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Production of cyclodextrin by poly-lysine fused *Bacillus macerans* cyclodextrin glycosyltransferase immobilized on cation exchanger

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Abstract

Bacillus macerans cyclodextrin glycosyltransferase (CGTase) fused with 10 lysine residues at its C-terminus (CGTK10ase) was immobilized onto a cation exchanger by ionic interaction and used to produce α-cyclodextrin (CD) from soluble starch. Poly-lysine fused immobilization increased the V_m of the immobilized CGTase by 40% without a change in K_m. The activation energies of thermal deactivation (E_a) were 41.4, 28.1, and 25.9 kcal mol^{-1}, respectively, for soluble wild-type (WT) CGTase, soluble CGTK10ase, and immobilized CGTK10ase, suggesting destabilization of CGTase by poly-lysine fusion and immobilization onto a cation exchanger. Maximum α-CD productivity of 539.4 g l^{-1} h^{-1} was obtained with 2% soluble starch solution which was constantly fed at a flow rate of 4.0 ml min^{-1} (D = 240 h^{-1}) in a continuous operation mode of a packed-bed reactor. The operational half-life of the packed-bed enzyme reactor was estimated 12 days at 25 °C and pH 6.0.

Keywords: CGTase; Cyclodextrin; Poly-lysine; Immobilization; Packed-bed enzyme reactor

1. Introduction

Cyclodextrins (CDs) are cyclic oligosaccharides composed of six, seven, or eight α-1,4-glycosidic-linked glucosyl residues (α-, β-, or γ-CD) and produced from starch or starch derivatives by cyclodextrin glycosyltransferase (CGTase, E.C. 2.4.1.19). CGTases convert starch to a mixture of CDs, glucose, maltose, and other oligosaccharides [1]. Moreover, the content of CDs depends on reaction conditions, substrate concentration, amount of enzyme, and source of CGTase [2]. *Bacillus macerans* CGTase mainly produces α-CD from starch with 75% selectivity [3–6].

Advantages of enzyme immobilization include a repeated use of an expensive enzyme, easy separation of the product, and greater variety of reactor design [7–10]. When an enzyme is attached to the support material, the functional groups, located at the active site of the enzyme and responsible for the catalytic reaction, should not be involved in the immobilization procedure and therefore remain potent [11,12]. Since mass transfer environment is also liable to be affected by immobilization, careful characterization of an immobilized enzyme is crucial before operation optimization. CGTase has been immobilized onto Diaion HP-20, IRA-93, and IRA-900 [2,8,13]. A recombinant *Escherichia coli* system expressing *B. macerans* CGTase fused with 10 lysine residues at its C-terminus (CGTK10) was constructed for the purpose of simple ion-exchange purification of CGTase with high purity [6].

In this report, the poly-lysine tag was employed for the immobilization of CGTase onto a cation exchanger, characterized for the enzymatic properties of the immobilized CGTK10ase. Finally, a packed-bed immobilized enzyme reactor was analyzed and optimized for maximum productivity of α-CD production from starch.

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2. Materials and methods

2.1. Expression and affinity purification of CGTase

Wild-type and poly-lysine fused CGTases were expressed in *E. coli* BL21(DE3): pLysE harboring the corresponding expression vectors, pTCGT1[5] and pTCGTK10 [6]. Culture conditions for the recombinant *E. coli* strains and procedures for crude enzyme preparation were described in detail in our previous report [6]. Epoxy-activated Sepharose 6B (Sigma Chemical Co., St. Louis, MO, USA) coupled with α-CD was prepared as described by Sundberg and Porath [9]. The matrix was packed into a glass column and the bound CGTase was eluted from the matrix with 1% β-CD solution.

2.2. Immobilization of CGTK10ase and continuous production of α-CD

SP-Sepharose 4 Fast Flow (Amersham Biosciences, Uppsala, Sweden) cation-exchange resin (1 ml) was washed twice with 5 ml of buffer A (10 mM sodium phosphate buffer (pH 6.0, 0.5 mM $CaCl_2$)), mixed with 2 ml of purified CGTK10ase (2.82 mg ml^{-1}) and then incubated at 4 °C for 4 h with gentle shaking. Cation-exchange resin immobilized with CGTK10ase was subjected to a column and washed with buffer A for 2 h. Concentrations and feed rates of soluble starch were varied to determine an optimal condition for α-CD production. A void volume of a packed-bed reactor was estimated 0.36 ml. The temperature of the packed-bed reactor was maintained at 25 °C by circulating temperature-controlled water.

2.3. Measurement of CGTase activity

CGTase activity was determined in terms of cyclizing activity with some modifications [14]. Colorimetric assay was based on the inclusion of methyl orange by α-cyclodextrin (α-CD), which resulted in a decreased absorbance at 520 nm. Fifty microlitres of the appropriately diluted enzyme solution was added to 1.45 ml premixed substrate solution. The final reaction mixture was composed of 0.1% (w/v) soluble starch and 0.1 mM methyl orange in imidazole–HCl buffer (pH 6.0) containing 5 mM $CaCl_2$. Soluble starch solution was always prepared immediately before assay. A decrease in absorbance at 520 nm was observed kinetically for 1 min using a spectrophotometer (UV-1601, Shimadzu, Kyoto, Japan). From the regressed kinetic slope, the amount of CD was calculated using a calibration curve determined with various amounts of CD. Based on the calibration curves, 1 mM change in α-CD concentration resulted in 0.545 changes in absorbance. One unit of CGTase activity was defined as the amount of enzyme required for the formation of 1 μmol CD/min under the specified conditions. In this experimental condition, 1 U/ml enzyme solution resulted in 0.012 absorbance changes. Usually, 1–5 U/ml enzyme solution gave linear decrease of absorbance in 1 min. Even though the absorbance change in a minute was relatively small (0.01–0.1), 60-points kinetic measurement followed by the linear regression yielded reliable calculation of enzyme activity. The specific activity was expressed as the activity per unit mass of protein.

2.4. Determination of kinetic parameters

The kinetic parameters for soluble WT CGTase, soluble CGTK10ase, and immobilized CGTK10ase were determined by incubating 50 μl (0.3 μg) of enzyme with 1450 μl of reaction mixture containing 0.1–2% soluble starch in imidazole–HCl buffer (pH 6.0). The data obtained were fitted to the Michaelis–Menten equation of substrate inhibition (Eq. (1)) using nonlinear least square regression. All kinetic parameters were estimated from three independent experiments.

$$v = \frac{V_m S}{K_m + S + \frac{S^2}{K_{is}}} \quad (1)$$

where V_m is the maximum reaction rate (g l^{-1} h^{-1}), K_m the Michaelis–Menten constant (g l^{-1}), S the substrate concentration (g l^{-1}), and K_{is} is the inhibition constant (g l^{-1}).

2.5. Thermal stability of CGTases

Thermal stability of soluble WT CGTase, soluble CGTK10ase, and immobilized CGTK10ase were measured at 20, 30, 40, and 50 °C, and the residual enzyme activities were determined after specific time of incubation. The deactivation constant (k_d) and activation energy (E_a) for thermal deactivation were estimated through fitting the data to the Arrhenius-type equation (Eq. (2)).

$$k_d = A_d \, e^{-E_a/RT} \quad (2)$$

2.6. Analysis of reaction products

Reaction products were analyzed by HPLC (Knauer, Berlin, Germany) equipped with the carbohydrate analysis column (Bio-Rad, Richmond, CA, USA). The samples were eluted at a flow rate of 1.0 ml min^{-1} using a mixture of acetonitrile and water (65:35, v/v) as a mobile phase.

3. Results and discussion

3.1. Properties of soluble WT CGTase, soluble CGTK10ase, and immobilized CGTK10ase

Kinetic parameters for the three types of CGTase were estimated from three independent experiments, averaged, and then summarized in Table 1. Maximum reaction rate (V_m) values were 1.59 g l^{-1} h^{-1} for soluble WT CGTase, 2.30 g l^{-1} h^{-1} for soluble CGTK10ase, and 2.38 g l^{-1} h^{-1} for immobilized CGTK10ase. It is interesting to note that V_m for

Table 1
Kinetic parameters for soluble wild-type (WT) CGTase, soluble CGTK10ase, and immobilized CGTK10ase

Type	Kinetic parameters[a]		
	K_m (g l^{-1})	K_{is} (g l^{-1})	V_m (g l^{-1} h^{-1})
Soluble WT CGTase	0.10	10.7	1.59
Soluble CGTK10ase	0.15	11.8	2.30
Immobilized CGTK10ase	0.16	14.0	2.38

[a] Estimation of all parameters was done by nonlinear fitting of the data to Michaelis–Menten equation of substrate inhibition.

the immobilized CGTK10ase was elevated by over 40% compared with soluble WT CGTase, suggesting the suitability of CGTK10ase for an immobilized enzyme reactor to produce α-CD. As shown in Table 1, K_m values were 0.10 g l^{-1} for soluble WT CGTase, 0.15 g l^{-1} for soluble CGTK10ase, and 0.16 g l^{-1} for immobilized CGTK10ase, indicating no serious diffusion limitation caused either by poly-lysine fusion or enzyme immobilization [12,15]. Although the K_m value of the immobilized CGTK10ase was slightly larger than that of the soluble WT CGTase, the experimental results showed that the immobilized CGTK10ase could yield reaction rate as high as the WT CGTase when saturated with the substrate. In all cases, optimum concentrations of soluble starch were estimated around 0.1% (w/v), which was compatible with the previous report in which the maximum yield of α-CD was obtained at low substrate concentration [1].

The results for immobilization have been described in our previous paper [16]. CGTK10ase immobilized on SP-Sepharose retained slightly higher specific activity within an experimental error range (∼5% higher than the free CGTK10ase) [16]. The unchanged specific activity of the CGTK10ase compared with free CGTK10ase might indicate no deterioration during the immobilization process. The immobilization yield of CGTK10ase varied from 0.65 to 0.95 depending on the immobilization conditions because an adsorption profile followed the Langmuir isotherm-like saturation curve.

The thermal stability of soluble WT CGTase, soluble CGTK10ase, and immobilized CGTK10ase were also measured at various temperatures. E_a values of soluble CGTK10ase and immobilized CGTK10ase were 26 and 28 kcal mol^{-1}, respectively, corresponding to about 47% decrease compared with that of soluble WT CGTase (Table 2). A significant reduction in E_a values indicated that both soluble CGTK10ase and immobilized CGTK10ase were unstable compared with the soluble WT CGTase. However, in the presence of Ca^{2+} the structure stabilizing metal of CGTase, immobilized CGTK10ase is as thermostable as soluble WT CGTase, whereas soluble WT CGTK10ase showed quite lower thermostability than the soluble WT CGTase [16]. The poly-lysine residues interact with various peptides and proteins such as β-amyloid peptide, ClpB chaperone, and HslU protease [17–19]. Perhaps, when CGTK10ase interacts with the fused poly-lysine during heat treatment in the absence of Ca^{2+}, the structurally unstable CGTK10ase is more sensitive to heat shock than WT CGTase in both soluble and immobilized conditions. Poly-lysine undergoes a conformational change from random coil to β-sheet by binding the positive charged lysine residues with the negative charged phosphatidic acid vesicles [20]. A report has shown that interactions are stronger for the β-sheet conformation of poly-lysine than other conformations [21]. In this respect, the β-sheet conformation of the fused poly-lysine probably induced by binding with a negative charged cation exchanger might exert negative effects on thermostability of the structurally unstable CGTK10ase, though the CGTK10ase is immobilized on a cation exchanger.

3.2. Continuous production of α-CD in packed-bed reactor

The three-dimensional structure of *B. macerans* CGTase shows that the CGTase consists of five domains: A, B, C, D, and E [22]. The catalytic domain (A domain) of CGTase is independently located at its N-terminal region from other domains (B, C, D, and E domains). Since CGTK10ase was fused with 10 lysine residues at the C-terminus of CGTase, the 10 lysine residues did not mask active sites of the catalytic domain, which was confirmed in the experimental results (Table 1). CGTK10ase could be displayed unidirectionally compared with other randomly directed immobilization methods. Further, specific enzyme activity did not decrease even when CGTK10ase was

Table 2
Parameters of thermal deactivation kinetics for soluble WT CGTase, soluble CGTK10ase, and immobilized CGTK10ase

Types	Thermal deactivation rate constant (k_d, h^{-1})			
	20 °C	30 °C	40 °C	50 °C
Soluble WT CGTase	1.76×10^{-3}	5.72×10^{-3}	1.70×10^{-1}	1.01×10^{-1}
Soluble CGTK10ase	3.03×10^{-3}	1.86×10^{-2}	2.05×10^{-1}	2.19×10^{-1}
Immobilized CGTK10ase	2.45×10^{-2}	7.05×10^{-2}	2.40×10^{-1}	2.24×10^{-1}

	Activation energy of thermal deactivation (E_a, kcal mol^{-1})
Soluble WT CGTase	41.4
Soluble CGTK10ase	28.1
Immobilized CGTK10ase	25.9

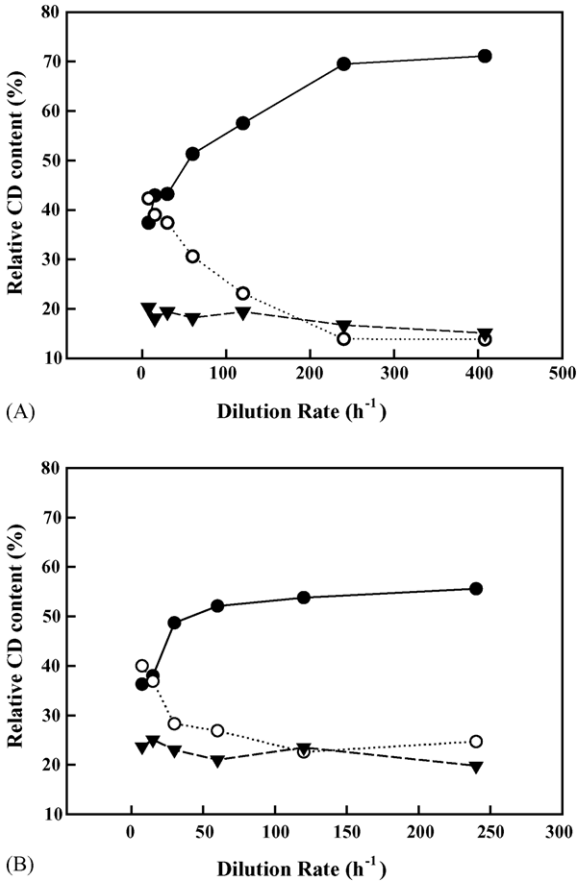

Fig. 1. Relative content of CDs in packed-bed column reactor at pH 6.0 and 25 °C. α-CD (●), β-CD (◯), and γ-CD (▼). Soluble starch solutions, 0.2% (w/v) for A and 2% (w/v) for B, were fed at various flow rates. Volume of packed-bed column reactor was 1 ml with 0.36 ml of void volume.

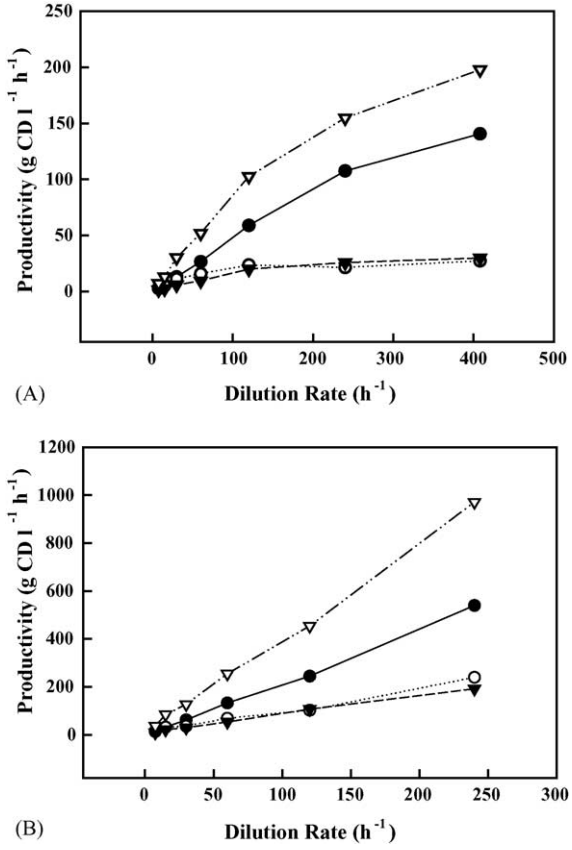

Fig. 2. Productivities of CDs in packed-bed column reactor at pH 6.0 and 25 °C. Total CD (▽), α-CD (●), β-CD (◯), and γ-CD (▼). Soluble starch solutions, 0.2% (w/v) for A and 2% (w/v) for B, were fed at various flow rates. Volume of packed-bed column reactor was 1 ml with 0.36 ml of void volume.

immobilized. Thus, CGTK10ase was immobilized onto a cation exchanger to continuously produce α-CD from soluble starch. A change in composition of produced CDs was observed due to variations in feed rate and concentration of soluble starch. Higher selectivity of α-CD was obtained at lower starch concentration in the feed. Although higher amounts of total CDs were obtained by elevating a substrate concentration from 0.2 to 2%, the relative content of α-CD in the product decreased as substrate concentrations increased. However, the relative content of α-CD gradually increased with increasing feed rate at a given concentration of soluble starch in the feed (Fig. 1). The reaction time of CGTase with the substrate obviously decreases with increasing feed rate. Thus, the increased α-CD ratio at higher feed rates might imply that the substrates are initially changed to the form suitable for α-CD production, and rapidly followed by the conversion to other forms suitable for the β- or γ-CDs [23]. The selectivity of *B. macerans* CGTase for α-CD varied with immobilization methods [24,25]. When CGTK10ase was immobilized on a cation exchanger, the produced α-CD ratio was higher than that of other immobilization methods using Amberite IRA-900 and chitosan. Maximum α-CD productivity of 539.4 g l^{-1} h^{-1} was obtained with 2% soluble starch solution which was constantly fed at a flow rate of 4.0 ml min^{-1} (Fig. 2) with dilution rate (*D*) of 240 h^{-1}. The α-CD productivity of the immobilized CGTase based on poly-lysine fusion was significantly high relative to the CGTase immobilized by other methods [24,25]. It might be due to a large amount of immobilized CGTK10ase on a cation exchanger. The above results indicated that a substrate feed rate could be used as a useful control parameter for continuous production of α-CD with high purity in a packed-bed enzyme reactor. Kim et al. achieved an enhanced productivity of transglycosylated xylitol by increasing substrate feed rates for the enzymatic action of CGTase [2].

Operational stability of the immobilized enzyme reactor and productivity of each CD were monitored at 25 °C for 13 days. Soluble starch (1%) was continuously fed at a flow rate of 0.5 ml min^{-1}. The yield of total CDs from soluble starch decreased to 44% after 13 days of operation, probably due to the inactivation of the immobilized enzyme (Fig. 3). It is quite interesting, however, to note that the relative content of α-CD in the product increased by 10% with the operation time. As is shown in Fig. 1, high feeding rate favored the selective production of α-CD. Possibly, CGTase deterioration

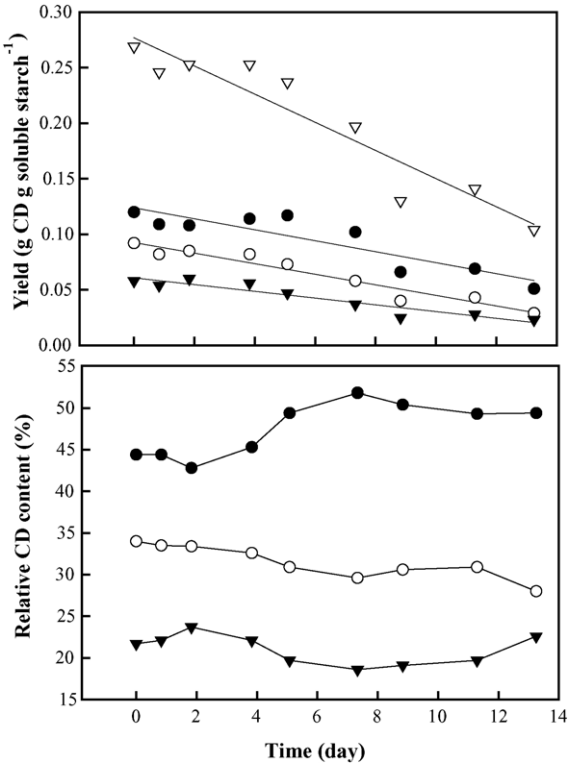

Fig. 3. Operational stability of packed-bed reactor at pH 6.0 and 25 °C. Total CD (▽), α-CD (●), β-CD (◯), and γ-CD (▼). Soluble starch solution (1%, w/v) was constantly fed at flow rate of 0.5 ml min^{-1}.

in a prolonged period of incubation made a similar effect to those from the elevated feeding rate, which lead to the less conversion of substrate form favorable of α-CD production to the substrates favorable of other CDs. Thus, it is suggested that the careful optimization of reaction conditions concerning reaction time, the amount of enzyme, feeding rate, and feeding concentration for the efficient production of α-CD.

Even though there was a significant decrease in enzymatic activity with prolonged incubation, we could maintain the yield of α-CD production by just lowering the substrate feed rate and concomitantly elevating the residence time of a substrate solution (data not shown). The half-life of the packed-bed reactor was estimated 12 days, which is comparable with *B. macerans* CGTase immobilized in a different way [13].

4. Conclusions

Poly-lysine fused CGTase was immobilized on a cation exchanger and was used to produce α-CD from soluble starch in a packed-bed reactor. The maximum reaction rates indicated that poly-lysine fusion and immobilization of CGTase did not cause a structural change of CGTase and diffusion limitation. For production of α-CD in the packed-bed reactor, the immobilized CGTase produced mainly α-CD at various process conditions with the half-life of 12 days. Scale-up studies will follow to implement the poly-lysine-based immobilization techniques in an industrial environment.

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