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Preparation of polymeric macroporous hydrogels for the immobilization of enzymes using an emulsion-gelation method

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ABSTRACT

Novel polymeric macroporous hydrogels were developed to entrap and immobilize lipase. Poly(ethylene glycol) methyl ether acrylate hydrogel was used as the support. The emulsion-gelation method using oil-in-water emulsions was used to simultaneously synthesize the hydrogels and entrap lipase in the randomly distributed, non-interconnected, sphere-like macropores, which were several micrometers in diameter. The lipase, immobilized within the macroporous hydrogel, successfully catalyzed the hydrolysis of triacetin without leakage of lipase or loss of activity during repeated use. The macroporous hydrogel-immobilized lipase exhibited higher activity than the lipase immobilized within a non-porous hydrogel, which indicates entrapment of lipase in the macropores without interference from the polymer and excellent diffusional permeability of macroporous hydrogel to substrate/product species.

1. Introduction

Enzymes are very promising alternatives to conventional industrial catalysts in industrial chemistry. They are extensively used in the selective syntheses of special medical or ingestible chemicals, which are typically carried out under mild conditions, achieve higher product purity and avoid the use of toxic solvents. Therefore, the use of enzymes is more energy efficient and environmentally friendly compared to traditional chemical treatments. For instance, enzymatic methods in biodiesel production can be used to address the issues of excessive energy requirements and catalyst removal [1]. For the purpose of reducing product cost, immobilization facilitates the efficient recovery and reuse of costly enzymes; immobilized enzymes can be used repeatedly or continuously in a variety of reactors, such as packed bed [2], fluidized bed [3], flow-through micro-reactor [4], and membrane [5,6]. Enzymes have been successfully immobilized on various water-insoluble supports, such as inorganic materials, synthetic polymeric gels, and natural macromolecules, through physical adsorption, covalent bonding, microencapsulation, or matrix entrapment. A suitable support for enzyme immobilization needs to provide improved activity and stability of the immobilized enzyme without permitting leakage or hindering the diffusion of substrate molecules and reaction products.

Synthetic polymeric gels make useful supports for enzyme immobilization. The gels are able to retain large amounts of solvent and allow substrate and product transport with minimum diffusion restriction. Furthermore, porous gels enhance the diffusivity of a solute that is comparable to its diffusivity in a solvent, which results in the apparent enhancement of the activity of the immobilized enzyme. Several types of porous gels have been developed, and the majority of the literature addresses gel microparticles with porous surface morphology prepared by suspension polymerization [7–13]. Gel microparticles could achieve rapid reactions; however, microparticles are difficult to handle when used in industrial applications. Bulk gels with connected macropores have been prepared by a freeze–thaw technique [14,15] and photolithography [16]. Generally, the immobilization of enzymes within porous gels is carried out by physical adsorption or covalent attachment to the prepared gels. Conventional methods to immobilize enzymes within porous gels require a complex multistep preparation process. Furthermore, conventional methods are associated with issues, such as the leakage of physically adsorbed enzymes and the limitation or required special treatment of polymeric gels applicable for covalent bonding. A critical problem to avoid is the activity loss of immobilized enzymes that occurs due to the conformational changes induced by the interaction and covalent linkage with the polymers.

In this study, we focus on polymeric macroporous hydrogels with randomly distributed, non-interconnected, sphere-like macropores that are several micrometers in diameter, which are created by a porogen technique, for the purpose of achieving improved activity of immobilized enzymes for hydrolysis reactions in aqueous media. A schematic of the macroporous hydrogels entrapping enzymes is shown in Fig. 1. They can be prepared by means of an emulsion-gelation method, which resembles the methods of our previous studies [17,18]. The method involves

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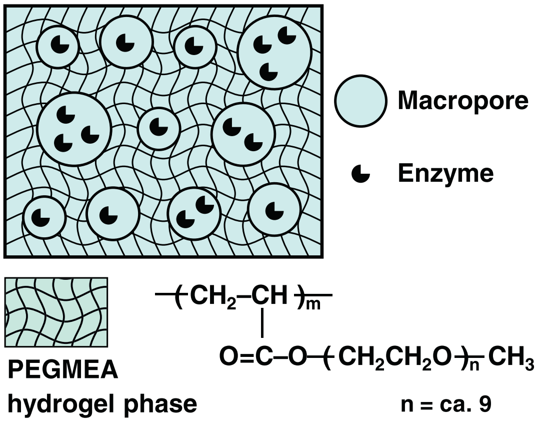

Fig. 1. Schematic diagram of the polymeric macroporous hydrogel containing enzymes and the chemical structure of poly(PEGMEA).

the following sequence: synthesizing polymeric hydrogels in an aqueous phase in an oil-in-water (O/W) emulsion by free-radical copolymerization of a monomer with a cross-linker, loading the oil-phase enzymes, and washing (removing) the dispersed oil microdroplets as a pore template (porogen), where enzymes could not pass through the polymer network and remain in the macropores. The polymerization of hydrogels and the entrapment of enzymes in the macropores are accomplished simultaneously using this method. Simultaneous polymerization and entrapment is also carried out during the conventional formation of non-porous hydrogels, however, reactive chemical species damage the enzymes, and the existence of enzymes hinders gelation. In contrast, oil media prevents damage to the enzymes during polymerization for the proposed macroporous hydrogels, and the polymerization proceeds regardless of the presence of enzymes. The proposed method improves upon conventional method in terms of its simplicity in the immobilization process and its applicability to many types of polymers and enzymes.

The unique structures of the proposed macroporous hydrogels can also contribute to improving results obtained for an immobilized enzyme reaction. Enzymes are entrapped and mildly retained in macropores at a near-native state without interacting with polymers. The proposed immobilization method can minimize the denaturation of enzymes and generate full biological functions of their active sites. Enzymes entrapped in macropores can possess a high level of activity and stability that are comparable to free enzymes in a solution. Furthermore, the proposed macroporous hydrogels are used as a bulk and have excellent diffusional permeability for substrate/product species.

The goals of this study are to develop and prepare by the emulsion-gelation method novel polymeric macroporous hydrogels that entrap enzymes and to characterize their enzyme-catalyzed reaction properties with respect to conventional non-porous hydrogels. We used poly(ethylene glycol) methyl ether acrylate (PEGMEA, Fig. 1) hydrogel as the support. The model enzyme reaction studied was the hydrolysis of triacetin to glycerin and acetic acid catalyzed by lipase. It is well known that lipase catalyzes various reactions such as the hydrolysis of triglycerides, the synthesis of esters, and transesterification. The internal structure of hydrogels was observed with a scanning electron microscope (SEM) and a confocal laser scanning microscope (CLSM).

2. Experimental

2.1. Reagents

PEGMEA (primary monomer), *N*,*N*′-methylenebisacrylamide (MBAA; cross-linker), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED; accelerator), and ammonium peroxodisulfate (APS; initiator) were used to form the continuous hydrogel phase. Oleyl alcohol was used as the oil phase and polyoxyethylene(20) sorbitan monolaurate (Tween 20, HLB value 16.7) was used as the surfactant. Lipase PS Amano SD (Amano Enzyme Inc.) was used as the enzyme and is composed of approximately 10 wt% lipase from *Burkholderia cepacia* and 90 wt% dextrin. Triacetin was used as the substrate. The phosphate buffer solution at pH = 7 was prepared using Na_2HPO_4 and KH_2PO_4. All chemicals were used without further purification. Fluorescein-4-isothiocyanate (FITC) was used as the fluorescent labeling reagent.

2.2. Preparation of hydrogels

The macroporous hydrogels were prepared by the emulsion-gelation method involving free-radical polymerization that was previously described [11,12]. A monomer solution (7.2 cm^3) containing PEGMEA, MBAA and TEMED and an initiator solution (0.8 cm^3) containing APS were prepared, for which the phosphate buffer solution was used as a solvent. The final concentrations of PEGMEA, MBAA, TEMED, and APS were 500, 100, 10, and 1 mol/m^3 in the aqueous phase, respectively. The oil phase was 2 cm^3 of oleyl alcohol containing 0.1 kg/m^3-oil Tween 20 and 27.5 kg/m^3-oil (or 5.5 kg/m^3-gel) Lipase PS Amano SD. These solutions were purged with nitrogen gas for 1 h. The oil phase was slowly added to the monomer solution in a test tube, then the initiator solution was injected into the monomer solution using a syringe. The O/W emulsion was immediately formed by mixing for 30 s with a tube mixer; the volume fraction of oil in the pre-gel emulsion was 0.2. The emulsion was allowed to stand, and the polymerization was performed in a tube with an inside diameter of 6 mm at 30 °C under a nitrogen atmosphere. The resultant gels were cut into discs with a 6 mm diameter and 2 mm length and were then thoroughly washed with ethanol followed by water. Gels with various lipase concentrations (11, 27.5, and 55 kg/m^3-gel Lipase PS Amano SD) were also prepared.

To serve as the control, a conventional non-porous hydrogel was prepared in the same manner without using the oil phase.

2.3. Lipase-catalyzed reactions

The activity of lipase immobilized within the hydrogels was determined using a typical batch method. A given amount of hydrogel (20 pieces containing 5.5 kg-Lipase PS Amano SD/m^3-gel) and 50 cm^3 phosphate buffer solution containing 2 wt% triacetin were mixed in a vial. The vial was placed in a water bath at 37 °C and was shaken throughout the experiments. An aliquot (1 cm^3) of the solution was taken at a specified time and titrated with a 10 mol/m^3 NaOH aqueous solution to determine the concentration of acetic acid produced from the reaction. The conversion of triacetin to acetic acid was determined from the concentration of acetic acid. The hydrogels were immersed in a new 2 wt% triacetin solution after the experiment, and the reaction was then repeated.

The reaction was carried out using hydrogels with various amounts of immobilized lipase and free lipase (6.2 mg) described in the previous paragraph with the total dosage of lipase equal in each experimental system.

2.4. Observation

The pre-gel emulsion without APS was placed between cover slides and was observed with a stereomicroscope.

The internal structure of the macroporous hydrogel was observed by the SEM. The sample gel specimen were prepared by freeze-drying and then coating them with platinum and palladium.