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Immobilization of glucose oxidase within calcium alginate gel capsules

A. Blandino *, M. Macías, D. Cantero

Biological and Enzymatic Reactors Research Group, Department of Chemical Engineering, Food Technology and Environmental Technologies, Faculty of Sciences, University of Cádiz (UCA), W 11 510 Puerto Real (Cádiz), Spain

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Abstract

Glucose oxidase (GOD) was encapsulated within calcium alginate gel capsules. The effects of gelation conditions on capsule characteristics such as thickness, percentage of enzyme leakage and encapsulation efficiency were studied and the optimal conditions for GOD encapsulation obtained. Oxidation of glucose to gluconic acid followed Michaelis–Menten kinetics.

Keywords: Glucose; Glucose oxidase; Calcium alginate; Capsules; Gelation conditions; Kinetics

1. Introduction

Enzymes are used as biocatalysts in the chemical, pharmaceutical and food industriest and as specific ligands in clinical and chemical analysis [1,2]. The ability to make high-cost enzymes reusable and stable has meant that immobilized enzymes have attracted a great deal of attention [3]. Effective enzyme immobilization can be achieved using several techniques, one of which is encapsulation within a gel matrix. This immobilization technique consists of enclosing the enzyme in an aqueous solution inside a semipermeable membrane capsule [4]. Basically, there are two main advantages of this immobilization method: the particle structure allows contact between the substrate and enzyme to be achieved in an appropriate way and, in addition, it is possible to immobilize several enzymes at the same time [5]. With regard to the different biopolymers that can be employed in the formation of the semipermeable membrane in capsules, alginate is one of the most frequently used [6,7] owing to the fact that the immobilization procedure is carried out under very mild conditions [8]. In molecular terms, alginate is an anionic linear polysaccharide with 1,4′-linked D-mannuronic acid and L-guluronic acid residues either as blocks of the same units or as a random sequence of the two sugar residues [9,10]. Two such sequences give rise to an 'egg-box' array upon contact with calciurn ions, forming an ordered gel structure [11]. This complex formation has been exploited and calcium alginate gels used for biocatalyst encapsulation matrices.

Glucose oxidase (E.C. No. 1.1.3.4) was chosen as a model enzyme in this study. In this respect, the enzymic oxidation of β-D-glucose to β-luconolactone and hydrogen peroxide by glucose oxidase (GOD) has been widely studied because of its considerable analytical and industrial applications [12,13]. In the vast majority of these applications glucose oxidase is fixed on different supports to yield derivatives with catalytic activities [14,15]. The support material, which plays an important role in the utility of the immobilized enzyme, should be readily available, non-toxic and should also avoid causing any deleterious alteration of the enzyme. In this context, the main objective of this work was to evaluate the use of calcium alginate gel capsules as a matrix for enzyme immobilization. Some of the capsule characteristics, such as thickness and the stability and diffusional properties of the gel membrane are examined. Furthermore, apparent kinetic parameters of the encapsulated GOD are compared with the intrinsic kinetic parameters of the soluble enzyme.

* Corresponding author. Tel.: +34-956-830907; fax: +34-956-016411.

E-mail address: ana.blandino@uca.es (A. Blandino).

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2. Materials and methods

2.1. Materials

Alginic acid salt obtained from the brown algae (*Laminaria hyperborea*) was purchased from Fluka BioChemika (Fluka art. no. 71238, Switzerland). The specifications of this product are the most suitable for immobilization of microorganisms and enzymes: molecular weight 100 000–200 000; pH (1% in water) 6.0–7.5; loss on drying $\leq 15\%$; ash ≤ 5 27%. A medium viscosity sodium salt of carboxymethylcellulose (CMQ was obtained from Fluka BioChemika (Fluka art. *no*. 21902). According to the manufacturers specifications, a 2% w/v solution has a viscosity range of 400–1000 mPa s at 25°C, a degree of substitution between 0.60 and 0.95, a loss on drying at 110°C of $< 15\%$ and a pH range (1% in water) of 6.5–8.0. Glucose oxidase (β-D-glucose:oxygen-l-oxidoreductase, EC 1.1.3.4.) type X-S from *Aspergillus niger*, 128 000 units per mg solid (69% protein), was purchased from Sigma Chemical Company (SIGMA, G 7141). Its molecular weight was 152 000 Da. D-glucose from Panreac (Spain) was used as the substrate (Panreac art. *no*. 141341). All other chemicals were commercially available products of reagent grade.

2.2. GOD encapsulation procedure

Calcium alginate capsules were prepared by extrusion using a simple one-step process [16]. Several sodium alginate solutions of different concentrations were prepared, according to the particular experiment to be performed, and were used as anionic solutions. For the preparation of cationic solutions, CMC was dissolved in different solutions of $CaCl_2$ to give a 3% w/v CMC solution. CMC was used as a non-gelling polymer to modulate the viscosity and density of the cationic solution in order to ensure the spherical shape of the capsules. The enzyme was dissolved in the cationic solution. Droplets of the cationic solution ($CaCl_2$/CMC/GOD) solution were dropped through a silicone tube, using a peristaltic pump, into 200 ml of sodium alginate solution. The sodium alginate solution was maintained under constant stirring (330 rpm), using a magnetic stirrer situated at the bottom of the vessel, in order to prevent the droplets from sticking together and to minimise the external mass transfer resistance. A dropping height of 10 cm. was used to ensure that spherical droplets were formed. The inner diameter of the silicon tube was 1.6 mm. The total dropping time was kept lower than 1% of the residence time of the capsules in the anionic solution in order to ensure that capsules were all formed over the same period. Once the $CaCl_2$/CMC/GOD solution had been dropped into the alginate solution, a capsular membrane formed instantaneously around each droplet due to the cross-linking of the interfacial alginate molecules by calcium cations. In each experiment, different times of gelation or periods in which capsules were formed were selected. Prior to the removal of capsules the sodium alginate solution was diluted more than four-fold by adding the appropriate amount of distilled water. This dilution of the alginate solution outside the capsules reduces the possibility of capsules sticking together when they are in close contact and also helps to stop the gelation process. After diluting the sodium alginate solution, the capsules were recovered by filtration using a Büchner funnel. With the aim of stabilising the calcium alginate membrane, the capsules were immediately transferred to a 1.3% w/v $CaCl_2$ solution and incubated for 15 min. Finally, the capsules were rinsed with distilled water to remove excess $CaCl_2$. All of the above procedures were carried out at 25°C.

2.3. Determination of GOD leakage from capsules

In order to assess GOD diffusion out of the calcium alginate capsules, 50 capsules were placed in a 100 ml Erlenmeyer flask with 50 ml of 50 MM calcium acetate buffer (pH 5. 1). The buffer solution was agitated at 600 rpm with a Teflon bar on a magnetic stirrer. It was confirmed from preliminary experiments that the liquid film resistance around the capsule could be neglected at the stirrer speed employed. The temperature was held constant at 25°C.

The percentage of diffusion of glucose oxidase was calculated from the measurement of protein concentration within the core of the capsules both before and after the diffusion experiments. These measurements were carried out by placing five capsules in five test tubes, one per test tube, along with 2 ml of buffer solution in each. The capsules were then cut in half in order to dissolve their contents. The percentage of glucose oxidase leakage from the capsules was analysed after 22 h of the process [17].

$$\text{Percentage of leakage} = \left(1 - \frac{[\text{GOD}]_{\text{core},t=22\ \text{h}}}{[\text{GOD}]_{\text{core},t=0\ \text{h}}}\right)\cdot 100 \quad (1)$$

2.4. GOD encapsulation efficiency

In order to assess the enzyme encapsulation efficiency, it was necessary to measure the protein concentration in the cationic solution and within the core of the capsules before the diffusion experiments. Measurements of protein concentration in the cationic solution were carried out by adding five droplets of this solution to five test tubes, one droplet per test tube, each containing 2 ml of buffer solution. The protein concentration in each sample was assayed. The protein con-

centration within the core of the capsule was determined according to the method described above [17].

$$\text{Encapsulation efficiency} = 100\ \frac{[\text{GOD}]_{\text{core},t=0\ \text{h}}}{[\text{GOD}]_{\text{drop}}} \quad (2)$$

2.5. Operation of the reactor and kinetic studies

The kinetics of oxidation of β-D-glucose by both free and immobilized GOD were studied in batch operation mode. The equipment consisted of an automatic, thermostatically controlled reactor (APPLIKON ADI 1030) equipped with an aeration system, mechanical agitation and sample collector. Automatic control was performed with a PID computer system. The reactor was 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 vvv 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

Activities of both free and immobilized GOD were assayed by measuring the concentration change of glucose with a spectrometric assay. The concentration of the substrate glucose was varied from 10 to 125 mM in the buffer solution. All solutions were freshly prepared in 50 mM calcium acetate buffer (pH 5.1) made up with distilled deionized water.

In all experiments the same protocol for starting up the bioreactor was followed. Initially, the glucose solution in calcium acetate buffer was introduced into the bioreactor and air was bubbled through the solution for 60 min. The oxygen concentration was measured using a Clark electrode. After this stabilization time, which was required for thermostatting the bioreactor and obtaining a saturation concentration of oxygen in the solutions, 200 capsules (or free enzyme) were added. The time at addition was taken as $t = 0$ in all the experiments carried out. At different time intervals, including $t = 0$, a 1 ml sample was taken from the reactor. For the experiments with free GOD, 0.2 ml of trichloroacetic acid (15% w/v) was added to stop the reaction.

One unit of glucose oxidase was defined as the amount of enzyme required to oxidise 1 μmol of β-D-glucose to D-gluconic acid and hydrogen peroxide per min at 35°C and pH 5.1.

2.6. Erosion of capsules

The percentage of erosion was calculated from the measurement of membrane thickness of the capsules both before and after the erosion experiments. In order to perform this process 200 capsules were placed in 3 l of calcium acetate buffer solution (50 mM, pH 5.1) in the automatic reactor employed for the kinetic studies. The equipment was operated with an aeration rate of 1 vvv, the temperature was maintained at 35°C and different stirring rates were assayed. The erosion of capsules was measured after 15 h.

2.7. Analytical procedures

Measurement of the external diameter of the capsules was carried out using a caliper after drying the surface of the capsules with filter paper. The membrane thickness was studied by cutting the capsules in half and carrying out measurements in at least four different locations on the membrane. The image processing software MIP 4 ADVANCED allowed the measurement of the membrane thickness on an image of each half capsule captured by a video camera connected to a microscope. Calibration of the system was carried out using the same software on an image of a Neubauer chamber of 0.0025 mm^2. The diameters and gel layer thicknesses reported here represent the average of the measurements performed on ten capsules obtained under the same experimental conditions. The values have a confidence interval of 95%.

The protein concentration in the samples was assessed by the Lowry technique, as modified by Peterson [18]. Each protein concentration determination was carried out in triplicate.

The glucose concentration was estimated by a dinitrosalicylic acid method [19]. For this technique 3 ml of dinitrosalicylic acid reagent was added to the sample and the mixture was heated on a boiling water bath for 15 min. The change in colour was measured using a spectrophotometer at a wavelength of 550 nm.

3. Results and discussion

3.1. Formation of capsules

Fig. 1 shows the relationship between membrane thickness and gelation time at different sodium alginate and $CaCl_2$ concentrations. For these experiments the concentrations of sodium alginate used were 0.5 and 1.0% w/v, while the concentrations of the cationic solution were fixed at 1.3 and 5.5% w/v $CaC1_2$. The thickness of the capsule membrane increased with increasing gelation time and then levelled off irrespective of the concentration of the sodium alginate and $CaCl_2$ solution employed for capsule formation. When a cationic solution containing calcium ions was dropped into anionic alginate, a spherical membrane was formed around the liquid core. Instantaneous diffusion of calcium ions through the interphasic membrane resulted in the progressive build-up of a calcium alginate layer around the core and an increase of the membrane thickness. No significant increase in membrane thick-

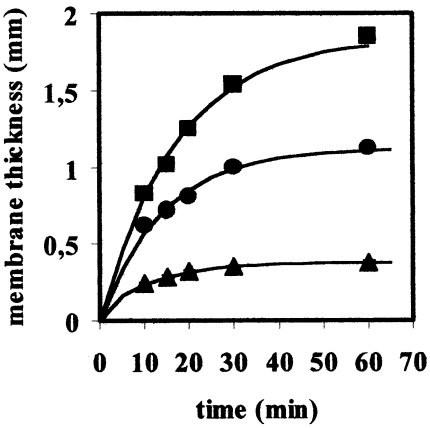

Fig. 1. Effects of gelation time, $CaCl_2$ and sodium alginate concentration on membrane thickness of capsules. Symbols: (●) sodium alginate 1.0% w/v and $CaCl_2$ 5.5% w/v; (■) sodium alginate 0.5% w/v and $CaCl_2$ 5.5% w/v; (▲) sodium alginate 0.5% w/v and $CaCl_2$ 1.3% w/v.

ness was observed after a certain time, indicating complete utilisation of calcium for the cross-linking of the alginate outer layer. This temporal evolution was also exhibited by the external diameter; however, the core diameter — obtained from the external diameter and membrane thickness — was always of the same order of magnitude (5.6 ± 0.1 mm) and was independent of the operational conditions (Tables 1 and 2).

On the other hand, on increasing the sodium alginate concentration of the solutions used in the capsule formation, the thickness of the membrane decreased at a given gelation time (Table 1). This effect was due presumably to the fact that on increasing the number of biopolymer molecules per unit solution volume in the vicinity of the core capsule, the number of binding sites for Ca^{2+} ions also increased. As a result, a more densely cross-linked gel structure would probably form and, consequently, have a lower thickness. In connection with this phenomenon, the capsules obtained from 1.0% w/v sodium alginate solutions were more resistant, from a mechanical point of view, than those obtained from 0.5% w/v solutions. Therefore, it can be stated that the use of 1.0% w/v sodium alginate solutions resulted in a more dense gel and justifies the supposition outlined above. For this reason an alginate concentration of 1.0% w/v was selected for the rest of the experiments carried out.

In other respects, on increasing the $CaCl_2$ concentration the thickness of the membrane increased at a given gelation time (Table 2). This result can be explained by the fact that an increase in the concentration of calcium ions initially contained in the core capsule will result in a higher concentration gradient between the core and the outside solution. This situation will favour the diffusion of Ca^{2+} ions from the core.

3.2. Percentage of GOD losses and encapsulation efficiency

The influence of $CaCl_2$ concentration on the percentage of GOD losses and encapsulation efficiency are

Table 1
Diameters and thicknesses of gel layer capsules as a function of time with different sodium alginate concentrations[a]

	Sodium alginate 0.5% w/v		Sodium alginate 1% w/v	
Time (min)	Diameter (mm)	Thickness (mm)	Diameter (mm)	Thickness (mm)
10	7.1 ± 0.1	0.82 ± 0.03	6.5 ± 0.3	0.55 ± 0.02
15	7.5 ± 0.1	1.01 ± 0.01	7.0 ± 0.1	0.67 ± 0.01
20	8.1 ± 0.2	1.25 ± 0.03	7.1 ± 0.2	0.76 ± 0.04
30	8.5 ± 0.1	1.54 ± 0.02	7.3 ± 0.1	0.89 ± 0.02
60	9.1 ± 0.1	1.85 ± 0.08	7.8 ± 0.1	1.15 ± 0.02

[a] $CaCl_2$ concentration was 5.5% w/v.

Table 2
Diameters and thicknesses of gel layer capsules as a function of time with different $CaCl_2$ concentrations[a]

	$CaCl_2$ 1.3% w/v		$CaCl_2$ 5.5% w/v	
Time (min)	Diameter (mm)	Thickness (mm)	Diameter (mm)	Thickness (mm)
10	6.15 ± 0.1	0.24 ± 0.01	7.1 ± 0.1	0.82 ± 0.03
15	6.2 ± 0.1	0.29 ± 0.01	7.5 ± 0.1	1.01 ± 0.01
20	6.3 ± 0.1	0.32 ± 0.01	8.1 ± 0.2	1.25 ± 0.03
30	6.4 ± 0.1	0.35 ± 0.01	8.5 ± 0.1	1.54 ± 0.02
60	6.45 ± 0.1	0.38 ± 0.01	9.1 ± 0.1	1.85 ± 0.08

[a] Sodium alginate concentration was 0.5% w/v.

Table 3
Percentage of enzyme losses and encapsulation efficiency as a function of calcium chloride concentration[a]

$[CaCl_2]$ (w/v)	GOD losses (%)	Encapsulation efficiency (%)
1.3	39 ± 4	70 ± 2
2.6	33 ± 5	71 ± 5
4	8 ± 3	85 ± 4
5.5	4 ± 2	95 ± 4

[a] Sodium alginate concentration was 1.0% w/v.

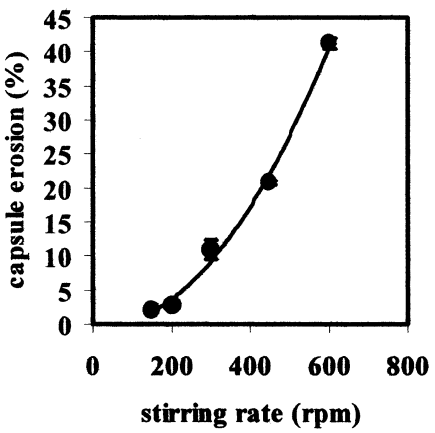

Fig. 2. Relationship between stirring rate and percentage of capsule disintegration.

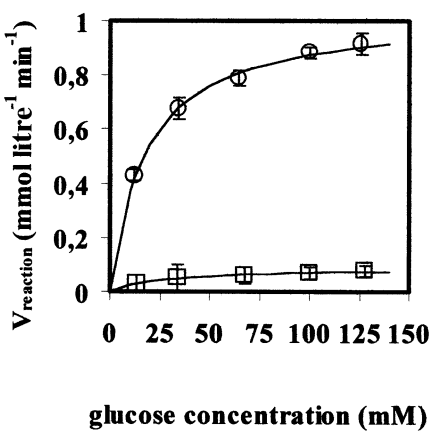

Fig. 3. Reaction rates of free (●) and encapsulated GOD (■) at different glucose concentrations. pH 5.1; temperature 35°C; 0.019 g free enzyme or 0.146 g GOD-immobilized calcium alginate gel capsules.

represented in Table 3. For these experiments, concentrations of $CaCl_2$ were varied in the range 1.3–5.5% w/v, while the concentration of the sodium alginate solution was fixed at 1.0% w/v. The use of concentrated $CaCl_2$ solutions significantly reduced the percentage of GOD that diffused out of the capsules. In this way, the combination of 1% w/v sodium alginate solutions with 5.5% w/v $CaCl_2$ solutions led to percentage enzyme losses of 4 ± 2. Moreover, the enzyme encapsulation efficiency, i.e. the percentage of GOD contained within the capsule in relation to the initial amount employed for the capsule formation, increased on increasing the $CaCl_2$ concentration. All of these results confirm that capsules obtained from concentrated biopolymer and cation solutions have a gel network that is more densely cross-linked and, therefore, have a smaller matrix mesh size. From these results, 1.0% w/v sodium alginate, 5.5% w/v calcium chloride and a gelation time of 1 h were selected as the optimum conditions for the formation of capsules. These experimental conditions were therefore used throughout the rest of the experiments. Under these conditions the capsules have a diameter in the range 7.8 ± 0.1 mm, a membrane thickness of 1.15 ± 0.02 mm and an encapsulation efficiency of 95 ± 4.

3.3. Capsule erosion

The membrane stability of the capsules was also evaluated. Fig. 2 shows the relationship between the percentage of gel erosion and stirring rate. The percentage of erosion increased dramatically with increasing stirring rate. This trend was expected given the fact that traction forces on capsules increased on increasing the stirring rate. However, no significant changes were observed in the percentage of gel erosion when the capsules were maintained in the reactor for a longer process time (25 h).

From the results obtained it is advisable to use stirring rates lower than 400 rpm for prolonged times in bioreactors with mechanical agitation.

3.4. Kinetic analysis

In order to study the kinetic effect of encapsulation, the initial rates of glucose oxidation reaction by the free and encapsulated GOD were measured at various glucose concentrations. Free enzyme (0.019 g) or calcium alginate capsules containing 0.146 g of GOD were used. In the present experiments, oxygen concentration was kept high (above 10 ppm throughout the assays) so as not to retard the oxidation rate of glucose. Thus, the initial reaction rate is expressed by Michaelis–Menten kinetics (Fig. 3). The same behaviour has been described previously when GOD was encapsulated within liposornes [20] and within transparent glass capsules [21].

The maximum reaction rate (V_{max}) and the Michaelis constant (K_m) of the free and encapsulated GOD were calculated, for air saturation, using a non-linear regression computer program. The results obtained are shown in Table 4. The decrease in the V_{max}, value caused by immobilisation is considered to result from the membrane within the capsules, which can offer

Table 4
Kinetic parameters for free and encapsulated GOD

GOD	V_{max} (mM min^{-1})	K_m (mM)	r^2
Free	1.03 ± 0.11	18 ± 6	0.99
Encapsulated	0.09 ± 0.01	30 ± 6	0.98

significant resistance to the transport of substrates. Therefore, the immobilization procedure limited accessibility of glucose molecules to the active sites of the enzyme and caused a decrease in the maximum reaction rate. The apparent K_m, of the encapsulated enzyme was almost twice as high as that of the enzyme in solution, indicating that the binding of substrates was weaker.

4. Conclusions

Calcium alginate gel capsules have been used as an enzyme immobilization matrix. The immobilization method has an advantage in that it is possible to tailor some of the capsule characteristics, such as thickness and percentage of enzyme leakage, by altering appropriate formulation conditions like gelation time and sodium alginate and $CaCl_2$ concentrations. The optimum conditions selected for the effective encapsulation of glucose oxidase were 1% w/v sodium alginate, 5.5% w/v $CaC1_2$ and 1 h gelation time. On using these conditions for capsule formation it is advisable to use stirring rates lower than 400 rpm for prolonged times in bioreactors with mechanical agitation. The apparent kinetic parameters of the encapsulated and free glucose oxidase were compared, and this showed that the Michaelis constant (K_m) of the immobilized GOD was higher than that of the free GOD, while there was a more pronounced difference in the maximum reaction rates (V_{max}).

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