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Characterisation of acidic heteroglycans from *Tremella fuciformis* Berk with cytokine stimulating activity

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Abstract

Four kinds of acidic heteroglycan, T3a–T3d, were isolated from the body of the fungus *Tremella fuciformis* Berk. The molecular weights of T3a–T3d were 550, 420, 55, and 48 kDa, respectively. Glycosidic linkage analysis showed that they had a mannan backbone consisting of 3-linked Man*p*, and side chains containing glucosyl, mannosyl, fucosyl, xylosyl, and glucuronic acid residues attached through O-2, O-4, or O-6 of about half of the backbone mannosyl residues. A partial acidic hydrolysate of T3a could be divided into a low-branching region (T3a-1, 2, 3, 4) mainly branched at the 2-position of 3-linked Man*p* in the mannan backbone, and a high-branching region (T3a-5A) branched at the 2,4- or 2,4,6-positions of 3-linked Man*p* in the backbone. The low-branching region, which is predominant in the backbone, was substituted with non-reducing terminal Glc*p*A, Fuc*p* and short side chains consisting of (1 → 6)-linked Glc*p* and (1 → 2)-linked Man*p* linked through C-2 of the mannan backbone. The high-branching region, which is a minor component of the backbone, was linked with long side chains of (1 → 6)-linked Glc*p* and (1 → 4)-linked Glc*p*A in their branching points. T3a–T3d were able to induce human monocytes to produce interleukin-1, interleukin-6, and tumor necrosis factor in vitro. The different fragments of the acidic hydrolysate of T3a (T3a-1, 2, 3, 4, 5A) also induced monocytes to secret interleukin-6 with high potency, indicating that the activity may be caused by a common structure,

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(1 → 3)-mannan in the four heteroglycans and their fragments. The change of molecular weight had no obvious influence on the activity of the heteroglycans. © 1996 Elsevier Science Ltd.

Keywords: *Tremella fuciformis*; Heteroglycans; Interleukin-1 (IL-1); Interleukin-6 (IL-6); Tumor necrosis factor (TNF)

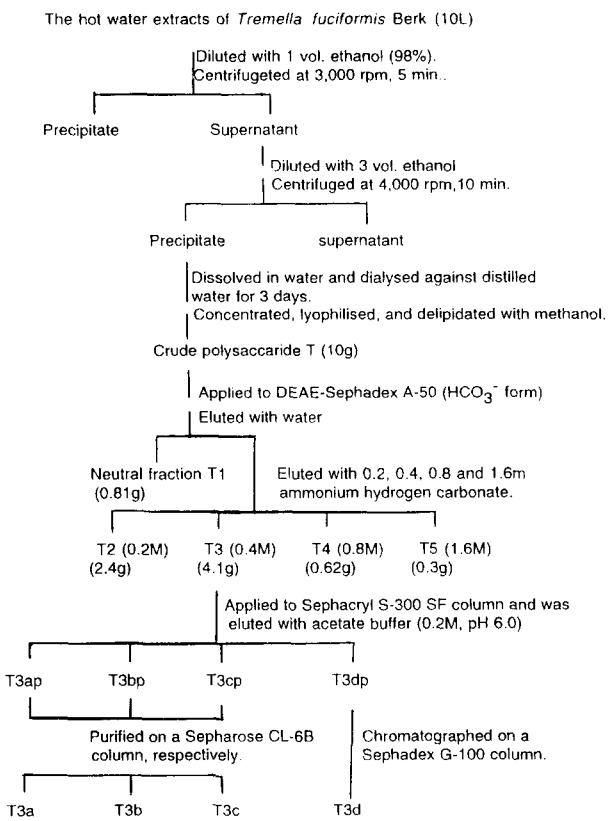
1. Introduction

The body of the fungus *Tremella fuciformis* Berk is a common food and traditional drug used clinically in China as a tonic. In recent years, it has been found that the polysaccharide fraction composed of acidic and neutral heteroglycans [1] has several pharmacological activities, such as enhancing host immune functions [2], anti-tumor activity [1], hypoglycemic activity [3], and as an anti-gastric ulcer agent [4]. The polysaccharide fraction has been used clinically in China in cancer patients treated with chemotherapy or radiotherapy to enhance their immune function [2]. Many polysaccharides have been found to be immunomodulatory, and may contribute to various therapeutic effects, such as anti-tumor or anti-inflammatory activities [5,6]. To study the relationship between structure and activity and to further evaluate the polysaccharides of *T. fuciformis* Berk in clinical use as immunomodulators, we report the structure and cytokine stimulating activities of four acidic heteroglycans.

2. Experimental

Materials.—The bodies of *T. fuciformis* Berk were cultivated in Fujian Province, China. DEAE-Sephadex A-50, Sephacryl S-300 SF, Sepharose CL-6B, and standard dextrans were obtained from Pharmacia. Bio-Gel P-2, Bio-Rad protein assay reagents were purchased from Bio-Rad; Sep-pak C_{18} cartridges from Water Associates Inc.; and Dowex 50X-200(H^+) cation-exchange resin from Aldrich chemical Co.

General methods.—Total carbohydrate, uronic acid, protein, and acetyl groups were assayed by the phenol–sulfuric acid [7], *m*-hydroxylbiphenyl [8], Lowry [9], and hydroxamic acid [10] method, respectively, using Man, GlcA, bovine serum albumin, and glucose pentaacetate as the respective standards. TLC was performed on cellulose-coated plastic sheets (Mark, 5577) in ethyl acetate–pyridine–acetic acid–water (5:5:1:3). Reducing sugar and uronic acid were detected with alkaline silver nitrate [11] and *p*-anisidine hydrochloride [12]. GLC was carried out at 180 °C using a Hewlett–Packard 5700A Gas Chromatograph equipped with a flame-ionisation detector and a glass column (3 mm i.d. × 200 cm) packed with 3% of OV-225 on Uniport HP or DB-1. Uronic acids were converted into their corresponding carboxyl-reduced products [13] and analysed by GLC as the alditol acetates. HPLC was performed [14] on a Simadzu 6A chromatograph equipped with a UV detector and differential refractometer. The molecular weight of each polysaccharide was analysed by HPLC equilibrated with water or 0.2 M NaCl on a PL–aquagel–OH column and was estimated from the calibration curve of standard dextrans. The content of LPS (lipopolysaccharide) in different samples was assayed with a toxicolour system Et-1 set (Seikagaku Co.).



Scheme 1.

Isolation and purification of water soluble polysaccharide.—The bodies of *T. fuciformis* Berk (500 g) were extracted with water for 6 h at 100 °C, and the residual materials were re-extracted twice in the same manner. The extracts were combined, concentrated to 10 L by rotary evaporation, and then treated as shown in Scheme 1 to obtain the purified acidic heteroglycans, T3a–T3d.

Methylation analysis.—Each sample was methylated once by the Hakomori method [15] purified by a Sep-Pak C_{18} cartridge [16], and then reduced with sodium borodeuteride in 95% ethanol–tetrahydrofuran (27:73) [17]. The resulting carboxyl-reduced, partially *O*-methylated polysaccharide was desalted (Dowex 50W) and then remethylated. The purified per-*O*-methylated polysaccharide was hydrolysed (2 M trifluoroacetic acid, 1 h, 121 °C), reduced (sodium borohydride, 1.5 h, RT), and acetylated (Ac_2O, 3 h, 121 °C). The resulting partially methylated alditol acetates were analysed by GLC and GLC–MS [18]. GLC–MS (70 eV) was performed [19] on a TRIO 1000 instrument equipped with a DB-1 column. The molar ratios were calibrated using the peak areas and response factors [20] of the flame-ionisation detector in GLC.

Partial hydrolysis of T3a.—T3a (50 mg) was hydrolysed with 1 M formic acid (1 h, 90 °C). After hydrolysis, the formic acid was removed by evaporation on a rotary

evaporator and the product was applied to a column (2.2 × 220 cm) of Sephadex G-50, equilibrated with water. Based on the elution pattern the hydrolysate was divided into five parts, T3a-1, 2, 3, 4, 5, and T3a-5 was further subdivided into T3a-5A and T3a-5B on a Bio-Gel P-2 column.

Cytokine stimulating activities in vitro.—Human monocytes were isolated from human A^+ blood buffy coat [21]. Monocytes were cultured in complete medium consisting of RPMI 1640 and 25% A^+ serum. Polysaccharide subfractions in different concentrations were added to the monocytes for 24 h before the supernatants were harvested, and the contents of IL-1, IL-6, and TNF was assayed as described in refs. [22,23]. The LPS (1 μg/mL) and PBS buffer (0.25 M, pH 7) were used as positive and negative controls. Results are presented as pg/mL ±SD of triplicate determination in Table 5.

3. Results and discussion

The delipidated polysaccharide fraction (Scheme 1) was composed of Fuc:Xyl:Ara:Man:Glc:Gal in the molar ratio 0.2:0.3:Trace:1.0:Trace:Trace and contained 24.3% of uronic acid and 0.88% of protein. After DEAE-Sephadex A-50 chromatography to obtain the unabsorbed fraction (T1), and absorbed fractions (T2–T5), an assay for monocyte stimulation showed that five fractions were active. Their compositional analysis is show in Table 1. T3 was further purified by gel-filtration chromatography, and the four purified polysaccharides obtained (Scheme 1). T3a–T3d, were analysed by glass-fiber electrophoresis in pyridineacetate buffer, pH 5.4, and HPLC eluted with 0.2 M NaCl. Each of the four purified polysaccharides showed a single spot on electrophoresis and a single peak on HPLC. They showed a similar content of the

Table 1
Properties of the polysaccharide fractions from *Tremella fuciformis* Berk

Chemical properties	T1	T2	T3	T4	T5
Carbohydrate (as Man)	82.3%	84.6%	80.4%	48.8%	51.5%
Uronic acid (as GlcA)	5.3%	26.5%	29.3%	28.7%	31.2%
Protein (as BSA)	8.0%	0.1%	0.1%	3.4%	3.9%
Acetyl group (as pentaacetates Glc)	0.6%	2.3%	1.5%	1.2%	1.6%
LPS (pg/mg as endotoxin)	22	20	21	22	20
Component sugars (mol%)					
Fuc	5	9	9	9	14
Xyl	6	15	14	19	8
Ar	–	–	–	–	14
Man	31	50	48	42	28
Glc	52	T [a]	T	T	T
Gal	–	–	–	–	3
GlcA	6	27	29	30	33

[a] T: Trace.

Table 2
Properties of T3a–T3d

Chemical properties	T3a	T3b	T3c	T3d
Carbohydrate (as Man)	80.1%	73.4%	80.4%	48.8%
Uronic acid (as GlcA)	24.6%	28.0%	15.9%	22.2%
Protein (as BSA)	–	–	0.2%	0.6%
Acetyl group (as pentaacetates Glc)	2.4%	2.0%	1.9%	1.4%
LPS (pg/mg as endotoxin)	22	20	23	25
Component sugars (mol%)				
Fuc	11	10	8	9
Xyl	10	9	10	8
Man	48	48	58	52
Glc	6	6	8	9
GlcA	25	28	16	22
Molecular weight (Tested by HPLC) (kDa)				
0.2 M NaCl	550	410	55	48

carbohydrate, protein, acetyl group, and component sugars but differed in their content of uronic acid and molecular weight (Table 2). Glycosidic linkage analysis (Table 3) showed that they all had (1 → 3)-D-mannan backbones, with the branching points at the 2-, 4-, or 6-position of the 3-linked Man*p*, but different amounts of 2-branched, 6-branched, 2,6-di-branched, and 2,4,6-tri-branched 3-linked Man*p* in their backbones. The side chains of the four polysaccharides consisted of 2-*O*-linked Man*p*, 6-*O*-linked Glc*p*, and 5-*O*-linked Xyl*f*, and terminating with Glc*p*A, Xyl*f*, and Fuc*p*.

Table 3
Methylation analysis of T3a–T3d

Glycosyl residue	Position of groups	Deduced glycosidic linkages	Composition (mol%)			
			T3a	T3b	T3c	T3d
Xyl	2,3,5	terminal (*f*)	2.7	1.5	2.3	2.2
	3,5	2	–	0.5	–	–
	2,3	5	1.6	0.4	1.3	0.6
Fuc	2,3,4	terminal (*p*)	10.0	8.9	7.1	9.0
Glc	2,3,4	6	4.1	6.0	7.9	1.3
Man	3,4,6	2	0.4	1.7	10.3	1.7
	2,4,6	3	32.2	34.6	44.8	37.4
	4,6	2,3	28.9	18.4	14.2	28.5
	2,4	3,6	1.1	2.9	1.6	1.5
	4	2,3,6	1.2	3.7	0.6	1.1
		2,3,4,6	0.5	–	1.1	0.5
GlcA[a]	2,3,4	terminal (*p*)	18.3	20.4	9.8	16.2

[a] Detected by 6,6-d_2.

Table 4
Methylation analysis of T3a-1, 2, 3, 4, 5A

Glycosyl residues	Position of groups	Deduced glycosidic linkages	Composition (mol%)				
			T3a-1	T3a-2	T3a-3	T3a-4	T3a-5A
Xyl	2,3	5	–	–	–	–	1.2
Fuc	2,3,4	terminal (*p*)	0.8	0.7	0.4	0.5	1.1
Glc	2,3,4	6	2.5	2.1	–	2.6	30.0
Man	3,4,6	2	1.8	1.8	1.9	3.6	–
	2,4,6	3	50.9	51.6	52.3	52.7	–
	4,6	2,3	21.8	21.0	20.0	18.5	–
	2,4	3,6	1.9	1.8	1.6	1.7	0.4
	4	2,3,6	1.0	0.9	0.5	0.6	4.5
		2,3,4,6	–	–	–	–	2.6
GlcA [a]	2,3,4	terminal (*p*)	19.3	20.2	23.2	19.9	24.1
	2,3	4	–	–	–	–	35.6

[a] Detected from *6,6-d_2*.

Table 5
Cytokine stimulating activities of T3a–T3d

Samples	Concentrations (μg/mL)	Content of cytokines (±SD) (pg/mL)		
		IL-1	IL-6	TNF
T3a	1000	1010 (±110)	4600 (±520)	1950 (±320)
	500	1750 (±150)	6500 (±350)	2300 (+230)
	250	1250 (±310)	6600 (±620)	2000 (±150)
	125	1500 (±32O)	5700 (±430)	2150 (±170)
	62.5	600 (±120)	5000 (±570)	1850 (±210)
T3b	1000	800 (±380)	2500 (±330)	1200 (±180)
	500	810 (±290)	5800 (±650)	1700 (±140)
	250	1150 (±140)	6100 (±440)	2450 (±190)
	125	1010 (±180)	5800 (±510)	2200 (±210)
	62.5	610 (±310)	5500 (±390)	2250 (±230)
T3c	1000	980 (±190)	5800 (±470)	2200 (±150)
	500	1300 (±280)	6200 (±590)	2350 (±190)
	250	1250 (±120)	5900 (±570)	2150 (±220)
	125	1050 (±180)	5600 (±440)	1950 (±140)
	62.5	750 (±310)	1800 (±350)	1800 (±180)
T3d	1000	630 (±120)	3300 (±520)	650 (±200)
	500	850 (±140)	5500 (±530)	750 (±150)
	250	800 (±180)	5600 (±620)	1150 (±140)
	125	610 (±210)	6150 (±600)	1800 (±180)
	62.5	980 (±80)	5900 (±490)	1780 (±190)
LPS	1.0	1050 (±210)	5800 (±570)	1400 (±150)
PBS buffer	0.25 M	0	36 (±12)	0

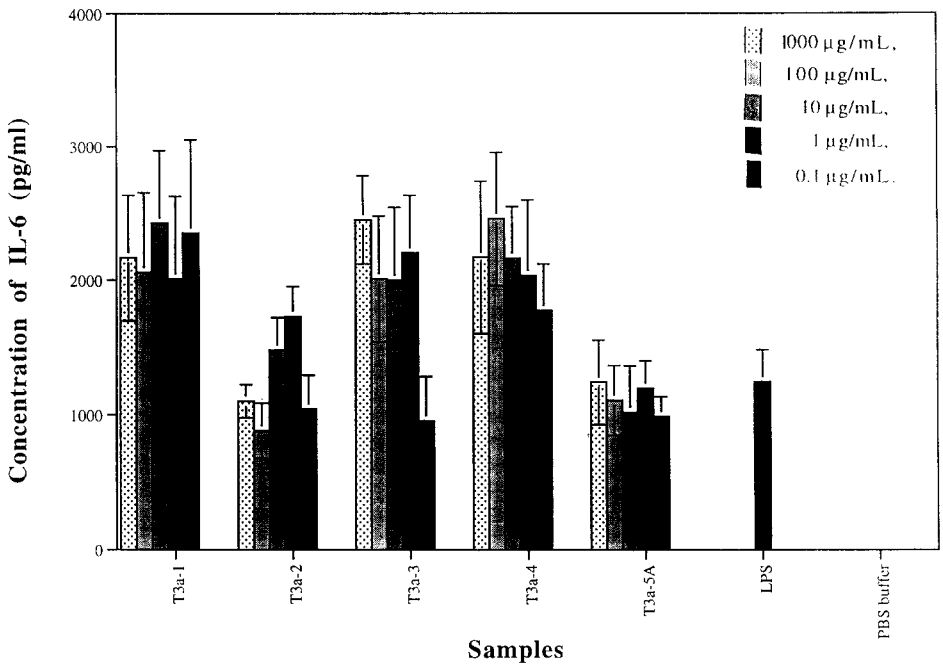


Fig. 1. Content of IL-6 in a supernatant of monocyte culture incubated with different concentrations of T3a-1, 2, 3, 4, and 5A. Positive control: LPS (1 μg/mL). Negative control: LPS-free PBS buffer. Concentrations of the samples are as shown in the figure.

T3a was hydrolysed with formic acid and the hydrolysate was isolated by Sephadex G-50 and Bio-Gel P-2 columns to obtain T3a-1, 2, 3, 4, 5A, and 5B. Neutral component sugar analysis showed that T3a-1, 2, 3, 4 contained Man mainly in addition to small amounts of Xyl, Fuc, or Glc, whereas T3a-5A and T3a-5B had a smaller amount of mannose than, respectively, Glc and Fuc. All fragments of the hydrolysate contained uronic acid tested quantitatively by TLC and GLC. The molecular weights of the fractions T3a-1, 2, 3, 4, and 5A were assayed by HPLC and shown to be 220, 145, 53, 21, and 3.5 kDa, respectively. The results of the methylation analysis are shown in Table 4. T3a-1, 2, 3, and 4 consisted of (1 → 3)-D-mannan in which about 30% of the 3-linked Man*p* was branched at the 2-position and was attached with non-reducing terminal Glc*p*A, Fuc*p*, or side chains of 6-*O*-linked Glc*p* and 2-*O*-linked Man*p* mainly, with the exception that 6-*O*-linked Glc*p* was absent in T3a-3. T3a-5A contained mainly 4-*O*-linked GlcA and 6-*O*-linked Glc*p* with 2,3,4,6-tetra-*O*-substituted Man*p* and 2,3,6-tri-*O*-substituted Man*p* indicating that T3a-5A is a high-branching region of T3a.

The four homogenous heteroglycans, T3a–T3d, stimulated human monocyte secreting IL-1, IL-6, and TNF with similar ability (Table 5). In the highest concentration (1 mg/mL), most of the samples suppressed the secretion of cytokines. T3a-1, 2, 3, 4, and 5A, fragments of T3a, also could induce human monocytes to produce IL-6 in the concentrations from 1 mg/mL to 0.1 μg/mL (Fig. 1) although they had very different molecular weights (220–3.5 kDa), suggesting that the molecular weight of the poly-

saccharides had no obviously influence on the activity and that the fragments possessed potential in clinical use. T3a-3, in which 6-*O*-linked Glc*p* was absent and non-reducing terminal Fuc*p* was negligible, still had significant activity, indicating that the α-D-mannan might be essential for expression of the activity.

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