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# PREPARATION AND CHARACTERIZATION OF β-D-FRUCTOFURANOSYL *O*-(α-D-GALACTOPYRANOSYL URONIC ACID)-(1→6)-*O*-α-D-GLUCOPYRANOSIDE AND *O*-(α-D-GALACTOPYRANOSYL URONIC ACID)-(1→6)-D-GLUCOSE*

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## ABSTRACT

β-D-Fructofuranosyl *O*-(α-D-galactopyranosyl uronic acid)-(1→6)-*O*-α-D-glucopyranoside (**3**) was prepared by treating raffinose with D-galactose oxidase, followed by hypoiodite oxidation of the resulting aldehyde. Mild acid hydrolysis of **3** gave *O*-(α-D-galactopyranosyl uronic acid)-(1→6)-D-glucose.

## INTRODUCTION

Affinity gels for the isolation of D-glucose-binding proteins have been prepared by coupling maltose[1] and cellobiose[2] *via* their reducing ends to various stationary phases. However, these gels are unsuitable for purifying those D-glucose-binding proteins which require the OH-1 group of D-glucose for recognition and binding. D-Glucose-transport proteins of animal cells are probably in this category as there is evidence that the OH-1 and OH-3 groups of D-glucose are involved in the interaction with the transporter[3]. Currently, there is no method available for coupling D-glucose saccharides to matrices in such a way that the reducing OH-1 group remains free. The present work reports the preparation of two affinity ligands, raffinuronic acid (**3**) and melibiouronic acid (**4**). Compound **3** was prepared by treating raffinose (**1**) with D-galactose oxidase, followed by hypoiodite oxidation of the resulting aldehyde. Melibiouronic acid (**4**) was obtained by mild acid hydrolysis of **3**. Coupling of **4** and **3** *via* the carboxyl group to appropriate supports (for example, to AH–Sepharose by the carbodiimide-catalyzed amide formation) would yield gels containing D-glucose (with a free reducing end) and sucrose units, respectively, which could be used for purifying the respective binding proteins (*viz.*, the glucose-transporter and sucrose-taste receptors).

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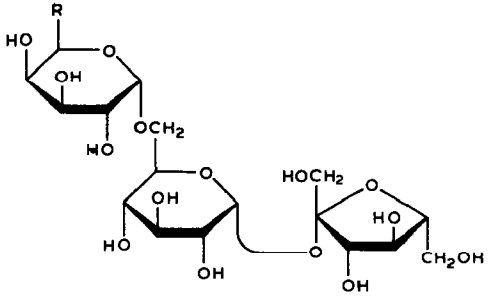
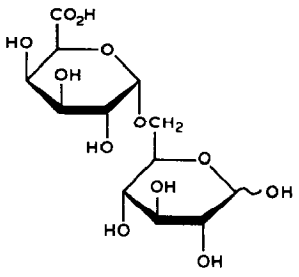
## RESULTS AND DISCUSSION

The published procedures for the oxidation of sugars by D-galactose oxidase use very large proportions of the enzymes[4,5]. In order to maximize the oxidation of raffinose (**1**) without using an excess of enzymes, the level of D-galactose oxidase was decreased 16-fold, and several trial experiments were carried out. It is well established that D-galactose oxidase is inactivated by one of its products, *i.e.*, hydrogen peroxide[6]. Therefore, the effect of adding either catalase or peroxidase as the peroxide-destroying enzyme was tested. In view of the report[6] that at room temperature D-galactose oxidase loses 10% of its activity within 8 h, the effect of adding the enzymes in one lot at the beginning of the reaction or in five equal aliquots at 24-h intervals was compared. The generation of the product (6″-*aldehydo* form of **1, 2**) was monitored by Dische's original carbazole–sulfuric acid assay[7]. In comparison to the modified carbazole assay[8] for uronic acids, the original assay distinguishes between galactose, galactodialdose, and galacturonic acid derivatives. Galactodialdose yields a greenish-brown chromogen having an absorption maximum at 550 nm and galacturonic acid a pink chromogen having an absorption maximum at 525 nm[9].



1 R = $CH_2OH$
2 R = CHO
3 R = $CO_2H$



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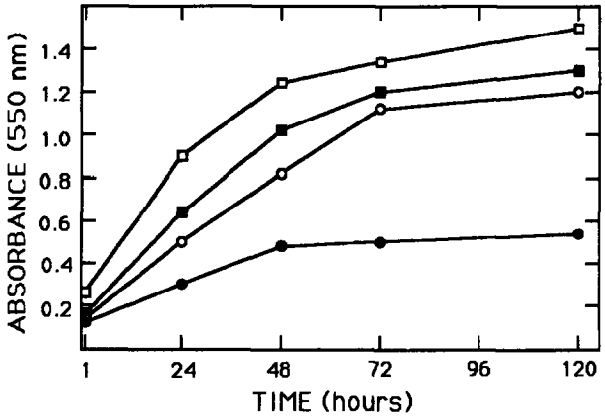


Fig. 1. Time course of the oxidation of raffinose (**1**) by D-galactose oxidase. D-Galactose oxidase (120 units) and catalase or peroxidase (1560 units each) were incubated with **1** (59.4 mg) in 20mM phosphate buffer (pH 7.0, 5.9 mL). After the addition of enzymes, the flask was flushed with oxygen, stoppered, and shaken at 30°. Aliquots of the incubation mixtures were removed at the indicated times and analyzed for aldehyde formation by Dische's original carbazole reaction[7]: (—■—■—) catalase was used, (—●—●—) peroxidase was used, (—○—○—) enzymes were added at 24-h intervals, and (—□—□—) concentration of D-galactose oxidase was doubled.

TABLE I

CONVERSION OF RAFFINOSE (**1**) INTO RAFFINURONIC ACID (**3**)

| *Preparation* | *$H_2O_2$-destroying enzyme* | *Amount of* **1** *used (mg)* | *Yield of* **3** | |
|---|---|---|---|---|
| | | | *mg* | *%* |
| 1 | Peroxidase | 47.2 | 16.9 | 35 |
| 2 | Peroxidase | 92.9 | 31.6 | 34 |
| 3 | Catalase | 59.4 | 31.0 | 52 |
| 4 | Peroxidase | 59.4 | 19.4 | 33 |
| 5 | Catalase | 178.4 | 95.2 | 53 |

The conversion of raffinose (**1**) into the 6″-*aldehydo* form (**2**) was greater in the presence of catalase than peroxidase (Fig. 1). Even though peroxidase has been used to promote decomposition of hydrogen peroxide[5], this enzyme requires a reduced acceptor (for example, 4,4′-diamino-3,3′-dimethoxybiphenyl) as a co-substrate (Galactostat kits are based on a coupled peroxidase–chromogen reaction). It is also evident that the rate of oxidation slows down after ~72 h. In one experiment, it was found that doubling the concentration of D-galactose oxidase improved the yield of oxidized **1** by ~25% (Fig. 1). However, in view of the high cost of this enzyme, the increase in yield was not considered economical. Addition of the enzymes at 24-h intervals did not increase the amount of sugar oxidized and so, in all subsequent incubations, the enzymes were added in one lot.

In preparative experiments, **1** was treated for 96 h with D-galactose oxidase

and catalase, and the product was directly oxidized with hypoiodite. Raffinuronic acid (**3**) was then purified by ion-exchange chromatography. The yield of **3** was in the range of 50–55% (Table I). The step limiting the yield of **3** was the D-galactose oxidase treatment, as hypoiodite oxidation resulted in the quantitative conversion of **2** into **3** (not shown). In preparative experiments where peroxidase was used instead of catalase, the yield of **3** was lower (33–35%) (see Table I), thus confirming the results obtained in the preliminary experiments.

A portion of purified **3** was chromatographed on a column of Bio-Gel P-2 (Fig. 2). It was eluted as a single peak which was shifted considerably from where **1** is eluted. The abnormal behavior of acidic sugars on the Bio-Gel column is apparently due to charge effects. We found that acidic sugars, such as *N*-acetylneuraminic acid (mol. wt. 309) and *N*-acetylneuraminyllactitol (mol. wt. 635), are eluted considerably later on Bio-Gel P-2, P-4, and P-6 columns than would be expected from their size (Fig. 2). The purified preparation of **3** gave a single spot when chromatographed on paper in solvent *C* and *D* and was detected with the silver nitrate or periodate–benzidine reagents, or Bromocresol Green. In the acidic solvents *A* and *B* and with development times of 14–48 h, both purified **3** as well as **1** gave streaky spots, possibly due to hydrolysis of the ketofuranosyl linkage (Fig. 3). Thin-layer chromatography of **3** also showed a single spot. Colorimetric assays showed that **3** contained an equimolar proportion of D-galacturonic acid and sucrose. From these results, it was clear that no degradation of the trisaccharide had occurred during the isolation procedure. In agreement, acid hydrolysis of **3** (2M

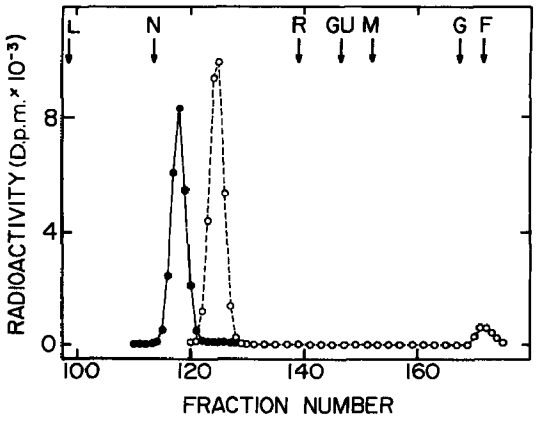


Fig. 2. Gel filtration of **3** before (—●—●—) and after (-O---O-) mild acid hydrolysis (see Experimental). The samples (0.8 mg of **3** or 1.0 mg of hydrolyzate of **3**) were applied to a 3 × 97 cm column of Bio-Gel P-2 (200–400 mesh) and eluted with 0.1M pyridine acetate buffer, pH 5.0. Fractions (2.4 mL) were collected and 0.3-mL aliquots were analyzed for radioactivity. Standards shown by the arrows are: (F) D-fructose, (G) D-glucose, (M) melibiose, (GU) D-galacturonic acid, (R) raffinose (**1**), (N) *N*-acetylneuraminic acid, and (L) *N*-acetylneuraminyllactitol.


Fig. 3. Paper chromatography of hydrolyzates of **3**. Samples of **3** (0.4 mg) were hydrolyzed with 50mM acetic acid (pH 2.9) at 100°. After 1, 2, 3, 4, and 5 h, a sample was removed and examined by paper chromatography, performed on Whatman 3MM paper in solvent *A* for 36 h. The sugars were detected with alkaline silver nitrate: Lane 1, raffinose (**1**) and D-glucose; lane 2, melibiouronic acid (**4**) and D-galactose; lanes 3–7, **3** hydrolyzed for 5, 4, 3, 2, and 1 h, respectively; and lane 8, **3** and D-fructose.

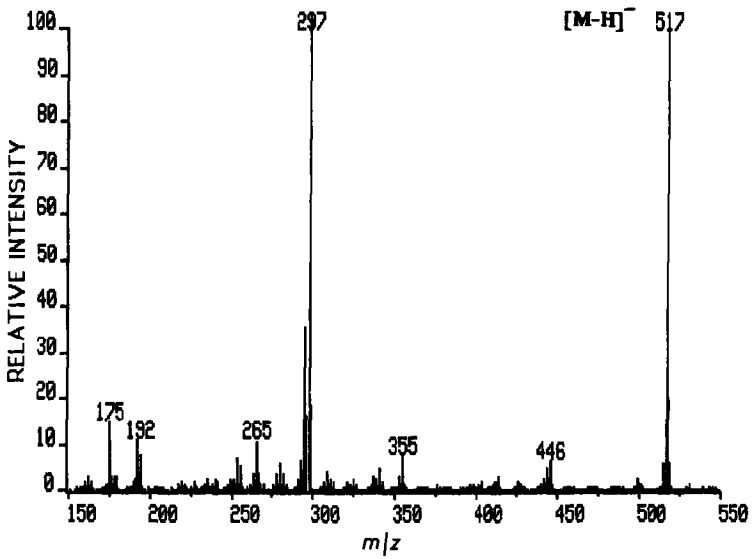


Fig. 4. F.a.b. mass spectrum of **3**. The spectrum was recorded with a ZAB SE mass spectrometer (VG analytical, Manchester, U.K.). The sample was loaded in a triethanolamine matrix and analyzed in the negative ion mode. This experiment was kindly performed by Drs. V. N. Reinhold and S. Santikarn, Dept. of Nutrition, Harvard School of Public Health, Boston, MA.

hydrochloric acid, 8 h), followed by paper chromatography in solvents *A* and *D* showed the presence of D-galacturonic acid but no D-galactose. The conversion of the D-galactopyranosyl into a D-galactopyranosyl uronic group was further confirmed by i.r. spectroscopy; this revealed an absorption in the 1600–1800 $cm^{-1}$ region identical with that of galacturonic acid, a carbonyl (C=O) stretching band at ~1725 $cm^{-1}$, characteristic of carboxyl groups. The f.a.b. mass spectrum of **3** (Fig. 4) showed the molecular ion, $(M - H)^-$ at *m/z* 517, giving rise to a series of ions specific for the proposed structure. The ion at *m/z* 355 was formed by the elimination of the D-fructose residue, and that at *m/z* 175 by the elimination of sucrose. These results and the absence of ions at *m/z* 501 or 503 clearly indicated the conversion of raffinose into **3** (the ions at *m/z* 295–298 are matrix ions).

For the quantitative conversion of **3** into melibiouronic acid (**4**) and D-fructose, both enzymic and acid hydrolytic conditions were tested. Sucrose, raffinose, and **3** were treated with invertase as described by Gascon and Lampen[10]. Invertase, which effectively hydrolyzed sucrose, was found to act poorly on raffinose and **3** (data not presented). The ketofuranosyl linkage is cleaved very readily with mild acid as compared to the aldopyranosyl and aldopyranosyl uronic acid linkages. The rate of hydrolysis of sucrose, raffinose, and **3** by 50mM acetic acid at 65 and 100° was monitored by measuring the increase in reducing sugars by the Park–Johnson assay[11]. The results indicated that the furanosyl bond in all three oligosaccharides was completely hydrolyzed at 100° within 3 h (data not presented). Examination of the products of the hydrolysis of **3** by paper chromatography (Fig. 3) showed no evidence for the release of melibiose, D-glucose, or D-galacturonic acid during this acid treatment. This supports the complete conversion of all the D-galactosyl into D-galactosyl uronic acid groups and the stability of the released melibiouronic acid to further degradation. A portion of the hydrolyzate was chromatographed on a Bio-Gel P-2 column (Fig. 2). The material that was eluted about seven fractions after that of **3** accounted for 70% by weight of the trisaccharide and was characterized as melibiouronic acid (**4**). The second peak, which co-emerged with monosaccharide standards, consisted of D-fructose and accounted for 30% by weight of **3**. No material was eluted at the trisaccharide position. Further, the only carbazole-positive peak detected was that of **4**, thus proving the absence of **3** and D-galacturonic acid. Thus, all of **3** was hydrolyzed to **4** and D-fructose with no destruction of the monosaccharides, a result in agreement with the paper chromatography data. Therefore, for the preparative isolation of **4**, **3** was hydrolyzed with 50mM acetic acid at 100° for 3 h, and the hydrolyzate applied to an anion-exchange column. In four experiments melibiouronic acid (**4**) was obtained with yields of 88, 97, 84, and 101%. Colorimetric analysis of the product for D-fructose was negative, indicating the absence of unhydrolyzed **3** in the final preparations. T.l.c., monitored by detection with sulfuric acid and paper chromatography in solvents *A*, *B*, *C*, and *D*, followed by staining with silver nitrate or Bromocresol Green, revealed single spots, confirming the purity of the preparation of **4**.

The direct oxidation of the *6''-aldehydo* compound **2** to **3** prevents or minimizes the formation of artifacts by side reactions, such as polymerization of the reactive aldehyde during isolation or subsequent storage. However, in one experiment the D-galactose oxidase-reaction mixture was directly chromatographed on a column of Bio-Gel P-2 to yield a product consisting of **2** and unoxidized **3**, but free of enzymes and buffer salts. Compound **2** was then coupled to AH–Sepharose by reductive amination in the presence of sodium cyanoborohydride[12]. This approach can be used to prepare gels containing sucrose components. However, it is not suitable for obtaining gels containing D-glucose with a free reducing group as subsequent mild acid hydrolysis to remove D-fructose appears to affect the structure of the Sepharose beads.

The same set of reactions described for **1** may be used to convert stachyose into the corresponding uronic acid derivative. Higher yields may be expected in this conversion as stachyose is oxidized[9] by D-galactose oxidase ~3.4-fold more rapidly than is **1**.

## EXPERIMENTAL

*Materials.* — D-Galactose oxidase (EC 1.1.3.9), peroxidase (Type 1) (EC 1.11.1.7), catalase (from bovine liver) (EC 1.11.1.6), and invertase (Grade VII) (EC 3.2.1.26) were purchased from Sigma Chemical Co. (St. Louis, MO). Raffinose was obtained from Eastman Kodak Co. (Rochester, NY). [$^3$H]Raffinose (290 GBq/mmol) was obtained from New England Nuclear (Boston, MA). Bio-Gel P-2, AG 50W-X8 cation-exchange resin, and AG 1-X8 anion-exchange resin were purchased from Bio-Rad Laboratories (Richmond, CA).

*Analytical methods.* — I.r. spectra were recorded with a Perkin–Elmer grating infrared spectrophotometer model 267 on vacuum-dried samples (0.5–1.0 mg), mixed with i.r.-grade KBr (200 mg) and pressed into a pellet. Uronic acid content was determined by the modified carbazole reaction[8] with D-galacturonic acid as the standard. The sucrose content in **3** was determined by the cysteine–carbazole reaction[13] for ketohexoses with sucrose as the standard. Paper chromatograms were developed by the descending technique on Whatman No. 1 or 3 MM paper; the following solvent systems (v/v) were used: (*A*) 18:3:1:4, ethyl acetate–acetic acid–formic acid–water, (*B*) 9:3:1:4, ethyl acetate–acetic acid–formic acid–water, (*C*) 10:4:3, ethyl acetate–pyridine–water, and (*D*) 5:5:1:3, ethyl acetate–pyridine–acetic acid–water. The sugars were detected with the alkaline $AgNO_3$ or periodate–benzidine reagents, and uronic acids also by reaction with Bromocresol Green. T.l.c. was done on Redi-coat-HK (silica gel with 0.3M $KH_2PO_4$) plates obtained from Supelco, Inc. (Bellefonte, PA). The plates were developed in paper-lined tanks by the ascending technique in 4:5:2, (v/v) butanol–acetone–water solvent system. After the solvent had reached the top, the plates were removed from the tank and air dried. This process was repeated two more times and the sugars were detected by charring with 50% $H_2SO_4$ at 120°. Liquid-

scintillation counting was performed with an Intertechnique model SL-4000 spectrometer, equipped with a d.p.m.-calculating module. Aqueous samples (0.3 or 1.0 mL) were mixed with ACS II counting scintillant (3 or 10 mL; Amersham Corp., Arlington Heights, IL) in plastic vials.

*Treatment of raffinose with* D-*galactose oxidase.* — Five separate preparative experiments were carried out. In some experiments, [$^3$H]raffinose was included for easy monitoring of the products. Details of one typical experiment are as follows: raffinose (**1**; 180 mg) and [$^3$H]raffinose (370 KBq) were dissolved in 20mM sodium phosphate buffer (12 mL), pH 7.0. D-Galactose oxidase (720 $\mu$L, 100 U/mL) in 20mM sodium phosphate buffer (pH 7.0) and catalase (470 $\mu$L, 996 U/mL) in 20mM sodium phosphate buffer (pH 7.0) were added to the raffinose solution at 0, 24, 48, 72, and 96 h. Thus, a total of 360 units of D-galactose oxidase and 2340 units of catalase were utilized. After each addition of enzymes, the flask was flushed with $O_2$, stoppered, and shaken at 30°. Alternatively, the total amount of the enzymes was added at the beginning of the reaction or peroxidase was used instead of catalase.

*Hypoiodite oxidation of compound* **2**. — In most experiments, compound **2** was not purified but was directly oxidized to the uronic acid derivative by a modification of the method of Schaffer and Isbell[14] as follows: A 100mM solution of $I_2$ in 0.25M KI (1.2 mL) was added to a stirred solution of D-galactose oxidase- and catalase-treated **1** (180 mg) in 20mM sodium phosphate buffer (18 mL), pH 7.0, followed by dropwise addition of 0.1M NaOH (1.8 mL). The addition of $I_2$ and NaOH was repeated four times. The oxidation was allowed to proceed at room temperature for a total of 20 min.

*Chromatographic purification of* **3**. — The mixture containing the oxidized raffinose was cooled in an ice bath and passed through a column containing AG 50W-X8 ($H^+$) cation-exchange resin (20 mL) at 4° in order to remove $Na^+$ and $K^+$. The effluent was collected in a stirred, aqueous slurry of $Ag_2CO_3$ (0.6 g) to precipitate $I^-$ and $PO_4^{3+}$. The solution was centrifuged and the supernatant, after being filtered through filter paper, was passed through a column packed with AG 50W-X8 ($H^+$) cation-exchange resin (10 mL) to remove excess $Ag^+$. The effluent was made neutral by the dropwise addition of M NaOH and applied to a column containing AG 1-X8 ($HCO_2^-$) anion-exchange resin (5 mL). The column was eluted with water (50 mL) to remove any unoxidized **1**. The bound compound **3** was eluted with M formic acid and the effluent was collected in 1-mL fractions. The fractions were assayed for radioactivity and for uronic acid. The positive fractions were pooled and lyophilized to yield 95 mg of product. Some preparations contained a u.v.-absorbing material which apparently was a contaminant arising from the enzymes. In order to remove this impurity, the lyophilized sugar was dissolved in water (3 mL) and applied to a Bio-Gel P-2 (200–400 mesh) column (3 × 97 cm). The column was eluted with water, and 3-mL fractions were collected and assayed for radioactivity and absorbance at 280 nm. The fractions free of the u.v.-absorbing material but containing radioactivity were pooled and lyophilized.

*Mild acid hydrolysis of* **3**. — A solution of raffinuronic acid (**3**) (40 mg/mL) in 50mM acetic acid (pH 2.9) was heated at 100° for 3 h. The hydrolyzate was lyophilized, the residue dissolved in water, the pH adjusted to 7.0, and the solution applied to a column of AG 1-X8 ($HCO_2^-$) anion-exchange resin. The water wash containing D-fructose was collected in bulk and lyophilized. Melibiouronic acid (**4**) was eluted with M formic acid and fractions (1 mL) were collected. The fractions containing uronic acid and radioactivity were pooled and lyophilized to recover **4**.

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