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Isolation and Analysis of a Novel Proteoglycan from *Zizyphus jujuba cv. Jinsixiaozao*

JIN-WEI LI[1,4]*, YI-YONG CHEN[2], SHAO-DONG DING[3] AND LIAN-FU ZHANG[1,4]

[1.] *School of Food Science and Technology, Jiangnan University, Wuxi 214036, China*
[2.] *Department of Biology and Food Engineering, Changshu Institute of Technology, Changshu 215500, China*
[3.] *Center of Testing and Analysis, Jiangnan University, Wuxi 214036, China*
[4.] *State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214036, China*

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ABSTRACT

Chinese jujube (*Zizyphus jujuba Miller*) is a well-known oriental medicinal plant that has various biological activities. In this study, a novel water-soluble proteoglycan (ZSG4b) was isolated from *Zizyphus jujuba cv. Jinsixiaozao* and its effect on the complement system was investigated. ZSG4b was extracted with hot water and purified by DEAE-Sepharose CL-6B and Sepharose CL-6B column chromatography. It was eluted as a single symmetrical narrow peak on high-performance gel-permeation chromatography (HPGPC) and the average molecular weight was estimated to be 1.4×10^5 Da. ZSG4b contained 83.5% glycan and 9.7% protein. Gas chromatography analysis result indicated that the glycan portion of ZSG4b was composed mainly of rhamnose, arabinose, mannose and galactose in a molar ratio of 13.8:4:3:8. It also contains 29% uronic acid as determined by *m*-hydroxybiphenyl method. The presence of galacturonic acid was estimated by the increase of galactose content in the carboxyl-reduced ZSG4b. The amino acid composition showed that it was rich in aspartic acid, glutamic acid, serine, threonine and leucine; the molar content of these five amino acids constituted 62.8% of the total amino acids. The β-elimination reaction demonstrated that the protein and glycan were linked by O-linkage. ZSG4b exhibited high anti-complement activity which was increased in accordance with the concentration below 120 μg/mL *in vitro*.

Key words: *Zizyphus jujuba cv. Jinsixiaozao*, proteoglycan, Fourier transforms infrared spectra, β-elimination reaction, anti-complement activity

INTRODUCTION

Chinese jujube (*Zizyphus jujuba Miller*) is indigenous to China with a history over four thousand years. It has been widely planted in re-forested areas within the Yellow River valley, and chosen as a variety compatible with the present ecology and economy[(1)]. The fruit of Chinese jujube is a kind of favorable and profitable fruit, and is much admired for its high nutritional value. It is customarily employed as a crude drug for analeptic and palliative purpose, and also used as food, food additive and flavor. Because of its broad pharmacological effects, it has been used for thousands of years in traditional Chinese medicinal prescriptions. The Chinese share of the world jujube production is about 90% and has increased in the last ten years due to demand for the food and pharmaceutical industries[(1)]. A steady rise in consumer demand was reflected by the expansion of Chinese production from 1.1 million tons in 1997 to 1.4 million tons in 2001. Most of the Chinese jujube has been consumed in fresh and dried forms, so research works have been done on the preservation and drying to enhance the quality of Chinese jujube[(2-5)].

The complement system is important in initiating inflammation, and its activation might induce opsonization, leukocyte activation, mast cell degranulation or lysis of target cells by the end-product of the cascade, which can damage invading cells by entering their lipid membrane. The acquired immune system is also stimulated by complement activation, thus bridging the innate and acquired immune system. There are also interactions between complement and other cascade systems such as coagulation and fibrinolysis. Due to the important physiological role of the complement system, its modulation is related to various diseases and considered as an interesting target for drug development[(6)].

In recent years, glycans extracted from plants and fungi in traditional Chinese medicine have been regarded as an important class of biological response modifiers. Some plant glycans are known to possess complement-modulating activity[(7-15)], which might be present in Chinese jujube. To our knowledge, however, the isolation, purification and characterization of glycans from Chinese jujube and subsequent evaluation of complement-modulating activity have not yet been reported.

In our previous papers, antioxidant activities of

* Author for correspondence. Fax: +0086-510-5807976;
E-mail: LJW6688@hotmail.com

Chinese jujube have been studied[16]. The aim of the present paper was to study the chemical composition and structural features of proteoglycan isolated from *Zizyphus jujuba cv. Jinsixiaozao*, by monosaccharide analysis, amino acid analysis, β-elimination and Fourier transforms infrared (FT-IR) spectra analysis. Its effect on the complement system was also investigated.

MATERIALS AND METHODS

I. *Materials and Equipments*

Zizyphus jujuba cv. Jinsixiaozao was obtained from the Research Institute of Jujube (Shandong, China). It was dried at 60°C in an oven for 48 hr after removal of seeds, ground to pass through an 1-mm screen and stored in a refrigerator.

II. *Proximate Composition Analysis*

Total carbohydrate was determined by the phenol-sulphuric acid colorimetric method using glucose as a standard[17]. Water content was determined by weight loss after drying by following the method described by Huang[18]. Fat content was determined using a Soxhlet apparatus according to the method described by Huang[18]. Crude fiber was measured according to the standard procedures[18]. Protein content was calculated from the nitrogen content (%N × 6.25) analyzed by Kjeldahl method. Ash was determined gravimetrically according to the standard procedures[18].

III. *Extraction Procedure*

Ground *Zizyphus jujuba cv. Jinsixiaozao* was refluxed with 95% ethanol at 70°C in water bath for 3 hr. Subsequently, the dried ethanol extract was extracted with distilled water at 80°C for 3 hr, filtered through gauze and centrifuged to remove water-insoluble materials. The aqueous extract was concentrated, and then mixed with four volumes of cold 95% ethanol for the isolation of glycans. The precipitate was recovered by centrifugation, washed sequentially with cold 80% ethanol, 95% ethanol, 100% ethanol and acetone, and finally lyophilized. Brown water-soluble glycans (ZSG) were thus obtained.

IV. *Anion Exchange Chromatography and Gel Permeation Chromatography*

ZSG were fractionated by anion-exchange chromatography on a DEAE-Sepharose CL-6B column (2.6 × 37 cm). The sample was dissolved in distilled water and left overnight. The solution was filtered and applied onto the column. The column was first eluted with 0.1 M NaAc (pH 5.0) at a flow rate of 1.25 mL/min. Then the elution was effected by a linear gradient (1500 mL) from 0 to 1.5 M NaCl in 0.1 M NaAc (pH 5.0). Fractions containing carbohydrate from the elution step were pooled and lyophilised.

The carbohydrate fraction was solubilized in water and further purified by size-exclusion chromatography on a Sepharose CL-6B column (2.6 × 160 cm), eluted with 0.02 M NaCl at a flow rate of 0.5 mL/min. In order to eliminate any residual contaminants, chromatography on a Sepharose CL-6B column was repeated; the fractions containing glycan were combined and concentrated, and then used as the purified proteoglycan (ZSG4b).

V. *Molecular Weight Determination*

The molecular weights of ZSG4b was determined by HPSEC, which was performed on a Waters HPLC system, including two serially linked Ultrahydrogel^{TM} Linear (ID 7.8 mm × 300 mm) columns, a Waters 2410 interferometric refractometer detector and an on-line degasser. The mobile phase was 0.1 M $NaNO_3$. A 20 μL sample (20 mg/mL) was injected in each run at room temperature and was eluted at a flow rate of 0.9 mL/min. The molecular weight was estimated by referring to the calibration curve made under the same conditions from Dextran T-series molecular weight standards (6100, 16,500, 26,290, 40,000, 84,000, 158,000 Da).

VI. *Protein Contents and Amino Acid Analysis*

The protein content of ZSG4b was determined by the method of Lowry *et al*.[19], using bovine serum albumin as the standard. The protein content in column fractions was determined by measuring the absorbance at 280 nm. The amino acids composition of ZSG4b was determined using an automatic amino acid analyzer (835-50, Hitachi) after acid hydrolysis.

VII. *Total Carbohydrate and Monosaccharides Analysis*

Total carbohydrate content of ZSG4b was determined according to the methods mentioned above. The uronic acid content was determined by *m*-hydroxybiphenyl assay using glucuronic acid as a standard[20]. The uronic acid of ZSG4b was reduced to the carboxyl-reduced glycan with sodium borodeuteride (NaBD4)[21], and then the carboxyl-reduced glycan was hydrolysed with 2 M trifluoroacetic acid (TFA). The corresponding alditol acetate was prepared as described below.

The composition of neutral monosaccharides was analyzed by gas chromatography. ZSG4b was dissolved in 2 M TFA and hydrolysed at 121°C for 6 hr in a sealed glass tube. Acetylation was carried out with 10 mg hydroxylamine hydrochloride and 0.5 mL of pyridine for 30 min at 90°C. Acetic anhydride (0.5 mL) was then added with continuous heating and the alditol acetate derivative (1-2 mL) was analyzed by gas chromatograph. The percentages of monosaccharides in the sample were calculated from the peak areas using response factors.

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VIII. *Analysis of Glycan-peptide Linkage*

The linkage of glycan-peptide was analyzed by β-elimination reaction[22,23]. The sample (4 mg) was dissolved in 4 mL of 0.2 M NaOH containing 1.0 M $NaBH_4$, incubated at 45°C for 30 min. The absorption spectrum was scanned from 200 nm to 500 nm with an UV-2102 spectrophotometer and compared with the sample (4 mg) in 5 mL of distilled water.

IX. *FT-IR Spectra Analysis*

FT-IR spectra of the samples were acquired at a resolution of 8 cm^{-1}. The samples were incorporated into KBr and pressed into a 1 mm pellet. Spectra were recorded at the absorbance mode from 4000 to 400 cm^{-1} on a Nicolet Nexus FT-IR spectrometer.

X. *Lymphocytes Proliferation and Anti-complement Activity In vitro*

The mitogenic activities of ZSG and its purified compounds were estimated according to the method described by Wang *et al.*[24]. The anti-complement activity was measured by the complement fixation test[25]. The anti-complement activity of ZSG4b was expressed as the inhibition percentage of the hemolysis of sheep erythrocytes at the TCH_{50} of the control and was calculated by the following equation:

$$\textit{Anti-complementary activity}\ (\%) = [\frac{A_{control} - A_{sample}}{A_{control}}] \times 100\%$$

Wherein, $A_{control}$ and A_{sample} are the absorbances at 542 nm of test sample and control, respectively.

RESULTS AND DISCUSSION

I. *Analysis of Proximate Composition and Some Chemical Components of Zizyphus jujuba cv. Jinsixiaozao*

The proximate composition and contents of some chemical components of *Zizyphus jujuba cv. Jinsixiaozao*, such as protein, fat, ash, carbohydrate, reducing sugar and fiber were listed in Table 1. Significant amounts of protein (5.01%), ash (2.26%), as well as fat (0.37%), were observed. The main constituents were carbohydrate (81.62%), including fiber (8.40%). It is concluded that *Zizyphus jujuba cv. Jinsixiaozao* is rich in carbohydrate, and contains less protein and ash.

II. *Extraction and Fractionation of Proteoglycan by Ion-exchange and Gel Permeation Chromatography*

Ground *Zizyphus jujuba cv. Jinsixiaozao* was refluxed with ethanol to deactivate the endogenous enzymes and remove some soluble materials, including free sugars, amino acids and some phenols. Then the dried ethanol-extracted residue was extracted with distilled water at 80°C. After dialysis and precipitation with ethanol, crude water-soluble glycan (ZSG) was obtained as brownish powder. ZSG was fractionated by anion-exchange chromatography (DEAE-Sepharose CL-6B) into four peaks, which were ZSG1 recovered from the NaAc-buffer eluate, and ZSG2, ZSG3 and ZSG4 recovered from the NaCl eluate (Figure 1). ZSG1 is likely a proteoglycan with a positive or no net charge at pH 5.0, while ZSG2 is apparently a simple glycan, instead of a proteoglycan. However, both of them did not show any enhancing effect on spleen emphocytes prolifiration, while ZSG4 markedly enhanced the lymphocytes proliferation (Table 2). Therefore, ZSG4 was applied to the Sepharose CL-6B column, eluted with 0.02 M NaCl as two peaks (Figure 2). In the Chromatogram, the distribution coefficients of the two peaks (ZSG4a and ZSG4b) were 0.59 and 0.72, respectively. The two peaks were individually pooled and lyophilized, resulting in white powder. ZSG4b had higher lymphocytes proliferation activity than ZSG4a (Table 2). ZSG4b was chromatographed on a Sephadex G-200 column and a single peak was obtained when eluted with 0.02 M NaCl (Figure 3), in which the protein and the sugar peaks appeared in the same fractions with proportional absorbance, indicating that ZSG4b was homogeneous. ZSG4b was also eluted as a single symmetrical narrow peak on high-performance gel-permeation chromatography (HPGPC),

Table 1. Chemical components of *Zizyphus jujuba cv. Jinsixiaozao*

Components	Dry w.t. (%)
Protein	5.01 ± 0.05
Fat	0.37 ± 0.01
Moisture	18.99 ± 1.23
Ash	2.26 ± 0.03
Carbohydrate	81.62 ± 3.12
Crude fiber	8.40 ± 0.54

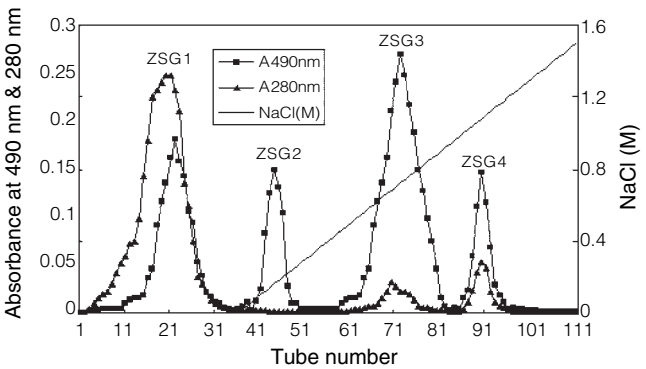

Figure 1. Chromatogram of ZSG on the DEAE-Sepharose CL-6B column eluted with a linear-gradient NaCl solution (18 mL/tube).

Table 2. Effect of ZSG1, ZSG2, ZSG3, ZSG4, ZSG4a and ZSG4b on proliferation of spleen lymphocyte *in vitro*

Sample	Concentration (μg/mL)	3H Thymidine incorporation
Control		40361 ± 1112
ZSG1	30	38361 ± 1097
	50	41035 ± 983
	100	41867 ± 1395
ZSG2	30	39876 ± 1950
	50	40533 ± 1884
	100	39575 ± 2275
ZSG3	30	40569 ± 1490
	50	49819 ± 2033
	100	68543 ± 3122
ZSG4	30	45761 ± 1387
	50	56543 ± 3009
	100	74041 ± 2980
ZSG4a	30	39976 ± 1158
	50	47761 ± 2134
	100	58219 ± 2737
ZSG4b	30	51321 ± 2935
	50	75632 ± 3384
	100	86536 ± 4321

Each value is expressed as mean ± standard deviation (n = 8).

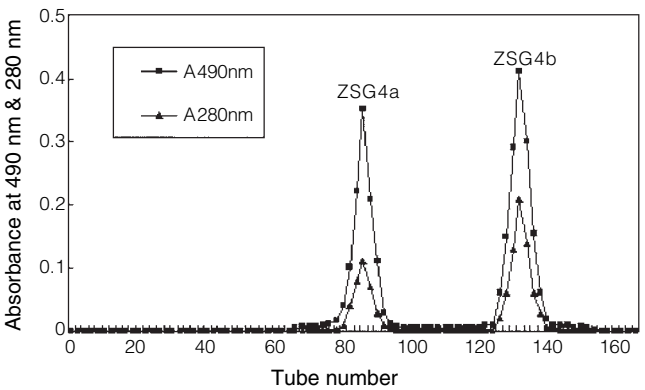

Figure 2. Chromatogram of ZSG4 on the Sepharose CL-6B column eluted with 0.02 M NaCl (4 mL/tube).

and the average molecular weight was estimated to be 1.4×10^5 Da by referring to Dextran T-series molecular weight standard (Figure 4).

III. *Carbohydrate Composition and Content*

The content of carbohydrate of ZSG4b was determined as 83.5%. GC analysis indicated that ZSG4b was composed mainly of rhamnose, arabinose, mannose and galactose in a molar ratio of 13.8:4:3:8. It also contained 29% uronic acid. ZSG4b was reduced with $NaBD_4$ to

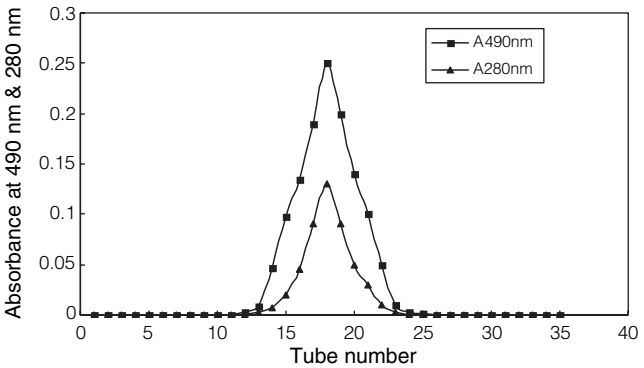

Figure 3. Chromatogram of ZSG4b on the Sephadex G-200 column by elution with 0.02 M NaCl (4 mL/tube).

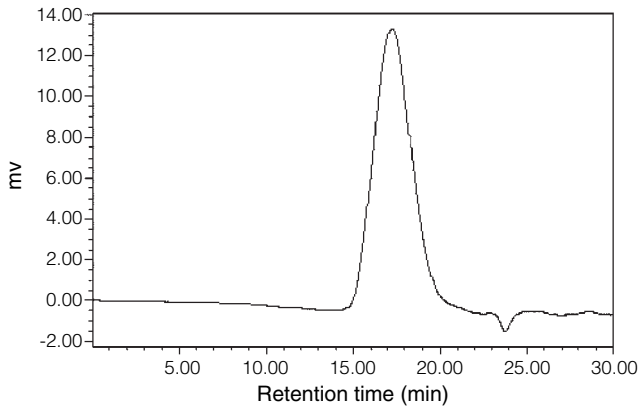

Figure 4. HPGPC profile of ZSG4b.

yield the carboxyl-reduced ZSG4b, and then hydrolysed with 2M TFA. The hydrolysate was derivatized and the alditol acetate derivative was analyzed by the method of the total cabohydrate and monosaccharides analysis. GC analysis showed an increase in the proportion to galactose compared to that in ZSG4b. The presence and content of galacturonic acid was estimated by the increase of galactose content in the carboxyl-reduced ZSG4b.

IV. *Protein Composition and Content*

The absorbance at 280 nm and positive reaction of ZSG4b in the Folin-phenol reaction indicated that ZSG4b contained a protein moiety. The content of protein of ZSG4b was determined as 9.7%. The data on amino acid composition of ZSG4b was listed in Table 3. It is rich in aspartic acid, glutamic acid, serine and threonine. Besides these amino acids, the molar ratio of leucine was also present in a fairly high value. These five amino acids constituted 62.8 % of the total amino acids.

V. *Linkage Analysis*

Carbohydrates are covalently linked through their

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Table 3. Amino acid composition of ZSG4b

Amino acid	Composition (mol %)
Alanine	4.23
Arginine	2.87
Aspartic acid	6.38
Cystine	0.55
Glutamic acid	10.02
Glycine	3.46
Histidine	1.51
Isoleucine	2.55
Leucine	5.52
Lysine	4.42
Methionine	0.50
Phenylalanine	2.82
Proline	3.56
Serine	15.49
Threonine	30.98
Tyrosine	1.59
Valine	3.55

hemiacetal hydroxyl to proteins primarily in two ways: (1) linkage to the amide nitrogen of asparagine to give N-glycosides; (2) linkage to the hydroxyl groups of serine or threonine to give O-glycosides. The O-glycosidic linkages, which link glycan with threonine and serine, are alkali labile. When treated with alkali, these O-glycosidic linkages are released by β-elimination to form unsaturated amino acids (dehydroaminobutyric acid and dehydroalanine, respectively) and the free sugar chain. The unsaturated amino acids have distinct absorbance near 240 nm. This method has been widely employed to analyze the type of linkages in proteoglycan[22]. The UV scanning spectra of ZSG4b with and without alkali treatment are shown in Figure 5. By comparison, the sample with alkali treatment did have distinct absorbance at 240 nm, showing that β-elimination reaction had taken place, which demonstrated that protein and carbohydrate were linked by O-linkage in ZSG4b.

VI. *FT-IR Spectrum*

The 1200-850 cm^{-1} region, which is referred to as the "finger print" region, is unique to a sample. For glycan this region allows the identification of major chemical groups in glycans: the position and intensity of the bands are specific for every glycan[26,27]. In order to confirm the identity of ZSG4b, it was analyzed by FT-IR. The FT-IR spectrum of ZSG4b is presented in Figure 6. The attributions of the main absorptions are characteristic of glycosidic structures and are related to CO stretching (1018 cm^{-1}); C–O–C stretching (1146 cm^{-1}) and

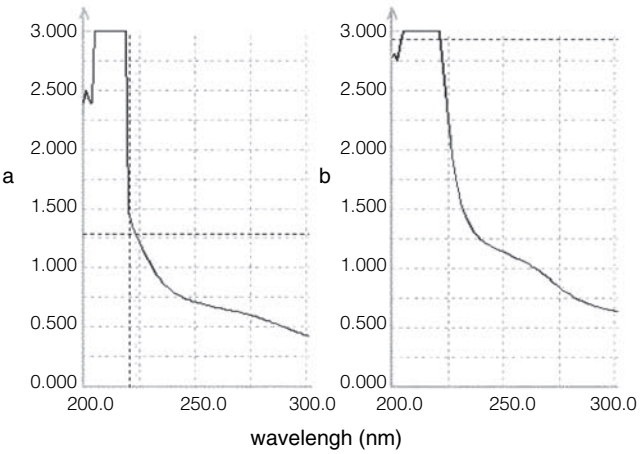

Figure 5. UV spectrum of ZSG4b (a, without alkali treatment; b, with alkali treatment).

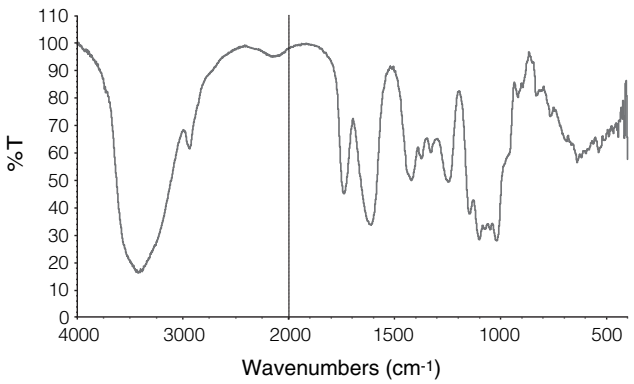

Figure 6. Fourier transform infrared spectra of ZSG4b.

anomeric C1H group vibration (918 cm^{-1}). Further analysis of the FT-IR spectra revealed that the broader band of absorption between 3600 and 2500 cm^{-1} was due to O–H stretching. The absorption at 3423 cm^{-1} is assigned to stretching of –OH groups and that one at 2938 cm^{-1} to the C–H stretching. Likewise, protein patterns were also observed with characteristic absorptions at 1612 and 1421 cm^{-1}. On the other hand, N–H vibration generally expected at 3400 cm^{-1} could be overlapped by OH stretch vibration at 3423 cm^{-1}. Protein structures could also be represented by the absorption at 1246 cm^{-1}, suggesting ZSG4b is a proteoglycan.

VII. *Anti-complement Activity In Vitro*

Anti-complement activity test is an *in vitro* test for the ability of the proteoglycan to interact with the complement cascade reaction. The complement system is part of the innate immune system and consists of a group of serum proteins, which are activated in a cascade mechanism. Activation might be initiated at the level of C3 by lipopolysaccharides (LPS) and micro-organisms (the alternative pathway) or at the level of Clq by immune

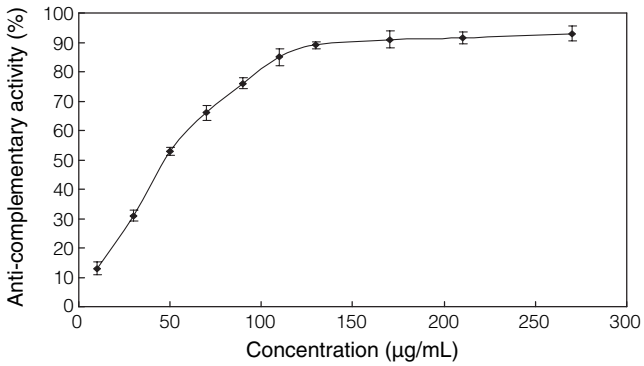

Figure 7. The anti-complement activity of ZSG4b.

complexes containing antigen and IgM or IgG antibodies (classical pathway) or by the binding to a mannose binding lectin present in serum (lectin pathway). ZSG4b had a potent anti-complement activity, 90% at a concentration of 210 μg/mL. From the dose-response curves in Figure 7, it was found that the anti-complement activity of ZSG4b was increased in accordance with the concentration below 120 μg/mL.

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