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Short communication

Use of chitosan–clay composite as immobilization support for improved activity and stability of β-glucosidase

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Abstract

Equal weights of cuttlebone chitosan and activated clay were mixed and prepared as the wet (without freeze-drying) and dried (with freeze-drying) beads. The resulting composite beads were then cross-linked with glutaraldehyde as the supports for β-glucosidase immobilization. The immobilization conditions including enzyme loading, immobilization time, cross-linking time, and the amount of glutaraldehye were optimized. The properties of the enzyme immobilized on wet and dried composites were compared. It was shown that the storage stability of immobilized enzyme was higher than that of free enzyme. The activity of immobilized enzyme on dried composite was higher those on wet chitosan beads and wet composite after being repeatedly used for 50 times. The pH and thermal stabilities of free and immobilized enzymes were also investigated.

Keywords: β-Glucosidase; Immobilization; Chitosan–clay composite; Stability

1. Introduction

β-Glucosidase (E.C.3.2.1.21) comes from widespread sources including bacteria, animals, and plants. It exhibits wide substrate specificity and is capable of cleaving β-glucosidic linkages of conjugated glucosides and disaccharides [1]. The substrates include some oligosaccharides such as cellotetraose and cellopentaose [2]. β-Glucosidase is able to hydrolyze cellooligosaccharides and cellobiose into glucose [3], and is capable of hydrolyzing anthocyanins that are the main coloring agents found in foods of vegetable origin [4,5]. The main interest in this enzyme is related to its possible applications in food processing industry (such as the production of wine and fruit juices) for improving organoleptic product properties [6]. Also, β-glucosidase is used in the synthesis of glycoconjugates by reversing the normal hydrolytic reaction [7,8].

Chitosan is the cheap, non-toxic, and not harmful support to the enzymes. It possesses many advantages such as excellent hydrophilicity, high porosity, and large adhesion area (>23 m^2/g in this work, for example); all of which lead to the low steric hindrance to the enzymes and thus a low mass transfer resistance. Chitosan has hydroxyl (–OH) and amino ($–NH_2$) groups, which easily link with enzymes [9] and, can be cross-linked with glutaraldehyde to prevent dissolution in acidic solutions (pH <2) [10]. When chitosan is prepared in the bead form, it can entrap twice as much of the enzymes, estimated according to the mass transported and the volume of reactive dyes sorbed onto the internal pores [11]. Cetinus and Oztop [12] reported that catalase immobilized on chitosan beads exhibits better thermal and pH stabilities than the free one, and tyrosinase, β-glucosidase, and acid phosphatase have high activities and long lifetimes when they are immobilized on chitosan beads [13–15]. The cross-linked chitosan beads are more applicable in biochemical engineering due to their greater mechanical strength; however, there are still operational defects; for example, the density is too close to that of water (causing it to float easily) and the texture is too soft. This will largely limit the industrial applications of chitosan.

The problems of chitosan beads outlined above may be solved when chitosan was mixed with some solid powders such as clays and activated carbons to increase its density and mechanical strength, thereby extending application possibilities [16–18]. Lai and Shin [17] have immobilized acid phosphatase on chitosan and montmorillonite to improve the phosphorus content of soil. In this work, the activated clay was added to chitosan/acetic acid slurry to prepare composite beads, in either wet (without

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freeze-drying) or dried (with freeze-drying) manner, to improve the properties of chitosan beads. These beads were then cross-linked with glutaraldehyde for β-glucosidase immobilization. The optimal immobilization conditions (enzyme loading, immobilization time, cross-linking time, and amount of glutaraldehye) were examined. Also, the operation stabilities of immobilized enzymes when used repeatedly (temperature, pH, storage, etc.) were studied and compared with those of free enzyme. This is of practical importance for further applications.

2. Materials and methods

2.1. Preparation of cross-linked composite beads

Chitosan and activated clay were prepared following the methods described previously [19]. Chitosan flakes (1 g) and activated clay (1 g) were dissolved in 1 M acetic acid (100 mL) and were agitated with a disperser (IKA, Ultra-Turrax T25 basic) at 24,000 rpm for 10 min. The yielded viscous solution was placed in a vacuum dryer for 3 h to remove air bubbles, and then was sprayed drop-wise through a syringe, at a constant rate, into a neutralizing solution containing 15% NaOH and 95% ethanol in a volume ratio of 4:1. The beads were left in solution for 1 day. The prepared beads were washed with deionized water until the solution was neutral. They are referred to "wet" composite beads. While they were further dried in a freeze-dryer (Eyela FD-550) for 6 h, they are referred to "dried" composite beads. The diameters of wet and dried composite beads, and wet pure chitosan beads were 3.2, 2.4, and 2.6 mm, respectively.

An aliquot of the wet or dried composite (0.05 g) was placed in a 100-mL vessel containing 50 mL of 5 g/L glutaraldehyde (Acros Co.). The solution was agitated at 150 rpm and 30 °C. After cross-linking for 2 h, the beads were washed thoroughly and stored in deionized water.

2.2. Immobilization of enzyme and activity determination

An amount of cross-linked wet or dried composite (0.05 g) was in contact with 2 mL of 1 mg/mL of β-glucosidase (Sigma, E.C.3.2.1.21, from almonds, 3.4 unit/mg) in a shaker for 18 h at 4 °C. After washing with deionized water three times, the immobilized beads were stored in a vessel containing 0.01 M of acetate buffer (pH 3.5) at 4 °C.

The activity of β-glucosidase in the solution was determined by adding 0.1 mL sample to 0.9 mL of 0.1 M acetate buffer (pH 3.5), which contains 5 mM of *p*-nitrophenyl β-D-glucopyranoside (Sigma Co., N7006) substrate [15]. The mixture was incubated with stirring at 25 °C for 1 min and stopped by adding 2 mL of 1 M Na_2CO_3. The absorbance of the product *p*-nitrophenol at 400 nm was measured using an UV/visible spectrophotometer (Jasco V-530), and the activity was calculated based on a molar extinction coefficient of 18,300 dm^3/(mol cm). One activity unit (U) of β-glucosidase is defined to be the amount of this enzyme required hydrolyzing 1 μmol of substrate per minute. The activity of β-glucosidase immobilized on the composite was similarly measured, except that 0.1 mL of the solution was replaced by 0.1 mL deionized water and a given amount of wet or dried composite.

2.3. Stability experiments

The thermal and pH stabilities of free and immobilized enzymes were examined by measuring the activity of enzyme after the enzyme had been in the solutions for 1 and 6 h, respectively, at different temperatures (15–75 °C) and different pH (2.5–6.0).

3. Results and discussions

3.1. Effect of enzyme loading and immobilization time on enzyme activity

The BET surface areas of cross-linked and non-crosslinked dried composite beads, and the cross-linked dried chitosan beads are measured to be 114.0, 36.8, and 23.3 m^2/g, respectively, in this work. This indicates that cross-linking step increases the surface area. Table 1 shows the effect of β-glucosidase loading on the enzyme activity. The activity of immobilized enzyme increases as β-glucosidase loading increases up to 30 mg/g chitosan; i.e., the immobilized enzymes have almost no activity variation in the loading range 30–50 mg/g chitosan. As shown in Table 1, the activity of dried-composite immobilization enzyme is 1.7–1.9 times higher than that of the wet one, and the remaining activity in the case of dried composite enzyme is higher than that in wet one. Normally, the activity of immobilized and free enzyme remaining in the solution (i.e., the remaining activity) in one immobilization run has opposite trends. This is not the case here. Repeated experiments showed the same tendency, as also seen in Table 2. One possible reason for such abnormal behavior is that the cross-linking agent glutaraldehyde, which is adhered on the interior surface of the beads and is harmful to enzyme activity [19], could be removed more

Table 1
Effect of enzyme loading on the activity of immobilized β-glucosidase

Support	Property	Enzyme loading (mg/g chitosan)				
		10	20	30	40	50
Wet composite	Activity unit (U)	216.4	274.0	286.8	286.3	289.0
	Remaining activity (%)	1.5	0.5	0.9	0.6	1.1
Dried composite	Activity unit (U)	394.6	480.8	507.2	512.4	511.9
	Remaining activity (%)	6.1	15.4	29.8	48.2	62.1