Crystallization and Characterization of an Esteroproteolytic Enzyme from Porcine Pancreas*

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An earlier paper (1) from this laboratory described the presence of a proesterase in porcine pancreas which could be activated by trypsin. The activity of the esterase toward esters of α-hydroxybenzoic acid was studied. The impure enzyme appeared to be different from other pancreatic enzymes known to exist as zymogens: the chymotrypsins, the carboxypeptidases, trypsin from bovine pancreas, and elastase from porcine pancreas. This report describes the isolation and crystallization of the active enzyme and a preliminary study of some of its properties. Crystalline preparations of the enzyme have been found to possess proteolytic as well as esteratic activity. An estimate of the Michaelis constant, $K_m$, and specific activity determinations of the enzyme the procedure described earlier (1) was used. For purified preparations of the enzyme the activity was determined by a continuous method as follows. One milliliter of the enzyme solution was placed in a 25-ml Erlenmeyer flask containing 3 ml of 0.4 M Tris buffer at pH 8.65, and 5 ml of water were added. The solution was equilibrated in a water bath at 37°C. One milliliter of a freshly prepared solution of substrate, 0.15 M in 50% ethanol, was then added. An aliquot of the mixture was used for the determination of optical density at 300 nm due to substances other than reaction products. Except at high enzyme concentrations, the rate of hydrolysis remained constant for 30 minutes.

Determinations of esterase activity with N-acetyl-L-tyrosine ethyl ester as substrate were done by the pH-Stat method (3). The equipment used was a Radiometer unit consisting of a Titirgraph (SBR2/SBU1) and a Type III automatic titrator. The substrate was dissolved in water and the final volume of the reaction mixture was 15 ml. No calcium salt was added. The titrating agent was 0.08 N NaOH. The pH of the reaction mixture was maintained between 8.05 and 8.10 at 37°C. No corrections were made for spontaneous hydrolysis, which was negligible. The rate of hydrolysis was determined from the slope of the curve during the first minute of reaction.

Proteolytic activity was determined with casein and poly-L-glutamic acid as substrates. With casein as substrate the proteolytic activity was determined by the Kunitz method (4) as modified by Wu and Laskowski (5).

Crystalline as well as amorphous preparations of the esterase catalyze the hydrolysis of poly-$\text{L}$-glutamic acid. The optimal pH for the hydrolysis of this substrate is between 4.6 and 5.6. The kinetics of hydrolysis was studied with the pH-Stat at pH 5.3 and at a temperature of 37°C by titration with 0.1 N NaOH, and by paper chromatography with a mixture of butanol, acetic acid, and water as the solvent. The reaction mixture contained 20 mg of the poly-$\text{L}$-glutamic acid and 300 $\mu$g of enzyme protein in 5 ml. Aliquots of the hydrolysate were applied to paper strips by a stripping technique. The paper strips were then developed by three successive passages of the solvent. The chromatograms after treatment with ninhydrin followed by Cu(NO$_3$)$_2$ were scanned with a Photovolt Varicord integrator to determine the relative amounts of components formed. Aliquots of the hydrolysate chromatographed at various times

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1 A detailed study of the enzymatic hydrolysis of poly-$\text{L}$-glutamic acid will be the subject of a later communication.
during the reaction always showed ten to twelve components, none of which was free glutamic acid. Both chromatographic and titration data showed that after 20 minutes under the above conditions, no further degradation occurred.

**Electrophoresis**—Electrophoretic analyses were done by the starch gel method of Smithies (6), and by the free boundary method.

In the starch gel procedures 0.02 M borate buffer, pH 9.0, was used to prepare the starch gel. The pH of the finished slab was between 8.4 and 8.6. Preparations of protein were applied to a slit in the horizontal slab by means of filter paper strips. The voltage gradient was approximately 5 volts per cm, and the time of electrophoresis was 15 to 17 hours. The temperature during electrophoresis was maintained between 2 and 5° by the circulation of fluid from a low temperature ethanol-water bath through the bed of the starch slab.

Fractionation of pancreatic extracts on the DEAE-cellulose column was followed routinely by means of starch gel electrophoresis. In order to determine which of the components was active, assays were made on extracts from the starch slab. Sections in an unstained slab corresponding to protein sections in a stained slab were removed and homogenized in a Potter-Elvehjem homogenizer with Tris buffer, pH 8.6, at 0-5°. The suspension was centrifuged at 10,000 × g for 20 minutes at 0°, which removed almost all the starch, and the supernatant fluid was assayed using valeryl salicylate as substrate.

Acetate buffers were used at various pH values and all at an ionic strength of 0.1. The temperature was 1.5°, and the potential gradient varied from 5.32 to 5.83 volts per cm. All operations were performed at 0-5°. A single mixing chamber with a volume of 500 ml was used, and all operations were performed at 0-5°. The pH 3.6 extract from 700 g of porcine pancreas was adjusted to pH 6.0 and centrifuged. The supernatant liquid, 2550 ml, was then added to the column. The buffers and their sequence were 1 liter of potassium phosphate buffer at pH 6.0 and ionic strength 0.005, and the following sodium acetate buffers (all at ionic strength 0.1): 4 liters at pH 5.6, 3 liters at pH 4.8, 0.5 liter at pH 4.5, and 3 liters at pH 3.9. The flow rate was 10 to 20 ml per minute until the effluent pH had decreased to 4.5, at which time the collection of 18-ml samples was begun, and the flow rate was decreased to 5 to 10 ml per minute. With the above sequence of buffers, the enzyme emerges from the column as the pH of the effluent decreases below 4.5. The location of the enzyme in the effluent fractions was determined simply by optical density readings at 280 μm.

The fractions containing the enzyme were combined and the solution was saturated with (NH₄)₂SO₄ or NaCl. The resulting precipitate was suspended in 20 to 25 ml of water in a Visking cellulose sac and dialyzed against water at 0-5° to remove the salt.

In order to investigate the active fraction further, it was again chromatographed on a DEAE-cellulose column. The dimensions of the column were 45 × 1.2 cm; it contained 4.0 to 4.1 of DEAE-cellulose. The column was equilibrated first with a pH 6.0 phosphate buffer, ionic strength 0.005. The active fraction (55 ml, pH 6.0) containing 28.64 mg of N was then added. The effluent pattern was developed with a series of acetate buffers from pH 5.5 to 4.0, all at ionic strength 0.1. A single mixing chamber with a volume of 500 ml was used. The results of such an experiment are shown in Fig. 1. Several of the fractions were combined as indicated in Fig. 1. In each combined fraction total nitrogen was determined, as well as specific activity toward the substrates, valeryl salicylate, N-acetyl-L-tyrosine ethyl ester, and casein. Table I shows the specific activities and recoveries of activities in the combined fractions.

**Crystallization of Enzyme**—Enzyme preparations from the preparative column have been crystallized a number of times. The crystallization is performed as follows. A suspension of the enzyme, which had been dialyzed exhaustively against water, and which contained 5 to 10 mg of protein per ml, is brought into solution by adjusting the pH to 5.8 to 6.2 by the addition of a few drops of borate buffer (pH 7.85, 0.1 M). The solution is filtered and then dialyzed against acetate buffer, pH 4.2 and ionic strength 0.005. The dialyze after a few hours yields a heavy opalescent suspension which, after several weeks at 0°
and frequent changes of the buffer, gradually changes into large
crystals (Fig. 2). Crystallization was also obtained in the presence
of ammonium sulfate at pH 4.3; needles were formed. However, in a number of attempts to repeat this type of crystal-
ization, only occasionally were we successful.

Different preparations of the enzyme crystallized under
identical conditions have yielded crystals as depicted in Fig. 2.
The electrophoretic properties, as well as activities toward the
various substrates, appear to be the same for all the crystalline
preparations.

RESULTS AND DISCUSSION

An effluent diagram obtained by the chromatography of the
active fraction is shown in Fig. 1. Inspection of this figure shows
that practically all the protein is eluted within a pH range of 4.6
to 4.3. The main component (Fractions V and VI) accounts
for 50% of the total nitrogen added to the column. This com-
ponent has been collected in several experiments and crystallized
(Fig. 2). The specific activities, as well as recoveries of activities
in the fractions indicated in Fig. 1, are shown in Table I. The
data indicate that no further separation of the enzymatic ac-
tivities, present in the active fraction, was obtained by a re-
chromatography; this suggests that the activity toward these
substrates is associated with the same protein or proteins. These
data show the possibility that the enzyme may exist in several
forms. Fig. 3 shows the effluent diagram obtained by chroma-
tography of the esterase which had been subjected to three
crystallizations. The column and elution procedures were the
same as those in Fig. 1 and are described in the text above. The
pattern suggests some heterogeneity, which may be due to
anomalous effects of the column. This result is in contrast to the
electrophoretic analysis (see below). In the latter case both
starch gel and free boundary electrophoreses indicate the prep-
arations to be homogeneous after three crystallizations. The
experimental work to date leads us to believe that this hetero-
genicity may be due to slightly different forms of the same enzyme.

The heterogeneity could be caused by the autoproteolytic effects
of the enzyme. Such effects are known to occur with \alpha\-chymo-
trypsin, giving rise to several forms of the same enzyme (7).

### Table I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Active fraction</th>
<th>I</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>VI</th>
<th>VII</th>
<th>VIII</th>
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<tbody>
<tr>
<td>Specific activity *</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valeryl salicylate X 10^{-3}</td>
<td>9.9</td>
<td>1.7</td>
<td>15.4</td>
<td>14.9</td>
<td>13.9</td>
<td>13.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-L-tyrosine ethyl ester X 10^{-3}</td>
<td>3.6</td>
<td>0.8</td>
<td>4.5</td>
<td>5.0</td>
<td>5.4</td>
<td>4.7</td>
<td>5.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein X 10^{3}</td>
<td>13.7</td>
<td>2.6</td>
<td>13.3</td>
<td>15.8</td>
<td>17.5</td>
<td>15.8</td>
<td>19.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% activity recovered</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valeryl salicylate</td>
<td>100</td>
<td>1</td>
<td>10</td>
<td>38</td>
<td>32</td>
<td>7</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Acetyl-L-tyrosine ethyl ester</td>
<td>100</td>
<td>1</td>
<td>12</td>
<td>34</td>
<td>31</td>
<td>7</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>100</td>
<td>1</td>
<td>10</td>
<td>28</td>
<td>27</td>
<td>6</td>
<td>10</td>
<td></td>
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</tr>
</tbody>
</table>

* Expressed as micromoles hydrolyzed per liter per 30 minutes per
  mg of N per ml.

† Expressed according to Kunitz, per μg of N.

Fig. 2. Crystals of the esterase after three crystallizations. A, crystals from active fraction from preparative column. Magnification, 70 X. B, crystals obtained after a chromatography of the same active fraction (Fractions V and VI, see Fig. 1). Magnification, 42 X.
In Table II the recovery and purification data of a fractionation on the preparative column are shown, beginning with the homogenate of pancreas and terminating with the preparation which had been crystallized three times. The results are typical and consistent with those of a large number of fractionation experiments.

Table III shows the specific activity of the active fraction, and of the enzyme after three crystallizations, toward the four substrates used throughout this investigation. The data indicate clearly that the specific activity after crystallization increases proportionately for each of the substrates. These results support the conclusions derived from the data in the rechromatography experiment described above; namely, that the activity toward these substrates is associated with the same enzyme protein.

Fig. 4 shows the electrophoretic behavior of a preparation of the esterase which had been crystallized three times, in free boundary and in starch gel electrophoresis. Both methods of electrophoresis indicate a homogeneous preparation. The low isoelectric point, 4.1 to 4.2, estimated from a plot of pH against mobility (Fig. 5) agrees well with the elution behavior of the enzyme on the DEAE-cellulose column.

The isoelectric point of the esterase is well below that of the known proteolytic enzymes. Trypsin, α-chymotrypsin, and chymotrypsin B, all from bovine pancreas, have isoelectric points of 10.8 (8), 8.3 (9), and 4.7 (9), respectively. Elastase from porcine pancreas has an isoelectric point of 9.5 (10, 11). Starch gel electrophoresis of trypsin and α-chymotrypsin at pH 8.4, with or without previous incubation with diisopropyl fluorophosphate, showed trypsin to be cationic and α-chymotrypsin to be essentially neutral. Chymotrypsin B from bovine pancreas, with a known isoelectric point of 4.7 (9), was studied in detail and compared with the esterase enzyme. Mixtures of the two enzymes subjected to electrophoresis at pH 8.5 yielded two well separated protein zones, with the esterase having the greater mobility. Electrophoresis after incubation with diisopropyl fluorophosphate yielded the same results.
Fig. 6 shows the specific activity of the esterase, α-chymotrypsin, and chymotrypsin B toward the substrate, valeryl salicylate. The data for chymotrypsin B were obtained with crystalline chymotrypsin B and crystalline chymotrypsinogen B. The latter was activated as described by Laskowski. The protein concentrations of chymotrypsin B and chymotrypsinogen B were determined from optical density data in conjunction with concentration factors recorded in the literature. Although both α-chymotrypsin and chymotrypsin B show some activity, it is of such a low order of magnitude that the $K_m$ could not be calculated with any degree of certainty from the Lineweaver-Burk plot. $K_m$ for the esterase, at pH 8.63 in 5% ethanol and at 37°, was calculated to be $2.7 \times 10^{-3}$ (Table IV).

N-Acetyl-L-tyrosine ethyl ester, a substrate which has been used extensively in kinetic studies of the chymotrypsins, is hydrolyzed by the esterase. Table IV shows the $K_m$ values of α-chymotrypsin and the esterase obtained with this substrate using the pH-Stat method. Experiments at different concentrations of substrate and enzymes have shown α-chymotrypsin to have a specific activity 4 to 5 times that of the esterase.

The esterase has proteolytic activity as determined by the Kunitz method with casein as the substrate. The proteolytic activity of the esterase toward casein is less than that of α-chymotrypsin (Table IV).

An interesting feature of the enzyme is its ability to hydrolyze rapidly poly-L-glutamic acid. This is in marked contrast to α-chymotrypsin and trypsin, both from bovine pancreas, which do not attack this substrate to any significant extent. The degradation products are peptides and no detectable amount of free glutamic acid is formed. The enzyme carboxypeptidase does hydrolyze poly-L-glutamic acid; however, the only product formed is glutamic acid. Work with crude pancreatic extracts has suggested the presence of an enzyme other than carboxypeptidase which does hydrolyze poly-L-glutamic acid. The pH optimum for the hydrolysis of poly-L-glutamic acid by the esterase is close to 5.3, which is well below the optimum for the hydrolysis of acetyl-L-tyrosine ethyl ester, pH 7.5, and valeryl salicylate, pH 8 to 9. A number of other poly-L-amino acids were studied as substrates for the enzyme; in all cases the hydrolysis was negligible. It is of interest that poly-n-aspartic acid was hydrolyzed very slowly if at all.

The behavior of the enzyme on the DEAE-cellulose column, as well as its electrophoretic and kinetic properties, suggests that it is different from the well characterized enzymes obtained to date from bovine and porcine pancreas. We are aware that some of these differences may be due to species protein specificity. The enzyme may be related to the chymotrypsins, particularly chymotrypsin B. This is presently being investigated by a detailed study of the molecular properties of the enzyme.

The possibility that the enzyme is an artifact produced during the preparative procedure does not seem likely. Preparations of the enzyme obtained by the present method, but under conditions deviating from those reported in this paper, appeared to be identical with those described here. Fractionation of the pancreatic extract with ethanol at low temperatures (−20°) yielded preparations of the enzyme with electrophoretic and kinetic properties that were in accord with those prepared by the column method.

* M. Laskowski, personal communication.
acid at pH 5.3, a substrate that is not attacked significantly by trypsin or \( \alpha \)-chymotrypsin.

REFERENCES

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