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ORIGINAL ARTICLE

Biochemical properties and cyclodextrin production profiles of isoforms of cyclodextrin glycosyltransferase

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Abstract Cyclodextrin glycosyltransferase (CGTase) catalyzes the conversion of starch to cyclodextrin (CD), an important host molecule for the study of host–guest interactions. CGTase from *Paenibacillus* sp. RB01 and its recombinant form showed the same isoform pattern. The three isoforms, two major (isoforms I and II) and one minor (isoform III), all had a different net charge but the same molecular mass. The aim of this work was to characterize the three isoforms, and especially to compare their CD production profiles. Isoforms I and II were separated on a FPLC Mono Q column and showed the same optimum pH (pH 5 for dextrinizing and pH 6–7 for cyclization activity) and optimum temperature (65–70 °C for both activities). However, the two isoforms differed in their catalytic efficiency of the coupling reaction with variable concentrations of the β-CD donor in the presence of a fixed amount of cellobiose acceptor, with k_{cat}/K_m values of 3.46×10^{-3} and 2.20×10^{-3} mM^{-1} min^{-1}, for isoforms I and II, respectively. Both isoforms I and II were found to have β-CGTase activity and gave a similar CD6:CD7:CD8 product ratio of 0.2:1.0:0.6, with an increase in the ratio of the small-ring to the large-ring CDs from 1.0:0.5 to 1.0:0.3 from a 6 to 24 h reaction time. However, in terms of maximal CD yields, the two isoforms differed in their optimal reaction temperature and time required, the optimal conditions being at 40 °C for 6 h for isoform I and at 60 °C for 24 h for isoform II.

Keywords *Paenibacillus* sp. RB01 · Cyclodextrin glycosyltransferase · Isoforms separation · Cyclodextrin production profile

Abbreviations

CGTase	Cyclodextrin glycosyltransferase
CD	Cyclodextrins
A11	*Paenibacillus* sp. A11
RB01	*Paenibacillus* sp. RB01
HPAEC	High performance anion exchange chromatography

Introduction

Cyclodextrin glycosyltransferase (CGTase, 4-α-D-glucan: 1,4-α-glucanotransferase, EC 2.4.1.19) is a member of the α-amylase family with a low hydrolytic activity. This enzyme catalyzes four different reactions: cyclization, coupling, disproportionation and hydrolysis. The main reaction is the formation of cyclic oligosaccharides product (cyclodextrins, CDs) from starch and related α-1,4-glucan substrates [1]. Three native forms of small-ring CDs, that is CD6 (α), CD7 (β) and CD8 (γ), are obtained. However, in the initial phase of product formation, large-ring CDs (CD > 9) are usually formed [2]. Due to their structural characteristics, CDs can accommodate various guest molecules to form soluble or insoluble inclusion complexes, and so improve the physicochemical properties, such as the stability and solubility, of the included guest molecules [1]. At

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present, CDs are extensively used in the food, cosmetic and pharmaceutical industries. Therefore, the research on the enzyme synthesizing CDs is of prime concern.

CGTase enzymes can be divided into three groups according to their main CD product formed (α-, β- or γ-CGTases). They are all extracellular enzymes produced by a diverse array of microorganisms [3], but the enzymes from different sources show different properties, such as the optimal and working pH and temperature range, their molecular mass and pI and the ratio of the CD products produced. Only a few CGTases have been reported to have multiple forms, they are mostly from the genus *Bacillus*, where from two to more than seven isoforms have been found [4, 5]. All the different isoforms reported show the same apparent size but different pI values, usually within 0.1–0.2 pH units, with the exception being those from *Bacillus* INMIA which show both size and charge differences [6].

The thermotolerant *Paenibacillus* sp. RB01 strain was isolated from the hot springs area of Ratchaburi province, Thailand [7]. The bacteria produce a CGTase activity in the temperature range of 30–45 °C. Although the bacteria grow best at 37 °C, their CGTase exerted the highest activity at 40 °C. The enzyme was purified and biochemically characterized [8], revealing a molecular size of 65 kDa on reducing SDS-PAGE. However, it displayed three distinct bands on native- and IEF-PAGE that suggested that the enzyme had three isoforms with small differences in net charge.

In the work reported here, the separation and characterization of the two main CGTase isoforms (I and II) was studied as recombinant proteins, with a special emphasis on the CD production profile. The third isoform was not obtained in sufficient yields to allow further characterization.

Materials and methods

Cloning, expression and partial purification of the CGTase from *Paenibacillus* sp. RB01

The *cgt* gene from *Paenibacillus* sp. RB01 was cloned by PCR from genomic DNA using the primers designed from the sequence deposited in the Genbank. Recombinant plasmids were prepared from ligation of the *Nco*I and *Xho*I digested PCR product and the pET19b vector, then introduced into the *E. coli* BL21 (DE3) host by electroporation. The colonies containing recombinant plasmids were selected on agar plate containing ampicillin and soluble starch. Then the plasmids harboring *cgt* were extracted and sequenced to confirm the cloned gene. For expression, the recombinant cells were grown in ampicillin-supplemented LB medium and the recombinant protein (rCGTase) was expressed by IPTG induction. The wild type CGTase from *Paenibacillus* sp. RB01, and the rCGTase from transformed BL21 (DE3) cells were both prepared from the culture supernatant and partial purification was performed by starch adsorption as reported [8].

Separation of CGTase isoforms

The isoforms of the cloned rCGTase were separated by anion-exchange chromatography using a Mono Q^{TM} HR 5/5 column (Amersham Biosciences, USA). The column was pre-equilibrated with 10 mM phosphate buffer pH 6.5. The concentrated partially purified CGTase solution was applied onto the column and then eluted with the equilibrating buffer at a flow rate of 1 mL/min until the A_{280} was about zero. A linear gradient of 0.5 M NaCl was then increased from 0 to 100% within 30 min. Fractions (0.5 mL) were collected and the A_{280} and dextrinizing activity profiles were measured. The isoform pattern of the enzyme was followed by running samples from each fraction on the native-PAGE, with both protein and activity staining. The fractions containing the highly purified isoform I were pooled while those containing a mixture of isoforms were rechromatographed on the same column and condition to obtain isoform II in higher purity.

Biochemical properties of CGTase isoforms

Native-PAGE

To investigate the separation profile of CGTase, a native-PAGE (7% (w/v) acrylamide resolving gel) was performed. The gels were stained with coomassie blue for protein and iodine solution for dextrinizing activity. In the activity staining, slightly modified from the method of Kobayashi et al. [9], the gels were soaked in 10 mL of 2% (w/v) potato soluble starch in 0.2 M phosphate buffer pH 6.0, at 40 °C for 10 min. The gels were then quickly rinsed several times with distilled water and I_2 solution (0.2% (w/v) I_2 in 2% (w/v) KI) was added for color development. A clear zone against the blue background represents a starch degrading activity.

Optimum pH and temperature

For evaluation of the optimum pH for enzyme activity, the universal buffer (citric acid, KH_2PO_4, H_3BO_3 and 5′,5′-diethylbarbituric acid (veronal), all at 40 mM) was used. The buffer was adjusted to desire pH by adding 0.2 M NaOH. The enzyme was incubated with 0.2% soluble starch at 70 °C for 10 min for dextrinizing activity or 6% soluble starch at 70 °C for 30 min for cyclization activity. The dextrinizing activity was followed by the starch-iodine method [10], whilst the

cyclization activity was assayed using phenolphthalein to detect β-CD as described [11]. For determination of the optimum temperature for CGTase activity, the assays were performed in the range from 30 to 80 °C in 50 mM phosphate buffer, pH 6.0 for dextrinizing activity or 10 mM Tris-HCl buffer, pH 7.0 for cyclization activity.

Kinetics parameters

The kinetic parameters of the enzyme coupling reaction were determined by pre-incubating various concentrations of β-cyclodextrin (0.5–15 mM) as the donor and 10 mM cellobiose as the glucosyl acceptor at 55 °C in 50 mM sodium acetate buffer, pH 6.0, for 5 min. The reaction was started with addition of the enzyme sample (0.38 and 0.51 pmol of isoforms I and II, respectively), incubated for 5 min, and then stopped by boiling. Subsequently, 4 units of glucoamylase from *Aspergillus niger* (67 U/mg) was added and incubated at 55 °C for 90 min to convert the linearized oligosaccharides to glucose. The released reducing sugars were evaluated by the DNS method [12]. The kinetic parameters K_m and V_{max} were determined from Lineweaver-Burk plot, then k_{cat} and k_{cat}/K_m values were calculated.

Detection of cyclodextrin products

The reaction was performed at 40/60 °C in 50 mM Tris-HCl buffer pH 7.0 using 4% (w/v) soluble starch (potato) as the substrate and 3 Units (Dextrinizing) of CGTase in a total volume of 10 mL. An aliquot (1 mL) of the sample was withdrawn at 6, 12 and 24 h, boiled to stop the reaction, cooled to room temperature and then the linear oligosaccharides were digested with 0.4 U glucoamylase for 6 h. The reaction was stopped by boiling for 10 min and then centrifuged at 8,000×g for 30 min. Each 25 µL sample of the supernatant was injected into a HPAEC-PAD. The elution was performed using an increasing $NaNO_3$ gradient (0–200 mM) in 150 mM NaOH, as described before [13]. The CD products were identified and quantitated by comparison with standard CDs.

Results and discussion

CGTase isoforms separation

The existence of three different natural CGTase isoforms in *Paenibacillus* sp. A11 was previously observed by resolution on two-dimensional SDS-PAGE, where the three isoforms showed the same apparent mass (SDS-PAGE) but different pI values (IEF) of 4.87, 4.75 and 4.55 [8, 14]. Those three isoforms were isolated by column chromatofocusing within the pH range of 6.2–4.0 [5]. However, due to the difficulty in isolating sufficient amounts of each of the native isoforms, this study used rCGTase. The construct, when expressed in the host *E. coli* cells, was shown to have the same isoform pattern as the native CGTase, except for the apparently reduced proportion of isoform III (Fig. 1a). The rCGTase enzyme was partially purified, and the isoforms were separated, by an anion exchange Mono Q column FF. The isoforms were eluted from the column by a linear 0–0.5 M NaCl gradient (Fig. 2a). Two major protein peaks that also corresponded to the two dextrinizing activity peaks in the initial phase of the gradient (fractions 9–20) were obtained. At around fractions 37–42, another major protein (A_{280}) peak was observed, but this had no detectable dextrinizing enzyme activity. Figure 2b shows the isoform pattern for fractions 9–20 (lanes 1–12, respectively) after native-PAGE resolution and staining for dextrinizing activity staining. Isoform I was mainly eluted at fractions 11–13 (Fig. 2a), although the activity was significant in tracks 1–7 (=fractions 9–15), which represents a low NaCl elution concentration of around 20–25 mM NaCl. Isoform II was mainly eluted at fractions 15–18 with a secondary elution shoulder in fractions 19–21, which concurs with its activity being found in lanes 7–12 (Fig. 2b; equivalent to fractions 15–20), and was most pure in the secondary shoulder (lanes 10–12 in Fig. 2b; equivalent to fractions 18–20), which represents a NaCl elution concentration of around 50 mM. Thus, fractions 13–17 are a mixture of the two isoforms.

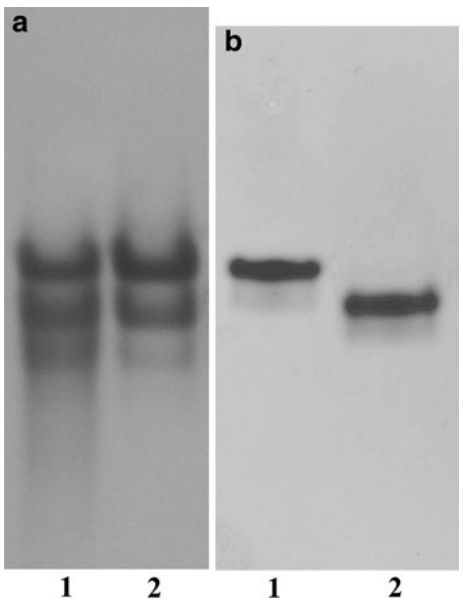

Fig. 1 Native-PAGE of (**a**) partially purified CGTase from *Paenibacillus* sp. RB01 (*lane 1*) compared with the cloned rCGTase expressed by the transformed *E. coli* BL21 (DE3) host cells (*lane 2*), and (**b**) the isolated rCGTase isoform I (*lane 1*) and isoform II (*lane 2*). Each lane was loaded with 5 µg of total protein

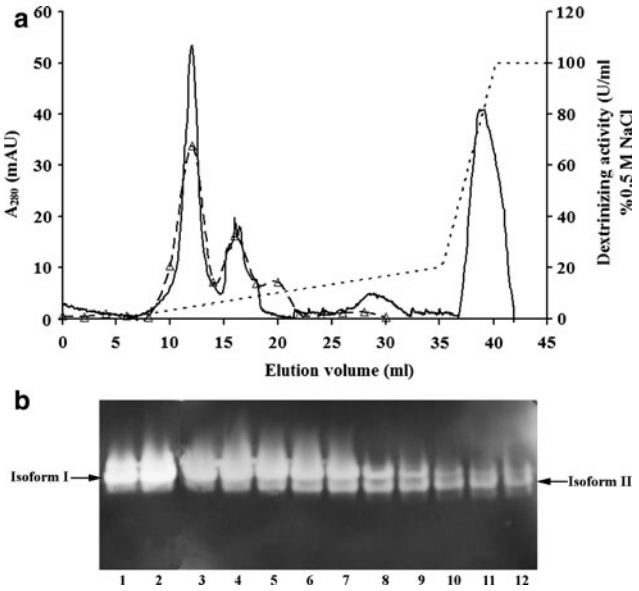

Fig. 2 **a** Chromatogram of rCGTase isoforms separation on a Mono Q FF column. — A_{280}, -▵- dextrinizing activity, - - - - % (v/v) of 0.5 M NaCl. **b** Isoform pattern after native-PAGE resolution of the eluted rCGTase fractions from the Mono Q FF column. Staining was by amylolytic (dextrinizing) activity, each lane was loaded with 0.6 units; *lanes 1–12* are fractions 9–20 from **a**

The very low enzyme activity peak in fractions 23–30 (Fig. 2a) is likely to be isoform III, but insufficient amounts were harvested for further characterization and study. Although the column profile reveals the two main activity peaks were well separated (Fig. 2a), native-PAGE analysis suggested the separation between isoforms I and II was rather poor (Fig. 2b), assuming that either of the two isoforms cannot change to the other after enrichment. The fractions which showed only isoform I were pooled and stored at −80 °C, while the rest of the fractions, which were a mixture of isoforms I and II were reloaded onto the column and separated under the same conditions to collect a higher purity isoform II preparation. The separated isoforms I and II that appeared closer to homogeneity (Fig. 1b), were then used in the next experiments.

Comparison of the biochemical properties of the rCGTase isoforms I and II

Optimum pH and temperature for enzyme activity

Both rCGTase isoforms I and II were found to be very similar in terms of their apparent in vitro optimal pH and temperature for the enzyme activity. For evaluation of the optimum pH for CGTase activity, the enzyme was incubated in the universal buffer at various pHs at 70 °C. Both isoforms showed the highest dextrinizing activity at pH 5.0, while at pH 6.0–8.0 the activity decreased to 80% of

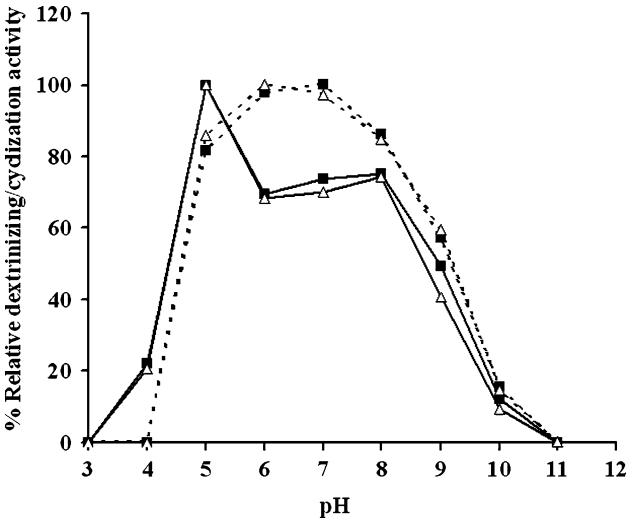

Fig. 3 Optimum pH of the isolated rCGTase isoforms I and II for **a** dextrinizing activity (-▪- Isoform I and -▵- Isoform II) and **b** cyclization activity (-▪- Isoform I and -▵- Isoform II). Data are representative of three independent repeats

that at pH 5.0. With respect to the cyclization activity, both isoforms showed an optimum pH of 6.0–7.0, while 80% of the relative activity was obtained at pH 5.0 and 8.0. The activity of both isoforms was completely lost at pH 3.0 and 10.0 (Fig. 3).

With respect to the temperature optimum, the two isoforms showed the highest dextrinizing and cyclization activities at temperatures of 65–70 °C when incubated in phosphate buffer pH 6.0 or Tris-HCl buffer pH 7.0 as described under "Methods" section (Fig. 4). However, a rather broad optimal temperature range for dextrinizing activity was observed in the range of 55–70 °C. The two

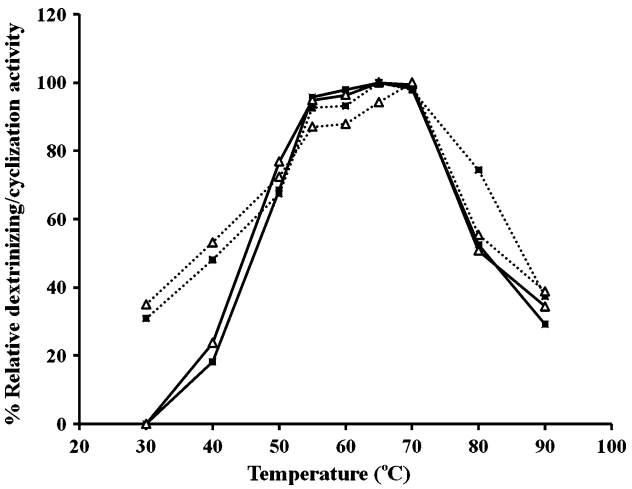

Fig. 4 Optimum temperature of the isolated rCGTase isoforms I and II for **a** dextrinizing activity (-▪- Isoform I and -▵- Isoform II) and **b** cyclization activity (-▪- Isoform I and -▵- Isoform II). Data are representative of three independent repeats

isoforms significantly lost both their dextrinizing and cyclization activities at temperatures above 70 °C, with only 25% residual activity at 90 °C.

Kinetic parameters

The kinetic parameters were evaluated by the coupling reaction, where the β-CD ring is opened and combined with the linear cellobiose oligosaccharide chain to produce a longer linear oligosaccharide [15]. This pair of donor–acceptor substrate molecules was chosen because this CGTase has already been shown to be a β-CGTase [8], and cellobiose has previously been determined to be an efficient acceptor [16]. Under these conditions both isoforms showed similar K_m and V_{max} values for β-CD (Table 1), but differed in their k_{cat} and the catalytic efficiency of the coupling reaction. The k_{cat}/K_m value of isoform II was almost 1.6-fold lower than that of isoform I. The two values were significantly different ($p < 0.05$, Student's t test). We, therefore, propose that different CGTase isoforms contribute to the different catalytic rates already observed.

Table 1 Kinetic parameters of isoforms I and II of CGTase from coupling reaction with various concentrations of β-CD donor and fixed concentration of cellobiose acceptor

CGTase	K_m (mM)	V_{max} (μmol min^{-1})	$k_{cat} \times 10^{-3}$ (min^{-1})	$k_{cat}/K_m \times 10^{-3}$ (mM^{-1} min^{-1})
Isoform I	1.30 ± 0.59	1.58 ± 0.52	4.46 ± 1.86	3.47 ± 0.15
Isoform II	1.09 ± 0.52	1.30 ± 0.91	2.55 ± 1.80	2.20 ± 0.60

Data are shown as the mean ± SD (n = 4)

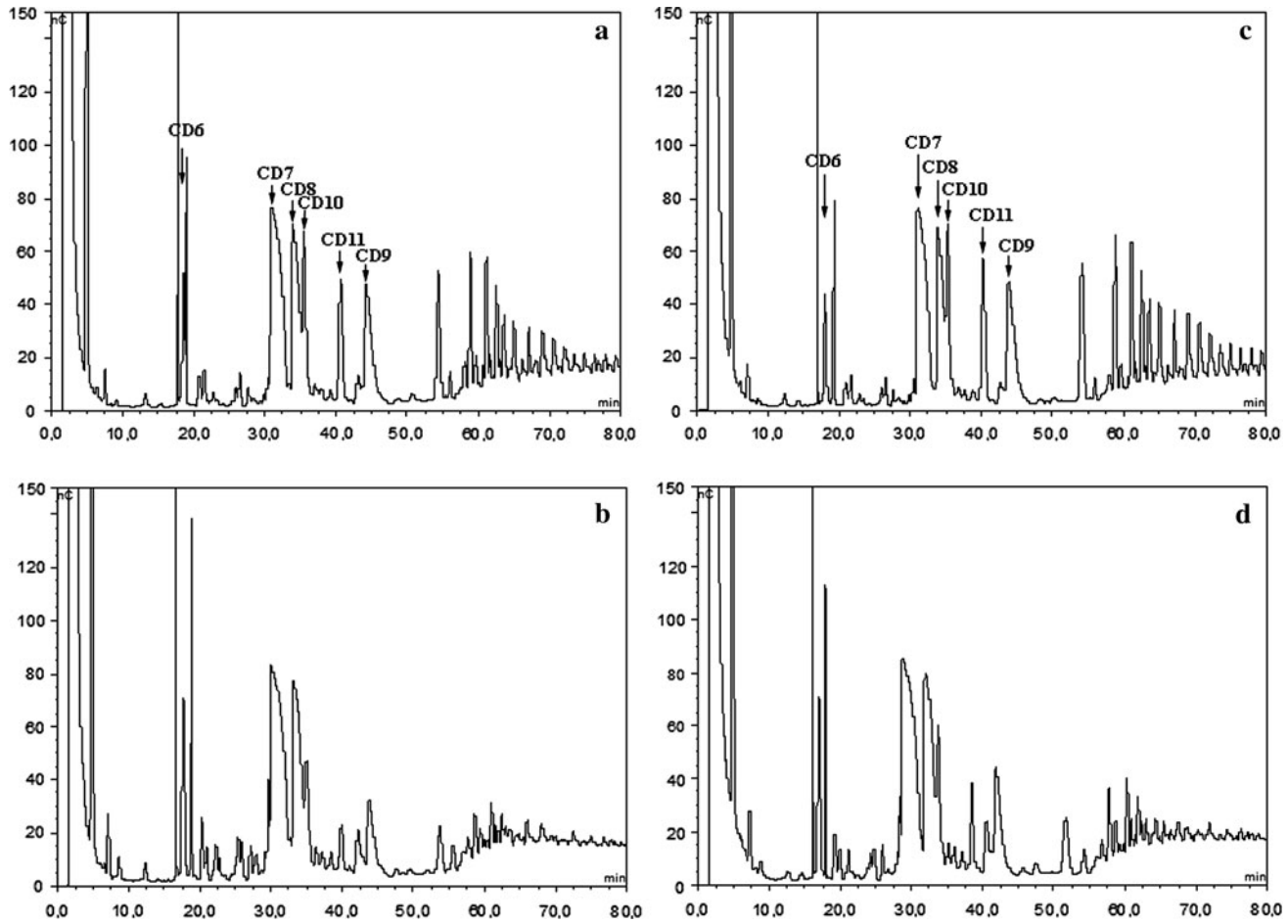

Fig. 5 HPAEC chromatograms of the CD products produced by the rCGTase (**a**, **b**) isoforms I and (**c**, **d**) isoform II. The enzyme (3 units) was mixed with 4% (w/v) soluble starch at pH 7.0 and incubated at 60 °C. The reaction mixture was withdrawn after 6 h (**a**, **c**) and 24 h (**b**, **d**) incubation and analyzed for the products. The chromatograms are representatives of three independent repeats

Table 2 Profile of CD production by isoforms I and II at different temperatures and reaction times from HPAEC analysis

Isoform	Time (h)	Temp (°C)	Ratio of each CD[a] (peak area)	Relative ratio of small-ring CDs[b]	Ratio of small-ring to large-ring CDs[c]	
					Peak area	Relative ratio
I	6	60	20:123:70:53:35:26	0.16:1.0:0.57	213:114	1.0:0.54
	12		27:135:78:39:29:17	0.20:1.0:0.56	240:85	1.0:0.35
	24		28:113:63:29:20:12	0.25:1.0:0.56	204:61	1.0:0.30
II	6	60	15:103:50:42:32:22	0.14:1.0:0.49	168:96	1.0:0.57
	12		21:104:61:34:26:14	0.20:1.0:0.59	186:74	1.0:0.40
	24		31:133:82:37:29:13	0.23:1.0:0.62	246:79	1.0:0.32
I	6	40	24:171:83:51:45:35	0.14:1.0:0.49	278:131	1.0:0.47
	12		28:151:82:41:31:23	0.19:1.0:0.54	261:95	1.0:0.36
	24		32:141:78:26:18:13	0.23:1.0:0.55	251:57	1.0:0.23
II	6	40	7:81:37:30:26:19	0.09:1.0:0.46	125:75	1.0:0.60
	12		19:114:54:37:26:23	0.17:1.0:0.47	187:86	1.0:0.46
	24		20:120:68:30:21:18	0.17:1.0:0.57	208:69	1.0:0.33

[a] CD6:CD7:CD8:CD9:CD10:CD11

[b] CD6:CD7:CD8

[c] CD6 + CD7 + CD8:CD9 + CD10 + CD11

Note: The three repeated experiments gave similar relative peak areas and product ratios

CD production profiles

When incubated with soluble starch as the substrate at 40 or 60 °C for 6 h, in addition to CD6–CD8 as products, both isoforms also produced a relatively high amount of large ring CDs (>CD9), but the proportion of these large-ring CDs was lower after 12 and 24 h of production (Fig. 5). The increase in the ratio of small- to large-ring CDs (CD6 to CD8 vs. CD9 to CD11) from 1.0:0.5 to 1.0:0.3 was observed from a 6 to 24 h reaction time for both isoforms (Table 2). This result agrees well with a previous report that CGTase can give rise to large-ring CDs in the initial phase of production [2].

It was observed that the product levels of CD6–CD8 increased at 24 h. The chromatogram of CD products obtained from isoform I was essentially the same as that for isoform II in the sense that CD7 (β-CD) was the main CD product at all time points. The observed ratios of CD6:CD7:CD8 at 24 h of 60 °C incubation, for isoforms I and II were very similar at 0.25:1.0:0.56 and 0.23:1.0:0.62, respectively (Table 2).

Interestingly, when the two rCGTase isoforms were analyzed for their ability to synthesize CDs, they showed a clear difference in the preference temperature and the time required for the highest CD production. Isoform I yielded the highest content of CD7 and total CD after a 6 h reaction time at 40 °C, whereas isoform II required 24 h at 60 °C for maximal productivity.

In conclusion, the rCGTase isoforms I and II had similar optimal pH and temperatures for enzymatic activities, but the catalytic efficiency values in the coupling reaction with CD7 were different. The two isoforms are β-CGTases and both gave a similar ratio of CD products. However, they showed a clear difference in the temperature and the time required for the highest production of CDs.

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