Note

Synthesis of Peptides Consisting of Essential Amino acids by a Reactor System Using Three Proteinases and an Organic Solvent

Yukitaka KIMURA,† Koji MURAYA, Yoichi ARAKI, Hiroyoshi MATSUOKA,†† Kazuhiro NAKANISHI,* and Ryuichi MATSUNO

Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606, Japan
** Department of Biotechnology, Faculty of Engineering, Okayama University, Tsushimanaka, Okayama 700, Japan*

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Peptides are physiologically, nutritionally, and physicochemically different from free amino acids and proteins. Some peptides, such as enkephalin and angiotensin, have biological activity; some, such as aspartame, have tastes. Dipeptides and tripeptides are assimilated at the brush-border membrane of the intestinal tract more quickly than free amino acids, for which the absorption mechanisms are different.[1] So peptization of free branched-chain amino acids, which are used to treat cirrhosis of the liver, might make this treatment more effective. Peptides can be also used as food supplements because their solubility and functions are different from those of proteins. Peptides in solution result in a lower osmotic pressure than the free amino acids with which they are constructed. Again, peptization helps nutritional substances to be absorbed *via* the intestines during illness.

Useful peptides are synthesized by chemical methods, enzymatic methods using proteinases, and genetic engineering. Chemical methods are usually used for the syntheses of oligopeptides. However, enzymatic methods[2] have some merits over chemical ones. Enzymatic methods can be done under mild conditions. A racemic mixture can be used as the substrate because proteinases have strict selectivity; there must be an L-amino acid at least one side of the cleavage site. Nonetheless, a few enzymatic methods are used for the commercial production of peptides. The reason is that there are only a few simple and efficient reactor systems.

We have proposed various kinds of reactors that use an organic solvent for peptide synthesis.[3-8] With proficient use of these reactors, a few reactors can synthesize various useful peptides. As an example of the reactor system, we here describe syntheses of the three peptides consisting of eight essential amino acids using only three proteinases used commonly: papain, α-chymotrypsin, and thermolysin. Additionally, another peptide containing L-histidine was synthesized by papain because a lot of evidences that L-histidine should be included in essential amino acids even for adults have been accumulated recently.[9] Taking account of the substrate specificities of these enzymes, the peptides chosen were Z-K(Z)I-OMe, Z-TWV-NH_2, Z-MLF-OMe, and Boc-H(Tos)V-OEt. The steps of the syntheses were as follows.

$$\text{Z-K(Z)} + \text{I-OMe} \xrightarrow{\text{Thermolysin}} \text{Z-K(Z)I-OMe} + H_2O \quad (1)$$

$$\text{Z-T} + \text{W-OEt} \xrightarrow{\text{Papain}} \text{Z-TW-OEt} + H_2O \quad (2)$$

$$\text{Z-TW-OEt} + \text{V-}NH_2 \xrightarrow{\alpha\text{-Chymotrypsin}} \text{Z-TWV-}NH_2 + \text{EtOH} \quad (3)$$

$$\text{Z-L} + \text{F-OMe} \xrightarrow{\text{Thermolysin}} \text{Z-LF-OMe} + H_2O \quad (4)$$

$$\text{Z-M} + \text{LF-OMe} \xrightarrow{\text{Thermolysin}} \text{Z-MLF-OMe} + H_2O \quad (5)$$

$$\text{Boc-H(Tos)} + \text{V-OEt} \xrightarrow{\text{Papain}} \text{Boc-H(Tos)V-OEt} + H_2O \quad (6)$$

Here, Z denotes *N*-(benzyloxycarbonyl)-, used as a protective group at the N-terminal or on the side chain of L-lysine. OMe, OEt, and NH_2, are methyl ester, ethyl ester, and amide, respectively; they were the protective groups at the C-terminal. Boc-H(Tos) is *tert*-(butyloxycarbonyl)-N^{im}-tosyl-L-histidine. The nine amino acids, written in the one-letter code, were L-amino acids.

Z-X, Z-K(Z), Boc-H(Tos), X-OEt·HCl, X-OMe·HCl, and X-NH_2·HCl (X = L-amino acid) were purchased from the Peptide Institute, Inc. (Osaka, Japan) or Kokusan Chemical Works, Ltd. (Tokyo, Japan) and used as substrates. Papain (EC 3.4.22.2; 2 × recrystallized), bovine

† To whom correspondence should be addressed.
†† Present address: *Ishihara Sangyo Kaisha Ltd., Kusatsu 525, Shiga, Japan.*

α-chymotrypsin (EC 3.4.21.1; 3 × recrystallized) obtained from Sigma Chemical Co. (U.S.A.), and thermolysin (EC 3.4.24.4; crystallized once) supplied by Daiwa Kasei K. K. (Osaka) were used without further purification. Immobilized thermolysin (IMT) and immobilized α-chymotrypsin (IMC) were prepared as described previously.[3,8] Papain was immobilized on mechanically rigid resins sieved through 24 and 42 meshes of Amberlite XAD-8[10] (from Japan Organo Co., Ltd.). One gram of the wet resin, which had been washed with ethanol and then a large amount of 0.1 M phosphate buffer (pH 7), was suspended in 12.5 ml of 0.1 M phosphate buffer, pH 7, containing 0.25 g of papain at 0°C. The suspension was shaken for 16 hr at 4°C and filtered on a G-2 glass filter. Immobilized papain (IMP) was washed with about 100 ml of the same buffer. About 70% of the enzyme protein added was absorbed to the XAD-8.

Unless otherwise specified, the peptides were synthesized by free enzyme in an aqueous-organic biphasic system or by an immobilized enzyme (IME) in an organic solvent as follows. In a biphasic system we used ethyl acetate for the organic phase, 0.05 M Mes–NaOH buffer (Mes = 2-(*N*-morpholino)ethanesulfonic acid) for the aqueous phase at pH 5–7, and 0.1 or 0.25 M Tris–HCl buffer for the aqueous phase at pH 7–10. To stabilize the proteinases, 5 mM $CaCl_2$ was added to the buffer for thermolysin, 20 mM $CaCl_2$ was added to the buffer for α-chymotrypsin, and 0.1% 2-mercaptoethanol was added to both phases for papain. Five milliliter of ethyl acetate saturated with a buffer, containing 50 mM Z-X, was mixed with 5 ml of a buffer saturated with ethyl acetate, containing 100 mM X-OMe·HCl, X-OEt·HCl, or X-NH_2·HCl) and 0.4% proteinase, and then the mixture was vigorously stirred with a magnet and incubated at 40°C. In peptide synthesis with an IME in an organic solvent, as the substrate solution, we used ethyl acetate saturated with a buffer, containing 50 mM Z-X and 100 mM X-OMe that had been desalted.[8]

Substrates and products were separated by high-performance liquid chromatography (HPLC; LC-6A; Shimadzu Corp., Kyoto, Japan) with an ODS column (4.6 × 150 mm column packed with Cosmosil $5P_{18}$-P; Nacalai Tesque, Inc., Kyoto, Japan) at the flow rate of 0.8 ml/min with a mixture of acetonitrile and water (1 : 1, v/v) adjusted to pH 2.5 with phosphoric acid. The eluted reactants were detected by a UV detector at 254 nm. The yield of product was calculated based on the amount of the limiting substrate, *i.e.*, substrate of lower concentration. In the experiments with equimolar substrates, the yields were also calculated based on the same substrates. Products were isolated by extraction with ethyl acetate and further purified by chromatography on an ODS column (2.5 × 12 cm). Then they were identified by amino acid analysis (K-101-AS; Kyowa Seimitsu Co., Ltd.) and fast atom bombardment mass spectrography (FAB-MS; Jeol JMS-DX-300).

Table I shows the yields with a free proteinase in a biphasic system and with an IME in an organic solvent. The yield of Z-K(Z)I-OMe in an aqueous-organic biphasic system was almost 100% at pH 5.8–7.6. In a reaction with IMT in an organic solvent, conversion to Z-K(Z)I-OMe was almost 100%. The product was hydrophobic, so the yield was very high.[4] When Z-K, which was not protected at the amide of the side chain, was used as the substrate, the yield of Z-KI-OMe with thermolysin was negligible under various reaction conditions tested. The reasons might be that Z-KI-OMe was hydrophilic and that L-lysine did not fit at the P_1 site of thermolysin, which is the carboxylic side of the cleavage site.

In the synthesis of Z-TW-OEt by papain, a large amount of polymers of L-tryptophan from W-OEt[11] and by-products such as Z-TWW-OEt were produced in a

Table I. YIELDS OF PEPTIDES SYNTHESIZED WITH A FREE PROTEINASE IN A BIPHASIC SYSTEM OR WITH AN IMMOBILIZED PROTEINASE IN AN ORGANIC SOLVENT

Products	Yield with free proteinase[a] (%)	Yield with immobilized proteinase[b] (%)
Z-K(Z)I-OMe	≃100[c] (88[d])	≃100[n] (91[o])
Z-TW-OEt	73[e] (35[f])	43[p] (21[q])
Z-TWV-NH_2	≃100[g] (3[h])	≃100[r] (≃100[s])
Z-LF-OMe	≃100[i] (84[j])	≃100[t] (84[u])
Z-MLF-OMe	≃100[k]	≃100[v]
Boc-H(Tos)V-OEt	82[l] (66[m])	91[w] (54[x])

Values in the parentheses are the yields in the experiments with equimolar substrates.

[a] In a biphasic system. [b] In an organic solvent; [c] 50 mM Z-K(Z), 100 mM I-OMe·HCl, pH 5.8–7.6, α = 1 (α, volume ratio of organic to aqueous phases). [d] 50 mM Z-K(Z) and I-OMe·HCl, pH 7.0, α = 1. [e] 200 mM Z-T, 50 mM W-OEt·HCl, pH 6.0, α = 1. [f] 50 mM Z-T and W-OEt·HCl, pH 6.0, α = 1. [g] 5 mM Z-TW-OEt, 1.5 M V-NH_2·HCl, pH 9.0, α = 2. [h] 5 mM Z-TW-OEt and V-NH_2·HCl, pH 8.7, α = 1. [i] 50 mM Z-L, 100 mM F-OMe·HCl, pH 6.0–7.0, α = 1. [j] 50 mM Z-L and F-OMe·HCl, pH 6.0, α = 1. [k] 50 mM Z-M, 50 mM LF-OMe·HCl, pH 6.0, α = 1. [l] 50 mM Boc-H(Tos), 100 mM V-OEt·HCl, pH 5.9, α = 1. [m] 50 mM Boc-H(Tos) and V-OEt·HCl, pH 5.8, α = 1. [n] 50 mM Z-K(Z), 100 mM I-OMe, 0.4 g of IMT in 3 ml of substrate solution (SS). [o] 50 mM Z-K(Z) and I-OMe, 0.3 g of IMT in 3 ml of SS. [p] 20 mM Z-T, 10 mM W-OEt, 0.2 g of IMP in 3 ml of SS. [q] 10 mM Z-T and W-OEt, 0.1 g of IMP in 2 ml of SS. [r] 5 mM Z-TW-OEt, 50 mM V-NH_2, 0.1 g of IMC in 2 ml of SS. [s] 5 mM Z-TW-OEt and V-NH_2, 0.1 g in 2 ml of SS. [t] 50 mM Z-L, 100 mM F-OMe, 0.5 g of IMT in 4 ml of SS. [u] 50 mM Z-L and F-OMe, 0.3 g of IMT in 3 ml of SS. [v] 50 mM Z-M, 50 mM LF-OMe, 1.0 g of IMT in 4 ml of SS. [w] 50 mM Boc-H(Tos), 100 mM V-OEt, 0.1 g of IMP in 2 ml of SS. [x] 50 mM Boc-H(Tos) and V-OEt, 0.1 g of IMP in 2 ml of SS.

buffer system, and the yield of Z-TW-OEt was 0.2%. In a biphasic system, the yield was increased to 55% when the volume ratio of the organic to aqueous phases, α, was 1 and when the pH of the aqueous phase was 6.0. The increase in the yield was due to the suppression of two reactions: one, the polymerization of tryptophan from W-OEt by papain (because the concentration of W-OEt in the aqueous phase was half that in the organic phase), and the other, the synthesis of by-products such as Z-TWW-OEt (for the same reason as in the synthesis of Z-GGFL-OEt).[6] The maximum yield of Z-TW-OEt was 73%. Though IMP was used, the yield did not increase. By the way, the yield of Z-TW-OEt was negligible in a biphasic system when α-chymotrypsin, thermolysin, pepsin (from Sigma), trypsin (from Wako Pure Chemical Industries, Ltd.), neutral proteinase (from Kyowa Hakko Kogyo Co., Ltd.), or subtilisin-A (from Novo Biolabs) was used.

The yield of Z-TWV-NH_2 was very high. In a biphasic system at pH 7 when α-chymotrypsin or IMC was used, synthesis did not proceed, contrary to our previous finding.[8] The partition coefficient (P value) of non-dissociated Z-TW-OEt was 2000, higher than that of Z-GF-OMe (600), so the concentration of Z-TW-OEt in the aqueous phase was low. α-Chymotrypsin has lower esterase activity at pH 7 than at pH 9–10.[12] The low concentration of Z-TW-OEt and the low activity of esterase probably caused the poor yield at pH 7.

According to the amino acids pattern for mature recommended by FAO/WHO/UNU in 1985,[9] it is more desirable to combine L-valine with KI than with TW. So we tried to synthesize a tripeptide containing L-lysine, L-isoleucine, and L-valine. No combination of these three could be produced. In hydrolysis by pepsin, the placement of L-valine or L-isoleucine in the P_1 site prevents peptides from being hydrolyzed even if an L-amino acid with suitable substrate specificity is located at the P'_1 site.[13] Thus, the β-methyl groups of these two amino acids may have interfered with the synthesis of the tripeptide consisting of L-valine, L-isoleucine, and L-lysine.

Z-LF-OMe was so hydrophobic that the yield was very high. The yield of Z-MLF-OMe was also high. Details of the synthesis of Z-MLF-OMe in which MLF induces chemotaxis of leucocytes will be published elsewhere. Thermolysin can hydrolyze two bonds of MLF. In the synthesis of Z-GGFL-OEt,[6] its relatively low P values gave rise to by-products. In the synthesis of Z-MLF-OMe, its high P value resulted in a high yield.

The yield of Boc-H(Tos)V-OEt was also high as shown in Table I because of its hydrophobicity. When the substrate without the protective group on the side chain of L-histidine, Z-H, was used, no product was detected.

Here, we gave an example of a simple, efficient reactor system that uses only three proteinases for the syntheses of four peptides consisting of essential amino acids and L-histidine.

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