CHAPTER III

RESULTS

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

The intracellular recombinant amylomaltase from *C. glutamicum* expressed in *E. coli* was prepared and purified by HisTrap affinity column chromatography as described in 2.6. The column profile (Figure 3.1) showed that the majority of protein was in unbound fractions and the enzyme with high starch transglucosylation activity (fraction 21-25) was eluted in the bound fraction. Fraction 22-24 were collected and dialyzed against 20 mM phosphate buffer, pH 7.4. The purification result is shown in Table 3.1. The specific activity of the purified enzyme was 54.7 U/mg protein. It was 48-fold purified with the yield of 55%.

After purification, the purity and the size of the enzyme were analyzed by SDS-PAGE (Figure 3.2). More than 15 protein bands were found in the crude enzyme fraction (lane 1) whereas a major band with 84 kDa of purified enzyme was observed in lane 2.

3.2 Determination of acceptor specificity and synthesis of glucoside products

Two groups of acceptor (saccharides and short chain alcohols) were analyzed for acceptor specificity by starch transglucosylation activity assay. For short chain alcohols, the effect of alcohol solvent on enzyme activity and stability was first investigated. The enzyme incubated with soluble potato starch in the presence of 1-30% (v/v) of various short chain alcohols at 30 °C for 1 hour. At start, less than 50%

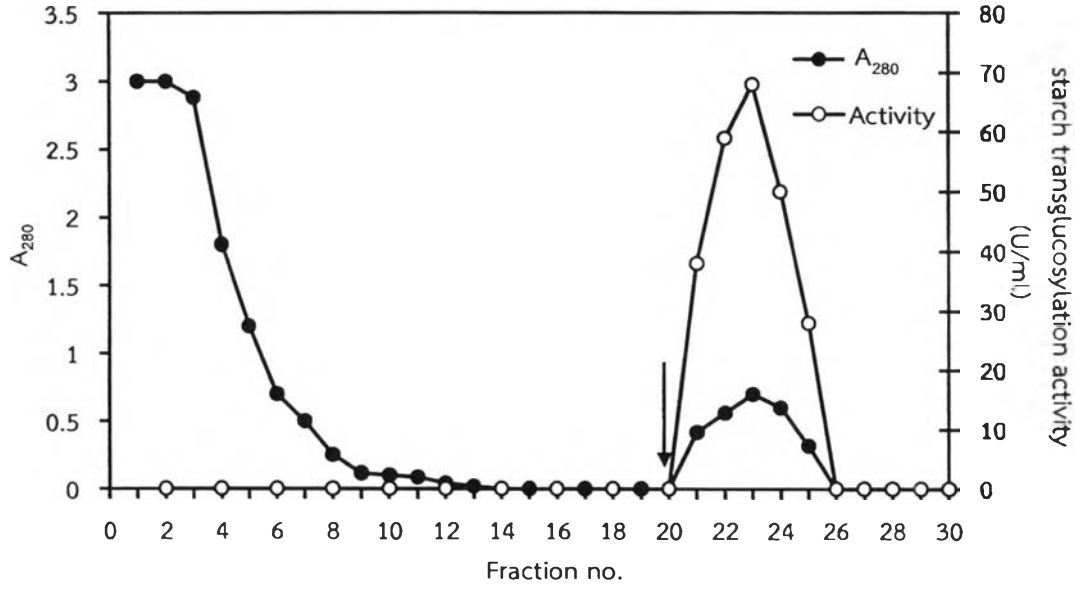


Figure 3.1 Purification chromatogram of amylomaltase from *C. glutamicum* by HisTrap affinity column

Crude amylomaltase was loaded onto the column (5×1 ml), the column was washed by binding buffer (0.5 M NaCl and 20 mM imidazole in 20 mM phosphate buffer, pH 7.4) and eluted by 500 mM imidazole in the same buffer. The flow rate was 1 ml/min and 3 ml of each fraction was collected. The arrow indicates where the elution starts.

Table 3.1 **Purification of recombinant amylomaltase from *C. glutamicum***

Purification Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Fold	Yield (%)
Crude enzyme	602	527	1.14	1	100
Purified enzyme	328	6.0	54.7	48.0	54.5

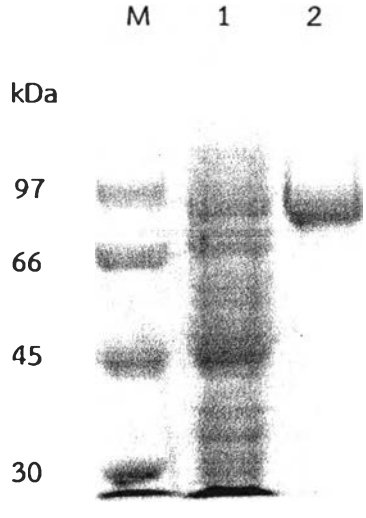


Figure 3.2 **Crude and purified recombinant amylomaltase from *C. glutamicum* as analyzed by 7.5% SDS-PAGE**

Lane M: Low molecular weight protein marker

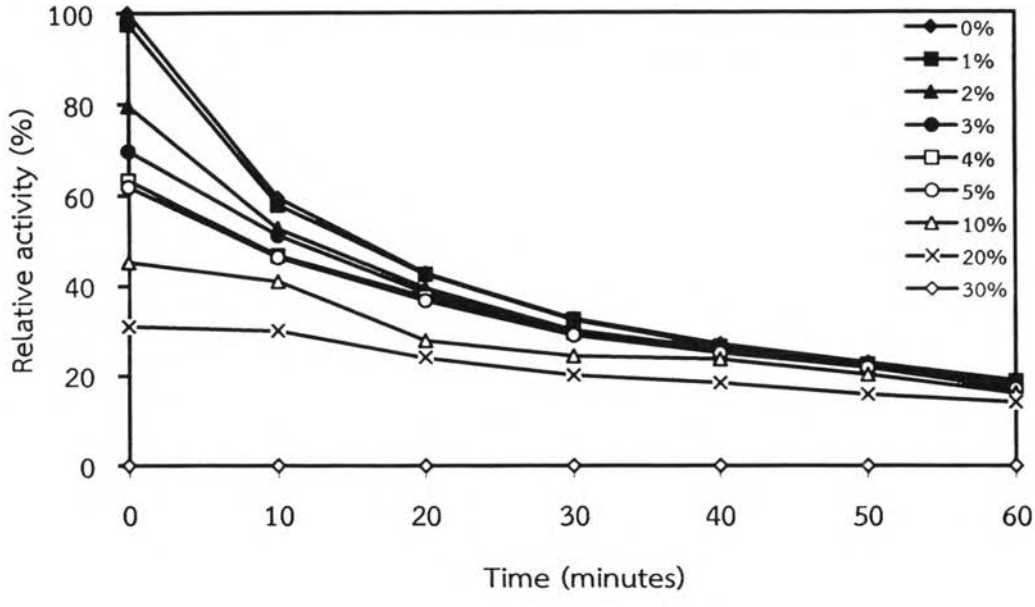
Lane 1: Crude enzyme 15 μg

Lane 2: Purified enzyme 3 μg

of relative activity was lost in the reaction containing 0-5% (v/v) methanol while up to 70% activity was decreased with 20% methanol and all activity was lost in presence of 30% methanol (Figure 3.3a). For enzyme stability, 20-30% of activity was remained in the presence of 1-20% (v/v) methanol. In the reaction containing ethanol at t_0, the activity was decreased about 60% in the reaction containing 10% (v/v) ethanol and the enzyme showed no activity in 20-30% (v/v) ethanol. After 60 minutes, the enzyme still had 20-40% activity in the presence of 0-10% (v/v) ethanol (Figure 3.3b). At initial time, the reaction containing propanol, 0-2% (v/v) propanol showed little effect on the activity whereas more than 2% (v/v) propanol showed pronounced effect. However, after 10 minutes, only 50% activity remained in the presence of 2% (v/v) propanol. And less than 20% relative activity was left in the presence of 3-30% (v/v) propanol after 60 minutes of incubation (Figure 3.4a). Finally, butanol showed the most effect on enzyme activity especially at the concentration higher than 1% (v/v) which led to no activity from the beginning of incubation to the end. Only in 1% (v/v) butanol that 35% of the relative activity could be detected after 60 minutes incubation (Figure 3.4b). From this experiment, at 10 minutes incubation time, the enzyme could still had some activity to catalyze transglucosylation reaction: 30-60% in ≤ 20% methanol; 30-80% in ≤ 10% ethanol; 55-65% in ≤ 2% propanol and 10-20% in ≤ 30% propanol; 60-80% in ≤ 1% butanol.

In determining acceptor specificity, the enzyme was incubated with 0.2% (w/v) soluble potato starch and 2.5 mM saccharide/1-20% (v/v) short chain alcohols (methanol to propanol, for butanol on 1% was used) in phosphate buffer, pH 6.0 at 30 °C for 10 minutes. As analyzed by starch transglucosylation activity, all of tested

(a)



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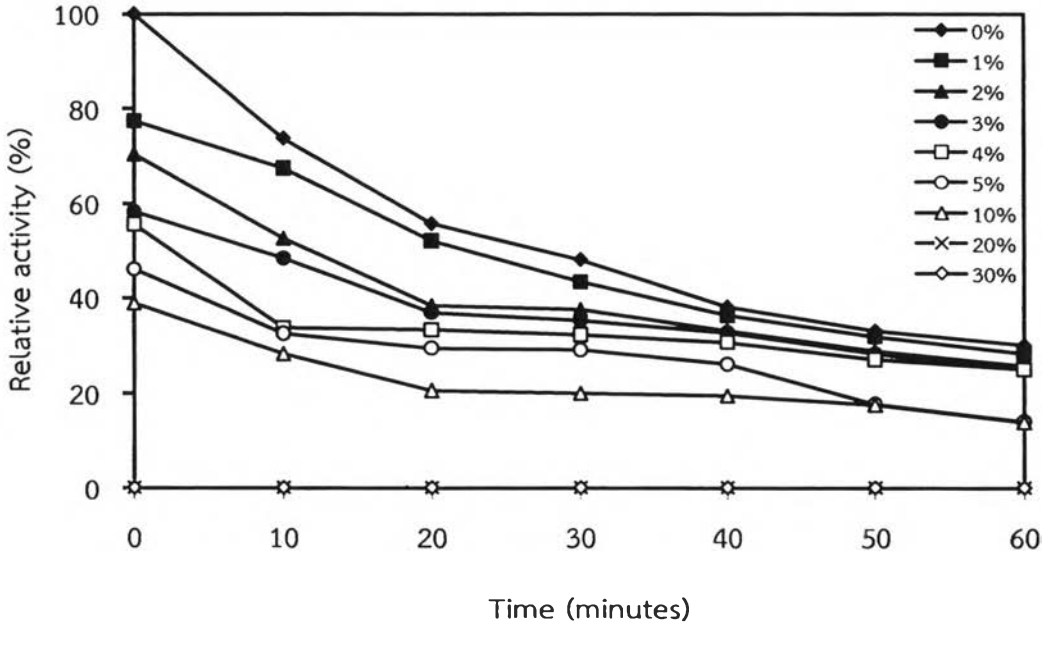
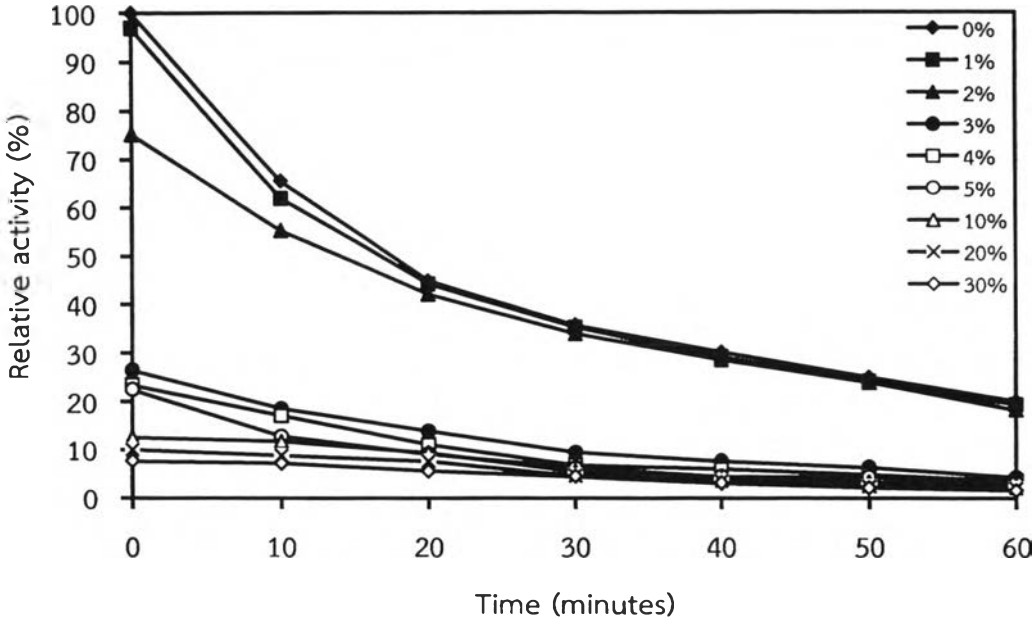


Figure 3.3 Enzyme stability in various concentrations (1-30%, v/v) of methanol (a) and ethanol (b)

(a)



(b)

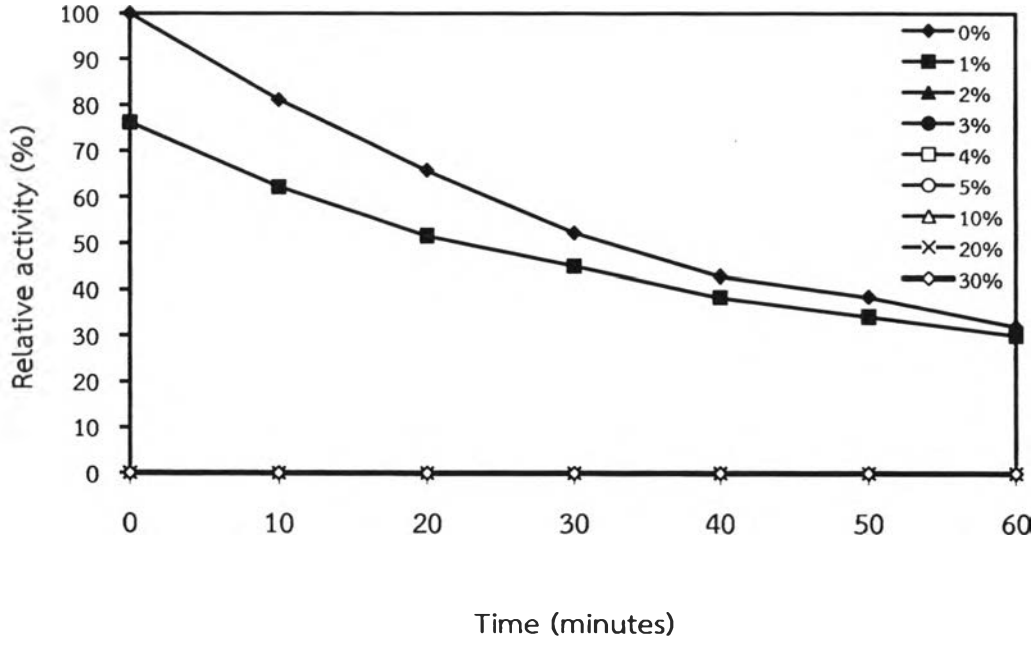


Figure 3.4 Enzyme stability in various concentrations (1-30%, v/v) of propanol (a) and butanol (b)

concentrations of short chain alcohols (methanol-butanol) demonstrated no activity indicating that they could not act as acceptor for amylomaltase. This is in contrast to saccharide acceptors in which most resulted in starch transglucosylation activity (Figure 3.5) suggesting that saccharide could act as an acceptor for this amylomaltase. In the experiment, monosaccharide subgroup was separated into four types: hexose, deoxy hexose, monosaccharide derivatives and pentose. For hexose, glucose was found to be the best acceptor and yielded the highest activity thus was set as 100% activity, followed by mannose, fructose and galactose with 24.7, 17.6 and 9.2% relative activity, respectively. Both fucose and rhamnose as deoxyhexose gave very low activity around 8%. For pentose sugar, arabinose and ribose resulted in 15-20% activity. For monosaccharide derivatives, ascorbic acid revealed the highest activity though of only about 25% whereas sugar alcohol like sorbitol and xylitol were poorer acceptor. Within the disaccharide subgroup, maltose gave the highest activity of 60%, followed by palatinose and sucrose with 36-37% relative activity while lactose yielded the least activity. Maltotriose and maltotetraose were the best of the trisaccharide and oligosaccharide subgroups and gave 46.4% and 27.3% relative activity, respectively. Maltohexaose and maltoheptaose did not give any activity.

The glucoside products synthesized by incubation of enzyme and short chain alcohol or flavonoid or saccharide acceptors with soluble potato starch donor were analyzed by TLC. On the TLC plate in Figure 3.6, standard glucose, maltose and maltotriose are shown in lane 1-3, respectively. Relative mobility (Rf) of glucose was higher than those of maltose and maltotriose, respectively. β-cyclodextrin moved slower than maltotriose (lane4) and soluble potato starch did not move at all

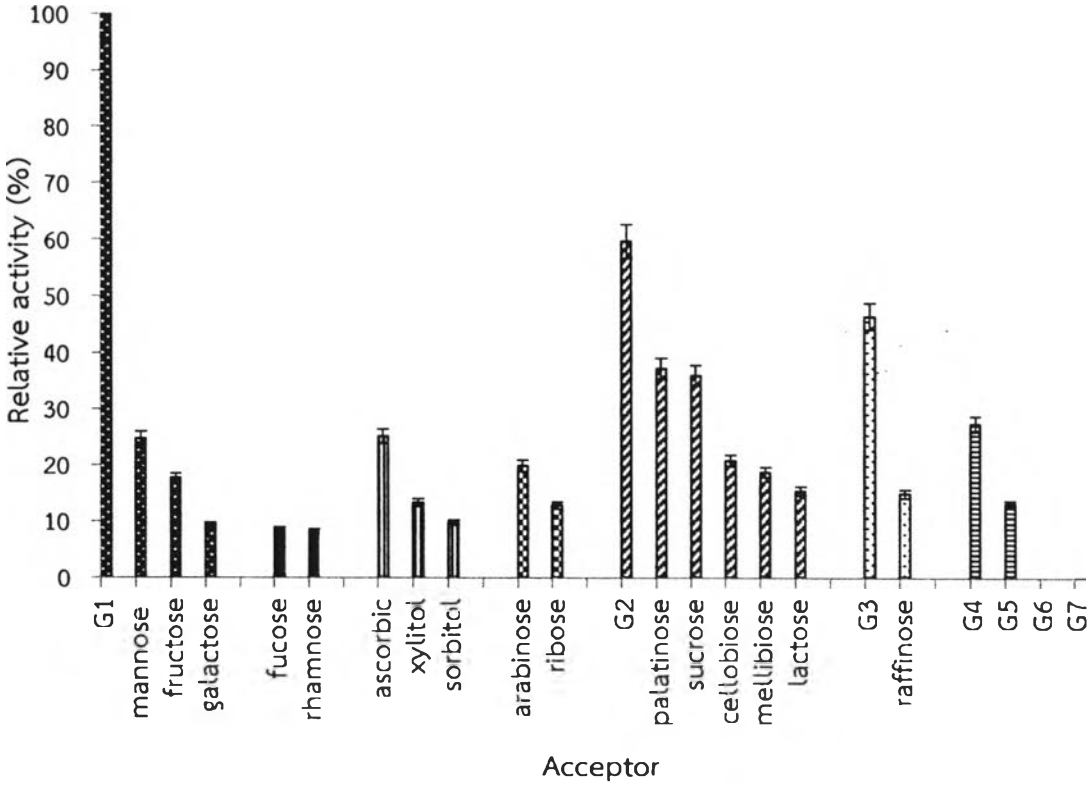


Figure 3.5 Relative starch transglucosylation activity of amylomaltase with different saccharide acceptors when 0.2% (w/v) soluble potato starch was used as the glucosyl donor

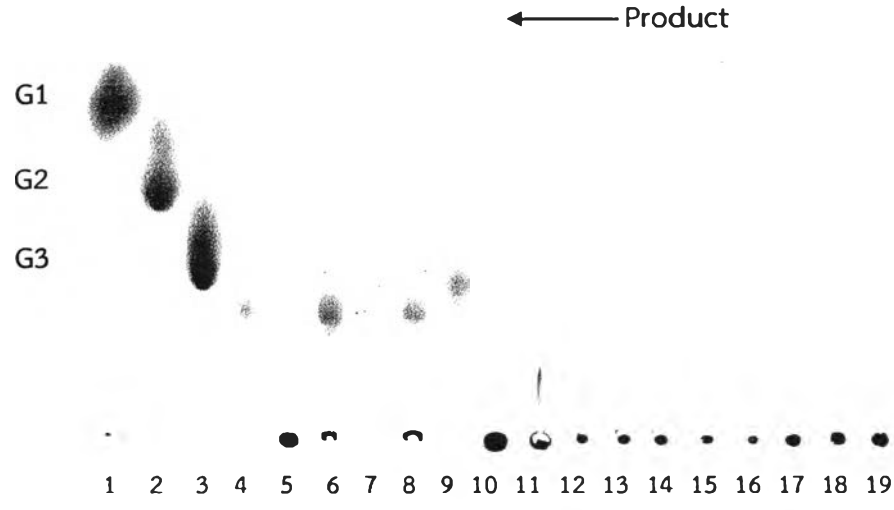


Figure 3.6 TLC chromatogram of reaction mixtures containing short chain alcohol acceptors using ethylacetate:acetic acid:water (3:1:1 by volume) as a mobile phase

Lane 1:	Glucose
Lane 2:	Maltose
Lane 3:	Maltotriose
Lane 4:	β-cyclodextrin
Lane 5:	Soluble potato starch
Lane 6-7:	Reaction of CGTase without acceptor at 0 and 24 hours
Lane 8-9:	Reaction of CGTase with 30% (v/v) methanol at 0 and 24 hours
Lane 10-11:	Reaction of amylomaltase without acceptor at 0 and 24 hours
Lane 12-13:	Reaction of amylomaltase with 1% (v/v) methanol at 0 and 24 hours
Lane 14-15:	Reaction of amylomaltase with 1% (v/v) ethanol at 0 and 24 hours
Lane 16-17:	Reaction of amylomaltase with 1% (v/v) propanol at 0 and 24 hours
Lane 18-19:	Reaction of amylomaltase with 1% (v/v) butanol at 0 and 24 hours

(lane5). The glucoside products synthesized by CGTase and amylomaltase were concurrently tested. Reaction without acceptor catalyzed by CGTase was tested as control to confirm glucoside products at 0 and 24 hours of incubation (lane 6-7). In lane 9, CGTase reaction with 30 % (v/v) methanol at 24 hours gave only one spot of glucoside product as shown by the arrow. For amylomaltase, the reaction without acceptor as control was tested at 0 and 24 hours and only spot of soluble potato starch was detected at the origin in lane 10 and 11. For the reaction mixtures with 1% methanol/ethanol/propanol/butanol, no glucoside product was found. When glusoside products synthesized by incubation of amylomaltase with soluble potato starch and various flavonoid acceptors were analyzed by TLC, all of flavonoid acceptors tested (hesperidin, naringin, pinostrobin, fisetin, epicatechin and epigallocatechin gallate) showed no glucoside products (Figure 3.7-3.9). Only the spot of flavonoid acceptor on the top and soluble potato starch at the origin of TLC plate were observed. In contrast, five types of epicatechin glucosides or epigallocatechin gallate glucosides in lane 9 and 11 (Figure 3.9) were detected in the reaction catalyzed by CGTase when β-CD was used as a glucosyl donor.

The saccharide acceptors with high relative starch transglucosylation activity were selected to qualitatively detect glucoside products by TLC. Glucoside products could be detected when glucose, maltotriose, maltotetraose, maltopentaose, mannose, palatinose and sucrose were used as the acceptor. However, fucose, ascorbic acid, arabinose, lactose, cellobiose and melibiose could not act as acceptor since glucoside product could not be detected. For glucose acceptor, at least four glucoside products were found at Rf 0.72, 0.66, 0.60 and 0.53 (Figure 3.10, Table 3.2)

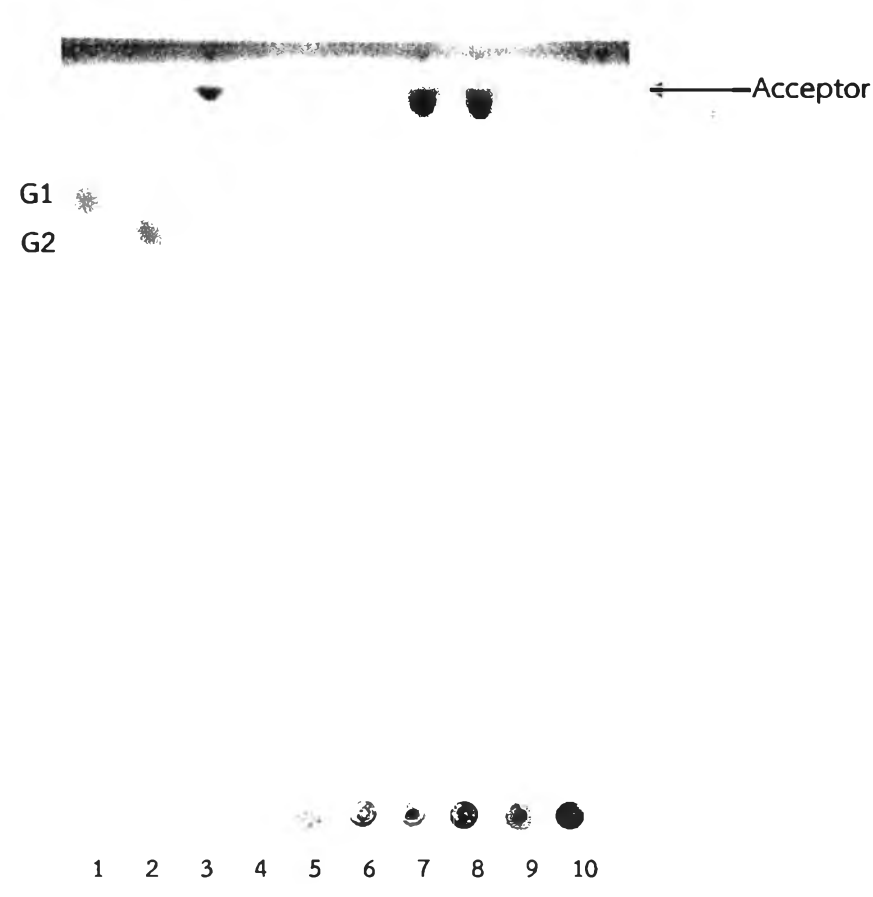


Figure 3.7 TLC chromatogram of reaction mixtures containing hesperidin and naringin acceptor using ethylacetate:acetic acid:water (3:1:1 by volume) as a mobile phase

Lane 1:	Glucose
Lane 2:	Maltose
Lane 3:	Naringin
Lane 4:	Hesperidin
Lane 5:	Soluble potato starch
Lane 6:	Reaction of amylomaltase without acceptor at 24 hours
Lane 7-8:	Reaction of amylomaltase with 2.5% (w/v) naringin at 0 and 24 hours
Lane 9-10:	Reaction of amylomaltase with 0.05% (w/v) hesperidin at 0 and 24 hours

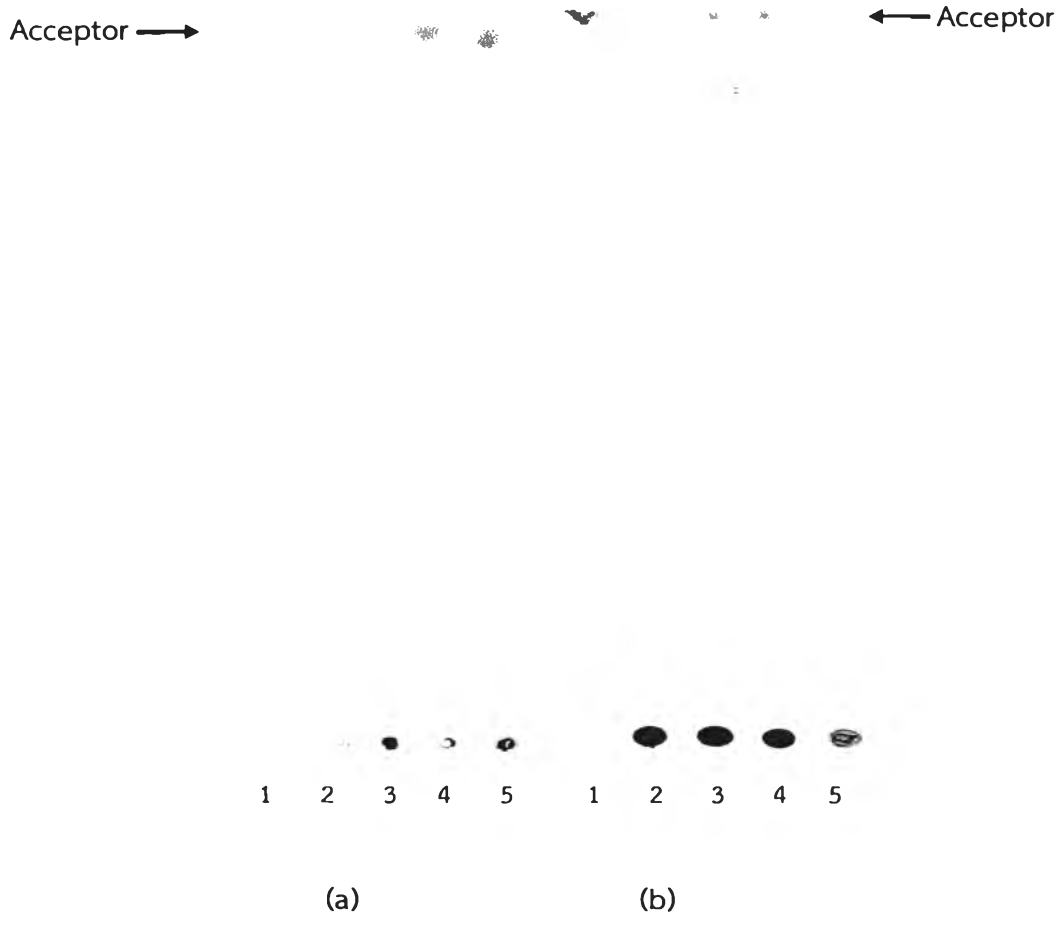


Figure 3.8 TLC chromatograms of reaction mixtures containing pinostrobin (a) and fisetin (b) acceptor using ethylacetate:acetic acid:water (3:1:1 by volume) as a mobile phase

Lane 1a:	Pinostrobin
Lane 2a:	Soluble potato starch
Lane 3:	Reaction of amylomaltase without acceptor at 24 hours
Lane 4a-5a:	Reaction of amylomaltase with 0.05% (w/v) pinostrobin at 0 and 24 hours
Lane 1b:	Fisetin
Lane 2b:	Reaction of amylomaltase without acceptor at 24 hours
Lane 3b-4b:	Reaction of amylomaltase with 0.05% (w/v) fisetin at 0 and 24 hours
Lane 5b:	Soluble potato starch

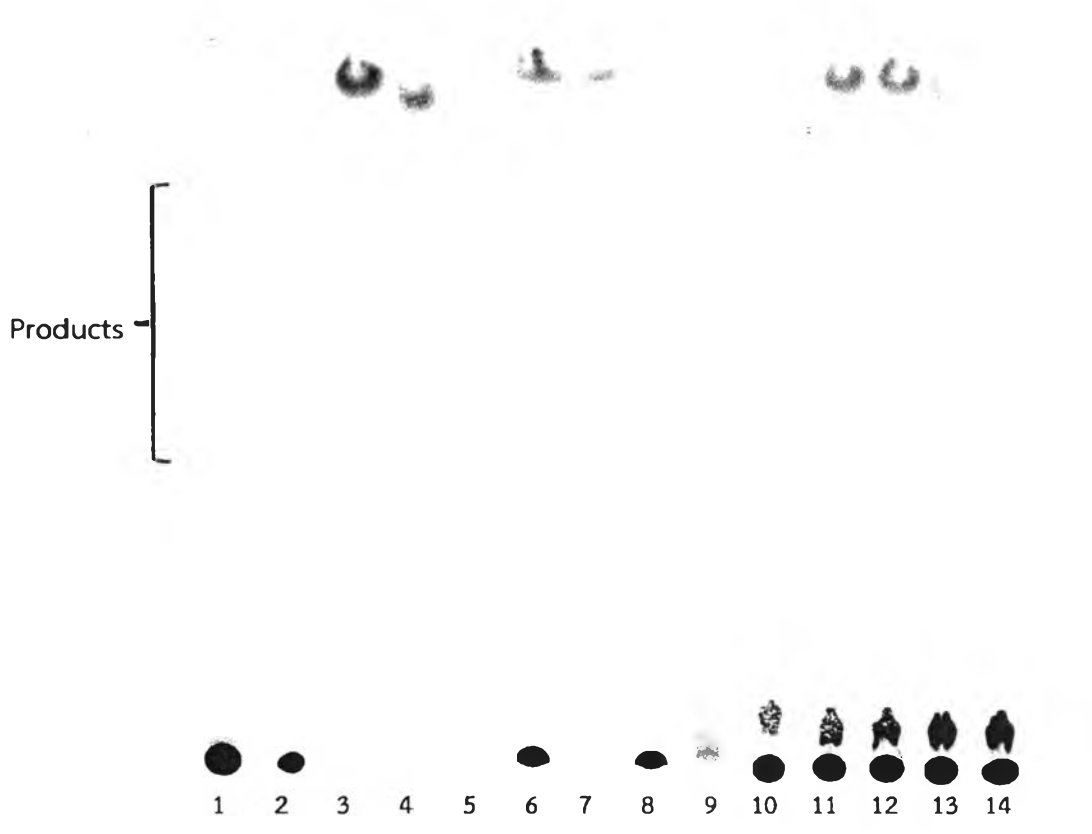


Figure 3.9 TLC chromatogram of reaction mixtures containing epicatechin and epigallocatechin gallate using ethylacetate:acetic acid:water(3:1:1 by volume) as a mobile phase

Lane 1:	β-cyclodextrin
Lane 2:	Soluble potato starch
Lane 3:	Epicatechin
Lane 4:	Epigallocatechin gallate
Lane 5:	Reaction of CGTase without acceptor at 24 hours
Lane 6-7:	Reaction of CGTase with 0.5% (w/v) epicatechin at 0, 24 hours
Lane 8-9:	Reaction of CGTase with 0.5% (w/v) epigallocatechin gallate at 0, 24 hours
Lane 10:	Reaction of amylomaltase without acceptor at 24 hours
Lane 11-12:	Reaction of amylomaltase with 0.5% (w/v) epicatechin at 0, 24 hours
Lane 13-14:	Reaction of amylomaltase with 0.5% (w/v) epigallocatechin gallate at 0, 24 hours

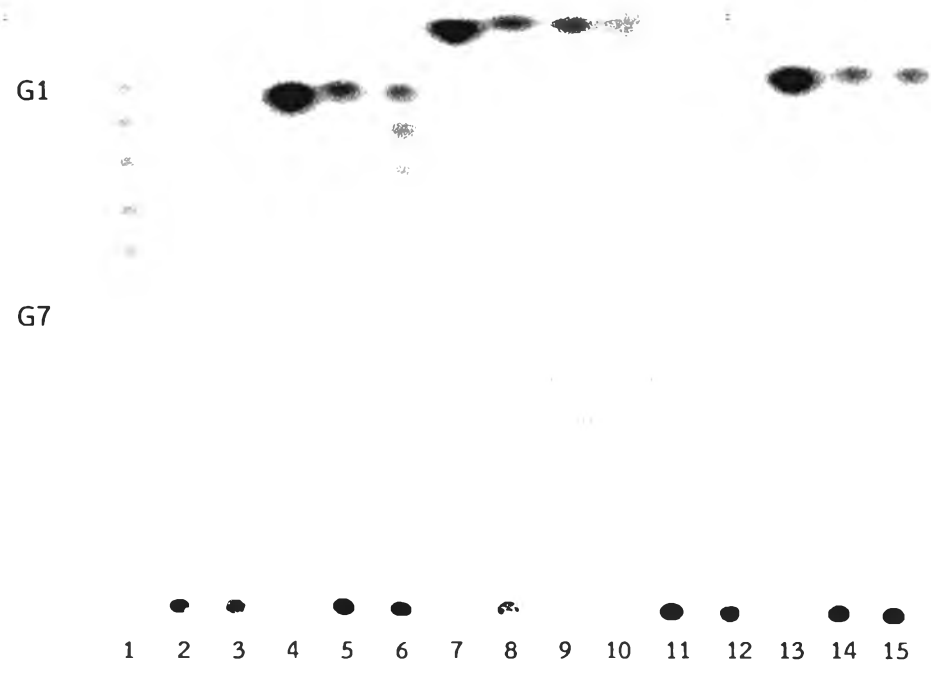


Figure 3.10 TLC chromatogram of reaction mixtures containing glucose, fucose, ascorbic acid and arabinose acceptor using butanol:pyridine:water (5:4:1 by volume) as a mobile phase

Lane 1:	Standard G1-G7
Lane 2:	Soluble potato starch
Lane 3:	Reaction of amylomaltase without acceptor at 24 hours
Lane 4:	Glucose
Lane 5-6:	Reaction of amylomaltase with 2.5 mM glucose at 0, 24 hours
Lane 7:	Fucose
Lane 8-9:	Reaction of amylomaltase with 2.5 mM fucose at 0, 24 hours
Lane 10:	Ascorbic acid
Lane 11-12:	Reaction of amylomaltase with 2.5 mM ascorbic acid at 0, 24 hours
Lane 13:	Arabinose
Lane 14-15:	Reaction of amylomaltase with 2.5 mM arabinose at 0, 24 hours

Table 3.2 Relative mobility (Rf) values of standard G1-G7 and glucoside products from glucose acceptor detected by TLC.

Standard/Acceptor	product	Rf
G1	-	0.78
G2	-	0.73
G3	-	0.67
G4	-	0.60
G5	-	0.53
G6	-	0.48
G7	-	0.45
Glucose	1 (G2)	0.72
	2 (G3)	0.66
	3 (G4)	0.60
	4 (G5)	0.53

while at least five glucoside products were detected when maltotriose/maltotetraose were used as the acceptor (Figure 3.11, Table 3.3), the products were found at Rf 0.78, 0.73, 0.65/0.58, 0.52 and 0.47. More than six glucoside products were detected from maltopentaose acceptor at Rf 0.76, 0.70, 0.60, 0.51, 0.38 and 0.31 whereas glucoside products from mannose could not be separated and identified (Figure 3.12). For palatinose acceptor, at least three glucoside products were observed at Rf 0.57, 0.50, 0.43 while at least four glucoside products from sucrose were detected at Rf 0.65, 0.58, 0.51 and 0.47 (Table 3.4).

3.3 Analysis of glucoside products by TLC and HPLC

As analyzed by TLC, several saccharides were good acceptors for this amylomaltase. Maltooligosaccharide acceptors (G1 to G5) and sucrose gave glucoside products as MOS and maltooligosylsucrose, respectively, these products are not new and their synthesis have been reported. Therefore the two remain acceptors which gave considerable products: mannose and palatinose, were selected for further analysis of glucoside products by TLC and HPLC.

TLC analysis was re-performed to confirm glucoside products from both acceptors, at least five mannose glucosides (MGs) and four PGs were detected (Figure 3.13). Then these glucoside products were analyzed by HPLC. For mannose acceptor, only soluble potato starch peak at Rt 20-26 minutes was found in the reaction without acceptor (Figure 3.14a) while peaks of soluble potato starch at Rt 20-26 minutes and mannose at Rt 77 minute were detected in reaction at 0 hour (Figure 3.14b) and nine peaks of glucoside products were observed at Rt 29, 31, 33, 35, 38, 41, 46, 51 and 58 minutes with 3.67% total yield calculated from the equation

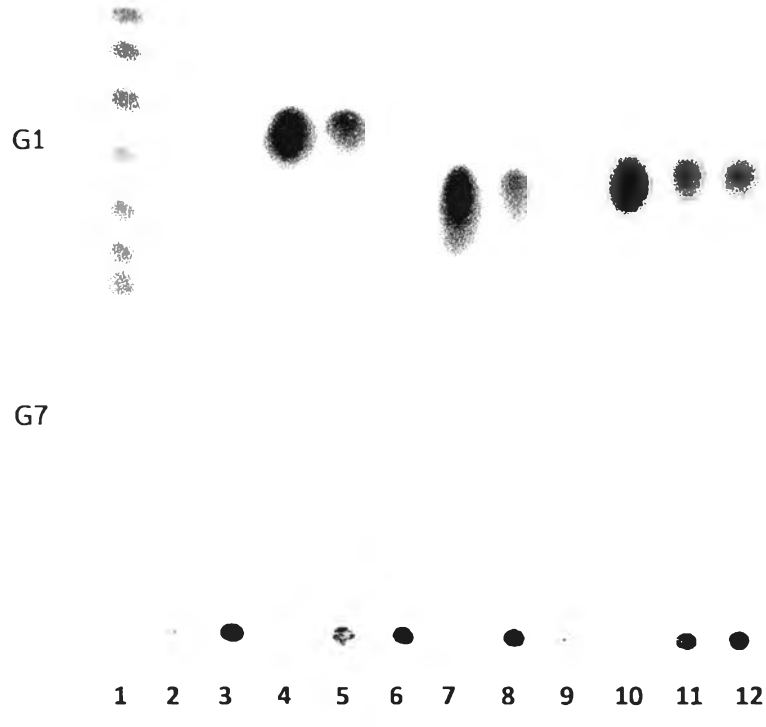


Figure 3.11 TLC chromatogram of reaction mixtures containing maltotriose, maltotetraose and lactose acceptor using butanol:pyridine:water (5:4:1 by volume) as a mobile phase

Lane 1:	Standard G1-G7
Lane 2:	Soluble potato starch
Lane 3:	Reaction of amylomaltase without acceptor at 24 hours
Lane 4:	Maltotriose
Lane 5-6:	Reaction of amylomaltase with 2.5 mM maltotriose at 0, 24 hours
Lane 7:	Maltotetraose
Lane 8-9:	Reaction of amylomaltase with 2.5 mM maltotetraose at 0, 24 hours
Lane 10:	Lactose
Lane 11-12:	Reaction of amylomaltase with 2.5 mM lactose at 0, 24 hours

Table 3.3 Relative mobility (Rf) values of standard G1-G7 and glucoside products from maltotriose and maltotetraose

Standard/Acceptor	product	Rf
G1	-	0.81
G2	-	0.76
G3	-	0.70
G4	-	0.62
G5	-	0.56
G6	-	0.50
G7	-	0.45
Maltotriose	1 (G2)	0.78
	2 (G3)	0.73
	3 (G4)	0.58
	4 (G5)	0.52
	5 (G6)	0.47
Maltotetraose	1 (G2)	0.79
	2 (G3)	0.73
	3 (G4)	0.65
	4 (G5)	0.52
	5 (G6)	0.47

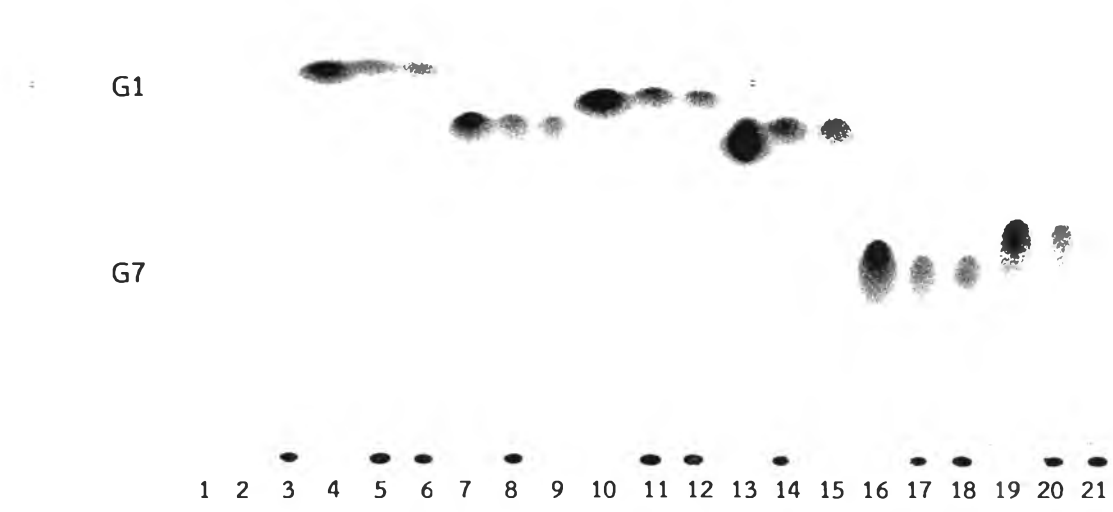


Figure 3.12 TLC chromatogram of reaction mixtures containing mannose, palatinose, sucrose, cellobiose, melibiose and maltopentaose using butanol:pyridine:water (5:4:1 by volume) as a mobile phase

Lane 1:	Standard G1-G7
Lane 2:	Soluble potato starch
Lane 3:	Reaction of amylomaltase without acceptor at 24 hours
Lane 4:	Mannose
Lane 5-6:	Reaction of amylomaltase with 2.5 mM mannose at 0, 24 hours
Lane 7:	Palatinose
Lane 8-9:	Reaction of amylomaltase with 2.5 mM palatinose at 0, 24 hours
Lane 10:	Sucrose
Lane 11-12:	Reaction of amylomaltase with 2.5 mM sucrose at 0, 24 hours
Lane 13:	Cellobiose
Lane 14-15:	Reaction of amylomaltase with 2.5 mM cellobiose at 0, 24 hours
Lane 16:	Melibiose
Lane 17-18:	Reaction of amylomaltase with 2.5 mM melibiose at 0, 24 hours
Lane 19:	Maltopentaose
Lane 20-21:	Reaction of amylomaltase with 2.5 mM maltpentaose at 0, 24 hours

Table 3.4 Relative mobility (Rf) values of standard G1-G7 and glucoside products from mannose, palatinose, sucrose and maltopentaose

Standard/Acceptor	product	Rf
G1	-	0.77
G2	-	0.71
G3	-	0.63
G4	-	0.55
G5	-	0.49
G6	-	0.44
G7	-	0.40
Mannose	Could not be separated	
Palatinose	1	0.57
	2	0.50
	3	0.43
Sucrose	1	0.65
	2	0.58
	3	0.51
	4	0.47
maltopentaose	1	0.76
	2	0.70
	3	0.60
	4	0.51
	5	0.38
	6	0.31

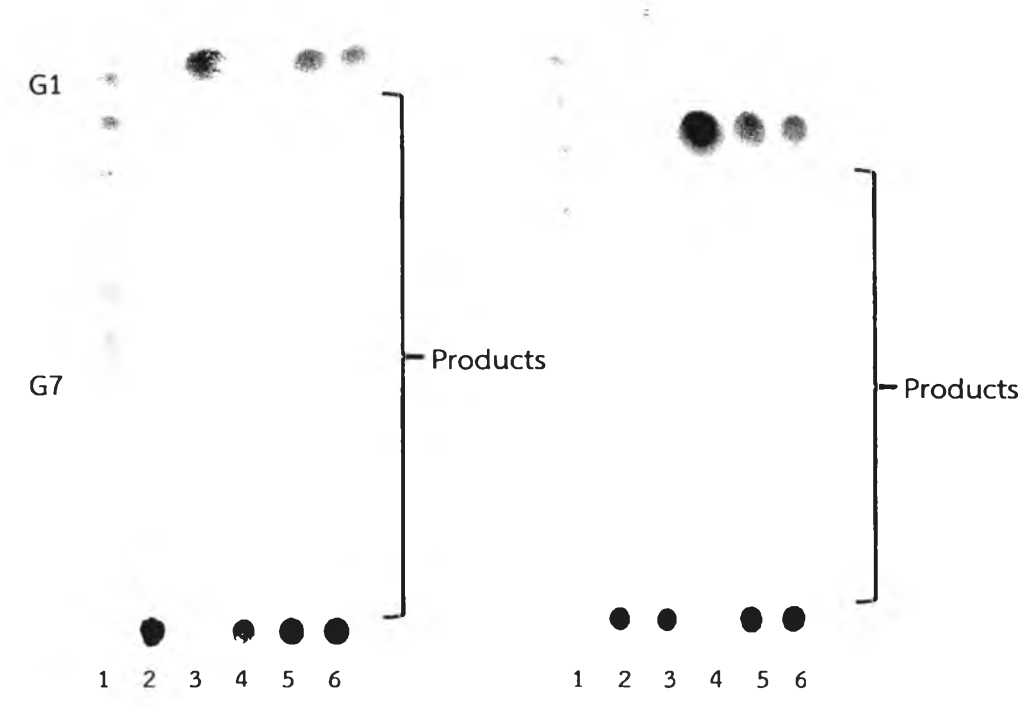


Figure 3.13 TLC chromatograms of glucoside products from mannose (a) and palatinose (b) acceptors using butanol:pyridine:water (5:4:1 by volume) as a mobile phase

Lane 1a:	Standard G1-G7
Lane 2a:	Soluble potato starch
Lane 3a:	Mannose
Lane 4a:	Reaction of amylomaltase without acceptor at 24 hours
Lane 5a-6a:	Reaction of amylomaltase with 2.5 mM mannose at 0, 24 hours
Lane 1b:	Standard G1-G7
Lane 2b:	Soluble potato starch
Lane 3b:	Palatinose
Lane 4b:	Reaction of amylomaltase without acceptor at 24 hours
Lane 5b-6b:	Reaction of amylomaltase with 2.5 mM palatinose at 0, 24 hours

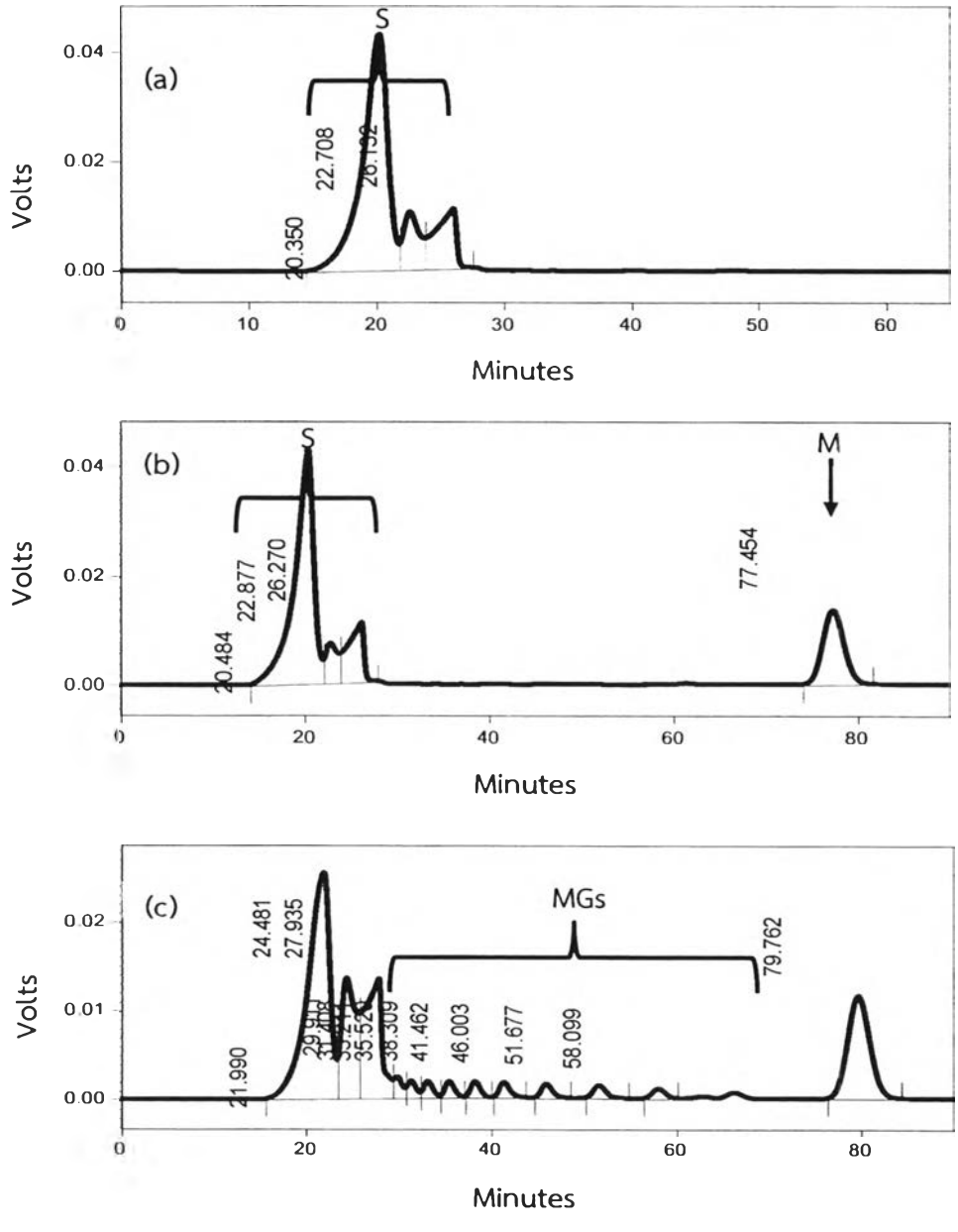


Figure 3.14 **HPLC chromatograms of glucoside products synthesized by amylomaltase using two substrates: soluble potato starch donor and mannose acceptor.** S = soluble potato starch, M = mannose, MGs = mannose glucosides

(a) Control reaction without mannose acceptor at 24 hours

(b-c) Glucoside products from reaction with mannose at 0, 24 hours

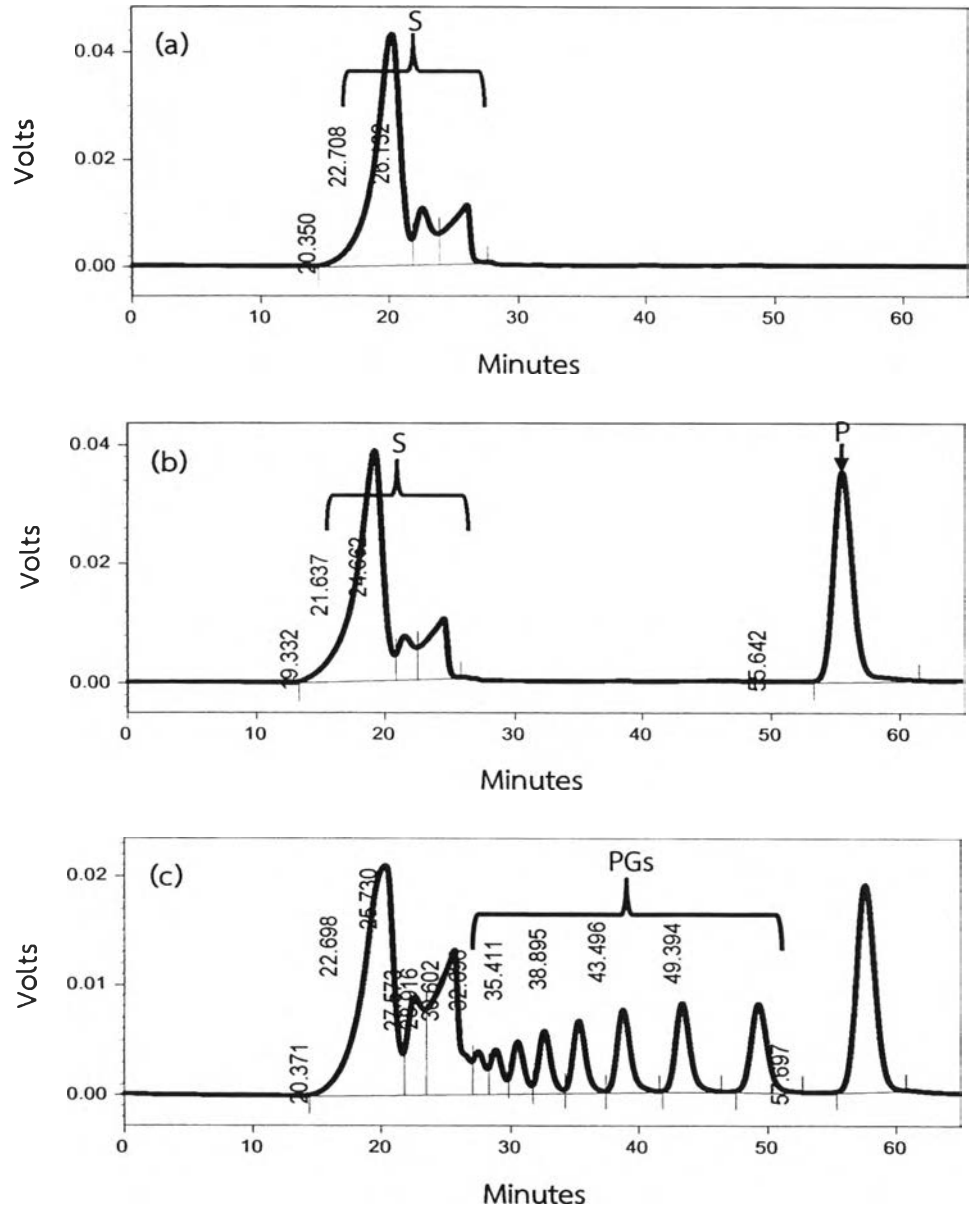


Figure 3.15 **HPLC chromatograms of glucoside products synthesized by amylomaltase using two substrates: soluble potato starch donor and palatinose.** S = soluble potato starch, P = palatinose, PGs = palatinose glucosides

(a) Control reaction without palatinose acceptor at 24 hours

(b-c) Glucoside products from reaction with palatinose at 0, 24 hours

described in 2.12 (Figure 3.14c). For palatinose acceptor, only soluble potato starch peak at Rt 20-26 minutes was found in the reaction without acceptor (Figure 3.15a) while two peaks of soluble potato starch at Rt 19-24 and palatinose at Rt 55 minute were observed in the reaction with palatinose acceptor at 0 hour (Figure 3.15b). At 24 hours incubation, eight peaks of glucoside products at Rt 27, 28, 30, 32, 35, 38, 43 and 49 minutes with total yield of about 53.6% were obtained (Figure 3.15c). The yield of glucoside products from palatinose acceptor was higher than that of mannose. Thus, palatinose was chosen as a suitable acceptor for amylomaltase.

3.4 Optimization of the synthesis of glucoside products

To optimize the synthetic process for highest yield of the glucoside products, four parameters were necessarily studied.

Effect of acceptor concentration

The condition was analyzed as described in 2.13. 5.0% (w/v) soluble potato starch was incubated with 3 U/ml of enzyme and various palatinose concentrations as acceptor at 30 °C for 24 hours. The peak area values of glucoside products resulted from HPLC was determined and plotted against palatinose concentration. The results are shown in Figure 3.16a, the peak area value was slightly increased in the reaction containing 0.0-2.5 mM palatinose. After that, peak area value was significantly increased and gave highest value in the reaction containing 7.5 mM palatinose. The peak area value was dramatically decreased at 15 mM palatinose. Thus 7.5 mM palatinose was chosen as optimal acceptor concentration.

Effect of donor concentration

The condition was analyzed as described in 2.13. Optimal acceptor concentration was incubated with 3 U/ml of enzyme and various soluble potato starch concentrations as glucosyl donor at 30 °C for 24 hours. It was found that the peak area value of glucoside products was sharply increased in the range of donor concentration of 0-2.0% (w/v). After that, the peak area value slightly dropped when donor concentration was increased (Figure 3.16b). From these results, though 2.0% (w/v) starch gave 10% higher products than 1.0% (w/v) starch, the 1.0% concentration was selected as the suitable donor concentration on account of easy handling of the reaction mixture.

Effect of enzyme concentration

Optimal acceptor and donor concentrations were incubated with various enzyme concentrations at 30 °C for 24 hours as described in 2.13. The peak area value of glucoside products in the reaction containing 0-1.0 U/ml of enzyme was significantly increased. Then the peak area value slightly increased in the reaction containing 1.0-5.0 U/ml enzyme and showed the highest value at 5.0 U/ml of enzyme (Figure 3.17a). After that it was rather constant in the reaction containing more than 5 U/ml enzyme. So the appropriate enzyme concentration was 5 U/ml.

Effect of incubation time

The reaction consisted of optimal acceptor, donor and enzyme concentrations was incubated at 30 °C for various incubation time as described in 2.13. The peak area of glucoside products from 0 to 6 hours of incubation was extremely increased. After that, the peak area value gradually and slightly increased

and showed the highest value at 24 hours incubation. However, the reaction incubated longer than 24 hours showed slowly decreasing of peak area value. From these results, a suitable time of incubation was 24 hours (Figure 3.17b).

3.5 Preliminary characterization of glucoside products

Preliminary characterization of the product was performed to confirm the linkage of PGs. Glucoside products were produced by incubation of enzyme with soluble potato starch as a donor and palatinose acceptor using optimal condition and the products were detected by HPLC. The reaction catalyzed by amylomaltase at 0 hour showed two peaks of soluble potato starch at Rt 16-23 minutes and palatinose at Rt 51 minutes (Figure 3.18a). At 24 hours, eight peaks of PGs at Rt 24, 26, 27, 29, 31, 34, 39 and 44 minutes were found (Figure 3.18b). Then the reaction catalyzed by amylomaltase at 24 hours was treated with 40 U/ml of glucoamylase from *Aspergillus niger* to hydrolyze α-1,4 glycosidic linkages of glucose units. All PG products disappeared and an additional peak of glucose appeared at Rt 62 minutes (Figure 3.18c). The yield of glucoside products using the optimized condition for synthesis obtained in Figure 3.18b was 67.2%.

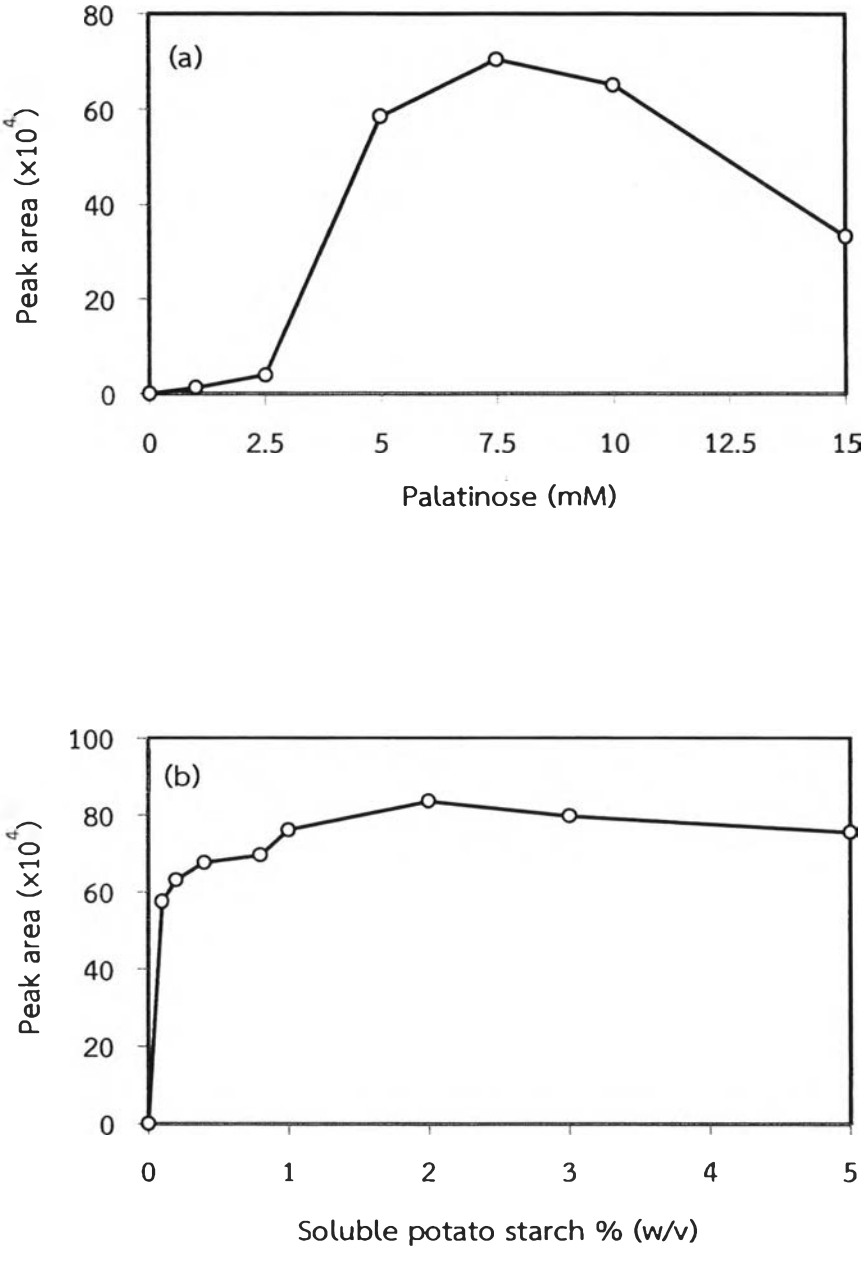


Figure 3.16 Effect of palatinose concentration (a) and soluble potato starch concentration (b) on the synthesis of glucoside products

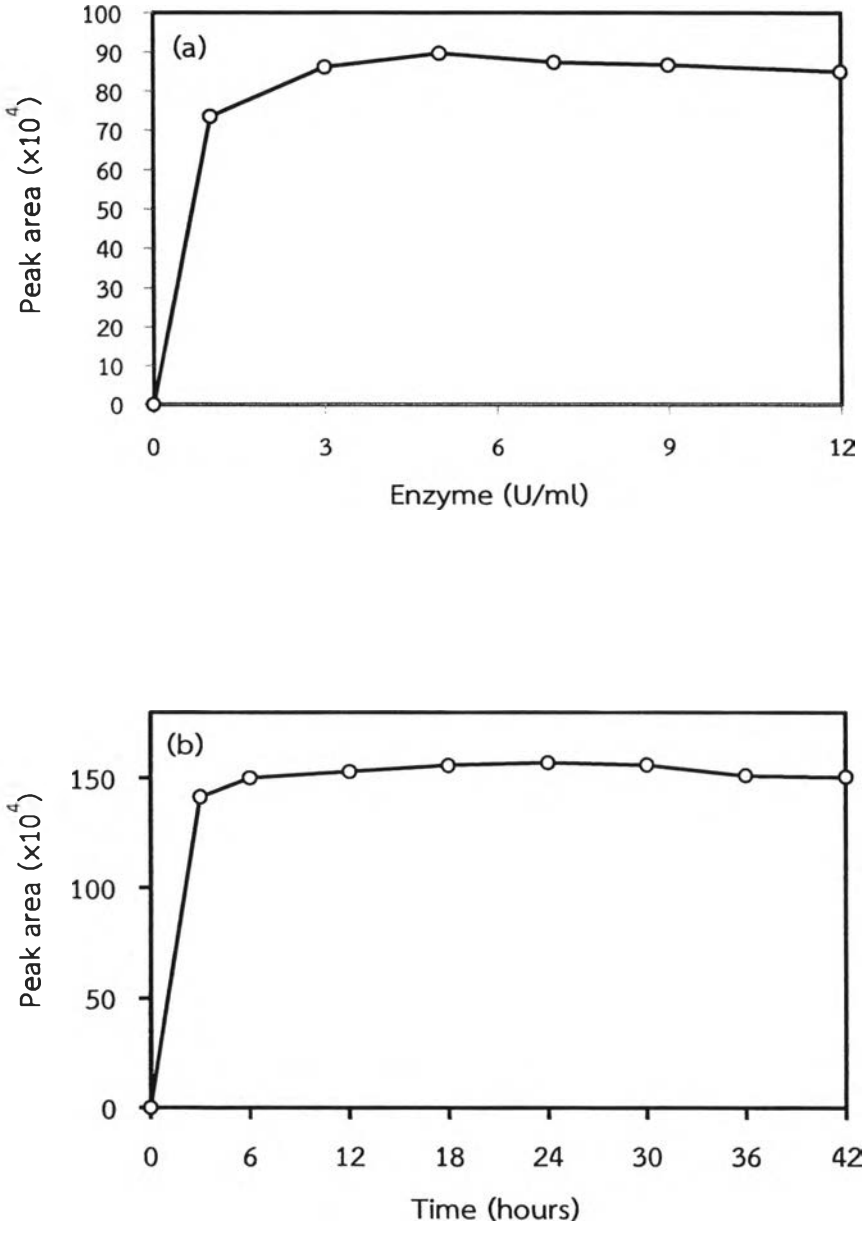


Figure 3.17 Effect of enzyme concentration (a) and incubation time (b) on the synthesis of glucoside products

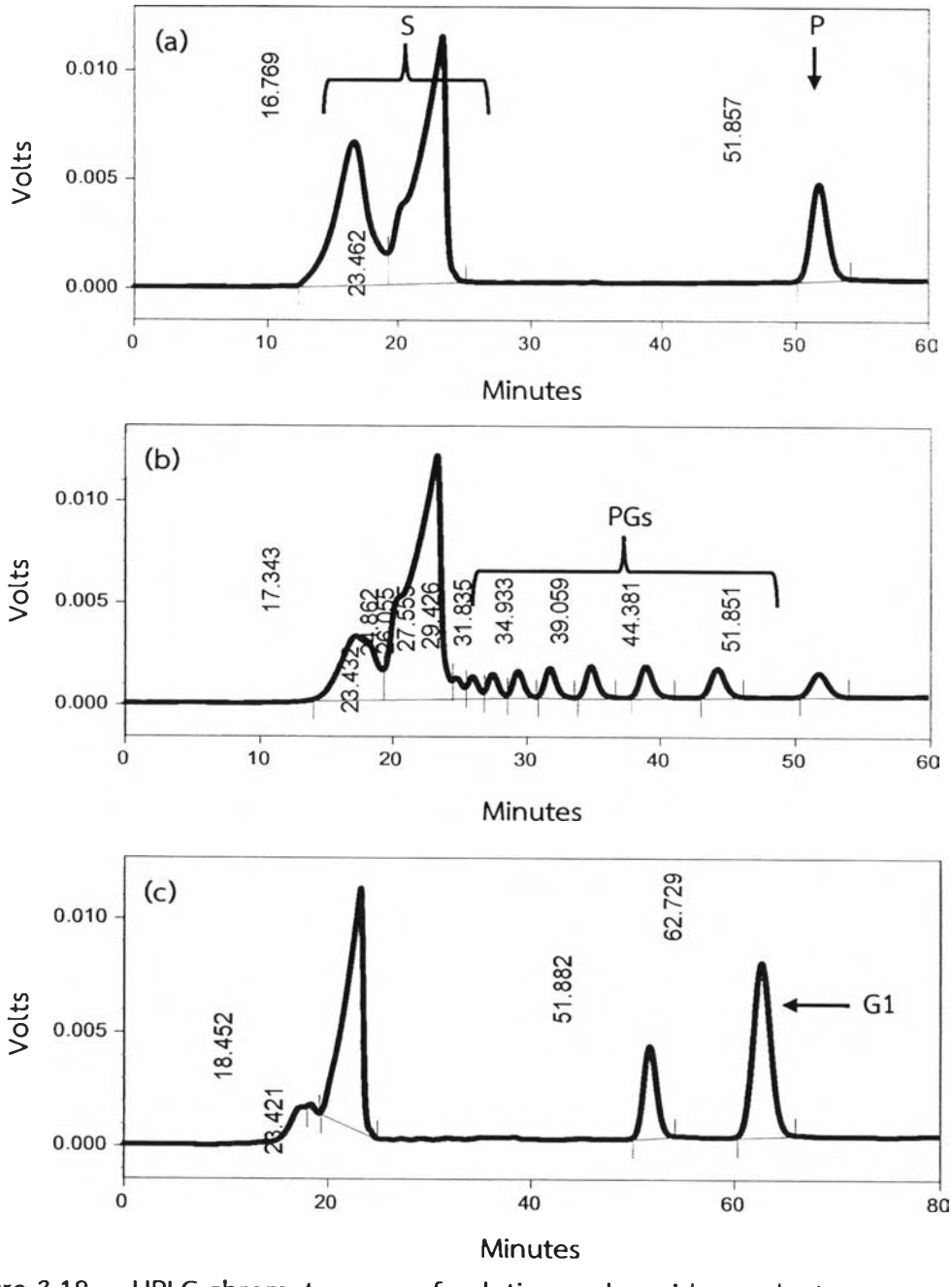


Figure 3.18 HPLC chromatograms of palatinose glucoside products

S = soluble potato starch, P = palatinose, PGs = palatinose glucosides, G1 = glucose

(a,b) Glucoside products from reaction catalyzed by amylomaltase at 0, 24 hours

(c) Glucoside products from b and treated with glucoamylase

3.6 Large scale preparation, product separation and identification

The reaction mixture was prepared at optimal condition and up scaled from 600 µl to 60 ml, the reaction was 100-fold enlarged. The remain soluble potato starch in the reaction was removed by centrifugation and supernatant was concentrated to 2.5 ml. Then sample was applied onto a Biogel-P2 column for separation of products, and sugar content was monitored. More than nine sharp peaks were observed on the Biogel-P2 profile (Figure 3.19) and fourteen Biogel-P2 peaks (a-n) were further identified by HPLC and HPAEC.

Analysis of PGs by HPLC (Figure 3.20) showed that soluble potato starch donor (Rt 18-21minutes) was observed in peak a and b from Biogel-P2 profile whereas glucoside products were found in peak c to m. However, every fraction of glucoside products still contained the peaks of remain soluble potato starch (Rt 18-21 minutes), and the late fractions (j to n) also contained palatinose (Rt 51 minutes). The largest PG was found at Rt 24 (peak c-f) and the smallest PG was detected at Rt 44 (peak g-f). From these results, soluble potato starch, PGs and palatinose could be separated by Biogel-P2 column and HPLC and eight peaks of PGs were observed at Rt 24, 26, 27, 29, 31, 34, 39 and 44 minutes.

HPAEC analysis was also used to further examine glucoside products compared with standard G1-G7. PGs in the peak c-h from Biogel-P2 column were further analyzed by HPAEC, the results showed that long chain of glucoside products (PG1-PG15) were observed in peak c-d while PG1-PG10 as short chain of glucoside products were detected in peak e-h. The result showed that PG1-PG15 could be separated by HPAEC and PG1 was a main product obtained (Figure 3.21).

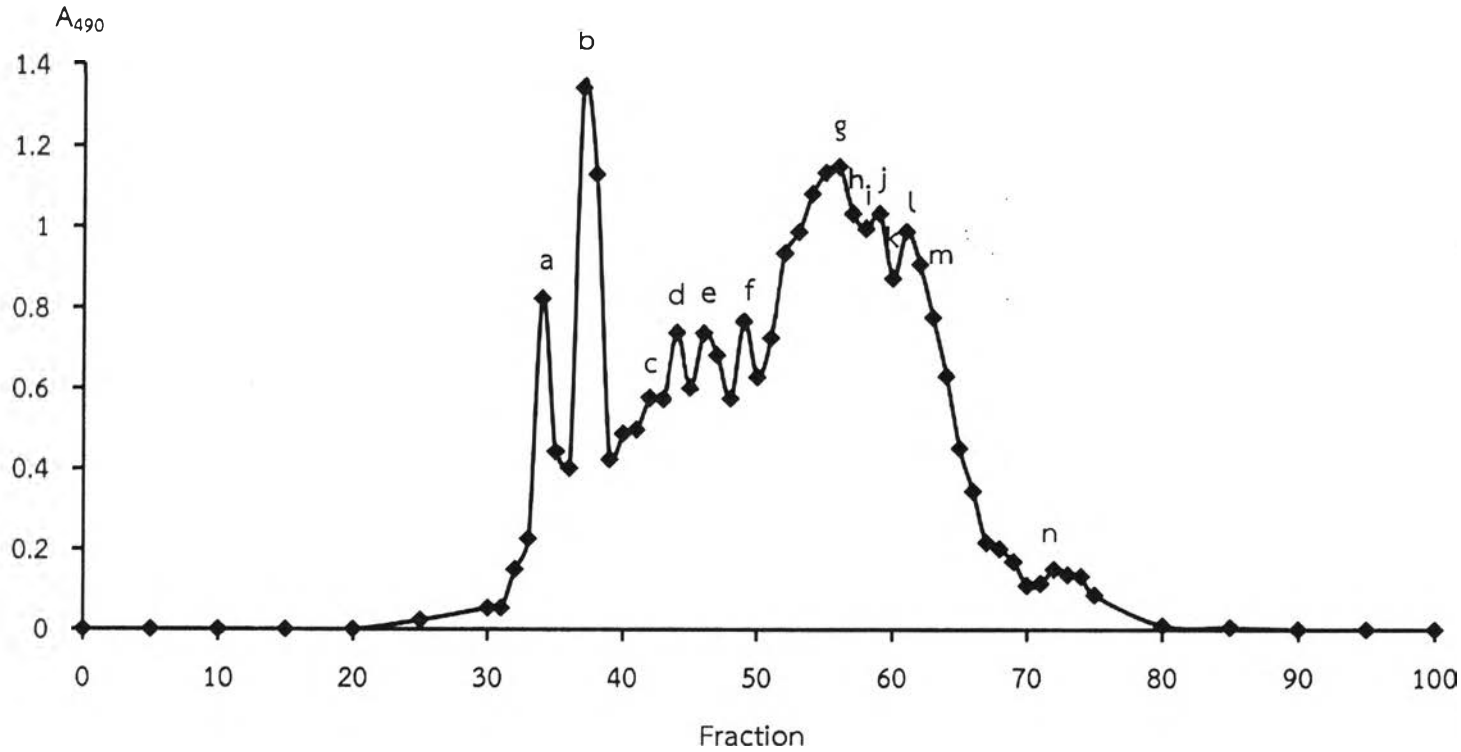


Figure 3.19 **Biogel-P2 profile (column size 1.2 x 97 cm) of palatinose glucosides.** Eluted with ultrapure water at a flow rate of 8 ml/hr at room temperature, fraction size was 1 ml. The sugar content was monitored spectrophotometrically by phenol-sulfuric acid method at 490 nm. Peaks a to n were collected.

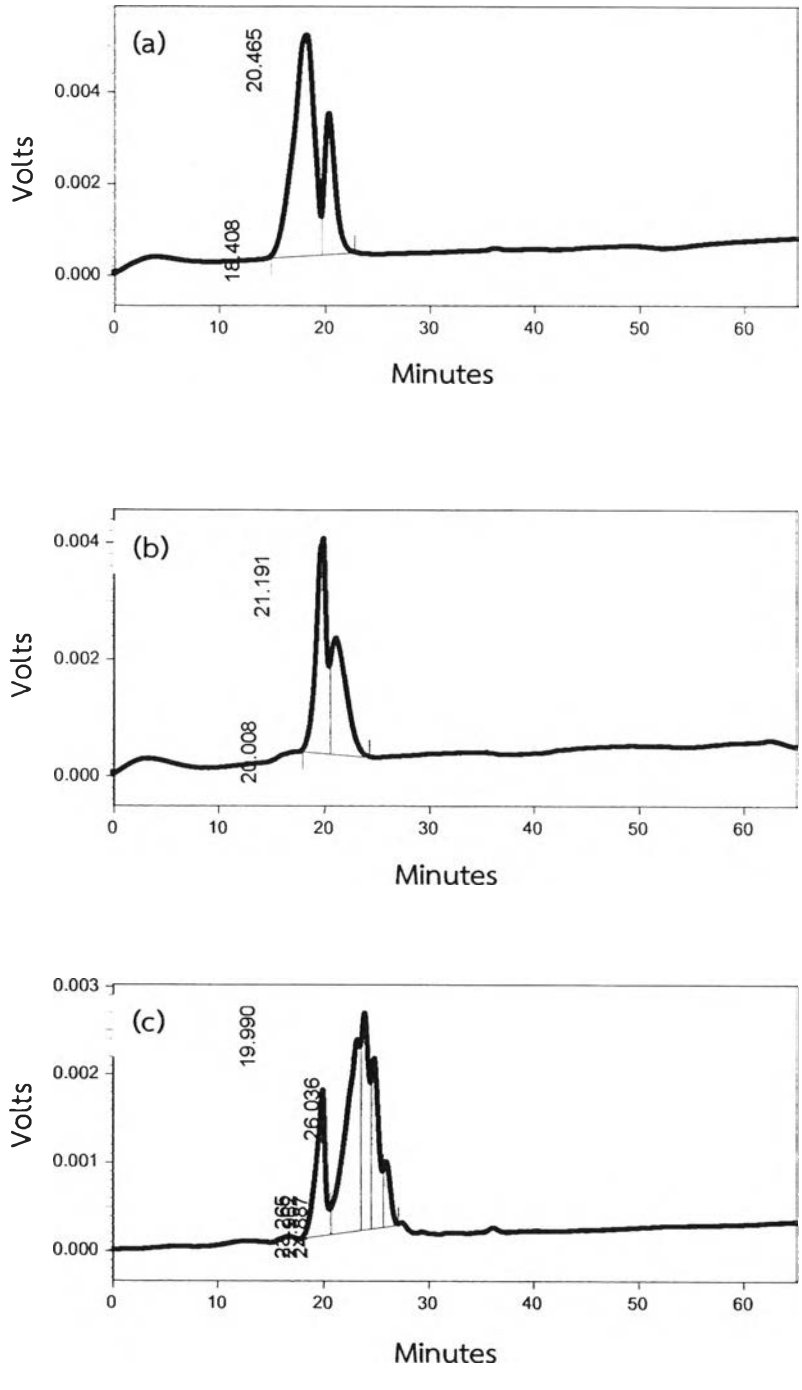


Figure 3.20 **HPLC chromatograms of palatinose glucosides previously separated by Biogel-P2 column.** (a) to (n) were separated peaks on Biogel column.

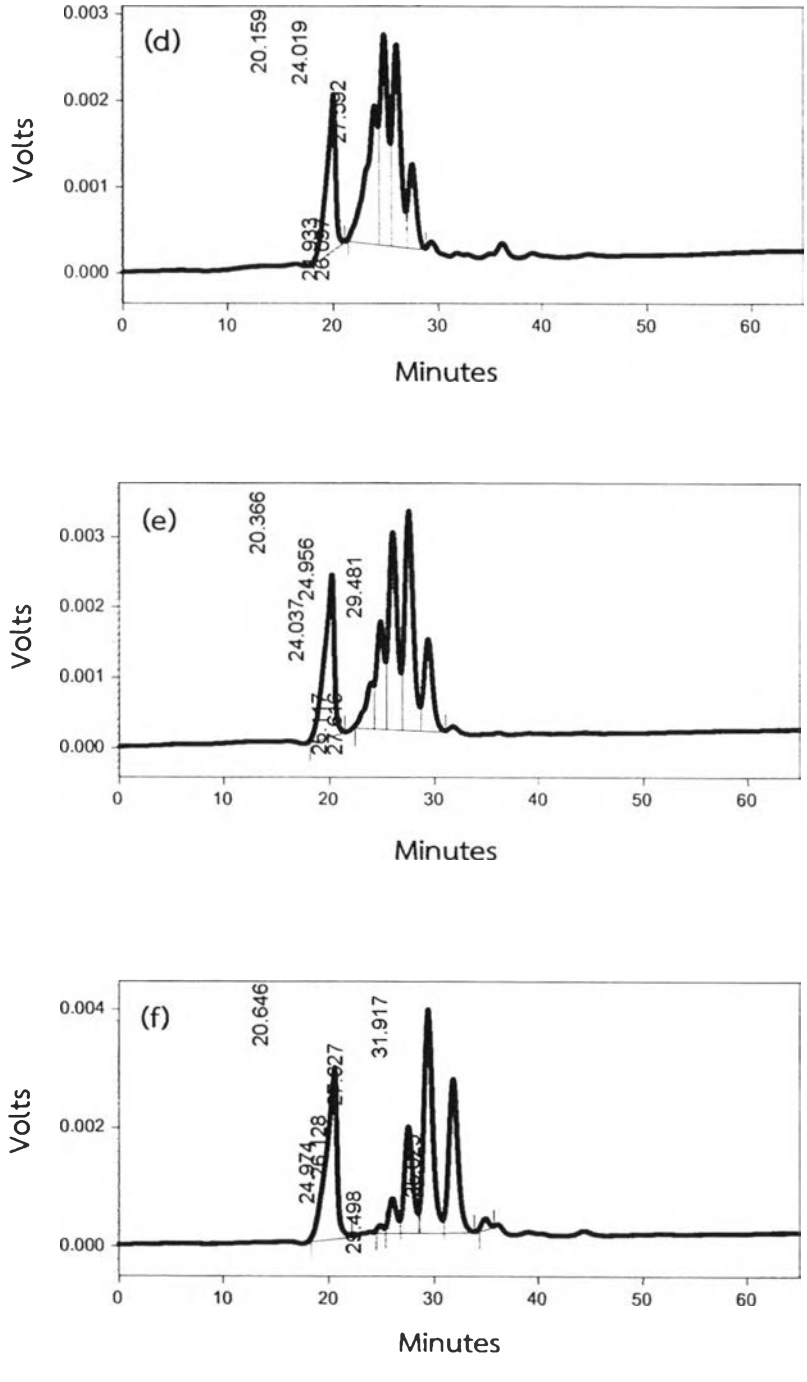


Figure 3.20 (continue) HPLC chromatograms of palatinose glucosides previously separated by Biogel-P2 column. (a) to (n) were separated peaks on Biogel column.

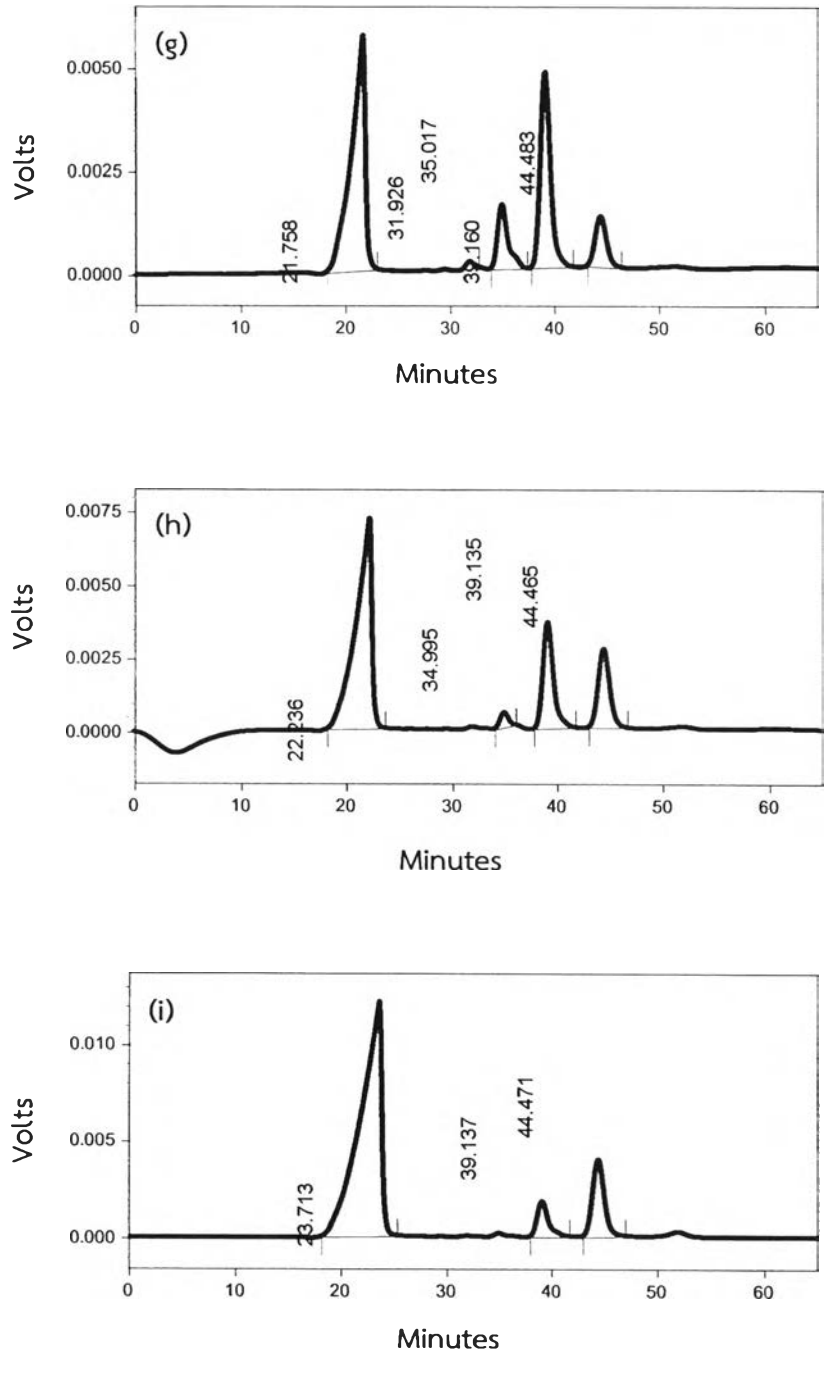


Figure 3.20 **(continue) HPLC chromatograms of palatinose glucosides previously separated by Biogel-P2 column.** (a) to (n) were separated peaks on Biogel column.

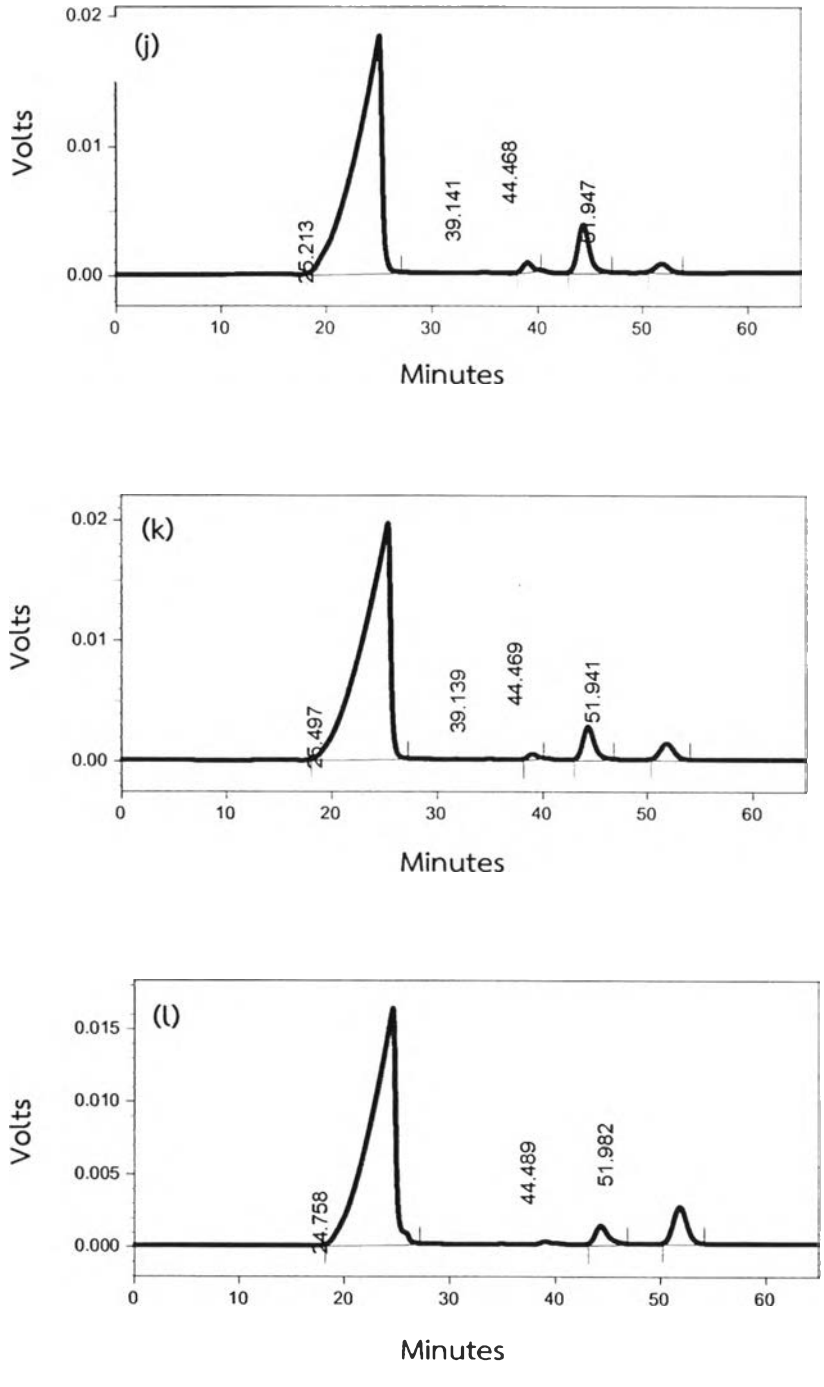


Figure 3.20 (continue) HPLC chromatograms of palatinose glucosides previously separated by Biogel-P2 column. (a) to (n) were separated peaks on Biogel column

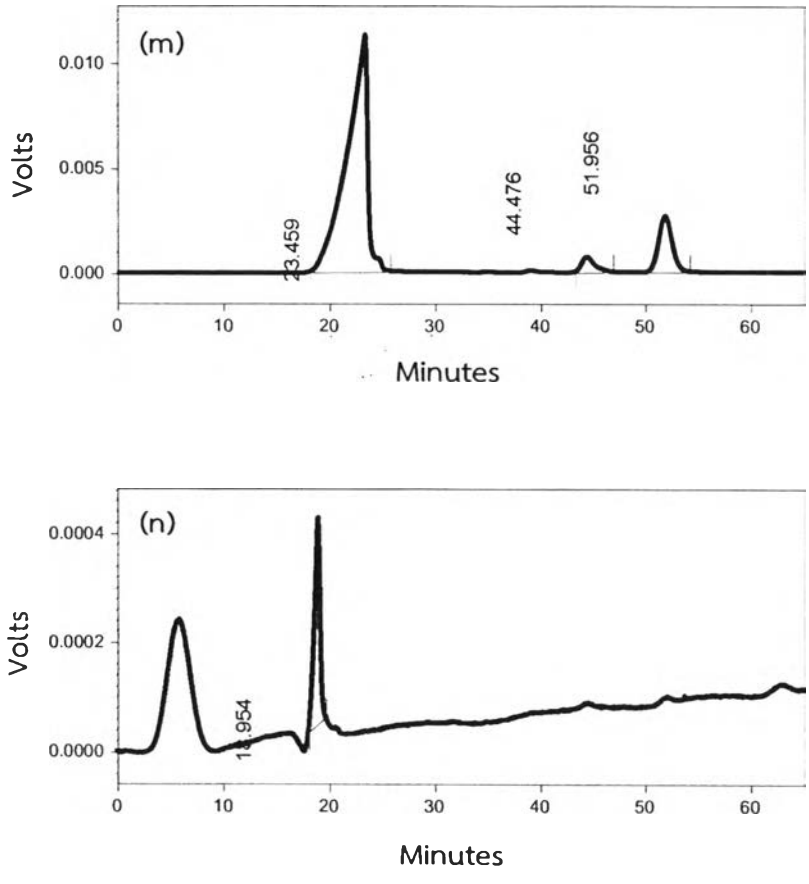


Figure 3.20 (continue) HPLC chromatograms of palatinose glucosides previously separated by Biogel-P2 column. (a) to (n) were separated peaks on Biogel column

Table 3.5 HPLC retention time (Rt) of palatinose glucosides previously separated by Biogel-P2 column.

Biogel-P2 Peak	HPLC retention time (Rt)
a	18, 20
b	20, 21
c	19, 23, 24, 26
d	20, 24, 26, 27
e	20, 24, 26, 27, 29
f	20, 24, 26, 27, 29, 31, 35
g	21, 31, 35, 39, 44
h	22, 34, 39, 44
i	22, 34, 39, 44
j	23, 39, 44
k	25, 39, 44, 51
l	24, 44, 51
m	23, 44, 51
n	18

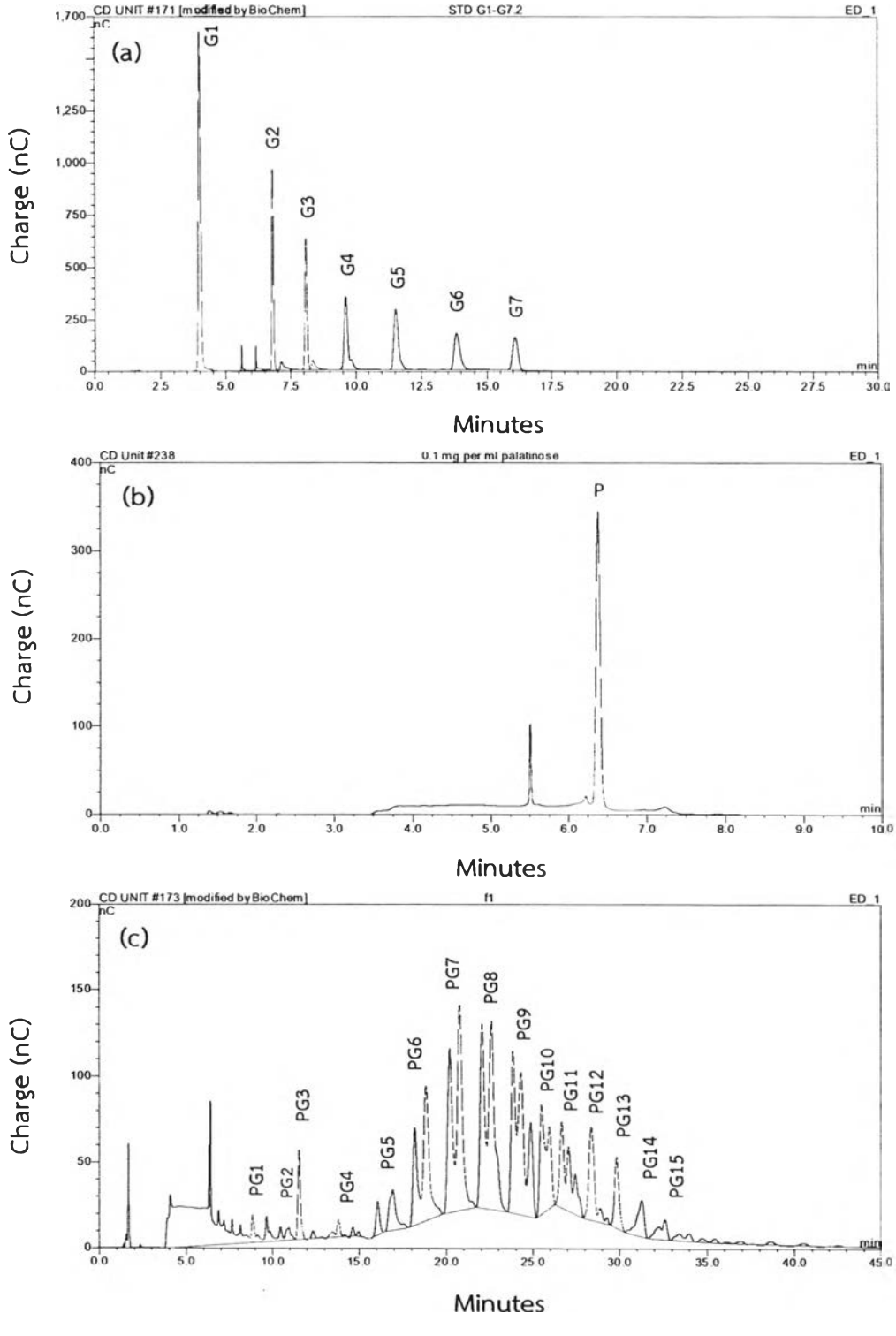


Figure 3.21 **HPAEC chromatograms of palatinose glucosides previously separated by Biogel-P2 column**. (a) standard G1-G7 (b) standard palatinose (c-h) separated peaks from Biogel-P2 column.

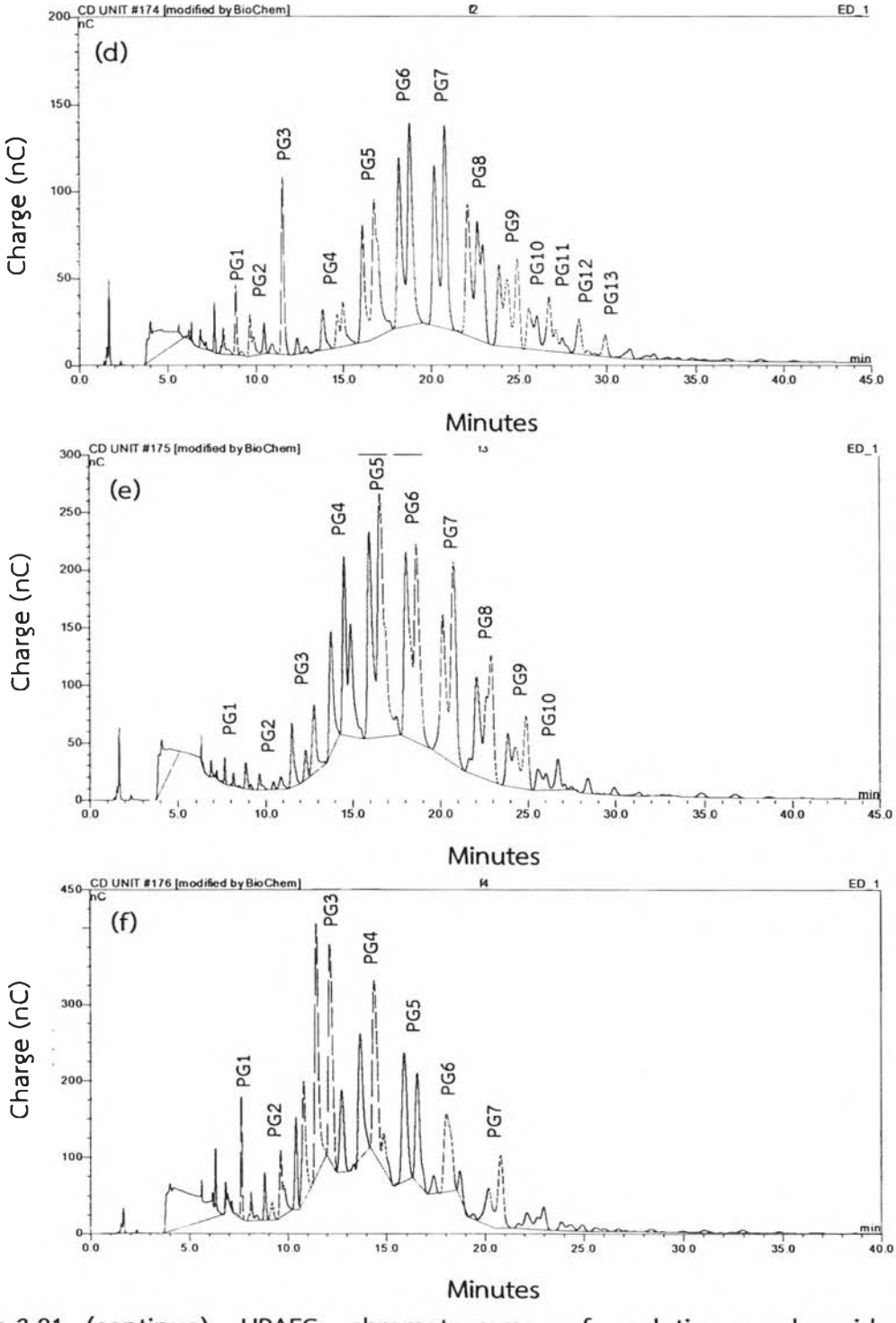


Figure 3.21 (continue) HPAEC chromatograms of palatinose glucosides previously separated by Biogel-P2 column. (a) standard G1-G7 (b) standard palatinose (c-h) separated peaks from Biogel-P2 column.

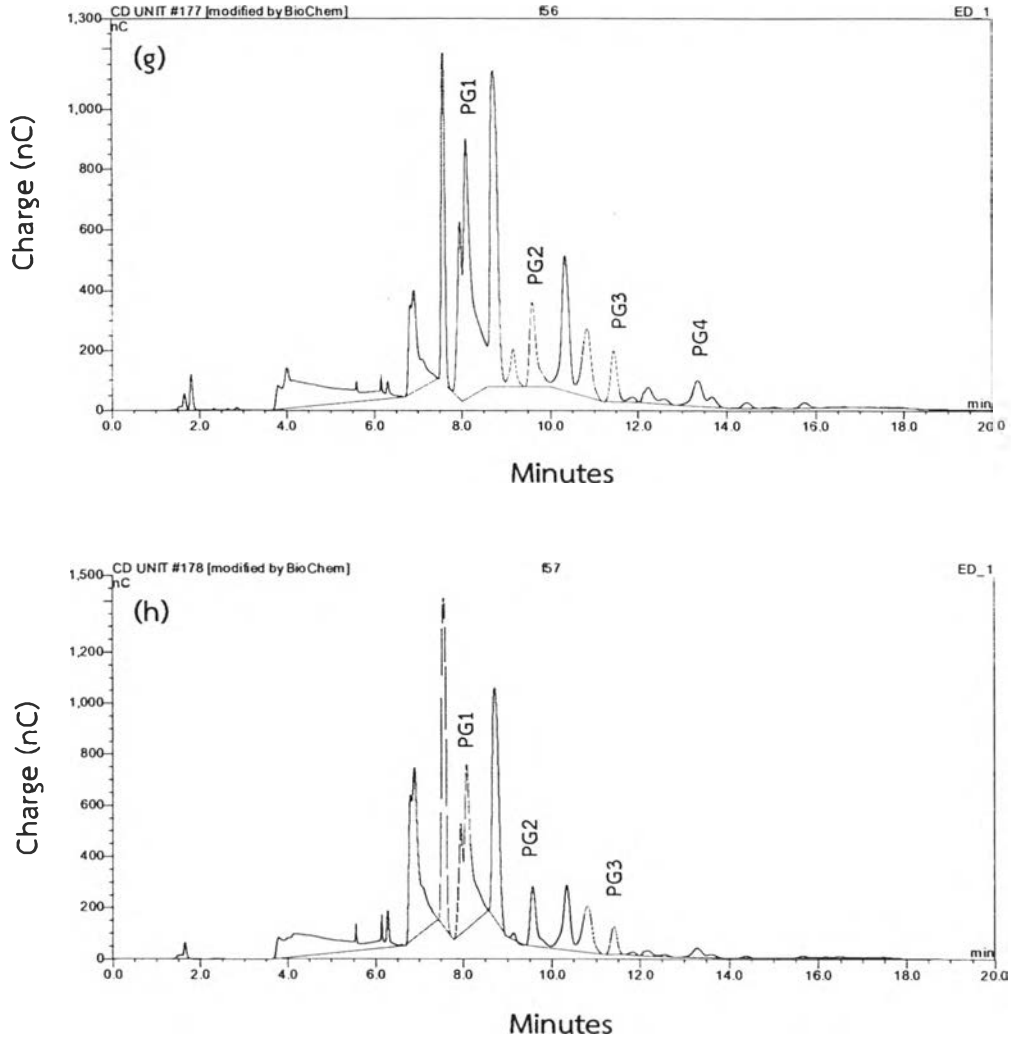


Figure 3.21 (continue) HPAEC chromatograms of palatinose glucosides previously separated by Biogel-P2 column. (a) standard G1-G7 (b) standard palatinose (c-h) separated peaks from Biogel-P2 column.

3.7 Characterization of palatinose glucoside products

3.7.1 Mass Spectrometry (MS)

The molecular weight of PGs were analyzed by TOF-MS as mentioned in 2.17.1. The size of PGs shown at m/z 527 and 689 of $[M+Na]^+$ were 504 and 666 Da (Figure 3.22b). This molecular weight was corresponded to the calculated size of PG1 and PG2 which contains palatinose linked with one and two glucose residues.

3.7.2 Nuclear Magnetic Resonance (NMR)

The structure of PGs was analyzed and compared to palatinose (Figure 3.23a) by ^{1}H-NMR. The result from ^{1}H-NMR spectrum showed the signal of α-1,6 at δ = 4.90 ppm and α-1,4 at δ = 5.34 ppm indicating that palatinose linked with glucose unit by an α-1,4 glycosidic bond (Figure 3.23b).

3.7.3 Sweetness test

The sweetness of palatinose and PGs was tested using sucrose as reference sweetener. The results showed that the sweetness of palatinose was closed to that of sucrose while the sweetness of short chain PGs and long chain PGs were about twice lower than palatinose. The sweetness of long chain PGs was about 10% less than of the short chain PGs (Table 3.6).

3.7.4 Hygroscopic test

Hygroscopicity was determined by measuring the increased weight of palatinose, short chai and long chain PGs. For palatinose (Figure 3.24), day 1-3, the weight was not increased until day 4 which increased for 2%. Then the weight was slightly increased and reached 6% in one week. The weight of short chain PGs was

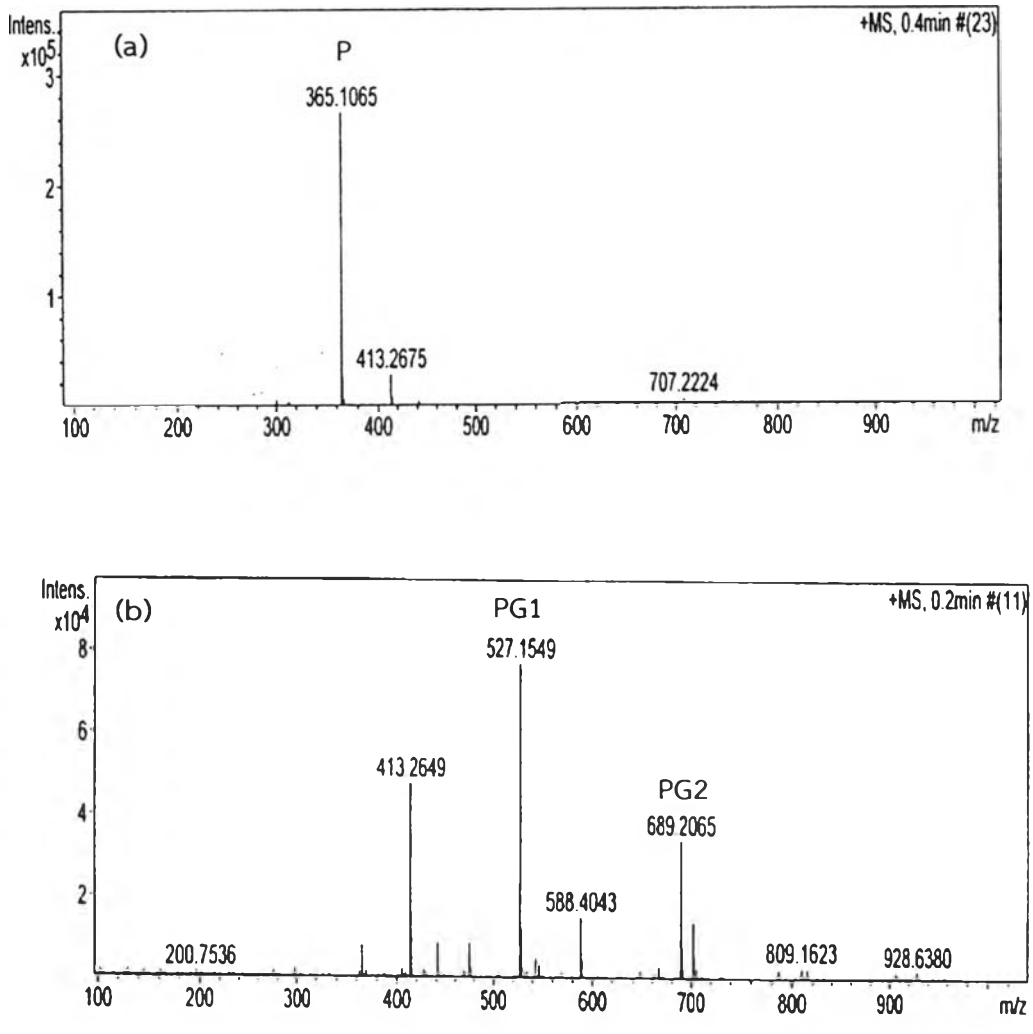


Figure 3.22 TOF mass spectrum of palatinose (a) and PG1 and PG2 (b)

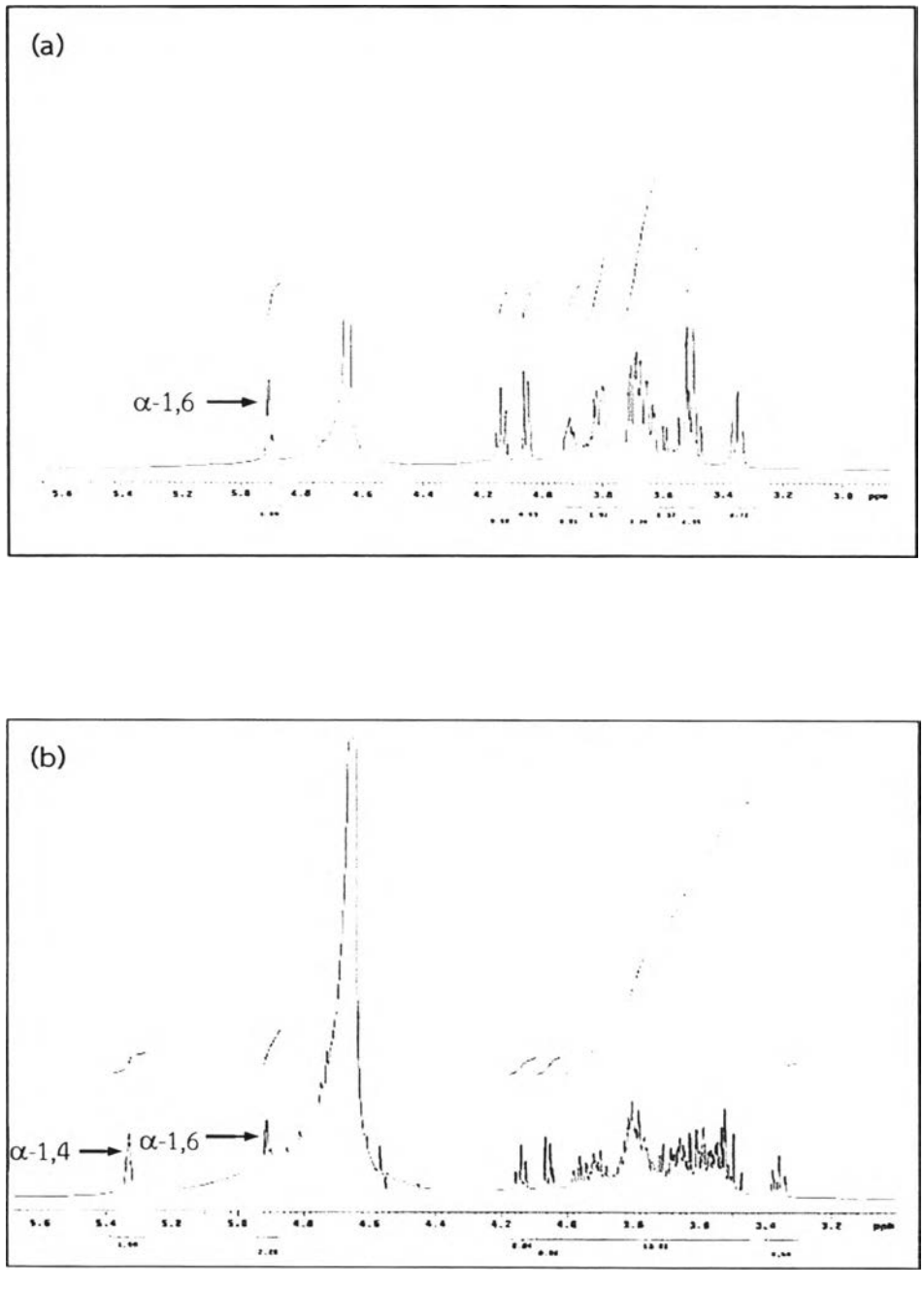


Figure 3.23 ^{1}H-NMR spectrum of palatinose (a) and PGs (b)

Table 3.6 Relative sweetness of palatinose glucoside products

Saccharide	Relative sweetness
Sucrose	1.0
Palatinose	0.97[a]
Short chain PGs	0.58[a], 0.61[b]
Long chain PGs	0.50[a], 0.53[b]

a = compared to sucrose

b = compared to palatinose

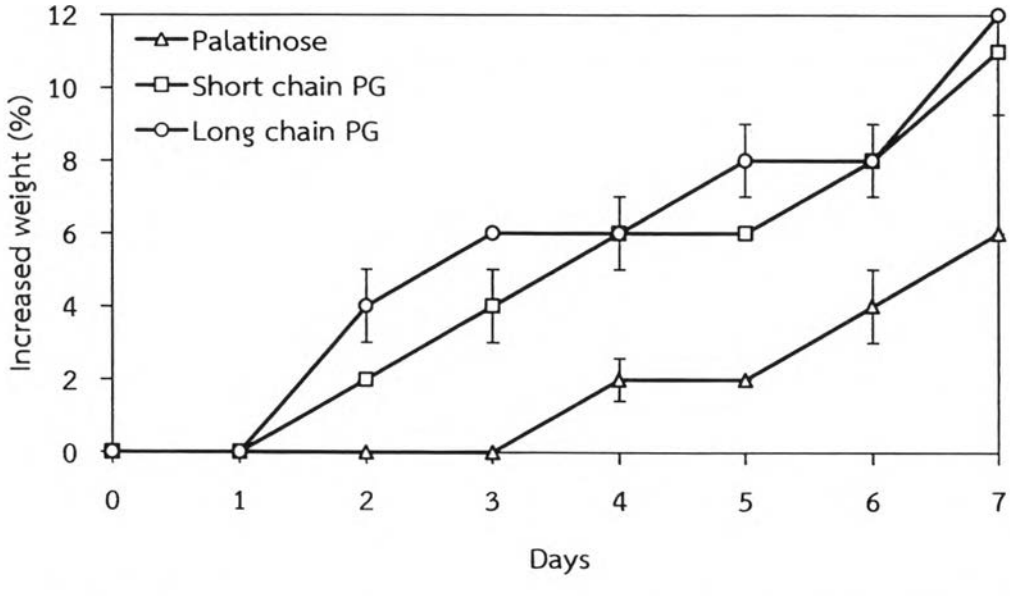


Figure 3.24 Weight increased of palatinose and both PGs from hygroscopic test

increased in day 2 for 2% and significantly increased 11% for one week. For long chain PGs, the weight was extremely increased from 0 to 6% in 3 days and it was constant in day 4. After that it was significantly increased 12% for one week.

3.7.5 Prebiotic activity

The hydrolysis by rat intestinal enzymes of palatinose and PGs was tested. Palatinose could be partially hydrolyzed and the peaks of glucose (3.8 minutes), fructose (4.2 minutes) and remained palatinose (6.2 minutes) were observed when compared HPAEC chromatogram of the reaction treated with digestible enzymes with that at t_0 (Figure 3.25b). Short chain PGs (Figure 3.26b) and long chain PGs (Figure 3.27b) could be digested by rat intestinal enzymes and the peaks of glucose and fructose were detected while peaks of short chain and long chain PGs were disappeared.

3.7.6 Antibacterial activity

Antibacterial activity of PGs against *E. coli and S. aureus* was tested using agar diffusion test. For *E. coli*, the zone of inhibition could not be observed on LB-agar plate containing streptomycin, palatinose, short chain or long chain PGs. For *S. aureus*, the inhibition zone also could not be observed on LB-agar with kanamycin, palatinose or both PGs. However, the clear zone on LB-agar plate with ampicillin of *E. coli* and *S. aureus* was found. The result demonstrated that palatinose and PGs had no antibacterial activity.

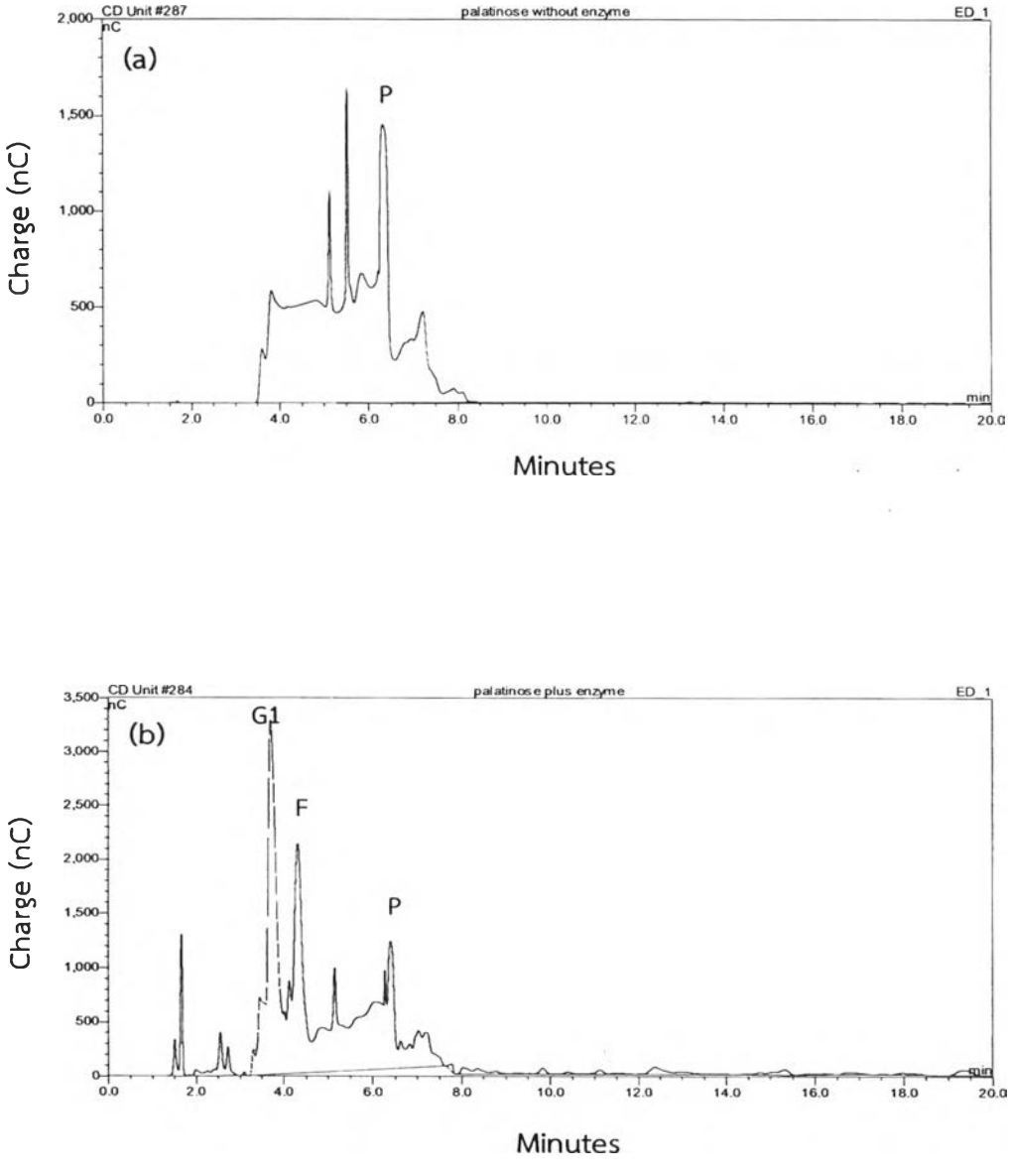


Figure 3.25 **HPAEC chromatograms of palatinose incubated without (a) and with rat intestinal enzymes (b).** P = palatinose, G1 = glucose, and F = fructose

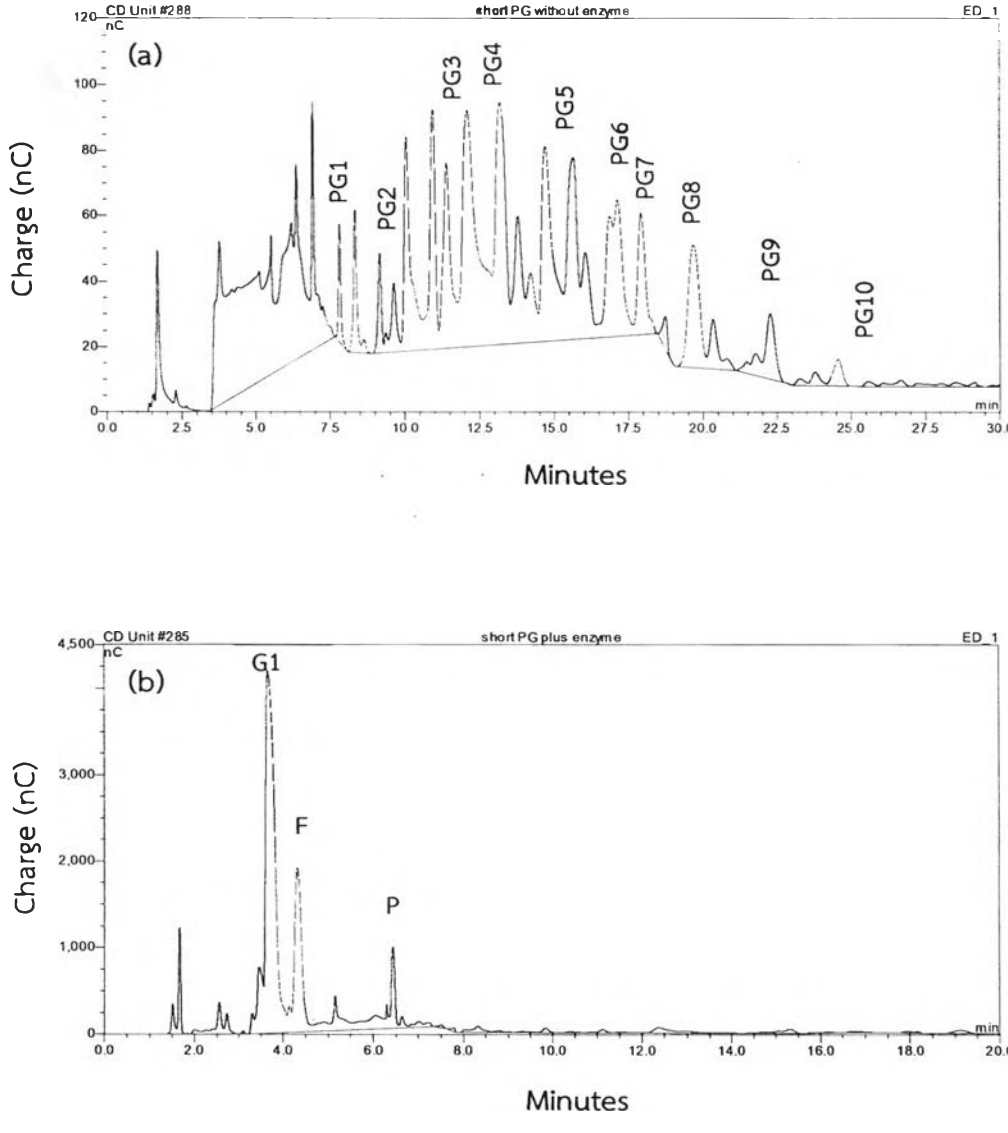


Figure 3.26 **HPAEC chromatograms of short chain PGs incubated without (a) and with rat intestinal enzymes (b).** PGs = palatinose glucosides, G1 = glucose, F = fructose, and P = palatinose

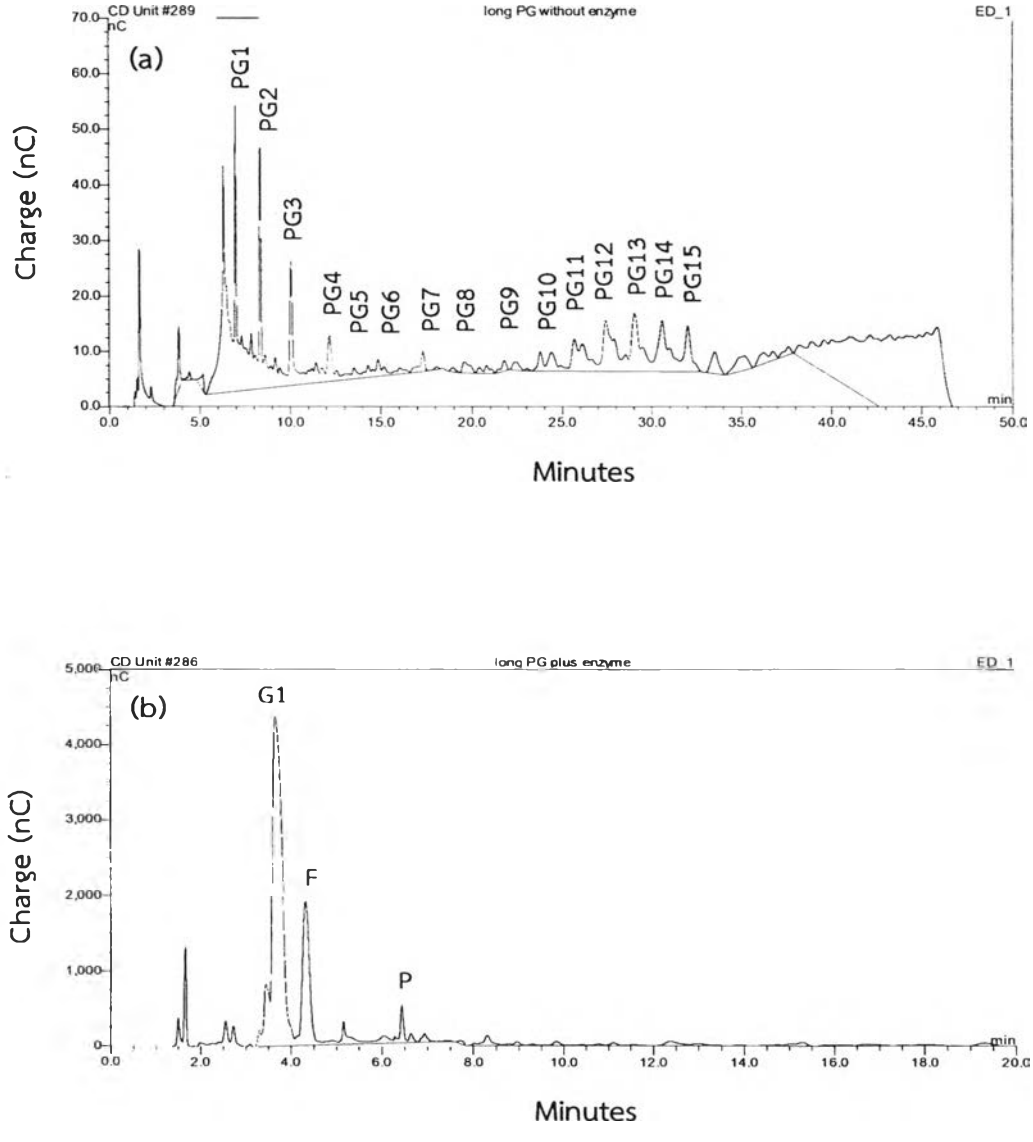


Figure 3.27 **HPAEC chromatograms of long chain PGs incubated without (a) and with rat intestinal enzymes (b).** PGs = palatinose glucosides, G1 = glucose, F = fructose, and P = palatinose