Screening for *Corynebacterium glutamicum* Amylomaltase Mutant to Altered Large-ring Cyclodextrin Production Profiles

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Abstract

Amylomaltase produces large-ring cyclodextrin (LR-CD), a cyclic α-1,4-glucan with high degree of polymerization (DP ≥ 16). LR-CD can enhance solubility, improve stability and assist protein refolding. LR-CD can form complexes with various guest molecules e.g. flavonoids and drugs which can be used in pharmaceutical, food and cosmetic industries. The aim of this research is to screen the mutated amylomaltase that show different LR-CD production profiles. Random mutagenesis by Error-prone PCR has been used to create various mutants of amylomaltase from *Corynebacterium glutamicum*. Initially, mutants were subjected to screen using glucose oxidase assay. The mutated enzymes were purified by DEAE FF^{TM} and the partial purified enzymes were subjected to LR-CDs synthesis. The LR-CD product profile was investigated by High-Performance Anion-Exchange Chromatography (HPAEC). 1E5 mutated enzyme showed the same product profile but produced higher amount of principal LR-CD (CD27 to CD29). H5 mutated enzyme exhibited two corresponding peaks of LR-CDs which were CD30-CD32 and CD34-CD36. These results indicated that 1E5 carried a mutated residue involved in an amount of LR-CD production while H5 contained a mutated residue involved in LR-CD product size. These two mutated enzymes will be further characterized.

Keywords: amylomaltase, large-ring cyclodextrin (LR-CD), *Corynebacterium glutamicum*

Introduction

Large-ring cyclodextrin (LR-CD), a cyclic structure of glucose oligomer linked via α-1,4 glycosidic bonds consisting of glucose more than 9 units (Endo et al. 2007). LR-CDs contain hydrophilic outside, thus dissolves in water, with non-polar cavity inside which provides a hydrophobic. Thus, they enable to form inclusion complexes with appropriate guest molecules resulting in various properties (Endo et al. 2002; Hashimoto et al. 2002; Kuriki et al. 2006). Amylomaltase (AM, E.C. 2.4.1.25), an intracellular 4-α-glucanotransferase (4αGTase), is a member of α-amylase family which has an ability to catalyze LR-CD formation through intramolecular and intermolecular transglycosylation interaction (Ven der Maarel et al. 2002). The enzyme has four different activities: disproportionation, cyclization, hydrolysis and coupling. (Takaha et al. 1999; Tomono et al. 2002). Previously, amylomaltase gene from *Corynebacterium glutamicum* (*Cg*AM) was cloned and expressed in *Escherichia coli* and recombinant *Cg*AM was purified and characterized (Srisimarat et al. 2011). In this research, random mutagenesis was carried out on *Cg*AM gene in order to search for mutated *Cg*AM with altered LR-

CD production ability. This will lead to understanding and improvement of amylomaltase for LR-CD production.

Methodology

Random mutagenesis of CgAM

Random mutagenesis was introduced into *C. glutamicum* amylomaltase gene by Mn^{2+} doping PCR mediated-random mutation (Lin-Georke at al. 1997). The random mutagenic PCRs were performed in a total volume of 50 ml along with 0.25 mM of each primer, 0.2 mM (dATP/dGTP), 1 mM (dCTP/dTTP), 0.05, 0.1 or 0.25 mM $MnCl_2$ (in three separately reactions), 1.5 mM $MgCl_2$, 1x *Taq* DNA polymerase buffer and 2.5 U *Taq* DNA polymerase. Thermal cycles were an initial 95°C for 3 min followed by 25 cycles of 95°C for 1 min, 55°C for 30 s and 72°C for 2 min. All mutated clones were transformed and expressed in *E.coli* BL21(DE3) using pET-17b expression vector.

Crude enzyme preparation for screening of mutated CgAM

The mutant *E.coli* BL21(DE3) harboring mutated amylomaltase gene were cultured in LB medium at 37°C with 250 rpm shaking. At OD_{600} = 0.4, isopropylthio-β-D-galactoside (IPTG) was added to final concentration of 0.4 mM and cells were harvested at 2 hours after IPTG induction. Crude enzymes were obtained after cell sonication.

Disproportionation activity assay

Disproportionation activity was determined by glucose oxidase assay, measuring the amount of free glucose in the reaction (Berham et al. 1972). Crude enzyme was incubated with 5% maltotriose at 30°C for 10 min and stopped by adding 1N HCl. After that, glucose oxidase reagent was added and incubated for 10 minutes at room temperature. The reaction mixture was measured at A_{505}. The mutated clones that contained residual disproportionation activity at least 80% of the wild-type enzyme's, were selected for further experiment.

Preparation and partial purification of CgAM

The selected *Cg*AM mutants were cultured in 500 ml of LB medium for enzyme production. Crude enzyme was obtained after cell sonication and was then purified by DEAE FF^{TM} column chromatography. Column was equilibrated with 50 mM phosphate buffer pH 7.4. Crude enzyme was then loaded onto the column. Step-wise elution was performed by adding elution buffer (50 mM phosphate buffer pH 7.4 with NaCl in range of 0.1M-1.0 M) to elute bound amylomaltase. Eluted protein fractions carrying starch transglycosylation activity were pooled and dialyzed against 50 mM phosphate buffer pH 7.4.

Starch transglycosylation activity assay

Starch transglycosylation activity was determined by iodine method measuring the starch decrease (Park et al. 2007). Partial purified enzyme was incubated with 0.2% (W/V) soluble starch and 1% maltose in 50 mM phosphate buffer pH 6.0 at 30°C for 10 min and stopped by boiling. Iodine solution (0.02% iodine in 0.2% potassium iodide) was added and A_{600} was measured. One unit was defined through the amount of enzyme that produced 1% reduction in blue color of starch-iodine complex per min.

Starch degradation activity assay

Starch degradation activity was determined by iodine method, measuring the reduction of starch (Park et al. 2007). Partial purified enzyme was incubated with 0.75% (W/V) soluble starch in 50 mM phosphate buffer pH 6.0 at 30°C for 10 min and stopped by adding 1N HCl. Iodine solution (0.005% iodine in 0.05% potassium iodide) was added and A_{660} was measured. One unit was the amount of enzyme required to degrade 1 mg/ml of soluble starch in 10 min.

LR-CDs synthesis

Partial purified enzyme of 0.05 U starch degradation activity was incubated with 2% (w/v) pea starch in 50 mM phosphate buffer pH 6.0 at 30°C for 6 hours under 150 rpm shaking. The reaction was stopped by boiling. Subsequently, 4 U of glucoamylase was added to assure that residual linear oligosaccharides were completely digested.

Analysis of LR-CDs production profile by High Performance Anion Exchange Chromatography (HPAEC)

LR-CDs reaction mixture was analyzed by HPAEC. A sample was loaded onto the Carbopac PA-100 column. Column was pre-equilibrated with and eluted with a linear gradient of sodium nitrate (0-2 min, increasing from 4% to 8%; 2-10 min, increasing from 8% to 18%; 10-20 min, increasing from 18% to 28%; 20-40 min, increasing from 28% to 35%; 40-55 min, increasing from 35% to 45%; 55-60 min, increasing from 45% to 63%) in 150 mM NaOH with a flow rate of 1 ml/min. The size of LR-CD product was compared with 0.8 mg/ml mixed standard cycloamylose (EZAKI glico).

Results and Discussions

Screening for mutated *Cg*AM using glucose oxidase assay

Random mutagenesis is a method by which random mutation points can be introduced on amylomaltase gene. These mutations may cause amylomaltase enzyme exhibiting distinct activity. Initially, 100 mutated clones were screened for disproportionation activity. Glucose oxidase assay was carried out. This assay is an enzymatic oxidation to determine glucose content from the reaction

$$\text{glucose} + O_2 + H_2O \rightarrow \text{gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} \rightarrow \text{quinoneimine} + 4H_2O$$

A mutant that displayed disproportionation activity at least 80% of wild-type (WT) enzyme activity was selected. In this work, there were 10 clones exhibited disproportionation activity at acceptable level (Table 1).

Table 1: Summary of mutated amylomaltase clones were screened using glucose oxidase assay

	Range of amylomaltase activity from mutants compared to wild type		
	Less than 80	80-100	More than 100
Number of clones	90	7	3

Preparation and Partial purification of mutated *Cg*AM

Starch transglycosylation activity and protein determination, the specific activity of crude WT, 1E5 and H5 *Cg*AM obtained were 2.286, 1.78, and 1.56 U/mg. Crude WT, 1E5 and H5 enzyme were purified by DEAE column chromatography to obtain partial purified amylomaltases. H5 and 1E5 enzymes were confirmed as a protein band at 84 kDa by SDS-PAGE which showed the same size as WT enzyme. The specific activity of partial purified WT, 1E5 and H5 were 15.11, 7.58 and 8.14 U/mg with the yield at 30.78, 22.33 and 19.98% respectively (Table 2).

Table 2: Purification table of WT, 1E5 and H5 *Cg*AM

Purification step	Total protein (mg)	Total activity* (U)	Specific activity* (U/mg)	Purification fold	Yield (%)
WT Crude extract	255	583.1	2.286	1	100
WT DEAE FF^{TM}	11.9	179.5	15.11	6.6	30.8
1E5 Crude extract	268	477	1.78	1	100
1E5 DEAE FF^{TM}	14.1	106.5	7.58	4.3	22.3
H5 Crude extract	344.8	537.9	1.56	1	100
H5 DEAE FF^{TM}	13.2	107.5	8.14	5.2	19.9

**activity assay was performed by starch transglycosylation assay*

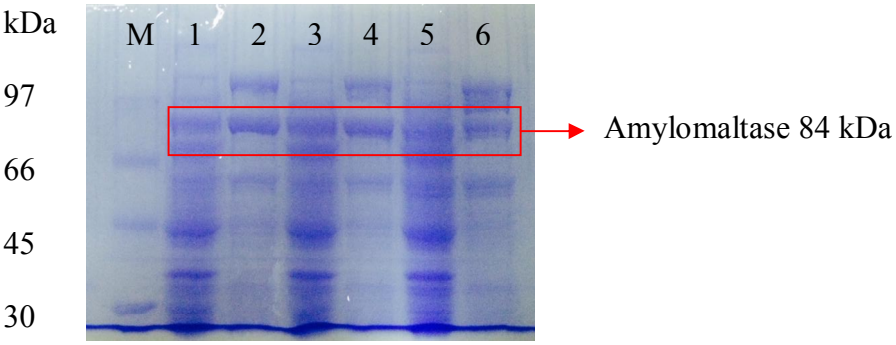

Figure 1: SDS-PAGE analysis of partial purified enzyme**.** Lane M is protein marker; Lane 1 is crude WT enzyme; Lane 2 is a partial purified WT enzyme; Lane 3 is crude 1E5 enzyme; Lane 4 is a partial purified 1E5 enzyme; Lane 5 is a crude H5 enzyme; Lane 6 is a partial purified H5 enzyme

HPAEC analysis of LR-CDs production profile

LR-CDs were produced by WT, 1E5 and H5 amylomaltase enzymes as described in experimental. LR-CDs were then analyzed by HPAEC. CD27 (cyclodextrin with 27 glucose monomers) to CD29 were principal LR-CD products of WT *Cg*AM. 1E5 enzyme showed the same LR-CDs profile as WT enzyme but produced higher amount of CD26 to CD35 while H5 enzyme produced principal CDs at CD30 to CD32 and CD34 to CD36 (Figure 2). This indicated that 1E5 and H5 enzymes carried mutated amino acid residues which are required for LR-CD formation. 1E5 *Cg*AM produced higher amount of principal LR-CD products than WT . This may due to 1E5 mutant has higher starch hydrolysis activity or lower coupling activity than WT *Cg*AM. Starch hydrolysis activity involves in starch digestion while coupling activity relates to LR-CDs hydrolysis.LR-CDs production profile of H5 mutant was different from that of WT enzyme. It is possible that H5 mutant carried mutated amino acid residues at substrate binding site.

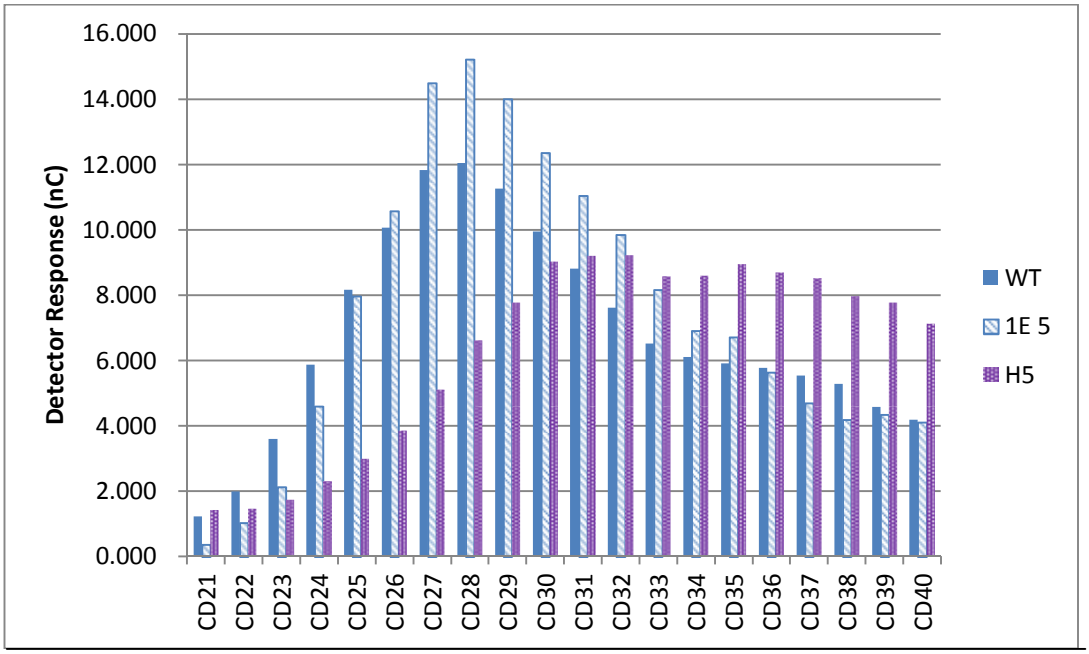

Figure 2: LR-CDs production profile of WT, 1E5 and H5 amylomaltases, analyzed by HPAEC. LR-CDs synthesis was performed using 0.05U starch degradation activity of *Cg*AM and 2% (w/v) pea starch in 50 mM phosphate buffer pH 6.0 at 30°C for 6 hours under 150 rpm shaking. Each CD number represented glucose monomers. Amount of CDs were determined by an electrocatalytic oxidation (nC) by HPAEC detector.

Conclusions

1E5 and H5 mutated *Cg*AM were screened by disproportionation activity and investigated for LR-CD production. H5 enzyme produced principal LR-CD product at CD30 to CD32 and CD34 to CD36 while 1E5 produced the same principal LR-CD products at CD27 to CD29 but at a higher amount of LR-CDs. These two mutated enzyme might carry a mutated residue involved in LR-CD formation. Thus, 1E5 and H5 *Cg*AM will be further characterized.

Acknowledgements

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