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Chemical characterization and immunological activities of an acidic polysaccharide isolated from the seeds of *Cuscuta chinensis* Lam

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KEY WORDS *Cuscuta chinensis* Lam; polysaccharides; nuclear magnetic resonance; T-lymphocytes; B-lymphocytes; antibodies

ABSTRACT

AIM: To study chemical characterization and immunological activities of an acidic polysaccharide, CHC-1, isolated and purified from the seeds of *Cuscuta chinensis* Lam. **METHODS**: Both chemical and spectral methods were used to investigate the chemical characterization of CHC-1. Effect of CHC-1 on the proliferation rates of T- and B-lymphocytes both *in vivo* and *in vitro*, and antibody production *in vivo* was measured at various concentrations of CHC-1. **RESULTS**: The molecular weight of CHC-1 was estimated to be more than 1.0×10^6. The analytical results of sugar components indicated that CHC-1 was composed of Rha, Ara, Gal, and GalA in a molar ratio of 0.8:1.0:1.5:0.3. Methylation analysis and ^1H, ^{13}C NMR further identified the linkages of the residues of CHC-1. CHC-1 0.1 g/L promoted remarkably the proliferation of T-cells and B-cells *in vitro*. CHC-1 25 mg/kg, 50 mg/kg caused an evident increase in spleen weight, lymphocyte proliferation, antibody production, etc. But its effect on IgG levels was not significant. **CONCLUSION**: CHC-1 is a highly branched heteropolysaccharide and possessed immune enhancement activities.

INTRODUCTION

Cuscutae semens, the dried seeds of *Cuscuta chinensis* Lam, are a traditional Chinese medicine, used as a tonic[1,2] to nourish the liver and kidneys, and to treat impotence and seminal emission. Moreover, it is considered to have an anti-tumour effect in Indian Traditional Medicine[3]. In addition, it has been reported that the crude polysaccharide of *Cuscuta chinensis* Lam causes lymphocyte agglutination, proliferation, and increase of [^{3}H]thymidine, [^{3}H]uridine as well as [^{3}H]leucine incorporation at different degrees[4]. An acidic polysaccharide, CHC-1, has been isolated from the alkali-extract of *Cuscutae* semens in our lab. Here we report the chemical characterization and immune enhancing properties of this polysaccharide.

MATERIALS AND METHODS

Materials Dried *Cuscutae* semens 5 kg were purchased from Shanghai Medicinal Materials Co and identified by Prof HUANG Xiu-Lan. Reagents: 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluene sulfonate (CMC) and trifluoroacetic acid (TFA) were purchased from Merck Co. Concanavalin A (ConA) and lipopolysaccharide (LPS) were from Sigma Co, and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were from Fluka. Medium RPMI-1640 was purchased from Gibco Laboratories. All RPMI-1640 media were supplemented with HEPES buffer 10 mmol/L, benzylpenicillin 100 kU/L, streptomycin 100 mg/L, *L*-glutamine 2 mmol/L, 2-mercaptoethanol 50 μmol/L, and 10 % fetal bovine serum, pH 7.2.

Preparation of CHC-1 The CHC-1 was isolated from the alkali-extract of *Cuscutae* semens by DEAE anion-exchange column and purified by Sephacryl S-300 gel filtration column.

Determination of the molecular weight The polysaccharide (2 mg) was dissolved in NaOH 0.001 mol/L, applied to a gel-filtration chromatographic column of Bio-Rad TSK40-TSK50, eluted with the same buffer and detected by a Refractive Index Detector. Preliminary calibration of the column was conducted using dextrans of different molecular weights.

Constituent analysis CHC-1 5 mg was dis-

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solved with 4 mL TFA 2 mol/L and hydrolyzed at 110 ℃ for 2 h, then the monosaccharides were reduced and converted to the corresponding alditol acetates as described by Blakency *et al*[5]. Analytical GLC was performed with a Shimadzu-9A gas chromatograph.

Reduction of uronic acid[6] CHC-1 20 mg was dissolved with distilled water and CMC was added. As the reaction proceeded the pH of the reaction mixture was maintained at 4.75 by automatic titration with HCl 0.1 mol/L using a ZD-2 pH-Stat which plotted the total volume of acid added *vs* time. All reactions were allowed to proceed for at least 3 h. After hydrogen ion uptake had ceased aqueous sodium borohydride solution 2 mol/L was added slowly. The pH of the mixture was maintained at 7.0 by automatic titration with HCl 4 mol/L in the pH-Stat. A total of 25 mL of the borohydride solution was usually required for reduction, which was completed in 60 min.

Methylation analysis Glycosyl-linkage positions were determined using a modification of the Hakomori procedure[7]. Per-methylated product was then hydrolyzed in TFA 2 mol/L (4 h, 110 ℃), reduced, and acetylated. The partially methylated alditol acetates obtained from these reactions were analyzed by FD 800 GC-MS.

NMR spectral studies NMR spectra were recorded at Bruker AM-400 Spectrometers in D_2O.

Lymphocyte proliferation test[13,14] ***in vivo*** ICR (♀) mice from Shanghai Experimental Animal Center (Grade Ⅱ, Certificate № 153), body weight (20 ± s 2) g, were divided randomly into 3 groups: control group (A), CHC-1 25 mg/kg group (B), CHC-1 50 mg/kg (C), ip × 4 d, respectively. The mice were killed on d 5. The body weight and weight of spleen and thymus were measured. The spleen cells were suspended to a final density of 5×10^9 cells/L in RPMI-1640 medium. Cells (100 μL/well) were added into 96-well plate in the presence of ConA (5 mg/L) or LPS (25 mg/L). After incubation at 37 ℃ in a humidified 5 % CO_2 incubator for 44 h, T- and B-lymphocyte proliferation was tested by MTT (5 g/L, 20 μL/well) assay. The plate was incubated for another 4 h and then the resolver (100 μL/well) was added. The absorbance was measured by DG-3022 ELISA at 570 nm.

Antibody production test[12] Effect on antibody production was measured by quantitative hemolysin spectrophotometry (QHS) assay. The mice were challenged by ip 5 % sheep red blood cell (SRBC) 0.2 mL per mouse on d 0. CHC-1 25 and 50 mg/kg were injected ip × 4 d to the mice and the mice were killed on d 5 as above mentioned. Fresh SRBC was washed thrice with PBS (pH = 7.2) and diluted at 1∶20. Spleen cell suspensions (2×10^{10} cells/L) were prepared. Cell suspensions 1 mL, SRBC 1 mL and serum of guinea-pig 1 mL were mixed, and incubated at 37 ℃ for 1.5 h and then centrifuged. Absorbance is preferred.

Serum IgG levels were measured by single immunodiffusion method. The diameters of samples in a rabbit anti-mouse serum plate diffusion ring were measured.

Lymphocyte proliferation test[13,14] ***in vitro*** The polysaccharide samples (0.001 – 0.1 g/L) were incubated with mouse splenocytes as described before. The absorbence was measured also by DG-3022 ELISA at 570 nm.

RESULTS

Structure studies

Isolation and purification of CHC-1 The crude polysaccharide CHC was extracted from *Cuscuta* semens by extraction with NaOH 1 mol/L. The CHC-1 was isolated from CHC by DEAE anion-exchange chromatography, then purified by gel filtration chromatography, dialyzed against distilled water, and lyophilized. In accordance with the method of the reference[8], CHC-1 was eluted as a single symmetrical peak corresponding to a molecular mass of more than 1.0×10^6 as determined by HPLC method, which indicated that the polysaccharide was homogeneous.

Monosaccharide constituents of CHC-1 The composition of the CHC-1 (Tab 1), determined by TLC analysis and GLC analysis as alditol acetate derivatives, indicated that it was composed of rhamnose, arabinose, and galactose in the molar ratio of 0.7∶1.0∶1.5. The absorption of IR at 1735.6 cm^{-1} and the result of TLC both proved that CHC-1 contained uronic acid. GLC

Tab 1. Sugar analysis of CHC-1.

Sugar	R_t^a (min)	Molar ratio	
		Native CHC-1	Reduced CHC-1
Rha	6.82	0.7	0.8
Ara	8.52	1.0	1.0
Gal	21.25	1.5	1.8
GalA	–	0.3	–

[a] Retention time (min) on a 5 % OV225/AW-DMC-chromosorb W column (80 – 100 mesh)

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analysis of the reduced CHC-1 showed rhamnose, arabinose, and galactose in the molar ratio of 0.8∶1.0∶1.8, which further confirmed it containing galacturonic acid. As a result, the relative proportions of rhamnose, arabinose, galactose, and galacturonic acid is 0.8∶1.0∶1.5∶0.3.

Linkage analysis of the CHC-1 Methylation analysis of the CHC-1 (Tab 2), together with ^{13}C NMR signals of anomeric carbons (Tab 3), showed that the galactose and rhamnose residues were in the pyranosidic form (Galp, Rhap) and the arabinose residues were in the furanosidic form (Araf). The residues were identified as 2,4-disubstituted Rhap, 5-substituted Araf, 3,5-disubstituted Araf, terminal Araf, 4-substituted Galp, 6-substituted Galp, and 3, 6-disubstituted Galp, respectively. When the polysaccharide was reduced with $NaBH_4$ and then methylated, subsequent analysis revealed that the components were the same as those in the former, but the proportion of 4-substituted Galp increased. Thus this residue existed partly in the polysaccharide as 4-substituted GalpA (A = uronic acid). Therefore, CHC-1 was a highly branched molecule with branch! points at 3,5-disubstituted Araf, 3,6-disubstituted Galp, and 2,4-disubstituted Rhap residues.

Tab 2. Methylation analysis of CHC-1.

Sugar	Molar ratio		Mass fragments (m/z)
	Native CHC-1	Reduced CHC-1	
3-Me Rha	11.98	10.35	43, 87, 101, 129, 143, 189,203
2,3,5-Me_3 Ara	10.50	11.42	43,71,87,101,117,129, 161
2,3-Me_2 Ara	11.29	13.83	43,87,101,117,129,189
2-Me Ara	5.70	3.34	43, 85, 117, 127, 159, 201,261
2,3,4,6-Me_4 Gal	6.26	4.20	43, 101, 117, 129, 145, 161,205
2,3,6-Me_3 Gal	11.55	19.30	43,71,87,101,117,131, 173,233
2,3,4-Me_3 Gal	17.22	17.64	43,71,87,101,117,129, 161,189,233
2,4-Me_2 Gal	10.54	11.06	43,71,87,117,129,159, 189,233

Tab 3. Characteristic signals of ^{13}C NMR of CHC-1.

Chemical shift (ppm)	Carbon assignment
176.77	–COOH of GalA
109.56 – 111.39	C-1 of α-*L*-Araf
104.75 – 106.50	C-1 of β-*D*-Galp
100.60	C-1 of α-*L*-Rhap
71.67	CH_2OH of 6-subsituted Gal or 5-substituted Ara
66.97	CH_2OH of 6-subsituted Gal or 5-substituted Ara
63.27	CH_2OH of Ara or Gal
19.08	CH_3 of Rha

NMR studies ^1H NMR signal at 1.35 ppm deduced the presence of Rhamnose. As 1D NMR spectrum showed complex signals in anomeric region, it was difficult to assign these signals. According to the references[9-11], ^{13}C NMR signals for anomeric carbons at 109.56 – 111.39, 104.75 – 106.50, 100.60 ppm showed that the configuration of the residues in the CHC-1 was α-*L*-Araf, β-*D*-Galp, and α-*L*-Rhap, respectively. Due to the poor resolution of the spectra and severe overlaps, it was difficult to assign all the carbon signals without any ambiguities. However, some chemical shifts of the residues were assigned (Tab 3) on combining the data from the former analysis.

Immunological activities CHC-1 25 mg/kg increased the body weight from (23 ± 1) g to (25 ± 2) g ($P < 0.05$) and spleen weight from 6.5 ± 0.7 mg/g to (11.8 ± 0.6) mg/g ($P < 0.001$). CHC-1 50 mg/kg only increased spleen weight from (6.7 ± 0.8) mg/g to (13.9 ± 1.7) mg/g body weight ($P < 0.01$). No marked changes in thymus weight in CHC-1 treated groups were observed.

Effect on antibody production CHC-1 25, 50 mg/kg elevated antibody production of spleen significantly (Tab 4).

Tab 4. Effect of ip CHC-1 on antibody formation *in vivo* by quantitative hemolysin spectrophotometry (QHS). $n = 6$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Dose (mg/kg)	*A*	IgG (g/L)
0 (Control)	54.4 ± 0.9	13.1 ± 4.1
25	62.9 ± 1.1^c	14.0 ± 1.7
50	62.6 ± 1.7^b	16.0 ± 1.5

No marked effect of CHC-1 was found on IgG level in serum (Tab 4).

Effect on lymphocyte proliferation CHC-1 promoted the proliferation of T-cells induced by ConA and the proliferation of B-cells induced by LPS. The results suggest that CHC-1 increase the proliferation rate of lymphocytes in the presence of mitogens (Tab 5).

Tab 5. Effect of ip CHC-1 on lymphocyte proliferation (MTT assay) in mice. $n = 6$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* Control.

Dose (mg/kg)	Absorbance T-cells	B-cells
0(Control)	0.30 ± 0.02	0.42 ± 0.01
25	0.44 ± 0.03^b	0.53 ± 0.01^c
50	0.47 ± 0.03^c	0.56 ± 0.03^b

In vitro test CHC-1 (0.001 – 0.1 g/L) was incubated with mouse splenocytes as described before. CHC-1 (0.01 – 0.1 g/L) had a promotive effect on T- and B-lymphocyte proliferation *in vitro* (Tab 6).

Tab 6. Effect of CHC-1 on proliferation of T- and B-cells by MTT assay *in vitro*. $n = 6$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* Control.

Dose (g/L)	Absorbance T-cells	B-cells
0(Control)	0.42 ± 0.02	0.43 ± 0.04
0.001	0.45 ± 0.02^b	0.94 ± 0.04^c
0.01	0.69 ± 0.02	0.71 ± 0.02
0.1	0.82 ± 0.02^b	0.82 ± 0.03^c

DISCUSSION

Based on the previous study, CHC-1 has been found to be a complex hetero-polysaccharide. It is composed of β-*D*-Gal, β-*D*-GalA, α-*L*-Ara, and α-*L*-Rha. The linkages of these residues were identified as 2,4-disubstituted Rhap, 5-substituted Araf, 3,5-disubstituted Araf, terminal Araf, 4-substituted Galp, 6-substituted Galp, 3, 6-disubstituted Galp, and 4-substituted GalpA, respectively. According to its components, CHC-1 belongs to the pectic polysaccharides. Usually, the structures of this kind of polysaccharides are very complicated.

CHC-1 increased antibody production, and raised ConA-induced T-lymphocyte proliferation and LPS-induced B-lymphocyte proliferation at 25 mg/kg and 50 mg/kg *in vivo*. Moreover, CHC-1 had a direct effect on T- and B-lymphocyte proliferation *in vitro*. These results indicate that CHC-1 is an immuno-stimulator. Since deficiency of immune function results in many diseases such as tumor, infection, aging, *etc*, it is of significance to further investigate the bio-activity of CHC-1. Moreover, it can be considered that many activities of *Cuscuta chinensis* Lam may be related to the immune enhancing activities of the polysaccharides, which it contains.

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酸性菟丝子多糖的化学特征和免疫活性

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关键词 菟丝子；多糖；核磁共振；T-淋巴细胞；B-淋巴细胞；抗体

目的：研究从菟丝子（*Cuscuta chinensis* Lam）中提取分离的酸性纯多糖CHC-1的结构特征及免疫活性．**方法**：利用化学方法和NMR光谱分析方法对CHC-1的结构特征进行了解析，并研究了CHC-1对小鼠抗体生成以及对淋巴细胞增殖率的影响．**结果**：CHC-1的分子量大于1.0×10^6．糖组份分析结果显示CHC-1是由Rha，Ara，Gal和GalA组成，比例为：0.8:1.0:1.5:0.3．甲基化结果和^1H，^{13}C NMR分析进一步确定了CHC-1中各残基的连接方式．CHC-1 0.1 g/L能在体外明显升高T，B细胞的增殖率，CHC-1 25，50 mg/kg显著增加小鼠的脾重，淋巴细胞的增殖率以及促进抗体生成．**结论**：CHC-1为一种多分支的杂多糖，并具有免疫增强活性．

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