CHAPTER IV

DISCUSSION

4.1 Preparation and purification of amylomaltase from *C. glutamicum*

Crude amylomaltase was collected and purified by HisTrap affinity column chromatography as in previous report (Srisimarat *et al.* 2011). This recombinant amylomaltase contains 10 histidine residues at the N-terminus. The imidazole ring of histidine can bind to nickel (II) attached at stationary phase of the column and other proteins with nonspecific interaction were washed by the binding buffer with low concentration of imidazole. After that, elution buffer with high concentration of imidazole was applied which led to elution of the enzyme (Schmitt *et al.* 1993). From the purification table (Table 3.1), crude and purified enzyme showed 1.14 and 54.7 U/mg protein, respectively and the purity was 48 folds with 54.5% recovery. The crude enzyme showed similar specific activity to that reported previously by our group (Srisimarat *et al.* 2011). However, the specific activity of the purified enzyme was approximately two times higher because the protein content in this work was lower. And the recovery was also two times higher. The size of the purified enzyme as analyzed by 7.5% SDS-PAGE was 84 kDa (Figure 3.2) which is the same as that previously reported (Srisimarat *et al.*, 2011).

4.2 Determination of acceptor specificity and the synthesis of glucoside products

In previous work on the enzymes of the 4α-glucanotransferase family, several studies on acceptor specificity and the synthesis of glucoside products catalyzed by CGTase have been reported. For example, hesperidin glucosides were synthesized by

CGTase from an alkalophilic *Bacillus* species using soluble starch as donor, the products showed higher water solubility compared to natural hesperidin. (Kometani *et al.* 1994). The maltooligosaccharides with 63-79% yield were synthesized by CGTase from *Thermoanaerobacter* sp. using soluble starch as donor and glucose as acceptor (Martin *et al.* 2000). The synthesis of alkyl glucosides by CGTase from *Paenibacillus* sp. RB01 through intermolecular transglucosylation (Chotipanang 2011, Katelakha 2012) to improve surfactant and emulsifying activity were reported. Epicatechin glucosides (Aramsangtienchai *et al.* 2011) and cellobiose glucosides (Wongsangwattana *et al.* 2010) were also synthesized by this enzyme to improve their properties such as solubility and antioxidant activity. In contrast to CGTase, only a few number of acceptor study of amylomaltase and synthesis of glucoside products have been reported. Amylomaltase from *E. coli* IFO 3806 prefers α-hydroxyl group of hexose at position C4 (Kitahata *et al.* 1989). In 2011, a novel amylomaltase from *C. glutamicum* with 20-25% amino acid sequence identity to those previously reported amylomaltase was characterized (Srisimarat *et al.* 2011). By this enzyme, maltooligosylsucrose with an anticariogenic property was synthesized using sucrose and raw tapioca starch as glucosyl acceptor and donor, respectively (Saehu *et al.* 2013). And *thermus* sp. amylomaltase in combination with transglucosidase were used to synthesize IMOs with prebiotic activity (Rudeekulthamrong *et al.* 2013).

In this work on the acceptor specificity of amylomaltase from *C. glutamicum*, three groups of acceptors were determined: short chain alcohols, flavonoids and saccharides. Since alcohol solvent may affect enzyme activity and stability, both properties of amylomaltase were first analyzed in 1-30% (v/v) short chain alcohols. It was found that after 10 minutes incubation, this enzyme still retained acceptable

level of activity enough for catalysis of starch transglucosylation reaction in 1-20% (v/v) methanol, 1-10% (v/v) ethanol, 1-30% (v/v) propanol and 1% (v/v) butanol. The relative activity of the enzyme was decreased when the concentration and incubation time were increased. Amylomaltase was less stable to organic solvent than CGTase, CGTase from *Paenibacillus* sp. RB01was reported to be stable in 1-50% (v/v) short chain alcohols (methanol-butanol) (Katelakha 2012). Our result showed that all concentrations of short chain alcohols (methanol-butanol) did not lead to starch transglucosylation activity of amylomaltase, they could not act as glucosyl acceptor. This is in contrast to CGTase, the enzyme from *Paenibacillus* sp. RB01 could effectively use short chain (methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, and 2-butanol) (Chotipanang 2011) and medium chain alcohols (pentanol, isopentanol, 2-hexanol and cyclohexanol) as acceptor in the coupling activity assay.

In contrast to alcohols, saccharides were able to function as glucosyl acceptor for starch transglucosylation activity of this amylomaltase. Glucose led to the highest activity of *C. glutamicum* amylomaltase, the same result was found with CGTases from *B. stearothermophilus, B. circulans and B. macerans* (Kitahata *et al.* 1992). We found that mannose, fructose and galactose (listed in decreasing order of making active enzyme) were less effective than glucose. By comparison of these hexose acceptors with glucose, the configuration of OH group at C2 of mannose and C4 of galactose were different. Fructose is a ketose sugar while glucose is an aldose sugar. This result thus suggested that the configuration of OH at C4 was most important for amylomaltase, a change in OH configuration at this position significantly affected on its relative activity. Furthermore, an aldose sugar was preferred. Fucose and rhamnose as deoxy hexose, they have methyl group instead of CH_2OH at C6

which might affect binding with the enzyme, thus resulting in decreasing activity to lower than that revealed by mannose. This finding indicated that OH at C6 position was more important than at C2. For pentose sugar, arabinose and ribose, they gave significantly lower activities than that by glucose suggesting that the hexose structure was required for a compound to be an effective acceptor of amylomaltase. Within the disaccharide subgroup, the relative activity gave by maltose (glucose, α-1,4-glucose) > palatinose (glucose, α-1,6-fructose) ≥ sucrose (glucose, α-1,2-fructose) > cellobiose (glucose, β-1,4-glucose) > melibiose (galactose, α-1,6-glucose) > lactose (galactose, α-1,4-glucose), this result suggested that amylomaltase preferred the glucose structure linked by α-1,4 glycosidic bond. For the trisaccharide subgroup, amylomaltase preferred maltotriose to raffinose (galactose, α-1,6-glucose, α-1,2-fructose), while maltotetraose was the most preferred acceptor in the oligosaccharide subgroup. In addition, maltopentaose was rather poor acceptor while maltohexaose and maltoheptaose could not act as acceptor. From the results of the four subgroups of saccharides, the relative activity provided could be ranked in the decreasing order as glucose > maltose > maltotriose > maltotetraose. It can be concluded that this amylomaltase prefers hexose structure of the maltooligosaccharide series with up to 4 glucose residues, similar result was also reported for the amylomaltase from *E.coli* IFO 3806 (Kitahata *et al.* 1989). And the α-hydroxyl groups of hexose at position C2, C4, C6 are suitable for the active site of amylomaltase. This is similar to the study of CGTase from *B. circulans* A11 (Wongsangwattana 2000), CGTases from *B. macerans*, *B. megaterium* (Kitahata 1988) and 4α-glucanotransferase from *P. Kodakaraensis* KOD1 (Tachibana *et al.* 2000) were reported to prefer free OH groups at C2, C3 and C4 with the same configuration as

glucose. For our amylomaltase, C3 configuration of glucose may also be required, however, the saccharides tested could not lead to this conclusion. D-allose with different configuration at C3 compared to glucose should be tested as acceptor to confirm the requirement of the enzyme for this position.

Glucoside products from flavonoids could not be analyzed by iodine method of the starch transglucosylation activity assay because the color of flavonoids interfered the color of starch-iodine complex. Thus the glucoside products from flavonoid acceptor were analyzed only by TLC.

4.3 Analysis of glucoside products by TLC and HPLC

The glucoside products from flavonoids and short chain alcohols were separated by TLC using ethylacetate:acetic:water (3:1:1 by volume) as a nonpolar solvent system (Chotipanang 2011). The silica-coated TLC plate acts as an adsorbent stationary phase while nonpolar solvent acts as a mobile phase so nonpolar compounds can be soluble in this solvent and moved with larger distance (larger Rf value) than polar compounds. For standard G1-G3 (Figure 3.6), G1 is less polar so it was highly solubilized in this solvent system and moved with larger distance and appeared on the top of TLC plate. G3 is more polar thus moved with smaller distance. Soluble potato starch donor which showed low solubility in this solvent was detected at the origin whereas short chain alcohols were observed at solvent front. A colleague in our research group found that methyl monoglucoside was produced in the transglucosylation reaction catalyzed by CGTase from *Paenibacillus* sp. RB01 using β-cyclodextrin as glucosyl donor and methanol as an acceptor (Chotipanang 2011), so this reaction was used in our study as positive control to confirm the formation of a glucoside product. From the result, methyl

monoglucoside spot was found above the spot of β-cyclodextrin in lane 9 (Figure 3.6). Nevertheless, there was no glucoside product produced by amylomaltase when short chain alcohols were used as acceptor (lane 13, 15, 17 and 19, Figure 3.6), the result of which correlated well with that from acceptor specificity experiment using starch tarnsglucosylation activity assay. All of the results suggested that short chain alcohols (methanol-butanol) could not act as acceptor when soluble potato starch was used as donor for this amylomaltase. This might be due to the small size and inappropriate geometry of short chain alcohols so they could not fit to the active site of amylomaltase. However, these alcohols could bind to CGTase active site as the glucoside products were observed (Chotipanang 2011, Katelakha 2012). Besides CGTase, other enzymes capable of synthesizing glucosides from alcohol acceptor are levansucrase (Kim *et al.* 2000), dextransucrase (Kim *et al.* 2009) and β-glucosidase (Lirdprapamongkol and Svasti 2000).

Several work on flavonoid glucosides synthesis have been reported. Hesperidin, neohesperidin and naringin glucosides were synthesized by CGTase from alkalophilic *Bacillus* species using soluble starch as a glucosyl donor in an alkaline condition. The enzyme transferred the glucosyl units to OH group at C4 of hesperidin and C3 of neohesperidin and naringin producing their glucoside products. These glucoside products showed higher water solubility and lower bitterness than parent acceptor (Kometani *et al.* 1994, Kometani *et al.* 1996). Furthermore, three types of epigallocatechin gallate glucosides produced by glucansucrase from *L. mesenteroides* B-1299CB have been reported with 144 folds higher antioxidant activity (Moon *et al.* 2006). In our work, flavonoids tested consisted of hesperidin, naringin, pinostrobin, fisetin, epicatechin and epigallocatechin gallate. The glucoside

products were separated by TLC using ethylacetate:acetic acid:water (3:1:1 by volume) as a solvent. All flavonoids tested were rather non polar compound so they migrated with larger Rf. In Figure 3.7, reactions with hesperidin or naringin at 24 hours were analyzed compared to the reaction without acceptor to confirm the glucoside products. The reaction without acceptor showed only the spot of soluble potato starch at the origin (lane 6) indicating that amylomaltase, without acceptor, could not transfer the glucosyl unit to other starch molecules to form linear oligosaccharides. Reaction with naringin (lane 8) and hesperidin (lane 10) gave no glucoside product, they could not act as an acceptor. Pinostrobin, fisetin (Figure 3.8), epicatechin and epigallocatechin gallate (Figure 3.9) gave similar result, no glucoside products could be observed (lane 14, 16). In contrast, glucosides of naringin, hesperidin, epicatechin and epigallocatechin gallate could be synthesized by CGTases (Kometani *et al.* 1994, Aramsangtienchai *et al.* 2011). In our work, CGTase from *Paenibacillus* sp. RB01was used as positive control, and five spots of glucoside products were observed (lane 7 and 9, Figure 3.9). It was then concluded that all flavonoids tested in this study could not act as an acceptor for this amylomaltase. The geometry of acceptor sites in CGTase and amylomaltase was different though the two enzymes catalyze similar reaction with four activities (Takaha and Smith 1999). The active site of CGTase from *B. circulans* strain 251 (Figure 4.1) has at least nine subsites with three conserved catalytic amino acid residues (Glu257, Asp299 and Asp 328) that involved in substrate binding and catalysis (van der Veen *et al.* 2001) while amylomaltase from *T. thermophilus* HB8 also has three conserved amino acid residues (Asp293, Glu340 and Asp 395) but with at least seven subsites. Binding residues in the two enzymes were not the same though catalytic residues were the same. For binding function,

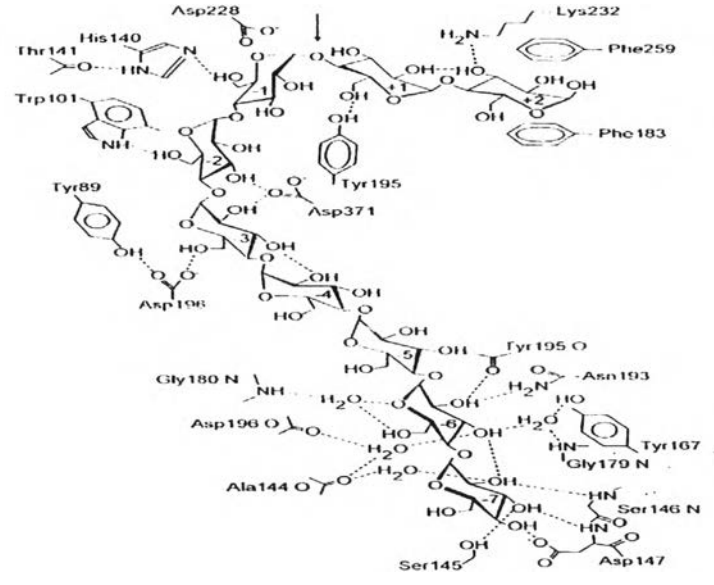
amylomaltase also has 250s loop above the active site that partially shield the active site for binding, and another loop at 470s was also proposed. However, CGTase does not have these loop structures. Having these unique loops, the structure might cause substrates like short chain alcohols and flavonoids tested in this study could not fit to the active site of amylomaltase (Kaper *et al.* 2007).

In conclusion, the results of acceptor specificity for short chain alcohols and flavonoids suggested that though they contain functional OH which is required for amylomaltase, these molecules could not fit to the enzyme active site which might be due to unsuitable size or nonfavorable interaction.

Glucoside products from saccharide acceptors were detected by TLC using butanol:pyridine:water (5:4:1 by volume) as a nonpolar solvent system (Saehu *et al.* 2013). Glucoside products were more polar than saccharide acceptors so they migrated with smaller Rf than parent saccharides. At least four types of glucoside products from glucose were detected in lane 6 (Figure 3.10) while more than five types of glucoside products from maltotriose and maltotetraose were observed in lane 6 and 9 (Figure 3.11). In Figure 3.12, the smear of glucoside products from mannose was found in lane 6 and at least 6, 3 and 4 spots of glucoside products from maltopentaose, palatinose and sucrose were detected in lane 9, 12, and 21, respectively. Mannose and palatinose were then selected as suitable acceptors for this amylomaltase to produce glucoside products on account of possibly new products with considerable amounts obtained.

Glucoside products from mannose and palatinose were separated by Resex RSO oligosaccharide Ag^{+} HPLC column using ion exclusion mechanism to separate

(a)



(b)

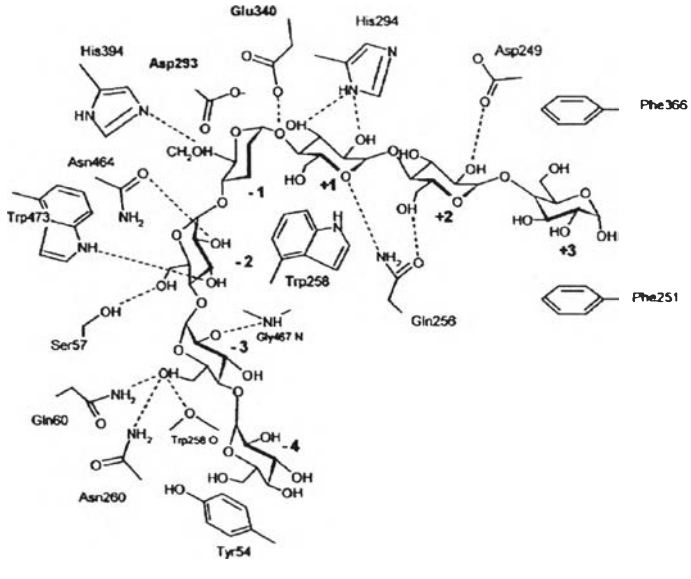


Figure 4.1 Schematic overviews of binding of maltononaose (G9) in the active site of CGTase from *B. circulans* strain 251 (a) and binding of maltoheptaose (G7) in the active site of amylomaltase from *T. thermophilus* HB8 (b).

neutral and ionic compounds (Papadoyannis and Samanidou 2005). The nonionic compounds entered to the pore of resins that linked by 4% crosslink sulfonated styrene-divinylbenzene sphere whereas ionic compounds were rejected. Then the nonionic compounds were eluted by ultrapure water. The yield of glucoside product from palatinose (53.6%) was significantly higher than that from mannose (3.67%) so palatinose was chosen as a suitable acceptor for the synthesis of glucosides products from this amylomaltase.

4.4 Optimization of the synthesis of glucoside products

The optimal temperature and pH of starch transglucosylation activity of amylomaltase from *C. glutamicum* have been reported. The enzyme displayed the maximum activity at 30 °C, pH 6.0 but it showed no activity at 50 °C (Srisimarat 2010). So, the optimal condition for synthesis of glucoside products by this amylomaltase was analyzed here by varying four parameters: concentration of acceptor, donor, enzyme and the incubation time. From the result, the optimized condition obtained for the synthesis of PGs was to incubate 5 U/ml enzyme with 7.5 mM palatinose and 1.0% (w/v) soluble potato starch in 50 mM phosphate buffer, pH 6.0 at 30 °C for 24 hours with 67.2% yield.

Preliminary characterization of glucoside products was performed by treating the products with glucoamylase from *A. niger* to investigate the linkage of PGs. Glucoamylase (1,4-α-glucosidase) is the exo-enzyme that mainly hydrolyzes α-1,4 linkage from the non-reducing end of oligosaccharides or starch molecules resulting in the release of glucose residues (Sauer *et al.* 2000). The enzyme can also hydrolyzes the α-1,6 linkage (Mertens and Skory 2007). Glucoamylase was added in the reaction mixture after the synthesis of PGs and the resulting sample was

analyzed by HPLC (Figure 3.18c). The chromatogram after glucoamylase treatment indicated that PGs were consisted of glucose residues connected to palatinose by an α-1,4 linkage. The yield of PGs obtained was 67.2% which was higher than the yield before optimization (53.6%). Using the same amylomaltase, maltooligosylsucrose was synthesized by this amylomaltase with a 81.7% transglucosylation yield using starch and sucrose as donor and acceptor substrates (Saehu *et al.* 2013). For other glucosides synthesized by different enzymes: 2-*O*-α-D-glucopyranosyl L-ascorbic acid catalyzed by CGTase showed a high 97% yield (Aga *et al.* 1991) while fructooligosaccharides with 49.8% yield were synthesized by fructofuranosidase (Markosyan *et al.* 2007). Thus the yield of PGs obtained in this study is acceptable.

4.5 Large scale preparation, product separation and identification

The reaction mixture was 100 times up scaled using the optimal condition and the reaction was concentrated to 2.5 ml before applying onto Biogel-P2 column to separate PGs. The separation was based on size. The stationary phase was polyacrylamide beads that formed by polymerization of N,N'-methylene-bis-acrylamide and the pore size was 40-50 μm. Biogel-P2 was effectively used to separate carbohydrates in the range of 100-1800 Da (Laboratories 2000). The small molecules could enter the pore while larger molecules were firstly eluted by filtrated ultrapure water. Eluted fractions were analyzed for sugar content by phenol-sulfuric method. Sulfuric acid was added to hydrolyze PGs to monosaccharides and the phenol interacted with these molecules producing orange color (Nielsen 2010). Soluble potato starch, PGs and palatinose were separated by this column which were confirmed by HPLC and HPAEC. PGs in peak c-m from Biogel column were

successfully separated and eight peaks of PGs were identified at Rt 24, 26, 27, 29, 31, 35, 39 and 44 by HPLC (Figure 3.20).

Moreover, peak c-h from HPLC with high concentrations of PGs were identified by HPAEC using Dionex CarboPac PA1 column. HPAEC is anionic exchange chromatography that is used to separate carbohydrate molecules through specific interaction between OH group of carbohydrate molecules and stationary phase. The separation based on size, composition, and linkage (Colleoni *et al.* 1999). Each following HPAEC peak represents addition of one glucose unit to preceding peak. The result showed that PG1-PG15 were identified (Figure 3.21) with the trisaccharide PG1 as a major product. Thus the largest PG identified in this study was PG15, resulting from addition of 15 glucose units, one by one, to the parent palatinose acceptor. The results here also suggested that HPAEC analysis demonstrated higher sensitivity of separation than HPLC.

Only one report on the enzymatic synthesis of PGs from palatinose has been found. In that study, the glucoside products from palatinose acceptor was synthesized by *T. brockii* Kojibiose phosphorylase. The products were separated by carbon-celite column chromatography and detected by HPAEC, in which the trisaccharide *O*-α-D-glucopyranosyl-(1,2)-*O*-α-D-glucopyranosyl-(1,6)-D-fructofura-nose) and tetrasaccharide (*O*-α-D-glucopyranosyl-(1,2)-*O*-α-D-glucopyranosyl-(1,2)-*O*- α-D-glucopyranosyl-(1,6)-D-fructofuranose) were main products (Takahashi *et al.* 2007). The linkage of the palatinose glucosides obtained was the α-1,2 glucosidic bond which was different from the PGs in this study in which the linkage was proved to be the α-1,4 by glucoamylase treatment and NMR result.

4.6 Characterization of glucoside products properties

MS and ^{1}H-NMR

The molecular weight of PGs were 504 Da and 666 Da confirm the trisaccharide and tetrasaccharide nature by NMR, the structure of PGs were palatinose connected to glucose units by α-1,4 linkage. The structure of PGs is as shown in Figure 4.2.

Sweetness test

The results showed that the sweetness of paltinose was about the same as sucrose. Interestingly, the sweetness of short chain and long chain PGs was lower than palatinose. The result agreed well with previous study that reported that the sweetness of long chain oligosaccharides was decreased when DP was increased (Nakakuki 1993). PGs were reported to be sweeter than fructooligosaccharides (Dominguez *et al.* 2014) and galactooligosaccharides (Tzortzis and Vulevic 2009), their sweetness was 0.3 times that of sucrose. The health benefit of low sweetness of PGs was apparent in using as sweetener in food and beverage products in replace of sucrose.

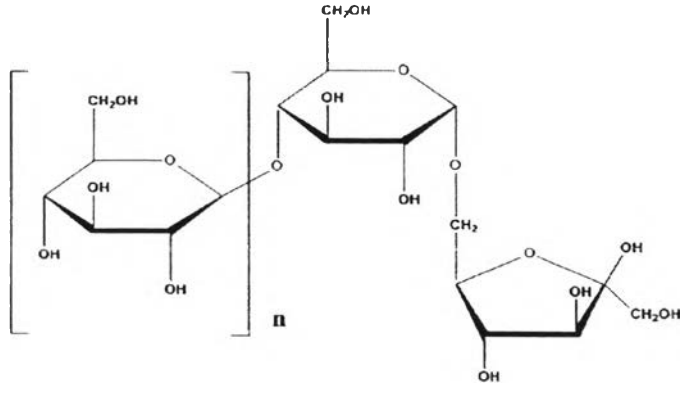


Figure 4.2 **The structure of palatinose glucosides $(PG)_n$,** n = 1-15 glucose units

Hygroscopic test

For one week of testing, the weight gain of long chain PGs was higher than those for short chain PGs and palatinose indicating that moisture was best adsorbed by long chain PGs. From previous reports, a disaccharide, neoagarobiose showed 11 times and 5 times higher in hygroscopic property than sodium hyaluronate and glycerol, respectively. For the hygroscopic property of oligosaccharides (DP2-DP7), the list in order is: DP3 > DP4 > = DP7 > DP5 > DP6 > DP2 (Donnelly *et al.* 1973). The substance with hygroscopic property such as sorbitol, sucrose and pectin were added to retain the moisture in food and cosmetic products such as in candy, cake, jam (Shin *et al.* 1988) and moisturizing cream (Kobayashi *et al.* 1997). PGs in this work could thus be used as a humectant in food and cosmetic products.

Prebiotic activity

A prebiotic is a non-digestible substance which stimulates the growth and activity of normal flora bacteria in the colon such as *Lactobacillus casei, Lactobacillus lactis, Bifidobacterium infantis and Bifidobacterium lactis* that effects on homeostasis of digestive system (Gibson and Mc Cartney 1998). At present, saccharide is the most interesting prebiotics due to health benefit such as prevention of pathogen colonization (Felley *et al.* 2001), lowering of serum cholesterol (Taylor and Williams 1998), and reduction of diarrhea (Rani 1998). In this study, the prebiotic property was tested by treating of palatinose and PGs with rat intestinal enzymes (Nimpiboon *et al.* 2011). The results showed that palatinose could be partially digested by these enzymes giving rise to glucose and fructose while glucosyl groups of both short chain and long chain PGs could be completely hydrolyzed. Rat

intestinal enzymes are mixture of α-amylase, α-glucosidase, glucoamylase, sucrase and isomaltase (Yang *et al.* 2005) so the α-1,4 linked-glucose from non-reducing ends of PGs could be completely hydrolyzed and glucose was released as product while the α-1,6 linkage between glucose and fructose in palatinose could be partially hydrolyzed. Thus palatinose and PGs have similar prebiotic activity.

Antibacterial activity

Palatinose, short chain and even long chain PGs showed no antibacterial property for *Escherichia coli* a gram negative and *Staphylococcus aureus* a gram positive. From previous studies, some saccharides were reported to have antibacterial activity. For example, maltotriose, maltotetraose, maltopentaose and maltohexaose at two concentrations tested (750 μg/ml and 3 mg/ml)showed antibacterial activity against *Erwinia carotovora* (Baetriz Serrano 1980), while combination of maltotetraose and adiposin-2 showed antibacterial activity against *Enterobacterialceae* (Kangouri *et al.* 1982). In another study, 0.5% chitooligosaccharides (DP3-DP6) completely inhibited the growth of *E. coli* (Jeon and Kim 2000).