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Communication to the Editor

Synthesis of Aspartame Precursor with an Immobilized Thermolysin in Mixed Organic Solvents

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N-(benzyloxycarbonyl)-L-aspartyl-L-phenylalanine methyl ester, a precursor of the synthetic sweetener, aspartame, was synthesized from *N*-(benzyloxycarbonyl)-L-aspartic acid and L-phenylalanine methyl ester with an immobilized thermolysin (EC 3.4.24.4) in the mixed organic solvent system of *tert*-amyl alcohol and ethyl acetate. A mixed solvent consisting of *tert*-amyl alcohol and ethyl acetate at a ratio of 33:67 (v/v) was found to be the most suitable with respect to synthetic rate and stability of the immobilized enzyme. The reaction continued to proceed quite successfully in a column reactor at 40°C and at a space velocity of 3.6 h^{-1} with a yield of 99%, using 40 m*M* Z-Asp and 200 m*M* PheOMe dissolved in the mixed solvent as the substrate. © 1995 John Wiley & Sons, Inc.

Key words: enzymatic synthesis • thermolysin • immobilized enzyme • aspartame precursor • organic solvent

INTRODUCTION

At present, a precursor of the synthetic sweetner aspartame is produced commercially, mostly by chemical syntheses and sometimes enzymatic methods, using the free enzyme.[5,7] Generally, chemical synthesis is not specific, and thus the yield is not high enough with an appreciable amount of byproducts.[5] The enzymatic synthesis is in principle advantageous over the chemical method: The reaction occurs specifically under a mild condition to give a high yield. However, the way to produce an aspartame precursor in a bioreactor system using an immobilized enzyme has not yet been established, but would enable the reuse of the enzyme and decrease the burden for downstream processing.

We have been engaged in the synthesis of *N*-(benzyloxycarbonyl)-L-aspartyl-L-phenylalanine methyl ester (Z-AspPheOMe), the aspartame precursor from L-phenylalanine methyl ester (PheOMe) and *N*-(benzyloxycarbonyl)-L-aspartic acid (Z-Asp) with an immobilized thermolysin. We have clarified the kinetics of the synthesis of the aspartame precursor[4] and various factors on the performance of the immobilized enzyme.[1–3,6] Previously,[1,2] we reported the effect of organic solvents on the behavior of the immobilized enzyme. Particularly, we elucidated in detail the kinetics and stability of the immobilized enzyme in ethyl acetate (EA)[1] and in *tert*-amyl alcohol (TA).[2] In TA, the immobilized enzyme is quite stable. In EA, it is stable when the PheOMe concentration is high enough in comparison with the Z-Asp concentration. However, it becomes labile in the high Z-Asp concentration regions. The synthetic rate in TA is higher than that in EA when the concentrations of Z-Asp and PheOMe are similarly high. However, as the PheOMe concentration increases relative to the Z-Asp concentration, the synthetic rate becomes higher in EA than in TA. Thus, in this study, we synthesized Z-AspPheOMe in the mixed solvent system consisting of TA and EA to have a continuous reaction in an immobilized enzyme column reactor with a high yield and stability.

MATERIALS AND METHODS

Materials

Crystalline thermolysin (1 × crystallized), donated from Daiwa Kasei K.K. (Osaka, Japan), was used without further purification. Z-Asp and PheOMe · HCl were purchased from Peptide Institute Inc. (Minoh, Japan) and Kokusan Chemical Works (Tokyo), respectively. PheOMe was prepared from PheOMe · HCl as described previously.[3] As a support for enzyme immobilization, Amberlite XAD-7 (Japan Organo Co., Tokyo) sieved at 24 and 42 meshes was used. Glutaraldehyde was obtained as a 25% aqueous solution from Nacalai Tesque (Kyoto, Japan). EA, TA, and 2-(*N*-morpholino)-ethane sulfonic acid (MES) were obtained from Wako Pure Chemical Industries (Osaka, Japan), and Dojin Laboratories (Kumamoto, Japan), respectively. All other reagents were purchased either from Nacalai Tesque or Wako.

Preparation of Immobilized Enzyme

Thermolysin was immobilized onto Amberlite XAD-7 by the same method as reported previously.[3] Before use the

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immobilized enzyme was equilibrated with 0.05 *M* MES-NaOH buffer, pH 6.0, containing 5 m*M* $CaCl_2$ (hereafter described as assay buffer).

Courses of Synthetic Reaction

Courses of the synthetic reaction were taken at 40°C at the immobilized enzyme concentration of 0.02 g/mL (wet weight), using 10 mL of EA, TA, and mixed solvents of TA and EA at various volume ratios, containing 40 to 200 m*M* Z-Asp and 200 m*M* PheOMe as the substrate. In the reaction in EA, the reaction mixture contained 3.5% assay buffer,[1] and in TA and mixed solvents the buffer content was 4%.[2] At appropriate times during the course of the reaction, a 0.2-mL portion of reaction mixture was taken, and analyzed with HPLC (LC-6A, Shimadzu Corp., Kyoto) equipped with an ODS column (STR ODS-H, Shimadzu) at 254 nm.[1,2]

Stability Test of Immobilized Enzyme

The immobilized enzyme was incubated at 40° to 70°C for 5 h in either EA, TA, or the mixed solvents of TA and EA at various volume ratios, containing 3.5% (in EA) or 4% assay buffer either with or without 200 m*M* Z-Asp and 200 m*M* D-phenylalanine methyl ester (D.C.-PheOMe). D.C.-PheOMe was used in place of L-phenylalanine methyl ester to prevent the occurrence of a reaction during incubation. After incubation, the immobilized enzyme was equilibrated with 0.1 *M* Tris-HCl buffer, pH 7.5, containing 5 m*M* $CaCl_2$ and stored overnight in the same buffer. Then, the remaining activity was measured.[3]

Continuous Reaction

Synthesis of Z-AspPheOMe was allowed to continue at 40°C. Forty millimolar Z-Asp and 200 m*M* PheOMe dissolved in mixed solvents of TA and EA at ratios of 33:67 (v/v) and 25:75 (v/v) and in EA containing 4% and 3.5% assay buffer were continuously fed into a glass column reactor (2.2 × 15 cm) packed with 12.5, 7.5, and 7.5 g (wet) of immobilized enzyme at flow rates of 36, 30, and 30 mL/h, respectively. Here, the space velocity based on the volume of the immobilized enzyme was calculated to be 3.6, 4.9, and 4.9 h^{-1}, respectively. The effluent from the column was taken at appropriate times and assayed by HPLC. At the end of the continuous reaction, the whole immobilized enzyme was taken from the column, and the remaining activity was assayed.[3]

RESULTS

Courses of Synthesis of Z-AspPheOMe

Figure 1 shows the course of Z-AspPheOMe synthesis, using 40 to 200 m*M* Z-Asp and 200 m*M* PheOMe as substrate, in EA, TA, and mixed solvents of TA and EA at ratios of 33:67 (v/v) and 25:75 (v/v), respectively. When the Z-Asp concentration was 200 m*M* (Fig. 1a) the synthetic rate was considerably low in all the solvents. Particularly, in EA the yield was only 5% after 51 h of incubation. As the volume fraction of TA increased, the reaction rate increased, and it was the highest in TA (100%): the yield was still 45% after 51 h of incubation. Thus, even in TA the synthetic rate is still too low to have a continuous reaction. When the Z-Asp concentration was 120 m*M* (Fig. 1b), the synthetic rate as well as the change in conversion increased compared with the results shown in Figure 1a. At the Z-Asp concentration of 80 m*M* (Fig. 1c), the synthetic rate was the highest in the mixed solvent at a ratio of 33:67 (v/v), and the lowest in TA. At the Z-Asp concentration of 40 m*M* (Fig 1d), the synthetic rate increased further in all the solvents. The increase was more pronounced in EA and mixed solvents at the ratios of 33:67 (v/v) and 25:75 (v/v) than in TA. In EA and mixed solvents at the ratios of 33:67 (v/v) and 25:75 (v/v), the yield reached higher than 95% after incubation for about 6 h. To examine the effect of the organic solvent composition, the initial synthetic rate was measured as a function of the volume fraction of EA, using 40 m*M* Z-Asp and 200 m*M* PheOMe as the substrate (Fig. 2). At volume fractions of EA lower than around 60%, the initial synthetic rate was low, and at that exceeding 60% it increased sharply. The maximum initial synthetic rate was obtained at EA volume fractions of 75% to 100%.

In Table I, the relative activities remaining after 51 h of reaction shown in Figure 1 are summarized. In EA, the remaining activity sharply decreased as the Z-Asp concentration increased in the presence of 200 m*M* PheOMe. The relative remaining activity was 83%, 78%, 32%, and 0.6% for Z-Asp concentrations of 40, 80, 120, and 200 mM. On the other hand, in TA, the relative remaining activity was 100% for all the concentrations of Z-Asp. The behaviors of the activity and stability in EA and TA could be explained by the different state of water around the enzyme molecules immobilized in the support.[1,2] In the mixed solvents of TA and EA, the stability of the immobilized enzyme was enhanced with the increase in the volume fraction of TA and with the decrease in the Z-Asp concentration. At 40 m*M* Z-Asp and 200 m*M* PheOMe, the relative remaining activity for the reaction in the mixed solvent at a ratio of 33:67 (v/v) was substantial at 100% except in the case of the Z-Asp concentration of 200 m*M*.

Stability of the Immobilized Enzyme

The immobilized enzyme was incubated for 5 h in EA, TA, and mixed solvents with and without the substrate (200 m*M* Z-Asp and 200 m*M* D.C.-PheOMe) to elucidate the stability of the immobilized enzyme in detail. Figure 3a shows the effect of organic solvent composition on the relative remaining activity measured after a 5-h incubation at different temperatures in the absence of the substrate. The immobilized enzyme was much more stable in TA than in EA as reported previously.[2] In the mixed solvents, the immobi-

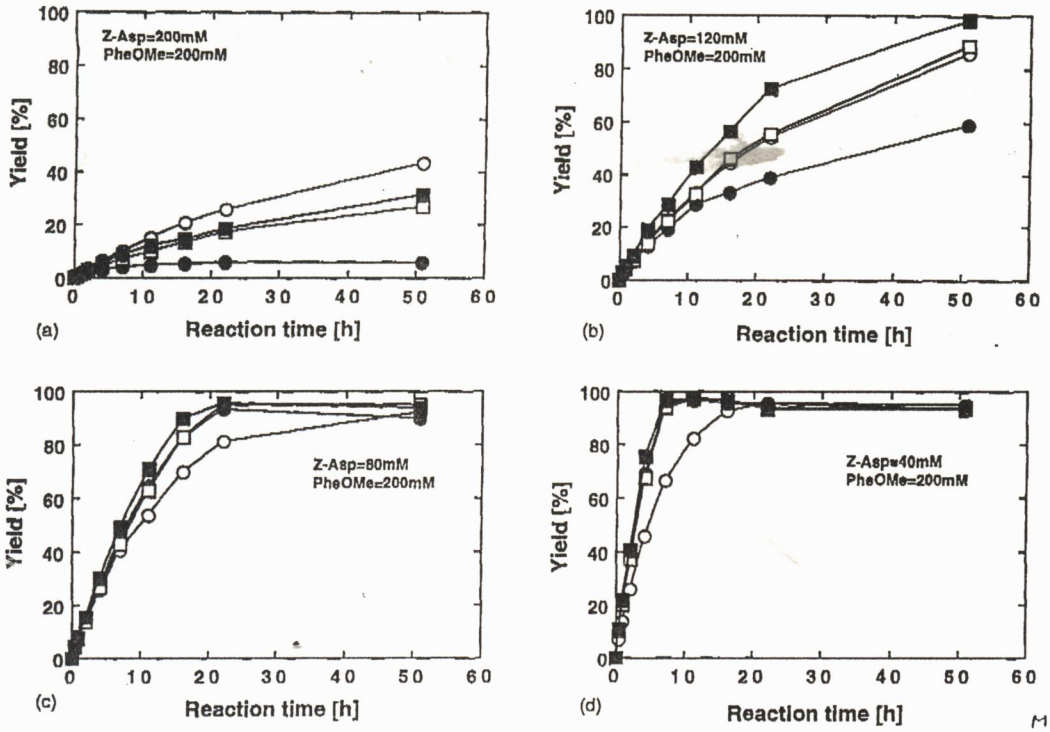

Figure 1. Synthesis of Z-AspPheOMe at 40°C. The initial concentration of Z-Asp was 200 m*M* (a), 120 m*M* (b), 80 m*M* (c), and 40 m*M* (d), in the presence of 200 m*M* PheOMe. The concentration of immobilized enzyme was 0.02 g/mL. The organic solvent used was EA (●), mixed solvents of TA and EA at ratios of 25:75 (v/v) (■) and 33:67 (v/v) (□), and TA (○), respectively.

lized enzyme was more stable with decreasing the volume fraction of EA (increasing the volume fraction of TA) as shown in Figure 3a. Up to 50°C, the immobilized enzyme was fully stable in the organic solvents with all the volume fractions of EA. At 70°C, the immobilized enzyme was stable for the volume fractions of EA up to 67% (volume fraction of TA = 33%). Figure 3b shows the relationship between the relative remaining activity and volume fraction of EA after a 5-h incubation in the presence of the substrate. The stability of the immobilized enzyme decreased, compared with that in the absence of the substrate. However, the immobilized enzyme was more stable at a smaller volume fraction of EA (increasing the volume fraction of TA) than in the absence of substrate (Fig. 3a). At 50°C the immobilized enzyme was stable up to the volume fractions of EA of 60%. At a higher incubation temperature, the stability of the immobilized enzyme decreased, and the extent of decrease was much more pronounced with increasing volume fraction of EA.

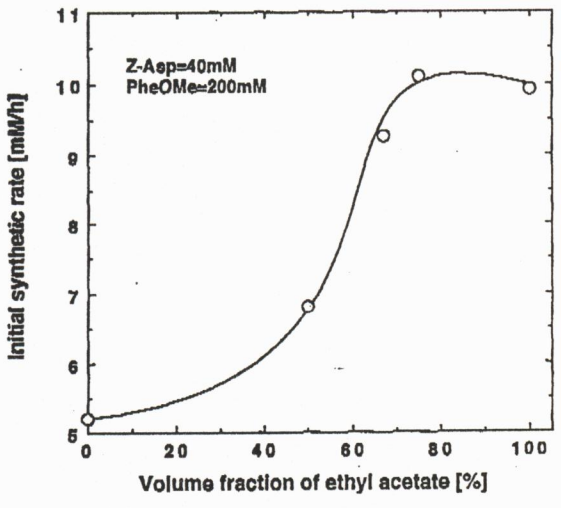

Figure 2. Effect of volume fraction of EA in the mixed solvents on the initial synthetic rate at 40°C. The concentrations of Z-Asp and PheOMe were 40 m*M* and 200 m*M*, respectively. The immobilized enzyme concentration was 0.02 g/mL.

Continuous Synthesis of Z-AspPheOMe

Based on the results shown in Figures 1 and 3, we did a continuous reaction in the mixed solvent of TA and EA at a

Table I. Relative activity remaining after 51 h of reaction.

Z-Asp concentration (m*M*)[a]	Relative remaining activity (%)			
	Ethyl acetate	25% *t*-Amyl alcohol[b]	33% *t*-Amyl alcohol[c]	*t*-Amyl alcohol
200	0.6	64	82	100
120	32	100	100	100
80	78	100	100	100
40	83	100	100	100

[a]The PheOMe concentration was 200 m*M*.
[b]The mixed solvent of 25% *t*-amyl alcohol and 75% ethyl acetate.
[c]The mixed solvent of 33% *t*-amyl alcohol and 67% ethyl acetate.

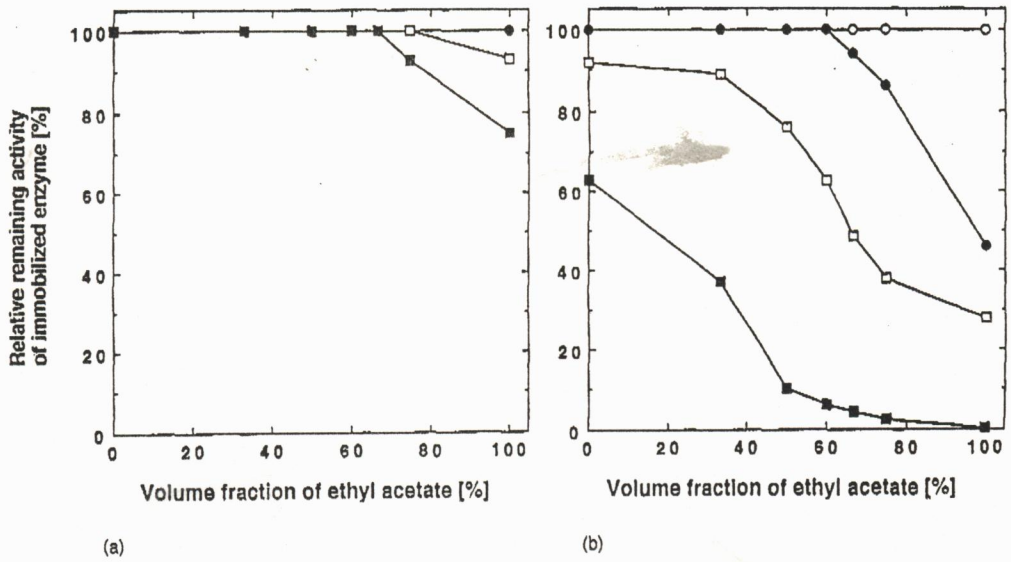

Figure 3. The effect of volume fraction of EA in the mixed solvent on the thermal stability of the immobilized enzyme in the absence (a) and in the presence (b) of substrate. The immobilized enzyme was incubated for 5 h at 40°C (○), 50°C (●), 60°C (□), and 70°C (■), respectively, and the remaining activity was measured.

ratio of 33:67 (v/v), using 40 m*M* Z-Asp and 200 m*M* PheOMe as substrate. Under these conditions, the synthetic rate was quite high (Fig. 1d) and the immobilized enzyme was sufficiently stable (Fig. 3). The yield reached 99% or higher at a retention time of around 20 min in a column reactor packed with 12.5 g of the immobilized enzyme as shown in Figure 4a. Based on these findings, the reaction was allowed to proceed continuously for 1 week in the mixed solvent at a ratio of 33:67 (v/v) at a space velocity of 3.6 h^{-1}. The yield with respect to the substrate Z-Asp concentration was maintained at around 99% for 1 week without formation of any precipitate as observed in ethyl acetate[3] as shown in Figure 4b. The relative activity remaining after a continuous reaction was higher than 98%. For comparison, we examined the continuous reaction in the mixed organic solvent of TA and EA at a ratio of 25:75 (v/v) and in EA at a space velocity of 4.9 h^{-1}. The activity remaining after a 1-week continuous reaction was 92% in the mixed solvent of TA and EA at a ratio of 25:75 (v/v). In EA it was only 80%, and a white precipitate, possibly L-phenylalanine, formed by a nonenzymatic decomposition of PheOMe, caused by the channeling of flow and sometimes plugging of the bed.

DISCUSSION

In previous studies,[1,2] we proposed that the difference in activity and stability of the immobilized enzyme in various organic solvents is due to the different state of water around the enzyme molecules immobilized inside the support. In EA, there may exist a water layer around the enzyme molecules immobilized, and that the pH in the layer can be determined by partition of the substrate between the water phase and the outer organic solvent phase.[1] As the Z-Asp concentration becomes high, the aqueous-phase pH decreases and, as a result, the stability as well as the synthetic rate decreases. In TA, the synthetic rate is less affected by the substrate concentration than in EA, probably because there may exist no clear water phase, which is in contrast to the case of EA.[2] Furthermore, in TA, the stability of the immobilized enzyme is considerably enhanced.[2] In this study, we synthesized Z-AspPheOMe in the mixed solvents of TA and EA. The mixture of TA and EA at a ratio of 33:67 (v/v) was found to be an optimal solvent for the synthesis of Z-AspPheOMe with an immobilized thermolysin from the viewpoint of the stability and activity of the immobilized enzyme. The immobilized enzyme showed a sufficiently high activity in the mixture of TA and EA at a ratio of 33:67 (v/v). Furthermore, the immobilized enzyme was much more stable in the mixed solvent than in EA and sufficiently stable to have a long-term continuous reaction as far as the Z-Asp concentration (40 m*M*) was low in comparison with the PheOMe concentration (200 m*M*). In the mixed solvent, at a ratio of 33:67 (v/v), the release of calcium ion from the immobilized enzyme was suppressed to almost the same extent as in TA.[2] After a 5-h incubation of 0.4 g (wet) of immobilized enzyme in 6 mL of mixed solvent containing 200 m*M* Z-Asp and 100 m*M* D,L-PheOMe, the amount of calcium released was 0.04 mg/g compared with 0.03 mg/g in TA. This is the reason why the immobilized enzyme was quite stable in the mixed solvent of TA and EA at a ratio of 33:67 (v/v). In EA, the amount of calcium released was about 0.18 mg/g.[2] The reaction was allowed to continue using 40 m*M* Z-Asp and 200 m*M* PheOMe as the substrate. PheOMe was used in excess compared with Z-Asp. Under such conditions the synthetic rate was sufficiently high: The synthetic rate increased linearly with increasing PheOMe concentration in the mixed solvent

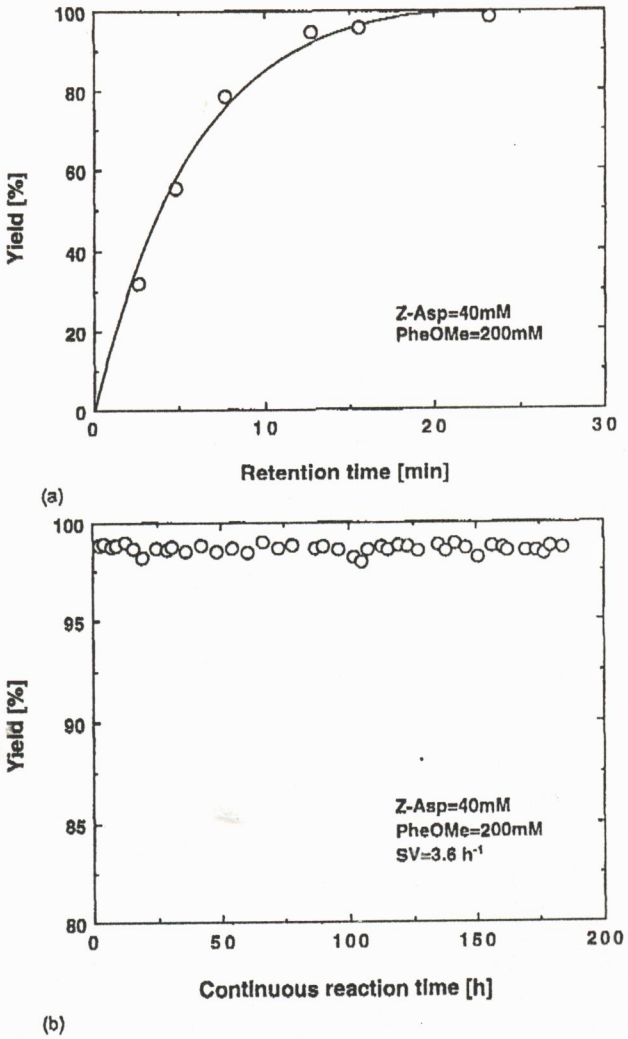

Figure 4. Effect of retention time on the yield of Z-AspPheOMe (a) and continuous synthesis of Z-AspPheOMe in a column reactor (b) at 40°C. The mixed solvent of TA and EA at a ratio of 33:67 (v/v), containing 40 m*M* Z-Asp and 200 m*M* PheOMe and 4% assay buffer, were continuously fed into a glass column packed with 12.5 g of the immobilized enzyme at various retention times (a). Synthesis of Z-AspPheOMe was allowed to continue in the same column at a space velocity of 3.6 h^{-1}.

of TA and EA at a ratio of 33:67 (v/v) (data not shown) in a way similar to that in TA[2] in the PheOMe concentration range up to 200 m*M*. Furthermore, the immobilized enzyme was sufficiently stable as described previously. The stability of the immobilized enzyme increased in the mixed solvent as the Z-Asp concentration became lower at a constant concentration of PheOMe (200 m*M*), as shown previously. The continuous reaction using 40 m*M* Z-Asp and 200 m*M* PheOMe as substrate in the mixed solvent of TA and EA, at a ratio of 33:67 (v/v), maintained a yield of around 99% with a relatively high space velocity of 3.6 h^{-1} for 1 week without any loss of activity. The productivity of Z-Asp-PheOMe synthesis for the continuous reaction done in the mixed solvent of 33:67 (v/v) was evaluated to be 1.17 g/(g wet immobilized enzyme) · day. This value was similar to that obtained with TA [1.0 g/(g · day)] as reported previously.[2] In the latter case, a higher Z-Asp concentration of 80 m*M* was used as the substrate in the presence of 200 m*M* PheOMe with a yield of around 85%.[2] Although there is no appreciable difference in the productivity in the two reaction conditions, a high yield obtained in the mixed solvent system would be advantageous to diminish the burden for downstream processing of the aspartame precursor, which is presumably the most time-consuming step for industrial production of aspartame. Since the fraction of Z-Asp in the reaction mixture is only 1% or less in the mixed organic solvent system, separation of Z-AspPheOMe would be simplified. The nonconverted PheOMe containing a very small amount of Z-Asp can be reused as the substrate. Thus, the target of our future project will be to establish an effective method for separation of Z-AspPheOMe and recovery of PheOMe as substrate and to construct an effective bioreactor system for production of the aspartame precursor.

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