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Fabrication of enzyme-entrapped composite and macroporous gel beads by suspension gelation combined with sedimentation polymerization

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ABSTRACT

Composite and macroporous gel beads for the immobilization of enzymes were produced by combining suspension gelation with sedimentation polymerization. Physically cross-linked calcium alginate microcapsules containing lipase were prepared by electrostatic atomization. Monodisperse millimeter-sized poly(ethylene glycol) diacrylate hydrogel (composite gel) beads were fabricated by polymerizing droplets of pre-gel aqueous suspension that contained microcapsules during their descent in silicone oil. Macroporous gel beads with entrapped lipase were prepared by treating the composite gel beads with a trisodium citrate aqueous solution. The lipase immobilized within the composite and macroporous gels successfully catalyzed the hydrolysis of *p*-nitrophenyl acetate over successive cycles of use. The kinetics of the enzymatic reaction was analyzed using the Michaelis–Menten equation.

1. Introduction

In situ entrapment of enzymes within polymeric gels is achieved by polymerizing a solution containing monomers, cross-linking monomers, and enzymes [1–6]. It has the advantages of being applicable to various types of enzymes and polymeric gels as well as a lower degree of enzyme deactivation than other immobilization methods, such as binding enzymes to a carrier and cross-linking enzymes together. However, *in situ* entrapment has disadvantages such as enzyme deactivation because of exposure to reactive chemical species during polymerization, leakage of enzymes entrapped within large-sized gel networks, and the low apparent reaction rate of small-sized gel networks in which the diffusion of substrates is limited. Bernfeld et al. reported a significant decrease in the activity of enzymes entrapped *in situ* during the synthesis of acrylamide gels [1]. Soni et al. also confirmed the deactivation of enzymes by exposing them to free radicals during polymerization; they prevented deactivation by adding enzymes to the pre-gel aqueous solution several minutes after initiating polymerization [6]. There are very few studies on controlling the extent of deactivation of *in situ* entrapped enzymes. Macroporous gels enhance the diffusivity of substrates, leading to an apparent increase in the activity of the enzyme-catalyzed reaction. Petrov et al. achieved *in situ* entrapment of enzymes within the gel-network of a macroporous gel, whose macropores were formed using cryohydrate as a porogen [7]. We previously achieved *in situ* entrapment of enzymes within the macropores of a macroporous gel, wherein the macropores were formed using oil droplets [8]. Other than these, there have been few reports on *in situ* entrapment of enzymes within the macropores of macroporous gels.

Here, we propose the use of the suspension-gelation method (Fig. 1), which was developed in our previous study [9], to prepare macroporous gels for immobilization of enzymes. First, enzyme-containing microcapsules are prepared. The microcapsule-containing polymeric hydrogel, *i.e.*, the composite gel, is then synthesized by polymerizing a pre-gel aqueous suspension containing the monomers, cross-linking monomers, and microcapsules. Finally, the macroporous gel with an entrapped enzyme is prepared by treating the composite gel with a chemical and breaking down the microcapsules. Using this technique, *in situ* entrapment of enzymes within the gels is achieved without requiring contact between the reactive chemical species and enzymes during polymerization. Since the enzymes remain in the macropores and do not interact with polymers, they remain in a near-native state, wherein the full biological function of their active sites could be realized.

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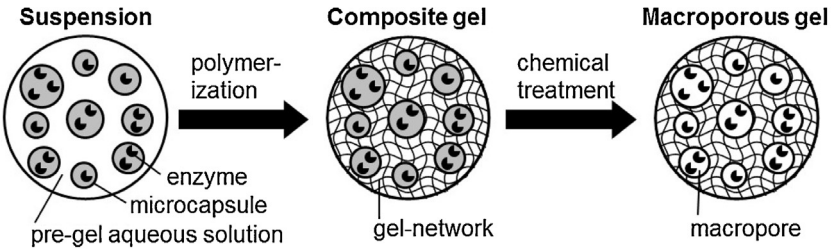

Fig. 1. Schematic diagram of the preparation of composite and macroporous gels with entrapped enzyme by suspension gelation.

The goal of this study is to develop an enzyme-entrapped composite and macroporous gel beads by using a novel production method that combines suspension gelation with sedimentation polymerization. Another goal is to characterize the activity of the entrapped enzyme. The calcium alginate microcapsules that entrapped lipase, a type of the hydrolase, were prepared by electrostatic atomization. Poly(ethylene glycol) diacrylate (PEGDA) hydrogel beads, which were used as the carrier, were prepared by sedimentation polymerization. Both methods are based on the falling-drop technique. The millimeter-sized gel beads contribute to the ease of handling and are suitable for industrial utilization in a fixed-bed column. The hydrolysis of *p*-nitrophenylacetate (*p*-NPA) to *p*-nitrophenol (*p*-NP) and acetic acid was used as the model enzymatic reaction [10–12]. The gel beads were characterized by observations using a scanning electron microscope (SEM) and a confocal-laser scanning microscope (CLSM), and by thermogravimetric analysis (TGA). The recyclability of the gels and the kinetics of the enzymatic reaction were also investigated.

2. Materials and methods

2.1. Preparation of gel beads entrapping lipase

The calcium alginate microcapsules containing lipase were prepared using the procedure reported in our previous study [9]. Sodium alginate (Wako Pure Chemical Industries, Ltd.) and Lipase PS Amano SD (Amano Enzyme Inc.; mixture of approximately 10 wt% lipase from *Burkholderia cepacia* and 90 wt% dextrin) were used. The weight-average molecular weights of sodium alginate and lipase were approximately 670,000 and 33,000 g/mol, respectively, measured by size-exclusion chromatography using pullulan as the standard. An aqueous solution containing sodium alginate (concentration: 5 kg/m^3) and Lipase PS Amano SD (concentration: 10 kg/m^3) was sprayed through a stainless-steel nozzle electrode by applying a voltage of 3.1 kV, and was collected in an aqueous solution containing calcium chloride (concentration: 15 kg/m^3) and Lipase PS Amano SD (concentration: 10 kg/m^3) at room temperature (*ca.* 20 °C). The diameters of the microcapsules were measured using a stereomicroscope.

The composite gel beads were prepared by sedimentation polymerization, using a modified version (Fig. 2) of the apparatus developed in our previous study [13]. The monomer aqueous suspension containing PEGDA, *N*,*N*,*N*',*N*'-tetramethylethylenediamine (TEMED; as an accelerator), and the microcapsules, and the initiator aqueous solution containing ammonium peroxodisulfate (APS) were prepared and purged with nitrogen gas for 1 h at room temperature. Silicone oil (KF-96-20c, Shin-Etsu Chemical Co., Ltd.) was placed in a glass reactor with an internal diameter of 41 mm (depth of oil: 780 mm) and purged with nitrogen gas for 2 h at room temperature. The agitated monomer aqueous suspension was fed by a syringe pump at a flow rate of 1.8 cm^3/min; the initiator solution was fed at a flow rate of 0.2 cm^3/min. The two solutions were mixed in the flow channel and the polymerization were then initiated, where the final concentrations of PEGDA, TEMED, and APS were 200, 40, and 20 mol/m^3, respectively, Lipase PS Amano SD was 3 kg/m^3 in the mixed pre-gel solution, and the volume fraction of microcapsules was 0.3. The mixed pre-gel solution was released dropwise through a nozzle with internal and external diameters of 2.60 and 5.10 mm, respectively. The pre-gel droplets sank in the silicone oil, which was circulated by flowing nitrogen gas and rolled onto the wall of the sloped reactor. The diameters of the as-synthesized gel beads were measured using a digital camera. The resultant composite gels were washed with water. Macroporous gels were then obtained by immersing the composite gels first in a trisodium citrate aqueous solution (concentration: 100 mol/m^3) and then in water.

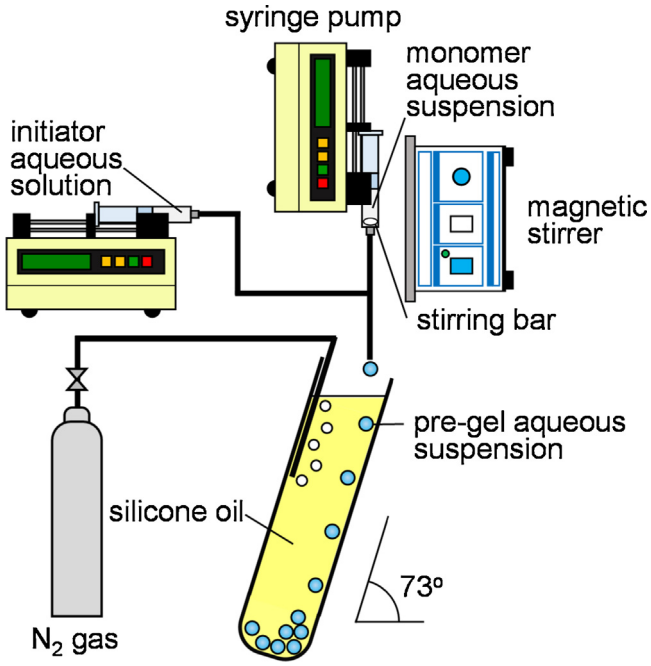

Fig. 2. Schematic diagram of the preparation of gel beads by sedimentation polymerization.

Conventional non-porous gel beads (concentration of Lipase PS Amano SD: 3 kg/m^3-gel) were prepared in the same manner without added microcapsules.

2.2. Hydrolysis of p-NPA

The gel (4 pieces) and phosphate buffer solution (concentration: 0.1 kmol/m^3; volume: 3 cm^3; pH: 6.8) were mixed in a cubic glass cell (dimensions: 10 × 10 × 45 mm). The cell was set in an ultra-violet and visible (UV–vis) spectrophotometer equipped with a temperature controller. Next, 1,4-dioxane containing *p*-NPA (0.03 cm^3) was added to the cell and hydrolysis was then initiated under agitation at 25 °C. The initial concentration, S_0, of