

## THE OXIDATION OF TERMINAL D-GALACTOFURANOSE RESIDUES OF A GALACTAN AND A GLYCOPROTEIN BY A D-GALACTOSE OXIDASE PREPARATION FROM *Dactylium dendroides*\*

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### ABSTRACT

A galactan, isolated from the unicellular organism *Prototheca zopfii*, and a glycoprotein from a hyphal cell-wall fraction of the fungus *Pithomyces chartarum* have been oxidised by a D-galactose oxidase preparation from *Dactylium dendroides*. The oxidised polymers were subsequently reduced with sodium borotritide. The site of oxidation was identified as C-6 of non-reducing D-galactofuranosyl residues in both polymers.

### INTRODUCTION

D-Galactose oxidase (D-galactose:oxygen oxidoreductase, EC 1.1.3.9) was first isolated as an extracellular enzyme secreted by the fungus *Polyporus circinatus*<sup>1</sup>, and was shown to catalyse the oxidation of D-galactose by molecular oxygen to produce *galacto*-hexodialdose and hydrogen peroxide<sup>2,3</sup>. In addition to D-galactose and 2-acetamido-2-deoxy-D-galactose, a number of D-galactosides and oligosaccharides or polysaccharides which contain D-galactose in the non-reducing, terminal position were oxidised. Recently, it was demonstrated that dihydroxyacetone is a better substrate for the enzyme than is D-galactose<sup>4</sup>. The stereochemistry of the dehydrogenation of the primary alcohol group of D-galactopyranose by D-galactose oxidase has been determined<sup>5</sup>. Using D-galactose-6-*d* and methyl  $\beta$ -D-galactopyranoside-6-*d*, it was established that the reaction involves removal of the 6-*pro-S*-hydrogen atom. From the measurement of the rates of oxidation associated with various axially attached 4-substituents, it was shown that the axial 4-hydroxyl group of D-galactopyranose does not play a hydrogen-bonding role primarily, but contributes a substituent of a size optimal for interaction with the enzyme<sup>6</sup>.

Use has been made of D-galactose oxidase as an analytical tool in structural studies of galactose-containing polysaccharides by further oxidation of the D-galactopyranosyl residues to D-galactopyranosyluronic acid residues<sup>7</sup>. Numerous studies

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\*Dedicated to the memory of Professor Edward J. Bourne.

have been reported on the introduction of a tritium label by reduction with sodium borotritide of the products of enzymic oxidation of glycoproteins and glycolipids to produce D-galactosyl-6-*t* and 2-acetamido-2-deoxy-D-galactosyl-6-*t* groups in the molecules<sup>8-12</sup>. The apparent lack of reactivity of the enzyme towards D-galactofuranosylglycerol from *Mycoplasma mycoides*<sup>13</sup>, and to galactocaralose<sup>14</sup> has been suggested as evidence for the galactofuranose nature of that sugar in these compounds.

Evidence is now presented that, in fact, D-galactose oxidase preparations are capable of oxidising C-6 of D-galactofuranose-containing residues of a fungal glycoprotein, isolated from a cell-wall fraction of *Pithomyces chartarum*, and of a galactan from *Prototheca zopfii*.

#### MATERIALS AND METHODS

**Glycoprotein sample.** — The purified glycoprotein (W1) from *Pithomyces chartarum* was used in all experiments<sup>15</sup>. This material migrated as one band on cellulose acetate electrophoresis. A sample of the galactan was purified from the cells of *Prototheca zopfii*<sup>16</sup>. D-Galactose oxidase from *Dactylium dendroides* was purchased from Worthington Biochemicals Ltd., and D-galactose dehydrogenase [D-galactose: NAD oxidoreductase EC 1.1.1.48] was purchased from Boehringer Corporation. Sodium borotritide was obtained from the Radiochemical Centre, Amersham.

**Analytical methods.** — Free D-galactose was estimated by the D-galactose dehydrogenase method<sup>17</sup>. Glycosidically bound D-galactose was estimated by using the same method after acidic hydrolysis. For the partial acidic hydrolysis of the glycoprotein, 0.01M HCl for 1 h at 100° was used. Hydrolysates were dialysed exhaustively against distilled water and the diffusates freeze-dried. Total carbohydrate was measured by using the orcinol-H<sub>2</sub>SO<sub>4</sub> procedure<sup>18</sup>. Qualitative paper chromatography was carried out with: *A*, ethyl acetate-pyridine-water (10:4:3); *B*, propan-1-ol-ethanol-water (14:2:7). T.l.c. was carried out on aluminium-backed Silica gel plates (Merck, A.G. Darmstadt) with *C*, benzene-ethanol (4:1). Gel-permeation chromatography was performed on columns (50 × 2 cm) of Sephadex G-25 with 1% sodium chloride as eluant.

**Oxidation reactions with D-galactose oxidase.** — Samples (1 mg) were dissolved in 2.0 ml of 0.1M sodium phosphate buffer (pH 7.0) to which chromogen (*o*-dianisidine, Worthington, Glucostat; 2 ml) and peroxidase (0.1 ml, 20 units) had been added. The reactions were started by the addition of D-galactose oxidase (20 units), and the extinctions at 420 nm were measured.

**Sodium borotritide reduction.** — Samples (10 mg) of the carbohydrates, after oxidation with D-galactose oxidase and adjustment to pH 8.0, were reduced with 800  $\mu$ Ci of NaBT<sub>4</sub> (0.1 ml) for 1 h, followed by an addition of NaBH<sub>4</sub> (10.5 mg). After 3 h at room temperature, the pH of the solution was adjusted to 5.0 with 0.5M acetic acid to destroy the excess reducing agent and then readjusted to pH 8.0 with 0.5M NaOH.

**Release of formaldehyde.** — The method was essentially that of Vaskovsky and

Isay<sup>19</sup>, based on the Nash procedure<sup>20</sup>. 0.2M Sodium metaperiodate (0.1 ml) was added to the carbohydrate (1 mg/ml). Oxidation was allowed to proceed at 4° in the dark. Samples (0.1 ml) were withdrawn at timed intervals, and the excess periodate was destroyed by addition of 0.1 ml of sodium arsenite (0.25M containing 0.4M H<sub>2</sub>SO<sub>4</sub>). The formaldehyde was then determined by using Nash's reagent<sup>20</sup>.

*Isolation of formaldehyde from the galactan from P. zopfii after oxidation with D-galactose oxidase and reduction with sodium borotritide.* — The polysaccharide (20 mg), after oxidation by D-galactose oxidase and reduction by sodium borotritide, was purified by gel-permeation chromatography. A sample (4 mg) was oxidised with sodium metaperiodate, and Nash's reagent was added as before. At the end of the incubation period, conc. ammonia (2 ml) was added and the resulting yellow solution was evaporated to dryness under reduced pressure. The lutidine derivative was then extracted from the residue with acetone, and the extract was concentrated to a small volume. After chromatographic separation of this product (t.l.c., silica gel, solvent C), the adsorbent was cut into 1-cm strips, which were then washed with acetone (5 ml) directly into scintillation vials to elute material from the strips. The solvent was removed under reduced pressure and a toluene-based scintillation fluid [5 ml, containing POPOP (0.1 g) and PPO (5 g)] was added to each vial, which was then counted in a Tricarb scintillation counter.

#### RESULTS AND DISCUSSION

The galactan synthesized by *Prototheca zopfii* has been shown to possess a branched structure with main chains of (1→6)-linked D-galactopyranose residues and ~10% of side chains containing terminal D-galactofuranosyl units<sup>16</sup>. The presence of the D-galactofuranose residues was established from the results of methylation analysis, partial hydrolysis with acid, and periodate oxidation. When this polysaccharide was treated with D-galactose oxidase in the presence of peroxidase and a chromogen, oxidation of the polysaccharide was complete after ~10 h; samples of the polysaccharide which had been subjected to mild hydrolysis with acid or to oxidation by D-galactose oxidase, followed by re-isolation of the polymer, showed essentially no oxidation (Fig. 1). This apparent oxidation was not due to the presence of free D-galactose as a contaminant of the polysaccharide sample, because a similar amount of chromogen was formed when the galactan was dialysed against water for 24 h before oxidation.

The oxidised galactan was reduced with sodium borotritide, thus incorporating a tritium label at C-6 of the non-reducing, terminal D-galactose residues which had been oxidised. No free D-galactose-6-*t* could be found in this product, indicating the absence of any hydrolase in the D-galactose oxidase preparation, and therefore supporting the theory that the polysaccharide was oxidised. Isolation of the galactan was achieved by gel-permeation chromatography, when it was demonstrated that the fractions containing radioactivity coincided with the polysaccharide (Fig. 2). These results are compatible with the enzymic oxidation of a polysaccharide molecule

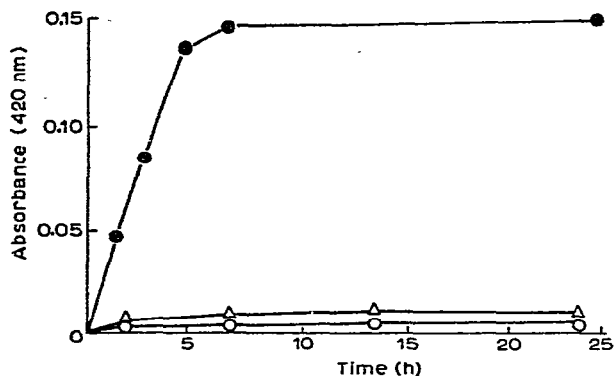


Fig. 1. Oxidation of *P. zopfii* galactan by D-galactose oxidase: ●—●, *P. zopfii* galactan; △—△, *P. zopfii* galactan after 24-h treatment with D-galactose oxidase; ○—○, *P. zopfii* galactan after mild, acidic hydrolysis.

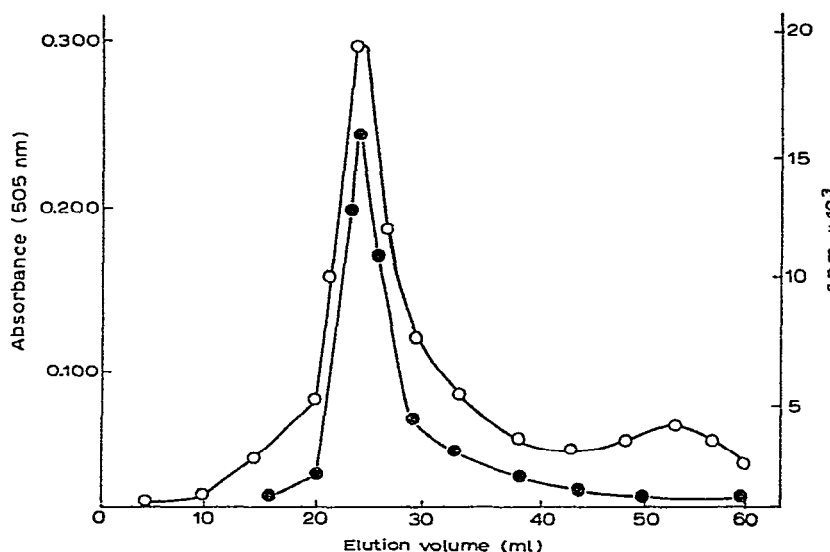


Fig. 2. Chromatographic separation of *P. zopfii* galactan after oxidation with D-galactose oxidase and reduction with sodium borotritide: ○—○, total carbohydrate; ●—●, radioactivity.

containing both D-galactopyranose and D-galactofuranose residues. The results of the structural studies on *P. zopfii* galactan did not indicate whether or not the non-reducing, terminal furanose residues were directly or indirectly attached to the main chain. Therefore, it is possible that a few furanose end-groups could be hydrolysed from the galactan during the isolation procedures, and D-galactopyranose end-groups would result if these furanose residues had been indirectly attached to the main chain.

When the tritiated galactan reacted with sodium metaperiodate, formaldehyde was released; after treatment with 2,4-pentanedione-ammonia, the formaldehyde was converted into the lutidine derivative, which was separated by thin-layer chromatography. The yellow derivative ( $R_F$  0.50) co-chromatographed with an authentic sample of 3,5-diacetyl-1,4-dihydro-2,6-lutidine. Exposure of the chromatographic plate to u.v. light discharged the colour of the derivative, thus eliminating quenching during radioactive counting. The major radioactive zone ( $R_F$  0.50) accounted for ~75% of the total radioactivity. Therefore, it appears that at least 75% of the D-galactose residues which had been oxidised in the galactan were furanoid, and can be accounted for in the production of 6-aldehydo-D-hexofuranosyl residues. This result provides additional evidence for the presence of D-galactofuranose residues in the polysaccharide.

The glycoprotein from *Pithomyces chartarum* was subjected to the same investigations. Treatment with D-galactose oxidase gave results identical to those obtained for the galactan (Fig. 1). Mild, acidic hydrolysis of the enzyme-oxidised and sodium borotritide-reduced glycoprotein released 80% of the total incorporated radioactivity, and this co-chromatographed (paper, solvent A) with D-galactose. After oxidation of the glycoprotein with D-galactose oxidase, the yield of formaldehyde released by periodate oxidation decreased to 65%, and the yield of D-galactose released on mild hydrolysis with acid decreased to 61% (Table I). Thus it appears that ~35% of the D-galactose residues were oxidised by the enzyme. This result is in agreement with previous reports on the oxidation of only 30% of the D-galactopyranose residues in the galactoglucomannan from *Picea engelmann*<sup>7</sup>.

TABLE I

RELEASE OF FORMALDEHYDE (PERIODATE OXIDATION) AND D-GALACTOSE (MILD, ACID HYDROLYSIS) FROM ALIQUOTS OF GLYCOPROTEIN FRACTIONS

Sample	Formaldehyde $\mu\text{mol/mg}$ (%)	D-Galactose $\mu\text{mol/mg}$ (%)
W1	0.67 (100)	0.87 (100)
gW1 <sup>a</sup>	0.44 (65)	0.53 (61)
aW1 <sup>b</sup>	0.044 (65)	—

<sup>a</sup>Glycoprotein oxidised by D-galactose oxidase for 6 h using standard conditions. <sup>b</sup>Glycoprotein treated with 0.01M HCl at 100° for 1 h.

The major portion of the radioactivity released by periodate oxidation of the glycoprotein was shown, after reaction of formaldehyde with 2,4-pentanedione, to have the same mobility (t.l.c.) as 3,5-diacetyl-1,4-dihydro-2,6-lutidine.

Since the enzyme was unable to oxidise either D-galactofuranosylglycerol<sup>13</sup> or methyl  $\alpha$ - or  $\beta$ -D-galactofuranoside, but did oxidise *Salmonella* T1 lipopolysaccharide, which contains terminal D-galactofuranosyl residues<sup>22</sup>, it appears that the affinity of the enzyme is towards substrates of large molecular weight.

It is not possible to comment on the homogeneity of the enzyme preparation, and no conclusions can be made as to whether D-galactose oxidase is an enzyme having a low specificity towards substrates of differing configuration, or whether distinct enzymes exist for the oxidation of D-galactopyranosyl and D-galactofuranosyl residues.

It appears that, in the two cases cited, the mode of action of D-galactose oxidase is in oxidising the C-6 hydroxymethyl group of D-galactofuranosyl residues to an aldehyde group. This result does not necessarily invalidate the previous reports on the use of this enzyme, in conjunction with sodium borotritide, for the incorporation of a label at C-6 of terminal D-galactopyranose residues, but indicates the possibility of oxidation of more than one type of D-galactose residue. In addition, the identification of the radioactive lutidine derivative from the periodate oxidation of a galactan-*t* produced by using this procedure can give confirmatory evidence for the existence of D-galactofuranosyl end-groups in the molecule.

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