Biochemical Engineering Journal 48 (2009) 124–131

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

A novel cyclodextrin glycosyltransferase from *Bacillus sphaericus* strain 41: Production, characterization and catalytic properties

Cristiane Moriwaki, Lívia Rosas Ferreira, Júlia Regina Tedesco Rodella, Graciette Matioli*

Pharmacy and Pharmacology Department, State University of Maringá, Av. Colombo, 5790, 87020-900 Maringá, PR, Brazil

ARTICLE INFO

Article history:
Received 28 April 2009
Received in revised form 16 August 2009
Accepted 3 September 2009

Keywords:
Bacillus sphaericus
Cyclodextrin glycosyltransferase
Enzyme
Microbial
Kinetics
Optimization

ABSTRACT

The alkalophilic *Bacillus sphaericus* strain 41 was isolated from soybean-soil culture, using a highly alkaline pH medium containing 1% Na_2CO_3. The cyclodextrin glycosyltransferase (CGTase) from this microorganism was purified up to 315-fold with a yield of 31%, by biospecific affinity-column chromatography using Sepharose 6B gel and β-cyclodextrin (β-CD) as the ligand. The molecular weight of the purified enzyme was estimated to be 59 kDa by SDS-PAGE. In addition to the cyclization, the CGTase showed disproportionation and coupling activities. For cyclization activity, the optimal pH was 6.0 and the temperature was 65 °C. The enzyme showed pH stability in the range of 6.0–7.0. Thermal deactivation was noticeable above 70 °C, and the enzyme was highly stable below 65 °C. The activation and deactivation energy for the production of β-CD were 9.4 kcal/mol and 28.0 kcal/mol, respectively. The influence of substrate concentration on the initial rate of CD production was studied, and the kinetic parameters were determined. The K_m was 0.0008 mol/L and V_{max} was 0.0631 mol of β-CD/(L h), using maltodextrin as substrate. The CGTase was strongly inhibited by the products, and produced a level of CDs reaching 22 g/L with a β-CD ratio of 54%. This enzyme produced α-, β- and γ-CD in the ratio of 0.40:1:0.45.

1. Introduction

Cyclodextrin glycosyltransferase (CGTase) is an important industrial enzyme, unique in its capacity to convert starch and related substrates into cyclodextrins (CDs) through cyclization, an intramolecular transglycosylation reaction. CGTase also catalyzes intermolecular transglycosylation reactions, such as coupling, in which the CD ring is cleaved and transferred to linear acceptors; and disproportionation, wherein two linear oligosaccharides are converted into linear oligosaccharides of different sizes. The CGTase possesses weak hydrolytic activity, in which water is the glycosyl acceptor [1].

CDs are cyclic maltooligosaccharides of six (α-CD), seven (β-CD), and eight (γ-CD) or more units of glucose joined by α-(1,4) bonds. The steric arrangement of glucose units in the CD molecule results in the shape of a hollow truncated cone with a hydrophilic external surface, which makes CDs water-soluble, and a hydrophobic internal cavity, which enables CDs to form inclusion complexes with various guest molecules [1,2]. Inclusion in CDs exerts a profound effect on the physicochemical properties of guest molecules, which explains their broad utilization in food, pharmaceuticals, cosmetics, and environmental protection, bioconversion and textile industries [3].

Another industrial CGTase application, in addition to the production of CDs by means of the cyclization reaction, is the stevioside glycosylation to reduce bitterness, improve sweetness and enhance water solubility [4]. CGTase can also be utilized in the preparation of baked products, in which the incorporation of the enzyme into the dough increases the volume and delays the process of bread staling during storage [5]. Yet, because of the inability of CGTase to bypass α-(1,6) bonds in gelatinized starches, degradation of these substrates leads to a reduction in viscosity without a corresponding decrease of the high-molecular character of starch. Thus, for surface sizing or coating of paper, the action of CGTase improves the writing quality of the paper, resulting in a glossy surface that is good for printing [1].

CGTase is generally an extracellular enzyme [6] found in bacteria, and was recently also discovered in archaea [7]. However, there is no published report about *Bacillus sphaericus* that produce CGTase. There are some published references about *B. sphaericus* that produce an intracellular cyclodextrinase (CDase), the enzyme that hydrolyzes CDs. Evidence has been presented that CDs are transported into the cytoplasm of microorganisms via a specific system, and that they are hydrolyzed inside the cell by intracellular CDase to form linear maltooligosaccharides for use as an energy source [8]. CDases of *B. sphaericus* E-244 and *B. sphaericus* ATCC 7055 were purified and characterized by Oguma et al. [9,10] and Galvin et al. [11], respectively.

Considering the innovative character of *B. sphaericus* CGTase, which has not been previously reported, and the diverse industrial

* Corresponding author. Tel.: +55 44 3261 4301; fax: +55 44 3261 4119.
E-mail addresses: gmatioli@uem.br, gramatioli@yahoo.com.br (G. Matioli).

1369-703X/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bej.2009.09.001

applications of the enzyme, the aim of the present study was to produce, purify, characterize and investigate the catalytic properties of CGTase from *B. sphaericus* strain 41, as well as to determine the optimum culture conditions for enhanced production of the enzyme.

2. Materials and methods

2.1. Materials

Cyclodextrins (α-, β- and γ-CD), phenolphthalein, bovine serum albumin, Coomassie blue dye, standard protein molecular-weight markers (SDS7B2) and Sepharose CL-6B were purchased from Sigma (St. Louis, MO, USA). Soluble starch from potato (article 101252), glucose and maltose were purchased from Merck (Darmstadt, Germany). Maltodextrin (Dextrin 10 from maize starch, article 31410) was obtained from Fluka (Buchs, Switzerland). Starches of rice, potato, cassava and corn (maize) were a kind gift from Claspar (Maringá, PR, Brazil). α-Amylase (Termamyl 120L) was purchased from Novo Nordisk (Denmark). All other chemicals used were of analytical grade.

2.2. Bacterial strain and culture conditions for CGTase production

Strain 41, identified by conventional classic taxonomy as *B. sphaericus*, was isolated from a sample of soil where soybeans were growing, in northern Paraná in southern Brazil, as described in our previous report [12]. The microorganism was suspended in sterile distilled water and cultured in solid medium, pH 10.3, with the following composition (% w/v): soluble starch 1.0, polypeptone 0.5, yeast extract 0.5, K_2HPO_4 0.1, $MgSO_4{\cdot}7H_2O$ 0.02, Congo red dye 0.01, Na_2CO_3 1.0 and agar 1.5. The culture plates were incubated at 37 °C for 48 h, and the colonies were then transferred to 500-mL Erlenmeyer flasks containing 250 mL of liquid culture medium with the same composition as the solid medium, except for the presence of agar, dye and the soluble starch at 2.0% (w/v). It was cultured at 37 °C for 48 h in an incubator under orbital shaking (120 rpm). In sequence, at a rate of 5% (v/v), 2000-mL Erlenmeyer flasks, containing 1000 mL of liquid culture medium were inoculated and the culture proceeded at 37 °C for 5 days, with stirring at 120 rpm. Then, the cells and the insoluble material were harvested from the culture medium by centrifugation (8800 × *g*, 15 min, 4 °C), and the cell-free supernatant was used as the source of the enzyme. A preliminary assay was carried out, and aliquots were collected periodically (0 h, 24 h, 48 h, 72 h, 96 h, 120 h and 144 h). Enzyme production increased gradually until the 5th day, and remained constant until the end of the assay.

2.3. Purification of CGTase

The proteins of the cell-free supernatant were precipitated by the addition of ammonium sulfate at 80% saturation. Next, vigorous centrifugation was conducted (8800 × *g*, 30 min, 4 °C) and the protein precipitate was dissolved in 10 mM Tris–HCl buffer, pH 8.0. The CGTase was purified by biospecific affinity-column chromatography using Sepharose CL-6B gel and β-CD as the ligand [13]. Three milliliters of fractions were collected, and each was assayed for CGTase activity and protein content. The fractions that showed CGTase activity were pooled and submitted to ultrafiltration in order to eliminate the β-CD used at elution. Protein content was determined by the method of Bradford [14], using bovine serum albumin as the standard.

2.4. Molecular weight

The molecular weight of the purified enzyme was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), according to Laemmli [15]. SDS-PAGE was performed on 12.5% polyacrylamide gel, using standard protein molecular-weight markers with molecular weights ranging from 26.6 kDa to 180.0 kDa. Protein bands were visualized by Coomassie blue dye.

2.5. CGTase activity assay

2.5.1. Cyclization

Cyclization activity (used as the standard enzyme assay) was measured as a function of the β-CD production rate, using maltodextrin as the substrate at 1% (w/v) in 50 mM Tris–HCl buffer and 5 mM $CaCl_2$, pH 8.0, at 50 °C. In six test tubes, 1 mL of substrate solution and 1 mL of enzyme solution were added and the tubes were incubated for 30 min at 50 °C. One test tube was removed every 5 min, and the CGTase was inactivated by heating the tube in boiling water for 10 min. The control was a reaction time of 0 min, and in this case the CGTase was first inactivated by boiling, and then the substrate solution was added. The β-CD concentration was determined by the colorimetric method, as will be described in Section 2.8.1, and the enzymatic activity was calculated from the slope of the β-CD concentration-versus-time curves, considering dilution, volume of enzyme and volume of reaction contained in the assay. One unit of activity (U) was defined as the amount of enzyme that produces 1 μmol of β-CD/min under the assay conditions.

2.5.2. Coupling

Coupling activity was measured according to the disappearance of β-CD in the presence of glucose. One milliliter of enzyme solution was added to six test tubes containing 1 mL of 1% (w/v) β-CD solution and 1% (w/v) glucose solution in 50 mM Tris–HCl buffer and 5 mM $CaCl_2$, pH 8.0, and these tubes were incubated for 30 min at 50 °C. One tube was removed every 5 min, and the CGTase was inactivated by heating the tube in boiling water for 10 min. For the control test tube, the procedure was the same as the cyclization activity. The amount of residual β-CD was determined by the colorimetric method, as will be described in Section 2.8.1. One unit of coupling activity was defined as the amount of enzyme that can convert 1 μmol of β-CD/min.

2.5.3. Disproportionation

Disproportionation activity was determined by the conversion of maltose into larger maltooligosaccharide oligomers. One milliliter of enzyme solution was added to a test tube containing 1 mL of 1% (w/v) maltose solution in 50 mM Tris–HCl buffer and 5 mM $CaCl_2$, pH 8.0, and this was incubated for 10 min at 50 °C. The maltose conversion was determined by HPLC. One unit of disproportionation activity was defined as the amount of enzyme that can convert 1 μmol of maltose/min.

2.6. Effects of the culture conditions on CGTase production

B. sphaericus strain 41 was cultured in solid medium and incubated at 37 °C for 48 h. After that period, the colonies were transferred to 250-mL Erlenmeyer flasks containing 100 mL of liquid culture medium, in order to obtain an absorbance of 1.0 at l = 660 nm. Five milliliters of this medium was transferred to 100 mL of each of seven liquid culture media, which were prepared containing soluble starch as a standard substrate, standard peptone (meat and casein) and several different peptone sources (meat, liver, tryptone, heart, lung and trachea). These were cultured at 37 °C for 5 days in an incubator under orbital shaking (120 rpm). Next, the liquid culture media were centrifuged (8800 × *g*, 15 min, 4 °C) and the cell-free supernatant was used to determine the enzyme activity. For estimation of cell growth, the pellets were appropriately diluted and used to determine the optical density (OD) at 660 nm.