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Preparation of a novel sulfated glycopeptide complex and inhibiting L1210 cell lines property in vitro

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ABSTRACT

A native glycopeptide, LZ-D-4, was purified from the fruiting bodies of *Ganoderma lucidum*, and its derivative, LZ-D-9, was prepared using sulfated agent (CSA: Pyr = 1:6). HPLC experiment showed that both of them were homogeneity and had molecular weights of 1.56×10^4 and 1.30×10^4 Da, respectively. Some properties including amino acid and monosaccharide compositions were investigated for the two compounds, along with anti-tumour bioactivity in vitro. The results indicated that two monosaccharides (Xyl and Ara) were completely destroyed and most of amino acids in LZ-D-4 were broken during sulfated process. Anti-tumour test in vitro showed that both complexes have properties to inhibit growth of L1210 cell lines and have similar bioactivities, but might have different mechanism.

1. Introduction

Sulfation is a preferred method used to modification of polysaccharide due to the positive impacts on polysaccharide biological functions (Wang, Yao, Guan, Wu, & Kennedy, 2005) including anti-tumour (Alekseyenko et al., 2007), antioxidant (Hu, Gen, Zhang, & Jiang, 2001; Ruperez, Ahrazem, & Leal, 2002), antithrombotic (Fonseca et al., 2008; Pinhal et al., 1994) properties, and so on. The sulfation of polysaccharide could not only improve the solubility but also expand chains of sulfated glucan in the aqueous solution, which could be improve the anti-tumour activity. (Zhang, Zhang, Zhou, Chen, & Zeng, 2000) *Ganoderma lucidum*, a well-known traditional Chinese medicine, from which more than two hundred polysaccharides/glycoconjugates have been isolated (Huie & Di, 2004). Except for a series of sulfated polysaccharides of β-D-(1 → 3)-glucan from spores of *G. lucidum* were prepared by Bao, Zhen, Ruan, and Fang (2002), no article dealing with sulfated polysaccharide/glycoprotein was found so far. So, in order to further research the bioactivity and chemical properties for sulfated polysaccharides/glycoconjugates from *G. lucidum*, in this work, a native glycopeptide was purified from *G. lucidum* fruiting bodies and a novel sulfated glycopeptide was obtained using chlorosulfonic acid and pyridine (CSA: Pyr = 1:6) as an esterifying agent and the anti-tumour bioactivities was investigated.

2. Materials and methods

2.1. Materials

Ganoderma lucidum fruiting bodies were collected in June, 2004 from Chongming district of Shanghai, People's Republic of China, and were authenticated by Prof. Taihui Li, Guangdong Provincial Key Laboratory of Microbial Culture Collection and Application, People's Republic of China. A voucher specimen had been deposited in the Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences (Accession No. LZ-2004-0119).

2.2. General analysis

The carbohydrate and peptide percentage were measured by the phenol–sulfuric acid method and BCA kits, respectively. IR spectra were recorded on a Bruker EQUIXOX55 spectrometer from 4000 to 400 cm^{-1}, and the frequency of scanning was 4 cm^{-1}.

2.3. Isolation and purification of native glycopeptide

About 5.0 kg air-dried fruiting bodies of *G. lucidum* were cut into small pieces before extracted with 10 vols 95% (v/v) EtOH for 24 h

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to get rid of lipid material. After removing lipid material, the residue was dried in sunshine, and extracted twice with 10 vol of distilled water for 2 h at 100 °C. Combined aqueous extracts were filtered using a hollow fiber cartridge fitted with membranes of different molecular weight cut-offs and the fraction D (designated as GLPD) (20.01 g, 0.40% yield) was collected.

Fraction D, was then dissolved in distilled water (concentration: 20 mg/mL) and applied to a DEAE-Sepharose Fast Flow column (XK 26 × 100 cm). The column was eluted first with distilled water and then with a 0–2 mol/L NaCl gradient (Fig. 1). Glycopeptide in the eluted fractions was detected using phenol–sulfuric acid method (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956), along with 280 nm detector. A fraction designated GLPDS1B was collected, and was freeze-dried, reconstituted in distilled water (10 mg/mL), and applied to a Sephacryl S-400 high-resolution column. Two polysaccharide peaks were detected using a refractive index detector (RID-10A, Shimadzu, Japan), and the fractions forming the second peak were collected and designated LZ-D-4.

2.4. Preparation of sulfated derivative

The sulfating agent was prepared using dry pyridine and chlorosulfonic acid (Yang, Gao, Han, & Tan, 2005; Yoshida et al., 1995). In brief, 100 mg LZ-D-4 was suspended in 20 mL of dry formamide at room temperature with vortex stirring, the sulfating reagent (CSA: Pyr = 1:6) was added. Then, the mixed solution was reacted under 50 °C for 5 h. After the reaction was finished, the mixture was cooled and neutralized with 4 mol/L NaOH solution. Finally, the product was dialyzed against distilled water for 72 h. The dialyzate was concentrated and freeze-dried and designated as LZ-D-9. The Sulfur content (S%) in the sulfated sample was determined according to method described by Schoniger (1956), and the degree of substitution (DS), which refers to the average number of sulfate residues on each monosaccharide residue, was calculated in line with method described by Zhang, Zhang, Wang, and Cheung (2003).

2.5. Homogeneity and molecular weight

Measurements were completed with HPSEC on a linked column of TSK PW4000 and PW3000, using 0.3 M $NaNO_3$ and 0.1 M NaH_2PO_4 as the mobile phase at a flow rate of 0.2 mL/min. The column temperature was kept at 35 °C and the signals were detected using a RI detector. All samples were prepared as 0.2% (w/v) solution, and 20 μL of solutions were analyzed in each run. The M_w of samples was determined and calibrated using standard Dextrans T-5, 12, 25, 50, 80, 150, and 270.

2.6. Quantification of amino acids

The composition of amino acid was analyzed according to the method described by Ye et al. (2008). Briefly, five milligrams in five milliliter of sample was digested in 6 M HCl at 110 °C for 24 h under nitrogen, the digestion solution was centrifuged and the amino acids for protein moiety was determined by HPAEC using a Dionex LC30 Amino Pac® PA-10 column (2 × 250 mm). The solution consisted of MilliQ water, 250 mM NaOH, and 1 M Na_2CO_3. The flow rate was 0.22 mL/min at 30 °C.

2.7. Sugar analysis

The process determining the monosaccharide was as follows: The sample was hydrolyzed with 2 M TFA at 110 °C for 3 h, and the HPAEC with a Dionex CarboPac™ PA-20 (3 × 150 mm) was applied to a Dionex detect the sugar composition. The column was eluted with 2 mM NaOH (0.45 mL/min). Monosaccharide components and percentage composition were determined using D-Gal, D-Glc, D-Ara, L-Fuc, L-Rha, D-Man, and D-Xyl standards (Ye et al., 2008).

2.8. β-Elimination reaction

About 5 mg LZ-D-4 was dissolved in 2 mL 0.2 mol/L NaOH solution, and the reaction was taken under 60 °C for 30 min. Then the solutions including before and after treatment with NaOH were scanned from 200 to 400 nm using UV detector. The *N*-glucosidic linkage always keeps stable under alkaline condition, while the *O*-glucosidic linkage is easy to be broken and its hydrolysates had obvious absorption at 240 nm (Tian & Zhang, 2005).

2.9. Anti-tumour experiment in vitro

L1210 cells (1 × 10^5 cell/mL) were incubated with 180 uL RPMI medium 1640 supplemented with 10% fetal bovine serum solution and 20 uL sample solutions (at concentrations of 0.05, 0.2, 0.5 mg/mL PBS buffer, respectively) under an atmosphere of 5% at 37 °C for 72 h. The tumour cells were continuously incubated for another 7 h after 20 uL Alamar Blue had been added. Then the solution was measured using a micro ELISA auto-reader 570 and 600 nm, respectively, and the inhibition rate was calculated in line with formula:

Inhibition rate (%) = {100 − [117,216 × $A_{\lambda 570}$ (sample) − 80,586 × $A_{\lambda 600}$ (sample)]/[117,216 × $A_{\lambda 570}$ (control) − 80,586 × $A_{\lambda 600}$(control)]} × 100%

3. Results and discussion

3.1. General analysis

The total yield of the sulfated product (LZ-D-9) was approximately 73%, and the content of sulfur was determined to be ~2.30%, so the DS value was calculated to be 0.12. Both of LZ-D-4 and LZ-D-9 appeared as single symmetrical peaks on HPLC and

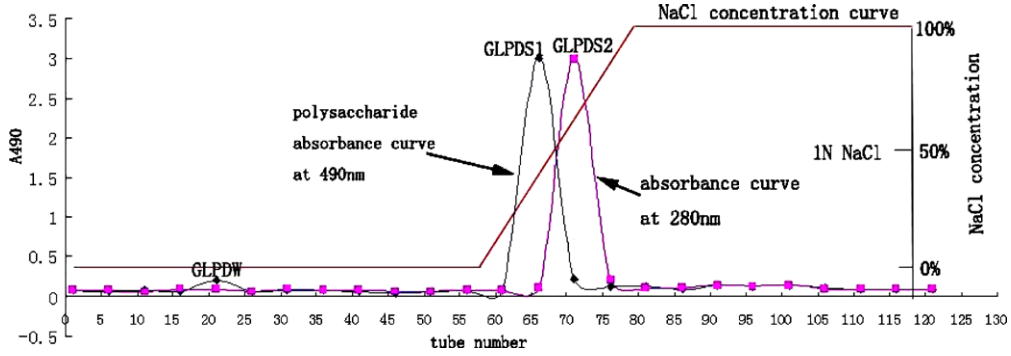

Fig. 1. The elution of GLPD by DEAE-Sepharose F. F. column chromatography.

had estimated molecular weight of 1.56×10^4 and 1.30×10^4 Da, respectively. Total carbohydrate of LZ-D-4 and LZ-D-9 were determined to be ~82% and ~88%, and peptide contents of them was estimated to be ~17% and ~4%, respectively.

3.2. Monosaccharide and amino acid analysis

HPAEC was applied to identify monosaccharide composition of the polysaccharide moiety and amino acids of peptide moiety in these two glycopeptides. The results were showed in the Tables 1 and 2, respectively.

Comparison of the monosaccharide composition and molar ratio indicated that the Ara and Xyl were broken completely when LZ-D-4 was treated with sulfated reagent. And molar ratio of monosaccharides in LZ-D-9 was different from that in LZ-D-4. The Glc was dominant in all monosaccharide compositions indicating residue Glc might be located on the core of polysaccharide moiety.

Amino acid composition analysis indicated that LZ-D-4 was rich in acidic amino acid of glutamic acid (11%) and aspartic acid (9%) and neutral amino acid of threonine (12%), alanine (12%), glycine(13%), and Serine (12%), together with some proportion for other amino acids including arginine (7%), valine (5%), proline (5%), leucine (4%), isoleucine (3%), and phenylalanine (3%), etc. However, treatment with sulfated reagent resulted in major decreases in the concentrations of arginine (77%), threonine (70%), serine (86%), pyenylalanine (87%), aspartic acid (83%), cystine (77%), and tyrosine (63%), and disappeared of other amino acid such as alanine, glycine, valine, glutamic acid, leucine, and isoleucine, etc.

3.3. FI-IR analysis

In the FT-IR spectrum of sulfated derivative, compared with that of the native glycopeptide LZ-D-4, two new absorption peaks found, one at 818 cm^{-1} representing a symmetrical C—O—S vibration associated with a SO_4 group and the other at 1253 cm^{-1} due to the presence of the bonds of S═O, indicating that the sulfation reaction had occurred (Bao et al., 2002).

3.4. Determination of the glycopeptide glycosylation

Generally, there are mainly two types of protein glycosylation: *N*-glycosylation and *O*-glycosylation (Geyer & Geyer, 2006). The carbohydrate percentage of complex LZ-D-4 was measured about 82% and the peptide content was about 17%. It was suggested that LZ-D-4 was a kind of glycopeptide. According to principle of the β-elimination reaction, the *N*-glucosidic linkage always keeps stable under low concentration alkaline condition, while the *O*-glucosidic linkage was easy to be broken and its hydrolysates had an absorption peak at 240 nm. (Tian & Zhang, 2005). In our research, the curve was very similar to one before treatment with 0.2 mol/L NaOH solution at 60 °C for 30 min and no ultraviolet absorbance peak at ca. 240 nm was found after the β-elimination reaction, revealing that the linkage between the polysaccharide moiety and peptide moiety of glycopeptide might belong to *N*-glucosidic linkage.

Table 1
The molar ratio of amino acids for polysaccharide moiety of glycopeptides LZ-D-4 and LZ-D-9.

	Fuc	Ara	Gal	Glc	Xyl	Man	GalA	GluA
LZ-D-4	1	0.24	5.70	16.01	0.58	6.29	1.71	6.67
LZ-D-9	1	–	7.09	28.58	–	8.96	1.24	5.22

–, not detected.

Table 2
The molar ratio of amino acids for protein moiety of glycopeptides LZ-D-4 and LZ-D-9.

Peak name	Ret. time (min)	Molar percentage	
		LZ-D-4	LZ-D-9
Arginine	2.0	9.84	2.24
Lysine	4.5	0.87	n.d.
Threonine	7.7	18.36	5.51
Alanine	8.2	18.08	n.d.
Glycine	8.6	19.74	n.d.
Valine	10.3	7.75	n.d.
Serine	11.5	17.41	2.44
Proline	12.5	8.62	n.d.
Isoleucine	15.3	4.37	n.d.
Leucine	16.4	6.42	n.d.
Histidine	24.2	2.20	n.d.
Phenylalanine	26.1	3.95	0.52
Glutamic acid	27.1	16.50	n.d.
Aspartic acide	27.3	14.25	2.44
Cystine	28.8	0.93	0.22
Tyrosine	31.0	1.47	0.55

n.d., not detected.

3.5. Anti-tumour test in vitro

Researches have confirmed that many kinds of polysaccharides have shown significant anti-tumour activities (Wong, Leung, Fung, & Choy, 1994) and low side-effects in vivo, and polysaccharides from the edible fungi exerted their anti-tumour ability via stimulating the immune response of the host organism, so, polysaccharides were regarded as biological response modifiers (BRMs) (Jimenez-Medina et al., 2008). However, some studies could be found that polysaccharide had potential properties to suppress tumour in vitro (Cui et al., 2007; Jimenez-Medina et al., 2008; Kwok et al., 2001) and different monosaccharide composition, peptide contents and structure of the polysaccharide had the different anti-tumour activities in vitro. In our research, a new native peptide–heteroglycan was isolated and its derivative which had a similar property to anti-tumour in vitro was produced. The in vitro inhibition ratios to the proliferation of L1210 cell lines by the two fractions at different concentrations were shown in Fig. 2. Both the samples exhibited certain inhibition ratios against tumour growth at all concentrations. Whereas 20–500 μg/mL concentration of LZ-D-9 inhibited tumour cell growth by 12–47%, inhibition rates for the same concentration range of LZ-D-4 ranged from 29% to 43%. The values compared with ~95% inhibition rate ($P < .05$) of cells treated with 0.5 μg/mL 5-flurouracil (a well-known anticancer agent served as the positive control). The results also suggested that the glycopeptides anti-tumour activities on L1210 cell lines related with the concentration of samples. When the concentrations of samples were below 200 μg/mL, the glycopeptide LZ-D-4

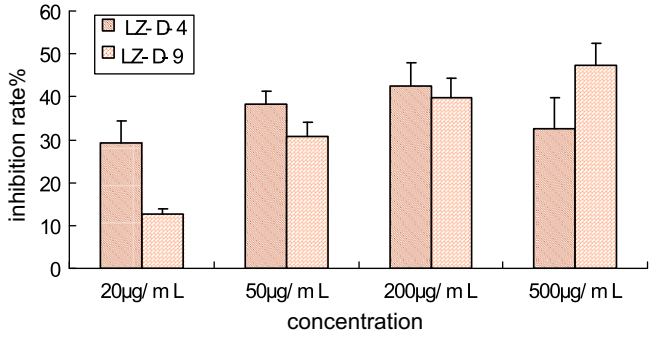

Fig. 2. Growth inhibitory effects of treatment with LZ-D-4 and LZ-D-9 on L1210 cell lines. The cells were treated with LZ-D-4 and LZ-D-9 for 72 h. Values are means ± SD of triplicate determinations.

had competence of anti-tumour slight higher than LZ-D-9. Otherwise, at the concentration of 500 μg/mL, LZ-D-9 had more inhibitory activity on the growth of tumour cells in vitro than fraction LZ-D-4. As above mentioned, the glycopeptide LZ-D-4 might be a conformation of single-helix and had a peptide content of 18%, which might enable it possess an anti-tumour ability. At the same time, the anti-tumour ability of the derivative might come from sulfated group and relatively expanded chain conformation (Tao, Zhang, & Cheung, 2006). The detailed mechanisms how the native glycopeptide and its derivative directly effect on tumour cells are under investigation in our laboratory.

4. Conclusions

A native glycopeptide was purified from the fruiting bodies of *G. lucidum* and its sulfated derivative prepared successfully with chlorosulfonic acid–pyridine complex (CSA: Pyr = 1:6) having a M_w of 1.30×10^4 Da with DS of 0.12. The composition changes were investigated before and after treatment with sulfated reagent. The results showed that the amino acid and monosaccharide were both affected during process. Both of the native glycopeptide LZ-D-4 sample and the sulfated derivative exhibited potent in vitro anti-tumour activities, which might be caused by different mechanism.

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