Characterisation, cloning and heterologous expression of the α-glucuronidase from *Aureobasidium pullulans*

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Summary

Xylanolytic accessory enzymes produced by the *endo*-β-1,4-xylanase overproducing, colour-variant strain of the euascomycetous fungus *Aureobasidium pullulans*, NRRL Y-2311-1, were studied. α-Glucuronidase activity was only induced during cultivation on carbon sources containing both xylose and glucuronic acid. An α-glucuronidase was partially purified from the supernatant of *A. pullulans* cultivated on birchwood glucuronoxylan. The enzyme had an apparent mobility on SDS-PAGE of 170 kDa, and after deglycosylation its mobility shifted to 118 kDa, indicating an extensively decorated protein. Maximal activity was measured at pH 3 in McIlvaine's phosphate-citrate buffer and at 40°C, and the enzyme was stable for 3 h at 40°C. The enzyme displayed substrate inhibition, and K_m- and K_i-values were calculated as 3.3 ± 0.29 mM and 9.8 ± 3.8 mM for aldotriouronic acid and 29.5 ± 7.6 mM and 29.0 ± 7.8 for aldobiouronic acid respectively.

PCR methods were used to clone the genes encoding an α-glucuronidase and an α-L-arabinofuranosidase of *A. pullulans* NRRL Y-2311-1. The deduced amino acid sequence of the α-glucuronidase encoding gene, *aguA*, shared greater than 60% identity with fungal glucuronidases and between 34% and 42% identity with bacterial α-glucuronidases, and it is member of family 67 of the glycoside hydrolases. The *aguA* gene encodes a protein of 836 amino acids with a putative secretion signal of 15 amino acids, resulting in a mature protein with a predicted molecular weight of 91 kDa. The gene was expressed in *S. cerevisiae* Y294 under control of the *ADH2* promoter and terminator. The heterologous α-glucuronidase was purified to homogeneity using Ni-chelate affinity chromatography, and it had an electrophoretic mobility of 120 kDa on SDS-PAGE. The enzyme was maximally active at 65°C and between pH 5 and pH 6. The enzyme was stable at 45°C, lost half of its activity after 22.5 minutes at 55 °C, and had a half-life of 5.6 min at 65 °C. It was stable at pH 4 and pH 6, and had a half-life of 17 min at pH 8. The enzyme had K_m-values in the millimolar range for the series from aldobiouronic acid to aldopentaouronic acid. It had the highest catalytic efficiency on aldobiouronic acid and the catalytic efficiency decreased with increasing chain-length of the oligosaccharide substrate.

The deduced amino acid sequence of the α-L-arabinofuranosidase gene, *abfA*, shared between 69% and 76% identity with family 54 α-arabinofuranosidases. The gene encodes a polypeptide of 498 amino acids with a putative signal peptide of 20 amino acids resulting in a mature protein with a calculated molecular weight of 49.9 kDa. It was expressed in *S. cerevisiae* Y294 and the heterologous enzyme was purified to homogeneity by gel filtration. It's size estimated by gel filtration was 36 kDa, and it had an apparent mobility of 49 kDa on SDS-PAGE. It showed maximal activity at 55°C and between pH 3.5 and pH 4. It was stable at 50°C and between pH 4 and pH 5. The enzyme had a K_m

for *p*-nitrophenyl α-arabinofuranoside of 3.7 ± 0.36 mM and a V_{max} of 34.8 ± 1.1 U/mg protein. It displayed 0.2 U/mg activity against *p*-nitrophenyl β-xylopyranoside.

Opsomming

Hierdie studie het gefokus op xilanolitiese ensieme van die *endo*-β-1,4-xilanase oorproduserende, kleur-variante ras van die euaskomiseet *Aureobasidium pullulans,* NRRL Y-2311-1. α-Glukuronidase-aktiwiteit is slegs geïnduseer tydens groei op koolstofbronne wat beide xilose en glukuronsuur bevat. α-Glukuronidase is gedeeltelik uit die supernatant van *A. pullulans* gekweek op berkehout glukuronoxilaan gesuiwer. Die ensiem se elekroforetiese mobiliteit met SDS-PAGE was 170 kDa en na deglikosilering het dit verskuif na 118 kDa, beduidend van 'n swaar geglikosileerde ensiem. Maksimum aktiwiteit is gemeet by pH 3 in McIlvaine se sitraat-fosfaat buffer en by 40°C. Die ensiem was stabiel by 40°C tydens 'n 3-uur inkubasie. Substraat inhibisie is bespeur, en die ensiem se K_m- en K_i-waardes vir aldotriouronsuur was onderskeidelik 3.3 ± 0.29 mM en 9.8 ± 3.8 mM en vir aldobiouronsuur was die waardes onderskeidelik 29.5 ± 7.6 mM en 29.0 ± 7.8 mM.

PKR metodes is benut om die gene vir α-glukuronidase en α-arabinofuranosidase te kloneer. Die afgeleide aminosuurvolgorde van die α-glukuronidase geen, *aguA*, was meer as 60% identies aan swam α-glukuronidases, en tussen 34% en 42% identies aan bakteriële α-glukuronidases, en dit is 'n lid van familie 67 van die glikosied hidrolases. Die *aguA* geen kodeer vir 'n proteïen van 836 amienosure met 'n sekresiesein van 15 amienosure, wat die produksie van 'n volwasse protein met 'n molekulêre gewig van 91 kDa tot gevolg het. Die geen is uitgedruk in *S. cerevisiae* Y294 onder beheer van die *ADH2* promoter en termineerder. Ni-chelaat affiniteitschromatografie is gebruik om die heteroloë α-glukuronidase te suiwer. Die elektroforetiese mobiliteit van die suiwer ensiem was 120 kDa met SDS-PAGE. Die ensiem het maksimale aktiwiteit by 65°C en tussen pH 5 en pH 6 getoon. Die ensiem was stabiel vir twee ure by 45°C, het die helfte van sy aktiwiteit binne 22.5 minute by 55 °C verloor, en het 'n halfleeftyd van 5.6 minute by 65 °C gehad. Dit was stabiel by pH 4 en pH 6 vir twee ure, en het 'n halfleeftyd van 17 minute by pH 8 gehad. Die ensiem het millimolaar K_m-waardes getoon vir die substraatreeks vanaf aldobiouronsuur tot aldopentaouronsuur. Dit het die hoogste katalitiese effektiwiteit vir aldobiuronsuur gehad en die katalitiese effektiwiteit het afgeneem met toenemende lengte van die oligosakkaried substraat.

Die afgeleide amienosuurvolgorde van die α-L-arabinofuranosidase geen, *abfA*, was tussen 69% en 76% identies aan familie 54 α-L-arabinofuranosidases. Die geen kodeer vir 'n proteïen van 498 amienosure met 'n seinpeptied van 20 aminosure, wat lei tot die produksie van 'n volwasse proteïen met 'n berekende molekulêre massa van 49.9 kDa. Die geen is uitgedruk in *S. cerevisiae* Y294 en die heteroloë ensiem is gesuiwer deur gel filtrasie. Die ensiem se geskatte molekulêre gewig met gel filtrasie was 36 kDa, en die ensiem se mobiliteit op SDS-PAGE was 49 kDa. Dit het maksimum aktiwiteit getoon by 55°C, en tussen pH 3.5 en pH 4. Dit was stabiel vir twee ure by 50°C en tussen

pH 4 en pH 5. Die ensiem se K_m vir *p*-nitrofeniel α-L-arabinofuranosied was 3.7 ± 0.36 mM en die V_{max} was 34.8 ± 1.1 U/mg proteïen. Die ensiem het aktiwiteit teen *p*-nitrofeniel β-D-xilopiranosied van 0.2 U/mg getoon.

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Chapter 1

Preface and scope of this thesis

Microbial lignocellulolytic enzymes have been the subject of intense study for the last 50 years, as a result of their great potential in several industrial applications (Bhat, 2000). They act by degrading the structure of the plant cell wall and thereby release nutrients or enable entry into the plant cell. The plant cell wall is a complex matrix that is built up of a number of organic polymers. Long cellulose microfibrils make up the main structural components of plant cell walls. Interspersed with the microfibrils, are found a variety of shorter non-cellulosic polysaccharides, including hemicelluloses and pectic polysaccharides. Finally, lignin acts as a glue-like filler material in which the microfibrils are embedded (Fengel and Wegener, 1989). Microbial lignocellulolytic enzyme systems consist of a collection of different glycoside hydrolases, carbohydrate esterases and peroxidases, each targeting a specific type of bond in the structure of the plant cell wall. Consequently, the actions of the lignocellulolytic enzymes are complementary, with new sites of action being generated in every successive degradation step (Dekker, 1989).

Every industrial application has its own unique requirements in terms of which components of the plant cell wall that need to be degraded or left intact. For instance, in the process of biobleaching, the complete degradation and removal of lignin is required with the least possible damage to cellulose microfibrils. The partial degradation of xylan is also required, as it is covalently linked to lignin, aiding in its removal. To this end, *endo*-β-1,4-xylanase has been employed successfully, while the presence of cellulolytic enzymes is not desirable (Viikari *et al.*, 1994). A more complete set of lignocellulolytic enzymes, including high cellulase activity is, on the other hand, required in the conversion of biomass to fuel ethanol and the pretreatment of lower grade animal feeds. In the first instance the complete conversion of the lignocellulosic substrate to its component monosaccharides is required, while the latter case requires a high degree of degradation of all the plant cell wall components to increase their bioavailability in the gastrointestinal tract (Bhat, 2000).

The study of lignocellulolytic enzymes has followed a few central themes. Numerous studies have reported the discovery of novel enzyme activities from different microorganisms and the carbon sources that induce their production as well as the characterization of their enzymatic properties and substrate range and specificity (Biely, 1993). Reaction mechanisms and determinants of specificity have also been probed using several substrate analogues (Birsan *et al.*, 1998). An important facet of this research has always been the synergistic effects that play a role when several of the lignocellulolytic enzymes act together to degrade polymeric substrates, and also the influence that

each enzyme's unique substrate specificity has on the collective action of a group of enzymes (Dekker, 1989).

The advent of modern molecular genetics has permitted the cloning of a large number of the genes encoding these enzymes, and in many cases homologues of the same gene from different microorganisms (Sapag *et al.*, 2002). Sequencing of upstream regions have also led to the identification of promoter and repressor elements responsible for the control of expression of these genes. Several of the cloned glycoside hydrolase genes have either been overexpressed in their native organism or a heterologously expressed in another host, giving access to larger quantities of the enzyme and/or allowing easier purification of the enzyme (Kulkarni *et al.*, 1999). Sequence information has led to the classification of the glycoside hydrolases and carbohydrate esterases into several families based on sequence identity, highlighting conserved properties and mechanisms among family members (Henrissat and Davies, 1997) (http://afmb.cnrs-mrs.fr/CAZY/). The crystal structure of several glycoside hydrolases have also been solved and this has led to additional insights into reaction mechanisms and modes of substrate and product binding by these enzymes (Törrönen and Rouvinen, 1997). Genetic engineering techniques along with the structural information about the glycoside hydrolases has allowed for the successful manipulation of glycoside hydrolases to obtain novel activities or slightly altered properties that are better suited to the application at hand (Kulkarni *et al.*, 1999). Investigation of the physiological role of lignocellulolytic enzymes in the organism that produces them has also recently received more attention (Shallom and Shoham, 2003).

Colour variants of the black yeast *A. pullulans* were initially isolated for their ability to secrete large quantities of *endo*-β-1,4-xylanase activity, and were thought to be useful in paper pulping, as they secrete no cellulolytic enzymes (Leathers, 1986). Four *endo*-β-1,4-xylanases were identified in the culture supernatant of *A. pullulans* NRRL Y-2311-1, two of them purified and characterized (Li *et al.*, 1993) and the *xynA* gene encoding these enzymes cloned (Li and Ljungdahl, 1994), and expressed in *S. cerevisiae* (Li and Ljungdahl, 1996). A β-xylosidase has also been isolated from *A. pullulans* NRRL Y-2311-1, and its action in combination with the *endo*-β-1,4-xylanase studied (Christov *et al.*, 1997). Very recently, a cinnamoyl esterase was also purified from the culture supernatant of *A. pullulans* NRRL Y-23111-1 and characterized in terms of its enzymatic properties (Rumbold *et al.*, 2003). The presence of α-arabinofuranosidase and acetyl esterase activity has also been noted in the culture supernatants of *A. pullulans* cultivated on lignocellulosic carbon sources (Myburgh *et al.*, 1991). Very little is, however, known about other xylanolytic accessory enzyme activities from this organism, and the picture of how it degrades and survives on xylan is incomplete.

The broad focus of the work preformed in this thesis was the isolation of xylanolytic accessory enzymes from *A. pullulans*, the cloning of the genes encoding some of the accessory enzymes, their expression in *S. cerevisiae*, and the study of the heterologous enzymes.

The specific aims of this thesis were:

1. To test for α-glucuronidase in the culture supernatants of *A. pullulans*, as well as to purify and characterize the enzyme.
2. To clone genes encoding xylanolytic accessory enzymes from *A. pullulans*, and to express them in *S. cerevisiae*.
3. To purify the heterologous accessory enzymes and to characterize them in terms of their basic biochemical and enzymatic properties.

This knowledge will enable us to form a more complete picture of xylan degradation by *A. pullulans*, and the enzymes it employs to this end. It will also add to the basic body of knowledge about lignocellulolytic enzymes, their relatedness, and their enzymatic properties.

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Chapter 2

Microbial xylanolytic enzymes: production, reaction mechanisms, classification and expression in baker's yeast

Introduction

Xylan is one of the main hemicelluloses found in plant cell walls, contributing to between 10 and 35% of the dry weight (Timmel, 1967; Dekker, 1989). The enzymatic degradation of xylan is a central to the carbon cycle in nature, and an important step in several industrial processes. Enzymatic hydrolysis of xylan has several advantages over chemical methods including reduced toxic waste production and greater specificity in the hydrolysis of particular chemical bonds. Xylan hydrolysing enzymes have found applications in several commercial processes including the bleaching of native as well as recycled paper pulp, the pre-digestion of lower grade animal feeds, the improvement of the consistency of dough for bread baking and the treatment of lignocellulosic wastes from the agricultural and food industry (Beg *et al.*, 2001). Xylanolytic enzymes are produced by microbial saprophytes and plant pathogens that use them to break down the components of the plant cell wall for the purpose of invasion and/or use of the sugar constituents for growth (Dekker, 1989). This review will focus on the ultrastructure and chemical composition of plant cell walls with particular emphasis on xylan as well as its enzymatic degradation. The classification, properties and function of the side-chain cleaving α-arabinofuranosidase and α-glucuronidase will be discussed in detail. The heterologous expression of glycoside hydrolases in the baker's yeast *Saccharomyces cerevisiae* formed an important part of this study and previous work in this field will be highlighted. Finally, the physiology and ecology of *Aureobasidium pullulans*, the euascomycetous fungus that was used as source of the enzymes in this study will be discussed.

The structure and chemistry of plant cell walls

Plant cell walls serve several functions, determining the size and shape of the cell, controlling the flow of water and nutrients, providing mechanical strength, providing a barrier against infection and activating defence systems upon invasion. As a whole, the plant cell wall can be viewed as a composite material. It contains extensive layered networks of long cellulose fibres and shorter hemicellulose fibres that are cemented in an amorphous meshwork of lignin (Bacic *et al.*, 1988). Three layers are discernible in the plant cell wall (Figure 2.1). The primary wall is the first layer formed during the growth phase of the cell and has to be rigid enough to withstand the turgor pressure in the plant, while being compliant enough to allow for longitudinal growth. It contains a loosely organised network of cellulose microfibrils and some hemicelluloses and is between 0.1 and 0.2 μm across. In the specialised load-bearing cells of the plant, such as the phloem and xylem and the

sclerenchymal fibre cells, a thick secondary wall is deposited at the end of the growth phase. The secondary wall itself consists of three layers. The outer layer contains a criss-cross network of cellulose microfibrils and is between 0.1 and 0.3 μm thick. In the thick middle layer of the secondary wall (1 to 5 μm), the cellulose microfibrils run nearly parallel to the vertical axis of the cell. The inner layer is composed of a thin layer (0.1 μm) of microfibrils that run perpendicular to the middle layer and form a flat helix around the cell. The middle lamella, which is found between the surfaces of the primary walls of individual cells, contains mainly lignin. Lignification occurs after secondary wall formation via free-radical polymerisation of phenylpropane subunits and consequently penetrates any remaining spaces in the primary and secondary walls encasing the fibres in a glue-like meshwork (Timmel, 1967; Reiter, 2002). The individual components of the cell wall will subsequently be considered in more detail.

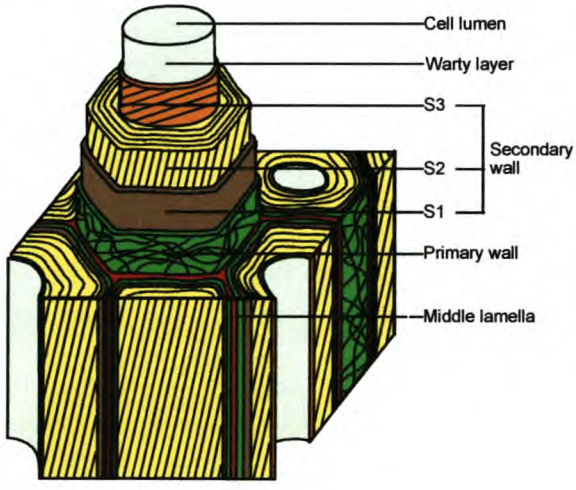

Figure 2.1. Schematic representation of the cell wall of a softwood tracheid of a hardwood fibre (Timmel, 1967). The middle lamella is shown in red the primary wall in green and the S1 layer in brown, the S2 layer in yellow and the S3 layer in orange. The lines represent the orientation of the cellulose fibres in the different cell wall layers.

Cellulose

Long cellulose microfibrils form the framework of the plant cell wall and are its main component, generally constituting between 40 and 50% of the dry weight of terrestrial plants. As cellulose is a constituent of all land plants it contributes to an estimated 40% of the organic matter found on earth. Cellulose is an entirely linear polysaccharide consisting of 1,4-linked β-D-glucopyranose units. The degree of polymerisation of cellulose is high and individual polymer chains usually contain between 10 000 and 15 000 glucose residues. The unbranched nature of the cellulose polymer, along with the fact that cellulose is composed of neutral carbohydrate moieties, allows for a high degree of intra- and intermolecular hydrogen bond formation. Cellulose is consequently found in nature as microfibrils of associated cellulose polymer chains that are mechanically strong, resistant to degradation and completely insoluble in water (Fengel and Wegener, 1989). The ordered structure of the cellulose microfibril and its orientation around the cell results from the manner in which cellulose is synthesised. Each microfibril contains 36 individual cellulose chains and assembles spontaneously during synthesis. Hexagonal rosette-like structures found in the plasma membrane are responsible for

cellulose synthesis and export. Each of the six rosette subunits is proposed to contain six 1,4-β-D-glucan synthases, resulting in the synthesis and export of 36 cellulose chains. The structure of the cellulose synthase complex allows for the ordered assembly of the individual chains into a microfibril and movement of the complex along the cellular cytoskeleton directs the orientation of the microfibrils around the cell (Reiter, 2002; Williamson *et al.*, 2002).

The hemicelluloses

Hemicellulose is a collective term describing a group of highly variable and substituted, alkali extractable, non-cellulosic polysaccharides. They constitute the second most abundant source of polysaccharide material in plant cell walls making up 20 to 30% of the dry weight. In contrast to cellulose, hemicelluloses have a low degree of polymerisation (>200 glycosidic units) and are usually soluble in alkaline solutions and sometimes even water. The carbohydrate components of hemicelluloses include xylose, mannose, glucose, galactose, arabinose and 4-*O*-methyl glucuronic acid. The hemicelluloses additionally contain esterified organic acids such as acetic acid and the cinnamic acids, ferulic and *p*-coumaric acid. Contrary to cellulose, the hemicelluloses are not synthesised at the plasma membrane, but in the Golgi cisternae and secreted by exocytosis (Brett and Waldron, 1996).

Xylan

Xylan consists of a linear chain of 1,4-linked β-D-xylopyranose units substituted with α-L-arabinofuranose, 4-*O*-methyl glucuronic acid, acetic acid esters and cinnamic acid esters depending on the plant species and tissue (Figure 2.2). In hardwoods, xylan generally contributes to between 15% and 30% of the dry weight and can be described as *O*-acetyl-4-*O*-methyl glucuronoxylan. About 70% of the xylose residues are esterified with acetyl groups at C2 or C3 or both, while one in ten xylose residues are substituted with α-1,2-linked 4-*O*-methyl glucuronic acid. Alkaline extraction of hardwood hemicelluloses saponifies the acetyl esters and if acetylated xylan is required extraction should be performed with DMSO or the deacetylated xylan can be chemically acetylated. The xylan of hardwood may contain two to three short side-branches per polymer molecule. The average degree of polymerisation of xylans isolated from hardwood has been between 100 and 200 xylose units (Timmel, 1967; Fengel and Wegener, 1989; Sjöström, 1993).

Softwoods contain 7% to 15% xylan on the basis of dry weight and can be described as arabino-4-*O*-methyl glucuronoxylan. Softwood xylans are generally more highly substituted with 4-*O*-methyl glucuronic acid, with every fifth or sixth xylose unit, on average, carrying a glucuronosyl side chain. Every eight or ninth xylose unit contains a α-1,3-linked arabinofuranose moiety in softwood xylan. Softwood xylans are shorter than hardwood xylans with an average degree of polymerisation of 70 to

130 xylose subunits and softwood xylans are not acetylated. Due to the much higher degree of lignification in softwoods compared to hardwoods, softwoods have to be delignified prior to the alkaline extraction of xylan (Timmel, 1967; Fengel and Wegener, 1989; Sjöström, 1993).

Xylans from several species in the commercially important Gramineae family, containing grasses and cereals, have been isolated and characterized. The xylan of grasses and cereals can be classified as arabino-4-*O*-methylglucuronoxylan, but additionally contains ferulic acid or *p*-coumaric acid esterified to C5 of the arabinofuranose side-chains. The position of the arabinofuranose moiety varies between C2 and C3 in xylans from the different Gramineae and doubly substituted xylose units with arabinofuranose on both C2 and C3 have also been noted (Wilkie, 1979; Bacic *et al.*, 1988). The degree of substitution with the cinnamic acids is relatively low. In wheat bran 1/150 xylose residues contains an arabinofuranose moiety esterified with ferulic acid, while in barley straw 1/120 and 1/240 of the xylose residues contain an arabinofuranose moiety esterified with ferulic and *p*-coumaric acid respectively (Mueller-Harvey and Hartley, 1986).

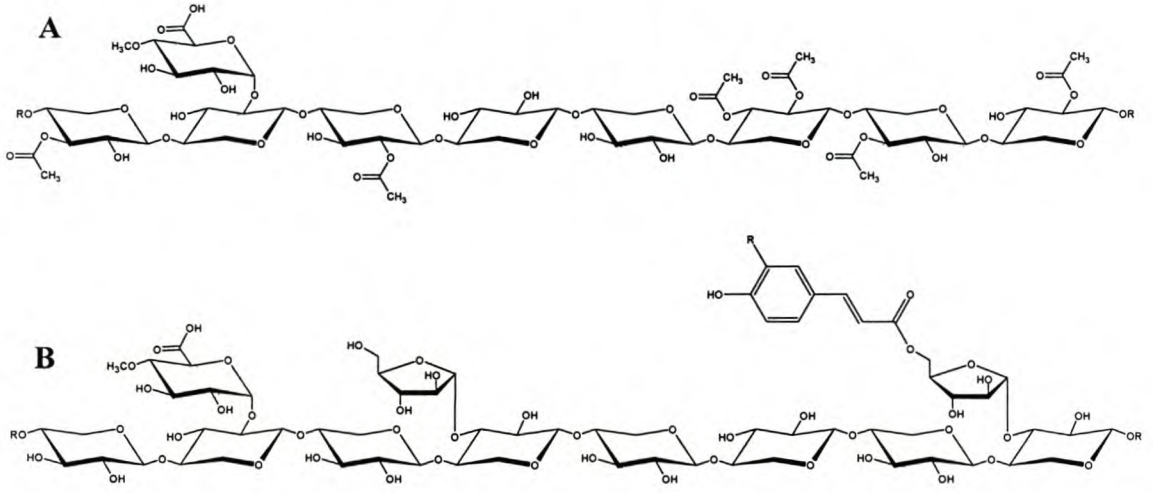

Figure 2.2. Schematic representation of a partial structure of **(A)** acetyl glucuronoxylan found in hardwood, showing the 1,4-linked β-D-xylopyranosyl backbone carrying an 1,2-linked 4-*O*-methyl-α-D-glucopyranuronosyl subtituent and *O*2- and *O*3-linked acetyl esters, and **(B)** arabinoglucuronoxylan found in grasses and cereals,. showing the 1,4-linked β-D-xylopyranosyl backbone carrying an 1,2-linked 4-*O*-methyl-α-D-glucopyranuronosyl subtituent and 1,3-linked α-L-arabinofuranosyl substituents. One α-L-arabinofuranosyl substituent contains an ester-linked cinnamic acid at the *O*5-position with R equivalent to H in *p*-coumaric acid and OCH_3 in ferulic acid (Fengel and Wegener, 1989).

Glucomannans

Glucomannans occur as a minor fraction in the cell walls of angiosperms (including hardwoods, grasses and cereals), constituting roughly 3% to 5% of the dry weight. Angiosperm glucomannans consist of an unsubstituted backbone of randomly distributed 1,4-linked β-D-glucopyranose and β-D-mannopyranose residues, the polymers are usually between 60 and 70 residues in length and they may be slightly branched. The ratio of mannose to glucose varies between 2:1 and 3:2 in different species.

O-acetyl-galactoglucomannans constitute the major fraction of gymnosperm (including softwoods) hemicelluloses and contribute to 20% to 25% of the dry weight. Gymnosperm glucomannans additionally contain 1,6-linked β-galactose side-chains with a ratio of galactose to glucose to mannose of 1:1:3, and acetyl groups ester-linked to the C2 and C3 positions of the mannose residues (Wilkie, 1979; Fengel and Wegener, 1989; Brett and Waldron, 1996).

Glucans

Glucans other than cellulose are known to occur in the cell walls of most plant species, although as minor components. Xyloglucan consists of a backbone of 1,4-linked β-glucopyranose subunits that are almost entirely substituted with 1,6-linked α-xylopyranose side chains. The xylose moieties may also contain 1,2-linked α-arabinofuranose, 1,2-linked β-galactopyranose or a disaccharide of α-1,2-fucopyranosyl-β-1,2-galactopyranose as further extensions of the side-chains. Xyloglucans are the most abundant hemicellulose in the primary cell walls of dicots. Callose consists of 1,3-linked β-glucose subunits, occurs in sieve cells of phloem and parenchyma cells of xylem tissue and may be found in close association with cellulose. The 1,3-1,4-linked β-glucans are associated with cell wall proteins in grasses and the 1,3- and 1,4-linkages occur in ratios between 1:1 and 1:2 in the polymer (Fengel and Wegener, 1989; Brett and Waldron, 1996).

Galactans

In larchwood, water-extractable arabinogalactan is one of the major hemicelluloses contributing to between 10 and 25% of the dry weight. It has a highly branched structure, consisting of backbone of 1,3-linked β-galactopyranose, substituted with single 1,6-linked β-glucuronic acid and 1,6-linked α-arabinofuranose units as well as longer side chains of only 1,6-linked β-galactopyranose, and 1,6-linked β-galactopyranose containing 1,3-linked α-arabinofuranose and β-arabinopyranose. Rhamnoarabinogalactans from hardwood have a backbone of 1,3-linked β-galactopyranose, but are additionally substituted with rhamnose residues and the molar ratio of galactose to arabinose to rhamnose is 17:10:2 (Timmel, 1967; Fengel and Wegener, 1989; Sjöström, 1993).

Pectin

Galacturonans

Galacturonans are pectic polysaccharides that consist of a backbone of 1,4-linked α-galacturonic acid interspersed with 1,2-linked α-rhamnose units. The α-rhamnose units occur at regular intervals and may carry long arabinan and galactan side chains linked to C4. The arabinan side-chains contain a backbone of 1,5-linked α-L-arabinofuranose that itself carries further brances of arabinan at C3. The C2 position of the α-L-arabinofuranosyl residues may also carry ferulic acid esters. The galactan side-

chains consist of a backbone of 1,4-linked β-D-galactose, and can also carry ferulic acid esters at the C6 position. The galacturonic acid residues in the rhamnogalacturonan backbone may also carry acetate esters at the C2 and C3 positions (Bacic *et al.*, 1988; Fengel and Wegener, 1989).

Arabinans

Arabinans occur as a minor pectic polysaccharide, and consist of a backbone of 1,5-linked α-arabinofuranose units substituted by single or multiple α-arabinofuranose units at the C2 and C3 positions (Bacic *et al.*, 1988; Williamson *et al.*, 2002).

Lignin

Lignin is an amorphous polymer of irregularly cross-linked phenyl propane units, and occurs in the cell walls of most higher plants, encrusting the cellulose fibres and providing the cell wall with additional structural rigidity. It is the second most abundant organic polymer on earth after cellulose. Lignin is formed by a peroxidase-catalysed free-radical polymerisation of *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol in the presence of hydrogen peroxide. Polymerisation occurs at the end of longitudinal cell growth and the polymerisation process continues as long as the cinnamyl alcohols and space is available in the cell wall. Lignin consequently acts as a glue-like filler material, occupying the spaces between individual microfibrils, displacing water molecules effectively turning the cell wall into an anhydrous environment. This results in an even greater degree of hydrogen bonding between the carbohydrate polymers of plant cell walls (Fengel and Wegener, 1989; Brett and Waldron, 1996).

Associations of xylan with other cell wall components

The preceding discussion of each of the cell wall components described their chemical composition after harsh isolation procedures. The properties of the polymers may be substantially different in the environment of the intact plant cell wall where they are free to interact with other components. Polysaccharides in solution form gels through extensive hydrogen bonding and van der Waal's interactions. The extent of non-covalent interaction of xylan with the other cell wall components is unknown as xylans are generally highly substituted, a characteristic that is known to interfere with gel formation. Intermolecular cross-links between xylan and lignin as well as between separate xylan polymer molecules exist and markedly affect the stability and degradability of the plant cell wall (Bacic *et al.*, 1988). Cross-links between separate xylan polymers occur as diferulate bridges formed by the ferulic acid side-chains on the arabinofuranose substituents of two xylan molecules. Ferulic acid moieties dimerised by a peroxidase catalysed oxidative coupling and diferulic acid has been released from purified xylan by saponification (Markwalder and Neukom, 1976). The carboxylate moiety of 4-O-methyl glucuronic acid in xylan was found to form ester bonds with the hydroxyls in

lignin. These bonds are alkali-labile and would not be detected in the normal extraction of xylan (Das *et al.*, 1984). Ether linkages between the hydroxyl groups of the ferulic acid components of xylan and lignin have also been shown to occur (Scalbert *et al.*, 1985). Other linkages that are possible, but have not been shown specifically in xylan include direct glycosidic linkages between reducing end xylose units and the lignin hydroxyls and direct ether linkages between the polysaccharide and lignin hydroxyls (Bacic *et al.*, 1988).

The biodegradation of xylan

As a result of the structural heterogeneity of xylan, the collective action of several glycoside hydrolases and esterases is needed for the complete degradation of the polymer (Figure 2.3). The degradation of the xylan backbone is achieved by the action of *endo*-β-1,4-xylanases (E.C. 3.2.1.8) that cleave the internal xylosidic bonds, releasing substituted and unsubstituted oligosaccharides of variable length. β-Xylosidase (E.C. 3.2.1.37) subsequently removes single xylose moieties from the non-reducing ends of unsubstituted xylo-oligosaccharides, while the xylanolytic accessory enzymes α-arabinofuranosidase (E.C. 3.2.1.55) and α-glucuronidase (E.C. 3.2.1.139) remove the side-chains from substituted xylo-oligosaccharides. Acetylxylan esterases (E.C. 3.1.1.6) as well as coumaric and ferulic acid esterases (E.C. 3.1.1.1) hydrolyse the esters of the respective organic acids (Dekker, 1989).

Synergy in xylan biodegradation

The combined action of the xylanolytic enzymes degrade the polymer to a much larger extent than the sum of each of the enzymes acting individually, indicating a synergistic effect. This is a result of both the structural complexity of the polysaccharide and the substrate specificity of the enzymes that degrade it. The presence of substituents on the xylan backbone generally acts as a steric hindrance to the action of the main-chain cleaving *endo*-β-1,4-xylanases. Additionally, most of the side-chain cleaving accessory enzymes have a clear preference for smaller oligosaccharides as substrates and in many cases only remove the substituents from the non-reducing ends of the oligosaccharide. Certain *endo*-β-1,4-xylanases are unable to hydrolyse the β-1,4-xylosidic linkage immediately adjacent to a substituted xylose residue, and release xylo-oligosaccharides containing the substituents on the internal xylose units. The action of β-xylosidase is required to remove any unsubstituted terminal xylose units, before the accessory enzymes can further process such oligosaccharides. Most β-xylosidases conversely cannot remove substituted non-reducing end xylose residues and require the presence of the accessory enzymes to remove any side chains. The most common degradation product of the *endo*-β-1,4-xylanases is xylobiose that cannot be degraded further by these enzymes and requires the action of β-xylosidase for complete degradation to D-xylose. The action of β-xylosidases also decreases as the length of the xylo-oligosaccharide increases from xylobiose and longer xylo-

oligosaccharides first have to be shortened by the *endo*-β-1,4-xylanases. The complete degradation of xylan therefore not only requires the presence of all the xylanolytic enzymes, but their co-operative action as well (Dekker, 1989; de Vries *et al.*, 2000; Beg *et al.*, 2001).

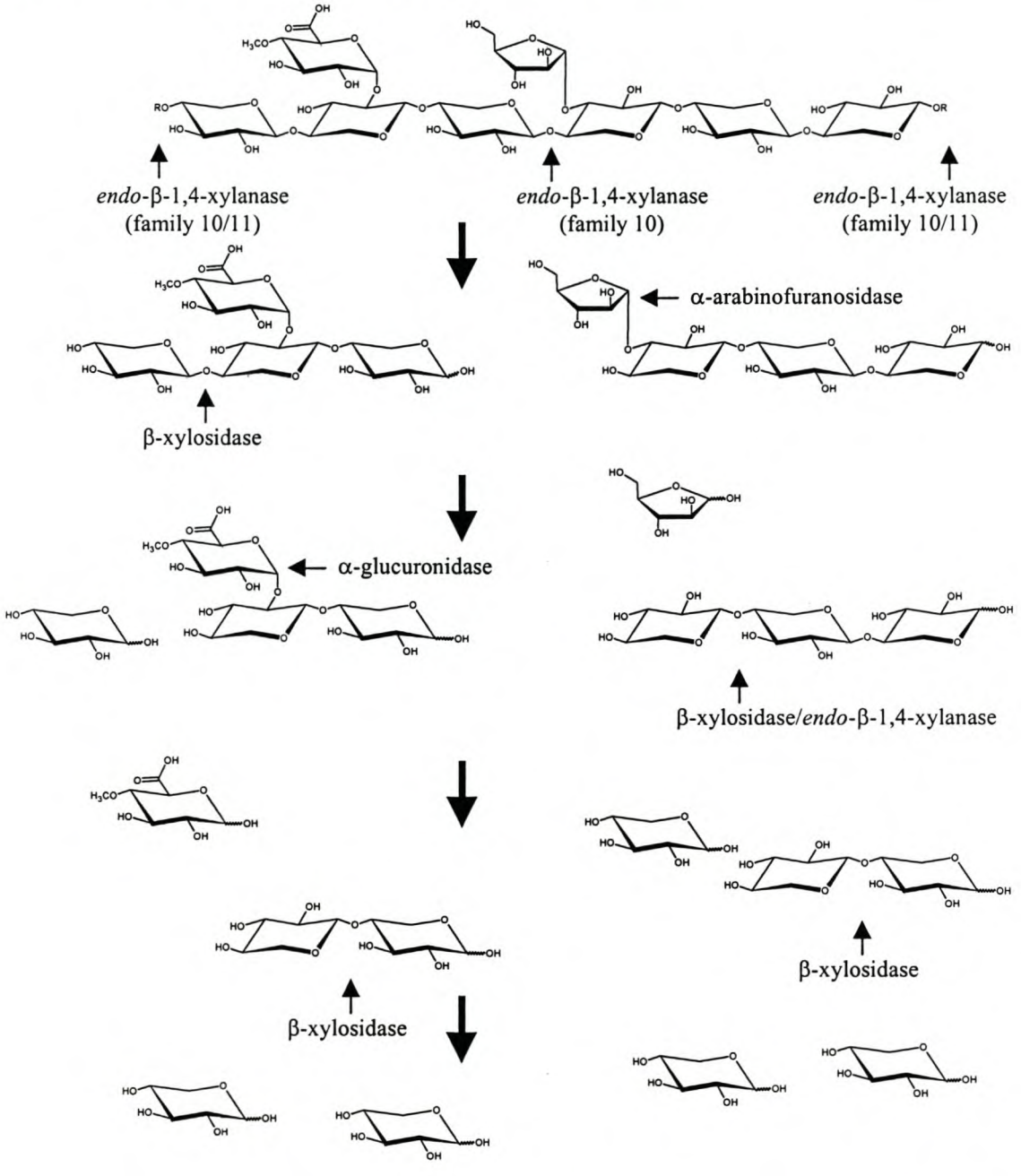

Figure 2.3. Schematic representation of the enzymatic degradation of a soluble arabino-4-*O*-methyl glucuronoxylan fragment, illustrating the substrate specificity of the various xylanolytic enzymes. R indicates the continuation of the β-1,4-linked xylose backbone. Family 10 endo-β-1,4-xylanases are more tolerant to substituents than family 11 endo-β-1,4-xylanases. In the first step the polymer is hydrolysed to smaller oligosaccharides by the action *endo*-β-1,4-xylanase. The aldouronic acid in the second step contains the substituent on an internal xylose moiety and requires the hydrolysis of the non-reducing end xylose unit by β-xylosidase before α-glucuronidase can remove the uronic acid substituent in the next step. The α-arabinose is situated on a terminal non-reducing end xylose unit and can be hydrolysed directly. β-xylosidase then completes the degradation to monosaccharides by cleaving non-reducing end xylose units from the unsubstituted oligosaccharides (Dekker, 1989).

Regulation of the expression of xylanolytic enzymes

The microbial production of xylanolytic enzymes generally requires growth on lignocellulosic substrates. Since neither cellulose nor xylan is able to cross the microbial cell wall, small molecules resulting from the degradation of both polymers by low levels of constitutively expressed glycoside hydrolases act as transcriptional inducers. The presence of permeases that allow the transport of the low molecular weight inducers across the cell wall is an additional requirement for the induction of these enzyme systems. The identity of the natural low molecular weight inducers seems to differ between microbial species, but they are all derived from the breakdown and metabolism of lignocellulosic material. The induction of the xylanolytic enzyme systems is repressed by the presence of glucose in the medium (Biely, 1993; Kulkarni *et al.*, 1999; Beg *et al.*, 2001).

The few detailed studies that investigated the effect of inducers and repressors of xylanolytic genes at the transcriptional level and analysed the upstream promoter regions of these genes have focussed on *Trichoderma reesei* and the Aspergilli. In spite of the similarity in the general pattern of induction of the xylanolytic genes by different carbon sources (responsiveness to lignocellulose and its breakdown products), there is also considerable variation in the extent to which each of the genes is induced by different carbon sources. This observation suggests that there are multiple and different transcription factors that control the expression of the different xylanolytic genes and that these genes possess different promoter structures (Margolles-Clark *et al.*, 1997; van Peij *et al.*, 1998; Mach and Zeilinger, 2003).

In a comprehensive study of the induction of hemicellulolytic genes in *T. reesei*, it was found that the entire complement of xylanolytic genes was induced by growth on xylan from three different sources or cellulose, respectively. Notably, the two *endo*-β-1,4-xylanase genes, *xyn1* and *xyn2*, differed in their response to the different xylans. The main small molecule inducers of the *endo*-β-1,4-xylanase genes were arabitol, xylobiose and sophorose, the β-1,2 isomer of cellobiose, and the product of β-glucosidase transglycosylation. The *xyn2* gene was responsive to cellobiose itself, whereas the *xyn1* gene was not. The *bxl1* gene, encoding a β-xylosidase, followed a similar pattern of expression as the *endo*-β-1,4-xylanases and was also responsive to arabinofuranose. The α-arabinofuranosidase gene *abf1* showed interesting regulatory features. It was only induced by xylan that was substituted with L-arabinose, even though it was induced by the same low molecular weight inducers as the *endo*-β-1,4-xylanases. Its expression was highly responsive to arabitol and it was the only gene besides *bxl1* that responded to L-arabinose. The gene encoding α-glucuronidase, *glr1*, showed a different pattern of induction. It was only responsive to cultivation on arabitol, xylobiose, cellulose and polymeric xylan. Finally, the acetylxylan esterase gene *axe1* was responsive to growth on cellulose, specifically 4-*O*-

methyl glucuronoxylan, sophorose and xylobiose. None of the xylanolytic genes were expressed in the presence of glucose and all of the xylanolytic genes except *xyn1* were induced by glucose depletion (Margolles-Clark *et al.*, 1997). From the above summary, it should be clear that regulation of the expression of each of the xylanolytic genes is probably controlled by different transcription factors, and that analysis of the upstream regions of each gene is necessary to further elucidate the mechanism of induction and repression. The distinct regulation of each of the xylanolytic genes likely allows for the optimal production of each enzyme only when it is required.

Several strategies including, sequencing of the regions upstream of the xylanolytic genes, deletion analysis, electrophoretic mobility shift assays and complementation of regulatory mutants by transformation with genomic libraries, have led to the identification of transcription factors and their binding motifs in *T. reesei* and the Aspergilli. In *T. reesei*, the most studied promoters are those of the *endo*-β-1,4-xylanase genes, *xyn1* and *xyn2*. Glucose repression of the *xyn1* promoter is regulated through the carbon catabolite repressor protein Cre1. The *xyn1* promoter contains an inverted repeat of the Cre1 consensus recognition motif, GGGGTC, and deletion of these sequences leads to gene expression at de-repressed levels. Induction of the *xyn1* gene is regulated through Xyr1, a homologue of the *A. niger* xylanolytic transcriptional activator XlnR. The XlnR recognition motif appears as an inverted repeat in the upstream regions of the *xyn1* gene, but the exact regulatory mechanism through Xyr1 is uncertain. Xyr1 may be permanently bound to its recognition sequence and induction repressed by the binding of an additional unknown factor, as the recognition motifs are permanently protected in footprinting assays, and mobility shift assays on the Xyr1 recognition sequences in the presence of cell extracts of glucose grown cultures shows lower mobility than expected. However, the consensus motif of the Xyr1 closely resembles that of Ace1, a transcription factor known to bind to the *cbh1* promoter, that may well play a role in *xyn1* regulation (Mach and Zeilinger, 2003).

As expected from the Northern analysis, the *xyn2* gene is regulated by a different set of transcription factors and the promoter region contains no Cre1 binding sites. Induction and repression appears to be regulated by binding of transcription factors to an upstream region of only 55 bp. An 11-bp *endo*-β-1,4-xylanase-activating element, GGGTAAATTG, was found to be occupied by the HAP2/3/5 complex and the cellulase regulator, Ace2, in both the induced and basal transcriptional states. A third unknown transcription factor is thought to bind the 4-bp motif AGAA, almost immediately upstream of the *endo*-β-1,4-xylanase-activating element and acts as a repressor (Mach and Zeilinger, 2003).

The xylanolytic regulator XlnR was first described in *A. niger* and it was shown by deletion and overexpression of the *xlnR* gene that this transcription factor induces nearly all the xylanolytic genes as well as two *endo*-glucanase genes in response to growth on xylose or xylan. Sequence analysis of the upstream promoter regions of several of the xylanolytic and cellulolytic genes revealed the

putative consensus binding sequence, GGCTAA (van Peij *et al.*, 1998). Subsequent analysis of the *aguA* gene promoter regions revealed that the true recognition sequence was GGCTAR (de Vries *et al.*, 2002). In a similar study, AoXlnR, the *A. oryzae* homologue of XlnR, was found to mediate the induction of several xylanolytic and cellulolytic genes in response to not only xylose and xylan, but also microcrystalline cellulose and cellobiose. The presence of several putative CreA binding sites was reported in the upstream regions of these genes (Marui *et al.*, 2002).

Our current knowledge of the transcriptional control of the xylanolytic genes comes from studies on only two genera of fungi. Studies in other fungi and bacteria are therefore needed to reveal if these mechanisms are of a more general nature. It is also clear that not all the transcription factors responsible for the induction of every xylanolytic gene are the same and they have not all been identified. Another aspect that needs to be addressed is the nature and identity of the signalling pathways responsible for the identifying the small molecule inducers relaying the signal to the nucleus.

The reaction mechanisms of glycoside hydrolases

Glycoside hydrolases follow two possible reaction mechanisms, one leading to retention of the anomeric configuration from substrate to product, and one leading to inversion of anomeric

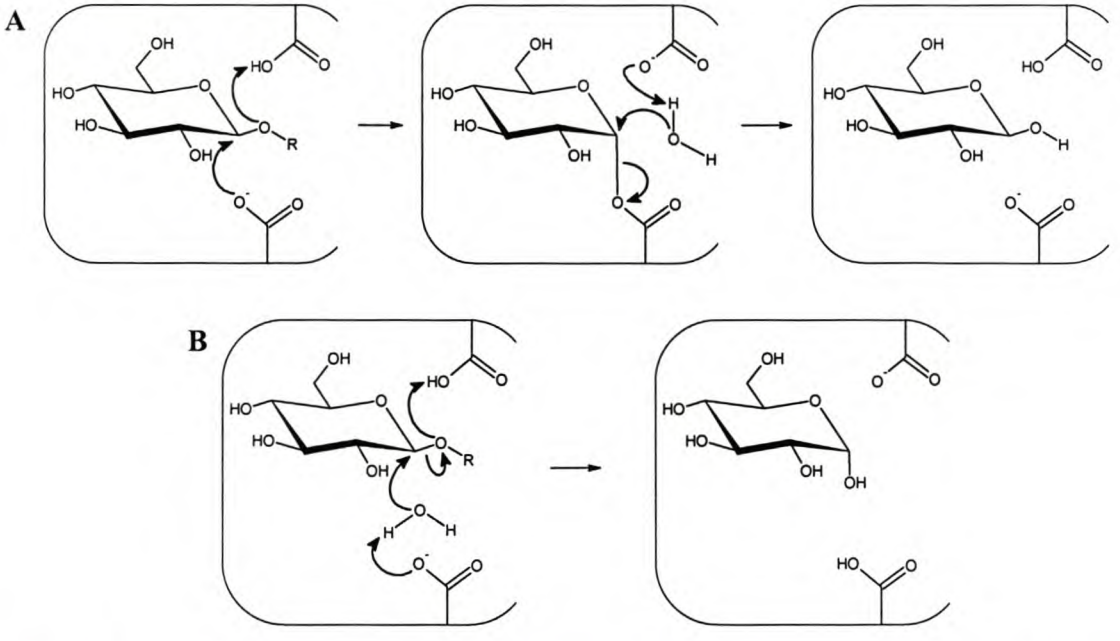

Figure 2.4. Schematic representation of the (A) retaining and (B) inverting reaction mechanisms followed by glycoside hydrolases (McCarter and Withers, 1994). In the first panel of A, the scheme shows the attack of the nucleophilic carboxylate on the anomeric carbon, concomitant with the transfer of a proton from the general acid to the glycosidic oxygen. The next panel shows the activation of water by the general base and its attack on the anomeric carbon and the transfer of the bond electrons back to the nucleophile, resulting in the release of product in the final panel. The first panel of B illustrates the activation of water by the catalytic base and its direct attack on the anomeric carbon, followed by the transfer of the bond electrons to the glycosidic bond oxygen as well as the transfer of a proton from the general acid to the glycosidic oxygen.

configuration in the product (Figure 2.4). Both mechanisms rely on the presence of a pair of carboxylate side-chains for catalysis. In the retaining enzymes, a two-step mechanism is followed in which the nucleophilic carboxylate attacks the anomeric carbon concomitant with the attack of the glycosidic oxygen on the proton of the general acid, leading to the formation of a glycoside-enzyme intermediate. Activation of water by extraction of a proton by the general base, and attack of the water oxygen on the anomeric carbon leads to completion of the reaction cycle and release of the product in the same anomeric state as the substrate. As a result of the existence of a covalently-bound glycoside-enzyme intermediate, glycosidic oxygens can also attack at the anomeric carbon in this transition state, leading to transglycosylation activity. The inverting glycoside hydrolases, on the other hand, follow a single step mechanism in which water, after activation by the catalytic base, directly attacks the anomeric carbon at the opposite surface of the glycosidic oxygen and the catalytic acid donates a proton to the leaving group oxygen. This class of enzymes is unable to perform transglycosylation reactions. Xylanolytic enzymes have been found to follow either reaction mechanism (McCarter and Withers, 1994).

The classification of the Carbohydrate Active enZYmes

The glycoside hydrolases can be classified according to a variety of criteria, including the IUBMB system, based on substrate specificity. Classification based on substrate specificity has certain shortfalls, such as the inability to cope with enzymes with multiple activities and the inability to distinguish between structurally different enzymes catalysing the same reaction. A classification system based on primary amino acid sequence was proposed in 1991 by Henrissat and co-workers, and subsequently updated to over 80 families of glycoside hydrolases and expanded to include the carbohydrate esterases. This system has the advantage that it can predict reaction mechanism, a feature dependant on the crystal structure and therefore the primary sequence of the polypeptide. Significant insights about the evolutionary history of the glycoside hydrolases and their structural domains can also be gained from such a classification system. Divergent evolution from a common ancestor can be inferred from the clustering of enzymes with different substrate specificity but similar amino acid sequences in the same group, while the existence of the same enzyme activity in several groups suggest evolutionary convergence (Henrissat and Davies, 1997).

The xylanolytic enzymes

endo-β-1,4-Xylanase

The main-chain cleaving *endo*-β-1,4-xylanases (E.C. 3.2.1.8) are the most intensively studied of the xylanolytic enzymes and they have been purified and/or cloned from a huge number of microorganisms found in a variety of environments. *Endo*-β-1,4-xylanases from mesophilic organisms usually have temperature optima between 40°C and 60°C, pH optima between 4 and 7, and

are stable between pH 3 and 10. Most of the sequenced *endo*-β-1,4-xylanases have been divided into families 10 and 11 of the glycoside hydrolases. Family 10 enzymes have molecular weights in excess of 30 kDa and acidic isoelectric points compared to the family 11 enzymes that have molecular weights between 20 and 24 kDa, and generally have alkaline isoelectric points. Bacteria generally produce multiple *endo*-β-1,4-xylanases of both families while fungi more commonly produce the lower molecular weight family 11 enzymes (Kulkarni *et al.*, 1999).

There are marked differences in overall three-dimensional structure as well as the active site architecture of the two families. As a result their substrate specificity and the structure of the products they release also differ. The active site of the family 10 *endo*-β-1,4-xylanases consists of an eightfold β/α-barrels and the overall structure of the enzyme resembles that of a shallow bowl with the catalytic residues situated in an open groove at its base. The family 11 enzymes consist of two β-sheets, stacked to form a sandwich, as well as a threefold α-helix. The structure of the enzyme is described as a partially closed right hand, with a loop between the β-sheets making up the thumb, and the active site situated in the deep cleft in the surface of the enzyme. Substrate binding subsites are labelled with negative integers from –1 in the direction of the non-reducing end and with positive integers from +1 in the direction of the reducing, with the cleavage occurring between the –1 and +1 subsites. The sugar-binding subsites usually contain aromatic side-chains aligned for π-stacking interactions with the sugar surfaces and the number of subsites greatly influences the minimum length of the oligosaccharide required for productive interaction with enzyme. Based on kinetic evidence, it has been proposed that the family 10 hydrolases contain 4 subsites while the family 11 hydrolases contain 5 or more (Jeffries, 1996; Törrönen and Rouvinen, 1997).

The more open active-site architecture of the family 10 enzymes and the lower number of substrate binding sites allow them to produce oligosaccharides of a lower degree of polymerisation than the family 11 enzymes and they are also more tolerant to the presence of substituents on the xylan backbone. The family 10 enzymes have the ability to hydrolyse the linkage between a substituted xyloside at the +1 subsite and an unsubstituted xyloside at the –1 subsite, releasing oligosaccharides with the substituent on the non-reducing end. Family 11 enzymes require an unsubstituted xyloside at the +1 subsite. There are also definite differences in the properties of the individual members of each family in terms of their substrate specificity, pH and temperature optima and stability and specific activity. The *endo*-β-1,4-xylanases of family 10 and 11 studied so far are strictly *endo*-acting enzymes and hydrolyse glycosidic linkages via a retaining mechanism (Biely *et al.*, 1997).

β-Xylosidase

β-Xylosidase (3.2.1.37) liberates single xylose residues from the non-reducing ends of xylo-oligosaccharides that are released during the enzymatic breakdown of the xylan polymer. They are generally most active against xylobiose, with activity decreasing with an increasing degree of polymerisation. The presence of side-chains inhibits β-xylosidase activity, especially if the substituents are located on the non-reducing end xylose unit (Viikari *et al.*, 1993). β-Xylosidase activity is currently found in members of families 3, 39, 43, 52 and 54. The occurrence of β-xylosidase activity in a large number of different groups, some with bifunctional activity, hints at convergent evolution, with the emergence of the same activity by several different routes.

The family 3 contains β-xylosidases, β-glucosidases, β-*N*-acetylhexosaminidases and α-arabinofuranosidases and activity proceeds via a retaining mechanism. The β-xylosidases found in this family have calculated monomeric molecular weights in the order of 87 kDa and isoelectric points around pH 5 and are mainly of fungal origin (http://afmb.cnrs-mrs.fr/CAZY/). The *Aspergillus nidulans* β-xylosidase, XlnD, was shown to be a dimer of 180 kDa by gel filtration and was active against *p*-nitrophenyl β-D-xylopyranoside with maximal activity at 50°C and pH 5 (Kumar and Ramon, 1996; Perez-Gonzalez *et al.*, 1998). The β-xylosidase of *T. reesei*, BXL1, released xylose from xylobiose and at a very slow rate from polymeric xylan, but did not show any α-arabinofuranosidase activity (Margolles-Clark *et al.*, 1996).

Family 39 contains β-xylosidases and α-iduronidases, and the members of this family follow a retaining mechanism. The family contains β-xylosidases of bacterial origin that have calculated monomeric molecular weights between 50 kDa and 60 kDa and isoelectric points between pH 5 and pH 6 (http://afmb.cnrs-mrs.fr/CAZY/). The *Thermoanaerobacterium saccharolyticum* β-xylosidase, XynB, is a produced as a monomeric protein of 55 kDa with pH and temperature optima of 5.5 and 70°C, respectively, when assayed with *p*-nitrophenyl β-D-xylopyranoside. This enzyme was inactive against xylan but displayed activity against xylobiose, xylotriose and xylopentaose (Lee and Zeikus, 1993).

Family 43 contains the bifunctional inverting α-arabinofuranosidases/β-xylosidases that will be discussed in the section on α-arabinofuranosidases activity.

Family 52 contains only β-xylosidases, mainly from different strains of *Geobacillus stearothermophilus*. The β-xylosidase of *G. stearothermophilus* T-6, XynB2, is a dimeric protein with a monomeric molecular weight of 79 kDa on SDS-PAGE and a native molecular weight of 157 kDa

by gel filtration. It has maximum activity at pH 5.6 to 6.3 and at 65°C when tested on *p*-nitrophenyl β-D-xylopyranoside and follows a retaining mechanism (Bravman *et al.*, 2001). Family 54 contains mainly α-arabinofuranosidases and a single bifunctional α-arabinofuranosidase/β-xylosidase from *Trichoderma koningii* G-39, with a calculated monomeric molecular weight of 51 kDa and iso-electric point of 5.9. Members of this family follow a retaining mechanism (http://afmb.cnrs-mrs.fr/CAZY/).

α-Arabinofuranosidase

α-Arabinofuranosidase (3.2.1.55) hydrolyses non-reducing end arabinose residues linked to the C2 or C3 positions and to a lesser degree the C5 position of several glycans, including arabinan, arabinogalactan and xylan. The exact classification of the α-arabinofuranosidases is more complicated than other xylanolytic glycoside hydrolases due to the variety of bonds that α-arabinofuranosidases could potentially degrade and their ability to act on several polymers. α-Arabinofuranosidase activity is found in families 3, 43, 51, 54 and 62 (http://afmb.cnrs-mrs.fr/CAZY/). The only family 3 member that shows α-arabinofuranosidase activity is a bifunctional enzyme that also possesses β-xylosidase activity and was cloned from *Thermoanaerobacter ethanolicus* (Mai *et al.*, 2000).

Family 43 contains bifunctional α-arabinofuranosidase/β-xylosidase activity and these enzymes follow an inverting mechanism (http://afmb.cnrs-mrs.fr/CAZY/). The β-xylosidase of *Bacillus pumilus*, XynB, has a calculated molecular weight of 61 kDa and an iso-electric point of 5.7. It has an apparent molecular weight of 56 kDa on SDS-PAGE and a native molecular weight of 190 kDa as determined by gel filtration, indicating a trimeric or tetrameric native structure. This enzyme was shown to be active against *p*-nitrophenyl β-D-xylopyranoside and xylobiose with optimal activity at pH 7 and 40°C (Xu *et al.*, 1991). XylB, isolated from *Butyrivibrio fibrisolvens* (Utt *et al.*, 1991), XylA, from *Clostridium stercorarium* (Sakka *et al.*, 1993), and XSA from *Selenomonas ruminantium* (Whitehead and Cotta, 2001) all have calculated monomeric molecular weights and iso-electric points similar to the *B. pumilus* XynB, but possess bifunctional β-xylosidase/α-arabinofuranosidase activity as measured with the corresponding *p*-nitrophenyl glycosides. The *C. stercorarium* enzyme exists as a tetramer with a native molecular weight of 220 kDa determined by gel filtration and optimal activity at pH7 and 65°C. Bifunctional β-xylosidase/α-arabinofuranosidase activity against xylan was confirmed for the *S. ruminantium* XYS in the presence of *endo*-β-1,4-xylanase activity. In light of the similarities between the *B. pumilus* XynB and the other bifunctional enzymes, it is tempting to speculate that it also has a bifunctional character and will show α-arabinofuranosidase activity if tested. Two lower molecular-weight bifunctional β-xylosidase/α-arabinofuranosidase enzymes with calculated monomeric molecular weights of 37 kDa and iso-electric points of 4.8, and significantly

higher sequence identity with each other than with the other bifunctional enzymes, namely the *Prevotella ruminicola* XynB (Gasparic *et al.*, 1995) and the *Bacteroides ovatus* XSA (Whitehead, 1995) are additional members of this family. The *P. ruminicola* XynB had activity against xylobiose, xylopentaose and birchwood xylan.

Family 51 consists of *endo*-glucanases and α-arabinofuranosidases and they follow a retaining mechanism. Many of the bacterial α-arabinofuranosidases found in this family are tetrameric enzymes with monomeric molecular weights around 57 kDa. They have a greater preference for *C2* and *C3*-linked side-chains over *O5*-linked side chains in a variety of polymers and oligomers, and are able to hydrolyse *p*-nitrophenyl α-L-arabinofuranoside (Schwarz *et al.*, 1995; Beylot *et al.*, 2001). There is definite variability in the substrate specificity of these enzymes. The *Streptomyces chartreusis* AbfI and the *Geobacillus stearothermophilus* T-6 AbfA have low activity against arabinoxylan, while the *Cytophaga xylanolytica* ArfI is inactive against arabinogalactan (Gilead and Shoham, 1995; Renner and Breznak, 1998; Matsuo *et al.*, 2000). The *S. chartreusis* AbfI is also unique in this family being the only member that contains a cellulose-binding domain. A multimeric α-arabinofuranosidase was cloned from *Streptomyces lividans*, with a monomeric molecular weight of 69 kDa and a native molecular weight of 380 kDa and similar substrate specificity to the *G. stearothermophilus* AbfA. It had no activity against arabinogalactan or oat spelt arabinoxylan, but acted on the breakdown products of the former when *endo*-β-1,4-xylanase activity was present. It was able to hydrolyse beet arabinan (Manin *et al.*, 1994). In contrast to the above-mentioned members of the family, the *Thermobacillus xylanolyticus* AbfD3 was highly active on wheat arabinoxylan, larchwood xylan and oat spelt xylan (Debeche *et al.*, 2000). An *A. niger* α-arabinofuranosidase, AbfA, that is specific for α-1,5 linked arabino-oligosaccharides is the only fungal member of this family (van der Veen *et al.*, 1991), however, many α-arabinofuranosidases from plants are also found in this family.

Family 54 is made up of a highly conserved group of fungal α-arabinofuranosidases with the ability to release arabinose from α-1,2, α-1,3 and α-1,5 linked positions on the non-reducing ends of a variety of oligosaccharides and polysaccharides such as the arabinans, arabinogalactans and arabinoxylan. These enzymes are active on *p*-nitrophenyl α-L-arabinofuranoside and in some cases, display low activity towards *p*-nitrophenyl β-D-xylopyranoside. Members of family 54 have been isolated and characterized from the Aspergilli (van der Veen *et al.*, 1991; Crous *et al.*, 1996; Gielkens *et al.*, 1999) and *Trichoderma reesei* (Margolles-Clark *et al.*, 1996) and these enzymes have monomeric molecular weights around 50 kDa. Enzymes of this family follow a retaining mechanism.

The α-arabinofuranosidases found in family 62 have been classified as arabinoxylan arabinofuranohydrolases based on their exclusive preference for arabinoxylan and its breakdown

products and the ability to release arabinose from the polymer without the need for prior degradation to oligosaccharides (Gielkens *et al.*, 1997). The family includes α-arabinofuranosidases from the Aspergilli and *Cochliolobus carbonum* with monomeric molecular weights around 35 kDa, as well as higher molecular weight bacterial α-arabinofuranosidases that have additional carbohydrate-binding domains. The *Cellvibrio japonicus* enzyme contains a carbohydrate binding module (Kellett *et al.*, 1990), while the α-arabinofuranosidases from *Streptomyces lividans* and *S. coelicolor* both contain a ricin B-type lectin domain that has the ability to bind to xylan (Vincent *et al.*, 1997). The reaction mechanism of this family has not yet been established.

α-Glucuronidase

α-Glucuronidase (E.C. 3.2.1.139) removes 4-*O*-methyl glucuronic acid substituents from the non-reducing ends of aldouronic acid oligomers, generated by the degradation of xylan by *endo*-β-1,4-xylanase or *endo*-β-1,4-xylanase and β-xylosidase. All of the α-glucuronidases tested so far have a requirement for the uronic acid to be located on the non-reducing end xylose of the oligosaccharide (Puls, 1992). α-Glucuronidase activity is found exclusively in family GH67, and it is the only activity found in this family. A putative α-glucuronidase with a high degree of sequence identity with family GH4 enzymes was cloned from *Thermotoga maritima* MSB8, and classified on its ability to hydrolyse *p*-nitrophenyl glucuronide (Suresh *et al.*, 2002). None of the microbial α-glucuronidases active on xylo-oligosaccharides can hydrolyse this substrate and the enzyme's activity against aldouronic acids was not determined. Its true function is likely still to be discovered. The bacterial α-glucuronidases are all dimeric proteins with monomeric molecular weights around 70 kDa and iso-electric points around 6, while the fungal enzymes are monomers with molecular weights between 90 and 130 kDa and iso-electric points around 5 (Puls, 1992). More detail on this enzyme will be provided in Chapter 3.

Acetyl xylan esterase and cinnamoyl esterases

Acetyl esterases cleave the acetic acid groups esterified to the C2 and/or C3 position on the xylan backbone, while the cinnamoyl esterases hydrolyse the *p*-coumaric and ferulic acids esterified to the C5 position of the arabinofuranose substituents of xylan. Most esterases have wide substrate specificity and the use of artificial substrates in the isolation and study of enzyme activity poses a problem for the proper classification of the esterases. Assays with chemically acetylated xylan or DMSO-extracted xylan revealed the existence of specific acetylxylan esterases. Acetylxylan esterases from different organism vary in their preference for polymeric xylan or xylo-oligosaccharides and in their specificity towards the position of the acetyl group on the xylose moiety. Synergy between *endo*-β-1,4-xylanase and acetylxylan esterase activity has been well established, with addition of acetyl

esterase activity allowing for the release of more and smaller oligosaccharides from xylan, and the addition of *endo*-β-1,4-xylanase increasing the amount of acetate released (Christov and Prior, 1993). Sequencing of the cloned genes has revealed great heterogeneity in the amino acid sequences of the acetyl esterases, and as a result acetylxylan esterases have been assigned to family 1 through 7 of the 13 carbohydrate esterase families, based on primary sequence and hydrophobic cluster analysis (http://afmb.cnrs-mrs.fr/CAZY/). Acetyl xylan esterases are secreted enzymes and the induction of enzyme activity occurs during growth of microorganisms on lignocellulosic substrates. High concentrations of xylose, and the presence of glucose act as inhibitors of the production of acetyl esterase activity. The cinnamoyl esterases from different organisms vary in their specificity towards the type of cinnamic acid released. Cinnamoyl esterases exist that are able to release both *p*-coumaric acid and ferulic acid although most have a preference for the one or the other. A cinnamoyl esterase with similar reaction rates on methyl ferulate and methyl *p*-coumarate has been isolated from the culture supernatant of *A. pullulans* NRRL Y-2311-1 (Rumbold *et al.*, 2003). The cinnamoyl esterases are small secreted enzymes that are inducible by growth of microorganisms on lignocellulosic substrates and show synergy with *endo*-β-1,4-xylanases in the degradation of xylan (Christov and Prior, 1993; Williamson *et al.*, 1998). All of the sequenced cinnamoyl esterases are currently classified in carbohydrate esterase family 1 of the carbohydrate active enzymes (http://afmb.cnrs-mrs.fr/CAZY/).

Expression of xylanolytic enzymes in baker's yeast

The baker's yeast *Saccharomyces cerevisiae* has been developed into an ideal host for the expression of eukaryotic proteins since the early 1980's. A very diverse collection of proteins from a wide variety of different organisms has been successfully expressed in this host. There are numerous reasons for interest in *S. cerevisiae* as a host for the production of heterologous proteins, many stemming from the inherent difficulties in expressing eukaryotic proteins in a prokaryotic host such as *Escherichia coli*. Proteins expressed in *E. coli* are often found in inclusion bodies and require the use of detergents to extract from cell lysates. Refolding of the protein into the native and/or active conformation after extraction is not guaranteed and is often troublesome (Romanos *et al.*, 1992; Hadfield *et al.*, 1993).

Post-translational modification differs substantially between prokaryotic and eukaryotic cells and the latter have unique polypeptide processing pathways along the endoplasmic reticulum and the Golgi-apparatus that are absent in prokaryotes. Endoproteolytic cleavage, multimer assembly, disulphide bridge formation and glycosylation are some of the important processes that occur along these pathways, allowing for the production of heterologous proteins that are structurally and functionally much more similar to their native forms. *S. cerevisiae* is able to secrete proteins that contain a

recognisable signal sequence. In cases where the yeast does not recognise the native signal sequence, it can be replaced by one of several secretion signals known to function in yeast, resulting in the secretion of a mature polypeptide of the correct length. The advantage of producing secreted heterologous proteins in yeast is that *S. cerevisiae* secretes very few of its own proteins into the culture medium, making subsequent purification of the heterologous protein much easier (Romanos *et al.*, 1992; Hadfield *et al.*, 1993).

S. cerevisiae has additional advantages as a host for heterologous protein production. It is used in the production of many foodstuffs and therefore has GRAS (generally regarded as safe) status and can be cultivated on simple and inexpensive media. It can be grown to high cell densities under controlled conditions leading to the increased production of the heterologous proteins. There are constraints to expression of heterologous proteins in baker's yeast. It lacks the capacity to splice introns from mRNA, and genes that are to be expressed therefore need to be derived from cDNA if the genomic counterparts contain any introns. Protein glycosylation differs markedly between different organisms, and *S. cerevisiae* is known to glycosylate proteins more extensively than most organisms. Proteins that need immunological activity or are required to have specific glycosidic structural recognition elements are troublesome if expressed in yeast. Glycosylation has not been shown to interfere with the normal functioning of heterologously produced enzymes (Romanos *et al.*, 1992; Hadfield *et al.*, 1993).

Expression vectors used in yeast are usually of the shuttle vector type that can be used to transform both *E. coli* and *S. cerevisiae*. Shuttle vectors have the advantage of being easy to manipulate in *E. coli* during the initial construction of the expression vector, and large amounts of the final construct can readily be prepared from *E. coli* before transformation of *S. cerevisiae*. They are usually derived from an existing *E. coli* vector and the naturally occurring yeast 2μ plasmid. The bacterial portion contains an origin of replication and a selection marker, in most cases an antibiotic resistance gene. The yeast 2μ plasmid is an autonomously replicating episomal plasmid containing several genes that are important for its stable inheritance and efficient segregation during cell division, and is maintained at around 10 to 40 copies per yeast cell. The minimum requirement for its use in a shuttle vector is the presence of the origin of replication and the *STB* locus. Host strains containing the native 2μ plasmid supply the plasmid-encoded protein products necessary for stable inheritance of the expression vector (Romanos *et al.*, 1992; Hadfield *et al.*, 1993).

Yeast expression vectors usually contain both yeast-derived promoter and terminator sequences with the heterologous open reading frame in between. These elements are essential for the correct transcription of the gene. The nature of the promoter determines when the gene will be transcribed and to a large degree, the abundance of the mRNA. The position of the TATA-box which is

conserved in most yeast promoters is one of the main determinants of where transcription is initiated. Promoters can be constitutive, such as the highly expressed *PGK1* promoter; inducible, such as the *GAL1* and *GAL10* promoters that are induced by galactose; or modulated by physiological conditions, such as the *ADH2* promoter that is induced by the change from aerobic to fermentative metabolism and glucose depletion. Translation of mRNA starts at the 5'-most ATG in the message and the average length of the non-coding leader sequences found in yeast mRNA's is ~50 nucleotides. Neither the length nor the sequence of the leader sequence has a major influence on the efficiency of transcription. A transcription terminator is necessary after the stop-codon in order to produce transcripts of a single size and is a strict requirement for efficient transcription. Premature termination of translation can occur when the open reading frame contains AT-rich regions. An important factor affecting mRNA stability and thus the production level of both intracellular and secreted proteins is the codon usage of the heterologous gene. Translation of messages with a different codon bias than that of *S. cerevisiae* results in the depletion of scarce tRNA's, quicker degradation of the message and ultimately lower production of the heterologous protein (Romanos *et al.*, 1992; Hadfield *et al.*, 1993).

Selectable markers are required for the stable propagation of episomal vectors in yeast. Auxotrophic selection markers are the most widely used and consist of genes on the plasmid that complement corresponding auxotrophic mutations in the yeast. These include *LEU2*, *TRP1*, *URA3* and *HIS3* and the yeast has to be cultured in defined medium lacking either leucine, tryptophan, uracil or histidine, respectively. Dominant selection markers are genes that encode resistance to antibiotics or other compounds and allow for selection in rich medium. Resistance markers for the antibiotic G418 and hygromycin are commonly used (Romanos *et al.*, 1992; Hadfield *et al.*, 1993).

The secretory pathway in *S. cerevisiae* is very similar to that of other eukaryotes. During the process of translation, the hydrophobic character of the secretion signal of the polypeptide being synthesised directs its own insertion through the lipid bilayer of the endoplasmic reticulum and effectively anchors the ribosome to the membrane. Translation continues into the lumen of the endoplasmic reticulum and at some point during this process the signal peptide is cleaved at a specific recognition sequence by a membrane-bound signal peptidase in the lumen of the ER. Finally, the completely synthesised peptide is exported to the Golgi-apparatus and subsequently via secretory vesicles to the exterior of the cell. Protein folding, disulphide bridge formation and glycosylation are carried out along this pathway. Secretion signals are rather diverse in nature, and many proteins from divergent organisms can be correctly cleaved and secreted by *S. cerevisiae*. Secretion signals have a few general features that are conserved. They usually consist of a core of about 20 hydrophobic residues with a positively charged amino acid at the N-terminus, and cleavage often occurs at the carboxyl side of a glycine or alanine that is followed by an acidic or basic residue (Mathews and van Holde, 1990; Romanos *et al.*, 1992; Hadfield *et al.*, 1993).

A huge number of glycoside hydrolases of both prokaryotic and eukaryotic origin have been expressed with great success in baker's yeast, including amylolytic, pectinolytic, cellulolytic and xylanolytic enzymes, and it seems that this class of enzymes as a whole are amenable to heterologous expression in yeast (Hadfield *et al.*, 1993). The small family 11 *endo*-β-1,4-xylanases are the xylanolytic enzymes that have been most commonly expressed in *S. cerevisiae*. The *Aspergillus kawachii xynC* gene was expressed under control of the constitutive *PGK1* promoter and terminator. The autoselective *fur1::LEU2* strain produced *endo*-β-1,4-xylanase activity at 18 U/ml when cultured in complex medium (Crous *et al.*, 1995). Two family 11 *Aspergillus nidulans endo*-β-1,4-xylanases, *xlnA* and *xlnB*, were expressed in *S. cerevisiae* with the yeast actin-gene promoter and their own signal peptides. The gene products were shown to be of the correct size as judged by SDS-PAGE. The amount of *endo*-β-1,4-xylanase activity produced by the recombinant yeast strains was not reported (Perez-Gonzalez *et al.*, 1996).

The *Trichoderma reesei xyn2* gene was expressed in two constructs, under control of either the *PGK1* promoter and terminator or the glucose-derepressable *ADH2* promoter and terminator in an autoselective *fur1::LEU2* recombinant *S. cerevisiae* strain. The enzyme was secreted efficiently indicating that the native Kex2-like cleavage site in the signal peptide was recognised by the *S. cerevisiae* endopeptidases. The biochemical properties of the recombinant enzyme resembled those of the enzyme isolated from *T. reesei*. In rich medium, expression under the control of the *PGK1* and *ADH2* promoters resulted in a yield of 9.6 U/ml and 72 U/ml, respectively (la Grange *et al.*, 1996). The *Bacillus pumilus endo*-β-1,4-xylanase, *xynA*, was expressed in a similar fashion. A yeast α-mating factor secretion signal was added to the N-terminus of the *xynA* open reading frame. Cultivation in complex medium resulted in the production of 0.27 U/ml and 0.51 U/ml of *endo*-β-1,4-xylanase activity by the two constructs respectively (Nuyens *et al.*, 2001). The *B. pumilus* β-xylosidase gene, *xynB*, fused to the yeast α-mating factor secretion signal was expressed under control of the *ADH2* promoter and terminator (la Grange *et al.*, 1997), and subsequently co-expressed with the *T. reesei xyn2* gene. Co-expression did not affect the amount of *endo*-β-1,4-xylanase produced, but resulted in an eightfold reduction in the amount of β-xylosidase in the culture supernatant. Co-expression of both enzymes did, however, result in a 25% greater release in reducing sugars from birchwood xylan compared to the *endo*-β-1,4-xylanase alone (la Grange *et al.*, 2000).

The *Cryptococcus albidus endo*-β-1,4-xylanase-encoding gene, *XLN*, is the only family 10 *endo*-β-1,4-xylanase that has been expressed in *S. cerevisiae*. It was expressed under control of its own promoter or the glucose-inducible yeast *ADH1* promoter and in both cases found to result in an enzyme that is similar to the native protein in terms of enzymatic properties as well as glycosylation.

Notably, glucose repression was active in *S. cerevisiae* when the gene's native promoter was used and expression of the genomic DNA copy of the gene that contains several introns did not yield any functional enzyme (Moreau *et al.*, 1992).

The *Aspergillus niger* α-arabinofuranosidase gene, *abfB*, was expressed in the *S. cerevisiae* laboratory strain Y294 (Crous *et al.*, 1996) and the *S. cerevisiae* commercial wine strain T_{73} (Sanchez-Torres *et al.*, 1996) under control of the constitutive *PGK1* and actin promoters respectively. In both cases the biochemical properties of the heterologously produced enzyme were very similar to the native protein. The laboratory strain Y294 produced a maximum of 1.4 U/ml of α-arabinofuranosidase activity and the wine strain T_{73} 0.68 U/ml. Expression and secretion of heterologously produced fungal xylanolytic enzymes is efficient enough to allow for activity screening of expression libraries (Saloheimo *et al.*, 1994). The *T. reesei abf1* and *bxl1* genes encoding α-arabinofuranosidase and β-xylosidase activity were isolated by screening a cDNA library in yeast for the ability to hydrolyse *p*-nitrophenyl α-L-arabinofuranoside. Their identities were confirmed by matching the predicted amino acid sequences to the partial sequences obtained from the purified and characterized enzymes (Margolles-Clark *et al.*, 1996).

The fact that so many microbial glycoside hydrolases and more specifically xylanolytic enzymes have been produced and secreted from *S. cerevisiae* suggests that it is an ideal host for the heterologous production of these enzymes. The *endo*-β-1,4-xylanase gene of *A. pullulans* was successfully expressed in *S. cerevisiae*, indicating that xylanolytic genes from the euascomycetous fungus should be ideal candidates for expression in baker's yeast (Li and Ljungdahl, 1996).

Physiology and ecology of *Aureobasidium pullulans*

Aureobasidium pullulans is a euascomycetous fungus and has been classified in the order Dothideales,

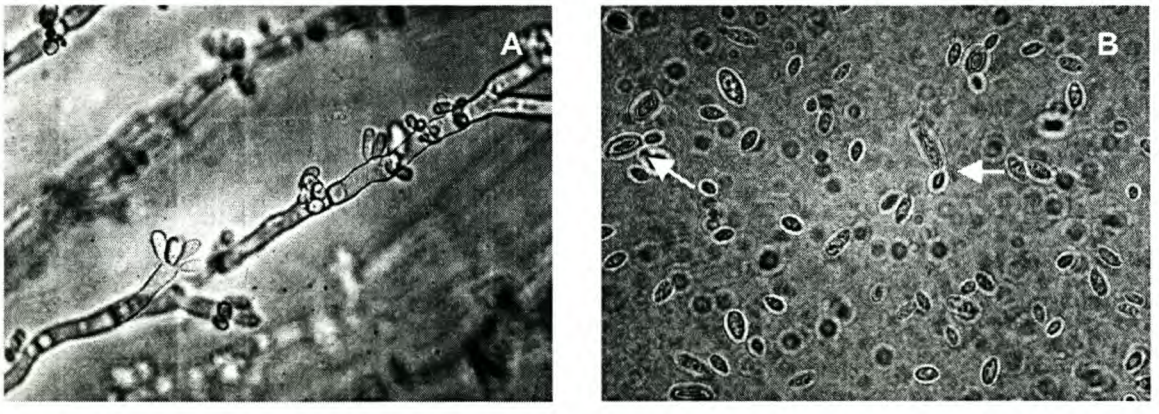

Figure 2.10. Micrographs of *Aureobasidium pullulans* growing as a mycelium **(A)** (http://www.doctorfungus.org/thefungi/img/aure_1.jpg) and as a budding yeast **(B)** (600× magnification). Arrows indicate cells in the process of budding.

family Dothideaceae (de Hoog, 2000) (Figure 2.10). A perfect stage has not been isolated for *A. pullulans*, and the anamorph displays dimorphism, being able to grow as a budding yeast or as a mycelial mass. Mycelia consist of septate hyphae that are hyaline and smooth with thin walls and they form chlamydospores. It is a representative of the black yeasts due to the production of darkly pigmented melanin. *A. pullulans* is most commonly found in temperate climates and has been isolated from a variety of soils and fresh-water and marine sediments all over the globe It is often associated with crop species and tropical fruit, and is a phytopathogen that can cause the softening of plant tissue (Deshpande *et al.*, 1992). *A. pullulans* has the capacity to cause opportunistic infection in humans and has been associated with cases of septicaemia (Kaczmarski *et al.*, 1986; Girardi *et al.*, 1993), abscesses of the spleen (Salkin *et al.*, 1986), corneal ulcers (Gupta *et al.*, 2001), localised cutaneous and subcutaneous infections following trauma (Redondo-Bellon *et al.*, 1997), and is also associated with peritoneal infections in patients with catheters (Clark *et al.*, 1995; Caporale *et al.*, 1996; Ibanez *et al.*, 1997).

Strains of *A. pullulans* have proven to be of considerable industrial importance. The organism produces an exo-polysaccharide slime called pullulan, consisting of entirely linear chains of α-1,4 and α-1,6-linked maltotriose and maltotetraose units. Pullulan has unique film-forming properties, making films that are thin, impermeable to oxygen and resistant to oils. This characteristic makes it suitable for use as a coating and packaging material in the pharmaceutical and food industries. The polymer also has good gelling properties and form highly viscous gels at relatively low concentrations, leading to its use in cosmetic emulsions, as an alternative thickener to starch, and in adhesives (Lee *et al.*, 1999; Roukas, 1999). A variety of other applications has been found for *A. pullulans*. It is capable of contributing to the biological clean-up of oil spills and it is a sensitive indicator of environmental pollution by heavy metals and other industrial pollution, with counts of the organism going up as pollution increases. The use of *A. pullulans* as a source of single cell protein has been suggested based partly on its ability to grow on a variety of cheap agricultural wastes. In the light of the organism's potential pathogenicity it is doubtful if this application would be preferable. *A. pullulans* has been applied in the biodegradation of agricultural and other lignocellulosic waste materials due to its ability to secrete large amounts of hydrolytic enzyme activity (Deshpande *et al.*, 1992).

Several strains of *A. pullulans* produce a large variety of enzymes with potential commercial application, many of which are hydrolytic enzymes involved in the breakdown of carbohydrate polymers. These include sucrase or α-fructofuranoside fructohydrolase, invertase used in production of invert sugar, the pectinolytic enzymes, polygalacturonase and pectin methyl-esterase used in the maceration of fruit pulp and the clarification of fruit juice, the amylolytic enzymes, α-amylase and glucoamylase used in the hydrolysis of starch to glucose and maltodextrins, and the xylanolytic enzymes discussed in detail above (Deshpande *et al.*, 1992).

Colour variants of *A. pullulans* lacking the typical pigmentation have been shown to be hyperproducers of xylanolytic enzymes. Strain NRRL Y2311-1 was found to secrete a hundred-fold more *endo*-β-1,4-xylanase than the typical pigmented strains of the organism (Leathers, 1986). The xylanolytic enzyme system of *A. pullulans* strain NRRL Y2311-1 has been the subject of intensive study and this strain was also found to produce the entire complement of mannanolytic enzymes, *endo*-β-1,4-mannanase, β-mannosidase, α-galactosidase and β-glucosidase (Kremnický and Biely, 1997). This strain produces a monomeric family 11 *endo*-β-1,4-xylanase with a molecular weight on SDS-PAGE of 22 and 25 kDa, indicating a differentially glycosylated protein (Li *et al.*, 1993). The *endo*-β-1,4-xylanase encoding gene, *xynA*, has been cloned and contains an open reading frame of 663 bp encoding a polypeptide of 23.5 kDa. The protein contains a 34 bp signal sequence and the mature enzyme has a predicted molecular weight of 20 kDa. Comparison of the genomic and cDNA sequences showed that the open reading frame contained a 59 bp intron (Li and Ljungdahl, 1994). The x*ynA* gene has been functionally expressed in *S. cerevisiae* under the inducible *GAL1* promoter and was produced as two proteins bands with molecular weights of 25 kDa and 27 kDa. The protein was secreted into the culture medium at a level of 26 U/ml, indicating that *S. cerevisiae* is capable of recognising the *A. pullulans* secretion signal, and correctly processing it along the secretory pathway. The *endo*-β-1,4-xylanase made up 82% of the total protein in the culture medium. Replacing the XynA secretion signal with the native yeast α-factor or invertase secretion signals led to poorer production and secretion of the enzyme (Li and Ljungdahl, 1996).

The xylanolytic accessory enzymes from *A. pullulans* NRRL Y2311-1 have been studied to a limited degree. The production of α-arabinofuranosidase, β-xylosidase and acetyl esterase activity in response to growth on xylose and xylan of different concentrations has been investigated (Myburgh *et al.*, 1991a), and the pH and temperature optima, as well ass stability of these enzymes in the crude supernatant of *A. pullulans* cultured on 1% oat spelt xylan has been determined (Myburgh *et al.*, 1991b). A purified dimeric β-xylosidase with a native molecular weight of 216 kDa and a purified 20 kDa *endo*-β-1,4-xylanase were found to be partially effective in removing xylan from dissolving pulp (Christov *et al.*, 1997). Another strain of *A. pullulans*, NRRL Y12974, has been found to produce a thermostable α-arabinofuranosidase. This enzyme was purified from the culture supernatant of *A. pullulans* NRRL Y12974 grown on oat spelt xylan. The protein was dimeric, with a monomeric molecular weight of 105 kDa on SDS-PAGE and a native molecular weight of 210 kDa on gel filtration. It had a temperature optimum of 75°C and a pH optimum of 4 to 4.5. The enzyme had a specific activity of 21.5 U/mg protein on *p*-nitrophenyl α-L-arabinofuranoside and debranched arabinan and arabinoxylan, but was inactive against arabinogalactan (Saha and Bothast, 1998). Evidence has also been found for the existence of inducible xylobiose and mannobiose permeases that

are responsible for the active transport of the corresponding disaccharides from the surroundings of the yeast cells (Kremnický and Biely, 1998). Further study of the xylanolytic accessory enzymes from *A. pullulans* will therefore broaden the knowledge base of these potentially useful enzymes and add insights into the mechanism of xylan degradation by this organism.

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Chapter 3
Microbial α-glucuronidases: Production, substrate specificity and reaction mechanism

Accepted in modified form as a chapter in an ACS volume on Lignocellulose Biodegradation

Abstract

Microbial α-glucuronidases play a crucial role in the enzymatic degradation of xylan in nature. They specifically hydrolyse 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 β-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 (Beg *et al.*, 2001). Xylan consists of a backbone of 1,4-linked β-xylose units substituted with arabinofuranose, 4-O-methyl glucuronic acid and acetyl esters (Puls, 1992). The complete degradation of xylan requires the concerted action of the main chain cleaving endo-α-1,4-xylanase, β-xylosidase as well as the side-chain hydrolysing enzymes, α-arabinofuranosidase, α-glucuronidase and acetylxylan esterase. Several α-glucuronidase enzymes have been purified and studied to date and their reaction mechanism 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.

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 (Puls *et al.*, 1987) (Figure 3.1). 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 (Fontana *et al.*, 1988). Alternatively polymeric xylan or native lignocellulosic substrates are used in conjunction with high amounts of *endo*-β-1,4-xylanase activity, generating the aldouronic acid substrates *in situ* (Puls, 1992). The colorimetric detection of

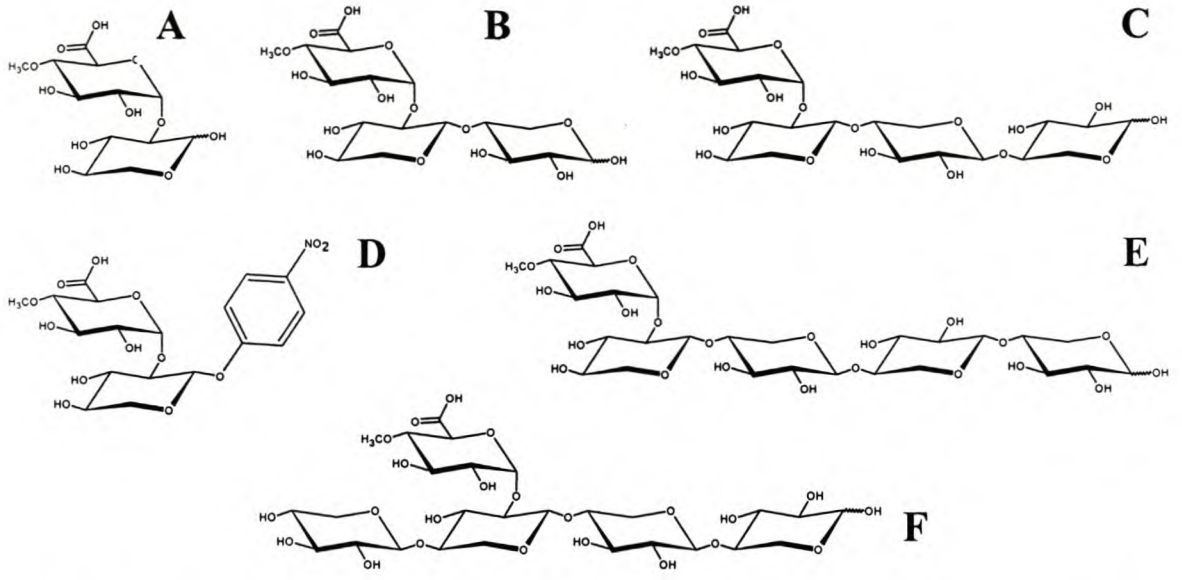

Figure 3.1. Structures of α-glucuronidase substrates. (A) aldobiouronic acid , (B) aldotriouronic acid, (C) aldotetraouronic acid, (D) 4-nitrophenyl 2-(4-*O*-methyl-α-D-glucopyranuronosyl)-β-D-xylopyranoside, (E) aldopentaouronic acid and (F) aldopentaouronic acid containing the glucuronosyl moiety on an internal xylose unit.

the 4-*O*-methylglucuronic acid released is the most commonly used method for determining α-glucuronidase activity (Khandke *et al.*, 1989). This method employs a modification of the Somogyi-Nelson procedure for the determination of reducing sugars (Nelson, 1944), using high salt and low copper concentrations, for the specific detection of uronic acids (Milner and Avigad, 1967). α-Glucuronidase activity has also been measured as the equivalent amount of xylobiose released from aldotriouronic acid by high-performance liquid chromatography (Puls *et al.*, 1987), by directly quantifying 4-*O*-methylglucuronic acid by high-performance liquid chromatography (Castanares *et al.*, 1995), as well as by gas-liquid chromatography of the silylated oxime derivatives of the reducing sugars released (Fontana *et al.*, 1988).

p-Nitrophenyl-glycosides have been used extensively as synthetic substrates in the assay of several glycosidase activities. Microbial α-glucuronidases are, however, unable to hydrolyse *p*-nitrophenyl-glucuronide and only α-glucuronidases from molluscs have been shown to be able to use it as a substrate (Zaide *et al.*, 2001; Bronnenmeier *et al.*, 1995; Kawabata *et al.*, 1995; Fontana *et al.*, 1988). A novel assay has been proposed using 4-nitrophenyl 2-(4-O-methyl-α-D-glucopyranuronosyl)-β-D-xylopyranoside as a substrate for α-glucuronidase, coupled to the hydrolysis of the *p*-nitrophenyl β-D-xylopyranoside product by excess exogenous β-xylosidase activity (Figure 3.1D). 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 routinely used colorimetric procedure, 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 (Biely *et al.*, 2000b), and it has already been used successfully in the characterisation of *Cellvibrio japonicus* α-glucuronidase (Nagy *et al.*, 2003). 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 (Nagy *et al.*, 2002). The same effect was observed in *Streptomyces olivochromogenes* and *Streptomyces flavogriseus* that were cultivated in the presence and absence of 1% oat spelt xylan or lignocellulosic material, although low activity was detectable without the inducers (Johnson *et al.*, 1988).

The oligomeric breakdown products of xylan, rather than the constituent monosaccharides, act as inducers for the production of α-glucuronidase activity (Nagy *et al.*, 2002; Shao *et al.*, 1995). 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 (Shao *et al.*, 1995). 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, as growth of *C. japonicus* on 1% glucose in addition to 1% oat spelt xylan led to complete suppression of the induction witnessed when it was grown on 1% oat spelt xylan as sole carbon source (Nagy *et al.*, 2002). In the case of *Fibrobacter succinogenes*, growth on pure cellulose leads to poorer induction of α-glucuronidase activity compared to growth on lignocellulose (Smith and Forsberg, 1991). 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 (Wood and Wilson, 1995).

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 (Margolles-Clarke, *et al.*, 1997).

A study on 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 (de Vries *et al.*, 1998). 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 mutant of XlnR (van Peij *et al.*, 1998). The transcription factor was found to recognise 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 a second, 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 (de Vries *et al.*, 2002).

The production of the complement of enzymes that degrades 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 are secreted, although the localisation of enzyme activity is not always reported.

There are exceptions where α-glucuronidase activity remains cell-associated or intracellular. Two notable examples are the intracellular α-glucuronidase from *Bacillus stearothermophilus* (Shulami *et al.*, 1999) and the cell surface associated α-glucuronidase from *Cellvibrio japonicus* (Nagy *et al.*, 2002). *B. stearothermophilus* protects substrate access by transporting aldotetraouronic acid, produced by the action of an extracellular *endo*-β-1,4-xylanase (*xynA*), across the cell wall via a putative four protein membrane transporter (*orf1-4*) that also demethylates the *O*4-position of the

aldouronic acid. It is subsequently cleaved to xylose and glucuronic acid by an intracellular α-glucuronidase (*aguA*), *endo*-β-1,4-xylanase (*xynA2*) and β-xylosidase (*xynB*). All of the genes for glucuronic acid utilisation were found on a single cluster in the genome of *B. stearothermophilus*. *C. japonicus* follows a different strategy by localising the xylanolytic accessory enzymes on its cell surface, thereby hydrolysing the small oligosaccharides, generated by the action of a secreted *endo*-β-1,4-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* (Uchida *et al.*, 1992), *Thermoanaerobacterium* spp. (Bronnenmeier *et al.*, 1995; Shao *et al.*, 1995) and *Clostridium stercorarium* (Bronnenmeier *et al.*, 1995).

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* (Dekker, 1983) and several microbial α-glucuronidases have been purified and studied to date (Table 3.1). Based on primary sequence, all of the known α-glucuronidases are assigned to family 67 of the glycoside hydrolases, 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 6.0. 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 dimeric (140 kDa) conformation (Ruile *et al.*, 1997). The pH optima of the bacterial α-glucuronidases, although acidic, are generally higher than 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 making direct comparisons between enzymes from different species difficult. 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 of the microbial α-glucuronidases.

Table 3.1. 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 (Uchida *et al.*, 1992)	150[a], 130[b]	4.8	60	1.4	0.37[4]	tetrao, not pNP-GlcU
Aspergillus niger (Uchida *et al.*, 1992)	150[a], 130[b]	4.8	60	4.7	0.47[4]	tetrao, not pNP-GlcU
Aspergillus tubingensis (de Vries *et al.*, 1998)	100[a], 107[b]	4.5-6.0	70	52	0.14[2/3]	trio, trace xylan
Phanerochaete chrysosporium (Castanares *et al.*, 1995)	112[b]	3.5	NR	4.5	NR	trio>tetrao, not bio, trace xylan
Schizophyllum commune (Tenkanen and Siika-aho, 2000)	125[b]	4.5-5.5	NR	18	NR	trio, xylan
Thermoascus aurianticus (Khandke *et al.*, 1989)	118[a], 117[b]	4.5	65	4.0	0.14[4]	bio to octao, xylan
Trichoderma reesei (Siika-aho *et al.*, 1994)	91[b]	4.5-6.0	NR	28	NR	trio>tetrao>pentao=bio, trace xylan
Helix pomatia (Kawabata *et al.*, 1995)	180[a], 97[b]	3.0	50	2.8	17.6[2]	pNP-GlcU, trio to pentao
Prokaryotes						
Bacillus stearothermophilus T-6 (Zaide *et al.*, 2001)	150[a], 78[b]	6.0	65	42	0.2[4]	tetrao, not pNP-GlcU, not xylan
Bacillus stearothermophilus 236 (Choi *et al.*, 2000)	161[a], 78[b]	6.5	40	15.3	0.78[3]	trio, not xylan
Cellvibrio japonicus (Nagy *et al.*, 2002)	150[a], 83[b]	6.3	NR	84.5	NR	pentao>tetrao>trio>bio, not xylan
Clostridium stercorarium (Bronnenmeier *et al.*, 1995)	124[a], 72[b]	6.0	NR	1.7	NR	tetrao, not pNP-GlcU, not xylan
Thermoanaerobacterium sp. (Shao *et al.*, 1995)	130[a], 74[b]	5.4	60	8.4	0.76[3]	bio>trio>tetrao, trace xylan
Thermoanaerobacterium saccharolyticum (Bronnenmeier *et al.*, 1995)	118[a], 71[b]	6.0	NR	10	NR	tetrao, not pNP-GlcU, not xylan
Thermotoga maritima (Ruile *et al.*, 1997)	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

Most of the microbial α-glucuronidases appear to have no or only very little activity against polymeric xylan compared to aldouronic acid oligomers. The only two exceptions are the α-glucuronidases from *Schizophyllum commune* (Tenkanen and Siika-aho, 2000) and *Thermoascus aurantiacus* (Khandke *et al.*, 1989). The latter enzyme was capable of releasing 4-*O*-methyl glucuronic acid from birchwood xylan at half the rate compared to 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 (Siika-aho *et al.*, 1994; Khandke *et al.*, 1989). The *T. reesei* enzyme was capable of releasing about five times more 4-*O*-methyl glucuronic acid from acetylated compared to deacetylated birch xylan while the *S. commune* enzyme was capable of releasing five times more 4-*O*-methyl glucuronic acid from the deacetylated compared to 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 (Nurizzo *et al.*, 2002). The opposite trend is observed for the α-glucuronidases from *Thermoanaerobacterium* sp., *Phanerochaete chrysosporium* and *T. reesei* (Shao *et al.*, 1995; Castanares *et al.*, 1995; Siika-aho *et al.*, 1994). This group of enzymes shows increasing activity as the chain length decreases up to aldotriouronic acid. However, all three enzymes differ in their preference for aldobiouronic acid. The *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 the *P. chrysosporium* enzyme cannot hydrolyse 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 hydrolyses aldobiouronic to aldooctaouronic acid at the same rate and maintains half the rate of activity on the xylan polymer (Khandke *et al.*, 1989).

It appears to be a general rule that α-glucuronidases have a preference for the 4-*O*-methyl glucuronic acid substituent 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 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 (Biely *et al.*, 2000b). 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, family 11 *endo*-β-1,4-xylanases 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 *endo*-β-1,4-xylanase (Siika-aho *et al.*, 1994). The *endo*-β-1,4-xylanases of family 11 release oligosaccharides with the substituents on non-terminal xylose units as they cannot access the xylosydic bond adjacent to substituted xylose units. β-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 on the terminal non-reducing end xylose unit.

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 centre (Figure 2.4). Both mechanisms involve an acid/base catalytic pair. The distance between the catalytic residues differs, being approximately 5 Å in enzymes following an

retaining mechanism, and between 9 Å and 10 Å in enzymes following an inverting mechanism, allowing for the presence of nucleophilic water for direct attack at the anomeric centre (Henrissat and Davies, 1997). 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 (Biely *et al.*, 2000a). 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-crystallised with the enzyme (Nurizzo *et al.*, 2002).

Crystal structure of α-glucuronidase

The crystal structure of GlcA67A, the α-glucuronidase of *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 (Figure 3.2). 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 (Nurizzo *et al.*, 2002).

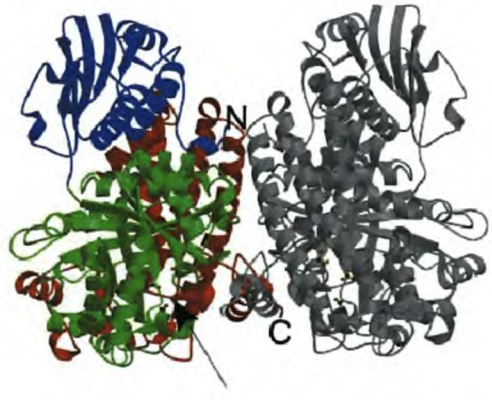

Figure 3.2. Ribbon diagram of the crystal structure of the *C. japonicus* α-glucuronidase, GlcA67A. The structure of the dimer is shown, with one of the monomers coloured according to domain. The *N*-terminal domain is shown in blue, the central catalytic domain in green and the *C*-terminal domain in red. The second monomer is shown in grey. The arrow indicates the position of the active site and the catalytic amino acids are shown in ball-and-stick format (Nurizzo *et al.*, 2002).

Co-crystallisation 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 (Nurizzo *et al.*, 2002). A catalytically inactive mutant of GlcA67A, E292A was used to characterise the enzyme-substrate complex and kinetic characterisation of mutants of evolutionarily invariant amino acids in GlcA67A further confirmed their role in substrate recognition (Nagy *et al.*, 2003) (Figure 3.3). The catalytic acid involved in the hydrolysis reaction was identified as Glu292 based on its position and distance relative to *O*2 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 fulfil this role and mutation of either amino acid to alanine results in a greater than 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 from. In this position the water molecule can be activated by either of the catalytic bases (Nagy *et al.*, 2003; Nurizzo *et al.*, 2002).

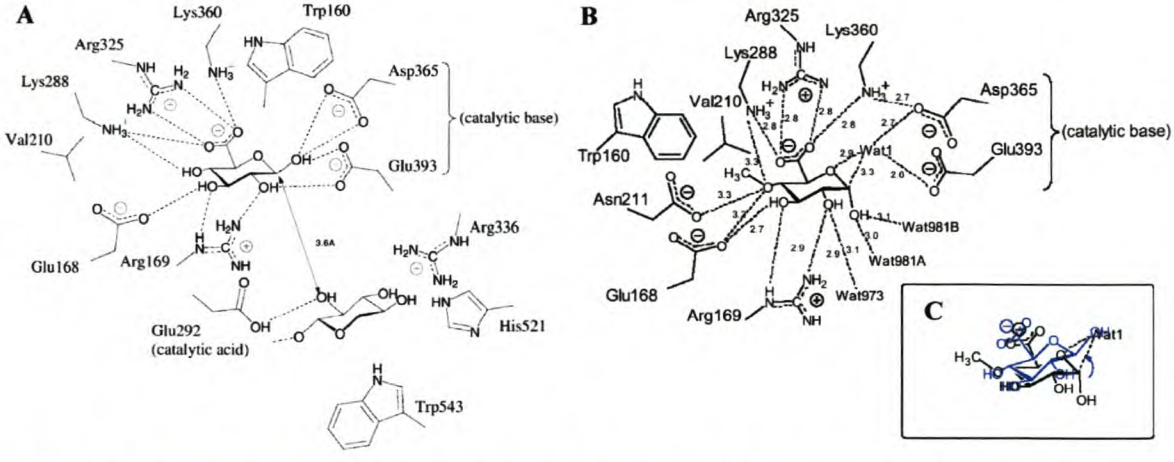

Figure 3.3. Schematic representation of the elements involved in substrate and product recognition. (A) The native enzyme in complex with glucuronic acid and xylotriose (Nurizzo *et al.*, 2002), (B) the E292A mutant in complex with the aldobiouronic acid and (C) the change in position of the pyranoid ring between the substrate (black) and product (blue) states (Nagy *et al.*, 2003).

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 (Figure 3.4). 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 hydrolyse 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, stabilising its position in the active centre. In the E292A mutant co-crystallised 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 (Nagy *et al.*, 2003; Nurizzo *et al.*, 2002).

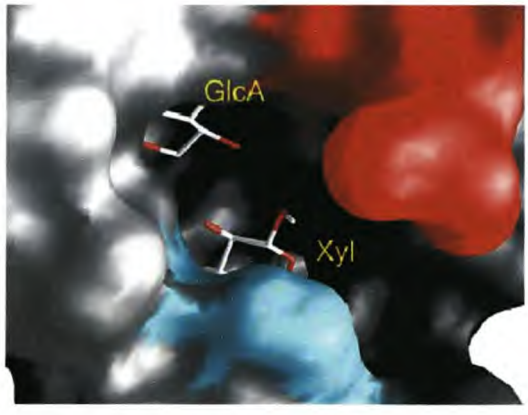

Figure 3.4. Surface representation of the active centre of the *Cellvibrio japonicus* α-glucuronidase showing the deep pocket in which the catalytic machinery is housed (Nurizzo *et al.*, 2002). The reaction products are shown as wire diagrams.

The 4-*O*-methyl glucuronic acid binding site is situated in the deepest part of the pocket and the carboxylate group is stabilised 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 a 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 that native GlcA67A has a twenty-fold higher K_i for glucose than for glucuronic acid (Nagy *et al.*, 2003; Nurizzo *et al.*, 2002).

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 a 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 centre 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 (Figure 3.4) (Nagy *et al.*, 2003; Nurizzo *et al.*, 2002).

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 (de Vries *et al.*, 2000; Tenkanen and Siika-aho, 2000; de Vries *et al.*, 1998; Castanares *et al.*, 1995; Siika-aho *et al.*, 1994). All of the studies addressed the effect of *endo*-β-1,4-xylanase and β-xylosidase activity on the release of 4-*O*-methyl glucuronic acid from xylan. As expected, the addition of either *endo*-β-1,4-xylanase or β-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 *endo*-β-1,4-xylanase and β-xylosidase results in a far greater release of 4-*O*-methyl glucuronic acid from xylan by α-glucuronidase than either enzyme on its own in combination with α-glucuronidase. The combined action of β-xylosidase and *endo*-β-1,4-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 (Tenkanen and Siika-aho, 2000; Castanares *et al.*, 1995).

The effect of α-glucuronidase activity on the release of reducing sugars from xylan by the action of *endo*-β-1,4-xylanase, β-xylosidase or a combination of the two has also been investigated (de Vries *et al.*, 2000; de Vries *et al.*, 1998; Siika-aho *et al.*, 1994). The degree to which α-glucuronidase enhances the release of reducing sugars from xylan is dependent on the nature of the *endo*-β-1,4-

xylanase that is used. The *T. reesei* α-glucuronidase enhanced reducing sugar release from xylan by the *T. reesei endo*-β-1,4-xylanase I to a much greater degree than the *T. reesei endo*-β-1,4-xylanase II. This result is unexpected as both these enzymes are classified in family 11 of the carbohydrate active enzymes and share a similar active site architecture (Siika-aho *et al.*, 1994). The effect of α-glucuronidase on the release of reducing sugars from xylan is also more pronounced when β-xylosidase acts alone compared to 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 on the terminal non-reducing end xylose unit will prevent it from hydrolysing the terminal xylose, whereas the *endo*-nature of *endo*-β-1,4-xylanase will allow it to hydrolyse β-1,4-xylosidic linkages that might exist further down the chain. The 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 hydrolysed after removal of the 4-*O*-methyl glucuronic acid side-chains (de Vries *et al.*, 2000; de Vries *et al.*, 1998; Siika-aho *et al.*, 1994).

The complete hydrolysis of xylan is dependent on the removal of all substituents from the backbone. α-Glucuronidases plays 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-glucuronidase have added another piece into the puzzle of lignocellulose biodegradation.

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Chapter 4

Partial purification and characterisation of an extracellular α-glucuronidase from the euascomycetous fungus *Aureobasidium pullulans* NRRL Y2311-1

Summary

The α-glucuronidase secreted by the *endo*-β-1,4-xylanase overproducing *Aureobasidium pullulans* strain, NRRL Y2311-1 was partially purified and characterized. Production of α-glucuronidase activity was induced by growth on a combination of xylose and glucuronic acid, as well as polymeric xylan and milled plant material. Maximal activity was observed after 3 days of cultivation in medium containing birchwood glucuronoxylan. The purification procedure consisted of ammonium sulphate precipitation, hydrophobic interaction, cation exchange and finally gel filtration. The enzyme ran as a diffuse band with an apparent molecular weight of 170 kDa on SDS-PAGE and migrated as a sharp band of 118 kDa after treatment with *N*-glycosidase F, indicating a high degree of asparagine-linked glycosylation. The enzyme had a K_m for aldotriouronic acid of 3.3 ± 0.9 mM and a K_m for aldobiouronic acid of 29.5 ± 7.6 mM. Substrate inhibition was observed and the inhibition constants are 9.8 ± 3.8 mM for aldotriouronic acid and 29.0 ± 7.8 for aldobiouronic acid. The enzyme was maximally active at pH 3 and 40°C. It was stable at 40°C for 3 h and had a half-life of 1 h at 50°C. Complete purification of the α-glucuronidase was hindered by a high degree of glycosylation of proteins secreted by *A. pullulans*, resulting in poor separation in almost all of the purification steps.

Introduction

α-Glucuronidase forms part of a larger collection of lignocellulolytic enzymes that microbial saprophytes and plant pathogens employ to degrade the intricate structure of plant cell walls. They specifically cleave the α-1,2 linkage between 4-*O*-methyl glucuronic acid (or glucuronic acid) side-chains and the xylose units of small oligosaccharides, liberated from polymeric xylan by the action of *endo*-β-1,4-xylanases. 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 (Beg *et al.*, 2001). Xylan is made up of a backbone of β-1,4-linked xylose units substituted with arabinose, 4-O-methyl glucuronic acid and acetyl groups (Figure 2.1) (Puls, 1992). The complete degradation of xylan requires the concerted action of the main chain cleaving *endo*-β-1,4-xylanase as well as the side-chain hydrolysing accessory enzymes, including α-glucuronidase. The work described in this chapter involves the partial purification of the α-glucuronidase from the culture supernatant of a colour variant strain of the euascomycetous fungus *A. pullulans*, NRRL Y2311-1, that was isolated for overproduction of *endo*-β-

1,4-xylanase activity (Leathers, 1986). The partially purified enzyme was characterized in terms of enzymatic properties and these results will be discussed in light of what is known about α-glucuronidases isolated from other fungal species.

Materials and methods

Microbial strains and culture conditions

A. pullulans strain NRRL Y2311-1 was obtained from the National Center for Agricultural Utilization Research (Peoria, Illinois, U.S.A.) and grown from frozen glycerol stock cultures on YPD plates (containing per litre, 10 g yeast extract (Difco), 20 g peptone (Difco), 20 g glucose and 20 g agar) for 3 days at 30°C. Enzyme production was achieved by cultivation of the organism in defined medium (containing per litre, 10 g birchwood xylan (Roth), 6.7 g yeast nitrogen base (Difco), 2 g L-asparagine and 5 g monobasic potassium phosphate) for 4 days at 30°C with shaking at 150 rpm. Defined medium was inoculated with 20 ml of a two day old culture grown in rich YPD medium. Several carbon sources were investigated for their ability to induce the production of xylanolytic enzymes.

Protein determination

Protein was determined by direct measurement of the preparations at 280 nm using bovine serum albumen as a standard (Ausubel *et al.*, 1995).

α-Glucuronidase assay

α-Glucuronidase activity was assayed by measuring the release of glucuronic acid or its 4-*O*-methyl derivative from aldotriouronic acid (4-*O*-methyl-D-glucopyranuronosyl-α-1,2-D-xylopyranosyl-β-1,4-D-xylopyranose) (Megazyme, Ireland). The amount of 4-*O*-methyl glucuronic acid released was quantified using the Milner-Avigad calorimetric method for the determination of hexuronic acids (Milner and Avigad, 1967). The reaction mixture consisted of 5 mM aldotriouronic acid, 50 mM formate buffer, pH 3, and an appropriate quantity of enzyme in a final volume of 100 µl. The reaction was terminated by adding 300 µl copper reagent and boiling for 15 min. Samples were cooled on ice and 200 µl arsenomolybdate reagent added to complete the colour reaction. Samples were transferred to a microtitre plate and absorbance read at 620 nm in an Anthos 2001 microtitre plate reader. A standard containing between 5 and 50 nmole glucuronic acid (Sigma) in buffer in a final volume of 100 µl was included with each assay. One unit of activity is defined as the amount of enzyme capable of releasing 1 µmole of glucuronic acid per min.

endo-β-1,4-Xylanase assay

endo-β-1,4-Xylanase activity was assayed by measuring the release of reducing sugars from birchwood glucuronoxylan (Roth, Germany) (Bailey *et al.*, 1992). Reducing sugars were quantified using dinitrosalicylic acid (DNS) as a colour reagent (Miller, 1959). The assay was performed by adding 50 µl of enzyme, appropriately diluted in citrate buffer, pH 6, to 450 µl of substrate solution (1% birchwood glucuronoxylan in 50 mM citrate buffer, pH 6), pre-incubated in a water bath at 50°C. The reaction was allowed to proceed for 5 min and terminated by the addition of 750 µl DNS colour reagent. Reaction mixtures were boiled for 10 min to allow for colour formation, cooled in ice water and absorbance at 540 nm measured. A standard containing between 50 and 500 nmole xylose in the substrate and reaction buffer was included with each determination. One unit of activity is defined as the amount of enzyme capable of releasing 1 µmole of reducing sugar per min.

β-Xylosidase assay

β-Xylosidase activity was determined by following the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-xylopyranoside (Sigma). The reaction mixture consisted of 5 mM *p*-nitrophenyl-β-D-xylopyranoside in 100 mM sodium acetate buffer, pH 5, and an appropriate amount of enzyme in a total volume of 100 µl. The reaction was allowed to proceed for 10 min at 40°C and terminated by the addition of 100 µl saturated sodium tetraborate. Samples were transferred to a microtitre plate and absorbance read at 414 nm in an Anthos 2001 microtitre plate reader. β-Xylosidase activity determined from the slope of the standard plot of absorbance as a function of *p*-nitrophenol (Sigma) concentration under the assay conditions. One unit of β-xylosidase activity is defined as the amount of enzyme capable of releasing 1 µmol *p*-nitrophenol from the substrate per min.

α-Arabinofuranosidase assay

α-Arabinofuranosidase activity was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-α-L-arabinofuranoside (Sigma). The reaction mixture consisted of 10 mM *p*-nitrophenyl-α-L-arabinofuranoside in 100 mM sodium acetate buffer, pH 5, and an appropriate amount of enzyme in a total volume of 100 µl. The reaction was allowed to proceed for 10 min at 40°C and terminated by the addition of 100 µl saturated sodium tetraborate. Samples were transferred to a microtitre plate and absorbance read at 414 nm in an Anthos 2001 microtitre plate reader. α-Arabinofuranosidase was activity determined from the slope of the standard plot of absorbance as a function of *p*-nitrophenol concentration under the assay conditions. One unit of α-arabinofuranosidase activity is defined as the amount of enzyme capable of releasing 1 µmol *p*-nitrophenol from the substrate per min.

Acetyl esterase assay

Acetyl esterase activity was assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenyl acetate (Sigma) as substrate (Biely *et al.*, 1996). Assays were performed at room temperature in microtitre plates in a final volume of 200 µl. A 50 mM stock of *p*-nitrophenyl acetate in dimethyl sulphoxide was diluted to 2 mM in 100 mM sodium phosphate buffer pH 6.5 immediately before use. The reaction was started by adding 100 µl of the prepared substrate to enzyme diluted to 100 µl in 100 mM sodium phosphate buffer pH 6.5. The change in absorbance at 414 nm was followed over time in an Anthos 2001 microtitre plate reader. Acetyl esterase activity was determined from the slope of the standard plot of absorbance as a function of *p*-nitrophenol concentration under the assay conditions. One unit of acetyl esterase activity is defined as the amount of enzyme capable of releasing 1 µmol *p*-nitrophenol from the substrate per min

SDS-polyacrylamide gel electrophoresis

Discontinuous sodium dodecyl sulphate gel electrophoresis was carried out using the Tris-glycine buffer system (Laemmli, 1970) with 7 to 10% polyacrylamide gels and Rainbow molecular weight markers (Amersham). Protein bands were visualised by silver staining (Blum *et al.*, 1987). Gels were soaked in fixing solution (50% methanol, 12% acetic acid, 0.0185% formaldehyde) for 30 min and washed twice for 10 min in 50% ethanol. Gels were pre-treated by soaking in 0.002% sodium thiosulphate pentahydrate for one min and washed with three changes of water for 30 seconds. Gels were subsequently soaked in 0.2% silver nitrate solution (containing 0.028% formaldehyde) for 10 min and given two quick washes with water. Gels were developed in 6% sodium carbonate, 0.0185% formaldehyde and 0.4 mg/L sodium thiosulphate pentahydrate. Development was terminated by decanting the developer and adding stop solution (50% methanol, 12% acetic acid).

Purification of the α-glucuronidase

Concentration of the culture supernatant by ultrafiltration

Culture supernatant was harvested after three days of growth by centrifugation at 10 000 rpm for 10 min in a Beckman J2-21 centrifuge using a JA-14 rotor. The decanted culture fluid was concentrated from 2 L to approximately 200 ml using a Minitan tangential flow ultrafiltration system (Millipore Corporation) fitted with a 10 kDa cut-off membrane. The preparation was further concentrated to approximately 20 ml in an Amicon ultrafiltration cell (Millipore Corporation) fitted with a 10 kDa cut-off membrane, and all subsequent concentration steps were performed similarly. Concentration was performed at 4°C and EDTA, phenyl methyl sulphonyl fluoride (Merck) and sodium azide were added to 5 mM, 1 mM and 0.02% (w/v), respectively, after concentration.

Ammonium sulphate precipitation

MOPS buffer at pH 7 was added to the preparation to a final concentration of 50 mM and the preparation made up to a volume of 40 ml. Protein was precipitated by slowly stirring in finely ground ammonium sulphate to 80% saturation at 4°C. The solution was left stirring for at least 1 h. Precipitated protein was sedimentented at 10 000 rpm for 30 min in a JA-20 rotor, the supernatant retained and dialysed overnight against distilled water and the preparation concentrated to 10 ml.

Hydrophobic interaction chromatography on Phenyl-sepharose

Chromatography was performed on Phenyl-sepharose Fast Flow resin (Pharmacia) packed in a 1.6 cm × 9.0 cm column (18 ml volume) coupled to a Biorad EconoPump chromatography system. The sample was applied to the column in 1 M ammonium sulphate and 100 mM sodium acetate, pH 5. The flow rate was adjusted to 1 ml/min and the column was washed with 1 M ammonium sulphate in 100 mM sodium acetate for 10 min. A gradient of 1 to 0 M ammonium sulphate in 100 mM sodium acetate, pH 5, was run over a period of 50 min. The column was eluted with 100 mM sodium acetate, pH 5, for an additional 20 min. Fractions were collected every 5 min (5 ml) for the duration of the run. Activity-containing fractions were pooled, dialysed overnight against de-ionised water and concentrated to 1.5 ml.

Gel filtration on Sephadex G200

Gel filtration was performed on Sephadex G200 (Sigma) packed in a 1.2 cm × 48 cm column (54 ml) in de-ionised water. After application, the sample was eluted at a flow rate of 0.2 ml/min and fractions of 0.5 ml collected. Activity-containing fractions were pooled and concentrated to 2 ml.

Cation exchange chromatography on CM-sepharose at pH 6

Cation exchange was performed at pH 6 on CM-Sepharose Fast Flow resin (Pharmacia) packed in a 1.5 cm × 3.5 cm column (6.2 ml) connected to a Biorad EconoPump chromatography system. The sample was applied in 20 mM *N*-morpholinoethane sulphonic acid (MES), pH 6. The flow rate was adjusted to 1 ml/min and fractions were collected every 2.5 min. The column was washed for 5 min with 20 mM MES, pH 6 and then eluted with a gradient of 0 to 1 M NaCl in 20 mM MES, pH 6 over 60 min. The column was washed with for an additional 10 min with to 1 M NaCl in 20 mM MES, pH 6. Activity-containing fractions were pooled, dialysed overnight against de-ionised water and concentrated to 1.3 ml.

Gel filtration on Sephadex G150

Gel filtration was performed on Sephadex G150 (Sigma) packed in a 1.5 cm × 50 cm column (85 ml) in de-ionised water. After application, the sample was eluted at a flow rate of 0.2 ml/min and 1 ml

fractions were collected. Activity-containing fractions were pooled and concentrated to 2 ml in an Amicon ultrafiltration cell with a 10 kDa cut-off membrane

Cation exchange chromatography on CM-sepharose at pH 4

Cation exchange was performed at pH 4 on CM-Sepharose Fast Flow packed in a 1.5 cm × 3.5 cm column (6.2 ml) connected to a Biorad EconoPump chromatography system. The sample was applied in 20 mM sodium lactate, pH 4. The flow rate was adjusted to 1 ml/min and fractions were collected every 2.5 min. The column was washed for 10 min with 20 mM sodium lactate, pH 4 and then eluted with a gradient of 0 to 1 M NaCl in 20 mM sodium lactate, pH 4 over 60 min. The column was washed with for an additional 10 min with 1 M NaCl in 20 mM sodium lactate, pH 4. Activity-containing fractions were pooled, dialysed overnight against de-ionised water and concentrated to 0.6 ml.

Characterisation of the partially purified α-glucuronidase

pH dependence of α-glucuronidase activity

The effect of pH on enzyme activity was investigated by assaying at pH values ranging between 2.2 and 8 using McIlvaine's phosphate-citrate buffer (McIlvaine, 1921). The reaction mixtures contained buffer (0.1 M citric acid and 0.2 M disodium phosphate mixed in various ratios) diluted 1:1 with the rest of the reaction components, 5 mM aldotriouronic acid and an appropriate amount of enzyme. Assays were performed at 40°C. Standards of between 10 and 100 µmole glucuronic acid were included for each pH point.

Temperature dependence of α-glucuronidase activity

The temperature dependence of enzyme activity was tested by assaying for activity between 20 and 70°C. The reaction mixture contained 50 mM formate buffer pH 3, 5 mM aldotriouronic acid and a suitable amount of enzyme, as described above.

Determination of temperature stability

The temperature stability of the enzyme was investigated by incubating the enzyme at pH 7 in 10 mM *N*-morpholinopropane sulphonic acid (MOPS) buffer between 20 and 60°C, taking samples at 30 min intervals and assaying for activity. The reaction mixture contained 50 mM formate buffer, pH 3, 5 mM aldotriouronic acid and a suitable amount of enzyme and assays were performed at 40°C as described above.

Investigation of kinetic properties

Kinetic constants of the enzyme for aldobiouronic acid and aldotriouronic acid (Megazyme, Ireland) were determined by assaying for activity at pH 3 and 40°C with a range of substrate concentrations. The concentration range for aldobiouronic acid was between 1 and 100 mM and the range for aldotriouronic acid was between 0.1 and 10 mM.

Deglycosylation with N-Glycosidase F

Removal of asparagine-linked glycan side-chains was achieved by treatment of the enzyme with a commercial preparation of *N*-glycosidase F (Roche). The protein preparation was incubated with denaturation buffer (containing ionic detergent, phosphate buffer, pH 8.6, and 1% 2-mercaptoethanol) for 3 min at 95°C and reaction buffer (containing phosphate buffer, pH 7.2 and non-ionic detergent) and 3 units of *N*-glycosidase F added. The reaction mixture incubated at 37°C for 1 h. The reaction was terminated by adding 30 µl of SDS-PAGE sample buffer and incubating at 95°C for 3 min. Treated and untreated samples were subsequently resolved by SDS-PAGE and silver stained as described above.

Results

The effect of culture conditions on the production of α-glucuronidase activity

The production of xylanolytic enzyme activities by *A. pullulans* on different carbon sources was investigated. *A. pullulans* was grown in defined medium with different carbon sources for three days and the activity of several xylanolytic enzymes measured in the culture supernatant. α-Glucuronidase activity (Figure 4.1A) was induced by growth of *A. pullulans* on polymeric xylan from oat spelts and birchwood, with the highest levels of about 20 mU/ml produced on birch xylan. A combination of the monosaccharides xylose and glucuronic acid also induced the production of α-glucuronidase activity, but neither of the sugars on its own was capable of doing so. Very little α-glucuronidase activity was detected after growth on sugarcane bagasse or wheat straw.

The production of other xylanolytic enzymes was also investigated including, *endo*-β-1,4-xylanase, β-xylosidase, α-arabinofuranosidase and acetyl esterase activity (Figure 4.1B to E). The glycoside hydrolases were all induced in a similar fashion. Xylose, the combination of xylose and glucuronic acid as well as the polymeric xylans induced the production of these activities and the induction was repressed by the presence of glucose. Enzyme production was poor on glucose, sugarcane bagasse and wheat straw. Notably, more α-arabinofuranosidase activity was produced when *A. pullulans* was grown on oat spelt xylan compared to birch xylan. Esterase activity was highly induced only by growth on the polymeric xylans. It was moderately induced on xylose, sugarcane bagasse and wheat

straw and only poorly induced by growth on any of the other monosaccharides or combinations thereof.

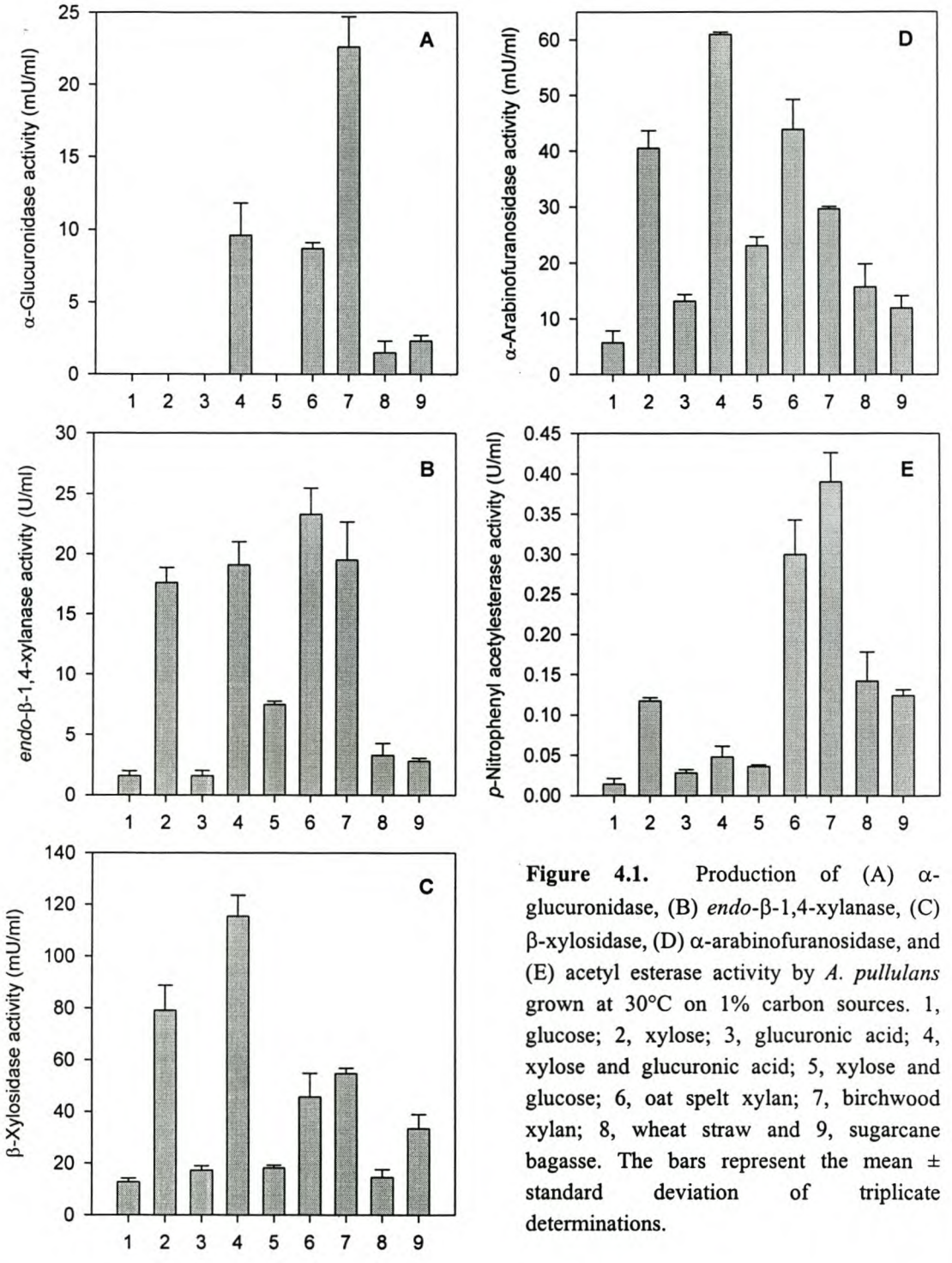

Figure 4.1. Production of (A) α-glucuronidase, (B) *endo*-β-1,4-xylanase, (C) β-xylosidase, (D) α-arabinofuranosidase, and (E) acetyl esterase activity by *A. pullulans* grown at 30°C on 1% carbon sources. 1, glucose; 2, xylose; 3, glucuronic acid; 4, xylose and glucuronic acid; 5, xylose and glucose; 6, oat spelt xylan; 7, birchwood xylan; 8, wheat straw and 9, sugarcane bagasse. The bars represent the mean ± standard deviation of triplicate determinations.

The time-course of the production of the xylanolytic enzyme activities by *A. pullulans* was investigated by culturing the fungus in defined medium containing 1% birchwood xylan and taking samples for assay every 8 h after inoculation (Figure 4.2). There was a lag of 32 h before any α-glucuronidase activity could be measured in the culture fluid and activity reached a maximum of about

22 mU/ml after 72 h and remained at that level for up to 120 h. All the other xylanolytic enzymes, except *endo*-β-1,4-xylanase activity, which started to decline after 72 h, followed a similar pattern of production over time.

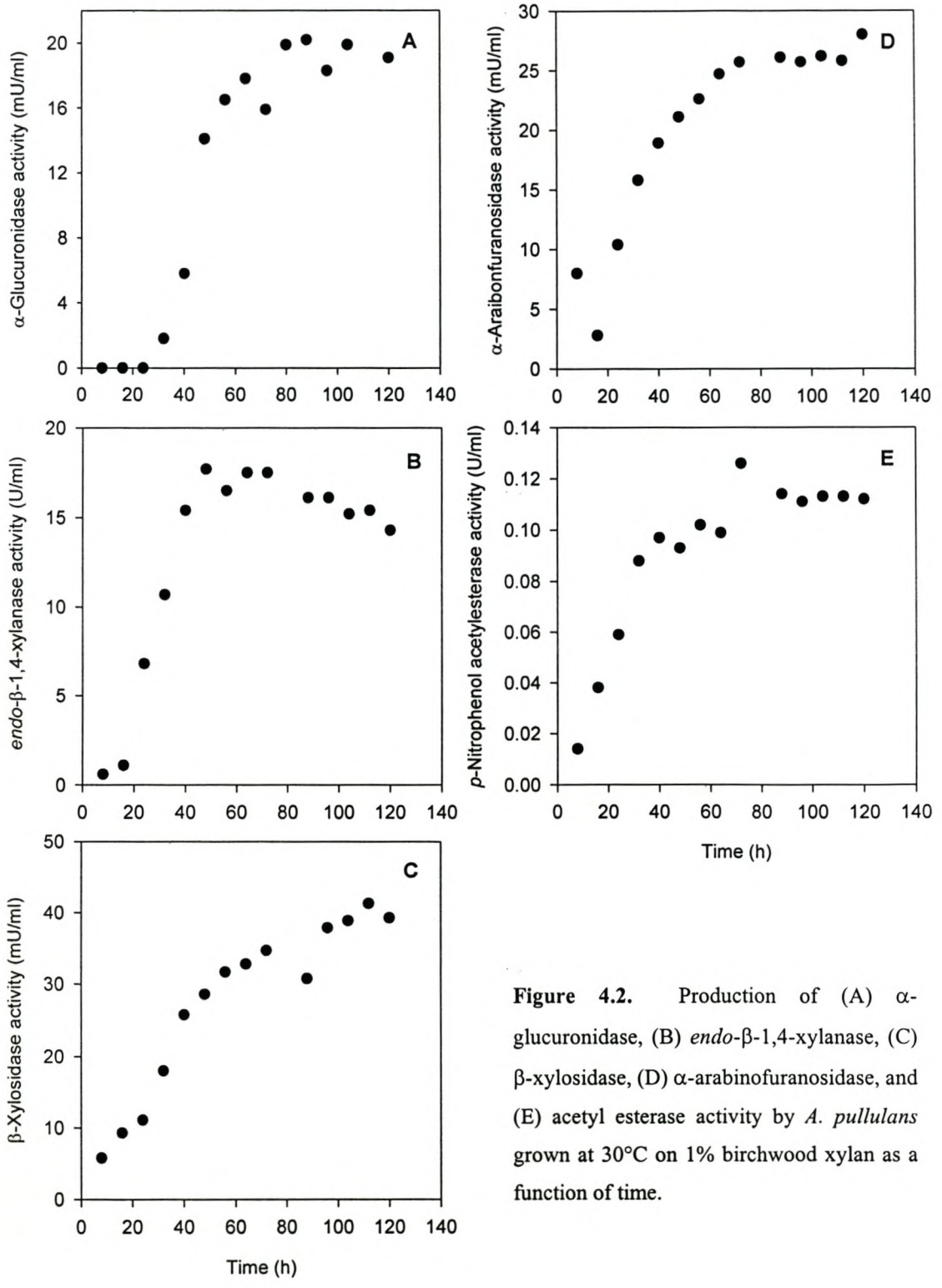

Figure 4.2. Production of (A) α-glucuronidase, (B) *endo*-β-1,4-xylanase, (C) β-xylosidase, (D) α-arabinofuranosidase, and (E) acetyl esterase activity by *A. pullulans* grown at 30°C on 1% birchwood xylan as a function of time.

Purification of the α-glucuronidase

Figure 4.3 shows an SDS-PAGE gel of the preparation after each purification step. The results of enzyme assays after each purification step are summarised in Table 4.1. The starting material for the purification procedure was the concentrated supernatant of an *A. pullulans* culture grown for 3 days in defined medium containing 1% birchwood xylan. The first purification step involved protein precipitation at 80% ammonium sulphate. About half the total protein and 95% of the *endo*-β-1,4-xylanase activity present in the concentrated culture supernatant were precipitated at this concentration of ammonium sulphate, compared to only 15% of the α-glucuronidase activity.

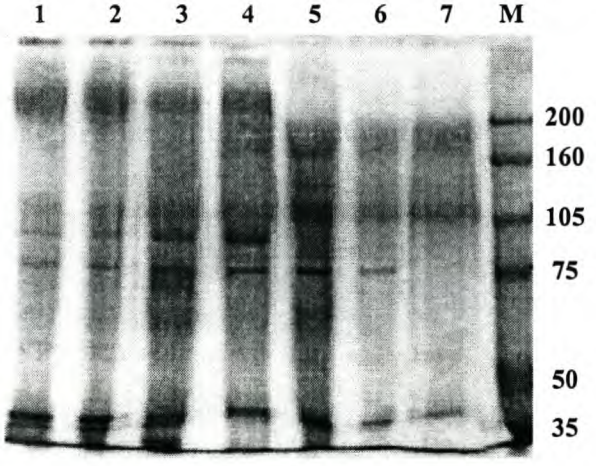

Figure 4.3. SDS-PAGE gel (7%) of the various protein preparations after each purification step. Lane 1 is the concentrated supernatant; Lane 2, the fraction not precipitated at 80% ammonium sulphate; Lane 3, the preparation after HIC on Phenyl-Sepharose; Lane 4, the preparation after gel filtration on Sephadex G200; Lane 5, the preparation after cation exchange on CM-Sepharose at pH 6; Lane 6, the preparation after gel filtration on Sephadex G150; Lane 7, the preparation after cation exchange on CM-Sepharose at pH 4. The right-hand lane is Rainbow molecular weight markers (Amersham).

Table 4.1. Purification of the *A. pullulans* α-glucuronidase.

Purification step	*αGlcU (U)*	*Protein (mg)*	*Spec Act (U/mg)*	*Yield (%)*	*Factor*	*βXyn (U)*	*βXyl (U)*	*αAraf (U)*	*AcE (U)*
Supernatant	42.3	1692	0.025	100	1	8860	36.6	22.0	293
Not precipitated	36.4	789	0.046	86	1.8	423	33.8	20.2	189
Phe-sepharose, pH5	29.6	59	050	70	20	270	1.5	3.1	52.3
Sephadex G200	26.6	8.6	3.1	63	124	32.3	0.7	0.9	32.8
CM-sepharose, pH 6	7.2	2.0	3.6	17	144	3.3	0.3	0.6	11.6
Sephadex G150	2.6	1.2	2.2	6.1	88	nd	0.09	0.05	3.6
CM-sepharose, pH 4	0.7	0.7	1.0	1.7	40	nd	0.02	0.008	0.3

1 U of activity is equal to 1 μmol of product formed per minute. Values given for measurements taken after fractions were pooled, dialysed and concentrated. αGlcU is α-glucuronidase; Spec Act, specific activity; βXyn, *endo*-β-1,4-xylanase; βXyl, β-xylosidase; αAraf, α-arabinofuranosidase, AcE, *p*-nitrophenyl acetyl esterase; nd, not detected.

Subsequently, the protein preparation was subjected to hydrophobic interaction chromatography on Phenyl-Sepharose at pH 5. This step was repeated four times on aliquots of the preparation to avoid overloading of the column matrix. The elution profile of the protein preparation is shown in Figure 4.4A. The α-glucuronidase activity was bound to the stationary phase in 1 M ammonium sulphate at pH 5 and was eluted by a gradient of decreasing ammonium sulphate. Most of the proteins were either washed through the column or bound poorly and were eluted at the initial stages of the ammonium sulphate gradient, as is evident from the large protein peak in the first seven fractions. The major

activity that co-eluted with the α-glucuronidase was *pNP*-acetyl esterase, although some *endo*-β-1,4-xylanase, β-xylosidase and α-arabinofuranosidase activity was also present in these fractions. Fractions 9 to 14, containing the bulk of the α-glucuronidase peak, were pooled, dialysed and concentrated, and used for analysis and further purification.

Gel filtration on Sephadex G200 was used as a size-separation technique in the following step. The concentrated protein preparation was loaded onto the column and the void volume eluted before 0.5 ml fractions were taken. The elution of the different xylanolytic enzyme activities from the column is shown in Figure 4.4B. α-Glucuronidase activity eluted in a very broad peak over 13 fractions (6.5 ml). Small amounts of α-arabinofuranosidase and β-xylosidase activity also appeared in these fractions. Significant amounts of *endo*-β-1,4-xylanase activity started to appear in the last two α-glucuronidase containing fractions. Fractions were not assayed for acetyl esterase activity or protein content. The α-glucuronidase containing fractions were pooled, dialysed and concentrated, and assayed for enzyme activity and protein content. Gel filtration on Sephadex G200 resulted in a marked decrease in the other xylanolytic enzyme activities with only a slight loss in α-glucuronidase activity. This step also resulted in the removal of bands on SDS-PAGE below 35 kDa as well as bands between 50 and 75 kDa and the appearance of a diffuse band migrating just below 200 kDa.

The next purification method used was cation exchange on CM-Sepharose at pH 6. Protein was bound in the absence of salt and eluted with a linear gradient up to 1 M NaCl. The elution profile of this step is shown in Figure 4.4C. Most of the protein as well as enzyme activity bound poorly to the matrix at this pH, and were eluted during the very early part of the salt gradient. *Endo*-β-1,4-xylanase activity was partly retained and eluted in two peaks. The fractions containing α-glucuronidase activity were pooled, dialysed and concentrated and analyzed. Cation exchange at pH 6 removed two bands on SDS-PAGE, one migrating at above 200 kDa and the other between 105 and 75 kDa. It also resulted in a tenfold decrease in *endo*-β-1,4-xylanase activity. A significant portion of the α-glucuronidase activity was lost during this step.

Sephadex G150 was used in the following gel filtration step. Fractions collected after elution of the void volume were assayed for activity. The elution profile is shown in Figure 4.4D. The α-glucuronidase activity eluted as a broad peak over 10 ml or ~25% of the column volume. The other xylanolytic activities eluted in similarly broad peaks, β-xylosidase activity in the same fractions as the α-glucuronidase activity, pNP-acetyl esterase activity 2 ml after the α-glucuronidase activity and α-arabinofuranosidase activity 4 ml after the α-glucuronidase activity. Fractions 23 to 28 were pooled, dialysed and concentrated for further purification. This resulted in the recovery of about one third of the α-glucuronidase activity and the complete removal of *endo*-β-1,4-xylanase activity from the

mixture. α-Arabinofuranosidase, β-xylosidase and pNP-esterase activities were also significantly less after this step. Only four bands were visible in the mixture after this gel filtration step, at ~170 kDa, ~110 kDa, 75 kDa and 35 kDa (Figure 4.3, lane 6).

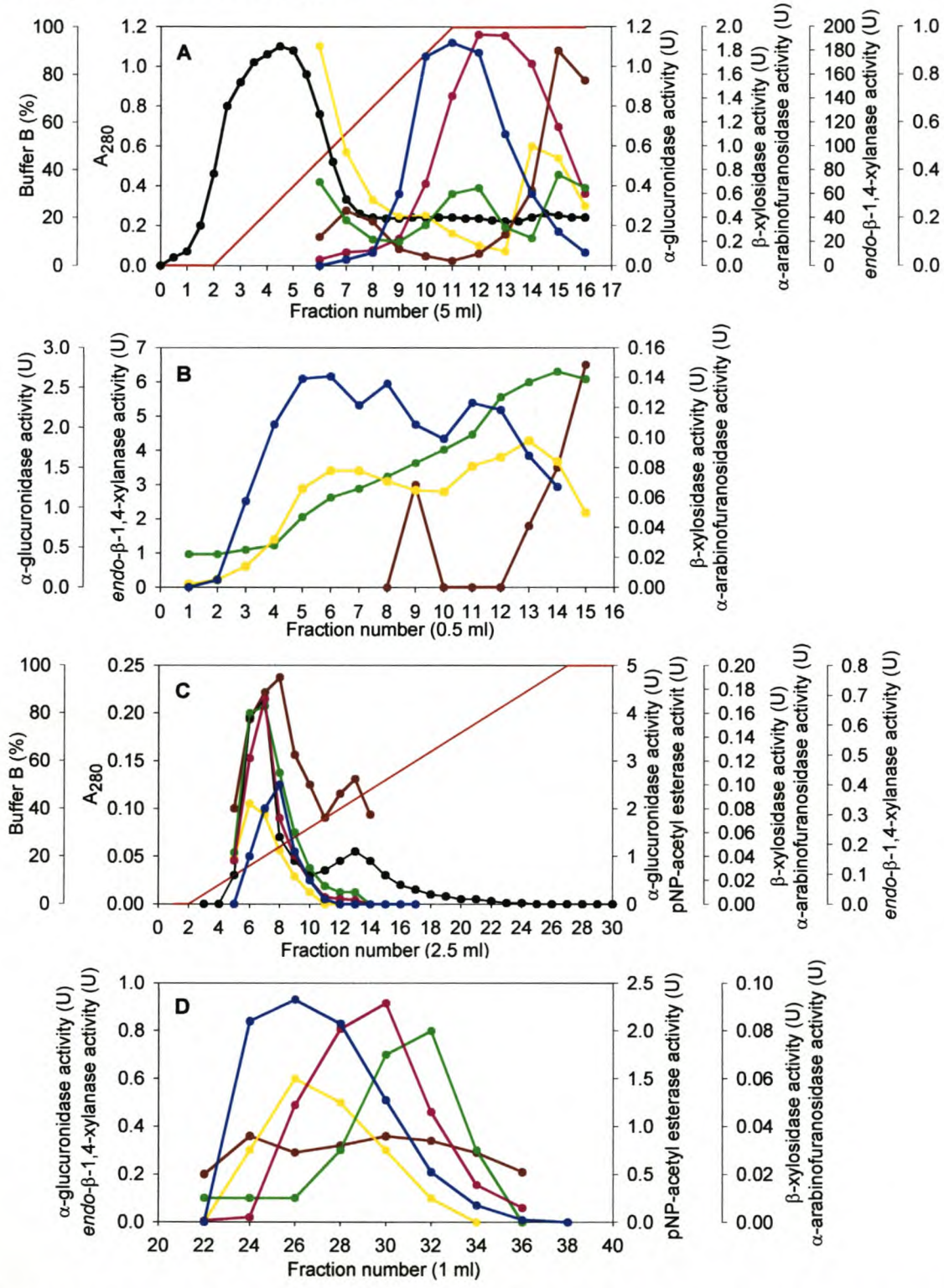

Figure 4.4. Elution profiles during purification of the *A. pullulans* α-glucuronidase. (A) Phenyl-sepharose at pH5. (B) Gel filtration on Sephadex G200. (C) CM-sepharose, pH 6. (D) Gel filtration on Sephadex G150. (E) CM-sepharose, pH 4. Black lines, absorbance at 280 nm; Red lines, % Buffer B; blue lines, α-glucuronidase activity; brown lines, *endo*-β-1,4-xylanase activity; yellow lines, β-xylosidase activity; green lines, α-arabinofuranosidase activity; pink lines, acetyl esterase activity.

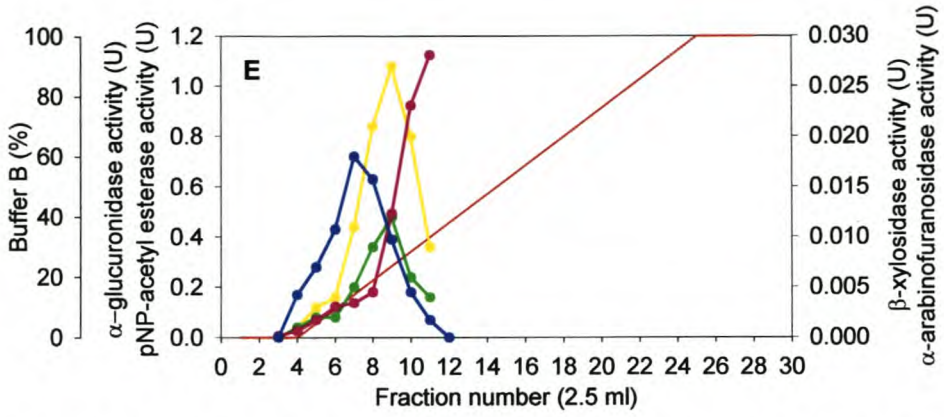

Figure 4.4 (continued). Elution profiles during purification of the *A. pullulans* α-glucuronidase. (A) Phenyl-sepharose at pH5. (B) Gel filtration on Sephadex G200. (C) CM-sepharose, pH 6. (D) Gel filtration on Sephadex G150. (E) CM-sepharose, pH 4. Black lines, absorbance at 280 nm; Red lines, % Buffer B; blue lines, α-glucuronidase activity; brown lines, *endo*-β-1,4-xylanase activity; yellow lines, β-xylosidase activity; green lines, α-arabinofuranosidase activity; pink lines, acetyl esterase activity.

The final purification step involved a second round of cation exchange on CM-sepharose, this time at pH 4. Protein was bound in 20 mM lactate buffer at pH 4 an eluted with a gradient of up to 1 M NaCl. The elution of the various enzyme activities is shown in Figure 4.4E. α-Glucuronidase activity bound poorly to the matrix and eluted first. It was followed by a peak containing β-xylosidase and α-arabinofuranosidase activity and subsequent peak containing pNP-acetyl esterase activity. There was significant overlap between the peaks containing the different activities. No *endo*-β-1,4-xylanase activity was detected in any of the fractions. Fractions 3 to 7, about half of the α-glucuronidase activity, but very little acetyl esterase activity were pooled, dialysed and concentrated. This step decreased α-arabinofuranosidase and β-xylosidase activity to barely detectable levels and decreased acetyl esterase activity by tenfold, whilst retaining 25% of the α-glucuronidase activity from the previous step. Only three bands were still visible on SDS-PAGE, two diffuse bands at ~170 kDa and ~110 kDa and a slight band at 35 kDa (Figure 4.3, lane 7). This preparation was subsequently used to characterise the α-glucuronidase from *A. pullulans*. The α-glucuronidase was purified 40-fold from the supernatant with a 1.7% yield.

Characterisation of the purified α-glucuronidase

Enzyme activity was maximal at pH 3 and activity was less than 50% of the maximum at pH 7 (Figure 4.5A). The temperature dependence of α-glucuronidase activity was investigated by measuring enzyme activity at temperatures ranging between 20 and 70°C. Maximal activity was observed at 40°C while no activity was detectable at 70°C (Figure 4.5B). The stability of the α-glucuronidase at different temperatures was investigated by incubating enzyme preparations for different lengths of time at temperatures from 20 to 60°C and then assaying for activity (Figure 4.5C). α-Glucuronidase

activity was stable up to 40°C for as long as 3 h and had a half-life of 60 min at 50°C. No activity remained after 30 min of incubation at 60°C.

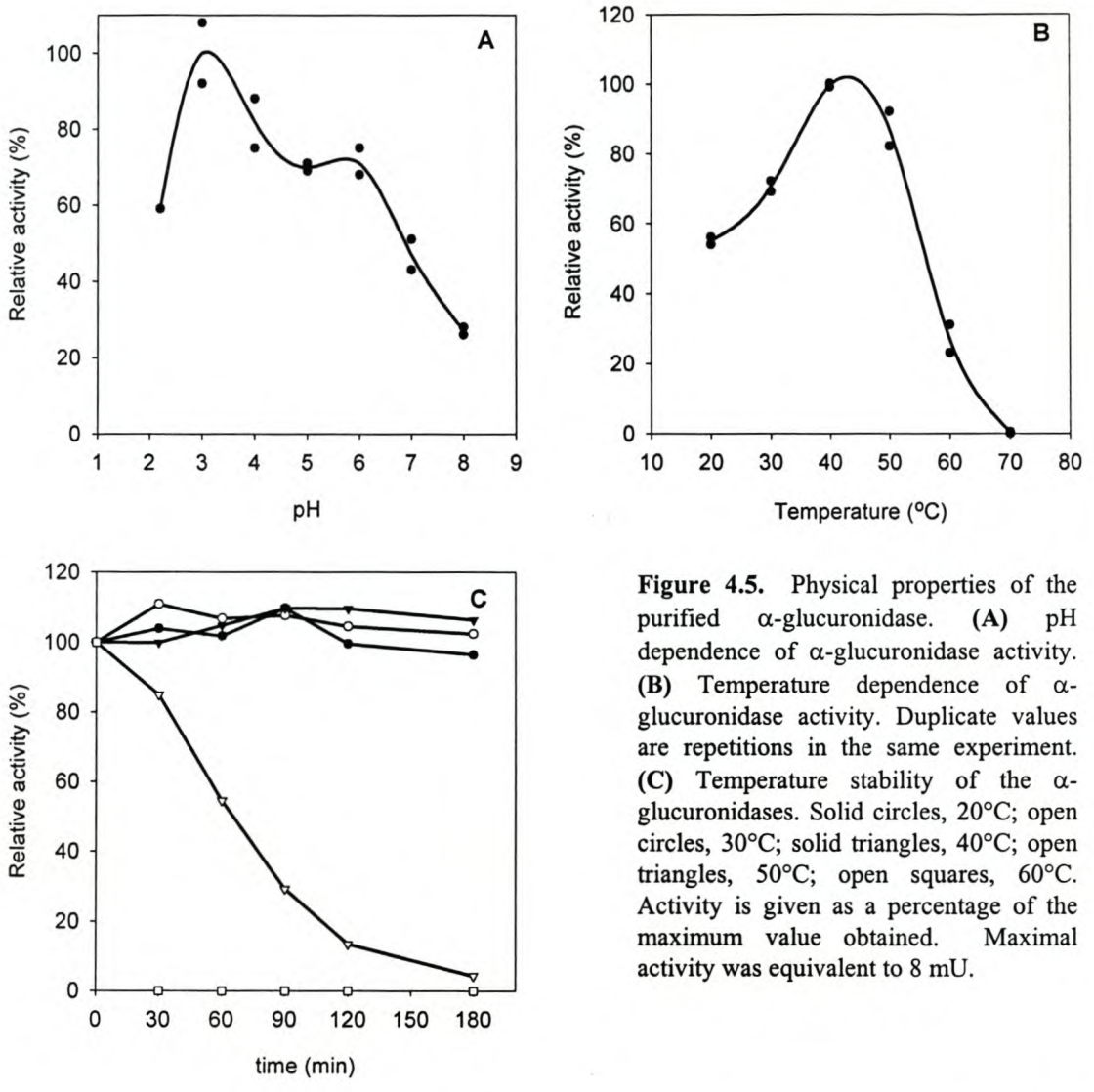

Figure 4.5. Physical properties of the purified α-glucuronidase. **(A)** pH dependence of α-glucuronidase activity. **(B)** Temperature dependence of α-glucuronidase activity. Duplicate values are repetitions in the same experiment. **(C)** Temperature stability of the α-glucuronidases. Solid circles, 20°C; open circles, 30°C; solid triangles, 40°C; open triangles, 50°C; open squares, 60°C. Activity is given as a percentage of the maximum value obtained. Maximal activity was equivalent to 8 mU.

Aldotriouronic acid and aldobiouronic acid were used to study the kinetic properties of the purified enzyme. Substrate titrations were performed with 0.1 to 10 mM aldotriouronic acid and with 1 to 100 mM aldobiouronic acid. Data was fitted with the equation describing substrate inhibition, $v = \frac{V_{max}[S]}{K_m + [S] + [S]^2/K_i}$ using the non-linear regression function of SigmaPlot (Jandel Scientific) (Figure 4.6). The enzyme showed a clear preference for the longer oligosaccharide substrate, with a K_m of 3.3 ± 0.9 mM for aldotriouronic acid compared to a K_m of 23.6 ± 10.1 for aldobiouronic acid. The inhibition constants for aldotriouronic acid and aldobiouronic acid were 9.8 ± 3.8 mM and 36.9 ± 17.3 respectively. Maximal reaction rates (V_{max}) were not calculated as the enzyme was only partially purified and protein measurements therefore did not reflect the true contribution of α-glucuronidase to the amount of protein in the preparation. Repeat experiments gave comparable results.

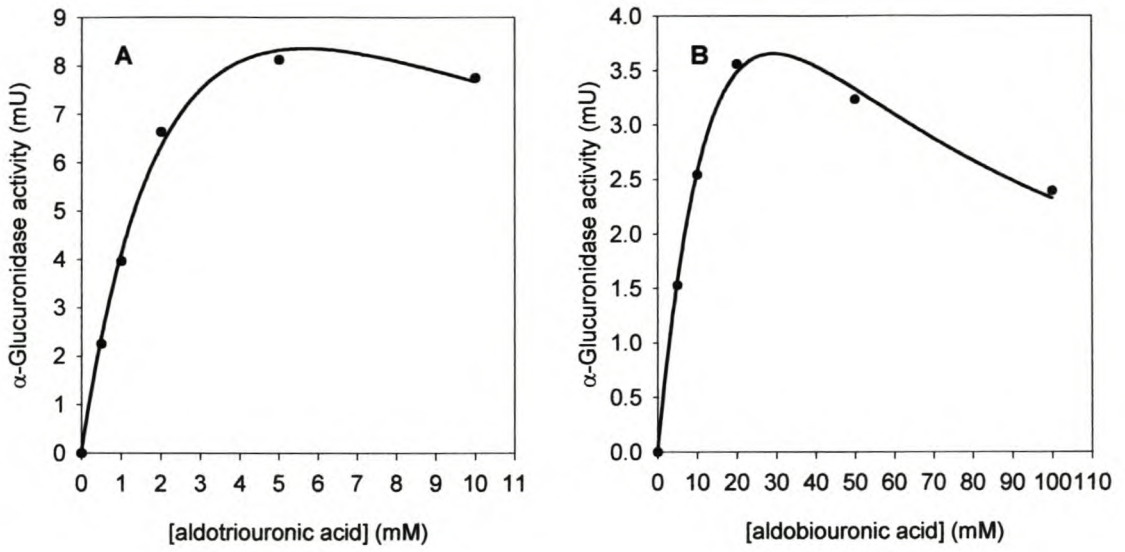

Figure 4.6. α-Glucuronidase activity as a function of substrate concentration. Assays were performed in formate buffer at 40°C and pH 3 for 5 min. **(A)** Titration with aldotriouronic acid. K_m was calculated as 3.3 ± 0.9 mM and K_i as 9.8 ± 3.8 mM. **(B)** Titration with aldobiouronic acid. The K_m was calculated as 29.5 ± 7.6 mM and K_i as 29.0 ± 7.8 mM.

The degree of asparagine-linked glycosylation of the α-glucuronidase was investigated by treatment of the preparation with *N*-glycosidase F. The preparation was deglycosylated under denaturing conditions, separated on SDS-PAGE and silver stained (Figure 4.7). Two diffuse bands, with an apparent mobility of 170 kDa and 110 kDa respectively, are visible in the untreated preparation (Lane 2). After deglycosylation, in addition to the 170 kDa and 110 kDa bands, two sharp bands with apparent mobilities of 118 kDa and 50 kDa were observed (Lane 1). A band containing the *N*-glycosidase F itself is visible migrating immediately behind the tracking dye front.

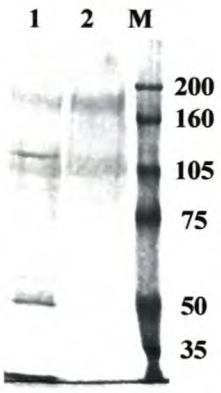

Figure 4.7. Deglycosylation of the partially purified α-glucuronidase preparation with *N*-glycosidase F. Samples were electrophoresed on a 7% SDS-PAGE gel and silver stained. Lane 1 is the preparation after treatment with *N*-glycosidase F under denaturing conditions while lane 2 is the untreated preparation. The lane marked M is a molecular weight marker (Rainbow marker, Amersham). Molecular weights are indicated in kDa.

Discussion

The objective of this part of the study was to purify and characterise the α-glucuronidase from a colour-variant strain of the saprophytic yeast-like fungus *A. pullulans*. Strain NRRL Y2311-1 of *A. pullulans* was originally isolated as a *endo*-β-1,4-xylanase overproducer (Leathers, 1986), and a

family 11 *endo*-β-1,4-xylanase has been purified from this strain (Li *et al.*, 1993), the gene cloned (Li and Ljungdahl, 1994) and expressed in *Saccharomyces cerevisiae* (Li and Ljungdahl, 1996). The xylanolytic accessory enzymes β-xylosidase, α-arabinofuranosidase and acetyl esterase have been detected in the culture supernatant of *A. pullulans* grown on either xylose or xylan (Myburgh *et al.*, 1991a), and characterized in terms of their pH and temperature optima and stability in the crude mixture (Myburgh *et al.*, 1991b). The work described in this chapter represents the first report on the isolation of an α-glucuronidase produced by *A. pullulans* strain NRRL Y2311-1.

It is well established that microbial xylanolytic enzyme systems are induced by the products of xylan degradation. Little xylanolytic activity is produced in the presence of glucose and/or the absence of xylan components. Xylanolytic activity is only induced in microorganisms once glucose is depleted or the small amount of constitutive xylanolytic activity produced by the microorganisms encounters xylan in the environment and starts degrading it. *A. pullulans* is no exception in this regard, as very low levels of the xylanolytic enzymes and no α-glucuronidase activity were detected when cultured in defined medium containing glucose (Figure 4.1). The presence of xylose in the medium markedly induced the production and secretion of all of the xylanolytic enzymes, except α-glucuronidase. Significant repression of the induction of xylanolytic activity was observed when both xylose and glucose were present in the medium. Glucuronic acid on its own did not act as an inducer of any xylanolytic enzymes, but a combination of glucuronic acid and xylose potently induced the production of all the xylanolytic carbohydrate hydrolases, including α-glucuronidase. Acetyl esterase activity was not significantly induced in response to cultivation in medium containing xylose and glucuronic acid. As expected, all of the xylanolytic enzymes were induced by growth in medium containing xylan from either birchwood or oat spelts. Very poor induction of the xylanolytic enzymes was noted on sugarcane bagasse and wheat straw, although this is possibly a reflection of the poor growth of *A. pullulans* on these carbon sources.

The results described above indicate that *A. pullulans* follows a similar pattern of induction and repression of its xylanolytic enzyme system as most previously studied fungi. Northern analysis of the *A. pullulans xynA* gene, encoding the *endo*-β-1,4-xylanase showed the same pattern, induction on xylose and xylan and repression thereof on glucose (Li and Ljungdahl, 1994). The upstream regions of this gene have not been analyzed for the presence of promoter or repressor binding sites. Induction and repression could well be mediated through homologues of the general carbon catabolite repressor protein CreA and the transcriptional activator XlnR as is the case in the Aspergilli (de Vries *et al.*, 2002; Marui *et al.*, 2002; van Peij *et al.*, 1998), and Cre1 as is the case for *Trichoderma reesei* (Margolles-Clark *et al.*, 1997). The induction pattern of α-glucuronidase activity in *A. pullulans* by different carbon sources differs from the other xylanolytic enzymes in that it is not induced by the

presence of xylose alone. It is only induced by growth on substrates that contain both xylose and glucuronic acid. The promoter region of this gene might contain a hitherto undescribed glucuronic acid (or 4-*O*-methyl glucuronic acid) responsive element. Evidence for the existence of an uronic acid responsive transcription factor was found by analysis of the expression of *aguA* from *A. niger* (de Vries *et al.*, 2002). The control of α-glucuronidase expression in *A. pullulans* is different from *A. tubingensis* (de Vries *et al.*, 1998) or *T. reesei* (Margolles-Clark *et al.*, 1997). In the former the α-glucuronidase gene is induced by growth on xylose, xylobiose and xylan and in the latter by growth on xylobiose, but not xylose.

It must be noted that the absolute amount of activity produced under the different culture conditions was used as basis for comparison of the levels of enzyme produced by *A. pullulans* cultivated on different carbon sources. This approach has some drawbacks, but is the result of difficulty with both protein and cell density measurements in the experimental set-up. The binding of Coomassie brilliant blue to the secreted proteins gave a gross underestimation of the amount of protein present (compared to absorbance at 280 nm) and gave highly variable results. This might be due to the fact that most of the proteins secreted by *A. pullulans* are highly glycosylated. Direct measurement of aromatic side-chains at 280 nm was also not suitable, as many of the growth substrates used in the experiment contained significant amounts of phenolic material. The particulate nature of many of the growth substrates interfered with the determination of cell density and the variable morphology of *A. pullulans* made the estimation of the cell numbers by direct counting difficult. Growth did appear to be vigorous on all of the monosaccharides as well as the two xylans tested, as the medium became notably turbid and the size of the cell pellets were comparable after centrifugation. Growth *per se* is quite possibly not the factor responsible for the differences in the production of the accessory enzymes, as very similar levels of *endo*-β-1,4-xylanase activity were produced by *A. pullulans* after cultivation on xylose, a combination of xylose and glucuronic acid, and the two different xylans. Growth was poor on both sugarcane bagasse and wheat straw and so was *endo*-β-1,4-xylanase production. The implication of this approach is that the smaller differences in the levels of each enzyme produced on the different carbon sources must be treated with caution. An alternative approach that has already been used with great success is to investigate transcript levels of the genes encoding xylanolytic enzymes. Results obtained with this approach can be normalised to total RNA extracted giving a much better indication of the degree of induction by different carbon sources.

The α-glucuronidase was partially purified from the culture supernatant of *A. pullulans* grown on birch xylan with a yield of 1.7% and 40-fold purification factor (Figure 4.3 and Table 4.1). The purification factor decreased in the last two purification steps, and the reason for this is unclear as both gel electrophoresis and enzyme assays show the removal of contaminating species. It is possible that

the decrease in the purification factor is the result of an assay artefact. The presence of *endo*-β-1,4-xylanase and β-xylosidase activity may have contributed to the release of additional reducing sugars from the aldouronic acids during the assay, resulting in an overestimate of amount of α-glucuronidase activity. The success of the purification procedure was that *endo*-β-1,4-xylanase activity, the principal component of the ensemble of secreted enzymes, was not detectable in the final preparation. The accessory enzyme activities were reduced to barely detectable levels and acetyl esterase activity was reduced a thousand-fold.

Two major protein components remained in the final preparation as judged by SDS-PAGE, both migrating as diffuse bands with apparent electrophoretic mobilities of 170 kDa and 110 kDa, respectively. The 170 kDa band is thought to corresponded with α-glucuronidase activity and the 110 kDa band with acetyl esterase activity, as the relative intensity of each band on SDS-PAGE consistently corresponded to the relative amounts of the respective activities present in the chromatographic fractions (not shown). In all of the chromatographic procedures, a substantial amount of acetyl esterase activity co-eluted with the α-glucuronidase activity (Figure 4.4) and repeating the same step did not result in removal any additional acetyl esterase activity. The diffuse appearance of the bands on SDS-PAGE is suggestive of extensively glycosylated proteins, and this was confirmed by treatment of the preparation with *N*-glycosidase F (Figure 4.7). The band corresponding to α-glucuronidase activity changed in electrophoretic mobility from 170 kDa to 118 kDa and the acetyl esterase band decreased from 110 kDa to 50 kDa after deglycosylation. The high degree of glycosylation of both α-glucuronidase and acetyl esterase activity clarifies why they are particularly difficult to separate chromatographically.

Enzymes secreted by *A. pullulans* are known to be extensively glycosylated, and this has caused great difficulty in purification of the mannanolytic enzymes from this organism (P. Biely, personal communication). In a mixture of highly glycosylated proteins, members of the same protein species behave less uniformly, as glycosylation is not entirely homogeneous, and different protein species in the mixture behave more uniformly due to the similar surface properties imparted by the extensive sugar side-chains (Scopes, 1994). This results in the elution of individual species as very broad peaks and poor separation between the components of the mixture, as is evident from the elution profiles in Figure 4.4. Ultimately, in spite of investigating many purification techniques under various conditions, the α-glucuronidase could not be purified to homogeneity using standard separation techniques.

The partially purified α-glucuronidase preparation contained no *endo*-β-1,4-xylanase activity, and only trace β-xylosidase activity and was used to characterise the enzyme in terms of its pH and

temperature optimum and kinetic properties. The *A. pullulans* α-glucuronidase has a pH optimum of 3, a temperature optimum of 40°C and is stable for up to 3 h at 40°C (Figure 4.5). The native enzyme has an apparent mobility on SDS-PAGE of 170 kDa and after deglycosylation it migrates at 118 kDa. In terms of physical properties, the *A. pullulans* α-glucuronidase is similar to previously characterized fungal α-glucuronidases (Table 2.1). These enzymes have molecular weights between 90 kDa and 130 kDa, judged by electrophoretic mobility, show maximum activity between 60°C and 70°C, and between pH 3.5 and pH 6.

The Michaelis-constants of the enzyme for aldobiouronic acid and aldotriouronic acid were determined (Figure 4.6). The enzyme had K_m of 3.3 ± 0.9 mM for aldotriouronic acid and 29.5 ± 7.6 mM for aldobiouronic acid. The Michaelis-constant for aldotriouronic acid is in the order of tenfold lower than that reported for other fungi. Substrate inhibition was observed with both aldouronic acids, a feature unique to the *A. pullulans* α-glucuronidase. The inhibition constants for aldotriouronic acid and aldobiouronic acid were 9.8 ± 3.8 mM and 29.0 ±7.8 mM respectively. The properties and structure of the *Cellvibrio japonicus* α-glucuronidase active site might offer an explanation for this phenomenon (Nurizzo *et al.*, 2002). The active centre is located in a deep cavity, with the glucuronate binding-site situated at the innermost part of the depression (Figure 3.4). The structure of active centre demands the sequential release of product, with the glucuronate following the xylo-oligosaccharide. Additionally, the aromatic side-chain of Trp543 that is involved in π-stacking interactions with the non-reducing end xylose unit retains an ordered structure after the xylo-oligosaccharide has diffused away and only glucuronate is bound. It is possible that this position is sufficiently "open" in the *A. pullulans* enzyme so that another substrate molecule could bind at the xylose binding site blocking the passage of the glucuronate, thereby effectively inhibiting the catalytic cycle. This explanation is speculative and will have to be tested experimentally to establish its validity.

This report presents the first isolation of an α-glucuronidase produced by *A. pullulans*, an enzyme that is likely to be a member of family 67 of the glycoside hydrolases based on its physical and kinetic properties. The difficulties in purifying the enzyme to homogeneity motivated an effort to clone and heterologously express the *A. pullulans* α-glucuronidase gene and further study its properties.

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Chapter 5

Cloning of the *Aureobasidium pullulans* α-glucuronidase gene, *aguA*, and expression in *Saccharomyces cerevisiae*

Abstract

The α-glucuronidase gene, *aguA*, of *Aureobasidium pullulans* NRRL Y-2311-1 was cloned by PCR methods and expressed in *Saccharomyces cerevisiae*. Based on amino acid sequence, AguA is a member of family 67 of the glycoside hydrolases. The amino acid sequence shares greater than 60% identity with fungal α-glucuronidases and shares between 34% and 42% identity with bacterial α-glucuronidases. The gene encodes a polypeptide of 836 amino acids that contains a putative signal peptide of 15 amino acids, resulting in a mature protein with a calculated molecular weight of 91.0 kDa and iso-electric point of 4.62. The gene was expressed in *S. cerevisiae* Y294 under control of the *ADH2* promoter and terminator. α-Glucuronidase activity was detected in the culture supernatant after 4 days of incubation, and accumulated in the medium to a level of 5 mU/ml (0.14 mg protein/L) after 9 days of incubation in defined medium, and 580 mU/ml after 8 days of cultivation in rich medium (16.6 mg protein/L). The heterologous α-glucuronidase was purified to homogeneity by Ni-chelate affinity chromatography. The heterologous protein had an electrophoretic mobility of 120 kDa on SDS-PAGE. It displayed maximal activity at 65°C, and between pH 5 and pH 6. The enzyme was stable at 45°C, lost half of its activity after 22.5 minutes at 55°C, and was rapidly inactivated at 65°C with a half-life of 5.6 min. It was stable at pH 4 and pH 6, and had a half-life of 17 min at pH 8. The enzyme had K_m-values in the millimolar range for the series of oligosaccharide substrates from aldobiouronic acid to aldopentaouronic acid. It had the highest catalytic efficiency on aldobiouronic acid and the catalytic efficiency decreased with increasing chain-length of the oligosaccharide substrate.

Introduction

Xylan is one of the main non-cellulose polysaccharides found in plant cell walls and contributes to between 10% and 35% of the dry weight depending on the plant species and the tissue type. Xylan consists of a backbone of 1,4-linked β-xylose units substituted with 1,2-linked α-4-*O*-methyl glucuronic acid, 1,3-linked α-arabinofuranose and acetic acid esters at position 2 and 3. Arabinofuranose side-chains may be further esterified at *O*5 with cinnamic acids (Timmel, 1967). The enzymatic degradation of xylan is central to the carbon cycle in nature and of industrial relevance.

The process involves the main-chain cleaving *endo*-β-1,4-xylanases, β-xylosidase, and the side-chain cleaving α-glucuronidase, α-arabinofuranosidase, acetylxylan esterases and cinnamoyl esterases (Beg *et al.*, 2001).

Several microbial α-glucuronidases have been purified and studied in terms of enzymatic properties (Puls, 1992). All of the sequenced α-glucuronidases have been classified into family 67 of the glycoside hydrolases (http://afmb.cnrs-mrs.fr/CAZY/). In terms of quaternary structure and primary sequence, microbial α-glucuronidases cluster into two groups within the family, defined by either bacterial or fungal origin. The bacterial enzymes have a dimeric quaternary structure with monomeric molecular weights of around 70 kDa, while the fungal enzymes are monomeric with molecular weights of around 90 kDa. Family 67 α-glucuronidases are highly conserved in terms of substrate specificity. They have an absolute requirement for the 4-*O*-methyl glucuronic acid substituent to be located on the terminal non-reducing xylopyranosyl unit of small xylo-oligosaccharides and are unable to hydrolyse *p*-nitrophenyl α-D-glucuronide (Puls, 1992). It appears that there is no set rule that applies to the length of the aldouronic acid substrate preferred by different microbial α-glucuronidases. Certain microbial α-glucuronidases have been reported to have trace activity against polymeric glucuronoxylan (Khandke *et al.*, 1989; Tenkanen and Siika-aho, 2000). The crystal structure of the *Cellvibrio japonicus* α-glucuronidase has recently been described and functional mutants of this enzyme studied in complex with substrate. Several structural elements that play a role in substrate recognition were discovered, all of which were found to be conserved in the alignments of the known α-glucuronidases, both of bacterial and fungal origin (Nagy *et al.*, 2003; Nurizzo *et al.*, 2002).

A. pullulans is an euascomycetous fungus (de Hoog, 2000) and colour variant strains of this organism have been isolated for the high-level production of xylanolytic enzymes (Leathers, 1986; Myburgh *et al.*, 1991b). The main *endo*-β-1,4-xylanase has also been purified (Li *et al.*, 1993), the *xynA* gene cloned (Li and Ljungdahl, 1994), and expressed in *S. cerevisiae* (Li and Ljungdahl, 1996). A highly homologous *endo*-β-1,4-xylanase gene has been cloned from the pigmented strain *A. pullulans* var *melanigenum*, and four consensus binding sites for the carbon catabolite repressor, CreA, were found in the upstream sequence of the gene (Ohta *et al.*, 2001). The presence of several xylanolytic accessory enzyme activities has been noted in the culture fluid of *A. pullulans* NRRL Y-2311-1 cultivated on lignocellulosic carbon sources (Myburgh *et al.*, 1991a). Here we present the first report of α-glucuronidase activity from *A. pullulans*, the cloning of the *aguA* gene, its expression in *S. cerevisiae* and the characterisation of the heterologous enzyme.

Materials and methods

Microbial strains and culture conditions

A. pullulans strain NRRL Y-2311-1 was obtained from the National Center for Agricultural Utilization Research (Peoria, Illinois, U.S.A.) and grown from frozen glycerol stock cultures on YPD media (containing per litre, 10 g yeast extract (Difco), 20 g peptone (Difco), 20 g glucose and for solid medium, 20 g agar) at 30°C and defined medium (containing per litre, 10 g birchwood xylan (Roth), 6.7 g yeast nitrogen base (Difco), 2 g L-asparagine and 5 g monobasic potassium phosphate). *Escherichia coli* XL1-Blue ((*recA1 endA1 gyrA96 thi-1hsdR17 supE44 relA1 lac*[F' *proAB lacI*^{q}*ZΔM15 Tn10* (Tet^{r})]^{c}) was obtained from Stratagene and routinely cultivated in LB medium (containing per litre, 5 g yeast extract, 10 g tryptone, 10 g NaCl and for solid medium, 20 g agar) and TB medium (containing per litre; 12 g tryptone, 24 g yeast extract, 4 ml glycerol and 100 mM phosphate buffer pH 7). *Saccharomyces cerevisiae* Y294 (α *leu2-3,112 ura3-52 his3 trp1-289*) (la Grange *et al.*, 1996) was routinely cultured in YPD medium or SC medium without uracil (containing per litre, 1.5 g amino acid mixture lacking uracil [2.5 g adenine, 2.4 g L-tryptophan, 1.2 g L-histidine, 1.2 g arginine-HCl, 1.2 g L-methionine, 1.8 g L-tyrosine, 3.6 g L-leucine, 1.8 g L-lysine-HCl, 3.0 g l-phenylalanine, 12 g L-threonine, 2.5 g proline, 2.5 g asparagine, 2.5 g alanine, 2.5 g isoleucine, 6 g aspartic acid, 6 g glutamic acid, 22.5 g serine and 9 g valine], 6.7 g yeast nitrogen base lacking amino acids and 20 g glucose, and for solid medium 20 g agar, medium adjusted to pH 6).

Primers

The following primers were used in the polymerase chain reaction (PCR) and sequencing (restriction endonuclease recognition sites doubly underlined, start and stop codons in bold and codons for hexahistidine tag underlined):

AGUdegF2:	AGYCACCTBGCYATGTCCA
AGUdegR2:	CCCAGCCRTTGTTRTCCTG
AGUinvL1:	ATACCGCGCTGTAGCTCTTGACCTG
AGUinvR1:	GCGGCAATCTCGGTATCCAAACTTTGAC
AGU-F3:	ATGCCAACGCATCTATCCTCTC
AGU-F4:	TGGCTCTGTATCAACTAGACTG
AGU-F5:	CGAACCTGGGCAAAGAC
AGU-F6:	GCTCGACGCATATGTCGCTGCTTAC
AGU-F7:	GTTGTGCAGATCAAATACGGACCG
AGU-R3:	ATAGTATCTCTGATTCCTGCTG
APaguA-L1:	**ATG**GATATATCCGCGAACCTG
APaguA-L2:	**ATG**CAACTTCTCACACTCTTTGC
APaguA-R1-XhoI:	AA<u>CTCGAG</u>**TTA**ATCAATCATTCCCTTCG
APaguA-R1-His6-XhoI:	AA<u>CTCGAG</u>**TTA**<u>ATGGTGATGGTGATGGTG</u>ATCAATCATTCCCTTCG
T7 forward:	TAATACGACTCACTATAGGG
SP6 reverse:	TATTTAGGTGACACTATAG

DNA isolation

Genomic DNA was extracted from *A. pullulans* according to a method described for baker's yeast (Hoffman and Winston, 1987) after cultivation in rich medium (1% yeast extract, 2% peptone, 2% glucose) for 48 hours. A 10 ml culture of *A. pullulans* was harvested by centrifugation and washed once with de-ionised water. The cell pellet was resuspended in 0.2 ml extraction buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris pH 8 and 1 mM EDTA) and vortexed briefly. Cells were lysed by adding 0.2 ml phenol: chloroform: isoamyl alcohol (25:24:1, v:v:v) and 0.3 g acid-washed glass beads, and were vortexed for 3 minutes. DNA was extracted with TE buffer (10 mM Tris pH 8, 1 mM EDTA), and the aqueous phase extracted with phenol: chloroform: isoamyl alcohol (25:24:1, v:v:v) until no more material was visible at the interface. DNA was precipitated by adding 1 ml ethanol to the aqueous phase and was sedimented by centrifugation at 13 000 rpm in a bench-top centrifuge. The DNA-containing pellet was resuspended in 0.4 ml TE buffer and 30 µg RNase A added to digest ribonucleic acid at 37°C for 5 minutes. DNA was precipitated by adding 10 µl 4 M ammonium acetate and 1 ml ethanol and was sedimented by centrifugation at 13 000 rpm. DNA was washed twice with 70% ethanol, dried and resuspended in 50 µl de-ionised water.

RNA isolation

RNA was extracted according to a previously published procedure with minor modifications (Laing and Pretorius, 1992), and standard precautions were taken while performing RNA manipulations (Ausubel *et al.*, 1995). RNA was extracted from a 5 ml *A. pullulans* culture grown for 4 days in defined medium containing 1% birchwood xylan. Cells were harvested by centrifugation and resuspended in diethyl pyrocarbonate (DEPC)-treated water, divided into 8 microfuge tubes and sedimented by centrifugation. Cells were resuspended in 300 µl STE buffer (250 mM Tris pH 7.2, 100 mM NaCl and 10 mM EDTA), and 300 µl phenol and 0.3 g acid washed glass beads were added. Preparations were vortexed for 45 seconds, 4 µl 10% SDS added, the preparations mixed and incubated on ice for 15 minutes. The mixture was extracted with 300 µl chloroform: isoamyl alcohol (24:1, v:v), and twice subsequently with phenol: chloroform: isoamyl alcohol (25:24:1, v:v:v). Nucleic acids were precipitated by adding 0.1 volume 5 M NaCl and 2 volumes 100% ethanol and incubating at –20°C for 2 hours. Precipitates were sedimented by centrifugation in a microfuge, washed twice with 70% ethanol and dried under vacuum. Precipitated material was resuspended in 50 µl DEPC-treated water and DNA was digested by adding 5U DNase, $MgCl_2$ to 100 mM and dithiothreitol to 10 mM and by incubating at 37°C for 30 minutes. RNA was extracted with phenol: chloroform: isoamyl alcohol, precipitated and washed as described above. Pellets were dissolved in 50 µl DEPC-treated water, the RNA quantified spectrophotometrically and evaluated by agarose gel electrophoresis. mRNA was reverse transcribed to cDNA using a First strand cDNA Synthesis kit

(Roche) with an oligo-p(dT)$_{15}$ primer and 1 µg total RNA by following the manufacturer's instructions.

Alignment of fungal α-glucuronidase genes

Coding nucleotide sequences of α-glucuronidases from *Aspergillus niger* (AJ290451), *Aspergillus tubingensis* (Y15405), *Talaromyces emersonii* (AF439788) and *Trichoderma reesei* (Z68706), were aligned using CLUSTAL-W based at the Network Protein Sequence Analysis server at the Institut Biologie et Chimie des Protéinis of the Centre National de la Recherche Scientifique in Toulouse, France (http://npsa-pbil.ibcp.fr/). Areas of highly conserved nucleotide sequence were used to design degenerate primers (AGUdegF2 and AGUdegR2).

PCR amplification of an internal section of the *aguA* gene using degenerate primers

Degenerate primers (AGUdegF2 and AGUdegR2) and 50 ng of genomic DNA were used for the amplification of an internal section of the *A. pullulans aguA* gene. Reaction mixtures for PCR consisted of template DNA, 1× reaction buffer, 1 U *Taq* DNA polymerase (Promega), 0.2 mM of each deoxynucleotide triphosphate (Sigma), 0.5 µM of each primer and between 1 and 4 mM $MgCl_2$ in a 50 µl reaction volume. A reagent blank was included for each reaction. PCR amplification was performed in a Hybaid PCR Sprint thermocycler using 40 cycles of amplification, with 45 seconds denaturing at 94°C, 45 seconds annealing at 40°C and 1 minute extension at 72°C. A 10 minute incubation at 72°C followed the last step and samples were maintained at 4°C after completion of the amplification cycle.

Molecular manipulation of PCR products

DNA manipulations were performed according to standard protocols (Ausubel *et al.*, 1995). PCR products were resolved by agarose gel electrophoresis and excised bands purified using a Wizard SV Gel and PCR Clean-Up System (Promega) or the freeze/squeeze method (Benson, 1984). Purified PCR products were ligated into the pGEM-T Easy vector (Promega) by following the manufacturer's instructions. *Escherichia coli* XL1-Blue was made competent by the $CaCl_2$-method (Ausubel *et al.*, 1995), transformed with the entire ligation reaction and transformed cells were plated on LB plates containing 100 mg/L ampicillin, 40 mg/L X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and 100 µmol/L IPTG (isopropyl-β-D-thiogalactopyranoside) and incubated at 37°C overnight. Small-scale plasmid preparations were performed after overnight cultivation of putative positive colonies in liquid TB medium containing 100 mg/L ampicillin (Birnboim and Doly, 1979). DNA sequencing was performed by the Central Analytical Facility of Stellenbosch University (http://www.sun.ac.za/saf/dna.html) on an ABI Prism 3100 Genetic Analyzer using a Big Dye

Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems), 0.5 µg plasmid DNA and 5 pmol primer.

Creation of circular DNA molecules for inverse PCR

Circular DNA molecules, which could serve as template for inverse PCR, were generated by partial digestion of *A. pullulans* genomic DNA followed by ligation at low DNA concentration (Ochman *et al.*, 1988; Triglia *et al.*, 1988). Partial digests of *A. pullulans* genomic DNA were made by incubating 10 µg DNA with 0.05 U/mg to 1 U/mg *Sau*3AI. Digests were performed in a total volume of 20 µl, and 2 µl aliquots were taken every 30 seconds after starting the reaction and inactivated in gel loading buffer at 65°C. The digested DNA was resolved on a 1% agarose gel and the areas corresponding to between 2000 and 5000 bp were excised and purified from the gel by the freeze/squeeze method (Benson, 1984). Intramolecular ligation was achieved by performing reactions at DNA concentration of 2.4 µg/ml. The reaction mixture contained 1.2 µg partially digested, size-selected DNA and 5 U of T4 DNA ligase (Roche) in its reaction buffer, and was incubated overnight at room temperature. DNA was precipitated and dissolved in 20 µl de-ionised water and 1 µl (~60 ng) was used in the subsequent PCR reactions.

PCR amplification of flanking regions of the *aguA* gene using inverse primers

Inverse primers (AGUinvL1 and AGUinvR1) and ~60 ng of circular DNA were used for the amplification of regions flanking the known internal section of the *A. pullulans aguA* gene. The composition of the reaction mixture was as described above. PCR amplification was performed using 35 cycles of amplification, with 45 seconds denaturing at 94°C, 45 seconds annealing at 55°C and 3 minutes extension at 72°C. A 10 minute incubation at 72°C followed the last step and samples were maintained at 4°C after completion of the amplification cycle. PCR products were resolved by agarose gel electrophoresis, cloned and sequenced as described above.

PCR amplification of the 3'-end of the *aguA* gene from cDNA

The 3'-end of the *aguA* gene was amplified using cDNA prepared from *A. pullulans* cultivated on birchwood xylan, and AGU-F4 and oligo$(dT)_{15}$ as primers. The composition of the reaction mixtures was as described above and 0.25 µl of the reverse transcription reaction was used as a template. The reaction cycle consisted of an initial denaturing step at 94°C for 2 minutes, followed by 40 cycles of denaturing at 94°C for 15 seconds, annealing at 35°C for 30 seconds and extension at 72°C for 45 seconds. PCR products were resolved by agarose gel electrophoresis, cloned and sequenced as described above.

PCR amplification of the entire *aguA* open reading frame

A 2589 bp fragment spanning the assembled putative open reading frame of the *A. pullulans aguA* gene was amplified from genomic DNA using AGU-F5 and AGU-R6 as primers. The Expand High Fidelity PCR system (Roche) was used to amplify the *aguA* gene and the products of 3 separate PCR reactions were independently cloned and sequenced. The reaction mixtures contained in a final volume of 50 µl, 1× reaction buffer, 100 ng template DNA, 0.5 µM of each primer, 0.2 mM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$ and 2.6 U of DNA polymerase. Amplification was achieved in 40 cycles of denaturing at 94°C for 15 seconds, annealing at 45°C for 30 seconds and extension at 72°C for 3 minutes, following an initial 2 minute denaturing step at 94°C. Thirty seconds was added to the extension step after every 10 amplification cycles. PCR products were resolved by agarose gel electrophoresis, cloned and sequenced as described above.

Heterologous expression of the *A. pullulans aguA* gene in *S. cerevisiae* Y294

Two putative open reading frames were identified in the sequenced DNA fragment, one containing a translation start site 45 bp upstream from the other. The longer open reading frame was designated *aguA** and the shorter *aguA* and both were amplified from genomic DNA using APaguA-L1 and APaguA-L2 as forward primers respectively. Both putative open reading frames were amplified coding for a polypeptide with or without an additional *C*-terminal hexahistidine tag using APaguA-R1-His6-XhoI and APaguA-R1-XhoI as reverse primers respectively. The four PCR products were designated *APaguA**, *APaguA*+His*, *APaguA* and *APaguA+His*. PCR reactions were performed with the Expand High Fidelity PCR System (Roche) using a 100 µl reaction volume and 200 ng template DNA. Amplification was achieved in 40 cycles of denaturing at 94°C for 15 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 3 minutes, following an initial 2 minute denaturing step at 94°C. Thirty seconds was added to the extension step after every 10 amplification cycles. Reaction mixtures were incubated for a further 10 minutes at 72°C following the completion of the entire amplification cycle. PCR products were resolved by agarose gel electrophoresis, cloned and sequenced as described above.

Each of the four gene constructs was digested from the pGEM-T Easy vector with *Eco*R1 (restriction site in multiple cloning site of the vector) and *Xho*1 (restriction site engineered into the reverse primer), their corresponding bands purified from agarose gels, ligated into pDLG1 (la Grange *et al.*, 1996), and used to transform *E. coli* XL1-Blue. Purified plasmids were examined by restriction digestion and used to transform *S. cerevisiae* Y294 using the lithium acetate/dimethyl sulphoxide procedure (Hill *et al.*, 1991). Transformed yeasts were plated on SC medium lacking uracil and cultured at 30°C until single colonies were discernible from the background. Transformants were picked and subsequently maintained on solid SC medium lacking uracil. Liquid SC-medium lacking

uracil was inoculated to 0.010 absorbance units at 600 nm from starter cultures grown from single colonies of *S. cerevisiae* Y294[pDLG1-APaguA*], *S. cerevisiae* Y294[pDLG1-APaguA*+His], *S. cerevisiae* Y294[pDLG1-APaguA], *S. cerevisiae* Y294[pDLG1-APaguA+His] as well as the plasmid control strain *S. cerevisiae* Y294[pDLG1], and cultured at 26°C with shaking at 150 rpm. Cell growth was monitored by measuring absorbance of the culture at 600 nm at regular intervals. α-Glucuronidase activity was measured in the culture supernatant and in lysates of cells disrupted by bead-beating as well as in the periplasmic space by spheroplasting. Bead beating was performed by vortexing cells with 425 to 600 micron glass beads after cells were washed twice in 50 mM acetate buffer pH 5. Cells were vortexed 5 times for 1 min followed by a 1 min incubation on ice. Cellular debris was sedimented by centrifugation and the supernatant resuspended in a volume of 50 mM acetate buffer pH 5 equivalent to the original sample. Spheroplasting of yeast cells was performed by incubating washed cells resuspended in 50 mM acetate buffer pH 5 with 1 mg Zymolyase for 6 h. The formation of spheroplasts was detected by microscopic inspection.

Generation of autoselective *S. cerevisiae* strains by *FUR1* disruption

The autoselective strain *S. cerevisiae* Y294[*fur1::LEU2* pDLG-APaguA+His] was generated by transforming *S. cerevisiae* Y294[pDLG-APaguA+His] with a linear 3270 bp NcoI-NsiI *fur1::LEU2* fragment digested from the plasmid pDF1 (la Grange *et al.*, 1996), as described above. Transformants were plated on SC-medium lacking both leucine and uracil. Transformants were screened by PCR for the replacement of the chromosomal *FUR1* gene with the disrupted *fur1::LEU2* allele. *FUR1*-disrupted strains were subsequently cultivated in YPD medium.

α-Glucuronidase assay

α-Glucuronidase activity was assayed by measuring the release of 4-*O*-methyl glucuronic acid from an aldouronic acid mixture (aldotetraouronic, aldotriouronic and aldobiouronic acids; 40:40:20) (Megazyme, Ireland). The amount of glucuronic acid released was quantified using the Milner-Avigad calorimetric method for the determination of hexuronic acids (Milner and Avigad, 1967). The reaction mixture consisted of 3 mg/ml of the aldouronic acid mixture, 50 mM acetate buffer pH 4.8, and an appropriate quantity of enzyme in a final volume of 100 µl. The reaction was terminated by adding 300 µl copper reagent and boiling for 15 min. Samples were cooled on ice and 200 µl arsenomolybdate reagent added to complete the colour reaction. Samples were quantified by measuring absorbance at 620 nm. A standard containing between 5 and 50 nmole glucuronic acid (Sigma) in buffer in a final volume of 100 µl was included with each assay. One unit of activity is defined as the amount of enzyme capable of releasing 1 µmole of glucuronic acid per min.

Protein determination

Protein content was estimated using the Bradford Coomassie Brilliant Blue dye binding method (Bradford, 1976) with commercial dye reagent (Biorad) and bovine serum albumin as standard.

SDS-polyacrylamide gel electrophoresis

Discontinuous sodium dodecyl sulphate gel electrophoresis was carried out using the Tris-glycine buffer system (Laemmli, 1970), 10% polyacrylamide gels and Rainbow molecular weight markers (Amersham). Protein bands were visualised by silver staining (Blum *et al.*, 1987).

Purification of the heterologous α-glucuronidase

Supernatants of cultures of *S. cerevisiae* Y294[pDLG1-APaguA+His], producing the α-glucuronidase containing the *C*-terminal hexahistidine tag were harvested by centrifugation at 5000 rpm in a JA-14 rotor after 8 days of cultivation. Supernatant was concentrated from 1 L to 4 ml by ultrafiltration using a Minitan tangential flow ultrafiltration system (Millipore Corporation) and an Amicon ultrafiltration cell (Millipore Corporation), both with 10 kDa cut-off membranes. The concentrated supernatant was made up to 300 mM NaCl and 100 mM Tris pH 8, applied to 0.1 ml Ni-NTA Agarose (Qiagen) in a hypodermic syringe. The column was washed with 1 ml loading buffer (100 mM Tris pH 8, 300 mM NaCl) followed by 1 ml 100 mM MES pH 5.9, and the α-glucuronidase eluted with 4 ml 100 mM acetate buffer pH 4. Fractions of 0.5 ml were collected, assayed for α-glucuronidase activity and analysed on SDS-PAGE.

Characterisation of the purified heterologous α-glucuronidase

Determination of temperature dependence

The temperature dependence of enzyme activity was tested by assaying for activity between 30°C and 75°C. The reaction mixture contained 50 mM acetate buffer pH 4.8, 3 mg/ml aldouronic acid mixture and an appropriate amount of enzyme.

Determination of pH dependence

The effect of pH on enzyme activity was investigated by assaying between pH 2.2 and pH 6 in 50 mM citrate buffer, and between pH 6 and pH 8 in 50 mM Tris-maleate buffer. Reactions were performed with 3 mg/ml aldouronic acid mixture and an appropriate amount of enzyme. Assays were performed at 40°C, and standards of between 5 and 50 μmole glucuronic acid were included for each pH point. Citrate was titrated with an equimolar amount of cupric sulphate and the pH of the terminated reactions adjusted to 4.8 by the addition of 1 M acetate buffer pH 4.8 before boiling.

Determination of temperature stability

The temperature stability of the enzyme was investigated by incubating the enzyme at 45°C, 55°C and 65°C, taking samples at 15 minute intervals and assaying for activity. The reaction mixture contained 50 mM acetate buffer pH 4.8, 3 mg/ml aldouronic acid mixture and a suitable amount of enzyme and assays were performed at 65°C.

Determination of pH stability

The short-term pH stability of the enzyme was tested by incubating the enzyme at 45°C with McIlvaine's phosphate-citrate buffer at pH 2.2, pH 4, pH 6 and pH 8, taking samples at 15 min intervals an assaying for activity in 200 mM acetate buffer pH 4.8 with 3 mg/ml aldouronic acid mixture at 65°C for 5 min.

Investigation of kinetic properties and substrate specificity

Kinetic constants of the enzyme for aldobiouronic acid (Megazyme), aldotriouronic acid (Megazyme), aldotetraouronic acid (kindly provided by P. Biely) and aldopentaouronic acid (Megazyme) were determined by assaying for activity at pH 4.8 and 40°C with a range of substrate concentrations. The enzyme was tested for its ability to hydrolyse an internally substituted aldopentaouronic acid (kindly provided by P. Biely) by incubating the same amount of enzyme used for the substrate titrations with 5 mM substrate in 50 mM acetate buffer pH 4.8 at 40°C for 12 h.

Results

Cloning of the *A. pullulans aguA* gene

The *A. pullulans aguA* gene was cloned by PCR methods. Degenerate primers were based on areas of highly conserved nucleotide sequence in alignments of known fungal α-glucuronidase genes, and were used to amplify an internal section of the *A. pullulans aguA* gene (Figure 5.1). The regions flanking

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                                    AGUdegF2: 5'-AGYCACCTBGCYATGTCCA-3'
AF439788        GTCAATGTCGGCACCAACACCACCTGGCTCGGAAGCCACCTGGCCATGTCCAATCTGTAT--
Z68706          ATCAATGTCGGCATGGACGATACGTGGCTAGGCAGTCACCTCGCCATGTCCAACATGTTT--
AJ290451        GTTAATGTGGGCACCAACAGAACATGGCTGGGTAGCCACCTTGCTATGTCCAATCTGTAC--
Y15405          GTTAATGTGGGCACTAACAGAACATGGCTGGGTAGCCACCTTGCTATGTCCAATCTGTAC--
                 * ***** ****   **   ** ***** ** ** ***** ** ********  ***

                                AGUdegR2: 3'-GTCCTRTTGTTRCCGACCC-5'
AF439788      --ACCCACTTCGGGCCCAATCCGGCCTCCCAGGACAACAACGGCTGGGGGCAATGGACCCGT
Z68706        --ACTCACTACGGTGCCAATCCCGCCTCTCAGGACAACAACGGCTGGGGCCAGTGGACTCGT
AJ290451      --ACCCACTATGGTCCAAATCCAGCTACACAGGATAACAATGGCTGGGGTCAATGGACACGT
Y15405        --ACTCACTATGGCCCAAACCCAGCTACACAGGATAACAATGGCTGGGGTCAATGGACACGT
                ** ****  **  * ** ** **  * ***** ***** ******** ** ***** ***
```

Figure 5.1. Degenerate primers based on highly conserved regions in the nucleotide sequences of the α-glucuronidase genes of *A. niger* (AJ290451), *A. tubingensis* (Y15405), *T. emersonii* (AF439788) and *T. reesei* (Z68706). Conserved nucleotides indicated with a star. The double dashes indicate breakage in the continuity of the sequences.

this internal section were amplified using inverse PCR (Figure 5.2). A region of 420 bp at the 3'-end of the *A. pullulans aguA* gene was amplified from cDNA using a gene-specific forward primer and an oligo(dT) reverse primer. The entire gene was subsequently amplified from genomic DNA and sequenced to confirm the sequence and assembly of the overlapping fragments.

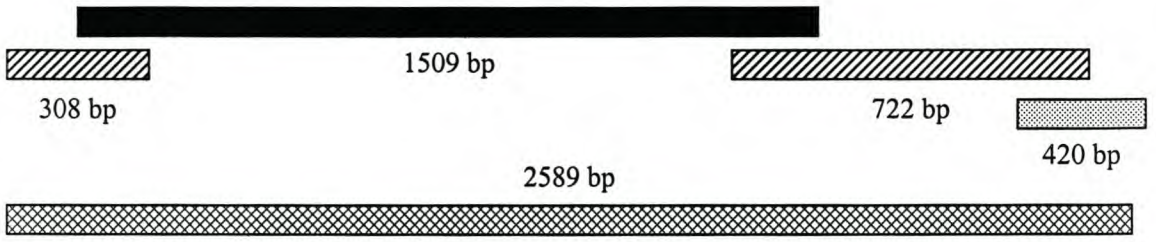


Figure 5.2. PCR amplification of the *A. pullulans aguA* gene. The black bar represents the area amplified from genomic DNA using degenerate primers, the hatched bars represent the areas amplified by inverse PCR and the dotted bar represents the area amplified from cDNA using a specific forward primer and an oligo(dT) reverse primer. The cross-hatched bar represents the entire open reading frame amplified from genomic DNA with specific primers. The size of each area is shown in base pairs.

The *A. pullulans aguA* gene consists of an intron-less open reading frame of 2508 nucleotides encoding a protein of 836 amino acids (Figure 5.3). The protein contains a putative secretion signal of 15 amino acids (von Heijne, 1986), resulting in the a mature protein with a predicted molecular weight of 91.0 kDa and a calculated iso-electric point of 4.62. An additional putative translation start site was found 45 bp upstream of the presumed translation start site. Initiation at this site would lead to the production of a mature protein with an additional 15 amino acids at the *N*-terminus. The signal peptide resulting from initiation at this putative translation start site contains many charged and polar amino acids, and therefore deviates from the consensus for signal peptides (von Heijne, 1986). A TATA-like element was found 55 bp upstream of the presumed translation start site with the sequence TATATAA. This deviates slightly from the consensus sequence TATAAA, however, promoter elements are less conserved in the filamentous fungi (Ballance, 1986). Twenty possible *N*-glycosylation sites were found in the deduced amino acid sequence of the *A. pullulans* α-glucuronidase (Gavel and von Heijne, 1990).

Database searches with the deduced amino acid sequence of the *aguA* gene matched significantly to family 67 glycoside hydrolases exclusively, indicating that the *aguA* gene encodes an α-glucuronidase. The *A.* pullulans α-glucuronidase amino acid sequence shared 66.5%, 66.1%, 65.3% and 62.9 identity with the α-glucuronidases from *T. emersonii*, *A. tubingensis*, *A. niger* and *T. reesei*, respectively. It shared between 34% and 42% identity with bacterial α-glucuronidases. A rooted tree constructed on the basis of amino acid sequences also showed that the *A. pullulans* α-glucuronidase was the most closely related to its homologue from *T. emersonii*, and was the most distantly related to

the bacterial α-glucuronidases (Figure 5.4). Sequence alignment showed that the catalytically important amino acids identified in the crystal structure of the *C. japonicus* α-glucuronidase were

```
-120                                                                    gatctgcatccccacatcatcgaaaagtgg
-90   tattccgcctgcgttatatcgccccgcatttgtggtatataatcgATGGATATATCCGCGAACCTGGGCAAAGACTTTTATCGCATCGAT
                                                    M  D  I  S  A  N  L  G  K  D  F  Y  R  I  D
1     ATGCAACTTCTCACACTCTTTGCGCTCTTCGGGCTTGCTCTTGCAGAGACTGGCCTTGACGGCTGGCTCCGATATGCCGCACTTCCATCT
1      M  Q  L  L  T  L  F  A  L  F  G  L  A  L  A  E  T  G  L  D  G  W  L  R  Y  A  A  L  P  S
91    TCGATATCATGGCCATCAGTTCCAAAGCACATTGTCGTCCTGAATACTACCGAAACAAGTCCTGTCTACACAGCAGGTCAAGAGCTACAG
31     S  I  S  W  P  S  V  P  K  H  I  V  V  L  N  T  T  E  T  S  P  V  Y  T  A  G  Q  E  L  Q
181   CGCGGTATCCAAAGCATTCTCGGTCAGAACTGCCGCTTTTCGACCAATAAGCCTGATGAATCTATCATCGTGGGCACGCTCGACGCATAT
61     R  G  I  Q  S  I  L  G  Q  N  C  R  F  S  T  N  K  P  D  E  S  I  I  V  G  T  L  D  A  Y
271   GTCGCTGCTTACGGCAATTTCAGCCAGTCTGTGAATCTCAAGGAAGACGGCTTCTGGCTGAGTACGGAAAACAATACCGTCCAGATTGTC
91     V  A  A  Y  G  N  F  S  Q  S  V  N  L  K  E  D  G  F  W  L  S  T  E  N  N  T  V  Q  I  V
361   GGTCAAAACGAACGAGGCGCCCTTTATGGAGCCTTTGAGTACTTGTCGATGCTGGCACAAGCGAACTTTTCCACTGTTGCATATGTATCT
121    G  Q  N  E  R  G  A  L  Y  G  A  F  E  Y  L  S  M  L  A  Q  A  N  F  S  T  V  A  Y  V  S
451   AATCCAGACGCCCCCATTCGATGGGTCAACCAGTGGGACAACCTCGACGGCAGTATCGAGCGAGGATACGGTGGCGCTTCGATATTCTTC
151    N  P  D  A  P  I  R  W  V  N  Q  W  D  N  L  D  G  S  I  E  R  G  Y  G  G  A  S  I  F  F
541   GCTAACGGCACCATCGTCAATGATCTCACCCGTGTGGCTGAGTATGCGCGTCTACTTGCCTCGGTGGGTATCAATGCCATTGTCATCAAC
181    A  N  G  T  I  V  N  D  L  T  R  V  A  E  Y  A  R  L  L  A  S  V  G  I  N  A  I  V  I  N
631   AATGTCAATGCCAACGCATCTATCCTCTCGCCACAGAACATCGACGGTGTTGGAAGAATCGCCGACACAATGCGGCCATATGGCGTGCAG
211    N  V  N  A  N  A  S  I  L  S  P  Q  N  I  D  G  V  G  R  I  A  D  T  M  R  P  Y  G  V  Q
721   ATTGGTCTCTCACTCTACTTTGCCTCTCCCACTTCTGGAGTTCAAGGGCAAGCCAATCTCACCACCTTTGACCCTCTCGACCCTGGAGTT
241    I  G  L  S  L  Y  F  A  S  P  T  S  G  V  Q  G  Q  A  N  L  T  T  F  D  P  L  D  P  G  V
811   GTGACCTGGTGGGGAAACGTTACCGATCAGATCTATGAGCGTATCCCGGACATGGCTGGCTACCTTGTCAAAGCCAACTCCGAGGGACAG
271    V  T  W  W  G  N  V  T  D  Q  I  Y  E  R  I  P  D  M  A  G  Y  L  V  K  A  N  S  E  G  Q
901   CCAGGACCGATCACGTATAATCGCACGCTTGCAGAAGGAGCTAATCTGTTCGCAAGAGCTCTGCAACCACATGGTGGCAGAGTACTTTTT
301    P  G  P  I  T  Y  N  R  T  L  A  E  G  A  N  L  F  A  R  A  L  Q  P  H  G  G  R  V  L  F
991   AGAGCCTTTGTCTATAACCAGTTGAATGAGAGTGACTGGAGAGCAGATCGGGCGAACGCCGCGGTCGACTTCTTCAAACCACTGGATGGC
331    R  A  F  V  Y  N  Q  L  N  E  S  D  W  R  A  D  R  A  N  A  A  V  D  F  F  K  P  L  D  G
1081  AAATTCGACGACAACGTTGTTGTGCAGATCAAATACGGACCGATTGATTTCCAAGTCAGAGAGCCTGCATCGCCGCTTTTCGCGAACTTG
361    K  F  D  D  N  V  V  V  Q  I  K  Y  G  P  I  D  F  Q  V  R  E  P  A  S  P  L  F  A  N  L
1171  TTCCATACCAACACGGCGATTGAACTGGAAGTTACTCAGGAATATCTGGGACAGCAGACTCATTTGGTATATCTTCCGCCCCTGTGGGAT
391    F  H  T  N  T  A  I  E  L  E  V  T  Q  E  Y  L  G  Q  Q  T  H  L  V  Y  L  P  P  L  W  D
1261  ACGGTCCTTGGCTTCGATATGCGCATCGACAACGAGACATCGCTGGTTCGAGACATCATAGCTGGTAGAACTTTCAACCGCTCACTTGGT
421    T  V  L  G  F  D  M  R  I  D  N  E  T  S  L  V  R  D  I  I  A  G  R  T  F  N  R  S  L  G
1351  GGCTACGCTGCAGTTGTCAATGTGGGAACAAACCAGACATGGCTCGGAAGTCATCTCTCCATGTCAAACTTCTACGCTTACGGTCGACTC
451    G  Y  A  A  V  V  N  V  G  T  N  Q  T  W  L  G  S  H  L  S  M  S  N  F  Y  A  Y  G  R  L
1441  GCATGGAACCCTACCGAAGACACTACCAAGATACACGAGGACTGGACACGCTTGACATTTGGTCTTGACCAGAATGTCGTTGATACCATT
481    A  W  N  P  T  E  D  T  T  K  I  H  E  D  W  T  R  L  T  F  G  L  D  Q  N  V  V  D  T  I
1531  ACTCATATGGCACTGGAGTCCTGGCCAGCATACGAAAACTACAGCGGCAATCTCGGTATCCAAACTTTGACTGACATCTTGTACACCCAT
511    T  H  M  A  L  E  S  W  P  A  Y  E  N  Y  S  G  N  L  G  I  Q  T  L  T  D  I  L  Y  T  H
1621  TTCGGGCCCAACCCGCAATCACAAGATAACAACGGCTGGGGTCAATGGACACGAGCCGATCACACGACGATCGGTATGGACAGGACTGTC
541    F  G  P  N  P  Q  S  Q  D  N  N  G  W  G  Q  W  T  R  A  D  H  T  T  I  G  M  D  R  T  V
1711  TCCAACGGCACAGGATATAGCGGAACCTATCCGCCCGAGGTTGCAGCGATGTATGAAAATATCGAGACTACACCAGACAATCTGCTGTTA
571    S  N  G  T  G  Y  S  G  T  Y  P  P  E  V  A  A  M  Y  E  N  I  E  T  T  P  D  N  L  L  L
1801  TGGTTCCACCACGTGAACTATACCCAAAAGCTCCACTCAGGCAAGACAGTCATTCAACACTTCTACGATGCTCACTATGCTGGCGCAGAC
601    W  F  H  H  V  N  Y  T  Q  K  L  H  S  G  K  T  V  I  Q  H  F  Y  D  A  H  Y  A  G  A  D
1891  ACCGCTCAGACATTCCCCGAGCAATGGCAGACGCTAGCAGGCAAGATTGACAGCCAACGTTTCGATGAGCAGCTGTATCGACTCAAGTAC
631    T  A  Q  T  F  P  E  Q  W  Q  T  L  A  G  K  I  D  S  Q  R  F  D  E  Q  L  Y  R  L  K  Y
1981  CAAGCTGGCCATTCACTTGTTTGGAGAGACGCCATCGTCAATTTCTACCACAACATCTCTGGCATTGCCGACGAGAACGGCAGAGTTGGC
661    Q  A  G  H  S  L  V  W  R  D  A  I  V  N  F  Y  H  N  I  S  G  I  A  D  E  N  G  R  V  G
2071  AACCATCCATACCGCATCGAAGCTGAGAGCATGACTTTGAATGGCTACGAACCATATGCTGTCAGTCCCTTCGAGACAGCATCAGATTAT
691    N  H  P  Y  R  I  E  A  E  S  M  T  L  N  G  Y  E  P  Y  A  V  S  P  F  E  T  A  S  D  Y
2161  TATGCAGTCGTCACTAGCGATAACAGTACGACTGGCTCTGTATCAACTAGACTGCATTTCCCAGCCGGTACTTACGATGTCGGTGTCAAT
721    Y  A  V  V  T  S  D  N  S  T  T  G  S  V  S  T  R  L  H  F  P  A  G  T  Y  D  V  G  V  N
2251  TTCTATGACATGTATGGAGGGATATCAAAGTATGAGATGTTGATCAATAACAAGACTGTTGCAACTTGGGCTGGAGATAGCGAGAACTAT
751    F  Y  D  M  Y  G  G  I  S  K  Y  E  M  L  I  N  N  K  T  V  A  T  W  A  G  D  S  E  N  Y
2341  CTCGGTCACACGCCTTCGATTTACTTGGATGGACATTCAGCAAGAAGGATCACGTTTACAGATGTCACAGTGAAGGAAGGCGATACACTG
781    L  G  H  T  P  S  I  Y  L  D  G  H  S  A  R  R  I  T  F  T  D  V  T  V  K  E  G  D  T  L
2431  GAAATTGTTGGGATTCCAAATGGCATTGAGCCTGCTCCGCTGGACTATGTGGTCTTCTTGCCGAAGGGAATGATTGATTGAcgatagcag
811    E  I  V  G  I  P  N  G  I  E  P  A  P  L  D  Y  V  V  F  L  P  K  G  M  I  D  *
2521  tggatgacttttaatcagcaggaatcagagatactat
```

Figure 5.3. Nucleotide sequence of the *A. pullulans aguA* gene with the deduced α-glucuronidase amino acid sequence in bold. Coding sequence is shown as capitals and non-coding sequence as small letters. The putative signal peptide is underlined. Conserved residues referred to in the text are boxed. N-linked glycosylation consensus sequences are dotted underlined. The putative TATA-box is doubly underlined. Sequences numbered from the presumed physiologically relevant ATG.

conserved in the *A. pullulans* α-glucuronidase (Figure 5.5). These amino acids were previously found to be invariant in all α-glucuronidases sequences (Nagy *et al.*, 2003; Nurizzo *et al.*, 2002).

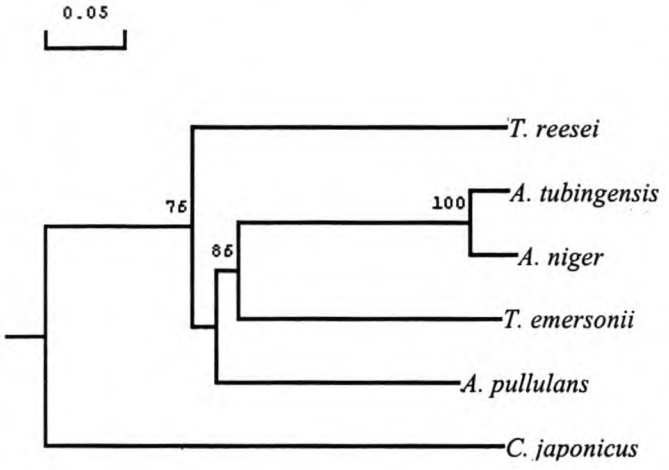


Figure 5.4. Rooted tree showing the phylogenetic relationships between the *A. pullulans* α-glucuronidase and microbial homologues. The tree was constructed on the basis of amino acid sequences. The values represent the percentage of 1000 bootstraps and the scale bar corresponds to 0.05 amino acid substitutions per site. The *C. japonicus* α-glucuronidase (AAL57752) was included as a representative of the bacterial enzymes. *T. reesei* (Q99024), *A. tubingensis* (O42814), *A. niger* (CAC38119), *T. emersonii* (AAL33576).

Expression of the *A. pullulans aguA* gene in *S. cerevisiae*

The *A. pullulans aguA* gene was expressed in *S. cerevisiae* Y294 in the episomal vector pDLG1, under control of the metabolically induced *ADH2* promoter and terminator. The longer of the two putative open reading frames, *APaguA**, was cloned as a 2566 bp *Eco*R1-Xho1 fragment containing the unmodified open reading frame, and as a 2575 bp *Eco*R1-Xho1 fragment engineered to code for an additional *C*-terminal hexahistidine tag. The shorter open reading frame, *APaguA*, was cloned as a 2527 bp *Eco*R1-Xho1 fragment containing the unmodified open reading frame, and as a 2545 bp *Eco*R1-Xho1 fragment, coding for an additional *C*-terminal hexahistidine tag (Figure 5.6).

In a trial experiment, cell lysis released 6.9 mU/mg and 11.1 mU/mg of α-glucuronidase activity from the [pDLG1-APaguA*]- and [pDLG1-APaguA*+His]-carrying strains, and 52.3 mU/mg and 72.8 mU/mg from the [pDLG1-APaguA]- and [pDLG1-APaguA+His]-carrying strains, respectively after 5 days of cultivation in defined medium. Work was continued with *S. cerevisiae* Y294[pDLG1-APaguA+His], as the more conventional signal peptide encoded by this construct resulted in higher yields of the enzyme in *S. cerevisiae*, and the His-tag did not negatively affect the intracellular accumulation of the enzyme. The production of cell-associated α-glucuronidase activity by *S. cerevisiae* Y294[pDLG1-APaguA+His], as well as cell density was followed over time in defined medium at 30°C (Figure 5.7B). Cultures reached stationary phase after 32 h of incubation and α-glucuronidase activity was detectable in lysates after 3 days of cultivation. Intracellular levels of enzyme activity reached a plateau of 25 mU/ml after 7 days. Equal amounts of α-glucuronidase activity were measured in the periplasmic fraction, released by spheroplasting, and the intracellular fractions, released by subsequent glass bead extraction, at the end of the experiment at 8 days. In a separate experiment, the extracellular accumulation of α-glucuronidase activity was measured (Figure

```
A. pullulans:   DGWLRYAALPSSISWPSVPKHIVVLNTTETSP-VYTAGQELQRGIQSILGQNCRFSTNK-
                D WLRY  +       +  K I  L+    SP +  A  ELQRG+  +L +       K
C. japonicus:   DMWLRYQPIADQTLLKTYQKQIRHLHVAGDSPTINAAAAELQRGLSGLLNKPIVARDEKL

A. pullulans:   PDESIIVGTLD-AYVAAYGNFSQSVN-LKEDGFWLS----TENNTVQIVGQNERGALYGA
                D S+++GT D + + A  N  + +  L  +G+ L      + + V +   ++ G LYG+
C. japonicus:   KDYSLVIGTPDNSPLIASLNLGERLQALGAEGYLLEQTRINKRHVVIVAANSDVGVLYGS

A. pullulans:   FEYLSML-AQANFSTVAYVSNPDAPIRWVNQWDNLDGSIERGYGGASIFFANGTIVNDLT
                F  L ++  Q     ++  S P    R VN WDNL+  +ERGY G S+ +  G++ N L
C. japonicus:   FHLLRLIQTQHALEKLSLSSAPRLQHRVVNHWDNLNRVVERGYAGLSL-WDWGSLPNYLA

A. pullulans:   -RVAEYARLL-------------------LSPQNIDGVGRIADTMRPYGVQIGLSLYFAS
                 R  +YAR+                    LS Q +  +  +AD  RPYG+++ LS+ F S
C. japonicus:   PRYTDYARINASLGINGTVINNVNADPRVLSDQFLQKIAALADAFRPYGIKMYLSINFNS

A. pullulans:   PTSGVQGQANLTTFDPLDPGVVTWWGNVTDQIYERIPDMAGYLVKANSEGQPGPITYNRT
                P    +   ++ T DPLDP V  WW     +IY  IPD  G+LVKA+SEGQPGP  Y R
C. japonicus:   P----RAFGDVDTADPLDPRVQQWWKTRAQKIYSYIPDFGGFLVKADSEGQPGPQGYGRD

A. pullulans:   LAEGANLFARALQPHGGRVLFRAFVYNQLNESDWRADRANAAVDFFKPLDGKFDDNVVVQ
                 AEGAN+ A AL+P GG V +RAFVY+   E     DR   A D F PLDGKF DNV++Q
C. japonicus:   HAEGANMLAAALKPFGGVVFWRAFVYHPDIE-----DRFRGAYDEFMPLDGKFADNVILQ

A. pullulans:   IKYGPIDFQVREPASPLFANLFHTNTAIELEVTQEYLGQQTHLVYLPPLWDTVLGFDMRI
                IK GPIDFQ REP S LFA +  TN  +E ++TQEY G  THL Y  PL++  L  +
C. japonicus:   IKNGPIDFQPREPFSALFAGMSRTNMMMEFQITQEYFGFATHLAYQGPLFEESLKTETHA

A. pullulans:   DNETSLVRDIIAGRTFNRSLGGYAAVVNVGTNQTWLGSHLSMSNFYAYGRLAWNPTEDTT
                  E S + +I+ G+ F     G A V+N GT++ W G     S++YA+GR+AW+
C. japonicus:   RGEGSTIGNILEGKVFKTRHTGMAGVINPGTDRNWTGHPFVQSSWYAFGRMAWDHQISAA

A. pullulans:   KIHEDWTRLTFGLDQNVVDTITHMALESWPAYENYSGNLGIQTLTDILYTHFGPNPQSQD
                   ++W R+TF      ++ +  M L S  A  NY   LG+  L      H+GP P + D
C. japonicus:   TAADEWLRMTFSNQPAFIEPVKQMMLVSREAGVNYRSPLGLTHLYS-QGDHYGPAPWT-D

A. pullulans:   NNGWGQWT-----RADHTTIGMDRTVSNGTGYSGTYPPEVAAMYENIETTPDNLLLWFHH
                +     WT     RA  T IG +RT   G+     YP  +A  + ++ + P++L+LWFHH
C. japonicus:   DLPRADWTAVYYHRASKTGIGFNRT-KTGSNALAQYPEPIAKAWGDLNSVPEDLILWFHH

A. pullulans:   VNYTQKLHSGKTVIQHFYDAHYAGADTAQTFPEQWQTLAGKIDSQRFDEQLYRLKYQAGH
                +++  ++ SG+ + Q     +Y G +  +     W      +D+ RF +    L+ Q
C. japonicus:   LSWDHRMQSGRNLWQELVHKYYQGVEQVRAMQRTWDQQEAYVDAARFAQVKALLQVQERE

A. pullulans:   SLVWRDAIVNFYHNISG
                ++ WR++ V ++ +++G
C. japonicus:   AVRWRNSCVLYFQSVAG
```

Figure 5.5. Alignment of the amino acid sequence of the *A. pullulans* α-glucuronidase with the *C. japonicus* GlcA67A. The consensus is indicated in between the sequences and + indicates a conservative change. Catalytically important residues identified in the crystal structure of the *C. japonicus* enzyme are boxed.(Nagy *et al.*, 2003).

5.7B). Growth was slower at the lower temperature, and cultures reached stationary phase after 40 h of incubation. Enzyme activity was detected in the culture supernatant after 4 days of growth and reached a steady state of about 5 mU/ml after 6 days. Extracellular accumulation of α-glucuronidase activity was measure in the autoselective strain *S. cerevisiae* Y294[*fur1*::*LEU3* pDLG1-APaguA+His], carrying the same plasmid, in rich medium at 26°C (Figure 5.7C). Cultures reached stationary phase after 48 h and activity was also detectable at this time point. The rate of accumulation of α-glucuronidase activity increased markedly after 4 days and did not slow by 8 days, at which time 0.58 mU/ml α-glucuronidase activity was measured in the culture supernatant.

Purification of the heterologous α-glucuronidase from the culture supernatant of *S. cerevisiae* Y294[pDLG1-APaguA+His]

The heterologous α-glucuronidase was purified 58-fold with a yield of 49% from the concentrated culture supernatant of *S. cerevisiae* Y294[pDLG1-APaguA+His] cultivated in defined medium by Ni-

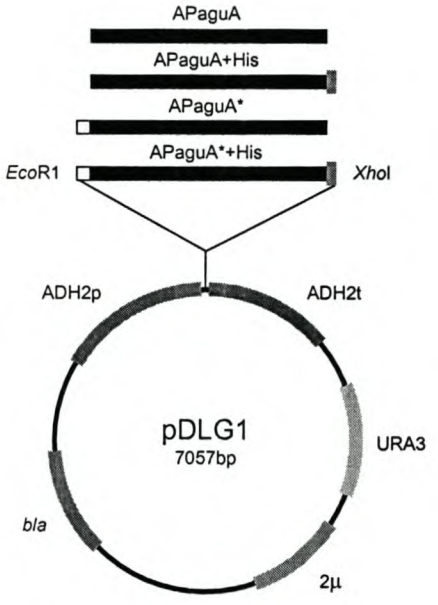
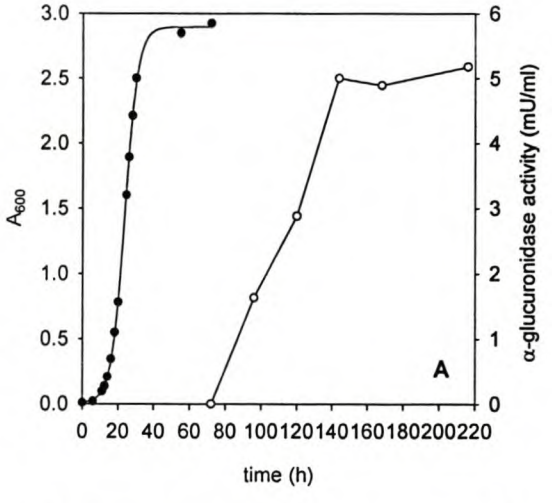
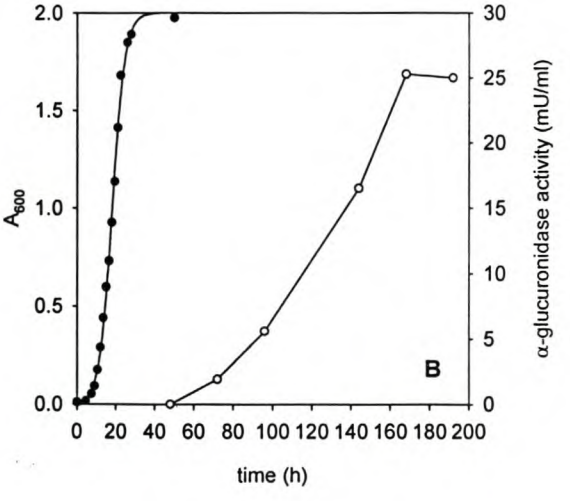


Figure 5.6. Schematic representation of the construction of the expression vectors pDLG1-APaguA (top bar), pDLG1-APaguA+His (second bar), pDLG1-APaguA* (third bar) and pDLG1-APaguA*+His (bottom bar). The additional 5'-end 45 bp in APaguA* are indicated by the white box, while the broad dark-grey box represents the hexahistidine tag. Key features of the base vector pDLG1 (circle) and their relative positions are highlighted





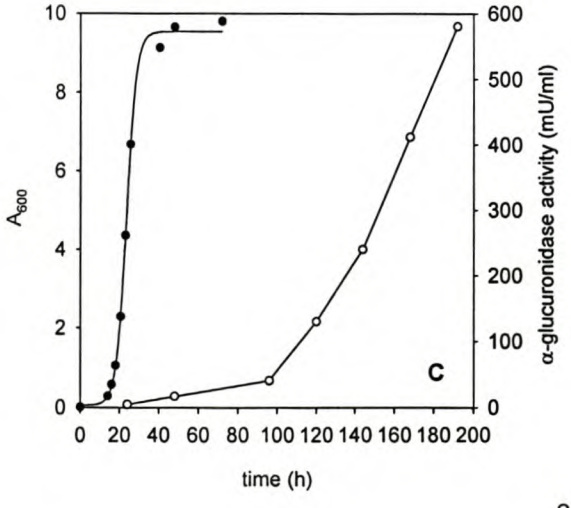


Figure 5.7. Production of the heterologous α-glucuronidase and cell density as a function of time. **(A)** Extracellular activity in SC-medium produced by *S. cerevisiae* Y294[pDLG1-APaguA+His] at 26°C. **(B)** Cell-associated activity in SC-medium produced by *S. cerevisiae* Y294[pDLG1-APaguA+His] at 30°C. **(C)** Extracellular activity produced by *S. cerevisiae* Y294[*fur1*::*LEU3* pDLG1-APaguA+His] in YPD-medium. Cell density was measured 600 nm (closed circles), and enzyme activity was measured (open circles).

chelate affinity chromatography. The purified enzyme had an apparent mobility of 120 kDa on SDS-PAGE and this was the only additional band visible in the culture supernatants of α-glucuronidase producing strain compared to the plasmid control strain carrying vector without insert (Figure 5.8).

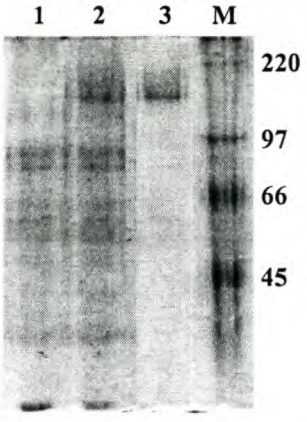


Figure 5.8. SDS-PAGE showing the production and purification of the heterologous α-glucuronidase. Lane 1 is the culture supernatant of the *S. cerevisiae* Y294[pDLG1] transformed with control plasmid only, lane 2 is the culture supernatant *S. cerevisiae* Y294[pDLG1-APagua+His] producing the α-glucuronidase, and lane 3 is the α-glucuronidase purified by Ni-chelate affinity chromatography and M indicates the molecular weight marker lane. Molecular weights of the standards are given in kDa.

Characterisation of the heterologous α-glucuronidase

Maximal activity was observed at 65°C (Figure 5.9A) and between pH 5 and pH 6 (Figure 5.9B). The enzyme was stable at 45°C, lost half of its activity within 22.5 min at 55°C and was rapidly inactivated at 65°C with a half-life of 5.6 min (Figure 5.9C). The enzyme was stable between pH 4 and pH 6, had a half-life of 17 min at pH 8 (Figure 5.9D), and was completely inactivated within 15 min at pH 2.2. Substrate concentration titrations were performed with aldobiouronic acid, aldotriouronic acid, aldotetraouronic acid and aldopentaouronic acid (Figure 5.10), and the kinetic constants of the enzyme for each aldouronic acid substrate determined (Table 5.1). Data for the titration with aldobiouronic acid were fitted with the Michaelis-Menten equation [$v = V_{max}[S]/(K_m + [S])$], while data for titrations with other aldouronic acids were fitted with the Michaelis-Menten equation describing substrate inhibition, $v = \frac{V_{max}[S]}{K_m + [S] + [S]^2/K_i}$, using the non-linear regression function of SigmaPlot (Jandel Scientific).

The enzyme showed an increasing reaction rate (and turnover number) with increasing chain length of the aldouronic acids. In contrast it showed a decreasing affinity for the substrate with increasing chain length of the aldouronic acids. Catalytic efficiency (k_{cat}/K_m) also decreased with increasing chain-length, and was the highest for aldobiouronic acid. The catalytic efficiencies for aldotriouronic acid and aldotetraouronic acid were nearly identical and about two-thirds of the value for aldobiouronic acid, while the catalytic efficiency for aldopentaouronic acid was markedly lower. Ordinary Michaelis-Menten kinetics were observed with aldobiouronic acid, while substrate inhibition was noted with longer aldouronic acid substrates. The enzyme had similar inhibition constants for aldotriouronic acid and aldotetraouronic acid. The inhibition constant for aldopentaouronic acid was 3

times higher than the inhibition constant for the two shorter aldouronic acids. The enzyme was unable to hydrolyse internally substituted aldopentaouronic acid in a 12 hour incubation at 40°C.

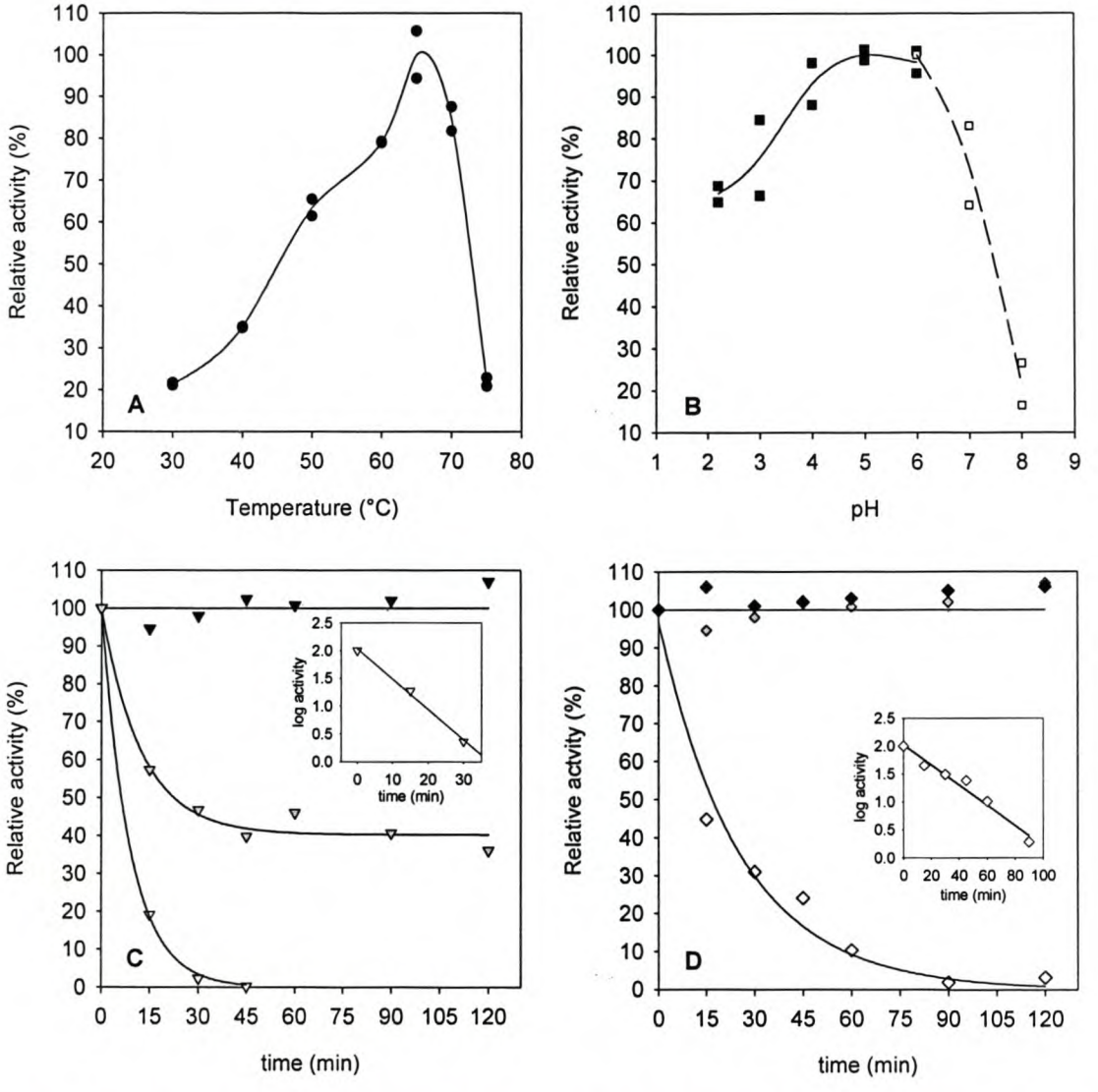


Figure 5.9. Physical properties of the purified heterologous α-glucuronidase. **(A)** Temperature dependence of α-glucuronidase activity. Assays performed at pH 4.8 for 5 min. **(B)** pH dependence of α-glucuronidase activity. Assays performed at 65°C for 5 min. Black squares, citrate buffer; white squares, Tris-glycine buffer. **(C)** Temperature stability of α-glucuronidase activity. Black triangles, 45°C; grey triangles, 55°C; white triangles, 65°C. **(D)** pH stability of α-glucuronidase activity. Black diamonds, pH 4; grey diamonds, pH6; white diamonds, pH8. Maximal activity is equal to 9 mU in all cases.

Discussion

As 4-*O*-methyl glucuronic acid is a substituent of all xylan species, α-glucuronidases form an integral part of the xylanolytic systems of microorganisms and play an important role in lignocellulose biodegradation (Dekker, 1989). α-Glucuronidase activity had been detected in the culture supernatants of the *endo*-β-1,4-xylanase overproducing euascomycetous fungus A. *pullulans* NRRL Y-2311-1 cultivated on carbon sources containing both glucuronic acid and xylose, suggesting the

presence of an α-glucuronidase encoding gene in this organism (Chapter 4). The *A. pullulans aguA* gene encoding an α-glucuronidase was cloned using homology-based PCR methods, as well as inverse

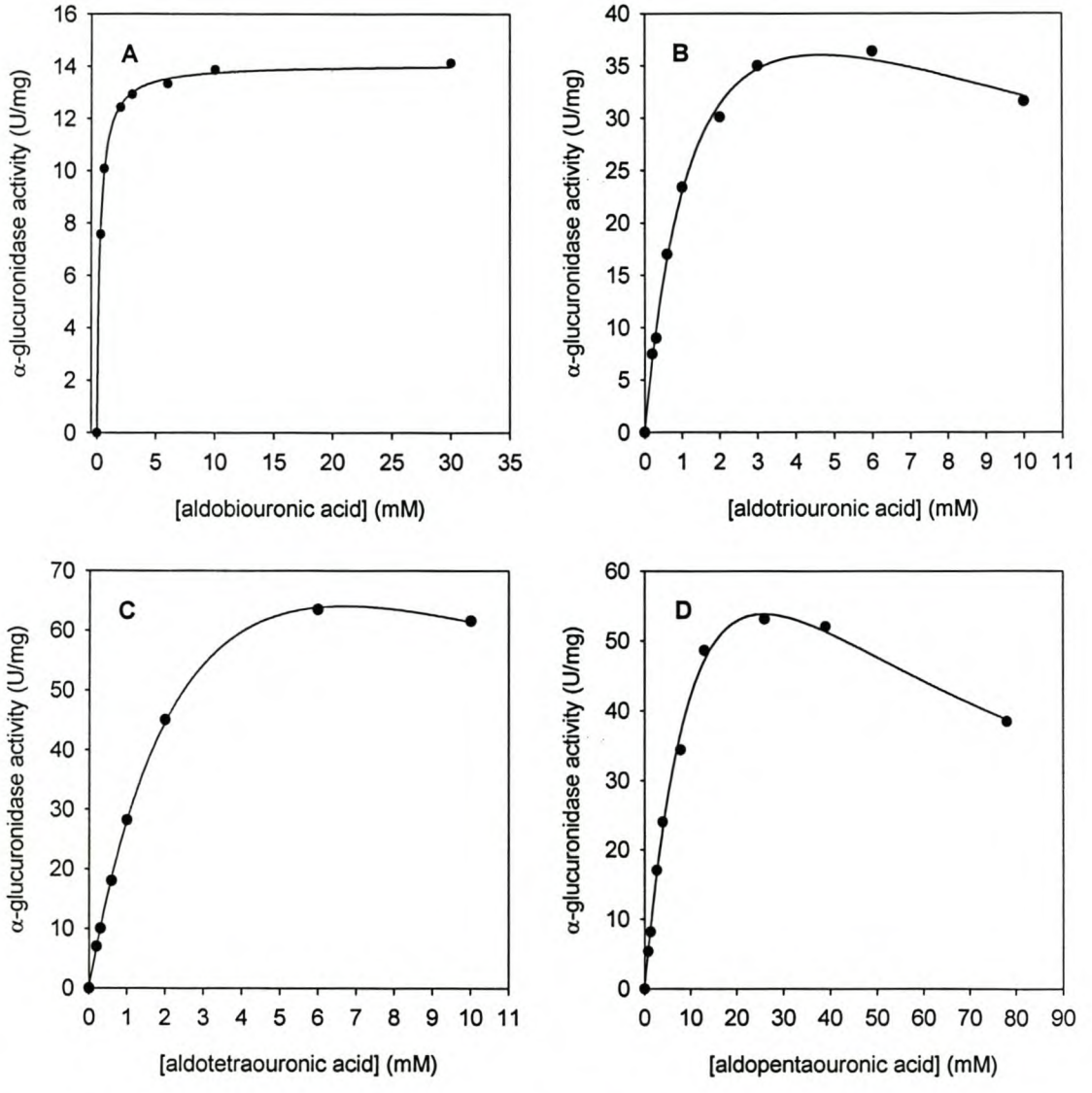


Figure 5.10. α-Glucuronidase activity as a function of substrate acid concentration. Assays were performed at pH 4.8 and 40°C for 5 minutes. **(A)** Aldobiouronic acid. **(B)** Aldotriouronic acid. **(C)** Aldotetraouronic acid. **(D)** Aldopentaouronic acid. Data were fitted with equations using the non-linear regression function of SigmaPlot.

Table 5.1. Kinetic constants of the *A. pullulans* α-glucuronidase for different aldouronic acids.

Substrate	V_{max}[a] (U/mg)	K_m[a] (mM)	K_i[a] (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}.s^{-1}$)
aldobiouronic acid	14.1 ± 0.08	0.25 ± 0.009	NI[b]	21	84
aldotriouronic acid	60.0 ± 4.3	1.54 ± 0.19	14.0 ± 2.8	90	58
aldotetraouronic acid	135 ± 6.2	3.70 ± 0.23	12.0 ± 1.2	202	55
aldopentaouronic acid	126 ± 14.1	17.4 ± 2.8	38.3 ± 7.5	189	11

[a] Mean ± standard deviation given for value calculated from fit
[b] No inhibition

PCR (Figure 5.1 and 5.2). α-Glucuronidase sequences are currently found exclusively in family 67 of the glycoside hydrolases and sequences cluster into two groups, depending on fungal or bacterial origin. Degenerate primers were therefore based on the fungal family 67 sequences, and the *A. pullulans aguA* gene is a new member of this family based on its primary sequence. The deduced amino acid sequence is closely related to its homologues from other fungi and is more distantly related to its bacterial homologues (Figure 5.4). The high degree of sequence identity (>60%) of the *A. pullulans* α-glucuronidase with its fungal homologues is expected, as these organisms are all classified in the Pezizomycotina, a subphylym of the Ascomycota (Domsch *et al.*, 1980). The *A. pullulans* α-glucuronidase is the most closely related to its homologues from *T. emersonii* and *A. tubenginsis* and *A. niger*, and is more distantly related the *T. reesei* α-glucuronidase (Figure 5.4).

Analysis of the upstream sequence revealed a TATA-like element 55 bp from the presumed physiologically relevant translation start site, although its sequence deviates slightly from the consensus sequence (Figure 5.3, doubly underlined). These elements have, however, been shown to be less conserved in fungi (Ballance, 1986). Amplification and sequencing of more upstream sequence may reveal common elements known to be involved in the control of lignocellulolytic genes in other fungi. CreA consensus sequences have been found in the *xynA* gene of *A. pullulans* var. *melanigenum*, and as induction of α-glucuronidase production by *A. pullulans* is repressed by the presence of glucose, it is possible this process is mediated through homologues of CreA. Two putative translation start sites were possible from the sequence of the *aguA* gene (Figure 5.3). The shorter open reading frame encoded a putative signal peptide of 15 amino acids that contained long stretches of hydrophobic amino acids, which is in agreement with our current knowledge of signal sequences (von Heijne, 1986), and is therefore thought to be the physiologically relevant open reading frame, at least for the secreted α-glucuronidase. Whether the longer open reading frame is translated, or is of physiological significance, such as encoding an intracellular or membrane-anchored variant of the enzyme, is difficult to predict. The mature protein contains 821 amino acids and has a predicted molecular weight of 91 kDa, and is very similar in size to its fungal homologues.

Several amino acids that were found to play key roles in catalysis and substrate recognition in the *C. japonicus* GlcA67A have been shown to be conserved in all of the α-glucuronidases sequenced so far (Nagy *et al.*, 2003; Nurizzo *et al.*, 2002). Alignment of the *A. pullulans* and *C. japonicus* GlcA67A sequences showed that the corresponding amino acids were also conserved in the *A. pullulans* α-glucuronidase (Figures 5.5), and we speculate that they play similar roles in the functioning of the enzyme (*C. japonicus* GlcA67A positions listed in brackets). The role of the catalytic acid is therefore likely fulfilled by Glu298(292), while the catalytic base/s most likely are Asp376(365) and/or Glu404(393). Trp162(160) and Trp556(543) probably serve as the sites of π-stacking interactions at

the surfaces of the 4-*O*-methyl glucuronosyl and the xylopyranosyl residues respectively. The carboxylate group of the glucuronosyl residue is likely stabilised by the presence of the three positively charged side-chains of Lys294(288), Arg 331(225) and Lys 371(360). The sequences surrounding these catalytically important amino acids are also largely conserved (Figure 5.3), giving further support to the likelihood of their respective roles in the *A. pullulans* enzyme. No counterpart was found for Val210 of GlcA67A in the *A. pullulans* enzyme, and it is possible that it accommodates the hydrophobic nature of the glucuronosyl methyl-group by different means. X-ray crystallography and mutational analysis would shed more light on the functioning of the *A. pullulans* α-glucuronidase on a molecular level.

Both putative open reading frames were successfully expressed in *S.* cerevisiae Y294 under control of the metabolically induced *ADH2* promoter and terminator, with or without *C*-terminal hexahistidine tags. α-Glucuronidase activity accumulated to a seven-fold higher level in yeast cells expressing the shorter open reading frame after five days in a trial experiment, and the hexahistidine tags did not appear to negatively influence production of the heterologous enzyme. Work was continued with *S. cerevisiae* Y294[pDLG1-APaguA+His] because of the higher overall yields and the ease of purification with the hexahistidine tag.

The production of α-glucuronidase activity by *S. cerevisiae* Y294[pDLG1-APaguA+His] cultivated in defined medium was followed over time in the cell-associated fraction and in the culture supernatant in two separate experiments (Figure 5.7 A and B). In both cases enzyme activity was only detectable 2 days after stationary phase was reached. The *ADH2* promoter is only induced once glucose becomes depleted and ethanol accumulates, explaining the lag in enzyme production. Activity reached a steady state level of 5 mU/ml in the culture supernatant after 6 days, and 25 mU/ml in the cell-associated fraction after 7 days. Activity in the cell-associated fraction was divided equally between the periplasmic and intracellular fractions. The five-fold lower level of α-glucuronidase activity in the medium and the substantial amount of activity in the periplasmic space suggests that access through the cell wall of the yeast might limit the effective secretion of the heterologous α-glucuronidase, most likely due to its large size. Cultivation of the autoselective strain *S. cerevisiae* Y294[*fur1*::*LEU3* pDLG1-APaguA+His], carrying the same vector, in rich medium led to a greater than hundred-fold level of α-glucuronidase activity (0.58 U/ml) in the culture supernatant after 8 days of cultivation (Figure 5.7C). This is despite an only threefold increase in cell density, suggesting that nutrient availability plays a substantial role in the production of the heterologous enzyme. Activity levels measured in the culture supernatant and the cell-associated fraction in defined medium, and the culture supernatant in rich medium corresponded to 0.14 mg/L, 0.71 mg/L and 16.6 mg/L, respectively. The levels of extracellular α-glucuronidase activity produced by *A. pullulans* grown on defined medium

containing 1% birchwood xylan was fivefold more (Figure 4.1) than the levels of extracellular of heterologous α-glucuronidase activity produced by the recombinant yeast strains. However, overall activity levels were similar, if the cell-associated fraction is taken into account.

Trial experiments indicated that in spite of the much higher yields of the enzyme in the cell-associated fraction in defined medium and the supernatant of rich medium, neither preparation was suitable for the convenient metal-affinity purification of the heterologous α-glucuronidase. Components of the rich medium chelated nickel, stripping it from the resin while intracellular proteins released by lysis gave background binding to the resin. As sufficient activity could be obtained from the supernatants of defined medium, these preparations were used. The heterologous α-glucuronidase was purified 58-fold by a single affinity-chromatography step on Ni-NTA sepharose with a yield of 49%, and was found to be electrophoretically pure (Figure 5.8). The enzyme migrated with an electrophoretic mobility of 120 kDa on SDS-PAGE and the band ran as a slight smear. The difference in the observed electrophoretic mobility and the calculated molecular weight of 91 kDa, suggests that the heterologous α-glucuronidase is substantially glycosylated in baker's yeast. This result is expected as the amino acid sequence of the *A. pullulans* α-glucuronidase contains twenty possible asparagine linked glycosylation sites (Gavel and von Heijne, 1990) (Figure 5.3), and as *S. cerevisiae* is known to extensively glycosylate secreted proteins (Romanos *et al.*, 1992). The native α-glucuronidase produced by *A. pullulans* is even more extensively decorated than heterologous enzyme and the partially purified enzyme had an apparent mobility of 170 kDa on SDS-PAGE (Chapter 4). The heterologous α-glucuronidase showed maximal activity at 65°C and between pH 5 and pH 6, and was stable at 45°C and between pH 4 and pH 6 for 2 hours (Figure 5.9) . These values are in agreement with what is known about the properties of other fungal α-glucuronidases and are very similar to the values obtained for the α-glucuronidases from *A. niger* and *A. tubingensis*, the two most closely related homologues of the *A. pullulans* α-glucuronidase that have been characterised (Table 3.1).

The substrate specificity of family 67 α-glucuronidases is an area about which there is still a lack of clarity. What is known with a large degree of certainty is that family 67 α-glucuronidases are only able to hydrolyse the glycosidic linkage between 4-*O*-methyl glucuronic acid and terminal non-reducing end xylopyranosyl units of aldouronic acid substrates. This is largely explained by the dead-end structure of the substrate binding site in the crystal structure of the *C. japonicus* GlcA67A (Nurizzo *et al.*, 2002). The *A. pullulans* α-glucuronidase showed no activity against an internally substituted aldopentaouronic acid and is therefore similar to its family 67 homologues in this regard. One property of the family 67 α-glucuronidases that shows a lack of uniformity is the preferred chain length of the aldouronic acid substrate. The *A. pullulans* α-glucuronidase displayed an increasing turnover number as the chain length increases from aldobiouronic acid to aldotetraouronic acid, but

conversely had a decreasing affinity for the substrate as the chain length increased. The result is that the catalytic efficiency of the enzyme also decreased with increasing chain length (Table 5.1). This trend is the direct opposite of what was observed for the *C. japonicus* GlcA67A (Nurizzo *et al.*, 2002). Co-crystallisation of catalytically inactive mutants of the *C. japonicus* enzymes with aldobiouronic acid revealed that only the 4-*O*-methyl glucuronosyl moiety had an order structure, while the position of the xylopyranosyl residue could not be defined. The W543A mutant of the enzyme also had four-fold more activity than the W160A mutant, indicating that the enzyme maintains more defined π-stacking interactions with the 4-*O*-methyl glucuronosyl moiety than with the xylopyranosyl moieties of the substrate. The less defined nature of interaction of these enzymes with the xylo-oligosaccharide portion of the aldouronic acid substrate may lead to a greater influence of local amino acid sequence variation, resulting in the variations in substrate preference observed in the family 67 α-glucuronidases.

Substrate inhibition was observed with all the aldouronic acids tested excepting aldobiouronic acid (Figures 5.10). The active site architecture of family 67 glycoside hydrolases offers some insights into why this might occur. The deep, enclosed nature of the substrate binding site requires the ordered release of product after completion of the hydrolysis, with the xylo-oligosaccharide portion departing first, followed by the 4-*O*-methyl glucuronic acid. If favourable interactions between the binding sites for the xylopyranosyl residues and a second substrate molecule are possible with only the 4-*O*-methyl glucuronic acid still bound to the enzyme, a dead-end complex will be formed and the enzyme will remain catalytically inactive until both molecules have dissociated. The relatively low affinities of the *A. pullulans* α-glucuronidase for the aldouronic acid substrates and its relatively low turnover increase the likelihood of such events occurring.

This report describes the cloning of a new family 67 α-glucuronidase gene from the euascomycetous fungus *A. pullulans*, and describes the basic properties of the heterologous enzyme expressed in *S. cerevisiae* as well as investigating the substrate range and specificity of the enzyme. Most of the interpretations of the kinetic data obtained for the enzyme are based on the crystal structure of the bacterial α-glucuronidase from *C. japonicus*. As all of the catalytically important amino acids are conserved in all family 67 α-glucuronidases, and indeed also in the newly described *A. pullulans* enzyme, and as these two enzymes share a reasonable degree of sequence identity, this approach is clearly a valid one. Caution is, however, needed in the direct extrapolation of structural interpretations of the properties of one enzyme to a closely related homologue. The crystal structure of a fungal α-glucuronidase will therefore be a great breakthrough in the study of α-glucuronidases in general and will definitely lead to additional insights in the mechanism and specificity of these enzymes.

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Chapter 6

Cloning of the *Aureobasidium pullulans* α-arabinofuranosidase gene, *abfA*, and expression in *Saccharomyces cerevisiae*

Abstract

The family 54 α-arabinofuranosidase gene of *Aureobasidium pullulans* NRRL Y-2311-1, *abfA*, was cloned by PCR methods and expressed in *Saccharomyces cerevisiae*. The deduced amino acid sequence of the AbfAp is highly conserved and showed between 69% and 76% identity with known glycoside hydrolase family 54 α-arabinofuranosidases. The gene encodes a polypeptide of 498 amino acids that contains a putative signal peptide of 20 amino acids resulting in a predicted mature protein with a calculated molecular weight of 49.9 kDa and iso-electric point of 4.97. It was expressed in *S. cerevisiae* Y294 in a multi-copy episomal vector under control of the metabolically induced *ADH2* promoter and terminator. Enzyme activity was detectable after 72 hours of growth and reached levels of 0.027 U/ml and 1.34 U/ml after 8 days in defined and rich medium, respectively. The heterologous enzyme was purified to homogeneity by gel filtration. It's size estimated by gel filtration was 36 kDa, and it had an apparent mobility of 49 kDa on SDS-PAGE, indicating a monomeric quaternary structure. It showed maximal activity at 55°C and between pH 3.5 and pH 4. It was stable at 50°C and between pH 4 and pH 5. The enzyme had a K_m for *p*-nitrophenyl α-L-arabinofuranoside of 3.7 ± 0.36 mM and a V_{max} of 34.8 ± 1.1 U/mg protein. It displayed trace β-xylosidase activity and activity against 10 mM *p*-nitrophenyl β-D-xylopyranoside was 0.2 U/mg.

Introduction

L-Arabinofuranose is a component of a number of plant cell wall polysaccharides, including arabinoxylan, arabinogalactan and arabinan. Arabinans contain α-L-arabinofuranose both as the 1,5-linked backbone, as well as the 1,2- and 1,3-linked side-chains, while α-L-arabinofuranose is found as 1,3- and 1,6-linked side-chains in arabinogalactans. Arabinoxylan is a major component of the cell walls of softwoods as well as grasses and cereals and consists of a backbone of 1,4-linked β-xylopyranose units that carries α-L-arabinofuranose substituents at the *O*-2 and *O*-3 positions (Bacic *et al.*, 1988). α-Arabinofuranosidases (E.C. 3.2.1.55) catalyse the hydrolysis of single α-L-arabinofuranose residues from the non-reducing ends of arabinose-containing polysaccharides and play an integral role in the biodegradation of hemicelluloses. They have been employed in numerous industrial applications, usually complementing the action of endo-β-1,4-xylanase in the enzymatic degradation of xylan. Specific applications have included the bioconversion of agricultural residues to fuel, the pre-treatment of paper pulp, the pre-treatment of lower grade animal feeds for increased digestibility and the enzymatic clarification of fruit juices (Saha, 2000).

Based on amino acid sequence, α-arabinofuranosidases activity has been classified into glycoside hydrolase families 43, 51, 54 and 62 (http://afmb.cnrs-mrs.fr/CAZY/). Family 54 α-arabinofuranosidases are capable of removing single 1,2-, 1,3- and 1,5-linked terminal non-reducing end α-L-arabinofuranoside residues from arabinan, arabinogalactan and arabinoxylan, as well as small oligosaccharides generated by the action of the corresponding *endo*-glycanases. Members of this family are capable of hydrolysing the synthetic aryl glycoside *p*-nitrophenyl α-L-arabinofuranoside and show trace activity against *p*-nitrophenyl β-D-xylopyranoside. They are monomeric proteins with predicted molecular weights around 50 kDa and have only been found in fungi.

The first family 54 α-L-arabinofuranosidase to be purified and characterized was the arabinofuranosidase B from *Aspergillus niger* (Tagawa and Kaji, 1988), and the *abfA* gene encoding this enzyme has been cloned (Flipphi *et al.*, 1993), and has been successfully expressed in *Saccharomyces cerevisiae* (Crous *et al.*, 1996). Homologues of this gene have been cloned from several Aspergilli, the enzyme activities characterized, and their expression pattern studied on different carbon sources. Transcription of the genes and production of the enzymes are induced by cultivation of carbon sources containing L-arabinofuranose as well as its catabolic intermediate L-arabitol. Their induction is repressed by the presence of glucose in the growth medium via the CreA response element (Gielkens *et al.*, 1999). Similarly, the main α-L-arabinofuranosidase from *Trichoderma reesei* was purified (Tagawa and Kaji, 1988), its substrate specificity studied (Kaneko *et al.*, 1998), the *abf1* gene encoding this enzyme cloned and expressed in *S. cerevisiae* (Margolles-Clark *et al.*, 1996). Recently the α-L-arabinofuranosidase from *Penicillium purpurogenum* α-L-arabinofuranosidase was also purified (Carvallo *et al.*, 2003), the *abf1* gene encoding this enzyme cloned and its expression in *S. cerevisiae* studied (de Ioannes *et al.*, 2000). Family 54 α-L-arabinofuranosidases are highly conserved in terms of their primary sequence, substrate specificity and regulation of their expression.

A. pullulans is an euascomycetous fungus and the colour variant strain NRRL Y-2311-1 has been isolated for the high-level production of xylanolytic enzymes (Leathers, 1986). The main *endo*-β-1,4-xylanase has also been purified (Li *et al.*, 1993), the *xynA* gene cloned (Li and Ljungdahl, 1994), and expressed in *S. cerevisiae* (Li and Ljungdahl, 1996). The presence of α-L-arabinofuranosidase activity has been noted in the culture fluid of *A. pullulans* NRRL Y-2311-1 cultivated on lignocellulosic carbon sources (Myburgh *et al.*, 1991a), and the enzyme activity characterized in the crude culture supernatant of this organism (Myburgh *et al.*, 1991b). Here we describe the cloning of the *A. pullulans* NRRL Y-2311-1 family 54 α-arabinofuranosidase gene, *abfA*, its expression in *S. cerevisiae*, as well as the purification and characterisation of the heterologous enzyme.

Materials and methods

Microbial strains and culture conditions

A. pullulans NRRL Y-2311-1, *Escherichia coli* XL1-Blue and *S. cerevisiae* Y294 were routinely cultured as described in Chapter 5.

Primers

The following primers were used in the polymerase chain reaction (PCR) and sequencing (restriction endonuclease recognition sites doubly underlined, start and stop codons in bold and codons for hexahistidine tag underlined):

ABFdegF1:	CCACCTGCCTCATCACCATHATHTAYGAYC
ABFdegR1:	GCCCTCCTTAGACATAGGRTTRTANCC
ABFdegF2:	ACCCTCAACGGCCAGAARGCNTAYGG
ABFdegR2:	TCATGACGCCCTCGTAGAANGTNCCYTG
ABFinvF3:	GGCATCGGTGGCGATAAC
ABFinvR3:	GCCTGATAAAGATGGTTTGCAC
ABF-F4:	CAACTTGGCAGGCGCTATTG
APabfA-F:	GATCTTCGCTGGAGATGTGC
APabfA-R:	CCCTATCGCCCGATAAGAG
APabfA-L1:	**ATG**CGTTCCAGGACGAACATC
APabfA-R1-BglII:	AAAGATCT**TTA**AGCAAAGCTGCTTCCAAC
APabfA-R1-His6-BglII:	AAAGATCT**TTA**ATGGTGATGGTGATGGTGAGCAAAGCTGCTTCCAAC
T7 forward:	TAATACGACTCACTATAGGG
SP6 reverse:	TATTTAGGTGACACTATAG

DNA isolation

Genomic DNA was extracted from *A. pullulans* as described in Chapter 5.

Alignment of known fungal α-arabinofuranosidases

Amino acid sequences of α-arabinofuranosidases from *Aspergillus awamori* (BAB21567), *Aspergillus nidulans* (CAA74084), *Aspergillus niger* (CAA01903), *Aspergillus oryzae* (BAB71803), *Penicillium purpurogenum* (AAK51551). *Trichoderma koningii* (AAA81024), and *Trichoderma reesei* (AAP57750), were aligned using CLUSTAL-W based at the Network Protein Sequence Analysis server at the Institut Biologie et Chimie des Protéinis of the Centre National de la Recherche Scientifique in Toulouse, France (http://npsa-pbil.ibcp.fr/). Areas of highly conserved amino acid sequence were used to design two sets of degenerate primers (ABFdegF1, ABFdegF2, ABFdegR1 and ABFdegR2). Primers were designed using the consensus-degenerate hybrid oligonucleotide primer design strategy (Rose *et al.*, 1998).

PCR amplification of an internal section of the *abfA* gene using degenerate primers

The four possible combinations of the degenerate primers ABFdegF1, ABFdegF2, ABFdegR1 and ABFdegR2 and 100 ng of genomic DNA were used for the amplification of an internal section of the *A. pullulans abfA* gene. Reaction mixtures for PCR consisted of template DNA, 1× reaction buffer, 1 U *Taq* DNA polymerase (Promega), 0.2 mM of each deoxynucleotide triphosphate (Sigma), 0.5 µM of each primer and 2 mM $MgCl_2$ in a 50 µl reaction volume. A reagent blank was included for each reaction. PCR amplification was performed in a Hybaid PCR Sprint thermocycler using 40 cycles of amplification, with 45 s denaturing at 94°C, 45 s annealing at 53°C and 1 min 30 s extension at 72°C. A 10 min incubation at 72°C followed the last step and samples were maintained at 4°C after completion of the amplification cycle.

Molecular manipulation of PCR products

DNA manipulations were performed according to standard protocols as described in Chapter 5.

Creation of circular DNA molecules for inverse PCR

Circular DNA molecules, which could serve as template for inverse PCR were prepared as described in Chapter 5.

PCR amplification of flanking regions of the *abfA* gene using inverse primers

Inverse primers (ABFinvF3 and ABFinvR3) and ~60 ng of circular DNA were used for the amplification of regions flanking the known internal section of the *A. pullulans abfA* gene. The composition of the reaction mixture was as described above. PCR amplification was performed using 35 cycles of amplification, with 45 s denaturing at 94°C, 45 s annealing at 50°C and 1 min 30 s extension at 72°C. A 10 min incubation at 72°C followed the last step and samples were maintained at 4°C after completion of the amplification cycle. PCR products were resolved by agarose gel electrophoresis, cloned and sequenced as described above.

PCR amplification of the entire *abfA* open reading frame

A 1816 bp fragment spanning the assembled putative open reading frame of the *A. pullulans abfA* gene was amplified from genomic DNA using APabfA-F and APabfA-R as primers. The Expand High Fidelity PCR system (Roche) was used to amplify the *abfA* gene and the products of 3 separate PCR reactions were independently cloned and sequenced. The reaction mixtures contained in a final volume of 50 µl, 1× reaction buffer, 100 ng template DNA, 0.5 µM of each primer, 0.2 mM of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$ and 2.6 U of DNA polymerase. Amplification was

achieved in 40 cycles of denaturing at 94°C for 15 s, annealing at 50°C for 30 s and extension at 72°C for 2 min, following an initial 2 min denaturing step at 94°C. Thirty seconds was added to the extension step after every 10 amplification cycles. PCR products were resolved by agarose gel electrophoresis, cloned and sequenced as described above.

Heterologous expression of the *A. pullulans abfA* gene in *S. cerevisiae* Y294

The putative open reading frame was amplified coding for a polypeptide with or without an additional *C*-terminal hexahistidine tag using APabfA-L1 as forward primer, and APabfA-R1-His6-BglII and APabfA-R1-BglII as reverse primers respectively. The PCR products were designated *APabfA* and *APabfA+His*. PCR reactions were performed with the Expand High Fidelity PCR System (Roche) using a 100 µl reaction volume and 200 ng template DNA. Amplification was achieved in 40 cycles of denaturing at 94°C for 15 s, annealing at 50°C for 30 s and extension at 72°C for 2 min, following an initial 2 min denaturing step at 94°C. Thirty seconds was added to the extension step after every 10 amplification cycles. Reaction mixtures were incubated for a further 10 min at 72°C following the completion of the entire amplification cycle. PCR products were resolved by agarose gel electrophoresis, cloned and sequenced as described above.

Both gene constructs were digested from the pGEM-T Easy vector with *Eco*R1 (restriction site in multiple cloning site of the vector) and *Bgl*II (restriction site engineered into the reverse primer), their corresponding bands purified from agarose gels, ligated into the multi-copy episomal shuttle-vector pDLG1 (la Grange *et al.*, 1996) (Figure 4.3) and used to transform *E. coli* XL1-Blue. Purified plasmids (pDLG1-APabfA and pDLG1-APabfA+His) were examined by restriction digestion and used to transform *S. cerevisiae* Y294 using the lithium acetate/dimethyl sulphoxide procedure (Hill *et al.*, 1991). Transformed yeasts were plated on SC medium lacking uracil and cultured at 30°C until single colonies were discernible from the background. Transformants were picked and subsequently maintained on solid SC medium lacking uracil. Liquid SC-medium lacking uracil was inoculated to 0.010 absorbance units at 600 nm from starter cultures grown from single colonies of *S. cerevisiae* Y294[pDLG1-APabfA] and *S. cerevisiae* Y294[pDLG1-APabfA+His], as well as the plasmid control strain *S. cerevisiae* Y294[pDLG1], and cultured at 26°C with shaking at 150 rpm.

Cell growth was monitored by measuring absorbance of the culture at 600 nm at regular intervals. α-Arabinofuranosidase activity was measured in the culture supernatant and in lysates of cells disrupted by bead-beating. α-Arabinofuranosidase activity was measured in the culture supernatant and in lysates of cells disrupted by bead-beating as well as in the periplasmic space by spheroplasting. Bead beating was performed by vortexing cells with 425 to 600 micron glass beads after cells were washed twice in 50 mM acetate buffer pH 5. Cells were vortexed 5 times for 1 min followed by a 1 min

incubation on ice. Cellular debris was sedimented by centrifugation and the supernatant resuspended in a volume of 50 mM acetate buffer pH 5 equivalent to the original sample. Spheroplasting of yeast cells was performed by incubating washed cells resuspended in 50 mM acetate buffer pH 5 with 1 mg Zymolyase for 6 h. The formation of spheroplasts was detected by microscopic inspection.

Generation of autoselective *S. cerevisiae* strains by *FUR1* disruption

The autoselective strain *S. cerevisiae* Y294[*fur1::LEU2* pDLG-*APabfA*] was generated by transforming *S. cerevisiae* Y294[pDLG-*APabfA*] with a linear 3270 bp NcoI-NsiI *fur1::LEU2* fragment digested from the plasmid pDF1 (la Grange *et al.*, 1996), as described above. Transformants were plated on SC-medium lacking both leucine and uracil. Transformants were screened by PCR for the replacement of the chromosomal *FUR1* gene with the disrupted *fur1::LEU2* allele. *FUR1*-disrupted strains were subsequently cultivated in YPD medium.

α-Arabinofuranosidase assay

α-Arabinofuranosidase activity was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-α-L-arabinofuranoside (Sigma). The reaction mixture consisted of 5 mM *p*-nitrophenyl-α-L-arabinofuranose in 50 mM sodium acetate buffer pH 4, and an appropriate amount of enzyme in a total volume of 100 µl. Reactions were performed at 55°C and terminated by the addition of 400 µl saturated sodium tetraborate, and the amount of product formed quantified by measuring absorbance at 405 nm. α-Arabinofuranosidase was activity determined from the slope of the standard plot of absorbance as a function of *p*-nitrophenol concentration under the assay conditions. One unit of α-arabinofuranosidase activity is defined as the amount of enzyme capable of releasing 1 µmol *p*-nitrophenol from the substrate under the assay conditions.

Protein determination

Protein content was estimated direct measurement of the preparation at 280 nm using bovine serum albumin as standard.

SDS-polyacrylamide gel electrophoresis

Discontinuous sodium dodecyl sulphate gel electrophoresis was carried out using the Tris-glycine buffer system (Laemmli, 1970), 10% polyacrylamide gels and Rainbow molecular weight markers (Amersham). Protein bands were visualised by silver staining (Blum *et al.*, 1987).

Expression, production and purification of the heterologous α-arabinofuranosidase

Culture supernatant of *S. cerevisiae* Y294[pDLG1-APabfA] was harvested by centrifugation at 5000 rpm in a JA-14 rotor after 8 days of cultivation. The supernatant was concentrated from 1 L to 0.6 ml by ultrafiltration using a Minitan tangential flow ultrafiltration system (Millipore Corporation), an Amicon ultrafiltration cell (Millipore Corporation) and an Amicon Centriplus centrifugal filter device (Millipore Corporations), all with 10 kDa cut-off membranes. Two hundred microlitre fractions of the concentrated supernatant of *S. cerevisiae* Y294[pDLG1-APabfA] were loaded onto a Superdex 75 HR 10/30 column (10 × 300 mm) connected to an Äkta Purifier chromatography system (Amersham Pharmacia Biotech) and eluted in 50 mM acetate buffer pH 4 and 300 mM NaCl at a flow rate of 0.5 ml/min . Fractions of 0.5 ml were collected, assayed for α-arabinofuranosidase activity and analysed on SDS-PAGE.

Characterisation of the purified heterologous α-arabinofuranosidase

Determination of temperature optimum

The temperature dependence of enzyme activity was tested by assaying for activity between 20 and 65°C. The reaction mixture contained 50 mM acetate buffer pH 4, 5 mM *p*-nitrophenyl α-L-arabinofuranoside and an appropriate amount of enzyme and assays were allowed to proceed for 5 min.

Determination of pH optimum

The effect of pH on α-arabinofuranosidase activity was investigated by assaying at pH points ranging between 2.2 and 6 in McIlvaine's phosphate-citrate buffer (McIlvaine, 1921). The reaction mixtures contained buffer (0.1 M citric acid and 0.2 M disodium phosphate mixed in various ratios) diluted 1:1 with the rest of the reaction mixture, 5 mM *p*-nitrophenyl α-L-arabinofuranoside and an appropriate amount of enzyme. Assays were performed at 55°C for 5 minutes.

Determination of temperature stability

The temperature stability of the enzyme was investigated by incubating the enzyme in 100 mM acetate buffer pH 4 at 50°C, 55°C and 60°C, taking samples at 15 min intervals and assaying for activity at 55°C for 5 min. Reaction mixtures contained 50 mM acetate buffer pH 4 and 5 mM *p*-nitrophenyl α-L-arabinofuranoside.

Determination of the pH stability

The effect of pH on the stability of the enzyme was investigated by incubating the enzyme in McIlvaine's phosphate-citrate buffer at pH values ranging from 2.2 to 7 and 50°C, taking samples at 15 min intervals and assaying for activity in 200 mM acetate buffer pH 4 and 55°C for 5 minutes.

Investigation of kinetic properties and substrate specificity

Kinetic constants of the enzyme for *p*-nitrophenyl α-L-arabinofuranoside were determined by assaying for activity at pH 4 and 55°C for 5 minutes, using substrate concentrations ranging from 0.2 to 30 mM . A substrate blank was included for each concentration point. Activity against *p*-nitrophenyl-β-D-xylopyranoside (Sigma) was assayed at 10 mM substrate with an identical amount of enzyme for 30 min at 50°C.

Results

Cloning of the *A. pullulans abfA* gene

The *A. pullulans abfA* gene was cloned by PCR methods. Degenerate primers were based on areas of highly conserved amino acid sequence in alignments of known family 54 α-arabinofuranosidases and were used to amplify an internal section of the *A. pullulans abfA* gene (Figure 6.1). The regions flanking this internal section were amplified using inverse PCR. The entire gene was subsequently amplified from genomic DNA and sequenced to confirm the sequence and assembly of the

```
                                                ABFdegF1
T_koningii      =VKRGSDGATTAISPLSSG-VANAAAQDAFCAGTTCLITIIYDQSGRGNHLREAPPGGFSG
P_purpurogenum =IKRGSDGATTTISPLSAGGVANAAAQDTFCANTTCLITIIYDQSGRGNHLTQAPPGGFSG
A_oryzae        =VKRGSDGSTTDIAPLSAGGVADAATQDSFCANTTCLITIIYDQSGRGNHLTQAPPGGFNG
T_reesei        =VKRGSDGATTAISPLSSG-VANAAAQDAFCAGTTCLITIIYDQSGRGNHLTQAPPGGFSG
A_kawachii      =LQRGSDDTTTTISPLTAGGIADASAQDTFCANTTCLITIIYDQSGNGNHLTQAPPGGFDG
A_awamori       =LQRGSDDTTTTISPLTAGGIADASAQDTFCANTTCLITIIYDQSGNGNHLTQAPPGGFDG
A_niger         =LQRGSDDTTTTISPLTAGGIADASAQDTFCANTTCLITIIYDQSGNGNHLTQAPPGGFDG
E_nidulans      =VQRASDGTTTTITPLSAGGVADASAQDAFCENTTCLITIIYDQSGNGNDLTQAPPGGFNG
                 ..* ** .** *.**..* .* *..**.** .************* ** * .****** *
                                ABFdegF2
T_koningii       PESNGYDNLASAIGAPVTLNGQKAYGVFVSPGTGYRNNAASGTAKGDAAEGMYAVLDGTH=
P_purpurogenum   PDTNGYDNLASAIGAPVTLNGQKAYGVFISPGTGYRNNAVSGTATGDAAEGMYAVLDGTH=
A_oryzae         PESNGYDNLASAVGAPVTLNGKKAYGVFMSPGTGYRNNAASGTATGDEAEGMYAVLDGTH=
T_reesei         PESNGYDNLASAIGAPVTLNGQKAYGVFVSPGTGYRNNAASGTAKGDAAEGMYAVLDGTH=
A_kawachii       PDTDGYDNLASAIGAPVTLNGQKAYGVFMSPGTGYRNNEATGTATGDEAEGMYAVLDGTH=
A_awamori        PDTDGYDNLASAIGAPVTLNGQKAYGVFMSPGTGYRNNEATGTATGDEAEGMYAVLDGTH=
A_niger          PDTDGYDNLASAIGAPVTLNGQKAYGVFMSPGTGYRNNEATGTATGDEAEGMYAVLDGTH=
E_nidulans       PDVGGYDNLAGAIGAPVTLNGKKAYGVFVSPGTGYRNNEAIGTATGDEPEGMYAVLDGTH=
                 *.  ****** *.********.******.*********   *** **  ***********
                                                                ABFdegR1
T_koningii      =NNAGDPSISYRFVTAAIKGQPNQWAIRGGNAASGSLSTFYSGARPQ-VSGYNPMSKEGAI
P_purpurogenum =NNAADPSVSYRFLSTIVKGESNQWAIRGGNAVSGSLSTYYSGARPS-ASGYNPMSKEGAI
A_oryzae        =NNAGDPSISYRFVTAVVKGEANQWSIRGANAASGSLSTYYSGARPS-ASGYNPMSKEGAI
T_reesei        =NNAGDPSISYRFVTAAIKGQPNQWAIRGGNAASGSLSTFYSGARPQ-VSGYNPMSKEGAI
A_kawachii      =YNSGDPSISYRFVTAAVKGGADKWAIRGANAASGSLSTYYSGARPD-YSGYNPMSKEGAI
A_awamori       =YNSGDPSISYRFVTAAVKGGADKWAIRGANAASGSLSTYYSGARPD-YSGYNPMSKEGAI
A_niger         =YNSGDPSISYRFVTAAVKGGADKWAIRGANAASGSLSTYYSGARPD-YSGYNPMSKEGAI
E_nidulans      =YNAGDPSISYRFVTAILKGGPNLWALRGGNAASGSLSTYYNGIRPTDASGYNPMSKEGAI
                  *. ***.****... .**    *..** ** ******.* * **   ************
                            ABFdegR2
T_koningii       ILGIGGDNSNGGQGTFYEGVMTSGYPSDATENSVQANIVAARYAVAPLTSGPALTVGSSI=
P_purpurogenum   ILGIGGDNSHGAQGTFYEGVMTSGYPSDATENSVQANIVAAKYAAGSLTSGPALTVGSSI=
A_oryzae         ILGIGGDNSNGAQGTFYEGVMTSGYPSDATENSVQADIVAAKYAIASLTSGPALTVGSSI=
T_reesei         ILGIGGDNSNGAQGTFYEGVMTSGYPSDATENSVQANIVAARYAVAPLTSGPALTVGSSI=
A_kawachii       ILGIGGDNSNGAQGTFYEGVMTSGYPSDDTENSVQENIVAAKYVVGSLVSGPSFTSGEVV=
A_awamori        ILGIGGYNSNGAQGTFYEGVMTSGYPSDDTENSVQENIVAAKYVVGSLVSGPSFTSGEVV=
A_niger          ILGIGGYNSNGAQGTFYEGVMTSGYPSDDTENSVQENIVAAKYVVGSLVSGPSFTSGEVV=
E_nidulans       ILGIGGDNSVSAQGTFYEGAMTDGYPDDATENSVQADIVAAKYATTSLISGPALTVGDTV=
                 ****** **   ******* ** *** * ******  ****.*    * ***. * *  .
```

Figure 6.1. CLUSTAL-W alignment of the several fungal α-arabinofuranosidases. A star indicates all amino acids identical at that position and a full-stop indicates a conservative change at that position. An equal sign indicates discontinuity in the alignment. Areas on which each primer was based are shaded.

overlapping fragments (Figure 6.2). The *A. pullulans abfA* gene consists of an intron-less open reading frame of 1494 nucleotides encoding a protein of 498 amino acids (Figure 6.3). The protein

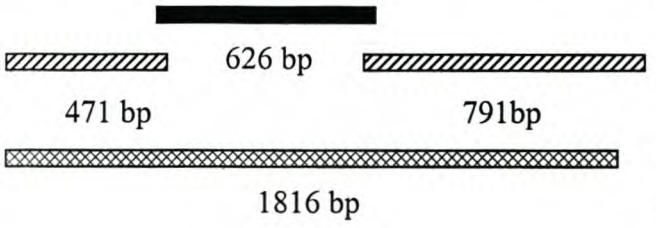

Figure 6.2. PCR amplification of the *A. pullulans abfA* gene. The black bar represents the area amplified from genomic DNA using degenerate primers, the hatched bars represent the areas amplified by inverse PCR and the cross-hatched bar represents the entire open reading frame amplified from genomic DNA with specific primers. The length of each area is shown in bp.

```
-185                                                                gatcc
-180        cgccggattacggaaaataacagagcgagttcgtatgcgatgatcttcgctggagatgtg
-120        ctacatccacagctcgaacataaatagagaagacaatgccgcctggctgtccaacatcaa
-60         ctcctctcatatccgcaagcttcctgtcaaccctcctcacagttcgctcatcactcaaac
  1          ATGCGTTCCAGGACGAACATCGCTCTTGGCCTAGCTGCCACTGGTTCCCTAGTCGCTGCC
  1          M  R  S  R  T  N  I  A  L  G  L  A  A  T  G  S  L  V  A  A
 61         GCGCCTTGCGATATCTATCAGAATGGCGGTACTCCTTGCGTAGCTGCTCACGGCACAACT
 21          A  P  C  D  I  Y  Q  N  G  G  T  P  C  V  A  A  H  G  T  T
121         CGCGCATTGTATGATTCCTACACTGGTCCTCTCTACCAACTTAAGAGAGGCTCAGATGGC
 41          R  A  L  Y  D  S  Y  T  G  P  L  Y  Q  L  K  R  G  S  D  G
181         ACTACGACCGATATTTCTCCTTTGTCTGCTGGTGGTGTTGCCAATGCTGCTGCTCAGGAC
 61          T  T  T  D  I  S  P  L  S  A  G  G  V  A  N  A  A  A  Q  D
241         TCTTTCTGCAAGGGTACTACCTGTCTTATCAGTATTATCTACGATCAGTCTGGGCGTGCA
 81          S  F  C  K  G  T  T  C  L  I  S  I  I  Y  D  Q  S  G  R  A
301         AACCATCTTTATCAGGCCCAGAAAGGTGCTTTCAGCGGACCAGATGTCAACGGAAACGAC
101          N  H  L  Y  Q  A  Q  K  G  A  F  S  G  P  D  V  N  G  N  D
361         AACTTGGCAGGCGCTATTGGAGCACCAGTGACTTTGAATGGCAAGAAGGCATATGGCGTG
121          N  L  A  G  A  I  G  A  P  V  T  L  N  G  K  K  A  Y  G  V
421         TTCATCTCGCCCGGCACTGGGTACAGAAACGACGAAGTCAGCGGCACGGCCACTGGAAAC
141          F  I  S  P  G  T  G  Y  R  N  D  E  V  S  G  T  A  T  G  N
481         GAACCTGAGGGCATGTATGCTGTTCTTGACGGCACTCATTACAACGATGCTTGCTGCTTT
161          E  P  E  G  M  Y  A  V  L  D  G  T  H  Y  N  D  A  C  C  F
541         GACTACGGAAACGCGGAAATCAGCAACACGGATACTGGTAACGGACATATGGAGGCCGTC
181          D  Y  G  N  A  E  I  S  N  T  D  T  G  N  G  H  M  E  A  V
601         TACTATGGTAACAACACGATTTGGGGCAGTGGCTCTGGCAGCGGTCCTTGGCTCATGGCC
201          Y  Y  G  N  N  T  I  W  G  S  G  S  G  S  G  P  W  L  M  A
661         GACCTTGAGAACGGTTTGTTCTCTGGCCAGGGTACCAAGCAGAACACTGCAGACCCTTCA
221          D  L  E  N  G  L  F  S  G  Q  G  T  K  Q  N  T  A  D  P  S
721         ATCTCCAACAGATTCTTCACCGGAATGGTCAAGGGAGAGCCTAACCAGTGGGCGCTTCGC
241          I  S  N  R  F  F  T  G  M  V  K  G  E  P  N  Q  W  A  L  R
781         GGTAGCAATGCCGCGTCCGGTTCCTTGTCGACCTACTACAGTGGCGCTCGTCCCACCGTC
261          G  S  N  A  A  S  G  S  L  S  T  Y  Y  S  G  A  R  P  T  V
841         GGCGGTTACAACCCCATGAGCCTCGAGGGCGCCATCATTCTTGGCATCGGTGGCGATAAC
281          G  G  Y  N  P  M  S  L  E  G  A  I  I  L  G  I  G  G  D  N
901         AGCAATGGCGCTCAGGGCACTTTCTATGAGGGGGTCATGACCTCGGGCTACCCGTCTGAT
301          S  N  G  A  Q  G  T  F  Y  E  G  V  M  T  S  G  Y  P  S  D
961         GCCACTGAAGCCTCGGTGCAGGCCAACATTGTGGCTGCGAAGTACGCTACCACATCTTTG
321          A  T  E  A  S  V  Q  A  N  I  V  A  A  K  Y  A  T  T  S  L
1021        AACACAGCACCACTCACTGTCGGCAACAAGATTTCGATCAAGGTGACCACCCCCGGCTAC
341          N  T  A  P  L  T  V  G  N  K  I  S  I  K  V  T  T  P  G  Y
1081        GACACCCGCTATCTGGCACACACCGGAGCCACCGTCAACACGCAGGTTGTCTCTTCATCT
361          D  T  R  Y  L  A  H  T  G  A  T  V  N  T  Q  V  V  S  S  S
1141        AGCGCGACTAGCCTCAAGCAGCAGGCCAGCTGGACTGTTCGCACAGGCCTCGGTAACAGC
381          S  A  T  S  L  K  Q  Q  A  S  W  T  V  R  T  G  L  G  N  S
1201        GGCTGTTACTCTTTCGAGTCGGTTGATACACCTGGAAGCTTCATCAGACACTACAACTTC
401          G  C  Y  S  F  E  S  V  D  T  P  G  S  F  I  R  H  Y  N  F
1261        CAGCTCCAGCTCAACGCGAATGACAACACCAAGGCTTTCAAGGAAGACGCGACTTTCTGC
421          Q  L  Q  L  N  A  N  D  N  T  K  A  F  K  E  D  A  T  F  C
1321        TCTCAGACCGGTCTTGTTACCGGCAACACTTTCAACTCGTGGAGCTACCCTGCCAAGTTC
441          S  Q  T  G  L  V  T  G  N  T  F  N  S  W  S  Y  P  A  K  F
1381        ATCCGTCACTACAACAATGTTGGATACATCGCCAGCAACGGTGGTGTTCACGACTTTGAC
461          I  R  H  Y  N  N  V  G  Y  I  A  S  N  G  G  V  H  D  F  D
1441        TCTGCTACAGGCTTCAACAACGATGTCAGCTTTGTGGTTGGAAGCAGCTTTGCTTAGatg
481          S  A  T  G  F  N  N  D  V  S  F  V  V  G  S  S  F  A  *
1501        taaaaggtcaggatgaatatgatggatgtttatgacaaaagaagttatgagtttgtagtt
1561        atggaatcttagctgtagcttttgaaagcctttgggatatcagatgtttgtctcttgttc
1621        atgtgccgttgcaaagaagaaaagaaggagcagcaagcagtgaggctcttatcgggcgat
1681        agggctagatc
```

Figure 6.3. Nucleotide sequence of the *A. pullulans abfA* gene with the deduced α-arabinofuranosidase amino acid sequence in bold. Coding sequence is shown as capitals and non-coding sequence as small letters. The putative signal peptide is underlined. The N-linked glycosylation consensus sequence is dotted underlined.

contains a putative secretion signal of 20 amino acids (von Heijne, 1986), resulting in a mature protein with a predicted molecular weight of 49.9 kDa and a calculated iso-electric point of 4.97. One possible *N*-glycosylation site was found in the deduced amino acid sequence of the *A. pullulans* α-arabinofuranosidase (Gavel and von Heijne, 1990). Based on amino acid sequence, AbfA is a member of family 54 of the glycoside hydrolases in the Carbohydrate Active enZYmes classification system (http://afmb.cnrs-mrs.fr/CAZY/) and its amino acid sequence shares between 69% and 76% identity with fungal α-arabinofuranosidases in this family. Construction of a rooted tree based on amino acid sequences showed that the *A. pullulans* α-arabinofuranosidase did not form part of any grouping in the tree, although there is a very high overall sequence similarity between these enzymes (Figure 54).

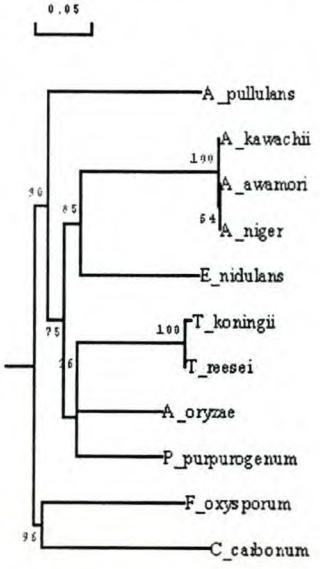

Figure 6.4. Rooted tree showing the phylogenetic relationships between the *A. pullulans* α-arabinofuranosidase and its family 54 homologues. The tree was constructed on the basis of amino acid sequences. The values represent the percentage of 1000 bootstraps and the scale bar corresponds to 0.05 amino acid substitutions per site. Sequences used: *Aureobasidium pullulans* (this work), *Aspergillus kawachii* (BAB96816), *Aspergillus awamori* (BAB21567), *Aspergillus niger* (CAA01903), *Emericella nidulans* (CAA74084), *Trichoderma koningii* (AAA81024), *Trichoderma reesei* (AAP57750), *Aspergillus oryzae* (BAB71803), *Penicillium purpurogenum* (AAK51551), *Fusarium oxysporum* (CAD90582) and *Cochliolobus carbonum* (AAG42253).

Expression of the *A. pullulans abfA* gene in *S. cerevisiae* Y294

The *A. pullulans abfA* gene was expressed in *S. cerevisiae* Y294 on the episomal vector pDLG1, under control of the metabolically induced *ADH2* promoter and terminator (Figure 6.5). Two constructs of

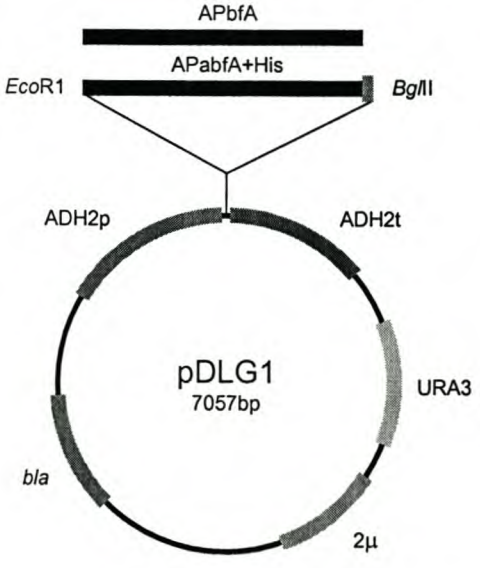

Figure 6.5. Schematic representation of the construction of the expression vectors pDLG1-APabfA (top bar) and pDLG1-APabfA+His (bottom bar). Key features of the base vector pDLG1 (circle) and their relative positions are highlighted.

the expression vector were made, the first encoding the native enzyme and the second coding for an additional *C*-terminal hexahistidine tag. In a trial experiment α-arabinofuranosidase activity was detectable in the culture supernatant of *S. cerevisiae* Y294[pDLG1-APabfA], expressing the enzyme lacking the hexahistidine tag, but not in the culture supernatant of *S. cerevisiae* Y294[pDLG1-APabfA+His], expressing the enzyme containing the hexahistidine tag. Production of α-arabinofuranosidase activity as well as cell-density was followed over time in defined medium (Figure 6.6A & B). In the strain producing the hexahistidine-containing enzyme, cell-associated activity was measured, and in the strain producing the unmodified enzyme, activity was measured in the supernatant. α-Arabinofuranosidase activity was accumulated at a similar rate in the supernatant and the cell-associated fractions of *S. cerevisiae* Y294[pDLG1-APabfA] and *S. cerevisiae* Y294[pDLG1-APabfA+His], respectively, and also reached a similar level of about 25 mU/ml after 7 days of cultivation. Activity was detectable after 3 days of cultivation, and the lag in production of the enzyme is the result of the induction of the *ADH2* promoter by glucose depletion and the accumulation of ethanol. The location of the hexahistidine-tagged α-arabinofuranosidase was most likely intracellular, as spheroplasting of the yeast cells did not release any activity. Activity was only

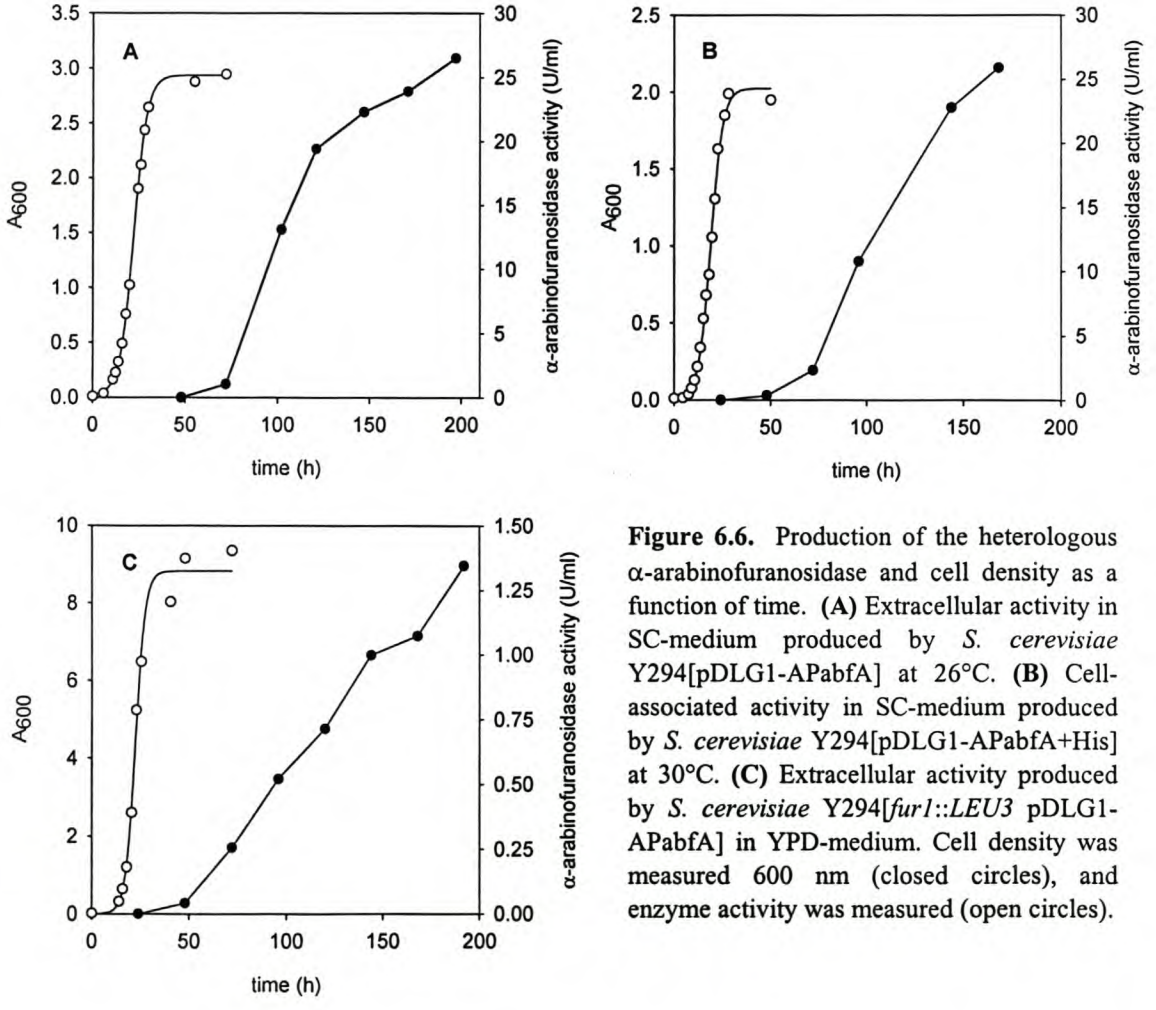

Figure 6.6. Production of the heterologous α-arabinofuranosidase and cell density as a function of time. **(A)** Extracellular activity in SC-medium produced by *S. cerevisiae* Y294[pDLG1-APabfA] at 26°C. **(B)** Cell-associated activity in SC-medium produced by *S. cerevisiae* Y294[pDLG1-APabfA+His] at 30°C. **(C)** Extracellular activity produced by *S. cerevisiae* Y294[*fur1*::*LEU3* pDLG1-APabfA] in YPD-medium. Cell density was measured 600 nm (closed circles), and enzyme activity was measured (open circles).

released upon subsequent glass-bead extraction. *S. cerevisiae* Y294[pDLG1-APabfA] was cultivated at 26°C, as the yield of the heterologous enzyme was inconsistent at 30°C, dropping from the normal levels after 8 days of cultivation of about 25 mU/ml to less than 2 mU/ml in separate experiments. The production of α-arabinofuranosidase activity by the autoselective strain *S. cerevisiae* Y294[*fur1*::*LEU3* pDLG1-APabfA], was studied in rich medium (Figure 6.6C). Cell density reached threefold higher levels at stationary phase, while α-arabinofuranosidase activity was more than fifty-fold higher after 8 days of cultivation. The levels of α-arabinofuranosidase activity produced in defined medium and rich medium corresponded to 1.25 mg/L protein and 67 mg/L protein, respectively.

Purification of the heterologous α-arabinofuranosidase from the culture supernatant of *S. cerevisiae* Y294[pDLG1-APabfA]

The heterologous α-arabinofuranosidase was purified from the concentrated supernatants of *S. cerevisiae* Y294[pDLG1-APabfA] by a single gel-filtration step (Figure 6.7A). The enzyme eluted at a molecular weight of 36 kDa upon gel filtration. On SDS-PAGE, an additional band with an apparent mobility of 49 kDa was visible in the supernatants of the enzyme-producing strain compared to the plasmid control strain and this band corresponded to the fractions containing α-arabinofuranosidase activity (Figure 6.7B). The enzyme was purified 7.2-fold with a 25.6% yield.

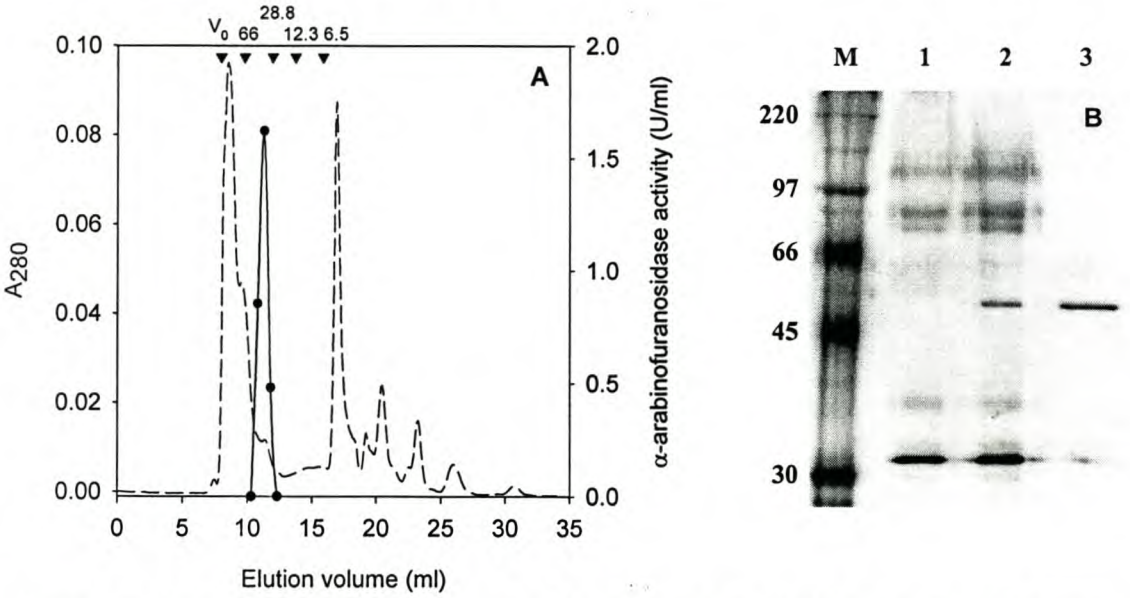

Figure 6.7. Purification of the heterologous α-arabinofuranosidase. **(A)** Elution profile of the concentrated supernatant of *S. cerevisiae* Y294[pDLG1-APabfA] on Superdex 75 HR 10/30. Absorbance at 280 nm is shown as a stippled line and α-arabinofuranosidase activity is shown as a solid line. Elution volumes of molecular weight standards indicated by triangles and molecular weights indicated in kDa (Bovine serum albumin, 66 kDa; carbonic anhydrase, 28.8 kDa; cytochrome C, 12.3 kDa and aprotin, 6.5 kDa). V_0 denotes the void volume. **(B)** SDS-PAGE of the concentrated supernatant of the plasmid control strain *S. cerevisiae* Y294[pDLG1] (lane 1), the α-arabinofuranosidase producing strain *S. cerevisiae* Y294[pDLG-APabfA] (lane 2), and the purified α-arabinofuranosidase (lane 3). The molecular weight marker lane is marked M, and molecular weights are given in kDa.

Characterisation of the purified heterologous α-arabinofuranosidase

The purified heterologous α-arabinofuranosidase was characterized in terms of its biochemical properties. The enzyme displayed maximal activity at 55°C (Figure 6.7) and between pH 3.5 and pH 4 under the assay conditions (Figure 6.7B). Activity was stable for up to 2 h at 50°C and activity was lost slowly at 55°C with a half-life of 145 min (Figure 6.7C). At 60°C the enzyme was rapidly inactivated and had a half-life of 7.8 min (Figure 6.7D). The enzyme was stable at pH 4 and pH 5 for a period of 2 hours, it had a half-life of 29 min at pH 3 and was completely inactivated within 15

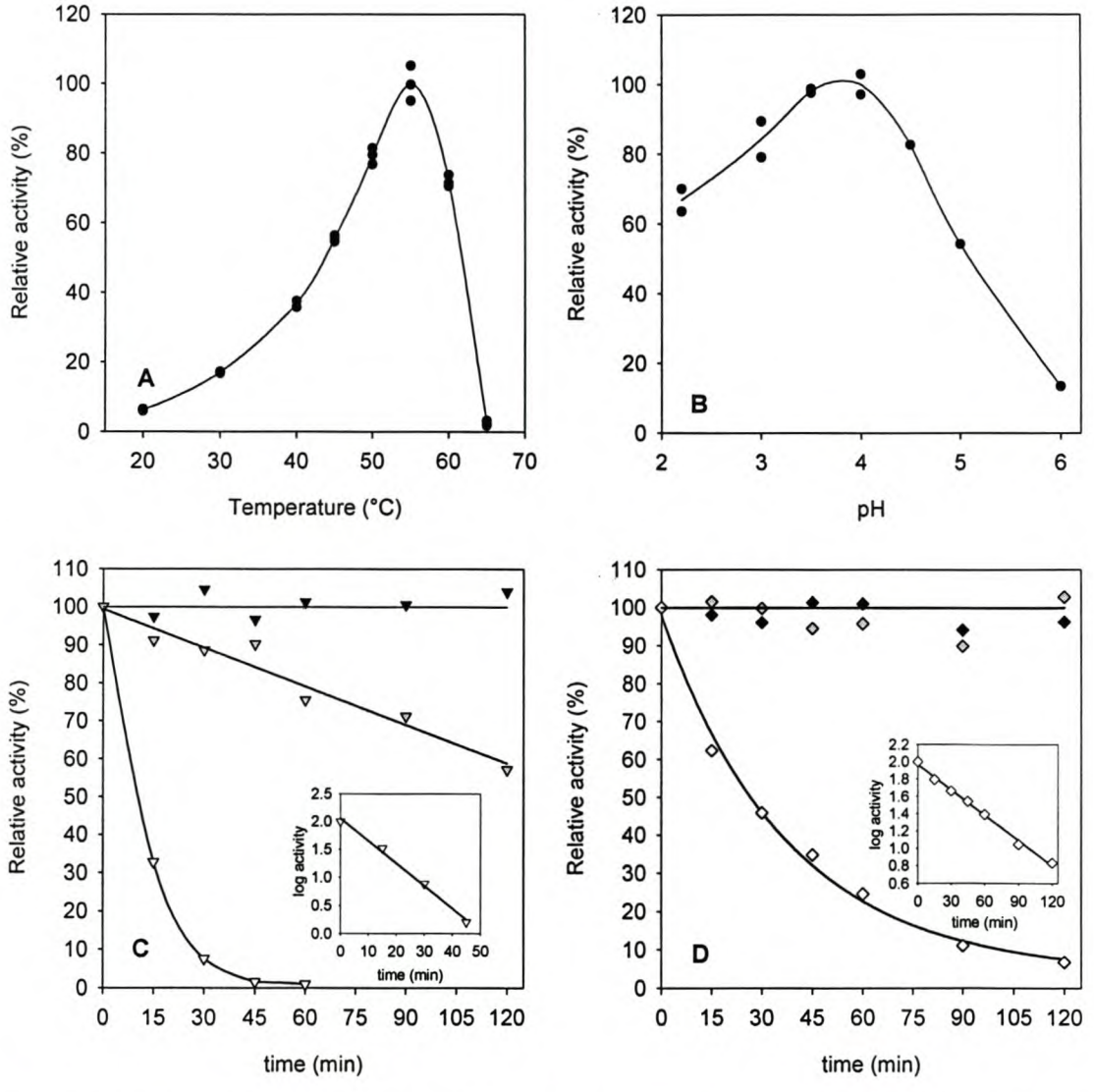

Figure 6.7. Physical properties of the purified α-arabinofuranosidase. **(A)** Temperature dependence of α-arabinofuranosidase activity. Assays were performed for 5 min in 50 mM acetate buffer pH 4. **(B)** pH dependence α-arabinofuranosidase activity. Assays were performed for 5 min at 55°C, using McIlvaine's phosphate-citrate buffer. Replicates within the same experiment are shown. **(C)** Temperature stability of the α-arabinofuranosidase activity. Black triangles, 50°C; grey triangles, 55°C; white triangles 60°C. **(D)** pH stability of α-arabinofuranosidase activity. Black diamonds, pH 4; grey diamonds, pH5; white diamonds, pH 6. Activity is given as a percentage of the maximum of 8 mU. The inserts show semi-log plots used to calculate half-lives.

minutes at pH 2.2, pH 6 and pH 7 (Figure 6.8). The enzyme had a K_m of 3.7 ± 0.36 mM for *p*-nitrophenyl α-L-arabinofuranoside and a V_{max} of 34.8 ± 1.1 U/mg protein at pH 4 and 55°C (Figure 6.8). The turnover number, k_{cat}, was calculated as 29 s^{-1} and the catalytic efficiency (k_{cat}/K_m) as 7.84 $s^{-1}mM^{-1}$. The enzyme displayed trace β-xylosidase activity and activity against 10 mM *p*-nitrophenyl β-D-xylopyranoside was 0.2 U/mg under identical reaction conditions.

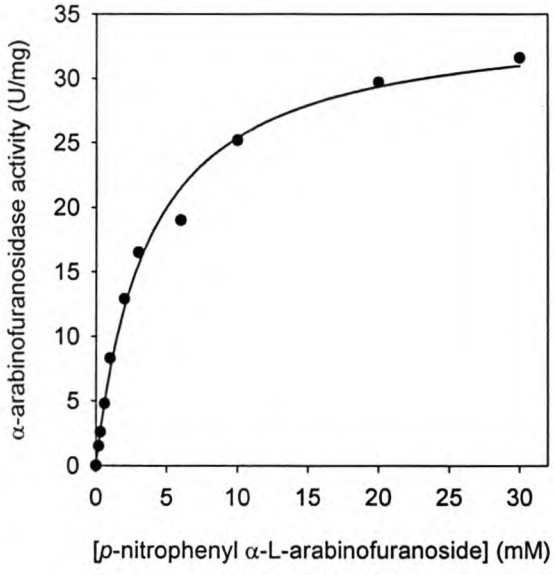

Figure 6.8. α-Arabinofuranosidase activity as a function of *p*-nitrophenyl α-L-arabinofuranoside concentration. Assays were performed for 5 minutes at 55°C and pH 4, and data fitted with the Michaelis-Menten equation, $v = V_{max}[S]/(K_m + [S])$. The K_m for the substrate was calculated as 3.7 ± 0.36 mM and V_{max} as 34.8 ± 1.1 U/mg protein.

Discussion

α-L-Arabinofuranosidase is an essential enzyme in the biodegradation of lignocellulosic material thereby playing an important role in the carbon cycle. A new glycoside hydrolase family 54 α-arabinofuranosidase gene has been cloned form the xylanase-overproducing strain of the euascomycetous fungus *A. pullulans* NRRL Y-2311-1, expressed in *S. cerevisiae* and the heterologous enzyme characterised. The *A. pullulans abfA* gene is highly conserved and shared between 69% and 76% sequence identity with other members of family 54. Members of this family are highly conserved in terms of primary sequence as well as their enzymatic properties. The *A. pullulans abfA* gene was cloned by homology based methods, using the sequences of known family 54 enzymes to design degenerate primers for PCR. The methodology employed to clone this gene therefore biased the process to discovering another member of this family.

S. cerevisiae has been used as a host for the production of several glycoside hydrolases (Romanos *et al.*, 1992) and was a good candidate for the heterologous expression of the *A. pullulans abfA* gene. The *A. pullulans* NRRL Y-2311-1 *endo*-β-1,4-xylanase gene, *xynA*, had been successfully expressed in baker's yeast, and the native secretion signal of the XynAp was recognised by *S. cerevisiae*, and the heterologous enzyme secreted into the culture medium (Li and Ljungdahl, 1996). Furthermore, the genes encoding two family 54 α-arabinofuranosidases, from *A. niger* (Crous *et al.*, 1996) and *T. reesei*

(Margolles-Clark *et al.*, 1996) had been expressed in *S. cerevisiae* and found to be effectively secreted. The high degree of sequence identity of the *A. pullulans* AbfAp with the other family 54 enzymes expressed in *S. cerevisiae* and the fact that an *A. pullulans* glycoside hydrolase gene had already been expressed in *S. cerevisiae* made it likely that this approach would be successful.

The heterologous α-arabinofuranosidase lacking the *C*-terminal hexahistidine tag was secreted into the culture medium, indicating that the native *A. pullulans* secretion signal was recognised by the post-translational processing machinery of *S. cerevisiae*. The *A. pullulans* α-arabinofuranosidase was produced at similar levels as the *A. niger* enzyme by *S. cerevisiae* in defined medium and rich medium (around 0.025 U/ml and 1.3 U/ml, respectively), although the former was expressed under the metabolically-induced *ADH2* promoter and the latter under the constitutive *PGK1* promoter (Crous *et al.*, 1996). The *A. pullulans* enzyme was produced in *S. cerevisiae* cultivated at 26°C, instead of 30°C, as was the case for the *A. niger* enzyme, as yields were inconsistent at the higher growth-temperature. This effect had been observed before in strains of *S. cerevisiae* expressing foreign proteins (Setati *et al.*, 2001). It is likely due to an overtaxing of the yeast cells that are already growing at their maximal rate by the additional stress of producing a foreign protein. The more than fifty-fold higher yield of in spite of an only threefold increase in cell density in rich medium compared to defined medium suggests that the availability of nutrients plays an important role in the production of the heterologous α-arabinofuranosidase.

The addition of a *C*-terminal hexahistidine-tag had a negative effect on the secretion of the enzyme into the culture medium. A very similar overall level of enzyme activity was produced by strains producing the His-tag containing enzyme, although no activity was detected in the supernatant of the strain producing the his-tagged protein after 5 days of cultivation. The location of the His-tag containing enzyme was most likely intracellular, as spheroplasting of the yeast cells did not release any activity into the medium. The charged nature of the hexahistidine tag could potentially interfere with the proper folding of the protein and its processing along the secretory pathway. The poor secretion of the His-tag containing α-arabinofuranosidase, combined with the lack of stability of the enzyme above pH 5, excluded Ni-chelate affinity chromatography as a convenient technique for purification of the heterologous α-arabinofuranosidase.

The unmodified α-arabinofuranosidase could conveniently be purified from the supernatant by gel filtration, due to the fact that *S. cerevisiae* secretes very few proteins when cultured in defined medium. A single gel filtration step on Superdex 75 was sufficient to purify the heterologous enzyme to homogeneity (Figures 6.5 & 5.6). The yield of the pure enzyme was 25.6% and it was purified 7.2-fold from the culture supernatant. Despite the much higher yields of the unmodified enzyme in rich

medium, the rapid purification of the enzyme by gel filtration was hindered by the presence of additional proteins in the medium. The purified enzyme had an apparent mobility of 49 kDa on SDS-PAGE compared to the predicted molecular weight of 49.9 kDa for the mature protein. Enzyme activity eluted at 36 kDa upon gel filtration on Superdex 75, indicating that it had a monomeric quaternary structure. Only one potential asparagine-linked secretion signal was found in the deduced amino acid sequence of the *A. pullulans* α-arabinofuranosidase, making it unlikely that the heterologous enzyme is at all glycosylated. Both *A. pullulans* and *S. cerevisiae* are known to extensively glycosylate proteins. The fact that the heterologous enzyme does not appear to be glycosylated could be due to lack of accessibility of the potential glycosylation site.

In terms of biochemical properties, the *A. pullulans* α-arabinofuranosidase is very similar to other family 54 enzymes. Maximal activity was observed at 55°C compared to values of around 60°C for the Aspergilli and 50°C for the *P. purpurogenum* Abf1p (Carvallo *et al.*, 2003; Saha, 2000). The *A. pullulans* α-arabinofuranosidase displayed maximal activity between pH 3.5 and pH 4 compared to values of pH 3.5 to pH 5.0 for most of the family 54 enzymes (Saha, 2000) . The *A. pullulans* α-arabinofuranosidase was only tested on synthetic aryl glycosides (Saha, 2000). It had a K_m for *p*-nitrophenyl α-L-arabinofuranoside of 3.7 mM, which is in line with K_m-values of other family 54 enzymes for this substrate in the millimolar range. It also displayed trace activity against *p*-nitrophenyl β-D-xylopyranoside, as was reported for the *T. reesei* (Margolles-Clark *et al.*, 1996) and *P. purpurogenum* enzymes (Carvallo *et al.*, 2003).

α-Arabinofuranosidase activity in the culture supernatant of *A. pullulans* NRRL Y-2311-1 was reported to have maximal activity at 60°C and pH 5, and to be stable at 60°C and pH 6 (Myburgh *et al.*, 1991b). This differs quite markedly from what was found for the heterologous enzyme, which showed maximal activity at 55°C and pH 3.5 to pH 4, and was stable at 50°C and between pH 4 and pH 5. It is possible that the behaviour of the enzyme in the mixture of proteins in the culture supernatant of *A. pullulans* is quite different from the pure enzyme on its own. It is also likely that *A. pullulans*, like *A. niger* may produce more enzymes from more than one family, that are all capable of hydrolysing *p*-nitrophenyl α-L-arabinofuranoside (Flipphi *et al.*, 1993), and that the properties observed in the crude supernatant are therefore the compound characteristics of several enzymes. Different glycosylation patterns in *S. cerevisiae* and *A. pullulans* may also contribute to differences between the native and heterologous enzyme. A thermostable α-arabinofuranosidase with a temperature optimum of 75°C was previously isolated from another colour-variant strain of *A. pullulans*, NRRL Y-12974 (Saha and Bothast, 1998). Whether this enzyme is a member of family 54 is debatable, however, as it shows a dimeric quaternary structure, with monomeric molecular weights

of 105 kDa and was unable to release arabinofuranose from arabinogalactan. No sequence information is available about this enzyme, making it difficult to establish this with certainty.

This study presents the first report of an α-arabinofuranosidase gene from the black yeast *A. pullulans*, and as such adds to our current knowledge of both family 54 glycoside hydrolases as well as the lignocellulolytic enzyme system of *A. pullulans*. The only lignocellulolytic gene that has been cloned from *A. pullulans* is *xynA*, encoding the main *endo*-β-1,4-xylanase (Li and Ljungdahl, 1994). Further investigation of the substrate specificity of the enzyme will reveal if the highly conserved primary sequence of members of this family also results in very similar substrate specificity. This should include testing for activity on arabinan, arabinogalactan and arabinoxylan, as well as short oligosaccharides derived from these polymers. This information will shed light on the substrate range of the enzyme as well as its preference for the kind of glycosidic linkage the arabinofuranosyl moiety is involved in. Sequencing of upstream areas of the *abfA* gene are also likely to reveal regulatory elements that may be involved in the control of gene expression, as very little is currently known about the control of expression of xylanolytic genes in *A. pullulans*.

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Chapter 7

General discussion and conclusion

Introduction

A. pullulans is an euascomycetous fungus that has proven useful in a variety of different industrial applications. The colour-variant strain of *A. pullulans*, NRRL Y-2311-1, was originally isolated for high-level *endo*-β-1,4-xylanase production, and this enzyme has also been purified and characterized and its gene cloned, and expressed in *S. cerevisiae*. The presence of β-xylosidase, α-arabinofuranosidase and *p*NP-acetyl esterase activity has also been noted in the culture supernatants of *A. pullulans* NRRL Y-2311-1 cultivated on xylan. Little further work has been done to study the xylanolytic enzyme system of this fungus. The work undertaken in this thesis endeavoured to gain more insight into the xylanolytic enzyme system of this fungus by investigating accessory enzyme activity, specifically α-glucuronidase and α-arabinofuranosidase. In addition, it attempted to expand the basic knowledge on both α-glucuronidase and α-arabinofuranosidase activity. Information about the xylanolytic accessory enzymes, in general, is still lacking in comparison to what is known about the main-chain cleaving *endo*-β-1,4-xylanases.

Purification of an α-glucuronidase from the supernatant of *A. pullulans* NRRL Y-2311-1

The initial focus of the work for this thesis was on the purification of an α-glucuronidase from the culture supernatant of *A. pullulans* NRRL Y-2311-1. *A. pullulans* had not previously been shown to secrete α-glucuronidase activity, although it was reasonable to expect it produce the whole spectrum of xylanolytic enzymes (Myburgh *et al.*, 1991). It was interesting to note that *A. pullulans* secreted α-glucuronidase activity, only when it was cultivated on carbon sources containing both glucuronic acid and xylose, but not either alone (Figure 4.1). This would imply the presence of genetic control elements responsive to the presence of both sugars. The control of expression of lignocellulolytic enzymes may vary widely between even related fungi, and most likely depends a great deal on their individual lifestyles. Transcription of the *A. niger aguA* gene was induced by the presence of xylose or xylan and induction was found to be mediated through the xylanolytic transcriptional activator XlnR (de Vries *et al.*, 2002). A second XlnR-independent element, sensitive to the presence of glucuronic acid or galacturonic acid was later described (de Vries, 2003).

The *A. pullulans* NRRL Y-2311-1 α-glucuronidase could only be partially purified from the culture supernatant using standard chromatography techniques (Figures 4.3 & 4.4). A very high degree of non-uniform behaviour of most of the enzyme activities tested, and especially the α- glucuronidase

was noted in all chromatography steps. This is, at least partly, explained by the tendency of *A. pullulans* to glycosylate its secreted proteins very heavily, and a high degree of glycosylation was also noted after treatment of the partially purified protein by *N*-glycosidase F (Figure 4.7). In terms of physical and enzymatic properties, the *A. pullulans* α-glucuronidase is very similar to other fungal α-glucuronidases (Table 3.1).

One property of the enzyme that has not been observed in other α-glucuronidases before is the occurrence of substrate inhibition (Figure 4.6). Repeat experiments unambiguously indicated that this observation was not an artifact due to variability in the assay procedure itself. Fitting of equations describing normal Michaelis-Menten kinetics also clearly showed this equation did not correctly describe the behaviour of the enzyme in response to increasing substrate concentration. Substrate inhibition has been shown for family 10 *endo*-β-1,4-xylanases, and it is possible that this is a common feature of glycoside hydrolases (Honda *et al.*, 2002). The active-site architecture of the *P. cellulosa* GlcA67A, the only α-glucuronidase with a resolved crystal structure, provides further clues as to how substrate inhibition might occur (Nurizzo *et al.*, 2002). The substrate binding site consists of a deep cleft, with the 4-*O*-methyl glucuronosyl binding site in the deepest part of this cavity, requiring the ordered and sequential release of first the xylo-oligosaccharide portion of the product, followed by the 4-*O*-methyl glucuronic acid. Binding of a second substrate molecule to the xylopyranosyl binding-site in the enzyme before the release of the 4-*O*-methyl glucuronic acid, essentially inactivating the enzyme. The high K_m-values of the enzyme for its substrates coupled to a relatively low turnover number, could potentially increase the likelihood of such complexes occurring at high substrate concentration.

Cloning of the *A. pullulans* NRRL Y-2311-1 *aguA* and *abfA* genes

The cloning of genes encoding the xylanolytic accessory enzymes was based on areas of conserved sequence identity between sequenced enzymes and the *A. pullulans* homologues. It was therefore expected that the *aguA* gene would be a family 67 glycoside hydrolase and that the *abfA* gene would be a family 54 glycoside hydrolase, as sequences from the corresponding families were used to design the degenerate primers for amplification of portions of each gene. This serves as a confirmation that *A. pullulans* carries a set of genes very similar to that of other fungi in order to degrade xylan. These methods, however, do not give any indication of the possibility of other novel xylan-degrading enzyme activities produced by *A. pullulans*.

One point worth expanding on concerns the design of degenerate primers for the PCR amplification of closely related homologues genes. Amplification of the *aguA* gene was attempted by a very straightforward, and perhaps naive strategy. Primers were simply based on areas of highly conserved

nucleotide sequence in alignments of known fungal α-glucuronidase genes. Bacterial enzymes were excluded because of the lower sequence identity between the fungal and bacterial enzymes. It was fortuitous that there were sufficiently long stretches of almost completely conserved nucleotide sequence to allow for the design of degenerate primers. A more sophisticated approach was followed in the design of the primers for the *abfA* gene. Primers were designed following the consensus-degenerate hybrid oligonucleotide primer (CODEHOP) strategy (Rose *et al.*, 1998). The 5'-end of the primer, that is more tolerant to mismatches, is invariant and designed using the most frequently used codon for the particular amino acid in that organism. The 3'-end which needs to be an exact match for efficient binding and extension is allowed to vary. This reduces the degeneracy of the primer and increases the chances of successfully amplifying the correct product. Both approaches were successful in amplifying the respective genes. Out of the four possible combinations of the four primers for the amplification of the *abfA* gene, three gave the correct product. PCR with the more specific *abfA* primers also gave a significantly smaller number of amplification products than the *aguA* primers, reducing the number of false positives that needed to be screened by sequencing. The CODEHOP approach therefore seems to be a good starting point in the amplification of closely related homologous sequences, but does not guarantee success. The same approach was used in an attempt to amplify the *A. pullulans* β-xylosidase gene, but failed, in spite of several attempts using different primer combinations, amplification conditions and nested PCR.

The use of inverse PCR to amplify the regions flanking the known internal sequences was particularly effective in obtaining the remaining parts of the open reading frames. It is a technique that is a little more than 10 years old and has been used for a variety of PCR applications, including the study of transposable elements (Triglia *et al.*, 1988; Ochman *et al.*, 1988). Part of the success can be attributed to the use of partial digestion and size-selection of the digests, before the ligation step. Partial digestion with a frequently-cutting restriction enzyme such as *Sau*3AI leads to the effective generation of a large collection of smaller DNA molecules, many of which would be of appropriate size for PCR amplification, once self-ligated. Digestion with enzymes with 6-bp recognition sequences theoretically cut once every 4096 bp (4^6), and as such generate molecules that are too large to easily amplify once self-ligated. Size-selection of the digests, by excision of the appropriately sized region from agarose gels, ensured that potential templates that were too small to yield significant amounts of sequence or too large to amplify efficiently were excluded. These two populations of potential template molecules would have put a drain on the resources in the subsequent PCR reactions and would have decreased the efficiency of amplification of the desired templates. The only disadvantage of this method is that it results in many differently sized potential circular templates and that from these, many PCR products are generated that have to be sequenced to obtain coverage of the entire open reading frame. The many overlapping sequences obtained in this way did, however, give greater confidence in the final assembly of the putative open reading frame. The 3'-most 300 bp of the *aguA*

gene were not part of any of the inverse PCR products and were easily and effectively amplified from cDNA using a specific forward primer and an oligo(dT) reverse primer. By doing so the 3'-end of the mRNA molecule as well as the poly-adenylation site were also identified.

The correct assembly of the putative open reading frames as well as the correct sequence of each of the genes were confirmed by amplifying each putative open reading frame with a set of specific primers from genomic DNA. High-fidelity DNA polymerase was used in the PCR reactions, and the products of three separate PCR reactions were individually cloned and sequenced. In both cases, all three of the sequences obtained from the whole open reading frame were identical, and only a few single base-pair differences were observed between the assembled sequence and sequence obtained from the whole open reading frame, and these were most likely the result of *Taq* polymerase errors in the initial PCR reactions.

Unfortunately, in both cases, little upstream sequence was obtained by inverse PCR, and only a putative TATA-like element could be found in the 120 bp upstream of the aguA gene, while no definite upstream regulatory sequences were discovered in the 185 bp upstream of the *abfA* gene. More information on the upstream sequences can be gained by using further inverse PCR with a new set of primers, located more closely to the translation start site or by using part of the gene to probe a genomic DNA library. Very little is known about the genetic elements involved in the transcriptional regulation of the *A. pullulans* xylanolytic enzyme genes, and so far only the presence of CreA consensus binding sequences has been noted in the upstream sequence of the *A. pullulans* var. *melanigenum xynA* gene (Ohta *et al.*, 2001). Investigations of this nature will almost certainly yield novel and interesting data. Northern blot analysis of the transcription levels of both genes in response to growth of the fungus on different carbon sources is also likely to reveal interesting information about the control of these genes, and is probably also worthwhile pursuing. Much more specific information can be gained by Northern analysis than by enzyme assay alone, although these analyses will most likely yield similar trends. It is also noteworthy that neither of the two cloned genes contained any introns, in contrast to the *A. pullulans xynA* gene, the only other sequenced xylanolytic gene from this fungus, which contains a single 59-bp intron (Li and Ljungdahl, 1994).

Expression of the *aguA* and *abfA* genes in *S. cerevisiae* Y294 and purification of the heterologous α-glucuronidase and α-arabinofuranosidase

The *A. pullulans aguA* and *abfA* genes were expressed in *S. cerevisiae* Y294 in the multicopy episomal plasmid vector pDLG1 under control of the derepressable *ADH2* promoter and terminator (Figures 5.6 & 6.5). This vector had been previously constructed and successfully used for the expression of glycoside hydrolase genes in *S. cerevisiae*, and efficient transcription of genes in this vector had been shown (la Grange *et al.*, 1996). Repression of the *ADH2* promoter by glucose in the medium allows

for the efficient growth of the yeast cultures to stationary phase before the strain of producing the heterologous protein is imposed on the yeast cells. The codon bias indices were calculated as 0.301 and 0.208 for the *abfA* gene and the *aguA* gene, respectively, and these values were sufficiently high to assume that tRNA's would not be limiting during production of the heterologous enzymes (Hadfield *et al.*, 1993). Production of both enzymes was inconsistent at 30°C, which is the optimal growth temperature for baker's yeast. Cultivation at 26°C led to more consistent yields of both enzymes, and was probably due less metabolic strain on the yeast at the lower temperature, resulting in the greater availability of resources for the production of the heterologous protein (Setati *et al.*, 2001).

It has been previously shown that the native secretion signal encoded by the *A. pullulans xynA* gene was recognized by *S. cerevisiae* and that the *endo*-β-1,4-xylanase was efficiently secreted into the culture medium (Li and Ljungdahl, 1996). The putative secretion signals of both enzymes contained basic amino acids at the *N*-terminal end, a hydrophobic core, and some polar amino acids towards the *C*-terminal end, and thus conformed to the rules for signal sequences (von Heijne, 1986). Both enzymes were secreted by *S. cerevisiae* and it is probable that the native secretion signals were recognized and correctly processed (Figures 4.5 & 5.4). Whether or not the cleavage sites for the enzymes in *S. cerevisiae* were identical to the cleavage sites in *A. pullulans* can only be resolved if *N*-terminal amino acid sequencing is performed on both the heterologous and the purified enzymes. A second translation start site, 45 bp upstream of the presumed ATG, was found in the sequence of the *aguA* gene. Initiation of translation at this start site leads to the addition of 15 amino acids, as well as deviation of the signal sequence from the consensus. The α-glucuronidase containing the additional 15 amino acids was, nonetheless, successfully produced by *S.* cerevisiae, albeit at a lower level. The possibility of a physiological role for this feature in *A. pullulans*, such as a cell associated or membrane-bound variant of the protein, cannot be excluded. Addition of an *N*-terminal hexahistidine tag interfered with secretion of the heterologous α-arabinofuranosidase, and all of the enzyme activity remained cell associated. It is possible that the charged nature of the extra histidine residues somehow interferes with correct processing of the heterologous protein along the secretory pathway. The instability of the heterologous α-arabinofuranosidase above pH 5 precluded the use of Ni-chelate affinity chromatography to purify the protein.

Disruption of the *FUR1* gene by homologous recombination with a *LEU2*-containing cassette was performed in the recombinant strains *S. cerevisiae* Y294[pDLG1-APaguA+His] and *S. cerevisiae* Y294[pDLG1-APabfA], generating autoselective strains, obliged to retain the plasmid vectors in rich medium (Romanos *et al.*, 1992). Cultivation of the autoselective strains in rich medium led to a threefold increase in cell density and a 50-fold and 100-fold increase in the accumulation of α-arabinofuranosidase activity and α-glucuronidase activity in the culture supernatant, respectively,

compared to growth in defined medium. These results clearly indicate that capacity of *S. cerevisiae* to produce heterologous proteins depends greatly on the availability of nutrients. In the case of the α-glucuronidase, the results also show that the efficient processing of the extracellular proteins along the secretory pathway is also markedly influenced by the availability of nutrients, especially if heterologous proteins are large. The autoselective strains were useful for obtaining large amounts of enzyme activity, but in both cases led to difficulties in purification. Unknown components present in the rich medium stripped nickel ions from the affinity resin, and additional proteins in the rich medium co-eluted with the α-arabinofuranosidase on gel filtration. In order to obtain pure enzymes work was continued with recombinant strains cultivated on defined medium, in spite of the much lower yields of both enzymes.

The heterologous α-glucuronidase was purified to homogeneity from the concentrated culture supernatant of *S. cerevisiae* cultivated on defined medium in a single Ni-chelate affinity chromatography step. The enzyme was purified 58-fold with a yield of 49%. A certain portion of the enzyme activity in the concentrated supernatant was unable to bind to the resin, regardless of multiple applications to the column or the use of fresh resin, and it is thought that this represents a pool of incompletely translated heterologous protein, that lacks the C-terminal hexahistidine tag, but still retains enzyme activity. An additional advantage of using Ni-chelate affinity chromatography with a protein carrying a C-terminal histidine tag is that only completely transcribed protein is obtained in this way. Ni-chelate affinity chromatography proved to be very convenient in the purification of the heterologous α-glucuronidase, as very little resin was needed, due to its high binding capacity, no special equipment was needed and purification could be achieved very quickly without excessive attention to detail in the chromatographic procedure. The heterologous α-arabinofuranosidase could be purified to homogeneity in a single gel filtration step with a 25% yield and 7.2-fold purification was achieved. This was possible when the enzyme was produced in defined medium, as *S. cerevisiae* secretes very few proteins of its own. The purified α-glucuronidase had an apparent mobility on SDS-PAGE of 120 kDa and its predicted molecular weight was 91 kDa, indicating that the protein was glycosylated by *S. cerevisiae*. This finding is expected as its amino acid sequence contains 22 possible *N*-linked glycosylation sites. The amino acid sequence of the α-arabinofuranosidase, in contrast, contained only one possible *N*-linked glycosylation site, and the mobility of the heterologous protein was also virtually identical to the predicted molecular weight of 49.9 kDa.

Enzymatic properties of the purified heterologous α-glucuronidase and α-arabinofuranosidase

The *A. pullulans aguA* gene is a member of family 67 of the glycoside hydrolases and it was therefore not surprising that the heterologous α-glucuronidase showed very similar biochemical properties to the other enzymes in this family (Table 3.1). What was surprising, was that the properties of the α-glucuronidase purified from the culture supernatant of *A. pullulans*, and the heterologous α-glucuronidase expressed in *S. cerevisiae* differed (Table 7.1). If it is assumed that both enzymes are encoded by the same gene there is a large difference in the degree of glycosylation between the protein produced in *A. pullulans* compared to the protein produced in *S. cerevisiae*. Whether this is a sufficient explanation for the observed differences is doubtable. It is also possible that *A. pullulans* produces two similar α-glucuronidases and that the one was purified and the gene for the other cloned. An α-glucuronidase with different properties to the family 67 enzymes and a unique *N*-terminal amino acid sequence has been described, and it is possible that other families of this enzyme exist (Tenkanen and Siika-aho, 2000). Unfortunately, attempts to perform *N*-terminal sequencing on the purified α-glucuronidase failed to give meaningful data. Amino acid sequence from the purified enzyme will definitely help clear up this issue.

Table 7.1. Properties of the purified and heterologous α-glucuronidases

	Purified α-glucuronidase	Heterologous α-glucuronidse
M_r on SDS-PAGE	170 kDa	120 kDa
Temperature optimum	40°C	65°C
pH optimum	3	5 to 6
Temperature stability	40°C	45°C
K_m aldobiouronic acid	29.5	0.25
K_i aldobiouronic acid	29.0	—
K_m aldotriouronic acid	3.3	1.54
K_i aldotriouronic acid	9.8	14.0

Due to the very high degree of sequence similarity between the *A. pullulans* α-arabinofuranosidase and other members of family 54 it was expected that they would be very similar in terms of enzymatic properties, and this was found to be the case. The heterologous α-arabinofuranosidase was only characterized towards the synthetic substrates *p*-nitrophenyl α-L-arabinofuranoside and *p*-nitrophenyl β-D-xylopyranoside and its specificity towards natural substrates warrants further investigation.

Conclusions

From the work done in this study, it can be concluded that:

1. The colour-variant strain of *A. pullulans* NRRL Y-2311-1 produces an extracellular α-glucuronidase in response to growth on carbon sources containing both xylose and glucuronic acid.
2. The purified α-glucuronidase is similar to the fungal enzymes found in family 67 of the glycoside hydrolases.
3. *A. pullulans* NRRL Y-2311-1 contains genes encoding a family 54 α-arabinofuranosidase and a family 67 α-glucuronidase.
4. *S. cerevisiae* can effectively express both genes and correctly process and secrete both enzymes.
5. The heterologous enzymes share a high degree of sequence identity with homologues in their respective families, and are also very similar in terms of enzymatic properties.

Future research

Many of the possibilities for further research have been highlighted during the course of this discussion. The approach used to clone the *abfA* and *aguA* genes can obviously be extended to clone the genes encoding other xylanolytic enzymes produced by *A. pullulans*. Knowledge of the sequence of the *abfA* and *aguA* from *A. pullulans* genes also makes it possible to obtain upstream promoter sequence and much information about how expression of the xylanolytic genes is regulated can be gained in this way. The substrate specificity of both enzymes was only given cursory examination in this report, and can obviously be expanded upon. The availability of suitable substrates with validated structures is always a hurdle in this research, and their isolation and structural analysis will require a great deal of work in such studies. Both enzymes are likely to have a synergistic contribution to the degradation of xylan and other lignocellulosic substrates in conjunction with other lignocellulolytic enzymes and is worth investigating. The high yields of both heterologous enzymes in rich medium also makes it possible to evaluate their usefulness in any industrial process were they might be applicable.

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