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Note

Conformation of the (1→3)-β-D-glucan in the sclerotia of *Sclerotinia sclerotiorum* IFO 9395 assessed by ^{13}C-c.p.–m.a.s. n.m.r. spectroscopy

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Ultrastructure is an important feature for the antitumor activity of (1→3)-β-D-glucans. Thus, the antitumor activity of pachyman (U-pachyman) is induced by treatment with urea[1]. Treatment of schizophyllan in low concentration at 150° degraded the helical conformation, produced small aggregates[2,3], and reduced the antitumor activity. The triple helical structure is thought to be an essential structural requirement for the antitumor activity of schizophyllan. The conformation of glucans can also be affected by the action of zymolyase, an endo-(1→3)-β-D-glucanase, produced by *Arthrobacter luteus*[4–8]. Thus, boiling treatment of pachyman increased the digestivity of zymolyase. The X-ray crystallography of curdlan, lentinan, scleroglucan, and schizophyllan strongly suggested the existence of a triple helical structure[9–12]. Saito *et al.*[13,14] have applied the cross polarisation–magic angle spinning (c.p.–m.a.s.) n.m.r. method to these (1→3)-β-D-glucans, and suggested that the chemical shift of the resonance of C-3 was strongly affected by the torsion angles[13,14] and that there were at least 4 types of solid state conformations, namely, I (curdlan-type), II (laminaran-type), III (laminaripentaose-type), and IV (dimethyl sulfoxide adduct). We have suggested[15] that, for the (1→3)-β-D-glucan fractions variously obtained from the fungus *Grifola frondosa*, native and helix conformations were present. In liquid culture, the glucans (LLFD, LELFD), synthesised as the native form, were transformed into the helix form by treatment with urea and/or sodium hydroxide. Comparison with the spectra reported by Saito *et al.*[13,14] suggests that the helix form corresponds to form I (curdlan type) and the native form to form II (laminaran type). Similar results have been obtained[16,17] with an antitumor (1→3)-β-D-glucan (SSG) branched at C-6 of alternate units in the main chain and produced by *Sclerotinia sclerotiorum* IFO 9395. We now report on the structure and conformation of the (1→3)-β-D-glucan of "sclerotia" produced by culturing *S. sclerotiorum* on an agar plate[18].

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S. sclerotiorum IFO 9395 was cultured on malts agar at 25°. After ~4 weeks, the sclerotia, in the form of black balls (~5 mm in diameter), were recovered, lyophilised, homogenised, and then extracted with water in an autoclave to give fraction TSHW. Extraction of the residue (TS-HWR) with aqueous 10% sodium hydroxide containing 5% of urea at 4° gave fraction TS-CAS, and neutralisation of the residue gave fraction TS-CAR.

TSHW contained ~65% of carbohydrate and ~35% of protein, and the component sugars were glucose and mannose in the molar ratio of 1:0.7. Methylation analysis gave mainly 2,3,4,6-tetra-, 2,4,6-tri-, and 2,4-di-*O*-methylglucose in the molar ratios of 1.0:1.0:0.8. The ^{13}C-n.m.r. spectrum of TSHW in 0.2M sodium hydroxide contained a signal for anomeric carbons at 104 p.p.m. Thus, TSHW contained a large proportion of (1→3)-β-D-glucan branched at alternate units of the main chain as in SSG. TS-CAS contained ~90% of carbohydrate and ~10% of protein, and the component sugars were glucose and mannose in the molar ratio 1:0.04. Methylation analysis gave 2,3,4,6-tetra-, 2,4,6-tri-, and 2,4-di-*O*-methylglucose in the molar ratios 1:1.3:0.8. Thus, the sclerotia were composed mainly of a (1→3)-β-D-glucan branched at alternate units of the main chain.

Fig. 1d shows the c.p.–m.a.s. n.m.r. spectrum of TSHW, which is similar to that of the native form of SSG (Fig. 1h). TSHW contained some mannan, but the relevant signals were not identified clearly because of low resolution. The spectrum of TS-CAS (Fig. 1e) is similar to that of the helix form of SSG (Fig. 1g). A glucan fraction partially purified from TS-CAS by DEAE-Sephadex chromatography (Fig. 1f) also had a spectrum similar to that of the helix form of SSG.

Fig. 1a shows the c.p.–m.a.s. n.m.r. spectrum of the sclerotia. Compared with the spectra of curdlan (Fig. 1i) and SSG (Fig. 1g,h), the signal for C-3 (86 p.p.m.) had a chemical shift similar to that of the native form of SSG. The sclerotia contained mannan and chitin as well as (1→3)-β-D-glucan (data not shown). On the basis of the molar ratio and the yield of TSHW and TS-CAS, the content of mannan was 8-fold less than the glucan conent. Thus, the contribution of the mannan moiety to the spectra of sclerotia would be small and the spectrum would reflect the conformation of the (1→3)-β-D-glucan. In order to eliminate the effect of the mannan, TS-HWR was prepared (see above), and its spectrum was similar to that of the sclerotia. It is assumed that the content of chitin in the sclerotia is low since the characteristic signals (carbonyl and methyl) were not detected. Thus, the spectrum of the sclerotia is thought to represent the (1→3)-β-D-glucan moiety and it is suggested to have the "native" conformation. In contrast, TS-CAS and the partially purified glucan each has a helix conformation. This observation is supported by the fact that treatment with urea and/or sodium hydroxide changed the conformation of SSG from native to helix[17].

The spectrum of TS-CAR was different from those of the sclerotia and TS-HWR, which is due, in part, to the treatment of the mushroom with aqueous sodium hydroxide. Whereas the conformation of the (1→3)-β-D-glucan in the sclerotia of *S. sclerotiorum* IFO 9395 and in the culture broth of *S. sclerotiorum*[17] is

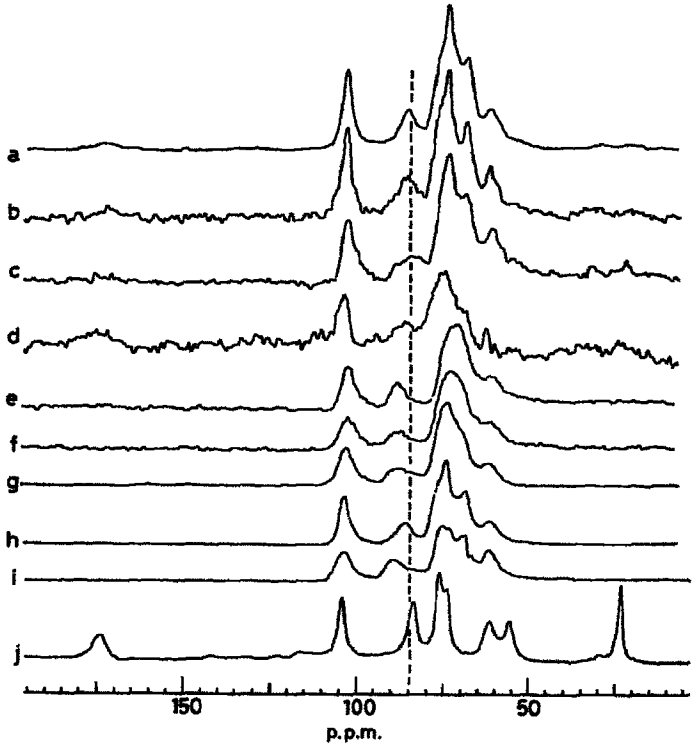

Fig. 1. ^{13}C-C.p.-m.a.s. n.m.r. spectra of the sclerotia and the polysaccharide fractions obtained from *Sclerotinia sclerotiorum* IFO 9395: a, sclerotia; b, hot-water extracted residue (TS-HWR); c, alkaline extracted residue (TS-CAR); d, hot-water extract (TSHW); e, cold alkaline extract (TS-CAS); f, (1→3)-β-D-glucan of TS-CAS partially purified by DEAE-Sephadex chromatography; g, helix form of SSG; h, native form of SSG; i, curdlan; j, chitin. Chemical shift data for the reference compounds: curdlan, C-1 104.6, C-2 73.9, C-3 90.1, C-4 70.2, C-5 76.1, C-6 62.5 p.p.m.; chitin, C-1 104.0, C-2 55.5, C-3 73.5, C-4 83.5, C-5 76.0, C-6 61.5, C-2 (methyl) 23.0, C-2 (carbonyl) 174.0 p.p.m.

the native form, the conformation of curdlan, a linear (1→3)-β-D-glucan obtained from *Alkaligenes faecalis*, is the helix form[14]. The nascent (1→3)-β-D-glucan segment is thought to have a random coiled conformation; when a certain chain length is reached, the native and helix forms develop. During the formation of ultrastructure, the side-chain moieties would interfere sterically with the formation of the helix form. Further, conformational differences between curdlan and the sclerotial glucan suggested that the biosynthesis of the branches proceeded concomitantly with the biosynthesis of the main chain.

EXPERIMENTAL

Production of sclerotia[18]. — *Sclerotinia sclerotiorum* IFO 9395 (Ascomycotina, Discomycetes, Helotiales) was purchased from the Institute for Fermentation (Osaka) and cultured on malt agar (Eiken Co. Ltd., Tokyo) plated

on 9-cm diameter dishes at 25°. After ~4 weeks (after the end of the mycelial growth), the sclerotia (in the form of a black ball, ~5 mm in diameter) was picked up from the plate and lyophilised (yield, 6.5 g/100 plates).

Preparation of the extracts. — The sclerotia was dispersed using a universal homogeniser HBA (Nihon Seiki Seisakusho Co., Tokyo) and then extracted with water (autoclave, 121°, 60 min, 3 times). The combined extracts were dialysed against water and then lyophilised to yield TSHW (15%). The residue was extracted with aqueous 10% sodium hydroxide containing 5% of urea at 4° for 24 h (6 times). The combined extracts were dialysed extensively against water and lyophilised to yield TS-CAS (54%). The residue was neutralised with acetic acid, washed extensively with water, and lyophilised to yield TS-CAR (20%).

DEAE-Sephadex chromatography. — A solution of TS-CAS (160 mg) in 8M urea (160 mL) was applied to a column (20 mL) of DEAE-Sephadex A 25 (Cl^- form) and eluted with 8M urea. The eluate was dialysed against water and then lyophilised (yield, 80%).

N.m.r. studies. — C.p.–m.a.s. ^{13}C-n.m.r. spectra were measured with a JEOL-FX 200 instrument, equipped with a c.p.–m.a.s. unit operating with the appropriate software. The spectra were measured with the use of a Dyflon rotor. Contact time, pulse interval, and number of pulses were 1 ms, 1–2 s, and 500–2000 scans, respectively. Chemical shifts relative to that of Me_4Si were determined by using the adamantane signal (29.5 p.p.m.). Solution ^{13}C-n.m.r. spectra were measured with a JEOL-FX 200 instrument equipped with a 10 ϕ sampling tube.

General methods. — Methylation was performed by the method of Hakomori[19]. Each product was treated with aqueous 90% formic acid at 100° for 10 h in a sealed tube. After evaporation of the formic acid, the residue was hydrolysed with M trifluoroacetic acid at 100° for 5 h and the hydrolysate was concentrated to dryness. The resulting partially methylated sugars were converted into the alditol acetate derivatives and subjected to g.l.c. on a glass column (200 × 0.3 cm) packed with 0.3% of OV-275/0.4% XF-1150, and a temperature programme of 120° → 190° at 2°/min. The molar ratio of each methylated derivative was calculated by reference to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol as 1.0.

Component sugars were converted into the corresponding alditol acetates and subjected to g.l.c. Optical rotations were measured with a DIP-4 (JASCO) instrument equipped with a 5-cm quartz cell. Sugar contents were determined as "anhydroglucose" by the phenol–sulfuric acid method, using D-glucose as standard[20]. Protein was determined by the Lowry–Folin method with bovine serum albumin as the reference[21].

REFERENCES

1 Y. Y. Maeda, J. Hamuro, Y. O. Yamada, K. Ishimura, and G. Chihara, in G. E. Wolstenholme and J. Knight (Eds.), *Immunopotentiation*, Elsevier, North-Holland, 1973, p. 259.
2 T. Yanaki, W. Itoh, and K. Tabata, *Agric. Biol. Chem.*, 50 (1986) 2145–2147.
3 T. Yanaki, K. Tabata, and T. Kojima, *Carbohydr. Polym.*, 5 (1985) 275–282.

4 K. KITAMURA, T. KANEKO, AND Y. YAMAMOTO, *Arch. Biochem. Biophys.*, 145 (1971) 402–404.
5 K. KITAMURA AND Y. YAMAMOTO, *Arch. Biochem. Biophys.*, 153 (1972) 403–406.
6 K. KITAMURA AND Y. YAMAMOTO, *Agric. Biol. Chem.*, 45 (1981) 1761–1766.
7 K. KITAMURA, *Agric. Biol. Chem.*, 46 (1982) 963–969.
8 K. KITAMURA, *Agric. Biol. Chem.*, 46 (1982) 2093–2099.
9 T. L. BLUHM AND A. SARKO, *Can. J. Chem.*, 55 (1977) 293–300.
10 Y. DESLANDES, R. H. MARCHESSAULT, AND A. SARKO, *Macromolecules*, 13 (1980) 1466–1471.
11 T. L. BLUHM, Y. DESLANDES, AND R. H. MARCHESAULT, *Carbohydr. Res.*, 100 (1982) 117–125.
12 Y. TAKAHASHI, T. KOBATAKE, AND H. SUZUKI, *Proc. Jpn. Carbohydr. Symp., 7th*, (1984) 58–59.
13 H. SAITO, R. TABETA, AND T. HARADA, *Chem. Lett.*, (1981) 571–574.
14 H. SAITO, R. TABETA, T. SASAKI, AND Y. YOSHIOKA, *Bull. Chem. Soc. Jpn.*, 59 (1986) 2093–2101.
15 N. OHNO, Y. ADACHI, I. SUZUKI, S. OIKAWA, K. SATO, Y. SUZUKI, M. OHSAWA, AND T. YADOMAE, *Chem. Pharm. Bull.*, 34 (1986) 2555–2560.
16 N. OHNO, I. SUZUKI, AND T. YADOMAE, *Chem. Pharm. Bull.*, 34 (1986) 1362–1365.
17 N. OHNO AND T. YADOMAE, *Carbohydr. Res.*, 159 (1987) 293–302.
18 N. OHNO, H. SHINOHARA, AND T. YADOMAE, *Chem. Pharm. Bull.*, 34 (1986) 5071–5078.
19 S. HAKOMORI, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
20 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
21 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265–275.