Coimmobilization of Malic Enzyme and Alanine Dehydrogenase on Organic–Inorganic Hybrid Gel Fibers and the Production of L-Alanine from Malic Acid Using the Fibers with Coenzyme Regeneration

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ABSTRACT: Malic enzyme (EC 1.1.1.39) and alanine dehydrogenase (EC 1.4.1.1) were entrap-immobilized on hybrid gel fibers of cellulose acetate (CA) and zirconium (Zr) alkoxide by air-gap wet spinning. The production of L-alanine from malic acid with coenzyme regeneration was examined with the enzymes immobilized on the fibers. The productivity of L-alanine of the immobilized enzymes decreased to approximately one-fifth of that of free enzymes, but the CA–Zr-fiber-immobilized enzymes retained a high level of productivity after repeated use. Reduced form of nicotinamide adenine dinucleotide (NADH) recycling also occurred effectively for the enzymes immobilized on the fiber. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 116: 2901–2905, 2010

Key words: composites; enzymes; fibers

INTRODUCTION

The development of a sol–gel process that synthesizes ceramics at low temperatures has made it possible to form novel organic–inorganic hybrids.[1–3] An application example of organic–inorganic hybrids is a support of functional materials such as biomolecules.[4–6] Immobilized biocatalysts have been applied to the production and conversion of various compounds, such as amino acids, peptides, enzymes, sugars, organic acids, antibiotics, steroids, nucleosides, nucleotides, lipids, terpenoids, fuels, and commodity chemicals. The applications of immobilized biocatalysts are now expanding along with the development of immobilization techniques and the improvement of biocatalysts.[7] We previously reported that an organic–inorganic hybrid gel fiber was easily formed between cellulose acetate (CA) and zirconium (Zr) alkoxide and that this fiber formation process could be used as a technique to entrap-immobilize enzymes (urease, invertase, β-galactosidase, or lipase).[8–12] The enzyme immobilized on the gel fiber showed stable activity for repeated cycles and over a long period of time compared to a conventionally immobilized enzyme.

Since the production of L-amino acid with immobilized aminoacylase was started in Japan in 1969, some amino acids have been industrially produced with immobilized enzymes or immobilized microorganisms.[13] These productions are simple reaction systems, but further complex reaction systems will be needed in the future to produce various amino acids with biocatalysts. Therefore, the immobilization of multienzymes will be needed for the complex reaction.

In this study, we tried to immobilize two enzymes (malic enzyme and alanine dehydrogenase) simultaneously on CA–Zr alkoxide gel fibers, and the production of L-alanine using the fibers was examined. L-Alanine is a typical amino acid and is used in food products, medicinal products, and cosmetics. Suye et al.[14] reported the production of L-alanine from malic acid with coenzyme regeneration. This is the malic enzyme-to-alanine dehydrogenase reaction that requires Reduced form of nicotinamide adenine dinucleotide (NADH) in the production of L-alanine (Fig. 1), which is favorable for NADH regeneration. Furthermore, pyruvate produced from the malic enzyme reaction is a substrate for the alanine dehydrogenase reaction.

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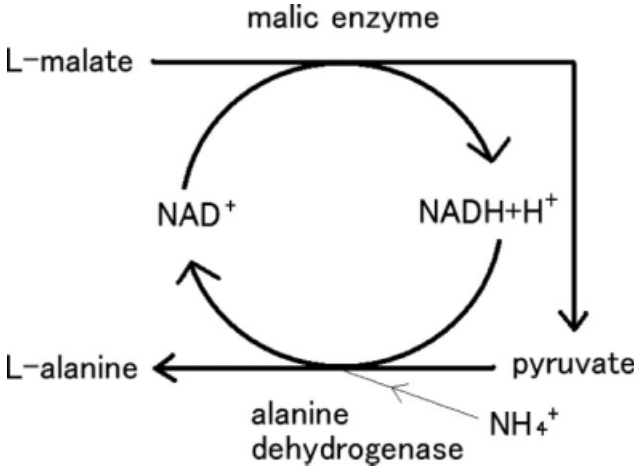

Figure 1 Production of L-alanine with NADH cycling.

Miyawaki et al.[15] performed NADH regeneration with an immobilized conjugated enzyme system of alcohol dehydrogenase and lactate dehydrogenase in a hollow-fiber-capillary reactor. Kajiwara and Maeda[16] coimmobilized malate dehydrogenase and formate dehydrogenase in poly(ethylene glycol) diacrylate gel and investigated the production of malate with NADH regeneration. Recently, Ozyilmaz and Tukel[17] reported a reaction with immobilized glucose oxidase and catalase onto magnesium silicate for coenzyme regeneration. However, there have been few reports on the immobilization of conjugate enzyme reaction systems with coenzyme regeneration, and the formation of immobilized multienzymes requires a more simple and convenient technique. Furthermore, there has been no report on NADH regeneration by an immobilized conjugated enzyme system on an organic–inorganic hybrid support. The high-efficiency and low-cost production of useful materials would be expected with the development of high-performance immobilized multienzymes.

EXPERIMENTAL

Materials

CA (weight-average molecular weight $\approx$ 45,000, acetyl content = 39.8%) was obtained from Wako Pure Chemicals Ind., Ltd. (Osaka, Japan). Zr butoxide was obtained from Aldrich Co., Ltd. (St. Louis, MO) Alanine dehydrogenase (EC 1.4.1.1, 55 units/mg, from *Bacillus stearothermophilus*) was purchased from Unitika, Ltd. (Tokyo, Japan). Malic enzyme (EC 1.1.1.39, 0.069 units/mg, from *Pseudomonas dimiuta* IFO 13182) was produced and partially purified by the method reported by Suye and coworkers.[14,18] oxidized form of nicotinamide adenine dinucleotide (NAD^+) was obtained from Nacalai Tesque, Inc. (Kyoto, Japan).

Enzyme immobilization on the CA–Zr alkoxide gel fibers

CA–Zr alkoxide gel-fiber-immobilized enzymes were formed by air-gap wet spinning. A 10 wt % CA acetone solution (CA = 1.11 g, acetone = 10 g, spinning solution) in which malic enzyme (0.7 units) and alanine dehydrogenase (7.0 units) were dispersed was extruded into a stirred 5 wt % Zr butoxide solution in acetone (coagulation solution) through a glass nozzle with N_2 compressed gas. Before the experiments, acetone was dehydrated with molecular sieves (3A 1/16, Wako Pure Chemicals Ind., Ltd.). After the solution was allowed to stand for 30 min, the resultant gel fiber was removed from the solution, washed with acetone and distilled water several times to remove the residual alkoxide, and stored overnight in 66.7 m*M* phosphate buffer (pH 7.0) at 5°C.

Production and analysis of L-alanine

The standard reaction solution (50 mL, pH 7.5 adjusted with 2*N* KOH before the addition of NAD^+) for L-alanine production contained 6.95 μmol (139 μ*M*) of NAD^+, 5 mmol (100 m*M*) of L-malic acid, 0.1065 mmol (2.13 m*M*) of $MgCl_2$, 5 mmol (100 m*M*) of NH_4Cl, and Tris-HCl buffer (50 m*M*, pH 7.5). The enzymes immobilized on the gel fiber were immersed in this solution, and the enzymatic reaction was started. The solutions were shaken at 70 stokes/min in a thermostatic water bath at 37°C.

The L-alanine produced was analyzed with a liquid chromatograph (TOSOH HLC-8020, Tokyo, Japan; column: ShodexAsahipak ES-502N, inside diameter = 100 × 7.6 mm, Showa Denko, Tokyo, Japan). The solvent was a 50 m*M* NaH_2PO_4 solution. The flow rate was 1.0 mL/min, and the elution followed at 210 nm.

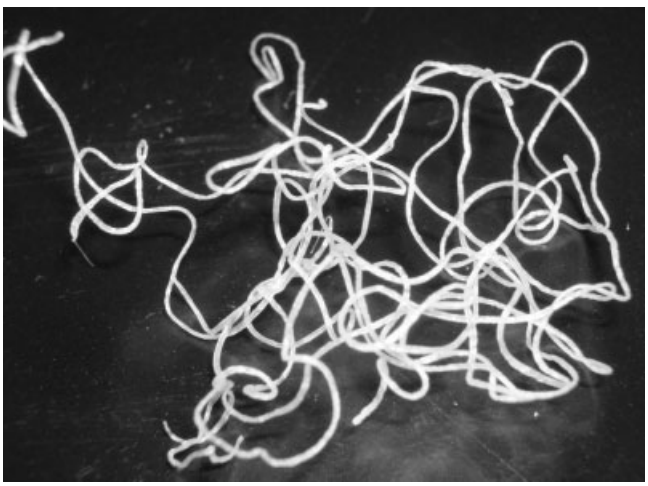

Figure 2 Photograph of the CA–Zr gel fiber-immobilized malic enzyme and alanine dehydrogenase.

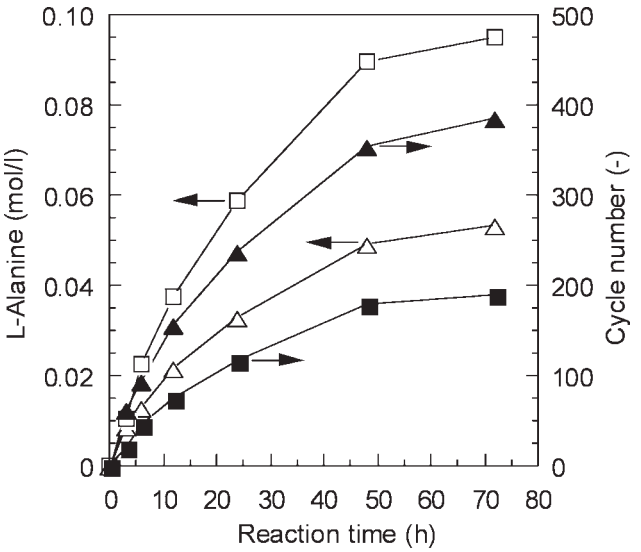

Figure 3 Effect of the reaction time on the L-alanine production and cycle number of NADH for the free-enzyme reaction systems: (□ ■) 500 and (△ ▲) 139 μ*M* NAD^+.

RESULTS AND DISCUSSION

Figure 2 shows a photograph of the CA–Zr-gel-fiber-immobilized malic enzyme and alanine dehydrogenase (fiber diameter ≈ 360 μm). The gel fiber was semitransparent and slightly elastic in water. It had a smooth surface with an absence of macropores. The CA–Zr gel fiber had good stability in common solvents, phosphate buffer solution, and electrolyte solution.[9] From Fourier transform infrared and X-ray photoelectron (XP) spectra, the fiber was considered to be formed by a coordinated interaction between Zr and the oxygen of the hydroxyl group or the carbonyl group on the CA molecules.[11] It is probable that malic enzyme and alanine dehydrogenase must have been entrap-immobilized randomly in the three-dimensional network of the CA–Zr gel. Because of the structural rigidity of the gel fiber, no compaction or large pressure drop in the flow column would occur if the fiber was used for a bioreactor.

Figure 3 shows the effect of the reaction time on the production of L-alanine with free enzymes and the cycle number of NADH regeneration. The number of L-alanine molecules divided by the number of NAD^+ molecules gives the cycle number. The amount of L-alanine produced increased with increasing reaction time. The yield of L-alanine with 25 μmol (500 μ*M*) of NAD^+ was higher than that with 139 μ*M* NAD^+. The conversion from malic acid to alanine with 500 μ*M* NAD^+ reached 95% in 72 h, and the cycle number was 190. The conversion with 139 μ*M* NAD^+ was 53% after 72 h, but the cycle number was a high value, 385. In this experiment, we found that the coenzyme functioned effectively in the case of a lower NAD^+ content (139 μ*M*).

Figure 4 shows the results of L-alanine production with immobilized enzymes on the CA–Zr gel fiber. Equal amounts of immobilized and free enzymes were used (0.7 units of malic enzyme and 7.0 units of alanine dehydrogenase), and the NAD^+ content was also the same, 139 μ*M*. The production of L-alanine with immobilized enzymes became approximately one-fifth that of free enzymes, but it was a high productivity. The cycle number was 94 at 72 h, which was considered a high performance for immobilized enzymes. The reasons for the decrease in the L-alanine productivity and cycle number with the immobilization were as follows: (1) the mobility of enzymes became low and (2) the enzymes immobilized inside the gel fiber did not participate in the reaction because the substrate and coenzyme may not have been dispersed in the fiber.

Figure 5 shows the effects of repeated use on the relative productivity of enzyme immobilization on the CA–Zr gel fibers. The relative productivity, 57.8%, was held for six reaction cycles. In our previous studies, enzymes (invertase, β-galactosidase, or lipase) did not leak from the CA–Zr gel fibers in significant amounts after repeated use and washings.[9,11,12] This was probably because of hard physical entrapment in the CA–Zr gel, which had tight gel networks, and/or because of the chelating properties of the Zr ion, which was used to couple the enzymes. The enzymes had amine and carboxyl groups. These were considered to act as ligands toward Zr.[9] Thus, the leakage of malic enzyme and alanine dehydrogenase from the gel fibers did not occur. Malic enzyme has a lower thermal stability[19] although alanine dehydrogenase shows a good thermal stability.[20] On the basis of these facts, it is likely

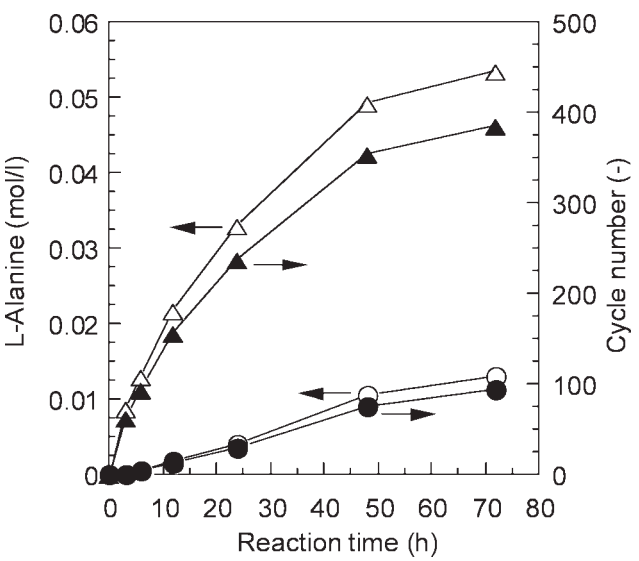

Figure 4 Effect of the reaction time on the L-alanine production and cycle number of NADH for the (△ ▲) free and (○ ●) immobilized enzymes. NAD^+ = 139 μ*M*.

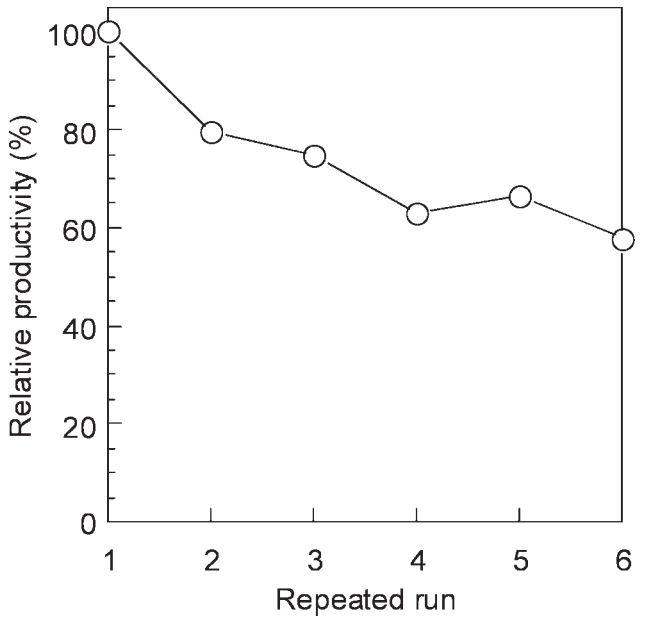

Figure 5 Effect of repeated runs on the relative productivity of L-alanine with enzyme-immobilized CA–Zr gel fibers (reaction time = 12 h). The initial productivity was 100%.

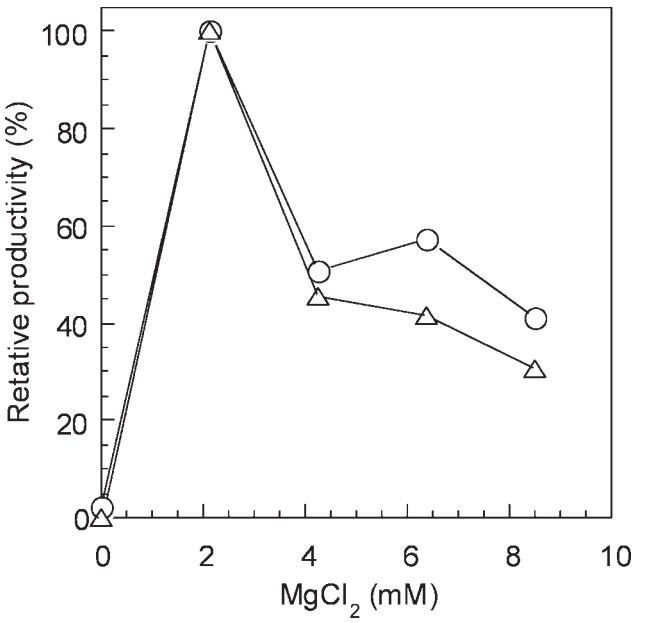

Figure 7 Effect of the $MgCl_2$ concentration on the relative productivity of L-alanine for the (△) free and (○) immobilized enzymes. The production at 2.13 m*M* was taken as the reference productivity.

that a decrease in the relative productivity depends on the thermal denaturation of malic enzyme.

Figure 6 shows the relationship between the specific surface area and L-alanine productivity of the fiber. In the calculations for the specific surface areas, we assumed that the fiber was a cylinder with a smooth surface. As shown in the figure, the alanine production increased with increasing specific surface area of the fiber (with decreases in the fiber diameter). These results suggest that the enzymatic reaction occurred in the neighborhood of the fiber surface and that the productivity for immobilized enzymes would become higher with the formation of fine-spun fibers such as nanofibers.

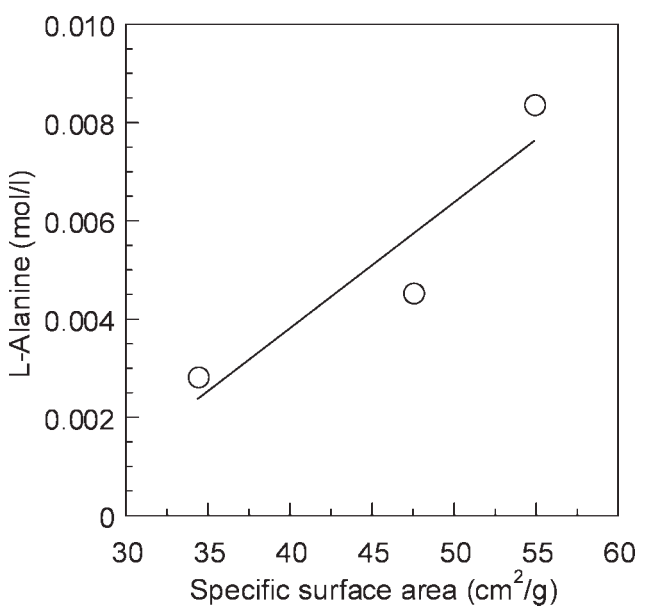

Figure 6 Effect of the specific surface area of the CA–Zr gel fibers on the L-alanine production (reaction time = 24 h).

$MgCl_2$ is a cofactor in the malic enzyme reaction. The reaction does not proceed without $MgCl_2$. Figure 7 shows the effects of $MgCl_2$ concentration in the reaction solution on the relative amount of L-alanine produced. The amounts at 2.13 m*M* were adopted as 100% for both enzymes. An inhibitory effect of $MgCl_2$ was observed at concentrations higher than 2.13 m*M* for both enzymes. A similar tendency was observed in the study in ref. 14, but the experimental conditions were different from than ours.

CONCLUSIONS

We succeeded in the coimmobilization of malic enzyme and alanine dehydrogenase on CA–Zr alkoxide hybrid gel fibers with a simple method. The productivity of L-alanine of the enzymes decreased with the immobilization, but the CA–Zr-fiber-immobilized enzymes retained a high level of productivity after repeated use. The regeneration of NADH also occurred effectively for the enzymes immobilized on the fiber. The next challenge will be immobilization of coenzyme on the fiber. The CA–Zr fiber would be a useful material as a support for multiple enzymes, and our method of simultaneous enzyme immobilization is a simple one that can be applied for practical purposes.

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