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Characterization of Cyclodextrin Glucanotransferase Produced by *Bacillus megaterium*

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Abstract Cyclodextrin glucanotransferase, produced by *Bacillus megaterium*, was characterized, and the biochemical properties of the purified enzyme were determined. The substrate specificity of the enzyme was tested with different α-1,4-glucans. Cyclodextrin glucanotransferase displayed maximum activity in the case of soluble starch, with a K_m value of 3.4 g/L. The optimal pH and temperature values for the cyclization reaction were 7.2 and 60 °C, respectively. The enzyme was stable at pH 6.0–10.5 and 30 °C. The enzyme activity was activated by Sr^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, and Cu^{2+}, and it was inhibited by $Zn^{2+}$and Ag^{+}. The molecular mass of cyclodextrin glucanotransferase was established to be 73,400 Da by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, 68,200 Da by gel chromatography, and 75,000 Da by mass spectrometry. The monomer form of the enzyme was confirmed by the analysis of the N-terminal amino acid sequence. Cyclodextrin glucanotransferase formed all three types of cyclodextrins, but the predominant product was β-cyclodextrin.

Keywords Cyclodextrin glucanotransferase · Cyclodextrins · Enzyme characterization · *Bacillus megaterium* · Application

Introduction

Cyclodextrin glucanotransferase (CGTase, EC 2.4.1.19) is a transglycosylating enzyme, which catalyzes four different reactions: cyclization, disproportionation, coupling, and hydrolysis. In the cyclization reaction, the enzyme transforms α-1,4-glucans to cyclic nonreducing oligosaccharides, composed of six, seven, or eight glucose units. They are called, respectively, α-, β-, and γ-cyclodextrins (CD). In the disproportionation reaction, the nonreducing end of a linear chain is transferred to an acceptor molecule, yielding a mixture of oligosaccharides of a different polymerization degree. In the coupling reaction,

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CGTase degrades CD via intramolecular transglycosylation, resulting in an accumulation of linear products. In addition, the enzyme displays a weak hydrolytic activity [1].

The main application of CGTase is for CD production [2]. The CD cyclic structure is characterized with a hydrophilic outer surface and a hydrophobic cavity, which allows the formation of inclusion complexes with different hydrophobic compounds. As a result, the physical and chemical properties of the guest molecules are altered. This unique ability of CD enables their broad application in food, pharmaceuticals, cosmetics, agriculture, analytical chemistry, and biotechnology [3, 4]. The demand of CD determines the need of their production.

CGTase is an extracellular, inducible enzyme produced only by microbial cells. CGTase biosynthesis was reported for different strains of *Bacillus* [1], *Klebsiella* [5, 6], *Paenibacillus* [7], *Brevibacterium* [8], *Thermococcus* [9], *Thermoanaerobacterium* [10], and *Anaerobranca* [11]. CGTases produced by *Bacillus macerans* strains [2, 12, 13] and alkaliphilic *Bacillus* species [14–16] are well studied, fully characterized, and used for CD production.

All isolated enzymes produce the three types of CD in different ratios [17]. CGTases forming predominantly a certain type of CD are considered to be perspective for use. Except for the product specificity, enzymes from different strains differ significantly in their biochemical properties. On the other hand, the process of CD production depends significantly on the CGTase used. For these reasons, characterization and application of new and weakly studied CGTases provide new perspectives for CD production.

The aim of the present research is the biochemical characterization of the rarely used CGTase, produced by a strain of *Bacillus megaterium*.

Materials and Methods

CGTase Production and Purification

A strain of *B. megaterium* from the collection of the department of biochemistry and molecular biology, UFT, Plovdiv, was used for CGTase preparation. The strain was cultivated on a slant agar medium, containing (g/L): meat-peptone broth 13.0, starch 10.0, and agar-agar 20.0. The pH of the medium was adjusted to 7.5. The tubes were incubated at 37 °C for 24 h and were stored at 4 °C up to 3 months. For inoculum preparation, the biomass from a slant-agar tube was transferred to a 500-mL Erlenmeyer flask, containing 50 mL potato extract with pH 7.5, and the strain was cultivated at 37 °C on a reciprocal shaker at 220 strokes per minute for 22 h. CGTase biosynthesis was carried out in 500-mL Erlenmeyer flasks, in a medium containing 100 mL potato extract and 0.5% (v/v) corn steep liquor with pH 7.5. Inoculum (1.0% v/v) was added, and the flasks were incubated at 37 °C on a reciprocal shaker at 220 strokes per minute for 72 h. Biomass was removed by centrifugation at 5,000×*g* for 20 min. The culture liquid was concentrated by ultrafiltration in Amicon cell with a PS 20000 membrane (Membrane Technologies). CGTase activity of the crude enzyme concentrate thus prepared was 1.61 U/mL.

CGTase purification was performed by a combination of three steps: adsorption on starch, ion exchange chromatography, and gel chromatography, as described previously [18]. The purified enzyme with CGTase activity of 0.308 U/mL and specific activity of 60.39 U/mg showed a single band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Estimation of CGTase Substrate Specificity

Substrate specificity of CGTase was investigated with the following substrates: potato soluble starch (Poland), amylopectin (Fluka), amylose (Fluka), and α- and γ-CD (Merck). The concentration of all tested substrates was 20.0 g/L.

Kinetic Constants Determination

The kinetic constants were determined with soluble potato starch as a substrate in concentration of 2.0–30.0 g/L at pH 6.0 by applying the Lineweaver–Burk transformation [19].

Effect of pH and Temperature on CGTase Activity

The effect of pH on CGTase activity was measured at pH 2.2–11.0 using 0.2 M citrate buffer (pH 2.2–9.0) and 0.2 M carbonate buffer (pH 9.0–11). The effect of temperature on CGTase activity was estimated in the range of 20–90 °C at pH 7.5.

Effect of pH and Temperature on CGTase Stability

The effect of pH on CGTase stability was investigated by incubating the enzyme in buffer solutions at 30 °C for 90 min and then assaying the enzyme activity. For determination of the thermal stability, CGTase was incubated at various temperatures, at pH 7.5 for 0.5–6.0 h.

Effect of Metal Ions and Reagents on CGTase Activity

The effect of metal ions and reagents on CGTase activity was estimated in the case of Na^+, K^+, Mg^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Fe^{3+}, Ca^{2+}, Pb^{2+}, Sr^{2+}, Ag^+, Hg^{2+}, ethylenediamine tetraacetic acid, SDS, ICH_2COOH, and NaN_3. CGTase was incubated in 2.0-mM reagents solutions at 30 °C for 30 min, and the enzyme activity was measured.

Determination of CGTase Molecular Mass and Amino Acid Sequence

CGTase molecular mass was determined by SDS-PAGE, gel chromatography, and mass spectrometry.

SDS-PAGE was performed by the method of Laemli [20] on Bio-Rad Mini-Protein system with 10.0% polyacrylamide gel at a constant current of 10 mA. The following protein calibration kit (Amersham Pharmacia Biotech.) was used: phosphorilase *b* (94,000 Da), bovine serum albumin (67,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (30,000 Da), trypsin inhibitor (20,100 Da), and α-lactalbumin (14,400 Da). The staining procedure was done with Coomassie Blue R-350.

Gel chromatography was carried out on a Protein Pak-300 column, using pepsin (35,000 Da), egg albumin (45,000 Da), human albumin (66,000 Da), and lactate dehydrogenase (140,000 Da) as standard molecular mass proteins. Elution was done with 0.5 M NaCl at 0.5 mL/min flow rate.

Determination of CGTase molecular mass by mass spectrometry was performed on a mass spectrometer PerSeptive Biosystems.

Amino acid sequence analysis was carried out on an automated sequenator HP 1100 high-performance liquid chromatography (HPLC) System 189.

Application of CGTase for CD Production

CGTase was tested for CD production with 50.0 g/L soluble potato starch as a substrate at the following reaction conditions: 2.0 U/g crude enzyme, pH 6.0, 60 °C, and 20 h.

Determination of CGTase Activity

CGTase activity was determined with phenolphthalein using Kestner's method [21] with modification [18]. A 20.0-g/L solution of soluble potato starch in phosphate buffer with pH 6.0 was used as a substrate. A mixture of 2.0 mL starch solution and 2.0 mL CGTase was incubated at 30 °C for 10 min. At the beginning and at the end of the reaction, samples of 0.2 mL were taken and were added to 2.0 mL phenolphthalein solution, containing 1 part 3.8 mM phenolphthalein in ethanol and 50 parts carbonate buffer with pH 10.5. The final volume was adjusted to 5.0 mL with distilled water. The absorbance at 550 nm was measured in relation to a blank sample, containing a mixture of water and phenolphthalein. β-CD concentration was calculated using a calibration curve. One unit of CGTase activity was defined as the amount of enzyme that forms 1 μmol β-CD for 1 min under the assay conditions.

Determination of α-, β-, and γ-CD Concentration

The concentration of α-, β-, and γ-CD was determined by HPLC system Shimadzu 20 AHT with a refractive index detector. For estimation of α- and γ-CD, YMC-Pack ODS-AQ column was used. The mobile phase was a mixture of methanol and water in a ratio of 3:97 with a flow rate of 1.3 mL/min, and the column temperature was 30 °C. β-CD were determined on Ultrahydrogel column, at 30 °C, and bidistilled water as a mobile phase with flow rate of 0.8 mL/min.

Results and Discussion

Substrate Specificity of CGTase

Substrate specificity of CGTase was tested with different α-1,4-glucans (Table 1). The enzyme showed maximum activity, when starch was used as a substrate. The activities displayed toward both starch fractions (amylose and amylopectin) were not equal. Amylopectin appeared to be a better substrate, ensuring maximum CGTase activity. On the contrary, in the case of amylose, the relative activity was only about 30%. This is probably due to the branched structure of amylopectin, which allows the enzyme reaction to

Table 1 Substrate specificity of CGTase.

Substrate	Relative activity (%)
Starch	100.0
Amylose	31.2
Amylopectin	99.7
α-CD	0.0
γ-CD	0.0

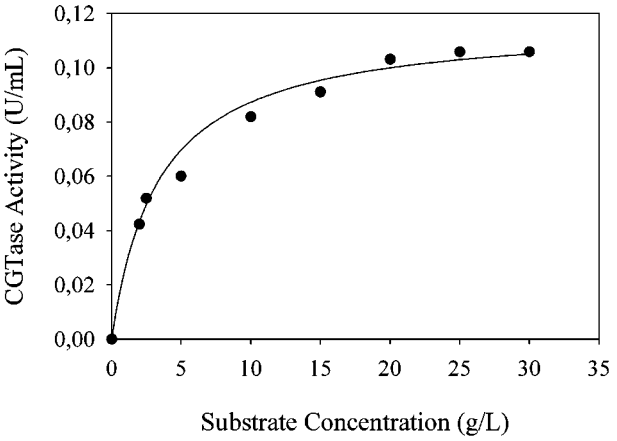

Fig. 1 Effect of substrate concentration on CGTase activity

start from a greater number of points in comparison to amylose. The same tendency was reported for other CGTases [22, 23]. Some CGTases were found to transform one type of CD to another [24, 25]. For these reasons, α- and γ-CD were tested as substrates for β-CGTase activity. The results showed that at the reaction conditions used, CGTase was not capable of degrading α- and γ-CD.

Table 2 Characterization of CGTases from different strains.

Strain	Optimum pH	Optimum temperature (°C)	pH stability	Thermal stability (°C)	K_m (g/L)	Molecular mass[a] (Da)	Predominant CD	Reference
B. megaterium	7.2	60	6.0–10.5	30	3.40	73,400	β	Present work
B. macerans 11	4.5	65	6.0–8.0	65	0.18	52,000	α	[12]
B. firmus No.37	6.0	65	Not cited	60	Not cited	77,600	β	[28]
B. firmus NCIM 5119	5.5–8.5	65	7.0–11.0	30	1.21	78,000	β	[16]
B. agaradhaerens LS-3C	9.0	55	5.4–11.0	40	21.20	110,000	β	[15]
Bacillus sp.277	5.0; 8.5	60	6.0–10.0	70	Not cited	69,000	β and γ	[29]
Bacillus sp. G_1	6.0	60	7.0–9.0	60	0.15	75,000	β	[30]
Paenibacillus sp. F8	7.5	50	6.0–8.0	40	Not cited	72,000	β	[31]
K. pneumoniae AS-22	7.0–7.5	45	6.0–9.0	35	1.35	75,000	α	[32]
Anaerobranca gottshalkii	6.0–9.0	65	Not cited	66	Not cited	78,000	α	[33]
Thermococcus sp.	5.0–5.5	90–100	Not cited	100	Not cited	83,000	α	[9]

[a] Determined by SDS-PAGE

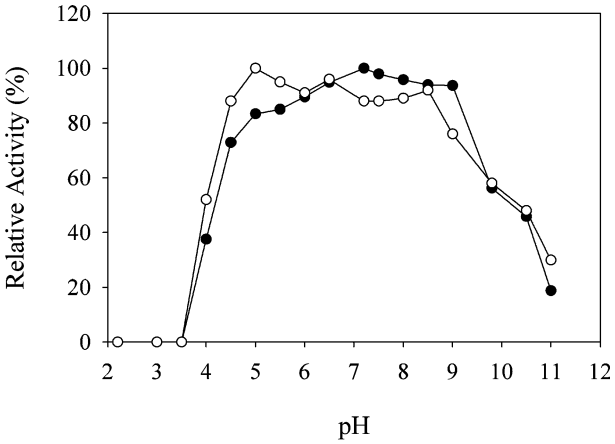

Fig. 2 Effect of pH on CGTase activity: *filled circles*, purified enzyme; *open circles*, crude enzyme

Kinetic Constants of the Cyclization Reaction

The effect of substrate concentration on CGTase activity is shown in Fig. 1. When starch concentration was raised up to 10.0 g/L, CGTase activity increased monotonically. When greater initial concentrations were applied, CGTase sensitivity to the amount of substrate decreased. This was probably due to substrate or product inhibition. The inhibition of the cyclization activity by the reaction products was established in a previous research (unpublished data). Product inhibition is characteristic for other CGTases as well [22, 26, 27]. On the basis of the experimental data, the kinetic constants of the cyclization reaction were calculated. The K_m value was found to be 3.4 g/L, and V_m was 0.117 μg min^{-1} mL^{-1}. The kinetic constants reported for other CGTases varied in a wide range (Table 2). In general, K_m of 3.4 g/L revealed a good affinity of the enzyme toward soluble starch.

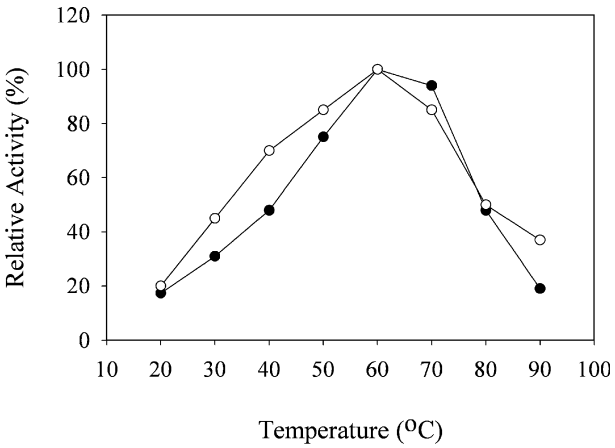

Fig. 3 Effect of temperature on CGTase activity: *filled circles*, purified enzyme; *open circles*, crude enzyme

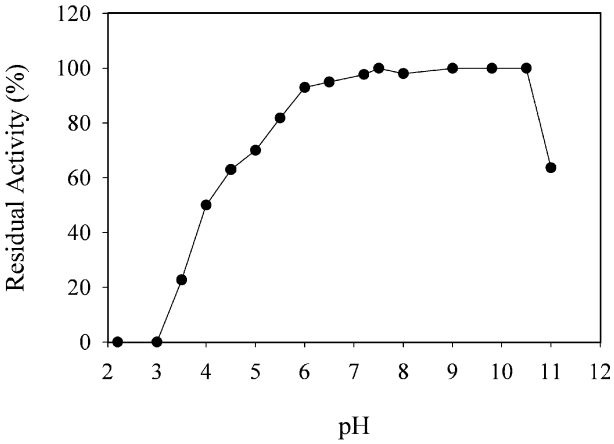

Fig. 4 Effect of pH on purified CGTase stability

Effect of pH and Temperature on CGTase Activity

The effect of pH on CGTase activity is presented in Fig. 2. For comparison, the data about the crude enzyme is included. The purified enzyme was active in a wide pH range: from 4.0 to 11.0 and displayed above 60% relative activity at pH 4.5–9.0. The optimum pH value was determined to be 7.2. Similar results were obtained for crude CGTase. Above 70% relative activity was observed in the pH range from 4.5 to 8.5. A slight difference was noticed in relation to the optimal pH value. For the crude enzyme, it was found to be pH 5.0, but high activity was registered also at pH 6.5 and 8.5. It should be noted that the high activity of CGTase from *B. megaterium* in a wide pH range is a positive feature of the enzyme, as it enables the performance of the CD production process without maintaining a constant pH.

The effect of temperature on CGTase activity is presented in Fig. 3. The optimal value was 60 °C for both enzyme preparations. Significant CGTase activity was detected at

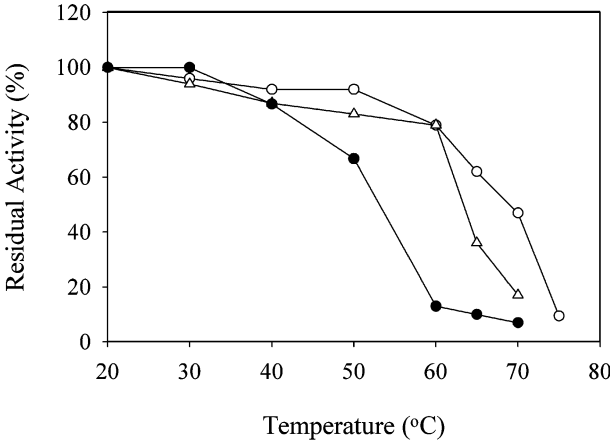

Fig. 5 Effect of temperature on CGTase stability: *filled circles*, purified enzyme, 0.5 h incubation time; *open circles*, crude enzyme, 2 h incubation time; *open triangles*, crude enzyme, 6 h incubation time

Table 3 Effect of reagents on CGTase activity.

Reagent	Residual activity (%)	Reagent	Residual activity (%)
None	100.0	$FeCl_3$	100.0
$SrCl_2$	116.7	$CaCl_2$	100.0
$MgCl_2$	115.9	ICH_2COOH	100.0
$CoCl_2$	114.8	NaN_3	100.0
$PbCl_2$	113.4	SDS	98.2
$MnCl_2$	112.7	Hg_2Cl_2	91.6
$CuSO_4$	111.0	EDTA	86.7
NaCl	104.9	$ZnSO_4$	83.5
KCl	101.4	$AgNO_3$	72.7

70 °C. Further increase in temperature led to a decrease in enzyme activity, probably because of thermal inactivation. However, the crude enzyme retained about 40% of its activity at 90 °C. This property could be used in the CD production process, as high temperatures brought certain advantages for the process.

In relation to the optimum pH and temperature values, CGTase from *B. megaterium* was similar to the enzymes produced by other species (Table 2).

Effect of pH and Temperature on CGTase Stability

CGTase was stable in a wide pH range (Fig. 4). The enzyme retained 100% of its activity, when incubated at pH 6.0–10.5. This is another positive feature of CGTase in relation to its application.

The thermal stability of CGTase is presented in Fig. 5. The purified enzyme was not characterized with high thermal stability. It was stable only up to 30 °C, when incubated for 30 min. In the temperature range of 40–50 °C, the residual activity remained above 50%, but the further increase in temperature led to a rapid decrease in enzyme activity. The crude CGTase showed higher resistance to thermal inactivation. Even when the incubation time was 6 h, the enzyme retained more than 80% of its initial activity up to 60 °C. The inactivation of CGTase at high temperatures should not be considered a disadvantage for CD production, as an increase in enzyme stability is expected in the presence of a substrate. A similar effect was reported for CGTase from *Bacillus firmus* NCIM 5119 [16]. Besides, the retaining of high enzyme activity for a long period of time in the case of crude CGTase is of benefit for the process.

Table 4 Molecular mass of CGTase.

Method	Molecular mass (Da)
SDS-PAGE	73,400
Gel chromatography	68,200
Mass spectrometry	75,000

Effect of Metal Ions and Reagents on CGTase Activity

The effect of various reagents on CGTase activity is presented in Table 3. A positive effect was observed in the presence of some metal ions, such as Sr^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, and Cu^{2+}. The increase in enzyme activity was not higher than 17%. In contrast to other CGTases [15, 34], the enzyme from *B. megaterium* was not affected by the presence of Ca^{2+}. CGTase activity was inhibited by $Zn^{2+}$and Ag^{+}. The unchanged enzyme activity in the presence of iodo acetic acid indicated the absence of thiol groups in the active site.

Molecular Mass and N-terminal Amino Acid Sequence of CGTase

CGTase molecular mass was determined by three methods (Table 4). The close values, obtained by the different methods (68,200–75,000 Da), indicated that CGTase is a monomer protein. This consideration was confirmed by the amino acid sequence analysis. The presence of only one N-terminal amino acid was established. The first 13 amino acids in the polypeptide chain were determined to be Ala, Pro, Asp, Thr, Ser, Val, Ser, Asn, Lys, Gln, Asn, Phe, and Ser.

Product Specificity

CGTase from *B. megaterium* was tested for the application in the CD production process. When soluble potato starch was used as a substrate, the total CD yield obtained after 20 h reaction time was about 49%. CGTase formed the three CD types, and the predominant product was β-CD. At the specified reaction conditions, the ratio of α/β/γ was 27:68:5.

It can be concluded that the biochemical properties of CGTase from *B. megaterium* enable the application of the enzyme for CD production.

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