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Wheat Carboxypeptidase-Catalyzed Peptide Synthesis by Aminolysis of *N*-Acyl Amino Acid Ester. Indication of the Acyl-Enzyme Mechanism

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Dipeptide synthesis from *N*-[3-(2-furyl)acryloyl]-acylated (Fua-) amino acid ethyl ester and amino acid amide catalyzed by wheat bran carboxypeptidase (carboxypeptidase W) was studied. The optimum pH for peptide formation was at pH 8; more than 60% of the initial *N*-Fua-L-phenylalanine ethyl ester was converted to *N*-Fua-L-phenylalanylglycinamide under optimum conditions. The dependence of peptide formation on the concentration of amino acid amide showed an apparent saturation; this could be successfully explained by a reaction scheme which involved an acylated enzyme attacked by an enzyme-bound amine. The results for a reaction in the presence of two different amine components also supported this mechanism. Gly-NH_2 had a better affinity to the acylated carboxypeptidase and the apparent dissociation constant ($K_{N(app)}$) was about 50 mM. These results were compared with those for those reactions catalyzed by carboxypeptidases from yeast and malt.

Serine carboxypeptidases [EC 3.4.16.1] from various origins[1-9] are characterized by the broad substrate specificity and by esterase and/or amidase activity in addition to intrinsic carboxypeptidase.[3,6-11] Some of the enzymes of this group have been successfully utilized for peptide synthesis from *N*-acyl amino acid esters and amino acid amides or esters.[12-14] In addition to these practical aspects, the aminolysis reaction and transpeptidation reactions[15] have been considered as indirect evidence for a reaction mechanism which includes an acylated intermediate. The existence of such an intermediate was observed by a stopped-flow technique in the case of carboxypeptidase Y.[16,17] A carboxypeptidase from wheat bran (carboxypeptidase W)[18] is considered to be a member of this group of enzymes; we have studied the kinetics of its reaction for peptide and ester hydrolyses.[19,20] In due course we found that this carboxypeptidase is useful for peptide synthesis by the aminolysis of an ester substrate. Here, we examine some fundamental points for such a utilization.

Experimental

Materials. Carboxypeptidase W was obtained from Pentel Co. Ltd. (Tokyo, Lot 68305031). Enzyme solutions were prepared by dissolving it in deionized water; it was stored as a concentrated solution (0.05 mM (1M=1 mol dm^{-3})) at 4 °C. The protein concentration was determined from the absorbance at 280 nm using $A^{1\%}_{1cm}$=19.6 and Mr=118000.[18] Carboxypeptidase Y was obtained from Oriental Yeast Co. (Osaka) and used as described before.[19] *N*-[3-(2-furyl)acryloyl] amino acids were synthesized by the *N*-hydoxysuccinimide method. Their analytical data have been described previously.[22-24] Other chemicals were of reagent grade and were used without further purifications.

Methods. Reactions were carried out at 37 °C in the presence of 0.2 M NaCl and 15% *N*,*N*-dimethylformamide, unless otherwise mentioned. The pH was adjusted by adding concd HCl or NaOH solutions. After incubation for the indicated time, a portion was taken out, quenched by mixing with a 5-fold volume of acetonitrile, filtered on Millipore FHLP013, and then analyzed on a reversed-phase chromatography (Waters HPLC 6000A-440 with Cosmosil 5C18). The eluent solution contained 45% CH_3CN, 50 mM Na_2SO_4 and approximately 5 mM H_3PO_4.

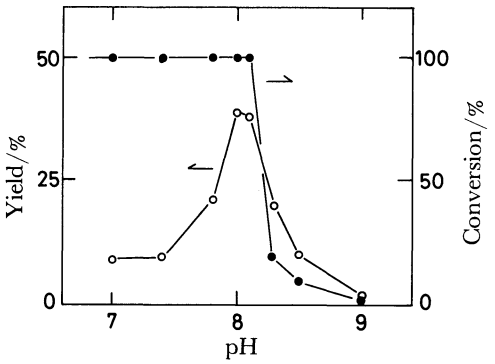

Fig. 1. pH Dependence of the carboxypeptidase W-catalyzed peptide formation from Fua-Phe-OEt and Leu-NH_2 at 37 °C. [enzyme]=0.5μM, [Fua-Phe-OEt]=2 mM, [Leu-NH_2]=0.1 M, [NaCl]=0.2 M, and 15% *N*,*N*-dimethylformamide. ○: Peptide yield, Fraction of product peptide relative to the initial amount of the ester. ●: Ester conversion, Fraction of hydrolyzed ester relative to the initial amount of the ester.

Results and Discussion

Figure 1 shows the pH dependence of the peptide formation from Fua-Phe-OEt and Leu-NH_2 after incubation for 5 h under the indicated conditions. They are given in a form of conversions (=(total consumption of the ester)/(initial amount of the ester)) and yield (=(produced amount of the dipeptide amide)/(initial amount of the ester)). Since the catalytic activity of carboxypeptidase W decreased at a pH above 8,[19] the conversion efficiency decreased above 8 and the maximum yield was obtained at a pH around 8. Similar pH profiles were obtained at different enzyme concentrations. This result is in contrast to

Table 1. Product Distribution in the Aminolysis Reaction of Fua-Phe-OEt Catalyzed by Carboxypeptidases W and Y with Several Nucleophiles at [Fua-Phe-OEt]=2 mM, [Nucleophile]=0.1 M, [enzyme]=0.5 mM, [NaCl]=0.2 M, 15% *N,N*-Dimethylformamide 37°C and pH 8.0

Nucleophile pK_a[a]	Carboxypeptidase W					Carboxypeptidase Y		
	Fua-Phe-Xxx-NH_2	Fua-Phe-Xxx	Fua-Phe	f_1/f_0[b]	$K_{N(app)}$(mM)	Fua-Phe-Xxx-NH_2	Fua-Phe-Xxx	Fua-Phe
Phe-NH_2 7.1	23	<1	77	0.30	350	<1	25	75
Leu-NH_2 7.6	38	0	62	0.61	170	19 (66)[c]	16 (15)	65 (19)
Gly-NH_2 7.9	63	0	37	1.7	50	33	0	67
Gly-Gly 8.1	<1	0	100	—	—			

a) from Ref. 25. b) $f_0=v_0/(v_0+v_1)$ as in Eq. 1. Therefore $f_1/f_0=v_1/v_0$. c) Numbers in parentheses were obtained at pH 9.5 in carbonate-hydrogencarbonate buffer (0.1 M).

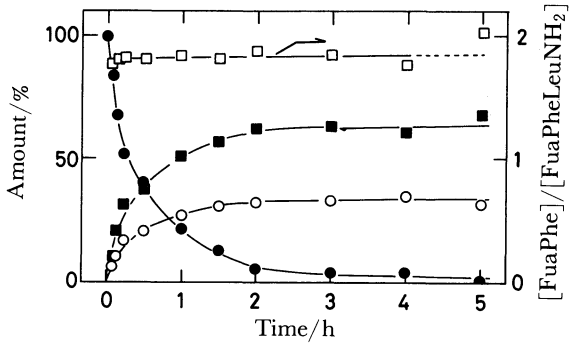

Fig. 2. Time course of the carboxypeptidase W-catalyzed peptide formation from Fua-Phe-OEt and Leu-NH_2 at 37°C. [Fua-Phe-OEt]=5 mM, [Leu-NH_2]=0.1 M, [enzyme]=0.1 μM, [NaCl]=0.2 M, 15% *N,N*-dimethylformamide and pH 8.0. ●: Fua-Phe-OEt, ■: Fua-Phe, ○: Fua-Phe-Leu-NH_2. □: Ratio of Fua-Phe and Fua-Phe-Leu-NH_2.

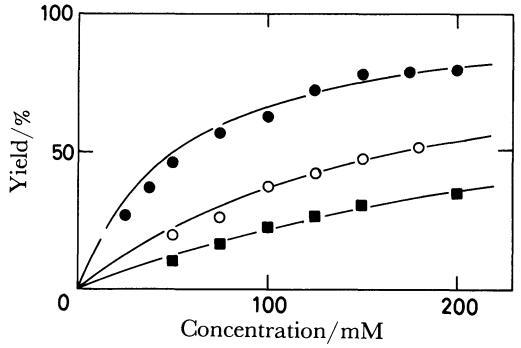

Fig. 3. Amino acid amide concentration dependence of peptide formation at 37°C. [Fua-Phe-OEt]=2 mM, [enzyme]=0.5 μM, [NaCl]=0.2 M, 15% *N,N*-dimethylformamide and pH 8.0. ●: With Gly-NH_2, ○: with Leu-NH_2, ■: with Phe-NH_2

that for carboxypeptidase Y-catalyzed peptide formation using the same substrates, where the yield increased monotonously (up to pH 10) with an increase in pH (Shima et al. unpublished result). At optimum pH, The time course of the reaction by carboxypeptidase W was as shown in Fig. 2. Under these conditions, the ratio (□) of the hydrolyzate (Fua-Phe) and the aminolysis product (Fua-Phe-Leu-NH_2) was practically constant during the reaction period. Table 1 shows the results for product analyses for reactions with different nucleophiles, together with the results for carboxypeptidase Y under similar conditions. As we have shown in a preceding paper,[20] the amidase activity of carboxypeptidase W is low and the production of *N*-acyldipeptide with carboxylate terminal is practically negligible in the present case. This result is also in contrast to the case of carboxypeptidase Y. Gly-Gly was not an effective nucleophile, while Gly-NH_2 was the best among three amino acid amides used here.

The amide concentration dependency of the peptide yield is shown in Fig. 3 for three amino acid amides. To explain the results we postulate a competition between the hydrolysis and the aminolysis of the acyl-enzyme intermediate, while taking account of a possible inhibitory action of added nucleophile, as shown in Eqs. 1—4:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\cdot S \xrightarrow{k_2} EA \quad (1)\ \text{[Acyl-enzyme formation]},$$

$$EA \xrightarrow{k_3} E + P_0 \quad (2)\ \text{[Hydrolysis of acyl-enzyme]},$$

$$EA + N \overset{K_N}{\rightleftharpoons} EA\cdot N \xrightarrow{k_n} E + P_1 \quad (3)\ \text{[Aminolysis]},$$

$$E + N \overset{K_N^i}{\rightleftharpoons} E\cdot N \quad (4)\ \text{[Competitive inhibition]}.$$

Here, the k_i's are the respective rate constants. S, P_i's, E, E·S, and EA are the substrate (ester), products (amino acid and peptide), enzyme, enzyme-substrate complex and acylated enzyme, respectively. EA·N and E·N are a complex formed from the acylated enzyme and the nucleophile and enzyme-nucleophile complex, respectively. K_N and K_N^i are the respective dissociation constants. These expressions are similar to those provided by Bender[26] and by Fastrez and Fersht[27] to explain the partitioning of the acyl-enzyme intermediate of chymotrypsin by water and an acceptor (nucleophile). However, the specific binding of the nucleophile to the enzyme active site is considered in the present case. They are also analogous to expressions given for the reaction of papain by Fink and Bender[28]; here, a reaction path which includes the ternary complex composed of EA and two molecules of N is out of the consideration. In a steady state analysis of the reaction, Eq. 1 is considered to be equivalent to

$$E + S \overset{K_m}{\rightleftharpoons} EA, \quad (5)$$

Table 2. Carboxypeptidase W-Catalyzed Aminolysis of Fua-Phe-OEt with two Amino Acid Amides (Xxx-NH_2 and Yyy-NH_2) at [Fua-Phe-OEt]=2 mM, [Xxx-NH_2]=[Yyy-NH_2]=0.1 M, 15% *N,N*-Dimethylformamide 37 °C and pH 8.0

Xxx-NH_2	Yyy-NH_2	Yield of Fua-Phe-Xxx-NH_2 (f_1) (%)	Yield of Fua-Phe-Yyy-NH_2 (f_1') (%)	f_1/f_1'[a]
Gly-NH_2	Phe-NH_2	61	7.8	7.8 (7.0)
Gly-NH_2	Leu-NH_2	57	14	4.1 (3.4)
Leu-NH_2	Phe-NH_2	29	14	2.1 (7.0)

a) Numbers in parentheses were calculated by Eq. 16.

where $K_m=\{(k_{-1}+k_2)/k_1\}\{k_3^*/(k_2+k_3^*)\}$ and $k_3^*=k_3+k_n[N]/K_N$. Then, we obtain the rates of the formation of two products (P_0 and P_1) as

$$v_0 = dP_0/dt = k_3([S]/K_m)[E]_0 / \{1 + (K_m/[S])(1 + [N]/K_N) + [N]/K_N^i\}, \quad (6)$$

and

$$v_1 = dP_1/dt = k_n([N]/K_N)([S]/K_m)[E]_0 / \{1 + (K_m/[S])(1 + [N]/K_N) + [N]/K_N^i\}. \quad (7)$$

Since [N] is in great excess to the enzyme in each case and there are no cross terms in Eqs. 6 and 7, the ratio of two reaction rates is constant during the reaction period (the case in Fig. 3). Therefore, these equations, though originally written for the initial state of the reaction, can be related to the fraction (f_1) of the aminolysis product over the total consumption of the ester substrate:

$$f_1 = v_1/(v_0 + v_1) = k_n([N]/K_N)/\{k_3 + k_n([N]/K_N)\} = 1/\{1 + (k_3/k_n)(K_N/[N])\}. \quad (8)$$

Equation 8 predicts that an apparent saturation is observed in the concentration dependence of the peptide yield and that the apparent saturation parameter ($K_{N(app)}$) ($=(k_3/k_n)K_N$) shows a total affinity of aminolysis. The observed concentration dependency was analyzed by a least-square method and $K_{N(app)}$ was evaluated (Table 1). Since the nucleophilicity of Gly-NH_2 is weakest, the smallest $K_{N(app)}$ value observed for this amide is due to the smallest K_N value, i.e., the affinity of Gly-NH_2 to the acylated enzyme is best among the three. This is in contrast to the fact that in the hydrolysis reaction K_m values of Fua-Phe-Leu and Fua-Phe-Phe are five- to seven-fold smaller than that of Fua-Phe-Gly at low pH.[19]

When two kinds of amino acid amides are present in a reaction mixture, the product distributions (fractions of two peptide products; f_1 and f_1') are listed in Table 2. For such systems we have to add two equations concerning the second amine component (N'):

$$EA + N' \overset{K_{N'}}{\rightleftharpoons} EA\cdot N' \xrightarrow{k_n'} E + P_1', \quad (9)$$

and

$$E + N' \overset{K_{N'}^i}{\rightleftharpoons} E\cdot N'. \quad (10)$$

In these equations $K_{N'}$, k_n', and $K_{N'}^i$ have similar meanings as K_N, k_n, and K_N^i, respectively.

Then, v_1, v_1', and v_0 are

$$v_0 = k_3([S]/K_m)[E]_0 / \{1 + (K_m/[S])(1 + [N]/K_N + [N']/K_{N'}) + [N]/K_N^i + [N']/K_{N'}^i\}, \quad (11)$$

$$v_1 = k_n([N]/K_N)([S]/K_m)[E]_0 / \{1 + (K_m/[S])(1 + [N]/K_N + [N']/K_{N'}) + [N]/K_N^i + [N']/K_{N'}^i\}, \quad (12)$$

and

$$v_1' = k_n'([N']/K_{N'})([S]/K_m)[E]_0 / \{1 + (K_m/[S])(1 + [N]/K_N + [N']/K_{N'}) + [N]/K_N^i + [N']/K_{N'}^i\}. \quad (13)$$

Also,

$$f_1 = k_n([N]/K_N)/\{k_3 + k_n([N]/K_N + k_n'([N']/K_{N'})\}, \quad (14)$$

and

$$f_1' = k_n'([N']/K_{N'})/\{k_3 + k_n([N]/K_N + k_n'([N']/K_{N'})\}. \quad (15)$$

This means that the ratio of the fractions for two amino acid amides is

$$f_1/f_1' = \{k_n([N]/K_N)\}/\{k_n'([N']/K_{N'})\}. \quad (16)$$

Where [N]=[N'], f_1/f_1' can be calculated from $K_{N(app)}$; the results are listed in parentheses. They are comparable to the experimental values. This supports the scheme presently considered for the reaction of carboxypeptidase W. Under similar conditions, carboxypeptidase Y gave more complex results: Fua-Phe-Phe was 37%, Fua-Phe-Gly-NH_2 18%, and Fua-Phe 45% at [Phe-NH_2]=[Gly-NH_2]=0.1 M. In this case an apparent "activation" of the amidase action on the once produced Fua-Phe-Phe-NH_2[20] was observed in the

presence of the second nucleophile (Gly-NH_2) (compare with the result listed in Table 1) and the present mechanism for carboxypeptidase W was not sufficient for explaining these results.

Thus, carboxypeptidase W was shown to have an efficient activity for a peptide synthesis from *N*-acyl amino acid ester and amino acid amide by an aminolysis of the acylated enzyme intermediate.

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