



This is an electronic reprint of the original article. This reprint may differ from the original in pagination and typographic detail.

Mollerup, Filip; Parikka, Kirsti; Vuong, Thu V.; Tenkanen, Maija; Master, Emma

Influence of a family 29 carbohydrate binding module on the activity of galactose oxidase from Fusarium graminearum

Published in: Biochimica et Biophysica Acta: General Subjects

DOI: 10.1016/j.bbagen.2015.10.023

Published: 01/02/2016

Document Version Peer-reviewed accepted author manuscript, also known as Final accepted manuscript or Post-print

Published under the following license: CC BY-NC-ND

Please cite the original version:

Mollerup, F., Parikka, K., Vuong, T. V., Tenkanen, M., & Master, E. (2016). Influence of a family 29 carbohydrate binding module on the activity of galactose oxidase from Fusarium graminearum. *Biochimica et Biophysica Acta: General Subjects*, *1860*(2), 354-362. https://doi.org/10.1016/j.bbagen.2015.10.023

This material is protected by copyright and other intellectual property rights, and duplication or sale of all or part of any of the repository collections is not permitted, except that material may be duplicated by you for your research use or educational purposes in electronic or print form. You must obtain permission for any other use. Electronic or print copies may not be offered, whether for sale or otherwise to anyone who is not an authorised user.

Influence of a family 29 carbohydrate binding module on the activity of galactose oxidase from *Fusarium graminearum*

Filip Mollerup¹, Kirsti Parikka², Thu V. Vuong³, Maija Tenkanen² and Emma Master^{1,3*}

¹Department of Biotechnology and Chemical Technology, Aalto University, 00076 Aalto, Finland

²Department of Food and Environmental Sciences, University of Helsinki, P.O. Box 27, Helsinki 00014, Finland

³ Department of Chemical Engineering and Applied Chemistry, University of Toronto, 200 College Street, Toronto, Ontario, M5S 3E5, Canada.

*Corresponding author:

Emma R. Master

Department of Chemical Engineering and Applied Chemistry

University of Toronto, 200 College Street

Toronto, Ontario, M5S 3E5, Canada.

Phone: +1 - 416-946-7861

Fax: +1 - 416-978-8605

E-mail: <u>emma.master@utoronto.ca</u>

ABSTRACT

Background

Galactose oxidase (GaO) selectively oxidizes the primary hydroxyl of galactose to a carbonyl, facilitating targeted chemical derivatization of galactose-containing polysaccharides, leading to renewable polymers with tailored physical and chemical properties. Here we investigate the impact of a family 29 glucomannan binding module on the activity and binding of GaO towards various polysaccharides. Specifically, CBM29-1-2 from *Piromyces equi* was separately linked to the N- and C-termini of GaO.

Results

Both GaO-CBM29 and CBM29-GaO were successfully expressed in *Pichia pastoris*, and demonstrated enhanced binding to galactomannan, galactoglucomannan and galactoxyloglucan. The position of the CBM29 fusion affected the enzyme function. Particularly, C-terminal fusion led to greatest increases in galactomannan binding and catalytic efficiency, where relative to wild-type GaO, k_{cat}/K_m values increased by 7.5 and 19.8 times on guar galactomannan and locust bean galactomannan, respectively. The fusion of CBM29 also induced oligomerization of GaO-CBM29.

Major conclusions

Similar to impacts of cellulose-binding modules associated with cellulolytic enzymes, increased substrate binding impeded the action of GaO fusions on more concentrated preparations of galactomannan, galactoglucomannan and galactoxyloglucan; this was especially true for GaO-CBM29. Given the N-terminal positioning of the native galactose-binding CBM32 in GaO, the varying impacts of N-terminal versus C-terminal fusion of CBM29-1-2 may reflect competing action of neighbouring CBMs.

General significance

This study thoroughly examines and discusses the effects of CBM fusion to nonlignocellulytic enzymes on soluble polysaccharides. Herein kinetics of GaO on galactose containing polysaccharides is presented for the first time.

Keywords: galactose oxidase, carbohydrate binding module, galactomannan, galactoglucomannan, galactoxyloglucan, fusion proteins

1. INTRODUCTION

Galactose 6-oxidase (GaO, D-galactose: oxygen 6-oxidoreductase, EC 1.1.13.9, CAZy family AA5) oxidizes a wide range of primary alcohols, including the C6 hydroxyl group of galactose, with concomitant reduction of dioxygen to hydrogen peroxide. GaO oxidation of primary alcohols to the corresponding aldehyde is catalyzed by a free radical-coupled copper complex, which is stabilized by an irreversible tyrosyl-cysteine thioether linkage that forms spontaneously in the presence of oxygen and reduced copper ions [1]. The mechanism of autocatalytic processing by GaO has been well characterized, and involves copper incorporation followed by cleavage of an N-terminal pro-sequence and formation of the thioether linkage between the side-chains of Tyr272 and Cys228 [1–4].

To confirm full activation of recombinantly expressed GaO, the electrophoretic mobility of the enzyme has been evaluated, as has the activity of GaO in the presence of an oxidant. In particular, since the thioether bond is resistant to reduction under normal SDS-PAGE conditions, self-induced maturation is observed by gel electrophoresis as an apparent loss in molecular weight of 3 kDa [4]. In addition to the fully activated Cu(II)-Tyr[•]-Cys complex, GaO can be in two alternative oxidation states: the fully reduced form (Cu(I)-Tyr-Cys), and the semi-reduced form (Cu(II)-Tyr-Cys). Accordingly, potassium ferricyanide has been added to enzyme preparations to ensure reactions are performed using fully activated GaO [2,5].

Structural characterizations reveal a shallow exposed copper complex in the active site of GaO [6]. This could explain why to date, GaO is the only oxidase reported to oxidize primary hydroxyls in galactose-containing polysaccharides, including tamarind seed galactoxyloglucan, guar galactomannan, and spruce galactoglucomannan [7]. This unique activity has been harnessed for formation of aero- and hydrogels [8,9] as well as chemoenzymatic modification of polysaccharides, whereby primary alcohols on terminal galactose

substituents are enzymatically oxidized allowing subsequent regioselective chemical derivatizations. For example, Parikka et al. (2012) [10] demonstrate that aldehyde groups introduced by GaO into polysaccharide substrates could be further oxidized to galacturonic acid derivatives, and that resulting polysaccharides bind more strongly to cellulose surfaces than (2,2,6,6-tetramethylpiperidin-1-yl)oxyl-oxidized polysaccharides (i.e. TEMPO mediated oxidation). That same year, Xu et al. [11] used GaO to introduce aldehyde functionalities to galactoxyloglucan, which allowed regioselective introduction of propargylamino groups that are amendable to "click chemistry" [12]. More recently, Leppänen et al. [13] demonstrated that aldehyde functionalities introduced to the C6-position of galactose in various hemicelluloses permits site-specific allylation, which also represent reactive "hot-spots" for further functionalizations. Despite the ability of GaO to facilitate regioselective modification of polysaccharides, most studies that have used GaO for this purpose have reported incomplete oxidation of terminal galactose units, especially in conditions where the polysaccharide is not fully soluble [10].

Adherence of carbohydrate-active enzymes to substrates is often mediated through associated carbohydrate-binding modules (CBMs), which can potentiate enzyme activity on insoluble substrates such as cellulose, especially when the insoluble substrate is also present at low concentrations [14]. Similar to cellulose binding modules, CBMs that bind hemicelluloses, including xylan and glucomannan, can alter the activity of the cognate catalytic domain. For example, appending a family 6 CBM or CBM22 to glycoside hydrolase (GH) family 10 xylanases can increase the activity of corresponding enzymes on insoluble oat spelt xylan [15,16], and retention of a CBM3 increases the activity of *Paenibacillus curdlanolyticus* Xyn10D on insoluble wheat arabinoxylan [17]. Likewise, linking a CBM2 to the C-terminus of *Thermotoga maritima* Cel74 promotes hydrolysis of microcrystalline cellulose (Avicel) [18], whereas the native CBM1 domain of *Trichoderma*

reesei Man5A enables hydrolysis of mannans immobilized to cellulose [19]. While most studies of CBM fusion on enzyme action demonstrate enhanced activity towards insoluble substrates, impacts on solubilized substrates have also been observed. For example, fusing a bacterial CBM2 to the C-terminus of the GH10 xylanase from *T. maritima* (XynB) was reported to double XynB activity towards soluble birchwood xylan [20], and CBM32 of *Clostridium thermocellum* Man5A reduces the transglycosylation activity observed for Man5A alone [21].

In the case of GaO, a family 32 CBM predicted to bind galactose tightly associates with the N-terminal end of the catalytic domain such that deletion of this binding module inactivates the enzyme [22]. Also relevant to binding galactose-containing polymers, the CBM29-1-2 tandem from *Piromyces equi* comprises two CBM29 modules that act synergistically to promote binding to a broad range of polysaccharides, including galactomannans and galactoglucomannans [23]. Structural characterization of CBM29-1 and CBM29-2 reveal that both hydrophobic stacking interactions and direct hydrogen bonds contribute to ligand binding, and that the narrow binding cleft and ligand-induced conformational changes observed for CBM29-1 could explain the lower affinity of CBM29-1 for carbohydrates compared to CBM29-2 [24]. Furthermore, mutagenesis studies of CBM29-2 that include complexes with cello- and mannohexaose, show that interactions between this module and the O2 of mannose or glucose minimally impact the overall binding energy of CBM29-2 [24,25]. This observation, along with weaker binding by CBM29-1 likely explains the broad range of polysaccharides recognized by CBM29-1-2 as a whole.

In *P. equi*, CBM29-1-2 is positioned at the C-terminus of a noncatalytic protein (NCP1) that forms part of a cellulase-hemicellulase complex. Herein we describe the first direct fusion between CBM29-1-2 and a carbohydrate-active enzyme. Specifically, we explore the impact of fusing CBM29-1-2 to the N- and C-terminus of GaO from *Fusarium*

graminearum, generating CBM29-GaO and GaO-CBM29, respectively. We hypothesized that CBM29-1-2 fusion would promote GaO binding to a broad range of polysaccharides, which in turn could increase the catalytic efficiency of GaO on galactose-containing substrates. We further hypothesized that by increasing GaO association with the substrate, higher final oxidation of targeted polysaccharides could be achieved. Accordingly, after confirming that the fused CBM29-1-2 promoted GaO binding to a series of galactose containing polysaccharides, we evaluated the influence of CBM29-1-2 binding on GaO-mediated oxidation of spruce galactoglucomannan, galactomannan from guar and locust bean, and tamarind galactoxyloglucan. These polysaccharides are similarly characterized by a β -(1 \rightarrow 4)-linked glycoside backbone comprised of glucose and/or mannose, but differ by varying degrees of terminally substituted galactose (Fig. 1).

This study showed that CBM29-1-2 fusion increased GaO binding and catalytic efficiency towards galactoxyloglucan, galactomannan and galactoglucomannan, where catalytic efficiency and binding to polysaccharides was particularly enhanced for the Cterminal fusion, GaO-CBM29. However, benefits of enhanced substrate binding and enzyme efficiency did not translate to higher enzyme performance as measured by end-point oxidation of the selected polysaccharides. Therefore, analogous to impacts of cellulosebinding modules on hydrolytic enzymes, the current assessment shows that the overall performance of CBM containing carbohydrate oxidases may be limited by enzyme dissociation. In addition to implications for CBM applications, the impact of CBM positioning on corresponding GaO variants will be discussed.

2. MATERIALS AND METHODS

2.1. Materials. Yeast extract, yeast nitrogen base and peptone were purchased from Lab M Limited (UK), whereas all other chemicals were reagent grade and purchased from Sigma Aldrich unless otherwise specified. Locust bean galactomannan was purchased from

Fluka, whereas galactoxyloglucan from tamarind seed was obtained from Megazyme, and guar galactomannan was obtained from Sigma Aldrich (batch 14K0168). Spruce galactoglucomannan was generously provided by Professor Stefan Wilför (Åbo Academy, Finland), and *Pichia pastoris* KM71H expressing wild-type GaO was kindly supplied by Professor H. Brumer (University of British Columbia) [26]. Reported molecular weights of polysaccharides used in this analysis are as follows: 29 kDa for spruce galactose glucomannan [27], 470 kDa for tamarind galactoxyloglucan, 2600 kDa for guar galactomannan [7], and 310 kDa for locust bean galactomannan (as stated by Sigma-Aldrich (G0753)).

2.2. Construction of fusion proteins. The amino acid sequence of galactose oxidase from *Fusarium graminearum* (P0CS93) and the family 29 carbohydrate binding module (CBM29-1-2) from *Piromyces equi* (AAK20910) were used to design GaO-CBM29 and CBM29-GaO fusions according to Spadiut et al. (2010) [26], where both lacked the N-terminal pro-sequence of native GaO, and contained a C-terminal His₆-tag as well as the following linker sequence: TPTKGATPTNTATPTKSATATPTRPSVPTNTPTNTPANTPM (see Fig. S1 for full protein sequences). Genetic constructs were designed based on the enzyme sequence and optimized for expression in *Pichia pastoris*. Gene sequences were then introduced into the pJ912 expression plasmid (DNA 2.0; USA), allowing expression in *P. pastoris* from the AOX1 methanol inducible promotor. The resulting pJ912 plasmids obtained from DNA 2.0 were transformed into *E. coli* XL-1 (Agilent Technologies, USA) for storage and regeneration, and transformed into *P. pastoris* SMD1168H (Invitrogen) by electroporation for protein production. Notably, *P. pastoris* was used to produce enzymes for the current study, given its reported advantages for scale-up over *E. coli* [26].

Colonies of *P. pastoris* transformants were induced on buffered methanol-complex agar plates (BMMY agar (w/v): 1% yeast extract, 2 % peptone, 2 % agar, 100 mM potassium

phosphate buffer (pH 6.0), 1.34 % yeast nitrogen base without amino acids (YNB), 4 x 10^{-5} % biotin, 0.5 % methanol), and then screened for protein expression by immuno-colony blot as previously described [28]. Integration of the plasmid into the *P. pastoris* genome was verified by colony PCR using gene specific primers.

2.3. Expression of GaO constructs. *P. pastoris* transformants expressing GaO, CBM29-GaO, or GaO-CBM29 were grown in shake flasks and a 2-L bioreactor system [29]. Briefly, for shake flask cultivations, selected *P. pastoris* transformants were induced to express CBM29-GaO or GaO-CBM29 at 15 °C for 4 days using buffered minimal methanol medium containing histidine (BMMH (w/v): 100 mM potassium phosphate buffer (pH 6.0), 1.34 % yeast nitrogen base without amino acids (YNB), 4 x 10⁻⁵ % biotin, 0.5 % methanol, 0.004% histidine); 0.5 % methanol was added every 24 h to replenish the inducer.

All fermentations were performed using a Biostat B Plus bioreactor (Sartorius), where cultivation conditions were based on Pichia Fermentation Process Guidelines provided by Invitrogen, with minor modifications [29]. Once acclimatized to methanol metabolism, the methanol flow rate was adjusted to 7.0 mL h⁻¹ (for SMD1168H transformants) or 2.7 mL h⁻¹ (for KM71H transformants) and DO was monitored to ensure that oxygen consumption was limited by methanol metabolism.

GaO-CBM29 and CBM29-GaO were purified from shake-flask cultivations using ammonium sulfate (up to 70 (w/v) % of saturation at 4 °C), followed by dissolving the resulting protein pellet in 50 mM sodium phosphate buffer (pH 7.0) before filtration and affinity purification using Ni-NTA resin (Qiagen) [29]. Fractions containing purified GaO-CBM29 were exchanged to 50 mM sodium phosphate buffer (pH 7.0), whereas fractions containing CBM29-GaO were adjusted to 1 M ammonium sulfate and then further purified by hydrophobic interaction chromatography (HIC) using a 10 mL phenyl sepharose fast flow column (GE Life Sciences) [29]. To purify enzymes from the bioreactor system, recovered

supernatant was adjusted to pH 7.5 and 1 M ammonium sulfate. The recombinant proteins were subsequently purified using a 10 mL phenyl sepharose FF column followed by Ni-NTA affinity resin [29]. To reach more than 95% homogeneity, CBM29-GaO samples were further adjusted to pH 7.0 and passed through a 1 mL DEAE-sepharose column (HiTrap DEAE FF, GE Life Sciences) [29].

In all cases, protein samples were transferred to 50 mM sodium phosphate buffer (pH 7.5) before measuring protein concentration using the Bradford method (Bio-Rad Laboratories, USA). To ensure full activation of GaO, purified wild-type GaO and GaO fusions were treated with copper sulfate and potassium ferricyanide as previously described [2,4]. Purified samples were then flash frozen and stored at -80°C.

2.4. Affinity gel electrophoresis. Binding of wild-type GaO and CBM29 fusions to guar and locust bean galactomannan, tamarind galactoxyloglucan, and spruce galactoglucomannan was examined by native affinity gel electrophoresis as described by Freelove et al. (2001) [23]. Briefly, native polyacrylamide gels prepared for these analyses contained 7.5 % (w/v) bis-acrylamide, 25 mM Tris/250 mM glycine buffer (pH 8.8) and 0.01% or 0.005% (w/v) of each polysaccharide. Approximately 5 µg of GaO and each fusion protein were loaded onto the gels in Commassie blue G-250 and then run at 10 mA/gel for 3 to 5 h. Phosphorylase B from rabbit muscle (5 µg, Sigma) was used as a reference for these analyses.

2.5. Analytical ultracentrifugation. Three dilutions of GaO and GaO-CBM29 proteins in 50 mM sodium phosphate pH 7.5 were spun at 7,000 and 9,000 as well as 11,000 rpm at 4 °C in a Beckman Optima XL-A analytical ultracentrifuge using an An-60 Ti rotor. The density of buffer and the partial specific volumes of proteins were calculated using SedNterp [30]. Data analysis was done with the Origin MicroCal XL-A/CL-I Data Analysis Software Package version 4.0.

2.6. GaO activity assay. The activity of wild-type GaO and GaO fusions was measured using the previously described chromogenic ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay [2]. The standard reaction mixture (final volume: 250 μ L) contained 7 U/mL horseradish peroxidase, 2 mM ABTS, and 300 mM galactose in 50 mM sodium phosphate buffer (pH 7.0); reactions were then initiated by adding 5 μ L of the purified enzyme sample diluted to accurately measure initial reaction rates. Reactions were monitored at 420 nm at 30 °C for 3 min. Hydrogen peroxide (from 5 x 10⁻⁴ to 5 x 10⁻² μ mole) was used to generate a standard curve. Each reaction was performed in triplicate, at minimum.

For kinetic analyses, 100 µL of a solution containing 4 mM ABTS and 15 units/mL horseradish peroxidase in 50 mM sodium phosphate buffer (pH 7.0) were mixed with 5 µL of enzyme (adjusted to 0.2 pmoles for galactose and 1.5 pmoles for polysaccharides) and then preheated to 30 °C. To initiate the reaction, 100 µL of substrate solution (in 50 mM sodium phosphate buffer, pH 7.0) was added to the sample and initial rates of reaction were followed by measuring absorbance at 420 nm. A standard curve was generated using hydrogen peroxide $(4.9*10^{-2} \text{ to } 4.9*10^{-4} \text{ µmoles})$ to quantify the oxidation of substrates. To determine apparent kinetic parameters using polysaccharides (galactoxyloglucan, galactoglucomannan, locust bean and guar galactomannan) final polysaccharide concentrations were between 0.005 -0.1 % (w/v). Kinetic parameters of wild-type GaO and CBM29-1-2 fusions for galactosecontaining polysaccharides calculated using the Michaelis-Menten equation in the Origin Software. Each reaction was performed in triplicate, at minimum. Molar content of galactose was calculated based on reported saccharide compositions for spruce galactoglucomannan [27], tamarind xyloglucan [31], and guar and locust bean galactomannan [32]. The apparent $K_{\rm m}$ values considering percent concentration of each polysaccharide is also indicated in Table 1.

2.7. Quartz crystal microbalance with dissipation (QCM-D). Locust bean galactomannan (0.5 % (w/v)) prepared in 50 mM sodium phosphate pH 7.5 was spin-coated on gold sensors (Q-Sense, Sweden) using a WS-400B-6NPP spin coater (Laurell Technologies, USA) following a previously reported protocol [33]. QCM-D experiments were performed using the Q-Sense E4 instrument (Q-Sense, Sweden). The coated sensors were washed and equilibrated with the binding buffer (50 mM sodium phosphate pH 7.5) before injecting the protein solutions (0.5 μ g/mL in the binding buffer). For the entire experiment, the flow rate was 0.1 mL/min and the temperature was maintained at 25 °C.

2.8. Determination of the degree of oxidation of polysaccharides. The oxidation of polysaccharides was done with high GaO load (1.5 U/mg galactose). Guar galactomannan, galactoglucomannan and galactoxyloglucan (0.1% w/v) were stirred in Milli-Q water for 24 h to ensure dissolution. The enzymes GaO, HRP, and catalase were added. The enzyme dosages and reaction conditions were based on previous studies [10]. The amount of GaO was related to the approximate amount of terminal galactose present in the polymer (1.5 U of GaO/mg of galactose). The dosages of HRP and catalase were 11 and 1870 U/mg of galactose, respectively. For example, 24 U of GaO, 181 U of HRP, and 30 kU of catalase were used in the oxidation of 40 mg of galactomannan containing about 16 mg of galactose (ca. 40% of total carbohydrates). Samples (1 ml; containing 1 mg of polysaccharide) were taken after 1, 3, 5, and 24 h, and the enzymes were inactivated by heating the samples in a boiling water bath for about 5 min. A previously developed method utilizing NaBD4 reduction and GC-MS analysis was utilized in the determination of the degree of oxidation was calculated as an average of the DOs of the duplicate samples.

2.9. Impact of pH and temperature. The standard activity assay was used to evaluate the impact of CBM29 fusion on the pH optimum and temperature stability of GaO.

The pH optima were determined by performing the standard GaO assay at pH 6.0 to 9.0 with increments of 0.5 pH units; buffers used included 100 mM sodium citrate buffer (pH 6.0 and 6.5), sodium phosphate buffer (pH 7.0 and 7.5), and Trizma•Cl buffer (pH 8.0 to 9.0). Each reaction was performed in triplicate, at minimum.

To investigate the potential of CBM29 to increase the temperature stability of GaO, 400 μ L solutions containing 2.0 μ M of enzyme in 50 mM sodium phosphate buffer (pH 7.0) were incubated in low protein-binding microtubes at 25°C to 65 °C for 2 h. After cooling reactions to 25 °C residual enzyme activity was measured using the standard GaO assay. Stability at room temperature was determined by measuring GaO activity after incubating 0.2 μ M of each protein for up to 72 h in the presence or absence of 0.75 mg/mL BSA. Each reaction was performed in triplicate, at minimum.

3. RESULTS AND DISCUSSION

3.1. Production of wild-type GaO and CBM29 fusions. Removing CBM32 from wild-type GaO leads to complete loss in enzyme activity [22]; we have also seen that substituting the native CBM32 for CBM29-1-2 does not regain GaO function [34]. Therefore, to investigate the influence of CBM29-1-2 fusion to GaO activity, the binding module was fused to either the N-terminus or C-terminus of GaO that retained the N-terminal CBM32 but omitted the native pro-sequence, thereby yielding CBM29-GaO and GaO-CBM29, respectively.

A detailed summary of recombinant GaO, CBM29-GaO, GaO-CBM29 expression in *P. pastoris* and subsequent purification is reported in Mollerup et al. [29]. Briefly, N-terminal or C-terminal fusion of CBM29-1-2 led to similar trends in protein production as previously observed for recombinant GaO expression in *P. pastoris* [26, 35]. The addition of copper sulfate to shake-flask cultivations doubled GaO-CBM29 activity (see Figure 1 in [29]),

whereas enzyme production in a bioreactor system increased the yield of CBM29-GaO and GaO-CBM29 by more than 12 times and 6 times, respectively; corresponding specific activities also increased by more than 20% (see Table 1 in [29]). Furthermore, the addition of 0.5 mM copper (II) sulfate to reaction mixtures containing purified GaO, CBM29-GaO, and GaO-CBM29, or treatment with potassium ferricyanide, did not increase corresponding specific activities, confirming that the purified proteins were in the fully oxidized and active state [Cu²⁺-Tyr•-Cys] (see Table 2 and Figure 5 in [29]). However, expression of the N-terminal construct led to co-expression of seemingly truncated versions of GaO-CBM29, which efficiently bound the Ni-NTA column through the remaining C-terminal his₆-tag. Ultimately, CBM29-GaO was successfully isolated using a DEAE column, albeit at comparatively low yield (see Figure 3 in [29]).

3.2. Improved binding to selected hetero-polysaccharides. Before investigating the impact of CBM29-1-2 fusion on GaO activity, the function of the fused CBM29-1-2 was first verified using affinity gel electrophoresis (AGE). All galactose-containing polysaccharides that would be later used in activity measurements were included in the AGE analysis to also uncover potential differences in CBM29-1-2 affinity towards each substrate. These polysaccharides were galactoxyloglucan from tamarind seed, galactomannan from guar and locust bean, and spruce galactoglucomannan (structures are depicted in Fig. 1).

The AGE analyses confirmed that CBM29-1-2 fusion to either the amino or carboxyl terminus of wild-type GaO increased GaO binding to tested polysaccharides, particularly guar and locust bean galactomannan (Fig. 2). Notably however, even though SDS-PAGE analyses showed N-terminal and C-terminal CBM29-1-2 fusions migrating as single bands with molecular weights consistent with calculated values (see Figures 2 and 3 in [29]), AGE resolved both proteins as two bands. This was especially apparent for GaO-CBM29, even in gels lacking polysaccharide. Flint et al. [36] observed ligand-mediated dimerization of the

E78R variant of CBM29-2. Therefore, to determine whether CBM29-1-2 could lead to oligomerization particularly when fused to the C-terminus of GaO, the oligomerization states of GaO and GaO-CBM29 were evaluated using analytical ultracentrifugation (AUC) in absence of substrate. The ratios of the oligomer mass to the theoretical monomer mass for GaO and GaO-CBM29 were 1.1:1 and 2.5:1 respectively, where in both cases the data clearly fit a monodisperse model (Fig. 3). In this case, the fractional ratio calculated for GaO-CBM29 might be explained by the presence of low quantities of either the dimeric or trimeric form of GaO-CBM29, which is also consistent with the detection of two distinct bands by AGE.

3.3. Activity and kinetics of GaO and fusion proteins. After confirming that fusion of CBM29-1-2 to the amino or carboxyl terminus of wild-type GaO did not affect the pH optimum of the enzyme, specific activities and kinetic parameters were determined using galactose and galactose-containing polysaccharides to evaluate the impact of CBM29-1-2 fusion on GaO activity. Because GaO specifically targets galactose, kinetic parameters were calculated based on galactose contents in each polysaccharide (Table 1). However, since this is the first time kinetic values for GaO on polysaccharides have been reported, corresponding apparent K_m values based on polysaccharide concentration (% (w/v)) are also presented (Table 1).

Although not initially expected, CBM29-1-2 fusion slightly increased the k_{cat} of GaO on galactose by approximately 1.5 fold (Table 1). However, K_m values also increased by a similar amount such that catalytic efficiencies were essentially unaltered. It is conceivable that CBM29-1-2 fusion influenced GaO folding, although the impact of expressing wild-type GaO and GaO fusions in KM71H versus SMD1168 strains respectively, can not be ruled out. Highest gains to wild-type GaO activity were observed after C-terminal CBM29-1-2 fusion, where catalytic efficiencies towards guar and locust bean galactomannan increased by up to

7.5 and 19.8 times, respectively (Table 1). Increases in catalytic efficiency were more modest after N-terminal fusion of CBM29-1-2, and in fact decreased 2-fold for galactoxyloglucan.

As summarized in Figure 1, galactose represents approximately 18 % of the sugars in tamarind galactoxyloglucan [30], approximately 33% and 20% of sugars in guar and locust bean galactomannan, respectively [31], and 10% (w/v) of the sugars in spruce galactoglucomannan [27]. In light of these differences, two particularly intriguing trends to emerge from the kinetic analyses were 1) out-performance of C-terminal CBM29-1-2 fusions over N-terminal constructs, and 2) higher gains in catalytic efficiency towards galactomannans compared to galactoxyloglucan and galactoglucomannan. Given the higher carbohydrate binding affinities reported for CBM29-2 compared to CBM29-1 [23,24], it is conceivable that binding preferences of neighbouring CBM29-2 and CBM32 is antagonistic, particularly when corresponding ligands are distantly positioned on the same polysaccharide molecule. This may explain the increase in K_m values of CBM29-GaO towards galactoxyloglucan, and presents the compelling possibility to utilize CBM fusions of GaO to probe the distribution of terminal galactose substituents in different polysaccharides. At the same time, highest gains in catalytic efficiency were observed for GaO-CBM29 towards locust bean galactomannan, which were largely explained by decreased K_m values. Since the galactose content in galactomannan from guar is nearly two times higher than that from locust bean, higher gains in catalytic efficiency using locust bean galactomannan suggests that the affinity of CBM29-1-2 towards mannan may be higher than the affinity of CBM32 from GaO towards galactose. Although direct comparisons of corresponding CBMs would be necessary to confirm this possibility, the corresponding hypothesis as well as potential antagonism between CBM32 and CBM29-1-2 modules linked to GaO, was further explored using quartz crystal balance with dissipation.

3.4. Quartz crystal microbalance with dissipation (QCM-D). When GaO-CBM29 and CBM29-GaO were passed in parallel over gold sensors coated with locust bean galactomannan, GaO-CBM29 led to a greater frequency decrease than its counterpart (Fig. 4), consistent with higher stable binding by GaO-CBM29. The comparatively strong affinity of GaO-CBM29 towards locust bean galactomannan was further evidenced by higher rates of adsorption as well as the additional drop in frequency observed when passing GaO-CBM29 over a locust bean galactomannan-coated sensor already treated with CBM29-GaO; a similar effect was not seen when CBM29-GaO was passed over GaO-CBM29 treated sensors (Fig. 4). Accordingly, consistent with the interpretation of kinetic analyses, the lower binding of CBM29-GaO to locust bean galactomannan than GaO-CBM29 as determined by QCM-D again predicts antagonism between the neighboring CBM29-2 and CBM32 in CBM29-GaO. Alternatively, the neighbouring CBM32 may disrupt the cooperative binding previously reported between CBM29-1 and CBM29-2 [23]. Of note, QCM-D did not detect binding of wild-type GaO to locust bean galactomannan (Fig. S2). This result confirms that binding of galactomannan by CBM29-GaO and GaO-CBBM29 was largely mediated by the appended CBM29 rather than the native CBM32, further suggesting that CBM29 affinity towards mannan is higher than the affinity of CBM32 towards galactose.

3.5. Degree of oxidation of galactose containing polysaccharides. Whereas CBM29-1-2 fusion improved the catalytic efficiency of GaO on most galactose containing polysaccharides, the extent of oxidation after prolonged reaction periods is also important to evaluate since it predicts how well each enzyme would perform in practical applications. Therefore, the degree of oxidation by wild-type GaO and CBM29-1-2 fusions was compared using spruce galactoglucomannan, galactoxyloglucan, and guar galactomannan. The final concentration of polysaccharides used in these experiments was 0.1% (w/v), which was required to facilitate analysis but notably also corresponded to highest substrate

concentrations used for kinetic analyses on completely dissolved polysaccharides. Because of the variable galactose content in the substrates, the results are presented as the degree of oxidized galactose on basis of total galactoses in each substrate (Fig. 5a) and on total carbohydrates (Fig. 5b).

The degree of oxidation was similar or lower for CBM29-GaO compared to wild-type GaO, particularly in the first 5 h of the reaction (Fig. 5a). By contrast, even though the catalytic efficiency of GaO-CBM29 on galactose containing polysaccharides was higher than CBM29-GaO and GaO, enzyme performance as measured by degree of oxidized polysaccharides after 24 h was lowest for GaO-CBM29 (Fig. 5a).

Lowest performance of GaO-CBM29 in these reaction conditions becomes particularly evident when comparing degrees of oxidation of galactoxyloglucan and galactoglucomannan after 1 h and 5 h. The loss of competitiveness observed during kinetic analysis of GaO-CBM29 is not surprising when recalling that substrate concentrations used in these experiments were above the apparent K_m values of the enzymes tested. Moreover, since galactose contents in galactoxyloglucan and galactoglucomannan are lower than in guar galactomannan, comparatively high affinity towards backbone sugars could limit GaO oxidation of galactoxyloglucan and galactoglucomannan when these substrates are present at high concentrations. This is because in these cases, enzyme performance would be especially limited by slow dissociation from the polysaccharide to access new oxidation sites. Accordingly, similar to studies employing cellulose binding modules to increase cellulase performance, gains in GaO activity upon fusion to a hemicellulose binding module were lost upon increasing substrate concentration, especially since substrate concentrations tested herein formed a homogeneous rather than insoluble suspension.

3.6. Effect of C-terminal CBM29-1-2 fusion on the temperature stability of GaO. In addition to impacting enzyme action on polymeric substrates, CBMs from other

thermophilic bacteria have been shown to increase the thermostability of fused enzymes from mesophilic eukaryotes [38]. Although C-terminal CBM29-1-2 fusion did not affect the stability of GaO after 2 h at 25 to 65 °C (Fig. S3), the GaO-CBM29 construct displayed higher stability when stored at room temperature compared to wild-type GaO. Specifically, wild-type GaO retained only 16% of initial activity after 24 h at room temperature, whereas nearly 50% of GaO-CBM29 remained active after 72 h at the same temperatures (results not shown). While activity losses were irreversible, it is well known that addition of BSA to storage buffer can increase the shelf-life of enzymes. Similarly, since stability at room temperature was compared using the same moles of enzyme, it is possible that the higher stability of GaO-CBM29 resulted from the additional mass contributed by the CBM. Consistent with this explanation, the addition of 0.75 mg/mL BSA did not improve the stability of GaO-CBM29, but led to only 30% loss of wild-type GaO activity after storage for 72 h at room temperature. Alternatively, anchoring the catalytic domain of GaO through the N-terminal CBM32 and C-terminal CBM29 could increase enzyme stability, as has been observed for rigid linker sequences appended to cellulolytic enzymes [39].

4. CONCLUSIONS

Functional production of recombinant CBM29-GaO and GaO-CBM29 was successfully completed using *Pichia pastoris*. Although CBM29-1-2 fusion did not appear to impact the maturation or full activation of GaO, the position of the CBM29-1-2 fusion affected protein yields. Moreover, while fusing CBM29-1-2 to either the N-terminus or Cterminus of GaO did not affect the temperature stability or pH optimum of GaO, CBM29-1-2 positioning affected GaO kinetics as well as binding to galactose-containing polysaccharides; CBM29-1-2 fusion also promoted oligomerization of GaO. Most significantly, C-terminal fusion led to greatest enhancements in polysaccharide binding, which also led to highest gains in catalytic efficiency compared to wild-type GaO and CBM29-GaO. Together with QCM-D studies, the kinetic analyses could be explained by possible antagonism between neighboring CBM29-2 and CBM32 modules in CBM29-GaO, or alternatively by disrupted cooperatively between CBM29-1 and CBM29-2 when CBM-29-2 is also flanked by CBM32. Clearly, a specific study of the CBMs alone and in comparison to the fusion proteins is required to resolve these possibilities.

Similar to impacts of cellulose-binding modules on hydrolytic enzyme activity, gains to GaO activity mediated by CBM29-1-2 were lost by increasing substrate concentration and reaction time. Under these conditions, the lower galactose content in galactoxyloglucan and spruce galactoglucomannan compared to guar galactomannan meant that the degree of polysaccharide oxidation was limited by enzyme detachment rather than binding. In summary, the impact of fusing CBM29-1-2 to the AA5 galactose oxidase was analogous to reported impacts of cellulose binding modules to glycoside hydrolases, where greatest gains are observed when transforming low concentrations of substrate. Our analyses also suggest that enzymes harboring different CBMs at the N- or C-terminus may provide useful molecular tools for polysaccharide characterization.

5. ACKNOWLEDGEMENTS

Funding was provided by: Academy of Finland (Contract No. 1252183). The authors of this

article also thank: M. Andberg, A. Frey, and O. Turunen for helpful discussions, as well as A.

Kankaanpää and H. Li for technical assistance.

6. REFERENCES

- [1] J. Whittaker, The radical chemistry of galactose oxidase, Arch. Biochem. Biophys. 433 (2005) 227–239. doi:10.1016/j.abb.2004.08.034.
- [2] A.J. Baron, C. Stevens, C. Wilmot, K.D. Seneviratne, V. Blakeley, D.M. Dooley, et al., Structure and mechanism of galactose oxidase: The free radical site, J. Biol. Chem. 269 (1994) 25095–25105.
- [3] N. Ito, S.E. Phillips, K.D. Yadav, P.F. Knowles, Crystal structure of a free radical enzyme, galactose oxidase, J. Mol. Biol. 238 (1994) 794–814. doi:10.1006/jmbi.1994.1335.
- [4] M. Rogers, A. Baron, M.J. McPherson, P.F. Knowles, D.M. Dooley, Galactose oxidase pro-sequence cleavage and cofactor assembly are self-processing reactions, J. Amercan Chem. Soc. 122 (2000) 990–991. doi:10.1021/ja993385y.
- [5] M. Rogers, E. Tyler, N. Akyumani, The stacking tryptophan of galactose oxidase: a second-coordination sphere residue that has profound effects on tyrosyl radical behavior and enzyme catalysis, Biochemistry. 46 (2007) 4606–4618. doi:10.1021/bi062139d.
- [6] N. Ito, S.E. Phillips, C. Stevens, Z.B. Ogel, M.J. McPherson, J.N. Keen, et al., Novel thioether bond revealed by a 1.7 A crystal structure of galactose oxidase, Nature. 350 (1991) 87–90. doi:10.1038/350087a0.
- [7] K. Parikka, A.-S.S. Leppänen, L. Pitkänen, M. Reunanen, S. Willför, M. Tenkanen, Oxidation of polysaccharides by galactose oxidase, J. Agric. Food Chem. 58 (2010) 262–71. doi:10.1021/jf902930t.
- [8] K. Parikka, F. Ansari, S. Hietala, M. Tenkanen, Thermally stable hydrogels from enzymatically oxidized polysaccharides, Food Hydrocoll. 26 (2012) 212–220. doi:10.1016/j.foodhyd.2011.05.012.
- [9] K.S. Mikkonen, K. Parikka, J.-P. Suuronen, A. Ghafar, R. Serimaa, M. Tenkanen, Enzymatic oxidation as a potential new route to produce polysaccharide aerogels, RSC Adv. 4 (2014) 11884. doi:10.1039/c3ra47440b.

- [10] K. Parikka, A.S. Leppänen, C. Xu, L. Pitkänen, P. Eronen, M. Österberg, et al., Functional and anionic cellulose-interacting polymers by selective chemo-enzymatic carboxylation of galactose-containing polysaccharides, Biomacromolecules. 13 (2012) 2418–2428. doi:10.1021/bm300679a.
- [11] C. Xu, O. Spadiut, A.C. Araújo, A. Nakhai, H. Brumer, Chemo-enzymatic assembly of clickable cellulose surfaces via multivalent polysaccharides, ChemSusChem. 5 (2012) 661–665. doi:10.1002/cssc.201100522.
- [12] H.C. Kolb, M.G. Finn, K.B. Sharpless, Click Chemistry: Diverse Chemical Function from a Few Good Reactions, Angew. Chemie - Int. Ed. 40 (2001) 2004–2021. doi:10.1002/1521-3773(20010601)40:11<2004::AID-ANIE2004>3.0.CO;2-5.
- [13] A.-S. Leppänen, C. Xu, K. Parikka, P. Eklund, R. Sjöholm, H. Brumer, et al., Targeted allylation and propargylation of galactose-containing polysaccharides in water, Carbohydr. Polym. 100 (2014) 46–54. doi:10.1016/j.carbpol.2012.11.053.
- [14] A. Várnai, M.R. Mäkelä, D.T. Djajadi, J. Rahikainen, A. Hatakka, L. Viikari, Carbohydrate-binding modules of fungal cellulases: Occurrence in nature, function, and relevance in industrial biomass conversion, Adv. Appl. Microbiol. 88 (2014) 103– 165. doi:10.1016/B978-0-12-800260-5.00004-8.
- [15] M.L. Selanere, R. Andersson, Cell wall composition of 1B/1R translocation wheat grains, J. Sci. Food Agric. 82 (2002) 538–545. doi:10.1002/jsfa.1070.
- [16] G. Mamo, R. Hatti-Kaul, B. Mattiasson, Fusion of carbohydrate binding modules from *Thermotoga neapolitana* with a family 10 xylanase from *Bacillus halodurans* S7, Extremophiles. 11 (2007) 169–77. doi:10.1007/s00792-006-0023-4.
- [17] M. Sakka, Y. Higashi, T. Kimura, K. Ratanakhanokchai, K. Sakka, Characterization of *Paenibacillus curdlanolyticus* B-6 Xyn10D, a xylanase that contains a family 3 carbohydrate-binding module, Appl. Environ. Microbiol. 77 (2011) 4260–4263. doi:10.1128/AEM.00226-11.
- [18] S.R. Chhabra, K.R. Shockley, S.B. Conners, K.L. Scott, R.D. Wolfinger, R.M. Kelly, Carbohydrate-induced differential gene expression patterns in the hyperthermophilic bacterium *Thermotoga maritima*, J. Biol. Chem. 278 (2003) 7540–52. doi:10.1074/jbc.M211748200.
- [19] P. Hägglund, T. Eriksson, A. Collén, W. Nerinckx, M. Claeyssens, H. Stålbrand, A cellulose-binding module of the *Trichoderma reesei* β-mannanase Man5A increases the mannan-hydrolysis of complex substrates, J. Biotechnol. 101 (2003) 37–48. doi:10.1016/S0168-1656(02)00290-0.
- [20] F.S. Kittur, S.L. Mangala, A.A. Rus'd, M. Kitaoka, H. Tsujibo, K. Hayashi, Fusion of family 2b carbohydrate-binding module increases the catalytic activity of a xylanase from *Thermotoga maritima* to soluble xylan, FEBS Lett. 549 (2003) 147–51. doi:10.1016/S0014-5793(03)00803-2.

- [21] K. Mizutani, M. Sakka, T. Kimura, K. Sakka, Essential role of a family-32 carbohydrate-binding module in substrate recognition by *Clostridium thermocellum* mannanase CtMan5A, FEBS Lett. 588 (2014) 1726–30. doi:10.1016/j.febslet.2014.03.022.
- [22] M. McPherson, C. Stevens, A. Baron, Galactose oxidase: Molecular analysis and mutagenesis studies, Biochem. Soc. Trans. 21 (1993).
- [23] A.C. Freelove, D.N. Bolam, P. White, G.P. Hazlewood, H.J. Gilbert, A novel carbohydrate-binding protein is a component of the plant cell wall-degrading complex of *Piromyces equi*, J. Biol. Chem. 276 (2001) 43010–7. doi:10.1074/jbc.M107143200.
- [24] J. Flint, D.N. Bolam, D. Nurizzo, E.J. Taylor, M.P. Williamson, C. Walters, et al., Probing the mechanism of ligand recognition in family 29 carbohydrate-binding modules, J. Biol. Chem. 280 (2005) 23718–23726. doi:10.1074/jbc.M501551200.
- [25] S.J. Charnock, D.N. Bolam, D. Nurizzo, L. Szabó, V.A. McKie, H.J. Gilbert, et al., Promiscuity in ligand-binding: The three-dimensional structure of a Piromyces carbohydrate-binding module, CBM29-2, in complex with cello- and mannohexaose., Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 14077–82. doi:10.1073/pnas.212516199.
- [26] O. Spadiut, L. Olsson, H. Brumer, A comparative summary of expression systems for the recombinant production of galactose oxidase, Microb. Cell Fact. 9 (2010) 1–13. doi:10.1186/1475-2859-9-68.
- [27] S. Willför, R. Sjöholm, C. Laine, M. Roslund, J. Hemming, B. Holmbom, Characterisation of water-soluble galactoglucomannans from Norway spruce wood and thermomechanical pulp, Carbohydr. Polym. 52 (2003) 175–187. doi:10.1016/S0144-8617(02)00288-6.
- [28] M. Foumani, Altered substrate specificity of the gluco-oligosaccharide oxidase from Acremonium strictum, Biotechnol. Bioeng. 108 (2011) 2261–2269. doi:10.1002/bit.23149.
- [29] Mollerup Filip, E. Master, Influence of a family 29 carbohydrate binding module on the recombinant production of galactose oxidase in *Pichia pastoris*, Data Br. (n.d.). In press.
- [30] T.M. Laue, B.D. Shah, T.M. Ridgeway, S.M. Pelletier, Computer-aided interpretation of analytical sedimentation data for proteins, in: S.E. Harding, A.J. Rowe, J.C. Horton (Eds.), Anal. Ultracentrifugation Biochem. Polym. Sci., The Royal Society of Chemistry, 1992: pp. 90–125.
- [31] W.S. York, L.K. Harvey, R. Guillen, P. Alberhseim, A.G. Darvill, Structural analysis of tamarind seed xyloglucan oligosaccharides using β-galactosidase digestion and spectroscopic methods, Carbohydr. Res. 248 (1993) 285–301. doi:10.1016/0008-6215(93)84135-S.

- [32] P.J.H. Daas, H.A. Schols, H.H.J. de Jongh, On the galactosyl distribution of commercial galactomannans, Carbohydr. Res. 329 (2000) 609–619. doi:10.1016/S0008-6215(00)00209-3.
- [33] T. V. Vuong, E.R. Master, Fusion of a xylan-binding module to gluco- oligosaccharide oxidase increases activity and promotes stable immobilization, PLoS One. 9 (2014). doi:10.1371/journal.pone.0095170.
- [34] Y. Mottiar, M.Sc. thesis., Univ. Toronto, Toronto, Ontario, Canada. (2012) 15–22.
- [35] G.E. Anasontzis, M. Salazar Penã, O. Spadiut, H. Brumer, L. Olsson, Effects of temperature and glycerol and methanol-feeding profiles on the production of recombinant galactose oxidase in *Pichia pastoris*, Biotechnol. Prog. 30 (2014) 728–35. doi:10.1002/btpr.1878.
- [36] J. Flint, D. Nurizzo, S.E. Harding, E. Longman, G.J. Davies, H.J. Gilbert, et al., Ligand-mediated dimerization of a carbohydrate-binding module reveals a novel mechanism for protein-carbohydrate recognition, J. Mol. Biol. 337 (2004) 417–426. doi:10.1016/j.jmb.2003.12.081.
- [37] M. V. Voinova, M. Jonson, B. Kasemo, "Missing mass" effect in biosensor's QCM applications, Biosens. Bioelectron. 17 (2002) 835–841. doi:10.1016/S0956-5663(02)00050-7.
- [38] S.P. Voutilainen, S. Nurmi-Rantala, M. Penttila, A. Koivula, Engineering chimeric thermostable GH7 cellobiohydrolases in *Saccharomyces cerevisiae*, Appl. Microbiol. Biotechnol. (2013) 1–11. doi:10.1007/s00253-013-5177-2.
- [39] G. Zou, S. Shi, Y. Jiang, J. van den Brink, R.P. de Vries, L. Chen, et al., Construction of a cellulase hyper-expression system in *Trichoderma reesei* by promoter and enzyme engineering, Microb. Cell Fact. 11 (2012) 21. doi:10.1186/1475-2859-11-21.

		GaO		GaO-CBM29					CBM29-GaO			
Substrate	k_{cat} (min ⁻¹)	K _m (mM) ^a	$k_{ m cat}/K_{ m m}$	<i>K</i> _m (wt%) ^b	k_{cat} (min ⁻¹)	$K_{\rm m}$ (mM) ^a	$k_{\rm cat}/K_m$	$K_{\rm m}$ (wt%) ^b	k_{cat} (min ⁻¹)	K _m (mM) ^a	$k_{ m cat}/K_m$	$K_{\rm m}$ (wt%) ^b
D-Galactose	$\begin{array}{c} 24 \times 10^3 \\ \pm 400 \end{array}$	$\begin{array}{c} 42.4 \pm \\ 3 \end{array}$	560 ± 33		$\begin{array}{c} 36 \times 10^3 \\ \pm 900 \end{array}$	60.4 ± 5	590 ± 36		$\begin{array}{c} 31 \times 10^3 \\ \pm \ 900 \end{array}$	56.0 ± 5	560 ± 40	
Galacto- glucomannan	195 ± 28	$\begin{array}{c} 0.16 \\ \pm \ 0.07 \end{array}$	1200 ± 500	28.5 ± 13	200 ± 13	$\begin{array}{c} 0.074 \\ \pm \ 0.02 \end{array}$	2700 ± 700	13.3 ± 4	239 ± 37	0.13 ± 0.08	$\begin{array}{c} 1800 \\ \pm 800 \end{array}$	23.4 ± 14
Galacto- xyloglucan	221 ± 7	$\begin{array}{c} 0.07 \\ \pm \ 0.01 \end{array}$	$\begin{array}{c} 3300 \\ \pm 400 \end{array}$	12.2 ± 2	274 ± 15	$\begin{array}{c} 0.076 \\ \pm \ 0.02 \end{array}$	$\begin{array}{c} 3600 \\ \pm \ 700 \end{array}$	13.7 ± 4	219 ± 10	$\begin{array}{c} 0.14 \\ \pm \ 0.02 \end{array}$	$\begin{array}{c} 1500 \\ \pm 200 \end{array}$	25.2 ± 4
Guar Galactomannan	311 ± 15	$\begin{array}{c} 0.22 \\ \pm \ 0.03 \end{array}$	$\begin{array}{c} 1400 \\ \pm 200 \end{array}$	39.6 ± 5	402 ± 11	$\begin{array}{c} 0.037 \\ \pm \ 0.005 \end{array}$	$\begin{array}{c} 11 \times 10^3 \\ \pm 2000 \end{array}$	6.7 ± 1	313 ± 15	$\begin{array}{c} 0.081 \\ \pm \ 0.02 \end{array}$	$\begin{array}{c} 3700 \\ \pm 800 \end{array}$	14.6 ± 4
Locust bean Galactomannan	258 ± 24	0.19 ± 0.06	$\begin{array}{c} 1400 \\ \pm \ 300 \end{array}$	34.2 ± 11	214 ± 9	$\begin{array}{c} 0.008 \\ \pm \ 0.002 \end{array}$	$\begin{array}{c} 27\times\!10^3\\\pm9000\end{array}$	$\begin{array}{c} 1.4 \pm \\ 0.4 \end{array}$	$\frac{382 \pm 24}{24}$	$\begin{array}{c} 0.084 \pm \\ 0.02 \end{array}$	$\begin{array}{c} 4600 \pm \\ 1000 \end{array}$	15.1 ± 4

Table 1. Kinetic parameters of galactose oxidase and CBM29 fusions on galactose-containing polysaccharides.

Errors indicate standard deviations; n=3.

^a $K_{\rm m}$ values based on calculated galactose concentration in reactions containing each polysaccharide (mM) [27, 30, 31]

^bApparent K_m values based on the final concentration of each polysaccharide (mg/L)

FIGURE LEGENDS

Fig 1. Molecular structures of galactoglucomannan, galactoxyloglucan, and

galactomannan. Galactoglucomannan from spruce comprises a β -(1→4)-linked backbone of mannopyranosides and glucopyranosides with a ratio of approximately 4:1, where approximately one in eight mannopyranoside units is decorated with α -(1→6)galactopyranoside [27]. Three out of four β -(1→4)-linked glucopyranoside sugars that make up galactoxyloglucan are substituted with α -(1→6)-xylopyranoside, and approximately half of these xylopyranoside units are further substituted by β -(1→2)-galactopyranoside [31]. Galactomannan from guar and locust bean comprise a β -(1→4)-mannopyranoside backbone that is substituted by α -(1→6)-galactopyranoside, where galactose accounts for approximately 33% and 20% of total sugar in guar and locust bean galactomannan, respectively [32].

Fig 2. Affinity gel electrophoresis of wild-type GaO and CBM29 fusions.

Gels contained (a) 0.01 and (b) 0.005 % (w/v) galactoglucomannan, galactoxyloglucan, guar galactomannan or locust bean galactomannan. Dotted line indicates the migration of the Phosphorylase b (PhB) reference. Lanes; 1: PhB, 2: CBM29-GaO, 3: GaO-CBM29, 4: GaO, 5: PhB.

Fig 3. Analytical ultracentrifugation for the oligomeric state of GaO and GaO-CBM29.

The sedimentation equilibrium of the sample was run at 4°C at speeds of 7,000 and 9,000 as well as 11,000 rpm. The red lines correspond to the fit of the data to a monodisperse monomeric (for GaO) and oligomeric (for GaO-CBM29) models using the apparent molecular mass of these proteins.

Fig 4. QCM-D frequency changes during the binding of GaO-CBM29 or CBM29-GaO with LBG-coated gold sensors. The protein solutions ($0.5 \mu g/mL$) were each passed over the coated sensors with a flow rate of 0.1 mL/min at 25 °C for 5 h. The solid lines represent frequency changes at the 3rd, 5th, 7th, 9th and 11th harmonic overtones of sensors first treated with CBM29-GaO; dash lines show corresponding frequency changes of sensors first coated with GaO-CBM29. Arrows indicate transition points within the experiment. The frequency changes observed for the CBM29 fusions corresponded to approximately 820 and 420 ng/cm² of bound mass for GaO-CBM29 and CBM29-GaO, respectively as calculated using the Voigt model [37].

Fig 5. Degree of oxidation (dOx) of galactose in polysaccharides treated with wild-type GaO and CBM29 fusions. The degree of (a) oxidized galactose units and (b) oxidized galactose of the total carbohydrate content, in each treatment of 0.1% (w/v) spruce galactoglucomannan, tamarind galactoxyloglucan and guar galactomannan.



Figure 1



Figure 2



Figure 3



Figure 4



Figure 5A



Figure 5B

Supplemental material for the manuscript:

Influence of a family 29 carbohydrate binding module on the activity of galactose oxidase from *Fusarium graminearum*

Filip Mollerup¹, Kirsti Parikka², Thu Vuong³, Maija Tenkanen² and Emma Master^{1,3*}

¹Department of Biotechnology and Chemical Technology, Aalto University, 00076 Aalto, Finland ²Department of Food and Environmental Sciences, University of Helsinki, P.O. Box 27, Helsinki 00014, Finland ³Department of Chemical Engineering and Applied Chemistry, University of Toronto, 200 College Street, Toronto, Ontario, M5S 3E5, Canada.

*Corresponding author:

Emma R. Master

Department of Chemical Engineering and Applied Chemistry

University of Toronto, 200 College Street

Toronto, Ontario, M5S 3E5, Canada.

Phone: +1 - 416-946-7861

Fax: +1 - 416-978-8605

E-mail: emma.master@utoronto.ca

A.1. SUPPLEMENTAL FIGURES

GaO

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGVSLEKREAEA ASAPIGSAISRNNWAVTCDSAQSGNECNKAIDGNKDTFWHTFYGANGDPKPPHTYTIDMKTTQNVNGLSMLPRQDGNQNGWIGR HEVYLSSDGTNWGSPVASGSWFADSTTKYSNFETRPARYVRLVAITEANGQPWTSIAEINVFQASSYTAPQPGLGRWGPTIDLPIVP AAAAIEPTSGRVLMWSSYRNDAFGGSPGGITLTSSWDPSTGIVSDRTVTVTKHDMFCPGISMDGNGQIVVTGGNDAKKTSLYDSSS DSWIPGPDMQVARGYQSSATMSDGRVFTIGGSWSGGVFEKNGEVYSPSSKTWTSLPNAKVNPMLTADKQGLYRSDNHAWLFGW KKGSVFQAGPSTAMNWYYTSGSGDVKSAGKRQSNRGVAPDAMCGNAVMYDAVKGKILTFGGSPDYQDSDATTNAHIITLGEPG TSPNTVFASNGLYFARTFHTSVVLPDGSTFITGGQRRGIPFEDSTPVFTPEIYVPEQDTFYKQNPNSIVRVYHSISLLLPDGRVFNGGG GLCGDCTTNHFDAQIFTPNYLYNSNGNLATRPKITRTSTQSVKVGGRITISTDSSISKASLIRYGTATHTVNTDQRRIPLTLTNNGGN SYSFQVPSDSGVALPGYWMLFVMNSAGVPSVASTIRVTQ<mark>HIHHHHH</mark>

GaO-CBM29

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGVSLEKREAEA ASAPIGSAISRNNWAVTCDSAQSGNECNKAIDGNKDTFWHTFYGANGDPKPPHTYTIDMKTTQNVNGLSMLPRQDGNQNGWIGR HEVYLSSDGTNWGSPVASGSWFADSTTKYSNFETRPARYVRLVAITEANGQPWTSIAEINVFQASSYTAPQPGLGRWGPTIDLPIVP AAAAIEPTSGRVLMWSSYRNDAFGGSPGGITLTSSWDPSTGIVSDRTVTVTKHDMFCPGISMDGNGQIVVTGGNDAKKTSLYDSSS DSWIPGPDMQVARGYQSSATMSDGRVFTIGGSWSGGVFEKNGEVYSPSSKTWTSLPNAKVNPMLTADKQGLYRSDNHAWLFGW KKGSVFQAGPSTAMNWYYTSGSGDVKSAGKRQSNRGVAPDAMCGNAVMYDAVKGKILTFGGSPDYQDSDATTNAHIITLGEPG TSPNTVFASNGLYFARTFHTSVVLPDGSTFITGGQRRGIPFEDSTPVFTPEIYVPEQDTFYKQNPNSIVRVYHSISLLLPDGRVFNGGG GLCGDCTTNHFDAQIFTPNYLYNSNGNLATRPKITRTSTQSVKVGGRITISTDSSISKASLIRYGTATHTVNTDQRRIPLTLTNNGGN SYSFQVPSDSGVALPGYWMLFVMNSAGVPSVASTIRVTQT**PTKGATPTNTATPTKSATATPTRPSVPTNTPNTPNTPVSATYSV** VGSFRSSSDFTEYKFEVDDEYQFDRIIVQDGPASNIPIYMRYIIVSTGSCDDFNPVVDTTKKVPVTTTTKKSNVRATYTVIFKNASGLP NGYDNWGWGCTLSYYGGAMIINPQEGKYGAVSLKRNSGSFRGGSLRFDMKNEGKVKILVENSEADEKFEVETISPSDEYVTYILD VDFDLPFDRIDFQDAPGNGDRIWIKNLVHSTGSADDFVDPINHHHHH

CBM29-GaO

MRFPSIFTAVLFAASSALAAPVNTTTEDETAQIPAEAVIGYSDLEGDFDVAVLPFSNSTNNGLLFINTTIASIAAKEEGVSLEKREAEA VSATYSVVYETGKKLNSGFDNWGWDSKMSFKDNSLVLTADPDEYGAISLKNLNSNYYGKGGCIYLQVKTETEGLVKVQGVRGY DETEAFNVGSFRSSSDFTEYKFEVDDEYQFDRIIVQDGPASNIPIYMRYIIYSTGSCDDFNPPVDTTKVPVTTTTKKSNVRATYTVIFK NASGLPNGYDNWGWGCTLSYYGGAMIINPQEGKYGAVSLKRNSGSFRGGSLRFDMKNEGKVKILVENSEADEKFEVETISPSDEY VTYILDVDFDLPFDRIDFQDAPGNGDRIWIKNLVHSTGSADDFVDPINTPTKGATPTNTATPTKSATATPTRPSVPTNTPTNTPANTP MASAPIGSAISRNNWAVTCDSAQSGNECNKAIDGNKDTFWHTFYGANGDPKPPHTYTIDMKTTQNVNGLSMLPRQDGNQNGWI GRHEVYLSSDGTNWGSPVASGSWFADSTTKYSNFETRPARYVRLVAITEANGQPWTSIAEINVFQASSYTAPQPGLGRWGPTIDLP IVPAAAAIEPTSGRVLMWSSYRNDAFGGSPGGITLTSSWDPSTGIVSDRTVTVTVKHDMFCPGISMDGNGQIVVTGGNDAKKTSLYD SSSDSWIPGPDMQVARGYQSSATMSDGRVFTIGGSWSGGVFEKNGEVYSPSSKTWTSLPNAKVNPMLTADKQGLYRSDNHAWLF GWKKGSVFQAGPSTAMNWYYTSGSGDVKSAGKRQSNRGVAPDAMCGNAVMYDAVKGKILTFGGSPDYQDSDATTNAHIITLG EPGTSPNTVFASNGLYFARTFHTSVVLPDGSTFITGGQRRGIPFEDSTPVFTPEIYVPEQDTFYKQNPNSIVRVYHSISLLLPDGRVFN GGGGLCGDCTTNHFDAQIFTPNYLYNSNGNLATRPKITRTSTQSVKVGGRITISTDSSISKASLIRYGTATHTVNTDQRRIPLTLTNN GGNSYSFQVPSDSGVALPGYWMLFVMNSAGVPSVASTIRVTQ

MFalpha1 (Saccharymyces cerivisae signal transduction

Predicted native CBM32 domain of GaO (Fusarium graminearum)

GaO catalytic domain (Fusarium graminearum)

TP-rich linker sequence

CBM29 (Piromyces equi)

His₆-tag

Fig S1. Sequence of GaO/CBM29 fusions. CBM29 was fused to the N- and C-terminus of GaO using the same 40 amino acid linker sequence. A His-tag was added to the C-terminus of all the proteins to facilitate purification.



Fig. S2. QCM-D frequency and dissipation changes during the GaO interaction with locust bean galactomannan-coated or bare gold sensors. The solid lines represent frequency (blue-like) and dissipation (orange-like) changes at the 3rd, 5th, 7th, 9th and 11th harmonic overtones when GaO ($0.5 \mu g/mL$) was injected at the flow rate of 0.1 mL/min at 25 °C over bare gold sensors while the dash lines show frequency and dissipation changes when the enzyme was injected over locust bean galactomannan-coated gold sensors.



at 25 to 65 °C. Symbols indicate wild-type GaO (squares), GaO-CBM29 (diamonds).