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RESEARCH PAPER

# Production of Long-chain Isomaltooligosaccharides from Maltotriose Using the Thermostable Amylomaltase and Transglucosidase Enzymes

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**Abstract** Amylomaltase and transglucosidase were combined to produce long-chain isomaltooligosaccharides (IMOs). IMOs are effective prebiotics that stimulate the growth of healthy bacteria in human intestines and thus promote better overall health. In this study, the p17bAMY amylomaltase was expressed from its gene, which had been directly isolated from soil samples, while transglucosidase was purchased and purified by a gel-filtration column. Crude amylomaltase was purified by heat treatment, Q-, and phenyl-sepharose column. The purified amylomaltase had a molecular weight of 57 kDa. Specificity on the substrates of the amylomaltase was also studied and it was found that this enzyme was able to catalyze transglucosylation activity using substrates $G_2$ to $G_7$. However, $G_3$ was the most preferred substrate for the enzyme. Here, $K_{m\text{-}G3}$ and $k_{cat}/K_m$ were 23 mM and $1.72 \times 10^8$ mM/min, respectively. Amylomaltase and transglucosidase were tested both alone and in combination on a $G_3$ substrate to study the efficient process for the IMOs production. The obtained products from the enzymatic reactions were monitored using the TLC analytical method and a densitometer. The amylomaltase led to products containing linear maltooligosaccharides, while the transglucosidase produced short-chain IMOs. Interestingly, when amylomaltase and transglucosidase were used in combination, long-chain IMOs with sizes larger than $IMO_4$ were observed under the determined condition.

**Keywords:** amylomaltase, isomaltooligosaccharides (IMOs), prebiotic, transglucosidase, transglucosylation

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## 1. Introduction

Amylomaltase (E.C. 2.4.1.25), an intracellular 4-α-glucanotransferase (4αGTase), is a well known member of the α-amylase family. The 4αGTase group is composed of 4 types of enzymes including cyclodextrin glycosyltransferase (E.C. 2.4.1.19) (type I), amylomaltase (type II), glycogen debranching enzyme (E.C. 3.2.1.33) (type III), and other 4αGTase (type IV) [1]. In this study, we focus on amylomaltase due to its wide area of potential applications in several industries [1]. Amylomaltase was firstly found in *Escherichia coli* as a maltose-inducible enzyme which is essential for the maltose metabolism [2], and the amylomaltase gene was later cloned from several bacteria, including *E. coli* [2], *Clostridium butyricum* NCIMB 7423 [3], hyperthermophilic archaeon *Thermococcus litoralis* [4], *Thermus aquaticus* ATCC 33923 [5], *Aquifex aeolicus* [6], *Pyrobaculum aerophilum* IM2 [7], *Thermus brockianus* [8], *Synechocystis* sp. PCC 6803 [9] and *Corynebacterium glutamicum* [10]. In addition, this enzyme is also found in plants such as potatoes, carrots and tomatoes. It is called a debranching enzyme (D-enzyme), and is concerned with starch metabolism [11]. Amylomaltase can catalyze four reactions such as disproportionation, cyclization, 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 minor activities of amylomaltase comprise cyclication, coupling and hydrolysis reactions. The cyclication reaction converts starch to large-ring cyclodextrin (LR-CDs). The coupling and hydrolysis reaction by which LR-CDs are cleaved and the obtained linear glucan are transferred to an acceptor or water molecule [5,10].

Recently, amylomaltases have been explored for their potential applications in the food, pharmaceutical, medical, and paper industries. Srisimarat *et al.* [10] reported on the use of amylomaltase from *C. glutamicum* in the production of LR-CDs. LR-CDs have high potential in stabilization and solubilization of large and insoluble or unstable drug molecules. Amylomaltase is also used in the production of a thermo reversible starch gel that is of commercial interest since it can be applied as a substitute for gelatin [7]. Moreover, amylomaltase has been reported to have an important role in the production of isomaltooligosaccharide (IMO) prebiotic when used in combination with maltogenic amylase [12]. IMOs are a non-digestible food ingredient which passes through the gastrointestinal tract without digestion by digestive enzymes. The prebiotics beneficially affect the host by selectively stimulating the growth and/or activity of non-pathogenic bacteria and a limited number of pathogenic bacteria in the colon, and thus improves host health [13]. The structure of an IMO is a branched oligosaccharide that contains a series of α-1,6 and α-1,4 linkages or only α-1,6 linkages such as panose and isomaltose. In the food industry, IMOs are commercially produced from starch using a combination of enzymes in a three-step process. Firstly, starch is liquefied by α-amylase (E.C. 3.2.1.1). Secondly, ß-amylase (E.C. 3.2.1.2) hydrolyses starch to maltooligosaccharides, and then α-glucosidase (E.C. 3.2.1.20) catalyzes the transglycosylation reaction converting the α-1,4 linkages to α-1,6 linkages [13]. Because of the higher consumer demand for IMOs prebiotics, numerous works on IMOs production have been carried out. For example, Lee *et al*. [12] studied the efficient production of IMOs by maltogenic amylase from *B. stearothermophilus* (BSMA) and α-glucanotransferase (α-GTase) from *Thermotoga maritima*. After 20 h of reaction, a 58% yield of IMO was obtained from 30% (w/v) liquefied corn syrup substrate when BSMA was used alone, and a 68% yield was obtained when BSMA was used in combination with α-GTase. In a later study, Goulas *et al*. [14] investigated the synthesis of IMOs from sucrose by the combined use of *Ln. mesenteroides* dextransucrase and *P. lilacinum* dextranase. In Goulas *et al*.'s study, a recycled ultrafiltration membrane reactor was used in order to develop a continuous synthesis process for IMOs production. Recently, Pan and Lee reported the production of high-purity IMO using the enzymatic conversion of transglucosidase and fermentation of yeast cells [15]. Both *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis*yeasts were able to remove digestible sugar such as glucose, maltose, and maltotriose from a mixture of low-purity IMOs to convert them into high-purity IMOs.

In this study, for the purpose of synthesizing the long-chain IMOs, an amylomaltase gene was directly cloned from soil bacterial DNA and expressed in *E. coli* BL21 (DE3) [16]. The cloned cells were then grown in a Luria-Bertani (LB) broth for enzyme production. The crude enzyme obtained from the cloned cells was purified, biochemically characterized, and used in combination with transglucosidase for producing long-chain IMOs.

## 2. Materials and Methods

### 2.1. Materials

Soluble potato starch, glucose ($G_1$), maltose ($G_2$), maltotriose ($G_3$), maltotetraose ($G_4$), maltopentaose ($G_5$), maltohexaose ($G_6$), maltoheptaose ($G_7$), isomaltose ($IMO_2$), isomaltotriose ($IMO_3$), panose and isomaltotetraose ($IMO_4$), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Yeast extract and tryptone were obtained from Difco (Detroit, USA). *Rhizopus* sp. Glucoamylase was purchased from Sorachim (Genas, France) and *Aspergillus niger* transglucosidase was obtained from Amano enzyme Inc. (Nagoya, Japan). HisTrap Q FF and phenyl HP columns were obtained as products of GE Healthcare (Buckinghamshire, UK). The commercial glucose oxidase kit originated from Human (*mti*-diagnostics GmbH, Germany). Silica gel 60 TLC plate and all other chemicals of analytical grade were obtained from Merck (Darmstadt, Germany) and Wako Chemicals GmbH (Neuss, Germany).

### 2.2. Bacterial strains and conditions for recombinant amylomaltase production

In our previous work, the amylomaltase gene was directly isolated from soil bacterial DNA using the soil DNA extraction kit (ISOIL) (Nippon gene, Toyama, Japan) to eliminate the need for bacterial culture [16]. This gene was cloned into a pET-17b vector to express the protein in *Escherichia coli* BL21 (DE3) and the resulting plasmid was named p17bAMY. In this study, the p17bAMY cell was grown in LB media containing 100 μg/mL ampicillin, and cultivated at 37°C. When $A_{600}$ of the culture medium reached between 0.4 and 0.55, 0.5 mM IPTG was added to the culture medium, which was then continually cultured at 37°C for 6 h. The recombinant cells were subsequently separated by centrifugation at 7,500 rpm for 30 min. The cells were washed twice with 25 mM sodium phosphate

buffer at pH 7.5, and then separated by centrifugation at 9,500 rpm for 45 min. The recombinant cells were resuspended using a 20 mM Tris-HCl buffer at pH 8.0, and disrupted by sonication with 6 mm diameter microtip of 40% amplitude for 3 cycles of 5 min pulse and 5 min pause. Cell debris was removed by centrifugation at 9,500 rpm for 45 min. The crude extract was determined for enzyme activities and protein concentration.

### 2.3. Amylomaltase assay and protein determination

#### *2.3.1. Starch transglycosylation activity*

The starch transglycosylation activity was measured using the iodine method [10]. The reaction mixture contained 250 μL of 0.2% (w/v) soluble potato starch, 50 μL of 1% (w/v) maltose, 100 μL of the enzyme and 600 μL of 50 mM phosphate buffer, pH 7.5. The reaction was performed at 70°C for 10 min and stopped by boiling at 100°C 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 then monitored. One unit of starch transglycosylation activity was defined as the amount of enzyme that produces a 1% reduction in the absorbance per minute under the described conditions.

#### *2.3.2. Disproportionation activity*

The disproportionation activity was measured using the glucose oxidase method [10]. The reaction mixture contained 30 μL of 5% (w/v) maltotriose and 100 μL of the enzyme in a 20 mM Tris-HCl buffer at pH 8.0. The reaction was generated at 70°C for 10 min and then stopped by boiling at 100°C for 10 min. Then, 10 μL aliquot from the reaction mixture was added to 1 mL of glucose oxidase reagent. The mixture was subsequently incubated at 37°C for 5 min and the absorbance at 500 nm was then measured. The glucose concentration was calculated from this equation 1:

$$\text{Glucose concentration (mmol/L)} = 5.55 \times (\Delta A_{Sample}/\Delta A_{Std.}) \quad (1)$$

One unit of disproportionation activity was defined as the amount of enzyme required for the production of 1 μmol of glucose per min under the described conditions.

#### *2.3.3. Protein determination*

The protein concentration was measured using a Bradford assay [17], with bovine serum albumin (BSA) used as the standard.

### 2.4. Purification of recombinant amylomaltase

Crude extract was heated at 70°C for 30 min. The protein precipitate was removed by centrifugation at 10,000 rpm for 45 min. The residual supernatant was determined as the starch transglycosylation activity and was further purified by HiTrap Q FF column chromatography (volume 1 mL) at 4°C. The HiTrap Q FF column chromatography was equilibrated with a starting buffer (20 mM Tris-HCl, pH 8.0) at the flow rate of 1 mL/min. The supernatant enzyme was then introduced to the column using a syringe at the flow rate of 1 mL/min. The unbound protein was washed with a starting buffer at the same flow rate. The bound protein was eluted with a linear 30 mL gradient of 0.0 ~ 0.5 M NaCl in a starting buffer at the flow rate of 1 mL/min. Active fractions from this column were pooled and loaded onto a HiTrap phenyl HP column chromatography (volume 1 mL) device, equilibrated with a starting buffer containing 1 M ammonium sulfate. The unbound protein was removed by washing with a starting buffer containing 1 M ammonium sulfate until $A_{280}$ of eluent decreased to a baseline. The bound protein was then eluted with an 80 mL gradient of decreasing ammonium sulfate concentration, ranging from 1 to 0 M in a starting buffer. The fractions having amylomaltase activity were collected as a purified pool and concentrated for subsequent studies.

### 2.5. Recombinant amylomaltase purity and molecular weight determination

Amylomaltase purity in each step of purification and its molecular weight as a pure protein were estimated by SDS-PAGE on a 10% polyacrylamide gel using the Mini protein II electrophoresis apparatus (Bio-Rad Laboratories, CA, USA). Protein bands were visualized using Coomassie blue staining [18]. The Precision Plus Protein™ Dual Color standards (Bio-Rad Laboratories, CA, USA) were used as markers.

### 2.6. Substrate specificity of recombinant amylomaltase

Various malto-oligosaccharide substrates at 50 mM concentration were incubated with amylomaltase (100 μL, 72 U/mL) in 20 mM phosphate buffer, pH 8.0 at 70°C for 30 min. The reaction was halted by boiling at 100°C for 10 min. Ten μL aliquot from the reaction mixture was then withdrawn for measuring the glucose oxidase activity as described in Section 2.3.2., and the result was expressed as relative activity.

### 2.7. Thinlayer chromatography (TLC) analysis

Substrate specificity of amylomaltase was also analyzed on TLC. The transglycosylation products were prepared as mentioned in Section 2.6. Five μL of standard $G_1$-$G_7$ (5 mg/mL) and ten μL of sample reaction were dropped on a silica gel plate. The TLC plate was then developed at ambient temperature for 5 h with mobile phase solvent of 1-propanol: ethyl acetate: water (7:1:2, v/v/v) in the TLC tank. Spots were made visible by dipping the plate in a

solution of ethanol: sulfuric acid (9:1, v/v), drying, and heating at 110°C for 15 min.

### 2.8. Kinetic studies of recombinant amylomaltase

Initial velocity studies for the disproportionation reaction of enzymes were carried out using the glucose oxidase method [10] as described. The concentrations of $G_2$-$G_4$ substrates varied from 0 to 200 mM. The Lineweaver-Burk of the initial velocity against malto-oligosaccharide concentration was plotted and kinetic parameters were then determined using the EnzFitter program, version 2.0.14.0 (Biosoft).

### 2.9. Isomaltooligosaccharides (IMOs) production

#### *2.9.1. Separation and quantitative determination of IMOs*

The synthesized IMO products were separated by TLC with a mobile phase system of acetonitrile: ethyl acetate: 1-propanol: distilled water (8.5:2:5:4, v/v/v/v) [19]. Following development, the IMO products were made visible by dipping the TLC plate into ethanol containing 10% (v/v) sulfuric acid. After air-drying, the TLC plate was placed in an oven for 15 min at 110°C. The densities of the IMO spots on the TLC plate were measured using a densitometer with the GS-800 program (Bio-Rad Laboratories, CA, USA) and compared with the IMO standard curve.

#### *2.9.2. Optimum conditions for IMOs production*

The effect of parameters on the IMOs production was studied. The reaction mixture was varied in each factor as follows: substrate concentration, incubation time, temperature, pH, and enzyme unit. After reaction incubation, the reaction mixture was halted by boiling at 100°C for 10 min, and the reaction mixture was then treated with 2 U glucoamylase at 40°C for 3 h to convert the linear oligosaccharides to glucose. The obtained IMO product was analyzed on a TLC plate as described in Section 2.9.1. The amount and distribution of the chain length of IMO were reported in units of mg/mL IMO and the best optimum condition was used for IMO production.

### 2.10. Mass spectrometry

A mass spectrometry device determines the molecular mass of chemical compounds by separating ionic molecules according to their mass-to-charge ratio (m/z). The molecular mass was calculated from equation 2:

$$(\text{molecular mass} + \text{number of protons})/\text{charge} = \text{mass-to-charge ratio (m/z)} \quad (2)$$

The sample was introduced to the mass spectrometry device. The Electrospray Ionization-Time of Flight Mass Spectrometry (ESI-TOF MS) profile was recorded on micro TOF at the Biological Service Unit of The National Center of Genetic Engineering and Biotechnology (BIOTECH), Pathum Thani, Thailand.

### 2.11. Nuclear magnetic resonance (NMR)

Structural identification of the IMO products produced by amylomaltase and transglucosidase was performed using $^1$H NMR with a Varian-UNITY plus a 500 NMR spectrometer operating at 500 MHz in $D_2O$ at ambient temperature at the Scientific and Technological Research Equipment Centre, Chulalongkorn University, Bangkok, Thailand. Chemical shifts were measured with sodium-4,4-dimethyl-4-silapentane sulfonite (DSS) as an internal standard.

## 3. Results and Discussion

### 3.1. Production and purification of recombinant amylomaltase

The p17bAMY recombinant cell was grown in LB broth containing 100 μg/mL ampicillin. When $A_{600}$ of the culture medium reached 0.4, 0.5 mM IPTG was added in the medium to induce amylomaltase production at 37°C for 6 h. The cells were then harvested and disrupted by sonication and the supernatant obtained was used as crude amylomaltase. The crude amylomaltase obtained was highly purified according to the following procedures: heat treatment, column chromatography on HiTrap Q FF column, and column chromatography on HiTrap phenyl HP column (Table 1). The enzyme was 79-fold purified with a 34% yield. The purified enzyme from the Phenyl HP column showed one band on 10% SDS-PAGE with the molecular mass of 57 kDa (Fig. 1), which corresponded to the size of the cloned gene (Fig. 2). Thus, the size of this enzyme was the same as that of the enzyme purified from the *Thermus*

**Table 1.** Purification of the p17bAMY recombinant amylomaltase

| Purification steps | Total protein (mg) | Total activity* (U) | Specific activity (U/mg) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude enzyme | 441 | 2,361 | 5 | 1 | 100 |
| Heat treatment | 46 | 2,173 | 47 | 9 | 92 |
| HiTrap Q FF column | 7 | 1,873 | 268 | 54 | 79 |
| HiTrap phenyl HP column | 2 | 793 | 397 | 79 | 34 |

*Assayed by starch transglycosylation activity.

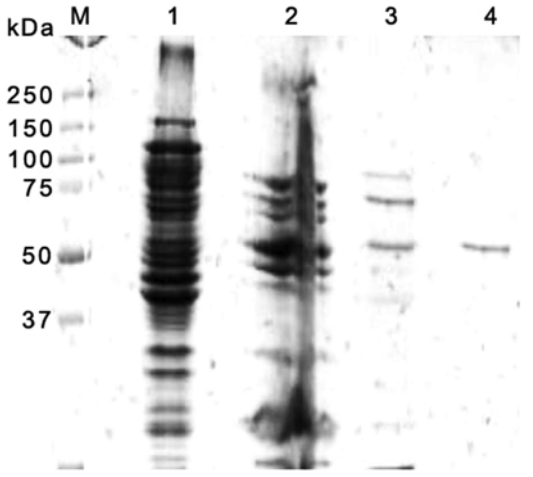


**Fig. 1.** SDS-PAGE analysis of p17bAMY recombinant amylomaltase in each step of purification on a 10% acrylamide gel. Lane M, protein markers; 1, crude enzyme; lane 2, heat treatment; lane 3, HiTrap Q FF column; and lane 4, HiTrap phenyl HP column.

sp. and *Synechocystis* sp. PCC6803 cell [5,8,9]. However, a larger size of amylomaltase in the range of 79 ~ 93 kDa has been reported from *Thermococcus litoralis, E. coli* IF03806 [4], and *C. glutamicum* [10].

### 3.2. Substrate specificity and transglycosylation products

In order to confirm the identity of the p17bAMY amylomaltase overexpressed in *E. coli* BL21 (DE3), the action of the purified enzyme and maltooligosaccharide ($G_2$-$G_7$) substrates was investigated from the amount of released glucose in each tube using the glucose oxidase method [10]. The result showed that $G_3$ was the best substrate of the enzyme, and the descending order of the preferred substrate was $G_3$>$G_4$>$G_5$>$G_6$>$G_7$>$G_2$. In addition, the pattern of the generated transglycosylation products from all of maltooligosaccharides ($G_1$-$G_7$) was also analyzed by TLC plate. The result showed that the amylomaltase could catalyze the disproportionation reaction from $G_2$ to $G_7$ substrates while $G_1$ could not be used as a substrate (Fig. 3). These results implied that the p17bAMY amylomaltase catalyzed the transfer of glucose or larger units from one 1,4-α-glucan to another, and required at least a maltose unit for the disproportionation reaction. Unlike the results for p17bAMY amylomaltase, Terada *et al.* [5] reported that while *T. aquaticus* amylomaltase catalyzed the transglycosylation of malto-oligosaccharides, larger molecules ($G_4$ and $G_5$) are more effective substrates than smaller molecules ($G_2$ and $G_3$).

### 3.3. Kinetic studies of recombinant amylomaltase

Kinetic parameters of the purified p17bAMY amylomaltase were examined with $G_2$, $G_3$, and $G_4$ substrates at 70°C. The

```
1    atggagcttccgcgcgcttatggtctgcttctccaccccacgagc
     M  E  L  P  R  A  Y  G  L  L  L  H  P  T  S
46   ctccccggcccctacggcgtcggcgtcctgggccaggaggcccgg
     L  P  G  P  Y  G  V  G  V  L  G  Q  E  A  R
91   gacttcctccgcttcctcaaggaggcaggggggcggtactggcag
     D  F  L  R  F  L  K  E  A  G  G  R  Y  W  Q
136  gtcctccccttgggccccacgggctatggcgactcccctaccag
     V  L  P  L  G  P  T  G  Y  G  D  S  P  Y  Q
181  tccttcagcgccttcgccggaaaccccctacctcatagacctgagg
     S  F  S  A  F  A  G  N  P  Y  L  I  D  L  R
226  cccctcgcggaaaggggctacgtgcgcctggaggaccccggcttc
     P  L  A  E  R  G  Y  V  R  L  E  D  P  G  F
271  ccccaaggccgggtggactacggcctcctctacgcctggaagtgg
     P  Q  G  R  V  D  Y  G  L  L  Y  A  W  K  W
316  cccgccctgaaggaggccttccggggcttcaaggaaaaggcctcc
     P  A  L  K  E  A  F  R  G  F  K  E  K  A  S
361  ccggaggagcgggaggccttcgccgccttccgggagagggaggcc
     P  E  E  R  E  A  F  A  A  F  R  E  R  E  A
406  tggtggctcgaggactacgccctcttcatggccctgaaggggcg
     W  W  L  E  D  Y  A  L  F  M  A  L  K  G  A
451  cacgggggcttccctggaaccggtggccccttcccctgcggaag
     H  G  G  L  P  W  N  R  W  P  L  P  L  R  K
496  cgggaagagaaggcccttagggaggcgaaaagcgccttggccgag
     R  E  E  K  A  L  R  E  A  K  S  A  L  A  E
541  gaggtggccttccacgccttcacccagtggctcttcttccgccag
     E  V  A  F  H  A  F  T  Q  W  L  F  F  R  Q
586  tgggggggccttgaaggcggaggccgaggcgttgggcatccggatc
     W  G  A  L  K  A  E  A  E  A  L  G  I  R  I
631  atcggggacatgcccatcttcgtggccgaggactccgccgaggtc
     I  G  D  M  P  I  F  V  A  E  D  S  A  E  V
676  tgggcccaccccgagtggtttcacctggacgaggagggccgcccc
     W  A  H  P  E  W  F  H  L  D  E  E  G  R  P
721  acggtggtggcgggggtgccccccgactacttctcggagacgggc
     T  V  V  A  G  V  P  P  D  Y  F  S  E  T  G
766  cagcgctggggcaaccccctttaccgctgggacgttttggagcgg
     Q  R  W  G  N  P  L  Y  R  W  D  V  L  E  R
811  gaggggttctccttctggatccgccgtctggagaaggccctggag
     E  G  F  S  F  W  I  R  R  L  E  K  A  L  E
856  ctcttccacctggtgcgcatagaccacttccgcggctttgaggcc
     L  F  H  L  V  R  I  D  H  F  R  G  F  E  A
901  tactgggagatccccgcaagctgccccacggcggtggaggggcgc
     Y  W  E  I  P  A  S  C  P  T  A  V  E  G  R
946  tgggtcaaggccccgggggagaagctcttccagaagatccaggag
     W  V  K  A  P  G  E  K  L  F  Q  K  I  Q  E
991  gtcttcggcgaggtccccgtcctcgccgaggacctgggggtcatc
     V  F  G  E  V  P  V  L  A  E  D  L  G  V  I
1036 acccccgaggtggaggccctgcgcgaccgcttcggccttcccggg
     T  P  E  V  E  A  L  R  D  R  F  G  L  P  G
1081 atgaaggtcctgcagttcgcctttgacgacgggatggaaaacccc
     M  K  V  L  Q  F  A  F  D  D  G  M  E  N  P
1126 ttcctcccccacaactaccctgcccacggccgggtggtggtctac
     F  L  P  H  N  Y  P  A  H  G  R  V  V  V  Y
1171 accggcacccacgacaacgacaccaccctgggctggtaccgcacg
     T  G  T  H  D  N  D  T  T  L  G  W  Y  R  T
1216 gccacccccccacgagaaggccttcatggcgcggtacctggcggac
     A  T  P  H  E  K  A  F  M  A  R  Y  L  A  D
1261 tgggggatcaccttccgggaagaggaggaggtgccctgggccctg
     W  G  I  T  F  R  E  E  E  E  V  P  W  A  L
1306 atgcacctggggatgaagtccgtggcccggctcgccgtctacccg
     M  H  L  G  M  K  S  V  A  R  L  A  V  Y  P
1351 gtgcaggacgtcctggccctgggcagcgaggcccggatgaactac
     V  Q  D  V  L  A  L  G  S  E  A  R  M  N  Y
1396 ccgggaaggccctcggggaactgggcctggcggctcctcccgggg
     P  G  R  P  S  G  N  W  A  W  R  L  L  P  G
1441 gagctttccccggagcacggggcgaggcttagggccatggccgag
     E  L  S  P  E  H  G  A  R  L  R  A  M  A  E
1486 gccacgggacggctgtaa 1503
     A  T  G  R  L
```

**Fig. 2.** Nucleotide and deduced amino acid sequence of the amylomaltase gene from p17bAMY plasmid. The three catalytic sites of amylomaltase are shaded and the start and stop codons are shown in bold type. The four conserved regions among the α-amylase family enzymes are underlined.

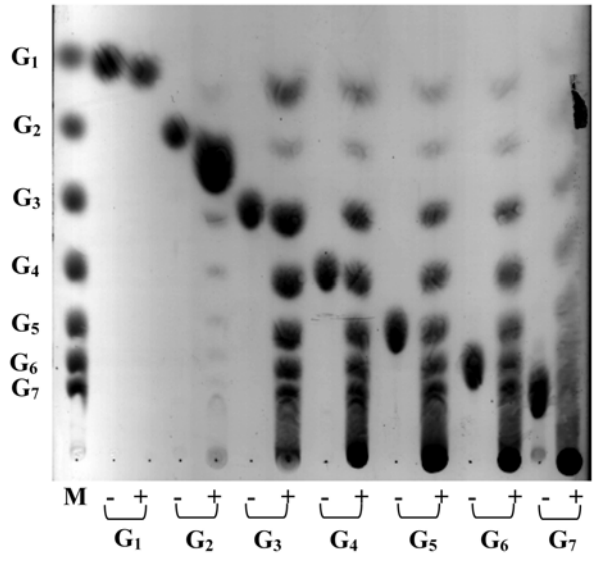


**Fig. 3.** TLC analysis of the reaction pattern of p17bAMY amylomaltase on maltooligosaccharides. Lane M, maltooligosaccharide standards; reaction mixtures on maltooligosaccharides ($G_1$-$G_7$) with (+) or without (-) p17bAMY amylomaltase.

**Table 2.** Kinetic parameters of amylomaltase with maltooligosaccharide substrates

| Substrate | $K_m$ (mM) | $k_{cat}$ (/min) | $k_{cat}/K_m$ (mM/min) |
|---|---|---|---|
| $G_2$ | 37 ± 3 | $3.32 \times 10^9$ | $8.96 \times 10^7$ |
| $G_3$ | 23 ± 2.5 | $4.00 \times 10^9$ | $1.72 \times 10^8$ |
| $G_4$ | 26 ± 3.2 | $4.20 \times 10^9$ | $1.59 \times 10^8$ |

obtained results, as shown in Table 2, indicated that the enzyme has a low affinity toward $G_2$ ($K_m$ = 37 mM). For the $G_3$ substrate, the $K_m$ and $k_{cat}/K_m$ values showed that $G_3$ was the best substrate, with an ability to provide the strongest binding and the highest catalytic efficiency. However, no significant differences were found in the turnover number ($k_{cat}$) of the $G_3$ and $G_4$ substrates. This pattern was similar to the kinetic properties of *P. aerophilumamylomaltase* [7]. On the other hand, a different result was observed for *A. aeolicus* amylomaltase [6]. The $K_m$, $k_{cat}$, and $k_{cat}/K_m$ values of *A. aeolicus* amylomaltase indicated that $G_5$ was the best substrate of the enzyme.

### 3.4. Production of IMOs using amylomaltase and transglucosidase

As an effort to develop the production of IMO, amylomaltase and transglucosidase were added to pure $G_3$ substrate under various conditions. The first effect of amylomaltase and transglucosidase activities was investigated from a series of reactions using $G_3$ substrate at various concentrations from 1 ~ 20% (w/v). The result showed that 20% (w/v) $G_3$

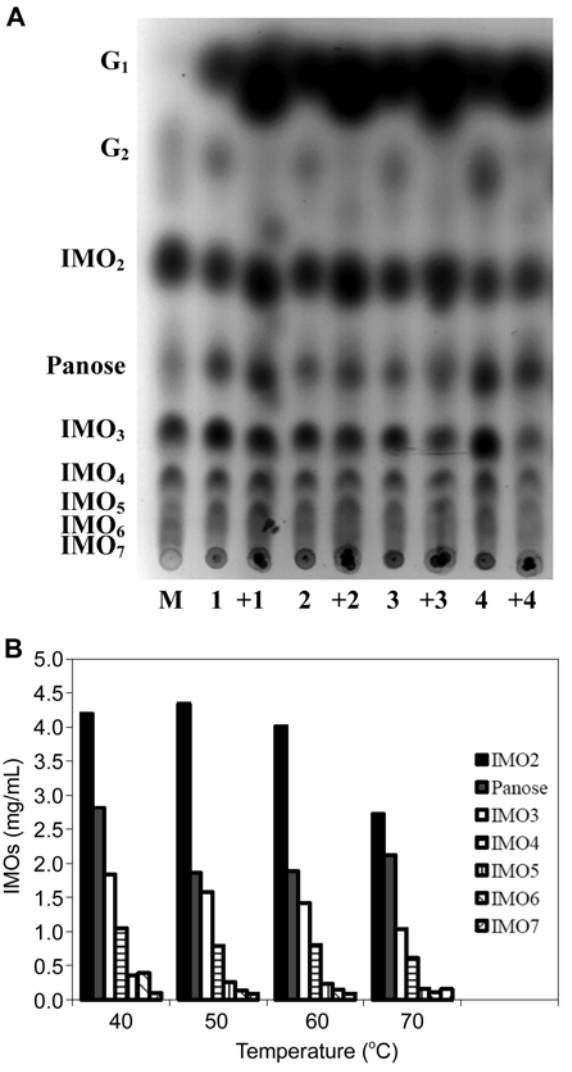


**Fig. 4.** (A) TLC analysis of oligosaccharide products from reaction mixture 20% (w/v) $G_3$, 8 U tranglucosidase and 1.5 U amylomaltase at various temperatures. Lane M: Standard IMOs and MOs, lane 1, 2, 3, 4: reaction product at 40, 50, 60, and 70°C, respectively, lane +1, +2, +3, +4: lanes 1, 2, 3, 4 after treatment with 2 U glucoamylase. (B) Distribution of chain lengths of IMOs after treatment with 2 U glucoamylase. Data are shown as the mean and are derived from 3 independent repeats.

substrate was the optimal concentration for the maximum IMOs production. The appropriate amount of each enzyme for the highest yield of IMOs was also examined. This experiment was carried out by varying the amount of transglucosidase at 0.5 ~ 8 U at different fixed amounts of amylomaltase in a phosphate buffer of pH 6.0. The results revealed that the optimal amount in co-operative action of amylomaltase and transglucosidase was determined to be 1.5 and 8 U, respectively. When using 1.5 more units of amylomaltase, the yield of IMOs product decreased. The decrease of formed IMOs might be the result of the higher unit of amylomaltase, increasing its hydrolytic activity by

which IMOs were hydrolyzed using water. This result correlated with the increase of glucose and maltose contents as the reaction proceeded at higher units of amylomaltase. Further, amylomaltase and transglucosidase were tested alone with $G_3$ substrate on a transglucosylation reaction. As a result, the amylomaltase alone led to products containing linear maltooligosaccharides (MOs), while the transglucosidase alone produced short-chain IMOs such as isomaltose ($IMO_2$), isomaltotriose ($IMO_3$), and isomaltotetraose ($IMO_4$). Interestingly, when amylomaltase and transglucosidase were combined, long-chain IMOs with sizes greater than $IMO_6$ were observed. At the optimum condition, when 1.5 U of amylomaltase and 8 U of transglucosidase were incubated with 20% (w/v) $G_3$ for 30 min at 40°C, IMO products were produced to 9.9 mg/mL with distribution of IMOs lengths from DP 2-7 (Figs. 4A and 4B). The cooperative action mode of the two enzymes on IMOs production indicated that under high $G_3$ concentration (20% w/v), transglucosidase could catalyze the glycosyl transfer reaction mainly through the α-1,6 linkage, and rarely through the α-1,4 linkage, while amylomaltase transferredthe glycosyl group from one α-1,4 glucan donor to another acceptor to form only the α-1,4 linkage [20-22,7]. Considering the facts and starting from $G_3$, amylomaltase was better able to catalyze the transglycosylation reaction using $G_3$ as the substrate than transglucosidase which preferred $G_2$ [23]. Thus, early in the reaction, the amylomaltase catalyzed the glycosyl or maltosyl transfer from one $G_3$ to another $G_3$ molecule and released $G_2$ and $G_1$ residues. The obtained $G_2$ and $G_1$ were then utilized easily by transglucosidase for the production of short-chain IMOs such as $IMO_2$, $IMO_3$, and panose through a α-1,6 transglycosylation reaction. Later in the reaction, amylomaltase converted the short-chain IMOs to long-chain IMOs; *i.e.* $IMO_4$-$IMO_7$. Therefore, the advantage of the method used in this study is the ability to control the chain lengths of IMO by changing the units of both enzymes and by providing a cost benefit for the alternative industrial production of IMO.

### 3.5. Mass spectrometry analysis

As a mean for determining the molecular weight, the obtained transglycosylation products were analyzed using mass spectrometry. The result showed that the peak values of the pseudomolecular ion $[M+Na]^+$ at *m/z* corresponded to the size of the IMO products: $IMO_2$, panose, $IMO_3$, $IMO_4$, $IMO_5$ and $IMO_6$ (Fig. 5). The proportion of obtained IMO decreased ~50% when the IMO length increased at each one unit of glucose. However, until now, the only report available is that of Lee *et al.* [12], which reported that the production of IMO has a degree of polymerization (DP) ranging from 2 to 6 using a cooperative reaction between alpha-glucanotransferase and maltogenic amylase. Generally, most of the IMOs (DP3-DP9) were produced from *L. mesenteroides* dextransucrase using sucrose as the substrate [24]. However, IMOs were synthesized from *L. mesenteroides* dextransucrase consisting of only α-1,6 glucosidic linkages. In contrast, the IMOs from amylomaltase and transglucosidase, having α-1,6 and α-1,4 glucosidic linkages, were more stable from digestive enzymes such as isomaltase than the IMOs of only the α-1,6 bond [25]. Moreover, Lee *et al.* [12] reported that IMOs containing α-1,6 and α-1,4 glucosidic linkages can be selectively fermented in the colon by beneficial bacteria such as bifidobacteria and lactobacilli and can also be used as substitute sugars for diabetics.

### 3.6. NMR analysis

The structure of transglycosylation products from the cooperative action of amylomaltase and transglucosidase

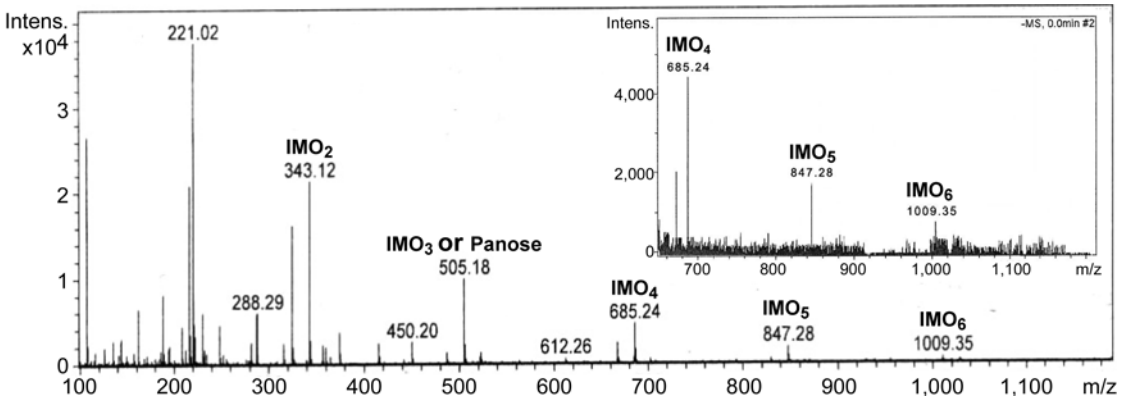


**Fig. 5.** Mass spectrum of the formed IMOs after the incubation of 20% (w/v) $G_3$, 8 U tranglucosidase, and 4.5 U amylomaltase for 30 min at 40°C and pH 6.0. The formed IMOs were analyzed by mass spectrometer after treatment with glucosidase. Enlarged view of $IMO_4$-$IMO_6$ is presented in the small picture.

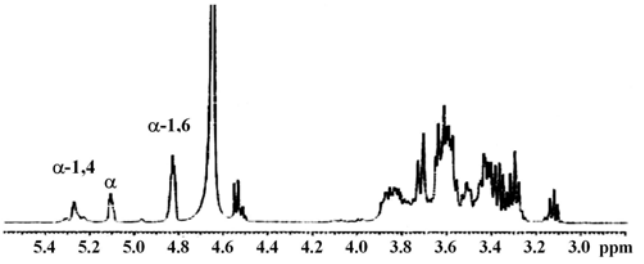


**Fig. 6.** $^{1}$H NMR spectrum of the formed IMOs from cooperative reaction of transglucosidase and amylomaltase with pure $G_3$. The formed IMOs were analyzed using $^{1}$H NMR spectrometer after treatment with glucosidase.

was confirmed by $^{1}$H NMR. The products from $G_3$'s transglycosylation reaction were identified to be oligosaccharides, having reducing end sugars with α-1,4 and α-1,6 glycosidic linkages, which appeared as prominent peaks at the chemical shifts of 5.25 and 4.96 ppm, respectively (Fig. 6). The peak's high area from the $^{1}$H NMR spectrum of reaction products suggested that the structure of formed IMOs (DP2-DP6) is mostly elongated with α-1,6 linkages and less with α-1,4 linkages.

## 4. Conclusion

Recently, various carbohydrate-modifying enzymes *i.e.* cyclodextrin glycosyltransferase, amylomaltase, and glycogen debranching enzyme, have received considerable attention in the food, pharmaceutical, and cosmetic industries. These enzymes are desirable for the production of useful carbohydrates such as cyclodextrin, prebiotc carbohydrates and branching oligosaccharides, among others. In this work, the amylomaltase gene from the p17bAMY recombinant cell was successfully expressed and purified by heat treatment, and the Q sepharose and phenyl sepharose columns. The purified thermostable amylomaltase and transglucosidase were tested both alone and in combination with $G_3$ to study their abilities on the production of IMOs. As results, the amylomaltase alone could produce transglycosylation products (maltooligosaccharides) from all of the maltooligosaccharides tested except $G_1$. However, when the amylomaltase was used in combination with transglucosidase, IMO products (DP2-DP6) were observed. The IMO's structure was identified by mass spectrometry and $^{1}$H NMR, which formed IMOs from the cooperative action of amylomaltase, while transglucosidase gave longer chains than the commercially available IMOs (≤ DP4). Kaneko *et al.* [25] reported that the long-chain IMOs are absorbed at a much lower degree and persist longer than the short-chain IMOs in the small intestine. Thus, there has been a tendency to prefer the long-chain IMOs over short-chain IMOs.

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