*Carbohydrate Research*, 184 (1988) 285–287
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands



## Note

# A (1→3,4)-linked β-D-glucan from the cell walls of regenerating tobacco protoplasts

ANDREAS HENSEL AND GERHARD FRANZ*

*University of Regensburg, Faculty of Chemistry and Pharmacy, Universitätsstraße 31, D-8400 Regensburg (F.R.G.)*

(Received May 9th, 1988; accepted for publication, July 12th, 1988)

Regenerating protoplasts are useful for studying cell-wall formation and polysaccharide biosynthesis[1]. For protoplasts from *Nicotiana tabacum*, the composition of the cell walls from leaf mesophyll cells and suspension-cultured cells are different[2]. The cell walls of suspension-cultured cells and tobacco leaves contain up to 40% of cellulose, whereas those from protoplasts, after regeneration for 4 days, consist of only 5% of cellulose besides other matrix polysaccharides[3]. The non-cellulosic polysaccharide fraction after methylation was found to consist of 65% of 4- and 3-linked β-D-glucose residues. L-Arabinose, D-galactose, D-mannose, and uronic acids were present in smaller amounts[1,4]. Therefore, two types of glucans may be present in the cell walls from regenerating tobacco protoplasts, namely, (*a*) lichenin-type polysaccharides [(1→3,4)-β-D-glucans], which are found mostly in monocotyledons[5–7], but are present also in one dicotyledon[8]; and (*b*) a mixture of callose-type glucans and short-chain (1→4)-β-D-glucans, as in the cell walls of regenerating *Vinca rosea* protoplasts[9].

The structure of the non-cellulosic glucan fraction from tobacco protoplast cell walls was unknown hitherto. For a detailed study of the non-cellulosic glucans from tobacco protoplasts, alpha-amylase- and pectinase-treated cell walls of protoplasts cultured for 3 days were methylated according to the method of Harris *et al.*[10]. G.l.c. of the partially methylated alditol acetates subsequently obtained gave peaks corresponding to products from 4- and 3-linked glucose residues in the ratio 4:1. The relatively high amount (11%) of terminal glucose residues indicated the glucans to have a low molecular weight.

The molecular weight of a hemicellulosic fraction, extracted with 0.1M sodium hydroxide and containing 80% of glucose residues, was estimated as 6,600 by gel-permeation chromatography on a Superose ™12 column and comparison

*Author for correspondence.

0008-6215/88/$ 03.50 

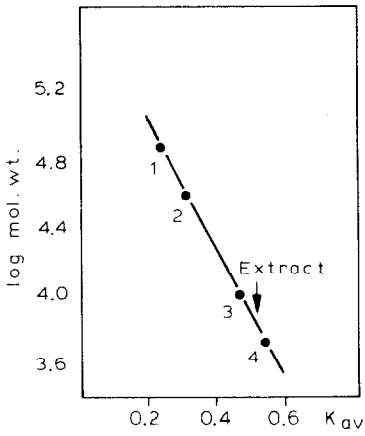


Fig. 1. Gel-permeation chromatography on Superose ™12 of an alkali extract from regenerating protoplasts after culture for 3 days. Eluant: 0.1M NaOH: mol. wts. of dextran standards: 1, 70,300; 2, 40,000; 3, 10,500; 4, 5,000.

with standard dextrans (Fig. 1).

The purified cell-wall fraction was treated with a glucanase (EC 3.2.1.73) from *Bacillus subtilis*, which hydrolyses β-(1→4) linkages exclusively, if neighbouring β-(1→3) linkages are present, thereby liberating mono-, di-, and oligo-saccharides[11,12]. T.l.c. of the enzymic hydrolysate of the tobacco protoplast cell-wall fraction revealed laminaribiose and minor amounts of glucose. The specificity of the glucan hydrolase was confirmed by its action on (1→3,4)-β-D-glucans from oat and barley, which also gave laminaribiose and glucose.

In contrast to the high-molecular mixed-linked glucans from monocotyledons, the glucan from the cell walls of *Nicotiana tabacum* protoplasts is only a short-chain matrix polysaccharide. It is not known whether this glucan is synthesized because of the non-physiological state of the protoplasts or if it is used by the cells as a cellulose precursor[21].

## EXPERIMENTAL

*Isolation and culture of protoplasts from* Nicotiana tabacum *var.* xanthi. — Protoplasts were isolated and cultured as previously described[4]. Protoplasts were kept in the culture medium described by Nagata and Takebe[13] at $3 \times 10^5$ cells/mL in the dark for 20 h, then illuminated with 2000 lux at 25°.

*Isolation of cell-wall material.* — Protoplasts were cultured for 3 days, then centrifuged for 2 min at 100*g*, washed three times with the culture medium, and homogenized in a Braun–Melsungen glass homogenizer. The cell-wall fraction was isolated by the method previously described[14].

Starch was removed with pancreatic alpha-amylase[16] (Sigma) and pectic substances were removed from the cell-wall fraction with Pectinol[17] (Röhm) at pH 4.5.

Polysaccharides were methylated according to Harris *et al.*[10]. The analysis of the partially methylated alditol acetates was carried out with a Hewlett–Packard GC 5890A with a mass-selective detector 5970B on a Durabond Fused-Silica capillary column (DB 225, 0.25 mm × 30 m) and a temperature program of 170→210° at 10°/min followed by an isothermic phase. The following products were obtained: 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol (13.4 min), 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol (19.3 min), and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol (20.7 min).

Gel-permeation chromatography was carried out on a column (1 × 30 cm) of Superose ™12 with the aid of an FPLC-System (Pharmacia), with 0.1M NaOH as eluent. Fractions were monitored by the anthrone method[18]. The column was calibrated with dextrans (Pharmacia) of mol. wts. 70,300, 40,000, 10,500, and 5,000 (see Fig. 1).

Enzymic degradation of the non-cellulosic glucans with *Bacillus subtilis* glucan hydrolase was performed according to the method of Anderson and Stone[19]. The products were subjected to t.l.c. on Silica Gel G 60 (Merck), using *A*, acetone–water (87:13); and *B*, acetonitrile–water (87:13); and detection with aniline–diphenylamine–phosphoric acid[20]. The respective $R_F$ values of glucose and laminaribiose were 0.69 and 0.61 in solvent *A*, and 0.53 and 0.36 in solvent *B*. Cellobiose was not detected.

ACKNOWLEDGMENTS

The *Bacillus subtilis* glucanase was a generous gift from Professor B. A. Stone (Bundoora, Australia). The authors thank Dr. W. Blaschek for discussion and the "Fonds der Chemischen Industrie" for financial support.

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