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

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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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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

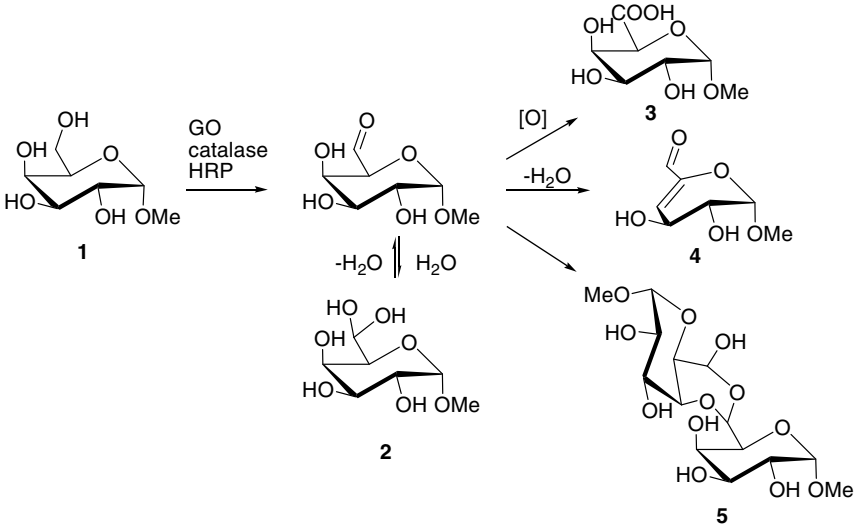
The peaks are referenced to internal acetone (D_2O) or the residual solvent peak at 39.5 ppm (DMSO-d_6).
[a] Recorded at 300 MHz.
[b] Recorded at 500 MHz.
[c] Compound **2** as a hydrate.
[d] No 2D spectra used in the assignment.

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.