Carboxypeptidase B

IV. PURIFICATION AND CHARACTERIZATION OF THE PORCINE ENZYME

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Several preceding reports from these laboratories (2-5) and elsewhere (6-8) have defined carboxypeptidase B as a pancreatic proteolytic enzyme capable of rapidly hydrolyzing peptide bonds to release certain carboxyl-terminal basic amino acids from peptides and proteins. The enzyme has been partially purified in the form of its inactive zymogen from bovine pancreas glands (3, 8).

In the present communication a convenient procedure for the preparation of purified active carboxypeptidase B from autolyzed porcine pancreas tissue is given in detail. Some of the chemical and molecular properties of this enzyme are also presented. A sensitive and convenient spectrophotometric assay for carboxypeptidase B activity is outlined.

EXPERIMENTAL PROCEDURE AND RESULTS

Assay Procedure and Definition of Units—The method employed to measure carboxypeptidase B activity is based on the difference spectra of hippuric acid relative to hippuryl-L-arginine. Procedures similar to the present one have been employed to estimate trypsin and chymotryptic activities (9). When the absorbancy of a 0.001 m solution of hippuric acid in 0.025 M Tris, pH 7.65, containing 0.1 M NaCl, was measured against a blank consisting of a 0.001 m solution of hippuryl-L-arginine (3) in the same buffered salt solution, a broad peak was observed in the ultraviolet region with a maximum at 254 mp. At this wave length the difference in absorbancy was 0.36 (1-cm cells).

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Routine assays were carried out in the following manner: 3 ml samples of a 0.001 m solution (prepared fresh daily) of hippuryl-L-arginine in buffered salt solution were placed in 1-cm quartz cells in the Beckman model DU spectrophotometer and the absorbancy was set to zero at 254 nm (slit width 0.4, photomultiplier at sensitivity position 4). Enzyme solution was introduced in a volume of from 5 to 20 μl, the absorbancy was immediately reset to zero with the sensitivity knob, and the increasing absorbancy was recorded at constant time for the desired length of time.

A solution of 0.001 m hippuric acid in buffered salt solution was routinely used as a reference standard during enzyme runs.

All assays were conducted at room temperature (25°). Apparent zero order kinetics was observed up to about 40% hydrolysis. Enzyme concentration was adjusted so as not to exceed 30% hydrolysis within 10 minutes. A plot of change in absorbancy at 254 nm with time for several enzyme concentrations is shown in Fig. 1. It is apparent from the data shown in this figure that the rate of substrate hydrolysis is directly proportional to enzyme concentration within the range employed.

Protein concentration was estimated by the trichloroacetic acid turbidimetric procedure (11) or from nondializable total nitrogen (micro-Kjeldahl) values. Units of activity are defined as the percent hydrolysis per minute at the 0.001 m substrate concentration, and specific activity is expressed as units per mg of protein.

Purification Procedure

Preparation of Acetone Powders—Frozen whole swine pancreas glands were cut into slices of approximately 5 mm thickness and spread in enameled trays to a depth of 2 to 4 cm. The trays were covered with heavy paper and the tissue was allowed to autolyze for 16 hours at room temperature. The entire autolysate was extracted with 4 volumes of acetone at room temperature for 1 minute in a Waring Blender at high speed and filtered rapidly with suction. The residue was re-extracted successively in the Blender twice with 4 volumes of acetone, once with 4 volumes of acetone-ether (1:1 by volume), and once with 4 volumes of ether and was spread out to dry at room temperature. These powders of auto-lyzed swine pancreas have been stored at 4° for several months without loss of carboxypeptidase B activity.

Preparation of Extracts—Fifty grams of the powder were extracted with 1 liter of distilled water at room temperature by gently swirling for 15 minutes in a 2-liter Erlenmeyer flask. The extract was then centrifuged and the supernatant was dialyzed against water in a cold room for 24 hours and the dialyzate was frozen at -15° and lyophilized. The lyophilized powder was used as a source of the enzyme.

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solved in 40 to 50 ml of 0.05 M ammonium sulfate (209 g per liter). The pH was maintained between 7.0 and 7.2 by the gradual addition of solid ammonium sulfate.

After a 30-minute equilibration period, the precipitate was collected by centrifugation (30 minutes at 14,600 \( \times g \)). The precipitate, dissolved in 40 to 50 ml of 0.05 M Tris, pH 7.25, was dialyzed overnight against distilled water.

**Ammonium Sulfate Fractionation**—The extract was cooled to 0 to 2°C (all subsequent operations were carried out at this temperature) and the pH adjusted to 7.0 to 7.2 with NaOH. Solid ammonium sulfate was gradually added with stirring to 0.35 saturation (289 g per liter). The pH was maintained between 7.0 and 7.2 by the addition of NaOH. The suspension was allowed to equilibrate for 30 minutes and the precipitate removed by centrifugation (15 minutes at 14,600 \( \times g \)). With the pH maintained as above, the supernatant fluid was brought to 0.6 saturation by the gradual addition of solid ammonium sulfate (146 g per liter). The suspension was stirred for 30 minutes and centrifuged (30 minutes at 14,600 \( \times g \)). The precipitate, dissolved in 40 to 50 ml of 0.05 M Tris, pH 7.25, was dialyzed overnight against distilled water.

**Batch Absorption and Elution from Diethylaminoethyl Cellulose**—After dialysis, one-ninth volume of 0.05 M Tris, pH 7.25, was added to the protein solution and the pH was adjusted to 7.25. Twenty-five grams of DEAE-cellulose (12) (0.72 to 0.82 meq per g equilibrated with 0.005 M Tris, pH 7.25, and sucked to damp-dryness on a Büchner funnel) were added and the mixture was stirred for 20 minutes. The cellulosic adsorbent was collected by suction filtration and washed four times by stirring for 2 to 3 minutes with 150 ml portions of 0.005 M Tris, pH 7.25. Finally, the enzyme was eluted from the adsorbent by stirring for 2 to 3 minutes with one 100 ml and two 50 ml portions of 0.1 M NaCl in 0.005 M Tris, pH 7.25. The combined eluates were brought to 0.65 saturation (43 g per 100 ml) by the gradual addition of solid ammonium sulfate without pH adjustment. After a 30-minute equilibration period, the precipitate was collected by centrifugation (30 minutes at 14,600 \( \times g \)) and suspended in 4 to 5 ml of 0.05 M Tris, pH 7.25. Upon dialysis against distilled water the protein went into solution. Usually a small amount of protein precipitated from solution during longer (overnight) dialysis and was removed by centrifugation (10 minutes at 10,000 \( \times g \)). Substantially all of the enzyme activity remained in the supernatant fluid. These protein solutions contained carboxypeptidase B which had approximately 85% of the maximal enzymatic activity. Storage of the frozen solutions at \(-10^\circ\) for as long as 3 months resulted in no loss of enzyme activity.

**Chromatography on DEAE-cellulose**—Batch extraction and elution were employed before column chromatography as a means of overcoming mechanical difficulties in operation of columns (excessive volumes of eluates and clogging of columns due to large quantities of strongly adsorbed material). The results of a small scale run are plotted in Fig. 2. Since contaminating proteins are eluted in close proximity to carboxypeptidase B, it is necessary to calculate specific activities of fractions and combine only those of high activity.

In the preparative procedure, 5 to 8 ml (150 to 200 mg of protein) of the carboxypeptidase B solution from the batch DEAE-cellulose step were placed on a \( 2 \times 20 \)-cm column of DEAE-cellulose (0.72 to 0.82 meq per g) which had been previously equilibrated with 0.005 M Tris, pH 7.5. The protein solution was washed into the column with several milliliters of the same buffer and the chromatogram was developed with 500 ml of 0.005 M Tris, pH 7.5, with a linear gradient (13) of NaCl from 0 to 0.2 M. Five-milliliter fractions were collected at a flow rate of 5 ml per minute. Fractions with specific activity of 18,000 and above were combined and brought to 0.65 saturation with solid ammonium sulfate without pH adjustment. The precipitate obtained upon centrifuging this suspension was taken up in 2 to 3 ml of 0.05 M Tris, pH 7.25, and dialyzed against several changes of large volumes of distilled water. Alternatively, the precipitate obtained after ammonium sulfate addition was dissolved in a minimum volume of water (3 to 5 ml) and the protein was freed of dissociable salt by gel-filtration (14) on a \( 1.5 \times 25 \)-cm column of Sephadex which had been equilibrated with water.

These clear, colorless solutions of carboxypeptidase B have been stored frozen for several months at \(-10^\circ\) without loss in activity. Substantial losses (25 to 45%) in activity occur upon lyophilization. The lyophilized enzyme has been stored in the cold or at room temperature for 2 weeks without further loss in activity.

The experimental procedure was that described by Longsworth (17), except that the initial boundary was formed in the center of the Tiselius cell channel by careful layering of dialysate onto the protein solution. This necessitated much less washing of the channel during the boundary-sharpening process.

Two solutions of carboxypeptidase B, 0.376 and 0.230%, in 0.05 M potassium phosphate buffer, pH 7.02, each gave diffusion coefficients ($D_{20,w}$) of $8.16 \times 10^{-7}$ cm$^2$ sec$^{-1}$. No significant heterogeneity was evident from the shape of the distribution curve.

**Partial Specific Volume**—The pycnometric determination of

In Table I is presented a typical protocol which shows total recoveries of activity and enrichment. A 16- to 17-fold purification over extracts of the acetone powder of autolyzed tissue has been accomplished.

<table>
<thead>
<tr>
<th>Purification stage</th>
<th>Total units</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Total recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water extract of acetone powder*</td>
<td>12.084</td>
<td>10,742</td>
<td>1,125</td>
<td>100</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$ precipitate</td>
<td>7.493</td>
<td>1,810</td>
<td>4,140</td>
<td>62</td>
</tr>
<tr>
<td>DEAE-cellulose, batch treatment</td>
<td>5.786</td>
<td>413</td>
<td>14,010</td>
<td>48</td>
</tr>
<tr>
<td>Supernatant solution after dialysis</td>
<td>5.496</td>
<td>345</td>
<td>15,930</td>
<td>45</td>
</tr>
<tr>
<td>Chromatography on DEAE-cellulose</td>
<td>3.316</td>
<td>180</td>
<td>18,420</td>
<td>27</td>
</tr>
</tbody>
</table>

* One unit is defined as per cent of hydrolysis per minute at a 0.001 M substrate concentration.

† One hundred grams of acetone-dried powder of autolyzed porcine pancreas.

**Properties of Carboxypeptidase B**

**Spectral Characteristics**—Carboxypeptidase B displays a typical protein absorption spectrum in the ultraviolet range with a maximal extinction at 278 mp and a 0.57 ratio of absorbances at 260 and 280 mp, respectively. The absorbancy index at 278 mp of a 1% solution was found to be 21.4 and the molar absorbancy index is $7.35 \times 10^3$ based on the 34,300 molecular weight value reported below.

**Electrophoresis**—The results of a typical electrophoresis run of the purified material are shown in Fig. 3. Under the conditions specified in the legend there was no sign of other components, although material taken only through the ammonium sulfate precipitation step showed six different electrophoretic fractions. The mobility of the descending boundary at 0$^\circ$ was computed to be $-2.38 \times 10^{-3}$ cm$^2$ volt$^{-1}$ sec$^{-1}$, (0.05 M potassium phosphate buffer, pH 7.02).

**Ultracentrifugation**—Sedimentation runs were carried out in the Spinco model E ultracentrifuge equipped with a phase plate diaphragm and the thermistor rotor temperature control. Fig. 4 illustrates the type of boundary obtained during sedimentation and shows the lack of secondary components. Three different runs at concentrations of 1.40, 0.70, and 0.35% gave sedimentation constants ($s_{20,w}$) of 3.28, 3.24, and $3.23 \times 10^{13}$ sec$^{-1}$, respectively (0.05 M potassium phosphate buffer, pH 7.02).

**Diffusion**—Diffusion studies were made in the Aminoos model B electrophoresis apparatus with carefully selected cells and the Rayleigh interferometer. The bath temperature was 3.25 ± 0.02$, ^\circ$C, low enough to prevent degradation of the enzyme over the period of 2 to 3 days necessary for extensive boundary spreading.
partial specific volume was made on a 1.63% solution of the enzyme at 25.0°, and gave a value of 0.720 ± 0.005 cm³ per g. This value compares with that of 0.73, calculated from the amino acid composition (18).

Molecular Weight—With the Svedberg equation and the experimentally determined values recorded here, the molecular weight of porcine carboxypeptidase B was calculated to be 34,300 ± 600.

Amino Acid Composition—A sample of purified carboxypeptidase B, which had been desalted by gel filtration (14), was lyophilized, equilibrated at room temperature, and analyzed for water (105°, 18 hours under high vacuum), ash (constant weight at 800°) and nitrogen (micro-Kjeldahl). The following values were obtained: moisture 8.2%, ash 2.5%, nitrogen 15.5% (corrected for moisture and ash). Samples of approximately 2 mg were hydrolyzed with 3 ml of triple distilled 6 N HCl under nitrogen in sealed Pyrex glass tubes at 105° for 24, 48, and 72 hours. After removal of excess HCl by one evaporation to dryness under reduced pressure (rotary evaporator) at 40–50°, the entire 2 mg sample was washed into the ion exchange column for amino acid analysis.

The amino acid analyses were performed on an automatic amino acid analyzer (19). Tryptophan was determined on a separate unhydrolyzed sample by an ultraviolet spectrophotometric method (20).

The results of these analyses appear in Tables II and III. It is apparent from the data recorded in Table II that only small losses of threonine and serine occurred during hydrolysis, approximately 2% and 5%, respectively, in 24 hours. Losses of cystine and methionine were greater, approaching 10% in 24 hours. Isoleucine and valine were incompletely released in 48 hours. The 72-hour values were accepted, although these values may still be slightly low. Ammonia increased at a more rapid rate than could be accounted for by the destruction of threonine and serine. The source of the increasing ammonia is not known, although it could conceivably have been formed through the destruction of tryptophan which is present in relatively large amounts. However, the discrepancy was not large except at 72 hours. This value, therefore, was not included in the calculation of the results.

All of the nitrogen in the sample of carboxypeptidase B was accounted for as amino acids (Table III). However, the recovery by weight was several per cent low (Table III). This result together with the rather high ash value of 2.5% indicates the presence of a significant amount of nonprotein material. The sample whose analysis appears in Table III was prepared by gel filtration (14) which would readily remove any dissociable, low molecular weight material. Similar results were obtained with another sample prepared for analysis by exhaustive dialysis. Whether the nonprotein material represents an impurity or an integral part of the enzyme is not clear from the present data.

Although it is not possible to calculate a reliable molecular weight value from the amino acid composition, the data are consistent with the molecular weight of 34,300 calculated from physical measurements. This value has been used to estimate the number of moles of each amino acid per mole of enzyme (Table III).

Metal Content and Inhibition of Enzyme Activity by Metal-

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### Table II

<table>
<thead>
<tr>
<th>Residue</th>
<th>Average change per 72 hr</th>
<th>Correction</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 hr</td>
<td>48 hr</td>
<td>72 hr</td>
</tr>
<tr>
<td>Half cystine</td>
<td>23.5</td>
<td>22.3</td>
</tr>
<tr>
<td>Methionine</td>
<td>15.7</td>
<td>14.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>90.6</td>
<td>67.3</td>
</tr>
<tr>
<td>Serine</td>
<td>56.1</td>
<td>52.6</td>
</tr>
<tr>
<td>Ammonia</td>
<td>35.1</td>
<td>35.0</td>
</tr>
<tr>
<td>Valine</td>
<td>52.8</td>
<td>56.2</td>
</tr>
</tbody>
</table>

---

### Table III

<table>
<thead>
<tr>
<th>Amino acid composition of carboxypeptidase B</th>
<th>Moles per 10⁶ g</th>
<th>Residue % of total N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>90.7</td>
<td>10.43</td>
</tr>
<tr>
<td>Threonine</td>
<td>84.6</td>
<td>8.55</td>
</tr>
<tr>
<td>Serine</td>
<td>49.0</td>
<td>4.26</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>69.3</td>
<td>8.94</td>
</tr>
<tr>
<td>Proline</td>
<td>37.0</td>
<td>3.59</td>
</tr>
<tr>
<td>Glycine</td>
<td>64.4</td>
<td>3.67</td>
</tr>
<tr>
<td>Alanine</td>
<td>70.4</td>
<td>5.00</td>
</tr>
<tr>
<td>Half cystine</td>
<td>21.4</td>
<td>2.19</td>
</tr>
<tr>
<td>Valine</td>
<td>30.3</td>
<td>3.00</td>
</tr>
<tr>
<td>Methionine</td>
<td>14.4</td>
<td>1.89</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>48.2</td>
<td>5.45</td>
</tr>
<tr>
<td>Leucine</td>
<td>63.7</td>
<td>7.20</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>57.3</td>
<td>9.35</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>33.3</td>
<td>4.90</td>
</tr>
<tr>
<td>Lysine</td>
<td>49.0</td>
<td>6.28</td>
</tr>
<tr>
<td>Histidine</td>
<td>16.3</td>
<td>2.25</td>
</tr>
<tr>
<td>Arginine</td>
<td>28.1</td>
<td>4.39</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>25.9</td>
<td>4.82</td>
</tr>
<tr>
<td>Amide N</td>
<td>(77.8)</td>
<td>7.03</td>
</tr>
</tbody>
</table>

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*T All amino acids not listed did not change significantly.
* The comparison should be on an equal weight basis. However, the sampling errors were considerably larger than the very minor error introduced by employing these units.
* To be applied to the 24-hour hydrolysate.
* The increase in ammonia is greater than can be accounted for by the destruction of threonine and serine, particularly between 48 and 72 hours. For purposes of correction, the gain in the first 24 hours is assumed to be the same as in the second 24 hours.
* Total change.

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chelating Agents—Significant quantities of zinc only were found in purified samples of carboxypeptidase B upon examination by x-ray fluorescence and by emission spectroscopy. Zinc was determined qualitatively by a chemical procedure utilizing the trichloroacetic acid precipitation technique (21). Samples (10 to 20 mg) of three different preparations of comparable enzymatic activity were employed for analysis. Based on the molecular weight of 34,300, the ratios of moles of zinc to moles of carboxypeptidase B were found to be 0.98, 1.01, and 1.04 for the three samples.

The enzymatic activity of purified carboxypeptidase B was inhibited by the metal-chelating agents 1,10-phenanthroline, 8-hydroxyquinoline-5-sulfonic acid, and 2,2'-dipyridyl. Preliminary inhibition-concentration relationships: 1,10-phenanthroline produced 15, 40, and 60% inhibition at 0.33, 0.66, and 1.0 x 10^-4 M, respectively; 8-hydroxyquinoline-5-sulfonic acid produced 10, 31, and 62% inhibition at 0.8, 1.7 and 3.3 x 10^-4 M, respectively; and 2,2'-dipyridyl produced 30, 72, and 100% inhibition at 3.3, 5.0, and 6.6 x 10^-4 M, respectively.

Ethylendiaminetetraacetic acid (0.01 M) had no effect on enzyme activity. Addition of low concentrations of 2,2'-dipyridyl (6.6 x 10^-4 M) resulted in a 25% increase in rate of hydrolysis, possibly due to the selective removal of traces of inhibiting metals in either the enzyme preparation or in the assay solution. A similar observation has been reported (22) for another metalloenzyme, yeast alcohol dehydrogenase.

**Discussion**

Experimental data are presented in this communication which show that carboxypeptidase B, purified from swine pancreas tissue by the outlined procedure, behaves as a homogenous protein with respect to ion exchange chromatography, moving boundary electrophoresis, sedimentation, and free diffusion analysis.

Certain of the chemical and molecular characteristics of carboxypeptidase B are notable, particularly as compared to carboxypeptidase A. A possible similarity in mechanism of action of the two carboxypeptidases has been previously suggested (2, 3, 23). This suggestion is further supported by the present finding that, like carboxypeptidase A (24), carboxypeptidase B contains one nondialyzable gram atom of zinc per mole. Although these metal studies can be considered only preliminary, the present evidence is consistent with the possibility that zinc is a structural and a functional component of the enzyme which participates in its enzyme action.

The molecular weight of the B enzyme is identical to that reported for carboxypeptidase A (25, 26). There is also a remarkable similarity in amino acid composition of the two enzymes (for carboxypeptidase A, see (20)). There are, however, distinct differences in the content of certain amino acids. Carboxypeptidase B has about one-half as much serine and several times as much methionine as the A enzyme. The cystine content of the B enzyme indicates the presence of four disulfide bonds whereas carboxypeptidase A apparently has two disulfide bonds. This suggests a considerable difference in structure. There is also a significantly larger quantity of amide nitrogen in the B enzyme than reported for carboxypeptidase A. These differences may be related to the observation that carboxypeptidase A is practically insoluble in water (27), whereas the B enzyme is very water soluble.

**Summary**

1. A procedure was developed for the purification of carboxypeptidase B from an acetone powder of autolyzed swine pancreas tissue. It includes water extraction of acetone powder, a preliminary fractionation with ammonium sulfate, and chromatography on diethylaminoethyl cellulose.

2. A sensitive and convenient spectrophotometric assay for carboxypeptidase B is presented. It is based on the change in absorbancy at 254 mp of hippuryl-L-arginine solutions during hydrolysis.

3. Carboxypeptidase B behaves as a homogeneous protein with respect to several physical chemical criteria. The molecular weight, as calculated from sedimentation analyses and diffusion measurements, is 34,300 ± 600.

4. The complete amino acid composition was determined. It is similar in many respects to that of bovine carboxypeptidase A, but differs in the lower content of serine and the higher content of sulfur-containing amino acids and amide nitrogen.

5. Carboxypeptidase B contains 1 atom of zinc per molecule. Metal chelation studies suggest that this metal is a functional part of the enzyme.

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**References**


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