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Dextran aldehyde coating of glucose oxidase immobilized on magnetic nanoparticles prevents its inactivation by gas bubbles

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Abstract

Glucose oxidase (GOX) was readily inactivated in stirred systems by the interaction between the enzyme and the hydrophobic interfaces of gas bubbles. Immobilization of the enzyme on glutaraldehyde-agarose (a porous support) under very mild conditions yielded preparations with similar thermostability as the soluble GOX. However, this immobilization permitted the enzyme to retain full stability in stirred systems due to lack of undesired interaction of the gas bubbles with the enzyme. Similar immobilization of the enzyme on magnetic nanoparticles, a non-porous support, presented very different results: here GOX was inactivated by the gas bubbles in a similar way to the soluble enzyme. This could be due to the fact that the enzyme is now immobilized on the external surface of the particles, and, therefore, it is fully exposed to the gas bubbles. The coating of the enzyme molecules with aldehyde dextran, while maintaining the enzyme thermostability, permitted to avoid enzyme inactivation by gas bubbles.

Keywords: Enzyme inactivation; Interaction with hydrophobic interfaces; Dextran coating of enzymes; Magnetic nanoparticles to immobilize enzymes; Glucose oxidase

1. Introduction

Enzyme immobilization is usually considered as a technique that increases the operational stability of enzymes by diverse reasons: the enzyme is dispersed and cannot aggregate, interaction with external interfaces is prevented [1–3] and the enzyme structure may be rigidified by multipoint covalent attachment [4–7]. Certainly, this happens if a porous support is used, where the enzyme is immobilized inside the pores and cannot interact with any other enzyme molecule or interface.

However, the interest in using magnetic particles as supports for immobilization of enzymes as biocatalysts is constantly increasing nowadays [8–10]. These supports have a very small size [9], and despite being non-porous, they permit to immobilize over 100 mg of protein/gram of wet particles. A small nanoparticle would be very difficult to recover and handle at industrial level. However, the use of magnetic nanoparticles may be a suitable solution to overcome such problems, the use of a magnet allowing a simple recovery of the catalyst [10,11].

In some instances, these non-porous supports may present some advantages compared to the use of porous supports. For example, they have no external diffusion problems. In fact, non-porous supports may be the only industrial option to use in solid–liquid systems (e.g., precipitated protein, agriculture wastes). However, by using these non-porous nanoparticles, the enzyme is immobilized on their external surface and therefore, the protective effect of the immobilization inside the pores of a porous support is lost.

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Thus, it is possible that an enzyme, immobilized on these nanoparticles, may suffer inactivation by the mechanisms described for soluble enzymes, especially through interaction with gas bubbles generated by strong stirring or bubbling of oxygen [12]. Inactivation by interfaces proceeds via destabilization of electrostatic, hydrophobic and hydrogen bonds of the protein, leading to the irreversible denaturation of the enzyme [13]. Recently, our group has described that covering soluble enzymes with polymers (e.g., aldehyde dextran) may greatly reduce the interaction of the enzymes with air–liquid interfaces, thus avoiding this inactivation mechanism [14]. Herein, by using glucose oxidase as a model enzyme, we have attempted to assess the protective effect on the inactivation of this protein by interaction with air–liquid interfaces promoted by the immobilization of the enzyme on the surface of a nanoparticle. Finally, we have studied if the dextran coating of the enzyme immobilized on these nanoparticles may be a suitable solution to overcome this problem.

2. Materials

Glucose oxidase (GOX) from *Aspergillus niger*, 2,2′-azino-bis(3-ethylbenzathiazoline-6-sulfonic acid) (ABTS), sodium borohydride, horseradish peroxidase, ethylendiamine and carbodiimide were purchased from Sigma (St. Luis, MO, U.S.A). Glucose was from Panreac (Barcelona, Spain). Sodium periodate was from Merck (Darmstadt, Germany). Dextran was from Fluka (Buchs, Switzerland). As a porous support, 6% cross-linked amino agarose beads (MANAE) (mean particle size 45–165 μm, mean pore size 70 nm) activated with 1 μmol/g was prepared as previously described in [15]. As a non-porous support, carboxylated magnetic nanoparticles (57 μequiv. of COOH/g, containing 54% ferrite) EM1-100/40 (this is the non-porous support) were supplied by Estapor Microspheres (Merck Co., France), having a mean particle diameter of 800 nm. Other reagents were of analytical grade.

3. Methods

3.1. Activity assay of GOX

Glucose oxidase activity was determined spectrophotometrically by an increase in absorbance at 414 nm resulting from the oxidation of ABTS through a peroxidase coupled system [16]. The reaction mixture consisted of 1 mL of 100 mM sodium phosphate buffer at pH 6.0, 0.5 mL of 1 M glucose, 0.1 mL of a 10 mg/mL ABTS solution prepared in distilled water and 0.1 mL of a 2 mg/mL peroxidase solution in 100 mM sodium phosphate buffer at pH 6.0.

One enzymatic unit causes the oxidation of one micromole of ABTS per minute at 25 °C and pH 6.0 under the specified conditions.

3.2. Preparation of glutaraldehyde agarose

Ten milliliters of 6% cross-linked-MANE-agarose activated with 1 μmol amino groups/mL were suspended in 90 mL of 11% glutaraldehyde (v/v), 100 mM sodium phosphate, pH 7. The gel was gently stirred for 14 h and then washed with a great excess of distilled water. This activated support was immediately used after its preparation [17].

3.3. Immobilization on glutaraldehyde agarose

Five grams of activated agarose was incubated with 10 mL of a GOX solution (13.5 UI/mL) in 25 mM sodium phosphate buffer at pH 7. Periodically, samples of supernatant and suspension were withdrawn and their activity was assayed as described above.

3.4. Preparation of aldehyde dextran

Aldehyde dextran (M_w 20,000) was prepared by complete oxidation with sodium periodate as previously described [17]. A 100 mL solution containing 3.33 g of dextran in distilled water was prepared. Then, 8 g of sodium periodate was added, which permitted the full oxidation of the dextran molecule, and the mixture was allowed to react at 25 °C. After 3 h, the solution was dialyzed against 50 vol of distilled water to eliminate the formaldehyde produced during the oxidation.

3.5. Activation of magnetic particles

One milliliter of commercial suspension of magnetic particles (10 mg/mL) was washed three times with 100 mL of distilled water during 30 min in an end-over-end rotation. After that, the particles were suspended in 100 mL of 1 M ethylendiamine solution at pH 6 containing 1 mM carbodiimide, to modify only 10% of the carboxyl groups in the support [18,19]. After 90 min of reaction at 25 °C, the particles were washed 5 times with 100 mL distilled water for 30 min.

The aminated particles thus obtained were incubated with a 10% glutaraldehyde solution in 200 mM sodium phosphate buffer, pH 7, during 16 h at 25 °C. After the incubation, the particles were washed thoroughly with distilled water.

3.6. Immobilization of GOX onto activated particles

20 mg of activated particles were incubated with 20 mL of 25 mM sodium phosphate buffer at pH 7 containing 7 UI/mL of GOX. The suspension was allowed to react at 25 °C and at different time intervals, samples of the supernatant as well as the suspension were withdrawn and measured for enzyme activity. As an end point for the immobilization, 35 mL of ice-cold 100 mM sodium bicarbonate pH 8.5 and sodium borohydride were added to reach a concentration of 1 mg/mL

to the solution in order to reduce the remaining aldehyde groups and the amine aldehyde bonds [20]). This solution was stirred for 15 min and then 50 mg of sodium borohydride were again added. Once again this solution was allowed to react for 15 min at 4 °C and after that the pH was adjusted at pH 7.

3.7. Modification of the immobilized GOX with dextran-aldehyde

Five milligrams of particles with immobilized GOX (5 mg GOX/g particle) was suspended in 0.1 M sodium phosphate buffer, pH 7, and incubated with 15 mL of aldehyde dextran (M_w 20,000) prepared as previously described, for 16 h at 25 °C. After that, 35 mL of cold (4 °C) 100 mM sodium bicarbonate, pH 8.5, and 50 mg of solid sodium borohydride were added to the solution in order to reduce the remaining aldehyde groups and the amine-aldehyde bonds [20]. This solution was stirred for 15 min and then 50 mg of sodium borohydride was again added. Once again this solution was allowed to react for 15 min at 4 °C and after that the pH was adjusted to 7.

3.8. Enzyme inactivation in strongly stirred aqueous systems

Fifty milliliters of 5 mM sodium phosphate buffer at pH 7 containing immobilized GOX modified and unmodified with dextrans, or soluble GOX (0.15 IU/mL) were placed in a cylindrical container (diameter 5 cm), and then mechanically stirred at 1200 rpm using a 4 cm diameter long mechanical stirrer in order to generate air–liquid interfaces. The experiment was carried out at pH 7 and 25 °C. Samples were withdrawn at different time intervals and the residual activity was measured as previously described. Controls were carried out in the same conditions without stirring with the unmodified enzyme.

All results reported represent averages of at least three experiments. Moreover, in all cases, the experimental error was not higher than 5%.

3.9. Thermal inactivation of different GOX preparations

The soluble and the immobilized enzyme were incubated at 50 °C. Periodically, samples were withdrawn and their remaining activities were assayed as described above. In all these assays, suspensions containing 0.03 IU/mL were incubated in 25 mM sodium phosphate buffer pH 7.0.

4. Results

4.1. Thermal stability of the different GOX preparations

Immobilization yields on glutaraldehyde agarose (porous and non-magnetic support) as well as on activated nanoparticles (non-porous and magnetic support) were quite poor in both cases due to the low activation of the used supports to prevent an intense multipoint covalent attachment between the enzyme and the support (approx. 25% of the added enzyme was immobilized after 24 h in both cases, retaining full activity).

Fig. 1A shows that the soluble enzyme and both immobilized preparations exhibit very similar thermostability, as intended by the low activation of the support and immobilization conditions utilized.

Therefore, the rigidity of the immobilized enzyme preparations was very similar to that of the soluble enzyme. Thus, we could study the effect of the immobilization of the enzyme on porous and non-porous supports on the stability of the enzyme under stirred systems.

4.2. Stability under stirred systems

The soluble enzyme was readily inactivated in the presence of gas bubbles under conditions where the enzyme was fully stable without stirring (result not shown), showing the adverse effect of the gas bubbles on the enzyme (Fig. 1B). This effect was not found using agarose-GOX (a porous support used for comparison purposes). The immobilized enzyme retained its full activity during stirring. Considering that the thermostability of this derivative and the soluble en-

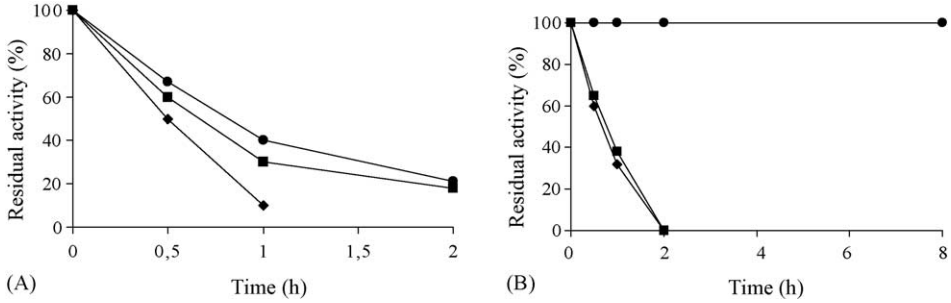

Fig. 1. (A) Thermal inactivation course of different GOX preparations. Experimental conditions were 50 °C and pH 7. Diamonds: Soluble GOX; Circles: GOX derivative prepared on poorly activated glutaraldehyde agarose. Squares: GOX derivative prepared on poorly activated glutaraldehyde magnetic particles. (B) Inactivation course of different Gox preparations under strong stirring. Diamonds: soluble GOX; Circles: GOX derivative prepared on poorly activated glutaraldehyde agarose. Squares: GOX derivative prepared on poorly activated glutaraldehyde magnetic particles. Further details are described in Section 3.

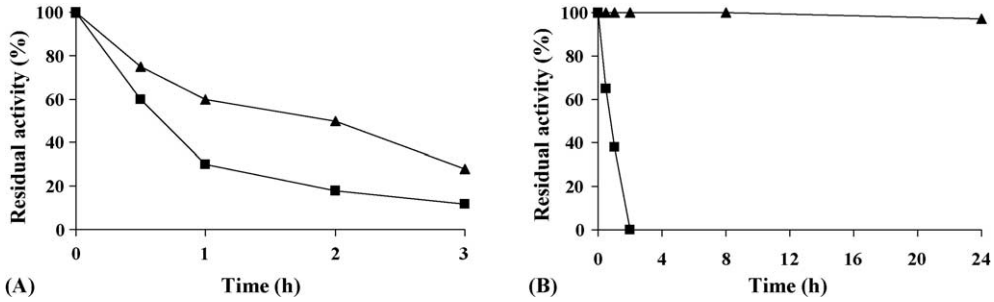

Fig. 2. (A) Effect of the dextran modification on the thermal stability of GOX immobilized on magnetic nanoparticles. Experimental conditions were 50 °C and pH 7. Squares: GOX immobilized on nanoparticles. Triangles: GOX immobilized on nanoparticles with dextran coating. (B) Effect of the dextran modification on the inactivation course of GOX immobilized on magnetic nanoparticles. Squares: GOX immobilized on nanoparticles. Triangles: GOX immobilized on nanoparticles with dextran coating. Further details are described in methods.

zyme were very similar, these differences should be attributed to the fact that the enzyme did not interact with the gas bubbles because it is inside the pores of the support.

However, the enzyme immobilized on nanoparticles was inactivated in a very similar fashion to that of the soluble enzyme in this stirred system (Fig. 1B). This implies that the mere immobilization of the enzyme on this non-porous support did not prevent the enzyme inactivation by the presence of gas bubbles.

4.3. Covering of GOX with aldehyde-dextran

Modification of glucose oxidase immobilized on magnetic nanoparticles, with 20 kDa dextran aldehyde did not cause a significant decrease in the enzyme activity (approx. 95% of the starting activity was recovered after the chemical treatment).

Fig. 2A shows that the enzyme thermostability increased slightly after the treatment. However, a significant stabilization of the enzyme in stirred systems can be observed in Fig. 2B. Therefore, this treatment could be used to prevent the enzyme inactivation by interaction with gas bubbles when the enzyme is immobilized on non-porous supports, in a similar fashion to the stabilization of soluble enzymes.

5. Conclusions

Immobilization of enzymes on magnetic nanoparticles is attracting a great interest in the last years, because they may have some advantages compared to preparations with solid porous supports, being the only alternative in some cases (e.g., solid or semisolid substrates). However, this methodology may not be useful to avoid some inactivation mechanisms of enzymes that were prevented by the use of porous supports. For example, interaction with gas bubbles is a critical inactivation factor using GOX in soluble form. The immobilization of the enzyme on porous supports is able to fully prevent this kind of inactivation. However, we show that the immobilization of enzymes on non-porous support has no effect on the enzyme stability in the presence of gas bubbling (despite it may seem obvious, this fact was not previously considered in any of the references using these type of supports to immobilize enzymes). Finally, we show that the dextran coating of the immobilized enzyme is able to prevent this inactivation cause, although the full coating may be more complicated by steric reasons (near to the support it is difficult that the dextran can react) [21]. These results should be of general applicability to other enzymes or polymers coating, and solve an important problem in any system where gas bubbling, strong stirring, etc. may be necessary.

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