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Structure of epiglucan, a highly side-chain/branched (1→3;1→6)-β-glucan from the micro fungus *Epicoccum nigrum* Ehrenb. ex Schlecht

Frank Schmid,[a] Bruce A. Stone,[b] Barbara M. McDougall,[a] Antony Bacic,[c] Kirstee L. Martin,[a] Robert T.C. Brownlee,[d] Eugene Chai,[e] Robert J. Seviour[a,*]

[a]*Biotechnology Research Centre, La Trobe University, Bendigo, Vic. 3550, Australia*
[b]*School of Biochemistry, La Trobe University, Bundoora, Vic. 3083, Australia*
[c]*Plant Cell Biology Research Centre, School of Botany, University of Melbourne, Melbourne, Vic. 3010, Australia*
[d]*Department of Chemistry, La Trobe University, Bundoora, Vic. 3083, Australia*
[e]*CRC for Industrial Plant Biopolymers, Department of Chemical Engineering, University of Melbourne, Melbourne, Vic. 3010, Australia*

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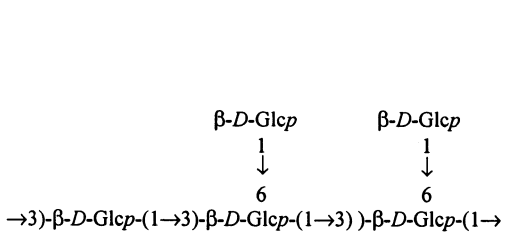
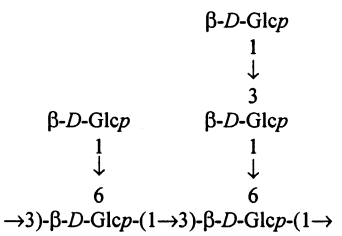
Abstract

The extracellular fungal polysaccharide, epiglucan, synthesised by *Epicoccum nigrum* is a side-chain/branched (1→3;1→6)-D-β-glucan. Methylation analysis, ^{13}C DEPT NMR and specific enzymic digestion data show slight variation in branching frequency among the epiglucans from the three strains examined. The (1→3)-β-linked backbone has (1→6)-β-linked branches at frequencies greater than the homologous glucans, scleroglucan and schizophyllan, from *Sclerotium* spp. and *Schizophyllum commune*, respectively. The structural analyses do not allow a distinction to be made between structures I and II.



I



II

Epiglucan displays non-Newtonian shear thinning rheological properties, typical of these glucans.

Keywords: Extracellular polysaccharide; (1→3;1→6)-β-glucan; *Epicoccum nigrum*

* Corresponding author. Tel.: +61-3-54447459; fax: +61-3-54447476.
E-mail address: r.seviour@bendigo.latrobe.edu.au (R.J. Seviour).

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1. Introduction

Many fungi produce extracellular glucan homopolymers with commercially important functional properties including emulsification and friction reduction.[1] Among these are the relatively rare linear α-glucans such as pullulan from *Aureobasidium pullulans*.[2] Some extracellular β-glucans, especially the (1→3)-β-glucans have useful rheological properties[3] and also exhibit antitumor activity, a property not shown by α-glucans, (1→4)-β-glucans or (1→6)-β-glucans.[4,5] This activity is believed to be related to the organisation of the (1→3)-β-linked backbone into a triple helix,[6] the frequency and complexity of side-branching and to their molecular weight.[4]

Although many fungal (1→3)-β-glucans have been described, only a few have been rigorously characterised, and so their chemical diversity and potentially useful functional properties are still poorly understood.[8] All those extracellular fungal β-glucans that have been characterised appear to possess structures similar to that of the *Claviceps purpurea* glucan,[9,10] in having a (1→3)-β-linked backbone with (1→6)-β-linked side branches. Other glucans belonging to this group are scleroglucan from *Sclerotium glucanicum*,[11] schizophyllan from *Schizophyllum commune*,[12] cinerean from *Botrytis cinerea*[13] and pestalotan from *Pestalotia* sp.[14] Although all have a (1→3)-β-D-glucopyranosyl linked backbone, their frequency of branching and length of their side branches differ. Thus *Claviceps* glucan, scleroglucan, cinerean and schizophyllan have a single (1→6)-β-D-glucopyranosyl side branched residue attached, on average, to every third glucosyl backbone residue.[10–13] By contrast, pestalotan is more frequently branched, with both single (1→6)-β-D-glucopyranosyl and less frequently (1→6)-β-D-glucopyranosyl disaccharide side branches attached, on average, to three of every five backbone residues.[14]

Gel-forming properties of culture media of *Epicoccum nigrum* were first reported by Bamford et al.,[15] but no attempt was made to identify the gel-forming polymer. Michel et al.[16] found that most *E. nigrum* strains synthesise an extracellular, ethanol-insoluble mucilage containing a β-linked glucan which they named epiglucan. Here we describe a detailed chemical characterisation of epiglucans from three strains of *E. nigrum*, and document some rheological properties for one of the epiglucans.

2. Results and discussion

All 36 isolates of *E. nigrum* from Bendigo produced extracellular polysaccharide in yields ranging from 0.3 to almost 35 g/L, which is higher than the yields of other fungal polysaccharides including β-glucans reported in the literature.[8] *E. nigrum*, strains E48 and E61, obtained from the Botany Department, ANU, Canberra, Australia,[17] and F19 from the Biotechnology Research Centre, La Trobe University, Bendigo, Australia, were included in this study to ascertain whether isolates from different locations produced similar or different polysaccharides. F19 was selected from the Bendigo isolates because of its high exopolysaccharide yield (35 g/L). GC–MS analyses of alditol acetate derivatives of products from complete acid hydrolysis of polysaccharides of all three *E. nigrum* isolates revealed only glucose. Glucosidic linkage configuration for F19 was exclusively of the β-type as determined from the IR spectrum (Fig. 1), which shows absorption at 891 cm^{-1} characteristic of the β anomer of glucopyranose. There was no absorption at 841 cm^{-1} indicating the absence of the α-glucopyranosyl residues.[7] The same features were observed for the glucans from strains E48 and E61, confirming the earlier observation of Michel et al.[16] The polysaccharides from each of the three isolates appeared to be fully methylated after three methylations. With fewer methylations, methylated alditol acetates indicating the presence of most of the other possible linkage types, and large amounts of glucitol hexaacetate were detected (results not shown). Products detected after three consecutive methylations and their ratios are shown in Table 1. These results clearly indicate that the epiglucans from F19, E48 and E61 all exhibit terminal Glcp, 3-Glcp and 3,6-Glcp residues. The F19 epiglucan appears to have the highest

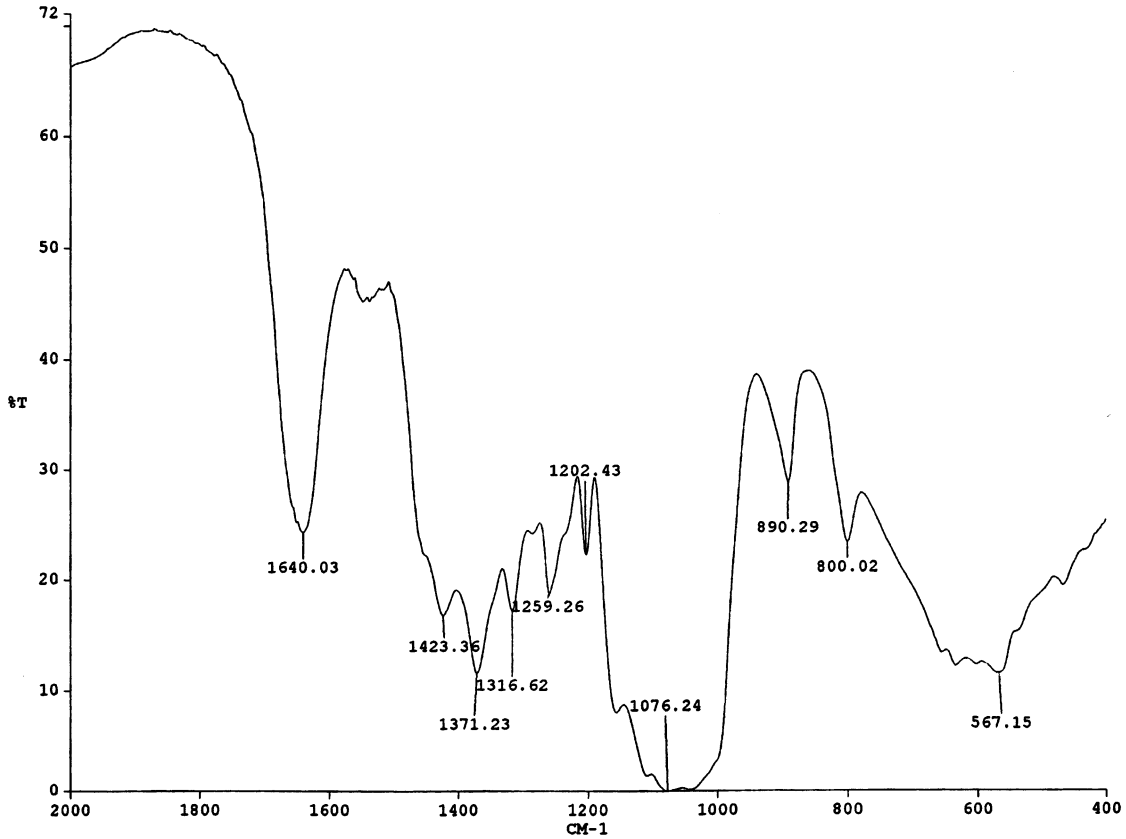


Fig. 1. Infra red spectrum of epiglucan, the extracellular polysaccharide produced by *E. nigrum* F19. Polysaccharides from strains E48 and E61 gave identical spectra.

branching frequency whereas the E48 and E61 epiglucans have slightly lower branching frequencies, indicating that there is some variation in branching frequency among the *E. nigrum* strains.

The ^{13}C NMR (DEPT 135) spectra for the three epiglucans were quite similar. The spectrum of the E48 epiglucan is shown in Fig. 2. The ^{13}C spectra for each polysaccharide closely resemble those published for other side-chain-branched $(1\rightarrow3;1\rightarrow6)$-β-glucans.[14,18,19] Further structural information was obtained using ^{13}C NMR (DEPT 135) spectroscopy that can distinguish carbons on the basis of the number of attached protons.[20] The assignments of the C-13 spectra of epiglucans for the three *Epicoccum* strains are shown in Table 2, and are based on the assignments of Rinaudo and Vincendon.[18] Unbranched C-6 ($-CH_2-OH$) resonate at approximately δ 61, whereas branched C-6 ($-CH_2-O-R$) resonate at approximately δ 68;[14,18,19] thus the presence of both peaks clearly indicates some branching at C-6. Although the peak at δ 68.5 overlaps with peaks from C-4 carbons (δ 68.8), it can be easily distinguished from the latter using a DEPT-135 sequence which edits the C-13 spectrum according to the number of attached protons. Signals from carbons that have one or three hydrogens attached appear in the opposite phase to signals from carbons that have two hydrogens attached to them. This method was particularly useful for separating and assigning the overlapping signals

Table 1
Products from methylation analyses of epiglucans produced by strains F19, E48 and E61

Methyl substitution	Linkage	F19 (mol%)	E48 (mol%)	E61 (mol%)
2,3,4,6-Glc*p* [a]	terminal	39.5 (0.95)	41.6 (1.1)	41.5 (1.1)
2,4,6-Glc*p*	1,3	19.6 (0.48)	21.9 (0.59)	23.1 (0.63)
2,4-Glc*p*	1,3,6	40.8 (1.0)	36.5 (1.0)	36.4 (1.0)

[a] 1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-hexopyranose. Approximate ratios are given in parentheses.

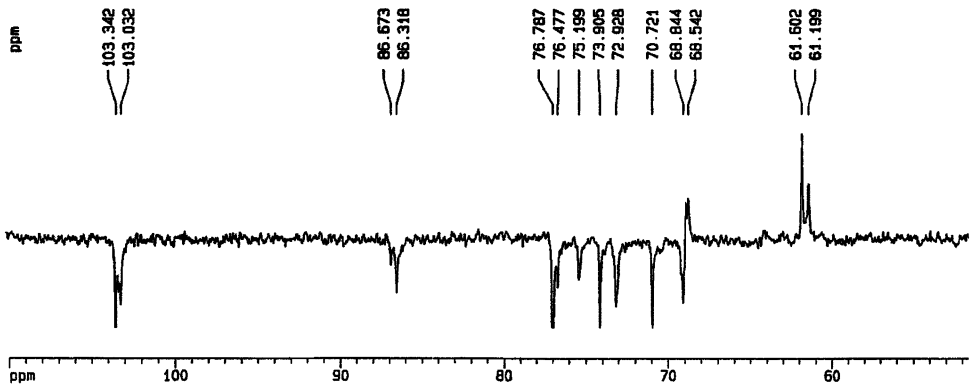


Fig. 2. ^{13}C NMR (DEPT 135) spectrum of the epiglucan produced by *E. nigrum* strain E48, measured in Me_2SO-d_6 at 100 °C.

Table 2
^{13}C NMR spectral assignments of epiglucans produced by strains F19, E48 and E61 [a]

Sugar residue	C-1	C-2	C-3	C-4	C-5	C-6
A, F	103.3 [b]	72.9	86.7 [b]	68.8	75.2	61.6 [b]
B, G	103.3 [b]	72.9	86.3 [b]	68.8	76.5	68.5
C, H	103.3 [b]	72.9	86.3 [b]	68.8	76.5	68.5
D, I	103.0 [b]	73.9	76.8	70.7	76.5	61.6 [b]
E, J	103.0 [b]	73.9	76.8	70.7	76.5	61.2 [b]

[a] A–E refers to residues shown in Fig. 3(a). F–J refers to residues shown in Fig. 3(b).
[b] Assignments could be reversed.

from C-4 carbons and C-6 carbons. The spectrum (Fig. 2) shows the signals from CH_2 carbons as positive and the CH carbons as negative. It also shows conclusively that the E48 epiglucan has branching at C-6, and this was found for the E61 and F19 epiglucans also.

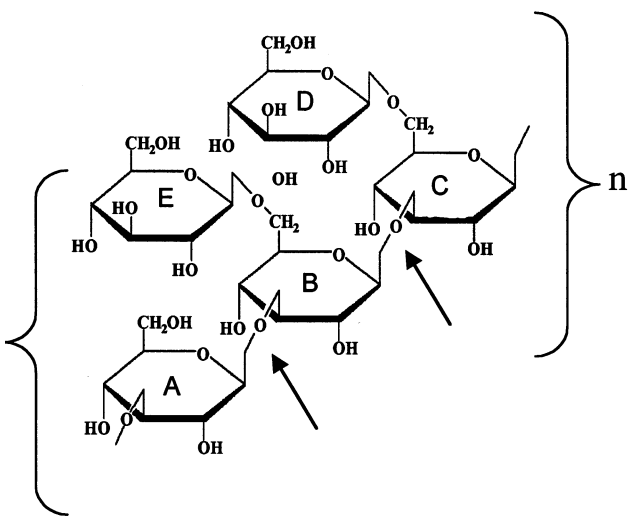
Approximate branch ratios could theoretically be obtained through a comparison of the C-6 branched (δ 68.5) and unbranched (δ 61.2–61.6). However, this would depend on accurately deconvoluting a series of overlapping peaks with unknown relaxation times. This was not attempted. Comparison of the intensities of these peaks with similar peaks in scleroglucan (data not shown) shows that all the epiglucans have considerably more branching than scleroglucan. Two possible structures, I and II, consistent with the methylation data and NMR spectra are presented in Fig. 3 (a) and (b).

The signals overlapped and were broad due to the high molecular mass and viscosity of the epiglucans, although viscosity, and hence the linewidths was reduced partially by measuring the spectra at an elevated temperature (373 K). Signals found at δ 68.5 are from the C-6 carbons which are O-substituted, while unsubstituted C-6 carbons show signals at δ 61.2–61.6. This interpretation follows the general rule that glycosylation of a carbon atom creates a downfield shift of 4–10 ppm for the α-carbon and an upfield shift of 1 ppm for the β-carbon.[18,21] C-3 carbons can also be distinguished by this rule, where O-substituted C-3 carbons of the glucan backbone (ABC for structure I in Fig. 3(a) and FGH for structure II in Fig. 3(b)) gave signals between δ 86.3–86.7 and the unsubstituted C-3 carbons from the side chain residues (DE for structure I in Fig. 3(a) and IJ for structure II in Fig. 3(b)) show signals at δ 76.5. These spectral data (Table 2) show the same features for all three epiglucans and are consistent with a (1→3)-β-linked backbone chain that has (1→6)-β-linked side groups attached.

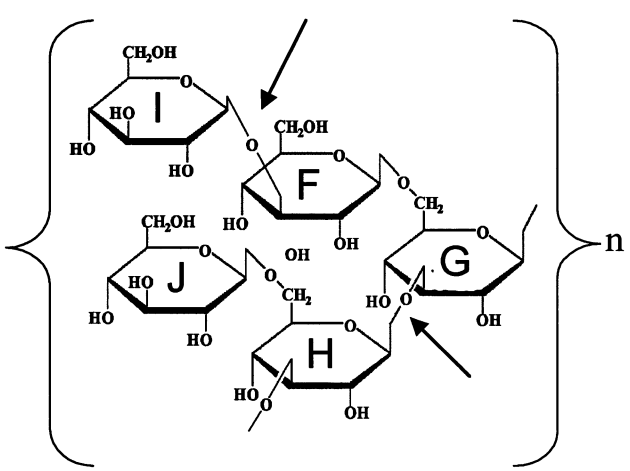
The ^{13}C NMR data does not reveal peaks corresponding to the α configuration of the anomeric carbon. α-Glc anomeric carbons would resonate at approximately δ 100.0 and anomeric β-glc carbons slightly downfield at about δ 104.0.[22] Peaks are only visible be-

tween δ 103.0 and 103.3 (Fig. 2), strongly suggesting that only β anomeric carbons are present.

When the epiglucans from the three isolates, F19, E48 and E61, were incubated with two purified fungal (1→3)-β-*exo*-glucanases, glucose from unbranched backbone residues, and gentiobiose from branched backbone residues were detected as the only hydrolysis products. The products of hydrolysis of laminarin from *Laminaria digitata* and scleroglucan from *S. glucanicum* were used as controls. On TLC analysis, laminarin, a (1→3;1→6)-β-glucan with (1→6)-branches at every 6–8 backbone



a - Proposed structure I for epiglucan



b - Alternative structure II for epiglucan

Fig. 3. Proposed structures I (a) and II (b) of the *E. nigrum* extracellular polysaccharides from methylation data and ^{13}C NMR. Arrows indicate possible (1→3)-β-*exo*-glucanase hydrolysis sites.

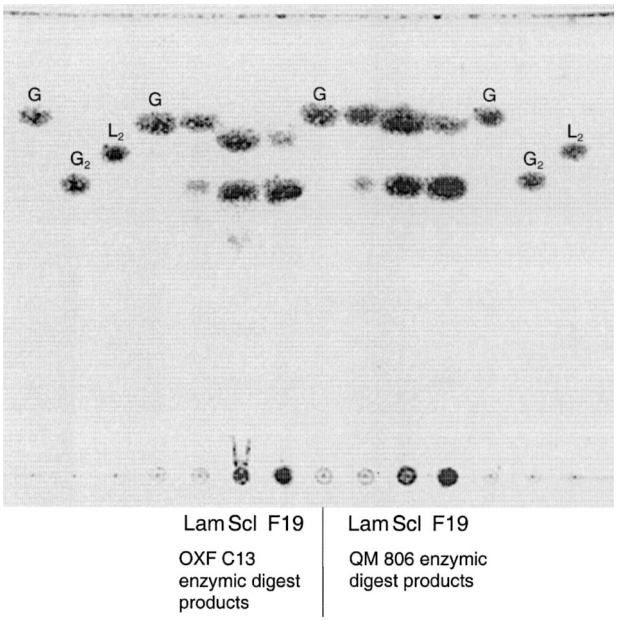


Fig. 4. TLC separation of products from enzymic hydrolysis of laminarin, scleroglucan and *E. nigrum* F19 extracellular polysaccharide using the (1→3)-β-*exo*-glucanases from *Acremonium* sp. (OXF C13) and *S. dimorphosporum*. Details are given in the text. G, glucose; G_2, gentiobiose; L_2, laminaribiose; Lam, laminarin; Scl, scleroglucan; OXF C13, *Acremonium* sp. (OXF C13); QM806, *S. dimorphosporum*.

Table 3
Quantification of enzymic digestion products from strain F19 epiglucan using a purified *Acremonium* sp. (OXF C13) (1→3)-β-*exo*-glucanase

Digestion products	Epiglucan (mol ratio)		
	F19	E48	E61
Glucose	1.0	1.0	1.0
Gentiobiose	1.9	1.4	1.8

residues,[7,23] yielded much more glucose than gentiobiose, as adjudged subjectively by visual appearance of the spot (Fig. 4). With scleroglucan, the intensities of the glucose and gentiobiose spots appeared to be approximately equal (Fig. 4), which is consistent with its higher branching frequency. For the epiglucans from F19, E48 and E61, the yields of gentiobiose after enzymic digestion (as assessed from relative intensities of the spots on TLC plates) were much higher than those of glucose, indicating that the frequency of side branching was higher than that in scleroglucan. Quantification of these products by anion exchange HPLC (Table 3) support this con-

clusion and shows that the side-branching frequencies are consistent with the methylation (Table 1) and ^{13}C NMR results for E61 and F19. However, the apparent branching frequency for E48 epiglucan as judged by glucose to gentiobiose ratio was reproducibly lower (1.4:1), and is not consistent with the MS and NMR data.

It was apparent that the epiglucans were not completely digested by a single enzyme treatment, which was thought to be due to the insolubility of the glucan in the aqueous digestion mixture. To increase solubility, the glucan was pretreated in 1 M NaOH before each digestion (see Section 4 for full details). When residual F19 epiglucan recovered from the enzymic digest was subjected to two further digestions after pretreatment, the glucose to gentiobiose ratio was the same as after the first hydrolysis, suggesting that the products generated reflected the overall structure of the glucan and not a fraction of it that was most susceptible to enzymic attack. The enzymic hydrolysis data also confirms that the glucose monomers are D- and not L-isomers. However, these data do not allow differentiation between the two possible structures for epiglucan given in Fig. 3 (a) and (b) since the (1→3)-β-*exo*-glucanase used would probably cleave at the sites indicated on Fig. 3 (a) and (b), and produce identical products, in the same ratios.

This epiglucan showed typical shear thinning behaviour (Fig. 5) similar to scleroglucan and schizophyllan.[3,24] The rheological properties of the dispersed epiglucan showed no significant changes over a temperature range of 10–60 °C (results not shown).

3. Conclusions

Infra-red spectroscopy, ^{13}C NMR and specific enzymic digestion data show conclusively that all the glucosidic linkages are in the β configuration. The data from methylation analysis and the supporting evidence from the ^{13}C NMR and enzymic digestion strongly indicate that epiglucan is a highly side-branched (1→3;1→6)-β-D-glucan. Two structures can be proposed: (a) structure I, with a (1→3)-β-linked backbone to which single (1→6)-β-linked branching residues are attached, on average, to two out of every three backbone residues; (b) structure II, also with a (1→3)-β-linked backbone but with side branches consisting either of single (1→6)-β-linked residues or (1→6)-β-linked laminaribiosyl residues, in a 1:1 ratio. ^{1}H COSY or TOCSY NMR of (1→3)-β-*endo*-glucanase hydrolysis products or periodate oxidation could be used to differentiate between structures I and II (Fig. 3(a) and (b)). In a similar study[25,26] periodate oxidation resolved the structure of the (1→3;1→6)-β-glucan from *Omphalia lapidescens*, the data favouring structure I over structure II.

There is no evidence that epiglucan or other side-branched fungal (1→3;1→6)-β-glucans are composed of regular repeating units as is found in certain bacterial extracellular polysaccharides, e.g., xanthan. Furthermore,

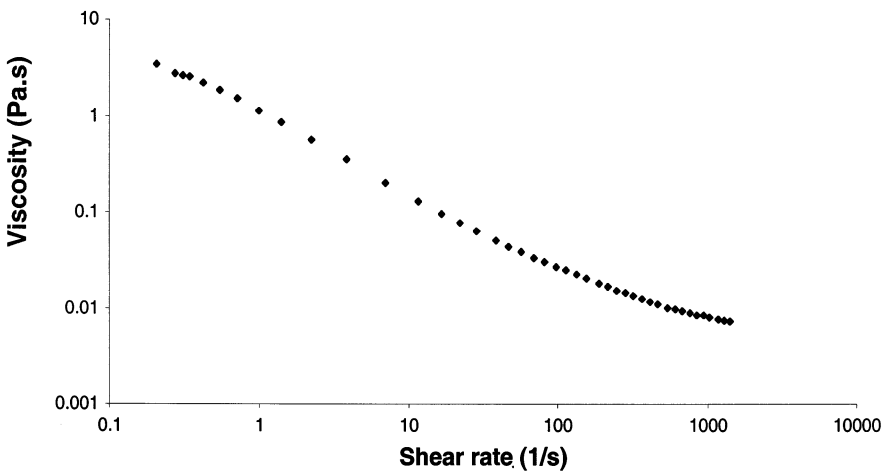


Fig. 5. Shear thinning properties of 0.5% (w/v) *E. nigrum* F19 extracellular polysaccharide at 20 °C. Details are given in the text.

the values obtained from the three *E. nigrum* strains suggest that there are slight structural variations between them. Their structures suggest that the frequency and extent of side branching is determined by the characteristics of the side-branch synthesising enzyme.[27] Epiglucan belongs to the *Claviceps* family of side-chain branched $(1\rightarrow3;1\rightarrow6)$-β-D-glucan polysaccharides. The epiglucans are more highly branched than scleroglucan or schizophyllan. They are similar to the wall polysaccharide from *O. lapidescens*,[25,26] and less branched than the *Pestolotia* sp. glucan in which three out of every five backbone residues are branched.[14] Rheological analysis indicates that epiglucan has shear thinning properties similar to those recorded for scleroglucan and schizophyllan.[3,24]

4. Experimental

E. nigrum, strains E48 and E61 were obtained from the Botany Department, ANU, Canberra, Australia.

Isolation of E. nigrum *strains and screening for extracellular polysaccharide production.*—A total of 36 strains of *E. nigrum* were isolated from the playing fields at La Trobe University, Bendigo, Vic., Australia by exposing malt extract agar (MEA, Oxoid) plates to the air above freshly cut grass for 5 min and incubating at 25 °C for 72 h. *E. nigrum* colonies were readily identified by colony colour and classification code.[17] All isolates were maintained on MEA slopes at 4 °C.

Cultures were screened for their ability to produce extracellular mucilage. Cells grown on 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 days in an orbital incubator (Paton Industries, South Australia, Australia) at 200 rpm. The cells were removed by filtration through Whatman GF/C filters, and polysaccharide was precipitated from suspension by addition of two parts cold abs EtOH, collected on dried preweighed Whatman GF/C filters and dried to constant weight.

Large scale preparation of extracellular polysaccharide.—Selected *E. nigrum* isolates (see results) were grown for 7–10 days in a 2.5 L LH airlift fermentor (LH Series 500, Enztech NSW, Australia) on a basal medium consisting of a mineral salts solution,[28] supplemented with K_2HPO_4 (15.0 g/L), Na_2HPO_4 (15.0 g/L), glucose (30g/L) and $NaNO_3$ (0.08 g/L total N). The polysaccharide was recovered by precipitation from the culture filtrates by the addition of two parts abs EtOH. The pH in the vessel was maintained at 6.5 by automatic addition of 2 M HCl or 2 M NaOH using an LH series 505A controller and an Ingold pH probe. A suspension of the polysaccharide was then extensively dialysed against frequent changes of distilled water over a period of 5 days at rt, freeze-dried (Dynavac, Freeze Drier, model FD12, Vaccum Technology Associates, Hingham, MA, USA) and finely powdered using a Spex Freezer Mill (Spex Industries Inc, Metuchen, NJ, USA).

Infra-red spectroscopy.—KBr disks were prepared by mixing 3 mg of powdered polysaccharide and 300 mg of KBr and analysed using a Perkin–Elmer FT-IR spectrometer (model 1720X).

Monosaccharide analysis.—Monosaccharides were determined as their alditol acetates after acid hydrolysis of the powdered polysaccharide with 2.5 M trifluoroacetic acid.[23] Alditol acetates were recovered in CH_2Cl_2 and analysed by gas chromatography–mass spectrometry on a Shimadzu GC-17A gas chromatograph with Shimadzu QP-5000 quadrapole mass spectrometer using a 30 m × 0.32 mm i.d. × 1.0 μm DB-1 glass capillary column (J & W Scientific) with He carrier gas. Initial column temperature was 160 °C, increasing to 180 °C at 6 °C/min, 180–204 °C at 1 °C/min and 204–260 °C at 10 °C/min. The internal standard was *myo*-inositol.

Methylation analysis.—Samples were methylated using the method of Ciucanu and Kerek[29] as described by Read et al.[23] with modifications. Polysaccharide (100 μg) was dissolved in Me_2SO (100 μL) by heating (100–120 °C) in an oil bath (High-Temp Bath, HTB-150-D, Thermoline, Australia) until dissolution was complete (several hours to sev-

eral days). The samples were permethylated three times.[23] To ensure complete acid hydrolysis after methylation, the duration of the digestion in 2.5 M TFA was extended to 8 h at 100 °C. Partially methylated alditol acetates were analysed by gas chromatography–mass spectrometry as described above.

^{13}C NMR.—^{13}C NMR data were obtained on a Bruker DRX400 NMR spectrometer operating at 100 MHz. The spectra were all collected at 373 K to reduce linewidth. The polysaccharide (20 mg) was fully dissolved after prolonged incubation only (2–4 days) at 100–120 °C in 1 mL of deuterated Me_2SO. Data were collected from a 0.6 mL aliquot of this solution in a 5 mm tube. The DEPT spectra were obtained at 373 K using the standard Bruker 'dept 135' pulse sequence, and 8–16 k transients were collected.

Enzymic digestion of epiglucan.—Epiglucan (100 μg) was dissolved in 50 mM AcONa buffer (50 μL, pH 5.0) and digested with the fungal (1→3)-β-*exo*-glucanases (EC 3.2.1.58) from *Sporotrichum dimorphosporum* (kindly supplied by the late Dr E.T. Reese, Natick, MA, USA) and *Acremonium* sp. (OXF C13) which was purified by FPLC to electrophoretic homogeneity, for up to 24 h at 37 °C. Enzyme activities ranged from 500 to 800 units, where one unit is defined as the amount of enzyme liberating one μmole of reducing sugars as glucose equivalents per minute. Reducing sugars were measured by the method of Somogyi–Nelson.[30,31] The reactions were stopped by heating at 100 °C for 10 min. Reaction products were identified after TLC of samples on silica gel 60 plates (E. Merck, Australia), developed twice in 2:1:1 EtOAc–AcOH–water (v/v).[32] Reducing sugars were detected with the orcinol reagent.[33] In some digests, the presumptive glucose spot ran slower than the standard (see Fig. 4). Its identity was confirmed by spiking the reaction mixtures with a glucose standard.

The reaction products were separated and quantified using high pH anion exchange HPLC, where 10–25 μL of sample was applied to a Dionex HPAEC with a CarboPac PA-1 column (4 × 250 mm; Dionex Corp., Sunnyvale, CA, USA). Products eluted in 150 mM NaOH (run isocratically) at a flow rate of 1 mL/min were monitored 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).

Since the epiglucans were dissolved poorly in aqueous media, prior to enzyme treatment they were pre-treated in 1 M NaOH (5 mg was dissolved in 1 mL 1 M NaOH at 60 °C for 30 min and then neutralised with 1 M HCl). Neutralisation caused an increase in viscosity of the glucan solution. Enzyme digests were performed at 37 °C for 4 h, in duplicate, using 400 μg of glucan mixture and 800 μL of enzyme (500–800 units). The reactions were stopped by boiling for 10 min. Residual glucan was precipitated by addition of 3 vols of cold EtOH and allowed to stand for 12 h at −20 °C, after which it was collected by centrifugation at 11,000*g* (Hermle Z160 M, Medos Company, Melbourne, Australia) for 10 min. The supernatant was removed and the residual glucan was washed twice more with 70% EtOH. All the supernatants were pooled and dried at 40 °C. Once dry, the residual material was dissolved in 200 μL of water and analysed by high pH anion-exchange HPLC. The residual glucan was digested twice more with NaOH treatment, between each enzymic digestion.

Rheological studies.—The polysaccharide produced by the F19 strain was dissolved in 1 M NaOH at 80 °C, then neutralised with 1 M HCl and adjusted to a final concentration of 0.5% (w/v) with water. Since epiglucan is insoluble in water after recovery from culture filtrate, the resulting solution was opaque and viscous, suggesting true dissolution had not occurred. Viscosity readings were obtained at 20 °C using a cone and plate system (angle 1:59:00 deg:min:s, diameter 6.0 cm) on a Carri-Med CSL^2 100 rheometer. The gap between the plate and the cone was 52 μm.

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