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Immobilization of Cyclodextringlycosyltransferase (CGTase) from *Bacillus firmus* in Commercial Chitosan

KELI C.A. SOBRAL, REGINA M.O. RODRIGUES, ROGÉRIO D. DE OLIVEIRA, FLÁVIO F. DE MORAES and GISELLA M. ZANIN*

Chemical Engineering Department, State University of Maringá, Av. Colombo 5790, 87020-900 – Maringá-PR, Brazil

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Abstract

The enzyme cyclodextringlycosyltransferase (CGTase) was immobilized in commercial chitosan with different methods of immobilization at different temperatures, with the aim of obtaining a product with improved activity and higher recovery of the free enzyme activity. Three immobilization methods were tested: adsorption, covalent bonding with γ-aminopropyltriethoxysilane (CB-γAPTS), and covalent bonding with hexamethylenediamine (BC-HEMDA). Two test conditions were used, 7 °C without agitation and 26 °C with stirring. The best results were obtained with the method that uses HEMDA as the bifunctional covalent binding agent, giving the highest immobilized enzyme specific activity, 0.263 μmol β-CD/min mg of protein, and highest enzyme activity recovery, 5.2%, when immobilization was carried out at 7 °C.

Introduction

Chitosan is an excellent support for immobilization because it is nontoxic, it can be found in different forms (powder, gel, fibers or membrane), it has high affinity for proteins, and it can be easily derivatized. Additionally, chitosan is cheap and readily available, because it is a derivative from chitin, which is a byproduct from the fish industry and one of the most common polysaccharides [1].

The immobilization of enzymes in polymers can facilitate the recovery of the free enzyme activity, maintain an appropriate pH, affinity for the substrate, and enzyme stability because in this case immobilization may cause benign changes in enzyme microenvironment, or in the of mobility of the protein. The extension of these changes depends upon the enzyme, support and reaction conditions used for immobilization. Among these factors, the choice of support is the most important factor. An ideal support for enzyme immobilization must be selected considering some essential properties, such as, chemical stability, hydrophilic behavior, rigidity, mechanical stability, large surface area, and resistance to microbial attack [2].

The enzyme cyclodextringlycosyltransferase (CGTase) has been immobilized in different supports and the great majority of these were inorganic supports. In the last decade, few studies were made concerning the immobilization of CGTase in chitosan. Yang and Su [3] immobilized the CGTase from Bacillus alkalophilic sp in chitosan by the method of covalent bonding with crosslinking by glutaraldehyde, and they have obtained 46% conversion of potato starch (5%) to cyclodextrins at 60 °C, pH 8.5. The main product was β-CD, 34%. In presence of ethanol conversion increased to 58.3%. Su and Yang [4] used the same kind of immobilized CGTase in presence of α-amylase and amyloglucosidase, also immobilized in chitosan and calcium alginate, respectively. They have used a continuous process with 1% non-hydrolyzed starch at 40 °C, pH 4.5 and obtained a starch conversion to cyclodextrins of 70%.

The aim of this work was to study chitosan as support for immobilizing the enzyme CGTase, by different methods of immobilization and different reaction conditions. CGTase from *Bacillus firmus* was immobilized on commercial chitosan by adsorption, covalent bonding with γ-aminopropyltriethoxysilane (CB-γAPTS), and covalent bonding with hexamethylenediamine (BC-HEMDA). Two test conditions were used, 7 °C without agitation and 26 °C with stirring. The resulting data is used to compare the immobilized enzymes percentage of protein fixation, specific immobilized enzyme activity, and activity recovery, in relation to the activity of the free enzyme.

Materials and methods

Enzyme

Cyclodextringlycosyltransferase (CGTase) from *Bacillus firmus* (strain No. 37) was produced according to the methodology of Matioli et al [5]. This enzyme specific activity, determined as given below in the section *Enzyme Activity*, is 5.1 μmol β-CD/min mg.

* Author for correspondence: E-mail: gisellazanin@maringa.com.br

Support

Chitosan of pharmaceutical grade was obtained from FARMACON Ltda, Maringá, Brazil, with particle size in the range of 40–65 mesh.

Enzyme Production

CGTase was cultivated in a shaker at 150 rpm with 750 mL of liquid medium, pH 10, and 37 °C. The standard cultivation medium was composed as follows (% w/v): soluble starch 1.0; polypeptone 0.5; yeast extract 0.5; potassium phosphate 0.1; magnesium phosphate 0.02; sodium carbonate 1.0, according to the methodology of Matioli *et al.* [5].

Enzyme Immobilization

Hydration of support

Chitosan was hydrated by successive washings with ethanol and acetone, each 10% in volume, in a Büchner funnel, using 10 mL of washing solution per g of support. Then, the support was washed with distilled water until neutral pH was obtained at the filtrate. It was next, kept under vacuum for 15 min, and dried at 60 °C for 24 h, as described by Pereira [6].

Physical adsorption

First, the hydrated support was exposed to vacuum for 15 min, then 5 mL of enzymatic solution was added per g of support, under vacuum. The enzyme concentration used was equivalent to 2.0 mg protein/g support. It was also added to the support 10 mL of a buffered medium containing distilled water and 20% of Tris-HCl buffer, pH 8, 0.01M and 10% of $CaCl_2$ 50 mM. The mixture of support and enzymatic solution was contacted at the desired temperature, either 7 or 26 °C, for 24 h. Completed the immobilization step, the immobilized particles were separated from the liquid in a Büchner funnel, in which suction was maintained for 5 min. The filtrate was collected and the concentration of protein was assayed. The immobilized CGTase (ICGTase) was washed 10 times with distilled water and the filtrates were also collect and assayed for protein. In the last suction operation, vacuum was maintained for 20 min. The immobilized enzyme humidity was determined by drying a sample at 105 °C for 15 h. The protein contents of all filtrates were used for calculating the amount of enzyme bound to the support, and from it, the percentage of protein recovered upon enzyme immobilization.

Covalent bonding with γ-aminopropyltriethoxysilane (CB-γAPTS)

Hydrated chitosan was directly activated with glutaraldehyde 2.5% (w/v) in sodium hydrogen phosphate buffer, pH 7.0, 0.1 M. The support was exposed to vacuum for 15 min and then the glutaraldehyde solution was slowly added under vacuum in a proportion of 15 ml/g support. The mixture of support and glutaraldehyde solution was contacted at 26 °C, for 1 h. Then, to eliminate excess glutaraldehyde, the derivatized chitosan was washed with distilled water until a neutral pH was obtained in the filtrate. In the last suction operation, vacuum was maintained for 20 min, and the humidity of the derivatized chitosan was determined by drying a sample at 105 °C for 15 h, according to the methodology described by Tardioli [7]. The CGTase enzyme was then immobilized by the same procedure as given for the physical adsorption method.

Covalent bonding with hexamethylenediamine (BC-HEMDA)

Approximately 2.5 g of hydrated support was exposed to vacuum for 15 min. Then, still under vacuum, a 2.0% (w/v) solution of HEMDA was added, up to complete immersion of support solids, giving 12.8 mL of solution/g support. The mixture of support and liquid was kept under agitation at 40 °C for 2 h. Then, the liquid solution was decanted and a 3% glutaraldehyde solution in hydrogen phosphate buffer, pH 7.0, 0.1 M, was added in the proportion of 12.8 mL of solution/g support. The suspension was kept under agitation at 26 °C for 15 min. Next, the support was transferred to a Büchner funnel and washed 10 times with the same volume of glutaraldehyde as given above. In the last suction step the derivatized support was kept under vacuum for 15 min, and dried at 105 °C for 15 h, as described by Pereira [6] for humidity determination. The CGTase enzyme was then immobilized by the same procedure as given for the physical adsorption method.

Enzyme activity

The activity of the free enzyme was determined at 50 °C by assaying the initial reaction rate of β-CD production, using the method of initial velocities [8]. The enzyme substrate was maltodextrin FLUKA, 10 g/L, in Tris-HCl buffer, pH 8.0, 0.01 M and 50 mM $CaCl_2$. The substrate solution (1.5 mL) was warmed to reaction temperature and mixed with the same volume of enzyme solution. Reaction was followed for 30 min and tubes were taken out of the thermostatic bath each 5 min. The enzyme was inactivated by boiling for 5 min, and the tubes were stocked at 4 °C for later β-CD assay [5].

The activity of the immobilized CGTase was determined also by assaying the initial reaction rate of β-CD production, using the method of initial velocities [8]. However, in this case, a batch reactor fitted with a stainless steel basket was used for holding the immobilized enzyme. The reaction was carried out at 50 °C with 50 mL of the same substrate solution described above. After the substrate solution reached the reaction temperature, a 1.5 mL sample was taken, this being the zero time point of the test, and the basket containing about 1.5 g of immobilized enzyme particles was introduced into the reactor. Samples of 1.5 mL were taken at 3-min intervals up to 18 min. Then, the basket was removed from the reactor and two more samples were collected, at time 21 and 28 min, respectively. All samples were treated as described above for the free enzyme.

Protein assay

The protein concentration of all enzyme solutions and collected filtrates was determined by the method of Bradford

Table 1. Results for the percentage of protein fixation, immobilized enzyme specific activity, and percentage of activity recovered for the immobilization of CGTase in chitosan

Test	Experimental conditions		Results		
	Method	Temperature (°C)	Protein fixation (%)	Specific activity (μmol β-CD/ min mg of protein)	Activity recovered (%)
1	Adsorption	7 *	41.9	0.180	3.5
2		26 **	38.2	0.183	3.6
3	CB-γAPTS	7 *	30.7	–	–
4		26 **	44.7	–	–
5	CB-HEMDA	7 *	89.7	0.263	5.2
6		26 **	98.4	0.127	2.5

* without agitation; ** with stirring.

[9], using Coomassie Brilliant Blue G-250, and BSA as standard protein.

β-CD assay

The concentration of β-CD was determined by the dye extinction colorimetric method with phenolphthalein, described by Vikmon [10], and modified by Hamon and Moraes [11].

Results and discussion

The results for the percentage of protein fixation, immobilized CGTase activity, and activity recovery are shown in Table I, for different methods and conditions used in the experimental procedures.

For immobilization by adsorption it was observed that for both conditions tested, namely, 7 °C without agitation and 26 °C with stirring, the results are very close, demonstrating that, within the range studied, either temperature or agitation have little effect on the immobilized CGTase (ICGTase) parameters.

The immobilization of CGTase by covalent bonding with γ-aminopropyltriethoxysilane (CB-γAPTS) has shown that this method is not appropriate for chitosan. Although acceptable percentages of protein fixation have been obtained, it was not possible to determine the ICGTase specific activity because excessive data scattering was obtained during activity measurements.

According to Bon [12], when silanization was applied to chitin, the reagent γ-APTS attacked chitin. Therefore, we believe that the same might be happening to chitosan, because chitin is the precursor of chitosan. When this immobilization method was applied to purified CGTase immobilized in controlled pore silica, and using maltodextrin as substrate (5 g/L) the results were [7]: ICGTase specific activity, 0,00185 μmol of β-CD/min mg of enzyme, percentage of protein fixation, 28.96%, percentage of activity recovery, 28.68%.

Table I shows that immobilization of CGTase by covalent bonding to chitosan with hexamethylenediamine (CB-HEMDA) has given the best results in this study for the conditions of immobilization reaction at 7 °C, without agitation. Other enzyme immobilization studies, that have immobilized glucoamylase and lipase in chitosan, have also observed that the method of covalent bonding with HEMDA leads to better results [6, 12].

Although the result with highest protein fixation, 98.4%, was observed with the covalent method of immobilization with HEMDA at 26 °C, this condition gave only half of the enzyme activity obtained with immobilization at 7 °C. A possible explanation for this result could be the formation of multiple layers of immobilized enzyme, leading to difficult access of the active site of the immobilized enzyme molecules by the substrate [6].

As Table I shows, the maximum percentage of activity recovered in the ICGTase was only 5.2%, and it occurred for the covalent method of immobilization with HEMDA at 7 °C. This low activity yield needs to be improved with further research studies.

Conclusion

Chitosan has shown the best results for immobilization of the enzyme CGTase when the covalent bonding method with hexamethylenediamine (BC-HEMDA) was used at 7 °C, without agitation. In these conditions, the immobilized CGTase preparation gave a protein recovery of 89.7%, specific enzyme activity of 0.263 μmol β-CD/min mg of protein, and 5.2% recovery of free enzyme activity. Further studies are warranted for improving immobilized CGTase activity and activity recovery.

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References

1. G. Spagna, R.N. Barbagallo, D. Casarini and P.G. Pifferi: *Enzyme and Microb. Technol.* **28**, 247 (2001).

2. M. Chellapandian and M.R.V. Krishnan: *Proc. Biochem.* **33**, 595 (1998).
3. Yang and C.S. Su: *J. Chem. Technol. Biotech.* **46**, 283 (1989).
4. C.S. Su and C.P. Yang: *J. Chem. Technol. Biotech.* **48**, 313 (1990).
5. G. Matioli, G.M. Zanin, F. Guimarães and F.F. de Moraes: *Appl. Biochem. Biotech.* **70-72**, 267 (1998).
6. E.B. Pereira: Máster Thesis, Universidade Estadual de Maringá, Maringá, PR, Brasil, 128 pp (1999).
7. P.W. Tardioli, G M. Zanin and F.F. Moares: *Appl. Biochem. Biotech.* **84-86**, 1003 (2000).
8. M. Dixon and E.C. Webb: *Enzymes*, 3rd, edn., Longman Group, London, Chap. 2 (1979).
9. M. Bradford: *Anal. Biochem.* **72**, 248 (1976).
10. M. Vikmon: in J. Szejtli (ed), *The First International Symposium on Cyclodextrin*, D. Rildel, Dordrecht, pp. 60–74 (1981).
11. V. Hamon and F. F. Moraes: in *Etude preliminaire a l'immobilsation de l'enzyme CGTase WACKER*, Laboratorie de Tecnologie Enzymatique. Université de Tecnologie de Compiègne (1990).
12. E. Bon, D.G. Freire, M.F. Mendes and V.F. Soares: *Biotech. Bioeng. Symp.* **14**, 447 (1984).