Identification and characterization of some *Aspergillus* pectinolytic glycoside hydrolases

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Joris S. Zandleven

Abstract

Pectinases are used for many food applications, in particular for the manufacture of fruit juices. However, the array of pectin modifying enzymes as available today is insufficient to completely degrade pectic polysaccharides from plants, which consequently can cause problems in food processing. As the genome sequence of *Aspergillus niger* indicated the presence of more pectin modifying enzymes than previously known, research was carried out to identify, produce, and characterize novel pectinases from this species.

From the complete inventory of the pectinolytic glycoside hydrolase family 28 of *A. niger* a new gene group of seven exo-acting enzymes was found. Three of these enzymes (PGXA, PGXB, PGXC) were biochemically identified from which it was demonstrated that PGXB and PGXC act as an exo-polygalacturonase while PGXA rather acts like an exo-xylogalacturonan hydrolase.

The xylogalacturonan hydrolase (XGH) was thoroughly investigated for its action towards a xylogalacturonan (XGA) derived from gum tragacanth by isolation and characterization of the produced oligosaccharides. Also XGH activity towards XGA in the saponified modified 'hairy' regions (MHR-s) of pectin from apples and potatoes was investigated. The enzyme predominantly released the di-saccharide GalAXyl from these substrates which illustrates the preference of XGH to act between two xylosylated GalA residues. However this enzyme was also able to release low substituted XGA oligosaccharides as well as linear GalA oligosaccharides, which shows its tolerance for unsubstituted GalA residues in its active site.

By using XGH as analytical tool, the presence of XGA could also be demonstrated in the stem and the leaves of *Arabidopsis thaliana*, which shows that the presence of this polymer is not strictly confined to storage tissues or reproductive organs of plants as was previously thought to be the case.

Contents

Aan mijn ouders

Chapter 1

General Introduction

1.1 Project background

The research presented in this thesis forms part of a larger project (Carbnet) which deals with the discovery of new carbohydrate modifying enzymes (CMEs) from *Aspergillus niger* and was funded by IOP ("Innovatief Onderzoeks Programma") Genomics. Within this project the laboratory of Food Chemistry focused on the exploration of novel pectinases.

At the start of the project, seven polygalacturonases (Parenicová *et al.*, 2000b) and two rhamnogalacturonases (Sykerbuyk *et al.*, 1997) from *A. niger* were known, which have been cloned, over-expressed and biochemically characterized. These enzymes are usually components of commercial *Aspergillus* pectinase preparations which are used by industry for high yield juice extraction, clarification, maceration, and liquefaction of fruit tissues. However, the range of pectic enzymes present in commercial preparations is still insufficient and after their application in processing of plant materials, still parts of undegradable pectin are left, for which no enzymes have been found yet. For instance, during ultra filtration of depectinized fruit juices, which has become a standard operation in juice clarification in order to produce sparkling clear juices such as apple juice (Rao *et al.*, 1987; Schobinger, 1988), fouling of the membranes occurs which is caused by the accumulation of highly branched parts of pectins (Schols *et al.*, 1990b). Another example is pectin enriched soybean meal which is commonly used as feed for livestocks. Pectin in soybean is poorly utilized by monogastric animals, and partial degradation of these polysaccharides by the addition of pectinases may improve the utilization by these animals. However so far, large parts of the pectin polysaccharides from soybean appeared to be resistant towards the existing spectrum of pectinases (Huisman *et al.*, 2001).

Recently the genome of *A. niger* has been sequenced by DSM Food Specialties (Delft, The Netherlands) from which it became clear that only a fraction of the potential of carbohydrate modifying enzymes (CMEs) produced by this fungus was currently explored. As a result, the Carbnet project was founded which aims at the discovery of all CMEs which include pectinases, amylases and fructanases. Also the regulation of gene expression of these enzymes was studied. Detailed information regarding the Carbnet project can also be found at URL: www.senternovem.nl/mmfiles/factsheet%20Aspergillus%20(IGE01021) _tcm24-34630.pdf.

With the use of functional genomics tools we are able to investigate the set of these CMEs present in *A. niger* in a rapid and efficient way compared to the conventional techniques used

in the past. The choice to scrutinize for new CMEs of *A. niger* was supported by the fact that this species is easy to handle when cultivating, and that it secretes its enzymes into the medium from which they are easy to recover. Moreover the newly found CMEs from *A. niger*, which have a "Generally Recognized As Safe" (GRAS) status, can be used safely for food applications. This Carbnet project provides insight into the regulation of the genes involved, the synergistic interactions of the enzymes, and the mode of action of these enzymes towards complex polysaccharides (Fig. 1).
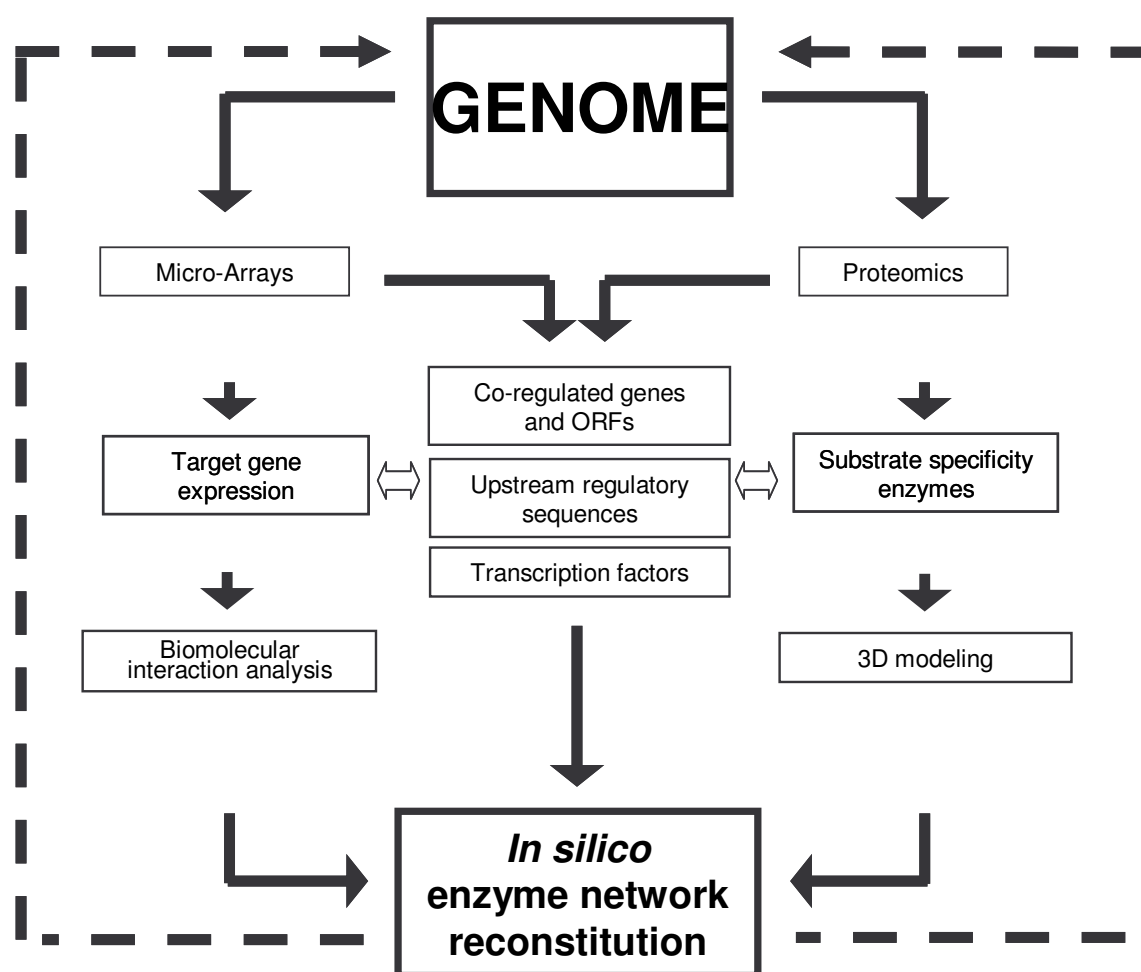
Figure 1. Overview of the Carbnet project

With the use of bioinformatics it is possible to mine the genome sequence of *A. niger* for novel CMEs by using the existing protein sequences of CMEs in the CAZy web-site database (at URL: http://afmb.cnrs-mrs.fr/~pdero/CAZY/db.html). From this, a database of all

annotated CMEs of *A. niger* can be created which forms the basis of this project. With the use of micro-array techniques (Fig. 1, Micro-Arrays) we are able to analyze which CMEs are expressed by this fungus when grown on simple carbon sources, such as D-glucose, D-galacturonic acid, D-xylose, as well as complex substrates, such as sugar beet pectin. By measuring the expression levels of the mRNA's and selecting those mRNA's which correspond to CMEs, we can study which CMEs are involved in what type of carbohydrate utilization (Fig. 1, Target gene expression). Similarly the expression of CMEs from *A. niger*, when grown on specific carbon sources, can also be followed at the protein level by proteomics (Fig. 1, Proteomics). The obtained CMEs of *A. niger* are identified by their amino acid sequence using nano-LC-nanospray MS-MS techniques in combination with the *A. niger* genome database and/ or other public protein databases.

The identity and the mode of action of novel CMEs are ultimately determined by investigation of their activity towards specific substrates (Fig. 1, Substrate specificity enzymes). After incubation of CMEs with these substrates, the obtained degradation products are analyzed for their structural features. The structure of the different products will be determined by HPLC techniques in combination with mass spectrometry. In addition, the catalytic mechanism of CMEs can be obtained from their 3D crystal structure (Fig. 1, 3D modeling), which is realized by crystallization of these enzymes followed by X-ray crystallography. The obtained data may enable us to build 3D structures of these CMEs which are compared to existing 3D models in literature. From this we are able to study the amino acid residues of these CMEs which are involved in catalysis of the corresponding substrate.

Newly found CMEs, including pectinases, from *A. niger* may be functionally important in the development of new and improved products and processes, such as to contribute to solve the ultrafiltration problem in apple juice manufacturing. Also the carbohydrate-degrading enzyme network of *A. niger* as a whole can be studied, which provides fundamental insight in the catabolytic mechanism of this species when grown on (complex) carbohydrates. In addition, these enzymes might be used as analytical tools in order to detect polysaccharides in different plant sources.

The literature review shown below will provide an introduction to *Aspergillus niger* in general, followed by the structural elements of pectin and characteristics of some (microbial) pectinases. Finally the aims of this thesis are described.

1.2 *Aspergillus*

The fungus *Aspergillus niger* (*A. niger*) is a member of the Deuteromycetes (Fungi Imperfecti) which stands for those fungi that reproduce asexually. This species belongs to the *A. niger* section *Nigri,* which are also known as the black aspergilli (Abarca *et al.*, 2004). The presence of this species has been reported in field situations and in stored foods such as peanuts, pecans, corn, apples, pears, peaches, grapes, strawberries, tomatoes and melons (Pitt and Hocking, 1985; Pitt and Hocking, 1997).

A. niger is extensively used by industry for the production of enzymes (for instance β-galactosidases, lipases, proteases, hemicellulases, cellulases, and pectinases) and organic acids. While pectinases are used for processing plant materials to food products, the organic acids, such as citric acid, are used in food and feed industry as flavor enhancers, acidifiers, stabilizers or preservatives (Magnuson and Lasure, 2004).

Since 1917, *Aspergillus niger* has been used for the production of citric acid (Raper and Fennel, 1965). Nowadays, around 700.000 - 1.000.000 tons of citric acid are produced per annum, which makes it the most important organic acid in quantitative terms (Ikram-ul *et al.*, 2004; Magnuson and Lasure, 2004; Prado *et al.*, 2005; Vandenberghe *et al.*, 2000). Around 70 % of total citric acid manufactured is used in diverse food and beverage products, while the remainder is mostly used for pharmaceutical formulations (Magnuson and Lasure, 2004; Prado *et al.*, 2005; Vandenberghe *et al.*, 2000).

Pectinases from fungal sources, especially from *A. niger*, are used commonly in the fruit juice industry and account, next to cellulases and hemicellulases, for approximately 20% of the one billion US dollar annual sales of all industrial enzymes (Bhat, 2000; Kashyap *et al.*, 2001). These enzymes are used in the production of sparkling clear juices, such as apple juice, pear juice, berrie juice, and grape juice, but also for the manufacture of juices with stable clouds, such as citrus juices and prune juices.

A. niger has been utilized for decades in the food industry without any negative impact on human health (Dijck *et al.*, 2003; Schuster *et al.*, 2002). Moreover, *A. niger* products are considered "Generally Regarded As Safe" (GRAS) by the United States Food and Drug Administration (FDA) (Oxenbøll, 1994; Abarca *et al.*, 2004; Schuster *et al.*, 2002). Another reason for *A. niger*'s success in the food industry is that it grows rapidly on cheap substrates

and secretes its enzymes into the medium, from which they are easy to recover (Oxenbøll, 1994).

To understand the function of pectinases from fungal sources, such as *A. niger*, towards pectic polysaccharides, first a general overview will be given of the plant cell polysaccharides, with emphasis on pectic polysaccharides. Then the function of pectinolytic enzymes will be described with special attention to those that belong to the glycoside hydrolase family 28 of *A. niger*.

2. Plant cell wall polysaccharides

Plant cells are surrounded by a "wall", which stands out from the plasma membrane. These cell walls enable regulation of cell expansion and strengthen them to osmotic stress. They also protect these cells from insects as well as from invading pathogenic fungi and bacteria (Carpita and Gibeaut, 1993).

Cell walls of growing plant tissues are predominantly composed of primary walls and middle lamella (Fig. 2). The primary cell wall mainly consists of polysaccharides and to a lesser extent of glycoproteins, phenolic esters, minerals and enzymes, while the middle lamella is a polysaccharide rich region between primary cell walls of adjacent cells (Fry, 1988; O' Neill and York, 2003). The most important polysaccharides in primary walls are cellulose, hemicellulose and pectin (O' Neill and York, 2003; Albersheim *et al.*, 1996; Gibeaut and Carpita, 1994; Zablackis *et al.*, 1995).

Cellulose is the most abundant plant polysaccharide which consist of β-$(1\rightarrow 4)$-linked D-glucan chains. These chains arrayed in cellulose microfibrils, are coated with hemicelluloses, such as xyloglycan and arabinoxylan. The hemicelluloses cross-link the cellulose microfibrils, which creates a cellulose/hemicellulose network (Albersheim *et al.*, 1996; Gibeaut and Carpita, 1994; O' Neill and York, 2003).

Pectin covers a diverse group of polysaccharides which are present in primary cell walls and middle lamella of plant cell walls (Voragen *et al.*, 2001). These polysaccharides function as "glue" that holds the cellulose microfibrils, hemicellulose and proteins together (Carpita and Gibeaut, 1993), however the exact nature of how these pectin polysaccharides are linked to other polymers is yet unknown (Mort, 2002).

2.1 Structural elements of pectin

Pectin is probably the most complex class of plant cell wall polysaccharides and consist of several monosaccharides (Vincken *et al.*, 2003). Generally pectin is comprised of two families of acidic polymers (Voragen *et al.*, 2001):

- Galacturonan, which include pectin molecules with exclusively α-(1,4)-linked D-galacturonic acid (GalA) residues in the backbone, such as homogalacturonan, xylogalacturonan and rhamnogalacturonan II. Rhamnogalacturonan II is a highly branched homogalacturonan which contains rare sugar residues (see further) and its name is therefore somewhat misleading, because it suggests that it contains a backbone of alternating rhamnose and D-galacturonic acid residues.

- Rhamnogalacturonan I, which has a backbone of alternating α-(1,2)-linked L-rhamnose and α-(1,4)-linked D-galacturonic acid residues.
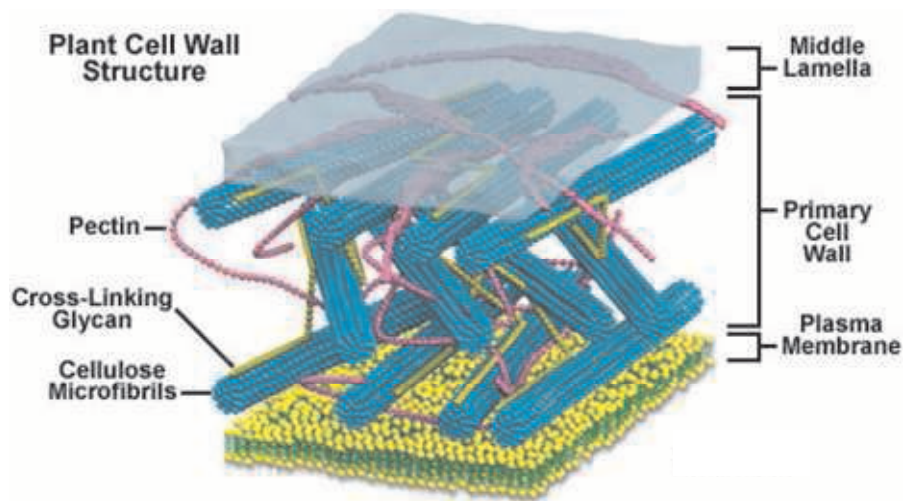
Figure 2. Model of the plant cell wall structure in higher plants. The cellulose microfibrils are cross-linked by glycans, predominantly xyloglucans. The cellulose/hemicellulose network is embedded in a matrix of pectic polysaccharides. Davidson, M.W. (2005) at URL: http://micro.magnet.fsu.edu/cells/plants/cellwall.html
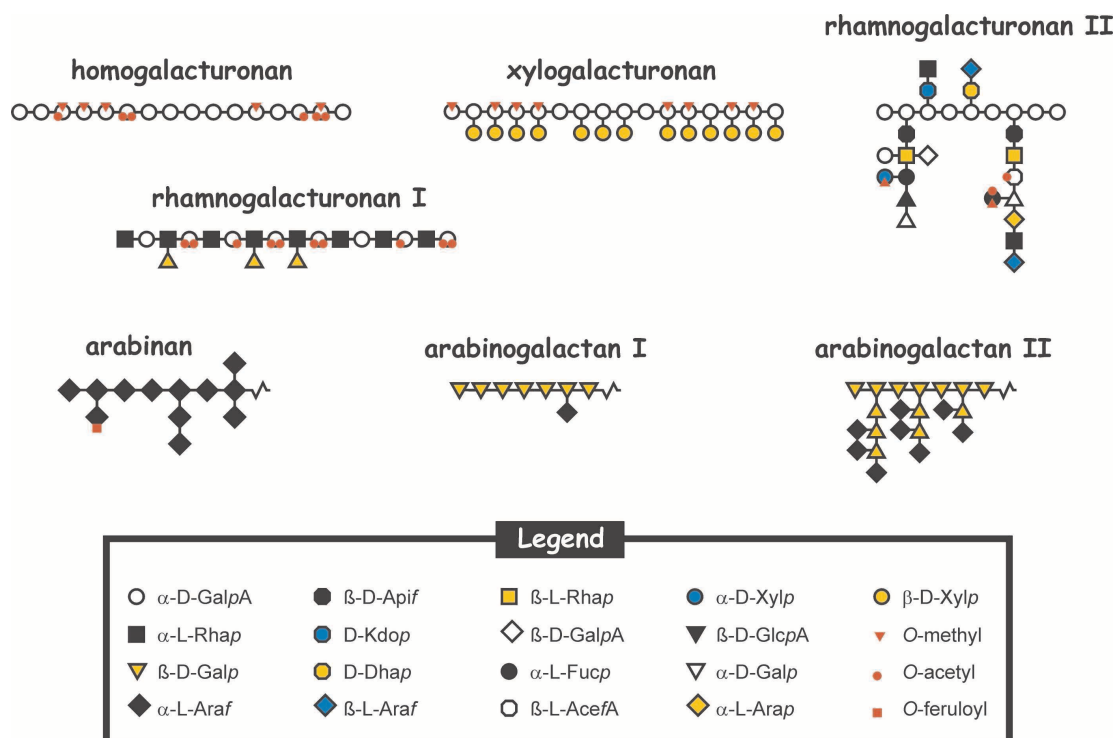
Figure 3. Schematic structure of pectic polysaccharides. For details of the symbols, see legend. The type of linkages present in the pectic polysaccharides are explained in the text (Vincken *et al*. 2003).

2.1.1 Homogalacturonan

Homogalacturonan (HG) is a linear chain of α-(1, 4)-linked D-galacturonic acid residues (Fig. 3) in which some of these residues can be methyl-esterified at the carboxylic acid group (e.g. C-6 position), and can carry acetyl groups on C-2 and C-3 (Albersheim *et al.*, 1996; O' Neill and York, 2003; Vincken *et al.*, 2003). It is the most predominant biopolymer in primary cell walls of dicotyledonous plants. Highly methyl-esterified HG polymers are also known as "pectin" while HG polymers with a low or no methyl-esterification are referred to as "pectic acid" (O' Neill and York, 2003). Particularly, methyl-esterified pectins have gained a lot of interest in the food industry (see section 2.2), because of their excellent gelling and stabilizing properties which are governed by their degree and pattern of methyl-esterification (Vincken *et al.*, 2003).

2.1.2 Xylogalacturonan

Xylogalacturonan (XGA) consist of a linear chain of α-(1, 4)-linked D-galacturonic acid residues in which β-D-xylose residues are β- (1, 3)-linked to part of the GalA residues (Fig. 3). This biopolymer has been detected in the walls of reproductive plant organs such as soybeans, kidney beans, peas, apple fruit, pear fruit, onions, carrot, pine pollen, cotton seed, and watermelon (Bouveng, 1965; Kikuchi *et al.*, 1996; Schols *et al.*, 1995; Schols and Voragen, 1996; Weightman *et al.*, 1994; Yu and Mort, 1996). Xylogalacturonan accounts for approximately 21% and 4% (w/w) of soybean pectin and apple pectin respectively (Voragen *et al.*, 2001), while it accounts for about 1.8 % (w/w) of the pea hulls (Le Goff *et al.*, 2001).

The degree of xylosylation in XGA varies between 25% (as isolated from watermelon) and 75% (as isolated from apple), whereas the degree of methylation ranges between 40-90% (Voragen *et al.*, 2001).

Exudates from gum tragacanth (from the *Astralagus* species) also contain a XGA-like polysaccharide (Fig. 4), which contains besides single β-D-xylose residues, also the disaccharide units α-L-fucose-(1, 2)-D-xylose and β-D-galactose-(1, 2)-D-xylose (Aspinall and Baillie, 1963).

$\rightarrow$ 4) -α-D-GalA (1$\rightarrow$4) α-D-GalA (1$\rightarrow$4) α-D-GalA (1$\rightarrow$ 4) α-D-GalA (1$\rightarrow$

```
        3                 3                          3
        ↑                 ↑                          ↑
        1                 1                          1
     β-D-Xyl           β-D-Xyl                    β-D-Xyl
                          2                          2
                          ↑                          ↑
                          1                          1
                       α-L-Fuc                    β-D-Gal
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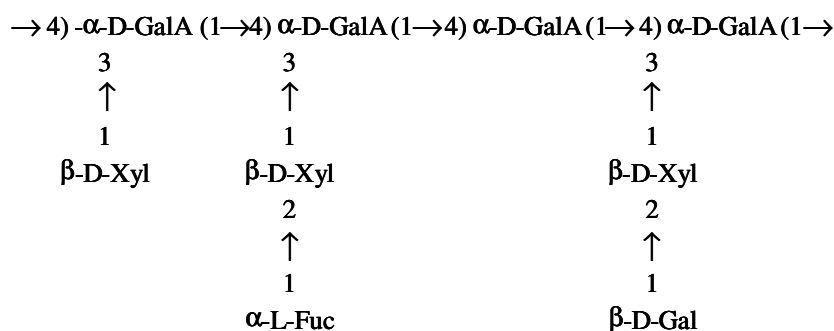
Figure 4. Structure of gum tragacanth according to Aspinall and Baillie (1963).

It has also been proposed that oligomeric side chains exist in XGA from apple, pea and soy pectin. These side chains are composed of 1,4-linked xylose residues (as in apple XGA; Oechslin *et al.*, 2003), 1,4- and 1,2-linked xylose residues (as in soy XGA; Nakamura *et al.*, 2002), and 1,2- and 1,3-linked xylose residues (as in pea XGA; Le Goff *et al.*, 2001).

2.1.3 Rhamnogalacturonan II

Rhamnogalacturonan II (RG-II) contains a backbone of about nine α-(1, 4)-linked D-galacturonic acid residues (Fig. 3) that carry four side chains which consist of a number of rare sugars, such as apiose, aceric acid, 2-keto-3-deoxy-D-manno-octulosonic acid (Kdo) and 2-keto-3-deoxy-D-lyxo-heptulosaric acid (Dha). This polymer has been found in many plants tissues and appears to be highly conserved in plant varieties (O' Neill and York, 2003; Schols and Voragen, 2002; Vincken *et al.*, 2003).

2.1.4 Rhamnogalacturonan I

Rhamnogalacturonan I (RG-I) can have a backbone composed of as many as 100 repeating units of alternating α-(1, 2)-linked rhamnose and α-(1, 4)-linked galacturonic acid residues (Fig. 3; Albersheim *et al.*, 1996; McNeil *et al.*, 1980). A part of the GalA residues may be methyl esterified and / or acetylated (McNeil *et al.*, 1984). Depending on the source of the cell walls, around 20 to 80% of the rhamnose residues are branched with neutral sugar side chains attached to C-4. These side chains can vary from one single galactose residue up to polymeric chains of 50 glycosyl residues or more composed of arabinose and / or arabinose and

galactose residues. Polymeric chains of arabinose are also known as arabinans, while those that contain both arabinose and galactose are referred to as arabinogalactan I (Schols and Voragen, 1996; Schols and Voragen, 2002; Vincken *et al.*, 2003).

Arabinans are composed of α-(1, 5)-linked L-arabinose residues with other α-L arabinose residues attached to about one-third of the backbone residues by 1,3 and/ or 1,2 bonds (Beldman *et al.*, 1997). There are two structurally different forms of arabinogalactans. Arabinogalactan type I is a linear chain of β-(1, 4)-linked D-galactose residues in which α-L-arabinose residues are attached to the *O*-3 of the galactose residues (Carpita and Gibeaut, 1993; Mohnen, 1999; Ridley *et al.*, 2001; Vincken *et al.*, 2003). Arabinogalactan type II are highly branched polysaccharides with ramified chains of β-D-galactose residues joined by 1,3 and 1,6 linkages. The exterior chains of arabinogalactan II, which is predominantly composed of β-(1, 6)- linked D-galactose residues, are mainly terminated with L-arabinose residues (Carpita and Gibeaut, 1993; Ridley *et al.*, 2001; Vincken *et al.*, 2003; Voragen *et al.*, 2001). Arabinogalactan II can also be associated with proteins, also known as arabinogalactan proteins (AGPs), and are as such not regarded as a structural component of the primary cell wall (Schols and Voragen, 2002; Vincken *et al.*, 2003; Voragen *et al.*, 2001) .

2.1.5 The macromolecular structure and biological functions of pectin in plant cell walls

Pectin can be represented as one molecule containing "smooth" and "hairy" regions. The "smooth" regions are mainly composed of HG while the "hairy" regions (HR) primarily comprise RG-I, XGA, arabinan, AG-I, and AG-II. When these "hairy" regions are extracted from plant tissues, by using mixtures of enzymes including pectinases, cellulases and hemicellulases, these regions can be partially degraded, and therefore the forthcoming material is referred to as modified "hairy" regions (MHR; Vincken *et al.*, 2003).

Based on fragmentation of MHR with the use of enzymes, a pectin model was proposed (Schols and Voragen, 1996; Vincken *et al.*, 2003). In this model, MHR is presented as three assembled subunits, namely a rhamnogalacturonan with side chains of single galactose residues, a rhamnogalacturonan with long arabinan and/ or AG-I side chains, and a XGA. Additionally it is thought that this latter polymer could be a continuation of HG. Although this model suggests that HG and XGA are present in the backbone of pectin, it is recently

speculated that these polymers could be also attached as side chains to a RG-I backbone (Vincken *et al.*, 2003).

During plant growth, several interactions between plant cell wall polysaccharides take place, which are likely involved in the development and strengthening of the cell wall. Studies have shown that two RG-II molecules have the ability to form a complex with boron, which can crosslink two HG molecules (Ishii and Matsunaga, 2001; Ishii, 1999). There are indications that the borate binding RG-II polysaccharides facilitate the development and growth of plant cell walls (Hu and Brown, 1994; Ishii and Matsunaga, 2001). As plant cells mature, a decrease in the degree of methyl-esterification of homogalacturonans it is often observed in pectin. As a consequence, two anti-parallel chains of pectin can condense in the cell wall by cross-linking with Ca^{2+} ions to form "junction zones", also known as multiple "eggbox", which contributes to cell wall strength (O' Neill and York, 2003). In cell walls of some plants the galactose and arabinose side chains in rhamnogalacturonans, as well as hemicelluloses may also be esterified with ferulic acids. Based on this, it is hypothesized that pectins could be linked to hemicellulose through the formation of dehydrodiferuloyl cross-links. These types of cross-links are thought to be involved in the regulation of cell wall growth (Ishii, 1997).

2.1.6 Influence of pectin on texture of ripening fruits

Pectin is present in large amounts in fruit cell walls and contributes to tissue strength of the fruit. During ripening of fruit, the tissue softens and this is often accompanied by the solubilization of the pectic polysaccharides, especially those in the middle lamella region.

It has been clearly observed that hydrolysis of α-(1, 4)-D-galacturonan linkages in pectin by the enzyme PG is one of the major causes of cell wall change during fruit softening (Gross and Wallner, 1979; Mafra *et al.*, 2001; Missang *et al.*, 2004; Redgewell *et al.*, 1997; Wakabayashi, 2000). Also pectin methyl-esterases (PME) are proposed to be involved in the ripening process of fruit tissues, which regulate the degradation of the pectin hydrolysis by PG (Mafra *et al.*, 2001; Missang *et al.*, 2004). Although yet not evidenced (Redgewell *et al.*, 1997), several authors also proposed that the degradation of (arabino-)galactans in cell walls of fruits may play a role in fruit softening, based on the decline of galactose and arabinose

during fruit ripening. This suggests that the (arabino)galactan side chains could regulate the activity of PG by restricting its access towards its corresponding substrate (Gross and Wallner, 1979; Wakabayashi, 2000).

2.2 Exploitation of pectins in the food industry

Pectins are of importance as a component of a "dietary fibre-rich diet". They have the ability to lower blood cholesterol levels, to act against diarrhea, to regulate and protect the gastrointestinal tract, to stimulate the immune system, and to function as detoxicants (Schols and Voragen, 2002; Voragen *et al.*, 1995; Yamada, 1996).

Extracted pectins are also known for their ability to form gels, which is a property that is widely used in the food industry. This type of pectin is manufactured industrially by hot acid extraction of dry apple pomace or citrus peels and contains primarily homogalacturonan (May, 2000; Voragen *et al.*, 2001). The gelling ability of pectins depends on the temperature, pH, ionic strength, and on the number of esterified carboxyl groups (i.e. degree of esterification). It is known that high methylated pectins (HM) have the ability to form a gel in the presence of high amounts of sugar and acid, which is commonly used for the production of marmalades, acid jams and jellies. These HM pectins have also found application as stabilizers and thickening agents in dairy products such as drinkable yoghurts, blends of milk and fruit juices, and acidified soybean milk products (Voragen *et al.*, 1995).

Pectins with a low methoxyl content (LM pectin) can gel in the presence of divalent cations, such as calcium (Ca^{2+}) and do not require sugar addition, which makes them useful for the production of "low-calorie" gelled products (Thibault and Ralet, 2001).

3. Pectinolytic enzymes

Pectinases are present in many higher plants and are produced by many microorganisms (Benen *et al.*, 1999; Benen *et al.*, 2002; Benen and Visser, 2003d; Voragen *et al.*, 1995; Whitaker, 1990). They can produce textural changes in fruits and vegetables during ripening, storage and processing, as a result of depolymerization, de-esterification and solubilization of pectic polysaccharides. Microbial pectinases can provide functions in fermented foods, but are

also produced industrially as processing aids in the food industry (Voragen *et al.*, 1995; Voragen *et al.*, 2001).

Owing to the complexity of pectin it can be easily realized that many pectinases are involved in fully degrading this polymer (Benen *et al.*, 2002). Among these pectinases are pectin methylesterases, pectin- and rhamnogalacturonan acetylesterases, polygalacturonases, rhamnogalacturonan hydrolases, and pectate-, pectin-, and rhamnogalacturonan lyases (Benen *et al.*, 1999).

Polygalacturonases cleave α-1,4-D galacturonan linkages in homogalacturonan (Fig. 5). These enzymes generally prefer non-methylesterified pectins and their activities decrease with increasing degree of methyl-esterification. Some polygalacturonases were found to be active towards LM pectins (Parenicová *et al.*, 2000a). Polygalacturonases can be divided into enzymes that hydrolyse the polymer by an *endo*-attack (*endo*-polygalacturonase; EC. 3.2.1.15), and those that act from the non-reducing end (Fig. 5), by releasing mono- or digalacturonic acid (*exo*-polygalacturonase; EC 3.2.1.67 and EC 3.2.1.82). Endo-polygalacturonases randomly attack their substrate and produce a number of galacturonic acid oligosaccharides, while exo-polygalacturonases cleave the chain in a zipper fashion (Benen *et al.*, 2002; Benen and Visser, 2003d).

Pectin and pectate lyases split α-1,4-D galacturonan linkages in HG by β-elimination and introduce a double bond between C4 and C5 of the newly formed non-reducing end. While pectin lyases (E.C. 4.2.2.10) prefer to act towards high methylated pectins, pectate lyases (endo acting (EC 4.2.2.2) and exo-acting (EC 4.2.2.9)) are most active towards pectate or LM pectins (Fig. 5; Benen *et al.*, 2002; Pilnik and Voragen, 1991).

Smooth regions

Exo-poly-galacturonase
Pectin methyl esterase
Polygalacturonase
Pectate lyase
Pectin acetyl esterase
pectinlyase

homogalacturonan

Gal   Xyl
GalA   Ac
Rha   Me

Hairy Regions regions

Xylogalacturonan hydrolase

RG-hydrolase   **RG-lyase**

RG-galacturonohydrolase

RG-acetyl esterase

RG-rhamnohydrolase

xylogalacturonan   **Rhamnogalacturonan I**
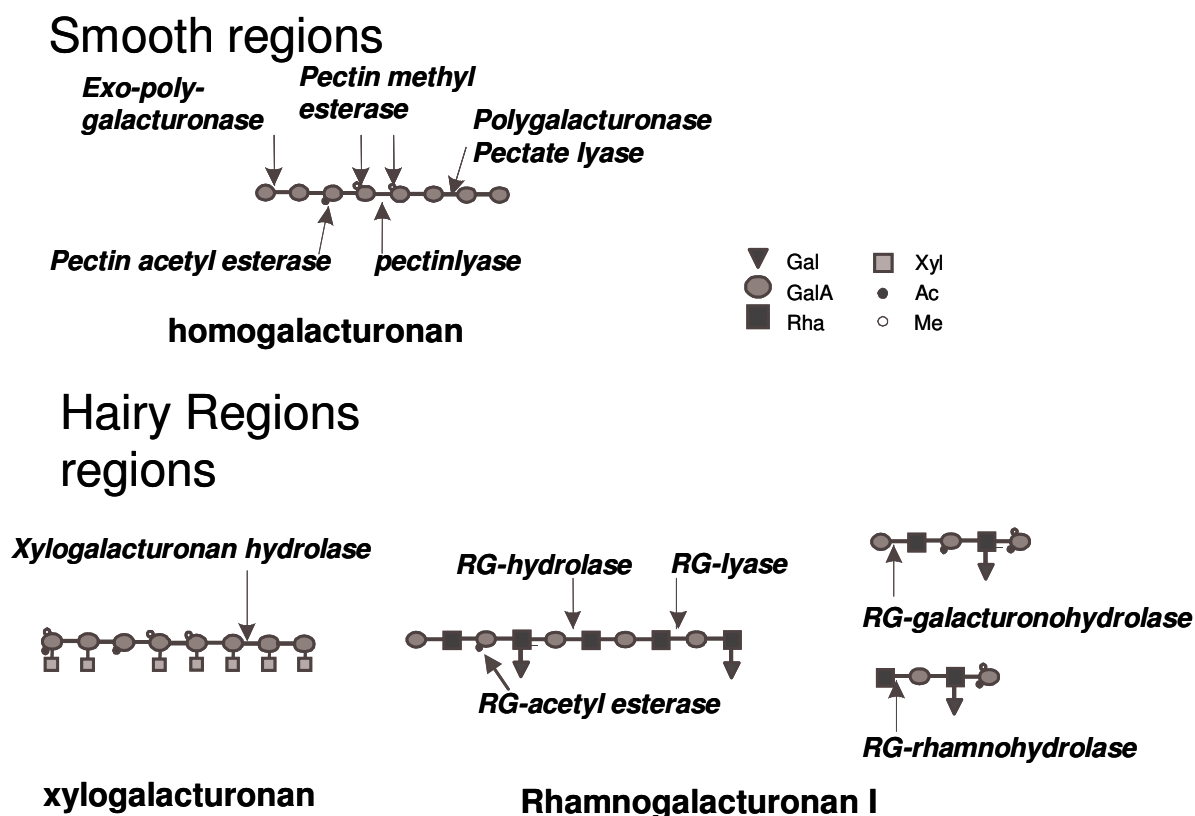
Figure 5. Schematic representation of the pectinases involved in the degradation of homogalacturonan, rhamnogalacturonan I and xylogalacturonan

Since 1990, a number of rhamnogalacturonan degrading enzymes have been reported (Kofod *et al.*, 1994; Mutter, 1997; Schols *et al.*, 1990). These enzymes can be divided into two different types of rhamnogalacturonan depolymerising enzymes, namely a rhamnogalacturonan hydrolase (EndoRGH) that cleaves α-galacturonic acid-(1,2)-α-rhamnose linkages, and a rhamnogalacturonan lyase (EndoRGL) that cleaves α-rhamnose-(1,4)-α-galacturonic acid linkages by β-elimination (Fig. 5; Voragen *et al.*, 2001; Beldman *et al.*, 1996; Benen *et al.*, 2002). Also two other enzymes have been reported to be specific for rhamnogalacturonan fragments, namely a rhamnogalacturonan rhamnohydrolase and a rhamnogalacturonan galacturonohydrolase, which remove rhamnose and galacturonic acid residues respectively from the non-reducing end (Fig. 5; Beldman *et al.*, 1996; Mutter *et al.*, 1998; Mutter *et al.*, 1994).

Pectin esterases (Fig. 4) are enzymes that are capable to hydrolyze methyl and acetyl groups from pectin and consist of three different classes, namely pectin methylesterases (PME; EC

3.1.1.11), pectin acetylesterases (PAE; EC 3.1.1.6) and rhamnogalacturonan acetyl esterases (RGAE).

Pectin methylesterases cleave off methyl esters which are present at the carboxylic function (C-6) in the backbone of homogalacturonan, which results in the formation of free carboxylic acid and methanol. The acetylesterases split off acetyl groups from C-2 and/ or C-3 from GalA residues and can be grouped in those that are restricted to homogalacturonan (PAE) and rhamnogalacturonan (RGAE) respectively (Fig. 5; Benen *et al.*, 2003c; Benen *et al.*, 2002; Voragen *et al.*, 1995; Voragen *et al.*, 2001).

Also a novel type of pectinase, xylogalacturonan hydrolase, has been found in *Aspergillus tubingensis*. This enzyme acts on xylogalacturonan by cleaving α-1,4-D galacturonan linkages in an endo-fashion (Fig. 5) and has a requirement for xylosyl side chains (Van der Vlugt-Bergmans *et al.*, 2000).

3.1 Microbial pectinases

Several pectinases have been isolated from microorganisms such as *Erwinia chrysanthemi*, *E. carotovora*, *Aspergillus niger*, *A. tubingensis* and *A. aculeatus* (Benen *et al.*, 2002). A list of different types of known microbial pectinases is presented in Table 1, along with their family number according to Coutinho and Henrissat (1999). The enzymes presented in Table 1 can also be found at the following URL: http://afmb.cnrs-mrs.fr/~pdero/CAZY/db.html. It should be noted that the assignment of these microbial pectinases into family numbers is based on amino acid sequence similarities rather than biochemical properties.

As shown in Table 1, the hydrolytic pectinases are grouped into glycoside hydrolase family 28 (GH28). As the amino acid sequence information of enzymes ExoRGR and ExoRGG is still unknown, these enzymes have still to be attributed to a proper family. The pectic lyases are more diverse and have been grouped into six different families. The pectin esterase PME has been grouped into family number CE8, whereas the pectin esterases PAE and RGAE have both been assigned into family number CE12.

Enzyme	Abbreviation	Family[1]
Endopolygalacturonase	EndoPG	GH28
Exopolygalacturonase	ExoPG	GH28
Endoxylogalacturonase	EndoXGH	GH28
Endorhamnogalacturonase	EndoRGH	GH28
RG rhamnohydrolase	ExoRGR	?
RG galacturonohydrolase	ExoRGG	?
Endopectate lyase	EndoPAL	L1
Endopectin lyase	EndoPL	L1
Exopectate lyase	ExoPAL	L2
Endopectate lyase	EndoPAL	L3
Endorhamnogalacturonan lyase	EndoRGL	L4
Endopectate lyase	EndoPAL	L9
Exopectate lyase	ExoPAL	L9
Endopectate lyase	EndoPAL	L10
Endorhamnogalacturonan lyase	EndoRGL	L11
Pectin methylesterase	PME	CE8
Pectin acetylesterase	PAE	CE12
RG acetylesterase	RGAE	CE12

Table 1. Overview of different types of microbial pectinases (Benen *et al.*, 2002). [1]GH, glycoside hydrolase; L, lyase; CE, carbohydrate esterase.

3.1.1 Hydrolysis mechanism of family 28 glycoside hydrolases

Glycoside hydrolases can degrade their corresponding substrate either by retaining or inversion of the anomeric configuration (Fig. 6). Inverting glycoside hydrolases utilize a direct displacement of the carbohydrate leaving group, which as a consequence results in the inversion of the anomeric configuration (hence α configuration becomes β or vice versa). Retaining glycoside hydrolases use a double-displacement mechanism with retention of the anomeric configuration. Both mechanisms use two carboxyl residues on opposite sites of the sugar plane which act as a general acid and a general base (for inverting glycoside hydrolases), or as a general acid/ base and a nucleophile/leaving group (for retaining glycoside hydrolases) respectively (Biely *et al.*, 1996; McCarter and Withers, 1994; Sinnott, 1990).
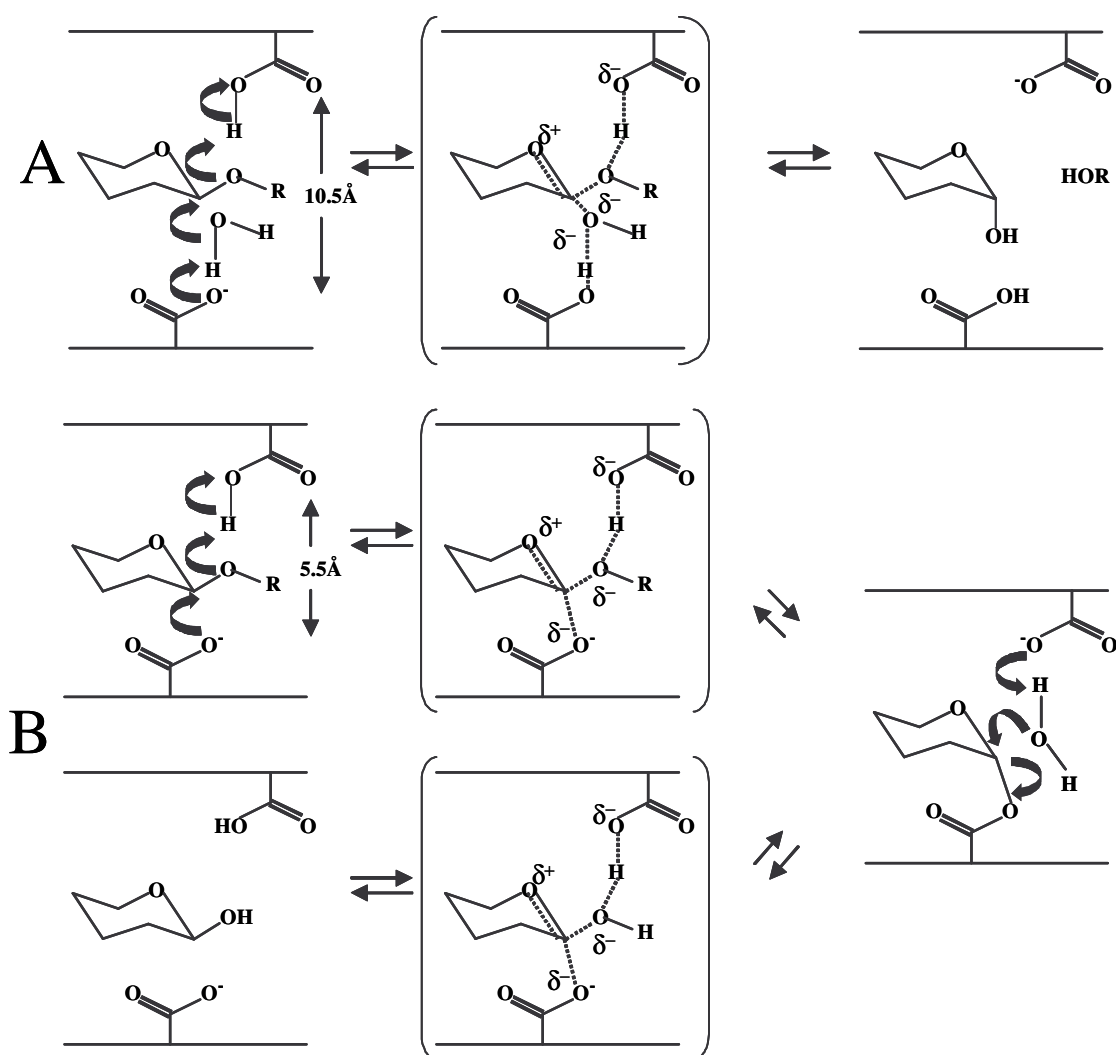
Figure 6. Schematic representation of the inverting (A) and retaining (B) mechanism of glycoside hydrolases. (Coutinho and Henrissat, 1999) at URL: http://www.cazy.org.

All hydrolytic pectinases that are grouped into glycoside hydrolase family 28 operate with an inverting mechanism. This glycosidase family includes all *Aspergillus* endo- and exo-polygalacturonases, and rhamnogalacturonan hydrolases, as well as the *Aspergillus tubingensis* xylogalacturonan hydrolase (Coutinho and Henrissat, 1999; de Vries and Visser, 2001; Markovic and Janecek, 2001).

The structure of glycoside hydrolase family 28 enzymes is typically known for its right-handed parallel β-helix. These enzymes usually contain 10 to 11 turns of β-strands that are coiled into four parallel β-sheets (Cho *et al.*, 2001). In Figure 7, an example of the structure

of the endo-PGII from *A. niger* is shown. The structure-function relationships of this enzyme will be discussed in more detail in the next paragraph.

3.1.2 Proposed structure-function relationships of family 28 glycoside hydrolases, using endo-polygalacturonase II from *A. niger*

At present, nine pectinolytic glycoside hydrolases from *A. niger* have been cloned and over-expressed, namely seven endo-polygalacturonases (Bussink *et al.*, 1991) and two rhamnogalacturonan hydrolases (Sykerbuyk *et al.*, 1997). Also a xylogalacturonan hydrolase gene was proven to be present in *A. niger* (Benen *et al.*, 2002).

The endo-polygalacturonase II (endo-PGII) from *A. niger* is considered to be a model enzyme for family 28 glycoside hydrolases. The elucidation of its three-dimensional structure (see Fig. 8, which shows the active site cleft of the endo-PGII) along with site-directed mutagenesis studies on this enzyme has gathered insight into its substrate specificity and catalytic properties (Armand *et al.*, 2000; Benen *et al.*, 2002; Pagès *et al.*, 2000).

Based on the bond-cleavage frequencies of endo-PGII towards a set of oligogalacturonates a provisional subsite map was constructed, which is composed of seven subsites (-5 to +2) (Benen *et al.*, 1999). Subsites are spots on or in the enzyme where the building blocks of a carbohydrate substrate binds and are numbered from the catalytic site that is located between subsites -1 and +1. These subsites are aligned in a linear array and the active site is located at a specific location between two subsites (Fig. 9). By convention, oligo- and polysaccharides are bound with their non-reducing end towards a subsite with a negative sign, and are bound in a productive way only when it covers subsites -1 and +1 (Benen *et al.*, 2002).

The subsites of *A. niger* endo-PGII involved in substrate binding and catalysis have been studied (Armand *et al.*, 2000; Pagès *et al.*, 2000). For this, six charged amino acid residues of endo-PGII, namely Asp180, Asp201, Asp202, His223, Arg256 and Lys258, which are strictly conserved in the active site among polygalacturonases, were subjected to site-directed mutagenesis. From the differences in bond-cleavage frequencies of the wild type endo-PGII and its mutants, along with the three dimensional structure of the wild type enzyme, it was suggested that Arg256 and Lys258 are involved in substrate binding. These residues were proposed to interact with adjacent galacturonic acid residues that occupy subsite -1 (Lys258) and +1 (Arg256) respectively (Armand *et al.*, 2000). The proposed function of these residues

was also supported by studies on the 3D structure of *A. aculeatus* endo-polygalacturonase and the modeled structure of this endo-polygalacturonase complexed with octagalacturonic acid (Cho *et al.*, 2001).
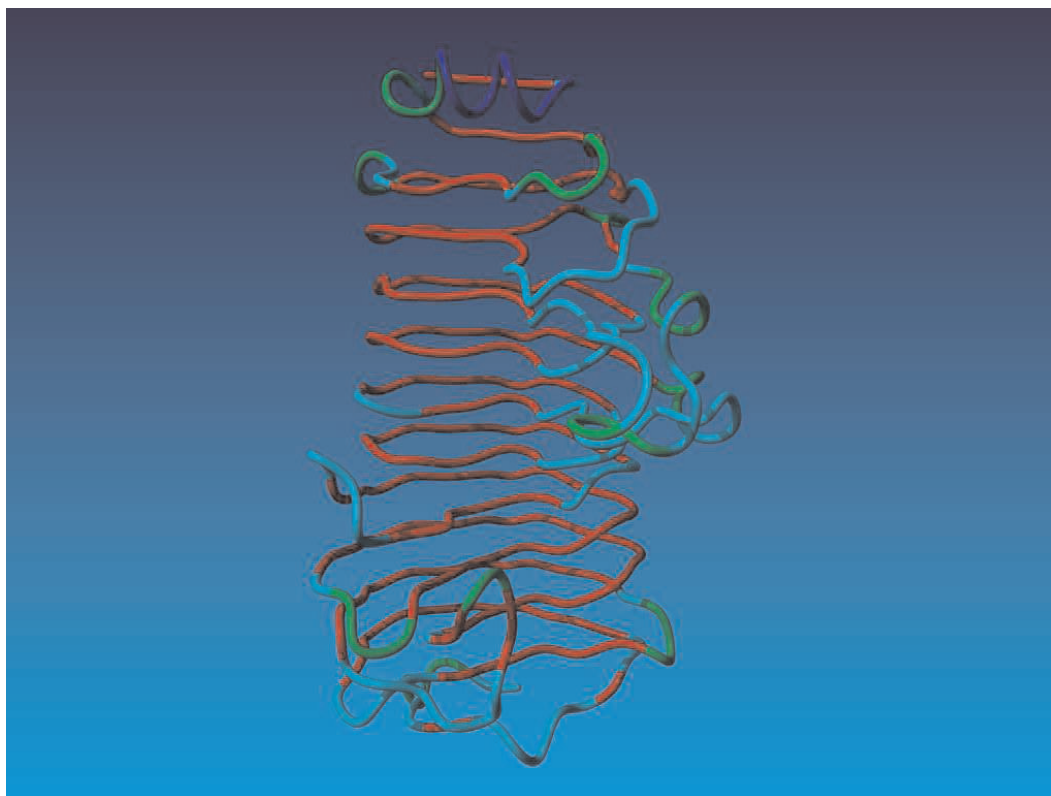
Figure 7. Three dimensional model of endo-polygalacturonase II (Armand *et al.,* 2000).
The alpha-helixes, beta-helixes, and loops of the enzyme are shown in dark blue, red and green respectively.

The function of His223 in substrate binding and catalysis is not yet clear. It has been proposed that His223 plays a role in maintaining the proper ionization state of Asp201, which is involved in catalysis (Armand *et al.*, 2000). Also Cho *et al.* (2001) proposed that it may function as a proton donor for this residue.

The three aspartate residues (Asp180, Asp201, and Asp202) are critical for catalysis and are clustered in the vicinity of subsites -1 and +1. While Asp180 with the assistance of Asp202 was proposed to act as a base to activate the bound water molecule, Asp201 was hypothesized to be the general acid that protonates the leaving group (Fig. 10; Armand *et al.*, 2000; Pagès *et al.*, 2000).
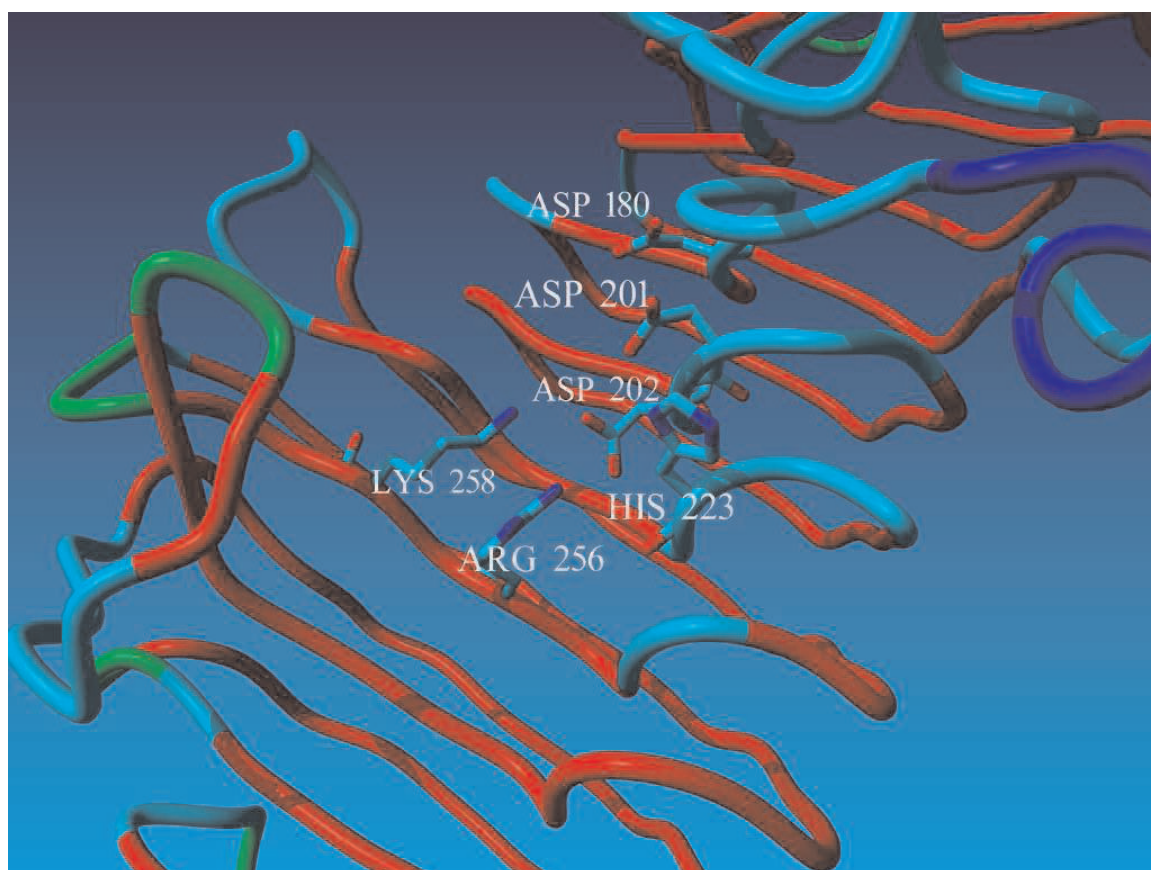
Figure 8. Three dimensional model of the active site cleft of endo-polygalacturonase II. The amino acid residues Asp180, Asp201, Asp202, His223, Arg256 and Lys258 (depicted as the side groups in the model) are involved in substrate binding and catalysis (Armand *et al.,* 2000).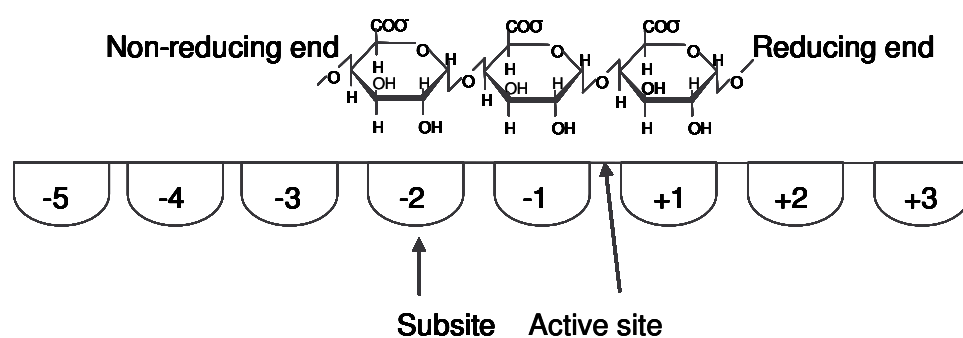 The alpha-helixes, beta-helixes, and loops of the enzyme are shown in dark blue, red and green respectively. The backbone, N atoms, and O atoms of the side groups of the amino acid residues are shown in pale blue, dark blue and red respectively.

Figure 9. Schematic illustration of a subsite array for polygalacturonase degrading enzymes.

Since the three aspartate residues in endo-PGII are conserved among family 28 glycoside hydrolases, the catalytic mechanism for endo-PGII (Fig. 10) could be applied for this entire family (van Santen *et al.*, 1999). Although in *A. aculeatus* rhamnosidase, which also belongs to the glycoside hydrolase family 28, Asp202 is replaced by a glutamate (Armand *et al.*, 2000; van Santen *et al.*, 1999), the side chain of this latter residue occurs approximately in the same position as the side chain of Asp202 (van Santen *et al.*, 1999).
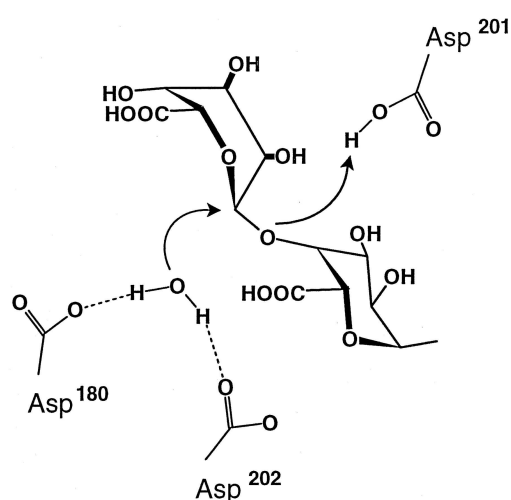
Figure 10. Schematic view of the proposed catalytic mechanism for family 28 glycoside hydrolases (van Santen *et al.*, 1999). The Asp201 acts as the acid (proton donor), whereas Asp180 and Asp202 activate the water molecule (which acts as the nucleophile). Numbering of the aspartate residues is according to endo-PGII.

3.1.3 Random acting and processive acting endo-polygalacturonases of *A. niger*

Seven endo-polygalacturonases from *A. niger* were studied in depth for their action towards polygalacturonic acid (Benen *et al.*, 1999; Parenicová *et al.*, 1998; Parenicová *et al.*, 2000a; Parenicová *et al.*, 2000b). The appearance and disappearance of the oligosaccharide products during hydrolysis of PGA are presented in a product progression profile (PPP). These PPPs are divided into two groups, namely the one being presented by endo-PGI and the one being presented by endo-PGII. The enzyme endo-PGII was demonstrated to act as a random acting enzyme as it produced large products at the early stage of the reaction. The enzyme endo-PGI was demonstrated not to be a fully random-acting enzyme (and thus a processive enzyme) based on the predominant formation of the monomer GalA at the initial stage of the reaction.

Also the action of this enzyme towards a hexamer (GalA$_6$) confirmed a processive behavior (Benen *et al.*, 1999; Benen *et al.*, 2003b).

With the aid of the amino acid sequence alignments of the *A. niger* endo-polygalacturonases, as well as the structure of endo-PGII, it was found that an arginine at position 96 (Arg96) in the endo-PGI group was probably accountable for the processive behavior of these enzymes. For the random acting enzymes (endo-PGII group) the Ser91 residue was found at the equivalent position of the processive enzymes. By replacement of Arg96 for a Ser96 in endo-PGI and Ser91 for a Arg91 in endo-PGII the processive behavior was interchanged between these two enzymes. While endo-PGI-R95S behaved as a random acting enzyme, endo-PGII-S91R possessed properties of a processive enzyme (van Pouderoyen *et al.*, 2003).

3.2 Function of pectinases in the food industry

Pectinases from food-grade fungi, such as *A. niger*, are used predominantly as processing aids in the manufacture of fruit juices. The application of these exogenous pectinolytic enzymes improves and influences the efficiency of the fruit juice process, which cannot be achieved alone by the endogenous enzymes that occur naturally in the fruit. Upon grinding of the raw fruit, pectinases are added which reduce the viscosity of the pectin-rich crude juice, also known as pulp enzyming, and therefore improve the processing capacity and the yield of the fruit juice (Benen and Voragen, 2003a).

While in the early 1930s enzyme mixtures of various glycosidases were used, nowadays it becomes possible to use tailor made enzyme preparations for specific applications. For instance, food-grade fungal enzyme preparations which mainly contain endo-polygalacturonase activity can be used to produce pulpy nectars in a process called maceration. These nectars are known for their higher content of fruit solids, pigments and nutrients compared to those which are processed thermo-mechanically (Voragen *et al.*, 1992). The production of fruit juices can also be achieved by liquefaction of the fruit tissues. In this process the fruit tissues are solubilized by using a broad spectrum of polysaccharide degrading enzymes, such as pectin, hemicellulose, and cellulose degrading enzymes (Grassin and Fauquembergue, 1996; Uhlig, 1998; Benen and Voragen, 2003a). The liquefaction technique is relatively simple and economical for the manufacture of high yields of clear or cloudy juices. It is particularly useful for those fruits, such as mango, guava and banana, from

which no juice can be obtained by pressing (Benen and Voragen, 2003a). Liquefaction also limits losses of nutrients which occurs when mechanical pressing is applied, as for instance for the manufacture of carrot juice (Voragen *et al.*, 1992).

4 Aim and outline of this thesis

The aim of this thesis was to biochemically identify and characterize pectinases, which belong to the glycoside hydrolase family 28 of *A. niger*, that became available in the Carbnet project. Special emphasis was put on the characterization of the xylogalacturonan hydrolase (XGH). This enzyme was discovered in *A. tubingenisis* (Van der Vlugt-Bergmans *et al.*, 2000), and subsequently cloned and over-expressed in the *A. niger* "PlugBug" (van Dijck, 1999). Although XGH originates from *A. tubingensis*, similar characteristics are also expected for XGH from *A. niger* as these enzymes have an amino acid sequence identity of 97 % (Chapter two).

Identification and characterization of the pectinolytic enzymes from this family was realized by investigating their activity towards several different pectin substrates which were chemically and/ or enzymatically modified. Other pectinolytic enzymes from *A. niger* such as pectin, pectate lyases, rhamnogalacturonan lyases, as well as accessory enzymes of this species, such as pectin esterases, endo- and exo-arabinases, were not covered in this thesis.

The entire set of the pectinolytic glycoside hydrolase family 28 from *A. niger*, as obtained by bioinformatics tools, micro-array techniques, protein sequencing, and biochemical identification methods using pectin substrates, is illustrated in Chapter two. As described in Chapter three, the enzyme xylogalacturonan hydrolase (XGH) was further investigated for its action towards a xylogalacturonan (XGA) derived from gum tragacanth in order to understand its mode of action towards this substrate analogue. As a side project of Carbnet, the action of XGH was further studied towards XGA in the saponified modified "hairy" regions of apple and potato pectin (Chapter four) which also enabled us to study the structural features of this polymer from both sources. Also the presence of XGA in plant cell walls (*Arabidopsis thaliana*), other than storage or reproductive tissues, was demonstrated by using this enzyme (Chapter five).

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Chapter 2

A new group of exo-acting family 28 glycoside hydrolases of *Aspergillus niger* that are involved in pectin degradation

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Abstract

The fungus *Aspergillus niger* is an industrial producer of pectin degrading enzymes. The recent solving of the genomic sequence of *A. niger* allowed an inventory of the entire genome of the fungus for potential carbohydrate degrading enzymes. By applying bioinformatics tools 12 new genes putatively encoding family 28 glycoside hydrolases were identified. Seven of the newly discovered genes form a new gene group, which we show to encode exo-acting pectinolytic glycoside hydrolases. This group includes four exo-polygalacturonan hydrolases (PGAX, PGXA, PGXB and PGXC) and three putative exo-rhamnogalacturonan hydrolases (RGXA, RGXB and RGXC). Biochemical identification using polygalacturonic acid and xylogalacturonan as substrates demonstrated that indeed PGXB and PGXC act as exo-polygalacturonases while PGXA acts as an exo-xylogalacturonan hydrolase.

The expression levels of all 21 genes were assessed by microarray analysis. The results from this study demonstrate that exo-acting glycoside hydrolases play a prominent role in pectin degradation.

Introduction

Pectin is a complex heteropolymer present in the middle lamella of the primary cell wall of plants. This biopolymer accounts for about one-third of the total cell wall material [1] and as such represents an important carbon source for bacteria and fungi. Pectin is composed of a number of distinct polysaccharides [2], such as homogalacturonan (HG), xylogalacturonan (XGA), rhamnogalacturonan I (RGI), and rhamnogalacturonan II (RGII). Rhamnogalacturonan carries short and long, linear and branched side chains which are built up from neutral sugars (e.g. arabinose, galactose).

Two pectin models exist, namely a pectin structure in which HG is an extension of RGI or vice versa and has neutral sugar side chains [3], and a pectin structure that is built from a backbone of RGI where HG and neutral sugars form the side chains [2].

HG, also known as the smooth region of pectin, is an unbranched polymer composed of 1,4-linked α-D-galacturonic acid residues. XGA, RGI and RGII are part of the branched or "hairy" regions of pectin [4]. In XGA xylose is linked at *O*-3 of the galacturonyl residues of the HG backbone. Rhamnogalacturonan I (RGI) is composed of a backbone of alternating α-(1, 2)-linked rhamnosyl and α-(1, 4)-linked galactosyluronic acid residues [2]. The rhamnosyl residues are branched with *O*-4 attached neutral sugar side chains which can vary from a single galactose residue up to polymeric chains of glycosyl residues composed of arabinose and / or arabinose and galactose residues. Rhamnogalacturonan II (RGII) is comprised of a backbone of approximately nine α-(1, 4)-linked D-galactosyluronic acid residues that carry four side chains, containing rare sugars, such as apiose and aceric acid [2].

The biodegradation of this complex and heterogeneous structure of pectin requires many different enzymatic activities. Exo- and endo-polygalacturonan hydrolases (exo- and endoPG), pectin lyases (PL) and pectate lyases (PLY) degrade HG. XGA can be degraded by xylogalacturonan hydrolase [5] and exo-PG [6, 7], whereas rhamnogalacturonan hydrolases (RGH) [8] and rhamnogalacturonan lyases (RGL) [9, 10] degrade RGI. In addition, the complete enzymatic depolymerisation of pectin requires the presence of different types of esterase activities [11].

The industrially used saprobic fungus *Aspergillus niger* is an excellent producer of pectinolytic enzymes which, unlike those produced by many other fungi of the same genus, enjoy a GRAS status. So far 9 genes encoding pectinolytic glycoside hydrolases produced by

A. niger have been cloned and the corresponding enzymes have been characterised in detail. The list includes seven different endopolygalacturonan hydrolases [12, and references herein], and two rhamnogalacturonan hydrolases [8], all belonging to family 28 of the general classification system of glycoside hydrolases [13].

The structures of two *A. niger* endo-polygalacturonan hydrolases, PGI and PGII, have been solved [14, 15]. This, together with the biochemical data obtained from site directed mutagenesis of strictly conserved amino acids, allowed the identification of the residues involved in catalysis, substrate binding, substrate specificity and mode of action of PGII [16-18].

After the recent sequencing of the genome of *A. niger*, it became clear that only a part of the pectinase spectrum is currently explored. To obtain a complete inventory of pectinolytic glycoside hydrolases produced by *A. niger*, we applied bioinformatics and functional genomics tools. We were able to identify a total of 21 genes which belong to family 28 glycoside hydrolases. Their transcriptional levels were assessed using custom made Affymetrix gene chip DNA microarrays. Proteins from four newly discovered genes, encoding for novel exo-activities, have been overexpressed and their activities have been biochemically identified.

Materials and methods:

Bioinformatics

Genome mining

The genome of *Aspergillus niger* strain CBS513.88, which is a natural derivative of strain NRRL3122, has been recently sequenced [19]. A list of accession numbers for currently available protein sequences belonging to family 28 glycoside was obtained from the CAZy web-server [20] at http://afmb.cnrs-mrs.fr/CAZY/. The corresponding 309 protein sequences were retrieved from the SWISS-PROT database [21] at http://www.expasy.org/sprot/ and used to build a hidden Markov model profile using the HMMER package [22] from http://hmmer.wustl.edu/. The genome of *A. niger* was screened with the obtained profile using the WISE 2 package [23] from http://www.ebi.ac.uk/Wise2/.

Sequence analyses

2D alignments of identified protein sequences were performed using the T-coffee program [24] and manually curated. Dendrograms and distance measurements were performed using the Mega 3 software package [25]. Sequences were compared using the pairwise analysis algorithm with distances only computed setting. Gaps/Missing Data were calculated by the pairwise deletion algorithm. Substitution model used was amino p-distance with all substitutions included. Homogeneous pattern among lineages and uniform rates among sites were applied. Dendrograms were constructed by using the Neighbour-Joining Method with the same distance parameters as described above. The nucleotide sequences reported in this paper have been submitted to the DDBJ, EMBL and GenBank® Nucleotide Sequence Databases under the accession numbers DQ374422 to DQ374431, DQ417225 and DQ417226.

Verification of intron positions by RT-PCR

Total RNA was isolated from frozen ground mycelia using TRIZOL® Reagent (Invitrogen™) according to the instructions of the supplier. RNA concentrations were estimated using a NanoDrop® ND-1000 Spectrophotometer. cDNAs of *pgx*C and *rgx*A were amplified by two step RT-PCR. In the first step, 1.5 µg total RNA from 2 h transfer cultures on D-galacturonic acid and L-rhamnose, respectively were used as a template in the reverse transcription reaction. Omniscript® reverse transcriptase (QIAGEN) was used as described by the supplier. Second step RT-PCR was performed with 1µl product from the reverse transcription reaction

using 2.5 units SuperTaq Plus polymerase (Sphaero Q, Leiden, The Netherlands) under standard PCR conditions. Prior to sequencing, two overlapping fragments per messenger were amplified with the following primers: for the amplification of pgxC 5'fragment: pgxCAFw 5'-CGTCATGTCTGTCTTCAAGG-3' and pgxCBRv 5'-TGATACTGTCGGTGATCTGG-3'; for the amplification of pgxC 3'fragment: pgxCDFw 5'-CACTGCGGTGCAGAATATAG-3' and pgxCCRv 5'-GCGTTCATGCAGATCACACT-3'; for the amplification of rgxA 5'fragment: rgxAAFw 5'-GGTATCGAGGTGAGCCAGGA-3' and rgxABRv 5'-TATGGTCGGATCGGTACGTG-3'; for the amplification of rgxA 3'fragment: rgxADFw 5'-GGCTGTTCTTGTCGATTCCT -3' and rgxACRv 5'-CAGCGACAGCTCACGAATTG -3'; RTPCR products were gel purified with QIAquick® gel extraction kit (QIAGEN) and outsourced to BaseClear Labservices (BaseClear B.V., Leiden, The Netherlands) for sequence determination.

Transcriptional profiling

Strains and Growth Conditions

Wild type strain *Aspergillus niger* N400 (CBS 120.49) was used in all transcriptional profiling experiments.

300 ml minimal medium [26], pH6.0, containing 0.1% (w/v) yeast extract and Vishniac trace elements [27] with 2% (w/v) D-fructose as a sole carbon source was inoculated with 10^6 spores/ml and cultivated at 30°C at 250 rpm in an orbital shaker. After 18 h of incubation mycelium was harvested on a Büchner funnel with nylon gauze, washed once with sterile 0.9% (w/v) NaCl and aliquots of 1.5 g (wet weight) mycelium were transferred to 50 ml minimal medium, pH6.0, 0.1% (w/v) yeast extract, Vishniac trace element solution and 1% (w/v) of the various sole carbon sources: D-fructose (Merck), D-glucose (Merck), D-galacturonic acid (Fluka Chemica), L- rhamnose (ACROS organics), D-xylose (Merck), D-sorbitol (Merck), polygalacturonic acid (United States, Biochemical Corp.) and sugar beet pectin (GENU, Copenhagen pectin). At 2, 4, 8 and 24 h after transfer mycelium was harvested on a Büchner funnel with nylon gauze and immediately stored at -70°C. The amount of monomeric sugars remaining in the culture fluid was assessed by standard HPLC techniques [7].

RNA manipulations and microarray processing

Before and during microarray processing RNA quality was verified by analysing aliquots with 1% TAE agarose gel electrophoresis and Agilent Bioanalyzer "Lab on chip" system (Agilent Technologies, Palo Alto, CA). Messenger RNA levels were assessed using custom made "dsmM_ANIGERa_coll" Affymetrix GeneChip® Microarrays which were kindly provided by DSM Food Specialties (Delft, The Netherlands).

Probe labeling, hybridisation and scanning

Total RNA from mycelium samples was amplified, labeled and hybridized strictly following the Affymetrix protocols for "Eukaryotic Target Preparation" and "Eukaryotic Target Hybridization". For probe array wash and stain, the "Antibody Amplification Washing and Staining Protocol" was used. Probe arrays were scanned with Agillent technologies G2500A Gene Array Scanner at pixel value 3 μm and wavelength 570 nm. Raw intensity measurements and present/absent calls were derived in Microarray Suite Software version MAS5 (Affymetrix, UK Ltd) after applying "Mask all outliers" algorithm. All chip data were scaled to an arbitrary target gene intensity of 500.

Cloning and overexpression of enzymes

PGXA, PGXB and RGXB were overexpressed in *A. niger* PlugBug® [28] and kindly supplied by DSM Food Specialities (Delft, The Netherlands).

Cloning and overexpression of PGXC

Molecular work was essentially carried out using standard techniques [29]. All enzymes were used according to the instructions provided by the supplier. *A. niger* genomic DNA was isolated as described [30] 1.5 kb PCR fragment containing the *rgx*C gene was amplified using primers PGXCFw 5'-TGG CAT GGC AAT TGG AGA CC-3' and PGXCRv 5'-GTG TGG CTT CCT GTG GAT GG-3'. The obtained product was used as a template in a subsequent nested PCR reaction using primers PGXC3NsiIFw 5'-GCA TCG TCA TGC ATG TCT TC-3'and PGXCNotIRv 5'-TGG CGG CGG CTG GAT GGC TTA-3' for the introduction of restriction sites NsiI and NotI at the ATG start codon and 73 bp downstream the stop codon of the pgxC gene respectively. Both PCR and nested PCR were performed using Pfu Turbo Polymerase (Stratagene). The nested PCR product was NsiI/NotI digested, gel-purified using

QIAquick® gel extraction kit (QIAGEN) and ligated in plasmid pIM3710 [31] resulting in pgxC overexpression vector pIM5150.

Transformation of *A. niger* NW188 (*csp*A1, *pyr*A6, *leu*A1, *prt*F28, *gox*C17) was performed as described [32] using 1 μg of pGW635 [33] and 20 μg of co-transforming pIM5150. Control transformation with 1 μg of pGW635 was performed in parallel. Positive transformants determination and enzyme production were performed as described [34] with the following modifications: STREAMLINE SP and Citrate buffer pH 3.8 were used instead of Sephadex DEAE and 10 mM piperazine/HCl. Following dialysis further purification steps were performed as described below.

Enzyme purification and gel electrophoresis

PGXA, PGXB, PGXC, and RGXB, were purified by dialysis overnight at 4°C against 50 mM NaAc (pH 5.7) and subsequent size-exclusion chromatography on a Hiload 16/60 Superdex 75 column (Amersham Pharmacia Biotech, Uppsalsa, Sweden) equilibrated with 50 mM NaAc (pH 5.7). The elution was carried out with the same buffer at a flow rate of 1 mL/min, and fractions of 2 mL were collected. Protein homogeneity was determined by SDS-PAGE on a 8-25% polyacrylamide gradient gel under reducing conditions, using the PhastSystem and the protocol of the supplier (Amersham Pharmacia Biotech, Uppsala, Sweden). The gels were stained by Coomassie Brilliant Blue. Low molecular mass standard proteins (14.4-94 kDa) were used to determine the molecular mass of the purified enzymes.

Substrates

PGA and XGA

Xylogalacturonan (XGA-25) was prepared from gum tragacanth by treatment with alkali and trifluoro acetic acid (TFA) and had a Xyl:GalA ratio of 1:4 [35]. Polygalacturonic acid (PGA) was from ICN Biomedials Inc., (Ohio, US). As substrates for rhamnosidase activity, p-nitrophenyl-α-L-rhamnopyranoside (pnp-Rha; Sigma Chemical Co, ST Louis, USA), naringin (Sigma Aldrich, Steinheim, Germany), and hesperidin (Fluka, Steinheim, Germany) were used.

Amidated and methylated pectin

Three commercial pectins, A, B and E extracted from lemon peels were obtained from Degussa Texturant Systems SAS (Baupte France). These pectins are partially methyl-esterified with a degree of methyl-esterification (DM) of 74%, 72 % and 30% respectively, where the distribution of methyl-esters is blockwise for pectin A and random for pectin B [36].

Two other commercial pectins, O5 and O27, were obtained from DANISCO. These pectins have a degree of amidation (DA) of 5% and 27% respectively. Prior to use, the methyl esters were removed from O5 and O27 by saponification [37]. Saponified amidated pectins are referred to as O5sap and O27sap.

Linear rhamnogalacturonan oligosaccharides

Saponified apple MHR (a-MHR-S) was prepared as described [38]. Enzymatic liquefaction of apples for the preparation of a-MHR-S was performed with a commercial enzyme preparation (Ultra-SP; Novozymes, Copenhagen, Denmark). This substrate was further treated with rhamnogalacturonan hydrolase (RGH) and fractionated by preparative size exclusion chromatography (HPSEC) as described [39]. Fractions 45-60, which contained oligomeric products, as determined by HPSEC analysis, were pooled and freeze-dried. As this material still contained some polymeric residue, further fractionation was performed by gel filtration on a Sephadex G50 column (GE Health Care, Uppsala, Sweden), using Millipore water as eluent at a flow rate of 1 mL/min. Fractions 89-100, which contained primarily oligosaccharides, as determined by HPSEC analysis, were pooled and subsequently freeze-dried. This pool is referred to as a-MHR-S-fr1. Since the activity of a RG-rhamnohydrolase and a RG-galacturonohydrolase towards rhamnogalacturonan oligosaccharides is hindered by Gal side chains [40], a-MHR-S-fr1 was treated with β-galactosidase [41] for 19 h at 40°C in 20 mM bis-Tris (pH 5). The final protein concentration was 2.2 μg/ml. The enzyme was inactivated by heating for 10 min at 100ºC. Subsequently, β-galactosidase treated a-MHR-S-fr1 (a-MHR-S-fr1-g) was desalted on a Biogel P2 column (Bio-Rad, CA, U.S.A.) using Millipore water as eluens at a flow rate of 1 mL/min. After desalting, a-MHR-S-fr1-g was analyzed by MALDI-TOF MS [39], for the presence of rhamnogalacturonan oligosaccharides. Four linear rhamnogalacturonan oligosaccharides were detected at m/z 539, m/z 685, m/z 861 and m/z 1007. Based on the mode action of RG hydrolase towards rhamnogalacturonan it is

concluded that the produced rhamnogalacturonan oligosaccharides with a m/z at 685 and m/z at 1007 correspond to Rha-GalA-Rha-GalA and GalA-Rha-GalA-Rha-GalA respectively. The rhamnogalacturonan oligosaccharides at m/z 539 and at m/z 861 correspond to GalA-Rha-GalA and GalA-Rha-GalA-Rha-GalA. As these latter two oligosaccharides cannot be produced directly from RG hydrolase treated rhamnogalacturonan it is assumed that these oligosaccharides are formed due to side activities in the RG hydrolase preparation, in particular RG-rhamnosidase activity

Enzyme activity measurements

PGXA, PGXB, PGXC and RGXB were analyzed for their activity towards 0.25% (w/v) PGA, 0.25% (w/v) XGA-25, and 0.25% (w/v) of the pectins, encoded as A, B, C, O5sap, and O27sap. Exo-PG from *Aspergillus tubingensis* [42] was included as a reference. The action of RGXB was also studied towards 0.25% (w/v) narigin, 0.25% (w/v) hespiridin, 0.02% (w/v) α-L-pnp-Rha, and 0.5% (w/v) a-MHR-S-Fr1-g. For comparison, this latter substrate was also treated with rhamnogalacturonan galacturonohydrolase from *A. aculeatus* [43]. All digests were made in 50 mM NaAc (pH 4.0) for 16 h at 30°C. The final protein concentration was 1 μg/mL. Enzymes were inactivated by heating the reaction mixtures for 10 min at 100°C. All digests were analyzed by HPSEC and HPAEC, as described [44], except for RG-galacturonohydrolase or RGXB treated a-MHR-S-Fr1-g which were analyzed by MALDI-TOF MS as described [39]. Products released from XGA-25 and PGA after 1 h of incubation with PGXA, PGXB, PGXC or Exo-PG were also analyzed by HPAEC.

The specific activity of PGXA, PGXB, PGXC and Exo-PG was determined towards 0.25% (w/v) PGA and 0.25% (w/v) XGA-25, as described [34]. Specific activity is in units per mg of enzyme (U/mg), where one unit is defined as one micromole of reducing sugar released per minute. The influence of pH on enzyme activity was determined as described [34].

Identification of RGXC protein sequence by MS Analysis

Tryptic digest of RG-galacturonohydrolase from A. aculeatus

Protein concentration of RG-galacturonohydrolase [43] was determined by the Bradford assay (Bio-Rad, Hercules, CA) and this enzyme was digested with trypsin as described [45]. The tryptic digest was subsequently acidified to a pH 3.0 by the addition of 10% formic acid. The sample was then analysed by nano-RPLC/MS/MS.

Nano-LC- nano-spray mass spectrometry

LC-MS/MS analysis of the tryptic peptides was performed on an integrated Famos/Switchos/Ultimate 1D/2D nano-flow HPLC system (LC Packings, a division of Dionex, San Francisco, CA, USA), as described [46] with the following adaptations: The peptides were first trapped on a LC Packings C18 precolumn (300 μm i.d. x 5 mm, 100 Å) and eluted after desalting onto a Pep Map C18 resolving column (3 μm, 100 Å, 75 μm I.D. x 15 cm, LC Packings, Dionex) using the following elution profile: a linear gradient of increasing acetonitrile concentration in water (5-30%) over 40 min, isocratic at 30% for 5 min, a linear gradient (30-95%) over 30 min, isocratic at 95% for 5 min. All eluents contained 0.05% formic acid as the ion-pairing agent.

The electrospray needle was operated with a voltage differential of 3.0 kV, and the heated desolvation capillary was held at 180°C. The specific mass-to-charge value of each peptide sequenced by tandem mass spectrometry was excluded dynamically from reanalysis for 2 min.

Analysis of MS/MS and MS3 data

Data analysis of each raw tandem spectrum was performed as described [47], with adaptations. Processed tandem mass spectra were correlated with the various public protein databases and the *A. niger* protein database (DSM, Delft, The Netherlands) using the program Bio Works 3.1 (Thermo Finnigan). The allowed mass tolerance range between expected and observed masses for search was +/- 1.0 Da for MS peaks, and +/- 0.1 Da for MS/MS fragment ions. The correlation results were then filtered using the value of the cross-correlation score and the matched sequence for each spectrum. For singly charged peptides, spectra with a cross-correlation score to a tryptic peptide ≥ 1.5 were retained. For multiply charged peptides, spectra with a cross-correlation to a tryptic peptide ≥ 2 were retained. All spectra with cross-correlation scores not meeting these criteria were eliminated from further consideration.

Results and discussion

Genome mining

Screening the genome sequence of *A. niger* with a hidden Markov model profile for glycoside hydrolase family 28 returned 21 significant alignments from which only 9 correspond to already known *A. niger* proteins. The start and the end of each alignment were extended to the nearest in frame start-codon and nearest stop-codon respectively. Further analysis of the Gene Wise output indicated possible frame shifts in the sequences of *pgx*C and *rgx*A therefore the corresponding cDNAs were sequenced full length. Comparison of the obtained cDNA sequences with the predicted gene models demonstrated the presence of one frame shift in the genomic sequence of *rgx*A and 3 frame shifts in the genomic sequence of the *pgx*C. These corrected sequences were used for further analysis.

Comparison of *A. niger* glycoside hydrolases

The protein sequence alignment of the complete *A. niger* glycoside hydrolase family 28 and the corresponding sequence distance dendrogram are presented in Figure 1.

B

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                340             360           380      +1 +1  400              420       +1-1    440
                *.  *            .**                  *                                 .
PGII  ---GGHNTDAFDVGNSVGVNIIK-PWVHNQDDCLAV--NSGENIWFTGGTCI-GGHGLSIGSVGVDRSN--NVVKNVTIEHSTVSNSEN-----AVRIKTIS--------- :234
PGI   ---GGHNTDGFDISESTGVYISG-ATVKNQDDCIAI--NSGESISFTGGTCS-GGHGLSIGSVGGRDD--NTVKNVTISDSTVSNSAN-----GVRIKTIY--------- :236
PGA   ---GGHNTDGFDISESTYITITG-ATVKNQDDCVAI--NSGENIYFSGGTCS-GGHGLSIGSVGGRDD--NTVKNVTFIDSTVSDSEN-----GVRIKTVY--------- :235
PGB   ----GHNTDAFDIGQSTYITIDG-ATVYNQDDCLAI--NSGEHITFTNGYCD-GGHGLSIGSIGGRSD--NTVNDVTISNSKVLNSQN-----GVRIKTIY--------- :233
PGE   --EAA-NTDGFDIGDSTYITITG-ANVYNQDDCVAV--NSGENIYFSGGVCS-GGHGLSIGSVGGRDD--NTVKNVTFYDSEIKSSQN-----GVRIKTIY--------- :240
PGC   -DLAA-NTDGFDIGESTYITITG-AEIYNQDDCVAI--NSGENIYFSASVCS-GGHGLSIGSVGGRDD--NTVKNVTFYDVNVLKSQQ-----AIRIKTIY--------- :241
PGD   GDPAAHNTDGFDIKQSDFLTLSN-SWVHNQDDCVAV--TSGSSIVVDNLYCY-GGHGLSIGSIGGKSN--NTTVDGVTFSNSQVINSEN-----GCRIKSNA--------- :381
XGH   --NPPKNTDGFDIGESTYVTITE-VTVVNDDDCVALKPSS-NYVTVDTISCT-GSHGISVGSLGKSSD--DSVKNIYVTGATMINSTK-----AAGIKTYPSGG------ :274
PGXC  -GSFPFNTDGFD-VEGTNIQITD-SIMYNGDDAIAVGADSHD TLFTRNTIGY-QTHGMSIGSLGKDPTDFANISNIRFDDVTVVDGLY------AARFKSWSG-------- :275
PGXA  -DAPAKNTDGWDIYRSSNIVIQD-SRIVNTDDCVSFKPNSTQIVI-QNLDCT-GSHGISVGSLGQYQGETDIVEDLYIYNISMTDASD-----VARIKVWPGVPA----- :294
PGAX  -DNEAKNTDGWDTYRSNNIVIQN-SVINNGDDCVSFKPNSTNILV-QNLHCN-GSHGISVGSLGQYKDEVDIVENVYVYNISMFNASD------MARIKVWPGTPS----- :289
PGXB  -SSDPANTDGMDSLDVDGVSFTN-MRIDVGDDCFSPKPNTTNIFV-QNMWCN-NTHGVSMGSIGQYAGEMDIIENVYIENVTLLNGQN-----GARLKAWAGQDV----- :306
RGXC  GDNWVQNTDGFDTMDATNIQLTN-FVYQGGDDCIAIKPRSYNIDI-QNVTCR-GGNGIAIGSLGQYL-EDSSVANIRVDKVNIIRYNED-MHNSAYLKTWVGALVPQSSY :284
RGXB  DGGSAQNTDGANTIYSKNITLTN-WEVVNGDDSISTKANSTDITI-ANCTFT-SGLGIAIGSIGQYNGAFETVERLKISNITYEKTTH-----AVYFKTWTGDQV----- :306
RGXA  EGEWVQNTDGIDTYRSDHITVTN-WVYQGGDDAVAFKGNSTNIHV-ENVTVY-GGPGIAFGSLGQYPDRTDI VENVTVRNVRVQPSFQRAMNSGVYFKSWIGVNY----- :288
RGA   ------GLDGIDVW-GSNIWVHD-VEVTNKDECVTVKSPA-NNILVESIYCN-WSGGCAMGSLGAD----TDITDILYRNVYTWSSNQ-----MYMIKSN---------- :256
RGB   ------GLDGIDVW-SNNIWVHD-VEVTNKDECVTVKSPS-KNILIESIYCN-WSGGCGMGSFGSD----TNVSDITYRNIY TWSSNN-----MMLIKSN---------- :256
RGD   ------GLDGIDIS-GANYWIHD-VEVTNGDECVTVKSPS-ANVRVENVFCN-HSGGCAMGSLGTD----TNISNIEFENIYTYNSTQ-----MYMIKSN---------- :257
RGC   ------ATDAIDVW-GENMWFHD-IEVSNGDECVTVKSPA-HNYLIENIYCN-LSGGTAIGSLGTG----TNISDIHYRNLYM-NQAD-----ACFLKSN---------- :255
RGE   ------GLDGIDLY-GGNIWIHL-VMVTNKDECVTSKTNS-HNFLIENIYCN-PSGGCAIGSLGSS----VNVTNILYRNVYTWDSNQ-----MMMIKTN---------- :258
RGF   ------SYDGIDAI-GTNYYIHD-NEVTNRDECVSVKSPS-HHALVENLVCNQAGSGVSIGSLNVS----AEISNIEARNISIIQGNN-----IAFIKTYP--------- :255
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                460          480          500              520          540
                             +1            .                                        #      #
PGII  ---------GATGSVSEITYSNIVMSGISDYGVVIQQD YEDGKPT----GKPTNGVTIQDVKLESVTGSVDSGA-----------------------TEIYLLCGS-GSC :307
PGI   ---------KETGDVSEITYSNIQLSGITDYGIVIEQD YENGSPT----GTPSTGIPITDVTVDGVTGTLEDDA-----------------------TQVYILCGD-GSC :309
PGA   ---------DATGTVEDITYSNIQLSGISDYGIVIEQD YENGDPT----GTPSNGVTISDVTLEDITGSVDSDA-----------------------VEIYILCGD-GSC :308
PGB   ---------GKTGTVENVKFEDITLSDISKYGIVVEQD YENGSPT----GTPTNGVKVEDITFKKVTGSVKSSG-----------------------TDIYILCGS-GSC :306
PGE   ---------GDTGSVSEVTYKEITLSDITDYGIVVEQN YDDTS------KSPTDGITIEDFVLDNVQGSVESSG-----------------------TNIYIVCGS-DSC :311
PGC   ---------GDTGSVSEVTYHEIAFSDATDYGIVIEQN YDDTS------KTPTTGVPITDFVLENIVGTCEDDD-----------------------CTEVYIACGS-GSC :313
PGD   ---------DTTGEVYNVKYENITLSGISDYGIDIQQD YENGAT----GDPTNGVKIENISFVNVKGTMSDGK-----------------------DYYILCGD-GSC :453
XGH   --------DHGTSTVSNVTFTDFTVDN-SDYAFQIQSCYGE-DDDYCE-ENPGNA-KLTDIVVSSFSGTTSDKY--------------------DPVVANIDCGSDGTC :351
PGXC  --------GTGLVKNVTWNNIRVFN VT-FPIFVTQSYSDQGASRSGTVNASSAVMMEDFTWSDFAGSINTYQPGDGSCVSDPCWYNVGLPNLKHTEALIIE CHTAQSC :374
PGXA  DTSGS-TSGGGLGRVRNVTYEHMQSENND-HIISVSQCYESKNQTMCDSY-P-SKLVIEDVLFKDFKGTTSK------------------KYDPEIGELTCSSPDVC :379
PGAX  ALSADLQGGGGSGSVKNITYDTALIDNVD-WAIEITQCYGQKNTTLCNEY-P-SSLTISDVHIKNFRGTTSG------------------SEDPYVGTIVCSSPDTC :375
PGXB  ---------GYGRINNVTYKNIQIQNTD-APIVLDQCYFDINATECAKY-P-SAVNITNILFENIWGSSSG-----------------KDGKIVADLVCSPDAVC :382
RGXC  E-SAGVPRGDGWGSIRNVLFSNFNVQGAS-AGPSISQDSGDN-----GSYAGTSKMSISNVAFVNFTGWVD-----------------TEKSVVSTVSCSEVHPC :365
RGXB  GYPPN-GGGGGLGYASDIVATNLKTNNLKGAPFTISQCTTFSGAS---GNCTNSKFQIRDLVFTDISGTTDS-----------------SDVASFQCSAVAPC :388
RGXA  GVPPN-GGGGGHGYVRNVSVENLRLKDVQ-LPVYIDTCLSYLFSENITQYCDTSTYE FEDLHFRNISGNGLA-------------TVTDYPGKNISFAVALLCSEKAPC :382
RGA   ---------GGSGTVNNTVLENFIGHGNA-YSLDVDSYWSSMTA------VDGDGVQLSNITFKNWKGTEADGA--------------------ERGPIKVVCSDTAPC :329
RGB   ---------GGSGFVENVLLENFIGHGNA-YSLDIDSYWASMSA------VDGDGVQLSNITVKNWK GTEAYGA--------------------ERGPVKVVCADGAPC :329
RGD   ---------GGNGTVTNCSFKNFIGYSNA-YMLDLDTYWGDES--------DGDGIKYENIGFENWKGTSSNGI--------------------QRSPIRILCPDANPC :328
RGC   ---------NGDGIVKNIIWENVIVHGGP-YPLAIDEAWGDDRG--------SVGVQVSNLTFRNWHGESVSA---------------------SRPVIRLQCDSDVPC :325
RGE   ---------GGLGNVSNIVFENFIGHGNV-NSLDLDSYWSSMNA------IDGVGIYYHNITIYNWTGTAIDGE---------------------TRPPIRVICPEDMPC :331
RGF   ---------GGSGYVKDVTFENFR SLNSL-YGLDINQYWQNTWE------PDTGSVTLSNLVFKNFSGSVADGA---------------------LRPPLYLFASDLTFA :328
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Figure 1. Comparative sequence analysis of *A. niger* family 28 glycoside hydrolases. (A) Dendrogram of *A. niger* family 28 glycoside hydrolases. EndoPG: endo-polygalacturonan hydrolases; XGH: xylogalacturonan hydrolase; Exo-PG: exo-polygalacturonan hydrolases; ExoRG: putative exo-rhamnogalacturonan hydrolases; EndoRG: endo- rhamnogalacturonan hydrolases. The average pair wise distance within the different functional groups of *A. niger* hydrolases is indicated in brackets. Biochemically identified enzymes are in bold. * - *A niger* enzyme amino acid sequence that are greater than or equal to 97% identical to that of a previously characterized *A. tubingensis* enzyme. (B) Excerpt of the multiple alignment of *A. niger* family 28 glycoside hydrolases. * - columns containing catalytic residues; # - Conserved cysteine bridge; +1 - columns containing residues involved in substrate binding at subsite +1; -1 - columns containing residues involved in substrate binding at subsite -1. The fully conserved amino acids Asp339, Asp361, and Lys428 are shown in bold and italics. The partially conserved amino acids Asp342, Asp362, His386, Ser389, Arg426, and Tyr479 are marked in grey.

Based on sequence similarity three major groups are observed (Fig. 1 A). The first group is composed of all previously known endopolygalacturonases (endoPG) of *A. niger*. No newly identified sequences are added to this group. The second group contains six newly discovered open reading frames (ORF) of *A. niger* of which one designated PGAX is a very close homolog of *A. tubingensis* exo-polygalacturonase (Exo-PG) [42] with 99% amino acid identity. Based on sequence similarity, the six ORFs can be divided into two sub-groups (Fig. 1A) each comprising three proteins. PGAX forms a subgroup with PGXA and PGXB,

therefore it is very likely that PGXA and PGXB are also exo-polygalacturonan hydrolases. RGXA, RGXB and RGXC form the second distinct subgroup and evidence is presented that they are similar to rhamnogalacturonan galacturonohydrolase [43] (see below). The conservation of intron positions within ORFs of both sub-groups further underpins this subdivision. The most deviating sequence from all 21 identified proteins is PGXC. Also, there are no introns in the encoding gene *pgx*C to support its classification. However, exo-polygalacturonan hydrolase activity was determined for the encoded enzyme (see below). The last group includes two previously characterised endo-acting *A. niger* proteins – rhamnogalacturonan hydrolase A (RGHA) and rhamnogalacturonan hydrolase B (RGHB) as well as 4 putative enzymes which we propose to be endo-rhamnogalacturonan hydrolases. Finally XGH, the strongly conserved homolog (97% amino acid identity) of xylogalacturonan hydrolase of *A. tubingensis* [5] did not group together with any of the other sequences.

Detailed inspection of the amino acid conservation between all 21 sequences revealed that only one of the 4 disulfide bridges between columns 544 and 550 of the multiple alignment (Fig. 1 B) demonstrated by the protein structures of PGII of *A. niger* [14] and RGA of *A. aculeatus* [48] is well conserved within family 28 glycoside hydrolases of *A. niger*. The other three cysteine bridges are well conserved in the groups of endoPGs and endoRGs but not in the exo-hydrolase group. Inspection of the catalytic residues revealed that all previously identified catalytic Asp residues are conserved within the *A. niger* family except for Asp362 (Fig. 1B). In the group of rhamnogalacturonan hydrolases Asp362 is replaced by Glu which is characteristic for this type of enzymatic activity [48]. The substrate binding Lys at alignment position 428 is 100% conserved while the second substrate binding residue Arg426 is present in all endoPG sequences and in only four of the seven proteins from the exo-group, *viz.* PGAX, PGXA, PGXB and PGXC. Armand and co-workers [16] have demonstrated by site directed mutagenesis that the corresponding Arg of *A. niger* PGII (Arg256) is essential for the proper orientation of the substrate in the catalytic cleft and mutations of this residue dramatically affect the substrate affinity of the enzyme. Interestingly, other amino acids shown to be involved in substrate binding at subsite +1, His386 and Ser389, are conserved within the same subset of sequences. The same is true for the Tyr479, which, in PGII, is involved in the stabilisation of the substrate at subsite +1 [16, 49]. The partial conservation of amino acids responsible for substrate specificity and binding within the exo-hydrolase group

further supports the suggestion that only PGXA, PGXB, PGXC and PGAX have exo-polygalacturonan hydrolase activity.

Identification of RGXC as a close homolog of *A. aculeatus* rhamnogalacturonan galacturonohydrolase

A rhamnogalacturonan galacturonohydrolase (RG-galacturonohydrolase), which specifically removes terminal galacturonosyl residues from rhamnogalacturonan I, has been isolated from the commercial mixture Pectinex Ultra SP produced by *A. aculeatus* [43]. In order to identify a possible *A. niger* homolog of this enzyme a tryptic digest of the *A. aculeatus* RG-galacturonohydrolase was analysed by MS. The obtained peptide masses were used to search the inferred *A. niger* proteome. At least 6 peptides perfectly matched the sequence of RGXC resulting in a total of 29% overall coverage of the protein (data not shown). Based on sequence similarity, RGXC is grouped together with RGXA and RGXB indicating that there are three candidate genes encoding proteins with exo-rhamnogalacturonan hydrolase activity.

Transcriptional profiling

The complex substrate sugar beet pectin (SBP) and polygalacturonic acid (PGA) were chosen as primary carbon sources to investigate expression of selected pectinolytic genes. Galacturonic acid, rhamnose and xylose represent the most abundant sugar residues present in homogalacturonan, rhamnogalacturonan and xylogalacturonan and were used to asses the effects on gene expression caused by simple well defined carbon sources. Fructose as a strong repressor of the expression of genes that are under carbon catabolite regulation (CCR) and sorbitol as a non-inducing sugar like alcohol, which does not affect the CCR mechanisms were chosen as control substrates. After transfer from 18 h pre-culture on 2 % fructose, mycelium was sampled at four time points during 24 h growth on the different substrates. The corresponding total RNA samples were used in microarray experiments to monitor the mRNA levels of family 28 glycoside hydrolase encoding genes (Fig 2).
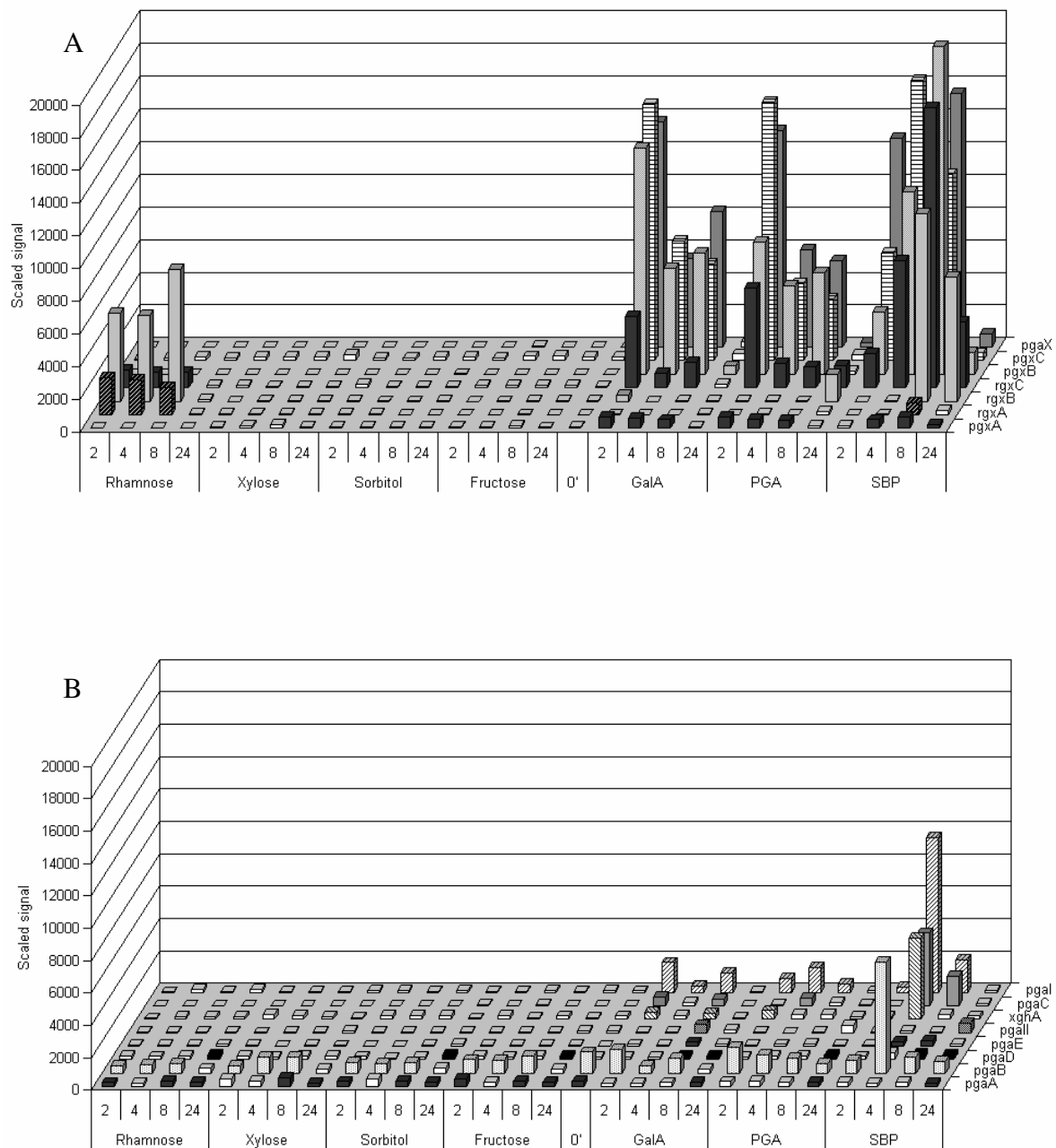
Figure 2. **Transcriptional levels of *A. niger* glycoside hydrolases – 24 hours time course on different carbon sources.**

A – endo-polygalacturonan hydrolases and xylogalacturonan hydrolase. B – exo-acting hydrolases.

Minor axis intervals - time points of harvesting after transfer from fructose (in hours);0' – 18 hours preculture on 2% (w/v) fructose; GalA – galacturonic acid; PGA – polygalacturonic acid; SBP – sugar beet pectin. White colour indicates absent call.

During growth on non-pectic carbon sources, *viz.* fructose and sorbitol, none of the genes encoding for enzymes from the exo-hydrolase group was transcribed. In contrast, transfer from fructose to galacturonic acid and PGA caused a strong induction peak in the first 2 h after transfer for *pga*X, *pgx*B and *pgx*C followed by a decrease to relatively moderate expression levels during the next 8 h and further reduction of the signal to background levels 24 h after transfer, coinciding with the depletion of carbon source. Although less strongly induced, *rgx*C displayed a similar transcriptional profile on galacturonic acid and PGA. This gene was also moderately expressed together with *rgx*A and *rgx*B after transfer to rhamnose (Fig. 2B). Interestingly *pgx*A showed only low expression levels on these carbon sources.

Transfer to SBP caused a more complex transcriptional pattern. *Pga*X was quickly induced and remained highly expressed reaching a maximum 8 h after transfer. *Pgx*C messenger levels reached a maximum 4 h after transfer. *Pgx*B and *rgx*C levels gradually increased and reached a maximum 8 h after transfer. The signals obtained for *rgx*C were much higher on sugar beet pectin compared to rhamnose or galacturonic acid as sole carbon source. SBP caused a similar effect on the induction of the *rgx*B gene. *Rgx*A messenger was hardly detected 8 h after transfer to SBP.

In contrast to the strong induction observed for genes from the exo-group, the transfer to galacturonic acid and PGA did not cause a strong effect on the expression of endopolygalacturonases. SBP moderately induced *pga*I, *pga*B and *pga*C 4 h after transfer, coinciding with the slight accumulation of extra-cellular free galacturonic acid (data not shown).

*Pga*A and *pga*B have been reported to be constitutively expressed [50] which is in agreement with the data obtained in this study (Fig. 2A). Yet a slight increase of *pga*B messenger was observed coinciding with the depletion of fructose during the time course of incubation suggesting that, although weak, *pga*B is under CCR control. In contrast, when pectic substrates were used, the signal of *pga*B decreased in time to reach a background level at 24 h (Fig. 2A). Instead, low levels of *pga*D messenger were detected. PGD, the protein encoded by *pga*D gene, has been proposed to be a cell wall attached oligogalacturonase with high tolerance towards methyl-esterified pectins [12]. HPLC measurements (data not shown) demonstrated that at the time points of expression of *pga*D free sugars are being depleted from the culture fluid, which together with its functional properties, suggests that the

expression of *pga*D may in part be due to carbon starvation. Still, during incubation on galacturonic acid and sugar beet pectin *pga*D messengers could be detected slightly earlier, 8 h after incubation.

Despite many efforts including several genetic screens [51], no specific transcriptional factors regulating the expression of pectinolytic glycoside hydrolases have been identified so far. The strong discrepancy between the general expression profiles observed for the exo- and the endo-hydrolase encoding genes suggests separate or loosely linked regulation mechanisms for both groups. Exo-polygalacturonases are well induced by galacturonic acid and the observed expression levels on pectin, although slightly more elevated, do not differ much from the levels observed on its monomeric substitute. Unexpectedly, galacturonic acid and PGA as a sole carbon source appeared to be only weak inducers for endo-polygalacturonases, while SBP caused profoundly higher expression of endo-PG. In addition, the expression pattern of all studied genes on SBP has a much more complex character compared to monomeric sugars, which suggests that a multi-factorial regulation system and not just galacturonic acid, is responsible for the induction of pectinolytic genes.

Biochemical identification of PGXA, PGXB, PGXC and RGXB

Purified PGXA, PGXB, PGXC and RGXB appeared as a single band upon SDS-PAGE (results not shown) and had an apparent molecular mass of 78 kDa, 67 kDa, 79 kDa, and 82 kDa respectively (Table 1). The difference between the measured and calculated molecular masses of these enzymes is most probably caused by a high degree of glycosylation, which has also been demonstrated previously for the exo-polygalacturonase (Exo-PG) from *A. tubingensis* [42].

Enzyme	Molecular mass (kDa)	Calculated molecular mass (kDa)	Specific activity towards PGA (U/mg)	Specific activity towards XGA (U/mg)	pH-optimum towards PGA
PGXA	78	47	7	25	3.5-4.0
PGXB	67	48	242	33	4.0-4.5
PGXC	79	45	223	202	3.5-4.0
Exo-PG	78[1]	47	230	17	4.2[1]
RGXB	82	50	n.d.	n.d.	n.d.

Table 1. Properties of PGXA, PGXB, PGXC, Exo-PG, and RGXB.

Specific activity of the enzymes were determined towards 0.25% (w/v) PGA and 0.25 % (w/v) XGA in 50 mM NaOAc (pH 4.0). The optimum pH for each enzyme was determined towards 0.25% (w/v) PGA in McIlvaine buffers (over a pH range of 2.5-7.5). n.d.: not determined. [1]As determined by Kester et al. (1996).

The action of PGXA, PGXB, PGXC and RGXB was studied towards PGA, XGA-25, and pectins with various degrees of methylation or amidation. As determined by HPSEC, only PGA and XGA-25 were degraded by PGXA, PGXB and PGXC. Similar results were observed for Exo-PG, which indicates that PGXA, PGXB and PGXC have an exo-polygalacturonase activity. RGXB was not active towards these GalA-containing substrates, indicating that this enzyme is not a polygalacturonase. To investigate whether RGXB exhibits exo-rhamnogalacturonan hydrolase activity, a mixture of rhamnogalacturonan oligosaccharides was treated with RGXB and analyzed by MALDI-TOF MS. These oligosaccharides were also treated with exo-acting rhamnogalacturonan galacturonohydrolase (RG-galacturonohydrolase) from *A. aculeatus* [43] for comparison. As determined by MALDI-TOF MS (results not shown), rhamnogalacturonan oligosaccharides GalA-Rha-GalA-Rha-GalA and GalA-Rha-GalA, at m/z 861 and m/z 539 respectively, disappeared upon treatment with RG-galacturonohydrolase, which implies the expected removal of the non-reducing GalA residue from these oligosaccharides [43]. The enzyme RGXB did not degrade these rhamnogalacturonan oligosaccharides. Also the rhamnogalacturonan oligosaccharides Rha-GalA-Rha-GalA-Rha-GalA at m/z 1007 and Rha-GalA-Rha-GalA at m/z 685 respectively were not attacked (results not shown). From these results it is concluded that RGXB does not act towards these rhamnogalacturonan oligosaccharides from the non-reducing end nor from the reducing end. However, this is still unclear for rhamnogalacturonan oligosaccharides with a rhamnose residue at the reducing end.

As RGXB was able to degrade pnp-Rha (results not shown), for now this enzyme will be named a pnp-rhamnohydrolase. In contrast to the pnp-rhamnohydrolase from *A. aculeatus* [40], RGXB did not hydrolyze hespiridin or naringin.

The enzymes PGXA, PGXB and PGXC were optimally active towards PGA at pH 3.5-4.0, pH 4.0-4.5 and pH 3.5-4.0, respectively (Table 1). pH optima for exo-polygalacturonases close to this value were previously reported by several investigators [42, 52, 53].

The specific activities of PGXA, PGXB, PGXC towards PGA and XGA-25 are presented in Table 1. Also the specific activity of *A. tubingensis* Exo-PG towards these polymers is included in this table for comparison. PGXB, PGXC and Exo-PG have a similar activity towards PGA. The specific activity of PGXB and Exo-PG towards XGA were less than towards PGA, which shows that these enzymes have a higher preference for PGA. However PGXC has no preference as it was equally active on both substrates. Compared to the other enzymes, PGXA has a significant lower specific activity towards PGA and it is the only enzyme that has a higher specific activity towards XGA-25 than towards PGA. This was also demonstrated by HPAEC analysis (see further).

As determined by HPSEC analysis (results not shown), PGA and XGA-25 were degraded by PGXA, PGXB and PGXC, respectively, without a dramatic decrease in the molecular weight of these polymers. These results were comparable to those obtained for Exo-PG and imply that PGXA, PGXB and PGXC degrade both polymers in an exo-fashion. This is further substantiated by analysis of the products by HPAEC. PGXA, PGXB and PGXC predominantly produce GalA from PGA, and a mixture of GalA and the disaccharide GalA-Xyl from XGA-25 at the early stage of the reaction (i.e. after 1 hour of incubation, see Fig. 3) as well as after prolonged incubation (results not shown). These results were similar to those observed for Exo-PG, thus, again confirming the exolytic mode of action of these enzymes as predicted from the sequence alignment.

A detailed inspection of the HPAEC results demonstrates that PGXA acts differently towards PGA and XGA-25 than PGXB, PGXC and Exo-PG. As shown in Figure 3A, the production of GalA from PGA (after 1 hour of incubation) was relatively low for PGXA, when compared to PGXB, PGXC and Exo-PG. These results are also in accordance with the specific activities of these enzymes towards PGA (Table 1). As illustrated in Figure 3B, compared to the other enzymes, PGXA also produced significantly more GalA-Xyl in ratio to GalA from XGA-25.

This shows that PGXA prefers to act towards xylosylated GalA residues of the substrate XGA-25. It can be concluded that PGXA is primarily behaving as an exo-xylogalacturonan hydrolase (and not an exo-polygalacturonase), but is also able to degrade polygalacturonic acid.
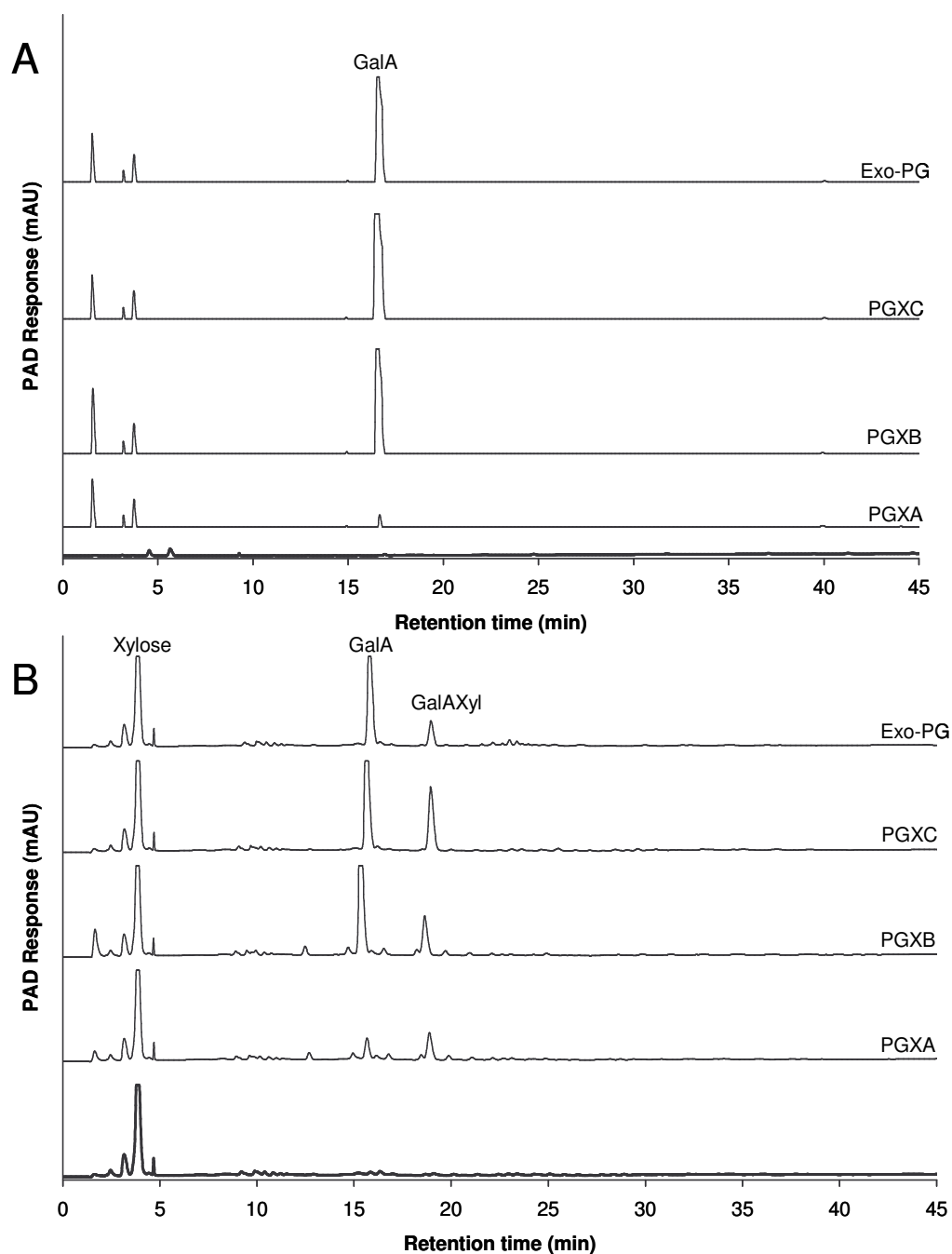
Figure 3 HPAEC of PGA (A) and XGA (B) hydrolysis after treatment for 1 h with PGXA, PGXB, PGXC and Exo-PG respectively. Spectra of untreated PGA and XGA are in bold.

Summary and conclusions

This paper presents for the first time an overview of the entire set of pectinolytic glycoside hydrolases encoded in the genome of *Aspergillus niger*.

In addition to the already well-studied gene group of seven endo-polygalacturonan hydrolases, we were able to extend the number of endo-rhamnogalacturonan hydrolases to six. Furthermore, a new gene group comprising seven genes encoding for exo-acting pectinolytic glycoside hydrolases was identified. In addition one gene encoding for a pectinolytic glycoside hydrolase was identified to be a xylogalacturonan hydrolase.

Subsequent sequence analysis of the new gene group in combination with transcriptional data allowed for the prediction of two subgroups of enzyme activities. Four genes were assessed as encoding the exo-polygalacturonan hydrolases – PGAX, PGXA, PGXB, PGXC and three genes as encoding the exo-rhamnogalacturonan hydrolases RGXA, RGXB and RGXC. PGAX encoded by the *A. niger* gene *pga*X is almost identical with the earlier described Exo-PG of *A. tubingensis.*

Biochemical characterisation of the three proposed exo-polygalacturonan hydrolases PGXA, PGXB and PGXC demonstrated that indeed all three enzymes were active towards homogalacturonan in an exo-fashion. Yet, while the biochemical properties of PGXB do not differ much from those of the Exo-PG of *A. tubingensis*, the data obtained for PGXA strongly indicate that this enzyme is a novel exo-xylogalacturonan hydrolase. Interestingly, PGXC appears to be an enzyme with reduced substrate specificity and remains unaffected in its activity when GalA residues of the substrate are substituted with xylose.

Additionally, the MS analysis of a tryptic digest of the RG galacturono-hydrolase produced by *A. aculeatus*, a protein for which no gene sequence is known, allowed the identification of its *A. niger* homolog RGXC.

Although RGXB showed some activity towards pnp-rhamnose we were not able to identify the natural substrate of this enzyme. Additional analysis is required to investigate the possible function of RGXA and RGXB. Yet, both enzymes posses the catalytic residues and the proper spacing required for the inverting mechanism characteristic for enzymes from family 28 glycoside hydrolases. The expression of the genes encoding these two enzymes on rhamnose and sugar beet pectin suggests their involvement in the degradation of pectin regions that contain rhamnose, most probably rhamnogalacturonan I.

The generally higher expression levels observed for genes encoding exo–acting glycoside hydrolases compared to those of genes encoding endo-activities, the strong initial rates of the reactions catalysed by them and the fact that *A. niger* is able to utilise galacturonic acid in its monomeric form suggest that exo-activities play a much more important physiological role than expected previously during pectin degradation.

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Chapter 3

Mode of action of Xylogalacturonan hydrolase towards xylogalacturonan and xylogalacturonan oligosaccharides

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Abstract

Xylogalacturonan hydrolase (XGH, GH 28) is an enzyme that is able to degrade xylogalacturonan (XGA), which is a polymer of α-D-galacturonic acid highly substituted with β-D-xylose. XGA is present in cell walls of various plants and exudates such as gum tragacanth.

XGA oligosaccharides were derived from a XGH digestion of gum tragacanth, then fractionated, and analyzed for their sugar composition and structure by MALDI-TOF MS and Nano-spray MS.

Several oligosaccharides from XGA were identified with different galacturonic acid/ xylose ratios including five oligosaccharide isomers. Although XGH can act as an endo-enzyme, product progression profiling showed that the disaccharide GalAXyl was predominantly produced from XGA by XGH, which indicated also an exolytic action. The latter was further supported by degradation studies of purified oligosaccharide $GalA_4Xyl_3$. It was shown that XGH acted from the non-reducing end towards the reducing end of this oligosaccharide showing the processive character of XGH.

The results from this study further show that although XGH prefers to act between two xylosidated GalA units it tolerates unsubstituted GalA units in its -1 and +1 subsites.

Introduction

Pectins are complex and highly heterogenous polysaccharides found in primary cell walls and intercellular regions of higher plants. These biopolymers contain α-(1→ 4)-linked D-galacturonic acid chains, called the smooth regions of pectins. Besides these linear chains, pectin further consists of the branched polysaccharides rhamnogalacturonan I, rhamonogalacturonan II and xylogalacturonan, which together are referred to as the hairy regions[1].

Xylogalacturonan (XGA) is an α-(1→ 4)-linked D-galacturonic acid chain, which is highly substituted with β-D-Xylose at the C-3 position (Fig. 1) [2].
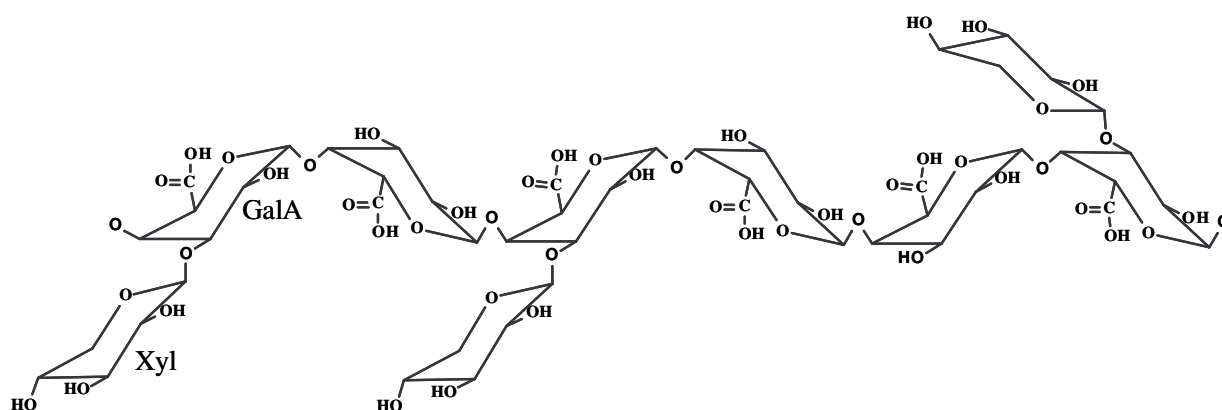
Figure 1. Schematic structure of xylogalacturonan (XGA).

This high molecular weight polymer is present in cell walls of various plants such as peas, soybeans, apple fruit, pear fruit, onions, cotton seeds, watermelons, and in exudates such as gum tragacanth from the *Astralagus* species [3].

Pectins play an important role in the food industry due to their excellent gelling, thickening and stabilizing properties. They are further believed to lower blood cholesterol levels, to protect the gastrointestinal tract and to stimulate the immune system [3, 4].

In some industrial food processes, such as the enzymatic clarification of fruit juices, it is crucial that pectins are completely degraded. For this process several pectinases are available and many of these enzymes have been characterised from a wide variety of microorganisms. Among the most commonly used pectinases are those produced by black aspergilli like *Aspergillus niger*. This organism is capable of secreting high levels of enzymes and its

products are 'generally recognized as safe' allowing them to be used in food applications [5, 6].

Several pectinases have been isolated from *A. niger* such as endo-polygalacturonases, endo-pectate lyases, endo-pectin lyases, pectin methyl esterases, which act on the smooth region of pectin, as well as rhamnogalacturonan acetylesterase, rhamnogalacturonases, arabinases and galactanases that act on the hairy regions of pectin. Most of these enzymes have been thoroughly characterised with respect to their mode of action on their corresponding substrates [7-10].

Despite the rich collection of pectinases in technical enzyme preparations obtained from *A. niger*, parts of the hairy regions of pectin remain resistant to degradation and cause membrane fouling in the ultrafiltration process for fruit juice clarification [10]. Being part of the pectic hairy regions, fouling is largely influenced by XGA. Little is known about pectinases that can degrade XGA, with the exception of exo-polygalacturonases from *Aspergillus sp.* [9, 11, 12] Recently, the enzyme xylogalacturonan hydrolase (XGH) was discovered in *Aspergillus tubigensis,* which acts specifically on XGA by cleaving the galacturonic acid backbone in an endo-fashion [5, 13]. This enzyme belongs to the pectin degrading glycoside hydrolase family 28 (GH 28), which includes polygalacturonases (PGs) and exo-polygalacturonases (exo-PGs), based on its amino acid sequence similarity towards this family [14, 15]. In addition the amino acid sequence of XGH contains functionally important residues equivalent to the four conserved active-site segments of PGs [15-17]. All family 28 members, including XGH, act with an inverting mechanism [18].

XGH has been characterised with respect to its kinetic parameters, temperature- and pH-effects and degradation of differently substituted (xylo-) galacturonans [11].

Here we report on the mode of action of XGH on its substrate XGA. This was realized by degradation of XGA by XGH, followed by fractionation and quantitation and analysis of the obtained oligosaccharides for their sugar abundance, composition and structure. Furthermore, the degradation of a defined XGA oligosaccharide was studied in a time course in order to determine the direction of action of XGH.

Experimental

Substrate

Two xylogalacturonans (XGAs) derived from acid-modified and alkali saponified gum tragacanth, were used. These XGAs had a Xyl:GalA ratio of 0.29 (XGA-29) and 0.47 (XGA-47) respectively [11].

Enzyme

The enzyme xylogalacturonan hydrolase (XGH) from *Aspergillus tubingensis* was cloned [5] and expressed in the *A. niger* "PlugBug" of DSM Food Specialities (Delft, the Netherlands). This crude enzyme preparation was purified [11]. The crude and purified enzyme preparation had a specific activity of 77 U/mg and 150 U/mg respectively.

Enzyme incubations

XGH was used to digest XGA-29 (final concentration of 1 mg/ml) for one h at 30 ℃. The final enzyme concentration was 3.17 µg/ml. The substrate was dissolved in water in a total volume of 10 ml and had a final pH of 3.6 without additional buffering. The enzyme was inactivated by heating the reaction mixture for 10 min at 100 ℃. The digested XGA was concentrated to 5 mg/ml, by freeze drying and redissolving in water, and was analyzed by HPSEC as described previously [19]. Subsequently, the digest was analyzed by HPAEC at pH 12 using pulsed amperometric detection (PAD) as described previously [20]. The elution of the XGA oligosaccharides by HPAEC was adapted as follows: a combination of two linear gradients was used starting with 0-600 mM sodium acetate in 100 mM NaOH for 50 min, followed by 600-1000 mM sodium acetate in 100 mM NaOH for 5 min.

Preparation of XGA oligosaccharides

For the preparation of XGA oligosaccharides, 300 mg XGA-29 was digested, as described above, and concentrated to 25 mg/ml prior to fractionation by HPAEC. All fractions were desalted by H[+]-Dowex AG 50W X8 (Biorad, Hercules, CA) treatment. Subsequently, the desalted fractions were analyzed by HPAEC for their purity and compared with the complete XGA digest for assignment. A GalA$_3$ standard (5mg/ml) was used for quantification.

MALDI-TOF mass spectrometry

The fractionated XGA oligosaccharides were analyzed by MALDI-TOF as described previously [19]. Two types of MS spectrometers were used: Voyager-DE RP Biospectrometry workstation (PerSeptive Biosystems Inc., Framingham, MA, USA) and Ultraflex TOF MS (Bruker Daltonics, Hamburg, Germany). The concentration of the XGA oligosaccharides ranged from 0.25 mg/ml to 1 mg/ml. The samples were mixed with a matrix solution (1 µl of sample in 1 µl of matrix) on a silver plate. The matrix solution was prepared by dissolving 10 mg of 2,5-dihydroxybenzoic acid in 1-ml mixture of acetonitrile/water (500:500 µl).

XGA oligosaccharides were identified based on their theoretical m/z masses. These masses were based on the GalA/Xyl ratio's, assuming total protonation of GalA and addition of one Na^+ or K^+.

Labeling

Labeling of the individual XGA oligosaccharides with ^{18}O at their reducing ends was performed as described [21]. The XGA samples were incubated at 40 ℃ for 12 days. The progress of labeling was followed by MALDI-TOF MS and the labeled XGA oligosaccharides were further structurally characterized by post-source decay (PSD) using the Ultraflex-TOF (Bruker Daltonics, Hamburg, Germany).

Nano-spray mass spectrometry

Dynamic nanospray MS was performed on a LCQ Ion-trap (Finnigan MAT 95, San Jose, CA) as described previously [19]. For MS analysis of XGA oligosaccharides settings were adapted as follows: the flow was set at 5 µl/min, the spray voltage at 4.5 kV and 20-30% relative collision energy for MS^2 and higher was applied.

Product progression profiling of XGH

A XGA-29 solution (1 mg/ml) was incubated with purified XGH (0.35 µg/ml, final concentration) at 30 °C. The digest had a total volume of 20 ml. At different time intervals (0-48 h) a sample was taken and incubated for 10 min at 100 ℃ to terminate the reaction. An extra enzyme dose (0.70 µg/ml, final concentration) was added to the digest after 24 h.

By freeze drying and redissolving in water, samples were concentrated five times and were subsequently analyzed by HPAEC along with identified XGA oligosaccharides and $GalA_3$ (5 mg/ml).

Preparation and degradation of oligosaccharide $GalA_4Xyl_3$

Another batch of XGA from gum tragacanth batch, similar to XGA-47, as described by Beldman *et al.* (2003), was degraded by XGH using the same conditions as above. This digest was fractionated by HPAEC as described before, and was isolated.

$GalA_4Xyl_3$ was labeled and structurally characterized by MALDI-TOF PSD. Subsequently, $GalA_4Xyl_3$ was degraded with purified XGH using the conditions as described previously. At different intervals (0-24 h) a sample was taken and incubated for 10 min at 100 ºC to terminate the reaction. An extra enzyme dose (0.70 μg/ml, final concentration) was added to the digest after 8 h. The progress of degradation in all samples was analyzed by MALDI-TOF MS using the Ultraflex TOF MS (Bruker Daltonics, Hamburg, Germany) as described.

Results and discussion

HPLC analysis of xylogalacturonan digest

Xylogalacturonan (XGA-29) was incubated with xylogalacturonan hydrolase (XGH) for 24 h and subsequently analyzed by HPSEC. An endo type of degradation of XGA was observed as shown previously [11], although analysis by HPAEC (Fig. 2) revealed the presence of more XGA oligosaccharides than reported before. This is explained by the application of higher concentrations of the degraded substrate onto the column. In addition, to the previously observed oligosaccharides, high molecular weight XGA products (shown as peaks 5-20) were noticeable after one hour of enzymatic degradation of XGA.

Identification of xylogalacturonan oligosaccharides

An XGA-29 digest was preparatively fractionated by HPAEC in order to isolate the individual oligomeric products. All fractions were subjected to HPAEC again to check their purity. By MALDI-TOF MS these purified XGA oligosaccharides were analyzed for their sugar composition. Figure 2 shows the HPAEC profile of the XGA digest from which eighteen XGA oligosaccharides were identified. Also the previously observed products Xyl and GalA as well as the di-mer GalAXyl were observed.

The XGA oligosaccharides identified have various GalA/Xyl ratios. In addition, five pairs of oligosaccharide isomers were found having a similar sugar composition but a different elution time upon HPAEC analysis. This can be explained by a different distribution of Xyl over the GalA backbone as the xylose side chain may be linked to a different GalA residue or to a different –OH group (C2 or C3) on the same GalA residue. These pairs of oligosaccharide isomers are shown as peaks 3 and 6, 7 and 10, 11 and 14, 14 and 16, and 15 and 18. To avoid confusion, the isomers are accentuated as shown in the table.

A measurable amount of xylose was present in the digest. This indicates that some xylose was released by enzymatic or chemical hydrolysis from XGA and/or the oligosaccharides during the digestion. The total amount of free xylose however was estimated to be only about 2 % of the total amount present in the substrate.
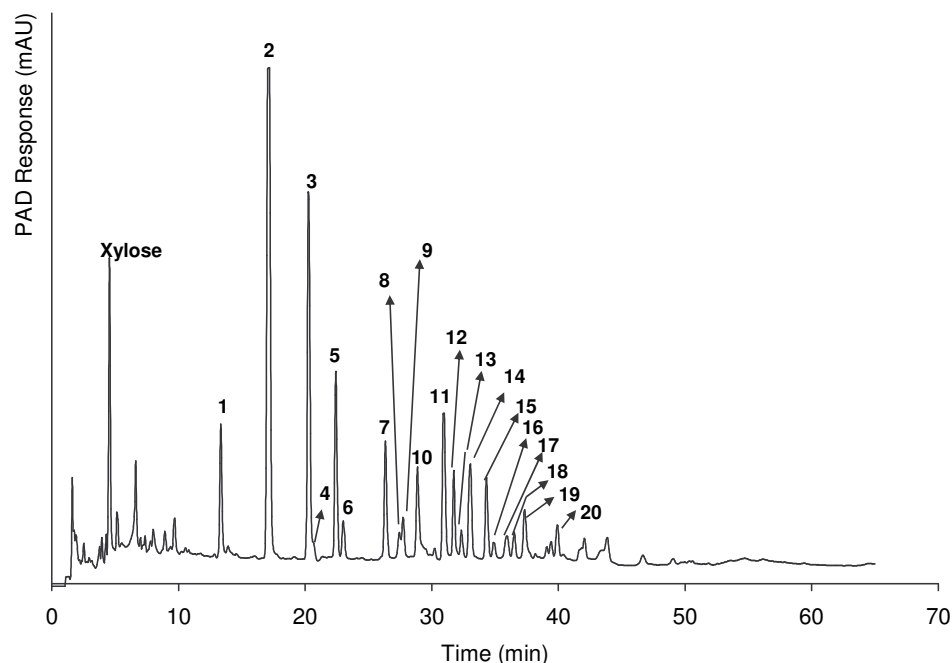
1) GalA (217)	5) GalA$_2$Xyl$_2$ (657)	9) GalA$_3$ (569)	13) GalA$_4$ (745)	17) GalA$_5$ (921)
2) GalAXyl (349)	6) GalA$_2$Xyl' (525)	10) GalA$_3$Xyl' (701)	14) GalA$_4$Xyl' + GalA$_5$Xyl$_2$ (877 + 1185)	18) GalA$_5$Xyl' (1053)
3) GalA$_2$Xyl (525)	7) GalA$_3$Xyl (701)	11) GalA$_4$Xyl (877)	15) GalA$_5$Xyl (1053)	19) GalA$_6$Xyl (1229)
4) GalA$_2$ (393)	8) GalA$_3$Xyl$_2$ (833)	12) GalA$_4$Xyl$_2$ (1009)	16) GalA$_5$Xyl$_2$' (1185)	20) GalA$_6$ (1097)

Figure 2. HPAEC elution profile of a XGA-29 digest.
The included table summarizes the newly identified XGA oligosaccharides (bold; see further). The m/z masses of the XGA oligosaccharides (including the addition of a H$^+$ and Na$^+$) are shown between brackets. The accentuated XGA oligosaccharides (') are isomers with different distribution of xylose over the GalA backbone

Structural characterization of XGA oligosaccharides

Oligosaccharides GalA$_2$Xyl, GalA$_2$Xyl', GalA$_3$Xyl, GalA$_3$Xyl$_2$ and GalA$_3$Xyl' as shown in Figure 2 were further analyzed for their structure. The oligosaccharide GalA$_4$Xyl$_3$ (derived from XGA-47) was also analyzed for its structure and used for detailed degradation analysis by XGH (see further).

For structural characterization, these oligosaccharides were ^{18}O-labeled at their reducing end by acid catalyzed exchange in H$_2$^{18}O, which will increase their size in m/z by 2. All labeled

XGA oligosaccharides and their corresponding fragments that contain the label will be indicated by an asterix (*).

The labeled oligosaccharides $GalA_2Xyl*$ and $GalA_2Xyl'*$ were subjected to nano-spray MS. Figure 3 shows a MS^2 spectrum of the fragmented parent ion at m/z 527, corresponding to the ^{18}O-labelled oligosaccharide $GalA_2Xyl$. The produced fragments were [GalA*] (m/z = 219), [GalAXyl*] (m/z= 315), [GalA$_2$*] (m/z=395) and [GalA$_2$Xyl-OH-H$^+$] (m/z= 507). The presence of fragment GalAXyl* showed that xylose was substituted at the reducing GalA unit. In the MS^2 spectrum of $GalA_2Xyl'*$ also the fragment GalAXyl* (m/z = 351) was found, which showed that in this oligosaccharide the xylose was also present at the reducing GalA.
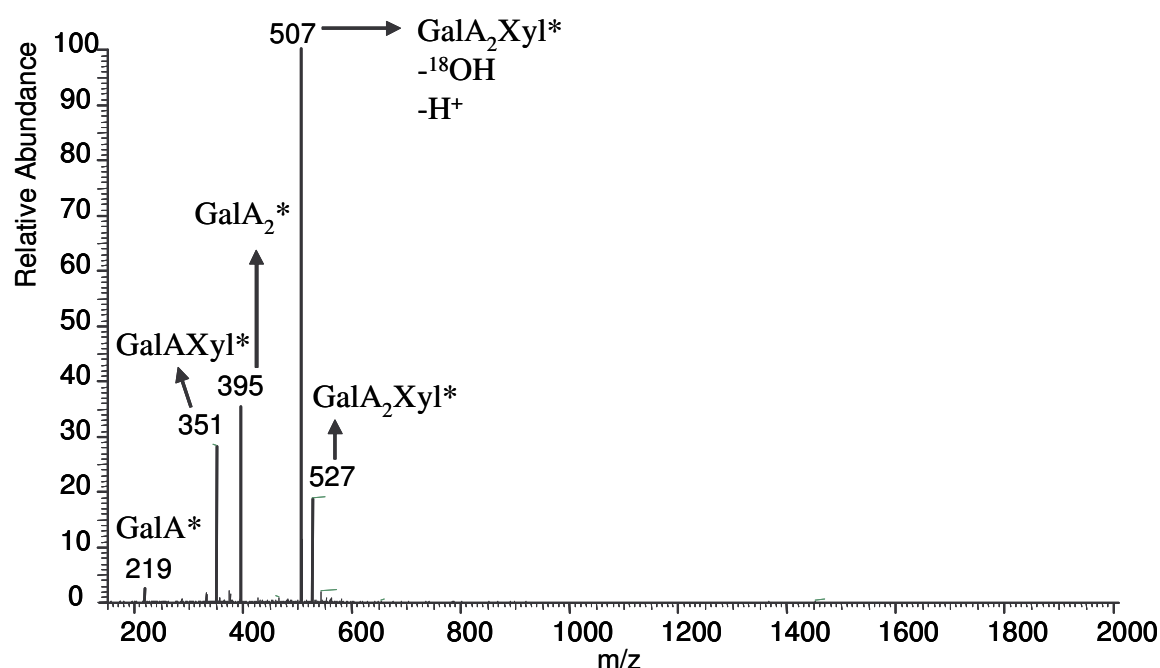

Figure 3. MS^2 spectrum of GalA$_2$Xyl* (m/z 527).

However, the MS^2 spectra showed a different ratio of fragments from $GalA_2Xyl*$ and $GalA_2Xyl'*$. This difference in fragmentation might be explained by different xylose linkages at the reducing GalA unit for these two oligosaccharides. Although xylose is known to be attached at the C-3 position of GalA in XGA, the presence of other types of linkages could occur, yet in small proportions [2]. Therefore we suggest that the major product $GalA_2Xyl$, shown as peak 3 in Figure 2, carries xylose at the C-3 position of the reducing GalA unit

while the minor product GalA$_2$Xyl', shown as peak 10, carries xylose at the C-2 position. The proposed structures of GalA$_2$Xyl and GalA$_2$Xyl' are presented in Figure 4.
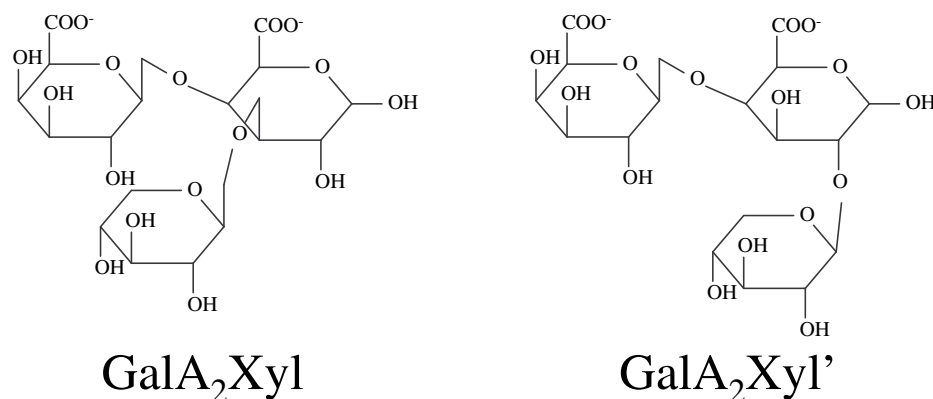
Figure 4. Proposed structures of XGA oligosaccharides GalA$_2$Xyl and GalA$_2$Xyl'.

It is likely that the difference in substitution with xylose at the reducing GalA of GalA$_2$Xyl and GalA$_2$Xyl' causes the different elution upon HPAEC. A similar phenomenon has been reported before for xylan oligosaccharides substituted with arabinose at either C-2 or C-3 of the xylose residue [22].

The other labeled oligosaccharides were analysed by MALDI-TOF using post-source decay (PSD). Figure 5 shows a PSD spectrum of the (fragmented) parent ion at m/z 703, corresponding to GalA$_3$Xyl*. In Table 1 the results are summarized for fragments that were observed with PSD of GalA$_3$Xyl* as well as of GalA$_3$Xyl'*, GalA$_3$Xyl$_2$ and GalA$_4$Xyl$_3$*.
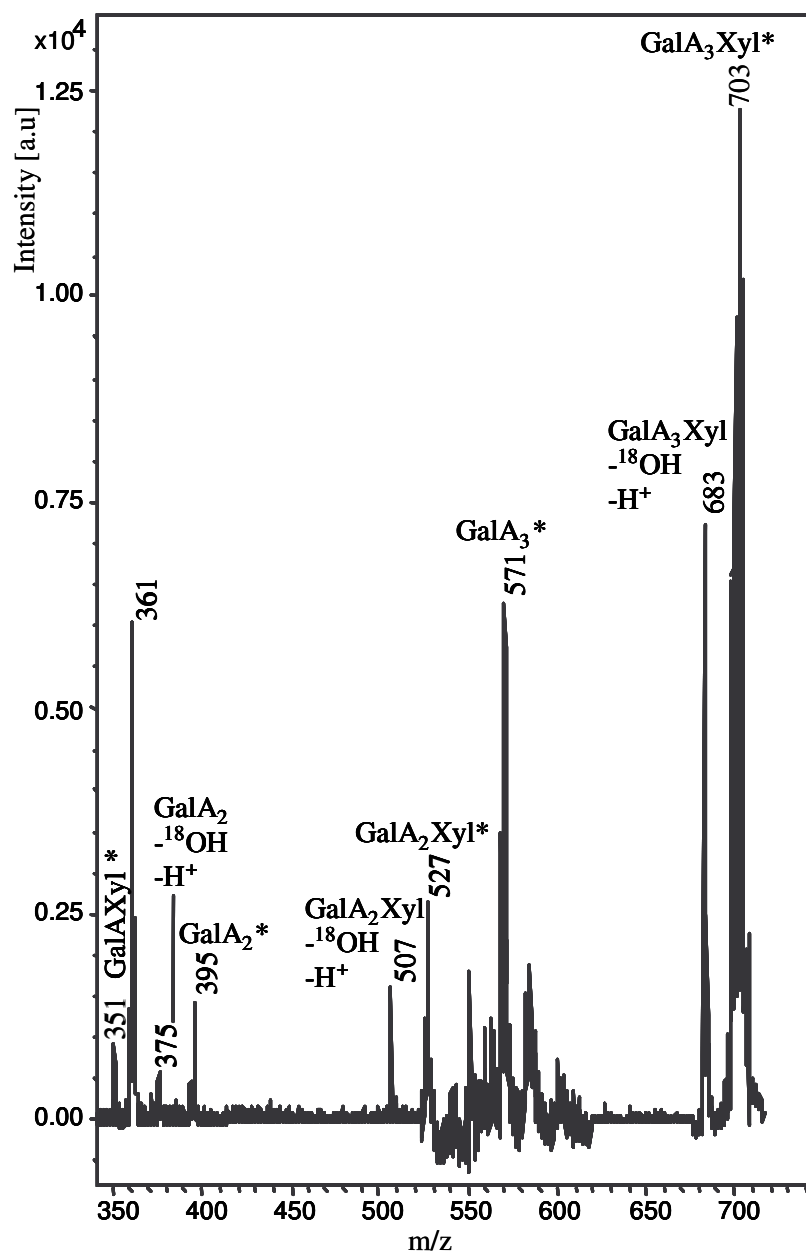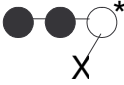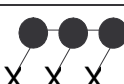
Figure 5. MALDI-TOF PSD spectrum of GalA$_3$Xyl* (m/z 703).

XGA oligosaccharide (**m/z**)	Observed XGA fragments (**m/z**)
●●○* with X GalA$_3$Xyl* (**703**)	[GalA$_3$Xyl*] (**703**); [GalA$_3$Xyl – ^{18}OH – H$^+$](**683**); [GalA$_3$*] (**571**); [GalA$_2$Xyl*] (**527**); [GalA$_2$Xyl– ^{18}OH – H$^+$](**507**); [GalA$_2$*] (**395**); [GalA$_2$ –^{18}OH – H$^+$](**375**); [GalAXyl*] (**351**)
●●○* with X GalA$_3$Xyl'* (**703**)	[GalA$_3$Xyl*] (**703**); [GalA$_3$Xyl -^{18}OH – H$^+$](**683**); [GalA$_3$*] (**571**); [GalA$_2$Xyl] (**525**); [GalA$_2$Xyl – OH – H$^+$] (**507**); [GalA$_2$*] (**395**); [GalA$_2$ – ^{18}OH – H$^+$](**375**); [GalAXyl] (**349**)
●●○ with X X GalA$_3$Xyl$_2$ (**833**)	[GalA$_3$Xyl$_2$] (**833**), [GalA$_3$Xyl$_2$-OH – H$^+$] (**815**); [GalA$_3$Xyl] (**701**); [GalA$_3$Xyl -OH – H$^+$](**683**); [GalA$_3$](**569**); [GalA$_2$Xyl] (**525**); [GalA$_2$Xyl-OH – H$^+$](**507**); [GalA$_2$] (**393**); [GalAXyl] (**349**)
●●●○* with X X X GalA$_4$Xyl$_3$* (**1143**)	[GalA$_4$Xyl$_3$*] (**1143**); [GalA$_4$Xyl$_2$*] (**1011**); [GalA$_4$Xyl$_2$] (**1009**); [GalA$_4$Xyl$_2$ –^{18}OH – H$^+$] v [GalA$_4$Xyl$_2$ –^{16}OH – H$^+$] (**991**); [GalA$_4$Xyl*] (**879**); [GalA$_3$Xyl$_2$*] (**835**), [GalA$_3$Xyl$_2$*] (**833**); [GalA$_3$Xyl$_2$ – ^{18}OH – H$^+$] v [GalA$_3$Xyl$_2$ – ^{16}OH – H$^+$] (**815**); [GalA$_3$Xyl*] (**703**); [GalA$_3$Xyl-^{18}OH – H$^+$] (**683**); [GalA$_2$Xyl$_2$] (**657**); [GalA$_3$](**569**); [GalA$_2$Xyl*] (**527**); [GalA$_2$Xyl-^{18}OH – H$^+$] (**507**); [GalAXyl] (**349**)

Table 1. Observed fragments derived from MALDI-TOF PSD spectra of labeled oligosaccharides GalA$_3$Xyl and GalA$_3$Xyl', GalA$_4$Xyl$_3$ and unlabeled oligosaccharide GalA$_3$Xyl$_2$ along with the drawn structures. GalA= •, xylose = X and reducing GalA= ○. The asterix (*) indicates the label at the reducing end of the oligosaccharide.

The derived structures were rationalized as follows.

GalA$_3$Xyl*: As shown in Figure 5 and Table 1 the presence of the GalAXyl* fragment indicates xylose substitution at the reducing GalA unit of GalA$_3$Xyl*. **GalA$_3$Xyl'***: PSD analysis of this oligosaccharide resulted in the appearance of fragment GalAXyl, but did not reveal fragments GalAXyl* and GalA$_2$Xyl*. The absence of the latter two fragments indicates that no xylose substitution occurs at the reducing and internal GalA units. Therefore other possibilities than xylose substitution at the non-reducing end of GalA$_3$Xyl'* can be excluded.

GalA$_3$Xyl$_2$: Labeling of this oligosaccharide failed for unknown reasons; however PSD analysis could resolve the structure of this unlabeled oligosaccharide. The absence of the GalA$_2$Xyl$_2$ fragment demonstrated that the xyloses are not substituted to two adjacent GalA units. Therefore the only possibility was substitution of the reducing and non-reducing end of GalA$_3$Xyl$_2$.

GalA$_4$Xyl$_3$*: PSD analysis on GalA$_4$Xyl$_3$* showed the absence of GalAXyl* which suggests a free reducing GalA unit. Therefore each of the other three GalA units must be substituted with a xylose.

Product progression profiling of XGH

The appearance of products during the degradation of XGA by XGH was studied. This was done by degrading XGA-29 with purified XGH for different time intervals followed by HPAEC analysis and quantification (Fig. 6). The co-eluting XGA oligosaccharides were plotted together in the product progression profiles.

It was assumed that the response factors of the individual oligosaccharides under the conditions of HPAEC were not substantially different from each other. This was based on HPAEC analysis of GalA and GalA oligosaccharides with a DP of 2 to DP 5 in equal molar concentrations for which no substantial differences in response factors were found (results not shown). In addition, from the literature it is known that only a small difference in response factor for GalA and GalAXyl exist [9]. This demonstrates that xylose substitution has no remarkable affect on the response factor of xylogalacturonan oligosaccharides.

The product progression profiles show a predominant production of GalAXyl with a minor production of linear oligosaccharides like $GalA_2$, $GalA_3$ and $GalA_4$ indicating that XGH prefers to act between two xylosylated GalA units. This would include that subsites –1 and +1 of XGH have a high affinity for xylosylated GalA units. Linear oligosaccharides were not degraded after prolonged digestion, which shows the dependency of XGH for xylose substitution as well as the absence of other galacturonosidases (i.e. exo-PG). To our knowledge only exo-PGs from *Aspergillus sp.* are known to accept xylose substituted galacturonic acid in its subsite –1, because they produce the dimer GalAXyl from XGA [12, 23]. Although exo-PG has a pronounced sequence similarity with XGH, this latter enzyme only contains a part of the conserved regions characteristic for exo-PGs. This indicates the uniqueness of XGH [17].
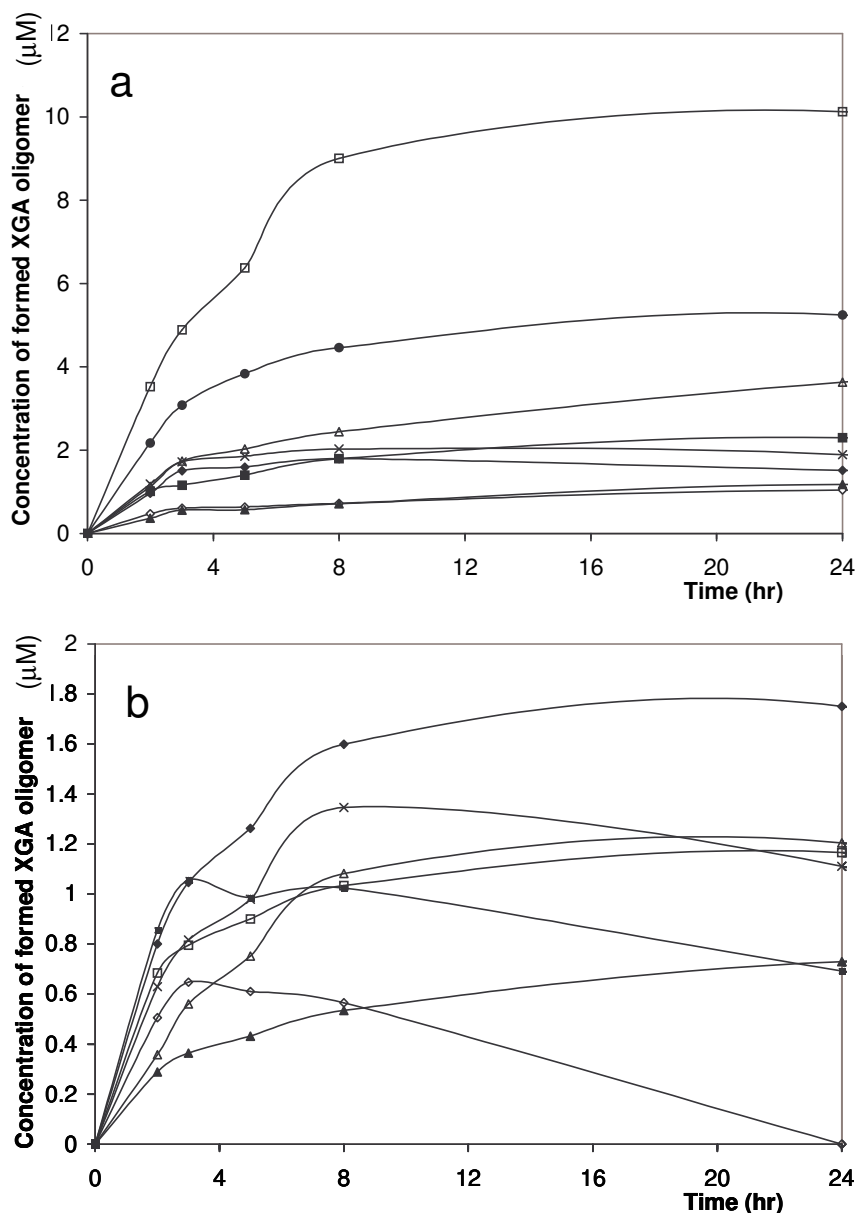
Figure 6. Product progression profiles of XGH on the XGA analog.
Figure 6a represents the products: GalA(Δ), GalAXyl($\square$), GalA$_2$Xyl($\bullet$), GalA$_2$($\lozenge$), GalA$_2$Xyl$_2$($\times$), GalA$_2$Xyl'($\blacktriangle$), GalA$_3$Xyl($\blacksquare$), GalA$_3$Xyl$_2$+ GalA$_3$($\blacklozenge$), while figure 6b represents the products: GalA$_3$Xyl'(Δ), GalA$_4$Xyl($\blacklozenge$), GalA$_4$Xyl$_2$($\blacksquare$), GalA$_4$($\blacktriangle$), GalA$_4$Xyl' + GalA$_5$Xyl$_2$ ($\times$), GalA$_5$Xyl ($\square$),GalA$_5$Xyl$_2$'($\lozenge$).Note the different scaling of 6a and 6b.

XGH can also act between two GalA units of which one is xylosylated. This is evidenced by the production of oligosaccharides such as GalA$_3$Xyl and GalA$_3$Xyl' (peak 7 and 10 in Fig. 2

respectively), that have no xylose substitution at the non-reducing or at the reducing end, respectively (Table 1).

As shown in Figure 6, $GalA_4Xyl_2$ was further degraded, which demonstrates that XGH prefers to act on XGA oligosaccharides with a backbone of at least four GalA units. In addition, $GalA_5Xyl_2'$ was degraded more efficiently than $GalA_4Xyl_2$, which demonstrates that an increase of the GalA backbone by one GalA unit also significantly increases the XGH activity. Furthermore, the rapid degradation of $GalA_5Xyl_2'$ is in line with the decline of the $GalA_4Xyl'$-$GalA_5Xyl_2$ pair in the progression profiles. $GalA_4Xyl$ and $GalA_5Xyl$ were not further degraded, which demonstrates that increased xylose substitution enhances XGH activity.

Degradation of $GalA_4Xyl_3$

In order to study the direction of action of XGH, a highly substituted oligosaccharide with at least four GalA units in the backbone that can potentially be hydrolyzed more than once by XGH is required. Furthermore, this oligosaccharide should have an asymmetrical structure with respect to xylose substitution for the recognition of the reducing end. As none of the oligosaccharides that were produced from XGA-29 met this requirement, XGA-47 with a higher degree of substitution with xylose was used to prepare the desired oligosaccharide. Partial degradation of this substrate resulted in the production of significant amounts of oligosaccharide $GalA_4Xyl_3$, which appeared suitable for further degradation studies. This oligosaccharide was purified to near homogeneity (>95%) from the XGA digest by preparative HPAEC and structurally characterized by MALDI-TOF PSD as described in the previous paragraph.

$GalA_4Xyl_3$ was degraded with purified XGH for different time intervals followed by MALDI-TOF MS analysis (results not shown). Degradation of the oligosaccharide $GalA_4Xyl_3$ for 5 min resulted in the formation of significant amounts of GalAXyl and $GalA_3Xyl_2$. After 24 hrs of incubation the main products left were $GalA_2Xyl$ and GalAXyl. This shows that XGH acts mainly from the non-reducing end of $GalA_4Xyl_3$ towards the reducing end, cleaving off GalAXyl step-by-step. Furthermore, the smallest substrate hydrolysable appeared to be $GalA_3Xyl_2$. Since a small amount of $GalA_2Xyl_2$ was also produced, it can be concluded that XGH also acts, however less favorably, at the internal glycosidic bond of $GalA_4Xyl_3$. This

mode of action is depicted schematically in Fig 7. The number of four subsites has been arbitrarily taken.
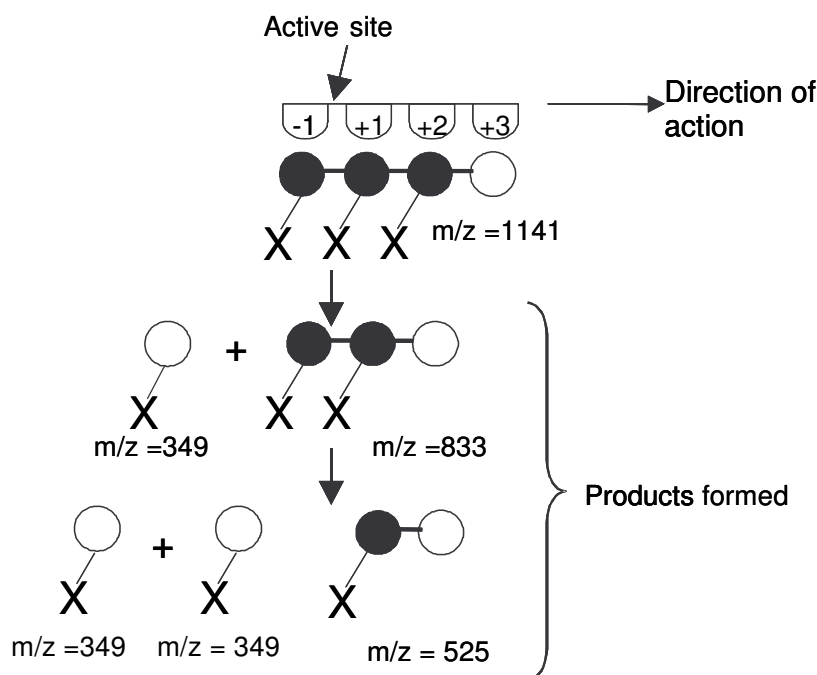
Figure 7. Proposed model of the action mechanism of XGH towards GalA$_4$Xyl$_3$.
GalA= •, xylose = X and reducing GalA= ○. The number of sub-sites is arbitrarily taken as 4.

Although XGH is recognized as an endo-enzyme, it primarily behaves in an exolytic way during degradation of XGA. Unlike endo-PG's that are known to attack the GalA chain randomly [8, 24], XGH acted on GalA$_4$Xyl$_3$ from the non-reducing end towards the reducing end, which is indicative for an exo acting enzyme [8, 9, 25].

The exo-character is also in accord with the higher sequence homology of XGH to exo-PGs than to endo PGs [5]. The stepwise release of GalAXyl from GalA$_4$Xyl$_3$ suggests a processive behavior of XGH.

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Chapter 4

Enzymatic degradation studies of xylogalacturonans from apple and
potato, using xylogalacturonan hydrolase

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Abstract

Action of xylogalacturonan hydrolase (XGH) towards xylogalacturonan (XGA) present in the
alkali saponified *"modified* hairy regions" from potato- and apple pectin was studied.
Analysis of enzymatic degradation products from XGA in these complex pectins
demonstrated that the degradable xylogalacturonans from both sources have a similar xylose
side chain distribution. The disaccharide β-D-Xyl-(1,3)-GalA was the predominant product
from these substrates.

The number of different enzymatic degradation products from xylogalacturonan present in
apple and potato pectin was much lower than the number of different products from a
xylogalacturonan derived from gum tragacanth. This suggests a relatively uniform distribution
of xylose in the degradable part of XGA from apple and potato pectin. In addition, dimeric
side chains of xylose were observed in digests of XGA from both pectins, which apparently
did not hinder the action of XGH. From this it is assumed that Xyl-Xyl as well as Xyl
substituted GalA residues are accepted in subsite −1 and +1 of xylogalacturonan hydrolase.

1. Introduction

Pectinases play an important role in processing plant materials to food products, such as depectinization of fruit juices, maceration of vegetables and fruit, and extraction of vegetable oils (Benen, van Alebeek, Voragen, and Visser, 2003; Grassin and Fauquembergue, 1996; Heldt-Hansen, Kofod, Budolfsen, Nielsen, Huttel, and Bladt, 1996; Thibault and Ralet, 2001). A whole array of pectinases has been isolated from various plants, bacteria and fungi, including *Aspergillus niger*. These enzymes can be divided into those acting on the 'smooth' regions and those acting on the ramified 'hairy' regions (HR) of pectin (Benen, Vincken, and van Alebeek, 2002; Vincken, Voragen, and Beldman, 2003b; Voragen, Beldman, and Schols, 2001). The HR, as isolated from apple, consists of xylogalacturonan and rhamnogalacturonan. In addition, the latter pectic polysaccharide can also be ramified with long neutral sugar side-chains (Vincken, Schols, Oomen, Beldman, Visser, and Voragen, 2003a; Schols and Voragen, 2002)

Xylogalacturonan (XGA) exists in various cell walls of plants (Thibault, 2001; Voragen *et al.*, 2001) and has been analyzed for its structure in several plant derived materials such as gum tragacanth (Aspinall and Baillie, 1963), apple pectin (Schols, Bakx, Schipper, and Voragen, 1995), soy pectin (Nakamura, Ruta, Maeda, Takao, and Nagamatsu, 2002) and pea pectin (Le Goff, Renard, Bonnin, and Thibault, 2001). In these pectins XGA consist of an α-$(1\rightarrow 4)$-linked D-Galacturonic acid chain, which is substituted at O-3 with β-D-xylose side chains (Thibault, 2001; Vincken *et al.*, 2003a). Oligomeric side chains of xylose are proposed to exist in XGA from apple, soy and pea pectin and contain 1,4-linked xylose residues (Oechslin, Lutz and Amado, 2003), 1,4- and 1,2-linked xylose residues (Nakamura *et al.*, 2002), and 1,2- and 1,3-linked xylose residues (Le Goff *et al.*, 2001) respectively, based on sugar linkage analysis.

As XGA contributes to membrane fouling in the ultra filtration process for fruit juice clarification (Herweijer, Vincken, Meeuwsen, van der Vlugt-Bergmans, Beldman, van Ooyen, Voragen, 2003; Van der Vlugt-Bergmans, Meeuwsen, Voragen, and van Ooyen, 2000), it is crucial that it is completely degraded during the preceding enzymatic treatment of the fruit pulp. Degradation of XGA is possible with exo-polygalacturonases (Beldman, van den Broek, Schols, Searle-van Leeuwen, van Laere, and Voragen, 1996; Kester, Benen, and Visser, 1999). In addition, the enzyme xylogalacturonan hydrolase (XGH), discovered in *Aspergillus*

tubingensis, is able to degrade XGA in an endo fashion (van der Vlugt-Bergmans, Meeuwsen, Voragen, and van Ooyen, 2000). The corresponding gene for XGH from *A. tubingensis* has been over-expressed in the *A. niger* "PlugBug" (van Dijck, 1999) and the forthcoming enzyme was subsequently purified (Beldman, Vincken, Meeuwsen, Herweijer, and Voragen, 2003). This enzyme has been studied for its pH- and temperature stability as well as its activity towards xylogalacturonans with different degrees of xylose substitution (Beldman *et al*. 2003). Additionally, the mode of action of this enzyme towards XGA from alkali and acid modified gum tragacanth has been investigated by analysis of the structure of the degradation products (Zandleven, Beldman, Bosveld, Benen, and Voragen, 2005). In this study, XGH is investigated with repect to its activity towards XGA present in apple and potato MHR. Also some aspects about the subsite model of XGH are discussed based on the structure of XGA oligosaccharides formed.

2. Experimental

2.1. Substrates

Saponified potato MHR (p-MHR-S) and saponified apple MHR (a-MHR-S) were prepared as described (Schols, Posthumus, and Voragen, 1990). Enzymatic liquefaction of apples for the preparation of a-MHR-S was performed with a commercial enzyme preparation (Ultra-SP; Novozymes, Copenhagen, Denmark). An amount of 7.5 g of this substrate was further treated with rhamnogalacturonan hydrolase (RGH) and fractionated by preparative size exclusion chromatography (SEC) in order to obtain a XGA enriched a-MHR-S substrate. This procedure was done as follows: a-MHR-S (5 mg/ml final concentration) was treated with purified RGH (Novozymes, Copenhagen, Denmark; Kofod, Kauppinen, Christgau, Andersen, Heldt-Hansen, Dorreich, Dalbfge, 1994; Schols *et al*., 1990) for 24 hrs at 40 °C in 50 mM NaOAc buffer (pH 5.6). The final enzyme concentration was 3.33 µg/ml. The enzyme was inactivated by heating the reaction mixture for 10 min at 100 ºC. Subsequently, the RGH treated a-MHR-S was dialyzed against water. A yield of 6.15 g RGH treated a-MHR-S was obtained after freeze-drying. A portion of 6 g from this material was applied onto a Superdex 30 PG column (5.5 L, 70 x 10 cm) using millipore water as eluent with a flow rate of 25 ml·min^{-1}. Two fractions, a-MHR-S-frA (pooled fractions 18-27) and a-MHR-S-frB (pooled fractions 27-41) respectively, were collected (Fig. 1) and subsequently freeze-dried. The fractions a-MHR-S-frA and a-MHR-S-frB gave a yield of 1.15 g and 2.0 g, respectively.

2.2. Neutral sugar composition and uronic acid content

p-MHR-S and a-MHR-S-frB were analyzed for their neutral sugar composition by gas chromatography (Englyst & Cummings, 1984), using inositol as an intern standard. The samples were treated with 72% (w/w) H_2SO_4 for 1 h at 30 °C, followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100 °C. Prior to analysis by gas chromatography, the released neutral sugars were converted to their alditol acetates.

The uronic acid content of p-MHR-S and a-MHR-S-frB was determined by an automated m-hydroxydiphenyl assay using an autoanalyser (Skalar Analytical BV, Breda, The Netherlands; Blumenkrantz and Asboe-Hansen, 1973; Thibault and Robin 1975).

2.3. HPSEC analysis

High-performance size-exclusion chromatography was performed on three TSK columns (7.8 mm ID x 30 cm per column) in a series of TSKGel G4000 PWXL, TSKGel G3000 PWXL and TSK2500 PWXL, in combination with a PWX-guard column. All columns were from TosoHaas (Japan).

Elution took place at 30 ℃ using 0.2 M sodium nitrate at a flow rate of 0.8 ml/min. The eluate was monitored using a refractive index (RI) detector. The calibration was performed using pullulan standards with a molecular weight of 404, 112, 47.3, 22.8, 5.9 kDa and GalA (0.196 kDa).

2.4 HPAEC at pH 12

High performance anion-exhange chromatography was performed on a Dionex system equipped with a CarboPac PA-1 column (4 mm ID x 250 mm) in combination with a CarboPac PA Guard column (3 mm ID x 25 mm) and pulsed amperometric detection (PAD). The elution (1 ml/min) of the oligosaccharides was performed by using a combination of two linear gradients, starting with 0-600 mM sodium acetate in 100 mM NaOH for 50 min, followed by 600-1000 mM sodium acetate in 100 mM NaOH for 5 min.

2.5. MALDI-TOF mass spectrometry

Prior to mass spectrometry analysis, samples were desalted by treatment with H^+-Dowex AG 50W X8 (Biorad, Hercules, CA), using a final concentration of 350 mg H^+-Dowex per ml digest. The desalted digests were mixed with a matrix solution (1 µl of sample in 1 µl of matrix) and applied on a MALDI sample plate. The matrix solution was prepared by dissolving 9 mg of 2,5- dihydroxybenzoic acid in a 1-ml mixture of acetonitrile:water (300 µl: 700 µl).

MALDI-TOF MS (Matrix-Assisted Laser Desorption/ Ionisation Time-Of-Flight Mass Spectrometry) analysis was performed using an Ultraflex workstation (Bruker Daltonics, Hamburg, Germany) equipped with a nitrogen laser of 337 nm. The mass spectrometer was selected for positive ions, which were accelerated by an electric field of 12 kV, after a delayed

extraction time of 200 ns. The ions were detected in the reflector mode. External calibration of the mass spectrometer was performed using a mixture of maltodextrines (mass range 365-2309).

2.6 Electrospray mass spectrometry

Electrospray MS was performed on a LTQ Ion-trap (Thermo, San Jose, CA). Sample was running through a transferring capillary (100 µm ID) and a spraying capillary with an ID of 50 µm at a flow rate of 1 µl/min. MS analysis was carried out in the positive mode using a spray voltage of 4.5 kV and a capillary temperature of 200 ºC. The capillary voltage and tube lens were set at 33 V and 225 V respectively. MS^2 and higher was executed using a window of 1 m/z and a relative collision energy of 20-30%.

2.7. Enzyme incubations

Purified XGH, prepared as described (Beldman *et al*., 2003), was used to degrade a-MHR-S-frA, a-MHR-S-frB and p-MHR-S (final concentration of 5 mg/ml). These substrates were dissolved in 50 mM NaOAc (pH 3.5) and incubated with XGH (final concentration of 0.35 µg/ml) for 20 h at 30 ºC. The enzyme had a specific activity of 150 U/mg. Subsequently, the enzyme was inactivated by heating the reaction mixtures for 10 min at 100 ºC. The digested substrates including their controls (i.e. a-MHR-S-frB and p-MHR-S, which were not treated with XGH) were desalted by treatment with H$^+$-Dowex AG 50W X8 (Biorad, Hercules, CA), using a final concentration of 350 mg H$^+$-Dowex per ml digest. The desalted samples were subsequently analyzed by HPSEC and HPAEC using a set of known XGA-oligosaccharides for identification (Zandleven *et al*., 2005).

2.8. Product analysis of XGH degraded a-MHR-S-frB and p-MHR-S at different time intervals

Oligosaccharide digests were prepared from a-MHR-S-frB and p-MHR-S as described above. At 0 min (blank), 30 min, 1h, and 20 h, a sample was taken and incubated at 100 ºC for 10

min to inactivate the enzyme. The obtained oligosaccharides were desalted and subsequently analyzed by HPAEC, and by MALDI-TOF MS or electrospray MS.

2.9 Fractionation of XGH degraded a-MHR-S-frB and p-MHR-S

A-MHR-S-frB and p-MHR-S were degraded by XGH using the same conditions as described in section "Enzyme incubations". These digests were fractionated by HPAEC on the Carbopac PA-1 column as described. Samples of the fractionated XGA oligosaccharides were desalted and analyzed by MALDI-TOF MS. Fractions containing GalAXyl$_2$ were pooled, desalted by H$^+$-Dowex AG 50W X8 (Biorad, Hercules, CA) and freezedried until further use.

2.10 Degradation of GalAXyl$_2$ by β-xylosidase

GalAXyl$_2$, as isolated from 25 mg of p-MHR-S and a-MHR-S-frB respectively, was degraded by β-(1,4)-xylosidase (E.C.3.2.1.37; GH 3) from *Aspergillus awamori*. The conditions were as follows: the total yield of GalAXyl$_2$ was dissolved in 500 µl 50 mM NaOAC (pH 4.0) and incubated with β-(1,4)-xylosidase (final concentration of 0.85 µg/ml) for 20 h at 30 °C. The enzyme preparation had a specific activity of 1.45 U/mg.

Degradation of GalAXyl$_2$ was analyzed by HPAEC and MALDI-TOF MS, as described in section 2.4 and 2.5 respectively.

3. Results and Discussion

3.1. Preparation of xylogalacturonan enriched fractions from a-MHR-S

In the current model for pectin, in particular for apple pectin, XGA is linked to rhamnogalacturonan I (Schols *et al*., 1995). To facilitate the isolation of XGA, a-MHR-S was treated with RGH and subsequently fractionated by size-exclusion column chromatography. From this, XGA enriched fractions were obtained that were used for further degradation by XGH.
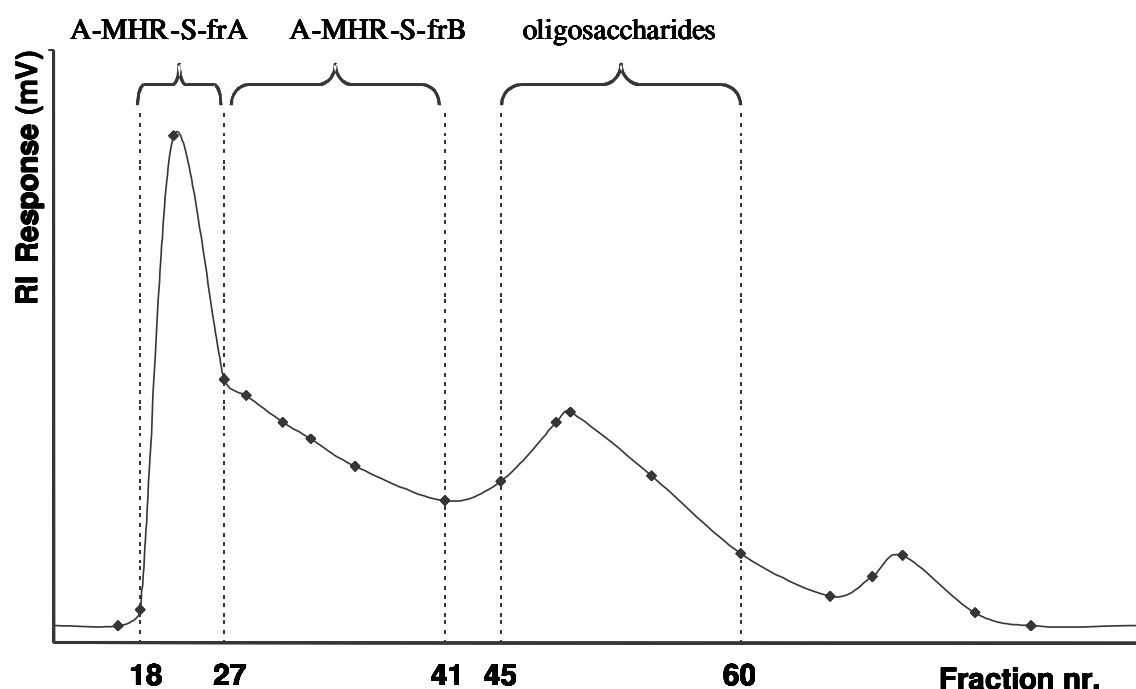
Figure 1. Size-exclusion chromatography of RG-hydrolase treated a-MHR-S on Superdex 30 PG.

As shown in Figure 1, column chromatography of RGH degraded a-MHR-S resulted in three distinct peaks, with a broad shoulder on the first peak. Fractions 18-27 and 27-41 were pooled and named a-MHR-S-frA and a-MHR-S-frB, respectively. As determined by HPSEC and HPAEC (results not shown), these fractions contained high molecular weight polysaccharides while fractions 45-60 contained oligosaccharides. The presence of high molecular weight XGA was further investigated in these polymer fractions by degradation with XGH and analysis of the products by HPLC. HPSEC and HPAEC analysis both demonstrated the formation of XGA oligosaccharides from a-MHR-S-frB (see section 3.3) while only minor

production of XGA oligosaccharides was observed from a-MHR-S-frA (results not shown). Apparently, this latter polymer fraction consists of RG-I structures with XGH-undegradable polysaccharides.

Based on the distinct formation of XGA oligosaccharides from a-MHR-S-frB it was decided to continue product analysis from this fraction only.

3.2 Sugar compositions of a-MHR-S-frB and p-MHR-S

The sugar compositions of a-MHR-S-frB and p-MHR-S are presented in Table 1. The amounts of xylose and galacturonic acid indicate that these substrates contain a significant amount of XGA. The arabinose content confirms the presence of long side chains of arabinan in rhamnogalacturonan I, in particular in the case for a-MHR-S-frB (Schols *et al*., 1990).

Substrate	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	AUA	(w/w %)*	Xyl:GalA
a-MHR-S-frB	3	0	61	9	0	9	0	19	75	0.47
p-MHR-S	5	0	18	12	1	15	1	49	60	0.24

Table 1. Sugar composition (mol%) of a-MHR-S-frB and p-MHR-S

* Percentage of carbohydrate in the sample, based on dry weight

3.3. HPLC analysis of XGH degraded a-MHR-S-frB and p-MHR-S

The action of XGH was studied towards XGA in a-MHR-S-frB and p-MHR-S. Degradation of these substrates was followed by HPSEC and HPAEC as shown in Figure 2 and 3, respectively.

From previous studies of RGH treated a-MHR-S (Schols *et al*., 1995) it is known that XGA, linked to rhamnogalacturonan, elutes in the first peak in the HPSEC chromatogram. This peak represents the high molecular weight fraction of RGH treated a-MHR-S-frB. Upon XGH treatment, this XGA-containing peak (which elutes around 19-22 min; Fig. 2), disappeared while in the case of XGH treatment of p-MHR-S only a partial decrease of the corresponding peak was observed. This shows that XGA degradation took place in both substrates. However,

the partial decrease of the XGA containing peak of p-MHR-S can be ascribed to the presence of XGH-resistant parts of XGA and / or other high molecular weight polysaccharides of RG-I.
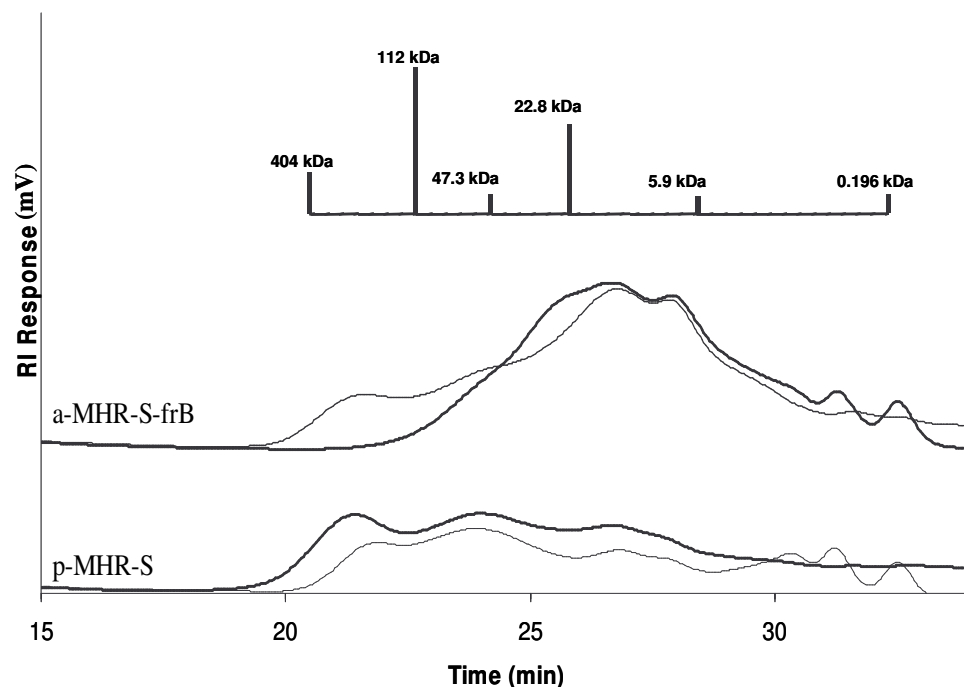
Figure 2. HPSEC of a-MHR-S-frB and p-MHR-S, respectively untreated (thin line) and treated for 20 hr with XGH (thick line). The ruler shows the molecular weights of the Pullunan standards (404, 112, 47.3, 22.8, and 5.9 kDa) and GalA (0.196 kDa).

As shown in Figure 2, three peaks appeared at retention times around 30-33 minutes upon XGH treatment of a-MHR-S-frB and p-MHR-S. The latter peak is eluting in the size range of GalA (i.e. around 32.5 min), while the other two peaks represent XGA oligosaccharides.

The formation of XGA oligosaccharides from a-MHR-S-frB and p-MHR-S was also analyzed by HPAEC. To identify these oligosaccharides, a mixture of XGA oligosaccharides with known structures, derived from gum tragacanth (XGA-29), was used (Zandleven *et al*., 2005; Fig 3). As shown in Figure 3, HPAEC analysis demonstrated the formation of XGA oligosaccharides from the XGH degradable parts of XGA in a-MHR-S-frB and p-MHR-S. A similar type of XGA oligosaccharide production was observed for both substrates. Compared to the large number of different oligosaccharides present in the gum tragacanth digest, only a few distinct XGA oligosaccharides were released from a-MHR-S-frB and p-MHR-S. The main product was the disaccharide GalAXyl, while a significant amount of $GalA_2Xyl_2$ and

minor quantities of GalA, GalA$_2$ and GalA$_2$Xyl were found. Other unidentified oligosaccharides, including an oligosaccharide eluting at 27.4 min (shown as peak Y) were also observed. Large XGA oligosaccharides as obtained from XGA-29 (which are shown as peaks 7-15; Fig. 3), were hardly produced from a-MHR-S-frB and p-MHR-S.
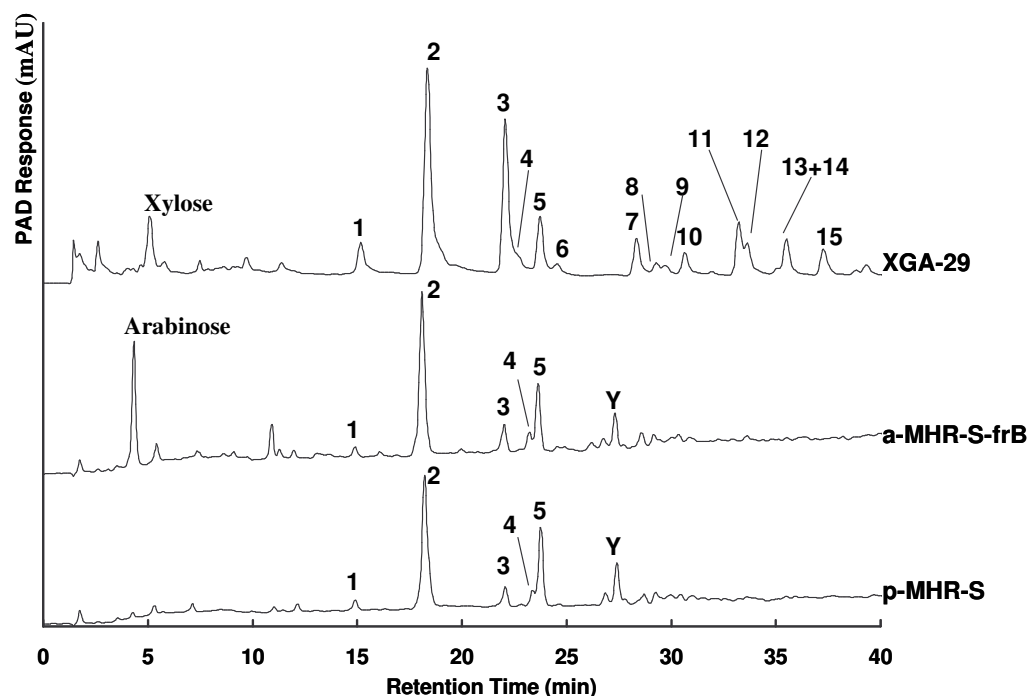
1) GalA	○	4) GalA$_2$	●–○	7) GalA$_3$Xyl	10) GalA$_3$Xyl'	13) GalA$_4$
2) GalAXyl	○ with X	5) GalA$_2$Xyl$_2$	●–○ with X X	8) GalA$_3$Xyl$_2$	11) GalA$_4$Xyl	14) GalA$_4$Xyl' + GalA$_5$Xyl$_2$
3) GalA$_2$Xyl	●–○ with X	6) GalA$_2$Xyl'	●–○ with X	9) GalA$_3$	12) GalA$_4$Xyl$_2$	15) GalA$_5$Xyl

Figure 3. HPAEC of XGH degraded XGA-29 (from gum tragacanth; used as standard), a-MHR-S-frB, and p-MHR-S. The table summarizes the XGA oligosaccharides of which some have been structurally characterized (Zandleven *et al*., 2005). The accentuated XGA oligosaccharides (') are isomers with different distribution of xylose over the GalA backbone. ● = GalA, ○ = reducing GalA and Y = XGA oligosaccharide with unknown structure.

The appearance of unknown oligosaccharides in the a-MHR-S-frB and p-MHR-S digests, necessitated further identification by MALDI-TOF MS and is described in section 3.4.

Free arabinose was also found in the a-MHR-S-frB digest. As the XGH preparation does not contain arabinofuranosidase, this arabinose is probably liberated by acid hydrolysis during the incubation temperature and inactivation step at 100 °C (pH 3.5) of the digestion mixture. Indeed a lower amount of free arabinose (about 60 % reduction) was detected by HPAEC analysis of a-MHR-S-frB which was not treated at 100 °C (results not shown). The formation of free arabinose was even further reduced (about 90 % reduction) when a-MHR-S-frB was incubated at pH 6.0.

The susceptibility for hydrolysis of the arabinosyl linkages at a low pH has also been reported before (Beldman, Schols, Pitson, Searle-van Leeuwen, Voragen, 1997). Also free arabinose was expected in the p-MHR-S digest, but could however not be detected by HPAEC analysis (results not shown). This is probably related to the lower arabinose content of this substrate (Table 1) as well as to structural differences of this arabinan (Beldman *et al.*, 1997).

3.4. Product analysis of XGH degradable parts of XGA from a-MHR-frB and p-MHR-S at different time intervals

a-MHR-S-frB and p-MHR-S were degraded by XGH for different time intervals and analyzed by HPAEC (Fig. 4) and MALDI-TOF MS (Fig. 5) for the presence of XGA oligosaccharides. Degradation of a-MHR-S-frB and p-MHR-S during 30 min and 1 h resulted mainly in the production of the disaccharide GalAXyl (Fig. 4), which was also reported before for XGH treatment of a-MHR-S (Beldman *et al.*, 2003) and XGA-29 from gum tragacanth (Zandleven *et al.*, 2005) respectively. In addition, a significant amount of $GalA_2Xyl_2$ was observed as degradation product from a-MHR-S-frB and p-MHR-S.

Comparing the HPAEC profiles of Figure 3 and 4 for the corresponding samples of a-MHR-S-frB and p-MHR-S (20 h of treatment with XGH) only some minor differences were observed, which falls within the range of experimental variance.

The HPAEC results were confirmed by MALDI-TOF MS analysis of the products from p-MHR-S (Fig. 5) and a-MHR-S-frB (results not shown) for the samples after 20 h of incubation. The MS spectra of both degraded substrates showed the main product GalAXyl, and significant amounts of $GalA_2Xyl_2$, $GalA_2$ and $GalA_2Xyl$. Two novel XGA oligosaccharides, $GalAXyl_2$, with a m/z of 481, and $GalA_2Xyl_3$, with a m/z of 789, were observed as products from both substrates. The formation of $GalAXyl_2$ and $GalA_2Xyl_3$ by

XGH suggests the presence of dimeric side chains in XGA of these pectic polysaccharides from apple and potato. In order to confirm this, the structure of GalAXyl$_2$ and GalA$_2$Xyl$_3$ was investigated further (see section 3.5).
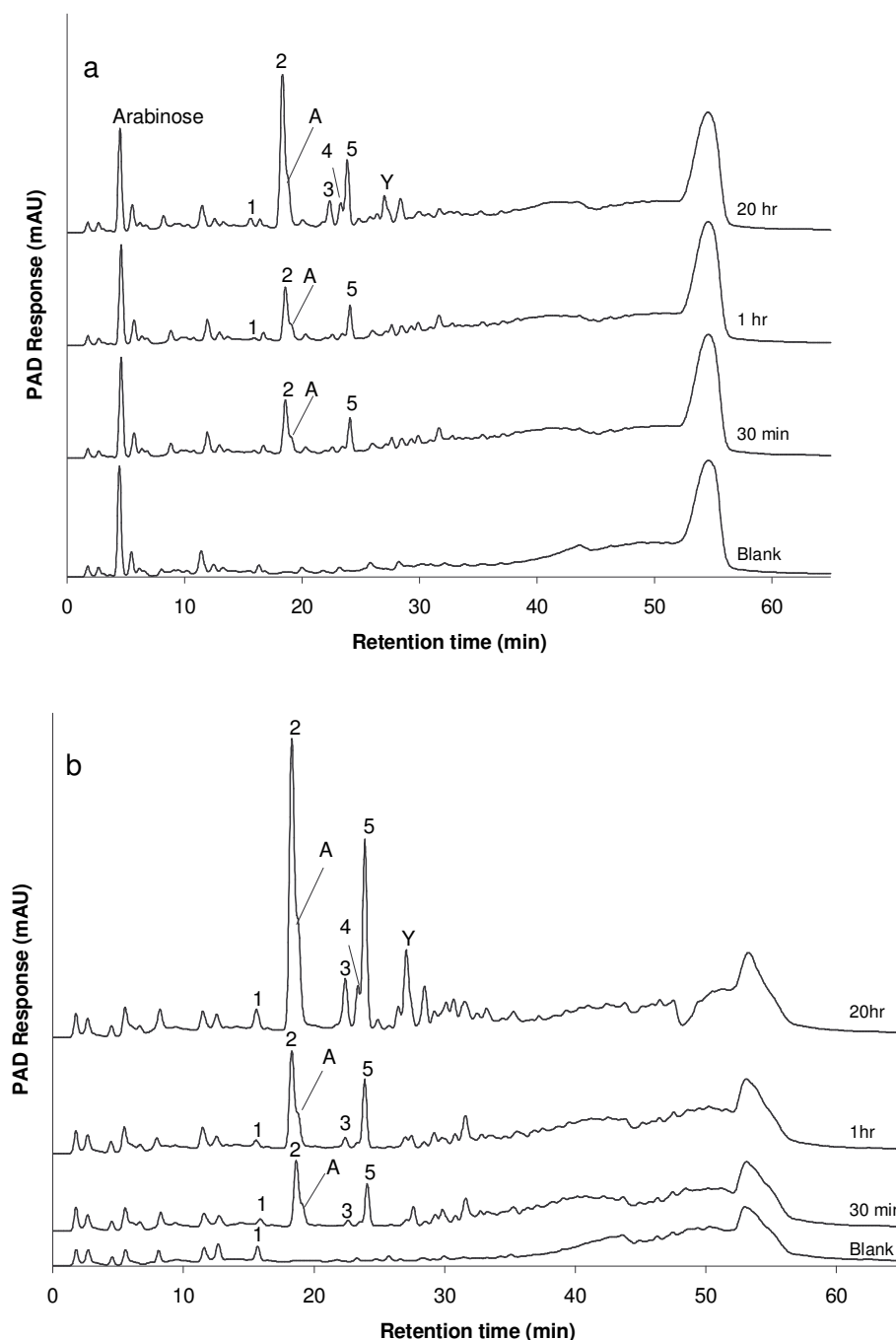
Figure 4. HPAEC of a) a-MHR-S-frB after treatment with XGH for 0 min (blank), 30 min, 1 h and 20 h; and b) p-MHR-S after treatment with XGH for 0 min (blank), 30 min, 1 h and 20 h. All samples were treated at 100° C for 10 minutes to inactivate the enzyme. Numbers refer to XGA oligosaccharides as shown in the table in Figure 3. A= GalAXyl$_2$, Y= XGA oligosaccharide with unknown structure.
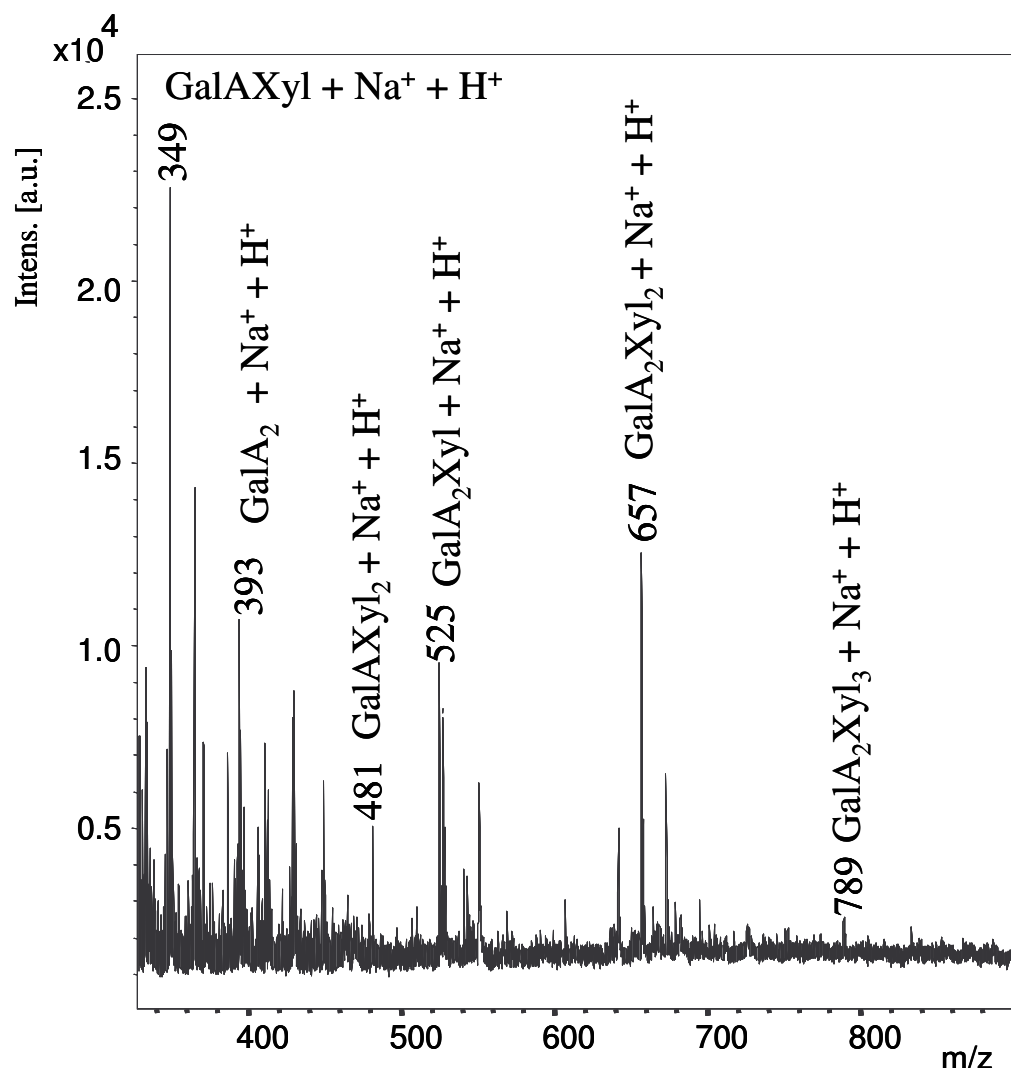
Figure 5. Positive mode MALDI-TOF MS spectrum of p-MHR-S after treatment with XGH for 20 h. The m/z masses of the XGA oligosaccharides include H^+ and Na^+.

As previously hypothesized, XGH is a processive enzyme which prefers to act between two xylosylated GalA units, based on its mode of action towards XGA from gum tragacanth (Zandleven *et al.*, 2005). This report confirms this hypothesis, based on the predominant production of GalAXyl by XGH treatment of a-MHR-S-frB and p-MHR-S. Furthermore, these results indicate that a rather regular distribution of xylose side chains over the galacturonan back-bone exists in the degradable parts of XGA of both sources. This is based on the relatively little variation in oligosaccharide products in the XGH digests.

Degradation of XGA from gum tragacanth by XGH showed a large number of different XGA oligosaccharides (Zandleven *et al.*, 2005), indicating a random distribution of xylose side chains. This is confirmed in the present study and can be explained by the preparation procedure of XGA from gum tragacanth, involving a mild TFA treatment (Beldman *et al.*, 2003), which leads to a random release of xylose residues from the XGA substrate.

The oligosaccharide $GalAXyl_2$ was identified in the digests by MALDI-TOF MS, but could not yet be identified in the corresponding HPAEC chromatograms of these samples. For this, XGH-degraded p-MHR-S was fractionated by HPAEC and the presence of $GalAXyl_2$ in the fractions was analyzed by MALDI-TOF MS. It was found that $GalAXyl_2$ and GalAXyl co-elute during HPAEC analysis (results not shown). However, due to experimental variance, in some cases these oligosaccharides could partly be separated by HPAEC, as for instance shown in Figure 4. In this figure $GalAXyl_2$ eluted slightly later than GalAXyl and is indicated as "shoulder" A. These results also demonstrate that the production of $GalAXyl_2$ from a-MHR-S-frB and p-MHR-S by XGH is significant. As shown in Figure 4, the peak height for this oligosaccharide was almost half of the peak height for GalAXyl, which implies that a substantial amount of dimeric side chains of xylose is present in xylogalacturonans from apple and potato pectin. In line with these results, it is hypothesized that peak Y (Fig 3. and Fig. 4) corresponds to $GalA_2Xyl_3$, based on the elution behavior of neighboring XGA oligosaccharides.

3.5. Investigation on the structure of $GalAXyl_2$ and $GalA_2Xyl_3$ from p-MHR-S and a-MHR-S-frB

The fine structure of $GalAXyl_2$ and $GalA_2Xyl_3$ in XGH digests of p-MHR-S and a-MHR-S-frB was studied by electrospray MS (Fig. 6 and 7). As shown in Figure 6, the presence of $GalAXyl_2$ is confirmed for p-MHR-S, as demonstrated by the fragmentation of this oligosaccharide in MS^2 mode. The appearance of a fragment at m/z 305, corresponding to two linked xyloses, demonstrates the dimeric side-chain in $GalAXyl_2$. This fragment was observed at a higher abundance when MS^3 analysis was performed on a fragmentation product of $GalAXyl_2$ at m/z 463, which corresponded to $GalAXyl_2$ with the loss of OH^- and H^+ (Fig 6; inserted panel).
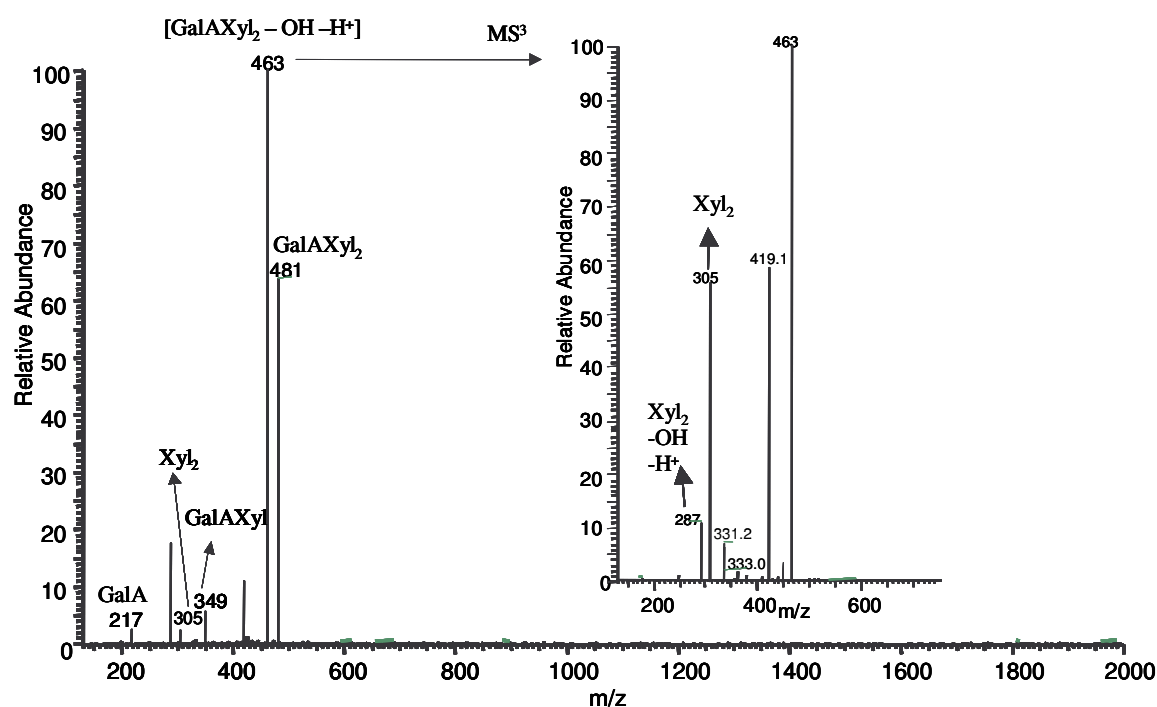
Figure 6. MS^2 scan of GalAXyl$_2$ at m/z 481 including a MS^3 scan of a fragment of GalAXyl$_2$ at m/z 463 (inserted panel). The *m/z* value of GalAXyl$_2$ include the addition of an H$^+$ and Na$^+$. The fragment at m/z 463 represents GalAXyl$_2$ with the loss of OH$^-$ and H$^+$.
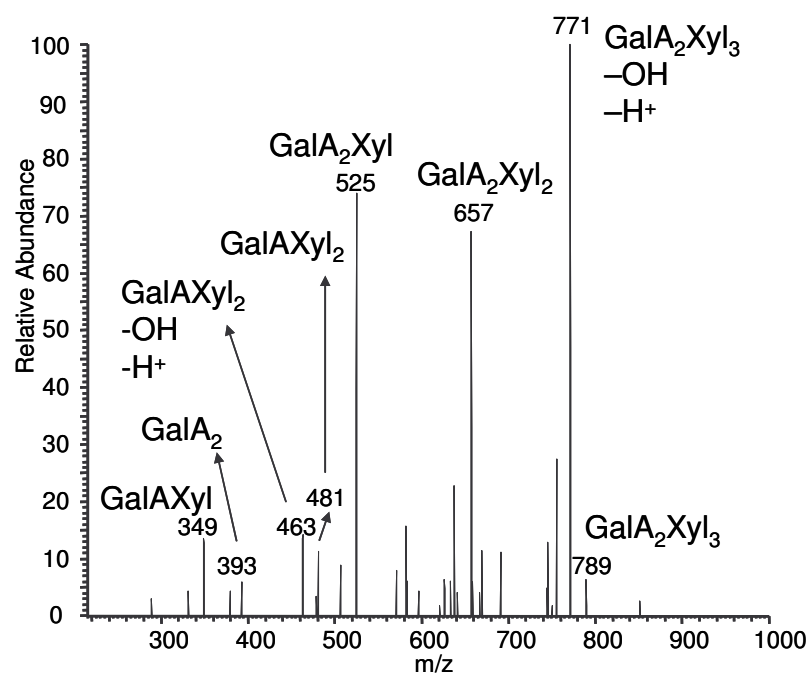
Figuur 7. MS^2 scan of GalA$_2$Xyl$_3$ at m/z 789. The *m/z* value of GalA$_2$Xyl$_3$ include the addition of an H$^+$ and Na$^+$.

An identical fragmentation spectrum was obtained for $GalAXyl_2$ that was formed by fragmentation of $GalA_2Xyl_3$ in the MS^2 mode (for the latter, see Fig. 7). This confirms that a dimeric side chain of xylose also exists in the XGA oligosaccharide $GalA_2Xyl_3$.

Similar results were acquired for $GalAXyl_2$ and $GalA_2Xyl_3$ present in the a-MHR-S-frB digest (results not shown), which points out that these dimeric side-chains of xylose are also present in XGA from apple pectin.

Based on linkage analysis, the existence of side chains longer than one xylose unit in XGA has been indicated for apple- (Oechslin *et al.*, 2003) and pea pectin (Le Goff *et al.*, 2001). In this report, the occurrence of dimeric side chains of xylose in XGA in apple- and potato pectin is demonstrated based on the structure of the oligosaccharides $GalAXyl_2$ and $GalA_2Xyl_3$.

It is generally accepted that the single terminal xylose is in the β-form and is linked at the O-3 position of GalA in XGA (Beldman *et al.*, 2003; Le Goff *et al.*, 2001; Schols *et al.*, 1995; Vincken *et al.*, 2003a). From this, we assume that the dimeric side chains in XGA from a-MHR-S-frB and p-MHR-S are also linked at this position.

The nature of the linkage between the xyloses in the dimeric side chains in XGA from apple and potato pectin is unknown. In order to obtain information about the anomeric form of the terminal xylose residue, we purified the oligosaccharide $GalAXyl_2$ from a p-MHR-S and a-MHR-S-frB digest to near homogeneity (>95%) by analytical HPAEC. Subsequently it was treated with β-(1,4) xylosidase from *Aspergillus awamori* and analyzed by HPAEC and MALDI-TOF MS (results not shown). $GalAXyl_2$ from both sources was degraded by this enzyme to free xylose and GalAXyl, which implies that the two xyloses are β-linked.

Linkage analysis of apple pectin (Schols *et al.*, 1995) and cellulase degraded cellulosic residues of apples (Oechslin *et al.*, 2003) respectively, show that 1,4 linked xylose residues prevail as non-terminal xylose in apple pectin. Schols *et al.* (1995) found that 90% of the total amount of non-terminal xylose residues exist as 1,4 linked xylose residues, whereas Oechslin *et al.* (2003) showed that exclusively 1,4 linked xylose residues are present. From these studies it is reasonable to assume that the dimeric side chains of xylose present in a-MHR-S-frB could also be 1,4 linked.

The fact that a-MHR-S-frB and p-MHR-S were successfully degraded by XGH with concomitant production of $GalAXyl_2$ and $GalA_2Xyl_3$ implies that the dimeric side chains in XGA from these sources did not hinder the action of the enzyme. We already showed that

xylosylated GalA units can accommodate subsites -1 and +1 of XGH (Zandleven *et al.*, 2005). This study indicates that GalA residues that have a side chain of two xylose moieties, can also fit in these subsites. This is illustrated in Figure 8, which shows the formation of GalAXyl$_2$ from a specific part of a XGA molecule, in two consecutive steps. Based on this model, it can be speculated that the dimeric side chains of xylose in XGA from apple and potato pectin are probably pointed towards the outside of the binding cleft of XGH, when the enzyme-substrate complex is formed.
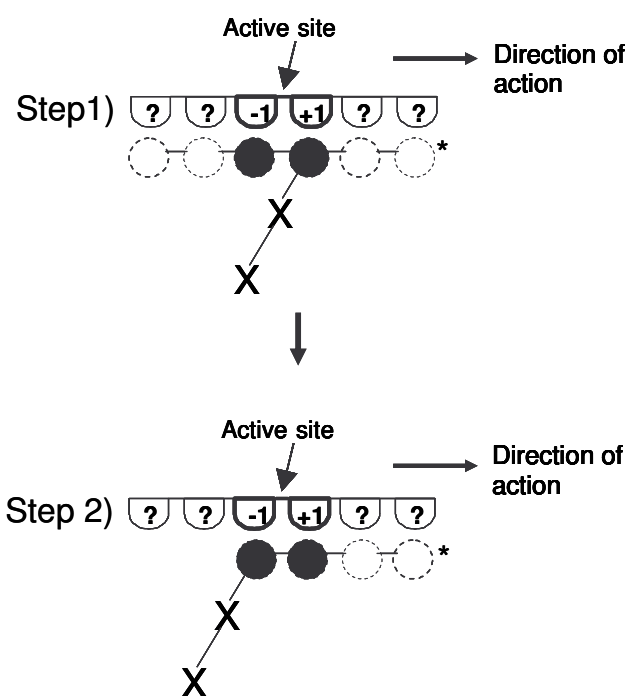
Figure 8. Representation of the proposed action of XGH to produce GalAXyl$_2$ from XGA in apple and potato pectin. GalA =•, xylose = X . The asterix (*) indicates the reducing end.

Conclusively, this study confirms the occurrence of XGA in apple and potato pectin by the use of XGH which specifically acts towards this polymer. The degradable part of XGA from both sources is similar in structure, contains a regular distribution of xylose side chains, and also contains dimeric side chains of xylose which are presumably β-1,4 linked in the case for apple pectin.

Acknowledgements

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Chapter 5

Xylogalacturonan exists in cell walls from various tissues of *Arabidopsis thaliana*

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Abstract

Evidence is presented for the presence of xylogalacturonan (XGA) in *Arabidopsis thaliana*. This evidence was obtained by extraction of pectin from the seeds, root, stem, young leaves and mature leaves of *A. thaliana*, followed by treatment of these pectin extracts with xylogalacturonan hydrolase (XGH). Upon enzymatic treatment, XGA oligosaccharides were primarily produced from pectin extracts obtained from the young and mature leaves and to a lesser extent from those originating from the stem of *A. thaliana*. The oligosaccharide $GalA_3Xyl$ was predominantly formed from these pectin extracts. No XGA oligosaccharides were detected in digests of pectin extracts from the seeds and roots.

A low number of different XGA oligosaccharides were obtained from pectins of *A. thaliana*; as was also observed in digests of XGA from apple and potato pectins. This indicates a uniform distribution of xylose in XGA from *A. thaliana*. The predominant production of $GalA_3Xyl$, as well as the release of linear GalA oligosaccharides pointed to a lower degree of xylose substitution in XGA from *A. thaliana* than in XGA from apple and potato.

The estimated amount of XGA accounted for approximately 2.5 %, 7 % and 6 % (w/w) of the total carbohydrate in the pectin fraction of the stem, young leaves and mature leaves respectively.

1. Introduction

Primary cell walls are the major textural components of plant-derived foods. The most important polysaccharides that account for 90 to 100% of the structural polymers of these cell walls are cellulose, hemicellulose and pectin (Albersheim *et al.*, 1996).

Cellulose is comprised of β-(1→ 4)-linked D-glucan while hemicelluloses primarily consists of xyloglucan and arabinoxylan (Albersheim *et al.*, 1996). Pectin is a heteropolysaccharide, which contains α-(1→ 4)-linked D-galacturonic acid chains (also known as the smooth regions of pectins) and the branched polysaccharides rhamnogalacturonan I, rhamnogalacturonan II, and xylogalacturonan (referred to as the "hairy" regions) (Benen *et al.*, 2002; Schols and Voragen, 1996; Vincken *et al.*, 2003).

Cellulose exists in plant cell walls as microfibrils, which are composed of long semicrystalline β-glucan chains (Gibeaut and Carpita, 1994). These microfibrils are cross-linked by hemicelluloses, such as xyloglucans that are believed to be a major factor in controlling the rate of cell-wall expansion (Gibeaut and Carpita, 1994; O'Neill and York, 2003; Zablackis *et al.*, 1995). The load-bearing cellulose-hemicellulose network is embedded in a matrix of pectic polysaccharides, which form a hydrated and crosslinked three-dimensional network (Gibeaut and Carpita, 1994; Knox, 2002).

Xylogalacturonan (XGA) is a chain of α-(1→ 4)-linked D-galacturonic acid, which is substituted with β-D-xylose at the O-3 position. It is suggested that this biopolymer is a side chain of RG-I (rhamnogalacturonan I) in the "hairy" regions of pectin (Vincken *et al.*, 2003).

The presence of XGA in plants has been reported in storage tissues of reproductive organs such as in cell walls of peas, soybeans, watermelons, apples, pears, onions, potato's, pine pollen, and cotton seeds (Albersheim *et al.*, 1996; Le Goff *et al.*, 2001; Nakamura *et al.*, 2002; Schols *et al.*, 1995; Thibault and Ralet, 2001; Vincken *et al.*, 2003; Voragen *et al.*, 2001; Zandleven *et al.*, 2006; Huisman *et al.*, 1999). Its presence has also been reported in exudates from trees, such as gum tragacanth from the *Astralagus species* (Aspinall and Baillie, 1963).

Previous studies demonstrated that leaf primary cell walls of *A. thaliana* contain homogalacturonan, RG-I, RG-II, xylan, xyloglucan and cellulose (Zablackis *et al.*, 1995). These polymeric structures were also suggested to be present in cell walls of the stem of this plant species (Gardner *et al.*, 2002). Additionally, mannan polysaccharides have also been reported in the cell walls of leaves and stem of *A. thaliana* (Handford *et al.*, 2003).

So far, the presence of other polysaccharides, such as XGA, has not been demonstrated in *A. thaliana* (Zablackis *et al.*, 1995), although it has been suggested that this polymer exists in root caps of this plant species. This was based on immunocytochemical analysis using an LM8 antibody that was raised against pea XGA (Willats *et al.*, 2004). Also Gardner *et al.* (2002) mentioned XGA as a probable xylose-containing polysaccharide in the stem of *A. thaliana*; however this was only based on the sugar composition of the alcohol insoluble residue (AIR) of the stems.

Recently, a gene (At5g33290) expected to encode a β-xylosyl transferase was identified in *A. thaliana*. Pectin isolated from a T-DNA insertion line having a T-DNA insertion in this particular gene was found to contain less xylose compared to the wild type (Sørensen *et al.*, unpublished data). We therefore hypothesized that pectin from *A. thaliana* may contain regions of XGA. To investigate this, pectin was extracted from cell wall material, prepared from the seeds, roots, young leaves and mature leaves of *A. thaliana*. The pectin fractions were analysed for their sugar composition and subsequently treated with xylogalacturonan hydrolase (XGH) to determine the presence of XGA. Based on the obtained XGA hydrolysis products, some structural characteristics of *A. thaliana* XGA are hypothesized. Also the amount of XGA liberated from these pectin fractions was estimated.

2. Results

2.1 Analysis of CWM

CWM was prepared from the seeds, roots, stem, young leaves and mature leaves of *Arabidopsis thaliana*. In line with other reports (Zablackis *et al.*, 1995), polysaccharides possibly lost during some of the preparation steps (i.e. starch and protein removal) were not considered as cell wall components.

These CWM was saponified to ensure full removal of methyl- and acetyl esters, which could interfere with subsequent enzymatic treatment. The sugar composition of the saponified CWM (CWM-s) samples is shown in Table 1.

	(mol %)								(w/w %)
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	AUA	Total carbohydrate
Mature leaves	3	1	8	9	5	7	33	35	49
Young leaves	2	1	7	8	6	8	37	33	47
Stem	1	0	6	20	7	5	43	19	57
Root	2	1	18	14	4	7	35	20	52
Seeds	8	1	18	8	3	11	27	25	20

Table 1. Sugar composition (mol %) and total carbohydrate content (w/w %) of CWM-s from mature leaves, young leaves, stem, root and seeds of *A. thaliana*

All the samples contained high amounts of glucose and significant amounts of xylose, which suggests the presence of cellulose, xylan and/ or xyloglucan. The presence of these polysaccharides was indicated before in cell walls of *A. thaliana* leaves (Zablackis *et al.*, 1995).

Besides glucose and xylose, also rhamnose, arabinose, galactose and in particular high amounts of galacturonic acid were detected, which implies that these samples also contain pectin. The presence of the major sugars, glucose and galacturonic acid has also been demonstrated previously for *A. thaliana* stems (Gardner *et al.*, 2002) and *A. thaliana* leaves (Zablackis *et al.*, 1995) although different pectin extraction techniques were used. The total carbohydrate content of the CWM-s from the different plant organs (Table 1) was relatively low, which is probably caused by the presence of non-polysaccharide materials, such as salts and proteins. Also the pectin fractions as well the pellet fractions, derived from these CWM-s,

have a low total carbohydrate content (see Table 2 and 3). This is probably due to similar reasons as well as the use of EDTA in the extraction buffer. Apparently, non-polysaccharide material (including EDTA) is not easily removed from the pectin and pellet fractions by dialysis. Despite this, the presence of non-polysaccharide materials in these fractions did not hinder the enzymatic degradation of XGA (see section 2.3)

2.2 Analysis of pectin extracts

To investigate the presence of xylogalacturonan (XGA) in the cell walls of seeds, roots, stem, young leaves and mature leaves of *A. thaliana* it was required to extract the pectin from CWM-s of these plant organs first. The solubilized material, as extracted from these CWM-s, which contain the pectic polysaccharides will be referred to as "pectin fractions". Figure 1 shows an example of a pectin extraction scheme (mature leaves). The amounts of dry matter and total carbohydrate in the CWM-s and the pectin fraction are also indicated. The calculated yields of dry matter of the pectin fractions were 52 %, 28 %, 31 %, 42%, and 35 % (w/w) of the CWM-s of the seeds, roots, stem, young leaves and mature leaves respectively.

CWM-s from mature leaves
105 mg dry matter → 49 % (w/w) total carbohydrate (= 51.5 mg)

Pellet fraction   Pectin fraction
                   36.8 mg dry matter → 28 % (w/w) total carbohydrate (=10.3 mg)

Yield of dry matter: $\dfrac{36.8 \text{ mg}}{105 \text{ mg}}$ *100 % ≈ 35 %

Yield of carbohydrate: $\dfrac{10.3 \text{ mg}}{51.5 \text{ mg}}$ *100 % ≈ 20 %

Treatment with XGH

0.6 mg XGA oligosaccharides

Yield of carbohydrate as oligosaccharides: $\dfrac{0.6 \text{ mg}}{10.29 \text{ mg}}$ * 100 % ≈ 6 %

Figure 1. Pectin extraction scheme of the mature leaves from *A. thaliana*, which includes the yield of total carbohydrate in the CWM-s and pectin fraction, as well as the yield of XGA oligosaccharides derived from the pectin fraction.

Based on the dry matter yields and the sugar compositions it was estimated that the yield of carbohydrate in the pectin fractions were 8 %, 16 %, 17 %, 21 % and 20 % (w/w) for the seeds, roots, stem, young leaves, and mature leaves respectively.

As expected for the method used (Voragen *et al.*, 1983), the sugar composition of all the pectin fractions shows that galacturonic acid is the major sugar (Table 2).

Pectin extracts	(mol %)								(w/w %)
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	AUA	Total Carbohydrate
Mature leaves	3	1	9	7	5	9	5	64	28
Young leaves	4	0	9	6	5	9	5	64	23
Stem	3	0	16	13	2	10	4	52	31
Root	2	1	10	6	5	10	3	63	29
Seeds	15	0	17	5	3	4	7	70	14

Table 2. Sugar composition (mol %) and total carbohydrate content (w/w %) of pectin extracts of CWM-s from mature leaves, young leaves, stem, root and seeds of *A. thaliana*.
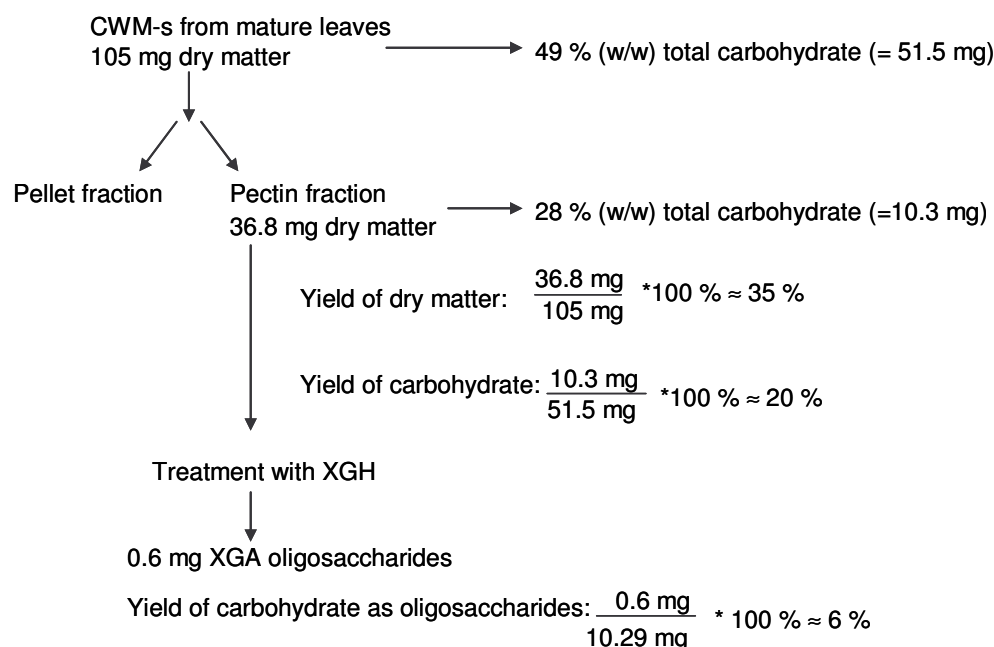
Compared to the sugar composition of the CWM-s samples, the amount of glucose is significantly reduced in the corresponding pectin fractions, which illustrates that pectin was selectively extracted.

Arabinose and galactose are the major neutral sugars in pectin fractions from the root, stem and leaves. This was also found in pectin extracts from several fruit and vegetables (Voragen *et al.*, 1983). The arabinose and galactose contents suggest that RG-I with side chains of arabinan and/ or arabinogalactan exists in these pectin fractions.

A relatively low content of galactose was observed in the pectin fraction from the seeds. On the other hand a high amount of rhamnose was found in this pectin fraction. This has also been reported before for the ammonium oxalate extracted mucilage from the seeds of *A. thaliana* (Usadel *et al.*, 2004). This implies that RG-I is a major component in pectin from the seeds of *A. thaliana*.

The presence of both galacturonic acid and xylose in all pectin fractions (Table 2) indicate that these fractions may contain XGA. This is further investigated by the use of XGH as described in section 3.3.

The major sugars in the pellet fractions of the different CWM-s extracts from roots, stem, young leaves and mature leaves were glucose and xylose (Table 3), which points to the potential presence of cellulose, xylan and/ or xyloglucan.

Pellets	(mol %)								(w/w %)
	Rha	Fuc	Ara	Xyl	Man	Gal	Glc	AUA	Total carbohydrate
Mature leaves	2	1	9	12	4	6	48	19	41
Young leaves	1	0	8	13	5	7	51	17	38
Stem	2	1	3	23	4	3	57	9	53
Root	2	2	18	14	3	7	44	10	41
Seeds	4	1	19	7	3	12	28	27	27

Table 3. Sugar composition (mol %) and total carbohydrate content (w/w %) of pellet fractions after pectin extraction of CWM-s from mature leaves, young leaves, stem, root and seeds of *A. thaliana*.

Besides glucose and xylose, these pellet fractions also contain uronic acid. Apart from glucose, the pellet fraction of the seeds is particularly rich in this monosaccharide. Based on the sugar composition of the pellet fractions it is suggested that uronic acid occurs as glucuronic acid as well as galacturonic acid in these fractions. This is based on the results shown in Table 3, in which the presence of arabinose, xylose and uronic acid indicate that these fractions contain the hemicellulosic polysaccharide glucuronoarabinoxylan. A similar conclusion was made by Zablackis *et al*. (1995). Besides this, also pectin related sugars were found in these pellet fractions, which point at the presence of galacturonic acid. Again, the total carbohydrate content of the pellet fractions was relatively low, which is probably due to reasons already mentioned above.

The pellet fraction from the seeds has a relatively higher content of rhamnose and galactose, compared to other pellet fractions, which indicates that this fraction contains a relatively high amount of rhamnogalacturonan. The relative galactose content of the pellet fraction from the seeds was 3 times higher than its corresponding pectin fraction, while the relative rhamnose content in this pellet fraction was approximately 4 times lower. Also an equal proportion of arabinose was found in the pellet, compared to its corresponding pectin fraction. These results indicate that, although a major part of the pectin was extracted, the pellet fraction of seeds still contains rhamnogalacturonan I with side chains of arabinans and/ or arabinogalactans.

2.3 Evidence for XGA in *A. thaliana*

The presence of XGA in the pectin fractions was investigated using the enzyme XGH as analytical tool. The pectin fractions were treated with XGH for 16 h, prior to analysis by HPAEC and MALDI-TOF MS.
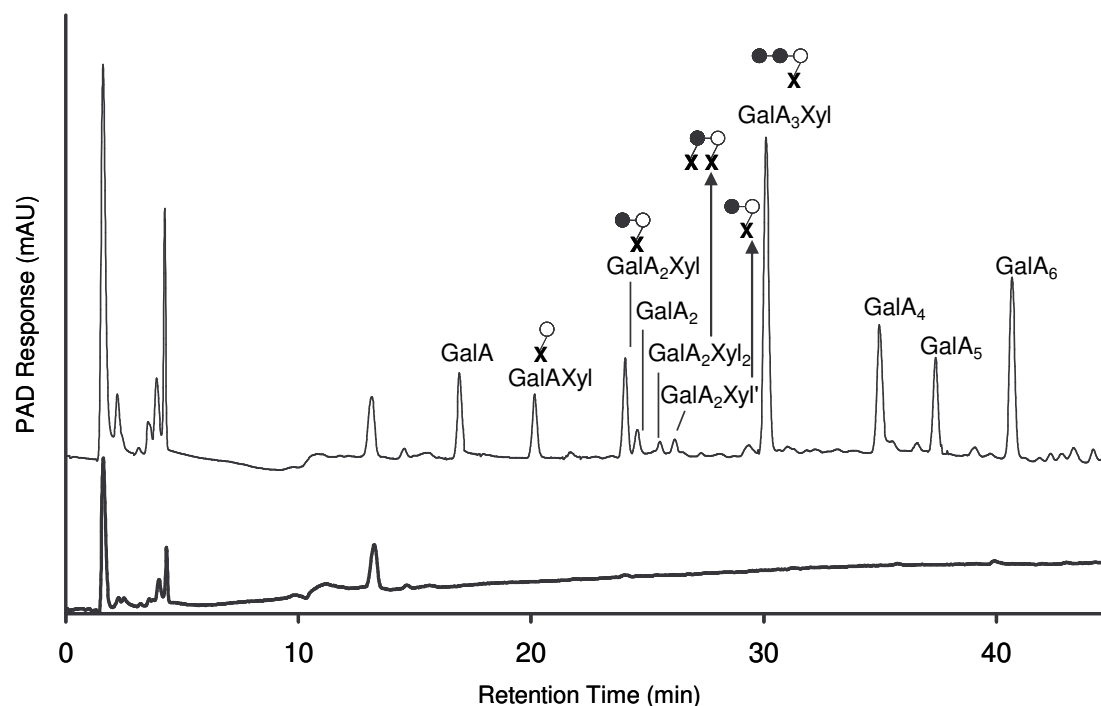
Figure 2. HPAEC of pectin from mature leaves of *A. thaliana*, untreated (bold line) and treated for 20 h with XGH (thin line). The structures of the XGA oligosaccharides, as characterized previously (Zandleven *et al.*, 2005), are also shown. The accentuated oligosaccharide $GalA_2Xyl$' is an isomer in which the xylose residue is probably linked to the O-2 of the reducing GalA, instead of the O-3 in $GalA_2Xyl$. ● = GalA, ○ = reducing GalA, X= xylose.

As demonstrated by HPAEC (Fig. 2), ten different oligosaccharides were identified in the mature leaf digest. The main product was $GalA_3Xyl$, while significant amounts of GalA, GalAXyl, $GalA_2Xyl$, $GalA_4$, $GalA_5$ and $GalA_6$ and minor quantities of $GalA_2$, $GalA_2Xyl_2$ and $GalA_2Xyl$' were observed. The accentuated oligosaccharide $GalA_2Xyl$' is an isomer in which the xylose residue is probably linked to the O-2 of the reducing GalA (Zandleven *et al.*, 2005).

Comparable results were acquired for young leaves (Fig. 3) and stem (results not shown), although a significantly lower level of the oligosaccharides $GalA_4$, $GalA_5$ and $GalA_6$ was released from pectin originating from the young leaves.
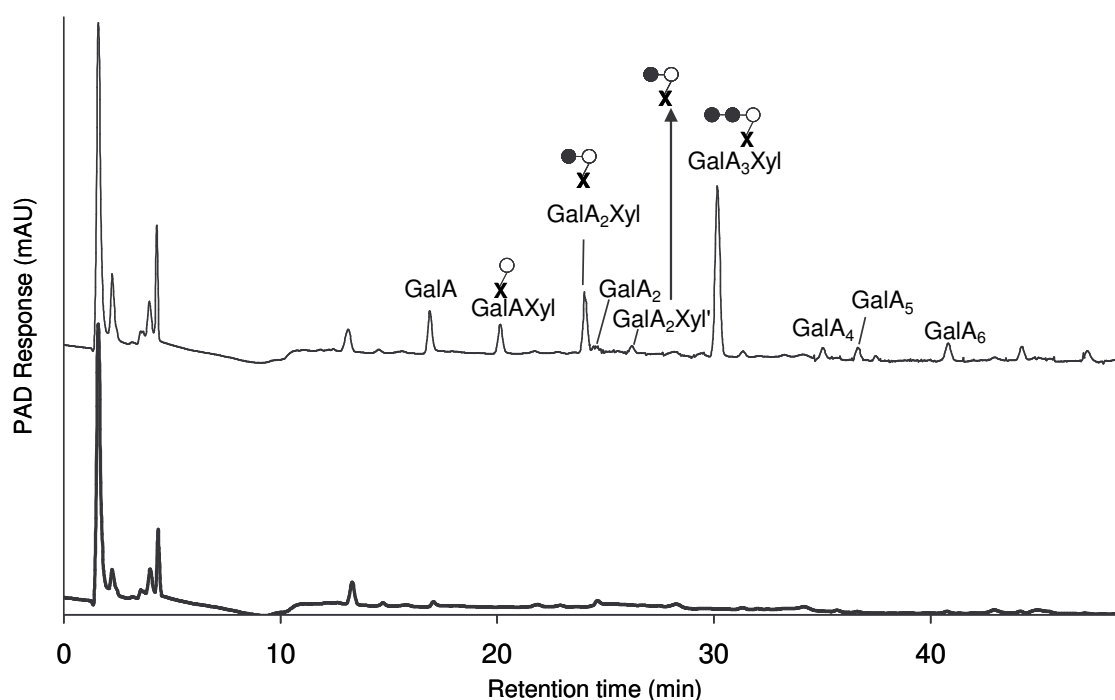
Figure 3. HPAEC of pectin from young leaves of *A. thaliana*, untreated (bold line) and treated for 20 h with XGH (thin line). The structures of the XGA oligosaccharides, as characterized previously (Zandleven *et al.*, 2005), are also shown. The accentuated oligosaccharide $GalA_2Xyl'$ is an isomer in which the xylose residue is probably linked to the O-2 of the reducing GalA, instead of the O-3 in $GalA_2Xyl$.

• = GalA, ○ = reducing GalA, X= xylose.

The formation of a series of oligosaccharides, as presented in Figures 2 and 3, was confirmed by MALDI-TOF MS (results not shown). From this it can be concluded that XGA is present in the stem, young leaves and mature leaves of *A. thaliana*. A low number of different XGA oligosaccharides were produced from these pectins, which was also observed for digests of XGA from apple and potato pectins (Zandleven *et al.*, 2006). This implies that the distribution of xylose side chains over the backbone in XGA from *A. thaliana* is relatively uniform. The

degree of xylose substitution in XGA from pectin from the stem and the leaves is lower than that of XGA from apple pectin and potato pectin. While XGH mainly released the disaccharide GalAXyl from these latter two sources, a predominant production of GalA$_3$Xyl was seen for the *A. thaliana* pectins. Also the relative amount of linear oligosaccharides in relation to branched GalA oligosaccharides was higher in the *A. thaliana* pectins than in pectins from apple and potato (Zandleven *et al.*, 2006).

The enzyme XGH was also able to release linear GalA oligosaccharides from the *A. thaliana* pectin fractions. Based on the fact that XGH degrades neither polygalacturonic acid (Beldman *et al.*, 2003) nor galacturonic acid oligosaccharides (Zandleven *et al.*, 2005) it is concluded that these linear GalA oligosaccharides are also products from XGA. Linear GalA oligosaccharides can be produced by XGH from unsubstituted regions of XGA by hydrolysis of the linkage in the galacturonan back-bone, next to a xylosylated GalA.

Although we already illustrated that subsites -1 and +1 of XGH can accommodate xylosylated GalA units (Zandleven *et al.*, 2005), this study indicates that these subsites show a preference for these xylosylated residues. This can be seen from the formation of XGA oligosaccharides, which all have a xylose substitution at the reducing end. It is possible that other subsites of the enzyme do not require or may not accept xylosylated GalA residues. This can be deduced from the significant formation of GalA oligosaccharides, especially from pectin of the mature leaves (Fig. 1). The production of the linear oligosaccharides GalA$_4$, GalA$_5$ and GalA$_6$ (which was especially high from the pectin of the mature leaves) implies that short regions of HG together with XGA exist in pectin from *A. thaliana*. The indication that XGA segments are interrupting the HG structural elements was also mentioned by Vincken *et al.* (2003).

By using HPAEC and MALDI-TOF MS analysis (results not shown), the presence of XGA could neither be demonstrated in pectin from the seeds nor from the roots of *A. thaliana*, although it has been indicated that XGA exists in root caps of *A. thaliana* (Willats *et al.*, 2004). Also the pellet fractions from the different plant organs contained some residual pectin material, but no XGA oligosaccharides were detected by HPAEC and MALDI-TOF MS analysis (results not shown) of these XGH-treated fractions. It is possible that the concentration of XGA is too low in these pectin samples to be detected by our methods. If this is the case, it is possible that xylose in these samples originates from xylose containing polysaccharides other than XGA. Alternatively, it is also possible that XGH-resistant XGA

exists in these pectin fractions. The occurrence of XGH-resistant XGA has also been observed before for pea XGA (Beldman *et al*., 2003).

The total amount of liberated XGA from the pectin fractions of the stem, young leaves and mature leaves was estimated. For this, the amount of each XGA oligosaccharide in the different pectin digests was quantified and the total amount determined by summation of these values. Linear GalA oligosaccharides, which were regarded as products from XGA, were also included in the calculations. An example of the yield of XGA oligosaccharides (% w/w) from the mature leaves is illustrated in Figure 1.

Approximately 2.5 %, 7 % and 6 % (w/w) of XGA related products could be liberated from the total carbohydrate present in the pectin fraction from the stem, young leaves and mature leaves respectively. Only the degradable part of XGA is taken into account for the calculation of the yield of XGA oligosaccharides. It cannot be ruled out that XGH resistant parts of XGA are also present in these pectin samples, which imply an underestimation of these values.

The results from this study clearly demonstrate the presence of XGA in the stem, young leaves and mature leaves of *A. thaliana*, although a relative lower amount of this polymer was detected in the stem compared to the leaves.

To our knowledge this is the first time that the occurrence of XGA has been demonstrated in plant material other than storage or reproductive tissues, and root caps. The outcome of this study may initiate investigations on the presence of XGA in other plant varieties, as well as the relationship between XGA structure and its function in different plant tissues.

3 Experimental

3.1. Plant material

Arabidopsis thaliana (L) Heyn. Ecotype Col-0 was used for all experiments.

Plants were grown in peat at an 8 h photoperiod at 100-120 µmol photons m^{-2} s^{-1}, 20 ^{o}C, 70 % relative humidity and watered using tap water when necessary.

Plant material (green tissue) was harvested as young leaves (small rosettes 10 to 12 leaf stage), mature leaves (12 weeks), stems (including flowers and silliques) and seeds. Roots were obtained from plants grown hydroponically according to Husted *et al*. (2002).

3.2 Preparation of cell wall material (CWM)

Cell wall material was prepared as follows: alcohol insoluble residue (AIR) was prepared as described (Fry, 1988) with adaptations. Tissue of interest was ground in liquid nitrogen with a mortar and pestle and boiled in 96% ethanol for 30 min. The supernatant was removed after centrifugation at 10.000 g for 5 min. The pellet was washed with 70% ethanol with subsequent centrifugation until it appeared free of chlorophyll. A final wash with 100% acetone was performed and the pellet was dried under vacuum.

Alcohol insoluble residue (AIR) was treated with enzymes for the removal of starch. For this, AIR was suspended in 10 volumes of a solution that had been pre-heated to 95°C containing 10 mM potassium phosphate buffer (pH 6.5), 1 mM $CaCl_2$, and 0.05% NaN_3. Starch was allowed to gelatinize for 30 s before addition of thermostable α-amylase (Megazyme, Bray, Ireland) to a final concentration of 1 U/ml. The suspension was incubated at 85°C for 15 min. After the incubation the sample was cooled to 25°C and amyloglucosidase and pullulanase (both from Megazyme) were added to a final concentration of 1 U/ml. The suspension was incubated for 16 h at 25 °C, with continuous shaking at 500 rpm. The suspension was centrifuged for 10 min at 6000 g. The pellet was washed with 50 ml of a solution containing 10 mM potassium phosphate (pH 6.5), 1 mM $CaCl_2$, and 0.05% NaN_3, centrifuged again at 6000 g for 10 min, and finally dried under vacuum. The de-starched cell wall material was extracted with phenol:acetic acid:water (2:1:1, v/v/v) for 3 h (1:10 (w/v) ratio between AIR

and phenol:acetic acid:water 2:1:1) at room temperature followed by centrifugation at 6000 g for 5 min. The pellet (CWM) was washed three times with water to remove phenol and extracted proteins and finally dried under vacuum.

3.3. Saponification of CWM

Pectin, as present in CWM from seeds, root, stem, young leaves and mature leaves was saponified in 0.1 M sodium hydroxide for 24 h at 4 ºC and subsequently neutralized with 0.1 M acetic acid. After neutralization, samples were dialyzed overnight against MilliQ water and freeze-dried until further use. Saponified CWM is referred to as CWM-s.

3.4. Pectin extraction from CWM-s

Pectin was extracted from 200 mg, 100 mg, 130 mg, 20 mg, and 105 mg of CWM-s from seeds, root, stem, young leaves and mature leaves of *A. thaliana* according to the method of Voragen *et al*. (1983). For this extraction a cold solution of 5 mM EDTA in 50 mM NaOH (4 °C) was used.

The obtained pectin and pellet fractions from each type of CWM-s were dialyzed overnight against distilled water. Aliquots of these fractions were freeze-dried prior to analysis of their sugar compositions (see section 2.5). The total concentration of soluble polysaccharides in the pectin fractions as well as the total concentration of the residue in the pellet fractions was calculated using the yield of dry material from the freeze dried aliquots of these fractions.

The remainder of the pectin fractions as well as the pellet fractions were dialyzed against 50 mM NaOAc (pH 3.5) prior to treatment by XGH, see section 2.7.

3.5. Neutral sugar composition and uronic acid content

CWM-s, as well as the corresponding pectin and pellet fractions, were analyzed for their neutral sugar compositions using gas chromatography, after derivatization to alditol acetates (Englyst and Cummings, 1984). Inositol was used as internal standard. The samples were hydrolyzed in 72% (w/w) H_2SO_4 for 1 h at 30 °C, followed by a treatment in 1 M H_2SO_4 for 3

h at 100 °C. The uronic acid content of the samples was determined by an automated m-hydroxydiphenyl assay (Blumenkrantz and Asboe-Hansen, 1973; Thibault and Robin, 1975).

3.6 Substrates and enzymes

Xylogalacturonan (XGA-29) was prepared from gum tragacanth by treatment with alkali and trifluoro acetic acid (TFA) as described (Beldman *et al.*, 2003). This XGA had a Xyl:GalA ratio of 0.29.

A set of XGA oligosaccharides with known structures and with different GalA/ Xyl ratios were obtained as described (Zandleven *et al.*, 2005).

The enzyme xylogalacturonan hydrolase (XGH) from *Aspergillus tubingensis* was cloned (Van der Vlugt-Bergmans *et al.*, 2000) and expressed in the *A. niger* "PlugBug" (DSM Food Specialities, Delft, the Netherlands). This enzyme was purified as described (Beldman *et al.*, 2003) and had a specific activity of 150 U/mg.

3.7. Enzyme incubations

One ml from the dialyzed pectin or pellet fraction (ranging in concentration between 1 to 6 mg/ml) from the different plant organs was incubated with XGH for 20 h at 30 °C. The final enzyme concentration was 0.35 µg/ml, which should be able to degrade all XGA possibly present in these samples during the incubation period. Subsequently, the enzyme was inactivated by heating the reaction mixtures for 10 min at 100 °C. The XGH treated samples were analyzed by HPAEC using a set of known XGA-oligosaccharides for identification (Zandleven *et al.*, 2005). GalA, $GalA_2$, and $GalA_3$, (all 10 mM) were taken as standards to calculate the concentration of the corresponding oligosaccharides, as well as of the xylogalacturonan oligosaccharides. For the calculation of the XGA oligosaccharides it was assumed that xylose substitution has no significant effect on the response factor of galacturonan oligosaccharides (Sakamoto *et al.*, 2002). The standard $GalA_3$ was also used to calculate concentrations of oligosaccharides with a degree of polymerization (DP) of 4 and higher, based on the experience that the differences in response factors for these larger oligosaccharides are relatively small.

3.8 MALDI-TOF mass spectrometry

XGH-treated pectin samples were desalted by treatment with H^+-Dowex AG 50W X8 (Biorad, Hercules, CA), using a final concentration of 350 mg H^+-Dowex per ml digest. The desalted digests were mixed with a matrix solution (1 µl of sample in 1 µl of matrix) and applied on a MALDI sample plate. The matrix solution was prepared by dissolving 9 mg of 2,5- dihydroxybenzoic acid in a 1-ml mixture of acetonitrile:water (300 µl: 700 µl).

MALDI-TOF MS analysis was performed using an Ultraflex workstation (Bruker Daltonics, Hamburg, Germany) equipped with a nitrogen laser of 337 nm. The mass spectrometer was selected for positive ions, which were accelerated by an electric field of 12 kV, after a delayed extraction time of 200 ns. The ions were detected in the reflector mode. External calibration of the mass spectrometer was performed using a mixture of maltodextrines (mass range 365 to 2309).

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Chapter 6

General discussion

Pectinases are used in processing of plant materials to food products, for instance fruit juices such as apple juice (Herweijer *et al.*, 2003). However, the array of pectin modifying enzymes as available today is still insufficient to degrade pectin completely. For example, highly branched parts of apple pectin remain resistant towards these pectinases and, as a consequence, cause fouling problems of the membranes during ultrafiltration of the apple juice (Schols *et al.*, 1990).

The genome of *A. niger*, which has been recently sequenced by DSM Food Specialties, showed that probably more carbohydrate modifying enzymes are present in this fungus than known so far, which implies that also the number of pectinases in *A. niger* is possibly underestimated. Therefore the goal of this thesis, which forms a part of the Carbnet project (Chapter 1), was to investigate the complete collection of pectinases from *A. niger*, with special attention to the Glycoside hydrolase family 28. Newly found pectinases were identified by functional genomics and proteomics, and subsequently biochemically characterized, particularly with respect to their substrate specificity and mode of action (Chapter 2). Also emphasis was put on the characterization of the xylogalacturonan hydrolase (XGH) towards a xylogalacturonan (XGA) derived from gum tragacanth (Chapter 3), and XGA in apple and potato pectin (Chapter 4). This enzyme was also used to detect XGA in plant tissues of *Arabidopsis thaliana* (Chapter 5). The results described in this thesis will be discussed in more detail in the following paragraphs.

The glycoside hydrolase family 28 of *Aspergillus niger*

Seven different endo-polygalacturonases (Parenicová *et al.*, 2000) and two rhamnogalacturonases from *A. niger* (Sykerbuyk *et al.*, 1997) have already been cloned and characterized in detail. These enzymes, which belong to the glycoside hydrolase family 28 of *A. niger*, were found by the use of Southern and western analysis (Parenicová *et al.*, 1998; Sykerbuyk *et al.*, 1997).

Recently the genome of *A. niger* became available to the Carbnet project and enabled us to use bioinformatics, microarray technology and proteomics, and provided a clear overview of all possible pectinolytic glycoside hydrolase family 28 enzymes of *A. niger*. This approach is more efficient compared to the conventional Southern and western blot techniques which are

labour-intensive and usually not always effective to discover novel enzymes; in this case for *A. niger* (Sykerbuyk *et al.*, 1997).

Besides the existing seven endo-polygalacturonases and 2 rhamnogalacturonan hydrolases (see Fig. 1 of Chapter 2) four new enzymes with possible rhamnogalacturonan hydrolase activity have been found. Also a new group of seven exo-acting enzymes were annotated of which four were identified as exo-polygalacturonases and three as exo-rhamnogalacturonases. Also one enzyme annotated as xylogalacturonan hydrolase (XGH) was found in this species.

Besides the *A. tubingensis* Exo-PG and XGH, also a known rhamnogalacturonan galacturonohydrolase (Mutter *et al.*, 1998a) from *A. aculeatus* enabled the identification of its *A. niger* homolog RGXC. As a consequence the RGXA and RGXB were annotated as exo-rhamnogalacturonases based on their sequence similarities with RGXC.

Subsequently the identity of these pectinases had to be confirmed by the use of biochemical methods in which the activity of these enzymes is tested towards a set of different pectin substrates. Indeed the enzymes PGXB and PGXC were identified as exo-polygalacturonases which agrees with their predicted function. However PGXA, which was predicted to be an exo-polygalacturonase, behaves rather like an exo-xylogalacturonan hydrolase as shown from its preference to act towards XGA (see Table 1 and Figure 3A, B of Chapter 2). Also PGXC, which was predicted to be an exo-polygalacturonase, appears to have a similar activity towards both PGA and XGA (see Table 1 of Chapter 2), which makes it debatable whether this enzyme is an exo-polygalacturonase or an exo-xylogalacturonase. This confirms that annotation based on bioinformatics is usually not sufficient for identification of enzymes.

Speculations on the role of some partly conserved amino acids among family 28 glycoside hydrolases of *A. niger*

While there are several different endo-acting enzymes known to degrade PGA and RG-I, apparently only one endo-acting enzyme (XGH) from *A. niger* is known that can degrade XGA. This signifies that XGH is rather a unique enzyme within the glycoside hydrolase family 28. XGH contains the amino acids Asp339, Asp361, Asp362, His386, and Lys428 (equivalent to Asp180, Asp201, Asp202, His223, and Lys258; endo-PGII numbering) that are conserved among all polygalacturonases (Fig. 1).

```
                199‡                    222‡                                    256‡
     340          360            380      +1      400                 420    +1     440
     *.  *             .  **            .   ↓↓  ↓                      .      ‡↓ ↓-1     .
PGII ---GGHNTDAFDVGNSVGVNIIK-PWVHNQDDCLAV--NSGENIWFTGGTCI -GGHGLSIGSVGVGDRSN --NVVKNVTIEHSTVSNSEN -----AVRIKTIS--------- :234
PGI  ---GGHNTDGFDISESTGVYISG-ATVKNQDDCIAI--NSGESISFTGGTCS -GGHGLSIGSVGGRDD --NTVKNVTISDSTVSNSAN -----GVRIKTIY--------- :236
PGA  ---GGHNTDGFDISESTYITITG-ATVKNQDDCVAI--NSGENIYFSGGTCS -GGHGLSIGSVGGRDD --NTVKNVTFIDSTVSDSEN -----GVRIKTVY--------- :235
PGB  ----GHNTDAFDIGQSTYITIDG-ATVYNQDDCLAI--NSGEHITFTNGYCD -GGHGLSIGSVGGRSD --NTVNDVTISNSKVLNSQN -----GVRIKTIY--------- :233
PGE  --EAA-NTDGFDIGDSTYITIT G-ANVYNQDDCVAV--NSGENIYFSGGVCS -GGHGLSIGSVGGRSD --NTVKNVTFYDSEIKSSQN -----GVRIKTIY--------- :240
PGC  -DLAA-NTDGFDIGESTYITITG-AEIYNQDDCVAI--NSGENIYFSASVCS -GGHGLSIGSVGGRDD --NTVKNVTFYDVNVLKSQQ -----AIRIKTIY--------- :241
PGD  GDPAAHNTDGFDIKQSDFLTLSN-SWVHNQDDCVAV--TSGSSIVVDNLYCY -GGHGLIGSIGGKSN --NTVDGVTFSNSQVINSEN -----GCRIKSNA--------- :381
XGH  --NPPKNTDGFDIGESTYVTITE-VTVVNDDDCVALKPSS-NYVTVDTISCT -GSHGISVGSLGKSSD --DSVKNIYVTGATMINSTK -----AAGIKTYPSGG------ :274
PGXC -GSFPFNTDGFD-VEGTNIQITD-SIMYNGDDAIAVGADSHD TLFTRNTIGY-QTHGMSIGSLGKDPTDFANISNIRFDDVTVVDGLY -----AARFKSWSG-------- :275
PGXA -DAPAKNTDGWDIYRSSNIVIQD-SRIVNTDDCVSFKPNSTQIVI-QNLDCT-GSHGISVGSLGQYQGETDIVEDLYIYNISMTDASD -----VARIKVWPGVPA----- :294
PGAX -DNEAKNTDGWDTYRSSNNIVIQN-SVINNGDDCVSFKPNSTNILV-QNLHCN-GSHGISVGSLGQYKDEVDIVENVYVYNISMFNASD -----MARIKVWPGTPS----- :289
PGXB -SSDPANTDGMDSLDVDGVSFTN-MRIDVGDDCFSPKPNTTNIFV-QNMWCN-NTHGVSMGSIGQYAGEMDIIENVYIENVTLLNGQN -----GARLKAWAGQDV----- :306
RGXC GDNWVQNTDGFDTMDATNIQLTN-FVYQGGDDCIAIKPRSYNIDI-QNVTCR-GGNGIAIGSLGQYL-EDSSVANIRVDKVNIIRYNED -MHNSAYLKTWVGALVPQSSY :284
RGXB DGGSAQNTDGANTIYSKNITLTN-WEVVNGDDSISTKANSTDITI-ANCTFT-SGLGIAIGSIGQYNGAFETVERLKISNITYEKTTH -----AVYFKTWTGDQV----- :306
RGXA EGEWVQNTDGIDTYRSDHITVTN-WVYQGGDDAVAFKGNSTNIHV-ENVTVY-GGPGIAFGSLGQYPDRTDI VENVTVRNVRVQPSFQRAMNSGVYFKSWIGVNY----- :288
RGA  ------GLDGIDVW-GSNIWVHD-VEVTNKDECVTVKSPA-NNILVESIYCN-WSGGCAMGSLGAD ----TDITDILYRNVYTWSSNQ -----MYMIKSN--------- :256
RGB  ------GLDGIDVW-SNNIWVHD-VEVTNKDECVTVKSPS-KNILIESIYCN-WSGGCGMGSFGSD ----TNVSDITYRNIYTWSSNN-----MMLIKSN--------- :256
RGD  ------GLDGIDIS-GANYWIHD-VEVTNGDECVTVKSPS-ANVRVENVFCN-HSGGCAMGSLGTD ----TNISNIEFENIYTYNSTQ -----MYMIKSN--------- :257
RGC  ------ATDAIDVW-GENMWFHD-IEVSNGDECVTVKSPA-HNYLIENIYCN-LSGGTAIGSLGTG ----TNISDIHYRNLYM-NQAD-----ACFLKSN--------- :255
RGE  ------GLDGIDLY-GGNIWIHL-VMVTNKDECVTSKTNS-HNFLIENIYCN-PSGGCAIGSLGSS ----VNVTNILYRNVYTWDSNQ -----MMMIKTN--------- :258
RGF  ------SYDGIDAI-GTNYYIHD-NEVTNRDECVSVKSPS-HHALVENLVCNQAGSGVSIGSLNVS ----AEISNIEARNISIIQGNN -----IAFIKTYP--------- :255
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                   460                 480               500                  520                540
                    .                   .      +1.         .                   .                    .    ‡    ‡
PGII ---------GATGSVSEITYSNIVMSGISDYGVVIQQD YEDGKPT----GKPTNGVTIQDVKLESVTGSVDSGA --------------------- TEIYLLCGS-GSC :307
PGI  ---------KETGDVSEITYSNIQLSGITDYGIVIEQD YENGSPT----GTPSTGIPITDVTVDGVTGLEDDA ---------------------- TQVYILCGD-GSC :309
PGA  ---------DATGTVEDITYSNIQLSGISDYGIVIEQD YENGDPT----GTPSNGVTISDVTLEDITGSVDSDA --------------------- VEIYILCGD-GSC :308
PGB  ---------GKTGTVENVKFEDITLSDISKYGIVVEQD YENGSPT----GTPTNGVKVEDITFKKVTGSVKSSG --------------------- TDIYILCGS-GSC :306
PGE  ---------GDTGSVSEVTYKEITLSDITDYGIVVEQN YDDTS------KSPTDGITIEDFVLDNVQGSVESSG --------------------- TNIYIVCGS-DSC :311
PGC  ---------GDTGSVSEVTYHEIAFSDATDYGIVIEQN YDDTS------KTPTTGVPITDFVLENIVGTCEDDD --------------------- CTEVYIACGS-GSC :313
PGD  ---------DTTGEVYNVKYENITLSGISDYGIDIQQD YENGGAT----GDPTNGVKIENISFVNVKGTMSDGK --------------------- DYYILCGD-GSC :453
XGH  ---------DHGTSTVSNVTFTDFTVDN-SDYAFQIQSC YGE-DDDYCE-ENPGNA-KLTDIVVSSFSGTTSDKY --------------------- DPVVANIDCGSDGTC :374
PGXC ----------GTGLVKNVTWNNIRVFN VT-FPIFVTQS YSDQGASRSGTVNASSAVMMEDFTWSDFAGSINTYQPGDGSCVSDPCWYNVGLPNLKHTEALIIE CHTAQSC :374
PGXA DTSGS-TSGGGLGRVRNVTYEHMQSENND -HIISVSQC YESKNQTMCDSY -P-SKLVIEDVLFKDFKGTTSK --------------------- KYDPEIGELTCSSPDVC :379
PGAX ALSADLQGGG GSGSVKNITYDTAL IDNVD -WAIEITQC YGQKNTTLCNEY -P-SSLTISDVHIKNFRGTTSG --------------------- SEDPYVGTIVCSSPDTC :375
PGXB ----------GYGRINNVTYKNIQIQNTD -APIVLDQC YFDINATECAKY -P-SAVNITNILFENIWGSSSG --------------------- KDGKIVADLVCSPDAVC :382
RGXC E-SAGVPRGDGWGSIRNVLFSNFNVQGAS -AGPSISQDSGDN-----GSYAGTSKMSISNVAFVNFTGWVD --------------------- TEKSVVSTVSCSEVHPC :365
RGXB GYPPN-GGGGGLGYASDIVATNLKTNNLKGAPFTISQCTTFSGAS ---GNCTNSKFQIRDLVFTDISGTTDS --------------------- SDVASFQCSAVAPC :388
RGXA GVPPN-GGGGGHGYVRNVSVENLRLKDVQ -LPVYIDTCLSYLFSENITQYCDTSTYE FEDLHFRNISGNGLA -------------- TVTDYPGKNISFAVALLCSEKAPC :382
RGA  ---------GGSGTVNNTVLENFIGHGNA -YSLDVDSYWSSMTA ------VDGDGVQLSNITFKNWKGTEADGA --------------------- ERGPIKVVCSDTAPC :329
RGB  ---------GGSGFVENVLLENFIGHGNA -YSLDIDSYWASMSA ------VDGDGVQLSNITVKNWK GTEAYGA --------------------- ERGPVKVVCADGAPC :329
RGD  ---------GGNGTVTNCSFKNFIGYSNS -YMLDLDTYWGDES --------DGDGIKYENIGFENWKGTSSNGI --------------------- QRSPIRILCPDANPC :328
RGC  ---------NGDGIVKNIIWENVIVHGGP -YPLAIDEAWGDDRG --------SVGVQVSNLTFRNWHGESVSA --------------------- SRPVIRLQCDSDVPC :325
RGE  ---------GGLGNVSNIVFENFIGHGNV -NSLDLDSYWSSMNA ------IDGVGIYYHNITIYNWTGTAIDGE --------------------- TRPPIRVICPEDMPC :331
RGF  ---------GGSGYVKDVTFENFR SLNSL-YGLDINQYWQNTWE ------PDTGSVTLSNLVFKNFSGSVADGA --------------------- LRPPLYLFASDLTFA :328
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Figure 1. Excerpt of the multiple alignment of *A. niger* family 28 glycoside hydrolases as previously shown in Chapter 2 of this thesis. The partly conserved amino acids at position 359, 385 and 426 of these enzymes are shown in bold and indicated with an arrow. ‡ = endo-PGII numbering

This has also been previously demonstrated for the *A. tubingenis* XGH (Van der Vlugt-Bergmans *et al.*, 2000). In Figure 1 it can be seen that the arginine residue (Arg426; equivalent to Arg256 in endo-PG numbering), which is known to be involved in substrate binding, is highly conserved among polygalacturonases while it is replaced for a glycine residue in XGH. With respect to this position, XGH discriminates itself from the polygalacturonase group.

Prior to discuss the role of some partly conserved amino acids in the pectinolytic glycoside hydrolase family 28, it is first assumed that the active site topology of these enzymes is highly similar to the topology of the active site described for the endo-polygalacturonase II of *A. niger* of which the 3D structure is known (Armand *et al.*, 2000; Markovic and Janecek, 2001). We viewed the active site topology of endo-PGII using the Yasara software package (version

6.7.15) and have replaced the arginine at position 256 (equivalent to Arg426 in Fig 1) in the 3D structure of this enzyme for a glycine residue (hence Gly256) in order to use it as a structure for XGH (see Fig. 2).

The Arg256 residue, which is present in all polygalacturonases, is involved in substrate binding in which the carboxyl group of GalA is hydrogen-bonded to the side chain of Arg256 (Cho *et al.*, 2001). In XGH, the glycine residue at this position does not posses any side chain and can therefore not form a hydrogen bond with a GalA residue. This may be one of the reasons why XGH lost its affinity for polygalacturonic acid.

It is debatable whether the Gly256 residue in XGH (Fig. 2) is responsible for the enzyme's interaction with **xylosylated** homogalacturonan to be able to act on the galacturonan backbone. However, because Gly256 in XGH does not contain a side chain it might create space in the active site cleft of the enzyme. This space may be sufficient to harbor xylosylated galacturonic acid residues, or even dimeric side chains of xylose (as discussed later). However we know that **exo**-polygalacturonases that, just like endo-polygalacturonases, posses the Arg256 residue can still act towards XGA. This suggests that an other amino acid (or amino acids) may also be involved in allowing the enzyme to accept xylosylated Gal residues in its active site. Such an amino acid should exist among XGH as well as exo-polygalacturonases and not in endo-polygalacturonases. As shown in Figure 1, at amino acid position 385 (equivalent to position 222 in endo-PGII numbering) all endo-polygalacturonases contain a glycine, while the exo-polygalacturonases possess either a serine or a threonine, and the XGH a serine. Serine and threonine are both amino acids with a hydroxyl group in a short side chain.

To study the possible roles of glycine and serine at position 222, we have viewed the structure of endo-PGII, by using the Yasarah software, and have replaced the Gly222 residue for a Ser222 residue (see Fig. 2). Unlike Gly222, which is common in all endo-polygalacturonases, a Ser 222 (as in XGH and some exo-polygalacturonases) is probably able to form a hydrogen bond with the side chain of asparigine199 (Asn199, see Fig 2). A similar hydrogen bond can also be suggested when Gly222 is replaced by a threonine (which was found in some exo-polygalacturonases). Although the Asp199 residue (equivalent to Asp359, see Fig. 1) appears to be present in most of the polygalacturonases, the enzyme PGXB forms an exception to this.

This enzyme contains a valine residue instead of an asparagine residue at position 199. In this case it is unlikely that a hydrogen bond can be formed between Val-199 and Thr222.

Although we suggest that the presence of a Ser or a Thr residue at position 222 in exo-polygalacturonases and XGH may be involved in changing the topological structure of the active site pockets in these enzymes, and thereby allowing these enzymes to accept xylosylated GalA, we still have no clear idea to which extent the active site of these enzymes is changed and how the 3D structure will look like.

In rhamnogalacturonan degrading enzymes of *A. niger* also a correlation can be made between the presence of glycine, serine and threonine at position 222 (equivalent to position 385; see Fig. 1) and the proposed functions of these enzymes. The enzymes RGXA, RGXB and RGXC, which are proposed to be exo-acting rhamnogalacturonases carry a glycine at position 222 while the proposed rhamnogalacturonan hydrolases (except for RGF which also possesses a glycine at position 222) carry a serine at this position. It is known that exo-acting rhamnogalacturonases are more active towards linear rhamnogalacturonan substrates (Mutter *et al.*, 1998a; Mutter *et al.*, 1994), while rhamnogalacturonan hydrolases can act towards galactosylated rhamnogalacturonan (Mutter *et al.*, 1994; Mutter *et al.*, 1998b; Schols *et al.*, 1994).

From this it can be speculated that pectinolytic glycoside hydrolases which possess the amino acids Ser222 or Thr222 (equivalent to Ser385 or Thr385; Fig 1) are involved in degrading branched substrates such as galactose substituted RG-I (as for rhamnogalacturonan hydrolases) or xylose substituted HG (as for exo-polygalacturonases and XGH), while those that contain a Gly222 (equivalent to Gly385; Fig. 1) are involved in the degradation of linear substrates such as RG-I (as for the exo-rhamnogalacturonases) and PGA (as for the endo-polygalacturonases). Although the proposed rhamnogalacturonan hydrolase RGF which contains a glycine at this position, forms an exception, it is possible that this enzyme preferably degrades non-galactosylated RG-I.

The suggested roles of these partially conserved residues at position 222 among glycoside hydrolase family 28 enzymes of *A. niger* (Fig. 1) must still be regarded as highly speculative. In order to clarify this, it is necessary to perform site-directed mutagenesis of the amino acids of interest. Particularly the Gly222 residue (as in endo-polygalacturonases and exo-rhamnogalacturonan hydrolases) should be replaced for a serine or a threonine, and the

Ser222 or Thr222 residue (as in XGH, exo-polygalacturonases and rhamnogalacturonan hydrolases) should be replaced by a glycine. In addition, the Gly256 residue in XGH should be replaced for an arginine to investigate the effect on its activity towards PGA and XGA. In addition, the determination of the 3D structure of XGH, complexed with a substrate analogue might clarify the possible role of Gly256.
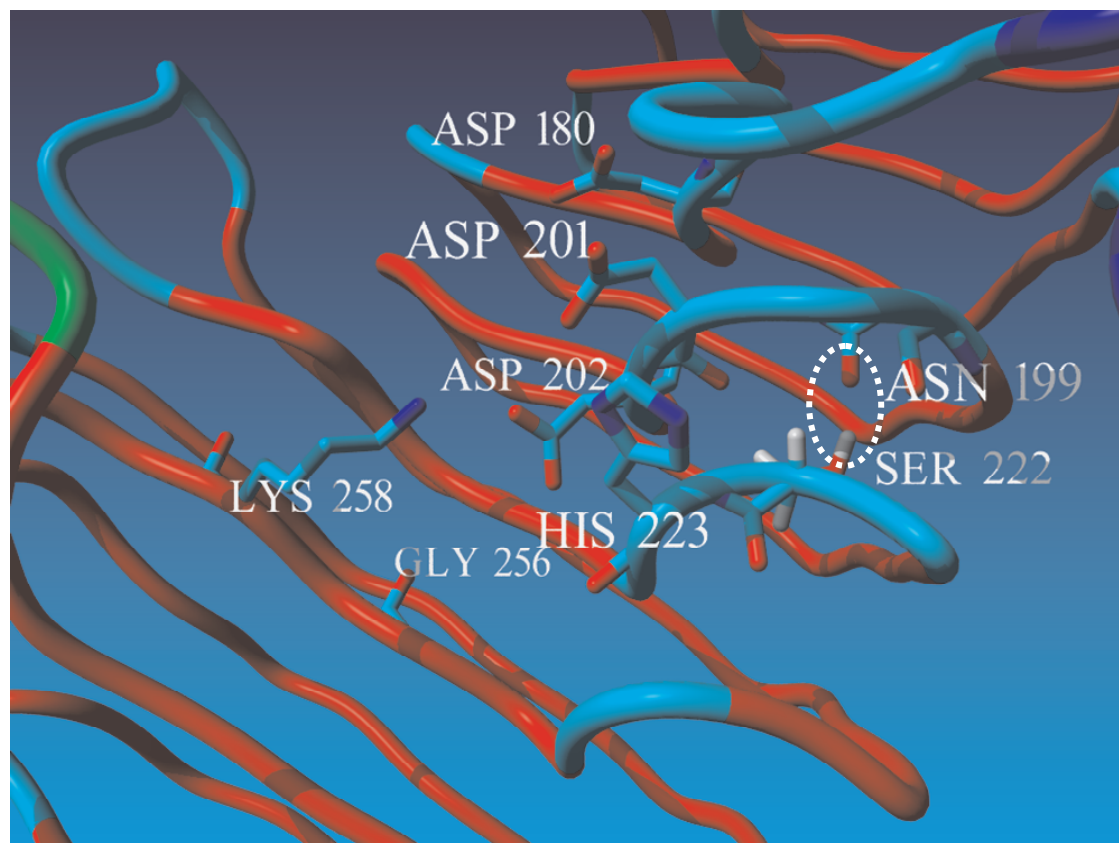
Figure 2. Adapted 3D structure of the active site cleft of Endo-PGII, as a model for XGH, which includes the catalytic amino acid residues Asp180, Asn199, and Asp201, the substrate binding amino acid residues His223 and Lys 258, and the amino acid residue Asn199. The substrate binding amino acid residue Arg256 is replaced by a glycine, while the amino acid residue Gly222 is replaced for a serine. The alpha helixes, beta-helixes, and loops of the enzymes are shown in dark blue, red and green respectively. The backbone, N atoms, and O atoms of the side groups of the amino acid residues are shown in pale blue, dark blue and red respectively. The H atoms of the side chains of Ser222 are shown in white. The dotted circle accentuates the possible formation of a hydrogen bond between the side groups of the amino acids Ser222 and Asn199.

Mode of action of xylogalacturonan hydrolase

The enzyme xylogalacturonan hydrolase was discovered in *A. tubingensis* as described by Van der Vlugt-Bergmans *et al.* (2000). This enzyme was reported to act on the galacturonic acid backbone by cleaving α-1,4-D galacturonan linkages in an endo-fashion and has a requirement for xylose side chains (Van der Vlugt-Bergmans *et al.*, 2000). Although this enzyme has been characterized with respect to its pH optimum, temperature optimum and kinetic properties (Beldman *et al.*, 2003), it was unclear how the xylose distribution on the galacturonic acid backbone affects the action of XGH. In order to clarify this, we studied the mode of action of XGH towards a XGA derived from gum tragacanth (Chapter 3), as well as towards XGA in the saponified modified 'hairy' regions (MHR-s) of apple pectin and potato pectin (Chapter 4). The demonstration of XGA in cell wall material from different tissues of *Arabidopsis thaliana*, which was achieved by the use of XGH, also enables us to make a few conclusions on the mode of action of this enzyme (Chapter 5). In this thesis research the mode of action of XGH is investigated by analysis of the structure of XGA oligosaccharides it releases from these substrates as well as to which extend these oligosaccharides are produced.

As XGA from gum tragacanth was already studied for its degradability by XGH (Beldman *et al.*, 2003), we commenced our work on this substrate. Upon degradation of a XGA derived from gum tragacanth (XGA-29) by the enzyme XGH, the released oligosaccharides were first preparatively separated by HPAEC and subsequently analyzed for their composition and structure by mass spectrometry (Chapter 3). Then the XGA-oligosaccharide composition was determined by HPAEC using the XGA-29 digest for assignment of the different peaks. All XGA products derived from XGA-29 with known structures are summarized in Table 1.

The XGA-29 digest was also used for identification of XGA oligosaccharides in digests of XGA in MHR-s of apple pectin and potato pectin (Chapter 4), and in cell wall material from the leaves and the stem of *A. thaliana* (Chapter 5). The released XGA oligosaccharides with known structures from these substrates are also summarized in Table 1.

	XGA products formed								
Source	**GalA**	**GalA-Xyl**	**GalA-Xyl$_2$**	**GalA$_2$-Xyl**	**GalA**	**GalA$_2$-Xyl$_2$**	**GalA$_2$-Xyl'**	**GalA$_3$-Xyl$_3$**	**GalA$_3$-Xyl**
XGA-29 (gum tragacanth)	X	**X**		X	X	X	X	X	X
MHR-s Apple pectin	X	**X**	X	X	X	X			
MHR-s Potato pectin	X	**X**	X	X	X	X			
CWM-s mature leaves (*A. thaliana*)	X	X		X	X	X	X		**X**

	XGA products formed						
Source	**GalA$_3$-Xyl$_2$**	**GalA$_3$**	**GalA$_4$-Xyl$_4$**	**GalA$_3$-Xyl'**	**GalA$_4$**	**GalA$_5$**	**GalA$_6$**
XGA-29 (gum tragacanth)	X	X	X	X	X	X	X
MHR-s Apple pectin							
MHR-s Potato pectin							
CWM-s mature leaves (*A. thaliana*)					X	X	X

Table 1. Summary of XGA products, with known structure, released from a XGA derived gum tragacanth (XGA-29), apple pectin, potato pectin and CWM-s of the mature leaves of *A. thaliana* respectively by XGH. These products are ordered based on their elution times upon HPAEC. Products which were found in these digests are indicated by "X". Those XGA products which were produced predominantly are marked with "**X**". • = GalA, X= xylose. The asterix (*) indicates the reducing end. The accentuated oligosaccharides GalA$_2$Xyl' and GalA$_3$Xyl' are isomers with different distribution of xylose over the GalA backbone.

It is clear that XGH tolerates XGAs which vary in their degree of xylose substitution as well as in the distribution of the xyloses. It can be concluded that XGA-29 has a random distribution of xyloses over the GalA backbone because of the high number of different XGA products (twenty, including five isomers) which were formed from this substrate (Chapter 3). The XGA in MHR-s of apple and potato pectin must have a more regular distribution of xylose over the GalA backbone as indicated by the limited number of different XGA products that were produced from these sources. XGA present in the cell wall of the leaves and the stem of *A. thaliana* have their xyloses also regularly distributed, however, compared to XGA-29 and XGA in MHR-s of apple and potato pectin, the degree of xylose substitution of XGAs from *A. thaliana* is rather low. This is based on the predominant release of the oligosaccharide GalA$_3$Xyl as well as the relatively high production of linear GalA oligosaccharides from this latter source.

Based on the products formed, a Xyl:GalA ratio between 1:2 to 1:3 can be estimated for XGA-29 (see Table 1) while a Xyl:GalA ratio of almost 1:1 is assessed for XGA in MHR-s of apple pectin. This latter ratio can also be estimated for XGA in MHR-s in potato pectin, because the XGA oligosaccharide production from this pectin source was similar to that of apple pectin. The XGA present in the cell wall of leaves and the stem of *A. thaliana* may have a Xyl:GalA ratio between 1:4 to 1:5.

Analysis of the XGA products released from XGA-29 and from XGA in MHR-s of apple and potato pectin shows that XGH prefers to act between two xylosylated GalA units, as deduced from the high production of the disaccharide GalAXyl (Chapter 3). The formation of XGA oligosaccharides that have no xylose substitution at the non-reducing end or at the reducing end, such as GalA$_3$Xyl, and GalA$_3$Xyl' (see Table 1), show that XGH can also act between two GalA residues of which only one is xylosylated. In addition, XGH also released GalA and linear GalA oligosaccharides, which were significantly present in digests from XGA-29 and in particular in XGA digests from the cell wall of the mature leaves (see Fig. 2 of Chapter 5) and the stem of *A. thaliana*. Although this suggests that XGH can also act between two unsubstituted GalA residues, no XGA oligosaccharides have been identified yet which only contain xylose substitution at the internal GalA residue(s). It has also been demonstrated from the product progression profiles (Chapter 3) that linear GalA oligosaccharides were not further degraded by XGH, as well as that polygalacturonic acid (PGA) is hardly degraded by

XGH (Beldman *et al.*, 2003). From this, it is concluded that these linear oligosaccharides are produced by XGH from unsubstituted regions of XGA by hydrolysis of the linkage in the galacturonan back-bone, next to a xylosylated GalA. This implies that XGH can only cleave the α-(1,4) glycosylic linkage between two GalA residues if at least one of these residues is xylosylated.

The ability of XGH to act towards these differently substituted XGAs can be explained if it is assumed that the enzyme accepts xylosylated GalA residues as well as unsubstituted GalA residues in its subsites -1 and +1. This is also illustrated in a model (see Fig. 3). The number of subsites of XGH is yet unknown, however based on the product progression profile of XGA-29 (Chapter 3) it can be seen that the oligosaccharide $GalA_4Xyl_2$ was the smallest oligosaccharide that could be degraded and that an increase in the backbone of such an oligosaccharide by one GalA unit, hence $GalA_5Xyl_2$, appeared to enhance the activity of the enzyme significantly. This suggests that 5 subsites of the enzyme can be involved in binding these GalA residues. It is possible that more than 5 subsites are involved; however so far we were unable to study the degradation of XGA oligosaccharides with a GalA backbone of more than 5 residues. It is also unclear which subsites, other than -1 and +1, are involved in binding these GalA residues. Therefore the subsite model which is shown in Figure 3 should be regarded as tentative.

Figure 3A illustrates the release of GalAXyl, which was prominently found in digests from XGA-29 and in digest of XGA from MHR-s of apple and potato pectin, and shows that subsites -1 and +1 of the enzyme can dock these residues. Also the formation of the oligosaccharides $GalA_3Xyl$ (Fig 3B) and $GalA_3Xyl'$ (Fig 3C), which have a xylose substitution at the reducing end and non-reducing end respectively, demonstrate that subsites -1 and +1 of the enzyme can also tolerate unsubstituted GalA residues. Figure 3D illustrates the probable release of a linear GalA oligosaccharide ($GalA_4$), which was produced significantly from the CWM-s of the mature leaves and stem of *A. thaliana*. This oligosaccharide is released from unsubstituted regions of XGA by hydrolysis of the linkage in the galacturonan back-bone, next to a xylosylated GalA as discussed.

From this model it is generally assumed that subsites -1 or +1 of the enzyme always need to bind a xylosylated GalA residue in order to act.
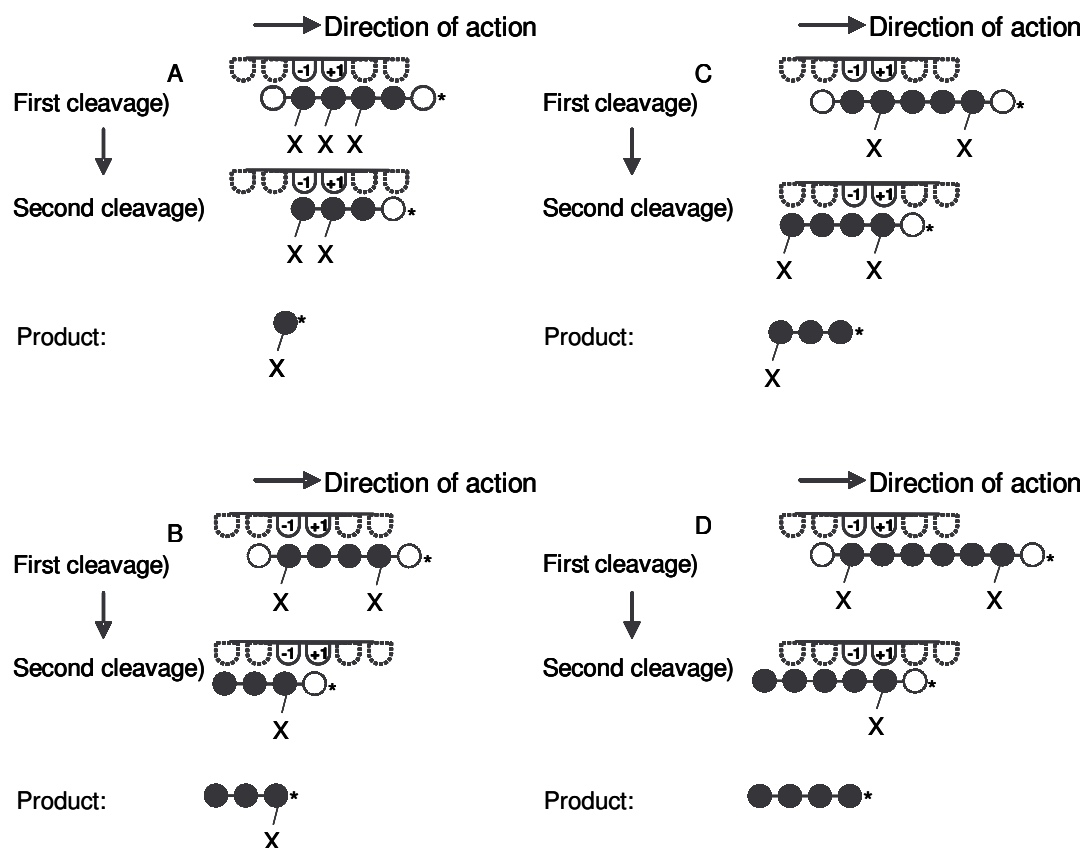
Figure 3. Representation of the proposed action of XGH towards some specific parts of XGA molecules which shows the release of the oligosaccharides GalAXyl (A), GalA₃Xyl (B), GalA₃Xyl' (C) and GalA₄ (D) (see Table 1). The oligosaccharides GalA$_3$Xyl (B) and GalA$_3$Xyl' (C) have a xylose substitution at the reducing end or at the non-reducing end respectively. The release of GalA$_4$ is demonstrated by hydrolysis of the linkage in the galacturonan back-bone, next to a xylosylated GalA. From this model, it can be envisaged that the subsites -1 and +1 of the enzyme accept xylosylated GalA and unsubstituted GalA residues. • = GalA, X= xylose, ○ = GalA of the continuing chain of the galacturonic acid backbone. The asterix (*) indicates the reducing end.

XGH produced also some other (unexpected) oligosaccharides when acting towards XGA-29 and XGA in MHR-s of apple and potato pectin. Here we can mention oligosaccharide GalA$_2$Xyl', as derived from XGA-29, which is proposed to carry the xylose at the C-2 position of the reducing GalA residue (Chapter 3). If this can be proven, it would mean that β-D-xylose substitution at the C-2 position in XGA does not hinder the action of XGH, and hence that these xylose side chains are also accepted (at least) in subsite -1 of the enzyme.

The enzyme also released the XGA oligosaccharides $GalAXyl_2$ and $GalA_2Xyl_3$ from XGA in MHR-s of apple and potato. Both oligosaccharides contain a dimeric side chain of xylose from which it is concluded that, besides xylose substituted GalA residues, also dimeric side chains of xylose are accepted in subsites -1 and +1 of XGH (Chapter 4). The dimeric side chains of xylose in XGA from MHR-s of apple and potato pectin may be pointed towards the outside of the binding cleft of XGH which facilitates the formation of the enzyme-substrate complex. However there may also be another explanation for the acceptance of these dimeric side chains. The glycine residue, which is present at position 256 of XGH (see Fig. 2) has no side chains and may therefore create sufficient space in the active site cleft of this enzyme to harbor, besides Xyl substituted GalA residues, also Xyl-Xyl substituted GalA residues.

The XGH described in this study shows some properties of both an endo-polygalacturonase and an exo-polygalacturonase. XGH degrades XGA in an endo fashion as demonstrated from the rapid decrease in the molecular weight of this substrate (Beldman *et al.*, 2003). This is also observed when polygalacturonic acid (PGA) is degraded by endo-polygalacturonases (endo-PGs). Besides this, processive endo-PGs are known, like endo-PGI, which predominantly produce GalA from PGA (Benen *et al.*, 1999; Benen *et al.*, 2003). It is possible that XGH is also a processive enzyme based on the predominant release of the disaccharide GalAXyl. Although XGH shows some similar properties with endo-polygalacturonases, these latter enzymes cannot degrade XGA while XGH and exo-polygalacturonases can. During degradation of XGA, the enzyme XGH behaves as an exo-acting enzyme, as illustrated by the stepwise degradation of the XGA oligosaccharide $GalA_4Xyl_3$ from the non-reducing end towards the reducing end (see Fig. 7 of Chapter 3).

Presence of XGA in different tissues of *Arabidopsis thaliana*

In this thesis it is evidenced that the presence of XGA is not strictly confined to storage tissues or reproductive organs, and root caps of plants (Albersheim *et al.*, 1996; Willats *et al.*, 2004). By using the well characterized xylogalacturonan hydrolase (XGH) it is possible to detect XGA oligosaccharides in pectin fractions obtained from the stem, young leaves and mature leaves of *Arabidopsis thaliana* (Chapter 5). No XGA is detected in pectin fractions from the roots and seeds of this plant species, which is probably due to the absence or a too

low concentration. However, using immunochemical techniques, Willats *et al.* (2004) indicated that XGA is present in root caps of *A. thaliana*. For this, a monoclonal antibody LM8 was used, which was originally raised against XGA from pea testea (Le Goff *et al.*, 2001). This anti-body was specifically bound to the root caps of *A. thaliana* which suggests that XGA is solely associated with these cells of the root and not with the root body. As these root caps are known to be loosely attached cells (Willats *et al.*, 2004) it is possible that these cells were lost during the preparation of CWM from the roots and may therefore, at least partly, explain why we did not detect XGA in the pectin fraction of this tissue.

In general, XGH can be used as a powerful tool to confirm the presence of XGA in plant cell walls. In addition, this enzyme also provides clues on the structure of XGA (e.g. degree of substitution and distribution of xyloses), which is based on the produced XGA oligosaccharides. However it should be remembered that with this method only the degradable part of XGA in the plant cell wall is investigated.

Since XGA has been detected for the first time in plant cell walls of the stem, young leaves and mature leaves of *A. thaliana* it may open up possibilities to generate anti-bodies towards XGA in these tissues. From this, the location of XGA in the cell wall of these tissues can be studied which may help us to understand the role of this polymer. Although the LM8 anti-body might also be useful for this, this anti-body appears to be specific for highly substituted XGA (Willats *et al.*, 2004). From this, it is unlikely that it will bind to a XGA with a low degree of xylose substitution as found in pectin from the stem, young leaves and mature leaves of *A. thaliana*. To generate anti-bodies with specificity for such a XGA, it is first necessary to isolate XGA from the pectin fractions of these tissues. This can be achieved by enzymatic degradation of non-XGA regions in pectin as described previously for the isolation of XGA from pea testea (Le Goff *et al.*, 2001) or from the MHR-s of apple pectin (Schols *et al.*, 1995). Fractions which are enriched in XGA can be identified by XGH degradation, and can be finally used for the generation of anti-bodies as described previously (Willats *et al.*, 2004).

Similarly XGA can be demonstrated in cell walls of several tissues of other plants using XGH or anti-bodies that are raised against XGA (e.g. from *A. thaliana*) as rapid screening tools.

Screening for novel pectinases from *A. niger*: future challenges and opportunities

Large parts of pectic polysaccharides from different plant sources remain resistant towards the spectrum of presently available pectinases. Besides the enzyme-resistant highly branched MHR regions of apple pectin, a few examples of some other enzyme-resistant plant polysaccharides will be discussed here, with special attention to xylogalacturonan.

The enzyme XGH is able to degrade XGA from different sources (gum tragacanth, apple potato, *A. thaliana*) which differed in their degree of xylose substitution as well as their distribution of the xylose side chains. Also dimeric side chains of xylose in XGA from apple and potato pectins did not hinder the action of the enzyme. However as reported by Beldman *et al.* (2003), XGA from pea testea remains highly resistant towards this enzyme. As shown from the proposed model of pea XGA (Le Goff *et al.*, 2004; Fig. 4) it is hard to understand why XGH cannot attack this polymer. It is unlikely that the degree of xylose substitution of pea XGA (65%) is the cause for this problem, because XGH is able to degrade low substituted (XGA-29, with ~ 30% of xylose substitution) as well as high substituted XGAs (XGA in MHR-s from apple and potato pectin with ~ 90% of xylose substitution). Pea XGA contains dimeric side chains of xylose, which are probably differently linked than the dimeric side chains in XGA from MHR-s of apple and potato pectin, and may therefore hinder the action of XGH. However, as there are only a few galacturonic acid residues in pea XGA that carry such side chains it is questionable if this explains why XGH cannot act towards pea XGA.

From this it can be concluded that the structure of pea XGA must be different than the structure of XGA as derived from gum tragacanth (XGA-29), and XGA in MHR-s of apple and potato pectin. This structure of pea XGA is probably more complex compared to the proposed structure as shown in Figure 4.
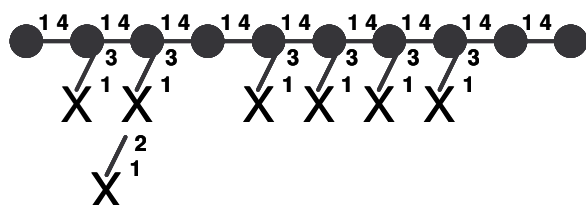

Figure 4. Proposed structure of xylogalacturonan from pea testea (Le Goff *et al.,* 2001).

• = GalA, X= xylose.

Willats *et al.* (2004) demonstrated that an exo-polygalacturonase from *A. aculeatus* was indeed able to release XGA products (GalA and GalAXyl) from pea XGA, however only little degradation of this polymer was observed. Probably the dimeric side chains of xylose in pea XGA hinder the action of this enzyme.

XGA regions in soybean are also hardly degraded by XGH (Huisman *et al.*, 2001). This is probably caused by the presence of oligomeric side chains of xylose in soybean XGA (Huisman *et al.*, 2001; Nakamura *et al.*, 2002), which hinder the enzyme to interact with this polymer. In addition it was also reported that further substitution of xylose residues with fucose may also be responsible for the resistance of soybean XGA towards XGH (Huisman, 2000a). An exo-polygalacturonase from *A. aculeatus* was shown to be more effective in the degradation of soybean XGA than XGH. Yet a large part of the XGA remains resistant towards this exo-polygalacturonase (Huisman *et al.*, 2001) which is probably due to similar reasons as described for XGH.

Besides XGA, most of the other pectic polysaccharides present in cell walls of soybean remain resistant to enzymatic degradation. For instance Huisman *et al.* (2000b) described a hypothetical structure of the rhamnogalacturonan regions of CDTA-extractable soybean pectic substances for which no enzymes are currently available to degrade it (Fig. 5).
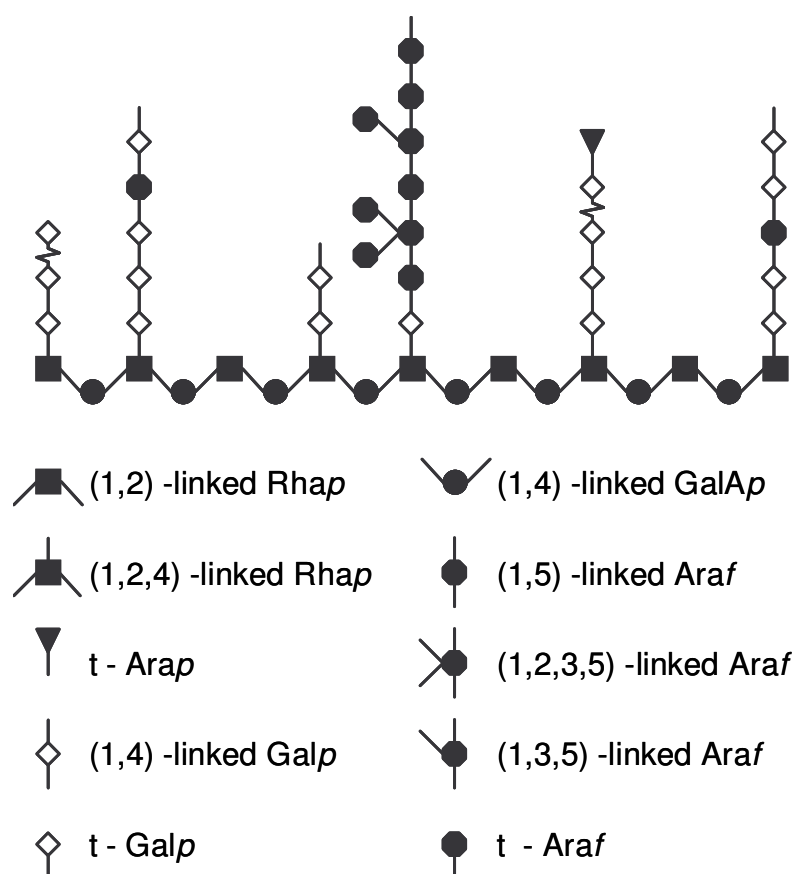
Figure 5. Proposed model of the rhamnogalacturonan regions of CDTA-extractable soybean pectic substances according to Huisman *et al.* (2000b).

These enzymes include for example arabinopyranosidases and rhamnogalacturonan hydrolases that act on side-chain containing rhamnogalacturonan.

It is obvious that many pectinases which might degrade such pectic polymers have yet to be found. In our approach, in which we screened the genome of *A. niger* for the entire pectinolytic glycoside hydrolase family 28, we already found potentially novel pectinases. As described in Chapter 2, a new gene family comprised of seven exo-acting enzymes and 4 new putative rhamnogalacturonan hydrolases have been found from this species. Probably these pectinases may aid in the degradation of those pectic polysaccharides for which no enzymes have been found yet. For example, these four novel putative rhamnogalacturonan hydrolases could be tested for their ability to degrade side-chain containing rhamnogalacturonan regions (as for instance in MHR-s of apple pectin or soybean pectin). Also the exo-polygalacturonases, especially PGXA which is presumed to be an exo-xylogalacturonan hydrolase, may improve the degradation of XGAs from pea testea or soybean.

In a similar way, the genome of *A. niger* can be explored for instance for the presence of assessory enzymes, which can aid in the degradation of arabinogalactan side chains in rhamnogalacturonan regions of soybean pectin (Fig. 5; Huisman, 2000b), such as arabinopyranosidases. Similarly the presence of xylosidases and fucosidases in this species can be investigated which may degrade the polymeric side chains in soybean XGA. As a consequence such a modified soybean XGA may become better degradable for XGH and/ or exo-polygalacturonases. A similar result may also be expected for pea XGA.

Alternatively it is possible to grow *A. niger* specifically on pectic fragments for which no enzymes have been found yet, for instance for those pectic polysaccharides as described above. Degradation of these substrates during fermentation can be readily followed by HPSEC analysis and those pectinases which are responsible for this can be obtained from the growth media. Also the expression levels of genes, which may correspond to pectinases that are involved in degradation of these substrates, can be measured by using micro-array techniques in a similar experiment as described in Chapter 2. Subsequently these genes which encode for putative novel pectinases can be cloned and expressed in a similar fashion as performed before (Chapter 2). Finally these (still putative) expressed pectinases can be biochemically identified and characterized by using specific pectic substrates.

These are just a few examples of this approach to search for those pectinases that are still needed for the complete degradation of pectin from different plant sources. This underpins the powerful use of the genome of *A. niger* to screen for such enzymes. These newly found enzymes from *A. niger*, which have a GRAS status, can be readily used for industrial applications.

Although *A. niger* is a potential source for novel pectinases, it cannot be excluded that this species lacks pectinases that are still required to fully degrade pectic material. It may therefore also be useful to investigate (novel) pectinases in other (fungal) sources such as *Aspergillus oryzae*. This species also has the advantage of having a GRAS status (Tailor and Richardson, 1979) as well as that its genome sequence is known (Coutinho and Henrissat, 1999). As *A. oryzae* is commonly used for the fermentation of soybeans (Hong *et al.*, 2004; Ling and Chou, 1996), to produce soy sauce, it may be an interesting source for pectinases that can degrade soybean pectin. In addition, in principle all other organisms of which the genome sequence is known can be mined for novel pectinases.

Ultimately, by obtaining the complete array of pectinases we can improve formulation of tailor-made enzyme preparations. As a result, pectins can be modified in a controlled manner which can be subsequently used for particular food applications.

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Summary

The subject of this thesis was to biochemically identify and characterize (novel) pectin degrading enzymes that belong to the glycoside hydrolase family 28 of *Aspergillus niger*. These pectin degrading enzymes became available by exploring the genome of *A. niger* for these enzymes, using functional genomics techniques.

Biochemical identification and characterization of these enzymes was executed by analysis of their activity towards a set of pectinolytic substrates. Special attention has been given to the characterization of the xylogalacturonan hydrolase towards a XGA derived from gum tragacanth and XGA from other plant sources.

In Chapter 2, a complete inventory of the pectinolytic glycoside hydrolase family 28 of *A. niger* is presented, which was obtained by applying bioinformatics tools on the genome sequence of this species. Besides the seven existing endo-polygalacturonases and two rhamnogalacturonases in *A. niger*, a new gene group of seven exo-acting enzymes was found, which included four exo-polygalacturonan hydrolases and three putative exo-rhamnogalacturonan hydrolases. Also four novel putative rhamnogalacturonan hydrolases were found in this species. Three exo-acting enzymes PGXA, PGXB and PGXC were biochemically identified by using PGA and XGA as substrates for which it was demonstrated that PGXB and PGXC act as an exo-polygalacturonase, while PGXA behaves as an exo-xylogalacturonan hydrolase.

The xylogalacturonan hydrolase (XGH) was investigated for its mode of action towards a XGA derived from gum tragacanth (XGA-29; Chapter 3). This was achieved by analysis of the structure of the XGA fragments released from XGA-29 as well as to which extend these oligosaccharides were produced. Upon degradation of XGA-29, several XGA oligosaccharides were formed with different galacturonic acid/ xylose ratios, including five oligosaccharide isomers.

Although XGH is known to be an endo-acting enzyme, product progression profiling illustrated a predominant production of the di-saccharide GalAXyl, which also indicates an exolytic action of this enzyme. The latter was also supported when XGH was investigated for its action towards a defined XGA oligosaccharide $GalA_4Xyl_3$ in a time course experiment.

From this, XGH was demonstrated to act from the non-reducing end towards the reducing end of this oligosaccharide.

Based on the released XGA products from XGA-29, in particular GalAXyl, it was shown that although XGH prefers to act between two xylosidated GalA units, it can also tolerate unsubstituted GalA units in its -1 and +1 subsites.

The action of XGH was also investigated towards XGAs in the saponified modified 'hairy' regions (MHR-s) of apple pectin and potato pectin (Chapter 4). Product analysis in digests of these XGAs illustrated that the degradable parts of XGA from both sources have a similar xylose side chain distribution. Once again, the di-saccharide GalAXyl was predominantly formed which underpins the preference of XGH to act between two xylosylated GalA units as well as its exolytic behavior. A significant lower number of different XGA products was released from these sources, compared to the number of different XGA products derived from XGA-29. This suggests a regular distribution of xylose over the galacturonan backbone in the degradable parts of XGA in MHR-s of apple and potato pectin. Also the oligosaccharides GalAXyl$_2$ and GalA$_2$Xyl$_3$ were observed in the digests of XGA from MHR-s of apple and potato pectin, which shows the presence of dimeric side chains of xylose (Xyl-Xyl) in these sources. These side chains did not hinder the action of XGH which points out that this enzyme can also accept Xyl-Xyl substituted GalA residues in subsites -1 and +1.

With the aid of XGH it was possible to detect XGA in different tissues of *Arabidopsis thaliana* (Chapter 5). This was achieved by extraction of the pectin from the seeds, roots, stem, young leaves and mature leaves of this species, followed by treatment of these pectin extracts with XGH. From this, XGA oligosaccharides were only detected in the stem and the leaves of *A. thaliana*. The estimated amount of XGA accounted for approximately 2.5 %, 7 % and 6 % (w/w) of the total carbohydrate in the pectin fraction of the stem, young leaves and mature leaves respectively. A low number of different XGA oligosaccharides was detected in pectin hydrolysates from the cell wall of different tissues of *A. thaliana*. This suggests a regular distribution of xylose in the degradable parts of XGA of these tissues, which was similar to our findings for XGA in MHR-s from apple and potato pectin. Also the XGA from these *A. thaliana* pectins contained a lower degree of xylose substitution compared to XGA in MHR-s from apple and potato pectin. The oligosaccharide GalA$_3$Xyl was predominantly released from these *A. thaliana* pectins, and the amount of linear GalA oligosaccharides in

relation to branched GalA oligosaccharides was higher in these *A. thaliana* pectins than in MHR-s from apple and potato.

The results described in this thesis are discussed in Chapter 6 with emphasis on the mode of action of XGH. Also some speculations are made on the roles of some partial conserved amino acids in the pectinolytic glycoside hydrolase family 28 of *A. niger*. In addition, the use of XGH as a functional tool for the detection of XGA in other plant varieties is discussed.

Finally the usefulness of our methods to explore the genome of *A. niger* for novel pectin degrading enzymes is discussed. Our approach (Chapter 2) is attractive to explore enzymes that may degrade those pectic polysaccharides, as derived for instance from soya and pea, which are still highly resistant towards the collection of pectin modifying enzymes as available today. As a consequence, the improved degradation of these polysaccharides may improve their utilization in industrial applications.

Samenvatting

Het in dit proefschrift beschreven onderzoek betrof de biochemische identificatie en karakterisatie van (nieuwe) pectine modificerende enzymen die behoren tot de familie 28 glycosyl hydrolasen van *Aspergillus niger*. Deze pectine modificerende enzymen kwamen tot onze beschikking door het genoom van *A. niger* te onderzoeken naar deze enzymen, met gebruikmaking van "functional genomics" technieken. Deze enzymen werden vervolgens biochemisch geïdentificeerd en gekarakteriseerd aan de hand van hun activiteit op bepaalde pectine substraten. Met nadruk was er aandacht besteed aan de karakterisatie van het enzym xylogalacturonaan hydrolase op een xylogalacturonaan (XGA) dat verkregen was van gum tragacanth, en op XGA uit andere plantenbronnen.

In Hoofdstuk 2 is de gehele inventaris van familie 28 glycosyl hydrolasen van *A. niger* gepresenteerd. Dit was gerealiseerd door gebruikmaking van "bioinformatics tools" op het genoom van *A. niger*. Naast de zeven bestaande endo-polygalacturonaan hydrolasen en de twee rhamnogalacturonaan hydrolasen van *A. niger* was er een nieuwe groep van zeven exo-enzymen gevonden. Deze nieuwe groep bestond uit vier exo-polygalacturonaan hydrolasen en drie vermoedelijke exo-rhamnogalacturonaan hydrolasen. Er waren ook vier nieuwe mogelijke rhamnogalacturonaan hydrolasen gevonden. Drie exo-enzymen (PGXA, PGXB en PGXC) werden biochemisch geïdentificeerd door hun activiteit te toetsen op polygalacturonaan (PGA) en xylogalacturonaan (XGA). Daaruit is gebleken dat PGXB en PGXC beiden werken als een exo-polygalacturonaan hydrolase, terwijl PGXA zich als een exo-xylogalacturonaan hydrolase gedraagt.

Xylogalacturonaan hydrolase (XGH) was bestudeerd voor zijn "mode of action" op xylogalacturonaan dat verkregen was uit gum tragacanth (XGA-29; Hoofdstuk 3). Dit werd gerealiseerd door de structuur van de gevormde XGA oligosacchariden te bepalen, evenals de mate waarin deze oligosacchariden werden gevormd. Na behandeling van XGA-29 met XGH werden er verscheidene XGA oligosacchariden geproduceerd met verschillende galacturonzuur/ xylose ratio's, inclusief vijf isomeren van deze oligosacchariden.

Ondanks dat XGH bekend staat als een endo-enzym, werd, zoals aangegeven in de product progressie profielen, het di-saccharide GalAXyl voornamelijk gevormd waaruit blijkt dat dit enzym ook exolytische eigenschappen bezit. Dit laatste werd nog eens bevestigd door de

wijze waarop XGH het XGA oligosaccharide GalA$_4$Xyl$_3$ afbrak. Het enzym brak dit oligosaccharide af in de richting van het niet-reducerend eind naar het reducerend eind.

Gebaseerd op de gevormde produkten uit XGA-29, met GalAXyl in het bijzonder, werd het duidelijk dat hoewel XGH prefereert om twee galacturonzuur eenheden te splitsen die beiden gesubstitueerd zijn met een xylose, dit enzym ook niet gesubstitueerde galacturonzuur eenheden kan accepteren in zijn -1 en +1 subsites.

De activiteit van XGH was ook onderzocht op XGA in verzeept "modified hairy regions" (MHR-s) van appel en aardappel pectine (Hoofdstuk 4). Uit product analyse van digesten van deze xylogalacturonanen was gedemonstreerd dat de afbreekbare delen van XGA uit beide bronnen eenzelfde distributie van xylose zijketens hebben. Ook werd het di-saccharide GalAXyl voornamelijk geproduceerd wat bevestigt dat XGH prefereert om twee ge-xylosyleerde galacturonzuur eenheden te splitsen en dat dit enzym exolytische eigenschappen bezit. XGH produceerde een laag aantal verschillende XGA oligosacchariden uit MHR-s van appel en aardappel pectine, vergeleken bij het aantal verschillende XGA oligosacchariden dat werd geproduceerd uit XGA-29. Daaruit kan worden verondersteld dat de afbreekbare delen van XGA uit MHR-s van appel en aardappel pectine een regelmatige distributie van xylose zijketens hebben.

Ook werden de oligosacchariden GalAXyl$_2$ en GalA$_2$Xyl$_3$ gevonden in digesten van XGA uit MHR-s van appel en aardappel pectine dat wees op de aanwezigheid van xylose di-meren als zijketens in XGA. Deze zijketens hadden geen remmende werking op de activiteit van XGH waaruit wordt verondersteld dat het enzym ook Xyl-Xyl gesubstitueerde galacturonzuur eenheden kan accepteren in zijn -1 en +1 subsites.

Met behulp van XGH was het mogelijk om XGA aan te kunnen tonen in verschillende weefsels van *Arabidopsis thaliana* (Hoofdstuk 5). Dit werd gedaan door eerst pectine selectief te extraheren uit de zaden, wortel, stam, jonge bladeren, en volwassen bladeren van deze plant. De pectine extracten uit deze weefsels werden vervolgens behandeld met het enzym. Hieruit werd geconstateerd dat alleen XGA oligosacchariden werden gevormd uit pectine extracten van de stam en de bladeren van *A. thaliana*. De geschatte hoeveelheid XGA was verantwoordelijk voor ongeveer 2.5 %, 7 %, en 6% (w/w) van de totale hoeveelheid carbohydraat in de pectine extracten van respectievelijk de stam, de jonge bladeren en de volwassen bladeren.

In de pectine hydrolysaten van de stam, jonge bladeren en volwassen bladeren van *A. thaliana* was een laag aantal verschillende XGA oligosacchariden gevonden. Dit wijst uit dat de afbreekbare delen van XGA, afkomstig uit deze weefsels, een regelmatige distributie van xylose bezitten, wat vergelijkbaar was met onze bevindingen voor XGA uit MHR-s van appel en aardappel pectine. Het XGA uit de pectine extracten van *A. thaliana* had ook een lagere graad van xylose substitutie vergeleken met XGA uit MHR-s van appel en aardappel pectine. Het oligosaccharide $GalA_3Xyl$ was voornamelijk geproduceerd uit de pectine extracten van *A. thaliana*. Ook de hoeveelheid lineaire GalA oligosacchariden in verhouding met vertakte GalA oligosacchariden was hoger in deze pectine extracten vergeleken met de gevormde XGA oligosacchariden uit MHR-s van appel en aardappel pectine.

De resultaten in dit proefschrift, in het bijzonder de "mode of action" van XGH, zijn bediscussieerd in Hoofdstuk 6. Ook is er gespeculeerd over de rol van een paar gedeeltelijk geconserveerde aminozuren in de familie 28 glycosyl hydrolasen van *A. niger*. Tevens is het gebruik van XGH als middel voor de detectie van XGA in andere plantensoorten besproken.

Ten slotte word het nut van onze aanpak, dat beschreven staat in Hoofdstuk 2, om het genoom van *A. niger* te onderzoeken naar nieuwe pectine afbrekende enzymen bediscussieerd. Deze aanpak is aantrekkelijk om enzymen op te sporen die pectines kunnen afbreken, uit bijvoorbeeld soya en erwt, die momenteel nog steeds moeilijk afbreekbaar zijn. Een verbetering in de afbraak van zulke pectines heeft als gevolg dat deze pectines beter zouden kunnen worden gebruikt voor industriële toepassingen.

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Curriculum Vitae

Joris Zandleven werd geboren op 1 mei 1976 te Eindhoven. Na het behalen van zijn HAVO-diploma aan het Van Mearlant Lyceum te Eindhoven begon hij met zijn studie Medische Microbiologie aan de Fontys Hogeschool Eindhoven in 1995. Tijdens deze studie heeft hij stage gelopen bij Numico Research B.V. te Wageningen en een afstudeerstage gelopen aan het EC Slater instituut te Amsterdam. In juli 1999 heeft hij zijn Bachelor diploma behaald. Vervolgens begon hij in augustus 2000 met een MSc studie aan de Strathclyde University in Glasgow waarbij hij een afstudeerstage heeft gelopen aan het Scottisch Crops Research Institute in Dundee. In september 2001 heeft hij zijn MSc diploma behaald.

In de periode van april 2002 tot en met augustus 2006 deed hij een promotie-onderzoek bij de leerstoelgroep Levensmiddelenchemie aan de Wageningen Universiteit, onder begeleiding van Prof. Dr. A.G.J. Voragen en Dr. Gerrit Beldman. Dit onderzoek dat werd uitgevoerd in deze periode is beschreven in dit proefschrift.

List of Publications

Full papers

- Zandleven J., Beldman, G., Bosveld, M., Benen, J., Voragen, A. (2005) Mode of action of Xylogalacturonan hydrolase towards xylogalacturonan and xylogalacturonan oligosaccharides. *Biochemical Journal* **387**, 719-725.

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- Martens-Uzunova, E[1]., Zandleven, J.S[1]., Benen, J.A.E., Awad, H., Kools, H.J., Beldman, G., Voragen, A.G.J., van den Berg, J.A., and Schaap, P.J. Exo-Acting Glycoside Hydrolases of *Aspergillus niger* Involved in Pectin Degradation. Biochemical Journal. In press.
  [1]Both authors contributed equally to this paper.

Abstracts

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Addendum

Overview of completed training activities

Discipline specific activities:

Courses:

VLAG Summer school glycosciences (Wageningen, June 2002)
Scientific Writing (2004)
Food Enzymology (2004)

Conferences:

Carbohydrate Bioengineering meeting (Groningen, 2003)
Seminar ICAT technique (Leiden, 2002)
Seminar 2D gel electrophoresis (Utrecht, 2002)
X-Cell Wall meeting (Sorrento, Italy, 2004)
Seminar Millipore (2004)
Seminar Leuven University (2004)

General Courses:

Food Chemistry PhD trip (USA, November 2002)
Food Chemistry PhD trip (Japan, December 2004)
PhD student week VLAG (2003)
Career Perspectives Course, Meijer & Meijaard, Wageningen, (2005)

Additional activities:

Preparation PhD proposal
Food Chemistry Seminars (Wageningen, 2002-2006)
Food Chemistry Colloquia (Wageningen, 2002-2006)
Carbnet meetings (Amersfoort, Utrecht, Wageningen, Haren, 2002-2006)