ISSN 1671-4083 Acta Pharmacol Sin 中国药理学报 2002 Feb; 23 (2): 162 - 166
http://www.chinaphar.com

Structural characterization and biological activities of SC4, an acidic polysaccharide from *Salvia chinensis*

R96 A

LIU Cui-Ping, FANG Ji-Nian[1], LI Xiao-Yu, XIAO Xiao-Qiu (*Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China*)

KEY WORDS *Salvia chinensis*; polysaccharides; molecular structure; T-lymphocytes; B-lymphocytes; PC12 cells; biomolecular nuclear magnetic resonance

ABSTRACT

AIM: To study the chemical characterization and some biological activities of an acidic polysaccharide, named SC4 from *Salvia chinensis*. **METHODS**: Chemical and spectral methods were employed to identify the structural characterization of SC4. Effect of SC4 on the proliferation of T- and B-lymphocytes both *in vivo* and *in vitro* was measured at various concentrations, and the protective effects of SC4 on PC12 cells against H_2O_2-induced injury was observed. **RESULTS**: SC4 was a highly branched polysaccharide with mean molecular weight of 4.5×10^5, composed of Rha, Xyl, Ser, Gal, and GalA in the molar ratio of 1.0:7.0:5.3:1.2:4.2. Methylation analysis and NMR identified the linkages of the residues of SC4. SC4 increased the B-lymphocyte proliferation and spleen weight remarkably while no obvious changes were examined in T-lymphocyte proliferation, thymus weight, and the level of IgG and C3 in blood of mice. Moreover, SC4 enhanced the PC12 cells viability after H_2O_2 treatment. **CONCLUSION**: SC4, the acidic polysaccharide with complicated structure, was a B-lymphocyte stimulator and protected PC12 cells at the concentration of 20 mg/L against H_2O_2-induced injury.

INTRODUCTION

Salvia chinensis Benth, a plant belonging to labiatae, is widely distributed in China, including Hubei, Sichuan, Guangxi, Guangdong, Hunan, and other provinces in eastern China. It has been used as a folk medicine for many years to treat cancer of the esophagus, phlegm panting, hepatitis, and red and white vaginal discharge, *etc*[1]. Up to now, many studies had been reported on the sterols, triterpenes, and phenolic acid from the plant[2-4]. However, its polysaccharides had been little studied. In this paper, an acidic polysaccharide, SC4, had been isolated from the aqueous-extract of *Salvia chinensis* Benth, and its immunological properties and protective function on PC12 cells against H_2O_2-induced injury were investigated.

MATERIALS AND METHODS

Materials Dried crude drug of *Salvia chinensis* Benth was purchased from Shanghai Corporation of Chinese Traditional Drugs and identified by Prof CHEN Ke-Li in Department of Pharmacy, Hubei College of Traditional Chinese Medicine. Reagents: 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulphonate (CMC) and trifluoroacetic acid (TFA) were obtained 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) was from Fluka. Medium RPMI-1640 was purchased from Gibco Laboratories.

Preparation of SC4 The crude drug of *Salvia chinensis* (2.0 kg) was defatted with 95 % alcohol and then extracted for 4 h with 3 × 60 volumes of hot water (80 - 90 ℃). The combined aqueous extract was deproteinated with trichloroacetic acid. After centrifugation, the supernatant solution was intensively dialyzed against running water for 3 d and then against distilled water for 1 d. The nondialyzable fraction was concentrated to 2.0 L under depressed pressure and 4 volumes of 95 % alcohol was added slowly and stirred at 4 ℃. Then the mixture was stored overnight at -10 ℃. The resulting precipitate was vacuum-dried at 40 ℃ and gave a dark brown powder of 40 g. A portion

[1] Correspondence to Prof FANG Ji-Nian.
Phn 86-21-6431-1833, ext 601. Fax 86-21-6437-0269.
E-mail jnfang@mail.shcnc.ac.cn
Received 2001-06-22 Accepted 2001-11-16

of the crude polysaccharide 10 g was applied to DEAE-cellulose column chromatography and eluted with water, followed by NaCl solution 0.2 and 0.3 mol/L. Based on the colorimetric test for total sugar with the phenol-H_2SO_4 method, the fraction eluted with NaCl solution 0.2 mol/L was further fractionated with alcohol of different concentrations. The resulting fraction obtained with alcohol 65 % was subjected to Sephacryl™ S-300 gel filtration chromatography repeatedly to give a polysaccharide, SC4 0.15 g. After eluted with NaCl 0.2 mol/L, whose homogeneity was indicated by high performance gel permeation chromatography (HPGPC) described below.

Determination of the homogeneity and molecular weight[5,6] The homogeneity was indicated by HPGPC as a single symmetric peak on Ultrahydrogel™ 500 and 2000 serial columns. Dextran T-series (T-700, 580, 500, 110, 80, 70, 40, 11, 9.3) were used to pre-calibrate the Waters LC system, fitted with 515 HPLC pump, in line degasser, 2410 RI detector, and Millennium^{32} software (version 3.05.01). NaAc 0.003 mol/L was employed as mobile phase at the flow rate of 0.5 mL/min. The column temperature was maintained at (30.0±0.1) ℃.

Sugar composition analysis SC4 5 mg was dissolved in 4 mL TFA 2 mol/L and hydrolyzed at 110 ℃ for 2 h. The resulting monosaccharides were analyzed by thin layer chromatography (TLC), then reduced and converted to the corresponding alditol acetates as described by Blakenney *et al*[7]. Analytical gas chromatography (GC) was performed with corresponding alditol acetates of standard samples as standards on a Shimadzu-9A gas chromatograph, equipped with a 5 % OV 225/AW-DMC-Chromosorb W (80-100 mesh) column.

Reduction of uronic acid[8] SC4 20 mg was dissolved in 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 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. The borohydride solution 25 mL was usually required for the reduction, which was completed in 60 min.

Methylation analysis Glycosyl linkages were determined using the methylation method described by Needs *et al*[9]. Per-methylated products were then hydrolyzed with TFA 2 mol/L at 110 ℃ for 4 h. The resulting hydrolysate was reduced and acetylated. The partially methylated alditol acetates were subjected to GC-MS analysis. The GC-MS spectra were obtained using a QP Class-5000 instrument (Shimadzu, Japan).

NMR analysis NMR spectrum was recorded with a DRX 400 spectrometer (Bruker Co Ltd, Switzerland) at room temperature in D_2O.

Lymphocyte proliferation test *in vivo*[10,11] ICR (♀) mice from Shanghai Experimental Animal Center (Grade Ⅱ, Certificate № 153) with body weight of 20 g ± *s* 2 g were divided randomly into three groups: control group (A), SC4 12.5 mg/kg group (B), SC4 25 mg/kg group (C), ip × 4 d, respectively. The mice were killed on d 5. The weight of body, spleen, and thymus was measured, respectively. The spleen cells were suspended to a final density of 5×10^9/L in RPMI-1640 medium supplemented with HEPES buffer 10 mmol/L, benzylpenicillin 100 kU/L, streptomycin 100 mg/L, *L*-glutamine 2 mmol/L, 2-mercaptomethanol 50 μmol/L, and 10 % fetal bovine serum, pH 7.2. 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 the humidified atmosphere containing 5 % CO_2 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 (*N*, *N*-Dimethyl formamide 50 % + sodium dodecysulfate 10 %, 100 μL/well) was added. The absorbance was measured by DG-3022 ELISA at 570 nm.

Lymphocyte proliferation test *in vitro*[10,11] The polysaccharide samples (0.001, 0.01, and 0.1 g/L) were incubated with mouse splenocytes as described above. The absorbance was measured by DG-3022 ELISA at 570 nm.

Antioxidant test on PC12 cells against H_2O_2 *in vitro*[12] PC12 cells were high passages from ATCC (American Type Culture Collection) and maintained at 37 ℃ in a humidified atmosphere containing 5 % CO_2. Cells were seeded into multi-well plates (Greiner) at a density of 2×10^7/L in phenolsulfonphthalein (phenol red) free RPMI-1640 medium (Gibco), supplemented with 10 % heat-inactivated bovine calf serum, penicillin 100 kU/L, streptomycin 100 mg/L, and *L*-glutamine 2 mmol/L. All experiments were carried out for 24-48 h after cells were seeded. Hydrogen peroxide (H_2O_2) 8.8

ISSN 1671-4083 Acta Pharmacol Sin 中国药理学报 2002 Feb; 23 (2)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

mol/L was stored at 4 ℃ until stock solutions 100 mmol/L were prepared in phosphate buffered saline (PBS) immediately before use. H_2O_2 100 mmol/L was further diluted for adding into the cultures. SC4 was dissolved and diluted with PBS. The PC12 cells were pre-incubated with SC4 for 2 h before H_2O_2 was added.

Cell survival was evaluated by MTT reduction at 6 h post-treatment with H_2O_2. MTT stock solution in PBS was added till the final concentration was up to 0.5 g/L. The incubation was continued for 4 h. Finally, an equal volume of a solution containing 50 % dimethylformamide and 20 % SDS, pH 4.8, was added. The mixtures were kept overnight and then the amount of MTT formazan was qualified by determining the absorbance at 570 nm and 630 nm using Universal Microplate Reader (Bio-Tek).

Data were evaluated for statistical significance with one-way ANOVA followed by Ducan's multiple range test using a computerized statistical package named STATPAK 4.11 (Northwest Analytical Inc, Portland, Oregon, USA).

RESULTS

Isolation and purification of SC4 The acidic polysaccharide, SC4, was isolated from the aqueous extract by DEAE anion-exchange chromatography with NaCl 0.2 mol/L as eluent, then purified by gel filtration chromatography repeatedly, dialyzed against distilled water, and lyophilized. The polysaccharide was eluted out as a single symmetric peak on Ultrahydrogel™ 500 and 2000 serial columns, corresponding to a molecular weight of 4.5×10^5, which suggested SC4 to be homogeneous.

Composition of SC4 The composition of SC4, determined by TLC[13] and GC as alditol acetates, indicated that SC4 consisted of Rha, Xyl, Ser, and Gal in the molar ratio of 1.0∶7.0∶5.3∶1.2. The strong absorbance in IR (Infrared) spectrum of SC4 at 1747.2 cm^{-1} and TLC analysis confirmed that SC4 contained uronic acid. GC analysis of reduced SC4 showed the presence of Rha, Xyl, Ser, and Gal in the molar ratio of 1.0∶7.0∶5.3∶5.4, which further revealed that SC4 contained galacturonic acid. As a result, SC4 was composed of Rha, Xyl, Ser, Gal, and GalA in the molar ratio of 1.0∶7.0∶5.3∶1.2∶4.2 (Tab 1).

Tab 1. Glycosyl-residue composition of SC4.

Glycosyl residue	Rt/min	Molar ratio	
		Native SC4	Reduced SC4
Rha	6.59	1.0	1.0
Xyl	8.55	7.0	7.0
Ser	16.77	5.3	5.3
Gal	22.84	1.2	5.4
GalA	-	4.2	

Linkage analysis of SC4 Methylation analysis of reduced SC4, together with ^{13}C NMR spectrum of SC4 showed that the rhamnosyl, xylosyl, and galactosyl residues were all in pyronosidic form and identified as terminal Rhap, 1,2- and 1,3-disubstituted Xylp, 1,4-disubstituted Galp, 1,3,4- and 1,3,6-trisubstituted Galp, respectively. Owing to the high amount of GalA in SC4, the methylation of native SC4 was not successful. However, it was possible to deduce the linkages of GalA according to the methylation analysis of reduced SC4. The linkages of Gal in reduced SC4 might include those of Gal and GalA. That is to say, Gal might exist in the form of 1,4-disubstituted Galp, 1,3,4- and 1,3,6-trisubstituted Galp, and GalA had the linkages of 1, 4-disubstituted GalAp, 1, 3, 4- and 1, 3, 6-trisubstituted GalAp (Tab 2).

Tab 2. Methylation analysis of reduced SC4.

Methylated sugars (as alditol acetates)	Type of linkage	Rt[1]/min	Molar ratio	Mass fragments (m/z)
2,3,4-Me3 Rha	1-linked Rhap	14.12	1.0	43,58,71,89,101,117,131,161,175
3,4-Me_2 Xyl	1,2-linked Xylp	15.97	0.8	43,59,71,87,101,117,159,161,189
2,4-Me_2 Xyl	1,3-linked Xylp	16.09	1.4	43,58,85,99,101,117,159
2,3,6-Me_3 Gal	1,4-linked Galp	16.71	1.1	43,71,87,99,101,113,117,129,161,173,233
2,4-Me_2 Gal	1,3,6-linked Galp	17.69	1.3	43,58,87,99,101,117,129,139,148,159,189,233
2,6-Me_2 Gal	1,3,4-linked Galp	18.29	1.0	43,58,87,97,99,117,129,143,159,185,203,231,305

1) obtained on an OV-17 column (30 m × 0.25 mm) at the line velocity of 26 ℃/s.

NMR studies Due to the severe overlaps of signals of carbons in the ^{13}C NMR spectrum of SC4, it was difficult to assign all signals without any ambiguities. According to the references, the signals at δ 176.3 and 173.4 in ^{13}C NMR revealed the presence of uronic acid and serine, assigned to the carbons of C = O of galacturonic acid and serine, respectively. In anomeric region, the signals at δ 106.4 – 106.3 and 102.1 – 101.5 indicated that the configurations of the residues in SC4 were β-*D*-Galp and β-*D*-GalAp, α-*L*-Xylp and α-*L*-Rhap, respectively. Based on the analysis above, some chemical shifts were assigned (Tab 3).

Tab 3. Characteristic signals in ^{13}C NMR of SC4.

Chemical shift	Carbon
176.3	-COOH of GalA
173.4	-COOH of Ser
106.4 – 106.3	C-1 of β-*D*-Galp and β-*D*-GalAp
102.1 – 101.5	C-1 of α-*L*-Xylp and Rhap
68.0	C-6 of 6-substituted β-*D*-Galp
55.8	*N*-substituted C of Ser
19.3	CH_3 of Rha

Lymphocyte proliferation *In vivo*, SC4 promoted the proliferation of B-cells induced by LPS remarkably while inhibited the proliferation of T-cells induced by ConA at 25 mg/kg (Tab 4). In addition, SC4 had no obvious promotive effect on T- and B-lymphocyte proliferation *in vitro* (Tab 5). It was suggested that the function of SC4 *in vivo* was different from that *in vitro* and SC4 inhibited cellular immunity but promoted humoral immunity. In addition, SC4 12.5 and 25 mg/kg increased the spleen weight (Tab 4) while did not affect the body weight, thymus weight, and the level of IgG and C3 in the blood of mice obviously.

Protective effect of SC4 on PC12 cells against H_2O_2 induced injury Active mitochondria of living cells can cleave MTT to produce formazan, the amount of which is correlated directly to the living cell number. Cell viability as determined by MTT reduction was markedly decreased after PC12 cultures were exposed to H_2O_2 200 μmol/L for 6 h (Tab 6), suggesting that PC12 cells were very sensitive to H_2O_2 injury. However, pretreatment with SC4 at the concentration of 20 mg/L enhanced the viability of PC12 cells *vs* H_2O_2 treated group. However, when the concentration is up to 40 mg/L, as shown in Tab 6, no obvious change in PC12 viability was observed in comparison with H_2O_2 treated group.

DISCUSSION

According to the present study, SC4 is composed of α-*L*-Xylp, α-*L*-Rhap, β-*D*-Galp, β-*D*-GalAp, and Ser with the linkages of 1-linked α-*L*-Rhap, 1,2- and 1,3-linked α-*L*-Xylp, 1,4- and 1,3,4-linked β-*D*-Galp and (or) β-*D*-GalAp, and 1, 3, 6-linked β-*D*-Galp, respectively. It belongs to the pectic polysaccharides of complicated structure. Serine-containing polysaccharide is not very common in plant, although serine is usual in the structural unit of chondroitin, heparin, and proteoglycan[14,15], *etc*. However, since the components with small molecules from *Salvia chinensis* are nearly all acidic, the serine-containing polysaccharide from the plant might have something to do with the acidic small molecules in the process of biological synthesis of these components.

As described above, SC4 inhibited the proliferation of T-lymphocyte cells at 25 mg/kg but promoted that of B-lymphocyte cells remarkably, revealing that SC4 was a B-lymphocyte cells stimulator. Moreover, SC4 protected PC12 cells obviously at the concentration of 20 mg/L against H_2O_2-induced injury while had no effect when the concentration increased, revealing that the protective function of polysaccharides on PC12 cells against H_2O_2-induced injury was closely related to sample concentrations.

Tab 4. Effect of ip SC4 on lymphocyte proliferation by MTT assay in mice. $n = 6$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

Dose/mg·kg^{-1}	A_{570} T-cells	A_{570} B-cells	Spleen weight (mg per 10 g)	Body weight/g
0 (Control)	0.62 ± 0.09	0.39 ± 0.02	70 ± 6	22 ± 2
12.5	0.46 ± 0.07	0.75 ± 0.02^c	126 ± 14^c	23 ± 1
25	0.21 ± 0.01^c	0.63 ± 0.02^c	151 ± 19^c	24 ± 2

ISSN 1671-4083 Acta Pharmacol Sin 中国药理学报 2002 Feb; 23 (2)
E-mail aps@mail.shcnc.ac.cn Phn/Fax 86-21-6474-2629

Tab 5. Effect of SC4 on proliferation of T- and B-cells by MTT assay *in vitro*. $n=6$. $\bar{x}\pm s$. $^bP<0.05$ *vs* control.

Concentration/ g·L^{-1}	A_{570} T-cells	B-cells
0 (Control)	0.542 ± 0.012	0.57 ± 0.03
0.001	0.593 ± 0.019^b	0.541 ± 0.010
0.01	0.518 ± 0.025	0.47 ± 0.05
0.1	0.560 ± 0.029	0.57 ± 0.03

Tab 6. Protective effect of SC4 against H_2O_2-induced injury in PC12 cells. $n=6$. $\bar{x}\pm s$. $^cP<0.01$ *vs* H_2O_2 group.

	Concentration	Absorbance
Control		0.2932 ± 0.013
H_2O_2	200 μmol/L	0.1154 ± 0.010
H_2O_2 + SC4	200 μmol/L + 20 mg/L	0.2028 ± 0.015^c
H_2O_2 + SC4	200 μmol/L + 40 mg/L	0.1470 ± 0.017

REFERENCES

1 Jiangsu New Medical College. Dictionary of Chinese Materia Medica. Shanghai: Shanghai Science and Technology Press; 1995. p 597-8.
2 Xu MJ, Lin YL, Shi JC, Cheng PY. Studies on the chemical compositions of *Salvia chinensis*. Chin Trad Herb Drugs 1987; 18: 46-6.
3 Qian TX, Li LN. Isosalvianolic acid C, a depside possessing a dibenzooxepin skeleton. Phytochemistry 1992; 31: 1068-70.
4 Qian TX, Yan ZH, Li LN. Mono-feruloyl-*R*, *R*-(+)-tartaric acid from *Salvia chinensis*. J Chin Pharm Sci 1993; 2: 148-50.
5 Wei YA, Fang JN. To determine the homogeneity and molecular weight of polysaccharides by HPGPC. Acta Pharm Sin 1989; 24: 532-6.
6 Alsop RM, Vlachogiannis GJ. Determination of the molecular weight of clinical dextran by gel chromatography on TSK PW TYPE columns. J Chromatogr 1982; 246: 227-40.
7 Blakenney AB, Harris PJ, Henry RJ, Stone BA. A simple and rapid preparation of alditol acetates for monosaccharide analysis. Carbohydr Res 1983; 113: 291-9.
8 Taylor RL, Conrad HE. Stoichiometric depolymerization of polyuronides and glycosaminoglycuronans to monosaccharides following reduction of their carbodiimide-activated carboxyl groups. Biochemistry 1972; 11: 1383-8.
9 Needs PW, Selvendran RR. Avoiding oxidative degradation during sodium hydroxydimethyloidine-mediated carbohydrate methylation in dimethyl sulfoxide. Carbohydr Res 1993; 245: 1-10.
10 Heek K, Reimann J, Kabelitz D, Hardt C, Wagner H. A rapid colorimetric assay for the determination of IL-2-producing helper T cell frequencies. J Immunol Methods 1985; 77: 237-46.
11 Zhou DH, Shen YS, Zhao MR. The application of MTT colorimetric assay to measure the proliferation of lymphocytes and the activities of rat/mouse IL-2. Chin J Immunol 1986; 2: 39-46.
12 Xiao XQ, Yang JW, Tang XC. Huperzine A protects rat pheochromocytoma cells against hydrogen peroxide-induced injury. Neurosci Lett 1999; 275: 73-6.
13 Liu CP, Dong Q, Fang JN. New simple method to detect uronic acids in polysaccharides. Chin Trad Herb Drugs 2001; 32: 404-7.
14 AmorPA, Yethon JA, Monteiro MA, Whitfield C. Assembly of the K40 antigen in *Escherichia coli*: identification of a novel enzyme responsible for addition of *L*-serine residues to the glycan backbone and its requirement for K40 polymerization. J Bacteriol 1999; 181: 772-80.
15 Yamada S, Sakamoto K, Tsuda H, Yoshida K, Sugiura M, Sugahara K. Structural studies of octasaccharides derived from the low-sulfated repeating disaccharide region and octasaccharide serines derived from the protein linkage region of porcine intestinal heparin. Biochemistry 1999; 38: 838-47.

石见穿酸性多糖 SC4 的结构特征和生物活性

刘翠平，方积年[1]，李晓玉，萧晓秋
(中国科学院上海生命科学研究院上海药物研究所，上海 200031，中国)

关键词 石见穿；多糖类；分子结构；T 淋巴细胞；B 淋巴细胞；PC12 细胞；活质分子核磁共振

目的：研究从石见穿(*Salvia chinensis*)中得到的酸性多糖 SC4 的化学结构和生物活性. **方法**：利用化学和光谱方法分析了 SC4 的结构特征，并观察了 SC4 对淋巴细胞增殖的影响以及增强 PC12 细胞对抗 H_2O_2 造成的损伤的作用. **结果**：SC4 的分子量为 4.5×10^5，由 Rha, Xyl, Ser, Gal 和 GalA 组成，摩尔比为 1.0:7.0:5.3:1.2:4.2. 甲基化和 ^{13}C NMR 进一步确定了 SC4 中各糖残基的连接方式. SC4 能显著促进 B 淋巴细胞的增殖，增加小鼠脾重，增强 PC12 细胞对抗 H_2O_2 造成的氧化损伤的能力. **结论**：SC4 为一多分枝的酸性多糖，其免疫活性的靶细胞为 B 淋巴细胞，并显著增强 PC12 细胞对抗 H_2O_2 造成的损伤的能力.

(责任编辑 朱倩蓉)