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Composition Analysis and Immunological Activities of the Oligosaccharides Isolated from *Cistanche Deserticola*

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Abstract. A oligosaccharide (CDOS) was obtained from *Cistanche deserticola* by alkali (pH=10) extraction, ethanol precipitation, and fractionated into two purified fractions (i.e.,CDOS-1 and CDOS-2)by Sephadex G-100 and Sephadex G-25 column filtration chromatography. The monosaccharide composition of the CDOS was assayed by high performance liquid chromatography (HPLC).It was found that CDOS-1 was only composed of sucrose and CDOS-2 was mainly composed of sucrose, rhamnose and mannitol, with a mole ratio of 1:0.73:3.61. Immunological tests indicated that CDOS presented significant effect on the mouse spleen index, increasing the phagocytosis activity of macrophages and stimulating antibody-producting cell proliferation. It is hoped that the CDOS will be developed into functional food or medicine.

Keywords: *Cistanche deserticola*, oligosaccharide, purification, composition, immunological activities.

1. Introduction

Cistanche deserticola Y.C.Ma. (Family *Orabanchaceae*) is a short parasitic plant native in the northwest of China. The whole dried plant (without flowers) is known as a tonic and is called "Rou Congrong".In oriental medicine,it is classified as sweet and salty in taste,warm in nature,and attributive to kidney and large intestine channels,with the function of invigorating the kidney and supplementing essence,moisturizing the intestine and relaxing bowels [1]-[3].The modern pharmacological study showed that it could prompt the DNA synthesis and delay the process of senility, increase the antioxidative [4]， prevent and treat cardiovascular diseases [3]. In addition,it could also cause analgesic and anti-inflammatory effects [5], enhances learning and memory by inducing nerve growth factor [6]. Some studies indicated that *C. deserticola* extracts could activate the phagocytic function of intra-abdominal macrophage in mice [7]-[9] and enhance body′s immunity [10].According to the previous studies, this plant contains multiple active constituents which including phenylethanoid glycosides, iridoids, lignans,saccharides, alkaloid etc. [11] As the *C. deserticola* phenylethanoid glycosides and polysaccharide are recognized as the major active components, numerous studies have focused on their structures and bioactivities during the past decades [12]-[17].As for the valuable oligosaccharides in *C. deserticola*, the reports are quite limited. Oligosaccharides, a short chain saccharide containing of homo- or hetero-sugars, are well known for their beneficial effects on human life and have been extensively utilized for long time [18]. Functional oligosaccharides, which have a physiological function such as low cariogenicity and bifidobacteria growth factor [19], improve the health of humans and animals.They have been utilized as a food ingredient. Recently, new functions of oligosaccharides, which have the ability to modulate the immune system in humans, animals,and fish, have been reported [20].In this paper,we report the first part of the results of the research program,the fractionation of the total oligosaccharides obtained from the alkali extract of *C. deserticola* by a combination of ultrafiltration and gel permeation chromatography,and the composition analysis of them by

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high-performance liquid chromatography (HPLC). In addition,we also present the immunological activities of the *C. deserticola* oligosaccharides.To our knowledge, there are few reports published about the immunostimulatory activity studies of *C. deserticola* oligosaccharides.

2. Experimental

2.1. Materials

The *C. deserticola* were cultivated and collected from Alxa League (Inner Mongolia, China). Kunming mice (GradeII,six weeks old) were purchased from the Pharmacology Experimental Center of Inner Mongolia University. Sephadex G-100，Sephadex G-25, trifluoroacetic acid (TFA), 1-phenyl-3-methyl-5-pyrazolone(PMP), D-glucose, D-galactose, D-fructose, D-xylose, D-mannose, D-galacturonic acid, D-glucuronic acid, sucrose, rhamnose,mannitol, fucose, rhamnose, were purchased from Sigma (St. Louis, MO, USA). Medium RPMI-1640 was purchased from Gibco Invitrogen Co. (San Diego, CA, USA). All other chemicals were of analytical grade.

2.2. Extraction of oligosaccharides

The dried bodies of *C. deserticola* were cut into smaller pieces and further ground into powder by a mill, were extracted with anhydrous ethanol (3×5000 ml) at 70 ° for 3 h under atmospheric pressure. A reflux condenser was fixed to remove lipids. The residue left was then extracted with alkali (pH=10) at 60 ℃ for 3 times (2h each time). After centrifugation (2000 g for 15 min, at 20 ℃), the supernatant was concentrated to one-tenth of the volume in a rotary evaporator under reduced pressure at 50 ℃ and filtered. Then the filtrate was deproteinized using the Sevag reagent [21], and decolored with activated carbon.

2.3. Isolation and purification of oligosaccharides

The freeze-dried crude oligosaccharides was dissolved in distilled water, centrifuged, and then the supernatant was purified by a Sephadex G-100 column (1×50 cm), equilibrated with ultrapure water. After loading with sample, the column was eluted with ultrapure water at a flow rate of 5 ml/min.Different fractions were collected using test tubes. Total carbohydrate content of each tube was measured at 490nm by phenol-H_2SO_4 method [22]. The water eluted solution was separated into two fractions CDOS-1 and CDOS-2. Two fractions ware respectively purified further on a Sephadex G-25 column (2.7×85 cm) by using ultrapure water (at a flow rate of 1 ml/min). After collecting the purified fraction, it was lyophilized.

2.4. Analysis of monosaccharide composition

The monosaccharide composition of the CDOs was achieved by HPLC analysis. The CDOs (2 mg) was hydrolyzed first with anhydrous methanol containing 2 M HCl at 80 ℃ for 16 h under a nitrogen atmosphere and then with 2 M TFA at 120 ℃ for 1 h. After TFA was removed by evaporation, the hydrolysates were subsequently derivatized with PMP according to the reported method [23] and analyzed by HPLC. HPLC separation was performed on the EF-2002 HPLC system (KNAUER company,Germany).The PMP derivatives were chromatographed using Sugar- PAK volumn (6.5×300mm ,Waters company,America), and the absorbance was measured at 245 nm. The injection volume was 20 μL, and the mobile phase, composed of PBS (solvent A) and acetonitrile (solvent B), was used for isocratic elution at the volume ratio of 82% (A) to 18% (B). Total HPLC run time was 40 min, and the flow rate was 0.5 mL/min.

2.5. Immunobiological Activities

2.5.1. Phagocytic function of monocyte macrophage

Sixty Kunming mice (GradeII,six weeks old) were purchased from the Pharmacology Experimental Center of Inner Mongolia University and were acclimatized for 1 week prior to use. All mice were randomly divided into four groups, consisting of a saline control group, high CDOs dosage group, moderate dosage group and low CDOs dosage group. The mice were injected intraperitoneally with 0.5mL oligosaccharides solution once per day for 5 days. The high,moderate, or low CDOs dosage group received 100, 50, or 25 mg/kg/bw CDOs, respectively;and 0.5ml saline was injected in the control group. On the seventh day a carbon particle clearance experiment was carried out,according to Hou [24], and the spleen index and thymus index were measured. Briefly, 0.05 mL/10 g/bw India ink was injected into each mouse through the vena

caudalis, then 20μL blood was obtained from the vena orbitalis posterior 3 and 7 minutes after the injection. The blood samples were placed in tubes with 2mL 0.1% Na_2CO_3, and OD values were measured at 600 nm.The clearance index (K), phagocytic index (α) and immune organ index were calculated as follows(1), t_2 and t_1 means 7 minutes and 3 minutes respectively.

2.5.2. The antibody-producting cell proliferation

Groups of Kunming mice (five per group) were immunized by intraperitoneal injection of 2 x 10^7 SRBC in 1.0 ml of PBS added with 50ug of test materials (none in the control). One week later, splenocytes (10^6 cells per 2 ml per well) from Kunming mice were cultured with or without test materials for 72 h in 10% RPMI 1640 medium under 5% CO_2 in air, in triplicate for each culture. The number of PFC against SRBC per 10^6 splenocytes was determined [25], [26].

$$K=\frac{(\log OD_3-\log OD_7)}{t_2-t_1},$$
$$\alpha=\frac{\text{body weight}}{(\text{spleen weight}+\text{liver weight})*\sqrt[3]{K}},$$
$$\text{Immune organ index}=\frac{\text{immune organ weight (mg)}}{\text{body weight (g)}}. \quad (1)$$

2.6. Statistical analysis

The data were expressed as mean values ±SD. The difference between tested groups and control was analyzed by Student's t test. $P < 0.05$ was considered to be significant.

3. Results and Discussion

3.1. Isolation and purification of oligosaccharides

CDOs was isolated from the alkali extract of the dried bodies of *C. deserticola* by a yield of 3.07%. Two fractions of CDOS-1 and CDOS-2 were isolated from distilled water elute by the Sephadex G-100 column, respectively (Fig.1). The purified fractions of CDOS-1 and CDOS-2 showed a single peak on Sephadex G-25 column, indicating that no other oligosaccharides was present in the sample. The results of monosaccharide compositions showed that CDOS-1 was only composed of sucrose (Fig.2) and CDOS-2 was mainly composed of sucrose, rhamnose and mannitol (Fig.3) , with a mole ratio of 1:0.73:3.61.

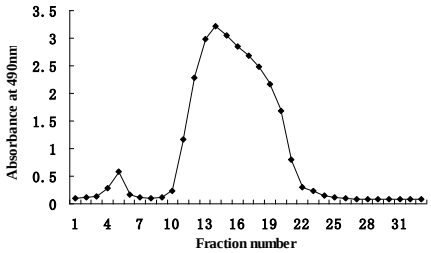

Fig. 1: The profile of CDO isolated from the dried bodies of C. deserticola on a Sephadex G-100 column eluted with distilled water at a flow rate of 5 mL/min.

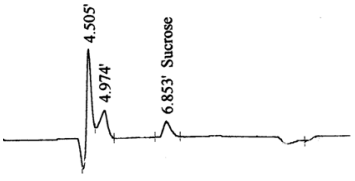

Fig. 2: The monosaccharide compositions of CDOS-1 analyzed by HPLC.

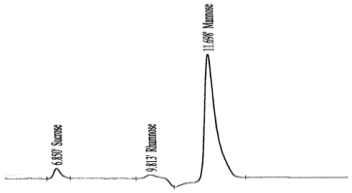

Fig. 3: The monosaccharide compositions of CDOS-2 analyzed by HPLC.

3.2. Immunobiological Activities of CDOs

Much in vivo and in vitro evidence has demonstrated that natural oligosaccharides displayed immunomodulating function by stimulating both cellular and humoral immunoresponse [27], [28]. In this

paper, 100 mg/kg/bw CDOs increased the mouse spleen index, but there were no significant differences in the thymus index between the treated groups and control groups(Table 1).Macrophages are an important component of host defenses against viral infection by inhibiting intracellular replication of viruses and by killing virusinfected cells [29].When activated, a variety of oxygen or nitrogen intermediates and cytokines are released from macrophages and participate in various important biological functions, such as anti-inflammatory and anti-tumor activities [30]-[32]. Therefore, phagocytic activity of macrophages is an important indicator of organism immune functions. In this study, the moderate and high doses of CDOs increased the phagocytosis activity of macrophages (Table1). Antibody-producting cell proliferation induced by CDOs was studied by examination of the increase in hemolytic PFC in the spleens of Kunming mice which were immunized with SRBC plus the test specimen. Results indicated that moderate and high doses of CDOs significantly enhance antibody-producting cell proliferation (Table2). High doses of CDOs caused a highly significant increase of PFC count ($P < 0.01$).

Table 1: Effects of CDOs on the spleen and thymus indices and phagocytosis of MΦ of mouse. Data represent means ± SD (n = 6).

group	Spleen index (mg/g/bw)	Thymus index (mg/g/bw)	Phagocytic index index α of MΦ
Control	2.87±0.82	2.37 ±0.94	3.97 ±1.45
Low dose	4.20 ±1.053	2.18 ±0.85	4.44 ±1.13
Middle dose	5.64 ±0.83*	2.10 ±0.51	5.24 ±0.91**
High dose	6.86 ±1.07**	2.22 ±0.77	6.18 ±1.28**

Control: normal saline; Low dose group: 25mg/kg/bw CDOs; Moderate dose group: 50mg/kg/bw CDOs; High dose group: 100mg/kg/bw CDOs. Data were analyzed by one-way analysis of variance. * P <0 .05, **P <0.01, compared with the control group.

Table 2: Activity of CDOs to increase anti-SRBC hemolytic PFC count in the spleen of Kunming mice immunized with SRBC together with the test specimens[a]

index	Control	Low dose	Middle dose	High dose
Hemolytic PFC coun	3.97±1.45	4.43±1.33	5.44±1.13*	8.11±2.20**

a Kunming mice (five per group) received an intraperitoneal injection of 0.1 ml of PBS containing 50 μg of the test specimen and 2×10^7 SRBC. Seven days later, the splenocytes of each mouse were subjected to hemolytic PFC assays. Data are expressed as the mean hemolytic PFC count±standard error of the mean.** $p < 0.01$ versus untreated control.* $P < 0.05$ versus untreated control.

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