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Heptasaccharide and octasaccharide isolated from *Paris polyphylla* var. *yunnanensis* and their plant growth-regulatory activity

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Abstract

Two oligosaccharides, heptasaccharide (HS) and octasaccharide (OS), were isolated from the water extract of the rhizomes of *Paris polyphylla* var. *yunnanensis*. They were identified as linear oligomers, composed of glucose and mannose monomers, with the molecular formula of $C_{42}H_{72}O_{36}$ ($M = 1152$ and DP = 7) for HS and $C_{48}H_{82}O_{41}$ ($M = 1214$ and DP = 8) for OS (DP, degree of polymerization). Both had plant growth-regulatory activity at low concentrations, stimulating the shoot formation of *P. polyphylla* var. *yunnanensis* in culture at 2.5–10 mg/l, and the growth and saponin formation of *Panax japonicus* var. *major* hairy roots at 10–30 mg/l. This is the first report on these oligosaccharides isolated from *P. polyphylla* var. *yunnanensis* and their plant growth-regulatory activity.

Keywords: *Paris polyphylla* var. *yunannensis*; Trilliaceae; Oligosaccharide; Structure; Plant growth regulation

1. Introduction

Oligosaccharides isolated from the cell wall fragments of plants and fungi have been recognized as an important class of relatively new or 'non-traditional' plant hormones that function as signal molecules to regulate plant growth, development and defense responses [1–3]. These complex carbohydrates have diverse molecular structures and are therefore suited for encoding intricate biological information in plants. On the other hand, the molecular diversity and complexity of oligosaccharides make it a tedious task to isolate oligosaccharides from carbohydrate extracts of plant and fungal materials. Purification of the oligosaccharides and elucidation of the molecular structures is essential not only for identifying the bioactive constituents but also for understanding their functions in plant growth regulation. In addition to their regulatory activity in plants, oligosaccharides may also have biological activity in animals. In particular, some polysaccharides and oligosaccharides isolated from medicinal plants and fungi have shown immunomodulatory and anti-tumor activity in human and animals [4–6].

Paris polyphylla var. *yunnanensis* (Fr.) Hand-Mazz. is a perennial medicinal plant belonging to the Trilliaceae family, section *Euthyra* Franch, distributed mainly in the southwest of China [7]. The rhizome of *P. polyphylla* var. *yunnanensis* is used in traditional Chinese medicine as a hemostasis and antimicrobial agent. Its principal bioactive ingredients are thought to be some steroid saponins and polysaccharides [8,9]. As part of our program on the bioactive compounds from this herb, this work was undertaken to isolate the potentially bioactive oligosaccharides from the rhizomes of *P. polyphylla* var. *yunnanensis*. This article describes the structural characteristics of two oligosaccharides iso-

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lated and shows their stimulating effects on plant growth and secondary metabolite accumulation in plant tissue cultures.

2. Materials and methods

2.1. Isolation and purification of oligosaccharides from rhizomes of P. polyphylla var. yunnanensis

Fresh rhizomes or hypogeal stems of *P. polyphylla* var. *yunnanensis* were collected in Kunming, Yunnan Province of China, which were lyophilized and powdered. The dry rhizome powder was soaked in ethanol for 24 h at room temperature (~ 20 °C). After removing the ethanol by filtration, the remaining solid was extracted with hot water at 60 °C for three times, 8 h each. The water extract solution was concentrated to a small volume by evaporation in vacuo, and then subjected to ethanol precipitation (to remove large polysaccharides). The resultant ethanol–water solution was collected and concentrated by vacuum evaporation. The concentrated extract was collected for further purification of oligosaccharides.

To purify the oligosaccharides, the extract was applied to a Bio-Gel P-2 chromatographic column (3.5×40 cm) and eluted by distilled water. During the operation, the oligosaccharides eluted from the column were frequently checked with a HPTLC GF254 silica gel plate, mobile phase *n*-butanol/acetic acid/water 2:1:1 (v/v). The component spots were visualized with an α-naphthol–sulfuric acid solution, using sucrose as a reference. After repeated column chromatography, two main components, designated compound **1** and **2**, were isolated.

2.2. Structural characterization of oligosaccharides

The infrared (IR) spectra of compounds **1** and **2** were measured in KBr on a Perkin–Elmer IR-577 spectrometer (Perkin–Elmer, Shelton, CT). ^{1}H- and ^{13}C-NMR spectra were measured in D_2O on a Bruker AM-400 spectrophotometer (Bruker BioSpin, Billerica, MA); all chemical shift values were reported in δ (ppm). Negative-ion FAB-MS were measured on a VG Autospec 3000 mass spectrometer (Micromass, Manchester, UK).

Acetic methylated alditols of compounds **1** and **2** were prepared for negative-ion FAB-MS analysis to determine the molecular weight, and for GLC-MS analysis to further confirm the carbohydrate molecular structure of the compounds. The alditols were prepared and then completely methylated, according to the method of Larson et al. [10]. In brief, the compounds (each 3 mg) were hydrolyzed in trifluroroacetic acid, and the hydrolysis products were reduced into their alditols by sodium borohydride. The completely methylated products were partially hydrolyzed in 1 N HCl, and then acetified.

2.3. Micropropagation of P. polyphylla var. yunnanensis plantlets

P. polyphylla var. *yunnanensis* plant seeds were initially sterilized and treated with a temperature shift, and then kept in sterile and moisten sand for 3 to 4 months until germination. To obtain seedlings, the geminated seeds were transferred to hormone-free MS medium [11] with 8 g/l of agar, and incubated at 25 °C in the light of 1500 lux illumination intensity for 12 h, and then at 20 °C in the dark for 12 h, each day, at 80% relative humidity. About 30 days later, shoots were cut off the seedlings and then transferred to MS medium supplemented with 0.5 mg/l of α-naphtheleneacetic acid, 0.1 mg/l of 6-benzyl adenine and 8 g/l of agar. The shoot culture was maintained in the same conditions as for the seedlings, and subcultured every 60 days.

2.4. Culture of P. japonicus var. major hairy roots

Hairy roots of *Panax japonicus* var. *major* were induced by the infection of plant seedlings with pRi-containing *Agrobacterium rhizogenes* strain 15 834. The hairy roots were stored in hormone-free MS agar medium. More details of the hairy root culture have been given elsewhere [12]. The tests of the oligosaccharides on hairy root growth and saponin accumulation were carried out in suspension culture of the hairy roots in liquid MS medium on a rotary shaker at 110 rpm. The culture period was fixed at 40 days at which the culture usually reached a maximum dry weight and a stable saponin content. The hairy roots were harvested from the culture by filtration and dried by lyophilization. The total saponin content of hairy roots was isolated and quantified as described previously [13,14]. Briefly, the lyophilized root samples were powdered, and extracted with water-saturated *n*-butanol at room temperature in an ultrasonic cleaning bath. The *n*-butanol extract solution was collected and evaporated at 40 °C under vacuum to remove the solvent. The solid residue was redissolved in methanol and then applied to a thin-layer chromatography (TLC) plate (Silica Gel 60, UV254, 0.25-mm layer), using chloroform–methanol–water at 15:12:2 ratio as the mobile phase to isolate the total saponin. The total saponin obtained by the TLC was reacted with 5% vanillin in acetic acid and perchloric acid at 60 °C, forming a purple product with a peak absorbance at 560 nm, which is proportional to the concentration of total saponin.

3. Results

3.1. Structural characteristics of the two purified oligosaccharides

A crude oligosaccharide mixture from the rhizomes of *P. polyphylla* var. *yunnanensis* exhibited moderate stimulating effect on the growth and saponin synthesis of *P. japonicus* var. *major* hairy roots, and the multiplication of *P. polyphylla* var. *yunnanensis* shoots (data not shown). Bio-assay fractionation of the active oligosaccharides led to the isolation of two compounds, **1** and **2**. The major structural characteristics of the two compounds obtained from various spectral analyses were summarized in Table 1.

Compound **1** was identified as a heptasaccharide (HS). The molecular formula $C_{42}H_{72}O_{36}$ was derived from the negative-ion FAB-MS $(M-H)^-$ 1151 *m/z*. Its IR peak at 844 cm^{-1} is characteristic of an α-type glycosidic linkage. The ^{1}H-NMR chemical shift values of 4.85 and 5.40 ppm indicate the presence of both α and β sugar moieties. The ^{13}C-NMR data indicate mannose (Man) and glucose (Glc) monomers. The Man and Glc molecular composition was also supported by GC-MS analysis of the acetic methylated alditols (data not shown). The negative-ion FAB-MS data of the acetic methylated alditol derivatives further confirms the molecular weight. All these spectral characteristics conform to those of a HS.

Most of the spectral characteristics of compound **2** are identical or close to those of compound **1**, and only the negative-ion FAB-MS spectra of the compound and its acetic methylated alditols had a few minor differences. Based on these data, compound **2** was identified as an octasaccharide, with the molecular formula of $C_{48}H_{82}O_{41}$ according to the negative-ion FAB-MS $(M-H)^-$ 1313 *m/z*.

GLC-MS analysis of the acetic derivatives from the methylated alditols suggests only linear linkage between the monomers in the oligosaccharide compounds (Table 2). In combination with the data in Table 1, the structure of compound **1** was identified as D-Glc-(1-6)-β-D-Glc-(1-6)-β-D-Glc-(1-6)-β-D-Glc-(1-6)-β-D-Glc-(1-6)-β-D-Glc-(1-4)-α-D-Man, and that of **2** as D-Glc-(1-6)-β-D-Glc-(1-6)-β-D-Glc-(1-6)-β-D-Glc-(1-6)-β-D-Glc-(1-6)-β-D-Glc-(1-6)-β-D-Glc-(1-4)-α-D-Man. The degrees of polymerization (DP) of **1** and **2** were seven and eight, respectively.

3.2. Effects of *1* and *2* on P. polyphylla var. yunnanensis shoot multiplication

Both compounds had positive effect on the proliferation of *P. polyphylla* var. *yunnanensis* shoots (Table 3) at all concentrations applied, 2.5–20 mg/l. The effect was concentration-dependent, and most significant with compound **1** at 2.5 mg/l and **2** at 10 mg/l, respectively. However, these compounds did not significantly promote the elongation of *P. polyphylla* var. *yunnanensis*

Table 1
Physical and chemical characteristics of compounds **1** and **2**

Characteristics	Compound **1**	Compound **2**
Physical appearance	White powder	White powder
IR main peak	844 cm^{-1}	844 cm^{-1}
^{1}H-NMR chemical shift (ppm)	4.85, 5.40	4.85, 5.41
^{13}C-NMR chemical shift (ppm)	94.95 (-α-D-Man C_1) 98.85 (-β-D-Glc C_1) 102.67 (-β-D-Glc-C_1)	94.91 (-α-D-Man C_1) 98.81 (-β-D-Glc C_1) 102.60 (-β-D-Glc-C_1)
Negative-ion FAB-MS (*m/z*)	1151 $[M-H]^-$, 989, 827, 665, 503, 341, 179	1313 $[M-H]^-$, 1151, 989, 827, 665, 503, 341, 179
Negative-ion FAB-MS of acetic methylated alditols (*m/z*)	1473 $[M-H]^-$	1677 $[M-H]^-$

Table 2
Methylation analysis of the linkage regions of compounds **1** and **2** based on GLC-MS

Acetic derivatives of methylated alditols	Molar ratio		Mode of linkage
	Compound **1**	Compound **2**	
1,2,3,6-O–Me_4–Man	0.81	0.85	-4)D-Man
2,3,4,6-O–Me_4–Glc	1.2	1.2	-1)D-Glc
2,3,6-O–Me_3–Glc	5.1	5.8	-1)D-Glc-6-

Table 3
Effect of compounds **1** and **2** on multiplication of *P. polyphylla* var. *yunnanensis* shoots

Compound	Concentration (mg/l)	Number of shoot multiplication[a]
Control	0	2.5±1.0
Compound **1**	2.5	8.5±1.2*
	5	5.1±1.3**
	10	3.5±1.2***
	20	4.3±1.4***
Compound **2**	2.5	3.9±0.8***
	5	4.6±0.9**
	10	7.8±1.3*
	20	3.7±1.1***

*$P < 0.001$; **$P < 0.01$; *** $P < 0.05$, compared with control.
[a] Data presented as mean±S.E., triplicate measurements, 60 day culture.

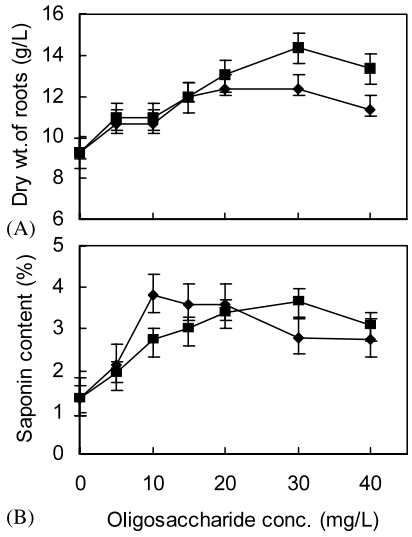

Fig. 1. Effect of compound **1** (–◆–) and **2** (–■–) on growth (A) and saponin production (B) of *P. japonicus* var. *major* hairy roots (over 40-day culture period).

shoots (data not shown). This is different from that reported by some others that oligosaccharides could promote the growth (elongation) of roots and shoots of some plants [15,16].

3.3. Effects of *1* and *2* on *P. japonicus var. major* hairy root culture

Both compounds significantly stimulated the hairy root growth and saponin accumulation of the culture (Fig. 1). Compound **2** at 30 mg/l in the culture medium had the most dramatic effect on the hairy root growth, yielding a dry weight 1.5 times of the control (14.4 g/l vs. 9.3 g/l) (Fig. 1A). Compound **1** had a more potent effect on saponin accumulation of the culture, and particularly at 10 mg/l, increased the saponin content of hairy roots by nearly twofold (3.80% vs. 1.33% of control).

4. Discussion

Two simple oligosaccharides isolated from the rhizomes of *P. polyphylla* var. *yunnanensis* have shown growth-regulatory activity in the shoot culture of the same plant species and the hairy root culture of a different species, *P. japonicus* var. *major*. This suggests that these oligosaccharides may be acting as the growth regulators in their originating species, and in other plant species. Many oligosaccharides derived from various sources, such as chitin and chitosan, and fungal and plant cell wall polysaccharides, act as elicitors or the signal molecules in plant defense responses to pathogens, inducing phytoalexin production [17]. The stimulation of secondary metabolite accumulation in plant tissue and cell cultures by pure or mixture oligosaccharides is mostly a result of such elicitor activity of these compounds. For example, oligosaccharides with DP 6 derived from chitin and chitosan potentiated methyl jasmonate-induced production of paclitaxel in *Taxus canadensis* cell culture [18]. In our previous study [13], oligosaccharides extracted from cultured *Panax ginseng* cells stimulated saponin accumulation in culture of *Panax nontoginseng* cells. Saponins belong to a class of triterpenoids (secondary metabolites) derived from the acetate–mevalonate metabolic pathway in plants.

Whilst most early studies on the biological activities of oligosaccharides were concerned with the effects of these molecules in the elicitation of plant defense responses to pathogenic attacks, many recent studies suggest more diverse and general roles of these molecules in regulating plant growth and development, such as the promotion of root growth [15,16] and flower formation [19]. In this work, the two oligosaccharides with DP 7 and 8 isolated from *P. polyphylla* var. *yunnanensis* stimulated saponin production as well as the growth of *P. japonicus* var. *major* hairy roots. These effects were more likely a result of hormonal action rather than elicitor action since the latter usually causes negative effect on growth while stimulating the secondary metabolite accumulation of plant tissues and cells in culture.

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