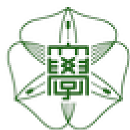
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Preparation and Properties of the Sei Whale Carboxypeptidase A

Tsutomu YONEDA*

Abstract

From the pancreas of a sei whale, *Balaenoptera borealis*, carboxypeptidase A was isolated and the preparation was homogeneous by analytical acrylamide disc electrophoresis. During the course of purification, the apparent specific activity of the enzyme rose to 270 folds of that of partially activated pancreatic fluid. The preparation was a typical metalloenzyme, which was inhibited by thiol reagents and chelating agents such as phenanthrolin. The whale carboxypeptidase A (WCPA) possesses optimum pH near 8.2 and optimal temperature at 50°C when carbobenzoxy-glycyl-L-phenylalanine was used as substrate. The specificity of the ultraviolet absorption spectrum of WCPA was similar to that of the bovine carboxypeptidase A.

Introduction

Among the numerous kinds of proteolytic enzymes, carboxypeptidase A is the most useful tool[1] for the structure analysis of the carboxyl terminal of proteins[2][3][4], since the enzyme splits the peptide linkages of carboxyl terminals in a regular sequence of amino acids.

The presence of carboxypeptidase A has been observed on eight kinds of animals; human being, dog, rat, cow, pig, lungfish, dogfish and starfish[5]. However, concerning the preparation of carboxypeptidase A, not so many kinds of animals could be pointed out as its source; only a few such as the well known bovine by Anson[6] and Neurath[7], porcine by Folk[8] and dogfish by Lacko[9].

The present paper describes the preparation of carboxypeptidase A from the sei whale pancreas and some properties of the enzyme.

Materials and Method

The frozen pancreas of a sei whale, *Balaenoptera bolealis*, was kept at –20°C until use as an enzyme source, which was caught off Nemuro in Hokkaido toward the end of June 1970. The carboxypetidase A was isolated by the method of Anson with some modifications[10].

Carbobenzoxy-glycyl-L-phenylalanine (CGP) was purchased from Sigma Chemical Co. For the substrate solution of the enzymatic reaction 0.004 M CGP in 0.05 M Tris-HCl, pH 7.4 was used through all the experiments.

The reagents for the acrylamide disc electrophoresis were obtained from Wako

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Pure Chemical Industries, Ltd. The procedure of the electrophoresis was according to the method of Ornstein and Davis[11] and its details were reported previously[12].

Ninhydrin reaction on L-alanine split from CGP was carried out according to the method of Yemm and Cocking[13] with some modifications; a preliminary equal volume of 0.2 M citrate buffer, pH 5.0 (21.008 g of $C_6H_8O_7 \cdot 2H_2O$ and 40.00 g of NaOH in 500 ml of water) and a ninhydrin-KCN-methyl cellosolve solution (50 ml of 5.0% ninhydrin in methyl cellosolve plus 250 ml of methyl cellosolve containing 5 ml of 0.01 M KCN) were combined. One milliliter of this solution and an aliquot, 0.1 ml, from the enzyme reaction system were mixed and heated at 100°C for 15 min., cooled for 5 min. and diluted with 3.0 ml of 60% ethanol. The enzymatic activity was represented as absorbance at 570 nm spectrophotometrically using Shimadzu's Beckman Spectrophotometer QR-50.

The protein concentration of preparations was estimated according to the ultraviolet absorption method; the absorbance of 1.94 at 280 nm represents 1 mg per ml of protein.

Phenanthrolin, disodium ethylenediaminetetraacetate dihydrate (EDTA), mercapto ethanol, L-cysteine, trisaminomethane (Tris), barbital sodium, citric acid and other inorganic reagents were also the products of Wako Pure Chemical Industries, Ltd.

Unless otherwise stated the proteins were handled under cooled conditions close to 4°C through all experiments.

Results and Discussion

The procedure of WCPA preparation was followed thus: Five hundred grams of a frozen whale pancreas was sliced and thawed for 3 days at 2°C and a partially autolyzed fluid was collected. The turbidity was spun down at 20,000×g for 30 min. The supernatant was made to alkaline with the addition of 1 M NaOH and adjusted to pH 7.8 then heated at 37°C for 1 hour for further autolysis. The activated fluid was acidified to pH 4.6 with 5 M acetic acid with stirring and immediately the solution was diluted to ten folds with distilled water. After standing overnight the supernatant was siphoned off and the precipitated residues were collected by centrifugation at 7,000×g for 30 min. then 25 grams (wet weight) of euglobulin were obtained.

The euglobulin precipitate was suspended in distilled water to a volume approximately one fifth to that of the pancreatic fluid. The pH of the suspension was adjusted to 6.0 with dropwise addition of freshly prepared 0.2 M $Ba(OH)_2$. After the pH became stabilized, the soluble proteins at this pH were centrifuged off at 20,000×g for 30 min. The precipitate was resuspended in distilled water the same volume as above, and 0.2 M $Ba(OH)_2$ was added with stirring to reach

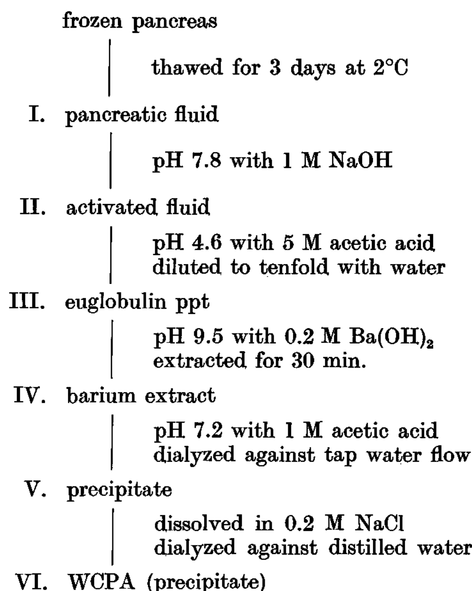

Fig. 1. Flow sheet shows the steps in the preparation of carboxypeptidase A from frozen pancreas of sei whale.

a stable pH of approximately 9.5. After standing for 30 min. the barium extracted solution was obtained as supernatant by centrifugation at 20,000×g for 30 min. The transparent supernatant solution was allowed to stand overnight after adjustment to 7.2 with 1 M acetic acid in a refrigerator at 4°C. The solution which became turbid with crystals was dialyzed in a Visking tube against a flow of tap water overnight. The precipitate was collected with centrifugation and dissolved in 0.2 M NaCl, a volume of one-tenth that of the pancreatic fluid. After centrifugation at 20,000×g for 30 min., the solution was dialyzed against distilled water overnight. Five hundred milligrams (wet weight) of WCPA was spund down above dialysate by centrifugation (Fig. 1). The preparation could be stored over a year until its being use for further experiment at -20°C without loss of its carboxypeptidase activity.

An analytical disc electrophoresis was carried out the following step: pancreatic fluid, activated fluid at pH 7.8, euglobulin, barium extract of euglobulin and final product of WCPA. For one gel column, 0.1 ml of each solution, in the case of precipitate a suitable amount product was dissolved in 0.1 ml of 0.2 M NaCl, which was combined and mixed with an equivolume of spacer gel solution for one sample gel. The electrophoretic conditions; 7.5% pore size, pH 9.4 were that of Ornstein and Davis and electrophoresis was carried out in the refrigerator at 2°C and 2 mA current for one gel column (Fig. 2). From their electropherograms, it was found that at the step of euglobulin only two kinds of proteins were presented

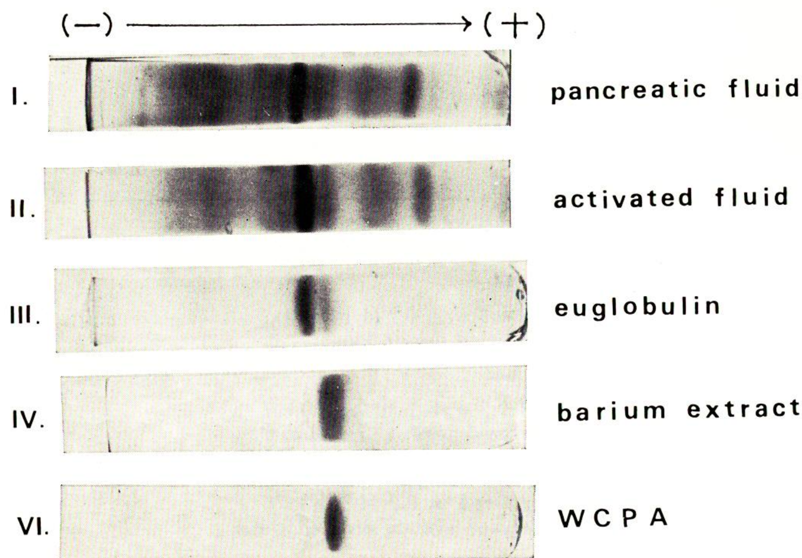

Fig. 2. Disc electropherograms of WCPA and the preparations at various steps during the course of its purification. Electrophoresis was carried out at pH 9.4 on 7.5% pore size gel and proteis were stained with amido black 10 B.

and the final step preparation of WCPA was a homogeneous protein by this analytical method.

The activity of the carboxypeptidase A was determined by measuring the concentration of L-phenylalanine split from the substrate (CGP). Generally the incubation was carried out in a small test tube at 25°C. A typical assay system was initiated to mix 0.5 ml of substrate solution; 0.004 M CGP in 0.05 M Tris-HCl, pH 7.4, and 0.5 ml of enzyme solution; 0.05–2.5 mg protein in 0.2 M NaCl. An aliquot (0.1 ml) from the incubating mixture at 3 min. intervals was taken and introduced into the test tube containing 1.0 ml of ninhydrincitrate buffer solution, which was freshly prepared combining equivolumes of a ninhydrin-KCN-methyl cellosolve solution and 0.2 M of citrate buffer, and heated for 15 min., cooled, diluted and measured the absorbance at 570 nm spectrophotometrically. The time course assays of the carboxypeptidase A activity took the following steps: pancreatic fluid, barium extract and WCPA (Fig. 3). From the results it is possible to calculate the level of specific activity, and WCPA was found to be 270 folds to that of the pancreatic fluid. But the apparent rising of the specific activity is due not only to the purification but also to the activation of the proenzyme of carboxypeptidase A.

The ultraviolet absorption spectrum of WCPA was compared with that of the bovine carboxypeptidase A, which was presented by Bargetzi et al.[14] (Fig. 4).

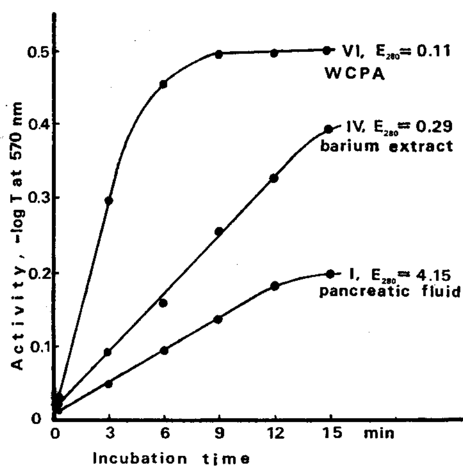

Fig. 3. The time course assays of carboxypeptidase A activity at three steps through the purification of the enzyme from frozen pancreas of sei whale. The protein concentration of each enzyme solution was represented as the extinction at 280 nm (E_{280}). Assay conditions are described in the text.

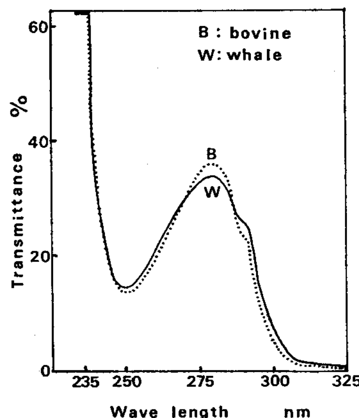

Fig. 4. Ultraviolet absorption spectra of sei whale carboxypeptidase A (W) and bovine carboxypeptidase A (B) which was represented by Bargetzi et al.

The specific absorption of WCPA agreed very closely to that of the bovine enzyme, showing a maximum absorption at 278 nm and a sholder at 290 nm belonging to their tyrosyl residues.

For an optimum pH assay 3.0 ml of 0.05 M sodium Veronal, pH 6.4–9.8, 0.5 ml of substrate solution and 0.1 ml of WCPA solution were combined and incubated for 3 min. at 25°C then 1.0 ml of ninhydrincitrate buffer solution, 5.0 ml of 60% ethanol were used. It was found that a pretty broad optimal pH near 8.2 (Fig. 5), that was a little more alkaline pH than that of bovine enzyme (pH 7.5–7.8), when CGP was used as substrate.

For the assay of optimal temperature, the typical enzyme assay system was employed at various temperatures, 10°–80°C, although the pH was kept at 8.2 with sodium Veronal. The temperature at 50°C was optimal for WCPA using CGP as substrate (Fig. 6).

To study the additive effect,[15] the enzyme reaction system was designed as follows; 0.5 ml of substrate solution, 0.4 ml of enzyme solution and 0.1 ml of additive solution. In this manner the concentration of the additives were adjusted to 2.5 mM or 5 mM when the enzymic reaction was going on. L-Cystein was more effective than mercapto ethanol (Fig. 7), and phenanthrolin, which was known as an excellent inhibitor of carboxypeptidase, showed a larger effect than that of EDTA (Fig. 8). Moreover the cobalt ion acts as activator whereas the

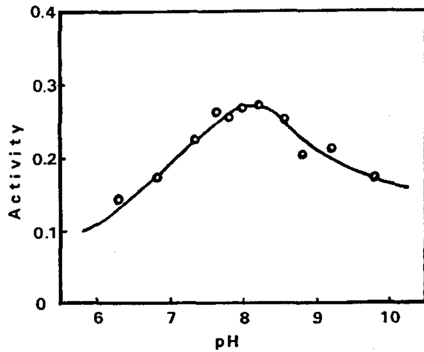

Fig. 5. Relation between pH and activity of sei whale carboxypeptidase A. The activity was shown by the extinction at 570 nm after the ninhydrin reaction, and the procedure is descrived in the text.

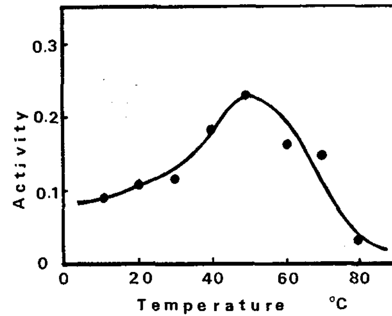

Fig. 6. Relation between temperature and activity of sei whale carboxypeptidase A. The activity was shown by the extinction at 570 nm after the ninhydrin reaction and the method is put in the text.

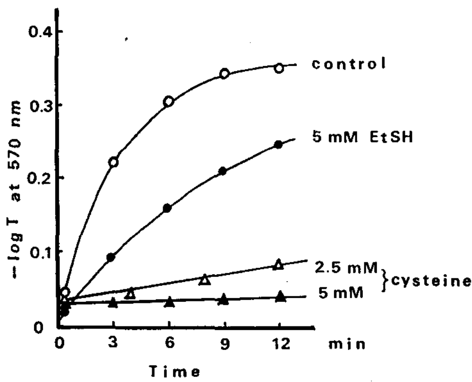

Fig. 7. Effect of thiol reagents on the activity of sei whale carboxypeptidase A.

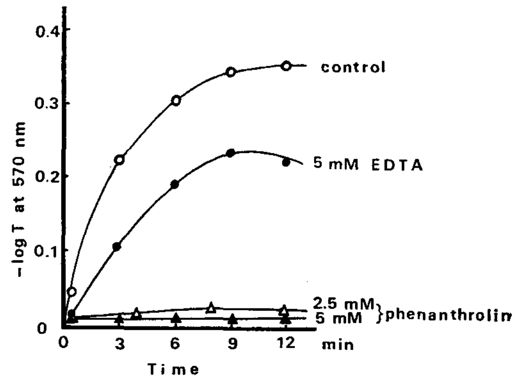

Fig. 8. Effect of chelating agents on the activity of sei whale carboxypeptidase A.

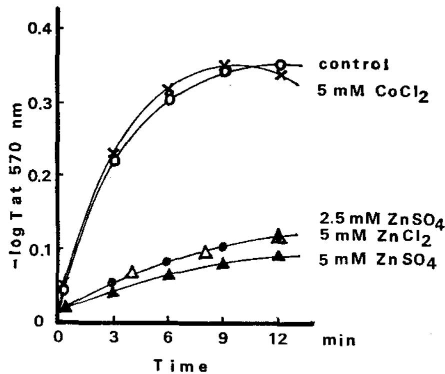

Fig. 9. Effect of metal ions on the activity of sei whale carboxypeptidase A.

zinc ion acts rather as inhibitor, but to explain the phenomenon further studies are expected. However, it is clear that WCPA is a metalloenzyme, in which the metal plays an important role in the catalysis.

Through these observations: the hydrolysis of CGP, the optimum pH, the ultraviolet absorption and the additive tests, it is almost certain that the isolated WCPA is a typical pancreatic carboxypeptidase A.

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