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Calcium Alginate Gel as Encapsulation Matrix for Coimmobilized Enzyme Systems

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Abstract

Encapsulation within calcium alginate gel capsules was used to produce a coimmobilized enzyme system. Glucose oxidase (GOD) and catalase (CAT) were chosen as model enzymes. The same values of V_{max} and $K_{m\ app}$ for the GOD encapsulated system and for the GOD-CAT coencapsulated system were calculated. When gel beads and capsules were compared, the same catalyst deactivation sequence for the two enzymes was observed. However, when capsules were employed as immobilization support, GOD efficiencies were higher than for the gel beads. These results were explained in terms of the structure of the capsules.

Index Entries: Calcium alginate; gel capsules; gel beads; coimmobilization; glucose oxidase; catalase.

Introduction

The immobilization of enzymes onto insoluble supports is an active topic of research in enzyme technology and is essential for the application of such systems to industrial processes. The general operational advantages of immobilized enzymes include reusability (especially if the enzymes are scarce or expensive), the possibility of batch or continuous operational modes, better-quality products (there should be little enzyme in the product requiring inactivation or downstream purification), a great variety of engineering designs for continuous processes, and the potential for greater efficiency in consecutive multistep reactions.

Effective enzyme immobilization can be achieved using several techniques, one of which is encapsulation within a gel matrix. In contrast to gel beads, capsules and microcapsules consist of a liquid core surrounded by

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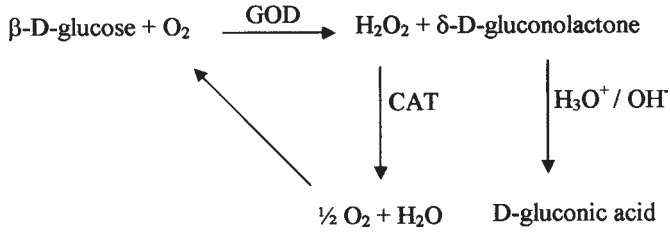

Fig. 1. Reaction mechanism of GOD/CAT system.

a semipermeable membrane that retains the biocatalyst inside *(1)*. There are two main advantages inherent in this immobilization method: first, the particle structure allows appropriate contact between the substrate and biocatalyst since the biocatalyst is in solution within the core capsule, and, second, it is possible to immobilize several enzymes at the same time *(2)*.

Of all the different polymers that can be employed for the encapsulation of biologically active materials, alginate is used extensively because it is biochemically inert and gels under mild conditions *(3)*. In addition, immobilization within calcium alginate gel capsules is a simple and inexpensive immobilization technique in terms of both capital and operational costs *(4)*. As a naturally occurring polysaccharide found in intercellular tissues of brown algae *(5)* and in some soil bacteria *(6)*, alginate is a linear polysaccharide of 1,4-glycosidically linked polymannuronate, polyguluronate, and polymannuronate-guluronate blocks *(7,8)*. In solution, alginates behave like flexible coils. However, on interaction with divalent metal ions—such as Ca^{2+}—they form ionotropic gels *(9)*. The gel is a porous structure that allows diffusion of substrates and products but retains the larger molecular weight encapsulant. All these characteristics have contributed to calcium alginate capsules finding wide-ranging applications in many different areas of science.

In this respect, we report here the use of this technique to produce a coimmobilized enzyme system. Glucose oxidase (GOD) and catalase (CAT) were chosen as model enzymes. Both enzymes are nature related because they participate in the enzymatic pool of those microorganisms able to oxidize glucose to gluconic acid (Fig. 1). The action of GOD produces gluconolactone and H_2O_2 from glucose. In aqueous solution, the gluconolactone converts spontaneously to gluconic acid. The H_2O_2, which causes the deactivation of both enzymes according to first-order kinetics *(10,11)*, is then split to oxygen and water with the aid of CAT.

In previous work, we studied the kinetics of oxidation of β-D-glucose by the two-enzyme system coimmobilized within calcium alginate gel beads *(12,13)*. The main objective of the present work was to compare the performance of the coimmobilized enzyme system GOD-CAT within calcium alginate gel beads and capsules.

Materials and Methods

Reagents

Sodium alginate from *Laminaria hyperborea* was provided by Fluka BioChemika, Switzerland (art. no. 71238). A medium-viscosity sodium salt of carboxymethylcellulose (CMC) was also obtained from Fluka BioChemika (art. no. 21902). Anhydrous $CaCl_2$ (purissimum grade) was used as the calcium salt for capsule formation (Panreac, Spain; art. no. 141219).

GOD (EC 1.1.3.4) type X-S from *Aspergillus niger*, 128,000 activity units/mg of solid (69% protein), was purchased from Sigma (G 7141). One unit of GOD was defined as the amount of enzyme required to oxidize 1 µmol of β-D-glucose to D-gluconic acid and H_2O_2/min at 35°C and pH 5.1 under air-saturated oxygen conditions. CAT (EC 1.11.1.6) from *A. niger*, 6600 activity units/mg of solid, was obtained from Sigma (C 3515). One unit of CAT was defined as the amount of enzyme required to decompose 1 µmol of H_2O_2/min at pH 7.0 and 25°C, while the H_2O_2 concentration falls from 10.3 to 9.2 m*M*.

A solution of D-(+)-glucose in a pH 5.1 buffer (acetic acid/calcium acetate) was used as the substrate (Panreac; art. no. 141341). All other chemicals were commercially available products of reagent grade.

Procedure for Immobilization of Enzymes

Calcium alginate capsules were prepared by extrusion as described in our previous study *(14)*. Alginate solution (1% [w/v]) was used as the anionic solution and 3% (w/v) CMC dissolved in 5.5% (w/v) $CaCl_2$ was employed as the cationic solution. The enzymes were dissolved in the cationic solution. Droplets of the cationic solution were dropped, through a silicone tube (1.6 mm in diameter) using a peristaltic pump, into 200 mL of sodium alginate solution under constant stirring (330 rpm). The dropping height was 10 cm and the gelation time, or period in which capsules were formed, was 1 h.

Under these conditions, the capsules have a diameter in the range of 7.8 ± 0.1 mm, a membrane thickness of 1.15 ± 0.02 mm, and a protein encapsulation efficiency (i.e., the percentage of protein contained within the capsule in relation to the initial amount employed for the capsule formation) of 95 ± 4.

Calcium alginate gel beads were also prepared by extrusion, but in this case, a 1% (w/v) sodium alginate solution was dropped into a 500-mL 5.5% $CaCl_2$ solution under constant stirring. The enzymes were dissolved in the anionic solution.

Reactor Operation and Start-Up

The performance of the coimmobilized GOD-CAT system was studied in both batch and continuous operation modes. The equipment for batch experiments consisted of an automatic, thermostatically controlled

reactor (APPLIKON ADI 1030) equipped with an aeration system, mechanical agitation, and a sample collector. Automatic control was achieved using a proportional, integral, and derivative (PID) computer system. The reactor was constructed of glass and had a capacity of 5.2 L and a working volume of 3 L. The equipment was operated with an aeration rate of 1 vvm and a stirring rate of 300 rpm. The temperature was kept constant at 35°C using a cooling/heating bath with an accuracy range of ±0.1°C.

A glass reactor (300-mL total volume with a working volume of 250 mL) in conjunction with mechanical stirring and a water jacket was used for continuous experiments. Temperature control was provided by a thermostated water bath. All connections were consisted of silicone tubes. The reactor was equipped with an aeration system, and samples were automatically taken at the reactor outlet by a fraction collector. The temperature of operation and the aeration and agitation rates were fixed at the same values as in the equipment used for batch experiments.

In all the experiments, the same protocol for bioreactor start-up was carried out. The solution of glucose in calcium acetate buffer was introduced into the bioreactor and the capsules were added (200 capsules for experiments in batch operation mode and 100 for continuous mode). This instant was considered $t = 0$ in all the experiments carried out. At $t = 0$ and subsequent different time intervals, 1 mL of sample was taken from the reactor and glucose and H_2O_2 concentrations were measured.

The concentration of the substrate glucose was varied from 10 to 125 m*M* in the buffer solution. The solutions were freshly prepared in 50 m*M* calcium acetate buffer (pH 5.1) made up with distilled, deionized water. All experiments were carried out under air-saturated oxygen conditions.

Analytical Methods

Glucose was determined by the dinitrosalicylic acid method *(15)*. H_2O_2 concentration was measured using the method described by Boltz and Howell *(16)*.

Results and Discussion

Performance of Coencapsulated GOD-CAT Enzyme System

To examine the performance of coencapsulated GOD-CAT enzyme, two kinds of experiments were carried out. For the first ones, the glucose concentration was varied in the range of 10–125 m*M*, while the activity ratio of GOD/CAT was kept constant at 6 (18,660 and 3110 activity units of GOD and CAT, respectively). These same experiments were carried out using the same activity units of single-enzyme GOD immobilized within the capsules (Fig. 2). In the second type of experiment, the GOD/CAT activity ratio was varied in the range of 4–24 for a fixed glucose concentration of 14 m*M*. The activity units of GOD were kept constant (Table 1). All these experiments were carried out in triplicate.

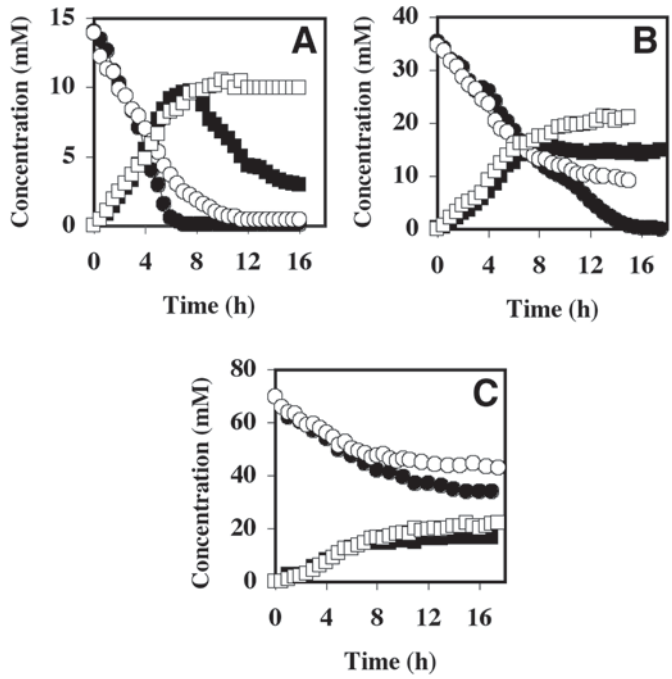

Fig. 2. Kinetics of glucose oxidation (○, ●) and H_2O_2 formation (□, ■) by encapsulated GOD alone (○, □) and coencapsulated GOD-CAT system (●, ■) for bulk glucose concentration of **(A)** 14, **(B)** 35, and **(C)** 70 m*M*.

Table 1
Relationship Between GOD/CAT Activity Ratio and GOD Efficiencies[a]

GOD/CAT	CAT (IU)	Total process time (h)	GOD efficiency · 10^5 (mmol/[L· IU · h])
∞	0	12.0	6.05 ± 0.04
24	777.5	11.5	6.24 ± 0.08
12	1555	10.0	7.03 ± 0.03
6	3110	7.5	9.50 ± 0.07
5	3732	7.0	9.85 ± 0.04
4	4665	7.0	9.90 ± 0.04

[a]$\alpha = 0.05$; $n = 3$.

As illustrated in Fig. 2, similar trends were apparent with respect to glucose concentration in the reactor for all the experiments carried out with the single-enzyme GOD. Three stages, corresponding to the different activity states of GOD, were clearly observed. In the first stage, the glucose concentration decreased in a linear fashion with time, indicating that the glucose oxidation occurred at a constant rate. It can be considered that, during this period, there was sufficient active enzyme to oxide glucose at the rate at which it was transported to the core. In this respect, Michaelis-

Menten kinetic studies were performed with both free and encapsulated GOD. It was estimated that the maximum reaction rate for glucose of the enzyme GOD in solution (V_{max}), in conditions of air saturation, was almost 10 times as high as that of the encapsulated enzyme. This decrease in the V_{max} value caused by immobilization was considered to result from the membrane within the capsules, which offered significant resistance to the transport of glucose *(14)*. After this first stage, a progressive decrease in the reaction rate was produced as a consequence of the irreversible deactivation that GOD suffered owing to the H_2O_2 generated within the capsules. Finally, at the third stage, glucose concentration remained constant in the reactor, indicating that most of the GOD enzyme was completely deactivated or that the glucose in the reactor had been exhausted.

The addition of CAT to the capsules did not change the initial rates of glucose oxidation and, therefore, the same values of the maximum reaction rate (V_{max} = 0.09 ± 0.01 m*M*/min) and the apparent Michaelis constant ($K_{m\,app}$ = 30 ± 6 m*M*) as for the GOD encapsulated system were calculated. Clearly, the use of less concentrated sodium alginate and $CaCl_2$ solutions for the formation of capsules would have diminished the mass transfer limitations. In this respect, it was confirmed that the capsules obtained from concentrated biopolymer and cation solutions had a gel membrane that was more densely crosslinked and, therefore, had a smaller matrix mesh size. Nevertheless, we kept sodium alginate and $CaCl_2$ concentrations at 1.0 and 5.5% (w/v), respectively, because it was experimentally determined that under these conditions the percentage of GOD leakage is negligible—4 ± 2 after 22 h in an agitated buffer solution—and CAT diffusion out of capsules was not observed *(14)*. After this first stage, the rate of glucose oxidation was found to be higher for the experiments carried out with the GOD-CAT enzyme system. As one would expect, the addition of CAT to the capsules increased the overall reaction rate. In the same way, the total process time required for complete oxidation of the glucose in the reactor was directly related to the amount of CAT contained within the capsules (Table 1). In this sense, reduction of the GOD/CAT activity ratio led to a decrease in the total process time or to an increase in GOD efficiency (mmol of oxidized D-glucose/[L · GOD activity units · h]). Therefore, the expected protective effect that CAT exerts on GOD activity was demonstrated once again.

On the other hand, it seems that there is an optimum GOD/CAT activity ratio for the oxidation process. In this sense, under the conditions assayed, reduction of the activity ratio from 6 to 4 had practically no effect on the GOD performance.

Comparisons Between Gel Beads and Capsules for Coimmobilization of GOD-CAT Enzyme System

To compare the reactor performance of the coencapsulated GOD-CAT enzyme system with the corresponding one for gel beads, several experi-

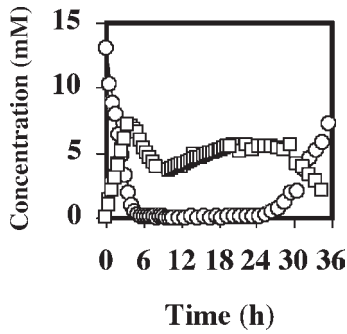

Fig. 3. Kinetics of glucose oxidation (○) and H_2O_2 formation (□) by coencapsulated GOD-CAT system in continuous operation mode. Bulk glucose concentration = 14 m*M*; GOD/CAT = 6; MRT = 3 h.

ments in continuous operation mode were carried out. These experiments were performed using the same stirred-tank reactor and under the same experimental conditions as previously reported for calcium alginate gel beads *(12)*. The results obtained for the coencapsulated GOD-CAT enzyme system for a fixed glucose concentration of 14 m*M*, a GOD/CAT activity ratio of 6, and a mean residence time (MRT) of 3 h are plotted in Fig. 3.

Initially, the glucose concentration decreased in a linear fashion with time and subsequently remained at a constant value very close to zero. It can be considered that, during these stages, GOD is in an active state and, consequently, all the glucose that diffuses into the capsule is oxidized. Nevertheless, about 27 h after the process had started, a progressive increase in glucose concentration was observed. This increase can be explained as being a consequence of the irreversible deactivation that GOD suffers owing to the H_2O_2 generated within the capsules.

Regarding H_2O_2 concentration in the reactor, four stages were observed. In the first stage, the concentration of H_2O_2 increased rapidly and reached a maximum value. Therefore, at this stage, the production of H_2O_2 by GOD is much faster than the rate at which it is consumed by CAT, and the H_2O_2 that is not decomposed within the capsules diffuses into the solution. In the second stage, a decrease in H_2O_2 concentration was observed, indicating that CAT decomposes the H_2O_2 formed in the capsules and also part of the H_2O_2 accumulated in the reactor. However, about 11 h after the process had started, the H_2O_2 concentration increased continuously in the solution, signifying that CAT was being deactivated by its own substrate. Nevertheless, at this stage the glucose had been exhausted in the reactor and, therefore, GOD was in an active state. From these results, it is believed that the H_2O_2 formed in the oxidation reaction deactivates CAT first. The same catalyst deactivation sequence was observed when calcium alginate gel beads were used as the immobilization support *(12)*. In the fourth stage, a decrease in H_2O_2 concentration was observed, and this was thought to be owing to washout phenomena.

When GOD was coencapsulated with CAT, GOD remained in an active state for 50, 27, and 18 h of process when MRTs of 5, 3, and 2 h were used, respectively. Under these conditions, a GOD efficiency of about 2.6×10^{-3} mmol of D-glucose/IU was estimated. However, when the GOD-CAT enzyme system was coimmobilized within calcium alginate gel beads, the system worked at a peak performance for the first 10 h of the process for an MRT of 3 h and a GOD/CAT activity ratio of 1.6. After this time the system gradually began to deactivate *(12)*. Under these conditions, a GOD efficiency almost three times lower than that for the coencapsulated enzyme system was estimated, unless the GOD/CAT activity ratio was lower. Therefore, the protective effect that CAT exerts on GOD activity is lower for the gel beads than for the capsules. This result can be explained in terms of the structure of capsules: since both enzymes are in solution within the liquid core, contact between the substrate and biocatalyst can be achieved in an appropriate way, and the two enzymes are in close contact. As a consequence, the protective effect that CAT exerts on GOD activity is enhanced. Thus, the advantage of liquid-core capsules for the coimmobilization of enzymes over conventional bead entrapment was confirmed.

References

1. Nigam, S. C., Tsao, I. F., Sakoda, A., and Wang, H. Y. (1988), *Biotechnol. Technol.* **2,** 271–276.
2. Chang, T. M. S., McIntosh, F. C., and Mason, F. G. (1966), *Can. J. Physiol. Pharmacol.* **44,** 115.
3. Skjak-Braek, G. and Martinsen, A. (1991), in *Seaweed Resources in Europe: Uses and Potential*, Guiry, M. D. and Blunden, G., eds., CRC Press, New York, p. 219.
4. Christenson, L., Dionne, K., and Lysaught, M. (1993), in *Fundamentals of Animal Cell Encapsulation*, Goosen, F. A., ed., CRC Press, New York, pp. 7–41.
5. Indergaard, M. and Skjak-Braek, G. (1987), *Hydrobiologia* **151/152,** 541–543.
6. Linker, A. and Jones, R. S. (1966), *J. Biol. Chem.* **241,** 3845–3851.
7. Haug, A. (1959), *Acta Chem. Scand.* **13,** 601–603.
8. Grasdalen, H., Larsen, H., and Smidsrød, O. (1981), *Carbohydr. Res.* **89,** 179–184.
9. Grant, G. T., Morris, E. R., Rees, D. A., Smith, P. J. C., and Thom, D. (1973), *FEBS Lett.* **32,** 195–200.
10. Tse, P. H. S. and Gough, D. A. (1987), *Biotechnol. Bioeng.* **29,** 705–713.
11. Malikkides, C. O. and Weiland, R. H. (1982), *Biotechnol. Bioeng.* **24,** 2419–2439.
12. Romero, L. E. and Cantero, D. (1998), in *Stability and Stabilization of Biocatalyst*, Ballesteros, A., Plou, F. J., Iborra, J. L., and Halling, P. J., eds., Elsevier, New York, pp. 107–112.
13. Romero, L. E., Macías, M., and Cantero, D. (1998), *Ingeniería Química* **10,** 189–193.
14. Blandino, A., Macías, M., and Cantero, D. (2000), *Process Biochem.* **36,** 601–606.
15. Miller, G. L. (1959), *Anal. Chem.* **31,** 426–428.
16. Boltz, D. F. and Howell, J. A. (1987), *Colorimetric Determination of Nonmetals*, John Wiley & Sons, New York.