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ON THE WATER-SOLUBLE HETEROGALACTAN FROM THE FRUIT BODIES OF *Lentinus edodes*

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ABSTRACT

The chemical structure of a heterogalactan isolated from the trichloroacetic acid extract of the fruit bodies of *Lentinus edodes* is reported It consists of a main chain of (1→6)-linked α-D-galactopyranose residues, part of which are substituted in the 2-position either with single L-fucopyranose or D-mannopyranose residues However, there is a possible alternative structure of a branched D-galactan in which most of the side-chains are terminated with L-fucose or D-mannose residues

INTRODUCTION

The fruit bodies of *Lentinus edodes* are the most popular edible mushroom in Japan and China About 65% of the dried fruit bodies consists of carbohydrates, most of which are present as polysaccharides, together with a lesser proportion of low molecular-weight carbohydrates such as mannitol or trehalose. In the previous publication[1], we reported the structures of two water-soluble polysaccharides isolated from the fruit bodies of *L edodes* One is an α-D-glucan of the glycogen type, having an average chain-length of about 5–6, and the other is a β-D-glucan containing (1→6)-, (1→3)-, and (1→4)-linked β-D-glucopyranose residues The present paper reports the isolation of a heterogalactan from the fruit bodies of *L edodes* and the results of structural studies on it

RESULTS AND DISCUSSION

Extraction of the dried fruit-bodies of *Lentinus edodes* with 3% trichloroacetic acid, followed by fractional precipitation with methanol, yielded two different types of polysaccharide Fraction *A*, precipitated at a 50% concentration of methanol, was characterized as an α-D-glucan of the glycogen type Fraction *B*, precipitated at a 75% concentration of methanol, gave D-galactose, D-glucose, L-fucose, and D-mannose on acid hydrolysis Fractional precipitation of this fraction with Cetavlon in borate buffer at different pH values yielded four subfractions. The first subfraction (*B*-1), recovered from the Cetavlon complex precipitated at pH 8 0, was shown to be a glucose-free

heterogalactan having $[\alpha]_D$ +102° On hydrolysis, it gave rise to D-galactose, L-fucose, and D-mannose in the molar ratio of 6 0.1 5 1 0 The homogeneity of the purified polysaccharide was examined by sedimentation analysis, zone electrophoresis, and gel filtration The polysaccharide gave only one peak on ultracentrifugation ($s = 1.55$), on zone electrophoresis (Fig 1), and on gel filtration (Fig 2) Hydrolysis of the methylated polysaccharide ($[\alpha]_D$ +81°, OCH_3, 42 5%) yielded a mixture of methylated sugars that were separated by preparative paper chromatography to give the methyl ethers listed in Table II As can be seen from Table II, all of the main sugars in the polysaccharide are present in the pyranoid form L-Fucose and D-mannose are present exclusively as terminal, non-reducing residues D-Galactose is found as non-reducing end-units and as 6-*O*- and 2,6-di-*O*-substituted units Occurrence of a small amount of mono-*O*-methyl-D-galactose indicates either incomplete methylation or demethylation during hydrolysis, and is probably not structurally significant However, the possibility of double branching cannot be completely neglected A stepwise, partial, acid hydrolysis was performed, with isolation of the fragments of low molecular weight between each step The hydrolysis products were fractionated on a charcoal–

TABLE I

COMPONENT SUGARS OF THE SUBFRACTIONS OBTAINED FROM THE CETAVLON PRECIPITATE OF FRACTION *B*

Fraction	*Weight (g)*	*Component sugars*[a]			
		Gal	*Fuc*	*Man*	*Glc*
Original sample (*B*)	4 3	++	+	+	+
B-1	1 5	++	+	+	−
B-2	0 8	++	+	+	+
B-3	0 2	−	−	−	++
B-4	1 0	−	−	−	++

[a] ++, Present in large proportion, +, present, −, absent

TABLE II

HYDROLYSIS PRODUCTS FROM THE METHYLATED HETEROGALACTAN

Methylated sugars	*Weight (mg)*	*Molar percent*	*Relative retention time of the methyl glycosides*	R_{Glc-Me_4}[b]
2,3,4,6-Me_4-Man[a]	171 8	11 6	1 43	96 7
2,3,4-Me_3-Fuc		19 3	0 75	88 6
2,3,4,6-Me_4-Gal		0 8	1 79	88 6
2,3,4-Me_3-Gal	223 7	40 3	8 11	72 3
3,4-Me_2-Gal	139 5	27 1		46 5
mono-Me-Gal	5 1	1 0		33 0

[a] 2,3,4,6-Me_4-Man = 2,3,4,6-tetra-*O*-methyl-D-mannose and so on [b] Relative to 2,3,4,6-tetra-*O*-methyl-D-glucose, solvent *C*

TABLE III

OLIGOSACCHARIDES FROM THE PARTIAL HYDROLYZATE

Oligosaccharide	*Yield (mg)*	$[\alpha]_D$	*Partial hydrolysis*	R_{Gal}[b]
(1→6)-α-galactobiose	44	139	Gal, Gal_2[a]	0 75
(1→6)-α-galactotriose	50	147	Gal, Gal_2, Gal_3	0 56
(1→6)-α-galactotetraose	58	165	Gal, Gal_2, Gal_3, Gal_4	0 40

[a]Gal_2 = α-(1→6)-galactobiose and so on [b]Relative to galactose, solvent *B*, with double development

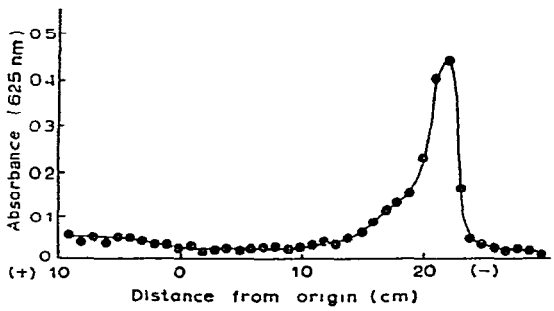

Fig 1 Zone electrophoresis of the heterogalactan Electrophoresis was performed on Toyo GA-100 glass filter-paper at 1,350 V, 70 min, with 0 1M sodium tetraborate (pH 9 3)

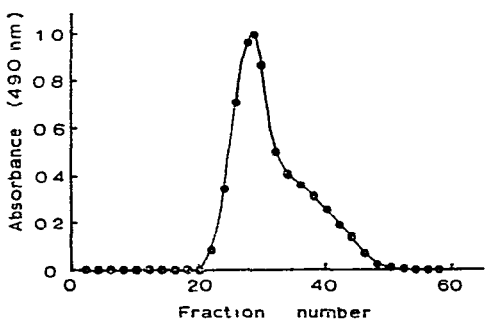

Fig 2 Elution pattern of the heterogalactan The Sephadex G-100 column (1 5 × 84 cm) was eluted with water

Celite column by stepwise elution with water and 2 5–15% ethanol The fragments found are listed in Table III, together with some of their properties. As shown in Table III, only galactose-containing oligosaccharides belonging to a homologous series were isolated from the partial acid-hydrolyzate Of these oligosaccharides, the first member of the homologous series was characterized as 6-*O*-α-D-galactopyranosyl-D-galactose

Treatment of the polysaccharide with sodium metaperiodate led to consumption of 1 58 moles of periodate and to release of 0 69 moles of formic acid per hexose residue Reduction of the oxidized product with sodium borohydride followed by acid hydrolysis gave no reducing sugars, suggesting the absence of 3-*O*-D-mannopyranosyl-L-fucopyranose residues, the latter have been commonly observed as side-chains of other *Basidiomycetes* heterogalactans[2–7]

On the basis of these results, it is possible to suggest that the heterogalactan of *L edodes* consists of a backbone of (1→6)-linked α-D-galactopyranose residues, part of which are substituted at C-2 by single L-fucose or D-mannose residues However, there is a possible alternative structure in which the main chain is partly branched, with side-chains of the same structure as the backbone and most, if not all, of the side chains terminated with L-fucose or D-mannose residues No information is available as yet on the configuration of the L-fucose and D-mannose residues

There have been several reports dealing with the isolation and characterization of heterogalactans of *Basidiomycetes*, such as *Polyporus giganteus*[2], *Armillaria mellea*[3,4], *Polyporus fomentarius*[5], *Polyporus igniarius*[5], *Polyporus pinicola*[6], and *Polyporus squamosus*[7] Most of these heterogalactans have a common structure consisting of a backbone of (1→6)-linked α-D-galactopyranose residues, part of which are substituted at C-2 either by L-fucopyranose or 3-*O*-α-D-mannopyranosyl-L-fucopyranose residues Some additional side-chains, such as 6-*O*-(3-*O*-methyl-α-D-galactopyranosyl)-D-galactopyranose[4], single D-galactopyranose[5,7], or short chains composed of (1→2)- and (1→3)-linked α-D-galactopyranose residues[7] are present in some of the heterogalactans The only exception is a heterogalactan from the fruit bodies of *Polyporus giganteus*[2], which consists of a backbone of (1→6)-linked β-D-galactopyranose residues

The *L edodes* heterogalactan has a backbone essentially similar to those of other *Basidiomycetes* heterogalactans However, this polysaccharide differs distinctly from the others in its side-chain structure Whereas part of the L-fucose is present as non-terminal residues in the side chains of the other heterogalactans, both L-fucose and D-mannose are present exclusively as terminal residues in the heterogalactan of *L edodes*

Other studies concerning the *L edodes* polysaccharides have been reported by Chihara *et al*[8,9], who isolated and characterized a β-D-glucan that they named lentinan Further studies on the other polysaccharides of *L edodes* are now in progress and the results will be published in near future

EXPERIMENTAL

General methods — All evaporations were performed under diminished pressure at 40–45° Melting points are not corrected Paper chromatography was performed on Toyo No 50 filter paper by descending or ascending methods with the following solvent systems (v/v) (*A*) 8 2:1 ethyl acetate–pyridine–water, (*B*) 65% 1-propanol, and (*C*) 4 1·5 1-butanol–ethanol–2% aqueous ammonia Preparative

paper-chromatography was carried out on Toyo No 527 thick filter-paper Neutral sugars and methylated sugars were detected on the chromatogram by spraying the paper with aniline hydrogen phthalate Gas–liquid chromatography (g l c) was effected with a Yanagimoto Model G-80 gas chromatograph fitted with a flame-ionization detector and a glass column (150 × 0 4 cm diameter) that had been packed with 10% of poly(diethyleneglycol succinate) on Diasolid-L Analysis was made at 170°, at a nitrogen flow-rate of 30 ml min $^{-1}$ The retention times of the methylated methyl glycosides are relative to that of methyl 2,3,4,6-tetra-*O*-methyl-*β*-D-glucoside Optical rotations were determined at 15–20° with a Nippon Bunko Model DIP-SL polarimeter. Ultracentrifugation analysis was performed with a Hitachi UCA-1A analytical ultracentrifuge Zone electrophoresis of the polysaccharides[10] was conducted on Toyo GA-100 glass filter-paper (5 × 60 cm) in 0 1M sodium tetraborate (pH 9 3), at 1,350 V for 70 min The filter paper was cut into strips (5 × 1 cm) that were eluted with deionized water The carbohydrates in the eluted solutions were determined by the anthrone method Complete acid hydrolysis of the polysaccharide was effected by heating the sample (10 mg) with M hydrochloric acid (1 ml) for 3 h in a boiling water-bath The hydrolyzate was neutralized with silver carbonate, deionized with Amberlite IR-120 resin (H^+ form), and evaporated to a syrup Analysis of the molar ratio of the component sugars was performed by paper chromatography The acid hydrolyzate was spotted on Toyo No 50 filter paper which was developed by the descending method with solvent *A* Zones corresponding to the sugars were cut off from the chromatogram according to the guide strips, and then eluted with water The eluted sugars were determined by the anthrone method

Isolation of the heterogalactan — The dried fruit-bodies of *L edodes** (1 kg) were dipped in 3% trichloroacetic acid (15 l) and disintegrated in a Waring blender Mixtures were kept at 5° overnight and then filtered. The filtrate was concentrated to about one half of the original volume and the polysaccharide precipitated with an equal volume of methanol The precipitate was centrifuged off, washed successively with methanol and ether, and air dried, yield 13 5 g (fraction *A*) The supernatant solution was treated with an equal volume of methanol (final concentration of methanol, 75%) to give 5 2 g of precipitate (fraction *B*) On hydrolysis, fraction *B* gave rise to D-galactose, D-glucose, D-mannose, and L-fucose whereas fraction *A* gave only D-glucose

A glucose-free heterogalactan was obtained by gradual precipitation of fraction *B* with cetyltrimethylammonium bromide (Cetavlon) in borate buffer at different pH values[11] Fraction *B* (4 3 g) was dissolved in water (200 ml) and treated with equal volumes of 0 15M Cetavlon and borate buffer (pH 8 0) The precipitate formed was washed with water, dissolved in 2M acetic acid, and the solution was poured into 3 volumes of methanol The precipitate was successively washed with methanol and ether, and air dried, yield 1 5 g (*B*-1) The supernatant at pH 8 0 was adjusted to pH 9 0 and the precipitate formed was treated by the foregoing procedure

*A commercial product purchasable in Japanese supermarkets

to give 0 8 g of an additional product (*B*-2) The supernatant at pH 9 0 was further adjusted to pH 10 0, and 0 2 g of polysaccharide (*B*-3) was recovered from the precipitate. The final supernatant, at pH 10,0, was poured into three volumes of methanol to give 1 0 g of precipitate (*B*-4) On hydrolysis, *B*-1 gave rise to galactose, mannose, and fucose in a molar ratio of 6 0 1 5 1 0, and no glucose was detected in the hydrolyzate The purified heterogalactan had $[\alpha]_D$ +102° (*c* 0 9, water) and contained a trace of nitrogen (0 19%) The results of the fractionation are given in Table I

Gel filtration — A solution of the polysaccharide (*B*-1) (5 mg) in 0 5 ml of water was applied to a column (84 × 1 5 cm in diam) of Sephadex G-100 The column was eluted with water and the effluent was collected in 3-ml fractions The carbohydrate content of each fraction was determined by the phenol–sulfuric acid method (Fig 2)

Periodate oxidation — Periodate oxidation was conducted with 0 02M sodium metaperiodate at 5° in the dark The periodate consumption was monitored spectrophotometrically[12], and the formic acid released was titrated with 0 01M sodium hydroxide after reduction of the excess of periodate with ethylene glycol The polysaccharide consumed 1 58 moles of periodate and released 0 69 mole of formic acid per hexose residue After completion of the oxidation, the oxidation product was submitted to reduction with sodium borohydride No reducing sugar could be detected in an acid hydrolyzate of the deionized reduction-product

Methylation analysis. — The polysaccharide (1 3 g) was dissolved in dimethyl sulfoxide (60 ml) and treated with methylsulfinyl carbanion solution that had been prepared by dissolving sodium hydride (0 8 g) in dimethyl sulfoxide (15 ml)[13] The mixture was stirred under nitrogen for 4 h at room temperature, and methyl iodide (4 ml) was then added The mixture was then stirred overnight, diluted with water, dialyzed, and the dialyzed material was evaporated to dryness Six methylations were required, to give a product (1.1 g) having $[\alpha]_D$ +81° (*c* 0 9, chloroform), and OCH_3 42 5% (calc , 44 1%). The methylated polysaccharide was hydrolyzed by the method of Garegg and Lindberg[14], it (600 mg) was suspended in 72% sulfuric acid (7 ml) and stirred for 2 5 h at 0–5° until it was dissolved The mixture was diluted with water (56 ml) and heated for 4 h at 100° The hydrolyzate was then neutralized with barium carbonate, deionized with Amberlite IR-120 resin (H^+ form), and evaporated to a syrup, yield 560 mg The hydrolyzate was resolved by preparative paper-chromatography with solvent system *C* For g l c , the methylated polysaccharide (5 mg) was heated in an sealed tube with 5% methanolic hydrogen chloride (0 5 ml) for 6 h at 100° The methanolyzate was neutralized with silver carbonate, and the filtrate was evaporated to dryness The residue was dissolved in a small amount of methanol and submitted to g l c

The methyl ethers from the degradation product of the methylated polysaccharide were characterized by paper-chromatographic comparison with authentic samples, by g l c of their methyl glycosides, and by demethylation with boron trichloride[15]. Yields and some properties of the hydrolysis products from the methylated polysaccharide are listed in Table II Furthermore, the major components were

characterized by preparation of crystalline derivatives The methylated sugars were characterized as follows

2,3,4,6-Tetra-*O*-methyl-D-mannose had $[\alpha]_D$ −1° (*c* 1 2, water) and gave an aniline derivative[16] having m p and mixed m p 142°.

2,3,4-Tri-*O*-methyl-L-fucose had $[\alpha]_D$ −100° (*c* 1 4, water) and gave an aniline derivative[17] having m p and mixed m p 132°

2,3,4-Tri-*O*-methyl-D-galactose* had $[\alpha]_D$ +104° (*c* 0 5, water) and gave an aniline derivative[18] having m p and mixed m p 163°

3,4-Di-*O*-methyl-D-galactose had $[\alpha]_D$ +93° (*c* 0 8, water) and gave galactose on demethylation It was conclusively characterized by g l c –mass spectroscopy of its alditol acetate by the courtesy of Prof B Lindberg

Partial acid hydrolysis of the heterogalactan — The polysaccharide (1 4 g) was submitted to successive hydrolytic treatments for 1 h at 100° (twice with 0 1M hydrochloric acid and twice with 0 2M hydrochloric acid) After each treatment, ethanol was added and the precipitate formed was submitted to further hydrolysis Hydrolyzates from each step were combined, neutralized with silver carbonate, and evaporated to give 1 2 g of syrup

The partial hydrolyzate (1 2 g) was dissolved in water and poured on a column of charcoal–Celite (20 g of each) The column was successively eluted with water and 2 5–15% ethanol The effluent was collected in 100-ml fractions which were examined by paper chromatography A list of the fragments obtained and some of their properties are given in Table III Each oligosaccharide gave rise only to galactose on hydrolysis and, on partial hydrolysis, the higher members of the series yielded the lower homologues These results suggest that these oligosaccharides belong to a homologous series The first member of the series had $[\alpha]_D$ +139° (*c* 0 9, water) Methylation of this sugar, followed by methanolysis, gave equimolecular proportions of the methyl glycosides of tetra-*O*-methyl- and tri-*O*-methyl-D-galactose, which were characterized by g l c as 2,3,4,6-tetra- and 2,3,4-tri-*O*-methylgalactose, respectively[20] Thus, the oligosaccharides obtained by partial acid-hydrolysis were shown to form a homologous series of (1→6)-linked α-D-galactose oligomers

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