0273-2289/96/5901—0001$06.50

Cholesterol Oxidation by Microencapsulated Cholesterol Oxidase

P. T. VASUDEVAN* AND TAO ZHOU

Department of Chemical Engineering, Kingsbury Hall, University of New Hampshire, Durham, NH 03824

Received April 25, 1995; Accepted May 8, 1995

ABSTRACT

Cholesterol oxidase from *Brevibacterium* was microencapsulated using a phase-separation technique. The stability of the enzyme during storage and the activity of the microencapsulated enzyme were determined and compared with the soluble enzyme. The effects of various parameters during microencapsulation on enzyme activity are discussed.

Index Entries: Cholesterol; cholesterol oxidase; *Brevibacterium;* microencapsulation.

INTRODUCTION

Cholesterol oxidase from *Brevibacterium* is a flavin-dependent enzyme that catalyzes the oxidation and isomerization of 3β-hydroxy steroids. The enzyme contains 1 mol of the cofactor flavin adenine dinucleotide (FAD)/mol of the protein. The highest enzyme activity is observed using cholesterol as a substrate. Cholesterol esterase and cholesterol oxidase are the two enzymes used in cholesterol assay. Trettnak and Wolfbeis (*1*) have developed a fiberoptic cholesterol biosensor in which cholesterol oxidase was covalently coupled on to a nylon membrane and the consumption of oxygen was measured by fluorescence microscopy. There are a number of advantages in immobilizing the enzyme for use in biosensors, and these are well known. We wanted to examine the effects of microencapsulating cholesterol oxidase in order to determine the stability

*Author to whom all correspondence and reprint requests should be addressed.

and activity of the enzyme on microencapsulation. Microencapsulation was considered since the enzyme can then be adapted to a variety of configurations and to specific processes. Potential applications include enzymatic assay of free cholesterol and also for separation of cholesterol from animal fats. Saponification of fats and oils is ineffective with constituents, such as sterols, tocopherols, and pigments. There is a growing consumer awareness about the effects of cholesterol, and many consumers wish to reduce their intake of cholesterol, a sterol found primarily in animal fats. Cholesterol can be partially separated from animal fats by crystallation, but the process is not economically viable. Cholesterol oxidase from *Brevibacterium* is relatively inexpensive compared to the enzyme from other sources, such as *Nocardia*. However, for obvious reasons, the enzyme cannot be used in the soluble form in such applications. Hence, we decided to examine the efficacy of microencapsulating cholesterol oxidase from *Brevibacterium*, and the results are reported in this article.

MATERIALS AND METHODS

Materials

Ash-free cholesterol (C-3292) and cholesterol oxidase from *Brevibacterium* (C-8153) were obtained from Sigma Chemical Company, St. Louis, MO. The activity of cholesterol oxidase was measured in International units and 1 U converts 1.0 mmol of cholesterol to 4-cholestene-3-one/min at pH 7.0 and at 37°C.

Materials needed for microencapsulating cholesterol oxidase are acetate buffer (pH = 5.0 at 25°C), glutaraldehyde crosslinking reagent, cellulose acetate butyrate (CAB), polyethylene imine (PEI), methylene chloride, and toluene. All these materials were obtained from Aldrich Chemical Company (Madison, WI). The zeolite (Zeolite W) was obtained from UOP, NJ.

Preparation of Buffer Solution

A so-called biological buffer solution of pH 7.4 at 37°C, the same pH value as that of human blood, was prepared by dissolving 1.360 g of KH_2PO_4 and 5.677 g of Na_2HPO_4 in 1 L of distilled and deionized water. After mixing this buffer with Triton X-100 at a ratio of buffer/triton = 9:1, we obtained a pH of 7.38–7.40 at 37°C.

Preparation of Cholesterol Solution

Cholesterol solution was prepared according to the following procedure: 500 mg of cholesterol were dissolved in 10 mL of Triton X-100 by slowly heating and stirring until the solution was clear, and 90 mL of the prepared buffer solution were then added. This gave a cholesterol concentration of 500 mg/dL.

Preparation of Soluble Enzyme Solution

A known amount of enzyme was weighed and dissolved in the buffer solution to obtain a cholesterol oxidase concentration of 1 U/50 μL. The solution was freshly made just before each experiment.

Preparation of Microencapsulated Enzyme Particles

Cholesterol oxidase was directly immobilized onto the zeolite particles, and the cellulose acetate butyrate microencapsulates containing the particles were made by a phase-separation technique (*2*). Details of the preparation are as follows.

For the enzyme preparation (cholesterol oxidase-zeolite sorbent [ZWO]), 100 U of cholesterol oxidase (approx 5.7 mg) and 500 mg of zeolite were suspended in 50 mL of acetate buffer (pH 5.0 at 20°C) under gentle stirring. Seven milliliters of glutaradehyde crosslinking reagent were added after 20 min, and the suspension was gently stirred for another 25 min. ZWO sorbent was then precipitated by centrifugation for 10 min. After decanting the acetate, the precipitated particles were mixed with 3 mL of the phosphate buffer and 100 mg of PEI, and the contents wer gently mixed to make a gel-like solution. This preparation was emulsified in 10 mL of an organic polymer solution (2.5% CAB solution, prepared by dissolving CAB in a solution containing 1.5:1 by volume of methylene chloride/toluene solvent) at room temperature. While stirring at 500 rpm, 8 mL of toluene were added dropwise (about 20–25 drops/min) to precipitate a CAB membrane around the ZWO sorbent microdroplets. After removal of the methylene chloride/toluene supernatant, the microcapsules were dried under vacuum for 2 h and then dried in a shallow plate under a ventilated hood for 4 h. The dried microcapsules were refrigerated in an air-tight container.

Experimental Procedure

A YSI Model 5300 Biological Oxygen Monitor was used in all the experiments. The system consisted of a 5300 Monitor, a 5301 Standard Bath Assembly, and two 5331 Standard Oxygen Probes. A chart recorded was connected to the monitor to collect experimental data. An oxygen cylinder (99.6% oxygen) was used to supply oxygen to the experiments. All experiments were conducted at a temperature of 37°C and pH of 7.4.

The following procedure was used in reactions catalyzed by soluble cholesterol oxidase. Five milliliters of the cholesterol sample solution were injected into the reaction chamber, and the solution was saturated with oxygen under agitation. Between 10 and 50 μL (0.2–1.0 U) of the cholesterol oxidase solution were then injected. The oxygen consumption in the first 30 s was used to determine the initial reaction rates.

For runs with microencapsulated enzyme, cholesterol sample solutions were saturated with oxygen while stirring and monitoring with the oxygen probe. A known amount of the microcapsulated particles (15.5–62

Table 1
Activity Data for Microencapsulated and Soluble Enzyme

Encapsulated enzyme			Soluble enzyme		Comparison
Encap. enz. mg	Theor. act. units	Init. rate m*M*/min	Sol. enz. U/mL	Init. rate m*M*/min	Actual act., encap. U
15.5	2.5	0.064	0.2	0.289	0.18
20.6	3.33	0.068	0.16	0.239	0.19
31.0	5.0	0.077	0.12	0.169	0.23
34.5	5.56	0.081	0.08	0.116	0.25
62.0	10.0	0.104	0.04	0.070	0.34

mg) containing the cholesterol oxidase was weighed in the reaction chambers. After setting up the reaction chambers in the bath, 5 mL of the oxygen-saturated sample solution were injected into the chamber, and immediately the plunger of the oxygen-probe was inserted to bring the liquid level between the lower end of the access slot and the overflow groove of the plunger, making sure that there were no gas bubbles in the solution. The stirrer brake was released to start the stirrer. On the chart recorder paper, a rise in oxygen level to a certain percentage (owing to higher oxygen level in the sample solution than that in air) followed by a decrease was observed. From this, the initial reaction rate was measured from the point where the oxygen level decreased steadily. These reaction rates were compared with that of the soluble enzyme-catalyzed reaction to determine the relative activity of the microencapsulated enzyme.

RESULTS AND DISCUSSION

The weight of the microencapsulated enzyme for one set of runs was found to be 620 mg (theoretical weight is 628.4 mg), and this encapsulated theoretically 100 U of cholesterol oxidase; 6.2 mg of the capsules should therefore theoretically contain 1 U of cholesterol oxidase if no enzyme is lost during the microencapsulation. The initial reaction rate catalyzed by encapsulated cholesterol oxidase was found to increase linearly with the amount of enzyme utilized, in agreement with the results for soluble cholesterol. The results are tabulated in Table 1. The correlation coefficient in both cases was found to be 0.995. The theoretical activity shown in Table 1 for the encapsulated enzyme refers to the maximum possible activity that can be achieved, assuming no enzyme is lost during microencapsulation, and also that there is no activity loss. The last column in Table 1 shows the actual activity of the microencapsulated enzyme. This was obtained by comparing rate data for the soluble and encapsulated enzymes. The rate data shown in Table 1 were found to be highly reproducible.

From Table 1, it appears that there is considerable loss in activity on microencapsulation. There are many reasons that could cause the low

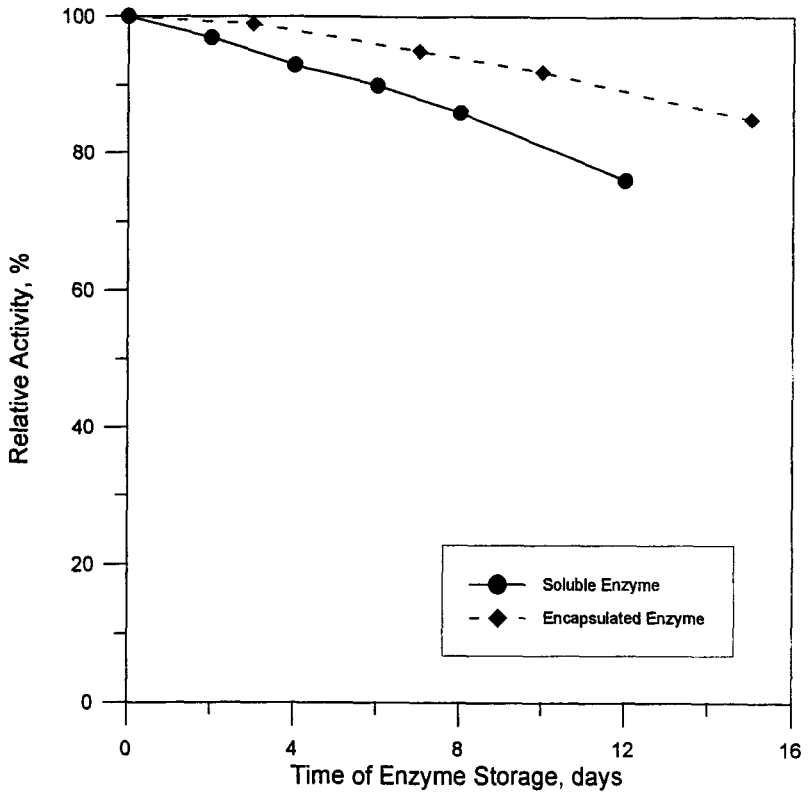

Fig. 1. Relative activity of encapsulated and soluble cholesterol oxidase. Enzymes were stored at 4°C.

efficiency. First, some protein might be lost during the preparation of the microcapsules. We believe that this is the most important reason, since in the preparation procedure outlined earlier, the acetate buffer is decanted, and enzyme that is not adsorbed on the zeolite or crosslinked will be removed with the buffer. Second, the enzyme in the microcapsules could lose some activity during the drying period since the cells need several hours to get rid of the moisture and organic solvent even under vacuum (this can be considerably reduced by lyophilization). Finally, reduction in activity can be attributed to diffusional limitations, since cholesterol molecules have to penetrate the capsule wall and the PEI has to reach the enwrapped cholesterol oxidase and be oxidized and isomerized there; the oxygen molecules have to diffuse in to regenerate the enzyme. The products molecules undergo the same path in an opposite direction.

The encapsulated cholesterol oxidase could be stored longer than the soluble forms of the enzyme. Figure 1 shows the relative activities of cholesterol oxidase in microcapsules and solution after 2–15 d of storing in refrigerator at 4°C. The relative activity is defined as the ratio of the activity of stored enzyme and freshly prepared enzyme. The encapsulated cholesterol oxidase could maintain 98% activity when stored at 4°C for 3 d as Fig. 1 indicates. Also, it is clear from this figure that the deactivation rate

is much faster for soluble enzyme compared to microencapsulated enzyme. The used encapsulated cholesterol oxidase is also reusable after washing, and had about 95% of its initial activity.

The conditions for preparation of the microencapsulated enzyme have to be carefully controlled. Glutaraldehyde, which is added after the immobilization of cholesterol oxidase on the zeolite surface to stabilize the enzyme within the cell, is also a poison to the enzyme. The amount of glutaraldehyde used, therefore, is critical to the process. The stirring speed has to be very slow in the immobilization process, since the enzyme is sensitive to shear forces. An experiment showed that even if the amount of cholesterol oxidase was doubled (to 200 U in a preparation) and other conditons maintained the same as described under Experimental Procedure, doubling the stirring speed (to about 300 rpm) resulted in an 80% loss in enzyme activity.

Experiments were conducted by using different amounts (50, 100, 150 mg) of PEI (50%) to study the effect on morphology. The experiment with 100 mg PEI gave the best results. With 50 mg of PEI, the particles were too small and often blocked the stirrer in the reactor, since the lesser amount of PEI could not gel the fine particles effectively. On the other hand, too much PEI resulted in bigger capsules, which resulted in diffusional problems of both substrate and product.

The stirring speed during the step of precipitating a membrane around the particles is also critical. The optimum stirring speed and rate of toluene addition to prevent gelling of PEI and to ensure that the particles were covered with a proper coating of CAB were found to be 500 rpm and 20–25 droplets/min, respectively. When the stirring speed was doubled (1000 rpm) in the precipitation step, we obtained only about 27% activity compared to the capsules prepared with a stirring speed of 500 rpm.

CONCLUSIONS

Cholesterol oxidase from *Brevibacterium* was microencapsulated using a phase-separation technique. The microencapsulated enzyme exhibited a higher storage stability than its soluble counterpart, and could be reused as well, retaining 95% of its initial activity. The loss in enzyme activity during microencapsulation is attributed mainly to the preparation procedure in which enzyme is probably carried with acetate buffer during decantation. In other words, we believe that only a fraction of the enzyme in the solution is microencapsulated. The stirrer speed, rate of toluene addition, and amount of PEI added during microencapsulation have to be carefully controlled in order to ensure that an enzyme with the highest possible activity is obtained.

REFERENCES

1. Trettnak, W. and Wolfbeis, O. S. (1990), *Anal. Biochem.* **184,** 124–127.
2. Chibata, I. (1978), *Immobilized Enzymes,* John Wiley, New York, p. 59.