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Characterization of the *Bacillus macerans* Cyclodextrin Glucanotransferase Overexpressed in *Escherichia coli*

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ABSTRACT

Cyclodextrin glucanotransferase (CGTase, EC 2. 4. 1. 19) converts starch and related α-1,4-glucans to cyclodextrin (CD). Our previous studies of the enzyme have suggested that E344 on the polypeptide is crucial to the enzyme activity. Mutational analysis of CGTase was performed to confirm this idea. Three mutant CGTases containing either E344D, E344K or E344L substitution were overexpressed in *Escherichia coli*. However, only the wild-type and E344D CGTases became soluble when expressed at 20 °C. These two enzymes were purified to homogeniety from *E. coli* cells after β-CD and Ni-NTA affinity chromatographies. The Km values of the authentic *Bacillus macerans* CGTase (2.10 mM), and of the wild-type (0.58 mM) and E344D (1.05 mM) CGTases purified from *E .coli* were different. The *k*cat values of the three CGTases were 99.8, 26.5 and 90.7 s^{-1}, respectively. The percentage of α-CD production was 18.4% for the authentic CGTase, 24.9% for the wild-type and 14.5% for the E344D CGTases purified from *E. coli*. The changes of both the coupling and cyclization activities of CGTase caused by E344D suggest that E344 is important to the catalytic function of CGTase.

Key Words: cyclodextrin glucanotransferase, site-directed mutation, overexpression, CD production

I. Introduction

Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) catalyzes the conversion of starch into cyclodextrins (CDs), which are capable of forming inclusion complexes with many compounds used in the food, cosmetic and pharmaceutical industries (Szejtli, 1982; Nagatomo, 1985; Pszczola, 1988; Staranes, 1990). The relationship between the structure of CGTase and its enzymatic properties has been studied by cloning, sequencing and comparing the genes encoding CGTases in different bacteria (French and Kobayashi, 1980; Binder *et al.*, 1986; Takano *et al.*, 1986; Kaneko *et al.*, 1987, 1990; Kimura *et al.*, 1987, 1989; Hofmann *et al.*, 1989; Hill *et al.*, 1990; Sin *et al.*, 1991; Kitamoto *et al.*, 1992; Paloheimo *et al.*, 1992). Four highly conserved regions were observed in the peptide sequences of starch-hydrolysing enzymes, including α-amylases and CGTases (Nakamura *et al.*, 1993). Fujiwara *et al.* (1992) found that the cyclization activity of CGTase was conferred by the NH_2-terminal region of the enzyme. Hellman *et al.* (1990) truncated the CGTase at its C-terminal region and observed a change of product composition toward γ-CD. Thus, the integral molecular structure of the enzyme appears to be essential for enzymatic activities. Furthermore, certain Glu, Asp, His residues in the highly conserved regions of CGTase were shown to be related to enzymatic activity (Klein *et al.*, 1992; Nakamura *et al.*, 1993). It was also found that Y195 in CGTase from *Bacillus circulan* strain 250 played an important role in the formation of cyclic products (Penninga *et al.*, 1995). Using Woodward's reagent K for chemical modification, we identified and located E344 in the active site of *B. macerans* CGTase and proposed that it is an essential amino acid residue for CGTase activity (Jeang and Fang, 1992). In this paper, we report the overexpression of the wild-type and E344D CGTases, as well as the kinetic parameters of both enzymes.

II. Materials and Methods

1. Bacterial Strains and Plasmids

B. macerans IAM1243 used for purification of authentic CGTase and construction of the mutant *cgt* gene was obtained from the Institute of Agricultural Microbiology of Tokyo University. *E. coli* M15 (*Nal*^{s} *Str*^{s} *rif*^{s} *lac*$^-$ *ara*$^-$ *gal*$^-$ *mtl*$^-$ *F*$^-$ *recA*$^+$ *uvr*$^+$) with pREP4 plasmid was used as a host

for the overexpression of CGTase. pUG-D, pUG-K and pUG-L were derived from pUC19 (Wung, 1994); they contained E344D, E344K and E344L *cgt* genes, respectively. pQE32, used as the overexpression vector, was purchased from QIAGEN (Chatsworth, CA, U.S.A.).

2. Preparation of Antibodies Against *B. macerans* CGTase

The methods used here for cultivation of *B. macerans* and purification of CGTase to homogeniety have been described previously (Jeang *et al.*, 1992). Antiserum of CGTase was prepared by means of four weekly intravenous injections into a New Zealand white rabbit with purified CGTase (500 μg) emulsified with Freunds' complete adjuvant for the first injection and with incomplete adjuvant for the subsequent injections. One hundred ml of blood from the immunized animal was collected 5 days after the last injection. After removal of blood cells, the antiserum was diluted with 2 volumes of phosphate buffer saline (NaCl, 0.14 M; KCl, 3 mM; Na_2HPO_4, 8 mM; KH_2PO_4, 1 mM). Then, ammonium sulfate was added slowly into the diluted antiserum to 50% saturation and stirred for one hour. The precipitated proteins were collected by centrifugation (12000 $\times$ g, 30 min) and dialyzed against distilled water overnight. Afterwards, Tris-HCl buffer (pH 8.0) was added into the protein solution to obtain a final concentration of 10 mM. The buffered protein solution was applied to a DEAE-Sepharose CL-6B column (2.6 X 35 cm) equilibrated with 10 mM Tris-HCl buffer. To purify IgG, the column was first eluted with the same buffer and then eluted with a linear gradient of NaCl (0 to 0.3 M) in the buffer. The IgG eluted in the first protein peak was collected, pooled and purified by adsorption with the cell lysate of *E. coli* XL1-Blue. The purified antibody was used for immunological detection of CGTases.

3. Overexpression and Purification of the Wild-type and Mutant CGTases

The procedure for construction of the overexpression plasmids is shown in Fig. 1. The chromosomal DNA of *B. macerans* and the pUG-D, pUG-K and pUG-L plasmids were used as templates for the synthesis of wild-type, E344D, E344K, and E344L *cgt* genes by polymerase chain reaction, respectively. The forward primer was 5'-ATAGGATCCAGGAGGGTACCATAATGTCACCCGATACGAGCGTG-3', and the reverse primer was 3'-CACCTGACCGTTTTAATTCGCCAGCTGTACATC-5'. To construct plasmids which were able to overexpress CGTases, the pQE32 plasmid and the *cgt* genes synthesized by PCR were digested with *Sal* I and *Bam* HI, ligated, and transformed into *E. coli* M15. This construction produced a fusion protein with 18 extra amino acids residues (i.e., MRGSHHHHHHGIQEGTIM) at the N-terminus of CGTase. The recombinant plasmids which possessed the wild-type and mutated *cgt* genes (E344D, E344K and E344L) were called pQG, pQG-D, pQG-K and pQG-L, respectively.

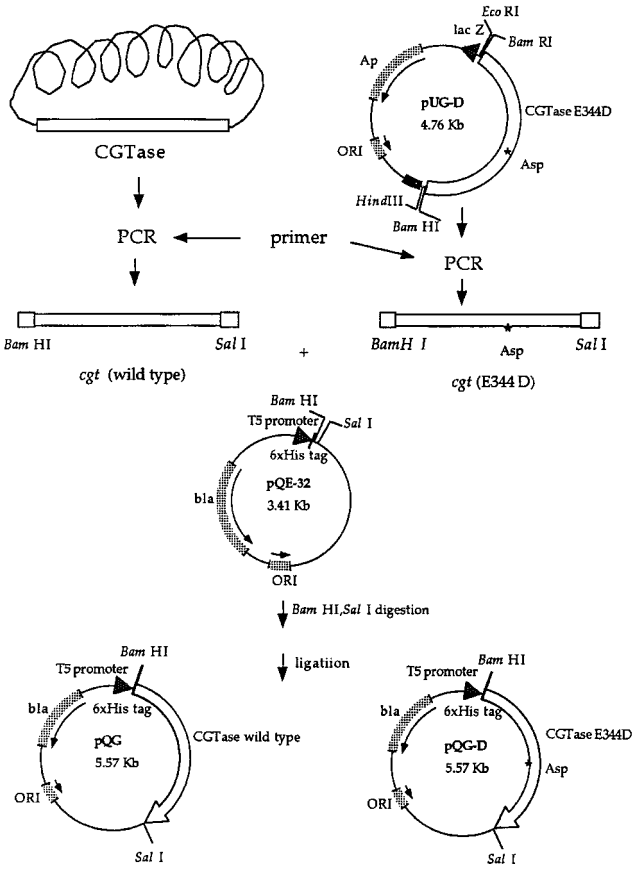

Fig. 1. Construction of the overexpression plasmids of CGTases. The wild-type and mutant *cgt* genes were obtained by PCR using *B. macerans* chromosome DNA and the plasmid, pUG-D, as templates. The forward primer contained a *Bam* HI cutting site, and the reverse primer contained a *Sal* I site. After digestion with *Bam* HI and *Sal* I, the *cgt* genes were cloned into the compatible sites of pQE32 plasmid to generate pQG and pQG-D.

To overexpress CGTase, one ml of overnight culture was inoculated into 100 ml of LB liquid medium and grown at 37 ºC, 170 rpm. When the culture reached a cell density (A_{660}) of about 0.6-1.0, various amounts of IPTG were added to induce the synthesis of CGTase, and the culture was continuously grown under the same conditions for 5 hr. To obtain a soluble form of the enzyme, the pregrowing culture was cooled at 4 ºC for half an hour before IPTG induction. Then, the induced culture, which contained 1 mM IPTG, was incubated at 20 ºC and 170 rpm for 5 hr. Afterwards, the culture was centrifuged at 4000 $\times$ g for 20 min at room temperature; the supernatant was decanted, and the cell pellet was washed twice with distilled water. After the cell pellets was resuspended in 10 ml of 20 mM Tris-

HCl buffer (pH 7.2) containing 0.2 mM PMSF, the cell sample was sonicated using an ultrasonicator (Ultrasonic Processor XL of Misonic Inc.) and clarified by centrifugation. The supernatant thus obtained was then diluted to 100 ml with Tris-HCl buffer. This solution was referred to as the crude enzyme extract.

Affinity chromatographic purification of CGTase using β-CD-conjugated Sepharose CL-6B column has been described previously (Jeang *et al.*, 1992). Briefly, the fractions with enzymatic activity were collected and dialyzed against 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl. The dialyzed enzyme solution was then applied onto a Ni-NTA column equilibrated with the above phosphate buffer at a flow rate of 12 ml/hr. After the column was washed with 50 mM phosphate buffer (pH 6.0) containing 300 mM NaCl and 10% glycerol, the bound enzyme was eluted with a linear gradient of imidazole from 0 to 0.5 M in 30 ml of the same washing buffer. The fractions which contained CGTase activity were collected.

4. Enzyme Assay

The method used for enzyme assay was reported previously (Jeang *et al.*,1992). Both coupling and starch digesting activities of CGTase were measured.

5. Polyacrylamide Gel Electrophoresis of Proteins

Disc-PAGE was performed using 7.5% polyacrylamide gel in Tris-glycine buffer as described by Laemmli (1970). SDS-PAGE of the overexpressed proteins was carried out according to the procedure of Studier (1973). For activity staining, the gel was washed twice with distilled water after disc-PAGE, soaked with 0.02 M acetate buffer (pH 5.5) for 20 min, transferred to the same buffer containing 3% soluble starch and then incubated at 40 ºC for 2 hr. After enzymatic reaction and after the excess starch was washed off from the gel surface using acetate buffer (0.02 M, pH 5.5), the gel was stained with a solution containing 3% potassium iodine and 1.3% I_2.

III. Results

1. Overexpression of CGTase in *E. coli*

Overexpression of CGTase in *E. coli* harboring the pQG plasmid was examined by Coomassie Brilliant Blue staining and immunobloting of the total cell lysate (Fig. 2). A protein with mobility consistent with that of the authentic *B. macerans* CGTase was observed when IPTG was added to the culture at a final concentration of 1 mM (Fig. 2 (a): lanes 4-8). This protein reacted positively with the antibody raised against CGTase (Fig. 2(b): lanes 4-8). However, no coupling activity of CGTase was detected in the supernatant of the *E. coli* cell homogenate (data not shown). TEM examination of *E. coli* cells which overproduced the CGTases (Fig. 3) revealed that this was due to the fact that most of the overexpressed CGTases formed inclusion bodies in *E. coli* cells. To increase the production of CGTase in the soluble form, the *E. coli* culture was incubated at a relatively low temperature (20 ºC) as suggested by Schein (1989). Just as expected, the amount of soluble CGTase increased considerably (Fig. 4: lane 6). Similarly, soluble E344D CGTase was obtained when it was overexpressed in *E. coli* at a low temperature (data not shown). The E344K and E344L CGTases were also overexpressed in *E. coli*. The extent of their expression was similar to that of wild-type and E344d CGTase; however, only trace amounts of soluble CGTases were obtained when the cultures were cultivated at 20 ºC. Probably, this was attributable to the replacement of E344 on CGTase with K or L, which resulted in exposure of the internal hydrophobic region of the mutant CGTases and, thus, their aggregation.

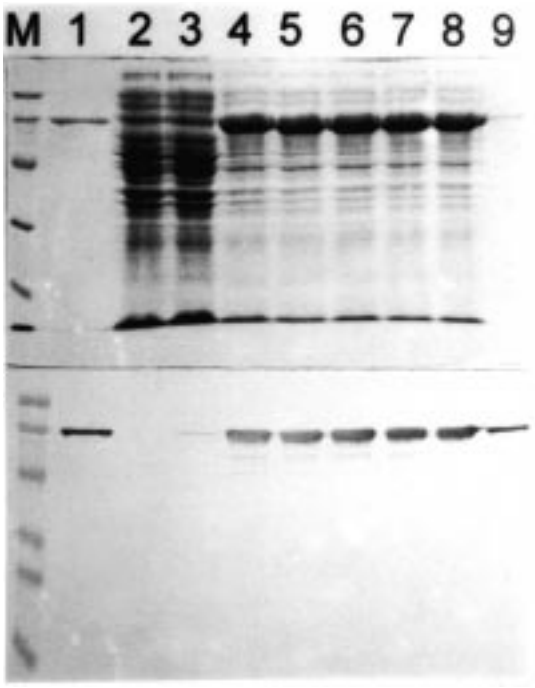

Fig. 2. SDS-PAGE (a) and Western blot (b) analyses of CGTase induced by different amounts of IPTG. (a) Protein samples loaded onto the 10% SDS-polyacrylamide gel were electrophoresed and stained with Coomassie Brilliant Blue. (b) The proteins on the polyacrylamide gel were transblotted onto a piece of nitrocellulose paper and detected immunologically with antibodies raised against *B. macerans* CGTase. Lane M : low molecular weight standard; lanes 1 and 9 : purified CGTase from *B. macerans*; lane 2 : 10 μl of total cell lysate of *E. coli* M15 harboring pQE32; lanes 3, 4, 5, 6, 7 and 8 : 10 μl (in (a)) or 3 μl (in (b))of total cell lysate of *E. coli* M15 harboring pQG induced by 0, 1, 2, 4, 6 and 8 mM IPTG, respectively.

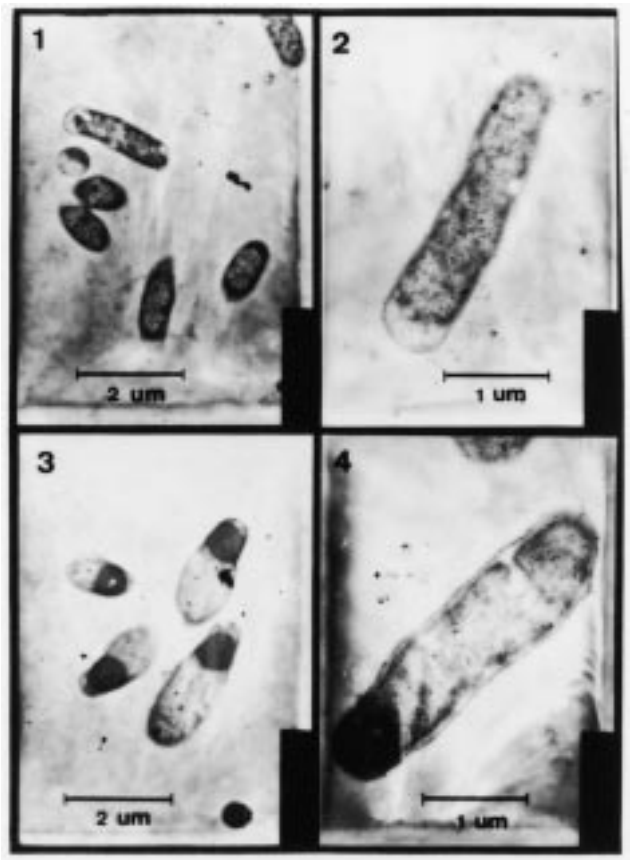

Fig. 3. TEM examination of the CGTase inclusion bodies in *E. coli*. Panels 1 and 2 : *E. coli* M15 harboring pQE32; panels 3 and 4 : *E. coli* M15 harboring pQG grown for 5 hr after IPTG induction.

To semi-quantify the amount of CGTase produced in *E. coli*, we measured the enzyme unit (*a*) of a known amount (*W*, mg) of *B. macerans* CGTase and determined the total enzyme units (*b*) in the soluble fraction of cell lysate from a fixed volume (*c*, ml) of *E. coli* culture. Calculation using the equation X (mg/l) = $W \times b/a \times 1000/c$ revealed that the yields of the wild-type and E344D CGTases in *E. coli* were 103.6 mg/l and 125 mg/l, respectively. Both of them were about 20-fold higher than that secreted in the culture of *B. macerans*. The wild-type and E344D CGTases constituted 54 % and 60 % of the total cellular proteins of *E. coli* M15, respectively, as examined by scanning the SDS-PAGE gel with a Bio-Image scanner.

2. Purification and Characterization of the Wild-type and Mutant CGTases Overexpressed in *E. coli*

After the crude enzyme solution was passed through two affinity columns as described in the Methods section, the wild-type and the E344D CGTases with 18 extra amino acid residues at their N-termini were purified. The fusion proteins were referred to as the wild-type and E344D CGTases purified from *E. coli*. Both of the purified enzyme displayed a single protein band on the native PAGE gel after staining with Coomassie Brilliant Blue (Fig. 5(a)). Zymograms of the two CGTases purified from *E. coli* showed that they both had starch-digesting activity, as did the authentic *B. macerans* CGTase (Fig. 5(b)). Although different amounts of purified CGTases were applied, both protein and activity staining revealed the purity of the enzyme samples.

The results of Ouchterlory double fusion analysis showed that there were similar bands of protein precipitate between the antiserum of *B. macerans* CGTase and different CGTases (data not shown). These results suggest that the antigenicities of the *E. coli* wild-type and E344D CGTases are similar to that of the authentic *B. macerans* CGTase.

The *B. macerans* CGTase, the wild-type and the E344 CGTases purified from *E. coli* were allowed to act indi-

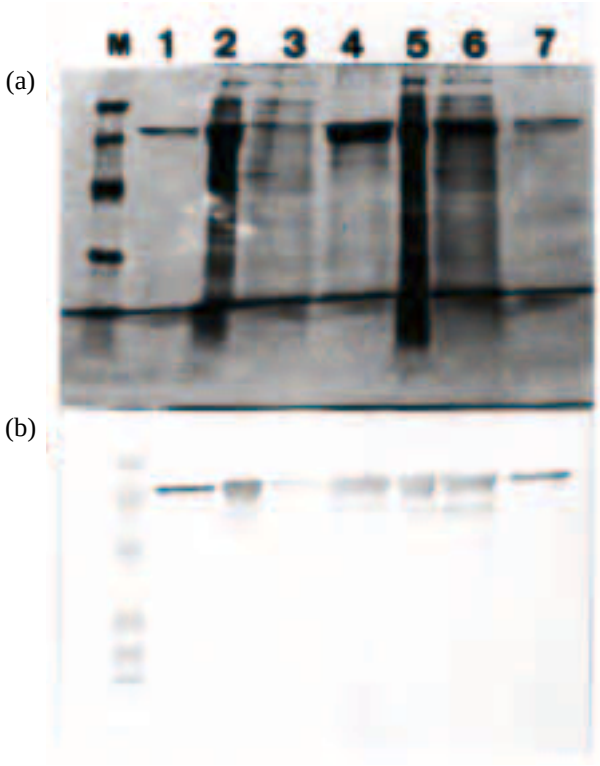

Fig. 4. Effect of the incubation temperature on the forms of wild-type CGTase overexpressed from *E. coli* M15 harboring pQG. (a) Protein samples loaded onto the 10% SDS-polyacrylamide gel were electrophoresed and stained with Coomassie Brilliant Blue. (b) The proteins on the polyacrylamide gel were transblotted onto an NC paper and detected immunologically with antibodies raised against *B. macerans* CGTase. Lane M : low molecular weight standard; lane 1 : *B. macerans* CGTase; lanes 2, 3 and 4 : total, soluble and insoluble fraction, respectively, of *E. coli* cell lysate prepared from cell samples incubated at 37 ºC; lanes 5, 6 and 7 : total, soluble and insoluble fraction, respectively, of *E. coli* cell lysate prepared from cell samples incubated at 20 ºC.

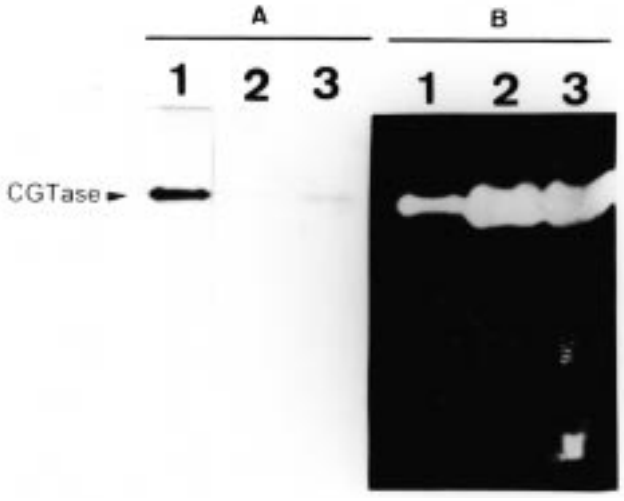

Fig. 5. Purity and activity of the wild-type and mutant CGTases. Disc-PAGE was performed on a 7.5% polyacrylamide gel. The protein samples were loaded onto a polyacrylamide gel, electrophoresed, then (a) stained with Coomassie Brilliant Blue, (b) soaked in the ice cold acetate buffer (0.02 M, pH 5.0), and then stained with a solution containing 3% KI and 1.3% I_2. Lanes 1, 2 and 3 : *B. macerans* CGTase, wild-type and E344D CGTase from *E. coli*, respectively.

vidually with various concentrations of α-CD to measure their enzymatic coupling activities. Table 1 shows the kinetic parameters of the three CGTases. The authentic *B. macerans* CGTase (2.10 mM, 99.8 s^{-1}) and the wild-type CGTase (0.58 mM, 26.5 s^{-1}) purified from *E. coli* had different Km and *k*cat values; however, their catalytic efficiencies, *k*cat/Km, were similar. Since the *E. coli* overexpressed CGTase was different from the authentic *B. macerans* CGTase in the N-terminal region, we suspected that the difference of catalytic mechanisms between these two enzymes was due to the structural change of the overexpressed CGTase in its N-terminal domain. Moreover, the *k*cat/Km value of E344D CGTase was 2-fold higher than that of wild-type CGTase, suggesting that the E344D substitution in the mutant enzyme led to a higher coupling efficiency.

Table 1. Kinetic Parameters of Different CGTases

	Km (mM)	*k*cat (s^{-1})	*k*cat/Km($M^{-1}s^{-1}$)
B. macerans CGTase	2.10±0.11	9.98×10^1	4.75×10^4
wild-type CGTase from *E. coli*	0.58±0.05	2.65×10^1	4.57×10^4
E344D CGTase from *E. coli*	1.05±0.06	9.07×10^1	8.64×10^4

Notes: These parameters were determined by means of a coupling reaction of CGTase. A fixed amount of CGTase was added to the reaction mixture, which contained various concentrations of α-CD (0. 5, 1.0, 2.5, 5.0, 7.5, 10.0 or 12.5 mM), 20 mM sucrose, 0.1 mg amyloglucosidase, and 20 mM Tris-HCl buffer (pH 7.5). The glucose produced was estimated using the colorimetric method of Daniel and Neal (1967) with dinitrosylisilic acid as reagent.

The cyclodextrins produced by different CGTases while acting on soluble starch are shown in Fig. 6. The conversion rates of total CDs were 50.5%, 46.1% and 42. 5% for the authentic *B. macerans* CGTase, the wild-type and E344D CGTases purified from *E. coli*, respectively. In addition, the conversion rates for α-CD were 18.4%, 24. 9% and 14.5% for the three enzymes, respectively. α-CD was the major product of cyclization reaction in the early stage for all three enzymes. The change in the amount of α-CD and β-CD during incubation was similar to that reported for other α-CGTases (Kaneko *et al.*, 1990).

IV. Discussion

We found previously that no other starch-digesting enzymes were copurified with CGTase expressed in *E. coli* after β-CD affinity chromatography (Wung, 1994). However, the advantage was lost in the purification of CGTases overexpressed in *E. coli* M15. This is because two different forms of CGTase were expressed in *E. coli* cells harboring pQG and pQG-D plasmids, owing to the presence of an extra ribosomal binding site in front of the *cgt* gene on the plasmids. Thus, an extra Ni-NTA affinity column was designed to selectively purify the CGTase with 6x His residues fused at the N-terminus of the protein. Based on the purification data, we found that this was the predominant form of CGTase overexpressed in *E. coli*. This result suggests that the 5' most ribosomal binding site of the *cgt* gene was used more frequently than the one adjacent to the *cgt* gene during translation.

It was proposed by Kubota *et al.* (1991) that the substrate binding-sites on CGTase are located in both domains A and D of the enzyme. The differences in the Km and *k*cat values of coupling reaction between the authentic *B. macerans* CGTase and the wild-type CGTase purified from *E. coli* indicate that the 6x His and the extra amino acids residues at the N-terminus of the *E. coli* overexpressed CGTase might affect the structure of domain A. The influence of this potential structural change was also observed in the cyclization activity of CGTase. The maximal yields of α-CD by these two CGTases were about the same: 36. 4% and 36.7%, respectively (Fig. 6). However, the durations of time required for the maximal yields of α-CD were different; it was longer for the CGTase with peptide fusion at the N-terminus. We suspected that this was caused by the poor substrate-binding capability or the slower turn over rate of the fusion CGTase. The slowest declining rate of α-CD in the reaction mixture containing wild-type CGTase purified from *E. coli* might have been due to the smallest *k*cat value of the coupling reaction of this enzyme (Table 1). The cyclization rate of the E344D CGTase was highest among the three; however, the production of α-CD by this enzyme was not as high as that of the wild-type CGTase. The remaining α-CD in the reaction mixture after 48 hr in-

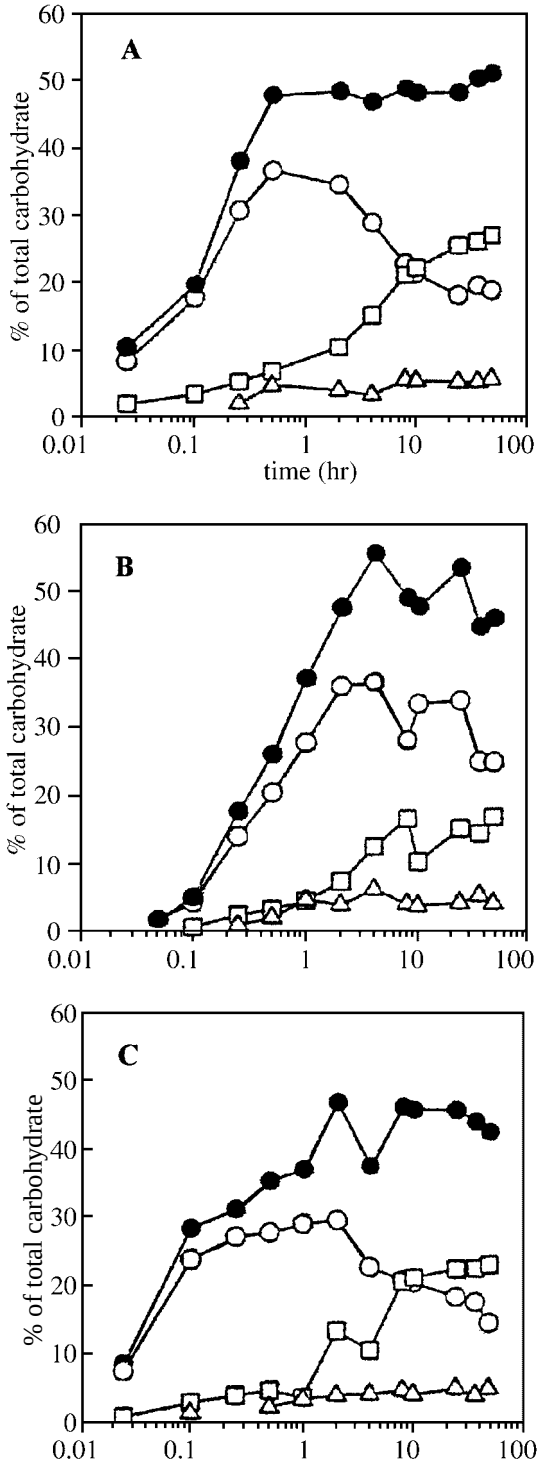

Fig. 6. The CD production of different CGTases. (a) CGTase purified from *B. macerans*, (b) CGTase purified from *E. coli* harboring pQG, (c) CGTase purified from *E. coli* harboring pQG-D. The CGTases were added separately to 1% soluble starch solution (E/S = 1/1000, by weight). At time intervals, reaction mixture was sampled and cleaned by means of methanol precipitation to remove unreacted starch. The supernatant was dried and redissolved in distilled water and taken for HPLC analysis. •—• : total CDs; ○—○ : α-CD; □—□ : β-CD; Δ—Δ : γ-CD.

cubation was 14.5% and 24.5% for E344D and wild-type CGTases, respectively. The higher catalytic efficiency of the mutant CGTase on coupling reaction might explain why only a smaller amount of α-CD remained after a longer period of incubation. The above-mentioned results revealed that E344 is not only responsible for substrate binding, but is also involved in catalytic function. Furthermore, the N-terminal region of this enzyme is involved in both substrate binding and catalytic activities of CGTase. The coincident changes of coupling (Table 1) and cyclization reactions (Fig. 6) imply that the two reactions are carried out at the same location or at nearby location of the enzyme.

Acknowledgment

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於大腸桿菌中大量表現之*Bacillus macerans*環狀糊精葡萄糖基轉移酵素性質之研究

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摘 要

環狀糊精葡萄糖基轉移酵素(CGTase)可催化澱粉及α-1,4-葡萄糖聚醣轉變為環狀糊精(CD)。前此，以化學試劑WRK修飾的實驗中發現，源自Bacillus macerans之CGTase，其多胜G上第344位置的麩胺酸殘基與酵素之活性有關。故本實驗在此位置上進行定點突變以確認之。將三種突變*cgt*基因轉形於*Escherichia coli* M15可大量表現基因產物，於37劍下分別產生E344D, E344K及E344L CGTase的不溶體。降低轉形株之培養溫度至20劍時，僅原態及E344D CGTase大部分以可溶性狀態生成。以b-CD及Ni-NTA二種親和膠體層析，可自大腸桿菌轉形株中純化得原態及E344D CGTase。進行對α-CD偶合反應之動力學分析，得*Bacillus macerans*，原態及E344D CGTase之Km值分別為2.10，0.58及1.05 mM，而*k*cat值為99.8, 26.5及90.7 s□。此外，環化反應分析中發現三者α-CD之產量分別為18.4, 24.9及14.5%。由於E344D的突變造成CGTase對偶合反應及環化反應催化活性的變化，因此證實E344與酵素之催化功能有關。