Carbohydrate Research, 203 (1990) 103–107
Elsevier Science Publishers B.V., Amsterdam

Structure of an exocellular β-D-glucan from *Pediococcus* sp., a wine lactic bacteria*

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(Received July 28th, 1989; accepted for publication, October 25th, 1989)

ABSTRACT

Pediococcus sp. produces an exocellular slime containing exclusively D-glucose. The structure of the polysaccharide was determined by methylation analysis, Smith degradation, enzymic hydrolysis, and ^{13}C-n.m.r. spectroscopy as having a trisaccharide repeating unit, →3)-β-D-Glc*p*-(1→3)-[β-D-Glc*p*-(1→2)]-β-D-Glc*p*-(1→.

INTRODUCTION

Many bacterial species are able to synthesize exocellular polysaccharides[1–4]. Because of their industrial significance, the two polysaccharides that have been the most studied are the dextran produced by *Leuconostoc mesenteroïdes* and the xanthan produced by *Xanthomonas campestri*[5]. Recently, the biological role of exocellular polysaccharides produced by phytopathogenic bacteria (*Agrobacterium*, *Erwinia*, *Pseudomonas*, and *Rhizobium*) has been studied[6], but little is known about their molecular structures. Some lactic bacteria found in wine are exocellular polysaccharide producers. By increasing the viscosity of the wine, these strains are responsible for wine ropiness and were first described by Pasteur[7] and Laborde[8]. More recently, this effect was studied by Lonvaud and Joyeux[9] who isolated from ropy wines (characterized by their "oily" pouring properties) several strains; these were identified as *Pediococcus ceverisiae* and are able to produce exocellular polysaccharides in a synthetic medium. We report herein the structure of an exocellular D-glucan produced by a *Pediococcus* sp. strain.

* This investigation was supported by the Institut d'Oenologie (director Professor P. Ribereau-Gayon), by Novo Ferments (Basel, Switzerland), by the Centre National de la Recherche Scientifique (Unité Mixte de Recherche du C.N.R.S. 111, director Professor J. Montreuil), and by the Université des Sciences et Techniques de Lille Flandres-Artois.
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0008-6215/90/$ 03.50

EXPERIMENTAL

Microbiological techniques. — The *Pediococcus* sp. strains isolated from ropy wines[10] were cultured on a modified Carr liquid medium containing per L: yeast extract (Difco, purified from polysaccharides by ethanol precipitation; 4 g), casaaminoacids (Difco; 5 g), D-glucose (5 g), L-malic acid (5 g), KH_2PO_4 (0.6 g), KCl (0.45 g), $CaCl_2{\cdot}2H_2O$ (0.13 g), $MgSO_4{\cdot}7H_2O$ (0.13 g), and $MnSO_4{\cdot}H_2O$ (0.003 g). The pH was ajusted to 4.5 and the solution autoclaved for 15 min at 110°. Ethanol was added (100 $mL.L^{-1}$ of synthetic medium) and a 1-L flask was inoculated with a 48-h preculture grown on the same medium and incubated at 25°. After 12 days, the bacteria were centrifuged off (20 min, 7500*g*) and the supernatant solution dialyzed against distilled water.

Purification of the polysaccharides. — The exocellular polysaccharides were isolated by ethanol precipitation (3 parts of ethanol per part of medium). After 48 h, the filamentous D-glucan was collected by centrifugation (20 min, 7500 *g*), rinsed three times with 3:1 ethanol–water, solubilized in water by use of ultrasonic waves, and purified by chromatography in a column (2 × 20 cm) of DEAE-Sepharose CL 6B (Pharmacia Fine Chemicals, Uppsala, Sweden), eluted with water and 0.5M NaCl at 0.5 $mL.min^{-1}$. Fractions were collected and tested for sugar content with the phenol–H_2SO_4 reagent. Fractions reacting positively were combined and lyophilized. The yield of polysaccharide was 120 $mg.L^{-1}$ of culture.

Molecular weight determination. — The molecular weight was determined by chromatography in a column (1.6 × 74 cm) of Sephacryl S 400 (Pharmacia Fine Chemical) using 0.1M NaCl as eluant (29.5 $mL.h^{-1}$). Fractions (6.4 mL) were collected and tested with the phenol-H_2SO_4 reagent. The column was calibrated with several dextran T samples (Pharmacia T-10, T-40, T-70, and T-500).

^{13}C-N.m.r. spectroscopy. — ^{13}C-N.m.r. spectra were recorded at 100 MHz with a Bruker AM-400 WB spectrometer coupled with an Aspect 3000 calculator (Centre Commun de Mesures, USTL-FA). The sample (50 $mg.mL^{-1}$ of D_2O) was analyzed at 80° with the standard program POWGATE (1H broad-band with composite-pulse decoupling, $D_1 = 0.1$s, $PW = 90° = 6\ \mu$s; $S_1 = S_2 = 1$ W). Spectral width was 26000 Hz for 32K frequency-domain and time-domain data points. Chemical shifts are expressed relative to the signal of internal sodium 4,4-dimethyl-4-sila-(2,3-$^2H_4$)pentanoate (δ 0.0) with an accuracy of 0.1 p.p.m.

Carbohydrate analysis. — A sample (200 μg) of polysaccharide lyophilized in the presence of *myo*-inositol (internal standard, 100 μg) was hydrolyzed with 4M trifluoroacetic acid for 4 h at 100° in a Sovirel tube. The monosaccharides produced were per(trimethylsilyl)ated and analyzed by g.l.c. in a capillary column of (50 × 0.34 mm i.d.) of CP Sil 5CB with the temperature being raised $2°.min^{-1}$ from 80 to 260°.

Methylation analysis. — The polysaccharide was permethylated as described by Paz Parente *et al.*[11]. The permethylated polysaccharide was methanolyzed and the partially methylated methyl glycosides were peracetylated with 1:5 (v/v) pyridine–acetic anhydride overnight at room temperature. The partially methylated and acetylated

methyl glycosides were separated by g.l.c. and analyzed by g.l.c.–m.s. under the conditions described by Fournet *et al.*[12].

Smith degradation. — Periodate oxidation, reduction, and mild hydrolysis with dilute acid were performed as described by Johnson *et al.*[13]: 50 mM $NaIB_4$ (26 mL) was added to the polysaccharide solution (33 mg in 13 mL of 0.1M sodium acetate, pH 5.0). The mixture was stored for 8 days at 4°. An excess of 1,2-ethanediol (240 mg) was added and, after 30 min, the mixture was dialyzed against distilled water for 48 h at 4°. The reduction was carried out with $NaBH_4$ (350 mg, 24 h, 20°), and the solution was dialyzed and treated with 50mM H_2SO_4 for 24 h at 20°. The precipitate obtained on neutralization was centrifuged off.

Enzymic hydrolysis. — The insoluble, periodate-resistant polysaccharide was digested with a commercial D-glucanase preparation (Glucanex, Novo Ferment AG, Switzerland) obtained from *Trichoderma* containing 10 units.mg^{-1} of exo-(1→3)-β-D-glucanase (EC 3.2.1.58). A suspension of the D-glucan (25 mg) in a solution (10 mL) containing 10 units of glucanase in 0.01M sodium acetate buffer (pH 5.0) was incubated for 24 h at 35°. After heat inactivation (100°, 5 min) and centrifugation, the digest (4 mL) was applied to a column of Sephadex G 15 (Pharmacia Fine Chemicals) and eluted with water.

RESULTS AND DISCUSSION

Gel filtration, on a Sephacryl S 400 column, of the exocellular polysaccharide of *Pediococcus* sp. eluted from a DEAE-Sepharose column with water indicated an average mol. wt. of 800 000. G.l.c. of the per(trimethylsilyl)ated monosaccharides obtained after hydrolysis showed the presence only of glucose. Analysis by g.l.c.–m.s. of the methanolyzed, permethylated polysaccharide showed the presence of methyl 2,3,4,6-tetra-, 2,4,6-tri-, and 4,6-di-*O*-methylglucoside (as acetates) in equimolar proportions (Table I). These results indicated that the D-glucan from *Pediococcus* sp. is constituted by a trisaccharide repeating unit having a (1→3)-linked backbone and a (1→2)-linked branch of one of D-glucopyranosyl group.(1).

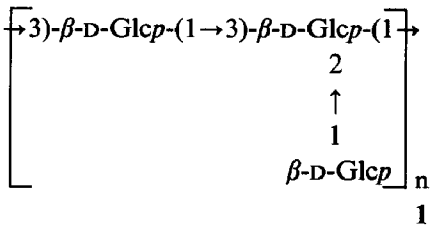
TABLE I

G.l.c. analysis of methyl ethers obtained from methylated D-glucan of *Pediococcus* sp.

Parameters	*O-methyl ethers*					
	2,3,4,6-		*2,4,6-*[a]		*4,6-*[a]	
	α	*β*	*α*	*β*	*α*	*β*
Retention time (min)	16.6	14.5	28.0	24.8	31.7	30.8
Pick height (cm)	10.5	2.1	8.4	2.5	9.2	3.5
Molar ratio[b]	1.1		1		1.1	

[a] As methyl *O*-acetylglycosides.

[b] Values are given relative to one residue of methyl 2,4,6-tri-*O*-methyl-D-glucoside.



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This structure **1** was confirmed by the study of the insoluble, periodate-resistant polysaccharide obtained by Smith degradation of the native exopolysaccharide. This insoluble D-glucan was hydrolyzed by the exo-(1→3)-β-D-glucanase of *Trichoderma* sp. (Glucanex). The elution profile of the digest applied to a Sephadex G-15 column showed mainly D-glucose and a small proportion of incompletly digested D-glucan. Methylation analysis of the periodate-resistant polysaccharide gave methyl 2,4,6-tri-*O*-methylglucoside, which indicated a linear (1→3)-linked sequence, thus confirming that the branched D-glucopyranosyl group was linked to the D-glucopyranosyl residues of the backbone by a (1→2)-linkage.

Structure **1** was also confirmed by the indentification in the ^{13}C-n.m.r. spectrum (Fig. 1) of 18 resonances including in the anomeric region three resolved signals of nearly equal intensities at δ 105.10, 104.32, and 104.26 which could be attributed to C-1 of β-D-glucose residues.

The extracellular β-D-glucan of *Pediococcus* sp. may be structurally related to more highly branched (1→3,1→2)-β-D-glucans, such as the capsular polysaccharide from *Streptococcus pneumoniae* type 37 which has a single (1→2)-linked β-D-glucopyranosyl group attached to each D-glucose unit of the main chain[14]. The exocellular D-glucan is responsible for some aspects of wine deterioration (ropiness). It is also structurally related to scleroglucans, such as the (1→3,1→6)-β-D-glucan produced by *Botrytis cinerea*[15], the only difference being the nature of the linkage of the side chains,

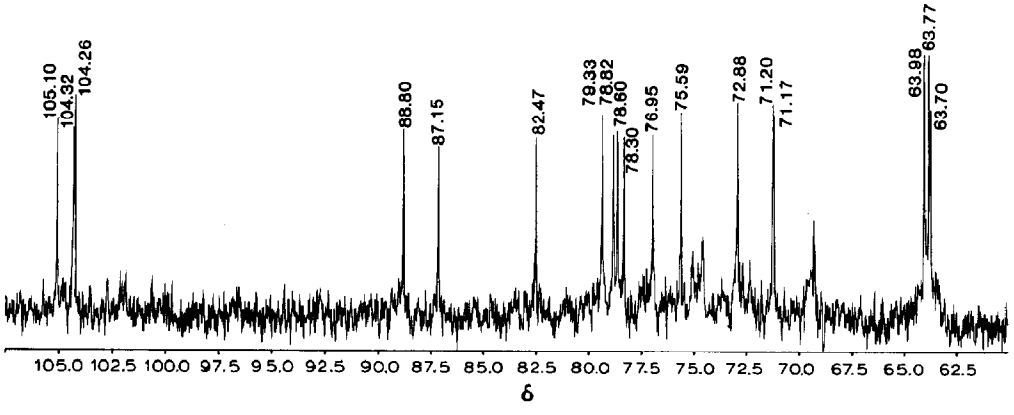

Fig. 1. ^{13}C-N.m.r. spectrum of the polysaccharide isolated from *Pediococcus* sp. culture.

(1→2) instead of (1→6). This difference may explain the greater resistance of the native *Pediococcus* glucan to the action of the exo-(1→3)-β-D-glucanase, as compared to the D-glucan of *B. cinerea*.

ACKNOWLEDGMENTS

The authors thank Jean-Michel Wieruszeski for help in the n.m.r. analysis, and the Conseil Régional du Nord/Pas de Calais, the Centre National de la Recherche Scientifique, the Ministère de la Recherche et de l'Enseignement Supérieur, the Ministère de l'Education Nationale, and the Association pour la Recherche sur le Cancer for their contribution in the acquisition of the 400-MHz n.m.r. apparatus.

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