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Enzyme immobilization in a biomimetic silica support

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Robust immobilization techniques that preserve the activity of biomolecules have many potential applications[1–8]. Silicates, primarily in the form of sol-gel composites or functionalized mesoporous silica, have been used to encapsulate a wide variety of biomolecules[1,4–8] but the harsh conditions required for chemical synthesis limit their applicability[1,8]. Silaffin polypeptides from diatoms catalyze the formation of silica *in vitro* at neutral pH and ambient temperature and pressure[9]. Here we show that butyrylcholinesterase entrapped during the precipitation of silica nanospheres retained all of its activity. Ninety percent of the soluble enzyme was immobilized, and the immobilized enzyme was substantially more stable than the free enzyme. The mechanical properties of silica nanospheres facilitated application in a flow-through reactor. The use of biosilica for enzyme immobilization combines the excellent support properties of a silica matrix with a benign immobilization method that retains enzyme activity.

We determined the suitability of biosilica as an immobilization matrix with the enzyme butyrylcholinesterase. The activity of butyrylcholinesterase entrapped in biosilica was compared with that of the free enzyme to determine the effect of immobilization. The biosilicification reaction mixture consists of silicic acid (hydrolyzed tetramethyl orthosilicate) and a silica-condensing synthetic peptide (R5). The R5 peptide (H_2N-SSKKSGSYSGSKGSKR RIL-COOH) is the repeat unit of the silaffin polypeptide previously identified from the diatom *Cylindrotheca fusiformis*. It catalyzes the precipitation of silica within seconds when added to a solution of silicic acid[10]. The resulting material is a network of fused spherical silica particles with an average diameter of 500 nm. After immobilization of the enzyme, 90% (±7.2) of the initial free enzyme activity was detected in the biosilica spheres. The remaining enzyme activity (5.4% ± 4.9) and protein was detected in the supernatant and wash fractions, which indicated negligible loss of enzyme activity during immobilization.

The high efficiency of the immobilization technique can be partly attributed to the mild conditions, which minimize enzyme denaturation. In comparison, when we immobilized butyrylcholinesterase in a sol-gel according to a previously reported method[11], less than 10% of the initial enzyme activity remained (data not shown). Our results were consistent with the 85% decrease in butyrylcholinesterase activity upon sol-gel immobilization reported in the earlier study[11]. In another study, butyrylcholinesterase immobilized in a sol-gel matrix lost 70% of the initial activity when the sol-gel was stored for 30 d[12]. Given the low immobilization efficiency of the sol-gel method, we did not pursue the comparison further.

We investigated the efficiency of immobilization at a range of enzyme concentrations to determine the immobilization capacity of the biosilica nanospheres. The immobilization efficiency was reproducible for enzyme concentrations up to 2 mg/ml. At 4 mg/ml the loading was reduced to 70%. The biosilicification reaction yields approximately 1.2 mg of silica from a 100 µl reaction mixture. The calculated capacity for enzyme loading in the biosilica nanospheres was 220 mg enzyme/g silica (20% w/w). The enzyme loading in conventional sol-gel protocols is usually limited to 0.1–5% (w/w), owing primarily to the hydrophobicity of alkyl silicates and the denaturing effect of alcohol byproducts and also as a result of protein aggregation at elevated concentrations[8]. Enzyme loadings as high as 20% (w/w) have also been reported with polyglyceryl silicate xerogels, in which some of the limitations of conventional sol-gels have been overcome by using biocompatible substrates that exhibit increased water solubility[8].

We analyzed the biosilica nanospheres containing the immobilized butyrylcholinesterase by scanning electron microscopy (SEM) to determine their morphology and size distribution (**Fig. 1**). This analysis revealed a matrix of fused silica nanospheres with an average diameter of 500 nm, in agreement with previous observations[9,13].

The high yield of enzyme immobilization raised the question of whether the butyrylcholinesterase was physically entrapped within the silica nanospheres during the precipitation process or simply adsorbed to the surface of the particles. Biosilica nanospheres were prepared without enzyme and then incubated with a solution of butyrylcholinesterase (at the same enzyme concentration and reaction time as used in the immobilization reaction). The resulting biosilica nanospheres were washed and all of the initial enzyme activity was detected in the wash fractions. This indicated that our enzyme immobilization protocol physically entrapped and immobilized the enzyme within the biosilica nanospheres during the silica formation. This conclusion was confirmed by the absence of leaching of the enzyme during continuous use.

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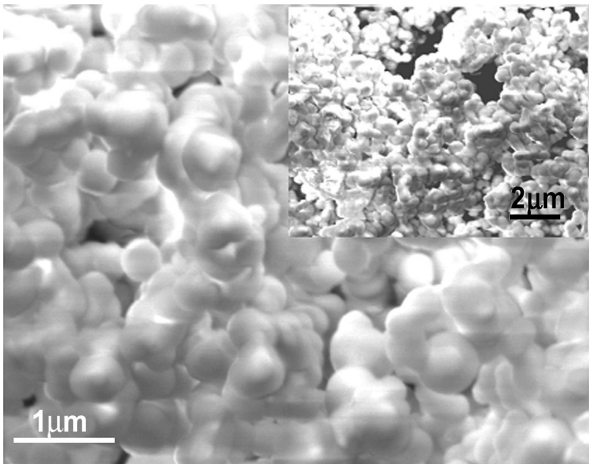

Figure 1 SEM micrograph of biosilica-immobilized butyrylcholinesterase.

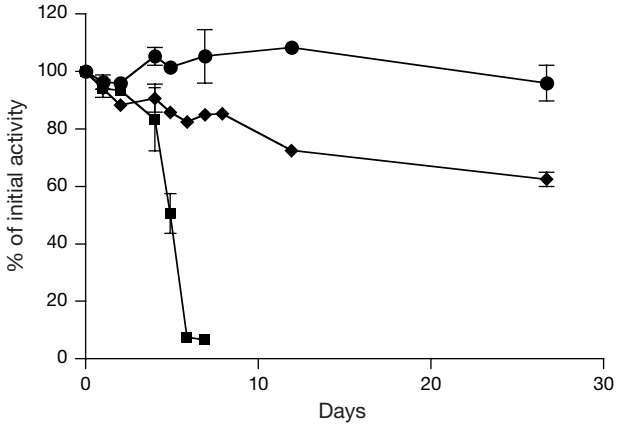

Figure 2 Stability of butyrylcholinesterase activity at 25 °C in free and biosilica-immobilized enzyme systems. Free enzyme (■), free enzyme with antibiotic solution (◆) and biosilica-immobilized enzyme (●).

The stability of biosilica-immobilized enzymes at room temperature is critical if the system is to be suitable for a wide range of applications. We therefore evaluated the stability of the enzyme during storage at 25 °C over a 30-d period for (i) free enzyme in buffer solution, (ii) free enzyme in buffer plus an antibiotic solution and (iii) biosilica-immobilized enzyme in buffer solution (**Fig. 2**). The immobilized enzyme retained 100% of the initial activity when stored in aqueous buffer for 30 d. The free enzyme lost activity rapidly. The stability of the free enzyme was prolonged by the addition of an antibiotic solution, which indicates that the loss of the activity was probably due to microbial degradation. The addition of the same antibiotic solution to the biosilica did not change the enzyme's stability. When the biosilica-immobilized enzyme was dried under vacuum before storage at 25 °C, the activity was again retained for 30 d. These results indicate that biosilica-immobilization of the enzyme provided a stable environment and prevented the loss of activity that occurs when the enzyme is stored in solution at room temperature.

We investigated the thermostability of free and biosilica-immobilized enzyme to determine whether the biosilica nanospheres could protect the immobilized enzyme from thermal denaturation. The enzyme in solution was denatured by incubation at 65 °C for 1 h (85% decrease in activity compared to a control), in agreement with previously reported observations[14]. In contrast, the biosilica-immobilized enzyme retained 100% of its activity when incubated under the same conditions (65 °C for 1 h). Enhanced thermostability for sol-gel composites[8,15] has been attributed to the stabilizing effect of the support matrix, which prevents the extensive conformational changes typical of thermal denaturation. In contrast, immobilization of butyrylcholinesterase onto a polyacrylamide-type bead did not significantly improve the heat stability of the enzyme[16]. The ability to retain enzyme activity at high temperatures expands the range of conditions suitable for enzyme function and provides a number of processing advantages such as reduced risk of microbial contamination, lower viscosity, improved transfer rates and improved substrate solubility[17].

A stable, immobilized enzyme system is suitable for a number of applications that would not be feasible with a soluble enzyme system. One such application is a flow-through 'mini-reactor' system whereby the immobilized enzyme can be continually reused over an extended period of time. We prepared biosilica-entrapped butyrylcholinesterase in two alternative systems: (i) a fluidized bed system and (ii) a packed-bed system (**Fig. 3**).

In both the fluidized-bed and packed-bed systems with a flow rate of 1 ml/min, complete conversion of the indophenyl acetate solution continued for over 1,000 column volumes (500 ml) of substrate solution with no significant loss in enzyme activity or conversion efficiency. In the fluidized-bed system at 2 ml/min, the reaction rate could be sustained for over 2,000 column volumes of substrate solution, again with little loss in activity, but the conversion efficiency was lower (~50%) because of the lower retention time within the column. In the packed-bed system, however, the conversion rate decreased with time (**Fig. 3**). The loss of activity could not be attributed to washout of the protein because no protein was detected in the eluate. When the packing material was removed, resuspended and returned to the column, the conversion rate returned to its original maximum and the activity decreased again over time, which showed that the immobilized enzyme was still present and active (**Fig. 3**). The silica spheres from the packed-bed columns formed a solid plug that showed initial signs of cracking. We conclude that the enzyme activity was not reduced during the continuous flow, but rather that the overall retention time was lowered, owing to packing of the silica particles and subsequent formation of channels within the packing material. The mechanical stability of the biosilica-immobilized enzyme indicates that it can be used in flow-through applications, but the configuration of the apparatus remains to be optimized. For batch systems, the immobilized biocatalyst can be recovered from the reaction medium and reused, reducing cost.

Our preliminary investigations indicate that several other enzymes can be immobilized using the biosilica entrapment method. The ability to immobilize a variety of biomolecules offers new opportunities to combine disparate biological functionalities into new architectures. The biomimetic silicification process opens up a simple route to a structurally defined nanoporous support matrix, which may provide the basis for the development of biosensors, decontamination systems and immobilized enzyme reactors. Additionally, the physical morphology of the biosilica structures can be manipulated by variations in the reaction environment[13], allowing the shape to be tailored to the application. For example, frits or filters containing immobilized enzymes could be incorporated into specific devices for detection or catalysis.

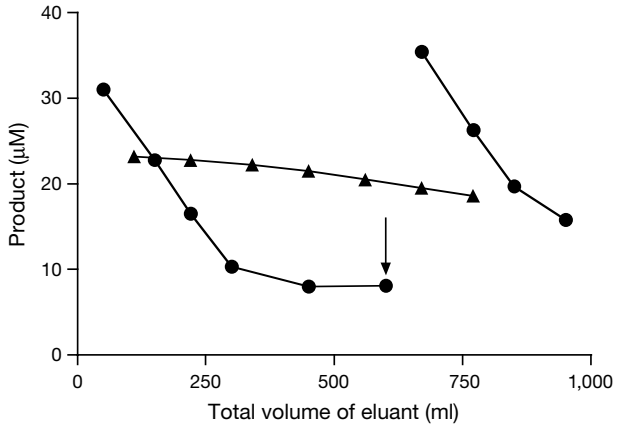

Figure 3 Stability of biosilica-immobilized butyrylcholinesterase in a flow-through system. Activity of biosilica-immobilized butyrylcholinesterase was compared in packed-bed (●) and fluidized-bed systems (▲). The concentration of the hydrolysis product (absorbance at 630 nm) was measured in the eluate. The feed solution of indophenyl acetate (2×10^{-4}M) was supplied at a flow-rate of 2 ml/min. At the point indicated (↓) the biosilica was removed from the column, washed by centrifugation and returned to the column.

METHODS

Enzyme and reagents. Butyrylcholinesterase (EC 3.1.1.8) was from Sigma (highly purified lyophilized powder from equine serum containing approximately 50% protein and activity of 1,200 units per mg protein). Enzyme stock solutions were prepared by dissolving the lyophilized enzyme in cholinesterase-specific buffer (0.1 N NaOH, 0.1 M KH_2PO_4, pH 8)[18]. The stock solutions were divided into aliquots of 50 U/ml based on the nominal units of activity as designated by the manufacturer. All other chemicals were of analytical grade and obtained from Sigma-Aldrich. The synthetic peptide, R5, was obtained from New England Peptides.

Preparation of biosilica, with entrapped enzyme. A stock solution of the R5 peptide (100 mg/ml) was prepared in deionized water. Silicic acid was prepared by hydrolyzing tetramethyl orthosilicate (TMOS) in 1 mM hydrochloric acid to give a final concentration of 1 M. The precipitation mixture consisted of 80 μl of butyrylcholinesterase stock solution (3.5 μg protein), 10 μl of hydrolyzed TMOS (final concentration of 10 mg/ml) and 10 μl of peptide stock. The mixture was agitated for 5 min at room temperature (22 °C). The resultant silica particles were removed by centrifugation for 10 s (14,000*g*) and then washed twice with deionized water. For long-term stability experiments, duplicates of free enzyme and biosilica-immobilized enzyme were prepared with and without the addition of an antibiotic and antimycotic solution (Sigma) at a final concentration of 10 ml/L.

For SEM analysis, the silica precipitate was washed with distilled water and mounted onto a carbon tape secured to an aluminum stub and observed under a Phillips XL30 FEG environmental scanning microscope.

Determination of butyrylcholinesterase enzyme activity. Butyrylcholinesterase activity was measured spectrophotometrically at 630 nm as described[18] using indophenyl acetate (2×10^{-4} M) as a substrate and a cholinesterase-specific buffer. At pH 8.0, cholinesterases hydrolyze the yellow indophenyl acetate to a blue reaction product (4-(4-hydroxy-phenylimino)-cyclohexa-2,5-dienone). The absorptivity of the product was determined to be 8,100/M/cm based on an assumption of complete conversion by butyrylcholinesterase. Silica particles were removed by centrifugation (10 s at 14,000g) before determination of absorbance. All assays were done at room temperature unless otherwise stated. Protein concentration was determined by using a bicinchonic acid protein assay kit (Pierce Biotechnology).

Continuous flow experiments. For continuous flow experiments, 0.5 ml polyethylene solid-phase extraction tubes (Supelco) were packed with biosilica particles from a 1 ml reaction mixture containing enzyme at a final concentration of 50 U/ml. The silica particles were supported by 20 μm polyethylene frits (Supelco). A packed-bed reactor system and a fluidized-bed reactor system were investigated. The packed column was prepared by enclosing the biosilica tightly between the frits. The column was washed with 5 ml of cholinesterase-specific buffer before use. The indophenyl acetate solution (2×10^{-4} M final concentration in cholinesterase-specific buffer, pH 8) was pumped through a 0.22 μM filter and then through the column at a fixed rate. The effluent was collected in 10 ml fractions in a fraction collector before determination of the absorbance of the blue reaction product. The movement of the biosilica nanospheres in the fluidized-bed column was created by the motion of liquid flowing upwards through the column; the volume in the fluidized-bed reactor was 0.5 ml. The quantity of biosilica-immobilized enzyme was the same in both cases. The entire apparatus was enclosed within an incubator and maintained at 25 °C.

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