Simultaneous Purification of Carboxypeptidase A, Carboxypeptidase B and Chymotrypsin C from Autolyzed Porcine Pancreas Glands

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Carboxypeptidases A and B and chymotrypsin C from autolyzed porcine pancreas glands were simultaneously purified by extraction of acetone powder with water, ammonium sulfate precipitation, and chromatography on DEAE-cellulose. Then carboxypeptidases A and B were freed from contaminated endopeptidase such as elastase and chymotrypsin C by affinity chromatography on D-phenylalanine-CH-Sepharose 4B and D-arginine-CH-Sepharose 4B, respectively. Chymotrypsin C was freed from carboxypeptidases A and B by passage through D-phenylalanine-CH-Sepharose 4B. These carboxypeptidases A and B can be used for carboxyl-terminal analysis of peptides without prior treatment with diisopropyl phosphorofluoridate to inactivate contaminated pancreatic endopeptidases.

Keywords Carboxypeptidase, chymotrypsin, affinity chromatography, porcine pancreas glands

Carboxypeptidases A and B and chymotrypsin C from autolyzed porcine pancreas glands have been separately isolated by the methods of Folk et al.1-5 Bovine and porcine carboxypeptidases A and B purified by the usual methods including ion exchange chromatography are contaminated with a trace amount of pancreatic endopeptidases. Thus the treatment of carboxypeptidases A and B with diisopropyl phosphorofluoridate has been essential for the use of carboxyl-terminal analysis to inactivate the contaminated endopeptidases.4,6 In the last fifteen years, affinity chromatography has greatly facilitated the isolation of enzymes. This principle has also been applied to the isolation of carboxypeptidases A and B that are activated with trypsin from the proenzymes (zymogens) A and B.7-15 Commercial preparations of the bovine carboxypeptidase A from autolyzed pancreas glands are composed of several active forms which differ mainly in their amino-terminal regions.16 During the study of simultaneous purification of porcine carboxypeptidases A and B from autolyzed pancreas glands, we found that chymotrypsin C could also be purified at the same time. We report a simultaneous purification method for these three enzymes. The method consists of the following four steps: extraction of the autolyzed and dried pancreatic powder with water, fractionation of the extract with ammonium sulfate precipitation, separation of the precipitate by chromatography on DEAE-cellulose, and final purification of the proteases with affinity chromatography for carboxypeptidase A and chymotrypsin C on D-phenylalanine-CH-Sepharose 4B and for B on D-arginine-CH-Sepharose 4B.

Experimental

Materials
Z-Gly-LPhe-OH, Bz-Gly-LArg-OH, Bz-LAla-OMe, Tos-LArg-OMe, and Bz-LTyr-OEt were purchased from Peptide Laboratory (Osaka, Japan). DE-11 and DE-32 were from Whatman. CH-Sepharose 4B and CM-SephadeX C-50 were products from Pharmacia Fine Chemicals. Porcine elastase, twice recrystallized, was obtained from Worthington Biochemicals.

Enzymatic assays
Carboxypeptidase A activity toward Z-Gly-1Phe-OH was assayed by the method of Whitaker et al.17 Carboxypeptidase B activity toward Bz-Gly-LArg-OH was assayed by the method of Folk.4 Chymotrypsin C activity toward Bz-LLeu-OEt was assayed by a modification of the method of Folk.18 Elastase activity toward Bz-LAla-OMe was assayed as described by Shotton.19 One unit of activity was defined as the amount of enzyme that catalyzed the hydrolysis of one µmol substrate/min.

Determination of protein
Protein concentration in solutions was determined
by the measurement of the absorbance at 280 nm. The amounts of carboxypeptidases A and B, elastase, and chymotrypsin C were determined from the absorbance at 280 nm using the values of $E_{280}^\text{cm}=19.6$, 21.4, 22.2, and 25.0, respectively.1,3,19,18

Preparation of the adsorbents, $\alpha$-Phenylalanine-CH-Sepharose 4B and $\alpha$-Arginine-CH-Sepharose 4B

CH-Sepharose 4B was converted to the N-hydroxysuccinimide ester, which was coupled with either $\alpha$-phenylalanine or $\alpha$-arginine by the method of Cuatrecasas and Parikh.20 The $\alpha$-amino acid concentration of the gel was determined to be 5.01 mM for phenylalanine and 3.05 mM for arginine by amino acid analysis.

Purification of enzymes

Acetone powder was prepared from the autolyzed porcine pancreas glands.13 Acetone powder was extracted with water as described by Folk.5 The extract was precipitated at pH 7.1 with 0.6 saturated ammonium sulfate. The precipitate was dissolved in 10 mM Tris-HCl buffer, pH 8.0. The solution was dialyzed against the same buffer. The dialyzed solution was chromatographed on DE-11 (Fig. 1). Active fractions of carboxypeptidases A and B, and chymotrypsin C were separately pooled, precipitated with ammonium sulfate, and rechromatographed on DE-32. For further purification of carboxypeptidases A and B, the active enzymes eluted from DE-32 were precipitated with ammonium sulfate, dissolved in 50 mM Tris-acetate buffer, pH 6.0, and dialyzed against the same buffer. The dialyzed carboxypeptidases A and B were chromatographed on $\alpha$-phenylalanine-CH-Sepharose 4B and $\alpha$-arginine-CH-Sepharose 4B, respectively (Fig. 2 and 3). The active chymotrypsin C eluted from DE-32 was precipitated, and dissolved in

![Fig. 1 Chromatographic separation of carboxypeptidases A and B, and chymotrypsin C from autolyzed porcine pancreas glands on DE-11. The dialyzed solutions of ammonium sulfate precipitate in 10 mM Tris-HCl buffer, pH 8.0, were chromatographed on a column (3.2x40 cm) of DE-11 with 1000 ml linear gradient of 0 to 0.45 M NaCl in 10 mM Tris-HCl, pH 8.0. The gradient of NaCl is shown by the broken line. Fractions (15.0 ml) were collected at a flow rate of 240 ml/h. 1-4 indicate the peaks of elastase, carboxypeptidases B and A, and chymotrypsin C, respectively.](image1)

![Fig. 2 Elution pattern of porcine carboxypeptidase A on $\alpha$-phenylalanine-CH-Sepharose 4B. The carboxypeptidase A eluted from DE-32 (54.5 mg protein) was applied to a column (1.0x3.0 cm) of $\alpha$-phenylalanine-CH-Sepharose 4B equilibrated with 50 mM Tris-acetate buffer, pH 6.0; at point A the eluant was changed to 2.0 M NaCl/50 mM Tris-acetate buffer, pH 6.0; at point B the eluant was changed to 50 mM Tris-HCl buffer, pH 9.0; at point C the eluant was changed to 2.0 M NaCl/50 mM Tris-HCl buffer, pH 9.0. Fractions (3.0 ml) were collected at a flow rate of 21 ml/h.](image2)

![Fig. 3 Elution pattern of porcine carboxypeptidase B on $\alpha$-Arginine-CH-Sepharose 4B. The carboxypeptidase B eluted from DE-32 (46.7 mg protein) was applied to a column (1.0x3.0 cm) of $\alpha$-arginine-CH-Sepharose 4B equilibrated with 50 mM Tris-acetate buffer, pH 6.0; at point A the eluant was changed to 2.0 M NaCl/50 mM Tris-acetate buffer, pH 6.0; at point B the eluant was changed to 2.0 M NaCl/50 mM Tris-HCl buffer, pH 9.0. Fractions (3.0 ml) were collected at a flow rate of 21 ml/h.](image3)
the same manner as that of carboxypeptidases. The solution was desalted on Sephadex G-25, and applied to a column of D-phenylalanine-CH-Sepharose 4B to remove carboxypeptidases A and B (Fig. 4). The fractions passed through the column were rapidly chromatographed on CM-Sephadex C-50.21

Results and Discussion

Figure 1 shows a typical elution pattern of carboxypeptidases A and B, and chymotrypsin C from the autolyzed porcine pancreas glands, on a column of DE-11. Carboxypeptidases A and B were contaminated with chymotrypsin C and elastase.

Carboxypeptidase A was freed from endopeptidases by affinity chromatography on D-phenylalanine-CH-Sepharose 4B (Fig. 2): Nonbinding proteins including chymotrypsin C were eluted with 50 mM Tris-acetate buffer, pH 6.0: Carboxypeptidase B and elastase were desorbed with 2.0 M NaCl/50 mM Tris-acetate buffer, pH 6.0. Finally, carboxypeptidase A was eluted with 50 mM Tris-HCl buffer, pH 9.0.

Carboxypeptidase B was freed from endopeptidases by affinity chromatography on D-arginine-CH-Sepharose 4B (Fig. 3): Nonbinding proteins including elastase and chymotrypsin C were eluted with 50 mM Tris-acetate buffer, pH 6.0: Carboxypeptidase B and elastase were desorbed with 2.0 M NaCl/50 mM Tris-acetate buffer, pH 6.0. Finally, carboxypeptidase B was eluted with 50 mM NaCl/50 mM Tris-HCl buffer, pH 9.0.

Chymotrypsin C was freed from carboxypeptidases by affinity chromatography on D-phenylalanine-CH-Sepharose 4B (Fig. 4): The chymotrypsin C eluted from DE-32 (440 mg protein) was applied to a column (0.95X7.0 cm) of D-phenylalanine-CH-Sepharose 4B in 50 mM Tris-acetate buffer, pH 6.0. At the arrow, the eluant was changed to 50 mM Tris-HCl buffer, pH 9.0. Fractions (9.5 ml) were collected at a flow rate of 138 ml/h.

Table 1 Summary of purification of carboxypeptidase A. Crude extract, dialyzed solution of ammonium sulfate precipitate

<table>
<thead>
<tr>
<th>Volume/ml</th>
<th>Total activity (units)</th>
<th>Specific activity (units/µmol)</th>
<th>Yield, %</th>
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<tr>
<td>662</td>
<td>26500</td>
<td>76300</td>
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Table 2 Summary of purification of carboxypeptidase B. Crude extract, dialyzed solution of ammonium sulfate precipitate

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Table 3 Summary of purification of chymotrypsin C. Crude extract, dialyzed solution of ammonium sulfate precipitate

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A and B by passing through a column of D-phenylalanine-CH-Sepharose 4B (Fig. 4), and then chromatographed on CM-C-50 to separate some active forms of the enzyme.21

The purification procedures of carboxypeptidases A and B, and chymotrypsin C are summarized in Tables 1, 2, and 3, respectively. The yields of carboxy-
peptidases A and B, and chymotrypsin C were 373 mg, 143 mg, and 292 mg, respectively, per 100 g of acetone powder.

N-terminal analysis by the dinitrophenylation method\textsuperscript{22} and SDS–polyacrylamide gel electrophoresis by the method of O’Farrel\textsuperscript{23} showed that the carboxypeptidase A was mainly composed of three active forms, each with an apparent molecular weight (Mr) of 32000 (Fig. 5).\textsuperscript{1,2} Similarly, the carboxypeptidase B was mainly composed of three active forms, of which the major form has a Mr of 34000 similar to that of Folk’s carboxypeptidase B (Fig. 5).\textsuperscript{3} The nicked form of carboxypeptidase B, corresponding to bovine B\textsubscript{1} and B\textsubscript{2}\textsuperscript{24}, was not found in our preparations. The chymotrypsin C was composed of one major and three minor active forms\textsuperscript{21} (not shown).

The affinity-chromatographically purified carboxypeptidases A and B showed no endopeptidase activity toward such sensitive substrates as Tos-LArg-OMe\textsuperscript{25}, Bz-LTyr-OEt\textsuperscript{26}, Bz-LLeu-OEt\textsuperscript{18}, Bz-LAla-OMe\textsuperscript{19}, or the chymotryptic, trypsic, and thermolysin peptides of boar protamine.\textsuperscript{27} Therefore, the carboxypeptidases A and B were completely freed from contaminated endopeptidases, and also separated from each other by affinity chromatography. Accordingly, these enzymes A and B can be used for carboxyl-terminal analysis of peptides without prior treatment with diisopropyl phosphorofluoridate to inactivate contaminated pancreatic endopeptidases.

As the reagent for the C-terminal sequencing of proteins, porcine carboxypeptidases A and B seem better than those of bovine origin. Porcine enzymes A and B have higher solubility and both are very stable and easier to handle: they can be kept frozen at \(-20^\circ\text{C}\) for at least one year without loss in activity; they show no loss in activity after several thawings and refreezings.\textsuperscript{1,4} The procedure described here can also be applicable to purify the commercial preparations of the porcine enzymes.

References


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