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Reaction of phosphorylase-a with α-D-glucose 1-phosphate and maltodextrin acceptors to give products with degree of polymerization 6–89

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ABSTRACT

A series of linear glucan saccharides (GS) with defined quantity and degree of polymerization (DP) were synthesized from α-D-glucose 1-phosphate (α-D-Glc 1-P) by phosphorylase-a. The GS product fractions with average DP 11, 22, 38, 52, 60, 70, and 79 were measured by HPSEC-ELSD system. Then the same seven fractions were resolved into individual peaks with DP: 6–14, 10–32, 27–55, 37–67, 44–75, 49–83 and 53–89 by HPAEC-PAD system. Results showed that measurement of α-D-Glc 1-P amount consuming during GS synthesis by both systems enable calculation of reaction yield. The reaction yield for the 24 h biosynthesis of the GS product was 25.3% (measured by HPSEC-ELSD) or 29.1% (measured by HPAEC-PAD). The HPSEC-ELSD and HPAEC-PAD systems were also successfully used for phosphorylase-a activity measurement in order to perform its kinetic characterization. This study established feasible systems for preparation of various sizes of the GS with defined DP and quantity as well as characterization of phosphorylase-a kinetics.

1. Introduction

Glucose polymers exist in nature in various forms. Amylose is a linear polymer of α-1,4-linked glucose, and is a minor component of starch. Amylopectin, the major component of starch is a polymer, where short amylose chains are connected with α-1,6 bound to form a cluster structure. A starch granule, higher plant energy reservoir, contains about 20–30% amylose and 70–80% amylopectin (Chen & Bergman, 2007). Glycogen is also a branched glucan, but differs from amylopectin in number and organization of α-1,6-branched links and the absence of a cluster structure. Glycogen serves as a form of energy storage in animals and some microorganisms (Kajiura, Takata, Kuriki, & Kitamura, 2010). Glucose polymers also exist in nature in cyclic form, e.g. cyclic α-1,4 glucans with degree of polymerization (DP) 6–8 are well known as cyclodextrins (Yang et al., 2006).

Glucose polymers possess different physical and biomedical properties. Amylose is applicable to various medical treatments as absorbable surgical suture, plasters, and tourniquets (Masao, Jun'ichi, & Yasushi, 2005). Oral administration of enzymatically synthesized α-1,6 branched α-1,4-glycogen (ESG) significantly prolonged the survival time of Meth A tumor-bearing mice. The ESG also significantly stimulated macrophage-like cells (J774.1), leading to augmented production of nitric oxide and tumor necrosis factor-α (Ryoyama, Kidachi, Yamaguchi, Kajiura, & Takata, 2004). Glycogen from clams has been reported to have antitumor activity, although the activity depends on the extraction methods (Takaya et al., 1998).

There is increasing interest in engineering glucose polymers with well-defined structure and properties in order to find novel applications that cannot be achieved using currently available glucose polymers. A linear glucan is one of the most interesting targets since it is able to form an inclusion complex with various guest molecules. Therefore it acts as a potentially useful functional material (Ohdan, Fujii, Yanase, Takaha, & Kuriki, 2006). Amylose can be obtained from starch by separating amylose and amylopectin. However, natural amylose is heterogeneous in terms of its molecular size, the number of α-1,6-branched links, and the extent of contamination by amylopectin. Therefore natural amylose is not suitable for the linear glucan application studies (Kadokawa, 2012).

The aim of this study was to develop a method for the preparation of glucans with essentially linear structure and specific DPs from an excess of α-D-glucose 1-phosphate (α-D-Glc 1-P) by phosphorylase-a, and to analyze their size and chain-length distribution. The length of glucan saccharide (GS) products was determined by high-performance liquid chromatography (HPLC) system: multi-mode

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size exclusion chromatography (SEC) coupled with an evaporative light-scattering detector (ELSD). Then, the chain-length distribution of prepared glucans was analyzed by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC–PAD). In addition, the HPSEC–ELSD and HPAEC–PAD systems were utilized for measurement of GS synthesis reaction yield and phosphorylase-a kinetic characterization. The method presented herein is suitable for preparation, production and analysis of linear glucans with well-defined DP in high quality, making it possible to conduct further studies on their bioactivities.

2. Materials and methods

2.1. Chemicals and reagents

Phosphorylase-a [E.C.2.4.1.1] from rabbit muscle (Cat. no. 9032–10-4), α-D-Glc 1-P, citric acid and sodium hydroxide for buffer preparation, galactose (180 Da) and malto-oligosaccharides (maltose, 342 Da; maltotriose, 504 Da; maltotetraose, 666 Da; maltopentaose, 828 Da; maltohexaose, 990 Da; maltoheptaose, 1152 Da) used in HPLC calibration and as acceptors, and HPLC-grade solvents were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Pullulan standards (P-5, 5900 Da; P-10, 11,800 Da; P-20, 23,600 Da) also used in HPLC calibration were from Showa Denko K. K. Shodex Group (Kanagawa, Japan). For HPAEC eluents preparation, sodium hydroxide was bought from Merck KGaA (Darmstadt, Germany) and sodium acetate was from Sigma–Aldrich Co. Deuterium oxide for NMR analysis was also from Sigma–Aldrich Co.

2.2. Synthesis of glucan saccharides (GS)

To obtain defined elongated chain length, reaction mixtures containing 25 mM α-D-Glc 1-P, 100 mM Na-citrate buffer (pH 7.0), phosphorylase-a ($2\,U \times ml^{-1}$), and 80 μM acceptors (maltopentaose, maltohexaose or maltoheptaose) were incubated at 37 °C and controlled for 1, 2, 4, 6, 8, 16 and 24 h. The reaction mixtures were purified by ultrafiltration ($14{,}000 \times g$, 10 min) with Microcon Ultracel YM-30 membrane (Millipore, Bedford, MA, USA) to eliminate phosphorylase-a. For HPSEC-ELSD and NH2-HPLC-ELSD analyses, the reaction mixtures were purified by passing through Sephadex G-25 resin (Sigma–Aldrich Co.) to remove salts.

2.3. Product detection by HPSEC-ELSD and NH2-HPLC-ELSD systems

The HPSEC-ELSD system consisted of a pump PU-1580 from Jasco (Tokyo, Japan), an Asahipak GS-320 HQ multi-mode column (300×7.6 mm) preceded by an Asahipak GS-2G 7B guard column (50×7.6 mm) (Shodex, Showa Denko, Tokyo, Japan), and an ELSD ZAM 3000 (Schambeck SFD GmbH, Bad Honnef, Germany). The temperature settings for the column oven and detector nebulizer were 30 °C and 80 °C, respectively. Reaction mixture was injected via a CO-150 sampler with a 20 μL sample loop (Rheodyne, Cotati, CA, USA). The mobile phase for the HPSEC was isocratic (70:30 water-MeOH) with flow-rate of 0.4 ml/min.

For the NH2-HPLC-ELSD system, the same pump, ELSD, injector sampler and sample loop were used as above. However, an Asahipak NH2 P-50 4 E multi-mode column (250×4.6 mm) and an Asahipak NH2P-50G 4 A (10×4.6 mm) (Shodex, Showa Denko, Tokyo, Japan) were used, where the temperature settings for the column oven and detector nebulizer were 40 °C and 80 °C, respectively. The mobile phase was isocratic (65:35 MeCN-water) with flow-rate of 1 ml/min. The commercial galactose, malto-oligomers with DP 2–7 and pullulan with M_w 5900–23,600 Da were used as calibration standards. Chrom Manager 5.8 software from Analytical Based Development Center (Taichung, Taiwan) was used for online data monitoring and analysis.

2.4. Product detection by HPAEC-PAD system

The Dionex HPAEC-PAD system consisted of ICS-3000 single pump (SP), ICS-3000 detector/chromatography module (DC) with injection valve containing 11.65 μL sample loop, and an electrochemical detector with cell containing Au working and Ag/AgCl reference electrodes (Thermo Fisher Scientific Inc., Waltham, MA, USA). Waveform pulse potentials and durations were as follows: $E_1 = 0.05$ V ($t_0 = 0$ s); $E_2 = 0.05$ V ($t_1 = 0.2$ s); $E_3 = 0.05$ V ($t_2 = 0.4$ s); $E_4 = 0.075$ V ($t_3 = 0.41$ s); $E_5 = 0.075$ V ($t_4 = 0.6$ s); $E_6 = -0.15$ V ($t_5 = 0.61$ s); $E_7 = -0.15$ V ($t_6 = 1$ s). The columns CarboPac™ PA1 guard (PA1 guard) (2×50 mm), CarboPac™ PA1 analytical column (PA1) (2×250 mm) and CarboPac™ PA100 analytical column (PA100) (2×250 mm) were connected in series. The temperature for the column oven was 25 °C. Eluents were 165 mM NaOH solution as eluent A and 82.5 mM NaOH solution containing 412 mM NaOAc as eluent B. The optimal conditions found for the chain-length distribution monitoring was: 0–150 min, linear gradient from 47.5% to 81.3% eluent B; 150–200 min, linear gradient to 83.5% eluent B; 200–250 min, linear gradient to 92.5% eluent B, and 250–300 min, isocratic elution of 92.5% eluent B. The flow rate for these conditions was 0.225 ml/min. Chromeleon 6.80 software from Thermo Fisher Scientific Inc. was used for online data monitoring and analysis. The synthesized GS fractions were purified by passing through carbohydrate membrane desalter CMD™ 300, 4 mm (Thermo Fisher Scientific Inc.), to exchange ≥99.5% of the sodium ions for hydrogen ions.

2.5. ^{1}H nuclear magnetic resonance spectroscopy (^{1}H NMR)

^{1}H NMR spectroscopy was conducted in the Instrumentation Center at National Taiwan University (Taipei, Taiwan). The fractions F1-m6 (Figs. 1B-a and 3B) and F2-m6 (Figs. 1B-b and 3C) were collected at time intervals 14–17 min using HPSEC as well as 18.5–150 min using HPAEC-PAD. Samples were prepared according to the published method in which 1 mg of maltoheptaose, F1-m6 or F2-m6 was dissolved in 0.8 ml of D_2O. The dissolved sample was filtered by 0.22 μm membrane and loaded into a NES-600 tube (178×5 mm) (Optima Inc., Tokyo, Japan). The NMR spectra were recorded on a Bruker AVIII 500 MHz spectrometer (Yen et al., 2005). The maltoheptaose spectra were compared with theoretical maltoheptaose spectrum predicted by MestReNova software 8.1.1 (Mestrelab Research, Santiago de Compostela, Spain).

2.6. Kinetic characterization of phosphorylase-a

Henri–Michaelis–Menten constant (K_m) and maximum enzyme velocity (V_{max}) were determined from Henri–Michaelis–Menten and Lineweaver–Burk plots. Kinetic data were obtained by measuring the rate of GS synthesis by phosphorylase-a ($2\,U \times ml^{-1}$) for 1 h, under the standard assay conditions, using a maltohexaose as the acceptor and a substrate concentration ([S]) 3 mM–25 mM α-D-Glc 1-P. The time of the assay was short enough to limit substrate utilizations below 5% (Segel, 1976; Kazłowski, Kazłowska, Pan, & Ko, 2011). For kinetics measurement by HPSEC-ELSD the same system and method were used as described in Section 2.3. The GS products measured by HPSEC-ELSD were calculated from maltoheptaose standard concentrations vs. peak area trendline: $Y = 8087.7x$; Y, peak area and x, concentration. The HPAEC-PAD kinetics measurement was performed with the instruments and eluents described in Section 2.4 except that the only columns PA1 guard (2×50 mm) and PA1 (2×250 mm) were used. The flow rate was 0.25 ml/min. The gradient system used for this experiment was: 0–90 min, linear from 47.5% to 92.5% eluent B. The products measured by HPAEC-PAD were calculated from maltoheptaose standard concentrations vs. peak area trendlines:

$Y = 0.075x - 0.2567$ for product concentration 10–50 μg/ml, and $Y = 0.015x + 3.25$ for product concentration 50–60 μg/ml; Y, peak area and x, concentration.

3. Results

3.1. Establishing the calibrations of glucan saccharides (GS)

In the HPSEC-ELSD system, calibration curves constructed from retention time vs. M_w of the maltoheptaose, pullulan P-5 (5900 Da), pullulan P-10 (11,800 Da), pullulan P-20 (23,600 Da) standards were used to extrapolate into the DP of GS. The DP of GS peaks were calculated based on calibration lines: $Y = -1{,}220.6x + 21{,}212$ for GS with M_w 1150–5900 Da; $Y = -5{,}667.6x + 77{,}000$ for GS with M_w 5900–11,800 Da; and $Y = -12{,}500x + 155{,}600$ for GS with M_w 11,800–23,600 Da; Y, M_w and x, retention time. Calibration curve constructed from standard concentration vs. peak area was used for quantification of α-D-Glc 1-P based on the linear trendline: $Y = 5792.5x + 524.4$; Y, peak area and x, α-D-Glc 1-P concentration. In the NH2-HPLC-ELSD system the GS were identified by retention time comparison between identified peaks and commercial maltooligosaccharide standards (DP 2–7). For the peaks with DP 8–14, the DP was estimated assuming homologous series, adding one glucose residue for each peak (Fredriksson, Andersson, Koch, & Åman, 1997). In the HPAEC-PAD system maltohexaose and maltoheptaose were used to determine the retention time of the peaks with DP 6 and 7, respectively. For the GS with DP 6–13 each oligosaccharide was regarded as two adjacent peaks what was a result of structural changes in part of synthesized GS during analysis in alkaline solution. For the peaks with DP ≥ 14, the DP was estimated adding one glucose residue for each peak. Calibration line constructed from α-D-Glc 1-P standard concentrations vs. peak area was used for quantification of α-D-Glc 1-P ($Y = 65.42x + 161.34$; Y, peak area and x, concentration).

3.2. Determining the DP of GS products by HPSEC-ELSD, NH2-HPLC-ELSD and HPAEC-PAD systems

In order to obtain elongated chain length of GS, reaction mixture containing α-D-Glc 1-P, Na-citrate buffer, phosphorylase-a (GP; EC 2.4.1.1; 1,4-α-glucan:phospahate, α-D-glucosyltransferase) and acceptors (maltopentaose, maltohexaose or maltoheptaose) was incubated at 37 °C and controlled for: 1 h (fraction 1, F1), 2 h (F2), 4 h (F3), 6 h (F4), 8 h (F5), 16 h (F6) and 24 h (F7). A progressive DP increasing of GS products was clearly shown when the synthesis reaction time was extended (Fig. 1).

The HPSEC system, calibrated with the large number of standards providing an accurate description of the non-linear relationship between DP and retention time (Fredriksson et al., 1997). The average DP for the fractions: F1-m5 (maltopentaose was used as the acceptor to initiate the reaction), F2-m5, F3-m5, F4-m5, F5-m5, F6-m5 and F7-m5 were: DP 7, DP 16, DP 32, DP 47, DP 55, DP 67 and DP73, respectively (Fig. 1A); for the fractions: F1-m6 (maltohexaose was used as the acceptor), F2-m6, F3-m6, F4-m6, F5-m6, F6-m6 and F7-m6 were: DP 11, DP 22, DP 38, DP 52, DP 60, DP 70 and DP 79, respectively (Fig. 1B); as well as for the fractions: F1-m7 (maltoheptaose was used as the acceptor), F2-m7, F3-m7, F4-m7, F5-m7, F6-m7 and F7-m7 were: DP 13, DP 23, DP 41, DP 53, DP 61, DP 74 and DP 82, respectively (Fig. 1C). The fractions: F1-m5, F1-m6 and F1-m7 were also analyzed by NH2-HPLC-ELSD system and showed apparent individual peak resolutions (Fig. 2). However, the NH2-HPLC-ELSD precluded analysis of the GS products with DP ≥ 15 because the products with M_w larger than 2,300 Da were excluded by the system.

The GS products: F1-m6, F2-m6, F3-m6, F4-m6, F5-m6, F6-m6 and F7-m6 were resolved into individual peaks by the HPAEC-PAD system with DP: 6–13, 9–32, 25–55, 36–66, 44–73, 49–84 and 53–89, respectively (Fig. 3). It is apparent that HPAEC-PAD chromatograms illustrating individual peak distribution of each fraction were well correlated with their HPSEC-ELSD equivalents (Fig. 1B vs. Fig. 3). Partition of the GS into two peaks, clearly shown in maltohexaose and maltoheptaose standards (Fig. 3A), DP 6–13 (Fig. 3B) and DP 9–13 (Fig. 3C) as well as the partition of GS into peak and shoulder, shown in DP 14–25 (Fig. 3C) were considered as the result of structure changes in a part of analyzed GS in alkaline solution.

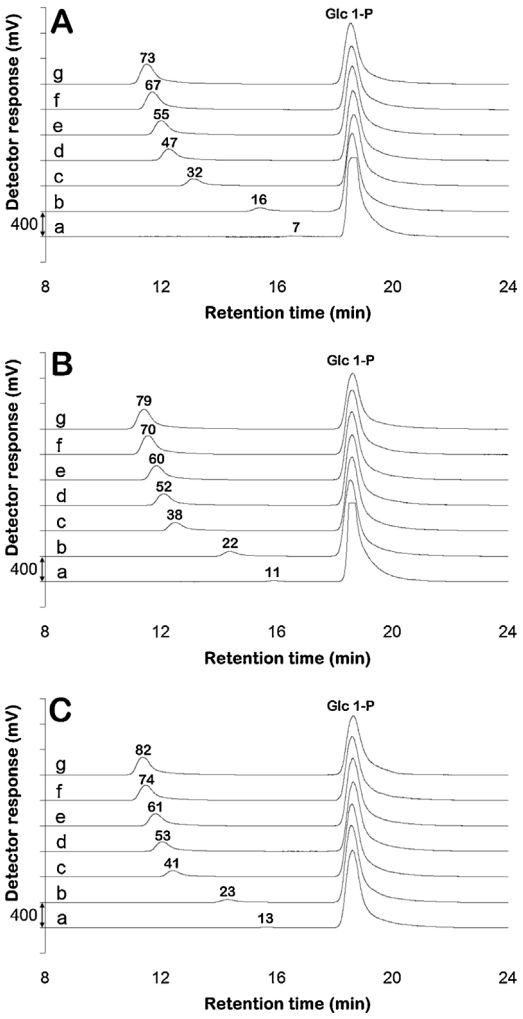

Fig. 1. HPSEC-ELSD chromatograms of GS fractions synthesized by phosphorylase-a from α-D-Glc 1-P with: maltopentaose (A), maltohexaose (B), maltoheptaose (C) as acceptors and incubated for 1–24 h (a–g). Numbers above the peaks (7–82) indicate the average DP of GS. The chromatographic conditions are described in Section 2.3.

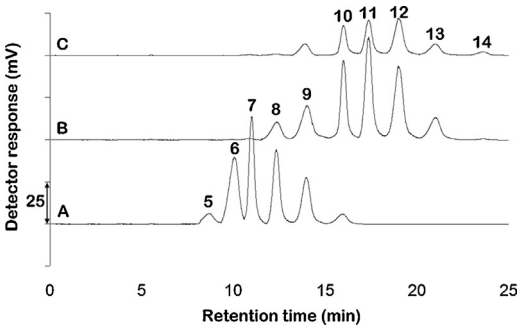

Fig. 2. NH2-HPLC-ELSD chromatograms of GS fractions synthesized by phosphorylase-a from α-D-Glc 1-P with: maltopentaose (A), maltohexaose (B), maltoheptaose (C) as acceptors and incubated for 1 h. Numbers above the peaks (5–14) indicate the DP of GS. The chromatographic conditions are described in Section 2.3.

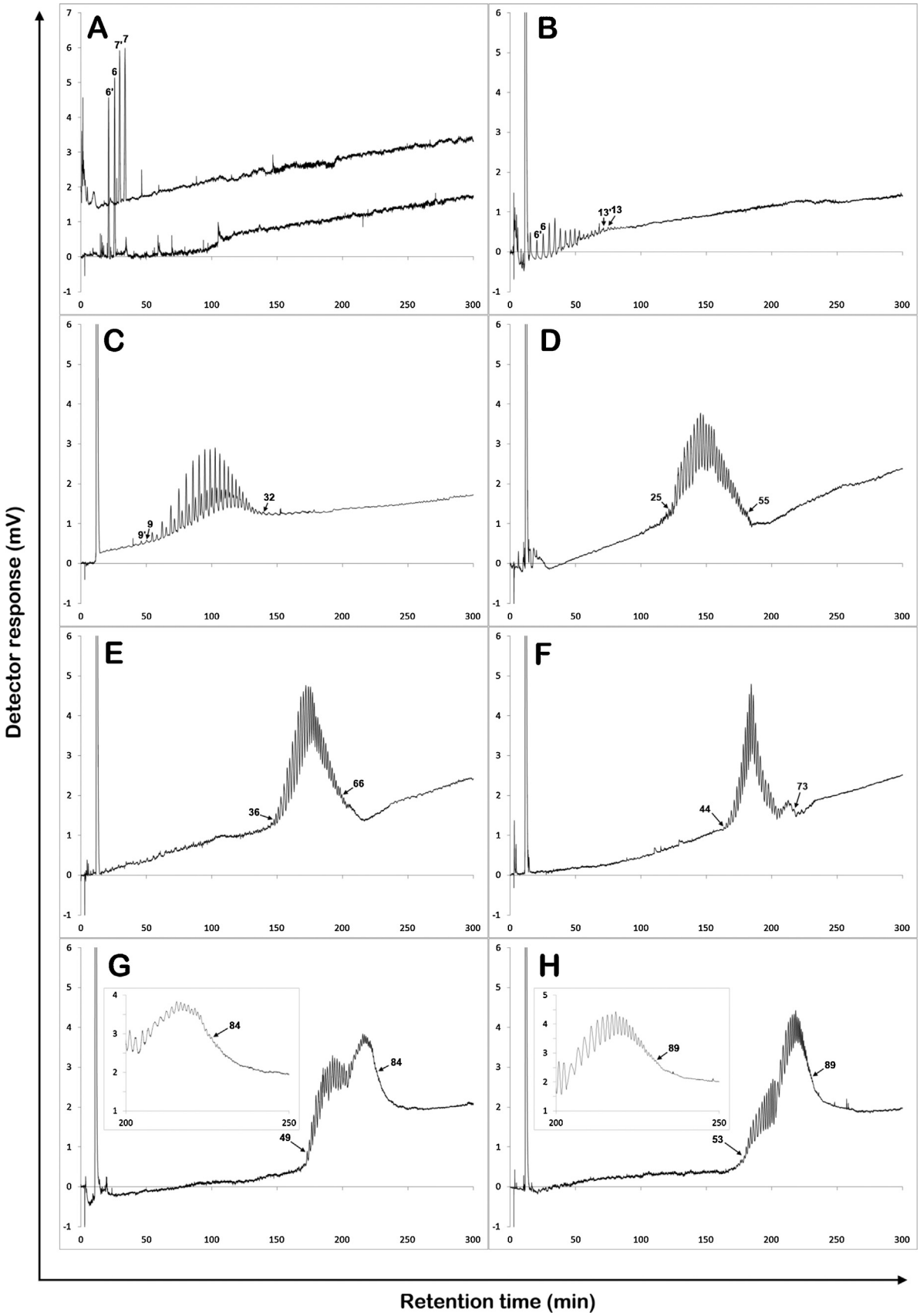

Fig. 3. HPAEC-PAD chromatograms of maltohexaose (A,a) and maltoheptaose (A,b) standards, and GS fractions synthesized by phosphorylase-a from α-D-Glc 1-P with maltohexaose as the acceptor and incubated for 1–24 h (B–H). Numbers above the peaks (6–89) indicate the DP of GS. Peaks designed as 6', 7', 9' and 13' correspond to structure modified GS in alkaline condition. Inlets: close-up chromatograms of GS with DP 65–84 (G) and 65–89 (H). The chromatographic conditions are described in Section 2.4. HPAEC-PAD chain-length distributions by peak areas of the GS fractions (B–H) are presented in Appendix Figure S1.

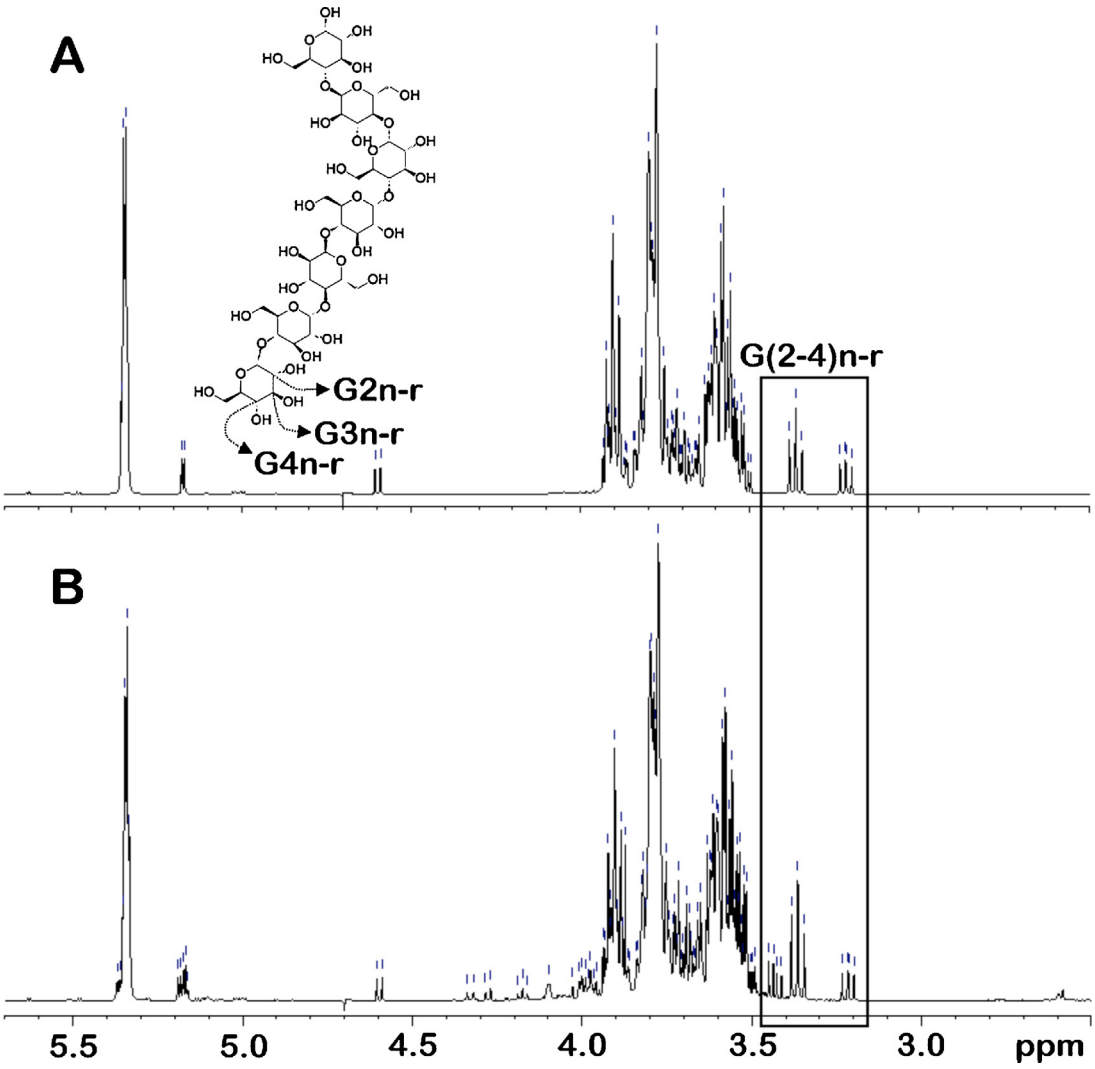

Fig. 4. ^{1}H NMR (500 MHz, Deuterium Oxide) spectra of maltoheptaose (A) and maltoheptaose collected after HPAEC-PAD analysis (B). (A): δ 5.33 (m, 7H), 4.60 (d, J = 7.5 Hz, 1H), 4.05–3.86 (m, 10H), 3.85–3.49 (m, 23H), 3.40–3.19 (m, 6H); (B): δ 5.33 (m, 7H), 4.60 (d, J = 7.3 Hz, 1H), 4.12–3.86 (m, 10H), 3.85–3.48 (m, 23H), 3.46–3.19 (m, 6H). Abbreviations: G, α-D-glucopyranose; n-r, non-reducing end; numbers from 2 to 4 refer to place of protons.

In order to confirm this phenomenon, maltoheptaose standard as well as F1-m6 and F2-m6 obtained after phosphorylase-a synthesis were analyzed by ^{1}H NMR. Their NMR spectra were compared with spectra of samples collected after HPAEC-PAD monitoring. For both, maltoheptaose standard and maltoheptaose collected after HPAEC-PAD monitoring 46 from 55 proton signals expected for maltoheptaose were identified. It was apparent that the distinct differences between the maltoheptaose standard and the maltoheptaose collected after HPAEC-PAD monitoring are localized in the G2α, G3α and G4α resonance environment on the α-D-glucopyranosyl non-reducing end (n-r) (Fig. 4). The ^{1}H NMR spectra showed that the maltoheptaose standard had signals for G2α n-r–G4α n-r distributed at δ 3.19–3.40 ppm, m; however the maltoheptaose collected after HPAEC-PAD monitoring had signals extension for G2α n-r-G4α n-r at δ 3.19–3.46 ppm, m (Fig. 4). The same phenomenon was observed also for F1-m6 and F2-m6 (Appendix Figure S2).

α-D-Glc 1-P consumption for GS synthesis reaction also was measured by these two chromatographic systems (Fig. 5). Results showed an apparently larger standard error when the G 1-P was measured by HPAEC-PAD in comparison to HPSEC-ELSD (Fig. 5); nevertheless both systems enable calculation of GS synthesis reaction yield by phosphorylase-a (Table 1).

3.3. Kinetics

For precise measurement of enzyme kinetics for phosphorylase-a, a fixed amount of enzyme (2 U × ml^{-1}) and reaction time (1 h)

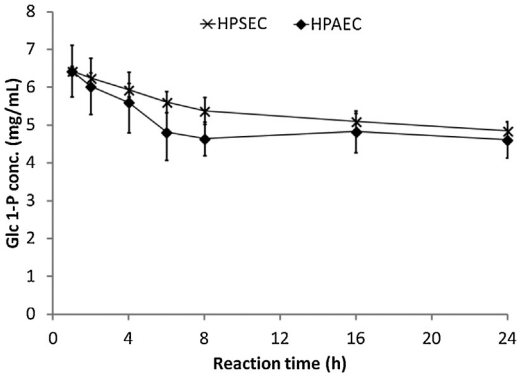

Fig. 5. α-D-Glc 1-P consumption for GS synthesis reaction by phosphorylase-a measured by HPSEC-ELSD and HPAEC-PAD. Concentrations (mg/ml) of α-D-Glc 1-P were calculated in the same way as in Table 1.

Table 1
The α-D-Glc 1-P consumption and reaction yield of GS synthesis reaction by phosphorylase-a monitored by HPSEC-ELSD and HPAEC-PAD systems.

Reaction time (h)	HPSEC-ELSD		HPAEC-PAD	
	α-D-Glc 1-P consumption (mg/ml)[a]	Reaction yield (%)[b]	α-D-Glc 1-P consumption (mg/ml)[a]	Reaction yield (%)[b]
1	0.06	0.9	0.07	1.0
2	0.26	4.1	0.47	7.2
4	0.57	8.8	0.90	13.9
6	0.89	13.7	1.68	25.9
8	1.12	17.3	1.86	28.6
12	1.40	21.6	1.67	25.7
24	1.65	25.3	1.89	29.1

[a] α-D-Glc 1-P consumption: [α-D-Glc 1-P input into reaction mixture (mg/ml) – residual α-D-Glc 1-P after synthesis reaction (mg/ml)] Concentrations (mg/ml) of α-D-Glc 1-P were calculated from the standard curves: $Y = 5792.5x + 524.4$ for HPSEC-ELSD and $65.42x + 161.34$ for HPAEC-PAD; Y, peak area and x, α-D-Glc 1-P concentration.

[b] Reaction yield: [α-D-Glc 1-P input into reaction mixture (mg/ml) – α-D-Glc 1-P consumed during synthesis reaction (mg/ml)]/ [α-D-Glc 1-P input into reaction mixture (mg/ml)] × 100%.

were chosen in order to limit substrate utilization below 5% (Fig. 6). The K_m (mM) and V_{max} (μg/min) kinetic parameters measured by HPSEC-ELSD were equal to 1.76 and 12.75, respectively. The kinetic parameters measured by HPAEC-PAD were: $K_m = 2.02$ and $V_{max} = 16.40$. Some differences in the K_m and V_{max} parameters between these 2 methods might be caused from that in the HPAEC-PAD system, the synthesized GS product during 1 h showed apparent individual peak resolution (DP 7–15). Therefore, an individual calibration curves constructed from standard concentration vs. peak area for each GS with DP7–15 is required for precise analysis.

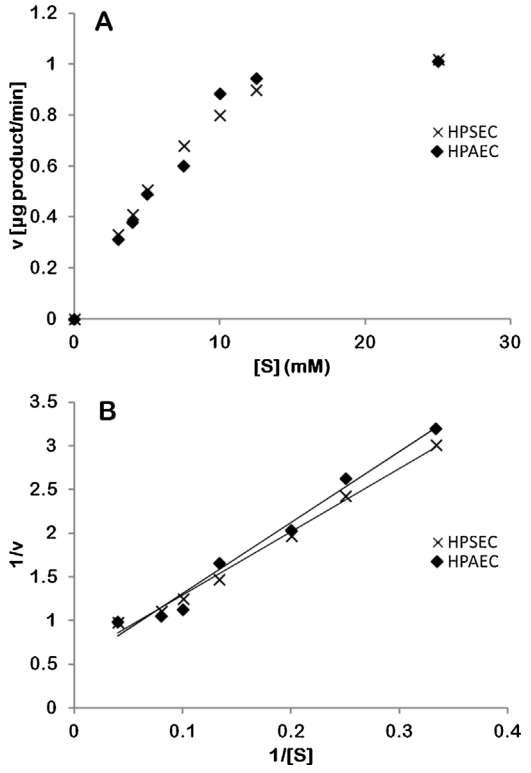

Fig. 6. Henri–Michaelis–Menten (A) and Lineweaver–Burk (B) plots for phosphorylase-a kinetics measurement by HPSEC-ELSD and HPAEC-PAD using α-D-Glc 1-P as a substrate. The α-D-Glc 1-P synthesis was catalyzed by phosphorylase-a (2 U × 1 ml^{-1} of α-D-Glc 1-P substrate [S] range of 3–25 mM) for 1 h, under the standard assay conditions, described in the Section 2.6.

Due to lack of maltooligosaccharide commercial standards with DP 8–15, the concentrations of synthesized oligosaccharides were approximately calculated from the maltoheptaose standard concentration vs. peak area calibration curve.

4. Discussion

Phosphorylase is an important allosteric enzyme in carbohydrate metabolism (Kazłowski, Chen, Chao, Lai, & Ko, 2013). The phosphorylase enzymes from different sources differ in their regulatory mechanisms and their natural substrates. However, all known phosphorylases share catalytic and structural properties. Glucan phosphorylase (GP; EC 2.4.1.1; 1,4-α-glucan:phospahate, α-D-glucosyltransferase) is a unique enzyme that produces GS with desired average M_w. Linear glucans can be synthesized when α-D-Glc 1-P is in excess, and where a glucan synthetic reaction predominates (Ohdan et al., 2006). Although all α-glucan phosphorylases belong to a large, highly homologous group that includes starch phosphorylases from plants, glycogen phosphorylases from bacteria, yeasts and animals, and maltodextrin phosphorylases from bacteria, these enzymes from distinct origins differ in their modes of regulation and substrate preferences (Palm, Goerl, & Burger, 1985).

Hanes (1940) found that potato phosphorylase transferred glucose units from α-D-Glc 1-P to the nonreducing ends of starch. Starting with only α-D-Glc 1-P and the phosphorylase, there was no reaction and a starch-primer was required for reaction (Hanes, 1940). Then, several dozen papers were published from 1953 to 2012 where primers were required for starch and GS synthesis (French & Wild, 1953; Robyt & Mukerjea, 2013). However, Mukerjea & Robyt (2005) studied the effects of adding different concentrations of maltodextrins: maltose, maltotriose, and maltododecaose to maize, wheat, and rice starch granules in the presence of ADP-[^{14}C]Glc, and found that the maltodextrins inhibited starch biosynthesis, rather than stimulating it, as would be expected for the addition of increasing concentrations of primers (Mukerjea & Robyt, 2005). The major product in the presence of maltose was maltotriose, with decreasing amounts of maltotetraose-maltononaose, and the major products in the presence of maltotriose was maltotetraose and maltopentaose, with decreasing amounts of maltohexaose-maltononaose. Therefore, it was concluded that the maltodextrins were acting as acceptors for transglycosylation side-reactions, rather than acting as primers for polymerization to give starch chains (Mukerjea & Robyt, 2005; Robyt & Mukerjea, 2013).

Primer (actually a term: putative primer is used for follow-up discussion, because as mentioned above, it is rather acceptor of the glucosyl residue from α-D-Glc 1-P; Robyt & Mukerjea, 2013) specificity of potato phosphorylase was investigated long ago. French and Wild (1953) studied different chain length saccharides to be putative primers for potato phosphorylase synthetic ability. Maltotriose was the shortest oligosaccharide working as the putative primer for the potato phosphorylase; however, maltooligosaccharides with DP > 3 was verified as so much better putative primers than the maltotriose for this reaction (French & Wild, 1953). Synthetic amyloses of average DP in the range 6–568 were prepared by the enzymatic method presented by French & Wild (1953) with the maltotetraose as the putative primer; however, the DP was only approximately determined by an iodine-staining method (Bailey & Whelan, 1961). Subsequently, the GP from potatoes extended the linear polymer of α-1,4-linked glucose using maltotetraose as a putative primer was utilized for industrial applications, but this process has been hampered by its low thermostability (Yanase, Takata, Fujii, Takaha, & Kuriki, 2005). In order to obtain GP suitable for industrial use, Yanase et al. (2005) introduced random mutations into the potato GP gene, and obtained enzyme with improved thermostability. This enzyme produces linear glucans

with maximum M_w 1400 kDa and yield 60.2% (Yanase et al., 2005). The α-1,4 linear glucan was synthesized by thermostable GP from *Thermus aquaticus* at 70 °C. The product of this reaction was characterized by narrow distribution, indicating no product precipitation and maximum M_w 124 kDa. In this experiment, however, the chain-length distribution was not presented (Fujii et al., 2003). Also linear-glucan was prepared with maltohexaose as a putative primer using rabbit muscle phosphorylase-a. However, only two different length of glucan products with average DP 46 and 85 were obtained (Blennow, Bay-Smidt, Wischmann, Olsen, & Lindberg Møller, 1998). Our work is the methodology that attempts to acquire pure, relatively short GS with DP about 11–82 for further use. This methodology may be also useful for GS preparation using glucan phosphorylases from other sources, e.g. plants, bacteria or yeast. The solvent system for HPSEC-ELSD may be also replaced by 100% water, which we presented in our previous publication (Kazłowski, Pan, & Ko, 2008).

Different detection approaches commonly used in carbohydrate analysis adapted for determining glucose oligo- and polysaccharide content were reported in previous reports. Chain-length is one of the key parameters, and HPSEC has been generally employed to estimate the saccharide size (Hanashiro, Abe, & Hizukuri, 1996). The chain length of amylopectin shows basically bimodal fraction distribution, F1 (long B chain, DP ≥ 35) and F2 (A chain, DP 6–18 and short B chain, DP 19–34) by HPSEC (Hizukuri, 1985; Nagamine & Komae, 1996). However, HPSEC could not separate individual chains greater than DP 16 (Kazłowski et al., 2008). HPLC connected with an NH2-bonded silica column using a refractive index was demonstrated to be a useful method for separating chains up to DP 30 due to the poor solubility of the large carbohydrates in reversed-phase eluents (Dionex application note 67: Determination of plant-derived natural oligo- and polysaccharides). However, this is not sufficient for analysis of the chain-length distribution of longer polysaccharides, e.g. amylopectin, where the main part of the chains achieving DP about 100 (Hanashiro et al., 1996). Another method for determining chain-length distribution of saccharides such as debranched starch is fluorophore-assisted carbohydrate electrophoresis (FACE) (Jiang, Campbell, Blanco, & Jane, 2010a). FACE is an analytical method for characterizing carbohydrate chain length that has been applied to neutral, charged, and N-linked oligosaccharides and that has been implemented using capillary electrophoresis; however, it has a limited maximum detectable chain-length at about DP 80 (Jiang, Srichuwong, Campbell, & Jane, 2010b).

Previously HPAEC-PAD was applied to determine many types of carbohydrates in a large variety of samples. Carbohydrates were separated with a PA1, strong anion-exchange column, specifically designed to separate mono- and oligosaccharides (Lee, 1996). Koizumi and co-workers presented that HPAEC-PAD with the PA1 could be used to separate linear α- or β-D-gluco-oligosaccharides and -polysaccharides with DP 50 (Koizumi, Kubota, Tanimoto, & Okada, 1989). Then, the HPAEC-PAD use for separation of glucose polymers (wheat amylopectin) with DP up to 81 was reported. This result was obtained by utilization of two PA1 columns (4 × 250 mm) connected in series (Hanashiro et al., 1996). Recently, analysis of the long chains of the sweet potato amylopectins (DP > 36) showing two subgroups: with peaks DP 45–48, and peak or shoulder around DP 72 was reported. However, for the subgroup at DP around 72, the chains longer than DP at around 60 were not resolved as individual peaks in HPAEC system (column: PA-100, 4 × 250 mm) (Zhu, Corke, & Bertoft, 2011). The chain length with DP higher than 60 was only approximately obtained by continuous dividing the chromatogram into corresponding peaks until reaching the baseline (Bertoft, 2004). According to our knowledge, this work is the first where HPAEC-PAD chromatography is used for the chain-length distribution analysis with DP ≤ 89. This performance was obtained by connecting PA1 and PA100 columns in series. The PA100 has a smaller latex particle size and increased latex cross-linking compared to the PA1. These differences yield more efficient chromatography and 10% less column capacity (Dionex application note 67: Determination of plant-derived natural oligo- and polysaccharides). The connection of PA1 and PA100 instead of two PA1 columns appears to result in the identification of a few more peaks in GS size than was reported previously (Hanashiro et al., 1996).

The limitation of the HPAEC-PAD system is that the alkaline condition use during analysis may cause the structure changes of saccharides. Koizumi and co-workers described that the shift of β-D-gluco-oligosaccharides and -polysaccharides to the smaller molecules was the result of the epimerization and degradation of maltooligosaccharides in alkaline solution (Koizumi, Fukuda, & Hizukuri, 1991). Also, the maltoheptaose was indicated to change into other molecules with retention times shorter than that of maltoheptaose during HPAEC-PAD analysis (Nagamine & Komae, 1996).

In conclusion, this study has established feasible biosynthesis of series of GS under controlled reactions of phosphorylase-a with α-D-glucose 1-phosphate and maltodextrin acceptors, and their length was monitored by HPSEC-ELSD and HPAEC-PAD systems. The GS of defined DP, quantity and high purity were successfully prepared for further testing of their biological activity potentials. Also, both systems enable measurement of phosphorylase-a activity, therefore these systems would also be applicable in kinetic characterization of phosphorylase enzyme from other natural sources such as mungbean.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.01.101.

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