Journal of Pharmaceutical and Biomedical Analysis 55 (2011) 977–983

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Comparison of polysaccharides from different *Dendrobium* using saccharide mapping

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ARTICLE INFO

Article history:
Received 8 March 2011
Received in revised form 22 March 2011
Accepted 25 March 2011
Available online 6 April 2011

Keywords:
Dendrobium
Polysaccharides
Saccharide mapping
Enzymatic digestion
High-performance size-exclusion chromatography (HPSEC)

ABSTRACT

Multiple species of *Dendrobium* are widely used as *Shihu*, a well known Chinese herb, for medicinal purpose in China. Small molecules such as phenols, alkaloids and coumarins are obviously varied in different species of *Dendrobium*. But there are few reports on polysaccharides, one of major active components, from *Dendrobium*. In this study, polysaccharides from different species or locations of *Dendrobium* were compared using saccharide mapping. The results showed that polysaccharides of *Dendrobium* from different species or locations were obviously varied in spite of they had some similar characters, which is helpful to control the quality of *Dendrobium*.

1. Introduction

The plants of *Dendrobium* genus, with more than 1100 species, are widely distributed throughout Asia, Europe and Australia. There are 78 species of *Dendrobium* plants found in China [1], and about 30 species of them, well known as *Shihu* in China, are employed in traditional or folk medicine [2]. According to the record of China Pharmacopeia (2010 version), *Dendrobium nobile* Lindl., *D. chrysotoxum* Lindl., *D. fimbriatum* Hook., and the related species of *Dendrobium* genus are all officially used as *Shihu*. Due to multiple origins, their active compounds including coumarins [3,4], phenols [2,5,6] and alkaloids [7] were greatly varied [8]. Actually, polysaccharides in *Dendrobium* have also been demonstrated their various beneficial effects, such as antioxidant, anti-hyperglycemic [9,10], immuno-stimulating [11,12] and antitumor [13] activities. However, to the best of our knowledge, there is no report on the specific characters of polysaccharides, due to the complexity, in different species or locations of *Dendrobium*, though compositional monosaccharides in several species of *Dendrobium* were investigated [14].

In this study, polysaccharides from eight *Dendrobium* samples with different species or locations were first compared using saccharides mapping, a method developed base on their carbohydrase enzymatic digestion properties and chromatographic characteristics of the enzymatic hydrolysates in our lab [15].

2. Experimental

2.1. Chemicals, reagents and materials

Eight samples of *Shihu*, including *D. huoshanense* Tang et Cheng and *D. officinale* Kimura et Migo from Anhui, *D. fimbriatum* Hook., *D. Chrysanthum* Lindl., *D. nobile* Lindl., and *D. officinale* Kimura et Migo from Yunnan, *D. nobile* Lindl. from Guizhou and *D. officinale* Kimura et Migo from Zhejiang, were collected in 2008 and 2009 by ourselves. The botanical origin of material was identified by Professor Dongxia Shen from China Pharmaceutical University and Yunnan Jinling Botanical Medicine Co., Ltd., Simao, China, and the voucher specimens were deposited at the Institute of Chinese Medical Sciences, University of Macau, Macao, China.

Acetonitrile and ammonium acetate for HPLC analysis were purchased from Merck (Darmstadt, Germany) and Riedel-de Haën, (Seelze, Germany), respectively. Deionized water was prepared by Millipore Milli Q-Plus system (Millipore, Bedford, MA). Sodium acetate, sodium phosphate monobasic and sodium phosphate dibasic from Riedel-de Haën were used in preparation of buffer solution for enzymatic digestion of polysaccharides. 1-phenyl-3-methyl-5-pyrazolone (PMP) was purchased from Sigma (St. Louis, MO, USA).

Abbreviations: HPSEC, high-performance size-exclusion chromatography; PMP, 1-phenyl-3-methyl-5-pyrazolone.

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doi:10.1016/j.jpba.2011.03.041

Table 1
Conditions for enzymatic hydrolysis modified from the operation manual.

Enzyme	EC number	Buffer solution	pH	Temperature (°C)
Arabinanase	3.2.1.99	50 mM sodium acetate	4.0	40
Xylanase	3.2.1.8	25 mM sodium acetate	4.7	40
1,4-β-D-Galactanase	3.2.1.89	25 mM sodium acetate	4.0	40
Cellulase	3.2.1.4	25 mM sodium acetate	4.5	40
Pectinase	3.2.1.15	50 mM sodium acetate	5.5	40
β-Mannanase	3.2.1.78	50 mM sodium acetate	4.5	40
1,3-β-Glucanase	3.2.1.39	50 mM sodium acetate	6.0	40
Lichenase	3.2.1.73	25 mM sodium phosphate	6.5	40
α-Amylase	3.2.1.1	100 mM sodium acetate	7.0	40
Isoamylase	3.2.1.68	100 mM sodium acetate	4.0	40

D-galacturonic acid monohydrate (GalA), D-glucuronic acid (GlcA), D-arabinose (Ara), D-mannose (Man), D-galactose (Gal), D-glucose (Glc) were purchased from Fluka (Buchs, France). L-Rhamnose monohydrate (Rha), D-xylose (Xyl), maltose (Malt), pectinase (endopolygalacturonase, EC 3.2.1.15), cellulase (endo-1,4-β-D-glucanase, EC 3.2.1.4) and α-amylase (EC 3.2.1.1) were purchased from Sigma (St. Louis, MO, USA). Endo-arabinanase (EC 3.2.1.99), isoamylase (glycogen 6-glucanohydrolase, EC 3.2.1.68),

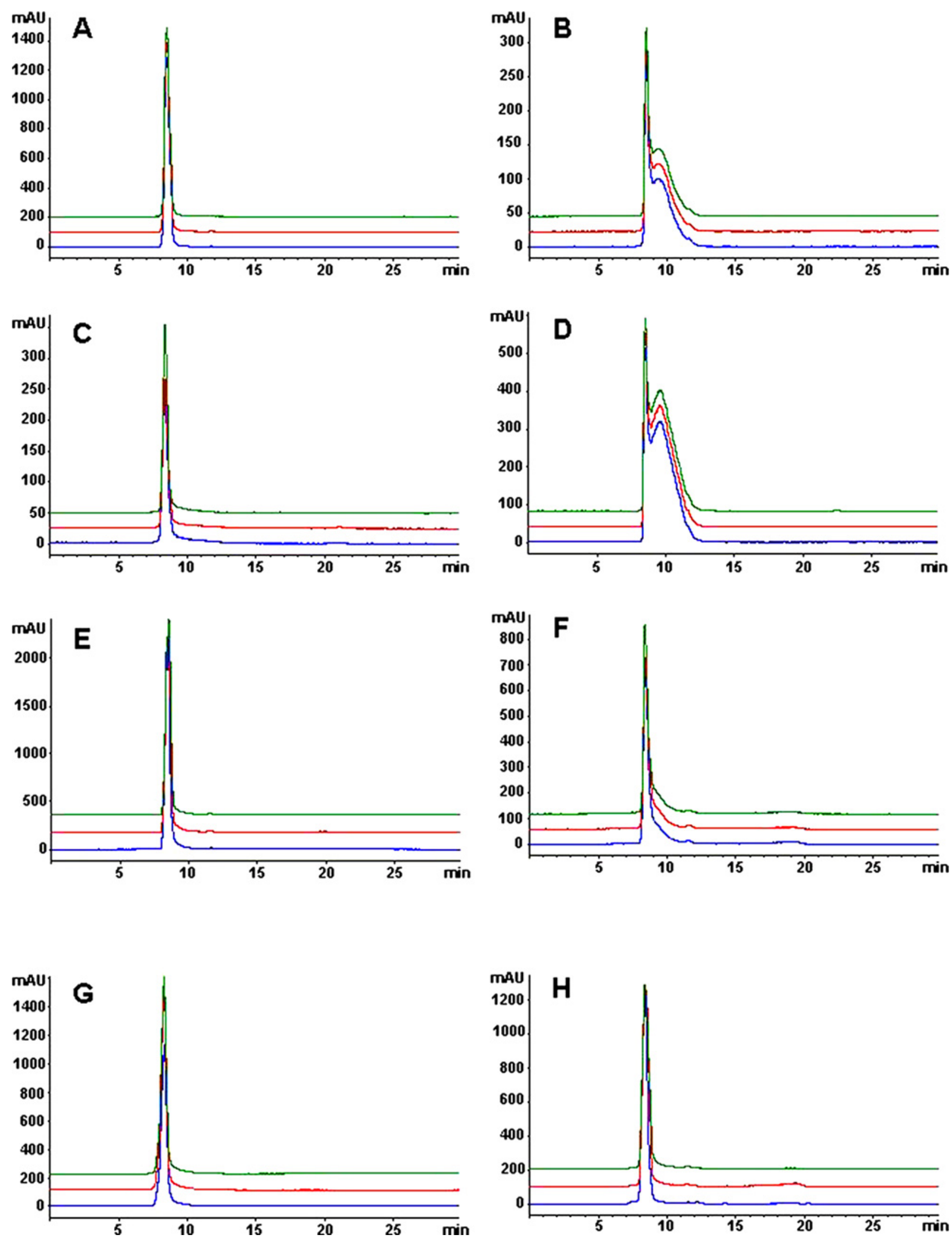

Fig. 1. HPSEC-ELSD profiles of three individual extracted polysaccharides from investigated *Dendrobium* spp. The samples were from different locations of China. (A) *D. huoshanense* from Anhui; (B) *D. fimbriatum* from Yunnan; (C–E) *D. officinale* from Anhui, Yunnan and Zhejiang, respectively; (F) *D. chrysanthum* from Yunnan; (G and H) *D. nobile* from Guizhou and Yunnan, respectively.

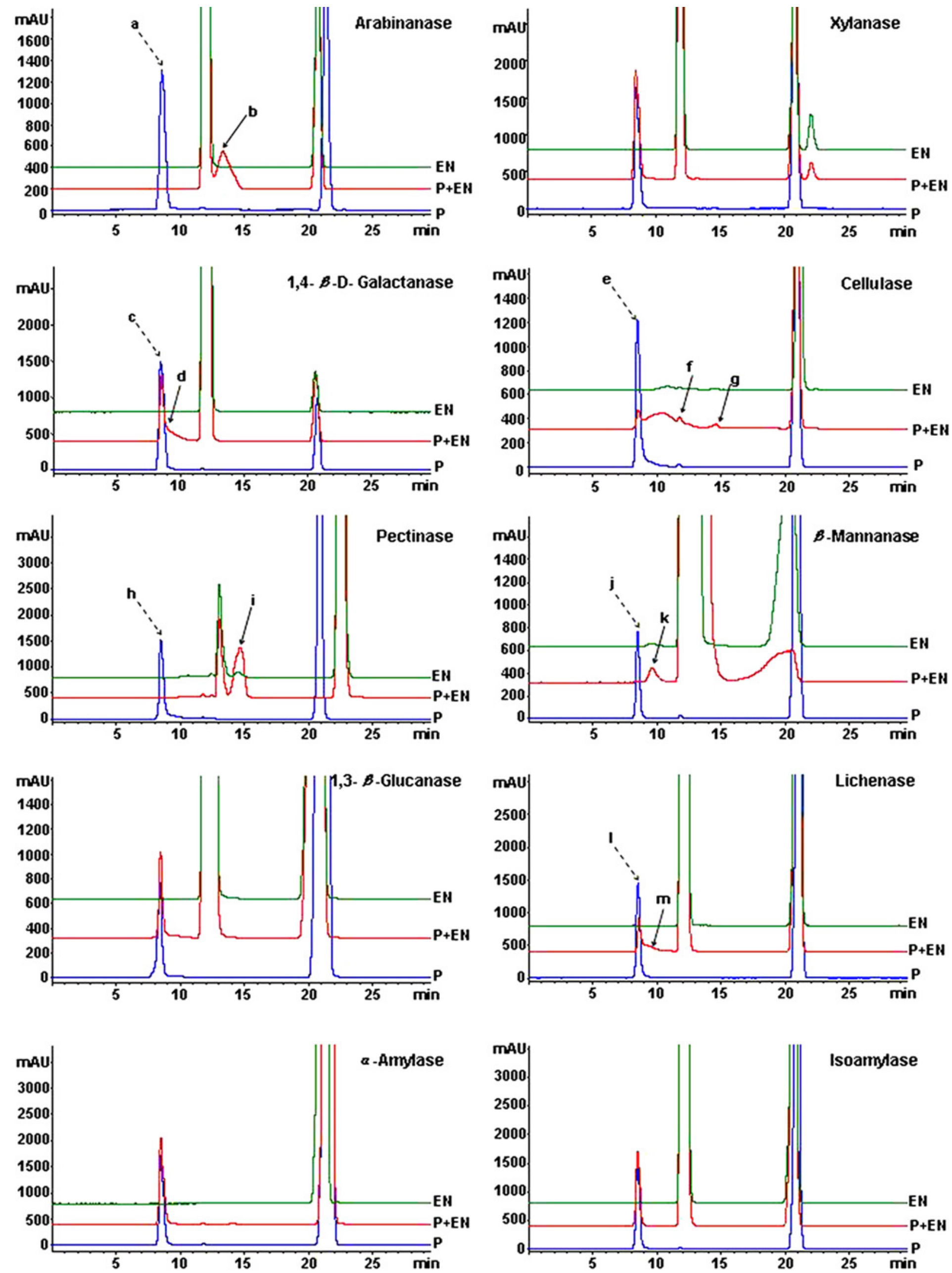

Fig. 2. HPSEC-ELSD profiles of polysaccharides of *D. huoshanense* treated with (P + EN) or without (P) selected enzymes (EN). Peaks a–m were changed peaks during enzymatic hydrolysis.

xylanase (EC 3.2.1.8), endo-1,4-β-D-galactanase (EC 3.2.1.89), β-1,3-D-glucanase (endo-1,3-β-D-glucanase, EC3.2.1.39), lichenase (EC 3.2.1.73) and β-mannanase (EC 3.2.1.78) were obtained from Megazyme (Wicklow, Ireland).

2.2. Preparation of polysaccharides from Dendrobium

Dried powders of *Shihu* (0.20 g) were immersed in 10 mL deionized water and refluxed in a Syncore parallel reactor (Büchi, Switzerland) for 1 h at the temperature of 100 °C with stirring at 120 rpm. After centrifugation at 5000 × *g* for 10 min (Allegra X-15R, Beckman Coulter, Fullerton, CA), an aliquot of 5 mL supernatant was precipitated by addition of ethanol to final concentration of 75% (*v*/*v*), and stayed overnight (12 h) under 4 °C. After centrifugation (5000 × *g*) for 10 min, the precipitate was heated on water bath (60 °C) to remove residual ethanol. The dried extract was dissolved in 5 mL hot water (60 °C), then the low molecular weight compounds were removed using ultra centrifugal filters [molecular weight cut-off (MWCO) = 10 kDa] (Millipore, Billerica, MA) by centrifugation at 4000 × *g* in duplicates (15 min each). Finally, the remains were dissolved in 4 mL water and centrifugated (5000 × *g*) for 5 min to remove the indissoluble residue. The supernatant was collected and the content of polysaccharides was determined using phenol-sulfuric acid assay with glucose as reference. The content of polysaccharides, calculated as glucose [15], in the solution was adjusted to about 0.75 mg/mL before high-performance size-exclusion chromatography (HPSEC) analysis and further treatment.

2.3. Enzymatic digestion

Polysaccharide solution (100 μL) was mixed with certain enzyme (the final concentration was 15 U/mL) in a total volume

Table 2
Response of polysaccharides from different *Dendrobium* to selected enzymatic digestion.

Enzyme	Polysaccharides							
	DHP[a] (AH[b])	DFP (YN)	DCP (YN)	DNP (YN)	DNP (GZ)	DOP (YN)	DOP (AH)	DOP (ZJ)
Arabinanase	+[c]	+	+	+	+	+	+	+
Xylanase	–[c]	+	+	–	–	–	+	–
1,4-β-D-Galactanase	+	+	+	+	+	+	+	+
Cellulase	+	+	+	+	+	+	+	+
Pectinase	+	+	+	+	+	+	+	+
β-Mannanase	+	+	+	+	+	+	+	+
1,3-β-Glucanase	–	+	+	+	–	–	+	+
Lichenase	+	+	+	+	+	+	+	+
α-Amylase	–	+	–	–	+	–	–	–
Isoamylase	–	–	–	–	+	–	–	–

[a] DHP, DFP, DCP, DNP, and DOP are polysaccharides from *D. huoshanense*, *D. fimbriatum*, *D. chrysanthum*, *D. nobile* and *D. officinale*, respectively
[b] AH, YN, GZ and ZJ are Anhui, Yunnan, Guizhou and Zhejiang, respectively.
[c] +, positive response; –, negative response.

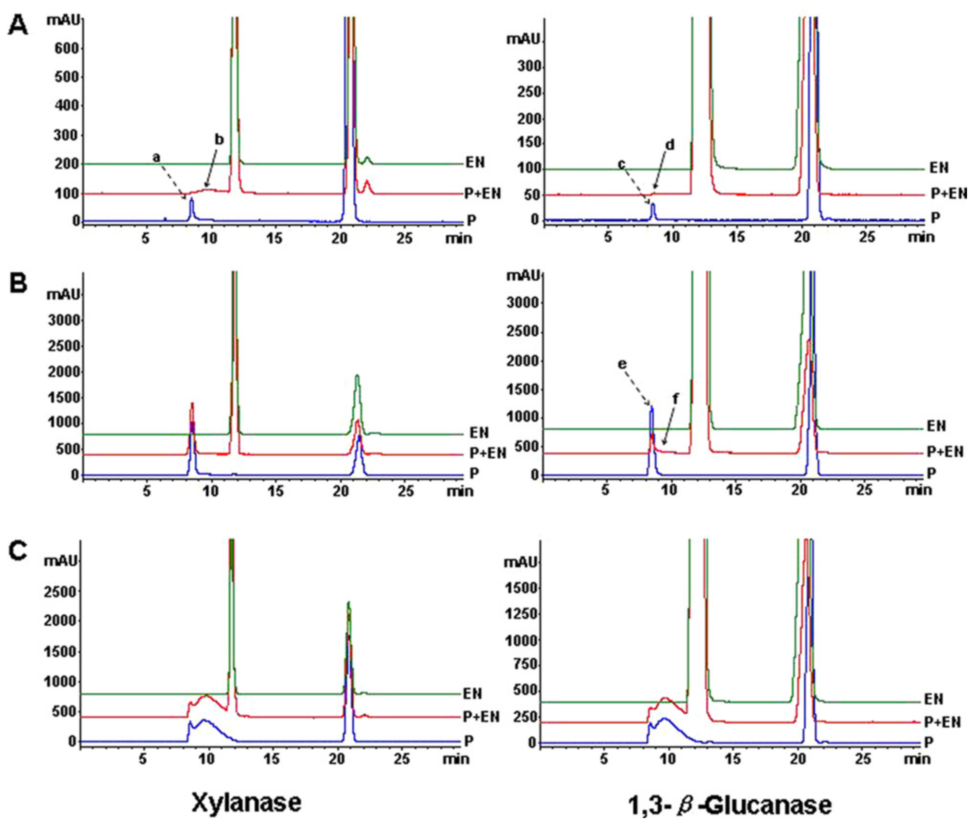

Fig. 3. HPSEC-ELSD profiles of polysaccharides from *D. officinale* from (A) Anhui, (B) Zhejing and (C) Yunnan treated with (P + EN) or without (P) selected enzymes (EN). Peaks a–f were changed peaks during enzymatic hydrolysis.

of 200 μL and digested overnight (12 h) under the optimum conditions (Table 1). Then the mixture was heated at 80 °C for 1 h to stop the enzymatic digestion. After centrifugation at 15700 × *g* (5415 D, Eppendorf AG) for 20 min, the supernatants were applied to HPSEC-DAD-ELSD analysis or derivatization followed by HPLC-DAD-MS analysis. Deionized water instead of polysaccharide solution, treated as mentioned above, was used as blank control.

2.4. Derivatization with PMP reagent

The derivatization was carried out referring to previous study in our lab [15] with minor modifications. Briefly, the enzymatic hydrolysate (600 μL) was mixed with the same volume of NH_3 solution, and then 0.5 M PMP methanolic solution (200 μL). The mixture was allowed to react (70 °C water bath for 30 min) and then was cooled to room temperature with addition of water (2000 μL). The solution was blown to dryness under 50 °C by nitrogen evaporators (Organomation Associates, Inc., Berlin, MA, USA), then repeatedly added water (2000 μL) and dried twice to remove NH_3. The residue was mixed with the mixture of water and chloroform (1 mL each). After vigorous shaking and centrifugation at 15700 × *g* for 5 min (5415 D, Eppendorf AG), organic phase was discarded to remove the excess reagents. The operation was performed in triplicates, and finally the aqueous layer was filtered through a 0.45 μm syringe filter (Agilent Technologies) before HPLC-DAD-MS analysis. A standard solution, containing six monosaccharides (Rha, Ara, Xyl, Man, Glc and Gal), two uronic acids (GlcA and GalA) and one disaccharide (Malt), was also treated as mentioned above for reference.

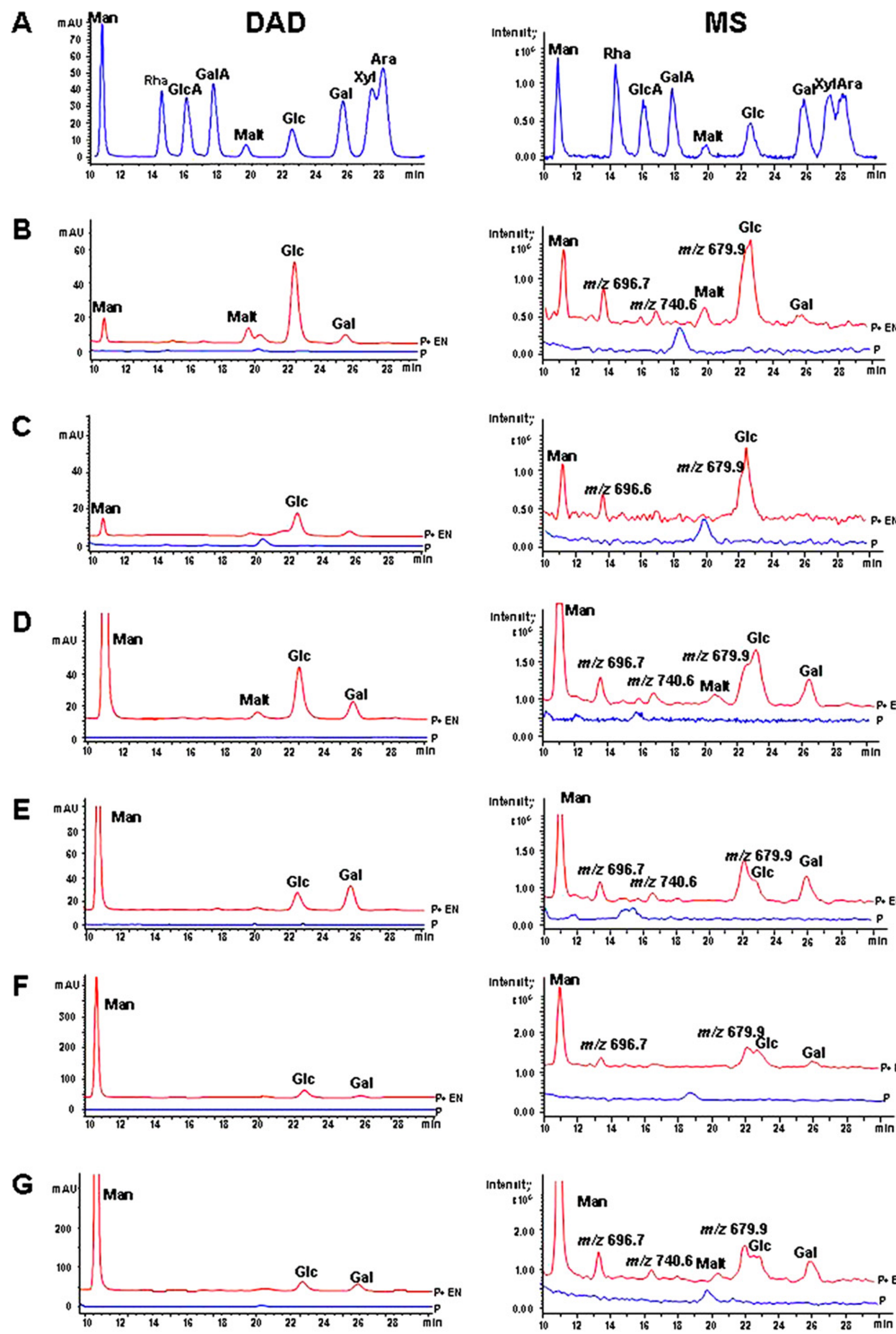

Fig. 4. HPLC chromatograms of PMP derivatized (A) standard saccharides, hydrolysates of polysaccharides from (B) *D. officinale* (Yunnan), (C) *D. huoshanense* (Anhui), (D) *D. chrysanthum* (Yunnan), (E) *D. officinale* (Anhui), (F) *D. officinale* (Zhejiang) and (G) *D. nobile* (Yunnan) treated with (P + EN) or without (P) selected enzymes (EN) detected by DAD (UV 245 nm) and MS detection (TIC). Cellulose was selected for sample B and C, and pectinase was employed for sample D–G.

2.5. HPSEC-DAD-ELSD analysis

The analysis was performed on an Agilent 1100 series LC/DAD system (Agilent Technologies, Palo Alto, CA) coupled with ELSD. The separation was achieved on a TSK G-3000PW$_{XL}$ column (300 mm × 7.8 mm i.d., 10 μm) operated at 30 °C. Ammonium acetate aqueous solution (20 mM) was used as mobile phase at a flow rate of 0.6 mL/min. DAD was set at 260 nm and 280 nm. The signal from ELSD was transmitted to Agilent Chemstation for processing through an Agilent 35900E interface. The parameters of ELSD were set as follows: the drift tube temperature was 110 °C and nebulizer nitrogen gas flow-rate was at 3.0 L/min, impact off mode. An aliquot of 10 μL solution was injected for analysis.

2.6. HPLC-DAD-MS characterization

The PMP derivates of saccharides derived from enzymatic hydrolysate of polysaccharides were analyzed using HPLC-DAD-MS. Samples (10 μL) were injected onto a Zorbax Eclipse XDB-C$_{18}$ column (150 mm × 4.6 mm i.d., 5 μm) operated at 25 °C. The separation was achieved using gradient elution with 20 mM ammonium acetate aqueous solution (A) and acetonitrile (B) at a flow rate of 1.0 mL/min: 0–1 min, 13–17% B; 1–30 min, 17% B. UV detection wavelength was set at 245 nm. MS spectra were acquired in positive ion mode. The full scan mass spectra were obtained from *m/z* 100 to 1500. ESI-MS conditions were as follows: dry gas (N_2) 8 L/min, dry temperature 350 °C, nebulizer pressure, 45 psi. ESI-MS/MS conditions: isolation width 4, fragment amplification 1.5 V, compound stability 50%. Identification of saccharides was achieved by comparison of their MS data with those of standard compounds. The same sample without related enzyme treatment was also used for parallel analysis as control to confirm the saccharides in polysaccharides hydrolysates were derived from the enzymatic hydrolysis.

3. Results and discussion

3.1. Repeatability of polysaccharides preparation from Dendrobium

Water extraction and ethanol precipitation is a conventional method for preparation of polysaccharides from medicinal plants, which has also been widely used in *Shihu* [9,11,14,16–18]. Low molecular weight compounds might co-precipitate with polysaccharides were removed by ultrafiltration (MWCO = 10 kDa) in this study. Actually, preparation of polysaccharides from *Shihu* with good repeatability is crucial for ensuring the accurate results. Fig. 1 showed that three individual polysaccharides prepared from the same sample had good repeatability. Furthermore, UV 260 nm and 280 nm were also selected for monitoring conjugated nucleic acid and/or peptide in this study, and the major peaks had no obvious absorbance under the investigated conditions (data not shown).

3.2. Enzymatic digestion characters of polysaccharides from Dendrobium

It has been reported that polysaccharides from *Dendrobium* spp. are usually consist of glucose, galactose, mannose, xylose, arabinose, rhamnose, glucuronic acid and galacturonic acid [12,14,17,18]. Moreover, (1 → 4)-β-D-Glc*p*, (1 → 4)-α-D-Glc*p*, (1 → 6)-α-D-Glc*p* and (1 → 4)-β-D-Man*p* are widely existed in glycans from *Dendrobium* [11,12,16,19]. Therefore, cellulase, lichenase, α-amylase, isoamylase and β-mannanase were selected for enzymatic hydrolysis of the polysaccharides. Besides, arabinanase, xylanase, 1,4-β-D-galactanase, pectinase and 1, 3-β-glucanase were also used.

Fig. 2 showed HPSEC-ELSD profiles of polysaccharides from *D. huoshanense* before and after selected enzymatic digestion. The results showed that four enzymes, i.e. xylanase, 1,3-β-D-glucanase, amylases and isoamylase, had no significant effects on the polysaccharides. But arabinanase (peak a diminished and peak b found), 1,4-β-D-galactanase (peak c → peak d), cellulase (peak e diminished and peak f and g found), pectinase (peak h diminish and peak i found), β-mannanase (peak j diminish and peak k found), and lichenase (peak l diminished and peak m found) can certainly hydrolyze the fraction of polysaccharides. The results suggested that polysaccharides from *Dendrobium* may be consist of arabinose, galactose, glucose, galactosyluronic acid and mannose with, at least partial, 1,5-α-arabinofuranosidic, (1 → 4)-β-D-galactosidic, (1 → 4)-β-D-glucosidic, (1 → 4)-α-D-galactosiduronic, 1,4-β-D-mannosidic linkages (Table 2).

3.3. Comparison of polysaccharides from different Dendrobium

As shown in Table 2, besides the common characters mentioned above, polysaccharides form different *Dendrobium* have their specific characteristics. For example, *D. fimbriatum* from Yunnan and *D. nobile* from Guizhou contained starch, while *D. fimbriatum* and *D. chrysanthum* from Yunnan and *D. officinale* from Anhui had xylose and 1,3-β-glucosidic linkage. These differences may exist in the samples of different species or the samples of same species from different locations. Actually, three samples of *D. officinale*, respectively, from Yunnan, Anhui and Zhejiang could be discriminated based on their response to xylanase and 1,3-β-glucanase (Table 2 and Fig. 3). While four species of *Dendrobium*, including *D. fimbriatum*, *D. chrysanthum*, *D. nobile* and *D. officinale*, from Yunnan could also be distinguished according to their polysaccharides to the responses of xylanase, 1,3-β-glucanase and α-amylase digestion (Table 2).

Indeed, among 8 investigated samples, polysaccharides, respectively, from *D. huoshanense* (Anhui) and *D. officinale* (Yunnan), *D. chrysanthum* (Yunnan) and *D. officinale* (Anhui), as well as *D. nobile* (Yunnan) and *D. officinale* (Zhejiang) had the same responses to endo-carbohydrases, but their hydrolysates, after derivatization with PMP, could be discriminated using LC-DAD-MS. Fig. 4 showed that the difference of polysaccharides between *D. officinale* (Yunnan) and *D. huoshanense* (Anhui), *D. chrysanthum* (Yunnan) and *D. officinale* (Anhui), *D. nobile* (Yunnan) and *D. officinale* (Zhejiang). The further study is in progress.

4. Conclusions

The polysaccharides from different species or different locations of *Dendrobium* were first compared using saccharides mapping. The results showed that polysaccharides of *Dendrobium* from different species or locations were obviously varied in spite of they had some similar characters, which is helpful to control the quality of *Dendrobium*.

Acknowledgements

The research was partially supported by grants from the Science and Technology Development Fund of Macao (028/2006/A2) and University of Macau (UL015/09-Y3) to S.P. Li.

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