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Expression and Characterization of a Recombinant *Fusarium* spp. Galactose Oxidase

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Abstract

The *Fusarium* spp. *(Dactylium dendroides)* galactose oxidase was expressed in *Aspergillus oryzae* and *Fusarium venenatum* hosts. Under the control of an *A. niger* α-amylase or a *Fusarium* trypsin promoter, high level galactose oxidase expression was achieved. The recombinant oxidase expressed in the *A. oryzae* host was purified and characterized. The purified enzyme had a molecular weight of 66 kDa on sodium dodecyl sulfate-polymerase gel electrophoresis (SDS-PAGE) and 0.4 mol copper atom per mole protein. The stoichiometry increased to 1.2 after a Cu saturation. Based on a peroxidase-coupled assay, the enzyme preparation showed an activity of 440 turnover per second toward D-galactose (0.1 *M*) at pH 7 and 20°C. The enzyme had an optimal temperature of 60°C at pH 6.0 and an activation free Gibbs energy of 33 kJ/mol. A series of D-galactose derivatives was tested as the reducing substrate for the oxidase. The difference in activity was interpreted by the stereospecificity of the oxidase toward the substituents in the pyranose substrate, particularly on the C5 and the cyclic hemiacetal O sites. The recombinant oxidase could act on some galactose-containing polysaccharides, such as guar gum, but was not able to oxidize several common redox compounds that lacked a primary alcohol functional group.

Index Entries: Galactose oxidase; expression; specificity; *Fusarium*.

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Introduction

Galactose oxidase (GOase; EC. 1.1.3.9) is a Cu-containing enzyme that catalyzes the oxidation of primary alcohols (particularly the OH group at the C6 site in D-galactose [Gal]) to aldehydes with the concomitant reduction of O_2 to H_2O_2. Extensive physical and chemical characterizations, including X-ray crystal structure determination and site-directed mutagenesis, have elucidated the fundamental aspects of the structure-function relationship of the enzyme *(1–6)*. Recently, GOase is receiving increased attention for its potential applications as a diagnostic reagent or industrial biocatalyst *(5)*. Attractive industrial applications of GOase involve the biotransformation of glyco-biopolymers (glycolipids, glycoproteins, polysaccharides) into desirable materials such as sweeteners, flavorants, or paper strength additives *(7)*. The high stereospecificity, mild operation conditions, and environmental-friendliness of a GOase-based bio-oxidative system holds advantages over conventional chemical systems that are often hard to control, non-specific, costly, or hazardous.

To further explore its potential for biotechnological applications, we investigated the heterologous expression and the stereospecificity of *Fusarium* spp. (*Dactylium dendroides*) GOase with approaches that were different from those reported in previous literature. Our *Aspergillus oryzae* and *Fusarium venenatum* expression systems produced recombinant GOase (rGOase) at levels significantly higher than those of the *A. nidulans* and *Escherichia coli* hosts previously reported *(8)*. Our kinetic study of a series of D-Gal derivatives showed a high stereospecificity at cyclic hemiacetal O site (in addition to the well-known C3, C4, C5, and C6 sites) for the pyranose-GOase interaction.

Materials and Methods

Materials and Instruments

Chemicals used as buffers and substrates were commercial products of at least reagent grade. Horseradish peroxidase (HRP) was from Sigma (St. Louis, MO, USA) (type VI-A, 1310 U/mg) and recombinant *Coprinus cinereus* peroxidase (CiP) was purified in our laboratories. The protocols for molecular biology experiments (including restriction digests, DNA ligations, gel electrophoresis, and DNA preparations) were adapted from either the instructions of the manufacturer (e.g., Stratagene; La Jolla, CA, USA) or standard procedures. All oligonucleotides were synthesized with an Applied Biosystems (Foster City, CA, USA) 294 DNA/RNA synthesizer. DNA sequence was determined using Prizm dye terminator chemistry on an Applied Biosystems 377XL DNA sequencer. A primer walking strategy was used to generate the nucleotide sequence of entire genes. The polymerase chain reaction (PCR) was performed on a Perkin Elmer (Foster City, CA, USA) 9600 thermocycler. *N*-terminal amino acid sequencing was carried out in an Applied Biosystems 476A Protein Sequencer following SDS-

PAGE and electroblotting onto PVDF membrane. The spectral data were recorded on either a Shimadzu (Columbia, MD, USA) UV160U spectrophotometer with 1-cm quartz cuvet, a Hewlett-Packard (Mountain View, CA, USA) 8452A diode array spectrophotometer, or a microplate (Molecular Devices Thermomax; Sunnyvale, CA, USA) reader in 96-well (Costar, serocluster grade; Cambridge, MA, USA) microplates. Chromatography was done with a Pharmacia FPLC (Piscataway, NJ, USA) and electrophoresis was made with a Bio-Rad (Hercules, CA, USA) or a Novex (San Diego, CA, USA) cell and ready gels. The differential calorimetry scanning was made on a MicroCal VP-DSC instrument (Northampton, MA, USA).

Enzymic Assays

GOase activity was measured by a coupled assay in which the H_2O_2 generation [from D(+)-Gal and O_2 under the catalysis of GOase] was detected by the oxidation of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) under the catalysis of a peroxidase such as HRP *(8)* or CiP. Typical assay solutions contained 0.06–0.1 *M* Gal, 17 μg/mL HRP or 25 μg/mL CiP, 1 m*M* ABTS, 0.1 *M* sodium phosphate (pH 7.0) or 0.4 *M* phosphate-citrate buffer (pH 6.0), and GOase capable of producing 0.03–3 U of absorbance change at 405 nm in 1-cm path length cuvet. The extinction coefficient of 105 mM^{-1}/cm at 280 nm *(8)* was used to calculate the specific activity. The plate assay applied for screening active transformants was made on minimal media plates containing 1% maltose (as the carbon source) and 2.5 m*M* *o*-anisidine. Transformants were grown on the plates and after 3 d, the plates were flooded with solution containing 20 m*M* Na-phosphate, pH 7.0, 100 m*M* Gal, and 20 μg/mL HRP. After 30 min at room temperature, the plates were decanted and incubated at room temperature overnight for purple color development.

The pH-activity profile was determined in phosphate-citrate buffer or Britton and Robinson buffer (pH 2.7–11, made by mixing 0.1 *M* boric acid, 0.1 *M* acetic acid, 0.1 *M* phosphoric acid with 0.5 *M* NaOH to desired pH). To measure the thermal activity, GOase in 0.4 *M* phosphate/citrate buffer (pH 6.0, 0.45 mL) was incubated with 60 m*M* D-Gal at various temperatures. After 5 min, the solution was chilled on ice and the accumulated H_2O_2 was determined by mixing the solution with 0.45 mL 25 μg/mL CiP and 1 m*M* ABTS. To measure the pH stability, GOase was incubated in 0.15 *M* phosphate/citrate buffers of pH 3.0–9.0 at 30°C for 2 h before being diluted 10-fold and assayed at 30°C with 60 m*M* D-Gal in 0.4 *M* phosphate/citrate buffer of pH 6.0. To measure the thermal stability, 7 n*M* GOase in 0.1 *M* sodium phosphate, pH 7.0, was incubated at 20, 34, 42, 50, 60, and 68°C, respectively, for 30 min before being assayed at 25°C with 0.1 *M* D-Gal in 0.1 *M* sodium phosphate, pH 7.0.

The effect of exogenous Cu(II) in solution on GOase activity was assayed in 20 m*M* PIPES-NaOH, pH 6 with the presence of 0, 0.2, or 5 m*M* $Cu(NO_3)_2$. The $K_m(O_2)$ was determined with an Oxi3000 Oximeter (MTW)

O_2 electrode at 30°C. The solution contained 60 m*M* D-Gal in 4 mL 0.15 *M* phosphate/citrate buffer of pH 6.0.

Fusarium *GOase Gene Isolation*

The full-length GOase gene was amplified from *Fusarium* spp. (NRLL 2903) genomic DNA. The two synthetic oligonucleotide primer pairs used to amplify the gene were atgATGAAACACCTTTTA ACACTCGCT and ttgggcccTCACTGAGTAACGCGAATCGTCGA, atttaaatATGAAACACCTTTTAACACTCGC and ttaattaaTCACTGAGT AACGCGAATCGTCG (capital letters: coding sequence). About 50 pmoles of each primer were used in a PCR reaction containing 1 µg of *Fusarium* genomic DNA, 1X Pwo buffer (Boehringer; Indianapolis, IN, USA), 2 µL of 10 m*M* blend of dATP, dTTP, dGTP, and dCTP (Perkin Elmer), and 1.25 U Pwo polymerase (Boehringer). The amplification conditions were one cycle at 94°C for 2 min, 55°C for 30 s, and 72°C for 1 min; 9 cycles at 94°C for 15 s, 55°C for 30 s, and 72°C for 1 min; 15 cycles at 94°C for 15 s, 55°C for 30 s, and 72°C for 1 min, with an extension of 20 s/cycle; and a final cycle of 94°C for 15 s, 55°C for 30 s, and 72°C for 7 min. The reaction was chilled with a 4°C soak cycle. The PCR amplification created two different 2-kb products that contained the GOase gene as verified by DNA sequencing on both strands using the primer walking technique with dye-terminator chemistry. The reaction products were isolated on a 1% agarose gel and the DNA bands were excised and purified using Qiaex II (Qiagen; Valencia, CA, USA).

Expression in A. oryzae

The 2 kb PCR product that had a 5' ATG and 3' *Apa*I site was digested with *Apa*I and cloned into pMWR3SAN *(8)* resulting in the expression plasmid pEJG23 (Fig. 1). It contained the *A. oryzae* α-amylase (TAKA) promoter and terminator, and was co-transformed with pToC90 (containing *amdS* selection marker) into a protease-deficient *A. oryzae* host strain JaL 228 (kindly provided by J. Lehmbeck of Novo Nordisk A/S) using methods previously reported *(9)*. Based on the growth on COVE medium using acetamide as sole nitrogen source, 39 transformants were recovered, regrown on individual COVE plate, then transferred to a GOase indicator plate. The untransformed host was used as control. Positive transformants (11) were detected by the brown color development on the agar plate and spore purified by patching isolated colonies to new COVE plates twice in succession. Spore stocks of each positive transformant were made with 0.008% Tween-20. One milliliter of each spore stock, along with the untransformed host, was added to 20 mL MY50/carbenicillin (50 µg/mL) in 125-mL glass shake flasks and incubated at 34°C under 150 rpm agitation. After 4, 6, and 7 d of incubation, 500 µL culture broth was removed from each flask and assayed for GOase activity.

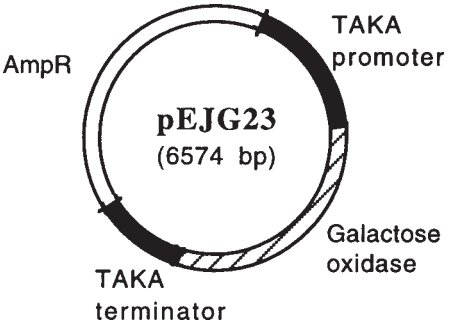

Fig. 1. The plasmid pEJG23 for the expression of GOase in *A. oryzae*.

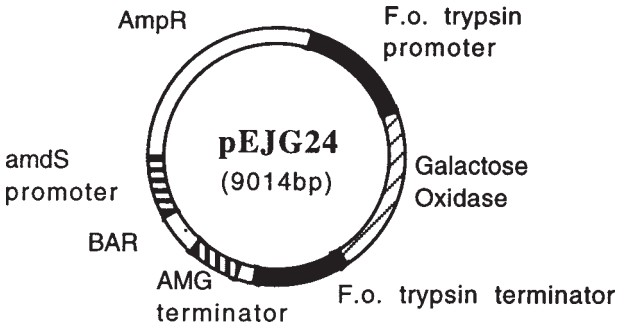

Fig. 2. The plasmid pEJG24 for the expression of GOase in *F. venenatum.*

Expression in Fusarium venenatum

The 2-kb PCR product containing a 5' *Swa*I and a 3' *Pac*I site was first cloned into the pCR-Blunt plasmid obtained from Invitrogen (Carlsbad, CA, USA). After digesting with *Swa*I/*Pac*I, a 2-kb reaction product was isolated and then cloned into pDM181, resulting in the expression plasmid pEJG24 (Fig. 2). The plasmid, containing *F. oxyporum* trypsin promoter and terminator as well as BAR selectable marker, was introduced into *F. venenatum* host CC1-3 using methods previously reported *(10)*. Based on the growth on phosphinotricine BASTA (a phosphorylated α-aminobutyric acid-based herbicide from Hoechst; Strasburg, France), 61 transformants were recovered and then grown on individual Vogel's plate containing NO_3^- and BASTA at room temperature for two weeks in plastic bags. The transformants were grown on fresh Vogel's plates containing NO_3^- and BASTA for 1 wk at room temperature in plastic bags. A plug from each transformant or the CC1-3 control was then inoculated into individual plastic 125-mL shake flasks containing 30 mL M400Da (50 g/L of maltodextrin, 2 g/L of $MgSO_4$, 2 g/L of KH_2PO_4, 4 g/L of citric acid, 2 g/L of urea, 1 mL/L of COVE trace metals), and incubated at 30°C, for 7 d with 150 rpm agitation.

Purification

The purification comprised ultrafiltration, anion-exchange, and cation-exchange chromatography. The cell-free culture broth (pH 7.1, 23 mS conductivity) was filtered with Whatman #2 paper and subjected to an ultrafiltration on an Amicon (Bedford, MA, USA) Spiral-Concentrator (S1Y10 membrane cartridge). The washed/concentrated broth (pH 7.1, 1 mS conductivity) was then loaded onto a Q-Sepharose column (XK26, 180 mL gel, preequilibrated with 10 m*M* Tris-HCl, pH 7.5 [buffer A]). The active fraction was eluted by a linear gradient of buffer A plus 2 *M* of NaCl (buffer B). After being pooled, washed (to 1 mS conductivity), and concentrated, the GOase enriched solution was applied to an SP-Sepharose column (XK-16, 60 mL gel, preequilibrated with buffer A). The elution of the weakly bound GOase by buffer B yielded a GOase preparation with apparent electrophoretic homogeneity as judged by SDS-PAGE.

The photometric Cu titration was made with 2,2'-biquinoline *(11)*. The Cu saturation of GOase was carried out by incubating 0.7 m*M* GOase with 3.2 m*M* $Cu(NO_3)_2$ in 20 m*M* PIPES-NaOH, pH 6.0, at 25°C for 3 h *(8)*.

Results and Discussion

Heterologous Expression of GOase

The *Fusarium* GOase was successfully expressed and secreted in our *F. venenatum* host, under the control of the *F. oxysporum* trypsin promoter, and in our *A. oryzae* host, under the control of the TAKA-amylase promoter. The GOase expressed in *A. oryzae* was purified to apparent electrophoretic homogeneity by sequential ultrafiltration, anion-exchange, and cation-exchange chromatography. Overall a 44% recovery yield and an 89-fold purification were obtained. Based on the activity, a yield near 0.1 g/L at shake-flask level was found for the expression in *A. oryzae* host. The yields were considerably higher than the 0.005–0.05 g/L yields reported for the expression of GOase in *A. nidulans* or *E. coli (8)*, and higher than the heterologous expression of another Cu protein family, laccase, in *A. oryzae*, *Trichoderma reesei*, or *Saccharomyces cerevisiae (9,12,13)*. Our result with *F. venenatum* indicated the potential of this novel host for general heterologous expression *(10)*.

Molecular Properties of the Purified GOase

On SDS-PAGE, rGOase showed a band at approx 66 kDa. Because a 68.5 kDa has been deduced from the DNA sequence and observed for the wild-type GOase *(14)*, the rGOase was likely not glycosylated by either host. *N*-terminal sequencing showed a terminus of APIGSAIS–. Comparing with the predicted DNA sequence and the protein sequence determined for the wt-GOase, it seemed that a pro(di)peptide (AS–) was processed by the *A. oryzae* host for the maturation of rGOase.

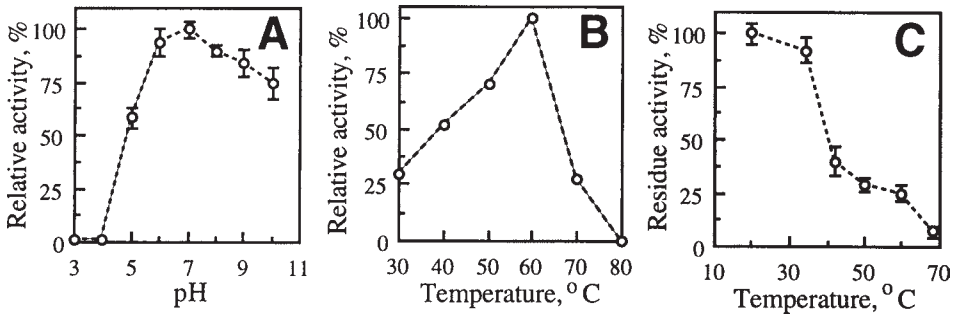

Fig. 3. **(A)** The pH dependence of rGOase activity. **(B)** The thermal activity of rGOase. **(C)** The thermal stability of rGOase.

The solution of the rGOase purified from the *A. oryzae* host had a pink-greenish appearance. In 10 m*M* Tris-HCl, pH 7.7, the rGOase showed a UV-visible spectrum with maxima at 272, 436, and 625 nm. The relative absorbances at these wavelengths were 127, 1.0, and 1.1, respectively, suggesting a reduced state *(5)*. The biquinoline titration yielded a 0.4 Cu/protein stoichiometry. After an incubation with $Cu(NO_3)_2$ *(8)*, a 1.2 Cu/protein stoichiometry was observed. The low Cu content before the saturation might arise from an insufficient/ineffective Cu incorporation during the fermentation or from a Cu loss during the purification.

Enzymatic Properties of rGOase

As shown in Fig. 3A, rGOase has an optimal pH of 7.0 as assayed by the coupled HRP/ABTS method, similar to the value reported for the wild-type enzyme *(3)*. The enzyme had an apparent K_m(D-Gal) of 83 m*M* and an apparent $K_m(O_2)$ of 0.22 m*M*, which were smaller than the values reported for the wild type *(3)*. Assuming that the oxidation of each galactose molecule or the concomitant production of one H_2O_2 molecule was coupled to the generation of two $ABTS^+$ radicals, the pre- and post-Cu-saturated rGOase oxidized galactose (0.1 *M*) at 200 and 440 turnover/s, respectively, at pH 7.0 and 20°C. The addition of up to 5 m*M* $Cu(NO_3)_2$ into the assay solution of the purified rGOase did not yield an immediate increase of activity, indicating the need for prolonged incubation (3 h) to incorporate Cu(II) into the apo-GOase. Figure 3B shows the thermal activity of rGOase at pH 6. From 30 to 60°C (the optimal temperature), the rate increase corresponded to an apparent activation free energy $\Delta G^{\neq}$ of 33 kJ/mol. At the same pH, differential calorimetry scanning yielded a phase-transition (protein unfolding) temperature T_d of 70°C. rGOase was stable at 30°C at pH 6.0–8.5 for at least 2 h. However, the thermal stability of rGOase was rather low (Fig. 3C). Although GOase is very resistant to chemical denaturation (such as by 6 *M* urea *[5]*), its thermal stability was apparently quite low.

An apparent activation of rGOase by CiP was observed. The continuous monitoring of rGOase/Gal-generated H_2O_2 by the coupled CiP/ABTS method yielded an apparent rate, measured by both $ABTS^+$ generation or

Table 1
Relative Activity (%) of rGOase Toward Various Reducing Substrates

D(+)-Galactose (0.1 *M*)	100	α-D-Talose (0.1 *M*)	67
CH_3-β-D-Galactoside (60 m*M*)	148	D-Mannose (60 m*M*)	<0.02
Galactosamine (50 m*M*)	46	D-Xylose (60 m*M*)	0.3
Gal-6-PO_4Na_2 (0.1 *M*)	<0.02	L-Glucose (0.1 *M*)	0.3
Gal-6-SO_3Na (0.1 *M*)	0.4	L-Gulose (0.1 *M*)	<0.02
Gal-4-SO_3Na (0.1 *M*)	0.4	D-Psicose (89 m*M*)	<0.02
D(+)-Glucose (0.1 *M*)	0.2	D-Fructose (60 m*M*)	<0.02
CH_3-α-D-Glucopyranoside (60 m*M*)	2.5	L-Sorbose (60 m*M*)	<0.02
CH_3-β-D-Glucopyranoside (60 m*M*)	1.5	Raffinose (60 m*M*)	103
2-Deoxy-D-Glc (60 m*M*)	<0.02	β-Lactose (0.1 *M*)	15
Glc-6-PO_4Na_2 (60 m*M*)	<0.02	Sucrose (0.1 *M*)	0.3
Polygalacturonic acid (0.167% w/v)	<0.02	Maltose (0.1 *M*)	0.2
Locust bean gum (0.167% w/v)	<0.02	D(+)-Cellobiose (0.1 *M*)	0.2
Guar gum (0.167% w/v)	4.1	Methyl syringate (2 m*M*)	<0.02
o-$(C_6H_4)(NH_2)_2$ (2 m*M*)	0.02	$C_6H_5CH_2NH_2$ (0.9 *M*)	<0.02
10-$C_2H_5CO_2H$-phenathiozine (2 m*M*)	<0.02	Acetone (0.1 *M*)	<0.02
L-Lysine methyl ester (0.15 *M*)	<0.02	ABTS (2 m*M*)	0.3

O_2 consumption, 13-fold higher than that observed by the "end-point" method in which CiP/ABTS was added to the assay solution 5 min after the rGOase-catalyzed D-Gal oxidation started. This effect was caused by the peroxidase alone. It has been previously reported that various oxidants, such as peroxidase and $K_3Fe(CN)_6$, could activate GOase *(5)*. However, $K_3Fe(CN)_6$ did not activate rGOase under our conditions.

Specificity for the Reducing Substrates

Table 1 shows the relative activity of rGOase toward various Gal derivatives and alternative reducing substrates. The activity of D-talose (Tal) was 67% of that of its C2 epimer D-Gal, similar to the value reported for wt-GOase (*5*). The relatively high activity of D-Tal or D-galactosamine indicated that the stereo configuration of the –OH or the substitution by $–NH_2$ at the C2 site had only minor effect on the activity of rGOase. In contrast, the low activities of D-glucose (Glc), 2-deoxy-D-Glc, D-mannose, or D-Gal-4-SO_3Na indicated that the stereo configuration of the –OH or the substitution by $–SO_3^-$ at the C4 site had a major effect. The low activities of D-Gal-6-SO_3Na and D-Gal-6-PO_4Na_2 (as well as D-Glc-6-PO_4Na_2) indicated that the substitution of the –OH at the C6 site by the bulky, charged SO_3^- or PO_4^{2-} inactivated the *pro-S* C6 H abstraction ability of rGOase, likely due to the unfavorable substitution effect on the steric/electrostatic complementarity. The significant activities of CH_3-galactoside and raffinose (as well as lactose and guar gum) indicated that rGOase could oxidize Gal derivatives with bulky substitutes at the C1 site.

By superposing their cyclic hemiacetal O atoms at the C4 site of D-pyranose, α-L-Glc or α-L-gulose (Gul) could adapt respectively a chair con-

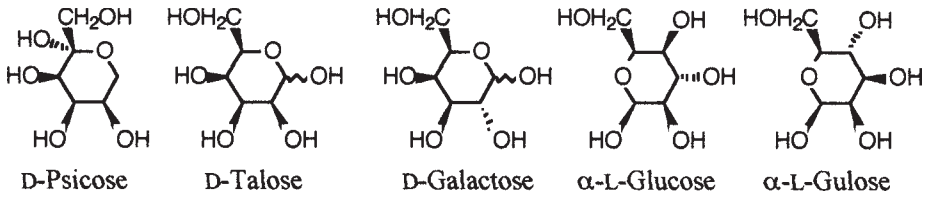

Fig. 4. The structure of the carbohydrate substrates tested for rGOase.

figuration similar to 4-deoxy-α- or 4-deoxy-β-D-Tal in terms of their OH stereo configurations at the sites corresponding to the C1, C2, C3, and C6 sites in D-Tal (Fig. 4). A docking of α-L-Glc and α-L-Gul by such orientation in the substrate pocket of GOase might reveal the space/electrostatic complementarity at the C4 and the cyclic hemiacetal O sites between D-Gal (or D-Tal) and GOase. Because 4-deoxy-D-Gal can be 60% as active as D-Gal *(3)*, the effect of positioning the cyclic hemiacetal O of α-L-Glc and α-L-Gul at the C4 site of D-Tal (or D-Gal) should be small. Hence the low activities of α-L-Glc and α-L-Gul could be attributed to their C4 centers that would take the cyclic hemiacetal O site in D-Gal as shown in Fig. 4: In this case, the H-bond found between the W290 of GOase and the cyclic hemiacetal O of D-Gal would be lost and the stereo projections of the C4-OH in α-L-Glc and α-L-Gul would cause severe steric hindrances inside the substrate pocket, because normally the hemiacetal O of D-Gal is in close van der Waals contact with the hydrophobic wall of the substrate pocket in GOase *(15)*. A molecular graphic analysis showed that although the cyclic O of a docked D-Gal points toward the solvent medium, its replacement by a C-OH would lead to a steric hindrance between the OH and either Y329 or F194 of GOase depending on the projection of the OH.

Being a ketohexose, D-psicose (Psi) can cyclize into a pyranose. As shown in Fig. 4, the "psicopyranose" could mimic 1-deoxy-D-Tal except that the former has a "C5"-OH. Lack of an OH group at the C1 site in D-Tal (or D-Gal) is not expected to significantly impact the substrate activity *(6)*. If D-Psi could bind to GOase by the D-Tal-mimicking chair configuration shown in Fig. 4, then its low activity might be attributed to the steric effect of its OH (derived from the carbonyl) at the site corresponding to the C5 site in D-Gal, similar to the severe steric hindrance between the C6-CH_2OH in L-Gal and the substrate pocket in GOase *(6)*. The results on all the monosaccharides tested indicated that the stereospecificity of GOase toward pyranoses is highly sensitive at the C3, C4, C5, C6, and the cyclic hemiacetal O sites, but less so at the C1 and C2 sites, as previously proposed *(5,16)*.

In addition to the mono- and disaccharides, we also tested the activity of rGOase on various non-carbohydrate redox compounds, including aliphatic/aromatic amine/hydroxy and heterocyclic compounds. Under our conditions, rGOase exhibited very poor activity in oxidizing these substances (along with other carbohydrates that had configurations significantly different from that of D-gal), similar to the results published before

(3). This is consistent with the mechanism in which a high degree molecular recognition between the reducing substrate and GOase is pre-requisite and that the substrate oxidation is controlled by the (*pro-S*) C-H bond cleavage at the C6 site *(15)*. The stereospecificity and methylene H abstraction mechanism, combined with the low redox potential at the Cu site in GOase (0.3 V *[3]*), apparently preclude GOase from being a "blue" Cu oxidase-type enzyme capable of oxidizing nonprimary alcohol redox compounds using an electron transfer-controlled mechanism.

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