Protease-Catalyzed Peptide Formation under High Pressure

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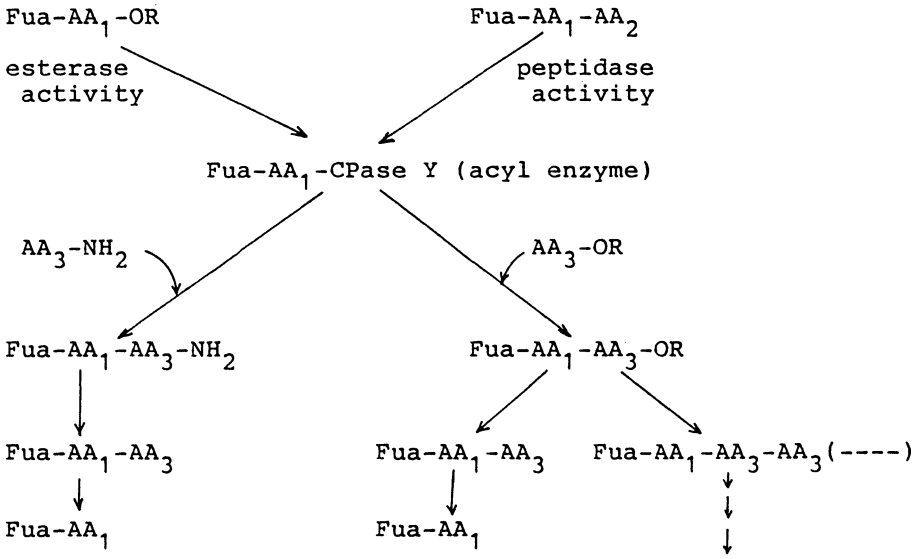
The effect of high pressure on peptide formation by the catalysis of carboxypeptidase Y (substitution of ester or peptide by amino acid derivative) or by thermolysin (condensation of *N*-acylamino acid and amino acid amide) was studied. The carboxypeptidase Y-catalyzed substitution reaction of *N*-[3-(2-furyl)acryloyl]-phenylalanine ethyl ester with glycinamide or phenylalaninamide showed a six-fold higher total peptide yield at 200 MPa than at atmospheric pressure. In the case of the reaction of *N*-acyldipeptide and amino acid amide, both the peptide yield and substitution efficiency were improved at elevated pressure and the wasteful hydrolysis of the substrate was highly depressed by increasing pressure. The pressure was also effective to get rid of the substrate inhibition by the amino acid ester in the reaction between the *N*-acylamino acid ester and the amino acid ester and to yield much dipeptide ester at high pressure. An improvement of the peptide yield by pressure for the reaction of thermolysin was observed in a combination of less specific substrates, *N*-benzyloxycarbonyl-L-aspartic acid and phenylalanine methyl ester, since the high catalytic activity of this enzyme under elevated pressure was significant only in the case that the peptide yield was kinetic-controlled.

During the course of our studies on the reaction mechanisms of several proteases by examining the influence of hydrostatic pressure on the reactions,[1-3] we found that the pressure affected one type of activity of some enzymes in a considerably different manner from the other, depending on the nature of the scissile bond of the substrate. Also, a certain enzyme show a considerable apparent pressure-activation. One example of the former is carboxypeptidase from yeast (CPase Y),[2] which is grouped into serine carboxypeptidase.[4] The intrinsic carboxypeptidase activity of this enzyme (Eq. 1, where AA_i and R denote amino acid residue and alkyl group, respectively) was highly depressed by increasing the pressure, while an esterase (Eq. 2) or amidase (Eq. 3) reaction was less influenced by pressure; e.g., at 100 MPa the rate of hydrolysis of *N*-[3-(2-furyl)acryloyl]phenylalanine ethyl ester and amide was 85 and 75%, respectively, of that at atmospheric circumstance, while that of *N*-[3-(2-furyl)-acryloyl]glycylphenylalanine was decreased to less than 15% by the same pressure increase (pH 6, 25 °C). This resulted mainly from the difference in the ionic nature of the substrate and the discouraged electrostatic interaction at the elevated pressure to avoid any increase in the molar volume due to liberated hydration water around ionic groups.

$$---AA_1\text{-}AA_2 \longrightarrow ---AA_1 + AA_2 \quad (1)$$
$$---AA_1\text{-}OR \longrightarrow ---AA_1 + ROH \quad (2)$$
$$---AA_1\text{-}NH_2 \longrightarrow ---AA_1 + NH_3 \quad (3)$$

An example of the apparent pressure-activation was seen in the case of thermolysin, a zinc-containing



Scheme 1. Possible pathways of peptide formation and degradation of the primary product in CPase Y system.
AA_i, amino acid residue; R, alkyl group.

endoprotease from *B. thermoproteolyticus*. A pressure increase of up to 100 MPa gave about a 15-fold higher second-order rate for the hydrolysis of *N*-acyldipeptide amide catalyzed by this enzyme.[3] This could be explained in terms of a change of the structure and a subsequent change in the hydration state of the protein coupled with the progress of the reaction. These findings led us to inspect the pressure effect on the protease-catalyzed peptide formation reactions, since both of these enzymes are known to catalyze such kinds of reactions.[5] Actually, in a preliminary study we found a strong influence of pressure on a peptide formation reaction by CPase Y.[6]

In this report we describe the pressure effect on various types of peptide formation reactions catalyzed by CPase Y and thermolysin. With CPase Y, peptides can be formed by a nucleophilic attack by an amino acid derivative on the acylated intermediate[7] (Scheme 1), which is derived from either an ester substrate[8] or a peptide substrate.[9] Nucleophile can be either an amino acid amide (X=NH_2) or an amino acid ester (X=OR). The reverse reaction of the hydrolysis, starting from e.g. *N*-acylamino acid and amino acid (Eq. 4), might also give peptides.[10] With thermolysin, peptide can be formed only by a reaction of this type and the carboxyl part of AA_2 should be capped (-X).

$$---AA_1 + AA_2(-X) \longrightarrow ---AA_1\text{-}AA_2(-X) \quad (4)$$

Experimental

Materials. CPase Y was kindly donated by Oriental Yeast Co. (Osaka, Japan) (Lot 21003001) and thermolysin was obtained from Daiwa Kasei Co. (Osaka, Japan) (Lot T4KB31,32). *N*-[3-(2-Furyl)acryloyl]amino acids (Fua-amino acids) and *N*-[3-(2-furyl)acryloyl]dipeptides including the standard samples for the chromatographic analysis were prepared by the *N*-hydroxysuccinimide method,[11] as previously reported.[1-3] This chromophoric acyl group was chosen since it facilitates product detection and since we had made some kinetic studies on the proteases by using this type of substrate. The principle obtained from the present results may be applicable to substrates with other acyl groups. Buffer reagents were purchased from Dojindo Laboratories (Kumamoto, Japan) and other amino acid derivatives were of commercial reagent grade. The authentic sample of *N*-benzyloxycarbonyl-Asp-Phe methyl ester (Cbz-Asp-Phe-OMe) was a kind gift from Prof. Y. Okahata, Tokyo Institute of Technology, Japan.

Methods. The reaction solution contained an appropriate amount of organic solvent (depending on the reaction species; dimethyl sulfoxide [DMSO], *N,N*-dimethylformamide [DMFA], or acetonitrile [MeCN]), enzyme and substrates, buffered at described pH with 2-morpholinoethanesulfonic acid, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid, 2-amino-2-hydroxy-methyl-1,3-propanediol, 3-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-1-propanesulfonic acid, or 3-cyclohexylamino-1-propanesulfonic acid.

It was divided into two parts; one part was sealed in a small polypropylene-made bottle, placed in a stainless-steel pressure vessel located in a thermostated bath, while the other part was incubated under atmospheric pressure at a controlled temperature. Kerosene was used as the pressure medium.

After a prescribed period the reaction mixture was taken out from each part of the solution and quenched by adding 5 volumes of a mixture of acetonitrile and 5 mM-aqueous phosphoric acid (1M=1 mol dm^{-3}). Portions were then analyzed by liquid chromatography (Shimadzu LC6A-Cosmosil 5C18-P). The eluent was a 40—60% mixture of acetonitrile with 5 mM of aqueous phosphate (pH 2.5—3) containing 50 mM Na_2SO_4 or triethylamine. The amount of each species was calculated from the integration of the eluent curve (Shimadzu Chromatopak C-R6A) after corrections with the extinction coefficient measured on a spectrophotometer (Uvidec 610-B) independently.

Results and Discussion

Carboxypeptidase Y. Table 1 shows the product distribution of the CPase Y-catalyzed reaction of Fua-Phe-OEt with Gly-NH_2 or Phe-NH_2 at atmospheric pressure and at 200 MPa. With Gly-NH_2, Fua-Phe, and Fua-Phe-Gly-NH_2 were detected as the products and the yield of the dipeptide amide was larger at high pressure. As reported before,[12] this enzyme shows carboxamidopeptidase activity towards Fua-Phe-Gly-NH_2 and, therefore, the primarily produced dipeptide amide can be directly hydrolyzed to Fua-Phe and Gly-NH_2. With Phe-NH_2, a dipeptide having free carboxylate (Fua-Phe-Phe), was also detected in the reaction mixture. This *N*-acyldipeptide was produced by the amidase activity on the primary product, Fua-Phe-Phe-NH_2. Thus, Fua-Phe was produced by the hydrolysis of either Fua-Phe-OEt or Fua-Phe-Phe (Eq. 5). (For Fua-Phe-Phe-NH_2 CPase Y shows no carboxamidopeptidase activity.[1])

Table 1. Effect of Pressure on CPase Y-Catalyzed Formation of Dipeptide Amide from Fua-Phe-OEt and Amino Acid Amide[a]

Nucleophile	Pressure	Yield (%)		
	MPa	Fua-Phe	Fua-Phe-Xxx-NH_2	Fua-Phe-Xxx
Gly-NH_2	0.1	84.6	15.4	0
	200	60.6	39.4	0
Phe-NH_2	0.1	91.2	2.3	6.5
	200	45.4	16.1	38.5

a) pH 8.0, [CPaseY]=0.6 μM, [Fua-Phe-OEt]= 2 mM, [Xxx-NH_2·HCl]= 0.1 M, 15 (v/v)% DMFA, 3 h reaction at 30 °C.

Table 2. Effect of Pressure on CPase Y-Catalyzed Hydrolysis of Fua-Dipeptide Amide[a)]

Substrate	pH	Pressure	Product yield/%[b)]		
		MPa	Fua-Xxx	Fua-Xxx-Yyy	Fua-Xxx-Yyy-NH_2
Fua-Gly-Phe-NH_2	5.0	0.1	54.6	0.2	45.3
		100	24.5	38.1	37.5
	7.5	0.1	84.7	0.5	14.8
		100	54.2	22.4	23.4
Fua-Phe-Gly-NH_2	5.0	0.1	46.7	0.0	53.3
		100	41.5	0.0	58.5
	7.5	0.1	53.5	0.0	46.0
		100	52.8	0.0	46.9
Fua-Phe-Phe-NH_2	5.0	0.1	75.4	14.8	9.8
		100	65.9	26.1	8.0
	7.5	0.1	60.9	39.2	0.0
		100	57.0	43.0	0.0

a) [Substrate]=0.25 mM, [CPase Y]=0.5 μM, 30 °C, 2.0 (v/v)% DMSO. b) Amount of each product found in the reaction mixture after 3 h incubation. Xxx and Yyy indicate Gly or Phe.

Table 3. Effect of Pressure on CPase Y-Catalyzed Dipeptide Formation from Fua-Gly-Phe and Leu-NH_2[a)]

[Enzyme]	pH	Pressure	Product yield/%				Ratio[b)]
μM		MPa	Fua-Gly-Phe	Fua-Gly	Fua-Gly-Leu-NH_2	Fua-Gly-Leu	
1.0	6.5	0.1	0	88.5	11.5	0	11.5
		100	4.3	68.5	22.6	4.5	23.4
	7.0	0.1	0	91.6	8.4	0	8.4
		100	42.3	40.5	14.0	3.2	24.3
2.0	6.5	0.1	0	95.7	4.3	0	4.3
		100	0	78.4	18.3	3.2	18.3
	7.0	0.1	0	96.1	3.9	0	3.9
		100	17.1	63.2	16.6	3.1	20.0

a) [Fua-Gly-Phe]=0.25 mM, [Leu-NH_2·HCl]=0.1 M, 3 h reaction at 35 °C, 2.5 (v/v)% DMFA.
b) (amount of Fua-Gly-Leu-NH_2)/(total hydrolyzed substrate).

$$\text{Fua-Phe-Phe-NH}_2 \rightarrow \text{Fua-Phe-Phe} \rightarrow \text{Fua-Phe} \quad (5)$$
$$\text{NH}_3 \qquad\qquad \text{Phe}$$

At 200 MPa, the total peptide yield was increased approximately six-fold. This result could be explained by considering the effect of pressure on the hydrolysis of Fua-dipeptide amides by this enzyme (Table 2). By increasing the pressure, the carboxypeptidase activity was retarded more rigorously than the amidase or the carboxamidopeptidase activity and, as a result, a dipeptide with a carboxylate end was obtained in higher yield.

An acyl-enzyme can be also produced from an *N*-acyldipeptide substrate which is attacked by amino acid amide to form an *N*-acyldipeptide amide. A time course of such a reaction under atmospheric pressure is shown in Fig. 1. After 3 h the reaction was completed. Table 3 shows the effect of pressure on such a peptide formation reaction: namely, the product distribution of a combination of Fua-Gly-Phe+Leu-NH_2 after 3 h reaction at 0.1 and 100 MPa. At elevated pressure the yield of Fua-Gly-Leu-NH_2 increased. At the same time the amount of remaining unreacted substrate, Fua-Gly-Phe, also increased, which was brought about by a strong retardation of the peptidase activity by pressure. This means that the pressure depressed the wasteful hydrolysis of the substrate.

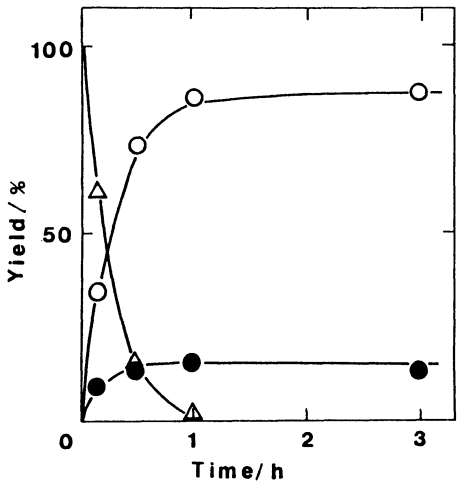


Fig. 1. Time course of CPase Y-catalyzed peptide formation from Fua-Gly-Phe and Leu-NH_2. Relative amounts of Fua-Gly (○), Fua-Gly-Phe (△), and Fua-Gly-Leu-NH_2 (●) are shown. [Fua-Gly-Phe]= 0.25 mM, [Leu-NH_2·HCl]=0.1 M, [enzyme]=1.0 μM, pH 6.5 (0.1 M Hepes), 2.5%(v/v) DMFA, 35 °C.

When the enzyme concentration was increased, *N*-

Table 4. Effect of Pressure on CPase Y-Catalyzed Dipeptide Formation from Fua-Phe-OEt and Phe-OEt[a)]

[Phe-OEt·HCl]	Pressure	Product yield/%					
M	MPa	Fua-Phe-OEt	Fua-Phe	Fua-(Phe)$_2$-OEt	Fua-(Phe)$_2$	Fua-(Phe)$_3$-OEt	Fua-(Phe)$_3$
0.05	0.1	0.2	53.2	1.4	40.9	0	4.3
	100	0.1	54.4	2.2	38.9	0	4.4
0.08	0.1	8.4	40.7	4.2	39.9	0.7	6.1
	100	0.1	47.5	10.5	36.0	0.6	5.1
0.1	0.1	37.0	24.4	13.8	20.0	2.1	2.3
	100	0.7	45.2	19.9	28.8	1.8	3.4
0.15	0.1	83.7	5.4	8.5	2.1	0.2	0
	100	17.8	37.4	28.9	12.9	1.3	0.9

a) [Fua-Phe-OEt]=1.0 mM, 30 °C, pH 9.5, [CPase Y]=9.5 nM, 7.0 (v/v)% MeCN, reaction time 3 h.

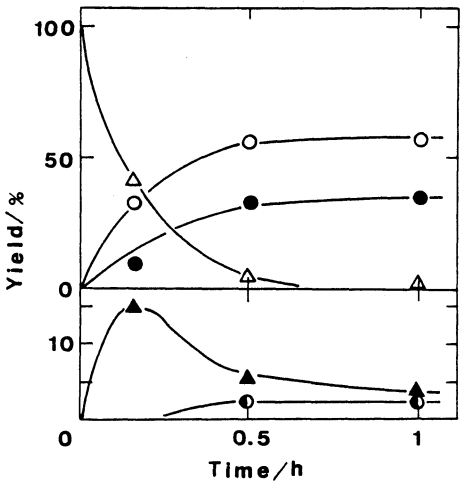


Fig. 2. Time course of CPase Y-catalyzed peptide formation from Fua-Phe-OEt and Phe-OEt. Relative amounts of Fua-Phe (○), Fua-Phe-OEt (△), Fua-Phe-Phe (●), Fua-Phe-Phe-OEt (▲), and Fua-Phe-Phe-Phe (◐) are shown.
[Fua-Phe-OEt]=1.0 mM, [Phe-OEt·HCl]=0.05 M, [enzyme]=10 nM, pH 9.5, 7.0%(v/v) MeCN, 30 °C, 0.1 M NaCl.

acyldipeptide (Fua-Gly-Leu) appeared in the product, due to the amidase activity of this enzyme on the once produced dipeptide amide coupled with the highly depressed carboxypeptidase activity at elevated pressure. With this carboxypeptide used as an acyl donor, however, the peptide yield was relatively low, at most around 30%, even at elevated pressure.

Next, the reaction between *N*-acylamino acid ester and amino acid ester was examined. In this case the peptide ester once produced can further act as a substrate[8)] and the peptide chain can be extended, as shown in Fig. 2. Table 4 shows an example of the effect of pressure on such a reaction; Fua-Phe-OEt+Phe-OEt. At low Phe-OEt concentration the effect was practically negligible, since the esterase activity was affected by pressure to a smaller extent. Most of the product was *N*-acyldipeptide and small portions were dipeptide ester and tripeptide ester.

When the nucleophile (Phe-OEt) concentration was increased in order to increase the substitution efficiency, the amount of unreacted substrate increased, since this component also acted as a kind of inhibitor, of which the inhibition constant was estimated to be about 10 mM from the reported data.[4)] The pressure was highly effective to get rid of such a substrate inhibition and the yield of the dipeptide ester product was increased under high pressure.

Table 5. Effect of Pressure on Thermolysin-Catalyzed Peptide Formation Reaction

Reaction	pH	Pressure	Product yield
		MPa	%
Fua-Gly+Leu-NH_2[a)]	7.0	0.1	83.0
		200	52.1
	8.0	0.1	78.0
		200	62.6
Cbz-Asp+Phe-OMe[b)]	6.0	0.1	6.1
		150	35.8
	6.0	0.1	12.8[c)]
		150	55.8[c)]
	7.0	0.1	15.3
		150	33.6

a) [Fua-Gly]=1 mM, [Leu-NH_2·HCl]=50 mM, [TLN]=1.1 μM, 30 °C, 5 (v/v)% DMSO. Incubation time 6 h. b) [Cbz-Asp]=5 mM, [Phe-OMe·HCl]=100 mM, [TLN]=5 μM, 30 °C, 5 (v/v)% DMSO 50 (v/v)% MeCN. Incubation time 4 h. c) [TLN]=7.5 μM, 35 °C. Other conditions are as above.

Thermolysin. This enzyme effectively catalyzes the condensation reaction of *N*-acylamino acid and amino acid amide. Table 5 shows the result on the effect of pressure on thermolysin-catalyzed peptide condensation.

For a reaction to form a specific dipeptide substrate, such as Fua-Gly+Leu-NH_2→Fua-Gly-Leu-NH_2 (the second-order rate constant of the hydrolysis of this substrate by thermolysin is about 2×10^4 $M^{-1}s^{-1}$ at pH 6.5, 25 °C), the condensation efficiency is very high, much higher than other proteases when we compare their hydrolytic activities.[13)] Though the condensed product did not precipitate under the present experimental conditions (the concentration of the acyl substrate was set well below the solubility of the product; monophasic system), the reaction proceeded fast (as shown in Fig. 3-a) and the product yield was deter-

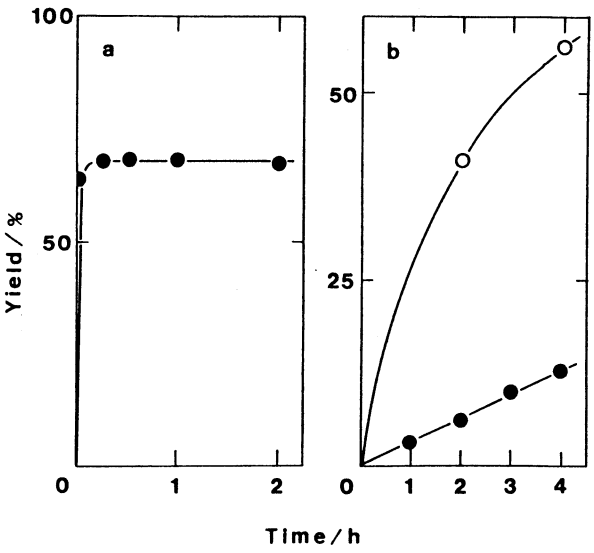


Fig. 3. Time course of thermolysin-catalyzed peptide formation from Fua-Gly+Leu-NH_2 (a) and Cbz-Asp+Phe-OMe (b). Yield of *N*-acyldipeptide amide (a) or ester (b) is shown.
(a) [Fua-Gly]=1mM, [Leu-NH_2·HCl]=0.1M, [TLN]=1.0 μM, pH 7.0 (Hepes 0.1M), 5% DMSO, 37°C.
(b) [Cbz-Asp]=5mM, [Phe-OMe·HCl]=0.1 M. [TLN]=7.5 μM, pH 6.0, 5% DMSO, 50% MeCN, 35°C.
●, at 0.1MPa; O, at 150 MPa.

mined mostly by the equilibrium of the reaction (thermodynamic-controlled). At increased pressure the electrostriction of the water around the carboxylate and ammonium ions of the *N*-acylamino acid and amino acid amide, respectively, is favored in order to decrease the partial volumes in the system which in turn forces the reaction to proceed towards the dissociated (hydrolyzed) side and results in a lower yield of the peptide formation. At such a pressure as discussed in the present study the volume change contribution of enzyme protein structure is far less significant and that of the hydration change of the substrate (or protein) plays dominant roles.[14]

On the contrary the combination of Cbz-Asp and Phe-OMe (which is known as a reaction to produce a precursor of a sweetener[15]) is 'non-specific' to this enzyme and the reaction proceeded slowly (Fig. 3-b). Thus, the yield is determined rather "kinetically." In such a case the higher catalytic activity performed by the increased pressure is sufficient to exceed the negative factor given by the substrate hydration and, hence, a nearly six-fold increase in the peptide formation was observed under the present conditions.

These results indicate that pressure can be an important parameter in improving the peptide yield in a reaction catalyzed by proteolytic enzymes; this principle will be applied to other systems with different types of the substrate and enzyme. Though the pressure had been used to influence and improve various organic (or inorganic) syntheses,[16] the present example shows that the effect of pressure is exerted through molecular recognition and a reaction by a macromolecular catalyst which can amplify the effect to enable the control of reactions at relatively low pressure.

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