Cyclodextrin Production from Amaranth Starch by Cyclodextrin Glycosyltransferase Produced by *Paenibacillus macerans* CCM 2012

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Abstract

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Cyclodextrins (CDs) are synthesised by bacterial extracellular enzym cyclodextrin glycosyltransferase (CGTase, E.C. 2.4.1.19) from starch or starch derivatives. The production of α-, β-, and γ-CDs by CGTase from *Paenibacillus macerans* CCM 2012 was studied in regard to the effect of the starch source (amaranth, maize) on the yield of CDs. CGTase was produced by a 3-day sterile cultivation in the laboratory Bench-top fermentor BiostatB under aerobic conditions. CGTase was partially purified by ammonium sulfate precipitation at 60% saturation. Electrophoretic analysis (SDS-PAGE) of the isolated CGTase enzyme was carried out according to the method by Laemmli (1970), the apparent molecular weight was in the range from 105 kDa to 114 kDa. All the commercially important α-, β-, and γ-CDs were detected chromatographically after the hydrolysis of the maize and amaranth (*Amaranthus cruentus*) starches with the isolated enzyme. The amaranth starch appears to be an excellent substrate for CDs production because of the high dispersibility, high starch-granule susceptibility to amylases, and the exceptionally high amylopectin content.

Keywords: *Amaranthus craentus*; CGTase enzyme; starch substrates; transglycosylation reactions

The first reference to cyclodextrins (CDs) was made in a publication of Villiers (1891). CDs are cyclic non-reducing oligosaccharides composed of (α-1.4)-linked α-D-glucopyranose units with a relatively hydrophobic central cavity and a hydrophilic outer surface.

As a result of their molecular structure and shape, they possess a unique ability to act as molecular containers by entrapping the guest molecules in their internal cavity. CDs are typical host molecules and may trap a great variety of molecules having the size of one or two benzene rings, or even larger ones carrying a side chain of comparable size, to form crystalline inclusion complexes (Szejtli 1982). The formation of the inclusion complexes modifies the physical and chemical properties of the host molecule, mostly in terms of water solubility. The most abundant natural CDs are α-cyclodextrin (α-CD), β-cyclodextrin (β-CD), and γ-cyclodextrine (γ-CD), contain-

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ing 6, 7, and 8 glucopyranose units, respectively. In recent years, CDs have been recognised as important ingredients for food, cosmetic, and pharmaceutical industries.

Cyclodextrin glycosyltransferases (CGTase, E.C. 2.4.1.19), enzymes coming from various bacteria, mostly *Bacillus* sp., catalyse the CDs formation from starch or starch derivatives via an inter- and intramolecural transglycosylation reactions. CGTases generally produce a mixture of CDs, glucose, maltose, and other oligosaccharides with different polymerisation degrees (Qi & Zimmermann 2005). Although α-, β-, and γ-CDs are the major products of CGTase, it has been known that trace amounts of larger cyclic glucans (δ-, ε-, ζ-, η-, and θ-CD) are also present in the reaction mixture (French *et al*. 1965). The bacterial strain *Bacillus macerans* is the most frequently used source of the CGTase enzyme.

Various types of starch can be used as the substrates for CGTase including corn and potato starches (Kim *et al*. 1997). Starch consists of amylopectin and amylose. Amylose is an essentially linear molecule in which the D-glucose units are linked by α-(1.4) glycosidic links. Amylopectin contains α-(1.4)-linked glucose and α-(1.6) linkages, resulting in a branched structure. Amylose and amylopectin molecules are organised into quasicrystalline macromolecular aggregates called starch granules. The size, shape, and structure of these granules vary substantially among botanical sources. The proportions of amylose and amylopectin in starches also vary with their source, but generally are in the range of 20% to 30% of amylose in normal cereal starch. Both can serve as materials for CDs formation, but amylopectin gives higher yields than amylose, because the reaction with CGTase begins at the non-reducting end of the starch molecule.

Amaranth starch is an interesting alternative substrate for CDs production. Amaranth constitutes an important part of the diet in areas of Latin America, Africa, and Asia. Amaranth starches are of the waxy, or glutinous, type and consist of spherical, angular, or polygonal granules with an exceptionally small size ranging from 1 μ to 3 μ in diameter, which helps to better dispersion (Okuno & Sakaguchi 1981; Stone & Lorenz 1984). Amylose content in the amaranth starches is exceptionally low, in the range 0–14% (Becker *et al*. 1981; Okuno & Sakaguchi 1981; Sugimoto *et al*. 1981). The amaranth starch granules have high susceptibility to amylases and unique functional properties (Goering 1967).

The aim of the present work is to investigate the production of α-, β- and γ-CDs by CGTase from *Paenibacillus macerans*, in regard to the effect of the starch source (amaranth, corn) on the yield of CDs.

MATERIAL AND METHODS

Microorganism and inoculum preparation. The strain of *Paenibacillus macerans* CCM 2012 (formerly *Bacillus macerans*) (Ash *et al*. 1993), purchased from the Czech Collection of Microorganism in the freeze-dried form, was used for the CGTase preparation. The strain was cultivated on Petri dishes with agar medium containing: M1383 Bacillus medium 6 g/l, M091 plate count agar (both HiMedia Laboratories Pvt. Ltd., Mumbai, India) 23.5 g/l, soluble starch sec. Leulier (Lach-Ner, s.r.o., Neratovice, Czech Republic) 2 g/l, distilled water 1 litre.

After adjusting the final pH to 7, the medium was sterilised by autoclaving at 121°C for 15 minutes. The Petri dishes were incubated at 30°C for 72 hours.

CGTase production. The inoculum was transferred from the Petri dishes to the laboratory Bench-top fermentor BiostatB (B.Braun Biotech Internatioanal GmbH, Melsungen, Germany). The composition of the fermentation medium was as follows: RM 004b malt extract powder (HiMedia Laboratories Pvt. Ltd., Mumbai, India) 30 g/l, soluble starch sec. Leulier (Lach-Ner s.r.o., Neratovice, Czech Republic) 5 g/l, calcium carbonate: 2.5 g/l, ammonium sulphate 2.5 g/l, M1383 Bacillus medium 1 g/l, M 255 Malt Extract Broth (both HiMedia Laboratories Pvt. Ltd., Mumbai, India): 0.5 g/l, calcium chloride 0.25 g/l medium was sterilised by autoclaving at 121°C for 15 minutes. The suitable conditions for the bacteria growth were as follows: initial pH 6.5, temperature 32°C, initial medium volume 2 l, inoculum volume 1% (v/v), air flow 6 l/min, stirrer system speed 500 rpm. An automatic feed control program was created to control the fermentation conditions. A starch containing solution (1%, w/v, soluble starch sec. Leulier; 6%, w/v, malt extract powder RM 004b) feed-pump turned on when pH value increased above 7 and the pump turned off above pH value of 7.5. Each time when pH decreased to 6, an

alkali ammonium sulphate containing solution (10%, w/v, ammonium sulphate; 1%, w/v, M1383 Bacillus medium – pH adjusted to 9.0 by NaOH) feed pump turned on until pH value reached 6.5. High-level pH indicated a reduced metabolism due to carbon exhaustion, whereas low pH value was caused by depletion of the nitrogen sources.

After 3 day's fermentation, the medium was centrifuged at 23 700× g, 5°C, for 15 minutes. The complete supernatant containing the crude enzyme was collected. The CGTase was separated from the supernatant by precipitation with ammonium sulphate at 60% saturation. The precipitate was collected by centrifugation at 2600× g, 5°C, for 15 min, repeatedly washed and dissolved in 100 ml of 0.1M citrate buffer, pH 5.6. The solution contained 9.52% w/v of crude protein, as determined by the Kjeldahl method.

Cyclodextrin production. The native amaranth starch isolated from *Amaranthus cruentus* grain (AMR Amaranth, Blansko, Czech Republic), containing mainly amylopectin and from 3.9 to 5.7% amylose (Hoover *et al*. 1998); corn starch (Sigma-Aldrich, Steinheim, Germany; S4126 unmodified regular corn starch) containing approximately 73% amylopectin and 27% amylose were used as substrates for the cyclodextrin production. The Megazyme total starch assay procedure (Noack, Prague, Czech Republic) was used to determine the purity of the native amaranth starch. The sample contained 85.1% of the total starch. Both types of starch were suspended in 50 ml 0.02M citrate buffer to reach the total starch concentration 70 g/l. The suspensions obtained were sterilised by autoclaving at 121°C for 15 minutes. The sterilised starch solutions were cooled to 49°C and 1 ml of the CGTase solution, prepared as described above, was added to each of them. The mixtures were incubated with 0.05 ml toluene at 49°C for 24 h in 100-ml Erlenmeyer flasks.

Gel permeation chromatography analysis of the isolated CGTase enzyme. GP-HPLC separations were conducted using Shimadzu apparatus equipped with a binary pump (LC-10AD), a degasser (DGU-14A), a photodiode array Shimadzu UV-VIS detector (SPD-M6A), and a column oven (CTO-10A) with a manual injector 7125 containing a 20 µl sample loop. Superdex 75 10/300 GL (300 × 10 mm) column (Amersham Biosciences AB, Little Chalfont, UK) was used. Isocratic elution was performed at 30°C with 0.1M Na_2HPO_4 as eluent-flow rate (0.5 ml/min).

Electrophoretic analysis of the isolated CGTase enzyme. Polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulphate (SDS-PAGE) was carried out according to the method by Laemmli (1970) using SigmaMarker™ wide range, 6500–200 000 Da, standard mixture to estimate the apparent molecular weight of the CGTase enzyme. The experiments were carried out in reductive (5% v/v mercaptoethanol) and non-reductive conditions in parallel.

Determination of α-CD, β-CD, γ-CD. The concentrations of α-, β-, and γ-CDs formed in the reaction mixture were determined by high performance liquid chromatography (HPLC) system Ecom (České Meziříči, Czech Republic) according to the method described by Rashid *et al*. (2002). The reaction mixture was filtered through a membrane (Microfiltre-nylon) with the pore size of 0.45 µm. The filtered sample (injection volume 40 µl) was separated on TSK gel Amide-80 (4.6 mm i.d. × 25.0 cm/l; Tosoh, Tokyo, Japan) column and eluted with acetonitrile–water (60:40 v/v) at 1 ml/minute. The flow cell was set at 30°C, and the products were detected with a refractive index detector. The method of standard addition was used to determine the concentrations of the individual CDs. Pure α-, β-, and γ-CD standards were purchased from Sigma-Aldrich (Steinheim, Germany).

RESULTS AND DISCUSSION

SDS-PAGE gel electrophoresis showed the presence of a protein with an apparent molecular weight ca 105 kDa (10% gel) or 114 kDa (12% gel) accompanied by several minor proteins with significantly lower molecular weights (Figure 1). This protein, supposed to be CGTase, was a markedly predominant component of the precipitate obtained from the fermentation medium by ammonium sulphate precipitation, as confirmed also by gel permeation chromatography (result not shown). The apparent molecular weight of the CGTase determined as described above is in contradiction with the published results. There is a good agreement in the results of molecular mass determination of the native form of the extracellular CGTase from *Paenibacillus macerans,* which is approximately 74 kDa (Fortagy 1983; Steighardt & Kleine 1993; Kim *et al.* 2005). The *Paenibacillus macerans* CGTase in the fermentation media was usually present in the form of one single polypeptide chain but the dimeric form of the enzyme was also

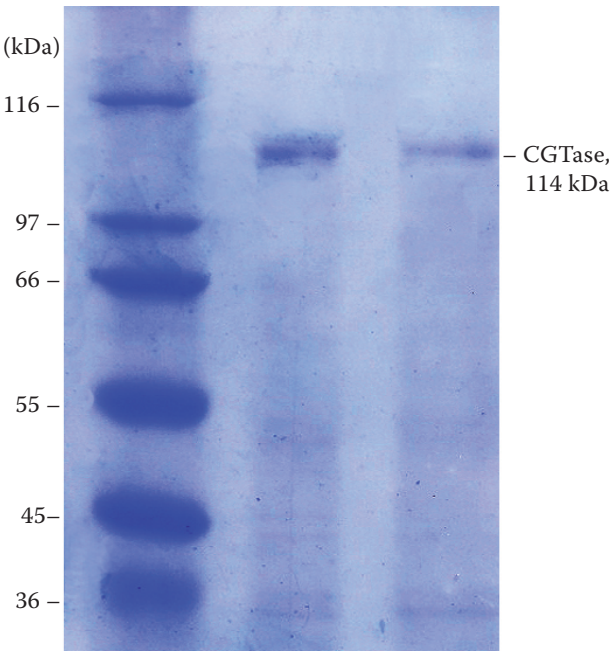
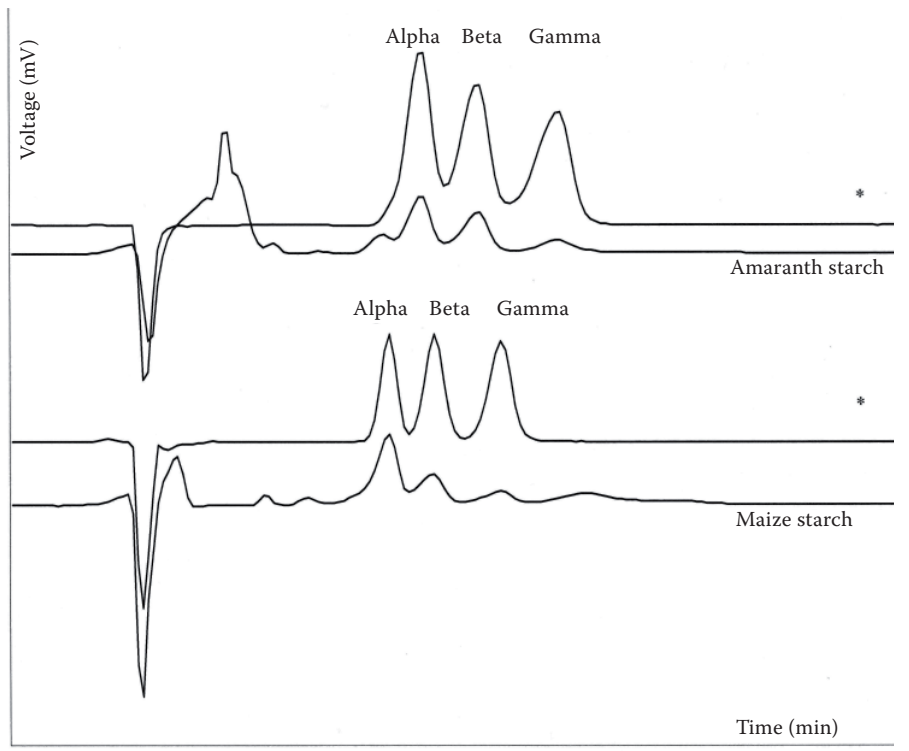


Figure 1. 12% SDS-PAGE of the standard mixture SigmaMarker (TM) wide range (line1) and the isolated extracellular proteins containing CGTase enzyme in reductive (line 2) and non-reductive conditions (line 3)

described (Takano *et al.* 1986). The *cgt* gene encoding α-cyclodextrin glycosyltransferase (α-CGTase) from *Paenibacillus macerans* strain JFB05-01 was expressed even in *Escherichia coli*. The recombinant enzyme was then purified and characterised (Kim *et al.* 2005; Li *et al.* 2009a). Therefore, the CGTase isolated in our study was probably in a form of aggregates or complexes with an unidentified component with well controlled size and stable even under the reductive conditions.

Since all known wild-type CGTases produce a mixture of α-, β-, and γ-CDs, they have been further classified into α-, β-, and γ-CGTases according to their major cyclodextrin products during the initial phase of the reaction. The α-CGTase from *Paenibacillus macerans* is most commonly used for the commercial production of α-cyclodextrin. Although it produces mainly α-CD during the initial stage of starch conversion and is one of the few α-CGTases identified, the proportion of α-CD in the total cyclodextrin products usually decereses after prolonged incubation (Li *et al.* 2009b). The CGTase from *B. macerans* IFO 3490 produced α-, β-, and γ-CDs in the ratio of 2.7:1:1 in the first 10 min of the enzyme reaction. The ratio of the CDs was almost constant regardless of the pH range (4.0–8.5) of the reaction system (Tonkova 1998). The changes of amino acid residues at certain positions in the catalytic domain of CGTases to different amino acids have also been shown to affect the CDs products ratio (Nakamura *et al.* 1994; Rimphanitchayakit *et al.* 2005; Li *et al.* 2009b).

The results of our chromatographic determination of α-, β-, and γ-CDs are shown in Figure 2. The



*mixture of standard samples of the individual CDs

Figure 2. Normal phase chromatograms of the hydrolysates of the amaranth and corn (maize) starches containing the individual CDs

Table 1. Comparison of CDs yields from the amaranth and corn starches (70 g/l)

	Amarath starch		Corn starch	
	(g/l ± SD)	yield (%)	(g/l ± SD)	yield (%)
α- CD	6.46 ± 0.071	9.23	5.88 ± 0.094	8.40
β- CD	6.13 ± 0.092	8.76	2.84 ± 0,059	4.06
γ- CD	2.98 ± 0,084	4.25	1.47 ± 0.087	2.10
∑ CD	15.57	22.24	10.19	14.56

comparison of the CDs yields from the amaranth and corn starches is shown in Table 1. Total CDs yield is significantly higher in the case of the amaranth starch (22.24%) in comparison with that of corn (14.56%). The reasons for this difference can be the higher amylopectin content, higher dispersibility, and higher starch-granule susceptibility to amylases of the amaranth starch (Tomita *et al*. 1981). There are also significant differences in the distribution of the individual CDs. Higher relative proportions of β-CD and γ-CD were observed using the amaranth starch. The altered product profile is a result of different compositions and characteristics of the starch substrates. Further study should be done to confirm these surprising results and to explain the causes of this change.

On the chromatogram of the corn starch hydrolysate shown in Figure 2, a small peak of δ-CD is probably also noticeable. No similar peak appears on the chromatogram of the amaranth starch. However, the identity of the peak was not confirmed because δ-CD standards are not available commercially. Further characterisation of this product was outside the frame of our study.

CONCLUSION

The amaranth starch appears to be an excellent substrate for CDs production because of the high dispersibility, high starch-granule susceptibility to amylases, and exceptionally high amylopectin content. We will continue with further purification and characterisation of the purified CGTase enzyme.

References

Ash C., Priest F.G., Collins M.D. (1993): Molecular identification of rRNA group 3 bacilli using a PCR probe test. Antonie van Leeuwenhoek, **64**: 253–260.

Becker R., Wheeler E.L., Lorenz K., Stafford A.E., Grosjean O.K., Betschart A.A., Saunders R.M. (1981): A compositional study of amaranth grain. Journal of Food Science**, 46**: 1175–1180.

Fortagy W.M. (1983): Microbial amylases. In: Fortagy W.M. (ed.): Microbial Enzymes and Biotechnology. Applied Science Publishers, Essex: 1–92.

French D., Pulley A.O., Effenberger J.A., Rougvie M.A., Abdullah M. (1965): Studies on the Schardinger dextrins. XII. The molecular size and structure of the delta-, epsilon-, zeta-, and eta-dextrins. Archives of Biochemistry and Biophysics, **111**: 153–160.

Goering K.J. (1967): New starches. II. The properties of the starch chunks from *Amaranthus retroflexus*. Cereal Chemistry, **44**: 245–252.

Hoover R., Sinnott A.W., Perera C. (1998): Physicochemical characterization of starches from amaranthus cruentus grains. Starch, **50**: 456–463.

Kim T.J., Kim B.C., Lee H.S. (1997): Production of cyclodextrin using raw corn starch without a pretreatment. Enzyme and Microbial Technology, **20**: 506–509.

Kim S.-G., Kweon D.-H., Lee D.-H., Park Y.-Ch., Seo J.-H. (2005): Coexpression of folding accessory proteins for production of active cyclodextrin glycosyltransferase of *Bacillus macerans* in recombinant *Escherichia coli*. Protein Expression and Purification, **44**: 426–432.

Laemmli U.K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, **227**: 680–685.

Li Z., Li B., Gu Z., Du G., Wu J., Chen J. (2009a): Extracellular expression and biochemical characterization of α-cyclodextrin glysosyltransferase from *Paenibacillus macerans*. Carbohydrate Research, **345**: 886–892.

Li Z., Zhang J., Wang M., Gu Z., Du G., Li J., Chen J. (2009b): Mutations at subsite –3 in cyclodextrin glycosyltransferase from *Paenibacillus macerans* enhancing α-cyclodextrin specificity. Applied Microbiology and Biotechnology, **83**: 483–490.

Nakamura A., Haga K., Yamana K. (1994): Four aromatic residues in the active center of cyclodextrin glucanotransferase from alkalophilic *Bacillus* sp. 1011: Effects of replacements on substrate binding and cyclization characteristics. Biochemistry, **33**: 9929–9936.

Okuno K., Sakaguchi S. (1981): Glutinous and non-glutinous starches in perisperm of grain amaranths. Cereal Chemistry, **63**: 273–276.

Qi Q., Zimmermann W. (2005): Cyclodextrin glucanotransferase: from gene to applications. Applied Microbiology and Biotechnology, **66**: 475–485.

Rashid N., Cornista J., Ezaki S., Fukui T., Atomi H., Imanaka T. (2002): Characterization of an archaeal

cyclodextrin glucanotransferase with a novel C-terminal domain. Journal of Bacteriology, **184**: 777–784.

Rimphanitchayakit V., Tonozuka T., Sekano Y. (2005): Construction of chimeric cyclodextrin glucanotransferases from *Bacillus circulans* A11 and *Paenibacillus macerans* IAM1243 and analysis of their product specificity. Carbohydrate Research, **340**: 2279–2289.

Steighardt J., Kleine R. (1993): Production and immobilization of a proteinase-reduced CGTase. Applied Microbiology and Biotechnology, **39**: 63–69.

Stone L.A., Lorenz K. (1984): The starch of Amaranthus – Physicochemical properties and functional characteristics. Starch, **36**: 232.

Sugimoto Y., Yamada K., Sakamoto S. (1981): Some properties of normal and waxy-type starches of *Amaranthus hypochondriacus* L. Starch, **33**: 112.

Szejtli J. (1982): Cyclodextrins and their Inclusion Complexes. Akademiai Kiado, Budapest.

Takano T., Fukuda M., Monma M., Kobayashi S., Kainuma K., Yamane K. (1986): Molecular cloning, DNA nucleotide sequencing and expression in *Bacillus subtilis* cells of the *Bacillus macerans* cyclodextrin-glucanotransferase gene. Journal of Bacteriology, **166**: 1118–1122.

Tomita Y., Sugimoto Y., Sakomoto S., Fuwa H. (1981): Some properties of starches of grain amaranths and several millets. Journal of Nutritional Science and Vitaminology, **27**: 471.

Tonkova A. (1998): Bacterial cyclodextrin glucanotransferase. Enzyme and Microbial Technology, **22**: 678–686.

Villiers A. (1891): Sur la fermentation de la fecule par l'action du ferment butyrique. Comptes rendus, **112**: 536.

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