Functional Capsule Membranes. Part 29.[1] Thermolysin-immobilized Capsule Membranes as Bioreactors in the Synthesis of a Dipeptide (Precursor of Aspartame) in an Organic Solvent

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Thermolysin (TLN) was covalently immobilized onto a large, ultrathin nylon capsule membrane grafted with poly-[*p*-(aminomethyl)styrene] using glutaraldehyde. When the TLN-immobilized capsule containing a buffer solution (pH 7) in the inner aqueous phase was immersed in a chloroform solution of *N*-benzyloxycarbonyl-L-aspartic acid (Z-L-Asp) and L-phenylalanine methyl ester (L-PheOMe) with shaking at 40 °C, the dipeptide (Z-L-Asp-L-PheOMe) was produced efficiently in the outer chloroform solution. From the Lineweaver–Burk plot, condensation in the aqueous–organic solvent involves initial binding of Z-L-Asp to the enzyme to form the Z-L-Asp–enzyme complex and then attack by L-PheOMe on the complex as the rate-determining step to form the peptide linkage. The TLN–capsule system can be used repeatedly without denaturation of protein structures by organic solvents because the enzyme on the capsule membrane is protected by buffer solutions coming from the inside. The enzyme-immobilized capsule membrane is a new bioreactor in aqueous–organic heterophases.

Capsule membranes have been widely used for encapsulation of solids or liquids in order to protect, separate, and aid in storage, and for sustained release of medicaments.[2] Therefore, permeation or release behaviour through the capsule membrane has been extensively investigated.[3] For example, we have reported that permeability of the nylon capsule, whose porous membrane is corked with lipid bilayers[4] or surface-grafted with linear polymers,[5] is reversibly controlled by outside effects such as temperature, photoirradiation, pH, and electric field. Recently, we have developed a new application of polymer-grafted nylon capsule membranes for a reactor in liquid–liquid phases:[6,7] 'onium salt polymers grafted onto a capsule membrane act as a phase-transfer catalyst accelerating nucleophilic substitution for substrates in the inner organic phase and reagents in the outer aqueous phase.

In the past few years, much attention has been devoted to the protease-catalysed synthesis of peptides in an organic solvent using a reverse hydrolysis reaction.[8] For example, the dipeptide of *N*-benzyloxycarbonyl-L-aspartyl-L-phenylalanine methyl ester (Z-L-Asp-L-PheOMe) can be prepared by the reverse catalysis of thermolysin in an organic solvent containing a small amount of aqueous buffer solution. In this reaction, several methods have been tried to shift the equilibrium toward the condensation product in the reverse enzyme reaction: (i) the hydrophobic product is deposited and separated from the aqueous reaction mixture,[9] (ii) the water-in-oil emulsion system in which the enzyme is dissolved in the inner buffer solution and protected from the outer organic solvent,[8b,10] and (iii) the enzyme adsorbed physically into the aqueous ion-exchange resin is suspended in an organic solvent.[11,12] However, these methods have some disadvantages such as (i) the problem of the separation of an enzyme from the precipitated product, (ii) the problem of denaturation of the enzyme by organic solvents, and (iii) low reactivity because of the slow diffusion of substrates and products into the ion-exchange resin.

In this paper, we report that the thermolysin(TLN)-immobilized capsule membrane (TLN-capsule) is an effective bioreactor in the synthesis of Z-L-Asp-L-PheOMe, the precursor of the synthetic sweetener aspartame, from *N*-benzyloxycarbonyl-L-aspartic acid (Z-L-Asp) and L-phenylalanine methyl ester (L-PheOMe): the dipeptide is produced in the outer chloroform solution when the TLN-capsule containing a buffer solution in the inner aqueous core is immersed in the chloroform solution of Z-L-Asp and L-PheOMe at 40 °C. A schematic illustration is shown in Figure 1. The enzyme immobilized onto a capsule membrane always exists in the interface between the inner aqueous and the outer organic phase and is wet with buffer solutions excluded from the inner phase, and hence can retain high reactivity without denaturation in organic solvents.

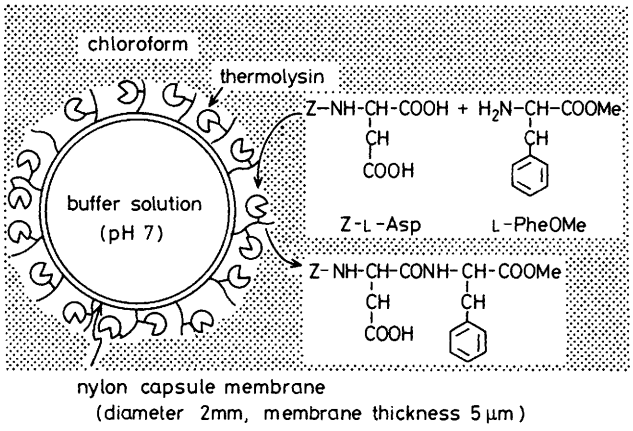

Figure 1. A schematic illustration of dipeptide synthesis catalysed by a thermolysin-immobilized capsule membrane in heterophases. The enzyme was covalently immobilized using glutaraldehyde with poly-[*p*-(aminomethyl)styrene] grafted onto the capsule membrane

Experimental

Materials.—*N*-Benzyloxycarbonylaspartic acid (Z-L-Asp and Z-D-Asp), phenylalanine methyl ester (L-PheOMe and D-PheOMe), and *N*-furylacryloylglycyl-L-leucinamide (Fua-Gly-LeuNH_2) were kindly donated by Ajinomoto Co. Ltd. (Tokyo), Dr. K. Oyama (Toyo Soda Ltd., Tokyo), and Professor S. Kunugi (Fukui University), respectively. Crystalline thermolysin (enzyme activity 8080 p.u. mg^{-1}) was purchased from Daiwa Kasei Ltd. and was recrystallized according to the literature procedure.[13] *p*-(Aminomethyl)styrene was prepared from *p*-(chloromethyl)styrene (Seimi Chemical, Yokohama) by a Gabriel synthesis, b.p. 54—56 °C at 0.2 mmHg. Analytical grades of other reagents were used without further purification.

Thermolysin-immobilized Capsule Membranes (TLN-capsule).—Large, porous, and ultrathin nylon-2,12 capsule membranes (diameter 2.5 mm; membrane thickness 5 μm; dry weight, 25 ± 2 μg) were prepared according to the method described previously.[4–7] The nylon capsules were surface-grafted with *p*-(aminomethyl)styrene in an degassed, acidic aqueous solution at 40 °C by photopolymerization with irradiation by an Ushio 500 W super-high-pressure lamp for 2 h. The capsules were washed with an excess of methanol and acidic water to remove unchanged monomer and non-grafted polymer, and dialysed against 0.2M-NaCl aqueous solution. After complete hydrolysis of polymer-grafted nylon capsules in strong acid conditions at 60 °C for 1 day, it was confirmed from weighing capsules before and after polymerization, elemental analysis, and gel-permeation chromatography of the residual graft-polymer that 200—210 μg (1.5×10^{-6} unit mol) of linear poly-(*p*-aminomethyl)styrene were grafted per capsule (average degree of polymerization 10^3).

Immobilization of thermolysin onto the capsule membrane was carried out as follows. Thermolysin (0.15 g) was dissolved in ice-cold 0.02M-2-(*N*-morpholino)ethanesulphonate buffer (5 ml; pH 6.0) containing 5M-NaBr and 17mM-$CaCl_2$. Addition of the high concentration of NaBr increases the solubility of thermolysin more than 50-fold.[12] To this enzyme solution 200 pieces of capsule membrane grafted with poly-(*p*-aminomethyl)-styrene and 50% glutaraldehyde (0.25 g) were added and stirred at 4 °C for 12 h. The capsules were washed successively and dialysed against 0.2M-buffer (pH 7.0) containing 0.01M-$CaCl_2$. The amount of immobilized thermolysin was estimated to be 19 ± 1 μg [(5.8 ± 0.3) × 10^{-10} mol] per capsule from weighing capsules before and after immobilization.

Measurements of Enzyme Activity in Aqueous Solutions.—Hydrolytic activities of free enzyme and thermolysin-immobilized capsule membrane were determined against a chromophoric substrate, *N*-furylacryoylglycyl-L-leucinamide (Fua-Gly-$LeuNH_2$) in aqueous solution.[14] Enzyme reactions were followed by a u.v.–visible spectrophotometer (Shimadzu W-240, Shimadzu Seisakusho Ltd., Kyoto) at 322 nm where the difference spectrum is a maximum for the substrate and products (Fua-Gly and $LeuNH_2$) (Δε 2 500 M^{-1} cm^{-1}). The hydrolysis reactions were studied in 3 ml of 0.1M-buffer solutions (pH 4—9, 0.01M-$CaCl_2$) containing free TLN or a TLN-capsule after the addition of dimethyl sulphoxide solution (50 μl) of substrates at 40 °C.

Peptide Synthesis in Organic Solvent.—The condensation reaction catalysed by the TLN-capsule was carried out as follows. Ten pieces of the capsule containing 0.2M-morpholino-ethanesulphonate buffer (pH 7; 10 μl) in the inner aqueous phase were shaken in a chloroform solution (1 ml) of Z-L-Asp 1—8mM) and excess of L-PheOMe (30—400mM) at 40 °C. In the case of the free TLN, the buffer solution (100 μl) containing the enzyme was suspended in a chloroform solution (1 ml) of the substrates with shaking. Within the prescribed time interval, the production of the dipeptide (Z-L-Asp-L-PheOMe) and the reduction of substrates (Z-L-Asp and excess of L-PheOMe) in the outer chloroform solution were monitored by h.p.l.c. [instrument, Toyo Soda HLC-803C with u.v. detector (at 258 nm); column, TSK gel ODS-120T; eluant, phosphate buffer (pH 2.5)—acetonitrile 6:4]. Identification of Z-L-Asp-L-PheOMe was by comparison of the h.p.l.c. retention time and i.r. spectrum with those of an authentic sample.

Results and Discussion

Activity of Immobilized Enzyme in Aqueous Solution.—In order to determine the effect of the immobilization of thermolysin (TLN) onto the capsule membrane, the hydrolytic activity of the free TLN and the TLN-capsule was first studied using *N*-furylacryloylglycyl-L-leucinamide (Fua-Gly-$LeuNH_2$) as substrate in 0.1M-Tris buffer solution containing 0.01M-$CaCl_2$.[14] The hydrolytic activity of the free TLN (1.9×10^{-7}M)

Table 1. Kinetic parameters of thermolysin-catalysed hydrolysis and peptide synthesis at 40 °C

	Hydrolysis of Fua-Gly-$LeuNH_2$[a]			Synthesis of Z-L-Asp-L-PheOMe in chloroform	
Enzyme	$10^4 K_m$/M	k_{cat}/s^{-1}	$k_{cat}K_m^{-1}$/dm^3 mol^{-1} s^{-1}	$10^3 K$/ mol dm^{-3}	k_2/dm^3 mol^{-1} s^{-1}
Free TLN	2.94	5.55	18 900	6.67	0.147
TLN-capsule	5.76	1.15	2 000	9.08	0.129

[a] 0.1M Hepes buffer containing 0.01M-$CaCl_2$ (pH 6.6).

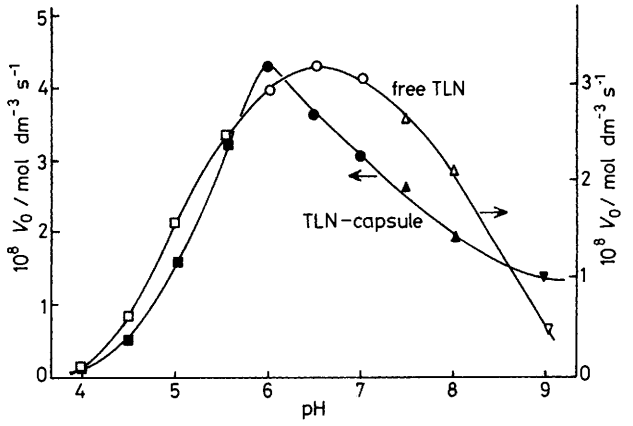

Figure 2. pH Dependences of hydrolytic activity of thermolysin for Fua-Gly-$LeuNH_2$ substrates at 40 °C. [free TLN] 1.9×10^{-7}M, [TLN-capsule] capsule immobilized with 5.8×10^{-10} mol TLN, [substrate] 1.30×10^{-4}M, 0.2M-buffer solution containing 0.01M-$CaCl_2$: (□,■) acetate, (○,●) morpholinoethanesulphonate, (△,▲) Tris, and (▽,▼) glycine buffers

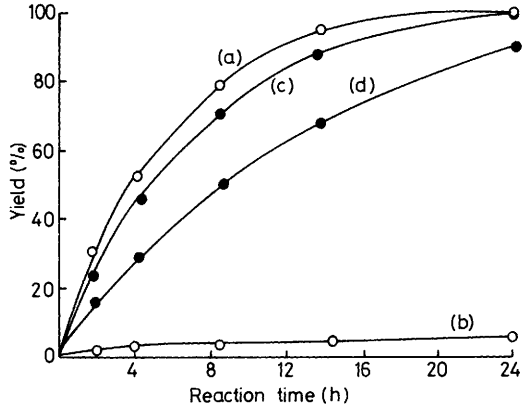

Figure 3. Peptide synthesis catalysed by thermolysin at 40 °C [Z-L-Asp] 6.0×10^{-3}M, [L-PheOMe] 0.3M, [free TLN] 5.8×10^{-9} mol in 100 μl of buffer solution, [TLN-capsule], 10 pieces of the capsule immobilized with 5.8×10^{-10} mol of TLN. (a) Free TLN in buffer–chloroform suspension, (b) free TLN in buffer solution (pH 7), (c) TLN-capsule in chloroform, (d) broken pieces of TLN-capsule in chloroform

Table 2. Peptide synthesis catalysed by thermolysin at 45 °C[a]

Enzyme	Substrates: Z-L-Asp	Substrates: L-PheOMe	$10^8 v_0$/mol dm^{-3} s^{-1}	Yield after 24 h (%)
Free TLN in buffer solution (pH 7)	Both in buffer solution		1.3	4.0
Free TLN in buffer–chloroform	Both in chloroform		11	100
Broken pieces of TLN-capsule in buffer–chloroform	Both in chloroform		6.2	90
TLN-capsule containing buffer solution suspended in chloroform	Both in chloroform		9.5	100
	In buffer solution	In chloroform	3.7	100
	In chloroform	In buffer solution[b]	0.5	50

[a] [Z-L-Asp] 6.0 × 10^{-3}M, [L-PheOMe] 0.3M, free TLN 5.8 × 10^{-9} mol in 100 μl of buffer solution, TLN-capsule 10 pieces of the capsule immobilized with 5.8 × 10^{-10} mol of TLN. [b] L-PheOMe was dissolved in buffer solution as an HCl salt.

and the TLN-capsule (5.8 × 10^{-10} mol per capsule) was studied in buffer solution (3 ml) for the various concentrations of Fua-Gly-LeuNH$_2$ [(6.5—26) × 10^{-5}M] at 40 °C. From Lineweaver–Burk plots, Michaelis constants (K_m), the rate constant (k_{cat}), and the second-order rate constants (k_{cat}/K_m) of the free TLN and the TLN-capsule were obtained and summarized in Table 1.

For a TLN-capsule the k_{cat} value decreased and the K_m value increased by a factor of 2—5 compared with the corresponding values for free TLN. Since most of the poly-[*p*-(aminomethyl)-styrene] grafted onto the capsule membrane is protonated at pH 6.6 and the membrane surface near the enzyme becomes hydrophilic, the relatively hydrophobic substrate (Fua-Gly-LeuNH$_2$) has difficulty in forming a complex with the enzyme and the K_m value of the TLN-capsule may increase relative to a free enzyme. The decrease in k_{cat} value for the immobilized thermolysin is explained by the slight denaturation of the protein resulting from covalent immobilization onto the nylon capsule membrane. As a result, the second-order rate constants (k_{cat}/K_m), the apparent activity of the enzyme for the TLN-capsule, was 9.5-times smaller than that for the free enzyme. The hydrolytic activity of thermolysin on the capsule did not alter for 2—3 months when stored in buffer solutions at 5 °C. In general, when the enzyme is immobilized covalently on the support, the stability is increased but the activity is decreased compared with free enzyme.

The pH dependence of the initial velocity (v_0) for the TLN-capsule and free TLN are shown in Figure 2. The optimum pH showing the highest activity of the TLN-capsule is in a lower region (pH 6) and shows a sharper pH–rate profile than that of free TLN, probably because of the acidic, protonated poly-[*p*-(aminomethyl)styrene] graft-polymers near the enzyme.

Peptide Synthesis in Organic Solvents.—Protease is known to synthesize peptides using reverse hydrolysis in an organic solvent by shifting the equilibria toward the condensation products. The synthesis of Z-L-Asp-L-PheOMe was carried out as follows. Ten pieces of the TLN-capsule containing buffer solution (10 μl) in the inner aqueous core was immersed in chloroform solution (1 ml) of substrates (Z-L-Asp and L-PheOMe). When free TLN or broken pieces of the TLN-capsule were employed, the buffer solution (100 μl) of TLN was suspended in chloroform (1 ml). The reactions were performed at 40 °C with vigorous shaking. Figure 3 shows typical time courses of the production of Z-L-Asp-L-PheOMe by various reaction methods. The initial velocity (v_0) obtained from the initial slope in Figure 3 and the yield after 24 h are summarized in Table 2. When TLN-immobilized capsule membranes containing buffer solution in the inner aqueous phase were shaken in a chloroform solution of the two substrates, the yield of the dipeptide in the outer chloroform solution obtained by h.p.l.c. increased proportionally with time and reached 100% conversion after 24 h (v_0 9.5 × 10^{-8} mol dm^{-3} s^{-1}). The reactivity of the TLN-capsule was the same as that of free TLN dissolved in buffer solution and suspended in chloroform (v_0 11 × 10^{-8} mol dm^{-3} s^{-1}). Although the hydrolytic acitivity of TLN was decreased by a factor of 9.4 by immobilization onto the capsule membrane (see Table 1), the activity in the synthesis of the dipeptide seemed not to be decreased by immobilization. When the TLN-immobilized capsule membrane was broken into small pieces and suspended in biphasic aqueous–organic solutions, the initial velocity decreased to two-thirds that of a TLN-capsule containing a buffer solution in the core. This means that the enzyme is at the interface between the inner buffer solution and the outer chloroform solution in order to synthesize efficiently the dipeptide from substrates solubilized in the chloroform solution. In aqueous solution, free TLN, as well as the TLN-capsule, showed very limited activity for peptide synthesis because it is difficult to shift the equilibrium of the enzyme reaction towards the condensation product and the dipeptide (if produced) is easily hydrolysed in aqueous solution.

The nature of the organic solvent might affect the rate and yield of the condensation reaction. Four organic solvents, toluene, n-hexane, carbon tetrachloride, and ethyl acetate, other than chloroform were tested. Z-L-Asp and L-PheOMe (6mM and 300mM, respectively) were not soluble in toluene, n-hexane, or carbon tetrachloride saturated with buffer solution. When ethyl acetate saturated with buffer solution was employed for the outer organic solvent, the inner aqueous buffer solution of the TLN-capsule leaked easily to the outer phase and the capsule shrank within 1 h. The initial velocity and the yield after 24 h were very small (v_0 2.4 × 10^{-8} mol dm^{-3} s^{-1} and 15%, respectively) compared with those in chloroform (see Table 2). The activity of the TLN-capsule in ethyl acetate decreased because thermolysin on the capsule membrane was surrounded by buffer solutions leaking from the inside and could not approach the substrates in the organic solvent.

Reaction Mechanism for Dipeptide Synthesis.—Thermolysin is a widely studied neutral metalloendoproteinase, but its mode in the reverse reaction in organic solvents is not yet well understood.

The effect of the enzyme concentration of the TLN-capsule (number of capsules) on the initial velocity of the synthesis of Z-L-Asp-L-PheOMe is shown in Figure 4. The initial velocity increased linearly upon increasing the number of TLN-capsules. Figure 5 shows the effect of the concentration of L-PheOMe in chloroform solution on the initial velocity and on the yield of dipeptide after 24 h when 10 pieces of the TLN-capsule were employed at 40 °C. The initial velocity (v_0) linearly increased with the concentration of L-PheOMe in the range 0.15—0.35M with no indication of rate saturation. It should be noted that two plots of initial velocity deviated from the linear relation and

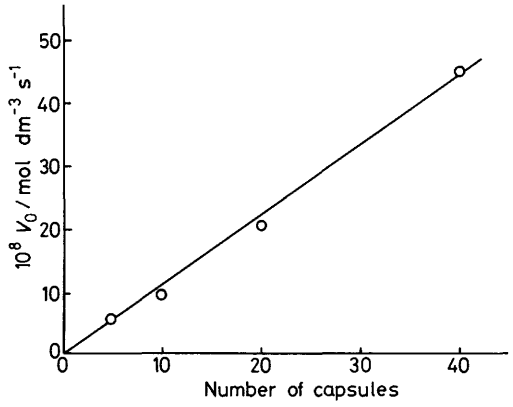

Figure 4. Effect of enzyme concentration (number of TLN-capsules) on the initial velocity of peptide synthesis at 40 °C. [Z-L-Asp] 6.0 × 10^{-3}M, [L-PheOMe] 0.3M, TLN immobilized, 5.8 × 10^{-10} mol per capsule

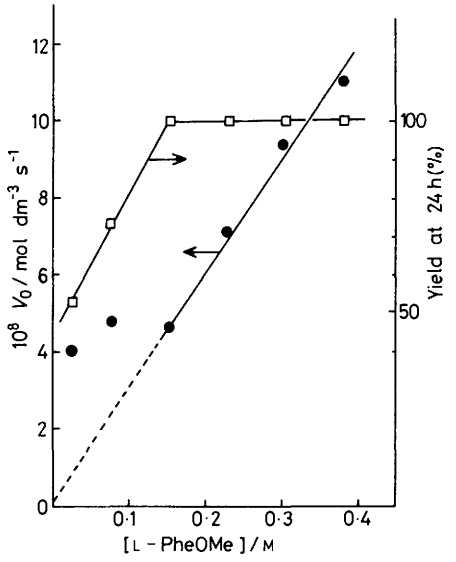

Figure 5. Effect of concentration of L-PheOMe on initial velocity and on yield of dipeptide at 24 h catalysed by TLN-capsule at 40 °C. [Z-L-Asp] 6.0 × 10^{-3}M, TLN 5.8 × 10^{-9} mol (10 pieces of TLN-capsule)

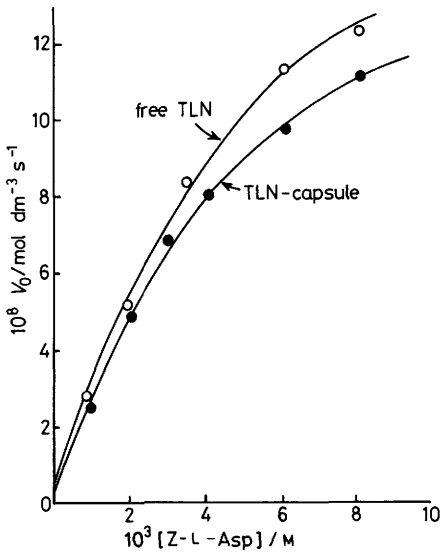

Figure 6. Effect of the concentration of Z-L-Asp on initial velocity of peptide synthesis catalysed by thermolysin at 40 °C. [L-PheOMe] 0.3M, free TLN 5.8 × 10^{-9} mol in 100 μl of buffer solution, TLN-capsule, 10 pieces of capsule immobilized with 5.8 × 10^{-10} mol of TLN

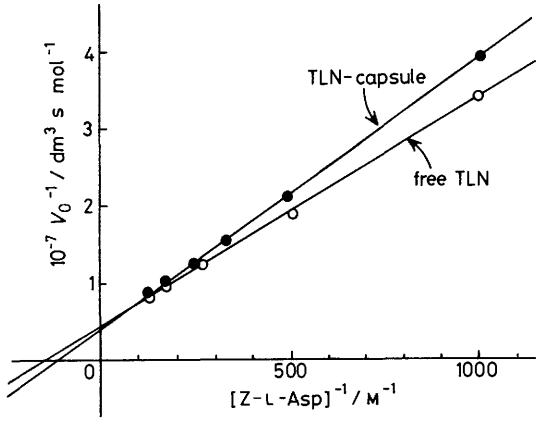

Figure 7. Lineweaver–Burk plots for Figure 6

the yield was very low in the range [L-PheOMe] 0.02—0.07M. For a relatively low concentration of L-PheOMe, the pH of the inner buffer solution was slowly decreased from 7 to 4 because of the partition of the other substrate Z-L-Asp containing free carboxylic acid into the inner buffer solution. The pH of the inner buffer solution decreases from 7 to 6 in the inital phase of the reaction and the initial velocity (v_0) increases as the optimum pH is 6 (Figure 2). However, the yield after 24 h decreased since the pH value of the inner buffer solution changed to 4 in the latter phase of the reaction.

When racemic DL-PheOMe was employed instead of the L-isomer, the slope of the initial velocity–[DL-PheOMe] plot is half that of Figure 5. Thus the rate with L-PheOMe is not affected by addition of the D-isomer.

A plot of [Z-L-Asp] against the initial velocity showed rate saturation, typical Michaelis–Menten behaviour, as shown in Figure 6, when both the TLN-capsule and free TLN were employed in chloroform. Rate retardation was observed when Z-D-Asp coexisted with the L-isomer, indicating that Z-D-Asp acts as an inhibitor in the peptide synthesis. Lineweaver–Burk plots of $1/v_0$ against 1/[Z-L-Asp] for the TLN-capsule and free TLN show linear correlations as shown in Figure 7.

The observations that the rate increases linearly with the concentration of L-PheOMe and thermolysin and that that Lineweaver–Burk plot for Z-L-Asp yields a straight line resemble the kinetic results reported by Oyama *et al.* for a dipeptide synthesis catalysed by free TLN in aqueous media[15] and reported by Chance for the reaction of horseradish peroxidase.[16] These facts suggest that the condensation of Z-L-Asp with L-PheOMe catalysed by the TLN-capsule in an organic solvent occurs basically *via* the reaction mechanism (1) and (2).[15,16] This also applies to free TLN in chloroform.

$$\text{Z-L-Asp} + \text{enzyme} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{Z-L-Asp–enzyme} \quad (1)$$

$$\text{Z-L-Asp–enzyme} + \text{L-PheOMe} \xrightarrow{k_2} \text{Z-L-Asp-L-PheOMe} + \text{enzyme} \quad (2)$$

Thus, condensation involves initial binding of Z-L-Asp to the enzyme to form the Z-L-Asp–enzyme complex. L-PheOMe then

attacks the complex as the rate-determining step to form the dipeptide. Although the exact nature of the Z-L-Asp–enzyme complex cannot be described at this moment because of the limited information available, Oyama *et al.* have proposed that the reaction proceeds *via* attack of the amino group of L-PheOMe on the α-carboxy group of Z-L-Asp to produce the peptide bond directly (the reverse of general base-catalysed hydrolysis of a peptide bond) in aqueous media.[15]

The rate of the dipeptide synthesis can be expressed by equations (3) and (4) where $K = k_{-1}/k_{+1}$. The K and k_2 values

$$v_o = \frac{k_2[E_o][\text{Z-L-Asp}][\text{L-PheOMe}]}{K + [\text{Z-L-Asp}]} \quad (3)$$

$$\frac{1}{v_o} = \frac{K}{k_2[E_o][\text{L-PheOMe}]} \cdot \frac{1}{[\text{Z-L-Asp}]} + \frac{1}{k_2[E_o][\text{L-PheOMe}]} \quad (4)$$

for the TLN-capsule and free TLN were obtained from the slope and the intercept of Figure 7, respectively, and are summarized in Table 1. Both K and k_2 values for the TLN-capsule in chloroform gave similar values to those for the free TLN in biphasic aqueous–chloroform solution, although the hydrolytic activity of the TLN-capsule was much decreased compared with free TLN in buffer solution. Thus, the activity of the condensation catalysed by thermolysin in chloroform was not apparently reduced by immobilization onto the capsule membrane. Although the activity of thermolysin may be reduced by covalent immobilization onto the capsule membrane due to the denaturation of the protein, the enzyme on the capsule membrane is always wet by the soaked buffer solution from the inside and is protected from the outer chloroform solution. On the other hand, free thermolysin solubilized in the buffer-in-chloroform emulsion may be easily denatured by the organic solvent and the reactivity is reduced compared with that of TLN on the capsule membrane. As a result, the TLN-capsule showed similar reactivity to free thermolysin in chloroform.

Repeat Usage of TLN-capsules.—In order to permit the continuous production of dipeptides and the long-term stability of the TLN-capsule, condensation was repeated as follows. Ten pieces of the TLN-capsule were immersed in a chloroform solution of the substrates ([Z-L-Asp] 6.0×10^{-3}M, [L-PheOMe] 0.3M) for 24 h, then picked up, and immersed again in a new chloroform solution of the substrates. This procedure was repeated sevaral times. In the case of free TLN, the buffer solution of enzyme was dispersed in a chloroform solution of the substrates with shaking for 24 h, and then Z-L-Asp (6.0×10^{-3}M) was added in order to continue the synthesis of the dipeptide. The products accumulated in chloroform were hardly hydrolysed upon repeated reaction and did not cause inhibition of formation of the product. The yields of Z-L-Asp-L-PheOMe in chloroform solution for each 24 h period were plotted against the repeated runs (Figure 8).

In the case of free TLN, the yield was much decreased in the repeated runs and dipeptide was hardly produced by the third run. On the other hand, the yield barely decreased for each run and an 80% yield was obtained even after nine recycles when the TLN-capsule was employed. When broken pieces of the TLN-capsule were employed in the aqueous–organic phase, the yield decreased gradually with repeated reaction and the dipeptide was barely produced by the fifth run. These results indicate that free TLN or TLN bound to membrane fragments are denatured by chloroform in biphasic aqueous–organic systems and cannot be reused. In contrast, TLN immobilized on a capsule is protected from organic solvent by the soaked buffer solution on the inside and can be used repeatedly without reducing the activity. Thus, although the initial rate of peptide synthesis by the TLN-capsule is of the same order as that by free TLN in organic solvent, the TLN-capsule can be reused for several runs.

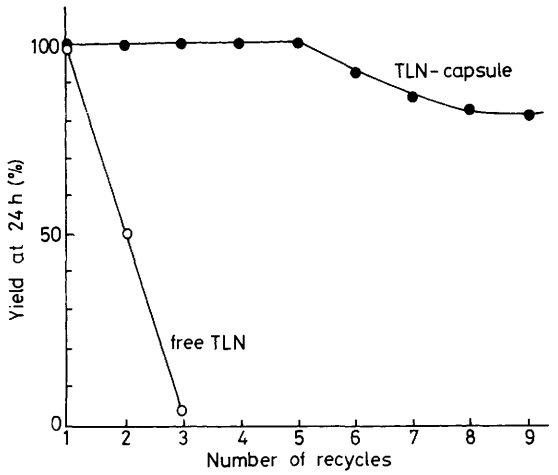

Figure 8. Repeated usage of TLN-capsule in peptide synthesis at 40 °C. [Z-L-Asp] 6.0×10^{-3}M, [L-PheOMe] 0.3M TLN 5.8×10^{-9} mol (ten pieces of TLN-capsule)

Variation of Substrate Feeds.—In the experiments above, both substrates were fed from the outer chloroform solution. We also tried to feed substrates from the inner aqueous phase of the TLN-capsule. (i) Z-L-Asp and L-PheOMe were dissolved in the inner buffer and the outer chloroform solution, respectively and (ii) Z-L-Asp was dissolved in the outer organic phase and L-PheOMe was dissolved as a HCl salt in the inner buffer solution. The initial velocity and the yield after 24 h of both these cases are shown in Table 2. When either Z-L-Asp or L-PheOMe was stored in the inner buffer solution of the TLN-capsule, the initial velocity and yield were decreased compared with the results when both substrates were dissolved in the chloroform solution. Thus, Z-L-Asp dissolved in the inner buffer solution was not much transferred to the chloroform phase because of the low partition coefficient of Z-L-Asp for the organic solvent. When the L-PheOMe, HCl salt was dissolved in the inner buffer solution, the pH value of the inner phase decreased with time and reached 4 after 24 h, because L-PheOMe, HCl was transferred to the organic solvent as neutral L-PheOMe, and HCl remained in the inner aqueous phase. These results show that both substrates should be fed from the outer organic phase in order to obtain a high initial rate and high yield.

Conclusions

When a thermolysin-immobilized capsule membrane containing buffer solution in the inner phase was shaken in a chloroform solution of Z-L-Asp and L-PheOMe, the dipeptide was produced efficiently in the outer chloroform solution. Thermolysin could be used continuously without denaturation of the protein by immobilization on the capsule membrane.

The enzyme-immobilized capsule membrane has the following features as a new type of bioreactor in heterophases. (1) The enzyme on the capsule membrane exists near the interface between the inner aqueous and the outer organic solution, and is protected from the organic solvent by the soaked buffer solution from the inside. (2) The enzyme-immobilized capsule can be easily separated from substrates and products in the outer organic phase, and can be used repeatedly without de-

naturation by organic solvents. (3) There is no induction period for the reaction caused by swelling of supports, which has frequently been observed in gel-supported enzymes.

The enzyme-immobilized capsule membrane provides a new bioreactor for the synthesis of various biologically active compounds in heterogeneous reactions. Further applications such as lipase- and carboxypeptidase-immobilized capsule membranes are in progress in this laboratory.

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