Journal of Pharmaceutical and Biomedical Analysis 51 (2010) 590–598

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Discrimination of polysaccharides from traditional Chinese medicines using saccharide mapping—Enzymatic digestion followed by chromatographic analysis

J. Guan [a], S.P. Li [a,b,*]

[a] Institute of Chinese Medical Sciences, University of Macau, Macao SAR, China
[b] Jiangsu Key Laboratory for TCM Formulae Research, Nanjing University of Chinese Medicine, Nanjing, China

ARTICLE INFO

Article history:
Received 10 August 2009
Received in revised form
13 September 2009
Accepted 19 September 2009
Available online 27 September 2009

Keywords:
Polysaccharide
Saccharide mapping
Enzymatic digestion
Traditional Chinese Medicines
Chromatographic analysis

ABSTRACT

Polysaccharides isolated from traditional Chinese medicines (TCMs) exhibit multiple pharmacological activities. However, quality control of polysaccharides is a challenge because of their complicate structure and macro-molecular mass. In this study, saccharide mapping based on specific enzymatic digestion of polysaccharides and chromatographic analysis was proposed to discriminate the polysaccharides from different TCMs. Endo-carbohydrase such as glucanase, arabinanase, xylanase, galactanase, cellulase, amylase and pectinase were used for enzymatic digestion of polysaccharides from 9 TCMs namely *Panax ginseng*, *P. notoginseng*, *P. quinquefolium*, *Cordyceps sinensis*, *C. militaris*, *Ganoderma lucidum*, *G. sinense*, *Astragalus membranaceus* and *Angelica sinensis*. By using high performance size exclusion chromatography (HPSEC) as well as derivatization with 1-Phenyl-3-methyl-5-pyrazolone (PMP) and HPLC analysis, the enzymatic hydrolysis properties of polysaccharides and their saccharide mapping were determined. The polysaccharides from 9 TCMs were firstly successfully distinguished based on their characteristic saccharide maps, which is helpful to improve the quality control of polysaccharides.

1. Introduction

Carbohydrate drugs have long been interested by pharmaceutical companies and biotech firms for their therapeutic effects [1]. As a class of carbohydrates with the degree of polymerization (DP) more than 10, polysaccharides are usually considered as main substrates for energy metabolism [2,3]. In fact, botanical polysaccharides such as water-soluble polysaccharides from traditional Chinese medicines (TCMs) have attracted a great deal of attention in last decades because of their broad spectrum of therapeutic properties and relatively low toxicity [4–7]. Indeed, immuno-modulation, anti-tumor, anti-oxidation, anti-virus, and anti-inflammatory bioactivities have been presented by many polysaccharides extracted from medicinal fungi and plants, such as *Cordyceps* sp. [8,9], *Ganoderma* sp. [10–15], *Panax* sp. [16–18], *Angelica sinensis* [19–21] and *Astragalus* [22,23]. However, quality control of polysaccharides is a challenge because of their complicate structure and macro-molecular mass. Since the activity of polysaccharides always shows strong relationship with their molecular mass [24,25], monosaccharide composition [26,27], configuration and position of glycosidic linkages [10,28], even the triple-helix conformation [29], the determination of these physical and chemical properties using chromatographic and electromigratic methods is the job for qualitative analysis of polysaccharides [30]. However, up to date, few reports focus on the discrimination of polysaccharides origin, which is crucial for quality control of polysaccharides from TCMs.

Generally, it is complex, difficult and time consuming to obtain structural information though it is unambiguous identification of polysaccharides. Currently, the fingerprints of high-performance thin-layer chromatography (HPTLC) [31] or gas chromatography (GC) [32] based on the constituent saccharides of polysaccharides, as well as chromatographic analysis of hydrolysates derived from total or partial acid hydrolysates, have been used for characterization of polysaccharides [33,34]. However, the selectivity of acid hydrolysis is poor, and the ratio of monosaccharide obtained in acid hydrolysate may be not in accordance with that in polysaccharides due to the degradation under acidic conditions [35,36]. Enzymatic digestion is specific, which has been used to find the novel characteristics of polysaccharides [37–40]. Actually, peptide mapping, the combination of specific enzymatic hydrolysis and characterization of hydrolysates, has been proved to be valuable for identification of protein [41,42]. Similarly, "saccharide mapping" based on carbohydrase hydrolysis followed by chromatographic analysis may be a powerful tool for characterization of polysaccharides.

In this study, polysaccharides from 9 traditional Chinese medicines, including *Panax ginseng*, *P. notoginseng*, *P. quinquefolium*, *Cordyceps sinensis*, *C. militaris*, *Ganoderma lucidum*, *G. sinense*, *Astra-*

* Corresponding author. Tel.: +853 8397 4692; fax: +853 2884 1358.
E-mail addresses: spli@umac.mo, lishaoping@hotmail.com (S.P. Li).

0731-7085/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2009.09.026

Table 1
Digestion conditions for enzymatic hydrolysis modified from operation manual of Megazyme and Sigma Co.

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
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
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 buffer	6.5	40

galus membranaceus and *Angelica sinensis* were firstly discriminated based on their carbohydrase enzymatic hydrolysis properties and chromatographic characteristics of enzymatic hydrolysates, i.e. saccharide mapping.

2. Experimental

2.1. Chemicals, reagents and materials

P. ginseng (PG), *P. notoginseng* (PN), *P. quinquefolium* (PQ) and *C. sinensis* (CS) were purchased from Zhong-qiao Corporation (Macau, China); *G. lucidum* (GL) and *G. sinense* (GS) were collected from Jinzhai, Anhui Province; *A. membranaceus* (AM) and *A. sinensis* (AS) were collected from Shanxi and Gansu provinces, respectively. The botanical origin of the materials mentioned above was identified by corresponding author. Mycelia of *C. militaris* (CM) were fermented in our lab (the fungus strain was from The Chinese Academy of Agricultural Sciences). All voucher specimens were deposited at the Institute of Chinese Medical Sciences, University of Macau, Macao, China.

D-galacturonic acid monohydrate (GalA), D-glucuronic acid (GlcA), D-arabinose (Ara), D-mannose (Man), D-galactose (Gal) and D-glucose (Glc) were purchased from Fluka (Buchs, France). Blue dextran 2000, 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). Acetonitrile for HPLC was purchased from Merck (Darmstadt, Germany). Ammonium acetate was obtained from Riedel-de Haën (Seelze, Germany). Deionized water was prepared by Millipore Milli Q-Plus system (Millipore, Bedford, MA, USA). Sodium acetate, sodium phosphate monobasic and sodium phosphate dibasic from Riedel-de Haën were used in preparation of buffer solution for enzymatic digestion. Endo-arabinanase (EC 3.2.1.99), isoamylase (glycogen 6-glucanohydrolase, EC 3.2.1.68), 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). 1-Phenyl-3-methyl-5-pyrazolone (PMP) was a gift from Dr. Song Yue of Agilent Technologies Co., Ltd. (Shanghai, China), and recrystallization twice using methanol before use. Other reagents not mentioned here were of analytical-reagent grade from standard sources.

2.2. Preparation of polysaccharides from TCMs

The powders of medicinal material were immersed with 20-folds volume deionized water and refluxed in a Syncore parallel reactor (Büchi, Switzerland) for 1 h at the temperature of 100 °C with stir at 120 rpm. An aliquot of 5 mL extract solution was centrifuged at 4713 × *g* for 10 min (Allegra X-15R, Beckman Coulter, Fullerton, CA), and the supernatant was precipitated by addition of ethanol to final concentration of 75% (v/v), which stayed overnight (12 h) under 4 °C. After centrifugation (4713 × *g* for 10 min), the precipitate was washed with 4 mL of 95% ethanol twice and then ethanol was removed on water bath (60 °C). The dried residue was redissolved in 5 mL hot water (60 °C), then after centrifugation, the supernatant was collected and its polysaccharides were quantitatively determined using phenol-sulfuric acid assay [43] with glucose as reference standard. The polysaccharides solution was adjusted to about 0.15 mg/mL of polysaccharides calculated as glucose, and used for HPSEC analysis and further treatment.

2.3. Enzymatic digestion

Polysaccharide solution (1000 μL) was mixed with certain enzyme (the final concentration of arabinanase, xylanase, 1,4-β-D-galactanase, cellulase, pectinase, α-amylase and isoamylase were 1.4 U/mL, 4.5 U/mL, 9.4 U/mL, 10 U/mL, 95 U/mL, 12.5 U/mL and 10 U/mL, respectively) in 1.5 mL Eppendorf tubes (Eppendorf AG, Hamburg, Germany) and digested overnight (≥12 h) under the optimum conditions (Table 1). Then the mixture was boiled at 85 °C for 30 min (Eppendorf Thermomixer Comfort, Eppendorf AG) to stop the enzyme digestion. The supernatant was applied for HPSEC analysis and derivatization, respectively, after centrifugation (5415 D, Eppendorf AG) at 15,700 × *g* for 30 min. Deionized water instead of polysaccharide solution, treated as mentioned above, was used as blank control.

2.4. Derivatization with PMP reagent

The sugar derivatization followed previous reports [44–46] with modifications. Briefly, the enzymatic hydrolysate (600 μL) was mixed with the same volume of NH_3 solution, and then 0.5 mol/L PMP methanolic solution (200 μL). The mixture was allowed to react on the Syncore parallel reactor (70 °C for 30 min) and then was cooled to room temperature with addition of water (2000 μL). The solution was vacuum evaporated to dryness under 50 °C, then repeatedly water was added (2000 μL) and dried twice to remove NH_3. The residue was dissolved in water and chloroform (1 mL each). After vigorous shaking and centrifugation at 15,700 × *g* for 5 min, organic phase was discarded to remove the excess reagents. The operation was performed triplicates, and finally the aqueous layer was filtered through a 0.45 μm syringe filter (Agilent Technologies) before liquid chromatography-diode array detector-mass spectrometry (LC–DAD–MS) analysis. A standard solution, containing 6 monosaccharides (Rha, Ara, Xyl, Man, Glc and Gal, ~1 mg/mL each), 2 uronic acids (GlcA and GalA, ~1 mg/mL each) as well as maltose (Malt, ~1 mg/mL), was also treated as mentioned above for reference.