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Structure and assembly of epiglucan, the extracellular (1→3;1→6)-β-glucan produced by the fungus *Epicoccum nigrum* strain F19

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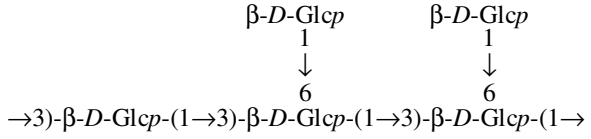
Abstract—In a previous article [*Carbohydr. Res.* **2001**, *331*, 163–171] two different structures for the possible modular repeating unit of the extracellular β-glucan, epiglucan produced by the fungus *Epicoccum nigrum* strain F19 were proposed. Clarifying which was the more likely one was considered essential before attempts were made to understand how epiglucan was assembled by this fungus. Data from Smith degradation analyses of epiglucan were consistent with the repeating unit of structure I, where single glucosyl residues are attached by (1→6)-β-linkages to two out of every three glucosyl residues in the (1→3)-β-linked glucan backbone. Repeated Smith degradations of ^{14}C-glucose labelled epiglucan showed that chain elongation occurred from its non-reducing end. Side chain insertion into the growing glucan was followed by analysis of real time incorporation of ^{13}C-glucose into epiglucan by ^{13}C NMR, and ^{14}C-glucose by enzymic digestion of the synthesised ^{14}C-epiglucan. All data obtained were consistent with the view that single (1→6)-β-linked glucosyl side residues are inserted simultaneously as the glucan backbone elongates.



Keywords: β-Glucan assembly; Epiglucan elongation; Side chain insertion; Extracellular polysaccharide; NMR spectroscopy; Biosynthesis; Radioisotope labelling

1. Introduction

Previous investigations into the assembly of linear cell wall and mucilage fungal glucans have concentrated mainly on understanding their direction of elongation, and the purification and characterisation of the glucan synthases involved.[1,2] Many fungal extracellular glucans are branched, which adds another level of complexity to such studies, as both the timing and enzymology of side branch insertion into the backbone glucan need to be considered. Batra et al.[3] studied the direction of elongation and timing of side branch insertion of the extracellular (1→3;1→6)-β-glucan from *Sclerotium rolfsii*. Cultures of *S. rolfsii* were pulsed with ^{14}C-glucose, the glucan isolated and the relative proportions of ^{14}C-glucose incorporated into reducing and non-reducing ends

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compared by measuring the radioactivity in the products after sequential Smith degradations. ^{14}C-Glucose was preferentially incorporated at the non-reducing end, thus indicating (1→3)-β-glucan backbone extension from this end.[3]

Several mechanisms to explain how side branch insertion is timed in relation to backbone elongation have been proposed for glucan assembly (Fig. 1), and all occur at the non-reducing end.[3,4] Mechanism A (Fig. 1A) has received some support from Rau[5] who hypothesised that single chains of (1→6)-β-linked side chain residues on (1→3)-β-glucans from *Schizophyllum commune* are attached after the linear glucans are transported into cell walls, but no experimental data were presented to support this hypothesis. Data for *S. rolfsii* indicated that single side chain residues were incorporated simultaneously with glucan backbone elongation, supporting mechanism B (Fig. 1B).[3] However, the methodology used was not sensitive enough to detect the mode of incorporation of ^{14}C-glucose in the early stages of glucan synthesis, thus giving little indication of the timing of side chain incorporation. The third mechanism (Fig. 1C) involves the transfer of pre-synthesised building blocks added to the growing glucan. This mechanism would be analogous to the one that introduces intra-chain (1→6)-β-linkages as has been reported for the yeasts *Candida albicans* and *Saccharomyces cerevisiae*, and the filamentous fungus *Aspergillus fumigatus*.[6–9]

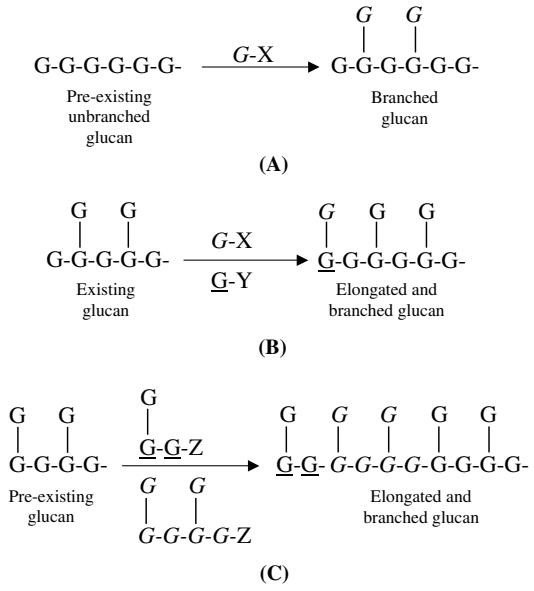

Figure 1. Possible mechanisms by which single side chain glucose residues could be incorporated into a growing (1→3)-β-glucan backbone. Examples assume that elongation occurs from the non-reducing end of the newly synthesised β-glucan. G refers to glucose residues. X and Y refer to a nucleoside diphosphate saccharide activator. (A) Sequential—glucan backbone is elongating first and then side branch glucose insertion. (B) Concurrent—glucan backbone elongation and then side branch glucose insertion. (C) Assembly of oligosaccharides followed by transfer to the growing glucan chain.

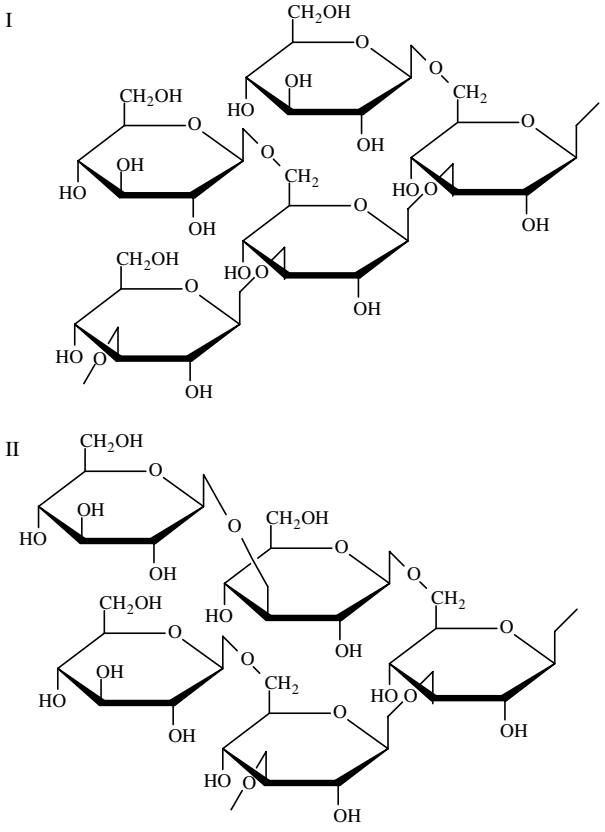

Figure 2. Possible modular repeating units for epiglucan structures I and II elucidated by Schmid et al.[10]

Briefly, laminaribiose residues from an existing (1→3)-β-glucan or laminarioligosaccharide are removed by a glucanosyltransferase, and transferred to the non-reducing end of a newly synthesised (1→3)-β-glucan via (1→6)-β-linked intra-chain branches.[6] However, no gene products have been identified that introduce (1→6)-β-linked side chain residues, and at present how other side-branched fungal extracellular β-glucans are assembled is not understood.

We report here the differentiation between the two proposed modular repeating units of epiglucan[10] (Fig. 2), its direction of elongation, and timing of side chain insertion during its synthesis by the fungus *Epicoccum nigrum* strain F19.

2. Results and discussion

2.1. Differentiation of the structure of the repeating unit of epiglucan

Smith degradation provides structural information, and together with methylation analyses, and enzymatic hydrolysis has been used to elucidate structures of fungal glucans, as for example with coriolan,[11] *Grifora umbellata* glucans,[12] *Claviceps* glucan[13] and scleroglucan.[14] Oxidation/degradation reactions have also been applied suc-

cessfully to clarify unequivocally structural ambiguities of other side chain branched (1→3)-β-glucans. Methylation analysis of the glucan produced by *Omphalia lapidescens* did not allow a distinction between two possible structures of its repeating unit.[15] In one, only single side chain branches were present, while the other had both disaccharide and monosaccharide side chain branches. Subsequently Saito et al.[16] used Smith degradation to de-branch the glucan, and resolve its true modular repeating unit. We have applied the same experimental approach to the epiglucan from *E. nigrum* strain F19.

The recovered epiglucan was subjected to two Smith degradations, with scleroglucan, a side chain branched glucan of known structure as a control. Each glucan was analysed in duplicate. A single degradation was insufficient to differentiate between the two possible epiglucan structures, as the theoretical amount of periodate consumed (0.8 mol IO_4^-/mol anhydro-glucose) and formic acid produced (0.4 mol formic acid/mol anhydro-glucose) would be the same for both structures I and II (Fig. 2). A second degradation provided the necessary information. Since single side branches still remain, the theoretical amount of periodate consumed for structure II is 0.66 mol IO_4^-/mol anhydro-glucose, whereas structure I would consume little periodate, since only a small number of non-reducing ends are present at levels ultimately determined by the length of the now-linear polymer.[17] The first degradation gave values (Table 1) very close to the theoretical values for periodate consumption and formic acid release. After the second degradation, very little periodate was consumed and more importantly, it was markedly less than the theoretical value of 0.66 mol IO_4^-/mol anhydro-glucose required for structure II (Fig. 2). The second degradation consumed approximately 88% (for F19) and 81% (for scleroglucan) less periodate compared to the first degradation, indicating that a large number of non-reducing glucose residues were removed from both glucans in the first degradation. The low amount of periodate consumed after the second oxidation strongly suggests that the original non-reducing glucose residues were from single side branch residues attached to the main chain. Estimations of branching frequencies from the first oxidation data suggest that F19 and scleroglucan have branching frequencies of about 2:2.8 and 1:2.8, respectively. The value for scleroglucan is consistent with the reported branching frequency, where one out of every three backbone residues carries a single branching residue.[14] Thus, oxidation data for the epiglucan indicates that it is a highly branched (1→3;1→6)-β-glucan with single side branches, on average, at two of every three backbone residues as in Structure I (Fig. 2). With this structural information it was then possible to investigate both the direction of main chain elongation and timing of side chain insertion, as discussed next.

Table 1. Results of periodate consumed and formic acid released after two Smith degradations of *Epicoccum nigrum* strain F19 epiglucan, with scleroglucan (Scl) used as a control

	1st degradation		2nd degradation	
	mol IO_4^{-1}/ mol anh-glc	mol formic acid/ mol anh-glc	mol IO_4^{-1}/ mol anh-glc	mol formic acid/ mol anh-glc
F19	0.87	0.41	0.10	0.05
Scl	0.52	0.26	0.10	0.05

Values were calculated from duplicates.
IO_4^{-1}—periodate, anh-glc—anhydro-glucose.

2.2. Direction of elongation of the *E. nigrum* strain F19 epiglucan

Batra et al.[3] used sequential Smith degradations to determine the direction of chain elongation of scleroglucan produced by *S. rolfsii*. Pulse-labelled glucan was produced by adding uniformly labelled ^{14}C-glucose to cultures of *S. rolfsii* and subsequent analysis of the reaction products allowed quantification and comparisons of the specific activities in the side branches, and the reducing and non-reducing ends of the main chain. After the first Smith degradation, the reducing-end glucose is converted to an arabinitol residue and the non-reducing end glucose residue is removed (Fig. 3). Glycerol is released as a reaction product from the non-reducing end, and its ^{14}C-specific activity, on a molar basis, is equal to that of the glucose residue at the non-reducing end. After a second degradation, the reducing end arabinitol residue is converted to a glycerol residue, which is not susceptible to oxidation (Fig. 3). The non-reducing end glucose is once again removed to expose a new glucose residue. The oxidation/degradation reaction when repeated, reveals a new glucose at the non-reducing end. To determine the specific activity of the reducing end glycerol, the glucan was fully hydrolysed and the distribution of the ^{14}C-activity determined (Fig. 3). The direction of elongation was determined by comparing which end had a higher ^{14}C-activity. Elongation from the non-reducing end is indicated by a higher activity of ^{14}C-glycerol liberated from the non-reducing end compared to ^{14}C-glycerol liberated from the reducing end.[3]

The ^{14}C-activities of glycerol released from the non-reducing ends after each Smith degradation, and from reducing ends after complete acid hydrolysis of epiglucan are given in Table 2. Data show that 5 min after the addition of the ^{14}C-glucose, an extracellular polysaccharide preferentially labelled at one end was generated. Comparing ^{14}C-activities of glycerol released from the non-reducing and reducing ends after the third Smith degradation is consistent with a view that elongation of epiglucan occurs at the non-reducing end, since incorporation of ^{14}C-glucose at this non-reducing end was about 5.5 times higher than at the reducing end. Chain

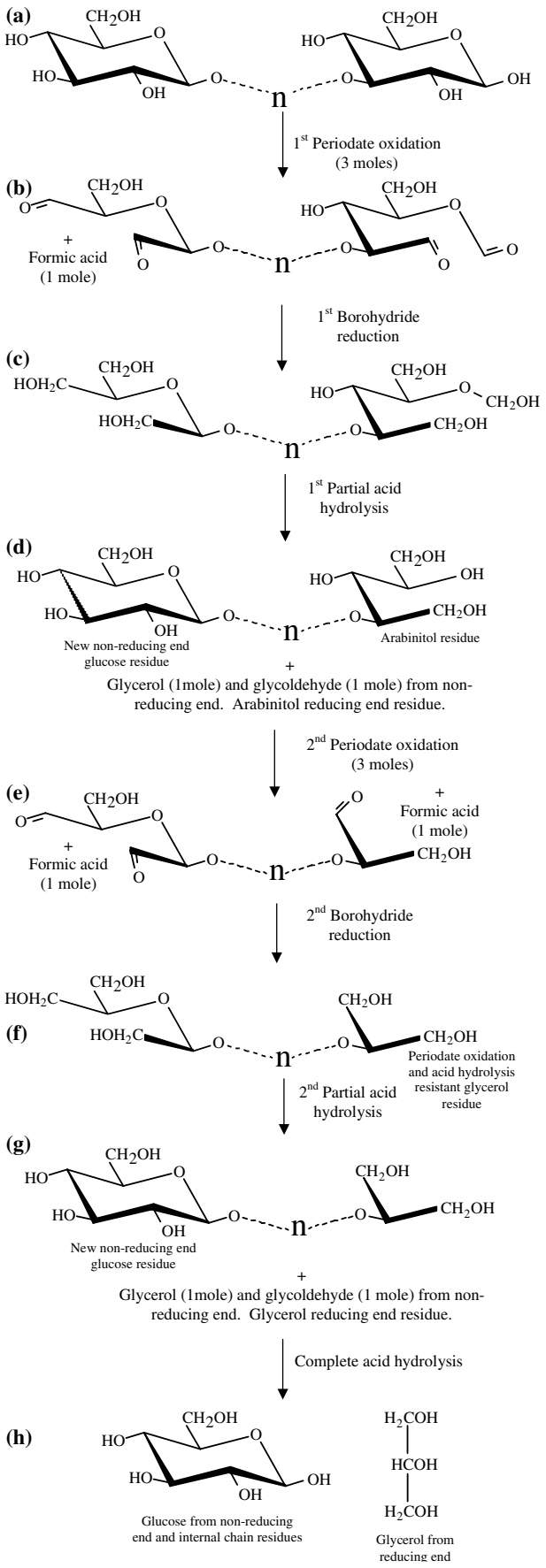

Table 2. Activities of ^{14}C-glycerol (cpm mmol^{-1}) released from the non-reducing ends of ^{14}C-labelled epiglucan after three Smith degradations

		Smith degradation number		
		1	2	3
Non-reducing end	F19, rep 1	56,132	53,732	42,740
	F19, rep 2	59,942	59,053	50,148
Reducing end	F19, rep 1			6435
	F19, rep 2			8474

Reducing end glycerol activities were determined after complete acid hydrolysis of the glucan remaining from the third Smith degradation. Data were obtained from duplicate determinations.
rep—replicate number.

elongation at the non-reducing end occurs during synthesis of the *S. rolfsii* extracellular glucan,[3] and the cell wall (1→3)-β-glucan of *S. cerevisiae*.[2] These observations might suggest that fungal extracellular (1→3)-β-glucans are generally assembled by transfer of glucose, probably from uridine 5′-diphosphoglucose, to the non-reducing end of the growing glucan chain.[18] However, biosynthesis of other extracellular (1→3)-β-glucans from other fungi needs to be investigated before any such generalisations can be made.

2.3. Determination of the kinetics of side chain incorporation mechanism versus real time ^{13}C NMR analysis

^{13}C NMR analysis was used to follow the relative incorporation of ^{13}C-carbon in real time for each of the carbons assigned previously.[10] The hypothesis was that carbons of ^{13}C-glucose-enriched residues in the glucan would appear as more intense peaks in the NMR spectra than carbons of unenriched residues. Samples of ^{13}C-labelled epiglucan were taken during the time course of incorporation and analysed by ^{13}C NMR. The assignments of carbons for the ^{13}C-epiglucans after ^{13}C NMR analysis (spectra shown in Fig. 4) were identical with those published by Schmid et al.[10] confirming the structure of epiglucan produced in this experiment. The small number of transients (4000–6000) needed to obtain spec-

Figure 3. Repeated Smith degradation reactions at the non-reducing and reducing ends of a (1→3;1→6)-β-glucan. **n** represents (1→3)-β-linked main chain residues. After the first degradation (a–d), the reducing end glucose residue is converted to an arabinitol residue. The non-reducing end glucose is cleaved to reveal a new glucose residue. After the second degradation (e–g), the reducing end arabinitol residue is converted to a stably bound glycerol residue, which does not undergo any further oxidation. The non-reducing end glucose is once again cleaved to reveal a new glucose residue. The degradation reactions can be repeated multiple times, each time revealing a new glucose residue at the non-reducing end. After complete acid hydrolysis (h), all glycosidic linkages are cleaved and glucose is released from the non-reducing end and from the internal chain residues, glycerol is released from the reducing end (from Stone and Clarke[30]).

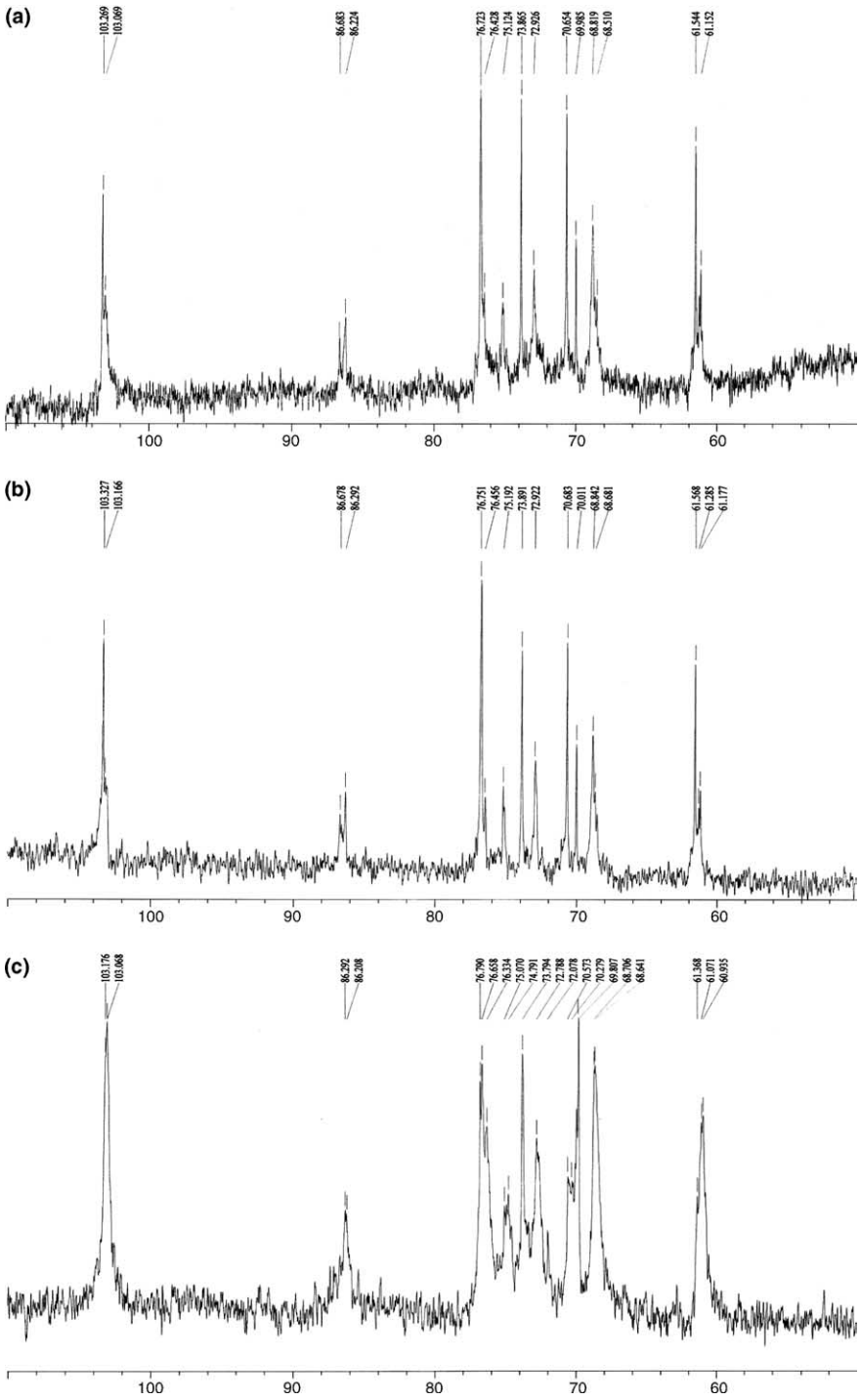

Figure 4. ^{13}C NMR spectra of glucose-U-$^{13}C_6$ enriched epiglucan samples taken after (a) 15 min (15 mg), (b) 30 min (15 mg), (c) 45 min (15 mg), (d) 60 min (20 mg) and (e) 90 min (20 mg) for determining the possible timing of side branch insertion. Samples were dissolved in deuterated dimethylsulfoxide (1 ml) and an average of 4000–6000 transients were collected at 373 K.

tra for the ^{13}C-enriched epiglucan samples indicated that ^{13}C-glucose was incorporated into the glucan, since 17,000–30,000 transients were required to give similar ^{13}C-spectra for the unenriched epiglucan previously.[10] Timing of side branch insertion is most readily reflected as changes in peak intensities of the C-6 unbranched (δ 61.0–61.6) and C-6 branched (δ 68.6–68.8) carbons with time, as these C-atoms are directly involved in branched C-1 to C-6 linkages.[19,20] The spectra (Fig. 4a–e) show no obvious differences in the peak intensities of the branched or unbranched C-6 carbons at different sampling times.

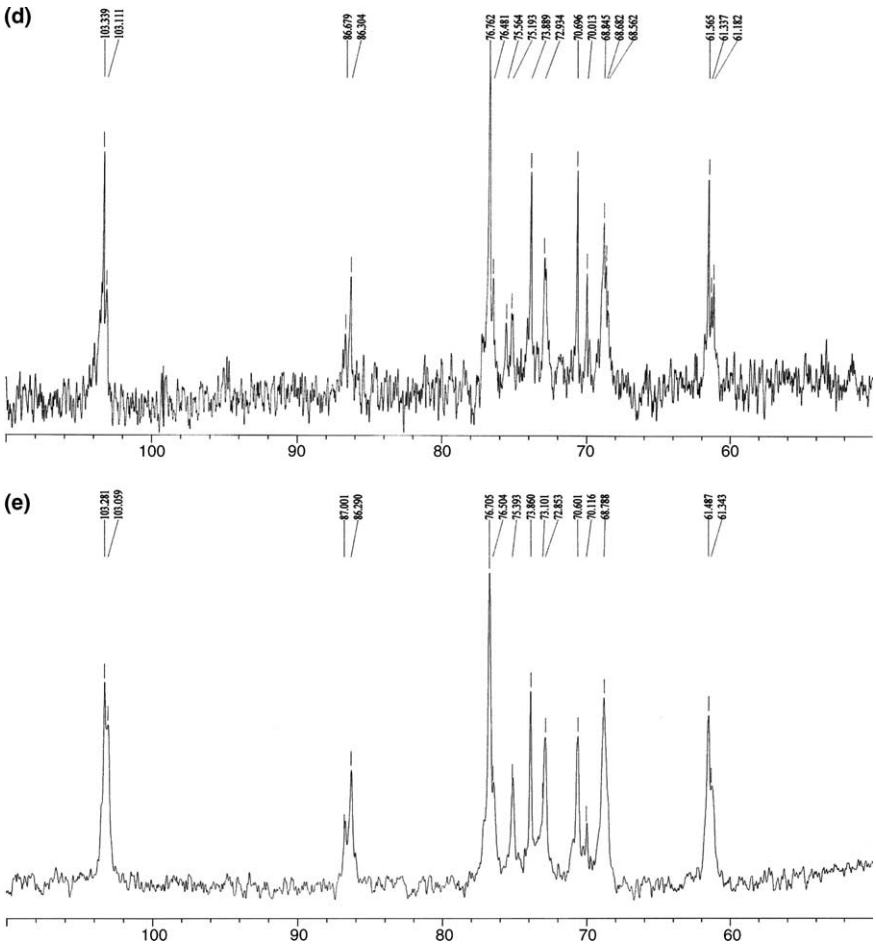

Figure 4. (*continued*)

This absence of differences in peak intensities is consistent with the conclusion that side chain residues are simultaneously attached as the glucan backbone elongates. However, it is possible that detectable differences in side chain insertion may have occurred before the first sample (15 min) was analysed by ^{13}C NMR, and so the data are not conclusive. In situ real time ^{13}C NMR analysis was not attempted as the volume of the NMR probe available was not large enough to contain the volume of biomass and medium (>500 ml) required to analyse newly synthesised ^{13}C-labelled polysaccharide. An alternative approach to confirm timing of side branch insertion was taken.

2.4. Determination of the kinetics of side chain incorporation by enzymatic digestion of real time ^{14}C-labelled polysaccharide samples

Because of the increased sensitivity of ^{14}C-analysis, incorporation of ^{14}C-glucose into the polysaccharide can be detected much earlier after ^{14}C-glucose addition, and at much shorter incubation time intervals than with ^{13}C-glucose based incorporation experiments. The sample times chosen were considered frequent enough to distinguish between the incorporation of ^{14}C-glucose into the backbone chain and the side branches. The hypothesis was that specific activities (cpm mol^{-1}) of lower than 2:1 for the gentiobiose/glucose ratio would indicate that incorporation of labelled glucose was higher in the backbone than in the side branches, suggesting that incorporation of the side chain residues does not occur simultaneously with main backbone chain elongation. Further it is assumed that the (1→3)-β-glucan chain is synthesised from the supplied ^{14}C-glucose, and/or diluted ^{14}C-glucose branch residues are added to the chain afterwards following mechanism A (Fig. 1). On the other hand, a ratio of 2:1 would indicate that levels of ^{14}C-glucose incorporation into both side and main chains are the same, indicating that side chains are incorporated as the glucan elongates as in mechanisms B and C (Fig. 1). A ratio greater than 2:1 would suggest that ^{14}C-glucose side chain residues are added to a pre-existing non- or partially ^{14}C-labelled (1→3)-β-glucan (mechanism A, Fig. 1). The ratios of the specific ^{14}C-activity incorporated into gentiobiose and glucose for each time sample after addition of ^{14}C-glucose (1 min intervals = 11 samples, 2 min intervals = 5 samples, 5 min intervals = 7 samples, 10 min intervals = 4 samples, 30 min intervals = 2 samples and

60 min intervals = 4 samples) were always about 1.9:1. As no differences in these ratios were detected over the course of the experiment even at the earliest sampling times, the data support the view that incorporation of the side chain glucose residues occurs simultaneously with the addition of backbone glucose residues.

3. Conclusions

The data presented here do not support mechanism A (Fig. 1) where side chains are added after the main chain is synthesised. The method of biosynthesis of epiglucan seems the same as for scleroglucan, with elongation from the non-reducing end, and side chains incorporated simultaneously.[3] Thus epiglucan is probably assembled by either mechanism B or C (Fig. 1). However, it is not possible to differentiate between these, as biosynthesis of a branched glucan by each of these mechanisms would lead to an equal incorporation of ^{14}C-glucose into the side chains and backbone residues.

Nor is it possible yet to speculate sensibly about the possible enzymology of epiglucan biosynthesis in *E. nigrum* strain F19. In *Streptococcus pneumoniae* type 37, the capsular glucan consists of a (1→3)-β-linked backbone with single (1→2)-β-linked side branches attached to each backbone residue.[21] Evidence suggests it is synthesised by only a single protein (Tts), which can catalyse formation of both β-glucosidic linkages.[22] Although several proteins have been identified from *C. albicans*, *S. cerevisiae* and *A. fumigatus* that introduce intra-chain (1→6)-β-linkage into (1→3)-β-glucan chains,[23] there is no evidence for any proteins linking a single (1→6)-β-glucosidic side residue onto (1→3)-β-glucan chains in any fungi. A detailed description of the enzymology of epiglucan biosynthesis would allow mechanisms B and C to be distinguished.

4. Experimental

4.1. Smith degradation of both unlabelled and ^{14}C-labelled epiglucans

Sodium periodate (5 ml, 30 mM) was added to 50 mg epiglucan dissolved in 5 ml reverse osmosis water (RO), and incubated at 4 °C on a plate shaker (Orbital Mixer, Ratek, South Australia). Periodate consumption was followed by the spectroscopic method of Aspinall and Ferrier,[24] and the reaction terminated by addition of 500 μl ethylene glycol once periodate consumption had ceased.[3] The oxidised glucan was recovered by addition of 3 vol of cold 95% ethanol, centrifuged (20,000*g*, 10 min, 4 °C) and washed three times with 2 ml cold 95% ethanol. It was then reduced by dissolving in 3 ml 2 M NH_2OH and 1 ml of 1 M $NaBH_4$ (made up in 2 M NH_2OH), with incubation at 50 °C overnight. Any remaining $NaBH_4$ was destroyed by addition of glacial acetic acid until bubbling ceased. The reduced epiglucan was precipitated with 3 vol of cold 95% ethanol and recovered by centrifugation. This material was then partially hydrolysed for 10 h at room temperature by adding 2 ml 0.5 M HCl, thereby releasing glycerol from the non-reducing ends (Fig. 3). The solution was neutralised with 0.5 M NaOH, and the epiglucan precipitated with 3 vol cold 95% ethanol, centrifuged and washed twice with 2 ml cold 95% ethanol. Supernatants were combined and stored at 4 °C for glycerol analysis. Any precipitated material was lyophilised, and the Smith degradation repeated twice more. Formic acid was detected and quantified using the Shimadzu LC-10Ai Ion Chromatograph System fitted with a Shimadzu SPD-M10AVP Diode Array Detector. Samples (10 μl) were injected onto a Shodex Ionpak KC-811 column fitted with a Shodex Ionpak KC-810P guard column (Shoko Co. Ltd., Tokyo, Japan). The mobile phase used was 0.1% H_3PO_4/H_2O at a flow rate of 1.0 ml min^{-1} and a column temperature of 40 °C. Glycerol was determined enzymically using the glycerokinase method (Triglyceride, GPO-Trinder, Reagent A, 337-40A, Sigma Diagnostics). Samples were analysed in duplicate.

For the determination of direction of elongation, the remaining precipitated ^{14}C-labelled glucan material was fully hydrolysed with 2.5 M trifluoroacetic acid (TFA) at 100 °C for 10 h, and the TFA removed by evaporation under nitrogen in a water bath at 50 °C.[25] Residues containing glucose released from the main chain and glycerol released from the reducing end, were dissolved in 500 μl of water, developed twice by TLC (Silica gel 60, Merck, Australia)[26] in ethyl acetate/acetic acid/water (2:1:1 v/v), and sugars visualised and identified with orcinol reagent[27] against known standards. The glycerol spots were scraped off the plate and eluted three times with 1 ml water, fractions combined and concentrated to 300 μl in a rotary evaporator at 50 °C before being transferred to microscintillation plates. The glycerol released from the non-reducing and reducing ends was quantified as described above and their ^{14}C-activity measured by scintillation counting as described in Section 4.5.

4.2. Production of ^{14}C-epiglucan for determination of chain elongation direction by Smith degradation

Flasks containing cultures of *E. nigrum* strain F19 grown on basal medium consisting of (l^{-1}), 0.5 g KCl, 0.5 g $MgSO_4$, 0.01 g $FeSO_4$, 1.0 g K_2HPO_4, supplemented with 40 g glucose l^{-1} and 0.05 g nitrogen l^{-1} as $NaNO_3$, were incubated on an orbital incubator (200 rpm, 28 °C). When they were actively producing polysaccharide, as determined by visual assessment of increasing viscosity of the culture medium (approximately 48–72 h), biomass was recovered by

centrifugation (20,000*g*, 10 min, 4 °C) and washed three times with water to remove any polysaccharide already synthesised. Washed cells were resuspended (50 g wet weight) in 100 ml fresh basal media without any added glucose or nitrogen and incubated with shaking (200 rpm) at 28 °C for 30 min to allow the cells to recover from the centrifugation steps. These cultures were then supplemented with 0.5% w/v unlabelled glucose and incubated for a further 30 min under the same conditions to allow synthesis of low levels of unlabelled polysaccharide. Then 2.22 MBq of uniformly labelled ^{14}C-glucose (D-[U-^{14}C]glucose, CFB96-25OUCI, Amersham Biosciences, Australia) was added and cultures incubated (200 rpm, 28 °C) for a further 5 min to produce epiglucan predominantly labelled at the elongating end. Cultures were rapidly chilled to 4 °C and biomass removed by centrifugation (20,000*g*, 10 min, 4 °C). The supernatant was dialysed overnight against running RO water at 25 °C to remove any free ^{14}C-glucose that might interfere in subsequent analyses, and lyophilised to yield the ^{14}C-labelled epiglucan. This material was then subjected to three Smith degradations as described above.

4.3. Production of ^{13}C-labelled epiglucan for ^{13}C NMR spectroscopic analysis

Biomass (300 g wet weight) prepared as described earlier (Section 4.2) was transferred to a 5 l fermentation vessel (Braun Biostat MD, Sartorius, Melbourne, Australia) containing 4 l of the same basal medium used above (Section 4.2) supplemented with 0.05% glucose, but no nitrogen source. The fermentor was operated at 28 °C, 600 rpm, an aeration rate of 0.4 vvm and no pH control. After 30 min, 600 mg of ^{13}C-glucose (D-glucose-U-$^{13}C_6$, CLM-1396, Cambridge Isotope Laboratories, Andover, MA, USA) was added to the medium. Samples of appropriate volumes for harvesting epiglucan were taken after 15 min (1000 ml), 30 min (1000 ml), 45 min (800 ml), 60 min (600 ml) and 90 min (400 ml). Cell free supernatants from these samples were dialysed overnight against running RO water and then freeze dried. Recovered epiglucan residues (15–20 mg) were dissolved in 1 ml deuterated dimethylsulfoxide for ^{13}C NMR analysis. ^{13}C NMR spectra were obtained on a Bruker DRX400 NMR Spectrometer operating at 400 MHz. Data were collected from a 0.6 ml sample of these epiglucan solutions in a 5 mm probe. The spectra were all collected at 373 K in order to reduce line width.

4.4. Enzymatic digestion of ^{14}C-labelled epiglucan for determination of the possible side chain incorporation mechanism

Uniformly labelled ^{14}C-glucose (1.48 MBq) was added to a biomass suspension of *E. nigrum* strain F19, as described earlier (Section 4.2). Samples (2 ml) were removed at frequent intervals and immediately heated at 100 °C for 10 min, centrifuged (11,000*g*, 10 min) and washed twice with 2 ml water. Supernatants were combined, dialysed overnight against running RO water and lyophilised. The lyophilised polysaccharide was dissolved in 500 μl water with heating at 90 °C for 10 h. After cooling, this material was digested with the (1→3)-β-glucan exo-hydrolase (EC 3.2.1.58) from *Acremonium blochii* strain OXF C13[28] (2000–3200 units in 400 ml of 50 mM sodium acetate buffer, pH 5.0, 37 °C, 24 h). Any remaining undigested polysaccharide was recovered after precipitation by addition of 2 vol of cold 95% ethanol followed by centrifugation, and the precipitate washed twice with 200 μl of cold 95% ethanol. Supernatants were combined and stored at −20 °C. This enzyme digestion was repeated further three times, and solubilised products added to those obtained earlier. The final pooled supernatants for each sample were dried at 40 °C in a rotary evaporator and redissolved in 100 μl of RO water. Digestion products were fractionated using a Dionex HPAEC and a CarboPac PA-1 column (4 × 250 mm; Dionex Corp.). Samples 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 ($t_1 = 50$ ms, $V_1 = 100$ mV; $t_2 = 100$ ms, $V_2 = 600$ mV; $t_3 = 50$ ms, $V_3 = -600$ mV). Dried purified fractions were transferred to microscintillation plates, and ^{14}C-activities determined by scintillation counting as described in Section 4.5.

4.5. Scintillation counting

^{14}C-scintillation counting used a 1450 Microbeta Plus Liquid Scintillation Counter (Wallac, Finland). A 700 μl aliquot of scintillation fluid (Optiphase 'Super Mix', Wallac, Finland) was added to microscintillation plates (PET 24 Well Sample Plate, 1450–402, Wallac) containing ^{14}C-labelled material, mixed for 30 min on a plate shaker (Delfia Plate Shaker, Wallac, Finland) and counted for 10 min/sample after a 30 min delay to minimise the effect of any other energy absorbed during plate handling such as light that may cause the scintillation cocktail to fluoresce.[29]

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