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A Novel *Colletotrichum graminicola* Raffinose Oxidase in the AA5 Family

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ABSTRACT We describe here the identification and characterization of a copper radical oxidase from auxiliary activities family 5 (AA5_2) that was distinguished by showing preferential activity toward raffinose. Despite the biotechnological potential of carbohydrate oxidases from family AA5, very few members have been characterized. The gene encoding raffinose oxidase from *Colletotrichum graminicola* (CgRaOx; EC 1.1.3.—) was identified utilizing a bioinformatics approach based on the known modular structure of a characterized AA5_2 galactose oxidase. CgRaOx was expressed in *Pichia pastoris*, and the purified enzyme displayed the highest activity on the trisaccharide raffinose, whereas the activity on the disaccharide melibiose was three times lower and more than ten times lower activity was detected on D-galactose at a 300 mM substrate concentration. Thus, the substrate preference of CgRaOx was distinguished clearly from the substrate preferences of the known galactose oxidases. The site of oxidation for raffinose was studied by ¹H nuclear magnetic resonance and mass spectrometry, and we confirmed that the hydroxyl group at the C-6 position was oxidized to an aldehyde and that in addition uronic acid was produced as a side product. A new electrospray ionization mass spectrometry method for the identification of C-6 oxidized products was developed, and the formation mechanism of the uronic acid was studied. CgRaOx presented a novel activity pattern in the AA5 family.

IMPORTANCE Currently, there are only a few characterized members of the CAZy AA5 protein family. These enzymes are interesting from an application point of view because of their ability to utilize the cheap and abundant oxidant O₂ without the requirement of complex cofactors such as FAD or NAD(P). Here, we present the identification and characterization of a novel AA5 member from *Colletotrichum graminicola*. As discussed in the present study, the bioinformatics approach using the modular structure of galactose oxidase was successful in finding a C-6 hydroxyl carbohydrate oxidase having substrate preference for the trisaccharide raffinose. By the discovery of this activity, the diversity of the CAZy AA5 family is increasing.

KEYWORDS CAZy AA5, galactose oxidase, nuclear magnetic resonance, NMR, carbohydrate, EC 1.1.3.—

Enzymes that cleave or form oligo- and polysaccharides are classified in the sequence-based carbohydrate-active enzyme database (CAZy [<http://www.cazy.org>]). The sequences are linked to information about functionality (e.g., substrate specificity) and three-dimensional structure. The CAZy database was recently expanded to account for redox enzymes, leading to the auxiliary activities (AA) group. Currently, 13 AA families exist, comprising lignolytic oxidoreductases (families AA1, -2, -4, and -6), lytic polysaccharide monoxygenases (families AA9, -10, -11, and -13), carbohydrate

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oxidases (families AA3, -5, -7, and -8), and one family with unknown functionality (family AA12) (1).

Carbohydrate oxidases that employ molecular oxygen as the terminal electron acceptor are particularly interesting from an application point of view because of their ability to combine the cheap and abundant oxidant O₂ with high specificity and regioselectivity toward carbohydrate substrates. The biochemically characterized carbohydrate oxidases are mostly discovered in fungi. Their biological function remains largely unknown, although in many cases it has been linked to production of hydrogen peroxide for peroxidases involved in lignin degradation and, for supplying electrons, together with dehydrogenases, for lytic polysaccharide monoxygenases (2). Most of the known carbohydrate oxidoreductases oxidize the anomeric C-1 of mono- and disaccharides and contain a FAD cofactor (3). In the flavoprotein containing AA3 family, glucose oxidases (EC 1.1.3.4) are the best characterized members due to their use as industrial biocatalysts and diagnostic reagents (4).

In recent years, enzymes capable of oxidizing C-1 in different glucooligosaccharides have also been described, e.g., the AA7 glucooligosaccharide oxidase (EC 1.1.3.—) from *Sarocladium strictum* (former name *Acremonium strictum*). This FAD-containing enzyme reduces O₂ to hydrogen peroxide and oxidizes the C-1 of glucose, maltose, lactose, cellobiose and cello-, malto-, and xylooligosaccharides yielding the corresponding lactone/acid (5, 6). Other characterized oligosaccharide oxidases include the lactose oxidase from *Microdochium nivale* (3, 7), celooligosaccharide oxidases from *Sarocladium oryzae* (8) and *Paraconiothyrium* sp. (9), and chitooligosaccharide oxidase from *Fusarium graminearum* (10). Pyranose dehydrogenase (PDH; EC 1.1.99.29) and pyranose oxidases (EC 1.1.3.10) are able to oxidize secondary hydroxyl groups in carbohydrates into corresponding ketones (11, 12). PDH catalyzes the oxidation of a number of mono-, di-, and oligosaccharides in pyranose form. Depending on the source of the enzyme and structure of the substrate, PDH oxidizes mainly the C-2 and C-3, although oxidation at C-1 and dioxidation at C-2,3 or C-3,4 have also been reported (12–14).

Presently, the only enzyme reported to selectively oxidize primary hydroxyls in carbohydrates is galactose oxidase (GaOx, EC 1.1.3.9), which is a single copper metalloenzyme secreted by the fungus *Fusarium* spp. Although GaOx oxidizes a variety of primary alcohols, it displays strict regiospecificity to carbohydrates, oxidizing solely the C-6 of D-galactose to the corresponding aldehyde (15). Interestingly, galactose oxidase also acts on terminal, nonreducing galactoses in oligo- and polysaccharides (16). Because of its potential in numerous applications, galactose oxidase has been a subject for intense studies for many years (17–25). For example, galactosylated polysaccharides (e.g., galactoglucomannan and galactoxyloglucon) oxidized by galactose oxidase have been used in the preparation of thermally stable hydrogels (26) and novel aerogels (27).

The AA5 family comprises two subfamilies, AA5_1 and AA5_2. Glyoxal oxidase activity (EC 1.2.3.15) clusters within the subfamily AA5_1, and it oxidizes a number of aldehydes and α -hydroxy carbonyl compounds (28, 29). In contrast, galactose oxidase activity exemplified the AA5_2 subfamily, until the recent discovery of two novel alcohol oxidases (AlcOx) revealed new functionalities in the AA5_2 family (30).

The three-dimensional structure of galactose oxidase from *Fusarium graminearum* (FgGaOx) comprises a three-domain, mainly β -sheet structure, with a single copper ion (18). The N-terminal domain I is a β -sandwich consisting of eight β strands in a jelly-roll motif (31) and is reported to be a family 32 carbohydrate binding module predicted to bind galactose (31, 32). This domain is located distant from the copper site but is associated with the N-terminal end of domain II. Domain II, which is the largest, has a seven-bladed β -propeller fold and contains the active site located close to the solvent-exposed surface. Three of the four copper ligands (Tyr-272, Tyr-495, and His-496) are provided by domain II. The C-terminal domain III has a long finger of two antiparallel β strands that penetrates into the middle cavity of the domain II propeller and provides the fourth ligand for the copper (His518). Thus, the copper site is formed by elements from both domains II and III. The two-electron transfer reaction catalyzed by FgGaOx is performed by a thioether-bridged tyrosylcysteine residue

TABLE 1 Selected putative carbohydrate oxidase sequences

UniProt accession no.	Organism	Domain organization	Natural variation(s) in the native sequence
G4NG45	<i>Magnaporthe oryzae</i>	WSC+ Kelch + DUF1929	R330Y, W290F, Q406T, and P463L
E3R0R1	<i>Colletotrichum graminicola</i>	PAN + Kelch + DUF1929	W290Y, Q406S, and P463G
Q2U1I2	<i>Aspergillus oryzae</i>	F5_F8_typeC + Kelch + DUF1929	Q406D
Q4WH00	<i>Aspergillus fumigatus</i>	F5_F8_typeC+ glyoxal oxidase N + DUF1929	R330H and Q406E

formed between Tyr272 and Cys228, which is coordinated directly to the copper ion in the active site (18).

Here, we used a bioinformatics approach to screen for novel carbohydrate oxidases. Since we were looking for an enzyme oxidizing the primary hydroxyl group, galactose oxidase was used as a template in the screen. By utilizing the modular structure, as well as essential catalytic and substrate interacting residues of *FgGaOx*, a putative carbohydrate oxidase from a plant-pathogenic fungus *Colletotrichum graminicola* was identified. The enzyme (*CgRaOx*) was purified from recombinant expression in *Pichia pastoris*, and its characterization revealed a unique substrate profile that further diversified the catalytic functionality of the AA5_2 subdivision.

RESULTS

Selection of carbohydrate oxidase targets. When beginning our study, biochemically characterized enzymes from the AA5_2 subfamily were exclusively from *Fusarium* species and displayed galactose oxidase activity, which oxidizes the C-6 hydroxyl of galactose. Particularly, the *Fusarium graminearum* (also known as *Gibberella zeae*) galactose oxidase sequence (I1S2N3, GAOA_GIBZE, *FgGaOx*) was chosen as the template to search for novel AA5_2 activities within publicly available sequence databases. According to the Pfam database (<http://pfam.xfam.org/>) three Pfam-A domains are recognized in *FgGaOx*: a F5_F8_typeC domain (PF00754) associated with the N-terminal domain, a Kelch_1 domain (PF01344), and a DUF1929 domain (PF09118) mostly found in sugar-utilizing enzymes. The seven bladed β -propeller structure of *FgGaOx* contains seven tandem Kelch sequence motifs, in which each motif forms a single four-stranded β -sheet, or a propeller blade (18, 31).

The active site with the essential copper is located in the second of the three domains, which is composed of repetitive Kelch motifs. A stretch of 22 residues from the DUF1929 domain forms a finger penetrating the propeller axis and providing the fourth copper ligand. Thus, the presence of the two domains forming the active site is a prerequisite for a functional enzyme. We used the Pfam database to search for proteins with similar domain architecture as *FgGaOx* and found 16 sequences with identical architecture. These sequences, all from various *Fusarium* species, most probably encoded proteins very similar to the known *FgGaOx* and were therefore excluded from this study. Instead, 70 sequences having different combinations of the three Pfam domains found in *FgGaOx* (PF00754, PF01344, and PF09118), as well as the Pfam domain found in the N-terminal domain of glyoxal oxidase (PF07250), were collected. From this collection, those that lacked residues predicted to act as copper ligands or else lacked the cysteine involved in the Tyr-Cys thioether bond were excluded from the set. Previous protein engineering and docking studies of *FgGaOx* identified Arg330, Phe464, Gln406, Phe194, Trp290, Tyr405, and Pro463 as potentially important for substrate binding (24, 33, 34). Therefore, sequences that differed at one or more of these positions, relative to the *FgGaOx* residues, were selected.

Altogether, four sequences were chosen for further studies: those from *Magnaporthe oryzae* (G4NG45), *Aspergillus oryzae* (Q2U1I2), *Aspergillus fumigatus* (Q4WH00), and *Colletotrichum graminicola* strain M1.001/M2/FGSC 10212 (E3R0R1). They share 34, 45, 52, and 30% protein sequence identity, respectively, with *FgGaOx*. All four sequences have a three domain structure and contain the DUF1929 domain as the C-terminal domain (Table 1). Two of the sequences were listed in the CAZy database in the AA5 family (G4NG45 and E3R0R1). Q2U1I2 and Q4WH00 have an F5_F8_typeC (PF00754)

N-terminal domain, similar to *FgGaOx*, whereas in *G4NG45* the domain was replaced by a WSC (PF01822) domain that has been suggested to be involved in carbohydrate binding. In *E3R0R1*, a PAN_1 domain (PF00024) suggested to be involved in either protein-protein interaction or protein-carbohydrate interactions was positioned at the N terminus. In the central domain, the Kelch motifs were found in all sequences, except in *Q4WH00*, where it was replaced by the glyoxal oxidase-N domain. The sequences had natural variations in their native sequence at one to four positions suggested as important for substrate binding (Table 1).

The signal sequences of the four chosen putative carbohydrate oxidase encoding genes were removed and replaced by the *Saccharomyces cerevisiae* α -factor signal peptide. The enzymes were expressed in *Pichia pastoris* in shake flask cultivations, and the buffer-exchanged culture supernatants were analyzed for oxidase activity on 20 sugars and simple aldehydes using an ABTS [2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid)] assay (data not shown). Activity was detected in only one of the recombinant *Pichia* strains, i.e., the strain expressing the *C. graminicola* putative carbohydrate oxidase encoding gene (*E3R0R1*). The enzyme in the culture supernatant was found to catalyze oxidation of the trisaccharide raffinose, and since the gene (having the locus tag identifier GRLG_11847) was not annotated, we adopted the gene name *raox*, for raffinose oxidase, and the corresponding phenotype *CgRaOx* according to its preferred substrate. A control strain containing the empty plasmid did not exhibit an oxidase activity on any of the tested sugars.

Sequence analysis of *CgRaOx*. Based on the sequence homology, *CgRaOx* was found to belong to the family AA5_2 subfamily (Fig. 1). The three other carbohydrate oxidase candidates were also identified as AA5_2 members and, interestingly, the gene for *G4NG45* showed high sequence identity (ca. 57%) with the two recently discovered novel alcohol oxidases (AlcOx) (30). The full-length sequence of *CgRaOx* is 913 amino acids (aa), and the predicted native signal sequence is 23 residues long. The sequence was originally annotated as a Kelch domain-containing protein. Using the *CgRaOx* amino acid sequence for a BLASTP search in the NCBI database (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), orthologs were found from example *Ustilago maydis* (XP_011389156.1) and *Pseudozyma antarctica* (GAK65151.1), with sequence identities of 66 and 58%, respectively, both annotated as galactose oxidase precursors.

CgRaOx (913 aa) had 29% sequence identity with *FgGaOx* (680 aa) over the full-length sequence and 41% sequence identity over domains 2 and 3 (see Fig. S1 in the supplemental material). In particular, Cys465 and Tyr509 in *CgRaOx* (numbering without the signal sequence) corresponded to Cys228 and Tyr272 in *FgGaOx* that form the thioether linkage, whereas Tyr732, His733, and His831 in *CgRaOx* corresponded to residues in *FgGaOx* that coordinate the essential Cu^{2+} . Moreover, three positions predicted to participate in substrate binding differed between *CgRaOx* and *FgGaOx*, namely, Trp290, Gln406, and Pro463 in *FgGaOx* were Tyr527, Ser644, and Gly700 in *CgRaOx*, respectively.

Production, purification and characterization of *CgRaOx*. SDS-PAGE analysis of the purified, recombinant *CgRaOx* produced in shake flask cultivations revealed a major band of approximately 130 kDa with an estimated purity of 80% (Fig. 2A). Approximately 0.8 mg of purified protein was obtained from a 1-liter cell culture, with a specific activity on raffinose of $0.07 \mu\text{mol min}^{-1} \text{mg}^{-1}$ (determined using the HRP/Ampex Red assay). In an effort to increase the yield of *CgRaOx*, the enzyme was also produced in a bioreactor system. The highest *CgRaOx* activity was achieved at the end of the 84-h cultivation (see Fig. S2 in the supplemental material), and 6.7 mg of protein from 1 liter of cell culture could be purified in two chromatographic steps (Fig. 2A). Like previous bioreactor productions of AA5_2 oxidases, the activity of the purified *CgRaOx* was not increased by incubation with copper sulfate or subsequently potassium ferricyanide, indicating that *CgRaOx* was fully coordinated by Cu(II) ions incorporated from the fermentation media (35, 36). The specific activity of the *CgRaOx* sample from the bioreactor was $3.0 \mu\text{mol min}^{-1} \text{mg}^{-1}$ on raffinose, which is ~ 40 times higher than for

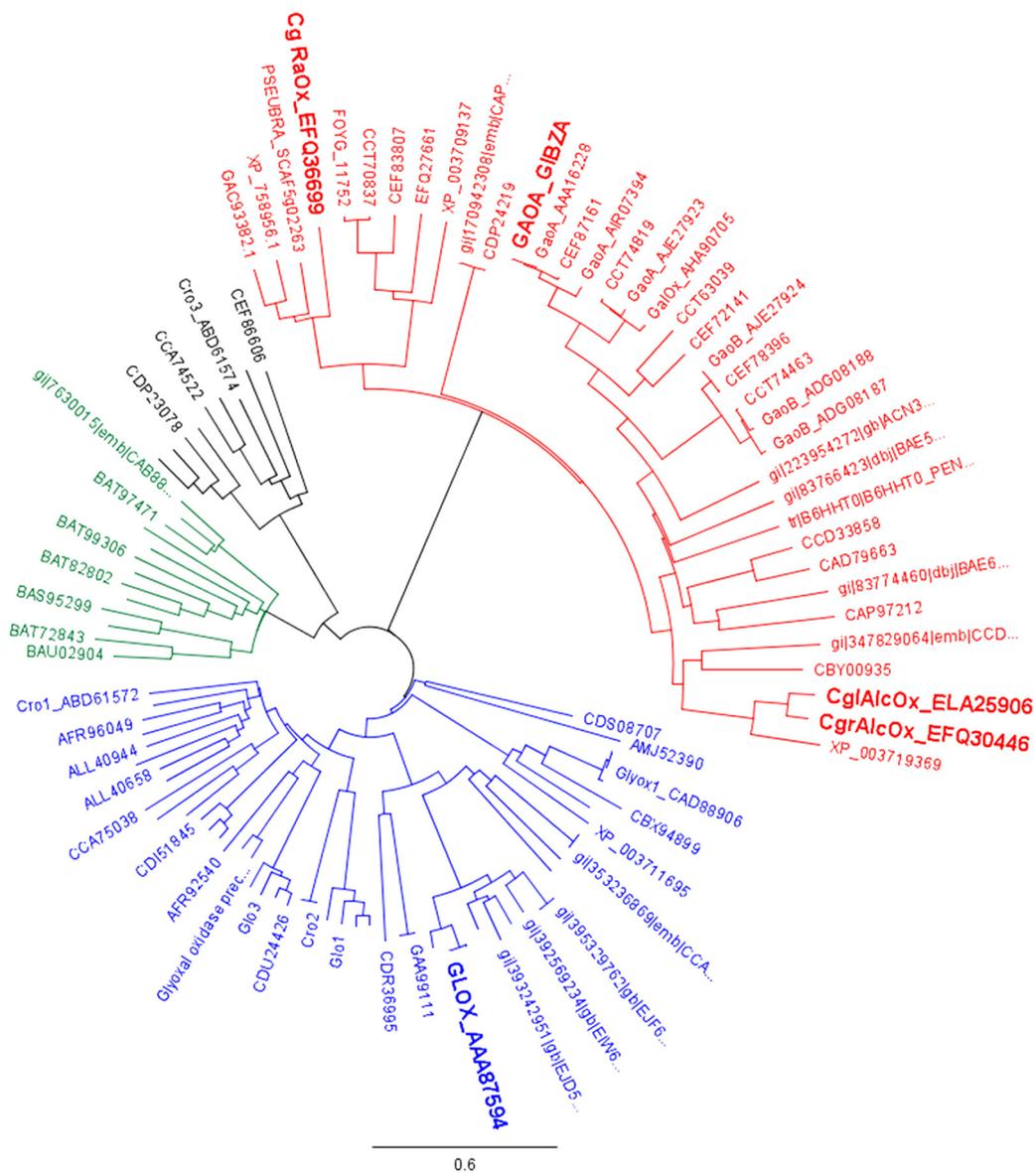


FIG 1 Phylogenetic tree of eukaryotic AA5 family members. The eukaryotic sequences in CAZy_AA5 family were aligned by CLUSTALW. Sequences that were indicated as partial were removed. Phylogenetic analysis showed that the 110 sequences clustered into two main groups: the galactose oxidase (AA5_2; shown in red) and glyoxal oxidase (AA5_1; shown in blue) subfamilies. The black and green branches are undefined due to the lack of any biochemically characterized members. The sequences mentioned in this study are indicated in boldface: CgRaOx_EFQ36699 for *Colletotrichum graminicola* RaOx, GAO_GIBZA for *Fusarium graminearum* GaOx, CgAlcOx_ELA25906 for *Colletotrichum gloeosporioides* alcohol oxidase, CgrAlcOx_EFQ30446 for *Colletotrichum graminicola*, and GLOX_AAA87594 for *Phanerochaete chrysosporium* glyoxal oxidase.

the enzyme produced in the shake flask cultivation. Using raffinose as the substrate, the consumed oxygen measured by an oxygen electrode confirmed that CgRaOx is an oxidase (see Fig. S3A in the supplemental material). Treatment with Endo F1 reduced the molecular mass of the recombinant CgRaOx to 90 kDa (Fig. 2B), which is consistent with the predicted molecular mass based on amino acid sequence; the identity of the purified protein was also confirmed by mass spectrometry. Notably, deglycosylation did not affect the activity of CgRaOx on raffinose (data not shown).

Of the tested sugar substrates, CgRaOx exhibited the highest activity on raffinose and melibiose and showed a low activity on D-galactose, but it displayed no activity on D-glucose, D-xylose, L-arabinose, fructose, sucrose, lactose, or the tetrasaccharide stachyose (Fig. 3). In addition, no activity was detected on the polysaccharides guar

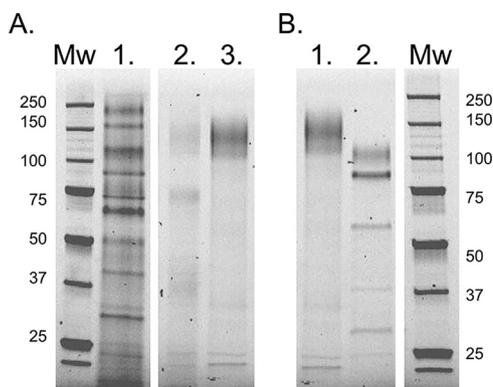


FIG 2 SDS-PAGE of purification steps and deglycosylation of *Colletotrichum graminicola* RaOx. (A) Figure lanes show molecular mass marker proteins in kDa. Lane 1, concentrated culture supernatant (21 μ g protein); lane 2, pooled protein fractions after phenyl Sepharose (3 μ g); lane 3, pooled protein after DEAE (3 μ g). Total protein in each lane indicated in parentheses. (B) The CgRaOx enzyme was deglycosylated with Endo F1 and analyzed before (lane 1) and after the treatment (lane 2). The molecular mass marker proteins (250, 150, 100, 75, 50, 37, and 25 kDa) are in lane Mw. The proteins were visualized using the Bio-Rad's Criterion stain-free gel imaging system (Bio-Rad). The gels shown have been spliced for labeling purposes.

galactomannan and tamarind galactoxyloglucan (measured at 2.5 mg/ml). Notably, oxidation of raffinose, melibiose or D-galactose by CgRaOx did not show saturation behavior even up to 400 mM substrate (Fig. 4). Nevertheless, apparent kinetic constants using the Michaelis-Menten equation could be calculated for raffinose and melibiose. The affinity was higher (i.e., showing a lower apparent K_m value) on raffinose (apparent $K_m = 480$ mM) than on melibiose (apparent $K_m = 590$ mM), resulting in a 3.5 times higher apparent catalytic efficiency on raffinose (0.0288 mM $^{-1}$ s $^{-1}$) than on melibiose (0.0077 mM $^{-1}$ s $^{-1}$) (Fig. 4). To compare the catalytic efficiency (k_{cat}/K_m) of all substrates, the initial rate at a substrate concentration much lower than the K_m was determined. This result showed that the catalytic efficiency for melibiose was 3.5-fold lower, and for D-galactose it was ~20-fold lower than for the best sugar substrate raffinose.

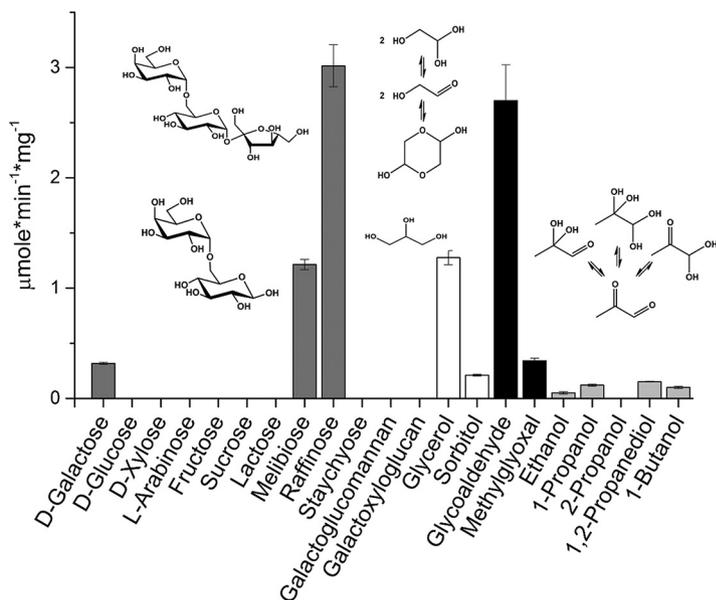
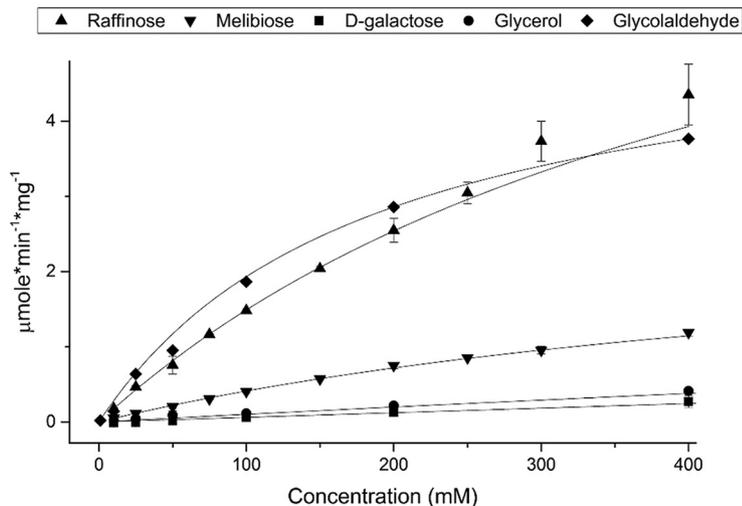


FIG 3 Substrate specificity of CgRaOx. CgRaOx was incubated with 19 different mono- and oligosaccharides, sugar alcohols, simple alcohols, or aldehydes at pH 7.5. All substrates were tested at 300 mM, except for stachyose (75 mM) and galactoglucomannan and xyloglucomannan (2.5 mg/ml). Prior to the addition of substrate, CgRaOx (0.45 to 1.1 pmol) was incubated for 30 min at 30°C in the presence of HRP (7.5 U/ml) and ABTS (2 mM). The final reaction volume was 205 μ l, and the activity was determined by continuously reading the absorbance at 420 nm.



Substrate	Michaelis-Menten			Linear
	k_{cat}, s^{-1}	K_M, mM	$v = V_{max} * \frac{[S]}{K_M + [S]}$	$\frac{v}{[E]} = \frac{k_{cat}}{K_M} * [S]$
Raffinose	13.86 ± 1.20	481.8 ± 49.11	0.0288 ± 0.005	0.0470 ± 0.002
Melibiose	4.52 ± 0.43	586.6 ± 67.06	0.0077 ± 0.002	0.0126 ± 0.002
D-galactose	na	na	na	0.0024 ± 4.5E-05
Glycerol	na	na	na	0.0033 ± 0.000
Glycolaldehyde	8.81 ± 0.01	184.9 ± 0.30	0.0476 ± 0.000	0.0608 ± 0.004

FIG 4 Kinetic behavior of CgRaOx on raffinose, melibiose, D-galactose, glycerol, and glycolaldehyde. The measurements were performed with 2.5 to 400 mM substrate at pH 7.5 and 22°C, and the oxidation was monitored using the colorimetric HRP/ABTS assay. The error bars show the standard errors of the mean ($n = 4$). In the figure, fitted Michaelis-Menten curves for raffinose, melibiose, and glycolaldehyde are shown. Since D-galactose and glycerol did not show saturation behavior even at substrate concentrations up to 1 M, the curves for these substrates were fitted to a reduced linear kinetics model. The apparent kinetic values for CgRaOx are presented in the table below the graph. The data for raffinose, melibiose, and glycolaldehyde were fitted using the Michaelis-Menten model. For comparison, the data for all substrates were fitted to the reduced linear kinetics model for substrate concentrations significantly lower the expected K_m values, i.e., 0 to 100 mM for raffinose and glycolaldehyde and 0 to 200 mM for melibiose, D-galactose, and glycerol. na, not applicable.

CgRaOx was also tested on substrates used to measure the activity of glycolaldehydes from subfamily AA5_1 (29). At a 300 mM substrate concentration, CgRaOx catalyzed oxidation of the glycolaldehyde dimer with 93% relative activity to raffinose. Glycerol and methylglyoxal were oxidized to a lesser degree (17 and 13% activities compared to raffinose, respectively; Fig. 3 and 4). The oxidation of the glycolaldehyde dimer followed Michaelis-Menten kinetics (Fig. 4), where the k_{cat} on glycolaldehyde ($k_{cat} = 8.8 s^{-1}$) was lower than that for raffinose (apparent $k_{cat} = 13.9 s^{-1}$). However, K_m values of CgRaOx on glycolaldehyde ($K_m = 185 mM$) were lower than those measured using raffinose (apparent $K_m = 480 mM$). Two recently published alcohol oxidases from AA5_2 showed activity toward a range of simple primary alcohols but not on sugars (30). Therefore, CgRaOx was also tested on a range of simple alcohols (Fig. 3). The corresponding activities were low compared to the tested sugars, validating that CgRaOx is a carbohydrate oxidase.

The optimal pH for the purified CgRaOx was 8.0 (see Fig. S3A in the supplemental material). At pH 9.0 CgRaOx retained 88% of its activity, whereas the activity was only 55% at pH 6.0. The stability of purified CgRaOx was measured at three temperatures for up to 22 h. The enzyme remained almost fully active after 6 h at 40°C, whereas at 50°C the enzyme was inactivated already in 15 min: the $t_{1/2}$ was 13 h at 40°C and 5 h at 45°C (see Fig. S3B in the supplemental material). The purified CgRaOx was stored in 50 mM Tris-Cl (pH 7.5), and it retained more than 90% of its activity after several months of storage at -80°C (data not shown).

Conversion to raffinose aldehyde and site of oxidation. The conversion of raffinose to the aldehyde derivative was studied by varying the enzyme dose and reaction time, and analyzing the products with nuclear magnetic resonance (NMR) and electrospray ionization mass spectrometry (ESI-MS). A low CgRaOx dose, such as 3.3 $\mu\text{g}/\text{mg}$ substrate, yielded a low degree of oxidation after 40 h. When a high dose of CgRaOx (up to 200 $\mu\text{g}/\text{mg}$ substrate) was applied, the conversion over 40 h increased by approximately 80% (Fig. 5 and 6); however, raffinose was still present, and side products had also formed.

The products were studied with NMR spectroscopy, comparing the results to a FgGaOx-catalyzed oxidation of methyl α -D-galactopyranoside. The main product in FgGaOx catalyzed reactions is a C-6 aldehyde (37). In aqueous solutions, the C-6 aldehydes exist as hydrates (geminal diols), and the doublet at 5.05 ppm was typical for the H-6" of the hydrate (37). A similar doublet was observed in the CgRaOx reaction (Fig. 5), confirming that the site of CgRaOx oxidation was the C-6 of the terminal galactose as well. Similarly to the FgGaOx catalyzed oxidation of methyl α -D-galactopyranoside (37), the doublet at 6.14 ppm corresponded to the olefinic hydrogen at C-4 of an α,β -unsaturated C-6 aldehyde product (Fig. 5). The chemical shifts of H-1" of the starting material and aldehyde product overlapped at 4.95 ppm, but the chemical shift of H-1" of the α,β -unsaturated side product at 5.2 ppm clearly differed from the others. In addition to the α,β -unsaturated aldehyde, a further oxidation product, a uronic acid, was also detected in the methyl α -D-galactopyranoside oxidation (37). Unfortunately, all of the chemical shifts of the acid overlapped with the other chemical shifts of the mixture of CgRaOx raffinose oxidation products, but its presence was confirmed by further mass spectrometry analysis (see below).

Oxidation products were also characterized with ESI-MS in negative mode as chlorine adducts. Negative mode has been found to be more informative than the positive mode in the structural analysis of oligosaccharides by ESI tandem mass spectrometry (ESI-MS/MS) (38). The detection of the negatively charged uronic acid was straightforward in its anionic form. The m/z values of the products corresponded to the molecules identified by NMR analysis (Fig. 6), as detailed below. Some starting material (Fig. 6A) was still present (m/z 539). The aldehyde product as such was not observed; instead, it was detected in a minor peak as a hydrate (Fig. 6B) (m/z 555 as a chlorine adduct). Interestingly, the main detected aldehyde form was a hemiacetal with methanol (Fig. 6E) (m/z 569 as a chlorine adduct) present in the ESI-MS solvent. The methanol hemiacetal was also found in the FgGaOx-catalyzed positive-control reaction. The α,β -unsaturated product (Fig. 6C) was seen as a chlorine adduct (m/z 519) and the uronic acid in its anionic form (m/z 517). All of these ions (except for product C in Fig. 6) and their MS/MS fragmentation patterns (Fig. 7) corresponded to those observed in the positive-control oxidation with FgGaOx (see Fig. S4 in the supplemental material).

We expected that the mechanism for the formation of the further oxidation product, uronic acid, was that CgRaOx oxidizes the aldehyde in its hydrate form. The mechanism was studied by conducting the CgRaOx reaction in H_2^{18}O . Upon hydrate formation, the aldehyde obtains a hydroxyl group from water, in this case the labeled H_2^{18}O . Since the hydrate formation is reversible, it is also possible that both hydroxyl groups of the hydrate contain the heavy isotope of oxygen. Thus, if the uronic acid was formed by the oxidation of the hydrate, as we expected, it would also contain one or two atoms of ^{18}O . On the other hand, if the aldehyde was oxidized directly using molecular oxygen, no ^{18}O labeled uronic acid would be present. ESI-MS analysis of the acidic fraction of the product mixture showed m/z for the uronic acid in its anionic form, thus containing two ^{18}O atoms (for the unlabeled form, the m/z was 517), and confirmed that CgRaOx oxidized the hydrate to the uronic acid (Fig. 8). Similarly, FgGaOx oxidized α -methyl galactopyranoside to corresponding galacturonic acid in H_2^{18}O (see Fig. S5 in the supplemental material).

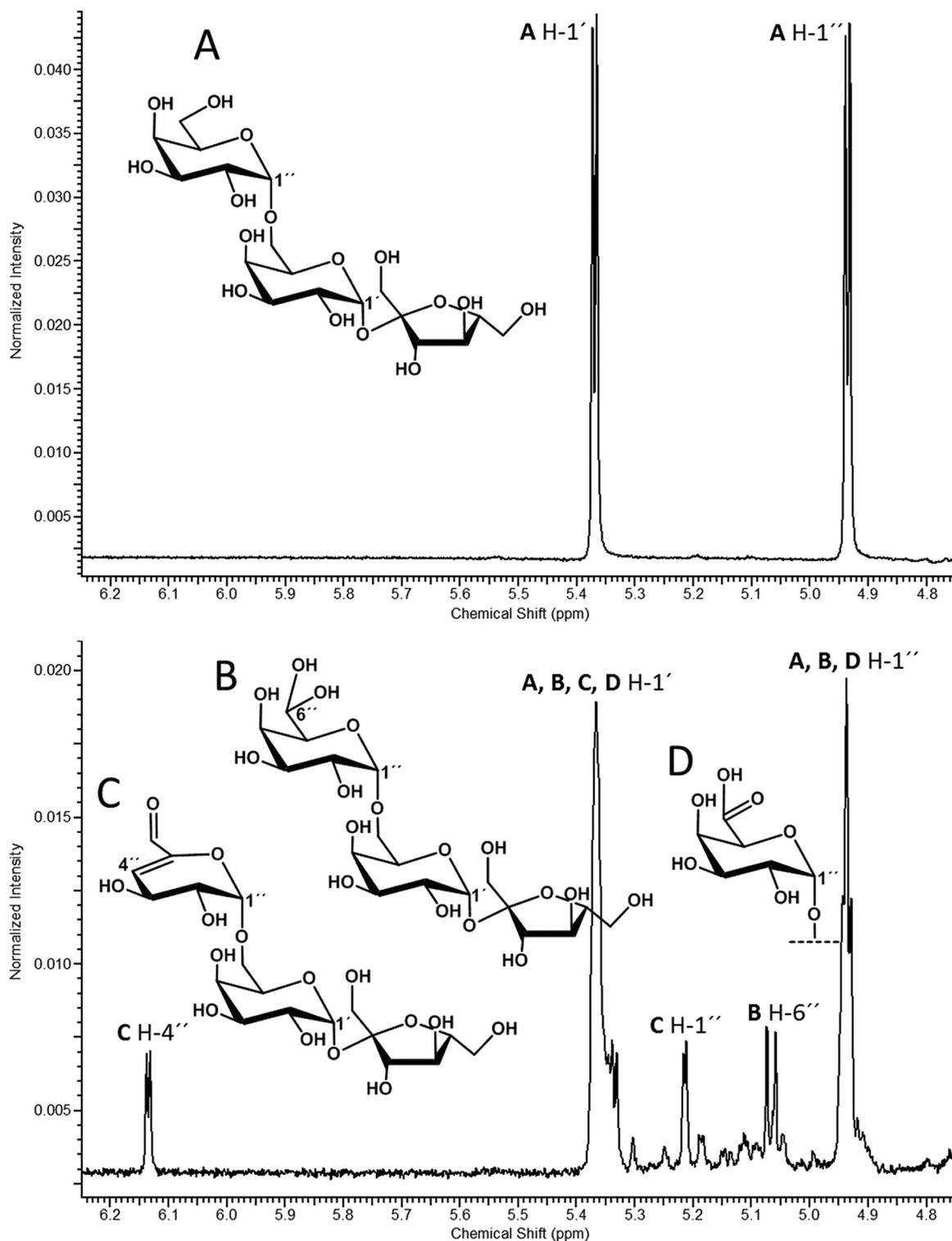


FIG 5 Partial ¹H NMR spectra (in D₂O, referenced to the residual water signal) of starting material raffinose (A) and the CgRaOx-catalyzed product mixture containing raffinose, raffinose aldehyde in hydrate form (B), the water elimination product of the aldehyde (C), and the uronic acid derivative (D).

DISCUSSION

For a long time, the only characterized member of the AA5_2 subfamily was galactose 6-oxidase from *Fusarium graminearum*. Recently, four additional galactose oxidases from *Fusarium* spp. have been added to the subfamily (39), followed by the

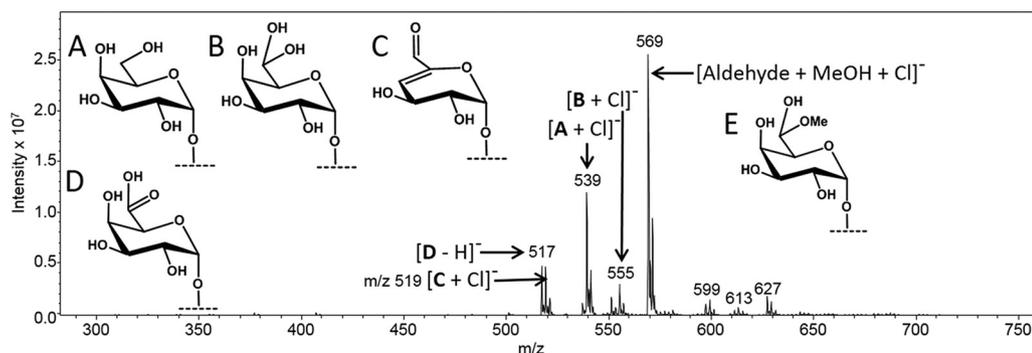


FIG 6 Negative-mode ESI-MS (Cl^- adducts) spectrum of the products of raffinose oxidation by CgRaOx. (A) Raffinose; (B) raffinose aldehyde in hydrate form; (C) water elimination product of the aldehyde; (D) uronic acid derivative; (E) hemiacetal formed from aldehyde and methanol (MeOH).

discovery of *Colletotrichum graminicola* and *C. gloeosporioides* alcohol oxidases (CgrAlcOx and CgAlcOx), which also belong to the AA5_2 subfamily (30).

The sequence of CgRaOx characterized here is a AA5_2 carbohydrate oxidase with a distinct substrate profile. Three of the seven amino acid residues predicted to participate in substrate binding distinguished CgRaOx from FgGaOx. Trp290 in FgGaOx is replaced by Tyr527 in CgRaOx. Besides the tyrosine at Trp290, CgRaOx differs from galactose oxidase by having a serine (Ser644) at Gln406 and a glycine (Gly725) at Pro463. Previous studies of FgGaOx have shown that substituting Trp290 with a phenylalanine in combination with Arg330 to lysine and Gln406 to tyrosine introduced activity on D-glucose but greatly compromised the catalytic efficiency of the enzyme (33). Although two of these positions are naturally substituted in CgRaOx, no activity was detected on glucose. This might be due to the retained Arg574. The corresponding residue in FgGaOx, i.e., Arg330, has been reported to form hydrogen bonds to the C-3 and C-4 hydroxyls of galactose and thus, together with the copper-ligand Tyr495, is the main explanation for the exclusion of glucose as a substrate. In CgrAlcOx the Trp290 is replaced by a phenylalanine, which Yin et al. suggest enhances the ability of the enzyme to oxidize alcohols (30). However, CgRaOx, with a tyrosine at Trp290, clearly showed greater activity on sugars than on alcohols.

The N-terminal domain in CgRaOx has a PAN domain motif that has been found in proteins mediating protein-protein or carbohydrate-protein interactions (40). Therefore, the PAN domain in CgRaOx might have the same role as the F5_F8_type C motif found in galactose oxidase, which is classified as a family 32 N-terminal carbohydrate-binding module possibly promoting binding to galactose (31, 32).

Only apparent kinetic parameters for CgRaOx could be determined, since saturation was not obtained even at 400 mM sugar concentrations. The apparent kinetic parameters, however, showed the catalytic efficiency to be higher on raffinose than on melibiose (Fig. 4). The kinetics also showed that D-galactose was a poor substrate for CgRaOx. Notably, the K_m value of FgGaOx on D-galactose is also rather high and has been reported at 40 to 100 mM (34, 35, 41). Moreover, it was surprising that CgRaOx did not oxidize stachyose, given that stachyose [Gal- α -(1 \rightarrow 6)-Gal- α -(1 \rightarrow 6)-Glc- α -(1 \rightarrow 2)- β -Fru] differs from raffinose [Gal- α -(1 \rightarrow 6)-Glc- α -(1 \rightarrow 2)- β -Fru] only by one galactose residue. No activity on galactosylated polysaccharides (galactoxylogucan and galactomannan) was detected either. The results suggest that only short oligosaccharides (di- and trisaccharides) are oxidized by the enzyme. In addition, the lack of activity on lactose [Gal- β -(1 \rightarrow 4)-Glc] and stachyose indicated that CgRaOx seemingly prefers substrates where α -(1 \rightarrow 6)-D-glucose occupies the monosaccharide unit next to the oxidized galactose residue. This is also supported by much higher activity on raffinose and melibiose [Gal- α -(1 \rightarrow 6)-Glc] than on D-galactose. Earlier characterizations of galactose oxidases from *Fusarium* spp. show a similar preference for galacto- α -(1 \rightarrow 6)-substituted oligosaccharides, suggesting that the enzymes have evolved toward this

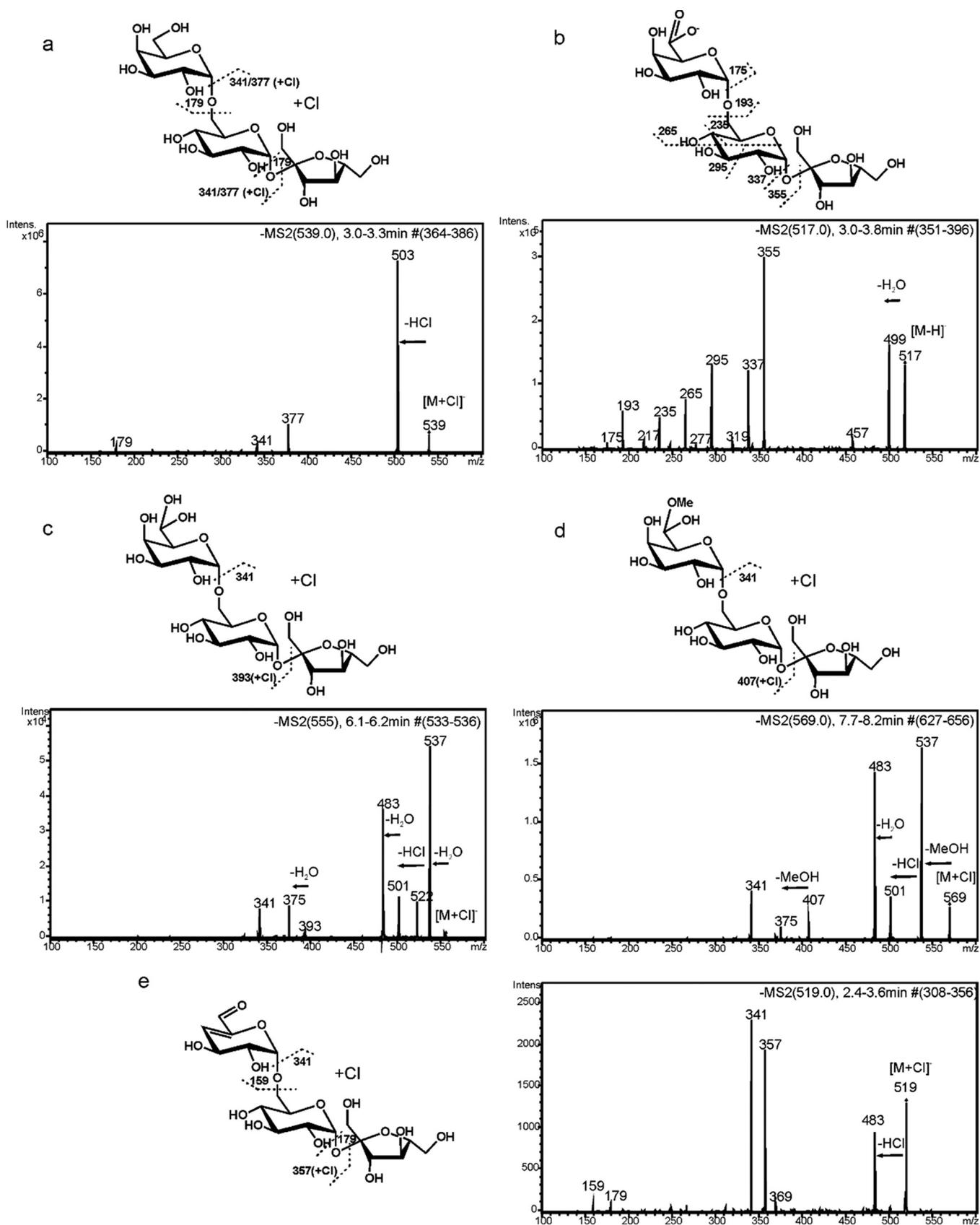


FIG 7 Negative-mode ESI-MS/MS spectrum and fragmentation patterns of the products of raffinose oxidation by CgRaOx. (a) Raffinose; (b) ionic acid derivative; (c) raffinose aldehyde in hydrate form; (d) hemiacetal formed from aldehyde and methanol; (e) water elimination product of the aldehyde.

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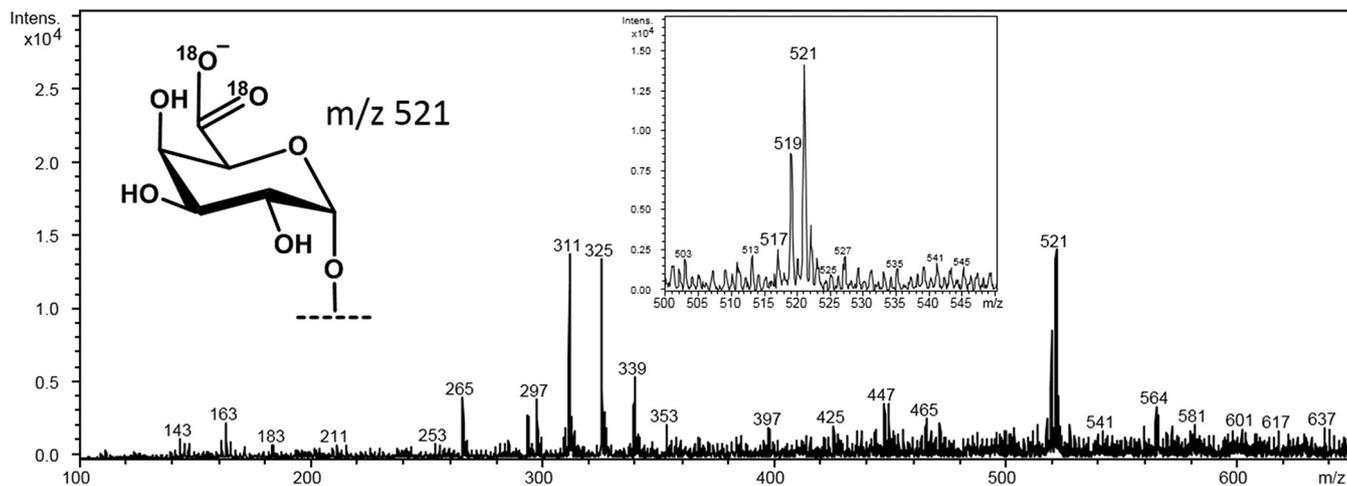


FIG 8 Negative-mode electrospray ionization-ion trap mass spectrometry (ESI-IT-MS) spectrum of the acidic fraction of the products of raffinose oxidation in H_2^{18}O by CgRaOx. The uronic acid derivative of raffinose contains two ^{18}O atoms.

specificity rather than galacto- β -(1 \rightarrow 4)-substituted substrates such as lactose (39, 42). Moreover, since the activity on raffinose was significantly higher than on melibiose (Fig. 3), the substrate preference was presumably also dependent on the third monosaccharide residue. The substrate specificity of CgRaOx was clearly different from the substrate specificity of FgGaOx, which catalyzes oxidation of D-galactosyl-containing saccharides ranging from the monosaccharide D-galactose up to polysaccharides, including the oligosaccharides stachyose, raffinose, and melibiose (reviewed in reference 25). For some galactose oxidases, e.g., a recombinant *Fusarium* GaOx expressed in *Aspergillus oryzae* (43) and a galactose oxidase from *Fusarium sambucinum* (39), the specific activity or catalytic efficiency has been reported to be higher on raffinose than on D-galactose. Nevertheless, in contrast to CgRaOx, both the recombinant *Fusarium* GaOx and the enzyme from *F. sambucinum* oxidize D-galactose very efficiently (39, 43). Compared to these two enzymes, the specific activity of CgRaOx is low. However, the unique substrate specificity profile, with a high preference for raffinose and a moderate preference for melibiose but a low preference for D-galactose, makes CgRaOx the first characterized member of family AA5_2 that displays distinguished functionality toward raffinose, thus giving rise to the naming of the enzyme.

In addition to carbohydrates, CgRaOx oxidized the glycolaldehyde dimer and glycerol. Glycolaldehyde in solution is known to exist in several different forms. The dimeric glycolaldehyde ring is interconverting between several different forms before reaching equilibrium where the hydrate (geminal diol) form of the substrate is dominant (44). Since only freshly solubilized glycolaldehyde dimer was accepted as a substrate, we assume that the dimeric structure, containing two hydroxyl groups, is the form that is oxidized by the enzyme. The glycolaldehyde may not be a natural substrate, since less than 10% of aqueous glycolaldehyde exists in the dimeric ring form, and so the observed activity might be due to the structural resemblance to a sugar ring. Different from the recently discovered alcohol oxidases of the AA5_2 family, CgRaOx showed only very low activity on the tested aliphatic alcohols and thus clearly is a carbohydrate oxidase (30). The sequence identities of CgRaOx with the two alcohol oxidases were only 29 and 30% for CgrAlcOx and CglAlcOx, respectively.

Our findings on the raffinose oxidase and the two recently discovered alcohol oxidases (30) indicate that the AA5_2 family is more diverse than previously anticipated. Common to all known AA5_2 carbohydrate oxidases is that they target the C-6 hydroxyl of galactose exclusively (monomer or in oligo- or polysaccharides), they require an unsubstituted C-4 hydroxyl on the oxidized galactose, and they carry an inherent N-terminal noncatalytic domain embedded with the structure of the catalytic domain. Considering the clearly different N-terminal domain of CgRaOx compared to galactose

oxidase and the absence of this domain in alcohol oxidase, it is possible that the type or absence of the N-terminal noncatalytic domain could be connected to the substrate specificity of the oxidases in AA5_2.

The physiological role for CgRaOx is unknown. The function could be to catalyze the production of the biologically important hydrogen peroxide in plant pathogens. Raffinose oxidase was discovered from the plant-pathogenic fungus *C. graminicola*, which colonizes and infects grasses and many grain crops. The trisaccharide raffinose can be found in many beans and vegetables (e.g., cabbage, brussels sprouts, broccoli, and asparagus), as well as cereals, where the free raffinose content in wheat bran is reported as >1% (45). Intriguingly, the accumulation of raffinose and stachyose, which is formed from raffinose by stachyose synthase, has been connected to biotic stress response in plants (46). Raffinose and stachyose are confirmed to have reactive oxygen species scavenging properties in plant cell membranes and thus contribute to cell membrane stability. Thus, it is conceivable that the CgRaOx-catalyzed oxidization of raffinose could inhibit synthesis of stachyose in plant cell walls and thereby diminish biotic stress response.

The ¹H NMR spectra of the oxidized raffinose showed that the hydroxyl group at C-6 of galactose was oxidized to an aldehyde. However, the C-6 aldehyde was not detected as such, but rather as a hydrate (geminal diol) in aqueous solution (37). Furthermore, when diluted in methanol-H₂O solution for the mass spectrometry analysis, the aldehyde did not form a hydrate at significant amount but preferably a hemiacetal with methanol, which was thus the main peak observed in the mass spectrometry spectrum (Fig. 5). The presence of methanol-hemiacetals, and their further MS/MS fragmentation, could indeed be used as a novel method to unambiguously identify C-6 aldehydes or C-2/C-3 ketones and thereby distinguish them from C-1 oxidized carboxylic acids, which have same *m/z* values as corresponding hydrates.

Uronic acid was formed as a side product in the CgRaOx-catalyzed reaction, as similarly observed earlier in the FgGaOx-catalyzed oxidation of methyl α -D-galactopyranoside (37). The mechanism of formation of the acid was the oxidation of hydrate form of the aldehyde product, as shown by performing oxidation in H₂¹⁸O. The FgGaOx-catalyzed reaction conditions could be controlled to avoid the formation of the acid (37), and most probably such reaction conditions could also be found for the CgRaOx-catalyzed reaction. The amount of uronic acid seemed to increase by increasing the dosage of RaOx and reaction time. Chemical oxidation procedures, where the oxidation to carboxylic acid proceeds by oxidizing the hydrate, such as TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl)-mediated reactions, are reported to produce either aldehydes or carboxylic acids, depending on the reaction conditions (47).

In summary, a bioinformatics approach, using the modular structure, as well as essential catalytic and substrate interacting residues of FgGaOx as selection criteria, was successfully used to find potential C-6 hydroxyl carbohydrate oxidases. A novel raffinose oxidase from *C. graminicola* (CgRaOx) was identified and, together with galactose oxidase, is the only carbohydrate oxidase known to oxidize a primary hydroxyl group in carbohydrates. CgRaOx was shown to have a unique substrate specificity pattern, which to our knowledge has not been reported for any other enzyme. The discovery of this novel activity profile underscores the diversity of activities yet to be found within the AA5_2 subfamily.

MATERIALS AND METHODS

Chemicals and enzymes. Basal salt medium (BSM), PTM1 trace salts, ABTS, and 10-acetyl-3,7-dihydroxyphenoxazine (AmplexRed) were purchased from Sigma-Aldrich. Raffinose and stachyose were purchased from Carbosynth, Ltd., Compton, UK, and guar galactomannan and tamarind galactoxyloglucan were obtained from Megazyme. All other substrates were purchased from Sigma-Aldrich at high-purity grade.

Galactose oxidase from *Fusarium* spp., which was produced recombinantly in *Pichia pastoris* was a gift from Hercules (Hercules Incorporated, Barneveld, The Netherlands). Horseradish peroxidase (HRP) type II and catalase C30 from bovine liver were purchased from Aldrich.

Cloning of potential carbohydrate oxidases. Four genes encoding putative carbohydrate oxidase enzymes were obtained as synthetic genes and codon optimized for *Pichia pastoris* (GeneArt, Germany).

The native signal sequences of the genes, predicted using SignalP (<http://www.cbs.dtu.dk/services/SignalP/>), were removed, and the genes were ligated into a linearized (EcoRI/KpnI digestion) pPICZ α A vector (Invitrogen) as a fusion with the *S. cerevisiae* α -factor signal peptide at the N terminus for efficient secretion into the medium. The bacterial cloning steps were carried out in *Escherichia coli* TOP10. The resulting plasmids, containing the genes for the putative carbohydrate oxidases from *Magnaporthe oryzae* (G4NG45), *Aspergillus oryzae* (Q2U112), *Aspergillus fumigatus* (Q4WH00), and *Colletotrichum graminicola* (E3R0R1) (the identifiers refer to the UniProt resource [<http://www.uniprot.org/>]), as well as the control plasmid pPICZ α A, were introduced by electroporation into the *P. pastoris* X-33 strain according to the manufacturer's protocol (Invitrogen).

Culture conditions. For small-scale cultivations, *P. pastoris* yeast strains were cultured in 50 ml of BMGY culture medium (2.0% peptone, 1.0% yeast extract, 1.3% YNB, 0.0004% biotin, 100 mM potassium phosphate [pH 6.0] and 1.0% glycerol) in 250-ml Erlenmeyer flasks at 250 rpm and 30°C with shaking until the optical density at 600 nm (OD_{600}) was 2 to 6. The cells were harvested by centrifugation at $3,000 \times g$ for 5 min, and then the cells were resuspended in BMMY culture medium (the same as BMGY but with glycerol replaced by methanol) to induce the expression of recombinant proteins. Methanol was added to the culture to a final concentration of 0.5% every 24 h. The cultivation was continued for 4 to 8 days, and samples were taken daily. Shake flask cultivations in a larger scale were performed with 200 ml of BMMY medium, supplemented with 0.5 mM $CuSO_4$, in 2-liter baffled shake flasks at 24°C for 8 days.

The bioreactor cultivation was based on Invitrogen *Pichia* fermentation process guidelines, with minor modifications (35). The bioreactor cultivation was performed in an autoclavable Biostat B Plus bioreactor (Sartorius), with a maximum working volume of 2 liters. All process control data were recorded in MFCswin (B. Braun Biotech International). The bioreactor was equipped with online pH and oxygen probes from Hamilton.

Inoculation cultures in 100 ml of yeast extract-peptone-dextrose were grown overnight at 30°C to an OD_{600} of 3.5. The cells were centrifuged ($3,000 \times g$, 5 min) and suspended in BSM before inoculation of the reactor. The initial fermentation volume was 1 liter of BSM, with an initial 4% glycerol concentration as the sole energy and carbon source. The pH was maintained at 5.5 by the automatic addition of 15% ammonium hydroxide, which also served as a nitrogen source. The dissolved oxygen concentration (DO%) was maintained above 40% by a cascade control, initially increasing the stirrer speed from 300 to 1,200 rpm and subsequently increasing the atmospheric airflow from 0.5 to 3.0 vessel volumes per minute. When the maximum oxygen transfer capacity of the reactor was reached, the DO% fell below 40% in the presence of an adequate substrate concentration. Antifoam was added automatically on demand.

Upon depletion of glycerol from the BSM medium the glycerol fed-batch phase was initiated manually by feeding a 50% glycerol mix into the reactor, while maintaining a substrate-limited growth rate. The glycerol feed rate was manually adjusted during the fed-batch phase to maintain a DO% between 25 and 35% at maximum oxygen transfer. For induction, the glycerol feed was stopped, and the DO% was allowed to increase $>40\%$, until minimal oxygen transfer was achieved, and glycerol was depleted from the reactor. After complete glycerol depletion, methanol feeding was started at a flow rate of 5 ml h^{-1} . Stopping the feed pump and measuring the time before the DO% rapidly increases verified a substrate limited growth rate. During the induction stage, the temperature was lowered to 20°C. Induction was kept for 84 h, during which time a total of 239 g of methanol was fed to the reactor. Ongoing adjustment of the methanol feed-rate was done to maintain the DO% between 25 and 35% at maximum oxygen transfer.

Purification of *C. graminicola* raffinose oxidase expressed in *P. pastoris*. *C. graminicola* raffinose oxidase (CgRaOx) was either expressed in shake flasks or in bioreactor cultivation as described above. The cultivation supernatant from shake flasks (3.4 liters) was concentrated to 360 ml using a Millipore Prep/Scale-TFF cartridge, and the buffer was changed to 50 mM Tris-Cl (pH 7.5). The concentrated supernatant was adjusted to a final concentration of 1 M $(NH_4)_2SO_4$ and applied on a 20-ml HiPrep Phenyl FF Sepharose column equilibrated with 1 M $(NH_4)_2SO_4$ in 50 mM Tris-Cl (pH 7.5). The protein was eluted with a linear gradient [1 to 0 M $(NH_4)_2SO_4$ in 50 mM Tris-Cl; pH 7.5], followed by elution with water. The active fractions showing a band of a correct size in SDS-PAGE analysis were pooled, and the buffer was changed to 50 mM sodium acetate (pH 5.5) by using a PD-10 column (GE Healthcare) and loaded onto a 5-ml HiTrap DEAE Sepharose FF column (equilibrated with 50 mM sodium acetate [pH 5.5]). The enzyme was eluted with a linear gradient (0 to 200 mM NaCl; 20 column volumes), followed by stepwise elution at 200 mM and 500 mM NaCl. The active fractions were combined and stored at -80°C in 50 mM Tris-Cl (pH 7.5).

Supernatant from the bioreactor cultivation was harvested by centrifugation ($13,680 \times g$, 60 min, 4°C), and the pH was adjusted to 7.5 by slowly adding 4 M NaOH, which caused heavy salt precipitation. The precipitated BSM salts were removed by centrifugation, and the clarified solution was adjusted to a final concentration of 1 M $(NH_4)_2SO_4$. The pH was confirmed prior filtration (Millipore Sterivex-GP PES filter unit [0.22- μm pore size]). Raffinose oxidase was purified from the clarified cultivation supernatant similarly as for the enzyme produced in shake flasks (see above).

The protein concentration was determined by the method described by Lowry et al. (48) with BSA as the standard, using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA). The purity of the enzymes was monitored by SDS-PAGE (10% Criterion stain-free gel; Bio-Rad), and the proteins were visualized using the Bio-Rad's Criterion stain-free gel imaging system.

The purified CgRaOx was also identified by peptide mass fingerprint mapping with matrix-assisted laser desorption ionization–time of flight mass spectrometry after alkylation and trypsin digestion. Protein samples with BSA as a control were separated on a 12% SDS-PAGE gel (Bio-Rad Laboratories)

stained with PageBlue Coomassie stain (Fermentas), followed by in-gel digestion with trypsin. The peptide extraction from gel and sample preparations was performed as described previously (50).

Enzyme activity determination. The carbohydrate oxidase activity in crude culture supernatants (50 μ l) was measured with 250 to 500 mM substrate in 50 mM Tris-Cl (pH 7) at 22°C, and the formation of hydrogen peroxide (H_2O_2) was coupled to an HRP-linked reaction (20) using 2 mM ABTS and 10 μ g/ml HRP. The used sugars and aldehydes were as follows: D-glucose (500 mM), D-galactose (500 mM), D-mannose (500 mM), D-fucose (325 mM), D-fructose (500 mM), D-xylose (500 mM), L-arabinose (500 mM), D-ribose (500 mM), D-arabinose (500 mM), sucrose (300 mM), lactose (300 mM), maltose (500 mM), raffinose (420 mM), maltotriose, stachyose (270 mM), cellobiose (270 mM), melibiose (300 mM), dihydroxyacetone phosphate, glycolaldehyde dimer, and guar galactomannan (2.5 mg/ml). The concentrations used are indicated in parentheses.

The carbohydrate oxidase activity of the purified enzyme on various sugars was monitored using a coupled HRP/ABTS photometric assay, with the exception of the enzyme purified from shake flask expression. For this enzyme batch, ABTS was replaced with the fluorometric Amplex Red reagent, as described below.

For substrate specificity measurements, 300 mM substrate, 2 mM ABTS, and 7.5 U/ml HRP were used. Specifically, 5 μ l (1.3 pmol) of enzyme purified from the bioreactor cultivation was preincubated in 40 μ l of 40 mM morpholinepropanesulfonic acid buffer (pH 7.5) containing 10 mM ABTS and 37.5 U/ml HRP at 30°C for 30 min to ensure a homogenous solution of fully oxidized (active) enzyme. To initiate the reaction, 160 μ l of 500 mM substrate solution was added, and the oxidized ABTS was monitored for 10 min at 420 nm. The activity was calculated using the molar absorbance coefficient for ABTS (ϵ): 36,000 $M^{-1} cm^{-1}$. The substrates tested were D-glucose, D-galactose, D-xylose, L-arabinose, lactose, sucrose, fructose, melibiose, raffinose, stachyose, glycolaldehyde dimer (freshly prepared), methylglyoxal, sorbitol, glycerol, ethanol, 1-propanol, 2-propanol, 1,2-propanediol, and 1-butanol. Water-soluble tamarind galactoxylogucan and guar galactomannan were tested at 0.1% (wt/vol). Substrate incubated with HRP (7.5 U/ml) and ABTS (2 mM) in the absence of enzyme was used as a blank sample. All samples were tested in quartets, and the activity is reported as the average values.

Kinetic parameters on raffinose, melibiose, D-galactose, glycerol, and glycolaldehyde (freshly prepared) were determined by measuring the activity of 1.3 pmol or 6.6 pmol of enzyme (purified from the bioreactor cultivation), according to the method describe above, using 10 to 400 mM substrate. All samples were tested in quartets, and the activity is reported as the average values. The kinetic data were calculated using the nonlinear Michaelis-Menten or a linear curve fit function in OriginPro 2016 (OriginLab Corporation). The standard deviation from sample replicates was used as instrumental weights for the y axis (activity).

For the enzyme purified from the shake flask cultivation, the more sensitive fluorometric HRP/Amplex Red assay was used. In the assay 0.025 μ g of purified enzyme was incubated with 2.5 to 50 mM the sugar substrate in 50 mM Tris-Cl (pH 7.5) at 22°C in a total volume of 50 μ l. After a 30-min incubation, 50 μ l of a reaction mix containing 2 μ g/ml HRP and 0.1 μ M Amplex Red dissolved in 50 mM Tris-Cl (pH 7.5) was added. Resorufin fluorescence was monitored in a Varioskan microtiterplate reader at λ_{ex} 540 nm and λ_{em} 585 nm. The produced H_2O_2 was calculated from a H_2O_2 standard curve.

pH optimum and temperature stability. To permit direct measurement of CgRaOx activity as a function of pH, the pH optimum of CgRaOx on raffinose was measured by monitoring the consumption of dissolved oxygen during the enzymatic reaction. After initiation of the reaction by addition of the enzyme, oxygen consumption was monitored for 5 h using an oxygen electrode (FIBOX 3 fiber-optic oxygen meter; PreSens, Regensburg, Germany). Raffinose (50 mM) was incubated with 20 μ g of enzyme in McIlvaine buffer (pH 4 to 6), Tris-Cl buffer (pH 7 to 8), and NaOH-glycine buffer (pH 9 and 10). The measurements were carried out under constant agitation in a 2-ml volume in fully sealed flasks to avoid entry of oxygen into the reaction mixture.

The temperature stability of CgRaOx was determined by incubating 0.5 μ g of enzyme in 50 mM Tris-Cl buffer (pH 7.5) at 40, 45, and 50°C. The enzyme was incubated for various time periods (up to 22 h), and the residual activity was measured with 10 mM raffinose as the substrate in 50 mM Tris-Cl buffer (pH 7.5) by measuring the formation of hydrogen peroxide as previously described.

Deglycosylation. Deglycosylation of raffinose oxidase was carried out using Endo F1 endoglycosidase (Sigma, USA) according to the manufacturer's description. CgRaOx was incubated at 37°C for 1 and 3 h with EndoF1 endoglycosidase in 50 mM sodium phosphate buffer (pH 5.5).

Identification of the product and determination of the site of oxidation by NMR spectroscopy and ESI-MS. Raffinose (3.3 mg, 33 mM) was dissolved in 5 mM Tris-Cl (pH 7.5, 200 μ l). The solution was treated with the enzymes CgRaOx (3.3 μ g/mg substrate), HRP (0.05 U/mg substrate), and catalase (11.2 U/mg substrate) and then stirred for 18 to 66 h at room temperature. HRP and catalase were added to potentially activate the enzyme similarly as in galactose oxidase (27, 37). High-dosage experiments were conducted to increase the conversion of raffinose to the product. Specifically, raffinose (5 mg, 10 mM) was dissolved in 5 mM Tris-HCl (1 ml), and enzyme dosages were up to 200 μ g of CgRaOx/mg of substrate, 1 U of HRP/mg of substrate, and 115 U of catalase/mg of substrate. The reactions were stirred for 18 to 66 h at room temperature. A positive-control reaction (to produce aldehyde) was performed by treating raffinose (dissolved in Milli-Q water instead of buffer) with galactose oxidase (0.5 U/mg substrate), HRP (1 U/mg substrate), and catalase (115 U/mg substrate) under the same conditions (16, 37). High-dosage reactions were also conducted using $H_2^{18}O$ (97 atom % ^{18}O ; Sigma-Aldrich) as a solvent.

1H NMR spectra were obtained using a Varian Innova 500 spectrometer (Varian NMR Systems, Palo Alto, CA) operating at 500 MHz. The NMR sample (ca. 5 mg/ml) was dissolved in D_2O and placed into an

NMR tube without filtration. The spectrum was collected at 25°C, and chemical shifts were referenced to the residual water signal.

The reaction products of CgRaOX-treated raffinose were analyzed by ESI-MS and ESI-MS/MS. The samples were purified and fractionated by using a Hypersep Hypercarb porous graphitized carbon column (Thermo Scientific, Waltham, MA) (49). A 250- μ l sample was diluted to 1 ml and applied to the column, and then salts were removed by elution with distilled water. Next, half of the samples were eluted with 0.1% trifluoroacetic acid in 50% acetonitrile to collect all products in same fraction, and the other half of the samples were fractionated to neutral and acidic products by elution first with 50% acetonitrile and then with 0.1% trifluoroacetic acid in 50% acetonitrile. The samples were dried under nitrogen gas flow for 10 min and freeze-dried overnight. The purified reaction products were dissolved to 250 μ l of distilled water and analyzed using an Agilent XCT Plus ion trap mass spectrometer (Agilent Technology, Palo Alto, CA) or Bruker Esquire-LC quadrupole ion trap mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany). Electrospray ionization was performed in negative mode, and nitrogen gas was used as both the nebulizing and drying gas. The ionization parameters were as follows: the drying gas temperature was 325°C, the drying gas flow was 4 liters/min, the capillary voltage was 3,200 V, and the nebulizer pressure was 10 lb/in² (for the Bruker ion trap) and 15 lb/in² (for the Agilent ion trap). The ion trap parameters were automatically set by the molecular mass of raffinose. For analysis, 2 μ l of sample was diluted in 200 μ l of 50% methanol and 1% HCOOH. Between 5 and 10 μ g of ammonium chloride was added to form chloride adduct ions [M+Cl]⁻. Samples were injected to the ion source with syringe using infusion pump at a flow rate of 300 μ l/h.

Accession number(s). The accession numbers for the sequences deposited in GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) were KX792119, KX792120, KX792121, and KX792122 for G4NG45, Q2U112, Q4WH00, and E3R0R1, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01383-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.7 MB.

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