

Continuous Synthesis of a Tripeptide by Successive Condensation and Transesterification Catalyzed by Two Immobilized Proteinases in Organic Solvent

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The tripeptide Z-GlyPheLeuNH₂ was continuously synthesized in a high yield from three amino acid derivatives, Z-Gly, PheOMe, and LeuNH₂, by immobilized thermolysin (IMT) and immobilized α -chymotrypsin (IMC) in an organic solvent, ethyl acetate. The optimal conditions for the synthesis of Z-GlyPheOMe were established theoretically. The yield of Z-GlyPheOMe with IMT in ethyl acetate saturated with buffer was more than 88% after continuous synthesis for 116 hr.

The optimal conditions for the synthesis of Z-GlyPheLeuNH₂ from Z-GlyPheOMe and LeuNH₂ by IMC through transesterification was established in batch reaction experiments. When the concentration of water in the reaction solution was 17–20 μ l/ml, the activity of IMC was highest. The equilibrium between the water concentration in the reaction solution and that in the resin used for enzyme immobilization depended on the resin and was not affected by the presence of the enzyme immobilized. Z-GlyPheLeuNH₂ was synthesized from Z-GlyPheOMe and LeuNH₂ with a yield of 100%, by continuous reaction for 160 hr.

The reactor for synthesis of this tripeptide was efficient and stable because of the use of transesterification and the choice of an appropriate organic solvent. The series plug-flow reactor was successfully operated for 220 hr with a yield of more than 80%. The residual activity of IMT was 94%, and that of IMC was 100%.

Many biologically active peptides have been discovered, some of which are parts of the amino acid sequence in dietary proteins such as casein.¹⁾ Dipeptides and tripeptides are assimilated by the brush-border membrane of the intestinal tract more quickly than free amino acids, for which the absorption mechanism is different.²⁾ In this sense, peptides are superior to the free amino acids with which they are constructed. In addition, peptides cause

a low osmotic pressure in solution. These characteristics are desirable for nutritional substances to be absorbed *via* the intestine during illness.

The synthesis of such useful peptides through the reverse reaction of hydrolysis by proteinase has often been reported.³⁾ The enzymatic method has some merits over the chemical one. However, chemical synthesis is usually used when the scale is industrial. A few pep-

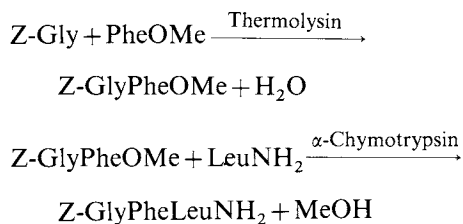
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Abbreviations: HPLC, high performance liquid chromatography; IMC, immobilized α -chymotrypsin; IME, immobilized enzyme; IMT, immobilized thermolysin; LeuNH₂, L-leucine amide; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PheOMe, L-phenylalanine methyl ester; Z-FF-M, *N*-(benzyloxycarbonyl)-L-phenylalanyl L-phenylalanine methyl ester; Z-GFL-NH₂, *N*-(benzyloxycarbonyl)-glycyl L-phenylalanyl L-leucine amide; Z-Gly, *N*-(benzyloxycarbonyl)-glycine; Z-GlyPheLeuNH₂, *N*-(benzyloxycarbonyl)-glycyl L-phenylalanyl L-leucine amide; Z-GlyPheOMe, *N*-(benzyloxycarbonyl)-glycyl L-phenylalanine methyl ester; Z-Phe, *N*-(benzyloxycarbonyl)-L-phenylalanine; Z-PhePheOMe, *N*-(benzyloxycarbonyl)-L-phenylalanyl L-phenylalanine methyl ester.

tides have been produced by the enzymatic method in a commercial or pilot scale.⁴⁾ To make the enzymatic method more popular, a lot of models of more concrete and efficient reactors have to be proposed.

Here, we report on an efficient and stable reactor for the synthesis of a tripeptide that made use of two main reactions. One was transesterification, and the other was the reaction in an organic solvent with an immobilized enzyme (IME). Transesterification by a proteinase such as α -chymotrypsin or papain has been used in the synthesis of peptides for half a century.⁵⁻⁷⁾ Recently there are reports of more unusual uses of transesterification.⁸⁾ Transesterification has some merits when used in a reactor. The reaction proceeds very fast, about one hundred times as fast as condensation. In addition, the protective group at the C-terminal, such as methyl ester, need not be removed before peptide synthesis. On the other hand, enzyme reactions in organic solvents also have advantages.⁹⁻¹²⁾ The synthesis of peptides in an organic solvent immiscible with water decreases the reaction rate, but increases the yield.^{13,14)} The activity and stability of enzymes in an immiscible organic solvent depends on the concentration of water.^{15,16)} The effects of the water concentration on activity of IME were also studied here.

Z-GlyPheLeuNH₂ was chosen as the tripeptide product for the testing of our reaction system. Phenylalanine and leucine are essential amino acids and glycine is a compound needed for the synthesis of physiologically important substances; it affects the solubility of peptides. The synthetic procedure was as follows.



Z-GlyPheOMe is continuously synthesized through a condensation reaction in a column containing immobilized thermolysin with the

organic solvent ethyl acetate as the carrier. The product was directly introduced to the second column of immobilized α -chymotrypsin with LeuNH₂ dissolved in ethyl acetate containing an appropriate amount of water and the final product was formed through the transesterification reaction.

Materials and Methods

Materials. Crystalline thermolysin (EC 3.4.24.4, crystallized once) supplied by Daiwa Kasei K.K. (Osaka, Japan) and bovine α -chymotrypsin (EC 3.4.21.1, recrystallized three times) purchased from Sigma Chemical Co. (U.S.A.) were used without further purification. Z-Gly and Z-Phe were obtained from Peptide Institute, Inc. (Osaka, Japan). PheOMe·HCl was a product of Kokusan Chemical Works (Tokyo, Japan). LeuNH₂·HCl was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). As a reagent in the buffer, 2-(*N*-morpholino)-ethanesulfonic acid (Mes) from Dojin Chemical Ltd. (Kumamoto, Japan) was used. The mechanically rigid resins for preparation of the immobilized enzyme were Amberlite XAD-2, XAD-7, XAD-8, IRC-50, IR-120, 200C, 252 (Japan Organo Co., Ltd.), Dowex MSC-1 (Dow Chemical Co.), and Merckogel SI-1000 (E. Merck). All other chemicals were of analytical grade, and were obtained either from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) or Nacalai.

Assay of the reactants. Substrates and products were separated by HPLC (LC-6A; Shimadzu Corp., Kyoto, Japan) with an ODS column (4.6 × 150 mm Cosmosil 5C₁₈-P packed column; Nacalai) at the flow rate of 0.8 ml/min using a mixture of acetonitrile and water (6:4, v/v) adjusted to pH 2.5 with phosphoric acid. The eluted reactants were detected by a UV detector at 254 nm. Thus, reactants were detected as *N*-benzyloxycarbonyl or phenylalanine residues. The concentration of LeuNH₂ was measured by the ninhydrin reaction.

Preparation of immobilized enzyme. Amberlite XAD-7 was sieved through 24 and 42 meshes and thermolysin was immobilized onto it by cross-linking after adsorption as described previously.¹⁷⁾ The enzyme activity of immobilized thermolysin (IMT) used was represented as μ moles of Z-PhePheOMe synthesized by 1 g wet IMT in a minute. Since this method of immobilization is simple, immobilized α -chymotrypsin (IMC) was prepared with a slight modification as follows. The mechanically rigid resins (all listed above) were sieved through 24 and 42 meshes and washed first with ethanol and then with a large amount of 0.025 M Tris-HCl buffer containing 20 mM CaCl₂, pH 7.5. Then 375 mg of crystalline α -chymotrypsin was dissolved in 7.5 ml of ice-cold 0.025 M Tris-HCl buffer containing 20 mM CaCl₂, pH 7.5. This enzyme solution was mixed

with 1.5 g of the wet resin in a 30-ml vial and gently shaken for 17 hr at 4°C. Then, half of the solution was removed from the suspension, and the rest was mixed with 3.75 ml of 25% glutaraldehyde (Nacalai) and shaken for 3 hr at 4°C for cross-linking of the enzyme molecules. The resultant enzyme-bound resin was first washed with chilled 0.1 M Tris-HCl buffer, pH 7.5, containing 5 mM CaCl₂ and 1 M NaCl, and then washed with the same buffer without NaCl on a G-2 glass filter, and stored at 4°C. All of the filtrate and washing waste produced during the preparation of the immobilized enzyme were pooled and the protein concentration was measured by the biuret method and the Lowry-Folin method.

Freeze-dried IMC was prepared as follows. First, 1.3 g of IMC was incubated in 0.1 M Tris-HCl buffer containing 20 mM CaCl₂, pH 9. The mixture was filtered on a G-2 glass filter and frozen on a watch glass with dry ice. The frozen IMC was dried in a thick glass apparatus under reduced pressure for 2 hr. The weight of the freeze-dried IMC was 59.4% that of the wet IMC.

Preparation of PheOMe and LeuNH₂. First, 50 mmol of PheOMe·HCl (or LeuNH₂·HCl) was dissolved in 100 ml of water containing 25 mmol of Na₂CO₃. This solution was mixed with 100 ml of chloroform in a separatory funnel, and then chloroform, which contained PheOMe (or LeuNH₂), was removed and put into a Erlenmeyer flask. The procedure was done two more times, and about 300 ml of chloroform was collected. Chloroform dehydrated with MgSO₄ was evaporated under reduced pressure, giving PheOMe as oil (or LeuNH₂ as a white powder).

Batch reaction for the synthesis of Z-GlyPheOMe in an organic solvent. First, 50 mM Mes-NaOH buffer, pH 7, containing 5 mM CaCl₂ was mixed with ethyl acetate at 40°C in a separatory funnel. We used Mes-NaOH buffer to incubate IMT and ethyl acetate to dissolve substrates as the reaction solution. Then, 0.2 g of the IMT, which had been incubated in the saturated Mes-NaOH buffer, was mixed in a vial with 4 ml of ethyl acetate containing 40 mM Z-Gly and 80 mM PheOMe, and the mixture was incubated at 40°C with shaking. At times, 0.2-ml portions of the organic solution were sampled and analyzed by HPLC.

Continuous synthesis of Z-GlyPheOMe. The method for the continuous synthesis of Z-GlyPheOMe was as same as that described in our previous paper.¹⁷⁾ Before the start of a continuous reaction, the IMT was equilibrated with saturated Mes-NaOH buffer, pH 7. In the continuous reaction, saturated ethyl acetate containing 40 mM Z-Gly and 80 mM PheOMe was fed into a glass column (11 × 150 mm) that contained 4.6 g (wet weight) of IMT at the flow rate of 0.75 ml/hr with a pump (MD-82, Yamazen Corp., Osaka, Japan). The space time, obtained by dividing the bed volume of IME by the feed rate, was 10 hr. A sealed calibrated cylinder with the substrate solution and

the column with the IMT were incubated in a water bath at 25°C. The effluent from the column was taken at times and analyzed by HPLC. Additionally, the yields were measured at various flow rates.

Batch reaction for the synthesis of Z-GlyPheLeuNH₂ in an organic solvent. Unless otherwise specified, the procedure was as follows. Before IMC was used in the reaction, it was equilibrated with 0.1 M Tris-HCl buffer, pH 9, containing 20 mM CaCl₂ for 20 min with shaking at 25°C. IMC was filtered onto a G-2 glass filter to remove buffer, and 0.1 g of the IMC was weighed in a 20-ml vial. Then, 2 ml of ethyl acetate, which was dehydrated with molecular sieves 3A, containing 5 mM Z-GlyPheOMe and 50 mM LeuNH₂ was added to the vial, and the mixture was incubated at 25°C with shaking. At times, 0.2-ml portions were sampled and analyzed by HPLC. The enzyme activity was assayed by the increase of the yield in 40 min and represented as μ moles of Z-GlyPheLeuNH₂ synthesized by 1 g wet IMC in a minute. The equilibrium yield was the yield at 24 hr, because the reaction was almost finished in 3 hr and the yield did not change after 24 hr.

Z-GlyPheLeuNH₂ was identified by amino acid analysis and ¹H-NMR after purification as follows. The reaction solution was collected when the yield was 100%, and washed twice with 5% citrate solution, water, 5% liquid ammonia, and water in a separatory funnel. The ethyl acetate, which contained Z-GlyPheLeuNH₂, was dehydrated with MgSO₄ and evaporated under reduced pressure. From the results of amino acid analysis, Gly:Phe:Leu was 1.02:1:0.99. ¹H-NMR data of Z-GlyPheLeuNH₂ was ¹H-NMR δ^{TMS} (CD₃OD) ppm: 0.89 (3H, d, *J* = 5.5 Hz), 0.92 (3H, d, *J* = 5.8 Hz), *ca.* 1.6 (3H, m), 2.96 (1H, dd, *J* = 14.0, 8.5 Hz), 3.16 (1H, dd, *J* = 14.0, 5.8 Hz), 3.67 (1H, d, *J* = 16.8 Hz), 3.77 (1H, d, *J* = 16.8 Hz), 4.34 (1H, m), 4.60 (1H, dd, *J* = 8.2, 5.5 Hz), 5.07 (2H, s), 7.2–7.3 (10H, m).

Measurement of dependence of enzyme activity on water concentrations in synthesis of Z-GlyPheLeuNH₂. To estimate the dependence of the enzyme activity on the water concentrations in the reaction system, 0–100 μ l of water was added with a microsyringe to a vial at the same time as when dehydrated ethyl acetate with substrates was put into the vial that contained IMC (on Dowex MSC-1) to start a batch reaction for the synthesis of Z-GlyPheLeuNH₂. To measure water concentrations in the reaction solution, a 0.5-ml portion of the solution was put into a 10-ml vial with 0.5 ml of dehydrated solvent ML (Mitsubishi Kasei Corp.). Then 0.25 ml of the mixture was injected through a microsyringe into a titration vessel of a Karl Fischer moisture meter (MKS-1s; Kyoto Electronics, Kyoto, Japan). To measure water concentrations in the resin with IMC, IMC was filtered out onto a dried G-2 glass filter after sampling at 40 min for an assay of the enzyme activity, and then all of the IMC was put into a 10-ml vial. After the addition of 1 ml of

dehydrated solvent ML, the mixture in the vial was shaken for 20 min, and all of the mixture (resin plus solution) was injected through a 10-ml pipette with a rubber bulb into the titration vessel. For titration, Karl Fischer reagent SS (Mitsubishi Kasei) was used. Ten microliters of water passed through a microsyringe was titrated to the end point, and standardization of the Karl Fischer reagent SS was calculated as: $F=10/A$, where F is the titer of the reagent ($\mu\text{l H}_2\text{O/ml}$), and A is the volume of the reagent consumed in the titration of $10\ \mu\text{l}$ of water. The dry weight of the resin was calculated by subtraction of the weight of water in the resin from the wet weight of the resin.

Continuous synthesis of Z-GlyPheLeuNH₂. Before the start of a continuous reaction, the IMC was equilibrated with 0.1 M Tris-HCl buffer, pH 9.0, containing 20 mM CaCl₂. In the continuous reaction, dehydrated ethyl acetate containing 5 mM Z-GlyPheOMe, 25 mM LeuNH₂, and 1.8% water (v/v) was fed into a glass column (11 × 150 mm) that contained 1.5 g (wet weight) of the IMC, at the flow rate of 4.1 ml/hr (space time, 28 min) with a pump (MD-82). The sealed calibrated cylinder containing the substrate solution and the column with the IMC were incubated in a water bath at 25°C. The effluent from the column was sampled at times for measurements of the yield of Z-GlyPheLeuNH₂ (by HPLC), the water concentration (by the Karl Fischer moisture meter), and the concentration of LeuNH₂ (by the ninhydrin method). After 160 hr, the IMC was sampled for measurement of the concentration of the water in the resin by the moisture meter, and washed thoroughly with 0.025 M Tris-HCl buffer, pH 7.5, containing 20 mM CaCl₂ in a vial. The residual activity of IMC was measured by a batch reaction of synthesis of Z-GlyPheLeuNH₂.

Continuous synthesis of Z-GlyPheLeuNH₂ from Z-Gly, PheOMe, and LeuNH₂. Before the start of a continuous reaction, the IMT and the IMC were equilibrated as described above. For the continuous reaction, ethyl acetate saturated with 50 mM Mes-NaOH buffer (pH 7) containing 5 mM CaCl₂, containing 40 mM Z-Gly and 80 mM PheOMe was fed into a first glass column (11 × 150 mm), which contained 5.0 g (wet weight) of IMT, at the flow rate of 1.2 ml/hr (space time, 6.8 hr) with a pump (MD-82). When the yield of Z-GlyPheOMe became constant, ethyl acetate containing 28.6 mM LeuNH₂ and 1.6% water (v/v) plus the effluent from the first column were fed into a second glass column (11 × 150 mm), which contained 2.5 g (wet weight) of the IMC (Fig. 1). The flow rate of the effluent from the second column was 8.0 ml/hr, and the space time in the IMC was 19 min. According to our calculations, the concentration of water in the substrate solution for the second column was 1.80–1.85% (v/v). The sealed calibrated cylinders containing the substrate solution and the columns with IMT and IMC were incubated in water baths at 25°C. The yield of Z-GlyPheLeuNH₂, the water concentrations, and the con-

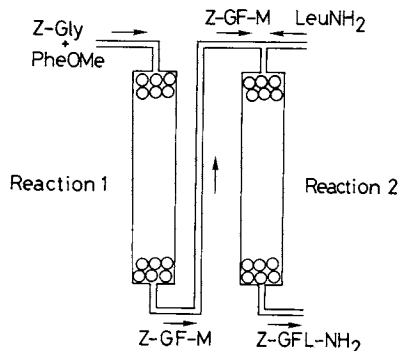


Fig. 1. Reactor System for Continuous Synthesis of Z-GlyPheLeuNH₂ from Z-Gly, PheOMe, and LeuNH₂.

Z-GF-M, Z-GlyPheOMe; Z-GFL-NH₂, Z-GlyPheLeuNH₂.

centration of LeuNH₂ were measured as described above. After 220 hr, about 0.5 g of IMC was sampled to measure the concentration of water in the resin, and IMT and residual IMC were washed thoroughly as described above. The residual activity of IMT was measured by the synthesis rate of Z-PhePheOMe in a biphasic system,¹⁷⁾ and that of IMC was measured as described in the previous section.

Results and Discussion

Calculation of the equilibrium yield of Z-GlyPheOMe

Previously, we elucidated the kinetics and equilibrium of the enzymatic synthesis of peptides in aqueous/organic biphasic systems,¹⁴⁾ and found that the equilibrium yield in a biphasic system depended on the pH in the aqueous phase and could be calculated. Here, we calculated the equilibrium yield of Z-GlyPheOMe at various pHs in the same way. The values of coefficients needed for calculation were measured; the negative log of the dissociation constants of the substrates ($\text{p}K_{\text{aZ-Gly}}=3.56$ and $\text{p}K_{\text{aPheOMe}}=7.00$) and the partition coefficient of the non-ionized form of the substrates and condensation product ($P_{\text{Z-Gly}}=25$, $P_{\text{PheOMe}}=20$, and $P_{\text{Z-GlyPheOMe}}=600$). From the results of calculation, the optimum pH is 5.4–5.5, and the yield was 94% when the ratio of the organic phase volume to the aqueous phase volume, α , was 40 and the initial concentrations of Z-Gly and

PheOMe were 40 and 80 mm.

According to another calculation, the pH in the aqueous phase was 5.6 when 40 mm Z-Gly, 80 mm PheOMe, and 0.05 M Mes-NaOH buffer, pH 7.0, were used, and α was equal to 40. After 90% of the Z-GlyPheOMe was converted, the pH increased to 6.1. So a high yield would be expected under these conditions.

Synthesis of Z-GlyPheOMe

In batch reactions, the yield was 97%, which was consistent with the calculated value. Table I shows the relationship between yield and space time in a plug flow reactor. It takes more than 5 hr to attain 90% yield of Z-GlyPheOMe. Figure 2 shows the yield of continuous synthesis of Z-GlyPheOMe from Z-Gly and PheOMe. For 120 hr, the yields were more than 88%. The yield was calculated based on the amount of Z-Gly.

Table I. RELATIONSHIP BETWEEN YIELD AND SPACE TIME IN A PLUG FLOW REACTOR

	Space time	Yield (%)
Reaction 1	3.2 hr ^a	55
	5.0 hr	89
	10.0 hr	98
Reaction 2	5 min	53
	8 min	74
	15 min	92

Reaction 1; Z-Gly + PheOMe \rightarrow Z-GlyPheOMe + H₂O. IMT, 1.73 μ mol-Z-FF-M/g-wet-IMT \cdot min, 4.6 g.

^a 2.9 g.

Reaction 2; Z-GlyPheOMe + LeuNH₂ \rightarrow Z-GlyPheLeuNH₂ + MeOH. IMC, 0.77 μ mol-Z-GFL-NH₂/g-wet-IMC \cdot min, 1.1 g.

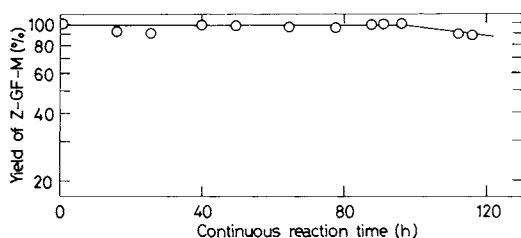


Fig. 2. Continuous Synthesis of Z-GlyPheOMe. IMT, 1.73 μ mol-Z-FF-M/g-wet-IMT \cdot min.

Batch reaction for the synthesis of Z-Gly-PheLeuNH₂

Table II shows the yields of Z-Gly-PheLeuNH₂ in 1 hr with α -chymotrypsin immobilized on various resins. IMC on Dowex MSC-1, a cation exchanger, synthesized Z-GlyPheLeuNH₂ with the highest yield and was easy to work with. About 30% of added enzyme protein was adsorbed to the resin.

Figure 3 shows the effects of the pH of the Tris-HCl buffer for incubating IMC, temperature, and the concentration of LeuNH₂ on the enzyme activity and the equilibrium yield with respect to Z-GlyPheOMe. Within the experi-

Table II. YIELDS OF Z-GlyPheLeuNH₂ WITH α -CHYMOTRYPSIN IMMOBILIZED ON VARIOUS RESINS

Resin	Yield at 1 hr (%)	
Amberlite	XAD-2	55.6
	XAD-7	58.3
	XAD-8	70.6
	IRC-50	18.9
	IR-120	0
	200C	84.7
	252	4.1
Dowex	MSC-1	96.3
Merckogel	SI-1000	89.8

0.45 g wet IMC, 5 mm Z-GlyPheOMe, and 50 mm LeuNH₂ in 2 ml of ethyl acetate.

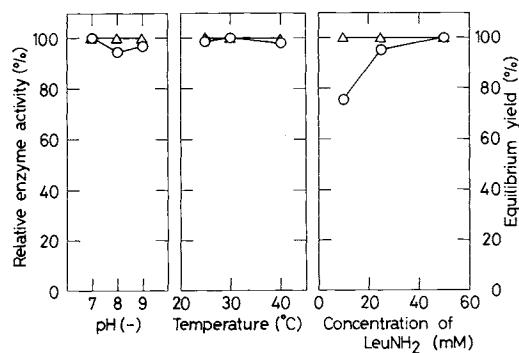


Fig. 3. Effects of pH in Preincubating Buffer, Temperature and Concentration of LeuNH₂ on Enzyme Activity and Equilibrium Yield.

O, relative enzyme activity; Δ , equilibrium yield. IMC for the experiments about pH and concentration of LeuNH₂, 1.50 μ mol-Z-GFL-NH₂/g-wet-IMC \cdot min. IMC for the experiment about temperature, 1.30 μ mol-Z-GFL-NH₂/g-wet-IMC \cdot min.

mental range, the pH of the buffer for incubation of the IMC did not affect activity much (left). The activity of IMC and the equilibrium yield were independent of temperature (middle). However, the initial rate of reaction in a biphasic system with free α -chymotrypsin at 37°C was about twice that at 30°C. These results suggest that the diffusion resistance of the substrate, Z-GlyPheOMe, into the inside of the resin affected the reaction rate. The concentration of LeuNH₂ did not affect the equilibrium yield, although the enzyme activity increased as the concentration increased.

The equilibrium relationship between the water concentration of the reaction solution and that in the resin is shown in Fig. 4. The relationship for resin without enzyme is similar to the relationships for IMC and freeze-dried IMC. This result shows that the nature of the resin decides the equilibrium of the water concentration. When the concentration of water in the solution exceeded the saturated concentration of water in ethyl acetate (32.4 $\mu\text{l/ml}$, 25°C), the concentration of water in

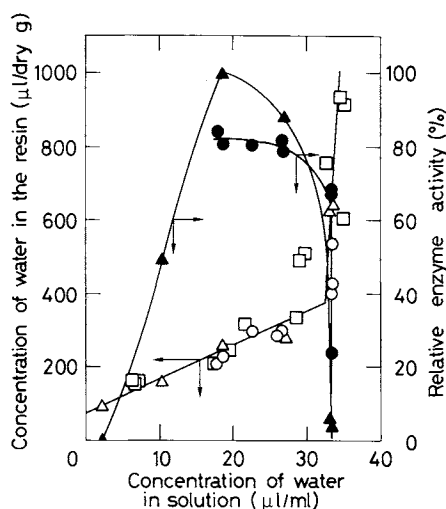


Fig. 4. Effects of Concentration of Water in Reaction Solution on Enzyme Activity and Concentration of Water in the Resin (Dowex MSC-1).

○, △, and □ show the relationship of the concentration of the water between in solution and in the resin. ● and ▲ show the relative enzyme activity at various concentrations of water (○, ●), IMC, 1.54 $\mu\text{mol-Z-GFL-NH}_2/\text{g-wet-IMC} \cdot \text{min}$; (△, ▲), freeze-dried IMC; (□), resin without enzyme.

the resin increased rapidly. The water phase appeared in a vial for the synthetic reaction with the further addition of water. The optimum water concentration for enzyme activity was 17–20 $\mu\text{l/ml}$, as shown in Fig. 4. As the concentration of the water in solution increased over this optimum concentration, the enzyme activity decreased. Then it decreases rapidly and concomitantly with a sudden increase in the water concentration in the resin over the saturated water concentration in solution. This result can be explained as follows. At a low concentration, the small amount of water around the enzyme does not allow the enzyme to act.¹⁵⁾ At a high concentration, the reaction rate will be controlled by the diffusion rate of Z-GlyPheOMe in the aqueous phase inside the resin. A small amount of Z-GlyPheOMe and a large amount of LeuNH₂ are partitioned to the aqueous phase because the partition coefficients of Z-GlyPheOMe and non-ionized LeuNH₂ to the organic phase are 600 and 0.1, respectively. Another evidence is that the reaction rate with ground IMC even at a higher water concentration, 32.3 $\mu\text{l/ml}$, was 97.4% that with IMC at the optimum water concentration. On the other hand, the equilibrium yields were 100% whatever the water concentration.

Continuous synthesis of Z-GlyPheLeuNH₂

Figure 5 shows the yield of Z-GlyPheLeuNH₂ from Z-GlyPheOMe and LeuNH₂

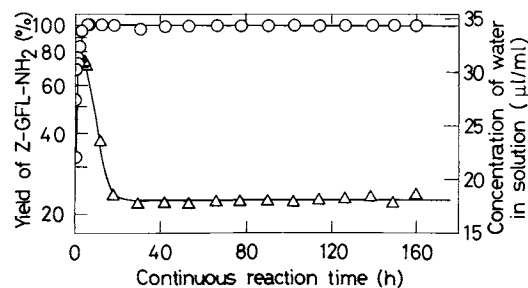


Fig. 5. Yield of Z-GlyPheLeuNH₂ from Z-GlyPheOMe and LeuNH₂ and the Concentration of the Water in Effluent during Continuous Reaction.

○, the yield of Z-GlyPheLeuNH₂; △, the concentration of the water in effluent. IMC, 1.95 $\mu\text{mol-Z-GFL-NH}_2/\text{g-wet-IMC} \cdot \text{min}$.

and the concentration of water in the effluent on continuous synthesis. IMC only needs a short time to attain 90% yield as shown in Table I. A high yield of Z-GlyPheLeuNH₂ with respect to Z-GlyPheOMe was maintained for 160 hr. At the early stage of the reaction, water from the IMC flowed out, then the concentration of water in the effluent became the same as that in the substrate solution. After the reaction ended, the concentration of water in the IMC was 192 μ l/g dry IMC. This value is almost optimum for the enzyme activity. The results showed that an equilibrium of water concentration between in the reaction solution and in IMC was established and was helpful in keeping the IMC active. In the experiment with substrate solution containing 0.75% water (v/v), a white powder (probably Z-GlyPheLeuNH₂) was precipitated in the bed of the IMC, and flow stopped.

Continuous synthesis of Z-GlyPheLeuNH₂ from Z-Gly, PheOMe, and LeuNH₂

The course of continuous synthesis of Z-GlyPheLeuNH₂ from Z-Gly, PheOMe, and LeuNH₂ is shown in Fig. 6. For 9 days, the yield of Z-GlyPheLeuNH₂ with respect to Z-Gly was kept at more than 80%. This value is higher than values in other reports.^{18,19} The concentration of water in the effluent was scattered between 16.1 and 19.1 μ l/ml (Fig. 6). After continuous synthesis, the concentration of the water in the IMC was 167 μ l/g dry IMC,

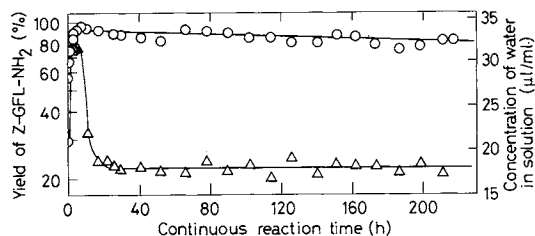


Fig. 6. Yield of Z-GlyPheLeuNH₂ from Z-Gly, PheOMe, and LeuNH₂ and the Concentration of the Water in Effluent during Continuous Reaction.

○, the yield of Z-GlyPheLeuNH₂; △, the concentration of the water in effluent. IMT, 2.96 μ mol-Z-FF-M/g-wet-IMT·min. IMC, 1.62 μ mol-Z-GFL-NH₂/g-wet-IMC·min.

which was slightly lower than the optimum value. The residual activity of IMT was 94%, and that of IMC was 100%.

The slightly low yields at the late stage of continuous synthesis would be due to some inactivation of IMT and to the concentration of the water in the IMC being somewhat low. The inactivation, caused by removal of calcium from the thermolysin, can be overcome by the addition of CaCl₂ to the substrate solution.¹⁷⁾ The lack of water could be surmounted by the checking of the concentration of the water in the substrate solution before the solution was fed into the second column as described in *Continuous synthesis of Z-GlyPheLeuNH₂*. If this is done, the yield would increase to nearly 100%.

The product, Z-GlyPheLeuNH₂, was completely dissolved in the ethyl acetate solution, and could be easily separated from Z-Gly, PheOMe, and LeuNH₂ by washing with acid or basic solution. We proposed a method for the continuous synthesis of a tripeptide from three amino acid derivatives in this paper. This method was efficient because of the use of transesterification and an organic solvent system, and the reaction proceeded safely at room temperature.

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