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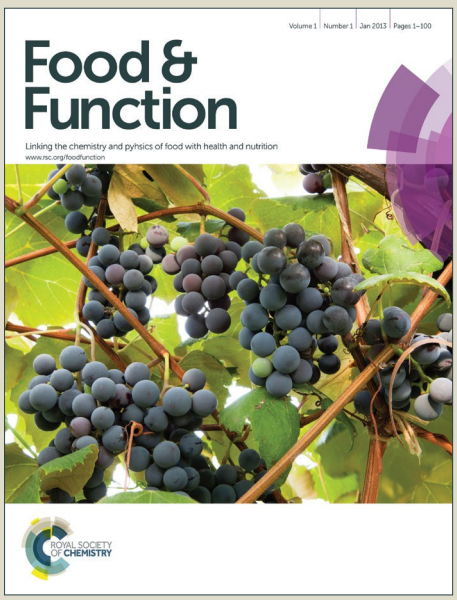

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Title:

Intestinal immunomodulating activity and structural characterization of a new polysaccharide from stems of *Dendrobium officinale*

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ABSTRACT:

A homogeneous polysaccharide fraction (DOP-W3-b) with highly intestinal immunomodulating activity was obtained from the stems of *Dendrobium officinale* through a bioactivity-guided sequential isolation procedure based on the screening of Peyer's patch-mediated immunomodulating activity. Oral administration experiments of mice showed that DOP-W3-b could effectively regulate intestinal mucosal immune activity by changing intestinal mucosal structures, promoting the secretions of cytokines from Peyer's patches (PPs) and mesenteric lymph nodes (MLNs), and increasing the production of secretory immunoglobulin A (sIgA) in lamina propria. Structure analysis indicated that DOP-W3-b was composed of mannose and glucose in the molar ratio of 4.5 with a relatively low molecular weight of 1.543×10^4 Da, and its repeat unit contained a backbone consisting of β-(1→4)-D-Man*p*, β-(1→4)-D-Glc*p* and β-(1→3,6)-D-Man*p* residues, a branch consisting of β-(1→4)-D-Man*p*, β-(1→4)-D-Glc*p* and terminal β-D-Glc*p*, and *O*-acetyl groups attached to *O*-2 of β-(1→4)-D-Man*p*. These results suggested that DOP-W3-b was a new polysaccharide with an essential potential for modulating body's immune functions.

1. Introduction

The intestinal mucosa is a crucial barrier for host defense against invading pathogens and food antigens from the luminal sides.[1] The gut-associated lymphoid tissues including Peyer's patches and isolated lymphoid follicles exist in the intestinal mucosa and play important roles in regulating both mucosal immune system and systemic immune system.[2] In particular, Peyer's patches, which are composed of follicle-associated epithelium containing specialized epithelial cells-M cells and highly specialized lymphoid follicles containing numerous B-cells, T-cells, dendritic cells and macrophages, have been reported to be an essential inductive sites for initiating the intestinal mucosal immune response.[3] Accumulating evidence has demonstrated that the activated lymphocytes in Peyer's patches would migrate into immune systemic circulation through mesenteric lymph nodes and finally home to the effective sites such as the lamina propria of intestinal mucosa to regulate mucosal and systemic immune responses that lead to the improvement of defense systems or immunocompromized situations.[4]

Dendrobium officinale Kimura et Migo (Orchidaceae) is a traditionally edible medicinal plant in China and has been used as raw materials to prepare beverages, porridges and soups in daily life for centuries.[5] It has been claimed that eating *D. officinale* will help to improve the healthy situations of the body to prevent some chronic diseases, such as hepatic dysfunction, hyperglycemia and immunological disorders.[6] Recently, polysaccharides from *D. officinale* have been reported as its major active constituents to exert a variety of pharmacological activities.[7] Among activities, the immunomodulating effects of *D. officinale* polysaccharides have attracted a lot of attention and the structures of six polysaccharide fractions have been identified.[5,8-11] However, because the immnunomodulating effects of these polysaccharide fractions were individually evaluated using *in vitro* splenic cell or macrophage model after the isolation and purification of single fraction, it is uncertain whether or not they are the most effective fractions in total polysaccharide extracts of *D. officinale*. Furthermore, *D. officinale* and its extracts are customarily consumed through the oral route. Since the

polysaccharides in herbs are difficult to be absorbed into blood circulation by the gastrointestinal tract because of their high molecular weights and especial glycosidic linkages that are different from those of the starch, it is possible for polysaccharides to exhibit their immunomodulating effects by interacting with the intestinal mucosal immune system.[12] However, it is also unclear whether or not those identified polysaccharide fractions from *D. officinale* effectively exert their immunomodulating functions after oral administration as reported in *in vitro* cell model.[5,8-11]

In the present study, we aimed at finding out homogeneous polysaccharide fractions with high intestinal immunomodulating activity from *D. officinale* stems. Firstly, immunomodulating polysaccharide fractions were isolated and purified based on a bioactivity-guided strategy using *in vitro* Peyer's patch-mediated immunomodulating activity assay. Then, the isolated immunomodulating polysaccharide fraction was orally administrated to mice to assess its *in vivo* immune functions. Lastly, the structure features of the isolated immunomodulating polysaccharide fraction were elucidated by HPGPC, FT-IR, GC, methylation analysis and NMR techniques.

2. Materials and methods

2.1 Plant materials and reagents

The stems of *Dendrobium officinale* were collected from Huoshan, China, in December of 2012. DEAE-Cellulose and Sephadex G-100 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dextrans (5.0, 25.0, 80.0, 150.0, 470.0, 610.0 kDa) and different monosaccharide standards were purchased from Fluka (St, Louis, MO, USA). RPMI 1640 medium, Hank's balanced salt solution (HBSS) and fetal bovine serum (FBS) were purchased from Gibco BRL Co. (Grand Island, NY, USA). Alamar Blue™ was from Alamar Biosciences Inc. (Sacramento, CA, USA) and goat anti-mouse FITC-IgA from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). ELISA kits for the determination of IFN-γ, IL-2 and IL-4 were from R&D Systems Inc. (Minneapolis, MN, USA). Chromogenic end-point tachypleus amebocyte lysate

(TAL) assay kit was purchased from Xiamen Horseshoe Crab Reagent Manufactory (Xiamen, China). All reagents used in this study were analytical grade.

2.2 Animals

Female ICR mice (6-8 weeks old) weighing 23 ± 2 g were purchased from the Experimental Animal Center of Anhui Medical University, China. They were housed in cages in a specific pathogen-free animal room and had free access to laboratory chow and water under a 12 h light/dark cycle and a relative humidity of 60 ± 5% at 24±1 °C. All procedures related to the animal experiments conformed to the internationally accepted principles as found in the Guidelines for Care and Use of Experimental Animals issued by the government of China and were approved by the Animal Care Review Committee, Hefei University of Technology.

2.3 Isolation and purification of polysaccharides

Stems of *D. officinale* were extracted and fractionated through a bioactivity-guided sequential isolation procedure based on the screening of Peyer's patch-mediated immunomodulating activity (S1). In brief, the dry powder of stems was extracted with 80% ethanol (1 : 60, g : mL) for 12 h by constant stirring at room temperature, and was filtered and centrifugated (3000 *g*, 10 min) to give the supernatant, which was concentrated in a vacuum rotary evaporator under the reduced pressure at 60 °C and lyophilized to obtain ethanol extract (A). The residue was extracted twice with distilled water (1 : 60, g : mL) at 100 ℃ for 2 h. Then, the part of the combined supernatant was concentrated and lyophilized to give water extract (B). The other was concentrated and precipitated with 4-fold volumes of anhydrous ethanol at 4 °C overnight. The resulting supernatant was lyophilized to give ethanol extract (C) and the precipitates to give polysaccharide extract (D). Crude polysaccharide (E) was obtained by deproteinization of (D) using the Sevag method.[13] Subsequently, (E) was dialyzed (molecular weight cutoff of 3500 Da) against running water and distilled water for 2 d and the non-dialyzable portion was lyophilized to give *D. officinale* polysaccharide (DOP, *i.e.* F). DOP was dissolved in distilled water, then applied to a DEAE-Cellulose column (1.6 cm × 60 cm) and eluted with water and 0.05 M NaCl solutions to give two polysaccharide fractions (DOP-W and DOP-S),

respectively. DOP-W was further precipitated with 40% ethanol, 60% ethanol and 80% ethanol in turn and DOP-W1, DOP-W2 and DOP-W3 were gained, repectively. Finally, the intestinal immunomodulating DOP-W3 was fractionated by Sephadex G-100 column (1.0 cm × 80 cm) to give three sub-fractions (DOP-W3-a, DOP-W3-b and DOP-W3-c). During extraction and fractionation, *in vitro* Peyer's patch-mediated immunomodulating activity assay was used for the assessment of different extracts and the detailed method was described in the following section. At the same time, the level of LPS in polysaccharides was measured using the limulus test with the chromogenic end-point tachypleus amebocyte lysate (TAL) assay kit according to the manufacturer's instruction.

2.4 Measurement of *in vitro* intestinal immunomodulating activity

The intestinal immunomodulating activity was measured according to the enhanced proliferation of bone marrow cells stimulated by the suspension of Peyer's patch cells of female ICR mice using the method of Suh *et al*.[2] In short, Peyer's patch cells from the small intestine of mice were suspended in RPMI-1640 medium supplemented with 5% FBS and regulated to a density of 2×10^6 cells/mL. Then, a 180 μL aliquot of cell suspension was dispensed into 96-well flat bottom microculture plates and cultured with test samples (20 μL) at 0, 50, 100 and 200 μg/mL at 37 °C under a humidified atmosphere of 5% CO_2-95% air. After 5 days of culture, the resulting cell supernatant was harvested by centrifugation (3000 *g*, 10 min). Subsequently, a 50 μL aliquot of cell supernatant was added to stimulate the proliferation of bone marrow cells cultured in 96-well flat bottom microculture plates, where each well contained 100 μL cell suspension (2.5×10^5 cells/mL) and 50 μL RPMI-1640 medium supplemented with 5% FBS. The proliferation of bone marrow cells was performed for 6 days under the same circumstance as above and was measured by Alamar Blue™ reduction assay as described by Hong *et al*.[14]

2.5 Measurement of *in vivo* intestinal immunomodulating activity

After adaptive feeding, the mice were randomly divided into 3 groups (10 mice in each). Group 1 was control group, in which the mice were administered orally with distilled water.

Others were experimental groups, in which the mice were administered orally once per day with aqueous solutions of DOP-W3-b for 3 or 7 days. According to the reported dosages,[2,14,15] DOP-W3-b was used at 500 mg/kg BW for group 2 and 2 g/kg BW for group 3. Then, mice were sacrificed by cervical dislocation after ether anesthesia and Peyer's patches (PPs) and mesenteric lymph nodes (MLNs) from the small intestine were isolated to prepare cell suspensions according to the method of Matsumoto *et al.*[16] Meanwhile, body and organ (spleen, thymus and liver) weights were measured to calculate the organ index. PPs and MLNs cell suspensions were respectively resuspended in RPMI-1640 medium supplemented with 5% FBS at a density of 2.5×10^6 cells/mL and cultured into 96-well flat bottom microculture plates with 500 μL of aliquots at 37 °C in a humidified atmosphere of 5% CO_2-95% air. After 3 d, the supernatant was recovered and the amount of IFN-γ, IL-2 and IL-4 were determined using commercially available ELISA kits according to manufacture's instruction. The proliferation of bone marrow cells was also determined after 6 days of stimulation by the supernatant of Peyer's patch cells that cultured for 5 days as described previously.

At the same time, the ileal segments of mice were taken and fixed in 10% formalin for 12 h, embedded in paraffin, serially sectioned at a thickness of 5 μm, routinely stained with hematoxylin-eosin and observed under light microscopy for structure analysis and goblet cell counting. IgA^+ cells in lamina propria were detected by immunofluorescence histochemistry according to the method of Medrano *et al.*[15] Briefly, histological sections were treated with PBS containing 10% FBS (v/v) to block nonspecific protein binding and IgA^+ cells were determined by using the monospecific antibody anti-mouse IgA-FITC under fluorescence microscopy in a LEICA SP5 (Leica Microsystems, Wetzlar GmbH, Germany).

2.6 HPGPC, FTIR and NMR analysis

The homogeneity and molecular weight of DOP-W3-b were determined by HPGPC, which was performed on Agilent 1260 Infinity system equipped with a refractive index detector and a tandem columns consisting of TSK G4000PWXL column (7.8 mm × 300 mm) and TSK

G5000PWXL column (7.8 mm × 300 mm) under the circumstance of 30 ℃. For each run, a 20 μL aliquot of sample was injected and eluted by double distilled water at the flow rate of 0.5 mL/min. The molecular weight of DOP-W3-b was calculated according to the calibration curve of standard dextrans. FTIR of DOP-W3-b was recorded on a Nicolet 6700 spectrometer (Thermo Nicolet, Madison, WI, USA) in a range of 4000-400 cm^{-1}. All NMR spectra including ^{1}H NMR, ^{13}C NMR, ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) and ^{1}H-^{13}C heteronuclear multiple quantum coherence (HMBC) were recorded on a Bruker Avance AV400 spectrometer using standard Bruker NMR software (Bruker, Billerica, MA, USA). In addition, the differential scanning calorimetric (DSC) experiment was performed using a Perkin–Elmer TA-Q200 Series DSC Thermal Analysis System (TA, USA) to determine the thermodynamic properties of polysaccharide, and the Congo red assay was carried out to identify the conformational structure of polysaccharide according to the method of Xu *et al*.[17]

2.7 Monosaccharide composition Analysis

According to our previous report,[18] DOP-W3-b was hydrolyzed by 2M trifluoroacetic acid (TFA) at 110 °C for 2 h, reduced with $NaBH_4$ at room temperature for 3 h, acetylated with mixture of pyridine and acetic anhydride (1:1, v/v) at 100 °C for 1 h and analyzed by gas chromatography (GC) to obtain monosaccharide compositions.

2.8 Methylation Analysis

According to the method described by Needs and Selvendran,[19] the dried DOP-W3-b using P_2O_5 was dissolved in dimethyl sulfoxide, added with NaOH, treated with an ultrasonic wave for 2 h and methylated by methyl iodide. Then, the methylated products were hydrolyzed with 2 M TFA at 110 °C for 4 h and reduced with sodium borodeuteride (Alfa Aesar, MA, USA) into partially methylated alditol acetates, which were analysed by GC-MS.

2.9 Statistical Analysis

All results were expressed as means ± SD and the differences between different experimental groups were tested using one-way analysis of variance (ANOVA). A value of $p < 0.05$ was

considered statistical significance.

3. Results

3.1 Bioactivity-guided isolation and purification of polysaccharides

Since *D. officinale* is traditionally consumed through the oral route to exert its healthy effects, an *in vitro* Peyer's patch-mediated immunomodulating activity assay was used to guide the isolation and purification of polysaccharides from its stems.

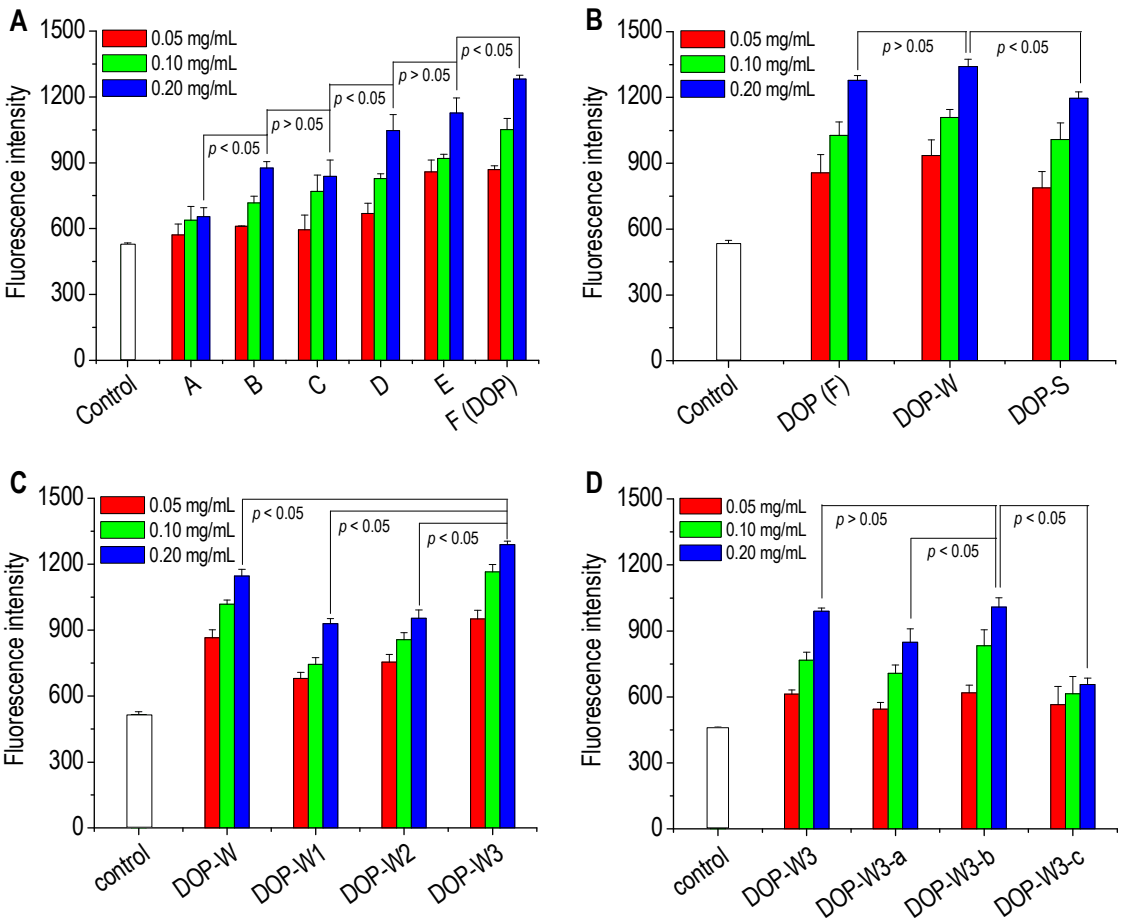

Fig.1 Peyer's patch-mediated intestinal immunomodulating activity of different extracts (**A**) and polysaccharide fractions from DEAE-ellulose anion-exhange chromatography (**B**), sequential ethanol preipitation (**C**) and Sephadex G-100 gel chromatography (**D**) of *Dendrobium officinal* stems. Extracts A, B, C, D, E, F (DOP) and different fractions of DOP including DOP-W, DOP-S, DOP-W1, DOP-W2, DOP-W3, DOP-W3-a, DOP-W3-b and DOP-W3-c are specified in Materials and Methods. Significant differences were compared between different groups at the level of $p < 0.05$.

As shown in Fig. 1A, different extracts obtained from *D. officinale* stems by a sequential

extraction procedure (S1) exhibited different immunomodulating activity. As compared to ethanol soluble extract (A), water soluble extract (B) had higher activity, which was 1.34 folds of that of extract (A) at the concentration of 0.2 mg/mL. With the increase of polysaccharide contents, extracts showed a trend to have enhancing activity. Among these extracts (B, C, D, E and F), crude *D. officinale* polysaccharides (DOP, *i.e.* F) with an average yield of 91.8 mg/g dry stems displayed the highest immunomodulating activity, which was 48.3%, 52.9%, 22.4% and 13.7% higher than those of extract (B), extract (C), extract (D) and extract (E) at the concentration of 0.2 mg/mL, respectively. DOP contained both neutral (DOP-W, yield: 92.18%) and acidic (DOP-S, yield: 3.07%) polysaccharide fractions, which were separated on DEAE-Cellulose anion-exchange column eluted by distilled water and 0.05 M NaCl (S2), respectively. Peyer's patch-mediated immunomodulating activity assay found that the activity of DOP-W was similar to that of DOP, but was higher than that of DOP-S (Fig. 1B). DOP-W was further precipitated with 40%, 60% and 80% ethanol in turn to give main activity fractions. As compared to DOP-W, although DOP-W1 (yield: 5.56%) from 40% ethanol precipitation and DOP-W2 (yield: 32.65%) from 60% ethanol precipitation showed lower immunomodulating activities, DOP-W3 (yield: 43.70%) from 80% ethanol precipitation remained higher activity (Fig. 1C). Subsequently, DOP-W3 with high immunomodulating activity was purified on Sephadex G-100 column and three sub-fractions including DOP-W3-a (yield: 11.31%), DOP-W3-b (yield: 17.27%) and DOP-W3-c (yield: 37.39%) were obtained. Of these three sub-fractions, DOP-W3-b expressed the most potent activity, which was close to that of DOP-W3, 18.9% higher than that of DOP-W3-a and 53.5% higher than that of DOP-W3-c at the concentration of 0.2 mg/mL (Fig. 1D). Thus, with the evaluation of *in vitro* Peyer's patch-mediated immunomodulating activity, a highly intestinal immunomodulating polysaccharide fraction, DOP-W3-b, was screened out from the water soluble extracts of *D. officinale* stems.

3.2 The *in vivo* intestinal immunomodulating effect of DOP-W3-b

The body and organ weights of mice after oral administration of DOP-W3-b were measured

and the significant increases in the weight gain and organ (spleen, thymus and liver) indices of mice appeared after 3 d or 7 d of oral administration with DOP-W3-b (S3). Subsequently, the proliferation of bone marrow cells by the supernatant from Peyer's patch cells of ICR mice orally administrated with polysaccharide was investigated to assess the *in vivo* immunomodulating activity of DOP-W3-b. As shown in Fig. 2, DOP-W3-b time- and dose-dependently stimulated the proliferation of bone marrow cells by the oral administration of 0.5 g/kg or 2 g/kg for consecutive seven days. Compared to the control, the proliferation of bone marrow cells by Peyer's patch cell supernatant was increased by 19.8% on the third day and 34.2% on the seventh day with 0.5 g/kg/day oral administration, and by 35.2% on the third day and 71.7% on the seventh day with 2.0 g/kg/day oral administration, suggesting DOP-W3-b is a potent modulator for the production of hematopoietic growth factor.

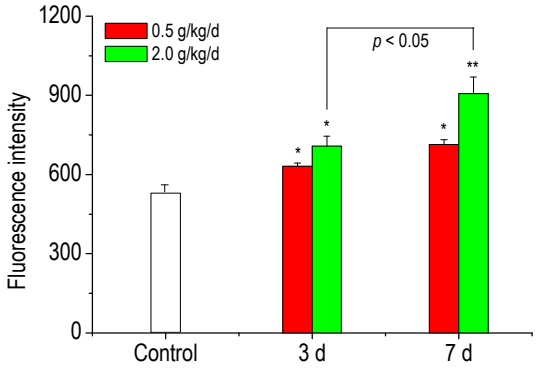

Fig.2 Effects of oral administration period and doses of DOP-W3-b on Peyer's patch-mediated intestinal immunomodulating activity. * and ** indicate the significant difference of $p < 0.05$ and $p < 0.01$ as compared with the control, respectively.

Table 1 Production of cytokines by lymphocytes from Peyer's patches (PPs) and mesenteric lymph nodes (MLNs) of the mice orally administrated with DOP-W3-b.

Day	Polysaccharide (g/kg/day)	IFN-γ (ng/L)		IL-4 (pg/mL)		IFN-γ/IL-4	
		PPs	MLNs	PPs	MLNs	PPs	MLNs
	Control	335.4±11.9	419.3±13.8	142.6±2.6	145.7±3.4	2.35	2.88
3	0.5	345.7±15.5	437.4±10.2	148.6±1.3	113.8±2.9	2.33	3.84
	2.0	372.7±18.4*	503.3±39.2**	158.9±3.4*	129.9±8.5	2.35	3.87
7	0.5	371.7±12.3*	463.9±41.1*	153.1±2.2*	120.7±2.3	2.43	3.84
	2.0	465.8±13.5**	542.3±35.8**	184.6±1.8**	137.8±1.2	2.52	3.94

* and ** indicate significant differences at the level of $p < 0.05$ and $p < 0.01$ as compared with the control, respectively.

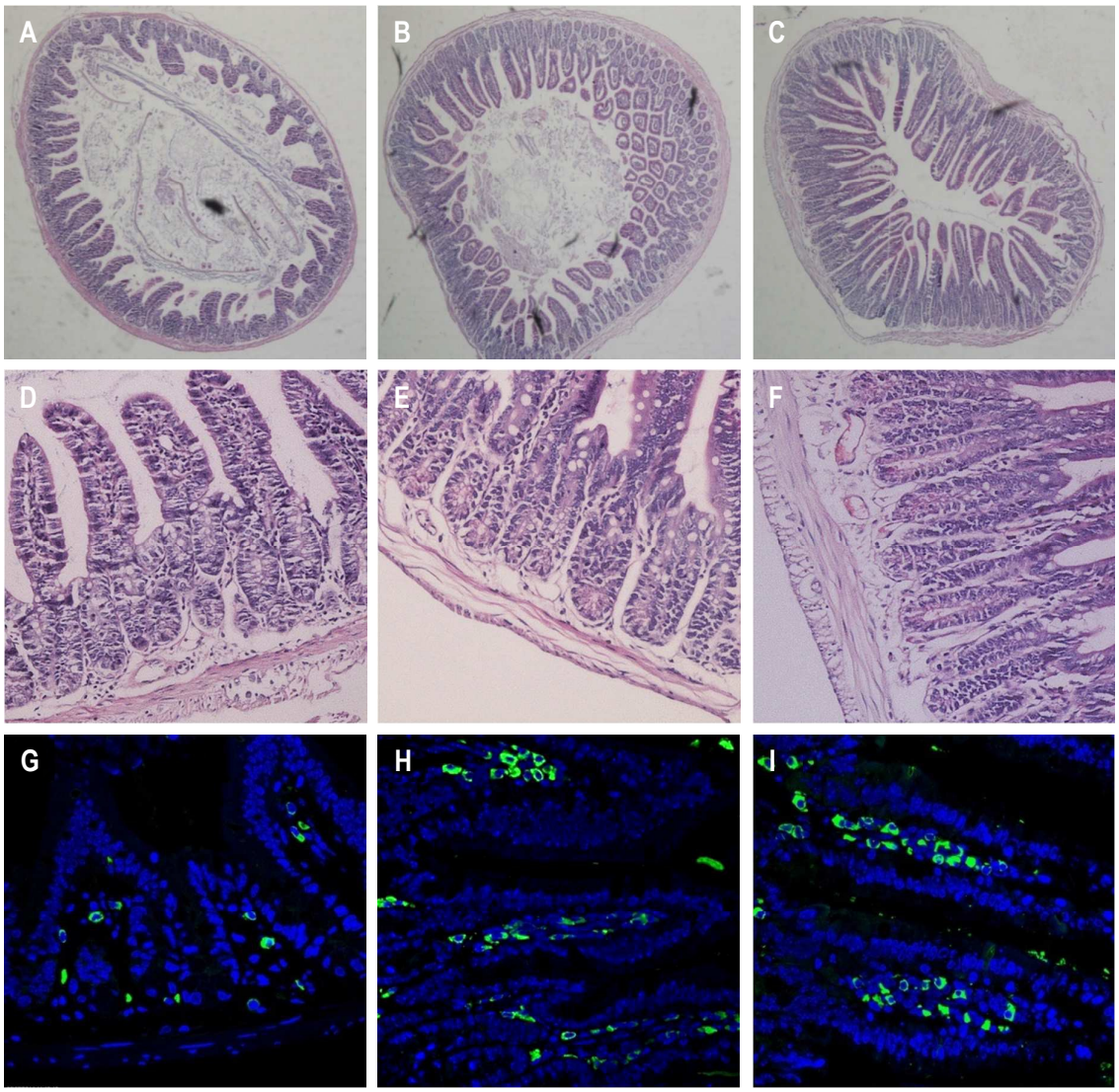

Fig.3 Circumference images and IgA$^+$ cells of small intestines from untreated mice (A, D and G) and mice orally administrated with 2.0 g/kg DOP-W3-b for 3 days (B, E and H) and 7 days (C, F and I). The hematoxylin and eosin (HE) staining images of small intestines are taken under 40 times (A, B and C) and 200 times (D, E and F), respectively. The immunofluorescence staining images for IgA$^+$ are taken under 200 times (G, H and I).

Then, the immunomodulating activity of DOP-W3-b was further analyzed based on the determination of cytokine levels in Peyer's patches (PPs) and mesenteric lymph nodes (MLNs) from mice orally administrated with or without polysaccharide. Table 1 shows that the oral administration of DOP-W3-b remarkably increased the secretion of IFN-γ and IL-4 from PPs as well as IFN-γ secretion from MLNs at the dose of 0.5 g/kg for 7 days and 2 g/kg for 3 days

and 7 days. Meanwhile, DOP-W3-b caused the regression of IL-4 production to some extent from MLNs although this regression is not significant as compared to the control. Further analysis found that the ratios of IFN-γ to IL-4 slightly increased in PPs but markedly increased in MLNs as compared to the control.

Lastly, the ileal histological observation was used to exam the effects of DOP-W3-b on the immune function of small intestines. HE staining of ileal sections showed a relatively great change in the morphological structures of intestinal mucosas between the mice of different groups (Fig. 3A to 3F). Very obviously, in comparison with the control group, mice orally administrated with 2.0 g/kg DOP-W3-b for 3d and 7d had longer villi and deeper crypts in addition to tidily and tightly arranged epithelial columnar cells in the ileum, where a significant increment of mucus producing cells (goblet cells) was observed in mice orally administrated with DOP-W3-b (S4). The immunofluorescence staining with goat anti-mouse IgA-FITC antibody exhibited that the oral administration of mice with 2.0 g/kg DOP-W3-b for 3 or 7 days significantly increased the numbers of IgA^+ cells in the ileum as compared to the control mice (Fig. 3G, 3H and 3I).

3.3 Structural characterization of DOP-W3-b

HPGPC analysis showed that DOP-W3-b was a homogeneous polysaccharide fraction evidenced by the existence of only a single and symmetric peak and its average molecular weight was 1.543×10^4 Da estimated by using dextrans of known molecular weights as standards (Fig. 4A). The qualitative DSC study also proved the homogeneity of DOP-W3-b with weak regularity, which was demonstrated by only a heat absorption peak at 290 °C without the occurrence of heat release peak (Fig. 4B). Congo red test displayed that within the tested NaOH concentrations ranging from 0.0 to 0.1 mol/L, the maximum absorption of Congo Red mixed with DOP-W3-b shifted to longer wavelengths as compared to Congo Red alone, indicated that DOP-W3-b has a certain binding with Congo Red. However, the mixture of Congo Red and DOP-W3-b did not appear a metastable absorption zone, suggesting the presence of no triple-helical conformation in DOP-W3-b solution (Fig. 4C). FT-IR spectrum

showed that DOP-W3-b was rich in carbohydrates (Fig. 4D). The peaks at around 3400 and 2890 cm^{-1} represented the stretching vibration of OH and C-H in sugar ring.[20,21] The peaks at around 1740, 1380, 1250 cm^{-1} were due to the valence vibration of C=O, the symmetric C-H bending vibration of the methyl group and the C-O vibration of O-acetyl groups, respectively.[11,22] The peak at around 1030 cm^{-1} could be assigned to the C–O vibration C-O-C and C-OH in sugar ring.[23,24] The peak at 897 cm^{-1}, similar to the characteristic absorptions of β-type glycosidic linkages in the reported polysaccharides from *D. officinale*,[8,10,11,22] indicated that β-configuration also existed in DOP-W3-b. The peaks around 870 cm^{-1} and 810 cm^{-1} have been recognized respectively from the in-phase stretching and the deformation of the equatorial C2-H in the mannose pyranosyl ring.[22] Thus, the absorption at 877 cm^{-1} and 814 cm^{-1} in DOP-W3-b suggested the existence of mannose.

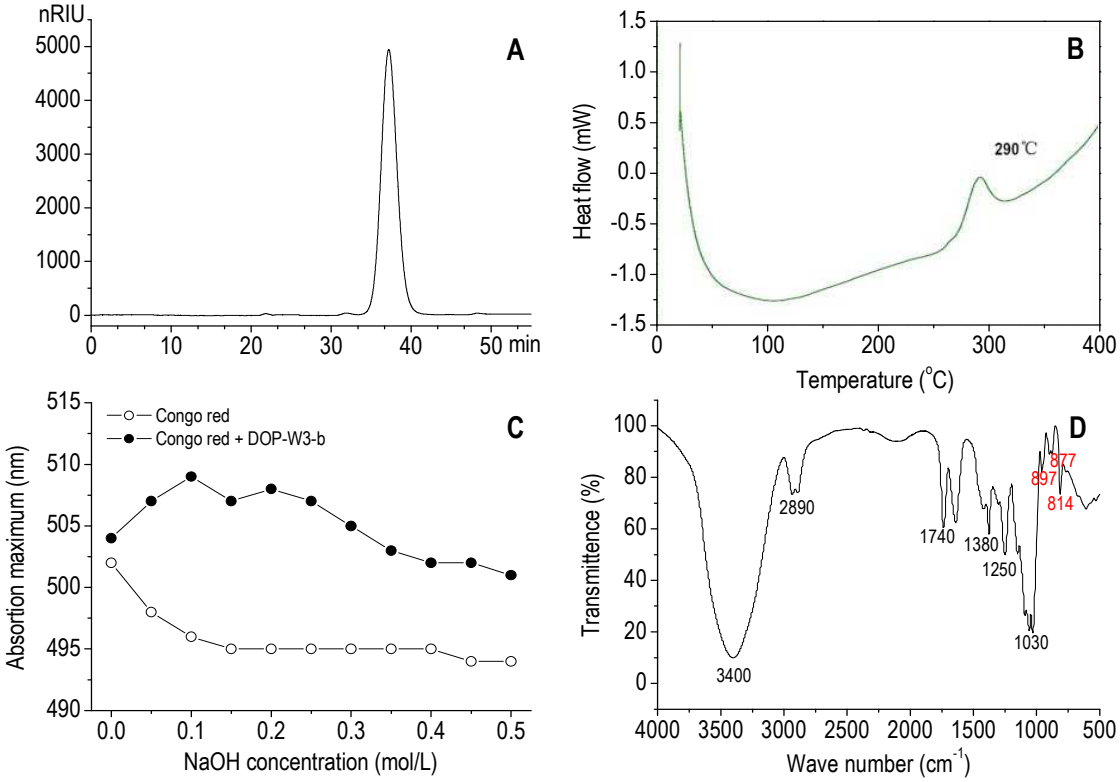

Fig.4 HPGPC (A), DSC (B), Congo red absorption (C) and FT-IR spectra of DOP-W3-b.

Monosaccharide compositions of DOP-W3-b were analyzed by GC to indicate that it was composed of mannose and glucose in a molar ratio of 4.5 : 1.0. Methylation analysis was used

to elucidated the linkage types of mannose and glucose in DOP-W3-b and the presence of four partially methylated alditol acetates, namely 2,3,6-Me_3-D-Man*p*, 2,4-Me_2-D-Man*p*, 2,3,6-Me_3-D-Glc*p* and 2,3,4,6-Me_4-D-Glc*p*, proved that the linkages of sugar resides were (1→4)-linked D-Man*p*, (1→3,6)-linked D-Man*p*, (1→4)-linked D-Glc*p* and nonreducing end D-Glc*p* in the molar ratio of 31.3 : 1.0 : 5.8 : 1.3 (Table 2 and S5). Besides, a trace of 3,6-Me_2-D-Man*p* was also found in the methylation analysis of DOP-W3-b.

Table 2 Major glycosidic linkage analysis of DOP-W3-b

Methylated sugar	Linkage types	Mass fragments (m/z)	Retention time (min)	Molar ratio
2,4-Me_2-Man*p*	1,3,6-linked Man*p*	43, 59, 74, 87, 101,118, 129, 143, 160, 174, 189, 202, 217, 234, 245	19.173	1.0
2,3,6-Me_3-Man*p*	1,4-linked Man*p*	43, 59, 71, 88, 102, 118, 131, 143, 162, 173, 191, 204, 217, 233, 246, 264	15.242	31.3
2,3,6-Me_3-Glc*p*	1,4-linked Glc*p*	43, 59, 71, 88, 102, 118, 131, 143, 162, 173, 191, 204, 217, 233, 246	16.576	5.8
2,3,4,6-Me_4-Glc*p*	1-linked Glc*p*	43, 59, 71, 87, 101, 118, 129, 145, 161, 172, 191, 207	12.739	1.3

The structure features of DOP-W3-b were further characterized by ^{13}C NMR, ^{1}H NMR, HSQC and HMBC techniques. The ^{13}C NMR spectrum of DOP-W3-b presented five anomeric carbon signals at δ 105.0, δ 102.6, δ 102.1, δ 101.6 and δ 101.1 ppm (Fig. 5A), which were designated as A, B, C, D and E based on their decreasing chemical shifts (Table 3). Frontal four signals were assigned to (1→3,6)-linked-Man*p* (A), (1→4)-linked-Man*p* (B), (1→4)-linked-Glc*p* (C) and 1-linked-Glc*p* (D) according to the literature data.[8,9,25,26] The signal δ 20.3 ppm at the high field and the signal δ 175.9 ppm at the low field of ^{13}C NMR spectrum were from the methyl carbons and carbonyl carbons of *O*-acetyl groups, respectively.[9,11,22] Data from literatures implies that the substitution of 2/3-OH by acetyl groups in the (1→4)-linked-Man*p* will result in the up-field shift of anomeric carbon signal.[8,9,27] Thus, the signal at δ 101.1 ppm in ^{13}CNMR, which was lower than that of (1→4)-linked-Man*p* (δ 102.6 ppm), could be assigned to the (1→4)-2/3-*O*-acetyl-linked-Man*p*. Considering the existence of 3,6-Me_2-Man*p* in methylation

analysis, it may be deduced that the signal at δ 101.1 ppm was from the anomeric carbon of (1→4)-2-*O*-acetyl-linked-Man*p*.

In the HSQC spectrum (Fig. 5B), on the basis of the anomeric carbon signals, the cross-peaks at δ 4.47/105.0, δ 4.73/102.6, δ 4.68/102.1, δ 4.78/101.6 and δ 4.82/101.1 ppm were assigned to the chemical shifts of H-1/C-1 of A, B, C, D and E. Correspondingly, five anomeric proton signals at δ 4.47, δ 4.73, δ 4.68, δ 4.78 and δ 4.82 ppm from A, B, C, D and E were found in the ^{1}H NMR spectrum (Fig. 5C), where the signal at δ 2.16 ppm confirmed the existence of *O*-acetyl groups. Since the signals from anomeric hydrogens were less than 5.0 ppm and from anomeric carbon were greater than 101.0 ppm, it was deduced that both mannose and glucose existed as β-configuration in DOP-W3-b. According the relevant literature data, the rest signals of sugar residues were summarized in Table 3.[8,9,25,27,28]

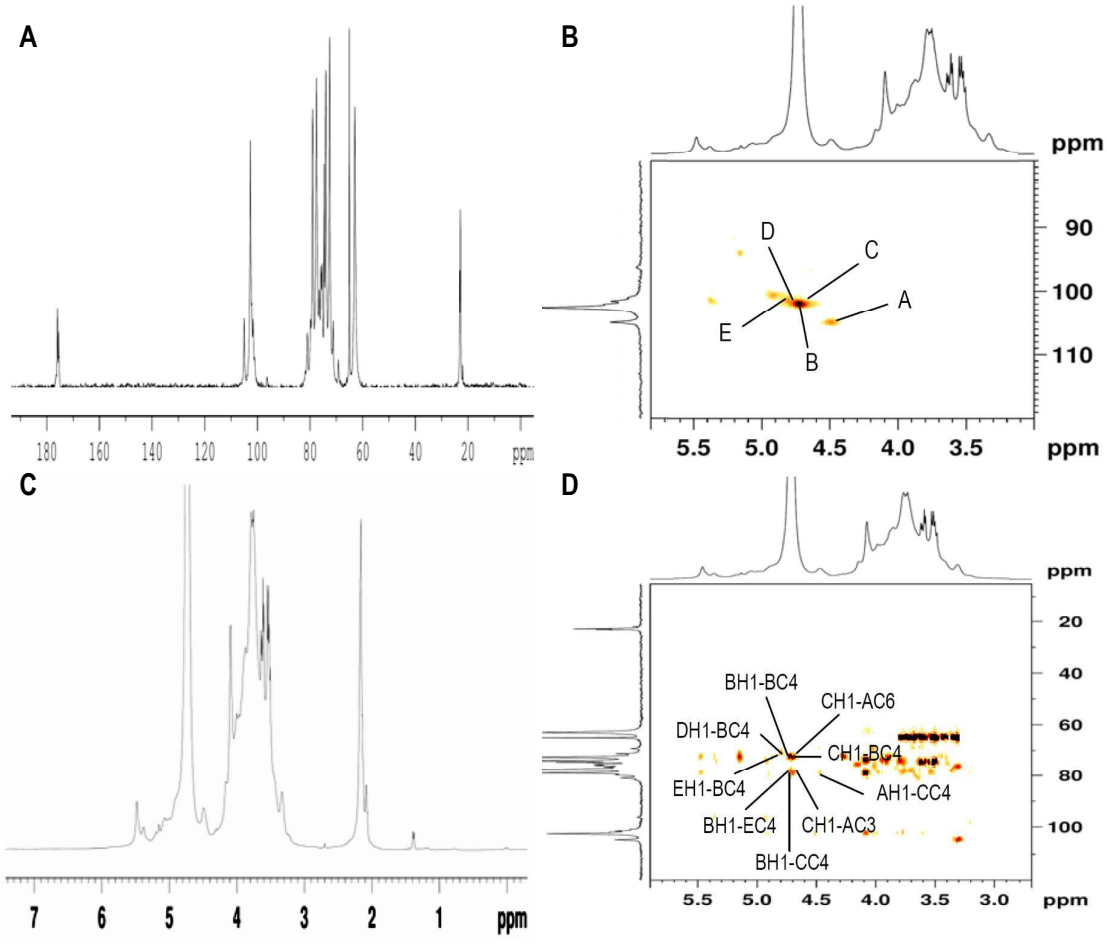

Fig.5 ^{13}C NMR (A), HSQC (B), ^{1}H NMR (C) and HMBC (D) spectra of DOP-W3-b.

The linkage sequence of different sugar residues in DOP-W3-b was inferred from the inter-residue correlations between the anomeric protons and carbons in the HMBC spectrum (Fig. 5D). The cross signal at δ 4.73/72.0, δ 4.73/78.0, δ 4.73/79.8, δ 4.68/78.0, δ 4.68/72.0, δ 4.68/71.0, δ 4.82/72.0, δ 4.78/72.0 and δ 4.47/79.8 indicated the linkages of B H1 and B C4, B H1 and E C4, B H1 and C C4, C H1 and A C3, C H1 and B C4, C H1 and A C6, E H1 and B C4, D H1 and B C4, and A H1 and C C4. Therefore, the repeat unit of DOP-W3-b can be proposed to have a backbone consisting of β-(1→4)-D-Man*p* residues, β-(1→4)-D-Glc*p* residues and β-(1→3, 6)-D-Man*p* residues, a branch of consisting of β-(1→4)-D-Man*p*, β-(1→4)-D-Glc*p* and terminal β-D-Glc*p*, and some *O*-acetyl groups attached to *O*-2 of β-(1→4)-D-Man*p*.

Table 3 Chemical shifts of the signals in ^{1}H NMR and ^{13}C NMR of DOP-W3-b

Sugar residues		1	2	3	4	5	6
(A) →3,6)-β-D-Man*p*-(1→	H	4.47	3.38	3.58	3.74	3.72	3.90
	C	105.0	74.8	78.0	72.4	76.3	71.0
(B) →4)-β-D-Man*p*-(1→	H	4.73	4.09	3.96	3.77	3.68	3.86
	C	102.6	71.2	76.2	72.0	74.3	62.0
(C) → 4)-β-D-Glc*p*-(1→	H	4.68	3.45	3.58	3.60	3.78	3.80
	C	102.1	74.5	75.3	79.8	75.7	61.0
(D) β-D-Glc*p*-(1→	H	4.78	3.39	3.57	3.30	3.53	3.77
	C	101.6	75.2	76.7	72.9	76.0	62.1
(E) →4)-2-*O*-acetyl-β-D-Man*p*-(1→	H	4.82	5.40	4.01	3.79	3.70	3.94
	C	101.1	72.4	71.5	78.0	74.4	61.5

4. Discussion

The large number of evidence has suggested that natural polysaccharides from edible plants possess significant health-improving functions including immunomodulating effects without toxicity and side effects, which has attracted the growing attention to developing functional foods with these polysaccharides.[29] In order to obtain polysaccharides with definite bioactivities and chemical structures, two ordinary strategies are used for the extraction of polysaccharides. The first method is to isolate and purify polysaccharides before the

evaluation of bioactivity and the second method is to obtain purified polysaccharides under the guidance of activity evaluation.[29,7] Although six different homogeneous polysaccharide fractions have been isolated from *D. officinale* stems using the first method and five of them have been shown to have an *in vitro* immunomodulatory activity by different labs,[5,8-11] it is impossible to say that they are the most effective polysaccharide fractions in *D. officinale* stems when the procedure of polysaccharide isolation is not guided by the bioactivity evaluation. In the current study, a homogeneous polysaccharide (DOP-W3-b) with significantly high immunomodulatory activity was obtained through a bioactivity-guided sequential isolation procedure (Fig. 1 and S1) based on the screening of Peyer's patch-mediated immunomodulating activity, which have been used to guide the isolation of immunomodulatory polysaccharides from different medicinal plants because the oral route is a traditional way for the exertion of their immune efficacies and Peyer's patches containing various immune cells are the site to mediate immune responses for intestinal antigens.[2,30-32] The newly obtained DOP-W3-b has an average molecular weight of 1.54×10^4 Da, which is evidently low as compared with those reported *D. officinale* polysaccharides,[5,8-11] suggesting that DOP-W3-b is a new immunomodulatory polysaccharide.

The reports from *in vitro* experiments have described the polysaccharides from *D. officinale* possessed multiple immunomodulating functions, which included the enhanced phagocytic activity, NO secretion and cytokine production in macrophage RAW 246.7 cells and THP-1 cells,[5,10,11,33,34] the inhibited expression of TNF-α-induced apoptotic factors in human salivary gland cell line A-253,[35] and the increased proliferation of spleen cells and cytotoxicity of NK cells.[5] The *in vivo* experiments have also shown that the polysaccharides of *D. officinale* not only significantly enhance cellular immunity, nonspecific immunity and IFN-γ production by splenocytes in mice,[12] but also effectively alleviate the disorder of Sjögren's syndrome (a chronic autoimmune disease) both in patients and in animal model through improving the expression and translocation of aquaporin 5 via activating M3 muscarinic receptors.[36-39] Our study demonstrated that DOP-W3-b after oral administration of

healthy mice may exert immunomodulating functions through regulating the production of IFN-γ and IL-4 from small intestinal PPs and MLNs (Table 1), changing the intestinal mucosal morphological structure and increasing the production of secretory immunoglobulin A (sIgA) in lamina propria (Fig. 3), implying that DOP-W3-b expressed its activities both in cellular immunity and in humoral immunity. It is well known that IFN-γ is Th1-associated cytokine and IL-4 is Th2-associated cytokine and the balance of Th1/Th2 plays a pivotal role in regulating the balance of cellular immunity and humoral immunity. Thus, our results suggested that DOP-W3-b exerted immunomodulating effects possibly by changing the balance of Th1/Th2 as indicated by the obvious increase in the ratio of IFN-γ to IL-4 in MLNs of mice after the oral administration of DOP-W3-b (Table 1).

In order to exclude the effect of LPS that is a common impurity in polysaccharides to stimulate the immune system, The limulus test of DOP-W3-b with the chromogenic end-point tachypleus amebocyte lysate (TAL) assay kit showed that LPS content was 0.000454 ± 0.000036 EU/mL in DOP-W3-b (0.2 mg/mL) and 0.000437 ± 0.000039 EU/mL in the control medium for *in vitro* assay. Similarly, LPS content was 0.005558 ± 0.000036 EU/mL in DOP-W3-b (2.0 g/kg) and 0.005541 ± 0.000039 EU/mL in the control vehicle for *in vivo* assay. Since no significant difference is found in LPS contents between the DOP-W3-b and the control and the control did not display intestinal immunomodulating activity *in vitro* and *in vivo*, we may conclude that the activity of DOP-W3-b as used in our study did not originate from LPS contamination.

It has been extensively suggested that the immunomodulating actions of polysaccharides depend on their molecular weights and chemical structures.[40] In many cases, different polysaccharide fractions with different molecular weights and structural features can be obtained from the extracts of a specific material. In the current study, both molecular weight determination and chemical structure analysis indicated that although DOP-W3-b and those reported *D. officinale* polysaccharides[5,8-11] are all composed of mannose and glucose, DOP-W3-b has distinct differences in detailed structural features including the molar ratio,

glycosidic bond types and linkage sequences of different sugar residues (Fig. 4, Fig. 5, Table 2 and Table 3). In particular, the structure analysis showed that DOP-W3-b is a branched polysaccharide while all reported polysaccharides from *D. officinale* are linear polysaccharides. However, whether the diverse structural features of different *D. officinale* polysaccharides endow them with variable immunomodulating properties needs to be further studied.

5. Conclusions

Using a bioactivity-guided sequential isolation procedure, a new homogenous polysaccharide (DOP-W3-b) with high immunomodulating activity was obtained from *D. officinale* stems. DOP-W3-b had a relatively low molecular weight of 1.543×10^4 Da and its repeat unit was composed of a backbone consisting of β-(1→4)-D-Man*p* residues, β-(1→4)-D-Glc*p* residues and β-(1→3,6)-Man*p* residues and a branch of consisting of β-(1→4)-D-Man*p*, β-(1→4)-D-Glc*p* and terminal β-D-Glc*p*, with *O*-acetyl groups attached to *O*-2 of β-(1→4)-D-Man*p*. Oral administration of DOP-W3-b could effectively regulate the small intestinal immune function through modulating intestinal mucosal structures, influencing the Th1- and Th2- associated cytokines production and promoting the secretion of sIgA in lamina propria in mice.

Abbreviations

D. officinale	*Dendrobium officinale*
DSC	Differential Scanning Calorimetric
FBS	Fetal bovine serum
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
HBSS	Hank's balanced salt solution
HE	Hematoxylin-eosin

HMBC	^{1}H-^{13}C heteronuclear multiple quantum coherence
HPGPC	High performance gel permeation chromatogram
HSQC	^{1}H-^{13}C heteronuclear single quantum coherence
MLNs	Mesenteric lymph nodes
PPs	Peyer's patches
PBS	Phosphate buffer saline
sIgA	Secretory immunoglobulin A
TFA	Trifluoroacetic acid

Conflicts of interest

The authors declare no conflict of interest.

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A newly branched *Dendrobium officinale* polysaccharide (DOP-W3-b) with highly intestinal immunomodulating activity and a relatively low molecular weight was obtained through a bioactivity-guided sequential isolation procedure.