

Expression and Characterization of Amylomaltase Gene Involved in the Large-Ring Cyclodextrin and Isomalto-Oligosaccharide Production

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Abstract

Amylomaltase catalyzes a cyclization reaction to produce large-ring cyclodextrins (LR-CDs) from starch, which can be used in pharmaceutical industry. In this study, the amylomaltase gene was sub-cloned from p17bAMY recombinant plasmid into a pET-19b vector containing an NH₂-terminal His-tag. The maximum expression was obtained when the recombinant cells were cultured at 37°C for 4 h with 0.8 mM IPTG. The amylomaltase was purified up to 47-fold by Ni-NTA column and the specific activity of enzyme was 122 units/mg protein with a 44% yield. The relative molecular mass of purified enzyme was 60 kDa by 10% SDS-PAGE. In addition, the enzyme prefers to use pea starch as a substrate in the production of LR-CDs with degrees of polymerization from 23-37 glucose units. Besides LR-CD production, amylomaltase when used in co-action with transglucosidase could also produce long chain isomalto-oligosaccharides (IMOs) from pea starch for serving as prebiotic substance.

Keywords: Amylomaltase; Cyclization; Disproportionation; Large-ring cyclodextrins (LR-CDs); Isomalto-oligosaccharide (IMO)

1. Introduction

Amylomaltase (EC 2.4.1.25) is a member of the 4- α -glucanotransferase (4 α GTase) group within the α -amylase family [1-3]. This enzyme is unique in the ability to convert starch to LR-CDs with a degree of polymerization (DP) of 16 glucose units upwards through the cyclization reaction. The enzyme can also catalyze

another three types of activities including disproportionation, coupling and hydrolysis reactions. Most amylomaltases have relatively high disproportionation activity whereby the glycosyl group is transferred from one α -1,4-glucan molecule (donor) to another molecule (acceptor). The other two activities are of minor to amylomaltase. The coupling and hydrolysis catalyze reaction by which LR-

CDs are cleaved, and the obtained linear glucan is transferred to an acceptor or water molecule, respectively [3]. Presently, amylomaltase has received interest due to beneficial use in producing several valuable products; functional oligosaccharides (prebiotics), a thermoreversible starch product with gelatin-like properties, and LR-CDs [4-5].

LR-CDs are the oligomers of anhydroglucose units, which could link to form a single helical V-amylose structure with α -1,4-glycosidic bonds. LR-CD molecules with an outside hydrophilic surface and an inside hydrophobic central cavity can form inclusion complexes with hydrophobic guest molecules. The interaction between the guest and LR-CD molecule leads to an increase of the solubility of the guest compound. Consequently, an elevation of stabilization toward the effects of light, heat, and oxidation occurred. Thus, LR-CDs have been suggested for application in the agricultural, cosmetic, food, and pharmaceutical industries [6-9].

IMOs, another product of the amylomaltase, are non-digestible prebiotic oligosaccharides which pass through the gastrointestinal tract without digestion by digestive enzymes. The prebiotic IMOs have beneficially affected the host by selectively stimulating the growth or activity of non-pathogenic bacteria and limiting a number of pathogenic bacteria in the colon. As reported, long-chain IMO was more effective in increasing the microflora such as *Bifidobacteria* and *Lactobacillus* than the short-chain IMO [10].

In microorganisms, amylomaltase was first found in *Escherichia coli* as a maltose-inducible enzyme, which is essential for the metabolism of maltose [11]. The amylomaltase gene has been cloned from several bacteria and archaea, e.g., *Clostridium butyricum* NCIMB 7423, *Thermus aquaticus* ATCC33923, *Aquifex aeolicus* and *Corynebacterium glutamicum* [12-15]. However, the presence of LR-CDs-producing amylomaltase has been reported only in the

three bacterial strains; *T. aquaticus* ATCC 33923, *A. aeolicus* and *C. glutamicum*. In plants, the enzyme is known as a disproportioning enzyme or D-enzyme. It was first found in potato tubers [16], spinach leaves [17] and arabidopsis leaves [18]. Plant D-enzyme or amylomaltase is assumed to be involved in starch metabolism, however, its precise role in this process is not clear.

Previously, the amylomaltase gene was directly isolated from soil DNA which collected from Ban Nong Khrok hot spring in Thailand without bacterial cultivation [19]. The gene was first constructed in pET-17b as p17bAMY plasmid and its deduced amino acid sequence showed 99, 86 and 84% similarities with that of the amylomaltases from *Thermus thermophilus* ATCC 33923 (formerly named as *T. aquaticus* ATCC 33923) [13], *T. aquaticus*YT-1 [20] and *T. brockianus* [21], respectively. However, the purified amylomaltase that transcribed from p17bAMY plasmid gave a low yield and inconvenient purification. Thus, in order to increase the expression and purification yield, in this study, the amylomaltase gene was sub-cloned into pET-19b containing an NH₂-terminal His-tag. Its expression under the stronger T7/*lac* promoter was performed while optimizing the concentration of the IPTG inducer. In addition, the biochemical characteristics of the purified enzyme and its use for the production of LR-CDs and IMOs are also studied.

2. Materials and Methods

2.1 Bacterial strains, plasmids and chemicals

Escherichia coli BL21 (DE3) was purchased from BioLab (UK). The pET-19b expression vector was obtained from Novagen (Germany). Restriction enzymes, DNA ligase and DNA polymerase were the products of New England Biolabs Inc. (USA). HisTrap FFTM column was obtained from GE Healthcare (UK). Soluble potato starch, glucose, malto-oligosaccharides and bovine serum albumin were purchased from Sigma

(USA). Yeast extract and tryptone were obtained from Difco (USA). *Rhizopus* sp. glucoamylase was purchased from Fluka (Germany). LR-CD standards (CD22 to CD50) were kindly provided by Prof. T. Endo, Hoshi University, Tokyo (Japan). Pea starch was kindly provided by Emsland-Stärke GmbH (Germany). The commercial glucose oxidase kit was from Human GmbH (Germany). All other chemicals used were of analytical grade.

2.2 p19bAMY culture

The p19bAMY, a pET-19b based plasmid containing the amylomaltase gene from *Thermus* sp. was transformed into *E. coli* BL21 (DE3) [19]. Transformed *E. coli* BL21 (DE3) cells were transferred from 50% glycerol stock into LB medium containing 100 µg/ml of ampicillin and grown at 37°C with rotary shaking overnight to be used as a starter culture.

2.3 Optimization of amylomaltase gene expression

The starter culture was transferred into 300 ml of the LB medium and cultured under the same conditions. When A_{600} reached 0.5, the transformed cells were induced by IPTG at different concentrations and times. After completed induction, the cells were harvested by centrifugation at 4,750 rpm for 30 min and then washed by 20 mM sodium phosphate buffer, pH 7.4. The transformed cells were resuspended in the same buffer and then disrupted by sonication at 40% amplitude for 3 cycles of 5 min pulse and 5 min pause (VibraCell™ VCX130, Sonics, U.S.A.). Cell debris was removed by centrifugation at 10,200 rpm for 2 h. The supernatant was collected for starch transglycosylation activity [19,20] and protein concentration [22]

2.4 Enzyme assay

2.4.1 Starch transglycosylation activity

The starch transglycosylation activity was measured by the iodine method [20]. Briefly, the reaction mixture contained 250 µl of 0.2% (w/v) potato soluble starch, 50 µl of 1% (w/v) maltose, 100 µl of the enzyme and

600 µl of 20 mM phosphate buffer (pH 7.4). The reaction was performed at 70°C for 10 min and stopped by boiling for 10 min. Then, 100 µl aliquot was withdrawn and mixed with 1 ml of iodine solution (0.02% (w/v) I_2 in 0.2% (w/v) KI), and the absorbance at 600 nm was monitored. One unit of starch transglycosylation activity was defined as the amount of enzyme that produces a 1% reduction in the absorbance per min under the described conditions.

2.4.2 Disproportionation activity

The disproportionation activity was measured by the glucose oxidase method [23]. One unit of disproportionation activity was defined as the amount of enzyme required for the production of 1 µmole of glucose per min under the described conditions.

2.4.3 Cyclization activity

The cyclization activity was measured by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis [24]. The reaction mixture contained 1.25 ml of 0.5% (w/v) pea starch, 250 µl of the enzyme (0.24 mg/ml) in 20 mM phosphate buffer (pH 7.4). The reaction was performed at 70°C for 3 h and then stopped by boiling for 10 min. After cooling, 2 U of glucoamylase was added and incubated at 40°C for 3 h before being inactivated by boiling for 10 min. The reaction mixture was then cool down and analyzed by HPAEC-PAD (Dionex, U.S.A.). One unit of cyclization activity was defined as the amount of enzyme required for the production of 1 nC of CD29 product per min under the described conditions.

2.4.4 Coupling activity

The coupling activity, a reverse reaction of cyclization activity, was measured by the glucose oxidase method [23]. One unit of coupling activity was defined as the amount of enzyme that reduced 1 µmole of glucose per min under the assay conditions used.

2.4.5 Hydrolytic activity

The hydrolytic activity was measured by the bicinchoninic acid assay (BCA) [25]. The reaction mixture contained 30 µl of 5

mg/ml mixture of the CDs (CD22-CD50), 200 μ l of the enzyme (0.24 mg/ml) in 20 mM phosphate buffer (pH 7.4). The reaction was performed at 70°C for 1 h and then stopped by adding 30 μ l of 1 N HCl. Next, 1 ml of the BCA reagent was added, incubated at 80°C for 25 min and inactivated on ice for 5 min prior to measuring the absorbance at 562 nm. One unit of hydrolytic activity was defined as the amount of enzyme required for the production of 1 μ mole reducing glucose per min under the described conditions.

2.5 Purification of amylomaltase enzyme

2.5.1 Cell cultivation and crude extract preparation

1% starter inoculum was transferred into 2.1 liters of LB medium and cultured at 37°C with 250 rpm rotary shaking (Gyromax™ 700, CA). When A_{600} of the culture reached 0.5, IPTG was added to a final concentration of 0.8 mM to induce amylomaltase production and the cultivation was then continued at 37°C for 4 h. After 4 h cultivation, cells were harvested and then disrupted by sonication (VibraCell™ VCX130, Sonics, U.S.A.). Cell debris was removed by centrifugation at 10,200 rpm for 2 h. The supernatant was collected for determination of starch transglycosylation activity and protein assay.

2.5.2 Purification of recombinant amylomaltase

The crude enzyme from the p19bAMY transformed cells was purified by HisTrap FF™ column chromatography. The HisTrap FF™ column (0.7 x 2.5 cm) was equilibrated with 5 columns volume of 20 mM phosphate buffer, pH 7.4 containing 0.5 M NaCl and 20 mM imidazole at flow rate 1 ml/min. The bound proteins were eluted by 500 mM imidazole in the same buffer at the flow rate of 1 ml/min. Fractions of 2 ml were collected. The active fractions were pooled for determination of starch transglycosylation activity and protein assay.

2.6 Analytical gel electrophoresis

The denaturing gel was performed according to the method of Bollag *et al* [26]. The slab gel system consisted of 0.1% SDS (w/v) in 10% separating and 5.0% stacking gel with Tris-glycine buffer, pH 8.0 containing 0.1% SDS as electrode buffer. After electrophoresis, the gel was stained by Coomassie® Brilliant Blue R-250 (Bio-Rad, U.S.A.) and then destained by distilled water.

Native polyacrylamide gel electrophoresis (Native-PAGE) was also used to identify amylomaltase in crude and HisTrapFF™ samples on 10% polyacrylamide gel, and stained for proteins and starch transglycosylation activity [27, 28]. After electrophoresis, the gel was soaked in 10 ml of staining solution containing 2.0% (w/v) potato soluble starch and 1% (w/v) maltose in 20 mM phosphate buffer, pH 7.4 at 70°C for 10 min. It was then quickly rinsed with distilled water and immersed in 10 ml of iodine solution (0.2% (w/v) I_2 in 2.0% (w/v) KI) and left for color development at room temperature. The clear zone on the dark brown background indicates starch-degrading activity.

2.7 Synthesis of LR-CDs

The optimum conditions for the production of LR-CDs were considered and defined in terms of obtaining the highest percentage yield of LR-CD products, as determined from the HPAEC-PAD results. The effects of different types of starch substrate and varying the substrate concentrations (0.5-2.5% (w/v)), incubation time (0.5-8 h), temperature (40-80°C), pH (5.0-8.0) and amylomaltase unit (10-50 U/ml, starch transglycosylation activity) were all investigated. After completion of the incubation period, all tested reactions were inactivated by boiling. Subsequently, 2 U glucoamylase was added to the reaction mixture and then incubated at 40°C for 3 h prior to HPAEC-PAD analysis.

2.8 Synthesis of IMO products

The optimum conditions for the production of IMOs were considered and

defined in terms of obtaining the highest percentage yield of IMO products, as determined from the HPAEC-PAD results. The effects of starch substrate type, pullulanase-treated pea starch concentration (15-35% (w/v)), incubation time (0.5-7 h), temperature (40-80°C), pH (5.0-8.0), transglucosidase (3-15 U/ml, starch transglycosylation activity) and amylomaltase (40-120 U/ml, starch transglycosylation activity) were all investigated. After completion of the incubation period, all tested reactions were inactivated by boiling. Subsequently, 2 U glucoamylase was added to the reaction mixture and then incubated at 40°C for 3 h prior to HPAEC-PAD analysis.

2.9 HPAEC-PAD analysis

2.9.1 LR-CD analysis

HPAEC-PAD was carried out on a Dionex model ICS 3000 system (Dionex, USA) using a Carbopac PA-100 column (4x250 mm, Dionex, USA) [21,29] to analyze and quantify the CD products. A 25- μ l sample was loaded onto the column and eluted with a linear gradient of sodium nitrate [4% (0-2 min), 4-13% (2-13 min), 13-35% (13-35 min), 35-45% (35-40 min), and 45-100% (40-50 min)] in 150 mM NaOH at a flow rate of 1 ml/min. The LR-CD products were identified by comparison with the standard LR-CDs.

2.9.2 IMO analysis

The long chain IMO products were analyzed by HPAEC-PAD using Cabopac PA1 column (0.4 x 25 cm, Dionex, U.S.A.) and an electrical detector (ECD40, Dionex). Two buffer, buffer A (150 mM NaOH in water) and buffer B (600 mM sodium acetate in buffer A, were used for the elution with a 0-30% (v/v) gradient of buffer B at a flow rate of 1 ml/min. A 25- μ l sample was injected to the column. Some of the standards (IMO2-IMO8 and panose) were used for calibration.

3. Results and Discussion

3.1 Optimization of amylomaltase gene expression

In this study, the amylomaltase transformants were grown in LB medium

containing ampicillin. The highest expression was found when IPTG was added to a final concentration of 0.8 mM and further grown for 4 h at 37°C.

3.2 Purification of amylomaltase enzyme

The expression level of crude enzyme showed a specific activity of 2.6 U/mg protein (Table 1). After HisTrap FF™ column chromatography, the enzyme purity was up to 47-fold with a yield of 44% and a specific activity was 122 U/mg protein (Table 1). Figure 1 showed the purification profile of column chromatography and the arrow indicated where elution of the bound proteins started. The other specific activity assays of the purified enzyme were also determined as shown in Table 2. In previous reports, amylomaltase from *Synechocystis* sp. PCC6803 was 2.9-fold purified with 84.5% yield [30] and that from *T. brockianus* was 35-fold purified with 67% yield after heat treatment and Ni²⁺-NTA column chromatography [21].

Table 1. Purification of amylomaltase from the p19bAMY transformed cell.

Purification step	Total protein ^b (mg)	Total activity ^c (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude enzyme ^a	1155	3004	2.6	1	100
HisTrap FF™	11	1331	122	46.9	44.3

^a Crude extract was prepared from 2.1 liters (9.5 g wet weight) of cell culture

^b Assayed by Bradford's method

^c Assayed by starch transglycosylation activity

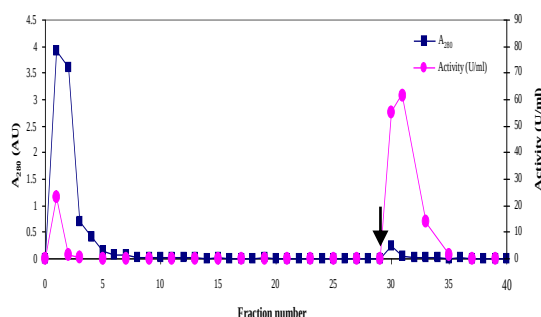


Fig.1. Purification profile of wild-type amyloamylase from the p19bAMY transformed cell by HisTrap FFTM column chromatography.

Table 2. Specific activities of amyloamylase^a.

Enzyme activity	Specific activity (U/mg)
Starch transglycosylation	122 ± 0.13
Disproportionation	28.5 ± 0.49
Cyclization	1.12 ± 0.03
Coupling	0.90 ± 0.04
Hydrolytic	0.014 ± 0.002

^a Data shown are the mean ± SD as were derived from three independent experiments.

3.3 Analytical gel electrophoresis

The relative molecular weight of the amyloamylase containing his-tag residues from recombinant clones was 60 kDa on 10% SDS-PAGE (Figure 2). In comparison, the molecular weight of the enzyme had the size similar to 4αGTase from *T. aquaticus* (57 kDa) [13], *Synechocystis* sp. PCC 6803 (57 kDa) [30] and potato D-enzyme in potatoes (60 kDa) [31], but had the different size from those enzymes from *Thermococcus litoralis* (79 kDa) [32], *C. glutamicum* (84 kDa) [15], and *E. coli* IFO 3806 (93 kDa) [33].

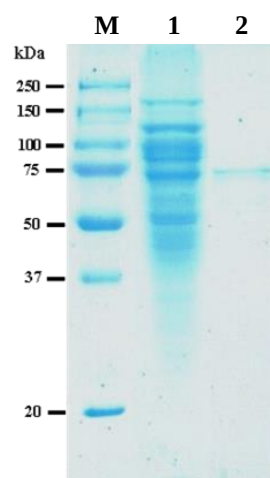


Fig.2. SDS-PAGE analysis of amyloamylase in each purification step.

Lane M: Molecular weight marker proteins (myosin 250 kDa, phosphorylase 150 kDa, β-galactosidase 100 kDa, bovine serum albumin 75 kDa, ovalbumin 50 kDa carbonic anhydrase 37 kDa and trypsin inhibitor 20 kDa) , Lane 1: Crude extract 30 μg, Lane 2: HisTrapFFTM column 2.5 μg

The starch transglycosylation activity staining after native-PAGE showed the clear zone on the dark brown background, which obviously indicates starch-degrading activity (Fig.3.).

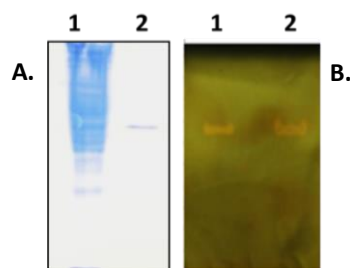


Fig.3. Native-PAGE analysis of amyloamylase.

(A.) Coomassie® Brilliant Blue R-250 staining (Lane 1: Crude extract 30 µg, Lane 2: HisTrapFF™ column 5 µg) (B.) Activity staining (Lane 1: Crude extract 2 U, Lane 2: HisTrapFF™ 2 U)

3.4 Synthesis of LR-CDs

The effect of different types of starch substrate was first performed by incubation of 10 U/ml amylomaltase (starch transglycosylation activity) with 1% (w/v) of each starch substrates at 70°C for 3 h. The results showed that pea starch was the best substrate for producing LR-CDs when compared with other starch substrates (Fig.4.A). In addition, other factors that affected LR-CD synthesis were also investigated such as the substrate concentrations (0.5-2.5% (w/v)), incubation time (0.5-8 h), temperature (40-80°C), pH (5-8), and amylomaltase unit (10-50 U/ml, starch transglycosylation activity) as shown in Fig.4.

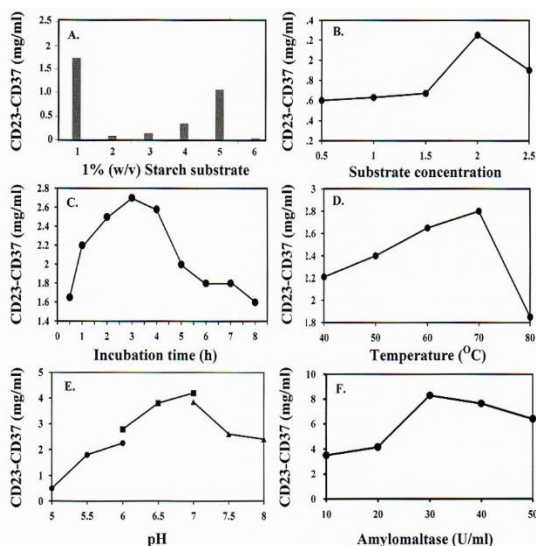


Fig.4. Effects of (A.) types of starch substrate: 1–pea, 2–corn, 3–tapioca 4–soluble potato, 5–soluble tapioca, and 6–amylopectin, (B) substrate concentration, (C.) incubation time, (D.) temperature, (E.) pH: acetate buffer (circle), phosphate buffer (square) and Tris-glycine buffer (triangle) were used, and (F.) amylomaltase concentration on LR-CD synthesis by the use of amylomaltase. The

amount of LR-CD product was analyzed by HPAEC-PAD on the basis of its standard curve using peak area. Data were averaged from triplicate experiments.

Normally, the 4αGTase can catalyze intra-molecular transglycosylation or cyclization reaction to form cyclodextrin (CD). Small CDs (CD6-CD8) are produced by CGTase while large CDs are produced by amylomaltase or D-enzyme [2,3]. After optimization of LR-CD production, the result showed that the best condition was incubation of 2% (w/v) pea starch with 30 U/ml amylomaltase in 20 mM phosphate buffer at a pH of 7.0 and 70°C for 3 h. It seems that the highest CD of the enzyme was CD29 (Fig.5.).

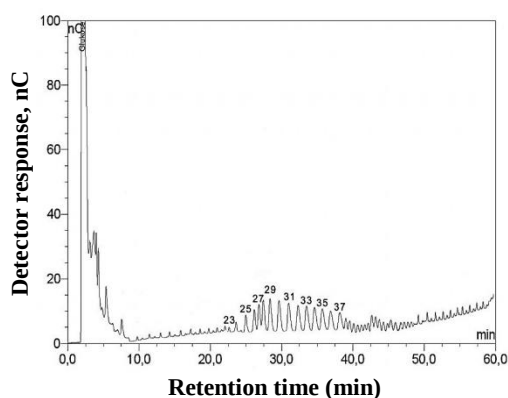


Fig.5. HPAEC-PAD analysis of LR-CD synthesized from 30 U/ml amylomaltase with 2.0% (w/v) pea starch at 70°C for 3 h. The peak numbers indicate DP of LR-CDs.

3.5 Synthesis of IMO products

In this study, the different types of starch substrate were performed and found that the best substrate for producing IMOs was pea starch due to its high ratio of amylose/amylopectin. To obtain the highest yield of long-chain IMOs, the optimum condition for the production of IMOs was performed under various factors such as substrate concentration, incubation time, temperature, pH and enzyme units. The synthesized products from reaction mixture

were monitored using HPAEC-PAD analysis. It was found that the highest IMO yield was obtained when 30% (w/v) hydrolyzing-pea starch was incubated with 120 U of amyloamylase and 6 U of transglucosidase at 40°C for 30 min (Figure 6). The long-chain IMO production by co-action of amyloamylase and transglucosidase at optimum condition was shown in Fig.7.

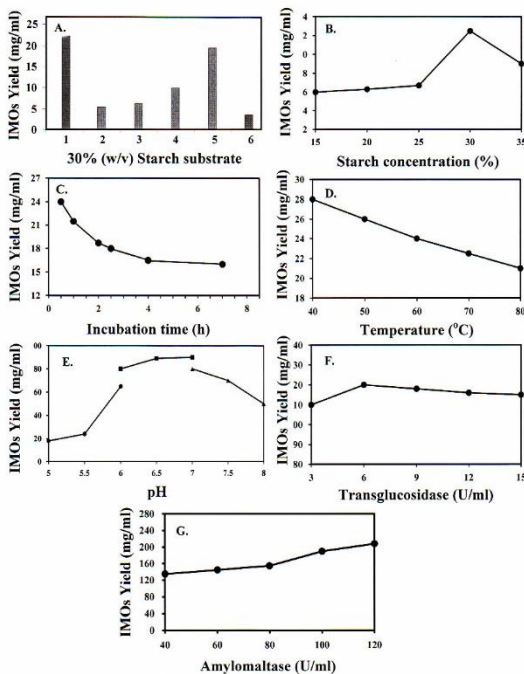


Fig.6. Effects of (A.) types of starch substrate: 1–pea, 2–corn, 3–tapioca 4–soluble potato, 5–soluble tapioca, and 6–amylopectin, (B) substrate concentration, (C.) incubation time, (D.) temperature, (E.) pH: acetate buffer (circle), phosphate buffer (square) and Tris-glycine buffer (triangle) were used, (F.) transglucosidase concentration, and (G.) amyloamylase concentration on IMO synthesis by the combined use of amyloamylase with transglucosidase. The amount of IMO product was analyzed by HPAEC-PAD on the basis of its standard curve using peak area. Data were averaged from triplicate experiments.

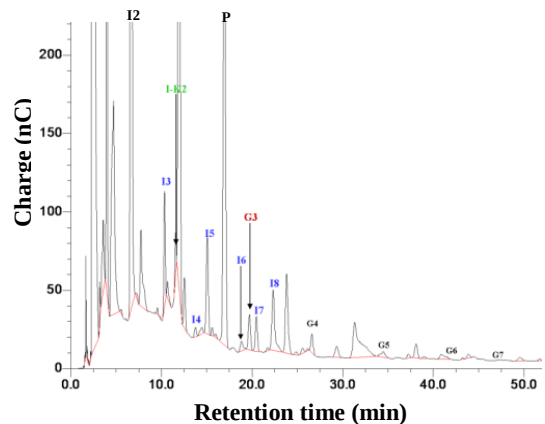


Fig.7. HPAEC-PAD analysis of long-chain IMOs synthesized from co-action of amyloamylase and transglucosidase with 30% (w/v) pullulanase-treated pea starch at optimum condition (40°C, pH 7.0) for 0.5 h. The $I_{(n)}$ and $G_{(n)}$, n indicate DP of IMO and malto-oligosaccharide (MOS), respectively. Other abbreviations: P, panose and I-K2 (2-O- α -isomaltosyl-D-glucose), speculate the name according to structure and DP.

Basically, the transglucosidase plays the role in synthesis to form the α -1, 6 glycosidic linkage between glucose unit by disproportionation reaction while amyloamylase involved synthesis of the α -1, 4 bond. Therefore, the co-action of both enzymes gave IMO products in the range of IMO2-IMO8. Several research groups have investigated the IMOs production. Duan *et al.* [34] reported the synthesis of IMOs (IMO2-IMO4) from maltose using α -glucosidase (EC 3.2.1.20) from *A. carbonarius* CCRC 30414. Lee *et al.* [4] have studied efficient production of IMOs by using the combination of an alpha-glucanotransferase (EC 2.4.1.25) from *T. maritima* and a maltogenic amylase from *B. stearothermophilus* (BSMA) to produce IMOs from corn starch. The result has been showed that the IMO content (IMO2-IMO7) was 68% and contained relatively larger IMOs compared with the obtained products from the reaction without α -glucanotransferase. Tanriseven and Dogan [35] produced IMOs by using the alginate-immobilized

dextran sucrose from *L. mesenteroides* B-512F with sucrose and glucose as substrates. The IMOs with more than DP2 were synthesized.

4. Conclusion

In conclusion, the amylomaltase gene that directly isolated from soil DNA was expressed in *E. coli* BL21 (DE3) using the pET-19b vector. The crude recombinant enzyme was highly purified to homogeneity by a one-step affinity column chromatography. This is the first report on alkalo-thermophilic amylomaltase. The enzyme was able to catalyze a cyclization reaction to produce LR-CDs from pea starch. The enzyme also can be used to produce long chain IMOs. Both products of the enzyme would be highly potential to be used in the food, pharmaceutical and chemical industries.

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