (25), pig renal gluminase (26), and spectrin (27). Amyloid fibrils (28) and the filamentous protein associated with neurofibrillary tangles in Alzheimer disease (29), Jacob–Creutzfeld disease (30), and scrapie (31), and in objects called "prions" (32) are similar in that they can be seen in the form of helical and pseudo-helical threads, often consisting of two filaments. There are, in addition, a wide range of cytoplasmic filaments that seem comparable in dimensions to the thread protein described here. However, with the exception of insulin and glucagon, all the work done on this variety of fibrous forms indicates a much higher monomer molecular weight, very different solubility properties, different amino acid composition, and, where sequences are known, there appears to be no homology with the 48

![Fig. 5. Zymogram of NaDodSO4/polyacrylamide gel electrophoresis incorporating gelatin in the running gel. Lane 1, molecular mass markers (kDa); lane 2, bovine trypsin; lane 3, purified PTP. Dark bands are Coomassie blue stained, nonproteolytic protein. Light bands reflect nonstaining region of gelatinolyis by migrating proteolytic activity.](image)

![Fig. 6. Amino acid sequence of A and B chains of purified bovine PTP.](image)

**Table 1. Amino acid composition of bovine PTP**

<table>
<thead>
<tr>
<th>Amino acid residue</th>
<th>Residues per 1000*</th>
<th>Residues per mol†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid‡</td>
<td>144.2 ± 2</td>
<td>23</td>
</tr>
<tr>
<td>Threonine</td>
<td>43.7 ± 1</td>
<td>7</td>
</tr>
<tr>
<td>Serine</td>
<td>147.9 ± 6</td>
<td>23</td>
</tr>
<tr>
<td>Glutamic acid‡</td>
<td>74.3 ± 6</td>
<td>12</td>
</tr>
<tr>
<td>Proline</td>
<td>53 ± 2</td>
<td>8</td>
</tr>
<tr>
<td>Glycine</td>
<td>69.6 ± 3</td>
<td>11</td>
</tr>
<tr>
<td>Alanine</td>
<td>65.6 ± 3</td>
<td>10</td>
</tr>
<tr>
<td>½ Cystine</td>
<td>38 ± 6</td>
<td>6</td>
</tr>
<tr>
<td>Valine</td>
<td>41.9 ± 2</td>
<td>7</td>
</tr>
<tr>
<td>Methionine</td>
<td>14.6 ± 1</td>
<td>2</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>33.1 ± 1</td>
<td>5</td>
</tr>
<tr>
<td>Leucine</td>
<td>72.7 ± 2</td>
<td>11</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>41.1 ± 1</td>
<td>6</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>22.1 ± 2</td>
<td>3</td>
</tr>
<tr>
<td>Lysine</td>
<td>35.3 ± 1</td>
<td>6</td>
</tr>
<tr>
<td>Histidine</td>
<td>27.5 ± 1</td>
<td>4</td>
</tr>
<tr>
<td>Arginine</td>
<td>34.7 ± 1</td>
<td>5</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>49.4 ± 5</td>
<td>8</td>
</tr>
</tbody>
</table>

Carbohydrate <0.1 mol/mol
Fatty acid <0.1 mol/mol
Phosphate (bound) <0.1 mol/mol

*Mean ± SD, n = 10.
†Calculated on the basis of a molecular mass of 17.8 kDa.
‡Includes amides.
NH₂-terminal residues of chain A and the 35 residues of chain B reported here. A computer-based survey performed by the National Biomedical Research Foundation (Washington, DC) on a data base of 2914 protein sequences (as of May 1984 and updated with regard to mammalian proteins as of Feb. 1985), comparing fragments of 25 residues in length with both A and B chains, failed to reveal any homologies except that of chain A with the human PTP (33).

Reduction and alkylation produced two components from the unreduced 17.8-kDa protein, the larger of which was estimated at about 14.5 kDa. It is thus possible that the small, labeled component at the solvent front, the B chain, is about 3 kDa, its full size. The accompanying slower faint band persisted through the final purification procedures and was labeled by radioalkylation, suggesting that it is related in some way to the thread protein. It accounts for no more than 10% of the total protein.

We (33) have isolated a homologous protein from acid extracts of human pancreas, using the extraction procedure described in this paper for bovine pancreas, and have also observed it in large amounts in pancreatic fluid obtained from normal subjects and patients with pancreatic disease. These threads, however, are single stranded in all cases at the electron microscopic level. They have very similar solubility characteristics and are related but not identical in amino acid composition. In contrast with bovine threads, they consist of a single polypeptide chain with 58% sequence homology to the NH₂-terminal 45 residues of the A chain of the bovine protein. Of interest is the homologous location of all three cysteine residues in both species and the absence of the disulfide-linked B chain from the human PTP.

As described in 1951 (2), relatively large amounts of helical threads, as much as 9–14% of total protein, could be found in sediments of various commercially obtained, sterile crystallized trypsinogen preparations, which led to the conclusion that the "phenomenon may represent a fibrous transformation of trypsinogen, a component or contaminant thereof."

The amino acid sequences reported here are not homologous with any part of the published complete primary structure of bovine trypsinogen (34). In a recent publication (35) dealing with what appears to be a related protein in human pancreatic fluid, the authors conclude that their protein (called by them "protein X") is a degradation product of trypsinogen. Since it is related by amino acid composition and sequence of 13 NH₂-terminal residues (36) to the A chain of the bovine thread protein described here, it is unlikely to be related to trypsinogen. Whether or not bovine or human PTP is a hydrolysis product of a larger precursor molecule is yet to be determined.

Of some interest is the absence of carbohydrate, fatty acid, and phosphate in the protein described here, indicating little or no post-translational modification. Thus, bovine PTP differs significantly from another recently reported protein of nearly the same size found in human pancreatic fluid and pancreatic stones, called pancreatic stone protein (PSP), which was said to be a phosphoprotein (37).

Efforts to isolate the PTP from bovine liver and kidney by the same extraction procedures used for pancreas did not succeed. Preliminary immunohistochemical studies using monoclonal antibodies to the bovine thread protein (unpublished data) localized it to the acinar cells of the pancreas and failed to show it in liver. More extensive immunohistochemical examination of a wide range of organs in the human, using monoclonal antibodies to the human thread protein, further specifies its unique localization to the pancreatic acinar cells (33).

The insolubility of this protein in the neutral pH range suggests that it may be present in the pancreas in filamentous form, perhaps as a structural element of secretion granules, perhaps dispersed in lysosomes where the pH may be low enough to keep it in solution, at least in part. It may be part of a larger soluble protein and may be an insoluble product of enzymatic processing.

Physiologic experiments have not yet been done at the organismic or cellular level and speculations as to function would be fruitless without some indicative data. At this time we are simply describing an interesting, apparently not previously observed, bovine pancreatic protein, still in search of a function.

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