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NMR characterization of the structure of a β-(1→3)-D-glucan isolate from cultured fruit bodies of *Sparassis crispa*

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Abstract—SCG, a purified β-D-glucan, obtained from *Sparassis crispa*, exhibits various biological activities including an antitumor effect, enhancement of the hematopoietic response in cyclophosphamide-induced leukopenic mice, and induction of the production of cytokines. The mechanisms of these effects have been extensively investigated; however, an unambiguous structural characterization of SCG is yet to be achieved. It is well accepted that the biological effects of β-glucan depend on its primary structures, conformation, and molecular weight. In the present study, we examine the difference of biological effects among β-glucans, elucidate the primary structure of SCG, and compare with SPG from *Schizophyllum commune* using NMR spectroscopy. Our data reveal that SCG but not SPG induce cytokine production from bone marrow-derived dendritic cells (BMDCs) and their major structural units are a β-(1→3)-D-glucan backbone with single β-(1→6)-D-glucosyl side branching units every three residues.

Keywords: *Sparassis crispa*; *Schizophyllum commune*; β-D-Glucan; NMR; Polysaccharide

1. Introduction

A well-known biologic response modifier (BRM) widely distributed in nature, β-glucan is important for the treatment of cancer and infectious diseases in both modern medicine and traditional oriental therapies, and as a dietary substance, because of its antitumor and immunomodulating properties.[1–5] For instance, lentinan from *Lentinus edodes*[6] and sonifilan (SPG) from *Schizophyllum commune* (*S. commune*)[7] have been used clinically for cancer therapy in Japan. A variety of β-glucans differing in structures have been isolated from various sources. The differences are in the primary structure, including the degree of branching (DB), degree of polymerization (DP) and linkage type, conformation, for example, triple helix, single helix, and random coil structures, and molecular weight.[8–10]

Sparassis crispa (*S. crispa*) is an edible/medicinal mushroom that recently became cultivable in Japan. Following preliminary investigations, the β-glucan content of *S. crispa* was found to be extensive (up to 43.6% of the dry weight of the fruiting bodies), as measured by the enzyme method of the Japan Food Research Laboratories (Tokyo). Additionally, polysaccharide fractions were prepared from cultured *S. cripsa*, and the structure and activities of the extracts were examined. In a previous result, polysaccharide fractions of *S. crispa* mainly consisted of 6-branched β-(1→3)-D-glucan and had immunopotentiating action.[11,12] Thus, *S. crispa* was determined to be a good material for preparing β-glucan with a high yield. We also previously obtained purified β-glucan SCG from crude polysaccharide extracts of *S. crispa*, and examined its biological and pharmacologic activity. SCG exhibits not only an antitumor effect[13] but also various biological activities, enhancing the hematopoietic response in cyclophosphamide-induced leukopenic mice by intraperitoneal and

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oral routes over a wide range of concentrations[14–16] and the response to human peripheral blood mononuclear cells (PBMCs), and inducing the production of cytokines.[17–20] The mechanisms of these effects have also been investigated extensively. An important element required to understand the mechanism of SCG action is the structural analysis of the glucan; however, the precise structure of β-glucan, SCG, is not clear. A previous structural analysis was conducted using 1D-^{13}C NMR spectroscopy and methylation.[11] One-dimensional-^{13}C NMR spectra are often used for the structural analysis of β-glucans because of good signal dispersion compared with 1D-^{1}H NMR spectroscopy; however, the acquirable structural information is limited because of low sensitivity and loss of coupling information. Thus, additional analysis using 1D-^{1}H and both homo- and hetero-nuclear 2D-NMR is vital for accurate structural elucidation of β-glucan, SCG. It is well established that the biological effects of β-glucan depend on its primary structure, conformation, and molecular weight. Therefore, the structural characterization of SCG is important not only to promote clinical usage for cancer therapy, but also for understanding the mechanisms of its biological effects.

In the present study, we report differences in the biological effects among β-glucans and the primary structure of purified β-glucan SCG and SPG, and completely assign all protons and carbons using 1D- and 2D-NMR spectroscopy including DQF-COSY, TOCSY, NOESY, 2D-selective TOCSY-DQFCOSY, ^{13}C-edited HSQC, HSQC-TOCSY, HMBC, and H2BC experiments.

2. Results and discussion

We recently showed that BMDCs potently induced various cytokines such as tumor necrosis factor-α (TNF-α), interleukin-12 (IL-12), and interferon-γ (IFN-γ) by a purified β-D-glucan, SCG. In addition, we also indicated that the production of those cytokines was completely abolished in a representative β-glucan receptor, dectin-1-knockout BMDCs, indicating that dectin-1 is required for cytokine induction in BMDCs after SCG stimulation.[21] We first compared these biological effects with SPG, which is a typical β-(1→3)-D-glucan possessing single β-(1→6)-D-glucosyl side branching units every three residues. As shown in Figure 1, the production of IL-6 and TNF-α from BMDCs was significantly increased by SCG, whereas these effects were not observed by SPG.

To clarify the reasons for these differences in the biological effects between SCG and SPG, we next examined the structure of SCG using GLC and NMR spectroscopy. Sugar composition analysis showed that the polysaccharide SCG is mainly composed of glucose with a slight amount of mannose in a molar ratio of 100:4 (data not shown). The 1D-^{1}H NMR spectrum of β-glucans in a mixed solvent of Me_2SO-d_6/D_2O (6:1) at 70 °C is shown in Figure 2. The anomeric region (δ_H 4.2–4.8 ppm) of both spectra contained four signals, three of which overlapped (δ_H 4.55 ppm). The other peak was a well-resolved doublet resonance (δ_H 4.254 ppm). The overlap of the anomeric doublets was confirmed in other NMR experiments, including a COSY experiment. The four sugar residues in SCG and SPG were arbitrarily labeled A1, A2, A3, and B, as described in Figure 2. On the basis of their observed chemical shifts, $^{3}J_{H1,H2}$ and $^{1}J_{H1,C1}$ (Table 1), all residues were assigned as β-hexapyranosyl residues. The 1D-^{13}C NMR spectra in Figure 3 show two signals in the anomeric region (δ_C 95–110 ppm) and are assigned to residues A1, A2, and A3 (δ_C 102.96 and 103.12 ppm) and residue B (δ_C 103.08 and 103.03 ppm), SCG and SPG, respectively, which were confirmed by cross-peaks in the ^{1}H, ^{13}C HSQC spectrum.

All ^{1}H resonances in the ^{1}H NMR spectra were assigned by means of COSY, TOCSY, and 2D-selective

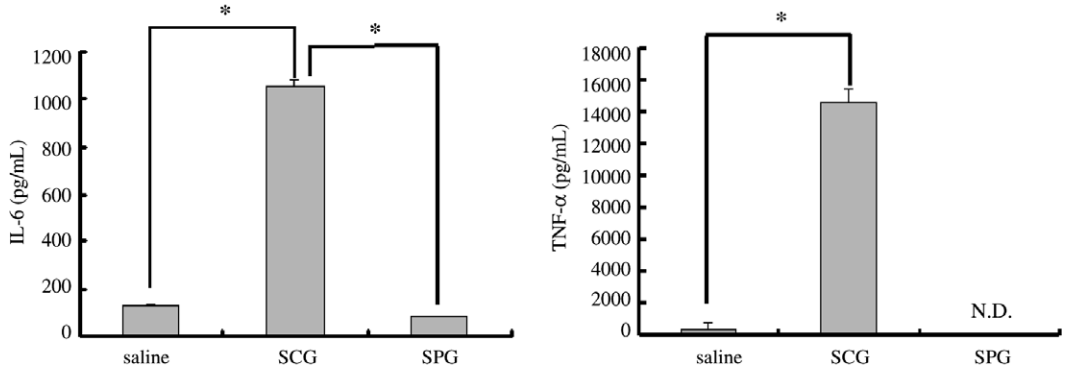

Figure 1. IL-6 and TNF-α production by BMDCs stimulated with SCG and SPG in DBA/2 mice in vitro. Bone marrow-derived dendritic cells (BMDCs) from DBA/2 mice were stimulated with SCG (100 μg/ml) or SPG (100 μg/ml) and cultured for 48 h. After incubation, the supernatant was collected. The concentration of IL-6 and TNF-α in the supernatant was determined by ELISA. Data show one of three experiments performed with similar results, each evaluating four mice per group. Values represent the mean ± SD. Significant difference, $^{*}p < 0.01$.

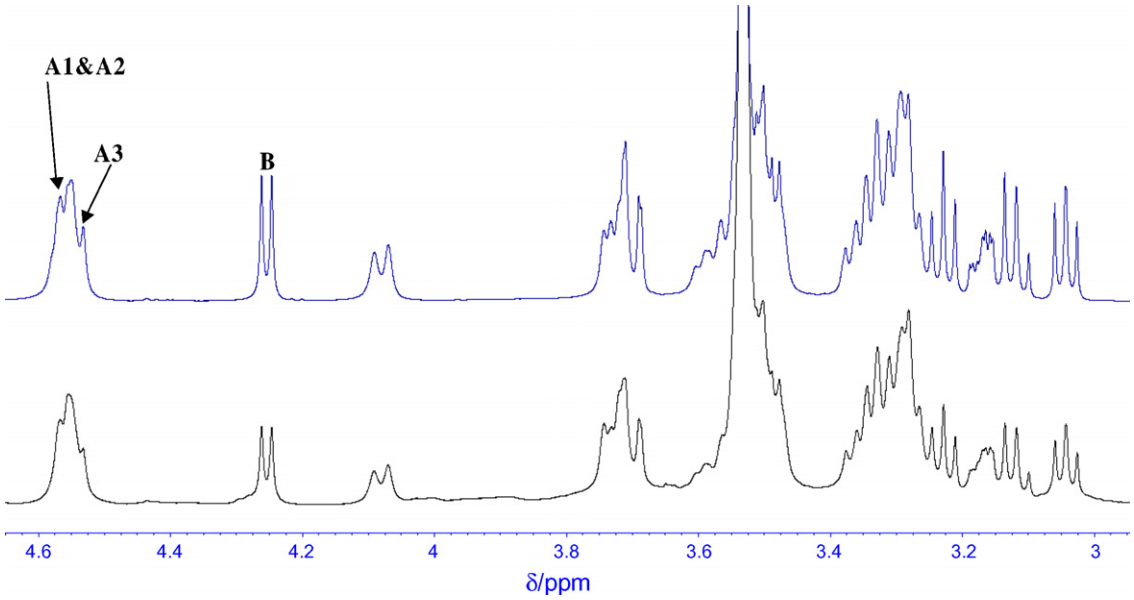

Figure 2. One-dimensional-^{1}H NMR spectra of β-glucans. 1D-^{1}H NMR experiments (upper: SPG, lower: SCG) at 70 °C.

Table 1. Coupling constants ($^{1}J_{H1,C1}$ and $^{3}J_{H1,H2}$) for SCG and SPG

	Residue			
	Al	A2	A3	B
SCG				
δ_H (ppm)	4.561	4.561	4.544	4.254
$^{1}J_{H1,C1}$ (Hz)	155.1	155.1	155.1	162.1
$^{3}J_{H1,H2}$ (Hz)	n.d.	n.d.	n.d.	7.6
SPG				
δ_H (ppm)	4.561	4.561	4.544	4.254
$^{1}J_{H1,C1}$ (Hz)	155.0	155.0	155.0	162.8
$^{3}J_{H1,H2}$ (Hz)	n.d.	n.d.	n.d.	7.5

n.d.: not detected due to heavy overlapping.

TOCSY-DQFCOSY experiments. In the 2D-TOCSY spectra of SCG and SPG, a complete series of cross-peaks between B H-1 and B H-2, 3, 4, 5, 6a, 6b was observed, as well as between A1, A2, A3 H-1 and A1, A2, A3 H-2, 3, 4, 5, 6a, 6b. These assignments were also examined using 1D-TOCSY experiments, but H2 to H5 resonances of A1, A2, and A3 residues in SCG and SPG were ambiguous due to a severe overlap of neighboring protons. These ambiguous assignments were further examined using 2D-selective TOCSY-DQFCOSY. As a result, all protons in SCG and SPG were assigned unambiguously. In addition, the ^{13}C-edited HSQC spectrum combined with HSQC-TOCSY using various mixing times (30–120 ms) for TOCSY spinlock and H2BC experiments allowed complete assignment of the ^{13}C spectrum. Tables 2 and 3 summarize the ^{1}H and ^{13}C NMR spectral assignments of SCG and SPG, respectively. These assignments are based on COSY, TOCSY,

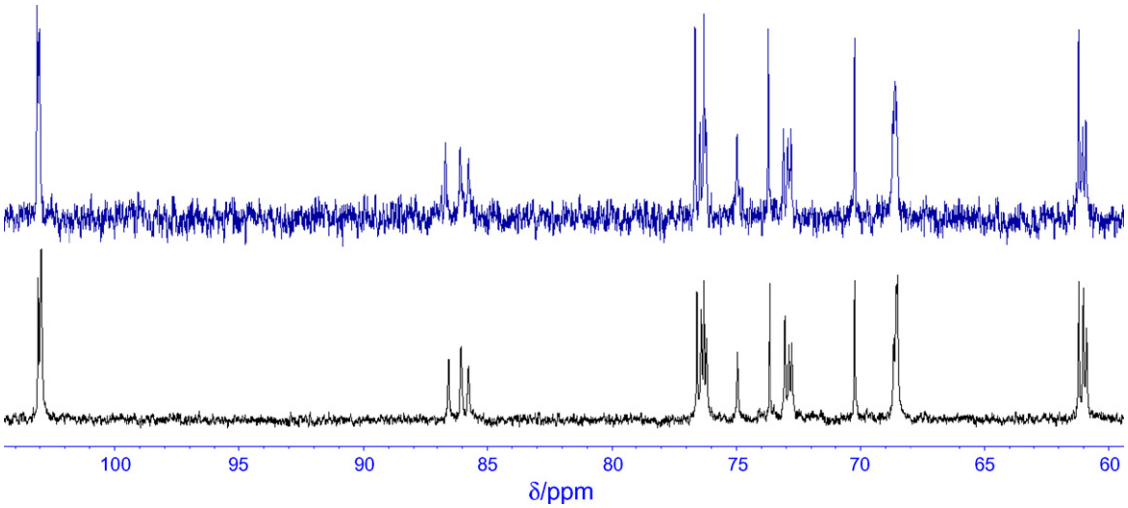

Figure 3. One-dimensional-^{13}C NMR spectra of β-glucans. Power-gated 1D-^{13}C NMR experiments (upper: SPG, lower: SCG) at 70 °C.

Table 2. Chemical shifts (ppm) of ^{1}H and ^{13}C NMR signals for SCG

Sugar residue	$^{1}H/^{13}C$						
	1	2	3	4	5	6a	6b
→3)-β-D-Glc*p*-(1→	4.561	3.327	3.520	3.280	3.309	3.724	3.477
A1	102.96	72.88	86.55	68.53	76.41	61.01	
→3)-β-D-Glc*p*-(1→	4.561	3.327	3.520	3.280	3.309	3.724	3.477
A2	102.96	72.76	86.06	68.53	76.19	60.89	
→3,6)-β-D-Glc*p*-(1→	4.544	3.349	3.540	3.264	3.520	4.081	3.585
A3	102.96	73.04	85.76	68.53	74.96	68.68	
β-D-Glc*p*-(1→	4.254	3.043	3.228	3.111	3.165	3.700	3.495
B	103.08	73.66	76.30	70.25	76.58	61.20	

Table 3. Chemical shifts (ppm) of ^{1}H and ^{13}C NMR signals for SPG

Sugar residue	$^{1}H/^{13}C$						
	1	2	3	4	5	6a	6b
→3)-β-D-Glc*p*-(1→	4.561	3.330	3.520	3.264	3.290	3.732	3.479
A1	103.12	73.09	86.68	68.63	76.46	60.92	
→3)-β-D-Glc*p*-(1→	4.561	3.330	3.520	3.264	3.290	3.732	3.479
A2	103.12	72.92	86.09	68.63	76.22	61.04	
→3,6)-β-D-Glc*p*-(1→	4.544	3.361	3.538	3.310	3.541	4.081	3.587
A3	103.12	72.80	85.75	68.57	74.97	68.72	
β-D-Glc*p*-(1→	4.254	3.043	3.228	3.118	3.171	3.701	3.495
B	103.03	73.72	76.30	70.25	76.65	61.21	

2D-selective TOCSY-DQFCOSY, HSQC, HSQC-TOCSY, and H2BC spectra. These assignments of anomeric ^{1}H and all ^{13}C signals were essentially in accordance with the values of similar compounds reported in the literature.[10]

In the spectra of SCG and SPG, the four cross-peaks (between B H-1 and A3 H-6, between A1 H-1 and A2 H-3, between A2 H-1 and A3 H-3, and between A3 H-1 and A1 H-3), which are shown in the ^{1}H, ^{13}C-HMBC spectra (Fig. 4), are assigned to four glycosidic linkages,

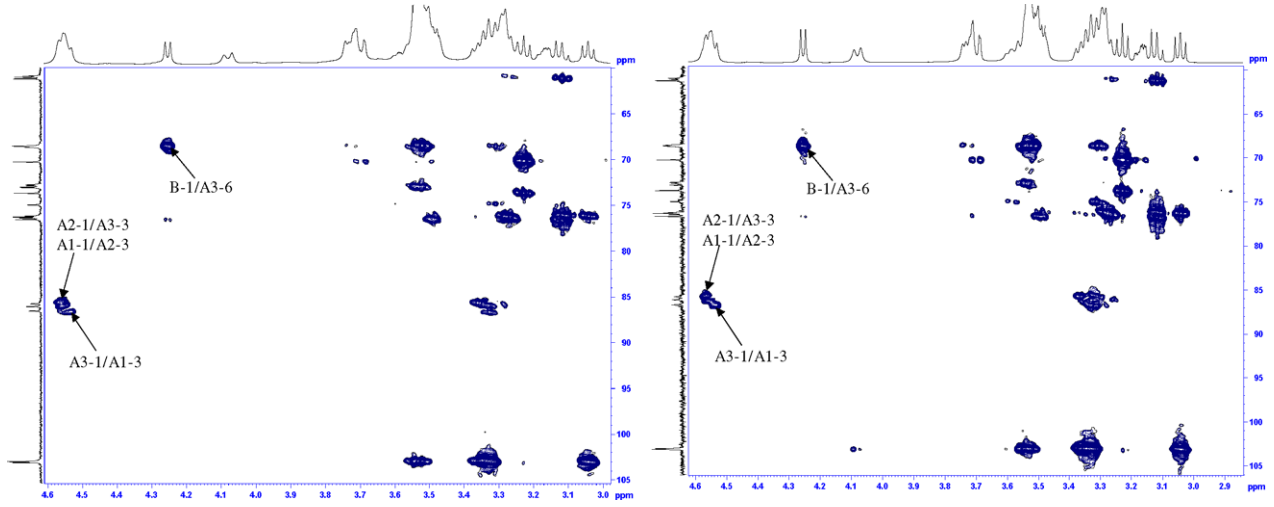

Figure 4. ^{1}H, ^{13}C-HMBC spectra of β-glucans. ^{1}H, ^{13}C-HMBC experiments (left: SCG, right: SPG) at 70 °C.

as indicated by B(1→6)A3, A1(1→3)A2, A2(1→3)A3, and A3(1→3)A1, respectively. These results were also confirmed by NOESY experiments (data not shown).

It is of note that the intensities of C-6 resonances of residue B between SCG and SPG are quite different compared with residue A1 and A2; however, the ratio of the main chain (namely, residue A) to side chain (namely, residue B) was 3:1 when calculated from the more accurate integrities of anomeric protons. These results might be due to the difference in the relaxation time caused by the difference of molecular motility around β-(1→3)-main chain between SCG and SPG.

The above results strongly suggested that the primary structure of purified β-glucan SCG obtained from the fruiting bodies of *S. crispa* contains a β-(1→3)- and β-(1→6)-glucans unit, as shown in Figure 5. These structural units are confirmed using not only NMR spectroscopy but also the resistance against formolysis and zymolyase digestion.[22] In the primary structure, it is essentially identical to β-glucan SPG from *S. commune*, which has been used clinically for cancer therapy in Japan.

In spite of the fine structural characterization of SCG and SPG in the present study, we could not clarify the molecular information of why the biological activity of these glucans was so different. During a decade of study, the biological activity of β-glucans was found to be regulated by various structural factors, such as the primary structure, molecular weight, solubility, and conformation.[8,9] From the viewpoint of component sugars, SCG contained a trace amount of mannose; however, we have recently shown that the activity of SCG disappeared in dectin-1 knockout mice, which are a representative receptor for β-glucan,[21] thus, the activity of SCG is definitely dependent on the major component, β-linked glucosyl unit. From the viewpoint of conformation, SPG is well known to have a complete triple helix structure, characterized by a gel filtration study under the physiological conditions, laser scattering, the reactivity to limulus factor G, and so on.[23] In contrast, SCG might contain an irregular single helical segment in addition to the triple helix segment, because of using sodium hydroxide treatment during the production process which destroys the native triple helical conformation and in general, its complete refolding is subsequently quite difficult. The irregular single helical segment in aqueous physiological solution gradually aggregated to form interchain interactions, thus the molecular weight of SCG under physiological conditions might be quite high (MW = over 2,000,000, whereas MW = 450,000 for SPG) to make viscous gel. The viscosity of SPG and SCG is actually quite different. Falch et al.[24] reported the relationship between molecular weight and activities of scleroglucan. Their report indicated that the TNF-α inducing ability of scleroglucans depended on their molecular weight, with the low molecular weight and high molecular weight samples stimulating monocytes to produce TNF-α most efficiently. However, in our data, the possibility of differences in molecular weight between SCG and SPG attributed to different biological activities was not ruled out; therefore, additional experiments were needed. In addition, SPG is a product of extracellular polysaccharide of *S. commune*, and SCG is an extract of the fruit body. β-Glucan of the fruit body is in general more complicated, producing a tree-like structure in addition to a simple repeating unit structure. We have already demonstrated that various activities including inducible nitric oxide synthase (iNOS) and nitric oxide (NO) synthesis in vivo and in vitro significantly differ between triple helical SPG and single helical SPG (alkaline-treated SPG, SPG-OH).[25–29] Namely, it was suggested that the single helical conformer of β-glucan was dominant in cytokine production and the subsequent up-regulation of iNOS and NO synthesis. This is also indicated that the conformational differences between SCG (possibly containing an irregular single helical segment in addition to the triple helix segment) and SPG (complete triple helix structure) could be contributed to differences in biological activities between SCG and SPG. This possibility has also not yet been ruled out.

From the possibilities described above, the molecular mechanism of the differences between SPG and SCG could not be clarified in the present study; hence, fine structural difference analyses to understand the differences between SCG and SPG are still needed. These analyses would be carried out using ultra-high field (at 900 MHz and beyond) NMR experiments[30] because the expected increase in spectral dispersion and improvement in sensitivity are observed.

In the present study, we revealed that SCG but not SPG potently induced the production of cytokine from BMDCs and clearly elucidated the primary structure of purified β-glucan SCG from *S. crispa*, which is a β-(1→3)-D-glucan backbone with single β-(1→6)-D-glucosyl side branching units every three residues. This structural finding is, at least in part, useful for revealing the precise mechanisms of the biological activities of purified β-glucan SCG and promoting food-based therapy.

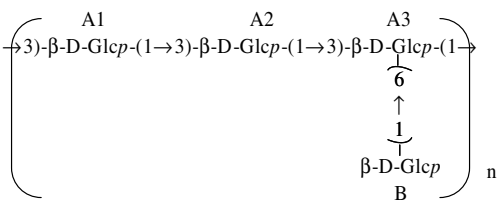

Figure 5. Structure of SCG from *S. crispa* and SPG from *S. commune*. Chemical structure of β-glucans analyzed in this study. Glc*p*: glucopyranose.

3. Experimental

3.1. Mice and materials

Male DBA/2 mice were purchased from Japan SLC. The mice were housed in a specific pathogen-free (SPF) environment and were used at 6–8 weeks of age.

Fruiting bodies of *S. crispa* were cultured by Minahealth Co., Ltd, Tokyo. SPG was purchased from Kaken Pharmaceutical Co., Ltd, Tokyo. D_2O (deuteration degree min 99.96%) and Me_2SO-d_6 (99.96%) were purchased from Merck.

3.2. Preparation of SCG

SCG was prepared as shown in Figure 6. Briefly, air-dried and powdered *S. crispa* was defatted with ethanol (4 °C for 2 days) and then extracted with hot water by autoclaving (121 °C for 2 h). The resulting residue was extracted with cold alkali (10% NaOH/5% urea at 4 °C for 2 days) and hot alkali (10% NaOH/5% urea at 65 °C for 1 h). Polysaccharide fractions of alkali extract were collected after extensive dialysis. Cold alkali extract dissolved in 8 M urea was applied to a DEAE-Sephadex A25 (Cl^-) column equilibrated with 8 M urea, and the passed-through fraction was collected and extensively dialyzed against tap and distilled water, and then lyophilized. Elemental analysis (C:H:N = 39.70:6.83:0.09) indicates that SCG is a highly pure carbohydrate and does not contain endotoxins (<12.5 pg/mg).

3.3. Cell preparation and cytokine production stimulated with β-glucan

Bone marrow cells were removed from the femurs and tibiae of 6- to 8-week-old male DBA/2 mice, and BMDCs were prepared as described.[21] SCG (100 μg/ml) or SPG (100 μg/ml) was added on day 5 after cultivation with granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-4 (IL-4). On day 7, culture supernatants were collected for cytokine assay.

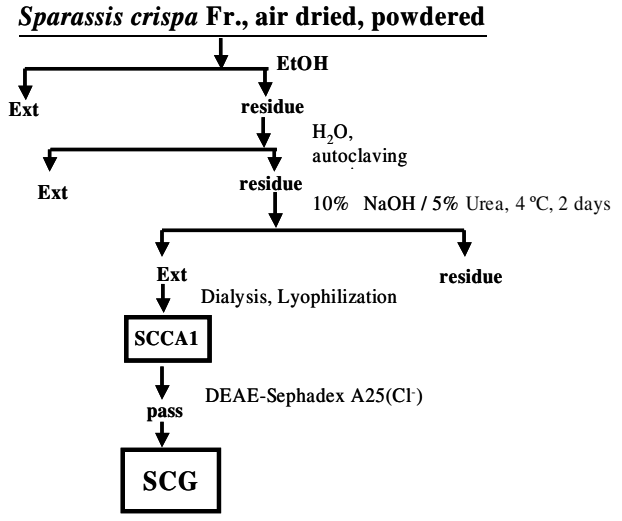

Figure 6. Scheme of extraction and purification of SCG from *S. crispa*.

3.4. Measurement of cytokines

For IL-6 and TNF-α, 96-well microtiter ELISA plates were coated with capture antibody for rat antimouse IL-6 mAb or rat antimouse TNF-α mAb (all from PharMingen). The plates were washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST) and blocked with 0.5% bovine serum albumin (BSA) containing PBST (BPBST) at 37 °C for 40 min. After washing, the plates were incubated with rMuIL-6, rMuTNF-α (all from PharMingen), or 50 μl test sample at 37 °C for 40 min. The plates were washed with PBST and then treated with antibody for biotinylated rat antimouse IL-6 mAb or biotinylated rat antimouse TNF-α mAb (all from PharMingen) in BPBST. Subsequently, the plates were treated with peroxidase-conjugated streptavidin and developed with a 3,3′,5,5′-tetramethylbenzidine (TMB) substrate system. Color development was stopped with 1 N phosphoric acid, and the optical density (OD) was measured at 450 nm.

3.5. NMR spectroscopy

Exchangeable protons were removed by suspending SCG in D_2O, and lyophilizing. This exchange process was repeated three times. All spectra were recorded in a mixed solvent Me_2SO-d_6/D_2O (6:1) (15 mg/mL) at 70 °C by the method of Kim et al.[10] on a Bruker Avance 500 spectrometer equipped with a TXI-*xyz* three gradient probe for the detection of ^1H or a BBO-*z* gradient probe for the detection of ^{13}C. Chemical shifts are given in parts per million, using the internal Me_2SO signal ($\delta_H = 2.53$ ppm) for ^1H and internal Me_2SO ($\delta_C = 39.5$ ppm) for ^{13}C as references. The 1D-^1H experiment was performed using the Bruker standard pulse sequence with 5000 Hz in 64 K complex data points. The relaxation delay was $5T_1$ in order to calculate accurate signal integrations. Prior to Fourier Transformation, zero filling was used four times, and noise was reduced using the TRAF function. The 1D-^{13}C experiment was performed using the Bruker standard pulse sequence with 30,581 Hz in 64 K complex data points. Prior to Fourier Transformation, zero filling was used four times, and noise was reduced using exponential multiplication. One-dimensional-TOCSY spectra were recorded with various mixing times using the Bruker standard pulse sequence with 5000 Hz in 64 K complex data points. Prior to Fourier Transformation, zero filling was used four times, and noise was reduced using exponential multiplication. One-dimensional-NOESY

was performed using the Bruker standard pulse sequence with 5000 Hz in 64 K complex data points. The mixing time for NOE growth was 200 ms. Prior to Fourier Transformation, zero filling was used four times, and noise was reduced using exponential multiplication. The shape of the selective excitation pulse for 1D-TOCSY, 1D-NOESY, and 2D-selective TOCSY-DQFCOSY was RE-BURP, and the duration of the pulse was set to 40 ms for H-1 of 4.25 ppm or 80 ms for H-1 of 4.55 ppm and H-6 of 4.11 ppm. Two-dimensional ^{1}H, ^{1}H-correlation spectroscopy (COSY) was conducted with 512 increments of 4096 data points with 8 scans per t_1 increment using the Bruker standard pulse sequence. The spectral width was 2694 Hz in each dimension. Two-dimensional total correlation spectroscopy (TOCSY) was carried out with a mixing time for TOCSY spinlock of 210 ms using the pulse sequence of Griesinger et al.[31] to suppress ROE signals. The spectral width was 4006 Hz in each dimension, and 512 increments of 4096 data points with 16 scans per t_1 increment were recorded. Two-dimensional nuclear overhauser effect spectroscopy (NOESY) was conducted with a mixing time of 200 ms using the Bruker standard pulse sequence. The spectral width was 4006 Hz in each dimension, and 512 increments of 4096 data points with 48 scans per t_1 increment were recorded. Two-dimensional-selective TOCSY-DQFCOSY was performed with 512 increments of 2048 data points with 32 scans per t_1 increment using pulse sequences of Sato et al.[32,33] The spectral width was 1947 Hz in each dimension. Two-dimensional ^{13}C-edited hetero-nuclear single quantum coherence spectroscopy (HSQC) was conducted with 512 increments of 2048 data points with 32 scans per t_1 increment using the Bruker standard pulse sequence. The spectral width was 3501 Hz for t_2 and 12,500 Hz for t_1. Two-dimensional ^{1}H, ^{13}C-hetero-nuclear multiple bond coherence spectroscopy (HMBC) was performed with 256 increments of 2048 data points with 132 scans per t_1 increment using the Bruker standard pulse sequence. The delay time for the evolution of long-range couplings was set to 62.5 ms (optimize for 8 Hz). The spectral width was 3501 Hz for t_2 and 12,500 Hz for t_1. Two-dimensional ^{1}H, ^{13}C-HSQC-TOCSY was conducted with 256 increments of 2048 data points with 36 scans per t_1 increment using the Bruker standard pulse sequence. The spectral width was 3501 Hz for t_2 and 12,500 Hz for t_1. Two-dimensional hetero-nuclear 2-bond correlation spectroscopy (H2BC) was carried out with 512 increments of 2048 data points with 128 scans per t_1 increment using the pulse sequence of Nyberg et al.[34–36] The spectral width was 5000 Hz for t_2 and 26,000 Hz for t_1. All 2D experiments were zero filled to 2k and 2k in both dimensions prior to Fourier transformation. A square cosine-bell window function was applied in both dimensions, except in the COSY experiment. A sine-bell window function was applied in both dimensions for the COSY experiment.

3.6. Statistical analysis

The results are expressed as the means ± standard deviation (SD). The significance of differences between the means was measured by Student's *t*-test.

Acknowledgments

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