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The Active Site of Carboxypeptidase C_U. I. Evidence for Serine in the Active Sites of Carboxypeptidases C_{Ua} and C_{Ub}

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Carboxypeptidases C_{Ua} and C_{Ub} were both inactivated with incorporation of 1 mol ^{32}P-labeled diisopropyl fluorophosphate per mol enzyme. The amino acid residue that reacted with this reagent was a serine residue in the active site of each enzyme. The amino acid sequence around this reactive serine residue was determined to be Glu-Gly-Asp-Ser-Gly-Gly-Glu-Leu for both enzymes by sequence analysis of three radioactive peptides isolated from partial acid hydrolysates of the ^{32}P-labeled enzymes.

Keywords——carboxypeptidase; *Citrus unshiu* MARC; reactive serine residue; amino acid sequence; serine protease

The exocarp of mandarin orange (*Citrus unshiu* MARC.) contains non-specific carboxyl-terminal exopeptidases which we call by the generic name "carboxypeptidase C_U." We have previously isolated and characterized two different enzymes of this type named carboxypeptidase C_{Ua} and C_{Ub}, with molecular weights of 96000 and 112000, respectively.[1] Both enzymes liberate most amino acids, including proline, from the C-termini of peptide chains and are therefore suitable for sequence analysis of proteins. The enzymes are diisopropyl fluorophosphate (DFP)-sensitive, like other plant carboxypeptidases,[3] and differ in specificity from the pancreatic metalloenzymes, carboxypeptidases A [EC 3.4.12.3.] and B [EC 3.4.12.4]. Carboxypeptidases C_{Ua} and C_{Ub} are also inactivated by phenylmethanesulfonyl fluoride, glycine ethyl ester in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, *p*-bromophenacyl bromide, phenylglyoxal, $HgCl_2$, $AgNO_3$, and CH_3HgCl but not by other metal ions, anions, ethylenediaminetetraacetic acid, or *o*-phenanthroline. These observations suggest that the enzymes are serine proteases having essential aspartic (or glutamic) acid, arginine, and histidine residues besides reactive serine residues, and may possess unique active site structures which differ from those of carboxypeptidases A and B.

The present paper describes the identification of the reactive serine residues in the active sites of carboxypeptidases C_{Ua} and C_{Ub} and the determination of the amino acid sequences around the serine residues.

Experimental

Material——Carboxypeptidases C_{Ua} and C_{Ub} were purified from the exocarp of *Citrus unshiu* MARC. by the method described previously.[1] Benzyloxycarbonyl-L-glutamyl-L-phenylalanine (Z-Glu-Phe) was obtained from the Peptide Research Foundation, Osaka. DFP and 5-dimethylamino-1-naphthalenesulfonyl(dansyl) chloride were purchased from Sigma Chemical Co., St. Louis, Missouri. [^{32}P] DFP (specific radioactivity, 37.7 μCi/μmol) was a product of the Radiochemical Centre, Amersham, England. Standard amino acids and reagents for amino acid analysis were obtained from Wako Pure Chemical Industries, Osaka. Other reagents and organic solvents used were of analytical grade.

Enzyme Assay——Carboxypeptidase activity was assayed with Z-Glu-Phe as a substrate, as described in a previous paper.[1] The protein concentration of enzyme solutions was determined by the Folin-Lowry method at 660 nm[4] with bovine serum albumin as a protein standard.

Preparation of [^{32}P]DFP-treated Enzymes and Their Partial Acid Hydrolysis——The enzymes (9—10 mg

of protein) were incubated with 1 μ mol of [^{32}P]DFP (37.7 μCi) for 60 min at 35°C in 3 ml of 0.1 M citrate buffer, pH 5.5. An equal volume of [^{32}P]DFP was added to the mixtures and the incubation was continued for an additional 60 min. In order to inhibit the enzymes completely, unlabeled DFP (1 μmol) was added and the mixtures were maintained for 60 min at 35°C. This procedure was repeated twice. The mixtures were then passed through a column (2×35 cm) of SephadexG-25 with distilled water as an eluant in order to remove the excess reagent. Fractions containing ^{32}P-labeled protein were collected and lyophilized, and their radioactivity was determined with an Aloka LSC-502 liquid scintillation counter. The ^{32}P-labeled enzymes obtained were partially hydrolyzed for 40 min at 110°C in 0.5 ml of 6 N HCl according to the method of Light.[5] The hydrolysates were diluted with 2 ml of distilled water, lyophilized, and subjected to high voltage paper electrophoresis.

Amino Acid Analysis——The ^{32}P-labeled peptides separated by high voltage paper electrophoresis were hydrolyzed in 0.2 ml of 6 N HCl in evacuated, sealed tubes for 24 h at 110°C. Amino acid analysis of the acid hydrolysates was performed on a single-column Hitachi KLA-5 amino acid analyzer. The content of tryptophan was determined on alkali-hydrolyzed samples by the method of Noltman *et al.*[6]

Sequence Determination——The amino-terminal sequences of the ^{32}P-labeled peptides were analyzed by the dansyl-Edman technique.[7] Their carboxyl-terminal sequences were determined by using carboxypeptidase C_{Ua} as follows. The peptides (10—20 nmol), after their amino termini had been protected with benzyloxycarbonyl groups,[8] were hydrolyzed with 0.4 nmol of carboxypeptidase C_{Ua} for 6 h at 35°C in 0.1 ml of 0.1 M citrate buffer, pH 5.5. The enzymatic reaction was stopped by the addition of 0.9 ml of 0.2 M citrate buffer, pH 2.2, and the mixture was applied directly to the amino acid analyzer.

Results

Preparation of [^{32}P]DFP-treated Enzymes

Incorporation of ^{32}P was determined on aliquots of [^{32}P]DFP-treated enzymes by counting of Cerenkov radiation[9] after the removal of excess reagents by gel filtration on Sephadex G-25. Carboxypeptidases C_{Ua} and C_{Ub} were both inactivated with incorporation of 1 mol [^{32}P]DFP per mol enzyme. The yields of [^{32}P]DFP-treated carboxypeptidases C_{Ua} and C_{Ub} were 95 and 90%, respectively.

Separation of ^{32}P-Labeled Peptides

^{32}P-Labeled peptides were separated from the partial acid hydrolysates of ^{32}P-labeled

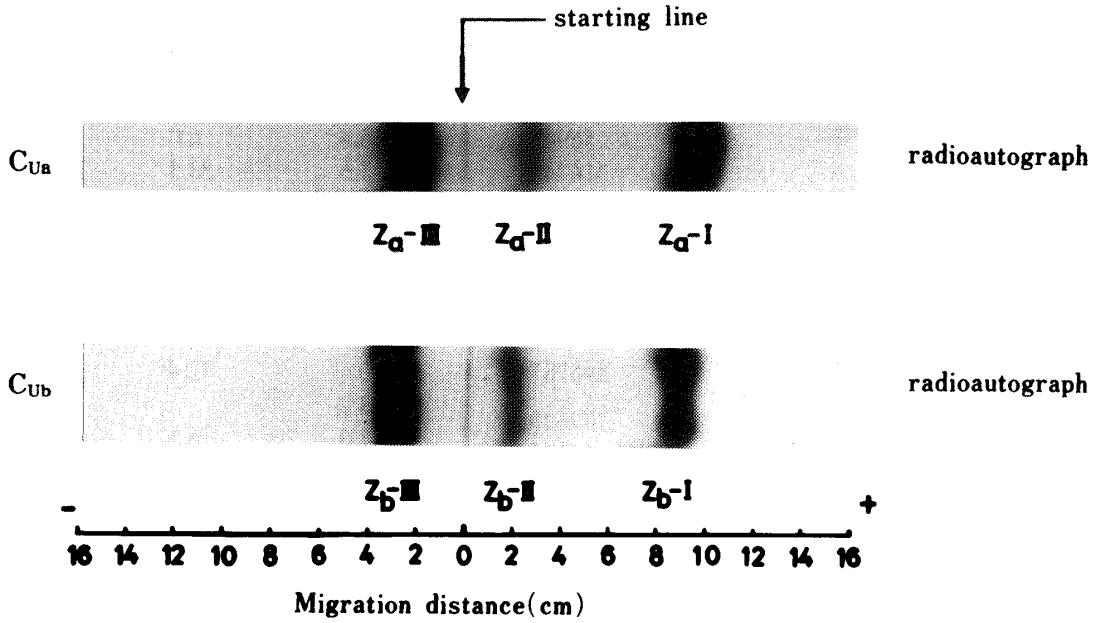

Fig. 1. High Voltage Paper Electrophoresis of Partial Acid Hydrolysates of [^{32}P] DFE-treated Carboxypeptidases C_{Ua} and C_{Ub}

The lyophilized partial hydrolysates were dissolved in 0.2 ml of 1 N formic acid (pH 1.8) and applied to starting lines drawn 20 cm from the anode-side edges of sheets (10 × 68 cm) of Whatman No. 3 MM filter paper. Electrophoresis was carried out for 90 min at 80 V per cm with 1 N formic acid in a Fuji Riken high voltage electrophoresis apparatus equipped with a metal cooling plate. Radioautographs were taken on medical X-ray films (24 h exposure).

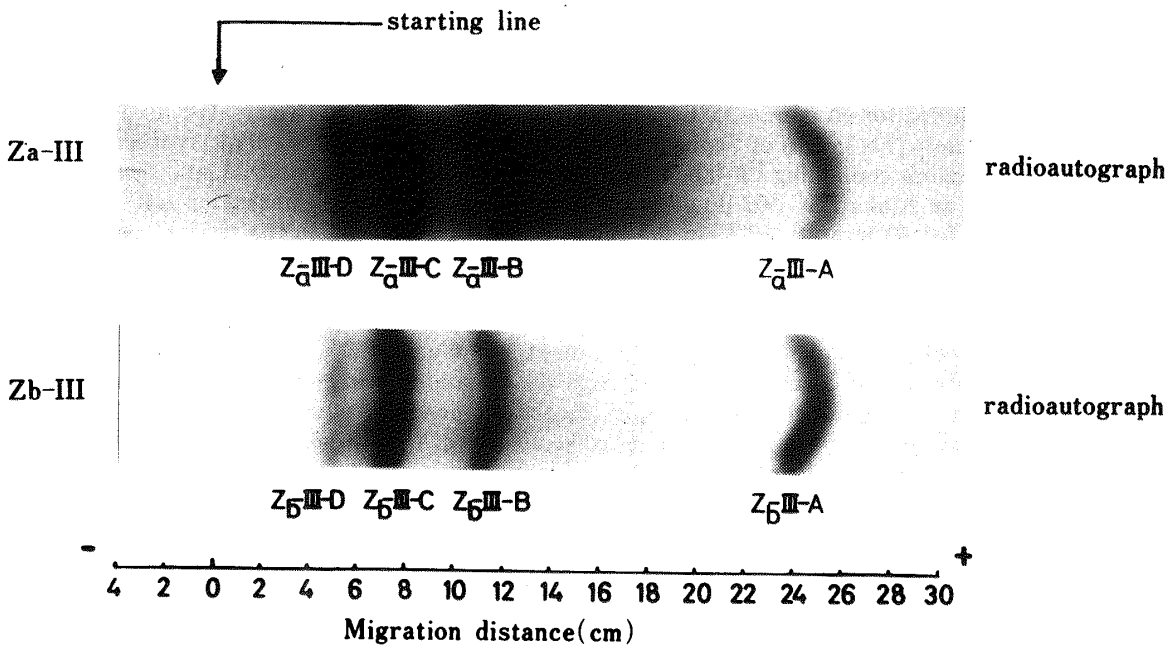

Fig. 2. Paper Electrophoresis of Za-III and Zb-III0

Radioactive peptide fractions isolated by paper electrophoresis at pH 1.8 (Fig. 1) were applied to starting lines drawn 15 cm from the anode-side edges of sheets (10 × 68 cm) of Whatman No. 3 MM filter paper. Electrophoresis was carried out for 180 min at 140 V per cm in pyridine–acetic acid–water (1 : 10 : 89), pH 3.6. Radioautographs were taken on medical X-ray films (48 h exposure).

TABLE I. Radioactivities and Yields of Peptides from [^{32}P] DFP-treated Carboxypeptidases C_{Ua} and C_{Ub}

	Radioactivity (cpm)	Relative radioactivity (%)	Peptide[a] (nmol)
[^{32}P]DFP-treated carboxypeptidase C_{Ua}	889726	100	94.1[b]
Za-I	360339	40.5	
Za-II	56942	6.4	6.0
Za-III	316748	35.6	33.5
Za-III-A	80075	9.0	
Za-III-B	64064	7.2	6.8
Za-III-C	62281	7.0	6.6
Za-III-D	57832	6.5	6.1
[^{32}P]DFP-treated carboxypeptidase C_{Ub}	816787	100	72.4[b]
Zb-I	189495	23.2	
Zb-II	57175	7.0	5.1
Zb-III	454134	55.6	40.3
Zb-III-A	82490	10.1	
Zb-III-B	133135	16.3	11.8
Zb-III-C	72694	8.9	6.4
Zb-III-D	85763	10.5	7.6

a) The yields of peptides were calculated from specific radioactivity measurements.
b) The molecular weights of carboxypeptidases C_{Ua} and C_{Ub} were taken as 96000 and 112000, respectively.[1]

carboxypeptidases C_{Ua} and C_{Ub} by high voltage paper electrophoresis at pH 1.8, as shown in Fig. 1. Three main radioactive fractions, Za-I, -II, and -III, were obtained from the hydrolysate of carboxypeptidase C_{Ua} as with carboxypeptidase C_{Ub}, which gave fractions Zb-I,-II, and

-III. Fractions Za-I and Zb-I were ninhydrin-negative, whereas the others were positive. Fractions Za-II and Zb-II were identified as *O*-phosphorylserine by comparison with an authentic standard.

The peptide fractions, Za-III and Zb-III, were further purified by paper electrophoresis at pH3.6, each yielding four radioactive bands, Za-III-A, -B, -C, and -D and Zb-III-A, -B, -C, and -D, respectively, as shown in Fig. 2.

Amino acids and peptides on the paper were detected with ninhydrin. The peptides were eluted from the paper with 30% acetic acid and lyophilized. Table I shows the radioactivity and yield of each fraction.

Amino Acid Compositions

The results of amino acid analysis of the radioactive fractions are summarized in Table II. The six radioactive peptide fractions, *i.e.* Za-III-B, -C, and -D from carboxypeptidase C_{Ua}

TABLE II. Amino Acid Compositions of Peptides from [^{32}P]DFP-treated Carboxypeptidases C_{Ua} and C_{Ub}

	Peptides[a]	Amino acid composition (mol of amino acid per mol of peptide)
C_{Ua}	Za-I	None (^{32}Pi[b])
	Za-II	Ser 1.0
	Za-III-A	None (^{32}Pi)
	Za-III-B	Asp 0.8, Ser 1.1, Glu 1.2, Gly 2.0
	Za-III-C	Asp 1.0, Ser 1.3, Glu 1.7, Gly 2.6
	Za-III-D	Asp 1.2, Ser 1.3, Glu 1.7, Gly 2.6, Leu 1.2
C_{Ub}	Zb-I	None (^{32}Pi)
	Zb-II	Ser 1.0
	Zb-III-A	None (^{32}Pi)
	Zb-III-B	Asp 1.0, Ser 1.2, Glu 1.1, Gly 2.0
	Zb-III-C	Asp 1.0, Ser 1.2, Glu 1.7, Gly 2.6
	Zb-III-D	Asp 1.0, Ser 1.3, Glu 1.7, Gly 2.9, Leu 1.2

a) Za and Zb refer to the rabioactive fractions obtained by high voltage paper electrophoresis of [^{32}P] DFP-treated carboxypeptidases C_{Ua} and C_{Ub}, respectively.
b) Inorganic phosphate.

TABLE III. Amino Acid Sequences Around the Reactive Serine Residues of Carboxypeptidases C_{Ua} and C_{Ub}

		Glu-Gly-Asp-Ser[a]-Gly-Gly-Glu-Leu
C_{Ua}	Za-III-B	⇁ ⇁ ⇁⇀ ⇁⇀ ⇁⇀
	Za-III-C	⇁ ⇁ ⇁ ⇁↽ ↽ ↽ ↽
	Za-III-D	⇁ ⇁ ⇁ ⇁ ⇁⇀ ⇁⇀ ↽ ↽

		Glu-Gly-Asp-Ser[a]-Gly-Gly-Glu-Leu
C_{ub}	Zb-III-B	⇁ ⇁ ⇁⇀ ⇁⇀ ⇁⇀
	Zb-III-C	⇁ ⇁ ⇁ ⇁↽ ↽ ↽ ↽
	Zb-III-D	⇁ ⇁ ⇁ ⇁ ⇁⇀ ⇁⇀ ↽ ↽

Amino acid sequences were determined by the dansyl-Edman method (⇁) and with carboxypeptidase C_{Ua} (↽).
a) Reactive serine residue.

TABLE IV. Amino Acid Sequences Around the Reactive Serine Residues of Typical Serine Proteases

Enzymes	Amino acid sequences	
Carboxypeptidase C_{Ua}	-Glu-Gly-Asp-Ser[a)]	-Gly-Gly-Glu-Leu-
Carboxypeptidase C_{Ub}	-Glu-Gly-Asp-Ser	-Gly-Gly-Glu-Leu-
Carboxypeptidase Y	-Ala-Gly-Glu-Ser	-Tyr-Ala-His-Gly-[10]
Trypsin (bovine)	-Gln-Gly-Asp-Ser	-Gly-Gly-Pro-Val-[11]
Trypsin (pig)	-Gln-Gly-Asp-Ser	-Gly-Gly-Pro-Val-[12]
Chymotrypsin (bovine)	-Met-Gly-Asp-Ser	-Gly-Gly-Pro-Leu-[13]
Elastase (pig)	-Gln-Gly-Asp-Ser	-Gly-Gly-Pro-Leu-[14]
Plasmin (human)	-Gln-Gly-Asp-Ser	-Gly-Gly-Pro-Leu-[15]
Thrombin (bovine)	-Glu-Gly-Asp-Ser	-Gly-Gly-Pro-Phe-[16]
Subtilisin BPN'	-Asn-Gly-Thr-Ser	-Met-Ala-Ser-Pro-[17]

a) Reactive serine residue.

and Zb-III-B, -C, and -D from carboxypeptidase C_{Ub}, each contained approximately one reactive serine residue. This shows that they had been obtained in a sufficiently homogeneous state to allow sequence analysis. No amino acid was detected in fraction Za-I, Zb-I, Za-III-A, or Zb-III-A.

Sequence Analysis of ^{32}P-Labeled Peptides

The amino acid sequences of the ^{32}P-labeled peptides are summarized in Table III. The amino acid sequences are: Asp-Ser-Gly-Gly-Glu for Za-III-B and Zb-III-B, Glu-Gly-Asp-Ser-Gly-Gly-Glu for Za-III-C and Zb-III-C, and Glu-Gly-Asp-Ser-Gly-Gly-Glu-Leu for Za-III-D and Zb-III-D. The glutamic and aspartic acid residues of these peptides do not seem to have been derived from glutamine and asparagine residues, respectively, since the deamination of the latter two was not observed on partial acid hydrolysis under the conditions described in "Experimental." The amino acid sequences around the reactive serine residues of carboxypeptidases C_{Ua} and C_{Ub} are thus determined to be Glu-Gly-Asp-Ser-Gly-Gly-Glu-Leu.

Discussion

In a previous paper,[1,2] we reported the purification and characteristics of carboxypeptidases C_{Ua} and C_{Ub} from the exocarp of *Citrus unshiu* MARC. The molecular weights of carboxypeptidases C_{Ua} and C_{Ub} were 96000 and 112000, respectively, as determined by ultracentrifugal analysis. Besides the differences in molecular weight, where were differences between the two enzymes in the numbers of amino acid and carbohydrate residues, behavior on diethylaminoethyl (DEAE)-cellulose chromatography, rates of hydrolysis of synthetic substrates, and kinetic parameters for the hydrolysis of Z-Glu-Phe in the presence and absence of inhibitors. Both enzymes were also different from carboxypeptidase C_N, which had been isolated from *Citrus natsudaidai* HAYATA and assumed to be a serine protease, in the chemical and enzymatic properties mentioned above, although an overall similarity exists among the three. A similarity was also seen between carboxypeptidases C_{Ua} and C_{Ub} in the inhibition pattern by chemical reagents.[2]

We have postulated from the results of modification studies[2] that carboxypeptidases C_{Ua} and C_{Ub} are serine proteases and not metalloenzymes like the pancreatic carboxypeptidases, and that sulfhydryl groups are not essential for their activities, in contrast with baker's yeast carboxypeptidase Y, which requires a sulfhydryl group as well as a reactive serine residue for its activity.[10] In the present study, reactive serine residues in the active sites of carboxypeptidases C_{Ua} and C_{Ub} were identified, and the amino acid sequences around the serine residues were determined. Table IV shows the amino acid sequences around the reactive serine

residues of typical serine proteases.

The sequences of seven amino acid residues around the reactive serine residues of carboxypeptidases C_{Ua} and C_{Ub} are similar to those of trypsin, chymotrypsin, elastase, and thrombin. In contrast, carboxypeptidase Y, which has a specificity similar to those of carboxypeptidases C_{Ua} and C_{Ub}, possesses a sequence different from those of the latter two.

Besides the serine residue, they have an aspartic acid and a histidine residue in the catalytic site, except for carboxypeptidase Y, which also has an essential sulfhydryl group in its active site. These finding suggests that carboxypeptidases C_{Ua} and C_{Ub} may have catalytic site structures similar to those of trypsin, chymotrypsin, and related enzymes. On the other hand, since carboxypeptidases C_{Ua} and C_{Ub} are not inhibited by L-(1-tosylamide-2-phenyl)-ethyl chloromethyl ketone or 1-chloro-3-tosylamide-7-amino-2-heptanone, a specific inhibitor of chymotrypsin or trypsin, respectively, and since they are not inhibited by soybean trypsin inhibitor, their binding sites seem to be different from those of chymotrypsin and trypsin, though direct proof of this remains to be obtained.

The present results provide direct evidence that carboxypeptidases C_{Ua} and C_{Ub} are "serine carboxypeptidases" [EC 3.4.16.1] as defined in Enzyme Nomenclature (1978), Nomenclature Committee of the International Union of Biochemistry.

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