



**HAL**  
open science

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

Elena S Martens-Uzunova, Joris S Zandleven, Jaques Ae Benen, Hanem Awad, Harrie J Kools, Gerrit Beldman, Alphons Gj Voragen, Johan A van den Berg, Peter J Schaap

► **To cite this version:**

Elena S Martens-Uzunova, Joris S Zandleven, Jaques Ae Benen, Hanem Awad, Harrie J Kools, et al.. A new group of exo-acting family 28 glycoside hydrolases of *Aspergillus niger* that are involved in pectin degradation. *Biochemical Journal*, 2006, 400 (1), pp.43-52. 10.1042/BJ20060703 . hal-00478593

**HAL Id: hal-00478593**

**<https://hal.science/hal-00478593v1>**

Submitted on 30 Apr 2010

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

Elena S. Martens-Uzunova<sup>1\*</sup>, Joris S. Zandleven<sup>1†</sup>, Jaques A.E. Benen\*, Hanem Awad<sup>†</sup>, Harrie J. Kools\*, Gerrit Beldman<sup>†</sup>, Alphons G.J. Voragen<sup>†</sup>, Johan A. van den Berg\*, and Peter J. Schaap\*‡

\*Section Fungal Genomics, Department of Microbiology, Wageningen University, Dreijenlaan 2, 6703 HA Wageningen, The Netherlands. <sup>†</sup> Laboratory of Food Chemistry, Department of Agrotechnology and Food Sciences, Wageningen University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands. <sup>1</sup> These authors contributed equally to this work.

‡To whom correspondence should be addressed (e-mail: Peter.schaap@wur.nl).

**Short title:** Exo-acting pectinolytic glycoside hydrolases of *A. niger*

## SUMMARY

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 exopolygalacturonases 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.

Keywords: *Aspergillus niger*, pectin degradation, glycoside hydrolase, transcriptional profiling, microarray, nano-LC-nanospray mass spectrometry

Abbreviations used: MALDI-TOF MS, matrix assisted laser desorption ionization time of flight mass spectrometry; HPSEC, high performance size exclusion chromatography; HPAEC, high performance anion exchange chromatography; PAD, pulsed amperometric detection; RG, rhamnogalacturonan; HG, homogalacturonan; XGA, xylogalacturonan; a-MHR-S, saponified apple modified "hairy" regions

## INTRODUCTION

Pectin is a complex heteropolymer present in the middle lamella and 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 (RG I) and rhamnogalacturonan II (RG II). Particularly rhamnogalacturonan carry 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  $\alpha$ -D-galacturonic acid residues. XGA, RG I and RG II 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  $\alpha$ -(1, 2)-linked rhamnosyl and  $\alpha$ -(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  $\alpha$ -(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 endo-xylogalacturonan hydrolases [5] and exoPG [6, 7], whereas rhamnogalacturonan hydrolases (RGH) [8] and rhamnogalacturonan lyases (RGL) [9, 10] degrade RG I. 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 *pgxC* and *rgxA* 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: *pgxC*AFw 5'-CGTCATGTCTGTCTTCAAGG-3' and *pgxC*BRv 5'-TGATACTGTCGGTGATCTGG-3'; for the amplification of *pgxC* 3'fragment: *pgxC*DFw 5'-CACTGCGGTGCAGAATATAG-3' and *pgxC*CRv 5'-GCGTTCATGCAGATCACACT-3'; for the amplification of *rgxA* 5'fragment: *rgxA*AFw 5'-GGTATCGAGGTGAGCCAGGA-3' and *rgxA*BRv 5'-TATGGTTCGGATCGGTACGTG-3'; for the amplification of *rgxA* 3'fragment: *rgxA*DFw 5'-GGCTGTTCTTGTTCGATTCCCT -3' and *rgxA*CRv 5'-

CAGCGACAGCTCACGAATTG -3'; RT-PCR 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<sup>6</sup> 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 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 Agilent technologies G2500A Gene Array Scanner at pixel value 3µm and wavelength 570nm. 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 Specialties (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] 1.5kb

PCR fragment containing the *rgxC* 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 (*cspA1*, *pyrA6*, *leuA1*, *prtF28*, *goxC17*) 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, Uppsala, 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 trifluoroacetic acid (TFA) and had a Xyl:GalA ratio of 1:4 [35]. Polygalacturonic acid (PGA) was from ICN Biomedicals Inc., (Ohio, US). As substrates for rhamnosidase activity, p-nitrophenyl- $\alpha$ -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 (Baupre 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 gelfiltration 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  $\beta$ -galactosidase [41] for 19 hr at 40 °C in 20 mM bis-Tris (pH 5). The final protein concentration was 2.2  $\mu$ g/ml. The enzyme was inactivated by heating for 10 min at 100 °C. Subsequently,  $\beta$ -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 eluents 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. ExoPG 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) hesperidin, 0.02% (w/v)  $\alpha$ -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  $\mu$ g/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 hour of incubation with PGXA, PGXB, PGXC or Exo-PG were also analyzed by HPAEC.

The specific activity of PGXA, PGXB, PGXC and ExoPG 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 digested with trypsin as described [45]. The tryptic digest was subsequently acidified to a pH of 3 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  $\mu$ m i.d. x 5 mm, 100 Å) and eluted after desalting onto a Pep Map C18 resolving column (3  $\mu$ m, 100 Å, 75  $\mu$ m 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  $\geq 1.5$  were retained. For multiply charged peptides, spectra with a cross-correlation to a tryptic peptide  $\geq 2$  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 *pgxC* and *rgxA* 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 *rgxA* and 3 frame shifts in the genomic sequence of the *pgxC*. 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. 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 (ExoPG) [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 exopolygalacturonan 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 *pgxC* to support its classification. However, exopolygalacturonan 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 (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 activity, specifically removing terminal galacturonosyl residues from rhamnogalacturonan I, has been characterised in 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* protein 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 assess 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).

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 *pgaX*, *pgxB* and *pgxC* 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, *rgxC* displayed a similar transcriptional profile on galacturonic acid and PGA. This gene was also moderately expressed together with *rgxA* and *rgxB* after transfer to rhamnose (Fig. 2B). Interestingly *pgxA* showed only low expression levels on these carbon sources.

Transfer to SBP caused a more complex transcriptional pattern. *PgaX* was quickly induced and remained highly expressed reaching a maximum 8 h after transfer. *PgxC* messenger levels reached a maximum 4 h after transfer. *PgxB* and *rgxC* levels gradually increased and reached a maximum 8 hours after transfer. The signals obtained for *rgxC* 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 *rgxB* gene. *RgxA* 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 *pgaI*, *pgxB* and *pgxC* 4 h after transfer, coinciding with the slight accumulation of extra-cellular free galacturonic acid (data not shown).

*PgaA* and *pgxB* 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 *pgxB* messenger was observed coinciding with the depletion of fructose during the time course of incubation suggesting that, although weak, *pgxB* is under CCR control. In contrast, when pectic substrates were used, the signal of *pgxB* decreased in time to reach a background level at 24 h (Fig. 2A). Instead, low levels of *pgaD* messenger were detected. PGD, the protein encoded by *pgaD* 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 *pgaD* free sugars are being depleted from the culture fluid, which together with its functional properties, suggests that the expression of *pgaD* may in part be due to carbon starvation. Still, during incubation on galacturonic acid and sugar beet pectin *pgaD* 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. Exopolygalacturonases 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 endopolygalacturonases, 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].

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 hydrolyse hesperidin 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* ExoPG 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 ExoPG 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.



## 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 family of seven endo-polygalacturonan hydrolases, we were able to extend the number of endo-rhamnogalacturonan hydrolases to six. Furthermore, a new gene family 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 *pgaX* is almost identical with the earlier described ExoPG 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 ExoPG 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 possess 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.

## ACKNOWLEDGEMENTS

This research was supported by Senter IOP, The Netherlands (IGE01021).

We would like to thank Esther Tichon for cloning the *pgxC* gene. We also thank Margaret Bosveld for the preparation of linear rhamnogalacturonan oligosaccharides.

## REFERENCES

- 1 Carpita, N. C. and Gibeaut, D. M. (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. *Plant J.* **3**, 1-30
- 2 Vincken, J.-P., Schols, H.A., Oomen, R.J.F.J., Beldman, G., Visser, R.G.F., Voragen, A.G.J. (2003) Pectin-The hairy thing. In *Advances in Pectin and Pectinase Research* (Voragen, A. G. J., Schols, H., Visser, R., ed.), pp. 47-59, Kluwer Academic Publishers, Dordrecht
- 3 Voragen, A. G. J., Pilnik, W., Thibault, J. -F., Axelos, M. A. V., and Renard, C. M. G. C. (1995) Pectins. In *Food Polysaccharides* (Stephen, A. M., ed.), pp. 287-339, Marcel Dekker, Inc, New York-Basel-Hing Kong
- 4 Schols, H. A., Voragen, A.G.J. (1996) Complex Pectins: Structure elucidation using enzymes. In *Pectins and pectinases* (Visser, J., Voragen, A.G.J., ed.), pp. 3-19, Elsevier Science B.V., Amsterdam
- 5 Van der Vlugt-Bergmans, C. J. B., Meeuwssen, P.J.A., Voragen, A.G.J., and van Ooyen, A.J.J. (2000) Endo-Xylogalacturonan hydrolase, a Novel Pectinolytic Enzyme. *Appl. Environ. Microbiol.* **66**, 36-41
- 6 Beldman, G., van den Broek, L.A.M., Schols, H.A., Searle-van Leeuwen, M.J.F., van Laere, K.M.J., Voragen, A.G.J. (1996) An exogalacturonase from *Aspergillus aculeatus* able to degrade Xylogalacturonan. *Biotechnol. Lett.* **18**, 707-712
- 7 Kester, H. C., Benen, J. A. and Visser, J. (1999) The exopolygalacturonase from *Aspergillus tubingensis* is also active on xylogalacturonan. *Biotechnol. Appl. Biochem.* **30**, 53-57
- 8 Suykerbuyk, M. E., Kester, H. C., Schaap, P. J., Stam, H., Musters, W. and Visser, J. (1997) Cloning and characterization of two rhamnogalacturonan hydrolase genes from *Aspergillus niger*. *Appl. Environ. Microbiol.* **63**, 2507-2515
- 9 Kofod, L. V., Kauppinen, S., Christgau, S., Andersen, L. N., Heldt-Hansen, H. P., Dorreich, K. and Dalboge, H. (1994) Cloning and characterization of two structurally and functionally divergent rhamnogalacturonases from *Aspergillus aculeatus*. *J. Biol. Chem.* **269**, 29182-29189
- 10 Azadi, P., O'Neill, M. A., Bergmann, C., Darvill, A. G. and Albersheim, P. (1995) The backbone of the pectic polysaccharide rhamnogalacturonan I is cleaved by an endohydrolase and an endolyase. *Glycobiol.* **5**, 783-789
- 11 de Vries, R. P. and Visser, J. (2001) *Aspergillus* Enzymes Involved in Degradation of Plant Cell Wall Polysaccharides. *Microbiol. Mol. Biol. Rev.* **65**, 497-522
- 12 Parenicova, L., Kester, H. C., Benen, J. A. and Visser, J. (2000) Characterization of a novel endopolygalacturonase from *Aspergillus niger* with unique kinetic properties. *FEBS Lett.* **467**, 333-336
- 13 Henrissat, B. and Davies, G. (1997) Structural and sequence-based classification of glycoside hydrolases. *Curr. Opin. Struc. Biol.* **7**, 637
- 14 van Santen, Y., Benen, J. A. E., Schroter, K.-H., Kalk, K. H., Armand, S., Visser, J. and Dijkstra, B. W. (1999) 1.68-Å Crystal Structure of Endopolygalacturonase II from *Aspergillus niger* and Identification of Active Site Residues by Site-directed Mutagenesis. *J. Biol. Chem.* **274**, 30474-30480
- 15 van Pouderooyen, G., Snijder, H. J., Benen, J. A. E. and Dijkstra, B. W. (2003) Structural insights into the processivity of endopolygalacturonase I from *Aspergillus niger*. *FEBS Lett.* **554**, 462
- 16 Armand, S., Wagemaker, M. J. M., Sanchez-Torres, P., Kester, H. C. M., van Santen, Y., Dijkstra, B. W., Visser, J. and Benen, J. A. E. (2000) The Active Site Topology of *Aspergillus niger* Endopolygalacturonase II as Studied by Site-directed Mutagenesis. *J. Biol. Chem.* **275**, 691-696
- 17 Pages, S., Heijne, W. H., Kester, H. C., Visser, J. and Benen, J. A. (2000) Subsite mapping of *Aspergillus niger* endopolygalacturonase II by site-directed mutagenesis. *J. Biol. Chem.* **275**, 29348-29353
- 18 Pages, S., Kester, H. C., Visser, J. and Benen, J. A. (2001) Changing a single amino acid residue switches processive and non-processive behavior of *Aspergillus niger* endopolygalacturonase I and II. *J. Biol. Chem.* **276**, 33652-33656
- 19 Groot, G., Peij, N. v. and Ooyen, A. v. (2002) Exploring and exploiting the *Aspergillus niger* genome. In *Annual Meeting of the Society for Industrial Microbiology August 2002*
- 20 Coutinho, P. M. H., B. (1999) Carbohydrate-Active Enzymes server at URL: <http://afmb.cnrs-mrs.fr/CAZY/index.html>.

- 21 Bairoch, A. and Apweiler, R. (2000) The SWISS-PROT protein sequence database and its supplement TrEMBL in 2000. *Nucl. Acids Res.* **28**, 45-48
- 22 Durbin, R., Eddy, S., Krogh, A. and Mitchison, G. (1998) *Biological Sequence Analysis: Probabilistic Models of Proteins and Nucleic Acids. A tutorial introduction to hidden Markov models and other probabilistic modelling approaches in computational sequence analysis.* Cambridge University Press
- 23 Birney, E., Clamp, M. and Durbin, R. (2004) GeneWise and Genomewise. *Genome Res.* **14**, 988-995
- 24 Notredame, C., Higgins, D. G. and Heringa, J. (2000) T-coffee: a novel method for fast and accurate multiple sequence alignment. *J. Mol. Biol.* **302**, 205
- 25 Kumar, S., Tamura, K. and Nei, M. (2004) MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief. Bioinform.* **5**, 150-163
- 26 Pontecorvo, G., Roper, J. A., Hemmons, L. M., Macdonald, K. D. and Bufton, A. W. J. (1953) The genetics of *Aspergillus nidulans*. *Adv. Genet.* **5**, 141
- 27 Vishniac, W. and Santer, M. (1957) The thiobacilli. *Bacteriological Reviews* **21**, 195-213
- 28 van Dijck, P. W. M. (1999) Made by Genetic Engineering: A series of articles to promote a better understanding of the use of genetic engineering. *J. Biotechnol.* **67**, 77
- 29 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- 30 de Graaff, L., van den Broek, H. and Visser, J. (1988) Isolation and transformation of the pyruvate kinase gene of *Aspergillus nidulans*. *Curr. Genet.* **13**, 315-321
- 31 Benen, J. A. E., Kester, H. C. M. and Visser, J. (1999) Kinetic characterization of *Aspergillus niger* N400 endopolygalacturonases I, II and C. *Eur. J. Biochem.* **259**, 577-585
- 32 Kusters-van Someren, M. A., Harmsen, J. A., Kester, H. C. and Visser, J. (1991) Structure of the *Aspergillus niger pelA* gene and its expression in *Aspergillus niger* and *Aspergillus nidulans*. *Curr. Genet.* **20**, 293
- 33 Goosen, T., Bloemheuvel, G., Gysler, C., de Bie, D. A., van den Broek, H. W. and Swart, K. (1987) Transformation of *Aspergillus niger* using the homologous orotidine-5'-phosphate-decarboxylase gene. *Curr. Genet.* **11**, 499
- 34 Parenicova, L., Benen, J. A., Kester, H. C. and Visser, J. (1998) pgaE encodes a fourth member of the endopolygalacturonase gene family from *Aspergillus niger*. *Eur. J. Biochem.* **251**, 72-80
- 35 Beldman, G., Vincken, J.-P., Meeuwssen, P.J.A., Herweijer, M., Voragen, A.G.J. (2003) Degradation of differently substituted Xylogalacturonans by Endoxylogalacturonan Hydrolase and Endopolygalacturonases. *Biocatal. Biotransform.* **21**, 189-198
- 36 Guillotin, S. E., Bakx, E. J., Boulenguer, P., Mazoyer, J., Schols, H. A. and Voragen, A. G. J. (2005) Populations having different GalA blocks characteristics are present in commercial pectins which are chemically similar but have different functionalities. *Carbohydr. Polym.* **60**, 391
- 37 Guillotin, S. E. (2005) Studies on the intra- and intermolecular distributions of substituents in commercial pectins. pp. 155, Wageningen University, Wageningen
- 38 Schols, H. A., Posthumus, M.A., Voragen, A.G.J. (1990) Hairy (ramified) regions of pectins. Part I. Structural features of hairy regions of pectins isolated from apple juice produced by the liquefaction process. *Carbohydr. Res.* **206**, 117-129
- 39 Zandleven, J., Beldman, G., Bosveld, M., Benen, J., Voragen, A. (2006) Enzymatic degradation studies of xylogalacturonans from apple and potato, using XGH. *Carbohydrate Polymers* **accepted**
- 40 Mutter, M., Beldman, G., Schols, H.A. and Voragen, A.G.J. (1994) Rhamnogalacturonan  $\alpha$ -L-rhamnopyranosylhydrolase. A novel enzyme specific for the terminal non-reducing rhamnosyl unit in rhamnogalacturonan regions of pectin. *Plant Physiol.* **106**, 241-250
- 41 van de Vis, J. W. (1994) Characterization and mode of action of enzymes degrading galactan structures of arabinogalactans. In Wageningen University, pp. 161, Wageningen University, Wageningen
- 42 Kester, H. C., Kusters-van Someren, M. A., Muller, Y. and Visser, J. (1996) Primary structure and characterization of an exopolygalacturonase from *Aspergillus tubingensis*. *Eur. J. Biochem.* **240**, 738-746
- 43 Mutter, M., Beldman, G., Pitson, S.M., Schols, H.A. and Voragen, A.G.J. (1998) Rhamnogalacturonan  $\alpha$ -D-Galactopyranosyluronohydrolase. An enzyme that specifically removes the terminal nonreducing galacturonosyl residue in rhamnogalacturonan regions of pectin. *Plant Physiol.* **117**, 153-163

- 44 Zandleven, J., Beldman, G., Bosveld, M., Benen, J., Voragen, A. (2005) Mode of action of Xylogalacturonan hydrolase towards xylogalacturonan and xylogalacturonan oligosaccharides. *Biochem. J.* **387**, 719-725
- 45 Gao, J., Opiteck, G. J., Friedrichs, M. S., Dongre, A. R. and Hefta, S. A. (2003) Changes in the Protein Expression of Yeast as a Function of Carbon Source. *J. Proteome Res.* **2**, 643-649
- 46 VerBerkmoes, N. C., Hervey, W. J., Shah, M., Land, M., Hauser, L., Larimer, F. W., VanBerkel, G. J. and Goeringer, D. E. (2005) Evaluation of "Shotgun" Proteomics for Identification of Biological Threat Agents in Complex Environmental Matrixes: Experimental Simulations. *Anal. Chem.* **77**, 923-932
- 47 Link, A. J., Eng, J., Schieltz, D. M., Carmack, E., Mize, G. J., Morris, D. R., Garvik, B. M. and Yates, J. R. (1999) Direct analysis of protein complexes using mass spectrometry. *Nat. Biotech.* **17**, 676-682
- 48 Petersen, T. N., Kauppinen, S. and Larsen, S. (1997) The crystal structure of rhamnogalacturonase A from *Aspergillus aculeatus*: a right-handed parallel beta-helix. *Struc.* **5**, 533
- 49 Cho, S., Lee, S., Shin, W., Cho, S. W., Lee, S. and Shin, W. (2001) The X-ray structure of *Aspergillus aculeatus* polygalacturonase and a modeled structure of the polygalacturonase-octagalacturonate complex. *J. Mol. Biol.* **311**, 863-878
- 50 Parenicova, L., Benen, J. K., H. C., Kester Hc Fau - Visser, J. and Visser, J. (2000) pgaA and pgaB encode two constitutively expressed endopolygalacturonases of *Aspergillus niger*. *Biochem. J.* **345**, 637-644
- 51 Parenicová, L. (2000) Pectinases of *Aspergillus niger*: a molecular and biochemical characterisation. pp. 200, Wageningen University, Wageningen
- 52 Hara, T., Lim, J.Y. & Ueda, S. (1984) Purification and some properties of exopolygalacturonase from *Aspergillus niger* cultured in the medium containing satsuma mandarin peel. *J. Jpn. Soc. Food Sci. Technol.-Nippon shok* **31**, 581-586
- 53 Sakamoto, T., Bonnin, E., Quemener, B., Thibault, J.-F. (2002) Purification and characterisation of two exo-polygalacturonases from *Aspergillus niger* able to degrade xylogalacturonan and acetylated homogalacturonan. *Biochim. Biophys. Acta* **1572**, 10-18

## Legends to the Figures

**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; ExoPG: exopolygalacturonan 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 complete multiple alignment of *A. niger* family 28 glycoside hydrolases is provided as a supplementary figure (see Supplementary Fig1).

**Figure 2. Transcriptional levels of *A. niger* glycoside hydrolases – 24 hours time course on different carbon sources.**

A – endo-polygalacturonan hydrolases and endo-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.

**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.

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

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. <sup>1</sup>As determined by Kester et al. (1996).

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
ExoPG	78 <sup>1</sup>	47	230	17	4.2 <sup>1</sup>
RGXB	82	50	n.d.	n.d.	n.d.

Figure 1 A

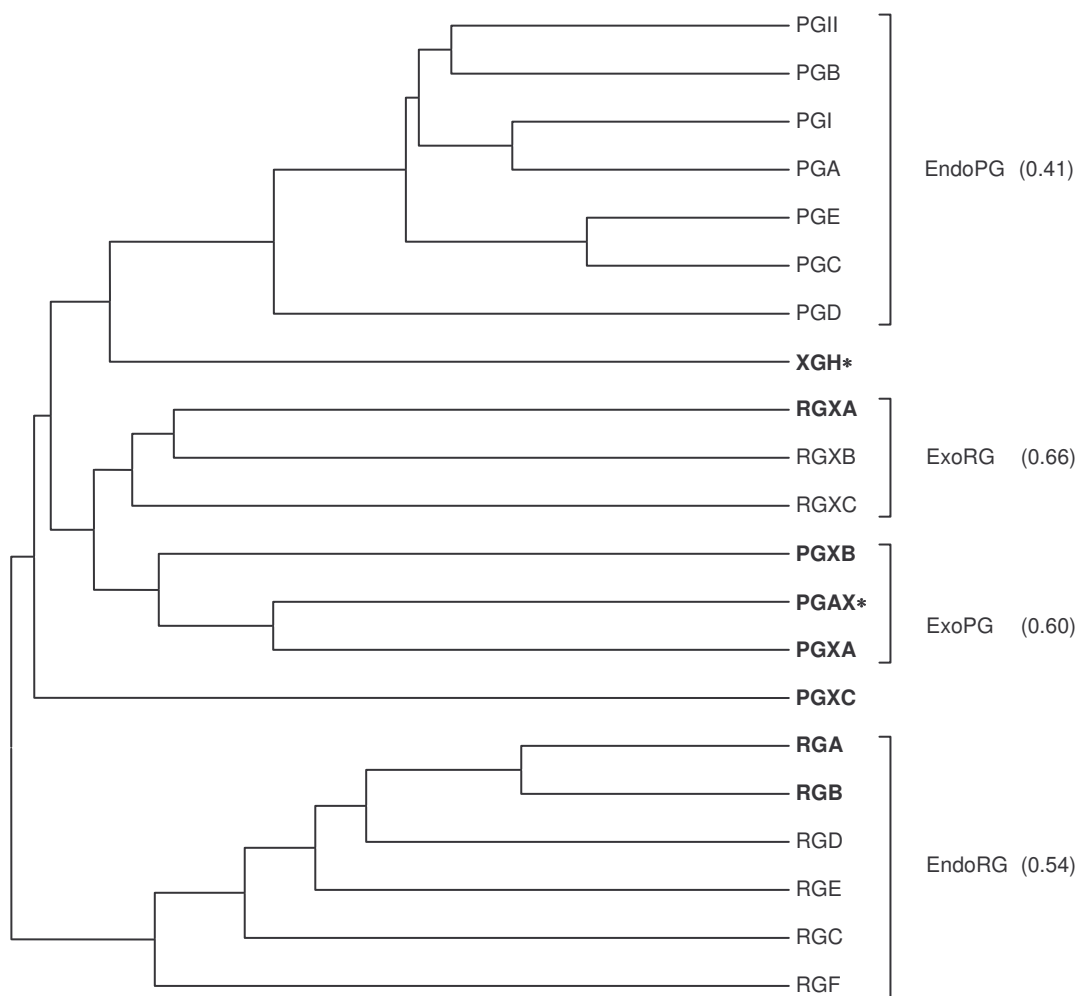




Figure 1 B

```

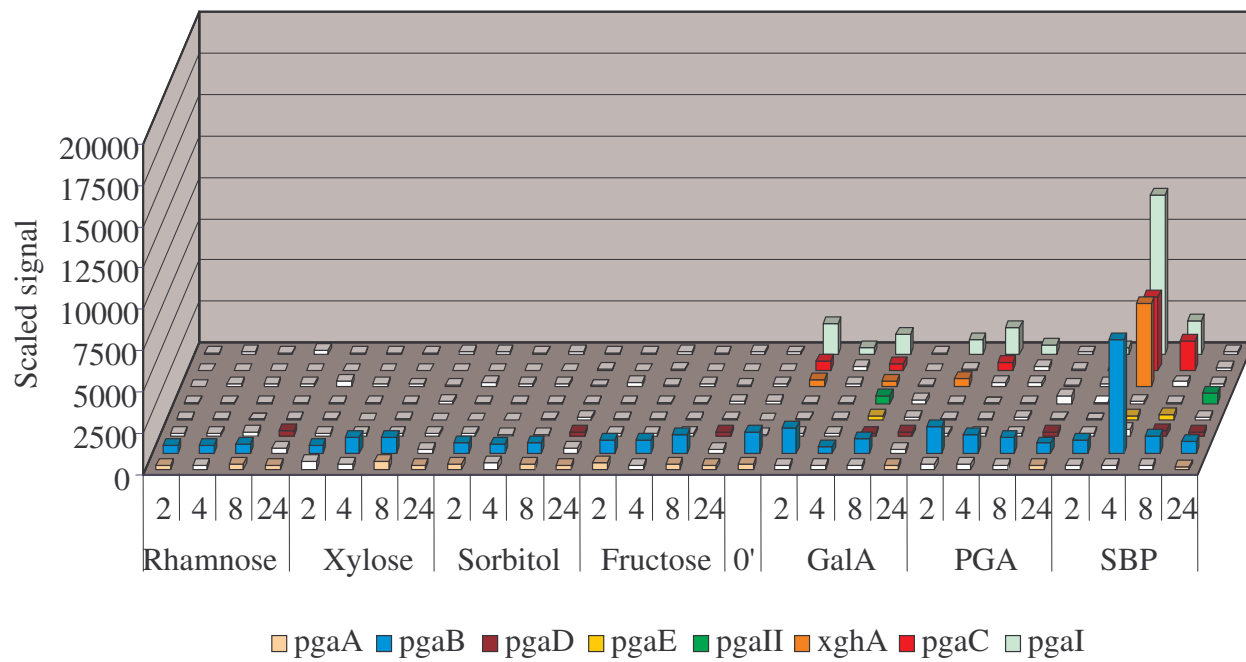
340          360          380          400          420          440
*  *          **          +1 +1          +1-1          .
PGII ---GGHNTDAPDVGNSVGVNIK-PWVHNDCLAV--NSGENIWF TGGTCTI-GGHGLSIGSVGDRSN--NVVKNVTIEHSTVSNSEN-----AVRIKTIIS----- :234
PGI ---GGHNTDGFDISESTGVYISG-ATVKNQDDCIAI--NSGESISFTGGTCS-GGHGLSIGSVGGRRDD--NTVKNVTISDSTVSNAN-----GVRIKTIY----- :236
PGA ---GGHNTDGFDISESTYITITG-ATVKNQDDCVAI--NSGENIYFSGGTCS-GGHGLSIGSVGGRRDD--NTVKNVTIFDSTVSDSEN-----GVRITKTVY----- :235
PGB ---GHNTDAPDIQGSTYITIDG-ATVYNQDDCLAI--NSGEHIFTNGYCD-GGHGLSIGSIGGRSD--NTVNDVTISNSKVLNSQN-----GVRITKIY----- :233
PGE --EAA-NTDGFIDGSDTYITITG-ANVYNQDDCVAV--NSGENIYFSGGVCS-GGHGLSIGSVGGRRSD--NTVKNVTFYDSEIKSSQN-----GVRIKTIY----- :240
PGC --DLAA-NTDGFIDGSESTYITITG-AEINYNDQDDCVAI--NSGENIYFSAVCS-GGHGLSIGSVGGRRDD--NTVKNVTFYDNNVLSKQQ-----ATRIKTIY----- :241
PGD GDPAAHNTDGFDIKQSDFLTLN-SWVHNDQDDCVAV--TSGSSIVVDNLYCY-GGHGLSIGSIGGKSN--NTVDGVTFSSQVINSEN-----GCRISKNA----- :381
XGH ---NPPKNTDGFIDGSESTYVITE-VTVVNDQDDCVALKPSS-NVVTVDITISCT-GSHGISVSGSLGKSSD--DSVKNIYVTGATMINSTK-----AAGIKTYPSSG----- :274
PGXC -GSFPFNTDGFID-VEGTNIQITD-SIMYNGDDAIAVGADSHDILFTRNITGY-QTHGMSIGSLGKDPDFANISNIRFDDVTVVDGLY-----AARFKTWSG----- :275
PGXA -DAPAKNTDGDWIDYRSDNIVIQD-SRIVNTDQCVSFKPNSTQIVI-QNLDCD-GSHGISVSGSLGQYQGETDIVEDLYIYNISMTDASD-----VARIKTVWPGVPA----- :294
PGAX -DNEAKNTDGDWIDYRSDNIVIQD-SVINNDQCVSFKPNSTNIIIV-QNLHNC-GSHGISVSGSLGQYKDEVDIVENVYVYNI SMFNASD-----MARIKTVWPGTSP----- :289
PGXB -SSDPANTDGMDSLDVDGVSFN-MRIDVQDDCFSPKPNSTNIFV-QNMWCN-NTHGVSMGSIQYAGEMDIENVTYIENVTLLNQN-----GARLKAWAGQDV----- :306
RGXC GDNWVQNTDGFIDMTADNIQLTN-FVYQGGDDCIAIKPRSYNIDI-QNVTCR-GGNGIAIGSLGQYL-EDSSVANIRVDKVNIRYRVED-MHNSAYLKITWVGALVPSQSSY :284
RGXB DGGSAQNTDGGANTYISKNIITLTN-WEVYNGDDSI STKANSTDIIT-ANCTFT-SGLGIAIGSLGQYNGAFETVERLKI SNITYEKTH-----AVYFKTWTGQDV----- :306
RGXA EGEWVQNTDGDITYRSDHIVTN-WVYQGGDDAVAFKGNSTNIIHV-ENVTYV-GGPGIAFGSLGQYPRDIDIVENVTVRNVVQPSFORAMNSGVYFRTSWGVNY----- :288
RGA -----GLDGDIVW-GSNIWVHD-VEVTNKDECVTVKSPA-NNILVESIYCN-WSGGCAMGSLGAD----TDITDILYRNVTWSSNQ-----MYMIKSN----- :256
RGB -----GLDGDIVW-SNIIWVHD-VEVTNKDECVTVKSPS-KNILIESIYCN-WSGGCAMGSPGSD----TNVSDITRYNIYTWSSNN-----MMLIKSN----- :256
RGD -----GLDGDIDIS-GANYWIHD-VEVTNKDECVTVKSPS-ANVRVENVFCN-HSGGCAMGSLGTD----TNISNIEFENIYTYNSTQ-----MYMIKSN----- :257
RGC -----ATDAIDVW-GENMWFHD-IEVSNQDECVTVKSPA-HNYLIENIYCN-LSGGTAIGSLGTG----TNISDIHYRNLYM-NOAD-----ACFLIKSN----- :255
RGE -----GLDGDIDLY-GENIWIHL-VMVTNKDECVTSKTNS-HNFLIENIYCN-PSGGCAIGSLGSS----VNVNTIYRNVTWDSNQ-----MMMIKTN----- :258
RGF -----SYDGDIAI-GTNYIYHD-NEVTNRDECVSVKSPS-HHALVENLVCNQAGSGVSI GSLNVS----AEISNIEARNISIIQGN-----IAFIKTYP----- :255

          460          480          500          520          540
          +1          #          #
PGII -----GATGSVSEITYSNIVMSGISDYGVVQQDYEDGKPT----GKPTNGVTIQDKVLESVTGSVDSGA-----TEIYLLCGS-GSC :307
PGI -----KETGDVSEITYSNIQLSGITDYGIVIEQDYENGSPD----GTPSTGPIITDVTVDGVTGLLEDDA-----TQVYIILCGD-GSC :309
PGA -----DATGTVEDITYSNIQLSGISDYGIVIEQDYENGDPD----GTPSNGVTISDVTLEDITGSVDSDA-----VEIYIILCGD-GSC :308
PGB -----GKTGTVENVKFEDITLSDISKYGVVEQDYENGSPD----GTPNGVKVEDITFKKVTGSVKSSG-----TDIYIILCGS-GSC :306
PGE -----GDTGSVSEVYKEITLSDITDYGIVVEQNYDDTS----KSPDGTITIEDFVLDNVQGSVESG-----TNIYIVCGS-DSC :311
PGC -----GDTGSVSEVYTHEIAPSDATDYGIVIEQNYDDTS----KPTTGVPIITDFVLENIVGICEDDD-----CTEVYIACGD-GSC :313
PGD -----DITGTEVYNVYENITLSDISDYGIDIQDYENGSGAT----GDPTNGVKIENISFVNVKGTMSDGG-----DYYIILCGD-GSC :453
XGH -----DHGTSTVSNVTFDFTVDN-SDYAFQIQSCYGE-DDDYCE-ENFGNA-KLTDIVVSSFSGTTSDKY-----DPVVANIDCGSDGT :351
PGXC -----GTGLVKNVTWNNIRFENVF-FPIFVTQSYSDQGASRSGTVNASSAVMDEFWTSDFAGSINTYQPDGSGCVSDPCWYVNLPLNKHTEALIEICHTAQSC :374
PGXA DTSGS-TSGGGLGRVRNVYEHMQSENND-HIISVSQCYESKNQTMCDYSY-P-SKLVIEDVLFKDFKGTTSK-----KYDPEIGELTCSFPDVC :379
PGAX ALSADLQGGGGSGSVKNITYDATALDNVD-WAIEITQCYGQKNTTLCNEY-P-SSLTISDVHINKNFRGTTSG-----SEDPYVGTIVCSPDTC :375
PGXB -----CYGRINNVYKNIQIQNTD-APIVLDQCYFDINATECAKY-P-SAVNITNIFENIWGSSSG-----KDGKIVADLVCSPPDVC :382
RGXC E-SAGVPRGDGWSIRNVLSNFNVQGS-AGPSISQDSGDN----GSYAGTSKMSISNVAFVNFVGTWVD-----TEKSVVSTVSCSEVHPC :365
RGXB GYPPN-GGGGGLGYASDIVATNLKTNLKGAPFTISQCTTFSGAS--GNCNLSKFIIRDLVFTDISGTTDS-----SDVASFCVSAVAP :388
RGXA GVPPN-GGGGGHGYVRNVSVENLRKDVQ-LPVYIDTCLSYLSEMITQYCDTSTYEFEDLHFRNISGNGLA-----TVTDYPGKNISFAVALLCSEKAPC :382
RGA -----GGSGTVNNTVLENFIGHNA-YSLDVSYSWSSMTA-----VDGDGVQLSNI TFKNWKGT EADGA-----ERGPVKVVCSDTAPC :329
RGB -----GGSGFVENVLENFIGHNA-YSLDIDSYWASMSA-----VDGDGVQLSNI TFKNWKGT EAYGA-----ERGPVKVVCADGAPC :329
RGD -----GGNGTVTNCSEFKNFI GYSNA-YMLDLDIYWGDES-----DGDGIKENIGFENWKGTSSNGI-----QRSPIRILCPDANPC :328
RGC -----NGDGVKNI IWENVIVHGGP-YPLAIDEAWGDDRG-----SVGVQVSNLTFRNWHGESVSA-----SRPVIRLQCDSDVPC :325
RGE -----GGLGMVSNIVFENFIGHNV-NSLDLDSYWSSMNA-----IDGVGIYHNITL YNWTGTAIDGE-----TRPPIRVICPEDMPC :331
RGF -----GGSGYVKDVTFENFRSLNSL-YGLDINQYWQNTWE-----PDTGSVTLNLFVKNFSGSVADGA-----LRPPLYFASDLTFA :328

```

Figure 2

A



B

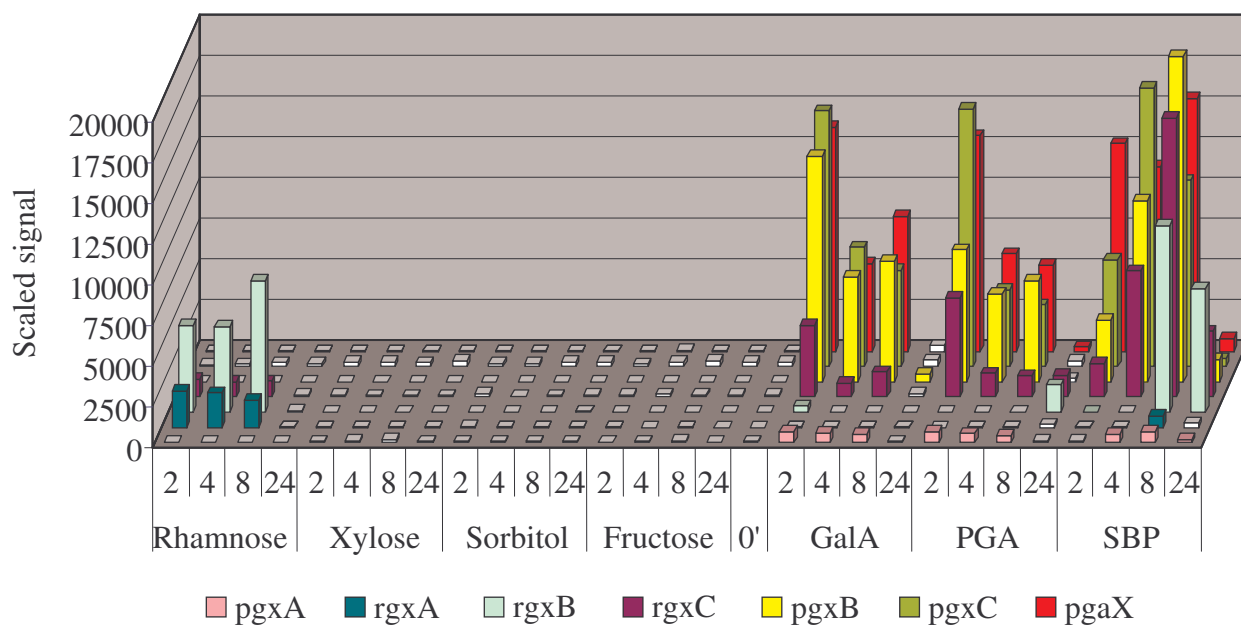


Figure 3 A.

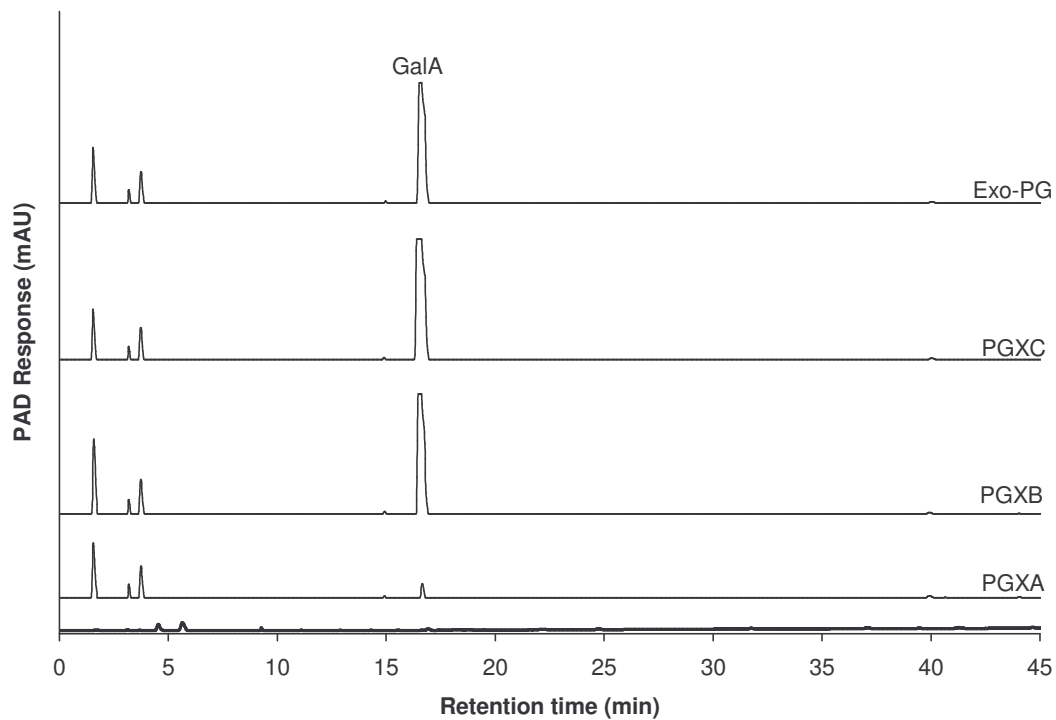


Figure 3 B.

