Available online at www.sciencedirect.com
ScienceDirect
Carbohydrate Research 342 (2007) 2481–2483
Carbohydrate RESEARCH

Note

Characterisation of the extracellular polysaccharides produced by isolates of the fungus *Acremonium*

Frank Schmid,[a] Frances Separovic,[b] Barbara M. McDougall,[a] Bruce A. Stone,[c] Robert T. C. Brownlee[d] and Robert J. Seviour[a,*]

[a]*Biotechnology Research Centre, La Trobe University, Bendigo, VIC 3550, Australia*
[b]*School of Chemistry, Bio21 Institute, The University of Melbourne, VIC 3010, Australia*
[c]*School of Molecular Sciences, La Trobe University, Bundoora, VIC 3086, Australia*
[d]*Department of Chemistry, La Trobe University, Bundoora, VIC 3086, Australia*

Received 18 August 2006; received in revised form 4 June 2007; accepted 18 June 2007
Available online 28 June 2007

Abstract—Solid state ^{13}C NMR studies of the extracellular glucans from the fungi *Acremonium persicinum* C38 (QM107a) and *Acremonium* sp. strain C106 indicated a backbone of (1→3)-β-linked glucosyl residues with single (1→6)-β-linked glucosyl side branches for both glucans. Analyses of enzymatic digestion products suggested that the average branching frequency for the *A. persicinum* glucan (66.7% branched) was much higher than that of the *Acremonium* sp. strain C106 glucan (28.6% branched). The solid state ^{13}C NMR spectra also indicated that both glucans are amorphous polymers with no crystalline regions, and the individual chains are probably arranged as triple helices.

Keywords: *Acremonium*; (1→3,1→6)-β-glucans; Solid state ^{13}C NMR; Fungal extracellular polysaccharide; Enzymatic hydrolysis

Extracellular polysaccharide production by the fungus *Acremonium diospyri* was first reported by Seviour and Hensgen.[1] This mucilaginous material was analysed by infrared spectroscopy, and gave a spectrum consistent with that of a β-D-glucan. A later survey of several *Acremonium* spp. showed that most produced extracellular, ethanol-insoluble mucilaginous material.[2] Structural characterisation, restricted to monosaccharide and infrared analyses, confirmed that all were β-glucans,[2] but no additional structural information was obtained. Here, we describe an NMR structural analysis of extracellular polysaccharides from two strains of *Acremonium* (*A. persicinum* and *Acremonium* strain C106). Monosaccharide analysis and determination of anomeric configurations of *Acremonium* glucans were not repeated as these data were reported previously by Stasinopoulos and Seviour.[2]

Although the methylation data indicated some branching involving the C-6 carbon of the main chain residues of these glucans, neither conventional nor DEPT 135 ^{13}C NMR[3] data could confirm this convincingly (data not shown). Therefore, solid state NMR was used since these glucans could be analysed in a solid, dehydrated form overcoming any solubility problems. Spectra for the *A. persicinum* and *Acremonium* strain C106 glucans are shown in Figure 1a and b, respectively. These spectra are almost identical to that reported for scleroglucan[4] and based on this similarity, the *Acremonium* glucans are most likely (1→3)-β-glucans with single (1→6)-β-linked side branches. Signals corresponding to C=O (δ 174.0) and quaternary alkyl (δ 30.1–46.4) groups, which were detected in the *Acremonium* strain C106 glucan spectrum, were not seen in the *A. persicinum* glucan spectrum. These probably reflect impurities introduced during glucan handling, since no evidence for similar material was seen with either of the glucans in their solution ^{13}C NMR spectra (spectra not shown). The broad signals in the spectra of both

* Corresponding author. Tel.: +61 3 54447459; fax: +61 3 54447476; e-mail: r.seviour@bendigo.latrobe.edu.au

0008-6215/$ - see front matter © 2007 Published by Elsevier Ltd.
doi:10.1016/j.carres.2007.06.018

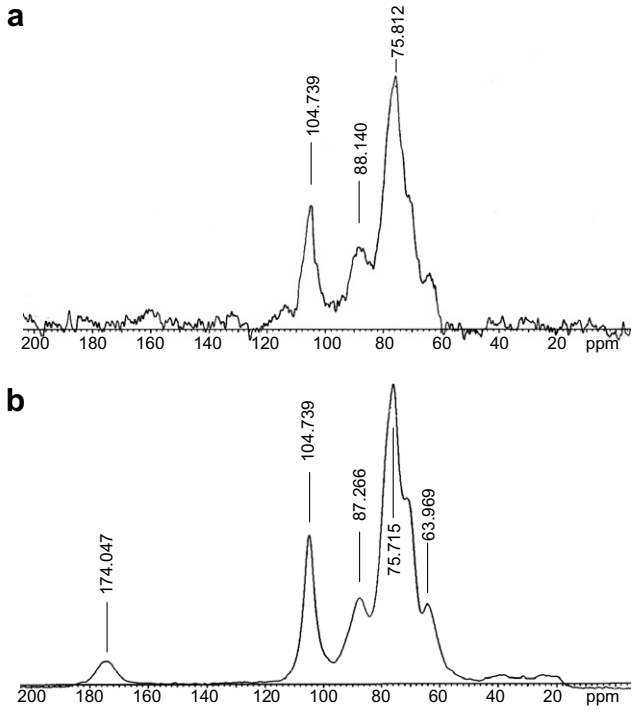

Figure 1. Solid state CP/MAS ^{13}C NMR spectra of the extracellular glucans produced by (a) *Acremonium persicinum* C38 and (b) *Acremonium* strain C106.

glucans suggest that they are amorphous polymers, and do not contain ordered crystalline regions, which would have resulted in much narrower linewidths.[4] Five major peaks were seen for both. Thus, C-1 and C-3 carbon signals are visible at about δ 104–105 and δ 87, respectively. The C-2 and C-5 carbons appear as very broad signals at about δ 75.7–76.3. A slight shoulder is visible at about δ 70 and corresponds to both C-4 and branched C-6 carbons, while the signals for the unbranched C-6 carbons appear at around δ 64. Although the solid state ^{13}C NMR data suggest that these glucans are similar to scleroglucan,[4] their branching frequencies were not convincingly revealed from these spectra. However, as postulated for scleroglucan, the spectra are consistent with a triple helical arrangement for both the *Acremonium* glucan chains, again based on similarities between their spectra.[4]

To determine the extent of side-chain-branching in these glucans, both were digested with the purified (1→3)-β-glucan *exo*-hydrolase (EC 3.2.1.58) from *Acremonium blochii* strain OXF C13.[5,6] The only products detected after prolonged periods of enzymatic digestion (24 h) were glucose and gentiobiose, and the quantitative data (Table 1) suggest that branching frequencies may differ between these two *Acremonium* glucans. Whereas the *A. persicinum* glucan appears to branch on average two out of every three backbone residues, as seen with epiglucan,[5] the *Acremonium* strain C106

Table 1. Quantification of enzymatic digestion products from *Acremonium* glucans using a purified (1→3)-β-glucan *exo*-hydrolase from *Acremonium blochii* (OXF C13)

Digestion products	Glucan (mol ratio)	
	A. persicinum	*Acremonium* sp. strain C106
Glucose	1.0	5.0
Gentiobiose	1.9	2.0

Products were separated on a Dionex HPAEC with a CarboPac PA-1 column and quantified by pulsed amperometric detection against standards of glucose and gentiobiose. Data represent duplicate analyses.

glucan seems to branch less frequently, with an average branching frequency of two out of every seven glucose residues. Glucans from closely related strains of other fungal species appear to differ in their branching frequencies, as characterised in *Botrytis cinerea*[7,8] and *Pleurotus* sp.[9,10] These enzymatic data alone do not rule out the possible existence of side branches consisting of more than one glucose residue, especially since some (1→3)-β-glucan *exo*-hydrolases may digest short oligosaccharides side chains.[11] Smith degradation attempted to clarify this, but the *Acremonium* glucans were resistant to periodate oxidation, and no structural data were obtained.

In conclusion, although the branching frequencies could not be determined clearly from the methylation or solution ^{13}C NMR data, the backbone in both *Acremonium* glucans appears to consist of (1→3)-β-linked glucose residues, probably with single (1→6)-β-linked side residues attached, but apparently at different frequencies in the two strains. Solid state ^{13}C NMR data indicated that these glucans probably form triple helices upon dehydration as is observed with scleroglucan and schizophyllan.[11] In the latter, the (1→6)-linked side chains are situated outside the triple helically wound (1→3)-β-glucan backbone and although these polysaccharides, like the unbranched (1→3)-β-glucan, curdlan, form gels, they are softer probably due to the dislocating interactions of the side chains.[11] The degree of interaction between the triple helices will depend on the number and distribution of the side chains. The insolubility of the *Acremonium* glucans after dehydration presumably results from the strong interactions between the triple helices and may explain the problems encountered here with methylation, solution ^{13}C NMR, and Smith degradation analyses.

The biological role of the capsular and secreted (1→3, 1→6)-β-glucans formed by *Acremonium* spp. and many other soil microfungi is uncertain. As hydrophilic, gel-forming polymers they may function in protecting the underlying hyphae from desiccation and, in the case of species pathogenic to plants, may be involved in eliciting host responses. There is evidence that in some spe-

cies, the (1→3, 1→6)-β-glucans are mobilised by secreted (1→3)-β-glucan hydrolases and the digestion products absorbed and utilised in wall synthesis.[11] The (1→3, 1→6)-β-glucans of the type produced by *Acremonium* spp. are well-recognised as activators of the mammalian innate immune system and have potential clinical applications as antitumour agents.[12]

1. Experimental

1.1. Growth of cultures and recovery of extracellular polysaccharide

A. persicinum (QM 107a) and *Acremonium* sp. strain C106 (LTUB C106) were maintained as soil cultures in the La Trobe University, Bendigo Culture Collection at 4 °C. Cells grown on malt extract agar (MEA) slopes for 72 h at 25 °C were suspended in 10 mL of sterile distilled water by gently scraping the surface of the slope, and the suspension was transferred to 250 mL conical flasks containing 50 mL of Czapek Dox broth (Oxoid). Flasks were incubated at 28 °C for 7 d in an orbital incubator (Paton Industries, SA, Australia) at 200 rpm. Biomass was removed from culture broths by centrifugation at 20,000*g* at 25 °C for 10 min. Polysaccharide was precipitated from the supernatant by addition of 2 vol of cold (4 °C) 95% ethanol, and the precipitated material was dialysed extensively against reverse osmosis (RO) water at room temperature (20 °C) to remove residual low molecular weight material. The dialysed polysaccharide was lyophilised and powdered in a Spex Freezer Mill (Spex Industries Inc., Metuchen, NJ, USA) operating for 1 min at three quarters of maximum impact frequency.

1.2. Solid state ^{13}C NMR analysis of extracellular polysaccharides

Solid state CP/MAS ^{13}C NMR spectra[13,14] were obtained on a Varian (Palo Alto, USA) Inova 300 spectrometer operating at 75.4 MHz. Polysaccharides (~50 mg) were analysed in a 5 mm rotor at a spinning speed of 7200 Hz, and for each spectrum 6400 transients were collected. The ^{1}H radiofrequency field strength was set to give a 90° pulse duration of 4 μs. The contact time and recycle delay were set at 1 ms and 2 s, respectively. Chemical shifts were calibrated relative to tetramethylsilane set at 0 ppm. Spectral width and line broadening were set at 30 kHz and 50–75 Hz, respectively.

1.3. Enzymatic digestion of extracellular polysaccharides

Each polysaccharide was digested with the fungal (1→3)-β-glucan *exo*-hydrolase (EC 3.2.1.58) from *A. blochii* OXF C13,[6] which was purified by FPLC to electrophoretic homogeneity, under conditions as described by Schmid et al.[5] Digest products were separated and quantified with a Dionex HPAEC and a CarboPac PA-1 column (4 × 250 mm; Dionex Corp.). Samples (10–20 μL) were eluted in 150 mM NaOH (run isocratically) at a flow rate of 1 mL min^{-1}, and products were detected by pulsed amperometric detection (Dionex) ($t_1 = 50$ ms, $V_1 = 100$ mV; $t_2 = 100$ ms, $V_2 = 600$ mV; $t_3 = 50$ ms, $V_3 = -600$ mV). Sugars were identified against standards run under identical conditions.

Acknowledgement

F. Schmid wishes to acknowledge receipt of a La Trobe University Ph.D. scholarship which supported this study.

References

1. Seviour, R. J.; Hensgen, K. *FEMS Microbiol Lett.* **1983**, *16*, 343–347.
2. Stasinopoulos, S. J.; Seviour, R. J. *Mycol. Res.* **1989**, *92*, 55–60.
3. Claridge, T. D. W. *High-resolution NMR techniques in organic chemistry*; Pergamon Press: New York, USA, 1999.
4. Bardet, M.; Rousseau, A.; Vincedon, M. *Magn. Reson. Chem.* **1993**, *31*, 887–892.
5. Schmid, F.; Stone, B. A.; McDougall, B. M.; Bacic, A.; Martin, K. L.; Brownlee, R. T. C.; Chai, E.; Seviour, R. J. *Carbohydr. Res.* **2001**, *331*, 163–171.
6. Martin, K. L.; McDougall, B. M.; Unkles, S. E.; Seviour, R. J. *Mycol. Res.* **2006**, *110*, 66–74.
7. Leal, J. A.; Rupérez, P.; Gomez-Miranda, B. *Trans. Brit. Mycol. Soc.* **1979**, *72*, 172–176.
8. Dubourdieu, D.; Ribereau-Gayon, P.; Fournet, B. *Carbohydr. Res.* **1981**, *93*, 294–299.
9. Burns, P. J.; Yeo, P.; Keshavarz, T.; Roller, S.; Evans, C. S. *Enzyme Microb. Technol.* **1994**, *16*, 566–572.
10. Gutiérrez, A.; Prieto, A.; Martínez, A. T. *Carbohydr. Res.* **1996**, *281*, 143–154.
11. Stone, B. A.; Clarke, A. E. *Chemistry and Biology of (1→3)-β-Glucans*; La Trobe University Press: Melbourne, 1992.
12. Chen, Z.; Seviour, R. *Mycol. Res.*, in press.
13. Dodd, A.; Separovic, F. An introduction to biological solid state NMR. In *Supramolecular Structure and Function 8*; Pifat-Mrzljak, G., Ed.; Kluwer Academic/Plenum: New York, USA, 2004; pp 145–156.
14. Drechsler, A.; Separovic, F. *IUBMB Life* **2003**, *55*, 515–523.