# Modification of Galactose Oxidase to Introduce Glucose 6-Oxidase Activity

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The selective oxidation of the 6-hydroxy group of D-glucose to introduce an aldehyde functionality is not catalyzed by known oxidase enzymes. Selective functionalization at the glucose C-6 position in oligo- and polysaccharides is a synthetically useful reaction that would greatly facilitate further chemical modifications for food, pharmaceutical, and materials applications. We chose the fungal enzyme galactose oxidase (D-galactose: oxygen 6-oxidoreductase, EC 1.1.3.9) as a starting point to try to generate a glucose 6-oxidase. A well-characterized copper-containing radical enzyme, galactose oxidase (GOase) oxidizes various primary alcohols to their corresponding aldehydes, with the reduction of oxygen to hydrogen peroxide.[1–5] Native GOase is highly active towards the 6-OH group of D-galactose but essentially inactive towards D-glucose. Glucose apparently cannot bind at the active site since concentrations as high as 1 M have no effect on the activity of the enzyme towards D-galactose.[6]

We first attempted to generate activity towards D-glucose by random point mutagenesis. After screening more than 30 000 clones, however, we observed no improvement in D-glucose activity, although both the expression level and thermostability were enhanced.[7] Careful examination of GOase activity shows that it is at least one million times less active towards D-glucose than towards D-galactose. We therefore concluded that the desired novel activity would require significant remodeling of the active site and is not accessible by point mutagenesis.

The crystal structure of GOase has been solved and a substrate binding model has been proposed based on a molecular docking experiment.[3, 5] According to the model, Arg330 forms hydrogen bonds with the hydroxy groups of substrate C-4 and C-3 atoms, while Gln406 forms an additional hydrogen bond with the C-2 hydroxy group of the substrate. In addition, a hydrophobic wall in the pocket that contains Phe194 and Phe464 interacts with the D-galactose backbone atoms C-6, C-5, and C-4. Trp290, which has been proposed to stabilize the radical form of GOase,[5] is believed to play a key role in restricting entry to the active center (Figure 1).[8]

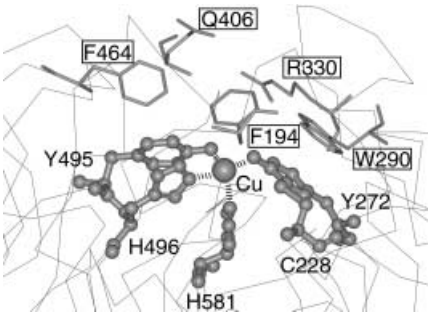


***Figure 1.*** *Residues R330, Q406, F464, F194, and W290, which were targeted for saturation mutagenesis (in stick form), and key active site residues (in ball and stick form) in the GOase crystal structure.[3, 5] The mutagenized residues are in a position to control substrate access to the active site.*

We constructed a combinatorial library by saturation mutagenesis of the Arg330, Phe464, and Gln406 residues of GOase mutant A3.E7, which is expressed more efficiently and is more stable than the wildtype enzyme in *Escherischia coli*.[7] More than 10 000 colonies were screened. Over 95 % were inactive towards galactose and none was more active than the parent. One mutant (M-RQ: mutations = R330K, Q406T) was identified that has 20 times greater activity than the parent enzyme towards D-glucose. Saturation mutagenesis of F194 in M-RQ generated no further improvement in activity towards D-glucose.

Saturation mutagenesis of Trp290 in the parent A3.E7 generated mutant M-W (W290F), with tenfold improved activity towards D-glucose. Again, no mutants with enhanced D-galactose activity were identified. Introduction of the W290F mutation into mutant M-RQ produced the mutant M-RQW, with 100-fold increased activity towards D-glucose compared to A3.E7. As expected, activity towards glucose came at a significant cost to the natural activity of the enzyme towards galactose, which was decreased 1000-fold in M-RQW compared to the parent enzyme.

Oxidation of D-galactose by GOase generates a dialdehyde whose tendency to form polymers complicates analysis. We therefore analyzed the product of the M-RQW-catalyzed oxidation of methyl-$\beta$-D-gluco-pyranoside. Figure 2 shows thin-layer chromatograms of the reaction mixture and the silica-gel purified product. We used Bial's reagent and Purpald reagent to detect the presence of sugars and aldehydes, respectively. All product components were stained upon treatment of the TLC plates with Purpald reagent, a diagnostic for aldehydes. A single major product was found. Trace byproducts are believed to be the dimer and $\alpha-\beta$ elimination product of the sugar aldehyde.[9, 10] The appearance of byproducts after product purification indicates that these substances are generated nonenzymatically. Byproducts with the same retention times were observed in the reaction of native GOase (Sigma) with methyl-$\beta$-D-galactopyranoside (data not shown).

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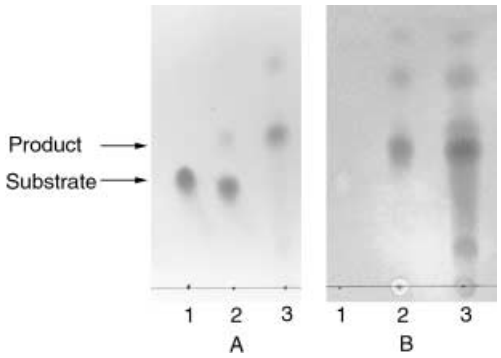


***Figure 2.*** *Thin layer chromatograms of the reaction mixture for methyl-β-D-gluco-pyranoside oxidation by engineered GOase M-RQW. TLC plates were stained with Bial's reagent for detection of sugars (A) and Purpald reagent for detection of aldehydes (B). Line 1: substrate; Line 2: reaction mixture; Line 3: purified product.*

$^{13}C$ NMR spectroscopy revealed that the chemical shift of the C-6 atom moved from 61 ppm in the substrate to 89 ppm in the product. An identical shift change was observed in the aldehyde product of methyl-β-D-galacto-pyranoside oxidation by native GOase. $^{1}H$ NMR spectra also showed formation of the aldehyde at the C-6 atom by the appearance of a signal with a chemical shift of 5.25 ppm. No oxidation was detected at other positions. Oxidation of D-glucose by mutant M-RQW is thus specific to the 6-OH group.

The activities of M-RQW and native GOase towards various alcohols (Table 1) show substantially different substrate specificities. M-RQW oxidizes several substrates towards which the native enzyme is inactive. These substrates include D-glucose and its derivatives as well as several aliphatic secondary alcohols. The oxidation of secondary alcohols by GOase has not been reported previously. GC – MS analysis of the reaction mixture that contained M-RQW with 2-butanol showed the appearance of a new peak whose identity was confirmed as 2-butanone.

***Table 1.*** *Relative rates of reaction for native GOase and mutant M-RQW.*

| Substrate | Native GOase[a][b] | Mutant M-RQW[a] |
|---|---|---|
| D-Galactose | 100 | 100 |
| D-Glucose | 0 | 4.8 |
| 2-Deoxy-D-glucopyranose | 0 | 3.9 |
| Methyl-β-D-glucopyranoside | 0 | 3.3 |
| D-Mannose | 0 | 0.002 |
| β-D-Lactose | 48 | 18.7 |
| L-Galactose | 0 | 1.5 |
| 2-Propene-1-ol | 0.3 | 2500 |
| D-Maltose | 0 | 0.45 |
| Amylose | 0 | 0.23 |
| 2-Pyridine methanol | 0.6 | 180000 |
| 3-Pyridine methanol | 5 | 35000 |
| 4-Pyridine methanol | 1.3 | 81000 |
| 1,3-dihyroxy-2-propanone | 200 | 1600 |
| 2-Butanol | 0 | 0.3 |
| 3-Buten-2-ol | 0 | 6.9 |

[a] Activities are reported relative to D-galactose (100). [b] The native enzyme is 1000 times more active towards D-galactose than the mutant.

Various primary alcohols with a carbonyl or aromatic group in the α-position, which includes 3-pyridylcarbinol and dihydroxyacetone, are also better substrates for the mutant than for the native enzyme. M-RQW is very efficient for some of these substrates; for example, M-RQW has higher specific activity towards 2-pyridylcarbinol than the native enzyme has towards D-galactose. M-RQW is also more active than native GOase towards dihydroxyacetone, the best substrate for native GOase, mainly as a result of a more than sixfold decrease in the Michaelis constant, $K_m$ (2.7 mM for M-RQW compared with 17 mM for the native enzyme). Apparently, accessibility to the active site is significantly augmented in the mutant to create a more broadly specific oxidase, and this broad specificity has not necessarily undermined the catalytic activity of the enzyme.

D-galactose is still a fairly good substrate for M-RQW, which is approximately 20 times more active towards D-galactose than towards D-glucose. The activity of M-RQW towards D-mannose is more than 2000 times lower than its activity for the 2-epimer of D-mannose, D-glucose, which demonstrates how important the configuration at the C-2 atom is for D-glucose binding. 2-Deoxy-D-glucose is a comparably good substrate to D-glucose.

The mutant M-RQW is a glucose 6-oxidase with a regioselectivity that has not been reported in nature. Glucose 1-oxidase, glucose 2-oxidase (pyranose oxidase), glucose 2,3-dehydrogenase, and galactose 6-oxidase (GOase) are all produced by fungi and presumably function to generate $H_2O_2$ to facilitate lignin degradation. There is evidence that *Pseudogluconobacter saccharoketogenes* produces an enzyme that oxidizes the hydroxymethyl group of the terminal glucose residue of a cyclomaltooligosaccharide to produce a carboxylic acid.[11] We cannot offer any good reason why the oxidation of glucose at the 6-hydroxy group to make aldehydes is unknown in nature. Combinatorial mutagenesis of GOase, however, and screening for activity towards glucose has generated an enzyme with a low but significant level of this activity (1.6 U mg$^{-1}$). The M-RQW enzyme is now a possible starting point for further improvement to make a glucose 6-oxidase for synthetic applications.

## Experimental Section

**Materials**: All chemicals were reagent grade or better. 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), D-galactose, and horseradish peroxidase (HRP) were from Sigma (St. Louis, MO). *E. coli* strain BL21(DE3) was purchased from Novagen (Madison, WI) and the production of plasmid pGAO-36, which contains the GOase mutant A3.E7, was described previously.[7] Restriction enzymes and ligase were obtained from Boehringer Mannheim (Indianapolis, IN), Life Technologies (Grand Island, NY), or New England Biolabs (Beverly, MA). A site-directed mutagenesis kit (Quick-Change) was purchased from Stratagene (La Jolla, CA).

**Library construction and screening**: The library Lib-RFQ, which contained species with random mutations at three sites (R330, Q406, and F464), was constructed by using the Quick-Change kit according to the standard protocol and with plasmid pGAO-036, which carries the GOase mutant A3.E7. The primers used to construct the libraries are: 5′-GCTGACAAGCAAGGATTGTACNN(G/C)TCAGACAACCACGCG-

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TGG-3′ and 5′-CCACGCGTGGTTGTCTGA(G/C)NNGTACAATCCTTG-CTTGTCAGC-3′ (for Lib-R), 5′-GGCCAACGACGTATTCCGNN(G/C)GAG-GATTCAACCCCG-3′ and 5′-CGGGGTTGAATCCTC(G/C)NNCGGAATTC-CACGTCGTTGGCC-3′ (for Lib-RF), 5′-GGTTGTGGCGTCAGAGTC(G/C)-NNATAATCTGGGGAGGCGGC-3′ and 5′-GCCGCCTCCCCAGATTAT-NN(G/C)GACTCTGACGCCACAACC-3′ (for Lib-RFQ), 5′-GCGGTCTT-CATATCGCAATGATGCANN(G/C)GAAGGATCCCCTGGTGG-3′ and 5′-CAACCAGGGGATCCTTC(G/C)NNTGCATCATTGCGATATGAAGACCAC-3′ (for Lib-RFQF), and 5′-CCATTGGAGGCTCCNN(G/C)AGCGGTGGCGTA-TTTGAGAAGAATGGCG-3′ and 5′-CGCCATTCTTCTCAAATACGCCA-CCGCT(G/C)NNGGAGCCTCCAATGG-3′ (for Lib-W). PCR conditions were as follows: 95 °C for 30 s, 18 cycles at 95 °C for 30 s, 55 °C for 60 s, and 68 °C for 9.5 min. The PCR products were treated with restriction enzyme *Dpn*I then the mixtures were purified with a Qiagen PCR purification kit before being transformed into BL21(DE3) by electroporation. The transformation and cell cultivation were described previously.[7] Aliquots of the cell extracts were reacted with D-galactose and D-glucose at pH 7.0. The initial rate (for the D-galactose activity assay) or endpoint (for the D-glucose activity assay) of $H_2O_2$ formation was recorded by monitoring the HRP catalyzed oxidation of ABTS at 405 nm on a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA).

**Protein purification and characterization**: *E. coli* cultivation and cell disruption were performed as previously described.[7] The samples were then loaded onto a diethylaminoethyl–cellulose column and eluted with sodium phosphate (NaPi; 50 mM, pH 7.0) at 4 °C. The active fractions were pooled and concentrated in a stirred ultrafiltration cell (Amicon Corp., Beverly, MA). The concentrated samples were dialyzed against NaPi (10 mM, pH 7.3) overnight and loaded onto a cellulose phosphate column. After washing with NaPi (500 mL; 10 mM, pH 7.3), the samples were further eluted with a linear gradient of NaPi (270 mL, 10 mM; 270 mL, 100 mM; pH 7.3). The active fractions were collected and concentrated by ultrafiltration. The concentrated samples were dialyzed against NaPi (100 mM, pH 7.0) overnight prior to storage at −80 °C. The purified protein ran as a single band in SDS-PAGE (Novex, San Diego, CA). Protein concentrations were determined from the absorbance at 280 nm ($\varepsilon = 1.05 \times 10^5\,M^{-1}\,cm^{-1}$) and corrected by a factor of 16/15[5] for M-RQW. The activity of the purified enzyme was measured by using an ABTS–HRP coupled assay in an NaPi solution (1 mL; 50 mM, pH 7.0) that contained HRP (13 U), ABTS (2.6 mg), and D-glucose (250 mM).[7] One unit of activity is the amount of enzyme that is able to produce 1 $\mu$mol product per minute under the reaction conditions.

**Oxidation of methyl-$\beta$-D-galacto-pyranoside, methyl-$\beta$-D-gluco-pyranoside, and 2-butanol**: Oxidation of methyl-$\beta$-D-galacto-pyranoside with native GOase was performed in an NaPi solution (5 mL; 50 mM, pH 7.0) that contained substrate (300 mM), GOase (95 U; Sigma), catalase (700 U; Sigma), and $CuSO_4$ (0.5 mM). Oxidation of methyl-$\beta$-D-gluco-pyranoside with M-RQW was performed in an NaPi solution (2 mL; 50 mM, pH 7.0) that contained substrate (200 mM), mutant (1.8 U), catalase (1700 U), and $CuSO_4$ (0.5 mM). Both reactions were performed at room temperature with vigorous stirring. 2-Butanol oxidation by M-RQW was performed at room temperature in an NaPi solution (100 µL; 50 mM, pH 7.0) that contained substrate (50 mM), $CuSO_4$ (0.5 mM), mutant GOase (0.9 U), and catalase (150 U).

**GC–MS analysis of the 2-butanol oxidation reaction**: GC–MS analysis was performed on an HP 6890 series GC system with an HP 5973 mass-selective detector and an Rtx-1 column (60 m × 0.3 mm × 5 µm, Restek, Belletontane, PA). Helium was used as the carrier gas with a flow rate of 1 mL min$^{-1}$. The mass spectrometer was operated in the scan mode (8.17 scans sec$^{-1}$) for the mass range 40–200 amu. The GC system was temperature programmed as follows: initial temperature 70 °C, raised at 5 °C min$^{-1}$ to 100 °C and at 20 °C min$^{-1}$ to 200 °C. The inlet and transfer line temperatures were both 200 °C.

**TLC and NMR analysis of the methyl-$\beta$-D-galacto-pyranoside and methyl-$\beta$-D-gluco-pyranoside oxidation products**: TLC was used to monitor the oxidation reactions. Chromatograms were developed on silica gel plates (250 µm, Whatman, Maidstone, Kent, England) with chloroform/methanol (4:1) as the solvent. The plates were dried before they were immersed in Bial's reagent (orcinol/ferric chloride with 3 volumes of ethanol; Sigma)[12] and visualized by heating at 120 °C for 5 min. Aldehydes were visualized by spraying the TLC plates with Purpald solution (Sigma; 2% in 1 M NaOH).[13]

A silica gel column (40 µm flash, Baker, Phillipsburg, NJ) was used to isolate the reaction products. The reaction mixtures were filtered by using centrifugal filter devices (Millipore, Bedford, MA) to remove the enzymes. The filtered reaction mixtures (10 mL) were loaded into the column directly and eluted with chloroform/methanol (4:1) and the eluted fractions were checked with TLC plates. The fractions that contained product were pooled and the solvent was evaporated. The crystallized samples were dissolved in $D_2O$ and stored at 4 °C. Purpald reagent was used to monitor the formation of aldehydes.

$^1$H 1D and 2D COSY NMR spectra and $^{13}$C NMR spectra were recorded on a Varian-500 instrument. Methyl-$\beta$-D-6-aldo-gluco-pyranoside: $^1$H NMR (500 MHz, $D_2O$, 300 K): $\delta = 5.28$ (d, 1H, H-1), 4.4 (d, 1H, H-6), 3.6 (s, 3H, $CH_3$), 3.52 (m, 2H, H-3, H-4), 3.42 (m, 1H, H-2), 3.29 (m, 1H, H-5) ppm; $^{13}$C NMR (75 MHz, $D_2O$, 300 K): $\delta = 103.6$ (C-1), 88.1 (C-6), 76.67 (C-5), 75.81 (C-3), 73.14 (C-2), 70.32 (C-4), 57.43 ($CH_3$) ppm.

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