Journal of Microbiology and Biotechnology
Vol. 4, No. 3, 204-209, 1994

Synthesis of an Aspartame Precursor Using Immobilized Thermolysin in an Organic Solvent

AHN, KYUNG SEOP, IN YOUNG LEE, IK HWAN KIM, YOUNG HOON PARK AND SUN BOK LEE[1]*

Genetic Engineering Research Institute, KIST, P.O. Box 17, Taedok Science Town, Taejon 305-606, Korea
[1]Department of Chemical Engineering, Pohang University of Science and Technology, P.O. Box 125, Pohang 790-600, Korea

The synthesis of N-(benzyloxycarbonyl)-L-aspartyl-L-phenylalanine methylester (Z-APM), a precursor of aspartame, from N-(benzyloxycarbonyl)-L-aspartic acid (Z-Asp) and L-phenylalanine methylester hydrochloride(L-PM·HCl) was investigated in a saturated-ethylacetate single phase system using immobilized thermolysin. Among the various supports tested, glyceryl-CPG was found to be most efficient for retaining enzyme activity. The enzyme immobilized onto glyceryl-CPG also showed the highest activity for Z-APM synthesis in saturated ethyl acetate. Z-APM conversion yield in saturated ethylacetate was half of that obtained in an ethylacetate-buffer two-phase system under the same reaction conditions. However, as the mole ratio of L-PM·HCl to Z-Asp was increased to 4.0, the conversion yield reached 95 %. When continuous synthesis of Z-APM was carried out in a plug flow reactor (PFR) with 80 mM of L-PM·HCl and 20 mM of Z-Asp in saturated ethylacetate (pH 5.5), more than 95 % of Z-Asp was converted to Z-APM with a space velocity of 1.16 hr^{-1} at 40°C. Although the operational stability in PFR was reduced rapidly, more than 80% of initial activity was maintained in CSTR even after a week of operation.

Aspartame (L-aspartyl-L-phenylalanine methyl ester), a dipeptide sweetner, has mainly been synthesized using chemical methods (2, 9). Such chemical processes, however, produce not only α-aspartame but β-aspartame which tends to leave a bitter taste. On the other hand, the enzymatic processes can synthesize only the α-form of aspartame due to specific selectivity of enzymes. Isowa *et al.* (6) have reported the thermolysin-catalyzed condensation reaction of N-(benzyloxycarbonyl)-L-aspartic acid (Z-Asp) with L-phenylalanine methylester (L-PM) in aqueous solution for the synthesis of N-(benzyloxycarbonyl)-L-aspartyl-L-phenylalanine methyl ester (Z-APM). This method enables us to obtain Z-APM in large quantity but it has disadvantages such as excess consumption of L-PM and requirement for an additional separation step due to the formation of an additive compound between Z-APM and L-PM (Z-APM·L-PM).

For the synthesis of a condensation product using hydrolytic enzymes, the use of an organic solvent is becoming a prevailing method (3, 10, 15-17). The rationale for this is that the condensation reaction can be driven toward product formation as the product formed in aqueous phase could mainly partition to organic phase. In addition, the organic solvent system has concomitant advantages such as prevention of the product hydrolysis by reverse reaction and easy recovery of product with high efficiency (1, 5, 7). In the case of aspartame synthesis, many attempts have been devoted to the synthesis of Z-APM in organic solvent system (4, 11-13, 18) since Oyama *et al.* (14) have reported the synthesis of aspartame presursor in an organic solvent system. In our previous report (8), we have investigated the thermolysin-catalyzed Z-APM synthesis in ethylacetate-water two-phase system. In this system, partitioning of the substrates and the product, which determines the reaction equilibrium, was found to be greatly affected by the pH of the reaction medium and the volume ratio of the organic solvent to the aqueous buffer solution. Through the optimization of the reaction conditions Z-APM was synthesized with more than 90% conversion yield in organic two-phase system using soluble thermolysin.

From the economic point of view, continuous synthesis of Z-APM with immobilized enzymes would be advantageous due to the reusability of the enzymes and

*Corresponding author
Key words: aspartame precursor, enzymatic process, organic single phase

easy recovery of the product. In this study, therefore, we have investigated the synthesis of Z-APM in organic single phase using immobilized thermolysin. For this purpose, immobilized enzyme with high activity and stability was first prepared, and studies on the optimization of Z-APM synthesis were followed. Finally, continuous synthesis of Z-APM was carried out in a plug flow reactor (PFR) and in a continuous stirred tank reactor (CSTR), and the performance of these two reactor types was compared.

MATERIALS AND METHODS

Materials

Crystalline thermolysin (EC 3.4.24.4), bezyloxycarbonyl-aspartic acid(Z-Asp), and L-phenylalanine methylester hydrochloride (L-PM·HCl) were purchased from Sigma (USA) and used for Z-APM synthesis. LC grade of ethylacetate was used as a reaction medium. Acetonitrile and water from Baxter (USA) were used for HPLC mobile phase. Glyceryl-CPG, Sephadex G-25, Amberlite XAD-4 and XAD-7 celite, and kaoline (all from Sigma) were used as supporting materials for enzyme immobilization.

Analytical Methods

Z-Asp, L-PM·HCl, L-phenylalanine and Z-APM were analyzed using HPLC (Hitachi L-3000) on a reversed phase column (μ-Bondapak C18, 30 cm×3.9 mm) with a flow rate of 0.8 ml/min at 220 nm. Mobile phase was made by mixing acetonitrile and water (60 : 40 v/v) and the pH was adjusted to 2.5 with phosphoric acid. Z-APM synthetic activity of immobilized enzyme was determined with 20 mM Z-Asp and 20 mM L-PM·HCl dissolved in saturated ethyl acetate and the reaction was carried out for 3 hrs at 40°C.

Preparation of Immobilized Enzymes

Immobilized enzymes were prepared by adsorption of the enzyme onto the solid supports. Celite, kaoline, glyceryl-CPG, Sephadex G-25, Amberlite XAD-4 and XAD-7 were tested as support materials. 0.1 gram of thermolysin was dissolved in 100 ml of 50 mM Tris buffer (pH 7.0) containing 0.5% of $CaCl_2$. Ten grams of cleaned support materials were added to the enzyme solution and then shaken gently for 5 hrs. After filtration, the enzyme-bound resin was washed successively with the buffer solution. The efficiency of the immobilization was determined by measuring the protein content and the enzyme activity in the filtrates. The immobilized enzyme was stored at 4°C before use.

Synthesis of Z-APM in Saturated Ethylacetate

Reaction medium was prepared by saturating ethylacetate with 50 mM MES buffer (pH 5.5) at 40°C. After substrates were dissolved in saturated ethylacetate, the final pH of the reaction mixture was readjusted to 5.5 with NaOH. 200 mg of immobilized enzyme was put into 2 ml of substrate solution preincubated at 40°C, and the reaction was carried out at 40°C with stirring.

Continuous Synthesis of Z-APM in a PFR and a CSTR

Continuous reactions in both plug flow reactor (PFR) and continuous stirred tank reactor (CSTR) were carried out with 20 mM Z-Asp and 80 mM L-PM·HCl in saturated ethylacetate (pH 5.5). Substrate solution incubated at 40°C was fed continuously from the bottom of the PFR (10.5×70 mm) loaded with 2.3 g (wet weight) of the immobilized enzyme. For the continuous reaction in a CSTR, 3.0 g (wet weight) of immobilized enzyme was suspended in 20 ml of substrate solution dissolved in saturated ethylacetate.

RESULTS AND DISCUSSION

Activity and Stability of Immobilized Thermolysin

For the continuous synthesis of Z-APM in organic solvent, selection of proper immobilization matrix which shows higher enzyme activity and longer operational stability is a prerequisite step. Previously, Amberlite XAD (11, 14), Toyopearl (14), glass beads (14), and polyurethane (18) have been used as support materials for the synthesis of Z-APM in organic solvent using immobilized thermolysin.

Table 1 shows an enzyme activity of thermolysin immobilized onto various support materials by physical adsorption. Among the various support materials tested, glyceryl-CPG, celite, and Sephadex G-25 retained relatively higher enzyme activities for Z-APM synthesis both in aqueous buffer solution and in saturated ethylacetate. In addition to the immobilization efficiency, the stability of immobilized thermolysin was measured by incubating in saturated ethylacetate equilibriated with 50 mM

Table 1. Enzyme Activity Retention of Thermolysin Immobilized onto Various Supports

Support	Retained enzyme activity (%)*	
	Buffer	Ethylacetate
Glyceryl-CPG	55	33
Celite	45	24
Sephadex G-25	47	14
Kaolin	25	12
Amberlite XAD-4	13	11
Amberlite XAD-7	11	13

*After immobilization, Z-APM synthetic reaction was carried out using immobilized enzymes with 20 mM Z-Asp and 20 mM L-PM·HCl in 50 mM MES buffer (pH 5.5) or saturated ethylacetate for 3 hrs at 40°C. The activity retention of immobilized enzyme was expressed as the ratio to total activity of initial soluble enzymes in buffer solution.

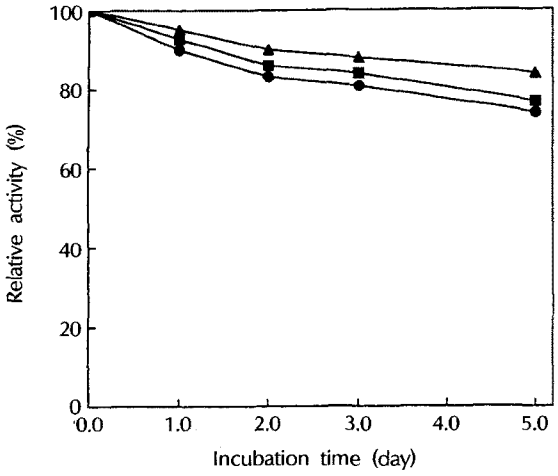

Fig. 1 Stability of immobilized thermolysin in organic phase. All immobilized enzymes were incubated in saturated ethyl acetate at 40°C. (■) Sephadex G-25, (▲) Glyceryl-CPG, (●) Celite.

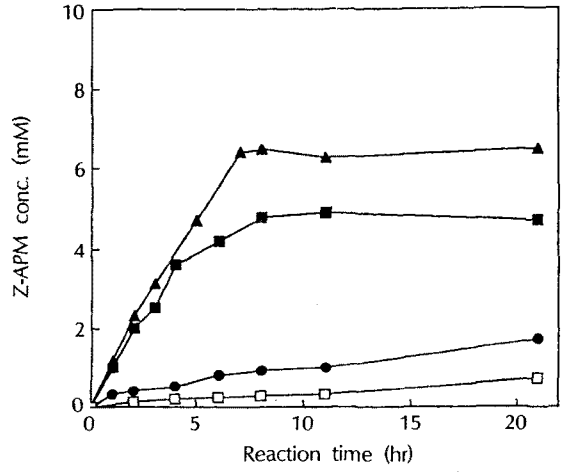

Fig. 2. Z-APM synthesis by immobilized thermolysin on various supports in saturated ethyl acetate.
Reactions were carried out with 20 mM Z-Asp and 40 mM L-PM·HCl at 40°C. (▲) glyceryl-CPG, (■) celite, (●) Sephadex G-25, (□) Amberlite XAD-7.

of MES buffer (pH 5.5). As shown in Fig. 1, the enzyme immobilized onto glyceryl-CPG maintained more than 85% of the initial activity during a 5 day incubation in saturated ethylacetate at 40°C.

In order to examine the activity of immobilized enzyme in saturated ethylacetate, Z-APM synthetic reactions were carried out using various immobilized enzymes. As shown in Fig. 2, thermolysin immobilized onto glyceryl-CPG showed highest reaction rate and Z-APM synthetic activity in saturated ethylacetate among tested in this work. Relatively low activities of the enzymes immobilized onto Amberlite XAD-7 and Sephadex G-25 may be due to brittleness of the matrix of resins

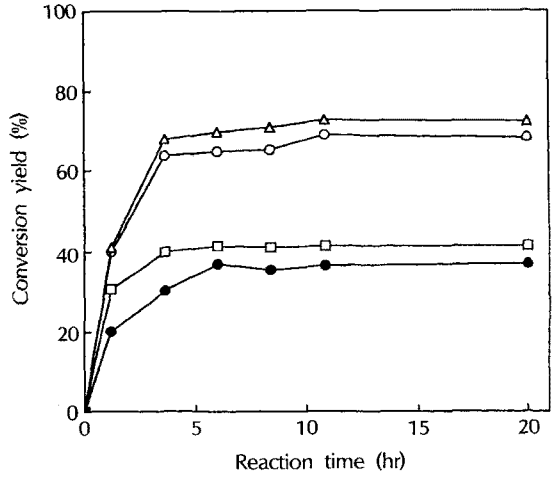

Fig 3. Effect of volume ratio of organic phase to water phase (α) on Z-APM synthesis.
Z-APM synthetic reactions were carried out with 20 mM Z-Asp and 40 mM L-PM·HCl at 40°C for 20 hrs. (○) α=1, (△) α=4, (□) a=9, (●) saturated ethylacetate (α=∞).

in saturated ethylacetate while the high enzyme activity of glyceryl-CPG enzyme seemed to be due to its sufficient hardness and inertness in organic solvent. Based on these results, glyceryl-CPG was chosen as an immobilization matrix for the synthesis of Z-APM in saturated ethylacetate reaction medium.

Synthesis of Z-APM in Organic Solvent System

The reaction medium composed of organic solvent saturated with buffer solution could be regarded as an extreme case of organic-buffer two-phase system. In this regard, the effect of the volume ratio of organic solvent to buffer (α) on Z-APM synthesis was investigated prior to optimize Z-APM synthesis in an organic single phase system.

Fig. 3 shows conversion yields of Z-Asp to Z-APM at various volume ratio of ethylacetate to buffer when glyceryl-CPG immobilized enzymes were used. It was observed that maximum conversion yield for Z-APM synthesis was obtained at the volume ratio (α) of 4. The effect of the volume ratio on Z-APM synthesis obtained with immobilized enzymes was very similar to that observed previously with soluble enzymes (8). This indicates that the effect of the volume ratio on partitioning of the substrates and the product is irrespective of whether enzymes are immobilized or not. The results shown in Fig. 3 also indicate that the conversion yield in organic single phase (saturated ethylacetate) is half of that obtained in organic two-phase system at the volume ratio of 4. This may reflect that water content in saturated ethylacetate is not sufficent for enzyme hydration and/or solubilization of substrate. It should be noted, however, that the activity of the immobilized

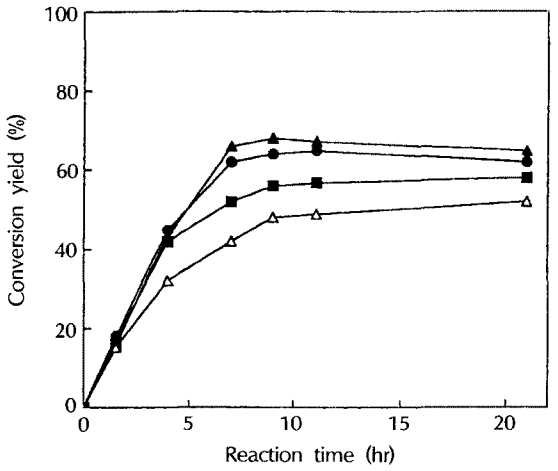

Fig. 4. Effect of pH adjustment of immobilized enzyme on Z-APM synthesis.
Reactions were carried out with 20 mM Z-Asp and 40 mM L-PM·HCl in saturated ethyl acetate (pH 5.5) at 40°C. MES buffer (50 mM) with different pH value was used to adjust the pH. The immobilized enzymes were soaked ovemight to buffer solution. (●) pH 5.0, (▲) pH 5.5, (■) pH 6.2,(△) pH 7.1.

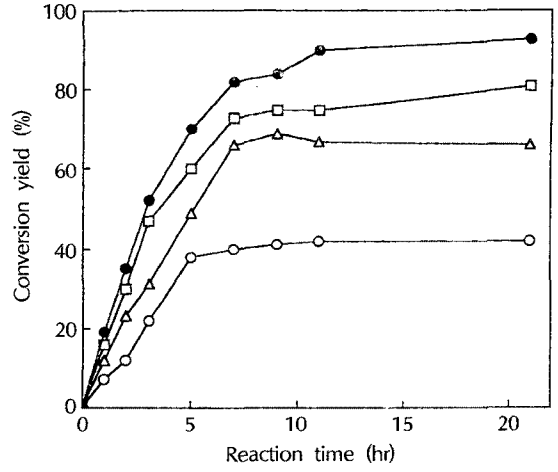

Fig. 5. Effect of substrate mole ratio on Z-APM synthesis.
Substrates were dissolved in saturated ethyl acetate and the pH was adjusted to 5.5. Moles of L-PM·HCl and Z-Asp are: (●) 80/20 mM, (□) 60/20 mM, (△) 40/20 mM, (○) 20/20 mM.

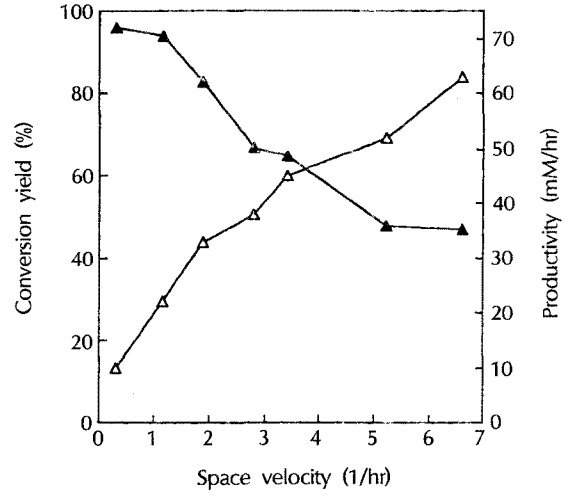

Fig. 6. Effect of space velocity on continuous Z-APM synthesis in PFR.
Z-APM synthesis was carried out with 20 mM Z-Asp and 80 mM L-PM·HCl in saturated ethyl acetate (pH 5.5) at 40°C. (▲) conversion yield, (△) productivity.

enzyme is considerably higher than that of soluble enzyme which shows very little activity in organic single phase due to the aggregation of enzyme molecules in organic solvent.

Synthesis of Z-APM in Saturated Ethylacetate

In order to optimize the synthesis of Z-APM in saturated ethylacetate, we investigated the effects of pH of the buffer solution where immobilized glyceryl-CPG thermolysin was prepared . The highest conversion yield of Z-APM synthesis was obtained when the immobilized enzyme was prepared at pH 5.5 (Fig. 4). This result agrees well with the optimal pH value obtained from the separate experiments with glyceryl-CPG immobilized enzymes (data not shown). As suggested by Zaks *et al.* (19), the ionization state of an immobilized enzyme in organic single-phase system could be directly influenced by the pH employed in immobilization step. In addition, as shown in our previous report (8), the pH of the reaction medium significantly affect the partitioning of product and substrates. In view of these regards, it was believed to be important to maintain the pH of immobilized enzyme at optimal conditions. Therefore, the immobilized enzyme was soaked in the buffer solution of pH 5.5 and used for subsequent experiments.

Our previous study (8) showed that there was an optimal mole ratio of L-PM·HCl to Z-Asp for the synthesis of Z-APM in an organic two-phase system. As a consequence, the effect of substrate mole ratio on Z-APM synthesis was also investigated in an organic single phase using immobilized enzyme. As shown in Fig. 5, conversion yield of Z-APM was increased as the mole ratio of L-PM·HCl to Z-Asp was increased, and it reached 95% when the mole ratio of L-PM·HCl to Z-Asp was 4.0. From the result of higher optimal mole ratio in organic single phase compared to that in the organic two-phase system (8), it can be deduced that higher amount of L-PM·HCl would be required for a reaction medium containing extremely low water content due to relatively lower solubility of substrates in organic solvent.

Continuous Synthesis of Z-APM

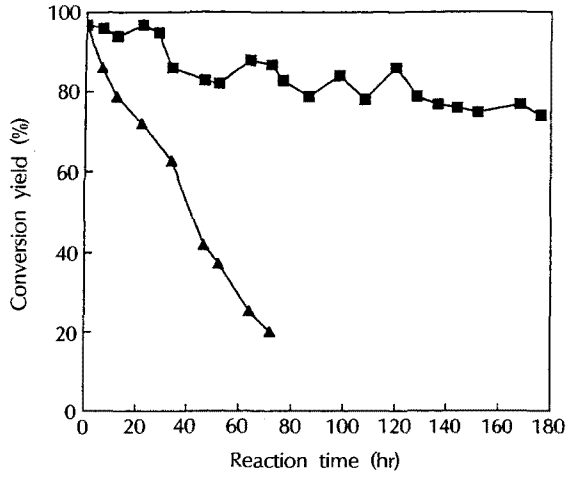

Fig. 7. Continuous synthesis of Z-APM with immobilized thermolysin.
Continuous reactions were carried out with 20 mM Z-Asp and 80 mM L-PM·HCl in saturated ethyl acetate (pH 5.5) at space velocity of 1.16 hr^{-1} in PFR (▲) and CSTR (■).

Finally, the performance of CSTR and PFR was compared in order to select suitable enzyme reactor configuration for Z-APM synthesis under previously optimized reaction conditions. A continuous reaction was first carried out at 40°C with a plug flow reactor (PFR). A substrate solution containing 20 mM Z-Asp and 80 mM L-PM·HCl dissolved in saturated ethylacetate was fed continuously into the reactor with varying the flow rates. As shown in Fig. 6, more than 95% of Z-Asp was converted to Z-APM at the space velocity of lower than 1.16 hr^{-1}. As expected, the conversion yield was gradually decreased with increasing the space velocity.

When PFR was used for Z-APM synthesis, the conversion yield rapidly decreased to 40% after 2 days of operation from the start of the reaction (Fig. 7). Previously, Nakanish *et al.* (11) have reported that the synthesis of Z-APM in PFR was unsuitable due to the inactivation of immobilized enzyme. They supposed that the main cause of the enzyme inactivation in PFR was the removal of calcium ions due to a relatively high Z-Asp concentration in PFR.

We also tried a continuous Z-APM synthesis using a continuously stirred tank reactor (CSTR). In the case of CSTR, the conversion yield was more than 80% even after a week under the same conditions employed for PFR (Fig. 7). The more efficient reactor performance of CSTR was expected because the Z-Asp concentration could be kept at a sufficiently low level in CSTR. Because of the lower level of Z-Asp concentration the calcium ions, metal ions required for the activity of thermolysin, might not be depleted in CSTR system. The rapid loss of enzyme activity of the immobilized enzymes in PFR may be caused, in part, by a severe channeling of the flow during the continuous operation.

Acknowledgement

This work was supported by grants from the Ministry of Science and Technology.

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(Received May 7, 1994)