Chapter 14

Microbial α-Glucuronidases

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Microbial α-glucuronidases play a crucial role in the enzymatic degradation of xylan in nature. They specifically hydrolyze the α-1,2-linkage between 4-*O*-methyl glucuronic acid and the xylose units of small oligosaccharides, liberated from polymeric xylan by the action of *endo*-β-1,4-xylanases. This review will focus on the assay for α-glucuronidase activity, the control of production of α-glucuronidase activity by microorganisms, their biochemical properties and substrate specificity, as well as the recently described crystal-structure of a microbial α-glucuronidase.

Introduction

Xylan is one of the main non-cellulosic polysaccharides found in plant cell walls and makes up between 10 and 35% of the dry weight. It is an abundant and renewable carbon source and its hydrolysis is important to many commercial processes. The hydrolysis of xylan is also central to the carbon cycle in nature, making the sugars locked up in the intact plant cell wall available for growth (*1*). Xylan consists of a backbone of 1,4-linked β-xylose units substituted with arabinofuranose, 4-*O*-methyl glucuronic acid and acetyl

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esters (*2*). The complete degradation of xylan requires the concerted action of the main chain cleaving *endo*-β-1,4-xylanase, β-xylosidase as well as the side-chain hydrolyzing enzymes, α-arabinofuranosidase, α-glucuronidase and acetylxylan esterase. Several α-glucuronidase enzymes have been purified and studied to date and the reaction mechanism has been elucidated. Sequence data has led to the clustering of microbial α-glucuronidases into a single family and the first crystal structure of a microbial α-glucuronidase has resulted in additional insight into the biochemical properties of these enzymes.

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Enzyme assay

The assay for α-glucuronidase activity is based on the hydrolysis of the α-1,2 bond between 4-*O*-methylglucuronic acid and the xylose unit of small xylo-oligosaccharides (*3*). These substrates are prepared from acid or enzymatic hydrolyzates of xylan using anion exchange chromatography to separate the acidic and neutral oligosaccharides followed by gel filtration to separate the different aldouronic acids (*4*). Alternatively polymeric xylan or native lignocellulosic substrates are used in conjunction with high amounts of xylanase activity, generating the aldouronic acid substrates *in situ* (*2*). The colorimetric detection of the 4-*O*-methylglucuronic acid released is the most commonly used method for determining α-glucuronidase activity (*5*). This method employs a modification of the Somogyi-Nelson procedure for the determination of reducing sugars (*6*), using high salt and low copper concentrations, for the specific detection of uronic acids (*7*). α-Glucuronidase activity has also been measured by determining the amount of product released by high-performance liquid chromatography (*3, 8*), and by gas-liquid chromatography (*4*).

p-Nitrophenyl-glycosides have been used extensively as synthetic substrates in the assay of several glycosidase activities. Microbial α-glucuronidases are; however, unable to hydrolyze *p*-nitrophenyl-glucuronide and only α-glucuronidases from mollusks have been shown to be able to use it as a substrate (*4,9-11*). A novel assay has been proposed using 4-nitrophenyl 2-(4-O-methyl-α-D-glucopyranuronosyl)-β-D-xylopyranoside as a substrate. Its hydrolysis by α-glucuronidase is coupled with the hydrolysis of the *p*-nitrophenyl xylopyranoside product by excess exogenous β-xylosidase activity. The aldobiouronic acid part of the structure serves as the recognition element in the substrate and the presence of the *p*-nitrophenyl group does not appear to hinder substrate binding or hydrolysis of the α-1,2 glycosidic bonds. This assay offers an improvement over the colorimetric procedure, routinely used, as it is not sensitive to the presence of salts or other reducing sugars in reaction mixture. The assay has the additional advantage of being highly specific, as no

p-nitrophenol is produced in the absence of α-glucuronidase activity (*12*), and it has already been used successfully in the characterization of *Cellvibrio japonicus* α-glucuronidase (*13*). The utility of the assay is at present limited by the availability of the substrate and the requirement for a pure β-xylosidase.

Production of α-glucuronidase activity by microorganisms

Several studies have been performed on different microorganisms in which the effect of carbon source on the production of α-glucuronidase activity has been investigated. These studies seldom use the same collection of carbon sources, making direct comparisons difficult. There are definite differences between microorganisms in terms of induction of α-glucuronidase activity, although a few general principles apply. The production of α-glucuronidase by most microorganisms requires growth in the presence of xylan-based or lignocellulosic carbon sources, while low levels of α-glucuronidase activity are constitutively produced in the absence thereof. This notion is supported by the fact that every α-glucuronidase purified so far was from an organism cultivated on polymeric xylan or lignocellulosic material. *C. japonicus* cultivated in Luria-Bertani broth produced α-glucuronidase activity in the presence of several xylan polymers, but no activity in the absence thereof (*14*). The same effect was observed for *Streptomyces olivochromogenes* and *Streptomyces flavogriseus* cultivated in the presence and absence of 1% oat spelt xylan or lignocellulosic material, although low activity was detectable without the inducers (*15*).

The oligomeric breakdown products of xylan, rather than the constituent monosaccharides, act as inducers for the production of α-glucuronidase activity (*14,16*). A sevenfold increase in α-glucuronidase activity in the culture filtrate of a *Thermoanaerobacterium* sp. was noted when polymeric xylan was used as carbon source instead of xylose (*16*). Similarly, no α-glucuronidase activity was found when *C. japonicus* was cultivated on glucuronic acid. Notably, induction of α-glucuronidase is repressed by the presence of glucose. α-Glucuronidase activity was induced when *C. japonicus* was grown on 1% oat spelt xylan as sole carbon source. However, production of the enzyme was completely repressed when the medium contained 1% glucose in addition to 1% oat spelt xylan (*14*). In the case of *Fibrobacter succinogenes*, growth on pure cellulose leads to poorer induction of α-glucuronidase activity compared to growth on lignocellulose (*17*). Contrary to these findings, no difference in activity was observed in the rumen anaerobic fungus *Piromonas communis* when grown on cellulose compared to when grown on xylan (*18*).

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Northern analysis supports the enzyme activity data. The *T. reesei glr1* gene was the most highly induced by growth on xylobiose, an oligosaccharide breakdown product of xylan, and not induced at all by growth on xylose. This induction was repressed by the presence of glucose. The glucose repressor protein Cre1 is most likely not involved in glucose-induced repression of the transcription of the *glr1* gene, as the *glr1* transcript was not detectable in the *T. reesei cre*1-1-mutant strain Rut-C30 when cultivated on glucose. Induction of the *glr1* gene was also observed on glucuronoxylan from beech, arabinoxylan from oat spelts and unsubstituted xylan from birch, as well as cellulose and arabitol. Induction of the *glr1* gene was also observed in glucose-depleted medium.

A study of the effect of different carbon sources on the transcript levels of the *aguA* gene of *Aspergillus tubingensis* showed that transcription was induced by the presence of xylose, xylobiose and birchwood xylan but not glucuronic acid in the culture medium (*19*). The transcription of the *aguA* gene was attenuated in the presence of glucose in the medium in addition to xylose and completely inhibited when glucose was added with xylan. In *A. niger*, induction of *aguA* by xylose and xylan is mediated through XlnR, a transcriptional activator that controls the expression of several xylanolytic enzymes. No induction of the *aguA* gene was observed in a strain containing a functional mutant of *xlnR* (*20*). The transcription factor was found to recognize a divergent XlnR binding site in the promoter region of the *aguA* gene. Two functional CreA binding sites were additionally found in the areas upstream of the *aguA* coding sequence, indicating that glucose repression is mediated through CreA in *A. tubingensis*. Evidence was also found for an XlnR-independent regulatory system that induces the transcription of the *aguA* gene in response to glucuronic acid or galacturonic acid, the former being a product of xylan degradation (*21*).

The production of the complement of enzymes that degrade xylan to catabolizable monosaccharides requires a substantial energy investment on the part of the organism that produces them. The cellular location of the accessory enzymes produced by each organism is therefore likely a reflection of the strategy it follows to gain preferential access to the nutrients released from the degradation of xylan and its lifestyle. Most of the α-glucuronidases studied thus far appear to be secreted although investigation of the exact localization of the enzyme activity is not always performed or reported.

There are exceptions where the activity remains cell-associated or intracellular. Two notable examples are the intracellular α-glucuronidase from

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Bacillus stearothermophilus (*22*) and the cell surface associated α-glucuronidase from *C. japonicus* (*14*). *B. stearothermophilus* protects substrate access by transporting aldotetraouronic acid, produced by the action of an extracellular xylanase (*xynA*), across the cell wall via a putative four protein membrane transporter (*orf1-4*) that also demethylates *O*4 of the aldouronic acid. It is subsequently cleaved to xylose and glucuronic acid by an intracellular α-glucuronidase (*aguA*), xylanase (*xynA2*) and β-xylosidase (*xynB*). All of the genes for glucuronic acid utilization were found on a single cluster in the genome of the organism. *C. japonicus* follows a different strategy by localizing the xylanolytic accessory enzymes on its cell surface, thereby hydrolyzing the small oligosaccharides, generated by the action of a secreted xylanase, to monosaccharides in close proximity to the membrane transporters that carry them across the cell wall. Other cell-associated α-glucuronidases have been purified from *Aspergillus niger* (*23*), *Thermoanaerobacterium* spp. (*9,16*) and *Clostridium stercorarium* (*9*).

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Biochemical properties of purified α-glucuronidases

The existence of an enzyme capable of releasing glucuronic acid from glucuronoxylan was first observed in the culture supernatant of *T. reesei* (*24*) and several microbial α-glucuronidases have been purified and studied to date (Table 1). Based on primary sequence, all of the known α-glucuronidases are assigned to family 67 of the Carbohydrate Active enZYmes, and they are the sole members of this family (http://afmb.cnrs-mrs.fr/CAZY/). There are two distinct groups in this family based on sequence similarity and quaternary structure. The fungal α-glucuronidases are monomeric proteins with molecular weights between 90 and 150 kDa and acidic pH optima, ranging from 3.0 to 4.8. In contrast, bacterial α-glucuronidases are dimeric proteins with subunits with monomeric molecular weights of around 70 kDa. An exception is the recombinantly expressed *Thermotoga maritima* enzyme that shows a variable oligomeric structure in response to changing salt concentrations. At low salt concentrations the enzyme occurs as an oligomer with a molecular weight in excess of 630 kDa, while in the presence of high salt concentrations the enzyme shows both a hexameric (450 kDa) and a dimeric (140 kDa) conformation (*25*). The pH optima for the bacterial α-glucuronidases, although acidic, are generally higher than those for the fungal α-glucuronidases, ranging from 5.4 to 6.5.

The kinetic properties of several of the purified α-glucuronidases have been investigated. Several aldouronic acids have been used as substrates, which makes direct comparisons between enzymes from different species difficult.

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Table 1. Properties of purified α-glucuronidases

Organism	M_r *(kDa)*	*pH*[c]	*T*[c] *(°C)*	V_{max} *(U/mg)*	K_m *(mM)*	*Substrate specificity**
Eukaryotes						
Aspergillus niger (23)	150[a], 130[b]	4.8	60	1.4	0.37[4]	tetrao, not pNP-GlcU
Aspergillus niger (23)	150[a], 130[b]	4.8	60	4.7	0.47[4]	tetrao, not pNP-GlcU
Aspergillus tubingensis (19)	100[a], 107[b]	4.5-6.0	70	52	0.14[2/3]	trio, trace xylan
Phanerochaete chrysosporium (8)	112[b]	3.5	nr	4.5	nr	trio>tetrao, not bio, trace xylan
Shizophyllum commune (26)	125[b]	4.5-5.5	nr	18	nr	trio, xylan
Thermoascus aurianticus (5)	118[a], 117[b]	4.5	65	4.0	0.14[4]	bio to octao, xylan
Trichoderma reesei (27)	91[b]	4.5-6.0	nr	28	nr	trio>tetrao>pentao=bio, trace xylan
Helix pomatia (28)	180[a], 97[b]	3.0	50	2.8	17.6[2]	pNP-GlcU, trio to pentao
Prokaryotes						
Bacillus stearothermophillus (11)	150[a], 78[b]	6.0	65	42	0.2[4]	tetrao, not pNP-GlcU, not xylan
Bacillus stearothermophillus (29)	161[a], 78[b]	6.5	40	15.3	0.78[3]	trio, not xylan
Cellvibrio japonicus (14)	150[a], 83[b]	6.3	nr	84.5	nr	pentao>tetrao>trio>bio, not xylan
Clostridium stercorarium (9)	124[a], 72[b]	6.0	nr	1.7	nr	tetrao, not pNP-GlcU, not xylan
Thermoanaerobacterium sp. *(16)*	130[a], 74[b]	5.4	60	8.4	0.76[3]	bio>trio>tetrao, trace xylan
Thermoanaerobacterium saccharolyticum (9)	118[a], 71[b]	6.0	nr	10	nr	tetrao, not pNP-GlcU, not xylan
Thermotoga maritima (25)	140[a], 79[b]	6.3	85	31	0.95[3]	trio

[a] determined by gel filtration, [b] determined by SDS-PAGE, [c] pH and temperature optima

nr – not reported

the superscripted number above the K_m-value indicates the degree of polymerisation of the aldouronic acid substrate used

* substrates tested and substrate preference where known, only prefix of aldouronic acids given

The specific activities for the purified enzymes range between 1.4 and 84.5 U/mg protein and the α-glucuronidases have K_m-values for aldouronic acids oligomers in the millimolar range. As a result of the complex structure of the xylan polymer and the variety of possible hydrolysis products, a number of issues arise in terms of substrate specificity.

Most of the microbial α-glucuronidases appear to have only very little or no activity against polymeric xylan compared to aldouronic acid oligomers. The only two exceptions are the α-glucuronidases from *Schizophyllum commune* (*26*) and *Thermoascus aurantiacus* (*5*). The latter enzyme was capable of releasing 4-*O*-methyl glucuronic acid from birchwood xylan at half the rate observed for aldotriouronic acid. Paradoxical results have been found by two studies that investigated the effect of the presence of substituents on the release of 4-*O*-methyl glucuronic acid from birch glucuronoxylan (*5,27*). *T. reesei* enzyme was capable of releasing about five times more 4-*O*-methyl glucuronic acid from acetylated than deacetylated birch xylan. Conversely, *S. commune* enzyme was capable of releasing five times more 4-*O*-methyl glucuronic acid from deacetylated than acetylated glucuronoxylan. The latter result would fit better with the current paradigm on xylan degradation, as it is believed that the presence of substituents limits accessibility of the enzyme to neighbouring glycosidic linkages.

The length of the aldouronic acid oligosaccharide may also have a marked effect on activity. The *C. japonicus* enzyme shows a preference for longer substrates and turnover number increases two-fold in the range from aldobiouronic to aldopentaouronic acid (*30*). The opposite trend is observed for the α-glucuronidases from *Thermoanaerobacterium* sp., *Phanerochaete chrysosporium* and *T. reesei* (*8,16,27*). This group of enzymes shows increasing activity as the chain length decreases to aldotriouronic acid. However, all three enzymes differ in their preference for aldobiouronic acid. *Thermoanaerobacterium* sp. α-glucuronidase shows its highest reaction rate on aldobiouronic acid, while the *T. reesei* enzyme has its lowest reaction rate on this substrate and *P. chrysosporium* enzyme cannot hydrolyze it at all. Chain length may also not influence activity to a significant degree, as is the case for the *T. aurantiacus* enzyme. This α-glucuronidase hydrolyzes aldobiouronic to aldooctaouronic acid at the same rate and maintains half of the rate of activity it has on aldouronic acids on the xylan polymer (*5*).

It appears to be a general rule that α-glucuronidases have a preference for 4-*O*-methyl glucuronic acid substituents on the terminal non-reducing end xylose unit of the oligosaccharide and almost all of the studies on substrate specificity have used substrates of this nature. Only one study so far shows

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direct evidence to this effect. The α-glucuronidase from *A. tubingensis* was found to lack activity against two aldouronic acid substrates containing non-terminal 4-*O*-methyl glucuronic acid substituents (*12*). Additional evidence for this fact comes from studies on the effect of combinations of α-glucuronidase and other xylanolytic enzymes on the amount of 4-*O*-methyl glucuronic acid released from xylan. In a study on the *T. reesei* α-glucuronidase, it was observed that only the combination of α-glucuronidase, xylanase and β-xylosidase resulted in release of significant amounts of 4-*O*-methyl glucuronic acid. Very little 4-*O*-methyl glucuronic acid was released by the combination of only α-glucuronidase and xylanase (*27*). Xylanases of family 11, such as those used in this study, release oligosaccharides with the substituents on non-terminal xylose units as they cannot access the xylosydic bond adjacent to substituted xylose units. Xylosidase activity is strictly required to remove the unsubstituted non-reducing end xylose units, resulting in the production of aldouronic acid oligomers carrying the 4-*O*-methyl glucuronic acid linked to the terminal non-reducing end xylose unit.

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Reaction mechanism of α-glucuronidase

Glycoside hydrolases follow either a retaining mechanism, involving two steps and an enzyme-bound glycoside intermediate, or an inverting mechanism involving a single step direct displacement by water at the anomeric center. Both mechanisms involve an acid/base catalytic pair. The distance between the catalytic residues differs, being approximately 5Å in enzymes following a retaining mechanism, and between 9Å and 10Å in enzymes following an inverting mechanism, allowing for the presence of nucleophilic water for direct attack at the anomeric center (*31*). The *A. tubingensis* α-glucuronidase enzyme is the only α-glucuronidase that has been investigated specifically in terms of its reaction mechanism. The authors used proton-NMR to show that the α-anomer of the 4-*O*-methyl glucuronosyl residue in the aldotetraouronic acid substrate was converted to the β-anomer in the free 4-*O*-methyl glucuronic acid that was produced, indicating an inverting reaction mechanism (*12*). The highly conserved nature of the family 67 α-glucuronidases makes it likely that they all follow the same mechanism and this notion is supported by crystallographic data on the distance between the catalytic carboxylates in the *C. japonicus* α-glucuronidase, as well as the anomeric configuration of the reaction products co-crystallized with the enzyme (*30*).

Crystal structure of α-glucuronidase

The crystal structure of GlcA67A, the α-glucuronidase from *C. japonicus* has recently been solved and has added significant insight into the reaction mechanism and substrate specificity of this enzyme. The enzyme is a dimer, consisting of two identical subunits, each containing three domains. The enzyme can be described as being butterfly-shaped when it is oriented so that the dimer-surface runs vertically along the central axis, with the N-terminus at the top and the C-terminus at the bottom. The N-terminal domain forms the "top wing of the butterfly" and contains a two-layer β-sandwich, with each β-sheet made up of five strands. It also contains two small helices that interact with the central catalytic domain. The central domain itself is situated below the N-terminal domain and makes up the "bottom wing of the butterfly". It has a $(\beta/\alpha)_8$ barrel structure and contains the catalytic residues on the C-terminal side of the protein. The C-terminal domain is situated behind the catalytic domain, covering one of its faces, and is primarily made up of α-helices. It forms the largest part of the dimer surface and also has interactions with the α-helices of the N-terminal domain (*30*).

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Co-crystallization of native GlcA67A with the reaction products glucuronic acid, xylobiose and a combination of glucuronic acid and xylotriose revealed the position and identity of the catalytic acid and several of the interactions that contribute to substrate specificity (Figure 1) (*30*). A catalytically inactive mutant of GlcA67A, E292A was used to characterize the enzyme-substrate complex and kinetic characterization of mutants of evolutionarily invariant amino acids in GlcA67A further confirmed their role in substrate recognition (*13*). The catalytic acid involved in the hydrolysis reaction was identified as Glu292 based on its position and distance relative to *O2* of the terminal xylose unit. No acidic side-chain apart from Glu292 was found to be close enough to donate a proton to the glycosidic-bond oxygen. A greater than million-fold decrease in activity was observed after mutation of this residue to alanine or cysteine. The identity of the catalytic base that activates water for nucleophilic attack at the anomeric carbon is uncertain. Both Asp365 and Glu393 are ideally positioned at the β-face of the glucuronic acid to fulfill this role and mutation of either amino acid to alanine results in a greater than a million-fold reduction in activity. The position of the glucuronic acid product in the native enzyme is very similar to that of the 4-*O*-methyl glucuronic acid substrate in the E292A mutant. Both are in a $^4C_1$ chair conformation and apart from the change from the α-anomer in the substrate to the β-anomer in the product, the pyranoid ring is also rotated by 27° around the position of the C4 carbon. The position of the C1 atom in the product, 1.4 Å further away from the catalytic base/s and the α-position of the hydroxyl, create the ideal position for a water molecule to attack

C1. In this position the water molecule can be activated by either of the catalytic bases (*13,30*).

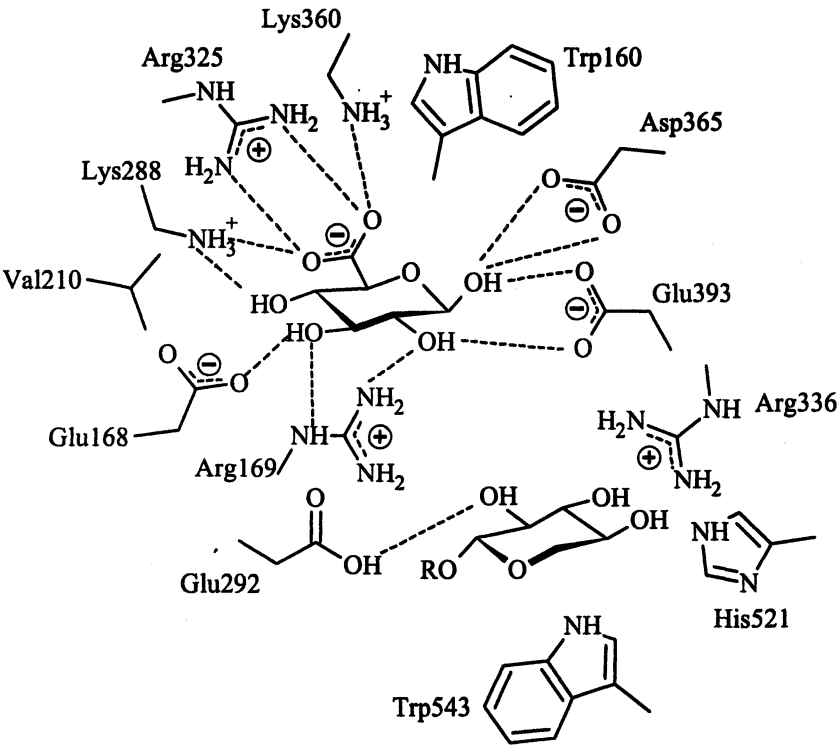

Figure 1. Schematic representation of the recognition elements involved in the binding of the reaction products to *C. japonicus* α-glucuronidase, GlcA67A (*14*).

The substrate binding site of the enzyme consists of a deep, partially hydrophobic pocket, with the specific recognition elements for glucuronic acid and the terminal non-reducing end xylose unit situated in the deepest recesses of the pocket. The terminal xylose unit is completely enclosed at the non-reducing end by Tyr329 and Arg336, the side-chain of the latter being able to form hydrogen bonds with *O*3 and *O*4 of this xylose unit. This feature explains the inability of the *C. japonicus* α-glucuronidase to hydrolyze 4-*O*-methyl glucuronic acid side-chains from internally positioned xylose units. The terminal xylose unit is also in an ideal position to form π-stacking interactions with Trp543, stabilizing its position in the active center. In the E292A mutant

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co-crystallized with aldotriouronic acid, the position of the non-reducing end xylose unit could not be determined, as it had no ordered structure. This finding might challenge the importance of the role of the xylose-Trp543 interaction in substrate recognition. Kinetic analysis of the W543A mutant of GlcA67A, however, revealed a 10^5-fold reduction in catalytic efficiency, confirming the essential nature of this interaction (*13,30*).

The 4-*O*-methyl glucuronic acid binding site is situated in the deepest part of the pocket and the carboxylate group is stabilized by three basic amino acids, namely Lys288, Arg325 and Lys 360. The importance of these interactions was confirmed by studying the kinetic properties of the three mutants K288A, R325A and K360A. Removal of any of these positively-charged side-chains led to at least 10^4-fold reduction in the catalytic efficiency. The most important of these interactions is that of the guanidinium group of Arg325 with the 4-*O*-methyl glucuronic acid carboxylate. It showed the largest reduction in catalytic efficiency when mutated, is geometrically and spatially in the most complementary position relative to the carboxylate group, and also has a higher pK_a in solution than the lysine amino groups. Further evidence for the importance of the 4-*O*-methyl glucuronic acid carboxylate in substrate recognition comes from the observation native GlcA67A has a twenty-fold higher K_i for glucose than for glucuronic acid (*13,30*).

Another structural recognition element is the methyl-group of 4-*O*-methyl glucuronic acid. In the crystal structure of the E292A mutant in association with its substrate, it was noted that the methyl-group is buried in a hydrophobic sheath made up by Trp160 and Val210. The role of the conserved Val210 is not clear as mutation of this amino acid to polar residues such as serine or asparagine did not have the same impact on catalytic efficiency as many of the other mutants studied. The inhibition constant of the native enzyme for glucuronic acid was, however, ~20 times lower than either V210N or V210S confirming the importance of a non-polar side-chain in close proximity to the methyl group. Trp160 is also ideally positioned to form π-stacking interactions with the surface of the pyranoid ring and the W160A mutant showed a 10^6-fold reduction in catalytic efficiency. The positioning of the glucuronic acid deeper in the active center in relation to the xylo-oligosaccharide implies the sequential release of first the xylo-oligosaccharide and thereafter the glucuronic acid and may have important implications in terms of the kinetic properties of the enzyme (*13,30*).

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The role of α-glucuronidase in xylan degradation

Few in-depth studies have investigated the synergistic role of α-glucuronidase in the enzymatic degradation of xylan (*8,19,26,27,32*). All of the studies addressed the effect of xylanase and xylosidase activity on the release of 4-*O*-methyl glucuronic acid from xylan. As expected, the addition of either xylanase or xylosidase activity to the reaction significantly increases the amount of 4-*O*-methyl glucuronic acid released from the xylan polymer by α-glucuronidase. The addition of both xylanase and xylosidase to α-glucuronidase resulted in a far greater release of 4-*O*-methyl glucuronic acid from xylan than the addition of either only xylanase or only β-xylosidase to α-glucuronidase. The combined action of xylosidase and xylanase leads to the release of small xylo-oligosaccharides substituted with 4-*O*-methyl glucuronic acid on the non-reducing end xylose unit. This type of compound has been shown to be the preferred substrate for most α-glucuronidases and the above-mentioned findings are thus in line with this view. It stands to reason that other substituents on the xylan backbone, such as acetyl and arabinofuranose groups, could hinder the access of the α-glucuronidase to the α-1,2 glycosidic bond. The addition of side-chain cleaving enzymes increased the amount of 4-*O*-methyl glucuronic acid released by α-glucuronidase, whether α-glucuronidase was applied alone or in combination with β-xylosidase or *endo*-β-1,4-xylanase or both (*8,26*).

The effect of α-glucuronidase activity on the release of reducing sugars from xylan by the action of xylanase, xylosidase or a combination of the two has also been investigated (*19,27,32*). The degree to which α-glucuronidase enhances the release of reducing sugars from xylan is dependent on the nature of the xylanase that is used. The addition of *T. reesei* α-glucuronidase to *T. reesei endo*-β-1,4-xylanase I enhanced the release of reducing sugars from xylan to a much greater degree than the addition of *T. reesei* α-glucuronidase to *T. reesei endo*-β-1,4-xylanase II. This result is unexpected as both *endo*-β-1,4-xylanases belong to family 11 of the carbohydrate active enzymes and share a similar active site architecture (*27*). The effect of α-glucuronidase on the release of reducing sugars from xylan is also more pronounced when β-xylosidase acts alone than when *endo*-β-1,4-xylanase acts alone or in combination with β-xylosidase. This can be attributed to the difference in the nature of the bonds that β-xylosidase and *endo*-β-1,4-xylanase attack. β-xylosidase is an exo-acting enzyme and the presence of any substituents linked to the terminal non-reducing end xylose unit will prevent it from hydrolyzing the terminal xylose, whereas the endo nature of xylanase will allow it to hydrolyze β-1,4-xylosidic linkages that might exist further down the chain. The

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relatively low degree of substitution of xylan with 4-*O*-methyl glucuronic acid (1/10 xylose units in hardwood xylan and 1/5 xylose units in softwood xylan) likely allows for a high degree of depolymerization by a combination of *endo*-β-1,4-xylanase and β-xylosidase. This leaves few xylosidic linkages to be hydrolyzed after removal of the 4-*O*-methyl glucuronic acid side-chains (*19,27,32*).

The complete hydrolysis of xylan is dependent on the removal of all substituents from the backbone. α-Glucuronidases play a critical role in this process by making additional sites available for the further action of *endo*-β-1,4-xylanases and β-xylosidases. The accumulated knowledge of the enzymatic properties and functioning of α-glucuronidases, as well as the elucidation of the crystal structure of a bacterial α-glucuonidase have added another piece into the puzzle of lignocellulose biodegradation.

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