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Immobilization of *Paenibacillus macerans* NRRL B-3186 cyclodextrin glucosyltransferase and properties of the immobilized enzyme

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Abstract

Cyclodextrin glucosyltransferase from *Paenibacillus macerans* NRRL B-3186 was immobilized on aminated polyvinylchloride (PVC) by covalent binding with a bifunctional agent (glutaraldehyde). The immobilized activity was affected by the length of the hydrocarbon chain attached to the PVC matrix, the amount of the protein loaded on the PVC carrier, and glutaraldehyde concentration. The activity of the immobilized enzyme was 121 units/gram carrier, the specific activity calculated on bound protein basis was 48% of the soluble enzyme. Compared to the free enzyme, the immobilized form exhibited: a higher optimal reaction temperature and energy of activation, a higher K_m (Michaelis constant) and lower V_{max} (maximal reaction rate), improved thermal stability and resistance to chemical denaturation. The operational stability was evaluated in repeated batch process and the immobilized enzyme retained about 85% of the initial catalytic activity after being used for 14 cycles.

Keywords: Cyclodextrin glucosyltransferase; Immobilization; Properties

1. Introduction

Cyclodextrin glucosyltransferase (CGTase) [α-1,4-glucan-4-glucosyltransferase, cyclizing, EC 2.4.1.19] catalyzes the formation of cyclodextrins (CD) from starch and related carbohydrates such as amylose, amylopectin, and malto-oligosaccharides [1]. The CD which is a ring structure molecule built up of 6, 7, or 8 glucopyranose units is referred to as α-, β-, or γ-Cds. Depending on the major product of the cyclization reaction, CGTase are characterized as α-, β-, or γ-CGTase. The cyclic products can form versatile inclusion complexes with many organic and inorganic compounds, which property is of particular importance given the widespread applications of CDs in the pharmaceutical, food, and chemical industries [2–4].

For the industrial-scale production of CDs, conventional batch production methods which directly utilize soluble CGTase have been mainly adopted. Continuous production of CD using immobilized CGTase would have several advantages including allowing reuse of expensive CGTase, obtaining an homogenous CD product, simplifying product purification process and providing opportunities for scaling up.

The activities of the immobilized CGTase so far reported are low when compared to those reported for other amylolytic enzymes [5–10]. The present study deals with the immobilization of *Paenibacillus macerans* NRRL B-3186 CGTase by covalent binding on polyvinylchloride (PVC). The changes of the characteristic features of the enzyme brought about by immobilization have been studied. The catalytic properties and stability of the immobilized enzyme have been compared to those of the free enzyme.

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2. Materials and methods

2.1. Microorganism

The cyclodextrin glucosyltransferase (CGTase) producing strain of *P. macerans* (NRRL B-3186) was obtained from the Northern Regional Research Laboratory (NRRL), Peoria, IL 61604, USA.

2.2. Enzyme production

P. macerans NRRL B-3186 was grown on potato dextrose medium (PD) with the following composition (g/litre): potato slices, 400; glucose, 10. The pH was adjusted to 6.5. Cultivation was made in 250 ml Erlenmeyer flasks, each containing 50 ml of sterile medium. The inoculum 2%, v/v, (10^8–10^9 cells/ml) was transferred to the culture medium and the flasks were incubated at 37°C for 72 h on a rotary shaker. The culture medium was centrifuged ($4500 \times g$) for 15 min in a refrigerated centrifuge. The clear supernatant was concentrated at 20°C and fractionated at 80% saturation with ammonium sulfate. This partially purified enzyme (specific activity 14.0 unit/mg protein) was used for the preparation of the immobilized enzyme.

2.3. Enzyme immobilization

Polyvinylchloride (PVC) was obtained from Amreia Petrochemical Company, Alexandria, Egypt (emulsion grade, k-value = 67). It was aminated with alkyldiamine and then coupled to glutaraldehyde (GA) as described by Cheng and Shaw [11]. A known amount of powdered CGTase dissolved in 5 ml of phosphate buffer (0.05 M, pH 6.0) was added to a suspension of one gram of activated PVC in 25 ml of phosphate buffer (0.05 M, pH 6.0). The mixture was stirred at 4°C for 12 h. The unbound enzyme was removed from the carrier by washing with 0.05 M phosphate buffer (pH 6.0) until no activity or soluble protein was detected in the washings.

2.4. Enzyme assays

CGTase was determined by the method reported by Nogrady et al. [12]. The reaction mixture, containing 40 mg of water-soluble starch (Sigma) in 1.0 ml phosphate buffer (0.05 M, pH 6.0) and 0.1 ml of the enzyme solution or weight sample of the immobilized enzyme was incubated at 60°C for 30 min. The reaction was stopped by the addition of 3.5 ml of 40 mM NaOH solution, and then 0.5 ml 0.02% (w/v) phenolphthalein solution prepared in 5 mM Na_2CO_3 was added. After standing for 15 min at room temperature, absorbance at 550 nm was read. One unit of enzyme activity (U) is defined as the amount of the enzyme forming one mg of CD per h under the assay conditions.

Amylase activity was determined according to the Bergmann et al. [13] by estimating the released reducing sugars from 1.0% saline starch in 0.2 M phosphate buffer (pH 6.0) by the method of Somogyi [14]. One unit of enzyme activity (U) is defined as the amount of the enzyme forming 1 μmol of reducing sugars (as glucose) per min under the assay conditions.

2.5. Preparation of liquefied starch

Soluble starch solutions (5%, w/v) were liquefied with 20 U α-amylase (Novo) per g of starch at pH 6.0 and 50°C for different time intervals (10–60 min) to obtain dextrose equivalent (DE) levels [9]. DE is defined as the percentage of reducing sugars to maximum convertible sugars.

2.6. Properties of the free and immobilized CGTase

2.6.1. Effect of pH

The effect of pH on free and immobilized CGTase was studied using citrate buffer (0.05 M, pH 3.5–5.5), citrate–phosphate buffer (0.05 M, pH 6–7.5), and phosphate buffer (0.05 M, pH 7.0–8.0).

2.6.2. pH stability

The pH stability of the free and immobilized enzymes was examined after preincubating enzyme samples at 25°C for 60 min at different pH(s), followed by adjusting the pH to the value of the standard assay system. The residual activity was assayed under the standard conditions. Acetate buffer (0.05 M, pH 3.5–6.0), Tris–HCl (0.05 M, pH 7–8), and glycine–NaOH (0.05 M, pH 9–10) were used for enzyme stability treatment.

2.6.3. Thermal stability

Enzyme samples were incubated with phosphate buffer (0.05 M, pH 6.0) at a designated temperature (50–70°C) for 1–8 h. The residual activity was assayed under the standard conditions.

2.6.4. Protein estimation

Protein was estimated using the method of Lowry et al. [15] using bovine serum albumin as standard. The protein content of the immobilized enzyme was calculated by subtracting the amount of unbound protein from the originally added protein.

2.6.5. Operational stability of the immobilized CGTase

One gram of PVC immobilized CGTase (wet) containing about 47 U of *P. macerans* CGTase was incubated with 20 ml of 5% (w/v) starch in phosphate buffer (0.05 M, pH 6.0) at 60°C for 20 min. At the end of the reaction, the immobilized enzyme was collected by centrifugation ($2500 \times g$) for 10 min, washed with distilled water, and resuspended in 20 ml of freshly

Table 1
Effect of spacer group length on the recovered activity of the immobilized *P. macerans* CGTase

Spacer group	Enzyme added		Enzyme coupled		Specific activity (U/mg coupled protein)	Recovered activity (%)
	Protein (mg/g carrier)	Activity (U/ g carrier)	Protein (mg/g carrier)	Activity (U/g carrier)		
1,2-Diaminoethane-GA	10.0	140	9.00	54.0	6.00	38.57
1,6-Diaminohexane-GA	10.0	140	9.12	63.0	6.90	45.00
1,10-Diaminodecane-GA	10.0	140	9.20	63.8	6.93	45.57

prepared substrate to start a new run. The supernatant was assayed for CD.

2.7. Precipitation of cyclodextrin crystals

This was carried out by the addition of *n*-propanol to a final concentration of 60%. The mixture was left for 5–6 h at 4°C. The precipitated CD crystals were examined microscopically and by thin layer chromatography as reported by Lee et al. [16] using *n*-butanol/ethanol/water (4:3:3 v/v) as a developing solvent. The plate was stained with 1% methanolic iondine.

The results reported are the means of at least four separate experiments.

3. Results and discussion

3.1. Enzyme immobilization

The amount of activity retained of the immobilized CGTase on PVC was affected by the length of the hydrocarbon chain attached to the matrix. The results in Table 1 indicate that the immobilized activity with 1,2-diaminoethan (spacer length of two carbons + GA) was about 86 and 84% of those with 1,6-diaminohexane (spacer length of six carbons + GA) and 1,10-diaminodecane (spacer length of ten carbons + GA), respectively. However, the amount of bound protein with all preparations was about the same (9.0–9.2 mg/g carrier). The observed increase in the immobilization yield with the increase of the hydrocarbon chain can be attributed to the increase of the local surface area of the support. Consequently, steric hindrance in the immediate vicinity of the enzyme molecules was reduced. This hindrance renders the active site less accessible to the substrate. A similar explanation has been given by other investigators [17,18].

Variation of CGTase concentration attached to the carrier altered the apparent specific activity of the immobilized enzyme. The specific CGTase activity was constant at a level of 6.8 U mg bound protein up to a loading (substitution) level of 18 mg protein/g carrier but decreased to 61% of this activity at a substitution level of 36 mg protein/g carrier (Table 2). This decrease in the specific activity with increasing substitution on the surface of the carrier could be attributed to protein crowding, which may impair or prevent the proper conformational changes required for catalysis [19]. In addition, excessive packing of the enzyme would also lead to the development of steric effects around the enzyme active sites [20]. In order to obtain the highest specific activity possible (about 6.8 U/mg protein), the final CGTase substitution was designed to be in the range of 18 mg protein/g carrier.

Table 2
Maximal loading of *P. macerans* CGTase on aminated PVC[a]

Enzyme added		Enzyme coupled		Specific activity (U/ mg coupled protein)	Recovered activity (%)
Protein (mg/g carrier)	Activity (U/ g carrier)	Protein (mg /g carrier)	Activity (U/g carrier)		
10	140	9.2	63	6.84	45.0
20	280	18.0	121	6.72	42.8
30	420	27.0	148	5.48	35.2
40	560	36.0	154	4.27	27.5

[a] Polyvinlychloride aminated with 1,6-diaminohexane.

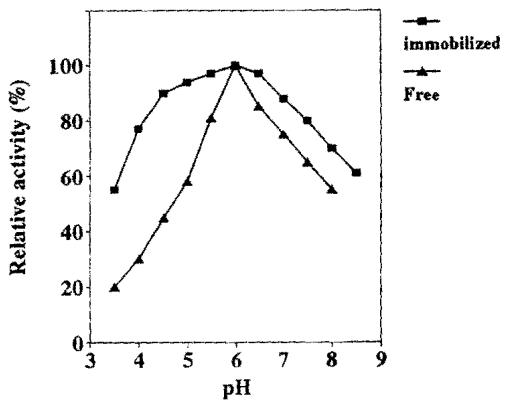

Fig. 1. Effect of pH on the activity of free and PVC immobilized *P. macerans* CGTase.

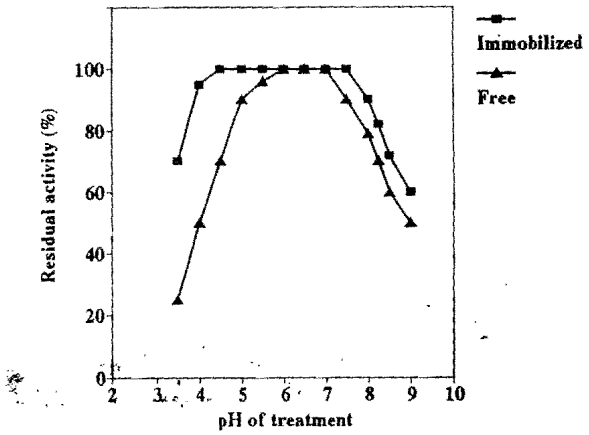

Fig. 2. pH stability of free and immobilized *P. macerans* CGTase.

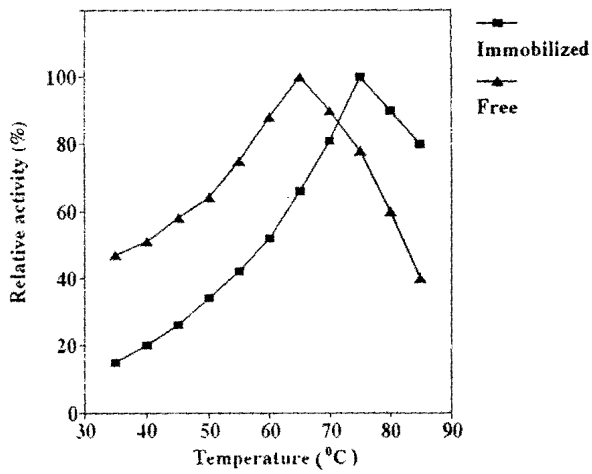

Fig. 3. Effect of temperature of the activity of free and immobilized *P. macerans* CGTase.

The optimum glutaraldehyde concentration for CGTase immobilization on PVC was at the level of 0.2% (v/v), beyond which the immobilized activity fell sharply.

When immobilization was carried out in the presence of 1.0% starch, the retained activity increased by 9.6%. This may be attributed to the protection of the enzyme active sites by the substrate during the immobilization processes. These results are in line with those previously reported for the immobilized CGTase [21] and dextransucrase [22].

3.2. Comparison of the catalytic properties of the free and immobilized CGTase

Both the free and the immobilized enzymes had an optimum pH of 6.0, but the optimum of the immobilized CGTase was broader at lower pH values (Fig. 1). Ivony et al. [5] reported that the positively charged carriers (as in the present case) displace the optimum pH of the enzymes attached to them to lower pH values. The pH stability of the immobilized CGTase (Fig. 2) was also shifted to a more acidic range (pH 3.5–7.5) than that of the free enzyme (4.5–7.0). Different pH profiles of the immobilized CGTase have been obtained depending on the carrier chosen and on the immobilization method applied [5,8,9,21,23].

The free enzyme had an optimum temperature at 60°C, whereas the optimum temperature of the immobilized CGTase was shifted to 75°C (Fig. 3). The temperature data were plotted in the form of Arrhenius plots (Fig. 4). The slope of a logarithmic Arrhenius plots is related to the activation energy (E_a) for the molecule by the relationship: slope = $E_a/2.303R$, where R is the gas constant. The plots for both the free and immobilized enzymes were linear and the calculated values of activation energy were equal to 5.1 and 9.8 kcal/mol for the free and the immobilized CGTase, respectively. The higher value of the activation energy obtained for the immobilized CGTase indicates that the applied immobilization procedure introduced changes in the structure of the enzyme molecule which impeded the enzyme catalyzed reaction. Similar results were reported previously for other immobilized CGTase [8].

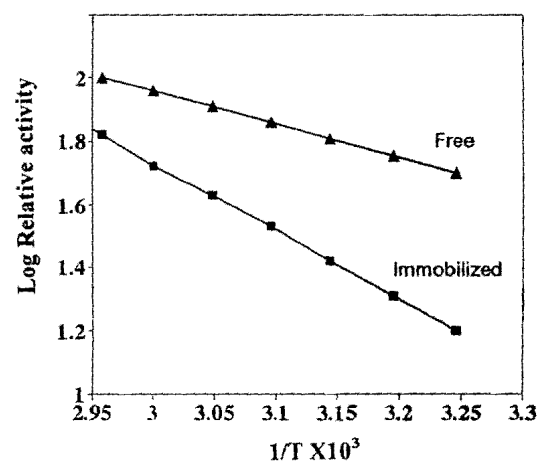

Fig. 4. Arrhenius plots from the data in Fig. 3.

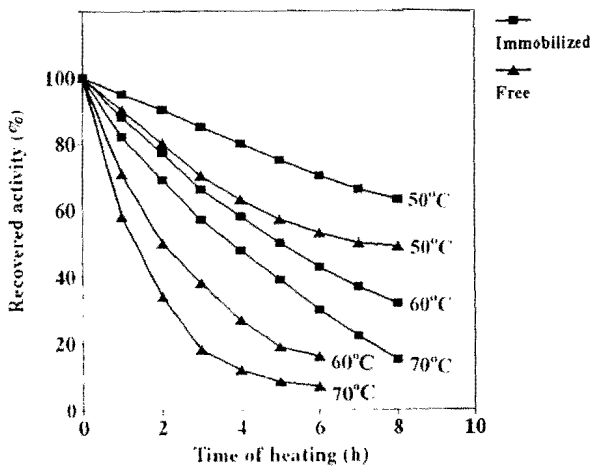

Fig. 5. Heat inactivation of free and immobilized *P. macerans* CGTase.

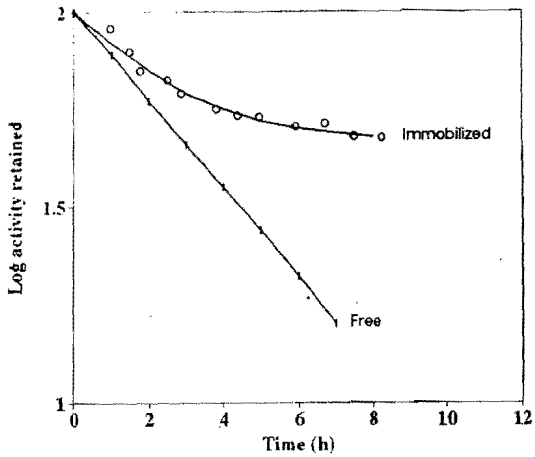

Fig. 6. Log of activity retained at 60°C as a function of time for free and immobilized *P. macerans* CGTase.

Table 3
Substrate specificity of soluble and immobilized CGTase

Substrate	Relative activity (%)	
	Free	Immobilized
Soluble starch	100	100
Corn starch	89	90
Potato starch	100	97
Rice starch	92	90
Amylose	101	103
Amylopectin	87	83
Partially liquefied starch, dextrose equivalent (DE):		
25	103	124
20	105	128
15	106	132
10	109	127
8	27	31

In contrast, cases where CGTase activation energy decreased after immobilization have also been reported [5,23].

The rates of heat inactivation of soluble and immobilized CGTase were investigated in the temperature range between 40 and 70°C (Fig. 5). In general, the immobilization process of the enzyme on PVC protected the enzyme against heat inactivation. For example, the calculated half-life value shows that the heat inactivation of the immobilized enzyme at 50, 60 and 70°C was 10.0, 6.0, and 4.0 h, respectively, whereas at these temperatures the free enzyme was less stable (half-lives of 6.0, 2.2, and 1.2 h, respectively). The immobilized *P. macerans* enzyme NRRL B-3186 immobilized on aminated PVC by covalent binding with GA appears to be more thermostable than the immobilized CGTases from *Bacillus macerans* [5,8] and *Bacillus* sp. [6]. When the logarithm of the activity retained was plotted against time, the free enzyme gave straight-line plots, suggesting a first-order reaction while this was not the case with the immobilized enzyme (Fig. 6). A possible explanation for the non first-order reaction may be due to cross linking to other enzyme molecules and to the support, causing heterogeneity in thermal stability. The results are in agreement with those reported by Desai et al. [24].

Linewaver–Burk plots of free and immobilized CGTase gave K_m values of 1.2 and 2.8 mg/ml, respectively, with starch. The reduction in affinity for the substrate on immobilization of the enzyme may be due to conformational changes on immobilization and /or mass transfer effects. The V_{max} values (maximal reaction rate) of the immobilized enzyme (10.5 mg CD/mg protein per h) was within 52.5% of the solution rate (20 mg CD/mg protein per h). An increase in K_m value and a decrease in the maximal reaction rate of CGTases after the immobilization process have been reported [23].

The substrate specificity of *P. macerans* CGTase immobilized on PVC was tested on various substrates and the results are shown in Table 3. The relative activities of the immobilized form on soluble starch, corn starch, potato starch, rice starch, amylose, amylopectin did not significantly differ from those of the free CGTase. On the other hand, the relative activities of the immobilized CGTase on dextrin or partially liquified starch (α-amylase treated starch) of different dextrose equivalent, were 1.2–1.3 times higher than those with soluble starch. This observation may be due to the higher diffusion rates of these low molecular weight substrates to be in contact with the immobilized systems. Similar observations were previously reported for other immobilized CGTases [8,9,23].

The effects of surfactants and various metal ions on the activity of the immobilized CGTase as compared to the effect of the native enzyme are listed in Table 4. The immobilized CGTase activity was not reduced in the

Table 4
Effect of surfactants and various metal ions on the activity of free and immobilized *P. macerans* CGTase

Substance	Concentration	Relative activity (%)	
		Free	Immobilized
None	–	100	100
Tween 80	0.01%	89	100
Tween 40	0.01%	83	98
Triton X 100	0.01%	92	100
Sodium dodecyl sulfate	0.1%	44	91
$CaCl_2$	10 mM	100	100
$CuSO_4$	10 mM	67	87
$FeSO_4$	10 mM	44	79
$HgCl_2$	10 mM	41	68
$CoCl_2$	10 mM	88	94

presence of various surfactants while the free enzyme showed remarkable loss of activity in the presence of sodium dodecyl sulfate. Calcium ions showed no action on the free or immobilized enzymes. On the other hand, Cu^{2+}, Co^{2+}, Fe^{2+} and Hg^{2+} adversely affected the activity of both the native and immobilized enzymes. However, it was observed that the inhibitory effects of these ions were less pronounced with the immobilized enzyme. This may be due to the protection of the immobilized enzyme by the carrier and this protection may result from structural changes in the enzyme molecule introduced by the immobilization procedure and consequently, lower the accessibility of the inhibiting ions to the active site of the enzyme. Similar results have been reported for other immobilized amylases [25].

3.3. Operational stability

The operational stability of the immobilized *P. macerans* CGTase immobilized on PVC was evaluated in repeated batch process. The results (Fig. 7) indicated that the stability of the immobilized enzyme in repeated use. Thus, the immobilized enzyme produced a good yield of CD (45% substrate conversion) with high 85% of the initial catalytic activity after 14 cycles.

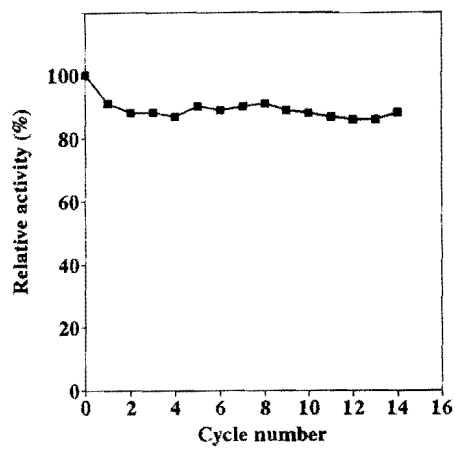

Fig. 7. Operational stability of PVC immobilized *P. macerans* CGTase in repeated use. The reaction mixture is described in the text.

3.4. Cyclodextrin formation

The enzyme reaction under the assay conditions (12 U/g substrate) resulted in about 9.5% of starch conversion (1% starch) and the CD produced was mainly of the β-type. As the enzyme concentration increased to 20 U/ g substrate, the yield of CD was increased to 15.6%. The CDs precipitated from this reaction mixture were of the α- (20%) and β- (80%)types as detected by thin-layer chromatography.

The results revealed that the immobilized *P. macerans* CGTase immobilized on aminated PVC showed a resistance against thermal and chemical denaturation, increased tolerance to wide pH range and could be reused for CD production. In addition, the immobilized enzyme acquired a higher temperature optima (75°C). This provides an additional advantage for practical application, which reduces the probability of microbial attack for the reaction mixtures. All these criteria could therefore, be successfully utilized in the practical application for continuous production of CDs.

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