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Production of Cyclodextrins by CGTase from *Bacillus clausii* Using Different Starches as Substrates

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Abstract Cyclodextrins (CDs) are cyclic oligasaccharides composed by D-glucose monomers joined by α-1,4-D glicosidic linkages. The main types of CDs are α-, β- and γ-CDs consisting of cycles of six, seven, and eight glucose monomers, respectively. Their ability to form inclusion complexes is the most important characteristic, allowing their wide industrial application. The physical property of the CD-complexed compound can be altered to improve stability, volatility, solubility, or bio-availability. The cyclomaltodextrin glucanotransferase (CGTase, EC 2.4.1.19) is an enzyme capable of converting starch into CD molecules. In this work, the CGTase produced by *Bacillus clausii* strain E16 was used to produce CD from maltodextrin and different starches (commercial soluble starch, corn, cassava, sweet potato, and waxy corn starches) as substrates. It was observed that the substrate sources influence the kind of CD obtained and that this CGTase displays a β-CGTase action, presenting a better conversion of soluble starch at 1.0%, of which 80% was converted in CDs. The ratio of total CD produced was 0:0.89:0.11 for α/β/γ. It was also observed that root and tuber starches were more accessible to CGTase action than seed starch under the studied conditions.

Keywords CGTase · Cyclodextrin · *Bacillus clausii* · Soluble starch · Corn starch · Cassava starch · Sweet potato starch · Waxy corn starch

Introduction

Cyclomaltodextrin glucanotranferase (CGTase, EC 2.4.1.19) is a member of the α-amylase family (family 13) of glycosylhydrolases, an important group of starch

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processing enzymes (1). This group of enzymes exhibits a broad diversity in reaction specificities. However, while amylases generally hydrolyze glucosidic bonds in the starch molecules, CGTases catalyse mainly transglycosylation reactions, with hydrolysis being a minor activity (1–3). The major activity is intramolecular transglycosylation or cyclization reaction, leading to the formation of nonreducing cyclic oligosaccharides, named cyclodextrins (CDs). The main types of CDs are α-, β-, and γ-CDs consisting of six, seven, and eight glucose monomers in cycles, respectively. Their structure has a hydrophilic outer surface and a hydrophobic cavity. Because of their ability to form inclusion complexes with many organic molecules, CDs and their derivatives have become increasingly useful in pharmaceutical, food, cosmetics, analytical chemistry, agriculture, and biotechnology (4–6).

The majority of the CGTases usually produce a mixture of α-, β-, and γ-CD, and the product ratio can vary depending on condition and reaction time. These require CDs purification and separation, which becomes a rather elaborate, costly, and time-consuming part of an industrial production process. Hence, efforts are being made not only to increase the CDs yield but also to improve conditions of enzymatic reaction of the CGTases toward a particular CD. Protein engineering of the enzyme has promising results for changing the product specificity (7, 8). On the other hand, addition of selective complexant agents and organic solvents to the reaction mixture has also shown to significantly influence the ratios of α-, β-, and γ-CDs and their yields. Many works have shown that different substrates can determine the kind of product obtained from enzymatic reaction CDs (9–16).

In this work, the profile of CD production by the action of CGTase from *Bacillus clausii* strain E16 in soluble starch and maltodextrin was studied. Furthermore, the action of this CGTase in the presence of other starch botanical sources such as cassava starch, sweet potato starch, corn starch, and waxy corn starch were analyzed.

Materials and Methods

Materials

CDs (α-, β-, γ-CD), glucoamylase, and maltodextrin (dextrose equivalent 13.0–17.0) were purchased from Sigma (St. Louis, MO). Soluble starch was obtained from Mallinckrodt (Paris, France), corn and cassava starches were obtained from Cargil (São Paulo, Brazil), and sweet potato and waxy corn starches were donated by Starch Laboratory of the Food Science and Technology Department (IBILCE-UNESP, SP, Brazil). Yeast extract was obtained from Difco (Detroit, USA), and peptone was obtained from Biobrás (Minas Gerais, Brazil). Other chemicals of analytical grade were obtained from Merck (Darmstadt, Germany).

Bacterial Strain and CGTase Production

CGTase used in this study was obtained from *B. clausii* E16 that was isolated and identified by Alves-Prado et al. (17, 18). The microorganism was grown in shake flasks (250 mL) containing culture medium (50 mL) composed (g/L) of soluble starch 13.4, peptone 4.9, yeast extract 5.9, K_2HPO_4 1.0, $MgSO_4.7H_2O$ 0.2, and Na_2CO_3 12.5 (separately sterilized), pH 10.1 (18), on a rotatory shaker at 200 cycles per min. After 48 h, the bacterial cells were harvested by centrifuging at 10,000×*g* for 10 min at 5 °C, and the clear supernatants were used as crude enzyme.

Enzymatic Assay

Two methods were used; one is the iodine method that was used to determine dextrinization or the ratio of hydrolysis of the starch, and the other is the phenolphthalein method that was used to determine CD formation. Starch-dextrinizing activity was determined in accordance with Fuwa (19) and Pongasawasdi and Yagisawa (20) with slight modifications. The reaction mixture containing 100 μL of diluted enzyme aliquot and 300 μL of 0.5% soluble starch prepared in 0.1 M acetate buffer, pH 5.5, was incubated at 55 °C for 10 min. The enzyme reaction was stopped by the addition of 4.0 mL of 0.2 M HCl solution. Then, 0.5 mL of iodine solution (0.3 g/L I_2 and 3.0 g/L KI) was added to form an amylose–iodine complex with residual amylose. The final volume was adjusted to 10 mL with distilled water. The absorbance of the blue color of the amylose–iodine complex was measured by spectrophotometer at 700 nm, and a decrease in absorbance was verified, when compared to a control tube with heat-inactivated enzyme. One unit of enzyme activity was defined as the quantity of enzyme that reduces the blue color of the starch–iodine complex by 10% per minute.

CGTase activity was measured as β-CD-forming activity based on the phenolphthalein method (21) with slight modifications as described in Alves-Prado et al. (18). One hundred microliters of diluted enzyme aliquot was added to 800 μL of 1% soluble starch prepared in 100 mM acetate buffer, pH 5.5, and incubated at 55°C for 10 min. The enzyme reaction was stopped by the addition of 4.0 mL of 0.25 M Na_2CO_3 solution, and 0.1 mL of 1 mM phenolphthalein solution was added to the reaction mixture. The absorbance was measured at 550 nm, and a decrease in absorbance was compared to a control reaction mixture with inactive enzyme (100°C for 30 min). One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of β-CD per minute using a standard curve with β-CD.

Protein Determination

Protein concentration was estimated according to the Hartree–Lowry method, using bovine serum albumin as the standard (22).

CGTase Purification

The CGTase was produced in 500-mL Erlenmeyer flasks, containing 80 mL culture medium with soluble starch as substrate, in a rotary shaker at 35 °C, for 48 h at 200 cycles per min. The cells were removed from the culture by centrifugation at 10,000×*g* for 10 min at 5 °C. The supernatant containing crude CGTase was concentrated by ultrafiltration using the Pellicon® system (Millipore, Beldford, MA). The concentrated CGTase was subjected to a gel filtration chromatography on a Sephadex superfine G-50 column (2.6 cm diameter× 100 cm length) pre-equilibrated with 20 mM Tris–HCl buffer (pH 7.5), containing 20 mM NaCl. The elution was carried out in the same buffer at a flow rate of 0.3 mL/min at room temperature, and 4 mL fractions were collected using a fraction collector (Pharmacia Biotech Frac-100, Sweden). The active CGTase fractions were concentrated by Centricon® (amicon bioseparations, Millipore). The partially purified CGTase was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (23).

Effect of Soluble Starch and Maltodextrin on CD Formation

Soluble starch and maltodextrin (1.0 and 2.5% w/v) prepared in acetate buffer, 100 mM, pH 5.5, were homogenized by heating in a boiling water bath. Four hundred microliters of

enzyme (adjusted to 10 U of dextrinizing activity per gram of substrate) was added to 100 mL of each substrate, and it was incubated in a shaker under agitation of 100 cycles per min at 55 °C for 24 h. Aliquots of 1 mL were transferred to 2-mL tubes, closed, and immediately heated in a boiling water bath. All samples were submitted to hydrolysis with amyloglucosidase (Sigma) and filtered through a 0.45-μm cellulose acetate membrane (Millipore). The CD formed in the reaction mixture was detected by high-performance liquid chromatography (HPLC). All experiments were done in triplicate.

Effect of Kind of Starches on CD Formation

The CGTase action was evaluated on cassava starch, sweet potato starch, corn starch, and waxy corn starch. Substrates' concentrations were at 2.5%. Enzyme, conditions, and quantity were conducted as described above, except that aliquot samples were withdrawn periodically until 24 h. In an independent experiment, starches were gelatinized by autoclave process. The percentage of starch converted into CDs was calculated by ratio of total grams of CDs formed divided per gram of starch and multiplied per 100.

CD Quantification

The CD formed in the reaction mixture was detected by HPLC. Conditions for HPLC were based on that described by Sato et al. (24). The HPLC system consisted of Jasco PU 990 pump (Jasco, Japan) connected to a Shodex RI 72 refractive index detector. In this system, it was connected a Zorbax-NH_2 column (250×4.6 mm, 5 μm, Aligent Technologies) installed in a column oven Dionex STH 585 (Dionex Softron GmbH, Germany) at 35 °C. It was used a mixture of acetonitrile/water (65:35 v/v) with the flow rate of 0.8 mL/min. All experiments were done in triplicate.

Results and Discussion

CGTase Purification

After 48 h of fermentation, the supernatant from the *B. clausii* strain E16 culture was used for partial purification of the CGTase. The supernatant containing crude enzyme was first concentrated by ultrafiltration and subsequently purified by gel filtration. The gel filtration profile and purity degree of enzyme is shown in Fig. 1.

Effect of Soluble Starch and Maltodextrin on CD Formation

CD production was evaluated on 1.0 and 2.5% of maltodextrin and soluble starch by CGTases action. After 24 h, the products formed were analyzed by HPLC. The ratio conversion in α-, β-, and γ-CD of the maltodextrin and soluble starch hydrolyzed by CGTase from *B. clausii* strain E16 is shown in Table 1.

It can be observed that there are differences in quantity and types of CD formed in accordance with the substrate used for CGTase action. In the presence of maltodextrin, the CD production was of three types, α-, β-, and γ-CD, while in soluble starch, there was production of only two types of CDs, β- and γ-CD, and no α-CD was observed. Many other CGTase studies have shown similar results, with the production of the β-CD

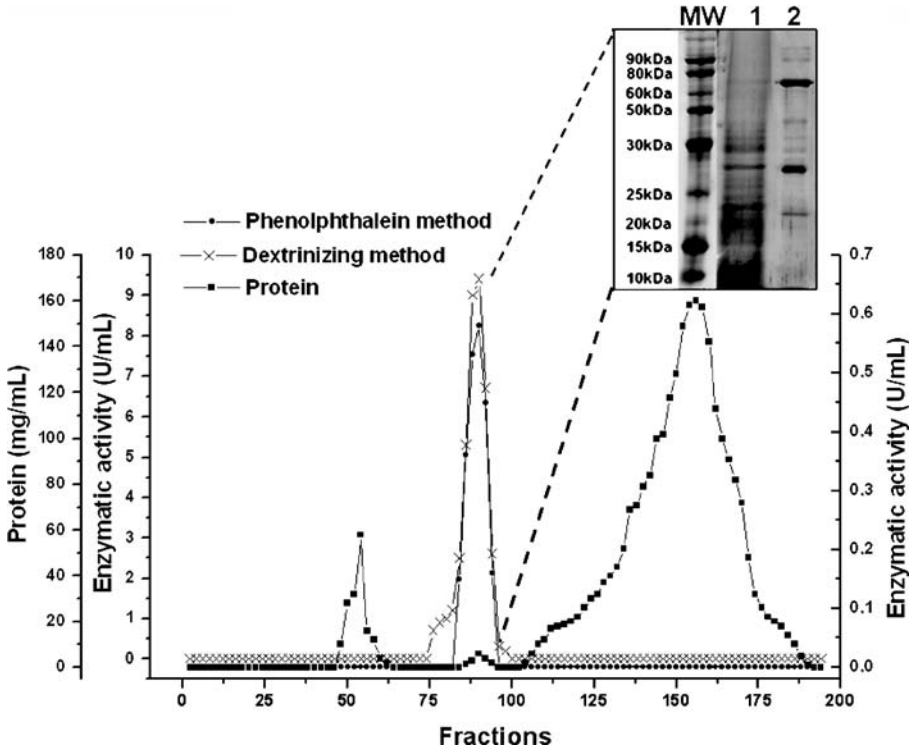

Fig. 1 Gel filtration process profiles on purification of the CGTase from *Bacillus clausii* strain E16, with illustration of electrophorese SDS-PAGE. MW: molecular weight; 1: crude CGTase; 2: partially purified CGTase

prevailing (9, 12, 13, 15, 16, 27, 28, 29). The better conversions were observed on 1.0% soluble starch, which showed a major conversion (80%) or in 2.5% maltodextrin (41%; Table 1). CGTase from *B. clausii* strain E16 produced preferentially, on soluble starch, β-(89%) and γ-CD (11%) in higher concentrations, similar to CGTase from *Bacillus* sp. AL-6 (25) that produces only β- and γ-CD in the presence of soluble starch. The authors have observed that it was possible to improve γ-CD yield when ethanol was added to the reaction mixture of hydrolysis. Charoenlap et al. (26) reported a CGTase from *B. circulans* (TISTR 907) as being highly specific for β-CD formation because its distributions of α-, β-, and γ-CDs were 7, 65, and 28%, respectively, from soluble starch. In this respect, we can also consider CGTase from *B. clausii* strain E16 as specific for β-CD formation. The

Table 1 Effect of maltodextrin and soluble starch on CDs production by CGTase from *Bacillus clausii* strain E16, after 24 h of hydrolysis.

Substrate	CD production (mg/mL)				CD
	α-CD	β-CD	γ-CD	Total	Conversion (%)
Maltodextrin 1.0%	0.41±0.03	1.20±0.13	0.51±0.07	2.12	21.0
Maltodextrin 2.5%	1.64±0.06	5.27±0.11	3.46±0.13	10.37	41.0
Soluble starch 1.0%	0	7.09±0.10	0.90±0.20	7.99	80.0
Soluble starch 2.5%	0	3.09±0.14	0.73±0.13	3.82	15.2

GTase from the *B. circulans* NRRL B380 using soluble starch as a substrate exhibited only 40% distribution of β-CD (27).

Effect of Kind of Starch on CD Formation

Soluble starch was a better substrate than maltodextrin for CD production, so other substrates were also used for CD production. The content and structural characteristics of amylose and amylopectin present in starches may vary depending of their botanical sources. So, it was interesting to investigate this parameter regarding to CD formation under CGTase action. Cassava starch, sweet potato starch, corn starch and waxy corn starch were used.

Generally, starches contain about 20 to 30% of amylose and 70 to 80% of amylopectin, and these concentrations change with the botanical source of starch. Cassava starch, sweet potato starch, corn starch, and waxy corn starch showed, respectively, 17.0, 20.7, 25, and less than 1% of amylose (28, 29, 30). The ratio amylose/amylopectin is an important factor to consider for CD production. The helicoidal structure of amylose with loops of six to seven glucose units can contribute with action of CGTase on α- and β-CD formation (2).

In Table 2, it can be observed that after 24 h of hydrolysis, 22 and 21% of cassava starch and sweet potato starch, respectively, were converted into β- and γ-CD. Corn starch (7.3%) was converted into β- and γ-CD, and only 1.5% of waxy corn starch was converted into β-CD.

In the results shown, cassava starch and sweet potato starch were more susceptible to CGTase action than corn starch, which has the highest percentage of amylose. However, another factor that should be considered is the lipid concentration of the starch. Root starches (cassava) and tuber starches (sweet potato) show low lipid quantities, less than 0.1%, while in cereal starches (corn), the lipid quantities are high, around 0.5 to 1.0% (30, 31, 32). Most of the lipid components of cereal grains are concentrated in the germ. The lipids of the endosperm have been classified as starch lipids, which are those associated with starch granule, and nonstarch lipids, which are those contained in the spherosome dispersed throughout the endosperm. Therefore, in cereal starches, amylose and lipid may form a complex, amylose–lipid, which is very stable and dissociates only at very high temperatures (30, 31, 33). The lower action of CGTase on corn starch can be due to the fact that part of amylose is engaged in lipid complexation. To analyze this hypothesis, an intense thermal treatment during homogenization of starches was done. Cassava, sweet potato, and corn starches were homogenized in a boiling water bath (Table 2 and Fig. 2) or were homogenized in a boiling water bath and then submitted to a gelatinization process in autoclave for 10 min at 121 °C and 1 atm (Table 3 and Fig. 3). Then, the starch solutions were submitted to CGTase action.

Table 2 Effects of starches from different botanical sources on CD production by CGTase from *Bacillus clausii* strain E16, homogenized by heating in a boiling water bath.

Botanical sources (2.5%)	CD production (mg/mL)				CD conversion (%)
	α-CD	β-CD	γ-CD	Total	
Cassava starch	0	4.75±0.28	0.73±0.11	5.48	22.0
Sweet potato starch	0	4.39±0.17	0.81±0.10	5.20	21.0
Corn starch	0	1.83±0.13	0	1.83	7.3
Waxy corn starch	0	0.30±0.06	0	0.30	1.5

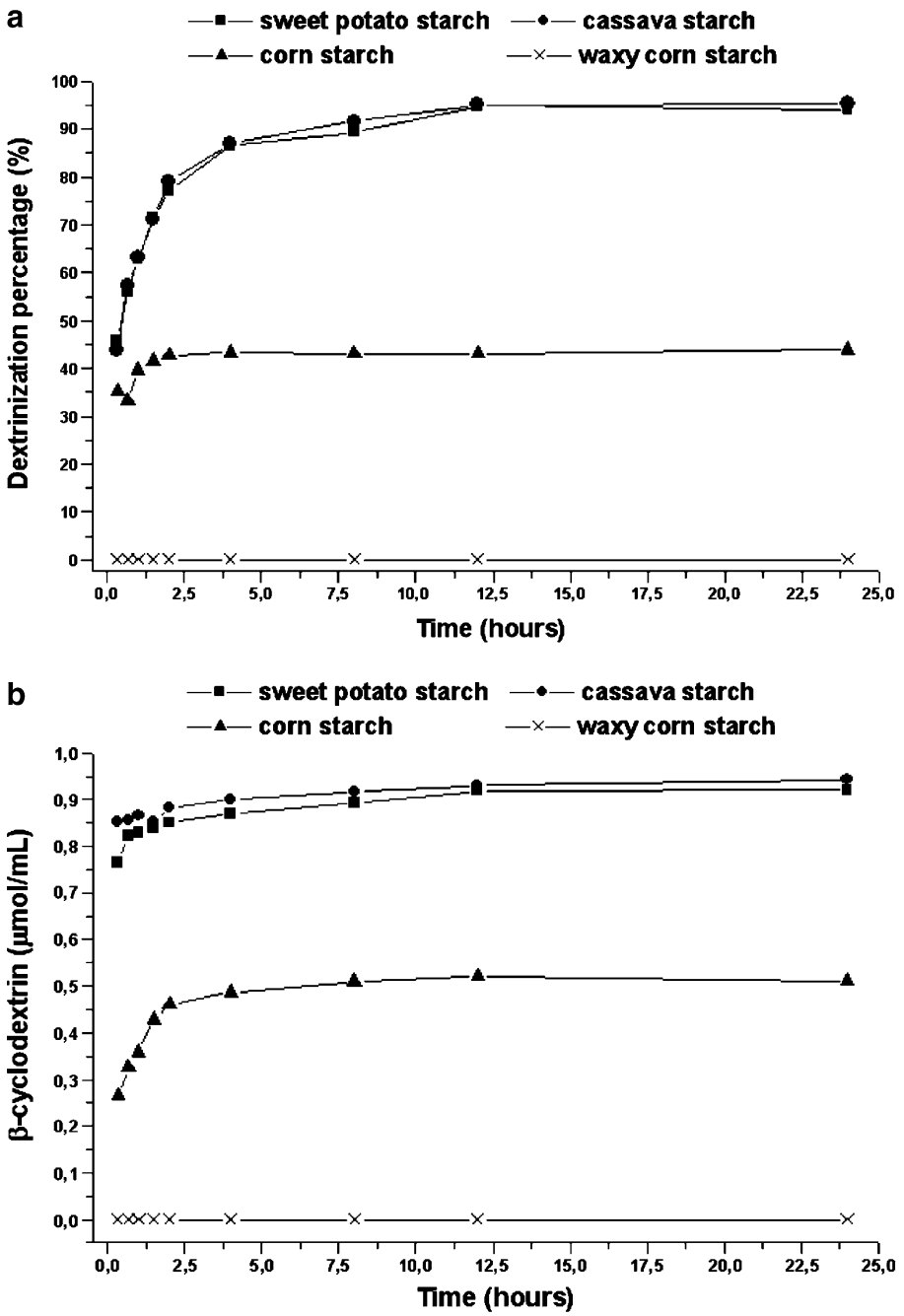

Fig. 2 CD production by action of CGTase from *Bacillus clausii* strain E16 on starches from different botanical sources. **a** Dextrinization percentage, **b** CD formation

Table 3 Effect of starches from different botanical sources on CD production by CGTase from *Bacillus clausii* strain E16, gelatinized by autoclave.

Botanical sources (2.5%)	CD production (mg/mL)				CD conversion (%)
	α-CD	β-CD	γ-CD	Total	
Cassava starch	0	4.92±0.21	1.03±0.11	5.95	24.0
Sweet potato starch	0	5.21±0.37	1.05±0.07	6.26	25.0
Corn starch	0	2.60±0.35	0.20±0.09	2.80	11.0
Waxy corn starch	0	1.58±0.12	0	1.58	6.3

Analyses of the time course of CD formation after gelatinization in boiling water bath through dextrizing (a) and phenolphthalein (b) methods are shown in Fig. 2. There was a better CD formation in cassava and sweet potato starches. At 4 h, it was shown that the homogenization in boiling water bath (conventional process) yield a maximum dextrinization of 95% for cassava and sweet potato starches, 42% for corn starch, and 0% for waxy corn starch. The time course of dextrinization with the gelatinization in boiling water bath followed by autoclaving (Fig. 3) showed an increased percentage of dextrinization (10%) for cassava and sweet potato starches. For corn starch, the dextrinization percentage increased 79% (42 to 79) in regard to conventional homogenization processes, and also, there was a conversion (25%) from waxy corn starch. The same characteristic was observed in regard to β-CD formation (Fig. 3b), in which an increase in conversion of 8% (0.88 to 0.95) for cassava and sweet potato starches and 66% (0.48 to 0.8) for corn starch were observed, and there was also a small conversion for waxy corn starch (0.15 μmol/mL). Therefore, these results suggest that the amylose–lipid complex present in corn starch granules reduces the CGTase action.

Table 3 shows the CD produced after 24 h quantified by HPLC. The data show a better CD production under the new homogenization condition when compared with heating in a boiling water bath. It was also observed that there was a significant increase in CD conversion when corn starch was used (2.6 against 1.8 mg/L, for β-CD). The increase in total CD yield was approximately 50%, and some γ-CD (0.2 g/L) could be quantified. The increase in total CD for cassava and sweet potato starch was lower, 8 and 20%, respectively. The ratio of CD production for α-, β-, and γ-types from cassava and sweet potato starches were similar, 0:0.83:0.16, and for corn starch, it was 0:0.93:0.07. Furthermore, there was increase in ratio of CD conversion with all starches gelatinized by autoclave. These results reinforce the idea that the amylose–lipid complex present in cereal starches can influence CGTase action and thus CD formation. Gawande and Patkar (34) have observed a low CD conversion using CGTase from *Klebsiella pneumoniae* AS-22 on a corn starch solution. Using CGTase from *Brevibacterium* sp no. 9605, Mori et al. (10, 11) have observed 1% lower conversion when using corn starch than when using sweet potato starch. Similar results were observed by Goel and Nene (12) who obtained a better CD conversion using CGTase from *Bacillus firmus* in cassava starch than in corn starch.

In accordance with these results, it can be said that CD formation by CGTase from *B. clausii* strain E16 depends not only on the amount of amylose but also on the ability of amylose and lip to form a amylose–lipid complex in the starch. Corn starch showed an increase of β- and γ-CD production when gelatinized in an autoclave. Production of γ-CD was not observed when this starch was homogenized by heating in a boiling water bath. This demonstrates that the gelatinization process of starch is an important factor with regard to CD production process. Waxy corn starch, which is rich in amylopectin, also produced a small amount of β-CD. A mix of α-, β-, and γ-CDs was produced when CGTases from *K.*

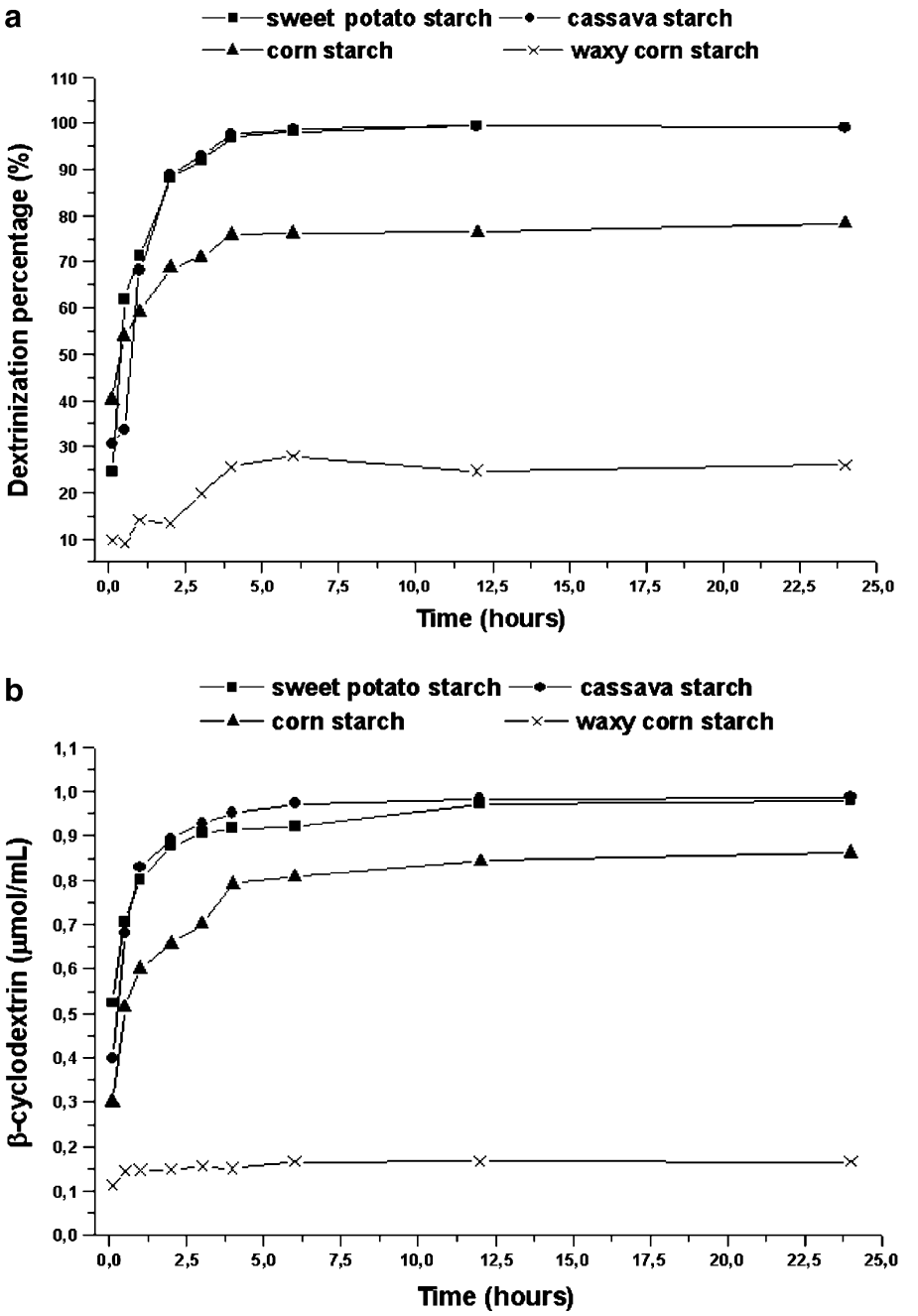

Fig. 3 CD production by action of CGTase from *Bacillus clausii* strain E16 on starches from different botanical sources, gelatinized on autoclave. **a** dextrinization percentage; **b** CD formation

pneumoniae AS-22 (34) and *Brevibacterium* sp. no. 9605 (10) were used. However, the CGTases from *B. firmus* (12, 29, 34) and *B. clausii* E16 produced only β- and γ-CD from different starches. The formation of only two types of CDs during hydrolysis processes is important in the separation process because it is easier to separate the CDs produced, which is interesting for industrial application.

Conclusions

CGTase from *B. clausii* strain E16 was specific for β-CD formation displaying a β-CGTase action. The distributions of α-, β-, and γ-CDs were 0, 89, and 11%, respectively, on soluble starch. The starches from different botanical sources influenced quantities and types of CD formed. It was also observed that root and tuber starches were more accessible to CGTase action. The process of starch homogenization can interfere whit the CGTase action and, consequently, on the CD formation. The gelatinization of starches by the autoclave process improves the CD production, mainly for cereal starches.

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