Improved Immobilized Enzyme Systems Using Spherical Micro Silica Sol-Gel Enzyme Beads

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Abstract Spherical micro silica sol-gel immobilized enzyme beads were prepared in an emulsion system using cyclohexanone and Triton-X 114. The beads were used for the *in situ* immobilization of transaminase, trypsin, and lipase. Immobilization during the sol to gel phase transition was investigated to determine the effect of the emulsifying solvents, surfactants, and mixing process on the formation of spherical micro sol-gel enzyme beads and their catalytic activity. The different combinations of sol-gel precursors affected both activity and the stability of the enzymes, which suggests that each enzyme has a unique preference for the silica gel matrix dependent upon the characteristics of the precursors. The resulting enzyme-entrapped micron-sized beads were characterized and utilized for several enzyme reaction cycles. These results indicated improved stability compared to the conventional crushed form silica sol-gel immobilized enzyme systems.

Keywords: sol-gel immobilization, microemulsion, surfactant, Triton-X 114, cyclohexanone, transaminase, lipase, trypsin

INTRODUCTION

The immobilization of enzymes offers many advantages, including multiple reuse, easy separation, and improved stability for efficient biotransformation and biodegradation [1-5]. Among various immobilization methods, sol-gel immobilization using silane compounds such as tetramethoxysilane (TMOS) has been applied for the preparation of thin film biosensors and for the immobilization of biocatalysts used in the biosynthesis of natural products and anti-fouling materials [6-10]. The most common silica sol-gel encapsulated enzyme systems have been produced in crushed powder form from the dried xerogel state [11-16], or sol-gel coatings with enzymes or antibodies affixed to various solid material surfaces [17-21]. The crushing of the silica particles, however, yields irregular shapes and sizes and makes the process of scale-up very difficult. Although spray-drying [22] and microwave assisted sol-gel methods [23] were attempted for the fabrication of spherical silica beads, these procedures also required an elevated temperature that is not suitable for *in situ* enzyme immobilization. Microparticle technology has significant importance for the development of new drug delivery methods [24]. Meanwhile, a sol-gel emulsion technology has been developed that combines the emulsion and sol-gel technology and used to prepare monodispersed silica particles for drug delivery [25-28]. The sol-gel emulsion process should be suitable for the immobilization of biological molecules such as antibodies and enzymes because of the prevailing ambient temperatures and relatively mild processing conditions. We report an optimized sol-gel emulsion process involving organic solvents for *in situ* enzyme immobilization using spherical silica beads and demonstrate the effects of different silica sol-gel precursors on enzyme activities.

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MATERIALS AND METHODS

Chemicals and Enzymes

The ω-transaminase from *Vibrio fluvialis* (ωATVf) was purified according to the procedures described elsewhere [29]. The lipase from *Candida rugosa* and trypsin from porcine pancreas were purchased from Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

Enzyme Immobilization and Assays

A magnetic stirrer from Hanna Instrument (Model HI 303N, Woonsocket, RI, USA) that displays digital RPM values was used for controlled mixing. Analysis of ω-transaminase activity was performed by measuring the concentration of acetophenone and 1-phenylethanol [30]. The lipase and trypsin assays were achieved by monitoring hydrolysis of *p*-nitrophenylacetanilide (pNPA), and *N*-benzoyl-*L*-arginine-4-nitroanilide (BAPNA), respectively [31,32].

Enzyme immobilization was achieved by mixing the sol-gel solution containing the enzyme with solvent containing the surfactant [27]. Mixtures of TMOS and various other silane compounds (methyltrimethoxysilane (MTMS), vinyltriethoxysilane (VTES), phenyltrimethoxysilane (PTMS), 3-glycidyloxypropyltrimethoxysilane (GTMS), dimethyldimethoxysilane (DMDMS), and hexadecyltrimethoxysilane (HTMS)) were used as building blocks for the corresponding gel matrices. Typically, to a 1 mL of enzyme solution (0.1 mg protein/mL of 0.1 M potassium phosphate buffer, pH 7.5), 1 mL of TMOS and 1 mL of the other specified silane partner were added and mixed by repetitious manual pipeting. After 30 sec, the mixture was added to 100 mL of cyclohexanone containing 10% of surfactant. After 5 min of further mixing, the sol-gel beads were collected by filtration, washed four times with distilled water, and stored at 4°C until needed.

RESULTS AND DISCUSSION

Optimization of Gelation Conditions

To optimize gelation procedure, multiple immobilization conditions were compared by varying the ratio of the precursors, water content, and pH of the mixture. Gelation time assessment was performed in the pH range of 6.5 to 7.5, observing remaining immobilized enzyme activity of ωATVf using TMOS, TEOS, and MTMS as model precursors. As gelation is an acid or base-catalyzed reaction, the time for gelation was dependent upon pH *e.g.*, gelation was completed in several hours at pH 6.5, compared to a few minutes at pH 7.5. The results indicated that the gelation of TMOS/MTMS/H_2O (1:1:1, v/v) at pH 7.5 yielded the highest remaining catalytic activity, and was completed within three minutes (data not shown). Thus, this condition was used for all subsequent experiments.

Effect of the Emulsifying Solvents, Surfactants, and Mixing Process for the Formation of Sol-Gel Beads and their Effects on the Stability of the Immobilized Enzyme

There are two important parameters for selecting the ideal organic solvents used in the preparation of sol-gel beads, polarity and viscosity. The polarity of solvents is important because water-immiscible solvents are the primary candidates to make distinctive phase-separation with the aqueous precursor and enzyme mixture. The viscosity of the solvent is important to the control of the bead size in the emulsification process. We chose several non-polar solvents for the formation of emulsion and compared the differences in performance for the bead formation. In comparing the solvents according to viscosity, a solvent with low viscosity (η) such as cyclohexane (η= 0.89) could not make an emulsion mixture as the shape of the sol-gel beads was not uniform compared to the more viscous solvents such as *n*-hexanol (η= 4.57) and *n*-heptanol (η= 5.82) (data not shown). Ultimately, uniform spherical sol-gel beads were obtained by using cyclohexanone (η= 2.02) with medium viscosity.

Table 1. Surfactant effects on bead formation, bead size and enzyme stability

Surfactant	Bead formation	Stability[a] (%)	Bead size (μm)	HLB
W/O surfactant	no[b]	(92)[b]	(100~200)[b]	–
CTAB	no	N/A[c]	N/A[c]	–
Tween 20	no	N/A	N/A	16.7
Triton-X 100	yes	32	1~10	13.5
Triton-X 114	yes	91	1~10	12.5
Triton-X 405	yes	<1	0.1~1	17.9
Triton SP 135	yes	99	10~100	8.0
Triton SP 190	yes	90	10~100	13.0

[a]Stability of the ωATVf in sol-gel beads was measured by comparing the enzyme activity after five consecutive reactions with 25 mM of phenylethylamine and 25 mM of pyruvate at 37°C for 15 h.
[b]Irregular shape of silica particles were obtained without surfactant for the sol-gel immobilization of enzyme. The stability and the particle size were measured to compare with those of sol-gel beads prepared with surfactant.
[c]The stability and the bead size were not available as no bead formation was observed with CTAB and Tween 20.

We examined the surfactants to verify the effect of different surfactants with a differing hydrophilic lipophilic balance (HLB) value on the activity of the enzyme (Table 1). The stability of immobilized enzyme was greatest using the surfactants with a relatively low HLB (<13), and smaller beads were obtained (1~10 μm) using a Triton-X series surfactant while maintaining stable emulsion mixtures. Although the commercial acid-splittable Triton-SP surfactants yielded higher stability for the resulting sol-gel immobilized enzymes, the emulsion was less stable and larger beads were obtained with a relatively broad size distribution. Considering the bead size that resulted and the product stability, Triton X-114 was chosen as the best surfactant for subsequent experiments.

The extent and the process of mixing play a crucial role in the shape and the size of the beads prepared by the emulsion system. At low rpm (Fig. 1A), irregular beads were obtained with a relatively large average particle size (around 100 μm), whereas beads prepared at high rpm were more uniform, spherical and smaller (1~10 μm, Fig. 1B). In spite of the differences in the shape and the size of the beads, the enzyme activities of the two bead preparations were not significantly different, which suggests that the mass transfer limitations of the substrates and products are not significant in the sol-gel beads of approximately 100 μm or smaller diameter.

Effect of Functional Groups of the Precursors Used in the Sol-Gel Preparations

Precursors of different functional groups were com-

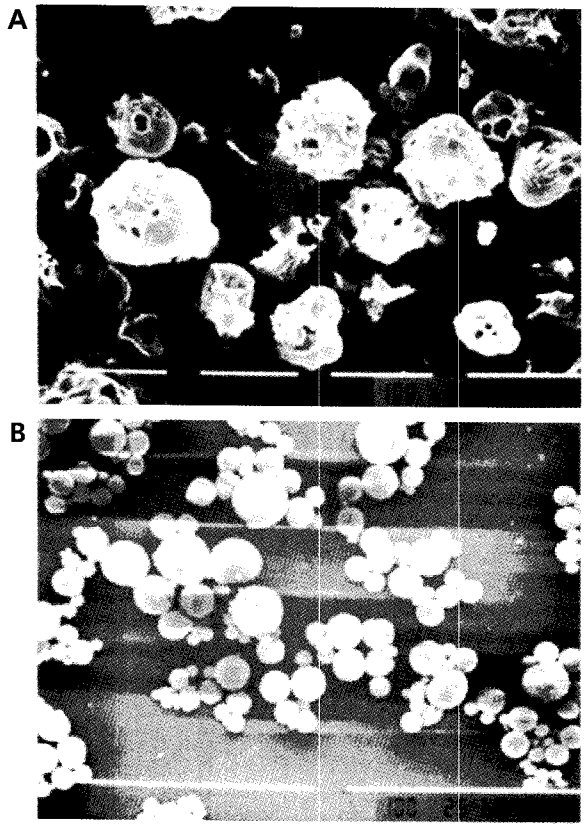

Fig. 1. SEM pictures of sol-gel beads prepared for different stirring speeds; (A) 180 rpm and (B) 500 rpm. The pictures were obtained after drying the bead samples overnight at room temperature. Scale bars in both figures indicate 100 μm.

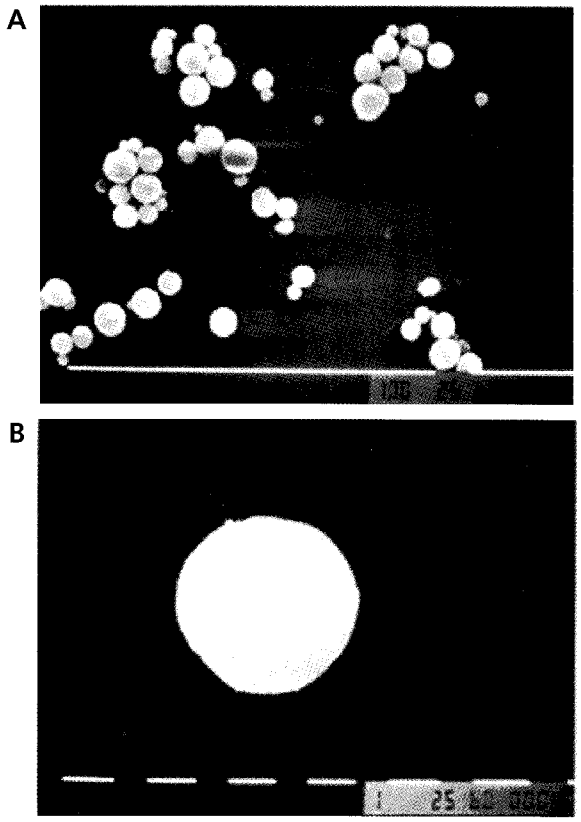

Fig. 2. SEM pictures of sol-gel beads prepared with VTES-TMOS. (A) Scale bar indicates 100 μm; (B) scale bar indicates 1 μm.

pared to aid the investigation of the microenvironmental effects on the shape, pore size, and enzyme activities of the beads. When precursors such as MTMS, PTMS, VTES, GTMS, HTMS, and DMDMS were mixed with TMOS to make sol-gel beads at 500 rpm, the diameters of the spherical beads approximated 1~10 μm, and no significant differences in bead size distribution and shape were observed by scanning electron microscopy (data not shown). The pore size of the beads was measured at 38.3 ± 1.3 Å and was seemingly unrelated to the kind of the mixture, except for beads prepared with TMOS only (14.5 Å pore size). Among the mixtures analyzed, TMOS-VTES produced beads with the narrowest size distribution compared to other combinations (Fig. 2).

The enzyme activities of the sol-gel immobilized ωATVf, lipase and trypsin beads were compared to identify the effect of precursor pairs. The effect of three different precursors, MTMS, PTMS, and VTES, are shown in Fig. 3, wherein these precursors were chosen as they were successfully used to produce spherical gel particles and were characterized by significant differences on the activity of entrapped ωATVf; GTMS and HTMS showed similar effects with MTMS on the activity of entrapped ωATVf. However, with DMDMS, bead formation was not favorable, and the activity of the entrapped ωATVf was slightly lower than with MTMS. Lipase showed the highest activity in the beads made of MTMS-TMOS, while trypsin and ωATVf yielded the highest activity with a VTES-TMOS combination. These results indirectly indicate that each enzyme has a favorable precursor pair and those subtle changes in the microenvironment of each bead, using different precursor pairs, could affect the ultimate enzyme activity in micro sol-gel beads.

Stability of Immobilized Enzyme in Micro-Sol Gel Beads

The stability of the ωATVf in sol-gel beads was measured with beads prepared under optimized condition (*i.e.* 1:1 mixture of TMOS and MTMS in cyclohexanone with Triton-X 114 (10%, v/v) mixed with the enzyme at 500 rpm). After five consecutive reactions with 25 mM of phenylethylamine and 25 mM of pyruvate at 37°C for 15 h, the remaining catalytic activity exceeded 91%. This result suggests that the activity of sol-gel immobilized enzymes were effectively retained in the beads during the reaction period, and the enzyme stability was maintained.

The immobilized ωATVf prepared by the emulsion method showed much enhancement on the activity and the stability compared to conventional crushed xerogel silica matrix immobilization. To prepare a crushed xerogel silica matrix, aging is known to be an essential process [27]. When we measured the enzyme activity of

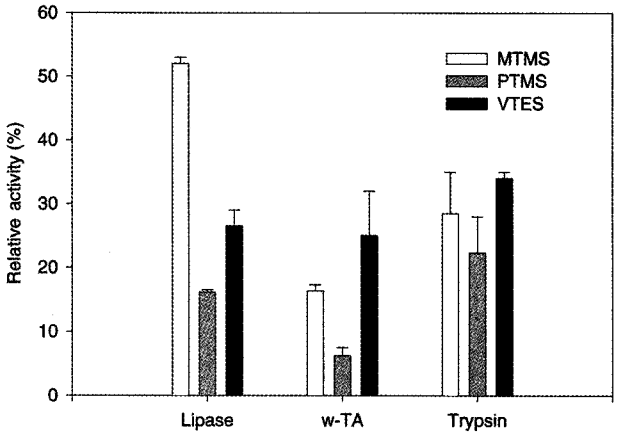

Fig. 3. Effects of different partner precursors of TMOS on the enzyme activities of the beads. Relative activities were measured and compared to that of the total free enzyme activity used for immobilization.

the crushed powder-form silica matrixes and varying the aging period from 1 to 3.5 h at room temperature, the enzyme activity of the powder matrixes was shown to be drastically decreased to 53% and to 31% compared to that of sol-gel immobilized enzyme beads by the emulsion process.

CONCLUSIONS

In this article, we report on a micro-emulsion silane mixture system that was developed to make and control the micron size of immobilized spherical enzyme beads. This study suggested that each enzyme has a unique preference of silica sol-gel precursor depending upon chemical, physical, and structural characteristics. While lipase showed the highest activity with MTMS as a precursor, trypsin and ωATVf showed their highest activities with VTMS. More hydrophobic PTMS showed the lowest activity for all enzymes studied and reported. As the pore size of prepared sol-gel particles were not affected by the use of different sol-gel precursors, the difference in enzyme activity could be attributed to their physicochemical characteristics, though it was not fully revealed in this article.

Silica sol-gel immobilization methods have been demonstrated successfully by several authors using the xerogel method [9-16]. Although their microscopic observations of the xero-gel showed an inner network of spherical particles [13], bulk xerogel enzyme agglomerates should be crushed into small particles for practical use, which usually resulted in the failure of large-scale production. The use of an emulsion process allowed us to prepare relatively regular sized active microenzyme immobilized beads. The stability of enzyme was influenced by the surfactant used to make the emulsion, yet relatively stable immobilized sol-gel enzyme beads could be obtained by using Triton X-114 and cyclohexanone as surfactant and solvent in an organic phase.

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