Physico-chemical Properties and Bioactivities of a Glycoconjugate LbGp5B from *Lycium barbarum* L.

PENG, Xue-Mei[a](彭雪梅) **QI, Chun-Hui**[b](齐春会) **TIAN, Geng-Yuan**[*,a](田庚元)
ZHANG, Yong-Xiang[b](张永详)

[a]*State Key Laboratory of Bio-organic & Natural Products Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, Shanghai 200032, China*

[b] *Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences, Beijing 100850, China*

A glycoconjugate designated as LbGp5B was isolated from the fruit of *Lycium barbarum* L. and purified to homogeneity by gel filtration. LbGp5B is composed of rhamnose (Rha), arabinose (Ara), galactose (Gal), glucose (Glc), galacturonic acid (GalA) and seventeen amino acids. The molecular weight of LbGp5B was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and by matrix-assisted laser desorption/ionization (MALDI) time of fight (TOF) mass spectrometry (MS). The preliminary experiments showed that LbGp5B promoted splenocyte proliferation in mice and inhibited the peroxidation of low density lipoprotein (LDL).

Keywords *Lycium barbarum L.*, glycoconjugate, activity

Introduction

Lycium barbarum L., a famous traditional Chinese herbal medicine which has functions of "nourishing the kidney and producing essence, nourishing the liver and brightening eyes", has been widely used as anti-aging health food since 2300 years ago. In the past years, our continuing effort was to isolate homogeneous glycoconjugates from the fruit of *Lycium barbarum* L. and characterize their structures as exactly as possible. Until now five glycoconjugates have been isolated. The structures of Lbp1—Lbp4 have been elucidated.[1-5] it was found that glycoconjugates showed high immunoactivity.[6] However, owing to the difficulty in collection of glycoconjugate Lbp5, it has not been studied. In this paper, we reported for the first time the isolation and purification of LbGp5B, its physico-chemical properties and bioactivities.

Experimental

Material

Lycium barbarum L. was the product of Ningxia Huizu Autonomous Region, People's Republic of China. Sephadex G-100 and CM-Sephadex C-50 were purchased from Ammersham Pharmacia Biotech. Sinapinic acid, α-cyano-4-hydroxycinnamic acid, 2,5-dihydroxybenzoic acid, concanavalin A (ConA), and lipopolysaccharide (LPS) were from Sigma. RPMI-1640 culture medium, penicillium, streptomycin, Hepes and glutamine were the products of Life Technologies Inc. (GIBCO BRL). ^{3}H-TdR was provided by Beijing Institute of Atomic Energy with 20 Ci/mol relative activity.

General

Concentrations were performed under diminished pressure at a bath temperature not exceeding 45℃. The spectrophotometer 722 for colorimetric analysis was the product of Shanghai Third Analytical Instrument Factory. High performance liquid chromatography (HPLC) was performed on Shimadzu LC-10AD equipped with BIO-

* E-mail: tiangy@pub.sioc.ac.cn

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SEP-S4000 exclusion column using water as solvent (0.4 mL/min), and the elute was monitored by RI detector. MALDI-TOF MS was conducted on BIFLEX™ III with Auto Xecute™ software. Capillary electrophoresis (CE) was performed on Water Quanta 4000 E using 0.1 mol/L boric acid-sodium hydroxide buffer (pH = 10) as solvent, detected at 20 V and 254 nm. Gas chromatography of the alditol acetate derivative was conducted on Varian VISTA 402 equipped with 3% OV-225 capillary column (0.3 mm × 25 m). The GC oven was held at 65℃ for 1 min before being increased to 290℃ at a rate of 8℃/min.

Isolation and purification of LbGp5B

The air-dried *Lycium barbarum* L. (500 g) was dispersed in 1500 mL of distilled water overnight, and filtrated. The residue was further dispersed in 750 mL of water for 6 h. At the same time, the pH of the mixture was kept at 7 with 1 mol/L NaOH with vigorous stirring. The extraction was concentrated to a small volume under diminished pressure at a bath temperature not exceeding 45℃, and then centrifuged. The supernatant was treated by 4 volume of 95% alcohol, and deposited overnight. After centrifugation, the precipitate was collected and dissolved in a small quantity of water. Savage reagent (chloroform: *n*-butanol = 4:1 (*V*/*V*)) was used to remove the dissociated proteins in solution. After being dialyzed against tap water for 2 d, the supernatant was vacuum-dried. The crude glycoconjugate named LBP was thus obtained. LBP was chromatographied on DEAE-Cellulose column eluted with water and then a linear $NaHCO_3$ gradient up to 0.5 mol/L, monitored by phenol-sulfuric acid assay at 490 nm[7] and by UV absorption at 280 nm. The fraction eluted with 0.5 mol/L was named Lbp5, which was further chromatographied on Sephadex G-100 column (0.75 cm × 115 cm) eluted with 0.1 mol/L NaCl at a flow rate of 0.8 ml/L, monitored by the same methods as above.

Determination of the molecular weight (M_r)

MALDI mass spectra were recorded on a BIFLEX™ III MALDI-TOF mass spectrometer. A solution of analyte mixed with sinapinic acid, α-cyano-4-hydroxycinnamic acid or 2, 5-dihydroxybenzoic acid and bovine insulin was applied to the sample plate and air-dried. Mass calibration was performed using the molecular ions from the bovine serum albumin at 66437 Da, bovine insulin at 5734.5 Da, and the matrix at 379.1 or peptide at 1209.3 Da as internal standards. Raw data were analyzed by using computer software provided by the manufacturer and are reported as average masses.

SDS-polyacrylamide gel electrophoresis

SDS-PAGE was performed according to the method of Laemmli.[8] Tris(hydrozymethyl)aminomethane – glycin (pH = 8.3) was used as running buffer. The electric current was controlled below 40 mA. The voltage of concentrated gel was 80 V, while that of separated gel was 140 V. After electrophoresis, proteins in the gel were stained with 0.1% by Coomassie blue brilliant R-250, 10% acetic acid and 30% methanol for 30 min and the destain was carried out in 10% acetic acid and 30% methanol.

Monosaccharide composition of LbGp5B

The sample was hydrolyzed in 1.0 mol/L H_2SO_4 at 100℃ for 4 h, neutralized with barium carbonate, reduced to alditol by $NaBH_4$ with trace ammonia solution (25%), and acetylated with acetic anhydride/pyridine (*V* : *V* = 1 : 1) at room temperature overnight.[9] The alditol acetate derivative after hydrolysis was analyzed by GC.

Effect of LbGp5B *on splenocyte proliferation*

^{3}H-TdR incorporation assay method[10] was used. Splenocytes were prepared from the spleens of mice killed by cervical dislocation under sterile condition. The mice spleens were gently pressed to release splenocytes and passed through stainless mesh (50 μm) and washed . Splenocytes were released by teasing into RPMI-1640 medium supplemented with 10% fetal calf serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin. The viable cells were then counted using a trypan blue dye exclusion test and were adjusted to 1×10^6 cell/mL for culture. The single cell suspending liquid was plated in a 96-well plate. After being cultured for 18 h, various concentrations of LbGp5B or (and) ConA/LPS were added to each well and cultured for another 72 h. Then ^{3}H-TdR was added to each well and

incubated for another 2.5 h. RPMI-1640 medium was used instead of LbGp5B in control group. The result was expressed in mean.

Inhibitory effect of LDL *oxidation*

After being isolated and extracted from human plasm, LDL oxidation was performed with a modification of the method described by Esterbauer *et al*.[11] Incubation was performed with 400 g/mL LDL protein supplemented with water solution of each sample. Oxidation was initiated by the addition of Cu^{2+} with different concentration as a pro oxidant, at the same time, LbGp5B was added. Thereafter, LDL was incubated at 37℃ for 18 h and dialyzed at 4℃ for 48 h with phosphate buffer saline (PBS). Then the resultant was added to 2 mL of 0.335% thiobarbituric acid (dissolved in 10% trichloroacetic acid), heated for 15 min in 95℃. When cooled to room temperature, the thiobarbituric acid reactive substance (TBARS) was extracted with 2 mL of *n*-butanol. The fluorescent absorption was detected at 535 nm.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed according to the method of Amoresano.[12] Tris-HCl (pH = 8.6) was used as running buffer and the concentration of agarose in gel was 0.5%. The electric current was controlled below 9 mA. After electrophoresis, proteins in the gel were stained with sudan black B for 30 min.

Results and discussion

Lbp5 (327 mg) was isolated from *Lycium barbarum* L. (500 mg) as experimental described. After being futher chromatographied on Sephadex G-100 column, Lbp5 was separated into three parts (Fig. 1). Using CM-Sephadex C-50 column, sub-fraction Lbp5B was purified to obtain a glycoconjugate named LbGp5B (Fig. 2). HPLC and CE detected the homogeneity of LbGp5B.

Elemental analysis of LbGp5B was C 43.82%, H 6.90% and N 9.18%. Neutral monosaccharide composition of LbGp5B was determined by gas chromatography. Rha, Ara, Glc and Gal were detected in a molar ratio of 0.1:1:1.2:0.3. Its galacturonic acid content is up to 9.0% as determined by *m*-hydroxydiphenyl-sulfuric acid assay. The result of amino acid analysis was showed in Table 1.

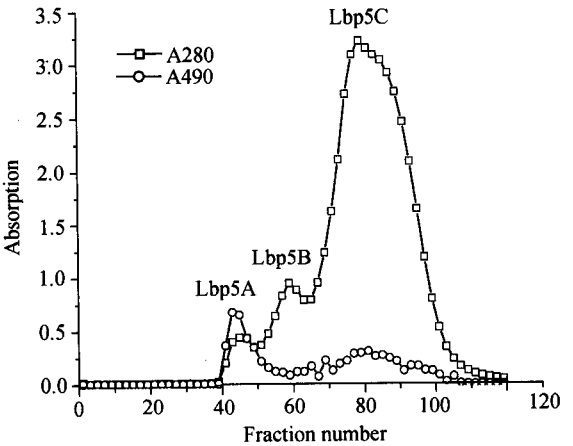

Fig. 1 Isolation of Lbp5 on Sephadex G-100 column (0.75 cm × 115 cm) eluted with 0.1 mol/L NaCl at a flow rate of 0.5 mL/L, 7 min/tube. A280: adsorbtion at 280 nm; A490: adsorbtion at 490 nm.

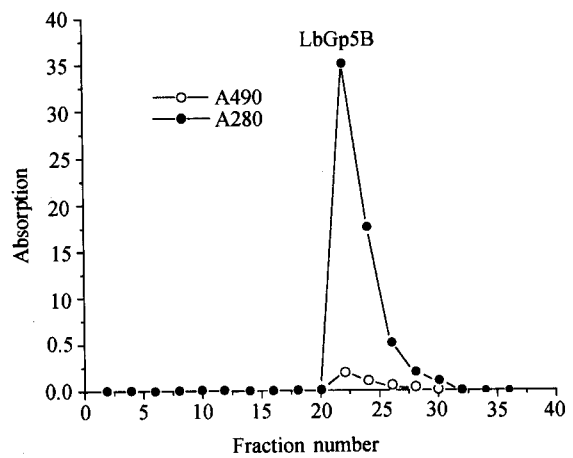

Fig. 2 Purification of Lbp5B on CM-Sephadex C-50 column (0.75 cm × 115 cm) eluted with 0.1 mol/L NaCl at a flow rate of 0.5 mL/L, 7 min/tube. A280: adsorbtion at 280 nm; A490: adsorbtion at 490 nm.

Table 1 Amino acid content in LbGp5B

Amino acid	Asp	Thr	Ser	Glu	Pro	Gly
Content (%)	13.70	5.76	6.95	11.32	4.87	1.055
Amino acid	Ala	Cys	Val	Met	Ile	Leu
Content (%)	6.46	1.91	6.54	0.23	4.17	8.65
Amino acid	Tyr	Phe	Lys	His	Arg	
Content (%)	3.65	3.97	5.91	1.19	4.18	

To determine the molecular weight (M_r), two kinds of methods were used and they came to the same conclusion. First, LbGp5B was directly analyzed by mass spectrometric methodologies in order to obtain the information on the overall molecular mass and the distribution of glycoforms.[12] Unfortunately, attempts to measure the accurate molecular mass of the glycoconjugate by electrospray mass spectrometry (ESMS) were probably unsuccessful because of the high content of glycans. LbGp5B was then analyzed by MALDI-TOF-MS producing the spectrum shown in Fig. 3. Two pairs of broad peaks could be identified in the spectrum, each pair corresponding to the MH^+ and MH_2^{2+} ions, respectively. The broadening of the signals was very likely due both to a large heterogeneity of the glycoforms and to the presence of unresolved photochemically generated matrix adduct ions.[13] A molecular mass centered at about 21567 Da could be expected. Second, SDS-polyacrylamide gel electrophoresis analysis of the purified LbGp5B showed that the purified fraction is homogeous, giving a single band of 23.7 kDa (Fig. 4).

As measured using ^{3}H-TdR incorporation assay, LbGp5B could promote splenocyte proliferation in LACA mice whenever ConA was used as stimulant or not. The same situation appeared when LPS was used (Table 2).

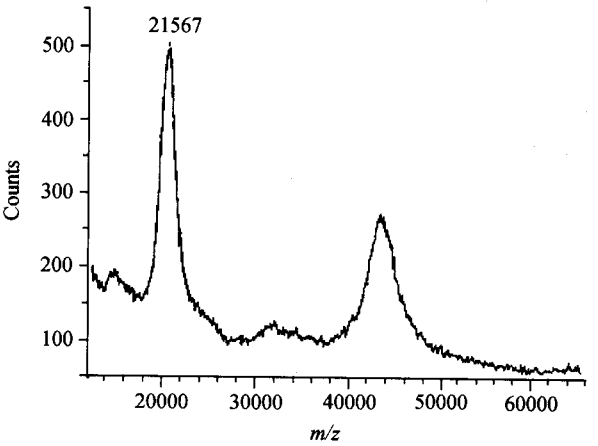

Fig. 3 MALDI/MS spectrum of LbGp5B. The single- and double-charged ions are reported.

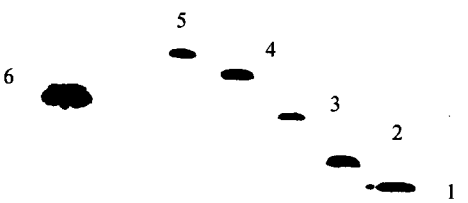

Fig. 4 SDS-PAGE of LbGp5B. The molecular weight markers are: (1) Albumin Bovine (66000); (2) Albumin Egg (45000); (3) Trypingen (24000); (4) β-Lactoglobulin (18400); (5) Lysozyme (14300); (6) LbGp5B (23700).

Table 2 Effect of LbGp5B on splenocyte proliferation in mice *in vitro*

Group	Dose (μg/L)	^{3}H-TdR incorporation (cpm, $x \pm s$)		
		(−)	ConA (+)	LPS (+)
Control	0	1166.5 ± 217.3	30823.5 ± 4085.4	7071.5 ± 1094.2
LbGp5B	10	936.5 ± 272.0[a]	41534.5 ± 3334.9[a]	8571.0 ± 822.8
	50	2465.5 ± 569.3[a]	37647.5 ± 7409.4	10610.5 ± 1175.1[a]
	100	5125.5 ± 1838.4[a]	48674.5 ± 4810.3[a]	11131.5 ± 982.0[a]

[a] $P < 0.01$ vs. control (0), t-test, $n = 3$.

LDL oxidation was induced by incubation of the LDL solution with metal ions, which catalyze a LDL peroxidation process. Accumulation of TBARS in LDL indicates the transformation of native LDL to oxidized LDL. After incubation of LDL for 18 h with Cu^{2+} ion, TBARS formation in the LDL was increased 6 fold compared with that before the incubation. However, after adding 10 μg/L LbGp5B to the LDL oxidation system, the LDL peroxidation was almost completely suppressed. The inhibitory effect showed a dose-dependent inhibition (Fig. 5).

After being oxidized, LDL carried more anion so that the migrating rate of its agarose gel electrophoresis was fastened.[14-15] The migrating rate was reduced in different degree when LbGp5B was added. It revealed that LbGp5B could directly inhibit the oxidation of LDL.

It is well established that the oxidation of LDL takes place as a key event in the development of atherosclerosis (AS).[16] The oxidized LDL could speed up

the formation of atherosclerosis rapidly. At present, two kinds of medicine are used to cure AS, one of them is anti-oxidizing medicine, which could inhibit or slow down the occurrence and development of AS by inhibiting LDL oxidation. Our results suggest the possibility that LbGp5B is useful for prevention of atherscleosis.

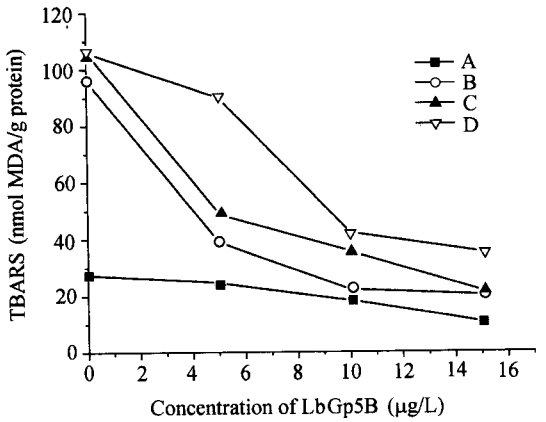

Fig. 5 Effect of LbGp5B on the oxidation of LDL. Cu^{2+} concentration: A: 3 μmol/L; B: 6 μmol/L; C: 9 μmol/L; D: 12 μmol/L.

In conclusion, LbGp5B exhibited significant immuno-modulaory activities and anti-LDL peroxidation effects in the present study. The structure and various biological activities of LbGp5B are of considerable interest for the food industry and for medicine. Further work is needed to determine the structure of LbGp5B.

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