THE PRESENCE OF ZINC IN CARBOXYPEPTIDASE C AND COUPLING OF THE ENZYME TO SEPHAROSE AND SEPHADEX

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1. Introduction

Carboxypeptidase C was discovered in the peels of citrus fruits by Zuber [1], who purified the enzyme and studied some of its properties. The enzyme has also been prepared from orange leaves [2]. It hydrolyzes most carboxyl-terminal amino acid residues but has a preference for aromatic residues. Unlike similar enzymes of animal origin it can split peptide bonds on both the carboxyl- and imino-side of proline [3].

The wide specificity makes carboxypeptidase C very suitable for sequence analysis of proteins and peptides [2]. In such work it would be convenient to use matrix bound enzyme, and we have therefore coupled it to Sepharose and Sephadex beads. An immobilized carboxypeptidase might also find technical application by debittering protein hydrolysates intended for beveridges. During our study we found that the enzyme contains carbohydrate and zinc which has not previously been reported.

2. Materials and methods

2.1. *Assay procedures*

Enzyme activities were determined in 1 ml 0.1 M sodium citrate buffer, pH 5.3, containing 0.5 mM CBZ-Glu-Tyr. After incubation at 37°C, the reaction was stopped by 0.2 ml of 0.3 M NaOH and 1 ml of ninhydrin solution [4] was then added. The reaction mixture was immediately boiled for 15 min, diluted with 50% ethanol and the absorbance read at 570 nm. One unit of enzyme is defined as the amount which liberates 1 μmole of tyrosine per min under these conditions.

2.2. *Enzyme purification*

We purified the enzyme from peels of various kinds of oranges some of which had higher activities than others. Two-hundred gram portions of peel were homogenized in 250 ml of a solution of 2.3% (w/v) NaCl in water. Extraction of only the outer, yellow part (flavedo) of the peels did not give appreciable higher specific enzyme activity in the extracts or lower carbohydrate content in the purified enzyme. The extract, which had a pH of about 4.5, was filtered and ammonium sulfate added to 70% saturation. The precipitate was dissolved in a small volume of 0.025 M sodium citrate buffer, pH 5.3. Alternatively, the extract was concentrated to about 50 ml in a Diaflo Hollow Fibre Cartride PM-10, after which several portions of 200 ml citrate buffer were added and the concentrations repeated. This procedure gave an almost colourless solution and no loss in enzyme activity.

The concentrated enzyme solution was passed through a column of Bio-Gel P-10 equilibrated and eluted with 0.025 M citrate buffer, pH 5.3. This removed impurities of low molecular weight including most remaining pigments (fig. 1). Fractions containing carboxypeptidase were pooled and concentrated in a Diaflo cell with a PM-10 filter and then chromatographed on Sephadex G-200 in 0.1 M citrate buffer (fig. 2). The fractions eluting at about two void volumes contained carboxypeptidase, and these were pooled, concentrated and rechromatographed on the same column. After two or three such gel filtrations a symmetrical peak was obtained, and polyacrylamide electrophoresis at pH 8.7 showed that the enzyme was essentially free of contaminating protein.

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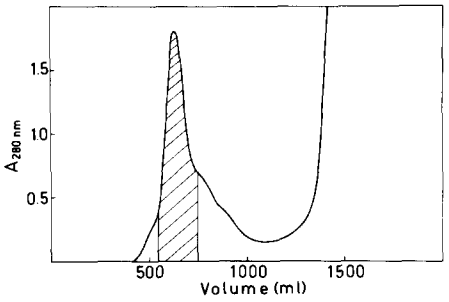

Fig. 1. Chromatography of crude, concentrated extract of orange peels on Bio Gel P-10 in 0.025 M sodium citrate buffer, pH 5.3. Fractions corresponding to the hatched area had highest carboxypeptidase activity and were pooled.

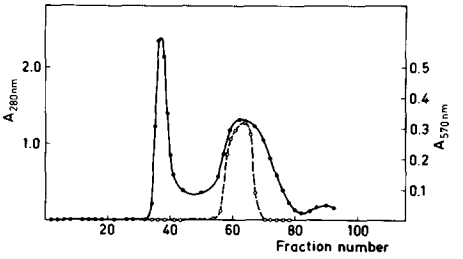

Fig. 2. Chromatography of concentrated material from Bio Gel P-10 on Sephadex G-200 in 0.1 M sodium citrate buffer, pH 5.3. Sample volume: 8 ml. Fraction volume: 5 ml.(●-●-●) absorbance at 280 nm.(o-o-o)carboxypeptidase activity measured as ninhydrin colour at 570 nm.

2.3. *Zinc determinations*

Protein solutions were analyzed by the atomic absorption method [5] by direct injection into a flame of air–acetylen. Corrections were made for the small amount of zinc present in the buffer solutions against which the enzyme samples were dialyzed before analysis.

3. Results and discussion

3.1. *Some properties of the enzyme*

The N-terminal amino acid of the enzyme was determined by dansylation [6, 7]. High voltage electrophoresis and polyamide layer chromatography of the hydrolysate gave only one spot corresponding to bis-dansyl-lysine, indicating that the enzyme was free from contaminating protein. Analysis of acid hydrolysates by the orcinol method showed that the enzyme contained up to 30% carbohydrate. Some of this could be removed by chromatography on CM-Sephadex C-50 before or after the gel filtration step, but the sugar content could not be reduced below about 10%. Amino acid analyses were done on two enzyme preparations which were homogenous in polyacrylamide electrophoresis. The amino acid composition differs from that of either carboxypeptidase A or B, showing more acidic amino acids, phenylalanine and proline, but less sulfur-containing amino acids. Glucoseamine, galactose and two unidentified sugars were also present in the hydrolysates.

Zuber [1] found that the enzyme was active after dialysis against EDTA or *o*-phenenthroline and concluded that it does not require metal ions in contrast to the animal carboxypeptidases which are zinc proteins. We could confirm that the enzyme was active in presence of these complexing agents, but found that it contained zinc. Analyses were done on two enzyme samples of which one had been dialyzed for two days against citrate buffer, pH 5.3, containing 0.01 M *o*-phenanthroline and the other had been dialyzed against only buffer. Both samples contained 3.6 μg zinc per mg protein. This corresponds to 5–7 gram-atoms of zinc per mole of enzyme based on the molecular weight 120 000–150 000 estimated by Zuber [1]. Polyacrylamide electrophoresis in presence of sodium dodecylsulfate [8] revealed a component which was estimated to have a molecular weight of only about 20 000. It is possible that the enzyme is composed of subunits, but we can not yet say if each of these contains one zinc atom.

When one batch of enzyme was purified by the procedure described above, its zinc content was analyzed after several of the purification steps, and the results are given in table 1. The observed increase in zinc content during purification is a further indication that the metal is an integral part of the enzyme.

Several attempts were made to remove the zinc from carboxypeptidase C by prolonged dialysis in the pH range 4 to 7 or by electrodialysis, but these were not successful. The enzyme was stable in 6 M urea, but dialysis against such solutions in 0.1 M citrate buffer, pH 5.3, containing 10^{-3} M *o*-phenanthroline did not give zinc-free protein. However, when the enzyme

Table 1
Zinc content of preparations of carboxypeptidase C

Purification step	μg Zn/g protein
Bio-Gel P-10	1370
Sephadex G-200 1st run	1850
Sephadex G-200 2nd run	2500
Sephadex G-200 3rd run	3400

was dialyzed against a similar solution which in addition contained 10^{-2} M mercaptoethanol to reduce disulfide bonds in the protein, its zinc content was only 0.40 μg per mg protein, and the enzyme was inactivated. These results indicate that the metal is very firmly bound, but further work is necessary to determine if it has a catalytic function.

3.2. *Coupling experiments*

The purified enzyme was coupled to cyanogen bromide activated Sepharose 2 B according to the procedure of Ernback, Porath and Axén [9]. After incubation for 16 hr at 4°C and pH 7, about 85% of the added protein had reacted. The enzyme-gel contained about 100 μg protein per mg dry weight, but some of the activity was lost during the coupling reaction, probably owing to the instability of the enzyme above pH 6. The immobilized enzyme retained activity in citrate buffer pH 5.3 for several weeks, and assays could be performed by the usual procedure with the modification that the gel particles were shaken during the assay and then allowed to settle before samples were taken for ninhydrin analysis. Small amounts of enzyme-gel were also incubated with various peptides, and samples of the supernatant solution analyzed by dansylation and subsequent high voltage electrophoresis. The following compounds were hydrolyzed: N-CBZ-Glu-Phe, N-CBZ-Ile-Phe, Ala-Pro-Gly (to give Gly) Leu-Gly-Val-Ala (to give Ala and Val) and Gly-Pro-Gly-Leu-Pro (to give Pro and Leu). The coupled enzyme was found to be active in presence of 8 M urea, which would be valuable in sequence analysis work, but attempts to get a quantitative analysis of the amino acids liberated under these conditions were not successful.

The recently developed technique for immobilizing proteins by means of isocyanides [10] was also tried on carboxypeptidase C, because this method is well suited for enzymes which are most stable at acidic pH. For this purpose, 125 mg of CM Sephadex C-50 (Pharmacia Fine Chemicals, Uppsala) was equilibrated with 0.05 M sodium phosphate buffer, pH 5.8. The gel (about 5 ml) was suspended in 10 ml of the same buffer, and 10 μl of acetaldehyde and 25 μl of cyclohexyl isocyanide (EGA-Chemie KG, Steinheim/Albuch, West Germany) and 2 ml of an enzyme solution containing about 10 mg (490 mU) of protein were added. The suspension was stirred in an ice bath for 18 hr, and the gel was then packed in a small column and washed for two days, first with phosphate buffer, and then with 0.1 M sodium citrate buffer, pH 5.3, containing 0.8 M NaCl. The first wash had a low carboxypeptidase activity, but no leakage of enzyme was detected in a final wash buffer which consisted of only 0.1 M citrate. Samples of the gel were assayed with CBZ-Glu-Tyr and found to have an activity of about 40 mU per ml sedimented gel. The recovery of enzyme activity amounted to about 45% of that in the enzyme solution applied to the gel.

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