ISSN 1330-9862
(FTB-2236)

scientific note

New Polysaccharide Compounds Derived from Submerged Culture of *Ganoderma lucidum* and *Lycium barbarum*

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Received: February 8, 2009
Accepted: July 14, 2009

Summary

Lycium barbarum and *Ganoderma lucidum*, highly valued Chinese medicinal fruit and mushroom, have attracted more and more attention because of the antitumour activities they have shown, and polysaccharides are considered their most important functional constituent. Forming new compounds of *L. barbarum* polysaccharides, isolated from dried *L. barbarum* fruits, and *G. lucidum* polysaccharides, derived from a submerged culture of *G. lucidum*, has been investigated in this paper. Our data illustrate that two polysaccharide compounds, fermented polysaccharides and mixed polysaccharides, at the appropriate ratio and concentration have stronger free radical scavenging ability than the single polysaccharide. Ion exchange chromatography and high-performance liquid chromatography (HPLC) analyses indicated that the components of the new polysaccharide compounds had changed compared to those of the single polysaccharide. In addition, similar fractions were shared with the two polysaccharide compounds. Hence, the findings demonstrate that these new polysaccharide compounds might have stronger bioactivity than a single polysaccharide and could be obtained more easily by fermentation.

Key words: *Ganoderma lucidum*, *Lycium barbarum*, polysaccharide compounds, free radical scavenging activity

Introduction

Lycium barbarum, a well-known traditional Chinese medicinal plant, was documented in a traditional Chinese medicinal monograph about 2300 years ago and has long been used in prevention and treatment of various human diseases (*1–3*). Nowadays, *L. barbarum* fruits have been widely used as a popular functional food with a large variety of beneficial effects, such as reducing blood glucose and serum lipids, antiaging, immunomodulating, anticancer, and so on (*4–7*). Some constituents of *L. barbarum* fruits have been chemically investigated, especially *L. barbarum* polysaccharide components, which are the most important functional factor (*8*). Five polysaccharides (LbGp1–LbGp5) have been isolated and structurally documented so far (*9*).

Ganoderma lucidum, a basidiomycete white rot fungus belonging to the Ganodermataceae family, has been widely used for medicinal purposes to promote health and longevity in China, Japan and Korea for thousands of years (*10*). This mushroom is considered to be a popular folk medicine for prevention or treatment of various diseases including hepatitis, hypertension, hypercholesterolemia, gastric cancer, arthritis and bronchitis. Recent studies on this fungus have also revealed its various positive biological activities, including antitumour and hypoglycemic activity, or anti-inflammatory effects and cytotoxicity towards hepatoma cells. The dried powder of *G. lucidum* is currently used worldwide as dietary supplement (*11,12*). The most attractive property of *G. lucidum* is its antitumour effect, which has been demonstrated to be mainly associated with its polysaccharides,

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or protein/peptide-bound polysaccharide fraction (*13*). *G. lucidum* polysaccharides can suppress the activity of colon cancer cells and they seem to act as a potent chemopreventive agent for colon carcinogenesis (*14*). Moreover, although *G. lucidum* is traditionally cultivated in solid culture, submerged fermentation technology for mycelial cultures of *G. lucidum* has been developed recently and has attracted much attention as a promising alternative because it has none of the disadvantages of solid culture, such as the long time required to cultivate the fruiting body of *G. lucidum*, the difficulty of controlling the quality of the product, and the high susceptibility of the culture to environmental changes (*15–17*). In this study, polysaccharides from *G. lucidum* have been obtained by fermentation in the submerged culture.

Although polysaccharides from both *L. barbarum* and *G. lucidum* have similar and important pharmacological functions, reports on using them together to obtain polysaccharide compounds with higher bioactivity are very rare (*18*). The objectives of our study are to evaluate whether two polysaccharide compounds, fermented polysaccharides and mixed polysaccharides, have better bioactivity than a single polysaccharide, and to assess whether the structure of the two polysaccharide compounds is similar and if they can be obtained by fermentation.

Materials and Methods

Materials and microorganism

Lycium barbarum fruits, growing in Ningxia, a well-known production area for *L. barbarum* in China, were from Ning Xia Chinese Wolfberry Group Company (Ningxia, PR China) and purchased from a local market. Strain *Ganoderma lucidum* CCDM 2267 used in this study, which was preserved in potato sucrose agar slant at 4 °C, was provided by Huazhong Agricultural University, Wuhan, PR China.

Chemicals

Phenol was from Guangzhou BoLi Bio-Tech Co., Ltd. (Guangzhou, PR China). Oil of vitriol, chloroform and methanol were obtained from Wuhan Yatai Chemical Reagent Co., Ltd. (Wuhan, PR China). Ethanol (95 %) was purchased from Tianjing Standard Science and Technology Co., Ltd. (Tianjing, PR China). Other chemicals and reagents used were obtained from Tianjing Damao Chemical Instrument Supply Station (Tianjing, PR China). The water used in the experiment was deionized water.

Media

Three different growth media were used and the compositions of these media were (in g/L): potato sucrose agar slant medium (PSAM)-potato extract 20, sucrose 2 and agar 2; potato sucrose liquid medium (PSLM)-potato extract 200, sucrose 20, without agar; *L. barbarum* submerged medium (LBSM)-*L. barbarum* juice 0.05, $MgSO_4$ 0.225 and KNO_3 0.159, bran extract 1 %, pH natural. The *L. barbarum* juice was obtained by cleaning the good berries, submerging them and crushing, after which the seeds were filtered to obtain the juice.

Extraction of polysaccharides

Extraction of *L. barbarum* polysaccharides

L. barbarum berries were dried at 60 °C and ground to fine power. The powder samples were refluxed to remove lipids with methanol/chloroform solvent (1:2) (by volume). After filtering, the residues were air-dried, refluxed twice again with 80 % ethanol, extracted three times in boiled water for 2, 1 and 0.5 h, respectively, and filtered. The combined filtrates were concentrated by a rotary evaporator at 60 °C and precipitated using 95 % ethanol, 100 % ethanol and acetone, respectively. After filtering and centrifuging, the precipitate was collected and vacuum-dried. The dried *L. barbarum* polysaccharides obtained were stored in a refrigerator for future use.

Extraction of *G. lucidum* polysaccharides

After a five-day incubation in PSLM, the mycelia of *G. lucidum* CCDM 2267 were obtained and filtered, then the filtrates (V1) were combined. The obtained mycelia were dried at 35 °C and powdered, then the powder was extracted three times with distilled water for 1 h and filtered again to obtain the filtrates (V2). The two combined filtrates (V1 and V2) were concentrated, refluxed with methanol/chloroform (1:2, by volume) for 4 h and then chloroform/*n*-butanol (4:1, by volume) for 4 h, concentrated again and precipitated using 95 % ethanol. The precipitate was dissolved in water and dialyzed for 24 h, then centrifuged and concentrated. At last, the precipitate was collected and vacuum-dried. The dried *G. lucidum* polysaccharides obtained were stored in a refrigerator for future use.

Extraction of fermented polysaccharides

Actively growing mycelia of *G. lucidum* CCDM 2267 were obtained from a newly prepared agar plate culture after it was incubated at 28 °C for 7 days. The mycelia (10 %, by volume) were inoculated into a 250-mL Erlenmeyer flask containing 100 mL of LBSM and incubated at 150 rpm and 28 °C for 5 days. The sample was attained and filtered using a filter fabric after homogenization. The residue was dried at 35 °C and extracted with distilled water three times, the combined extract and filtrates were concentrated with rotary evaporator, refluxed with methanol/chloroform (1:2, by volume) for 4 h and chloroform/*n*-butanol (4:1, by volume) for 4 h, then concentrated and precipitated using 95 % ethanol. The precipitate was dissolved in water and dialyzed for 24 h, then centrifuged and concentrated. Finally, the precipitate was collected and vacuum-dried. The dried fermented polysaccharides obtained were stored in a refrigerator for future use.

Production of compounded polysaccharides

Mixed polysaccharides

Mixed with the polysaccharides from *L. barbarum* and *G. lucidum* at the mass ratios of 1:1, 1:3, 1:5, 3:1 and 5:1, these mixtures were prepared in different concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 g/L) to determine the free radical scavenging ability, and the components of the new polysaccharide compounds (LBP/GLP=1:1) were further studied.

Fermented polysaccharides

G. lucidum CCDM 2267 was inoculated into the submerged medium (LBSM) with *L. barbarum* polysaccharides by fermentation. The process of biological combining of polysaccharides was as follows: active mycelia of *G. lucidum* CCDM 2267 at 10 % (by volume) were inoculated into a 250-mL Erlenmeyer flask containing 100 mL of LBSM and incubated at 28 °C (150 rpm) for 5 days. The fermented polysaccharides, extracted from the mycelia and filtrates, were used to determine the free radical scavenging ability.

Purification and detection of polysaccharides

Column chromatography was performed with DEAE--cellulose (2.6×30 cm). Volumes of 10 mL of the extracts (different polysaccharides obtained) at 400 mg/L were applied. The column was eluted with deionized water, NaCl at different concentrations (0.05, 0.10 and 0.50 M), $NaHCO_3$ (0.3 M) and NaOH (0.1 M) successively at the flow rate of 30 mL/h. The isolated fractions were measured by the phenol-sulphuric acid method. Finally, different fractions of isolated polysaccharides were obtained and lyophilized for 48 h for other assays.

The obtained fractions were analyzed with a high performance liquid chromatography (HPLC) system (Agilent 1100) equipped with Aqueous GPC Start up Kit column and eluted with distilled water at a flow rate of 1.0 mL/min at 20 °C. The separated components were monitored by a refractive index detector. The column was calibrated with different molecular mass standard glucosans from Sigma-Aldrich, USA and a standard curve was then established (*19*).

Analysis of free radical scavenging ability

This assay was based on the scavenging of stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals by polysaccharide. The free-radical scavenging activity of extracts was assayed by the DPPH method as described by Abe *et al.* (*20*).

Measurement of polysaccharides

The amount of polysaccharides was determined with the phenol-sulphuric acid method, using glucose as the standard (*21*).

Statistical analysis of the data

The results were presented as mean±S.D. of three determinations. Statistical analyses were performed using *t*-test and one way analysis of variance. Multiple comparisons of means were done by the LSD (least significance difference) test. A probability value of $p<0.05$ was considered significant.

Results and Discussions

Extraction of different polysaccharides

As shown in Table 1, significant difference was observed in the content as well as the purity of different polysaccharides extracted. In detail, the polysaccharide content of *L. barbarum* fruit is 0.131 g/g and the purity is 75.82 %, in addition, the contents of *G. lucidum* polysaccharides and fermented polysaccharides were 1.262 and 2.831 g/L, and their purities were 93.71 and 94.11 %, respectively. The extract content and purity of fermented polysaccharides have a bigger increase than those of *G. lucidum* polysaccharides, possibly because the strain *G. lucidum* CCDM 2267 was cultured in two different media (PSLM and LSDM).

Table 1. Content and purity of different extracted polysaccharides

	G. lucidum polysaccharides	*L. barbarum* polysaccharides	Fermented polysaccharides
Content	1.262 g/L	0.131 g/g	2.831 g/L
Purity/%	93.71	75.82	94.11

Free radical scavenging ability of mixed polysaccharides

It is noteworthy that free radical scavenging ability of mixed polysaccharides is stronger than that of single polysaccharide at the same concentration as shown in Table 2. For instance, the free radical scavenging rates of single polysaccharides from *L. barbarum* and *G. lucidum* at the concentration of 0.4 mg/mL are 26.67 and 25.08 %, respectively, while 32.06, 29.18, 28.24 and 31.64 % of free radical scavenging could be obtained when these polysaccharides form a compound at different ratios (LBP/GLP=1:1, 1:3, 3:1 and 1:5, respectively) at the same concentration. However, the free radical scavenging ability of mixed polysaccharides might be worse than of a single polysaccharide at the same concentration, for example, when the concentration is 0.1 mg/mL, the radical scavenging activity of polysaccharide compound (LBP/GLP=3:1) is 11.76 %, but the corresponding radical scavenging abilities of polysaccharides from *L. barbarum* and *G. lucidum* are 12.11 and 14.71 %, respectively. These results indicate that mixed polysaccharides could show stronger scavenging ability at the appropriate concentration and ratios.

Liu *et al.* (*22,23*) reported that the antitumour effects of polysaccharide compounds from fungi are better than a single polysaccharide. In addition, Lonseny *et al.* (*24*) also documented similar views. Our study as well suggests that the mixed polysaccharides show stronger bioactivity, the reasons might be the following: one is the synergism of different components; the other is the change of conformation or configuration of polysaccharide compounds, because the bioactivity of polysaccharides is in correlation with their conformation (*25*). It is known that the different conformations of polysaccharides coexist when polysaccharides are submerged in water, then high polymers are formed by combination of hydrogen bonds among different polysaccharides. Alternatively, the cooperation among different polysaccharides might be obtained, which leads to the improvement of bioactivity (*23–26*). However, the question is why the mixed polysaccharides have stronger bioactivity only at optimum ratio and concentration. According to the theory of traditional Chinese medicine, the compatibility of prescriptions is the key. The dose and concentration are the most important factors in the treatment of an illness

Table 2. Free radical scavenging activity of single polysaccharides and mixed polysaccharides

γ_0/(mg/mL)	Free radical scavenging of different polysaccharides/%						
	LBP	GLP	LBP/GLP (1:1)	LBP/GLP (1:3)	LBP/GLP (3:1)	LBP/GLP (1:5)	LBP/GLP (5:1)
0.1	14.71±2.42	12.11±0.30	23.75±0.91	21.30±1.11	11.76±2.90	26.66±1.91	8.14±1.01
0.2	19.05±3.63	17.04±0.91	25.77±0.60	24.29±0.21	18.82±1.31	27.25±3.60	11.30±2.30
0.3	24.34±2.11	21.65±1.21	28.33±1.11	28.22±2.01	21.50±2.21	30.69±1.61	17.85±2.20
0.4	26.67±1.91	25.08±0.20	32.06±1.41	29.18±0.00	28.24±1.10	31.64±1.20	22.37±1.91
0.5	28.57±0.00	28.30±0.00	32.80±0.00	31.84±1.61	29.63±0.30	35.19±1.30	28.14±0.00
0.6	31.64±0.20	31.40±0.10	34.61±0.51	34.61±0.51	30.37±2.10	36.02±1.40	30.40±2.90
0.7	32.28±0.70	32.69±0.10	36.10±1.61	36.10±0.31	34.76±0.41	39.57±2.31	32.09±3.10
0.8	31.22±0.30	32.69±0.70	38.87±0.31	38.02±0.51	37.65±4.01	38.74±0.90	37.29±1.71
0.9	33.65±0.91	34.73±1.01	39.40±2.01	37.81±0.91	39.14±0.61	37.56±0.90	39.89±1.20
1.0	32.80±0.30	36.33±2.01	38.34±0.31	36.00±5.01	40.43±1.21	37.20±3.31	40.45±1.41

γ_0 – concentration, LBP – *Lycium barbarum* polysaccharides, GLP – *Ganoderma lucidum* polysaccharides

although the reasons responsible for that have not been fully elucidated (*27,28*). The polysaccharides from *L. barbarum* and *G. lucidum* are also used as traditional Chinese medicine; the compatibility law may also be applied in forming new polysaccharide compounds. Therefore, the optimum ratio and concentration are necessary.

Free radical scavenging ability of fermented polysaccharides

The bioactivity of fermented polysaccharides from *G. lucidum* CCDM 2267 fermented in LSDM is shown in Fig. 1. At the concentrations of 0.1 and 0.2 mg/mL, compared to a single polysaccharide from *L. barbarum* or *G. lucidum*, the fermented polysaccharides result in a significant increase of free radical scavenging rate, which is 19.76 and 23.02 %, respectively. Fig. 1 also shows that the radical scavenging activity could reach up to 41.3 % at the concentration of 0.9 mg/mL and the free radical scavenging rate increases with the increase of concentration, although the radical scavenging activity decreases at the concentration of 1.0 compared with 0.9 mg/mL. In addition, at the concentration ranging from 0.3 to 0.8 mg/mL, the fermented polysaccharides show similar bioactivity of radical scavenging activity with single *G. lucidum* or *L. barbarum* polysaccharide. These results suggest that fermented polysaccharides show better activity only at optimum concentration. The improved bioactivity of fermented polysaccharides might be due to the structural change.

Purification of different polysaccharides

Crude polysaccharides were suspended in water with stirring. The insoluble polysaccharides were removed by filtration and soluble polysaccharides were separated by ion-exchange chromatography on DEAE-cellulose. Chromatography results of different polysaccharides are shown in Fig. 2. Four and three peak fractions appeared in eluted profiles of polysaccharides from *L. barbarum* and *G. lucidum*, respectively. In contrast, new fractions appear on the eluted profiles of mixed compounds (LBP/GLP=1:1) and fermented polysaccharides, whose fraction amounts are six (C1, C2, C3, C4, C5 and C6) and five (F1, F2, F3, F4 and F5), respectively. The results suggest that the mixed and fermented polysaccharides had different fractions from the single polysaccharides and the reasons might be due to the interaction among groups or branched chains of polysaccharides in the process of mixing and fermentation (*29–30*), which leads to the change of conformation or structure of polysaccharide components of the compounds.

To investigate the structural characteristics of mixed and fermented polysaccharides, the concentrations of polysaccharides in the eluate were determined. Different peak fractions of mixed polysaccharides (LBP/GLP=1:1) and fermented polysaccharides, with different molecular mass, were collected for further analyses.

HPLC analysis of mixed and fermented polysaccharides

Some papers have reported the molecular mass of different fractions from *L. barbarum* and *G. lucidum* polysaccharides (*9,12,29*), while in this paper, only the dis-

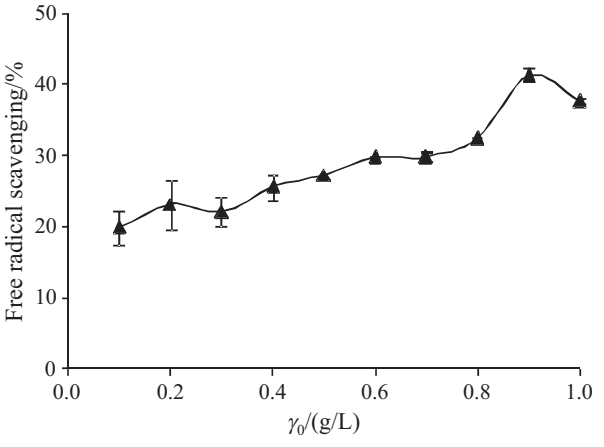

Fig. 1. Free radical scavenging of the fermented polysaccharides at different concentrations

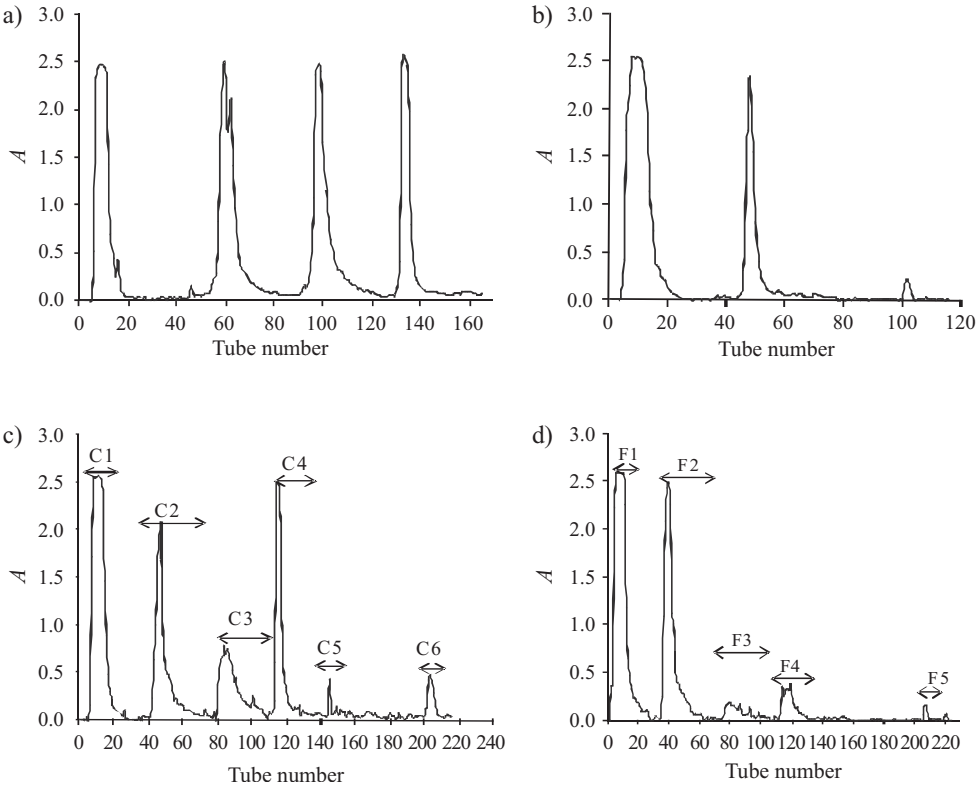

Fig. 2. Elution profiles of different polysaccharides extracted using DEAE-cellulose column (graphs a, b, c and d are elution profiles of *Lycium barbarum* polysaccharides, *Ganoderma lucidum* polysaccharides, mixed polysaccharides (LBP/GLP=1:1) and fermented polysaccharides, respectively; C1, C2, C3, C4, C5 and C6 indicate different fractions of mixed polysaccharides (LBP/GLP=1:1); F1, F2, F3, F4 and F5 are different fractions of fermented polysaccharides)

crimination between different fractions from mixed (LBP/GLP=1:1) and fermented polysaccharides was considered.

HPLC was applied to elucidate the relative molecular mass (M_r) of the polysaccharides. Different independent peaks were identified and the M_r values were determined; the first three fractions of mixed (LBP/GLP=1:1) and fermented polysaccharides are shown in Fig. 3. There are two or more peaks in Figs. 3a–d, which suggests that these collected fractions are not pure. According to the glucosan standard, the M_r of $F1_{(6.97)}$, $F1_{(9.472)}$, $F2_{(6.928)}$ and $F2_{(8.507)}$ are 764.54, 504.03, 851.14 and 12.86 kDa respectively. Figs. 3b and 3d show that the corresponding M_r are 1305.3, 333.47, 796.16 and 15 kDa, when the retention times of fermented polysaccharide are 6.759, 9.393, 6.954 and 8.434 min respectively. Fractions F3 and C3 share similar M_r, which are 10.12 and 9.11 kDa, respectively. Generally, the bioactivity of polysaccharides is significantly correlated with the relative molecular mass. Polysaccharides with high relative molecular mass can increase water solubility and result in more effective antitumour activity (*31*). It could be speculated that the obtained mixed and fermented polysaccharides have other important bioactivities besides free radical scavenging ability. From the descriptions above, it can be concluded that the mixed polysaccharides have some similar fractions as fermented polysaccharides, and polysaccharide compounds have improved bioactivity.

Different micromolecular fractions were obtained, for example, fractions F4 and C4 have similar M_r, which are 2.26 and 2.36 kDa. Fig. 4 shows that fractions F5 and C6 cannot be determined because their amounts or mixed micromolecular fractions are very low. The similar M_r could also be reflected in mixed and fermented polysaccharides in Fig. 4.

Fig. 5 shows HPLC profile of the fifth fraction (C5) of mixed polysaccharides (LBP/GLP=1:1), and its M_r is 1.16 kDa. However, the fraction does not exist in fermented polysaccharides. Compared with Fig. 2, there is an obvious decrease in the absorbance of fractions F3, F4 and F5, in comparison with the corresponding similar fractions of C3, C4 and C6. In addition, C5 disappears from the eluted profiles of fermented polysaccharides, suggesting that these similar fractions might be utilized by *G. lucidum* CCDM 2267 to satisfy its own growth.

Comparing all fractions of mixed and fermented polysaccharides, the results also indicate that similar polysaccharide fractions are shared by both polysaccharide compounds. However, during the process of forming compounds of polysaccharides, the extraction of a single polysaccharide needs to be done and long time is required for purification, but the fermented polysaccharides have none of the weaknesses stated above. Apparently, the fermented polysaccharides could be attained more easily although the fermentation time should be controlled carefully, because *G. lucidum* CCDM 2267 can utilize some polysaccharides to provide nutrition for its own growth. Hence, polysaccharide compounds attained

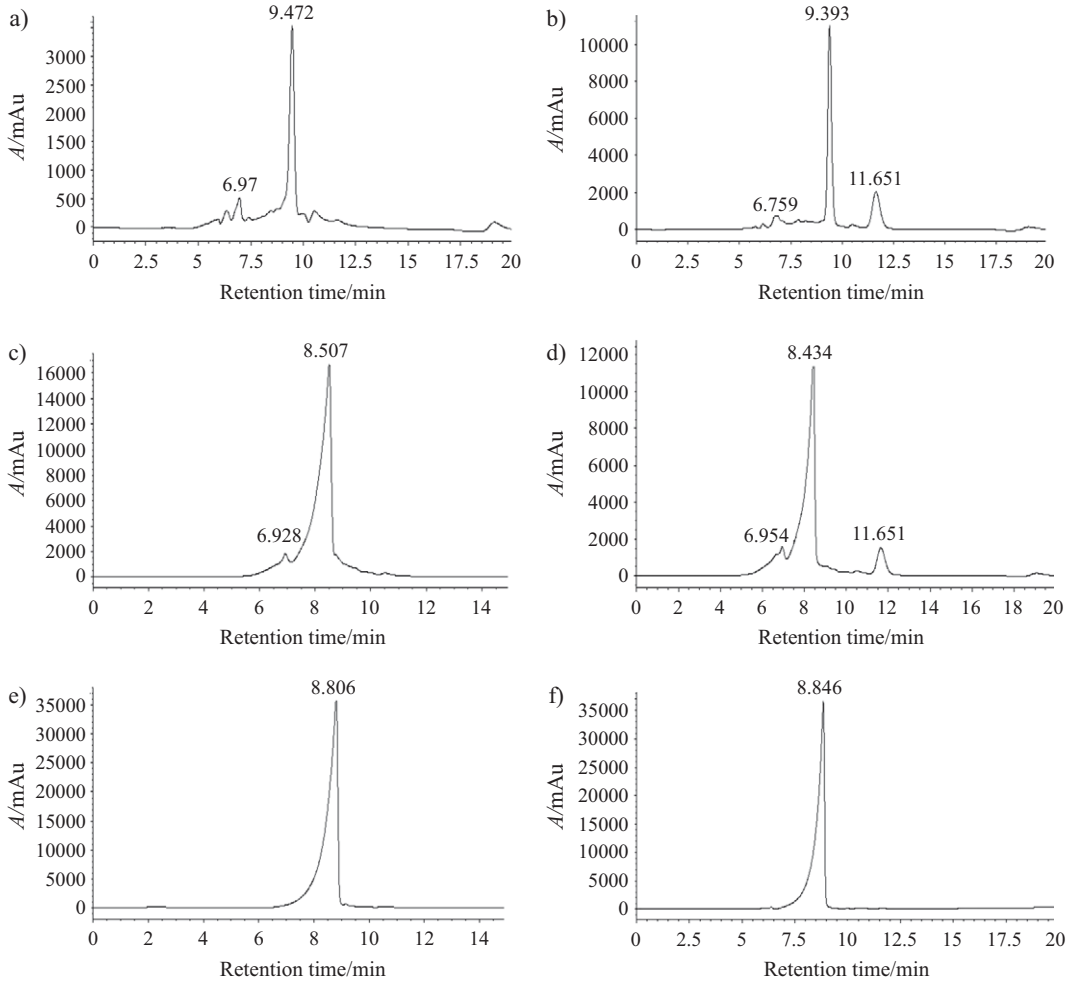

Fig. 3. HPLC profiles of the first three fractions of mixed polysaccharides (LBP/GLP=1:1) and fermented polysaccharides (graphs a, b, c, d, e and f are HPLC profiles of F1, C1, F2, C2, F3 and C3, respectively)

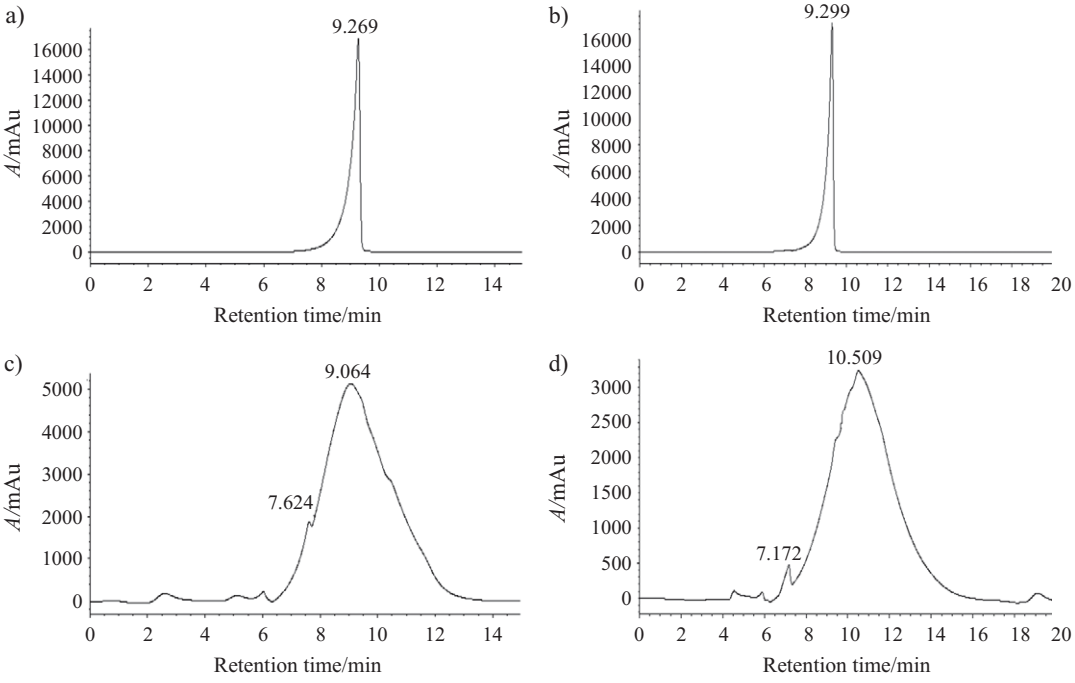

Fig. 4. HPLC profiles of different fractions of mixed polysaccharides (LBP/GLP=1:1) and fermented polysaccharides (graphs a, b, c and d are HPLC profiles of F4, C4, F5 and C6, respectively)

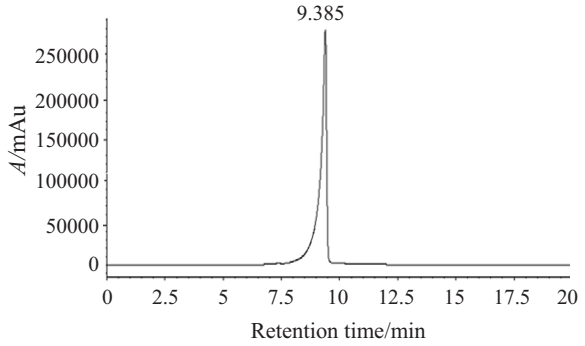

Fig. 5. HPLC profile of the fifth fraction (C5) of mixed polysaccharides (LBP/GLP=1:1)

by fermentation have a good potential application in industry to obtain high bioactivity products for medicinal uses.

Conclusions

In this work, two polysaccharide compounds, fermented polysaccharides and mixed polysaccharides, were tested and compared. The two compounds showed stronger free scavenging ability than that of a single polysaccharide. Compared to the single polysaccharide, the polysaccharide compounds have different fractions revealed by ion exchange chromatography on DEAE-cellulose. Furthermore, HPLC analysis of two polysaccharide compounds shows that they have similar fractions. Since fermented polysaccharides could be obtained more easily than the mixed polysaccharides, fermentation might be used as a good way to obtain polysaccharides with higher bioactivity. Further studies should be carried out to elucidate the structures of the two polysaccharide compounds, and bioactivity analysis through animal experiments will provide further evidence to apply these polysaccharides in the food industry.

Acknowledgement

The work was supported by 863 Advance Technologies Research Program Fund (No. 2007AA021301). The authors gratefully acknowledge the valuable assistance of the Testing Center of the Huazhong University of Science and Technology, PR China.

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