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

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**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$_2$O$_2$-induced injury was observed. **RESULTS:** SC4 was a highly branched polysaccharide with mean molecular weight of $4.5 \times 10^6$, 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$_2$O$_2$ 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$_2$O$_2$-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 paining, hepatitis, and red and white vaginal discharge, etc. 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$_2$O$_2$-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-morpholinooethyl) carbodiimide metho-$p$-toluenesulphonate (CMC) and trifluoroacetic acid (TFA) were obtained from Merck Co. Concanaavalin 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 x 60 volumes of hot water ($80 - 90$ °C). The combined aqueous extract was deproteinized 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 °C. Then the mixture was stored overnight at -10 °C. The resulting precipitate was vacuum-dried at 40 °C and gave a dark brown powder of 40 g. A portion
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\textsuperscript{TM} S-300
gel filtration chromatography repeatedly to give a polysac-
charide, 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\textsuperscript{3,4,5,6}** The homogeneity was indicated by
HPGPC as a single symmetric peak on UltraHydrogel\textsuperscript{TM} 500 and 2000 serial columns. Dextran
T-series (T-700, 500, 500, 110, 80, 70, 40, 11, 9.3)
were used to pre-calibrate the Waters LC gas system, fitted
with 515 HPLC pump, in line degasser, 2410 RI
detector, and Millennium\textsuperscript{TM} 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) °C.

**Sugar composition analysis** SC4 5 mg was
dissolved in 4 mL TFA 2 mol/L and hydrolyzed at
110 °C 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 Blakenery et al\textsuperscript{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\textsuperscript{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
takeup 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\textsuperscript{9}. Per-methylated products were then
hydrolyzed with TFA 2 mol/L at 110 °C 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\textsuperscript{10-11}** ICR (\(\frac{\text{♂}}{\text{♀}}\)) mice from Shanghai Experimental Animal
Center (Grade II; Certificate No 153) with body weight
of 20 g ± 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 x 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 x 10^6/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-mercaptoethanol 50
mmol/L, and 10% fetal bovine serum, pH 7.2. Cells
(100 \(\mu\)L/well) were added into 96-well plate in the
presence of ConA (5 mg/L) or LPS (25 mg/L). After
incubation at 37 °C in the humidified atmosphere
containing 5% CO_2 for 44 h, T- and B-lymphocyte
proliferation was tested by MTT (5 g/L, 20 \(\mu\)L/well)
assay. The plate was incubated for another 4 h and then
the color was measured (\(N, N\)-Dimethyl formamide 50 % + sodium
dodecylsulfate 10 %, 100 \(\mu\)L/well) was added. The
absorbance was measured by DG-3022 ELISA at 570 nm.

**Lymphocyte proliferation test in vitro\textsuperscript{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 vivo\textsuperscript{12}**. PC12 cells were high passages from ATCC
(American Type Culture Collection) and maintained at
37 °C in a humidified atmosphere containing 5% CO_2.
Cells were seeded into multi-well plates (Greiner) at a
density of 2 x 10^4/L in phenol-sulfphthalein (phenol
red) free RPMI-1640 medium (Glbo), 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
mol/L was stored at 4°C until stock solutions 100 mmol/L were prepared in phosphate buffered saline (PBS) immediately before use. H₂O₂ 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 H₂O₂ was added.

Cell survival was evaluated by MTT reduction at 6 h post-treatment with H₂O₂. 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 Duncan'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 x 10⁵, which suggested SC4 to be homogeneous.

Composition of SC4 The composition of SC4, determined by TLC[18] and GC as aldito 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).


<table>
<thead>
<tr>
<th>Glycosyl residue</th>
<th>Rt/min</th>
<th>Native SC4</th>
<th>Reduced SC4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rha</td>
<td>6.59</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Xyl</td>
<td>8.55</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Ser</td>
<td>16.77</td>
<td>5.3</td>
<td>5.3</td>
</tr>
<tr>
<td>Gal</td>
<td>22.84</td>
<td>1.2</td>
<td>5.4</td>
</tr>
<tr>
<td>GalA</td>
<td>-</td>
<td>4.2</td>
<td></td>
</tr>
</tbody>
</table>

Linkage analysis of SC4 Methylation analysis of reduced SC4, together with 13C NMR spectrum of SC4 showed that the rhamnlosyl, xylosyl, and galactosyl residues were all in pyranosidic 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 Gal, 1,3, 4- and 1,3,6-trisubstituted GalA (Tab 2).


<table>
<thead>
<tr>
<th>Methylated sugars (as aldito acetates)</th>
<th>Type of linkage</th>
<th>Rt¹ / min</th>
<th>Molar ratio</th>
<th>Mass fragments (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4-Me3 Rha</td>
<td>1-linked Rhap</td>
<td>14.12</td>
<td>1.0</td>
<td>43, 58, 71, 89, 101, 117, 131, 161, 175</td>
</tr>
<tr>
<td>3,4-Me2 Xyl</td>
<td>1,2-linked Xylp</td>
<td>15.97</td>
<td>0.8</td>
<td>43, 59, 71, 87, 101, 117, 159, 161, 189</td>
</tr>
<tr>
<td>2,4-Me2 Xyl</td>
<td>1,3-linked Xylp</td>
<td>16.09</td>
<td>1.4</td>
<td>43, 58, 85, 99, 101, 117, 159</td>
</tr>
<tr>
<td>2,3,6-Me3 Gal</td>
<td>1,4-linked Galp</td>
<td>16.71</td>
<td>1.1</td>
<td>43, 71, 87, 99, 101, 113, 117, 129, 161, 173, 223</td>
</tr>
<tr>
<td>2,4-Me2 Gal</td>
<td>1,3,6-linked Galp</td>
<td>17.69</td>
<td>1.3</td>
<td>43, 88, 97, 99, 117, 129, 143, 159, 185, 203, 231, 305</td>
</tr>
<tr>
<td>2,6-Me2 Gal</td>
<td>1,3,4-linked Galp</td>
<td>18.29</td>
<td>1.0</td>
<td>43, 38, 87, 97, 99, 117, 129, 143, 159, 185, 203, 231, 305</td>
</tr>
</tbody>
</table>

¹) obtained on an OV-17 column (30 m x 0.25 mm) at the line velocity of 26 °C/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 $\delta$ 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 anomic region, the signals at $\delta$ 106.4 - 106.3 and 102.1 - 101.5 indicated that the configurations of the residues in SC4 were $\beta$-D-Galp and $\beta$-D-GalAp, $\alpha$-L-Xylp and $\alpha$-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.

<table>
<thead>
<tr>
<th>Chemical shift</th>
<th>Carbon</th>
</tr>
</thead>
<tbody>
<tr>
<td>176.3</td>
<td>-COOH of GalA</td>
</tr>
<tr>
<td>173.4</td>
<td>-COOH of Ser</td>
</tr>
<tr>
<td>106.4 - 106.3</td>
<td>C-1 of $\beta$-D-Galp and $\beta$-D-GalAp</td>
</tr>
<tr>
<td>102.1 - 101.5</td>
<td>C-1 of $\alpha$-L-Xylp and Rhap</td>
</tr>
<tr>
<td>68.0</td>
<td>C-6 of 6-substituted $\beta$-D-Galp</td>
</tr>
<tr>
<td>53.8</td>
<td>N-substituted C of Ser</td>
</tr>
<tr>
<td>19.3</td>
<td>CH$_3$ of Rhap</td>
</tr>
</tbody>
</table>

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$_2$O$_2$ 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$_2$O$_2$ 200 $\mu$mol/L for 6 h (Tab 6), suggesting that PC12 cells were very sensitive to H$_2$O$_2$ injury. However, pretreatment with SC4 at the concentration of 20 mg/L enhanced the viability of PC12 cells vs H$_2$O$_2$ 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$_2$O$_2$ treated group.

DISCUSSION

According to the present study, SC4 is composed of $\alpha$-L-Xylp, $\alpha$-L-Rhap, $\beta$-D-Galp, $\beta$-D-GalAp, and Ser with the linkages of $1$-linked $\alpha$-L-Rhap, $1,2$- and $1,3$-linked $\alpha$-L-Xylp, $1,4$- and $1,3,4$-linked $\beta$-D-Galp and (or) $\beta$-D-GalAp, and $1,3,4$-linked $\beta$-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$_2$O$_2$-induced injury while had no effect when the concentration increased, revealing that the protective function of polysaccharides on PC12 cells against H$_2$O$_2$-induced injury was closely related to sample concentrations.

Tab 4. Effect of ip SC4 on lymphocyte proliferation by MTT assay in mice. $n=6$. $x \pm s$. $P<0.01$ vs control.

<table>
<thead>
<tr>
<th>Dose/mg·kg$^{-1}$</th>
<th>T-cells $A_{530}$</th>
<th>B-cells $A_{530}$</th>
<th>Spleen weight (mg per 10 g)</th>
<th>Body weight/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (Control)</td>
<td>0.62 ± 0.09</td>
<td>0.39 ± 0.02</td>
<td>70 ± 6</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>12.5</td>
<td>0.46 ± 0.07</td>
<td>0.75 ± 0.02</td>
<td>120 ± 14</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>25</td>
<td>0.21 ± 0.01$^a$</td>
<td>0.63 ± 0.02</td>
<td>151 ± 19$^a$</td>
<td>24 ± 2</td>
</tr>
</tbody>
</table>
#### Tab 5. Effect of SC4 on proliferation of T- and B-cells by MTT assay in vitro. n = 6. x ± s. \( P < 0.05 \) vs control.

<table>
<thead>
<tr>
<th>Concentration/g·L⁻¹</th>
<th>T-cells</th>
<th>B-cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control (0)</strong></td>
<td>0.542 ± 0.012</td>
<td>0.57 ± 0.03</td>
</tr>
<tr>
<td>0.001</td>
<td>0.593 ± 0.014</td>
<td>0.541 ± 0.010</td>
</tr>
<tr>
<td>0.01</td>
<td>0.518 ± 0.025</td>
<td>0.47 ± 0.05</td>
</tr>
<tr>
<td>0.1</td>
<td>0.560 ± 0.029</td>
<td>0.57 ± 0.03</td>
</tr>
</tbody>
</table>

#### Tab 6. Protective effect of SC4 against H₂O₂-induced injury in PC12 cells. n = 6. x ± s. \( P < 0.01 \) vs H₂O₂ group.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.2032 ± 0.013</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>0.1154 ± 0.010</td>
</tr>
<tr>
<td>H₂O₂ + SC4</td>
<td>0.2028 ± 0.015</td>
</tr>
<tr>
<td>H₂O₂ + SC4</td>
<td>0.1470 ± 0.017</td>
</tr>
</tbody>
</table>

### REFERENCES

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### 石见穿糖酸性多糖 SC4 的结构特征和生物活性

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**关键词** 石穿糖；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 H₂O₂ 造成的损伤的作用。**结果**：SC4 的分子量为 4.5 × 10⁵。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¹³C NMR 进一步确认了 SC4 中各糖基残基的连接方式。SC4 能显著促进 B 淋巴细胞的增殖，增加小鼠脾脏，增强 PC12 细胞对抗 H₂O₂ 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 H₂O₂ 造成的损伤的能力。

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