Chem. Pharm. Bull. 28(12)3479—3487(1980)

Carboxypeptidases from the Exocarp of Mandarin Orange (*Citrus unshiu* Marc.). II.[1] Chemical and Enzymatic Properties of Carboxypeptidases C_{Ua} and C_{Ub}

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(Received May 19, 1980)

Carboxypeptidases C_{Ua} and C_{Ub} were both shown to be glycoproteins by amino acid and carbohydrate analyses. The numbers of amino acid, hexose, and hexosamine residues were 811, 26 (as galactose), and 6 (as glucosamine), respectively, for carboxypeptidase C_{Ua} and 868, 42, and 8, respectively, for carboxypeptidase C_{Ub}. Both enzymes were almost completely inhibited by sodium dodecylsulfate and cetyltrimethyl ammonium bromide, but not by Brij-35, Tween 80, or Triton X-100. The enzymes were also inactivated by glycine ethyl ester in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, *p*-bromophenacyl bromide, phenylglyoxal, $AgNO_3$, CH_3HgCl, urea, and guanidine. When the enzymes were incubated with ^{203}Hg-labeled *p*-chloromercuribenzoic acid ([^{203}Hg]PCMB), approximately 0.2 mol of [^{203}Hg]PCMB was incorporated into them at pH 5.5 with no change in the enzymatic activity. After the denaturation of the enzymes with urea and guanidine, the incorporation of [^{203}Hg]PCMB increased to 2—3 mol per mol of enzyme.

The values of K_m, V_{max}, and k_p for the hydrolysis of Z–Glu–Phe were 7.7×10^{-2} M, 222 μmol/l·min·mg, and 2.4×10^4 min^{-1}, respectively, for carboxypeptidase C_{Ua} and 7.1×10^{-2} M, 125 μmol/l·min·mg, and 1.6×10^4 min^{-1}, respectively, for carboxypeptidase C_{Ub}. Diisopropylfluorophosphate, phenylmethanesulfonyl fluoride and $HgCl_2$ inactivated the enzymes with decreases in the values of V_{max} and k_p but with no change in that of K_m, indicating that they are noncompetitive inhibitors of both enzymes. 3-Phenylpropionic acid, which inhibited the enzymes reversibly, increased the value of K_m without changing those of V_{max} and k_p and thus is a competitive inhibitor.

Keywords——carboxypeptidase; *Citrus unshiu* Marc.; amino acid composition; stability; inhibition; chemical modification; kinetic parameter

Carboxypeptidases C_{Ua} and C_{Ub} [EC 3.4.16.1], both isolated from the exocarp of *Citrus unshiu* Marc., are carboxyl-terminal exopeptidases with broad specificity.[1] Their ability to liberate proline as well as other neutral, acidic, and basic amino acids from the carboxyl-terminus of a peptide chain makes them especially useful for sequence analysis. The molecular weights of carboxypeptidases C_{Ua} and C_{Ub} are 96000 and 112000, respectively, as determined by ultracentrifugal analysis. The enzymes are stable in the pH range from 4.5 to 5.5, but are quickly denatured at pH below 4.0 or over 7.0. They are strongly inhibited by diisopropylfluorophosphate (DFP) and $HgCl_2$.

Carboxypeptidases possessing similar specificity have been isolated from orange fruit[3] and leaves[4] and the exocarp of *Citrus natsudaidai* Hayata.[5] The enzymes from the former two sources are called carboxypeptidase C, and the enzyme from the last is called carboxypeptidase C_N. So far, however, the amino acid composition and the partial structure of the

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3) H. Zuber, *Nature* (London), **201**, 613 (1964); H. Zuber, *Hoppe-Seyler's Z. Physiol. Chem.*, **349**, 1337 (1968).
4) B. Sprössler, H.-D. Heilman, E. Grampp, and H. Uhlig, *Hoppe-Seyler's Z. Physiol. Chem.*, **352**, 1524 (1971).

active center have been reported only for carboxypeptidase C_N.[5c,d]

In the case of *Citrus unshiu* MARC., two carboxypeptidases were isolated from the exocarp. This is different from the other cases, in which only one carboxypeptidase was isolated from a single citrus source.

The present work was undertaken to determine some chemical and enzymatic properties of carboxypeptidases C_{Ua} and C_{Ub} in order to compare the characteristics of these enzymes with those of carboxypeptidase C_N and other carboxypeptidases. Modification studies were also carried out to identify the amino acid residues participating in the active centers of the enzymes.

Carboxypeptidases C_{Ua} and C_{Ub} were shown to be different from each other and also from carboxypeptidase C_N in amino acid and carbohydrate compositions and kinetic parameters. In most cases, the inhibitors and modifying agents tested exerted similar influences on carboxypeptidases C_{Ua} and C_{Ub}. The results of modification experiments indicated that serine, arginine, and aspartic or glutamic acid residues were possibly involved in the active centers of both enzymes.

Experimental

Materials——Benzyloxycarbonyl-L-glutamyl-L-phenylalanine (Z–Glu–Phe) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were obtained from the Peptide Research Foundation, Osaka. Glycine ethyl ester (Gly–OEt) and *p*-bromophenacyl bromide (BPB) were products of Nakarai Chemical, Co., Kyoto. DFP was obtained from Sigma Chemicals Co., St. Louis, Missouri. ^{203}Hg-labeled *p*-chloromercuribenzoic acid ([^{203}Hg]PCMB) was a product of the Radiochemical Centre, England. Phenylmethanesulfonyl fluoride (PMSF) was obtained from Eastman Kodak Co., Rochester, New York. Other reagents and organic solvents used were of analytical grade.

Enzymes——Carboxypeptidases C_{Ua} and C_{Ub} were purified from the exocarp of *Citrus unshiu* MARC. by the method described previously.[1] Their homogeneity was confirmed by both disc electrophoresis and ultracentrifugal analysis. By this purification method, each enzyme was obtained in the form of a solution in 0.1 M citrate buffer, pH 5.5, and was used as such or after dilution with the same buffer to desired concentrations. This buffer was used throughout the present work, unless otherwise specified. When it was necessary to replace the buffer, solutions of the enzyme were dialyzed against the required buffer.

Carboxypeptidase Assay——Carboxypeptidase activity was determined as described in a previous paper with Z–Glu–Phe as a substrate.[1] The specific activities of carboxypeptidases C_{Ua} and C_{Ub} were 33.4 and 26.8 units per mg of protein, respectively, towards this substrate. (One unit is defined as the amount of enzyme required to liberate 1 μmol of phenylalanine from the substrate per min).

Protein Determination——The protein concentration of enzyme solutions was determined by absorbance measurement at 280 nm with a Hitachi 101 spectrophotometer, unless otherwise stated. Bovine serum albumin was used as a protein standard.

Amino Acid Analysis——The enzymes (0.5—0.7 mg) were hydrolyzed with 1 ml of 6 N HCl under a vacuum in sealed Pyrex glass tubes at 110° for 24, 48, 72, and 96 hr. After removal of the HCl under reduced pressure over NaOH pellets, the hydrolysates were dissolved separately in 2 ml of 0.2 M citrate buffer, pH 2.2, and the amino acid concentrations in the solutions were determined with a Hitachi KLA-5 amino acid analyzer. Hexosamine was estimated simultaneously in this analysis. The content of tryptophan was determined on separate, unhydrolyzed samples by the ultraviolet spectrophotometric method.[6]

Carbohydrate Analysis——Carbohydrates were identified by thin-layer chromatography on Silica gel G plates (250 μm in thickness and 20 × 20 cm in size) with *n*-propanol–water (7 : 1) as a developing solvent,[5a,7] after hydrolysis of the enzymes in 3 N HCl at 100° for 24 hr under reduced pressure. The carbohydrates were located with a 2.5% solution of aniline hydrogen phthalate in *n*-butanol-saturated water. The content

5) *a*) Y. Kubota, S. Shoji, T. Funakoshi, and H. Ueki, *J. Biochem.*, **74**, 757 (1973); *b*) Y. Kubota, S. Shoji, T. Funakoshi, and H. Ueki, *J. Biochem.*, **76**, 375 (1974); *c*) S. Shoji and Y. Kubota, "Proceedings of the 13th Symposium on Peptide Chemistry," ed. by S. Yamada, Protein Research Foundation, Osaka, 1975, p. 94; *d*) S. Shoji and Y. Kubota, "Proceedings of the 14th Symposium on Peptide Chemistry," ed. by T. Nakajima, Protein Research Foundation, Osaka, 1976, p. 155.

6) G.H. Beaven and E.R. Holiday, "Advances in Protein Chemistry," Vol. VII, ed. by M.L. Anson, K. Bailey, and J.T. Edsall, Academic Press, New York, 1952, pp. 319—386.

7) A.E. Gal, *Anal. Biochem.*, **24**, 452 (1968).

of hexose was estimated by the methods of Morris[8] and Dreywood.[9]

Reaction with [^{203}Hg]PCMB——The enzymes (1 mg of protein) were incubated with 0.33 mmol of [^{203}Hg]-PCMB (1 μCi) at 35° for 120 min in 2 ml of 0.1 M citrate buffer, pH 5.5 or 7.0. After incubation, aliquots of 10 μl of the mixtures were withdrawn and assayed for enzymatic activity. The remains of the mixtures were dialyzed against 0.1 M citrate buffer, pH 5.5, and then against deionized water, for 3 days in each case. After dialysis, the protein concentration was determined by the method of Lowry *et al.*,[10] and the radioactivity with a Well type liquid scintillation counter. Control experiments were carried out under the same conditions except for omission of [^{203}Hg]PCMB. In other experiments, the enzymes were denatured with urea and guanidine in the following way prior to modification with [^{203}Hg]PCMB. Enzyme solutions in 0.1 M citrate buffer, pH 5.5, were dialyzed against 8 M urea and 6 M guanidine at 4° for 48 hr and then redialyzed against the citrate buffer under the same conditions. The redialyzed, denatured enzymes were then made to react with [^{203}Hg]PCMB as described above.

Results

Ultraviolet Absorption Spectra and Isoelectric Point

Carboxypeptidases C_{Ua} and C_{Ub} showed a typical absorbancy maximum near 280 nm and a minimum near 255 nm. The isoelectric points of carboxypeptidases C_{Ua} and C_{Ub} were determined to be pH 4.8 and 4.7, respectively, by cellulose acetate paper electrophoresis according to the method of Kubota and Ueki.[11]

Amino Acid and Carbohydrate Compositions

The amino acid compositions of carboxypeptidases C_{Ua} and C_{Ub} are given in Table I. The numbers of amino acid residues in Table I were calculated from the values obtained by extraporating the observed values of amino acids at four different hydrolysis times to zero time and on the basis of molecular weights, of 96000 and 112000 for carboxypeptidases C_{Ua} and C_{Ub}, respectively.

The hydrolysates of carboxypeptidases C_{Ua} and C_{Ub} each gave two carbohydrate spots with *Rf* values of 0.50 and 0.22, corresponding to those of authentic samples of galactose and glucosamine, respectively, on thin-layer chromatograms. The hexose content was therefore estimated with galactose as a reference standard. The contents of amino acid, hexose and hexosamine residues were 93.6, 4.4 (as galactose), and 1.0% (as glucosamine), respectively, for carboxypeptidase C_{Ua} and 86.7, 6.0 and 1.1%, respectively, for carboxypeptidase C_{Ub}. Carboxypeptidase C_{Ua} thus contains 811 amino acid residues, 26 galactose residues, and 6 glucosamine residues, and C_{Ub} comprises 868 amino acid residues, 42 galactose residues, and 8 glucosamine residues.

Stability

Desalting of carboxypeptidases C_{Ua} and C_{Ub} by dialysis caused losses of 74 and 92%, respectively, and lyophilization after desalting produced losses of 96 and 99%, respectively, of their original activities. When stored at 4° in the citrate buffer, they were quite stable and retained more than 85% of their original activities even after 3 months. These conditions are suitable for the storage of the enzymes.

Effects of Detergents on Carboxypeptidase Activity

Sodium dodecylsulfate (SDS), Tween 80, cetyltrimethyl ammonium bromide (CTMA), Triton X-100, and Brij-35 were used as detergents. The substrate, Z–Glu–Phe, was dissolved at a concentration of 0.02 M in citrate buffer containing the detergents at concentrations of 1—10%. Mixtures of 0.1 ml each of detergent solutions (2—20%) and enzyme solutions

8) D.L. Morris, *Science*, **107**, 254 (1948).
9) R. Dreywood, *Ind. Eng. Chem. Anal. Ed.*, **18**, 499 (1946).
10) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
11) Y. Kubota and H. Ueki, *J. Biochem.*, **64**, 405 (1968).

Table I. Amino Acid and Carbohydrate Compositions of Carboxypeptidases C_{Ua} and C_{Ub}

Amino acid or carbohydrate	Number of residues[a]		
	C_{Ua}	C_{Ub}	C_N[b]
Tryptophan[c]	13	12	6
Lysine	55	52	29
Histidine	25	26	18
Arginine	23	34	44
Aspartic acid	84	94	83
Threonine	42	52	37
Serine	63	57	52
Glutamic acid	78	78	46
Proline	41	56	14
Glycine	83	75	43
Alanine	73	83	50
Half-cystine	6	6	6
Valine	50	48	44
Methionine	8	11	7
Isoleucine	32	33	58
Leucine	65	74	93
Tyrosine	34	42	37
Phenylalanine	36	35	36
Total	811	868	703
Hexosamine[d]	6	8	5
Hexose[e]	26	42	32
Sum of amino acid and carbohydrate	843	918	740

a) Calculated on the basis of molecular weights of 96000 and 112000 for carboxypeptidases C_{Ua} and C_{Ub}, respectively.[1]
b) Carboxypeptidase C_N, cited from a previous paper.[5b]
c) Determined by the ultraviolet spectrophotometric method.[6]
d) Calculated as glucosamine. See the text.
e) Estimated by the methods of Morris[8] and Dreywood[9] as galactose. See the text.

(4 μg of C_{Ua} and 5 μg of C_{Ub}) in the citrate buffer were incubated at 35° for 30 min and assayed with substrate solutions having the same detergent concentrations as the mixtures.

SDS and CTMA almost completely inhibited the enzymes at final concentrations of 1 and 2%, respectively. Tween 80, Triton X-100, and Brij-35, on the other hand, showed only slight or no inhibition in the final concentration range of 1—10%.

Effect of Phenylalanine Analogs on Carboxypeptidase Activity

N-Acetyl-D-phenylalanine, 3-phenylpropionic acid (3-PPA), indole-3-acetic acid, *p*-nitrophenylacetic acid, *p*-hydroxyphenylpyruvic acid, and phenylacetic acid were used as phenylalanine analogs. Inhibition experiments with these analogs were carried out as described in a previous paper.[5b] Mixtures of 0.2 ml each of analog solutions and enzyme solutions (7 μg of C_{Ua} and 10 μg C_{Ub}) were incubated at 35° for 30 min at final analog concentrations of 1—50 mM. Only weak or no inhibition was found at a concentration of 1 mM of the analogs. At 5 mM, all the analogs distinctly inhibited the enzymes, with the exception of N-acetyl-D-phenylalanine, which had practically no influence on the enzymatic activity at any concentration. The degree of inhibition increased with increasing concentration of the analogs. 3-PPA showed the strongest inhibition at every concentration. At 50 mM, the remaining activity relative to the activity of the control, which was obtained without addition of the analogs, was approximately 5% for 3-PPA and was in the range of 18—41% for the other analogs, depending on their structures. The decreased activities of the enzymes after

treatment with 3-PPA were restored to the original levels on dialysis against the citrate buffer at 4° for 48 hr.

Effects of Urea and Guanidine on Carboxypeptidase Activity

Enzyme solutions (0.5 ml, 11 μg of C_{Ua} and 9 μg of C_{Ub}) were dialyzed against citrate buffer containing 1—8 M urea or 1—6 M guanidine at 4° for 48 hr and assayed for enzymatic activity. Both enzymes were inactivated by urea and guanidine, and their activities decreased with increasing concentration of the denaturants. Carboxypeptidases C_{Ua} and C_{Ub} lost 97 and 94%, respectively, of their original activities on dialysis against 8 M urea. When dialyzed against 4 and 6 M guanidine, they lost approximately 93% of their original activities. The enzymes were not fully reactivated after removal of the urea and guanidine by dialysis.

Reaction with [^{203}Hg]PCMB

Table II summarizes the results of the modification experiments with [^{203}Hg]PCMB. When the intact enzymes were incubated with [^{203}Hg]PCMB at pH 5.5, approximately 0.2 mol of [^{203}Hg]PCMB was incorporated into 1 mol of each of the enzymes without any loss of enzymatic activity. At pH 7.0, approximately 0.2 mol of [^{203}Hg]PCMB was again incorporated into the enzymes. In this case, however, the activities of carboxypeptidases C_{Ua} and C_{Ub} decreased to 23 and 11%, respectively, of those of the controls. The incorporation of [^{203}Hg]-PCMB increased on denaturation of the enzymes with urea and guanidine. The amounts of [^{203}Hg]PCMB bound to urea-denatured and guanidine-denatured carboxypeptidase C_{Ua} were 2.0 and 1.5 mol, respectively. The incorporation increased more markedly with denatured carboxypeptidase C_{Ub}; 3.0 and 2.8 mol of [^{203}Hg]PCMB were bound to urea-denatured and guanidine-denatured carboxypeptidase C_{Ub}, respectively.

Table II. Reactions of Carboxypeptidases C_{Ua} and C_{Ub} with [^{203}Hg]PCMB

	Enzyme	pH	Concentration of enzyme (nmol/ml)	Relative[a] activity (%)	PCMB incorporated (mol/mol of enzyme)
C_{Ua}	Intact	5.5	2.8	107	0.19
		7.0	2.7	23	0.20
	Urea-denatured	5.5	0.64	—[b]	2.0
	Guanidine-denatured	5.5	0.68	—	1.5
C_{Ub}	Intact	5.5	2.2	106	0.17
		7.0	2.0	11	0.23
	Urea-denatured	5.5	0.32	—[b]	3.0
	Guanidine-denatured	5.5	0.32	—	2.8

a) Relative enzymatic activity after reaction with [^{203}Hg]PCMB, expressed as a percentage of the activity of the control.
b) Not assayed, since urea and guanidine had almost completely inactivated the enzymes.

Effect of Chemical Modification on Carboxypeptidase Activity

The enzymes were made to react with a variety of chemical reagents under the conditions described in Table III and in a previous paper.[5b] Carboxypeptidases C_{Ua} and C_{Ub} were inhibited by DFP and PMSF, and the degree of inhibition increased with increasing concentration of the inhibitors. No or only slight activity remained after incubation with 1 mM DFP or PMSF. These inhibitors are considered to react with the hydroxyl group of a reactive serine residue.[12]

Both enzymes were strongly inhibited by $HgCl_2$. They lost 95% or more of their activities when the concentration of $HgCl_2$ was raised from 0.01 to 0.1 mM. At 1 mM $HgCl_2$, they were

12) E.F. Jansen and A.K. Balls, *J. Biol. Chem.*, **194**, 721 (1952); D.E. Fahrney and A.M. Gold, *J. Am. Chem. Soc.*, **85**, 997 (1963).

completely inactivated. The enzymes were also inactivated by $AgNO_3$ and CH_3HgCl, but to somewhat lesser extents than by $HgCl_2$. When carboxypeptidases C_{Ua} and C_{Ub} were incubated with 1 mM $AgNO_3$, their activities decreased to 3 and 7%, respectively, of the activities of the controls. The activities of carboxypeptidases C_{Ua} and C_{Ub} decreased to 27 and 24%, respectively, after incubation with 1 mM CH_3HgCl. The enzymes inactivated by these reagents could not be reactivated by dialysis against citrate buffer containing 1 mM EDTA.

Carboxyl groups in the enzymes were modified both with BPB and with Gly–OEt in the presence of EDC. Both enzymes were partially inactivated by BPB, and their activities decreased with increase in the incubation time. After incubation for 24 hr, the activities of carboxypeptidases C_{Ua} and C_{Ub} decreased to 40 and 45%, respectively, of those of the controls. Incubation of the enzymes with Gly–OEt and EDC also resulted in a loss of enzymatic activity. They almost completely lost their activities after 24 hr.

Phenylglyoxal, a modifier of arginine residues, inactivated the enzymes at pH 7.0. At pH 5.5, however, no or only weak inhibition was found even after incubation for 24 hr.

2-Hydroxy-5-nitrobenzyl bromide (HNBB), a modifier of tryptophan residues, showed practically no effect on the activities of the enzymes after incubation for 6 or 24 hr.

Kinetic Parameters

Table IV lists the kinetic parameters of carboxypeptidases C_{Ua} and C_{Ub} in the presence and absence of inhibitors. K_m and V_{max} for carboxypeptidase C_{Ua} were similar to, and nearly

Table III. Effects of Chemical Modifications on Carboxypeptidases C_{Ua} and C_{Ub}

Reagent	Incubation			Relative activity[a] (%)	
	Time (hr)	Temp. (°)	Concentration of reagent (mM)	C_{Ua}	C_{Ub}
DFP	2	35	0.01	66	71
	2	35	0.1	30	37
	2	35	1	0	0
PMSF	2	35	0.01	53	61
	2	35	0.1	34	48
	2	35	1	3	5
$HgCl_2$	0.5	35	0.01	81	84
	0.5	35	0.1	5	3
	0.5	35	1	0	0
CH_3HgCl	2	35	0.01	83	99
	2	35	0.1	79	46
	2	35	1	27	24
$AgNO_3$	2	35	0.01	45	59
	2	35	0.1	33	27
	2	35	1	3	7
BPB	2	35	1	79	76
	24	35	1	40	45
Gly–OEt (with EDC)	2	25	1000 (EDC, 150)	62	77
	24	25	1000 (EDC, 150)	0	0
Phenylglyoxal	6	25	30	98	92
	24	25	30	89	76
	6[b]	25	30	30	18
	24[b]	25	30	6	4
HNBB	6	25	10	89	97
	24	25	10	107	104

The enzymes were incubated with the reagents at pH 5.5 in 0.1 M citrate buffer, unless otherwise indicated, and assayed with Z-Glu-Phe as a substrate.

a) Expressed as a percentage of the activity of the control, which was determined under the same conditions except for omission of the reagents.

b) Incubated at pH 7.0 in 0.2 M ethylmorpholine buffer.

TABLE IV. Kinetic Parameters of Carboxypeptidases C_{Ua} and C_{Ub} for the Hydrolysis of Z–Glu–Phe in the Presence and Absence of Inhibitors

Enzyme	Inhibitor	Concentration of inhibitor (mM)	K_m ($10^2 \times$M)	V_{max} (μmol/l·min·mg)	k_p[a] ($10^{-4} \times min^{-1}$)
C_{Ua}	None	0	7.7	222	2.4
	3-PPA	1	12.5	222	2.4
	PMSF	0.1	7.1	154	1.7
	DFP	0.1	7.1	105	1.1
	$HgCl_2$	0.02	7.1	91	1.0
C_{Ub}	None	0	7.1	125	1.6
	3-PPA	1	8.3	125	1.6
	PMSF	0.1	6.7	71	0.9
	DFP	0.1	6.7	63	0.8
	$HgCl_2$	0.02	6.3	38	0.5

After the enzymes had been incubated with or without the inhibitors in 2 ml of 0.1 M citrate buffer, pH 5.5, at 35° for 30 min, aliquots of 0.25 ml were withdrawn from the incubation mixtures and made to react with 0.5 ml of 0.012—0.020 M Z-Glu-Phe at 50° for 5 min. During this time, aliquots of 0.1 ml were withdrawn at intervals of 1 min to determine the amount of phenylalanine liberated by the ninhydrin method.[5a]

a) Molecular activity, the number of substrate molecules hydrolyzed/mol of enzyme/min.

twice as large as the corresponding parameters for carboxypeptidase C_{Ub}, respectively. The value of molecular activity, k_p, was also higher for carboxypeptidase C_{Ua} than for carboxypeptidase C_{Ub}. Inactivation of the enzymes with PMSF, DFP, and $HgCl_2$ resulted in decreases in the values of V_{max} and k_p, whereas that of K_m remained unchanged. 3-PPA increased the value of K_m without changing those of V_{max} and k_p.

Discussion

In a previous paper,[1] we reported the purification of carboxypeptidases C_{Ua} and C_{Ub} from the exocarp of *Citrus unshiu* MARC. This was the first report demonstrating the presence of two carboxypeptidases in a single citrus source. The enzymes were separated from each other by ion-exchange chromatography on DEAE-cellulose. Besides the difference in behavior on DEAE-cellulose chromatography, there were differences between the two enzymes in molecular weight and rates of hydrolysis of synthetic substrates.

In the present study, the amino acid and carbohydrate compositions and some enzymatic properties of carboxypeptidases C_{Ua} and C_{Ub} have been determined and compared. The values of isoelectric point, pH 4.8 and 4.7 for carboxypeptidases C_{Ua} and C_{Ub}, respectively, are close to those of pH 4.3 for carboxypeptidase C_N[5b] and pH 4.5 for carboxypeptidase C from orange leaves.[4] There is some difference between carboxypeptidases C_{Ua} and C_{Ub} in the contents of amino acid, hexose, and hexosamine residues, although an overall similarity exists. The amino acid and carbohydrate compositions of both enzymes are significantly different from those of carboxypeptidase C_N, as shown in Table I.

Carboxypeptidases C_{Ua} and C_{Ub} were strongly inhibited by SDS and CTMA, whereas little or no effect was found with Triton X-100, Tween 80, or Brij-35. The latter three can therefore be used as solubilizers for less soluble substrates. Both enzymes were susceptible to denaturation by urea and guanidine and were not reactivated after removal of the reagents by dialysis. The enzyme molecule may undergo an irreversible structural alteration under the influence of urea and guanidine, resulting in marked loss of enzymatic activity.

Among the phenylalanine analogs tested, 3-PPA showed the strongest inhibition. The decreased activities of the enzymes after treatment with 3-PPA were restored to the original levels on dialysis, indicating that this analog is a competitive inhibitor of the enzymes.

In a previous paper,[1] we reported that PCMB decreased the activity of carboxypeptidase C_{Ub} to 75% of that of the control but excerted practically no effect on carboxypeptidase C_{Ua} on incubation at pH 5.5 and 35 for 120 min. In the present paper, we have re-examined the effect of PCMB on the enzymes by use of the ^{203}Hg-labeled compound. When the enzymes were incubated with [^{203}Hg]PCMB at pH 5.5 or 7.0 in 0.1 M citrate buffer, only 0.2 mol of [^{203}Hg]-PCMB was incorporated into 1 mol of each of the enzymes. At pH 5.5, neither of the enzymes was inactivated by PCMB. This finding is somewhat different from the previous observations. This is due probably to the difference in the concentration of PCMB, since the final PCMB concentration used previously was 0.5 mM instead of 0.17 mM in the present experiment. When the enzymes were incubated with [^{203}Hg]PCMB at pH 7.0, their activities decreased significantly. This seems to be attributable to the instability of the enzymes in the neutral and alkaline pH regions.[1] At pH 7.0, some structural or conformational change may have occurred in the enzyme molecule, resulting in the decrease in enzymatic activity.

Approximately 2 and 3 mol of [^{203}Hg]PCMB were incorporated into carboxypeptidases C_{Ua} and C_{Ub}, respectively, after denaturation with urea or guanidine. These findings suggest that the intact enzymes, which possess sulfhydryl groups buried in the molecule, scarcely react with PCMB, whereas the denatured enzymes, in which a part of the buried sulfhydryl groups would have appeared on the surface of the molecule, tend to react with PCMB. As we described previously,[1] the enzymes were not inhibited by NEM, which usually rapidly inhibits plant sulfhydryl proteases such as papain.[13] Moreover, carboxypeptidases C_{Ua} and C_{Ub} were not inactivated by either MIA or DTT. Sulfhydryl groups in the enzymes, therefore, are not likely to be essential for enzymatic activity.

$HgCl_2$, $AgNO_3$, and CH_3HgCl inactivated the enzymes irreversibly, suggesting that they are non-competitive inhibitors. $HgCl_2$ is known to react with sulfhydryl,[14] imidazole and carboxyl groups.[15] Chambers *et al.*[16] reported from the results of an X-ray crystallographic study that the primary silver ion binding site on trypsin was at the active center between the carboxyl group of Asp-102 and the δ-nitrogen of His-57. In the case of carboxypeptidases C_{Ua} and C_{Ub}, sulfhydryl groups are not likely to be essential for their activities as stated above. It is probable, therefore, that these metal ions are bound between the carboxyl group of an aspartic or glutamic acid residue and the imidazole group of a histidine residue in the active center of the enzymes, as with trypsin.

We reported previously that carboxypeptidases C_{Ua} and C_{Ub} were serine proteases and not metalloenzymes like the pancreatic carboxypeptidases.[1] In the present study, the activities of carboxypeptidases C_{Ua} and C_{Ub} decreased on modification with DFP, PMSF, BPB, Gly–OEt (in the presence of EDC), and phenylglyoxal. These findings suggest that the hydroxyl groups of serine residues, the carboxyl groups of aspartic or glutamic acid residues, and the guanidyl groups of arginine residues may be involved in the catalytic or binding sites of the enzymes, though direct proof remains to be obtained.

There is some difference between carboxypeptidases C_{Ua} and C_{Ub} in the kinetic parameters for the hydrolysis of Z–Glu–Phe in the presence and absence of inhibitors. The values of 7.7×10^{-2} and 7.1×10^{-2} M for the K_m of carboxypeptidases C_{Ua} and C_{Ub}, respectively, are slightly higher than the value of 4.0×10^{-2} M for carboxypeptidase C_N. These values are all quite high when compared to those for other nonspecific carboxypeptidases[3,17] which generally

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have values of the order of 10^{-3} M or even lower.

The kinetic properties of carboxypeptidases C_{Ua} and C_{Ub} seem to be somewhat different from those of other carboxypeptidases having similar specificity. The differences may result from different structures and natures of the binding sites specific to individual enzymes, though differences in assay conditions and substrates should also be taken into account. The kinetic data in Table IV show that PMSF and DFP as well as $HgCl_2$ are noncompetitive inhibitors of the enzymes, as they are with carboxypeptidase C_N.[5b] The data also show that 3-PPA is a competitive inhibitor of carboxypeptidases C_{Ua} and C_{Ub}, as it is with both carboxypeptidase C_N[5b] and carboxypeptidase A.[18]

Like carboxypeptidases C_{Ua} and C_{Ub}, several carboxypeptidases have been shown to occur in multiple forms.[17a,19] It seems likely that the occurrence of multiple forms of carboxypeptidases in a single organ, tissue, or cell is common in animals, plants, and microorganisms, though the differences in their functions remain to be elucidated. The possibility should also be considered that the multiple forms originate from a single carboxypeptidase or its zymogen through enzymatic cleavage during the extraction.

Acknowledgement This work was supported in part by a grant from the Ministry of Education, Science and Culture of Japan.

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