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Evaluation of Supports and Methods for Immobilization of Enzyme Cyclodextringlycosyltransferase

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Abstract

An experimental design with factorial planning was used for the immobilization of the enzyme cyclodextringlycosyltransferase (CGTase) from *Bacillus firmus* (strain no. 37) to select the best combination of support, method of immobilization, and conditions that gives primarily higher average values for the specific immobilized enzyme activity, and secondarily, higher average values for the percentage of protein fixation. The experimental design factors were as follows: supports—controlled-pore silica, chitosan, and alumina; immobilization methods—adsorption, and two covalent bonding methods, either with γ-aminopropyltriethoxysilane or hexamethylenediamine (HEMDA); conditions—7°C without agitation and 26°C with stirring. The best combination of factors that lead to higher average values of the response variables was obtained with immobilization of CGTase in silica with HEMDA at 7°C. However, immobilization in chitosan at 7°C gave the highest immobilized CGTase specific activity, 0.25 μmole of β-CD/(min·mg protein). Physical adsorption gave low specific enzyme activities, and, in general, a high load of enzyme leads to lower specific enzyme activity.

Index Entries: Cyclodextringlycosyltransferase; immobilized enzyme; controlled-pore silica; alumina; chitosan.

Introduction

Immobilized Enzymes

Enzymes are largely used as biocatalysts in the chemical, pharmaceutical ,and food industries, and also as specific ligands in chemical and

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clinical analyses *(1)*. Since the recovery and reuse of free enzyme in solution is limited by high processing cost, enzyme immobilization has been pursued as an alternative technology for lower enzyme usage and higher product throughput by weight of enzyme used. Although immobilized enzymes can be easily reused many times in a batch reactor, their activity might be limited by the immobilization technique and the presence of mass transfer limitations *(2)*. An optimum support must contain a large surface area per unit volume (or mass), in which it should be able to immobilize a large quantity of enzyme and offer little diffusional resistance to either substrate or product, and it should be easily available, nontoxic, and of low cost. The number of commercial applications of immobilized enzymes has been growing steadily, and various enzymes have already been immobilized in different polymers by several different techniques, mainly in the form of granules or membranes (e.g., hollow fibers), which are used for large-scale applications *(3,4)*.

Immobilized Cyclodextringlycosyltransferase

Immobilization of the enzyme cyclodextringlycosyltransferase (CGTase) has been pursued as a means of reducing the production cost of cyclodextrins (CDs) from starch *(5)*. CDs have many industrial applications, but many more would become commercially feasible if the price of CDs could be lowered. Today's main CD applications are as complexants for holding fragrances and flavors in the chemical, food, agricultural, and pharmaceutical industries, and as emulsion stabilizers in food products, jet printer inks, and so on. They have also specialized applications such as catalysts, and in chromatographic separation of optical enantiomers *(6,7)*.

There are not many publications describing immobilization of CGTase. Tardioli et al. *(5)* have immobilized highly purified CGTase in controlled-pore silica by covalent binding with γ-aminopropyltriethoxysilane (CB-γ-APTS) and obtained a specific activity for the immobilized CGTase (ICGTase) of 0.00185 μmol of β-CD/(min.mg of enzyme) at pH 8.0, 50°C, 28.96% protein fixation and 28.68% activity recovery, using maltodextrin (5 g/L) as substrate. Yang and Su *(8)* immobilized the CGTase from *Bacillus alkalophilic* sp in chitosan by the method of covalent bonding with cross-linking by glutaraldehyde and obtained 46% conversion of potato starch (5%) to CDs at 60°C, pH 8.5. The main product was β-CD (34%). In the presence of ethanol, conversion increased to 58.3%. Abdel-Naby *(9)* immobilized CGTase from *Paenibacillus macerans* NRRLB-3186 in aminated polyvinylchloride (PVC) of three different hydrocarbon chain lengths, by covalent binding with glutaraldehyde as a bifunctional reagent. The best results were obtained with PVC of the largest chain length, in which 45.57% of activity was retained, and best results for operational stability were found at pH 6.0 and 75°C. In the reaction for CD production, ICGTase has shown higher activation energy than the free enzyme *(9)*.

Because the type of support and the method of immobilization used are two important factors when enzymes are immobilized *(10)*, we decided to

study various supports and enzyme immobilization methods for the CGTase from *Bacillus firmus* (strain no. 37), seeking the most appropriate combination of support, method of immobilization, and conditions that give primarily higher average values for the specific immobilized enzyme activity, and, secondarily, higher average values for the percentage of protein fixation.

Materials and Methods

Enzyme

CGTase from *B. firmus* (strain no. 37) was produced according to the methodology of Matioli et al. *(11)*. The enzyme was cultivated at 37°C, pH 10.0, and 150 rpm, in 750 mL of liquid medium containing: 1.0 (w/v) soluble starch, 0.5 (w/v) polypeptone, 0.5 (w/v) yeast extract, 0.1 (w/v) potassium phosphate, 0.02 (w/v) magnesium sulfate, and 1.0 (w/v) sodium carbonate. The cell-free culture medium had a specific enzyme activity of 5.1 μmol of β-CD/(min·mg), determined as given in the section CGTase Activity.

Supports

Chitosan of pharmaceutical grade was obtained from Farmacon (Maringa, PR, Brazil), with a particle diameter in the range of 0.212–0.425 mm. Controlled-pore silica (0.35-nm mean pore diameter) was supplied by Sucrerie Vanciennes (Crepy in Vallois, France) with a mean diameter of 0.42 mm. Alumina (Aluminum oxide 90, acid activated) was supplied by Merck (Darmstadt, Germany) as a fine powder.

Enzyme Immobilization: Cleaning and Hydration of Supports

Controlled-pore silica and alumina were cleaned and hydrated with diluted nitric acid (1:20) applied at 10 mL of acid/g of support. The mixtures were agitated for 1 h at 82°C. Then the solids were washed with distilled water in a Büchner funnel up to neutral pH in the filtrate and vacuum dried for 15 min. The supports were later dried at 105°C for 15 h.

Chitosan was hydrated with successive washings with ethanol-acetone-water (10:10:80) at 10 mL of solution/g of support, washed with distilled water in a Büchner funnel up to neutral pH in the filtrate, and vacuum dried for 15 min. Then, the hydrated chitosan was dried at 60°C for 24 h *(12)*.

Immobilization by Adsorption

The cleaned and hydrated support was kept under vacuum for 15 min, and an enzymatic solution of CGTase (2 mg of protein/g of support) was added at 5 mL/g of support or, in the case of chitosan, at 10 mL of a buffered solution containing 2 m*M* Tris-HCl buffer, pH 8.0, and 50 m*M* $CaCl_2$. The mixture of support and enzymatic solution were incubated for 24 h, at either 7 or 26°C (as was the case according to the experimental design to be shown). The mixture was then separated in a Büchner funnel and vacuum dried for 5 min. The filtrate was collected and a sample of it was stocked at 4°C for later protein analysis. The solids of the immobi-

lized enzyme were washed 10 times with distilled water and the filtrates were collected for later protein analysis. After the last washing, the immobilized enzyme was vacuum-dried for 20 min. A sample of the vacuum dried solids was taken and dried 15 h at 105°C for determination of the immobilized enzyme humidity. The value of protein concentration in all filtrates was used for determination of the amount of protein that was not fixed in the support, and from it, the percentage of protein fixation in the immobilization procedure.

Immobilization by CB-γ-APTS

Support Silanization

Silica and alumina supports were silanized with a 0.5% (v/v) solution of γ-APTS, pH 3.0 to 4.0, added in the proportion of 3 mL of solution/g of support. The solids and solution were agitated for 5 min at room temperature and then kept at 75°C for 3 h. Later, the silanized support was vacuum washed with distilled water to remove excess γ-APTS and dried at 105°C for 15 h. Chitosan, being an organic support, was not silanized.

Support Activation

Silanized supports were activated with a glutaraldehyde solution (2.5% [v/v] in 0.1 *M* sodium hydrogen phosphate buffer, pH 7.0; 3 mL of solution/g of solid). Support solids were kept under vacuum for 15 min, and still under vacuum, the glutaraldehyde solution was added slowly up to complete immersion of the solid. For chitosan, 15 mL of glutaraldehyde solution/g of solid was used instead. The mixture of support and activating solution was agitated at 26°C for 1 h. Then the solids were washed with distilled water up to neutral pH to eliminate excess glutaraldehyde and vacuum dried for 20 min. CGTase was immobilized with the same procedure as described in the section Immobilization by Adsorption. The humidity of the immobilized enzyme was determined by drying a sample at 105°C for 15 h *(5)*.

Immobilization by Covalent Bonding with Hexamethylenediamine

Approximately 2.5 g of cleaned and hydrated support was kept under vacuum for 15 min and then a solution of Hexamethylenediamine (HEMDA) (2.0% [w/v]) was added under vacuum in the proportion of 12.8 mL of solution/g of solid. The mixture was kept under agitation for 2 h at 40°C. Later, the liquid was decanted and an equal volume of glutaraldehyde solution (3% [w/v] in 0.1 *M* sodium hydrogen phosphate buffer, pH 7.0) was added. The mixture was agitated for 15 min at 26°C. Next, the suspension was transferred to a Büchner funnel and washed up to neutral pH (10 successive washes of 12.8 mL of distilled water/g of support), and the solids were vacuum dried for 15 min. A sample of the immobilized enzyme was dried for 15 h at 105°C to determine humidity *(12)*. CGTase was then immobilized with the same procedure as described in the section Immobilization by Adsorption.

CGTase 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 *(13)*. The enzyme substrate was 10 g/L of maltodextrin (Fluka, Sigma-Aldrich, Buchs, Switzerland) in Tris-HCl buffer (pH 8.0), 0.01 *M* and 50 m*M* $CaCl_2$. The substrate solution (1.5 mL) was warmed to reaction temperature and mixed with an equal volume of enzyme solution. The reaction was allowed to proceed 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 also determined by assaying the initial reaction rate of β-CD production using the method of initial velocities *(13)*. However, in this case, a batch reactor fitted with a stainless steel basket was used to hold the immobilized enzyme. The reaction was carried out at 50°C with 50 mL of the same substrate solution just described. After the substrate solution reached the reaction temperature, a 1.5-mL sample was taken, this being the time zero 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 for the free enzyme.

β-*CD Assay*

The concentration of β-CD was determined by the dye extinction colorimetric method with phenolphthalein, described by Vikmon *(14)*, and modified by Hamon and Moraes *(15)*.

Protein Assay

The protein concentration of all enzyme solutions and collected filtrates was determined by the Bradford *(16)* method, using Coomassie Blue G-250, and bovine serum albumin as the standard protein.

Experimental Design

Evaluation of Supports and Immobilization Methods for CGTase

For evaluation of support/method influence on the immobilization of CGTase, we chose a complete ($3^2 \times 2^1$) factorial experimental design with two qualitative factors (support and method) and one quantitative factor (temperature). The factors and their levels were as follows: support (factor X1)—silica (level 1), chitosan (level 0), and alumina (level –1); methods (factor X2)—physical adsorption (level 1), CB-γ-APTS (level 0), and CB-HEMDA (level –1); and temperature (factor X3)—7°C without agitation (level 1) and 26°C with stirring (level –1). The response variables chosen were the percentage of protein fixation into the support used for

Table 1
Matrix for Full Factorial Design in Immobilization of Enzyme CGTase

	Factor level code[a]			Real factor level		
Run	X1	X2	X3	Support	Method	Temperature (°C)
1	1	1	1	Silica	Physical Adsorption	26
2	1	1	–1	Silica	Physical Adsorption	7
3	1	0	1	Silica	CB-γ-APTS	26
4	1	0	–1	Silica	CB-γ-APTS	7
5	1	–1	1	Silica	CB-HEMDA	26
6	1	–1	–1	Silica	CB-HEMDA	7
7	0	1	1	Chitosan	Physical Adsorption	26
8	0	1	–1	Chitosan	Physical Adsorption	7
9	0	0	1	Chitosan	CB-γ-APTS	26
10	0	0	–1	Chitosan	CB-γ-APTS	7
11	0	–1	1	Chitosan	CB-HEMDA	26
12	0	–1	–1	Chitosan	CB-HEMDA	7
13	–1	1	1	Alumina	Physical Adsorption	26
14	–1	1	–1	Alumina	Physical Adsorption	7
15	–1	0	1	Alumina	CB-γ-APTS	26
16	–1	0	–1	Alumina	CB-γ-APTS	7
17	–1	–1	1	Alumina	CB-HEMDA	26
18	–1	–1	–1	Alumina	CB-HEMDA	7

[a]X1, support, X2, method, X3, temperature.

the immobilization of CGTase, and the specific activity of the immobilized enzyme. Table 1 presents the experimental design in detail.

According to the experimental design, all 18 runs were made in triplicate and carried out randomly. The mass ratio of enzyme to support at the immobilization step was kept constant at 2.0 mg of protein/g of support.

Results and Discussion

The influence of the factors—support, immobilization method, and temperature—was evaluated based primarily on the response variable: specific activity of immobilized enzyme.

ICGTase specific activity was calculated from the initial velocity data for β-CD production and the mass of protein fixed per gram of support. The latter and the percentage of protein fixation were calculated from the difference between the amount of enzyme offered for immobilization and that recovered in the washings of the ICGTase.

Table 2 presents the matrix of experimental results for the factorial design runs and shows the results for ICGTase specific activity and percentage of protein fixation. The combination of immobilization method,

Table 2
Matrix of Experimental Results from Experimental Design
Applied to Immobilization of Enzyme CGTase

	Experimental conditions			Results for ICGTase	
Run	Support	Method	Temperature (°C)	Protein fixation (%)	Specific activity (U/mg of protein)
1	Silica	Physical Adsorption	26	40.13	0.15
2	Silica	Physical Adsorption	7	55.37	0.14
3	Silica	CB-γ-APTS	26	81.67	0.19
4	Silica	CB-γ-APTS	7	57.77	0.16
5	Silica	CB-HEMDA	26	84.13	0.15
6	Silica	CB-HEMDA	7	67.60	0.20
7	Chitosan	Physical Adsorption	26	39.37	0.10
8	Chitosan	Physical Adsorption	7	40.27	0.15
9	Chitosan	CB-γ-APTS	26	44.67	—
10	Chitosan	CB-γ-APTS	7	30.67	—
11	Chitosan	CB-HEMDA	26	98.37	0.10
12	Chitosan	CB-HEMDA	7	87.57	0.25
13	Alumina	Physical Adsorption	26	92.50	0.12
14	Alumina	Physical Adsorption	7	77.33	0.18
15	Alumina	CB-γ-APTS	26	69.90	0.06
16	Alumina	CB-γ-APTS	7	68.23	0.11
17	Alumina	CB-HEMDA	26	69.13	0.08
18	Alumina	CB-HEMDA	7	66.13	0.10

support, and temperature that led to the single highest ICGTase specific activity was the immobilization of CGTase in chitosan by covalent bonding with HEMDA (CB-HEMDA) at 7°C. For this condition, the ICGTase specific activity was 0.25 U/mg of protein and the percentage of protein fixation was high, 87.57%.

However, since the goal of our study was to determine the level of factors that gives primarily higher average values of the ICGTase specific activity, we applied the analysis of variance (ANOVA) to the full model using the software SAS for Windows® version 6.12. The results for the effect of each factor and their interactions on the response variable ICGTase specific activity are presented in Table 3. Because the immobilization of CGTase in chitosan by CB-γ-APTS did not produce significant results, the set of data resulting from this combination was removed from this analysis.

Allowing a level of statistical significance (p value) of 0.05, the results of the ANOVA shown in Table 3 allow us to conclude that the significant factors for attaining higher ICGTase specific activity are support (X1) and temperature (X3), which gave p values of 0.0057 and 0.0006, respectively.

Table 3
ANOVA Applied to Complete Model for ICGTase Specific Activity

Factor	Degrees of freedom	Sum of squares	Mean square	*F* value[a]	*p* value[b]
X1	2	0.0238	0.0119	5.93	<0.0057
X2	2	0.0022	0.0011	0.56	0.5783
X3	1	0.0280	0.0280	13.95	<0.0006
X1 · X2	1	0.0216	0.0216	10.75	<0.0022
X1 · X3	1	0.0081	0.0081	4.03	0.0518
X2 · X3	1	0.0022	0.0022	1.08	0.3044
X1 · X2 · X3	1	0.0074	0.0074	3.66	0.0634
Error	38	0.0763	0.0020		
Total	47	0.1696			

[a] *F* value = mean square of treatment/mean square of error.
[b] *p* value = level of statistical significance.

Table 4
Comparison of Difference Between Average Values of Response Variable ICGTase Specific Activity for Factors Whose Levels are Statistically Significant

Factors	Levels	Difference between average values of ICGTase specific activity (u/mg of protein)
Support	1 and –1	0.050
Temperature	–1 and 1	0.048

According to this analysis, and within the range of the factorial design, the choice of immobilization method is not significant to obtain high ICGTase specific activity, but its interaction with the support is (X1 · X2; $p = 0.0022$). The other interactions were not statistically significant.

The average values of the response variable (ICGTase specific activity) were analyzed next, for the levels of the factors that were of significance (support and temperature), to determine whether there were significant differences between response variable average values. Tukey test, with a level of significance of 5%, was used and the results are shown in Table 4.

As Table 4 shows, the average values of ICGTase specific activity differ with statistical significance when alumina (support, level 1) is changed to silica (support, level –1), the difference being 0.050. In addition, when the temperature level 1 (26°C) is changed to temperature level –1 (7°C), there was also a significant difference, 0.0483. Hence, the choice of silica as support and the temperature of immobilization of 7°C, without agitation, led to high average values of ICGTase specific activity. In relation to the immobilization method, there was not a significant difference

Table 5
Average Value of Response Variables Specific Activity of ICGTase and Percentage of Protein Fixation for All Levels and Factors of Experimental Design Applied to Immobilization of Enzyme CGTase

	Average values for ICGTase specific activity (U/mg of protein)			Average values for protein fixation (%)		
Level	Support	Method	Temperature	Support	Method	Temperature
1	0.164	0.147	0.12	64.44	57.49	68.87
0	0.152	0.128	—	56.82	58.82	—
–1	0.114	0.148	0.17	73.87	78.82	61.22

between the average values of ICGTase specific activity for the different immobilization methods used.

To proceed in the selection of the most appropriate factors, the choice of immobilization method was based on the second response variable: the percentage of protein fixation. Table 5 shows the average values for the response variables for each level of all factors. The immobilization method that led to the highest average value for the percentage of protein fixation (78.82%) was CB- HEMDA (immobilization method, level –1).

Therefore, the statistical analysis showed that the combination of factors including immobilization of CGTase in silica at 7°C by CB-HEMDA results both in higher average values for the ICGTase specific activity, and in higher average values for the percentage of protein fixation.

Further analysis of the data displayed in Table 1 shows that a too high percentage of protein fixation is correlated with a lower ICGTase specific activity. This is probably the result of steric hindrance of the active site of ICGTase, because multiple enzyme layers may be formed, or excessive crowding of the enzyme molecules into the support pores, making access to the active sites difficult. In addition, the higher temperature (26°C) with agitation that leads, in general, to higher percentage of protein fixation is also associated with lower ICGTase specific activity. These two results indicate the importance of extending this study to a range of lower enzyme to support mass ratio, below 2.0 mg of protein/g of support. A problem of major concern, however, is the fact that for all factor combinations of this study, the recovery of enzymatic activity for the ICGTase was lower than 5%, i.e., maximum ICGTase specific activity/free enzyme specific activity $\times$ 100 < 5%. This may be the result of not using a sufficiently purified enzyme, but it could also indicate that for greater activity recovery, a controlled-pore structure with larger pores should be used. The latter hypothesis is founded on the facts that CDs are large molecules, some linear maltooligosaccharides synthesized by CGTase are of large chain size *(17)*, and the molecule of CGTase from *B. firmus* has a mol wt of 75 kDa *(11)*.

Immobilization of CGTase by CB-γ-APTS did not give good results, and this combination should be removed from further studies. According to Bon et al. *(18)*, when silanization was applied to chitin, the reagent γ-APTS attacked chitin. The same might be happening to chitosan, because chitin is the precursor of chitosan.

Conclusion

Statistical analysis of the experimental design data indicates that the most important factors for guaranteeing, primarily, higher average values for the immobilized CGTase enzyme specific activities and, secondarily, higher average values for the percentage of protein fixation is the immobilization of CGTase in silica by the method of CB-HEMDA at 7°C, without agitation. However, the highest ICGTase enzyme specific activity, 0.25 U/mg of protein, occurred for immobilization in chitosan by the method of CB-HEMDA at 7°C, without agitation.

Further immobilization studies with CGTase should explore lower enzyme-to-support mass ratios (< 2 mg of enzyme/g of support), supports with larger controlled-pore sizes (> 0.35 nm), and new immobilization methods seeking higher recovery of the free enzyme activity.

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