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Application of Carboxypeptidase Cu to Amino Acid Sequence Analysis. I. Preparation and Enzymatic Properties of Immobilized Carboxypeptidase C_{Ua}

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Carboxypeptidase C_{Ua}, isolated from the exocarp of *Citrus unshiu* Marc., was bound to CNBr-activated agarose with coupling yields of 74—99%. The immobilized enzyme possessed 77—122% of the activity of the native enzyme and was stable to repeated assays and prolonged storage. It showed a broad substrate specificity similar to that of the native enzyme and liberated amino acids including proline sequentially from the C-termini of angiotensin I, bradykinin potentiator C, the oxidized B chain of bovine insulin, and bovine plasma albumin.

Keywords——carboxypeptidase; *Citrus unshiu*; immobilization; substrate specificity; stability; sequence analysis

Carboxypeptidase C_{Ua} [EC 3.4.16.1], isolated from the exocarp of *Citrus unshiu* Marc., is a serine protease having the ability to liberate proline as well as other neutral, acidic, and basic amino acids from the C-termini of peptide chains.[1] This broad specificity makes the enzyme especially useful for amino acid sequence analysis.

Immobilization of this enzyme is expected to provide a valuable tool for sequence analysis, since the immobilized enzyme can be readily removed by filtration or centrifugation at any stage of digestion and can be used repeatedly. Immobilization of carboxypeptidases was first reported by Grubhofer and Schleith,[2] who coupled pancreatic carboxypeptidase A with a diazotized poly-*p*-aminostyrene. Recently, Royer *et al.*[3] have described immobilized preparations of carboxypeptidase Y from baker's yeast. No attempt, however, has so far been made to immobilize carboxypeptidase C type enzymes, except for carboxypeptidase C_N.[4] We have previously attempted to immobilize carboxypeptidase C_N from *Citrus natsudaidai* Hayata by conjugating it to aminoethyl cellulose through glutaraldehyde. The specific activity of the immobilized enzyme obtained was only 24—60% of the original activity and decreased gradually on repeated assays. To overcome these disadvantages, we have prepared immobilized carboxypeptidase C_{Ua} by coupling the enzyme to agarose as a solid support. The usefulness of this preparation in the sequencing of several peptide and protein substrates was demonstrated.

Experimental

Enzymes——Carboxypeptidase C_{Ua} was purified from the exocarp of *Citrus unshiu* Marc. by the method described previously.[1a] By this purification method, the enzyme was obtained in the form of a solution in 0.1 M citrate buffer, pH 5.5, and it was stored at 4°C in the same buffer. When it was necessary to replace the buffer, solutions of the enzyme were dialyzed against the required buffer.

Materials——Agarose was obtained from Dojindo Laboratories, Kumamoto; CNBr from Kishida Chemical Co., Osaka; carbobenzoxy(Z)- and benzoyl(Bz)-dipeptides, angiotensin I, and bradykinin potentiator C from the Peptide Research Foundation, Osaka; oxidized B chain of insulin from Boehringer Mannheim, Germany; bovine plasma albumin from Armour Pharmaceutical Co., Illinois. Other reagents and organic solvents used were of analytical grade.

Enzyme Assays——The activity of the native enzyme was determined as described in a previous paper.[5] The immobilized enzyme was assayed as follows. Mixtures of 0.6 ml of substrate solution and 0.3 ml of enzyme suspension containing 0.15 ml of immobilized enzyme were incubated for 10 min at 50°C with shaking at 160 times/min. After the enzymatic reaction had been stopped by chilling the mixtures in ice-water, the incubation mixtures were centrifuged at 1500×g for 2 min to remove the immobilized enzyme, and aliquots of 0.1 ml were withdrawn from the supernatants for the assay of enzymatic activity.

Coupling of Carboxypeptidase C_{Ua} with Agarose——Chemical fixation of carboxypeptidase C_{Ua} to agarose was carried out as follows. Agarose gel was activated with CNBr according to the method of Axén[6] and immediately washed on a glass filter successively with ice water and 0.02 M citrate buffer, pH 5.5, at 4°C until free from unreacted CNBr. Carboxypeptidase C_{Ua} was added to the suspension of the activated gel, and the mixture was stirred gently for 24 h at 4°C. The coupled product was then filtered through a glass filter, and the protein concentration of the filtrate was determined by the Cu-Folin method.[7] The reaction product remaining on the glass filter was transferred into a beaker, suspended in 0.02 M citrate buffer, pH 5.5, containing 0.1 M L-lysine, and stirred gently for 2 h at 4°C in order to block the excess activated agarose. The product was then washed on a glass filter with 0.02 M citrate buffer, pH 5.5, at 4°C until free from unreacted L-lysine. The immobilized enzyme obtained was suspended in the same buffer and stored at 4°C until use.

Determination of Bound Enzyme——The amount of fixed enzyme was calculated by subtracting the amount of unfixed enzyme from that of starting enzyme.

Hydrolysis of Peptides and Proteins——Immobilized carboxypeptidase C_{Ua} was applied to C-terminal sequence analysis as follows. Angiotensin I (60 nmol), bradykinin potentiator C (100 nmol), oxidized insulin B chain (100 nmol), and bovine plasma albumin (60 nmol) were incubated separately with 0.2 ml of immobilized enzyme (0.16 nmol of protein) at 35°C in 1 ml of 0.1 M citrate buffer, pH 5.5, with shaking at 160 times/min. After incubation, the reaction mixture was chilled in ice-water, and the supernatant was separated by centrifugation. Aliquots of 0.5 ml were then withdrawn from the supernatants and added to 1.5 ml of 0.2 M citrate buffer, pH 2.2, in the cases of the former two substrates or to 0.5 ml of 20% trichloroacetic acid in the cases of the latter two substrates. The concentrations of amino acids in the solutions were determined with a Hitachi KLA-5 amino acid analyzer after centrifugation at 1500×g for 15 min when necessary.

Results

Protein Content and Activity of Immobilized Carboxypeptidase C_{Ua}

The protein contents and activities of four immobilized carboxypeptidase C_{Ua} derivatives which were prepared under different conditions are shown in Table I. The coupling efficiency ranged from 74 to 99%. The immobilized enzyme possessed 77—122% of the activity of the native enzyme.

Table I. Immobilization of Carboxypeptidase C_{Ua}

	Immobilized carboxypeptidase C_{Ua} preparations			
	I	II	III	IV
CNBr-activated agarose (ml of settled gel)	17	30	8.2	43
Enzyme (mg)	1.4	2.7	1.9	1.4
Reaction time (h)	22	12	24	24
Enzyme on gel (μg/ml of settled gel)	78	67	232	32
Coupling efficiency (%)[a]	95	74	99	99
Specific activity (units/mg of protein)				
Native enzyme	77	55	87	70
Immobilized enzyme	60	67	81	54
Relative activity (%)[b]	78	122	93	77
Recovery of activity	74	90	92	76

a) Coupling efficiency is experessed as the ratio in percent of the amount of enzyme bound to agarose to that used in the coupling reaction.
b) Relative activity is expressed as a ratio in percent of the specific activity of immobilized enzyme to that of native enzyme.

Substrate Specificity

Table II shows the relative activites of immobilized and native carboxypeptidase C_{Ua} toward *N*-substituted dipeptides. The specific activities of the immobilized and native enzymes toward Z–Glu–Phe were 68.7 and 62.9 units per mg protein, respectively. Immobilized carboxypeptidase C_{Ua} hydrolyzed Z–Glu–Phe and Z–Glu–Tyr more rapidly than the others, as did native carboxypeptidase C_{Ua}. Both enzymes released proline from Z–Phe–Pro, Z–Gly–Pro, Z–Pro–Pro, and Z–Glu–Pro. Their hydrolysis rates are influenced to some degree by the penultimate residues at the C-terminal ends of peptides. Both enzymes also released basic amino acids, lysine and arginine, from Bz–Gly–Arg and Bz–Gly–Lys, respectively.

TABLE II. Substrate Specificity of Carboxypeptidase C_{Ua}

No.	Substrate	Relative activity (%)	
		Immobilized carboxypeptidase C_{Ua}	Native carboxypeptidase C_{Ua}
1	Z–Glu–Phe	100	100
2	Z–Glu–Tyr	55.5	45.2
3	Z–Gly–Phe	3.6	1.8
4	Z–Gly–Leu	11.8	8.3
5	Z–Glu–Pro	0.62	0.14
6	Z–Phe–Pro	10.6	5.8
7	Z–Gly–Pro	0.90	0.25
8	Z–Pro–Pro	0.90	0.16
9	Bz–Gly–Arg	2.4	1.3
10	Bz–Gly–Lys	4.4	3.8

Substrates were dissolved in 0.1 M citrate buffer, pH 5.5, at 20 mM concentration. Mixtures of 0.6 ml of substrate solution and 0.3 ml of enzyme suspension (38.9 μg of protein/ml of immobilized enzyme gel) were incubated at 50°C for 10 min (Nos. 1, 2) or 30 min (Nos. 3—10). In the case of native enzyme, mixtures of 0.2 ml of substrate solution and 0.1 ml of enzyme solution (64.5 μg of protein/ml) were incubated as above. Relative activity is expressed as a percentage of the activity toward Z–Glu–Phe.

Effects of pH and Temperature on Carboxypeptidase Activity

When immobilized carboxypeptidase C_{Ua} was incubated with 20 mM Z–Glu–Phe for 10 min at pH 4.5—7.0 and temperatures between 30 and 70°C, its activity was found to be maximal at pH 5.5 and at a temperature of 50°C. These values are identical with those of native carboxypeptidase C_{Ua}.[1)]

The remaining activities of immobilized and native carboxypeptidase C_{Ua} were 84—100% and 60—100% of their original activities, respectively, when the enzymes were left standing in 0.1 M citrate buffer, pH 3.5—7.0 for 24 h at 4°C. The enzymes were most stable at pH 5.5, which is their optimal pH. At extreme pH values of 2.5 and 8.0, the enzymes lost most of their activities, which were not restored by replacement of the buffers with 0.1 M citrate buffer, pH 5.5.

The thermal stabilities of the enzymes are shown in Fig. 1. Both enzymes were stable at below 40°C but both almost completely lost their activities on pretreatment at 70°C. The immobilized enzyme seems to be resistant to denaturation by heat in the temperature range from 40 to 60°C.

When the immobilized enzyme was preincubated at pH 5.5 and 35°C, its activity did not change during preincubation for 48 h. The native enzyme, however, was inactivated gradually and lost 27% of its original activity after 24 h. At 50°C, the activity of the immobilized enzyme did not change during preincubation for 6 h. After this time, it was inactivated

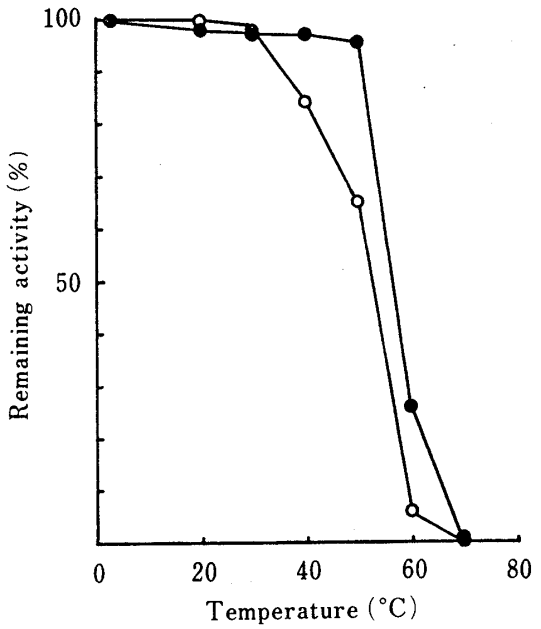

Fig. 1. Thermal Stabilities of Immobilized and Native Carboxypeptidase C_{Ua}

The enzymes were left standing in 0.1 M citrate buffer, pH 5.5, for 1 h at 4—70°C. After this pretreatment, enzymatic activity was determined by incubation with 20 mM Z-Glu-Phe in the same buffer for 10 min at 50°C. ●, immobilized carboxypeptidase C_{Ua}; ○, native carboxypeptidase C_{Ua}.

slowly and lost 52% of its original activity after 24 h. The native enzyme was inactivated rapidly at 50°C and lost 92% of its original activity after 24 h.

Stabilities to Repeated Assays, Lyophilization, and Prolonged Storage

Immobilized carboxypeptidase C_{Ua} was stable to repeated assays. It retained the original activity after twenty successive assays, each performed for 10 min at 50°C. Lyophilization before and after desalting, however, caused losses of 84 and 99% of its original activity, respectively. When stored at 4°C in 0.02 M citrate buffer, pH 5.5, it was quite stable and retained the original activity even after 9 months. The enzyme was therefore stored under these conditions.

Effects of Detergents, Urea, and Guanidine on Carboxypeptidase Activity

Sodium dodecyl sulfate (SDS), cetyltrimethyl ammonium bromide (CTMA), Tween 80, and Brij-35 were used as detergents. Immobilized carboxypeptidase C_{Ua} was preincubated with 1—10% detergent for 60 min at 35°C and assayed with 0.02 M Z-Glu-Phe as a substrate. SDS and CTMA almost completely inhibited the enzymes at final concentrations of 1 and 4%, respectively. The activity of the enzyme was decreased to 52% by Triton X-100 at a final concentration of 10%, whereas Tween 80 and Brij-35 showed only slight or no inhibition in the final concentration range of 1—10%.

The enzyme was also inactivated by reaction with 1—8 M urea or 1—6 M guanidine for 48 h at 4°C, and its activity decreased with increasing concentration of the denaturants. The enzyme completely lost its original activity on treatment with 8 M urea or 4 M guanidine and was not fully reactivated after removal of the denaturants.

Hydrolysis of Peptides and Proteins

Immobilized carboxypeptidase C_{Ua} hydrolyzed human angiotensin I sequentially from its C-terminus as shown in Table III. The first six residues, leucine, histidine, phenylalanine, proline, histidine, and isoleucine, were liberated in fairly large amounts after incubation for 25 min.

The enzyme also hydrolyzed bradykinin potentiator C having the amino acid sequence <Glu–Gly–Leu–Pro–Pro–Gly–Pro–Pro–Ile–Pro–Pro, which is not readily hydrolyzed by other carboxypeptidases. After incubation for 1 h, 0.26 mol proline and 0.04 mol isoleucine per mol of substrate were found in the incubation mixture, demonstrating the cleavage of the first Pro–Pro bond by the enzyme. The enzyme removed 0.88 mol proline, 0.36 mol isoleucine, 0.036 mol glycine, and 0.024 mol leucine per mol of substrate from the C-terminus after 24 h. This indicates that the peptide bonds Ile–Pro, Pro–Ile, Gly–Pro, Pro–Gly, and Leu–Pro were hydrolyzed and also that leucine was liberated from the resulting N-terminal tripeptide <Glu–Gly–Leu.

As Table IV shows, C-terminal alanine and the next basic residue, lysine, were released

Table III. Hydrolysis of Angiotensin I by Immobilized Carboxypeptidase C_{Ua}

Amino acid	Mol per mol of substrate				
	Incubation time (min)				
	2	5	15	20	25
Leucine	0.565	0.679	0.758	0.767	0.876
Histidine	0.239	0.319	0.621	0.736	0.914
Phenylalanine	0	0.127	0.380	0.571	0.713
Proline	0	0	0	0	0.263
Isoleucine	0	0	0	0	0.047

The amino acid sequence of angiotensin I is Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu. The reaction was carried out in 0.1 M citrate buffer, pH 5.5, at 35°C with a substrate concentration of 60 nmol/ml and a substrate-to-enzyme ratio (mol/mol) of 375 to 1. The procedures are described in detail in the text.

Table IV. Hydrolysis of Oxidized B Chain of Bovine Insulin by Immobilized Carboxypeptidase C_{Ua}

Amino acid	Mol per mol of substrate				
	Incubation time (h)				
	0.5	1	1.5	2	3
Alanine	0.361	0.413	0.429	0.491	0.528
Lysine	0.063	0.095	0.152	0.199	0.239
Proline	0	0	0.104	0.185	0.208
Threonine	0	0	0.103	0.165	0.206
Tyrosine	0	0	0.098	0.128	0.146
Phenylalanine	0	0	0.034	0.075	0.113
Glycine	0	0	0	0.032	0.054

The amino acid sequence around the C-terminal is –Glu–Arg–Gly–Phe–Phe–Tyr–Thr–Pro–Lys–Ala. The reaction was carried out in 0.1M pyridine acetate buffer, pH 5.5, at 35°C with a substrate concentration of 100 nmol/ml and a substrate-to-enzyme ratio (mol/mol) of 625 to 1. The procedures are described in detail in the text.

from the oxidized B chain of bovine insulin by immobilized carboxypeptidase C_{Ua} after incubation for 30 min. Glycine, the eleventh residue from the C-terminus of the substrate, was also liberated after 2 h.

The enzyme also hydrolyzed sequentially bovine plasma albumin which has the C-terminal sequence –Pro–Lys–Leu–Val–Val–Ser–Thr–Gln–Thr–Ala–Leu–Ala. Prior to hydrolysis, the substrate was purified by gel filtration on Sephadex G-100 to homogeneity as judged from the result of disc electrophoresis at pH 7.0. The enzyme removed 0.160 mol alanine and 0.082 mol leucine per mol of substrate from the C-terminus after incubation for 1 h. Hydrolysis proceeded sequentially, and the eleventh residue, lysine, was released after 6 h.

Discussion

Since Grubhofer and Schleith successfully immobilized some enzymes by coupling them with diazotized polyaminostyrene,[8] a number of immobilized enzyme preparations have been reported. As for the immobilization of carboxypeptidases, the coupling of pancreatic carboxypeptidase A with diazotized polyaminostyrene was first reported by the above authors.[2] However, the product showed low enzymatic activity, and was poorly characterized. Royer *et al.*[3] have sucessfully performed the coupling of carboxypeptidase Y from baker's yeast, a non-specific exopeptidase like carboxypeptidase C_{Ua}, with CNBr-activated Sepharose or

carboxyalkyl-Sepharose through ethylenediamine, with diazotized arylamine-glass through glycyltyrosine, and with CL–Sepharose through concanavalin A.

We have previously prepared an immobilized derivative of carboxypeptidase C_N, which was isolated from *Citrus natsudaidai* HAYATA and has specificity similar to that of carboxypeptidase C_{Ua}, by conjugating it to aminoethyl cellulose through glutaraldehyde. In the present study, immobilized carboxypeptidase C_{Ua} retaining 77—122% of the activity of the native enzyme toward Z–Glu–Phe was obtained with efficiencies ranging from 74 to 99% by direct coupling of the enzyme with agarose. The coupling efficiency of carboxypeptidase C_{Ua} with agarose was better than that of carboxypeptidase C_N with aminoethyl cellulose[4] and also those of immobilized ethylenediamine- and immobilized glycyltyrosine-carboxypeptidase Y.[3] It was, on the other hand, similar to that of carboxypeptidase Y coupled with CL–Sepharose through concanavalin A.[3] On the whole, the retained activity of immobilized carboxypeptidase C_{Ua} was considerably higher than those of immobilized carboxypeptidases C_N[4] and Y,[3] though the differences in experimental conditions do not permit direct comparison.

Immobilized carboxypeptidase C_{Ua} showed broad substrate specificity similar to that of the native enzyme, and had the ability to release proline from Z–Gly–Pro, which is not readily hydrolyzed by other carboxypeptidases. The specific activity of immobilized carboxypeptidase C_{Ua} toward Z–Glu–Phe was 92% of that of the native enzyme. The relative activities of the former toward the substrates tested, expressed as percentages of the activity toward Z–Glu–Phe, were higher than those of the latter. It is noteworthy that the activities of immobilized carboxypeptidase C_{Ua} to release proline from the N-substituted dipeptides were distinctly higher than those of the native enzyme, although the rate of hydrolysis was low in every instance. These findings suggest that immobilization of carboxypeptidase C_{Ua} may have caused some minor changes in the affinity of the enzyme for the substrates.

The optimal pH and temperature of carboxypeptidase C_{Ua} did not change on immobilization. On the other hand, immobilization appears to have increased the stability of the enzyme to pH, heat, and prolonged preincubation. Immobilized carboxypeptidase C_{Ua} was so stable that its original activity was retained even after twenty successive assays.

Immobilized carboxypeptidase C_{Ua} was strongly inhibited by SDS and CTMA, whereas Tween 80 or Brij-35 had little or no effect. The latter two can therefore be used as solubilizers for less soluble substrates, as in the case of the native enzyme. Triton X-100 decreased the activity of immobilized carboxypeptidase C_{Ua} gradually, and the inhibition increased with increasing concentration of the detergent. The native enzyme was not inhibited by Triton X-100.[1b] The immobilized enzyme was denature by both urea and guanidine and was not reactivated after removal of the reagents by replacement of the buffer with fresh 0.02 M citrate buffer, pH 5.5, as the native enzyme was not. Binding of carboxypeptidase C_{Ua} to the solid support, agarose, seems to have had practically no effect on the susceptibility of the enzyme to denaturation by either urea or guanidine.

Like the native enzyme, immobilized carboxypeptidase C_{Ua} released not only neutral, acidic, and basic amino acids but also proline from the C-termini of peptide chains, and it also sequentially hydrolyzed a protein substrate, bovine plasma albumin (molecular weight, 68000), beginning with its C-terminal residue. The application of immobilized preparations of non-specific serine carboxypeptidases for sequence analysis has so far been reported only for carboxypeptidase C_N[4] and carboxypeptidase Y,[3b] which were used for analyses of oxidized lysozyme and ribonuclease A, respectively. The specific activities toward Z-dipeptides of immobilized carboxypeptidase C_{Ua} were distinctly higher than those of immobilized carboxypeptidase C_N However, relatively little information is available on the substrate specificity of immobilized carboxypeptidase Y. Immobilized carboxypeptidase C_{Ua} having high enzymatic activity was easily prepared with good efficiency by direct coupling with CNBr-activated agarose, unlike carboxypeptidases C_N and Y, which require a specific ligand and modification of the

enzyme, respectively, before coupling with the solid supports. Furthermore, immobilized carboxypeptidase C_{Ua} is so stable that it can be utilized repeatedly. These advantages should make this immobilized enzyme useful for the C-terminal sequence analysis of peptides and proteins.

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