

Structural Features of an Anti-Diabetic Polysaccharide (TAP) from *Tremella aurantia*

Tadashi KIIHO,^{*a} Takashi KOBAYASHI,^a Hotaka MORIMOTO,^a Shigeyuki USUI,^a Shigeo UKAI,^a Kazuyuki HIRANO,^a Koichi AIZAWA,^b and Takahiro INAKUMA^b

Gifu Pharmaceutical University,^a 5-6-1 Mitahora-higashi, Gifu 502-8585, Japan and Research Institute, Kagome Co., Ltd.,^b 17 Nishitomiya, Nishinasuno-machi, Nasu-gun, Tochigi 329-2762, Japan.

Received April 20, 2000; accepted July 3, 2000

The structure of an anti-diabetic polysaccharide (TAP) obtained from the fruiting bodies of *Tremella aurantia* was investigated by methylation analysis, Smith degradation, partial acid hydrolysis, ¹³C-NMR spectrometry, and enzymatic digestion. The results suggested that TAP was composed of (1→3)-linked α-D-mannopyranosyl residues as a backbone, some of which were substituted at position 2 with (1→3)-linked β-D-xylopyranose side chains and with β-D-glucopyransyluronic acid at position 4 linked to terminal α-D-mannopyranose.

Key words *Tremella aurantia*; polysaccharide; structure; anti-diabetes

We previously reported that an acidic polysaccharide (TAP) isolated from the fruiting bodies of *Tremella aurantia* was composed of mannose, xylose, glucuronic acid, and glucose (molar ratio, 4:2:1:0.3), and contained *O*-acetyl groups (2.2%) in the molecule, and exhibited a significant hypoglycemic activity in diabetic mouse models following intraperitoneal administration and suppressed hyperglycemia by continuous oral administration.¹⁾ Furthermore, we reported that TAP was effective towards the activity of a key hepatic enzyme and plasma cholesterol levels.²⁾

In the present study, the structural features of TAP were investigated by a combination of chemical, enzymatic, and spectroscopic methods.

A TAP and its carboxyl-reduced polysaccharide (TAP-R), in which the carboxyl groups of glucuronic acid residues were reduced with a water-soluble carbodiimide and sodium borohydride, were fully methylated and the products hydrolyzed with acid; this converted sugars into partially methylated alditol acetates.³⁾ The results of GC and GC-MS spectrometry are shown in Table 1. Since the 2,3,6-tri-*O*-methyl-D-glucose derivative peak was only present in trace amounts in TAP, and was greatly increased in its carboxyl-reduced product TAP-R, the methylated sugar should be derived from (1→4)-linked glucuronic acid residues. The identification of 2,3,4-tri-*O*-methyl-D-xylose, 2,3,4,6-tetra-*O*-methyl-D-mannose, and 2,3,4,6-tetra-*O*-methyl-D-glucose indicates that the non-reducing terminal residues were D-xylopyranose, D-mannopyranose, and small amounts

of glucose. The identification of 4,6-di-*O*-methyl-D-mannose shows the presence of branching (1→2,3)-linked D-mannopyranosyl residues.

The de-*O*-acetylated polysaccharide (TAP-D) obtained by alkali treatment and its carboxyl-reduced polysaccharide (TAP-D-R) were subsequently oxidized with periodate and reduced with sodium borohydride to give the polyalcohols. In the hydrolysate of the polyalcohols (the first Smith degradation products), the molar ratio of xylose was reduced by half and the glucuronic acid disappeared, and the first Smith degradation product of TAP-D-R yielded erythritol (derived from 1,4-linked hexose), in addition to mannose, xylose and a trace amount of glucose. Furthermore, the partially degraded polysaccharide obtained by mild-acid hydrolysis of the polyalcohol was again oxidized, reduced, and mildly hydrolyzed to give the second controlled Smith degradation product (TAP-D-2CS). TAP-D-2CS was found to be only composed of (1→3)-linked α-D-mannopyranose by methylation analysis and IR spectrum (820 cm⁻¹). These results suggest the presence of side chains consisting of (1→3)-linked xylose residues attached to terminal xylose residues, and a core of (1→3)-linked α-D-mannopyranosyl residues.

The ¹³C-NMR spectra of TAP and TAP-R in dimethylsulfoxide (DMSO)-*d*₆ (Fig. 1) were compared with data in the literature.^{4,5,6)} The lower field signals at 171.6 and 170.5 ppm were assigned to the carbonyl of glucuronic acid and *O*-acetyl groups. The signals at 104.4, 102.9, and 100.3 ppm in the anomeric region could be attributed to C-1 of β-D-xylose,

Table 1. GC and GC-MS of Alditol Acetate Derived from Methylated Product of Carboxyl-Reduced TAP (TAP-R)

Methylated sugar (as alditol acetate)	T ^{a)}		Primary mass fragments (<i>m/z</i>)	Molar ratio	Mode of linkage
	Column A	Column B			
2,3,4-Me ₃ -Xyl	0.62	0.64	117, 161	2	[Xylp]1→
2,3,4,6-Me ₄ -Man	0.99	1.00	45, 117, 161, 205	2	[Manp]1→
2,3,4,6-Me ₄ -Glc	1.00	1.00	45, 117, 161, 205	0.3	[Glc]1→
2,4-Me ₂ -Xyl	1.08	1.34	117, 233	2	→3[Xylp]1→
2,4,6-Me ₃ -Man	1.84	2.17	45, 117, 161, 233	3	→3[Manp]1→
2,3,6-Me ₃ -Glc ^{b)}	2.18	2.49	45, 117, 233	2	→4[Glc]1→
4,6-Me ₂ -Man	2.81	3.51	45, 161, 261	4	→2,3[Manp]1→

a) Relative retention time with respect to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. Column A: DB 225 (capillary column), column B: 3% ECNSS-M. b) This was a slight amount in methylation analysis of native polysaccharide (TAP).

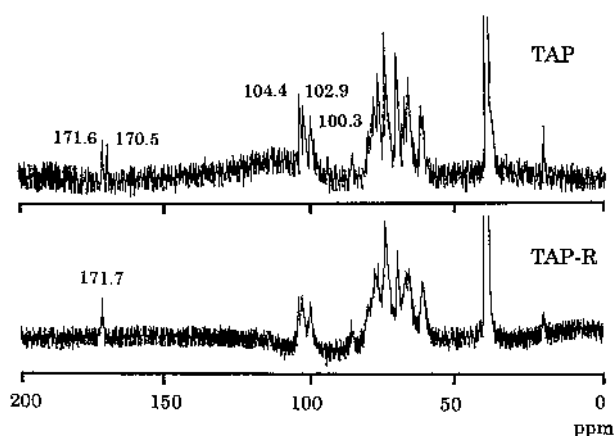
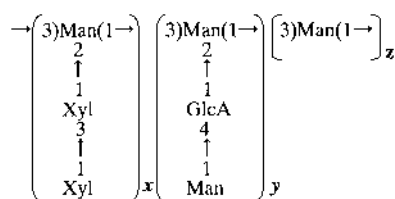


Fig. 1. ^{13}C -NMR Spectra of TAP and TAP-R in $\text{DMSO}-d_6$.



$$x : y : z = 2 : 2 : 3$$

Man = α -D-mannopyranose

Xyl = β -D-xylopyranose

GlcA = β -D-glucuronic acid

Fig. 2. A Possible Repeating Unit of TAP

α -D-glucuronic acid, and α -D-mannose, respectively.

The degraded polysaccharide (TAP-PH) prepared by partial acid hydrolysis showed a higher $[\alpha]_D + 12^\circ$ in water than the native polysaccharide (TAP) ($[\alpha]_D - 7^\circ$), and had half the amount of xylose, although the amounts of the other component sugars were not reduced. Consequently, it may be assumed that xylose exists as a β -D-linked sugar side chain.

We attempted to attack the polymer with an exo-type enzyme, α -mannosidase from jack bean. The de-O-acetylated and carboxyl-reduced glucuronic acid (TAP-D-R) derivative of TAP was used to facilitate the enzymatic digestion, then the removed sugars were analyzed by HPLC as *p*-aminobenzoic ethyl ester (ABEE)-derivatives. Mannose was identified as the removed sugar. It could thus be postulated that α -D-mannose exists as the non-reducing terminal residues of the side chains.

From the foregoing results, TAP seems to be constructed with a (1 \rightarrow 3)-linked α -D-mannopyranosyl backbone, some of which is substituted at the position 2 with (1 \rightarrow 3)-linked β -D-xylopyranose side chains and with β -D-glucopyranosyluronic acid at position 4 linked to terminal α -D-mannopyranose, as shown in Fig. 2. The non-reducing terminal α -D-mannopyranosyl residues may contribute to the potent hypoglycemic activity of TAP in diabetic mouse models. TAP an acidic polysaccharide obtained from the fruiting bodies of *T. aurantia*, was shown to have a similar structural unit, except for the terminal mannopyranosyl residues, to the polysaccharides of *T. fuciformis*,⁷⁾ *Auricularia auricula-judae*,⁸⁾ *T. mesenterica*⁹⁾ belonging to the same Heterobasidiae, and capsular polysaccharides of *Cryptococcus neoformans*⁶⁾ showing

a certain chemotaxonomic relationship to *Tremella* species.⁷⁾

Experimental

Specific rotations were measured with a JASCO DIP-4 automatic polarimeter. IR spectra were recorded on a JASCO A-102 spectrometer. GC was carried out a Shimadzu GC-4CM apparatus and a Shimadzu GC-15A instrument, equipped with a hydrogen-flame ionization detector. GC-MS was conducted using a Hewlett-Packard Model 5890II combined GC-MS spectrometry system equipped with a fused-silica capillary column (0.25 cm \times 30 m, film thickness 0.25 μm) of DB 225. ^{13}C -NMR spectroscopy was performed on a JEOL FT-NMR EX-400 spectrometer in $\text{DMSO}-d_6$ (50 mg/0.5 ml) at room temperature. The HPLC analysis was carried out using a Shimadzu LC-9A HPLC system equipped with a JASCO FP-920 fluorescence detector.

De-O-acetylation The polysaccharide was stirred in 0.1 M sodium methoxide-methanol solution for 8 h. The product collected by centrifugation was dissolved in water, dialyzed against water, and lyophilized to give the de-O-acetylated polysaccharide (TAP-D) in 95% yield, which showed no acetyl absorption band (1730 cm^{-1}) in the IR spectrum.

Carboxyl Reduction Carboxyl-reduction of glucuronic acid in the polysaccharide was performed according to the method of Taylor *et al.*,¹⁰⁾ using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and sodium borohydride as previously described.¹¹⁾ The reduction process was repeated 5 times until the signal at 171.6 ppm in the ^{13}C -NMR spectrum disappeared. Carboxyl-reduced polysaccharide (TAP-R) and de-O-acetyl and carboxyl-reduced polysaccharide (TAP-D-R) were obtained in 86 and 81% yields from TAP, respectively.

Methylation Analysis The polysaccharide was methylated 3 times by the Hakomori method as previously described.¹²⁾ The fully methylated polysaccharide was heated successively with 90% formic acid for 8 h at 100 $^\circ\text{C}$ and 2 M trifluoroacetic acid for 8 h at 100 $^\circ\text{C}$. The partially methylated sugars were converted into alditol acetate derivatives. The resulting partially methylated alditol acetates were analyzed by GC (3% ECNSS-M column at 175 $^\circ\text{C}$ and DB 225 capillary column at 180 $^\circ\text{C}$) and GC-MS spectrometry, as previously described.¹³⁾

Smith Degradation Each polysaccharide (TAP-D and TAP-D-R) was oxidized with 25 mM sodium periodate at 4 $^\circ\text{C}$ for 2 d, and reduced with sodium borohydride, as previously described,¹³⁾ to give the polyalcohols in 59% and 60% yields, respectively. A part of the polyalcohol was mildly hydrolyzed with 0.1 M acetic acid at pH 2.0 adjusted with 0.1 M HCl for 35 min at 100 $^\circ\text{C}$, and further oxidized similarly as above, then reduced to give the second polyalcohol. Each polyalcohol was hydrolyzed with 2 M sulfuric acid for 6 h at 100 $^\circ\text{C}$, and the hydrolysates (the first and second Smith degradation products: TAP-D-2CS) were analyzed by GC as previously described.

Partial Acid Hydrolysis The polysaccharide (TAP) was partially hydrolyzed with 0.1 M HCl for 6 h at 100 $^\circ\text{C}$ and the neutralized solution dialyzed against water using cellulose tubing (Viscose Corp.). The non-dialyzable solution was lyophilized to give degraded polysaccharide (TAP-PH) in 73% yield.

Enzymatic Digestion and Sugar Analysis by HPLC After a solution of TAP-D-R (100 μg) in 50 mM acetate buffer (pH 5.0, 100 μl) was incubated with α -mannosidase from jack bean (0.5 mg, 9 units, Sigma) for 24 h at 37 $^\circ\text{C}$, the reaction mixture was heated in a boiling water bath for 3 min, and centrifuged. Sugars were analyzed by HPLC as ABEE-derivatives, according to the method of Yasuno *et al.*¹⁴⁾ The supernatant (20 μl) was reacted with ABEE reagent (40 μl , Honen Corp.) for 1 h at 80 $^\circ\text{C}$, and the solution was washed with chloroform. The aqueous layer was injected into a Honepak C18 column (75 \times 4.6 mm, Honen Corp.) at a flow rate of 1.0 ml/min at 30 $^\circ\text{C}$, and detected by fluorescence monitoring (excitation 305 nm, emission 360 nm) with 0.2 M potassium borate buffer (pH 8.9) containing 7% acetonitrile as eluent. The ABEE-converted mannose was detected at 10.6 min.

References

- 1) Kiho T., Morimoto H., Sakushima M., Usui S., Ukai S., *Biol. Pharm. Bull.*, **18**, 1627—1629 (1995).
- 2) Kiho T., Morimoto H., Kobayashi T., Usui S., Ukai S., Aizawa K., Inakuma T., *Biosci. Biotech. Biochem.*, **62**, 417—419 (2000).
- 3) Björndall H., Hellerqvist C. G., Lindberg B., Svensson S., *Angew. Chem. Int. Ed. Engl.*, **9**, 610—619 (1970).
- 4) Gorin P. A. J., Barreto-Bergter E., Travassos L. R., *Carbohydr. Res.*, **95**, 205—218 (1981).
- 5) Kogan G., Pavliak V., Masler L., *Carbohydr. Res.*, **172**, 243—253

- (1988).
- 6) Bacon B. E., Cherniak R., Kwon-Cheng K. J., Jacobson E. S., *Carbohydr. Res.*, **283**, 95—110 (1996).
 - 7) a) Ukai S., Hirose K., Kiho T., Hara C., *Chem. Pharm. Bull.*, **25**, 338—344 (1977); Ukai S., Kiho T., Hara C., *ibid.*, **26**, 3871—3876 (1978). b) Gao Q., Seljelid R., Chen H., Jiang R., *Carbohydr. Res.*, **288**, 135—142 (1996).
 - 8) Ukai S., Morisaki S., Goto M., Kiho T., Hara C., Hirose K., *Chem. Pharm. Bull.*, **30**, 635—643 (1982); Sone Y., Kakuta M., Misaki A., *Agric. Biol. Chem.*, **42**, 417—425 (1978).
 - 9) Fraser C. G., Jennings H. J., Moyna P., *Can. J. Biochem.*, **51**, 219—224 (1973).
 - 10) Taylor R. L., Shively J. E., Conrad H. E., *Methods Carbohydr. Chem.*, **VII**, 149—151 (1976).
 - 11) Kiho T., Tsujimura Y., Sakushima M., Usui S., Ukai S., *Yakugaku Zasshi*, **114**, 308—315 (1994).
 - 12) Kiho T., Ito M., Nagai K., Hara C., Ukai S., *Chem. Pharm. Bull.*, **37**, 2770—2772 (1989).
 - 13) Kiho T., Itahashi S., Sakushima M., Matsunaga T., Usui S., Ukai S., Mori H., Sakamoto Y., Ishiguro Y., *Biol. Pharm. Bull.*, **20**, 118—121 (1997).
 - 14) Yasuno S., Murata T., Kokubo K., Yamaguchi T., Kamei M., *Biosci. Biotech. Biochem.*, **61**, 1944—1946 (1997); Yasuno S., Kokubo K., Kamei M., *ibid.*, **63**, 1353—1359 (1999).