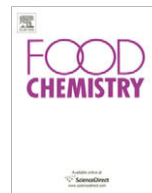
Food Chemistry 116 (2009) 595–603

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem



Analytical Methods

Chromatographic determination of polysaccharides in *Lycium barbarum* Linnaeus

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ARTICLE INFO

Article history:
Received 18 November 2008
Received in revised form 19 February 2009
Accepted 1 March 2009

Keywords:
Polysaccharide
Lycium barbarum Linnaeus
High-performance size exclusion chromatography
Gas chromatography

ABSTRACT

Polysaccharides in *Lycium barbarum* Linnaeus have been shown to be effective in preventing cancer. The objectives of this study were to develop an appropriate method for molecular weight determination of polysaccharides in *L. barbarum*. The most suitable analytical condition was: a volume-ratio of *L. barbarum* sample to deionized water at 1:10, followed by shaking in a 100 °C water bath for 30 min, concentrating to 50 mL and adding 250 mL of 95% ethanol for precipitation at −20 °C for 8 h, hydrolysing protein with 2.5 U/mL of proteinase at pH 8 and 60 °C for 4 h and separating polysaccharide into five fractions by high-performance size exclusion chromatography (HPSEC) with the molecular weight of two major fractions being 79,250 and 24,468 Da. Analysis of monosaccharides by gas chromatography (GC) indicated the presence of rhamnose, arabinose, xylose, mannose, glucose and galactose, with the molar ratio at 0.3:2.7:0.3:0.2:2.7:0.9, respectively.

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1. Introduction

Lycium barbarum Linnaeus, a traditional Chinese medicinal plant containing many functional components such as carotenoids, flavonoids and polysaccharides, has been shown to be effective in the prevention of chronic diseases (Gan, Zhang, Yang, & Xu, 2004; Inbaraj et al., 2008). The anti-proliferation effect of cancer cells and enhancement of immunity were demonstrated for both flavonoids and polysaccharides (Caltagirone et al., 2000; Huie & Di, 2004; Marchand, 2002; Wang & Fang, 2004). Therefore, it is important to learn about the amount and variety of these functional components.

The composition of carotenoids and flavonoids in *L. barbarum* Linnaeus has been well documented (Inbaraj et al., 2008; Qian, Liu, & Huang, 2004). However, the polysaccharide constituents still remained uncertain because of its complexity. Polysaccharide is widely distributed in edible plants and Chinese herbs and is composed of a minimum number of 100 monosaccharides, with molecular weight (MW) ranged from 10,000 to 1,449,000 Da (Zhang, Cheung, & Zhang, 2001 Apirattananusorn, Tongta, Cui, & Wang, 2008; Yapo & Koffi, 2008). The extraction of water-soluble polysaccharide is often carried out by hot water at 95–100 °C, followed by precipitation of unwanted substances such as protein and collection of supernatant after centrifugation for subsequent analysis (Huie & Di, 2004). For the alkaline-soluble polysaccharide, it was extracted with sodium hydroxide at 0.1–1.0 M instead of hot water (Huie & Di, 2004). Then the supernatant was subjected to enzymatic treatment, dialysis, gel filtration or ion exchange chromatography for further purification and determination of MW (Sun, Tang, Gu, & Li, 2005; Yang et al., 2006).

Shi, Sheng, Yang, and Hu (2007) reported that the highest yield of polysaccharide (44.8 g/kg) in *Chlorella pyrenoidosa* was achieved at 400 W of ultrasound for 800 s and then followed by incubation in water bath at 100 °C for 4 h in 80% ethanol. Two polysaccharide fractions were separated from the extracts of *C. pyrenoidosa* by using a Sepharose 4B column with the average MW being 81,877 Da and 1749 Da. GC analysis showed the majority of monosaccharide in both fractions were mannose with a low level of glucose. Yet, the major flaw of this study is that the number of polysaccharide fractions and monosaccharides separated was inadequate.

In another study the *C. pyrenoidosa* polysaccharides were fractionated using an ultrafiltration membrane with MW cut-off of 30 kDa and temperature at 40 °C and pressure at 14.0 psi followed by anion-exchange chromatography and size exclusion chromatography (Sheng et al., 2007). Two fractions were obtained with the MW being 69,658 Da and 109,406 Da, both of which were composed of rhamnose, mannose, glucose, and galactose. This outcome was different from that reported by Shi et al. (2007) using the same sample, which could be accounted for by the difference in extraction, purification and separation technique used.

Yang, Zhao, Yang, and Ruan (2008) determined water-soluble polysaccharide from *Gynostemma pentaphyllum* herb tea by hot-water extraction and ethanol precipitation. The monosaccharide was analysed by capillary zone electrophoresis, with glucose being present in largest amount, followed by galactose, arabinose, rhamnose, galacturonic acid, xylose, mannose and glucuronic acid.

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doi:10.1016/j.foodchem.2009.03.015

However, the purification step was inadequate and no MW was determined. The acidic polysaccharide in pumpkin was obtained by hot water at 80 °C for 2 h, followed by concentrating under vacuum, precipitating with 95% ethanol overnight, centrifugation, dialysis, and deproteination (Yang, Zhao, & Lv, 2007). A high-performance capillary electrophoresis method was used to determine the monosaccharide composition, with glucose showing the highest amount, followed by glucuronic acid, galactose, arabinose, xylose and rhamnose. But, no MW was measured.

A new polysaccharide with MW > 10^6 in the peel of *Passiflora liguralis* fruits was obtained through extraction by KOH for 3 days, centrifugation, precipitation with ethanol overnight, dialysis for 3 days, freeze drying and purification using a Sepharose DEAE CL-6B column (Tommonaro et al., 2007). The monosaccharide composition was shown to be xylose, glucose, galactose, galactosamine and fucose. Yet, the extraction step is lengthy. The analysis of monosaccharides has been previously achieved by paper chromatography (PC), thin-layer chromatography (TLC), gas chromatography (GC) and high-performance liquid chromatography (HPLC). Both PC and TLC are easy to operate, however, a lack in accuracy, reproducibility and resolution power was reported (Wang & Fang, 2004). Both normal-phase and reversed-phase HPLC are also adopted for analysis of monosaccharides, with the latter being inferior in separation power (Wang & Fang, 2004). Therefore, a NH_2 or carbohydrate column was frequently employed for separation of sugars by HPLC (Wang & Fang, 2004). In addition, GC was often used for monosaccharide separation because of its high resolution and short retention time (Wang & Fang, 2004). In view of the impact of polysaccharides on human health, this study was undertaken to develop a better method for extraction, purification and separation of polysaccharides in *L. barbarum* Linnaeus.

2. Materials and methods

2.1. Materials

A total of 12 kg of *L. barbarum* L. was purchased from a local drug store in Taipei, Taiwan. The Cosmosil 5 Diol-300-II (300 × 7.5 mm I.D., 5 μm particle size) column used to determine MW was from Nacalai Co (Kyoto, Japan). The capillary column HP-5MS (30 × 0.25 mm I.D., 0.25 μm film thickness, 5% diphenylpolysiloxane, 95% dimethylpolysiloxane, ultra low bleed) was from Agilent Technologies (CA, USA). Deionized water was made using a Mill-Q water purification system from Millipore (Bedford, MA, USA). Monosaccharide standards including glucose, galactose and myo-inositol (internal standard) were from Sigma (St. Louis, MO, USA), while arabinose, rhamnose, mannose and xylose were from Nacalai Co. The derivatizing agent pyridine was from J.T. Baker (Phillipsburg, NJ, USA). Hydroxylamine hydrochloride was from Fluka Chemical (Buchs, Switzerland). Sulphuric acid (95–97% purity), trifluoroacetic aicd, boric acid, methyl red, bromophenol blue, sodium hydroxide and catalizador Kjeldahl (Cu–Se) were from Panreac Co (Barcelona, Spain). Ethanol (95%) was from Taiwan Tobacco and Wine Co. Acetic anhydride, bovine serum albumin and proteinase (Type VIII from *Bacillus* L) were from Sigma. Anhydrous sodium sulphate was from Nacalai Co.

2.2. Instrumentation

The HPLC instrument is composed of a G1379A degasser, a G1311A quaternary pump, a G1312A binary pump (Agilent Technologies) and an Alltech ELSD 800 detector (Deerfield, IL, USA). The spectrophotometer (DU 640) was from Beckman (Fullerton, CA, USA). The GC system (model 6890 G1530A) equipped with flame ionisation detector (FID) was from Agilent Technologies. The homogenizer (890-68) was from Oster Co (Wisconsin, USA). The high-speed centrifuge (Sorvall RC5C) was from Du Pont (Wilmington, Delaware, USA). The rotary evaporator (N-1) was from Eyela (Tokyo, Japan). The pH metre (SP-701) was from San-Tai Co (Taipei, Taiwan).

2.3. Determination of basic composition of L. barbarum Linnaeus

The basic components in *L. barbarum* L. samples, including moisture, protein, fat and ash were determined using AOAC official method (1997). Briefly, moisture was analysed by placing 1-g sample in an oven at 105 °C and drying for 3–5 h to constant weight, and moisture (%) was calculated based on the difference of sample weight before and after drying divided by the original sample weight. Protein was analysed by placing 0.5 g of sample in a flask and mixed with 2.5 g of catalyst (Cu–Se) and 5 mL of sulphuric acid (36 N), after which the mixture was heated until a clear solution formed, followed by distillation by adding 10 mL of 40% NaOH gradually to liberate ammonia, and the distillate was collected with 25 mL of 4% boric acid solution containing indicator for titration with 0.01 N hydrochloric acid until colour changed. The protein content (%) was calculated using a formula as described by Association of Official Analytical Chemists (AOAC) (1997). Fat was analysed by wrapping 5 g of sample in a round-shape filter paper and dried at 130 °C for 2 h, after which the paper was placed in a flask and 40-mL of petroleum ether was added, followed by transferring the flask to a Soxlet extractor and heated at 135 °C for 2 h. The fat content (%) was calculated based on the difference of sample weight before and after drying divided by the original sample weight. Ash was analysed by placing 1.0 g of sample on a porcelain crucible and heating in a furnace at 550–600 °C overnight until grey or white colour formed, followed by cooling and heating again for 2 h. The ash content (%) was calculated based on the difference of sample weight before and after heating divided by the original sample weight.

2.4. Comparison of extraction efficiency of crude polysaccharide

The crude polysaccharide was determined based on a method by Sun et al. (2005) and was modified. A 10 g of *L. barbarum* L. sample was mixed with 50, 100, 150 or 200 mL of deionized water and homogenised for 1 min, after which the mixture was heated in hot water bath (80, 90 or 100 °C) for 10, 30, 60, 120 or 180 min for extraction, followed by centrifuging at 6000 rpm for 25 min and filtering through a filter paper to remove impurities. Then the crude polysaccharide extract was concentrated under vacuum at 40 °C to a small volume and diluted to 50 mL with deionized water, and a volume of 50, 150, 200, 250, 300, 350 or 400 mL of 95% ethanol was added for protein precipitation for 2, 4, 8 or 12 h at 25, −4 or −20 °C. The solution was centrifuged again to remove supernatant, and the precipitate was vacuum dried at 40 °C to obtain the crude polysaccharide, which was ground into powder and dissolved in 1000 mL of deionized water for use. In addition, prior to extraction, *L. barbarum* L. sample was also mixed with 100 mL of 50%, 80% and 95% ethanol in a 90 °C water bath for 2 h separately to remove water-soluble flavonoids and the sample was dried in an oven for subsequent analysis to compare the extraction efficiency of crude polysaccharide. During the experiment, one parameter was changed with the other two parameters fixed at the same time for evaluation of extraction efficiency.

2.5. Determination of total sugar in crude polysaccharide

The total sugar in crude polysaccharide of *L. barbarum* L. samples was determined using a phenol–sulphuric acid method as described by Dubois, Gilles, Hamilton, Rebers, and Smith (1956). A

0.2-mL of crude polysaccharide solution was mixed with 0.2 mL of 5% phenol solution and 1-mL of concentrated sulphuric acid was added, after which the mixture was shaken for 30 min and the absorbance was measured at 490 nm. The total sugar was calculated based on the standard curve of glucose, which was prepared by using six concentrations of 10, 20, 30, 40, 50 and 100 μg/mL.

2.6. Determination of protein

The protein content in *L. barbarum* L. sample was determined using a Lowry method (Lowry, Rosenbrough, Farr, & Randall, 1951). Six concentrations of 25, 50, 100, 150, 200 and 300 μg/mL of bovine serum albumin (BSA) were prepared, and 0.5 mL each was collected and mixed with 1-mL of reagent (a mixture of 1 mL of 4% Na–K tartrate, 10 mL of 2 N sodium hydroxide, 1 mL of 2% copper sulphate and 4 g of sodium carbonate and diluted to 100 mL with deionized water), followed by shaking and reacting at room temperature for 10 min, and adding 0.2 mL of Folin-Ciocalteu phenol. After thorough mixing, the solution was settled for 30 min, and the absorbance was measured at 750 nm. The BSA standard curve was obtained by plotting concentration against absorbance. For protein determination in *L. barbarum* L. samples, a 500 μL of crude polysaccharide solution was prepared and the absorbance was measured at 750 nm using the same method. The protein content was calculated based on the BSA standard curve and was expressed as μg/mL equivalent.

2.7. Removal of protein by protease

Because of presence of protein in *L. barbarum* L. samples, protein has to be removed to avoid subsequent interference in determining MW of polysaccharide. A 0.2 g of crude polysaccharide powder was dissolved in 40 mL of buffered phosphoric acid solution (50 mM and pH 8), and the solution was heated in a water bath at 60 °C for 5 min, after which 1-mL of 100, 200, and 300 unit proteinase (Type VIII from *Bacillus L*) was added separately and continued to heat in a water bath at 60 °C for 0.5–12 h. After the desired reaction time was reached, 40 mL of trichloroacetic acid (5%) was added to terminate the reaction. Then the mixture was cooled immediately so that the unreacted free protein and protein–polysaccharide complex were precipitated separately. After settling for 30 min and centrifuging at 10,000*g* for 15 min to remove protein, the soluble peptide in the supernatant was determined using Lowry method (Lowry et al., 1951) with absorbance measured at 750 nm.

2.8. Removal of protein by Sevag method

The removal of protein by Sevag method was also used to compare with that by proteinase. A 0.2 g of crude polysaccharide powder was dissolved in 40-mL of deionized water, after which a portion (5 mL) was collected and mixed with 5 mL of chloroform/*n*-butanol (4:1, v/v). The solution was shaken vigorously at room temperature for 15 min and then centrifuged at 6000 rpm for 10 min. The supernatant was collected and the protein content was analysed using Lowry method (Lowry et al., 1951), after which the supernatant was repeatedly washed with 5 mL of chloroform/*n*-butanol 1–6 times for determination of protein and polysaccharide losses. In addition, the polysaccharide content in the supernatant was also measured using a phenol–sulphuric acid method (Dubois et al., 1956).

2.9. Purification of polysaccharide by dialysis

Dialysis was used in this study to remove unwanted salts and impurities with MW < 3500. Initially the dialysis membrane was cut into a size of 10 × 5.4 cm and immersed in deionized water for 30 min, followed by soaking again in 0.1% of EDTA (50 °C) for 30 min. This procedure was repeated three times and then washed with deionized water for use. A 40 mL of deproteinated polysaccharide solution treated with proteinase was poured into a dialysis bag, which was placed in a deionized water-containing flask to flow. Dialysis was proceeded for 3 h, during which the dialysis solution was stirred and replaced 2–3 times to complete the desalting process. The dialysed solution was further concentrated to 40 mL, and a 200-mL of 95% ethanol was added for precipitation at −20 °C for 8 h, followed by vacuum drying and ground into powder (deproteinated powder).

2.10. Bleaching of polysaccharide by active carbon

After enzymatic hydrolysis of crude polysaccharide by proteinase, the presence of a deep yellow brown colour in polysaccharide may interfere with the subsequent determination of MW. Thus, a further bleaching treatment by active carbon is necessary. A bleaching method based on Zhao, Li, and Xiao (2005) was modified. One gram of deproteinated polysaccharide powder was mixed with 50 mL of deionized water, and the solution was added with six levels of 50, 150, 250, 350, 450 and 600 mg of active carbon separately. After shaking vigorously at room temperature for 15 min, the solution was centrifuged at 6500 rpm for 15 min, and the supernatant was collected with the absorbance measured at 450 nm for determination of polysaccharide loss.

2.11. Determination of molecular weight

A Cosmosil 5 Diol-300-II column (300 × 7.5 mm I.D., particle size 5 μm) was used to separate polysaccharide into several fractions of different molecular weight with a mobile phase of formic acid solution (pH 3), and detection was carried out using ELSD with drift temperature at 90 °C, gas pressure at 3 bar, column temperature at 40 °C and flow rate at 1 mL/min. As reported by the manufacturer, this column is appropriate to separate water-soluble polymers with MW ranged from 500 to 30,000 Da. The MW was determined base on the standard curve of pulluan with known MW.

2.12. Analysis of monosaccharides by GC

Internal standard myo-inositol was dissolved in deionized water for a concentration of 1000 μg/mL. Six concentrations of 25, 50, 100, 200, 400 and 600 μg/mL were prepared for glucose, arabinose and galactose separately. Likewise, six concentrations of 10, 20, 40, 60, 80 and 100 μg/mL were prepared for rhamnose, xylose and mannose separately. All the monosaccharide solutions were each contained 200 μg/mL of myo-inositol.

Polysaccharides in *L. barbarum* L. were hydrolysed using a method based on Benhura and Chidewe (2002) and was modified. One mL of purified polysaccharide solution was poured into a 10-mL screw-capped tube and 2 M trifluoroacetic acid was added, after which the mixture was reacted in a 120 °C oven for 3 h, followed by cooling to room temperature and centrifuging at 6000 rpm for 5 min, and the supernatant was collected. Monosaccharides were derivatized based on a method by Macías-Rodríguez, Quero, and López (2002) and Ye, Yan, Xu, and Chen (2006) and was modified. A 200-μL of monosaccharide standard solution or a 2-mL of hydrolysed polysaccharide solution was evaporated to dryness under vacuum separately and dissolved in 300 μL of hydroxylamine hydrochloride solution (60 mg in 1-mL of pyridine), followed by ultrasonication for 20 s, oximization in a 90 °C water bath for 30 min and cooling to room temperature. Then a 300 μL of acetic anhydride was added and the mixture was shaken homogeneously, after which the solution was subjected to acetylation in

a 90 °C water bath for 30 min and cooled to room temperature for formation of aldonitrile peracetylated derivatives. Next, 500 μL of chloroform was added and the solution was washed with 500 μL of deionized water 3–4 times. The lower layer (organic layer) was collected and passed through anhydrous sodium sulphate to remove residual moisture, evaporated to dryness under nitrogen and dissolved in 100 μL of chloroform for use. A 1-μL of chloroform solution was injected into GC, and the standard curve was prepared by plotting concentration ratio of monosaccharide to internal standard against its area ratio. The regression equation and correlation coefficient (R^2) of each standard curve were automatically determined with an EXCEL software system. The regression equations for arabinose, glucose, galactose, rhamnose, xylose and mannose were $y = 0.691x + 0.0073$, $y = 0.6815x + 0.0112$, $y = 0.7143x + 0.0065$, $y = 0.6843x + 0.0032$, $y = 0.7519x + 0.0036$ and $y = 0.7688x + 0.0048$, respectively, with R^2 ranged from 0.9963 to 0.9998, indicating a high linearity correlation was achieved for all the standard curves.

The GC condition used for separation of monosaccharides was based on a method by Macías-Rodríguez et al. (2002) and was modified. A capillary column HP-5MS (30 m × 0.25 mm I.D., 0.25-μm film thickness) was used with injector temperature at 270 °C, detector (FID) temperature at 300 °C, split ratio at 1:1, and flow rate at 0.3 mL/min with helium as carrier gas. The temperature in the oven was programmed as follows: 150 °C in the beginning, maintained for 3 min, increased to 270 °C at 10 °C/min and maintained for 15 min.

3. Results and discussion

3.1. Basic composition of *L. barbarum* L.

The basic components of *L. barbarum L.* sample contained carbohydrate in largest amount (51.23%), followed by moisture (25.80%), protein (15.96%), ash (5.58%) and fat (1.43%).

3.2. Extraction efficiency of total sugar and crude polysaccharide

Table 1 shows the extraction efficiency of total sugar and crude polysaccharide as affected by solvent volume, extraction time and temperature. The extraction efficiency was evaluated by mixing 10 g of sample and 50, 100, 150, and 200 mL of deionized water with a ratio of 1:5, 1:10, 1:15 and 1:20 separately, after which each solution was heated in hot water bath (80, 90 or 100 °C) for 10, 30, 60, 120, or 180 min, followed by concentration under vacuum at 40 °C and diluted to 50 mL with deionized

Table 1
Effect of different extraction and purification conditions as well as pretreatment with ethanol on crude polysaccharide content in *Lycium barbarum*.

Treatment conditions	Total sugar (g/g lycium)[c,d]	Crude polysaccharide (mg/g lycium)[c,d]	
Extraction conditions			
Volume ratio (v/v)[a]			
1:5	0.47 ± 0.01^{C}	12.30 ± 1.06^{C}	
1:10	0.53 ± 0.02^{B}	14.00 ± 1.73^{BC}	
1:15	0.55 ± 0.01^{B}	15.66 ± 1.57^{AB}	
1:20	0.58 ± 0.00^{A}	16.24 ± 1.82^{A}	
Extraction time (min)			
10	0.50 ± 0.03^{B}	17.62 ± 1.04^{B}	
30	0.54 ± 0.04^{A}	21.50 ± 1.61^{A}	
60	0.56 ± 0.01^{A}	21.14 ± 1.17^{A}	
120	0.54 ± 0.03^{A}	20.57 ± 0.40^{A}	
180	0.56 ± 0.02^{A}	19.69 ± 1.35^{A}	
Extraction temp. (°C)			
80	0.57 ± 0.02^{A}	22.53 ± 0.37^{B}	
90	0.57 ± 0.02^{A}	24.22 ± 0.94^{B}	
100	0.58 ± 0.02^{A}	27.30 ± 1.79^{A}	
Purification conditions			
Volume ratio (v/v)[b]			
1:2	–	33.64 ± 1.12^{D}	
1:3	–	26.44 ± 0.51^{E}	
1:4	–	38.67 ± 1.36^{C}	
1:5	–	47.28 ± 1.38^{A}	
1:6	–	40.94 ± 2.47^{B}	
1:7	–	42.01 ± 1.39^{B}	
1:8	–	41.89 ± 0.50^{B}	
Precipitation time (h)			
2	–	36.84 ± 0.35^{C}	
4	–	43.78 ± 0.77^{B}	
8	–	46.80 ± 0.57^{A}	
12	–	43.54 ± 0.59^{B}	
Precipitation temp. (°C)			
25	–	40.74 ± 1.00^{C}	
−4	–	45.73 ± 0.98^{B}	
−20	–	57.19 ± 0.29^{A}	
Pretreatment with EtOH prior to heating (90 °C, 2 h)			
		Ethanol portion	Hot water portion
Without pretreatment with EtOH	–	–	47.28 ± 1.38^{A}
With pretreatment			
95% EtOH	–	2.14 ± 0.20^{C}	32.99 ± 0.93^{B}
80% EtOH	–	15.05 ± 1.00^{B}	15.57 ± 0.62^{C}
50% EtOH	–	44.21 ± 0.34^{A}	8.53 ± 0.39^{D}

[a] Sample to water ratio.
[b] Sample to ethanol ratio.
[c] Average of duplicate analyses ± standard deviation.
[d] Symbols bearing different letters (A–D) in the same column within each treatment condition are significantly different ($P < 0.05$).

water. A volume of 95% ethanol (100, 150, 200, 250, 300, 350 or 400 mL) was added for precipitation for 2, 4, 8 and 12 h at various temperatures (25, −4, −20 °C) separately to obtain crude polysaccharide. Total sugar was determined using a phenol–sulphuric acid method as described in the method section (Dubois et al., 1956). Both total sugar and crude polysaccharide showed an increased trend following a rise in the ratio of sample to water, with a peak level of 0.58 g/g and 16.24 mg/g being attained, respectively, for the ratio at 1:20. However, a ratio of sample to solvent at 1:10 was selected for crude polysaccharide extraction instead of 1:20 to reduce solvent consumption and maintain a high level of crude polysaccharide. Also, an extraction time of 30-min was found to generate the highest content of total sugar and crude polysaccharide at 0.54 g/g and 21.50 mg/g, respectively. Of the various extraction temperatures, a large yield of total sugar and crude polysaccharide was obtained at 100 °C, which equalled 0.58 g/g and 27.30 mg/g, respectively.

The effect of volume ratio (sample to solvent), precipitation time and temperature on crude polysaccharide content in *L. barbarum* L. is shown in Table 1 as well. Following extraction of crude polysaccharide by hot water, ethanol is often added for crude polysaccharide precipitation. Of the various volume ratios of sample to ethanol, a ratio of 1:5 was shown to result in the highest content of crude polysaccharide (47.28 mg/g), followed by a ratio of 1:7, 1:8, 1:6, 1:4, 1:2 and 1:3. It is interesting to note that the polysaccharide content was reduced for a high ratio at 1:8 and 1:7, which equalled 41.89 and 42.01 mg/g, respectively. This is probably because of presence of a high number of hydroxyl groups in polysaccharide, which may interact with hydroxyl group of ethanol so that both were in a soluble state (Li & Fan, 2005). The effect of ethanol precipitation time on crude polysaccharide content was seldom investigated in the literature. Our study indicated the crude polysaccharide content increased along with increasing precipitation time and a maximum (46.80 mg/g) was reached in 8 h, but declined to 43.54 mg/g in 12 h, which may be due to enhancement of polysaccharide solubility in ethanol after prolonged precipitation. For precipitation temperature, the crude polysaccharide content rose following a decline in temperature and a plateau (57.19 mg/g) was achieved at −20 °C. By combining the results shown above, the most appropriate extraction condition was as follows: sample to water ratio at 1:10, heated in a water bath at 100 °C for 30 min, concentrated to a small volume under vacuum, followed by adding 95% ethanol with a volume five times that of sample, and precipitating at −20 °C for 8 h. A high yield (5.7%) of polysaccharide was obtained by using this method, which was substantially higher than that reported by Gan et al. (2004) and Li, Zhou, and Li (2007), which equalled 3.35% and 1.9%, respectively.

3.3. Pretreatment of crude polysaccharide extraction with ethanol

Table 1 also shows the effect of various concentrations of ethanol on crude polysaccharide content in *L. barbarum* Linnaeus prior to extraction and heating in hot water bath. The pretreatment by different concentrations of ethanol was used to remove water-soluble impurities such as flavonoids to avoid subsequent interference in determining MW of polysaccharides. The polysaccharide pretreated with 50% ethanol was present at a much larger amount in ethanol portion than in water portion (Table 1), indicating a significant loss of polysaccharide could occur for this treatment. However, with pretreatment by 80% ethanol, the content of polysaccharide in the water portion rose substantially compared to that by 50% ethanol. (Table 1), implying a less loss of polysaccharide to occur for 80% ethanol. By raising the ethanol concentration to 95%, only a small amount of polysaccharide was found in the ethanol portion, and most polysaccharides were extracted in water portion. It is apparent that the lower the ethanol concentration, the more the content of polysaccharides in the ethanol portion, mainly because of the increased water content. In contrast, a high yield of polysaccharide (47.28 mg/g) was shown in hot water portion without ethanol pretreatment. Thus, in our subsequent experiment the pretreatment step with ethanol was eliminated to reduce extraction time and prevent polysaccharide loss.

Gan et al. (2004) used a solvent system of acetone/petroleum ether (1:1, v/v) to extract polysaccharide in *L. barbarum* L. three times, followed by adding 80% ethanol to dried residue three times, heating in 80 °C water every 2 h for four times and then concentrating, precipitating to remove protein with ethanol, centrifuging, dissolving precipitate in water and subjecting to anion-exchange chromatography to obtain one neutral polysaccharide fraction and three acidic polysaccharide fractions, dialysing each fraction for 3 days and purifying on a Sephadex G200 column. A yield of 0.297 g of an acidic polysaccharide was obtained and was composed of 63.56% neutral sugars, 24.80% acidic sugars and 7.63% proteins. In a similar study, Li et al. (2007) extracted dried *L. barbarum* L. in boiling water, followed by concentrating the combined extracts, deproteinating by Sevag method (1934), dialysing the aqueous fraction against distilled water for 2 days, concentrating the retentate, adding 95% ethanol for precipitation, centrifuging, and drying the precipitate under vacuum to give a powder product. Compared to our study, both methods by Gan et al. (2004) and Li et al. (2007) are lengthy and require a much longer dialysis time.

3.4. Purification of polysaccharide by removal of protein

During analysis of polysaccharides in plant sample, protein has to be removed to avoid subsequent interference in determining MW of polysaccharides. In our study both the Sevag method and the application of proteinase in removing protein were compared. Table 2 shows the deproteination efficiency of crude polysaccharides as affected by Sevag method. With deproteination six times, there was no significant difference in losses of protein and polysaccharide, which ranged from 19.41% to 21.99% and 8.43% to 11.93%, respectively. It may be postulated that the Sevag method was effective in removing free proteins, but not proteins combined with polysaccharides.

Proteinase such as chymotrypsin, trypsin and papain have been frequently used to hydrolyse protein (Liu et al., 2007; Park, Cheong, & Jung, 1996; Peng & Zhang, 2003). In our study proteinase (Type VIII from *Bacillus L.*) was selected for hydrolysis. Fig. 1 shows the effect of incubation time on protein hydrolysis in crude polysaccharide as affected by different proteinase concentrations. Under the same concentration, the amount of peptide soared following an increase in reaction time, implying a higher degree of hydrolysis occurred. In other words, the larger the concentration of proteinase, the more formation the peptides.

By concentrating the polysaccharide solution after protein hydrolysis and precipitating with 95% ethanol, the protein and total sugar content were determined by Lowry and phenol–sulphuric acid methods, respectively. It was revealed that about 96.7% protein was removed by proteinase, indicating most polysaccharide–bound proteins could be hydrolysed, which was much greater than that of Sevag method as only 19.41–21.99% of protein was removed. However, the polysaccharide loss climbed to 59.0%, which was also larger than that of Sevag method (8.43–11.93%), probably because of hydrolysis of glycoprotein by proteinase, resulting in a considerable loss of polysaccharide. Nevertheless, the deproteinated polysaccharide with proteinase was still adopted for MW determination as the polysaccharide loss should not affect the polysaccharide MW distribution. Therefore, the most appropriate hydrolysis condition was chosen to be proteinase with a concentration at 2.5 U/mL, pH 8, temperature 60 °C and reaction time 4 h.

Table 2
Deproteination efficiency and polysaccharide loss as affected by Sevag method.[a]

Frequency of polysaccharide washed with chloroform–*n*-butanol	Protein content (mg/g)	Protein loss[b] (%)	Polysaccharide content (mg/g)	Polysaccharide loss[b] (%)
0[c]	22.3	–	57.19	–
1	17.40 ± 0.49[A]	21.99 ± 2.21[A]	51.86 ± 0.43[A]	9.31 ± 0.76[A]
2	17.62 ± 0.64[A]	21.01 ± 2.87[A]	51.71 ± 2.79[A]	9.58 ± 4.89[A]
3	17.87 ± 0.52[A]	19.88 ± 2.34[A]	50.36 ± 2.01[A]	11.93 ± 3.52[A]
4	17.55 ± 0.30[A]	21.29 ± 1.36[A]	51.10 ± 1.77[A]	10.58 ± 3.17[A]
5	17.76 ± 0.37[A]	20.36 ± 1.64[A]	52.13 ± 1.86[A]	8.85 ± 3.24[A]
6	17.97 ± 0.07[A]	19.41 ± 0.30[A]	52.36 ± 1.55[A]	8.43 ± 2.70[A]

[a] Average of duplicate analyses ± standard deviation.
[b] Symbols bearing different letters in the same column are significantly different ($P < 0.05$).
[c] Polysaccharide without washing.

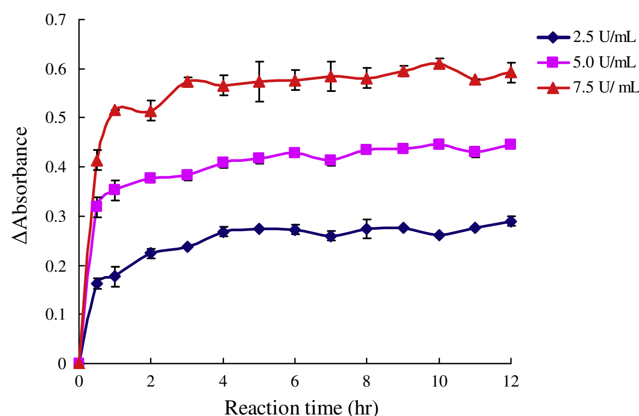

Fig. 1. Effect of proteinase concentrations on hydrolysis of protein in crude polysaccharides.

3.5. Bleaching of crude polysaccharide by active carbon

Because of presence of deep yellow brown colour of polysaccharide after protein hydrolysis, the removal of pigments by active carbon is necessary. Table 3 shows the effect of active carbon on decolorization of crude polysaccharide. Decolorization percentage was calculated based on the difference in absorbance with and without active carbon treatment divided by that without active carbon treatment. Following an increase in concentration of active carbon, the decolorization effect rose sharply as well, with a plateau of 68.39% decolorization being accomplished at 9 mg/mL, and remained almost the same at 12 mg/mL. Meanwhile, the polysaccharide loss was also raised from 8.24% to 30.28% for active carbon at a concentration of 1 and 12 mg/mL, respectively. This phenomenon clearly demonstrated the capability of active carbon to absorb pigment and polysaccharide simultaneously during bleaching process. Thus, the decolorized polysaccharide was not used for further experiment to reduce polysaccharide loss and shorten purification time. Instead, the deproteinated polysaccharides obtained by proteinase were used for subsequent separation for MW determination.

3.6. Determination of molecular weight of polysaccharide

A Cosmosil 5Diol-300-II column (300 × 7.5 mm I.D., 5 μm) containing silica gel was used to separate polysaccharide into several fractions with different molecular weight. For MW detection, the standard pulluan (Shodex P-83) with different MW, P-200 (21.2×10^4), P-100 (11.2×10^4), P-50 (4.73×10^4), P-20 (2.28×10^4), P-10 (1.18×10^4) and P-5 (0.59×10^4) was prepared and 20-μL was injected for HPSEC (High-Performance Size Exclusion Chromatography) analysis. Then the pulluan standard curve was obtained by plotting retention time against log MW. The log MW for MW 21.2×10^4, 11.2×10^4, 4.73×10^4, 2.28×10^4, 1.18×10^4 and 0.59×10^4 were 5.3263, 5.0492, 4.6748, 4.3579, 4.0718 and 3.7708, respectively, and the retention times were 5.620, 6.158, 7.161, 8.346, 9.045, and 9.627 min. The regression equation was $y = -0.3654x + 7.3403$ with a high correlation of $R^2 = 0.9919$ obtained for the standard curve. Fig. 2A shows the HPSEC chromatogram of polysaccharide in water extract, and some more peaks were present than in crude polysaccharide. (Fig. 2B), with the MW of most peaks being lower than 5900 Da. Obviously this should be caused by the ethanol precipitation step being not carried out for polysaccharides in water extract. In Figs. 2A and B, peak 1 denotes the polysaccharide fraction with MW at about 83,576 Da, peak 2 with MW at approximately 34,467 Da and peaks 3–6 with MW smaller than 5900 Da. Fig. 2C shows the HPSEC chromatogram of purified polysaccharides pretreated with 50% ethanol in a 90 °C water bath for 2 h, followed by heating in a 100 °C water bath for 30 min and adding ethanol for precipitation, centrifugation and vacuum drying. Only three peaks were present, corre-

Table 3
Effect of active carbon on decolorization of deproteinated polysaccharides and polysaccharide loss.[a,b]

Active carbon (mg/mL)	Decolorization percentage[c] (%)	Polysaccharide (mg/mL)	Polysaccharide loss[d] (%)
–	0.00 ± 0.00[F]	1.66 ± 0.01[A]	0.00 ± 0.00[G]
1	25.18 ± 0.01[E]	1.52 ± 0.01[B]	8.24 ± 0.43[F]
3	48.08 ± 0.07[D]	1.41 ± 0.00[C]	15.20 ± 0.04[E]
5	58.14 ± 0.39[C]	1.33 ± 0.01[D]	19.40 ± 0.17[D]
7	60.79 ± 0.56[B]	1.32 ± 0.03[D]	21.03 ± 0.85[C]
9	68.39 ± 0.00[A]	1.24 ± 0.01[E]	25.07 ± 0.85[B]
12	68.03 ± 0.02[A]	1.16 ± 0.00[F]	30.08 ± 0.09[A]

[a] Average of duplicate analyses ± standard deviation.
[b] Symbols bearing different letters (A–F) in the same column are significantly different ($P < 0.05$).
[c] Decolorization percentage = $(A_i - A_s) \times 100/A_i$, A_i: absorbance of initial polysaccharide at 450 nm; A_s: absorbance of bleached polysaccharide at 450 nm.
[d] Polysaccharide loss = $(A_i - A_s) \times 100/A_i$, A_i: absorbance of initial polysaccharide at 490 nm by phenol–sulphuric acid method; A_s: absorbance of bleached polysaccharide at 490 nm by phenol–sulphuric acid method.

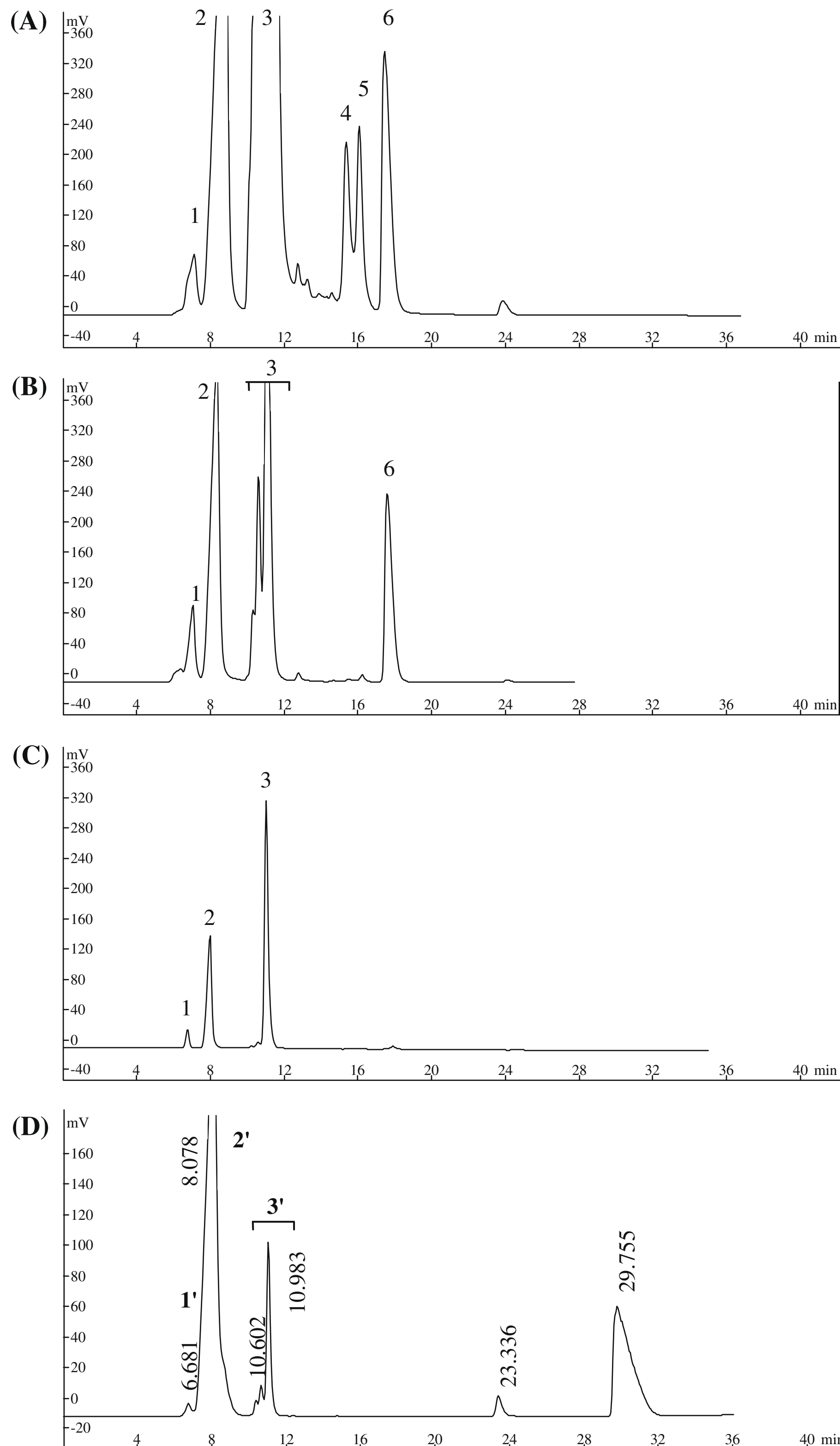

Fig. 2. Molecular weight distribution of polysaccharides from the fruit of *L. barbarum* (A) water extract, (B) crude polysaccharide, (C) crude polysaccharide pretreated with ethanol, (D) deproteinated polysaccharide.

sponding to a retention time of 6.713, 7.933, and 10.964 min, respectively. The MW of the peaks 1 and 2 were 77,156 and 27,642 Da, respectively, based on the standard curve of pulluan, but the MW of peak 3 was not determined as it was out of the linear range of the pulluan standard curve. This outcome clear revealed the pretreatment of sample with 50% ethanol to be effective in removing polysaccharides with small MW. This result is different from a report by Liang (2002), who showed the average MW of the water-soluble polysaccharide to be smaller than 10,000 Da, which is probably caused by the difference in conditions

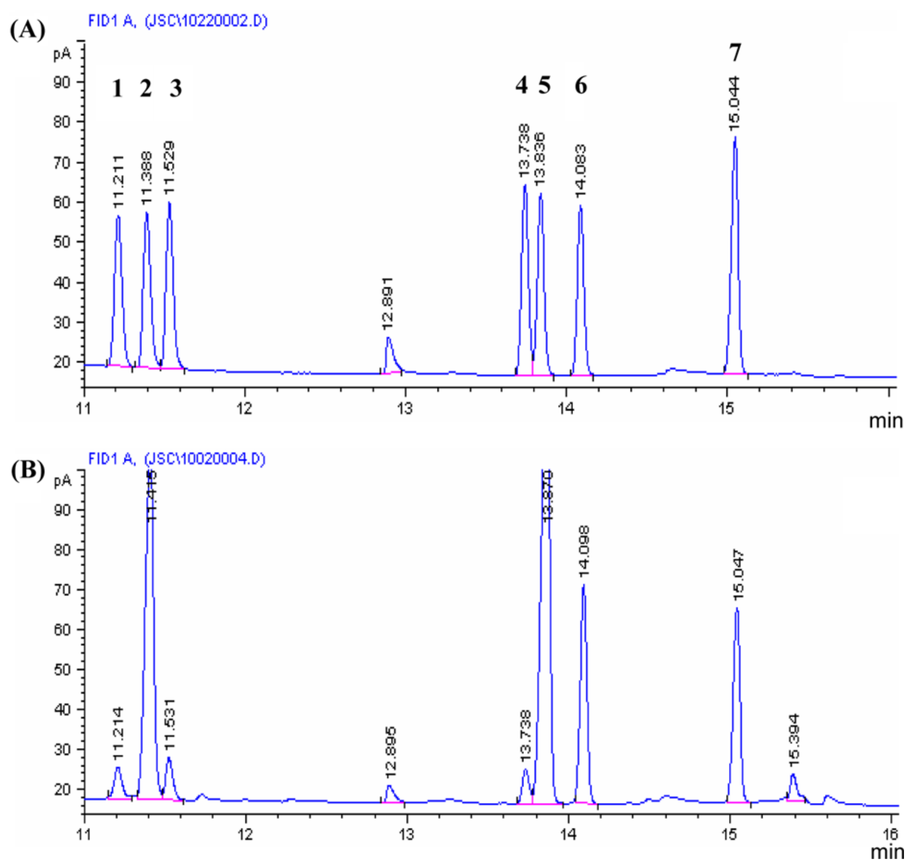

Fig. 3. GC chromatogram of monosaccharides (A) monosaccharide standards and internal standard myo-inositol; (B) monosaccharides in polysaccharide of *L. barbarum* sample and internal standard. Peaks: (1) rhamnose; (2) arabinose; (3) xylose; (4) mannose; (5) glucose; (6) galactose and (7) myo-inositol.

of extraction, purification and separation. Fig. 2D shows the HPSEC chromatogram of deproteinated polysaccharide, which was obtained by treatment of crude polysaccharide with proteinase at 60 °C and pH 8 for 4 h, with the MW of two major peaks 1′ and 2′ being 79,250 and 24,468 Da, respectively. Compared to the HPSEC chromatogram of crude polysaccharide (Fig. 2B), one more peak with retention time at 29.755 min was present, which may be due to the formation of polysaccharide with small MW through hydrolysis of glycoprotein.

3.7. GC analysis of monosaccharides

Fig. 3 shows the GC chromatogram of rhamnose, arabinose, xylose, mannose, glucose and galactose standards (Fig. 3A) and those in *L. barbarum* L. sample (Fig. 3B). By comparing retention times of unknown peaks with reference sugar standards and cochromatography with added standards, six monosaccharides including rhamnose, arabinose, xylose, mannose, glucose and galactose were identified, with the retention times being 11.21, 11.39, 11.52, 13.73, 13.83 and 14.08 min, respectively, and the molar ratio at 0.3:2.7:0.3:0.2:2.7:0.9. This result clearly demonstrated that both arabinose and glucose were the dominant monosaccharides in *L. barbarum* L. In a similar study, Li et al. (2007) reported the presence of rhamnose, arabinose, xylose, fucose, glucose and galactose in polysaccharide of *L. barbarum* L. with the molar ratio at 1:2.14:1.07:2.29:3.59:10.06, implying both glucose and galactose were the major sugars. This outcome is different from our experiment as no fucose was detected in the *L. barbarum* L. sample. The difference in molar ratio of monosaccharides and the presence of mannose in our *L. barbarum* L. sample should be caused by variation in variety of *L. barbarum* sample and the analytical condition employed.

4. Conclusion

In conclusion, the most appropriate extraction and purification condition for determination of polysaccharide in *L. barbarum* L. was as follows: A volume ratio of *L. barbarum* L. sample to deionized water at 1:10, followed by shaking at 100 °C for 30 min, concentrating to a small volume under vacuum, adding fivefold volume of 95% ethanol for precipitation for 8 h at −20 °C, hydrolysing protein with 2.5 U/mL of proteinase at pH 8 and 60 °C for 4 h and separating polysaccharide into two fractions by HPSEC with the MW being 79,250 and 24,468 Da. A maximum bleaching (68.39%) of crude polysaccharide was attained by active carbon at 9 mg/mL, however, a substantial loss of polysaccharide also occurred. Polysaccharide was shown to be composed of rhamnose, arabinose, xylose, mannose, glucose and galactose.

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