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Altered product specificity of a cyclodextrin glycosyltransferase by molecular imprinting with cyclomaltododecaose

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Cyclodextrin glycosyltransferases (CGTases), members of glycoside hydrolase family 13, catalyze the conversion of amylose to cyclodextrins (CDs), circular α-(1,4)-linked glucopyranose oligosaccharides of different ring sizes. The CD containing 12 α-D-glucopyranose residues was preferentially synthesized by molecular imprinting of CGTase from *Paenibacillus* sp. A11 with cyclomaltododecaose (CD_{12}) as the template molecule. The imprinted CGTase was stabilized by cross-linking of the derivatized protein. A high proportion of CD_{12} and larger CDs was obtained with the imprinted enzyme in an aqueous medium. The molecular imprinted CGTase showed an increased catalytic efficiency of the CD_{12}-forming cyclization reaction, while decreased k_{cat}/K_m values of the reverse ring-opening reaction were observed. The maximum yield of CD_{12} was obtained when the imprinted CGTase was reacted with amylose at 40°C for 30 min. Molecular imprinting proved to be an effective means toward increase in the yield of large-ring CDs of a specific size in the biocatalytic production of these interesting novel host compounds for molecular encapsulations. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: molecular imprinting; cross-linked proteins; cyclodextrin glycosyltransferase; cyclomaltododecaose; product specificity

INTRODUCTION

Cyclodextrins (CDs) are macrocyclic oligosaccharides with a hydrophobic interior cavity and a hydrophilic outer surface. CDs can form inclusion complexes with apolar guest compounds by molecular encapsulation. CD_6, CD_7, and CD_8 (α-, β-, and γ-CD) composed of 6, 7, and 8 α-D-glucopyranose residues, respectively, have therefore found important applications in the food and pharmaceutical industries (Szejtli, 2004). CDs are produced by the conversion of amylose by cyclodextrin glycosyltransferase (CGTase; EC 2.4.1.19). This enzyme is a member of glycoside hydrolase family 13, part of the α-amylase superfamily. CGTase is found in various microorganisms, mainly *Bacillus* species (Pongsawasdi and Yagisawa, 1988; Larsen *et al*., 1998). Similar to the other members of the glycoside hydrolase family, CGTase employs an α-retaining glycosidic bond cleavage reaction mechanism involving the formation of a covalent intermediate (Uitdehaag *et al*., 2001). The enzyme catalyzes three transglycosylation reactions (Qi and Zimmermann, 2005). The cyclization reaction is an intramolecular transglycosylation, which provides cyclic oligosaccharides from linear glucan substrates. The coupling reaction opens the CD prior to the transfer of the linear oligosaccharide to another linear oligosacharide as the acceptor molecule. In the disproportionation reaction, a linear oligosaccharide is transferred to another linear acceptor molecule by an intermolecular transglycosylation reaction. CGTases also catalyze the hydrolysis of glycosidic linkages; however, the activity is low.

A mixture of CD_6, CD_7, and CD_8, as well as small amounts of large-ring CDs ($\geq CD_9$) is formed by the cyclization reaction of CGTases (Pulley and French, 1961; French *et al*., 1965; Terada *et al*., 1997, 2001). After binding the glucan chain, the enzyme cleaves an α-1,4-glycosidic bond and forms the covalently bound intermediate. The non-reducing end of the resulting malto-oligosaccharide then moves to its reducing end to form the cyclic product. This is considered the rate-limiting step of CD formation (Uitdehaag *et al*., 2001).

Large-ring CDs may find applications as novel host compounds for larger guest molecules unable to form complexes with CD_6, CD_7, and CD_8 (Endo *et al*., 2002). However, due to their low abundance in enzymatic synthesis reactions and the requirement of tedious purification procedures, only a limited amount of

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Abbreviations used: *BSA, bovine serum albumin; CD, cyclodextrin; CGTase, cyclodextrin glycosyltransferase; HPAEC, high performance anion exchange chromatography; TNBS, 2,4,6-trinitrobenzene sulfonic acid.*

information is presently available on their properties. While CD_6, CD_7, and CD_8 show a doughnut-shaped form composed of cis-oriented glucopyranose units, large-ring CDs containing more than nine glucopyranose units exhibit different structures. Strains in their macrocycles cause a trans-orientation of diametrically opposed glucopyranose units stabilized by hydrogen bonds resulting in "band flips" and "kinks" in butterfly-like ring structures. The crystal structures of CD_{12} and CD_{14} are shown in Figure 1 (Saenger *et al*., 1998).

Although studies aiming to increase the product specificity of CGTase by protein engineering have revealed insight into the mechanism of the cyclization reaction, an enzyme that preferentially synthesizes a single CD has not been reported yet. Molecular imprinting is an attractive alternative approach to alter the properties of enzymes at the protein level with regard to their catalytic activity and substrate specificity (Rich and Dordick, 1997). A native enzyme can maintain its conformation it attained in the presence of a template molecule in an aqueous environment when it is transferred to a non-aqueous environment. The required stability of the imprinted properties of the enzyme allowing subsequent synthesis reactions in an aqueous environment can be achieved by cross-linking the derivatized enzyme using a radical polymerization procedure (Peißker and Fischer, 1999; Schoevaart *et al*., 2004, Vaidya *et al*., 2004). We have previously shown that the product specificity of a CGTase could be efficiently shifted by using this methodology (Kaulpiboon *et al*., 2007).

In this study, we describe the molecular imprinting of *Paenibacillus* sp. A11 CGTase to manipulate the enzyme to preferentially produce large-ring CDs. We used cyclomaltododecaose (CD_{12}) as the template molecule and compared the product specificity of the imprinted and immobilized enzyme with the native enzyme using different reaction conditions.

MATERIALS AND METHODS

Materials and enzymes

CD_6, CD_7, CD_8, maltotriose (G_3), methyl-α-D-glucopyranoside, bovine serum albumin (BSA), phenolphthalein, itaconic anhydride, 2,4,6-trinitrobenzene sulfonic acid (TNBS), 2,2′-azobis(2-methylpropionitrile), ethylene glycol dimethacrylate, water-free cyclohexane, and *n*-propanol were from Sigma-Aldrich Chemie GmbH (Munich, Germany). Dimethylsulfoxide was from Grüssing GmbH (Filsum, Germany). Amylose (pea starch, degree of polymerization = 4 000) was obtained from Emsland!Stärke GmbH (Emlichheim, Germany). Single large-ring CDs (CD_9–CD_{24}) were kindly provided by H. Ueda and T. Endo, Hoshi University, Tokyo, Japan. A large-ring CD mixture consisting of CD_6–CD_{24} was prepared as described previously (Qi *et al*., 2004). *Rhizopus* sp. glucoamylase was obtained from Toyobo Co., Ltd. (Osaka, Japan). *Paenibacillus* sp. A11 CGTase was purified using starch adsorption and ion exchange chromatography on a DEAE-Toyopearl 650 M column (Tosoh Corporation, Tokyo, Japan). The enzyme had a specific activity of 5 000 U/mg as determined by its dextrinizing activity (Rojtinnakorn *et al*., 2001).

Determination of the cyclization and coupling activity of CGTase

The cyclization activity of the native and imprinted enzymes was determined by incubating the enzyme (2.5 μg) with amylose at concentrations of 0.03–2.0 mg/ml in 10 mM potassium phosphate buffer, pH 6.0 containing 50% (v/v) of dimethylsulfoxide. The reaction mixture was incubated for 30 min at 40°C or 60°C. The reaction was stopped by boiling the mixture for 10 min. The amount of CD_{12} formed was determined by high performance anion exchange chromatography (HPAEC) using a calibration

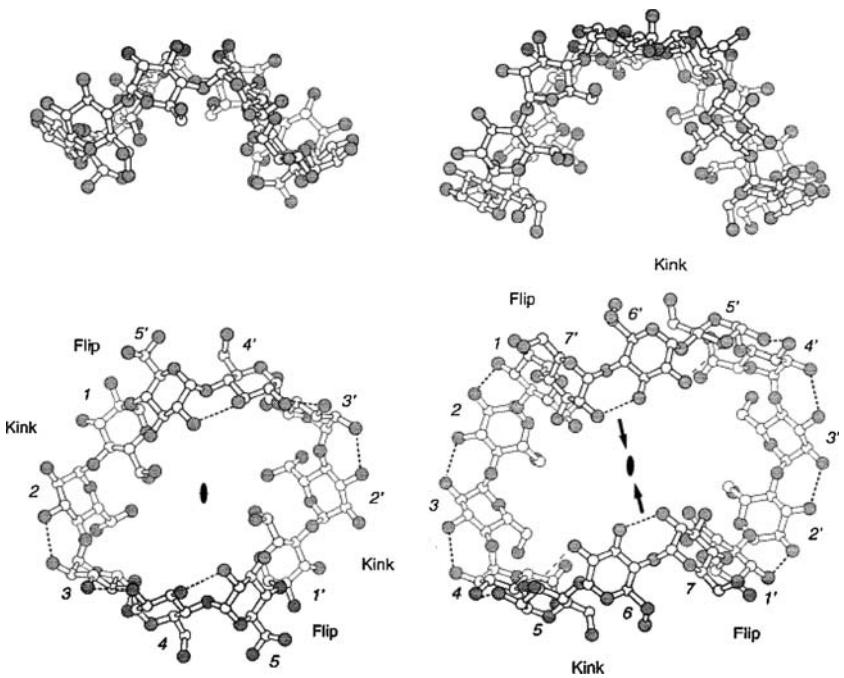

Figure 1. Structures of CD_{10} (left) and CD_{14} (right) in top and side views. Steric strain in these large-ring CDs is relieved by the introduction of flips and kinks (marked) (Saenger *et al*., 1998).

curve with an authentic standard. The coupling activity was determined by adding 0.03–2.0 mg/ml of a CD_6–CD_{24} mixture as the donor substrate and 60 mM methyl-α-D-glucopyranoside as the acceptor substrate. Potassium phosphate buffer (10 mM, pH 6.0) was added to obtain a total volume of 0.5 ml. The reaction was started by adding CGTase (2.5 μg). After incubation at 40°C or 60°C for 5 min, the reaction was stopped by boiling the sample. Subsequently, *Rhizopus* sp. glucoamylase (0.385 U) was added to convert linear oligosaccharides to glucopyranose at 40°C for 30 min. The released reducing sugars were determined using the dinitrosalicylic acid method (Bernfeld, 1955). The time course of CD_{12} synthesis was determined by incubating the CGTase with amylose at 40°C and 60°C for up to 24 h. To study the effect of reaction temperature on the cyclization and coupling activity of the native and imprinted enzymes, the reactions were performed for 30 min at temperatures between 30°C and 80°C. Lineweaver-Burk diagrams of the initial velocity against substrate concentration were plotted and kinetic parameters were determined using the EnzFitter program, v 2.0.14.0 (Biosoft, Cambridge, UK). Data presented are the mean ± SD of three independent repeats. The protein concentrations of the enzyme samples were determined using BSA as the standard (Bradford, 1976).

Analysis of CDs

HPAEC with pulsed amperometric detection was carried out using a DX-600 system (Dionex Corp., Sunnyvale, USA) to analyze and quantify the CD products formed. A Carbopac PA-100 analytical column (4 × 250 mm, Dionex Corp., Sunnyvale, USA) was used. A sample (25 μl) was injected and eluted with a linear gradient of sodium nitrate (0–10 min, increasing from 0% to 4%; 10–12 min, 4%; 12–32 min, increasing from 4% to 8%; 32–48 min, increasing from 8% to 9%; 48–59 min, increasing from 9% to 18%; 59–79 min, increasing from 18% to 28%) in 150 mM NaOH with a flow rate of 1 ml/min (Kaulpiboon *et al*., 2007). The amounts of CD_6 to CD_{24} were quantified by comparison with standard curves of authentic CD_6 to CD_{24} samples.

Derivatization of CGTase by acylation with itaconic anhydride

A sample of CGTase (6 mg) in 10 ml of 50 mM phosphate buffer, pH 6.0 was acylated with 30 mg of itaconic anhydride. The mixture was stirred at 4°C for 60 min. The pH was monitored and maintained at 6.0 using 3 M NaOH. Unreacted itaconic anhydride and other low-molecular mass compounds were removed by gel filtration (HiTrap desalting column, Amersham Pharmacia, Uppsala, Sweden) with distilled water as the eluent. The active CGTase fractions were combined and freeze-dried.

Determination of free amino groups in the CGTase

The relative amounts of amino groups of the native and covalently derivatized CGTase samples were determined according to Habeeb (1966) and Hall *et al*. (1973) with TNBS. To 0.3 ml of native or derivatized protein solution (0.5 mg/ml), 0. ml of $NaHCO_3$ (4%, w/v) and 0.3 ml of TNBS (0.1%, v/v) were added. The samples were placed in a thermomixer set at 1 000 rpm and 37°C. After 60 min, 0.47 ml of 1 M HCl was added and the absorption was measured at 335 nm against a blank treated as above containing 0.3 ml of deionized water instead of the protein solution.

Imprinting of the derivatized CGTase

Dry derivatized enzyme (15 mg) and CD_{12} (1 mg) were dissolved in 1 ml of 10 mM potassium phosphate buffer, pH 5.5. The mixture was incubated at 25°C for 30 min. The CGTase-CD_{12} complex was precipitated by adding 4 ml of *n*-propanol cooled to −20°C. The precipitate was collected by centrifugation at 11 000 rpm for 15 min at 4°C. The pellet was washed three times with 1 ml of *n*-propanol cooled to −20°C, freeze-dried, and stored at −20°C.

Cross-linking of the imprinted derivatized CGTase

Imprinted derivatized CGTase (10 mg) was suspended in 1 ml of water-free cyclohexane in an ultrasonication bath for 15 min. To this suspension was added 4 mg of 2,2́-azobis(2-methylpropionitrile) and 200 μl of ethylene glycol dimethacrylate. The radical polymerization was performed by UV irradiation ($\lambda = 312$ nm) at 25°C for 2 h. The resulting polymer was kept at 4°C for 12 h. The white polymer obtained was washed with 2 ml of cyclohexane, followed by 50 mM potassium phosphate buffer, pH 6.0, and freeze-dried.

Synthesis of CDs with native and CD_{12}-imprinted CGTase

The native and imprinted CGTase (2 U of cyclization activity) were incubated with 2.5 ml of 1.5% (w/v) pea starch in 0.2 M potassium phosphate buffer, pH 6.0 at 40°C for 30 min or 24 h. The reaction was stopped by boiling for 10 min. Glucoamylase (2 U) was added to convert linear oligosaccharides to glucopyranose. After 3 h of incubation, the glucoamylase activity was inactivated by boiling for 10 min.

RESULTS AND DISCUSSION

A comparison of the cyclic products formed following incubation of the native and the CD_{12}-imprinted and immobilized CGTase with amylose at 40°C for 30 min showed a drastic shift in the size distribution of the CDs obtained with the imprinted enzyme (Figure 2 and Table 1). While the native enzyme predominantly

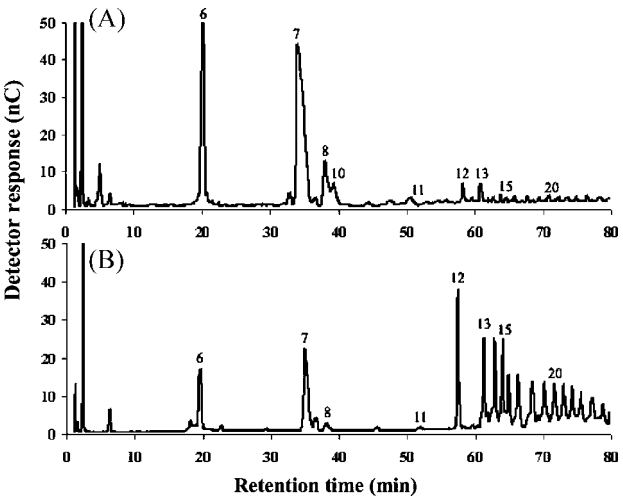

Figure 2. HPAEC–PAD analysis of CDs formed at 40°C after a reaction time of 40 min by the native (A) and CD_{12}-imprinted CGTase from *Paenibacillus* sp. A11 (B). The numbers above the peaks indicate the size of the CD.

Table 1. Relative amounts of different sized CDs formed with native and CD_{12}-imprinted CGTase

Enzyme	CD_6	CD_7	CD_8	CD_{12}	$CD_{13–25}$
Native CGTase	38.7 ± 0.6	53.9 ± 2.6	3.3 ± 0.3	1.6 ± 0.3	3.11 ± 0.3
CD_{12}-imprinted CGTase	10.0 ± 0.5	18.5 ± 1.1	9.5 ± 0.5	7.5 ± 0.5	54.5 ± 2.2

produced CD_7, the CD_{12}-imprinted CGTase formed CD_{12} as the main product. The amounts of large-ring CDs (CD_{13}–CD_{25}) obtained with the CD_{12}-imprinted and immobilized CGTase were also considerably higher compared to the native enzyme. The native and the imprinted CGTases both had an optimum temperature of 40°C for the cyclization reaction, while an optimum reaction temperature of 60°C was found for the coupling reaction of both enzymes (Figure 3). Analysis by HPAEC of the CDs formed by the native and imprinted CGTase at 40°C, 50°C, and 60°C further supported these results (Figures 4 and 5). The highest yields of CDs obtained with both native and imprinted CGTases were obtained at 40°C. This can be explained by the fact that the coupling activity of the enzyme resulting in the linearization of CD is lower at 40°C, the optimum temperature of the cyclization reaction (Qi *et al*., 2004).

To study the influence of reaction time on CD_{12} yield, the time course of CD_{12} synthesis by native and imprinted CGTases was compared at both reaction temperatures (Figure 6). The highest yields of CD_{12} were obtained using the CD_{12}-imprinted CGTase incubated at 40°C for 30 min. At longer reaction times, the amount of CD_{12} gradually declined due to the coupling activity of the enzyme using large-ring CDs as the substrate.

It has been shown previously that the reaction time and temperature have a strong impact on the yield and composition of large-ring CDs using CGTases (Endo *et al*., 2007). By performing the reaction at the optimal reaction time and temperature for the cyclization reaction, the yield of large-ring CDs obtained using the imprinted CGTase could thus be considerably increased.

To further characterize the properties of the imprinted CGTase, we compared the Michaelis–Menten constant (K_m), turnover rate (k_{cat}), and catalytic efficiency (k_{cat}/K_m) for the cyclization and coupling reactions with the native enzyme (Figure 7, Table 2). The CD_{12}-imprinted CGTase showed an increased catalytic efficiency of its CD_{12}-forming cyclization activity compared to the native enzyme. This effect was observed at 40°C as well as 60°C, which is

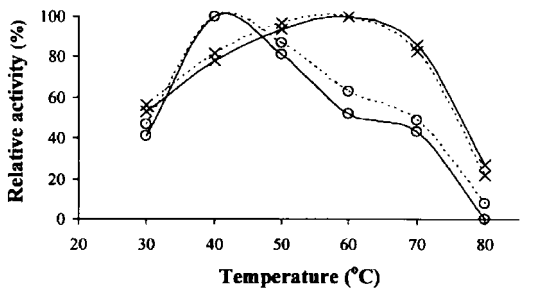

Figure 3. Comparison of the cyclization (o) and coupling (x) activity of the native (solid line) and CD_{12}-imprinted (dotted line) CGTase from *Paenibacillus* sp. A11 at different reaction temperatures.

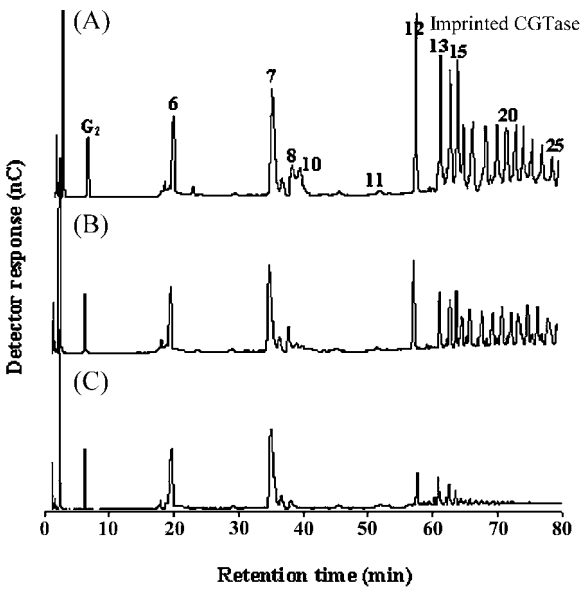

Figure 4. HPAEC analysis of CDs formed by the imprinted CGTase from *Paenibacillus* sp. A11 at 40°C (A), 50°C (B), and 60°C (C) and a reaction time of 30 min. The numbers above the peaks indicate the size of the CD. Maltose (G_2) was used as internal standard.

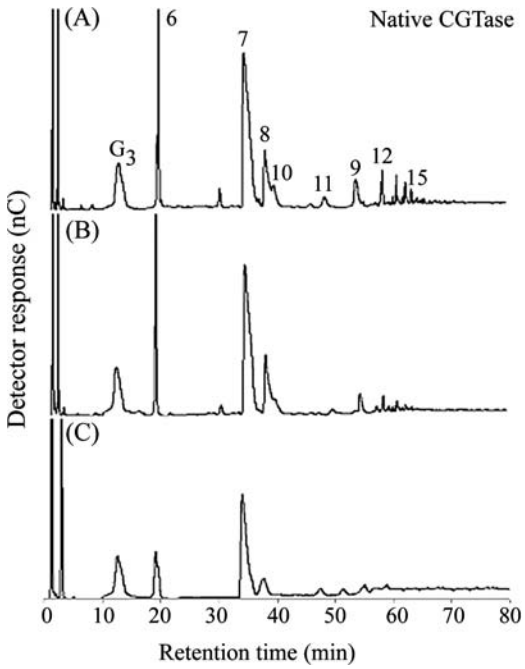

Figure 5. HPAEC analysis of CDs formed by the native CGTase from *Paenibacillus* sp. A11 at 40°C (A), 50°C (B), and 60°C (C) and a reaction time of 30 min. The numbers above the peaks indicate the size of the CD. Maltotriose (G_3) was used as internal standard.

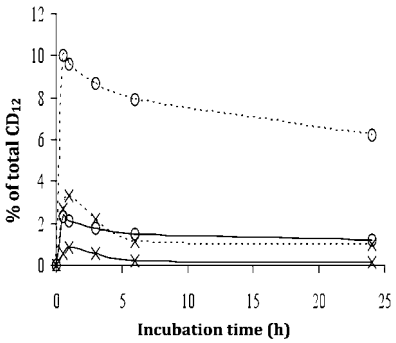

Figure 6. Time course of the synthesis of CD_{12} by the native (solid line) and CD_{12}-imprinted (dotted line) CGTase from *Paenibacillus* sp. A11 at 40°C (o) and 60°C (x).

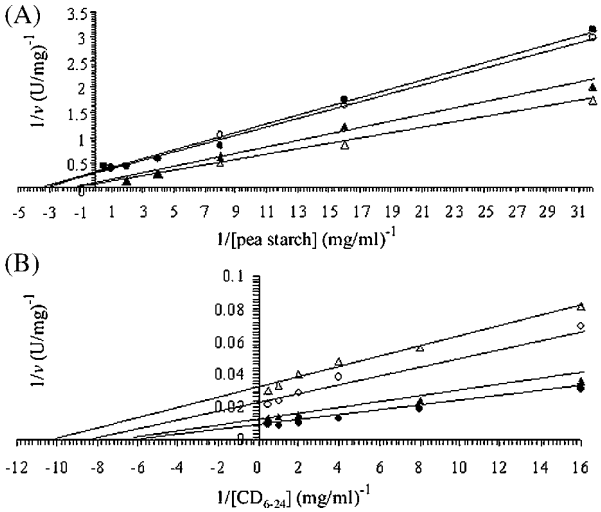

Figure 7. Lineweaver-Burk plots of the cyclization (A) and coupling reactions of the native and imprinted CGTase at 40°C and 60°C. Native CGTase (40°C), ▲; imprinted CGTase (40°C), Δ; native CGTase (60°C), ●; imprinted CGTase (60°C), ○.

above the optimum temperature for the cyclization reaction. The lower K_m and higher k_{cat} values obtained with the imprinted CGTase indicated a better binding and a more efficient turnover of the glucan chain to form CD_{12} in the cyclization reaction.

The catalytic efficiency of the reverse coupling reaction of the CD_{12}-imprinted CGTase was lower compared to the native CGTase at both 40°C and 60°C. As expected from the results described above, the efficiency of the coupling activity of both enzymes was higher at its optimum temperature of 60°C compared to 40°C. However, the imprinted CGTase showed a lower k_{cat}/K_m value of the coupling reaction compared to the native enzyme, further contributing to the high yield of large-ring CDs observed as products.

The product specificity of CGTase has been attributed to several factors (Kelly *et al*., 2009). Mutation experiments at the central active site of CGTase have been reported to result in changed CD product size profiles. However, interactions at oligosaccharide-binding sub-sites located close to the catalytic site apparently play a more important role in the determination of the size of the CD formed (Goh *et al*., 2009; Costa *et al*., 2009). By dissolving the CGTase in the presence of CD_{12}, the enzyme adopted a conformation favorable for the binding and cyclization of linear glucan chains of a specific size resulting in the formation of large-ring CDs, predominantly CD_{12}. The pronounced difference in the structure of the small CDs (CD_6–CD_8) and CD_{12} used as template could explain the higher level of large-ring CDs and the markedly reduced amounts of small CDs formed by the imprinted CGTase.

By covalently cross-linking the imprinted CGTase in a non-aqueous solvent, this conformation was maintained and provided sufficient stability to employ the manipulated enzyme subsequently in an aqueous medium required for the synthesis reactions, where the imprinted enzyme retained a higher activity toward the formation of the desired CD product of a specific size.

CONCLUSIONS

We have imprinted a CGTase from *Paenibacillus* sp. A11 producing CD_7 as the main product with CD_{12} and covalently stabilized the enzyme by cross-linking. As a result, the CD_{12}-imprinted CGTase synthesized a much higher ratio of CD_{12} and other large-ring CDs compared to the native enzyme. The results indicate that the product specificity of the CGTase could be altered by molecular imprinting it with a CD of a specific size. Furthermore, the yield of the desired CD could be further increased by optimizing the reaction conditions with regard to reaction temperature and time.

Acknowledgements

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Table 2. CD_{12}-cyclization and coupling activity catalyzed by the native and CD_{12}-imprinted CGTase from *Paenibacillus* sp. A11 at different reaction temperatures.

	40°C			60°C		
Reaction temperature	K_m (mg/ml)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1})(mg/ml)$^{-1}$	K_m (mg/ml)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1})(mg/ml)$^{-1}$
Cyclization reaction						
Native CGTase	0.83 ± 0.03	17.05 ± 2.8	2.05×10^1	0.33 ± 0.03	5.12 ± 0.5	1.55×10^1
CD_{12}-imprinted CGTase	0.71 ± 0.04	23.33 ± 4.2	3.29×10^1	0.31 ± 0.02	6.79 ± 0.8	2.19×10^1
Coupling reaction						
Native CGTase	0.16 ± 0.05	106.79 ± 7.1	6.67×10^2	0.17 ± 0.06	150.82 ± 5.1	8.87×10^2
CD_{12}-imprinted CGTase	0.10 ± 0.03	41.03 ± 1.3	4.10×10^2	0.12 ± 0.01	58.27 ± 2.6	4.85×10^2

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