CrossMark

ORIGINAL ARTICLE

Improved activity of β-cyclodextrin glycosyltransferase from *Bacillus* sp. *N-227* via mutagenesis of the conserved residues

Hua Wang[1] · Wenxi Zhou[2] · Hua Li[1] · Bu Rie[1] · Chunhong Piao[3]

Abstract β-Cyclodextrin glycosyltransferase (β-CGTase) belongs to the α-amylase family of enzymes and converts starch to cyclic oligosaccharides called β-cyclodextrins (β-CD). The β-CGTase from *alkalophilic Bacillus* sp. *N-227* was separately mutagenized to give three site-directed β-CGTase mutants, Y127F, R254F and D355R, that showed enhanced cyclization activity towards a starch substrate from 1.64 to 2.1-folds. Kinetic studies indicate that the mutants had higher affinity towards the substrate than the wild type β-CGTase. The Y127F mutant had the highest affinity which was indicated by the lowest K_m of 15.30 mM and the highest catalytic activity. Increasing hydrophobicity around the catalytic center appeared to favor the cyclization activity of the mutants. The β-CGTase and the three mutants showed optimal enzyme activity at 60 °C and pH 6.0. All the enzymes were stable for at least 60 min across a wide pH range (5.0–7.0).

Keywords β-Cyclodextrin glycosyltransferase · Cyclization activity · Optimum pH · Optimum temperature · Site-directed mutagenesis

✉ Hua Wang
  doccnrb@163.com

1 College of Life Science, Inner Mongolia University for Nationalities, Tongliao 028000, Inner Mongolia, China

2 Tongliao Academy of Agricultural Sciences, Tongliao 028000, Inner Mongolia, China

3 College of Food Science and Engineering, Jilin Agricultural University, Changchun 130118, China

Introduction

Cyclodextrin glycosyltransferase (EC 2.4.1.19, CGTase) belongs to the α-amylase family of enzymes (Bautista et al. 2012; Janecek and Sevcik 1999; MacGregor et al. 2001), which catalyzes three type reactions towards starch, namely cyclization to yield cyclodextrins (CDs), hydrolysis, and disproportionation (van der Veen et al. 2000b, c). Catalysis of cyclization is the primary function of CGTase (Leemhuis et al. 2010). The well-characterized forms of CDs are α-, β- and γ-CD that consist of six, seven and eight–D glucose units, respectively (Goh et al. 2009). Among them, the β-CD harbors a hydrophobic internal cavity and a hydrophilic outer surface, which endows β-CD with the water-soluble capacity (Szejtli 1990).

These properties make β-CD highly attractive for diverse applications in the field of industry, cavitation, food, medicine, as well as cosmetic (Astray et al. 2010; Slominska et al. 2002; Stella and He 2008; Szente and Szejtli 2004). The widespread use of β-CD suggests the importance for controlling the activity of β-CGTase. Previous studies have shown that mutations in the amino residues of β-CGTase influence the enzyme activity of β-CGTase at different levels (Leemhuis et al. 2002a, 2003b, 2004a, b; Penninga et al. 1995; van der Veen et al. 2000a). W652G amino acid substitution of β-CGTase in *Bacillus firmus var. alkalophilus* enhanced the cyclization activity and decreased the hydrolytic activity of β-CGTase (Hyun-Dong et al. 2000). Contrarily, mutant A230V CGTase in *Bacillus circulans* strain 251 improved the hydrolytic activity but reduced cyclization activity (Leemhuis et al. 2003a). Additionally, Dijkhuizen and collages uncovered that mutation of tyrosine 195, which located in the central active site cleft of CGTase, significantly suppressed the cyclodextrin formation (Penninga

مدينة الملك عبدالعزيز للعلوم والتقنية KACST

Springer

et al. 1995). The amino acid side chain at conserved Phe260 residues also regulated the hydrolytic activity of CGTase. Mutation of Phe260 converts CGTase from a transglycosylase into a starch hydrolase (Leemhuis et al. 2002a). Beyond these findings, the conserved residue Asp135 was reported to be required for the proper conformation of amino residues in the catalytic sites and activity (Leemhuis et al. 2003b). Results from the Leemhuis.H. demonstrated that substrate-binding site mutations of CGTase including Y167F, G179L, G180L, N193G and N193L exhibited decreased β-CD production, disproportionation as well as coupling reactions (Leemhuis et al. 2002b). All these findings suggested that amino acid mutations in the conserved and critical sites regulate the activity of CGTase. Therefore, it is desirable to prepare, characterize and study other novel mutants that may improve the activity of β-CGTase.

The structure of β-CGTase consists of five domains (Klein et al. 1992). Domains A and B are responsible for the catalytic activity of CGTase, while domains C and E mediate the binding to raw starch (Lawson et al. 1994; Penninga et al. 1996). The function of domain D remains to be illustrated. It was reported that Arg204 plus the three catalytic residues Asp206, Glu230 as well as Asp297 are totally conserved in the α-amylase family (Janecek 2002). Additionally, seven conserved sequence regions covering strand beta 2, beta 3, beta 4, beta 5, beta 7 and beta 8 of the catalytic (beta/alpha)(8)-barrel as well as the C-terminus of domain B have been identified (Janecek 2002). These conserved regions contain the catalytic, specificity and substrate-binding residues (Janecek 2002). The crystal structure of β-CGTase from *Bacillus circulans* strain 251 has been resolved (Lawson et al. 1994). The β-CGTase from *Bacillus* sp. N-227 in this study has identical amino acid sequence as the β-CGTase from *Bacillus circulans*. A Swiss Model structure based on a template (PDB ID: 1-pamB) is available. The three-dimensional structure of the β-CGTase revealed a 'pocket' or substrate-binding cleft which is the activity center of the enzyme. We report here three mutants of this β-CGTase, Y127F, R254F and D355R corresponding to variant positions located in Domain A, a part of the catalytic center, and their improved catalytic activity towards cyclization of starch.

Materials and methods

Strain and culture condition

The wild type β-CGTase from *Bacillus* sp. N-227 (GenBank accession DQ631916) and *Escherichia coli* strain BL21 (DE3) was provided by the Technology Center of Bright Dairy & Food Co. Ltd. (Shanghai, China). The β-

CGTase expression plasmid was based on the pET-28b vector and also generated by the Technology Center (The β-CGTase bacterial expression plasmid (pET-28b-β-CGTase-6xHis) which expresses the β-CGTase-6xHis fusion protein was purchased from the Technology Center).

Culture conditions

The seed medium components, 1% tryptone, 1% NaCl, and 0.5% yeast extract, were dissolved in distilled water in a total volume of 1 L, and then sterilized by autoclaving at 118 °C for 20 min.

Introduction of mutations by using overlap extension PCR

Overlap extension PCR is a well-established PCR amplification method that can be used to introduce and recover mutations in specific DNA sequences without the requirement for restriction enzyme sites (Angelaccio and di Patti 2002).

Primers

Based on the homologous nucleotide sequences of the wild type β-CGTase from *Bacillus* sp. N-227 (GenBank accession DQ631916), four pairs of primers were designed using Primer Premier 6.0 and Oligo 6.0 software. Three of the 4 pairs of primers had the desired substitution (Table 1).

Overlap extension PCR analysis

Overlap extension PCR was performed using the ABI Step One Plus system (Applied Biosystems) followed by melting curve analysis with the cycling programs described below. For the first round of PCR, pET-28b (pET-28b-β-CGTase-6xHis) was used as the template for production of ORF-1 and ORF-2. Initial denaturation was at 94 °C for 5 min, followed by 35 cycles of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 90 s. A final extension of 72 °C for 10 min was performed, followed by storage of products at 4 °C. A mixture of ORF-1 and ORF-2 was used as the template to produce the mutated gene fragment during the second round of PCR. The procedure involved initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 10 min. Taq polymerase was then added into the system, and the reaction was continued at 72 °C for a further 30 min. The samples were stored at 4 °C.

In vitro protein synthesis

A gel extraction kit (TIGEN Biotech) was used to isolate the PCR fragments which were then ligated into the

Table 1 Primers used in overlap extension PCR

Primers	Primer sequence (5′ → 3′)	
F1	CAAGCCATGGAAAGATTTATGAAA	β-CGTase
R2	ATTAT CTCGAGCTATTA AGGCTGCC	
F(YF)2	TGAGCTTATCACGGCttcTGGGCGCGGGACTCA	Y127F
R(YF)1	TGAGTCCCGCGCCCAgaaGCCGTGATAAGCTCA	
F(RF)2	TCGGGGTTGACGGCATTttcGTGGACGCGGTCAAGC	R254F
R(RF)1	GCTTGACCGCGTCCACgaaAATGCCGTCAACCCCGA	
F(DR)2	CTTCATCGACAATCATcgcATGGAGCGTTTCCACAC	D355R
R(DR)1	GTGTGGAAACGCTCCATgcgATGATTGTCGATGAAG	

pMD18-T. Simple vector *E. coli* DH5α was transformed with these plasmids using the heat shock method. Blue–white selection was conducted on flat LB plates containing ampicillin (100 mg/mL) to select recombinant colonies for expansion in liquid culture. Recovered plasmids with the correct restriction pattern were sent to Sangon Biotech for gene sequencing. The sequence analysis confirmed that cDNAs from pMD18-T were subcloned to pET-28b and introduced into *E.coli* BL21 (DE3) for protein expression.

Determination of a mutation point of the β-CGTase gene

The three bacterial strains expressing the β-CGTase mutants were named Y127F, R254F and D355R. Recombinant proteins were purified and subjected to western blotting using mouse anti-6-His monoclonal antibody. The medium containing the β-CGTase expressing strains was collected after shake cultivation for 8 h, and then 1% lactose was added overnight for induction of protein expression. After shake cultivation for an additional 8 h, a solution containing 0.5% Triton X-100 and 1% glycine was added, before centrifugation at 6000×*g*, 4 °C for 10 min. The supernatant was dialyzed against 4 L of a 20 mM phosphate buffer, pH 7.0 at 4 °C for 24 h and then loaded onto a His-Tag affinity column (General Electrics) for purification. The expression was confirmed by SDS-PAGE and western blotting.

Enzyme activity assays

The enzyme activity assays were conducted with soluble corn starch as a substrate and measured spectroscopically. The reaction medium (0.01 mL of the enzyme solution, 0.2 mL of 0.2% starch solution, 0.2 mL of a 0.2 M glycine-NaOH buffer pH 9.0) was incubated in a water bath at 40 °C for 10 min (Rimphanitchayakit et al. 2005). 100 μL of 4 mM iodine in 30 mM potassium iodide was added to the reaction and diluted to 10 mL with water. The starch iodine complex formation was quantified by measuring absorbance at 700 nm. One unit of enzyme activity was defined as the amount that elicited a 10% reduction in absorbance (Gawande et al. 2003). In the formula below, 'a' is the optical density (OD/min) of the reaction from the control group, and 'b' is the OD of the reaction from the sample.

$$U/\text{mL} = \frac{a-b}{a} \times 1000 \times \text{dilution factor}$$

Activity-pH profile

The optimum pH of the purified enzymes was determined by replacing 0.2 M glycine-NaOH buffer (pH 9.0) with either 0.2 M sodium acetate buffer (pH 4–5), 0.2 M sodium phosphate buffer (pH 6–7), or glycine-NaOH buffer (pH 8–10). Reactions were performed following the previous procedures described for the enzyme activity assay.

Activity–temperature profile

The optimum temperature of the purified enzymes was determined by incubating the reaction mixture at different temperatures, ranging from 30 to 80 °C for 10 min. Reactions were performed following the previous procedures described for the enzyme activity assay.

pH stability profile

The pH stability of the enzymes was measured by incubating 0.1 mL pure enzyme with 0.2 mL of 0.2 M sodium acetate buffer (pH 4–5), 0.2 M sodium phosphate buffer (pH 6–7) or glycine-NaOH buffer (pH 8–10), at 60 °C, and without substrate for 60 min. The CGTase assay procedure described above was followed to determine the residual activity of the enzyme.

Thermal stability profile

The thermal stability of the enzymes was measured by incubating 0.1 mL pure enzyme with 0.2 mL buffer (0.2 M sodium phosphate buffer, pH 6.0) without substrate at different temperatures (30–80 °C) for 60 min. The

standard CGTase activity assays described above was used to determine the residual activity of each enzyme (Jeang et al. 2005).

Kinetic assays

The K_m and V_{max} values for the purified enzymes were determined by incubating 100 µL of enzyme (0.5 µg) in 200 µL of 0.2 M phosphate buffer (pH 6.0) with soluble starch solution (0.4–6.0 mg/mL) at 60 °C for 10 min. The kinetic parameters of K_m and V_{max} were obtained by a nonlinear least-square fitting procedure using the Michaelis–Menten equation and the curve fitting software (Origin 8.0).

Results

Generation of the β-CGTase mutants

The overlap extension PCR was used for site-directed mutagenesis of the generation of the β-CGTase mutants, and three mutagenic primers were designed at three key positions of β-CGTase. As shown in Fig. 1a, the first-round PCR using primers F1 and R1 yielded a pattern containing 6 different bands following agarose gel electrophoresis. Lanes 1 and 2 were the two fragments of mutant gene of R254F, the fragments length was, respectively, 762 and 1377 bp; lanes 3 and 4 were the two fragments of mutant gene of D355R, the fragments length was, respectively, 1065 and 1074 bp; lanes 5 and 6 were the two fragments of mutant gene of Y127F, the fragments length was, respectively, 381 and 1758 bp. The second round extension PCR

using primers F2 and R2 yielded complete mutant genes. Agarose electrophoresis analysis of the mutation genes showed that there were three specific bands of 2.1 kbp (from lanes 2 to 4), respectively. The size of the mutant genes was similar with genes of β-CGTase (lane 1) (Fig. 1b).

We prepared the amino sequence alignment of β-CGTase from *Bacillus* sp. (142676), *Bacillus circulans* (39420) and *Paenibacillus* sp. Xw-6-66 (452182092). The location of each mutant within the primary amino acid sequence of the β-CGTase is shown in Fig. 2, where the 5 domains of β-CGTase and the mutation sites were highlighted.

We examined the gene products of the cloned β-CGTases fragment and its mutants by in vitro protein synthesis using an *E. coli* extract system. The recombinant expression plasmids were transformed into *E. coli* BL21 (DE3). Following induction of protein expression overnight using 1% lactose, we lysed the bacteria and obtained the mutant proteins, performed SDS-PAGE analysis of the proteins and the purifications (Fig. 3a–c). A band of about 70 kDa for the wild type β-CGTase (lane 1, Fig. 3a, b) and all mutant constructs (lanes 2–4, Fig. 3a, b) were observed. The novel combination of the modern molecular approaches used in this study for generation of β-CGTase mutants seems to be suitable for quick, reliable and simple. Mutant β-CGTase was constructed into the pET-28a vector that harbors His-tag. To detect the expression of mutant β-CGTase, Western blot analysis was performed with anti-His antibody. Western blotting for expression of the fusion His-tagged recombinant protein was shown in Fig. 3c. A band at about 70 kDa was observed for the wild type β-CGTase (lane 3) and the mutants (lanes 4-6), while the lane 1 and lane 2 were obtained with *E. coli* strain BL21 (DE3) and pET-28b.
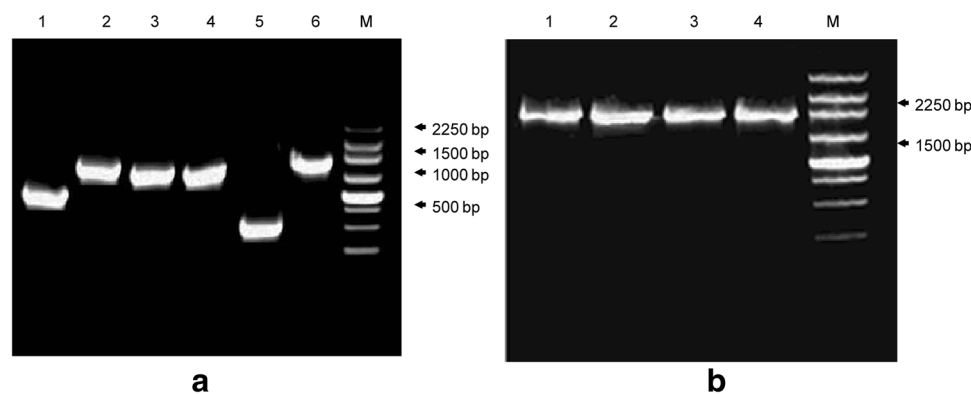

Fig. 1 Verification of the mutant constructs generation. **a** Band pattern observed after first round PCRs using primers F1 and R1. *Lane M* molecular weight marker (1–250 bp); *lanes 1* and *2* ORF-1 and ORF-2 of F substituted for R254; *lanes 3* and *4* ORF-1 and ORF-2 of R substituted for D355; *lanes 5* and *6* ORF-1 and ORF-2 of F

substituted for Y127. **b** Band pattern observed after second-round PCRs using primers F2 and R2. *Lane M* molecular weight marker (1–250 bp); *lane 1* the wild type β-CGTase; *lane 2* F substituted for Y127; *lane 3* R substituted for R254; *lane 4* F substituted for D355
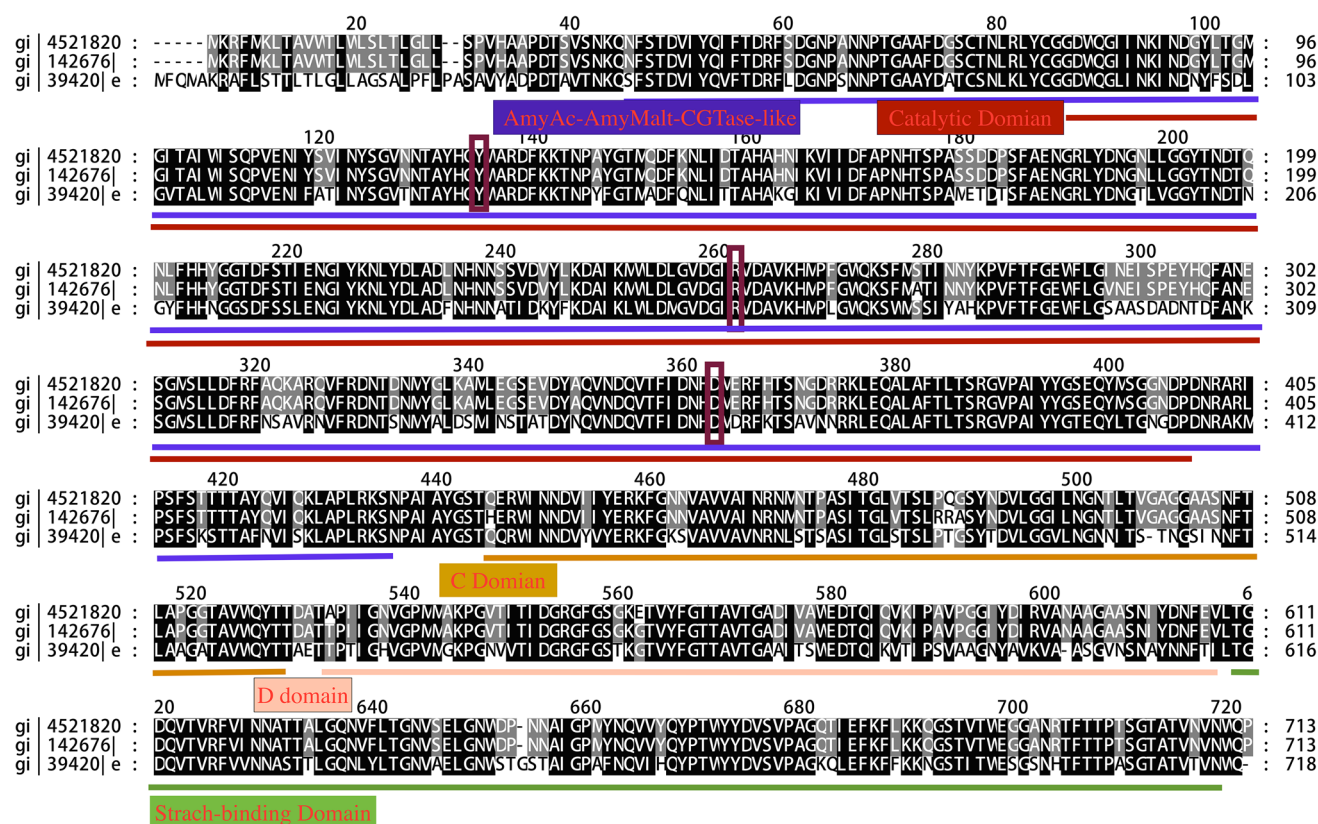
Fig. 2 Amino acid sequence alignment of the β-CGTases. The conserved domains of β-CGTases are shown with *different colors*. The mutation sites Y127, R254 and D355 are highlighted with *red box*

Enzyme activity assays

We tested relative enzyme activity of the wild type β-CGTase and Y127F, R254F, and D355R mutant β-CGTases and the results are listed in Tables 2 and 3. The data showed that cyclization activity of the mutant β-CGTases was increased to 2130–1660 U/mL compared to 1012 U/mL for the wild type β-CGTase, indicating a 1.6 to 2.1-fold activity enhancement.

The kinetic parameters for the β-CGTase and its mutants Y127F, R254F, and D355R were measured using the starch substrate and they are tabulated in Table 3. The V_{max} values in the range of 22.02 and 21.41 µg/min for the mutant proteins were higher than 21.00 µg/min for the parent wild type β-CGTase. Meanwhile, the observed k_{cat} values varying between 73.40 and 71.37/s for the mutant β-CGTases were also higher than the number of 70.00/s for the wild type β-CGTase. The ratio of k_{cat}/K_m for Y127F, R254F, and D355R variants was 4.80, 4.04, and 4.10/s/mM, respectively, compared to 3.65/s/mM for the wild type β-CGTase. The ratio suggested how efficiently a catalytic enzyme converts a substrate into products. A higher ratio indicates a higher efficiency of the enzyme.

The increased ratio of 1.15, 0.39, and 0.45 for the mutants compared to the wild type β-CGTase indicates enhanced catalytic activity for the mutants which was consistent with the enzyme activity assay.

The Michaelis constant K_m is an indicator of the substrate's affinity for the enzyme. As shown in Table 3, the observed K_m was lowered by 3.90, 1.40, and 1.80 mM for the Y127F, R254F, and D355R mutants to 15.30, 17.80, and 17.40 mM from 19.20 mM for the wild type β-CGTase. This observation indicates that all the three mutants had higher affinity towards the starch substrate than the parent β-CGTase.

Determination of pH optima using a starch substrate

The activity of the wild type and mutant forms of the β-CGTase as a function of pH was examined over a pH range of 4–8 in a universal buffer using the starch substrate (Fig. 4a). The activity-pH profiles for the Y127F, R254F, and D355R mutants were similar to that for the wild type β-CGTase. The highest activity was observed at pH 6.0 for all the four enzymes, indicating limited influence of the single site mutation on the activity–pH relationship.

Fig. 3 SDS-PAGE and western blotting validation of the mutant enzyme expression and purification. **a** SDS-PAGE validation of mutant enzyme expression; **b** SDS-PAGE validation of mutant enzyme purification; *Lane M* molecular weight marker of protein; *lane 1* the wild type β-CGTase; *lane 2* Y127F; *lanes 3* R254F; *lane 4* D355R. **c** Western blotting for expression of the wild type and mutant proteins; *Lane M* molecular weight marker of protein; *lane 1 Escherichia coli* strain BL21 (DE3); *lane 2* pET-28b; *lane 3* the wild type β-CGTase; *lane 4* Y127F mutant; *lane 5* R254F mutant; *lane 6* D355R mutant
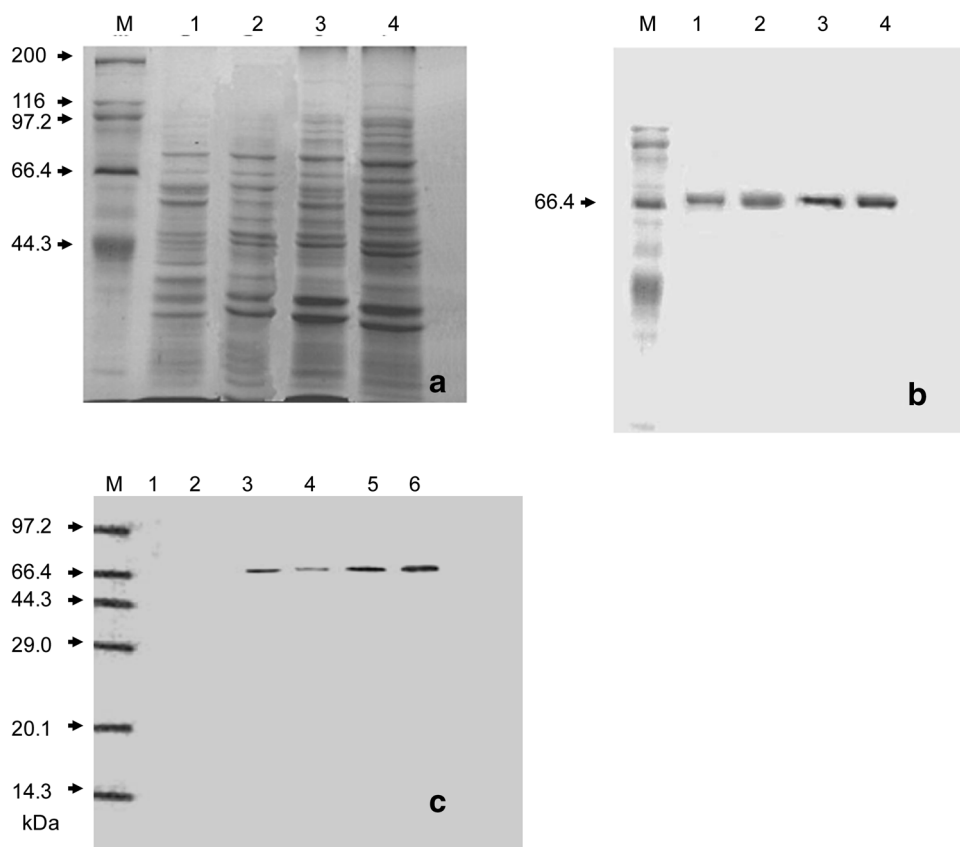
Table 2 Enzymatic activity of wild type β-CGTase and the Y127F, R254F, and D355R mutants

	Total activity (U/mL)	Relative value[a]
β-CGTase	1012.33 ± 1.528	1.00
Y127F	2130.27 ± 1.598*	2.10
R254F	1985.46 ± 2.006*	1.96
D355R	1660.75 ± 1.720*	1.64

* The mean difference is significant at the $P < 0.01$ level

[a] The ratio of total activity to mutants and β-CGTase. Experiments were performed in triplicate

Table 3 Kinetic parameters of β-CGTase and mutants

Bacterial strain	K_m (mM)	V_{max} (µg/min)	k_{cat} (per s)	k_{cat}/K_m (per s/mM)
β-CGTase	19.20 ± 1.747	21.00 ± 1.185	70.00 ± 3.950	3.65
Y127F	15.30 ± 1.236	22.02 ± 1.146	73.40 ± 3.820	4.80
R254F	17.80 ± 1.327	21.60 ± 1.284	72.00 ± 4.280	4.04
D355R	17.40 ± 1.556	21.41 ± 0.974	71.37 ± 3.247	4.10

pH stability using a starch substrate

The activity of each enzyme without pre-incubation in the buffer at pH 6.0 was defined as 100%. All the enzymes, including the wild type, were relatively stable at pH 4–7 with residual activity of more than 90%. However, they all became unstable at higher pH as indicated by the residual activity dropping from 85% at pH 8.0 to less than 30% at pH 10. The stability as a function of pH for the wild type and mutant forms of the β-CGTase was tested between pH 4 and pH 10 in a universal buffer (Fig. 4b). It is important to note that all mutants yielded stability
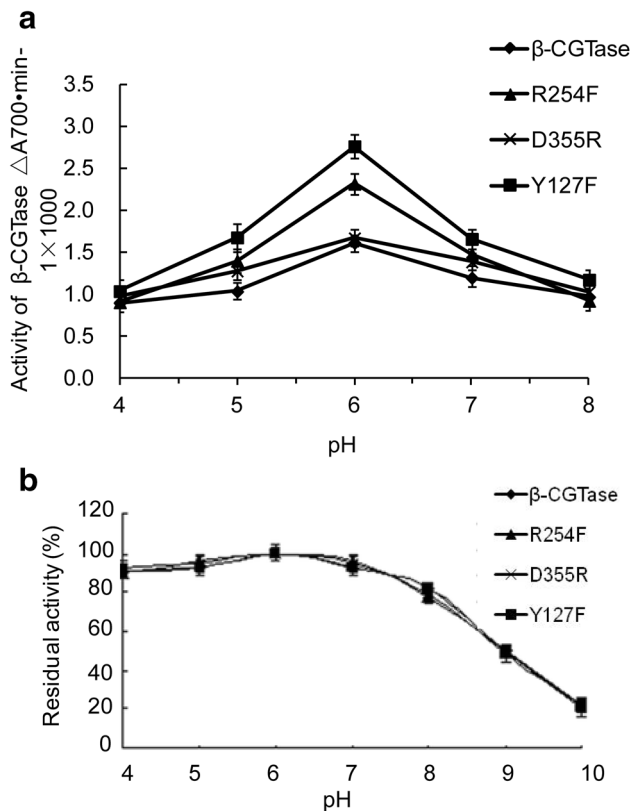
Fig. 4 Activity–pH profiles and stability of the wild type and mutant β-CGTases. **a** Activity–pH profiles of the wild type and mutant β-CGTases; **b** stability–pH of the wild type and mutant β-CGTases. The activity of the wild type β-CGTase and its mutants was measured using 0.01 mL of the enzyme solution mixed with 0.2 mL of 0.2% starch solution in a 0.2 M universal buffer at 37 °C for 2 h

curves which were very similar to that of the wild type enzyme ($P > 0.05$).

Activity–temperature profile

The optimal temperatures and thermostability of mutants and were compared to β-CGTase (Fig. 5a, b). The effect of temperature on the β-CGTase activity was investigated using the starch substrate at temperatures between 30 and 80 °C for 10 min at the optimal pH 6.0 for each enzyme (Fig. 5a). The optimal temperature for all the enzymes including the wild type β-CGTase was 60 °C. The activity–temperature profiles for the wild type β-CGTase and the mutants were very similar.

They were both optimally active at 30–60 °C, at temperatures from 30 to 60 °C, all the four β-CGTases retained at least 90% of the original activity for at least 1 h (Fig. 5b). Enzymatic activity gradually decreased when temperature exceeded 60 °C for all the enzymes. The observed residual activity at 70 °C was about 40%, and the activity decreased to 25% at 80 °C. Thus, the mutation did not have significant negative effect on stability when compared to the wild type β-CGTase ($P > 0.05$); the
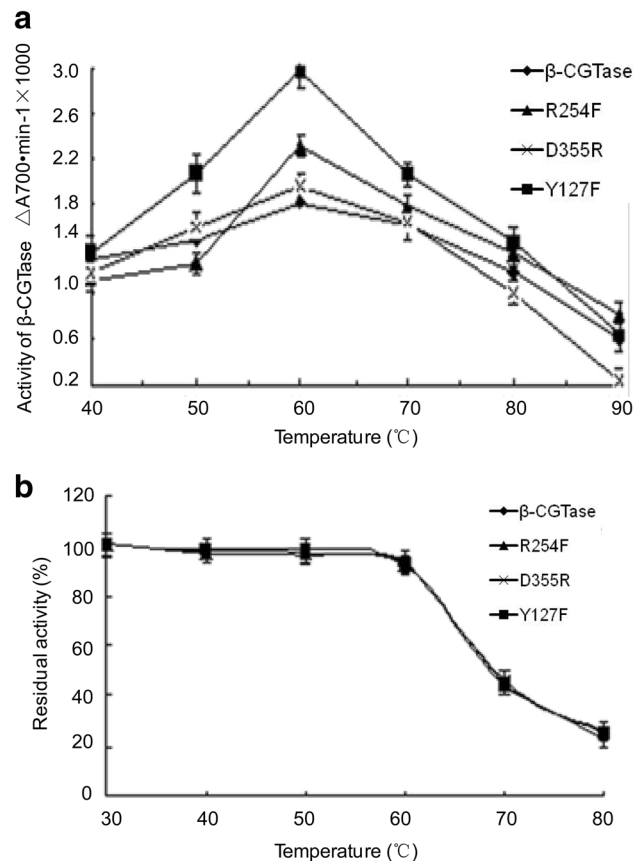

Fig. 5 Activity–temperature profiles and thermal stability for the wild type and mutant β-CGTases. **a** Activity–temperature profiles of the wild type β-CGTase and its mutants; **b** thermal stability of the wild type β-CGTase and its mutants

cyclization assays were performed as described (Penninga et al. 1995).

Discussion

β-CGTase is one of the important α-amylase family, and CGTase is the first enzyme of all the transglycosylases. In general, the cyclization activity of CGTases is much higher than the disproportionation and hydrolysis activities.

In this work, we engineered the mutants of β-CGTase by site-directed mutagenesis with the attempt to improve catalytic cyclization activity of β-CGTase. The three mutants (Y127F, R254F, and D355R) showed enhanced activity of β-CGTase. Interestingly, these mutant residues were all involved in the activity center of the enzyme, and they were located at the bottom of the 'pocket' in the N-terminal of the β-CGTase. The NH$_2$-terminal region of CGTase was important for cyclization characteristics, so the mutations would improve the activity of β-CGTase (Penninga et al. 1995). Fujiwara et al. (1992a, b) constructed the CGTase gene from *Bacilus stearothermophilus*

Fig. 6 Schematic representation of the reactions catalyzed by CGTase. After bond cleavage a covalently bound reaction enzyme glucosyl intermediate is formed. In the second step of the reaction the reaction intermediate is transferred to an acceptor molecule. In the cyclization reaction, the terminal OH^{-4} group of the covalently linked oligosaccharide is used as acceptor, whereas water or a second sugar is used as acceptors in the hydrolysis and disproportionation reactions, respectively. This figure has been adapted from Ref. Leemhuis et al. (2004a)
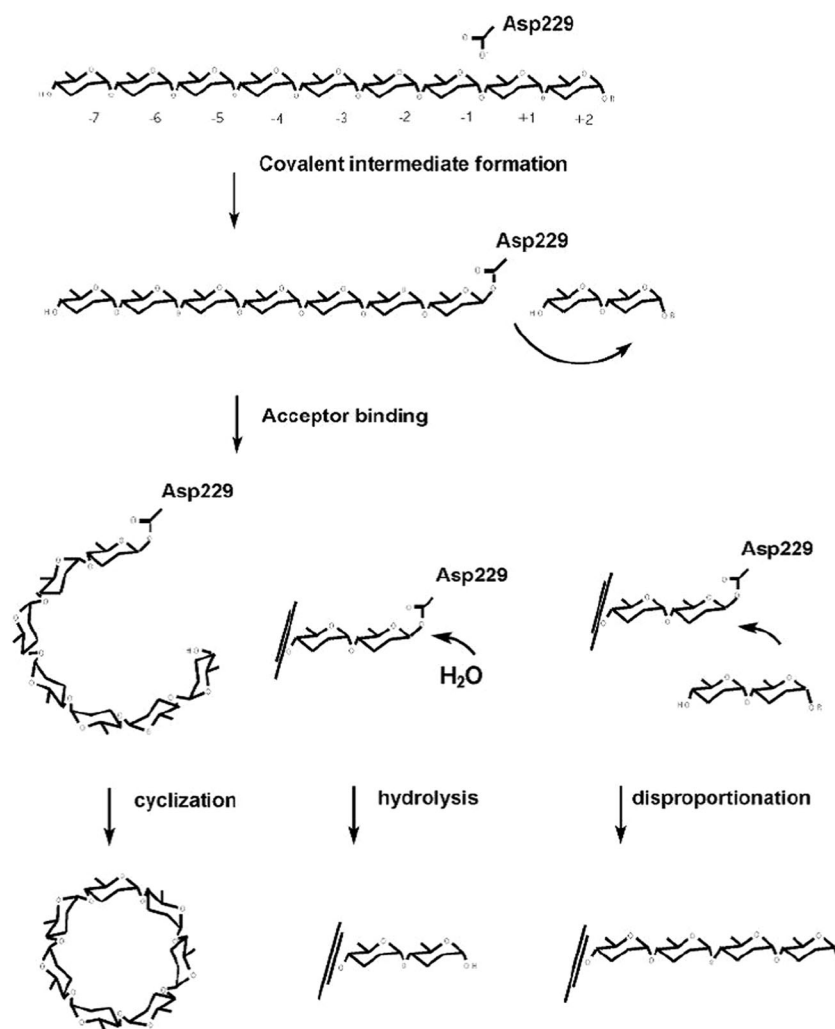
No2. Cgtl-F191Y (Phe at position 191 was replaced by Tyr) was constructed for the analysis of the NH_2-terminal region. The industrial production of cyclodextrins might be improved by the construction of mutant CGTase with improved activity of β-CGTase.

Each of these mutations was predicted to alter the hydrophobicity of a conserved domain within the β-CGTase by Support Vector Machine. Since single site mutations of the β-CGTase occurred at three different sites (127, 254, and 355) and their relative positions in the substrate-binding cleft (pocket) were expected to have different influence on the incoming starch substrate, it is difficult to discuss the activity–structure relationship. However, analysis of the observed cyclization activity of each of the three mutants and their wild type β-CGTase displayed a trend that increasing hydrophobicity of the catalytic center domain appeared to favor the cyclization activity of the mutants. The D355R mutant β-CGTase replacing a hydrophilic aspartic acid (D, hydropathy index = HI = −3.5) with a hydrophilic arginine (R, HI = −4.5) enhanced the enzyme activity to 1.6-folds. More significant enzyme activity enhancements (1.9 and 2.1-fold) were observed for the Y127F and R254F mutant β-CGTases where a hydrophilic tyrosine (Y, HI = −1.3) or arginine (R) was replaced with a hydrophobic phenylalanine (F, HI = 2.8), respectively.

It was assumed that the cyclization was taken by nucleophilic attack, and the key amino acid residues in the cyclization reaction were aspartic acid 229 in the cyclization reaction (Wind et al. 1998). When aspartic acid 229 nucleophilic attack C1 as shown in Fig. 6, the carboxyl ionization, the arginine with positive charge took the place of the negatively charged aspartic acid 355, the existence of positive charge was more advantageous to ASP229 nucleophilic attack C1, and conducive to Asp229 nucleophilic attack C1, and conducive to the substrate cyclization (Chung et al. 1998; Kragh et al. 2010).

In mutant of R254F, phenylalanine with a pair of conjugate lone pair electrons was advantageous to the

hydrophobic stacking effect and conducive to the stability of the seventh sugar molecules cyclization. For the mutant β-CGTase-Y127F, the hydrophobic phenylalanine blocked the entering H_2O into the active center, which restrained the hydrolysis and enhanced the cyclization. This result is similar with previous report (Leemhuis et al. 2003c).

The mutant proteins were successfully expressed and purified. The molecular weight of the purified mutant proteins was about 70 kDa, which was consistent with the expected values as reported by Nomoto et al. (1986). These enzymes present common properties with the majority of the reported CGTases which are monomeric with a molecular mass ranging from 60 to 110 kDa (Biwer et al. 2002; Nomoto et al. 1986).

The optimum pH of the mutants was pH 6.0, which was in accord with some other CGTases from alkalophilic *Bacillus* sp. G1 (Cao et al. 2005) and *B. stearothermophilus* ET1 (Sian et al. 2005). However, the mutants showed lower activity at pH 4.0 and 8.0, suggested that the mutants and wild type of β-CGTase required a near-neutral pH range to perform its reaction. Extreme pH values were not suitable for the enzyme to carry out cyclization activity. Most of the reported CGTases exhibited optimum pH range from 5.0 to 8.0 (Bovetto et al. 1992; Chung et al. 1998; Tachibana et al. 1999; Sian et al. 2005), but the enzyme from *Brevi-bacterium* sp. *no. 9605* possessed a higher optimum pH value at 10.0 (Cao et al. 2005).

The pH stability for cyclization is broad, in the range of pH 4.5–9.0, indicating that the mutants have not changed the pH-dependent activity of β-CGTase. A similar observation implying that the mutants of β-CGTase were related to pH stability has been also reported by Kimura et al. who constructed mutants by deleting the C-terminus of the CGTase from the *alkalophilic Bacillus* sp. *#1011* (Mori et al. 1994).

The optimal temperature for all the enzymes including the wild type β-CGTase was 60 °C, and they were both optimally active at 30–60 °C. The activity–temperature profiles for the wild type β-CGTase and the mutants were very similar. Studies by other researchers on CGTase from *B. autolyticus 11149*, *B. stearothermophilus* (Tomita et al. 1993) and *B. circulans E 192* (Chung et al. 1998) also found 60 °C was the optimum temperature, which is in agreement with mutants. And the enzymes had not a higher temperature stability compared to CGTase from *alkalophilic Bacillus* sp. G1 (Cao et al. 2005).

In vitro site-directed mutagenesis was performed to introduce Y127, R254 and D355 mutations in a conserved region of the β-CGTase enzyme. The mutant proteins were successfully expressed and purified. The Y127F, R254F, and D355R mutant β-CGTases exhibited a similar optimal temperature, optimal pH, thermal stability, and pH stability as the wild type β-CGTase. However, all the three mutants displayed a higher activity towards the corn starch substrate than the wild type β-CGTase. Increasing hydrophobicity around the substrate-binding cleft of the catalytic center appeared to enhance the cyclization activity of the enzymes.

Compliance with ethical standards

Conflict of interest All the authors declare that they have no conflicts of interest regarding this paper.

References

Angelaccio S, di Patti MCB (2002) Site-directed mutagenesis by the megaprimer PCR method: variations on a theme for simultaneous introduction of multiple mutations. Anal Biochem 306:346–349

Astray G, Mejuto JC, Morales J, Rial-Otero R, Simal-Gandara J (2010) Factors controlling flavors binding constants to cyclodextrins and their applications in foods. Food Res Int 43:1212–1218

Bautista V, Esclapez J, Perez-Pomares F, Martinez-Espinosa RM, Camacho M, Bonete MJ (2012) Cyclodextrin glycosyltransferase: a key enzyme in the assimilation of starch by the halophilic archaeon Haloferax mediterranei. Extremophiles 16:147–159

Biwer A, Antranikian G, Heinzle E (2002) Enzymatic production of cyclodextrins. Appl Microbiol Biot 59:609–617

Bovetto LJ, Villette JR, Fontaine IF, Sicard PJ, Bouquelet SJL (1992) Cyclomaltodextrin Glucanotransferase from *Bacillus-Circulans* E-192.2. Action Patterns Biotechnol Appl Bioc 15:59–68

Cao XZ, Jin ZY, Wang X, Chen F (2005) A novel cyclodextrin glycosyltransferase from an alkalophilic *Bacillus* species: purification and characterization. Food Res Int 38:309–314

Chung HJ et al (1998) Characterization of a thermostable cyclodextrin glucanotransferase isolated from *Bacillus* stearothermophilus ET1. J Agric Food Chem 46:952–959

Fujiwara S, Kakihara H, Sakaguchi K, Imanaka T (1992a) Analysis of mutations in cyclodextrin glucanotransferase from *Bacillus-stearothermophilus* which affect cyclization characteristics and thermostability. J Bacteriol 174:7478–7481

Fujiwara S, Kakihara H, Woo KB, Lejeune A, Kanemoto M, Sakaguchi K, Imanaka T (1992b) Cyclization characteristics of cyclodextrin glucanotransferase are conferred by the nh2-terminal region of the enzyme. Appl Environ Microb 58:4016–4025

Gawande BN, Sonawane AM, Jogdand VV, Patkar AY (2003) Optimization of cyclodextrin glycosyltransferase production from *Klebsiella pneumoniae* AS-22 in batch, fed-batch, and continuous cultures. Biotechnol Prog 19(6):1697–1702

Goh KM, Mahadi NM, Hassan O, Rahman RNZRA, Illias RM (2009) A predominant beta-CGTase G1 engineered to elucidate the relationship between protein structure and product specificity. J Mol Catal B-Enzym 57:270–277

Hyun-Dong S, Park TH, Lee YH (2000) Site-directed mutagenesis and functional analysis of maltose-binding site of beta-cyclodextrin glucanotransferase from *Bacillus* firmus var. alkalophilus. Biotechnol Lett 22:115–121

Janecek S (2002) How many conserved sequence regions are there in the alpha-amylase family? Biologia 57:29–41

Janecek S, Sevcik J (1999) The evolution of starch-binding domain. FEBS Lett 456:119–125

Jeang CL, Lin DG, Hsieh SH (2005) Characterization of cyclodextrin glycosyltransferase of the same gene expressed from *Bacillus macerans*, *Bacillus subtilis*, and *Escherichia coli*. J Agr Food Chem 53:6301–6304

Klein C, Hollender J, Bender H, Schulz GE (1992) Catalytic Center of cyclodextrin glycosyltransferase derived from X-ray structure-analysis combined with site-directed mutagenesis. Biochemistry 31:8740–8746

Kragh KM, Leemhuis H, Dijkhuizen L, Dijkstra BW (2010) Cyclodextrin glycosyltransferase (CGTase) polypeptides with modified hydrolysis activity. United States Patent. Lubbert Dijkhuizen, Zuidlaren, Cambridge

Lawson CL, Van montfort R, Strokopytov B, Rozeboom HJ, Kalk KH, De vries GE, Penninga D, Dijkhuizen L, Dijkstra BW (1994) Nucleotide-sequence and X-ray structure of cyclodextrin glycosyltransferase from *Bacillus-circulans* strain-251 in a maltose-dependent crystal form. J Mol Biol 236:590–600

Leemhuis H, Dijkstra BW, Dijkhuizen L (2002a) Mutations converting cyclodextrin glycosyltransferase from a transglycosylase into a starch hydrolase. FEBS Lett 514:189–192

Leemhuis H, Uitdehaag JCM, Rozeboom HJ, Dijkstra BW, Dijkhuizen L (2002b) The remote substrate binding subsite-6 in cyclodextrin-glycosyltransferase controls the transferase activity of the enzyme via an induced-fit mechanism. J Biol Chem 277:1113–1119

Leemhuis H, Kragh KM, Dijkstra BW, Dijkhuizen L (2003a) Engineering cyclodextrin glycosyltransferase into a starch hydrolase with a high exo-specificity. J Biotechnol 103:203–212

Leemhuis H, Rozeboom HJ, Dijkstra BW, Dijkhuizen L (2003b) The fully conserved Asp residue in conserved sequence region I of the alpha-amylase family is crucial for the catalytic site architecture and activity. FEBS Lett 541:47–51

Leemhuis H, Rozeboom HJ, Wilbrink M, Euverink GJW, Dijkstra BW, Dijkhuizen L (2003c) Conversion of cyclodextrin glycosyltransferase into a starch hydrolase by directed evolution: the role of alanine 230 in acceptor subsite + 1. Biochemistry 42:7518–7526

Leemhuis H, Rozeboom HJ, Dijkstra BW, Dijkhuizen L (2004a) Improved thermostability of *Bacillus circulans* cyclodextrin glycosyltransferase by the introduction of a salt bridge. Proteins 54:128–134

Leemhuis H, Wehmeier UF, Dijkhuizen L (2004b) Single amino acid mutations interchange the reaction specificities of cyclodextrin glycosyltransferase and the acarbose-modifying enzyme acarviosyl transferase. Biochemistry 43:13204–13213

Leemhuis H, Kelly RM, Dijkhuizen L (2010) Engineering of cyclodextrin glucanotransferases and the impact for biotechnological applications. Appl Microbiol Biot 85:823–835

MacGregor EA, Janecek S, Svensson B (2001) Relationship of sequence and structure to specificity in the alpha-amylase family of enzymes. Biochim Biophys Acta 1546:1–20

Mori S, Hirose S, Oya T, Kitahata S (1994) Purification and properties of cyclodextrin glucanotransferase from *Brevibacterium* Sp No-9605. Biosci Biotech Bioch 58:1968–1972

Nomoto M, Chen CC, Sheu DC, (1986) Purification and characterization of cyclodextrin glucanotransferase from an alkalophilic bacterium of Taiwan. Agric Biol Chem 50(11):2701–2707

Penninga D, Strokopytov B, Rozeboom HJ, Lawson CL, Dijkstra BW, Bergsma J, Dijkhuizen L (1995) Site-directed mutations in tyrosine-195 of cyclodextrin glycosyltransferase from *Bacillus-circulans* strain-251 affect activity and product specificity. Biochemistry 34:3368–3376

Penninga D, van der Veen BA, Knegtel RM, van Hijum SA, Rozeboom HJ, Kalk KH, Dijkstra BW, Dijkhuizen L (1996) The raw starch binding domain of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251. J Biol Chem 271:32777–32784

Rimphanitchayakit V, Tonozuka T, Sakano Y (2005) Construction of chimeric cyclodextrin glucanotransferases from *Bacillus circulans* A11 and *Paenibacillus macerans* IAM1243 and analysis of their product specificity. Carbohydr Res 340(14):2279–2289

Sian HK, Said M, Hassan O, Kamaruddin K, Ismail AF, Rahman RA, Mahmood NAN, Illias RM (2005) Purification and characterization of cyclodextrin glucanotransferase from *alkalophilic Bacillus* sp G1. Process Biochem 40:1101–1111

Slominska L, Szostek A, Grzekowiak A (2002) Studies on enzymatic continuous production of cyclodextrins in an ultrafiltration membrane bioreactor. Carbohydr Polym 50:423–428

Stella VJ, He QR (2008) Cyclodextrins. Toxicol Pathol 36:30–42

Szejtli J (1990) The Cyclodextrins and their applications in biotechnology. Carbohydr Polym 12:375–392

Szente L, Szejtli J (2004) Cyclodextrins as food ingredients. Trends Food Sci Tech 15:137–142

Tachibana Y, Kuramura A, Shirasaka N, Suzuki Y, Yamamoto T, Fujiwara S, Takagi M, Imanaka T (1999) Purification and characterization of an extremely thermostable cyclomaltodextrin glucanotransferase from a newly isolated hyperthermophilic archaeon, a *Thermococcus* sp. Appl Environ Microb 65:1991–1997

Tomita K, Kaneda M, Kawamura K, Nakanishi K (1993) Purification and properties of a cyclodextrin glucanotransferase from *Bacillus*-autolyticus-11149 and selective formation of beta-cyclodextrin. J Ferment Bioeng 75:89–92

van der Veen BA, Uitdehaag JCM, Dijkstra BW, Dijkhuizen L (2000a) The role of arginine 47 in the cyclization and coupling reactions of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251—implications for product inhibition and product specificity. Eur J Biochem 267:3432–3441

van der Veen BA, Uitdehaag JCM, Penninga D, van Alebeek GJWM, Smith LM, Dijkstra BW, Dijkhuizen L (2000b) Rational design of cyclodextrin glycosyltransferase from *Bacillus circulans* strain 251 to increase alpha-cyclodextrin production. J Mol Biol 296:1027–1038

van der Veen BA, van Alebeek GJWM, Uitdehaag JCM, Dijkstra BW, Dijkhuizen L (2000c) The three transglycosylation reactions catalyzed by cyclodextrin glycosyltransferase from *Bacillus circulans* (strain 251) proceed via different kinetic mechanisms. Eur J Biochem 267:658–665

Wind RD, Uitdehaag JCM, Buitelaar RM, Dijkstra BW, Dijkhuizen L (1998) Engineering of cyclodextrin product specificity and pH optima of the thermostable cyclodextrin glycosyltransferase from Thermoanaerobacterium thermosulfurigenes EM1. J Biol Chem 273:5771–5779