Exploring flavin-containing carbohydrate oxidases

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EXPLORING FLAVIN-CONTAINING CARBOHYDRATE OXIDASES

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Chapter 1

Biotechnological applications of carbohydrate oxidases

Alessandro R. Ferrari and Marco W. Fraaije

Background

On the 22nd of April 2016, one-hundred-seventy-five state members of the United Nations Framework Convention on Climate Change met in Paris to sign an agreement that might be the last rescue call in the constant battle of modern civilization against its own nemesis: global warming. By virtue of this agreement, efforts will be made to limit the increase of global temperature to 1.5 degrees Celsius [1].

The increase in greenhouse gas emission is one of the causes pointed out as responsible for global warming. One of the most abundant greenhouse gases is CO_2 whose emission dramatically increased since the industrial revolution. Its emission continues to increase exponentially year by year and the current levels have not been seen on planet Earth since millions of years [2]. Around 40% of the greenhouse gas emission can be attributed to industrial processes, agricultural byproducts and biomass burning. The remaining 60% is due to energy production, fossil fuel retrieval, processing and distribution, fuel used for transportation and finally by residential and commercial infrastructures [3]. Industrial processes are a very conspicuous source of greenhouse gases. For instance, the combustion of all carbon-based fuels to generate energy produces CO_2 as byproduct. Furthermore often toxic and harmful waste compounds are produced that may pose a threat to the environment and to the living beings.

While some biomass is currently used to produce biofuels, most of it is burned as it is very recalcitrant to chemical modification. In the recent years, several efforts have been made to try to render industrial chemistry more sustainable with an approach called "green chemistry". Ruled by 12 principles, the green chemistry approach aims at developing chemical processes and products which have the least impact possible on the environment. This is accomplished by minimizing waste and energy requirements, using renewable and less hazardous chemicals and developing safer and biodegradable products [4]. In this approach, enzymes are often good tools that make the fulfillment of these principles easier. Enzymes can be used as catalysts not only to perform industrial

processes in an environmental sustainable way but also to convert biomass into valuable compounds.

Enzymes can have several advantages over chemical processes in an industrial setting: 1) reactions are performed at atmospheric pressure; 2) required temperatures are significantly lower; 3) production of toxic and harmful byproducts is limited. These features will make a process performed enzymatically more environmentally sustainable because energy requirements are lower, no toxic waste is produced and the handling of the industrial plant is safer since required conditions are not threatening for the safety of the worker.

Biomass is currently being exploited through thermochemical routes. These include gasification, pyrolysis and torrefaction which use elevated temperatures to convert biomass in biofuels or valuable chemicals [5]. An alternative to these methods could be the biochemical route in which enzymes either isolated or as part of GMOs are used to extract value from biomass in two ways. In the first approach, as pre-treatment, lignocellulosic material is broken down, using enzyme cocktails, into its constituent components which are then used for producing biofuels. The second approach is the conversion of mono- or oligosaccharides from carbohydrates coming from plant biomass into valuable products that can be used in a wide array of different applications.

Among all the classes of enzymes so far discovered, this chapter will focus on the role of oxidases in developing environmental sustainable processes. Oxidases are also the central theme of this thesis. In particular, in this introductory chapter we will focus our attention on the application of carbohydrate oxidases by analyzing the patent literature.

Carbohydrate Oxidases

Carbohydrate oxidases are enzymes belonging to the oxidoreductase family. They can selectively oxidize carbohydrates

while using molecular oxygen as electron acceptor. They differ from dehydrogenases, which also catalyze oxidations, since these utilize other molecules as electron acceptors. In a few cases, oxidases will reduce molecular oxygen to harmless water [6], [7] while in some rare cases superoxide is generated [8]. Yet, most oxidases generate hydrogen peroxide as by-product by a two electron reduction of O_2. In fact, production of hydrogen peroxide by oxidases is exploited in a number of applications as we will see in the following paragraphs.

The transfer of electrons from carbohydrate molecules to dioxygen, instead of reducing alternative cofactors involved in the respiratory chain, is peculiar and inefficient from an energetic point of view. Normally electrons are used to generate energy through the respiratory chain by creating a proton gradient which is ultimately used by ATP synthase to generate ATP.

Carbohydrate oxidases evade this process by taking electrons from potential energy sources and transferring them directly to O_2, generating potentially toxic compounds (e.g. hydrogen peroxide, superoxide). This is probably the reason why, in nature, dehydrogenases are far more abundant than oxidases. Yet, many organisms produce oxidases despite the putative detrimental effects discussed above. Especially fungi secrete various oxidases.

It has been hypothesized that the physiological role of oxidases in nature, aside efficient oxidation of organic molecules, might accomplish two purposes: 1) generating hydrogen peroxide or superoxide as way to outcompete other microorganisms in the same ecological niche; 2) producing hydrogen peroxide to serve peroxidases in the degradation of lignin in a synergistic effort to liberate more resources that can be used as energy.

Since amino acids are poor in mediating redox reactions, oxidases evolved to be equipped with a tightly bound cofactor. Two main families of oxidases can be identified in nature: 1) copper-containing oxidases and 2) flavin-containing oxidases. For an extensive review on each oxidase family, the reader is referred to recent reviews [9], [10]. Among the group of the carbohydrate oxidases, at the time of

this writing, most enzymes belong to the flavin-dependent family of oxidases. Proteins belonging to this family are also called flavoprotein oxidases. One notable exception is the copper containing galactose oxidase from the fungus *Dactylium dendroide*.

In the case of flavoprotein carbohydrate oxidases, the FAD cofactor acts as an electron shuttle by abstracting two electrons from a CH–OH moiety of their substrate and transferring it to O_2. The flavin cofactor can be non-covalently, monocovalently or bicovalently bound to the protein. The type of cofactor binding can be often predicted by analyzing the protein sequence of a flavoproteins oxidase [11]. Flavoprotein oxidases with a bicovalently bound FAD show higher catalytic efficiency on bulky substrates such as secondary metabolites and oligosaccharides. This can be explained by the fact that a double covalently bound FAD allows the protein to evolve a relatively open substrate binding pocket thereby accommodating bulkier substrates [12].

The majority of the so far identified carbohydrate oxidases target the anomeric carbon (C1) of the substrate which results in the formation of a lactone. This subsequently may spontaneously hydrolyze in the corresponding aldonic acid. There are two exceptions to this: 1) pyranose 2-oxidase from *Polyporus obtusus* oxidizes its substrates at position C2 or C3 (when the C2 hydroxyl is absent) with the concomitant production of the corresponding ketoaldose; 2) Dbv29 from *Nonomuraea* sp. ATCC 39727 oxidizes carbohydrate moieties at the C6 position.

In recent years several crystal structures of flavin-containing carbohydrate oxidases were resolved. By knowing the molecular structure of carbohydrate oxidases, more insights were gained in the understanding of substrate binding and the mechanism of the oxidation reaction. This can be exploited in enzyme engineering strategies to improve properties of the oxidases such as thermostability, cosolvent tolerance or to change the catalytic scope as we will see in Chapter 2 of this thesis.

Taking glucooligosaccharide oxidase (GOOX) from *Acremonium strictum* as example carbohydrate oxidase we will now describe the structural properties in relation to substrate binding and mechanism of action. GOOX belongs to the vanillyl alcohol oxidase (VAO) family of flavoproteins which is rich in covalent flavoprotein oxidases [13]. The enzyme is composed of two major domains: an FAD binding domain (F domain) and a substrate binding domain (S domain) (Fig. 1). The F domain is formed by the N- and C-termini which fold into two subdomains packed against each other accommodating the FAD cofactor.

The carbohydrate binding groove that is formed by the S domain is made of a large seven-strand antiparallel β-sheet that is positioned over the isoalloxazine ring of the FAD cofactor. Cys^{130} and His^{70} are covalently bound to the isoalloxazine ring of the FAD respectively at the C6 and 8α-methyl group forming the 6-S-cysteinyl, 8α-N1-histidyl FAD (Figure 1). The substrate is positioned close to the reactive part of the flavin cofactor by stacking interactions with Tyr^{300} and Trp^{351} and the pyranose ring (Figure 2A). This is a common interaction for protein-carbohydrate recognition as seen in other carbohydrate oxidases such as lactose oxidase (PDB: 3RJ8), xylooligosaccharide oxidase (PDB: 5L6F), chitooligosaccharide oxidase (data not published).

As like other bicovalent flavoproteins, GOOX has an open carbohydrate-binding groove which allows it to utilize oligosaccharides efficiently. In fact, the non-reducing ends of the pyranose rings stick out into the solvent (Figure 2B).

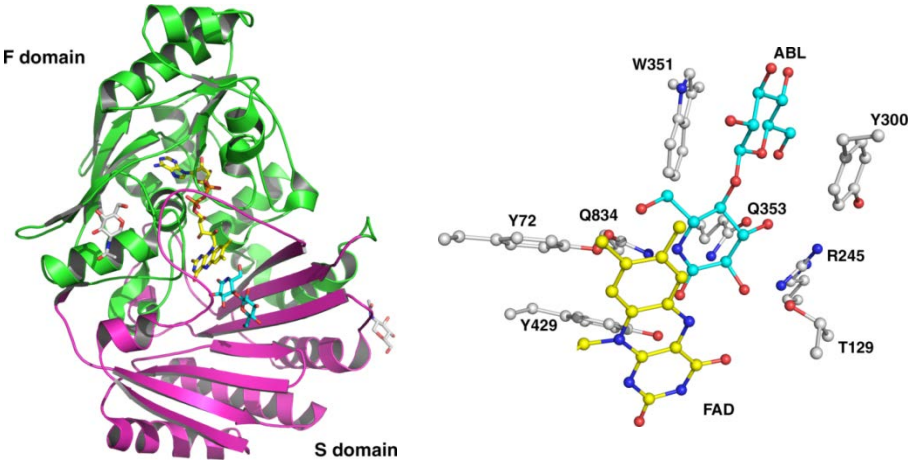

Figure 1: Left: glucooligosaccharide oxidase is made of two domains: the FAD-binding (F) domain and the substrate binding (S) domain. In this representation, the F domain is in green and the S domain is in magenta. In cyan, the substrate analogue (2R,3R,4R,5R)-4,5-dihydroxy-2-(hydroxymethyl)-6-oxopiperidin-3-yl-beta-D-glucopyranoside, in yellow the cofactor FAD, and in grey N-acetyl-D-glucosamine molecules are shown. Right: The active site of glucooligosaccharide oxidase. The stacking interactions of W351 and Y300 keep the substrate in place (cyan). PDB code: 2AXR.

The proposed mechanism of the reaction catalyzed by GOOX is based on two half-reactions. In the reductive half-reaction, most likely Tyr^{429} abstracts a proton from the OH^{1} group of the substrate. This is hypothesized to be facilitated by Asp^{355} which forms a hydrogen bond with Tyr^{429} through a molecule of water lowering the pKa of Tyr^{429}. A hydride is then transferred from the C^{1} of the substrate to the N^{5} of the FAD cofactor. If glucose is used as substrate, glucono-1,5-lactone is formed which is spontaneously hydrolyzed to gluconic acid. In the oxidative half-reaction, molecular oxygen is reduced by the reduced FAD to hydrogen peroxide after which the enzyme is ready for a new cycle of catalysis[14]. This mechanism is probably conserved among the other related carbohydrate oxidases of the VAO family that act on oligosaccharides and explains the exquisite regioselectivity. In fact, by multiple sequence alignment, the catalytic Tyr and Asp appear to be conserved in carbohydrate oxidase from *Microdochium nivale*, chitooligosaccharide oxidase from *Fusarium graminearum*,

xylooligosaccharide oxidase from *Myceliophthora thermophila* and hexose oxidase from *Chondrus crispus*.

Table 1. Overview of sequence-related bicovalent flavoprotein oxidases acting on carbohydrates.

Name	Organism	Preferred substrate	X-ray structure
Glucooligosaccharide oxidase (GOOX)	*Acremonium strictum*	Maltooligosaccharides, xylooligosaccharides, glucooligosaccharides	2AXR
Lactose oxidase (LaO)	*Microdochium nivale*	maltooligosaccharides, glucooligosaccharides, lactose	3RJ8
Chitooligosaccharide oxidase (ChitO)	*Fusarium graminearum*	chitooligosaccharides	-
Xylooligosaccharide oxidase (XylO)	*Myceliophthora thermophila*	xylooligosaccharides	5L6F
Hexose oxidase (HOX)	*Chondrus crispus*	glucose, galactose	-

Since the discovery and characterization of the first carbohydrate oxidases, a multitude of scientific papers have been published and a conspicuous number of patent applications have been deposited and granted. At the moment of this writing (June 2016), a search on PubMed for the query "glucose oxidase" gives 6553 results with papers having glucose oxidase in either the title or abstract. Furthermore glucose oxidase (GOX) was one of the first enzymes being used in a biotechnological application. It was employed in the development of a glucose biosensor by Clark and Ann Lyons already in 1962 [15].

Carbohydrate oxidases are often relatively stable enzymes that do not require expensive cofactors as only atmospheric oxygen is required for their functioning. Also, the use of relatively cheap and non-toxic carbohydrates is attractive. This explains their broad applicability as reflected in the number of patents. Their commercial exploitation relies on three core functionalities that this class of enzymes is capable to deliver: 1) production of hydrogen peroxide; 2) removal of molecular oxygen; 3) selective production of oxidized carbohydrates. In the following sections we describe each related

application area and we are going illustrate how it is being exploited by showing examples taken from patent literature.

Exploitation of H_2O_2 production by carbohydrate oxidases

One of the biggest economic reasons for which carbohydrate oxidases are being widely patented and used relies on their ability to produce hydrogen peroxide. In addition, carbohydrate oxidases offer the advantage to generate hydrogen peroxide in situ and "on demand" which means that conditions can be tweaked to obtain a tunable production. Based on these premises, four areas of applications based on the production of H_2O_2 by carbohydrate oxidases can be identified: 1) oxidases used for bleaching purposes, 2) oxidases to perform oxidations, 3) oxidases exploited for antiseptic effects, and 4) oxidases integrated in biosensors.

Carbohydrate oxidases as oxidizing and bleaching agents

Of the approximately 2.2 million metric tons of hydrogen peroxide produced globally every year, 60% is used for bleaching purposes: 50 % is used for pulp/paper bleaching and 10% for textile bleaching [16]. Stains, raw cotton, paper and pulp contain chromophores that present an absorption spectrum in the visible range. Bleaching agents such as hydrogen peroxide can decolor the substrate by chemical modification of chromophores that either makes them water-soluble, thus removable by washing, or by shifting the wavelength of absorbance outside of the visible region, making *de facto* the chromophore not visible. Bleaching is also required in other applications such as bread making, healthcare (hair color-bleaching, dental care) and laundry detergents.

For bleaching purposes, traditionally, halogen-containing chemicals were used. Despite being cost-effective they pose several environmental and health threats. When used in pulp bleaching processes, halogenated products will end up in the waste stream. When used in bread making, substances such as potassium

bromate pose a serious threat to human safety as this very substance has been shown to be mutagenic [17]. Carbohydrate oxidases represent a valuable alternative to chemical bleaching agents. In fact several carbohydrate oxidases are currently patented for bleaching purposes. GOX is the foremost used carbohydrate oxidase partly due to its thorough characterization performed in several decades of research since its discovery and also due to ease of production, the low cost of the substrate glucose and the role of glucose as marker. In the patent literature we can find GOX used to bleach flour by addition of glucose to start the reaction [18] or a toothpaste composition with glucose and GOX to promote hydrogen peroxide production resulting in teeth whitening [19]. Cellobiose oxidase is used to produce hydrogen peroxide out of pre-treated paper pulp [20]. Removal of highly colored stains such as carotenoids, and/or lignin-derived stains can be achieved by a plethora of carbohydrate oxidases patented to be used in a detergent composition [21], [22]. Carbohydrate oxidases have also been patented to bleach raw sugar in order to obtain refined white sugar which has higher commercial value [23].

The oxidative power of enzyme-generated H_2O_2 is not only used for bleaching purposes. In fact, especially in the bread making industry, enzyme-catalyzed oxidation is exploited to confer particular properties to the dough by improving its handling qualities and its gluten strength which in the end improves the final texture of the bread. GOX has been shown to improve bread's texture and volume. This has been shown to be a result of H_2O_2 production that gives a more elastic and viscous dough. Although the mechanisms behind these effects have not been completely elucidated yet, one hypothesis is that hydrogen peroxide oxidizes the thiol groups in the gluten proteins forming disulfide bonds. Nevertheless, GOX is known to cause crosslinking of dough proteins and to increase viscosity in the water soluble part of the dough [24]–[27].

The amount of glucose in dough is limited and dependent of the amount of cellulases used in the preparation. This limits the application of GOX to addition of exogenous glucose which

increases costs of the preparation. Therefore, several investigations aimed at testing different oxidases to improve bread making have been undertaken. Ideally the carbohydrate oxidases should act on sugars already present in the mixture and at high levels. Successful attempts in using hexose oxidase [28] and pyranose oxidase [29] have been published and patent applications granted for the use of GOOX and lactose oxidase [30], pyranose oxidase [31], hexose oxidase [32] and galactose oxidase [33] in improving the qualities of bread dough.

Carbohydrate oxidases as antimicrobial agents

Hydrogen peroxide has intrinsic antibacterial properties. By oxidizing bacterial cellular components and in particular the sulfhydryl groups of enzymes, it significantly impairs their structure/functionality leading ultimately to inhibition of growth or cell death. In the salivary, mammary and lachrymal secretions of mammals, hydrogen peroxide is part of the lactoperoxidase system which produces the very potent radical hypothiocyanite from thiocyanate. This system has been proven to be bacteriostatic and bactericidal to several varieties of Gram-negative and Gram-positive bacteria [34].

The production of H_2O_2 by carbohydrate oxidases or its combination with the lactoperoxidase system is being exploited as antimicrobial agent in a variety of different applications. As already reported, toothpaste compositions that include GOX have been patented. This has the advantage of using saccharides already present on the teeth coming from the eaten food. The use of GOX will also remove excess glucose from the mouth with the added benefit of limiting the carbon source used by bacteria for proliferation. In two patents, a carbohydrate oxidase is coupled to the lactoperoxidase system to deliver extra antimicrobial power [35], [36]. Bacterial proliferation in the mouth is detrimental not only because it might cause tooth decay but it may also cause bad breath. Therefore carbohydrate oxidases by themselves or in combination with the lactoperoxidase system have been patented in compositions to prevent or treat bad breath [37], [38].

Carbohydrate oxidases are also being used as microbial control agents in food packaging. Here not only they reduce the amount of oxygen, limiting the growth of aerobic microorganisms, but also produce hydrogen peroxide to limit bacterial proliferation [39], [40].

When the topical application of high concentrations of H_2O_2 is difficult or needs to be dosed, in situ production by carbohydrate oxidases may be a solution. For instance a nasal spray for the treatment of common cold includes carbohydrate oxidase together with its respective substrate in order to deliver H_2O_2 in the nasal cavity creating a high local concentration of oxidant [41]. In another patent, carbohydrate oxidases are used to deliver hydrogen peroxide in the vaginal tract to prevent the growth of Gram-negative bacteria [42].

Carbohydrate oxidases as biosensors

The production of hydrogen peroxide by a carbohydrate oxidase can be exploited indirectly in a coupled assay to detect/measure activity of a carbohydrate active enzyme. The product of the first enzyme can be converted by the carbohydrate oxidase which releases H_2O_2. This can be used by a peroxidase to convert a chromogen in a colored compound. This production can be followed in time and will be linearly related to the activity of the first enzyme (given that the carbohydrate oxidase and the peroxidase are in excess).

In this very thesis, in Chapter 2 such a system is reported. A chitooligosaccharide oxidase oxidises the products formed by chitinases and/or cellulases. A system is described in which the combination of these enzymes together with a horseradish peroxidase is used to create an assay to detect cellulase or chitinase activity. It was shown that it works also on complex mixtures of substrates such as shrimp shells and straw.

It is worth mentioning that several biosensors have been realized by coupling oxidases to electrodes. The already mentioned GOX was the pioneering application of this principle after which many others

have followed. For a more in-depth overview on the use of carbohydrate oxidases as biosensors, the reader is referred to two recent reviews [43], [44].

Carbohydrate oxidases as antioxidants

Despite being fundamental for life, dioxygen is a molecule that needs to be constantly battled with. In fact, molecular oxygen is prone to form reactive oxygen species (ROS). These are highly reactive free radicals that tend to give electrons to biological molecules, initiating a propagation reaction which ultimately leads to the alteration of the biomolecule with change of structure and/or loss of function [45].

ROS can pose a threat to human health and they play an important role in the pathogenesis of various serious diseases, such as neurodegenerative disorders, cancer, cardiovascular diseases, atherosclerosis, cataracts, and inflammation [46], [47]. ROS can also alter the taste and flavor of foods which can lead to rancid taste [24], [48].

Several patents have been granted to use carbohydrate oxidases as a prophylactic system to remove oxygen from foodstuff preventing the conversion of the aroma in off-flavors or rancid tastes [49]. For instance, GOX is used together with glucose to remove oxygen dissolved in the water used to grind soya beans to make soy milk. In this way, off-flavors are not developed and no subsequent boiling step is required [50]. In another case, during the processing of coffee used to make read-to-drink beverages, the exposure to oxygen makes the coffee lose its fresh, clean flavor and aroma and bitter, acid flavors develop. This is avoided by including GOX and glucose to the preparation [51].

Peppermint oil contained in chewing gum is also susceptible to oxidation which leads to loss of freshness. In a patent dated 1957,

GOX and glucose are added to the composition to remove oxygen and avoid the loss of flavor [52].

In another patent, a combination of amylase and carbohydrate oxidase from *Microdochium nivale* is used to generate maltose in the food product itself. This is subsequently converted to maltobionate by the oxidase with the consequent removal of oxygen [53]. This shows a smart usage of a carbohydrate oxidase which uses the molecules from the food itself for its functionality. Another patent which is based upon similar premises is one for a composition of an aldehyde/xanthine oxidase with an alcohol oxidase and a catalase that remove malodorous aldehydes and alcohols that are formed upon oxidation of fish oil. In this way they create a negative feedback loop that not only removes the bad smelling compounds but also their trigger which is molecular oxygen [54].

The presence of oxygen does not constitute an issue only for foods producer. An examination of the patent literature shows indeed patents that cover other fields of industry. For instance, due to regulatory and environmental issues aerosol products need to reduce their volatile organic content level. This has involved a reduction in the amount of solvent in many products and an increase in the water content. This makes containers more prone to rusting. Traditionally anti-corrosion chemicals such as borates, benzoates, molybdate, special surfactants (such as sodium lauroyl sarcosinate), sodium nitrite and morpholine and silicates were used to prevent corrosion of the container but many pose health and environmental risks. A composition of GOX and glucose are patented to remove water-dissolved oxygen from cans containing aerosol products, therefore preventing rusting [55].

Another less obvious field where oxygen can be problematic is ink jet printing. The presence of dissolved air in the ink can cause runability problems for inkjet printers. The air dissolved in the cartridge may form small bubbles within feed tubes in the printer print head, disrupting ink flow. Traditional methods of degassing involve flushing the ink with helium gas or subjecting the ink to a vacuum. These methods are certainly effective but only acutely.

Gaseous compounds such as dioxygen can indeed easily diffuse through the cartridge material and eventually re-saturate the ink. Galactose oxidase and GOX together with their respective substrates have been patented as ink additive to remove dissolved oxygen from inkjet printer cartridges [56].

Use of carbohydrate oxidases for carbohydrates modifications

So far the exploitation of the production of H_2O_2 and of the removal of molecular oxygen have been addressed. Now the focus will be shifted towards the removal of carbohydrates and their conversion into valuable products.

Carbohydrates are hydrophilic molecules rich in chiral centers. Their derivatization would be desirable since it can increase their value. Given the richness of (pro-)chiral centers, chemical modifications are hardly the solution to achieve this goal. Due to lack of selectivity, chemical derivatization of carbohydrates, when available, is a time-consuming effort. Here lies the power of using carbohydrate oxidases. Enzymes have the advantage of not only working at mild conditions but also of being highly stereo- and regioselective.

It is very attractive to modifiy oligo- and polysaccharides as this would result in new properties of the material. There is one example in the patent literature where a galactose oxidase is used to functionalize galactomannan. The OH groups at position C6 are converted to highly reactive aldehydes which could be used for further functionalizations. In the patent specifically the use as paper additive is described [57].

Aldonic acids produced from mono- or oligosaccharides, at pH values above their pK_a, present a negative charge. As a consequence, these molecules can chelate positively charged ions. This is exploited in two different ways in the patent literature. Aldonic acids can be used as carriers for metals for an alopecia treatment where they are used to carry zinc [58]. They have been

patented for a topical treatment in which they are used to carry different molecules to improve skin quality [59]. Also oxidized carbohydrates are patented to be used in a beverage as carriers for calcium, magnesium and amino acids [60].

Maltodextrins oxidized at the C1 position are patented in a composition for a detergent as chelators for calcium ions and/or transition metals. By forming soluble complexes, the oxidized maltodextrins prevent the metal ions to form precipitates or scale [61]. In the pulp and paper industry sediment build-up on equipment surfaces is a serious problem. This is mostly caused by accumulation of calcium salts (e.g., calcium carbonate and calcium sulphate) that contribute to the formation of scale. Treatment of process or waste water with carbohydrate oxidases reduces this problem by two means: 1) aldonic acids can facilitate precipitation, and/or improve the settling behavior, of suspended material; 2) they can compete, as complexing or chelating agents, with carbonate or oxalate, for the cationic calcium ions, which results in a decreased formation of calcium salts [62].

Aldonic acids can also be exploited for their intrinsic pH lowering effect. These acids can contribute substantially to lowering the pH of the medium in which they are produced. Based on this, Kraft Foods patented a very elegant process that uses lactose oxidase for cheese making. By producing lactobionic acid *in situ*, the acidification of the cheese happens directly, without the need of rennet and/or starter culture with lactic acid bacteria. Since lactobionic acid is produced using the lactose already present in milk, this will result in a product with reduced levels of lactose resulting in cheese products for lactose intolerant people [63].

Finally, carbohydrate oxidases can be used as a tool to remove carbohydrates from a mixture. For instance, during the production of wine, the sugar in the must is converted into alcohol by yeasts. By reducing the amount of sugar in the must, reduced-alcohol wine can be obtained. GOX has been indeed patented for this purpose with the added advantage that O_2 removal and H_2O_2 production will prevent the spoilage of the wine [64], [65].

In the paper and pulp industry, another common problem is the production of bad smell. Bad smell in these facilities can be due to several components. One of them is the conversion of carbohydrates in the waste water by contaminating microorganisms in smelly short chain fatty acids. One way to tackle this problem is presented in a patent of 2003 from Novozymes in which carbohydrate oxidases are used to convert the remaining carbohydrates in molecules that cannot be further converted in short chain fatty acids [66].

The carcinogenic compound acrylamide can be formed at high temperatures during the preparation of food products by a reaction between reducing sugars and amino acids. Again Novozymes in 2002 patented a solution to circumvent this problem. By including a carbohydrate oxidase in dough or by immersing potatoes in a liquid solution comprising a carbohydrate oxidase, the amount of reducing sugars is diminished thus reducing the risk of acrylamide formation [67].

Concluding remarks

In this chapter we looked into the wide range of potential applications carbohydrate oxidases can be used for. Exploitation of carbohydrate oxidases on large scale is limited by the costs of production and processing of these enzymes. One way to overcome this limitation can be the immobilization of the enzymes that will allow not only easy reutilization but also will make enzyme removal seamless. So far different techniques have been employed to successfully immobilize GOX [68], pyranose oxidase [69], hexose oxidase [70], galactose oxidase [71] and GOOX [72].

Nowadays, there are various approaches by which enzymes can engineered. This allows the fine tuning of carbohydrate oxidase to become for example more stable, more active at a low pH or more active on a new carbohydrate substrate. Yet, more sophisticated enzyme engineering approaches may introduce properties that are valuable for biotechnological applications. An interesting development may be brought by the exploration of carbohydrate

binding modules (CBM). So far 67 different protein families of CBMs have been identified. They can target different types of carbohydrate molecules ranging from starch to cellulose to chitin. The creation of carbohydrate oxidases fused to a CBM will allow: 1) easier and harmless immobilization; 2) delivery of the enzyme to targeted locations, therefore 3) dramatically increasing the local concentration of enzyme which will result in a more efficient action. Such enzyme fusion engineering approach has recently been explored [73], [74]. With the availability of various carbohydrate oxidases and a great number of CBMs this approach shows great promise.

Aim and outline of the thesis

The work described in this thesis aimed at the discovery, characterization and engineering of novel oxidases. The Netherlands Organisation for Scientific Research (NWO) provided funding for this research, in the framework of the TASC Technology Area Biomass. Key partners for the projects have been the companies DuPont Industrial Biosciences and AVEBE.

In Chapter 2, chitooligosaccharide oxidase from *Fusarium graminearum* (ChitO) is used to create an assay to detect chitinase or cellulase activity. ChitO can oxidize the products formed by chitinases and its mutant ChitO Q268R acts on the products formed by cellulases. A peroxidase is used to convert the formed hydrogen peroxide and two chromogens into a pink color whose production can be monitored in real time. The developed method is easy to use and very sensitive.

In Chapter 3, the engineering of ChitO was undertaken with the aim to explore its catalytic activity and to change its substrate scope. By structural comparison with other known carbohydrate oxidases, several residues potentially involved in substrate binding were selected for mutagenesis. Mutant enzymes were expressed, purified and characterized. By combining promising mutations we obtained one mutant with the highest catalytic activity towards N-acetyl-glucosamine ever reported in the literature. Another variant,

combining three mutations, has higher efficiency towards cellobiose, lactose and maltose compared to the wild-type enzyme.

In Chapter 4, the discovery and characterization of a xylooligosaccharide oxidase (XylO) from the thermophilic fungus *Myceliophthora thermophila* is described. XylO is a unique carbohydrate oxidase since it can only oxidize xylobiose and larger oligomers while being very inefficient at oxidizing cellodextrins, maltodextrins and lactose. The enzyme has been characterized and the crystal structure resolved.

In Chapter 5, the discovery, attempted characterization and the crystal structures of two oxidases from the thermophilic fungus *Myceliophthora thermophila* is described. The two oxidases belong to a cluster of proteins relatively unrelated to other known oxidases. Their crystal structures reveal unique active site features. Using the stopped-flow technique it could be confirmed that the proteins represent oxidases as they can use dioxygen as electron acceptor. However, despite attempts at finding out their substrate scope, their catalytic role remains enigmatic.

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Chapter 2

A fast, sensitive and easy colorimetric assay for chitinase and cellulase activity detection

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Abstract

***Background*:** Most of the current colorimetric methods for detection of chitinase or cellulase activities on the insoluble natural polymers chitin and cellulose depend on a chemical redox reaction. The reaction involves the reducing ends of the hydrolytic products. The Schales' procedure and the 3,5-dinitrosalicylic acid (DNS) method are two examples of these methods that are commonly used. However, these methods lack sensitivity and present practical difficulties of usage in high-throughput screening assays as they require boiling or heating steps for color development.

Results: We report a novel method for colorimetric detection of chitinase and cellulase activity. The assay is based on the use of two oxidases: wild-type chito-oligosaccharide oxidase, ChitO, and a mutant thereof, ChitO-Q268R. ChitO was used for chitinase while ChitO-Q268R was used for cellulase activity detection. These oxidases release hydrogen peroxide upon the oxidation of chitinase- or cellulase-produced hydrolytic products. The hydrogen peroxide produced can be monitored using a second enzyme, horseradish peroxidase (HRP), and a chromogenic peroxidase substrate. The developed ChitO-based assay can detect chitinase activity as low as 10 µU within 15 minutes assay time. Similarly, cellulase activity can be detected in the range of 6 – 375 mU. A linear response was observed when applying the ChitO-based assay for detecting individual chito-oligosaccharides and cello-oligosaccharides. The detection limits for these compounds ranged from 5-25 µM. In contrast to other commonly used methods, the Schales' procedure and the DNS method, no boiling or heating is needed in the ChitO-based assays. The method was also evaluated for detecting hydrolytic activity on biomass-derived substrates, i.e. wheat straw as a source of cellulose and shrimp shells as a source of chitin.

Conclusion: The ChitO-based assay has clear advantages for the detection of chitinase and cellulase activity over the conventional Schales' procedure and DNS method. The detection limit is lower and there is no requirement for harsh conditions for the development of the signal. The assay also involves fewer and easy handling steps. There is no need for boiling to develop the color and results are available within 15 minutes. These aforementioned features render this newly developed assay method highly suitable for applications in biorefinery related research.

Background

Enzymatic degradation of cellulose and chitin is a hot research topic due to its potential for efficient utilization of the energy and carbon content of these polymers [1]. Chitin and cellulose are highly abundant and natural polymers of 1,4-β linked sugar units of either N-acetyl-D-glucose amine or D-glucose, respectively. Chitin and cellulose share similarities in both structure and the enzymatic degradation mechanism. Generally, four groups of enzymes interact in the polymer degradation process: a) exoenzymes that are active on both ends of the polymer chain, b) endo-enzymes that attack easily accessible glycosidic bonds or amorphous regions in the polymer chain, c) dimer hydrolases i.e. β-glucosidases or chitobiosidase that hydrolyse oligosaccharides, and d) lytic polysaccharide monoxygenases that introduce breaks in the crystalline region of the polymer chain and facilitate polymer unpacking [2-4]. A final mixture of monomeric, dimeric and oligomeric carbohydrate units is produced which are commonly utilized for detection purposes. Using the reducing end functionalities in this mixture, a reaction with redox reagents develops a measurable color.

For detection of chitinolytic or cellulolytic activities, both soluble and insoluble substrates either natural or chemically modified are used. For example, assessment of chitinase activity can be accomplished with solubilized substrates such as ethylene glycol chitin, carboxymethyl chitin, and 6-O-hydroxypropyl-chitin or insoluble modified chitin substrates such as chitin-azure and tritium-labeled chitin [2,5]. However, the use of native unmodified substrates is highly preferred compared to the use of surrogate substrates that are chemically modified. To monitor the enzymatic activity, the reducing sugars released by the action of enzymes are determined colorimetrically. The common colorimetric methods currently used for measuring the reducing sugar content are the 3,5-dinitrosalicylic acid (DNS) method and the ferricyanide-based Schales' procedure [4,6,7]. The reduction of inorganic oxidants such as ferricyanide or cupric ions by the aldehyde/hemiacetal groups of

the reducing sugar ends leads to color change that can be measured spectrophotometrically. However there are several drawbacks of these methods such as: a) use of alkaline medium which destroys part of the reducing sugars, b) the necessity for heating or boiling for color development, c) the long reaction time, d) insensitivity at lower range of sugar concentrations, and e) difficulty in use in high-throughput screening [8,9].

Chito-oligosaccharide oxidase (ChitO) identified in the genome of *Fusarium graminearum* is the first discovered oxidase capable of the oxidation of chito-oligosaccharides [10, 11]. The oxidation takes place at the substrate C1 hydroxyl moiety leading to formation of equimolar amounts of H_2O_2 and the corresponding lactone. The produced lactone hydrolyzes spontaneously to the corresponding aldonic acids. ChitO display excellent activity on the substrates N-acetyl-D-glucosamine, chitobiose, chitotriose and chitotetraose with k_{cat} values of around 6 s^{-1} and K_M values below 10 mM (respectively 6.3, 0.30, 0.26, and 0.25 mM) [11]. The wild-type ChitO displays very poor activity towards cellulose-derived oligosaccharides. However, by a structure-inspired enzyme engineering approach, we have designed a mutant i.e ChitO-Q268R that displays a much higher catalytic efficiency towards cello-oligosaccharides [11]. The mutant enzyme displays k_{cat} values of around 7 s^{-1} for glucose, cellobiose, cellotriose and cellotetraose while the K_M values varies to some extend (respectively 182, 22, 6.5, and 20 mM) [11]. The ChitO-Q268R displays a poor catalytic efficiency for the chito-oligosaccharides. With these two oxidase variants, ChitO (selective for N-acetyl-glucosamine derivatives) and ChitO-Q268R (selective for glucose derivatives), it is feasible to efficiently oxidize chitin- or cellulose-derived hydrolytic products. This inspired us to explore the use of ChitO for assay development.

In the current report we present a ChitO-based assay by which chitinase and cellulase activities can be detected in a quick, sensitive and facile method. The approach takes advantage of the hydrogen peroxide generated by ChitO or ChitO-Q268R when acting on products formed by hydrolytic activity of chitinases or cellulases,

respectively. The well-established horseradish peroxidase (HRP) colorimetric assay was used for the detection of the produced H_2O_2. The use of these oxidases in combination with HRP constitutes a fast and sensitive method to detect chitinases and cellulases activity, without the necessity of a boiling step, commonly employed in other methods.

Results and Discussion

ChitO-based assay and Schales' method for chitinase detection

The chitinase ChitO-based assay is based on the oxidation of the chito-oligosaccharides by ChitO which are formed by the action of the chitinases on the chitin. Upon oxidation of these substrates, a stoichiometric amount of H_2O_2 is produced by reduction of molecular oxygen. The hydrogen peroxide is used by HRP to convert 4-aminoantipyrine (AAP) and 3,5-dichloro-2-hydroxy-benzenesulfonic acid (DCHBS) into a pink and stable compound [12]. As a result, the intensity of the pink color is proportional to the concentration of the available ChitO substrates. To test our assay for the detection of chitinase activity, a chitinase from *Streptomyces griseus* and colloid chitin as a substrate were used. Colloidal chitin is a natural unmodified substrate, easy to prepare, and convenient for pipetting compared to chitin flakes. Varying amounts of chitinase were incubated with colloid chitin for 60 minutes to allow degradation of the chitin. Subsequently, the ChitO assay components (i.e. ChitO, AAP, DCHBS, and HRP) were added to the incubations in the 96-well microtiter plate. Development of a clear pink color is indicative for chitinase activity. By measuring the absorbance at 515 nm, the activity of ChitO, and hence the activity of chitinase, could be determined. A clear relationship was observed between the measured absorbance and increasing units of chitinase (Figure 1). In fact, the data show a saturation curve which can be nicely fitted with a simple hyperbolic formula:

$A=A_{max}.[chitinase]/(x+[chitinase])$, $R^2=0.996$

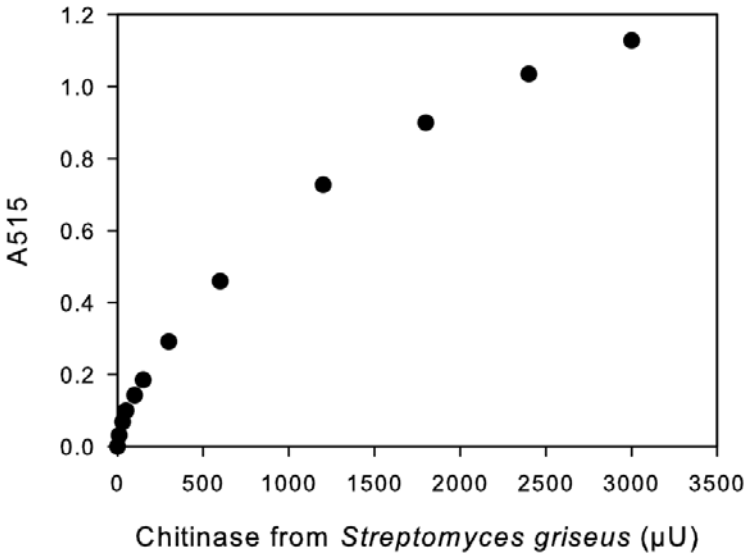

Figure 1: Application of the ChitO-based assay for detection the hydrolytic products of chitinase using colloid chitin as a substrate. The average of the absorbance values at 515 nm of the triplicates was subtracted with the averaged blank and plotted.

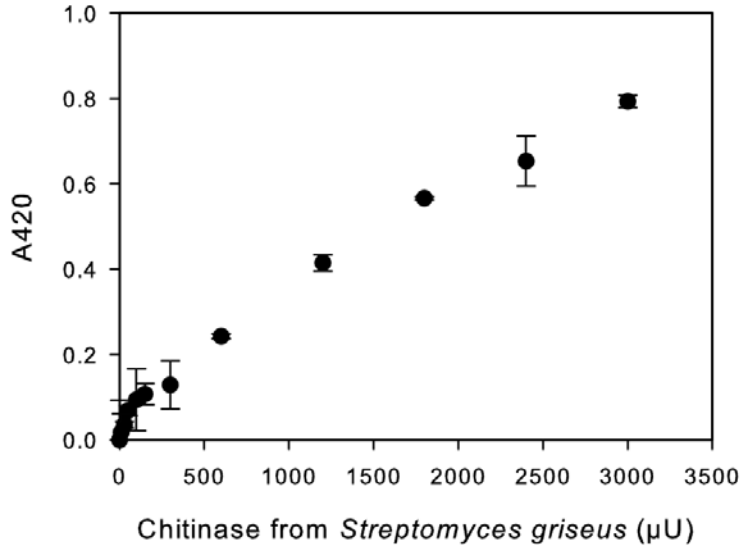

Figure 2: Application of the Schales' procedure to detect the hydrolytic products of chitinase using colloid chitin as a substrate. For each sample, the average of the absorbance at 420 nm was subtracted from the averaged blank value and plotted.

Interestingly, the assay could detect as low as 10 µU of chitinase with an assay time of only 15 min and using 0.12 U ChitO (p-value < 1%). The blank reaction (colloidal chitin incubated without chitinase) revealed that colloidal chitin itself is a very poor substrate for ChitO. For such incubation a very weak signal (A_{515} = 0.12) was recorded and used as a blank. The reproducibility of the ChitO-based assay was assessed by comparing the corrected absorbance values on nine replicates of colloidal chitin treated with 50 µU of chitinase, to nine replicates of not treated colloidal chitin (Figure 3). The assay showed high reproducibility with a low standard deviation (< 0.3 %) for both samples.

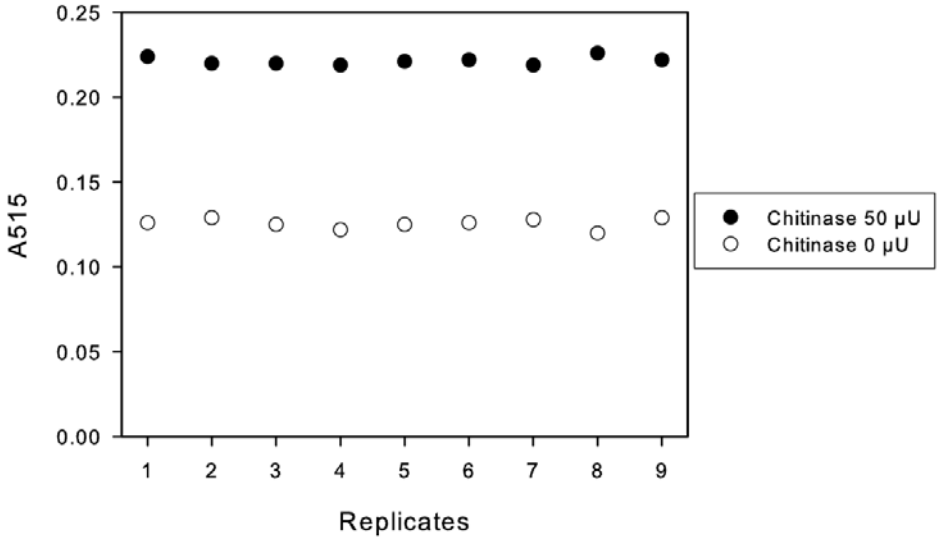

Figure 3: Reproducibility of the ChitO-based assay with chitinase. Absorbance values of the ChitO-based assay from nine replicates of colloidal chitin treated for 1 hour with 50 µU of chitinase from Streptomyces griseus were plotted against nine replicates of untreated colloidal chitin under the same assay conditions.

For benchmarking, we compared the ChitO-based assay to the Schales' procedure since it is one of the most common methods for detection of chitinase activity [7,13]. The Schales' reagent is yellow in color and reaction with reducing sugars results in color fading, which can be measured at 420 nm. Figure 2 shows the absorbance signal obtained in relation to the concentration of chitinase. A

chitinase activity of 600 µU was found to be the lowest detection limit (p-value < 3%). This value is 60 times higher than the detection limit of the ChitO-based assay (10 µU) indicating a higher sensitivity in favor of the ChitO-assay. In addition, the recorded signal intensity of the ChitO assay was higher, approximately 2-fold, than Schales' procedure. This can be concluded from comparing the signal responses in Figures 1 and 3, particularly when considering the range of 600 µU – 3000 µU chitinase. It is important to note that the boiling step, that is an essential step in the Schales' procedure, is omitted from the ChitO assay which represents one of the main advantages (Figure 4).

Figure 4: Comparison of the outline of the Schales' procedure and the developed ChitO-based assay. Schales' reagent, starting with a yellow color, reacts with the reducing sugars obtained from chitinase activity and after boiling a fading of the yellow color can be monitored at 420 nm. In the ChitO-based assay, the development of the pink product doesn't require any boiling step and will be visible in short time, depending on the concentration of oligomers in the reaction and the amount of ChitO used. Pictures were edited to improve contrast.

ChitO-based assay for cellulase detection

To adapt the ChitO-based assay for monitoring activity of cellulolytic enzymes, a ChitO mutant (ChitO-Q268R) was used instead of wild-type ChitO. ChitO-Q268R has a higher enzymatic efficiency toward glucose, cellobiose, cellotriose and cellotetraose compared to wild-type ChitO. We applied the assay using the same conditions as for detection of chitinase activity. As a model cellulase, an endocellulase from *Aspergillus niger* was used with a filter paper as a substrate. Endoglucanases typically hydrolyze accessible parts of the cellulose polymer and generate new chain ends. The generated cellotetraose and lower fragments will be substrates for ChitO-Q268R and consequently will allow H_2O_2 generation and development of the pink colored product. The signal intensity, which is based on endocellulase activity, depends on the fraction of accessible β-glycosidic bonds in the substrate.

It was gratifying to see that using ChitO-Q268R in combination with HRP resulted in a clear and immediate color development. As was found for the ChitO-based chitinase assay, a direct proportional relationship of the absorbance to the amount of cellulase units was observed (Figure 5).

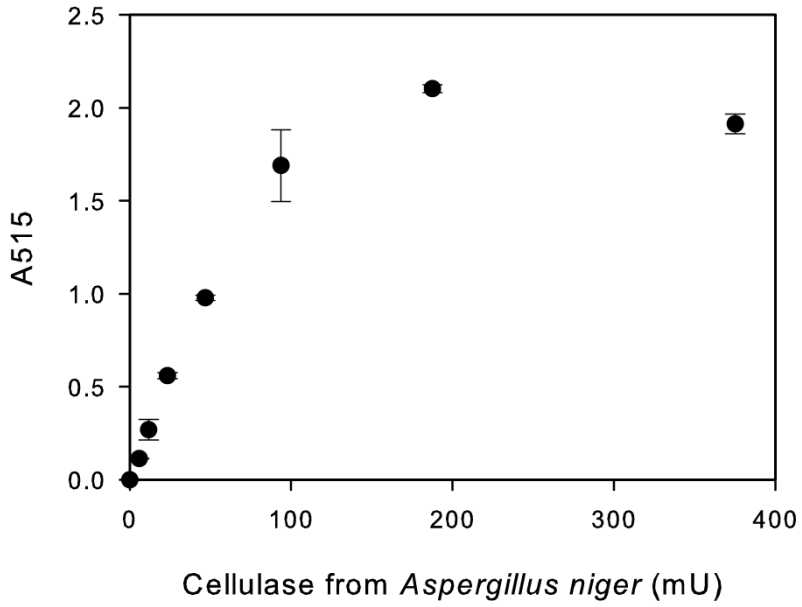

Figure 5: Application of ChitO-based assay for the detection of the hydrolytic products of a cellulase from Aspergillus niger using filter paper as a substrate. The mutant ChitO-Q268R was used instead of the wild-type ChitO. The average absorbance values at 515 nm of the triplicates was subtracted with the averaged blank and plotted.

The response curve started to level off when using >100 mU of the hydrolase. The lowest tested amount of endocellulase was 6 mU which could be detected with an assay time of 15 min using 0.13 U of ChitO-Q268R (p-value < 0.5%). The commonly used colorimetric reagent to measure the cellulose saccharification is DNS [14]. Drawbacks of this method are many such as non-reproducibility, complexity of reagents preparations and time-consuming. It requires also a strict control of temperature for proper color development and stability [15]. Moreover the use of toxic reagents and phenolic compounds in large amounts makes it not a very environmentally-friendly method. Trials have been made to improve the DNS assay, such as reducing the amount of reagents used and adapting it to a microtiter plate assay. However, heating or boiling is still required in all of these approaches [16]. Both the DNS assay and the ChitO-based cellulase assay cannot distinguish between the contributions given by the different sugars presents in the reaction mixture. However the ChitO-based assay does not require alkaline

medium and harsh treatment as in DNS, which results in degradation of the sugar content and decreased sensitivity [15,16]. On the whole, the assay represents a faster, high-throughput and "green" method for cellulase detection when compared with the established DNS assay.

ChitO-based assays: detecting defined substrates

The color that develops in the aforementioned assay experiments is a sum of the ChitO activity on a mixture of different hydrolytic products produced by chitinase or cellulase activity. In order to identify the sensitivity of the assay for individual hydrolysis products, response curves were determined. Two sets of compounds were tested: chito-ologosaccharides and cello-oligosaccharides. The experiments were performed at pH 6 and 5, respectively, similar to the ChitO-chitinase and ChitO-cellulase detection experiments. Figure 6A shows a direct response of the signal when testing varying concentrations of N-acetyl-D-glucosamine, chitobiose and chitotetraose, representatives of the chitin degradation products. The limit of detection for N-acetyl-D-glucosamine, chitobiose and chitotetraose was 5 µM (p-value < 5%). Based on the observed slopes, N-acetyl-D-glucosamine showed the highest signal response followed by chitobiose and chitotetraose. A similar trend has also been found with the Schales' method and has been described in literature by Horn and Eijsink 2004 [9]. The second set of compounds tested represented cellulose degradation products: glucose, cellobiose and cellotetraose. Figure 6B shows a direct response of the ChitO assay signal to the increasing concentration of the compounds. The limit of detection was 25 µM for glucose and 10 µM for cellobiose and cellotetraose (p-value < 5%). No specific trend of signal response to the compound's length was observed. Cellobiose showed the highest signal response followed by cellotetraose and glucose.

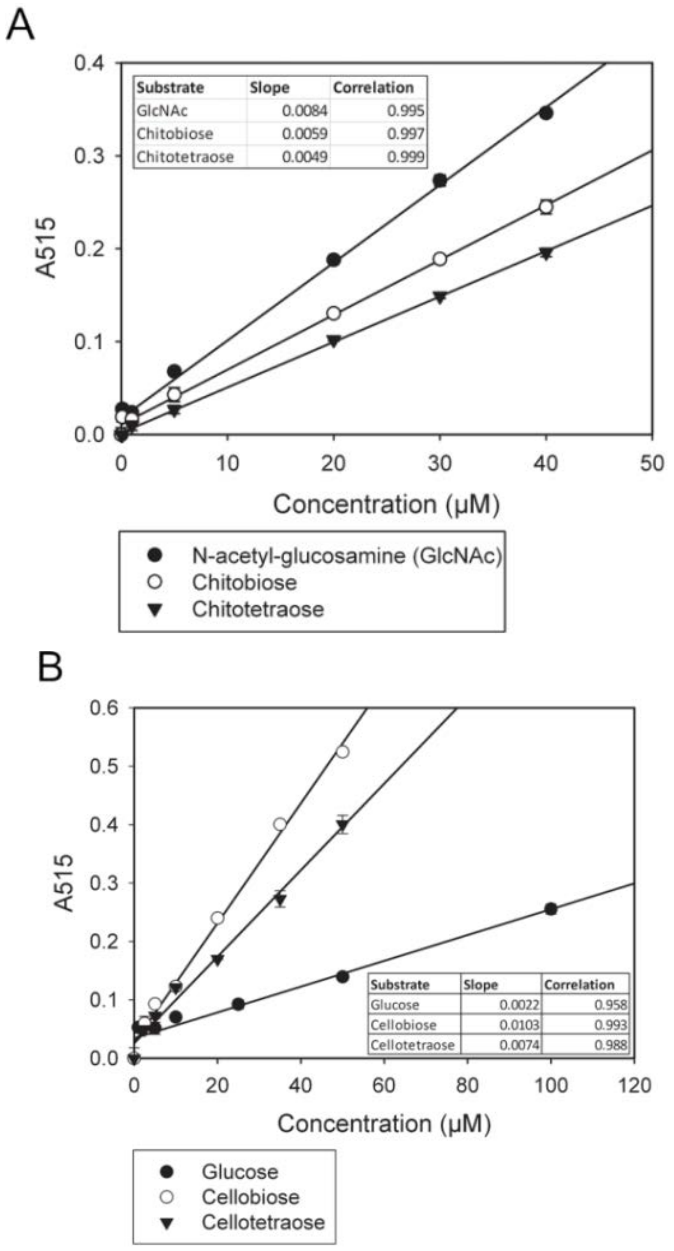

Figure 6: The signal response of the ChitO-based assays to when tested with varying concentrations of A) N-acetyl-glucosamine, chitobiose and chitotetraose and B) glucose, cellobiose and cellotetraose.

ChitO-based assays: monitoring hydrolysis of complex natural substrates

The ChitO assay showed applicability for detection of chitinase and cellulase activity on processed substrates such as colloid chitin and filter paper. We have tested the applicability of the assay on unprocessed and complex materials i.e. ground shrimp shell and wheat straw. In both cases a strong signal is observed (Figure 7). The assay was found to be very specific as the blank reactions did not yield any significant signal. The measured absorbance values for the triplicate samples showed only marginal differences which confirm the above results of the assay reproducibility.

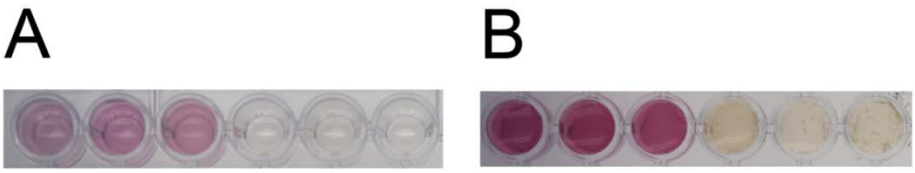

Figure 7: Test of ChitO-based assay on real substrates: A) shrimps' shell treated with chitinase from Streptomyces griseus, and B) straw treated with cellulase from Aspergillus niger. Triplicates of the substrate treated with the hydrolase (left) are compared with triplicates of non-treated substrate (right). Photos were edited to improve the contrast.

In the context of comparing the ChitO-based assay to the Schales' procedure, the reagents availability should also be addressed. The oxidases used in the ChitO-based assay are expressed in a standard expression system using *Escherichia coli* as host. The enzymes are stable at room temperature and active under the assay pH condition. A His-tag has been fused to the recombinant enzymes to facilitate the purification process. Expression in *E. coli* and subsequent purification can yield 40 mg (170 U) of purified protein per liter culture [11]. Considering the low amount of ChitO used in the present experiments (0.12 U per sample), a one liter culture provides sufficient ChitO for assaying >1400 samples.

Several strategies can be foreseen for further development of the ChitO-based assay. The formation of H_2O_2, that is a reactive oxidative

species, can be used for detection by highly sensitive techniques. For example, an amperometric redox polymer-based biosensor replacing the colorimetric reagents can be utilized as has been done for cellobiose dehydrogenase [17]. Alternatively, the use of a fluorescent dye such as Amplex Red for H_2O_2 detections will enable the ChitO-based assay to work in real time analyses and turbid materials such as soil samples. The present study has shown the applicability of ChitO-based assay for cellulose or chitinase activity detection. However it can find also a potential application in food industry, for example for the detection of chitin and chitosan content in edible mushroom [18].

Conclusion

We have developed ChitO-based assays that are very sensitive in detecting chitinase or cellulase activities. It allows the chromogenic detection of 10 µU of chitinase activity and 6 mU of endocellulase activity in just 15 minutes. The heating or boiling steps required for the Schales' procedure or DNS method are not necessary, which renders the ChitO-based method extremely easy. It was also demonstrated that the method can be used to detect chitin- or cellulose-derived carbohydrates and monitoring hydrolysis of complex natural material. The assay is highly suitable for high-throughput approaches and its versatility makes it a powerful tool for the discovery, engineering or optimizing of enzymes involved in the field of biorefinery research.

Methods

Chemicals

Chitinase from *Streptomyces griseus*, HRP, cellulase from *Aspergillus niger*, N-acetyl-D-glucosamine, 3,5-dichloro-2-hydroxybenzenesulfonic acid sodium salt were purchased from Sigma-Aldrich.

One unit of HRP is defined as the amount of enzyme that will form 1.0 mg of purpurogallin from pyrogallol in 20 sec at pH 6.0 at 20 ˚C. One unit of chitinase is defined as the amount of enzyme that liberates 1.0 mg of N-acetyl-D-glucosamine from chitin per hour at pH 6.0 at 25 ˚C in a 2 hour assay. One unit of cellulase is defined as the amount of enzyme that liberates 1.0 µmole of glucose from cellulose in one hour at pH 5.0 at 37 ˚C. 4-aminoantipyrine was purchased from Acros Organics. D-glucose monohydrate was purchased from Merck and cellotetraose, chitobiose, chitotetraose were purchased from Dextra, United Kingdom. Cellobiose (purity > 98%) was purchased from TCI Europe. Whatman filter paper grade 1 was purchased from GE Healthcare Life Sciences. *Escherichia coli* ORIGAMI2 DE3 was purchased from Emd Millipore and pET-SUMO vector was obtained from Invitrogen.

Colloidal chitin preparation

Colloidal chitin was prepared according to Chia-Rui et al., 2010 [8]. Briefly, 4.0 g of chitin was suspended in 37% HCl for 50 min and then 1.0 L of distilled water was slowly added. The colloid was centrifuged and the pellet washed with distilled water several times and then sterilized by autoclaving. Before each experiment, the amount of colloidal chitin to use was washed three times with MilliQ water and then resuspended in 50 mM phosphate buffer, reaching a pH value around 6.0.

ChitO production and purification

The protein expression and purification was based on the methods previously described by Heuts et al [11] with some modifications. Briefly, ChitO and ChitO-Q268R encoding genes were cloned in the pET-SUMO vector resulting in the expression of fusion proteins with a polyhistidine and a SUMO tag at the N-terminal end. Expression was carried out in *Escherichia coli* ORIGAMI2 DE3 (Emd Millipore) for 69 hours at 17˚C after which cells were harvested and sonicated in lysis buffer (50 mM Tris/HCl pH 7.6; 0.5 M NaCl; 10 mM imidazole). After ultra-centrifugation, the cell-free extract was incubated for 1 hour with 1.0 mL of Ni-Sepharose (GE Healthcare) pre-equilibrated

with lysis buffer. After washing with increasing concentrations of imidazole, the proteins were eluted with 0.5 M of imidazole. The samples were de-salted through Econo-Pac 10DG Desalting column (Biorad) and concentrated with Amicon Ultra (Millipore). The protein concentration was determined as previously described [11]. One Unit of wild-type ChitO is defined as the amount of enzyme that catalyzes the conversion of 1 µmol of chitobiose per minute. One Unit of ChitO-Q268R is defined as the amount of enzyme that catalyzes the conversion of 1 µmol of cellobiose per minute.

ChitO-based assay for chitinase detection

Increasing units of chitinase solution (10 µU – 3000 µU) was incubated with colloid chitin, 3.0 mg/ml final concentration, in 96-well microtiter plate to a final volume of 200 µL. The reactions were buffered with 50 mM KPi pH 6.0 and were kept at 30˚C on a shaking incubator for 1 hour. The 96-well microtiter plate was briefly centrifuged at 4˚C and 100 µL of supernatant was transferred to a new plate. Then, the ChitO assay components were added to the supernatant in the following order: 20 µL AAP (1 mM), 20 µL DCHBS (10 mM), 4 µL HRP (200 U/ml), 6 µL ChitO (20 U/mL), and 50 µL of 50 mM KPi pH 6.0 to reach a volume of 200 µL. The assay was incubated for 15 minutes at room temperature to allow the formation of the pink product. All measurements were run in triplicates. The plates were read for absorbance at 515 nm. The values were corrected for both the path length and the blank (substrate in buffer) and the means of each triplicate were plotted. Samples not treated with chitinase were used as negative control. In order to rule out the probability of continuous enzymatic chitinase activity during the assay, the signal intensity from boiled samples were compared to non-boiled samples and the signal difference was found to be statistically insignificant.

Schales' procedure for chitinase detection

A series of increasing units of chitinase solutions were incubated with colloid chitin in a 96-well microtiter plate in a similar setting to the ChitO assay described above. The microtiter plate was briefly

centrifuged at 4 ˚C and 100 µL of supernatant was transferred in a new plate. A volume of 100 µL of Schales' reagent (a solution of 0.5 M sodium carbonate and 0.5 g/L potassium ferricyanide in water) was added. The plate covered in aluminum foil was incubated at 100˚C for 15 min and, after cooling down, read for absorbance at 420 nm. As positive control, 50 mM of N-acetyl-D-glucosamine was used.

ChitO-based assay for cellulase detection

Whatman filter paper number 1 was used as a substrate for the cellulase activity detection. The filter paper was cut into 0.5 cm diameter discs with a common office hole-puncher and accommodated on the bottom of the 96-well microtiter plate. The cellulase from *Aspergillus niger* was dissolved in 50 mM sodium-citrate buffer pH 5.0 in different amounts (6 mU – 375 mU) and 200 µL of the solution was incubated with filter paper for 1 hour at 37 ˚C in a shaking incubator. The microtiter plate was briefly centrifuged at 4˚C and 100 µL of supernatant was transferred to a new plate. For detection of cellulose activity or detecting cellulose-derived sugars, the following components were added: 20 µL AAP (1 mM), 20 µL DCHBS (10 mM), 4 µL HRP (200 U/ml), 6 µL ChitO-Q268R (20 U/mL), and 50 µL of 50 mM KPi pH 6.0 to reach a final volume of 200 µL. The assay was incubated for 15 minutes at room temperature to allow the formation of the pink product. All measurements were performed in triplicates. Samples not treated with cellulase were used as negative control. Similarly to ChitO-based chitinase assay experiments, a comparison of the signal obtained from boiled samples including cellulase to non-boiled samples showed no statistically significant difference.

ChitO-based assay for detecting defined sugars

Two sets of compounds were used in the experiments. The first set comprised increasing concentrations (0.1 – 40 µM) of N-acetyl-D-glucosamine, chitobiose, and chitotetraose. The second set comprised various concentrations of glucose (1 – 100 µM), cellobiose (2.5 – 50 µM) and cellotetraose (2.5 – 50 µM). A volume of

100 µL of compound solution was put in the well of the microtiter plate, followed by addition of ChitO or ChitO-Q268R reagents as described above to a final volume of 200 µL.

ChitO-based assay for monitoring hydrolysis of complex natural substrate

Shrimps were purchased from the local market. The shells were peeled off, dried and blended in a common blender resulting in flocks of heterogeneous size. Wheat straw was purchased from a local supermarket, blended and sieved through a metallic sieve to obtain a small-particle powder. The ChitO assay was run in 96-well microtiter plate using 10 mg of the ground shrimps shell or wheat straw as substrate and chitinase (29 mU) or cellulase (500 mU) respectively. The plates were incubated at 30° C and 37° C respectively for 1h. The plates were centrifuged, and 100 µL of the supernatant was transferred to another plate. The color development using either ChitO or ChitO-Q268R reagents was performed as described above.

Statistical analysis

Significance was determined according to the Student's t-test using Excel software (Microsoft): p-values were accepted when $p< 0.05$.

List of abbreviations

CC: colloidal chitin; DNS: 3,5-dinitrosalicylic acid; ChitO: chito-oligosaccharide oxidase from *Fusarium graminearum*; HRP: horseradish peroxidase; AAP: 4-aminoantipyrine; DCHBS: 3,5-dichloro-2-hydroxybenzenesulfonic acid; GlcNAc: N-acetyl-D-glucosamine.

Competing interest

The authors declare that they have no competing interests.

Authors' contributions

MWF and ARF suggested the idea, ARF designed and performed the experiments and wrote the first draft of the manuscript, YG and MWF discussed the results and wrote the manuscript. All authors read and approved the final manuscript.

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Chapter 3

Expanding the substrate scope of chitooligosaccharide oxidase from *Fusarium graminearum* by structure-inspired mutagenesis

Alessandro R. Ferrari, Misun Lee, and Marco W. Fraaije

This chapter is based on:
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Abstract

Chitooligosaccharide oxidase from *Fusarium graminearum* (ChitO) oxidizes N-acetyl-D-glucosamine (GlcNAc) and its oligomers with high efficiency at the C1-hydroxyl moiety while it shows poor or no activity with other carbohydrates. By sequence and structural comparison with other known carbohydrate oxidases (glucooligosaccharide oxidase from *Acremonium strictum* and lactose oxidase from *Microdochium nivale*) eleven mutants were designed to redirect the catalytic scope of ChitO for improved oxidation of lactose, cellobiose and maltose. The catalytic properties of the most interesting mutants were further improved by combining single mutations. This has resulted in the creation of a set of ChitO variants that display totally different substrate tolerances. One ChitO variant shows a dramatic improvement in catalytic efficiency towards oxidation of glucose, cellobiose, lactose and maltose. We also describe a ChitO variant with the highest catalytic efficiency in GlcNAc oxidation so far reported in the literature.

Introduction

With the current efforts in valorizing plant biomass, enzymes are increasingly considered as valuable biocatalysts. Except for hydrolytic enzymes, e.g. cellulases to degrade cellulose, also oxidative enzymes have shown to be highly relevant to improve biorefinery processes [1]. While the recently discovered lytic polysaccharide monooxygenases are highly effective in cleaving polysaccharide chains (e.g. cellulose or chitin), oxidases acting on mono- or oligosaccharides can also be used to valorize carbohydrates [2]. Among the known and classified carbohydrate oxidases, chitooligosaccharide oxidase from *Fusarium graminearum* (ChitO) is the only one known so far to oxidize N-acetylated carbohydrates. N-acetyl-D-glucosamine (GlcNAc), the constituent element of the polymer chitin, and oligomers of it were identified as the best substrates of ChitO. The oxidation occurs at the C1-hydroxyl moiety leading to the formation of the corresponding lactone which subsequently hydrolyses to the respective aldonic acid. Remarkably, the enzyme shows higher catalytic efficiency, due to a lower K_M, towards oligomers of GlcNAc [3]. Another peculiar feature of ChitO is the mode of cofactor binding: it contains a FAD cofactor that is tethered to two amino acids, His^{94} and Cys^{154}. In most flavin-containing proteins the flavin cofactor is noncovalently bound or covalently linked via one protein-flavin linkage. Bicovalent binding of a flavin cofactor has been observed in a selected group of sequence-related flavoproteins that all belong to the vanillyl-alcohol oxidase (VAO) flavoprotein family [4]. It has been suggested that, based on the observed substrate acceptance profiles of the so-called bicovalent flavoproteins, the bicovalent anchoring of the cofactor has facilitated a rather open active site that allows interaction with bulky substrates [5]. This is in line with the observation that ChitO accepts various chitooligosaccharide oligomers.

In a previous study, a point mutation in the active site of ChitO, Q268R, changed the substrate specificity of the enzyme from chitooligosaccharide towards glucooligosaccharides. The improved

efficiency was due to a dramatic decrease in K_M for non-N-acetylated oligosaccharides, such as lactose and maltose, while the k_{cat} values were hardly affected (Heuts et al., 2007). The mutation was made on the basis of a sequence comparison with glucooligosaccharide oxidase from *Acremonium strictum* (GOOX) [6] and lactose oxidase from *Microdochium nivale* (LaO) [7] which both contain an arginine at the respective position. Both oxidases primarily show activity towards glucooligosaccharides. Although the effect of this single mutation was remarkable, the Q268R ChitO mutant still displays a relatively low catalytic efficiency towards glucooligosaccharides when compared to GOOX and LaO. This indicates that the difference in substrate recognition by these oxidases is determined by multiple residues. Using the sequence similarities between ChitO, GOOX, and LaO, and the available crystal structures of the latter two oxidases, the residues that form the oligosaccharide binding pocket of these flavoprotein oxidases is known. Such knowledge can be exploited for the rational design of carbohydrate oxidases with tailor made substrate acceptance profiles.

In this study we describe the successful redesign of ChitO through semi-random model-inspired redesign. Specifically, by combining single mutations we created a ChitO variant which shows a dramatic improvement in catalytic efficiency towards oxidation of glucose, cellobiose, lactose and maltose. Significantly, we also describe a ChitO variant with the highest catalytic efficiency towards oxidation of GlcNAc so far reported in the literature.

Results and discussion

Design of ChitO Variants

ChitO shows the highest catalytic efficiency towards GlcNAc and its oligomers [3]. In order to alter its substrate scope, with a focus on improving activity towards cellobiose, lactose and maltose, variants of ChitO were designed. Based on multiple-sequence alignment and inspection of the modelled ChitO structure in comparison with the crystal structures of GOOX (PDB: 2AXR) and LaO (PDB: 3RJA), eight residues were selected for site directed mutagenesis (Figure 1.). The selection criteria were the following: 1) residues were considered when involved in forming the substrate binding pocket, and 2) residues were only selected when different compared with the structurally related residues in GOOX and LaO. In total, eleven mutations were designed: M170Y, D279E/S, G321Y/N, G270E, F319S, D337E, W373Y/F, S410R. The mutations were created to either 1) replace the selected residue in ChitO with the residue type found in either GOOX or LaO; or to 2) change the electrostatic properties of the mutated residue (e.g. M170Y, G321Y/N, D279S).

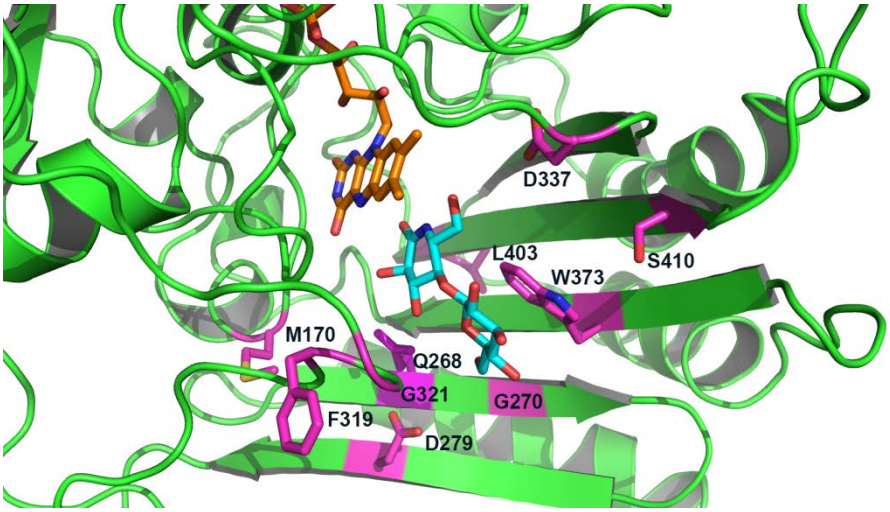

Figure 1: Overview of the mutations in the modeled structure of ChitO, with the FAD cofactor (carbon atoms in orange) and the target residues (carbon atoms in magenta) in sticks. The substrate analogue ((2R,3R,4R,5R)-4,5-dihydroxy-2-(hydroxymethyl)-6-oxopiperidin-3-yl beta-D-glucopyranoside with carbon atoms in cyan) is shown as bound in the crystal structure of glucooligosaccharide oxidase (PDB:2AXR).

The mutations had no significant influence on the expression level as all the variants were successfully expressed in *Escherichia coli* and purified through nickel affinity chromatography yielding three to four milligrams of pure protein per liter culture. Also FAD incorporation was not affected by the mutations as confirmed by the bright yellow color of the samples and by the inspection under UV light of the SDS PAGE gels containing the purified enzymes after incubation in performic acid and 5% acetic acid. This suggests that the various mutant proteins are correctly folded as FAD incorporation is a self-catalytic process which would not occur in a misfolded apo protein [5]. Also, the flavin absorbance spectra did not change significantly (Supplementary information) which is due to the fact that the targeted residue do not have any direct interactions with the isoalloxazine ring of the FAD cofactor (Figure 1).

Substrate screening

A panel of twenty-three industrially relevant substrates comprising monosaccharides, disaccharides, tetrasaccharides, cyclodextrins

and polysaccharides (Supplementary information) was created in order to test the activity of the newly designed variants. Specific activity of the purified eleven ChitO variants was measured using 50 mM and 5 mM of substrates. When compared with wild-type ChitO most of the variants decreased in activity on all tested substrates while still displaying the highest activity on GlcNAc. The W373Y and W373F mutants revealed the largest decrease in catalytic activity. They displayed extremely low or no activity for the tested substrates. This is in line with the observation that the respective residue in GOOX is in direct contact with the substrate analog (PDB: 2AXR). Interestingly, in a recent enzyme engineering study, Voung et al. [8] found that the substitution of a tryptophan at the corresponding residue in a variant of GOOX, GOOX_VN [9] to alanine resulted in a drastically increased K_M for cello-oligosaccharides [8]. It suggests that the tryptophan at this residue is important for ChitO to correctly position the substrates for hydride transfer to the FAD cofactor. LaO has a phenylalanine at the corresponding residue which may serve a similar role in supporting substrate binding. It is interesting to note that chitinases utilize tryptophans for substrate recognition and its activity is severely impaired when such residue is replaced with a phenylalanine or alanine [10].

Furthermore, two of the twelve mutants, G270E and S410R, showed a significantly higher specific activity towards lactose, cellobiose and GlcNAc. To precisely determine the improvement of the catalytic activity, these two mutants were analyzed in more detail.

Steady State Kinetic Analysis

To evaluate the activity improvement of the two engineered ChitO mutants S410R and G270E their K_M, k_{cat} and the catalytic efficiency (k_{cat}/K_M) were compared to those of wild-type ChitO and Q268R. Both ChitO mutants S410R and G270E increased the catalytic efficiency for cellobiose 3- and 2-fold, respectively (Table I). Also the activity towards lactose was significantly improved (3- and 6.5-fold, respectively) (Table I). Although both ChitO variants, containing a single residue substitution, improved the catalytic efficiency towards cellobiose and lactose, they did not reach the effect of the mutant

Q268R. Wild-type ChitO catalyzes the oxidation of chito-oligosaccharides most efficiently among the tested substrates. In previous work done by Heuts et al. the catalytic efficiency towards GlcNAc turned out to be 1016 $M^{-1}s^{-1}$ and for the oligomers even about 20 times higher [3]. The mutants S410R and G270E improved the catalytic efficiency on GlcNAc by 1.6 and 3.8-fold, respectively. Interestingly, while for G270E the k_{cat} slightly decreased and for S410R the k_{cat} remained as for wild-type, the K_M values decreased for both mutants 5- and 2-fold, respectively (Table I).

The substitution S410R corresponds to the arginine at position 391 in LaO [7]. Inspection of the crystal structure of LaO in complex with a substrate analogue shows how Arg391 is part of the substrate binding pocket. The two terminal nitrogen atoms of the guanidinium group are pointing towards the C2 and C3 hydroxyl groups of the second ring of the carbohydrate moiety of the bound substrate analog. Therefore the introduction of an Arg instead of a Ser in that position in ChitO might contribute to a better positioning of the substrate which lowers the K_M value. It is interesting to note that, unlike in ChitO, the substitution of this residue to an asparagine in a variant of GOOX, GOOX_V [8], leading to S388N, resulted in a two times lower k_{cat} for cellobiose [8].

The mutation G270E overlaps with the glutamate at position 247 in GOOX. From the crystal structure of GOOX in complex with a substrate analogue it can be concluded that the hydroxyl group of the C6 of the second ring of the carbohydrate moiety is aligned with one of the terminal oxygens of the glutamate. Therefore, the introduced Glu in ChitO might contribute to the positioning of the substrate analogous to E247 in GOOX. Furthermore, it may also result in repositioning or surrounding amino acids using water molecules as bridges for hydrogen bonds.

In summary, the changes introduced by the mutations G270E and S410R show, similar to the Q268R mutant, a trend in decreasing K_M values for the tested substrates while only G270E decreases k_{cat} values too.

*Table I. Michaelis-Menten kinetic parameters for ChitO wild-type (WT) and single mutants Q268R, G270E, S410R and combination thereof Q268R/G270E, G270E/S410R and Q268R/G270E/S410R on different carbohydrates. Kinetics parameters of glucooligosaccharide oxidase from Acremonium strictum (GOOX) and lactose oxidase from Microdochium nivale (LaO) are also included for comparison. *: Data from GOOX_VN (Foumani et al., 2011). n/a: not available.*

Substrate	Kinetic Parameters	GOOX (Lee et al., 2005)	LaO (Xu et al., 2001)	WT [3]	Q268R [3]	G270E	S410R	Q268R/ S410R	G270E/ S410R	Q268R/ G270E/ S410R
GlcNAc	k_{cat} (s^{-1})	8.08 ± 0.2*	n/a	6.4 ± 0.06	9.8 ± 0.39	4.7 ± 0.04	5.95 ± 0.1	7.2 ± 0.15	7.2 ± 0.12	7.7 ± 0.31
	K_M (mM)	340 ± 17*	n/a	6.3 ± 0.28	128 ± 10.6	1.2 ± 0.04	3.5 ± 0.18	44.4 ± 2.7	0.4 ± 0.03	19.6 ± 1.8
	K_i (mM)	n/a*	n/a	-	-	-	956 ± 190	-	-	863 ± 192
	k_{cat}/K_M (M^{-1} s^{-1})	24*	n/a	1016	77	3879	1700	163	16628	393
Glucose	k_{cat} (s^{-1})	9.1 ± 0.26	4	4.0 ± 0.13	7.4 ± 0.35	4.7 ± 0.18	4.1 ± 0.36	5.7 ± 0.12	5.8 ± 0.22	6.4 ± 0.13
	K_M (mM)	8.12 ± 0.16	42	752 ± 36	182 ± 18	529.2 ± 32.9	707.3 ± 92.8	96.9 ± 5.8	575.0 ± 35.4	49.9 ± 3.45
	k_{cat}/K_M (M^{-1} s^{-1})	1120	95	5	41	9	6	59	10	127
Cellobiose	k_{cat} (s^{-1})	5.36 ± 0.18	12	>0.6	6.7 ± 0.44	3.8 ± 0.15	5.8 ± 0.22	6.2 ± 0.24	5.7 ± 0.2	6.5 ± 0.09
	K_M (mM)	0.048 ± 0.08	59	>178	22.0 ± 3.3	60.6 ± 4.71	59.0 ± 4.39	2.3 ± 0.23	26.7 ± 2.53	1.19 ± 0.06

	K_i (mM)	n/a	n/a	-	-	-	-	94.9 ± 11.0	-	366 ± 39.1
	k_{cat}/K_M (M^{-1} s^{-1})	111667	203	32	305	63	99	2683	213	6483
Lactose	k_{cat} (s^{-1})	13.65 ± 0.41	n/a	4.6 ± 0.14	5.6 ± 0.11	4.5 ± 0.15	6.7 ± 0.49	8.0 ± 0.27	5.6 ± 0.09	10.4 ± 0.4
	K_M (mM)	0.066 ± 0.008	0.066	192 ± 9.2	13.0 ± 0.89	59.7 ± 4.38	42.8 ± 5.12	1.8 ± 0.15	12.0 ± 0.79	0.84 ± 0.08
	K_i (mM)	n/a	n/a	-	-	-	496 ± 168	51.7 ± 4.8	-	36.7 ± 5.3
	k_{cat}/K_M (M^{-1} s^{-1})	206818	n/a	24	431	76	156	4444	463	12381
Maltose	k_{cat} (s^{-1})	8.85 ± 0.27	6	7.6 ± 0.56	9.3 ± 0.23	4.7 ± 0.06	4.4	6.9 ± 0.08	5.0 ± 0.13	8.8 ± 0.18
	K_M (mM)	2.47 ± 0.05	11	332 ± 52	31.0 ± 2.5	71.4 ± 2.2	257.8	34.9 ± 1.30	170.0 ± 8.46	25 ± 1.7
	k_{cat}/K_M (M^{-1} s^{-1})	3583	545	23	300	66	17	198	29	352

Combining single mutations

In order to improve the catalytic efficiency on cellobiose, lactose and maltose, which are poor substrate for wild-type ChitO, multiple residue-substituted ChitO variants with a combination of Q268R, G270E and S410R were constructed. By combining these mutations, possibly synergetic effects on the substrate specificity enzyme activity could be achieved. Three combinations (Q268R/S410R, G270E/S410R and Q268R/G270E/S410R) were constructed, expressed and purified using the same methods used for the single mutants. The mutant Q268R/G270E was not constructed as the effect of the single mutant G270E on catalytic efficiency for the oxidation of cellobiose and lactose was generally lower when compared to the S410R mutation.

Expression of the holo form of the ChitO variants and the purity of the proteins were confirmed by SDS-PAGE analysis. Intriguingly, as was observed for the single mutants, the double and triple mutants all exhibited similar k_{cat} values for the tested compounds. This indicates that the mutations do not affect the rate-limiting step(s) of the oxidase. Yet, major effects of the combined mutations are observed when considering the K_M values.

When considering GlcNac, the best monosaccharide substrate for wild-type ChitO, the G270E/S410R mutant stands out with a >15 fold higher catalytic efficiency. It shows that the beneficial effects of the single mutations (3.8 and 1.6 fold increase in catalytic efficiency for G270E and S410R, respectively) can be combined such that a highly efficient GlcNac oxidase is created. Of all generated ChitO variants, the G270E/S410R mutant is the best ChitO variant for the oxidation of GlcNac. In fact, this ChitO mutant has the highest catalytic efficiency for GlcNAc oxidation so far reported in the literature. Similar synergistic effects of multiple mutations were seen for other substrates and ChitO variants. For example, the single mutants, Q268R and S410R, showed a modest increase (~10 fold) in catalytic efficiency on cellobiose and lactose when compared to wild-type ChitO while the respective double mutant is ~100 folds more

efficient (Table I). Also the catalytic efficiency of G270E/S410R ChitO for cellobiose and lactose oxidation was increased significantly compared to both single mutants. Yet, this double mutant is inferior when compared with the other mutant. This reinforces the important role of the Arg^{268} in recognizing substrates as cellobiose, lactose and maltose as previously reported (Heuts et al., 2007). It is noteworthy how the presence of this mutation drastically reduces K_M for all the tested substrates except GlcNAc (Table I).

The combination of all three mutations leads to a very potent lactose and cellobiose oxidase (200 and 500 fold higher catalytic efficiency, respectively) (Table I). This nicely shows the synergistic effects of the individual mutations. Remarkable is the high catalytic efficiency on lactose which is mainly due to the lowest K_M reported in this study (0.8 mM), which is 240-times lower than wild-type ChitO. The triple ChitO mutant is also the best variant for glucose and maltose as substrates. However, in line with the effects observed for the single mutants, the triple ChitO mutant still exhibits a rather high K_M values (≥25 mM) for these latter two carbohydrates.

It is worth noting that for some of the most efficient mutant-substrate combinations, substrate inhibition of different intensity was observed (GlcNAc, cellobiose and lactose with the triple mutant, cellobiose and lactose with the Q268R/S410R mutant, and GlcNAc and lactose with the S410R mutant, see Supplementary information for the plots). Similar substrate inhibition kinetics have been reported for GOOX and LaO [8], [11].

Determination of melting temperatures of ChitO variants

The effect of the mutations on the thermostability of the protein was also tested. The apparent melting temperatures of wild-type ChitO and ChitO variants were measured by using the ThermoFluor assay using SYPRO® Orange as unfolding reported dye. It was gratifying to observe that all mutants display a similar or improved thermostability. The data suggest that the S410R and G270E mutations do not significantly affect the thermostability of ChitO. However, interestingly, the Q268R mutation enhances the

thermostability as evidenced by an increase in apparent melting temperature in all mutants that carry this mutation. The effect seems most prominent in the single Q268R mutant with an apparent melting temperature that is almost 3˚C higher than wild-type ChitO (Table II).

Table II: Apparent melting temperatures of the ChitO variants as measured by ThermoFluor.

Variants	T_m' (ºC)
WT	53.5
Q268R	56.3
G270E	53.3
S410R	53.0
Q268R/S410R	55.0
G270E/S410R	53.3
Q268R/G270E/S410R	55.5

Conclusions

The substrate scope of ChitO was successfully changed through site directed mutagenesis. By creating a relatively small set of ChitO mutants, several variants were created that display superior activities with several carbohydrates when compared with the wild-type enzyme. This was achieved by focusing on ChitO residues that are part of the proposed substrate binding pocket, since one amino acid substitution in the active site of the proteins often leads to dramatic change of substrate specificity and the activity of the enzyme [12]. By comparing the sequence and the structure of the active site of ChitO with those of the closest homologues, several different residues were predicted to be responsible for the different substrate scope of ChitO.

The successful expression, purification and covalent incorporation of the FAD cofactor for all generated ChitO variants indicate that the mutations did not disturb the folding of the enzyme.

This study has revealed that Gly270 and Ser410 are involved in substrate recognition by ChitO while the other mutated residues appear to be less important in tuning the substrate specificity. The S410R mutation reduced the K_M values for all the substrates tested, including GlcNAc. This result is intriguing since this residue is relatively far from the docked substrate analog with the shortest distance of 10 Å between these moieties in the modeled ChitO structure (Figure 1). S410 is also quite distant from Q268, which was previously shown to be important in substrate recognition. The replacement of S410 by a relatively large and charged arginine may introduce new and favorable interactions for carbohydrates to bind to ChitO.

Similar to the S410R ChitO mutant, the mutation G270E in ChitO also did not affect the catalytic rate for any of the substrates tested while it significantly decreased the K_M values. While G270 is close to Q268 both in sequence and in structure, the effects of the respective mutations are not similar for all substrates. For example, while the Q268R mutant displays a 20-fold higher K_M for GlcNac, the respective K_M value for the G270E mutant decreased 5-fold. These data illustrate that the three identified residues, Q268, G270, and S410, contribute individually to shaping and creating favorable interactions with substrates. This is in line with the observation that by combining the single mutants Q268R, G270E and S410R, oxidases could be created with vastly different substrate specificities. The created mutants display synergistic effects concerning the catalytic efficiency of the double and triple ChitO mutants (Table I). In particular, the effect of Q268R/S410R on cellobiose oxidation and of the triple mutation on lactose oxidation showed a drastic synergistic effect of the single mutants.

The catalytic efficiencies of wild-type ChitO for the disaccharides cellobiose, lactose and maltose are all rather low. In a previous studies on ChitO, it was assumed that the enzyme has no preference

on the type of glycosidic bonds [3]. However, in this study the change of catalytic efficiency observed for maltose by various mutations differed significantly from that for cellobiose and lactose. In contrast to the drastic increase of catalytic efficiency for cellobiose and lactose by the combining mutations, the catalytic efficiency on maltose did not increase or increased only to a small extent. In other words, the created ChitO variants show preference for disaccharides with β-1,4-glycosidic bonds. A similar preference is found for GOOX [13] .

The thermostability of ChitO was not significantly affected by the G270E and S410R mutations. This result was somewhat expected as the amino acid are surface exposed. However, intriguingly, the Q268R mutation resulted in a marked thermostabilisation. In any case, the determined apparent melting temperatures are all above 50 ˚C which indicates that ChitO is a rather robust enzyme.

Having identified Gln268, Gly270 and Ser410 as key residues in determining the substrate specificity of ChitO, more elaborated mutagenesis approaches can be undertaken to generate libraries of variants with different substrate scopes and different efficiency. Yet, in order to obtain more insight into the molecular interactions of carbohydrates and ChitO, it is desirable to have a crystal structure of this fungal carbohydrate oxidase. Such structural information will also help to better understand the precise mechanism of the oxidation reactions catalyzed by the enzyme. For this purpose, we are currently performing crystallization studies.

The popularity of flavoprotein oxidases as biocatalysts in industries is increasing due to their ability to carry out regio- and/or enantioselective oxidation reactions for which they merely need molecular oxygen as an electron acceptor [14], [15]. Carbohydrate oxidases in particular are used in various industries including the medical, chemical, food and feed industries [2], [16]. Chitooligosaccharide oxidase is a promising candidate for broadening the oxidase-based applications as this study shows that it can be tuned to act on specific carbohydrates. For instance, an effective lactose oxidase could be used to produce lactobionic acid

which is a valuable compound for wide range of applications [11], [17]–[20]. The ability of ChitO to oxidize monosaccharides and disaccharides even at low substrate concentrations can also be exploited for sensing applications or developing new assays by which hydrolytic activity can be measured. Recently, we successfully developed an assay to detect chitinase or cellulase activity, using ChitO and ChitO Q268R respectively [21]. The development of ChitO variants with relatively lower K_M values for several oligosaccharides, reported in this study, will contribute to an even more sensitive assay for detection of amongst others chitinase and/or cellulase activities. The property of ChitO variants with increased activities towards cellobiose and a low activity for glucose suggests that these enzyme variants could be part of enzyme blends for saccharification of biomass analogous to the use of cellobiose dehydrogenase [22].

In this study we explored the catalytic scope of the ChitO by site directed mutagenesis. Variants were created with improved catalytic efficiency towards GlcNac, lactose, cellobiose and maltose. By this, residues that tune substrate specificity in ChitO and related carbohydrate flavoprotein were identified. These novel insights can be used for engineering tailor-made carbohydrate oxidases.

Conflict of Interest Disclosure

The authors declare no conflict of interest.

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Materials and Methods

Bacterial Strains

The *E. coli* TOP10 strain was used for the selection and storing of constructed plasmids. ChitO and its variants were expressed in *E. coli* ORIGAMI2 (DE3) from Emd Millipore.

Modelling, molecular docking and mutant design and site-directed mutagenesis

The sequence of ChitO was aligned with the sequences of its homologues GOOX and LAO using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and Geneious (Biomatters Ltd). The model structure of ChitO was obtained with SWISS-MODEL [23], [24] using the default parameters. The FAD cofactor was imported from the crystal structure 2AXR after superimposition in the software Pymol (http://www.pymol.org/). The substrate analogue (2R,3R,4R,5R)-4,5-dihydroxy-2-(hydroxymethyl)-6-oxopiperidin-3-yl beta-D-glucopyranoside was docked in the modelled ChitO with the softare YASARA (http://www.yasara.org) using the macro dock_runensemble. The pose in which the anomeric carbon of the substrate analogue was the closest to the FAD N5 was selected as best. Models of the ChitO mutants G270E, S410R and the triple mutant Q268/G270E/S410R were obtained by using YASARA using the modelled structure of ChitO as starting point.

Primers for site-directed mutagenesis were designed using the QuikChange® Primer Design Program (http://www.genomics.agilent.com/primerDesignProgram.jsp) and are listed in the Supplementary information.

A total of 20 µl of PCR reaction volume was used which included 1 µl of each forward and reverse primer (10µM), 1 µl of template vector pET-SUMO/ChitO [21] and 10 µl of Pfu Ultra Hot Start PCR master mix (Agilent Technologies). The concentration of pET-SUMO/ChitO

used as the template was 30 ng/µl. The negative control reaction was performed, which excluded primers. The PCR reaction was performed using the following protocol: Initial denaturation for 5 minutes at 94˚C; 18 cycles of denaturation for 1 minute at 94˚C, annealing for 30 seconds at 55˚C and extension for 13 minutes at 68˚C; final extension for 5 minutes at 68˚C. After the PCR reaction was finished, 1 µl of DpnI restriction enzyme (New England BioLabs) was added and the mixture was incubated at 37˚C overnight to degrade the parental plasmids. Mutants were confirmed by sequencing performed at GATC Biotech (Constance, Germany).

Protein expression and purification

The expression and purification of ChitO and its variants was performed as previously described [21]. Briefly, expression was carried out in *E. coli* ORIGAMI2 DE3 (EMD Millipore) for 69 hours at 17˚C after which cells were harvested and sonicated in lysis buffer (50 mM Tris/HCl pH 7.6; 0.5 M NaCl; 10 mM imidazole). After ultra-centrifugation, the cell-free extract was incubated for 1 hour with 1.0 mL of Ni Sepharose (GE Healthcare) pre-equilibrated with lysis buffer. After washing with increasing concentrations of imidazole, the proteins were eluted with 0.5 M of imidazole. The samples were desalted through Econo-Pac 10DG desalting columns (Bio- Rad, Hercules, CA, USA) and concentrated with Amicon Ultra-15 30 KDa (EMD Millipore). The protein concentration was determined as previously described [3].

Substrate screening and kinetic analysis

All reactions were performed at 25-28˚C and the pH of the reaction solutions was maintained at 7.6 using 50 mM potassium-phosphate buffer. To be able to monitor the activity of the purified proteins, the horseradish peroxidase coupled assay was conducted. The horseradish peroxidase oxidizes 4-aminoantipyrine (AAP) and 3,5-dichloro-2-hydroxybenzensulfonic acid (DCHBS) using hydrogen peroxide produced by the oxidation reaction of ChitO and releases a pink to purple colored product. An excess of horseradish peroxidase was used in order to determine the reaction rate of ChitO on the

substrates tested by means of the production rate of the colored substance measured by the light absorption at 515 nm. The reactions were measured with the Synergy Mx microplate reader (BioTek). The reaction mixtures were prepared in a 96-well microplate with a final volume of 200 µl per well, containing substrates of ChitO, 50 mM potassium-phosphate buffer pH 7.6, 0.10 mM AAP, 1.0 mM DCHBS, 4.0 units of horseradish peroxidase (HRP) and 20 nM of ChitO. 8.0 units of HRP were used when the production rate of the colored product was more than 5 s^{-1}, to avoid HRP being the rate limiting factor. All the components were added manually, except for ChitO. ChitO (100 µL) was added as last component in an automated manner in the microplate reader to ensure effective mixing and to initiate the reactions fast and reproducibly.

For the substrate screening, each substrate (23 different carbohydrates and derivatives, see supplementary information) solution with two different concentrations was prepared in the 50 mM potassium-phosphate buffer pH 7.6. For the kinetic assays, various concentration ranges of substrates were used depending on the activity of the protein on them. The reactions were performed at least in triplicate and the activity was measured for 3 minutes for each reaction with 5 seconds intervals. The measured initial activity, in the first minute of the reaction, at each substrate concentration was converted to k_{obs} using the following equation where v is the corrected production rate (A/sec), 26 mM^{-1} cm^{-1} is the extinction coefficient of the pink product, while a pathlength of 1 cm was employed.

$$k_{obs} = \frac{\mathrm{v}}{26\mathrm{mM}^{-1}\mathrm{cm}^{-1} \times \text{enzyme concentration}(\mathrm{mM}) \times 1\mathrm{cm}}$$

Using Microsoft Excel, the calculated k_{obs} values were plotted against the concentrations of the substrates and the k_{cat} and K_M were derived from fitting of the curve with the hyperbolic function $y = (k_{cat} * x) / (K_M + x)$. Where substrate inhibition was observed, data were fitted with the following equation: $y = (V_{max} * x) / (K_M + x * (1 + x / K_i))$ (Copeland, 2000).

Determination of the apparent melting temperature

To determine the thermal stability of the ChitO variants, the Thermofluor assay using SYPRO® Orange was conducted. 0.1% SYPRO Orange dye was added to the 40 µM protein solutions. Using CFX96 Real-Time System combined with C1000 Touch Thermal Cycler (BioRad), the intensity of the fluorescence was measured at a temperature gradient of 20 to 99 °C. The software generated fluorescence plots and derivative plots (fluorescence changes against temperature). The temperature for the lowest values of the peaks from the derivative plots were taken as apparent melting temperatures. The estimated error for the determined apparent melting temperatures is 0.5 °C.

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Supplementary Information

Substrates panel

The panel of substrates used for the screening was composed of glucose, galactose, fructose, mannose, xylose, arabinose, N-acetyl-D-glucosamine, sucrose, maltose, lactose, sorbitol, xylitol, cellobiose, cellotetraose, α-cyclodextrin, β-cyclodextrin, arabinan, chitosan, chitin, starch, maltotetraose, glycerol, hydroxymethylfurfural.

Primers

D279E_fw-5'GAACGGATACAGCGTCGAGGGTGCTTATATCGGTG3'

D279E_rev-5'CACCGATATAAGCACCCTCGACGCTGTATCCGTTC3'

D279S_fw-5'-
CTAAGAACGGATACAGCGTCAGTGGTGCTTATATCGGTGATG-3'

D279S_rev-5'-
CATCACCGATATAAGCACCACTGACGCTGTATCCGTTCTTAG3´

D337E_fw-5'-CATACGACGCACATGAGAACTTCTACGCCAGCA-3'

D337E_rev-5'-TGCTGGCGTAGAAGTTCTCATGTGCGTCGTATG-3'

G270E_fw-5'-
GAGATCACCATGCAGATGGAGGTCTCTAAGAACGGATACA-3'

G270E_rev-5'-
TGTATCCGTTCTTAGAGACCTCCATCTGCATGGTGATCTC-3'

G321Y_fw-5'-
CTTGTTACTCACTTCGCCTATGCTGGCGTCAACGTCAA-3'

G321Y_rev-5'-
TTGACGTTGACGCCAGCATAGGCGAAGTGAGTAACAAG-3'

G321N_fw-5'-
CTTGTTACTCACTTCGCCAATGCTGGCGTCAACGTCAA-3'

G321N_rev-5'-
TTGACGTTGACGCCAGCATTGGCGAAGTGAGTAACAAG-3'

M170Y_fw-5'-CACGGAGGCTACGGATATGTCGCCCGCAAGCAC-3'

M170Y_rev-5'-GTGCTTGCGGGCGACATATCCGTAGCCTCCGTG-3'

S410R_fw-5'-CAATTCTACGACAGGGTTGCCGCCACTGC-3'

S410R_rev-5'-GCAGTGGCGGCAACCCTGTCGTAGAATTG-3'

F319S_fw-5'-GGTCTTGTTACTCACAGCGCCGGTGCTGGCG-3'

F319S_rev-5'-CGCCAGCACCGGCGCTGTGAGTAACAAGACC-3'

W373F_fw-5'-
GCAGCAGCCACTCTTGGTTCTTGCAAATGGACATCACC-3'

W373F_rev-5'-
GGTGATGTCCATTTGCAAGAACCAAGAGTGGCTGCTGC-3'

W373Y_fw-5'-
GCAGCAGCCACTCTTGGTATTTGCAAATGGACATCACC-3'

W373Y_rev-5'-
GGTGATGTCCATTTGCAAATACCAAGAGTGGCTGCTGC-3'

L403W_fw-5'-
GTCCACCGTGACACCCTCTGGCTCTTCCAATTCTACGACA-3'

L403W_rev-5'-
TGTCGTAGAATTGGAAGAGCCAGAGGGTGTCACGGTGGAC-3'

The double mutant ChitO Q268R/S410R was constructed using the wild-type pET SUMO/chito as a template and both primers of Q268R and S410R. The previously constructed primer for Q268R by

[3] was obtained and the primer sequences are Q268R_fw 5´-CCTGCCGAGATCACCATGCGCATGGGTGTCTCTAAGAACG-3´ and Q268R_rev 5´-CGTTCTTAGAGACACCCATGCGCATGGTGATCTCGGCAGG-3´. For the mutant Q268R/G270E and Q268R/G270E/S410R an additional primer for Q268R/G270E was designed since the two mutation sites Gln268 and Gly270 are too close to each other. The sequence of the primers are Q268R/G270E_fw-5'-CTGCCGAGATCACCATGCGGATGGAG GTCTCTAAGAACGGATAC-3' and Q268R/G270E_rev-5'-GTATCCGTTCTTAGAGACCTCC ATCCGCATGGTGATCTC-3'. ChitO Q268R/G270E and the triple mutant Q268R/G270E/S410R were constructed with wild-type pET SUMO-chito and pET SUMO-chito S410R as a template respectively and the primers for Q268R/G270E were used.

Steady state kinetic plots

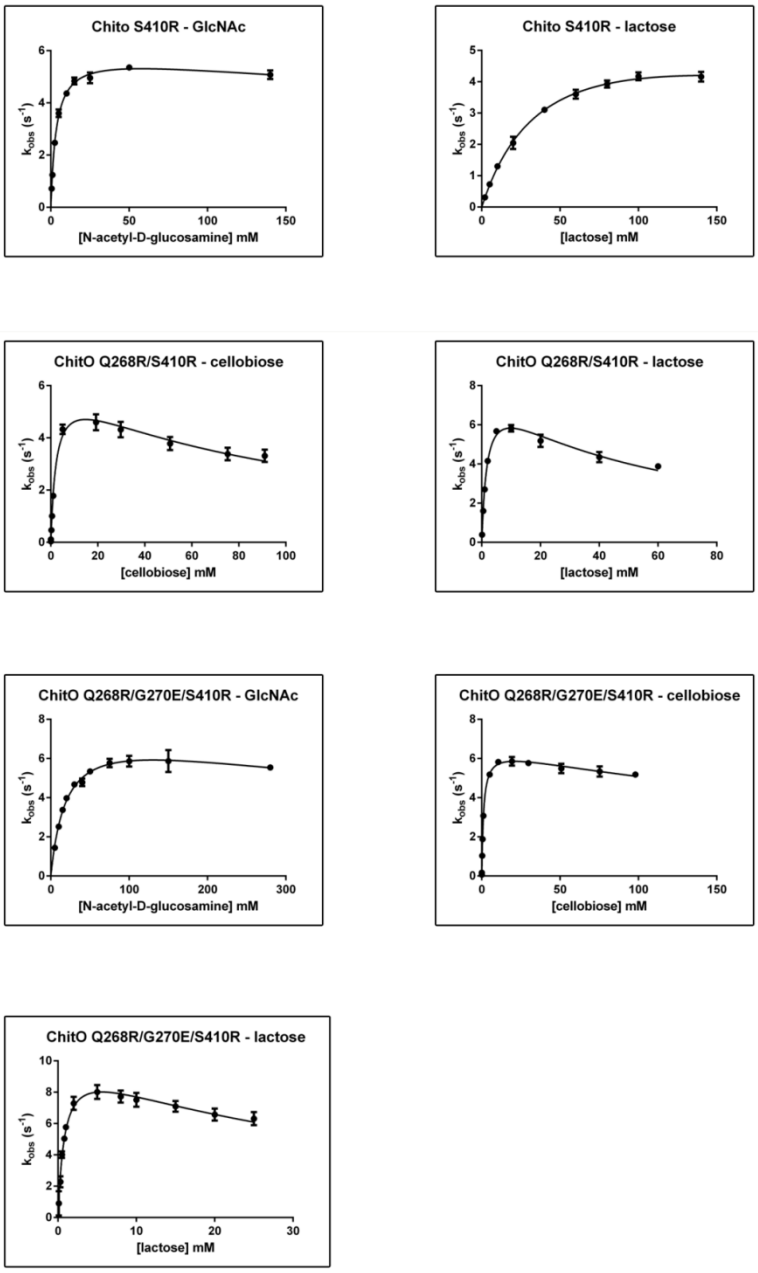

Fig. S1. Steady-state kinetic data of ChitO mutants that displayed substrate inhibition.

Some mutants of ChitO showed different degrees of substrate inhibition as can be seen in the plots above. Fitting of data was performed using the provided equation for substrate inhibition (See Material and Methods).

Flavin absorbance spectra

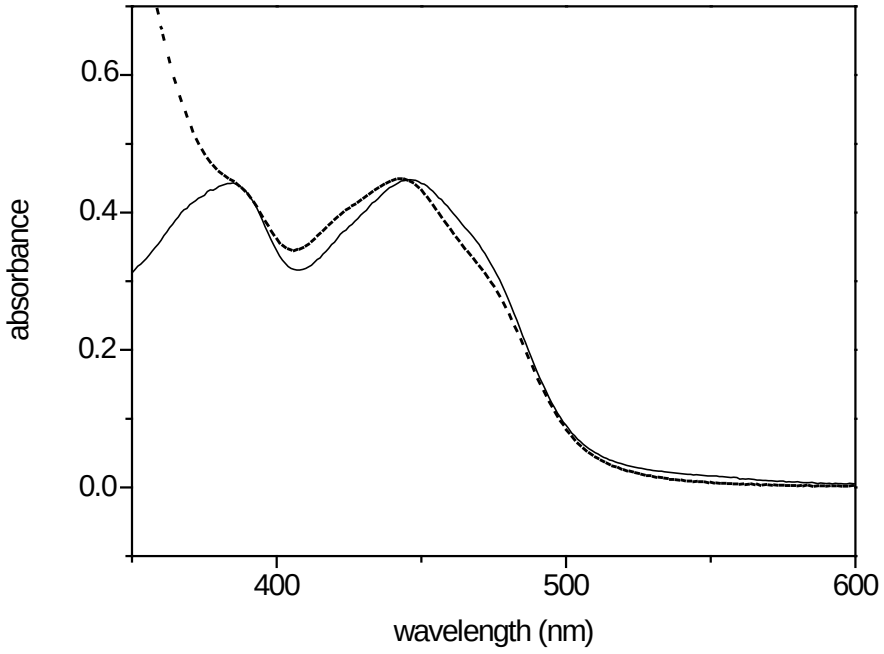

Fig. S2. Comparison of flavin absorbance spectrum of wild-type ChitO (38.8 µM, taken from Heuts et al. 2008) with the triple mutant ChitO Q268R/G270E/S410R (normalized for 38.8 µM).

The relatively high absorbance below 400 nm is due to the presence of the 4a-spirohydantoin FAD degradation product formed during the purification process as has also been observed for sequence-related the berberine bridge enzyme (Winkler et al., 2008). The content of this inactivated FAD derivated in the ChitO preparations differed from batch to batch for wild-type and mutant enzymes. However, enzyme concentrations were estimated based on the known extinction coefficient for the oxidized FAD cofactor of

wild-type ChitO at 445 nm, therefore only the concentration of intact FAD, thus active enzyme, was used to determine the turnover rates.

References for supplementary information

Heuts DPHM, Janssen DB, Fraaije MW. 2007. Changing the substrate specificity of a chitooligosaccharide oxidase from *Fusarium graminearum* by model-inspired site-directed mutagenesis. FEBS Lett. 581:4905–9.

Winkler A, Lyskowski A, Riedl S, Puhl M, Kutchan TM, Macheroux P, Gruber K. 2008. A concerted mechanism for berberine bridge enzyme. Nat. Chem. Biol., 12: 739–41.

Chapter 4

Discovery of a xylooligosaccharide oxidase from *Myceliophthora thermophila C1*

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Abstract

By inspection of the predicted proteome of the fungus *Myceliophthora thermophila* C1 for vanillyl-alcohol oxidase (VAO)-type flavoprotein oxidases, a putative oligosaccharide oxidase was identified. By homologous expression and subsequent purification the respective protein could be obtained. The protein was found to contain a bicovalently bound FAD cofactor. By screening a large number of carbohydrates, several mono- and oligosaccharides could be identified as substrate. The enzyme exhibits a strong substrate preference towards xylooligosaccharides, hence it is named xylooligosaccharide oxidase (XylO). Chemical analyses of the product formed upon oxidation of xylobiose revealed that the oxidation occurs at C1, yielding xylobionate as product. By elucidation of several XylO crystal structures (in complex with a substrate mimic, xylose and xylobiose), the residues that tune the unique substrate specificity and regioselectivity could be identified. The discovery of this novel oligosaccharide oxidase reveals that the VAO-type flavoprotein family harbors oxidases tuned for specific oligosaccharides. The unique substrate profile of XylO hints to a role in the degradation of xylan derived oligosaccharides by the fungus *M. thermophila* C1.

Introduction

Nature has developed a profusion of enzymes to produce, modify and degrade carbohydrates. These enzymes usually outcompete chemical approaches for carbohydrate conversions as they are highly selective and effective. Enzymes involved in carbohydrate modifications are thoroughly studied as they can be handy biocatalytic tools in developing new biotechnological applications. Especially with the growing research efforts with the aim to develop efficient biorefinery processes, it is attractive to have a well-equipped toolbox of enzymes active on carbohydrates (1).

The Carbohydrate Active enZymes (CAZy) database provides a very good overview on the classes and individual enzymes that are implicated in catalyzing carbohydrate chemistry. While the database is very rich in non-redox enzymes (~500,000 members), which include glycoside hydrolases and glycosyl transferases, relatively few redox enzymes are included (~10,000 members). In fact, only in recent years CAZy has included the so-called Auxiliary Activity (AA) families which represent the enzymes that assist in carbohydrate modifications through performing oxidations or reductions. One of these families is AA7 which contains flavoprotein oxidases acting on oligosaccharides (www.CAZy.org/AA7_all.html). Currently this family includes only a few characterized enzymes: glucooligosaccharide oxidase (GluO) from *Acremonium strictum* (2), chitooligosaccharide oxidase (ChitO) from *Fusarium graminearum* (3) and lactose oxidase (LaO) from *Microdochium nivale* (4). These fungal oxidases, all acting on different kinds of oligosaccharides, share a common structural fold as they belong to the vanillyl-alcohol oxidase (VAO) flavoprotein family (5). They also have in common that they selectively oxidize oligosaccharides at the C1 position (2, 3, 6). This results in formation of the corresponding lactones which typically undergo spontaneous hydrolysis. The electrons obtained through the oxidation of the carbohydrates are used by the enzyme to reduce molecular oxygen thereby generating hydrogen peroxide.

These redox reactions are facilitated by an FAD flavin cofactor that is covalently tethered to the enzymes via two covalent linkages.

Detailed studies on GluO and ChitO have revealed that these oxidases accept a wide range of mono- and oligosaccharides but are most efficient with oligosaccharides. Furthermore, they are able to discriminate between different types of oligosaccharides. For example, ChitO is very selective in oxidizing chitooligosaccharides while it only poorly accepts a limited number of other oligosaccharides. Elucidation of the structures of GluO and LaO and model-inspired engineering of ChitO has revealed the molecular basis for the observed oligosaccharide selectivities of these oxidases. The structures display a rather open active site close to the surface which allows binding of large oligosaccharides (7). As a result of the open character of the active site, the redox-active isoalloxazine moiety of the flavin cofactor is relatively solvent exposed. This provides an explanation for why these flavoproteins have the flavin cofactor covalently anchored via two amino acids (8). Through the bicovalent linkage, the cofactor can still be positioned for catalyzing oxidations while in other flavoproteins the amino acids are used to bind and position the cofactor. The open active site architecture in oligosaccharide oxidases is different from carbohydrate oxidases that preferentially act on monosaccharides. For example, glucose oxidase and pyranose oxidase have very occluded active sites (9, 10). Still, the nature and positioning of a number of residues that form the carbohydrate binding groove dictates which oligosaccharides can bind in such a way that the flavin cofactor is able to oxidize the substrate. The role of these residues in substrate acceptance was clearly demonstrated by engineering ChitO: by replacing three residues an efficient lactose oxidase could be generated from ChitO (11). This shows that the nature of the amino acids lining the carbohydrate binding groove in oligosaccharide oxidases determines the substrate specificity.

In this study we report on the discovery, characterization and crystal structures of a novel flavoprotein oxidase belonging to the VAO-family which is primarily active towards xylooligosaccharides (XOS):

xylooligosaccharide oxidase (XylO) from the thermophilic fungus *Myceliophthora thermophila* C1. By determining the kinetic and catalytic properties and elucidating its crystal structure, our study provides detailed insight into the distinctive enzyme properties of XylO. The results show also that XylO can be used for generating XOS-based aldonic acids.

Results

Homology analysis

We have recently identified a stretch of conserved residues that correlates with the presence of a bicovalently bound FAD in flavoprotein sequences that belong to the VAO flavoprotein family (12). As all currently known oligosaccharide oxidases contain FAD bound via such unique bicovalent attachment, we used this motif as filter in a PHI-BLAST search. We decided to search specifically the predicted proteome of *M. thermophila* C1 using the ChitO sequence as query. The targeted fungus is a rich source of thermostable and biocatalytically interesting enzymes (13). Thirteen putative bicovalent flavoprotein sequences were found in the predicted proteome of this fungus and through multiple sequence alignment two of them were found to cluster with ChitO, GluO and LaO. One of these two putative oxidases (KX139007), which shared 41%, 44% and 47% protein sequence identity with ChitO, GluO and LaO respectively, was selected for further studies. The corresponding gene encodes for a 497 amino acids protein whose first 16 amino acids encode for a signal sequence identifying the putative oxidase as an extracellular protein. The presence of the conserved FAD linking residues, His94 and Cys155 (Figure 1) strongly suggests that the respective protein contains a bicovalently bound FAD.

```
XylO    ANPKSGGHSYASFGLGGEDGHL (35) EEGKRAFSHGTCPGVGVGGHSLHGGFGFSSHSHGL 178
LaO     VSAKSGGHSYASFGFGGENGHL (36) DKYGRAISHGTCPGVGISGHFAHGGFGFSSHMHGL 155
ChitO   ISAKSGGHSYTSLGFGGEDGHL (34) NQGKRALAHGTCPGVGLGGHALHGGYGMVARKHGL 177
GluO    ISAKGGGHSYGSYGFGGEDGHL (34) DQGNRALSHGTCPAVGVGGHVLGGGYGFATHTHGL 178
         ..*.**H** * *:***:***::    ::  **::***C*.**:.**   **:*: :: ***
```

Figure 1: Partial sequence alignment of XylO, LaO, ChitO, and GluO showing the conservation around the two FAD-linking amino acids. The respective histidine and cysteine are in bold and in the consensus line.

Purification

From 50 mL of concentrated fermentation supernatant, around 80 mg of yellow-colored XylO was purified after three steps of purification. Purified XylO runs as one single band on SDS-PAGE at around 60 kDa. This does not agree with the predicted size of 53 kDa which suggests that the protein has undergone post-translational modifications, potentially glycosylations. The protein displayed clear fluorescence when, upon SDS-PAGE, the polyacrylamide gel was illuminated by UV light. The fluorescence was more intense when incubating the gel for 5 min in 5% acetic acid which confirmed the presence of covalently bound FAD. The purified enzyme displays a typical flavin spectrum, with absorbance maxima at 442 and 387 nm (Figure 2). Upon addition of 0.1% SDS, the flavin spectrum undergoes changes similar to the ones observed for unfolded berberine bridge enzyme (14), which also contains a bicovalently bound FAD cofactor.

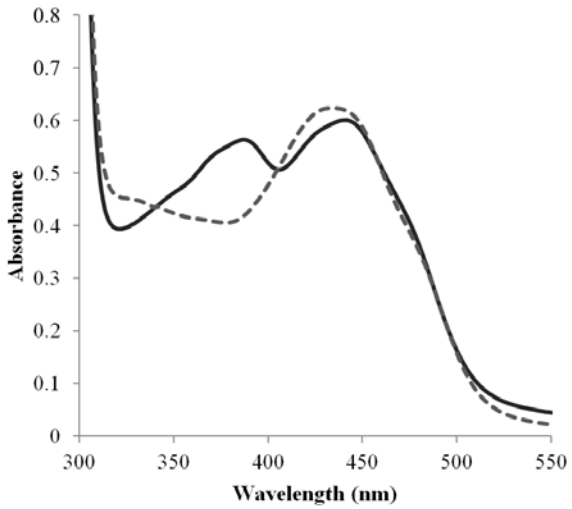


Figure 2: Spectrum of 52 µM native XylO (solid line) and unfolded XylO upon addition of 0.1% SDS (dashed line), in 50 mM potassium phosphate buffer pH 6.0.

Substrate identification

To establish the substrate profile of XylO, 23 carbohydrates (glucose, galactose, fructose, mannose, xylose, l-arabinose, N-acetyl-d-glucosamine, sucrose, maltose, lactose, sorbitol, xylitol, raffinose, cellobiose, cellotetraose, α-cyclodextrin, β-cyclodextrin, arabitol, chitosan, chitin, starch, maltotetraose, glycerol) were tested as substrates at 50 mM and 5 mM (for cyclodextrins, cellotetraose and maltotetraose the concentrations tested were 1 mM and 0.5 mM) using the HRP-based assay. After 1 min incubation at 25 ˚C with 1 µM of XylO, oxidase activity could be clearly observed for xylose, cellobiose, lactose, arabinose through formation of a clear pink color. A weak signal could also be detected for galactose, maltose and cellotetraose. Interestingly, while L-arabinose did show some activity, 1,5-α-L-arabinobiose did not. Based on the observed substrate profile, the steady state kinetics parameters were determined for xylose, cellobiose and lactose (Table 2). Intriguingly, while the k_{cat} values are in the same order of magnitude when compared with other known oligosaccharide oxidases (3, 15–17) the K_M values are several orders of magnitude higher. All oligosaccharide flavoprotein oxidases are known to perform better

on oligosaccharides when compared with the monosaccharides. This is probably due to their more open active site on the surface and the presence of an oligosaccharide binding groove (3, 4, 7, 16). Since XylO displayed very poor activity on cellobiose, no detectable activity on maltotetraose, and xylose had the lowest K_M value among the tested carbohydrates (which did not include xylose-based oligosaccharides), as a next step several XOS were tested as substrates (xylobiose, xylotriose, and xylotetraose). This revealed that XylO is a highly efficient oxidase for XOS (Table 2). While the k_{cat} values were similar for all identified XylO substrates, the K_M values dramatically decreased for all tested XOS. While for xylose a K_M of around 400 mM was found, the K_M values for xylobiose, xylotriose, and xylotetraose were around 3 orders of magnitude lower (Table 2). With k_{cat} values of 11.0-11.5 s^{-1} and K_M values in the (sub)millimolar range, XylO displays similar kinetic properties as those reported for ChitO and GluO in combination with their best oligosaccharide substrates (3, 17). The kinetic data clearly show that XylO prefers pentose-based oligosaccharides over hexose-based oligo-saccharides. This sets XylO apart from all other known oligosaccharide oxidases (16–20).

While the HRP-based assay confirms that XylO is using dioxygen as electron acceptor, acting as an oxidase, we also determined the apparent affinity towards dioxygen. By analyzing oxygen depletion curves, a K_M for oxygen of 0.13 mM could be determined. This shows that at standard atmospheric conditions (0.24 mM dioxygen in buffer solution), XylO is operating below saturation of the electron acceptor.

Table 2. Steady state kinetic parameters of XylO determined for cellobiose, lactose, xylose, and xylooligosaccharides. Data are representative of three independent experiments.

	k_{cat} (s^{-1})	K_M (M)	k_{cat}/K_M (M^{-1} s^{-1})
cellobiose	5.4 ± 0.3	0.342 ± 0.03	16
lactose	4.8 ± 0.6	0.532 ± 0.071	9
xylose	5.2 ± 0.4	0.359 ± 0.051	15
xylobiose	11.5 ± 0.5	0.00115 ± 0.00008	10000
xylotriose	11.0 ± 0.3	0.00069 ± 0.00004	16000
xylotetraose	11.2 ± 0.3	0.00043 ± 0.00003	26000

General enzyme properties

Having identified efficient substrates for XylO, we determined several enzyme properties by using xylobiose as substrate. To determine the pH optimum for activity, oxidase activity over a pH range from 4 to 8 was measured (Figure S3). This revealed that XylO is most active at pH 7, while it is still quite active at pH 6 (80%) and moderately active at pH 8 (33%).

M. thermophila strains are able to survive and replicate at temperatures higher than 45 ˚C (21–23). Therefore, many enzymes from this fungus have to tolerate relatively high temperatures. The thermostability of XylO was tested with the thermofluor assay using SYPRO orange as unfolding reporter dye (24). The apparent melting temperature was found to be 65˚C which indicates that it is indeed a thermostable enzyme. The temperature optimum for activity was also determined between 20 and 60˚C using an excess of HRP to compensate for loss of activity of this enzyme at higher temperatures (Figure S4). The optimal temperature for enzyme activity is 30˚C while it still displays 50% activity at 60˚C.

Product identification

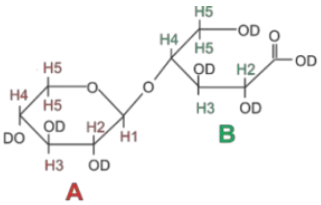
For determining the site of oxidation by XylO, the product formed upon conversion of xylobiose was determined. For product identification NMR and GC methods were used. The NMR spectra revealed the presence of a disaccharide as product. The two monosaccharide units were labeled A and B according to decreasing chemical shift values of their protons. The region (δ 4.6 – 3.0) in the 1D ^{1}H-NMR spectrum contained eleven well-resolved signals (Figure 3, Table 3). Based on observed ^{1}H chemical shifts and $^{3}J_{1,2}$ coupling constant values, residue A ($^{3}J_{1,2}$ 8.5 Hz) was assigned to have the β-anomeric configuration. The 2D COSY spectrum also allowed the assignment of the sequential order of the chemical shifts belonging to residue A and residue B. All ^{13}C chemical shifts could be assigned using a 2D ^{13}C-^{1}H HSQC spectrum in combination with 2D COSY (Figure 3). The downfield shift of B-4 (δ 83.6) is indicative for 4-substitution of residue B. Further evidence for the interpretation of the HSQC spectrum and the determination of the glycosidic linkage is available in the HMBC spectrum where intraresidual two- and three-bond ^{1}H-^{13}C couplings and inter-residual three-bond connectivities over the glycosidic linkages could be assigned (Figure 3). The absence of anomeric signals for residue B fits with a xyluronic acid residue. The NMR data are fully consistent with formation of xylobionic acid as oxidation product.

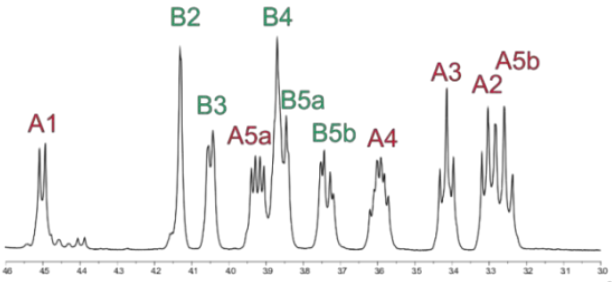
The structure of the xylobionate was further confirmed by GC-MS analysis. The GC chromatogram of the intact xylobionate shows one major peak and the respective MS spectrum showed a fragmentation pattern fitting xylobionate (Supplemental Information, Figure S1). After methanolysis the GC chromatogram of TMS-derivatives showed four peaks which were also identified by MS (Supplemental Information, Figure S2). One minor peak corresponded with TMS-derivatives of the methyl-glycoside of α- and β-xylofuranosides, two peaks corresponded with the methyl-glycosides of α- and β-xylopyranosides, and one peak corresponded with the methylated xyluronic acid. The GC data

confirm that XylO oxidizes xylobiose into xylobionic acid by C1 oxidation of the reducing end.

Table 3. Observed 1H and 13C chemical shifts (ppm) for the product formed upon conversion of xylobiose by XylO (D2O, 25 ˚C). The ppm values are relative to the signal of internal acetone (δ 2.225 for 1H and δ 31.07 for 13C).

Residue	H-1 C-1	H-2 C-2	H-3 C-3	H-4 C-4	H-5a C-5	H-5b
A (monosaccharide at non-reducing end)	4.505 104.8	3.304 75.5	3.414 78.2	3.596 71.6	3.923 67.5	3.257 67.5
B (monosaccharide at reducing end)	-	4.153 73.9	4.057 73.8	3.875 83.6	3.857 62.8	3.738 62.8


H5
OD
O
H4
H5
OD
H2
OD
H5
H4
H5
O
O
OD
H2
H1
DO
H3
OD
H3
OD
A
B


B2
B4
A3
A5b
A2
B3
B5a
A1
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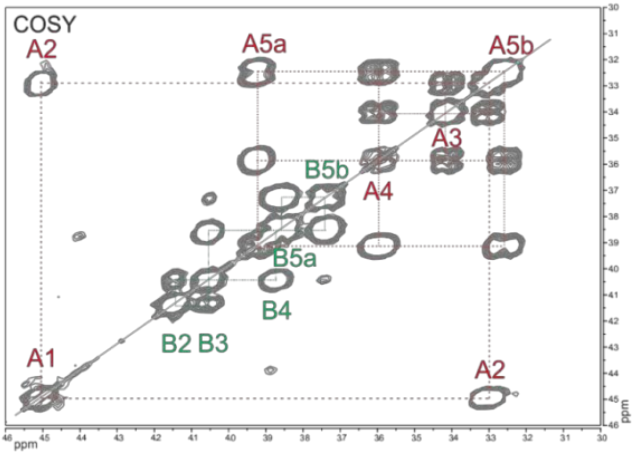
B5b
A4


COSY
A2
A5a
A5b
A3
B5b
A4
B5a
B4
B2 B3
A1
A2
ppm

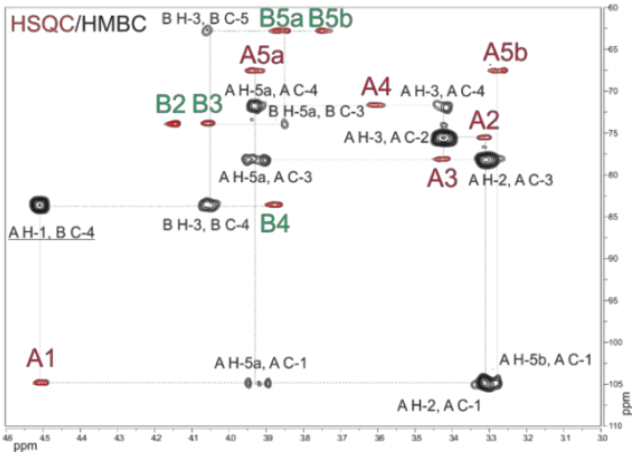

HSQC/HMBC
B H-3, B C-5
B5a B5b
A5a
A5b
A H-5a, A C-4
A4
A H-3, A C-4
B2 B3
B H-5a, B C-3
A2
A H-3, A C-2
A H-5a, A C-3
A3
A H-2, A C-3
A H-1, B C-4
B H-3, B C-4
B4
A1
A H-5a, A C-1
A H-5b, A C-1
A H-2, A C-1
ppm

Figure 3: The 1D 1H-NMR spectrum, the 2D 1H-1H COSY spectrum and the 2D 13C-1H HSQC (red peaks)/HMBC (black) spectra of the product formed upon conversion of xylobiose by XylO, recorded in D2O at 25 °C. In the HMBC spectrum, the cross-peak confirming the glycosidic linkage is underlined. The structure of the xylobionate, including the atom annotation, is also presented.

Oxidation of complex substrates

In the literature only few papers report on using carbohydrate oxidases to oxidize complex polysaccharide substrates, i.e. LaO (4) and galactose oxidase (25). As XylO was found to act on XOS, we tested the oxidase with various complex carbohydrate-containing mixtures that vary in xylan content: beechwood xylan, wheat arabinoxylan, oat flour, rye whole grain flour and barley flakes. The targeted mixtures (1% w/v) were first separated into soluble and insoluble fractions and each of them was tested in duplicate. Using the HRP-based assay specific activities towards the different potential substrate could be determined (Table 4). No activity was detected on insoluble substrates suggesting the necessity for the reducing ends of the poly- or oligosaccharides to be in solution. Testing the soluble fractions of oat flour and barley flakes did not yield any oxidase activity, while the soluble fractions of the xylans and rye flour revealed significant oxidase activity. The observed preference is in line with the fact that, when compared with barley and oat, rye grains are relatively rich in arabinoxylan. In contrast, oat and barley are relatively rich in glucans (26, 27). Thus the observed activities are in agreement with the above reported substrate profile for XylO. To further probe the use of XylO for oxidizing xylan derived XOS, we prepared hydrolyzed wheat arabinoxylan which comprises a population of XOS of different lengths substituted with 2-, 3- or 2,3-linked arabinose residues. The observation that hydrolysis of wheat arabinoxylan resulted in a 32-fold increase in XylO activity suggests that XylO is also able to accept arabinose-decorated XOS.

Table 4. Oxidation activity of XylO on the soluble and insoluble fractions of complex natural substrates. The HRP-based assay was performed at 30 °C. ND: not detected. NT: not tested. Data are representative of three independent experiments.

	specific activity (U/mg)	
	soluble	**insoluble**
beechwood xylan	0.50 ± 0.06	ND
wheat arabinoxylan	0.43 ± 0.08	ND
hydrolyzed wheat arabinoxylan[a]	13.8 ± 0.85	NT
oat flour	ND	ND
rye whole grain flour	1.03 ± 0.15	ND
barley flakes	ND	ND

Overall structure and active site of XylO

In order to understand the molecular basis by which XylO specifically accepts xylooligosaccharides, we set out to elucidate the crystal structure of XylO. Well-diffracting crystals could be obtained using PEG6000 as precipitant and the crystal structure of XylO was determined to a resolution of 1.93 Å using molecular replacement. The final model comprises amino acid residues 25 – 497, 1 FAD molecule, 7 N-acetylglucosamine (GlcNac) molecules, 1 MES molecule and 383 water molecules with *R/Rfree* 15.9 / 19.5 % (Table 1). The XylO structure is composed of two distinct domains (Figure 4). The FAD binding domain (FAD domain) comprises residues 25 – 231, and 447 – 497, while the substrate binding domain (S domain) comprises residues 232 – 446. The FAD domain contains two subdomains, both of which have an α/β-fold. The first subdomain (res. 8 – 113) is comprised of three parallel beta strands (β1 – β3), flanked on one side by two α-helices (α1 – α2) and on the other side by one α -helix (α3). The second, larger subdomain (res. 114 – 231 and 447 – 497) contains five antiparallel β-strands (β4 – β9), surrounded by six α-helices (α4 – α7) and (α13 – α14). The FAD binding pocket is embedded between the two subdomains. The S domain contains a seven stranded anti-parallel β-sheet (β10 – β16) flanked on the outside of the protein by 4 α-helices (α8 – α9 and α11 – α12) and on the inside by one α-helix (α10). N-linked GlcNAc

moieties could be modeled in electron density extending from Asn233, Asn245 and Asn289. Similarly, at Asn117 and Asn192 two GlcNAc residues were visible. Between the first and third α-helix in the first subdomain a conserved disulfide bridge (Cys30-Cys79) is present, which probably stabilizes the N-terminal helix.

The XylO structure is very similar to the structures of other structurally characterized VAO family members. The most similar structures are that of GluO from *Acremonium strictum* (28) (44 % sequence identity, PDB code 2AXR) and LaO from *Microdochium nivale* (48 % sequence identity, PDB code 3RJ8). The rmsd of GluO with XylO is 1.1 Å on 445 C α-atoms while the rmsd of LaO with XylO is 1.0 Å on 463 C α-atoms. Structural similarity to a lesser extent was observed with a glucose dehydrogenase from *Phleum pretense* (29) (PDB code 4PVE), the Bermuda grass isoallergen Cyn d 4 (PDB code 4DNS) (30), EncM from *Streptomyces maritimus* (31) (PDB code 3W8W) and a berberine bridge enzyme (ATBBE15) from *Arabidopsis thaliana* (32) (PDB code 4UD8). These proteins have 24-27% sequence identity when compared with XylO and superimpose with a rmsd of ~1.9 Å. Among these structures the FAD domains are very well conserved, the S domains are less well conserved, reflecting different substrate specificities.

Table 1. Data collection and refinement statistics. Values in parentheses are for the highest resolution shell.

Diffraction data	Native	Xylose	Xylobiose
Wavelength (Å)	1.54	1.54	1.54
Resolution range (Å)	53.6 – 1.93	53.5 – 1.79	53.4 – 1.80
Spacegroup	*C2*	*C2*	*C2*
Cell dimensions (Å) *a*, *b*, *c*, *β*	124.0, 59.4, 68.7, 90.6	123.3, 59.4, 68.5, 91.0	123.6, 59.2, 68.5, 90.8
Unique reflections	37594 (2327)	46206 (2069)	45931 (2430)
Wilson *B* factor (Å^2)	7.1	12.8	11.3
Completeness (%)	99.4 (91.4)	98.3 (78.6)	95.6 (70.1)
Overall I/σ (I)	11.8 (2.4)	11.0 (2.6)	11.4 (3.3)
R_{merge} (%)	16.3 (67.3)	8.3 (40.7)	7.8 (27.4)
R_{pim} (%)	7.1 (32.9)	4.9 (26.8)	4.8 (20.8)
***R*/ *Rfree* (%)**	15.9 / 19.5	17.3 / 20.7	15.2 / 18.4
R.m.s. deviations from ideal values			
Bond lengths (Å)	0.007	0.009	0.008
Bond angles (°)	1.30	1.39	1.45
Protein residues	473	473	473
FAD molecules	1	1	1
Ligand molecules		4 xylose	4 xylobiose
Water molecules	383	405	400
PDB accession ID	5K8E	5L6F	5L6G

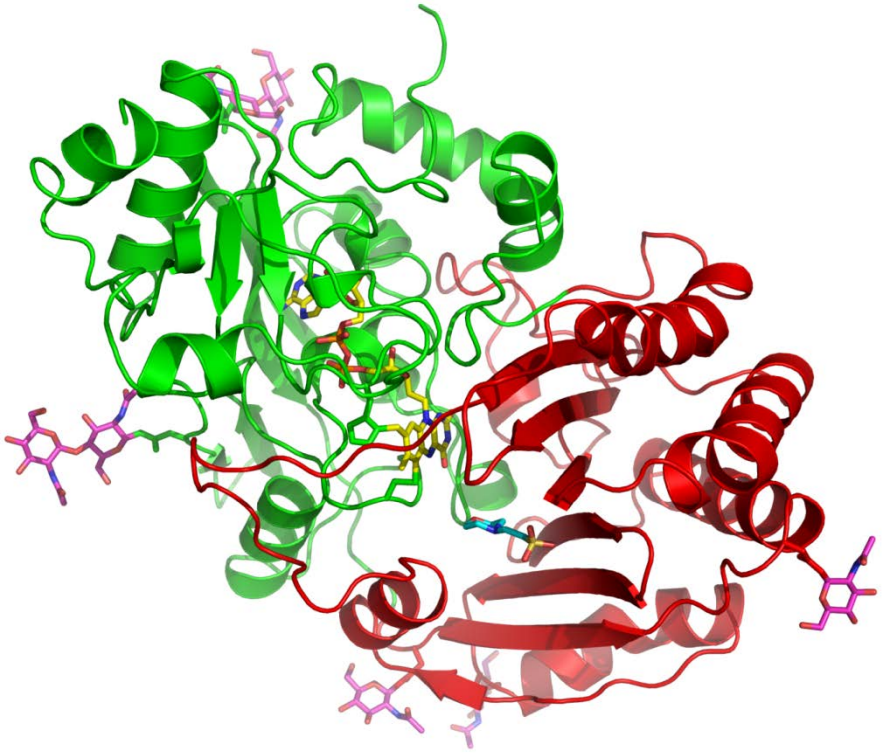


Figure 4: Crystal structure of XylO. A: overall structure with FAD domain in green and the substrate domain in red. The FAD flavin cofactor is in yellow sticks while the MES molecule is in cyan sticks. The N-linked N-acetylglucosamine moieties are also highlighted in stick representation (magenta). B: MES bound in the -2 substrate binding pocket. C: Xylose bound in the -1 substrate binding pocket. D: Xylobiose bound in the -1 and -2 substrate binding pockets. The Fo - Fc electron density omit maps of the MES, xylose and xylobiose molecules are contoured at 2σ are shown as gray mesh and were calculated with the ligands omitted.

XylO contains a bicovalently linked FAD, 6-S-cysteinyl, 8α-N1-histidyl FAD. Such a covalently tethered FAD cofactor was first observed in GluO (7). The isoalloxazine ring of the cofactor is covalently linked with the C6 atom to the Sγ of Cys155 and with the 8 α-methyl group to the Nδ1 of His94 (Figure S5). The access to the open active site is in a cleft in the S domain made up by the seven-stranded β-sheet and an α-helix-loop structure (res. 315 – 341). XylO has a similar open carbohydrate-binding groove as present in GluO and LaO, allowing mono-, di- and oligosaccharides to bind with the

non-reducing end of the sugar residue exposed to the solvent. Interestingly, at the entrance to the active site the morphilino ring of a MES molecule is bound between Tyr325 and Tyr376, acting as a clamp and mimicking a monoosaccharide binding at the -2 substrate site (Figure S5). Tyr325 and Trp376 stack on the morpholino ring with an interplanar distance of ~4.0 Å. For obtaining a structure with a substrate bound, crystals were soaked with xylose and xylobiose. In the two substrate complex structures, xylose is bound in the -1 subsite, next to the flavin cofactor, while xylobiose occupies the -1 and -2 subsites (Figure S6 and 4D). The observation that substrate-soaked XylO crystals lost their yellow appearance suggests that the complexed structures reflect the complex of flavin-reduced XylO with bound xylose or xylobiose. The xylose C1 atom, the site of oxidation, is bound in front of the flavin N5 with a distance of 3.4 Å and an angle with the N5–N10 atoms of 108°. Such geometry of substrate binding is common among flavoprotein oxidases that catalyze alcohol oxidations by a hydride transfer from the substrate to the N5 of the flavin cofactor (33). The xylose O1 atom interacts with Tyr451-OH (2.5 Å), Glu412-OE1 (2.9 Å) and a water molecule. The endocyclic-O makes close contacts with the carboxylate group of Glu412 (2.3 / 3.1 Å). The equatorial OH2 group forms hydrogen bonds with Thr154-OG1 (2.7 Å), Arg272-NH1 (2.8 Å), the isoalloxazine O4 (3.4 Å) and a water molecule, the equatorial OH3 and OH4, both with 2 water molecules. The xylobiose C1 is positioned slightly closer to flavin N5 (at 3.2 Å) and has a slightly larger angle of 112° when compared with the xylose-bound structure. Interactions of OH1, O5, OH2 and OH3 with the protein are similar as in the xylose bound structure. The xylose moiety in the -2 subsite has π-π stacking interaction, similar to the morpholino ring of the MES molecule, and a hydrogen bond to one water molecule. The xylobiose and MES binding induces no significant conformational change at the -2 subsite, except for the side chain of Tyr325. The phenyl ring rotates towards the bound sugar to optimize the stacking interactions. This rotation upon substrate binding is not observed in GluO and LaO, the latter having an Asn instead of Tyr325.

The active sites of XylO, GluO and LaO are very similar. Tyr96, Thr154, Arg272, Tyr325 (Asn in LaO), and Tyr451 are identical among the three enzymes. Some other residues clearly differ among the three enzymes: Leu 274 (Glu in GluO and Tyr in LaO), Tyr376 (Trp in GluO, Phe in LaO), Ile378 (Gln in GluO and LaO), and Glu412 (Gln in GluO and LaO). Tyr376-OH may prohibit binding of the C6-moiety of the aldohexoses in the active site as it is situated deeper in the -1 subsite compared to the Trp and Phe residues in Glu and LaO, respectively. Furthermore, two hydrophobic residues, Leu274 and Ile378 reside in the -2 subsite and thereby contribute to the selectivity towards xylooligosaccharides. At the same positions in GluO and LaO two polar residues, capable of accommodating the hydroxyl at the C6 position of the sugar moiety, are situated.

The substrate-soaked crystals also revealed auxiliary substrate binding sites at the XylO surface. Three additional binding sites for xylose are observed near Arg56-Phe426-Gly455, Trp182 and Asn369-Lys370. One additional binding site for xylobiose is detected near Trp259-Gly263 and the N-terminus-Asp28-Glu29-Arg41 from a symmetry related XylO molecule. The second binding site is near Arg56-Phe426-Gly455. Interestingly the xylobiose in the 3rd binding site near Glu104-Arg458-Glu484-Tyr487 has stacking interaction with the xylobiose in the 2nd binding site from a symmetry related molecule.

Discussion

The XylO from *M. thermophila* C1 is the first carbohydrate oxidase discovered so far to be specific for xylooligosaccharides. The elucidation of several XylO crystal structures has provided insight into the molecular basis for its highly specific substrate preference. While the overall structure and active site architecture of XylO resembles that of the so far characterized oligosaccharide oxidases, three residues in the substrate binding groove were found to be unique in XylO: Tyr376, Leu274 and Ile378. These residues seem to play a role in preventing aldohexoses to bind to XylO. Future

mutagenesis studies on one of the known oligosaccharide oxidases might be able to prove our hypothesis.

Hemicellulose constitutes the second most abundant polysaccharide after cellulose in plant biomass. Among all hemicellulose components, xylan is the most abundant one. Xylan is an heteropolysaccharide with a β-(1,4)-linked xylose-based backbone, often carrying other carbohydrate or aromatic moieties such as L-arabinose, D-galactose, feruloyl and glucuronic acid residues or acetyl moieties. By treating the xylan portion of hemicellulose chemically or enzymatically or by a combination of the two, xylooligosaccharides (XOS) can be generated. Depending on the source of the xylan, the generated XOS can vary in degree of polymerization and substitutions (34). XOS are currently used as valuable dietary fibers and prebiotics (35). Because of the configuration of their glycosidic bonds, XOS resist hydrolysis by salivary and intestinal digestive enzymes and arrive unmodified in the colon where they are fermented by anaerobic bacteria. XOS are also known to act as xylanase and cellulase inhibitors (36-38). For efficient degradation of plant biomass, efficient degradation or modification of XOS is desirable. As *M. thermophila* C1 is known to be equipped with an enzyme arsenal to degrade plant biomass, XylO may be part of a strategy to take out the inhibitory effects of XOS. The highly specific substrate acceptance profile of XylO towards XOS would provide the fungus a good biocatalytic tool to alleviate cellulase inhibition during biomass conversion. Except for a role in degrading or modifying hydrolysed xylan, the oxidase may also serve as hydrogen peroxide producing biocatalyst. Many fungi, including *M. thermophila* strains, secrete peroxidases which fully depend on the in-situ production of hydrogen peroxide for their activity. Therefore, being a secreted oxidase, XylO may utilize XOS to serve extracellular peroxidases. It is interesting to note that the role of XylO does not seem to be generic among fungi as only very few close homologs can be found in the genome sequence database. Clearly, future studies are needed to elucidate the exact role of XylO.

Through its rather narrow substrate range XylO may develop as a valuable biocatalyst. Given its exquisite specificity for XOS and very poor activity with cellobiose, XylO might be used in xylanase activity assays similar to the cellulose and chitinase assays that we previously described. By the combination of ChitO and HRP we developed a fast and sensitive assay to determine activity of chitinases and cellulases exploiting the specificity of ChitO for the products of hydrolysis of chitin and cellulose (39). Due to the low K_M values of XylO for XOS substrates, a sensitive xylanase assay involving XylO should be feasible. Furthermore XylO could be used to derivatize XOS. XylO introduces a carboxylic moiety to any xylooligosaccharide, resulting in the production of aldonic acids which may represent valuable compounds (40). Future studies will reveal the true potential of XylO as biocatalyst.

Experimental procedures

Bioinformatic analysis

The protein sequence of ChitO (accession number: XP_391174.1) was used to perform PHI-BLAST with the pattern G-x-C-x(6)-G-x(4)-GG on the genome of *M. thermophila* using the non-redundant genome database of NCBI. The used sequence pattern will limit the hits for protein sequences predicted to contain a bicovalent flavin cofactor (Kopacz & Fraaije, 2014).

Signal sequence identification was performed through the SignalP server (www.cbs.dtu.dk/services/SignalP/) (41).

Cloning and expression

The putative oligosaccharide oxidase encoding gene (GenBank accession number: KX139007) was amplified from genomic *M. thermophila* C1 DNA and cloned into a C1 expression vector. The expression cassette, consisting of the gene of interest under the control of the Pchi promoter and the cbh terminator, was obtained from the vector and used transform a low protease/(hemi-)cellulase

free *M. thermophila* C1 expression host in a co-transformation with the auxotrophic marker *pyr5* (42). Sixty randomly integrated transformants were grown in microtiter plates (43) and screened for XylO production levels in the culture broth by analyzing the flavin fluorescence upon SDS-PAGE (44). The transformant showing the highest levels of fluorescent polypeptide was grown in a fed-batch fermentation to produce XylO on a larger scale. The strain was grown aerobically in 2 L fermentors in mineral medium, containing glucose as carbon source, ammonium sulfate as nitrogen source and trace elements for the essential salts. After biomass formation, the enzyme was produced under glucose limiting conditions at pH 6.0 and 32 ˚C (45). The supernatant containing the enzyme was centrifuged and filtrated to remove cell biomass, concentrated 4 fold and dialyzed against 20 mM acetate pH 5.0.

Purification

The concentrated culture supernatant was exchanged with 20 mM Tris/HCl pH 7.6 and then loaded on HiTrap Q-sepharose column (5 mL) pre-equilibrated with the same buffer. The purification was performed on an ÄKTA system while monitoring absorbance values at 280 nm and 445 nm to detect flavoproteins. After applying the protein sample, the column was washed with buffer until the A_{280} reached the baseline. Subsequent elution was performed by using a gradient (0-2 M NaCl in 20 mM Tris/HCl pH 7.6) in 60 min at 3 mL/min. Fractions containing XylO were pooled and buffer exchanged to 50 mM acetate buffer pH 5.6. The sample was diluted 1:2 with a solution of 50 mM acetate buffer pH 5.6 containing 3.0 M ammonium sulfate and incubated at 4˚C while stirring for 40 min. Centrifugation at 4000x g for 30 min allowed the removal of precipitated proteins. After filtration of the supernatant through a 0.45 µM filter, it was applied to a HiTrap Phenyl-sepharose column (5 mL) pre-equilibrated with 50 mM acetate buffer pH 5.6 containing 1.5 M ammonium sulfate. The column was washed with the equilibration buffer until the absorbance at 280 nm reached the baseline. Elution was performed by using a gradient (1.5 – 0 M ammonium sulfate in 50 mM acetate buffer pH 5.6) in 60 min at 3

mL/min. Fractions that displayed absorbance at 445 nm (eluting at around 1.4 M ammonium sulfate) were pooled, concentrated, and loaded on a Superdex 200 column. For pre-equilibration and elution of the Superdex column a 50 mM acetate buffer pH 5.6 containing 100 mM NaCl was used. The fractions containing XylO activity were pooled and concentrated.

Crystallization, data collection, structure determination and refinement

Initial vapor-diffusion crystallization experiments were performed using a Mosquito crystallization robot (TTP Labtech). In a typical experiment, 0.1 µL screening solution was added to 0.1 µL protein solution (9.6 mg/mL) on a 96-well MRC2 plate (Molecular Dimensions); reservoir wells contained 50 µL screening solution. The screening solutions used for the experiments were PACT and JCSG+ (Molecular Dimensions). XylO crystals appeared after 3-4 days of incubation at 294 K in solutions containing PEG with pH 4.5 to 6.0. Crystallization conditions were optimized using hanging-drop set-ups with 20% PEG6000, 0.2 M ammonium chloride and 0.1 M MES pH 6.0 as precipitant, and drops containing 1 µL protein solution and 1 µL reservoir solution. Crystals grown from sodium acetate buffer at pH 5.5 showed better morphology but diffracted worse.

Before data collection, crystals were briefly soaked in a cryoprotectant solution, consisting of 20% glycerol, 20% PEG6000, 0.2 M ammonium chloride and 0.1 M MES pH 6.0. Ligand complexes were obtained by soaking in 2 M xylose or xylobiose, replacing glycerol as cryoprotectant, for 1 minute. X-ray diffraction data were collected on an in-house MarDTB Goniostat System using Cu-*Kα* radiation from a Bruker MicrostarH rotating-anode generator equipped with HeliosMX mirrors. Intensity data were processed using XDS(46). XylO crystals belong to space group *C*2 with one monomer of 54 kDa in the asymmetric unit. The V_M is 2.3 $Å^3$/Da (47) with a solvent content of 46%. Data collection statistics are listed in Table 1. The structure of the XylO was determined by the molecular replacement method using Phaser (48) with mixed model

coordinates of GluO (7) (PDB code:1ZR6) and LaO (PDB code: 3RJ8) as the search model.

ARP/wARP (49) was used for automatic building and the model was refined with REFMAC5 (50). Coot (51) was used for manual rebuilding and map inspection. In *2mFo-DFc* and *mFo-DFc* maps electron density was present for N-glycosylation and the ligands xylose and xylobiose. The quality of the models was analyzed with MolProbity (52). Five Translation/Libration/Screw groups were used in the last rounds of refinement. Atomic coordinates and experimental structure factor amplitudes have been deposited in the Protein Data Bank (PDB) (entries 5K8E for the holoprotein structure, 5L6F for the xylose bound structure and 5L6G for the xylobiose bound structure).

Analysis of kinetic and stability properties

All experiments were performed in 50 mM potassium phosphate buffer pH 6.0. The absorbance spectrum of XylO was recorded before and after addition of 0.1% (w/v) SDS. The extinction coefficient of XylO was determined using the extinction coefficient of 6-S-cysteinyl bound FMN (ε_{445} = 11.6 mM^{-1} cm^{-1}): 11.5 mM^{-1} cm^{-1} (53). Oxidase activity of XylO was detected by coupling H_2O_2 production to the oxidation of 4-aminoantipyrine and 3,5-dichloro-2-hydroxybenzenesulfonic acid by horseradish peroxidase (HRP). The formation of the resulting pink/purple colored product can be followed at 515 nm (ε_{515} = 28 mM^{-1} cm^{-1}). The reaction mixture contained 50 mM potassium-phosphate buffer, pH 6.0, 0.1 mM 4-aminoantipyrine, 1.0 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid, 4.0 units HRP and 30 nM of XylO. Substrate screening was performed using 23 different carbohydrates comprising mono-, di-, tetra-, poly- and cyclooligosaccharides. Potential substrates were tested in duplicate using concentrations of 50 mM and 5.0 mM for mono- and disaccharides, and 1.0 mM and 0.5 mM for tetra- and cyclo-oligosaccharides.

Enzyme kinetic parameters were determined at 25˚C for several carbohydrates using different ranges of substrate concentrations: glucose (30 mM – 1.5 M), cellobiose (7.0 mM – 262 mM), lactose (14

mM – 525 mM), xylose (10 mM – 560 mM), xylobiose (0.05 mM – 1.0 mM), xylotriose (0.03 mM – 2.0 mM), xylotetraose (0.025 mM – 1.0 mM). For each substrate twelve different substrate concentrations were measured at least in triplicate. Initial rates were obtained by measuring product formation for one minute every two seconds. The temperature optimum for activity was measured in duplicate in a temperature range from 20 to 60˚ C with 25 nM purified XylO and 5.0 mM xylobiose. Reactions were performed as described above in duplicate with the only difference that 127 U/mL of HRP were used so that HRP would not be the limiting factor in the assay at high temperatures. Relative activities to the highest k_{obs} were plotted against temperature.

The pH optimum was determined using the universal buffer solution designed by Britton and Robinson (54) from pH 4.1 to 8.3. k_{obs} values were measured by monitoring the initial dioxygen consumption rates with 400 nM purified XylO and 5.0 mM of xylobiose in duplicate for each pH value. Dioxygen concentrations were monitored with REDFLASH sensor spots and a Firesting O_2 detector and light source (Pyroscience, Aachen, Germany). For measuring dioxygen concentration dependent kinetic data, the depletion of molecular oxygen was monitored at pH 7.0 in Britton and Robinson buffer using 400 nM purified XylO, 5.0 mM xylobiose and 0.24 mM dioxygen as starting concentrations. The dioxygen depletion kinetic data were analyzed using the Michaelis Menten formula (55).

The thermostability of XylO was assessed with the Thermofluor assay using SYPRO orange as dye (24). The method is based on the fluorescence increase of the dye SYPRO orange upon binding to the hydrophobic interior of proteins which becomes exposed as the protein unfolds. The fluorescence increase was monitored by recording the emission at 575 nm while exciting at 490 nm and increasing the temperature by 1 ˚C/min. 20 µL of purified XylO (1 mg/mL) were tested in triplicate in 50 mM acetate buffer pH 5.6. The apparent melting temperature was determined by plotting the first derivative of the observed fluorescence versus temperature (56).

Oxidation of complex substrates

Wheat flour, rye whole grain flour, oat flour, and barley flakes were purchased at the local supermarket. Xylan from beachwood was obtained from TCI Chemicals while wheat arabinoxylan was from MEGAZYMES. Hydrolyzed wheat arabinoxylan was prepared by incubating 50 mg/mL wheat arabinoxylan in 50 mM MES pH 5.6 with 8.5 U of β-xylanase M1 from *Trichoderma viride* (MEGAZYMES) at 50 ˚C upon overnight shaking at 700 rpm. Subsequently the samples were spun down and 20 µL of the supernatant was used for the HRP-based assay.

Solutions of 4% (w/v) in 50 mM acetate buffer pH 5.6 of the above mentioned flours, xylan and wheat arabinoxylan were incubated at room temperature while shaking for 48 h. Samples were centrifuged for 45 min at 3000x g and the insoluble part was washed three times in acetate buffer. Subsequently, the sample was resuspended in a volume to achieve 20% (w/v). Samples at 0.1% and 1% of both the soluble and insoluble fractions were tested using the above described HRP assay using 30 nM of XylO. Also, 1% of insoluble substrates were incubated overnight at 37˚C while shaking at 2000 rpm with 1.0 µM of XylO. The samples were then spun down and the supernatant tested with the HRP assay to detect formation of hydrogen peroxide.

Oxygen measurements

The affinity for oxygen was determined using 400 nM of XylO and 5 mM xylobiose in an air tight cuvette. The oxygen concentration was measured during catalysis with REDFLASH sensor spots and a Firestring O_2 detector and light source (Pyroscience, Aachen, Germany). The slope of the oxygen decrease in time was plotted against the concentration of oxygen. The curve was fitted using a hyperbolic function: $v = k_{cat} \cdot [O_2] / K_{ox} + [O_2]$.

NMR and GC-MS analysis

Conversion of 10 mM xylobiose was performed for 210 min at 50 ˚C in 50 mM potassium phosphate buffer pH 6.0 using 1.0 µM XylO. NMR samples were exchanged twice with D_2O (99.9 atom %, Cambridge Isotope Laboratories, Inc., Andover, MA) with intermediate lyophilisation and finally dissolved in 0.6 mL D_2O. 1D/2D 500-MHz 1H NMR spectra and 125-MHz 13C NMR spectra were recorded in D_2O, containing acetone as internal standard (δ1H 2.225, δ13C 31.07). 1D and 2D NMR spectra were recorded on a Varian Inova 500 spectrometer (NMR Department, University of Groningen, The Netherlands) at a probe temperature of 25 ˚C. Suppression of the HOD signal was achieved by applying a Wet1D pulse sequence. 2D COSY data were collected in 200 increments of 4000 complex data points. Natural abundance 2D 13C–1H HSQC and HMBC experiments were recorded without decoupling during acquisition of the 1H FID. For GC-MS analysis, the product was analyzed intact, as well as after methanolysis (1.0 N methanolic HCl, 24 h, 85 ˚C) as a trimethylsilyl (TMS) ethers derivative (1:1:5 hexamethyldisilazane, trimethylchlorosilane, pyridine; 30 min, RT) by GC-EI-MS on a QP2010 Plus instrument (Shimadzu, 's-Hertogenbosch, The Netherlands), using a ZB-1HT column (Phenomenex B.V., Utrecht, The Netherlands). Separation was achieved using a temperature gradient: 140-240 ˚C.

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Author contributions: ARF performed the bioinformatics analysis that led to the identification of XylO. ARF performed designed and performed of the experimental work with help in the interpretation of the data by MWF. ARF wrote the first draft of the manuscript. MWF wrote the final version. HJR crystallized and resolved the 3D structure of XylO and wrote the corresponding part in this manuscript. JMD performed the experimental part to identify the product of XylO. JMD and SSvL analyzed the NMR and GC/MS results of the conversion and wrote the corresponding part in the manuscript. ASCV performed the experimental work concerning the cloning and expression of XylO. MJK wrote the cloning and expression section in this manuscript. MJK, JV and MWF contributed with concept and design of the studies and with interpretation of the data.

Abbreviations: xylooligosaccharide oxidase (XylO), vanillyl alcohol oxidase (VAO), glucooligosaccharide (GluO), lactose oxidase (LaO), chitooligosaccharide oxidase (ChitO), xylooligosaccharides (XOS), horseradish peroxidase (HRP), trimethylsilyl (TMS).

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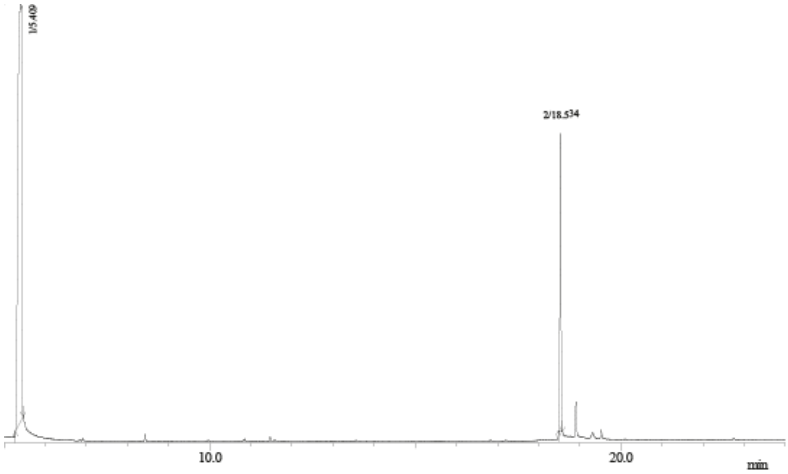
properties of a highly engineered halohydrin dehalogenase. *Chembiochem*. 14, 870–81

Supplementary information

Substrates used for screening: glucose, galactose, fructose, mannose, xylose, l-arabinose, N-acetyl-d-glucosamine, sucrose, maltose, lactose, sorbitol, xylitol, raffinose, cellobiose, cellotetraose, α-cyclodextrin, β-cyclodextrin, arabitol, chitosan, chitin, starch, maltotetraose, glycerol.

Figure S1. GC-MS chromatogram of TMS-derivative of xylobionate product (top) with MS-fragmentation spectrum of peak @ 18.534 min (bottom).



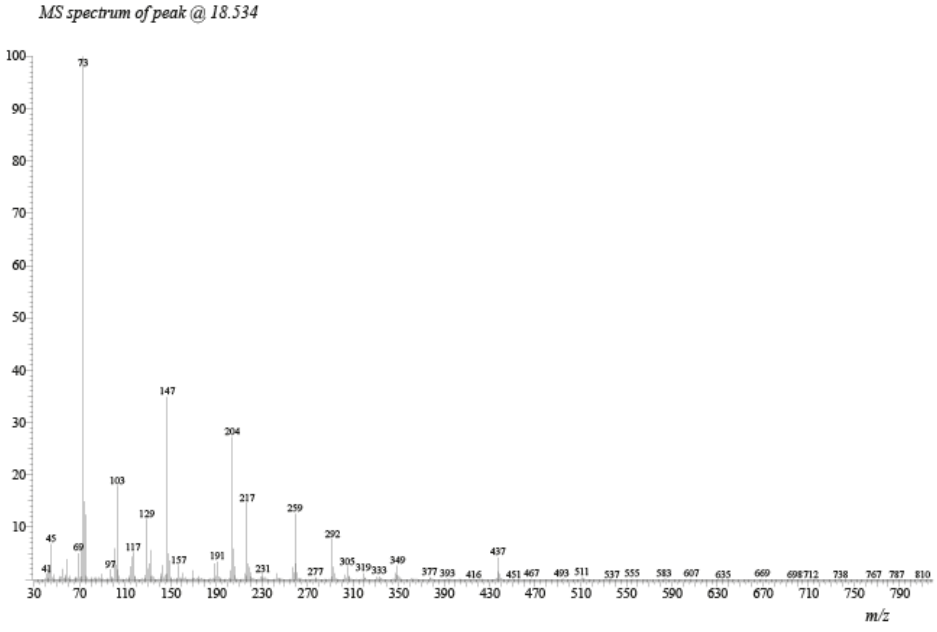
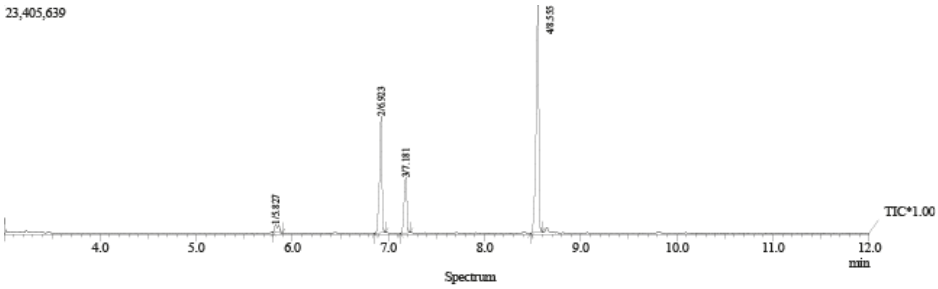


Figure S2. GC-MS chromatogram of TMS-derivatives of methyl-glycosides obtained by methanolysis of the xylobionate product (top) with MS-fragmentation spectrum of peaks @ 5.827 min, 6.923 min, 7.181 min and 8.555 min (bottom).



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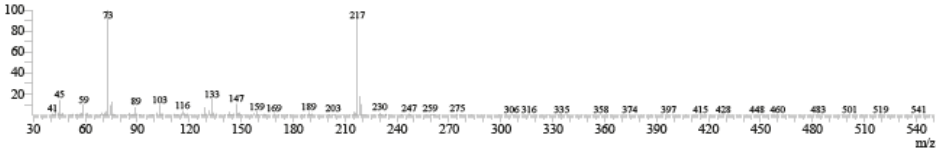

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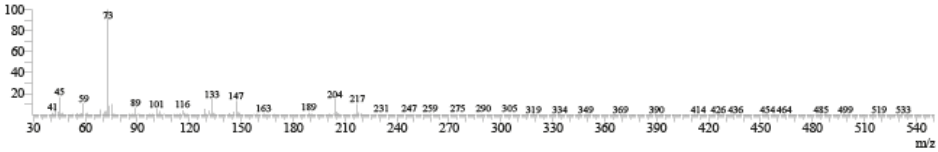

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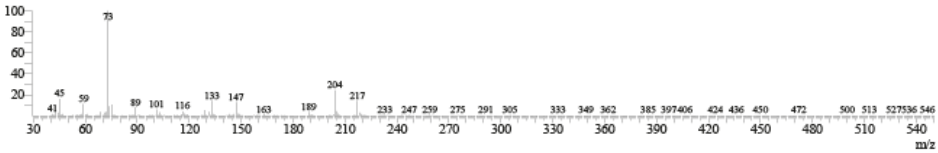

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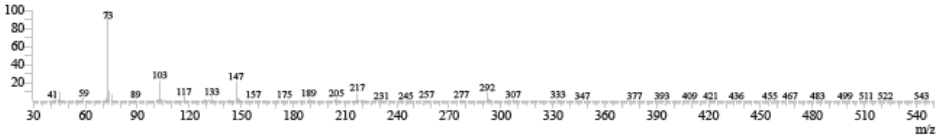


Figure S3. XylO pH optimum for activity.

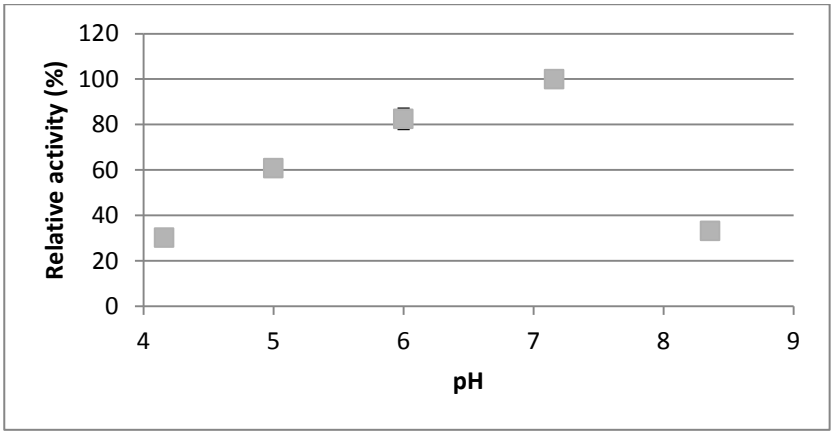


Figure S4. XylO temperature optimum for activity.

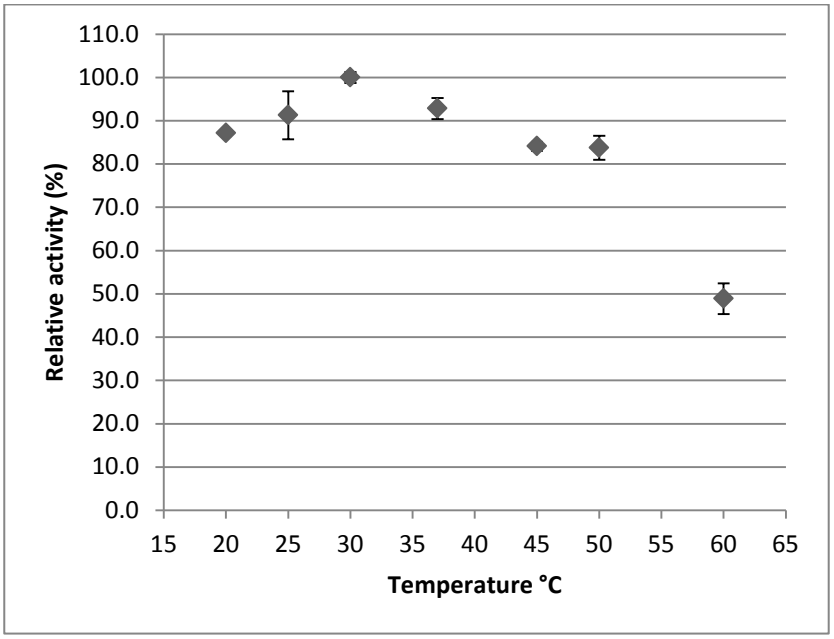


Figure S5. XylO active site with MES bound.

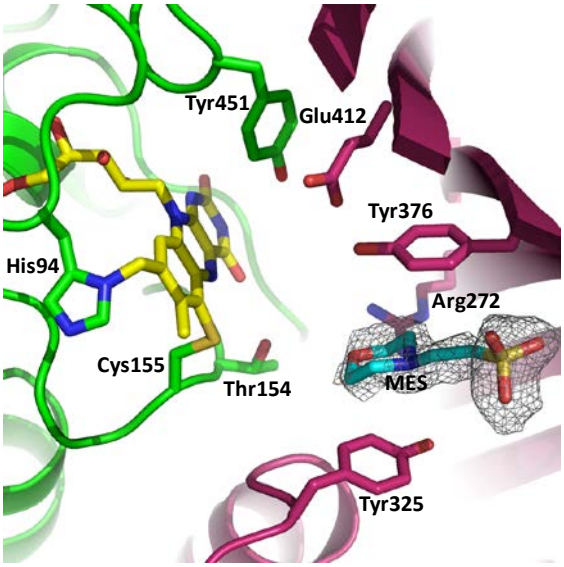


Figure S6. XylO active site with xylose bound.

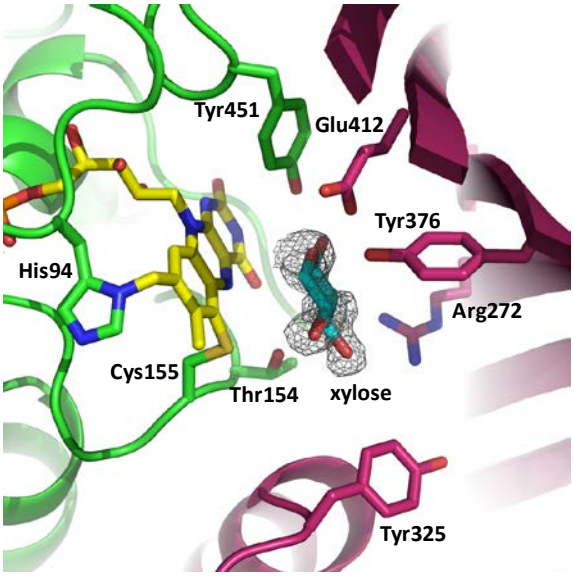
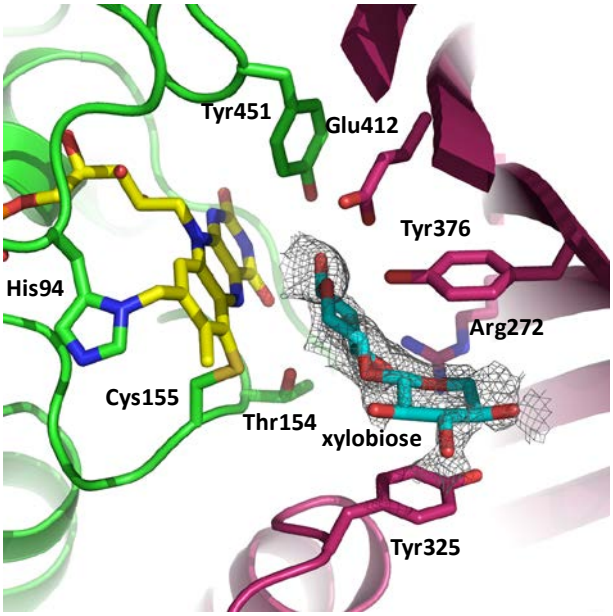


Figure S7. XylO active site with xylobiose bound.



Chapter 5

Characterization of two VAO-type flavoprotein oxidases from *Myceliophthora thermophila*

Alessandro R. Ferrari, Henriëtte J. Rozeboom, Aniek S.C. Vugts, Martijn J. Koetsier, Robert Floor, and Marco W. Fraaije

Abstract

The VAO flavoprotein family consists mostly of oxidoreductases harboring a covalently linked flavin cofactor. The linkage can be either monocovalent at position 8 with a histidine or tyrosine or bicovalent at position 8 with a histidine and at position 6 with a cysteine. Bicovalently bound flavoproteins show a preference for bulkier substrates such as oligosaccharides or secondary metabolites. The genome of the thermophilic fungus *Myceliophthora thermophila* C1 was found to be rich in genes encoding putative covalent VAO-type flavoproteins. Enzymes from this fungus have the advantage of being rather thermostable and homologous overexpression in *M. thermophila* C1 is feasible. Recently we discovered a new and VAO-type carbohydrate oxidase from this fungus: xylooligosaccharide oxidase. In this study, two other putative VAO-type oxidases, protein sequence XP_003663615 (MtVAO615) and XP_003665713 (MtVAO713), were expressed in *M. thermophila* C1, purified and characterized. Enzyme MtVAO615 was found to contain a bicovalently bound FAD, while enzyme MtVAO713 contained a monocovalent histidyl-bound FAD. The crystal structures of both proteins were obtained which revealed atypical active site architectures. It could be experimentally verified that both proteins, when reduced, rapidly react with molecular oxygen, a hallmark of flavoprotein oxidases. A large panel of alcohols, including carbohydrates, steroids and secondary alcohols were tested as potential substrates. Only for enzyme MtVAO713 low oxidase activity was discovered towards ricinoleic acid.

Introduction

Oxidases are enzymes capable of performing selective oxidative reactions using molecular oxygen as electron acceptor. They represent valuable and cost-effective biotechnological tools for industrial applications since they do not require expensive coenzymes but just molecular oxygen. To catalyze oxidations, oxidases typically employ a copper ion or a flavin as cofactor. Known examples of the former are galactose oxidase and laccases. The flavin-containing group of oxidases is more abundant. Oxidases in this category contain mostly a flavin adenine dinucleotide (FAD) as prosthetic group with a minority containing flavin mononucleotide (FMN). Based on sequence homology and the available structural information, six families of flavoprotein oxidases have been identified [1].

The FAD/FMN cofactor can be bound to the protein either non-covalently or covalently. The elucidation of the crystal structure of vanillyl alcohol oxidase (VAO) showed for the first time the FAD cofactor covalently bound to a protein via a histidine residue [2]. Members of the VAO flavoprotein family share a common overall structure which is composed of two domains: a conserved FAD binding domain that binds the adenine part of the FAD cofactor and a variable cap domain that covers the isoalloxazine ring of the cofactor and forms the substrate binding pocket [3]. In 2005 the crystal structure of another member of the VAO-type family, glucooligosaccharide oxidase (GOOX), was elucidated. This revealed for the first time a flavin cofactor covalently linked to two amino acids [4]. By having the FAD cofactor anchored by two covalent bonds, these bicovalent flavoproteins proteins seem to have evolved a rather open active site which allows them to accept bulky molecules as substrates [5]. It has also been shown that covalent tethering of the FAD to the protein increases the flavin redox potential, with bicovalent flavoproteins having the highest redox potentials [5]. With such high redox potentials, molecular oxygen is one of the few natural electron acceptors that covalent

flavoproteins can use. This is probably the reason why most covalent VAO-type flavoproteins are oxidases [1].

Among the VAO-type oxidases, a great variety of substrates are accepted [6]. Some oxidases are part of gene clusters involved in tailoring secondary metabolites. Therefore they accept relatively complex biomolecules as substrates. Examples of these are Dbv29 that acts on the precursor of the glycopeptide A40926 [7], aclacinomycin oxidase which catalyzes the oxidation of a C-O bond and the dehydrogenation of the sugar moiety of aclacinomycin precursors [8], and the tirandamycin oxidase that catalyzes the oxidation of a secondary alcohol moiety of a tirandamycin intermediate [9]. Also the recently discovered subfamily of VAO-type carbohydrate oxidases, such as xylooligosaccharide oxidase (XylO), chitooligosaccharide oxidase (ChitO) [10] or GOOX [11], act preferably on bulky oligosaccharides while monosaccharides are poorly recognized. The majority of VAO-type oxidases perform alcohol oxidations (EC 1.1.3.x) resulting in the production of aldehydes, ketones or lactones. Nonetheless, VAO, the prototype enzyme of this flavoprotein family, is not only able to perform alcohol oxidations but it is also capable to perform amine oxidations, hydroxylations and ether bond cleavage reactions [12]. Beside the aforementioned reactions, the VAO family includes also oxidases capable of C-C bound formation [13]. One known example is reticuline oxidase (also known as berberine bridge enzyme) involved in the biosynthetic pathway of plant isoquinoline alkaloids [14].

In an effort to discover novel oxidative biocatalysts acting towards biomass or biomass derived compounds, we specifically investigated the genome of the thermophilic fungus *M. thermophila*. This fungus is known to convert plant biomass and is a producer of a plethora of biomass converting enzymes. In addition, an effective *M. thermophila* expression system has been developed, enabling the production of high levels of recombinant proteins through homologous (and heterologous) expression. As *M. thermophila* is a thermophilic fungus, its proteins would withstand higher temperatures, making them optimal choices for industrial purposes.

We recently reported on the discovery and the elucidated crystal structure of a new VAO-type oxidase: xylooligosaccharide oxidase (XylO) from *M. thermophila* XylO has a similar active site when compared to previously determined structures of other known VAO-type carbohydrate oxidases such as glucooligosaccharide oxidase from *Acremonium strictum* (GOOX) and lactose oxidase from *Microdochium nivale* (LaO). Yet, for each of these enzymes, delicate differences in active site residues result in different substrate acceptance profiles. During the work that led to the discovery of XylO, other putative VAO-type oxidases were identified in the predicted proteome of *M. thermophila.* In this study we report on the characterization of two of these VAO-type flavoproteins.

Results and Discussion

Identification of *M. thermophila* genes encoding putative VAO-type flavoprotein oxidases

Using the sequence of the bicovalent flavoprotein ChitO as query, a BLAST search in the predicted proteome of *M. thermophila* was performed. Nineteen putative VAO-type flavoproteins were identified and subsequently aligned together with characterized VAO-type flavoproteins. Upon multiple sequence alignment of the recovered protein sequences with sequences from VAO-type flavoproteins for which a crystal structure was elucidated, a phylogenetic tree was built

Given their high similarity with MtVAO713, two additional sequences were included in the tree: an alcohol oxidase from *Hypomyces subiculosus* (accession number: ACD39759) and ecdysteroid-22-oxidase from *Nomuraea rileyi* (accession number: BAM11133). Thirteen clades can be identified in this tree. Eight clades mainly consist of known flavoproteins (clades I, III, IV, V, VI, VIII, XI, and XII). Clade V consists of the characterized carbohydrate oxidases, which includes XylO, while also another homolog (XP_003661939.1) of *M. thermophila* is part of this group. This suggests that *M. thermophila*

contains another carbohydrate oxidase. Other VAO-type proteins from *M. thermophila* are found in clades I, II, V, VII, IX, X, and XIII.

Through SignalP (http://www.cbs.dtu.dk/services/SignalP/), eleven proteins of *M. thermophila* were predicted to contain a signal sequence: XylO, XP_003661939, XP_003663615, XP_003663619, XP_003662540, XP_003661824, XP_003663565, XP_003660778, XP_003665713, XP_003660023, XP_003659997.

Interestingly the majority of these protein are predicted to be bicovalently bound while only four (XP_003662540, XP_003665713, XP_003660023, XP_003659997) are predicted to be monocovalently bound.

We selected two flavoproteins for further studies: XP_003663615 (MtVAO615), a putative bicovalent flavoprotein, and XP_003665713 (MtVAO713), a putative monocovalent flavoprotein. Interestingly, MtVAO713 has a Ser at the position where normally a Cys is present in bicovalent flavoproteins, forming the Cys-FAD linkage.

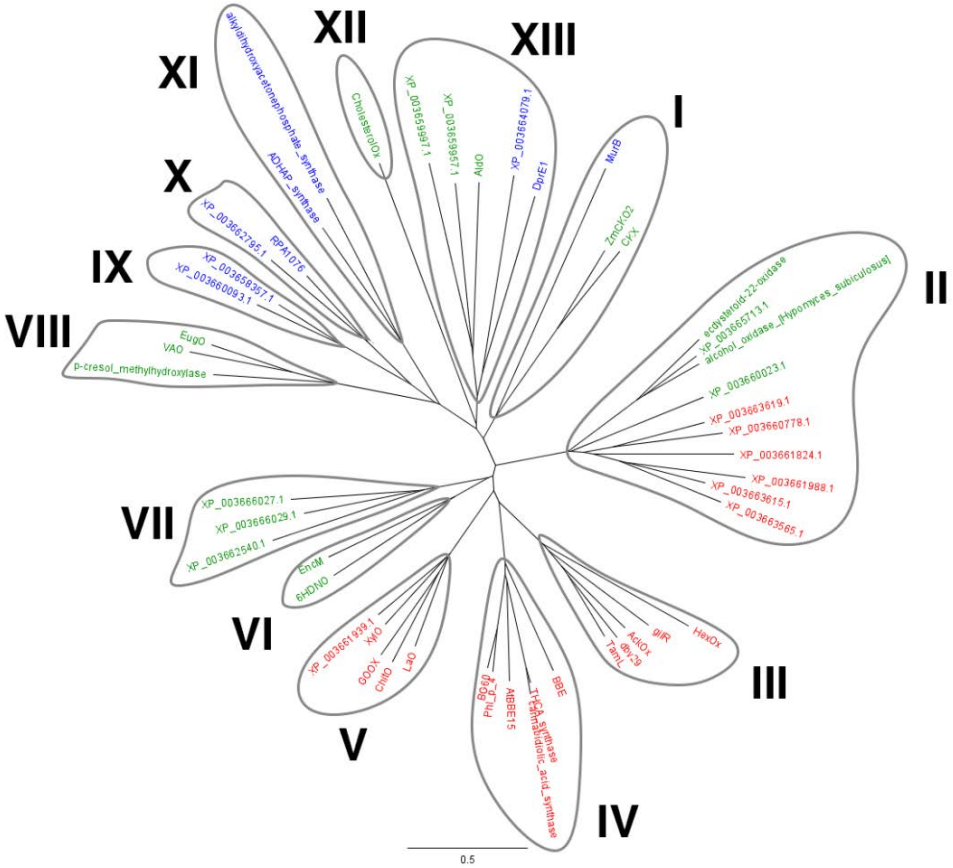


Figure 1: Phylogenetic tree of the VAO-type flavoproteins in the genome of M. thermophila supplemented with sequences of VAO-type proteins for which structures have been determined. Some covalent flavoproteins for which a structure is not available are also included in the tree: alcohol oxidase from Hypomyces subiculosus (ACD39759), ecdysteroid-22-oxidase from Nomuraea rileyi (BAM11133), and ChitO: chitooligosaccharide oxidase from Fusarium graminearum (XP_011325372). HexOx: hexose oxidase from Chondrus crispus (AAB49376), GilR: oxidoreductase from Streptomyces griseoflavus (Q7X2G7), AckOx: aclacinomycin oxidoreductase from Streptomyces galilaeus (ABI15166), dbv29: oxidase from Nonomuraea sp. ATCC 39727 (CAD91224), TamL: oxidase from Streptomyces sp. 307-9 (ADC79636), BBE: reticuline oxidase from Eschscholzia californica (P30986), THCA_synthase: Δ1-tetrahydrocannabinolic acid synthase from Cannabis sativa (Q8GTB6), cannabidiolic_acid_synthase: cannabidiolic acid synthase from Cannabis sativa (AKC34419), Phl_p_4: major pollen allergen Phl p 4 from Phleum pretense (P43213), BG60: oxidoreductase BG60 from Cynodon dactylon (AAS02108), LaO: carbohydrate oxidase from Microdochium nivale (3RJA), GOOX: glucooligosaccharide oxidase from Acremonium strictum (2AXR), , XylO: xylooligosaccharide oxidase from M. thermophila (XP_003663758), MurB: MurB from Pseudomonas aeruginosa (4JAY), PCMH: p-cresol

methylhydroxylase from Pseudomonas putida (P09788), VAO: vanillyl alcohol oxidase from Penicillium simplicissimum (P56216), EugO: eugenol oxidase from Rhodococcus jostii (5FXD), AtBBE15: AtBBE15 from Arabidopsis thaliana (4UD8), HDNO: 6-hydroxy-D-nicotine oxidase from Arthrobacter oxydans (P08159), EncM: EncM from Streptomyces maritimus (Q9KHK2), CholesterolOx: cholesterol oxidase from Brevibacterium sterolicum (1I19), AldO: alditol oxidase from Streptomyces coelicolor (2VFR), DprE1: decaprenylphosphoryl-beta-D-ribose oxidase from Mycobacterium tuberculosis (4FDN), ADHAP_synthase: mammalian alkyldihydroxyacetonephosphate synthase from Cavia porcellus (4BBY), alkyldihydroxyacetonephosphate_synthase: alkyldihydroxyacetonephosphate synthase from Dictyostelium discoideum (2UUU), RPA1076: a putative dehydrogenase from Rhodopseudomonas palustris CGA009 (3PM9), ZmCKO2: cytokinin oxidase/dehydrogenase 2 from Zea mays (4ML8), CKX: cytokinin oxidase/dehydrogenase from Arabidopsis thaliana AT5G21482 (2EXR). The phylogenetic tree was generated with Geneious, version 7.0 (http://www.geneious.com). In red: bicovalent flavoproteins. In green: monocovalent flavoproteins. In blue: non covalent flavoproteins.

Purification

MtVAO615

From 50 mL of concentrated fermentation supernatant, around 20 mg of MtVAO615 was obtained after two purification steps. The purified protein displays a bright yellow color and exhibits and absorbance spectrum with maxima at 349 and 446 nm (*Figure 2*A). The spectral features (the 349 nm peak is rather broad and the 446 nm peak has a pronounced should at around 480nm) are reminiscent of those of bicovalent flavoproteins. The purified protein runs in SDS-PAGE as a single band at around 70 kDa while the expected size is predicted to be 60 kDa. This indicates the presence of post-translational modifications such as glycosylation. Upon incubation of the gel with 5% acetic acid, the protein displayed clear fluorescence, confirming the presence of covalently bound FAD.

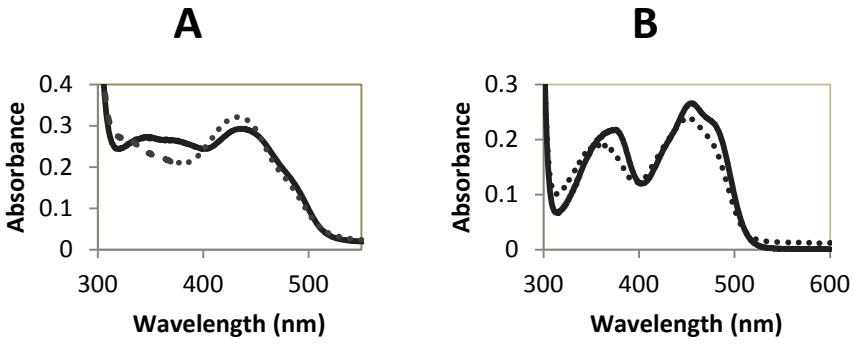


Figure 2: A) Spectrum of 26 µM native MtVAO615 (solid line) and unfolded MtVAO615 upon addition of 0.1% SDS and heating the sample at 80°C for 5 min (dashed line), in 20 mM Tris/Cl pH 7.6. B) Spectrum of 20 µM native MtVAO713 (solid line) and unfolded MtVAO713 upon addition of 0.1% SDS and heating the sample at 80°C for 5 min (dashed line), in 20 mM Tris/Cl pH 7.6.

MtVAO713

From 50 mL of concentrated fermentation supernatant, around 45 mg of MtVAO713 was obtained after two purification steps. The purified protein showed a bright yellow color and its absorbance spectrum displays maxima at 372 and 454 nm (Figure 2B). The absorbance spectrum is remarkably different from the one of MtVAO615 and hints to a non- or monocovalently bound flavin cofactor.

On SDS-PAGE the purified fraction runs as a single band at around 60 kDa which is the expected size for this protein. This suggests that this protein is not or not significantly glycosylated. Similar to MtVAO615, the protein displayed clear fluorescence upon SDS-PAGE and acetic acid treatment, indicating the presence of covalently bound FAD.

Stopped flow experiment and redox potential determination

After having purified both proteins and having confirmed the presence of a covalent flavin cofactor, we set out to determine whether the proteins can function as oxidases. The typical characteristic of an oxidase is its capability to use molecular oxygen

as electron acceptor. To test if the two purified proteins are oxidases, we first reduced anaerobically their flavin cofactor with the reducing agent sodium dithionite. This could be witnessed by the disappearance of the yellow color of the oxidized flavin. After full reduction of the proteins, the proteins were mixed with a buffer containing dioxygen. By using a stopped-flow machine, the reappearance of oxidized flavin could be monitored in millisecond time scale.

When mixing the reduced proteins with dioxygen, in both cases a rapid reoxidation of the reduced flavin was observed. Kinetic analysis of the spectral scans revealed that the reoxidation occurs in two (MtVAO713) or three (MtVAO615) steps. In both cases reappearance of the fully oxidized flavin spectrum mainly occurs in the first fast process with rates of 14.2±0.1 s^{-1} and 75.0±0.1 s^{-1}, respectively. The subsequent kinetic events resulted in minor spectral changes which may reflect formation of flavin radical species or damaged flavin cofactor species, due to the intensity of the light source used for diode array detection. Therefore, they are probably irrelevant kinetic events. As a solution of dioxygen saturated buffer was used for reoxidation, the bimolecular rate constants could be calculated: 2.4 10^4 $M^{-1}s^{-1}$ for MtVAO713 and 1.3 10^5 $M^{-1}s^{-1}$ for MtVAO615. These rates are in the same range as those of known flavoprotein oxidases [15]. When taking into account that the rate of reoxidation is often even enhanced when product is still bound to the active site, these data show that both proteins are true flavoprotein oxidases.

The redox potential of the FAD in both enzymes was investigated by using the xanthine oxidase-based method [16]. Using this method, a full reduction of both enzymes could be observed with no significant formation of a radical species. We were able to determine a redox potential of +23 mV for MtVAO713 using methylene blue (+11 mV) as reference dye. Such a relatively high redox potential is typical for monocovalent flavoproteins. For MtVAO615 we were unable to calculate the redox potential since the redox potentials of the tested dyes were too far off from the one

of the enzyme. In the redox titrations MtVAO615 became nearly fully reduced before thionin acetate (+64 mV) was reduced while with 2,6-dichloro indophenol (+217 mV) it was the other way around. No other suitable reference dyes could be obtained. These observations indicate that the redox potential of MtVAO615 is extremely high for a flavoprotein, around 120-160 mV, which is close to the values reported for other bicovalent flavoproteins [5].

Substrate screening

Once the oxidase activity was confirmed for both flavoproteins, we set out to explore their substrate scope. First, a panel of twenty-three carbohydrates (see Supplementary Information) was tested using the HRP-based assay. This assay couples the production of hydrogen peroxide, which is formed upon substrate oxidation by an oxidase, with the HRP-catalyzed formation of a purple product. Unfortunately, MtVAO615 nor MtVAO713 showed any activity towards the carbohydrates tested.

As can be seen in the phylogenetic tree (Figure 1), MtVAO713 is relatively closely related to an alcohol oxidase from *Hypomyces subiculosus* (GeneBank accession number: ACD39759.1; 59% sequence identity) [17] and the ecdysteroid-22-oxidase from *Nomuraea rileyi* (GeneBank accession number: BAM11133.1; 52% sequence identity) [18]. Both fungal enzymes are acting on rather bulky secondary alcohols. Hinted by this, a plethora of steroids and secondary alcohols were tested by either the HRP assay or GC/MS in order to identify potential substrates. This revealed one substrate for MtVAO713: ricinoleic acid. Ricinoleic acid is the major component of the castor oil which is obtained from the pressing of the seeds of the plant *Ricinus communis*. It is an unsaturated omega-9 fatty acid hydroxylated in position 12. The oxidation in position 12 results in the formation of 12-ketooleic acid. Recently, a Korean group reported on the successful whole cell conversion of ricinoleic acid to 12-ketooleic acid by using a recombinant strain of *Corynebacterium glutamicum* expressing a secondary alcohol dehydrogenase from *Micrococcus luteus* [19]. The conversion of ricinoleic acid by

MtVAO713 was confirmed by GC/MS analysis. Formation of 12-ketooleic acid could be measured. Nevertheless, the conversion rate is very poor as only 0.5% of 5 mM ricinoleic acid was converted in 16 hours by 8 µM of MtVAO713. This indicates that ricinoleic acid is not the optimal substrate for this enzyme. Yet, it confirms that MtVAO713 can act as an alcohol oxidase and that it can convert secondary alcohols, a rare oxidase activity. Several ricinoleic acid derivatives were tested to unravel which functional groups are important for activity. None of the tested related compounds (methyl ricinoleate, ricinoleyl alcohol, 12-hydroxystearic acid, 12-hydroxydodecanoic acid) was found to be converted as measured by GC/MS.

For MtVAO615 a similar approach did not bring any lead. When examining the phylogenetic tree, no close homologs could be identified for which any biochemical data are available. A BLAST search results in several homologs with putative functions which have not been characterized yet. Several steroids, secondary alcohols and other different molecules were tested as potential substrate but no activity could so far be identified. Also, complex mixtures of substrates were tested as ground rye, oats, wheat, barley, wheat straw, arabinan and wheat arabinoxylan. These substrate would provide not only oligo- and polysaccharides but also other components of the plant material. MtVAO615 did not show activity with these complex mixtures.

Crystal structures

To have a better insight into the properties of both oxidases, their crystal structures were determined. The structures of MtVAO615 and MtVAO713 were both determined by molecular replacement. The final model of MtVAO713 comprises 4 protein molecules with amino acid residues 27 – 598 and 4 FAD molecules. Electron density maps show the presence of N-glycosylation at Asn^{103} and Asn^{131}. The final R-factors are 19.8/23.7 (Rcryst/Rfree). The crystal structure of MtVAO615 with amino acid residues 27-574 was solved in two different space groups. The structure at pH 7.5 contains one

monomer while the structure at pH 5.0 contains two monomers. Rcryst and Rfree are 23.2/28.3 and 22.0/28.1 respectively. As already observed by SDS-PAGE, MtVAO615 is heavily glycosylated, showing electron density in 2Fo-Fc maps for N-glycosylation at Asn^{47}, Asn^{105}, Asn^{129}, Asn^{211}, Asn^{310}, Asn^{346} and Asn^{438}.

Both proteins belong to the VAO flavoprotein family. VAO-type proteins are composed of a FAD-binding domain and substrate domain [20]. MtVAO615 and MtVAO713 have 29% sequence identity and have an rmsd of 2.0 Å on 515 Cα atoms. The structural differences are mainly situated in loops on the exterior of the proteins. Sequence alignment indicated that MtVAO615 and MtVAO713 contained an extra N-terminal domain of about 100 amino acid residues compared to other VAO flavoproteins. However, the extension is only ~10 residues. The remainder of the extra residues is found in 2 large loops in the FAD binding domain, several smaller loops and the C-terminal extension of ~10 amino acid residues (Fig S1). The first 26 residues of both proteins are part of secretion signal sequences.

The MtVAO615 and MtVAO713 structures are similar to other structurally characterized VAO family members. The most similar structures to MtVAO615 are that of a flavoenzyme from *Streptomyces maritimus* (EncM, PDB code 3w8w, 22% sequence identity rmsd 2.2Å) [21], 6-hydroxy-D-nicotine oxidase from *Arthrobacter nicotinovorans* (6HDNO, PDB code 2bvf, 20% sequence identity, rmsd 2.3Å [22]), aclacinomycin oxidoreductase from *Streptomyces galilaeus* (AckOx, PDB code 2ipi, 22% sequence identity rmsd 2.4Å [8]), tirandamycin oxidase from *Streptomyces* sp. 307-9 (TamL, PDB code 2y08, 22% sequence identity, rmsd 2.3Å [23]) and lactose oxidase from *Microdochium nivale* (LaO, PDB code 3rj8, 21% sequence identity, rmsd 2.1Å, to be published). For MtVAO713 these values are 21% , 2.6Å (EncM); 20%, 2.8Å (6HDNO); 15%, 2.6Å (AckOx); 17%, 2.4Å (TamL); and 20%, 2.4Å (LaO). Structural similarity to MtVAO713 was highest for glucooligosaccharide oxidase from *Acremonium strictum* (GOOX, PDB code 1ZR6 19% seq. id., rmsd 2.3 Å [4]) and an oxidoreductase from *Streptomyces griseoflavus* (GilR, PDB

code 3POP, 18% seq. id., rmsd 2.5Å [24]). The FAD domains of both structures are well conserved while conservation in the S domains is much less, accounting for diverse substrate specificities (Fig S1).

Four disulfide bridges are present in MtVAO615 while in MtVAO713 six disulfide bridges exist. In both MtVAO615 and MtVAO713 the cofactor FAD is covalently tethered to the protein by covalent linkages. As expected, MtVAO615 harbors a bicovalently linked 6-S-cysteinyl-8α-N1-histidyl FAD while MtVAO713 has a monocovalently linked 8α-methyl-N1-histidyl FAD (Fig. S1). The isoalloxazine ring of the cofactor is covalently linked with the C6 atom to the Sγ of Cys^{222} and with the 8α-methyl group to the Nδ1 of His^{157} in MtVAO615 and His^{159} in MtVAO713. Cys^{222} in MtVAO615 is substituted for Ser^{231} in MtVAO713. All structural homologs have a covalently 8α-methyl-N1-histidyl FAD while at the Cys^{222}/Ser^{231} position, also Val or His residues are found (Fig S1).

The entrances to the active sites are shaped by the seven-stranded β-sheet of the substrate domain and α-helix11 with a loop (res. 391 – 413 for MtVAO615, res. 415 -433 for MtVAO713). The groove shaped substrate binding pocket of MtVAO615 is solvent accessible and contains, besides His^{157} and Cys^{222} which tether the FAD, Tyr^{159}, Thr^{221}, His^{237}, Thr^{341}, Tyr^{352}, Leu^{399}, cis-Pro^{401}, Ala^{402}, cis-Pro^{403}, Ala^{408}, Phe^{410}, Thr^{413}, Tyr^{449}, Asp^{451}, Leu^{475} Ala^{477}, Ala^{479}, Glu^{519} (Fig. 2A). The structure of the loop is formed by two cis-prolines and is probably important for substrate binding. On the other side of the active site the disulfide Cys^{450}-Cys^{476} stabilizes β-strands 17 and 18 with residues lining the active site.

The groove to the substrate binding pocket of MtVAO713 is broader than of MtVAO615 and is delimited by Tyr^{100}, Leu^{161}, Ser^{231}, His^{246}, Tyr^{352}, Phe^{354}, Leu^{425}, Asp^{427}, Ile^{435}, Ser^{433}, Val^{469}, Leu^{471}, Ile^{501} and Glu^{543} (Fig. 2B). The active site opposite to the FAD is very hydrophobic and is lined by several leucines and isoleucines. The hydrophobic nature of these residues is in line with the identified substrate ricinoleic acid, which is a very hydrophobic compound.

Table 1: Data collection and refinement statistics. Values in parentheses are for the highest resolution shell.

	MtVAO615	MtVAO615	MtVAO713
Diffraction data	pH 7.5	pH 5.0	
Wavelength (Å)	1.54	1.54	1.54
Resolution range (Å)	51.4-2.0	58.0-2.2	54.3-2.2
Spacegroup	$P2_12_12_1$	$P22_12_1$	$P2_1$
Cell dimensions (Å) *a*, *b*, *c*, *β*	59.7, 100.9, 111.8	62.4, 116.0, 198.7	83.2, 108.5, 136.0, 90.0
Number of unique reflections	45720 (2754)	71982 (4265)	120924 (5895)
Completeness (%)	98.1 (81.1)	99.5 (92.8)	98.8 (97.3)
Overall I/σ (I)	4.7 (1.0)	12.9 (3.7)	3.9 (1.6)
R_{merge} **(%)**	17.2 (98.6)	14.7 (51.4)	16.1 (48.3)
R_{pim} **(%)**	11.4 (70.7)	5.8 (22.5)	14.7 (47.9)
***R*/ *Rfree* (%)**	23.2 / 28.3	22.0 / 28.1	19.8 / 23.7
R.m.s. deviations from ideal values			
Bond lengths (Å)	0.009	0.007	0.010
Bond angles (°)	1.370	1.243	1.380
Protein residues	27-475	27-475 for both molecules	4 (30-598)
FAD molecule	1	2	4 x 1
Water molecules	389	931	1456
Glycerol molecules			8
NAG	9	3 (A), 6 (B)	4 x 3
β-mannose	2	-	-

Among all the structurally characterized bicovalent flavoproteins, a common feature is the wide opening of the active site on the surface. This contrasts with monocovalent or noncovalent flavoproteins in which the active site is more buried. This feature allows bicovalent flavoproteins to accommodate bulky substrates in their active site [5]. For instance, carbohydrate oxidases in which the FAD is bicovalently bound accept oligosaccharides with higher efficiency compared to monosaccharides[3], [10], [11]. Protein MtVAO713 and MtVAO615 are both an exception to this rule. Despite having a monocovalent FAD, the active site of MtVAO713 is rather open, similar to bicovalent flavoproteins (Figure 3A). MtVAO713 is also closely related to other bicovalent flavoproteins concerning its sequence and structure. This suggests that MtVAO713 has evolved from a bicovalent flavoprotein.

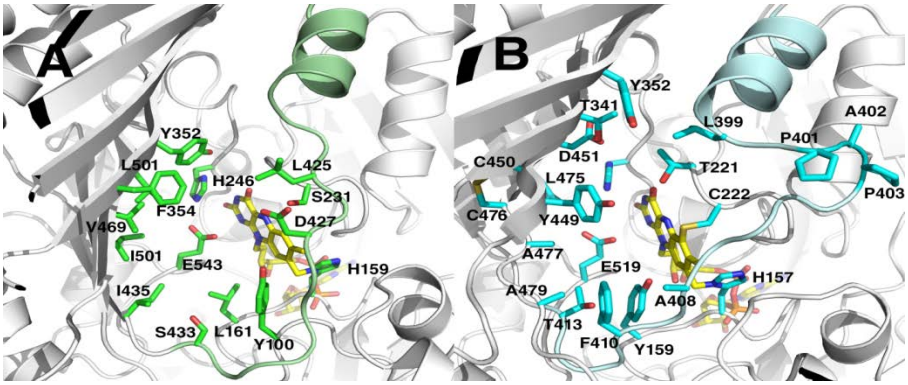


Figure 3: Side-by-side view of the overall structures of MtVAO713 and MtVAO615. The proteins consist of a FAD-binding (F) and a substrate-binding (S) domain. (A). Ribbon diagram showing MtVAO713 as a cartoon with helices colored in red and β-strands in yellow. The bound FAD (yellow) and the linking residues His157 and Cys222 (cyan) are shown in stick models. The disulfides bridges and the glycosylated residues (green) are displayed in stick representations and. (B) Ribbon diagram showing MtVAO615 as a cartoon in rainbow colors from the N-terminus in blue to the C-terminus in red. The bound FAD (yellow), the glycosylated residues (green) and the disulfides bridges are in stick representations.

On the other hand, despite having a bicovalently bound FAD cofactor, access to the active site of MtVAO615 is rather narrow (Figure 3B). In particular, Phe^{410} and Tyr^{449} seem to create a sort of funnel. It is worth to note that Phe^{410} is on a loop which runs longitudinally across the active site. While this loop is further away from the active site in the other sequence-related oxidases, in MtVAO615 it leans towards the active site contributing to narrowing the cavity. In physiological conditions and in the presence of the native substrate, the loop might be mobile to allow substrate binding.

Table 2: Amino acid per positions relative to MtVAO615 in the active site of structurally characterized VAO-type flavoproteins (in grey hydrophobic residues, in red negatively charged residues, in blue positively charged residues, in green polar residues). Question marks indicate dubious residues given the lack of structural similarity with MtVAO615. 1DII: p-cresol methylhydroxylase from Pseudomonas putida; 1VAO: vanillyl alcohol oxidase from Penicillium simplicissimum; MtVAO713: XP_003665713.1 from M. thermophila; MtVAO615: XP_003663615.1 from M. thermophila; 3POP: GilR from Streptomyces griseoflavus; 2IPI: aclacinomycin oxidoreductase from Streptomyces galilaeus; 5AWV: Dbv29 from Nonomuraea sp. ATCC 39727; 2Y08: TamL from Streptomyces sp. 307-9; 3D2D: reticuline oxidase from Eschscholzia californica; 3VTE: Δ1-tetrahydrocannabinolic acid synthase from Cannabis sativa; 4UD8: AtBBE15 from Arabidopsis thaliana; 3TSH: Phl p 4 from Phleum pretense; 4DNS: BG60 from Cynodon dactylon; 3RJ8: lactose oxidase from Microdochium nivale; 5L6F: xylooligosaccharide oxidase from M. thermophila; 2AXR: glucooligosaccharide oxidase from Acremonium strictum; ChitO: chitooligosaccharide oxidase from Fusarium graminearum; 2BVF: 6-hydroxy-D-nicotine oxidase from Pseudarthrobacter oxydans; 3W8W: EncM from Streptomyces maritimus; 1l19: cholesterol oxidase from Brevibacterium sterolicum; 2VFR: alditol oxidase from Streptomyces coelicolor; 4KW5: decaprenylphosphoryl-beta-D-ribose oxidase from Mycobacterium tuberculosis; 4BBY: alkyldihydroxyacetonephosphate synthase from Cavia porcellus.

Position relative to 615	159	237	451	475	477	519
1DII	F	V	I	H	V	Y (?)
1VAO	Y / S	V	T	H	I	Y (?)
5FXD	G	V	I	H	V	Y (?)
MtVAO713	L	H	L	V	I	E
MtVAO615	Y	H	D	L	A	E
3POP	G	Y	N	K	S	Y
2IPI	F	Y	Y	K	W	Y
5AWV	F	Y	I	K	N	Y
2Y08	Y	Y	I	K	V	Y
3D2D	Y	F	N	M	E	H
3VTE	A	Y	Y	E	W	Y
4UD8	Y	Y	N	K	Q	Y
3TSH	Y	F	D	N	Q	Y
4DNS	Y	F	D	N	Q	Y
3RJ8	Y	F	D	L	Q	Y
5L6F	Y	F	D	L	E	Y
2AXR	Y	Y	D	L	Q	Y
ChitO	Y	Y	D	L	Q	Y
2BVF	P	V	E	E	L	N
3W8W	M	F	L	V	N	F
1l19	W	H	R	W	N	K / E
2VFR	F	H	R	A	H	K
4KW5	Y	H	K	N	C	K
4BBY	S	H	T	I	P	H (?)

When inspecting the active site residues of both MtVAO615 and MtVAO713, striking differences between these two proteins and the known structurally resolved oxidases can be noted. Table 2 lists the active site residues of various VAO-type flavoproteins for which a crystal structure is available. This shows that, while MtVAO615 and MtVAO713 have a strikingly similar active architecture, their active site residues are very different from other VAO-type oxidases. For example, a structurally conserved Tyr often assists in catalysis in most VAO-type oxidases which is missing in MtVAO615 and MtVAO713: a Glu residue is present at this position, respectively Glu^{519} and Glu^{543} (Table 2). It has been proposed that Tyr^{429} in GOOX acts as a base which abstracts a proton from the O_1 of the substrate, contributing to the hydride transfer to FAD. A similar catalytic role is played by Tyr^{473} in Dbv29 [7], Tyr^{448} in GilR [24], Tyr^{447} in TamL [23], Tyr^{450} in aclacinomycin oxidoreductase (AckOx) [8] and Tyr^{484} in THCA synthase [25] (position '519' in Table 2). Asp^{355} in GOOX may assist in the proton transfer by lowering the pKa value of Tyr^{429} through a water molecule [4]. MtVAO615 has an Asp at a similar position, while MtVAO713 has a Leu (position 451 in Table 2). When comparing the active site residues, it seems that MtVAO615 and MtVAO713 represent a new subclass of oxidases with a totally new constellation of active site residues. While we could confirm oxidase activity on a secondary alcohol for MtVAO713, the real substrates for these oxidases still need to be identified. With the unique active sites, the oxidases may even catalyze unprecedented oxidative reactions as other sequence-related VAO-type oxidases have been shown to display various exotic oxidative reactions which include ether bond cleavage and C-C bond formation reactions.

Inspection of the structures of both oxidases revealed a feature that is in line with the efficient oxygen reactivity. Recently a so-called gatekeeper residue which tunes oxygen reactivity of VAO-type flavoproteins was identified on the *re*-side of the isoalloxazine ring [26]. While this residue is typically rather bulky in homologs that are poorly reactive with dioxygen, in these newly identified oxidases residues (Val225 in MtVAO615 and Pro234 in MtVAO713) are found that provide enough space for dioxygen to reach and react with the

reduced flavin cofactor. Taken together, the kinetic and structural data indicate that the two studied flavoproteins are bona vide oxidase.

Conclusions

In the present study we report the discovery and the structural characterization of two novel flavoprotein oxidases from the thermophilic fungus *M. thermophila*. Both present rather unique structural features in the active site. Only for MtVAO713 we could identify ricinoleic acid as substrate (although a very poor one) which paves the way for further exploration.

The elucidation of the crystal structures can be used to rational design a library of compounds to be virtually screened through automated docking. Unfortunately this approach suffers of several limitations: 1) when lacking a lead compound, the creation of the library of test compounds might be extremely challenging and time consuming; 2) the results of a docking procedure often only predict whether binding of the compound is likely while it does not guarantee whether the binding pose will be productive, enabling catalysis; 3) the current docking procedures lacks accuracy in predicting binding energies which is partly due to the fact that typically the enzyme structure is taken as a rigid configuration.

Another approach for identifying a substrate for a newly found enzyme is testing complex mixtures of natural sources, for example extracts of plant biomass can be used to screen by chemical analysis for chemical modifications. This approach is less specific but in this lies its advantage when no indication whatsoever is available as to which substrate would work with these novel enzymes. In particular for MtVAO713, extracts can be generated from flax and/or oats hulls and be tested with the enzyme. The analysis will present definitely challenges given the high heterogeneity of the sample. Pre-fractionation of the extract could be used to reduce the sample complexity. Alternatively, it may be tempting to use protein crystals to soak them in a (complex) mixture

of test compounds. When this is performed anaerobically, substrate will bind and by structure elucidation, the identity of tight binding substrates will be revealed.

Materials and Methods

Bioinformatic analysis

The sequence of ChitO (accession number: XP_011325372) was uses as query to perform a protein BLAST search on the "Non-redundant protein sequences" database with *M. thermophila* ATCC 42464 (taxid:573729) set as organism. Protein sequences were exported in the FASTA format and aligned with the software Geneious using default parameters. From the multiple sequence alignment, a phylogenetic tree was obtained with the following parameters: Genetic distance model: Jukes Cantor; Tree build method: Neighbor-Joining; Outgroup: No outgroup.

Transformation and Expression

Cloning and expression of MtVAO713 and MtVAO615 were performed as described in Chapter 4. The gene encoding MyThVAO713 was amplified from genomic *M. thermophila* ATCC 42464 DNA.

Purification

50 mL processed fermentation broths for each protein were received from DuPont Industrial Biosciences frozen at -20 °C. Samples were thawed on ice and buffer exchanged with 20 mM Tris/Cl pH 7.6. Subsequently they were loaded on pre-equilibrated 5 mL Hi-Trap Q-sepharose column using a FPLC purification system (AKTA Purifier). Absorbance was monitored at 280 nm, 445 nm and 600nm. The column was washed until absorbance at 280 nm reached baseline levels (≈ 10 column volumes). Both proteins were eluted at 10% of elution buffer (20 mM Tris/Cl pH 7.6 and 2 M NaCl).

Yellow fractions which displayed absorbance at 445 nm were collected, concentrated and subsequently loaded on a Superdex 200 column. 50 mM acetate buffer pH 5.6 containing 100 mM NaCl was used to equilibrate the column and for the elution. The yellow fractions displaying absorbance at 445 nm were collected and concentrated.

Crystallization

Crystallization, data collection and structure determination of MtVAO615

Initial vapour-diffusion crystallization experiments were performed using a Mosquito crystallization robot (TTP Labtech). In a typical experiment, 0.1 µL screening solution was added to 0.1 µL MtVAO615 protein solution (9.6 mg mL^{-1}) on a 96-well MRC2 plate (Molecular Dimensions); reservoir wells contained 50 µL screening solution. The screening solutions used for the experiments were PACT and JCSG+ (Molecular Dimensions). MtVAO615 crystals appeared after 3-30 d of incubation at 294 K in solutions containing PEG at pH 5 to 9. Crystallization conditions were optimized using hanging-drop set-ups containing 1 µL protein solution and 1 µl reservoir solution at 294K. Crystals grown from 11% PEG3350, 20% glycerol and 0.1 M Hepes pH 7.5 and from 20% PEG6000, 0.2 M NaCl and 0.1 M sodium acetate at pH 5.0 were used for crystal structure determination.

Before data collection, crystals were briefly soaked in a cryoprotectant solution, consisting of 20% glycerol, 25% PEG3350 and 0.1 M Hepes pH 7.5. X-ray diffraction data to 2.0 Å resolution were collected from a single cryo-cooled crystal mounted on an in-house MarDTB Goniostat System using Cu-Kα radiation from a Bruker MicrostarH rotating-anode generator equipped with HeliosMX mirrors. Intensity data were processed using iMosflm [27] . The diffraction patterns were anisotropic with a weak zone of decreased resolution (2.7 Å) resulting in poor statistics for the high resolution shell (Table 1). The crystals belong to spacegroup P212121 with one monomer of 54 kDa in the asymmetric unit. The VM is 2.7 Å3/Da [28] with a solvent content of 54%. Crystals grown in one

month at pH 5.0 were also cryocooled with 20% glycerol. They belonged to space group P22121 and diffracted to 2.2 Å resolution. With two monomers in the asymmetric unit the solvent content is 57% and VM is 2.9 $Å^3$/Da [28]. Intensity data were processed with XDS [29] . Data collection statistics are listed in Table 1.

Five structures were selected with highest identity to the C-terminal part of MtVAO615 i.e. 6HDNO (PDB code: 2BVF) [22], AckOx (PDB code: 2IPI)[8], TamL (PDB code: 2Y08) [9], EncM (PDB code: 3W8W)[21], and LaO, (PDB code: 3RJ8). No homology model could be obtained for the 100 N-terminal amino acids because of lack of sequence homology. Using these five structures as templates, homology models were generated with the SCWRL server [30]. Molecular replacement was performed with the program Phaser [31] using an ensemble of the five homology models.

Although Phaser was able to find a clear solution for the P212121 data with one monomer in the asymmetric unit, the calculated initial phases were not good enough to build the structure. This is probably caused by the low sequence identity and the anisotropically diffracting crystals. The phases calculated by Phaser for two monomers in the asymmetric unit in the P22121 data were sufficient for automatic model building by the Phase and build program in the PHENIX suite [32]. The refined model was used in determining the structure in the P212121 space group by Phaser.

Crystallization, data collection and structure determination of MtVAO713

Before crystallization of MtVAO713, an extra step of enzyme purification (and buffer exchange) was performed using a Superdex200 10/300 GL column (GE Healthcare), equilibrated with 20 mM Hepes buffer, pH 7.5, containing 150 mM NaCl. MtVAO713 eluted at a molecular weight of 53 kDa. Only the first fractions of the MtVAO713 peak containing FAD were pooled and concentrated. Dynamic light scattering (DLS) analysis (DynaPro NanoStar, Wyatt technology) indicates that the protein has a hydrodynamic radius of 3.38 nm, with an apparent molecular weight of 58 kDa and 5% polydispersity.

Crystallization conditions were screened using the Mosquito crystallization robot with a MtVAO713 solution of 10-23 mg ml^{-1} (see above). Many commercially available screens were tried but only with the Morpheus screen (Molecular Dimensions) thin yellow plate-like crystals appeared after 1 month of incubation at 294 K. Conditions were optimized using hanging-drop set-ups with 25% PEG3350 and 0.1 M Tris pH 8.5 - 9.0 as precipitant, and drops containing 1 µl protein solution (10 mg ml^{-1}) and 1 µl reservoir solution.

Crystals were soaked in a cryoprotectant prepared by the addition of 20 % (w/v) glycerol to the reservoir solution. X-ray diffraction data to 2.2 Å resolution were collected from a single cryo-cooled crystal mounted on the in-house X-ray source. Intensity data were processed using XDS [29]. The crystals (apparently) belong to space group P22121 with a=83.2Å, b=108.5Å and c=136.0Å and all angles 90°. With two monomers of 58 kDa in the asymmetric unit the VM is 2.4 $Å^3$/Da [28] with a solvent content of 48%.

MtVAO713 has 24% identity to MtVAO615 and this structure was used as a template for molecular replacement. The homology model was generated with the SCWRL server [30] and molecular replacement was performed with the program Phaser [31] . Phaser obtained a distinct solution for the first monomer but a solution for the second monomer could not be calculated. Analysis of the data revealed systematic absences for (0, k, 0) with k odd and for (0, 0, l) with l odd. However, the 00l reflections only spike at intermediate resolution displaying l = 2n presence, breaking down on lower and higher resolution suggesting a lower symmetry space group. After processing the data in space group P21, with a β angle of 90.00, Phaser was able to find four molecules in the asymmetric unit. Molecules A/D and B/C are related by an NCS operator that is close to a perfect twofold crystallographic rotation having P222 pseudosymmetry. The phases were good enough to build a model automatically with the Phase and build program in the PHENIX suite [32]. Data collection statistics are listed in Table 1.

Refinement

REFMAC5 was used for refinement of the models [33] and Coot [34] was used for manual rebuilding and map inspection. In the last rounds of refinement TLS groups were used. The quality of the models was analyzed with MolProbity [35] , secondary structure elements were assigned with DSSP [36] and Promals3D was used for structure alignment [37] . Figures were prepared with PyMOL (http://www/pymol.org) and ESPript [38]. Atomic coordinates and experimental structure factor amplitudes have been deposited in the Protein Data Bank (PDB) with PDB IDs 5xxx, and 5yyy and 5zzz for monomeric, dimeric MtVAO615 and MtVAO713, respectively.

Substrate screening

Carbohydrates and primary alcohol screening

The screening was performed as previously described [39]. In brief, oxidase activity of the two enzymes was detected by coupling H_2O_2 production to the oxidation of 4-aminoantipyrine and 3,5-dichloro-2-hydroxybenzenesulfonic acid by horseradish peroxidase (HRP). The formation of the resulting pink/purple colored product can be followed at 515 nm (ε_{515} = 26 mM^{-1} cm^{-1}). The reaction mixture for the carbohydrates/primary alcohol substrate screening contained 50 mM phosphate buffer, pH 7.6, 0.1 mM 4-aminoantipyrine, 1.0 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid, 4.0 units HRP and 1 µM of either enzymes. A panel of different carbohydrates comprising monomeric, dimeric, tetrameric, polymeric, cyclooligosaccharides and a range of primary alcohols were tested (see Supplementary information). Potential substrates were tested in duplicate using concentrations of 50 mM and 5.0 mM for mono- and disaccharides, and 1.0 mM and 0.5 mM for tetra- and cyclo-oligosaccharides. To exclude pH effect, xylobiose and cellobiose in 50 mM and 5.0 mM were tested in pH range from 4.0 to pH 9.0 using the Britton&Robinson buffer.

Complex mixtures screening

2 µM of either enzyme were used to detect oxidase activity with the HRP assay on complex mixtures of substrates. The assay was

performed in 50 mM acetate buffer pH 5.6 on 50 mg/mL ground rye, oats, wheat, barley, wheat straw, arabinan and wheat arabinoxylan.

Steroids and secondary alcohol screening

1 µM of MtVAO713 was used to perform the conversion of either 1% or 5 mM of a panel of steroids and secondary alcohols (Supplementary information). As buffer 50 mM Tris/Cl pH 8.0 and 10% DMSO as co-solvent were used. Reactions were performed overnight at 30 ˚C or 37 ˚C at 300 RPM in a final volume of 1 mL in a 2 mL Eppendorf tube. Reactions were extracted twice with ethyl acetate and injected in the GC/MS. Ricinoleic acid and derived substrates i.e. methyl ricinoleate and ricinoleyl alcohol, were further processed for derivatization with the TMS reagent. Samples were first evaporated and then resuspended in 60 µL of MTBE (methyl tert-butyl ether). Subsequently 60 µL of 1% TMS (trimethylsilyl) in BSTFA (N, O-bis(trimethylsilyl) trifluoroacetamide) were added. This was followed by incubation at 75 ˚C for 30 minutes to allow the derivatization. The final samples were injected into GC/MS.

GC/MS

The GC was performed on a HP1 column (dimethylpolysiloxan, 45 m x 0.25 mm x 0.25 µm) with Temperature gradient from 40-250 ˚C (5˚C/min) with a solvent cut-off of 6 min and a split ratio of 100. Ketooleic acid eluted at 36.9 minutes and ricinoleic acid at 37.2 minutes.

Stopped flow analysis

The reoxidation of both enzymes was monitored by measuring the absorbance change of the FAD in presence of saturating O_2 concentrations. A solution of approximately 40 µM of either enzyme was prepared in 50 mM phosphate buffer pH 7.6 to a final volume of 1 mL into a glass tube. The tube was flushed with N_2 for 10-15 minutes. Then, sodium dithionite (10 mM) was added until the enzyme solution became bleached. In another glass tube a solution of only buffer was flushed for 10-15 minutes with O_2. These solutions

were mixed 1 to 1 in an Applied Photophysics SX20 stopped-flow apparatus and re-oxidation was monitored for 1 second using a photo diode array (PDA) detector between 186 and 724 nm. The spectral scans were fitted using equations for double or triple exponential decays.

Redox potential determination

The redox potentials were determined by using the method described by Massey [16]. Reactions were performed in 50 mM potatassium phosphate buffer pH 7.0 at 25˚C. A cuvette with MtVAO615 (10 µM) or MtVAO713 (5 µM), xanthine (400 µM), methyl viologen (2-5 µM) and redox dye (5-10 µM) was made anaerobically by flushing with N_2 for 10-15 minutes. Subsequently, 50-60 µg of xanthine oxidase was added anaerobically to the cuvette and spectra were collected every 2 minutes during the reaction using a Jasco V-650 spectrophotometer. The redox potentials were calculated by plotting the log ([ox]/[red]) of the protein versus log([ox]/[red]) of the redox dye according to Minnaert et al [40]. As reference redox dyes we used methylene blue (E_M = +11 mV), thionin acetate (E_M = +64 mV) and 2,6-dichloro indophenol ($_{EM}$ = +217 mV).

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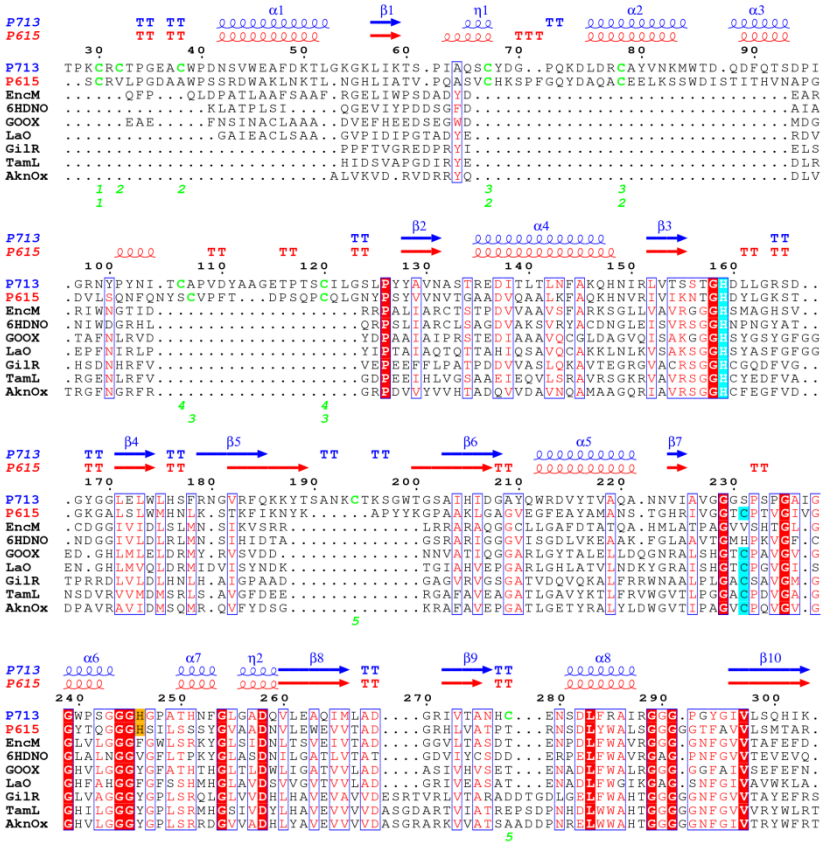
Supplementary information



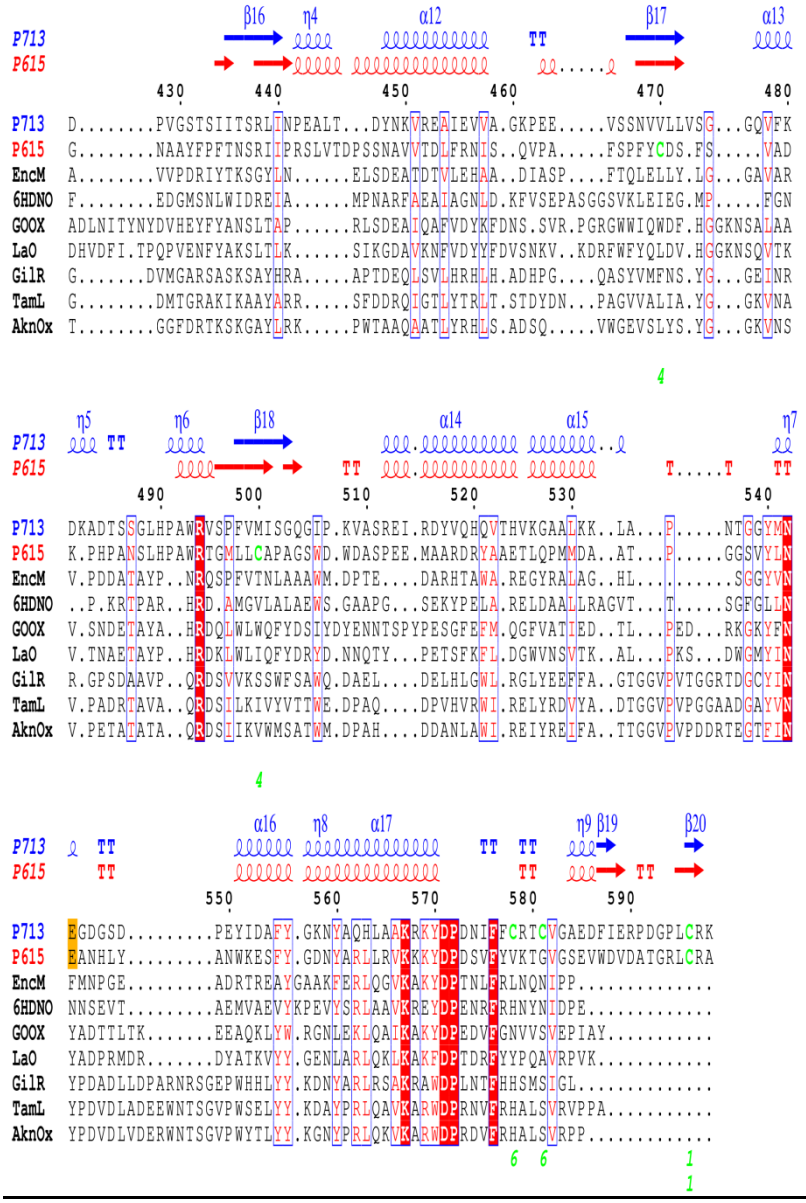


Figure S1: Multiple sequence alignment. Structure–based alignment of MtVAO713 and MtVAO615 from Streptomyces maritimus flavoenzyme (EncM, PDB code 3w8w) [21], Arthrobacter nicotinovorans 6-hydroxy- D-nicotine oxidase from (6HDNO, PDB code 2bvf) [22], Acremonium strictum glucooligosaccharide oxidase (GOOX, PDB code 1ZR6) [4], Microdochium nivale lactose oxidase (LaO, PDB code 3rj8), Streptomyces griseoflavus oxidoreductase (GilR, PDB code 3POP) [24], Streptomyces sp. 307-9 tirandamycin oxidase (TamL, PDB code 2y08) [23] and Streptomyces galilaeus

aclacinomycin oxidoreductase (AckOx, PDB code 2ipi) [8]. The structural alignment was made with Promals3D [37]. The secondary structure elements above the sequence alignment are those obtained from the crystal structure of MtVAO713 and MtVAO615. Residues involved in FAD binding have a cyan background color, identical residues have a red background color and similar residues have a red color. Residues with orange background are likely to be involved in catalysis. Disulfide linkages are indicated in green italics below the sequences. The figure was created with ESPript [38].

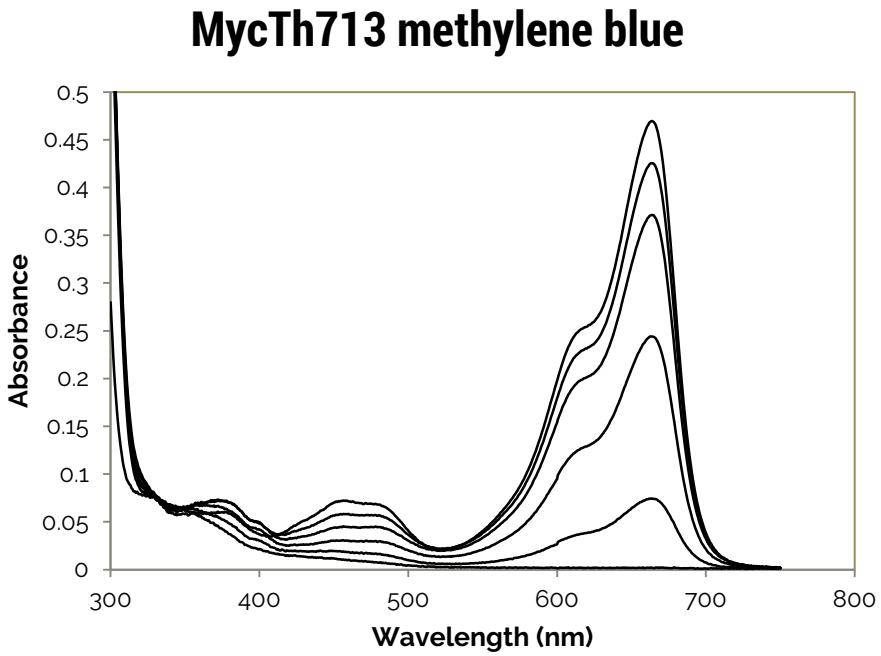


Fig S2: Spectrum of 5 µM MtVAO713 in the redox potential determination experiment with xanthine oxidase. The collected spectra are shown between time point 0 and time point at 34 minutes.

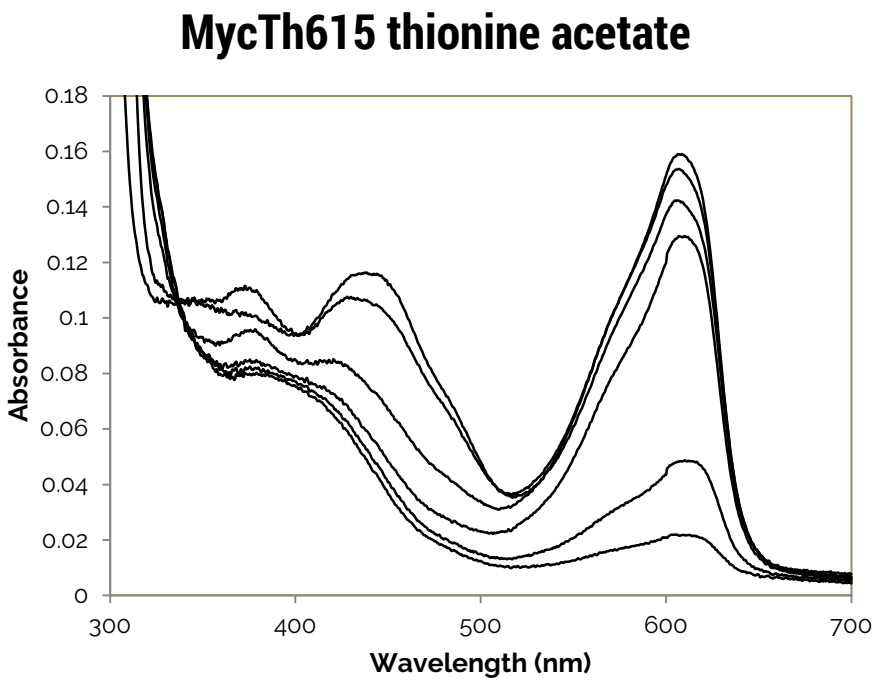


Fig S3: Spectrum of 10 µM MtVAO615 in the redox potential determination experiment with xanthine oxidase. The collected spectra are shown between time point 0 and time point at 88 minutes.

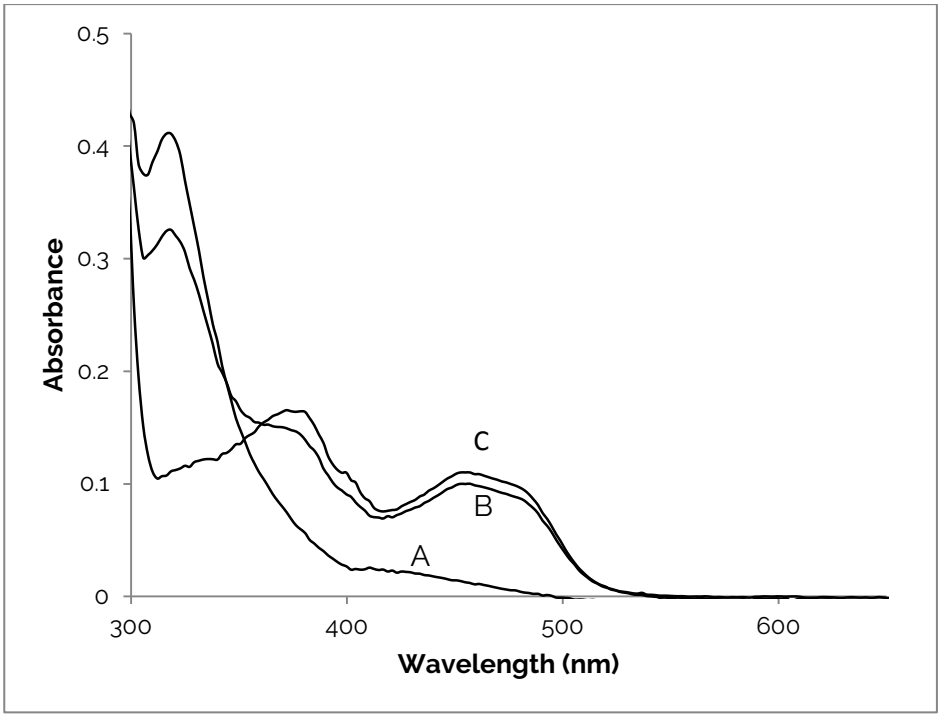


Fig S4: Deconvoluted absorption spectra of reduced (A), intermediate (B) and oxidized (C) MtVAO713 during reoxidation by molecular oxygen as measured by double-mixing stopped-flow spectrophotometry, after a delay time of 1.0 s.

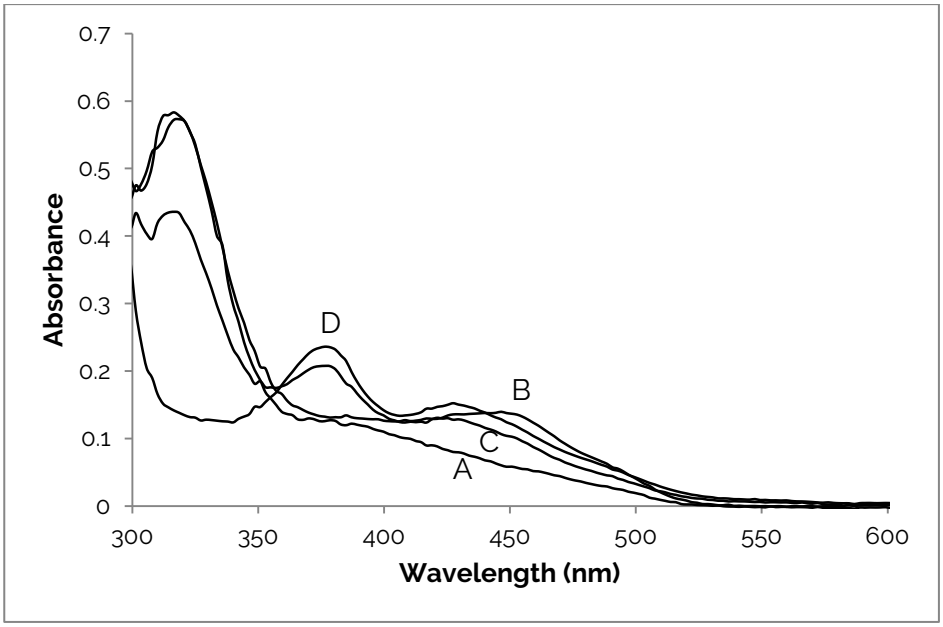


Fig S5: Deconvoluted absorption spectra of reduced (A), intermediate 1 (B), intermediate 2 (C) and oxidized (D) MtVAO615 during reoxidation by molecular oxygen as measured by double-mixing stopped-flow spectrophotometry, after a delay time of 1.0 s.

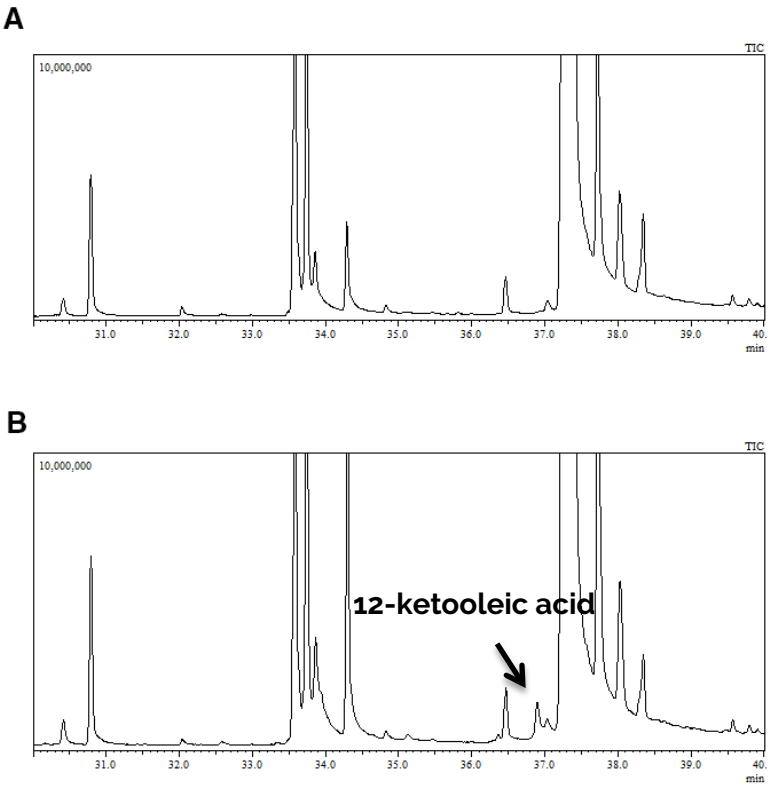


Fig S6: GC spectra of ricinoleic acid conversion by MtVAO713. A) Negative control without MtVAO713. B) Conversion with MtVAO713 present.

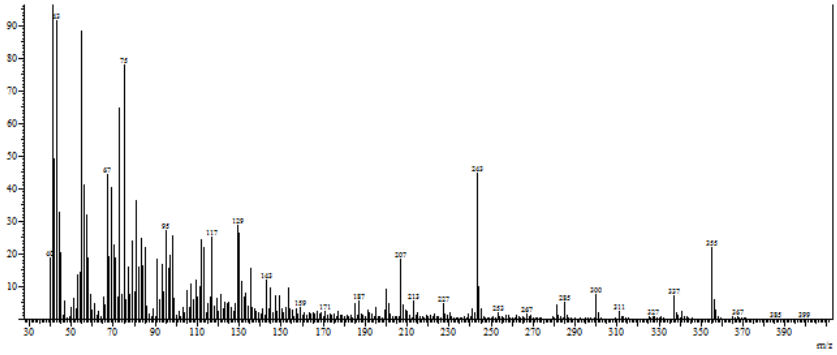


Figure S7: MS spectrum of the selected peak corresponding to 12-ketooleic acid.

List of substrates tested

MtVAO713: glucose, galactose, fructose, mannose, xylose, arabinose, N-acetyl-D-glucosamine, sucrose, maltose, lactose, sorbitol, xylitol, cellobiose, cellotetraose, α-cyclodextrin, β-cyclodextrin, arabinan, chitosan, chitin, starch, maltotetraose, glycerol, hydroxymethylfurfural, xylobiose, testosterone, cholesterol, Beta-ecdysone, 20-hydroxyecdysone, ergosterol, cholecalciferol, beta-sitosterol, stigmasterol, ricinoleic acid, methly ricinoleate, ricinoleyl alcohol, pinellic acid, cyclohexanol, 2-propanol, 2-butanol, 2-pentanol, 2-heptanol, 1-amino-2-propanol, 3-pentanol, 3-heptanol, 3-octanol.

MtVAO615: glucose, galactose, fructose, mannose, xylose, arabinose, N-acetyl-D-glucosamine, sucrose, maltose, lactose, sorbitol, xylitol, cellobiose, cellotetraose, α-cyclodextrin, β-cyclodextrin, arabinan, chitosan, chitin, starch, maltotetraose, glycerol, hydroxymethylfurfural, xylobiose, ethanol, benzyl alcohol, glucose-6-phosphate, galactose-1-phosphate, mannose-6-phosphate, fructose-6-phosphate, xylan, arabinan, arabinoxylan, rye whole grain flour, oats flour, rye refined flour, wheat flour, barley flakes, straw, N-acetyl muramate.

Chapter 6

Conclusions and future perspectives

Alessandro R. Ferrari and Marco W. Fraaije

Oxidases can be a valuable biotechnological tool. The oxidation reactions performed by oxidases can be stereoselective and regiospecific. In addition, molecular oxygen is used as cheap electron acceptor eliminating the need for expensive cofactors. Yet, there is a rather limited number of oxidases available for biotechnological applications. Therefore the discovery of novel oxidases is highly desirable.

This research aimed at discovering oxidases for novel applications in the processing of biomass or biomass-derived compounds. Specifically, carbohydrate oxidases capable to oxidize bulky substrates were the main target. Such enzymes typically belong to the VAO family of flavoproteins and have been shown to act on several components of the plant biomass. The derived oxidized molecules may have novel applications as is described in chapter 1.

Hydrolytic enzymes such as cellulases and xylanases are subjects of numerous investigations at the moment of this writing given the large interest in biofuels. Discovery of more efficient hydrolytic enzymes is highly desirable. The current method to determine the activity of these enzymes are not high-throughput, require harsh conditions and generate toxic waste. In chapter, 2 the development of a novel assay to detect and measure activity of biomass hydrolytic enzymes is reported. The so-called "ChitO assay" is based on an enzymatic cascade reaction: the hydrolytic activity is coupled to oxidase and peroxidase catalyzed reactions. Chitooligosaccharide oxidase (ChitO) from *Fusarium graminearum* and a variant thereof are used to oxidize the product formed upon hydrolysis activity of cellulases or chitinases. Due to the activity of ChitO, hydrogen peroxide is produced in the process which is used by horseradish peroxidase to convert two chromogens in a colored pink product. The development of this colored compound can be easily monitored in time. This assay represents a faster, highly sensitive, and more environmental-friendly alternative to traditional assays such as DNS and the Schale procedure. The assay can also easily be integrated in high-throughput screening protocols.

Carbohydrate oxidases of the VAO family typically share a very similar overall structure. Still they typically display very different substrate acceptance profiles. In order to understand the role of specific amino acids in determining the substrate scope of carbohydrate oxidases, ChitO was used as a model for rational protein engineering (Chapter 3). Sixteen model-inspired ChitO mutants were generated and their substrate scope explored. Three residues (Gln^{268}, Glu^{270} and Ser^{410}) were found to play a key role in determining the substrate specificity of ChitO. For example, the mutant G270E/S410R showed the highest N-acetyl-D-glucosamine oxidation activity ever reported. By mutating all three residues at the same time, a lactose oxidase was obtained. The insights obtained from this work can be used to predict the putative substrate scope of newly discovered carbohydrate oxidases.

In the process of discovering new enzymes from a particular organism, physiological features of the organism may already provide hints on in enzyme repertoire. The thermophilic fungus *M. thermophila C1* is a well characterized host of biomass acting enzymes. Furthermore through its ability to thrive at relatively high temperatures, it is a source of proteins which are typically quite thermostable. The genome of *M. thermophila* was screened for novel VAO-type oxidases using a bioinformatics approach. This revealed several putative VAO-type oxidase-encoding genes. In chapters 4 and 5 the discovery, the biochemical properties, and crystal structures of three novel flavoprotein oxidases are reported.

In chapter, 4 the xylooligosaccharide oxidase (XylO) from *M. thermophila C1* is described. With the identification of this novel carbohydrate oxidation, a new range of carbohydrates can be oxidized by the use of an oxidase. XylO shows a quite narrow substrate specificity with a strong preference towards xylobiose and higher oligomers of xylose. The enzyme also acts on derivatized xylooligosaccharides such as those generated by treatment of different kinds of xylan with a xylanase. Chemical analysis revealed that the oxidation takes place at the C1 position resulting in the formation of the corresponding aldonic acids. The elucidated crystal

structure confirmed the presence of a bicovalently bound FAD. Inspection of the active site provides some clues about the reasons for the narrow specificity of XylO. Tyr^{376}, Leu^{274} and Ile^{378} seem to play a role in preventing aldohexoses to bind to XylO. The insights obtained from this work add a further piece to the puzzle in understanding the molecular basis of substrate recognition by carbohydrate oxidases. This could help in future work to design *ad hoc* specificity by mutagenesis or *de novo*. XylO also represents a valuable new biocatalytic tool for enzymatic modification of xylose-based polymers.

Two other oxidases from *M. thermophila* are described in chapter 5. Purification of XP_003663615 (MtVAO615) and XP_003665713 (MtVAO713) confirmed that they also contain a covalent flavin cofactor. Stopped flow analysis revealed that they can be efficiently re-oxidized by molecular oxygen after being reduced. This confirms the two proteins are true flavoprotein oxidases. MtVAO615 is a bicovalent flavoprotein and MtVAO713 is a monocovalent flavoprotein. It has been reported so far that bicovalent flavoproteins have a more open active site compared to monocovalent flavoproteins. Elucidation of the crystal structure of both flavoproteins showed that in the case of MtVAO615 and MtVAO713 this does not hold true. MtVAO615 presents a quite narrow active site despite being bicovalent flavoprotein while MtVAO713 presents a rather open active site despite being a monocovalent flavoprotein. Both enzymes share an atypical composition of the residues in proximity to the FAD suggesting that both oxidases may catalyze unprecedented oxidation reactions. However, though a large number of compounds were tested, no good substrates could be identified for these newly discovered flavoprotein oxidases. It is worth noting that MtVAO713 has a very apolar active site. This might account for a substrate specificity that escaped our investigation which included mainly carbohydrates, alcohols and steroids as test compounds.

The work described in chapter 5 illustrates that even having high-resolution X-ray structures of enzymes available is in most cases not

sufficient yet to predict the substrate specificity and the type of catalyzed reaction. The advances of computational power and new computational methods might be able in the near future to solve these issues. By refinement of the computational techniques to elucidate the substrate scope of unknown enzymes and to predict the chemistry that will occur in the active site, the annotation of genome databases will be faster and cheaper. Such predictive computational tools would dramatically boost the discovery and application of novel enzymes tailored to specific needs.

Chapter 7

Nederlandse samenvatting

Alessandro R. Ferrari

This chapter was kindly translated by Friso S. Aalbers

In dit proefschrift presenteer ik het onderzoek dat ik onder begeleiding van Prof. Marco W. Fraaije heb verricht aan de enzym klasse genaamd oxidases.

Wat zijn enzymen?

Enzymen zijn de kleinste moleculaire machines die de natuur ooit heeft gemaakt. Deze onzichtbare (voor het blote oog) miniatuur bioreactoren zijn geëvolueerd om een specifieke functies uit te voeren met hoge precisie en spectaculaire prestaties. Echter, niet alle enzymen zijn hetzelfde, en sommigen zijn bijvoorbeeld sneller dan andere, net als dat er Ferrari en Opel auto's zijn.

Misschien weet u het niet, maar enzymen zijn overal. Het feit dat u nu aan het lezen bent en de informatie verwerkt in uw brein gebeurt door honderden als niet duizenden enzymen die werken in een meesterlijk nauwkeurig afgesteld ensemble. U leeft doordat de enzymen nutriënten opnemen uit voedsel en dit omzetten in bruikbare energie en bouwstenen voor uw cellen.

Niet alleen mensen, maar alle levende wezens bevatten enzymen. Zelfs de organismen die u niet kan zien. En dan bedoel ik bacteriën, gisten en schimmels. Die hebben vaak de meest interessante enzymen van alle. Hun enzymen kunnen taken volbrengen die voor (biotechnologische) toepassingen erg interessant zijn. Denk hierbij aan bier brouwen, biobrandstof maken, biosensoren creëren, afvalwater zuiveren, etc.

In het verleden was de chemie bepalend voor bijna ieder industrieel proces. Een chemisch proces was vaak de enige optie voor de industrie en de gevolgen ervan op het milieu werden vaak niet voor- of overzien. Dit is aan het veranderen en we realiseren ons meer en meer dat de manier waarop onze industrieën werken vaak niet duurzaam is en onze toekomst op deze planeet bedreigt. De energie verslindende processen dragen bij aan de emissie van broeikasgassen en productie van giftig afval dat niet alleen schadelijk is voor de ecosystemen waar ze in belandden maar ook

uiteindelijk een bedreiging voor ons. Zouden enzymen een van de oplossingen kunnen zijn voor dit probleem?

Enzymen bieden een waardevol "groen" alternatief voor (sommige) chemische processen en hebben vaak een aantal voordelen:

1. Reacties kunnen uitgevoerd worden bij atmosferische druk en mildere temperaturen ten opzichte van chemische processen.
2. Geen (of zeer weinig) gevaarlijk afval wordt gegenereerd.
3. Afgestemde reacties die met precisie in een stap kunnen volbrengen wat, op zijn best, meerdere stappen vereist met traditionele chemische processen.

Koolhydraat oxidases

De hoeveelheid enzymen die tot dusver zijn ontdekt is enorm. In mijn promotieonderzoek heb ik gewerkt aan enzymen die vallen onder de klasse genaamd oxidases, en specifiek de koolhydraat oxidases. Oxidases zijn enzymen die het weghalen van elektronen (oxidatie reactie) kunnen katalyseren. Zo kunnen koohydraat oxidases suikers oxideren met behulp van wat zuurstof.

Waarom zou dit nuttig kunnen zijn?

Er zijn verscheidene manieren waarop koolhydraat oxidases een zeer waardevol middel kunnen zijn vanuit een economisch oogpunt. Laten wij ze in detail bekijken.

Suikers met nieuwe eigenschappen

Door oxidatie krijgen moleculen nieuwe eigenschappen. Bijvoorbeeld kunnen geoxideerde suikers gebruikt worden om sommige mineralen te "vangen" (de technische term is "chelatie") zoals calcium, kalium of magnesium en hiermee heb je een manier om gezonde drankjes te maken die mineralen bevatten. Ook kunnen deze suikers gebruikt worden om het calcium in afvalwater te vangen, om kalkvorming te voorkomen.

Papier is gemaakt van cellulose vezels. Cellulose bestaat uit lange ketens van suiker moleculen. Door deze suikers te oxideren kan je papier krijgen met nieuwe eigenschappen (bijv. water afstotend, zelf-plakken, etc.).

Waterstof peroxide generator

Wanneer suikers worden geoxideerd, maken koolhydraat oxidases waterstof peroxide als bijproduct. Waarschijnlijk hebt u waterstof peroxide wel eens gebruikt of u gebruikt het nog steeds. Het is een oplossing die wordt gebruikt om wonden te desinfecteren. Waterstof peroxide zal de membranen en eiwitten van bacteriën afbreken en zodoende ze doden.

Op die wijze kunnen koolhydraat oxidases worden gebruikt als middel om sommige infecties tegen te gaan of in tandpasta's. Als je erover nadenkt, tanderosie wordt veroorzaakt door bacteriën die de suikers op je tanden verbruiken. Door tandpasta met een koolhydraat oxidase te gebruiken, zal je niet alleen de hoeveelheid bruikbare suikers voor bacteriën verminderen maar ook tegelijkertijd de bacteriën doden door de productie van waterstof peroxide.

De toepassingen van waterstof peroxide zijn niet beperkt tot het doden van bacteriën. Deze stof is ook een krachtig bleekmiddel. Het kan dingen witter maken door kleuren te verwijderen. Daarom kunnen koolhydraat oxidases gebruikt worden in wasmiddelen waarbij ze binden aan vlekken en waterstof peroxide maken om het te ontkleuren. Ook in de verwerking van bloem of witte suiker kunnen deze enzymen gebruikt worden om het preparaat witter te maken en meer verfijnd uit te laten zien.

Zoals u kunt zien, zijn we ver gekomen vanaf 1877 toen de Duitse fysioloog Wilhelm Kühne voor het eerst de term *enzym* gebruikte en beschreef als "de ongevormde of niet georganiseerde fermenten, wiens werking zonder aanwezigheid van organismen en buiten diezelfde kan plaatsvinden."

De onderzoeksresultaten

In hoofdstuk 2 van dit proefschrift beschrijf ik een nieuwe “groene” methode ter vervanging van twee traditionele assays die hedendaags veel gebruikt worden in de zoektocht naar effectieve enzymen om “groenere” brandstoffen mee te maken. Deze traditionele assays gebruiken hoge temperaturen en resulteren in giftig afval. Met de ontwikkelde “ChitO assay” bieden we een manier om het gehele proces meer milieuvriendelijk te maken door traditionele chemicaliën te vervangen met enzymen.

In hoofdstuk 3 beschouwen we niet alleen de kracht van het met kennis-gestuurde enzym engineering maar kunnen we ook zien hoe de specificiteit van een enzym slechts aan drie residuen kan worden toegeschreven. Door mutatie van drie aminozuren in het actieve centrum van een chitooligosaccharide oxidase, kon een lactose oxidase worden verkregen. Dit werk zou ook gebruikt kunnen worden om het mogelijke substraat bereik te voorspellen van nieuwe ontdekte koolhydraat oxidases.

In hoofdstuk 4 wordt dit principe verder onderbouwd. Een nieuw koolhydraat oxidase, XylO is ontdekt, gekarakteriseerd en de kristal structuur opgehelderd. XylO heeft een zeer grote gelijkenis met andere bekende koolhydraat oxidases, en toch kan het slechts een hele specifieke nieuwe klasse van suikers oxideren. Door het actieve centrum te analyseren kan worden gespeculeerd over de oorzaken van de beperkte specificiteit. Dit werk voegt een puzzelstuk toe over het substraat bereik van koolhydraat oxidases die kan helpen in toekomstig werk om *ad hoc* specificiteit te ontwerpen middels mutagenese of door *de novo* ontwerp.

Hoofdstuk 5 laat zien dat onderzoek onvoorspelbaar is qua resultaat en, ondanks je beste inspanningen, niet altijd een oplossing biedt voor een vraagstuk. Specifiek hebben we twee nieuwe oxidases ontdekt van de schimmel *Myceliophthora thermophila* waarvoor echter geen substraat gevonden kon worden. De opgehelderde atomaire structuren van deze oxidases geven een beeld van twee eigenaardige actieve centra die niet eerder zijn gezien bij oxidases.

Zou dit een nieuwe klasse van enzymen kunnen zijn? Toekomstige onderzoek zal het hopelijk uitwijzen.

Chapter 8

Riassunto in Italiano

Alessandro R. Ferrari

Questa tesi di dottorato rappresenta la condensazione di quattro anni di eccitanti scoperte, notti insonni, esperimenti riusciti al primo tentativo, esperimenti falliti al centesimo ma in ogni caso i migliori quattro anni della mia vita (fino ad'ora) sotto la supervisione del Prof. Marco Fraaije. Il tutto nella cittadina di Groningen in quella piccola nazione chiamata Olanda dove gli abitanti sono tutt'altro che piccoli. Ad averlo saputo prima, uno alto 167,5 cm (ci tengo a riportare i 0,5 cm) si trovava un'altra nazione in cui vivere.

In questo spazio cercherò, nel più breve tempo possibile di raccontarti, mio caro lettore, cosa ho fatto durante questi quattro anni. Perché, diciamocelo chiaramente, solo mia madre leggerà questa parte.

Innanzitutto devi capire cosa è un'enzima. Facile, immagina una fabbrica specializzata nel creare un solo prodotto. Oppure immagina una macchina capace di compiere un solo compito ma svolto con perfetta efficienza. Un enzima è tutto ciò ma in una scala talmente piccola che per poterlo vedere ti serve un microscopio elettronico super costosissimo e, di ciò che vedresti, comunque non capiresti nulla.

Un enzima è la più piccola macchina biologica che la natura ha creato. Una macchina in grado di compiere un certo processo in maniera efficiente, veloce e precisa.

Gli enzimi si trovano a migliaia nel nostro corpo (un'enzima ti aiuta a digerire la pasta, per esempio), negli animali, negli insetti, nei funghi e nei batteri. Inizialmente, gli enzimi per essere studiati, venivano estratti da questi organismi e isolati ma ciò richiedeva tantissimo tempo ed era letteralmente come trovare un ago in un pagliaio (ma ancora più difficile, se pensi quanto piccoli gli enzimi siano). Da quando hanno imparato a manipolare il DNA, gli scienziati hanno smesso di dipendere dagli organismi in cui gli enzimi vengono originalmente prodotti e iniziato a "trapiantare" gli enzimi in microorganismi che li possono creare più velocemente e abbondantemente. Questa tecnica si chiama clonaggio (non da confondere con clonazione, come quella della pecora Dolly).

Durante il mio lavoro ho prodotto enzimi di un fungo nel famoso batterio che noi tutti abbiamo nell'intestino, chiamato *Escherichia coli*. Ho preso il gene dell'enzima dal fungo e lo fatto "esprimere" (si dice così) in *Escherichia coli*. Gli scienziati usano abbondantemente questo batterio perché si riproduce molto velocemente (ogni 20 minuti si divide) ed è facilissimo da usare. Unico problema, la puzza nauseante che produce. Ma come sempre, in biologia, e nella vita in generale, bisogna scendere a compromessi.

Durante il mio dottorato ho lavorato su una classe specifica di enzimi chiamata *carbohydrate oxidases* che più o meno può essere tradotta in italiano come carboidrato ossidasi anche se non ho la più pallida idea se abbia appena inventato la parola o se esista davvero.

Questi enzimi prendono degli zuccheri e li convertono in composti acidi. Prima di darmi dello squilibrato, pensa che questo processo viene vastamente utilizzato da tantissime aziende nel mondo. Ne parlo vastamente nell'introduzione.

In ogni caso qui ti faccio un breve riassunto poiché mannaggiaallamiserianonsail'inglese.

Innanzitutto, quando questi enzimi convertono gli zucchieri in acidi, creano acqua ossigenata. L'acqua ossigenata, come ben sai, uccide i batteri. Quindi puoi immaginare come mettendo una carboidrato ossidasi in un dentifricio, non solo rimuovi dai denti gli zuccheri che verrebbero usati dai batteri per creare le carie, ma anche uccidi i batteri stessi usando l'acqua ossigenata prodotta. Forte, no?

L'acqua ossigenata ha anche altri vantaggi. Ad esempio è uno sbiancante. Nell'industria della carta che ti ricordo è fatta di cellulosa che a sua volta è fatta di zuccheri, le carboidrato ossidasi vengono usate per sbiancare la polpa che viene processata per divenire carta. Lo stesso vale per l'industria tessile, quella dei detergenti, e nella produzione di zucchero raffinato da quello di canna e persino nella produzione del pane.

L'uso delle carboidrato ossidasi ha un vantaggio rispetto ai tradizionali composti chimici. Per prima cosa non inquinano.

Secondariamente, non sono tossiche e possono essere usate liberamente nell'ambiente o in prodotti per il consumo umano (come il pane e lo zucchero appunto).

Queste sono solo alcune delle più importanti applicazioni delle carboidrato ossidasi ed il campo è piuttosto vasto.

Nello specifico io ho scoperto una nuova carboidrato ossidasi capace di funzionare solo su zuccheri che si ottengono dall'emicellulosa. Inoltre ho ingegnierizzato una carboidrato ossidasi cambiando lo zucchero che accetta, un po' tipo cambiare il motore ad una macchina per renderla più potente. Ho inoltre creato un metodo per scoprire enzimi che creano biofuel che si basa su una strategia più eco-sostenibile, comparata a quella che è attualmente in uso.

Grazie per aver letto fin qui.

Chiaramente, se hai letto fin qui, sei mia madre.

Appendices

Curriculum vitae

Alessandro R. Ferrari was born in Bari (Italy) on the 1st of June 1988. He first obtained a BSc degree in medical and pharmaceutical biotechnology at the University of Bari. During this time he spent 6 months at the National Council of Research (CNR) in Bari for an internship in the group of Prof. G. Pesole. In 2011 he participated at the iGEM competition working for 2 months at the École polytechnique fédérale de Lausanne in Lausanne (Switzerland) under the supervision of Prof. Bart Deplancke and Prof. Sebastian Maerkl. In 2012, as part of the MSc degree in medical biotechnology and molecular medicine at the University of Bari, he did his Masters project in the group of microbial physiology led by Prof. Lubbert Dijkhuizen at the University of Groningen. In December 2016 he joined, as PhD student, the group of Prof. Marco W. Fraaije. The research was part of the TASC Technology Area Biomass framework funded by the Netherlands Organization for Scientific Research (NOW). The research aimed at the discovery and characterization of carbohydrate oxidases for industrially relevant purposes.

List of publications

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When people think of the office they imagine boring and sad moments in a person's day. In the "Kings' Office" that couldn't have been the furthest away from the truth. Meneer Geoffrey, it was a real pleasure to have shared the office with you, you made me laugh a lot and it was too bad that it had been such a short time. Meneer Willem thanks for just being you, for your funny blunt comments and for inspiring me with your great graphic designs. Hemant, I'm really happy to have had you in the office: your permanent smile and steady calm were a great treasure especially in those "no-days". Lastly, to our new two office additions. Mohamed, thanks for your big loud laughter and Elisa thanks for your never ending speeches and our funny conversations.

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inveniet viam aut faciet.
— Seneca