The Crystallization and Partial Characterization of Porcine Trypsin*

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Since it was first isolated in crystalline form by Northrop and Kunitz in 1932 (1), bovine trypsin has been the object of considerable study (2, 3) which has culminated in a near elucidation of its complete structure (4). Except for a brief report by Ten-Broeck (5), that bovine and porcine trypsins were immunologically different, it is only within the last few years that trypsins from other animal species have begun to receive attention (6-13). Nord and coworkers (6, 7) have compared some of the physical, chemical, and catalytic properties of bovine, ovine, and porcine trypsin. Desnuelle's group (8-10) has studied the properties of pork trypsinogen with particular regard to its mode of activation to trypsin. Neither group used crystalline preparations of porcine trypsin for their studies but relied on chromatographic fractionation on carboxymethyl cellulose for achieving purity.

A preliminary report from this laboratory (11) has described the successful crystallization of porcine trypsin, followed shortly thereafter by a similar report from the Armour laboratories (13). As a prerequisite to a study of the chemical nature of the active site of porcine trypsin, it was necessary to establish the homogeneity of our crystalline preparation and to determine some of its more important physical and chemical parameters.

Experimental Procedure

Crystallization of Porcine Trypsin—The initial steps of purification were essentially the same as those described by Northrop, Kunitz, and Herriott (14) and involved precipitation of the protein from an acid extract of hog pancreas between 0.4 and 0.7 saturation with ammonium sulfate. A solution of this precipitate was allowed to undergo activation at pH 8 in the presence of 0.1 M CaCl₂ for 2 days at 4°. The protein precipitated by 0.7 saturation with ammonium sulfate was lyophilized and constituted the starting material for the crystallization procedure described below.

Of the lyophilized filter cake, 25 g were dissolved as completely as possible in 1 liter of 0.001 N HCl and centrifuged to remove any insoluble material. To the supernatant solution (Fraction I) was added sufficient solid ammonium sulfate to bring the solution to 0.4 saturation. Removal of the precipitate was facilitated by adding 2 to 3 g of Filter-Cel (Johns-Manville) followed by centrifugation. The precipitate was discarded and solid ammonium sulfate was added to the supernatant solution (Fraction II) to give 0.7 saturation. The precipitate was dissolved in a minimum amount of water (Fraction III), and a saturated solution of ammonium sulfate was added with stirring until 0.7 saturation was reached. After 1 to 2 hours at 4°, the precipitate was collected by suction filtration and washed with 10 to 20 ml of a saturated solution of magnesium sulfate. The moist filter cake was dissolved in 0.2 M borate buffer, pH 9, at a ratio of 1 ml of buffer for each gram of precipitate. When this solution was allowed to stand at 4° for several hours, mixed crystals of rectangular plates and rods appeared. After standing overnight in the cold, these crystals were collected by centrifugation. Occasionally, bipyramidal crystals were noted; the appearance of these crystals was similar to those described by Van Melle et al. (13) for their crystalline preparation of porcine trypsin. Recrystallization was effected by dissolving the first crop of crystals in 0.4 M borate buffer, pH 9, at a concentration of about 175 mg per ml (Fraction IV). Within a few hours, the long thin rods shown in Fig. 1 appeared, and these were collected by centrifugation, dissolved in 0.001 N HCl, dialyzed, and lyophilized (Fraction V).

Chromatography in CM-cellulose—For analytical studies, 10 to 15 mg of protein were dissolved in 1 ml of 0.05 M citrate buffer, pH 5.0, and applied to a column of CM-cellulose (0.9 x 30 cm) (Whatman) previously equilibrated against the same buffer. Buffers of the same molarity but of increasing pH were introduced in a stepwise fashion when no further protein was eluted by the preceding buffer. Tubes collected from each run were examined for protein by measuring their absorbance at 280 μm and assayed for activity on N-benzoyl-L-arginine ethyl ester and N-acetyltyrosine ethyl ester (see below). Tubes possessing activity were pooled (Fraction VI), dialyzed against 0.001 N HCl, and lyophilized.

Enzyme Assays—Tryptic and chymotryptic activities were measured on N-benzoyl-L-arginine ethyl ester and N-acetyltyrosine ethyl ester, respectively, by using the spectrophotometric procedure of Schwert and Takenaka (15). A unit of trypsin activity is arbitrarily defined as an absorbance change of 1 unit per minute and specific activity as the number of units per mg of protein. The chymotryptic content of the various trypsin preparations was calculated on the basis of the amount of crystalline α-chymotrypsin ( Worthington) which gave an equivalent level of chymotryptic activity. Protein was calculated from nitrogen determinations (16) and a value of 16.1% for the nitrogen content of crystalline porcine trypsin on a dry weight basis. Alternatively, absorbance readings at 280 μm were converted to

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* The abbreviation used is: CM, carboxymethyl.
Ultracentrifuge Studies—The sedimentation coefficient was calculated from data obtained with a Spinco model E ultracentrifuge. Molecular weight was determined by an approach to sedimentation equilibrium method making use of a Trautman plot (17, 18). Protein solutions were prepared in 0.1 M NaCl to give a concentration of 0.5% and placed in a 12-mm single sector cell. These were run at speeds of 15,220, 25,980, and 35,000 r.p.m. for 48 minutes at each speed, and photographs were taken at intervals of 8 minutes at a bar angle of 80°.

Electrophoresis—Moving boundary electrophoresis was conducted at 4° in a Klett apparatus of Longsworth design with a 2-ml micro-Tiselius cell. Disk electrophoresis was run in small columns of polyacrylamide gel by the procedure of Reisfeld, Lewis, and Williams (19). Photographs of the stained gels were taken with a Polaroid camera with the use of the setup described by Burns and Pollak (20).

Amino Acid Composition—Aliquots (0.1 ml) of a 3% solution of protein were hydrolyzed with 6 N HCl in evacuated, sealed tubes immersed in boiling toluene (110.8°) for periods of 12, 24, 44, and 66 hours. The hydrolysates were dried in a vacuum over pellets of NaOH, reconstituted in 5 ml of 0.2 M citrate buffer, pH 2.2, and 2-ml aliquots were applied to a Beckman/Spinco model 120 amino acid analyzer (21). Aliquots of the same protein solution were removed for the determination of total nitrogen (16), amide nitrogen (22), tryptophan (23, 24), and cysteic acid (25).

NH₂-terminal Amino Acid—The procedures described by Fraenkel-Conrat, Harris, and Levy (26) were used for the determination of NH₂-terminal amino acids after dinitrophenylation of the protein. Positive identification of the dinitrophenyl amino acids was made by hydrolysis with Ba(OH)₂ (27) followed by amino acid analysis.

RESULTS AND DISCUSSION

Chromatography on CM-cellulose—Chromatography on CM-cellulose has been used by a number of workers (6, 7, 9, 10, 28) for assessing the homogeneity of preparations of trypsin and trypsinogen. As shown in Fig. 2, when the twice crystallized preparation of porcine trypsin was applied to a column of CM-cellulose and eluted with 0.05 M citrate buffer, pH 5.0, a small amount of inactive material, constituting about 6% of the total protein, was not retained on the column. The remainder of the protein, however, emerged as a single peak when the pH of the buffer was raised to 5.5. The fact that each of the fractions comprising the active peak had very nearly the same specific activity is a good indication that this component was homogeneous. Rechromatography of this active component under the same conditions yielded a single peak which was located in essentially the same position. It would appear, therefore, that the small amount of impurity noted in the twice crystallized preparation was a true contaminant and did not arise as a consequence of autolysis during the course of chromatography as suggested by Charles et al. (10).

The homogeneity of the active component isolated by chromatography was verified by disk electrophoresis which indicated a single band (Fig. 3B). A twice crystallized preparation of beef
trypsin (Worthington) run at the same time for comparison showed the presence of two components (Fig. 3A). Reisfeld et al. (19) have likewise reported that their preparation of crystalline beef trypsin exhibited a second component in disk electrophoresis which they identified as chymotrypsin. The chymotryptic activity of our twice crystallized preparation of porcine trypsin was equivalent to a chymotrypsin level of 1.3%, a value which was reduced to 0.2% after chromatography. This low level of chymotryptic activity may represent the intrinsic ability of trypsin to hydrolyze chymotrypsin substrates (29) rather than contamination of the trypsin preparation with chymotrypsin.

Table I shows a typical purification of porcine trypsin including the final step involving chromatography in CM-cellulose. Since purity was of paramount concern in these studies, despite the low yield of the chromatographically purified enzyme (about 7% of the total activity present in the original filter cake), the column-purified material was employed in all subsequent experiments reported in this paper.

**Sedimentation Coefficient**—As depicted in the photographs shown in Fig. 4, porcine trypsin sedimented as a single symmetrical component when subjected to high speed centrifugation. A sedimentation coefficient (s20,w) of 2.77 S was calculated from the data provided by these pictures. This value may be compared to that of 2.5 S reported for bovine trypsin (30).

**Molecular Weight Determined by Sedimentation Equilibrium**—Previous studies with porcine trypsin have assumed its molecular weight to be the same as that of bovine trypsin, namely 24,000 (7). In the case of porcine trypsinogen, a molecular weight of 25,000 was calculated from its amino acid composition (10). Since a determination of the molecular weight by some physical method would obviously be desirable, an approach to sedimentation equilibrium method was conducted by the Archibald procedure as modified by Trautman (17) and Erlander and Foster (18). A value of $2.67 \times 10^{-7}$ was obtained for S/D, and this

![Fig. 3. Disk electrophoresis of beef and porcine trypsins. A, twice crystallized bovine trypsin (Worthington); B, twice crystallized porcine trypsin purified by chromatography on CM-cellulose.](http://www.jbc.org/)

![Fig. 4. Ultracentrifuge pattern of porcine trypsin. Twice crystallized porcine trypsin which had been purified by chromatography on CM-cellulose was run at 50,780 r.p.m. at a concentration of 1% in 0.005 M citrate buffer, pH 3.2. Photographs, from left to right, were taken at intervals of 8 minutes after full speed was attained. Temperatures: initial, 23.2°; final, 23.3°.](http://www.jbc.org/)
was employed in the Svedberg equation to calculate $M_{\text{app}}$, the apparent molecular weight of the protein

$$M_{\text{app}} = \frac{RT}{(1 - \varepsilon_0)} \left( \frac{S}{D} \right)$$

in which the letters and symbols have their usual significance.

The partial specific volume of the protein, $\varepsilon$, was calculated from the amino acid composition shown in Table II and the partial specific volumes of the individual amino acid residues as given by McMeekin, Grover, and Hipp (31). The $M_{\text{app}}$ thus cal-

**FIG. 5.** Electrophoresis pattern of porcine trypsin at pH 10.2. A 1% solution of twice crystallized and chromatographically purified porcine trypsin in 0.1 M glycine-0.05 M CaCl$_2$ buffer, pH 10.2, run for 450 minutes. Upper and lower patterns are the descending and ascending patterns, respectively.

**FIG. 6.** Electrophoretic mobility of porcine trypsin as a function of pH. Twice crystallized and chromatographically purified porcine trypsin in 1% solutions was run in 0.1 M diethylbarbiturate buffer, pH 9.2, 10.2, and 10.5. All buffers contained 0.05 M CaCl$_2$ to minimize autolysis. Field strengths varied from 3.46 to 3.90 volts per cm over a period of 4 to 7½ hours. Calculations of mobility were made from photographs which were projected with enlargement onto graph paper where they were traced manually.

### Table II

*Amino acid composition of porcine trypsin and comparison with bovine trypsin*

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Hydrolysis time</th>
<th>Amount</th>
<th>Amino acid residues per 100 g of protein</th>
<th>Nitrogen</th>
<th>Amino acid residues per 23,400 g of protein $^a$</th>
<th>Literature values $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 hrs</td>
<td>24 hrs</td>
<td>44 hrs</td>
<td>66 hrs</td>
<td>%</td>
<td>g of protein</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.676</td>
<td>0.655</td>
<td>0.664</td>
<td>0.673</td>
<td>0.664$^d$</td>
<td>4.72</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.151</td>
<td>0.183</td>
<td>0.165</td>
<td>0.167</td>
<td>0.187$^*</td>
<td>2.60</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.751</td>
<td>0.753</td>
<td>0.758</td>
<td>0.749</td>
<td>0.761$^d$</td>
<td>9.13</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.150</td>
<td>0.149</td>
<td>0.163</td>
<td>0.160</td>
<td>0.158$^d$</td>
<td>2.12</td>
</tr>
<tr>
<td>Asparagine</td>
<td>0.615</td>
<td>0.502</td>
<td>0.492</td>
<td>0.502</td>
<td>0.492$^d$</td>
<td>7.14</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.170</td>
<td>0.115</td>
<td>0.163</td>
<td>0.160</td>
<td>0.158$^d$</td>
<td>6.59</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.583</td>
<td>0.605</td>
<td>0.612</td>
<td>0.605</td>
<td>0.605$^d$</td>
<td>5.21</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.000</td>
<td>0.080</td>
<td>0.073</td>
<td>0.077</td>
<td>0.078$^d$</td>
<td>1.06</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.179</td>
<td>0.169</td>
<td>0.177</td>
<td>0.172</td>
<td>0.175$^d$</td>
<td>2.57</td>
</tr>
<tr>
<td>Proline</td>
<td>0.440</td>
<td>0.367</td>
<td>0.398</td>
<td>0.399</td>
<td>0.404$^d$</td>
<td>3.35</td>
</tr>
<tr>
<td>Serine</td>
<td>0.083</td>
<td>0.090</td>
<td>0.076</td>
<td>0.069</td>
<td>0.104$^e$</td>
<td>9.60</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.454</td>
<td>0.384</td>
<td>0.363</td>
<td>0.340</td>
<td>0.347$^d$</td>
<td>4.70</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.364</td>
<td>0.345</td>
<td>0.334</td>
<td>0.344</td>
<td>0.344$^d$</td>
<td>5.34</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.233</td>
<td>0.236</td>
<td>0.238</td>
<td>0.236</td>
<td>0.236$^d$</td>
<td>4.33</td>
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<tr>
<td>Valine</td>
<td>0.532</td>
<td>0.678</td>
<td>0.680</td>
<td>0.684</td>
<td>0.684$^d$</td>
<td>6.85</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.160</td>
<td>0.180</td>
<td>0.180</td>
<td>0.180</td>
<td>0.180$^d$</td>
<td>4.80</td>
</tr>
<tr>
<td>Total</td>
<td>97.40</td>
<td>102.20</td>
<td>219</td>
<td>221</td>
<td>222</td>
<td></td>
</tr>
</tbody>
</table>

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$^a$ Numbers in parentheses are number of residues to the nearest integer.

$^b$ Literature citations shown in parentheses.

$^c$ Trypsinogen data corrected for the release of an octapeptide (Phe-Pro-Thr-(Asp)-Lys) and a hexapeptide (Val-(Asp)-Lys) during the activation of the porcine and bovine zymogens, respectively (33).

$^d$ Average of values obtained for each period of hydrolysis.

$^e$ Maximum values obtained.

$^f$ Determined as cysteic acid after 22 hours of hydrolysis.

$^g$ Value extrapolated to zero time.

$^h$ Average of values of 0.264 by colorimetric method (23) and 0.220 by spectrophotometric method (24).

$^i$ Determined by the method of Hirs et al. (22).
calculated was 23,400 which is only slightly lower than the generally accepted value of 23,800 for beef trypsin (2).

Moving Boundary Electrophoresis—In Fig. 5 is shown a photograph of a representative electrophoretic pattern of porcine trypsin run at pH 10.2. The high degree of homogeneity which is apparent at this pH was also observed at pH values of 7.9, 9.2, and 10.5. A plot of electrophoretic mobility as a function of pH is shown in Fig. 6 in which an isoelectric point of 10.8 may be obtained by extrapolation to zero mobility. This value is somewhat higher than the value of 10.2 reported for porcine trypsin by Buck et al. (7) but agrees quite well with the isoelectric points of 10.4 to 10.8 which have been reported for beef trypsin (2, 7).

Amino Acid Composition—In Table II are summarized data pertaining to the amino acid composition of porcine trypsin as determined in this laboratory as well as the data for porcine and bovine trypsins reported by other investigators. Our composition data in general agree quite closely with those of Desnuelle's group (10), the principal difference being that we found six fewer aspartic acid residues. The average molecular weight calculated from our composition data and those of Desnuelle is approximately 23,000 which is in good agreement with the value of 23,400 determined by sedimentation equilibrium. The molecular weight of beef trypsin as calculated from the amino acid data of Holmman (32) gives a value of about 23,300 which is lower than the figure of 23,800 to 24,000 generally assigned to this enzyme (2, 7). A comparison of the composition of porcine and bovine trypsins shows notable differences in individual amino acids. In spite of this difference in the distribution of individual amino acids, the molecular weight of porcine trypsin seems to be almost identical with that of beef trypsin.

NH2-terminal Group—The homogeneity of our preparation of porcine trypsin was further verified by the finding that only dinitrophenyl isoleucine was recovered from the protein. This result confirms the report that both porcine and bovine trypsins have isoleucine as the NH2-terminal amino acid residue (10, 33). Both enzymes have also been reported to have a COOH-terminal aspartic acid residue. The average molecular weight calculated from our composition data and those of Desnuelle is approximately 23,000 which is in good agreement with the value of 23,400 determined by sedimentation equilibrium, was 23,400. Although bovine and porcine trypsins are very similar with respect to size, charge (isoelectric point, pH 10.8), and NH2-terminal residue (isoleucine), a significant difference in amino acid composition was noted.

Acknowledgment—We wish to acknowledge the invaluable assistance of Dr. Ronald Roberts in the use of the ultracentrifuge and in the calculations pertaining to the determination of molecular weight by an approach to sedimentation equilibrium.

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