Action of Serine Carboxypeptidases on Endopeptidase Substrates, *N*-Acyldipeptideamides

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Action patterns of two serine carboxypeptidases, one from yeast (Y) and the other from wheat bran (W), on *N*-[3-(2-furyl)acryloyl]-(Fua-) dipeptideamide substrates were examined by HPLC and amino acid analysis. In the reaction of the wheat enzyme the substrates were hydrolyzed to Fua-amino acid and no sufficient amount of Fua-dipeptides were detected on HPLC in the product mixtures. Very few or no free amino acids were observed by amino acid analysis. This indicates that the wheat enzyme exhibited carboxamidopeptidase activity on these substrates. On the contrary, carboxypeptidase Y gave Fua-amino acids and Fua-dipeptides as products, depending on the structure of the substrates. Accordingly, liberations of free amino acids were detected in some cases. This result shows that the yeast enzyme acts on some of the substrates in a two step manner: First by amidase and second by a carboxypeptidase activity. Based on these results the substrate binding mechanisms of these enzymes are discussed.

Serine carboxypeptidase [EC3.4.16.1] includes cathepsin A, and carboxypeptidases from yeast,[1] molds and plants.[2] They are considerably different from metallo-carboxypeptidases represented by carboxypeptidase A.[3,4] In particular, some of the serine carboxypeptidases show hydrolytic activities towards synthetic chymotrypsin-type substrates, such as Ac-Tyr-OEt and Bz-Tyr-NH-Np.[5-8] Recently, carboxamidopeptidase activities against several peptide hormones have also been reported,[9-12] further, we have found that *N*-substituted peptide amides with such a large 7-amino-4-methyl-2*H*-1-benzopyran-2-one can be hydrolyzed by, for example, an enzyme from yeast (carboxypeptidase Y).[13]

These endopeptidase-like activities of this class of enzyme, especially the esterase activity, have been utilized for peptide synthesis, by aminolysis of the ester or by transpeptidation.[14,15] However, this variety of activities, on the other hand, makes the prediction of the condensation product very complicated. Therefore, we investigate the hydrolysis pattern for the enzymes from yeast and wheat bran (carboxypeptidase W) on *N*-acyldipeptideamides in order to improve the design of the peptide formation by these enzymes. It would also be helpful to obtain an understanding of the substrate binding mechanisms of these enzymes.

Experimental

Materials. Five *N*-acyldipeptideamides (*N*-[3-(2-furyl)-acryloyl]-(Fua-) Gly-Phe-NH_2, Fua-Gly-Leu-NH_2, Fua-Phe-Gly-NH_2, Fua-Phe-Leu-NH_2, and Fua-Phe-Phe-NH_2) and corresponding Fua-dipeptides or Fua-amino acids were prepared as previously reported.[16] Carboxypeptidases Y and W were the same as those used in the previous studies.[13,17,18] The active concentration of the enzyme was determined by a steady state rate constant of the hydrolysis of Fua-Phe-Phe or Fua-Phe-OEt.[19] 2-Morpholinoethanesulfonic acid (Mes) and 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (Hepes) were from the Dohjindo Laboratories (Kumamoto). Other reagents were of commercial grade and were used without further purifications.

Methods. The absorbance spectrum was measured on a Uvidec 650-C spectrophotometer (Nippon Bunkou Co.; Tokyo). The reaction velocity of the hydrolysis of Fua-substrates at the bond including the carbonyl group of the substituted amino acid was measured by the absorbance decrease at 320—345 nm. The hydrolyzed product was analyzed by the high-pressure liquid chromatography of a Shimadzu LC-5A system attached to a Cosmosil 5C18 reversed-phase column. The absorbance was monitored at 305 nm through a UV-detector (Shimadzu SPD-1) and integrated (Shimadzu CR3A). The release of an amino acid or an amino acid amide was checked by an amino acid analysis on a Shimadzu LC3A-FLD1 system with fluorometric detection by the phthalaldehyde method.

Results and Discussion

The reactivity of carboxypeptidase W towards five Fua-dipeptideamide substrates was measured at pH 5—7.5 and 25 °C. Table 1 shows the amounts of products observed in the reaction mixtures after overnight incubation, relative to the initial amount of the substrate. This pH range was selected since carboxypeptidase W loses its catalytic activity above pH 8.[18] Among the five substrates studied, Fua-Phe-Leu-NH_2 was most susceptible to hydrolysis; only Fua-amino acid was observed as a product in the applied pH range and the pH dependency was not obvious. Fua-Phe-Phe-NH_2 and Fua-Phe-Gly-NH_2 were also susceptible and no sufficient amount of Fua-dipeptide was detected in the reaction mixture. Fua-Gly-Phe-NH_2 and Fua-Gly-Leu-NH_2 were less reactive. The former substrate gave some *N*-Fua dipeptide in the reaction mixture at high pH.

Table 2 shows the result for carboxypeptidase Y catalysis. This enzyme gave a somewhat different pattern from that for carboxypeptidase W. It hydrolyzed Fua-Phe-Leu-NH_2 and Fua-Phe-Phe-NH_2. The degree of hydrolysis was higher at higher pH. In the case of Fua-Phe-Phe-NH_2 nearly half of the product was Fua-dipeptide while in Fua-Phe-Leu-NH_2 only Fua-amino acid was the final product. Fua-Phe-

Table 1. The Product Yields after a Reaction of *N*-Acyldipeptideamides with Carboxypeptidase W at 25°C

Substrate \ pH	4		5		6		7		7.5	
Product[a]	A	D	A	D	A	D	A	D	A	D
Fua-Phe-Gly-NH_2	42	0	49	0	50	0	47	0	18	0
Fua-Phe-Leu-NH_2	76	0	81	0	77	0	91	0	80	0
Fua-Phe-Phe-NH_2	59	0	72	0	60	0	79	0	57	0
Fua-Gly-Leu-NH_2	6	0	3	0	3	0	2	0	0	0
Fua-Gly-Phe-NH_2	4	0	11	0	7	1	6	6	1	10

a) A: Fua-A_1, D: Fua-A_1-A_2; relative (%) to the initial amount of each substrate.

Table 2. The Product Yields after a Reaction of *N*-Acyldipeptideamides with Carboxypeptidase Y at 25°C

Substrate \ pH	4		5		6		7		7.5	
Product[a]	A	D	A	D	A	D	A	D	A	D
Fua-Phe-Gly-NH_2	0	0	5	0	4	0	39	0	25	0
Fua-Phe-Leu-NH_2	39	0	90	0	100	0	100	0	100	0
Fua-Phe-Phe-NH_2	19	0	68	0	65	31	58	42	44	56
Fua-Gly-Leu-NH_2	1	0	2	0	29	1	34	5	19	6
Fua-Gly-Phe-NH_2	1	0	3	4	22	1	32	6	22	6

a) A: Fua-A_1, D: Fua-A_1-A_2, relative (%) to the initial amount of each substrate.

Table 3. The Amount of Liberated Amino Acid Determined by Amino Acid Analysis

Enzyme	Carboxypeptidase Y		Carboxypeptidase W	
Substrate	pH	Amino Acid Fraction/%[a]	pH	Amino Acid Fraction/%[a]
Fua-Phe-Gly-NH_2	5.0	3.1	6.0	0.72
	7.5	0		
Fua-Phe-Leu-NH_2	5.0	55.0	6.0	0.13
	7.5	65.0		
Fua-Phe-Phe-NH_2	5.0	32.0	6.0	4.5
	7.5	38.0		
Fua-Gly-Leu-NH_2	5.0	11.0	6.0	0
	7.5	5.2		
Fua-Gly-Phe-NH_2	5.0	1.3	5.0	0
	7.5	6.4	7.0	0

a) Amount of liberated amino acid relative to the initial amount of each substrate.

Gly-NH_2 fell in the latter category, though its reactivity was not very high and Fua-Gly-Phe-NH_2 and Fua-Gly-Leu-NH_2 showed moderate reactivities and also gave Fua-dipetides as products.

To determine whether the Fua-amino acids observed in these reaction mixtures were produced directly from the substrates by carboxamidopeptidase activity or via Fua-dipeptide, i.e., hydrolyzed first by the amidase activity and successively by carboxypeptidase activity, some of the reaction mixtures were analyzed on an amino acid analysis system. Table 3 shows the detected amount of the corresponding free amino acid, relative to the initial amount of the substrate. In the case of carboxypeptidase W very few or no free amino acid was observed and these substrates were known to be hydrolyzed by this enzyme simply by carboxamidopeptidase activity. On the contrary, a sufficient amount of free amino acid was observed in some of the carboxypeptidase Y catalyzed hydrolyses. As in Table 2, Fua-Phe-Leu-NH_2 was completely hydrolyzed to *N*-Fua amino acid by carboxypeptidase Y at higher pH during the present reaction period; however, a major part of it was produced via two-step hydrolysis. The substrate was hydrolyzed once to Fua-Phe-Leu by amidase activity and then to Fua-Phe and Leu by intrinsic carboxypeptidase activity. Though this activity decreased with increasing pH,[7,20] it was still sufficient to hydrolyze the Fua-dipeptide produced

from the substrate, even at higher pH: at pH 8.0 and 25 °C, k_{cat}/K_m for the carboxypeptidase activity was in the order of 10^5 $M^{-1}s^{-1}$ (1M=1 mol dm^{-3}) for Fua-Phe-Leu.[21]

In the case of Fua-Phe-Phe-NH_2 the reaction proceeded in a similar fashion, but the amidase activity was higher than the carboxypeptidase activity at higher pH, with a considerable amount of the product accumulated at the state of Fua-Phe-Phe. At low pH where the amidase activity decreased,[7,12] a considerable portion of the substrate was left intact after the applied reaction time; only Fua-Phe was observed as a product. Therefore, the relative magnitude of the two activities determined the product distribution. Fua-Phe-Gly-NH_2 is a rather poor substrate of carboxypeptidase Y and was mostly hydrolyzed directly to Fua-Phe by carboxamidopeptidase activity. In Fua-Gly-Phe-NH_2 and Fua-Gly-Leu-NH_2 amidase activity was not very high and the two pathways were more or less concurrent.

The complexity of the hydrolysis of Fua-Phe-Leu-NH_2 by carboxypeptidase Y was reflected in the kinetics of the reaction observed by a change in the UV absorbance. The apparent second-order rate constant of the hydrolysis was obtained from the initial rate divided by both enzyme and substrate concentrations or by a pseudo-first-order analysis at $[E]_0$=50—100 nM and $[S]_0$=10—20 μM. It changed with pH as shown in Fig. 1 (○ and ◐) with an apparent maximum at about pH 7. In the pH range of 6.5—8, however, a semi-logarithmic plot of the absorbance change as a function of time shows a convex shape (insert in Fig. 1); the rate constants given in the figure were evaluated from the later portions of the plots. In contrast to this, the hydrolysis of the same substrate by carboxypeptidase W gave normal pseudo-first-order rate curves (though not shown here); the pH profile of the second-order rate constant (Fig. 1, ●) could be explained by the following simple equation assuming $k_{A(lim)}$=7.96×10^4 $M^{-1}s^{-1}$ and pK_a=6.60 ($k_{A(lim)}$: pH-independent (maximum) value of k_{cat}/K_m):

$$k_{cat}/K_m = k_{A(lim)}/(1 + [H^+]/K_a). \quad (1)$$

For a further consideration of the mechanism of serine carboxypeptidase, we cite here the previously reported change of the reaction parameters with the variation in the side-chain structures of *N*-acyl-dipeptides substrates (*N*-furylacryloyl-substrate for carboxypeptidase W[18] and *N*-benzyloxycarbonyl-substrate for carboxypeptidase Y[7]). Carboxypeptidase W has a relatively broader specificity, compared with carboxypeptidase Y, especially for the k_{cat} parameter. We note that there are some differences in the specificity of the carboxypeptidase activity and the carboxamidopeptidase or amidase activity.

These two carboxypeptidases were shown to exhibit fairly different pK_a values for peptidase activity, sug-

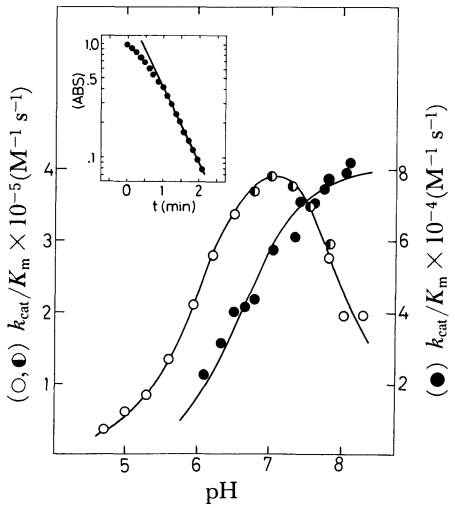

Fig. 1. pH Dependences of k_{cat}/K_m of the hydrolysis of FuaPheLeuNH_2 catalyzed by carboxypeptidase Y (○,◐) and by carboxypeptidase W (●) at 25 °C. [Enzyme]=50—100 nM, [substrate]=5—40 μM, 0.5% *N*,*N*-dimethylformamide, 0.1 M NaCl, 0.1 M Mes or Hepes. Insert; a semi-logarithmic plot of the kinetic data of carboxypeptidase Y at pH 7.0.

Table 4. Change in The Reaction Parameters with The Side Chain Structures of *N*-Acyl-Dipeptide Substrates for The Hydrolysis by Carboxypeptidase W and Carboxypeptidase Y[a]

Dipeptide sequence	Carboxypeptidase W			Carboxypeptidase Y		
	k_{cat}	K_m	k_{cat}/K_m[b]	k_{cat}	K_m	k_{cat}/K_m[b]
	s^{-1}	mM	10^4 $M^{-1}s^{-1}$	s^{-1}	mM	10^4 $M^{-1}s^{-1}$
-Gly-Phe	2.8	0.12	2.3(0.08)	2.2	1.7	0.13(0.04)
-Ala-Phe	39	0.18	22(0.74)	—	—	—
-Leu-Phe	28	0.033	84(2.9)	46	0.1	47(13)
-Phe-Phe	48	0.042	110(3.9)	420	0.5	84(23)
-Phe-Leu	72	0.064	120(4.1)	130	0.1	130(35)
-Phe-Pro	2.9	0.037	7.6(0.26)	23	0.7	3.4(0.93)
-Phe-Gly	93	0.32	29(1.0)	140	4.0	3.7(1.0)
-His-Leu	53	0.91	6.0(0.21)	1.4	1.8	0.078(0.02)

a) The reaction parameters of *N*-Fua-substrates at pH 4.0 and 25 °C (by Fukuda et al.[19]) for carboxypeptidase W and of *N*-CBZ-substrates at pH 6.5 and 25 °C (by Hayashi et al.[7] and Johansen et al. (-His-Leu)[22]) for carboxypeptidase Y. b) In parentheses are k_{cat}/K_m values relative to the value of -Phe-Gly.

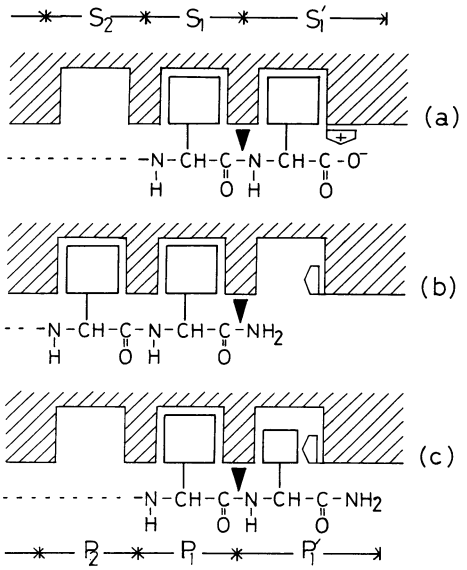

Fig. 2. Scheme of the substrate binding site of carboxypeptidase Y. S_i,S_i': The binding site for the C-terminal amino acid residue of the substrate. P_iP_i': The amino acid residues of the substrate in corresponing binding sites. Dark triangle shows the scissile point. (a) At low pH with a substrate having free carboxylate terminal and bulky amino acid residues. (b) At neutral pH with a substrate having amide end and bulky trerminal amino acid. (c) At neutral pH with a substrate having amide end and a ternial amino acid residue of a smaller size.

gesting that the anion-recognizing residue might be different. Our previous study[18] revealed that the catalytic sites for the carboxypeptidase activity and endopeptidase-like activity of carboxypeptidase W plausibly involved different functional groups. In view of the fact that the catalytic activity of carboxypeptidase Y involves a His residue,[4] and the apparent pK_a value in the substrate binding process[20] implies that this or another protonated His residue can act as an anion-recognition site of carboxypeptidase Y.

These considerations lead to a schematic picture of the substrate binding site of carboxypeptidase Y (Fig. 2): (a) The protonation of a His residue is essential for the binding of substrates having a carboxylate end. (b) At neutral and alkaline pH, the His residue loses its charge and some structural or conformational change might occur in the substrate-binding site. This process limits the size of the S_1' site and prevents the binding of a dipeptideamide substrate which has a bulky residue at P_1'. Therefore, such a substrate is not cleaved at the P_1-P_1' bond but rather at the terminal amide bond. (c) In the case of a dipeptide amide having a smaller residue at P_1', it penetrates into the corresponding position and a carboxamidopeptidase activity occurs. A neutral His residue can also be helpful for the binding of a moderate size residue at P_1'.

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