Biosynthetic origin and longevity in vivo of α-D-mannopyranosyl-(1 → 4)-α-D-glucuronopyranosyl-(1 → 2)-myo-inositol, an unusual extracellular oligosaccharide produced by cultured rose cells

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Abstract. A non-reducing trisaccharide, α-D-mannopyranosyl-(1 → 4)-α-D-glucuronopyranosyl-(1 → 2)-myo-inositol (MGI) accumulated in the spent medium of cell-suspension cultures of 'Paul’s Scarlet' rose (Rosa sp.) predominantly during the period of rapid cell growth. This trisaccharide was also produced by cultures of sycamore (Acer pseudoplatanus L.) but not by those of the graminaceous monocots maize (Zea mays L.) and tall fescue grass (Festuca arundinacea Schreb.). When added to cultured Rosa cells, [14C]MGI was neither taken up by the cells nor bound to the cell surface and was not metabolised extracellularly. When D-[6-14C]glucuronic acid was fed to cultured Rosa cells, extracellular [14C]MGI started to appear only after a 5-h lag period, compared with a 0.5-h lag period for labelling of extracellular polysaccharides. Furthermore, [14C]MGI continued to accumulate in the medium for at least 20 h after the accumulation of 14C-polymers had ceased. These observations indicate that extracellular MGI was produced from a slowly turning-over pool of a pre-formed intermediate. Structural considerations indicate that the intermediate could be a glucuronomannan or a phytyglycolipid (glycophosphoglycolipid). No Rosa polysaccharides could be found that generated MGI in the presence of living Rosa cells. We therefore favour phytyglycolipids as the probable biosynthetic origin of MGI.

Key words: Glucuronomannan – myo-Inositol – Oligosaccharide – Glucuronosylarabinose biosynthesis – Phytyglycolipid – Rosa (extracellular oligosaccharide)

Introduction

Oligosaccharins are those oligosaccharides that evoke biological responses when applied at low concentrations to living plant cells. They have been proposed to play important intercellular signalling roles in higher plants (York et al. 1984; Darvill et al. 1992; Aldington and Fry 1993, 1994; Auctová et al. 1995; Warneck et al. 1998). In studies of their physiological roles, it would be of great interest to investigate changes in the extraprotoplasmic concentrations of endogenous oligosaccharins, e.g. in response to environmental stimuli. However, such analyses are hindered by the low concentrations likely to be present in vivo. In addition, extraprotoplasmic fluid is not easily prepared in large volumes from plant organs. As an alternative, the spent medium of plant cell-suspension cultures can be collected in large volumes, facilitating the analysis of extraprotoplasmic oligosaccharides present at low concentration.

Plant cell cultures have been shown to accumulate in the culture medium biologically relevant concentrations of certain oligosaccharins, including those derived from xyloglucans (Fry 1986), pectins (Tani et al. 1992), N-linked glycoproteins (Priem et al. 1990; Priem and Gross 1992) and unidentified sources (Schröder and Knoop 1995; Roberts et al. 1997). We are investigating the secretion of oligosaccharins by cultured Rosa cells (García-Romera and Fry 1995, 1997), and recently discovered in this source a novel trisaccharide, α-D-mannopyranosyl-(1 → 4)-α-D-glucuronopyranosyl-(1 → 2)-myo-inositol (MGI) (Smith et al. 1999). In some experiments, MGI at 10−8–10−3 g/l significantly inhibited protein biosynthesis in Rosa cell cultures (Smith 1998), indicating that MGI is an oligosaccharin.

The trisaccharide MGI is highly unusual because it is a non-reducing compound with myo-inositol at the terminus. The biosynthetic origin of MGI is unclear. Extraprotoplasmic oligosaccharides can potentially arise by de-novo synthesis and secretion, or they can be generated secondarily by partial hydrolysis of a pre-formed precursor, e.g. a wall polysaccharide. The latter biosynthetic origin has been demonstrated for extrapro-
toplasmic XXFG, an oligosaccharin derived from xyloglucan (McDougall and Fry 1991). In the present work, we have investigated aspects of MGI metabolism: how the production of MGI changes during the culture growth cycle, the longevity of MGI in the presence of living plant cells, and whether MGI is synthesised de novo or via a pre-formed polymer.

Materials and methods

Plant cell-suspension cultures. Cell-suspension cultures of ‘Paul’s Scarlet’ rose (Rosa sp.) were maintained in a medium (Fry and Street 1980), initially at pH 6.1, that contained inorganic salts, D-glucose (20 g/l), 2,4-dichlorophenoxyacetic acid (1 mg/l), kinetin (0.5 mg/l) and Na2EDTA · 2H2O (7.4 mg/l). No inositol was added. The cells were sub-cultured fortnightly by 4-fold dilution. Cell-suspension cultures of sycamore (Acer pseudoplatanus L.), maize (Zea mays L.) and tall fescue grass (Festuca arundinacea Schreb.) were maintained as described by Stuart and Street (1969), Lorences and Fry (1991) and McDougall and Fry (1994), respectively.

Total oligosaccharides in spent medium of Rosa cells. Spent medium from a 14-d-old 800-ml Rosa culture was filtered through 53-μm nylon gauze. Some of the soluble extracellular polymers were precipitated by addition of an equal volume of ethanol and storage at 4 °C overnight. The precipitate was filtered off on nylon gauze and the ethanol removed from the filtrate in vacuo. The concentrated aqueous solution was analysed by gel-permeation chromatography.

Uronate-containing oligosaccharides in spent medium of Rosa cells. An inoculum (2.0 ml) of 1-week-old Rosa cell culture was added to 5 ml of fresh medium containing 3.7 MBq of [6-14C]glucuronic acid (100 μCi/ml; prepared as described before by Brown and Fry 1993) and incubated under standard conditions for a further 7 d. The culture was then filtered through nylon gauze, and the filtrate was analysed by gel-permeation chromatography.

Accumulation of extracellular MGI during the growth cycle of Rosa cultures. Duplicate 30-ml cultures of Rosa were harvested at intervals after sub-culture. Packed cell volume was determined after centrifugation at 1500 g for 5 min. The supernatant was dried in vacuo and the solutes were resuspended in 1.0 ml water and analysed by Dionex HPLC (Dionex, Camberley, UK) using eluent programme 1.

Survey of other cultures for MGI. Cell-suspension cultures of Acer, Zea and Festuca were harvested at 1 and 2 weeks after sub-culturing and the cells filtered off on nylon gauze. Some soluble extracellular polymers were precipitated by addition of an equal volume of ethanol and storage at 4 °C overnight. The precipitate was filtered off on nylon gauze and the ethanol removed from the filtrate in vacuo. The concentrated aqueous solution was analysed by Dionex HPLC using eluent programme 1.

Determination of the stability of [14C]MGI in vivo. Purified [GlC.A.14C]MGI was filter-sterilised and added to a 7-d-old Rosa culture (or fresh culture medium as a control) at about 7 Bq/ml. Aliquots (1 ml) were transferred to sterile 5-ml Petri dishes and incubated under standard growth conditions. At intervals, duplicate dishes were harvested: cells were filtered off on nylon gauze and washed with 2 × 1.0 ml of water. The total filtrate was analysed by paper chromatography. Material of Rf 0.0 was taken to be 14C-polymer. The [14C]MGI was recognised by co-migration with an α-(1 → 4)-trigalacturonic acid (GalA3) marker; the [14C]MGI was not accompanied by detectable [14C]GalA3, which can be resolved from MGI by paper electrophoresis (Garcia-Romera and Fry 1997; Smith et al. 1999).

Preparation of Rosa cells walls and soluble extracellular polysaccharides. Cells of Rosa (5 d after sub-culturing) were collected on nylon gauze, freeze-dried and repeatedly stirred overnight in fresh phenol/acetate acid/water (2:1:1, v/v/v; PAW) until the PAW contained no detectable protein (Fry 1988). The residue was then washed in 70% ethanol and repeatedly stirred overnight in fresh 90% dimethylsulphoxide until the extract contained no detectable starch. The final cell wall preparation was washed with 70% ethanol, then 100% acetone, and air-dried. Extracellular polysaccharides were collected from the initial culture filtrate by addition of 4 volumes of ethanol, incubation at 4 °C overnight and centrifugation at 1500 g for 10 min.

Preparation of [14C]-polysaccharides. A 1-week-old Rosa cell culture (50 ml) was supplied with 150 kBq of [6-14C]glucuronic acid and incubated under standard conditions for a further 7 d. Cell walls were freed of protein and starch as described above. 14C-Pectic polysaccharides were repeatedly extracted in 50 mM trans-1,2-diaminocyclohexane-N,N,N′,N′-tetra-acetic acid (CDTA) (Na +, pH 7.5), at room temperature overnight, until the extracts contained no more radioactivity. Further polysaccharide fractions were then extracted with 8 M urea [in 50 mM Hepes (Na +), pH 7.5, 6 M NaOH containing 1% NaBH4, and 6 M NaOH containing 4% H2BO3. In each case the extraction was repeated until no more radioactivity was detected in the supernatant. Each extract was de-salted on a Sephadex CL-4B column (150 ml bed volume; Sigma, Poole, UK) and freeze-dried.

Incubation of [14C]-polysaccharides with Rosa cells. The dried 14C-polysaccharides were resuspended in 0.25 ml of water, added to 6-d-old Rosa culture or fresh culture medium (10 ml in a 9-cm Petri dish) and incubated under standard conditions for 24 h. The cells were filtered off on nylon gauze and washed with 2 × 1.0 ml of water. The total filtrate was analysed by paper chromatography.

Search for GI units in soluble extracellular [14C]-polymers and [14C]-cells. From Rosa cultures that had been incubated with [6-14C]glucuronic acid for 25 h (see Fig. 4), the whole cells and the soluble extracellular polymers (= the origin of the paper chromatogram) were subjected to acid hydrolysis in 2 M trifluoroacetic acid (TFA). The products were analysed by paper chromatography. Radioactive material that co-migrated with authentic α-[4-14C]glucurononopyranosyl-(1 → 2)-myo-inositol ([14C]GI) was then eluted and subjected to electrophoresis.

Acid hydrolysis to release GI. Acid hydrolysis was conducted in 2 M TFA at 120 °C for 1 h. After removal of the TFA in vacuo, the hydrolysate was analysed by paper chromatography or HPLC (eluents programme 2).

Reference compounds for chromatography. The marker GalA3 was isolated from a pectinase digest of commercial polygalacturonic acid. Pure MGI and [14C]MGI were isolated as described by Smith et al. (1999). The [14C]GI was prepared from [14C]MGI by acid hydrolysis. Malto-oligosaccharides, α-glucuronic acid and α-glucuronolactone were from Sigma Chemical Co., Poole, UK.

Paper chromatography and electrophoresis. Chromatography was conducted on Whatman 3MM paper by the descending method for n-[6-14C]Glucuronic acid (filter-sterilised) was added at 3.5 kBq/ml, and aliquots of the culture (20 ml) were incubated in 5-cm Petri dishes under standard conditions. At intervals, duplicate dishes were harvested: cells were filtered off on nylon gauze and washed with 2 × 1.0 ml of water. The total filtrate was analysed by paper chromatography. Material of Rf 0.0 was taken to be 14C-polymer. The [14C]MGI was recognised by co-migration with an α-(1 → 4)-trigalacturonic acid (GalA3) marker; the [14C]MGI was not accompanied by detectable [14C]GalA3, which can be resolved from MGI by paper electrophoresis (Garcia-Romera and Fry 1997; Smith et al. 1999).

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