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Characteristics of Water-soluble Polysaccharide, Showing Inhibiting Activity on α-Glucosidase, in *Cordyceps militaris*

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Abstract Extract of water-soluble polysaccharide (CFWx), showing inhibiting activity on α-glucosidase, was prepared from the fruiting bodies of *Cordyceps militaris* by hot-water extraction, and ethanol precipitation. Chemical characteristics of CFWx were as follows: carbohydrate content 30% including 16% of uronic acid; 51% protein content; monosaccharide composition, Man:Glu:Gal (30:43:27); molecular weight 3-5×10^4. CFWx was further purified by ion-exchange, gel-permeation, and affinity chromatography and CFWx-AH-α fraction was isolated. Fundamental structure of CFWx-AH-α was deduced as α-(1→4)-D-glucan with α-(1→3)- and/or α-(1→6)-D-glycosidic side chains based on methylation analysis.

Keywords: *Cordyceps militaris*, chemical characteristics, fundamental structure, glucan, diabetes mellitus

Introduction

Occurrences of non-insulin dependent diabetes mellitus (NIDDM) are increasing with the ingestion of a large quantity of fatty foods, the lack of exercises and the undesirable living habits like smoking in the developed countries of the world. However, the pharmacological therapy of patients with diabetes has been surrounded by controversy because of peripheral insulin resistance in many type II patients. Current oral therapy using sulfonylurea derivatives, which primarily stimulate insulin secretion, often, fails to achieve the expected level of efficacy (1). Pharmaceuticals such as Troglitazone and Metformin have also been used to enhance peripheral insulin sensitivity (2). Although improved glycemic control with these drugs has been reported, potential adverse effects such as impairment of renal function, cardiogenic or septic shock, or liver failure need to be concerned (3). Thus, a more effective and safer treatment modality for type II patients needs to be further investigated, focusing on overcoming severe side effects. Under these circumstances there is a growing demand for preparing nutraceuticals with natural resources which may prevent or control diabetes mellitus. Currently mushrooms have been receiving much attention not only for their delicious tastes and flavors but their diverse biological activity to human beings. In paricular *Cordyceps militaris* has been reported as a source of alternative medicines. Its beneficial effects including antitumor, immunomodulating activities have been well demonstrated with its high level of hypoglycemic activity in a number of biochemical studies (4). CS-OHEP fraction from *Cordyceps sinensis,* being used in traditional Chinese medicine for a long time, exhibited significant hypoglycemic activity by peritoneal administration at a dose of 500 mg/kg in normal mice (5). Furthermore, CS-F30, a purified polysaccharide fraction of CS-OHEP, significantly lowered the glucose level at a dose of 50 mg/kg by peritoneal administration (6). In addition, the activity of key hepatic enzymes in glucose metabolism such as hepatic glucokinase, hexokinase, and glucose-6-phosphate dehydrogenase were significantly increased by intravenous administration of CS-F30 (6). Therefore, it is expected that continuous intake of *Cordyceps* preparations should be helpful for controlling diabetes to some extent.

In the present study, we prepared a water-soluble polysaccharide extract (CFWx) from *C. militaris* and examined its inhibiting activity on α-glucosidase. Thereafter, polysaccharide fraction was isolated from CFWx, and its chemical composition and fundamental structure were examined for its use as a potential nutraceutical.

Materials and Methods

Materials The freeze-dried fruiting bodies (100 g) of *Cordyceps militaris* were extracted for 3 hr with 800 mL of 85% ethanol 3 times. The residue was collected and then extracted for 3 hr with 800 mL of hot water 3 times. The water-soluble extract was concentrated to 250 mL and precipitated with 1,250 mL of ethanol. The precipitate was dialyzed and freeze-dried. The obtained fraction (17.1 g) was designated as CFWx and used for further experiments.

α-Glucosidase inhibitory assay The α-glucosidase inhibitory assay was performed according to the method described by Watanabe *et al.* (7). α-Glucosidase (yeast 0.7 U, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 100 mM phosphate buffer (pH 7.0) containing 2 g/L bovine serum albumin and 0.2 g/L NaN_3. A 50 μL of enzyme solution and 10 μL of sample were mixed in a well of a microtiter plate and measured for titer ($Abs_{405\ nm}$) at zero time with a microplate reader (Vmax; Molecular

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Devices, Sunnyvale, CA, USA). After incubation for 5 min, 50 μL of the substrate solution (2 mM of *p*-nitrophenyl-α-D-glucopyranoside) was added and incubated for another 5 min at room temperature. The absorbance of samples were measured and compared with the absorbance of the control (Acarbose; Sigma-Aldrich).

Isolation of glucan CFWx (0.1 g) was dissolved in 1 mL distilled water and passed through a diethyl aminoethyl (DEAE)-cellulose column (Macro-Prep DEAE Support, 2.5×40 cm, Bio-Rad Lab., Hercules, CA, USA) with 0-2 M NaCl gradient elution; 544 mL total elution volume; 6.8 mL volume of 1 fraction;1.25 mL/min flow rate. The ion exchanged fractions were gel-filtrated with Superose 6 10/300 GL column (1×30 cm; Amersham Biosciences, Piscataway, NJ, USA) with 0-0.5 M NaCl gradient elution; total elution volume, 60 mL; volume of 1 fraction, 1 mL; flow rate, 0.5 mL/min. Subsequently, the gel-filtrated fractions were separated into α-glucans (absorbed) and β-glucans (non-absorbed) through HI-Trap Con A column (1 mL column, Amersham Biosciences) with 0.1 M NaCl, 0.5 M methyl-α-D-glucopyranoside gradient elution; total elution volume, 27 mL; volume of 1 fraction, 1.5 mL; flow rate, 0.5-0.15-0.25-0.5 mL/min. The purification procedure of CFWx was repeated several times until collection of 0.1 g glucan fraction of CFWx.

General analyses Total sugar, protein, and uronic acid contents were measured by the phenol-sulfuric acid method (8), Lowry method (9), and Carbazole-sulfuric acid method (10), respectively. The molecular weight of each fraction was determined through gel filtration chromatography by comparing with the standard curve using standard dextrans (Amersham Pharmacia Biotech AB, Uppsala, Sweden).

Sugar analysis The sugar constituents of CFWx fraction were transformed into the corresponding alditol acetates (11) and detected by gas chromatography/mass spectrometry (GC/MS). Agilent 6890 GC was equipped with a 5973 MSD (Hewlett-Packard, Avondale, AZ, USA) and an ethylene glycol-coated fused silica capillary column (Supelcowax-10; 30 m×0.25 mm i.d., 0.25 μm film thickness, Supelco Inc., Supelco Park Bellefonte, PA, USA). Temperatures of the ion source and injector were 230°C at 70 eV, and 280°C, respectively. The oven temperature was raised from 100 to 280°C at 5°C/min. The flow rate of carrier gas (He) was 1 mL/min, and the split ratio was 1:10. The mass chromatogram of hexaacetyl hexitols was reconstructed with the most abundant ion (*m/z* 289) from hexaacetyl hexitols to exclude impurities during the estimation of relative quantities.

Determination of the position of glycosidic linkages Each polysaccaride fraction (5 mg) was methylated with 0.4 mL methyl sulfinyl methyl sodium at 25°C for 6 hr according to the Hakomori method (12). The obtained methylated polysaccharides were depolymerized with 90% formic acid at 105°C for 1.5 hr, followed by hydrolysis with 0.15 M H_2SO_4, and neutralized with barium carbonate. The hydrolyzed fraction was reduced with 10 mg $NaBH_4$ at 22°C for 20 hr and acetylated with an acetic anhydride in pyridine at 105°C for 2 hr. The partially methylated alditol acetates were detected by GC/MS under the same conditions as those applied to the sugar analysis.

Results and Discussion

Inhibition of α-glucosidase by CFWx Some of the edible mushrooms including *C. militaris* have been reported for their hypoglycemic effect in diabetic mouse models. Kwon *et al.* (13) reported all fractions of hot water extract of *C. militaris* showed mild hypoglycemic effect in streptozotocin (STZ)-induced diabetic rats by oral administartion (300 mg/kg). Despite their suggestion about hypoglycemic action that *C. militaris* has both insulin like and insulin promoting activity *in vivo*, inhibiting activity of *C. militaris* on α-glucosidase activity is still of great interest. The CFWx fraction obtained from *C. militaris* in this study inhibited α-glucosidase activity by 36.3% at a concentration of 0.5 mg/mL while acarbose inhibited the enzyme activity by 31.4% at the same level (Fig. 1). Kim *et al.* (14) reported the water extracts of *Phellinus linteus* and *Ganoderma lucidum* effectively inhibited maltase, sucrase, and α-amylase of rat intestine although their activities were weaker than that of acarbose. With the hypoglycemic effect of *C. militaris*, its association with peripheral insulin sensitization has been postulated. It means *C. militaris* may sensitize or stimulate insulin receptor activity, leading to an increase in peripheral glucose uptake, thereby overcoming insulin resistance. Hence, prolonged dietary intake of CFWx from *C. militaris* for α-glucosidase inhibitors or for peripheral insulin sensitization could lead to a positive effect on control of diabetes.

Isolation of polysaccharides CFWx was found to contain 30% of carbohydrate including 16% of uronic acid, 51.4% of protein, and 12.3% of ash (Table 1). Sugar analysis showed that the carbohydrate in CFWx was mainly composed of mannose, glucose, and galactose (30:43:27). Like other medicinal mushrooms such as *Agaricus blazei* and *P. linteus* special attention has been given to the polysaccharides or polysaccharides-protein complex of *C. militaris*. On that ground, it is of immense interest to

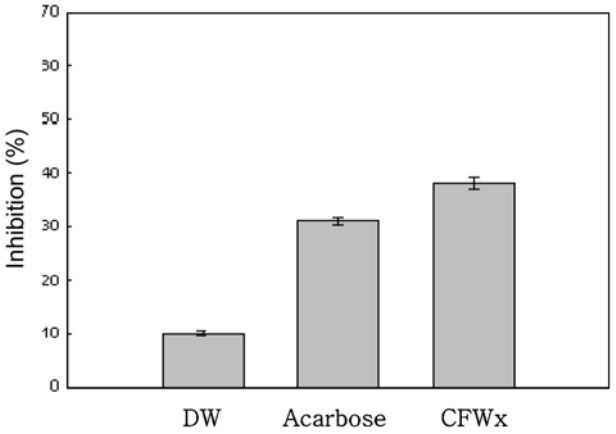

Fig. 1. Inhibitory effect of CFWx fraction from *Cordyceps militaris* on α-glucosidase activity. DW, distilled water, blank; Acarbose, positive control; CFWx, water-soluble polysaccharide extract of *C. militaris*.

Table 1. Proximate composition of water-soluble polysaccharide extract of *Cordyceps militaris* (CFWx)

	CFWx (%)
Total sugar	30.0±1.0
Protein	51.4±0.7
Ash	12.3±0.4
Moisture	2.4±0.2

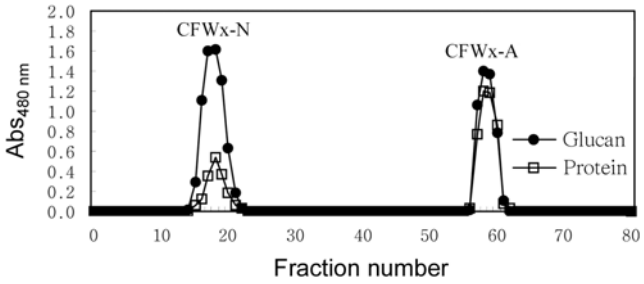

Fig. 2. Ion exchange chromatogram of CFWx fraction from *Cordyceps militaris*. CFWx-N, neutral polysaccharide fraction of CFWx; CFWx-A, acidic polysaccharide fraction of CFWx.

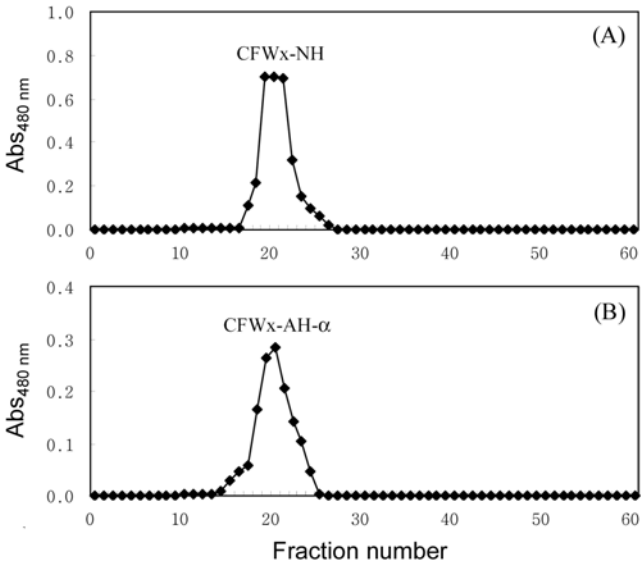

Fig. 3. Gel permeation chromatograms of CFWx-N (A) and CFWx-A (B) fraction from *Cordyceps militaris*. CFWx-NH, high molecular weight fraction of CFWx-N; CFWx-AH, high molecular weight fraction of CFWx-A.

isolate fractions of polysaccharide from CFWx and examine chemical features of them. For the separation of polysaccharides, the neutral fraction was separated from the acidic fraction on a DEAE-cellulose chromatography by 0-2 M gradient of aqueous NaCl (Fig. 2). The separated each fraction (CFWx-N, CFWx-A) was purified on Superose 6 10/300 GL column (1×30 cm) with 0.5 M NaCl as the eluent. The gel-filtered eluates (CFWx-NH, CFWx-AH) were symmetrically appeared according to the contents of carbohydrate (Fig. 3). CFWx-NH and CFWx-AH were further chromatographed into non-absorbed β type (CFWx-NH-β, CFWx-AH-β) and absorbed α type (CFWx-NH-α, CFWx-AH-α) through Hi-Trap Con A

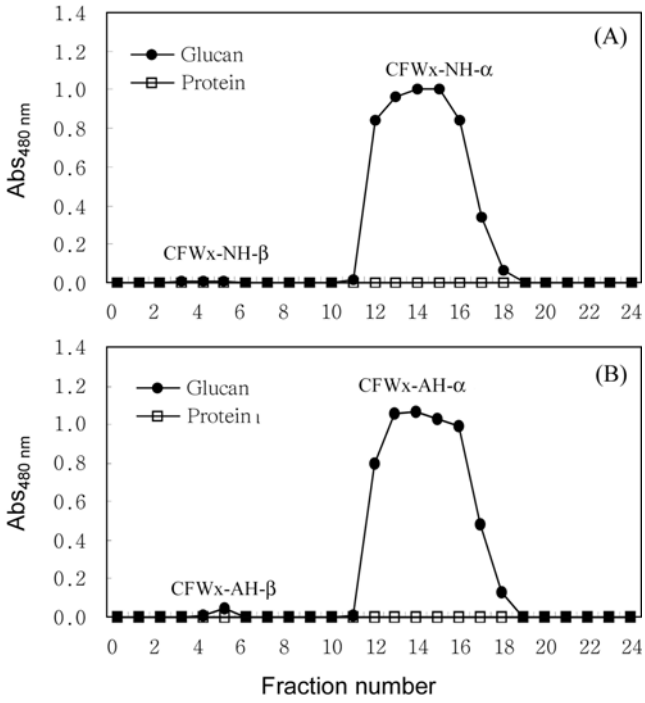

Fig. 4. Affinity chromatograms of CFWx-NH (A) and CFWx-AH (B) fraction from *Cordyceps militaris*. CFWx-NH-β, non-absorbed β fraction of CFWx-NH; CFWx-NH-α, absorbed α fraction of CFWx-NH; CFWx-AH-β, non-absorbed β fraction of CFWx-AH; CFWx-AH-α, absorbed α fraction of CFWx-AH.

(Fig. 4). The molecular weights of CFWx-NH and CFWx-AH were estimated to be 3-5×10^4 by referencing to the elution profile of the dextrans of known molecular weights. In case of *C. sinensis*, showing hypoglycemic activity, molecular weight of a neutral polysaccharide fraction (CS-F10) was reported to be about 1.5×10^4 (15).

Structural characterization The isolated polysaccharide fractions (CFWx-AH-α, CFWx-NH-α) from *C. militaris* were prepared for methylation analysis, and GC/MS spectrums were obtained for elucidating its structure. The GC/MS spectra collected were compared to the respective standard mass spectra reported by Janson *et al.* (16) and the fragmentation pattern of Chaplin (17). Interpretation of each spectrum was done according to the same procedure with previous report (18,19).

Methylation analyses of CFWx-NF-α and CFWx-AF-α showed 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol as the major peak (*m/z* 233, 173, 161, 129, 117, 101, 87, 43) (Fig. 5B). Additionally, peaks of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylhexitol derivatives (*m/z* 233, 161, 129, 117, 101, 43) (Fig. 5A) and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol (*m/z* 233, 189, 161, 129, 117, 101, 43) (Fig. 5C) revealed the presence of both (13)-linked and (16)-linked α-D-glucopyranosyl residues. The peak of 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylhexitol (*m/z* 261, 201, 161, 127, 117, 101, 43) (Fig. 5D) indicated that α-(1→3) or α-(1→6) residues were branched at C-6 of (1→4)-linked α-D-glucopyranosides. Particularly, all mass spectra obtained from the assigned peak in total ion chromatogram showed common mass fragment at *m/z* 117 with a base fragment at

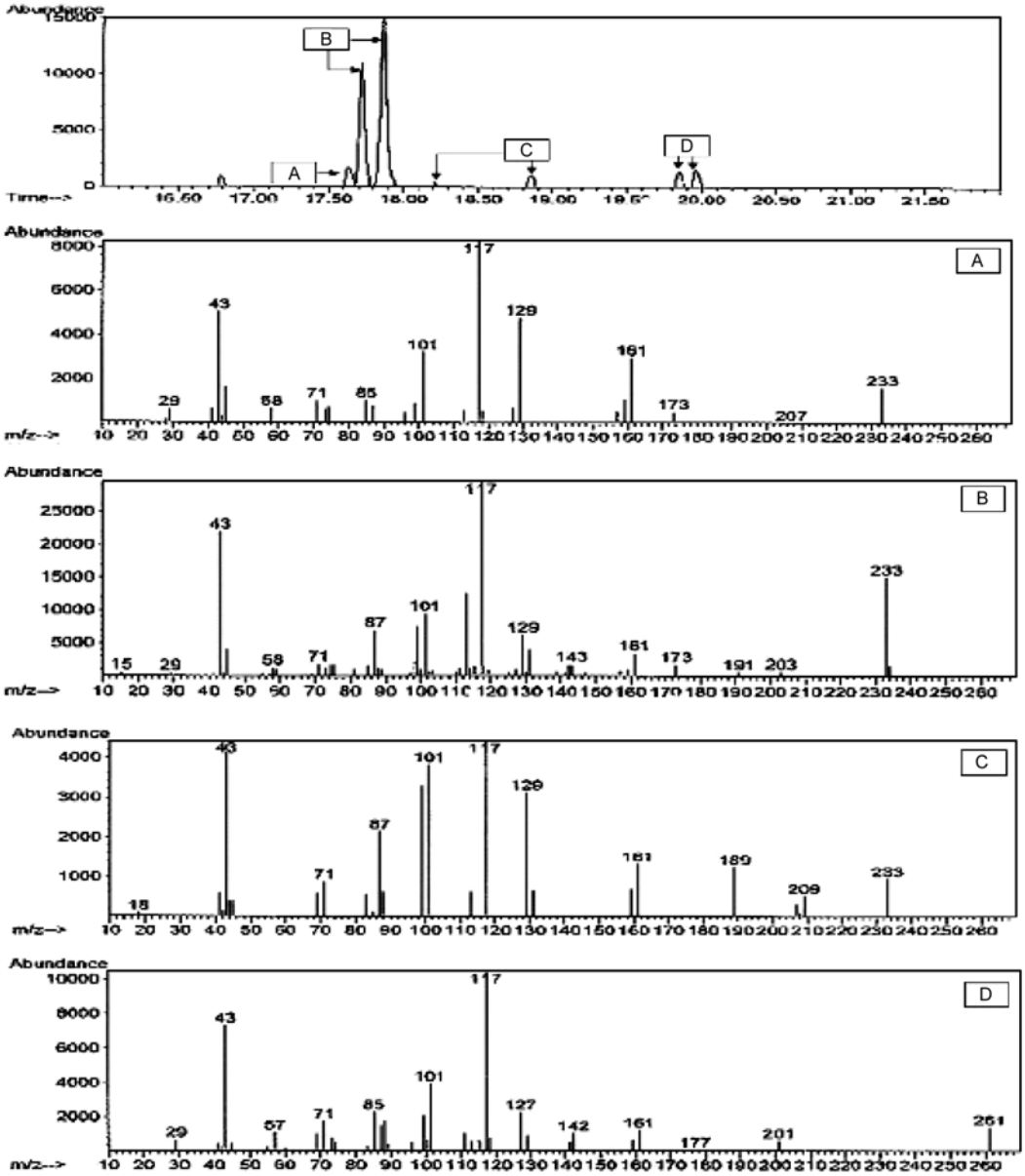

Fig. 5. GC-MS data of CFWx fraction from *Cordyceps militaris*. A, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylhexitol; B, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol; C, 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol; D, 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methylhexitol.

m/z 43. These results suggested a main backbone chain of CFWx-AF-α was formed with a (1→4)-linked α-D-glucopyranosides, branched with α-(1→3)-D-glucopyranosyl residues as well as α-(1→6)-D-glucopyranosyl residues.

Regrettably, there are not enough informations regarding the chemical structure of polysaccharide of *C. militaris*. Kiho *et al.* (15) stated that a polysaccharide fraction (CS-F10), showing the hypoglycemic effect, from the cultured mycelium of *C. sinensis* was composed of (1→5) and/or (1→6)-linked-D-galactopyranosyl residues with (1→2)-linked-D-mannopyranosyl branches. Unlike some medicinal mushrooms such as *A. blazei* and *P. linteus,* especially β-D-glucans were expected to be responsible for its anti-tumor activities (20), the authors deduced the fundamental structure of polysaccharide chain in *C. militaris* as α-(1→4)-D-heteroglycan with α-(1→3) and/or α-(1→6)-D-glycosidic side chains based on the characteristic mass fragment patterns obtained through the methylation analyses. Therefore, inhibiting activity of CFWx fraction obtained from *C. militaris* on α-glucosidase in this study seems to be related with its polysaccharide fractions (CFWx-AH-α, CFWx-NH-α) containing α-(1→4) heteroglycan with α-(1→3) and/or α-(1→6)-D-glycosidic side chains.

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