Molecular analysis of endo-rhamnogalacturonan hydrolases in *Aspergillus*

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Molecular analysis of endo-rhamnogalacturonan hydrolases in *Aspergillus*

Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus,
Dr. C.M. Karssen,
in het openbaar te verdedigen
op woensdag 7 mei 1997
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen

This research was supported by Unilever Research Laboratorium (Vlaardingen, The Netherlands)

Molecular analysis of endo-rhamnogalacturonan hydrolases in *Aspergillus* / Marjon ten Hoor-Suykerbuyk.
Thesis Wageningen - With summary in Dutch
ISBN 90-5485-706-4

Voor mijn vader en moeder
voor Gerhard en de kleine

Stellingen

1. De eenvoud van het bepalen van 'pectinase-activiteit' d.m.v. viscositeits-metingen en reducerende eindgroepen-bepalingen leidt tot de publikatie van irrelevante artikelen (*Solis-Pereira et al. (1993) Effects of different carbon sources on the synthesis of pectinase by Aspergillus niger in submerged and solid state fermentations. Appl Microbiol Biotechnol 39:36-41*).

2. Generalisatie van de aanwezigheid van RG-II strukturen in bioactieve pectines is nog geen garantie voor een actieve rol van deze strukturen in de heilzame werking van medicinale kruiden (*Hirano et al. (1994) Existence of a rhamnogalacturonan II-like region in bioactive pectins from medicinal herbs. Planta Med 60:450-454*).

3. Met het gebruik van steeds verfijndere moleculair-biologische technieken voor de classificatie van schimmels is het van belang een duidelijk onderscheid te maken tussen soortskenmerken en individuele kenmerken (Dit proefschrift).

4. Een literatuur referentie die niet genoemd wordt krijgt vaak meer aandacht dan één die wel genoemd wordt.

5. Het gebruik van oudere literatuurreferenties voor 'standaard'-technieken getuigt van luiheid en kan ten koste gaan van de reproduceerbaarheid van experimenten door anderen (SDS-PAGE: *Laemmli* 1970; Western blotting: *Burnette et al. 1981;* DNA-sequencing: *Sanger et al. 1977*).

6. Het gebruik van afkortingen in wetenschap en technologie is efficiënt, maar vooral ook verwarrend (*CMC*, carboxymethylcellulose; complement mediated cytotoxicity; chemistry, manufacturing and control; critical micel concentration).

7. Bij beslissingen omtrent de toelating van genetisch gemanipuleerde voedingsmiddelen zou de vrije keuze van de consument boven commerciële belangen moeten gaan.

8. Het kloneren van zoogdieren gaat ten koste van het unieke van een nieuw-geborene en dus van de biodiversiteit.

9. Het hier beschreven onderzoek laat sporen na.

Stellingen behorende bij het proefschrift
"Molecular analysis of endo-rhamnogalacturonan hydrolases
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Wageningen, 7 mei 1997

Contents

List of abbreviations

Ace	Aceric acid (3-*C*-carboxy-5-deoxy-L-xylose)
Api	Apiose (3-*C*-hydroxymethyl-D-glycero-tetrose)
Ara	L-arabinose
CNBr	Cyanogen bromide
DHA	3-deoxy-D-lyxo-2-heptulosaric acid
Fuc	L-fucose
Gal	D-galactose
Gal*p*A	D-galacturonic acid
Glu	D-glucose
Glc*p*A	D-glucuronic acid
*gox*C	Structural gene encoding glucose oxidase
KDO	2-keto-3-deoxy-D-manno-octulosonic acid
Man	L-mannose
Me-	Methylesterified
(M)HR	(Modified) hairy regions
PAE	Pectin acetylesterase
PEL	Pectate lyase
PG	Polygalacturonase
*pki*A	Pyruvate kinase encoding gene
PL	Pectin lyase
*pyr*A	Orotidine-5'-phosphate-decarboxylase encoding gene
PME	Pectin methylesterase
RFLP	Restriction fragment length polymorphism
RG-I,II	Rhamnogalacturonan I,II
Rha	L-rhamnose
RHG	Rhamnogalacturonan hydrolase
rhg	Rhamnogalacturonan hydrolase encoding gene
rRNA	Ribosomal RNA
Xyl	D-xylose

1 General introduction

Industrial application of pectinolytic enzymes

Pectinolytic enzymes are used in industrial processes, such as fruit juice production (Whitaker 1984) and wine making (Colagrande et al. 1994). For instance, the processing of apples to juice includes mechanical treatments, like pressing and ultrafiltration, and enzymatic treatments, like pulp enzyming, liquefaction, clarification and cloud stabilization (Voragen and Pilnik 1989). The enzymatic treatments are performed with crude pectinolytic enzyme preparations, which are commonly derived from nonpathogenic fungi, like *Aspergillus*, and therefore are generally regarded as safe (GRAS). Fungi, but also plants and bacteria, can degrade pectin by the production of a broad spectrum of pectinolytic enzymes. However, the ease of handling cultures and the advantages of an eukaryotic production system make fungi suitable organisms for various industrial purposes on a large scale.

The enzyme composition of a commercial preparation determines its effect on the yield of the juice or wine obtained, but also on characteristics as composition, viscosity, turbidity, colour and flavour thereof. Comparative analysis of fungal preparations which are currently used by different companies, revealed the presence of a variety of pectinolytic enzyme activities (Table 1). In addition, all of the preparations showed hemicellulase and cellulase activities, suggesting that complex substrates have been used as carbon source for fungal growth. Examples of carbon sources which are frequently used for the purpose of multiple enzyme production are milled soybean and wheat bran, because these contain the whole scala of plant polysaccharides (Hours and Sakai 1994).

Whereas most of the polymer degrading enzymes produced by fungi have favourable effects on fruit processing, some of them are undesired. An example is arabinan-haze formation in apple juices, which arises after debranching of arabinans by α-L-arabinofuranosidase activities also present in the enzyme preparation (Whitaker 1984). Commercial enzyme mixtures are therefore continuously optimized by adjusting media composition and growth conditions. Besides submerged cultivation also solid state fermentations of several fungal species are

Table 1 Enzyme activities in currently used industrial enzyme preparations from *Aspergillus*

Enzyme preparation	Source	Pectinolytic enzymes[1]					Hemicellulases[2]				Cellulases[3]	
		PG	PL	PME	PAE	RHG	AF	EA	EXL	XG	BG	CMC
Ultra SP	Novo Nordisk	+[4]	+	+	+ +	+ +	+	+	+	+	+	+
Biopectinase MKL	Quest Int.	±	+	+	±	±	±		+	+	+ +	±
Biopectinase OKL	Quest Int.	+	+ +	+ +	+	±	±	±	+	+ +	+ +	+ +
AP2 liquid	S. Nihon	+	+ +	+ + +	+	−	+	±	+	+	+ +	+ +
Cytolase M102	Genencor	+	+	±	+	+	+ +	±	+ +	+ +	+ +	+ +
Pearex	Miles	±	+	±	+	+	+	+	+	+		
Pectinase no.1	Moscow Int.	+ + +	+	±	+ +	±			+ +	+		
Pectine x 3XL	Novo Nordisk	+ +	+ +	+	+ +	+	+	±	+ +	+	+	+
Rapidase C80	Gist-brocades	+	+ +	+ +	+	+	+	+	+	+	+	±
Rapidase Press	Gist-brocades	+	+ +	+ +	+ +	+ +	+	+	+	+		±

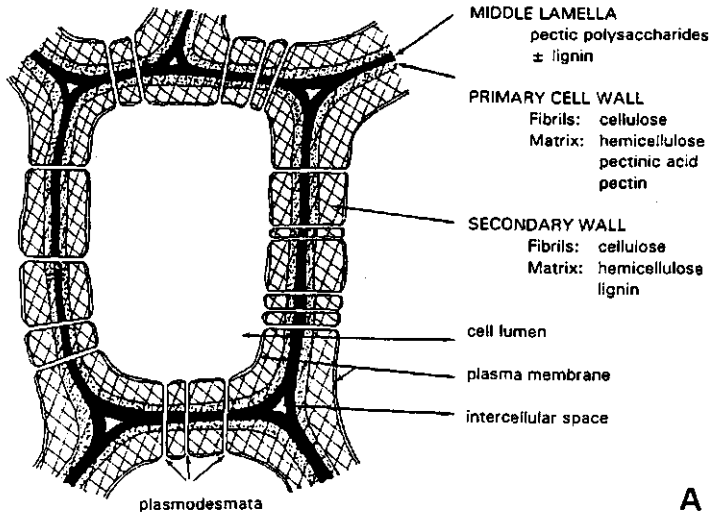
[1] See list of abbreviations [2] AF, arabinofuranosidase; EA, endo-arabinanase; EXL, endo-xylanase; XG, xyloglucanase [3] BG, β-glucosidase; CMC, carboxymethylcellulase [4] Enzyme activities are indicated as + + +, very high; + +, high; +, moderate; ±, low

being applied successfully to obtain pectinolytic enzyme preparations (Solís-Pereira et al. 1993; Roche et al. 1995). Likewise, intrinsic pectinolytic enzymes in plants can have undesired effects for other purposes. For instance, the production of polygalacturonases (PGs) during fruit ripening results in softening, which is unfavourable during the storage of fruits. This has led to the development of genetically modified, so-called Flavr Savr™ tomatoes, in which PG production levels are reduced by the introduction of antisense genes (Schuch 1994).

Differences between the enzyme mixtures in Table 1 are partly due to the use of different *Aspergillus* species for their preparation. Individual strains can be further improved by changing their genetic constitution, either by classical mutagenesis (random) or by genetic recombination (directed). For the latter, straightforward approach, enzymes with a desired activity are being identified in this strain (homologous) or in a strain belonging to another fungal or non-fungal species (heterologous), after which the corresponding genes are cloned and manipulated. Heterologous gene expression mostly occurs from an endogenous, well-known promoter (Upshall et al. 1992).

Analysis of pectin degradation products generated by the activity of individual enzymes progressively contributes to the structural elucidation of pectins (McCleary and Matheson 1986). Thus, purification and subsequent analysis of the pectinolytic enzymes PG, pectin lyase (PL) and pectin methylesterase (PME) from *A. niger* has contributed to our present knowledge about pectin structures, not only from apples (De Vries 1983), but also from grape berries (Saulnier and Thibault 1987) and carrots (Massiot and Thibault 1989). This knowledge in turn allows further adjustment of specific enzyme mixtures, which are required for appropriate processing of different fruits and vegetables.

Unlike PG, PL and PME, rhamnogalacturonan hydrolase activity is not common to all conventional enzyme preparations, although levels are progressively elevated by optimalization of growth conditions, as mentioned above (Table 1). For instance, the use of two foregoing enzyme preparations from *A. niger*, Biopectinase OS and LQ (Quest Int., The Netherlands), for apple processing



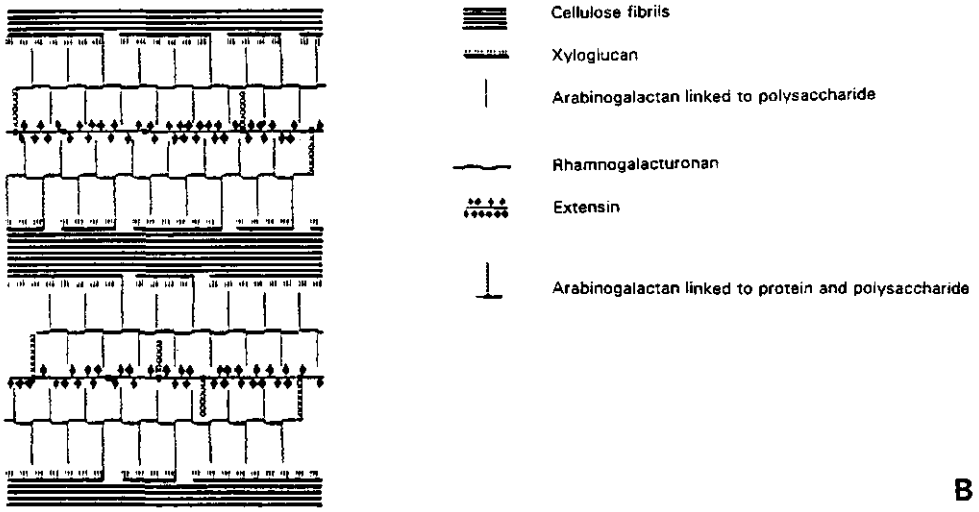


Fig. 1 **A** Schematic representation of the plant cell wall. **B** Structural model of the *Acer pseudoplatanus* primary cell wall, according to Keegstra et al. (1973). In this model xyloglucans form a monolayer, which is linked by hydrogen bonds to the cellulose microfibrils. Interaction between neighbouring microfibrils may occur by covalent attachment to the various other components: xyloglucans are attached to arabinogalactans, arabinogalactans to pectins, and pectins to the hydroxyproline-rich glycoprotein extensin

coincided with the attainment of residual pectin material in the final juice obtained, due to the absence of rhamnogalacturonan hydrolase activity in these enzyme preparations (Musters et al. 1993). At that time promising results were obtained with Pectinex Ultra SP, also called Ultra SP (Novo Nordisk, Denmark), originating from *A. aculeatus* and the effects were ascribed to rhamnogalacturonan hydrolase activity (Schols et al. 1990). Therefore, this type of enzymes has been further studied as described in this thesis. For a better understanding the attention will firstly be drawn to the structure and degradation of pectins in general.

Pectins

Localization and function of pectins in plant cell walls

All plant cells are surrounded by a cell wall, which provides the plant with a structural yet flexible framework. The primary cell wall, which is found in growing and dividing cells, can change into a secondary cell wall during differentiation (Dey and Brinson 1984). Between the cell walls of different cells an interstitial layer, the middle lamella, is found (Fig. 1A). The primary cell wall is composed of different polymers, 90% of which are polysaccharides and 10% are proteins (Dey and Brinson 1984). Secondary cell walls further contain the polyphenolic compound lignin, whereas cutin, which is composed of hydroxy fatty acids, constitutes the waxy external layer of plants (Cook and Rayner 1984).

Plant cell wall polysaccharides are divided into celluloses, hemicelluloses and pectins (Dey and Brinson 1984). Celluloses form microfibrillar structures, which are composed of polymerized glucan chains and constitute between 25% of primary cell walls up to 60% of woody, secondary cell walls (Dey and Brinson 1984; Fig. 1A).

Hemicelluloses form a group of heteropolysaccharides, including xylans, glucans and mannans, which are present in variable amounts in different plant families. For example, xyloglucans are the main hemicellulosic constituents of plants belonging to the Dicotyledonae and glucuronoarabinoxylans of those belonging

to the Monocotyledonae (Carpita and Gibeaut 1993). They are closely linked to cellulose microfibrils by hydrogen bonds, as stated by Dey and Brinson (1984). Other hemicelluloses, as glucomannans and galactomannans, primarily serve as storage polysaccharides in seeds.

The third major cell wall polysaccharide, pectin, is a complex heteropolysaccharide consisting of thirteen different monosaccharides. Two of these monosaccharides, galacturonic acid and rhamnose, are mainly found in the backbone of pectin, thus determined as homogalacturonan or rhamnogalacturonan, whereas the others are only found in the side chains. Galacturonic acid residues are partially methylated at C-6 and/or acetylated at C-2 or C-3.

Pectins constitute up to 35% of the primary cell wall and are the main component of the middle lamella (Fig. 1A). They also take part in the pectocellulose layer, which connects the external cuticle of plants with the epidermis (Cook and Rayner 1984). Pectins for industrial use are mostly derived from fruits and especially from the rinds of lemon and orange.

Comparison of pectins which were isolated from different fruits revealed variation in composition (Kravtchenko et al. 1992). Structural variation was also observed upon comparison of pectins from different locations within one plant. Pectins from the middle lamella and the cuticle are usually less branched and less methylated, thus leaving a higher amount of galacturonic acid residues which can interact via Ca^{2+} ions, than pectins from primary cell walls (Hwang et al. 1993; Alvarez et al. 1995). Pectin structures however are not static and changes in the degree of branching and the degree of methylation have been observed during fruit ripening (Fischer and Bennet 1991).

Pectins form a gel matrix in between the other cell wall constituents and thus provide a solid but flexible framework. The construction of a cell wall is illustrated by several models, which are principally based on an earlier model of Keegstra et al. (1973) (Fig. 1B). Besides a structural function, pectins also influence the wall porosity and by this they may control the access of for instance hydrolytic enzymes. Furthermore, pectins can modulate pH and ion balances because of their acidic character. Oligomers derived after pectin degradation can

serve as regulatory molecules both in processes of differentiation (Marfà et al. 1991) and in defense mechanisms against phytopathogens (Ryan 1988).

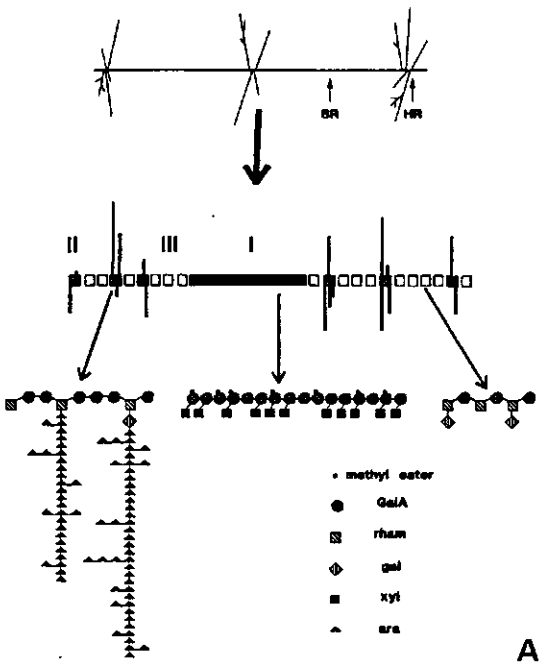
Chemical interactions of pectins

In the plant cell wall, pectin polymers are closely linked with other cell wall constituents, which explains their function as gel matrix. Side chains radially extend from the rhamnose residues within the rhamnogalacturonan part of pectin, thus forming the branched, 'hairy' regions (HR; Fig. 1B). Covalent linkages with proteins like extensin occur via these side chains, whereas hemicelluloses can also be directly linked to the backbone of HR, as was demonstrated for the major hemicellulose of apple cell walls, fucogalactoxyloglucan (Renard et al. 1991).

Although HRs and homogalacturonans have been independently isolated and characterized, they are presumably interconnected. An indication for this is the release of two types of rhamnogalacturonan structures from cell walls after degradation with polygalacturonase (An et al. 1994; Puvanesarajah et al. 1991) and likewise the release of homogalacturonan structures from cell walls after degradation with rhamnogalacturonan hydrolase (Renard et al. 1993).

Pectin molecules also interact with each other. Intermolecular ionic linkages can be formed indirectly via Ca^{2+} cations, forming bridges between different galacturonic acid residues. The 'egg-box'-like junction zone thus formed is the basis for gel-formation and is exploited by the preparation of jams and jellies from various fruits (Rolin and De Vries 1990). Stable junction zones require 15-20 contiguous galacturonic acid residues, interruptions taking place by residues which are methylated and by intercalating rhamnose residues. However, both the hydrophobic forces between methylated galacturonic acid residues and the presence of side chains linked to rhamnose residues contribute to the gelling properties of pectins (Burchard 1993; Oosterveld et al. 1995).

In addition, covalent linkages between different pectin molecules can be formed by dimerization of ferulic acid residues, which are esterified to arabinose and galactose residues in sugar beet pectin (Micard et al. 1994). However, no ferulic acid was found in apple pectin (Renard et al. 1990).



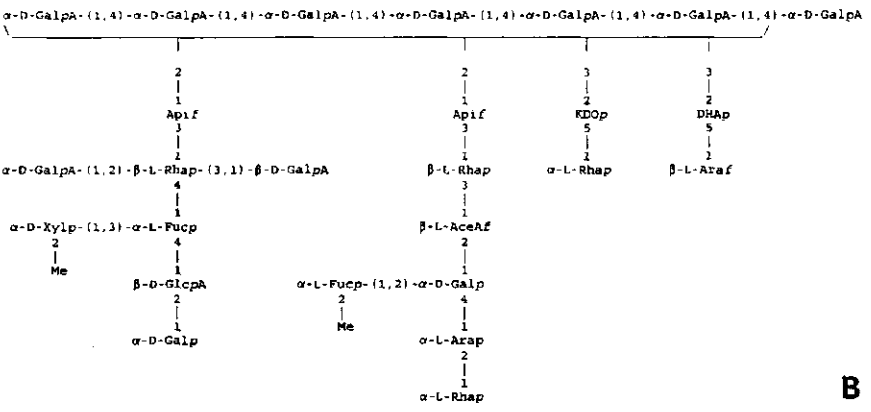


Fig. 2 A Hypothetical structure of apple pectin and of the prevailing modified hairy region population thereof, according to Schols (1995). SR, smooth regions; HR, hairy regions. Subunit I, xylogalacturonan; Subunit II, arabinan-rich stubs; Subunit III, oligomers. **B** Oligoglycosylfragments of rhamnogalacturonan II, according to Puvanesarajah et al. (1991). The particular backbone galacturonic acid residue to which each oligoglycosyl side chain is attached has not been determined

Structure of pectins

Pectins are acidic heteropolysaccharides, which include homogalacturonan, rhamnogalacturonan and the neutral polymers arabinan, galactan and arabinogalactan (Fig. 1B). Homogalacturonans have a backbone consisting of (1,4)-linked α-D-galacturonic acid residues, occasionally interrupted by single (1,2)-linked α-L-rhamnose residues. These interruptions cause an alteration in the three-dimensional structure by forming kinks. Depending on the degree of methylation of the galacturonic acid residues pectate, low methoxyl- and high methoxylpectins, are distinguished. The degree of acetylation in homogalacturonans is only limited, however branching with apiose and apobiose has been demonstrated in apiogalacturonans from *Lemna minor* (Hart and Kindel 1970) and with xylose in xylogalacturonans from soy bean (Kikuchi and Sugimoto 1976).

Rhamnogalacturonans are characterized by a backbone of alternating (1,4)-linked galacturonic acid and (1,2)-linked rhamnose residues. Here, about half of the rhamnose residues functions as an attachment point for complex arabinose- and galactose-containing side chains. These side chains might be synthesized independently from the backbone and linked afterwards in the cell wall (Hwang et al. 1993). Rhamnogalacturonan structures isolated from suspension-cultured sycamore cells (*Acer pseudoplatanus*) are named rhamnogalacturonan I (RG-I; McNeil et al. 1980). Rhamnogalacturonans isolated from apples and other fruits and vegetables are referred to as HR, because of their highly branched structure in contrast to the 'smooth' homogalacturonan regions (Schols 1995). HR isolated by the liquefaction method are called modified HR (MHR), because their structure may be altered by the enzymic treatments (Schols et al. 1990). In contrast to RG-I, where nearly equal amounts of rhamnose and galacturonic acid are present in the backbone, the rhamnose:galacturonic acid ratio in MHR varies from 0.41 in apple to 0.63 in leek. Detailed structural analysis of apple MHR revealed that about 20% of the galacturonic acid residues is substituted with *O*-acetyl-groups or xylosyl-groups, the latter of which are clustered within the xylogalacturonan subunit of MHR (Fig. 2A). A relatively low degree of methylation and traces of fucose, glucose and mannose are also found, however the

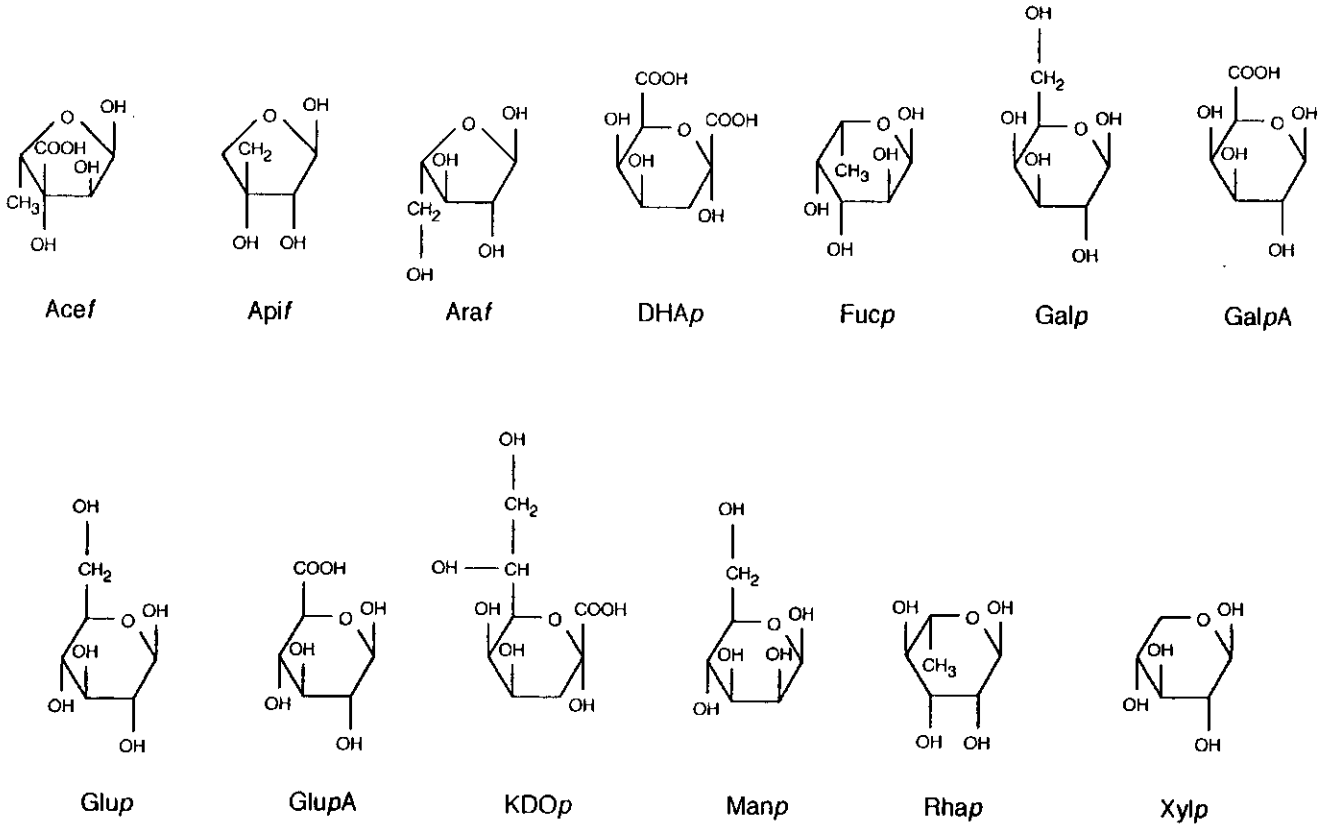


Fig. 3 Chemical structure of the monosaccharide constituents of pectin

site of linkage of these residues is not clear. In RG-I terminally linked fucose and xylose residues have been found associated with arabinose and galactose residues in the side chains. Additionally, glucuronic acid and 4-*O*-methyl-glucuronic acid residues are found, which are absent in MHR (An et al. 1994). Both sugars also take part of rhamnogalacturonans found in gum exudates, e.g. Karaya gum, and mucilages (Stephen 1983).

Another pectin structure isolated from sycamore cell walls by Darvill et al. (1978) is rhamnogalacturonan II (RG-II), which is in fact a short-chain substituted galacturonan (Fig. 2B). While a rhamnose:galacturonic acid ratio of 0.44 is found in RG-II, no rhamnose is present in the backbone, which is only composed of about seven galacturonic acid residues. Besides, some galacturonic acid residues are found in the side chains, together with arabinose, fucose, galactose, glucuronic acid and xylose residues. Some of the xylose and fucose residues are methylated to 2-*O*-methyl-xylose and 2-*O*-methyl-fucose, respectively (Darvill et al. 1978; Spellman et al. 1983). Other side chain constituents of RG-II are the more rare sugars 3-*C*-carboxy-5-deoxy-L-xylose (aceric acid), 3-*C*-hydroxymethyl-D-glycero-tetrose (apiose), 3-deoxy-D-lyxo-2-heptulosaric acid (DHA) and 2-keto-3-deoxy-D-manno-octulosonic acid (KDO) (see also Fig. 3).

In contrast to MHR and RG-I, where the rhamnose residues are either 1,2- or 1,2,4- linked, rhamnose residues in RG-II are highly substituted, being terminal, 1,3-, 1,3,4- or 1,2,3,4-linked. While the terminal linked rhamnose residues are in the α-configuration, as they are in RG-I and MHR, the other linkages are in the β-configuration. Substitutions take place with galacturonic acid at C-2, with galacturonic acid or aceric acid at C-3 and with fucose at C-4 of rhamnose. Rhamnose residues themselves are linked to C-2 of arabinose, C-3 of apiose or C-5 of KDO (Fig. 2B). Whereas the structure of the RG-II side chains is clear and the same oligomeric structures have been identified in the cell walls of various other plants, their site of linkage to the backbone has not yet been established. The presence of eleven different monosaccharides within one molecule, which is composed of about 60 residues in total, makes it structurally the most complex plant polysaccharide yet found.

Table 2 Main size and relative amount (dry mass) of pectin and its constituents homogalacturonan and rhamnogalacturonan in the primary cell walls of apple, sycamore cells and *Arabidopsis thaliana* leaves. MHR, modified hairy regions; RG-I and RG-II, rhamnogalacturonan I and II

Polymer	Molecular mass (kDa)	Plant source	% (w/w) pectin cell wall[1]	Reference
Pectin	80-200	Apple	35%	De Vries et al. 1983
		A. thaliana	42%	Zablackis et al. 1995
Homogalacturonan	21	Apple		Thibault et al. 1993
		A. thaliana	23%	Zablackis et al. 1995
Rhamnogalacturonan				
MHR		Apple	1.68%[1]	Schols 1995
RG-I	100-1000	Sycamore	7-14%	O'Neill et al. 1990
		A. thaliana	11%	Zablackis et al. 1995
RG-II	5	Sycamore	3%	O'Neill et al. 1990
		A. thaliana	8%	Zablackis et al. 1995

[1] Relative to the total apple mass, about 2% of which is composed of cell wall polysaccharides

The high resistance of RG-II structures to enzymic degradation allows their purification from plant material after fungal degradation. Thus, RG-II structures have been purified from apple juice (Pellerin et al. 1995) and even from the commercial enzyme preparation Pectinol AC, derived from *A. niger* after growth on pectin (York et al. 1985).

Comparison of the relative amount of the individual pectin components after isolation from the primary cell walls of plants, reveals that homogalacturonan is the highest and RG-II the least represented (Table 2). Values however vary with the plant source and the method of extraction used. Although pectins are highly heterogeneous in size, differences in molecular mass between the different constituents are an indication of their complexity. Apple MHR was chosen as a model substrate for the study described in this thesis.

Pectin metabolism

The biosynthesis of plant polysaccharides, among which pectins, is realized by the activity of several enzymes, however the exact mechanisms are not known (Fry 1988). Endogenous pectin degradation is performed by the activity of pectinolytic enzymes, some of which are produced constitutively whereas others are produced only during processes of growth, ripening and defence against infections (Wu et al. 1993; Fry 1995).

Plants form at least a part of the food supply of many organisms. Its constituent polysaccharides however are only partly degraded because of the limited enzyme production in most digestive systems. Endogenous enzymes of vertebrates and most invertebrates can only attack α-D-(1,4) glucosidic linkages, making starch the principal supplier of energy and α-amylase the major polysaccharide-degrading enzyme. In man α-amylase is secreted by the salivary gland and the pancreas, whereas the disaccharide degrading enzyme maltase is produced intracellularly by intestine epithelial cells, together with invertase and lactase. The degradation of the remaining polysaccharides is basically realized by the activity of enzymes produced by the microbial population of the intestine. Several plant polysaccharides can function as dietary fibres, which is manifested by a range of medicinal effects. Effects ascribed to pectins include the reduction of plasma-cholesterol levels and the activation of macrophages (cf. Pilnik and Rombouts 1985). Interaction with macrophage receptors has been demonstrated for the RG-II constituent KDO, which is also part of bacterial cell walls (Morrison et al. 1992). Possibly, this also explains the bioactivity of RG-II-like structures purified from Chinese and Japanese medicinal herbs (Hirano et al. 1994).

Exceptions among the vertebrates are formed by ruminants, because they predominantly digest non-starch polysaccharides like cellulose and pectin, albeit again with the help of bacteria and certain anaerobic fungi (Trinci et al. 1994).

Besides bacteria and fungi, the capacity to degrade pectin is restricted to some classes of protozoa, nematodes and insects.

Special about fungi is their exploitation of nearly all organic carbon sources, by the production of a large scala of enzymes. Among fungal species there is wide

variation in the ability to utilize carbon compounds of different type and location in dead and living plants. As mentioned, some cellulolytic fungi reside in the digestive apparatus of ruminants where they take care of the breakdown of celluloses in collaboration with bacteria. Lignolytic fungi have the enzymatic capacity for the complete breakdown of lignin, enabling wood to rot. On the other hand, a wide range of saprotrophic fungi, among which many species belonging to the genus *Aspergillus*, grow in soil where they produce a variety of pectinolytic enzymes in the presence of plant material. Many of these enzymes are also produced by plant pathogens, among which species belonging to the fungal genera *Botrytis*, *Fusarium* and *Sclerotinia* and the well studied bacterial genus *Erwinia*. Like other polymer-degrading enzymes pectinolytic enzymes are secreted by these organisms and act extracellularly. After pectin degradation small end-products can be absorbed through the microbial plasma membrane.

Pectinolytic enzymes and their encoding genes

Homogalacturonan degradation

The heterogeneity of pectin is reflected by the large group of pectinolytic enzymes, which are involved in its degradation. These enzymes can be classified into two major groups, viz. the methyl- and acetyl-esterases, which are responsible for modification of the polymer, and the depolymerases, which include endo- and exo-acting hydrolases and lyases. Well-studied are the homogalacturonan degrading enzymes PG, PL, PME and pectate lyase (PEL). These enzymes have been characterized in fungi, bacteria and plants, however up to now most PLs have been isolated from fungi and most PELs from bacteria. Recently, a pectin acetylesterase (PAE) active towards homogalacturonan of sugarbeet was isolated from the fungus *A. niger* (Searle van-Leeuwen et al. 1995).

For extensive cross-species analysis of pectinolytic enzymes in fungi, bacteria and plants specific activity staining methods have been developed. Besides, genomic and cDNA libraries are hybridized with genes encoding known enzymes

from other organisms as probes. In plants ripening-related enzymes can be explored via differential hybridisation of cDNA libraries prepared with mRNAs from ripe fruits. The functionality of new genes in ripening and in phytopathogenic processes can be easily controlled by elimination of the respective gene functions. This is achieved by co-expression of antisense mRNA in plants (Hall et al. 1993) and by insertion mutagenesis in *Erwinia* (Casadaban and Cohen 1979). Induced effects are analysed in the transgenic plants and the *Erwinia* mutants thus obtained. By these methods multiple isofunctional enzymes have been elucidated, which are encoded by gene families in each species. The genes presently characterized in isolates belonging to the fungal species *A. niger* and *A. tubingensis*, the bacterial species *Erwinia chrysanthemi* and *E. carotovora* and the plant species *Lycopersicon esculentum* (tomato), are summarized in Table 3. However, future elucidation of additional isoenzymes will certainly expand each of the pectinolytic gene families shown.

Up to now, the gene families encoding PEL in *E. carotovora* and PME in *E. chrysanthemi* and tomato comprise two to four different genes. Larger gene families characterized are those encoding PG and PL in *A. niger* and PEL in *E. chrysanthemi*. Each of these families is composed of up to seven genes and in some cases the multiplicity of isoenzymes is reflected at the genetic level by clustering of their encoding genes. Examples are the pectate lyase encoding genes *pel*A-*pel*E-*pel*D and *pel*B-*pel*C, which are clustered in the genome of *E. chrysanthemi* (Condemine and Robert-Baudouy 1991) and the pectin methylesterase encoding genes B8 and B16, which are associated with a third pectin methylesterase encoding gene in the genome of tomato (Hall et al 1994). The amino acid sequences deduced from two genes of the *pel*ADE cluster of *E. chrysanthemi* strain EC16, *pel*A and *pel*E, share 62% identity, whereas those from the *pel*BC cluster share 84% identity (Tamaki et al. 1988). The amino acid sequence deduced from the *pel*D gene, which has been characterized in strain B374, shares 60% and 81% identity, respectively with the *pel*A- and *pel*E-derived amino acid sequences in this strain (Van Gijsegem 1989). The amino acid sequences deduced from the tomato B8 and B16 genes even share 93%

Table 3 Genes encoding homogalacturonan degrading enzymes in the fungal species *A. niger* and *A. tubingensis*, the bacterial species *E. chrysanthemi* and *E. carotovora* and the plant species *Lycopersicon esculentum*

Species	Type	Enzyme	Gene	Reference
A. niger	CBS120.49	PG	*pga*I,II,C	Bussink 1992
	CBS120.49		*pga*E	Parenicova et al. 1995
	CBS120.49		*pga*A,B,D	Parenicova et al., unpubl
	RH5344		*pga*II[1]	Ruttkowski et al. 1990
A. tubingensis	NW756		*pga*II	Bussink 1992
		exoPG	*pga*X	Kester et al. 1996
A. niger	CBS120.49	PL	*pel*A,B,C,D	Kusters-van Someren 1991
	CBS120.49		*pel*E,F	Kusters-van Someren et al., unpubl
A. niger	RH5344	PME	*pme*	Khanh et al. 1991
	CBS120.49		*pme*	Visser et al. 1996
E. carotovora				
subsp *carotovora*	SCRI193	PEH	*peh*	Hinton et al. 1990
	SCC3193		*peh*A	Saarilahti et al. 1990
	71		*peh*-1	Liu et al. 1994
subsp *atroseptica*	EC		*peh*	Lei et al. 1992
E. chrysanthemi	EC16	exoPEH	*peh*X	He and Collmer 1990
E. chrysanthemi	EC16	PEL	*pel*B,E	Keen and Tamaki 1986
	EC16		*pel*A,C	Tamaki et al. 1988
	B374		*pel*A,D,E	Van Gijsegem 1989
	3937		*pel*A	Favey et al. 1992
	3937		*pel*B	
	3937		*pel*C	
	3937		*pel*D	
	3937		*pel*E	Reverchon et al. 1989
	3937		*pel*L	Lojkowska et al. 1995
	3937		*pel*Z	Pissavin et al. 1995
E. carotovora				
subsp *carotovora*	71		*pel*-1,2,X	Liu and Chatterjee, unpubl
			pel-3	Liu et al. 1994
	SCRI193		*pel*B,C	Hinton et al. 1989
	EC153		PL153	Trollinger et al. 1989
	ER		*pel*I	Ito et al. 1988
	ER		*pel*III	Yoshida et al. 1991
subsp *atroseptica*	EC		*pel*A	Lei et al. 1988
			*pel*B	Lei et al. 1987
E. chrysanthemi	3937	OGL	*ogl*	Reverchon et al. 1989
E. carotovora				
subsp *carotovora*	71	PL	*pnl*A	McEvoy et al. 1990
				Chatterjee et al. 1991
E. chrysanthemi	B374	PME	*pem*	Plastow 1988
	3937		*pem*	Laurent et al. 1993
			*pem*B	Shevchik et al. 1995
L. esculentum	Ailsa Craig	PG	pTOM6[1]	Grierson et al. 1986
	Ailsa Craig	PME	B16[1]	Ray et al. 1988
	Ailsa Craig		B8,16[1]	Hall et al. 1994
	Rutgers		PET1[1]	Harriman et al. 1991

[1] cDNA

identity (Hall et al. 1994). Because of the high degrees of similarity found within the different gene clusters, gene duplications are thought to underly the cluster phenomenon. Overall, only about 12% of the amino acids composing the *Erwinia pel*A,B,C,D and E derived sequences are conserved, pointing to independence of the two gene families (Hinton et al. 1989). Two other *pel* genes identified in *E. chrysanthemi*, *pel*L and *pel*Z, share neither significant homology with each other nor with the *pel*ADE and *pel*BC gene families. The close linkage of the *pel*Z gene with the *pel*BC gene cluster points to casual clustering, as also the close linkage of the *pem* gene with the *pel*ADE gene cluster (Hugouvieux-Cotte-Pattat et al. 1989).

In each of two *E. carotovora carotovora* strains, SCRI193 and EC153, an intracellular PEL encoding gene, *pel*B and PL153, respectively, has been identified. The amino acid sequences derived from these two genes share 98% identity (Hinton et al. 1989).

Three additional strains belonging to this subspecies, strains SCC3193, 71 and ER, the *E. carotovora atroseptica* strain EC and three *E. chrysanthemi* strains, EC16, B374 and 3937, are used for the characterization of pectinolytic enzymes. Likewise, two different species of tomato, designated Ailsa Craig and Rutgers, are used for the characterization of PME encoding genes, as well as two different *A. niger* strains, CBS120.49 and RH5344, for the characterization of *pme* and *pga*II. The concomitant aberrancies in gene structures found has led to confusion (Markovič and Jörnvall 1990). This emphasizes the need for proper classification methods, especially for strains which are used for industrial applications (see below).

Rhamnogalacturonan degradation

Liquefaction of apples with commercially used pectinolytic enzyme preparations for a long time resulted in pectic polymeric structures which remained after ultrafiltration of the final juice. Analysis of this residual material revealed that it is mainly composed of the HR of pectins, which are renamed to MHR because of possible enzymic modifications.

Structural analysis of MHR was initially performed by the use of enzymes with known mode of action, such as galactanase, β-galactosidase, arabinanase and arabinofuranosidase. However, MHR from both apple and sugar beet were largely resistant to these enzymes (De Vries 1983; Guillon and Thibault 1989). Schols et al. (1990) were the first to identify an enzyme activity which is capable of degrading the backbone of MHR, by using the *A. aculeatus* enzyme preparation Ultra SP from Novo Nordisk. The enzyme responsible for this activity, rhamnogalacturonan hydrolase A, was purified and characterized (Table 4). Structural analysis of MHR degradation products both revealed the rhamnogalacturonan hydrolase A specificity and simultaneously led to an adapted model structure of apple MHR (see Fig. 2A). Up to now five additional rhamnogalacturonan degrading enzymes have been purified from Ultra SP (see Table 4). For additional enzyme activities, which will contribute to complete MHR degradation, other enzyme preparations are considered.

The role of rhamnogalacturonan hydrolase A and other rhamnogalacturonan degrading enzymes in the structural elucidation of MHR

Rhamnogalacturonan hydrolase A hardly degrades native MHR, but enhanced activities can be obtained after saponification of the ester-linkages in MHR (Schols et al. 1990) or by combined incubation with rhamnogalacturonan acetylesterase. The latter enzyme has been purified both from Ultra SP (Table 4; Searle-van Leeuwen et al. 1992) and from *A. niger* after growth on pectin (Beldman et al. 1995).

The oligomeric MHR degradation products were analysed by high performance anion exchange chromatography, NMR spectroscopy and methylation analysis (Schols 1995). These analyses revealed eight different structures, consisting of four to nine sugar residues (Fig. 4). In all structures two or three rhamnose residues alternated with galacturonic acid residues, with rhamnose at the non-reducing end. From these results the specificity of rhamnogalacturonan hydrolase A for α-D-galacturonic acid-(1,2)-α-L-rhamnose linkages in MHR was

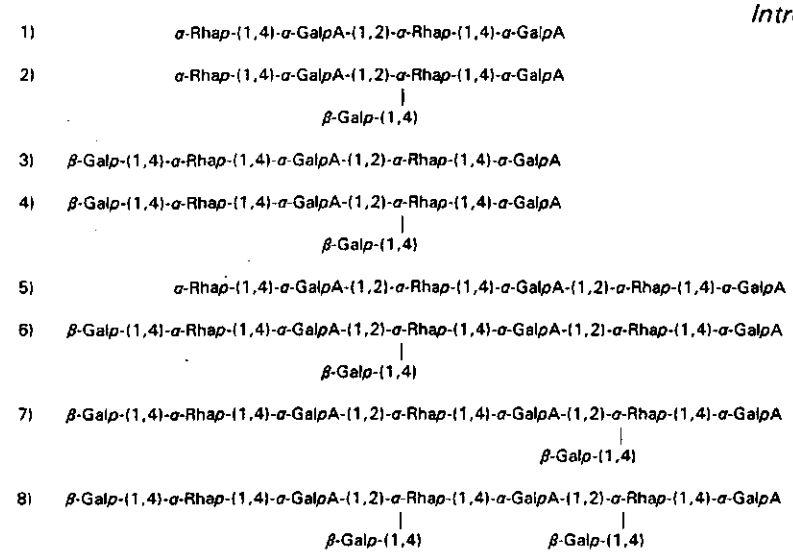


Fig. 4 Structure of the oligomers, obtained after degradation of saponified modified hairy regions from apple by rhamnogalacturonan hydrolase A, according to Schols (1995)

deduced. Resistance of the oligomers to further degradation by rhamnogalacturonan hydrolase A is partly due to interference by single unit galactose side chains, which are linked to half of the rhamnose residues. These side chains can be removed with β-galactosidase from *A. niger*, which has shown activity on both saponified MHR and its oligomeric degradation products (Table 4; Mutter et al. 1993). After this, the oligomers can be completely degraded by incubation with a partially purified enzyme fraction from Ultra SP, containing the enzymes rhamnogalacturonan rhamnohydrolase and rhamnogalacturonan galacturonohydrolase (Table 4; Mutter et al. 1993). Both enzymes have been purified and the presence of an additional rhamnogalacturonan galacturonohydrolase, acting from the reducing oligomeric end, is suggested by Schols et al. (1994).

High performance size exclusion chromatography analysis of the MHR digest revealed two additional populations of degradation products of higher molecular mass than the oligomeric products. These populations contained arabinan-rich stubs and xylogalacturonan fragments, respectively. Both structures are inclu-

Table 4 Properties of MHR degrading enzymes purified from Ultra SP[1]

Enzyme	Mass (kDa)	pH_{opt}	pI	T_{opt}	Bond cleaved	Mode of action	Reference
Rhamnogalacturonan hydrolase A	51	3-4		40-50	α-D-Gal*p*A-(1,2)-α-L-Rha*p*	endo	Schols et al. 1990
Rhamnogalacturonan acetylesterase	40	5.5		40			Searle-v.Leeuwen 1992
Rhamnogalacturonan rhamnohydrolase	84	4	4.9-5.4	60	α-L-Rha*p*-(1,4)-α-D-Gal*p*A	exo	Mutter et al. 1994
Rhamnogalacturonan galacturonohydrolase	67		4.8-5.0		α-D-Gal*p*A-(1,2)-α-L-Rha*p*	exo	Mutter et al. 1995
Exogalacturonase					α-D-Gal*p*A-(1,4)-α-D-Gal*p*A	exo	Beldman et al. 1996
Arabinanase	45	5.5			α-L-Ara*f*-(1,5)-α-L-Ara*f*	endo	Beldman et al. 1993
Arabinofuranosidase B1	37	3.0-3.5			α-L-Ara*f*(1,2/3)-α-L-Ara*f*	exo	Beldman et al. 1993
Arabinofuranosidase B2	37	4.0-4.5			α-L-Ara*f*-(1,2/3)-α-L-Ara*f*	exo	Beldman et al. 1993
Galactanase	42	4.0-4.25		50-55	β-D-Gal*p*-(1,4)-β-D-Gal*p*	endo	Van de Vis et al. 1991
Galactanase[2]	38	3.5-4.0	2.8	50-55	β-D-Gal*p*-(1,4)-β-D-Gal*p*		Lahaye et al. 1991

[1] Originates from *A. aculeatus* strain CBS101.43 or KSM510; [2] Originates from *A. niger* var. *aculeatus*

ded in the revised model structure of apple MHR, as depicted in Fig. 2A (Schols 1995).

Resistance of xylogalacturonans to rhamnogalacturonan hydrolase A degradation is understood, since the backbone is only scarcely interrupted by rhamnose residues. An exogalacturonase, which also has been purified from Ultra SP, is capable of degrading these structures to galacturonic acid and β-xylose-(1,3)-galacturonic acid dimers (Table 4; Beldman et al. 1996). This enzyme also showed activity on other rhamnogalacturonan structures, such as the soluble polysaccharides from soy bean (designated as SPS; Adler-Nissen et al. 1984) and gum tragacanth (Stephen 1983). Possibly, the dimeric products of MHR can be further degraded by β-xylosidase, which was successfully used for the degradation of soy bean polysaccharides.

Possible explanations for the resistance of the arabinan-rich stubs to rhamnogalacturonan hydrolase A degradation are their high level of branching and/or an irregular sequence of rhamnose and galacturonic acid residues in their backbone. Removal of the MHR side chains can be achieved with the enzymes arabinanase and α-L-arabinofuranosidase, both of which have been purified from Ultra SP and from *A. niger* (Beldman et al. 1993; Lerouge et al. 1993). Possibly also the galactanases purified from Ultra SP (Van de Vis et al. 1991) and from the enzyme preparation SP249, originating from *A. niger* var. *aculeatus* (Lahaye et al. 1991), contribute to the degradation of MHR side chains. The arabinanase, arabinofuranosidase and galactanase enzymes purified from *A. niger*, in combination with an additionally purified exo-β-galactanase, have the capacity to completely degrade the side chains of RG-I (Lerouge et al. 1993).

Rhamnogalacturonan hydrolase nomenclature

The nomenclature for rhamnogalacturonan hydrolases is not unambiguous. Before there was any knowledge about rhamnogalacturonan lyases and the degradation of linkages in the rhamnogalacturonan backbone by β-elimination instead of hydrolysis, the firstly identified rhamnogalacturonan hydrolase was

named rhamnogalacturonase A (RGaseA) by Schols et al. (1991). Subsequently, the newly characterized rhamnogalacturonan lyase was named RGaseB by Kofod et al. (1994), thus precluding consecutive naming of isoenzymes of both types in future. Likewise, the genes encoding RGaseA and RGaseB were named *rhg*A and *rhg*B, respectively. Afterwards, the enzymes RGaseA and RGaseB were renamed to RGHase and RGLase respectively, without changing the names of the respective genes (Azadi et al. 1996).

To avoid further confusion concerning rhamnogalacturonan hydrolase nomenclature, we have maintained the name *rhg* for the rhamnogalacturonan hydrolase encoding genes and for clarity we also used RHG for the corresponding enzymes.

Accessory enzymes

After liquefaction of apples with the enzyme preparation Ultra SP, still some material remained undegraded (Schols et al. 1991). Complete degradation of HR thus requires the activity of additional enzymes.

Minor amounts of fucose, glucose and mannose have been detected in HR, however their type of linkage is not clear (Schols 1995). Besides β-glucosidases no α-glucosidases have been identified in plants yet, making it very likely that also glucose residues taking part of HR are β-linked (Fry 1995). Most β-glucosidases, among which those from *A. aculeatus* are explored for the degradation of celluloses and glucosides (Sakamoto et al. 1985), however a putative accessory role in the degradation of HR cannot be excluded.

Bovine α-L-fucosidase has been successfully used for the degradation of RG-I side chains (Lerouge et al. 1993), but the same results can possibly be obtained with α-L-fucosidase from *Aspergillus* (Oxenbøll 1994). Additionally, the enzymes α-D-mannosidase (Inoue et al. 1995), β-glucuronidase (Oxenbøll 1994) and apiofuranosidase (Dupin et al. 1992), which have been characterized in different *Aspergillus* species, might act on HR, RG-I and RG-II, respectively. Further, the presence of methoxylgroups in HRs, though less than in homogalacturonans, possibly requires a specific pectin methylesterase, acting on HR.

Expression of pectinolytic enzymes

A battery of pectinolytic (iso)enzymes is involved in the degradation of pectin. Synergistic activities have been demonstrated between PME, which demethylates pectin into pectate, and either PG or PEL, both of which act on the latter substrate. As mentioned, the *pem* gene of *E. chrysanthemi* was found clustered with some of the *pel* genes in the genome. In the anaerobic bacteria *Clostridium thermosaccharolyticum* synergism between PME and PG is facilitated by extracellular complex-forming between these enzymes (Van Rijssel et al. 1993).

Both phenomena would suggest co-expression, however in most bacterial and fungal species pectinolytic enzymes are differentially expressed. Whereas expression of the majority of these enzymes is induced in the presence of pectin substrates, some are expressed constitutively. Examples are PG and PME from *Erwinia* (McMillan et al. 1994) and exo-pectinase from *A. niger*, the latter of which is only expressed in conidia (Aguilar and Huitrón 1993). In plants the expression of some pectinolytic enzymes is correlated with ripening, as was demonstrated for PG and PME in tomato (Bird et al. 1984; Harriman et al. 1991).

The spectrum of pectinolytic (iso)enzymes produced by *Erwinia* further depends on the source of pectin which is used as a substrate. Whereas most of the PEL, PL, PG and PME (iso)enzymes are produced on polygalacturonic acid, other variants are only produced in the presence of pectin, presumably because of its higher degree of methylation (McMillan et al. 1994). Besides, variable spectra of pectinolytic enzymes have been observed in fungal strains, which were grown either by solid state fermentation or by submerged cultivation (Hours and Sakai 1994).

In several fungal species variation in the onset of pectinolytic enzyme production has been demonstrated. For instance, PG and PEL from *A. nidulans* are produced sequentially during growth on polygalacturonic acid (Dean and Timberlake 1989), whereas pectinolytic enzyme activity in *A. niger* can be demonstrated during two intervals of growth on pectin (Schmidt et al. 1995). One of these intervals is growth-associated, whereas the other only starts after the end of

catabolite repression. Likewise, the hemibiotroph *Colletotrichum* produces pectinolytic enzymes mainly late during pathogenesis, whereas the necrotroph *Botrytis* already starts at the beginning, illustrating their different life styles (Dean and Timberlake 1989).

In order to achieve high and controlled expression levels of pectinolytic enzymes for industrial purposes, the endogenous transcription signals of the individual fungal genes are usually replaced by another, well-characterized and strong promoter. In such cases the accompanying fungal strain is also used for the production of the enzyme. However, the production of an enzyme from its own promoter in the strain of origin has several advantages. For instance, besides rhamnogalacturonan hydrolase A at least five additional rhamnogalacturonan degrading enzymes are produced simultaneously in *A. aculeatus*, as demonstrated by the preparation of Ultra SP (Table 4). Moreover, yet unidentified accessory pectinolytic enzymes might be produced as well under these circumstances. This makes *A. aculeatus* a convenient strain for the natural production of a variety of pectinolytic enzymes at a rather high level.

Strains can be improved by the successive integration of multiple copies of desired pectinase genes, whereas specific additional features can be introduced by applying mutagenesis. Eventually, desired properties of different strains can be recombined by somatic crossing.

Knowledge about mechanisms regulating the expression of pectinase genes in future will allow further manipulation and thus optimalization of pectinolytic enzyme production in *A. aculeatus*, without using heterologous gene promoters.

Regulation of pectinase gene expression

In plants, the induction of gene expression occurs mainly via wide domain control by hormones. Besides, galacturonic acid oligomers formed after pectin degradation, can play an important role as signalling molecules, especially when the degree of polymerization is between 10 and 15 (Albersheim and Darvill 1985).. Besides, post-transcriptional regulation of pectinolytic enzymes has been shown by the identification of the β-subunit of tomato PG (Watson et al. 1994),

the fungal PG inhibiting protein from the bean *Phaseolus vulgaris* (PGIP; Bergmann et al. 1994) and the PME inhibiting protein from the kiwi *Actinidia chinensis* (PMEI; Balestrieri et al. 1990).

Most of the pectinase genes characterized in fungi and bacteria are regulated at the transcriptional level, induction and repression taking place by pectin and glucose, respectively. Pectins from variable sources, like apple, beet, citrus, orange and soybean have been shown effective inducers. However, also the major endproduct formed after pectin degradation, galacturonic acid, has been successfully employed. Cooper and Wood (1975) already demonstrated that galacturonic acid is an effective inducer for the production of pectinolytic enzymes in fungi and since galacturonic acid induction is applied in various fungal and bacterial species. Recent examples are the induction of *pga*X in *A. niger* (Kester et al. 1996) and the induction of *pel* genes in *Erwinia* (McMillan et al. 1994). However, no pectinolytic enzyme production could be demonstrated after growth of either *A. nidulans* or *C. thermosaccharolyticum* on galacturonic acid (Dean and Timberlake 1989; Van Rijssel et al. 1993). In *C. thermosaccharolyticum* this can be explained by the absence of an appropriate transport system for galacturonic acid. In contrast, di- and tri-mers of galacturonic acid have been shown effective inducers. Presumably, these oligomers are degraded to galacturonic acid by an intracellularly produced oligo-PG (Van Rijssel et al. 1993).

Whereas little is known about the role of galacturonic acid in the regulation of gene expression in *Aspergillus* species, this is a well studied subject in *E. chrysanthemi*. Some mutant strains which are deficient in galacturonic acid catabolism also appeared ineffective in pectin degradation (Condemine et al. 1986). It turned out that accumulation of 2-keto-3-deoxygluconate (KDG), one of the intermediates of the galacturonic acid catabolic pathway, results in elevated levels of pectinolytic enzyme expression. Apparently, KDG plays a crucial role in the regulation of pectinase gene expression, thus explaining the failure in pectin degradation after mutation of KDG generating enzymes. Instead of induction, KDG acts indirectly via inhibition of the pectinase gene repressor kdgR

(Hugouvieux-Cotte-Pattat et al. 1994). The presence of a common kdgR binding sequence in the promoters of several pectinase genes of *E. chrysanthemi* is in agreement with this.

Nothing is known about regulation mechanisms involved in rhamnogalacturonan degradation in *A. aculeatus* and the potential role of catabolic intermediates. The large number of monosaccharide constituents of rhamnogalacturonan, which are putative end-products after degradation (Fig. 3), greatly extends the group of potential inducer molecules. The presence of a coordinated system for the regulation of rhamnogalacturonan and homogalacturonan degrading enzymes cannot be excluded.

Nomenclature and classification of *A. aculeatus* strains

Nomenclature of A. aculeatus strains

A. aculeatus strains are important producers of pectinolytic enzymes, as demonstrated by the successful application of the enzyme Ultra SP and the progressive purification of pectinolytic enzymes from this preparation (Table 4). Another experimental enzyme preparation from Novo Nordisk, SP249, originates from a strain referred to as *A. aculeatus* (Thibault and Rouau 1990) and *A. niger* var. *aculeatus* (Lahaye et al. 1991). Presumably this preparation is identical to Ultra SP. This would explain the similarities between the endogalactanases, which have been purified from the respective enzyme preparations (Table 4).

Other polymer degrading enzymes characterized in *A. aculeatus* are those required for the degradation of cellulose, another multi-enzyme system. Three β-glucosidases, endoglucanases and exoglucanases each have been isolated from *A. aculeatus* strain F50 (Sakamoto et al. 1985; Murao et al. 1988) and at least one of the β-glucosidase encoding genes has been cloned (Minamiguchi et al. 1995). The production of multiple polymer-degrading enzyme systems by this species might explain the phytopathogenic character of some *A. aculeatus* strains in nature (Adisa 1989).

The enzymes β-glucosidase and endo-glucanase have also been isolated from the related species *A. japonicus* (Sanyal et al. 1988), but the degree of similarity with the *A. aculeatus* enzymes is not known. Homogalacturonan degrading enzymes, PG and PL, have been characterized in *A. japonicus* strain ATCC 20236 (Ishii 1976).

Clarity about the relationship between different *A. aculeatus* strains and also between the species *A. aculeatus* and *A. japonicus* will allow more efficient use of different strains for tailored industrial applications. However, the development of proper classification methods is a prerequisite for this purpose.

History of classification of black Aspergilli

The genus *Aspergillus* is a group of filamentous fungi, most of which can only be reproduced asexually (Fungi Imperfecti), whereas others can also reproduce sexually (Ascomycetes). The latter were recognized as separate species and therefore carry different names (as shown in Table 5).

The name *Aspergillus* (rough head) was raised by Micheli in 1729 because of the typical conidial heads of these fungi, composed of divergent chains of spores. Eventually in the middle of the 19th Century the role of *Aspergilli* in processes of decay and disease in plants and human and their generation of antibiotics and other biochemical products began to be recognized. This sparked the search for identification methods, especially for strains used in industrial fermentations. Various taxonomic efforts were made by different authors. Firstly, *Aspergilli* were classified according to colony colour, by which the black *Aspergilli* could easily be distinguished from eight other groups. However, the introduction of additional morphological criteria for classification has led to the proposal of many different classification schemes by different authors. Some of them even distinguished different genera within the presently recognized genus *Aspergillus*.

At present the genus *Aspergillus* is divided into seven subgenera, which include nineteen different groups or sections in total (Samson 1994). Seven of these

Table 5 Classification of the genus *Aspergillus* according to Samson (1992, 1994). Both anamorph and teleomorph stages, representing the classes Fungi Imperfecti and Ascomycetes, respectively, are included. The teleomorphs carry their own genus names, as they were not recognized as *Aspergillus* strains in the past

Subgenus	Section	Number of species		
		Anamorph[1]	Teleomorph[2]	
Aspergillus	Aspergillus		19	Eurotium
	Restricti	3		
Fumigati	Fumigati	5	11	Neosartorya
	Cervini	4		
Ornati	Ornati	3	2	Hemicarpenteles
			2	Sclerocleista
			1	Warcupiella
Clavati	Clavati	4		
Nidulantes	Nidulantes	7	27[3]	Emericella
	Versicolores	23		
	Usti	5		
	Terrei	1		
	Flavipedes	1	3[4]	Fennellia
Circumdati	Wentii	3		
	Flavi	11		
	Nigri	6		
	Circumdati	16	2	Petromyces
	Candidi	1		
	Cremei	3	3	Chaetosartorya
	Sparsi	5		
Stilbothamnium	Stilbothamnium	5		

[1] And [2] are both represented within the genus *Aspergillus*; [3] Two of the teleomorphs are without an anamorph; [4] One of the teleomorphs is without an anamorph

sections belong to the subgenus Circumdati and include the section Nigri, which is also referred to as the 'black *Aspergilli*' (Table 5). During history both the total amount of groups distinguished within the genus *Aspergillus* and the amount of species distinguished within each of these groups have been highly changed by different authors. Species which were recognized within the group of the black *Aspergilli* are summarized in Table 6.

Thom and Church (1926) were the first who revised the genus *Aspergillus* and on the basis of morphological characteristics distinguished 69 species divided over eleven groups. The group of the black *Aspergilli* contained thirteen of these species (Table 6). In contrast to this, Mosseray (1934) recognized 35 different black *Aspergillus* species, eleven of which are listed in Table 6. Thom and Raper (1945) distinguished fourteen groups of *Aspergillus* species, with sixteen of the 77 species belonging to the black *Aspergillus* group. Raper and Fennel (1965) on their turn distinguished eighteen groups and 132 species, twelve of which belonged to the black *Aspergillus* group. Al-Musallam (1980) reduced the amount of black *Aspergillus* species to seven, however included eight varieties and additionally two formae. A recently performed extensive study of Samson (1992, 1994) led to the distinction of about 200 species, divided over the nineteen groups of Table 5. According to this classification the black *Aspergilli* only contained six different species, *A. niger*, *A. carbonarius*, *A. japonicus*, *A. tubingensis*, *A. heteromorphus* and *A. ellipticus* (Table 6). From these, *A. niger* and *A. carbonarius* were already distinguished by Thom and Church and consecutively also by the other authors. Apparently, strains belonging to these species can be distinguished by various classification methods. The species *A. japonicus* and *A. tubingensis* were introduced later by Mosseray. However, in contrast to the species *A. tubingensis*, which was subsequently only recognized by Raper and Fennel and by Samson, the species *A. japonicus* was recognized afterwards by all of the other authors. The remaining species *A. heteromorphus* and *A. ellipticus* were introduced by Raper and Fennel but were both maintained in the classification schemes of Al-Musallam and Samson. *A. helicotrix* was not

Table 6 Number of different species distinguished within the black *Aspergilli* by various authors

Species	Thom & Church	Mosseray[1]	Thom & Raper	Raper & Fennel	Al-Musallam	Samson
A. cinnamomeus[2]	+	+	+			
A. schiemanni[2]	+		+			
A. nanus[3]	+		+			
A. luchuensis	+		+			
A. violaceo-fuscus	+		+			
A. luteo-niger	+	+				
A. niger	+	+	+	+	+	+
A. phoenicis	+	+	+	+		
A. pulverulentus	+		+	+		
A. atropurpureus	+		+			
A. pulchellus	+	+				
A. carbonarius	+	+	+	+	+	+
A. fumaricus	+		+			
A. japonicus		+	+	+	+	+
A. atroviolaceus		+				
A. tubingensis		+		+		+
A. awamori		+	+	+		
A. ficuum		+		+		
A. foetidus			+	+	+	
A. miyakoensis			+			
A. fonsecaeus[4]			+			
A. aculeatus				+		
A. heteromorphus				+	+	+
A. ellipticus				+	+	+
A. helicotrix					+	

[1] Only 11 of the 35 species distinghuished are indicated; [2] Mutants of *A. niger*; [3] Identical to *A. subfuscus*; [4] Identical to *Sterigmatocystis fusca*

distinguished as a separate species by Samson, because high similarities were found with *A. ellipticus* (Kusters-van Someren 1991).

A. aculeatus strains thus were only distinguished as a separate species by Raper and Fennel. Al-Musallam (1980) could easily distinguish between uniseriate and biseriate species, which contain a single row and a double row of phialides, respectively and thus demarcated the uniseriates *A. japonicus* and *A. aculeatus* from the other species. However, instead of distinguishing *A. aculeatus* as a separate species, she only classified these strains as a variety of the species *A. japonicus*, named *A. japonicus* var. *aculeatus*. Finally, Samson could not distinguish between *A. aculeatus* and *A. japonicus* strains at all and thus omitted both the species *A. aculeatus* and the subclassification within the species *A. japonicus*. Thus, the currently used mor-phological criteria are not suitable for further classification of uniseriate strains, forcing the development of supplementary methods.

The heterogeneity within this group of strains will further increase in future by the continuous isolation of new strains and occasionally by mutation and degeneration events occurring during maintenance. As *Aspergillus* strains, among which the uniseriates, are more and more applied for industrial purposes, this stresses the need for proper and detailed strain identification methods. Historically, fungi are mainly classified on the basis of phenotypic characters. However, developments in molecular techniques nowadays provide additional methods directed at the genotypic level, which are effective in the determination of microheterogeneities between strains. One of these methods, RFLP analysis, can be directed to genes encoding proteins with different kinds of functions, which are differentially conserved among species. The RFLP method described in this thesis is suitable for the classification of *A. aculeatus* and *A. japonicus* strains, but with a few modifications this method will be applicable for the classification of a range of other *Aspergillus* species as well.

Aim and outline of the thesis

Most of the pectinolytic enzymes described so far only act on the homogalacturonan part of pectins, whereas a significant part of these structures is composed of rhamnogalacturonans. Therefore, there was an interest in novel enzymes, which could degrade these so-called hairy regions. *A. aculeatus*, a saprotrophic fungus belonging to the group of the black *Aspergilli*, appeared to be an effective producer of MHR degrading enzymes. At the time this work started only a rhamnogalacturonase (RHGA) was purified from the industrial enzyme preparation Ultra SP, which originates from an *A. aculeatus* strain (Schols et al. 1990). The work described in this thesis is aimed at the elucidation of the structure of rhamnogalacturonase encoding genes and of the mechanism of gene regulation, whereas overproduction of the corresponding enzymes was also studied.

Firstly, antibodies were generated against RHGA and a cDNA expression library was constructed, after which the *rhg*A gene was isolated from *A. aculeatus*, as described in Chapter 2.

Two rhamnogalacturonan hydrolase genes (*rhg*A and *rhg*B), which are homologous to *A. aculeatus rhg*A, were isolated from *A. niger*, as described in Chapter 3.

For the efficient expression of rhamnogalacturonase genes, the respective *rhg* gene promoters were replaced by the strong *A. awamori* xylanase promoter. This allowed detection of each of the gene products and the use of simple carbon sources for their induction. The rhamnogalacturonan hydrolases from *A. aculeatus* and *A. niger* were purified and their capacities in degrading apple hairy regions were compared. The induction characteristics of the respective rhamnogalacturonan hydrolase genes upon growth on apple pectin medium were analysed.

Rhamnogalacturonan hydrolase A expression was further optimized by selecting UV-induced *A. aculeatus* mutants, as described in Chapter 4. By using the *A. niger* glucose oxidase gene as reporter, expression could be monitored easily. Initially, this expression system was used for the investigation of rhamnogalac-

turonan hydrolase induction on other carbon compounds instead of apple pectin. Some of the mutants were subjected to more detailed analysis using somatic recombination.

As many different uniseriate strains, belonging either to the species *A. aculeatus* or to the species *A. japonicus*, are exploited for the production of pectinolytic enzymes, we investigated their relationship by RFLP analysis, as described in Chapter 5. Reference *A. niger* and *A. tubingensis* strains and additionally a few *A. carbonarius* strains were analysed at the same time, allowing comparison with biseriate black *Aspergillus* species.

References

Adisa VA (1989) Pectic enzymes of *Aspergillus aculeatus* associated with post-harvest deterioration of *Citrus sinensis* fruit. J Food Biochem 13:243-252

Adler-Nissen JL, Guertler H, Jensen GW, Olsen HAS, Riisgaard S, Schnelein M (1984) US Patent 4,478,939; Chem Abstr 102 (1985) 111481

Aguilar G, Huitrón C (1993) Conidial and mycelial-bound exo-pectinase of *Aspergillus* sp. FEMS Microbiol Lett 108:127-132

Albersheim P, Darvill AG (1985) Oligosaccharins. Sci Am 253:44-50

Al-Musallam A (1980) Revision of the black *Aspergillus* species. PhD Thesis, State University Utrecht

Alvarez M, Costa SC, Huber A, Baron M, Fontana JD (1995) The cuticle of the cactus *Cereus peruvianus* as a source of a homo-α-D-galacturonan. Appl Biochem Biotechnol 51/52:367-377

An J, O'Neill MA, Albersheim P, Darvill AG (1994) Isolation and structural characterization of β-D-glucosyluronic acid and 4-O-methyl-β-D-glucosyluronic acid-containing oligosaccharides from the cell-wall pectic polysaccharide, rhamnogalacturonan I. Carbohydr Res 252:235-243

Azadi P, O'Neill MA, Bergmann C, Darvill AG, Albersheim P (1996) The backbone of the pectic polysaccharide rhamnogalacturonan I is cleaved by an endohydrolase and an endolyase. Glycobiol 5:783-789

Balestrieri C, Castaldo D, Giovane A, Quagliuolo L, Servillo L (1990) A glycoprotein inhibitor of pectin methylesterase in kiwi fruit (*Actinidia chinensis*). Eur J Biochem 193:183-187

Beldman G, Searle-van Leeuwen MJF, Ruiter GA de, Siliha HA, Voragen AGJ (1993) Degradation of arabinans by arabinanases from *Aspergillus aculeatus* and *Aspergillus niger*. Carbohydr Polym 20:159-168

Beldman G, Mutter M, Searle-van Leeuwen MJF, Broek LAM van den, Schols HA, Voragen AGJ (1995) New enzymes towards pectic structures. In: Abstracts of the International symposium 'Pectins and Pectinases'. Elsevier, The Netherlands

Beldman G, Broek LAM van den, Schols HA, Searle-van Leeuwen MJF, Laere KMJ van, Voragen AGJ (1996) An exogalacturonase from *Aspergillus aculeatus* able to degrade xylogalacturonan. Biotechnol Lett 18:707-712

Bergmann CW, Ito Y, Singer D, Albersheim P, Darvill AG (1994) Polygalacturonase-inhibiting protein accumulates in *Phaseolus vulgaris* L. in response to wounding, elicitors and fungal infection. Plant J 5:625-634

Bird CR, Smith CJS, Ray JA, Moureau P, Bevan MW, Bird AS, Hughes S, Morris PC, Grierson D, Schuch W (1984) The tomato polygalacturonase gene and ripening-specific expression in transgenic plants. Plant Mol Biol 11:651-662

Burchard W (1993) Solution properties of plant polysaccharides as a function of their chemical structure. In: Meuser F, Manners DJ and Seibel W (eds) Plant polymeric carbohydrates. Royal Society of Chemistry, Cambridge, UK

Bussink (1992) The polygalacturonase-encoding gene family of *Aspergillus niger*. PhD Thesis, Agricultural University Wageningen

Carpita NC, Gibeaut DM (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

Casadaban MJ, Cohen SN (1979) Lactose genes fused to exogenous promoters in one step using a Mu-*lac* bacteriophage: in vivo probe for transcriptional control sequences. PNAS USA 76:4530-4533

Chatterjee A, McEvoy JL, Chambost JP, Blasco F, Chatterjee AK (1991) Nucleotide sequence and molecular characterization of *pnl*A, the structural gene for damage-inducible pectin lyase of *Erwinia carotovora* subsp. *carotovora*-71. J Bacteriol 3:1765-1769

Colagrande O, Silva A, Fumi MD (1994) Recent applications of biotechnology in wine production. Biotechnol Prog 10:2-18

Condemine G, Robert-Baudouy J (1991) Analysis of an *Erwinia chrysanthemi* gene cluster involved in pectin degradation. Mol Microbiol 5:2192-2202

Condemine G , Hugouvieux-Cotte-Pattat N, Robert-Baudouy J (1986) Isolation of *Erwinia chrysanthemi kdu*D mutants altered in pectin degradation. J Bacteriol 165:937-941

Cook RC, Rayner ADM (1984) Ecology of saprotrophic fungi. Longman Group Limited

Cooper RM, Wood RKS (1975) Regulation of synthesis of cell wall degrading enzymes by *Verticillium albo-atrum* and *Fusarium oxysporum* f.sp. *lycopersici*. Physiol Plant Pathol 5:135-156

Darvill AG, McNeil M, Albersheim P (1978) Structure of plant cell walls. VIII. A new pectic polysaccharide. Plant Physiol 62:418-422

Dean RA, Timberlake WE (1989) Production of cell wall-degrading enzymes by *Aspergillus nidulans*: A model system for fungal pathogenesis of plants. Plant Cell 1:265-273

De Vries (1983) Structural features of apple pectic substances. PhD Thesis, Agricultural University Wageningen

Dey PM, Brinson K (1984) Plant cell-walls. Adv Carbohydr Chem Biochem 42:265-382

Dupin I, Gunata Z, Sapis JC, Bayonove C (1992) Production of β-apiosidase by *Aspergillus niger*: partial purification, properties, and effect on terpenyl apiosylglucosides from grape. J Agric Food Chem 40:1886-1891

Favey S, Bourson C, Bertheau Y, Kotoujanski A, Boccara M (1992) Purification of the acidic pectate lyase and nucleotide sequence of the corresponding gene (*pel*A) of *Erwinia chrysanthemi strain* 3937. J Gen Microbiol 138:499-508

Fischer RL, Bennet AB (1991) Role of cell wall hydrolases in fruit ripening. Annu Rev Plant Physiol Plant Mol Biol 42:675-703

Fry SC (1988) The growing plant cell wall: chemical and metabolic analysis. Longman Scientific & Technical, Harlow, UK, pp 188-218

Fry SC (1995) Polysaccharide-modifying enzymes in the plant cell wall. Annu Rev Plant Physiol Plant Mol Biol 46:497-520

Grierson D, Tucker GA, Keen J, Ray J, Bird CR, Schuch W (1986) Sequencing and identification of a cDNA clone for tomato polygalacturonase. NAR 14:8595-8603

Guillon F, Thibault JF (1989) Enzymic hydrolysis of the "hairy" fragments of sugarbeet pectins. Carbohydr Res 190:97-108

Hall LN, Tucker GA, Smith CJS, Watson CF, Seymour GB, Bundick Y, Boniwell JM, Fletcher JD, Ray JA, Schuch W, Bird CR, Grierson D (1993) Antisense inhibition of pectin esterase gene expression in transgenic tomatoes. Plant J 3:121-129

Hall LN, Bird CR, Picton S, Tucker GA, Seymour GB, Grierson D (1994) Molecular characterization of cDNA clones representing pectin esterase isoenzymes from tomato. Plant Mol Biol 25:313-318

Harriman RW, Tieman DM, Handa AK (1991) Molecular cloning of tomato pectin methylesterase gene and its expression in Rutgers, Ripening Inhibitor, Non-ripening, and Never Ripe tomato fruits. Plant Physiol 97:80-87

Hart DA, Kindel PK (1970) Isolation and partial characterization of apiogalacturonans from the cell wall of *Lemna minor*. Biochem J 116:569-579

He SY, Collmer A (1990) Molecular cloning, nucleotide sequence, and marker exchange mutagenesis of the exo-poly-α-D-galacturonosidase-encoding *peh*X gene of *Erwinia chrysanthemi* EC16. J Bacteriol 172:4988-4995

Hinton JCD, Sidebotham JM, Gill DR, Salmond GPC (1989) Extracellular and periplasmic isoenzymes of pectate lyase from *Erwinia carotovora* subspecies *carotovora* belong to different gene families. Mol Microbiol 3:1785-1795

Hinton JCD, Gill DR, Lalo D, Plastow GS, Salmond GPC (1990) Sequence of the *peh* gene of *Erwinia carotovora*: homology between *Erwinia* and plant enzymes. Mol Microbiol 4:1029-1036

Hirano M, Kiyohara H, Yamada H (1994) Existence of a rhamnogalacturonan II-like region in bioactive pectins from medicinal herbs. Planta Med 60:450-454

Hours RA, Sakai T (1994) Protopectinase production in solid state culture of *Aspergillus awamori*. Biotechnol Lett 16:721-726

Hugouvieux-Cotte-Pattat N, Reverchon S, Robert-Baudouy J (1989) Expanded linkage map of *Erwinia chrysanthemi* strain 3937. Mol Microbiol 3:573-581

Hugouvieux-Cotte-Pattat N, Nasser W, Robert-Baudouy J (1994) Molecular characterization of the *Erwinia chrysanthemi kdg*K gene involved in pectin degradation. J Bacteriol 176:2386-2392

Hwang J, Pyun YR, Kokini JL (1993) Sidechains of pectins: some thoughts on their role in plant cell walls and foods. Food Hydrocolloids 7:39-53

Inoue T, Yoshida T, Ichishima E (1995) Molecular cloning and nucleotide sequence of the 1,2-α-D-mannosidase gene, *msd*S, from *Aspergillus saitoi* and expression of the gene in yeast cells. Biochim Biophys Acta 1253:141-145

Ishii S (1976) Enzymatic maceration of plant tissues by endo-pectin lyase and endo-polygalacturonase from *Aspergillus japonicus*. Phytopathol 66:281-289

Ito K, Kobayashi R, Nikaido N, Izaki K (1988) DNA structure of pectate lyase I gene cloned from *Erwinia carotovora*. Agric Biol Chem 52:479-487

Keegstra K, Talmadge KW, Bauer WD, Albersheim P (1973) The structure of plant cell walls. III. A model of the walls of suspension-cultured sycamore cells based on the interconnections of the macromolecular components. Plant Physiol 51:188-196

Keen NT, Tamaki S (1986) Structure of two pectate lyase genes from *Erwinia chrysanthemi* EC16 and their high-level expression in *Escherichia coli*. J Bacteriol 168:595-606

Kester HCM, Kusters-van Someren MA, Müller Y, Visser J (1996) Primary structure and characterization of an exopolygalacturonase from *Aspergillus tubingensis*. Eur J Biochem 240:738-746

Khanh NQ, Ruttkowski E, Leidinger K, Albrecht H, Gottschalk M (1991) Characterization and expression of a genomic pectin methylesterase-encoding gene in *Aspergillus niger*. Gene 106:71-77

Kikuchi T, Sugimoto H (1976) Detailed structure of an acidic polysaccharide in soy sauce, confirmed by use of two kinds of purified pectinases. Agr Biol Chem 40:87-92

Kofod LV, Kauppinen S, Christgau S, Andersen LN, Heldt-Hansen HP, Dörreich K, Dalbøge H (1994) Cloning and characterization of two structurally and functionally divergent rhamnogalacturonases from *Aspergillus aculeatus*. J Biol Chem 269:29182-29189

Kravtchenko TP, Voragen AGJ, Pilnik W (1992) Analytical comparison of three industrial pectin preparations. Carbohydr Pol 18:17-25

Kusters-van Someren MA (1991) Characterization of an *Aspergillus niger* pectin lyase gene family. PhD Thesis, State University Utrecht

Lahaye M, Vigouroux J, Thibault JF (1991) Endo-β-1,4-D-galactanase from *Aspergillus niger* var. *aculeatus*: Purification and some properties. Carbohydr Polym 15:431-444

Laurent F, Kotoujanski A, Labesse G, Bertheau Y (1993) Characterization and overexpression of the *pem* gene encoding pectin methylesterase of *Erwinia chrysanthemi* strain 3937. Gene 131:17-25

Lei SP, Lin HC, Wang SS, Callaway J, Wilcox G (1987) Characterization of the *Erwinia carotovora pel*B gene and its gene product pectate lyase. J Bacteriol 169:4379-4383

Lei SP, Lin HC, Wang SS, Wilcox G (1988) Characterization of the *Erwinia carotovora pel*A gene and its product pectate lyase A. Gene 62:159-164

Lei SP, Lin HC, Wang SS, Higaki P, Wilcox G (1992) Characterization of the *Erwinia carotovora peh* gene and its product polygalacturonase. Gene 117:119-124

Lerouge P, O'Neill MA, Darvill AG, Albersheim P (1993) Structural characterization of endo-glycanase-generated oligoglycosyl side chains of rhamnogalacturonan I. Carbohydr Res 243:359-371

Liu Y, Chatterjee A, Chatterjee AK (1994) Nucleotide sequence and expression of a novel pectate lyase gene (*pel*-3) and a closely linked endopolygalacturonase gene (*peh*-1) of *Erwinia carotovora* subsp. *carotovora* 71. Appl Environ Microbiol 60:2545-2552

Lojkowska E, Masclaux C, Boccara M, Robert-Baudouy J, Hugouvieux-Cotte-Pattat N (1995) Characterization of the *pel*L gene encoding a novel pectate lyase of *Erwinia chrysanthemi* 3937. Mol Microbiol 16:1183-1195

Marfà V, Gollin DJ, Eberhard S, Mohnen D, Darvill A, Albersheim P (1991) Oligogalacturonides are able to induce flowers to form on tobacco explants. Plant J 1:217-225

Markovič O, Jörnvall H (1990) Tomato and *Aspergillus niger* pectinesterases. Correlation of differences in reports: large species variations. Prot Seq Data Anal 3:513-515

Massiot P, Thibault JF (1989) Enzymic analysis of carrot cell-wall polysaccharides. Carbohydr Res 190:121-136

McCleary BV, Matheson NK (1986) Enzymic analysis of polysaccharide structure. Adv Carbohydr Chem Biochem 44:147-187

McEvoy JL, Murata H, Chatterjee AK (1990) Molecular cloning and characterization of an *Erwinia carotovora* subsp. *carotovora* pectin lyase gene that responds to DNA-damaging agents. J Bacteriol 172:3284-3289

McMillan GP, Barrett AM, Pérombelon MCM (1994) An isoelectric focusing study of the effect of methyl-esterified pectic substances on the production of extracellular pectin isoenzymes by soft rot *Erwinia* spp. J Appl Bacteriol 77:175-184

McNeil M, Darvill AG, Albersheim P (1980) Structure of plant cell walls. X. Rhamnogalacturonan I. A structurally complex pectic polysaccharide in the walls of suspension-cultured sycamore cells. Plant Physiol 66:1128-1134

McNeil M, Darvill AG, Fry SC, Albersheim P (1984) Structure and function of the primary cell walls of plants. Ann Rev Biochem 53:625-663

Micard V, Renard CMGC, Thibault JF (1994) Studies on enzymic release of ferulic acid from sugar-beet pulp. Lebensm Wiss Technol 27:59-66

Micheli PA (1729) Nova plantarum genera. 234 pp., illus. Florentiae.

Minamiguchi K, Ooi T, Kawaguchi T, Okada H, Murao S, Arai M (1995) Secretive expression of the *Aspergillus aculeatus* cellulase (F1-CMCase) by *Saccharomyces cerevisae*. J Ferm Bioeng 79:363-366

Morrison DC, Lei MG, Chen TY, Flebbe M, Halling J, Field S (1992) In: Friedman (ed) Microbial infections. Plenum Press, NY, pp 23-29

Mosseray R (1934) Les Aspergillus de la section "Niger" Thom et Church. La Cellule, tome XLIII, fasc.2

Murao S, Sakamoto R, Arai M (1988) Cellulases of *A. aculeatus*. Methods Enzymol 160:247-299

Musters W, Stam H, Suykerbuyk MEG, Visser J, Verbakel JMA (1993) Cloning and expression of DNA encoding a ripening form of a polypeptide having rhamnogalacturonase activity. Patent Appl no. 93201349.3

Mutter M, Beldman G, Schols HA, Voragen AGJ (1993) Enzymic degradation of hairy regions of pectin (abstract 107). J Cell Biochem 17A (Suppl):15

Mutter M, Beldman G, Schols HA, Voragen AGJ (1994) Rhamnogalacturonan *α*-L-Rhamnopyranohydrolase. Plant Physiol 106:241-250

Mutter M, Renard CMGC, Beldman G, Schols HA, Voragen AGJ (1995) Characterisation of RG degradation products of new RGases using RG-rhamnohydrolase and RG-galacturonohydrolase. In: Abstracts of the International symposium 'Pectins and Pectinases'. Elsevier, The Netherlands

O'Neill M, Albersheim P, Darvill AG (1990) The pectic polysaccharides of primary cell walls. In: Dey PM and Harborne JB (eds) Methods in plant biochemistry, Vol 2 Carbohydrates. Academic Press, London, pp 415-441

Oosterveld A, Beldman G, Bruijn JM de, Voragen AGJ (1995) Autoclave extraction of sugar beet pulp yields gel-forming pectic 'hairy regions'. In: Abstracts of the International symposium 'Pectins and Pectinases'. Elsevier, The Netherlands

Oxenbøll K (1994) *Aspergillus* enzymes and industrial uses. In: Powell KA, Renwick A and Peberdy JF (eds) The genus *Aspergillus*. Plenum Press, NY

Parenicova L, Benen JAE, Kester HCM, Visser J (1995) *pga*E - A fourth member of polygalacturonase encoding gene family of *Aspergillus niger*: cloning of *pga*E gene and basic biochemical characterisation of gene product. In: Abstracts of the International symposium 'Pectins and Pectinases'. Elsevier, The Netherlands

Pellerin P, Doco T, Brillouet J (1995) The pectic polysaccharide rhamnogalacturonan II is a major component of the polysaccharides present in fruit-derived products. In: Abstracts of the International symposium 'Pectins and Pectinases'. Elsevier, The Netherlands

Pilnik W, Rombouts FM (1985) Polysaccharides and food processing. Carbohydr Res 142:93-105

Pissavin C, Robert-Baudouy J, Hugouvieux-Cotte-Pattat N (1995) Identification of a seventh endo-pectate lyase produced by the phytopathogenic bacterium *Erwinia chrysanthemi* 3937. In: Abstracts of the International symposium 'Pectins and Pectinases'. Elsevier, The Netherlands

Plastow GS (1988) Molecular cloning and nucleotide sequence of the pectin methylesterase gene of *Erwinia chrysanthemi* B374. Mol Microbiol 2:247-254

Puvanesarajah V, Darvill AG, Albersheim P (1991) Structural characterization of two oligosaccharide fragments formed by the selective cleavage of rhamnogalacturonan II: evidence for the anomeric configuration and attachment sites of apiose and 3-deoxy-2-heptulosaric acid. Carbohydr Res 218:211-222

Raper KB, Fennell DI (1965) The genus *Aspergillus*. Williams and Wilkins Company, Baltimore

Ray J, Knapp J, Grierson D, Bird C, Schuch W (1988) Identification and sequence determination of a cDNA clone for tomato pectin esterase. Eur J Biochem 174:119-124

Renard CMGC, Thibault JF, Voragen AGJ, Broek LAM van den, Pilnik W (1993) Studies on apple protopectin VI: extraction of pectins from apple cell walls with rhamnogalacturonase. Carbohydr Pol 22:203-210

Renard CMGC, Voragen AGJ, Thibault JF, Pilnik W (1990) Studies on apple protopectin I: Extraction of insoluble pectin by chemical means. Carbohydr Pol 12:9-25

Renard CMGC, Voragen AGJ, Thibault JF, Pilnik W (1991) Studies on apple protopectin. IV: Apple xyloglucans and influence of pectin extraction treatments on their solubility. Carbohydr Pol 15:387-403

Reverchon S, Huang Y, Bourson C, Robert-Baudouy J (1989) Nucleotide sequence of the *Erwinia chrysanthemi ogl* and *pel*E genes, negatively regulated by the KdgR gene product. Gene 85:125-134

Roche N, Berna P, Desgranges C, Durand A (1995) Substrate use and production of α-L-arabinofuranosidase during solid-state culture of *Trichoderma reesei* on sugar beet pulp. Enzyme Microb Technol 17:935-941

Rolin C, Vries J de (1990) Pectin. In: Harris P (ed) Food gels. Elsevier Applied Science, London, NY, pp 401-434

Ruttkowski E, Labitzke R, Khahn NQ, Löffler F, Gottschalk M, Jany KD (1990) Cloning and DNA sequence analysis of a polygalacturonase cDNA from *Aspergillus niger* RH5344. Biochim Biophys Acta 1087:104-106

Ryan C (1988) Oligosaccharides as recognition signals for the expression of defensive genes in plants. Biochem 27:8879-8883

Saarilahti HT, Heino P, Pakkanen R, Kalkkinen N, Palva I, Palva ET (1990) Structural analysis of the *peh*A gene and characterization of its protein product, endopolygalacturonase, of *Erwinia carotovora* subspecies *carotovora*. Mol Microbiol 4:1037-1044

Sakamoto R, Kanamoto J, Arai M, Murao S (1985) Purification and physiochemical properties of three β-glucosidases from *Aspergillus aculeatus* No. F-50. Agric Biol Chem 49:1275-1281

Samson RA (1992) Current taxonomic schemes of the genus *Aspergillus* and its teleomorphs. In: Bennet JW and Klich MA (eds) *Aspergillus*: biology and industrial applications. Reed Publishing, USA, pp 355-390

Samson RA (1994) Taxonomy - Current concepts of *Aspergillus* systematics. In: Smith JE (ed) *Aspergillus*. Plenum Press, NY, pp 1-22

Sanyal A, Kundu R, Sinha S, Dube D (1988) Extracellular cellulolytic enzyme system of *Aspergillus japonicus*: 1. Effect of different carbon sources. Enzyme Microb Technol 10:85-90

Saulnier L, Thibault JF (1987) Enzymic degradation of isolated pectic substances and cell wall from pulp of grape berries. Carbohydr Pol 7:345-360

Schmidt O, Angermann H, Frommhold-Treu I, Hoppe K (1995) Experimental and theoretical investigations of submerged fermentation and synthesis of pectinolytic enzymes by *Aspergillus niger*. Appl Microbiol Biotechnol 43:424-430

Schols H (1995) Structural characterization of pectic hairy regions isolated from apple cell walls. PhD Thesis, Agricultural University Wageningen

Schols HA, Geraeds CJM, Searle-van Leeuwen MF, Kormelink FJM, Voragen AGJ (1990) Rhamnogalacturonase: a novel enzyme that degrades the hairy regions of pectins. Carbohydr Res 206:105-115

Schols HA, Veld PH in 't, Deelen W van, Voragen AGJ (1991) The effect of the manufacturing method on the characteristics of apple juice. Z Lebensm Unters Forsch 192:142-148

Schols HA, Mutter M, Voragen AGJ, Niessen WMA, Hoeven RAM van der, Greef J van der, Bruggink C (1994) The use of combined high-performance anion-exchange chromatography-thermospray mass spectrometry in the structural analysis of pectic oligosaccharides. Carbohydr Res 261:335-342

Schuch W (1994) Improving tomato quality through biotechnology. Food Technol 48:78-83

Searle-van Leeuwen MJF, Broek LAM van den, Schols HA, Beldman G, Voragen AGJ (1992) Rhamnogalacturonan acetylesterase: a novel enzyme from *Aspergillus aculeatus*, specific for the deacetylation of hairy (ramified) regions of pectins. Appl Microbiol Biotechnol 38:347-349

Searle-van Leeuwen MJF, Vincken J, Schipper D, Voragen AGJ, Beldman G (1995) Acetyl esterases from *Aspergillus niger*, purification and mode of action on pectins. In: Abstracts of the International symposium 'Pectins and Pectinases'. Elsevier, The Netherlands

Shevchik V, Condemine G, Hugouvieux-Cotte-Pattat N, Robert-Baudouy J (1995) Pectin methylesterase B of *Erwinia chrysanthemi*, the first pectinase characterised as a membrane lipoprotein. In: Abstracts of the International symposium 'Pectins and Pectinases'. Elsevier, The Netherlands

Solís-Pereira S, Favela-Torres E, Viniegra-González G, Gutiérrez-Rojas M (1993) Effects of different carbon sources on the synthesis of pectinase by *Aspergillus niger* in submerged and solid state fermentations. Appl Microbiol Biotechnol 39:36-41

Spellman MW, McNeil M, Darvill AG, Albersheim P (1983) Characterization of a structurally complex heptasaccharide isolated from the pectic polysaccharide rhamnogalacturonan II. Carbohydr Res 122:131-153

Stephen AM (1983) In: Aspinall GO (ed) The polysaccharides, Vol 2 Molecular biology. Acad Press, NY, pp 97-193

Tamaki SJ, Gold S, Robeson M, Manulis S, Keen NT (1988) Structure and organization of the *pel* genes from *Erwinia chrysanthemi* EC16. J Bacteriol 170:3468-3478

Thibault JF, Rouau X (1990) Studies on enzymic hydrolysis of polysaccharides in sugar beet pulp. Carbohydr Pol 13:1-16

Thibault JF, Renard CMGC, Axelos MAV, Roger P, Crépeau MJ (1993) Studies of the length of homogalacturonic regions in pectins by acid hydrolysis. Carbohydr Res 238:271-286

Thom C, Church MB (1926) The Aspergilli, pp. 1-272, illus. Baltimore.

Thom C, Raper KB (1945) A manual of the *Aspergilli*. Williams and Wilkins Company, Baltimore

Trinci APJ, Davies DR, Gull K, Lawrence MI, Nielsen BB, Rickers A, Theodorou MK (1994) Anaerobic fungi in herbivorous animals. Mycol Res 98:129-152

Trollinger D, Berry S, Belser W, Keen NT (1989) Cloning and characterization of a pectate lyase gene from *Erwinia carotovora* EC153. Mol Plant Microb Interact 2:17-25

Upshall A, Kumar AA, Kaushansky K, McKnight L (1992) Molecular manipulation of and heterologous protein secretion from filamentous fungi. In: Leong SA and Berka RM (eds) Molecular Industrial Mycology. Marcel Dekker Inc., NY, pp 31-44

Van de Vis JW, Searle-van Leeuwen MF, Siliha HA, Kormelink FJM, Voragen AGJ (1991) Purification and characterization of *endo*-1,4-β-D-galactanases from *Aspergillus niger* and *Aspergillus aculeatus*: use in combination with arabinases from *Aspergillus niger* in enzymatic conversion of potato arabinogalactan. Carbohydr Pol 16:167-187

Van Gijsegem F (1989) Relationship between the *pel* genes of the *pel*ADE cluster of *Erwinia chrysanthemi* strain B374. Mol Microbiol 3:1415-1424

Van Rijssel M, Gerwig GJ, Hansen TA (1993) Isolation and characterization of an extracellular glycosylated protein complex from *Clostridium thermosaccharolyticum* with pectin methylesterase and polygalacturonate hydrolase activity. Appl Environm Microbiol 59:828-836

Visser J, Suykerbuyk M, Kusters-van Someren M, Samson R, Schaap P (1996) Classification of black *Aspergilli* by RFLP analysis. In: Rossen L, Rubio V, Dawson MT and Frisvad JC (eds) Fungal Identification Techniques. EUR16510, EC SC-EC-EAEC Brussels-Luxembourg, pp194-200

Voragen AGJ, Pilnik W (1989) Pectin degrading enzymes in fruit and vegetable processing. ACS Symp Ser 389:93-115

Watson CF, Zheng L, DellaPenna D (1994) Reduction of tomato polygalacturonase β subunit expression affects pectin solubilization and degradation during fruit ripening. Plant Cell 6:1623-1634

Whitaker JR (1984) Pectic substances, pectic enzymes and haze formation in fruit juices. Enzyme Microb Technol 6:341-349

Wu Q, Szakacs-Dobozi M, Hemmat M, Hrazdina G (1993) Endopolygalacturonase in apples (*Malus domestica*) and its expression during fruit ripening. Plant Physiol 102:219-225

York WS, Darvill AG, McNeil M, Albersheim P (1985) 3-Deoxy-D-manno-2-octulosonic acid (KDO) is a component of rhamnogalacturonan II, a pectic polysaccharide in the primary cell wall of plants. Carbohydr Res 138:109-126

Yoshida A, Izuta M, Ito K, Kamio Y, Izaki K (1991) Cloning and characterization of the pectate lyase III gene of *Erwinia carotovora* Er. Agric Biol Chem 55:933-940

Zablackis E, Huang J, Müller B, Darvill AG, Albersheim P (1995) Characterization of the cell-wall polysaccharides of *Arabidopsis thaliana* leaves. Plant Physiol 107:1129-1138

2 Cloning, sequence and expression of the gene for rhamnogalacturonan hydrolase of *Aspergillus aculeatus*; a novel pectinolytic enzyme

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Appl Microbiol Biotechnol (1995) 43:861-870

Abstract Rhamnogalacturonan hydrolase A was purified from culture filtrate of *Aspergillus aculeatus* after growth in medium with sugar-beet pulp as carbon source. Purified protein was used to raise antibodies in mice and with the antiserum obtained a gene coding for rhamnogalacturonan hydrolase A (*rhg*A) was isolated from a λ cDNA expression library. The cloned *rhg*A gene has an open-reading frame of 1320 base pairs encoding a protein of 440 amino acids with a predicted molecular mass of 45 962 Da. The protein contains a potential signal peptidase cleavage site behind Gly-18 and three potential sites for *N*-glycosylation. Limited homology with *A. niger* polygalacturonase amino acid sequences is found. A genomic clone of *rhg*A was isolated from a recombinant phage λ genomic library. Comparison of the genomic and cDNA sequences revealed that the coding region of the gene is interrupted by three introns. Furthermore, amino acid sequences of four different peptides, derived from purified *A. aculeatus* rhamnogalacturonan hydrolase A, were also found in the deduced amino acid sequence of *rhg*A. *A. aculeatus* strains overexpressing rhamnogalacturonan hydrolase A were obtained by cotransformation using either the *A. niger pyr*A gene or the *A. aculeatus pyr*A gene as selection marker. For expression of rhamnogalacturonan hydrolase A in *A. awamori* the *A. awamori pyr*A gene was used as selection marker. Degradation patterns of modified hairy regions, determined by HPLC, show the recombinant rhamnogalacturonan hydrolase A to be active and the enzyme was found to have a positive effect in the apple hot-mash liquefaction process.

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Introduction

Pectin consists of 'smooth' regions of homogalacturonan and branched or 'hairy' regions with alternating galacturonic acid and rhamnose residues in the backbone. In the hairy regions most of the rhamnose residues are branched with complex side-chains consisting of arabinose-rich structures linked to galactose. The alternating galacturonic acid residues are methylated, acetylated or branched with xylose (Schols et al. 1990b). Complete degradation of pectin is a complex process requiring the interplay of many enzymes. To date a large number of pectinolytic enzymes of fungal origin have been purified (pectin esterase, pectin lyases, polygalacturonases) and the corresponding genes have been cloned mainly from *Aspergillus niger* (Harmsen et al. 1990; Bussink et al. 1992; Ruttkowski et al. 1991; Khanh et al. 1991). However, upon incubation of pectin with these enzymes the hairy regions remain undegraded. Complete degradation of pectins is of great importance for various industrial processes such as clarification of fruit juices (Biekman et al. 1990; Sreenath and Santhanam 1992). Recently it has been shown that the hairy regions of apple pectin can be further degraded by a newly purified enzyme called rhamnogalacturonan hydrolase A (Colquhoun et al. 1990; Schols et al. 1990a,b). This enzyme hydrolyzes α-D-galacturonopyranosyl-(1,2)-α-L-rhamnopyranosyl linkages in the backbone of the hairy regions of pectins.

Thus far many plant-cell-wall-degrading enzymes have been described that originate from *A. niger*. Far less is known about the production and characteristics of those enzymes in *Aspergillus aculeatus*. High rhamnogalacturonan hydrolase activity was demonstrated in a commercial preparation (Ultra SP) from *A. aculeatus*, from which the enzyme was purified (Schols et al. 1990a). Besides the purification of this rhamnogalacturonan hydrolase a rhamnogalacturonan acetylesterase (Searle-van Leeuwen et al. 1992) and endogalactanases have been purified from *A. aculeatus* (Lahaye et al. 1991; Van de Vis et al. 1991). In this report we describe the purification of rhamnogalacturonan hydrolase A, the cloning and characterization of the corresponding gene and (over)production of the enzyme in *A. aculeatus* and *Aspergillus awamori*.

Materials and methods

Preparation of antibodies and growth conditions. Purified rhamnogalacturonan hydrolase A was a gift from the Department of Food Chemistry and Microbiology and was used for the immunisation of two Balb/c mice by a procedure described before (Van der Veen et al. 1991). The antiserum thus generated could specifically detect rhamnogalacturonan hydrolase A in the culture filtrate of *A. aculeatus* CBS115.80 in Western blot analysis (Burnette 1981). Rhamnogalacturonan hydrolase A expression was obtained by growing *A. aculeatus* in minimal medium (MM; Pontecorvo et al. 1953) with sugar-beet pulp (1% w/v; CSM, Breda, The Netherlands) or brown-ribbon apple pectin (1% w/v; degree of esterification 72.8%; Obipektin, Bischofszell, Switzerland) as carbon source.

Purification, deglycosylation and amino acid sequencing of rhamnogalacturonan hydrolase A. For the purification of rhamnogalacturonan hydrolase A sugar-beet pulp medium was inoculated with 10^6 *A. aculeatus* spores per ml followed by growth at 30°C for 48 h. The culture filtrate was used for purification of rhamnogalacturonan hydrolase A. Column chromatography was carried out on DEAE-Sephadex A50, DEAE-Sepharose Fast Flow, Mono P HR 20/5 and Superose 12 columns, all obtained from Pharmacia LKB, Uppsala, Sweden. Details of the purification are given in Results. Deglycosylation of denatured rhamnogalacturonan hydrolase A with *N*-glycanase was carried out according to the instructions of the supplier (Genzyme, Cambridge, Massachusetts, USA). CNBr treatment and endoproteinase Lys-C digestion of rhamnogalacturonan hydrolase A were also performed according to the instructions of the supplier (Boehringer, Mannheim, Germany). Rhamnogalacturonan hydrolase A and derived protein fragments were subjected to amino acid sequencing using the method of Edman and an Applied Biosystems sequencer model 475 with an on-line phenylthiohydantoin-analyzer model 120A.

Construction and screening of a cDNA library of A. aculeatus. Mycelium from an overnight culture of *A. aculeatus* CBS115.80 in MM with 0.5% yeast extract, 0.2% casamino acids and 1% sucrose was transferred to MM with apple pectin (1% w/v) and allowed to grow for 24 h in a 2-l fermenter at 30°C and pH 4. Total RNA was isolated from mycelium harvested 3, 6 and 24 h after transfer by a standard method with guanidinium thiocyanate (Sambrook et al. 1989). The samples were centrifuged twice

over a CsCl cushion before the RNA was pooled. Poly(A)-rich RNA was then isolated using the polyATtract mRNA isolation kit (Promega, Madison, USA). The poly(A)-rich RNA obtained was subsequently used for the construction of a cDNA library using the λZAP cDNA synthesis kit (Stratagene, La Jolla, USA) according to the instructions of the supplier. For this, cDNA fragments, with a *Xho*I cohesive end flanking the poly(A) tail region and an *Eco*RI adaptor at the 5'end were prepared and used for directional cloning in the λZAPII vector, allowing expression of *lac*Z fusion genes. After packaging of the phages a primary library of $2.5x10^5$ plaque-forming units (pfu) was obtained. After amplification, this λ cDNA library was screened for rhamnogalacturonan hydrolase A expression. However, since no positive signals with the antiserum were obtained by plaque screening, we used colony screening as an alternative method for the selection of rhamnogalacturonan hydrolase A expressing clones. For this, inserts of λZAPII were excised and packaged by infection with helper phage R408 (Stratagene). The mixture of pBluescriptSK(+) phagemids obtained was used to infect competent *E. coli* BB4 cells (Stratagene), which were then plated on LB plates. Colonies were selected for ampicillin resistance, transferred to nitrocellulose filters (Schleicher & Schuell, Dassel, Germany), which were treated with a 10 mM isopropylthiogalactoside solution to induce the expression of *lac*Z fusion genes, and incubated for 3 h at 37°C. The filters were treated with 0.5 M NaOH containing 8 M urea to lyse the cells and to denature the induced proteins, and then neutralized to pH 7.5. Immunochemical screening was performed as described (Burnette 1981), using mouse anti-rhamnogalacturonan hydrolase antiserum, alkaline-phosphatase-conjugated goat anti-(mouse IgG) and the alkaline phosphatase assay according to the instructions of the manufacturer. Approximately $5x10^4$ colonies were screened.

Construction and screening of a genomic library of A. aculeatus. DNA was isolated from *A. aculeatus* CBS115.80 by the method of De Graaff et al. (1988). DNA partially digested with *Mbo*I was size-fractionated on a sucrose gradient and 8-kb to 21-kb fragments were cloned into the *Bam*HI site of λEMBL4 (Sambrook et al. 1989). After packaging, the recombinant phages obtained were used to infect competent LE392 cells for amplification. A genomic library containing $4x10^{10}$ pfu was obtained. A total of $5x10^4$ colonies were plated on LB plates for colony screening. A 0.5-kb *Xho*I cDNA fragment of pIM801 (Fig.1A) was used as a probe. Hybridization was carried out overnight in standard hybridization buffer (Sambrook et al. 1989) at 65°C, followed by

washing twice for 30 min with 2 x standard saline citrate (SSC) with 0.1% sodium dodecyl sulphate (SDS) and 0.1% sodium pyrophosphate at the same temperature (1 x SSC consists of 0.15 M sodium chloride and 0.015 M sodium citrate pH 7). The filters were exposed on Kodak X-Omat AR films (Eastman Kodak Company, USA) at – 70°C with an intensifying screen.

Northern blotting and primer extension. Total RNA was isolated from mycelium grown in MM with apple pectin, denatured with glyoxal and dimethylsulphoxide (Sambrook et al. 1989), subjected to electrophoresis and blotted onto Hybond N (Amersham, Buckinghamshire, England). Hybridization was carried out overnight in standard hybridization buffer at 60°C, and the filters were washed twice for 30 min with 2 x SSC containing 0.1% SDS and 0.1% sodium pyrophosphate at the same temperature. A 1.2-kb *Kpn*I-*Xho*I cDNA fragment of pIM801 (Fig. 1A) and a 0.9-kb *Eco*RI fragment of the *A. bisporus* 28S ribosomal RNA gene (Schaap and Visser, unpublished) were used as probes.

Primer extension was performed as described by Teeri et al. (1987) using poly(A)-rich RNA, isolated from mycelium grown in MM with apple pectin, and a 17-mer primer (5'AAAGAGCACGCATGATT3'), complementary to *rhg*A mRNA (position – 4 to 13).

E. coli strains, plasmids and DNA technology. Recombinant λ phages were propagated in *E. coli* LE392 (for λEMBL4) and in *E. coli* BB4 (for λZAPII) and plasmids in *E. coli* DH5α (BRL, Gaithersburg, USA). Standard methods were used for propagation and preparation of phage and plasmid DNA, transduction, plaque lift, Southern analysis and (sub)cloning (Sambrook et al. 1989). Sequencing of recombinant double-stranded plasmid DNA was performed using the T7 Sequencing kit (Pharmacia). Dedicated synthetic oligonucleotides were used as sequencing primers.

The plasmid pBluescriptSK(+) (Stratagene) was used for subcloning the *A. aculeatus rhg*A gene. Plasmid pIM801 (Fig. 1A) contains the complete cDNA sequence of *rhg*A. Plasmid pIM803 contains a genomic 3.9-kb *Bam*HI-*Sal*I fragment and comprises the complete *rhg*A gene and flanking sequences (Fig. 1B). pIM816 (Fig. 1C) contains a 2.5-kb fragment of the *A. awamori exl*A promoter in front of the *A. aculeatus rhg*A coding region (see below). The plasmid pGW635 contains the *A. niger* orotidine-5'-phosphate-decarboxylase gene (*pyr*A; Goosen et al. 1987), pIM805 contains the *A. aculeatus pyr*A gene (Suykerbuyk and Visser, unpublished) and pAW4-2A the *A. awamori pyr*A gene (Gouka et al. 1995).

Construction of a uridine auxotrophic mutant of A. aculeatus and Aspergillus transformations. Spores of the *A. aculeatus* CBS115.80 strain were UV-illuminated according to the method of Bos (1987). Conidiospore survival varied from 20% to 57%. Selection for pyrimidine mutations was performed using 5-fluoro-orotic acid resulting in NW212 (*pyr*A4). Strain NW216 (*csp*A1, *fwn*A1, *lys*A1) was obtained by three subsequent rounds of mutagenesis, using a standard filtration enrichment procedure (Uitzetter et al. 1986) for lysine auxotrophy as final step. The strains NW216 and NW212 were then crossed using the method described by Bos (1987). From the isolated heterozygous diploid colonies a few spores were directly plated onto CM-benomyl plates for haploidization. This resulted in the final acceptor strain NW217 (*pyr*A4, *csp*A1, *fwn*A1, *lys*A1) for transformation experiments. Standard methods (Goosen et al. 1987) were used for cotransformation of *A. aculeatus*, using either the *A. niger* or the *A. aculeatus pyr*A gene, and *A. awamori*, using the homologous *pyr*A gene as selection marker.

Construction and expression of the rhgA gene under control of the exlA promoter. For the construction of the *rhg*A gene under control of the xylanase promoter (*exl*A), the promoter region of *rhg*A was exchanged with the *exl*A promoter region by digesting pIM803 with *Bam*HI and *Nsi*I, removal of the *rhg*A-promoter-containing fragment and ligation of a fragment containing the *exl*A promoter (Fig. 1C). In order to position the *A. awamori exl*A promoter precisely adjacent to the *rhg*A coding region we used an adaptation of the splicing by overlap extension (SOE) technique (Horton et al. 1989). Therefore, one polymerase chain reaction (PCR) was performed using the cloned *exl*A promoter (Hessing et al. 1994) as a template and (5'CGAGGTTGTTCAAGCGT3') and (5'AGA AGGAAAAGAGCACGCATGATGATTGAAGAAAGCT3') as primers. In a second PCR the cloned *rhg*A gene was used as a template and (5'AGCTTTCTTCAATCATCATGCGTGCT CTTTTCCTTCT3') and (5'ATCATGTTCCCACTGGC3') as primers. The two partly overlapping PCR products were annealed and used as templates in combination with the two outer primers in a third PCR. The resulting fusion product was digested with *Nsi*I and *Kpn*I and ligated in the *exl*A-promoter–*rhg*A-gene hybrid digested with *Nsi*I and *Kpn*I. The resulting plasmid pIM816 (Fig. 1C) was cotransformed to the *pyr*A-deficient *A. awamori* strain NW208, using the *A. awamori pyr*A gene as selection marker. Transformants were tested for rhamnogalacturonan hydrolase expression by growing them for 24 h in MM with 0.5% sucrose and then adding 1% xylose to the medium. Rhamnogalacturonan hydrolase expression was detected after another 24 h of growth by Western blot analysis of the culture filtrate as described above.

Rhamnogalacturonan hydrolase activity and application. Rhamnogalacturonan hydrolase activity was determined by measuring the degradation of modified hairy regions from apples. For the isolation of modified hairy regions, Golden Delicious apples (10 kg) were crushed in a Magimix Cuisine System 3000 and pretreated with 0.05% (m/m) Biopectinase 200 L (Quest International, Naarden, The Netherlands) for 4 h at 55°C. After centrifugation at 8000 *g* for 30 min the supernatant was ultrafiltered and concentrated in a Pellicon microfiltration unit, which has a molecular mass cut-off value of 50 000 Da. The residue was dialysed and lyophilized. The modified hairy regions isolated in this way were used as a substrate for rhamnogalacturonan hydrolase A. The generation of degradation products after incubation of modified hairy regions with rhamnogalacturonan hydrolase A was used as a measure for rhamnogalacturonan hydrolase activity. A 5-ml sample of a 0.2% (w/v) solution of modified hairy regions in 0.05 M sodium acetate buffer (pH 5.0) was incubated with 10 μl enzyme preparation for 2 h at 50°C. The products formed were analysed on a Dionex BioLC/high-performance anion-exchange (HPAE) chromatography system using a CarboPac PA-1 anion-exchange column (25 cm x 4 mm) and a CarboPac PA-1 guard column. The column was loaded with 25 μl solution and eluted with a linear gradient of 0–0.5 M sodium acetate in 0.1 M sodium hydroxide over 50 min. The flow rate was 1.0 ml/min and the process was monitored using a pulsed electrochemical (PE) detector.

To test the use of rhamnogalacturonan hydrolase A in apple processing, apples were peeled, chopped, depipped and mashed to a fine purée. Apple mash was distributed into 500-g aliquots and preincubated at 55°C. The mashes were treated with enzyme preparations as described in the Results and incubated for 2 h at 55°C (hot-mash liquefaction). The viscosity of the hot mash was measured at regular intervals using a Brookfield viscosimeter with Helipath stand attachment and T-bar spindle. After 2 h of incubation a 30-g sample was removed from each mash and centrifuged for 20 min at 10 000 g. The volume and the Brix value of the juice were measured.

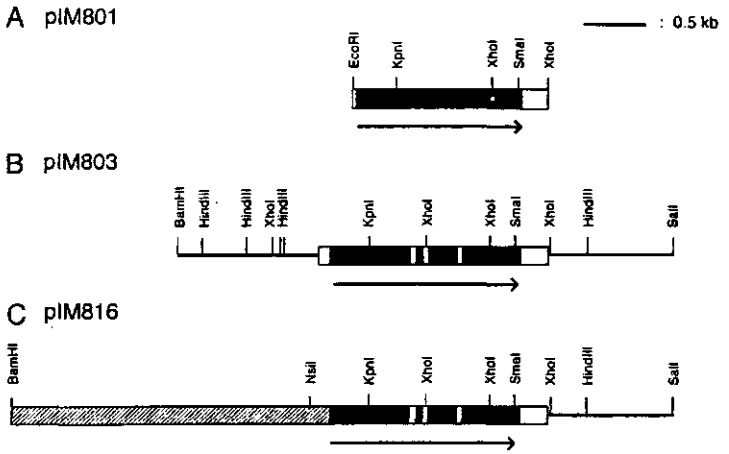


Fig. 1A–C Restriction map of an *A. aculeatus rhg*A cDNA clone (**A**) and genomic clone (**B,C**). Open reading frames are indicated as *closed bars*. **A** pIM801 contains 1.5 kb *rhg*A cDNA in pBluescriptSK(+). **B** pIM803 contains a 3.9-kb chromosomal *Bam*HI-*Sal*I fragment in pBluescriptSK(+). 5′- and 3′- untranslated sequences and introns are given as *open boxes*. **C** In pIM816 a 2.4-kb *Bam*HI-*Nsi*I fragment of the *A. awamori exl*A promoter is cloned before the *A. aculeatus rhg*A coding region. The *Nsi*I-*Kpn*I fragment is synthesized by the polymerase chain reaction to make the ATG fusion between the *exl*A promoter and the *rhg*A gene. The *exl*A promoter and leader sequences are indicated by a *hatched bar*

Results

Purification of rhamnogalacturonan hydrolase A

For the purification of rhamnogalacturonan hydrolase A *A. aculeatus* was grown in sugar-beet pulp medium for 48 h. The culture filtrate was diluted fivefold and the pH adjusted to 6.5 with sodium hydroxide. Subsequent concentration was performed by batchwise adsorption on DEAE-Sephadex A50 (100 g/l; equilibrated with 20 mM sodium phosphate buffer pH 6.5). Adsorbed protein was eluted by pulse elution with 20 mM sodium phosphate buffer pH 6.5 containing 0.5 M sodium chloride. The rhamnogalacturonan hydrolase containing fractions were selected by SDS-polyacrylamide gel electrophoresis (PAGE) followed by Western blot analysis, pooled and dialysed against water. Rhamnogalacturonan

hydrolase A was purified by using three different chromatographic steps as outlined in Fig. 2. After each chromatographic step rhamnogalacturonan hydrolase containing fractions were selected as described above, pooled and dialysed against the appropriate buffer. The repeated purification step with DEAE-Sepharose Fast Flow was required in order to achieve a good separation between rhamnogalacturonan hydrolase and an α-L-arabinofuranosidase activity. The removal of the α-L-arabinofuranosidase activity was confirmed by a specific enzyme assay (Van der Veen et al. 1991). Purification by chromatofocusing using a Mono P HR 20/5 column was performed twice. After adjustment of the pH to 6.0 with piperazine-HCl buffer the rhamnogalacturonan hydrolase containing fractions were loaded on the column and eluted with 40 ml 10% polybuffer 74/HCl, pH 3.5. The second elution was performed using 40 ml 8% polybuffer 74/HCl, pH 3.5, to obtain a less steep pH gradient. The final purification was achieved by gel-permeation chromatography on a Superose 12 column.

From a 2 l *A. aculeatus* culture containing 3.2 g protein, about 5 mg rhamnogalacturonan hydrolase A was obtained after purification. Analysis by SDS-PAGE revealed only one band of about 55 kDa and thus the enzyme appeared to be pure. The position of this band corresponds to the single band seen after Western blot analysis of culture filtrate. After *N*-glycanase treatment and SDS-PAGE analysis, a smaller protein band of about 46 kDa was visible (results not shown).

Isolation and characterization of cDNA and genomic clones encoding rhamnogalacturonan hydrolase A

RNA was isolated from *A. aculeatus* mycelium, transferred from sucrose to apple pectin medium, and subsequently used for the construction of a λZAPII cDNA library (for details see Materials and methods).

The initial screening of the cDNA library by immunological plaque screening was not successful, therefore we investigated whether purified, non-denatured, rhamnogalacturonan hydrolase A was recognized by the antiserum by spotting

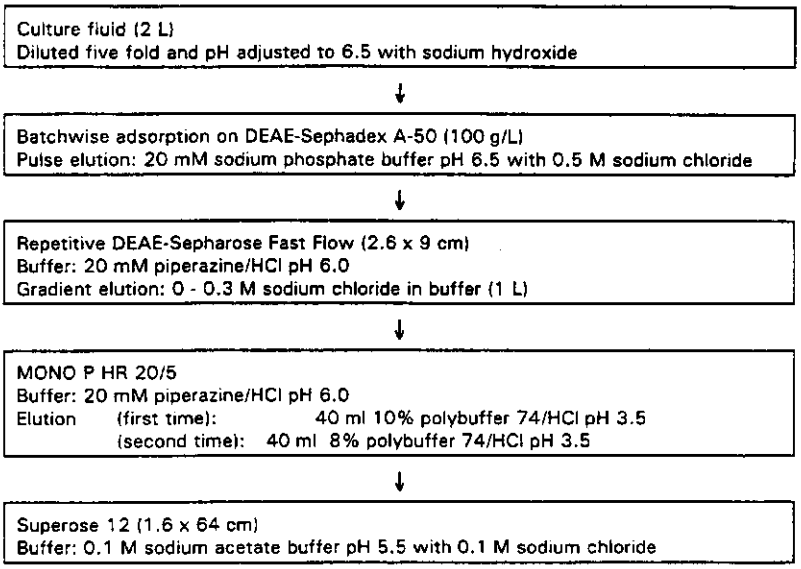


Fig. 2 Flow chart of the purification of rhamnogalacturonan hydrolase A from *A. aculeatus*. Details of the different purification steps are described in Results

the protein directly onto nitrocellulose. Unlike the results obtained with SDS-PAGE followed by Western analysis, no rhamnogalacturonan hydrolase A could be detected. Therefore small aliquots of the protein were pretreated with 2-mercaptoethanol, SDS, guanidinium thiocyanate and urea or denatured by heat at different temperatures, spotted onto nitrocellulose and then analysed by Western analysis. The conditions under which a clear signal could be obtained were pretreatment of rhamnogalacturonan hydrolase A with at least 6 M urea or heat denaturation prior to incubation with the antiserum.

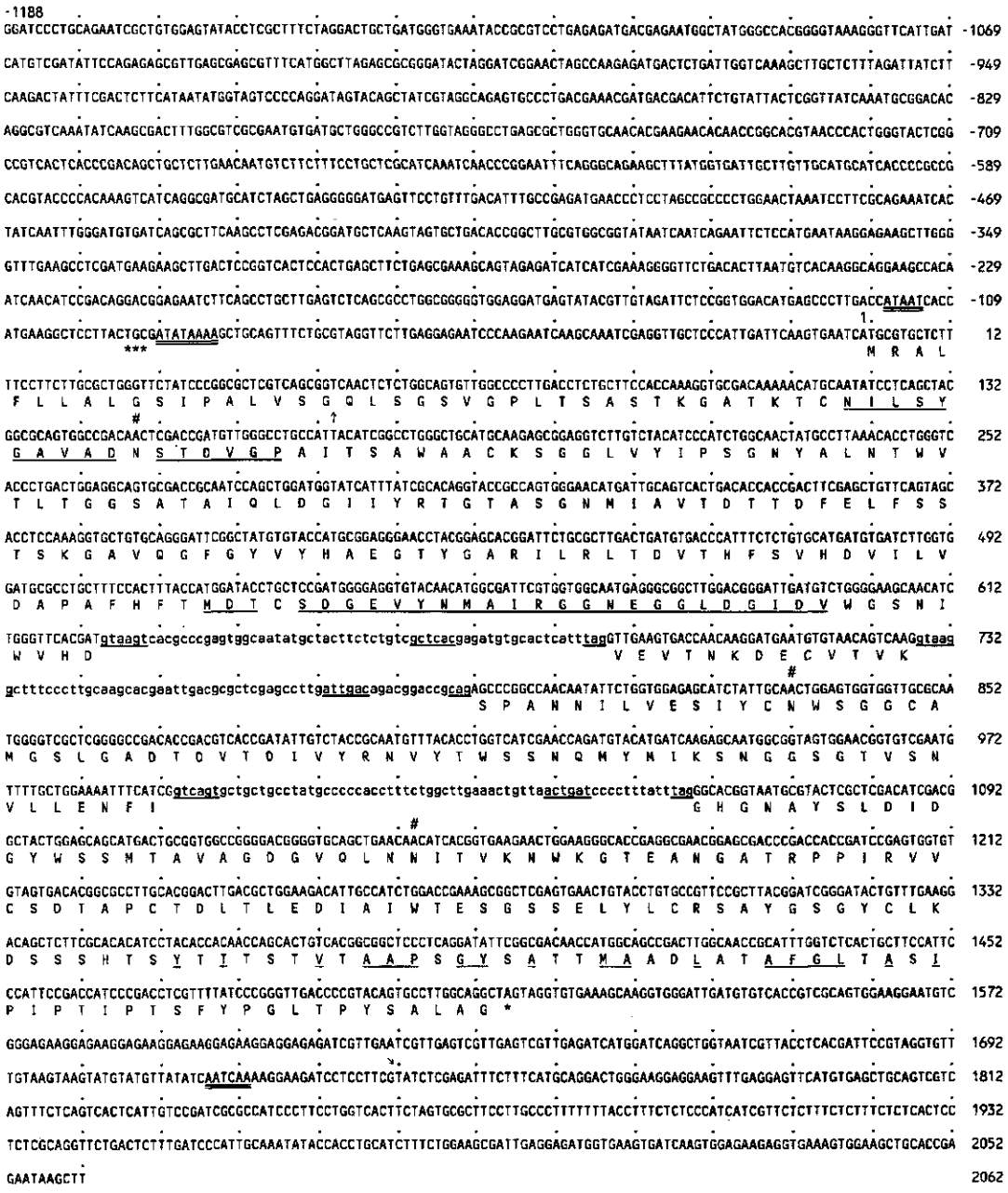
Therefore the inserts of λZAPII were excised and the colonies generated, containing recombinant pBluescriptSK(+) plasmids, were lysed in the presence of 8 M ureum followed by screening with the polyclonal antiserum. Screening of 10^5 colonies resulted in detection of 36 immunologically reactive clones. Restriction analysis of four independent clones revealed that two clones contained an insert of approximately 1.5 kb and two clones contained an insert of approximately 1

kb. All four clones contained the same 0.5-kb *Xho*I fragment (Fig. 1A). This *Xho*I fragment was used as a probe to screen an *A. aculeatus* genomic library in λEMBL4. Approximately $4x10^4$ plaques, estimated to represent about 20 times the size of the *Aspergillus* genome, were screened in duplicate. Eighteen hybridizing plaques were subjected to a rescreening procedure and remained positive. DNA of four positive clones and genomic DNA of *A. aculeatus* were subjected to Southern analysis, using a 1.2-kb *Kpn*I-*Xho*I fragment of pIM801 as a probe (Fig. 1A). Only one of the four phages contained the *Bam*HI, *Eco*RI, *Hin*dIII and *Sal*I fragments corresponding to the hybridizing genomic DNA fragments. A limited restriction map could be made, suggesting that the complete *rhg*A gene is present on a 3.9-kb *Bam*HI-*Sal*I fragment. Subcloning of this fragment in pBluescriptSK(+) resulted in pIM803 (Fig. 1B).

Both strands of the cDNA insert of pIM801 were completely sequenced and were found to comprise the entire coding region of the *rhg*A gene with a 24-bp leader sequence. The methionine at position 1 is followed by an open-reading frame comprised of 439 amino acids, encoding a putative protein of 46 kDa and a deduced isoelectric point of 4.2. A probable signal peptidase cleavage site is located between the glycine at position 18 and the glutamine at position 19 (Von Heijne 1986) and three consensus sites for N-linked glycosylation are found.

To confirm the reading frame of the *rhg*A cDNA sequence, purified rhamnogalacturonan hydrolase A was subjected to amino acid sequencing. However, we failed to determine the amino acid sequence of the N-terminal end of the mature protein. Therefore the protein was fragmented by CNBr treatment and endoproteinase Lys-C treatment. After separation by HPLC, selected fractions were subjected to amino acid sequence analysis. Four of the five amino acid sequences obtained, corresponding to two CNBr fragments (CNBr7 and 15) and two proteolytically obtained endoproteinase Lys-C fragments (Endolys5 and 7) were found within the deduced amino acid sequence of rhamnogalacturonan hydrolase A with the Endolys5 fragment starting at Lys-37 (Fig. 3).

The DNA sequence of the internal 3.3-kb *Bam*HI-*Hin*dIII fragment of the genomic



clone pIM803 (Fig. 1B) was determined in both strands and comprised the entire coding region of the *rhg*A gene and 1.2 kb of the promoter region. Comparison of the genomic DNA sequence with the cDNA sequence unambiguously identified the position and the size of three introns of respectively 64, 64 and 66 bp (Fig. 3).

5'- and 3'- non-translated sequences

A transcription start site of the *rhg*A gene was localized by primer extension analysis 91–93 bp upstream from the translation start codon (tacTGCgat) (Figs. 3, 4). In the surrounding sequence two putative TATA boxes were found, ATATAAAA at position –89 and ATAAT at position –117 from the ATG. This last sequence is located upstream from the transcription start site, which makes it more likely to function as a promoter element (Wobbe and Struhl 1990).
The polyadenylation site for the *rhg*A gene is located 224 bp downstream from the stop codon. A typical AATAAA sequence (AATCAAAA; Irniger and Braus 1994) is found 24 bp before the polyadenylation site.

Overexpression of the *rhg*A gene in *A. aculeatus* and *A. awamori*

In order to verify the presence of a complete and functional *rhg*A gene in pIM-803 and to generate a rhamnogalacturonan hydrolase overproducing strain, the uridine auxotroph *A. aculeatus* NW217 was cotransformed with pIM803 and pGW635 containing the *A. niger pyr*A gene or pIM805 containing the *A. aculeatus pyr*A gene. The resulting transformants were grown on sugar-beet pulp medium for 48 h. According to a Western analysis of dilutions of the culture

Fig. 3 Nucleotide sequence and deduced amino acid sequence of the *rhg*A gene of *A. aculeatus*. The amino acids determined from the CNBr and endoproteinase Lys-C fragments are *underlined*. ↑ The suggested signal peptidase cleavage site; # potential *N*-glycosylation sites. Intervening sequences are in *lower case* and intron-exon consensus sequences are indicated by *single underlining*, two putative TATA boxes and a putative polyadenylation signal are marked by *double underlining*. * Transcription start site; ↘ the polyadenylation site

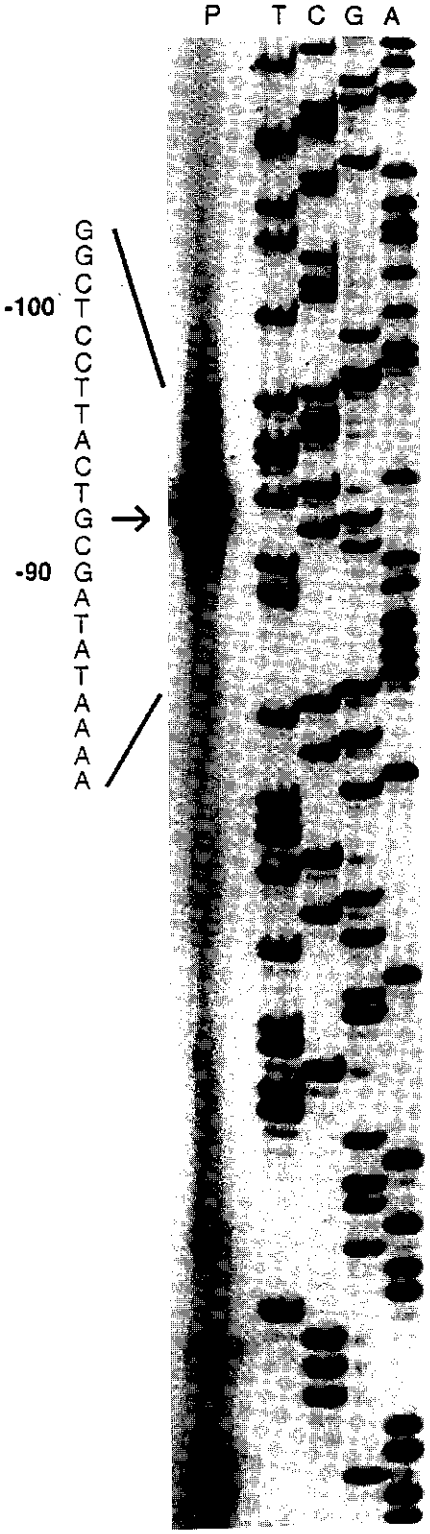


Fig. 4 Primer extension analysis of *A. aculeatus rhg*A mRNA. Primer extension products (*P*) were co-electrophoresed with sequencing reactions (*T,C,G,A*) carried out with the same primer (5'AAAGAGCACGCA TGATT3'). The sequencing reactions have been labelled in reverse order (TCGA) to show the sequence in the sense strand (corresponding to the RNA sequence) for easier interpretation. The transcription start site is marked with an *arrow*. Nucleotides between – 103 and – 82 are indicated

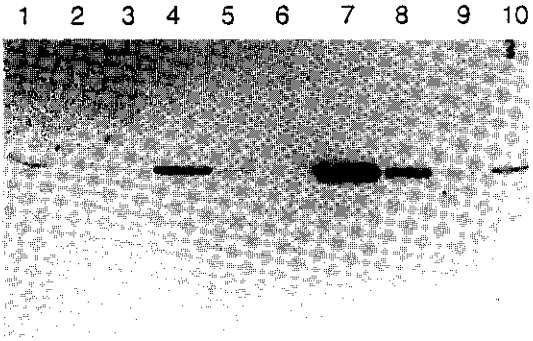


Fig. 5 Western analysis of culture filtrate of *A. aculeatus* wild type (*lanes* 1–3), *A. aculeatus* multicopy transformant NW217::pIM 803/75 (*lanes* 4–6) and *A. awamori* multicopy transformant NW208::pIM816/3 (*lanes* 7–9). The strains were grown for 24 h in minimal medium with 1% apple pectin (*A. aculeatus*) or 1% xylose (*A. awamori*) as carbon source. Medium samples were applied undiluted (*lanes* 1,4,7), 10 times diluted (*lanes* 2,5,8) or 100 times diluted (*lanes* 3,6,9). Lane 10 contains purified rhamnogalacturonan hydrolase A

filtrate rhamnogalacturonan hydrolase A was produced at a five to ten times higher level compared to the wild type strain in most of the transformants (e.g. NW217::pIM803/75; Fig. 5, lanes 4–6). Furthermore, when equivalent amounts of DNA were used a higher transformation efficiency was achieved with the *A. aculeatus pyr*A gene compared to the *A. niger pyr*A gene (results not shown).

In order to achieve higher rhamnogalacturonan hydrolase expression, *A. awamori* NW208 was cotransformed with pIM816, containing the *rhg*A gene under control of the *A. awamori exl*A promoter, and the homologous *pyr*A gene. Twelve transformants were grown in xylose medium (see Materials and methods) and the culture filtrates were analysed by Western blotting. Four of the analysed transformants (e.g. transformant NW208::pIM816/3; Fig. 5, lanes 7–9) produced rhamnogalacturonan hydrolase A at a level that was more than ten times higher than in the wild type *A. aculeatus* strain grown on beet pulp or pectin. In five other transformants no rhamnogalacturonan hydrolase A could be detected (results not shown), probably in this case only the *pyr*A gene had been integrated in the genome of these transformants. After cotransformation of *A. aculeatus* strain NW217 with pIM816 and pIM805 and cultivation of the transformants obtained in xylose medium, rhamnogalacturonan hydrolase A expression levels comparable to those in the *A. awamori* transformants were observed (results not shown).

To investigate the regulation of expression of *rhg*A in *A. aculeatus*, the wild type strain and multicopy transformant NW217::pIM803/75 were grown overnight in MM with 1% sucrose and transferred to MM with 1% apple pectin. RNA was isolated from mycelium of both strains just before and 6 h and 24 h after a shift to apple pectin and compared by Northern analysis. No *rhg*A expression could be detected in either strain grown on sucrose (Fig. 6A,B, lane 1). However, 6 h after the shift to apple pectin the *rhg*A mRNA level in the multicopy transformant was very high and declined to a low level after 24 h (Fig. 6A, lanes 2,3). In the wild type strain the *rhg*A mRNA was only visible 6 h after transfer, but at a lower level (Fig. 6B, lanes 2,3). The same blots were reprobed with part of the 28S ribosomal RNA gene of *A. bisporus* to verify equal loading

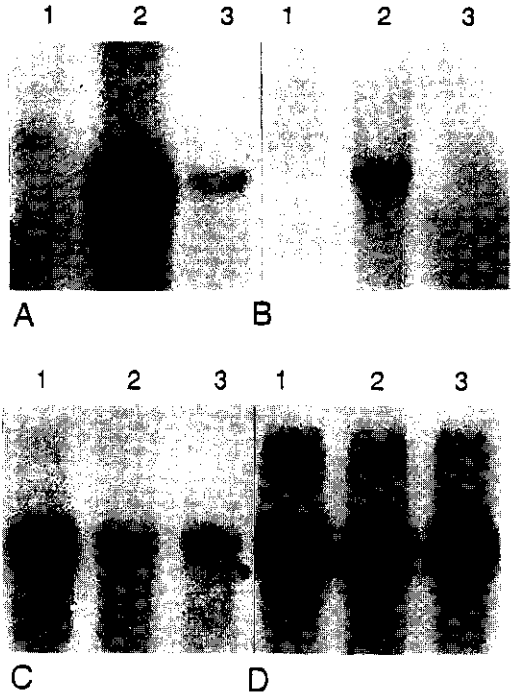


Fig. 6A-D Northern analysis of *A. aculeatus* transformant NW217::pIM803/75 (**A**) compared to the wild type (**B**). RNA was isolated from the mycelium before (*lanes* 1), 6 h after (*lanes* 2) and 24 h after (*lanes* 3) transfer of the corresponding strains to minimal medium (MM) with 1% apple pectin. Before transfer, the strains were grown for 24 h in MM with 1% sucrose. The location of the *rhg*A transcript is indicated with an *arrow* (**A,B**). The 28S ribosomal RNA level was determined as loading control (**C,D**)

(Fig. 6C,D). Figure 6C shows less RNA loaded in lanes 2 and 3, compared to lane 1. However, this does not influence the outcome of the results.

Rhamnogalacturonan hydrolase A activity and application

Rhamnogalacturonan hydrolase activity was demonstrated by modified hairy region degradation as described in Materials and methods. One of the *A. awamori* transformants (NW208::pIM816/3), in which high expression levels of *A. aculeatus* rhamnogalacturonan hydrolase A were obtained under the control of the *A. awamori exl*A promoter (see above), was grown on xylose medium as described in Materials and methods. Culture filtrate (10 μl) of this transformant was incubated with modified hairy regions for 2 h and analysed by HPAE chromatography analysis. Figure 7 shows the elution profile of isolated modified hairy regions without the addition of enzymes (Fig. 7A), upon incubation with

Biopectinase OS (Fig. 7B) and upon incubation with rhamnogalacturonan hydrolase A containing culture filtrate of *A. awamori* transformant NW208::pIM 816/3 (Fig. 7C). It is clear, that the isolated MHR can only be degraded by rhamnogalacturonan hydrolase A containing culture filtrate and not by Biopectinase. Using standard solutions it was shown that the peak at 23 min represents galacturonic acid. The other peaks of higher molecular mass could not be identified but presumably represent tetramers and hexamers containing rhamnose and galacturonic acid residues. As shown in Fig. 7 the addition of rhamnogalacturonan hydrolase A results in the appearance of various oligosaccharides from the substrate and hardly any galacturonic acid release, demonstrating the endo-activity of rhamnogalacturonan hydrolase A.

Application trials were performed by incubation of apple mash with Biopectinase LQ (Biopectinase OS supplemented with cellulases, 0.5 g/kg) in the presence or absence of culture filtrate of the rhamnogalacturonan hydrolase A producing wild type or transformant strains. For this, *A. aculeatus* wild type and transformant (NW217::pIM803/75) strains were grown on apple pectin and the *A. awamori* transformant (NW208::pIM816/3) strain was grown on xylose for 24 h as indicated in Materials and methods. A 3-ml sample of culture filtrate was added to the apple mash and juice yield was measured after 2 h of incubation. A 10% increase in yield at equal brix value was observed when rhamnogalacturonan hydrolase A was added. The effect of addition of rhamnogalacturonan hydrolase A in viscosity reduction of apple mash is shown in Fig. 8. Obviously the presence of rhamnogalacturonan hydrolase A has a positive effect on viscosity reduction especially in the initial phase of hot-mash liquefaction. The effect is most clearly demonstrated when rhamnogalacturonan hydrolase A from the *A. aculeatus* and *A. awamori* transformants is used as compared to that from the wild type strain, reflecting the higher production levels of these recombinant strains.

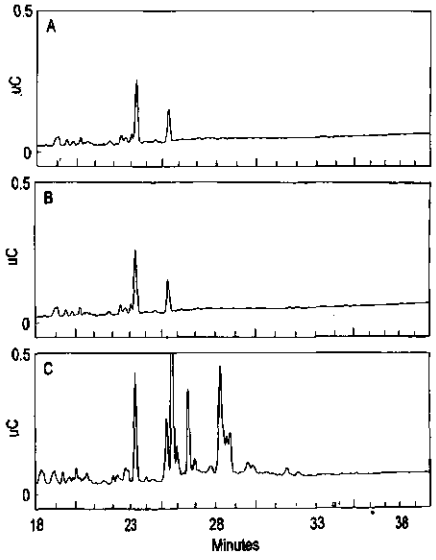


Fig. 7A-C High-performance anion-exchange chromatography elution profile of isolated modified hairy regions without addition of enzymes (**A**), with Biopectinase OS (**B**) and with rhamnogalacturonan hydrolase A containing culture filtrate from *A. awamori* transformant NW208::pIM816/3 (**C**). µC: µCoulomb

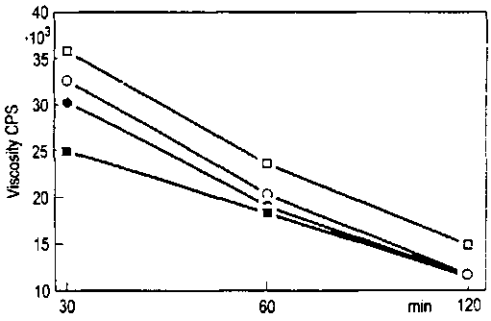


Fig. 8 The influence of rhamnogalacturonan hydrolase A produced by wild type and rhamnogalacturonan hydrolase A overproducing *Aspergillus* strains on viscosity reduction during apple hot-mash liquefaction. Apple mash was incubated with Biopectinase LQ alone (□) or in combination with rhamnogalacturonan hydrolase A, produced by *A. aculeatus* wild type (○) and transformant strain NW217::pIM803/75 (●), and *A. awamori* transformant strain NW208::pIM816/3 (■)

Discussion

Rhamnogalacturonan hydrolase A is a newly characterized pectinolytic enzyme, which can degrade hairy regions of pectin. As the amount of this enzyme is limited in commercial enzyme preparations, gene cloning provides an attractive tool to achieve rhamnogalacturonan hydrolase overproducing strains.

Our strategy for cloning the gene encoding *A. aculeatus* rhamnogalacturonan hydrolase A was to construct a cDNA library, using RNA from mycelium that had been grown in apple pectin medium. Despite the specificity of this library and the synthesis of cDNA molecules reaching at least up to 1.5 kb, there were difficulties with the immunological detection of rhamnogalacturonan hydrolase A

(fusion) proteins. Since rhamnogalacturonan hydrolase A could be visualized in the culture filtrate by SDS-PAGE and Western blot analysis, the most likely explanation for this phenomenon seemed to be a failure in the recognition of in *E. coli*-expressed rhamnogalacturonan hydrolase A (fusion) proteins by the antiserum. Indeed, the conformation of the rhamnogalacturonan hydrolase A protein appeared to define recognition by the antiserum, since purified rhamnogalacturonan hydrolase A could only be detected on blots after denaturation of the protein by boiling it or by treatment with 8 M urea. Therefore we decided to adapt the screening procedure in two ways. First, we randomly excised inserts of the λ cDNA library and, second, we added a denaturing step before the immunological screening of the obtained pBluescript plasmids. The use of urea for this purpose has been described before (Lebras et al. 1989). In this way we could detect rhamnogalacturonan hydrolase A (fusion) proteins with the antiserum and succeeded in cloning the gene coding for rhamnogalacturonan hydrolase A from the cDNA library of *A. aculeatus*. Evidence for this is that amino acid sequences of two CNBr and two endoproteinase Lys-C fragments of rhamnogalacturonan hydrolase A were present in the deduced amino acid sequence of the *rhg*A cDNA. Since no residues were found upon degradation of the N terminus of the purified protein, we concluded that the N terminus was blocked. Cleavage of the indicated putative signal sequence of 18 amino acids results in a mature protein with a glutamine at the N-terminal end. This glutamine residue can be modified to a pyroglutamine, as was previously described for a cellulase protein of *A. aculeatus* (Ooi et al. 1990). This modification is very resistant to Edman degradation and can be the cause of the problems encountered with sequencing the N terminus of rhamnogalacturonan hydrolase A.

The cDNA clone pIM801 contains the whole coding region of *rhg*A and a 24-bp leader sequence, as in the genomic *rhg*A sequence stop codons are present before the supposed ATG in all three frames. The region around the start codon conforms to the consensus sequence in eukaryotic genes (Kozak 1989).

The calculated molecular mass of rhamnogalacturonan hydrolase A is 45 962

Da, while after SDS-PAGE analysis a molecular mass of approximately 55 kDa is found. However, the lower calculated molecular mass corresponds well to the value for rhamnogalacturonan hydrolase A after *N*-glycanase treatment, making it very likely that the protein is glycosylated.

Three introns are found in the *rhg*A gene and the length of these introns conforms to those found in many other *Aspergillus* genes (Hawkins 1988). The nucleotide sequences of the intron-exon boundaries resemble the consensus sequences (Unkles 1992; Ballance 1986). In between the translation stop codon and the polyadenylation site the sequence GGAGAA is repeated five times. Similar repeats are found in trailer sequences of other fungal genes and it has been previously suggested that such repeats could play a role in polyadenylation site selection or mRNA stability (Frederick et al. 1990).

The increased expression of rhamnogalacturonan hydrolase A when the gene was integrated in multiple copies into the genome of *A. aculeatus* provides the evidence for the functionality of the cloned *rhg*A gene. Moreover, a proportionally higher rhamnogalacturonan hydrolase activity in fermentation cultures of transformant strains is demonstrated in a viscosity assay compared to the wild type strain using the same fermentation conditions. Besides, rhamnogalacturonan hydrolase activity in an *A. awamori* transformant is shown by formation of oligosaccharides from modified hairy regions. The HPAE chromatography profile obtained resembles that obtained by Schols et al. (1994).

The level of rhamnogalacturonan hydrolase A production in the multicopy transformant NW217::pIM803/75, as determined by Western blot analysis, is five to ten times higher than that in the wild type. This is in accordance with the copy number of the *rhg*A gene integrated in the genome of this transformant (data not shown).

Degradation of pectin requires the sequential attack by a variety of pectinolytic enzymes, with rhamnogalacturonan hydrolase A degrading the residual hairy regions. Northern analysis shows that the highest level of *rhg*A transcription is reached after 6 h of growth in apple pectin medium.

We compared the deduced amino acid sequence of *A. aculeatus* rhamnogalactu-

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RHGA 188  -GGNEGGL----DGIDVWGSN-IWVHDVEVTNKDECVTVKSPANNILVES 231
PGI  175  SDGDDNG-GHNTDGFDISESTGVYISGATVKNQDDCIAINS-GESISFTG 222
PGII 169  ADGDTQG-GHNTDAFDVGNSVGVNIIKPWVHNQDDCLAVNS-GENIWFTG 216
PGC  188  TDGDTDDLAANTDGFDIGESTYITITGAEIYNQDDCVAINS-GENIYFSA 236
           .*.. .     *..*. .*  . .    . *.*.*....* ...*  ..

RHGA 232  IYCNWSGGCAMGSLGADTD--VTDIVYRNVYTWSSNQMYMIKS-NGGSGT 278
PGI  223  GTCSGGHGLSIGSVGGRDDNTVKNVTISDSTVSNSANGVRIKTIYKETGD 272
PGII 217  GTCIGGHGLSIGSVGDRSNNVVKNVTIEHSTVSNSENAVRIKTISGATGS 266
PGC  237  SVCSGGHGLSIGSVGGRDDNTVKNVTFYDVNVLKSQQAIRIKTIYGDTGS 286
            *  . * ..**.*. ..  *.... .  . .*..  .**.   ..*.

RHGA 279  VSNVLLENF-IGHGNAYSLDIDGYW--SSMTAVAGDGVQLNNITVKNWKG 325
PGI  273  VSEITYSNIQLSGITDYGIVIEQDYENGSPTGTPSTGIPITDVTVDGVTG 322
PGII 267  VSEITYSNIVMSGISDYGVVIQQDYEDGKPTGKPTNGVTIQDVKLESVTG 316
PGC  287  VSEVTYHEIAFSDATDYGIVIEQNYDDTSKT--PTTGVPITDFVLENIVG 334
           **..   .. ..  ..*.. *.  .  .. *  ...*. . .  ...  *
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Fig. 9 Comparison of the amino acid sequence of rhamnogalacturonan hydrolase (RHG-A) of *A. aculeatus* with polygalacturonases PGI, PGII and PGC of *A. niger*. The most homologous region of the proteins is shown. Numbering is from the first amino acid of the signal peptide. * Identical amino acids; • similar amino acids. The amino acid residues in bold type are referred to in the text

ronan hydrolase A with those of the *A. niger* polygalacturonases PGI, PGII and PGC (Bussink et al. 1992). Whereas little overall homology is found (10.3%) after alignment by CLUSTAL (Higgins and Sharp 1988), residues which are conserved between rhamnogalacturonan hydrolase A and the polygalacturonases are particularly located in those regions, that were designated as relevant for polygalacturonase activity (Bussink et al. 1991). The possible candidates for carboxylate groups involved in catalysis in endo-PGII were postulated to correspond with aspartic acid residues at positions 180, 201 and 202. These three negative charges are conserved in rhamnogalacturonan hydrolase A (Asp-195, Asp-215 and Glu-216, Fig. 9). Also, the nearby aspartic residue at position 183 in PGII is conserved in rhamnogalacturonan hydrolase A (position 198). However, this residue was not included as a potential candidate because its equivalent is not found in tomato polygalacturonase (Bussink et al. 1991). Furthermore, two of the eight cysteine residues that are conserved between rhamnogalacturonan hydrolase A and the polygalacturonases, are located in this part of

the sequence. The positively charged sequence Arg-Ile-Lys (residues 256–258) in PGII, postulated to play a role in substrate binding, is not fully conserved in rhamnogalacturonan hydrolase A. The sequence reads in this case Met-Ile-Lys (residues 269–271). Another postulated active-site residue (His-223 in PGII), which is located in a conserved sequence Gly-His-Gly, present in all polygalacturonases, is absent in rhamnogalacturonan hydrolase A at the corresponding position, but is present at position 289–291. The homology observed between rhamnogalacturonan hydrolase A and endo-PG is relevant for further investigations of the catalytic mechanism and the different substrate specificities of these two pectinolytic hydrolases.

Note During the time the presently described *rhg*A gene has been characterized, a homologous *rhg*A gene from another *A. aculeatus* strain, CBS101.43, was cloned by Kofod et al. (1994). The nucleotide sequences of both genes share 96% identity, whereas only three of the deduced amino acids, located at the positions 3, 11 and 161, are different. The 5'- and 3'- non-translated sequences of the two *rhg*A genes share 87% and 77% identity, respectively. In the 3'- region this homology abruptly stops with the GGAGAA repeat described above. At the same location the sequence CATTGAGAT is repeated six times in the *rhg*A gene from strain CBS101.43

Acknowledgements This project was financed by Unilever Research Laboratorium. The authors thank Harry Kester and Peter van der Veen for purifying rhamnogalacturonan hydrolase A from *A. aculeatus*, Peter van de Vondervoort for construction of *A. aculeatus* strain NW217 and Henk Hennink, Diederik Schmedding and Ailin Casey from Quest International for performing MHR breakdown studies and application experiments

References

Ballance DJ (1986) Sequences important for gene expression in filamentous fungi. Yeast 2:229-236

Biekman ESA, Deelen W van, Voragen AGJ (1990) Enzymatic modification of potato and plant tissue. In: Dekkers JJ, Plas HC van der, Vuijk DH (eds) Agricultural biotechnology in focus in the Netherlands, PUDOC Wageningen, pp 183-187

Bos CJ (1987) Induction and isolation of mutants in fungi at low mutagen doses. Curr Genet 12:471-474

Burnette WN (1981) "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein. Anal Biochem 112:195-203

Bussink HJD, Buxton FP, Visser J (1991) Expression and sequence comparison of the *Aspergillus niger* and *Aspergillus tubigensis* genes encoding polygalacturonase II. Curr Genet 19:467-474

Bussink HJD, Buxton FP, Fraaye BA, Graaff LH de, Visser J (1992) The polygalacturonases of *Aspergillus niger* are encoded by a family of diverged genes. Eur J Biochem 208:83-90

Colquhoun IJ, Ruiter GA de, Schols HA, Voragen AGJ (1990) Identification by n.m.r. spectroscopy of oligosaccharides obtained by treatment of the hairy regions of apple pectin with rhamnogalacturonase. Carbohydr Res 206:131-144

De Graaff LH, Broek HWJ van den, Visser J (1988) Isolation and transformation of the pyruvate kinase gene of Aspergillus nidulans. Curr Genet 13:315-321

Frederick KR, Tung J, Emerick RS, Masiarz FR, Chamberlain SH, Vasavada A, Rosenberg S (1990) Glucose oxidase from *Aspergillus niger*. J Biol Chem 265:3793-3802

Goosen T, Bloemheuvel G, Gysler C, Bie DA de, Broek HWJ van den, Swart K (1987) Transformation of *Aspergillus niger* using the homologous orotidine-5'-phosphate-decarboxylase gene. Curr Genet 11:499-503

Gouka RJ, Hessing JGM, Stam H, Musters W, Hondel CAMJJ van den (1995) A novel strategy for the isolation of defined *pyr*G mutants and the development of a site-specific integration system for *Aspergillus awamori*. Curr Genet 27:536-540

Harmsen JAM, Kusters-van Someren MA, Visser J (1990) Cloning and expression of a second *Aspergillus niger* pectin lyase gene (*pel*A): indications of a pectin lyase gene family in *Aspergillus niger*. Curr Genet 18:161-166

Hawkins J (1988) A survey on intron and exon lengths. Nucleic Acids Res 16:9893-9908

Hessing JGM, Rotterdam C van, Verbakel JMA, Roza M, Maat J, Gorcom RFM van, Hondel CAMJJ van den (1994) Isolation and characterization of a 1,4-β-endoxylanase gene of *A. awamori*. Curr Genet 26:228-232

Higgins DG, Sharp PM (1988) CLUSTAL: a package for performing multiple sequence alignment on a microcomputer. Gene 73:237-244

Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77:61-68

Irniger S, Braus G (1994) Saturation mutagenesis of a polyadenylylation signal reveals a hexanucleotide element essential for mRNA 3' end formation in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 91:257-261

Khanh NQ, Ruttkowski E, Leidinger K, Albrecht H, Gottschalk M (1991) Characterization and expression of a genomic pectin methyl esterase-encoding gene in *Aspergillus niger*. Gene 106:71-77

Kofod LV, Kauppinen S, Christgau S, Andersen LN, Heldt-Hansen HP, Dörreich K, Dalbøge H (1994) Cloning and characterization of two structurally and functionally divergent rhamnogalacturonases from *Aspergillus aculeatus*. J Biol Chem 269:29182-29189

Kozak M (1989) The scanning model for translation: an update. J Cell Biol 108:229-241

Lahaye M, Vigouroux J, Thibault J (1991) Endo-β-1,4-D-galactanase from *Aspergillus niger* var. *aculeatus*: purification and some properties. Carbohydr Polymers 15:431-444

Lebras G, Teschner W, Devillebonne D, Garel JR (1989) Urea-induced inactivation, dissociation and unfolding of the allosteric phosphofructokinase from *Escherichia coli*. Biochemistry 28:6836-6841

Ooi T, Shinmyo A, Okada H, Hara S, Ikenaka T, Murao S, Arai M (1990) Cloning and sequence analysis of a cDNA for cellulase (FI-CMCase) from *A. aculeatus*. Curr Gen 18:217-222

Pontecorvo G, Roper JA, Hemmons LJ, MacDonald KD, Bufton AWJ (1953) The genetics of *Aspergillus nidulans*. Adv Genet 5:141-238

Ruttkowski E, Khanh NQ, Wientjes FJ, Gottschalk M (1991) Characterization of a polygalacturonase gene of *Aspergillus niger* RH5344. Mol Microbiol 5:1353-1361

Sambrook J, Fritsch E, Maniatis T (1989) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Schols HA, Geraeds CJM, Searle-van Leeuwen MF, Kormelink FJM, Voragen AGJ (1990a) Rhamnogalacturonase: a novel enzyme that degrades the hairy regions of pectins. Carbohydr Res 206:105-115

Schols HA, Posthumus MA, Voragen AGJ (1990b) Structural features of hairy regions of pectins isolated from apple juice produced by the liquefaction process. Carbohydr Res 206:117-129

Schols HA, Voragen AGJ, Colquhoun IJ (1994) Isolation and characterization of rhamnogalacturonan oligomers, liberated during degradation of pectic hairy regions by rhamnogalacturonase. Carbohydr Res 256:97-111

Searle-van Leeuwen MJF, Broek LAM van den, Schols HA, Beldman G, Voragen AGJ (1992) Rhamnogalacturonan acetylesterase: a novel enzyme from *Aspergillus aculeatus*, specific for the deacetylation of hairy (ramified) regions of pectins. Appl Microbiol Biotechnol 38:347-349

Sreenath HK, Santhanam K (1992) The use of commercial enzymes in white grape juice clarification. J Ferment Bioeng 73:241-243

Teeri T, Kumar V, Lehtovaara P, Knowles J (1987) Construction of cDNA libraries by blunt-end ligation: high frequency cloning of long cDNAs from filamentous fungi. Anal Biochem 164:60-67

Uitzetter JHAA, Bos CJ, Visser J (1986) Characterization of *Aspergillus nidulans* mutants in carbon metabolism isolated after D-galacturonate enrichment. J Gen Microbiol 132:1167-1172

Unkles SE (1992) Gene organization in industrial filamentous fungi. In: Kinghorn JR, Turner G (eds) Applied molecular genetics of filamentous fungi. Chapman London, pp 28-53

Van der Veen P, Flipphi MJA, Voragen AGJ, Visser J (1991) Induction, purification and characterization of arabinases produced by *Aspergillus niger*. Arch Microbiol 157:23-28

Van de Vis J, Searle-van Leeuwen M, Siliha H, Kormelink F, Voragen AGJ (1991) Purification and characterization of endo-1,4-β-D-galactanases from *Aspergillus niger* and *Aspergillus aculeatus*: use in combination with arabinases from *Aspergillus niger* in enzymic conversion of potato arabinogalactan. Carbohydr Polymers 16:167-187

Von Heijne G (1986) A new method for predicting signal sequence cleavage sites. Nucleic Acids Res 14:4683-4690

Wobbe CR, Struhl K (1990) Yeast and human TATA-binding proteins have nearly identical DNA sequence requirements for transcription in vitro. Mol Cell Biol 10:3859-3867

3 | Cloning and characterization of two rhamnogalacturonan hydrolase encoding genes from *Aspergillus niger*

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Abstract A rhamnogalacturonan hydrolase encoding gene of *Aspergillus aculeatus* was used as a probe for the cloning of two rhamnogalacturonan hydrolase encoding genes of *Aspergillus niger*. The corresponding proteins, rhamnogalacturonan hydrolase A and B are 78% and 72% identical with the *A. aculeatus* enzyme. In *A. niger* cultures which were shifted from growth on sucrose to growth on apple pectin as carbon source, the expression of the rhamnogalacturonan hydrolase A encoding gene (*rhg*A) was transiently induced after 3 h of growth on apple pectin. The rhamnogalacturonan hydrolase B encoding gene (*rhg*B) was not induced by apple pectin but was derepressed after 18 h of growth on either apple pectin or sucrose.

Gene fusions of the *A. niger rhg*A and *rhg*B coding regions with the strong and inducible *Aspergillus awamori exl*A promoter were used to obtain high-producing *A. awamori* transformants which were then used for the purification of the two *A. niger* rhamnogalacturonan hydrolases. High-performance anion-exchange chromatography of oligomeric degradation products showed that optimal degradation of isolated highly branched pectin fractions by *A. niger* rhamnogalacturonan hydrolase A and B occurred at pH 3.6 and pH 4.1 respectively. The specific activities of rhamnogalacturonan hydrolase A and B were then 0.9 U/mg and 0.4 U/mg respectively, which is significantly lower than the specific activity of *A. aculeatus* rhamnogalacturonan hydrolase (2.5 U/mg at an optimal pH of 4.5). However, compared to the A-enzymes, the *A. niger* B-enzyme appears to have a different substrate specificity since additional oligomers are formed.

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Introduction

Pectin is a heteropolysaccharide and a major constituent of the middle lamella of the primary cell wall of plants. It is composed of smooth homogalacturonan regions and highly branched or hairy regions. Hairy regions consist of (1,4)-linked *α*-D-galacturonic acid residues, alternating with (1,2)-linked *α*-L-rhamnose residues. About half of the rhamnose residues is branched with L-arabinosyl and/or D-galactosyl containing side chains. *O*-acetyl-groups and xylosyl-groups can be linked to galacturonic acid residues, of which some are methylated. Isolated hairy regions are referred to as modified hairy regions (MHR), since the isolation procedure may have altered their sugar composition and degree of methylation (Schols et al. 1990). Structural analysis of MHR from several plants revealed differences in the rhamnose:galacturonic acid ratio, varying between 0.41 in apple to 0.63 in leek, and differences in the degree of acetylation (Schols and Voragen 1994).

Other rhamnogalacturonan structures, designated RG-I and RG-II, have been isolated from sycamore cells (O'Neill et al. 1990). In RG-I an extremely high rhamnose:galacturonic acid ratio of 1 is found (Lau et al. 1985), whereas in RG-II aberrant *α*-D-galacturonic acid-(1,2)-*β*-L-rhamnose and *β*-D-galacturonic acid-(1,3)-*β*-L-rhamnose linkages are found together with many in pectin rare sugar residues in the side chains (Puvanesarajah et al. 1991).

Plants, fungi and bacteria degrade pectin by the production of various pectinolytic enzymes. There is interest in the application of these enzymes in industrial food processing such as clarification of fruit juices. Here it is important that pectins can be completely degraded (Whitaker 1984). Expression of enzymes acting on the smooth regions of pectins, such as polygalacturonases, pectin lyases and a pectin methylesterase, have been extensively studied in *Aspergillus niger* (Visser et al. 1994). Because of the complex structure, complete breakdown of hairy regions probably requires the (synergistic) action of several enzymes. Up to now two enzymes, which are able to split the rhamnogalacturonan backbone in MHR, have been isolated from *Aspergillus aculeatus* and the

corresponding genes have been cloned (Kofod et al. 1994; Suykerbuyk et al. 1995). One of these enzymes, a rhamnogalacturonan hydrolase, hydrolyzes α-D-galacturonic acid-(1,2)-α-L-rhamnose linkages in MHR (Schols et al. 1990), whereas the other enzyme, a rhamnogalacturonan lyase, degrades α-L-rhamnose-(1,4)-α-D-galacturonic acid linkages by β-elimination (Mutter et al. 1996). Both enzymes thus can degrade MHR, but enhanced activities of those enzymes are obtained after chemical pretreatment of the substrate. This can be achieved by saponification of ester-linkages or by removal of side chains with hydrochloric acid (Schols et al. 1990). The ester-bonds can also be hydrolyzed enzymatically by a rhamnogalacturonan acetylesterase (Searle-van Leeuwen et al. 1992).

The complex structure of MHR and the synergism between different MHR degrading enzymes, suggest a coordinated expression of the corresponding genes. We decided to investigate rhamnogalacturonan hydrolase gene expression in *A. niger*, because pectinolytic gene expression is genetically and physiologically better characterized in this organism than in other fungi (Visser et al. 1994). Molecular characterization of rhamnogalacturonan hydrolase encoding genes in *A. niger* will therefore contribute to the elucidation of regulatory mechanisms controlling pectinolytic gene expression in *A. niger* and will also allow comparison of enzyme structures in relation to substrate specificities of these enzymes in different species. Here we describe the cloning of two rhamnogalacturonan hydrolase encoding genes from *A. niger* as well as the overexpression, purification and characterization of the encoded enzymes.

Materials and methods

E. coli strains, plasmids and DNA technology. Recombinant λ phages were propagated in *E. coli* LE392 and plasmids in *E. coli* DH5α (Gibco BRL, Gaithersburg, USA). Standard methods were used for propagation and preparation of phage and plasmid DNA, transduction, plaque lift, Southern analysis and cloning (Sambrook et al. 1989). Sequencing was performed using the T7-Sequencing Kit (Pharmacia LKB, Uppsala, Sweden).

Standard and dedicated synthetic oligonucleotides were used as sequencing primers. Restriction enzymes were purchased from BRL.

Plasmid pIM860 contains a genomic 4.5-kb *Bam*HI fragment in pBluescriptSK(+) and comprises the complete *rhg*A gene of *A. niger* (Fig. 1). Plasmid pIM875 contains a genomic 4.5-kb *Eco*RI fragment in pUC19 and comprises the complete *rhg*B gene of *A. niger* (Fig. 1). The plasmid pGW635 contains the *A. niger* orotidine-5'-phosphate decarboxylase gene (*pyr*A) (Van den Hombergh et al. 1996) and pAW4-2A the *A. awamori pyr*A gene (Gouka et al. 1995).

Screening of a genomic DNA library of A. niger. A genomic library of *A. niger* N400 (CBS120.49) in λEMBL4 (Harmsen et al. 1990) was screened for the presence of rhamnogalacturonan hydrolase genes. Nylon filters (Hybond-N, Amersham, Buckinghamshire, England) containing a total of $4x10^4$ pfu were hybridized overnight at 58°C in a buffer containing 50 mM TrisHCl pH 7.4, 1 M sodium chloride, 10 mM EDTA pH 7, 0.5% (w/v) sodium dodecyl sulphate (SDS), 0.1% (w/v) sodium pyrophosphate, 10 x Denhardt solution (Sambrook et al. 1989) and was supplemented with salmon sperm DNA and polyadenylate RNA (both to a final concentration of 5 mg/ml). A 2.5-kb *Hin*dIII fragment encoding the *rhg*A gene of *A. aculeatus* was used as probe. The filters were washed twice for 30 min in a solution containing 2 x SSC, 0.1% (w/v) SDS and 0.1% (w/v) sodium pyrophosphate, at the same temperature. 20 x SSC consists of 3 M sodium chloride and 0.3 M sodium citrate.

Construction of the rhgA and rhgB fusion genes using the exlA promoter. For the construction of the *rhg*A and *rhg*B fusion genes under control of the endo-xylanase A promoter of *A. awamori* (*exl*A; Hessing et al. 1994), it was necessary to remove the *Eco*RI sites downstream from the *rhg*A and *rhg*B genes. In *rhg*A this was performed by ligation of the 2.3-kb *Pst*I-*Nsi*I fragment of pIM860 (see Fig. 1A) into the *Pst*I site of pUC19. A unique *Eco*RI site before the *rhg*B gene was realized by digestion of pIM875 with *Kpn*I and subsequent ligation. In this way the *Eco*RI site downstream of the *rhg*B gene was removed together with the 1.2-kb *Kpn*I fragment (Fig. 1C). Subsequently, a 1.6-kb *Eco*RI fragment of plasmid pUR2930, containing the *exl*A gene promoter, was ligated in front of the respective *rhg* genes resulting in the hybrid genes *exl*A-*rhg*A and *exl*A-*rhg*B. In order to position the *exl*A promoter precisely adjacent to the *rhg*A and *rhg*B coding regions we used an adaptation of the splicing by overlap extension tech-

nique (Horton et al. 1989). Therefore, PCR reactions were performed using the primers (5'CGAGGTTGTTCAAGCGT3'), (5'AGGATGGGAAGAGCAGG**CAT**GATGATTGAAGAAA GCT3'), (5'AGCTTTCTTCAATCATC**ATG**CCTGCTCTTCCCATCCT3')and (5'AGGTGTA AACGTTGCGG3') for *rhg*A and the primers (5'CGAGGTTGTTCAAGCGT3'), (5'GAGAG CTTGTCGAGAAG**CAT**GATGATTGAAGAAAGCT3'), (5'AGCTTTCTTCAATCATC**ATG**CT TCTCGACAAGCTCTC3') and (5'GCCGCCGTCCGTGCCAG3') for *rhg*B (the startcodons of *rhg*A and *rhg*B are in bold). The resulting *rhg*A sequences containing fusion product was digested with *Nsi*I and *Eco*RV and ligated into the *exl*A - *rhg*A hybrid gene digested with *Nsi*I and *Eco*RV. The *rhg*B sequences containing fusion product was digested with *Nsi*I and *Cla*I and ligated into the *exl*A - *rhg*B hybrid gene digested with *Nsi*I and *Cla*I. The resulting plasmids designated pIM836 and pIM846 respectively, are shown in Fig. 1.

Aspergillus transformation and rhamnogalacturonan hydrolase expression. Co-transformation was carried out as described (Kusters-van Someren et al. 1990). The plasmids pIM860 and pIM875 were used in combination with pGW635 for cotransformation of *A. niger* NW128 (*csp*A1, *pyr*A6, *gox*C17, *nic*A1; Witteveen et al. 1993). The plasmids pIM836 and pIM846 were used in combination with pAW4-2A for cotransformation of *A. awamori* NW208 (*pyr*A) originating from *A. awamori* CBS 115.52. Transformants were selected for uridine prototropy. For rhamnogalacturonan hydrolase overexpression in *A. niger*, transformants were grown in 250-ml erlenmeyer flasks with 50 ml minimal medium (MM; Pontecorvo et al. 1953), supplemented with 0.5% (w/v) yeast extract, 0.2% (w/v) casamino acids and 1% (w/v) sucrose. After 24 h of growth the mycelium of each transformant was transferred to MM with 1% (w/v) brown ribbon apple pectin (degree of esterification 72.8%; Obipektin, Bischofszell, Switzerland) as carbon source.

For Northern analysis of *A. niger* transformants two selected multi copy transformants designated NW128::pIM860/2 and NW128::pIM875/1, containing multiple copies of *rhg*A and *rhg*B, respectively, were grown in a 2-l fermenter with MM and 1% (w/v) sucrose at 30°C at pH 4.0. After 24 h of growth, the mycelial biomass was transferred to a fermenter with MM and 1% (w/v) apple pectin as carbon source, after which growth was continued under the same conditions.

A. awamori transformants were tested for rhamnogalacturonan hydrolase expression by growing them for 24 h in MM with 0.2% (w/v) yeast extract, 0.2% (w/v) casamino

acids and 0.5% (w/v) sucrose. To induce expression 1% (w/v) xylose was added to the medium, after which the cultures were allowed to grow for another 24 h.
Culture filtrates of *A. niger* and *A. awamori* were analysed by Western blot analysis, using antibodies raised against *A. aculeatus* rhamnogalacturonan hydrolase A (Suykerbuyk et al. 1995).

Southern and Northern analysis. Genomic DNA was isolated from *Aspergillus* wild type and transformant strains using the method of De Graaff et al. (1988). Hybridization conditions used for Southern analysis of genomic *A. niger* DNA were the same as described for the library screening.
Total RNA was isolated from powdered mycelium using Trizol (Gibco BRL), denatured with glyoxal and DMSO (Sambrook et al. 1989), subjected to electrophoresis and blotted onto Hybond N filters. The filters were hybridized overnight at 42°C in a solution containing 6 x SSC, 5 x Denhardt, 10% (w/v) dextran sulphate, 0.5% (w/v) SDS and 50% (v/v) formamide and washed twice for 30 min at 65°C in a solution containing 2 x SSC, 0.1% (w/v) SDS and 0.1% (w/v) sodium pyrophosphate. A 2.5-kb *Hin*dIII fragment of pIM860 and a 1.1-kb *Eco*RV fragment of pIM875 were used as probes. The relative specific activity of the *rhg*B probe was two times higher than that of the *rhg*A probe, as was determined by hybridization of the probes to different concentrations of the corresponding plasmids spotted onto Hybond N filters. Reprobing of the filters with a DNA fragment encoding 28S rRNA of *Agaricus bisporus* (Schaap et al. 1996) was performed to verify equal loading. For this, prehybridization and washing were performed at 60°C.

Purification and deglycosylation of A. niger rhamnogalacturonan hydrolases. A. awamori multi copy transformants were grown in 300 ml MM pH 6.0 with 0.2% (w/v) casamino acids, 0.2% (w/v) yeast extract and 0.5% (w/v) sucrose inoculated with 10^6 spores/ml in 1-l flasks for 24 h at 30°C in an orbital shaker at 250 rpm. Then xylose (3% w/v) was added to the cultures and growth was continued for 24 h. The culture filtrates were used for enzyme purification by column chromatography. Three columns were used, DEAE-Sephadex A50, DEAE-Sepharose Fast Flow and S-Sepharose Fast Flow, all of which were obtained from Pharmacia. Details of the purification are given under Results. Purification of the enzymes was monitored by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Molecular masses of the proteins

were determined on a 10% SDS- polyacrylamide gel, by comparison with protein test mixture 4 (Serva, Heidelberg, GFR). Protein concentrations were determined using the bicinchoninic acid method of Sigma Chemie (Bornem, Belgium). Deglycosylation of denatured rhamnogalacturonan hydrolase A and B with *N*-glycosidase F was carried out according to the instructions of the supplier (Boehringer, Mannheim, Germany).

Activity assay and determination of pH optima of A. niger rhamnogalacturonan hydrolases. Rhamnogalacturonan hydrolase activity was determined by measuring the degradation of saponified apple MHR purified from Golden Delicious apples as described before (Suykerbuyk et al. 1995). Incubation of 20 μl of enzyme with 0.5 ml of a 2% (w/v) saponified MHR solution in McIlvaine buffer was performed at 40°C. The pH optimum of the enzyme was determined by using McIlvaine buffers in the pH range 2.5 to 6.0. The enzymes were inactivated by boiling ten-fold diluted samples for 5 min. and 50 μl of this solution was loaded on a CarboPac PA-100 anion-exchange column (25 cm x 4 mm) (Dionex, Sunneyvale, California) for analysis of the products formed. Trigalacturonic acid was used as a standard. Elution was performed with a gradient of sodium acetate in 0.1 M sodium hydroxide, as described by Schols et al. (1994). The flow rate was 1.0 ml/min and the process was monitored using a pulsed electrochemical detector. One unit was defined as the amount of enzyme which produces 1 μmol/min of the oligomer corresponding to peak VIII (Fig. 7).

Nucleotide sequence accession numbers. The nucleotide sequences of the *rhg*A and *rhg*B gene and flanking regions have been deposited under EMBL accession no. X94220 and no. X94221, respectively.

Results

Isolation of the *rhg*A and *rhg*B genes from *A. niger*

The *rhg*A gene from *A. aculeatus* (Suykerbuyk et al. 1995) was used as a probe to detect hybridizing sequences in the *A. niger* genome. Southern blot analysis of the genomic DNA, using conditions of low stringency revealed multiple fragments, hybridizing with different intensity with this probe, in most of the restric-

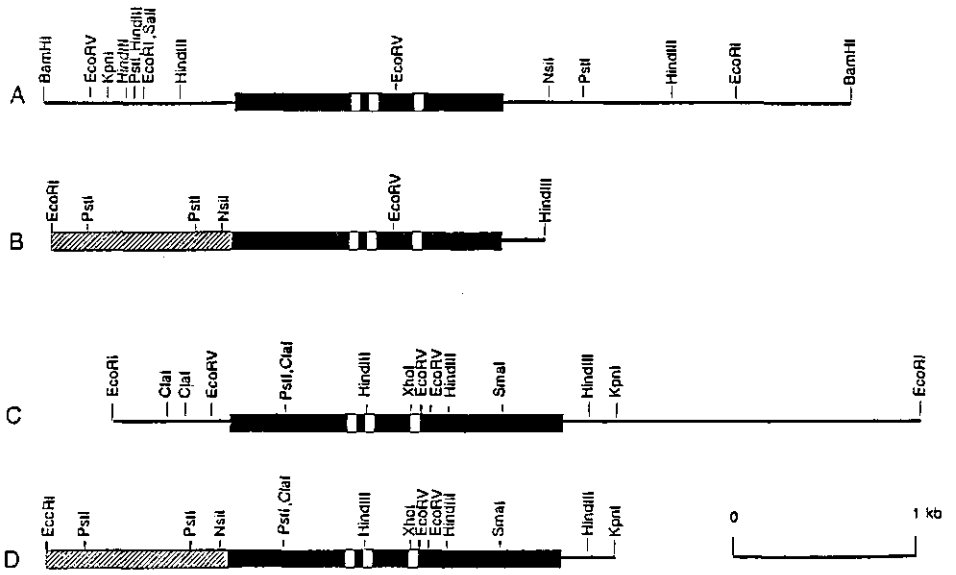


Fig. 1A-D Restriction map of *A. niger rhg*A and *rhg*B and fusion constructs. **A** pIM860 contains a 4.5-kb *Bam*HI-fragment of *rhg*A in pBluescriptSK(+). **B** In pIM836 a 1-kb fragment of the *exl*A promoter is cloned before the *rhg*A coding region. **C** pIM875 contains a 4.5-kb *Eco*RI fragment of *rhg*B in pUC19. **D** In pIM846 a 1-kb fragment of the *exl*A promoter is cloned before the *rhg*B coding region. Open reading frames are indicated as *filled bars* and introns as *open bars*. The *exl*A promoter and leader sequences are indicated by a *hatched bar*

tion digests. This suggested the presence of more than one rhamnogalacturonan hydrolase gene in this strain (Fig. 2). The same conditions were used to screen an *A. niger* genomic library, yielding 31 hybridizing phages. DNA of five individual phages was subjected to Southern analysis. The phages could be divided into two groups on the basis of restriction enzyme digestion pattern and intensity of hybridization signals. From each group a single hybridizing fragment was cloned, resulting in the plasmids pIM860 and pIM875 (Fig. 1), and both were sequenced. After comparison of the sequences with the sequence of *A. aculeatus rhg*A, highly similar open reading frames were found, which were designated *rhg*A and *rhg*B, respectively.

Structure of *A. niger* rhamnogalacturonan hydrolases

The open reading frames of the *rhg*A and *rhg*B genes (Fig. 3A and B) were deduced from the similarity of predicted amino acid sequences with the amino acid sequence of *A. aculeatus* rhamnogalacturonan hydrolase A. The two putative *rhg* genes are interrupted by three introns at the same positions as in *A. aculeatus rhg*A (Fig. 3). The deduced startcodons of both genes are preceded by stop codons in all frames and have a consensus A residue at the -3 position (Kozak 1989). Potential secretion signal cleavage sites (Von Heijne 1986) in the amino acid sequences were found between glycine 18 and glutamine 19 in the A-enzyme and between the two alanines at position 19 and 20 in the B-enzyme (Fig. 4). Both rhamnogalacturonan hydrolases contain consensus sites for *N*-linked glycosylation, six sites are present in the A-enzyme and four sites are found in the B-enzyme. The calculated molecular mass of the putative A-enzyme is 47075 Da with a deduced isoelectric point of 3.7. The calculated molecular mass of the putative B-enzyme is 57902 Da with a deduced isoelectric point of 3.9.

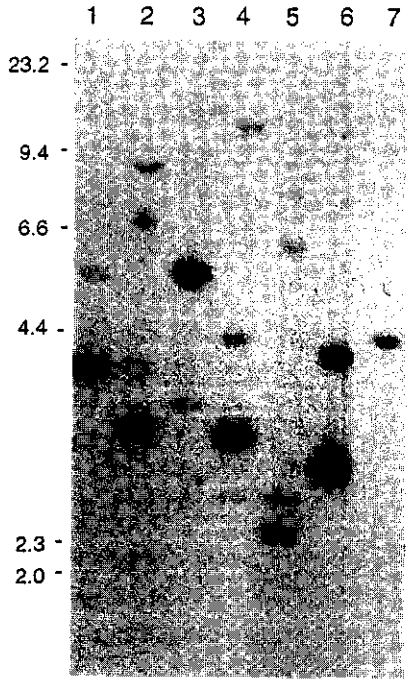
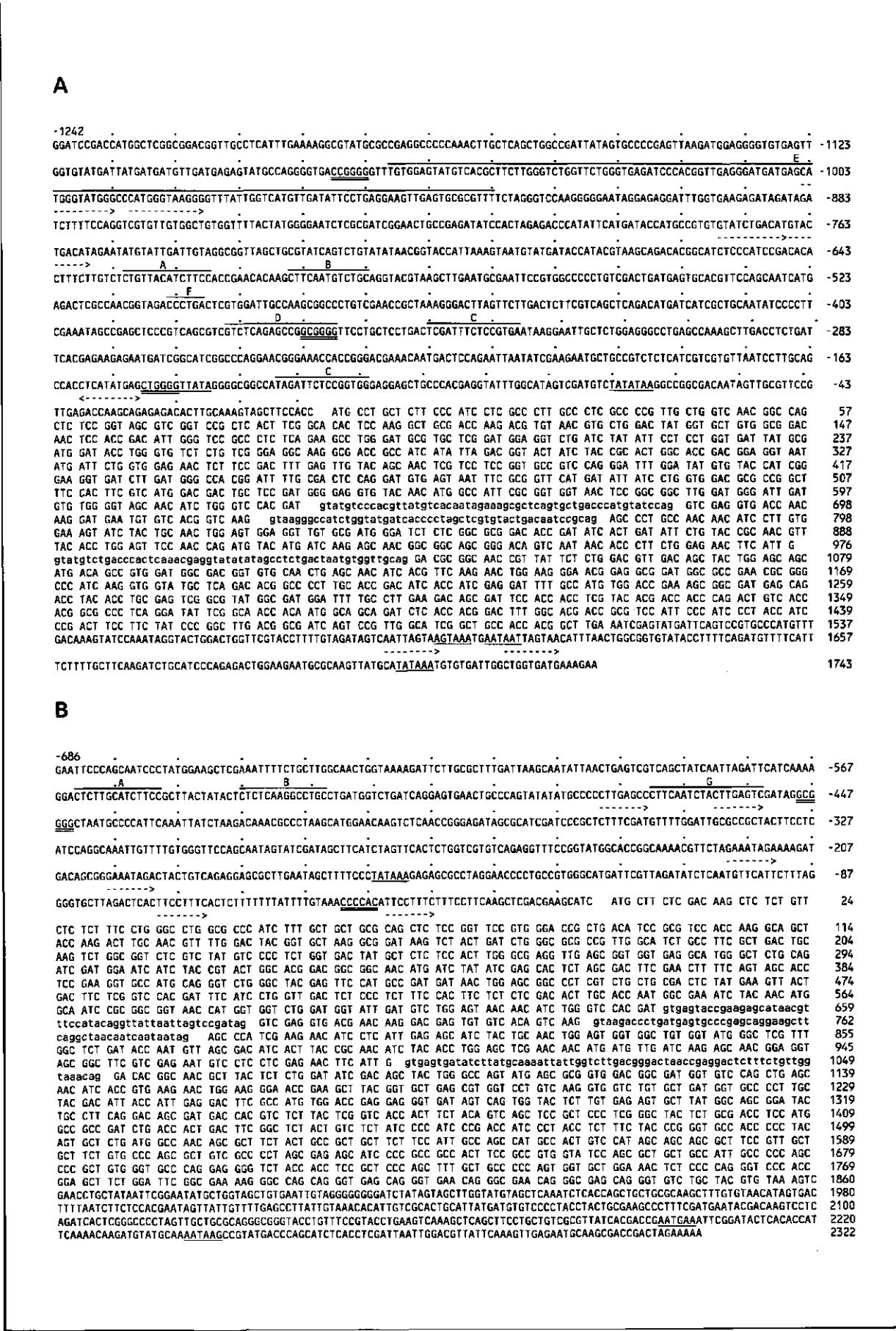


Fig. 2 Southern analysis of *A. niger* genomic DNA using a 2.5-kb *Hin*dIII fragment containing the *rhg*A gene of *A. aculeatus* as probe. DNA was digested with *Bam*HI-*Xho*I (*lane* 1), *Bam*HI-*Sal*I (*lane* 2), *Xho*I (*lane* 3), *Sal*I (*lane* 4), *Hin*dIII (*lane* 5), *Eco*RI (*lane* 6) and *Bam*HI (*lane* 7). Molecular mass markers (in kb), corresponding to lambda DNA digested with *Hin*dIII, are shown at the left


A
B

A high degree of homology is found between the rhamnogalacturonan hydrolase A proteins of *A. niger* and *A. aculeatus*, which share 78% identical amino acids. Similarly, rhamnogalacturonan hydrolase B shows 72% and 70% identity with the A-proteins of *A. aculeatus* and *A. niger*, respectively. Most conserved is the region between position 192 and position 246 in the amino acid sequence of *A. aculeatus* rhamnogalacturonan hydrolase A, which shows 100% and 87% identity with the corresponding regions in the *A. niger* A- and B-protein, respectively (Fig. 4A).

Comparison of the amino acid sequences also revealed a carboxyterminal extension of 112 amino acids in the *A. niger* B-protein. This region, which is rich in serine, alanine and proline residues, shows no similarities with rhamnogalacturonan hydrolases or with other proteins in the GenEMBL protein sequence data base. At the C-terminus the tripeptide sequence glycine-glutamic acid-glutamine (GEQ) is repeated four times.

Structure of non-coding sequences

Putative TATA boxes are found at positions -74 (TATATAA) and -142 (TTATA) before the startcodon of *rhg*A and at position -156 (TATAAA) before the startcodon of *rhg*B (Fig. 3). The *rhg*B promoter further contains a CT-rich region from position -71 to position -18, which may determine the position of transcription initiation (Unkles 1992).

In the 3' non-coding region of both *rhg* genes putative polyadenylation signals are found. These are located at 93 bp (AgTAAA), 101 bp (AATAAt) and 207 bp (tATAAA) downstream from the stopcodon of *rhg*A and at 340 bp (AATgAA)

Fig. 3 Nucleotide sequence of the *rhg*A (**A**) and the *rhg*B (**B**) gene of *A. niger*. Intervening and border sequences are in *lowercase* letters. Putative TATA boxes and putative polyadenylation signals are single *underlined*. Putative CREA binding sites are marked by *double underlining*. Sequences conserved between rhamnogalacturonan hydrolase gene promoters (box A-G) are marked by *overlining*. Various direct and inverted repeats in the 5'- and 3'- noncoding sequences are indicated by *arrows* underneath the sequence

A

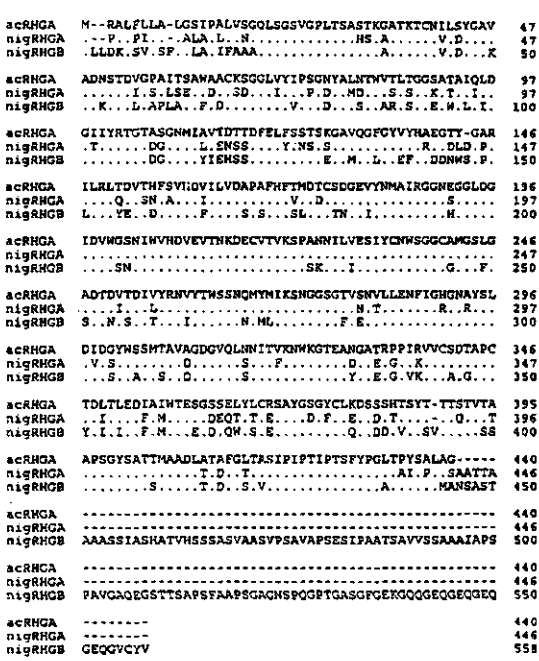
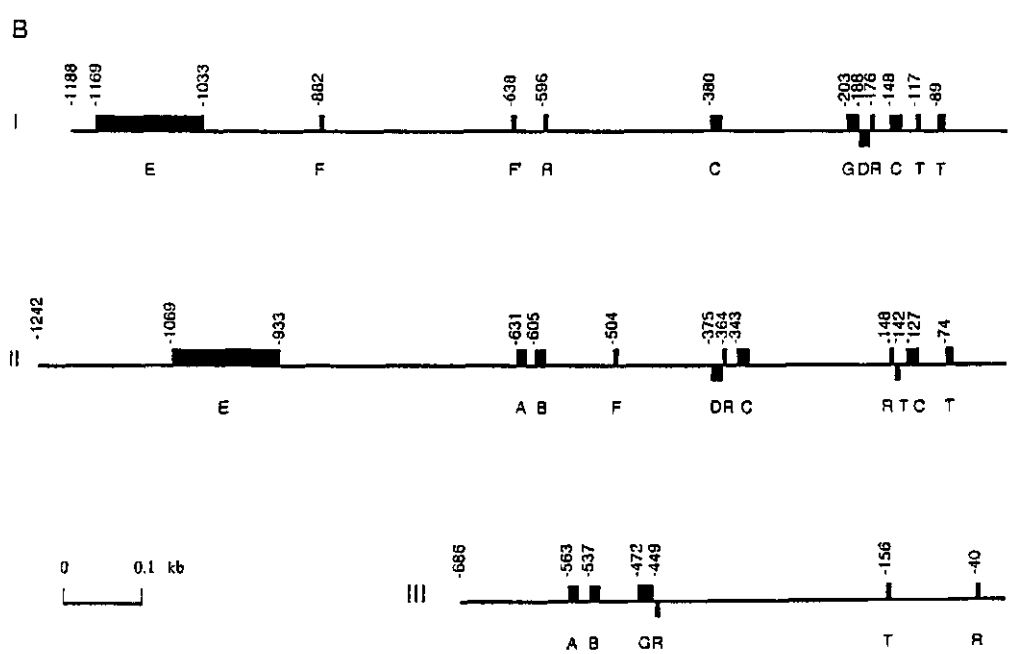


Fig. 4 Comparison of rhamnogalacturonan hydrolase amino acid sequences (**A**) and regulatory sequences (**B**). **A** Amino acid residues in *A. niger* rhamnogalacturonan hydrolase A (nigRHGA) and B (nigRHGB) identical to *A. aculeatus* rhamnogalacturonan hydrolase A (acRHGA) are indicated by a *dot*. Gaps (-) were introduced for optimal alignment. **B** Promoter sequences of *A. aculeatus rhg*A (I) and *A. niger rhg*A (II) and *rhg*B (III). Boxes, which are present in two different promoters, are marked by *A-G*. Putative TATA boxes are indicated by *T* and putative CREA binding sites by *R*. The distance between the 5′ border of each box and the respective startcodon (in bp) is indicated by *numbering*. The 3′ borders of boxes larger than 100 bp are also positioned by *numbering*. r, reversed

B



and 385 bp (AATAAg) downstream from the stopcodon of *rhg*B. Single mismatches in these signals with the AATAAA consensus sequence in higher eukaryotes (Irniger and Braus 1994) are here set in lowercase letters.

The 5' non-coding adjacent sequences of *rhg*A and *rhg*B share 47% identity. More than 500 bp upstream from the startcodons of both genes two boxes of 14 bp are found (box A and B), which show 86% and 79% sequence identity, respectively (Fig. 4B).

Surprisingly, more similarities are found between the *rhg*A promoters *A. aculeatus* and *A. niger*, which have 55% sequence identity over a region of 1188 bp. A box with the sequence TAGATTCTCCGGTGG (box C in Fig. 4B) is found in both promoter sequences, starting from position -127 in *A. niger rhg*A and from position -148 in *A. aculeatus rhg*A. In both genes this sequence is preceded within 25 bp by a putative CREA site which has the consensus sequence (G/C)(C/T)GG(G/A)G (indicated as box R; Kulmburg et al. 1993). The sequence of box C is repeated in *A. niger rhg*A starting at position -343 with four substitutions and in *A. aculeatus rhg*A starting at position -380 with five substitutions (Fig. 3).

Another box, 18 bp in size, starts 375 bp upstream from the startcodon of *A. niger rhg*A and shares 89% sequence identity with a box starting 188 bp upstream from the startcodon of *A. aculeatus rhg*A (box D in Fig. 4B). In both genes this sequence overlaps with another putative CREA binding site. Around 1 kb upstream from both startcodons a large region spanning 136 bp is found, which shows 77% sequence identity in both *rhg*A gene promoters (box E in Fig. 4B). Finally the CCCTGA box, a hexanucleotide sequence conserved in the upstream regions of several pectinase genes of *Aspergillus* (Bussink et al. 1992), was found once in *A. niger rhg*A and twice in *A. aculeatus rhg*A (box F in Fig. 4B).

Comparison of the promoter sequence of *A. niger rhg*B with those of *A. aculeatus rhg*A revealed only a 18 bp sequence starting from position -472 in *rhg*B (box G in Fig. 4B), which shows 83% identity with a sequence starting from position -203 in *rhg*A and which overlaps with box D in the *A. aculeatus* gene.

Expression of the *rhg*A and *rhg*B genes in *A. niger*

Western analysis of the culture filtrate of *A. niger* wild type after growth on apple pectin only revealed one faint band of about 72 kDa in size. To investigate whether this protein is encoded by the *rhg*A or the *rhg*B gene and to achieve higher expression levels of both rhamnogalacturonan hydrolases, the plasmids containing these genes, pIM860 and pIM875, were introduced into *A. niger* strain NW128 by cotransformation. One hundred of the transformants obtained were grown on apple pectin and the culture filtrates were subjected to Western analysis. In all transformants analysed only a very faint band, at the same position and with the same intensity as that in the wild type strain, was seen. Transformation of another host, *A. aculeatus* strain NW217, with either of the two genes, also did not result in elevated rhamnogalacturonan hydrolase expression levels (results not shown). However, Southern analysis of the genomic DNA of five *rhg*A and five *rhg*B *A. niger* transformants revealed the presence of multiple copies of the corresponding genes in each of these transformants. Therefore, we analysed rhamnogalacturonan hydrolase expression at the transcriptional level. Two of the transformants, containing 5 to 10 integrated copies of the respective *rhg* genes, were grown in a controlled shift experiment as described in Materials and methods. RNA levels at different time points of growth were compared by Northern analysis (Fig. 5). As expected, no *rhg*A expression was detected in the multicopy *rhg*A transformant before transfer from sucrose to apple pectin medium (Fig. 5A, lanes 1,2). Three hours after shifting to apple pectin medium the *rhg*A transcript was seen abundantly, but the expression already declined within 6 h after transfer to a low level (Fig. 5A, lanes 4 to 8). The size of the transcript, 1.8 kb, is somewhat larger than expected from the open reading frame of the *rhg*A gene.

In contrast, the *rhg*B transcript was detected after 18 h of growth on sucrose and declined thereafter (Fig. 5B, lanes 1,2). At this time point sucrose and glucose were depleted from the culture filtrate. Fructose was metabolized completely after 24 h of growth, just before the mycelium was transferred to apple

pectin medium (results not shown). After 18 h of growth on apple pectin the *rhg*B mRNA level increased again, to a slightly higher level than that seen after 18 h of growth on sucrose (Fig. 5B, lane 6). The size of the transcript, approximately 2 kb, would specify the B-enzyme.

Expression of the *rhg*A and *rhg*B genes in *A. awamori*

Expression levels obtained after multicopy integration of *rhg*A and *rhg*B in the *A. niger* genome were not high enough for detection by Western analysis. To achieve higher production levels, *A. awamori* strain NW208 was cotransformed with pIM836 or pIM846, containing the respective *rhg* genes under control of the *exl*A promoter. Rhamnogalacturonan hydrolase production was analysed by growth of the transformants on xylose and analysis of the culture filtrates by Western blotting (see Materials and methods).

In only one of the 47 *rhg*A transformants analysed an easily detectable band, about 72 kDa in size, was seen by Western blotting (Fig. 6A, lanes 1,2), whereas in the remaining transformants no protein could be detected (results not shown). Southern analysis of the genomic DNA of this and four other transformants revealed the presence of 20 and up to five *exl*A-*rhg*A copies integrated, respectively. Apparently, only the high copy transformant was able to produce detectable levels of rhamnogalacturonan hydrolase A.

In contrast, 15 of the 19 *rhg*B transformants analysed gave a clear band with a molecular mass of 87 kDa (Fig. 6A, lanes 3,4). In most of these transformants at least three additional protein bands were seen, varying in mass between 70 kDa and 87 kDa. Southern analysis of the genomic DNA of the highest producing transformant revealed that in this case only about five integrated copies of the *exl*A-*rhg*B fusion construct were present.

In agreement with the results obtained by Western analysis, Northern analysis of the RNA of both transformants revealed a low level of the *rhg*A transcript and a high level of the *rhg*B transcript (Fig. 5C and D).

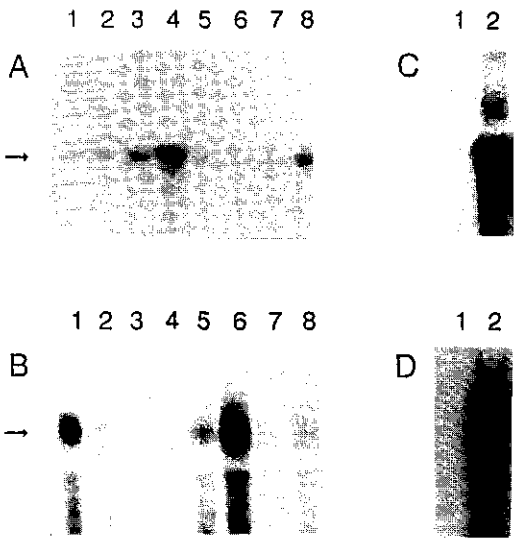


Fig. 5 Northern analysis of multi copy transformants. **A** *A. niger* NW128::pIM860/2 (*rhg*A), **B** NW128::pIM875/1 (*rhg*B), **C** *A. awamori* NW208::pIM836/5 (*exl*A-*rhg*A) and **D** NW208::pIM846/19 (*exl*A-*rhg*B). The *A. niger* strains were pregrown in minimal medium (MM) with 1% sucrose. RNA was isolated after 18 h and 24 h of growth (*lanes* 1,2) after which the mycelium was transferred to MM with 1% apple pectin. RNA was subsequently isolated 1 h, 3 h, 6 h, 18 h, 24 h and 30 h after transfer (*lanes* 3-8). The *A. awamori* strains were pregrown in MM with 0.5% sucrose after which 1% xylose was added to the medium. RNA was isolated before (*lanes* 1) and 6 h after xylose induction (*lanes* 2). The location of the *rhg*A and *rhg*B transcripts is indicated with an *arrow*

Purification and characterization of rhamnogalacturonan hydrolase A and B from *A. awamori* transformants

Multicopy *A. awamori* transformants were grown in xylose medium and purification of rhamnogalacturonan hydrolase A and B was performed as described before (Suykerbuyk et al. 1995) with a few modifications. Concentration of the culture filtrate was performed by batchwise adsorption on DEAE-Sephadex A-50 at pH 6.0. Adsorbed protein was eluted with 20 mM sodium phosphate buffer pH 6.0 containing 1 M sodium chloride. The rhamnogalacturonan hydrolase containing fractions were selected by SDS-PAGE, pooled and dialysed against

20 mM piperazine-HCl buffer pH 6.0. For purification of rhamnogalacturonan hydrolase A and B a DEAE-Sepharose Fast Flow column (2.5 x 25 cm) was used and elution was performed with a 1-l linear sodium chloride gradient (0-0.4 M) in buffer. Rhamnogalacturonan hydrolase containing fractions were dialysed against 10 mM sodium acetate buffer pH 4.0 and loaded onto a S-Sepharose Fast Flow column (2.5 x 25 cm) equilibrated with the same buffer. Elution was performed with a 1-l sodium chloride gradient (0-0.2 M) in buffer.

After purification about 3 mg of rhamnogalacturonan hydrolase A and about 40 mg of rhamnogalacturonan hydrolase B were obtained per litre of culture fluid. The higher yield of the B-enzyme is in agreement with the higher *rhg*B expression level as demonstrated by Western and Northern analysis.

SDS-PAGE of purified rhamnogalacturonan hydrolase A revealed only one protein band with a molecular mass of 72 kDa and thus the enzyme appeared to be pure (Fig. 6B, lane 1). The position of this band corresponded to the single band seen after Western blot analysis of culture filtrate. After *N*-glycanase treatment and SDS-PAGE analysis, a smaller protein band of 67 kDa was visible (Fig. 6B, lane 2).

After SDS-PAGE analysis of purified rhamnogalacturonan hydrolase B four bands were seen, varying in size between 70 kDa to 87 kDa (Fig. 6B, lane 3). These bands were also seen by Western analysis, however after purification the smallest instead of the largest band was predominant. *N*-glycanase treatment and SDS-PAGE analysis resulted in four bands in the range of 65 kDa to 82 kDa, suggesting that each of the proteins was reduced in mass by about 5 kDa (Fig. 6B, lane 4).

Rhamnogalacturonan hydrolase activity was demonstrated by degradation of saponified MHR. For this, approximately 1 μg of rhamnogalacturonan hydrolase A or B was incubated with saponified MHR and after 60 min samples were analysed by high performance anion exchange chromatography. Both rhamnogalacturonan hydrolase A and B were capable of hydrolyzing saponified MHR (Fig. 7B and C). In both profiles nine peaks were distinguished, which were also found after incubation of the same substrate with *A. aculeatus* rhamnogalactu-

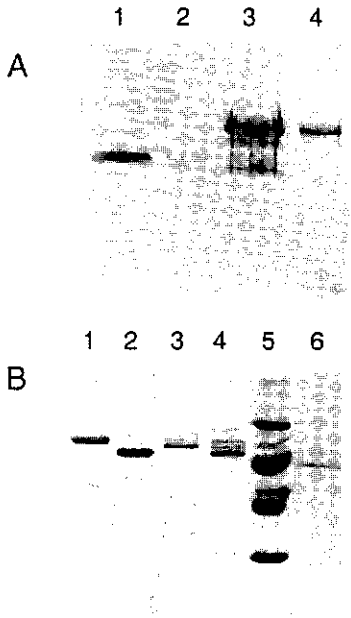


Fig. 6 Western analysis of the culture filtrates of *A. awamori* multicopy transformants (**A**) and SDS-PAGE of the purified rhamnogalacturonan hydrolases (**B**). **A** The transformants NW208::pIM836/5 (*lanes* 1,2) and NW208::pIM846/19 (*lanes* 3,4) were grown for 24 h in xylose medium. For Western analysis medium samples were applied directly (*lanes* 1,3) and 10 times diluted (*lanes* 2,4). **B** SDS-PAGE and Coomassie Brilliant Blue staining of rhamnogalacturonan hydrolase A before (*lane* 1) and after (*lane* 2) treatment with *N*-glycosidase F and of rhamnogalacturonan hydrolase B before (*lane* 3) and after (*lane* 4) treatment with *N*-glycosidase F. *Lane* 5 contains the S4 marker protein mixture and *lane* 6 contains rhamnogalacturonan hydrolase A from *A. aculeatus*

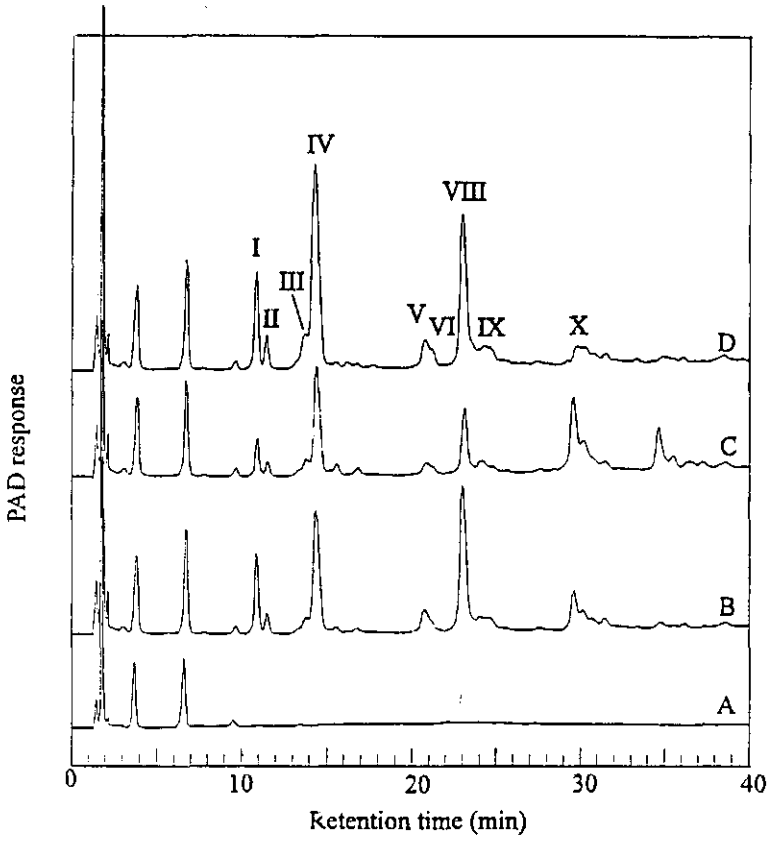
ronan hydrolase A (Fig. 7D). The oligomers corresponding to these peaks were analysed by Schols et al. (1994). Following their assignments four of these peaks consist of tetrameric backbones (I to IV). Peak I represents the unsubstituted backbone, peak II represents a tetrameric backbone substituted with galactose at the second rhamnose residue, peaks III and IV were copurified and predominantly contain substitution at both rhamnose residues while a fraction is only substituted at the first rhamnose residue. The five remaining peaks designated V, VI, VIII, IX and X consist of hexameric backbones. Peak V represents the unsubstituted backbone, peak VI is single substituted with galactose at the third rhamnose residue, peak VIII is double substituted with galactose, either at the first and second or at the first and third rhamnose residue while peak IX and X are triple substituted with galactose. Besides these oligomers, additional components with longer retention times were found in the profile of rhamnogalacturonan hydrolase B (Fig. 7C). More differences between rhamnogalacturonan hydrolase A and B were demonstrated when degradation of saponified MHR was followed in the time. Different final levels of the oligomers were obtained, e.g.

peak VIII, corresponding to the major hexamer, was formed to a lower level but with higher rate by rhamnogalacturonan hydrolase B as compared to rhamnogalacturonan hydrolase A (Fig. 7). Determination of the rate of product formation corresponding with increase of peak height of peak VIII in each profile revealed relative specific activities of 0.9 U/mg for rhamnogalacturonan hydrolase A, 0.4 U/mg for rhamnogalacturonan hydrolase B and 2.5 U/mg for *A. aculeatus* rhamnogalacturonan hydrolase A. For this, optimal pH conditions were determined for each enzyme which were pH 3.6 for the *A. niger* A-enzyme, pH 4.1 for the *A. niger* B-enzyme and pH 4.5 for the *A. aculeatus* rhamnogalacturonan hydrolase.

Discussion

The *A. niger* genome contains at least two rhamnogalacturonan hydrolase genes, *rhg*A and *rhg*B, which are homologous to the *A. aculeatus rhg*A gene. The encoded enzymes share 78% and 72% amino acid sequence identity respectively, with *A. aculeatus* rhamnogalacturonan hydrolase A. In addition, rhamnogalacturonan hydrolase B contains a C-terminal extension, which may have a function in substrate binding and in the localization or specificity of this enzyme. Alternatively, rhamnogalacturonan hydrolase B may have arisen from an imperfect gene duplication event. The presence of more genes belonging to this family cannot be excluded, considering the complex banding pattern seen after Southern analysis of the genomic DNA of *A. niger*. Recently, an enzyme activity capable of hydrolysis of α-D-galacturonopyranosyl-(1,2)-α-L-rhamnopyranosyl linkages has been demonstrated in a commercial β-galactosidase preparation from *A. niger* (An et al. 1994). Whether either one of the here described rhamnogalacturonan hydrolases is responsible for this activity, remains to be investigated. We only could detect a low level of rhamnogalacturonase A expression in the *A. niger* wild type strain.

In order to analyse the mode of action of the two *A. niger* rhamnogalacturonan hydrolases in the degradation of hairy regions, recombinant enzymes were puri-



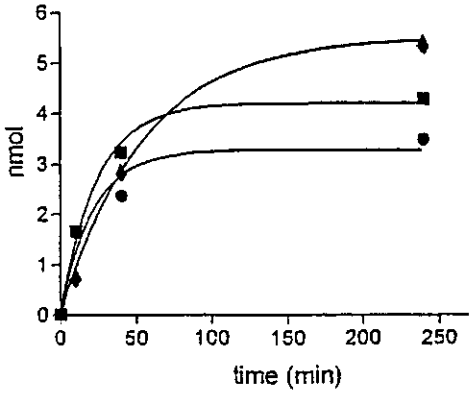


Fig. 7 High-performance anion-exchange chromatography elution profile of saponified MHR before (**A**) and after incubation for 60 min with *A. niger* rhamnogalacturonan hydrolase A (**B**), *A. niger* rhamnogalacturonan hydrolase B (**C**) and *A. aculeatus* rhamnogalacturonan hydrolase A (**D**) (upper part). Peaks I to VI and VIII to X are marked. Trigalacturonic acid was used as a standard.

Rate of formation of the degradation product represented by peak VIII. Specific enzyme activities were calculated from the initial rate of product formation. ♦, *A. niger* rhamnogalacturonan hydrolase A; ●, *A. niger* rhamnogalacturonan hydrolase B; ■, *A. aculeatus* rhamnogalacturonan hydrolase A

fied from *A. awamori*. High expression levels were found for the recombinant B-enzyme, which is in accordance with the results obtained with the *A. aculeatus rhg*A gene under control of the same *exl*A promoter (Suykerbuyk et al. 1995). The relative low level of rhamnogalacturonan hydrolase A expression in comparison with rhamnogalacturonan hydrolase B might be caused by a less stable *exl*A-*rhg*A fusion transcript. SDS-PAGE revealed that recombinant rhamnogalacturonan hydrolase B could be separated into four distinct bands, all of which reacted with the antibody. These four proteins behaved differently during the purification process since after purification the smallest instead of the largest band was predominant. The sizes of the bands of the A- and B-enzymes after *N*-glycanase treatment are not in accordance with their calculated molecular masses, 47075 Da and 57902 Da, respectively. However, the molecular masses of both proteins decreased further after treatment with sodium hydroxide, suggesting *O*-glycosylation (results not shown). In the A protein the threonine-rich C-terminal region may function as attachment site for *O*-glycosylation (Kobata 1992). The observed reduction of about 5 kD in mass of all B-enzymes after *N*-glycanase treatment and further compression of these B bands to one band after treatment with sodium hydroxide strongly suggests that all of these bands originate from one gene-product but different in their degree of *O*-glycosylation.

Rhamnogalacturonan hydrolase A and B activities of the recombinant enzymes were measured using saponified MHR from apple as a substrate. The high performance anion exchange chromatography profiles obtained after incubation of the A and B protein were quite similar to those obtained with *A. aculeatus* rhamnogalacturonan hydrolase A. Identification of the oligomers produced revealed that the latter enzyme acts on *α*-D-galacturonopyranosyl-(1,2)-*α*-L-rhamnopyranosyl linkages (Schols et al. 1994). Therefore we can conclude that the *A. niger* proteins are isofunctional in hydrolyzing these linkages.

The same method was used for establishing specific activities of the different rhamnogalacturonan hydrolases. The values obtained are not absolute but are derived from the initial rate of product formation of one of the possible products.

Another method for measuring pectinolytic enzyme activities, which is based on determination of reducing end groups, is found to be less sensitive for this purpose (Schols et al. 1990) as is also measurement of solubilized sugars using the carbazole/H_2SO_4 method (Sakamoto et al. 1994). The difference in amounts of oligomers released by rhamnogalacturonan hydrolase A and B after prolonged incubation times suggest different substrate specificities. In addition, more complex oligomers, which resist longer incubation times, are formed by rhamnogalacturonan hydrolase B.

In order to study the expression of these genes in *A. niger*, multicopy transformants were grown on apple pectin after pregrowth on sucrose. The induction pattern of *rhg*A shows an optimum within a few hours of growth on apple pectin, as was already shown for the *A. aculeatus rhg*A gene (Suykerbuyk et al. 1995). As no expression was seen during pregrowth on sucrose, the *rhg*A gene is like the *A. aculeatus* gene presumably repressed under these conditions. Possibly, the putative CREA sites in the promoter of this gene play a role in carbon catabolite repression (Cubero and Scazzocchio 1994). However, further research is required in order to determine their role in vivo.

The detection of *rhg*B mRNA after 18 h of growth on apple pectin or sucrose, suggests that the increase of expression of this gene is not the result from an apple pectin mediated induction but is the mere result of derepression, occurring after carbon source depletion. This suggests that this gene requires a different set of trans-acting factors, which is in agreement with its highly deviating promoter sequence. Only two boxes of 14 bp are conserved between the *A. niger* genes. Comparison with the *A. aculeatus rhg*A gene promoter revealed three boxes, box C, D and E, which are highly conserved between this promoter and the *A. niger rhg*A promoter and only one box of 18 bp (box G), which is highly conserved in the *rhg*B promoter and the *A. aculeatus rhg*A promoter. Computer analysis of other pectinase gene promoters however, did not reveal any homology with these boxes, which could point to specific function(s) in the regulation of transcription of rhamnogalacturonan hydrolase genes.

The production of a broad spectrum of oligomers by rhamnogalacturonan

hydrolase B in the presence of MHR may have a function in the liberation of inducer molecules, thus regulating the expression of other pectinolytic enzymes under starvation conditions. The different substrate specificity may be defined by the carboxyterminal extension within this enzyme. Possibly, interference of *rhg*A and *rhg*B expression can be established in transformants containing multiple copies of both *rhg* genes.

Acknowledgements This project was financed by the Unilever Research Laboratorium, Vlaardingen. We thank Dr. H. Schols for supplying the markers for high performance anion exchange chromatography analysis and J.W. Peters for the initial cloning of the genes

References

An J, Zhang L, O'Neill MA, Albersheim P, Darvill AG (1994) Isolation and structural characterization of *endo*-rhamnogalacturonase-generated fragments of the backbone of rhamnogalacturonan I. Carbohydr Res 264:83-96

Bussink HJD, Buxton FP, Fraaye BA, Graaff LH de, Visser J (1992) The polygalacturonases of *Aspergillus niger* are encoded by a family of diverged genes. Eur J Biochem 208:83-90

Cubero B, Scazzocchio C (1994) Two different, adjacent and divergent zinc finger binding sites are necessary for CREA-mediated carbon catabolite repression in the proline gene cluster of *Aspergillus nidulans*. EMBO J 13:407-415

De Graaff LH, Broek HWJ van den, Visser J (1988) Isolation and transformation of the pyruvate kinase gene of *Aspergillus nidulans*. Curr Genet 13:315-321

Gouka RJ, Hessing JGM, Stam H, Musters W, Hondel CAMJJ van den (1995) A novel strategy for the isolation of defined *pyr*G mutants and the development of a site-specific integration system for *Aspergillus awamori*. Curr Genet 27:536-540

Harmsen JAM, Kusters-van Someren MA, Visser J (1990) Cloning and expression of a second *Aspergillus niger* pectin lyase gene (*pel*A): indications of a pectin lyase gene family in *Aspergillus niger*. Curr Genet 18:161-166

Hessing JGM, Rotterdam C van, Verbakel JMA, Roza M, Maat J, Gorcom RFM van, Hondel CAMJJ van den (1994) Isolation and characterization of a 1,4-β-endoxylanase gene of *A. awamori*. Curr Genet 26:228-232

Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR (1989) Engineering hybrid genes without the use of restriction enzymes: Gene splicing by overlap extension. Gene 77:61-68

Irniger S, Braus G (1994) Saturation mutagenesis of a polyadenylylation signal reveals a hexanucleotide element essential for mRNA 3' end formation in *Saccharomyces cerevisiae*. Proc Natl Acad Sci USA 91:257-261

Kobata A (1992) Structures and functions of the sugar chains of glycoproteins. Eur J Biochem 209:483-501

Kofod LV, Kauppinen S, Christgau S, Andersen LN, Heldt-Hansen HP, Dörreich K, Dalbøge H (1994) Cloning and characterization of two structurally and functionally divergent rhamnogalacturonases from *Aspergillus aculeatus*. J Biol Chem 269:29182-29189

Kozak M (1989) The scanning model for translation: an update. J Cell Biol 108:229-241

Kulmburg P, Mathieu M, Dowzer C, Kelly J, Felenbok B (1993) Specific binding sites in the *alc*R and *alc*A promoters of the ethanol regulon for the CREA repressor mediating carbon catabolite repression in *Aspergillus nidulans*. Mol Microbiol 7:847-857

Kusters-van Someren MA, Harmsen JAM, Kester HCM, Visser J (1990) Structure of the *Aspergillus niger pel*A gene and its expression in *Aspergillus niger* and *Aspergillus nidulans*. Curr Genet 20:293-299

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685

Lau JM, McNeil M, Darvill AG, Albersheim P (1985) Structure of the backbone of rhamnogalacturonan I, a pectic polysaccharide in the primary cell walls of plants. Carbohydr Res 137:111-125

Mutter M, Colquhoun IJ, Schols HA, Beldman G, Voragen AGJ (1996) Rhamnogalacturonan α-L-rhamnopyranosyl-(1,4)-α-D-galactopyranosyluronide lyase. Plant Physiol 110:73-77

O'Neill M, Albersheim P and Darvill AG (1990) In:Dey PM (ed) Methods in plant biochemistry, Vol 2 Carbohydrates. Academic Press, London, pp 415-441

Pontecorvo G, Roper JA, Hemmons LJ, MacDonald KD, Bufton AWJ (1953) The genetics of *Aspergillus nidulans*. Adv Genet 5:141-238

Puvanesarajah V, Darvill AG, Albersheim P (1991) Structural characterization of two oligosaccharide fragments formed by the selective cleavage of rhamnogalacturonan II: evidence for the anomeric configuration and attachment sites of apiose and 3-deoxy-2-heptulosaric acid. Carbohydr Res 218:211-222

Sakamoto M, Shirane Y, Naribayashi I, Kimura K, Morishita N, Sakamoto T, Sakai T (1994) Purification and characterization of a rhamnogalacturonase with protopectinase activity from *Trametes sanguinea*. Eur J Biochem 226:285-291

Sambrook J, Fritsch E, Maniatis T (1989) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Schaap PJ, Müller Y, Baars JJP, Camp HJM op den, Sonnenberg ASM, Griensven LJLD van, Visser J (1996) Nucleotide sequence and expression of the $NADP^+$-dependent glutamate dehydrogenase (*gdh*A) from *Agaricus bisporus*. Mol Gen Genet 250:339-347

Schols HA, Geraeds CJM, Searle-van Leeuwen MF, Kormelink FJM, Voragen AGJ (1990) Rhamnogalacturonase: a novel enzyme that degrades the hairy regions of pectins. Carbohydr Res 206:105-115

Schols HA, Voragen AGJ (1994) Occurrence of pectic hairy regions in various plant cell wall materials and their degradability by rhamnogalacturonase. Carbohydr Res 256:83-95

Schols HA, Voragen AGJ, Colquhoun IJ (1994) Isolation and characterization of rhamnogalacturonan oligomers, liberated during degradation of pectic hairy regions by rhamnogalacturonase. Carbohydr Res 256:97-111

Searle-van Leeuwen MJF, Broek LAM van den, Schols HA, Beldman G, Voragen AGJ (1992) Rhamnogalacturonan acetylesterase: a novel enzyme from *Aspergillus aculeatus*, specific for the deacetylation of hairy (ramified) regions of pectins. Appl Microbiol Biotechnol 38:347-349

Suykerbuyk MEG, Schaap PJ, Stam H, Musters W, Visser J (1995) Cloning, sequence

and expression of the gene coding for rhamnogalacturonase (RHG) of *Aspergillus aculeatus*; a novel pectinolytic enzyme. Appl Microbiol Biotechnol 43:861-870

Unkles SE (1992) Gene organization in industrial filamentous fungi. In: Kinghorn JR, Turner G (eds) Applied molecular genetics of filamentous fungi. Chapman London, pp 28-53

Van den Hombergh et al. (1996) EMBL accession no. X96734

Visser J, Bussink HJ, Witteveen C (1994) Gene expression in filamentous fungi; expression of pectinases and glucose oxidase in *Aspergillus niger*. In: Smith A (ed) Gene expression in recombinant microorganisms. Marcel Dekker, Inc, New York, pp 241-308

Von Heijne G (1986) A new method for predicting signal sequence cleavage sites. Nucl Acids Res 14:4683-4690

Whitaker JR (1984) Pectic substances, pectic enzymes and haze formation in fruit juices. Enzyme Microb Technol 6:341-349

Witteveen CFB, Vondervoort PJI van de, Broeck HC van den, Engelenburg FAC van, Graaff LH de, Hillebrand MHBC, Schaap PJ, Visser J (1993) Induction of glucose oxidase, catalase and lactonase in *Aspergillus niger*. Curr Genet 21:408-416

4 | Identification of regulatory mutants of *Aspergillus aculeatus* affected in rhamnogalacturonan hydrolase expression

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Curr Genet (1996) 30:439-446

Abstract Rhamnogalacturonan hydrolase expression in *A. aculeatus* can be induced by pectin, but also by a combination of two constituent monosaccharides of pectin, rhamnose and galacturonic acid. The *rhg*A promoter was fused to the *A. niger* glucose oxidase coding sequence and a single copy of the hybrid gene was integrated at the *rhg*A locus in the genome of *A. aculeatus*. The gene product was subsequently used as reporter in a screening assay for the selection of rhamnogalacturonan hydrolase-overproducing mutant strains. At least four of the mutations were recessive and could be assigned to different loci. One mutation (*rgr*25) showed linkage with the *rhg*A locus. Inducible rhamnogalacturonan hydrolase expression levels of about 5-10 times that in the wild-type were found in the mutants *rgr*48, *rgr*25 and *rgr*34 after growth on a combination of rhamnose and galacturonic acid with or without fructose as a carbon source. In mutant *rgr*48 elevated levels of *rhg*A transcription were found.

Introduction

Rhamnogalacturonases are capable of degrading hairy regions and rhamnogalacturonan-I structures of pectin molecules, either by hydrolysis of α-D-galacturonic acid-(1,2)-α-L-rhamnose linkages (rhamnogalacturonan hydrolase) or by β-elimination of α-L-rhamnose-(1,4)-α-D-galacturonic acid linkages (rhamnogalacturonan lyase) between rhamnose and galacturonic acid residues in the backbone (Schols et al. 1990; Azadi et al. 1996; Mutter et al. 1996). A variety of rhamnogalacturonan degrading enzymes is produced by fungi and other organisms and this presumably reflects the structural complexity of hairy regions. Up to now

rhamnogalacturonan hydrolases have been isolated from *Aspergillus aculeatus* (Schols et al. 1990), *Trametes sanguinea* (Sakamoto et al. 1994) and tomato (Gross et al. 1995), whereas a rhamnogalacturonan lyase has been isolated from *A. aculeatus* (Mutter et al. 1996). Genes coding for rhamnogalacturonan hydrolase (*rhg*A) and for rhamnogalacturonan lyase (*rhg*B) have been isolated from *A. aculeatus* (Kofod et al. 1994; Suykerbuyk et al. 1995) and in *Aspergillus niger* two genes coding for rhamnogalacturonan hydrolase have been cloned (Suykerbuyk et al., unpublished results).

Little is known about the induction mechanism by which the different *rhg* genes are expressed. We have used apple pectin for the induction of the different rhamnogalacturonases in both *A. aculeatus* and *A. niger*, whereas Kofod and coworkers (1994) and Sakamoto and coworkers (1994) used milled soybean for the induction of the different rhamnogalacturonases in *A. aculeatus* and *T. sanguinea*, respectively. Although isolated, modified, hairy regions have been mostly studied in apple, they are widespread components of many other plants (Schols and Voragen 1994) and, therefore, a likely source of inducer molecules. In the present study we have investigated the induction of *rhg*A from *A. aculeatus* on different carbon sources. In addition we have generated mutants which are affected in rhamnogalacturonan hydrolase expression. To facilitate the screening procedure, we constructed a translational fusion between the *A. aculeatus rhg*A promoter and the *A. niger* glucose oxidase-encoding gene *gox*C (Witteveen et al. 1993). As *A. aculeatus* does not produce glucose oxidase (GOX) itself, and GOX is an extracellular enzyme which is mainly associated with the cell wall, it is a convenient reporter enzyme which can be easily detected at colony level in a plate assay (Witteveen et al. 1990; De Graaff et al. 1994). Here we describe the isolation and characterization of *A. aculeatus* mutants which show elevated levels of rhamnogalacturonan hydrolase expression.

Materials and methods

Strains and plasmids. Plasmids were propagated in *E. coli* DH5α (BRL, Gaithersburg, USA). Plasmid pIM803 contains a genomic 3.9-kb *Bam*HI-*Sal*I fragment encoding the *A. aculeatus rhg*A gene in pBluescriptSK(+) (Suykerbuyk et al. 1995). pIM732 contains a genomic 2.3-kb *Nsi*I fragment containing the *A. niger gox*C gene (Witteveen et al. 1993) cloned in the *Pst*I site of pUC19. pIM805 contains a genomic 3-kb *Bam*HI fragment encoding the *A. aculeatus pyr*A gene in pUC19 (Suykerbuyk and Visser, unpublished). The plasmids pIM806 and pIM826, the construction of which is described below, contain the glucose oxidase-expression tag. Plasmid pIM826 also contains the *pyr*A auxotrophic marker (Fig. 1A). *A. aculeatus* strain NW217 (*pyr*A4, *csp*A1, *fwn*A1, *lys*A1; Suykerbuyk et al. 1995), which was derived from *A. aculeatus* CBS115.80, was used for transformation experiments. One of its parent strains, NW212 (*pyr*A4), was used for crossing experiments (Suykerbuyk et al. 1995).

Aspergillus transformation, DNA isolation and DNA manipulation. Standard methods (Goosen et al. 1987) were employed for the transformation of *A. aculeatus* NW217. DNA was isolated from *A. aculeatus* transformants by the method of De Graaff et al. (1988). Standard methods were used for the propagation and preparation of plasmid DNA, Southern analysis, and cloning (Sambrook et al. 1989). Sequencing of recombinant double-stranded plasmid DNA was performed using the T7 Sequencing kit (Pharmacia LKB, Uppsala, Sweden).

Growth conditions and Western analysis. For rhamnogalacturonan hydrolase expression, minimal medium (MM; Pontecorvo et al. 1953) supplemented with 0.5% (w/v) yeast extract, 0.2% (w/v) casamino acids and 1% (w/v) sucrose or fructose was inoculated with spores of *A. aculeatus* strains in a concentration of 10^6 spores/ml. After 24 h of growth at 30°C the mycelium was transferred to MM (pH 6.0) with a combination of rhamnose and galacturonic acid with or without fructose (Janssen Pharmaceutica), or to MM (pH 4.0) with brown-ribbon apple pectin (degree of esterification 72.8%; Obipektin, Bischofszell, Switzerland) as a carbon source. Carbon sources were added to a final total concentration of 1%, except fructose which was added additionally to 0.2%. The rhamnose and galacturonic acid solutions (pH 6.0) were filter-sterilized and added separately. Rhamnogalacturonan hydrolase expression was detected by Western analy-

sis of the culture filtrates after 24 h of growth (Burnette 1981). For this a mouse polyclonal antibody raised against purified *A. aculeatus* rhamnogalacturonan hydrolase was used (Suykerbuyk et al. 1995).

Northern analysis. Total RNA was isolated from powdered mycelium by using Trizol reagents (Gibco BRL). Equal amounts of RNA were denatured with glyoxal and DMSO, subjected to electrophoresis and blotted onto Hybond N (Amersham, Buckinghamshire, England). The filters were prehybridized for 2 h in standard hybridization buffer (Sambrook et al. 1989) at 65°C, hybridized overnight in 10% (w/v) dextran sulphate, 5 x Denhardt, 6 x SSC, 0.5% (w/v) SDS and 50% (v/v) formamide at 42°C and washed twice for 30 min with 2 x SSC, 0.1% (w/v) SDS and 0.1% (w/v) sodium pyrophosphate at 65°C. A 2.5-kb *Hin*dIII fragment containing the *rhg*A gene of *A. aculeatus* was used as a probe. Re-probing of the filter with a DNA fragment encoding 28S rRNA of *Agaricus bisporus* (Schaap et al. 1996) was performed to verify equal loading. The same hybridization conditions were used for this, but washing was performed at 60°C.

Construction of the goxC gene under control of the rhgA promoter. In order to position the *A. aculeatus rhg*A promoter precisely adjacent to the *A. niger gox*C coding region we used an adaptation of the splicing by overlap extension technique (Horton et al. 1989). Therefore, one polymerase chain reaction (PCR) was performed using the cloned *rhg*A gene as a template and -108ATGAAGGCTCCTTACTG-92 and +17ACAAGGAGAGTCT GCATGATTCACTTGAATC-14 as primers (the startcodon is in bold). In a second PCR the cloned *gox*C gene was used as a template with -14GATTCAAGTGAATCATGCAGACTCTCC TTGT+17 and +312GACCTCCACCAGCGATG+296 as primers. The two partly overlapping PCR products were annealed and used as templates in combination with the two now outer primers (-108 to -92 and +296 to +312) in a third PCR. The resulting fusion product was digested with *Pst*I and *Sal*I and the obtained 208-bp fragment obtained was ligated in pBluescript and sequenced. Subsequently, a 1.1-kb *Pst*I fragment containing the *rhg*A promoter and a 1.9-kb *Sal*I fragment containing part of the *gox*C gene were ligated in respectively the *Pst*I and the *Sal*I site of this construct, resulting in plasmid pIM806. A 3-kb *Bam*HI fragment of pIM805, on which the *pyr*A gene is located, was ligated in the *Bam*HI site of pIM806, resulting in plasmid pIM826 (Fig. 1A).

Mutagenesis. Transformant NW217::pIM826/13, containing one copy of the reporter construct adjacent to the intact *rhg*A locus, was mutagenized. For this, 10 ml of a suspension containing 10^7 conidia per ml was irradiated in a petri dish with UV light (2 J/m^2 per s) for 90 s. This dose was determined to produce a spore viability of approximately 70%.

Mutant selection. The mutagenized spores were diluted and plated at a density of 10^4 and 10^5 viable spores per 9-cm diameter Petri dish, containing MM supplemented with 2.5 mM *O*-anisidine and 0.01% Triton X-100 and with 1% rhamnose or a combination of 0.5% rhamnose and 0.5% galacturonic acid, with or without 0.2% fructose, as a carbon source.

After 72 h of growth, glucose oxidase-producing colonies were visualized by the detection of H_2O_2 formation. These colonies were coloured brownish-red at the rim after the addition of 5 ml of a solution containing 50 mM glucose and 1 μg/ml horse radish peroxidase (Boehringer, Mannheim, Germany) in 100 mM sodium phosphate buffer, pH 6.0, to the plate.

Genetic analysis. Standard procedures used for genetic analysis of *A. aculeatus* mutant strains were as described by Bos et al. (1988). Somatic diploids were made by back-crossing each of the mutant strains with NW212. The somatic diploids obtained were purified and diploidy was verified by measuring the size of the spores, using a Coulter-counter (Coulter Electronics Nederland, Mijdrecht, The Netherlands). The diploids were directly transferred to complete medium supplemented with uridine and benomyl (0.08 μg/ml) for haploidization. Segregants were isolated, purified, and tested for all markers. In segregants which no longer carried the reporter construct, rhamnogalacturonan hydrolase expression was analysed to verify the presence of the respective rhamnogalacturonan hydrolase regulatory (*rgr*) mutations. Complementation of *rgr* mutations was tested in hetero-diploids, obtained by crossing strains carrying different mutations. As a control, homo-diploids were constructed by back-crossing of the mutant strains with their recombinant, reporter-less counterparts.

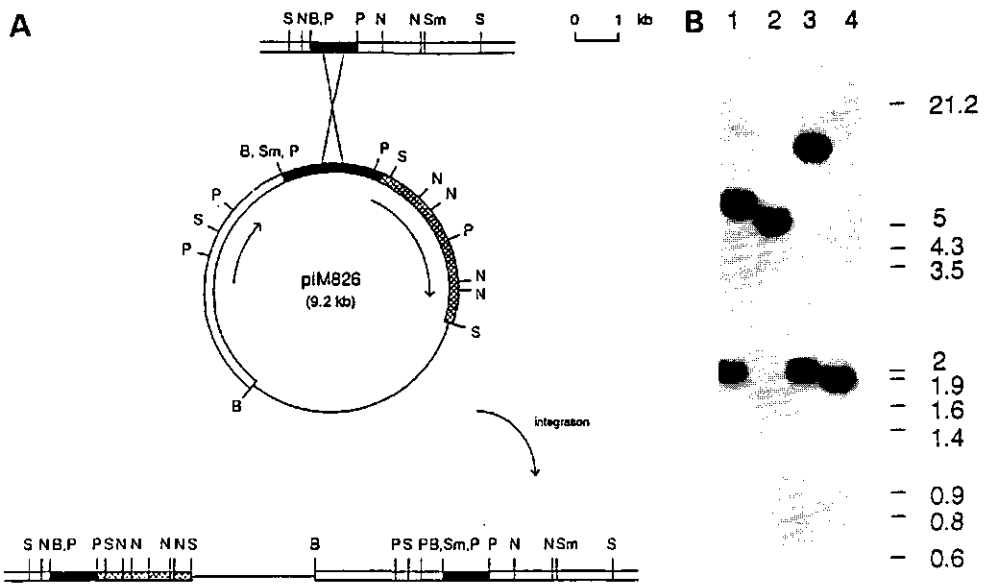


Fig. 1 A Integration scheme of pIM826 in the chromosomal DNA of *A. aculeatus*. The *rhg*A gene is indicated by a *filled box*, the *pyr*A gene by an *open box*, the *gox*C gene by a *hatched box* and pUC sequences by a *line*. Restriction enzyme-site abbreviations: *B BamHI*; *N NcoI*; *P PstI*; *S SalI*; *Sm SmaI*. **B** Southern analysis of *A. aculeatus* transformant NW217::pIM826/13. Genomic DNA of transformant (*lanes* 1,3) and host (*lanes* 2,4) strains was digested with *SalI* (*lanes* 1,2) or *NcoI* (*lanes* 3,4). The 1.1-kb *PstI* fragment containing the *rhg*A promoter of *A. aculeatus* was used as a probe. Marker sizes (kb) of lambda DNA digested with *Eco*RI-*Hind*III are shown at the right side of the membrane

Results and discussion

Rhamnogalacturonan hydrolase expression on different carbon sources

To investigate rhamnogalacturonan hydrolase expression on simple carbon sources, transformant NW217::pIM803/75, containing multiple copies of the *rhg*A gene in the genome (Suykerbuyk et al. 1995), was grown on MM with 1% sucrose for 24 h. The mycelium was then transferred to MM containing apple pectin, rhamnose, galacturonic acid, or a combination of both monosaccharides as a carbon source and allowed to grow for another 24 h. Western analysis of

the culture filtrates revealed that when rhamnose and galacturonic acid were used in combination the rhamnogalacturonan hydrolase was expressed to approximately the same level as on apple pectin (Fig. 2, lanes 1,4). However, when either rhamnose or galacturonic acid alone was used no rhamnogalacturonan hydrolase expression could be detected (Fig. 2, lanes 2,3). When the *A. aculeatus* host strain was used, the same results were obtained, albeit with lower expression levels (data not shown).

Expression of the reporter fusion plasmid in *A. aculeatus*

The construction of the translational fusion between the *rhg*A promoter and the *gox*C gene, resulting in plasmids pIM806 and pIM826, is described in the Materials and methods section. Initially, cotransformation of *A. aculeatus* strain NW217 was performed with pIM806, containing the *rhg*A::*gox*C reporter fusion, and the *pyr*A-containing plasmid pIM805. Transformants were obtained on uridine-deficient medium and analysed by the GOX-expression plate assay. Different levels of GOX expression were found in about 70% of the transformants, one of which NW217::pIM806/23, showed the highest level. Southern analysis of this transformant revealed the presence of 10-20 copies of the reporter construct integrated in the genome.

To determine whether the *gox*C reporter fusion gene responded appropriately to *rhg*A control, transformant NW217::pIM806/23 was plated on media containing

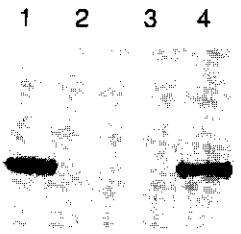


Fig. 2 Rhamnogalacturonan hydrolase expression in *A. aculeatus* transformant NW217::pIM803/75 as determined by Western analysis. The strain was grown for 24 h on 1% sucrose, after which the mycelium was transferred to media containing apple pectin (*lane* 1), rhamnose (*lane* 2), galacturonic acid (*lane* 3), or a combination of both monosaccharides (*lane* 4) as a carbon source

apple pectin or a combination of rhamnose and galacturonic acid as a carbon source. After 72 h of growth, colonies were stained for glucose oxidase activity. Clear staining was visible on the monosaccharide combination, whereas only a very low level of GOX expression was found on apple pectin (data not shown). By contrast, in the host strain the level of rhamnogalacturonan hydrolase expression after growth on apple pectin was the same as that on a combination of rhamnose and galacturonic acid (see above).

However, GOX expression in the transformant was inversely correlated with the pectin concentration in the plate (data not shown). Thus, pectin inhibited GOX expression or activity or else influenced GOX-activity staining by reacting with hydrogen peroxide. The highest expression was obtained with apple pectin in a concentration of 0.25% (w/v) at pH 6.0 and this low concentration was used in further experiments.

The same transformant was used for an analysis of the induction capacity of other monosaccharides, using GOX expression as a tag. For this, other reported hairy region constituents, arabinose, fucose, galactose, glucose and xylose, were tested in combination with rhamnose, with galacturonic acid, and with each other. GOX expression was found on media containing either fucose or galactose in combination with rhamnose or galacturonic acid, although at a much lower level than that on rhamnose and galacturonic acid. Although we cannot exclude carbon-catabolite repression in those cases, rhamnose and galacturonic acid apparently play a specific role in the regulation of rhamnogalacturonan hydrolase. This combination was therefore selected as a carbon source for the screening of rhamnogalacturonan hydrolase-overproducing mutants.

Isolation of mutants affected in rhamnogalacturonan hydrolase expression

For mutagenesis experiments we constructed a strain in which the fusion gene was integrated in a single copy at the *rhg*A locus of the *A. aculeatus* genome, leading to an intact copy of the reporter construct adjacent to an intact *rhg*A

gene. This ensures that the chromosomal environment of the *rhg*A promoter remains as much as possible the same (Fig. 1A). For this, strain NW217 was transformed with plasmid pIM826, in which the *pyr*A gene had been cloned as a selection marker. Genomic DNA of eight of the transformants obtained was digested with either *Sal*I or *Nco*I and hybridized with the *rhg*A promoter. Comparison of the respective banding patterns with those obtained in the auxotrophic host strain NW217 revealed one transformant, NW217::pIM826/13, in which the desired integration event had occurred. In this transformant two hybridizing bands, 1.9 kb and 4.8 kb in size, were shown in the *Sal*I digest, whereas a 4.5-kb hybridization band was found in the host strain (Fig. 1B). Substitution of the 4.5-kb by the 4.8-kb band was indicative of the insertion of the reporter fusion plasmid at the *rhg*A locus, whereas the presence of a single additional fragment, 1.9 kb in size, was expected in the case of single-copy integration. Likewise, in the *Nco*I digest a 1.8-kb band in the host strain was substituted by a 1.9-kb band in the transformant, which is indicative of the integration of the fusion gene at the *rhg*A locus. Furthermore, this transformant showed a single additional band, 8 kb in size, which is again indicative of single-copy integration. The integrity of the adjacent *rhg*A gene was confirmed with five additional restriction digests and by demonstrating rhamnogalacturonan hydrolase expression after growth on apple pectin (data not shown). In the GOX plate assay an inducible, low level of activity staining was found in this transformant, allowing detection of mutant colonies with increased GOX staining.

After mutagenesis of conidia by UV light and plating on different media, approximately 2 x 10^6 colonies were screened on agar plates. Mycelia of 59 colonies showed staining levels higher than that observed in the parental strain NW217:: pIM26/13 and were designated *rgr*1 to *rgr*59. From these, 35 colonies remained positive after a second screening, and rhamnogalacturonan hydrolase expression in these colonies was analysed in shake cultures containing media corresponding to those in the plate screening. Western analysis of the culture filtrate revealed 13 mutants in which rhamnogalacturonan hydrolase expression levels were co-elevated. These included 11 of the 26 mutants which were selected

Table 1 Glucose oxidase expression in an *A. aculeatus* transformant and derived mutants, obtained by UV treatment of *A. aculeatus* transformant strain NW217::pIM826/13. The mutant strains were grown on rhamnose (R), galacturonic acid (G), a combination of both monosaccharides with (RGF) or without (RG) fructose (F), or apple pectin [AP, 0.25% (w/v), pH 6.0], as a carbon source. The plates used for the first selection contained galacturonic acid, or a combination of rhamnose and galacturonic acid with or without fructose as a carbon source

Mutant	R	G	RG	RGF	AP	First selection
rgr48	++[1]	+++	++++	+++	+++++	RGF
rgr24	++	+++	++++	+++	+++++	RG
rgr21	+	++	++++	++	++	RG
rgr11	±	+	++++	-	++	G
rgr25	±	++	+++	++	++++	RG
rgr34	+	+	+++	+	+++	RG
rgr31	±	-	+++	++	+++	RG
rgr5	-	-	+++	+	+	RG
rgr27	-	-	++	±	+	RG
rgr8	-	-	++	-	+	RG
rgr23	-	-	++	-	+	RG
rgr36	-	-	++	-	+	RG
rgr37	-	-	++	-	+	RG
Transformant	-	-	+	-	+	

[1] Relative GOX expression levels are indicated as + + + + +, extremely good; + + + +, very good; + + +, good; + +, poor; +, very poor; -, no staining

on a combination of rhamnose and galacturonic acid, one of the seven mutants selected on the same medium supplemented with fructose, and one of the two mutants selected on galacturonic acid alone. These 13 mutants, which presumably contain mutations influencing the induction pathway of rhamnogalacturonan hydrolase expression, were further analysed by monitoring GOX expression on plates containing rhamnose, galacturonic acid, a combination of those sugars

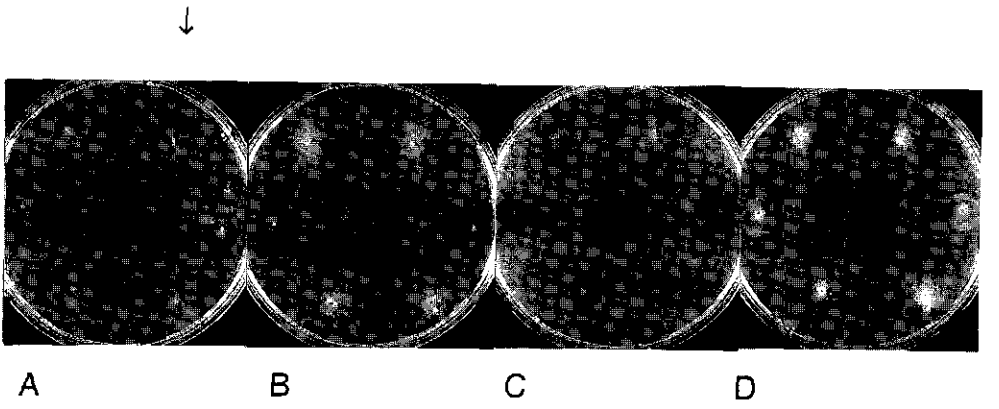


Fig. 3A-D Glucose oxidase (GOX) activity in the *A. aculeatus* host (clockwise indicated; *arrow = 1*), transformant (*6*) and five different mutant [NW217::pIM826/13 *rgr*8 (*3*), *rgr*25 (*2*), *rgr*27 (*4*), *rgr*34 (*5*) and *rgr*48 (*center*)] strains as determined by the GOX-expression plate assay. The strains were grown for 72 h on plates containing rhamnose (**A**), galacturonic acid (**B**), rhamnose and galacturonic acid (**C**), or rhamnose, galacturonic acid and fructose (**D**) as a carbon source

with or without fructose, or apple pectin as a carbon source. Four of the mutants showed expression levels which were slightly higher on a combination of rhamnose and galacturonic acid, whereas wild-type levels were found on the other substrates. In nine mutants elevated levels of GOX staining were found not only on a combination of both sugars, but to a variable extent also on either of the monosaccharides alone, on rhamnose and galacturonic acid in combination with fructose, and on apple pectin (Table 1). Five mutants with clearly different expression patterns, *rgr*8, *rgr*25, *rgr*27, *rgr*34 and *rgr*48 (Fig. 3), were selected for determination of rhamnogalacturonan hydrolase expression and genetic analysis.

Rhamnogalacturonan hydrolase expression in glucose oxidase-overproducing mutants

The five mutants were grown in MM with rhamnose, galacturonic acid or both, either with or without fructose, in shake cultures. Western analysis of the cul-

Table 2 Glucose oxidase (GOX) and rhamnogalacturonan hydrolase (RHG) expression in *A. aculeatus* transformant and derived mutant strains, as determined by plate assay (GOX) and Western analysis (RHG). The mutant strains were grown on rhamnose (R), galacturonic acid (G), or a combination of both monosaccharides with (RGF) or without (RG) fructose (F), as a carbon source

Mutant	R		G		RG		RGF	
	GOX	RHG	GOX	RHG	GOX	RHG	GOX	RHG
*rgr*48	+++[1]	++	++++	±	+++++	+++++	+++	++++
*rgr*25	-	±	+++	±	++++	++++	±	++++
*rgr*34	++	+	++	±	+++	++++	±	++++
*rgr*27	-	±	-	-	++	++	-	+
*rgr*8	-	-	-	-	++	++	-	-
Transformant	-	-	-	-	+	+	-	±

[1] Relative GOX expression levels are indicated as + + + + +, extremely good; + + + +, very good; + + +, good; + +, poor; +, very poor; -, no staining

ture filtrates revealed different phenotypes (Table 2), three of which, corresponding to the mutants *rgr*27, *rgr*25 and *rgr*48, are illustrated in Fig. 4. When a combination of both sugars was used as a carbon source, the expression levels of rhamnogalacturonan hydrolase corresponded to those of GOX in the respective mutants. On the other substrates, however, discrepancies were found between the relative expression levels obtained for rhamnogalacturonan hydrolase and glucose oxidase, most clearly demonstrated in mutant *rgr*48. Rhamnogalacturonan hydrolase was expressed to a low or even negligible level on rhamnose and galacturonic acid, respectively, whereas GOX expression was clearly visible on these substrates. On the other hand, when the sugars were used in combination with fructose, rhamnogalacturonan hydrolase was induced at a relatively higher level than glucose oxidase. This can be due to the different growth conditions used for the detection of the enzymes. Alternatively, the detection methods might not be suitable for quantitative measurements. Another

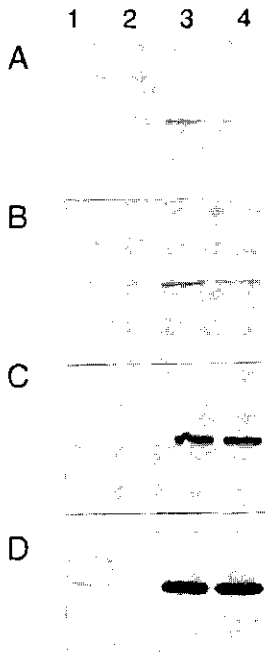


Fig. 4 Rhamnogalacturonan hydrolase expression in the *A. aculeatus* transformant (**A**) and three different mutant [NW217::pIM826/13 *rgr*27 (**B**), *rgr*25 (**C**) and *rgr*48 (**D**)] strains as determined by Western analysis. The strains were grown in cultures containing 1% sucrose for 24 h, after which the mycelium was transferred to rhamnose (*lanes* 1), galacturonic acid (*lanes* 2), rhamnose and galacturonic acid (*lanes* 3), or rhamnose, galacturonic acid, and fructose (*lanes* 4) as a carbon source

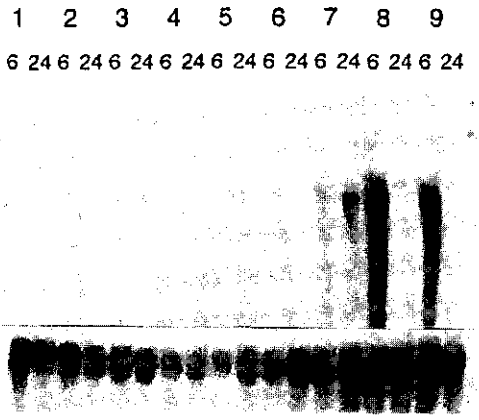


Fig. 5 Rhamnogalacturonan hydrolase expression in *A. aculeatus* host (*lanes* 1-3), transformant (*lanes* 4-6) and mutant (NW217::pIM826/13 *rgr*48, *lanes* 7-9) strains as determined by Northern analysis. All strains were grown on 1% fructose for 24 h, after which the mycelium was transferred to MM with apple pectin (*lanes* 1,4,7), rhamnose and galacturonic acid (*lanes* 2,5,8), or rhamnose, galacturonic acid and fructose (*lanes* 3,6,9). RNA was isolated from the mycelium 6 h and 24 h after induction. Hybridization was performed using the 2.5-kb *Hin*dIII fragment containing the *rhg*A gene of *A. aculeatus*. Lower panel: re-hybrization of the same blot with an *A. bisporus* 28S rRNA-encoding probe as a loading control

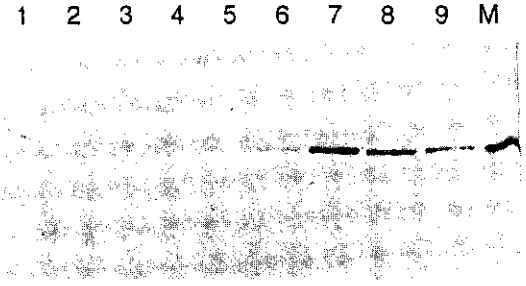


Fig. 6 Rhamnogalacturonan hydrolase expression in *A. aculeatus* host (*lanes* 1-3), transformant (*lanes* 4-6) and mutant (NW217::pIM826/13 *rgr*48, *lanes* 7-9) strains as determined by Western analysis. All strains were grown on 1% fructose for 24 h, after which the mycelium was transferred to MM with apple pectin (*lanes* 1,4,7), rhamnose and galacturonic acid (*lanes* 2,5,8), or rhamnose, galacturonic acid, and fructose (*lanes* 3,6,9). After growth for another 24 h the culture filtrates were analysed. *M*, purified rhamnogalacturonan hydrolase

possibility is the lack of some distant regulatory element(s) in the 1.1-kb promoter fragment present in the fusion construct.

In the mutants *rgr*25, *rgr*34, and *rgr*48 much higher rhamnogalacturonan hydrolase expression levels are found than in the transformant strain when a combination of both sugars was used as a carbon source, even in the presence of the repressing carbon source fructose. Possibly, the high rhamnogalacturonan hydrolase expression levels can also be obtained when lower amounts of the inducing sugars are used in combination with fructose.

Since *rgr*48 showed the most distinctive phenotype, this mutant was subjected to further analysis. To investigate the mechanism by which the factor, altered in *rgr*48, influences rhamnogalacturonan hydrolase expression, the *rhg*A transcription levels in this strain after growth on apple pectin and on a combination of both sugars with and without fructose were determined by Northern analysis and compared to those in the original strain NW217 and those in the parental strain. The *rhg*A transcript is clearly visible after 6 h of growth on rhamnose and galacturonic acid, either with or without fructose, and at a lower level on apple pectin, whereas only a barely visible, low level of transcript can be seen in the original strain (Fig. 5). This is in agreement with the results obtained by Western analysis (Fig. 6). Apparently, rhamnogalacturonan hydrolase expression in mutant *rgr*48 is influenced at the transcriptional level, making it very likely that in this case the mutagenized factor is, or else inhibits, a negatively acting regulatory element.

Genetic analysis of mutant strains

In order to perform complementation analysis between the five mutant strains *rgr*8, *rgr*25, *rgr*27, *rgr*34 and *rgr*48, which all contained the markers *pyr*A4, *csp*A1, *fwn*A1, *lys*A1 and *pyr*A$^+$/*gox*C$^+$ (originating from the plasmid), we had to remove some of the markers by back-crossing each individual mutant with *A. aculeatus* NW212 (*pyr*A4). Somatic diploids were obtained at a frequency of 10^{-5}, which is determined by spore-size measurements. This is comparable to

what has been found in *A. niger* (Bos et al. 1988); however, many of the diploids were unstable, as manifested by a high frequency of morphologically aberrant colonies after growth on MM or complete medium. The diploids were also very sensitive to benomyl, since optimal haploidization already occurred at a benomyl concentration of 0.08 μg/ml.

Diploids were recognized by black spores (*fwn*A$^+$) and prototrophic phenotype. The level of GOX expression in the five different diploids was lower than that found in the respective haploid mutant strains and slightly less than in the haploid parental strain (data not shown), indicating that each of the mutations was recessive.

After haploidization a variety of recombinants was obtained, in which the presence of the different genetic markers was established. Recombination frequencies between the different markers, including the respective mutations, were calculated. For this, more than 100 progenitors were analysed per crossing experiment.

Recombination frequencies found between the *lys*A$^+$ and the *pyr*A$^+$/*gox*C$^+$ phenotypes were 17%, 13%, 17%, 21% and 43% in five different crossing experiments. Apparently, the *lys*A1 marker is linked to the reporter construct and thus to the *rhg*A gene. The relatively high frequency of 43% is due to a mitotic crossing-over event in one particular diploid.

As expected, there is a very low level of recombination between the functional *pyr*A gene and the *gox*C gene ranging from 0% to 5% in five different crossing experiments. The occasional occurrence of recombination events between these closely linked markers was confirmed by demonstrating plasmid sequences, which surround the *pyr*A gene in pIM826, in reporter-less recombinants by Southern analysis (data not shown). Reversion of the *pyr*A4 mutation in these recombinants is therefore unlikely. Linkage has also been shown between *csp*A1 and *fwn*A1 (previous results). The recombination frequencies between the *rgr*25 mutation and each of the *lys*A1, *pyr*A$^+$/*gox*C$^+$ and *fwn*A1 markers was 18%, 25% and 58%, respectively. Recombination frequencies between the other *rgr* mutations and these markers were in the range of 44-59%, 63-77% and 35-

55%, respectively. From this, we concluded that *rgr*25 is linked to *lys*A1 and the reporter construct, resulting in the following linkage map of NW217::pIM-826/13: I, *rgr*25/*lys*A1/gox/pyr; II, *csp*A1/*fwn*A1; III, *pyr*A4. The other *rgr* mutations are not linked to any of these markers.

GOX non-producing recombinants (*pyr*A4, *fwn*A$^+$, *lys*A$^+$) could be isolated for all the mutants. The presence of the respective mutations in these recombinants was verified by Western analysis (data not shown). Four of the recombinants thus obtained were crossed with the five original mutant strains in independent experiments. Desired diploids were again recognized by their combined *fwn*A$^+$ and prototrophic phenotype. Analysis of GOX expression in these diploids revealed comparable levels, which were lower than those in the respective haploids, indicating independency of the five loci mutagenized.

Conclusions

Glucose oxidase expression is an easily scorable and novel phenotype in *A. aculeatus*. Single-copy integration of the reporter fusion at the *rhg*A locus allows a genetic analysis of the regulation of the rhamnogalacturonan hydrolase induction pathway.

Mutants selected on galacturonic acid, and a combination of rhamnose and galacturonic acid with and without fructose, showed common glucose oxidase expression patterns after growth on the respective carbon sources. Thirteen of the thirty five mutants analysed contained some form of trans-acting mutations, as proved by their influence on both the *rhg*A::*gox*C expression and the *rhg*A gene. The positive effect on rhamnogalacturonan hydrolase expression suggests that negatively acting factors are mutated. By parasexual genetics at least four different mutations, which were all found to be recessive, could be assigned to different loci, one of which (*rgr*25) is linked to the *rhg*A locus. Rhamnogalacturonan hydrolase expression levels of about 5-10 times wild-type level were shown in the mutants *rgr*25, *rgr*34 and *rgr*48 after growth on a combination of rhamnose and galacturonic acid, with or without fructose, as a carbon source.

This opens perspectives to still further increase the expression levels of rhamnogalacturonan hydrolase, and possibly other pectinolytic enzymes, to be realized by recombination of the different independent mutations obtained and by repeated transformation of reporter-less uridine-requiring recombinants.

Acknowledgements This project was financially supported by Unilever Research Laboratorium, Vlaardingen and Quest International, Naarden. We appreciate stimulating discussions with Dr. W. Musters and Dr. H. Stam

References

Azadi P, O'Neill MA, Bergmann C, Darvill AG, Albersheim P (1996) The backbone of the pectic polysaccharide rhamnogalacturonan I is cleaved by an endohydrolase and an endolyase. Glycobiol 5:783-789

Bos CJ, Debets AJM, Swart K, Huybers A, Kobus G, Slakhorst SM (1988) Genetic analysis and the construction of master strains for assignment of genes to six linkage groups in *Aspergillus niger*. Curr Genet 14:437-443

Burnette WN (1981) "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein. Anal Biochem 112:195-203

De Graaff LH, Broek HWJ van den, Visser J (1988) Isolation and transformation of the pyruvate kinase gene of *Aspergillus nidulans*. Curr Genet 13:315-321

De Graaff LH, Broeck HC van den, Ooijen AJJ van, Visser J (1994) Regulation of the xylanase-encoding *xln*A gene of *Aspergillus tubingensis*. Mol Microbiol 12:479-490

Goosen T, Bloemheuvel G, Gysler C, Bie DA de, Broek HWJ van den, Swart K (1987) Transformation of *Aspergillus niger* using the homologous orotidine-5'-phosphate-decarboxylase gene. Curr Genet 11:499-503

Gross KC, Starrett DA, Chen HJ (1995) Rhamnogalacturonase, α-galactosidase and β-galactosidase. Potential roles in fruit softening. Acta Hort 398:121-130

Horton RM, Hunt HD, Ho SN, Pullen JK, Pease LR (1989) Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene 77:61-68

Kofod LV, Kauppinen S, Christgau S, Andersen LN, Heldt-Hansen HP, Dörreich K, Dalbøge H (1994) Cloning and characterization of two structurally and functionally divergent rhamnogalacturonases from *Aspergillus aculeatus*. J Biol Chem 269:-29182-29189

Mutter M, Beldman G, Schols HA, Voragen AGJ (1994) Rhamnogalacturonan α-L-Rhamnopyranohydrolase. Plant Physiol 106:241-250

Mutter M, Colquhoun IJ, Schols HA, Beldman G, Voragen AGJ (1996) Rhamnogalacturonan α-L-rhamnopyranosyl-(1,4)-α-D-galactopyranosyluronide lyase, a new enzyme able to cleave RG regions of pectins. Plant Physiol 110:73-77

Pontecorvo G, Roper JA, Hemmons LJ, MacDonald KD, Bufton AWJ (1953) The genetics of *Aspergillus nidulans*. Adv Genet 5:141-238

Sakamoto M, Shirane Y, Naribayashi I, Kimura K, Morishita N, Sakamoto T, Sakai T (1994) Purification and characterization of a rhamnogalacturonase with protopectinase activity from *Trametes sanguinea*. Eur J Biochem 226:285-291

Sambrook J, Fritsch E, Maniatis T (1989) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Schaap PJ, Müller Y, Baars JJP, Camp HJM op den, Sonnenberg ASM, Griensven LJLD van, Visser J (1996) Nucleotide sequence and expression of the $NADP^+$-dependent glutamate dehydrogenase (*gdh*A) from *Agaricus bisporus*. Mol Gen Genet 250:339-347

Schols HA, Voragen AGJ (1994) Occurrence of pectic hairy regions in various plant cell wall materials and their degradability by rhamnogalacturonase. Carbohydr Res 256:83-95

Schols HA, Geraeds CJM, Searle-van Leeuwen MF, Kormelink FJM, Voragen AGJ (1990) Rhamnogalacturonase: a novel enzyme that degrades the hairy regions of pectins. Carbohydr Res 206:105-115

Suykerbuyk MEG, Schaap PJ, Stam H, Musters W, Visser J (1995) Cloning, sequence and expression of the gene coding for rhamnogalacturonase (RHG) of *Aspergillus aculeatus*; a novel pectinolytic enzyme. Appl Biotechnol Microbiol 43:861-870

Witteveen CFB, Vondervoort P van de, Swart K, Visser J (1990) Glucose oxidase overproducing and negative mutants of *Aspergillus niger*. Appl Microbiol Biotechnol 33:683-686

Witteveen CFB, Vondervoort PJI van de, Broeck HC van den, Engelenburg FAC van, Graaff LH de, Hillebrand MHBC, Schaap PJ, Visser J (1993) Induction of glucose oxidase, catalase and lactonase in *Aspergillus niger*. Curr Genet 21:408-416

5 Classification of black *Aspergilli* by RFLP analysis: Molecular criteria to identify *Aspergillus aculeatus*, *Aspergillus japonicus* and *Aspergillus carbonarius*

Abstract Restriction fragment length polymorphisms (RFLPs) were examined between 30 isolates of *Aspergillus japonicus*, *A. aculeatus* and *A. carbonarius* using a fragment of the 28S rDNA of *Agaricus bisporus*, the *A. niger pki*A gene and fragments of the *pel*A and *pel*D genes of *A. niger* as probes. With these probes, twenty-eight strains from two culture collections could be divided into three distinct groups based on their RFLP patterns. The RFLP patterns of the *A. carbonarius* group were homogeneous. Within the *A. japonicus* group significant polymorphisms were observed only with the *pel*D probe, whereas within the *A. aculeatus* group heterogeneity was detected with the *pel*A, the *pel*D and the *pki*A probe. The division however does not correspond for all strains with the taxonomical data based on morphological criteria. One *A. aculeatus* strain was not related to any of the three groups and may represent another species. One *A. carbonarius* strain showed the same patterns as the *A. tubingensis* reference strain. The molecular classification criteria developed here provide a simple and objective method for strain identification, using strains from public collections as reference strains. Analysis of other molecular criteria in these strains, will demonstrate to which extent this method will be applicable for broader use.

Introduction

A number of black *Aspergilli* (genus *Aspergillus*, section *Nigri*) are industrially exploited for the production of various extracellular enzymes and secondary metabolites, which are used in the food industry. Many of these products are generally recognized as safe (GRAS). Clear classification and identification of these strains can contribute to legal strain protection. The best characterized industrial organism of this section is *A. niger*. However, a continuous search for novel industrially important enzymes has drawn the attention to other black *Aspergilli*, e.g. *A. aculeatus*. From different strains of this species the identifi-

cation of novel pectinolytic enzymes, among which rhamnogalacturonan hydrolase, rhamnogalacturonan lyase and rhamnogalacturonan acetylesterase, and the cloning of their corresponding genes have been described recently (Searle-van Leeuwen et al. 1992; Kofod et al. 1994; Suykerbuyk et al. 1995). *A. aculeatus* and *A. japonicus* are both uniseriate black *Aspergillus* species (Al-Musallam 1980). They are however difficult to distinguish from each other using morphological criteria and this has led to confusion and the introduction of subclassification. Formal classification of the strains as *A. aculeatus* or *A. japonicus* is based on differences in vesicle size and conidiophore length according to Raper and Fennell (1965). They included the species *A. atroviolaceus*, *A. violaceo-fuscus* and *A. luchuensis*, which were distinguished previously by Mosseray (1934) and Thom and Raper (1945). However, since the morphological differences between *A. aculeatus* and *A. japonicus* are only minor, Al-Musallam treated both taxa at varietal level of *A. japonicus* (1980). The difficulties in distinguishing these taxa using morphological criteria suggests that additional measures of relatedness are required.

Classification of biseriate black *Aspergilli*, some of which are distinguished as a separate genus, called *Sterigmatocystis* by Cramer in 1859, is still problematic. Among these, *A. carbonarius* strains are not always correctly classified, in spite of distinct morphological features like large biseriate conidial heads containing large verrucose to tuberculate conidia.

The difficulties encountered in classification of these strains based on classical morphological characters might be circumvented by molecular genetic markers instead. Restriction fragment length polymorphism (RFLP) analysis has proven to be a useful method for classification of strains belonging to the *A. niger* aggregate within the Section *Nigri*. Kusters-van Someren et al. (1990, 1991) divided these strains into two distinct species, *A. niger* and *A. tubingensis*, by RFLP analysis of genomic DNA. Varga et al. (1994) confirmed these findings and distinguished a third species by RFLP analysis of mitochondrial DNA. The distinction between *A. niger* and *A. tubingensis* species was further supported by incompatibility between these species (Kusters-van Someren et al. 1991) and

the absence of the gene encoding the industrially important enzyme endo-xylanase A (XLNA) in *A. niger* (De Graaff et al. 1994). In contrast, ambiguous results were obtained about the status of a few *A. aculeatus*, *A. japonicus* and *A. carbonarius* strains included in the RFLP analysis (Kusters-van Someren et al. 1991).

In this study we used RFLP analysis for the classification of 30 black *Aspergillus* strains, including *A. aculeatus*, *A. atroviolaceus*, *A. japonicus*, *A. japonicus* var. *capillatus*, *A. luchuensis*, *A. violaceo-fuscus* mut. *grisea* and *A. carbonarius* strains and a *Sterigmatocystis fusca* strain, all obtained from public culture collections. As probes we used part of the ribosomal repeat unit of *Agaricus bisporus* and the pyruvate kinase gene of *A. niger* (*pki*A). Both probes contain highly conserved sequences, but hybridize with different parts of the genome, making them convenient RFLP markers for strain comparisons. The ribosomal repeat unit was used before for the classification of isolates belonging to the *A. niger* aggregate (Varga et al. 1994) but also of isolates belonging to the species *A. nidulans* (Varga and Croft 1994), *A. fumigatus* (Spreadbury et al. 1990) and *Fusarium* (Whitehead et al. 1992). Kusters-van Someren et al. (1990, 1991) used two other genes, encoding the industrially important pectin lyases A and D, as probes to classify strains belonging to the *A. niger* aggregate. As these genes appear to be less conserved these probes were used for subclassification. The RFLP patterns obtained with four different probes were analysed and compared with two reference strains, representing the species *A. niger* and *A. tubingensis* (Kusters-van Someren et al. 1990).

Materials and methods

Strains, culture conditions and DNA extraction. The strains used in this study were obtained from the Centraalbureau voor Schimmelcultures (CBS, Baarn, The Netherlands) and from the Agricultural Research Service of the United States Department of Agriculture (USDA, Peoria, USA; NRRL; Table 1), except for the

Table 1 *Aspergillus* strains used for RFLP analysis

Strain no.[1]	Original name	CBS/NRRL no.	Source	Collector
1	*A. japonicus* (T[2])	CBS114.51		Kominami K
2	*A. japonicus*	CBS568.65	Panama soil	Raper KB
3[3]	*A. japonicus*	NRRL1782	identical to 2	Banner 1941
4	*A. japonicus*	NRRL360	Puerto Rico soil	Johnson 1914
5	*A. japonicus*	NRRL2053	New Guinea tent cloth	White 1946
6	*A. japonicus*	CBS312.80		
7	*A. japonicus*	NRRL359		Blakeslee AF 1915
8	*A. japonicus* var. *capillatus*	CBS114.34	Man skin	Nakazawa R
9	*A. atroviolaceus* (T)	CBS522.89	Air Amsterdam	Mosseray R
10[3]	*A. atroviolaceus* (T)	CBS113.48	identical to 9	identical to 9
11	*A. atroviolaceo-fuscus* mut.*grisea* (T)	CBS122.35		Blochwitz A
12[3]	*A. atroviolaceo-fuscus* mut.*grisea* (T)	NRRL4880		identical to 11
13	*A. luchuensis*	CBS119.49	Lactua satica	Boedijn KB
14	*A. luchuensis*	CBS116.80	Palm oil Nigeria	Onions A
15	*A. aculeatus*	CBS101.43	Pterocarpus santalinus	
16	*A. aculeatus*	CBS172.66	Tropical soil	Raper KB
17[3]	*A. aculeatus*	NRRL5094	identical to 16	identical to 16
18	*A. aculeatus*	CBS114.80	Indian soil	Tiwari PP
19	*A. aculeatus*	CBS610.78	Tropical soil	Blakeslee 1915
20[3]	*A. aculeatus*	NRRL358	identical to 19	identical to 19
21	*A. aculeatus*	CBS186.67	Litter Zaire	
22	*A. aculeatus*	CBS308.80	Soil Nigeria	
23	*A. aculeatus*	CBS313.89	Soil Figi	Frisvad JC
24	*A. aculeatus*	CBS611.78	Tropical soil	
25	*A. carbonarius*	CBS111.80	S. Africa	Lawson EN
26	*A. carbonarius*	CBS112.80		Onions AHS
27	*A. carbonarius*	CBS113.80	Threobroma cacao Nigeria	Broadbent JA
28	*A. carbonarius* (NT[2])	CBS111.26	Paper	Church MB Fonseca F da
29	*A. carbonarius*	CBS127.49	Coffee arabica seeds	Sainclivier M
30	*Sterigmatocystis fusca*	CBS420.64		
31[4]	*A. niger*	CBS120.49		
32[4]	*A. tubingensis*	CBS643.92		

[1] Each strain is given a number which is used throughout this paper for clarity
[2] T, type strain; NT, neotype strain
[3] NRRL1782 and CBS568.65; CBS113.48 and CBS522.89; NRRL4880 and CBS122.35; NRRL5094 and CBS172.66; NRRL358 and CBS610.78 originate from the same substrate
[4] *A. niger* and *A. tubingensis* are used as reference strains

A. tubingensis strain CBS643.92, which was obtained from our own collection. All strains were grown in minimal medium (Pontecorvo et al. 1953) supplemented with 0.5% yeast extract, 0.2% casamino acids and 1% sucrose after inoculation with 10^6 conidia per ml. Incubation was at 30°C for 16 h using an orbital shaker at 250 rpm. Mycelium was harvested by filtration over cheese cloth and stored at -70°C before use. Genomic DNA was isolated from the mycelium as described by De Graaff et al. (1988).

RFLP analysis. Restriction enzymes were obtained from BRL (Gaithersburg, MD, USA). Five μg genomic DNA was digested overnight in a double digestion with *Kpn*I and *Xho*I or with *Pst*I and *Sal*I, using eighty units per restriction enzyme. Genomic DNA fragments were separated on 0.6% and 1% agarose gels, transferred to Hybond-N membranes (Amersham, Buckinghamshire, UK) and crosslinked by UV light. Prehybridization and hybridization were carried out for 2 and 16 h, respectively in standard hybridization buffer (Sambrook et al. 1989) at 60°C, followed by washing twice at the same temperature in a solution containing 2xSSC, 0.1% SDS and 0.1% sodium pyrophosphate.
A 0.9-kb *Eco*RI fragment containing part of the 28S ribosomal DNA of *Agaricus bisporus* (Schaap and Visser 1995), a 3.5-kb *Bam*HI-*Hin*dIII fragment containing the *A. niger* pyruvate kinase gene (*pki*A) (De Graaff et al. 1992), a 1.6-kb *Cla*I fragment containing part of the *A. niger* pectin lyase A gene (*pel*A) (Harmsen et al. 1990) and a 1.6-kb *Bam*HI-*Pst*I fragment containing the *A. niger pel*D gene (Gysler et al. 1990) were labelled by random priming (Feinberg and Vogelstein 1983) and used as probes in separate hybridization experiments.

Results and discussion

Four DNA fragments hybridizing with different loci and encoding functional genes, which differ in their degree of conservation between different species, were used as probes to detect DNA polymorphisms. The position of the four probes within the genes from which they were derived is indicated in Fig. 1. Genomic DNA samples of the strains were digested either with *Kpn*I and *Xho*I or with *Pst*I and *Sal*I. After separation of the fragments on 0.6% and 1% agarose gels and transfer of the DNA to nylon membranes, each blot was subjected to

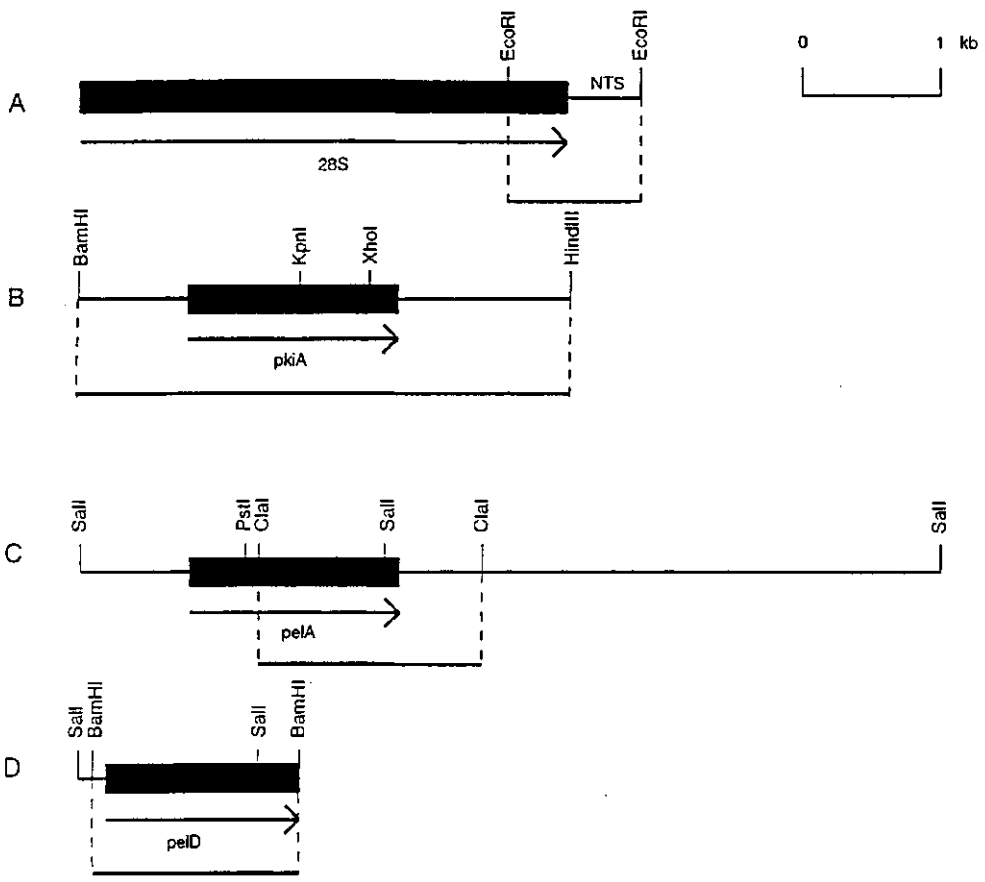


Fig. 1 Restriction maps of the 28S rDNA of *A. bisporus* (**A**) and the *pki*A (**B**), *pel*A (**C**) and *pel*D (**D**) genes of *A. niger*. The 28S rDNA and the coding regions of the genes are indicated by a *black bar*. The orientation of the genes is indicated with an *arrow*. Fragments which were used as probes are indicated by *solid lines*. NTS, non-transcribed spacer region

multiple hybridizations. Genomic DNA digested with *Kpn*I and *Xho*I was hybridized sequentially with the *A. niger pki*A probe and the *A. bisporus* 28S rDNA (28S) probe. Genomic DNA digested with *Pst*I and *Sal*I was hybridized sequentially with the *A niger pel*A probe, the related *A. niger pel*D probe and finally with the 28S probe. Each probe hybridized with one or more DNA fragments in the strains thus analysed (Figs. 2 and 3). The observed patterns for a particular

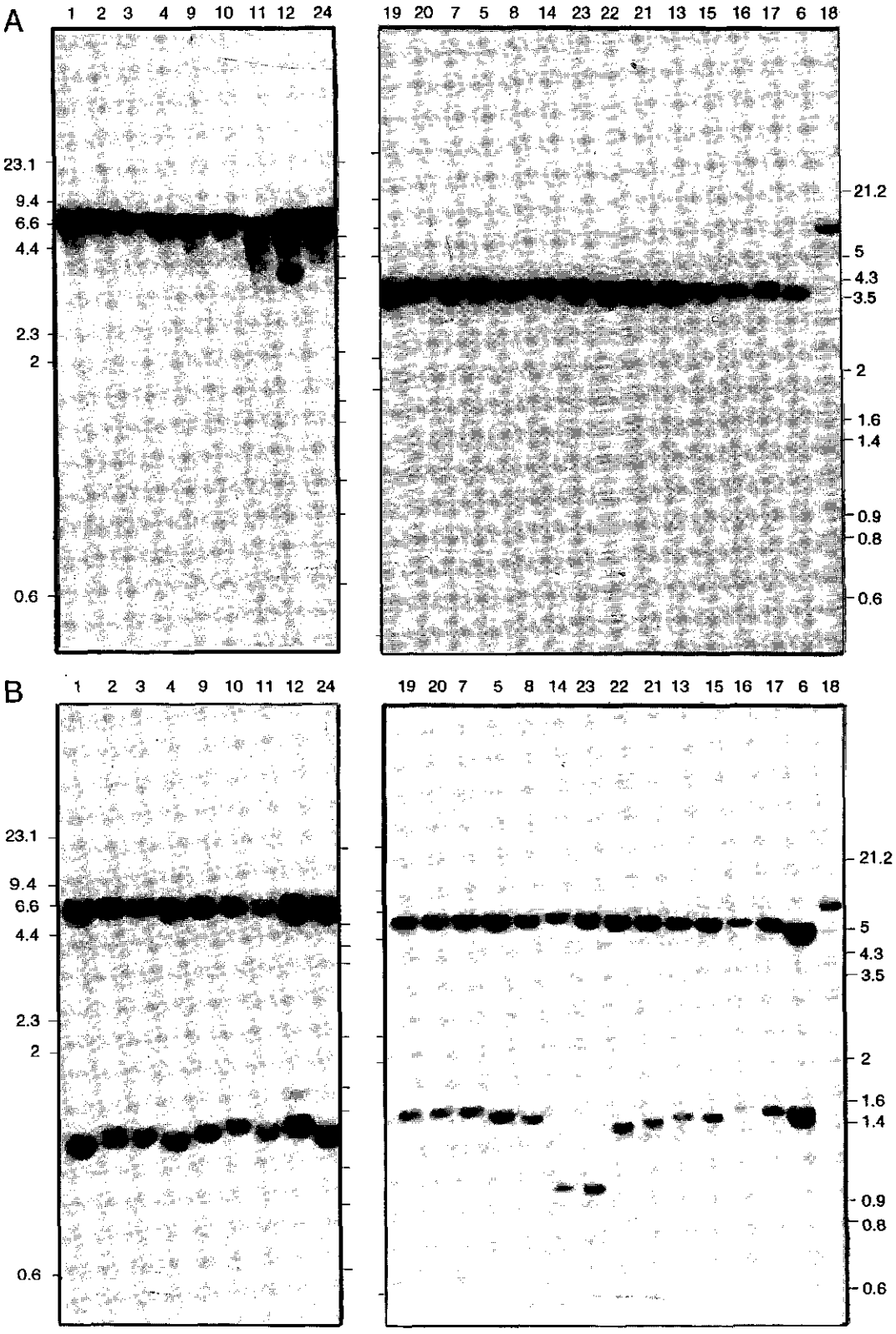
probe and digest are symbolized by Roman capitals and are shown in Table 2. In Table 3 the observed patterns for all the probes and digests used have been compiled for each individual strain.

Ribosomal DNA and *pki*A polymorphisms

After hybridization with the 28S probe simple patterns were observed (*cf* Fig. 2). The 28S probe hybridized with single bands of three different sizes (patterns A, B and C, Table 2A) after digesting the genomic DNA with *Kpn*I and *Xho*I. The patterns A and B were also seen in the *A. tubingensis* and *A. niger* reference strains, respectively. In one of the *A. atroviolaceo-fuscus* mut. *grisea* strains (strain 12) two bands were seen, one of which, band A, hybridized strongly whereas the other, band B, hybridized weakly (Fig. 2A). As this strain originates from the same *Aspergillus* isolate as strain 11, this might point to instability of the original *A. atroviolaceo-fuscus* mut. *grisea* strain after storage in two different culture collections.

After digestion of the genomic DNA with *Pst*I and *Sal*I and hybridization with the 28S probe thirteen different patterns were observed (Table 2A). All patterns except pattern J consisted of two fragments, hybridizing with equal intensity. The large fragment was either 5.5 kb or 6 kb in size, whereas the small fragment varied in size between 0.3 kb and 1.5 kb (Fig. 2B). Presumably this reflects heterogeneity in the non-transcribed spacer region (Fig. 1A).

On the basis of the results presented, several groups could be formed. Four of the five biseriate *A. carbonarius* strains except for strain 29, and the *S. fusca* strain formed a homogeneous group. In both digests this group was easily discernible from both the uniseriate and the reference strains (Table 3). On the basis of these digests *A. aculeatus* strain 18 could not be classified. However, since these results were obtained by RFLP patterns of only one locus, we also analysed a non-related locus using the *A. niger pki*A gene as probe (*cf* Fig. 1B). Nine different patterns, each composed of three hybridizing bands, were found with the *pki*A probe (Table 2A). Comparison of the size of these bands with that


A
1 2 3 4 9 10 11 12 24
19 20 7 5 8 14 23 22 21 13 15 16 17 6 18
23.1
9.4
6.6
4.4
2.3
2
0.6
21.2
5
4.3
3.5
2
1.6
1.4
0.9
0.8
0.6
B
1 2 3 4 9 10 11 12 24
19 20 7 5 8 14 23 22 21 13 15 16 17 6 18
23.1
9.4
6.6
4.4
2.3
2
0.6
21.2
5
4.3
3.5
2
1.6
1.4
0.9
0.8
0.6

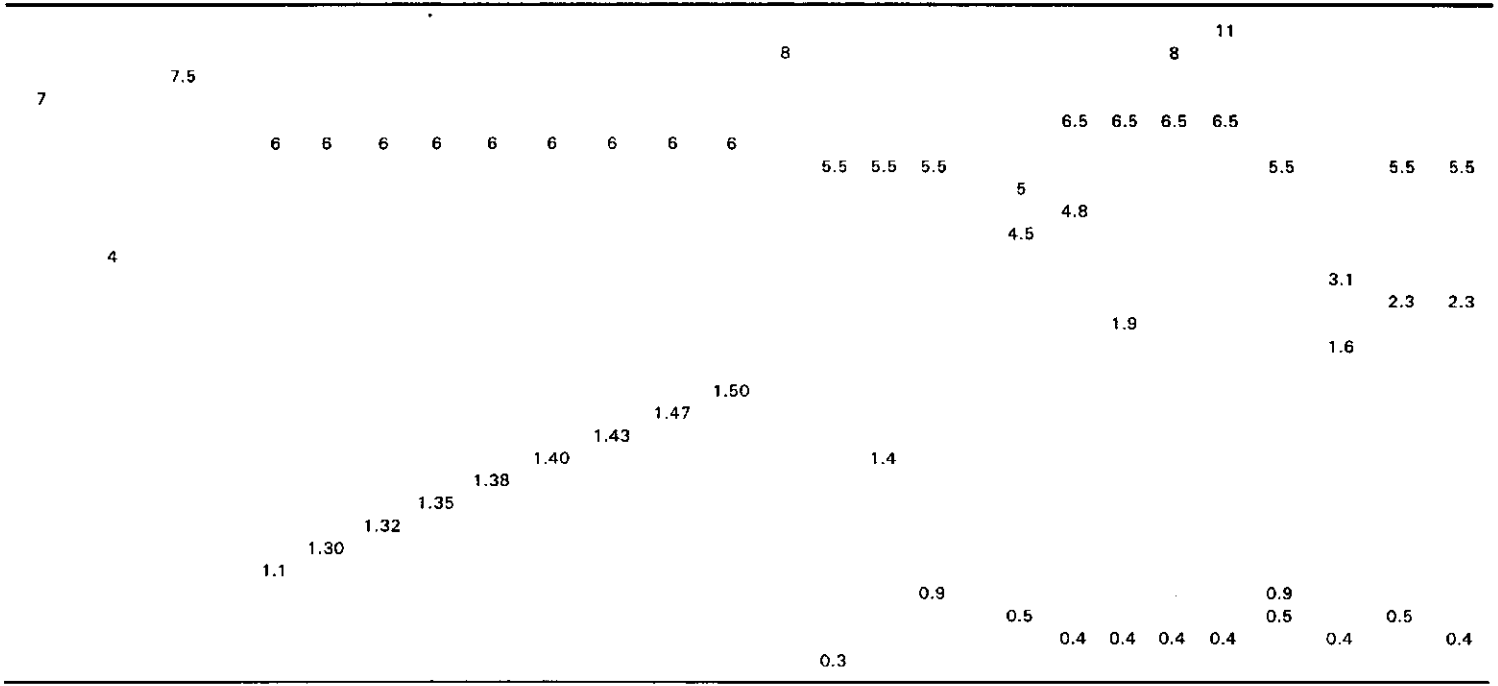
of the probe suggests that the smallest band (0.4 kb or 0.5 kb) represents an internal fragment in each homologous *pki*A gene, presuming that this gene is present in single copy in these strains. Exceptions are patterns C, F, G, H and I, in which two fragments are smaller than the probe size. The variability in size of the adjacent fragments presumably reflects sequence divergence in non-coding regions.

Pattern G is exclusively found in all *A. carbonarius* strains, except for strain 29, and in the *S. fusca* strain (Table 3). Since the 28S RFLP patterns of the *S. fusca* strain were also identical with these four *A. carbonarius* strains we suggest that this strain is classified as *A. carbonarius*. The *A. carbonarius* strain 29 showed the same pattern as the *A. tubingensis* reference strain with both the 28S and the *pki*A probe. Therefore classication of this strain as *A. tubingensis* seems more appropriate.

Pattern A was found with the *pki*A probe in a heterogeneous group of uniseriate strains. In Fig. 3A the results are shown of a separation of the fragments on a 0.6% agarose gel. The same set of strains showed, upon reprobing the same blot, a single band of 7 kb with the 28S probe (Table 3). However, when the genomic DNA was digested with *Pst*I and *Sal*I slight differences in size of the small hybridizing band were observed with this probe (Table 2A). As these sizes were not found in any of the other strains, these strains form a separate group. The group formed is composed of four *A. japonicus* strains, two *A. atroviolaceus* and two *A. violaceo-fuscus* mut. *grisea* strains and one *A. aculeatus* strain (Table 3). As the *A. japonicus* strains are in the majority and include the type strain CBS114.51, we suggest that the remaining strains in this group are classified as *A. japonicus*. Classification of the species *A. atroviolaceus* and *A.*

Fig. 2 Southern analysis of double digests of genomic DNA of the strains 1-24 with the *A. bisporus* 28S probe. Genomic DNA was double digested with *Kpn*I and *Xho*I (**A**) or with *Pst*I and *Sal*I (**B**). Gels used for electrophoresis contained 1% agarose. Strain numbers are as indicated in Table 1. Fragment sizes were determined using λDNA digested with *Hin*dIII and with *Hin*dIII and *Eco*RI as length marker. Marker sizes are shown in kb at the left side of the membranes (for λDNA digested with *Hin*dIII) and at the right side of the membranes (for λDNA digested with *Hin*dIII and *Eco*RI, excluding the 5.1-kb and the 1.9-kb fragments)

Table 2A Molecular sizes (kb)[1] of restriction fragments associated with the RFLP patterns for 28S rDNA and *pki*A



[1] Results are reproduced in at least four experiments

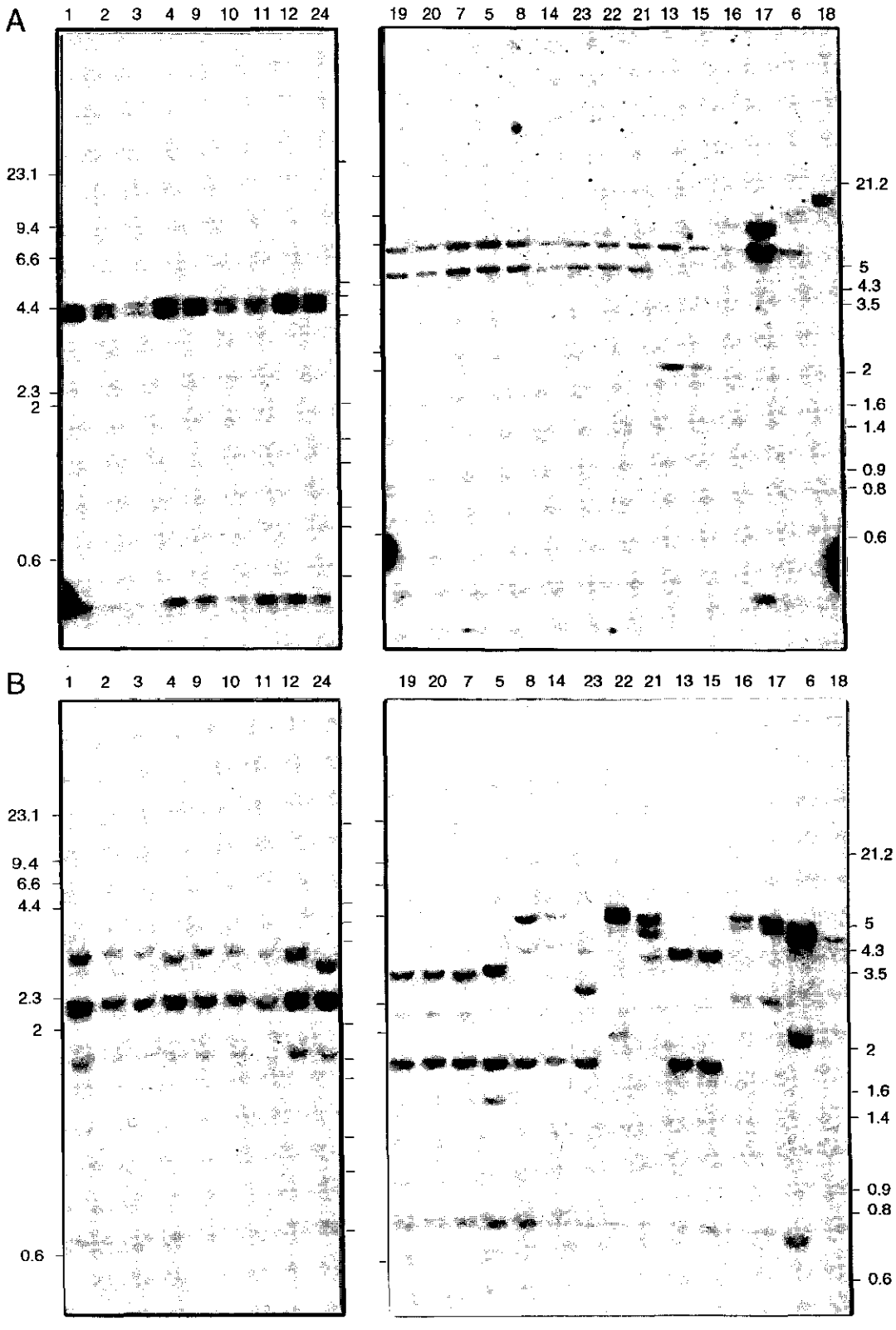
violaceo-fuscus mut. *grisea* as *A. japonicus* is in agreement with the findings of Al-Musallam, who synonymized these strains with *A. japonicus* var. *japonicus* (Al-Musallam 1980). The RFLP patterns however do not justify this subclassification. In contrast to our results, Kozakiewicz separated *A. atroviolaceus* from *A. japonicus* on the basis of conidial ornamentation and placed *A. aculeatus* as synonym (Kozakiewicz 1989).

The four remaining patterns obtained with the *pki*A probe, patterns B, C, D, and E are related (Table 2A). They share both an internal 0.4-kb and an adjacent 6.5-kb fragment of the homologous *pki*A genes (Fig. 3A). The fourteen strains demonstrating these patterns showed, upon reprobing the same blot, a single band of 4 kb with the 28S probe (Table 3). However, when the genomic DNA of these strains was digested with *Pst*I and *Sal*I slight differences in the size of the small hybridizing band were observed with this probe in twelve of these strains whereas two strains yielded pattern A (Fig. 2B and Table 2A). The group thus formed is composed of six *A. aculeatus*, three *A. japonicus*, one *A. japonicus* var. *capillatus* and two *A. luchuensis* strains (Table 3). As the *A. aculeatus* strains are in the majority, we suggest that the remaining strains in this group are reclassified as *A. aculeatus*.

In *A. aculeatus* strain 18 a unique pattern was found (pattern F, Table 2A). Unfortunately, the genomic DNA of this strain was not digested in the particular experiment shown in Fig. 3A. However, in Table 2A the results are presented that were obtained from a Southern analysis where a 1% agarose gel was used. This strain showed also a distinct pattern with the 28S probe when the genomic DNA was digested with *Pst*I and *Sal*I (pattern D, Fig. 2B and Table 2A) and thus could not be classified in any of the three groups formed (Table 3). Morphologically strain 18 also behaves aberrant in producing an abundant number of sclerotia.

RFLP analysis with the *A. niger pel*A and *pel*D genes as probe

A more refined analysis of the strains was done using the *A. niger pel*A gene as


A
1 2 3 4 9 10 11 12 24
19 20 7 5 8 14 23 22 21 13 15 16 17 6 18
23.1
9.4
6.6
4.4
2.3
2
0.6
21.2
5
4.3
3.5
2
1.6
1.4
0.9
0.8
0.6
B
1 2 3 4 9 10 11 12 24
19 20 7 5 8 14 23 22 21 13 15 16 17 6 18
23.1
9.4
6.6
4.4
2.3
2
0.6
21.2
5
4.3
3.5
2
1.6
1.4
0.9
0.8
0.6

probe (Fig. 1C). Sixteen different RFLP patterns were observed, each consisting of two to four bands. Estimated sizes of the hybridizing fragments are presented in Table 2B. In all patterns one of the bands hybridized weakly and this suggests that this fragment represents either a *pel*A border fragment or another, related gene in these strains. The existence of a family of *pel* genes in the strains analysed, just as in *A. niger*, could explain the complexity of the patterns observed. The most likely candidate for a hybridizing, related gene is the homolog of the *A. niger pel*D gene, which is most closely related with *pel*A in *A. niger*. Therefore the blots were reprobed with the *A. niger pel*D gene (Fig. 1D). In all patterns one band hybridized more strongly with the *pel*D probe, indeed indicating the existence of a second *pel* gene in these strains homologous to the *A. niger pel*D gene (Table 2B).

Although sixteen different hybridization patterns were observed, many of them are related. Patterns A, B and C consist of four bands, three of which have the same size. The fourth and largest band hybridized more strongly with the *A. niger pel*D probe. These patterns are restricted to the *A. japonicus* group identified with the 28S and *pki*A probes (Table 3).

The related patterns D, E, F, G and J were only observed in nine of the above identified *A. aculeatus* strains (Table 3). They share two bands, 0.7 kb and 1.7 kb in size, which hybridized strongly with the *pel*A probe (Table 2B). Pattern D was found in the strains 7, 19 and 20, which also show the same patterns with the 28S and the *pki*A probe, suggesting that they are closely related. Likewise, the strains 13 and 15, which both show pattern J and also are indistinguishable with the 28S and the *pki*A probe, are presumably closely related.

Fig. 3 Southern analysis of double digests of genomic DNA of the strains 1-24. (**A**) Genomic DNA double digested with *Kpn*I and *Xho*I was hybridized with the *A. niger pki*A probe. Gels used for electrophoresis contained 0.6% agarose. DNA of strain 17 was not digested in this particular experiment. (**B**) Genomic DNA double digested with *Pst*I and *Sal*I was hybridized with the *A. niger pel*A probe. Gels used for electrophoresis contained 1% agarose. Strain numbers are as indicated in Table 1. Fragment sizes were determined using λDNA digested with *Hin*dIII and with *Hin*dIII and *Eco*RI as length marker. Marker sizes are shown in kb at the left side of the membranes (for λDNA digested with *Hin*dIII) and at the right side of the membranes (for λDNA digested with *Hin*dIII and *Eco*RI, excluding the 5.1-kb and the 1.9-kb fragments)

Table 2B Molecular sizes (kb)[1] of restriction fragments associated with the RFLP patterns for *pel*A

*pel*A, *Pst*I-*Sal*I															
A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
							5.2								5.3
					5		5	5		5	5				
														4.8	
										4.5	4.5	4.5			
								4							
					3.5	3.5		3.5	3.5						
3.4[2]															
	3.2												3.2		
		3		3											
			2.9												
						2.6									
										2.5					
2.3[3]	2.3	2.3	2.3												
							2.1				2.1	2.1			
			1.7	1.7	1.7	1.7			1.7						
1.6	1.6	1.6													
				1.4									1.4	1.4	1.4
												1.3			
														1.1	
			0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7			
0.6	0.6	0.6													

[1] Results are reproduced in at least four experiments; [2] Fragments that give strong hybridization signals with the *pel*D probe are underlined;
[3] Fragments that give strong hybridization signals with the *pel*A probe are indicated in bold

In the related patterns H, I, K and L also a 0.7-kb fragment hybridized strongly with the *pel*A probe, together with a 5-kb fragment. Therefore it is suggested, that this small fragment represents an internal fragment of the homologous *pel*A genes in all the *A. aculeatus* strains. The other fragments, especially the one hybridizing preferentially with the *pel*D probe, were variable in size, indicating variability within the *A. aculeatus* group.

Pattern M was unique for *A. aculeatus* strain 18. Again this strain is incompatible with the proposed classification.

Pattern N consisted of only two fragments, one of which hybridized with the *pel*A probe and the other with the *pel*D probe. This pattern was restricted to the *A. carbonarius* strains identified above. In *A. carbonarius* strain 29 pattern P was found which is identical to that in *A. tubingensis*. This unambiguously identifies strain 29 as *A. tubingensis*.

In summary, by RFLP analysis in four different loci we were able to classify 28 out of 30 strains into three groups as shown in Table 3. Strains classified as *A. japonicus* are characterized by a 7-kb *Kpn*I-*Xho*I fragment hybridizing with the 28S rDNA probe and by three *Kpn*I-*Xho*I fragments of 0.5 kb, 4.5 kb and 5 kb hybridizing with the *pki*A probe and can clearly be separated from *A. aculeatus* strains. Furthermore, they are characterized by three *Pst*I-*Sal*I fragments, two of which (0.6 kb and 2.3 kb) hybridize more strongly with the *A. niger pel*A gene and one of which (3 kb, 3.2 kb or 3.4 kb) hybridizes more strongly with the *A. niger pel*D gene as probe.

Strains classified as *A. aculeatus* are all characterized by a 4-kb *Kpn*I-*Xho*I fragment hybridizing with the 28S probe and by three *Kpn*I-*Xho*I fragments hybridizing with the *pki*A probe, two of fixed size (6.5 kb and 0.4 kb) and one variable in size. The *A. aculeatus* strains have complex *Pst*I-*Sal*I RFLP patterns when probed with the less conserved *pel*A gene, but they all share one fragment of 0.7 kb and one fragment of either 1.7 kb or 5 kb, both hybridizing strongly with the *A. niger pel*A gene.

Finally, we have found that strains classified as *A. carbonarius* are easily charac-

Table 3 Proposed classification of 30 *Aspergillus* strains based on RFLP patterns[1] obtained with three independent probes

	CBS/NRRL no.	Strain no.	28S, *KpnI-XhoI*	28S, *PstI-SalI*	*pkiA*, *KpnI-XhoI*	*pelA*, *PstI-SalI*	Original name[2]
A. japonicus	CBS114.51	1	A	B	A	B	j (T[3])
	CBS568.65	2	A	C	A	A	j
	NRRL1782	3	A	C	A	A	j
	NRRL360	4	A	C	A	B	j
	CBS522.89	9	A	D	A	A	av (T)
	CBS113.48	10	A	E	A	A	av (T)
	CBS122.35	11	A	D	A	B	avg (T)
	NRRL4880	12	A	E	A	B	avg (T)
	CBS611.78	24	A	C	A	C	a
A. aculeatus	NRRL2053	5	B	H	B	E	j
	CBS312.80	6	B	I	E	L	j
	NRRL359	7	B	I	B	D	j
	CBS114.34	8	B	G	B	F	jc
	CBS119.49	13	B	H	C	J	lu
	CBS116.80	14	B	A	B	F	lu
	CBS101.43	15	B	H	C	J	a
	CBS172.66	16	B	I	D	K	a
	NRRL5094	17	B	I	D	K	a
	CBS610.78	19	B	I	B	D	a
	NRRL358	20	B	I	B	D	a
	CBS186.67	21	B	G	B	I	a
	CBS308.80	22	B	F	B	H	a
	CBS313.89	23	B	A	B	G	a
A. carbonarius	CBS111.80	25	C	K	G	N	c
	CBS112.80	26	C	K	G	N	c
	CBS113.80	27	C	K	G	N	c
	CBS111.26	28	C	K	G	N	c (NT[3])
	CBS420.64	30	C	K	G	N	sf
unknown	CBS114.80	18	C	J	F	M	a
A. niger	CBS120.49	31	B	L	H	O	n
A. tubingensis	CBS127.49	29	A	M	I	P	c
	CBS643.92	32	A	M	I	P	t

[1] The patterns, represented by Roman capitals, are specified in Table 2A and B; [2] a, *A. aculeatus*; av, *A. atroviolaceus*; avg, *A. atroviolaceo-fuscus* mut.*grisea*; c, *A. carbonarius*; j, *A. japonicus*; jc, *A. japonicus* var. *capillatus*; lu, *A. luchuensis*; n, *A. niger*; sf, *Sterigmatocystis fusca*; t, *A. tubingensis*; [3] T, type strain; NT, neotype strain

terized by a single 7.5-kb *Kpn*I-*Xho*I fragment and two *Pst*I-*Sal*I fragments of 0.3 kb and 5.5 kb hybridizing with the 28S probe and by three *Kpn*I-*Xho*I fragments of 0.4 kb, 1.6 kb and 3.1 kb hybridizing with the *pki*A probe. A relatively simple RFLP pattern, consisting of a 1.4-kb and a 3.2-kb *Pst*I-*Sal*I fragment, is found with the *pel*A gene as probe. Further, we have found that strain 29, CBS127.49 was erroneously classified as an *A. carbonarius* strain, and should be reclassified as *A. tubingensis*. However, we were not able to classify strain 18, CBS114.80, which was formerly classified as *A. aculeatus* by traditional identification methods. The molecular data rather suggest that this strain may represent a new *Aspergillus* species.

The results described above indicate that we have obtained a useful RFLP method for the classification of biotechnologically important *Aspergillus* strains, which are in use for the production of technical enzymes. This is not only important for strain identification, but also provides a useful guideline to direct screening programmes or to find legal strain protection. In future, other molecular criteria of these and other strains will be analysed in order to bring this method in a broader perspective.

References

Al-Musallam A (1980) Revision of the black *Aspergillus* species. Thesis, Rijksuniversiteit Utrecht, Utrecht

Cramer C (1859) Über eine neue Fadenpilzgattung: Sterigmatocystis Cramer. Vjschr naturf Ges Zürich 4:324-337

De Graaff LH, Broek HWJ van den, Visser J (1988) Isolation and transformation of the pyruvate kinase gene of *Aspergillus nidulans*. Curr Genet 13:315-321

De Graaff LH, Broek HWJ van den, Visser J (1992) Structure and characterization of the *Aspergillus niger* pyruvate kinase gene. Curr Genet 22:21-27

De Graaff LH, Broeck HC van den, Ooijen AJJ van, Visser J (1994) Regulation of the xylanase-encoding *xln*A gene of *Aspergillus tubingensis*. Mol Microbiol 12:479-490

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132:6-13

Gysler C, Harmsen JAM, Kester HCM, Visser J, Heim J (1990) Isolation and structure of the pectin lyase D-encoding gene from *Aspergillus niger*. Gene 89:101-108

Harmsen JAM, Kusters-van Someren, Visser J (1990) Cloning and expression of a second *Aspergillus niger* pectin lyase gene (*pel*A): indications of a pectin lyase gene family in *Aspergillus niger*. Curr Genet 18:161-166

Kofod LV, Kauppinen S, Christgau S, Andersen LN, Heldt-Hansen HP, Dörreich K,

Dalbøge H (1994) Cloning and characterization of two structurally and functionally divergent rhamnogalacturonases from *Aspergillus aculeatus*. J Biol Chem 269:-29182-29189

Kozakiewicz Z (1989) *Aspergillus* species on stored products. CAB International Mycological Institute UK

Kusters-van Someren MA, Samson RA, Visser J (1990) Variation in pectinolytic enzymes of the black Aspergilli: A biochemical and genetic approach. In: Samson RA and Pitt JI (eds) Modern concepts in *Penicillium* and *Aspergillus* classification. NATO ASI Series vol 185, Plenum Press, New York, pp 321-334

Kusters-van Someren MA, Samson RA, Visser J (1991) The use of RFLP analysis in classification of the black Aspergilli: reinterpretation of the *Aspergillus niger* aggregate. Curr Genet 19:21-26

Mosseray R (1934) Les Aspergillus de la section "Niger" Thom et Church. La Cellule, tome XLIII, fasc.2

Pontecorvo G, Roper JA, Hemmons LJ, MacDonald KD, Bufton AWJ (1953) The genetics of *Aspergillus nidulans*. Adv Genet 5:141-238

Raper KB, Fennell DI (1965) The genus *Aspergillus*. Williams and Wilkins Company, Baltimore

Sambrook J, Fritsch E, Maniatis T (1989) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Schaap PJ, Müller Y, Baars JJP, Camp HJM op den, Sonnenberg ASM, Griensven LJLD van, Visser J (1996) Nucleotide sequence and expression of the $NADP^+$-dependent glutamate dehydrogenase (*gdh*A) from *Agaricus bisporus*. Mol Gen Genet 250:339-347

Searle-van Leeuwen MJF, Broek LAM van den, Schols HA, Beldman G, Voragen AGJ (1992) Rhamnogalacturonan acetylesterase: a novel enzyme from *Aspergillus aculeatus*, specific for the deacetylation of hairy (ramified) regions of pectins. Appl Microbiol Biotechnol 38:347-349

Spreadbury CL, Bainbridge BW, Cohen J (1990) Restriction fragment length polymorphism in isolates of *Aspergillus fumigatus* probed with part of the intergenic spacer region from the ribosomal RNA gene complex of *Aspergillus nidulans*. J Gen Microbiol 136:1991-1994

Suykerbuyk MEG, Schaap PJ, Stam H, Musters W, Visser J (1995) Cloning, sequence and expression of the gene coding for rhamnogalacturonase (RHG) of *Aspergillus aculeatus*; a novel pectinolytic enzyme. Appl Microbiol Biotechnol 43:861-870

Thom C, Raper KB (1945) A manual of the *Aspergilli*. Williams and Wilkins Company, Baltimore

Varga J, Croft J (1994) Assignment of RFLP, RAPD and isoenzyme markers to *Aspergillus nidulans* chromosomes, using chromosome-substituted segregants of a hybrid of *A. nidulans* and *A. quadrilineatus*. Curr Genet 25:311-317

Varga J, Kevei F, Vriesema A, Debets F, Kozakiewicz Z, Croft J (1994) Mitochondrial DNA restriction fragment length polymorphisms in field isolates of the *Aspergillus niger* aggregate. Can J Microbiol 40:612-621

Whitehead DS, Coddington A, Lewis BG (1992) Classification of races by DNA polymorphism analysis and vegetative compatibility grouping in *Fusarium* f.sp. *pisi*. Physiol Mol Plant Pathol 41:295-305

Witteveen CBF, Vondervoort PJI van de, Broeck HC van den, Engelenburg FAC van, Graaff LH de, Hillebrand MHBC, Schaap PJ, Visser J (1993) Induction of glucose oxidase, catalase and lactonase in *Aspergillus niger*. Curr Genet 21:408-416

6 | Summary and conclusions

Processing of apples to juice involves the degradation of cell wall polysaccharides among which the heteropolymer pectin with complex 'hairy' regions. Fruit processing can be achieved by chemical methods, but enzymatic degradation is preferred, since it is extremely specific and can be performed under mild conditions. About twenty different plant glycosyltransferases are involved in pectin biosynthesis (Fry 1988) and it is therefore not surprising that an array of enzymes, many of which are produced by fungi, is required for their degradation.

The aim of this thesis was to investigate the genetic basis for the production of one such enzyme, rhamnogalacturonan hydrolase, in *Aspergillus*. The gene encoding rhamnogalacturonan hydrolase, which is present in the industrial enzyme preparation Ultra SP from *A. aculeatus*, was cloned (Chapter 2). This gene was used as a tool for the identification of related enzymes in *A. niger*, a well-known producer of pectinolytic enzymes (Chapter 3). The rhamnogalacturonan hydrolase expression system in *A. aculeatus* was analysed via classical mutagenesis, using the cloned gene as a tool (Chapter 4). The relationship between *A. aculeatus* strains and other black *Aspergilli* is studied at the molecular level by RFLP analysis (Chapter 5).

Rhamnogalacturonan hydrolases and their role in pectin degradation

The discovery of the endo-acting rhamnogalacturonan hydrolase, being part of the fungal enzyme preparation Ultra SP, promised a revolution in the pectin industry (Schols et al. 1990). Until then, MHR could only be modified enzymatically by the action of exo-glycosidases, which sequentially release side chain monosaccharides. Only endo-glucanases acting on homogalacturonan were known, like PG, PEL and PL (Fig. 1). However, a complex substrate like MHR can only be degraded efficiently by the additional activity of endo-glycanases. Resulting non-reducing ends of oligosaccharides formed can then serve as a substrate for several exo-acting enzymes, which is the basis for synergism. Alternatively, synergism has been demonstrated between rhamnogalacturonan

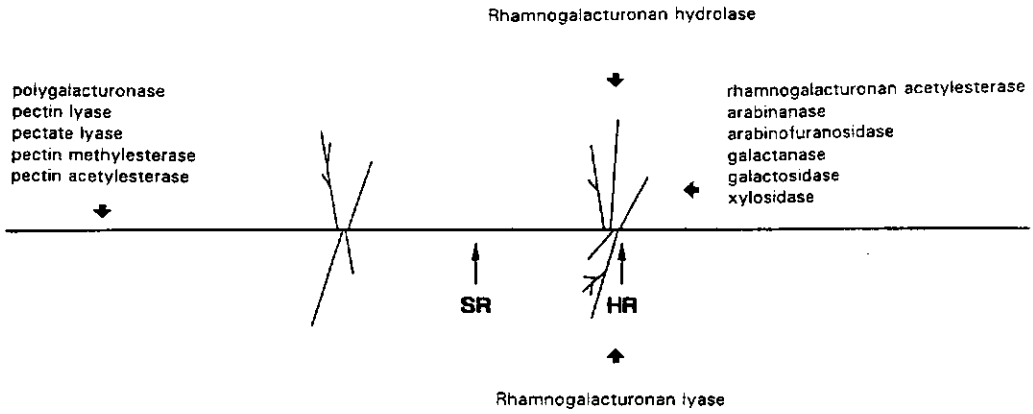


Fig. 1 Pectinolytic enzymes acting on the hairy regions (HR) and the smooth regions (SR) of pectin

hydrolase and rhamnogalacturonan acetylesterase, the latter of which is required before endo-degradation can occur (Searle-van Leeuwen et al. 1992). Synergistic activities have further been demonstrated between two exo-glycosidases, rhamnogalacturonan rhamnohydrolase and *A. niger* β-galactosidase, both acting on MHR degradation fragments released by rhamnogalacturonan hydrolase (Mutter et al. 1994).

The established structure of intermediate MHR-fragments, containing tetra- and hexameric and even larger backbone fragments, is not in agreement with the alternating nature of the backbone, in which galacturonic acid and rhamnose residues are connected by only two types of linkages, one of which is recognized by rhamnogalacturonan hydrolase (Schols 1995). Apparently, the linkages are not evenly cleaved by this enzyme, resulting in incomplete MHR degradation. This stresses the need for additional endo-acting enzymes with other specificities. In order to search for such enzymes, the gene encoding rhamnogalacturonan hydrolase in *A. aculeatus* was characterized. In Chapter 2 the isolation of the structural gene encoding rhamnogalacturonan hydrolase, *rhg*A, and the purification of the recombinant protein are described. Firstly, antibody screening of a cDNA expression library resulted in a cDNA clone, which was

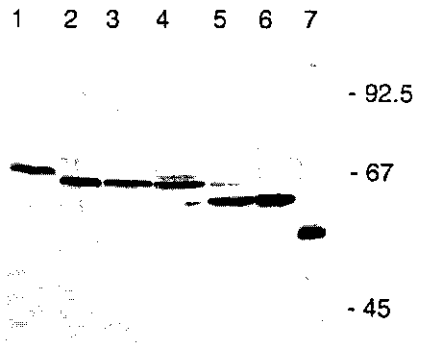


Fig. 2 Western analysis of the culture filtrate of *A. foetidus* strain CBS618.78 (*lane* 1), *A. niger* var. *nanus* strain CBS117.48 (*lane* 2), *A. niger* var. *phoenicis* strain CBS137.52 (*lane* 3), *A. niger* var. *hennebergii* strain CBS117.80 (*lane* 4) and *A. carbonarius* strains CBS112.80 (*lane* 5) and CBS420.64 (*lane* 6) after growth for 48 h on minimal medium with 1% (w/v) beet pulp. *Lane* 7 contains rhamnogalacturonan hydrolase from *A. aculeatus* strain CBS115.80. Species nomenclature is according to Al-Musallam (1980)

subsequently used as a probe for homologous screening of a genomic library. Rhamnogalacturonan hydrolases in other black *Aspergillus* species were identified by cross-hybridization of the genomic DNAs with the same probe. This revealed the presence of strong and weak hybridizing DNA fragments in all strains but most abundantly in *A. niger*, pointing to a gene family encoding isoenzymes.

Cross-reactivity with mouse polyclonal antibodies against rhamnogalacturonan hydrolase from *A. aculeatus* was demonstrated in all *A. aculeatus* and *A. japonicus* strains analysed so far (unpublished results) and also in other black *Aspergilli*, among which *A. foetidus*, *A. niger* varieties *nanus*, *phoenicis* and *hennebergii* and *A. carbonarius* strains, as illustrated in Fig. 2. The molecular mass of these proteins varies from 55 kDa in all *A. aculeatus* and *A. japonicus* strains to 65 kDa in *A. carbonarius*, 70 kDa in the different *A. niger* varieties and 75 kDa in *A. foetidus*. Thus, rhamnogalacturonan hydrolases appear to be widely spread within the black *Aspergilli*. Cross-reactivity of different proteins points to the recognition of a variety of epitopes by these antibodies, which is in agreement with the large number of protein fragments recognized after CNBr degradation of *A. aculeatus* rhamnogalacturonan hydrolase (unpublished results).

Table 1 Rhamnogalacturonan degrading enzymes and their encoding genes

Activity	Gene	Size (AA)	Enzyme	Production host	Mass (kDa)	pHopt	pI	Topt	Bond cleaved[1]	Reference
RG-hydrolase	*rhg*A[2]	440	RHGA	*A. aculeatus*	55	4.5	(4.2)[3]	(40)[4]	1-2	Chapter 2
	*rhg*A[5]	446	RHGA	*A. awamori*	72	3.6	(3.7)[3]	(40)[4]	1-2	Chapter 3
	*rhg*B[5]	558	RHGB	*A. awamori*	70-87	4.1	(3.9)[3]	(40)[4]	1-2	Chapter 3
			RGaseA	*A. aculeatus*	51	3-4	4.1-4.5	40-50	1-2	Schols et al. 1990
	*rhg*A[6]	440	rRGaseA	*A. oryzae*	62	3.5	4.5	30-50	1-2	Kofod et al. 1994
			rRGHase	*A. oryzae*	50.4				1-2	Azadi et al. 1996
			Protopectinase-T	*T. sanguinea*	55	4	8.1	50	1-2	Sakamoto et al. 1994
RG-lyase			RGaseB	*A. aculeatus*	57	6	5.1-5.3	50-60	1-4	Mutter et al. 1996
	*rhg*B[6]	527	rRGaseB	*A. oryzae*	55	6	5.2	50	1-4	Kofod et al. 1994
			rRGLase	*A. oryzae*	54.1				1-4	Azadi et al. 1996

[1] 1-2, α-D-GalpA-(1,2)-α-L-Rha; 1-4, α-L-Rha-(1,4)-α-D-GalpA; [2] *A. aculeatus* strain CBS115.80; [3] Calculated values are in brackets; [4] Actual temperatures are in brackets; [5] *A. niger* strain CBS120.49; [6] *A. aculeatus* strain CBS101.43; cDNA

Therefore we extended our analysis on rhamnogalacturonan hydrolase expression to the industrially well-known producer of pectinolytic enzymes *A. niger*, as described in Chapter 3. The *A. niger* genome was screened for sequences homologous to *A. aculeatus rhg*A by using this gene as a probe. This analysis revealed the presence of two genes, *rhg*A and *rhg*B, in this species (Table 1). The encoded enzymes, rhamnogalacturonan hydrolase A and B, show different degrees of amino acid sequence identity with rhamnogalacturonan hydrolase of *A. aculeatus*.

Comparison of the DNA sequences of the *rhg* genes from *A. niger* and *A. aculeatus* revealed identical sequences, not only in the coding regions but also in the adjacent promoter regions. For instance, a 15-bp-box is found with 100% identity in both *rhg*A gene promoters and a 130-bp upstream sequence shares 77% identity in both genes. Possibly, these elements have a regulatory role in the degradation of rhamnogalacturonan structures. The relatively low amount of rhamnogalacturonan in pectin, together with its high structural complexity (as described in Chapter 1), make it a less metabolizable pectic component than homogalacturonan, which is highly accessible to enzymatic degradation. Although this suggests sequential expression of the rhamnogalacturonan degrading enzymes after the homogalacturonan degrading enzymes, this is not confirmed by the early expression of *rhg*A, within 6 h after transfer of either *A. aculeatus* or *A. niger* to apple pectin. Expression of *rhg*B in *A. niger* could only be demonstrated at the mRNA level after multi-copy integration into the genome and was detected after 18 h of growth not only on apple pectin but also on sucrose.

The low expression levels in *A. niger* might be caused either by higher proteolytic activity in this species, or by a higher sensitivity to proteolytic degradation of the particular rhamnogalacturonan hydrolases. The latter explanation is more likely, as similar results are obtained after transformation of *A. aculeatus* with these genes.

The widespread application of an enzyme preparation like Ultra SP requires a qualified production system. In Chapters 2 and 3 a production system in *A.*

awamori is described, which is based on the endogenous endo-xylanase promoter. By this, rhamnogalacturonan hydrolases can be produced on xylose instead of apple pectin. An alternative system for the production of rhamnogalacturonan hydrolases is based on the glucoamylase promoter of *A. oryzae*, as described by Kofod et al. (1994) (Table 1). The advantage of a clean production system for the purification of enzymes is clearly demonstrated by the presence of contaminating enzyme activities after purification, either from *A. niger* or from apple, of an important exo-enzyme in rhamnogalacturonan degradation, β-galactosidase. Whereas the fruit variant of this enzyme was associated with β-D-fucosidase and α-L-arabinopyranosidase activities (Dick et al. 1990), the fungal variant was associated with rhamnogalacturonan hydrolase activity (An et al. 1994). However, instead of small amounts of impurities, the presence of multiple active sites in β-galactosidase might also explain this phenomenon.

Both the use of different host strains for enzyme purification and the use of different strains as a source of the encoding gene, might contribute to the differences in properties between the rhamnogalacturonan hydrolases encoded by *rhg*A of *A. aculeatus* (Table 1).

Comparison of enzyme activities of the purified *A. aculeatus* and *A. niger* rhamnogalacturonan hydrolases was performed by incubation with MHR and analysis of the generated degradation products. Both the formation of different final levels of oligomeric backbone fragments and of additional, larger oligomers by the *A. niger* B-enzyme point to differences in substrate specificity. Future structural analysis of the higher oligomeric fragments will reveal the sugar composition of these products. Inhibition of the B-enzyme by acetyl-groups or other side chains has not yet been investigated. However, the presence of additional monosaccharide constituents in some of the released products, originating either from the backbone or from the side chains of MHR, indicates a different degree of steric hindrance of the B-enzyme in comparison with the A-enzymes. Thus activity of the B-enzyme might be less dependent on side chain degrading enzymes, like those acting on arabinogalactan, in nature.

The specific activities of the three rhamnogalacturonan hydrolases are deduced

from the initial rate of product formation, as far as the hexameric double-substituted backbone fragment (corresponding to peak VIII in Fig. 7, Chapter 3) is concerned. Despite the relatively low expression levels of rhamnogalacturonan hydrolase A and B in *A. niger*, their specific activities amount to about one third and one fourth, respectively of the specific activity of *A. aculeatus* rhamnogalacturonan hydrolase. Because the overall amino acid sequence homology between the different enzymes is relatively high, no conclusion can be drawn about the function of particular conserved amino acids. Site-directed mutagenesis will be required in order to identify amino acids which are essential for rhamnogalacturonan hydrolase activity and substrate binding.

The only other rhamnogalacturonan hydrolase purified until now originates from an unrelated fungus, *Trametes sanguinea*, and is called Protopectinase-T. This enzyme shares most characteristics with *A. aculeatus* rhamnogalacturonan hydrolase, except for the relatively high pI of 8.1 (Table 1). The amino acid structure of this enzyme is not yet known, however its specificity is presumably similar to the *A. aculeatus* enzyme, as the same products are formed after MHR degradation (Sakamoto et al. 1994).

Another enzyme involved in rhamnogalacturonan degradation is rhamnogalacturonan lyase, acting on α-L-rhamnose-(1,4)-α-D-galacturonic acid linkages in the backbone. This enzyme shows no cross-reactivity with the antibodies raised against rhamnogalacturonan hydrolase, which is in agreement with its deviating amino acid sequence (Kofod et al. 1994).

Whereas rhamnogalacturonan hydrolase activity has been established in several other organisms not mentioned in Table 1, values cannot be easily compared, as different detection methods were used. Thus, Schols et al. (1990) determined the number of glycosidic linkages cleaved by rhamnogalacturonan hydrolase by comparing total molecular masses of MHR and the deduced amount of linkages before and after enzymic degradation. In turn, rhamnogalacturonan hydrolase activity in *T. sanguinea* was detected via measuring the release of solubilised pectin substances from lemon peel protopectin, using the carbazole/H_2SO_4 method (Sakamoto et al. 1994). Finally, Gross et al. (1995) determined rhamno-

galacturonan hydrolase activity in *Botrytis cinerea* and the fruits apple, grape and tomato indirectly, via measuring α-L-rhamnosidase activity. For this, they made use of the terminal rhamnose residues which are generated after rhamnogalacturonan hydrolase degradation of the substrate, polygalacturonic acid from orange. However, interference by endogenous exo-acting enzymes in the different organisms are not taken into account. Comparison with the specific activity of Ultra SP, also established with this method, revealed a 10 times higher value in the culture filtrate of *B. cinerea* and a 200 to 1000 times lower value in the different fruits. As *B. cinerea* RHG was only produced in a concentration of less than 25 ng per litre culture, detailed analysis of this enzyme after purification will require cloning and overexpression of the encoding gene (Chen et al. 1996). Additional criteria for rhamnogalacturonan hydrolase activity in *A. aculeatus* are the assessment of viscosity reduction of apple mash, as described in Chapter 2 and colony staining after growth on plates containing cross-linked, dyed (DLC-coupled) MHR complexes, which become stained after degradation (Kofod et al. 1994).

Regulation of rhamnogalacturonan hydrolase expression

Regulation mechanisms involved in pectinolysis are mostly studied in bacteria of the genus *Erwinia*. Many pectinolytic enzymes of *Erwinia* are expressed on (poly)galacturonic acid and a regulatory function is ascribed to one of the uronic acid intermediates, 2-keto-3-deoxygluconate (KDG; Condemine et al. 1984). Little is known about mechanisms regulating pectinolytic gene expression in *Aspergillus*. Simultaneous expression of a variety of MHR degrading enzymes in *A. aculeatus* after growth on pectin was demonstrated by the preparation of Ultra SP, indicating that pectin degradation by this species is more complete. For this, a well-coordinated regulation system will be required, which might specifically effect degradation of rhamnogalacturonan structures.

Knowledge about the gene structure of rhamnogalacturonan hydrolase, which has an important role in MHR degradation, allows various kinds of molecular approaches. A start for the identification of trans-factors, which are involved in

the regulation of such genes, has been made by UV mutagenesis of *A. aculeatus*. In Chapter 4 the isolation of mutants, which are positively affected in rhamnogalacturonan hydrolase expression is described. In order to facilitate screening procedures, glucose oxidase (GOX) from *A. niger* was used as reporter system.

Since apple pectin was successfully used as carbon source for the induction of rhamnogalacturonan hydrolases in liquid culture, it was also used for the growth and screening of mutants on agar plates. Resulting low levels of GOX staining were attributed to the use of this complex substrate, either by providing a background colour or by the containment of repressive free sugars, like glucose. Besides, pectin components might react with H_2O_2, the reactive byproduct formed after glucose oxidation which is essential for staining. H_2O_2 can also oxidize proteins, among which pectinolytic enzymes, a mechanism by which GOX producing, transgenic plants are thought to become resistant to *Erwinia* infections (Wu et al. 1995).

Fortunately, galacturonic acid and rhamnose are also capable of inducing *A. aculeatus rhg*A, which is in agreement with their abundance in rhamnogalacturonan structures. After a first screening for high GOX-producing mutants, co-elevation of rhamnogalacturonan hydrolase expression was verified by Western blot analysis, in order to eliminate cis-mutations within the reporter-construct. Among the selected mutants different GOX expression patterns were found on different carbon sources. Moreover, at least four of the mutations were assigned to different loci by complementation tests, indicating their genetic independence.

The involvement of four different loci in the repression of rhamnogalacturonan hydrolase genes points to a complex induction pathway. Future research will be focused on the identification of trans-factors and subsequent DNA binding studies. This will generate more information about the regulation of *rhg*A, including the role of rhamnose and galacturonic acid and their catabolic intermediates in signalling and the function of conserved boxes in *rhg* promoter sequences.

Classification of *A. aculeatus* and *A. japonicus* strains within the black *Aspergilli*

Rhamnogalacturonan hydrolases are industrially applied, requiring large-scale production systems. For the attainment of the GRAS status of these and also other pectinolytic enzymes, it is very important that the strains of origin are well-known and properly classified. This also allows reasonable selection of other, related species with desired properties, such as *A. japonicus*. As we now know, classification of at least some *A. aculeatus* strains is highly dependent on the method which is used for classification. Obviously, strain classification just on the basis of secondary morphological features is ambiguous, necessitating additional criteria at the molecular level.

In Chapter 5 a RFLP method is described, by which *A. aculeatus* strains can be clearly distinguished from other black *Aspergilli* as well as from *A. japonicus* strains, as illustrated in Fig. 3. Five of the strains analysed are reclassified from *A. aculeatus* to *A. japonicus* and vice versa, one strain probably represents a new taxon and *A. luchuensis*, *A. atroviolaceus* and *A. atroviolaceo-fuscus* mut. *grisea* cannot be distinguished as separate species. However, the group of *A. aculeatus* strains thus formed is heterogeneous in comparison with the group of *A. japonicus* strains. Ambiguous results obtained by the use of different methods suggest a high variety of and/or high variability within natural habitats occupied by these strains. As *A. aculeatus* and *A. japonicus* strains share some morphological features, the existence of a common phylogenetic ancestor strain, different from *A. niger*, is very likely.

The same RFLP method was used for the preliminary classification of *A. carbonarius* strains. This revealed high similarities between all but one of these strains and includes the *S. fusca* representant within this species. Like with the group of *A. japonicus* strains, a high degree of homogeneity was found in the group of *A. carbonarius* strains. With few modifications this RFLP method will be applicable for the classification of other *Aspergilli* and even of other fungal groups.

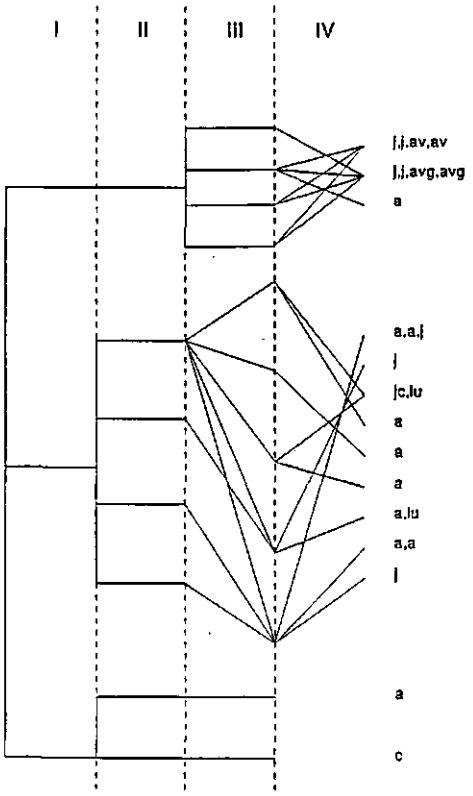


Fig. 3 Schematic representation of the classification of 30 black *Aspergilli* by RFLP analysis. Genomic DNA was hybridized with three different probes (I/III, II, IV) after digestion with two different restriction enzymes (I/II, III/IV). Links between different hybridization patterns are indicated by *lines*. a, *A. aculeatus*; av, *A. atroviolaceus*; avg, *A. atroviolaceo-fuscus* mut.*grisea*; c, *A. carbonarius*; j, *A. japonicus*; jc, *A. japonicus* var. *capillatus*; lu, *A. luchuensis*

References

Al-Musallam A (1980) Revision of the black *Aspergillus* species. PhD Thesis, State University Utrecht

An J, Zhang L, O'Neill MA, Albersheim P, Darvill AG (1994) Isolation and structural characterization of *endo*-rhamnogalacturonase-generated fragments of the backbone of rhamnogalacturonan I. *Carbohydr Res* 264:83-96

Azadi P, O'Neill MA, Bergmann C, Darvill AG, Albersheim P (1996) The backbone of the pectic polysaccharide rhamnogalacturonan I is cleaved by an endohydrolase and an endolyase. Glycobiol 5:783-789

Chen H-J, Starret DA, Zhou D, Tucker ML, Gross KC (1996) Cloning and characterization of rhamnogalacturonase A from *Botrytis cinerea*. Mol Plant Microbe Int (abstract J-6)

Condemine G, Hugouvieux-Cotte-Pattat N, Robert-Baudouy J (1984) An enzyme in the

pectinolytic pathway of *Erwinia chrysanthemi*: 2-keto-3-deoxygluconate oxidoreductase. J Gen Microbiol 130:2839-2844

Dick AJ, Opoku-Gyamfua A, DeMarco AC (1990) Glycosidases of apple fruit: A multifunctional β-galactosidase. Physiol Plantarum 80:250-256

Fry SC (1988) The growing plant cell wall: chemical and metabolic analysis. Longman Scientific & Technical, Harlow, UK, pp 188-218

Gross KC, Starrett DA, Chen HJ (1995) Rhamnogalacturonase, α-galactosidase, and β-galactosidase: Potential roles in fruit softening. Acta Hort 398:121-130

Kauppinen S, Christgau S, Kofod LV, Halkier T, Dörreich K, Dalbøge H (1995) Molecular cloning and characterization of a rhamnogalacturonan acetylesterase from *Aspergillus aculeatus*. J Biol Chem 270:27172-27178

Kofod LV, Kauppinen S, Christgau S, Andersen LN, Heldt-Hansen HP, Dörreich K, Dalbøge H (1994) Cloning and characterization of two structurally and functionally divergent rhamnogalacturonases from *Aspergillus aculeatus*. J Biol Chem 269:29182-29189

Mutter M, Beldman G, Schols HA, Voragen AGJ (1994) Rhamnogalacturonan α-L-Rhamnopyranohydrolase. Plant Physiol 106:241-250

Mutter M, Colquhoun IJ, Schols HA, Beldman G, Voragen AGJ (1996) Rhamnogalacturonan α-L-rhamnopyranosyl-(1,4)-α-D-galactopyranosyluronide lyase, a new enzyme able to cleave RG regions of pectins. Plant Physiol 110:73-77

Sakamoto M, Shirane Y, Naribayashi I, Kimura K, Morishita N, Sakamoto T, Sakai T (1994) Purification and characterization of a rhamnogalacturonase with protopectinase activity from *Trametes sanguinea*. Eur J Biochem 226:285-291

Schols H (1995) Structural characterization of pectic hairy regions isolated from apple cell walls. PhD Thesis, Agricultural University Wageningen

Schols HA, Geraeds CJM, Searle-van Leeuwen MF, Kormelink FJM, Voragen AGJ (1990) Rhamnogalacturonase: a novel enzyme that degrades the hairy regions of pectins. Carbohydr Res 206:105-115

Searle-van Leeuwen MJF, Broek LAM van den, Schols HA, Beldman G, Voragen AGJ (1992) Rhamnogalacturonan acetylesterase: a novel enzyme from *Aspergillus aculeatus*, specific for the deacetylation of hairy (ramified) regions of pectins. Appl Microbiol Biotechnol 38:347-349

Wu G, Shortt BJ, Lawrence EB, Levine EB, Fitzsimmons KC, Shah DM (1995) Disease resistance conferred by expression of a gene encoding H_2O_2-generating glucose oxidase in transgenic potato plants. Plant Cell 7:1357-1368

Samenvatting

Bij de enzymatische bereiding van appelsap spelen pectinolytische enzymen en met name de enzymen die inwerken op de 'hairy regions' van pectine (MHR), een belangrijke rol. Schimmels zijn belangrijke enzym-producenten en een aantal *Aspergillus* soorten worden veelvuldig gebruikt in de voedingsmiddelenindustrie. In dit proefschrift staat de afbraak van MHR door rhamnogalacturonan hydrolases centraal en de produktie van deze enzymen is uitgebreid onderzocht in zowel *A. aculeatus*, de producent van het commerciële enzympreparaat Ultra SP, als *A. niger*.

De waarneming van een endo-enzymactiviteit in Ultra SP, welke in staat is om MHR af te breken, betekende een doorbraak voor het inzicht in de wijze waarop pectine door schimmels wordt afgebroken. Tot dan toe waren alleen endo-enzymen bekend, die gericht zijn op de afbraak van homogalacturonan, zoals polygalacturonase, pectaat lyase en pectine lyase, en exo-enzymen, zoals galactosidase en arabinofuranosidase, die zich beperken tot de gedeeltelijke afbraak van MHR zijketens.

Bij afbraak van MHR door rhamnogalacturonan hydrolase, worden tetra- en hexa-saccharide fragmenten uit de hoofdketen vrijgemaakt. Dit is niet geheel in overeenstemming met een alternerende volgorde van rhamnose en galacturonzuur in de hoofdketen en de herkenning van één van beide typen bindingen tussen deze monosacchariden door rhamnogalacturonan hydrolase. Blijkbaar vindt geen complete afbraak plaats en zijn voor verdere afbraak additionele endo-enzymen met andere specificiteiten nodig.

In deze studie is allereerst het gen coderend voor rhamnogalacturonan hydrolase van *A. aculeatus*, *rhg*A, gecloneerd en het recombinant eiwit gezuiverd, zoals beschreven in hoofdstuk 2. Immunogene screening van een cDNA expressiebank resulteerde in een cDNA cloon, die vervolgens gebruikt werd als probe voor het screenen van een genomische bank.

Kruishybridizatie van genomisch DNA van andere schimmelsoorten behorend tot de sectie Nigri met deze probe, resulteerde in meerdere hybridizerende DNA fragmenten en dus potentiële gen-families in alle stammen, maar vooral in *A. niger*. In elk van de onderzochte stammen werd tevens kruisreactiviteit met het

polyclonale muize-antiserum, opgewekt tegen rhamnogalacturonan hydrolase van *A. aculeatus*, aangetoond. De betreffende eiwitten variëren in MW van 55 kDa in de verschillende onderzochte *A. aculeatus* en *A. japonicus* stammen tot 75 kDa in *A. foetidus*. De aanwezigheid van één rhamnogalacturonan hydrolase per stam leek niet in overeenstemming met de hybridizatie-patronen in de Southern analyse en tevens bleken deze enzymen algemeen binnen de sectie Nigri voor te komen.

Het onderzoek naar rhamnogalacturonan hydrolase expressie werd daarom voortgezet in *A. niger*, een belangrijke producent van pectinolytische enzymen. In hoofdstuk 3 wordt de screening van het *A. niger* genoom naar sequenties homoloog aan het *A. aculeatus rhg*A gen beschreven. *A. niger* blijkt in het bezit te zijn van twee verwante genen, *rhg*A en *rhg*B. De hiervan afgeleide aminozuur sequenties vertonen ook, zij het in verschillende mate, overeenkomst met *A. aculeatus* rhamnogalacturonan hydrolase.

Bij de vergelijking van de DNA sequenties bleken niet alleen de coderende gebieden, maar ook de aangrenzende, niet-coderende DNA sequenties homologieën te vertonen met het *A. aculeatus* gen. Beide *rhg*A promotoren hebben een box van 15 bp gemeenschappelijk en vertonen daarnaast 77% homologie over een traject van 130 bp. Mogelijk spelen deze elementen een regulerende rol bij de expressie van de verschillende rhamnogalacturonan hydrolase genen. De relatief geringe hoeveelheid rhamnogalacturonan in pectine in combinatie met de complexe structuur, maken het tot een moeilijk afbreekbaar substraat in vergelijking met bijv. homogalacturonan, dat vrij toegankelijk is voor enzymatische afbraak. Een verwachte opeenvolging in expressie van rhamnogalacturonan afbrekende enzymen na homogalacturonan afbrekende enzymen blijkt niet te worden bevestigd. De expressie van *rhg*A in zowel *A. aculeatus* als *A. niger* is relatief vroeg. De genen komen in beide stammen reeds tot expressie binnen 6 h na transfer naar appel pectine. Expressie van het *rhg*B gen in *A. niger* kan alleen worden aangetoond op mRNA niveau na multicopy integratie in het genoom en is zichtbaar na 18 h groei op appel pectine maar ook op sucrose.

Mogelijke verklaringen voor de lage expressie niveaus in *A. niger* zijn hoge pro-

teolytische activiteit in de gebruikte stam en hoge gevoeligheid voor proteolytische afbraak van de betreffende rhamnogalacturonan hydrolases. De laatste verklaring is het meest waarschijnlijk, daar vergelijkbare resultaten werden verkregen na transformatie van *A. aculeatus* met deze genen.

De toepassing van een enzym als rhamnogalacturonan hydrolase maakt een goed produktie systeem noodzakelijk. In de hoofdstukken 2 en 3 wordt een produktie systeem in *A. awamori* beschreven, dat gebaseerd is op de endogene endo-xylanase promoter. Dit maakt de produktie van rhamnogalacturonan hydrolases op xylose mogelijk i.p.v. op appel pectine, waardoor een zuiverder produkt verkregen wordt.

Enzymactiviteiten van de *A. aculeatus* en *A. niger* rhamnogalacturonan hydrolases zijn vergeleken d.m.v. incubatie met MHR als substraat en analyse van de verkregen afbraakprodukten. Het *A. niger* B-enzym blijkt extra oligomeren te vormen waarvan een aantal met hoger MW, duidend op een andere substraat specificiteit dan de A-enzymen. Remming van het B-enzym door acetyl-groepen of andere zijketens is nog niet onderzocht. Echter, de aanwezigheid van extra monosacchariden, afkomstig van hoofd- of zij-ketens van MHR, in sommige van de gevormde produkten, suggereert dat er verschillen in sterische hindering bestaan tussen het B-enzym en de A-enzymen. Mogelijk is het B-enzym minder afhankelijk van enzymen die bijv. de arabinogalactan zijketens afbreken.

De specifieke activiteit van de verschillende rhamnogalacturonan hydrolases is afgeleid van de initiële snelheid waarmee één van de hexameren (corresponderend met piek VIII in Fig. 7, hoofdstuk 3) gevormd wordt. Ondanks de relatief lage expressie-niveaus van rhamnogalacturonan hydrolase A en B in *A. niger*, vertonen deze enzymen een specifieke activiteit van respectievelijk 30% en 25% van die van *A. aculeatus* rhamnogalacturonan hydrolase. Vergelijking met gepubliceerde rhamnogalacturonan hydrolase-activiteiten, bepaald in andere organismen, wordt bemoeilijkt door het gebruik van verschillende detectie-methoden.

De hoge mate van conservering van aminozuursequenties in de verschillende enzymen maakt toekenning van potentiële functies aan specifieke aminozuren onmogelijk en behoeft verdere analyse d.m.v. 'site-directed' mutagenese.

In *Aspergillus* is nog niets bekend over mechanismen die de expressie van pectinolytische genen reguleren. De produktie van een breed scala aan MHR afbrekende enzymen in *A. aculeatus* na groei op pectine, duidt op een efficiënte pectine afbraak. Hiervoor is een goed-gecoördineerd regulatie-systeem, specifiek gericht op de afbraak van rhamnogalacturonan structuren, noodzakelijk. Kennis van de genstructuur van rhamnogalacturonan hydrolase maakt tal van moleculaire studies mogelijk. Een start ter identificatie van trans-factoren, die betrokken zijn bij de regulatie van deze genen, is gemaakt d.m.v. UV mutagenese van *A. aculeatus*. In hoofdstuk 4 wordt de isolatie van mutanten met verhoogde rhamnogalacturonan hydrolase expressie beschreven. De screening is uitgevoerd m.b.v. *A. niger* glucose oxidase als reporter systeem. Aangezien appel pectine een geschikte C-bron bleek voor de inductie van rhamnogalacturonan hydrolase in vloeibare kweken, werd dit substraat in eerste instantie ook gebruikt voor de screening van mutanten op agar medium. Echter de complexiteit van het substraat bracht een aantal nadelen met zich mee, zoals achtergrond kleuring, mogelijk represserende effecten door de aanwezigheid van andere suikers of inhibitie van de kleurreactie door interactie van pectine bestanddelen met H_2O_2. Een alternatieve C-bron voor de inductie van *rhg*A, gebaseerd op de combinatie van twee hoofdbestanddelen van rhamnogalacturonan, galacturonzuur en rhamnose, bleek wel bruikbaar. Mutanten met verschillende expressie patronen na groei op een aantal verschillende C-bronnen werden geselecteerd en vier van de mutaties konden d.m.v. complementatie-toetsen worden toegekend aan verschillende loci. Blijkbaar vindt regulatie van *rhg*A expressie in *A. aculeatus* plaats via een complex mechanisme, waarbij tenminste vier verschillende loci zijn betrokken. Identificatie van de betreffende trans-factoren zal bijdragen tot het ophelderen van het regulatie systeem en de rol van galacturonzuur en rhamnose daarin, maar ook mogelijkheden bieden tot een verdere verhoging van de enzymproduktie.

Aangezien rhamnogalacturonan hydrolases worden gebruikt in de levensmiddelenindustrie, is het belangrijk dat de identiteit van een geselecteerde produktiestam gewaarborgd is. Dit vereist een gedegen classificatie methode. Gezien de

arbitraire resultaten verkregen na classificatie van *A. aculeatus* stammen met de huidige methoden, welke veelal gebaseerd zijn op morfologische kenmerken, bestond de noodzaak tot een andere aanpak. In hoofdstuk 5 wordt een RFLP methode beschreven, waarmee *A. aculeatus* kan worden onderscheiden van een aantal andere soorten binnen de sectie Nigri, inclusief de morfologisch verwante *A. japonicus*. De groep van *A. aculeatus* stammen blijkt erg heterogeen te zijn vergeleken met bijv. de *A. japonicus* groep. Echter, een onderverdeling in variëteiten binnen beide groepen is op grond van deze classificatie niet langer gerechtvaardigd. Een aantal voormalige *A. aculeatus* en *A. japonicus* stammen wisselen van groep. Gezien de overeenkomstige morfologische kenmerken die beschreven zijn voor *A. aculeatus* en *A. japonicus* stammen, wordt een gemeenschappelijke phylogenetische voorouder, anders dan die van *A. niger*, waarschijnlijk geacht. Dezelfde RFLP methode is gebruikt voor het classificeren van *A. carbonarius* stammen en kan met geringe modificaties ook gebruikt worden voor de classificatie van andere *Aspergilli*.

Nawoord

Het proefschrift dat hier voor U ligt, is uit delen opgebouwd. Zoals zij nu een boek vormen, zo vormden zij mij, op een brede maar interessante weg. Onderweg hebben verschillende mensen mij bijgestaan, ieder op geheel eigen wijze. Daarom wil ik hierbij bovenal Jaap Visser bedanken, voor de mogelijkheden die hij mij geboden heeft en de inspiratie. Colja Laane ben ik dankbaar voor zijn bereidheid om promotor te zijn en voor zijn hulp bij de afronding van het proefschrift. Ook Hein Stam en Wouter Musters van Unilever wil ik bedanken voor hun bijdrage en de tijd die zij hebben vrijgemaakt om over de verschillende onderwerpen van dit proefschrift te discussiëren.

Verder bedank ik Peter Schaap voor de kritische beoordeling van mijn manuscripten. Een aantal andere mensen hebben bijgedragen aan de stimulerende werksfeer, dat zijn mijn lab- en kamergenoten. Margo, Wietse, Iris en de andere medewerkers van het 'Ciba-lab', bedankt hiervoor. Peter van de Vondervoort wil ik speciaal bedanken voor zijn hulp bij de genetische analyse van mutanten en Peter van der Veen voor zijn enthousiasme bij het oplossen van computerproblemen. Jan-Willem bedank ik voor zijn geweldige inzet tijdens zijn stageperiode.

Gerhard, jij hebt zo ongemerkt een niet te beschrijven bijdrage geleverd aan het tot stand komen van dit boekje. Jou en onze familieleden ben ik dankbaar voor het meeleven. Natuurlijk bedank ik ook alle anderen, die ik hier niet met name kan noemen, maar die wel een bijdrage hebben geleverd.

Curriculum vitae

Marjon ten Hoor-Suykerbuyk werd op 12 februari 1963 geboren in Vlaardingen. In 1981 behaalde ze aan het Bisschoppelijk College Schöndeln te Roermond haar Gymnasium β diploma, waarna werd begonnen met de studie Biologie aan de Rijksuniversiteit Utrecht. In 1984 werd het kandidaatsexamen B5* (Medische Biologie) behaald. De hieropvolgende doktoraalfase omvatte onderzoek binnen de vakgroep Experimentele Pathologie van het AZU (Prof.dr. W. den Otter en Dr. P. Roholl), het Koninklijk Instituut voor de Tropen te Amsterdam (Dr. W.J. Terpstra en Dr. J.E.R. Thole) en de vakgroep Moleculaire Celbiologie van de Rijksuniversiteit Utrecht (Prof.dr. H.O. Voorma en Dr. D. Schamhart). Het doktoraalexamen met 1^{e} graads lesbevoegdheid werd in november 1988 afgelegd.

Van december 1988 tot juni 1991 was zij werkzaam als wetenschappelijk onderzoeker bij de sectie Moleculaire Genetica en Gentechnologie van het Medisch Biologisch Laboratorium TNO te Rijswijk (Dr. C.A.M.J.J. van den Hondel en Ir. R.F.M. van Gorcom), waarna zij haar werkzaamheden kon voortzetten bij de sectie Moleculaire Genetica van Industriële Micro-organismen van de LU Wageningen (Dr. J. Visser). Het project werd gefinancierd door Unilever en heeft geleid tot de resultaten beschreven in dit proefschrift. In oktober 1996 is zij in dienst getreden als Project Supervisor Quality Control bij Centocor te Leiden.