International Journal of Biological Macromolecules 66 (2014) 46–51

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: www.elsevier.com/locate/ijbiomac

Modified chitosan microspheres in non-aggregated amylase immobilization

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ARTICLE INFO

Article history:
Received 13 August 2013
Received in revised form 4 February 2014
Accepted 10 February 2014
Available online 18 February 2014

Keywords:
Immobilization
Amylase
Microspheres
Chitosan

ABSTRACT

Immobilized enzymes are useful as reusable catalysts in industrial processes. In this study, α-amylase was used as a model enzyme to evaluate the propensity of synthesized porous chitosan microspheres as immobilization matrix. Chitosan microspheres were synthesized by grafting and covalent gelation technique using acrylamide (AAm) and glutaraldehyde (GA) as chemical agents, respectively. The synthesized chitosan-*cl*-poly(AAm) demonstrated amylase immobilization capacity of 350 mg/g. Furthermore, SEM results supported the porous microsphere structure for chitosan-*cl*-poly(AAm) with non-aggregated amylase immobilization, which accounts for comparable activity of immobilized amylase (3.28 μmol/ml/min) in contrast to free amylase (3.46 μmol/ml/min). The immobilized α-amylase was characterized for optimal pH and temperature activity and showed better resistance to temperature and pH inactivation in contrast to free amylase. The immobilized amylase retained more than 60% of its initial activity when stored at 4 °C for 30 days and retained 50% of its initial activity after seven successive repeated-use cycles. In conclusion, the study can be used as base for the immobilization of competent industrial biocatalysts in non-aggregated active structure.

1. Introduction

Amylases (α-amylase, β-amylase and glucoamylase) are among the most important industrial enzymes of technological promise in the food, paper and textile industries [1], detergents [2] and analytical chemistry [3]. But, the industrial use of such biocatalysts in enzyme reactors has certain practical problems i.e., high cost of isolation, purification of enzymes, structures instability once they are isolated from their natural environments and short operational lifetimes due to reaction conditions (organic solvents, elevated temperatures and pH can also cause the loss of activity due to aggregation or conformation loss) [4]. Three dimensional structure and conformation plays a crucial role in determining the catalytic efficiency of enzymes, thus eventually influencing their exploitability in biotechnological applications. The stability and good activity of the enzyme is an important concern in industrial biotechnology [5].

Immobilization of biocatalysts on solid support is very important to stabilize the structure of enzymes and hence their activities. Moreover, the heterogeneity of the immobilized enzyme systems facilitates easy recovery of enzyme and product, repetitive use of enzymes, continuous operation of enzymatic processes and decreased cost of processing. Enzyme immobilization on solid carriers is an important way to improve enzyme performance in industrial processes [6]. The physical and chemical characteristics (Pore size, hydrophilic/hydrophobic balance and surface chemistry) of support have been reported accountable in enzyme immobilization and its catalytic properties [7,8]. The mosoporous materials have been evaluated as most promising carriers for enzyme immobilization [9–12]. But, the synthetic and processing cost of mesoporous structure limits the applicability in the encapsulation of biomolecules [13,14]. Furthermore, the encapsulation in mesoporous structure may cause the aggregation of enzyme and failure of catalytic activity [15].

Chen et al. [16,17] have reported the significance of particulate support (particle size) in immobilization applications. Luo and Zhang [18] have reported the enhanced immobilization on microporous cellulose microspheres. An egg shell microporous membrane has also been reported effective in enzyme immobilization [19]. The chitosan have been extensively used due to the advantage of a great compatibility with the enzymes [20,21]. In view of that, the aim of this study was to find a simple and efficient

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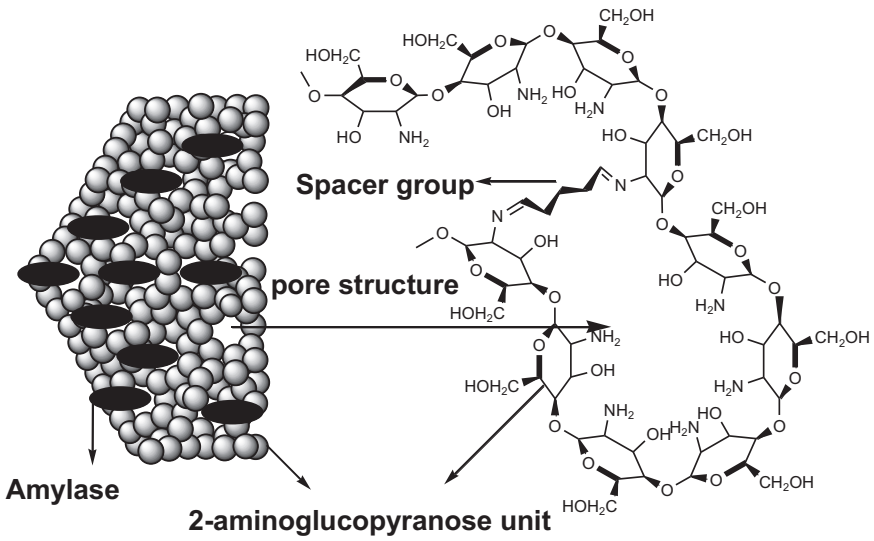
http://dx.doi.org/10.1016/j.ijbiomac.2014.02.022
0141-8130/



Scheme 1. Proposed structure of chitosan-*cl*-poly(AAm) with immobilized α-amylase.

method, to stabilize α-amylase with high yields and catalytic activity. Chitosan is the only cationic polysaccharide in nature derived from biomass. Its proteinaceous nature makes it worthy in enzyme immobilization [22]. However, chitosan potential as immobilization matrix can be enhanced via chemical modifications to realize the full potential of this versatile polysaccharide. Moreover, chitosan is amenable to chemical modifications due to versatility of chemical structure i.e., –OH, $–CONH_2$ and $–NH_2$ functional groups. Therefore, tailor made supports for enzyme immobilization can be produced with desired properties and uniform porosity by GA as spacer group.

2. Experimental

2.1. Material

α-Amylase (EC 3.2.1.1) was obtained from the HiMedia Laboratories. The other chemicals used for the study were of analytical grades and used as received. The weights were measured on Denver Balance having minimum readability of 0.01 mg.

2.2. Modification and characterization

The commercial chitosan was copolymerized with acrylamide (AAm) in the molar ratio of 1:1 using ammonium persulphate (APS, 2.0% (w/w) of chitosan and AAm) as initiator at 65 °C. After 2 h, the product was collected and washed with water and alcohol separately, respectively. The product was dried to a constant weight for further use. The efficiency of the product formation was evaluated as follows [23].

$$\%\text{Grafting Efficiency}(\%E) = \left(\frac{W_d}{W_r}\right) \times 100 \quad (1)$$

where W_d is the weight of the synthesized product (Chitosan-*g*-poly(AAm), where -*g*- is for grafting) and W_r is the weight of all reacting species including chitosan, AAm and APS. Chitosan-*g*-poly(AAm) based microspheres were synthesized by a covalent gelation technique using GA (10.0% (w/w) of chitosan and AAm). GA acts as a bifunctional crosslinker and in the process of crosslinking it generates pores by bridging the two chains of the chitosan through the amino groups of the later as shown in Scheme 1 [4] Chitosan-*g*-poly(AAm) (2.0% w/v) was dissolved in acetic acid (pH 5.0–6.0) and GA was added drop wise at room temperature with constant stirring using a mechanical stirrer for 6 h. Furthermore, the reaction setup was kept undisturbed for the next 24 h for completion and homogeneity of product by condensation reactions. The crosslinked microspheres were isolated by centrifugation and dried by vacuum till constant weight. The characterization of functionalized chitosan microspheres and enzyme loaded microspheres was carried out by X-ray diffraction method (XRD), Fourier transform infrared spectroscopy (FTIR) and scanning electron micrograph (SEM). FTIR spectra were recorded using KBr pellets on Perkin Elmer, SEMs on Joel JSM 6100 and XRD on JOEL-8030 X-ray diffractometer.

2.3. Immobilization of α-amylase

To the 10.0 ml buffered amylase (100 mg) solution (pH 7.0, 0.1 M) was added 0.250 g of modified chitosan microspheres. The mixture was stirred at 20 °C in a rotatory shaker for 24 h. The supernatant was used for protein quantification by Lowry method [24]. Enzyme-supported chitosan microspheres were separated by centrifugation, and the unbound enzyme was removed by washing with phosphate buffer (pH 7.0, 0.1 M) solution. The immobilized enzyme was dried at 30 °C for further use as biological catalyst.

2.4. Enzyme activity and reusability

The enzyme activity for free and immobilized enzyme was assessed via colorimetric analysis using 3,5-dinitrosalicylic acid (DNSA) reagent [25]. 3.5 mg of enzyme and its immobilized equivalent (in 10 mg of support) was added to 1.0 ml of 2.0% (w/v) starch solution in phosphate buffer (pH 7.0, 0.1 M), separately. The test sets were incubated at 35 °C for 30 min. To the incubated reaction set up 1.0 ml of DNSA was added and boiled for 10 min for color development. The optical density (OD) of the solution was determined at 550 nm in the UV–vis spectrophotometer (UV mini 1240 spectrophotometer). The effect of temperature (35 °C to 75 °C) on enzyme activity was studied at 30 min with 2.0% (w/v) starch in phosphate buffer (0.1 M, pH 7.0). The effect of pH (4.5 to 10) on enzyme activity in free and immobilized state was also studied at optimum 55 °C. The activity of free and immobilized amylase was calculated by using earlier reported formula [26]. For each test, the activity of enzyme at the beginning of reaction was taken as 100% residual activity [27].

The reusability for immobilized amylase was studied at 55 °C and for 30 min with 2.0% (w/v) of starch in phosphate buffer (pH 7.0, 0.1 M). After each activity measurement, the immobilized enzymes