The Effect of Pressure on Protease-catalysed Peptide Formation

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Peptide formation from an N-acyl amino acid ester and an amino acid amide using carboxypeptidase Y as a catalyst was shown to be considerably influenced by applying high pressure; at 150 MPa the peptide yield was almost five-fold higher than that at 0.1 MPa when $PheNH_2$ was used as the nucleophile.

Peptide synthesis using proteolytic enzymes has been shown to have several advantages.[1] Carboxypeptidase Y (CPase Y), isolated from yeast[2] and classified as a serine carboxypeptidase, is one of the enzymes used for such a purpose. This enzyme has a broad substrate specificity and shows esterase (equation 1) and amidase (equation 2) activities as well as its intrinsic carboxypeptidase activity (equation 3) (AA_i; amino acid residue).

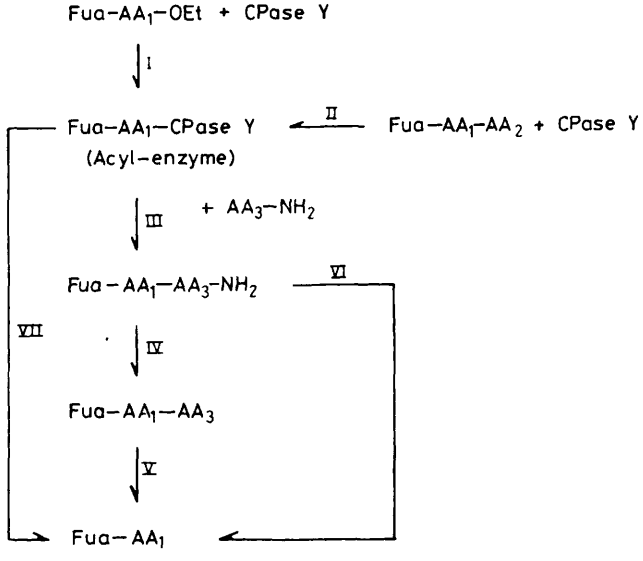
$$—AA_1–OR \longrightarrow —AA_1 + ROH \quad (1)$$

$$—AA_1–NH_2 \longrightarrow —AA_1 + NH_3 \quad (2)$$

$$—AA_1–AA_2 \longrightarrow —AA_1 + AA_2 \quad (3)$$

A peptide can be formed by aminolysis of an acylated intermediate[3] derived from an ester substrate[4] or by a transpeptidation,[5] as summarized in Scheme 1 for the case of N-acyl dipeptide formation. The relatively high amidase activity of this enzyme, however, results in hydrolysis of the initially produced peptide amide to a peptide having a free carboxylate group (process IV), which can be easily hydrolysed further to an N-acyl amino acid by the intrinsic carboxypeptidase activity (process V).

We have examined the reaction mechanisms of several proteases including serine carboxypeptidase by studying the influence of hydrostatic pressure on the reactions.[6,7] The effect of pressure on each type of activity of CPase Y was different depending on the nature of the scissile bond;[7] namely carboxypeptidase activity decreased drastically on increasing the pressure, while esterase and amidase reactions were less influenced by pressure changes. (At 25 °C and pH 6, an increase of pressure from 0.1 to 100 MPa reduced the second-order rate of the hydrolysis of Fua-Phe-OEt to 84.6%,† that of Fua-Phe-NH_2 to 75.7%, and that of Fua-Gly-Phe to 14.8%.)



Scheme 1. Reactions in CPase Y-catalysed dipeptide formation. For the sake of simplicity, the acylated enzymes formed during the processes IV, V, and VI are not explicitly described. Key: I, esterase activity; II, carboxypeptidase activity; III, aminolysis; IV, amidase activity; V, carboxypeptidase activity; VI, carboxamidopeptidase activity; VII, hydrolysis.

† Abbreviations used: Fua = *N*-[3-(2-furyl)acryloyl] Phe = phenylalanine, Gly = glycine.

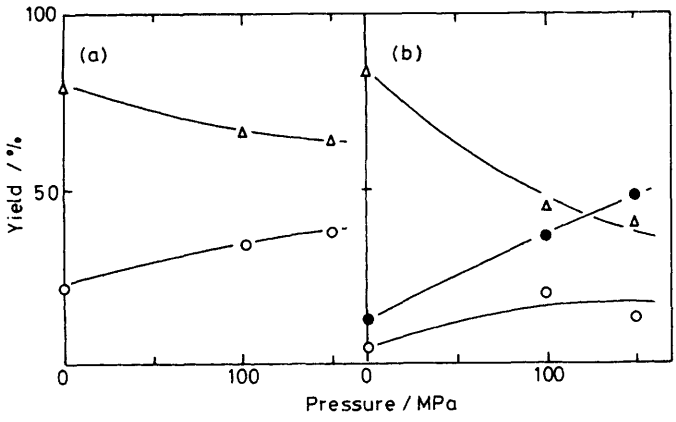


Figure 1. Effect of pressure on CPase Y-catalysed peptide formation at 30 °C. (a) Fua-Phe-OEt + Gly-NH_2, (b) Fua-Phe-OEt + Phe-NH_2. Relative amounts of Fua-Phe (△), Fua-Phe-Gly-NH_2 or Fua-Phe-Phe-NH_2 (○) and Fua-Phe-Phe (●) detected in the reaction mixture. [enzyme] = 0.6 μM, [Fua-Phe-OEt] = 20 mM, [amino acid amide] = 0.1 M, pH 8 (0.1 M Hepes), 0.2 M NaCl, 15% (v/v) *N*,*N*-dimethylformamide.

Therefore we investigated the idea that pressure can be used to control the yield of peptide formation catalysed by this enzyme. The idea originates from the principle suggested by Bresler some 40 years ago.[8] In this communication we describe the result of a relatively simple combination of substrates; peptide formation from Fua-Phe-OEt and Gly-NH_2 or Phe-NH_2.

Fua amino acids were prepared as previously reported.[9] CPase Y was kindly donated by the Oriental Yeast Co. (Osaka, Japan) (Lot 21003001). Peptide formation was carried out at 30 °C and pH 8.0 (Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethane-2-sulphonic acid, 0.1 M). The reaction solution usually contained 0.6 μM enzyme, 0.2 M NaCl, and 15% (v/v) *N*,*N*-dimethylformamide. The solution was sealed in a polypropylene bottle and placed in a stainless steel pressure vessel. After a prescribed period (usually 3 h) the reaction mixture was taken out and quenched by adding 5 volumes of acetonitrile. A portion was then analysed by liquid chromatography.

Figure 1 shows the pressure dependence of the product distribution. With Gly-NH_2, (Figure 1a), Fua-Phe and Fua-Phe-Gly-NH_2 were detected as the products, and the yield of the dipeptide amide was increased with increase in pressure. As reported previously,[10] this enzyme shows carboxamidopeptidase activity towards Fua-Phe-Gly-NH_2 and therefore the initially produced dipeptide amide can be directly converted into Fua-Phe (Scheme 1, process VI). Under high pressure, the velocities of processes VI and VII became relatively smaller than the formation of the acylated enzyme (I) and the aminolysis (III), and then the apparent yield of the dipeptide amide increases.

When Phe-NH_2 was used as the nucleophile, a dipeptide having free carboxylate (Fua-Phe-Phe) was also detected in the reaction mixture. This N-acyl dipeptide was produced by amidase activity (IV) on the primary product Fua-Phe-Phe-NH_2.[10] Fua-Phe was produced by the hydrolysis of Fua-Phe-CPase Y (VII) and of Fua-Phe-Phe (V). By applying a pressure of 150 MPa, the total peptide yield was increased approximately five-fold and the ratio of [Fua-Phe-Phe] to [Fua-Phe-Phe-NH_2] nearly doubled. This result is explained if the increase in pressure leads to retarding of the carboxypeptidase activity (V) to a larger extent than for the aminolysis reaction (III) or amidase activity (IV). Pressure showed the greatest inhibitory effect on process V, and the dipeptide with a free carboxylate end group was obtained in excess. This is consistent with independent results obtained on the effect of pressure on the hydrolysis of N-Fua dipeptide amides by this enzyme,[11] and reactions with other combinations of amino acids can be grouped into either of the two categories presented here.

These results indicate that pressure is an important parameter in improving the peptide yield in the reaction catalysed by CPase Y. Though pressure has often been used to influence and improve various organic (or inorganic) syntheses,[12] in the present example the effect of pressure can be amplified by molecular recognition and catalysis by a macromolecular catalyst and enables the reaction to be controlled with a relatively low pressure (only of the order of 100 MPa).

We acknowledge the advice and encouragement of Professors Norio Ise (Kyoto University) and Keizo Suzuki (Ritsumeikan University).

Received, 28th April 1987; Com. 575

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