Carbohydrate Research 344 (2009) 14–20

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Oxidation of methyl α-D-galactopyranoside by galactose oxidase: products formed and optimization of reaction conditions for production of aldehyde

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ARTICLE INFO

Article history:
Received 19 June 2008
Received in revised form 3 August 2008
Accepted 18 August 2008
Available online 24 August 2008

Keywords:
Galactose oxidase
Enzymatic oxidation
Methyl α-D-galactopyranoside
Methyl α-D-galacto-hexodialdo-1,5-pyranoside
Methyl 4-deoxy-α-D-*threo*-hex-4-enodialdo-1,5-pyranoside
D-Raffinose

ABSTRACT

The reaction conditions of galactose oxidase-catalyzed, targeted C-6 oxidation of galactose derivatives were optimized for aldehyde production and to minimize the formation of secondary products. Galactose oxidase, produced in transgenic *Pichia pastoris* carrying the galactose oxidase gene from *Fusarium* spp., was used as catalyst, methyl α-D-galactopyranoside as substrate, and reaction medium, temperature, concentration, and combinations of galactose oxidase, catalase, and horseradish peroxidase were used as variables. The reactions were followed by ^{1}H NMR spectroscopy and the main products isolated, characterized, and identified. An optimal combination of all the three enzymes gave aldehyde (methyl α-D-galacto-hexodialdo-1,5-pyranoside) in approximately 90% yield with a substrate concentration of 70 mM in water at 4 °C using air as oxygen source. Oxygen flushing of the reaction mixture was not necessary. The aldehyde existed as a hydrate in water. The main secondary products, a uronic acid (methyl α-D-galactopyranosiduronic acid) and an α,β-unsaturated aldehyde (methyl 4-deoxy-α-D-*threo*-hex-4-enodialdo-1,5-pyranoside), were observed for the first time to form in parallel. Formation of uronic acid seemed to be the result of impurities in the galactose oxidase preparation. ^{1}H and ^{13}C NMR data of the products are reported for the α,β-unsaturated aldehyde for the first time, and chemical shifts in DMSO-d_6 for all the products for the first time. Oxidation of D-raffinose (α-D-galactopyranosyl-(1-6)-α-D-glucopyranosyl-(1-2)-β-D-fructofuranoside) in the same optimum conditions also proceeded well, resulting in approximately 90% yield of the corresponding aldehyde.

1. Introduction

Galactose oxidase (GO, EC 1.1.3.9) is a single copper metalloenzyme that catalyses the oxidation of primary alcohols to corresponding aldehydes with strict regioselectivity.[1,2] The 65–68 kDa enzyme is secreted by fungus *Fusarium* spp. The corresponding gene has been isolated and expressed in *Aspergillus nidulans*,[3] *Pichia pastoris*,[4–6] and *Escherichia coli*[7] to improve the enzyme production. The three-dimensional structure revealing insight to the catalytic site has been solved.[3,8] However, the biological function of GO is unknown. In addition to galactose and various galactose-containing carbohydrates, alcohols with primary hydroxyl group, such as glycerol, salicyl alcohol, 1,3-propanediol, and xylitol, have been reported as substrates of GO.[9–11] The catalytic reaction of GO comprises oxidative and reductive half-reactions, using molecular oxygen as an electron acceptor and producing hydrogen peroxide. During these reactions, the enzyme alters between three different forms: an active, inactive, and fully reduced form. The active site of GO contains a tyrosine residue and a copper atom. In the active form of GO, the tyrosine is in a radical form and the copper atom at oxidation state +2.[1] Peroxidases are reported to enhance the action of GO by oxidizing the inactive form to the active radical form.[6,12] High concentrations of hydrogen peroxide are reported to inactivate GO and thus the presence of catalase, breaking down hydrogen peroxide, enhances the action of GO.[13]

The regioselectivity of GO is high for the galactose hydroxyl group at C-6. Thus various analytical techniques are based on GO. For example, lactose concentration of dairy products[14] and presence of glycoproteins in biomaterials[15] have been determined using GO biosensors. The C-6 oxidized galactose derivatives are valuable starting materials in various chemical conversions, and their production with GO is favorable compared to chemical catalysis. Protecting groups are not needed, and the reactions are performed in aqueous solutions. Oxidized galactose (galacto-hexodialdose) and lactose are, for example, potential protein cross-linkers, demonstrated by reaction with butylamine and by Amadori rearrangement of the product.[16,17] Immunoactive *N*-acetyl-D-galactosaminides have been synthesized starting from aldehydes obtained by GO-catalyzed oxidation.[18] The reaction of galacto-hexodialdopyranosides with formaldehyde gives metabolism-resistant 5-*C*-(hydroxymethyl)hexoses.[19] The GO-catalyzed reaction works as well when applied to galactose-containing polymers. Oxidized guar gum has potential applications in the paper industry, and this shows the possibilities for controlled chemical modification of galactomannans.[6,12,20]

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doi:10.1016/j.carres.2008.08.020

However, in addition to aldehydes, several side products have been reported to form in the oxidation with GO. The origin of these products is not clear. A dimeric product and an α,β-unsaturated aldehyde have been identified in the oxidation of methyl β-D-galactopyranoside.[21,22] Both were found after acetylation,[21,22] and the unsaturated aldehyde was also suggested to form in the reaction.[21] Oxidations of mono- and oligosaccharides are reported to produce the corresponding uronic acids.[23–25] In these studies, both low (2 U/mg)[23,25] and high (100 U/mg)[24] amounts of GO per mg of substrate produced uronic acids after varying reaction times, with yields as high as 90%.[24] In one case, up to 27% of oxidation product consisted of various unidentified components, which appeared when aqueous solution was allowed to stand for some time.[26]

In this work, GO-catalyzed oxidation was studied with the aim of controlling the degree of reaction, optimizing aldehyde production, and minimizing the formation of side products. The focus was especially in the use of a crude GO preparation produced by transgenic *P. pastoris*, having potential for industrial use. Methyl α-D-galactopyranoside was chosen as a substrate as it can be used as a model compound for carbohydrates having α-bonded galactosyl units (e.g., galactomannans). Reaction medium, temperature, substrate, and enzyme concentrations, and selected combinations of GO, catalase, and horseradish peroxidase (HRP) were used as variables in the reactions. Oxidation was followed by ^{1}H NMR spectroscopy and the main products were isolated, characterized, and identified. To ensure adequacy of the reaction for other galactose derivatives, the optimized conditions were tested with D-raffinose, which was oxidized to a corresponding aldehyde.

Table 1
Assignments of ^{1}H chemical shifts (ppm) of compounds **1**–**4** determined by 1D and 2D NMR experiments recorded at 300 MHz or 500 MHz in D_2O or DMSO-d_6 at 27 °C

Compound	H-1	H-2	H-3	H-4	H-5	H-6	–OMe
1 (D_2O)[b]	4.83	3.81	3.80	3.96	3.89	3.73–3.74	3.40
2 (D_2O)[b,c]	4.84	3.82	3.80	4.11	3.59	5.11	3.42
3 (D_2O)[b]	4.85	3.85	3.84	4.21	4.26	—	3.40
4 (D_2O)[b]	5.13	3.88	4.47	6.16	—	9.20	3.53
2 (DMSO-d_6)[a]	4.69	3.61	3.61	3.60	4.12	9.50	3.28
3 (DMSO-d_6)[a]	4.56	3.59	3.59	3.49	3.87	—	3.25
4 (DMSO-d_6)[b]	4.89	3.53	4.19	5.98	—	9.19	3.35

The peaks are referenced to internal acetone (D_2O) or the residual solvent peak at 2.50 ppm (DMSO-d_6).
[a] Recorded at 300 MHz.
[b] Recorded at 500 MHz.
[c] Compound **2** as a hydrate.

Table 2
Assignments of ^{13}C chemical shifts of compounds **1**–**4** determined by 1D and 2D NMR experiments recorded at 300 MHz or 500 MHz in D_2O or DMSO-d_6 at 27 °C

Compound	C-1	C-2	C-3	C-4	C-5	C-6	–OMe
1 (D_2O)[a]	100.0	68.4	69.8	69.5	71.0	61.5	55.3
2 (D_2O)[a,c]	102.2	70.8	72.1	71.4	75.2	91.2	57.9
3 (D_2O)[a,d]	99.9		68.4, 70.1, 71.3, 71.8			182.0	55.9
4 (D_2O)[a]	100.9	69.9	65.9	124.4	148.0	190.0	57.1
2 (DMSO-d_6)[a,d]	100.5		67.9, 68.8, 69.2, 76.0			200.5	55.1
3 (DMSO-d_6)[a,d]	100.1		68.3, 69.7, 70.3, 71.9			173.8	54.5
4 (DMSO-d_6)[b]	101.1	70.3	65.5	125.8	147.7	187.6	56.2

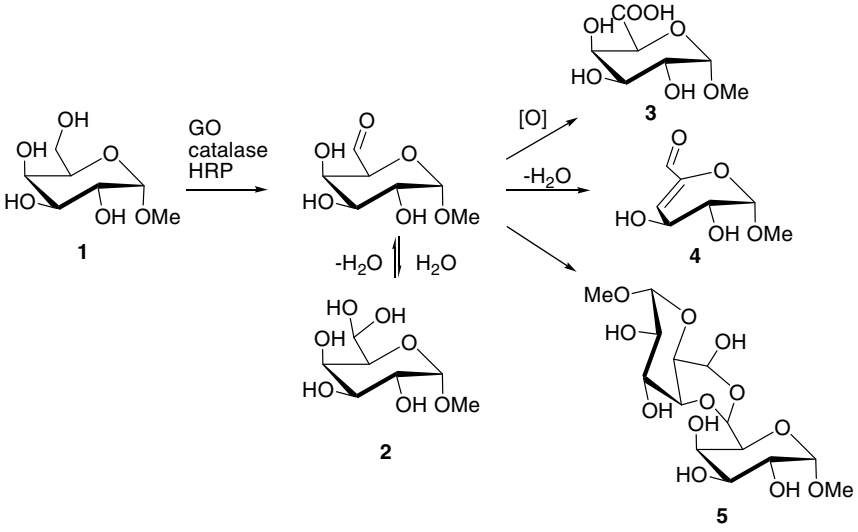
The peaks are referenced to internal acetone (D_2O) or the residual solvent peak at 39.5 ppm (DMSO-d_6).
[a] Recorded at 300 MHz.
[b] Recorded at 500 MHz.
[c] Compound **2** as a hydrate.
[d] No 2D spectra used in the assignment.

2. Results

2.1. The formation and structure of main and secondary products

The reaction conditions chosen for preliminary experiments were combined from the conditions reported earlier. Phosphate buffer, ranging from pH 6 to pH 7.3, and H_2O have been used as reaction media in GO-catalyzed reactions, and thus both were tested. Reaction temperatures studied were between 4 and 40 °C, as previously used for GO-catalyzed reactions. The reactions were first followed with thin-layer chromatography (TLC). Later on, ^{1}H NMR was found to be very useful as the formation of all of the products could be directly observed from the spectra, and their amounts estimated from the integrals of the proton signals.

The preliminary experiments showed one main product and several secondary products in the reaction mixture depending on the reaction conditions and, for example, duration of storage. The three most stable products were isolated by preparative TLC, and characterized by NMR (Tables 1 and 2) and mass spectroscopy. The main product of GO-catalyzed oxidation of methyl α-D-galac-



Scheme 1. Oxidation of methyl α-D-galactopyranoside and formation of secondary products. Aldehyde **2** occurs as a hydrate in H_2O. GO = galactose oxidase, HRP = horseradish peroxidase.

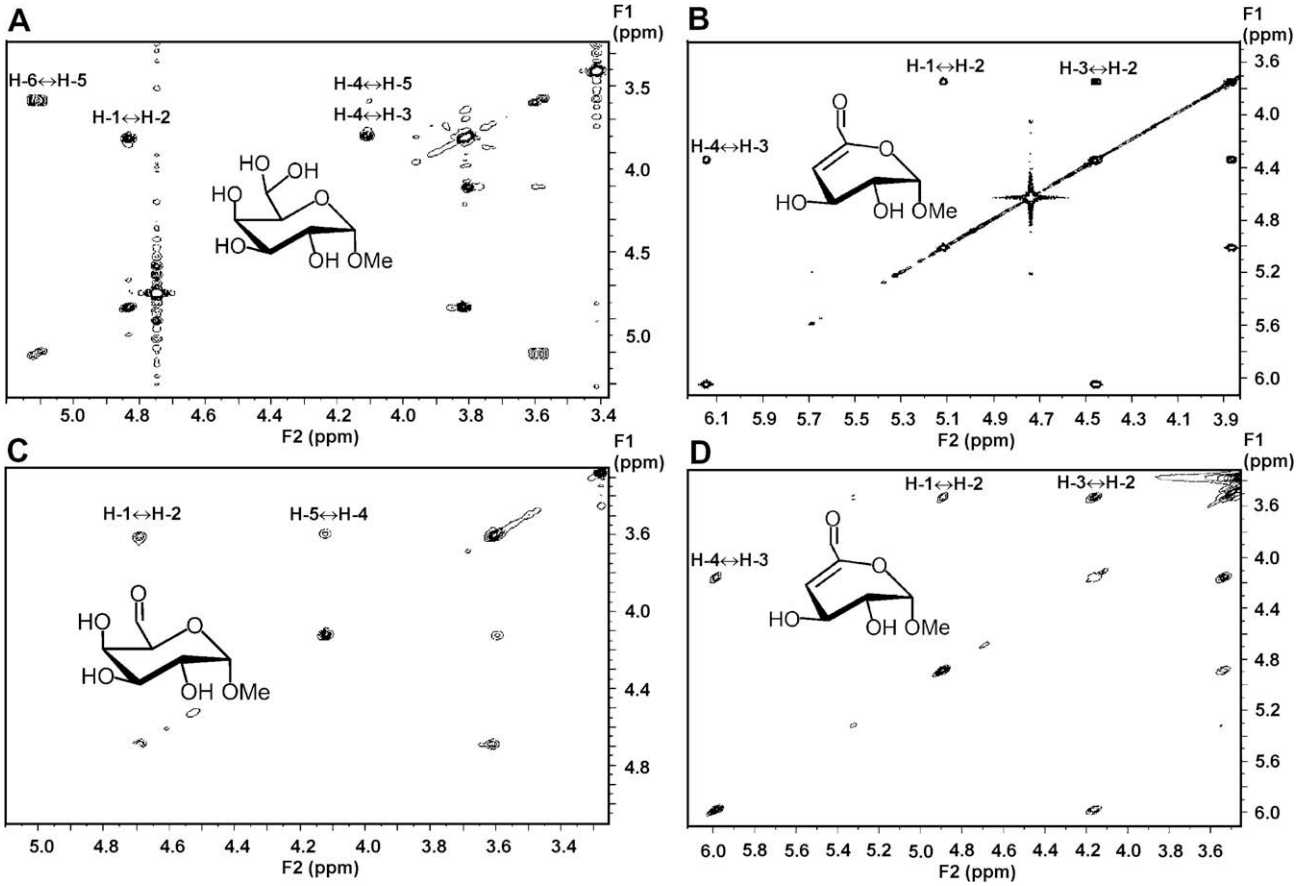


Figure 1. 2D COSY of **2** (A, C) and **4** (B, D). A and B: D_2O as a solvent; C and D: DMSO-d_6 as a solvent.

topyranoside **1** was clearly an aldehyde **2** (Scheme 1). As expected, **2** existed as a hydrate in D_2O, and NMR analysis of products in DMSO-d_6 confined the structure of **2**. The assignment of chemical shifts of the products in DMSO-d_6 might prove to be useful if more complex α-galactose derivatives were oxidized and analyzed, as in D_2O the chemical shift of hydrate H-6 is near the chemical shifts of anomeric protons. Two-dimensional NMR analyses were used in the assignment (examples of COSY spectra are shown in Fig. 1).

The main side products were found to be a uronic acid **3** and an α,β-unsaturated aldehyde **4**. A hemiacetal dimer **5** (Scheme 1), with a structure corresponding to an acetylated dimer reported earlier,[14,15] was suggested to exist in the reaction mixture based on the ^{1}H NMR and 2D COSY and TOCSY experiments of a mixture of **2** and secondary products. Mass spectra supported this, but the observed sodium adduct (m/z 407.3) could also have arisen from dimerization of **2** during ionization, which happened, for example, also to compound **3** ($[M+M+Na]^+$ adduct). According to the mass spectra, a low concentration of another hemiacetal, formed of **2** and **4**, may also have been present in the mixture (m/z $[M+Na]^+$ 389.3).

2.2. Following of the oxidation and optimization of reaction conditions

The formation of **2** and the secondary products was followed with ^{1}H NMR (D_2O as the solvent). As shown in Table 1, the chemical shifts of H-4, H-5, and H-6 of **2** differed from the chemical shifts of **1**. Of these three protons, H-5 provided the best distinction of the secondary products as well. Thus the relations of the integrals of H-5 of **2** and H-4 of **1**, which also differs relatively well from the other chemical shifts, were used in the calculation of the conversion (Fig. 2). The formation of **3** was observed from the appearance of its H-4 or H-5 at δ 4.21 or 4.26, respectively. Compound **4** did not form a hydrate in aqueous solution, thus being easily distinguished from the other compounds by way of H-6, in addition to the olefinic H-4.

The experiments resulted in good yield of **2** when all the three enzymes, GO (0.052 U/mg of substrate), catalase (112 U/mg), and

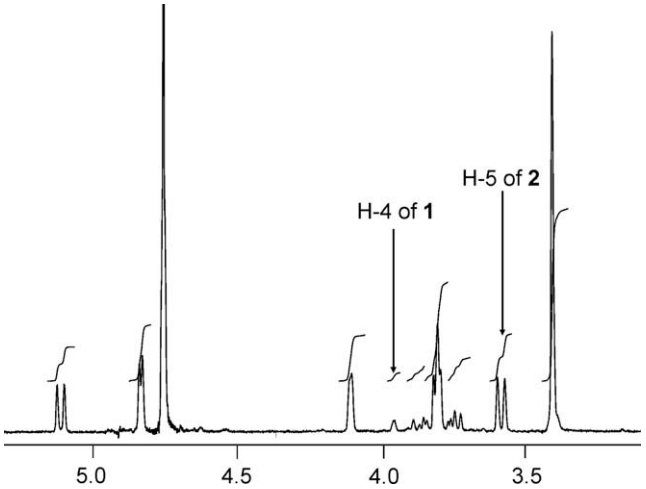


Figure 2. ^{1}H NMR spectrum of the reaction mixture was recorded at 300 MHz in D_2O at 25 °C after 5 h. The reaction was performed at 4 °C in water consisting of substrate **1** (13 mg/mL, 70 mM) and a combination of GO (0.052 U/mg), HRP (0.45 U/mg), and catalase (112 U/mg). The peaks are referenced to HDO.

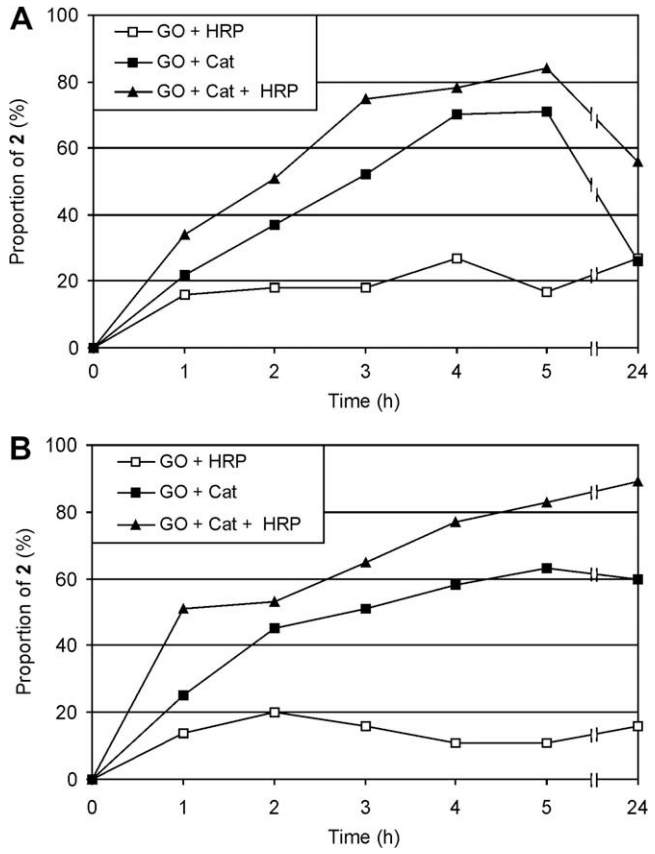


Figure 3. The molar proportion of aldehyde **2** in the reaction mixture after 1–24 h as analyzed by ^{1}H NMR spectroscopy. Reactions were performed at 4 °C in 25 mM sodium phosphate, pH 7, (A) and water (B) using 13 mg/mL (70 mM) substrate **1** and 0.052 U/mg GO, 0.45 U/mg HRP, and 112 U/mg catalase (Cat).

HRP (0.45 U/mg), were used in the oxidation of 70 mM (13 mg/mL) solution of **1** at 4 °C (Fig. 3). The best and almost quantitative yield was obtained after 24 h when the reaction was performed in water. Surprisingly the yield of **2** decreased drastically between 5 and 24 h when the reaction was done in sodium phosphate buffer, pH 7. Until 5 h the reactions proceeded similarly in water and buffer. HRP was needed for the best conversion. A higher amount of HRP (18 U/mg) had no effect on product formation. Catalase was a prerequisite for an efficient reaction, as expected. GO and HRP without catalase gave only 15–25% conversion to **2**. When less (10 U/mg) catalase was used, the reaction was not complete. An excess of catalase (1120 U/mg substrate) accelerated the reaction slightly, giving a 56% conversion to aldehyde after 1 h. The reaction proceeded approximately at the same rate at room temperature and at 4 °C. At 40 °C, oxidation started at a similar rate, but the proportion of **2** in the reaction mixture apparently remained at the same level after 3 h, and was only little higher after 24 h (75%, results not shown).

Table 3
The approximate molar proportion (%) of substrate **1**, aldehyde **2**, uronic acid **3**, elimination product **4**, and dimer **5** in selected reactions performed in 25 mM sodium phosphate buffer, pH 7, or water at 4 °C for 24 h

Reaction medium	Substrate (mg/mL)	GO (U/mg)	**1** (%)	**2** (%)	**3** (%)	**4** (%)	**5** (%)
Buffer pH 7	13	0.052	0–5	55	30 ± 5	—	0–10
Water	13	0.052	0–5	90	—	—	—
Water	13	1.04	—	20 ± 5	50 ± 5	20 ± 5	0–10
Water	93	0.052	70	30	—	—	—

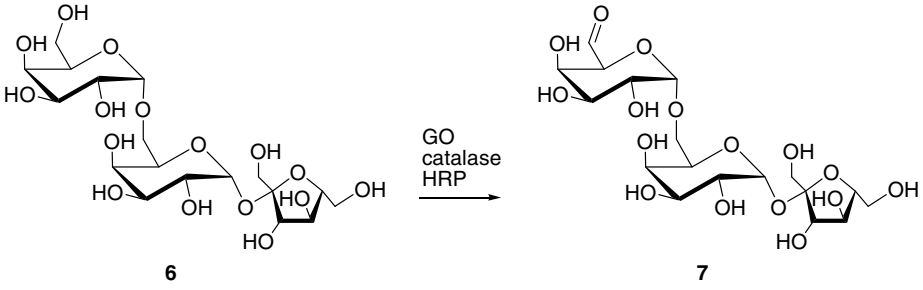
All reactions contained 0.45 U/mg HRP and 112 U/mg catalase (Cat).

Formation of secondary products depended on reaction conditions (Table 3). The presence of a significant amount of uronic acid **3** (Scheme 1) was observed after 24 h in the reactions performed in phosphate buffer, pH 7, and in the reactions performed in water when a higher amount of GO was used. For example, 1.04 U/mg of GO gave ca. 50% yield of **3** after 24 h (Table 3). The formation of other secondary products, such as the α,β-unsaturated aldehyde **4**, seemed to be slow in water too, or did not happen at all, whereas in the buffer, side products appeared more often. The secondary products were also often found after **2** was heated vigorously or stored in a concentrated solution. A decrease of pH was observed during the reactions especially in water, which might in some cases explain the enhanced formation of **4**. The β-hydroxy aldehydes are prone to elimination of water producing α,β-unsaturated aldehydes in acidic or alkaline conditions.

Suitability of the optimized conditions in oxidation of other galactose derivatives was tested with a trisaccharide, D-raffinose (**6**), which in the absence of a reducing end produced relatively simple proton spectra enabling the easy following of the reaction by NMR spectroscopy. Substrate **6** was converted to the corresponding aldehyde **7** in a good yield (Scheme 2; ca. 90% after 24 h) in water using the amounts of enzymes in the same ratio of units/mol of substrate as in the best oxidation of **1**. Samples taken after 1 and 5 h showed that the reaction proceeded at a rate comparable to that of **1**.

3. Discussion

Aldehyde was obtained in good yield in the reactions catalyzed by unpurified GO preparation in the optimized conditions. However, finding the optimal conditions was challenging as several variables, such as enzyme amounts, substrate concentration, reaction medium, and temperature, had effects on the yield and rate of conversion to the aldehyde or to the unwanted side products. The previous studies on GO-catalyzed oxidation have described various reaction conditions and several products have been observed, but



Scheme 2. Oxidation of D-raffinose. GO = galactose oxidase, HRP = horseradish peroxidase.

thorough investigation of the effects of reaction conditions on the compositions of product mixtures and on the relation of the conditions to the products has not previously been reported.

The combination of all the three enzymes, GO, catalase, and HRP, was found optimal for aldehyde production. Previously, the use of HRP has been reported only a few times, for example, in the oxidation of guar gum.[6] In our experiments, its presence and role in activating GO was important as the yield of **2** was 30% better in the presence of HRP than with GO and catalase only. Higher concentrations of catalase or HRP had no effect on the formation of side products. High yields of uronic acid were obtained only when an excess of GO preparation (1.04 U/mg substrate) was used. In contrast, the same and even higher amounts of GO and catalase per mg of substrate have been previously reported to result in an aldehyde. In one case, the absence of uronic acid was explained by the purity of GO used.[19] Our experiments with commercially available, partially purified GO agree that impurities in GO preparation might be the reason for the formation of a carboxylic acid. No uronic acid **3** was present after 24 h with the same, high (1.04 U/mg), or even higher GO:substrate ratio, when the partially purified GO was used.

It was not clear, what was the purity of GO used in the studies that reported high yields of uronic acids.[23–25] Prolonged incubation time such as 48–96 h is one of the proposed explanations for the acid formation.[24,25] We did not observe formation of **3** after such reaction times (e.g., 48 h) in the optimized conditions when water was used as the reaction medium, but when phosphate buffer, pH 7, was used, some carboxylic acid was formed already after 24 h. The impurities in GO preparation, such as enzymes producing uronic acids, might not be active in water, which had lower pH than the phosphate buffer, pH 7.

The formation of the other main side product **4**, or corresponding α,β-unsaturated aldehydes, has not been reported to happen during the GO-catalyzed oxidation, except once, when a minor amount was suggested to form in the reaction.[21] In other cases, α,β-unsaturated aldehydes have been found to form only during the acetylation of reaction products.[22,27] However, the degradation of various hexodialdopyranosides, including **2**, has been studied in phosphate and other buffers at pH 1.5–6.[28,29] At pH 4–6 the main degradation products were the α,β-unsaturated aldehydes, and **2** reacted in the elimination forming **4** considerably faster than the corresponding gluco-hexodialdopyranoside.[29] As **4** and related compounds are secondary products that form easily by water elimination, their formation can be a problem in the GO-catalyzed oxidation or in the following handling and treatments of aldehyde as water elimination is not so readily prevented as the formation of uronic acid.

Dilute substrate concentrations were found to be the best for aldehyde formation. With a higher substrate concentration, such as 0.5 M (93 mg/mL), when the enzyme:substrate ratio was the same as in the best conditions, conversion to aldehyde was much slower, for example, only 30% after 5 h. Scaling up the reaction to 1 g of substrate (70 mM, in water), with the same ratio of enzyme:substrate, worked well.

GO uses molecular oxygen as the electron acceptor. O_2 is converted to H_2O_2 when the enzyme is recycled back to its active form after the oxidation of an alcohol. Previously, oxygen flushing or bubbling have been used in some GO-catalyzed oxidations to ensure sufficient oxygen in the reaction mixture.[16,30] We did not find oxygen flushing necessary, as the reactions proceeded well in open flasks with magnetic stirring, with air as the oxygen source. Furthermore, oxygen is partially recycled back to the system when catalase degrades H_2O_2 formed in the reaction producing molecular oxygen and water.

The solubility of oxygen in water is inversely proportional to temperature. If equilibrium-state O_2 concentration in pure water was considered, there would be almost twice the amount of oxygen available in the reactions performed at 4 °C (ca. 0.40 mM O_2) than at 40 °C (ca. 0.22 mM O_2), and 1.5-fold than at room temperature (ca. 0.29 mM O_2).[31] This might explain why the reaction was not fastest at 40 °C. However, the enhanced enzymatic activity at the elevated temperature might have partly compensated for the loss of oxygen, as there was no significant difference in the rate of the reactions at 4 °C and at rt. The actual O_2 concentration also depends on the efficiency of stirring, which affects the rate of incorporation of new O_2 molecules from air to the reaction medium, and the amount of O_2 produced in situ by catalase. According to our measurements at rt, the amount of dissolved O_2 decreased fast immediately after the addition of GO to the reaction mixture, and remained low (ca. 0.02 mM) until 4–5 h, after which it started to increase, reaching 0.25 mM after 24 h. This was in accordance with the progress of the reaction as the conversion to aldehyde was quite complete after 5 h (ca. 85%).

The aldehyde derivatives of carbohydrates are interesting starting materials for further conversions and because of their possible interactions with other molecules. The corresponding uronic acid or α,β-unsaturated derivatives may not be a source for such a diverse selection of modifications. For example, when interactions between polysaccharides are desired, aldehydes are prone to formation of hemiacetal bonds. Aldehyde groups in a polysaccharide lead to changes in the physical properties, such as viscosity,[32] and improved interactions with cellulose fibers.[6] In addition to the advantages of selectivity, the oxidation with GO in water solutions is mild and does not lead to depolymerization. The substrate should have an unsubstituted hydroxyl group at C-4 to ensure the action of GO, thus potential polymers for GO oxidation contain, for example, 1,6-linked galactose units.

To summarize, aldehydes are obtained in good yields, and the formation of side products is minimized in an oxidation with a combination of GO with both catalase and HRP. In the optimized conditions, the oxidation is controlled and does not require purification of GO. The experiments with D-raffinose showed that the optimized reaction conditions are suitable for oligosaccharides and thus are expected to work well for polysaccharides that are substituted with galactose units with an unsubstituted hydroxyl group at C-4.

4. Experimental

4.1. Enzymes and substrates

Galactose oxidase was produced by *P. pastoris* carrying the gene encoding GO from *Fusarium* spp.[6] The GO preparation was a gift from Hercules (Barneweld, Netherlands) and it was used without further purification. According to the SDS–PAGE it contained one major 65 kDa protein. The protein content was 2.0 mg/mL as analyzed with the Bradford assay (Quick Start Bradford, Bio-Rad). As the activity of the GO preparation was not known, the reported specific activity of a similar preparation was used for the estimation of the GO amounts (26 U/mg).[16] Partially purified GO (G7907, 73 U/mg), methyl α-D-galactopyranoside, D-raffinose (α-D-galactopyranosyl-(1-6)-α-D-glucopyranosyl-(1-2)-β-D-fructofuranoside), horseradish peroxidase (P8250, Type II, 181 U/mg) and catalase (C30, from bovine liver, 22,000 U/mg) were purchased from Aldrich.

4.2. Analytical methods

The reactions were followed using thin-layer chromatography and NMR spectroscopy. The TLC analysis was carried out on Silica Gel 60 plates (Merck) using a mixture of MeOH/CH_2Cl_2/AcOH/H_2O

(10:20:1:1) as an eluent and a sample from the reaction mixture directly applied to the TLC plate. The carbohydrates were detected by spraying with $EtOH/H_2SO_4$, after which the plates were heated at 100 °C for 10 min.

NMR spectra were obtained with either a Varian Mercury 300 spectrometer or a Varian Inova 500 spectrometer (Varian NMR Systems, Palo Alto, CA, USA) operating at 300 MHz or 500 MHz, respectively. ^1H NMR spectroscopy was used for the optimization of reaction conditions and following the reaction, and both ^1H and ^{13}C NMR spectroscopies were used in the characterization of the isolated products. NMR samples, 1.3 mg/mL (following the reaction) or 10–15 mg/mL (characterization), were dissolved in D_2O or DMSO-d_6 and placed into NMR tubes without filtration. The spectra were run at 27 °C. The chemical shifts were referenced to internal acetone or HDO (D_2O) or the residual solvent peak (DMSO-d_6). Correlation NMR spectroscopy (COSY, HSQC, and TOCSY experiments) was used in the assignment of the spectra. ^1H spectra were acquired with a spectral width of 5000 Hz, 2.5 s acquisition time, and 16 scans. ^{13}C spectra were acquired with a spectral width of 20,000 Hz, 1.8 s acquisition time, and 12,920 repetitions. For COSY and TOCSY experiments, spectral widths of 5570 Hz and 5030 Hz, respectively, were employed in both dimensions and a matrix of 2048×256 complex points (8 scans per t_1 value) was collected. Data were zero-filled to 4096×512 and a 90° shifted sine-bell weighting function was employed in both dimensions prior to Fourier transformations. For HSQC experiments, spectral widths of 34,965 Hz in F_1 and 5320 Hz in F_2 were used. A matrix of 2048×256 complex points (64 scans per t_1 value) was collected. Data were zero-filled to 4096×512 and a 90° shifted sine-bell weighting function was employed in both dimensions prior to Fourier transformations.

Molar masses of the isolated products were also analyzed with electrospray ionization mass spectrometry (ESI-MS, positive ion mode). The analysis was carried out using an Agilent XCT Plus ion trap mass spectrometer. The samples were directly injected. The nebulizer gas (N_2) pressure was 30 psi, the drying gas flow and temperature were 8 L/min and 350 °C, respectively. The capillary voltage was set at 3270 V, capillary exit offset 121 V, skimmer 1 potential 40.0 V, and trap drive value 61.2. Spectra were recorded as averages of four, using ultra scan mode, and a scan range from 50 to 1500 m/z.

The amount of dissolved oxygen in the reaction mixture was measured using a Mettler-Toledo MO128 oxygen meter in mg/L mode at room temperature with magnetic stirring. The reaction mixture was transferred into a narrow container for the measurements due to the size of the oxygen meter probe.

4.3. Oxidation of methyl α-D-galactopyranoside

Methyl α-D-galactopyranoside **1** (in most experiments 0.1 g, 0.5 mmol) was dissolved in 7.5 mL of water or 25 mM sodium phosphate buffer, pH 7 (70 mM solution). Enzymes were added (e.g., combinations of GO, 0.052 U/mg; catalase, 112 U/mg; and/or HRP, 0.45 U/mg) and the mixture was stirred at 4 °C, rt or 40 °C for 24 h. GO and catalase dosages were chosen according to the literature and the dosage of HRP was chosen by recommendation of Dr. Sybe Hartmans. The experiments with the partially purified GO followed the same procedure with similar amounts of enzymes in relation to the substrate (methyl α-D-galactopyranoside **1**, 25 mg, 0.13 mmol, 65 mM; volume of the reaction mixture, 2 mL). Samples (100–200 μL) were taken after 1, 2, 3, 4, 5, and 24 h, heated in a boiling water bath for 3 min to inactivate the enzymes, evaporated with a rotary evaporator or a SC110A Speed Vac Plus concentrator equipped with a UVS400A Universal Vacuum System Plus with Vapornet (Savant Instruments, Inc., Holbrook, NY, USA), dissolved in D_2O, and placed in an NMR tube for the NMR analysis.

4.4. Isolation of products

A sample containing the oxidation products and the secondary products was applied to a preparative TLC (PLC) plate (Silica Gel 60). The PLC was eluted with $MeOH/CH_2Cl_2/AcOH/H_2O$ (10:20:1:1). The compounds were isolated based on their R_f values calculated from a TLC plate stained with $EtOH/H_2SO_4$. Each portion scratched from the PLC plate was ground in a mortar, stirred in 5–10 mL of H_2O for 5 min, filtered, and evaporated. Samples were dissolved in D_2O or DMSO-d_6 for NMR analysis. ^1H and ^{13}C NMR of **2**–**4** are presented in Tables 1 and 2. The R_f values were **2**, 0.60; **3**, 0.08; and **4**, 0.83. Mass spectra (ESI, positive ionization): **2** (present as hydrate and aldehyde forms), m/z 215.1 $[M(aldehyde)+Na]^+$, 233.1 $[M(hydrate)+Na]^+$, 407.3 $[M(aldehyde)+M(aldehyde)+Na]^+$, 443.3 $[M(hydrate)+M(hydrate)+Na]^+$; **3**, m/z 231.1 $[M+Na]^+$, 439.2 $[M+M+Na]^+$; **4**, m/z 197.1 $[M+Na]^+$, 215.3 $[M+H_2O+Na]+$, 374.3 $[M(dimer-H)+Na]^+$.

4.5. Oxidation of D-raffinose

D-Raffinose (pentahydrate, 0.3 g, 0.5 mmol) was dissolved in 7.5 mL of water to give a 70 mM solution, and GO (5.2 U), catalase (11150 U), and HRP (45 U) were added, after which the mixture was stirred at rt. Samples (100–200 μL) were taken after 1, 5, and 24 h, heated in a boiling water bath for 3 min to inactivate the enzymes, evaporated with a rotary evaporator, dissolved in D_2O, and placed in an NMR tube for the NMR analysis. ^1H NMR (300 MHz, D_2O) of the product **4** (as a hydrate): δ 5.40–5.39 (1H, d, J = 3.9 Hz, H-1′), 5.11–5.08 (1H, d, J = 7.5 Hz, H-6″), 4.97–4.96 (1H, d, J = 3.6 Hz, H-1″), 4.21–4.18 (1H, d, J = 8.7 Hz, H-3), 4.12–4.11 (1H, m, H-4″), 4.06–4.00 (1H, t, J = 8.7 Hz, H-4), 4.02–3.48 (14H, overlapping multiplets).

Acknowledgments

We thank Dr. Sybe Hartmans for supplying us with the *P. pastoris* galactose oxidase product, Ms. Marianne Mäki for the analysis of the protein content of the GO preparation, Professor Ilkka Kilpeläinen for the opportunity to run NMR spectra, Dr. Sami Heikkinen for assistance with running 2D NMR spectra, and Mr. Matti Wahlsten for assistance with running mass spectra. Ms. Ann-Sofie Leppänen, Dr. Stefan Willför, Dr. Patrik Eklund, and Professor Rainer Sjöholm are thanked for fruitful discussions. The study was funded by the Academy of Finland (contract no. 117765).

References

1. Whittaker, J. W. *Chem. Rev.* **2003**, *103*, 2347–2363.
2. Whittaker, J. W. *Arch. Biochem. Biophys.* **2005**, *433*, 227–239.
3. Baron, A. J.; Stevens, C.; Wilmot, C.; Seneviratne, K. D.; Blakeley, V.; Dooley, D. M.; Phillips, S. E. V.; Knowles, P. F.; McPherson, M. J. *J. Biol. Chem.* **1994**, *269*, 25095–25105.
4. Whittaker, M. M.; Whittaker, J. W. *Protein Expr. Purif.* **2000**, *20*, 105–111.
5. Wilkinson, D.; Akumanyi, N.; Hurtado-Guerrero, R.; Dawkes, H.; Knowles, P. F.; Phillips, S. E. V.; McPherson, M. *J. Protein Eng. Des. Sel.* **2004**, *17*, 141–148.
6. Hartmans, S.; De Vries, H. T.; Beijer, P.; Brady, R. L.; Hofbauer, M.; Haandrikman, A. J. Production of Oxidized Guar Galactomannan and its Applications in the Paper Industry. In *Hemicelluloses: Science and Technology*; Gatenholm, P., Tenkanen, M., Eds.; American Chemical Society: Washington, DC, 2004; pp 360–371.
7. Sun, L.; Petrounia, I. P.; Yagasaki, M.; Bandara, G.; Arnold, F. H. *Protein Eng.* **2001**, *14*, 699–704.
8. Firbank, S. J.; Rogers, M. S.; Wilmot, C. M.; Dooley, D. M.; Halcrow, M. A.; Knowles, P. F.; McPherson, M. J.; Phillips, S. E. V. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 12932–12937.

9. Hamilton, G. A.; De Jersey, J.; Adolf, P. K. Galactose Oxidase: The Complexities of a Simple Enzyme. In *Oxidases and Related Redox Systems*, King, T. E., Mason, H. S., Morrison, M. Eds.; Memphis, TN, 1971; pp 103–124.
10. Root, R. L.; Durrwachter, J. R.; Wong, C. H. *J. Am. Chem. Soc.* **1985**, *107*, 2997–2999.
11. Siebum, A.; van Wijk, A.; Schoevaart, R.; Kieboom, T. *J. Mol. Catal. B* **2006**, *41*, 141–145.
12. Brady, R. L.; Cheng, H. N.; Haandrikman, A. J.; Kuo, P.; Moore, A.; Riehle, R. J.; Lapre, J. A.; Mcnabola, W. T.; Nguyen, T. T.; Haandrikman, A. J.; Devries, H. T. Patent 2002012349, 2002.
13. Cooper, J. A. D.; Smith, W.; Bacila, M.; Medina, H. *J. Biol. Chem.* **1959**, *234*, 445–448.
14. Adanyi, N.; Szabo, E. E.; Varadi, M. Z. *Lebensm. Unters. Forsch.* **1999**, *209*, 220–226.
15. Kinoshita, M.; Inagake, K.; Kawabata, A.; Kuroda, R.; Oda, Y.; Kakehi, K. *Anal. Biochem.* **2000**, *284*, 87–92.
16. Schoevaart, R.; Kieboom, T. *Carbohydr. Res.* **2002**, *337*, 899–904.
17. van Wijk, A.; Siebum, A.; Schoevaart, R.; Kieboom, T. *Carbohydr. Res.* **2006**, *341*, 2921–2926.
18. Fialova, P.; Namdjou, D.; Ettrich, R.; Prikrylova, V.; Rauvolfova, J.; Krenek, K.; Kuzma, M.; Elling, L.; Bezouska, K.; Kren, V. *Adv. Synth. Catal.* **2005**, *347*, 997–1006.
19. Mazur, A. W.; Hiler, G. D., II *J. Org. Chem.* **1997**, *62*, 4471–4475.
20. Yalpani, M.; Hall, L. D. *J. Polym. Sci., Polym. Chem.* **1982**, *20*, 3399–3420.
21. Maradufu, A.; Perlin, A. S. *Carbohydr. Res.* **1974**, *32*, 127–136.
22. Ohrui, H.; Nishida, Y.; Higuchi, H.; Hori, H.; Meguro, H. *Can. J. Chem.* **1987**, *65*, 1145–1153.
23. Kelleher, F. M.; Bhavanandan, V. P. *J. Biol. Chem.* **1986**, *261*, 11045–11048.
24. Basu, S. S.; Dotson, G. D.; Raetz, C. R. *Anal. Biochem.* **2000**, *280*, 173–177.
25. Matsumura, S.; Kuroda, A.; Higaki, N.; Hiruta, Y.; Yoshikawa, S. *Chem. Lett.* **1988**, 1747–1750.
26. Bonnet, V.; Duval, R.; Rabiller, C. *J. Mol. Catal. B: Enzym.* **2003**, *24–25*, 9–16.
27. Maradufu, A.; Cree, G. M.; Perlin, A. S. *Can. J. Chem.* **1971**, *49*, 3429–3437.
28. Beving, H. F. G.; Theander, O. *Acta Chem. Scand. Ser. B* **1975**, *29*, 577–581.
29. Beving, H. F. G.; Theander, C. *Acta Chem. Scand. Ser. B* **1975**, *B29*, 641–646.
30. Mazur, A. W. Galactose Oxidase. Selected Properties and Synthetic Applications. In *Enzymes in Carbohydrate Synthesis*, Bednarski, M., Simon, E. S., Eds.; American Chemical Society: Washington, DC, 1991; pp 99–110.
31. Wilhelm, E.; Battino, R.; Wilcock, R. J. *Chem. Rev.* **1977**, *77*, 219–262.
32. Frollini, E.; Reed, W. F.; Milas, M.; Rinaudo, M. *Carbohydr. Polym.* **1995**, *27*, 129–135.