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Galactose oxidase and alcohol oxidase: Scope and limitations for the enzymatic synthesis of aldehydes

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Abstract

The utility of both galactose oxidase and alcohol oxidase for alcohol-to-aldehyde oxidation has been investigated, from a synthetic point of view. The speed of reaction and degree of conversion has been measured for 29 different primary alcohols. The two oxidative enzymes show complementary synthetic use, i.e. galactose oxidase for galactose-derived polyols and alcohol oxidase for aliphatic mono- and diols. Alcohol oxidase has been successfully used in combination with the aldolase DERA in a two-step, one-pot reaction cascade.
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1. Introduction

The current trend in chemistry towards 'green' reactions emphasizes the inherent advantages of catalytic methods. Biocatalytic methods, especially, promise a host of improvements compared to standard organic conversions [1]. Their capacity for highly (stereo-)selective transformations under mild, aqueous conditions, without the need for stoichiometric addition of heavy metal reagents or protection/deprotection schemes is practically unequalled within organic synthesis. With this in mind, we approached the selective conversion of several highly functionalized alcohols into the corresponding aldehydes as this oxidation reaction cannot be easily achieved by clean chemical means. In addition, the aldehydes obtained by enzymatic oxidation are useful as acceptor in subsequent enzymatic aldol reactions in a cascade mode without intermediate recovery steps.

Galactose oxidase (*D. dendroides*, E.C. 1.1.3.9.) and alcohol oxidase (*P. pastoris*, E.C. 1.1.3.13) were the biocatalysts of choice. Both enzymes are oxidases that do not require cofactors in the course of their catalytic cycles, but use molecular oxygen as an electron acceptor in the oxidation of alcohols to aldehydes (Fig. 1). The fact that they do not need stoichiometric addition of a cofactor like NADPH (or the presence of a second enzyme system to regenerate a catalytic amount of cofactor) makes oxidases more suitable for biocatalytic oxidations than the cofactor-dependent dehydrogenases.

Galactose oxidase (GO) is a copper-containing, free-radical catalyst that was first described in 1959 by Cooper et al. [2]. This publication triggered a steady stream of further publications detailing investigations into the enzyme's mechanism [3,4], its structure [5,6] and its range of possible substrates [7,8]. From the outset it was reported that the enzyme could transform a range of primary alcohols into the corresponding aldehydes, though at widely varying velocities.

Alcohol oxidase (AO) is a flavin-dependent, alcohol oxidizing protein originating in methylotropic yeasts and part of the GMC-oxidoreductase superfamily [9]. AO, like GO oxidizes a range of (aliphatic) primary alcohols to the corresponding aldehyde with concomitant production of H_2O_2. AO was earlier reported to have a range of unbranched aliphatic alcohols as its substrate [10–12].

Despite all the work carried out on the GO and AO systems, however, a clear picture on the limits of these systems' reactivities has failed to emerge and some ambiguities concerning the scope of GO especially remain [13]. Therefore, we have investigated the preparative scope and limitations of these enzymes on the basis of 30 different alcohol substrates.

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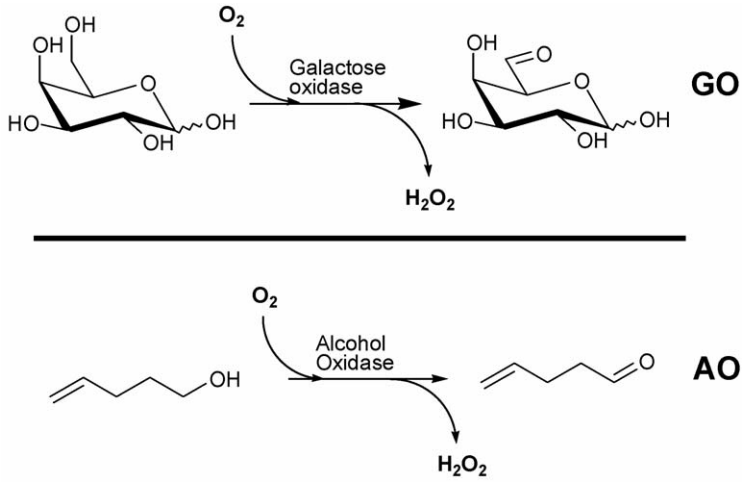


Fig. 1. The galactose oxidase (GO) and alcohol oxidase (AO) catalytic systems.

2. Experimental

^{1}H NMR spectra were recorded with a Jeol FX-200, a Bruker DPX-300 or a Bruker DPX 400 spectrometer, using methanol (CH_3OH: $\delta = 3.3$ ppm) or water (H_2O: $\delta = 4.8$ ppm) as an internal standard. ^{13}C noise-decoupled NMR spectra were recorded with a Jeol FX-200 at 50.1 MHz a Bruker DPX-300 spectrometer at 75.5 MHz and a Bruker DPX-400 at 100.7 MHz, using $CDCl_3$ ($\delta = 77$ ppm), CH_3OH ($\delta = 49$ ppm) or TSP (3-(trimethylsilyl)tetradeuteropropionic acid sodium salt, $\delta = 0$ ppm) as internal standard. All spectra were recorded in 10% D_2O in H_2O, except where noted otherwise. UV–vis-measurements were performed on a Perkin-Elmer Lambda 25 at 425 nm and at a temperature of 21 °C. Spots on thin-layer chromatography were detected with phosphomolybdic acid or $KMnO_4$-spraying. Enantiomeric excesses were determined by ^{1}H NMR using a chiral additive (*R*-(−)-1-phenyl-2,2,2-trifluoroethanol). All commercially available chemicals were purchased from Sigma–Aldrich, Acros or Fluka. All chemicals were used without further purification, unless stated otherwise. Galactose oxidase was a gift from Hercules (Barneveld, The Netherlands) and had the same specificity as the enzyme bought from Sigma. All other enzymes were bought from Sigma or Fluka.

2.1. General synthetic procedure

To 5 mL of a 200 mM solution of the substrate dissolved in 50 mM phosphate buffer 2 mg of catalase (Bovine liver, E.C. 1.11.1.6, 2440 units/mg) was added, followed by 30 units oxidase. The reaction mixture was brought under an oxygen atmosphere using a balloon and gently shaken on a rotatory shaker for 24 h. The conversion was determined by ^{1}H NMR.

2.1.1. GO

To 5 mL of a 200 mM solution of methyl α-D-galacto-pyranoside dissolved in 50 mM phosphate buffer (pH 7), 2 mg of catalase was added followed by 30 units galactose oxidase. The reaction mixture was brought under an oxygen atmosphere using a balloon and gently shaken on a rotatory shaker for 24 h (21 °C, 1 atm O_2). The conversion as determined by integration of the H1/H6 and C1/C6 signals of starting material and product using ^{1}H and ^{13}C NMR proved to be quantitative. Methyl α-D-galacto-hexodialdo-1,5-pyranoside could be isolated as an off-white powder in 95% yield by recrystallizing from 2-propanol/petroleum ether 40–60.

^{13}C NMR (75 MHz, 10% D_2O in H_2O): $\delta = 103.93$, 88.14 (C6, hydrate form), 76.85, 72.79, 70.69, 68.35, 57.20 (OCH_3) ppm.

2.1.2. AO

To 5 mL of a 200 mM solution of 4-penten-1-ol dissolved in 50 mM phosphate buffer (pH 7.4), 10 mg of catalase CLEA [14] (Cross-Linked Enzyme Aggregate) was added followed by 30 units alcohol oxidase. To the reaction mixture 5 mL of toluene was added and the two-phased reaction mixture was brought under an oxygen atmosphere using a balloon and gently shaken on a rotatory shaker for 24 h (21 °C, 1 atm O_2). The conversion was determined by GC, ^{1}H and ^{13}C NMR to be quantitative. The product could be isolated by separation of the two layers and extraction of the water layer with 5 mL diethyl ether. The combined organic layers were dried with $MgSO_4$ and evaporated in vacuo to half the volume, giving a 200 mM solution of 4-pentenal in toluene.

^{1}H NMR: (300 MHz, $CDCl_3$): $\delta = 9.69$ (1 H, t, H-1), 5.73 (1 H, m, H-4), 4.95 (2 H, m, H-5), 2.44 (2 H, m, H-2), 2.31 (2 H, m, H-3) ppm.

2.2. Reaction velocities

A hydrogen peroxide coupled assay similar to the one described in literature, was applied at pH 7 and 21 °C [7]. To a saturated solution of toluidine in 50 mM phosphate buffer (5 mg in 10 mL) was added 10 μL chloroperoxidase (*C. fumago*) obtained from Fluka (22429 units per mL). Subsequently 1 mL of the assay solution and 500 μL of a 150 mM substrate solution in 50 mM phosphate buffer were mixed in a cuvet and 1 unit of GO/AO was added, after which the mixture was rapidly homog-

enized and the UV–vis-absorbance at 425 nm was measured as a function of time. All substrates capable of mutarotation were dissolved 24 h before measurement.

2.3. Two-step, one-pot cascade reaction

To 10 mL of a 200 mM solution of 4-penten-1-ol dissolved in 50 mM phosphate buffer, 10 mg of catalase CLEA was added followed by 100 units alcohol oxidase. Acetone (4 mmol) was added subsequently, together with DERA (2-deoxyribo-5-phosphate aldolase (*E. coli* strain DH5α, E.C. 4.1.2.4, 500 units) and the mixture was brought under an oxygen atmosphere and put on a rotatory shaker (21 °C, 1 atm O_2). After 96 h, the solution was extracted with ethyl acetate and the collected organic layers were dried with $MgSO_4$. TLC and GC indicated that less than 10% of the starting material 4-penten-1-ol remained. After evaporation, the crude product was purified using column chromatography (PE/E 70/30) giving 30% yield of (*S*)-4-hydroxy-oct-7-en-2-one. The e.e. was 70%.

^{1}H NMR: (300 MHz, $CDCl_3$): $\delta = 5.80$ (1 H, m, H-7), 5.01 (2 H, m, H-8), 4.05 (1 H, m, H-4), 2.61 (2 H, m, H-3), 2.05 (5 H, m, H-1 + H-5), 1.68 (2 H, m, H-6) ppm.

^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 209.8$ (C2), 138.1 (C7), 114.9 (C8), 66.9 (C4), 49.9 (C3), 35.4 (C5), 30.7 (C1), 29.6 (C6) ppm.

3. Results and discussion

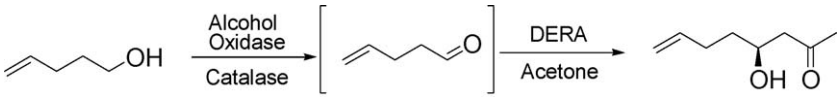
To test the synthetic utility of the oxidizing enzymes, 30 different (highly) functionalized primary alcohols were treated with GO and AO. To determine whether an alcohol was a substrate for either enzyme, they were tested with a chloroperoxidase-toluidine assay [7], from which the V_{rel} was measured. In this way a comparison with former literature data for GO was possible [15]. In addition, overall conversions have been determined by NMR after 48 h reaction. The data obtained above are summarized in Table 1.

The picture that emerges from the data in Table 1 is, broadly speaking, one of complementary reactivity. Galactose oxidase is an efficient oxidizing enzyme for substrates containing an accessible D-galactose moiety, as the oxidation of the galactosides, melibiose, raffinose, lactose and lactitol show. Unfortunately non-D-galactose based carbohydrates are no substrates. Galactose oxidase has a limited capacity for oxidizing shorter polyol chains which can be twisted to form a conformation similar to

Table 1
Relative initial reaction rates (normalized to 100 for α-D-galactose and methanol) and conversions (after 48 h) for GO and AO (phosphate buffer, pH 7, 21 °C, 1 atm O_2)

Nr.	Compound	V_{rel}GO (L)	V_{rel}GO	Conv-GO (%)	V_{rel}AO	ConvAO (%)
1	D-galactose	100	100	100	0	0
2	Methyl α-D-galactopyranoside	118, 125	98	100	0	0
3	Methyl β-D-galactopyranoside	227, 340	118	100	0	0
4	2-Deoxy-D-galactose	32	74	100	0	0
5	Lactose	2, 15	7	10	0	0
6	Lactitol	–	14	100	0	0
7	Lactulose	–	28	100	0	0
8	Lactobionic acid	–	9	20	–	–
9	Raffinose	154, 200	80	100	0	0
10	Locust Gum (galactomannan)	0	3	–	0	0
11	Melibiose	80, 162	95	100	0	0
12	Methyl β-D-mannopyranoside	0.03	0	0	–	–
13	Glycerol	–	0.02	1	0	0
14	Dihydroxyacetone[a]	540	126	>0	–	–
15	Hydroxyacetone[a]	4	2	>0	4	–
16	Benzyl alcohol	30	0.08	70	3	–
17	L-serine	–	0	0	0	0
18	2,2-Dimethyl-1,3-dioxolane-4-methanol (Solketal)	–	1.5	0	2	–
19	1-Chloro-2,3-propanediol	–	0.01	0	0	0
20	1,3-Propanediol	–	0.01	0	0	0
21	α-α-Diglycerol	–	0.01	0	0	0
22	D/L-threitol	–	2	0	4	–
23	1,4-Butanediol	–	0	0	2	–
24	1,2-Dihydroxybutane	–	0	5	0.1	–
25	1,2-Dihydroxybutene	–	0.4	–	2	0
26	*cis*-2-Butene-1,4-diol	–	0	0	0.5	–
27	3-Methylbutan-1-ol	–	0	0	2	–
28	4-Penten-1-ol	–	0	0	39	100
29	3-Buten-1-ol	–	0	0	37	50
30	Methanol	–	0	0	100	100

(–) corresponds to 'no data available/not measured'.
[a] Due to the complex mixture of products, the conversion could not be determined.



Scheme 1. A two-step, one-pot reaction, combining an oxidase and an aldolase.

the D-galactose C4–C6 fragment (e.g. glycerol). Despite its very low reactivity, Wong and co-workers [16] demonstrated the one-pot conversion of glycerol into aldol products using both GO and RhaD aldolase. Beyond these substrates its scope is severely limited and it shows no reactivity at all for the more hydrophobic primary alcohols like 4-penten-1-ol.

Alcohol oxidase on the other hand clearly prefers unbranched aliphatic primary alcohols. It effectively oxidizes short chain saturated and unsaturated primary alcohols. Introduction of further hydroxyl groups in the chain quickly decreases the effectivity of the system. The reaction velocity also decreases when the aliphatic tails become longer. Possible reasons are a diminished solubility in water of the substrate with increasing chain length and steric crowding of the active site, though the last argument is hard to verify without the availability of a crystal structure.

A comparison of different studies detailing the range of substrates oxidized by GO and the relative velocities of the oxidations compared to D-galactose, the natural substrate, shows marked deviations between articles [15,8]. In part, these differences can be explained by the choice of measuring method for observing the enzymatic reaction. E.g. measuring the speed of oxygen uptake by the system [17] compared with measuring increasing UV–vis-absorbance using a hydrogen peroxide detection system. Moreover, although many of the cited articles have looked at the initial activity and Km for different sets of substrates, most articles fail to take the total conversion at the end of the reaction into account. This is unfortunate because from a synthetic point of view the yield of a reaction is the most important parameter. Besides, as can be seen for the entries of lactobionic acid (Table 1, entry 8) and benzyl alcohol (Table 1, entry 16), initial reaction velocities do not always correlate well with the amount of conversion after 2 days.

If the GO results are compared with earlier studies, several discrepancies can be seen. Especially for those substrates that undergo mutarotation (galactose, lactose, etc.) different values can be found both within literature and compared to the results presented here. Reaction rates varying by a factor of more than five have been reported for lactose as substrate [18,7]. In part these different results depend on the time the system is allowed to equilibrate between the α and β forms. Not all earlier studies mention whether mutarotation was taken into account or how much time was used for equilibration of the anomers.

3.1. One-pot reaction cascades with oxidases

On a synthetic scale, the speed at which a substrate is oxidized with AO slowly decreases as substrate and product are starting to compete for the active site [10]. This can be prevented by the addition of an organic solvent that is immiscible with the water layer. The aldehyde that is formed in the reaction has a preference for the hydrophobic organic phase, leaving the active site of the enzyme available for its substrate, the alcohol, thus 'pulling' the reaction forward. Another way of removing the product aldehyde is converting it in a consecutive reaction step in the same pot.

As an example we have coupled an AO-catalyzed oxidation step with an aldol condensation catalyzed by DERA. In this way 4-pentenol was oxidized by the AO/catalase system in the presence of acetone and DERA to yield the aldol product in 30% after 5 days in 70% e.e. (Scheme 1). The limiting step was the DERA-mediated aldol condensation, as 4-pentenal and acetone were still present in the solution. Although DERA tolerates a range of acceptor substrates, the reaction velocity for non-natural substrates is often low, unfortunately. The experiment shows, however, that oxidase/aldolase one-pot reactions can be conveniently set up to prepare the chiral aldol products from a range of achiral primary alcohols.

4. Conclusions

Twenty-nine functionalized primary alcohols have been treated with GO and AO and have been tested on both reaction velocity and the extent of conversion to the corresponding aldehyde. The data show that both GO and AO have their own, roughly complementary, domains in which they are of synthetic use. GO remains largely limited to galactose-derived substrates. AO has a slightly larger scope of reactivity, but has a clear preference for unbranched, aliphatic, primary alcohols. Both GO and AO can be used for the preparation of functionalized aldehydes, under mild aqueous conditions, on a synthetically useful scale. A successful two-step cascade combining alcohol oxidase and an aldolase and leading to chiral aldol products has been developed.

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