Formation and Structure of Extracellular Glucans Produced by *Claviceps* Species

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SUMMARY

Submerged cultures of *Claviceps fusiformis* (Loveless) became progressively viscous during the incubation because of the production of an extracellular polysaccharide. The polysaccharide was shown to be a branched glucan with a $\beta 1 \rightarrow 3$ linked main chain having single glucopyranosyl units at intervals along it in the $\beta 1 \rightarrow 6$ configuration. For most of the media investigated growth at 27° and between pH 5 and 6 was accompanied by glucan production, which ceased when growth was arrested by lack of nutrient. The glucan was re-metabolized by the fungus only to a limited extent as the culture aged; the degree of branching varied during the culture period. The unit amount of glucan synthesized was not affected by numerous successive subculturings over a period of several months, but the product from the later cultures had a greater degree of branching. Branched glucans of similar structure to the above were also detected in low concentration in the natural sclerotia. The presence of $\beta 1 \rightarrow 3$ linked glucans in the cell walls of this and other fungal strains examined was also indicated.

INTRODUCTION

During studies on the production of clavine alkaloids in submerged culture by strains of *Claviceps fusiformis*, it was noted that the cultures became very viscous during incubation, to the extent that the fluid culture became practically immobile. This viscosity interfered with aeration (Chain, Gualandi & Morisi, 1966) and thus impeded the production of the alkaloids. It was therefore necessary to study the nature of the viscous metabolite in order to find conditions to prevent its formation; the present paper is concerned with a study of its production and of its structure. Preliminary studies showed that it was a polysaccharide of the glucan type.

Extracellular polysaccharides with various degrees of structural complexity frequently occur in fungal cultures and some of these have been shown to be glucans (for reviews see Clarke & Stone, 1963; Nordin & Kirkwood, 1965). Bouveng, Kiessling, Lindberg & McKay (1963) studied the structure and production of α - and β -linked glucans produced in shake cultures by *Pullularia pullulans* from a variety of sugar substrates. Davis, Rhodes & Shulke (1965) studied the production of glucans in shake culture by the Ascomycete fungi *Plectania occidentalis* and a *Helotium* species. Wallen, Rhodes & Shulke (1965) investigated the structure and physical properties of these glucans and found them to be branched polymers containing appreciable amounts of $\beta 1 \rightarrow 3$ linkages. Perlin & Taber (1963) and Vining & Nair (1966) have

found that cultures of *Claviceps* species give rise to polysaccharides. The former workers demonstrated that the polymer arising from *Claviceps purpurea* had a $\beta_1 \rightarrow 3$ linked primary chain with single glucose units attached in the 6 position ($\beta_1 \rightarrow 6$) at every fourth point, on average, along it.

METHODS

Organisms. Strain F 2047, used in most of these experiments, was derived by selection of a strain (no. B 37 received from Dr J. Renz of Sandoz Ltd., Basle, Switzerland), which had been isolated from a sclerotium on *Pennisetum typhoideum*, Rich. (millet) from the Tschad region of Africa. This organism has recently been described as *Claviceps fusiformis* (Loveless, 1967).

Other organisms used were: I.C. 49, related to *Claviceps*, isolated from sclerotia on Sorghum in Africa (received originally from M. C. Futrell, Zaria, Nigeria); F-114, isolated from sclerotia of *Claviceps litoralis* by Dr A. Tonolo, Istituto Superiore di Sanita, Rome; *Sclerotium rolfsii* (IM174210—Commonwealth Mycological Institute, London).

Sclerotia of *Claviceps fusiformis* were kindly supplied by the Director, Station Agricole de Sarid, Koudougou, Haute-Volta. The uninfected seeds of *Pennisetum typhoideum* (millet) were obtained from the same source.

Maintenance of the cultures. Stock cultures of F 2047 were prepared on potato glucose medium (see below) containing 2% agar and stored in the refrigerator.

Slope cultures for immediate use were prepared on the same medium at 24° and were transferred about once a month.

Submerged culture procedures

Media

The media used were as follows.

Medium I for seed production: a potato infusion was made by boiling 300 g. cut potatoes in 500 ml. water for 15 min.; after cooling the suspension was filtered through muslin and brought to pH 7 before addition of 30 g. glucose and making up to 1 l.

Medium II, a modification of the medium used by Stoll *et al.* (1954) for clavine alkaloid production: sucrose, 50 g.; ammonium succinate, 3.2 g.; KH_2PO_4 , 1.0 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 13 mg.; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 4 mg.; adjusted to pH 5.2 with concentrated ammonia, water to 1 l.

Medium III, as medium II, but at pH 5.5. Distilled water was used for shake-flask media, and tap water for fermenters (see below).

Sterilization conditions were as follows: for 100 ml. to 500 ml. Erlenmeyer flasks, 113° for 30 min.; for 1 l. to 3 l. flasks, 113° for 50 min.; for stirred fermenters containing 60 l. medium, 100° for 20 min. and 121° for 20 min.

Seed cultures

A suspension of mycelium and conidia obtained by shaking an agar slope culture with sterile water was used to inoculate seed cultures. These were maintained in shake culture at 27° and transferred every 3–4 days. For the first subculturings the seed culture was homogenized at speed 80 for 30 sec. in an Omni mixer. A 10% (v/v)

inoculum was used for all media. Washed inoculum was used for experiments on carbon source.

Fermentations in shake flasks

For seed and polysaccharide production, cotton-plugged 500 ml. Erlenmeyer flasks were placed on a rotary shaker at 27°. Under the conditions of agitation (200 rev./min., eccentric throw 10 cm.) the aeration rate in the absence of mycelium, measured by the method of Cooper, Fernstrom & Miller (1944), was equivalent to 30 ml. O₂ absorbed/100 ml. sulphite solution/hr.

For sampling, duplicate flasks were removed at intervals, and the course of fermentation followed by microscopic examination and by measurement of the following parameters: pH value, sugar utilization, ammonia utilization and dry weight of mycelium and polysaccharide. All experiments were at least duplicated.

Fermentations in stirred fermenters

Jacketed stainless-steel fermenters of 90 l. total capacity (Paladino, Ugolini & Chain, 1954) containing 60 l. medium II at 27° were used. Aeration was effected through a ring sparger with a sterile air flow of 30 l./min. for the first 24 hr and at 60 l./min. thereafter at an over-pressure of 1 atmosphere.

Agitation was provided with a top-driven stirrer rotating at 183 rev./min. and fitted with an 8-bladed turbine propeller (ratio diameter of fermenter:diameter of propeller, 3.5:1). Antifoam silicon R.D. (Midlands Silicones Ltd.) was added.

The fermenters were inoculated with 2 l. mycelial suspension grown in 3 l. shake flasks for 4 days at 25°.

Analytical techniques

Mycelial dry weight. As a standard procedure, the following was done: a sample of the culture was filtered through a filter paper supported by a nylon mat on a Hartley 3-piece Büchner funnel. The mycelium was washed twice with distilled water on the filter and then dried at 70° to constant weight.

Polysaccharide. The filtrate from above was mixed with 1–2 volumes of absolute ethanol and the precipitated polysaccharide collected on a glass rod and dried at 70° to constant weight. For structural studies, the precipitated polysaccharide was not immediately dried but resuspended in water to form a gel and then reprecipitated; this procedure was repeated several times (up to 6) before the final purified product was freeze-dried from aqueous suspension and weighed.

Total carbohydrate. Total carbohydrate was estimated by the anthrone method (Morris, 1948) and by use of the phenol+sulphuric acid reagent (Dubois *et al.* 1951, 1956).

Reducing sugar. This was determined by the method of Somogyi (1952).

Sucrose in culture filtrates was estimated by the picric acid method of Thomas & Dutcher (1924) modified as follows. To the aqueous 5 ml. sample was added 1 ml. 2N-NaOH and 4 ml. of the picric acid reagent; the mixture was heated for exactly 10 min., cooled and diluted to 25 ml. with distilled water before reading at 560 m μ (see below).

Glucose was specifically determined by a modified glucose oxidase method (Fleming & Pegler, 1963).

Nitrogen. Ammoniacal nitrogen was determined after distillation in the micro-Kjeldahl apparatus or Conway unit (Conway, 1962) followed by Nesslerization (Koch & Hanke, 1948), and, similarly, total nitrogen after digestion of the material with concentrated sulphuric acid and selenium.

Coloured complexes obtained were read at their respective maximum absorbencies in a Unicam SP. 500 spectrophotometer using a 1 cm. light path.

Chromatographic procedures. Compounds were separated by chromatography on paper (Whatman no. 1 or 3 MM) and on thin-layer plates with cellulose (Whatman CC 41). The following solvents were used: (a) butan-1-ol + pyridine + water (10 + 3 + 3, by vol.); (b) benzene + butan-1-ol + pyridine + water (1 + 5 + 3 + 3, by vol.); (c) ethyl acetate + pyridine + water (2 + 5 + 7, by vol.); (d) butan-1-ol + ethanol + water (4 + 1 + 1.9, by vol.); (e) propan-1-ol + ethyl acetate + water (7 + 1 + 2, 7 + 1 + 3 and 6 + 1 + 3, by vol.); (f) butan-1-ol + acetic acid + water (100 + 22 + 50, by vol.).

Paper electrophoresis was done by using Whatman 3 MM paper, at approximately 40 V./cm. in 0.05 M-borate, (pH 9.5) and 0.05 M-germanate (pH 10.7) buffers.

Detection reagents. Reducing sugars were detected with aniline hydrogen phthalate (Partridge, 1949), *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950) and benzidine (Bacon & Edelman, 1951); sugar alcohols were detected by using silver nitrate and sodium hydroxide (Trevelyan, Proctor & Harrison, 1950).

Identification of compounds. The following methods were used for identifying compounds: (1) Infra-red spectroscopy of solid films or nujol mulls measured with a Unicam SP. 200 G spectrophotometer. (2) Optical rotation recorded in a 1 dm. tube on a Perkin Elmer Model 141 polarimeter. (3) Chromatographic mobility. (4) M.p. and mixed m.p. in the case of crystalline products.

Enzymic methods. The following enzymes were used: a β -glucuronidase preparation from *Helix pomatia* (L'Industrie Biologique Française, S.A., Gennevilliers, France), β -glucosidase and maltase (Koch-Light Laboratories Ltd., Colnbrook, Bucks.), α -amylase (British Drug Houses, Poole, Dorset) and cellulase (Meiji Seika Kaisha Ltd., Tokyo, Japan); the specific $\text{exo-}\beta 1 \rightarrow 3$ -glucanase, derived from *Basidiomycete* QM806 (Reese & Mandels, 1959) was kindly given by Dr E. T. Reese (U.S. Army Natick Laboratories, Massachusetts).

Apart from the $\text{exo-}\beta 1 \rightarrow 3$ -glucanase, all enzyme reactions were done at 25° in 0.03 M-phosphate buffer (pH 6.0). The $\text{exo-}\beta 1 \rightarrow 3$ glucanase digests were done at 50° in 0.05 M-acetate buffer (pH 4.6). The products of enzymic hydrolysis were separated by chromatography in solvents *e* and *f*. Normally 1%, w/v (final), solution of substrate and 0.1% w/v (final) solution of enzyme were used; enzyme activity was terminated by addition of 0.01 M-mercuric chloride or immersion of the vessel containing the digest in boiling water for 5 min.

Incubations were for periods up to 24 hr.

Determination of branching in the polysaccharide. For accurate determinations of branching by using the $\text{exo-}\beta 1 \rightarrow 3$ -glucanase, the incubation time was decreased to 5 hr (see below: Table 8). A typical procedure was as follows. The products of incubation of 30 mg. glucan with the $\text{exo-}\beta 1 \rightarrow 3$ -glucanase were separated by chromatography (solvent *e*) eluted from the chromatogram with water and the major components characterized as their β -acetates (see above) to give: β -D-gentiobiose octaacetate (15.2 mg.), m.p. 194–195°, identical with an authentic specimen and β -D-glucose pentaacetate (25.2 mg.), m.p. 131–132°. Alternatively, the eluted compounds

were assayed by using the phenol + sulphuric acid reagent to determine the ratio of glucose to gentiobiose and hence the degree of branching.

Preparative and counting techniques using ^{14}C

Preparation of ^{14}C -labelled polysaccharides. Polysaccharide labelled with ^{14}C was prepared by growing the organism in the usual way on medium III but with 1% (w/v) sucrose, instead of 5%, containing 50 μC of $\text{U-}^{14}\text{C}$ sucrose/flask. The polysaccharide was isolated and purified as above.

Counting techniques. Products derived by enzymic hydrolysis of the radioactive glucan were separated by paper chromatography (solvent *e*) and the dried papers exposed to 'Kodirex No-screen' X-ray film. The autoradiograph obtained was used to determine the radioactive areas, which were removed, but into small pieces (10 $\times$ 10 mm.) and immersed in liquid scintillant (Bray, 1960). The radioactivity in the samples was determined on the paper in a Packard counter (model 3003). Corrections for variable efficiencies were made.

Procedures for structural determination of carbohydrates

Fractionation of extracellular polysaccharides. A sample (116.1 mg.) of purified polysaccharide from a 17-day culture was stirred with successive amounts of 5 N-sodium hydroxide (5 ml.) for 10 min. each time until all the material had dissolved. Each fraction was neutralized with acetic acid and the glucan, precipitated by addition of excess ethanol, was washed with ethanol, centrifuged and freeze-dried.

Fractionation of sclerotial polysaccharides. The powdered sclerotia (1 g.) were suspended in 100 ml. of 5% sodium hydroxide and stirred at 20° for 3 hr under a stream of nitrogen. After neutralization with 7.3 ml. glacial acetic acid, the extract was centrifuged for 1 hr at 12,000 g. The pale-brown opalescent supernatant fluid was brought with absolute ethanol to a final concentration of 70% (v/v) ethanol. The off-white flocculent precipitate was collected by centrifugation at 8000 g for 15 min., washed with 70% (v/v) ethanol in water and dried over CaCl_2 in vacuum. The dried residue (109 mg.) was free from sugars of low molecular weight as indicated by chromatography in solvents *e* and *f*.

Total hydrolysis of glucan. A typical procedure was as follows. A solution of glucan (70 mg.) in 1 ml. of 72% (w/v) concentrated sulphuric acid in water was stirred at 5° for 1 hr, after which water (25 ml.) was added and the solution was boiled for 4 hr. The filtrate, neutralized with barium hydroxide, was examined by paper chromatography (solvents *a* and *b*) and by paper electrophoresis in both buffers. After concentration in vacuum, acetylation of the residue (dried over phosphorus pentoxide) was done with acetic anhydride (0.7 ml.) and sodium acetate (50 mg.) by the method of Fischer (1916) and gave β -D-glucose pentaacetate, m.p. 130.5–131.0° (70 mg.), which was identical with an authentic specimen.

Partial hydrolysis of glucan. As the glucan proved intractable to partial hydrolysis in $\text{N-H}_2\text{SO}_4$, it was first dissolved by stirring overnight at 50° in 80% phosphoric acid (1 g./20 ml.). The dissolved glucan was precipitated at 5° by adding 10 ml. water followed by 90 ml. ethanol. The precipitate was collected by centrifugation at 8000 g for 15 min., washed with cold 75% (v/v) ethanol in water until neutral, and dried over CaCl_2 in vacuum to yield 390 mg. of white product. The treated glucan (2 mg.) was

hydrolysed with 100 μ l. of $\text{N-H}_2\text{SO}_4$ at 90° . Samples (10 μ l.) of the reaction mixture were removed at intervals over a period of 5 hr, neutralized with barium hydroxide and chromatographed in solvents *e* and *f*. Some of the products obtained were partially identified, after spraying the papers, by co-chromatography with known sugars in the above solvents.

Hydrolysis of the methylated glucan and identification of hydrolysis products

Methylated glucan (5.6 mg.—see below) was dissolved in 72% (w/v) concentrated sulphuric acid in water (0.1 ml.) and stirred at 0° for 1 hr. Water (1 ml.) was added and the solution was boiled for 4 hr. Examination of the hydrolysate, neutralized with barium hydroxide, by paper chromatography (solvent *d*) revealed components with the mobilities of a tetra-*O*-methyl, a tri-*O*-methyl and a di-*O*-methyl-D-glucose. The ratio of these components was determined by hypiodite oxidation (Chandra, Hirst, Jones & Percival, 1950) after separation by paper chromatography.

Methylated glucan (51.3 mg.) was hydrolysed essentially as above and the hydrolysis products isolated and identified after chromatography on Whatman 3 MM paper (solvent *d*). Three homogeneous fractions were obtained. Fraction A had the same mobility as 2,3,4,6-tetra-*O*-methyl-D-glucose and after isolation had a m.p. $91\text{--}94^\circ$. It proved identical to an authentic specimen. Fraction B had the same mobility as 2,4,6-tri-*O*-methyl-D-glucose. After isolation it had a m.p. $122\text{--}125^\circ$. It was identical with an authentic specimen, kindly given by Dr G. O. Aspinall (University of Edinburgh). Fraction C was chromatographically and electrophoretically homogeneous but could not be obtained crystalline. It was dissolved in water (0.5 ml.) and sodium borohydride (20 mg.) was added. After 18 hr the solution was neutralized to pH 6.5 with 2 *N*-acetic acid and sodium metaperiodate (50 mg.) added. After 3.75 hr at 3° , excess aqueous barium chloride was added. The filtered solution, which gave a positive test for formaldehyde with chromotropic acid reagent (MacFadyen, 1945), was extracted exhaustively with chloroform and the extract, dried with MgSO_4 , was evaporated. The product had a mobility of a di-*O*-methylpentose (solvent *d*). A solution of this product in dry dichloromethane (1 ml.) was demethylated with boron trichloride by the standard procedure (Allen, Bonner, Bourne & Saville, 1958). Examination of the product by paper chromatography (solvent *e*) and electrophoresis in both buffers revealed the presence of xylose only.

Methylation of the glucan. The glucan proved remarkably refractory to methylation. Eventually the following procedure was adopted (Wallenfels *et al.* 1963, and references cited therein). Glucan (128.5 mg.) was stirred with a solution of sodium borohydride (86 mg.) in water (20 ml.) for 12 hr and the resultant gel dissolved in 40% (w/v) aqueous sodium hydroxide (10 ml.) and stirred with dimethyl sulphate (4 ml.) at 20° for 18 hr. After warming to decompose excess dimethyl sulphate and cooling, the solution was dialysed against distilled water and concentrated by evaporation in vacuum at 40° . The above methylation was repeated twice and the final solution freeze-dried.

A solution of the partially methylated glucan in dimethyl formamide (20 ml.) was shaken with barium hydroxide (1 g.) and methyl iodide (5 ml.) at room temperature for 24 hr. A further amount of barium hydroxide (1 g.) and methyl iodide (5 ml.) was added and the mixture shaken for 24 hr. Water (200 ml.) was added and the suspension extracted with chloroform (5×50 ml.). The extracts, dried over MgSO_4 , were

evaporated to 1 ml. and addition of light petroleum (b.p. 60–80°) gave a precipitate (22.2 mg.), which was still incompletely methylated ($\nu_{\max}$ 3500 cm^{-1}).

A solution of the product in dimethyl sulphoxide (4 ml.) was shaken with barium oxide (1 g.) and methyl iodide (5 ml.) for 24 hr. This procedure was repeated and the product (20 mg.), isolated essentially as above, was free from absorption at 3500 cm^{-1} in its infra-red spectrum.

Periodate oxidations. The procedures were as follows.

(1) A sample of the glucan (12.5 mg.) was dispersed in water (5 ml.) and a solution of sodium metaperiodate (5 ml., 0.022 M) added. The suspension was stirred at 3° and samples (1 ml.) withdrawn at intervals for analysis for periodate uptake (Fleury & Lange, 1933) and formic acid liberation (Anderson, Greenwood & Hirst, 1955).

(2) Smith degradation (Goldstein, Hay, Lewis & Smith, 1959, 1965): a dispersion of the glucan (78.0 mg.) and sodium metaperiodate (144.9 mg.) in water (50 ml.) was stirred at 3° for 72 hr. Ethylene glycol (100 μl .) was added and after 0.5 hr the oxidized glucan was centrifuged down, washed with cold 50% (w/v) acetic acid and water and freeze-dried.

The resultant white solid (48.3 mg.) was suspended in water (10 ml.) and stirred overnight with sodium borohydride (100 mg.). Acetic acid (10 ml.) was added and the centrifuged precipitate was washed with 50% (v/v) aqueous ethanol. Sulphuric acid (0.2 N, 10 ml.) was added to the gel suspended in 10 ml. of water and the suspension stirred at room temperature for 24 hr. The precipitate was centrifuged down, washed well with water and freeze-dried to give 34 mg. of the degraded glucan.

Examination of the final supernatant fluid and washings, after neutralising with barium hydroxide, by paper chromatography (solvent *c*) showed the presence of glycerol only. After chromatography on Whatman 3 MM paper (solvent *c*) the glycerol was characterized as the tri-*p*-phenylazobenzoate m.p. 212–213°, prepared by the method of Baggett, Foster, Haines & Stacey (1960), which was identical with an authentic specimen prepared from glycerol (found on analysis: C, 71.1; H, 4.7; N, 11.8. $\text{C}_{42}\text{H}_{32}\text{O}_6\text{N}_6$ requires C, 70.4; H, 4.5; N, 11.7%).

Preparation of cell wall from mycelium

Suspensions of washed mycelium were shaken at about half the maximum amplitude in a Mickle disintegrator (Mickle Lab. Engineering Co., Surrey); each container held 0.75 g. wet wt mycelium, 5 ml. water and 5 ml. dry ballotini (no. 10). After 40 min. shaking, the hyphae were broken into short lengths and the cell contents had exuded. To free the cell walls from adhering cytoplasm, the process was repeated after the ballotini had been replaced by 4 ml. of 4% (w/v) sodium lauryl sulphate (pH 6.8), containing 10 mM-magnesium chloride. The large particles still remaining were allowed to sediment and the supernatant fluid decanted off. The cell walls were separated by centrifugation at 2000 *g* for 5 min. and washed by centrifugation 4 or 5 times with water. The residue was freeze-dried, yielding a white fluffy solid weighing about 50 mg. Microscopic observations, after staining with methylene blue and lactophenol cotton blue, indicated that the product was about 90% free from cell inclusions.

Fig. 1. *Claviceps fusiformis* strain F2047. Course of metabolism in stirred fermenters. Culture in medium II at 27° stirred at 183 rev./min. in a 60 l. fermenter with sterile air blown through at 30 l./min. for the first 24 hr and at 60 l./min. thereafter at an over-pressure of 1 atm. $\blacktriangle$, pH; $\bullet$, sucrose; $\circ$, ammonia nitrogen; $\blacksquare$, mycelium; $\square$, polysaccharide.

Fig. 2. *C. fusiformis* F2047. Course of metabolism in shake-flasks. Culture (100 ml. in 500 ml. Erlenmeyer flasks) shaken at 200 rev./min. in medium II at 27°. $\blacktriangle$, pH; $\bullet$, sucrose; $\circ$, ammonia nitrogen; $\blacksquare$, mycelium; $\square$, polysaccharide.

RESULTS

Physiological conditions controlling formation of the polysaccharide

In this work polysaccharide formation occurred between 24° and 27° and yields up to 4 g. dry wt mycelium/l. culture fluid were obtained.

The course of fermentation using medium II is given in Figs. 1 and 2. The polysaccharide was produced in shaken flasks and in the fermenters and reached maximum yields when growth ceased. In the fermenters growth was preceded by a long lag phase and the course of metabolism was slower. After the growing period, little or no decline

Table 1. *Claviceps fusiformis* strain F2047: effect of pH value of medium on polysaccharide production

Shake-flask culture at 27° in medium II at various pH values; harvested at 8 days.

pH value of culture		Yield of mycelium (g. dry wt/100 ml. culture)	Polysaccharide (mg./100 ml. culture)	Sporulation*
Initial	Final			
4.2	3.0	0.48	50	—
4.7	3.0	0.61	76	—
5.0	4.4	1.06	145	+
5.2	5.0	1.48	194	++
5.5	5.0	1.49	411	++++
5.7	5.0	1.67	353	++++
6.2	5.0	1.70	178	++++

*Arbitrary scale: no sporulation; + → ++++ degrees of sporulation.

Table 2. *Claviceps fusiformis* strain F2047: effect of ammonium succinate concentration on polysaccharide production

Shake-flask culture at 27° with various ammonium succinate concentrations (salts and sucrose concentration as in medium II); harvested at 8 days

Initial conc. of ammonium succinate in medium (%)	N (mg./ml.)	Mycelium (g./100 ml. culture)	Polysaccharide (mg./100 ml. culture)	Relative degrees of sporulation
0.13	0.24	1.191	85	—
0.21	0.39	1.191	86	—
0.32	0.59	1.489	209	++
0.41	0.76	1.408	210	++
0.54	1.00	1.196	263	++
0.64	1.18	1.196	266	++++

in the quantity of polysaccharide present in the culture was observed for at least 14 days (Fig. 2; Table 6) which indicated that it was not further metabolized by the fungus under the conditions used. This contrasts with the work of Davis *et al.* (1965), who found marked decreases in the polysaccharides produced by *Plectania occidentalis* and a *Helotium* species after 14 and about 2.5 days, respectively.

The effect of pH value on polysaccharide production in shake flasks is shown in Table 1; maximum production occurred between pH 5 and 6. The cultures were white below pH 5.2; with higher pH values a red pigment developed in increasing amounts.

Increasing the nitrogen concentration of the medium above a minimum value of

0.4 mg. N/ml. (Table 2) had little effect on polysaccharide production. Polysaccharide production was also independent, within fairly wide limits, of the sucrose concentration (Table 3). Sporulation occurred under most conditions towards the end of the incubation.

In addition to sucrose, the fungus utilized glucose, fructose, cellobiose and mannitol for growth, but not galactose, xylose, maltose, lactose, sorbitol, glycerol, starch or

Table 3. *Claviceps fusiformis* strain F 2047: effect of sucrose concentration on polysaccharide production

Shake-flask culture at 27° with various sucrose concentrations
(salts and ammonium succinate concentration as in medium II); harvested at 7 days.

Initial sucrose conc. in medium (%)	Mycelium (g./100 ml. culture)	Polysaccharide (mg./100 ml. culture)	Relative degree of sporulation
1	0.436	69	++++
2.5	1.272	105	+++
5	1.982	205	++
10	2.401	205	+

Table 4. *Claviceps fusiformis* strain F 2047: effect of carbon source on mycelial growth and polysaccharide production

Shake-flask culture at 27° with various carbon sources
(salts and ammonium succinate concentration as in medium II); harvested at 8 days.

Carbon source (5%, w/v, medium)	Mycelium (g./100 ml. culture)	Polysaccharide (mg./100 ml. culture)
Sucrose	1.60	224
Glucose	1.54	209
Fructose	1.39	215
Galactose	0.032	—
Xylose	0.032	—
Maltose	0.38	45
Lactose	0.047	—
Cellobiose	1.69	309
Mannitol	1.61	255
Sorbitol	0.12	—
Glycerol	0.13	27
Starch	0.21	16
Succinic acid	0.034	—

Table 5. *Claviceps fusiformis* strain F 2047: effect of nitrogen-source on polysaccharide production

Shake-flask culture at 27° with various nitrogen sources
(salts and sucrose concentration as in medium II); harvested at 8 days.

N source in medium (N = 0.75 g./l.)	Final pH value of medium	Mycelium (g./100 ml. culture)	Polysaccharide (mg./100 ml. culture)
Ammonium succinate	5.0	1.486	200
KNO ₃	6.4	0.904	—
Urea	6.2	1.792	Trace
Peptone	5.6	1.527	149
NH ₄ NO ₃	5.8	1.715	85

succinic acid (Table 4). As expected, polysaccharide production took place only when mycelial growth occurred. However, correlation between mycelial growth and polysaccharide production was not observed when the nitrogen source was varied while the carbon source (5% sucrose) was kept constant (Table 5). All nitrogen sources tested were utilized for growth but with nitrate or urea no polysaccharide production took place.

Structure of polysaccharide

A single specimen of the polysaccharide obtained from a 7-day shake-flask culture was used for the following structural studies. The purified freeze-dried product (sample A) was a white fibrous solid which had a total carbohydrate content of 97%, a total nitrogen content of 0.5% and, although it had been soluble in the original culture medium, was largely insoluble in water and only partially soluble in fresh culture medium and in 20% sucrose solution. Acid hydrolysis yielded only glucose as shown by paper chromatography and electrophoresis; the reducing power of the hydrolysate was equivalent to 88.5% anhydroglucose. D-glucose was isolated in good yield as the pentaacetate. Partial acid hydrolysis of this glucan at 90° yielded a series of oligosaccharides, two of which were tentatively identified by chromatography as laminaribiose and gentiobiose, which suggested the presence of $\beta 1 \rightarrow 3$ and $1 \rightarrow 6$ links. The optical rotation of the original material ($[\alpha]_D^{20} + 6.8^\circ \pm 0.3$ in 5 N-sodium hydroxide) and its infra-red spectrum ($\nu_{\max}$ 896 cm^{-1}) were further indications of β -linkages. Acid hydrolysis of the methylated glucan gave 2,3,4,6-tetra-O-methyl-D-glucose; 2,4,6-tri-O-methyl-D-glucose and 2,4-di-O-methyl-D-glucose in the ratio of 1.15:2.7:1.0; this indicated the presence of non-reducing glucopyranosyl end-groups, $1 \rightarrow 3$ glucopyranosyl linkages and 1,3,6 branch points, respectively, in the same ratio.

Periodate oxidation studies were in agreement with the methylation analysis. After 95.5 hr at 3° the glucan had consumed a limiting value of 0.51 mole/periodate/anhydrohexose unit and had liberated 0.20 mole formic acid. Only trace amounts of formaldehyde were detected. This is consistent with a structure containing either one non-reducing end-group or a $1 \rightarrow 6$ linkage for every five units: the 70% periodate resistant units obtained indicate $1 \rightarrow 3$ linkages or branch points with no vicinal hydroxyl groups. The slightly high value for the periodate consumption (0.5 mole) over that expected from the formic acid production (0.2 mole) remains to be explained.

Evidence for the fine structure of the glucan was obtained from a Smith degradation from which was isolated glycerol and a residual polymer only. The latter was not attacked further by periodate and its structure was confirmed enzymically (see below) as an unbranched linear $\beta 1 \rightarrow 3$ linked glucan.

The above results indicate that the original glucan consisted of chains of $1 \rightarrow 3$ linked β -D-glucopyranosyl units to which single β -D-glucopyranosyl units are attached by $6 \rightarrow 1$ linkages on every fourth unit, on average, along the chain. Supporting proof for the structure of the glucan was obtained from a concurrent series of enzymic investigations. The presence of β linkages was indicated by hydrolysis of the polymer to glucose by an enzyme preparation from *Helix pomatia* known to contain a specific β -glucosidase. Similarly, the polymer, and oligosaccharides obtained by its partial acid hydrolysis, were hydrolysed to glucose by a β -glucosidase from almonds but were unaffected by a fungal maltase and bacterial α -amylase. A partially purified fungal cellulase had only a slight hydrolytic action on this glucan which suggested that the linkages were not $\beta 1 \rightarrow 4$. Enzymic proof for the $\beta 1 \rightarrow 3$ nature of the linkages was

obtained by use of the specific $\text{exo-}\beta\text{I} \rightarrow 3\text{-glucanase}$, which hydrolysed the polymer to a mixture of glucose and gentiobiose in a molecular 3:1. The polymer ratio of derived by the Smith degradation (see above) gave only glucose on hydrolysis by this enzyme.

Glucan from stirred fermenters

The infra-red spectrum, optical rotation and products from complete acid hydrolysis and hydrolysis with the $\text{exo-}\beta\text{I} \rightarrow 3\text{-glucanase}$ obtained with the product from the stirred fermenter were identical with those found for the shake flask polysaccharide. The average glucose to gentiobiose ratio for the fermenter polysaccharide was 2.2:1.

Variations in glucan structure

Claviceps fusiformis strain F2047 did not lose its capacity for polysaccharide synthesis in submerged culture after many vegetative transfers (> 30) in contrast with the findings of Davis *et al.* (1965) with *Plectania occidentalis* and an *Helotium* species. However, variations in the structure of the *C. fusiformis* glucan were observed. Table

Table 6. *Claviceps fusiformis* strain F2047: variation in the degree of branching of the glucan during 20 days of incubation

Samples of the glucan, obtained from a shake-flask culture at 27° on medium II, were hydrolysed at 1% (w/v) in 100 $\mu\text{l.}$ of 0.05 M-acetate buffer (pH 4.6) with 0.1% (w/v) of $\text{exo-}\beta\text{I} \rightarrow 3\text{-glucanase}$ for 3 hr at 50°.

Time of growth (days)	Yield of glucan mg./100 ml. of culture	Molecular ratio of glucose to gentiobiose*
3	44	3.6:1
7	102	3.2:1
10	133	2.4:1
14	208	1.8:1
17	137	2.1:1
20	88	2.3:1

*Mean value obtained on products from enzyme hydrolysis. Ratio of units in main chain with branch to those without = 1:glucose/gentiobiose ratio.

6 shows that the degree of branching of the glucan, as measured by the ratio of the glucose to gentiobiose produced on enzymic hydrolysis with the specific $\text{exo-}\beta\text{I} \rightarrow 3\text{-glucanase}$, gradually increased to a maximum of one branch on every third residue after 14 days, then slowly declined. Furthermore, chemical and enzymic evidence suggested that over a period of months the overall degree of branching of polysaccharide produced after 10 days submerged culture had slowly increased from about one branch per four units to one per three units. The highest degree of branching corresponded to the highest degree of water solubility and lowest viscosity.

The variation in the degree of branching observed amongst these samples and between them and specimen A suggested the possibility that the glucan was not homogeneous but consisted of several components differing in their degrees of branching. However, fractionation experiments in N-sodium hydroxide using a single purified sample from a 17-day culture did not support this hypothesis. All fractions obtained were indistinguishable by infra-red spectroscopy and had approximately the same degree of branching, as indicated by hydrolysis with the $\text{exo-}\beta\text{I} \rightarrow 3\text{-glucanase}$ and measurement of the glucose and gentiobiose liberated (Table 7).

Tests on the polysaccharide derived from mycelium grown on media with various

carbon and nitrogen sources showed that neither had any effect on the degree of branching.

A sample of ^{14}C -glucan obtained from an ammonium succinate [$\text{U-}^{14}\text{C}$]sucrose (1 %) medium enabled an accurate check to be made on the degree of branching, as measured by the enzyme technique. The high value obtained at 24 hr (Table 8) was probably due to the fact that the $\text{exo-}\beta 1 \rightarrow 3$ -glucanase had a slight activity towards the $\beta 1 \rightarrow 6$ link, (already noted by Dr E. T. Reese, personal communication, 1966).

Table 7. *Claviceps fusiformis* strain F 2047: degree of branching of glucan fractionated in 5 N-sodium hydroxide

Sample of the glucan (116 mg.) obtained from a shake-flask culture at 27° on medium II, harvested at 17 days, fractionated as under 'Methods'; fractions hydrolysed at 1 % (w/v) in 100 μl . of 0.05 M-acetate buffer (pH 4.6) with 0.1 % w/v. of $\text{exo-}\beta 1 \rightarrow 3$ -glucanase for 3 hr at 50°.

Fraction	Weight of extracted polysaccharide in fraction (mg.)	Molecular ratio of glucose to gentiobiose
1	31.5	1.75:1
2	31.5	1.74:1
3	14.1	2.06:1
4	8.15	1.85:1
5	7.95	1.98:1
6	1.70	1.86:1

Table 8. *Claviceps fusiformis* strain F 2047: molecular ratio of the glucose to the gentiobiose liberated from [$\text{U-}^{14}\text{C}$]glucan during hydrolysis by $\text{exo-}\beta 1 \rightarrow 3$ -glucanase

Sample of the glucan, obtained from a shake-flask culture at 27° with 1 % (w/v) [$\text{U-}^{14}\text{C}$]sucrose containing 50 μC ./flask (salts and ammonium succinate concentration as in medium III), hydrolysed at 5 % (w/v) in 500 μl . of 0.05 M-acetate buffer (pH 4.6) with 0.1 % (w/v) of $\text{exo-}\beta 1 \rightarrow 3$ -glucanase at 50°.

Incubation period	Hydrolysis of glucan (%)	Molecular ratio of glucose to gentiobiose
0	0.90	—
3 min.	13.3	2.02:1
10 min.	35.7	2.03:1
40 min.	81.9	2.02:1
2 hr	98.5	2.09:1
5 hr	98.9	2.19:1
24 hr	99.3	2.44:1

Cell-wall glucans

Loosely filtered mycelium to which glucan still adhered was incubated with the $\text{exo-}\beta 1 \rightarrow 3$ glucanase to ascertain whether the glucan could be removed in this way from the hyphal walls. However, the dry weight of the mycelium after this treatment was lower than could be accounted for by removal of the glucan alone; moreover, microscopic observation clearly indicated that the cell walls had undergone some decomposition. Furthermore, the molecular ratio of glucose to gentiobiose in the enzyme digest was over 6:1, i.e. higher than in the glucan alone. Accordingly, cell-free samples of cell walls were prepared and subjected to enzymic hydrolysis by the $\beta 1 \rightarrow 3$ glucanase; they yielded glucose 26 %. Attempts to record the infra-red spectrum of the cell-wall material in nujol were not completely successful due to the highly particulate nature of the material; however, a small peak at 898 cm^{-1} and the absence of peaks

at 850 and 935 cm^{-1} indicated β linkage, which supports the evidence for the presence of chains of $\beta 1 \rightarrow 3$ linked glucose units in the walls.

The indication of $\beta 1 \rightarrow 3$ linked glucans in the walls of *Claviceps fusiformis* strain F2047 suggested that the natural sclerotium might contain similar polysaccharides. Sclerotia were ground and the resulting powder subjected to the action of the exo- $\beta 1 \rightarrow 3$ glucanase. No hydrolysis was observed. It was considered that this negative result might be due to the fact that potentially hydrolysable structures were not available to the enzyme because of the complex structure of the sclerotia. However, extraction of the sclerotial powder by alkali under nitrogen, followed by ethanol precipitation, yielded a product which represented 10.9% of the original and contained 58% total carbohydrate and 2.9% total-nitrogen. This extract was susceptible to prolonged enzymic hydrolysis and yielded glucose and gentiobiose in a ratio of approximately 2:1 (3.03 mg. were incubated with the exo- $\beta 1 \rightarrow 3$ glucanase for 20 hr and yielded after hydrolysis 0.94 mg. anhydroglucose). The glucose yield after hydrolysis indicated that the carbohydrate in the extract consisted of about 53% glucan containing $\beta 1 \rightarrow 3$ linkages and $\beta 1 \rightarrow 6$ branch points, in all representing 3.4% of the original dry sclerotia. Uninfected millet, used as a control, gave no indication of $\beta 1 \rightarrow 3$ glucans after extraction.

Allied organisms producing $\beta 1 \rightarrow 3$ glucans

During this work two other organisms related to *Claviceps* and a basidiomycete *Sclerotium rolfsii*, all of which produce polysaccharides, were tested for the presence of $\beta 1 \rightarrow 3$ linked glucans. Strain I.C. 49 was isolated from sclerotia on Sorghum in Africa; strain F-114 was isolated from sclerotia of *Claviceps litoralis*; the basidiomycete was kindly given by Dr B. E. J. Wheeler (Imperial College, London). All three fungi were found to produce $\beta 1 \rightarrow 3$ -linked glucans with $\beta 1 \rightarrow 6$ branches. Strain I.C. 49 gave a molar ratio of glucose to gentiobiose of about 4:1. The glucan produced by F-114 gave the highest degree of branching observed in the work (glucose:gentiobiose ratio about 1.5) and was the most water-soluble glucan. The presence of $\beta 1 \rightarrow 3$ -linked glucans from *S. rolfsii* has already been noted (Johnson *et al.* 1963); the culture used (IMI 74210—Commonwealth Mycological Institute, London) produced a glucan in large quantities after addition of 2% (v/v) ethanol to the culture medium (Dr B. E. J. Wheeler, personal communication, 1966); this glucan had a molar ratio of glucose to gentiobiose of about 3:1 and was very sparingly water-soluble.

Microscopic examination of the mycelia of the above organisms after treatment with the exo- $\beta 1 \rightarrow 3$ glucanase showed that the cell walls of all three organisms were attacked, thus suggesting that glucans with $\beta 1 \rightarrow 3$ linkages were present. The presence of $\beta 1 \rightarrow 3$ linked glucans in the cell walls of other fungi appears to be well established (Clarke & Stone, 1963; Horikoshi & Iida, 1964; Manners & Patterson, 1966; Wessels, 1966). Perlin & Taber (1963) noted that in some cases the glucan produced by *Claviceps purpurea* was attached to the mycelium, as has been found here for the glucan produced by the above strain of *Sclerotium rolfsii* and to a lesser extent the various strains of *Claviceps* tested. Evidence is accumulating to suggest that there may be a structural connexion between the cell walls and the glucan produced extracellularly.

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