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Production of a novel Cyclodextrin glycosyltransferase from *Bacillus* sp. SK13.002

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A β-cyclodextrin glycosyltransferase (β-CGTase) from a new alkalitolerant *Bacillus* sp. SK 13.002 strain which can significantly hydrolyze starch into short linear saccharides, in addition to production of cyclodextrins (CDs) was produced. The hydrolytic activity of this CGTase as a side reaction is thought to be due to partial retention of ancestral enzyme function from evolution over time. This CGTase is therefore another example of an enzyme at an intermediary stage in between ''true'' α-amylases and ''true'' CGTases. The strain also produced two CGTase isozymes which were purified to homogeneity using DEAE-Sepharose anion exchange chromatography and Superdex 75 gel filtration chromatography to show Molecular masses of 67.5 and 46.8 kDa on sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) respectively, which were confirmed by liquid chromatography/mass spectrometry (LC/MS) analysis to be 67.6 and 47.3 kDa, respectively. Both CGTase isozymes formed CDs from soluble starch. Most of the reported CGTases were composed of one single enzyme with only a few strains having CGTase isozymes showing different isoelectric points. Therefore, *Bacillus* sp. SK 13.002 strain is another *Bacillus* capable of producing CGTase isozymes which were individually purified to homogeneity.

Key words: Cyclodextrin glycosyltransferase, production, *Bacillus*, isozyme, cyclodextrin, purification.

INTRODUCTION

Cyclodextrin glycosyltransferases (CGTases; EC 2.4.1.19) are industrially important enzymes that produce cyclic α-(1, 4)-linked oligosaccharides called cyclodextrins (CDs) from starch (Parsiegla et al., 1998). They are extracellular induced enzymes produced by microbial cells, predominantly *Bacillus* species. However, production by a variety of other bacterial species has also been reported (Leemhuis et al., 2010). CGTase catalyzes mainly transglycosylation reactions (cyclization, coupling and disproportionation) but can also exhibit, to a lesser extent, *α*-amylase-like activity, hydrolyzing starch into short linear saccharides. The main products of CGTases are cyclic α-, β-, and γ-CDs, composed of 6, 7, or 8 glucose residues. CDs have numerous applications in the pharmaceutical, cosmetics, textile, food, as well as bioremediation and separation processes. Industrial production of CGTase became attractive when alkalophilic *Bacillus* species were introduced as producer organisms because of their high level of activity in wide pH and high temperature ranges (Atanasova et al., 2008; Martins et al., 2001).

CGTases are members of the large superfamily of

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α-amylase acting on starch and related α-glucans, called glycoside hydrolase family 13 (GH13). All family members share a conserved active site architecture along with four short conserved sequence regions embedded in a TIM $(\beta/\alpha)_8$ structural fold, indicating evolutionary diversification from a common enzyme ancestor (Janecek et al., 2003). All members either hydrolyse and/or transglycosylate *α*-glucosidic linkages to produce *α*-anomeric mono- and oligo-saccharides with catalysis proceeding via a double displacement mechanism (Uitdehaag et al., 1999). Continuous evolution resulted in enzyme intermediates partially retaining the initial ancestral function while catalyzing new functions (Aharoni et al., 2005). Over several evolutionary phases, aided by selective pressure, new enzyme function dominates while ancestral function decreases to a mere side reaction (Kazlauskas, 2005; Bornscheuer and Kazlauskas, 2004; Afriat et al., 2006). In some cases this has resulted in misidentification of CGTase enzymes (Wind et al., 1995).

There are many reports on the purification of CGTase enzymes, with most strategies used mainly involving adsorption of CGTase on starch followed by gel filtration. At present, CGTases from at least 50 host organisms have been identified and (partly) characterized, even though the genes for some have not been identified and sequenced yet (Leemhuis et al., 2010; Ramli et al., 2011). Most of the reported CGTases are monomeric, with those from *Bacillus* sp. ranging in Molecular mass between 33 and 110 kDa while those from *Micrococcus* sp. are between 85–120 kDa (Jemli et al., 2007; Alves-Prado et al., 2007; Martins and Hatti-Kaul, 2002). Only CGTase produced by *B. macerans* (Kobayashi et al., 1978), *B. licheniformis* (Aoki et al., 1988) and *Bacillus* sp. 562 (Yan and Lin, 1995) have been found to have subunits. However, CGTase isozymes have been reported from *Bacillus circulans* E 192 (Boveto et al., 1992), *Bacillus* sp.1070 (Volkova et al., 2001) and *Bacillus megaterium* strain no. 5 (Kitahata and Okada, 1974).

In the present work, we report on the production of two CGTase isozymes from a newly isolated alkalitolerant *Bacillus* sp. SK 13.002 strain with *α*-amylase-like activity, in addition to production of CDs. Both purified CGTase isozymes formed CDs from soluble starch.

MATERIALS AND METHODS

Materials

Soluble starch, β-Cyclodextrin, bovine serum albumin and all other analytical grade chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Electrophoresis reagents were bought from Bio-Rad Laboratories Inc. (Nanjing, China). Protein standard markers, DEAE-Sepharose CL 6B and Superdex 75 were procured from Sangon Biotech Co., Ltd (Shanghai, China).

Bacterial strain and culture conditions for CGTase production

Strain SK 13.002, identified as *Bacillus* sp. SK 13.002, was isolated from a soil sample by our laboratory. The gene sequences for this strain were deposited to the NCBI GenBank database under accession number GU570959.

The fermentation medium used for CGTase production contained per liter: 10 g soluble starch, 10 g soy peptone, 5 g yeast extract, 1 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$ and 8 g Na_2CO_3. A 3% (v/v) strain inoculum was transferred into a 250 ml conical flask containing the fermentation media and incubated at 37°C for 96 h with continuous orbital shaking at 200 rpm. Samples were taken at every 6 h interval for analysis. The cells were removed by centrifugation (10 000 rpm, 15 min, 4°C) and the supernatant used as the source of the crude enzyme and for activity assays. Estimation of cell growth as optical density was done at 600 nm. The experiments were done in triplicates.

CGTase activity assays

Cyclization activity

Cyclization activity was measured, according to a modified method by Savergave et al. (2008), as a function of the β-CD production rate, using soluble starch as substrate at 1% (w/v) in 50 mM Tris–HCl buffer, pH 7.0, at 65°C for 15 min. The β-CD produced in the assay was determined based on colour fading at 550 nm. One unit (U) of enzyme activity was defined as the amount of enzyme that produced 1 µmol of β-CD/min under the assay conditions.

Amylolytic activity

The starch hydrolyzing activity of CGTase was assayed by the method of Shiosaka and Bunya (1973). Two hundred microliter of the enzyme solution was incubated with 5 ml of 0.3% soluble starch in 20 mM acetate buffer (pH 5.5) containing l mM $CaCl_2$ at 40°C. After l0 min, 0.5 ml of the reaction mixture was pipetted out and added to 15 ml of 0.02 N H_2SO_4. To the mixture, 0.2 ml of 0.1 N I_2 solution was added, and then the color developed was measured at 660 nm. One unit of the enzyme activity was defined as the amount of the enzyme that catalyzed a 10% decrease of absorbance per min under the assay conditions.

CGTase concentration and precipitation

Concentrated enzyme solution was obtained using Millipore ultrafiltration system (Millipore Company, U.S.A) with a Molecular mass cut-off point of 10 kDa. The solution was then precipitated with 70% (w/v) saturated ammonium sulphate at 4°C. The precipitate was centrifuged at 10 000 rpm, 15 min, 4°C and the pellet dissolved in 20 mM Tris–HCl buffer, pH 8.0 before dialysis against the same buffer overnight at 4°C.

Purification of the CGTase and molecular mass

Crude CGTase solutions were then purified using DEAE-Sepharose CL 6B and Superdex 75 gel column, respectively. The Molecular mass of the purified enzymes was determined by SDS-PAGE, according to Laemmli (1970), using 4% acrylamide in the stacking gel and 12% acrylamide in the separating gel. Lysozyme (14.4 kDa), Trypsin inhibitor (20.1 kDa), Carbonic anhydrase (31 kDa), Rabbit actin (43 kDa), bovine serum albumin (66.2 kDa) and rabbit phosphorylase B (97.4 kDa) were used as standard protein Molecular mass markers. The gel was stained with 1% Coomassie Brilliant Blue R-250 (Bio-Rad). LC/MS analysis was done to confirm the Molecular masses of the gel filtration purified enzyme solutions.

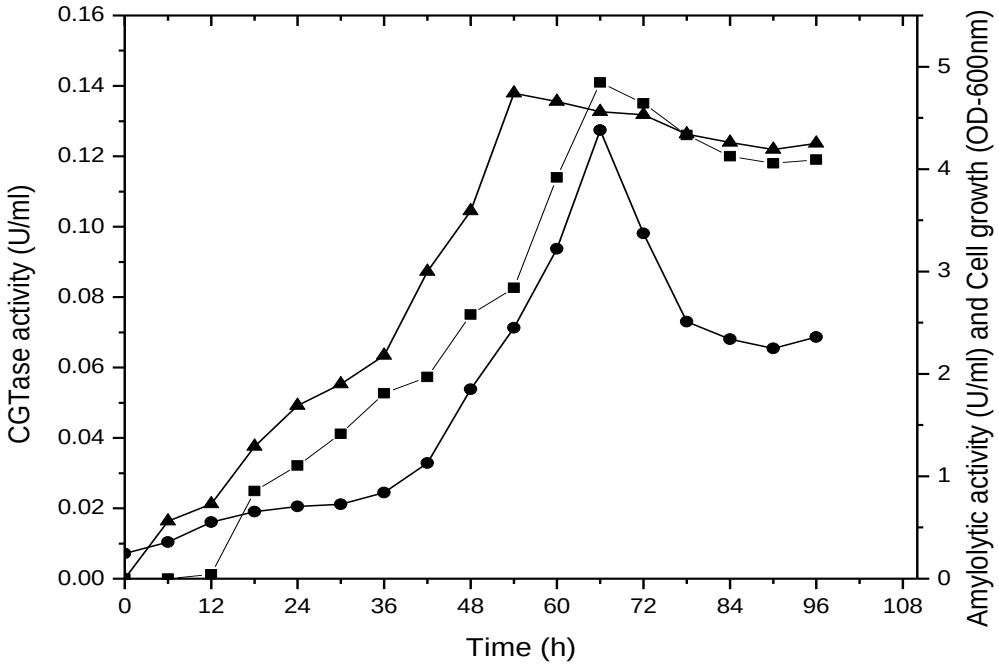

Figure 1. Cellular growth and CGTase production by *Bacillus* sp. SK13.002 strain. (▲: OD 600 nm; ■: CGTase activity, ●: Amylolytic activity).

RESULTS AND DISCUSSION

Growth and CGTase production from *Bacillus* sp. SK13.002

The strain grew well at a pH around neutral but produced very small amount of enzyme. However, it was also capable of growing in alkaline media with a pH around 10 and produced the highest amount of enzyme. This indicated that *Bacillus* sp. *SK13.002* is alkalitolerant. It produced predominantly β-CD from starch hence it was classified as a β-CGTase producer. The CGTase enzyme exhibited relatively low cyclization or protein expression as compared to several other CGTases. However, low cyclization activities have also been reported for CGTases from thermophilic *Thermoanaerobacter* sp. P4 (Avci and Donmez, 2009), *Bacillus agaradhaerens* LS-3C (Martins and Hatti-Kaul, 2003) and *Bacillus sphaericus* (Moriwaki et al., 2009).

As shown in Figure 1, within 12 h of fermentation, the amount of CGTase production was very small and could hardly be detected. However, it increased steadily until it reached the maximum of 0.141 U/ml after 66 h. There was also significant starch hydrolysis or amylolytic activity that proceeded alongside CGTase production which also reached the highest value of 4.38 U/ml at 66 h. The cell growth and the production of CGTase did not occur at the same rate as the CGTase production lagged behind. Although hydrolysis and cyclization are all performed at a unique active site in the enzyme, they proceed via different kinetic mechanisms (Martins and Hatti-Kaul, 2003; Van der Veen et al., 2000). This hydrolysis side reaction is undesirable, since it produces short saccharides that are responsible for accelerating the breakdown of the cyclodextrins during the coupling reaction, thus limiting the final product yields (Kelly et al., 2008). The short saccharides have also been found to repress extracellular enzyme production (Tonkova, 1998). Therefore, the decline in both cyclization and hydrolysis could be from the effects of these short saccharides. Sharp decline in starch hydrolysis could also indicate that most of the amylose was hydrolysed.

The *α*-amylase-like activity of *Bacillus.* sp. SK13.002 CGTase is thought to be due to partial retention of ancestral enzyme function from evolution over time. Studies of evolutionary relationships within the α-amylase family have provided evidence that CGTase enzymes evolved from α-amylases (Kelly et al., 2009a, b). Continuous enzyme evolution within this diverse family has resulted in intermediate enzymes having new functions while partially retaining ancestral function as a side reaction (Kelly et al., 2008; Janecek, 1995). The *Thermoanaerobacterium thermosulfurigenes* EM1 and *Bacillus* sp. B1018 CGTase enzymes were initially thought to be α-amylases because of their relatively high hydrolytic activity (Wind et al., 1995; Itkor et al., 1990).

Bacillus sp. SK13.002 CGTase is therefore an unusual type of CGTase, since besides CDs, significant amount of linear sugars were also formed from starch (data not shown). It is therefore another example of an enzyme at an

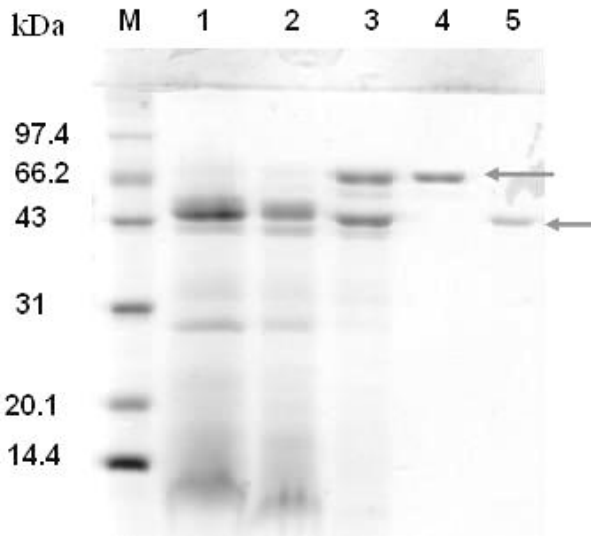

Figure 2. SDS-PAGE analysis of samples from various purification steps. Lane M, standard protein markers; lane 1, crude enzyme after ammonium sulphate precipitation; lane 2, sample precipitate after dialysis; lane 3, DEAE-Sepharose column chromatography; lane 4, Gel filtration CGTase-1; lane 5, Gel filtration CGTase-2.

intermediary stage in between ‘‘true’’ α-amylases and ‘‘true’’ CGTases.

Purification of CGTase and molecular mass

After 96 h of fermentation, the supernatant from *Bacillus* sp. SK13.002 culture was used for purification of CGTase. The ultrafiltration removed low Molecular mass proteins from the broth and resulted in increased specific activity. Concentration of the crude enzyme with 70% (w/v) saturated ammonium sulphate also increased specific activity and produced an 8.9 purification fold with 41.9% yield recovery. DEAE-Sepharose CL-6B column chromatography resulted in 15.1 purification fold and a percent recovery of 26.7. This purification step resulted in CGTase active fraction that showed two major bands on SDS-PAGE accompanied by some minor bands. Further purification of this CGTase active fraction using Superdex 75 gel filtration produced two CGTase isozymes, which appeared as single bands on SDS-PAGE (Figure 2). Each isozyme produced CDs when incubated with soluble starch (data not shown).

The Molecular masses of the purified CGTase isozymes were estimated to be 67.5 and 46.8 kDa by SDS-PAGE as shown in Figure 2, while LC/MS confirmation gave 67.6 and 47.3 kDa, respectively. Most of the reported CGTases are monomeric, with those from *Bacillus* sp. ranging in Molecular mass between 33 and 110 kDa while those from *Micrococcus* sp. are between 85–120 kDa (Alves-Prado et al., 2007; Martins and Hatti-Kaul, 2002). Only the CGTase produced by *B. macerans* (Kobayashi et al., 1978), *B. licheniformis* (Aoki et al., 1988) and *Bacillus* sp. 562 (Yan and Lin, 1995) have been found to have subunits on SDS-PAGE. However, the presence of two isoforms of the enzyme was not unexpected because similar data were obtained for other *Bacilli* CGTases. Two isozymes with pI 6.7 and 6.9 were obtained for CGTase from *Bacillus circulans* E 192 (Boveto et al., 1992), while *Bacillus* sp. 1070 (Volkova et al., 2001) CGTase isozymes displayed pI 5.1 and 5.3. Therefore *Bacillus* sp. SK13.002 strain is another *Bacillus* capable of producing CGTase isozymes which were individually purified to homogeneity.

Conclusion

A β-CGTase from a new alkalitolerant *Bacillus* sp. SK 13.002 strain which can significantly hydrolyze starch into short linear saccharides, in addition to production of CDs was produced. The enhanced hydrolytic activity of this CGTase is thought to be due to partial retention of ancestral enzyme function from evolution over time. The strain also produced two CGTases isozymes which were purified to homogeneity using both anion exchange chromatography and gel filtration and were found to have Molecular masses of 67.6 and 47.3 kDa by LC/MS analysis, respectively. A few strains are reported to produce CGTase isozymes hence our strain is also another *Bacillus* capable of producing CGTase isozymes. It is expected that more information will be gained about this CGTase isozymes from determination of the three-dimensional structure and amino acid residues and/or sequence regions which will allow detailed

comparison of structures with those of α-amylase and other CGTase proteins (Hofmann et al., 1989). Cloning and expression of the CGTase gene is necessary for obtaining sufficient enzyme for further characterization and making changes at the gene level to improve the critical features.

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