Isolation and Structure of an Extracellular Polysaccharide from *Streptomyces* sp. FERM-P1185

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(Received 21 July 1975; revised 4 December 1975)

SUMMARY

A streptomycete strain (FERM-P1185), isolated from soil, secreted a slime on glucose-asparagine agar, and produced viscous growth in liquid media containing peptone as nitrogen source. A purified polysaccharide isolated from the culture broth was composed of glucose and mannose units (molar ratio 1·87:1). Periodate oxidation, Smith degradation, methylation analysis, infrared and ^{13}C nuclear magnetic resonance spectra, indicated that this mannoglucan had a linear structure consisting of α -1,3- and α -1,4-linked glucopyranose and mannopyranose units.

INTRODUCTION

Much work has been done on extracellular polysaccharides produced by bacteria and fungi, but little attempt has been made to isolate these substances from streptomycetes. Recently, a *Streptomyces* sp. was isolated from soil, and found to produce an extracellular polysaccharide and a new antibiotic which inhibited plant pathogenic fungi, particularly *Piricularia oryzae* (Ōmura *et al.*, 1971).

This paper describes the characterization of the strain, and the isolation and structural examination of the polysaccharide.

METHODS

Characterization and cultivation. The organism, strain FERM-P1185, was described using the criteria applied in the International Streptomyces Project (Shirling & Gottlieb, 1966) and identified on the basis of *Bergey's Manual of Determinative Bacteriology* (Pridham & Tresner, 1974).

To produce the polysaccharide, an inoculum was pre-incubated in a medium containing (% w/v): glycerol, 1; starch, 1; soy bean meal, 2; CaCO_3 , 0·3; and tap water, pH 7·0; and shaken on a reciprocal shaker (120 rev./min) at 27 °C for 2 days. The bacterial suspension was then transferred to the production medium containing (% w/v): glucose, 6; peptone, 0·2; K_2HPO_4 , 0·1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0·05; CaCO_3 , 0·3; and tap water, pH 7·0; and shaken on a reciprocal shaker at 27 °C for 5 to 6 days.

Assay of mycelium growth. Broth culture (10 ml) was centrifuged for 10 min at 800 g and the wet mycelium weighed.

Assay of residual glucose. The amount of glucose remaining in the culture medium was determined by the *o*-toluidine method (Dubowski, 1962).

Analytical procedures. Ascending paper chromatography was performed using Toyo filter paper nos. 50 and 51, and the solvent systems: ethyl acetate–pyridine–water (10:4:3, by vol.), and ethyl acetate–pyridine–acetic acid–water (5:5:1:3, by vol.). Sugars were detected with alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950) and *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950), and the total hexose content determined using phenol–H₂SO₄ (Dubois *et al.*, 1956). Evaporations were carried out *in vacuo* at under 35 °C, and optical rotation determined at 22 °C using a Jasco (Tokyo, Japan) Dip-SL.

Gas–liquid chromatography (g.l.c.) was performed using a Shimadzu (Tokyo, Japan) GC-5A chromatograph with a glass column (0.3 cm × 200 cm) and a gas flow rate of 50 ml nitrogen/min. The column was packed with 5 % (w/w) ECNSS-M (cyanoethylsilicone polymer) on Chromosorb W (aw-dmcs, 60 to 80 mesh) and operated at 170 to 180 °C for identification of the component sugars and the Smith degradation products; it was packed with 3 % (w/w) silicone OV-210 on Chromosorb W (aw-dmcs, 60 to 80 mesh) and operated at 170 °C for identification of the *O*-acetyl-*O*-methyl alditols.

Mass spectra were recorded with a Hitachi RMU-7L at 70 eV ionization voltage. Infrared (i.r.) spectra were recorded with a Hitachi-215 spectrometer; proton magnetic resonance spectra with a JNM-MH-100 spectrometer, using sodium 2,2,3,3-tetradeutero-3-(trimethylsilyl) propionate (TSP) as internal standard; and ¹³C nuclear magnetic resonance spectra were measured at 25.1 MHz with a JEOL PS-100 spectrometer. All protons were decoupled. The polysaccharide was measured at a concentration in D₂O of 80 mg ml⁻¹. Chemical shifts were measured using dioxane [67.4 p.p.m. downfield from internal tetramethylsilane (TMS)] as an external standard, and were expressed relative to TMS. Spectra were determined after multiple scanning using a time-averaging device.

Chemicals. α-Amylase was purchased from Seikagaku-kogyo Co. (Japan), and pronase from Kaken Kagaku Co. (Japan).

Isolation and purification of the polysaccharide. The mycelium and insoluble materials were removed by centrifuging and crude preparations of the polysaccharide precipitated from the culture filtrate by adding 2 vols. acetone. The precipitate was dehydrated with acetone and dried; the yield was about 9 g/10 l culture broth.

The crude extracellular polysaccharide was dissolved in water and the solution adjusted to pH 7.6 with NaHCO₃. Pronase was added, and the solution was incubated for 2 days at 37 °C, and dialysed in Visking Cellophane tubing against running water for 2 days. The non-dialysable portion was concentrated to a small volume, and freed from pronase by shaking with chloroform–butyl alcohol (4:1, v/v) until no further gelatinous precipitate could be separated. After centrifuging, the aqueous solution was concentrated, and the product precipitated by addition of ethanol (yield was 13.6 % of the pre-digested material).

RESULTS

Characterization of the streptomycete strain

The sporophores were located on short side branches on the flexuous main hyphae and terminated in closed to partly open spirals (Spirales) of two or more coils. The spores were oval or ellipsoidal (0.5 to 0.8 μm × 1.0 to 1.5 μm) and had a smooth surface (Fig. 1). There was no evidence of sporangia, zoospores or sclerotia. The cultural and physiological characteristics of the strain are summarized in Tables 1 and 2.

The strain was classified in the white series, Group W; S; C–; SM (Pridham & Tresner, 1974), was considered to be similar to *Streptomyces rimosus* (Sobin, Finlay & Kane) Waksman (Waksman, 1953) and was assigned to the ‘rimosus’ subgroup. The properties of

Fig. 1. (a) Aerial mycelium and (b) spore chain morphology of *Streptomyces* sp. FERM-P1185 grown on oatmeal agar for 14 days at 27 °C.

Table 1. *Cultural characteristics of Streptomyces* sp. FERM-P1185

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose–nitrate agar	Poor, colourless	None	None
Glucose–asparagine agar	Good, spreading, slimy cream	None	None
Glycerol–calcium malate agar	Moderate, raised, pale yellow	None	None
Inorganic salts–starch agar	Restricted, spreading, colourless with light gold coloured centre	None	None
Tyrosine agar	Moderate, spreading, cream to butter yellow	None	Light gold
Nutrient agar	Moderate, cream	None	None
Yeast extract–malt extract agar	Good, raised, light ivory to old gold coloured centre	Thin, light ivory	Light gold
Oatmeal agar	Restricted, spreading, colourless	Thin, light ivory	None
Glucose–peptone–beef agar	Moderate, raised, light antique gold to light wheat	None	Melon yellow
Potato–glucose agar	Good, raised, light ivory to melon yellow	None	Pale yellow

the strain were in good agreement with those of the type strain, *S. rimosus* ISP5260, with respect to the following morphological and cultural characteristics: both formed light ivory coloured aerial hyphae on yeast extract–malt extract agar and on oatmeal agar; produced reddish growth on yeast extract–malt extract and inorganic salts–starch agars; and a soluble yellow pigment in glucose–peptone–beef and tyrosine agars. Strain FERM-P1185 produced a characteristic slime on glucose–asparagine agar but unlike *S. rimosus* ISP5260 it did not use arabinose or raffinose as sole sources of carbon and energy for growth.

Table 2. *Physiological properties of Streptomyces sp. FERM-P1185*

Property	Reaction
Melanin formation	Negative
Tyrosinase production	Negative
Degradation of starch	Negative
Hydrogen sulphide production	Negative
Nitrate reduction	Doubtful
Liquefaction of gelatin	Positive
Liquefaction of serum	Negative
Coagulation of milk	Positive (strong)
Peptonization of milk	Positive (weak)
Cellulolytic activity	Negative
Temperature range	No growth below 5 °C or above 50 °C
Sugars as sole source of carbon	
Glucose, fructose, inositol, mannitol	Good growth
Xylose	Poor growth
Arabinose, sucrose, rhamnose, raffinose	No growth

Fig. 2. Polysaccharide production by *Streptomyces sp. FERM-P1185*. Changes in the residual glucose (■) and polysaccharide (○) contents of the mycelium during its growth (●) in broth culture. pH changes (□) during growth are also recorded.

Production of the polysaccharide

The most effective carbon and nitrogen sources for the production of the polysaccharide were glucose (6 %, w/v) and peptone (0.2 %, w/v). Maximum growth was achieved after 3 to 4 days, and the production of the polysaccharide increased with the viscosity of the broth culture reaching a maximum (90 mg/100 ml) after 5 to 6 days (Fig. 2).

Properties of the purified polysaccharide

The purified polysaccharide (SM-P) gave a single spot on zone electrophoresis using Pevicon C-870 (polyvinyl resin, M & S Instruments, Japan) and 1 % borate buffer (pH 9.2); and gave a single peak on gel filtration on a Sepharose-6B column. The polysaccharide was then considered to be pure enough for structural studies. Purified SM-P, $[\alpha]_D^{20} + 16.2^\circ$ (c. 0.3 in water), did not give a blue colour with iodine, and contained 0.7 % nitrogen and 95.4 % total hexose.

Table 3. *Methyl ethers from the hydrolysate of the methylated polysaccharide*

Retention values of alditol acetate derivatives were measured by g.l.c. and are expressed relative to the mobility of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. Molar proportions of the methyl ethers were estimated from relative peak areas of the gas chromatogram.

Sugars	Relative retention	Molar proportion (%)
2,3,4,6-tetra- <i>O</i> -methyl hexose	1.00	2.16
2,4,6-tri- <i>O</i> -methyl hexose	1.78	50.2
2,3,6-tri- <i>O</i> -methyl hexose	2.23	47.6

Acid hydrolysis of the SM-P gave glucose and mannose in the molar ratio 1.87:1. In its i.r. spectrum, the SM-P had an absorption maximum at 840 cm⁻¹, which is characteristic of α -glycosidic linkages (Barker *et al.*, 1954). The proton magnetic resonance spectrum showed signals at 5.16 p.p.m., characteristic of an anomeric proton, but no signals of *O*-methyl, *O*-acetyl or other *O*-acyl groups. The SM-P did not react with α -amylase of the saccharifying type from *Bacillus subtilis*.

Periodate oxidation and Smith-type degradation

Purified SM-P (12.5 mg) was dissolved in water (10 ml), the total volume made up to 25 ml with 2 ml of 0.22 M-sodium periodate in distilled water, and the reaction allowed to proceed in the dark at 25 °C for 72 h. The number of moles of sodium periodate reduced per an-hydrohexose unit of the SM-P was 0.33 (after 1 h), 0.53 (2 h), 0.54 (3 h), 0.55 (6 h) and 0.55 (24 h), using the method of Malaprade (Hay, Lewis & Smith, 1965). No formic acid was detected (Whistler & Hickson, 1954). The polyalcohol, obtained as a syrup after the addition of ethylene glycol (0.5 ml), reduction with sodium borohydride (7 mg) and dialysis followed by concentration, was hydrolysed with 0.25 M-sulphuric acid (0.5 ml) for 5 h at 100 °C. The degradation products in the solution were converted into their alditol acetate derivatives by reduction with sodium borohydride, followed by acetylation using acetic anhydride-pyridine (1:1, v/v) (Sawardeker, Slonecker & Jeanes, 1965), and estimated by g.l.c. Molar ratios were calculated from the ratios of the areas under each peak in the chromatogram. Glucitol acetate, mannitol acetate, erythritol acetate and a trace of glycerol acetate were detected; and the molar proportions of the degradation products, glucose, mannose and erythritol, were calculated as 2.4:1.0:3.5.

Methylation

Purified SM-P was methylated four times using the method of Hakomori (1964). 2,3,4,6-Tetra-*O*-methyl, 2,3,6-tri-*O*-methyl, and 2,4,6-tri-*O*-methyl hexoses were detected in the acid hydrolysate by thin-layer chromatography using benzene-acetone (1:1, v/v) as solvent, and developing with the Molisch reagent (Dische, 1962). 1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl hexitol, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl hexitol (*m/e* 43, 45, 87, 101, 117, 129, 161), and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl hexitol (*m/e* 43, 45, 87, 99, 101, 113, 117, 233) were detected by gas-chromatographic mass spectrometry (Björndal, Lindberg & Svensson, 1967; Miyazaki & Naoi, 1975) of the alditol acetates derived from methylated SM-P. The molar proportions in the hydrolysate of methylated SM-P are shown in Table 3.

Analysis of oligosaccharides from controlled Smith-degradation products

SM-P (60 mg) was oxidized for 6 h with sodium periodate using the above procedure, and reduced with sodium borohydride for 18 h followed by dialysis. The oxidized and reduced

Fig. 3. The proton noise-decoupled ^{13}C Fourier transform n.m.r. spectrum of the polysaccharide at 25.1 MHz, recorded under the following conditions: saturated solution at 80 °C; solvent, D_2O ; internal D lock; pulse width, 12 μs .

Peaks 1 to 3 were assigned to anomeric carbon atoms; peak 8 to Glc(α) C-3; peak 9 to Man(α) C-3, Glc(α) C-2 and Glc(α) C-5; peak 10 to Glc(α) C-4 and Man(α) C-4; peak 12 to Man(α) C-6; and peak 13 to Glc(α) C-6. For assignment of other peaks, see Results. Peak 11 was the resonance from the standard dioxane (67.4 p.p.m. downfield of TMS).

SM-P was hydrolysed with 0.25 M-sulphuric acid (15 ml) at 100 °C for 2 h; this gave at least five oligosaccharides which had R_{glucose} values, i.e. R values relative to the mobility of D-glucose, of 0.65, 0.50, 0.31, 0.16 and 0.03. After air drying the paper chromatogram, the areas containing the two main fast-moving oligosaccharides were cut out and extracted with distilled water. The homogeneity of the two main oligosaccharides, O-1 (R_{glucose} , 0.65) and O-2 (R_{glucose} , 0.31), was examined by electrophoresis on Toyo Roshi glass fibre GH-100 in 0.042 M-borate buffer at pH 9.2 (1 mA cm^{-1}). Each gave a single spot, detected by Molisch reagent, which moved to the cathode. Oligosaccharides O-1 and O-2 were hydrolysed with 0.5 M-sulphuric acid at 100 °C for 5 h, and then each hydrolysate was subjected to g.l.c. on ECNSS-M. The molar ratios of the component sugars, estimated from relative peak areas of the chromatogram, were: for O-1, 2 glucose:1 mannose units; and for O-2, 2 glucose:3 mannose units. Both O-1 and O-2 showed absorption maxima in their i.r. spectra at 840 cm^{-1} characteristic of α -glycosidic linkages. When O-1 and O-2 were reduced with sodium borohydride, hydrolysed in 0.05 M-sulphuric acid and then acetylated, mannitol acetate was detected by g.l.c. in each hydrolysate.

Part of the oxidized and reduced SM-P was hydrolysed with 0.25 M-sulphuric acid at room temperature for 18 h, and produced two main oligosaccharides, O-1' (R_{glucose} , 0.52) and O-2' (R_{glucose} , 0.076). These were purified by paper chromatography and further hydrolysed with 0.005 M-sulphuric acid for 2 h at 100 °C. By this treatment, oligosaccharide O-1' produced glycerol, erythritol and oligosaccharide O-1 (R_{glucose} , 0.65); and oligosaccharide O-2' produced erythritol, glucose, mannose, oligosaccharide O-1, and two other oligosaccharides having R_{glucose} values of 0.23 and 0.06.

Fig. 4. The most probable sugar units in the polysaccharide SM-P.

¹³C Nuclear magnetic resonance spectrum

The ¹³C nuclear magnetic resonance spectrum of SM-P in D₂O showed 12 resonances, at 101.76, 101.79, 100.017, 81.475, 80.6, 77.59, 74.48, 73.03, 71.574, 70.895, 62.06, and 61.57 p.p.m. (Fig. 3). Those at 101.79 and 100.017 p.p.m. were assigned to two anomeric carbons at α-1,4- and α-1,3-linkages of glucopyranosyl residues, respectively: the position of these resonances is in good agreement with those seen in spectra of nigerose and maltose (Usui *et al.*, 1973). Peak 5, at 80.6 p.p.m., and peak 6, at 77.59 p.p.m., were assigned to the C-3 and C-4 resonances involved in the 3-substituted and 4-substituted glucopyranoses (Usui *et al.*, 1973), respectively. Peaks 8, 9, 10, 12, and 13 were assigned to the non-substituted carbons of glucopyranose and mannopyranose by comparison with spectra of known α-glucopyranose and α-mannopyranose (Johnson & Jankowski, 1972; Gorin & Mazurek, 1974). Peak 1, at 101.72 to 101.76 p.p.m., may be assigned to anomeric carbons in α-1,3- and α-1,4-linked mannopyranosyl residues, α-1,3- and α-1,4-linked glucopyranosyl-mannopyranosyl oligosaccharide moieties, and α-1,4-linked mannopyranosyl oligosaccharide (Gorin, 1973). Peaks 4 and 7 may be assigned to C-3 and C-4 involved in α-1,3- and α-1,4-linked glucopyranosyl and mannopyranosyl residues.

CONCLUSIONS

The purified extracellular polysaccharide of *Streptomyces* sp. strain FERM-P1185 contains glucose and mannose in the molar ratio 1.87:1. The presence of approximately equal proportions of 1,3- and 1,4-glycosidic linkages was shown by periodate oxidation, by methylation analysis and by ¹³C nuclear magnetic resonance spectroscopy. Smith-type degradation gave oligomeric fragments that contained both glucose and mannose; thus both sugars are present in the same polymer. Methylation analysis showed that the polysaccharide is unbranched and has an average chain length of 46 hexapyranose residues. We conclude that the polysaccharide is a linear polymer that contains structures of the type shown in Fig. 4.

Glucomannans with a β-1,4-linked linear structure have been isolated from many plants belonging to the families Orchidaceae, Liliaceae and Iridaceae and also from wood

hemicellulose (Aspinall, 1970) but glucomannans have not previously been isolated from streptomycetes; the mannoglucan from strain FERM-P1185 differs by possessing a linear structure consisting of approximately equal amounts of α -1,4- and α -1,3-linkages.

We thank Mrs O. Hayashi, K. Niwano, and K. Hata of Tokyo College of Pharmacy, and Ms Y. Miura of Kitasato University for technical assistance; and Mr K. Matsushita of the JEOL Co., Tokyo, for the ^{13}C n.m.r. measurements.

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