PECTINASES OF *ASPERGILLUS NIGER*:

A Molecular And Biochemical Characterisation

Propositions / Stellingen

1. The class of pectinases called 'polymethylgalacturonases' does exist.
   (Whitaker, J. R. (1991) Microbial pectolytic enzymes. In Microbial Enzymes and Biotechnology (eds. Fogarty, W. M. and Kelly, C. T.) Elsevier Applied Science, London and New York. pp. 133-175. This thesis.)

2. It is necessary to apply a 'global view' approach with respect to pectinase gene regulation in order to reveal the mechanism.
   (This thesis.)

3. Classic and modern fungal taxonomy approaches should get united - it would be profitable for both of them.
   (This thesis.)

4. The production of enzymes, which expression is driven by a promoter of 1,4-β-endoxylanase A gene of *Aspergillus awamori*, can be further optimised in fermentors by varying the inducer (D-xylose) concentration.
   (Gouka, R.J. *et al.* (1996) An expression system based on the promoter region of the *Aspergillus awamori* 1,4-β-endoxylanase A gene. Appl. Microbiol. Biotech. 46, 28-35. de Vries, R.P. *et al.* (1999) CreA modulates the XlnR induced expression on xylose of *Aspergillus niger* genes involved in xylan degradation. Res. Microbiol. 150, 281-285.)

5. The best source of inspiration for a researcher in the field of natural sciences is nature itself.

6. Sun has a great effect on our mood………on the average it rains 234 days a year in The Netherlands. This explains a lot about the Dutch mentality.

7. No matter how great the progress in communication technology, it won't change the fact that people are loosing the ability to 'communicate' with each other.

8. Avoid shortcuts. They always take too much time in the long run.

9. "There are two types of employees: Those, who do the work and those who take the credit. Try to be in the first group, there is much less competition there."
   (Indira Gandhi)

10. Sports take your physical energy but give you mental strength.

11. The word 'collective' in sports like volleyball should not be interpreted as 'social'. Then the word 'sport' would loose its meaning.

Propositions belonging to the thesis by Lucie Pařenicová entitled
Pectinases of *Aspergillus niger*: A molecular and biochemical characterisation
Wageningen, 25th of April 2000

Lucie Pařenicová

PECTINASES OF *ASPERGILLUS NIGER*:

A Molecular And Biochemical Characterisation

Proefschrift

ter verkrijging van de graad van doctor

op gezag van de rector mangificus

van Wageningen Universiteit,

Dr. C. M. Karssen,

in het openbaar te verdedigen

op dinsdag 25 april 2000

des namiddags te half twee de Aula.

věnováno rodičům,

Patrikovi a babičce
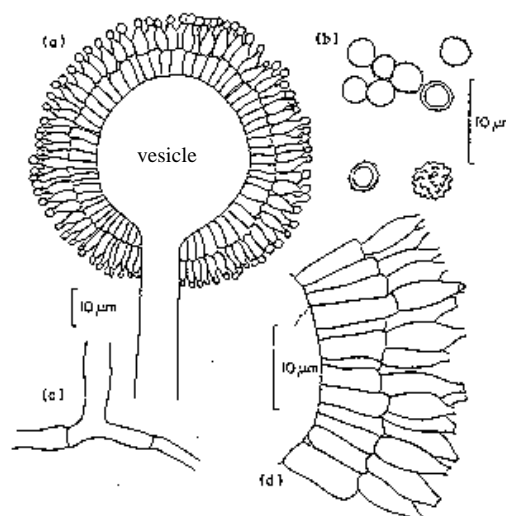
Contents

CHAPTER 1

General Introduction

1. *Aspergillus*

The species *Aspergillus niger* belongs to the division Eumycota, subdivision Deuteromycotina (Fungi Imperfecti) that comprises the fungi reproducing solely asexually by means of conidia [1]. Within the Deuteromycotina, this species belongs to the class of the Hyphomycetes, which produce conidiophores on any part of the mycelium, and more specifically to the genus *Aspergillus*. The genus *Aspergillus* is further divided into six subgenera. *A. niger* belongs to the subgenus *Circumdati*, section *Nigri* (the black aspergilli), which contains several other species as mentioned below.

Fig. 1. **Morphology of *Aspergillus*.** a) conidial head, b) conidia, c) foot cell, d) metulae and phialides (from left to right). From Onions *et al.* [2].

Aspergilli are characterised by a typical morphological structure (Fig. 1). A conidiophore, which includes the stalk and the conidial head, arises from specialised thick-wall mycelial structure called foot-cells. Conidiophores are non-septate, enlarged towards the apex and terminate in a globose, colourless to yellowish swelling (the vesicles). The vesicle bears over the whole surface 10 - 15 μm long, usually brown metulae, which themselves bear phialides. Conidia are produced successively from the tips of the phialides, thus forming unbranched dry chains. Conidia are smooth or ornamented, more or less globose and 4 - 5 μm in diameter but occasionally smaller [2].

1.1. The taxonomy of the black aspergilli

The origin of the name *Aspergillus* (rough head) dates back to the 18-th century, when Micheli (1729) [3] described a mould with conidiophores and heads bearing chains of spores. However, it took almost 200 years before a first taxonomic key for the classification of the aspergilli appeared [4]. The major criteria for the classification of an *Aspergillus* isolate were: i) colour of conidial heads, ii) stalk surface and colour, iii) shape of vesicles and iv) absence or presence of metulae. This allowed to separate 13 groups among the isolates. *Aspergillus niger* van Tieghem was classified as one of the Black *Aspergilli*, which also contained the biseriate species (carrying metulae and phialides) - *A. violaceo-fuscus* Gasperini, *A. luteo-*

niger Lutz, *A. phoenicis* Corda, *A. pulverulentus* McAlpine, *A. atropurpureus* A. Zimmermann, *A. pulchellus* Spegazzini and *A. carbonarius* Bainier, and the uniseriate species (containing only phialides) - *A. nanus* Montagne, *A. luchuensis* Inui, *A. perniciosus* Inui and *A. japonicus* Saito. However, imperfect filamentous fungi like the black aspergilli exhibit a high degree of variability in their morphological and physiological characteristics, often caused by the external growth conditions [5]. In the past this led to frequent changes of species names and to a re-classification of individual isolates. This tendency was clearly demonstrated in the work of Mosseray [6], who distinguished 35 species among isolates belonging to the *A. niger* group. Although fungal collections expanded in the years after, the number of species of the black aspergilli reduced. In Table 1 the changes in the classification of the genus *Aspergillus*, section *Nigri*, based on the morphological criteria since the year 1945, are indicated.

Thom and Raper [7] distinguished 17 different black aspergilli among them 8 and 4 species belonging to the biseriate *A. niger* and *A. carbonarius* series respectively, and 5 species belonging to the uniseriate *A. luchuensis* series. However, two of the uniseriate species, *viz. A. luchuensis* and *A. subfuscus*, were later re-classified by Raper and Fennell [8] as the biseriate *A. awamori* and *A. foetidus* var. *pallidus* species, respectively. These authors recognised 12 species of the black aspergilli, including four new species, *viz. A. tubingensis*, *A. ficuum*, *A. ellipticus* and *A. heteromorphus*. Al-Musallam [9] further reduced the number of species to 6 and the '*A. niger* aggregate' (with 6 varieties and 2 formae, *viz. A. niger* f. *hennebergii*, *A. awamori*, *A. phoenicis*, *A. phoenicis* f. *pulverulentus*, *A. nanus, A. usamii, A. intermedius*). Finally Samson [5] established six species based on morphological criteria.

The growing economical importance of the black aspergilli (see 1.2.) requires an unambiguous classification of an *Aspergillus* isolate prior to its application in biotechnological processes. This is often a difficult task, since many of the industrially important strains underwent a number of morphological changes in the process of 'domestication'. For the identification of isolates, for taxonomical purposes, and to define evolutionary relationships among closely related fungal species, molecular methods started to be widely used in the eighties. They included DNA complementarity, ribosomal RNA sequence comparison, and nuclear (nDNA) and mitochondrial DNA (mtDNA) restriction fragment length polymorphism (RFLP) analysis [10]. For the classification of the black aspergilli the main interest was in *A. niger* and related species. Kusters-van Someren and co-workers [11] examined twenty-three '*A. niger* aggregate' isolates and divided them into two

different species, *viz. A. niger* and *A. tubingensis*, based on the rDNA and pectin lyase A and B RFLP patterns. They also analysed representatives of other black aspergilli and concluded that *A. helicotrix* was a mutant variant of *A. ellipticus*, and that the *A. aculeatus* and *A. japonicus* taxa were identical.

Table 1. Overview (1945 - 1994) of the classification of the black aspergilli based on morphological criteria. The names in the parentheses represent the synonyms.

	Thom & Raper 1945	Raper & Fennell 1965	Al-Musallam 1980	Samson 1994
		A. tubingensis		*A. tubingensis*
	A. niger	*A. niger* (*A. fumaricus*)	*A. niger* & var.	*A. niger*
	A. foetidus	*A. foetidus* var. *pallidus* (*subfuscus*) var. *acidus*	*A. foetidus*	
	A. awamori	*A. awamori* (*A. miyakoensis, A. luchuensis*)		
	A. miyakoensis			
	A. phoenicis	*A. phoenicis*		
	A. pulvurulentus	*A. pulvurulentus*		
	A. niger mut. *cinnamomeus*			
	A. niger mut. *Schiemanni*			
		A. ficuum		
		A. ellipticus	*A. ellipticus*	*A. ellipticus*
		A. heteromorphus	*A. heteromorphus*	*A. heteromorphus*
			A. helicotrix	
	A. atropurpureus			
	A. fonsecaus			
	A. fumaricus			
	A. carbonarius	*A. carbonarius*	*A. carbonarius*	*A. carbonarius*
	A. luchuensis			
	A. japonicus	*A. japonicus* (*A. nanus*)	*A. japonicus* var. *japonicus* var. *aculeatus*	*A. japonicus*
	A. violaceus-fuscus			
		A. aculeatus (*A. violaceus-fuscus*)		
	A. nanus			
	A. subfuscus			

Row group labels (left margin, read vertically):
- Biseriate
  - *A. niger* series (small primary sterigmata and small conidia)
  - *A. carbonarius* series (conidia in excess of 5.0 μm)
- Uniseriate
  - *A. luchuensis* series

The separation of two species in the 'A. *niger* aggregate' was supported by the rDNA RFLP and random amplification of polymorphic DNA (RAPD) analyses described by Megnegneau *et al.* [12] and by the mtDNA and rDNA RFLP analyses by Varga *et al.* [13]. The latter author recently described a third taxon among field isolates of the 'A. *niger* aggregate', represented by six Brasilian strains [14]. Such analyses were extended to other representatives of the black aspergilli. The genotypical and phenotypical characterisation [15] of thirteen A. *carbonarius* isolates showed only small variations among their patterns, however, it allowed to distinguish one strain representing a new subspecies. A similar approach was used to analyse forty uniseriate isolates of A. *japonicus* and A. *aculeatus* species [16]. Although the authors did not observe any DNA polymorphisms upon RFLP analysis of the rDNA unit, these isolates showed a high degree of mtDNA polymorphism, which correlated with the RAPD patterns. However, the phenotypic patterns of these strains, based on isoenzyme analysis and carbon utilisation spectra, showed only little variation among the isolates. The dendogram based on the phenotypic characters of these strains exhibited only a limited correlation to that based on the genotypic characters.

Among the molecular and biochemical methods exploited, particularly RFLP analyses proved to be quick and reliable to establish the species identity of unknown black *Aspergillus* isolates.

1.2. Exploitation of aspergilli

Aspergilli are used by industry for the production of primary metabolites (organic acids, vitamins, fatty acids and amino acids) and enzymes as well as for bioconversion processes. Since 1917 A. *niger* strains are being used in the production of citric acid, a universally accepted additive with a broad range of applications in food, beverage, pharmaceutical and cosmetic industries and for industrial and technical processing [17]. Two species, *viz.* A. *niger* and A. *oryzae* (Section *Flavi*), prevail as enzyme producers [18]. While A. *niger* is mainly exploited in the area of food (bakery, starch industry) and beverages (wine and juice, brewery, distilling industry), A. *oryzae* is applied in fermentation of oriental sauces, such as soya sauce and miso [19]. Since both species have been used for years, many of their products obtained the GRAS (Generally Regarded As Safe) status.

The progress in fungal genetics, gene technology and protein engineering makes the aspergilli an attractive 'tool' for a number of biotechnological processes in different industries, one of these being the pectin industry. In this industry fungal pectinases are applied to generate specific pectic products.

2. Pectin

Pectin is a heteropolysaccharide found in the middle lamella and the primary cell wall of higher plants. Pectins function as a 'glue' that holds the other cell wall polysaccharides, like cellulose and hemicellulose (i.e. xyloglugan or glucuronarabinoxylan), and proteins, such as hydroxyproline-rich glycoprotein extensin, together (Fig. 2). Two antiparallel pectin chains can be condensed in the cell wall by cross-linking with Ca^{2+} ions to form 'junction zones' or the so-called multiple 'eggbox'. In some species pectins may be cross-linked to other pectins or non-cellulosic polysaccharides by ester linkages with dihydroxycinnamic acids such as diferulic acid [20]. In plants, pectins are present in all stages of development. The composition depends not only on the species but also on tissue, stage of growth and maturity, and growth conditions. Pectins are heterogeneous with respect to both chemical structure and molecular weight.

Fig. 2. **The type I primary cell wall of most flowering plants.** The cellulose microfibriles are interlaced with xyloglucan polymers, and this framework is embedded in a matrix of pectic polysaccharides, polygalacturonic acid and rhamnogalacturonan [20] (the figure was reproduced with the permission of Blackwell Science Ltd. and the Society for Experimental Biology).
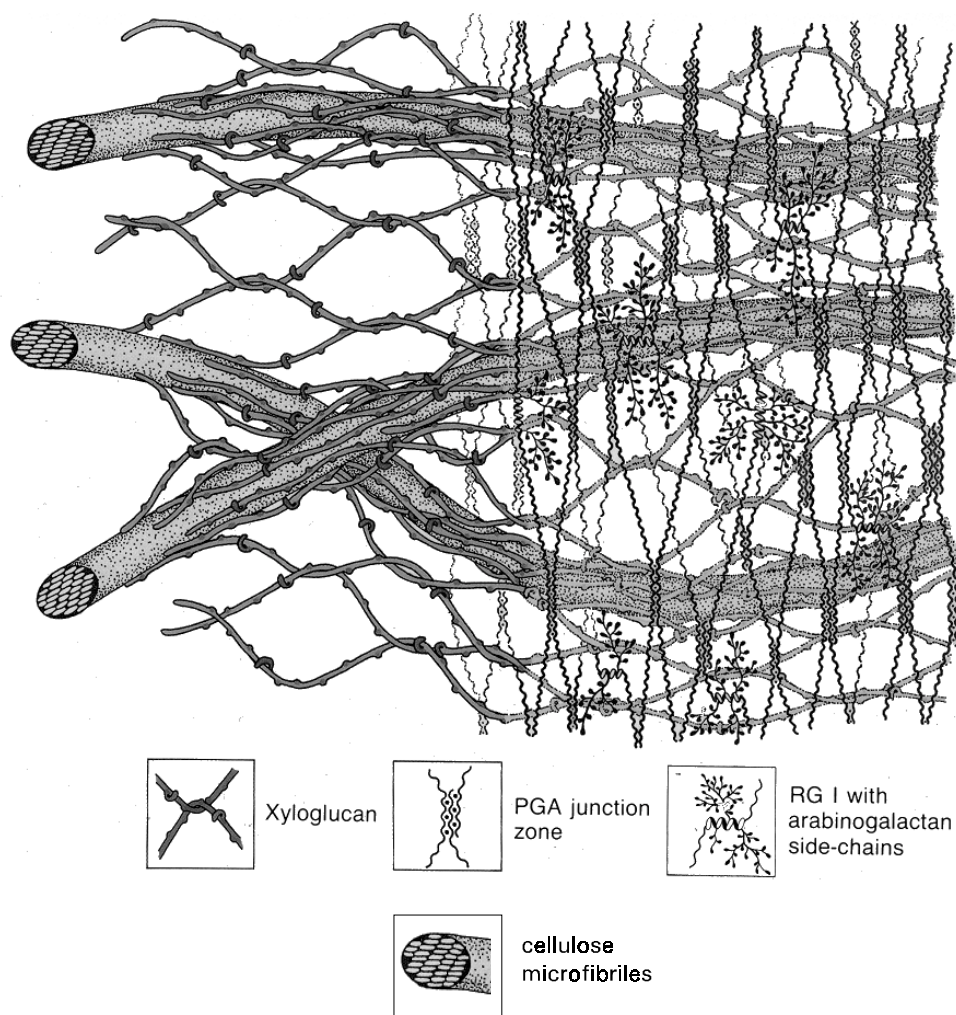
The main backbone of the pectin molecule is composed of a polymer of D-galacturonic acid, called homogalacturonan (homopolymer of [1→4]α-D-galactopyranosyluronic acid units with part of the carboxyl groups methylesterified, Fig. 3A) and rhamnogalacturonan I (heteropolymer of repeating [1→2]α-L-rhamnosyl-[1→4]α-D-galactosyluronic acid di-saccharide units, Fig. 3B) [21]. Apart from the rhamnogalacturonan I (RGI), the more complex rhamnogalacturonan II (RGII) structure has been identified in the primary wall of some plants [22], and is believed to play a role as a signal molecule in plant cell development rather than being a structural polymer [20]. The RG I is primarily responsible for the chemical and structural complexity of the pectic substances.

Fig. 3. **The main structures of pectic polysaccharides.** (A) Polygalacturonic acid composed of [1→4]α-D-galactopyranosyluronic acid units, partially methylesterified at C_6. (B) Rhamnogalacturonan I (RGI) contains repeating disaccharide units of [1→2]α-L-rhamnosyl-[1→4]α-D-galactosyluronic acid (GalA). The GalA units can be acetylated at secondary alkohols. About one half of rhamnosyl units of RGI carries side chains like arabinans, arabinogalactans and galactans [20].
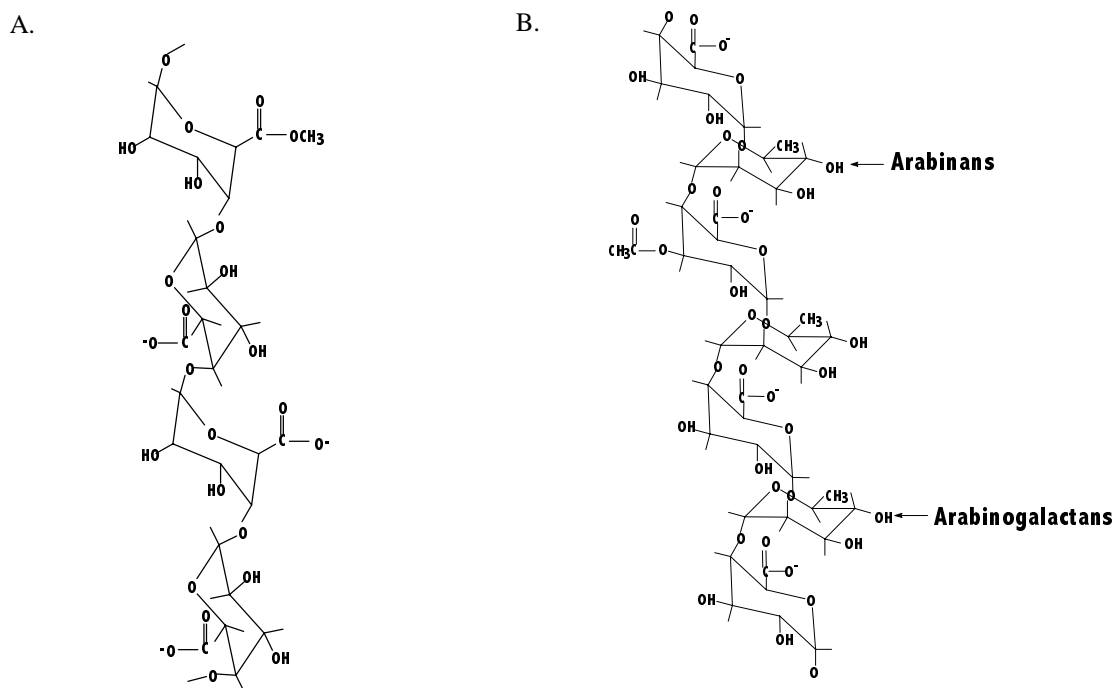
The pectin molecule is branched at the rhamnogalacturonan part by side chains like arabinans, galactans or arabinogalactans, which are linked by [1→4] linkages to rhamnose. In the main side chains, the arabinose units are α[1→5] linked and the galactose units are joined by β[1→4] linkages. Apart from these neutral sugars the side chains of pectins can also contain D-xylopyranose, D-glucopyranose and L-fucopyranose, whereas in RGII, D-apiose, 2-O-methyl-

D-xylose and 2-O-methyl-L-fucose are present [21]. In RG I the galacturonic acid residues are often acetylated at the C_2 or C_3 position, but acetylation was found also in the homogalacturonan region.

2.1. Applications of pectin

At present, the main sources of commercial pectin are apple pomace and citrus peels. They are extracted by hot acidified water and followed by chemical or enzymatic (using pectinases) processing. Pectins are used in many industries mainly because of their ability to form gels. Pectin use is allowed all around the world and was recommended as a safe additive by the joint FAO/WHO committee. It has a broad range of applications as a gelling agent, thickener, texturizer, emulsifier, stabiliser, fat or sugar replacer in low-calories foods, and as a component of many medicines. In medicine pectin is used mainly because of its anti-diarrhoea effect, for lowering of blood cholesterol levels and as a natural prophylactic substance against poisoning with toxic cations. Because of its biodegradable and recyclable character pectin films nowadays find more applications apart from those within the food and pharmaceutical industries.

The degree of polymerisation (DP) and degree of methylesterification (DE) determine the conditions of use. The DP of the pectin molecule varies from a few hundred to 1000 saccharide units, which corresponds to molecular weights from about 50,000 to 150,000 (http://www.herc.com/foodgums/pectin). Based on the number of the methylesterified galacturonic acid residues, pectins are classified as low (25-50 %) and high (50-80 %) methylesterified pectins. The low methylesterified (LM-) pectin is obtained from the high methylesterified (HM-) pectin usually by a treatment at mild acidic or alkaline conditions. In case ammonia is used, the LM-pectins can contain some amide groups at the C_6 position of the galacturonic acid units. The degree of methylation determines the gelling properties of pectin. In LM-pectin the gel is formed in the presence of Ca^{2+} (*viz*. the "eggbox" structure above) over a wide pH range, namely pH=1.0 to 7.0 or higher (http://www.herc.com/foodgums/pectin). In the HM-pectin gelling requires formation of interaction zones by hydrogen bonds and hydrophobic interactions of the methylester groups among the molecules [21]. This reaction requires quite a narrow pH range, around 3.0, and the presence of a cosolute (sucrose), at concentrations exceeding 55 % by weight. The textural properties of pectin gels are influenced notably by DE, but also by the sugar composition of the side chains, the degree of acetylation, the degree of amidation, and cross-linking of pectins.

About 80 % of the world production of HM-pectin are used in the manufacturing of jams and jellies. Other applications are found in the preparation of fruit drink concentrates and fruit juices. HM-pectin is known to prevent the formation of casein clumps in acidified dairy products at pH below the isoelectric pH (4.6). LM-pectin is used for instance in a combination with carrageenan to produce sugar-free jams for diabetics and in fruit preparations for yoghurt. The Ca^{2+} requirement of the LM-pectin is utilised in gelation of fruit/milk deserts (http://www.herc.com/foodgums/pectin).

3. Pectinases

Pectinases, or pectinolytic enzymes, are produced by a number of bacteria, yeast, fungi, protozoa, insects, nematodes and plants [23] in order to degrade (to obtain a carbon source) or to modify (in fruit ripening etc.) the heteropolysaccharide pectin. They can be classified, based on the type of linkages they attack, into the esterases, which saponify the substrate, and the depolymerases. The depolymerases can be subdivided based on the bond cleavage mechanism into the class of the hydrolases (hydrolytic cleavage) and the class of the lyases (β-elimination cleavage). Pectinases show different substrate specificity, but basically they can be separated into a group of homogalacturonan and a group of rhamnogalacturonan specific enzymes. Besides the main pectin backbone-degrading enzymes, the 'accessory' enzymes, active towards the side chains of pectin, are needed to fully accomplish pectin degradation.

Yet another class of pectin degrading enzymes is recognised by some authors. These enzymes called protopectinases convert insoluble protopectin into soluble pectin. However, as demonstrated for the protopectinase-encoding gene from *Trichosporum penicilliatum* [24], which shows a high sequence homology with endopolygalacturonases, this class of enzyme most probably represents a polygalacturonase or another defined pectinase activity (see below) with a high substrate specificity to protopectin.

Since the main part of this thesis deals with pectinases, especially endopolygalacturonases, of *A. niger*, the overview presented below will focus on the pectinase-encoding genes and the corresponding enzymes produced mainly by this micro-organism.

3.1. The homogalacturonan-degrading enzymes

A. niger produces a number of enzymes active on the homogalacturonan part of the pectin molecule. They include pectin methyl- and acetyl-esterase (EC 3.1.1.11 and EC 3.1.1.6), endopolygalacturonase (EC 3.2.1.15), exopolygalacturonase (EC 3.2.1.67), pectate lyase (EC

4.2.2.2) and pectin lyase (EC 4.2.2.10). While pectin lyase can utilise the naturally occurring methylesterified substrate, the activity of pectin methylesterase is required in order to produce low methylesterified pectin (pectate), the substrate for polygalacturonases and pectate lyase.

In food, feed and beverage industries pectinolytic enzyme preparations derived from *A. niger* belong to those most frequently applied. However, these preparations represent ill-characterised enzyme mixtures, which may cause difficulties in various applications or in case specific modifications of commercial pectins are required, i.e. in food gels. This has been one of the reasons to initiate research on *A. niger* pectinases in the early nineties. The programme focused on the identification and isolation of a broad spectrum of pectinase-encoding genes, the regulation of their expression, the overexpression and the biochemical characterisation of individual enzymes.

3.1.1. Polygalacturonases

Polygalacturonases together with rhamnogalacturonases (see 3.2.) belong to family 28 of glycosyl hydrolases [25]. Polygalacturonases can be divided into three groups. Endopolygalacturonases randomly attack the $[1\rightarrow4]\alpha$-glycosidic linkages of the polysaccharide chain producing a number of galacturonic acid oligomers, exopolygalacturonase type I (galacturonan 1,4-α-galacturonosidase) hydrolyses D-galacturonic acid from the non-reducing end, and exopolygalacturonase type II (exo-poly-α-galacturonosidase) releases digalacturonate from the non-reducing end of polygalacturonic acid. Endopolygalacturonases and exopolygalacturonases of type I were identified in fungi. Only two exopolygalacturonases of type II were isolated untill now from the bacteria *Erwinia chrysanthemi*, the strains EC16 and 3937 [26,27]. While the endo- and exo-polygalacturonases specifically hydrolyse polygalacturonate or pectin with a low degree of esterification, another class of polygalacturonases, endopolymethylgalacturonases, which were supposed to be active towards high-methylesterified pectin, has been described in the literature [23]. However, other researchers have never been able to demonstrate the enzymatic activity on this substrate, which most probably originated from the presence of contaminating pectin methylesterase or pectin lyase activities.

3.1.1.1. Polygalacturonase-encoding genes and families

Five different endopolygalacturonases (PG) and one exopolygalacturonase (PGX) were isolated from Pectinase K2B 078, a commercial *A. niger* pectinase preparation [28]. The five

PGs were purified to homogeneity and their physicochemical characterisation suggested that they may be encoded by five different genes. Two of these enzymes, named PGI and PGII, were found to be the most abundant in this pectinase preparation. Using a reverse genetics approach Bussink and coworkers [29] isolated the PGII-encoding gene (*pga*II) from *A. niger* N400 (= CBS 120.49). Similarly, the *pga*I gene was isolated [30]. In a search for new endopolygalacturonase-encoding genes an *A. niger* N400 genomic library was screened using a *pga*II-derived probe. In addition to *pga*I and *pga*II, five different *pga*II-related classes of hybridising phages (A-E) were identified, indicating the presence of an endopolygalacturonase-encoding gene family in *A. niger*. A molecular analysis of class C led to the isolation of the third PG-encoding gene, *pga*C [31].

Apart from the *A. niger pga* genes, PG-encoding genes were isolated from other aspergilli mainly by using a PCR-based approach or by heterologous screening of a DNA library with a fungal *pga* probe (Table 2). The structural features of the *pga* genes among the different aspergilli are highly conserved. This is reflected by a similar size of the enzymes encoded (362-383 amino acids) and by the exon-intron organisation. The endopolygalacturonase-encoding genes isolated from aspergilli today contain one to three introns, which interrupt the coding region at identical positions (Fig. 4, Table 2).

Table 2. **Overview of PG-encoding sequences from various aspergilli as present in the nucleotide sequence/protein databases**. + indicates that the protein was isolated and characterised (column 'enzyme purified'). The amino acid composition of the primary sequence is deduced from the *pga*-coding region based on the sequence homology and allows to calculate the molecular mass (Mw) of the protein. The gene type corresponds to the exon/intron organisation of *pga* genes as described in Fig. 4. d.s., direct submission. aa; amino acid.

Organism	Gene	Enzyme purified	GenBank/EMBL accession number	SwissProt/PIR/GP accession number	PDB/ crystal data	length aa	Mw	gene type	Ref.
A. aculeatus	*pga*I		AF054893			378	38 533	-	d.s.
A. flavus	*pec*A		U05015	P41749		363	37 464	II	[32]
	*pec*B		U05020	P41750		366	37 877	II	[32]
A. niger	*pga*I	+	X58892	P26213		368	38 108	II	[30]
	*pga*II	+	X58893	P26214	1CZF	362	37 684	I	[29]
	*pga*C	+	X64356	Q12554		383	40 502	III	[31]
A. oryzae	*pga*A	+	D14282	P35335		363	37 520	II	[33]
	*pga*B	+	AB007769			367	37 864	II	[34]
A. parasiticus	*pec*A		U17167 L23523	P49575		363	37 506	II	[35]
A. tubingensis	*pga*II		X58894	P19805		362	37 512	I	[36]
'*A. niger* RH5344'		+	X52903 X54146			362	37 512	I	[37]

This feature indicates the presence of a common ancestor of the *Aspergillus pga* genes. *pga* genes were also isolated from yeasts (*Kluyveromyces marxianus* [38] and *Saccharomyces cerevisiae* [39]), different species of *Penicillium* (*P. expansum* [40], *P. griseoroseum* [40], *P. janthinellum* [41] and *P. olsonii* [40]) and a number of phytopathogenic fungi like *Sclerotinia sclerotiorum* [42] and *Botrytis cinerea* [43], in which an endopolygalacturonase-encoding gene family was identified. Except for *Bcpg*3 from *B. cinerea*, which encodes a 514-amino acids long protein, the other fungal *pga* genes encode proteins of similar size as found in the aspergilli. This is not the case for bacterial and plant PG-encoding genes. In bacteria the length of the PGs ranges from 312 amino acid residues (*Agrobacterium tumefaciens,* [44]) to 529 (*Pseudomonas solanacearum,* [45]) or 1148 (*Thermoanaerobacter thermosulfurogenes* EM1, [40]). In plants enzymes were described from 365 (*Lycopersicum esculentum,* [46]) to 514 (*Cryptomeria japonica*, [47]) amino acid residues in length.

Fig. 4. **Schematic representation of the *pga* gene organisation in aspergilli.** The aspergilli *pga* genes present in databases contain one to three introns, the gene type is marked as I to III based on the number of introns present in the *A. niger pga* genes. The exon sequences corresponding to the pre- and pro- peptide (see 3.1.1.2.) are depicted. The amino acids preceding the mono- or di- basic cleavage site of the propeptide are below the sequences. On the left-hand side the length of unprocessed proteins is shown.
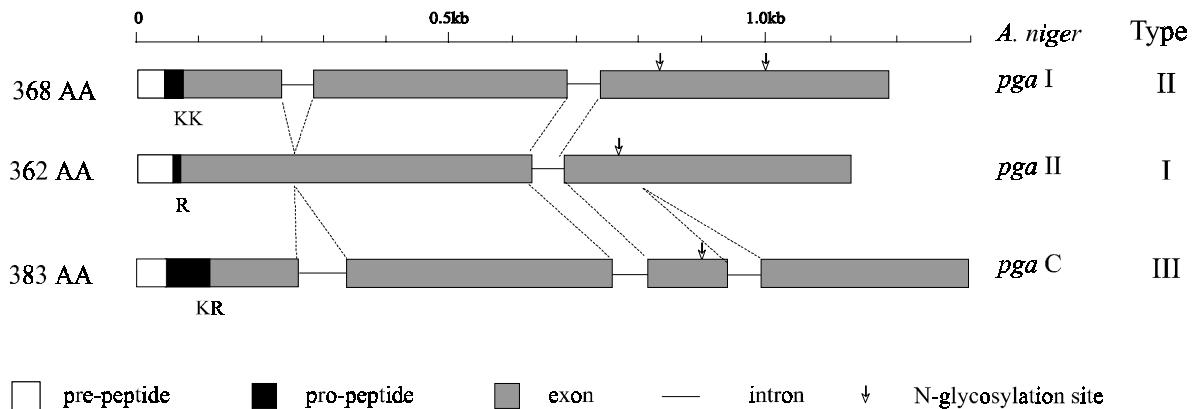
Unlike the PG-encoding genes, only three fungal exopolygalacturonase-encoding genes (*pgaX*) have been isolated so far from *A. tubingensis* [48], *Cochliobolus carbonum* [49] and *Fusarium oxysporum* [40]. The characterisation of *pgaX* from *A. tubingensis*, a species closely related to *A. niger*, showed that the protein encoded has only 13 % overall amino acid sequence identity with the *A. niger* endopolygalacturonases and is more closely related to plant endopolygalacturonases [48]. PGX-encoding genes have also been found in bacteria and plants, *viz. Erwinia chrysanthemi* [26,27], *Ralstonia solanacearum* [50], *Yersinia enterocolitica* [40], *Arabidopsis thaliana* [51] and *Zea mays* [52].

3.1.1.2. Physicochemical and biochemical properties of fungal polygalacturonases

Fungal polygalacturonases are mainly reported to be extracellular enzymes, often secreted in several isoforms. However, some authors described the presence of an exopolygalacturonase activity, which was associated with the conidia and the mycelium in *Aspergillus* spp. [53], or claimed the identification of intracellular polygalacturonases such as in *Penicillium frequentans* [54]. The latter results are doubtful since the authors used a supernatant of homogenised mycelia for the enzymatic assay and thus the 'intracellular' enzymatic activities might rather represent an activity of cell wall associated polygalacturonases.

The typical amino acid sequences of polygalacturonases contain a pre-peptide (signal peptide), and in the case of the endopolygalacturonases also a pro-peptide (see Fig. 4). The mature enzyme is produced after the cleavage of the pre(pro)peptide as demonstrated for some of the purified enzymes [29,30,37,48]. The function of the pro-peptide in the endopolygalacturonases is unknown, but was shown for enzymes like proteases [55] to play a role in the correct folding of the protein, in subcellular targeting or in acting as an (auto)inhibitor. Based on the derived amino acid sequences, *Aspergillus* polygalacturonases contain potential N-glycosylation sites (Asn-Xaa-Thr/Ser), which often occur at conserved positions (Fig. 4). It was shown by deglycosylation experiments that the N-glycosylation is partially responsible for the discrepancy between the calculated and the apparent molecular masses of the enzymes [30,48]. Recent studies to determine the glycosylation patterns of the recombinant PGI and PGII enzymes from *A. niger* [56,57] using a combination of mass spectrometric techniques, demonstrated that both enzymes possess one site occupied with the N-linked glycosyl structure of the high-mannose type, although PGI has two putative sites. No O-linked glycosylation occurred in these recombinant enzymes. The importance of the glycosylation for the *A. niger* endopolygalacturonase enzyme activity was demonstrated by Stratilová and coworkers [58]. They showed that deglycosylation of the enzyme, with N-glycosidase F under nondenaturating conditions, led to a complete inactivation.

Although the number of polygalacturonase-encoding nucleotide sequences present in the databases is increasing, reports about biochemical characterisation and kinetic studies of the proteins encoded are scarce. This is partially due to the difficulties encountered with the isolation of the enzymes. In the past several authors used commercially available *A. niger* pectinase preparations such as Pectinase [59], Pectinol [60], Pectinase K2B 078 [28] or Rohapect 5DL [37] to isolate and to characterise polygalacturonases. However, it is questionable whether the enzyme mixtures originate from a real *A. niger* isolate, since in case

of the *A. niger* RH5344 endopolygalacturonase it has been shown that the nucleotide sequence shares homology with *pga*II from *A. tubingensis* [36] to the extent that *A. niger* RH5344 is likely to be an *A. tubingensis* isolate.

Only recently, using recombinant DNA technology, the *Aspergillus* polygalacturonases have been successfully overproduced and purified to homogeneity. The *A. niger* RH5344 endopolygalacturonase was expressed under the control of the *ADH1* (alcohol dehydrogenase) promoter in *S. cerevisiae* yielding to 180 mg/l of the protein [61] and a relatively high level of expression (40-100 mg/l) was achieved in case of *A. oryzae* PGA and PGB and *A. niger* N400 pectinases by the homologous production under the control of a strong promoter, *viz.* those of *TEF1* encoding the *A. oryzae* translation-elongation factor 1α [34] and *pki*A encoding pyruvate kinase A from *A. niger* [48,62,68], respectively. Using this approach the purification of the enzyme can be further facilitated by the selection of a suitable strain and by modifying the growth conditions.

Table 3. Biochemical properties of some fungal polygalacturonases. The abbreviations $T_{opt.}$, $a_{spec.}$ and $pH_{opt.}$ correspond to temperature optimum, specific activity and pH optimum. K_m and $a_{spec.}$ were determined for pectate or polygalacturonate as substrates.

| source of enzyme		gene isolated	type of activity	pI	$T_{opt.}$	$a_{spec.}$ (U/mg of protein)	$pH_{opt.}$	K_m (mg/ml)	Ref.
A. niger	I[a]		ENDO	3.8			4.0		
	II[a]		ENDO				4.5		
	III[a]		ENDO	4.5			5.5	1.7	
A. niger	I[a]		ENDO			81	4.1		
	II[a]		ENDO			44	3.8		
A. niger			ENDO			75	5.0	0.54	
A. japonicus			ENDO			1362	4.5		
Botrytis cinerea			ENDO			2049	4.0	1.2	
Trichoderma koningii	I[b]		ENDO	6.41			5.0	0.8	
	II[b]		ENDO	6.57			5.0	0.85	
Verticillium albo-atrum			ENDO				6.5	1.5	
A. niger	I	+	ENDO	3.2-3.5		550	4.2	< 0.15	[28],
	II	+	ENDO	4.6-5.9		2760	4.2	< 0.15	[62]
	C	+	ENDO			24.9	4.2	< 0.15	
A. oryzae	A	+	ENDO		45 °C		5.0		[34]
	B	+	ENDO		55 °C		5.0		
A. carbonarius	I				55 °C	640	4.0		[64]
	II				50 °C	7000	4.1		
	III				55 °C	550	4.3		
A. tubingensis		+	EXO	3.7-4.4		255/262	4.2	3.1/3.2	[48]
Neurosporra crassa			ENDO		45 °C	299	6.0	5.0	[65]
Alternaria alternata			ENDO	8.8		12.4[V]	5.0	0.1	[66]
Fusarium oxysporum f.sp. *melonis*			EXO	6.4		8.8	5.0		[67]

* data obtained from a review [63]; [a] multiple forms; [b] isoenzymes, glycoproteins; [V] the specific activity described in viscosimetric units, otherwise 1U is defined as the amount of enzyme required to liberate 1 μmol of reducing equivalents per minute

The biochemical properties of several fungal polygalacturonases and their origin are summarised in Table 3. From the data presented it can be concluded, that fungal polygalacturonases are generally active under acidic pH conditions and that their temperature optimum is around 50 $^{\circ}$C. K_m values for polygalacturonic acid range from < 0.15 to 5 mg.ml^{-1} and the specific activities vary from 8.8 to 7000 U.mg^{-1} of protein. Polygalacturonases usually retain a low level of enzymatic activity when pectin with DE > 50 % is used as a substrate. Apart from hyphal fungal polygalacturonases those from yeast [69], bacteria and plants (reviewed by Pilnik and Rombouts [63]) have been biochemically characterised. They exhibit similar properties with respect to pH and temperature optima and K_m values for polygalacturonate as found for the fungal enzymes.

3.1.1.3. Mechanism of the glycosidic bond cleavage by polygalacturonases and the subsite concept

Glycosides hydrolyse their substrates either by inversion or retention of the anomeric configuration [70]. The inverting mechanism operates via a direct displacement of the carbohydrate leaving group by a water molecule, whereas the retaining, double-displacement mechanism, involves formation of a glycosyl-enzyme intermediate (see Fig. 5).

Fig. 5. Schematic representation of the hydrolysis mechanism of inverting (A) and retaining (B) glycosidases.
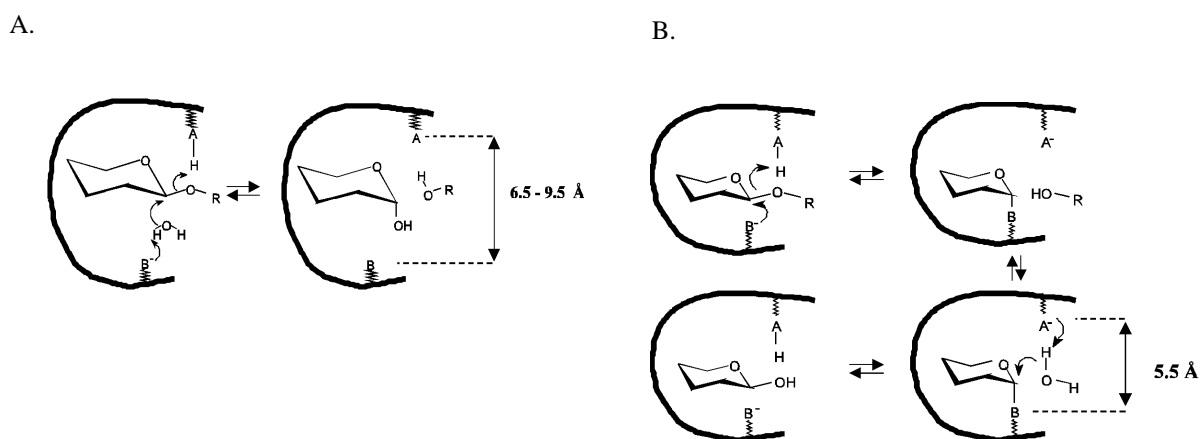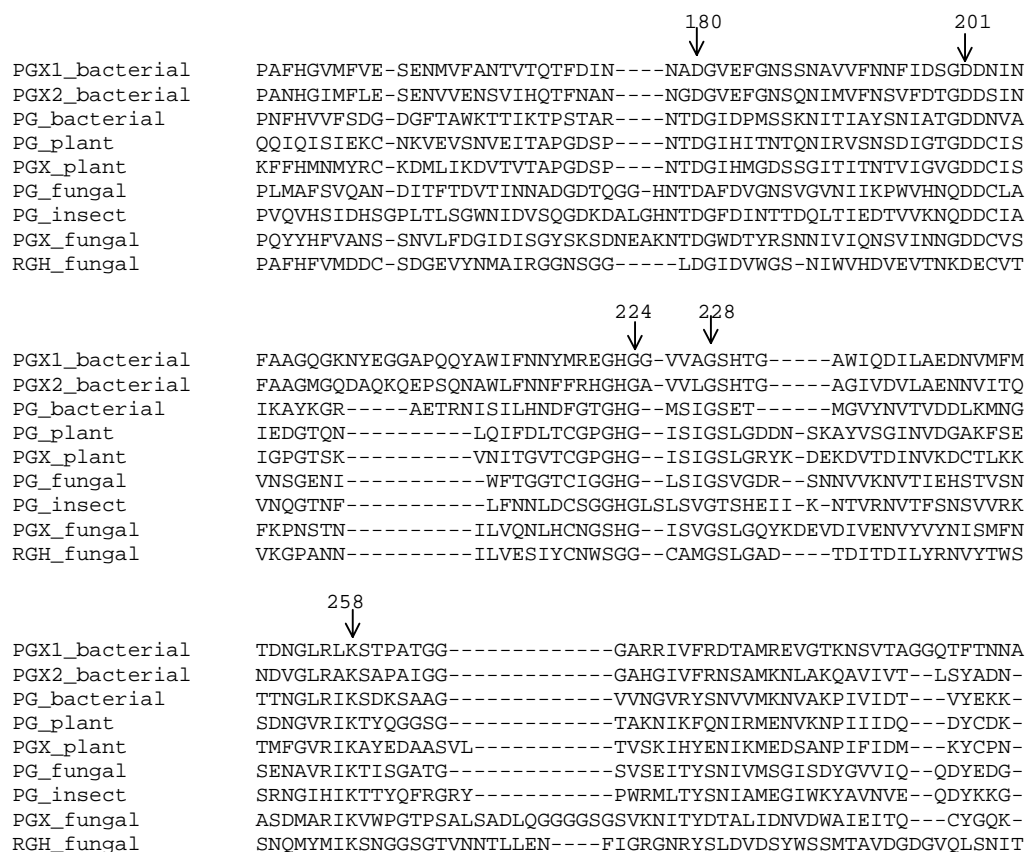

Both classes employ a pair of carboxylic acid residues in the active site, which act as a general acid and a general base (inverting mechanism) or as a general acid/base and a nucleophile/leaving group (retaining mechanism), respectively. In case of the *A. niger* PGI and

PGII and the *A. tubingensis* PGX it has been shown, that these enzymes belong to the class of inverting glycosidases [71].

In order to elucidate which amino acids in the *A. niger* endopolygalacturonases are involved in catalysis and binding of the substrate, different approaches including chemical modification of amino acid residues, pH-dependent photoinhibition and pH effects on kinetic data have been chosen in past. These studies identified a protonated histidine [72,73], at least one carboxylate group, either aspartic or glutamic acid [73,74], and tyrosine residue [75] to be involved in catalysis.

Fig. 6. A sequence alignment of family 28 hydrolases depicts the strictly conserved amino acid residues (arrows). For each species one representative was selected. The names correspond to the following sequences: PGX1_bacterial, *Ralstonia solanacearum* (U60106); PGX2_bacterial, *Erwinia chrysanthemi* (M31308); PG_bacterial, *Erwinia carotovora* (X51701); PG_plant, *Arabidopsis thaliana* (AJ002532); PGX_plant, *Zea mays* (X57627); PG_fungal, *A. niger* PGII (X58893); PG_insect, *Phaedon cochleariae* (Y17906); PGX_fungal, *A. tubingensis* (X99795); RGH_fungal, *A. niger* (X94220). The position of the amino acid residues is numbered according to the *A. niger* PGII sequence.

```
                                                          180              201
                                                           ↓                ↓
PGX1_bacterial    PAFHGVMFVE-SENMVFANTVTQTFDIN----NADGVEFGNSSNAVVFNNFIDSGDDNIN
PGX2_bacterial    PANHGIMFLE-SENVVENSVIHQTFNAN----NGDGVEFGNSQNIMVFNSVFDTGDDSIN
PG_bacterial      PNFHVVFSDG-DGFTAWKTTIKTPSTAR----NTDGIDPMSSKNITIAYSNIATGDDNVA
PG_plant          QQIQISIEKC-NKVEVSNVEITAPGDSP----NTDGIHITNTQNIRVSNSDIGTGDDCIS
PGX_plant         KFFHMNMYRC-KDMLIKDVTVTAPGDSP----NTDGIHMGDSSGITITNTVIGVGDDCIS
PG_fungal         PLMAFSVQAN-DITFTDVTINNADGDTQGG-HNTDAFDVGNSVGVNIIKPWVHNQDDCLA
PG_insect         PVQVHSIDHSGPLTLSGWNIDVSQGDKDALGHNTDGFDINTTDQLTIEDTVVKNQDDCIA
PGX_fungal        PQYYHFVANS-SNVLFDGIDISGYSKSDNEAKNTDGWDTYRSNNIVIQNSVINNGDDCVS
RGH_fungal        PAFHFVMDDC-SDGEVYNMAIRGGNSGG-----LDGIDVWGS-NIWVHDVEVTNKDECVT

                                              224   228
                                               ↓     ↓
PGX1_bacterial    FAAGQGKNYEGGAPQQYAWIFNNYMREGHGG-VVAGSHTG-----AWIQDILAEDNVMFM
PGX2_bacterial    FAAGMGQDAQKQEPSQNAWLFNNFFRHGHGA-VVLGSHTG-----AGIVDVLAENNVITQ
PG_bacterial      IKAYKGR-----AETRNISILHNDFGTGHG--MSIGSET------MGVYNVTVDDLKMNG
PG_plant          IEDGTQN----------LQIFDLTCGPGHG--ISIGSLGDDN-SKAYVSGINVDGAKFSE
PGX_plant         IGPGTSK----------VNITGVTCGPGHG--ISIGSLGRYK-DEKDVTDINVKDCTLKK
PG_fungal         VNSGENI----------WFTGGTCIGGHG--LSIGSVGDR--SNNVVKNVTIEHSTVSN
PG_insect         VNQGTNF----------LFNNLDCSGGHGLSLSVGTSHEII-K-NTVRNVTFSNSVVRK
PGX_fungal        FKPNSTN----------ILVQNLHCNGSHG--ISVGSLGQYKDEVDIVENVVYYNISMFN
RGH_fungal        VKGPANN----------ILVESIYCNWSGG--CAMGSLGAD----TDITDILYRNVYTWS

                         258
                          ↓
PGX1_bacterial    TDNGLRLKSTPATGG------------GARRIVFRDTAMREVGTKNSVTAGGQTFTNNA
PGX2_bacterial    NDVGLRAKSAPAIGG------------GAHGIVFRNSAMKNLAKQAVIVT--LSYADN-
PG_bacterial      TTNGLRIKSDKSAAG------------VVNGVRYSNVVMKNVAKPIVIDT---VYEKK-
PG_plant          SDNGVRIKTYQGGSG------------TAKNIKFQNIRMENVKNPIIIDQ---DYCDK-
PGX_plant         TMFGVRIKAYEDAASVL----------TVSKIHYENIKMEDSANPIFIDM---KYCPN-
PG_fungal         SENAVRIKTISGATG------------SVSEITYSNIVMSGISDYGVVIQ--QDYEDG-
PG_insect         SRNGIHIKTTYQFRGRY----------PWRMLTYSNIAMEGIWKYAVNVE--QDYKKG-
PGX_fungal        ASDMARIKVWPGTPSALSADLQGGGGSGSVKNITYDTALIDNVDWAIEITQ---CYGQK-
RGH_fungal        SNQMYMIKSNGGSGTVNNTLLEN----FIGRGNRYSLDVDSYWSSMTAVDGDGVQLSNIT
```

An important step towards the identification of the active site residues of glycosidases has been their classification into families on the basis of sequence similarity [25]. In case of the

family 28 of hydrolases, only five amino acids are strictly conserved among the enzymes from different species (Fig. 6), namely Asp-180, Asp-201, Gly-224, Gly-228 and Lys-258 (numbered according to *A. niger* PGII), which indicates their importance in catalysis and/or substrate binding. However, only a recent study on the active site topology of *A. niger* PGII by using a site-directed mutagenesis approach [76] together with the resolved crystal structure of this enzyme [77] definitely identified the catalytic residues, *viz.* Asp-180, Asp-201 and Asp-202. The model of the crystal structure of PGII (Fig. 7) shows the presence of a large cleft formed by a right handed parallel β-helix, which is about 8 Å wide and open at both ends in agreement with the endohydrolytic character of the enzyme.
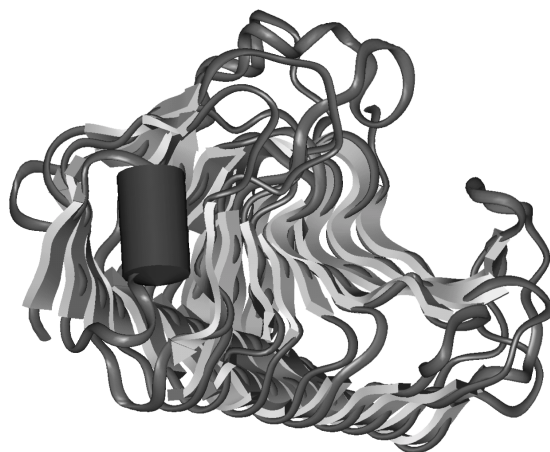

Fig. 7. The crystal structure of PGII from *A. niger* viewed from the N-terminal site through the cleft formed by β-helix.

The proposed mechanism for the hydrolysis of the α[1→4]-glycosidic bond by PGII now indicates that a water molecule, bound between Asp-180 and Asp-202 acts as the nucleophile, and that Asp-201 acts as a proton donor. All these residues are located at the bottom of the cleft. One histidine residue is conserved amongst the polygalacturonases (His-223, Fig. 6) but not in rhamnogalacturonases. The importance of this residue for catalysis, as indicated before, is explained by the close position to Asp-201, which makes it likely that these residues share a proton. Most probably this allows Asp-201 to be in the proper ionisation state to protonate the product. Similarly, three aspartic acid residues were determined in the active site of the *Erwinia caratovora* endopolygalacturonase crystal structure [78], whereas the *A. aculeatus* rhamnogalacturonase crystal data identified a glutamate residue at the position of Asp-202 (Fig. 6; [79]). Furthermore, the site-directed mutagenesis approach and the crystal data showed that Arg-256 and Lys-258 are primarily involved in substrate binding and are localised close to the active site.

It has been assumed that the active site of polymer-degrading enzymes with an endo-action pattern consists of several binding subsites complementary to the monomeric units of the polymer (Fig. 8). Indeed, the crystal structure of both the fungal and the bacterial endopolygalacturonase [77,78] support this hypothesis about the active site architecture.

Fig. 8. A schematic representation of an active site of a polysaccharide
-degrading enzyme consisting of several subsites, each of which
accommodates a monomeric unit of the polymer.
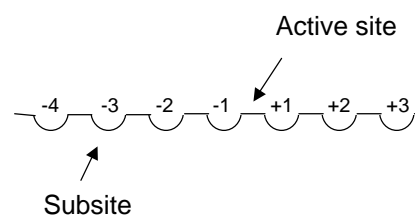
Although the fungal endopolygalacturonases share overall a high degree of sequence identity, their hydrolysis rates and mode of action on polygalacturonic acid and on oligomeric substrates can differ remarkably. In the pioneering work of Rexová-Benková and Heinrichová *et al.* [59,80] the oligogalacturonates of different chain length (n = 2-6) and their reduced equivalents were used to address the question, how many subsites were present. This approach led to a classification of the endopolygalacturonases into 3 classes based on the product formation. The reduced oligomer data enabled to identify at which position from the reducing end of the substrate hydrolysis occurred.

Unfortunately, these studies found only limited follow-up by other research groups. The degradation of modified pectins by PGs of different origin revealed that methylesters and acetyl groups limit their action, however to a different extent [81]. This study pointed out the importance of the structure of the sugar moiety of the polymer for catalysis. In case of the *A. niger* PGs, the detailed kinetic studies of recombinant PGI, II and C led to disclose some important differences among the members of the family [62]. While all the enzyme are composed of at least seven subsites, they differ in the bond cleavage frequencies on (reduced and unsaturated) galacturonic acid oligomers and in the level of degradation of pectins, which can be explained by different subsite affinities and architecture, respectively. None of the enzymes was able to degrade digalacturonate. Moreover, PGI and PGC show processive behaviour on polygalacturonate from the start of the reaction. Furthermore, the low specific activity of PGC on polygalacturonate (Table 3) suggested that polygalacturonate is not the natural substrate of the enzyme [62]. These results are important for understanding the *in vivo* role of the individual members of the endopolygalacturonase-encoding gene family for the

fungus, i.e. the degradation of specific parts of the pectin molecule, a quick generation of low molecular mass inducer etc. However, they also open new possibilities for industrial applications.

Unlike the *A. niger* PGs, PGX from *A. tubingensis* removes a galacturonic acid monomer from the non-reducing end of polygalacturonate [48]. Recently an additional activity of this enzyme was discovered, when exploiting PGX in the preparation of apple-pectin hairy regions. It releases xylogalacturonate from the xylogalacturonan part [82,83] of the pectin molecule.

3.1.1.4. Another look at polygalacturonases

Polygalacturonases, each can have a distinct role for the organism, which produces them. While in saprophytes like *A. niger*, they are important to provide nutrients from the environment by degrading pectin, in the case of phytopathogenic micro-organisms, they also participate in degradation of the plant cell wall to aid penetrating cells and spreading through plant tissue. Apart from pectinases other cell wall-degrading enzymes (CWDE) like xylanases, cutinases, glucanases and galactanases are being produced by phytopathogens during infection [84]. Pectinases, however, are typically produced first and in the largest amounts, and are the only CWDE capable of macerating plant tissue and killing plant cells on their own [85]. To establish the role of pectinases in pathogenicity is one of the main interests of researchers in phytopathology. The targeted disruption of the two major extracellular polygalacturonases in the maize pathogen *C. carbonum* [49] expressing only a residual polygalacturonase activity ($<$ 1 %) showed that the mutant strain was still pathogenic. Similarly, the disruption of *pga*1 in *B. cinerea* [86] and deletion of pectate lyase A-E (*pel*), exo-pectin lyase and pectin methylesterase (*pme*) genes in *E. chrysanthemi* [87] led only to a decrease in secondary infection (i.e. growth of the lesion beyond the inoculation spot), and a decrease of the ability to macerate plant tissue, respectively. These results demonstrate the involvement of pectinases in the pathogenesis, however they also show the complexity of the infection process, which probably requires a number of CWDE activities to be activated simultaneously.

In plants, obviously, polygalacturonases have to have another function. They are involved in ripening of fruits, formation of abscission zones and in the pollen tube development [88]. A number of polygalacturonase-encoding gene families have been reported from various plants, i.e. melon, maize, peach etc. [88], and they were divided based on the sequence homology over three clades (A-C). Clades A and B represent PGs identified in fruit and abscission

zones, while clade C, the most distantly related, includes exclusively PGs from pollen, which are classified as exo-polygalacturonases. Because the plant PGs are expressed in a wide range of developmental contexts, it is assumed that each distinct gene product has a special biochemical function [88].

3.1.2. Pectin and pectate lyases

The lyases catalyse eliminative cleavage of the α[1→4]-glycosidic bond between the (methylesterified) galacturonic acid residues in pectin or pectate to give Δ4,5 unsaturated oligosaccharides at the non-reducing end of the product (Fig. 9).

Fig. 9. α[1→4]-glycosidic bond cleavage by pectate and pectin lyase of the homo-galacturonan part of the pectin molecule.
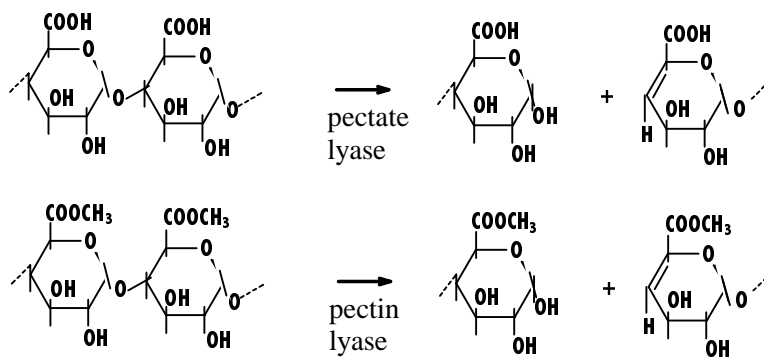
Until now the *pel* genes have only been reported to occur in bacteria and fungi. In *A. niger*, as for the endopolygalacturonases, a pectin lyase-encoding gene family was identified [89]. Four of the members, *viz. pel*A, *pel*B, *pel*C and *pel*D, were characterised at the molecular level [68,90,91; Kusters-van Someren, unpublished results]. Recently, a gene encoding pectate lyase (*ply*) from *A. niger* was isolated, the corresponding protein was overexpressed, purified to homogeneity and biochemically thoroughly characterised [92]. Pectate lyases are present also in other fungi, *viz. A. nidulans* [93], *Colletotrichum gloeosporioides* [94] and *Mycosphaerella pinodes* [40], and in plants, *viz. Nicotina tabacum* [95] and *Lycopersicon esculentum* [96]. However, the bacterial pectate lyase-encoding genes and their products are among the best characterised, mainly because of their role in pathogenesis, i.e. *E. chrysanthemi* and *E. carotovora*. Pectin lyases are usually active at pH lower than 8.0 and pectate lyases at pH 8-10. The latter enzymes have an absolute requirement for Ca^{2+} ions for catalysis. While the best substrate for pectin lyases is pectin with a high degree of esterification, pectate lyases are most active towards pectate or pectins with a low degree of esterification (20-50 %) (reviewed by Whitaker [23]).

3.1.3. Pectin methyl- and acetyl-esterases

Pectin methylesterase (PME) acts on pectin to remove methoxyl groups from the carboxyl group of the galacturonate unit and acetyl esterases remove the acetyl groups from the C_2 or C_3 position in galacturonate producing a secondary alcohol and acetate (see Fig. 10). Both enzymatic activities are necessary to produce a substrate accessible for pectin depolymerising enzymes. PME-encoding genes were isolated from various fungi, *viz. A. niger* [97,98], *A. aculeatus* [99] and *A. oryzae* [100], plants and bacteria.

Fig. 10.
Deesterification reactions of pectin catalysed by pectin methyl- and acetyl-esterase.
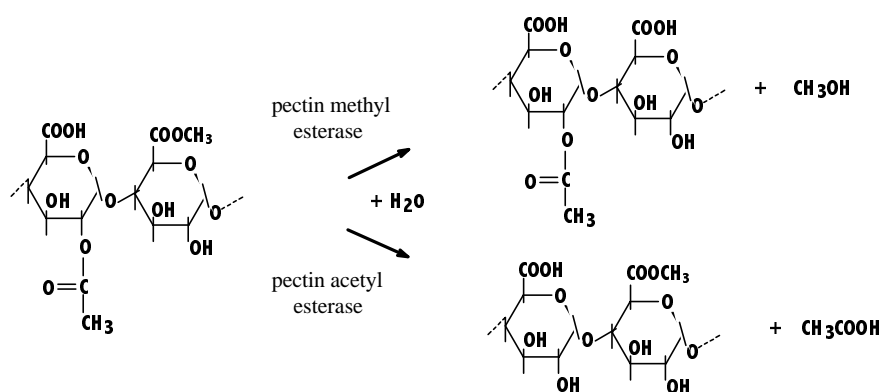
A number of PMEs was purified and biochemically characterised (reviewed by Whitaker [23]). The main difference between fungal and plant PMEs is their mode of action on pectins. While the fungal PMEs deesterify the substrate randomly, plant PMEs produce blocks of deesterified regions [101] (reviewed by Pilnik and Rombouts [63]). Recently, the mode of action of *A. niger* PME on (partially) methylated galacturonic acid oligomers was studied in detail using a combination of high performance anion exchange chromatography (HPAEC) and matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-MS) techniques [98]. Although acetylation of galacturonic acid occurs in both homo- and rhamno-galacturonan parts of the pectin molecule, so far only the genes encoding enzymes active in the RGI part of pectin have been isolated from two fungal species, *A. aculeatus* [102] and *A. niger* [103].

3.2. Rhamnogalacturonan I-degrading enzymes

These enzymes represent a recently formed group of pectinases, for the first time described by Schols *et al.* [83]. Until now four enzymes with a specific activity towards the RGI part of the pectin molecule have been reported from aspergilli, *viz.* rhamnogalacturonan hydrolase (RG-hydrolase) from *A. aculeatus* [104-106] and *A. niger* [107], rhamnogalacturonan lyase (RG-

lyase) from *A. aculeatus* [105,108], rhamnogalacturonan rhamnohydrolase (RG-rhamnohydrolase) from *A. aculeatus* [109] and rhamnogalacturonan galacturonohydrolase (RG-galacturonohydrolase) from *A. aculeatus* [110]. All enzymes are active towards the subunit of RGI, which contains strictly alternating galacturonic acid (GalA) - rhamnose (Rha) residues. RG-hydrolase and RG-lyase are endo-acting enzymes, the first one splits the GalA-Rha linkages, while the second one cleaves Rha-GalA bonds, leaving Δ4,5 unsaturated GalA at the non-reducing end. RG-rhamnohydrolase and RG-galacturonohydrolase are exo-acting enzymes, which release Rha or saturated GalA from the non-reducing end of RGI respectively (see Fig. 11). RG-galacturonohydrolase in not able to split the unsaturated GalA residue from the non-reducing end. Recently, a gene, *asd-1*, important in ascus development was isolated from *Neurosporra crassa*, which has a high sequence homology with the RG-lyase-encoding gene from *A. aculeatus* [111].

Fig. 11. Schematic representation of the RGI region of pectin containing strictly alternating GalA and Rha residues and of the activities of different rhamnogalacturonan degrading enzymes (adapted from Mutter [112]).
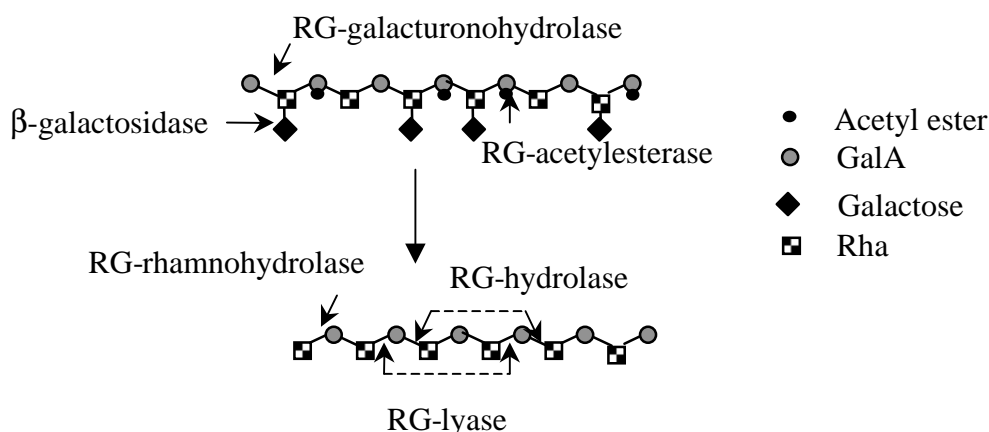
3.3. Accessory enzymes

As mentioned before (see section 2) the RGI region of pectin is branched at Rha by side chains of arabinans, arabinogalactans and galactans. Furthermore, pectins are cross-linked to other cell wall components via ester bonds of ferulic and coumaric acids and the hydroxyl functions at C_2 or C_6 of arabinose and galactose in the side chain, respectively. The enzymes, which degrade this pectic structure, are called accessory enzymes and include α-arabinofuranosidase, endoarabinase, β-galactosidase, endogalactanase and feruloyl and *p*-coumaroyl esterases (reviewed by de Vries [113]). Several studies demonstrated a synergy

between the accessory and main chain degrading enzymes leading to an enhancement of degradation of pectin [102-103].

3.4. Exploitation of pectinases in industry

Pectinases have been applied for more than 60 years in the food and beverage industry [114]. The main micro-organism exploited for the production of commercial pectinase preparation is *A. niger* (see also 1.2.). Pectinases mainly serve to prepare fruit and vegetables juices, such as: sparkling clear juices (apple, pear, grape), juices with clouds (citrus, prune, tomato juice and nectars), and juices, where the intent is to preserve the integrity of the plant cells by selectively hydrolysing the polysaccharides of the middle lamella. While in the case of the production of clear juices the pectinolytic enzymes are added to increase yield during pressing and for clarification, the stabilisation of clouds in orange juices is achieved by use of pectic enzymes with high levels of polygalacturonase activity [115]. Another field of application of pectinolytic enzymes is in the preparation of modified pectins, which are nowadays widely exploited (see 2.1.).

Commercial pectinolytic preparations derived from *A. niger* contain a large number of enzymes and therefore the results observed cannot easily be attributed to the action of a single enzyme or a class of enzymes [115]. Therefore, there is a need for the identification of the individual contributing enzyme activities to obtain more specific technical enzyme preparations and to facilitate the development of new applications.

4. Regulation of pectinase genes expression in fungi

Synthesis of polysaccharidases is usually induced by a monomeric or dimeric degradation product of the polymeric substrate. Further, synthesis of polysaccharidases is usually controlled by carbon repression, which inhibits synthesis of enzymes involved in polymer degradation when a more easily metabolisable carbon source is present [116-117]. The regulation of expression might occur at the transcriptional and translational level. In fungi the regulatory systems involved in (hemi-)cellulose degradation are among the best studied. In *A. niger* it was shown that the xylanolytic system is induced in the presence of D-xylose [118] and the regulation of the expression occurs at the transcriptional level via the activator XLNR [119], which coregulates the expression of genes encoding xylanases and many other cell-wall degrading enzymes, such as endoglucanases, cellobiohydrolases, ferulic acid esterase and

others [120-121]. Furthermore, it was shown that higher concentrations of xylose repress XLNR mediated induction and this effect appeared to be modulated via CREA, a wide domain carbon repressing protein [122]. The expression of arabinases from *A. niger* is also controlled at the level of transcription and L-arabinose and L-arabitol were identified as inducers [123].

The expression of pectinases in *A. niger* was studied mainly with the purpose to optimise the growth conditions to increase the yield of enzymes. These studies usually compare the production of pectinases in solid-state and submerged fermentation (SsF and SmF, respectively) under different physiological conditions, i.e. variable composition of culture media, pH, temperature and days of cultivation. Generally, in SsF the higher production of pectinases was observed on pectin between 1-2 days after inoculation and the synthesis of enzymes was less sensitive to carbon repression compared to SmF [124-125]. The lower pH (pH 3-4) favoured PG production on complex substrates like citrus pectin [126-127] and wheat bran [128], and variation in the pectinolytic enzyme composition was also noticed. Pectin, polygalacturonic and galacturonic acids induced synthesis of PME and PG in *A. niger* by increasing transcription, whereas glucose was found to repress the synthesis of both enzymes by a carbon repression mechanism that occurs at the translational level [125]. Another study, using fed-batch cultures with pectin supplemented with galacturonic acid (GalA) or glucose, demonstrated that a high concentration of glucose had a repressing effect and a high concentration of GalA had a negatively modulating feedback effect on the production of pectinases [129].

Compared to the fungal (hemi-)cellulases the knowledge about the molecular basis of the induction of the pectinolytic system in *A. niger* is poor. In *A. niger* N400 a low level of expression of PGI and PGII was detected on pectin by western blot analysis and it was shown that multicopy transformants of the PGI-, PGII- and PGC-encoding genes produced higher amounts of the corresponding proteins [29-31]. *A. niger* and *A. tubingensis pga*II gene expression was detected in the presence of 1 % pectin and 1 % sugar beet pulp and repressed on 2 % glucose. Furthermore, the *A. tubingensis pga*II multicopy transformant of *A. niger* showed the same induction pattern as in *A. tubingensis* suggesting a common regulatory system operating in both species [36]. Similar patterns of expression, i.e. pectin induced and sucrose repressed, were observed for the pectin lyases of *A. niger* N400 [68,91].

A promoter deletion study of the *pga*II gene demonstrated that the gene is subject to positive control, which is not limiting for its overexpression, and identified a 223 bp promoter

fragment responsible for the high expression of PGII on pectin [130]. A comparison of this promoter DNA sequence with the yeast regulatory DNA sequences revealed a region of high identity with the upstream activation site UAS2 of the *Saccharomyces cerevisiae CYC1* gene, which is under control of the HAP2/3/4 system. A similar sequence was identified in the *pga*I and *pga*C genes from which the *A. niger pga*-consensus, 5' TYATTGGTGGA 3', was derived [31]. The *A. niger* pectinase gene promoters were also analysed for the presence of a hexanucleotide sequence [31]. The sequence 5' CCCTGA 3' occurs at least once in all of these genes. However, further experimental evidence to prove the function of both DNA-promoter sequences is missing.

The pectinase induction was also studied in other fungal species. In *A. aculeatus*, the RGHA-encoding gene (*rgh*A) was found to be expressed on apple pectin and a combination of GalA and rhamnose. Furthermore, the possibility of negatively acting factors involved in *rgh*A expression was suggested [131]. The pectate lyase-encoding gene, *pel*A, from *A. nidulans* was induced on polygalacturonic acid and repressed on glucose. The glucose-mediated repression might be regulated by CREA as demonstrated by gel mobility shift assay using the *pel*A promoter fragment and the CREA protein [93]. *A. parasiticus* and *A. flavus* contain a *pga* gene being expressed on sucrose and pectin [32,35]. In *A. flavus*, additionally, also a pectin-induced *pga* gene is present. In *A. tubingensis* expression of the exopolygalacturonase-encoding gene was detected in the presence of GalA in the medium and was absent when glucose was added [48]. A differential expression of the *pga*-gene family was detected in the phytopathogenic fungus *B. cinerea* grown in liquid culture [43,132]. Each of the six members of the gene family showed a specific expression pattern depending on the carbon source used (glucose, GalA, polygalacturonic acid or pectin) and the pH of the medium, which suggests a specific role of the individual gene products for the fungus. Other pectin constituents such as arabinose and rhamnose were found to induce the PG expression in *Colletotrichum lindemuthianum* [133]. An interesting observation was done in a study of pectin lyase (PL) induction in *Penicillium griseoreoseum*. Yeast extract at concentrations as low as 0.0075 % (w/v) significantly promoted PL induction and was responsible for an increase of the cAMP level inside of the cells, suggesting the involvement of this second messenger in the biosynthesis of this enzyme [134-135].

The overview presented above demonstrates that a number of factors, i.e. carbon source, pH of the medium, cAMP, catabolite repression and aeration, can be involved in fungal pectinase expression. This can be expected in view of the much better understood prokaryotic pectinase

regulatory system as studied in *Erwinia* spp (reviewed by Hugouvieux-Cotte-Pattat *et al.* [136]). The pectinase genes as well as the genes involved in pectin catabolism and pectinase secretion are controlled by a repressor, KDGR. The intermediates of GalA catabolism, among which 2-keto-3-deoxygluconate (KDG), were identified as inducers of the pectinolytic system. Besides KDGR, a number of other factors affect the expression. The expression of several genes is controlled by cAMP-mediated catabolite repression (in the presence of glucose and pectin catabolic products), the PECS-PECM and PECT regulatory proteins, and by some global regulatory proteins such as the cAMP receptor protein (CRP) [137]. A number of other loci which might be involved in the regulation of the pectinase genes in *Erwinia* spp. are currently being investigated. The environmental factors such as osmolarity, temperature, oxygen and nitrogen availability, iron deprivation and DNA-damaging agents differentially affect the pectinase expression [136].

Aim and outline of the thesis

This thesis deals with two major topics - *Aspergillus* and pectinases. *A. niger* has over twenty genes, amongst which two gene families, which encode enzymes involved in the degradation of pectin. This broad spectrum of pectinases most probably reflects the complexity of the substrate. It is therefore likely that the endopolygalacturonase and pectin lyase-encoding gene families encode enzymes with different biochemical properties and therefore they can have also different physiological functions for the fungus.

The pectinolytic enzymes of *A. niger* are important in many biotechnological processes, however, they are usually applied as ill-characterised enzyme mixtures. The isolation of the genes encoding these enzymes, the biochemical characterisation of the individual pectinolytic activities and their overproduction is important to design 'tailor-made' enzyme preparations containing only the essential enzymes to obtain a specific product.

The endopolygalacturonases-encoding gene family of *A. niger* contains seven members. Three of them (*pga*I, II and C) were previously characterised at the molecular level and recently a thorough kinetic study of the recombinant PGI, II and C was published [62]. In Chapters 2, 3 and 4 the isolation of the genes *pga*E, *pga*A and *pga*B, and *pga*D, encoding four new PGs from *A. niger* is described. To analyse each of the members of the family in detail, the studies include northern analysis of their expression on various carbon sources, overproduction of the individual enzymes using a strong glycolytic promoter, and their purification and biochemical characterisation. The differences in substrate specificity are addressed in kinetic studies using different polymeric (polygalacturonate and pectins) and oligomeric substrates. Based on the gene expression data and biochemical characterisation of the corresponding enzyme, the possible role of the individual PGs in the degradation of the complex pectin molecule and for the fungus is discussed.

The available pectinase-encoding genes and the *A. niger* mutant collection present in our laboratory, allowed for the first time to investigate pectinase gene expression in fungi in a comprehensive and extensive way (Chapter 5). In this pioneering study different carbon sources were tested in order to elucidate the molecular basis of the pectinase inducer(s). Furthermore, two pectinase promoters, *viz. pgx* (exopolygalacturonase) and *pel*A (pectin lyase A), were exploited for the construction of promoter-reporter gene plasmids in order to isolate pectinase regulatory mutants of *A. niger*. The *pel*A promoter was further used in a deletion study and for the development of a screening method to test properties of different inducers in a quick and effective way.

Apart from *A. niger* other black *Aspergilllus* isolates are being nowadays applied in the biotechnological processes, however prior to their use the taxonomical position must be firmly established. The last two chapters (Chapter 6 and 7) describe a reliable way to identify unknown black *Aspergillus* isolates using molecular and/or biochemical techniques. They are a follow-up of the initial study of Kusters-van Someren *et al.* [11], who recognised two separate species within the '*A. niger* aggregate', *A. niger and A. tubingensis*, and pointed out the possibility to include *A. tubingensis* on the list of species with an accepted status as an enzyme producer. The identification has been now extended for other isolates closely related to *A. niger*, *viz. A. phoenicis*, *A. foetidus* and *A. awamori* (Chapter 6). The uniseriate species of the black aspergilli were thoroughly examined using two molecular approaches (rDNA sequencing and RFLPs) and one biochemical technique (Chapter 7). The classification of the closely related *A. japonicus* and *A. aculeatus* taxa is also discussed in an evolutionary context.

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CHAPTER 2

*pga*E encodes a fourth member of the endopolygalacturonase gene family from *Aspergillus niger*

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Abstract

In the present study, the molecular and basic biochemical characterization of endopolygalacturonase E, the fourth *Aspergillus niger* N400 endopolygalacturonase, is reported. The entire endopolygalacturonase E gene consists of 1293 bp interrupted by three short introns (50, 50 and 59 bp, respectively) as concluded from the cDNA sequence. The deduced amino acid sequence comprises 378 residues that include 39 N-terminal amino acids of the prepropeptide. The calculated M_r and pI of the mature protein are 35 584 and 3.6, respectively.

Compared with the other endopolygalacturonases from *A. niger* N400, the mature protein endopolygalacturonase E has the highest sequence identity with endopolygalacturonase C (77.6 %) followed by endopolygalacturonase I (57.6 %) and endopolygalacturonase II (54.3 %).

For overproduction of endopolygalacturonase E an *A. niger* multicopy strain was used that was transformed with a promoter gene fusion construct that directs expression from the glycolytic *A. niger* pyruvate kinase promoter. The enzyme was purified and characterized as an endopolygalacturonase based on product analysis after polygalacturonate hydrolysis and on bond cleavage frequencies of oligogalacturonates of different degree of polymerization (n= 2-7). The pH optimum was 3.8. K_m and V_{max} for polygalacturonate hydrolysis were 2.5±0.4 mg . ml^{-1} and 1.33 ± 0.18 µkat . mg^{-1}, respectively. A subsite map was calculated by the combination of the methods of Suganuma *et al*. (J. Biochem., 84, 293-316 (1978)) and Nitta *et al*. (J. Biochem. 69, 567-576 (1971)). This indicated that the enzyme was composed of at least five subsites.

Introduction

Endopolygalacturonases (poly (1,4-α-D-galacturonide) glycanohydrolase, EC 3.2.1.15) are involved in the degradation of pectin, a heteropolysaccharide found in the middle lamella and the primary plant cell wall, by hydrolyzing the $\alpha-1,4$ glycosidic bonds between adjacent α-D galacturonic acid residues present in the main chain. The pectinolytic enzymes can have a different role in nature depending on the organism producing them. They can act as enzymes important for fruit ripening (Pilnik and Rombouts, 1980), or as virulence factors when produced by phytopathogenic microorganisms (Collmer and Keen, 1986; Crawford and Kolattukudy, 1987) or they can serve as enzymes involved in decay of plant tissue when produced by saprophytes.

The main potential of the pectinolytic enzymes produced by the saprophytic fungus *Aspergillus niger* is their wide application in the food and beverage industry, for instance in the preparation of juices. Application of individual pectinases or defined cocktails of pectinases, amongst which endo- and exo-polygalacturonases, methyl- and acetyl-esterases, pectin and pectate lyases and rhamnogalacturonan hydrolases and lyases are found, can enhance the controlled breakdown of pectin and may lead to new well-defined pectic substances and better control of processes involving pectinases. Therefore the investigation of new enzymes participating in pectin degradation may extend the classic industrial repertoire of pectinolytic enzymes but may also help to understand how they evolved different substrate specificity, especially in case of the known pectinase gene families.

Bussink *et al.* (1990, 1991, 1992b) demonstrated the presence of 7 different endopolygalacturonase (*pga*) genes in *A. niger* N400 of which three genes *pga*I, *pga*II and *pga*C have already been characterized. Here we report the molecular cloning and sequence of the fourth endopolygalacturonase-encoding gene, *pga*E, and the biochemical characterization of the corresponding enzyme.

Abbreviations:

pga - polygalacturonase-encoding gene; *pki*A, pyruvate kinase-encoding gene; *pki*A-*pga*E, promoter-gene fusion of *pki*A promoter and *pga*E gene; CREA - carbon catabolite repressing protein A,(Gal$p$A)$_n$, oligogalacturonate with degree of polymerization (n); HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection.

Materials and Methods

Strains, phages and plasmids. The *Aspergillus niger* strain NW156 (*csp*A1, *pyr*A6, *leu*-13, *prt*F28) which is derived from *A. niger* N400 (CBS 120.49) was used for transformation. Classes of *pga*II hybridizing recombinant phages were described by Bussink *et al.* (1992b). Phagemid pBluescriptSK$^+$ (Short *et al.*, 1988) was used for general cloning experiments. Plasmid pGEM-T (Promega Corporation) served for cloning of PCR fragments. *E.coli* DH5α F'[F'/*end*A1 *hsd*R17 (r$_K^-$ m$_K^+$) *sup*E44 *thi*-1 *rec*A1 *gyr*A (Na^r) *rel*A1 Δ(*lac*IZYA-*arg*F)U169 *deo*R (φ80d*lac*Δ(*1ac*Z)M15] (Woodcock et al., 1989) was used for cloning throughout. λ phages were propagated in *E. coli* LE392 [F' *e14*$^-$ (*Mcr*A$^-$) *hsd*R514 (r$_K^-$m$_K^+$) *sup*E44 *sup*F58 Δ(*lac*IZY)6 *gal*K2 *gal*T22 *met*B1 *trp*R55] (Sambrook *et al.*, 1989). Plasmid pGW1800 (Bussink *et al.*, 1990) containing the *A. niger pga*II gene was used for isolation of the 1.3-kb *Bam*HI-*Bgl*II fragment to be used as the *pga* probe. pGW635 contains the *A. niger pyr*A gene (Goosen *et al.*, 1989) encoding orotidine-5'-phosphate decarboxylase and was used as a selection marker (restoration of the uridine prototrophy) in a cotransformation of *A. niger* NW156.

Manipulation of DNA and RNA. Isolation of phage and plasmid DNA, isolation of RNA and other molecular manipulations were carried out essentially as described by Sambrook *et al.* (1989). *A. niger* chromosomal DNA was isolated according to de Graaff *et al.* (1988). Restriction enzymes were used as recommended by the supplier (GibcoBRL). The standard hybridization conditions as described by Sambrook *et al.* (1989) were used for Southern blots.

The nucleotide sequence was determined by the dideoxy-nucleotide chain-termination method (Sanger *et al.*, 1977) using the ^{T7}Sequencing^{TM} Kit (Pharmacia) as described in the protocol. Computer analysis was done using the program PC/Gene (IntelliGenetics Inc.) and the sequence analysis software package of the Genetics Computer Group Inc. (Madison Wisconsin).

The promoter gene fusion (*pki-pga*E) was made in plasmid pPROM-H containing the 0.7-kb upstream region of the *pki*A gene encoding the pyruvate kinase of *A. niger* (de Graaff *et al.*, 1992). This plasmid was constructed in the following way. A *Nsi*I linker (5'-TGCATGCATGCA-3', Stratagene) was introduced into the *Hinc*II site of plasmid pUC18 (Yanisch-Perron *et al.*, 1985) yielding plasmid pUC18HN. Next a 0.7-kb fragment containing the promoter sequence of the *pki*A gene was isolated from plasmid pPK-PLB (Kusters-van Someren *et al.*, 1992) by digesting with *Bam*HI and *Nsi*I and introduced into the *Bam*HI and *Nsi*I sites of pUC18HN. The *Nsi*I site is located in such a way that the ATG sequence of the recognition sequence corresponds to the start codon of the gene to be expressed. For the construction of the *pki-pga*E gene fusion, pUC18HN was digested with *Nsi*I and *Hind*III, which is localized downstream of *Nsi*I in the pUC18HN polylinker and ligated with the 146 bp *Nsi*I-*Xho*I PCR generated N-terminal *pga*E fragment (for the explanation see below) and the 1.5-kb *Xho*I-*Hind*III fragment (see Fig.1) containing the remainder of the *pga*E gene and downstream sequence yielding plasmid pIM3803.

Site directed mutagenesis and generation of cDNA. PCR was carried out using the GibcoBRL Taq DNA polymerase kit. For construction of the *pki*A-*pga*E promoter fusion two primers were designed: PRIMER 1 - a thirty residue 5' TTCAACATGCAT-ACTTCTAGCTCGGTGATC 3' located in position -6 to 23 of the *pga*E gene, which allows introduction of a *Nsi*I site at the start codon, and PRIMER 2 - an eighteen-residue 5' GGCACTGCCACGTCGGAC 3' that anneals at position 215 to 198 (see Fig. 2). 200 pmole of each primer were used. The PCR cycler was programmed as follows: denaturation for 5 min at 95 ^{o}C, 30 cycles with the following settings: 1 min

denaturation at 95 ^{o}C, 1 min annealing at 57 ^{o}C and 1 min extension at 72 ^{o}C. The final extension was performed for 5 min at 72 ^{o}C.

cDNA was generated essentially according to Gilliland *et al.* (1990). In the first step, a reverse transcriptase reaction using the reverse transcriptase from moloney murine leukemia virus and reaction buffers as described by the supplier (Gibco BRL) was performed on 5 μg of total RNA isolated from an *A. niger pki-pga*E transformant (639.44) using a specific 3' end primer. This PRIMER 3 is a twenty-two-residue 5' CGATAGTCCATGGTTGCAGAT 3', which is located in the 3' non-coding region (position 1414-1393, see Fig.2). In the second step, the reverse transcriptase reaction was used for PCR employing the second, 5'end specific primer (PRIMER 4). This is a twenty-residue 5' AGCTCGGTGATCGGCCTAAC 3' starting at position 12 of the *pga*E gene. The same PCR programme was used as described above with the annealing temperature set at 54 ^{o}C.

Transformation of *A. niger* and analysis of endopolygalacturonase E production. *A. niger* NW156 was transformed as described (Kusters-van Someren *et al.*, 1991), using 1 μg of pGW635 and 20 μg of cotransforming pIM3803. Individual colonies resulting from the transformation were replated to generate spores. Spores were harvested and used to inoculate minimal medium (Pontecorvo *et al.*, 1953), pH 6.0, containing 1 % (mass/vol.) fructose, 0.1 % (mass/vol.) yeast extract, 0.02 % (mass/vol.) leucine, and Vishniac's trace element solution (Vishniac and Santer, 1957) at 2 x 10^6 spores ml^{-1}. Cultivation was performed in 50 ml cultures in a Gallenkamp rotary shaker set at 250 revolutions per minute at 30 ^{o}C. Culture fluid samples were withdrawn after 24 h and 48 h of cultivation. Following removal of mycelium by filtration over a nylon membrane filtrates were analyzed for endopolygalacturonase E by SDS/PAGE.

Production and purification of endopolygalacturonase E. *pki-pga*E transformant 639.44, producing the highest amount of endopolygalacturonase E (see Results and Discussion section), was used for large-scale production of the enzyme. To minimize contaminating proteins, the yeast extract added was dialyzed prior to addition to the medium. For this, a 20 % (mass/vol.) yeast extract solution was dialyzed for 16 h at 4 ^{o}C against ten volumes of water. The dialysate served for medium preparation while the retentate was discarded. 15 1-l flasks containing 330 ml of the same medium as described above, except for 2 % (mass/vol.) fructose instead of 1 % (mass/vol.), were used to cultivate transformant 639.44. After 22 h of cultivation, the culture fluid was collected by filtration, diluted two-fold by the addition of water to reduce the ionic strength and adjusted to pH 6.0. Next, 100 g of pre-swollen DEAE Sephadex A-50 was added to the filtrate and allowed to bind endopolygalacturonase E under gentle stirring at 4 ^{o}C for 16 h. The matrix was recovered by filtration and subsequently endopolygalacturonase E was eluted by a pulse of 1.0 M NaCl in 10 mM piperazine/HCl, pH 6.0. Following extensive dialysis against 10 mM Piperazine/HCl, pH 6.0, the enzyme was loaded onto a DEAE-Sepharose Fast Flow column (2.6 cm × 20 cm) and eluted with a 1600-ml linear gradient from 0-1.0 M NaCl in the piperazine/HCl buffer. Fractions containing endopolygalacturonase E that eluted between 600 and 650 mM NaCl, were pooled and dialyzed extensively against 50 mM sodium acetate, pH 5.0. For storage at 4 ^{o}C sodium azide [0.02 % (mass/vol.)] was added.

Enzyme assay. Endopolygalacturonase activity was routinely assayed at 30 ^{o}C in 0.5 ml 50 mM sodium acetate, pH 4.2, using 0.25 % (mass/vol.) polygalacturonate (United States Biochemical Corp) as a substrate. Samples (50 μl) were taken at regular intervals and the reaction was stopped by pipetting the sample into 1.0 ml 0.5 M Na_2CO_3. The release of reducing sugars was determined

according to Stephens *et al.* (1974) using D-galacturonate as a standard. To determine the pH optimum, the 50 mM sodium acetate buffer was replaced by McIlvaine buffers. Determination of bond cleavage frequencies was performed using 0.50 mM (reduced) oligogalacturonates of different degree of polymerization (n = 2 - 8) and 50 μM oligogalacturonates of degree of polymerization n = 2 - 7 in 50 mM sodium acetate, pH 4.2 at 30 $^\circ$C. Reactions were stopped by the addition of an equal volume of 2.0 mM Tris, 50 mM NaOH which resulted in a final pH of 8.3-8.5. Stopping the reaction at a higher pH resulted in spontaneous breakdown products of the oligogalacturonates as shown in control experiments. For the determination of hydrolysis rates of (reduced) oligogalacturonates of various degree of polymerization, identical conditions were used as described above while samples were taken and stopped at regular intervals. To determine V_{max} and K_m values of endopolygalacturonase E for tetragalacturonate, the experimental setup was the same, but various concentrations of substrate (0.08-2.0 mM) were used. Analysis and quantitation of the reaction products were performed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (see below).

Analytical methods. The protein content of purified endopolygalacturonase E was estimated by spectrophotometry. The molar extinction coefficient, 67 800 M^{-1} . cm^{-1}, was calculated by the method of Edelhoch (1967) based on the Trp, Tyr and Cys content taken from the deduced primary sequence (Fig. 2). It should be noted that the enzyme was not unfolded in 6 M guanidinium chloride prior to absorbance determination, which may result in underestimation of the actual protein content. The purity of the enzyme preparation was monitored by SDS/PAGE. The gel was stained with Coomassie brilliant blue R250. The protein molecular mass was estimated by SDS/PAGE calibrated with protein test mixture 4 (Serva).

Oligogalacturonates were isolated as described by Kester and Visser (1990). Reduced oligogalacturonates were prepared according to the method of Omran et al. (1986). Quantitation of the oligomers was performed with the m-hydroxydiphenyl colorimetric assay for uronic acids according to Ahmem El Rayah and Labavitch (1977). Reaction products were analyzed on a Dionex BioLC high-performance chromatography system using a CarboPac PA-100 anion-exchange column (25 cm × 4 mm) equilibrated with 0.1 M NaOH, 0.15 M sodium acetate using pulsed amperometric detection with settings for potential and pulse sequence for carbohydrate detection as recommended by the manufacturer. The samples loaded were eluted isocratically for 5 min followed by a linear gradient of 0.15-0.75 M sodium acetate in 0.1 M NaOH at 1 ml/min in 20 min. Products were quantitated by using mixtures containing either oligogalacturonates or reduced oligogalacturonates with degree of polymerization from n = 2 - 8 at 0.10 mM each for calibration and by an internal standard of 0.10 mM glucuronic acid (eluting between monogalacturonate and digalacturonate) with 50 μl injections.

Results and Discussion

Isolation of the gene encoding endopolygalacturonase E. Bussink *et al.* (1992 b) have shown that apart from the three known *pga* genes (*pga*I, II and C), four additional *pga* genes were likely to be present in the *A. niger* N400 genome. This was suggested by Southern and western analysis. One of these potential genes, *pga*E, is located on a λ-clone designated λE6.

This clone was further analyzed here. For this, λE6 DNA was digested with several restriction enzymes and analyzed via Southern blots using a probe comprising most of the *pga*II coding sequence (data not shown). Based on these data, a partial restriction map was established which revealed that the *pga*E gene is located on a 3.0-kb *Eco*RI fragment (see Fig. 1). This fragment was subcloned into pBluescriptSK$^+$ in both orientations yielding plasmids pIM3801 and pIM3802, which were used for nucleotide sequence analysis.

Fig. 1. Localization of *pga*E within a 3.0-kb *Eco*RI genomic fragment. The *Xho* I-*Hind* III fragment containing the gene was used for the construction of the promoter-gene fusion as described in the Materials and Methods section.
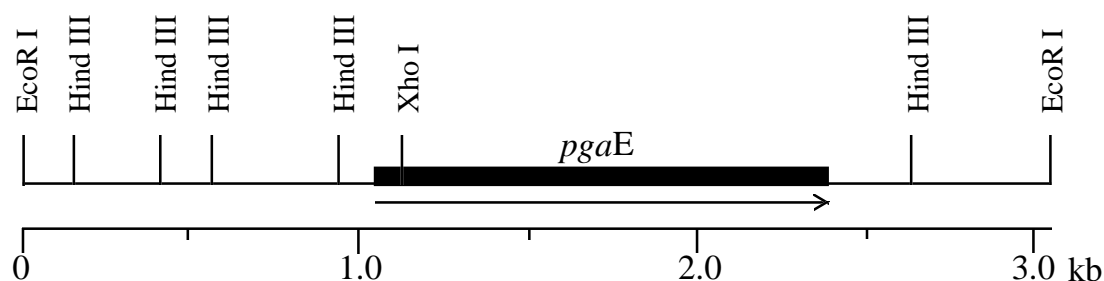
The nucleotide sequence of *pga*E. The entire 3.0-kb *Eco*RI fragment was sequenced over both strands (Fig. 2). After comparison of the sequence data with sequences in the gene and protein databases, the exact location of *pga*E, encoding a new polygalacturonase, was established. The 3.0-kb *Eco*RI fragment comprises 1045 bp of promoter region, 1293 bp of the *pga*E coding region, and 683 bp of 3'end non-coding region. Based on similarities with other polygalacturonase-encoding genes, the presence of three introns was predicted. To confirm these introns, the sequence of the cDNA of *pga*E was determined. For this, mRNA was isolated from an *A. niger* transformant containing a promoter-gene fusion of the strong glycolytic *pki*A promoter with *pga*E (see below). The mRNA isolated from one of these transformants grown on a medium with 2 % (mass/vol.) fructose as a sole source of carbon was used to generate *pga*E cDNA as described under Materials and Methods. Sequence analysis of the cDNA synthesized between the two primers (PRIMERS 3 and 4) demonstrated that the three predicted introns were correctly spliced. The nucleotide sequences of all three introns show the consensus 5' splice site GTRNGY and 3' splice site YAG (Unkles, 1992). Furthermore, sequences resembling the conserved lariat sequence, 5' NNCTRAY 3', which is

characteristic for other fungal introns, are also present with changes in position 5 and 7 of intron A and C, respectively (see Fig. 2). Analysis of the promoter region revealed a putative TATA box (TATAAAA) starting at position -98 bp from the translation start codon and two CAAT boxes (-105 CAAT and -118 CCAAT) further upstream. A pyrimidin-rich region usually thought to be involved in a correct start of transcription (Gurr *et al.*, 1987) is not present in the upstream sequence of the *pga*E gene. The nucleotides surrounding the translation start codon follow the rule of Kozak (1983): A is found in position -3 and the first triplet after the translation initiation codon starts with a purine.

Based on results of the *pga*II promoter deletion studies (Bussink *et al.*, 1992 a), a sequence 5' GACCGTTCATTGGTGGAACTAGC 3' resembling the upstream activation site of *CYC*1 gene of *Saccharomyces cerevisiae* was identified in the *pga*II promoter that appeared to be responsible for high level of gene expression. A similar, although shorter sequence (5' TYATTGGTGGA 3') was found in the promoter region of the *pga*I and *pga*C genes (Bussink *et al.*, 1992 b). This sequence, which is an extended version of the consensus sequence in yeast genes that are under control of the HAP2/3/4 system (Hahn, S. and Guarente, L., 1988; Bowman *et al.*, 1992), is not present in the promoter of *pga*E.

Because the expression of pectinolytic genes is repressed on media containing glucose, the *pga*E promoter was analyzed for the presence of the consensus sequence 5' SYGGRG 3'(Cubero and Scazzocchio, 1994), which represents the DNA binding domain for CREA. Six putative binding sites (see Fig. 2) were identified in the *pga*E promoter. In a recent study (Espeso and Peñalva, 1994), it was shown that binding of CREA is context independent only in case of the 5' GYGGGG 3' sequence. This motif is present once in the *pga*E promoter, at position -258 bp from the translation start codon. However, the exact role of the potential CREA binding sites in the regulation of *pga*E expression has to be established in future experiments.

A search for consensus sequences involved in termination of transcription in the 3' non-coding region revealed an ATAA sequence at position 283 bp from the translation stop codon. In higher eukaryotes, the consensus AATAAA sequence has been found before the poly(A) site, but in lower eukaryotes a similar sequence is often truncated or missing (Unkles, 1992).

Fig. 2. Nucleotide sequence of *pga*E of *A. niger* N400 and deduced amino acid sequence. Bold-face italicized hexanucleotide sequences in the promoter region highlight (in the opposite direction) the conserved hexanucleotide sequence 5' CCCTGA 3' found in other promoters of pectinolytic genes in *A. niger* (Bussink *et al.*, 1992 b). Single underlined nucleotides correspond to the 5' SYGGGG 3' CREA DNA binding domain. CAAT boxes are double underlined and the TATAAA box is in bold face. The processing sites in the prepro-sequence are indicated by >> for the putative signal peptidase cleavage site and with << for the experimentally determined dibasic cleavage site. Asn258, which is likely to be N- glycosylated, is also depicted (#). 5' and 3' splice sites and the lariat sequences within introns are underlined. In the 3'end non-coding region, the possible polyadenylation signal is marked by bold face lower typescript. The nucleotide sequence is deposited in the EMBL nucleotide sequence database under accession number Y14386.

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                                    gaattccccggatcaatcattgattgattaagggattgattgactgaataggaat  -991
ggcttgcttttgaacatggcttcccccatgatgtcaggcaacagaccagggcagtcatgtgccgttcatggggcagcacttgactgccat  -901
ggccaactaggtccgaaattaccatgcgatatctgctaagcttatgataatcagggtcagataatacaacccttggttggttgacctaa  -811
tcatcatacacgctttgattataaccaattttgctggtcacatggatgtctggctacccctgtgctgtctgggcttcaccacgagtattg  -721
accaagattggtcttctctgaataccctctgtctttgacaagccgtgcgcttccaataagtgcctccattgtaggagagatgttggacag  -631
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ataaccccagcatccgatcgccgaccccatcggacaatggagtcctcaggggcaacatactacagtgacggctgcctcaagtacagtgac  -361
cggtataatttcccaccactcatcgctgcacgccccgggtaaccggatgaggtttactgaaggcattgcgcgtcaggcacgggaaatata  -271
gtgtggcgctccgtggggagacagaggcacgcagaagtcctgcagaaactatcacattgtcccgcgcgctacggcagttcttccgccgga  -181
gctgcgaagtgccgtccacagaaagccgggagatattccaccacagagacagcagagatgccccaatgatacattcaatgggtataaaac  -91
gcacttattccgccagccaagaaagcccaagaggaaccaagacattgactaaaagcttgtctcaaaccttgatctgcgagtgtcttcaac  -1
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ATGGTGACTTCTAGCTCGGTGATCGGCCTAACGCTCTGGGCCGCACTGGTCAGTGCGAGCCCCGTTGCCGATCCGCTGGTGACCCCCGCC   90
  M  V  T  S  S  S  V  I  G  L  T  L  W  A  A  L  V  S  A  S  P  V  A  D  P  L  V  T  P  A      30
                                                                                    >>
CCGAAGCTCGAGGATCTGGAGAAGCGCGCCAACCTCCTGCACTTTCTCCGGCTCTGAGGGGGCCTCGTCTGCCAGCAAGTCCAAGACCTCG  180
  P  K  L  E  D  L  E  K  R  A  T  S  C  T  F  S  G  S  E  G  A  S  S  S  A  S  K  S  K  T  S    60
                     <<
TGCTCCACCATCGTGCTGTCCGACGTGGCAGTGCCCTCAGGCACAACGTTGGATCTGACCGATCTGAACGACGGGACTCACgtaagttac   270
  C  S  T  I  V  L  S  D  V  A  V  P  S  G  T  T  L  D  L  T  D  L  N  D  G  T  H                  87
tgcgcctcgacgcgcgattcttcgcatctcacaatgaacagGTCATCTTCGAAGGCGAAACCCACTTCGGTTACGAGGAATGGAGCGGAC   360
         intron A                          V  I  F  E  G  E  T  H  F  G  Y  E  E  W  S  G      103
CTCTTGTCTCAGTCTCCGGAACTGACATCACCGTCACCGGGGCCGACGGCGCCTATCTCAACGGTGACGGCAGTCGTTGGTGGGATGGCG   450
  P  L  V  S  V  S  G  T  D  I  T  V  T  G  A  D  G  A  Y  L  N  G  D  G  S  R  W  W  D  G       133
AGGGCAGCAATGGCGGCAAGACGAAGCCCAAGTTCTTCTACGCCCATGATCTGACCTCGTCCACGATCAGCGGTATCTACATCCAGAACT   540
  E  G  S  N  G  G  K  T  K  P  K  F  F  Y  A  H  D  L  T  S  S  T  I  S  G  I  Y  I  Q  N       163
CGCCGGTGCAGGTGTTCAGCATTGACGGGTCGACGTATCTTACTATGGAGGACATCACCATTGACAACACGGATGGCGATGATGGCGAGG   630
  S  P  V  Q  V  F  S  I  D  G  S  T  Y  L  T  M  E  D  I  T  I  D  N  T  D  G  D  D  G  E       193
CGGCCAACACCGATGGATTTGACATTGGCGATAGCACGTACATTACCATCACGGGCGCCAATGTATACAACCAAGATGACTGTGTGGCGG   720
  A  A  N  T  D  G  F  D  I  G  D  S  T  Y  I  T  I  T  G  A  N  V  Y  N  Q  D  D  C  V  A       223
TGAACTCGGGCGAGgtgggtagaccgtcctttaagctacggatgtggacactgatgggaaccagAACATTTACTTTTCGGGCGGCGTCTG   810
  V  N  S  G  E            intron B                                N  I  Y  F  S  G  G  V  C     237
CTCTGGTGGCCACGGGCTGTCGATCGGTTCCGTTGGCGGTCGGAGTGACAACACCGTGAAGAACGTGACCTTCTACGATTCGGATATCAA   900
  S  G  G  H  G  L  S  I  G  S  V  G  G  R  S  D  N  T  V  K  N  V  T  F  Y  D  S  D  I  K       267
                                                                 #
GAGCTCTCAGAACGgtaagttatattccgtcaaggttgtagttcggtgcgatcacatgctaagcggcgtatagGAGTCCGCATCAAGACC   990
  S  S  Q  N                    intron C                                    G  V  R  I  K  T     277
ATCTACGGCGACACTGGGTCGGTCAGTGAGGTCACCTACAAGGAGATCACCCTGTCCGATATCACCGACTATGGCATTGTGGTGGAGCAG  1080
  I  Y  G  D  T  G  S  V  S  E  V  T  Y  K  E  I  T  L  S  D  I  T  D  Y  G  I  V  V  E  Q       307
AACTATGACGATACGAGCGAGTCGCCGACCGATGGAATCACCATCGAAGACTTTGTCCTGGACAATGTGCAGGGCAGTGTGGAGAGCTCG  1170
  N  Y  D  D  T  S  E  S  P  T  D  G  I  T  I  E  D  F  V  L  D  N  V  Q  G  S  V  E  S  S       337
GGCACCAACATCTACATCGTCTGCGGATCAGACAGCTGCACGGACTGGACTTGACGGATGTGGATGTGACTGGAGGGAAGACCAGCTCC  1260
  G  T  N  I  Y  I  V  C  G  S  D  S  C  T  D  W  T  W  T  D  V  D  V  T  G  G  K  T  S  S       367
GACTGTGAGAATGTGCCGGACGATATCAGCTGTTAGcagctgggcaggactatggggggtaacaagctgtattatttgagagtattgttga  1350
  D  C  E  N  V  P  D  D  I  S  C  *                                                             378
gagaagagtggattgagttgactaggagacaatgtaggtttttgatctgcaaccatggactatcgttctttgtgtctcaatggatagcagt  1440
cgatcattgatgaatgctgctatgatcatcactgatagtcaaattgttccattgtaaaggttctactttacctcggcccactgagtaccc   1530
acagagccctcgtaggcagggaaccattctccctgtttcccctttgagataagctttcactgattggtgggaatataagccttgggggttg  1620
cagacagaggccgcacgccacttctacaacagctcctcaacgacgcaacaaagacacaggacatggatatatacagatctctcaatgag   1710
tacttccactaaaattaagtccctccgatttccaatacaatgaggaaccggcagagtactgaaatcccgtcataaccttgtctgttcatt   1800
gcatgatcccaacaacccaattaggctttacacttccaggtctgcaaaccctgtctgggcagactcttggccagcttaagatttccagtt   1890
ccctgctataggacaggcctgccaacagcgtgttccaaaggttgacggtcacaccacatgtcgaaccatattccagagccgaattc       1976
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Deduced protein sequence of endopolygalacturonase E and comparison with other poly-galacturonases. Based on a comparison of the derived amino acid sequence from *pga*E with sequences of the other fungal polygalacturonases it is assumed that endopolygalacturonase E is synthesized as a preproprotein with a 39-amino-acid extension.

For the other polygalacturonases from *A. niger* (Bussink *et al.*, 1990; 1991; 1992 b), a two-step maturation was suggested: cleavage at the von Heijne (1986) peptidase cleavage site followed by monobasic endopeptidase processing (endopolygalacturonase II) or dibasic endopeptidase processing (endopolygalacturonase I, C) in analogy to the Kex2 protease of *S. cerevisiae* (Calmels *et al.*, 1991) as evidenced by peptide sequencing. Likewise, endopolygalacturonase E contains a signal peptidase site between Ala19 and Ser20 and a dibasic cleavage site after Lys38 and Arg39 suggesting that the mature enzyme starts at position 40. This was fully confirmed by sequencing 20 N-terminal amino acid residues of the purified mature endopolygalacturonase E, a sequence that starts with A T S C T F (compare Fig. 2).

The deduced amino acid sequence of the mature enzyme results in a calculated molecular mass of 35 584 Da and a calculated isoelectric point of 3.6. Screening of the amino acid sequence of endopolygalacturonase E for N-glycosylation sites (Asn-Xaa-Thr/Ser) revealed Asn258 as the only possible candidate (see Fig. 2). This residue is conserved in all four polygalacturonases from *A. niger*. Only in endopolygalacturonase I is an additional N-glycosylation site found in the C-terminal part of the protein (Asn299).

A comparison of three endopolygalacturonases (endopolygalacturonase I, endopolygalacturonase II, endopolygalacturonase C) produced by *A. niger* N400 was reported by Visser *et al.* (1994). Based on the deduced amino acid sequence endopolygalacturonase E shows highest similarity (77.6 %) with endopolygalacturonase C followed by endopolygalacturonase I (57.6 %) and endopolygalacturonase II (54.3 %).

Expression of endopolygalacturonase E. Under the growth conditions applied until now, batch cultures with orange pectin, sugar beet pectin, polygalacturonate and galacturonate, no *pga*E-specific mRNA could be detected in *A. niger* N400, neither by northern analysis nor by RT-PCR. However, the conservation of transcriptional elements, the correct splicing when the gene is transcribed under the control of a strong promoter and the fact that the enzyme is active, although with low specific activity with the substrate used (see below), do not support the view that *pga*E should be considered as a pseudogene. The failure to detect specific low-

abundancy mRNA of complex systems has been noted before, for instance in ligninolytic system of *Phanerochaete chrysosporium* (Broda *et al.*, 1996).

Overproduction of endopolygalacturonase E. For isolation of endopolygalacturonase E a *pki*A-*pga*E construct was made and used for transformation of *A. niger* NW156 as described in the Material and Methods section. The *pki* promoter allows to produce the specific pectinase by cultivation on media with glucose or another glycolytic carbon source that represses expression of other pectinolytic genes (Maldonado *et al.*, 1989). A similar approach has previously been successfully used for the overproduction of other pectinolytic enzymes, *viz.* pectin lyase B from *A. niger* (Kusters-van Someren *et al.*, 1992) and exo-polygalacturonase from *A. tubingensis* (Kester *et al.*, 1996).

Out of fourteen *pki-pga*E transformants, 30 μl culture fluid samples taken after 24 h and 48 h of cultivation were analyzed by SDS/PAGE for production of endopolygalacturo-nase E. At both time points, several transformants showed a band of approximately 60 kDa migrating slightly slower than endopolygalacturonase I (data not shown). This observation is not in agreement with the calculated molecular mass. A similar effect has been described also for endopolygalacturonase I and for endopolygalacturonase C (Bussink *et al.*, 1991; 1992 b) and is probably caused by O- and/or N-linked glycosylation of the enzyme as was shown for endopolygalacturonase C by the effects of both NaOH and endoF treatment (Kester, H. C. M., unpublished results) and for endopolygalacturonase I by glycosylation analysis (Bergmann, C., personal communication). No bands were visible in the lane that contained a control sample from the host strain itself, *viz. A. niger* NW 156.

One of the *pki-pga*E transformants, *A. niger* 639.44, producing the highest amount of the 60 kDa band was used for purification of the enzyme. Since there was no further increase in the amount of endopolygalacturonase E produced after 24 h of growth under the conditions chosen (see Materials and Methods section), large scale cultivation was performed for 22 h. Approximately 200 mg endopolygalacturonase E were obtained from 5 l culture fluid by a simple two-step chromatography purification.

Characterization of endopolygalacturonase E. In a standard assay, performed at pH 4.2, endopolygalacturonase E has low specific activity, 500 nkat . mg^{-1}, as compared to 8.8 μkat . mg^{-1} and 45.8 μkat . mg^{-1} for endopolygalacturonase I and II, respectively (Kester and Visser, 1990). Therefore, both the pH optimum and temperature optimum were determined. From

Table 1, it is clear that the pH optimum is only slightly more acidic (0.3 pH units) than used in the standard assay.

Table 1. Temperature and pH optimum of endopolygalacturonase E. The temperature optimum was determined in 50 mM sodium acetate, pH 4.2, using 0.25 % (mass/vol.) polygalacturonate. The pH optimum was determined in McIlvaine buffers using 0.25 % (mass/vol.) polygalacturonate at 30 °C.

| Temperature	Endopolygalacturonas E activity	pH	Endopolygalacturonase E activity
(°C)	nkat. mg^{-1}		nkat. mg^{-1}
30	500	2.35	318
46	1264	2.71	404
60	2117	3.64	605
65	1740	3.88	637
		4.11	538
		4.30	358
		4.63	117
		4.89	0

Despite the fact that the enzyme is almost fourfold as active at 60 ^{o}C as at 30 ^{o}C, the activity is still quite low. Since the standard assay conditions are performed at 0.25 % (mass/vol.) polygalacturonate, a concentration which is ten times higher than the K_m values for endopolygalacturonase I and II (Kester, H. C. M., unpublished results), the K_m and V_{max} for endopolygalacturonase E were determined. At pH 4.2 and 30 ^{o}C, K_m and V_{max} values for polygalacturonate hydrolysis are 2.5 ± 0.4 mg . ml^{-1} and 1.33 ± 0.18 μkat . mg^{-1}, respectively. These results show that polygalacturonate is a poor substrate for endopolygalacturonase E when compared to endopolygalacturonases I and II (Kester and Visser, 1990).

Mode of action of endopolygalacturonase E. To clarify whether endopolygalacturonase E is an endo- or exo-acting enzyme, the products released upon hydrolysis of the polymeric substrate were followed in a time-course experiment by HPAEC-PAD analysis. In the case of an exo-type of mechanism, only monomers or dimers will accumulate in time. In the case of an endo-type of reaction, the polymeric chain will be attacked randomly, which results in a transient accumulation of larger oligomers that are converted to smaller oligomers upon

prolonged reaction. Fig. 3 shows that during initial stages of hydrolysis (up to 1 h) mainly oligogalacturonates with a higher degree of polymerization are formed, which are then gradually converted into lower oligomers resulting in mono-, di- and trigalacturonate with a strong excess of monogalacturonate after 24 h of incubation (data not shown). These results clearly demonstrate that endopolygalacturonase E is an endo-acting enzyme.

Fig. 3. Time course of products formed by endopolygalacturonase E acting on polygalacturonate. Compilation of products produced during the first 4 h of hydrolysis of 1 % (mass/vol.) polygalacturonate in 1 ml 50 mM sodium acetate, pH 4.2, at 30 °C; 50 µl samples were analyzed by HPAEC-PAD. 36 µg of endopolygalacturonase E were incubated in 0.55 ml 0.25 % (mass/vol.) polygalacturonate, 50 mM sodium acetate, pH 4.2, at 30 °C. G1, $(GalpA)_1$; G2, $(GalpA)_2$; G3, $(GalpA)_3$; G4, $(GalpA)_4$; G5, $(GalpA)_5$; G6-G8, $(GalpA)_{6-8}$.
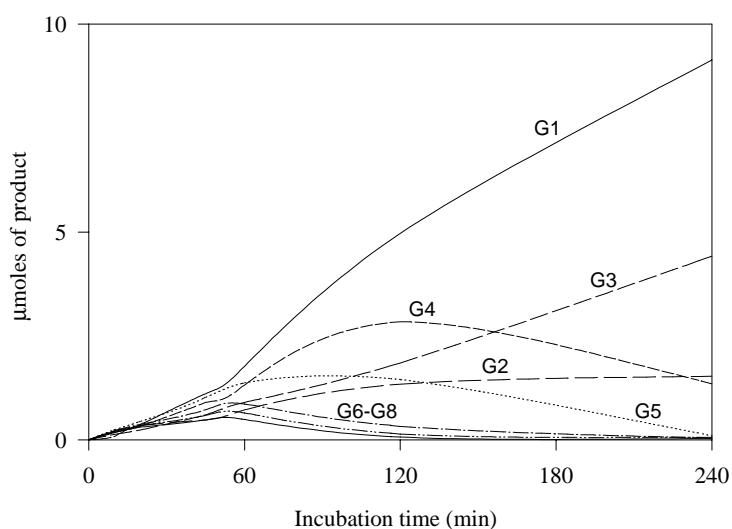
To understand the nature of the distribution of the final products, mono-, di- and trigalacturonate, the mode of action of endopolygalacturonase E on oligogalacturonates of defined chain length was investigated. We also included $NaBH_4$-reduced oligogalacturonates of similar length to elucidate the binding mode of the substrates with respect to the active site. In Table 2, the data of the mode of action study are compiled as the bond cleavage frequencies of reduced oligogalacturonates (0.5 mM) by endopolygalacturonase E. To minimize the potential risk of taking secondary cleavage products into account for determination of bond cleavage frequencies, which would lead to erroneous ratios, only data corresponding to less than 5 % hydrolysis and showing linear progression were considered. Also, both hydrolysis products from a particular binding mode were quantitated, and evaluated for being equal in

amount, except for reduced (GalA*p*)$_1$, the reduced monomer. In the latter case, only the non-reduced oligomer was considered. By following this procedure, only first generation products were analyzed. In addition, it should be noted here that endopolygalacturonase E is an inverting hydrolase (Sinnott, 1990) as it belongs to the same family 28 of hydrolases as endopolygalacturonases I and II and exopolygalacturonase (Henrissat, 1991), which were shown to act by inversion of configuration by Biely *et al.* (1996) excluding transglycosilation or condensation.

The smallest reduced oligogalacturonate hydrolyzed by endopolygalacturonase E is reduced (Gal*p*A)$_4$, which is cleaved into (Gal*p*A)$_3$ and reduced (Gal*p*A)$_1$ and in (Gal*p*A)$_2$ and reduced (Gal*p*A)$_2$ in almost equal ratio. The rate of the hydrolysis is very slow. For reduced (Gal*p*A)$_5$, the hydrolysis is much faster and results in formation of a very small amount of (Gal*p*A)$_4$ [and reduced (Gal*p*A)$_1$] and to an almost complete conversion in (Gal*p*A)$_3$ and reduced (Gal*p*A)$_2$ (96 %). Hydrolysis of reduced (Gal*p*A)$_6$ and reduced (GalA*p*)$_7$ is again faster then hydrolysis of reduced (Gal*p*A)$_5$. The main reduced product formed from reduced (Gal*p*A)$_6$ and reduced (Gal*p*A)$_7$ is still reduced (Gal*p*A)$_2$, but from reduce (Gal*p*A)$_8$ also reduced (Gal*p*A)$_3$ and reduced (Gal*p*A)$_4$ arise. Taken all these data together, it is clear that binding of reduced oligogalacturonates up to $n = 8$ is such that hydrolysis takes place at the reduced end. Therefore, it is concluded that the hydrolysis of non-reduced oligogalacturonates also takes place from the reducing end. The corresponding bond cleavage frequencies at 0.50 mM oligomer concentration are listed in Table 2. (Gal*p*A)$_3$ and (Gal*p*A)$_4$ are exclusively hydrolyzed at the first glycosidic linkage from the reducing end. Oligomers (Gal*p*A)$_5$ to (Gal*p*A)$_7$ are also hydrolyzed mainly at the first glycosidic linkage from the reducing end and to a small extent at the second and third glycosidic bond with increasing efficiency as the degree of polymerization increases from $n = 5$ to 7. Data for (Gal*p*A)$_8$ could not be obtained by this method because the contribution of secondary cleavage products was significant in this case. Inspection of preferred cleavage of all non-reduced oligogalacturonates tested reveals that formation of monomers is favoured over formation of dimers. As (Gal*p*A)$_3$ is also a substrate for endopolygalacturonase E the final products to be expected from polygalacturonate hydrolysis are monomers and dimers with a large excess of monomers. This is reflected in the progression curves (Fig. 3) although a considerable amount of (Gal*p*A)$_3$ is present. After 24 h of incubation, (Gal*p*A)$_3$ is converted into (Gal*p*A)$_1$ and (Gal*p*A)$_2$ (data not shown).

Table 2. Bond cleavage frequencies, hydrolysis rates and k_0/K_m values recalculated to 1 M endopolygalacturonase E acting on (reduced) oligogalacturonates of defined length. Assay conditions are as follows: 500 μM (reduced) oligogalacturonates or 50 μM oligogalacturonates were incubated with endopolygalacturonase E in 0.5 ml 50 mM sodium acetate, pH 4.2 At timed intervals, 50-μl aliqouts were withdrawn and mixed with 50-μl stopmix to raise the pH to 8.3-8.5. Products were analyzed and quantitated by HPAEC-PAD as described in the Materials and Methods section. The asterisk indicates the reduced end (alditol). Non-reduced oligogalacturonates at 500 μM were used to calculate rates and non-reduced oligogalacturonates at 50 μM were used to calculate k_0/K_m values. The boldface typescript indicates the reducing end. Bond cleavage frequencies are given in percentages.

n																rate ($nkat \cdot mg^{-1}$)	$k_0/K_m \times 10^{-6}$ ($min^{-1} \cdot M^{-1}$)
3											**G**	-	**G**	100	**G**	1.3	
4									**G**	-	**G**	-	**G**	100	**G**	121.7	
5							**G**	-	**G**	-	**G**	6	**G**	94	**G**	220.0	
6					**G**	-	**G**	-	**G**	3	**G**	17	**G**	80	**G**	293.4	
7			**G**	-	**G**	-	**G**	-	**G**	20	**G**	30	**G**	50	**G**	353.4	
3											**G**	-	**G**	100	**G**		4.5×10^{-3}
4									**G**	-	**G**	-	**G**	100	**G**		1.3
5							**G**	-	**G**	-	**G**	4	**G**	96	**G**		7.0
6						-	**G**	-	**G**	4	**G**	14	**G**	82	**G**		4.9
7			**G**	-	**G**	-	**G**	-	**G**	22	**G**	26	**G**	52	**G**		5.6
4									**G**	-	**G**	46	**G**	54	**G***		
5							**G**	-	**G**	-	**G**	96	**G**	4	**G***		
6					**G**	-	**G**	-	**G**	11	**G**	89	**G**	-	**G***		
7			**G**	-	**G**	-	**G**	6	**G**	34	**G**	59	**G**	-	**G***		
8	**G**	-	**G**	-	**G**	-	**G**	26	**G**	37	**G**	37	**G**	-	**G***		

The data presented for oligogalacturonate hydrolysis suggest that endopolygalacturonase E is an exopolygalacturonase, especially for $n < 6$. However, Fig. 3 and the cleavage frequencies of oligogalacturonates for $n > 5$ clearly show that this enzyme is an endopolygalacturonase. The exolytic character of the enzyme may be caused by the substrates used, which may not be the natural substrates of this enzyme (see below).

Calculation of the subsite map for endopolygalacturonase E. The overall hydrolysis of the non-reduced oligogalacturonates proceeded faster than of the reduced counterparts. Also, an increase in hydrolysis rate was observed for both type of substrates with increasing degree of polymerization. The increase of the reaction rate with increasing chain length is a common feature for glycosidases with multiple subsites for binding of the substrate.

Two methods have been devised in the past to assess the number and the binding energies of the individual subsites (Thoma *et al.*, 1971; Hiromi *et al.*, 1973). More recently, Suganuma *et al.* (1978) presented a simplified version of the theory that has made it possible to calculate subsite maps for endo-acting enzymes from bond cleavage frequencies and the parameter k_0/K_m in which k_0 equals V_{max}/e_0 (e_0 is the initial enzyme concentration). The parameter k_0/K_m can be obtained from the slope of a plot of $\ln S_0/S_t$ vs time in which S_0 is the initial substrate concentration and S_t, the substrate concentration at time (t) and can be used to calculate the affinity of a subsite A_{i+1} for the substrate according to the following equation (Suganuma *et al.*, 1978):

$$A_{i+1} = RT \ \ln((k_0/K_m)_{n+1} e_0 \times (P_{i+1}/\sum_{i+1}^{n} P_{i+1}) / (k_0/K_m)_n e_0 \times (P_i/\sum_{i}^{n} P_i)) \ (1)$$

For endopolygalacturonase E, k_0/K_m values recalculated to 1 M of enzyme were calculated from the slopes in $\ln S_0/S_t$ versus time plots for oligomers ranging from degree of polymerization $n = 3$ - 7 at 50 µM S_0 each (Table 2). The $\ln S_0/S_t$ versus time plots were linear over the complete period (up to 80 % conversion) for all oligomers used. Due to the low product concentrations at the substrate concentration used, which hampers accurate quantitation, product ratios were determined at 40-50 % conversion of the substrate whereby secondary cleavage events were corrected for based on the stochiometry of product pairs (Table 2). Comparison of the cleavage ratios obtained at 500 µM and 50 µM reveals only minor changes. The 50 µM S_0 used for each oligomer is most likely well below K_m for each oligomer since for (Gal*p*A)$_4$ K_m is 0.46 mM (see below) while for the higher oligomers (Gal*p*A)$_6$ and (Gal*p*A)$_7$, covering probably all subsites, the hydrolysis rate at 500 µM (293.4 nkat . mg^{-1} and 353.4 nkat . mg^{-1}, respectively) is still well below V_{max} (1330 nkat . mg^{-1}) on polymeric substrate indicating that the 500 µM used is by far not saturating making the data obtained at 50 µM suitable for the calculation of a subsite map according to Eqn (1).

In Fig. 4, the subsite map is presented as a histogram of subsite affinities. Since the method applied does not allow calculation of the binding affinity of the subsites covered in the productive mode of the smallest oligomer hydrolyzed, *in casu* (Gal*p*A)$_3$ covering subsites -2, -1, and +1, the total affinity of these three subsites was calculated from V_{max} and the K_m for (Gal*p*A)$_4$ which has only one productive binding mode (V_{max} = 302 nkat mg^{-1} and K_m = 0.46 mM, see Table 2) and the intrinsic rate constant k_{int} (47.5 s^{-1}) obtained from the V_{max} value for polymeric substrate (covering all sites), according to $\Delta G = -RT\ln K_p + 10$ kJ (Nitta et al., 1971) in which $K_p = V_{max}$ (GalA*p*)$_4$/ K_m (GalA*p*)$_4$ (e_0)/k_{int} and +10 kJ . mol^{-1} to correct for the free energy of mixing. The value thus obtained, -15.5 kJ . mol^{-1}, was corrected for the contribution of subsite -3 (A$_{-3}$ = 14.4 kJ . mol^{-1}) as obtained previously (see Fig. 4) since (GalA*p*)$_4$ also covers subsite -3, yielding the affinity for subsites -2, -1 and +1: A$_{-2,-1,1}$ = 1.1 kJ . mol^{-1}. Thus, subsites which accommodate (Gal*p*A)$_3$ in the productive mode have apparently a low affinity for this substrate, which may reflect the very slow rate of hydrolysis of this substrate and also the strong increase in hydrolysis rate of (Gal*p*A)$_4$ (two orders of magnitude) when subsite -3 (A$_{-3}$ = 14.4 kJ . mol^{-1}) is also covered.

Fig. 4. Subsite map of endopolygalacturonase E depicting binding affinities of galacturonate residues to individual subsites. Subsite affinities were calculated as described in the text using data from Table 2.
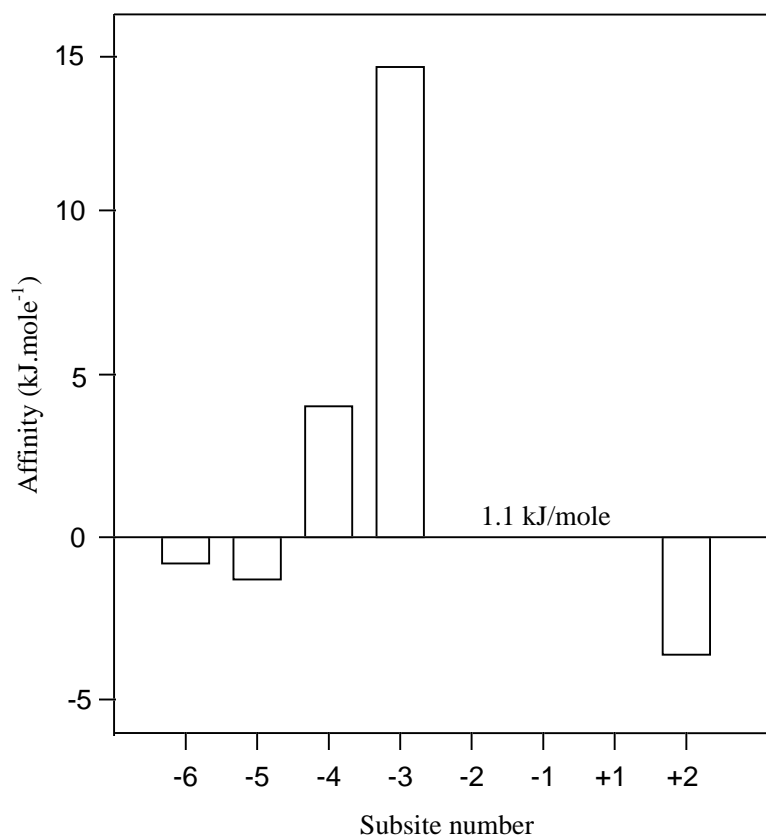
The affinity histogram reveals that effective binding for oligogalacturonates occurs at least from subsite -4 to +1, which suggests that there are five subsites on the enzyme. The low affinity at subsite -5 is likely too small to constitute a significant binding. It should, however, be pointed out that polygalacturonate may not be the true substrate of this enzyme and that possibly more subsites are involved in the binding of the preferred substrate, which is as yet unknown.

An indication that polygalacturonase E has preference for another substrate than polygalacturonates is given by the following. As can be inferred from Table 2, endopolygalacturonase E is able to hydrolyze reduced (Gal*p*A)$_4$ and reduced (Gal*p*A)$_5$ at the first glycosidic linkage counting from the reduced end, albeit at a slow rate. As the first residue is an alditol, which has a completely open structure, there is apparently no need for an intact galacturonate ring structure for reduced (Gal*p*A)$_4$ and reduced (Gal*p*A)$_5$ to bind at subsite +1 or, in other words, subsite +1 might require another sugar moiety than galacturonate or a substituted galacturonate moiety. This phenomenon may also explain the slow overall hydrolysis rate of polygalacturonate and the relatively high K_m for this substrate.

Kester and Visser (1990) reported the isolation of six different polygalacturonases from a commercial *A. niger* pectinase preparation K2B 078 (Rapidase, Gist Brocades) one of which appeared to be an exopolygalacturonase, whereas the other five were endopolygalacturonases, endopolygalacturonase I, II, IIIA, IIIB, and IV. The endopolygalacturonases were characterized with respect to specific activity and mode of action on oligogalacturonates (*n* = 2-7). Enzymes I and II, constituting the bulk of endopolygalacturonases present showed highest activity on polygalacturonate and were designated major endopolygalacturonases while the remaining endopolygalacturonases were designated the minor endopolygalacturonases. Comparison of the data described for the minor enzymes with those reported here for endopolygalacturonase E clearly shows that endopolygalacturonase E is not one of minor endopolygalacturonases.

Taking for endopolygalacturonase E into account that (a) K_m and V_{max} values for polygalacturonate show that this is a poor substrate, (b) subsite +1 can accommodate an alditol, (c) the total affinity of subsites -2 to +1 is small, and (d) the enzyme has not been detected in a pectinase preparation, it is strongly suggested that, despite the high sequence identity to endopolygalacturonases I and II that prefer polygalacturonate as a substrate, endopolygalacturonase E is active toward an unidentified part of the pectin molecule and is expressed at very low levels.

Currently, experiments are in progress using more complex substrates to identify the natural substrate for endopolygalacturonase E.

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CHAPTER 3

*pga*A and *pga*B encode two constitutively expressed endopolygalacturonases of *Aspergillus niger*

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Abstract

*pga*A and *pga*B, two genes encoding endopolygalacturonases (PGs, EC 3.2.1.15) A and B, were isolated from a phage genomic library of *Aspergillus niger* N400. The 1167 bp protein coding region of the *pga*A gene is interrupted by one intron, whereas the 1234 bp coding region of the *pga*B gene contains two introns. The corresponding proteins, PGA and PGB, consist of 370 and 362 amino acid residues respectively.

Northern-blot analysis revealed that *pga*A and *pga*B-specific mRNA accumulate in mycelia grown on sucrose. mRNAs are also present upon transfer to media containing D-galacturonic acid and pectin.

Recombinant PGA and PGB were characterized with respect to pH optimum, activity on polygalacturonic acid, and mode of action and kinetics on oligogalacturonates of different chain length (n = 3-7). At their pH optimum the specific activities in a standard assay for PGA (pH 4.2) and PGB (pH 5.0) were 16.5 μkat.mg^{-1} and 8.3 μkat.mg^{-1} respectively. Product progression analysis, using polygalacturonate as a substrate, revealed a random cleavage pattern for both enzymes and indicated processive behaviour for PGA. This result was confirmed by analysis of the mode of action using oligogalacturonates. Processivity was observed when the degree of polymerization of the substrate exceeded 6. Using pectins of various degrees of esterification it was shown that PGA and PGB both prefer partially methylated substrates.

Introduction

Aspergillus niger is a saprophytic micro-organism producing a vast number of extracellular enzymes involved in degradation of plant cell-wall material. Pectin is one of the major heteropolysaccharides found in the middle lamella and the primary plant cell wall, and has an important function in the structural organization of other cell-wall components, such as cellulose and hemicellulose [1].

A. niger enzymes degrading the homogalacturonan part of the pectin molecule include several pectin lyases [2-4], a single pectate lyase (J.A.E Benen, H.C.M Kester, L. Pařenicová and J.Visser, unpublished work), pectin methylesterase [5] and a family of endopolyga-lacturonases (EC 3.2.1.15) (PGs) [6-9].

At present, more than 30 different fungal *pga* sequences have been deposited in the Databases, the majority of which have been determined for genes from phytopathogenic micro-organisms. As in *A. niger* [8], PG-encoding gene families were discovered in the phytopathogenic fungi *Sclerotinia sclerotiorum* [10] and *Botrytis cinerea* [11]. However, little is known about the role of the different PGs in these micro-organisms. Only recently, studies on *pga* gene expression in the phytopathogenic fungi *Colletotrichum lindemuthianum* [12] and *A. flavus* [13], and the analysis of secreted PG isoforms during the growth of *S. sclerotiorum* on polygalacturonic acid [14] showed differential expression of these genes and secretion of the proteins. These results suggest that, although fungal PGs often show a high degree of sequence identity, their physiological function is different.

In order to understand the nature of the individual *A. niger* PG-encoding genes and their corresponding enzymes, we decided to analyse all of these, in detail, at the molecular and biochemical level. The *A. niger pga* gene family consists of seven different genes [8]. To date, the genes *pga*I [7], *pga*II [6], *pga*C [8] and *pga*E [9] as well as the corresponding enzymes [9,15] have been characterized. In the present study, two novel *pga* genes of *A. niger*, *pga*A and *pga*B, and the two enzymes they encode are described.

Abbreviations:

CREA - carbon catabolite repressing protein A; $(GalpA)_n$, oligogalacturonate with n degrees of polymerization; PG, endopolygalacturonase; BCF, bond-cleavage frequency; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection.

EXPERIMENTAL

Strains. For induction studies, the wild type laboratory strain *A. niger* N400 (= CBS 120.49) was used. The *A. niger* strain NW188 (*csp*A1, *pyr*A6, *leu*A1, *prt*F28, *gox*C17), which is derived from *A. niger* N400, was used for transformation of expression plasmids. The *A. niger* multicopy transformants, 617.42-1 and 617.49-15 of pIM3763 and pIM3773 (for a description see below), were used for overproduction and purification of PGA and PGB respectively. For cloning and propagation of plasmids and phages, *Escherichia coli* DH5α [16] and *E. coli* LE392 [17], were used.

Phages and plasmids. Isolation of recombinant *pga*II phages was described by Bussink *et al.* [8]. The phagemid pBluescriptSK⁺ [18] and plasmids pUC18 [19], pGEM-7 (Promega, Madison, WI, U.S.A.) and pEMBL19 [20] were used for subcloning of chromosomal DNA fragments or the construction of expression plasmids. The pGEM-T Easy kit (Promega) was used for cloning of PCR fragments.

Plasmid pGW635 carrying the *pyr*A gene which encodes orotidine-5`-phosphate decarboxylase from *A. niger* [21], served as a selection marker for restoration of uridine prototrophy. pGW1800 [6], encoding the *pga*II gene, and pIM2130 [22], containing a part of *A. niger* 18S gene were used for isolation of fragments for probes.

The plasmids pIM3760, pIM3761, pIM3762 and pIM3770 (Figure 1) carry the originally subcloned *pga*II hybridizing fragments of the *pga*A and *pga*B genes. pIM3760 contains the 5.0 kb *Eco*RI *pga*A fragment in pEMBL19 with the 5' of the gene oriented towards the *lac*Z translational start. In pIM3761 the orientation is reversed. pIM3762 contains the 4.5 kb *Hind*III *pga*A fragment in pBluescriptSK⁺. pIM3770 carries the 3.8 kb *Eco*RI *pga*B fragment in pBluescriptSK⁺.

Promoter-gene fusions. The expression plasmids containing the *pki*A promoter-gene fusion were constructed in the following way. The 0.7 kb *Bam*HI-*Nsi*I fragment of the *pki*A promoter was isolated from pUC18HN [9]. In the next step, pIM3760 served as a template to introduce the *Nsi*I restriction site at the translational start of *pga*A by PCR using *Taq* polymerase (Pharmacia Biotech., Uppsala, Sweden). For the PCR synthesis of the *Nsi*I-*Kpn*I *pga*A fragment (see Figure 1), two primers were designed: primer *pga*A-*Nsi*, a nineteen-residue long oligonucleotide, 5`CGCAATCATGCATTCTGCC 3`, localized at position 1907-1925 bp, and primer *pga*A-*Kpn*, a twenty-residue long oligonucleotide, 5`AGGTACCGCCGGAGAAGTAG 3`, annealing at position 2640-2621 bp of the *pga*A gene. The PCR conditions were: denaturation for 5 min at 95 °C, 30 cycles of 30 s denaturation at 95 °C, 30 s annealing at 60 °C, and 1 min extension at 72 °C. The final extension time was increased to 5 min. The resulting 734 bp *pga*A PCR fragment was checked by sequencing for PCR errors. The *pki*A promoter *Bam*HI-*Nsi*I fragment and the *pga*A *Nsi*I-*Kpn*I fragments were ligated into pGEM-7. In the final ligation, the 2.5 kb *Kpn*I-*Eco*RI fragment (see Figure 1), containing the remainder of the *pga*A gene and its downstream sequence, was cloned into pGEM-7 harbouring the *pki*A-*pga*A fragment resulting in pIM3763. For construction of pIM3773, the *pki*A-*pga*B expression plasmid, the presence of the *Nsi*I site localized at the translational start of the *pga*B gene was exploited. The 1.6 kb *Xho*I fragment (see Figure 1) containing the *pga*B promoter sequence and the 5` end of the *pga*B coding sequence was subcloned into pBluescriptSK⁺. In the next step, the *pga*B promoter part of this subclone was replaced by the 0.7 kb *Bam*HI-*Nsi*I fragment of the *pki*A promoter from pUC18HN. The final construct was obtained after ligation of the 1.3 kb *Xho*I fragment, consisting of the *pga*B gene and an additional 170 bp of sequence downstream of the 3' end of *pga*B.

Figure 1. Restriction map of the 5.8 kb *Eco*RI/*Hind*III λA43 and the 3.8 kb *Eco*RI λB4 fragments.

The black (exons) and white (introns) blocks depict the position of the *pga*A and *pga*B coding region. The arrows indicate the 5'→3' orientation of the genes. The fine dotted lines beneath the restriction maps indicate those parts of the λ phage resulting in pIM plasmids (pIM3762, pIM3760/pIM3761 and pIM3770). The nucleotide sequence data for *pga*A and *pga*B have been deposited with the EMBL, GenBank and DDBJ Databases under accession numbers Y18804 and Y18805 respectively.
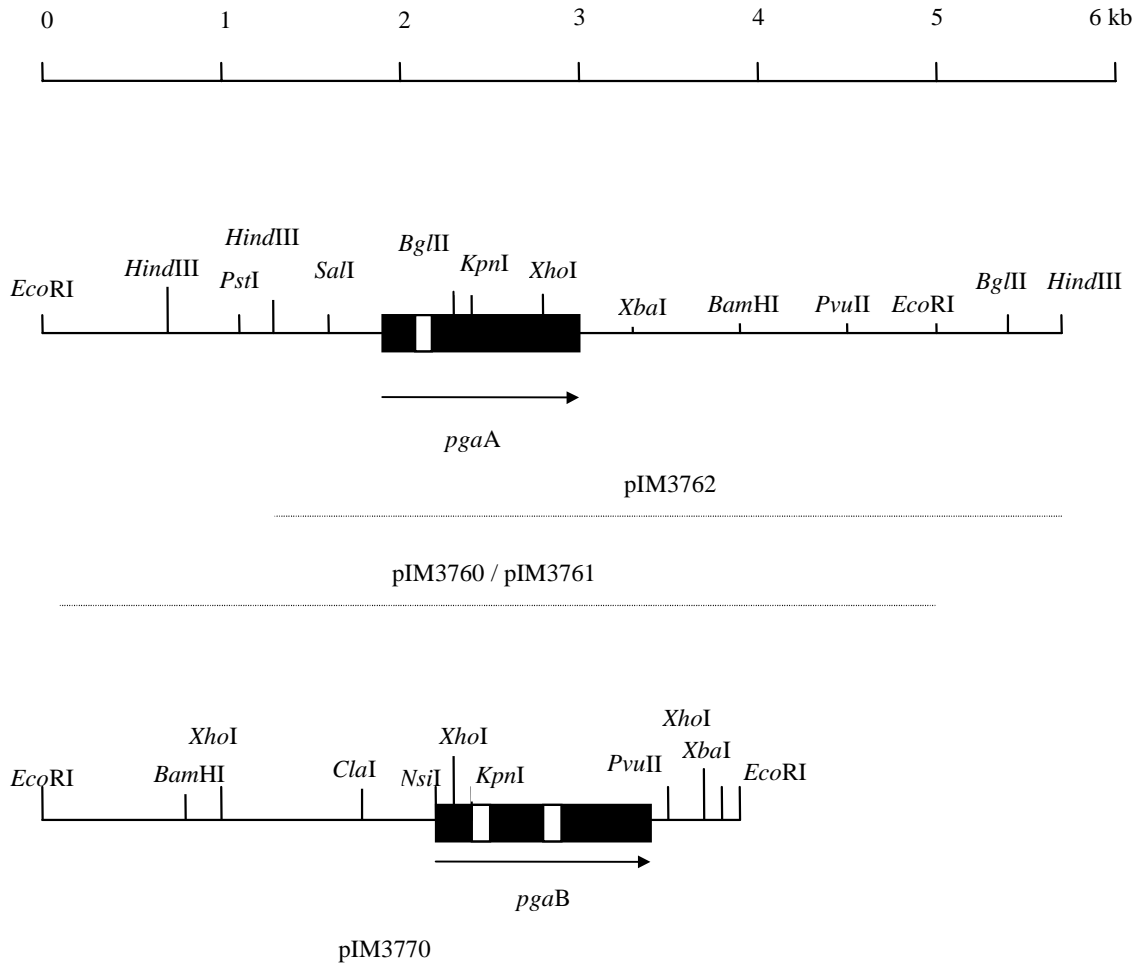
DNA and RNA manipulations. The phage and plasmid DNA isolation and other molecular techniques were essentially carried out as described by Sambrook *et al.* [17]. The restriction enzymes were used as described by the supplier (Gibco, BRL, Life Technologies Inc., Gaithersburg, U.S.A.). The nucleotide sequences were determined using either the Cy5^{TM} AutoCycle Sequencing Kit (Pharmacia Biotech.) or the Cy5^{TM}-dATP Labeling Mix (Pharmacia Biotech.) with universal and reverse primers or with gene-specific primers respectively. The reactions were analysed with an ALFexpress^{TM} DNA sequencer. Computer analysis was done using the program GeneRunner (Hastings Software, Inc., Hastings, NY, U.S.A.).

The total RNA was isolated using TRIzol^{TM} Reagent according to the supplier (Gibco BRL). The RNA concentration was estimated spectrophotometrically at 260 nm. RNA (20 μg) was loaded on to and separated in 1.2 % (w/v) agarose gel under glyoxal denaturation conditions [17]. After electrophoresis, the gels were capillary blotted overnight onto Hybond-N^{TM} membranes (Amersham International, Little Chalfont, Bucks., U.K.).

Prehybridization and hybridization of Southern blots were carried out at 56 ^{o}C or 68 ^{o}C for heterologous or homologous screening respectively [17]. Northern blots were hybridized at 42 ^{o}C in hybridization buffer containing 50 % (v/v) of formamide. For *pga*A the 335 bp *Xho*I-*Xba*I fragment, localized in the 3' part of the gene, and the 3' non-translated region was used as a probe, and for *pga*B the *Nsi*I-*Kpn*I 200 bp fragment from the 5' end of the gene served this purpose (see Figure 1). Transformation of *A. niger* was done as described before [3], using 1 µg of pGW635 and 20 µg of co-transforming plasmid DNA.

cDNA synthesis. The *A. niger* transformants, 617.42-1 and 617.49-15, were grown for 18 h in minimal medium containing 3 % (w/v) fructose. Total RNA isolated from the mycelium was used for the partial cDNA synthesis by reverse transcription-PCR. The reactions were performed according to Gilliland *et al.* [23] with minor modifications; 1 µg of RNA was used in the synthesis of the first strand cDNA, using the Moloney murine leukaemia virus reverse transcriptase under the conditions described by the supplier (Gibco BRL). Gene-specific primers were used. In the case of *pga*A, primer *pga*A-*Kpn* was used in the first reaction (see above), and for *pga*B a new primer was designed, a nineteen-residue long oligonucleotide, 5'AGTGCCAGTCTTGCCGTAG 3', annealing at position 3161-3143 bp. In the second step, the cDNA synthesis was completed by PCR, employing forward primers annealing in the 5' region of the respective gene. For *pga*A, a twenty-residue long oligonucleotide, 5' TGTTCTGCCTTGCGACCCTG 3', annealing at position 1933-1952 bp, and for *pga*B, an eighteen-residue long oligonucleotide, 5' GCATTTCCTCCAGAACGC 3', annealing at position 2227-2244 bp, was used. The amount used of each primer was 100 pmoles. The PCR cycler was programmed as described above with the following changes in the annealing temperature. For *pga*A a temperature of 43.5 ^{o}C was used and for *pga*B the temperature was 50 ^{o}C. The resulting PCR products were checked by sequencing.

Induction studies. *A. niger* N400 mycelium was pre-grown for 20 h in Erlenmeyer flasks with 200 ml of minimal medium containing per litre: 6.0 g NaNO$_3$, 1.5 g KH$_2$PO$_4$, 0.5 g MgSO$_4$.7 H$_2$O, 0.5 g KCl, trace elements [24], 0.1 % (w/v) dialysed yeast extract [9] and 1 % (w/v) sucrose. The flasks were inoculated with 10^6 spores.ml^{-1} and the cultures grown at 30 ^{o}C in an orbital shaker at 250 rev./min. The pre-grown mycelium was harvested using a Büchner funnel with nylon gauze, extensively washed with sterilized saline (9 g/l NaCl) and water. Aliquots (3 g) of wet mycelium were then transferred to 250 ml Erlenmeyer flasks containing 50 ml of minimal medium and 1 % (w/v) of a carbon source as specified in Figure 2. Sucrose, sugar beet pectin (Copenhagen Pectin A/S, Lille Skensved, Denmark) and polygalacturonic acid were added to the media before autoclaving, whereas D-galacturonic acid and L-rhamnose were filter-sterilized and added after autoclaving. The starting pH of the cultures was adjusted to pH 6.0.

Enzyme purification and characterization. The PGA and PGB overproducing transformants were selected as described previously [9]. The transformants, 617.42-1 and 617.49-15, producing the highest amount of PGA and PGB respectively, were used for a large scale purification under the same culture conditions as applied for overproduction of PGE [9]. The PGA was purified using the same chromatographic steps as applied for the purification of PGE [9]. For the isolation of PGB, the culture fluid was diluted three-fold with water and the pH was adjusted to 4.0. Next, pre-swollen CM-Sephadex C-50 (100 g) were added and stirred for 16 h at 4 ^{o}C. Bound PGB was eluted from the matrix by a pulse of 1M NaCl in 20 mM sodium acetate buffer, pH 4.0, followed by extensive dialysis against the same buffer. For final purification, the enzyme was loaded on to a S-Sepharose Fast Flow

column (2.6 cm x 20 cm) and eluted with a 1200 ml linear 0 - 0.8 M NaCl gradient in buffer. Enzyme-containing fractions were pooled, dialysed against 50 mM sodium acetate buffer, pH 5, and stored at 4 ^{o}C in the presence of 0.02 % (w/v) NaN$_3$. The purity of both enzyme preparations was monitored by SDS/PAGE and the gels were stained with Coomassie Brilliant Blue R 250. The molecular masses were estimated by SDS/PAGE; the gel was calibrated with protein test mixture 4 (Serva, Boehringer Ingelheim, Germany). The protein concentrations were calculated from the molecular extinction coefficients using the method of Edelhoch [25].

PG activity was routinely assayed as described for PGE [9], using 50 mM sodium acetate buffer pH 4.2 or pH 5.0 for PGA and PGB respectively. The pH optima and the effect of the degree of methylation of the substrate on activity were determined as described before [15]. Product-progression analysis on polygalacturonate and pectins with various degree of esterification, as well as the determination of bond-cleavage frequencies on oligogalacturonates with different degree of polymerization (DP = 2-8), was performed as described previously [9].

Results and Discussion

Subcloning of the *pga*A and *pga*B genes. Phages λA43 and λB4, representatives of the classes A and B [8] respectively, were selected for Southern blotting using the 1.3 kb *Bam*HI/*Bgl*II *pga*II-specific probe (see the Experimental section). The λA43 analysis revealed a 4.5 kb *Hind*III and a 5.0 kb *Eco*RI fragments, both hybridizing strongly (Figure 1). For subcloning of the 3.8 kb *Eco*RI-hybridizing fragment from λB4 (Figure 1) the same strategy was employed, which resulted in pIM3770.

Analysis of nucleotide sequences of *pga*A and *pga*B. The entire 5.8 kb *Eco*RI-*Hind*III subclone derived from λA43 contains 1167 bp of coding sequence and 1913- bp upstream and 2700- bp downstream sequence. The *pga*B nucleotide sequence includes 2224 bp of the 5' non-coding region, 1234 bp of the *pga*B coding sequence and 330 bp of the 3' non-coding region (Figure 1).

Based on the sequence alignment of *pga*A and *pga*B with other fungal *pga* genes present in the EMBL Gene Database, introns of 54 bp in length for *pga*A and 70 and 74 bp for *pga*B were predicted, which were confirmed by cDNA sequencing of the regions indicated in Figure 1. The intron boundaries in both genes follow the consensus sequences found for filamentous fungal introns [26,27]. When compared to other genes, the *A. niger pga* genes are highly identical in structural organization of introns and exons with those of *A. tubingensis* [28], *A. oryzae* [29], *A. flavus* [13] and *A. parasiticus* [30].

5' and 3' non-transcribed regions of *pga*A and *pga*B genes. According to Gurr *et al.* [27] the transcription start point of filamentous fungal genes is often preceded or followed by CT-rich motifs, and contains TATAAA and CAAT boxes. In the case of the *pga*A promoter, a TATATAA sequence, a modified version of a TATAAA box, was found at position -160 bp upstream from the translation start point, and three CAAT boxes were found between -192 and -4 bp. In case of the *pga*B promoter, no CAAT or TATAAA boxes could be detected close to the translation start point, but a CT-rich region was present at the position -91 to -44 bp [27]. Both of the genes fulfil the Kozak rule [31] in that a purine (adenosine) is present at position -3 bp from the AUG translation initiation codon.

It was previously shown for pectinolytic genes from other fungi, like a pectate lyase from *A. nidulans* [32] and an exopolygalacturonase from *A. tubingensis* [33] that (poly-) galacturonic acid had a positive, whereas glucose had a negative effect on the expression. The promoters of both the *pga*A and *pga*B genes were analysed for the presence of specific DNA sequences, such as consensus CREA (carbon catabolite repressing protein A) binding sites [34-36] and the 5' TYATTGGTGGA 3' sequence. The latter was proposed [8,37] to play a role in the induced expression of the *pga*II gene from *A. niger*.

Ten and six consensus CREA binding sites were found at a distance from –325 and –185 from the translation start codon to the far upstream sequence of the *pga*A and *pga*B promoters respectively. Furthermore, in the *pga*A 5' promoter region (-1525 bp from the ATG), the sequence 5' TGATTGGT 3', resembling the *pga*II 5' TYATTGGTGGA 3' sequence [37], was found on the opposite DNA strand.

The search for a (truncated) polyadenylation signal, (A)AUAAA [27], revealed the sequence, AUAAA, 253 bp downstream of the *pga*A-gene translation stop codon, but a similar motif was not observed in the *pga*B sequence.


Influence of the various carbon sources on *pga*A and *pga*B expression. *pga*A- and *pga*B-specific probes were checked for cross-hybridization with other *A. niger pga* genes by a dot-blot analysis, under stringent hybridization conditions, using 5 ng of plasmid DNA containing those genes. Although some background hybridization was detected, the intensity of the signal corresponding to the specific gene was much higher.

For the expression study several carbon sources were selected, which included the monomeric sugars present in the backbone of pectin, D-galacturonic acid and L-rhamnose, as well as the polymeric substrates, polygalacturonic acid and sugar beet pectin. Sucrose was used in order

to test whether *pga*A and *pga*B gene expression was affected by carbon catabolite repression. The results of this analysis are presented in Figure 2.

Figure 2. Northern-blot analysis of the effect of the carbon source on *pga*A and *pga*B expression.
The mycelium was pre-grown overnight for 20 h in minimal medium containing 1 % (w/v) sucrose and the medium was then changed to a minimal medium containing 1 % (w/v) of D-galacturonic acid (gaa), 1 % (w/v) D-galacturonic acid and 1 % (w/v) L-rhamnose (gaa+rha), 1 % (w/v) polygalacturonic acid (pga), 1 % (w/v) sugar beet pectin (SBP) or 1 % (w/v) sucrose. The mycelium was harvested at 2, 6 and 10 h after the change of medium. As a loading control the 18S rDNA fragment was used for reprobing of the blots.
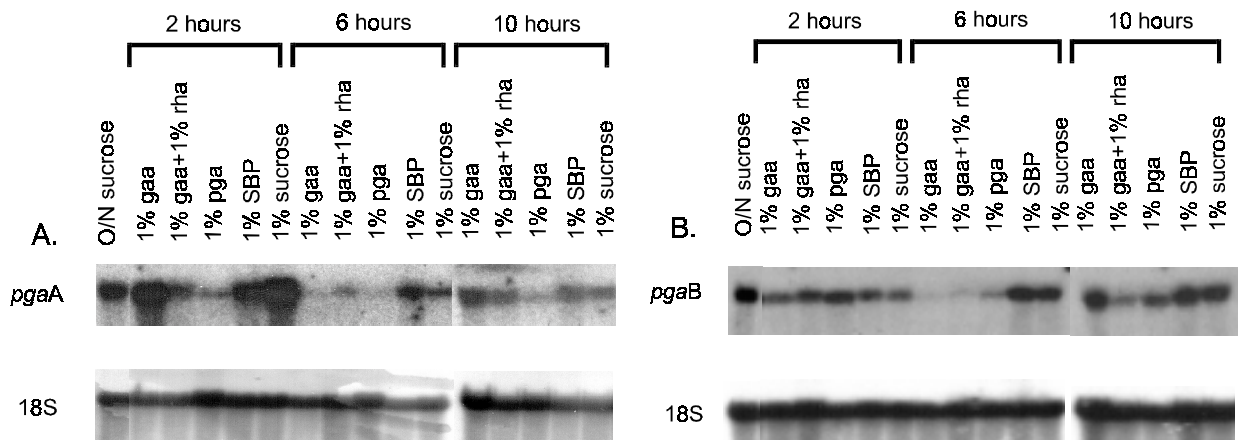
It is clear from the results shown in Figure 2 that *pga*A- and *pga*B-specific mRNA were both already present in mycelia of *A. niger* pre-grown overnight on sucrose. After the medium was replaced with fresh sucrose-containing medium the signal of the *pga*A and *pga*B mRNA remained high or even increased. Using the various pectic substrates, the expression patterns were only straightforward when the complex sugar beet pectin, which contains high amounts of neutral sugars as L-arabinose, D-galactose and D-xylose, was used. The two pentoses constitute more easy metabolized substrates than D-galacturonic acid and L-rhamnose, and therefore the expression of *pga*A and *pga*B is probably similar to the expression on sucrose. The same feature, the constitutive expression on glucose and pectin, was previously reported for the *pec*A genes from *A. flavus* [13] and *A. parasiticus* [30], which also encode a PG. Interestingly, the derived amino acid sequences of these PGs share the highest sequence similarity and phylogenetic relatedness with the *A. niger* PGA and PGB [11].

From the results presented in Figure 2, it is obvious that, using defined pectic constituents like D-galacturonic acid, D-galacturonic acid in combination with L-rhamnose, and

polygalacturonic acid, the expression levels of both, *pga*A and *pga*B genes differ depending on the substrate and time of induction. Whereas the level of the *pga*B transcript 2 h after the change of medium was similar on all carbon sources tested, there was an increase in the level of *pga*A transcription not only on sucrose but also in the presence of D-galacturonic acid. The high transcription signal detected on D-galacturonic acid might be related to a DNA sequence resembling the *pga*II 5' TYATTGGTGGA 3' upstream-activating sequence [8,37] in the 5' part of the *pga*A promoter. This sequence is not present in the *pga*B promoter. The *pga*A transcript levels on D-galacturonic acid were apparently lower when L-rhamnose was present simultaneously. Only when polygalacturonic acid was used as a substrate, was *pga*A transcription reduced 2 h after the change of medium. HPAEC-PAD analysis of the culture medium (results not shown) indicated that the concentration of D-galacturonic acid released was low and thus probably not sufficient to sustain proper growth and *pga*A expression. The high level of *pga*A mRNA on pectin could be the combined effect of the induction by D-galacturonic acid and the release of other sugars, such as L-arabinose and D-xylose, during pectin a degradation, thus providing a better carbon source for growth.

The transcription of both *pga*A and *pga*B was barely detectable 6 h after the change of medium to D-galacturonic acid, D-galacturonic acid and L-rhamnose and polygalacturonic acid. At the present time we do not have a satisfactory explanation for these results. The fact that *pga*A and *pga*B transcription was affected simultaneously suggests that the fungus had not yet adapted its metabolism to the utilization of D-galacturonic acid. An increase in mRNA levels after 10 h is in agreement with such a growth-related response, whereas modulation of expression 2 h after the change of medium seems to be *pga*A specific.

The presence of both the *pga*A and *pga*B transcripts on all carbon sources tested demonstrates the constitutive expression of these genes.

PGA- and PGB-derived amino acid sequences. The *pga*A and *pga*B genes encode proteins of 370 and 362 amino acids respectively. As observed earlier for the PGI and PGE from *A. niger* [7,9], both enzymes are most likely synthesized as prepro-enzymes. The cleavage of the signal peptide probably occurs at Ala[19] and Ala[20], based on the '(-3,-1)-rule' [38], in both PGA and PGB. The pro-sequences are most likely cleaved by a KEX2-like dibasic peptidase [39] after Lys-Arg residues at position 32 or 26 of PGA and PGB respectively. The mature PGA consists of 338 amino acid residues, a calculated relative molecular mass of 35497 and an isoelectric point of 3.43. The N-terminal processing of PGB leads to a 336 amino-acid-long

protein with a predicted relative molecular mass of 35188 and an isoelectric point of 6.19. Both enzymes were analysed for the presence of putative N-glycosylation sites (Asn-Xaa-Thr/Ser). In PGA such a site is located at Asn[214] of the mature protein and in mature PGB, these sites were found at Asn[265] and Asn[308] respectively. The N-glycosylation site of PGA is strictly conserved in all four *A. niger* PGs previously characterized [6-9]. However, the first N-glycosylation site of PGB can only be found in PGI [7], whereas the second site is unique.

Biochemical characterization of PGA and PGB

Specific activities. A standard assay performed at pH 4.2 for PGA and pH 5.0 for PGB, resulted in specific activities of 16.5 μkat.mg^{-1} and 8.3 μkat.mg^{-1} for PGA and PGB respectively. These specific activities were in the same order as those reported for PGI and PGII, 13.8 μkat.mg^{-1} and 36.5 μkat.mg^{-1} respectively [15], but much higher than those reported for PGC, 0.42 μkat.mg^{-1} [15] and PGE, 0.5 μkat.mg^{-1} [9]. This indicates, that like PGI and PGII, PGA and PGB prefer homogalacturonan as a substrate.

pH optima, V_{max} (app) and K_m (app) for PGA and PGB. The pH optima for PGA and PGB were 4.0 and 5.0 respectively (Figure 3). Since PGA and PGB are constitutively expressed in *A. niger*, their distinct complementary pH optima seem to ensure pectin degradation by the fungus over a wider pH range.

**Figure 3. pH optima for PGA and PGB acting on polygalacturonate as substrate.**

The pH optima for PGA (●) and PGB (○) were determined in McIlvaine buffers using 0.25 % (w/v) polygalacturonic acid as the substrate at 30 °C.
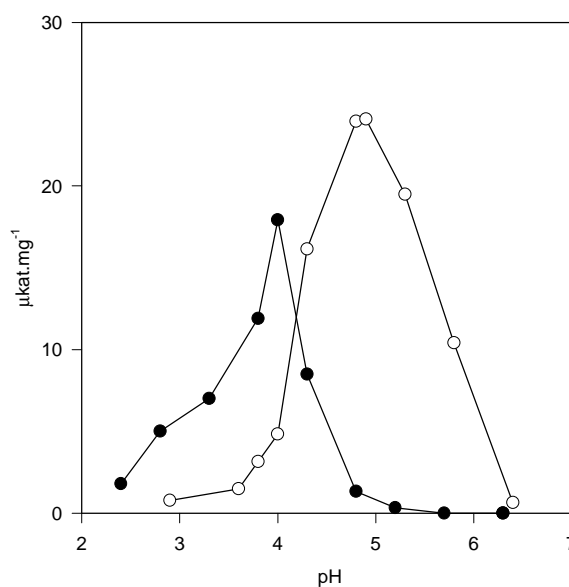
Comparison of the specific activities at the pH optima in McIlvaine buffer to those in sodium acetate buffer (standard assay) revealed a much higher specific activity (27.5 µkat.mg^{-1}) for PGB in McIlvaine buffer, whereas for PGA and PGs I, II [15], and E [9] comparable values were found in McIlvaine buffer and sodium acetate buffer. Since McIlvaine buffers are high in ionic strength, the effect of the addition of NaCl to the standard assay mixture on the specific activity of PGB was studied (Figure 4). The specific activity of PGB increased to 30.9 µkat.mg^{-1} at 175 mM NaCl.

Figure 4. The effect of [NaCl] on the activity of PGB.

PGB activity was assayed in a standard assay procedure with NaCl added as indicated.
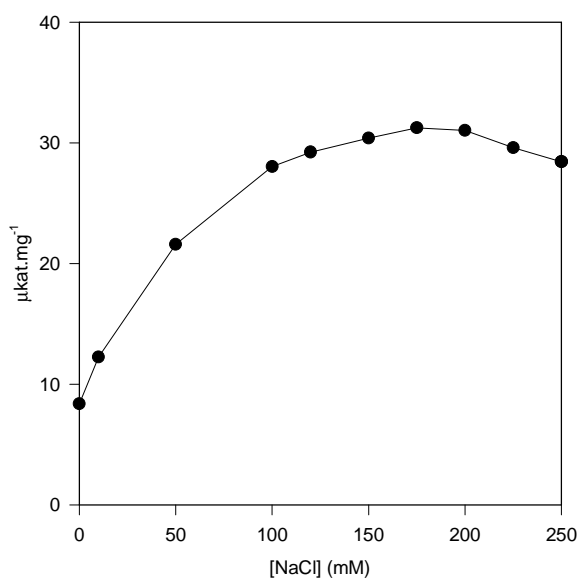
Due to sensitivity limits of the assay for reducing end groups, the K_m (app) value could not be accurately determined for PGA, but was < 0.15 mg.ml^{-1}. V_{max} (app) for PGA was calculated to be 18 µkat.mg^{-1}. In view of the low K_m (app), the pH optimum for PGA represents V_{max} (app) versus pH plot (Figure 3). The narrow profile of the plot suggests that only one ionizing group determines effective catalysis.

For PGB, the formation of reducing end groups, routinely determined at 10, 20, 30 and 40 min in a standard assay, was not linear at low substrate concentrations (< 1 mg.ml^{-1}), precluding accurate determination of the reaction rate. Thus, K_m (app) and V_{max} (app) values for PGB using polygalacturonic acid could not be calculated. This indicated that PGB is likely to be a true endo-acting enzyme, which prefers a polymer substrate and thus depletes the polymeric substrate during the time of the assay. The degree of polymerization of the particular polygalacturonic acid preparation was 150 [33]. When a substrate with a degree of polymerization of 215, such as lemon pectin with 7 % methylesterification (lemon 7), was used formation of reducing end groups was linear over the 40 min interval. The V_{max} (app) for

PGB using lemon 7 was calculated as 14.1 μkat.mg^{-1} and K_m (app) as 0.9 mg.ml^{-1}. At 2.5 mg.ml^{-1} lemon 7, the activity in a standard assay was 10.8 μkat.mg^{-1}. This activity was 1.2-fold higher than the activity observed when polygalacturonic acid, which also gave a linear response in reducing end groups, was used at the same substrate concentration. It is therefore possible that other factors, besides the higher degree of polymerization, contribute to the higher activity and linear progression. Those factors may be the presence of small amounts of salt(s) or the methylation itself (see below).

PGA and PGB prefer methylated substrates. It appeared that PGA and PGB were more active on partly methylated pectin (Table 1). However, for PGB the preference for methylated pectin changed into a tolerance for methylation when 200 mM NaCl was included in the assay. The preference/tolerance of the constitutively produced PGA and PGB for partially methylated substrate in combination with their complementary pH optima would allow the fungus to quickly and effectively respond to the presence of pectic substances once more favourable carbon sources are depleted. Both PGA and PGB differ, with respect to their sensitivity for methylation, from PGs I, II, C and E [15], which showed a clear preference for non-methylated substrate.

Table 1. Effect of the degree of methylation of pectin on the specific activity of PGA and PGB.

The rate of hydrolysis was determined during initial linear stages of the enzyme reaction by the determination of reducing-end groups. 0.25 % (w/v) of lemon pectin was used in 50 mM sodium-acetate, pH 4.2 (PGA) or pH 5.0 (PGB) at 30 °C. *, 200 mM NaCl added to the assay mixture.

PG	Degree of esterification (%)					
	0	7	22	45	60	75
	relative specific activity					
A	100	125	135	52	7	6
B	100	150	168	131	62	27
B*	100	98	92	45	18	16

Figure 5. Progression of products formed by PGA and PGB.

Products produced during the first 2-4 h of hydrolysis, at 30 °C, of: (**A**) 1 % (w/v) polygalacturonate in 1 ml 50 mM sodium acetate, pH 5.0, containing 490 ng PGB; (**B**) 1 % (w/v) polygalacturonate in 1 ml 50 mM sodium acetate, pH 4.2, containing 220 ng PGA; (**C**) 1 % (w/v) lemon pectin with a degree of esterification of 22 % in 1 ml of 50 mM sodium acetate buffer, pH 4.2, containing 220 ng PGA; (**D**) 1 % (w/v) lemon pectin with a degree of esterification of 45 % in 1 ml of 50 mM sodium acetate buffer, pH 4.2, containing 220 ng PGA. Samples (50 μl) samples were analysed by HPAEC-PAD. ●, (Gal$p$A)$_1$; ○, (Gal$p$A)$_2$; ■, (Gal$p$A)$_3$; □, (Gal$p$A)$_4$; ▲, (Gal$p$A)$_5$; △, (Gal$p$A)$_6$.
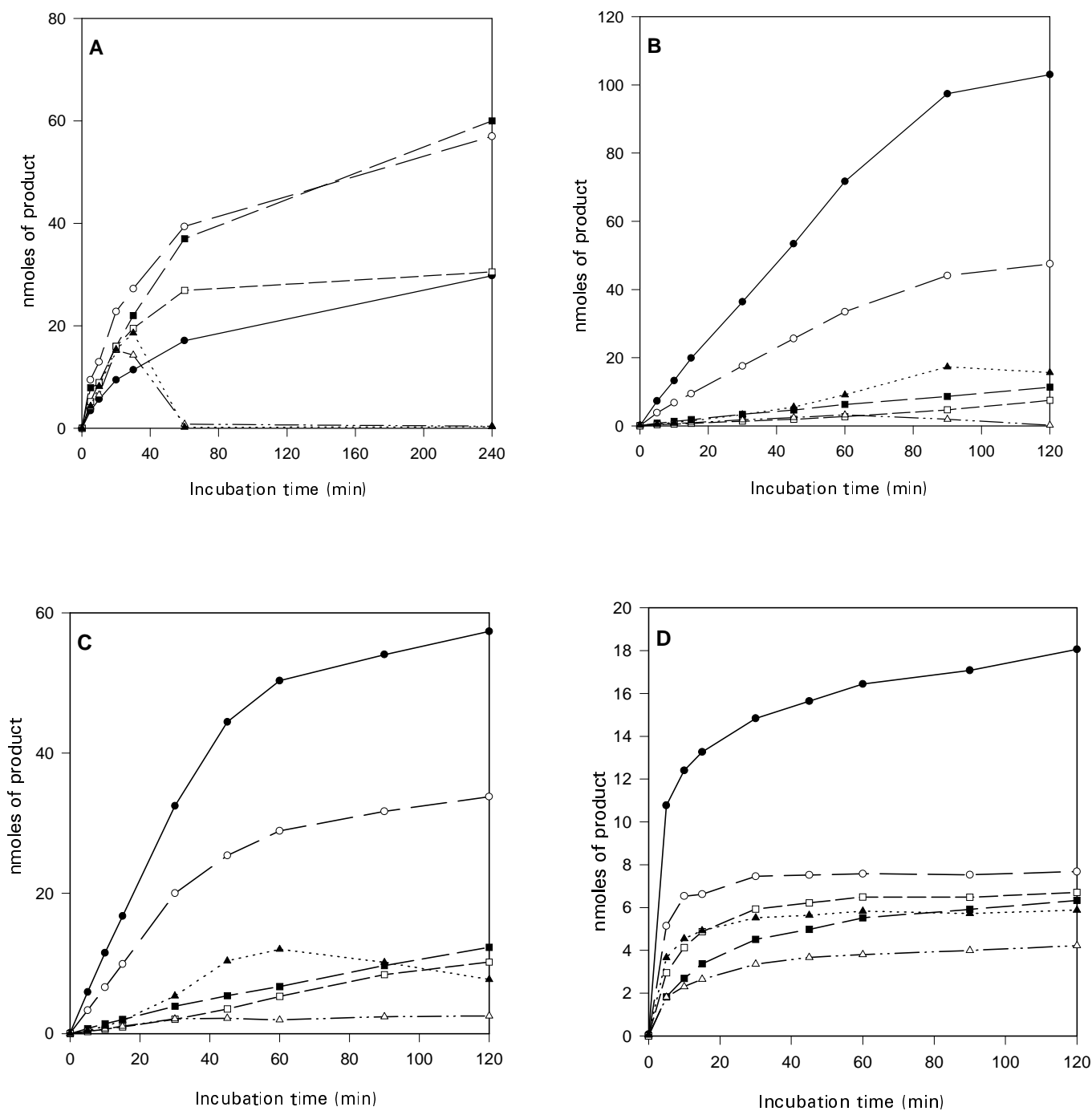
PGA and PGB are endo-acting enzymes. The product progression during polygalacturonic acid hydrolysis (Figures 5A and 5B) showed that both enzymes are endo-acting, since a transient accumulation of oligogalacturonates with degree of polymerization > 5 was observed. These were gradually converted to oligogalacturonates with a degree of polymerization < 5. For PGB, the transient accumulation of higher oligogalacturonates is more pronounced than for PGA. The product progression of PGB resembles the product progression of PGII and PGE, both randomly acting PGs [9,15]. However, PGA behaved more like PGI and PGC in this respect, displaying a strong increase of $(GalpA)_1$ from the start of the reaction. It was shown previously that this type of product progression originated from processive behaviour on oligogalacturonates, with $n > 5$ or $n > 6$ for PGI and PGC respectively [15].

For PGB, the effect of the addition of 200 mM NaCl to the reaction mixture on the product progression was investigated, and lemon pectins with 7, 22, 45, 60 and 75 % esterification were also included in this study. Apart from the expected increased rate of hydrolysis upon addition of 200 mM NaCl, which was corrected for by higher dilution of PGB, no effect on the product progression on polygalacturonic acid as a substrate was observed (not shown). The same observation was made for the series of lemon pectins. However, differences in product progression were observed, depending on the pectin used (results not shown). Generally, the higher the degree of esterification, the slower the formation of the oligogalacturonates with $n < 5$. Based on these results, it can be concluded that an increase in ionic strength for PGB results in a general increase in hydrolysis, probably by increasing the affinity for the substrate without increasing the affinity at a particular subsite.

Comparison of the product progression for PGA, using polygalacturonic acid, lemon pectin 22 and 45, revealed a more even accumulation of oligogalacturonates for the partially methylated substrates, particularly lemon pectin 45 (see Figures 5C and 5D). Thus the processivity of PGA is lessened when methylated substrates are used, indicating that not all subsites can equally well accommodate a methylated galacturonate moiety.

Mode of action of PGA and PGB

Using reduced $(GalpA)_n$, $n = 3-8$, it was made plausible that PGs I, II, C and E attacked oligogalacturonates from the reducing end [9,15], whereas exopolygalacturonase attacked the substrate from the non-reducing end [33]. Similarly, using reduced $(GalpA)_6$ as a substrate, and comparing the bond-cleavage frequencies (BCFs) with those obtained for non-reduced

$(GalpA)_6$, it was inferred that PGA and PGB also hydrolysed the oligogalacturonates from the reducing end (Table 2). As for the other *A. niger* PGs, $(GalpA)_3$ was the smallest substrate hydrolysed.

Inspection of the BCFs for PGA revealed that, except for $(GalpA)_5$, the first glycosidic linkage from the reducing end was preferentially hydrolysed. For $(GalpA)_7$ and $(GalpA)_8$, bond cleavage frequencies could not be determined because of deviation from stoichiometry of product pairs. A similar observation was made for PGI and PGC [15]. Furthermore, PGA and PGI displayed exactly the same BCFs up to $n = 5$. Also, both PGA and PGI showed a decreased rate of hydrolysis for $(GalpA)_5$ when compared with $(GalpA)_4$. The opposite would be expected since the rates increased again for $n = 6$, which indicates that more than 5 subsites are present on the enzymes (seven subsites were estimated for PGI). For PGI the decreased rate of hydrolysis of $(GalpA)_5$ was ascribed to competitive inhibition due to non-productive binding from subsites -5 to -1. This was also in agreement with the observed processive behaviour for PGI on $(GalpA)_6$. Likewise, $(GalpA)_5$ may be a competitive inhibitor for PGA. This is corroborated by the fact that, for $(GalpA)_6$, an extreme preference for binding from subsites -5 to +1 was observed, thus highlighting the high affinity of subsites -5 to -1 where non-productive binding of $(GalpA)_5$ most likely occurs.

For PGA, processive behaviour occurred only from $(GalpA)_7$ onward, as shown in Figure 6, where a ratio plot for $(GalpA)_7$ hydrolysis according to Robyt and French [40] is presented. This plot shows the non-coincidence for the $(GalpA)_6$ and $(GalpA)_1$ formation at the early stages of the reaction, which demonstrates the processive behaviour. The origin of this processivity resides in the high affinity of subsites -5 to -1 and the extreme bias for $(GalpA)_6$ hydrolysis in $(GalpA)_5$-$(GalpA)_1$ mode. Those properties make a shift of $(GalpA)_6$-bound unproductively at sites -6 to -1 (resulting from $(GalpA)_7$ hydrolysis) to sites -5 to +1 very likely.

Inspection of the BCFs for PGB shows that the pattern is typical for an endo-acting enzyme. The observed increased hydrolysis rate upon increasing chain length is also in agreement with this and suggests that the number of subsites is at least seven. Of all *A. niger* PGs studied in this respect [9,15], PGB shows the strongest preference for the second and the third glycosidic linkage from the reducing end, which is indicative of high affinity at subsites +2 and +3.

The BCFs and rates for PGA and PGB presented in Table 2 can well account for the observed product progression on polygalacturonate.

Table 2. BCFs and hydrolysis rates for PGA and PGB acting on (reduced) oligogalacturonates of defined length.

Assay conditions: 500 μM (reduced) oligogalacturonates were incubated with PGA or PGB in 0.5 ml 50 mM sodium acetate pH 4.2 or pH 5.0 respectively. At timed intervals, 50 μl aliqouts were withdrawn and mixed with 50 μl stopmix (2.0 mM Tris/HCl, 50 mM NaOH) to raise the pH to 8.3-8.5. Products were analysed and quantitated by HPAEC-PAD, as described in the Experimental section. G indicates each galacturonate residue with the BCFs (in percentages) given below the relevant bond. The boldface typescript (**G**) indicates the reducing end of the polymer and the asterisk (*) indicates the reduced end (galactonate).

(Gal*p*A)$_n$																Rate (μkat.mg^{-1})
PGA																
6					G	-	G	-	G	-	G	-	G	-	G*	1.07
										51		49				
3											G	-	G	-	G	0.0245
														100		
4									G	-	G	-	G	-	G	1.32
												3		97		
5							G	-	G	-	G	-	G	-	G	1.18
												46		54		
6					G	-	G	-	G	-	G	-	G	-	G	6.54
										1		3		96		
7			G	-	G	-	G	-	G	-	G	-	G	-	G	7.22
PGB																
6					G	-	G	-	G	-	G	-	G	-	G*	0.0022
								6		94						
3											G	-	G	-	G	0.0058
														100		
4									G	-	G	-	G	-	G	1.14
												2		98		
5							G	-	G	-	G	-	G	-	G	1.96
												90		10		
6					G	-	G	-	G	-	G	-	G	-	G	3.74
										50		27		23		
7			G	-	G	-	G	-	G	-	G	-	G	-	G	5.05
										46		47		7		
8	G	-	G	-	G	-	G	-	G	-	G	-	G	-	G	7.19
								15		29		53		3		

Figure 6. Ratio plot (Gal*p*A)$_7$ hydrolysis by PGA. Diagnostic plot for the occurrence of processivity according to Robyt, J.F. and French, D. [40]. The ratio of product formation, [(Gal*p*A)$_1$] (○) and [(Gal*p*A)$_6$] (●) relative to the initial substrate [(Gal*p*A)$_7$], is plotted against the progress of the hydrolysis reaction represented as ln [(Gal*p*A)$_7^{t=0}$]/[(Gal*p*A)$_7$].
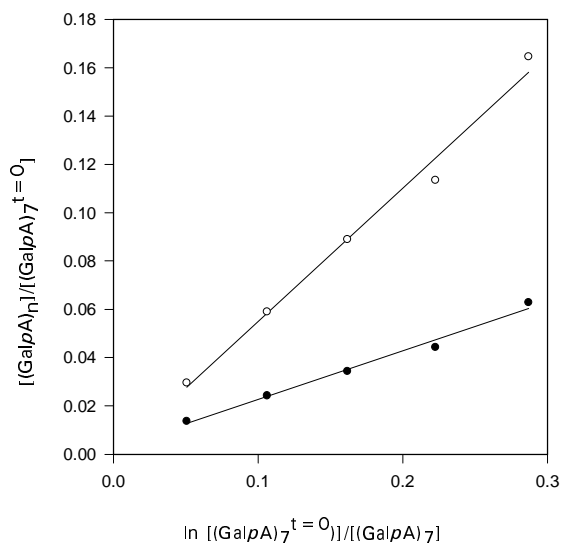
We now have characterized six out seven *A. niger* PGs. Previously it was suggested that PGI and PGII, being the most active enzymes, would be responsible for the major substrate supply for the fungus, whereas PGC and PGE would attack regions in the pectin molecule that are unaccessible for PGI and PGII. The constitutive expression of PGA and PGB in combination with their preference for a methylated substrate and the complementing pH optima, identifies those enzymes as 'scouting' enzymes which allow rapid adaptation of the fungus to pectic substrates.

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CHAPTER 4

*pga*D encodes a new type of endopolygalacturonase from *Aspergillus niger*

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Abstract

We isolated and characterized a new type of endopolygalacturonase encoded by the *pga*D gene from *Aspergillus niger*. The primary structure of endopolygalacturonase D differs from that of other *A. niger* endopolygalacturonases by a 136 amino acid residues long N-terminal extension. The expression of *pga*D in *A. niger* was detectable only by using RT-PCR in a *pga*D multicopy transformant grown on pectic substrate. Biochemical analysis demonstrated extreme processive behavior of the enzyme on oligomers longer than five galacturonate units. Furthermore, endopolygalacturonase D is the only *A. niger* endopolygalacturonase capable of hydrolyzing digalacturonate. It is tentatively concluded that the enzyme is composed of four subsites. The physiological role of endopolygalacturonase D is discussed.

Introduction

Endopolygalacturonases (E.C. 3.2.1.15) belong to a large group of enzymes, called pectinases, which are produced by a number of phytopathogenic and saprophytic micro-organisms in order to degrade pectin, a complex plant cell wall polysaccharide. Polygalacturonases are known as enzymes mainly hydrolyzing the demethylated backbone of the pectin molecule, *viz.* pectate, consisting of 1,4-α-linked D-galacturonic acid units.

In the saprophytic fungus *Aspergillus niger*, a multigene family encoding endopolygalacturonases (PGs) was identified [1]. Thus far six out of seven members of the endopolygalacturonase-encoding gene family have been sequenced [1-5] and the corresponding enzymes biochemically characterized [4-6]. Although the mature *A. niger* endopolygalacturonases share overall 66-85 % of amino acid identity, they exhibit quite different enzymatic properties. The specific activity of the *A. niger* PGs on polygalacturonate ranges from 415 nkat.mg^{-1} for PGC to 36.5 µkat.mg^{-1} for PGII. Furthermore, PGA and PGB appeared to prefer partially methylated substrates (*viz.* pectin with 22–45 % degree of esterification (DE)) in contrast to the other PGs, which tolerate methylation to various extent. The six *A. niger* endopolygalacturonases further differ in their pH optima (ranging from 3.9 to 5.0) and their mode of action on oligogalacturonates of defined chain length. In addition, initial studies of the expression of the endopolygalacturonase genes in *A. niger* indicated differences in their regulation at the transcriptional level as well [4-5].

One of our questions addresses the understanding, why *A. niger* has seven genes encoding one type of enzyme, an endopolygalacturonase.

A recent phylogenetic analysis of 35 related fungal endopolygalacturonases present in the database [7] showed that the *A. niger* endopolygalacturonases fall into four out of five monophyletic groups. Since each of the groups consists of endopolygalacturonases from different fungi, it was suggested that the ancestor gene of each of the five groups existed prior the divergence of these fungal species. This observation together with the results of our studies on the properties of the individual *A. niger* endopolygalacturonases suggest, that each one of the individual enzymes has its own physiological function for the fungus.

Here we present the study of a new type of endopolygalacturonase, which fits this view.

Abbreviations:
pga - polygalacturonase-encoding gene; PG - endopolygalacturonase; CREA - carbon catabolite repressing protein A; (Gal$p$A)$_n$, oligogalacturonate with degree of polymerization (n); r(Gal$p$A)$_n$, reduced oligogalacturonate with degree of polymerization (n); DE - degree of esterification; BCF - bond cleavage frequency; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; EP complex - enzyme-product complex.

2. Materials and Methods

Bacterial and fungal strains. For the propagation of phages and plasmids the *E. coli* strains LE392 [8] and DH5α [9] respectively, were used. For transformation of aspergilli the following strains were chosen: *A. niger* NW188 (*csp*A1, *pyr*A6, *leu*A1, *prt*F28, *gox*C17) and *A. nidulans* G191 (*paba*A1, *pyr*G89, *fwn*A1, *ua*Y9). The induction studies were performed using the wild type *A. niger* strain N400, the *A. niger* NW188 multicopy transformants of pIM3790 (NW188::pIM3790/61) (for plasmids see below) and the host *A. nidulans* G191 and its multicopy transformant of pIM3790 (G191::pIM3790/2.14). PGD was purified from the NW188::pIM3793/30 *A. niger* transformant.

Fig. 1: The partial restriction map of the 5.0-kb *Xho*I insert of pIM3790. The black and grey bars depict the position of *pga*D and the truncated ORF of the YAN9_SCHPO like protein, the arrows indicate the 5'→3' orientation of the genes. The capital letters correspond to the following restriction enzymes: X=*Xho*I, B=*Bam*HI, P=*Pst*I and H=*Hind*III.
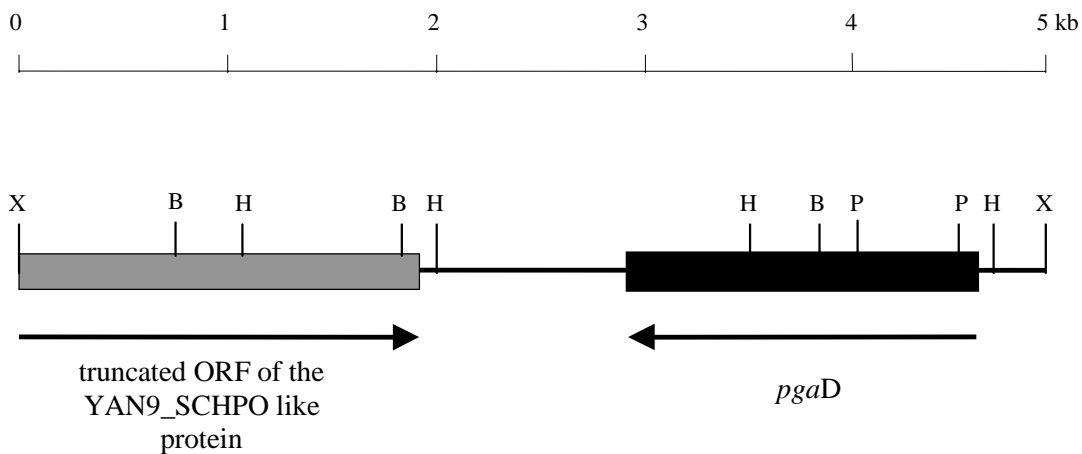
Phages and plasmids. Isolation of the *pga*II recombinant phages λD8 and λD11 was described by Bussink *et al.* [1]. For routine subcloning of DNA fragments and construction of the plasmids the phagemids pBluescriptSK+ and KS+ [10] were used. The pGEM-T Easy kit (Promega, Madison, WI, USA) was used for cloning of PCR fragments.

In the co-transformation of aspergilli pGW635 containing the *A. niger pyr*A [11] was used to restore the uridine prototrophy.

pGW1800 [2] and pIM3790 (see below) harboring the *pga*II gene, and the *pga*D gene respectively, were used for isolation of DNA fragments which served as probes. pIM3790 contains the originally subcloned 5.0 kb *Xho*I fragment of phage λD11 (see text and Fig. 1) and was used for further subcloning and sequencing.

pIM4700, the expression cassette comprising the pyruvate kinase A [12], *pki*A, promoter and suitable cloning sites downstream of the promoter was a gift of Dr. Jos Wubben and was constructed in the following way. At the first step a *Nsi*I site was introduced into the *Sst*II site of pBluescriptKS+. The plasmid was digested with *Sst*II and the protruding 3' ends were polished by T4 DNA polymerase action (Gibco BRL, Life Technologies Inc., Gaithersburg, USA). In the next step, the

dephosphorylation of the linear plasmid was done with calf intestinal alkaline phosphatase according to the manufacturer's recommendations (Gibco BRL). A *Nsi*I linker (5'- TGCATGCATGCA - 3', Stratagene Cloning Systems, La Jolla, CA, USA) was ligated into the dephoshorylated *Sst*II site and the resulting *Nsi*I site containing plasmid pBluescriptKNS$^+$ was confirmed by sequencing. In the next step, the 0.7-kb *Sst*I/*Nsi*I fragment of the *pki*A promoter was isolated from pPROM-H [4] and ligated into *Sst*I/*Nsi*I digested pBluescriptKNS$^+$, resulting in pIM4700.

pIM3793 containing the *pki*A-*pga*D fusion was constructed in the following way. pIM3790 was used in a PCR to generate a 309 bp *pga*D fragment in which the *Nsi*I restriction site was introduced at the translation start point. Two primers were designed: the forward (*Nsi*I) primer, a twenty-two-residue oligomer 5' CTTTTCATCATGATGCATCGCA 3', located at positions 383 to 404 bp and the reverse primer, a nineteen-residue oligomer 5' TACCTTGCTGCTGCTGGAG 3', annealing at the complementary DNA strand at the position 691 to 672 bp. The PCR cycler was programmed as follows: 5 min denaturation at 95 $^{\circ}$C and 30 cycli of 1 min denaturation at 95 $^{\circ}$C, 45 sec annealing at 51 $^{\circ}$C and 1 min extention at 72 $^{\circ}$C. The final extension was 10 min at 72 $^{\circ}$C. 35 pmoles of each primer were used in the reaction. The PCR fragment was isolated and checked for sequence mistakes. The final construct was made by the ligation of *pga*D fragments into pIM4700 in three steps. First the 200 bp *Nsi*I/*Pst*I fragment (see Fig. 1), released from the 309-bp *pga*D PCR fragment was ligated into *Nsi*I/*Pst*I digested pIM4700. Next, the 4.2-kb *Pst*I/*Xho*I fragment, isolated from pIM3790 (see Fig.1), was ligated into 3' of the *pki*A-*pga*D intermediate vector. The construction of pIM3793 was completed by the insertion of the 0.4-kb *Pst*I (Fig.1) fragment and checked for the correct orientation by restriction analysis.

DNA and RNA manipulations. The phage and plasmid DNA isolations and other molecular techniques were essentially carried out as described by Sambrook *et al.* [8]. The restriction enzymes were used as recommended by the supplier (Gibco BRL). The nucleotide sequence data were determined using either the Cy5^{TM} AutoCycle Sequencing Kit (Pharmacia Biotech. AB, Uppsala, Sweden) or the Cy5^{TM}-dATP Labeling Mix (Pharmacia Biotech.) with universal and reverse primers or with gene specific primers respectively. The reactions were analyzed with an ALFexpress^{TM} DNA sequencer (Pharmacia Biotech.). Computer analysis was done using the program GeneRunner (Hastings Software, Inc., Hastings, NY, U.S.A.).

Total RNA was isolated using TRIzol^{TM} Reagent according to the supplier (Gibco BRL). The RNA concentration was estimated spectrophotometrically at 260 nm. 10 µg or 15 µg of RNA were separated on 1.2 % (mass/vol.) agarose gel under glyoxal denaturation conditions [8]. After electrophoresis gels were capillary blotted overnight onto Hybond-N^{TM} membranes (Amersham International plc, Little Chalfont, Buckinghamshire, UK). For the transfer of RNA 10 x SSC buffer was used (1 x SSC: 0.15 M NaCl, 0.03 M tri-sodium citrate dihydrate, pH 7.0). After transfer the membranes were washed in 2 x SSC, dried and cross-linked by UV for 3 min.

The hybridization of Southern and northern blots was performed essentially as described by Sambrook *et al.* [8]. Northern blots were hybridized at 42 $^{\circ}$C in hybridization buffer containing 50 % (vol./vol.) of formamide. Southern blots were washed twice at 65 $^{\circ}$C for 30 min in buffers containing 0.5 % (mass/vol.) SDS and 2 x SSC and 0.5 x SSC. For the northern blots, the same washing regime was used with buffers containing 0.1 % (mass/vol.) SDS and 2 x, 1 x, 0.5 x and 0.2 x SSC, respectively.

For the transformation of aspergilli 1 µg of selection plasmid (pGW635) and 20 µg of cotransforming plasmid were used. The transformation was performed as described by Kusters-van Someren *et al.* [13].

RT-PCR. To verify the 5' end of the *pga*D gene and to determine the *pga*D expression in *A. niger*, RT-PCR was used. The RNA samples were obtained from mycelia of *A. nidulans* and *A. niger* strains grown under conditions as described below. All RNA samples were treated with the RQ1 RNase free DNase according to the protocol of the supplier (Promega). The RT-PCRs were performed essentially according to Gilliland *et al.* [14] with small modifications. For the synthesis of the first cDNA strand, the *pga*D specific primer "cDNA⁻", a nineteen-residue oligomer 5' ACCCGTGAGAT-CGATGGTG 3', annealing at positions 1014 bp to 996 bp, was used. The ss-cDNA product was then divided over three PCR tubes, each containing a different forward primer, *viz.* primers "cDNA1$^+$": an eighteen-residue oligomer 5' ATGCATCATCACGCTTTG 3', "cDNA2$^+$": a nineteen-residue oligomer 5' ATCTGCTCCTGCTTCCACC 3' and "cDNA3$^+$": a twenty-residue oligomer 5' TCCCTAGTA-ACTTCACACTC 3', annealing at positions 708-726 bp, 637-655 bp and 349-368 bp respectively. 100 pmoles of each primer were used in the reactions. For the synthesis of the second cDNA strand the PCR cycler was programmed as follows: 5 min denaturation at 95 ^{o}C, 30 cycli of 1 min denaturation at 95 ^{o}C, 1 min annealing at 47 ^{o}C, 51 ^{o}C or 52 ^{o}C for the reaction with the forward primers "cDNA1$^+$", "cDNA2$^+$" and "cDNA3$^+$" respectively, and 1 min extension at 72 ^{o}C. The final extension was done for 5 min at 72 ^{o}C. The amplified fragments were purified, cloned into pGEM-T Easy and sequenced. All PCRs were performed using the Pharmacia Taq-polymerase according to the manufacturer's instructions (Pharmacia Biotech.).

***pga*D expression studies.** In order to compare the transcription of *pga*D in different strains (wild type and *pga*D multicopy transformants), the mycelium was grown under the following conditions. 250 ml Erlenmeyer flasks with 50 ml of minimal medium containing per liter: 4.0 g NH_4Cl, 15 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 0.1 % (mass/vol.) dialyzed yeast extract, trace elements [15], supplements required and as a carbon source 3 % (mass/vol.) sucrose or a 2 % (mass/vol.) mixture (1:1) of Obipektin apple pectin, DE 61.2 % (Bischofszell, Switzerland) and fine ground sugar beet pulp, were inoculated directly with 10^6 spores/ml or with 3 g wet mycelia, pregrown for 20 hours. The pregrowth was done under the same culture conditions with 1.5 % (mass/vol.) sucrose as a sole carbon source. The mycelium was washed with sterile saline (8 g/l NaCl) and water prior to the shift. The starting pH was adjusted to pH 6.0. The temperature for growth was set at 37 ^{o}C and 30 ^{o}C for *A. nidulans* and *A. niger* strains respectively. The cultures were grown in an orbital shaker at 250 rpm. 20 and 40 hours after inoculation or shift, the mycelium was harvested on a Büchner funnel with a nylon gauze, washed with saline and immediately stored at -70 ^{o}C.

Selection of PGD overproducing transformants. The overproducing *A. niger pga*D transformants were selected as follows: 0.1 g of washed mycelia of the transformed and control strains, grown under the conditions described below, were incubated for 1 hour at 30 ^{o}C in an Eppendorf tube containing per 0.5 ml: 50 mM sodium-acetate buffer pH 4.2 and 0.25 % (mass/vol) polygalacturonic acid. The reaction was stopped by boiling the sample for 4 min and the degradation products were analyzed by HPAEC-PAD as described before [4]. The HPAEC-PAD profiles of the *A. niger pga*D transformants were compared with the untransformed parental strain. In a number of transformants only monogalacturonate and digalacturonate were detected following the incubation, while in the control strain also longer galacturonic acid oligomers were still present. The strains showing only mono- and di-galacturonate were considered as putative *pga*D transformants and further analyzed genetically (see the text).

Purification of PGD. Seven 1l Erlenmeyer flasks with 300 ml of minimal medium supplemented with leucine (0.02 % (mass/vol.)) were inoculated with 10^6 spores/ml of *A. niger* NW188::pIM3793/30 and cultivated at 30 ^{o}C for 22 h at 250 rpm in an orbital shaker. The composition of the minimal media was based on Pontecorvo *et al.* [16], except that NaNO₃, serving as the nitrogen source, was replaced by NH₄Cl (4 g/l). 2 % (mass/vol.) fructose served as the carbon source. The culture fluid was collected upon harvesting of the mycelia by filtration through a Büchner funnel with nylon gauze and diluted 5-fold with water. The pH was adjusted to 6.0. In the next step, batchwise adsorption of PGD to DEAE Streamline (Pharmacia Biotech.) was followed by elution with a pulse of 0.01 M piperazine/HCl buffer, pH 6.0 containing 1 M NaCl. The fractions with PG activity were pooled and extensively dialyzed against the same buffer without NaCl. The dialysate was loaded onto a Source 30Q column (1.5 x 8 cm, Pharmacia Biotech) equilibrated with 0.01 M piperazine/HCl, pH 6.0. Elution was performed with a 300 ml linear gradient (0 – 1 M NaCl) in the same buffer and 5 ml fractions were collected. PG containing fractions were pooled, diluted 5-fold with 0.01 M piperazine/HCl, pH 6.0 buffer and re-chromatographed on the same column. Elution was performed with a 250 ml linear gradient (0 – 0.5 M NaCl) in 0.01 M piperazine/HCl, pH 6.0. Fractions containing pure PGD, as judged by SDS-PAGE, were pooled and dialyzed against 0.05 M sodium acetate buffer, pH 5.0. The final yield from a 2.1 l culture was 19 mg of PGD. The enzyme was stored at 4 ^{o}C in the presence of 0.02 % (mass/vol.) sodium azide.

Enzyme assays and analytical methods. PGD activity on polygalacturonate, the determination of the pH optimum, the enzymatic activity on lemon pectins with 7, 22, 45, 60 and 75 % DE (Copenhagen Pectin A/S, Lille Skensved, Denmark), the product progression on polygalacturonate, determination of bond cleavage frequencies (BCF) on (reduced) oligogalacturonates were all performed as described before [4,6]. Assays on specifically methylesterified di- and tri-galacturonates, preparation of substrates and product analysis were described by Kester *et al.* [17]. The molar extinction coefficient for PGD, 38070 $M^{-1}.cm^{-1}$, was calculated by the method of Edelhoch [18]. The purity of PGD was monitored by SDS/PAGE. Gels were stained with Coomassie brilliant blue R250 and the molecular mass was estimated using molecular weight markers, test mixture 4 (Serva, Boehringer Ingelheim, Germany). The N-terminal sequencing was done at Eurosequence bv, Groningen, The Netherlands, using automated Edman degradation.

Deglycosylation. PGD was denatured by heating for 5 min at 100 ^{o}C in 0.05 M sodium phosphate buffer, pH 8.5, 0.1 % (mass/vol.) SDS, 0.1 M 2-mercaptoethanol and 0.01 M EDTA. N-deglycosylation was performed by incubation of denatured PGD (20 µg) with N-glycosidase F (3 U, Boehringer Mannheim, Germany) in the presence of 0.5 % (vol./vol.) Nonidet-P40 or 0.5 % (vol./vol.) Triton X-100, for 20 h at 37 ^{o}C. O-deglycosylation was performed by incubation of the purified PGD in 0.1 M NaOH, at room temperature for 1 h, followed by neutralization with 0.1 M HCl. The deglycosylation was monitored by determination of the protein molecular mass by SDS-PAGE.

3. Results and Discussion

Characterization of λD8 and λD11 phages. According to Bussink and coworkers [1], the *pga*II hybridizing recombinant phages of class D, isolated from the *A. niger* N400 phage

genomic library, might contain more than one endopolygalacturonase-encoding gene. Southern blot analysis of restriction enzyme digested λD8 and λD11 DNA, using the 1.3-kb *Bam*HI/*Bgl*II fragment of pGW1800 as a probe, revealed no differences in the restriction pattern of the DNA fragments containing the putative *pga*D. We considered the *pga*D harboring fragment of these phages as identical and continued with subcloning and sequencing of the λD11 phage. The partial restriction map of the 5.0-kb *Xho*I *pga*D subclone, pIM3790, is depicted in Fig.1.

Fig. 2: A comparison of the gene organisation of *pga*D, the endopolygalacturonase from *F. moniliforme* and *Bcpg*3 from *B. cinerea*.
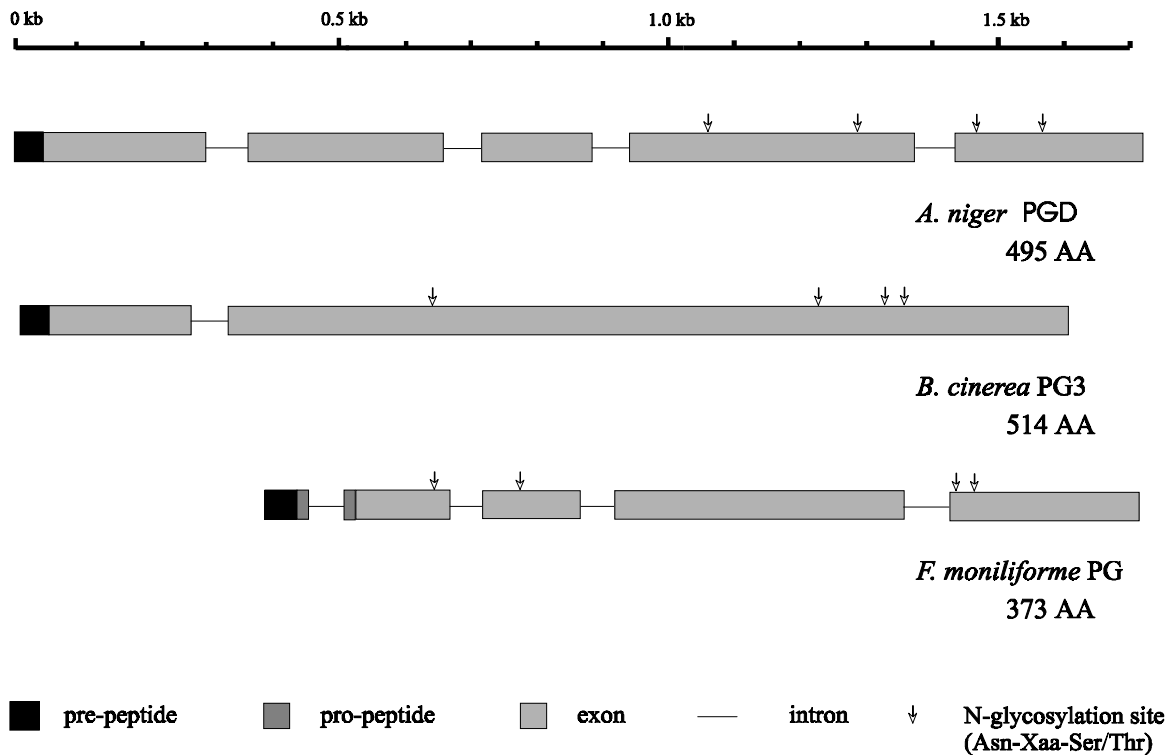
Analysis of the nucleotide sequence of pIM3790. The nucleotide sequence data obtained from pIM3790 subclones revealed the presence of a new endopolygalacturonase-encoding gene, *pga*D. The 2371 bp sequence from the *Xho*I site located at the 3' end of pIM3790 (see Fig. 1), containing 1716 bp of the protein coding region and 394 bp and 261 bp of the 5' and 3' non-coding sequences respectively, was determined over two strands. The length of the *pga*D-coding region exceeds that of the other *pga* genes from *A. niger* by a more than 400 bp at the 5' part of the gene. The remainder of the 5.0-kb *Xho*I insert of pIM3790 was sequenced over one strand. The truncated ORF at the 5' end with a high sequence identity to

Schizosaccharomyces pombe hypothetical 73.1 KD C3H1.09C transmembrane protein (accession number: Q10074,YAN9_SCHPO) was found in the opposite orientation to *pga*D in pIM3790 (see Fig. 1).

*pga*D shares the highest sequence similarity with the *Fusarium moniliforme* endopolygalacturonase gene [19] and the *Bcpg*3 gene from *Botrytis cinerea* [7]. This is also demonstrated by the similar exon and intron organization, when *pga*D is compared to the *F. moniliforme* endopolygalacturonase-encoding gene (see Fig. 2). Thus, the *pga*D gene structure deviates from the overall highly conserved structural gene organization of the other members of the endopolygalacturonase-encoding gene family from *A. niger* [1-5]. So far, out of more than 30 fungal *pga* sequences present to date in the database, only the *A. niger pga*D and the *Bcpg*3 of *B. cinerea* are longer.

Based on the nucleotide sequence homology with the *F. moniliforme pga* (Fig. 2), the presence of introns B, C and D was predicted. Intron A, localized in the 5' end of *pga*D, and the translation start point were confirmed by RT-PCR as described in the Materials and Methods section. The RNA samples from the mycelia of the *A. niger* and *A. nidulans pga*D multicopy transformants were used. While in the case of *A. niger* cDNA only one mode of splicing for intron A was observed at 690 bp, *viz.* at 5' GTGAGT, in the *A. nidulans* cDNA amplified fragments two modes of splicing of intron A occurred (Fig. 3). In *A. nidulans* splicing was also found at sequence position 5' GTAGTG, three nucleotides upstream of the previously mentioned splicing site. Both spliced forms of the *A. nidulans pga*D cDNA were amplified from one RNA population, thus demonstrating its heterogeneity. This shift of three nucleotides leads to the loss of one amino acid, valine. Since we did not observe the alternative splicing in the 5' end of intron A in *A. niger*, we can not make at this moment any conclusions about the possible function of a one amino acid deletion in the N-terminal part of the protein. The remaining introns B, C, and D, were found in identical positions as the corresponding introns in the *F. moniliforme* endopolygalacturonase (Fig. 2), and they comply to the fungal consensus 5' GTPuNGPy and 3' PyAG of splice sites [20], except for one change in the 5' part of intron C (*viz.* 5' GTACGA).

Fig. 3: The nucleotide sequence of *pga*D and the derived amino acid sequence of PGD (on the opposite page). In the promoter region the small italicized hexanucleotides indicate the putative 5' SYGGRG 3' CREA binding site [23], the bold small letters show the (C)CAAT boxes [20] and the TATAA box is depicted in capitals. The 5' and 3' splicing sites of intron A are in bold and italics. The putative poly-A site [20] is italicized in the terminator sequence. The beginning and the sequenced residues of the mature PGD are depicted in capital italic letters. The putative N-glycosylation sites (**Asn**-Xaa-Thr/Ser) are in bold.

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                          ctcgagctcggctctggaacgctgactgccgacggtgccaatcaaccag    49
caagtcaggacccctgctggacgctatgcgggggccgaccgaacgttctaaccaaagttgtctccact           118
caagcagaagatgggtcggttcacgatgcatccatgctaagctccggaggaggtggctgagcttagctt           187
attgaatcttgacttccggctagaccaacggtactgtggttctcggctctactgatccgatgctatcaa          256
tctatcaTATAAgaatgctgggttgtcgacggctgtccatggttgaaatctggaattcaacgccctcat          325
tgtccttccttttttcacttcgtttccctagtaacttcacactctttagtctttcattctttttcatcatg       394

ATGAAGCGCAGCGCGCTCCTTGCCTCTTTCCTTCCTCTCGCCCTGGGGGTGCGACAGCGCTGAGACTCAC        463
 M   K   R   S   A   L   L   A   S   F   L   P   L   A   L   G   C   D   S   A   E   T   H      23
TCCTGTGCCTCTGCCTTTTCCGTCTCCTCTGCGGCCGCCGCTAGCTTCTGCGCCACCTTCACGGCGAGC        532
 S   C   A   S   A   F   S   V   S   S   A   A   A   A   S   F   C   A   T   F   T   A   S      46
ACCGTGACTGCCACGACTGGCGTGCCGGATGTCTTCCTTTCCAACTGTGACTACAAGACCAAGCACCTC        601
 T   V   T   A   T   T   G   V   P   D   V   F   L   S   N   C   D   Y   K   T   K   H   L      69
TCCAGTGCCTGCAGCTGCCTGGGCACCGCCGATGGATCTGCTCCTGCTTCCACCCCGGCTGCCCCGGCA        670
 S   S   A   C   S   C   L   G   T   A   D   G   S   A   P   A   S   T   P   A   A   P   A      92
GTCTCCAGCAGCAGCAAGgtagtgagtttaccatttagatgcatcatcacgctttgattactgattcag        739
 V   S   S   S   S   K   (V)                           intron A                       99
tctagGGCAAGGCACCTGCTGTTGCAGCCACTCGCACCTCGGCGGATCCCGACCACTTTCCACACCACTG     808
        G   K   A   P   A   V   A   A   T   R   T   S   A   I   P   T   T   F   H   T   T     120
CCGTTCGTGTGCCCCTGTCCACCTCGGCAGCGGCGGCTGTGACCTCCCAGGCTGTCCTCCCGGCGCAGA     877
 A   V   R   V   P   L   S   T   S   A   A   A   A   V   T   S   Q   A   V   L   P   A   Q     143
GCTCCGTGGCAGGCAACGGAGGCACTACCTGCACAGTGACTGAGTACGCTTCGATCTCCTCTGCCGTCG     946
 S   S   V   A   G   N   G   G   T   T   C   T   V   T   E   Y   A   S   I   S   S   A   V     166
CATCCTGCTCCAACATCCTCCTGTCCAACATCAACGCCCTGCATCGAGCACCATCGATCTCACGGGTC     1015
 A   S   C   S   N   I   L   L   S   N   I   N   A   P   A   S   S   T   I   D   L   T   G     189
TGCAGACCGGTGCGGCTGTCATCTTTGCTGGTGAAACCgtaagtcatatccttcctatattaggggcaa     1084
 L   Q   T   G   A   A   V   I   F   A   G   E   T                          intron B         202
ccgccatactaacggagagtccgacagACCTTCGGTGACACCTACGACAGCGACTTCGACCCCATTGTC     1153
                             T   F   G   D   T   Y   D   S   D   F   D   P   I   V           216
ATCTCCGGTACCGACGTGACCATCACCGGTGAGGAGGGACATGTCATCAACGGTAACGGTGAAGCCTAC     1222
 I   S   G   T   D   V   T   I   T   G   E   E   G   H   V   I   N   G   N   G   E   A   Y     239
TGGGACGGCGAGGGATCCAACGGCGCACACGGACAAgtacgagcctaccaccacagccctttcatgaaga     1291
 W   D   G   E   G   S   N   G   G   Q   D   K                            intron C           251
accctactcatagctcctaccagGCCCGACCACTTCATTGTCGTCAAGGACATGTACAACTCCAAGATC     1360
                        P   D   H   F   I   V   V   K   D   M   Y   N   S   K   I           266
GAGAACCTCAACATCCTGAACTGGCCCGTCCACTGCTTCGAGATCGAGAACACCGAGTACCTGACCATC     1429
 E   N   L   N   I   L   N   W   P   V   H   C   F   E   I   E   N   T   E   Y   L   T   I     289
TCCGGCCTGATCCTCAACAACACCGCCGGTGACGCCGCCAACAGCAAGAGTGACGGCGACCCGGCCGCC     1498
 S   G   L   I   L   N   N   T   A   G   D   A   A   N   S   K   S   D   G   D   P   A   A     312
CACAACACCGACGGCTTCGACATCAAGCAGAGCGACTTCCTGACCCTCTCCAACTCCTGGGTGCACAAC     1567
 H   N   T   D   G   F   D   I   K   Q   S   D   F   L   T   L   S   N   S   W   V   H   N     335
CAGGACGACTGTGTTGCCGTTACCAGCGGTAGCTCGATCGTCGTCGACAACCTCTACTGCTACGGTGGT     1636
 Q   D   D   C   V   A   V   T   S   G   S   S   I   V   V   D   N   L   Y   C   Y   G   G     358
CACGGTCTCTCCATCGGCTCCATCGGCGGCAAGAGCAACAACACCGTCGACGGCGTCACCTTCTCCAAC     1705
 H   G   L   S   I   G   S   I   G   G   K   S   N   N   T   V   D   G   V   T   F   S   N     381
TCCCAGGTCATTAACAGCGAGAACGGCTGCCGCATCAAGTCTAATGCTGATACCACTGGCGAGgttagt     1774
 S   Q   V   I   N   S   E   N   G   C   R   I   K   S   N   A   D   T   T   G   E           402
ccatcctatcttaaccatcaacatcttattattctaaccaccgagcaaatacagGTCTACAACGTCAAG     1843
             intron D                                          V   Y   N   V   K           407
TACGAAAACATCACTCTGTCCGGCATCAGCGACTACGGCATCGACATCCAGCAGGACTACGAGAACGGT     1912
 Y   E   N   I   T   L   S   G   I   S   D   Y   G   I   D   I   Q   Q   D   Y   E   N   G     430
GGTGCTACCGGTGACCCGACCAACGGCGTCAAGATCGAGAACATCTCTTTCGTCAATGTCAAGGGTACT     1981
 G   A   T   G   D   P   T   N   G   V   K   I   E   N   I   S   F   V   N   V   K   G   T     453
ATGAGCGACGGCAAGGATTACTACATCCTCTGTGGTGATGGCAGCTGCTCTAACTTTGTCTTCACTGAT     2050
 M   S   D   G   K   D   Y   Y   I   L   C   G   D   G   S   C   S   N   F   V   F   T   D     476
GTCGACATCACCGGTGGTAGCGATGATAGCTGCAACTACCCTCTTCGGGATGCCCTTAAatcatgggt     2119
 V   D   I   T   G   G   S   D   D   S   C   N   Y   P   S   S   G   C   P   #               495
tgtgtgggttgcgcagtaggtgaagggggttggaaatactgaattgtatagtatctatctctctctattt     2188
tgatttcttgctttcctgggatgctgtggaccagcggattaggatagtaaaaccacctagttggtttca     2257
tttcaatggagggttcattatccattgtatatactattacataaacaataattttgtaggtgtataagg    2326
gggaagtacattctttgtagatgtgacagacatggtcaagtctac                            2395
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Expression of *pga*D in *A. niger* and *A. nidulans*. The expression of *pga*D was studied in wild type *A. niger*, and the pIM3790/61 and pIM3790/2.14 multicopy transformants of *A. niger* and *A. nidulans* respectively, under various growth conditions as described in Materials and Methods. Previous studies [5] revealed, that two members of the *A. niger* endopolygalacturonase-encoding gene family, *pga*A and *pga*B, are constitutively expressed. However, *pga*E mRNA could not be detected in a wild type *A. niger* strain, nor in an *A. niger* multicopy transformant, using both northern blot and RT-PCR analyses [4]. By northern blot analysis, the *pga*D specific mRNA could only be detected in the *A. nidulans* multicopy G191::pIM3790/2.14 transformant, at both time points (20 and 40 h) and under all growth conditions (sucrose or the mixture of pectin and sugar beet pulp) tested. No signal was detected in the wild type and the NW188::pIM3790/61 *pga*D multicopy transformant of *A. niger* (data not shown).

Fig. 4: Southern blot analysis of the pIM3790 *A. niger* and *A. nidulans* (A) and the pIM3793 *A. niger* (B) multicopy transformants. The lanes 1-6 in panel A correspond to: 1) *A. niger* N400; 2) *A. niger* NW188::pIM3790/61; 3) *A. niger* NW188::pIM3790/72 and 4) *A. niger* NW188::pIM3790/76 multicopy transformants; 5) *A. nidulans* G191 and 6) *A. nidulans* G191::pIM3790/2.14 multicopy transformant. The chromosomal DNA was digested with *Eco*RI. The arrow indicates the hybridising band of the linear pIM3790 plasmid. In panel B the lanes 1-6 correspond to *Hind*III restricted chromosomal DNA of the multicopy transformants of *A. niger*: 1) NW188::pIM3793/17; 2) NW188::pIM3793/30; 3) NW188::pIM3793/35; 4) NW188::pIM3793/36 and 5) NW188::pIM3793/37 and 6) *A. niger* N400. In both experiments the 1.3-kb *Hinc*II *pga*D fragment comprising the coding region was used as a probe. On the right hand side of the blots the position of fragments from λDNA digested with *Hind*III and *Eco*RI is depicted.
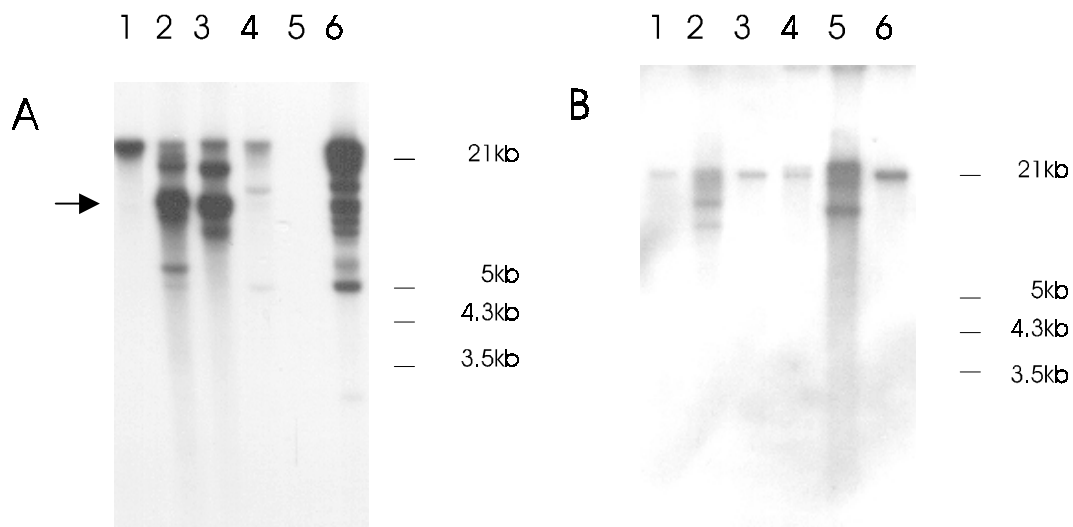
However, when the RNA sample of the *A. niger* NW188::pIM3790/61 *pga*D multicopy transformant, grown for 20 h after the shift to minimal medium containing the mixture of apple pectin and sugar beet pulp, was used in the RT-PCR, a *pgaD* cDNA fragment was generated (see above). This demonstrates the presence of *pga*D mRNA in the *A. niger pga*D multicopy transformant, although at a very low level. Since the length of the *pga*D promoter is sufficient to ensure the start of the transcription and both transformants contain a high copy number of pIM3790 (see Fig. 4), the changes in the *pga*D expression levels might be due to the differences in transcriptional regulatory mechanisms in *A. niger* and *A. nidulans*, acting within the 394 bp of the *pga*D promoter. Similar differences in the expression of another pectinolytic gene from *A. niger*, *pel*A encoding pectin lyase, were already previously reported for *A. niger* and *A. nidulans* [13].

The derived primary sequence of PGD. Based on the *pga*D nucleotide sequence, the derived PGD consists of 495 amino acid residues. The calculated relative molecular mass is 50 788 and the isoelectric point approximately 4.1. A signal peptide cleavage site was predicted in PGD after the first 16 N-terminal amino acid residues [21] and confirmed by N-terminal sequencing of the purified enzyme. Four possible N-glycosylation sites (Asn-Xaa-Ser/Thr) were found in PGD (see Fig. 2). Based on the N- and O-deglycosylation experiments, only O-glycosylation of PGD seemed to occur. The O-deglycosylation treatment shifts the molecular mass of the purified PGD (see below) on a SDS-PAGE gel from a smeared >100 kDa protein band to a diffused band with an approximate molecular mass of 62 kDa (data not shown).

The PGD of *A. niger* and BCPG3 of *B. cinerea* stand out from the primary structure of the other fungal PGs [7] by an approximately 140 amino acid long N-terminal extension. One of the possible functions of this N-terminal extension could be to anchor the enzyme in a membrane. We used two computer programs, *viz.* SOSUI (http://azusa.proteome. bio.tuat.ac.jp/sosui/) and PHDhtm (http://dodo.cpmc.columbia.edu/predictprotein), to predict the localization of possible transmembrane helices within the N-terminal extension. The SOSUI prediction, which is based on the physicochemical properties of amino acid sequences, characterized PGD as a soluble protein, whereas the PHDhtm program, which uses the evolutionary information as input to the network system, predicted one or two (based on the variable input matrix) transmembrane helices in the N-terminal extension of PGD. Because these results were controversial, we decided to overproduce and purify PGD and to raise a specific antibody to localize the enzyme.

Localization of PGD. Initially the *A. nidulans* G191::pIM3790/2.14 transformant was used to overproduce and isolate PGD. Although a high level of mRNA (data not shown) was detected under the growth conditions tested (see Materials and Methods) no endopolygalacturonase activity was found in the culture fluid. However, upon sonification of the mycelia a low level of endopolygalacturonase activity was detected, when compared to *A. nidulans* G191. This indicated that PGD might be localized intracellulary or could be attached to the cell wall. To obtain higher levels of expression, the *pki*A promoter was used to drive the *pga*D transcription, and the *pki*A-*pga*D construct was used to transform *A. niger*. PGD was purified from the culture fluid of one of these transformants, although in much lower yield compared to the other *A. niger* PGs overproduced the same way [4-5]. However, the selection procedure of the transformants suggested that the enzyme might be attached to the cell wall (see Materials and Methods). The purified PGD was used to raise the specific polyclonal antibody in a mouse. The PGD antibody was applied in immuno-gold labeling experiments to localize the enzyme. The preliminary data show that PGD is secreted but remains attached to the fungal cell wall.

Fig. 5: Progression of products formed by PGD on polygalacturonate. Hydrolysis of 0.25 % (mass/vol.) polygalacturonate in 1 ml 50 mM sodium-acetate pH 4.2 (0.76 µg PGD) at 30 °C. 50 µl samples were analyzed by HPAEC-PAD. ●, $(GalpA)_1$; ■, $(GalpA)_2$; ▲, $(GalpA)_3$; ▼, $(GalpA)_4$; ◆, $(GalpA)_{5-7}$.
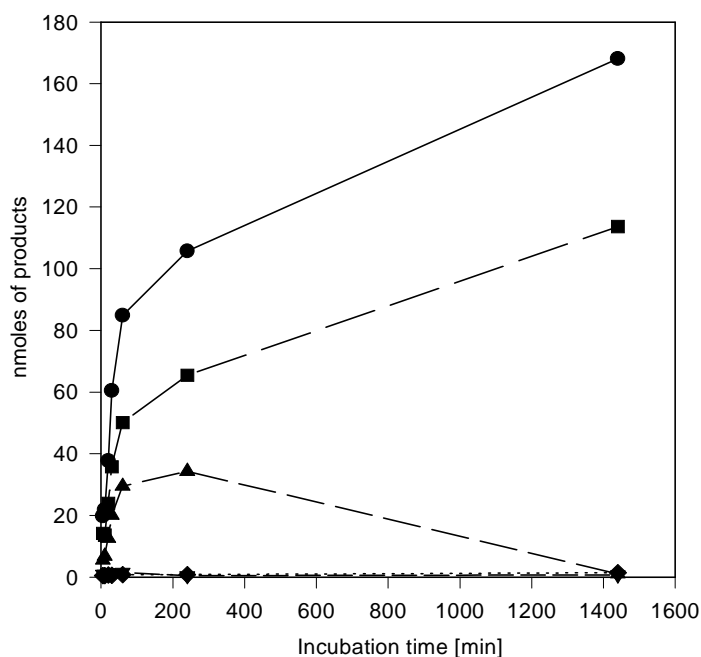
Biochemical characterization of PGD. The pH optimum of the purified PGD is around 4.2. However, this enzyme retains more than 30 % of its activity over a broad pH interval, from 2.4 to 5.2. The specific activity of PGD, determined at 30 $^\circ$C and pH 4.2, is 1.55 µkat.mg^{-1}. The kinetic parameters $K_{m\ app}$ and $V_{max\ app}$ measured under identical reaction conditions, are 0.2 $\pm$

0.03 mg.ml^{-1} and 1.57 $\pm$ 0.05 µkat.mg^{-1}, respectively. The relatively low specific activity of PGD on polygalacturonate suggests that polygalacturonic acid is not the optimal substrate for this enzyme.

Mode of action of PGD on polymeric and oligomeric substrates. The product progression for PGD using polygalacturonate as a substrate (Fig. 5) is characterized by a very small increase of oligogalacturonides ((Gal$p$A)$_n$) with longer chain length (n > 3) and a prominent increase of Gal$p$A and (Gal$p$A)$_2$. This type of action is typical for an endolytic enzyme with a strong processive behavior [5-6]. Of the four processive *A. niger* PGs (I, A, C and D), the enzyme described here is the most profound in this respect. This is also reflected by the bond cleavage frequencies using (Gal$p$A)$_n$ (n = 2-7). In Table 1 the bond cleavage frequencies on the (reduced) oligogalacturonate series (r(Gal$p$A)$_n$) are listed. Cleavage of r(Gal$p$A)$_n$ occurred exclusively at the second glycosidic bond from the reduced end up to n = 5, suggesting that subsite +1 can not accommodate a reduced galacturonate moiety. For r(Gal$p$A)$_6$ the third glycosidic linkage was the preferred bond hydrolyzed. The preference to hydrolyze the second glycosidic bond from the reduced end suggests, that, like the other *A. niger* PGs, PGD attacks the chain from the reducing end of the substrate. Interestingly, in contrast to the other *A. niger* PGs [4-6], PGD is able to hydrolyze the r(Gal$p$A)$_3$, but at a very low rate (0.17 nkat.mg^{-1}).

Hydrolysis of (Gal$p$A)$_2$ is also unique to PGD. Thus, unlike the other *A. niger* PGs which all require subsites –2 to +1 to be filled for catalysis, PGD only requires subsites –1 and +1 to be occupied. Comparison of the rates of hydrolysis of (Gal$p$A)$_n$ at 50 and 500 µM reveals, that, except for the dimer, the $K_{m\ app}$ values are below 50 µM. In fact, the rates at 50 µM are even somewhat higher than at 500 µM suggesting substrate inhibition as was observed for PGI [6]. At 50 µM (Gal$p$A)$_n$ the rate of hydrolysis of (Gal$p$A)$_n$ increased up to n = 4, and decreased for (Gal$p$A)$_5$ and (Gal$p$A)$_6$. At 500 µM substrate a similar tendency was observed. At both substrate concentrations the strongest rate increase (15-fold) was recorded upon occupying subsite –3, *viz.* (Gal$p$A)$_4$. The same rate increase was also recorded for r(Gal$p$A)$_5$ and r(Gal$p$A)$_6$ which also cover subsite –3. Moreover, binding of r(Gal$p$A)$_6$ at subsite –4 appears not favourable, this in contrast to PGI, II, A, C and E [4-6]. From these data and the product progression on polygalacturonic acid it is tentatively concluded that PGD is composed of only four functional subsites stretching from –3 to +1. At present we can not satisfactory explain the higher rates of hydrolysis of the reduced oligomers.

Table 1. Bond cleavage frequencies and hydrolysis rates of PGD on (reduced) oligogalacturonates of defined length. Assay conditions are: 500 μM (reduced) oligogalacturonates or 50 μM oligogalacturonates in 500 μl 50 mM sodium acetate, pH 4.2, were incubated with PGD. The calculation of the hydrolysis rates was based on the rate of product formation ([a]), rate of substrate consumption ([b]) and the rate of formation of reduced oligogalacturonates ([c]). Products were analyzed and quantified by HPAEC-PAD as described before [4]. The reducing end of the oligogalacturonates is indicated in bold. The asterisk indicates the reduced galacturonate. Bond cleavage frequencies are given in percentages and were determined from the 500 μM data. PGD is processive on (Gal$p$A)$_5$.

n														μkat. mg^{-1} 500μM	50μM
2											G	– (100)	**G**	0.67×10^{-3}[a]	0.67×10^{-4}[a]
3									G	–	G	– (100)	**G**	0.28×10^{-1}[a]	0.33×10^{-1}[a]
4							G	–	G	– (1)	G	– (99)	**G**	0.43[a]	0.53[a]
5					G	–	G	–	G	– (58)	G	– (42)	**G**	0.38[b]	0.46[b]
6			G	–	G	–	G	– (processive)	G	–	G	–	**G**	0.42[b]	0.33[b]
7	G	–	G	–	G	–	G	– (processive)	G	–	G	–	**G**	0.26[b]	
2											G	–	**G***	0	
3									G	–	G	– (100)	**G***	0.17×10^{-3}[c]	
4							G	–	G	–	G	– (100)	**G***	0.53×10^{-1}[c]	
5					G	–	G	–	G	–	G	– (100)	**G***	0.78[c]	
6			G	–	G	–	G	– (64)	G	– (16)	G	–	**G***	0.75[c]	

The mode of action analysis for (Gal$p$A)$_5$ to (Gal$p$A)$_7$ revealed deviation from stoichiometry of product pairs as expected for a processive enzyme. A ratio plot according to Robyt and French [22] for (Gal$p$A)$_5$ hydrolysis (data not shown), indeed demonstrated processive behavior on (Gal$p$A)$_5$. Due to the fact that processive behavior was observed already on (Gal$p$A)$_5$ and the strong preference for hydrolysis of (Gal$p$A)$_4$ at the first glycosidic linkage from the reducing end allowed for the calculation of the initial binding modes for (Gal$p$A)$_5$ as presented in Table 1. (Gal$p$A)$_2$ formation from (Gal$p$A)$_5$ served as the identifier of the first event in that particular mode and thus for the calculation of that particular frequency (58 %). From this the frequency of the binding mode that in a first event would result in the formation of Gal$p$A and (Gal$p$A)$_4$ logically followed (42 %). Next, the difference between the amount of

$(GalpA)_2$ and $(GalpA)_3$ served for the calculation of the degree of processivity. This learned that 66 % of the binding modes of $(GalpA)_5$ in $(GalpA)_4$ - $GalpA$ mode resulted in processive attack. The decrease of hydrolysis observed for $(GalpA)_{5-7}$ compared to $(GalpA)_4$ may be caused by the temporal tying up of the enzyme in the unproductive EP complex prior to the multiple attack.

Pectin degradation and specific activities on mono-methylesterified di- and tri-oligogalacturonates. The enzyme exhibits a relatively high activity on pectins with methylesterification up to 45 %, *viz.* 1.05 µkat.mg^{-1}. However, it appears that PGD prefers unmethylated substrate like PGI, II, C and E, although, compared to these enzymes, PGD shows the highest tolerance towards methylesterification.

Recently the use of synthetic mono-methylesterified di- and tri-galacturonates allowed us to study the tolerance for a methylgalacturonate binding to the subsites around the catalytic site of the individual *A. niger* PGs [17]. In contrast with the other *A. niger* PGs, PGD is able to hydrolyze all mono-methylesterified di- and tri-galacturonates (Table 2). This demonstrates that PGD can accommodate a methylated galacturonic acid at subsite -1 whereas the other enzymes were unable to do so [17]. It should however be noted that PGD was not able to split the fully methylated di- and tri-galacturonates. These data may explain the ability of PGD to still hydrolyze efficiently highly methylesterified pectins.

Table 2. Hydrolysis rates for *A. niger* PGD on mono-methylesterified di- and tri-galacturonates. Substrates (0.05 mM) dissolved in 300 µl 50 mM sodium acetate buffer pH 4.2 were incubated with 15 µg of PGD at 30 °C. The reaction rates were determined as described before [17].

Substrate	nkat.mg^{-1}	Relative rate (%)
$(GalpA)_2$	0.83×10^{-1}	100
1-methyl $(GalpA)_2$	0.12×10^{-1}	14
2-methyl $(GalpA)_2$	0.08×10^{-1}	10
$(GalpA)_3$	33.8	100
1-methyl $(GalpA)_3$	0.25×10^{-1}	0.07
2-methyl $(GalpA)_3$	0.03×10^{-1}	0.01
3-methyl $(GalpA)_3$	0.45	1.3

The biological function of PGD is most likely to generate galacturonic acid from the oligogalacturonates released from the pectin by the other PGs. This is based on the ability to hydrolyze the galacturonic acid dimer, the processivity of the enzyme to become evident starting from (Gal$p$A)$_5$ onwards, the likely presence of only four subsites, and the tolerance for a methylated galacturonic acid residue around the active site. We therefore propose that PGD is in fact an oligogalacturonate hydrolase.

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CHAPTER 5

Exploring the regulation of the expression of pectinase-encoding genes from *Aspergillus niger*

Abstract

The expression of several genes encoding enzymes active towards different parts of pectin was studied in *Aspergillus niger* using pectin or its monosaccharide constituents, D-galacturonic acid and L-rhamnose, as a carbon source. D-galacturonic acid appeared to induce a number of pectinase-encoding genes, including exopolygalacturonase (*pgx*), pectin lyase A (*pelA*), pectate lyase A (*plyA*), pectin methylesterase (*pme*) and rhamnogalacturonan lyase A (*rglA*). Sugar beet pectin was responsible for a strong induction of rhamnogalacturonan hydrolase A (*rghA*) 24 hours after the transfer of pregrown mycelia. Based on the specific induction profiles on D-galacturonic acid, the selected pectinase-encoding genes were divided over 3 groups. Expression of these genes was not detected when *A. niger* was grown on sucrose.

The 939 bp of the *pgx* and 1123 bp of the *pelA* promoter regions, were selected for the construction of promoter-reporter gene plasmids. The *pyrA* gene encoding orotidine-5'-phosphate-decarboxylase served as the reporter, in order to isolate *A. niger* pectinase regulatory mutants. Both constructs were introduced into an *A. niger pyrA* auxotrophic mutant and UV mutagenized. Although several categories of mutants expressing *pyrA*⁻ phenotype under inducing conditions were isolated, none of them was a regulatory mutant. In case of the *pelA* construct this approach was hampered by a previously introduced mutation in the *A. niger pyrA* auxotrophic host strain, which was responsible for decreasing the *pelA* expression. Several potential *pgx* regulatory mutants were characterised as either a mutation in the D-galacturonic acid utilisation pathway or as a *cis*-acting mutation in the *pgx-pyrA* construct. The latter case led to the identification of a 263 bp *pgx* promoter fragment responsible for induction by D-galacturonic acid.

In order to identify the region of the *pelA* promoter involved in the induction by D-galacturonic acid, a promoter deletion study of *pelA* was initiated. The *pelA* promoter fragments were fused with the *goxC* (glucose oxidase) reporter gene and the induced GOX activity was screened using a plate assay. The 721 bp fragment located -379 bp from the *pelA* translation start point up to -1100 bp was identified as necessary for a high level of *pelA* expression on D-galacturonic acid. Using protoplasts of a low copy number *pelA-goxC A. niger* transformant we found, that D-galacturonic acid uptake is inducible by pectin or its constituent(s). Furthermore, the increase of the D-galacturonic acid concentration (from 5 to 50 mM) enhanced the GOX expression under the control of the *pelA* promoter. However, a low level of GOX expression in the *pelA-goxC A. niger* transformant was also detected on 1 % (w/v) D-xylose, 1 % (w/v) D-glucose or 1 % (w/v) D-sorbitol in a plate assay and with 5 mM and 50 mM L-rhamnose or D-xylose in the protoplast assay.

Introduction

Aspergillus niger is an important industrial micro-organism used for the production of extracellular enzymes like proteases, cellulases, hemicellulases and pectinases. Many of its products hold the GRAS (generally regarded as safe) status and therefore they find a broad range of applications in the food, feed and beverage industry. Pectinases are enzymes able to degrade the complex heteropolysaccharide pectin, the major constituent of the middle lamella and the primary cell wall of higher plants. In industry they are usually used as poorly characterised enzyme mixtures produced by black aspergilli species, *e.g.* Pectinex (Novo Nordisk Biotech. Inc., Davis, CA) or Rapidase (DSM Gist brocades, Delft) from *A. niger*.

It is obvious, that due to the industrial importance of *A. niger*, the main interest of researchers is to study physiological conditions of growth in order to obtain the best production of pectinolytic enzymes. For instance, a much higher production of polygalacturonase (PG) and pectin methylesterase (PME) was found during solid state fermentation (SsF) of *A. niger* than during submerged fermentation (SmF) [1-2]. Moreover, the enzyme pattern of PGs and PMEs varied based on the fermentation system used. The SsF-grown mycelia, in both studies, were found to be more tolerant to glucose mediated carbon catabolite repression during pectinase production. Lower pH and a more complex carbon source, like sugar beet pulp or orange peel, stimulated PG [3-4] over PME [5] synthesis in the SsF of *A. niger* and *A. foetidus* respectively. Sensitivity of a pectinase regulatory system towards environmental conditions was also demonstrated by the effect of pH of the storage media on multiple forms of PGs produced by *A. niger* [6].

So far, twenty genes encoding enzymes active on pectin have been isolated from *A. niger* N400 (= CBS 120.49). They include two gene families of pectin lyases [7-9] and endopolygalacturonases [10-15] respectively, a pectate lyase-encoding gene [16], exopolygalacturonase (Benen, unpublished), pectin methylesterase (Kusters-van Someren, unpublished), rhamnogalacturonan hydrolase A and B [17], rhamnogalacturonan lyase (Benen, unpublished) and rhamnogalacturonan acetylesterase [18] genes. However, little is known about the molecular basis of the regulation of the pectinase-encoding genes in *A. niger*. Some genes, i.e. *pga*A and *pga*B, were found to be constitutively expressed [14], while others like *pga*II [20] and *pel*A and B [8-9] are induced by pectin and repressed in the presence of glucose. Promoter deletion studies of *pga*II led to the identification of a 220 bp promoter fragment, 576 bp upstream from the translation start point, containing an UAS similar to the yeast HAP2/3/4 binding site, responsible for high expression of PGII on pectin [20]. Furthermore, upon screening of the promoters of the endopolygalacturonase- and pectin lyase-encoding genes for

hexanucleotide sequences, the sequence 5' CCCTGA 3', was found to be conserved among these genes [12]. However, in both cases the additional experimental evidence of the significance of the proposed regulatory sequences is missing.

The number of the pectinase genes available in combination with our large laboratory collection of *A. niger* mutants, allowed us to initiate this study of the regulation of the expression of pectinase genes. Our aim was to investigate, whether the individual genes, encoding the enzymes acting on the different parts of the pectin molecule, are expressed in a concerted and/or consecutive manner, depending on the gradually released inducer(s) during the pectin breakdown. Since the main sugar constituent of the pectin backbone is D-galacturonic acid (galA), it was interesting to study, whether this is the real molecular inducer of the *A. niger* pectinolytic system, or whether the different pectinase genes are induced by various carbon sources. The recent cloning of the *xln*R gene encoding the major regulatory protein of the *A. niger* xylanolytic system [21] in our laboratory prompted us to exploit a similar strategy, based on the generation of regulator negative mutants, suitable to isolate gene(s) encoding the pectinase regulator(s) by complementation. Finally, *gox*C encoding glucose oxidase was chosen as a reporter system for a *pel*A promoter deletion study and for development of a screening method to identify the low molecular weight inducer(s) of the *pel*A expression.

The results of the various approaches to study the regulation of the pectinase genes expression are presented here.

Abbreviations: *pga* - endopolygalacturonase, *pgx* - exopolygalacturonase, *pel* - pectin lyase, *pme* - pectin methylesterase, *ply* - pectate lyase, *rhg* - rhamnogalacturonan hydrolase A, *rgl* - rhamnogalacturonan lyase, *gox*C - glucose oxidase, and *pyr*A - orotidine 5'-phosphate-decarboxylase encoding genes; galA - D-galacturonic acid; xyl - D-xylose; ara - L-arabinose; rha - L-rhamnose; gluA - D-glucuronic acid; gal - D-galactose; HPAEC-PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; FOA - 5-fluoro-orotic acid; *uri* - uridine; SM - supplemented minimal medium; CMS - supplemented complete medium; PROTO SM - protoplast stabilising medium; PG - endopolygalacturonase encoded by *pga*; PME - pectin methylesterase encoded by *pme*; GOX - glucose oxidase encoded by *gox*C.

Materials and Methods

Strains and plasmids. The *A. niger* strains used and constructed during this study are listed in Table 1. *E. coli* DH5α [22] was used for cloning throughout. The phagemid pBluescriptSK$^+$ [23] and pGEM-7 (Promega, Madison, U.S.A.) were used for cloning of DNA fragments or the construction of promoter-reporter gene plasmids. pGEM-T Easy (Promega) served for cloning of PCR fragments. Plasmids used

for the isolation of DNA fragments, to be used as probes, and those constructed during this study are mentioned in Table 2.

Table 1. The *Aspergillus niger* strains used in this study.

Strain	Genotype	Reference
N400 = CBS 120.49		laboratory collection
N402	*csp*A1	[38]
N888	*csp*A1, *pyr*A5, *phe*A1, *fwn*A	laboratory collection
NW219	*csp*A1, *nic*A1, *leu*A1, *pyr*A6	[44]
NW133	*csp*A1, *leu*A1, *pyr*A6, *gox*C17	[45]
NW219::pIM3900::2.4.8 (PELA 2.4.8)	*csp*A1, *nic*A1, *leu*A1, *pyr*A6, *pel*A-*pyr*A (3-4 copies)	this study
NW219::pIM3815::1.5 (PGX 1.5)	*csp*A1, *nic*A1, *leu*A1, *pyr*A6, *pgx*-*pyr*A (2 copies)	this study
NW219::pIM3815::1.5/X1-X3, 848.2, 848.5, 848.9, 848.10, 848.11, 848.12, 848.14, 848.16	potential *pgx* regulatory mutants, derivatives of PGX 1.5	this study
848.4, 848.6, 848.8, 848.13, 848.15	galacturonic acid non-utilising mutants, derivatives of PGX 1.5	this study
NW219:pIM3900::2.4.8/A1-A12	potential *pel*A regulatory mutants, derivatives of PELA 2.4.8	this study
NW133::pIM3820::5/14/19/37/40	*csp*A1, *leu*A1, *pyr*A6, *gox*C17, *pel*A-*gox*C	this study
NW133::pIM3821::2/9/19/26/32/40	*csp*A1, *leu*A1, *pyr*A6, *gox*C17, Δ*pel*A-*gox*C	this study

Growth conditions and transformation of *A. niger.* All media used in the expression studies were based on minimal medium (SM), which contains per liter: 6.0 g NaNO$_3$, 1.5 g KH$_2$PO$_4$, 0.5 g KCl, 0.5 g MgSO$_4$, 0.1 % (w/v) dialysed yeast extract [13], trace elements [24], pH 6.0 and necessary supplements. The SM plates contained 15 g/l agar. The culture media were inoculated with 10^6 spores/ml and incubated in a rotary shaker at 250 rpm at 30 ^{o}C. After 20 hours of growth, the mycelia were harvested on a Büchner funnel with a nylon gauze, washed with sterilised saline (9 g/l NaCl) and water and subsequently 3 g of the wet mycelia were transferred to different media. 1 % (w/v) of the various carbon sources were used in these experiments as indicated in the text. 200 ml of SM in 1 l or 50 ml SM in 250 ml Erlenmeyer flasks were used for pregrowing the mycelia and in the transfer experiments respectively. The mycelia were collected at time points indicated in the text and immediately stored at -70 ^{o}C.

A. niger NW133 was transformed with 10 μg of pIM3820 or pIM3821 according to the previously described protocol [8]. 1 μg of pGW635 served as the cotransforming plasmid to restore the uridine prototrophy. In case of the *A. niger* NW219 transformation with pIM3815 and pIM3900, the same protocol was followed with the following modification. Sucrose, which normally serves as an osmotic stabiliser of the protoplasts in a medium, was replaced by 1.33 M D-sorbitol supplemented with 1 % (w/v) galA as an inducer. The amounts of the plasmids used for the transformation were 1 or 5 μg for

pIM3815 and 2 μg or 3 μg for pIM3900, respectively. In the latter case transformation media of pH4 and pH6 were used.

Table 2. **Plasmids and constructs.** The localisation of the fragment within the coding region is mentioned in case of the endopolygalacturonase-encoding genes.

Plasmid	Gene / chromosome	Fragment(s) used as probe or for cloning purposes	Reference
pIM3700	*pga*I / II	280 bp *Nsi*I/*Xba*I (5' end) (probe)	[46]
pGW1800	*pga*II / III	300 bp *Acc*I/*Kpn*I (5' end) (probe)	[10]
pIM3760	*pga*A / V	330 bp *Xho*I/*Xba*I (3' end) (probe)	[14]
pIM3773	*pga*B / IV	200 bp *Nsi*I/*Kpn*I (5' end) (probe)	[14]
pIM3780	*pga*C / VII	275 bp *Nsi*I/*Sma*I (5' end) (probe)	[46]
pIM3790	*pga*D / I	130 bp *Cla*I/*Sst*I (3' end) (probe)	[15]
pIM3803	*pga*E / II	280 bp *Nsi*I/*Xho*I (5' end) (probe)	[13]
pGW820	*pel*A / I	1.6 kb *Cla*I (probe)	[8]
pIM3810	*pgx*	1.7 kb *Pst*I (probe)	Benen, unpublished
pIM3811	*pgx*	1.1 kb *Pst*I subclone of pIM3810	this study
pIM3640	*ply*A	0.9 kb *Xho*I/*Pst*I (probe)	[16]
pIM306	*pme* / VI	0.9 kb *Nsi*I/*Xho*I (probe)	Kusters-van Someren, unpublished
pIM860	*rhg*A / VII	1.2 kb *Eco*RV/*Hind*III (probe)	[17]
pIM3650	*rgl*A	1.4 kb *Bam*HI (probe)	Benen, unpublished
pGW635	*pyr*A	0.4 kb *Hind*III (probe)	[39]
pIM2131	28S	0.9 kb EcoRI (probe)	[47]
pIM2130	18S	0.7 kb *Eco*RI (probe)	[48]
pIM503	*gox*C		[30]
pIM3815	*pgx-pyr*A	the translational fusion of the 0.95 kb *Eco*RI/*Nco*I (used also as a probe) *pgx* promoter and *pyr*A	this study
pIM3900	*pel*A-*pyr*A	the translational fusion of the 1.12 kb *Sal*I/*Nsi*I *pel*A promoter and *pyr*A	Sánchez-Torres, unpublished
pIM3820	*pel*A-*gox*C	the translational fusion of the 1.1 kb *Eco*RV/*Nsi*I *pel*A promoter and *gox*C; as a probe the 2.1 kb *Eco*RI *pel*A-*gox*C fragment was used	this study
pIM3821	Δ*pel*A-*gox*C	2.5 kb *Cla*I/*Pvu*II Δ*pel*A-*gox*C fragment of pIM3820 in pBluescriptSK⁺ digested with *Cla*I/*Hinc*II; it contains 379 bp *Cla*I/*Nsi*I fragment of the *pel*A promoter	this study
pIM4647	*pep*A-*pyr*A	the 1.3 kb *pep*A promoter fragment fused with *pyr*A	Lenouvel, unpublished

UV mutagenesis and phenotypic characterisation of the mutants. To select for *A. niger* pectinase regulatory mutants, conidia of NW219::pIM3815::1.5 and NW219::pIM3900::2.4.8 (see Table 1) were UV irradiated (40 kJ/cm² for 90 sec) and plated on a selection medium (see below) in concentrations of 2×10^5 or 10^6 spores. The uridine auxotrophic mutants were selected on SM plates containing 1 % (w/v) galA, 10 mM uridine and 1 mg/ml or 1.2 mg/ml 5-fluoro-orotic acid (FOA) for the

NW219::pIM3815::1.5 or NW219::pIM3900::2.4.8 *A. niger* strains respectively. This selection method for uridine auxotrophic mutants is based on the protocol described for yeast [25]. The potential *pgx* and *pel*A regulatory mutants were rescued on plates containing complete medium (CMS) with uridine and further tested on plates containing SM (without yeast extract) with carbon sources as indicated in the text. The composition of CMS is the following: SM with 1 % (w/v) glucose, 0.2 % (w/v) neopeptone, 0.1 % (w/v) yeast extract, 0.1 % (w/v) casamino acids (vitamins free), 0.03 % (w/v) yeast ribonucleic acids and 0.2 % (v/v) vitamins solution (per 100 ml: 10 mg thiamine, 100 mg riboflavine, 10 mg *p*-aminobenzoic acid, 100 mg nicotinamide, 50 mg pyridoxine-HCl, 10 mg panthothenic acid and 2 mg biotine).

RNA and DNA manipulations. Total RNA was isolated from powdered mycelia using TRIzol^{TM} reagent (Gibco BRL, Life Technologies Inc., Gaithersburg, USA). The RNA concentration was estimated spectrophotometrically at 260 nm. For northern analysis 20 μg of RNA were separated on a 1.2 % (w/v) agarose gel under glyoxal denaturation conditions [26]. The gels were capillary blotted overnight onto Hybond-N^{TM} membranes (Amersham International plc., Little Chalfont, Buckinghamshire, UK) using 10 x SSC buffer (1 x SSC: 0.15 M NaCl, 0.03 M tri-sodium citrate dihydrate, pH 7.0). After transfer the blots were rinsed in 2 x SSC buffer, air-dried and crosslinked by UV for 3 min. Hybridisation was carried out overnight under homologous conditions [26] using 50 % (v/v) formamide in the hybridisation buffer. The blots were washed for 30 min at 65 ^{o}C in buffers containing 0.1 % (w/v) SDS and SSC buffer in the following concentrations: 2 x, 1 x, or 0.5 x. Fungal genomic DNA was isolated as described by de Graaff and co-workers [27]. Southern blot analysis was performed according to a standard protocol [26] using 5 μg of DNA for a digest.

The manipulation with plasmid DNA was essentially performed as described by Sambrook *et al.* [26]. Sequencing reactions were done using the Thermo-Sequenase fluorescent-labelled primer cycle sequencing kit (Amersham) with the universal or reverse primers. Subsequently they were analysed on an ALFexpress sequencer (Pharmacia Biotech. AB, Uppsala, Sweden). Computer sequence analysis was done using the program GeneRunner (Hastings Software, Inc., Hastings, NY, U.S.A.).

Assignment of linkage groups to the pectinase genes. Several of the pectinase-encoding genes were assigned to one of the eight chromosomes in *A. niger* (Table 2) on the basis of contour-clamped homogeneous electric field electrophoresis followed by Southern analysis of a set of five tester strains with linkage group-specific electrophoretic mobility variation [28].

PCR. To determine the low abundance *pga*E transcript the total RNA from the mycelium grown under the conditions described above was used in RT-PCR. The reaction was performed using the Moloney murine leukaemia virus reverse transcriptase (Gibco BRL) according to Gilliland *et al.* [29] with minor modifications. Prior to the reaction an additional step of RNA purification, a chloroform extraction, was included. The amount of RNA in the first reaction of the cDNA synthesis was varied, 1 μg or 10 μg were used in the total volume of 20 μl. Gene specific primers were used. In the first step, 100 pmol of PRIMER 3, 5' CGATAGTCCATGGTTGCAGAT 3' [13], annealing in the 3' non-coding region of *pga*E and in the second step 100 pmol of the cDNA specific primer I3, a twenty-seven residue long oligonucleotide, 5' GATATCAAGAGCTCTCAGAACGGAGTC 3', overlapping the third *pga*E intron, were used. 2 μl of the reverse transcriptase reaction product served as a template for PCR. The PCR conditions were: denaturation for 5 min at 95 ^{o}C, 30 cycles of 1 min denaturation at 95 ^{o}C, 1 min annealing at 51 ^{o}C and 1 min extension at 72 ^{o}C. In the final step the extension time was increased to 10 min.

For the construction of the *pgx-pyr*A fusion the following strategy was employed. pIM3811 (Table 2), containing the 939 bp of the *pgx* promoter and some 5' sequence of the *pgx* coding region, served for the introduction of the *Nco*I restriction site at the translation start point of *pgx*, using a specific primer in the PCR. 35 pmoles of *pgx Nco*I reverse primer, a thirty-five residue oligonucleotide, 5' TCCCATGGTGAGAGATT-GACAGGTGACAAGTCCGG 3', and of M13 (universal) primer (Stratagene Cloning Systems, La Jolla, CA, USA), annealing upstream from the *pgx* promoter in pIM3811 were used to amplify the 1045 bp *pgx* promoter fragment. The PCR-cycler was programmed as follows: denaturation for 5 min at 95 ^{o}C, 30 cycles of 1 min denatu-ration at 95 ^{o}C, 1 min annealing at 55 ^{o}C and 1 min extension at 72 ^{o}C. The final extension was 10 min. The PCR fragment was digested with *Eco*RI and *Nco*I to yield a 945 bp fragment. This fragment was exchanged with the 1.3 kb *Eco*RI/*Nco*I *pep*A promoter of pIM4647 (Table 2). The final *pgx-pyr*A construct, pIM3815, was checked by restriction analysis and by sequencing of the 5' end and the 3' end of the insert.

The *pel*A-*gox*C construct was made in the following way. A *Nsi*I restriction site was introduced in the translation start point of *gox*C in pIM503 (Tab. 2) by PCR using a forward *Nsi*I *gox*C primer, an eighteen residue long oligonucleotide, 5' CCATCATGCATACTCTCC 3'. The second primer, *gox*C reverse, an eighteen residue long oligonucleotide, 5' AGGATGGACTTCATTCCG 3', is localised 994 bp downstream of the *gox*C translation start point. 35 pmoles of both primers were used in the reaction. The PCR was done under the following conditions: the initial denaturation for 5 min at 95 ^{o}C, 30 cycles of 1 min denaturation at 95 ^{o}C, 1 min annealing at 42 ^{o}C and 1 min extension at 72 ^{o}C. The final extension was 10 min. The resulting fragment was cloned into pGEM-T Easy and sequenced. In the next step pGEM-7 was digested with *Nsi*I and *Eco*RI and ligated together with the 867 bp *Nsi*I/*Eco*RI *gox*C PCR fragment. The 1.12 kb *Nsi*I fragment of the *pel*A promoter was released from pIM3900 (Table 2) and cloned into the *Nsi*I restricted pGEM-7 containing the 867 bp of the 5'end of the *gox*C coding region, yielding the *pel*A-*gox*C intermediate plasmid. The correct orientation of the *pel*A *Nsi*I promoter fragment was checked by restriction analysis. The final *pel*A-*gox*C construct, pIM3820, was made by ligation of two fragments: the 4.3 kb *Sma*I/*Sal*I fragment of pIM503 [30] containing the main part of the *gox*C coding region in pUC9, and the 1.1 kb *Eco*RV/*Sal*I *pel*A promoter-5' end *gox*C fragment isolated from the pGEM-7 *pel*A-*gox*C intermediate plasmid. The *Eco*RV *pel*A site is localised 20 bp downstream of the 5' *Nsi*I site of the *pel*A promoter and was ligated into the *Sma*I restriction site of pIM503 polylinker.

The PCR's were performed using *Taq* polymerase (Pharmacia) according to the supplier's instructions. All the PCR products were checked by sequencing.

Glucose oxidase (GOX) plate and protoplast assays. The GOX activity in the plate assay was detected according to Witteveen *et al.* [31]; o-anisidine was added to the medium to a final concentration of 2.5 mM. *A. niger* NW133::pIM3820 and NW133::pIM3821 transformants were grown for 2 days at 30 ^{o}C on SM plates (without yeast extract) containing different carbon sources prior to the GOX activity staining.

For the GOX protoplast assay mycelia were pregrown for 16 hours in 1l Erlenmeyer flasks containing 250 ml SM supplemented with 0.5 % (w/v) yeast extract, 0.2 % (w/v) casamino acids and 2 % (w/v) sucrose or 1.5 % (w/v) sugar beet pectin as a carbon source. After harvesting, 1 g of the mycelium was resuspended in 10 ml of PROTO SM1 (SM without yeast extract + 0.8 M KCl + 20 mM CaCl$_2$) or PROTO SM2 (SM without yeast extract + 1.33 M D-sorbitol + 20 mM CaCl$_2$) media containing 0.1 g of Novozyme 234 (Novo Nordisk A/S, Copenhagen, Denmark). The mix was incubated further at 37 ^{o}C in a rotary shaker at 140 rpm, until more than 1×10^8 protoplasts were present. The mycelial debris was filtered over a glass funnel plugged with glass wool and the protoplasts were collected by centrifugation

at 1500 x *g* for 10 min at 4 °C. Subsequently they were washed two times with 20 ml of cold PROTO SM1 or PROTO SM2 media, resuspended at a concentration of 10^8 protoplasts/ml and kept on ice. Induction of *gox*C under the control of the *pel*A promoter with different carbon sources was carried out for 3 hours at 30 °C. A 1.5 ml Eppendorf tube contained 200 μl of the incubation mix with the following composition: 5 x 10^5 protoplasts, the carbon source to be tested, and the PROTO SM1 or PROTO SM2 medium. The tubes were kept open during the incubation. After 3 hours the protoplasts were lysed by adding 200 μl of the lysis mix (25 mM sodium-phosphate buffer pH=6, 2.5 mM *o*-dianisidine, 130 mM glucose and 2.5 μg/ml horse radish peroxidase (Boehringer, Mannheim, Germany)). After 1 min the content of the tube was briefly centrifuged. 100 μl of the reaction mixture were pipetted per well of an ELISA plate. The plate was then incubated at 37 °C in the microtiterplate reader (Thermo Max, Molecular Devices, Wokingham, UK). The GOX activity was assayed spectrophotometrically after the lysis of the protoplasts and quantified as the increase of A_{490} in time. In each experiment three different treatments were included: C1, the incubation mix without the carbon source; C2, the incubation mix without the carbon source and with 0.1 mg/ml of cycloheximide (for an inhibition of *de novo* protein synthesis) and C3, C2 with the carbon source added.

Results and Discussion

The induction pattern of the pectinase-encoding genes of *A. niger*. Pectin is the natural substrate of pectinases. Sugar beet pectin, for which several structural elucidation studies have been carried out [32-34] and which is quite complex, was selected as the pilot carbon source in this induction study.

Figure 1. Northern blot analysis of sugar beet pectin grown mycelia of *A. niger* N400. The numbers at the top correspond to: 0 = the overnight pregrown mycelium on 1.5 % (w/v) sucrose. Below the figure the pH of the medium at the time of harvesting is listed. The 28S rDNA fragment was used for reprobing of the blots in order to check for the loading.
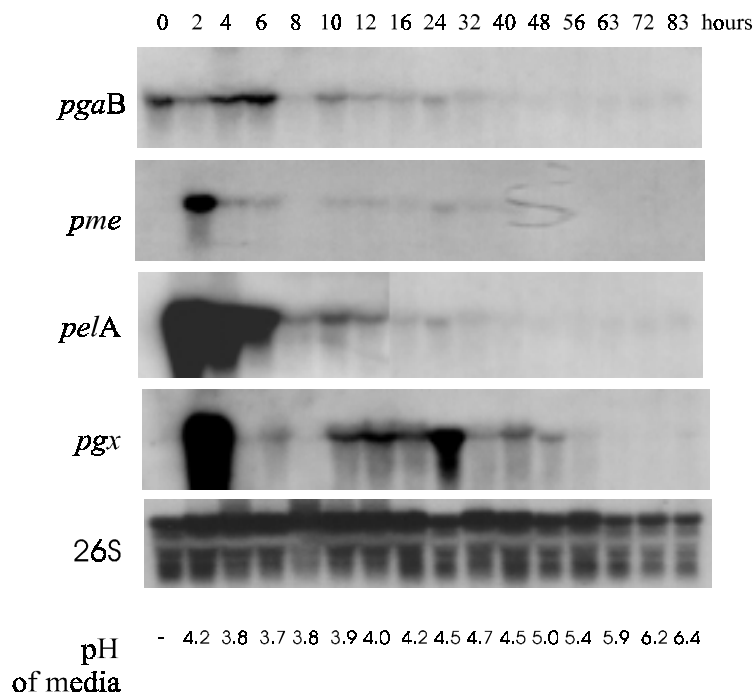
The wild type *A. niger* N400 RNA samples were isolated from the mycelia harvested at several time points during 83 hours of growth. Seven *pga*'s, *pme*, *pgx* and *pel*A (Table 2), which encode the enzymes active on the homogalacturonan region of pectin, were used for probing of the northern blots. Fig. 1 shows the expression pattern of four pectinase-encoding genes: *pga*B, *pgx*, *pme* and *pel*A.

Each gene has a typical induction profile. While the *pga*B transcription is not repressed by sucrose, the *pel*A, *pme* and *pgx* transcripts do not accumulate under these growth conditions. The transfer of the mycelia to the pectin-containing medium quickly triggers the *pgx*, *pel*A and *pme* expression. Furthermore, the level of the individual gene transcripts changes during the time of incubation (see Fig. 1). *pga*B mRNA first remains at a low, constant level, vanishes in the 8 hour sample to reappear at a low level in the 10 to 32 hour samples. *pme* is quickly induced at 2 hours and the level of the transcript subsequently drops and stays low up to 40 hours. At 8 hours the *pme* mRNA is not present. The *pel*A transcription is high during the first 6 hours after the transfer and then slowly decreases. The *pgx* mRNA level strongly increases immediately after the transfer, then drops and a second peak of induction appears at 24 hours. As for the expression of the remaining *pga* genes, only the *pga*A and *pga*I mRNAs could be detected under the growth conditions tested (data not shown). *pga*A was found to be constitutively expressed and the *pga*I transcription was induced by pectin and repressed in the presence of sucrose (for more details on *pga*E, *pga*A, *pga*B, and *pga*D expression see Chapters 2, 3 and 4). Since the selected genes encode enzymes acting on specific parts of the homogalacturonan region of the complex pectin molecule (see Fig. 2), it is possible, that their

Figure 2. The schematic representation of the homogalacturonan and rhamnogalacturonan regions of pectin (based on [41]). The enzymatic activities of different *A. niger* pectinases on the specific part of the pectin molecule is depicted. The abbreviations correspond to: PGX - exopolygalacturonase, PG I, II, A-E - endopolygalacturonase, PME - pectin methyl esterase, PLY - pectate lyase, PEL A - pectin lyase, RGH A - rhamnogalacturonan hydrolase, RGL - rhamnogalacturonan lyase.
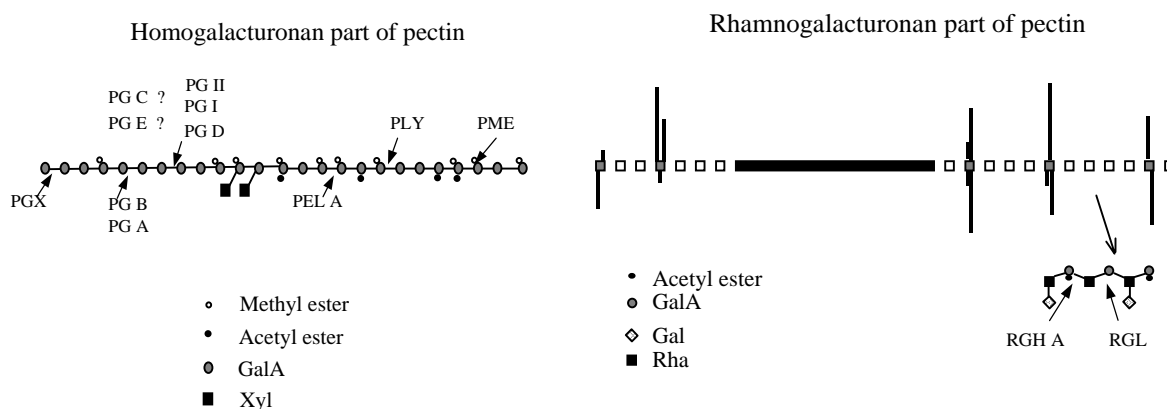
expression pattern reflects the stage of the pectin decomposition requiring a certain type of enzymatic activity to be expressed, *viz.* demethylation of pectin in the beginning by pectin methylesterase.

Figure 3. Northern blot analysis of *A. niger* **N400 mycelia grown in the presence of different carbon sources.** The upper part of the figure shows the expression patterns of *pel*A and *pgx* at five different time points and on five different media. The lower part shows the expression of *pme*, *rgl*A, *ply*A and *rhg*A at four time points and on selected media. The numbers correspond to: 0 = pregrown mycelium on 1.5 % (w/v) sucrose prior the shift, 1-5 = RNA samples isolated from SM after the shift containing 1 % (w/v) galA (1), 1 % (w/v) galA + 1 % (w/v) rha (2), 1 % (w/v) polygalacturonic acid (3), 1 % (w/v) sugar beet pectin (4) or 1 % (w/v) sucrose (5) respectively. The harvesting times after the shift of the pregrown mycelia are indicated at the top of the picture. At the right side-hand the type of the induction profile of the individual genes is depicted. The 18S rDNA fragment was used in reprobing as a loading control.
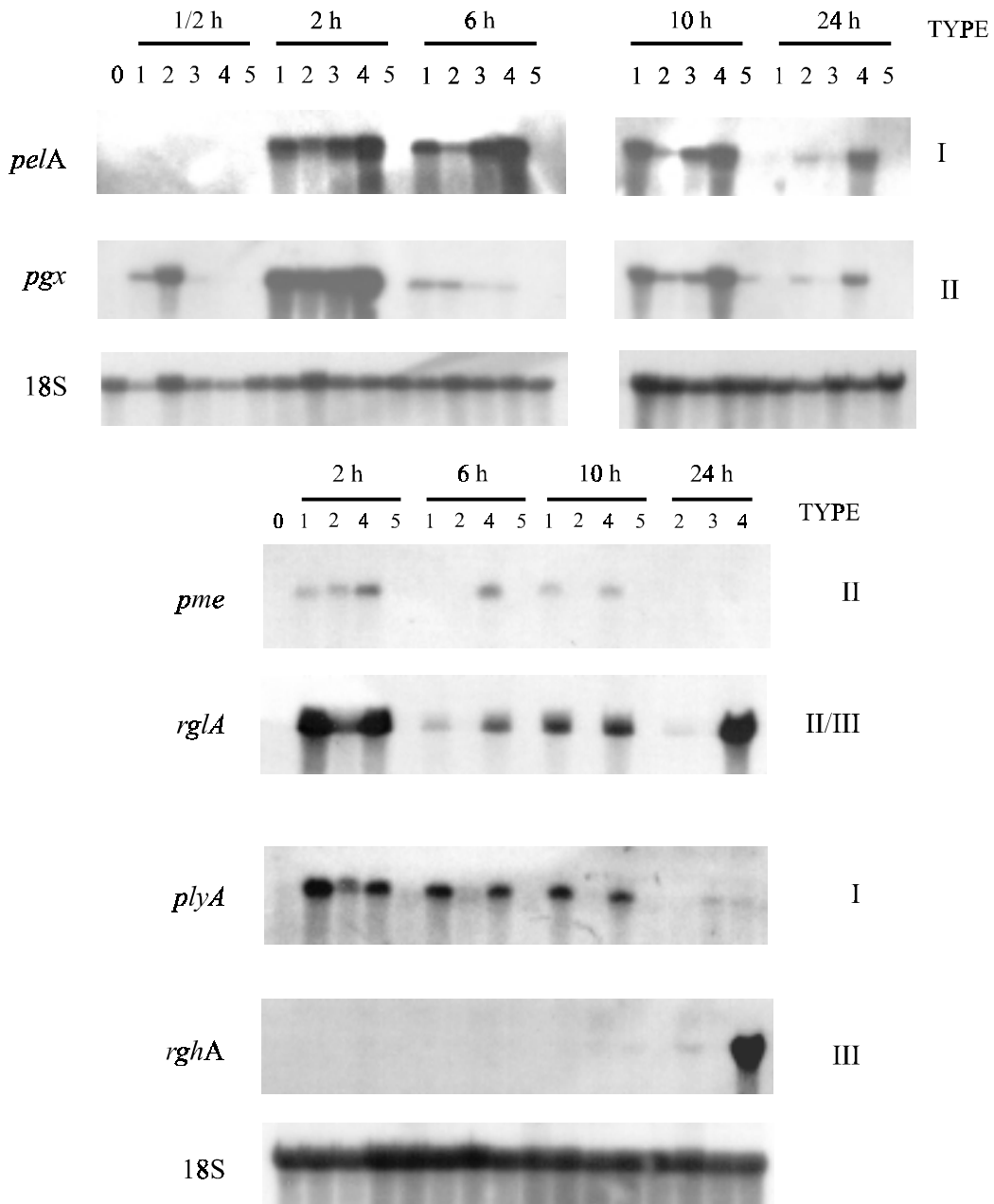
In order to elucidate the basis of the molecular inducer of the pectinase-encoding genes, the constituents of the pectin backbone - D-galacturonic acid (galA) and L-rhamnose (rha) - were used as the carbon source. Polygalacturonic acid and sugar beet pectin were included for comparison of the inductive effect. Sucrose was used to monitor the carbon catabolite repression of the expression of the genes. A set of six pectinase-encoding genes was selected encoding enzymes acting on the homogalacturonan (*pme*, *pgx*, *pel*A and *ply*A) and on the rhamnogalacturonan parts (*rgl*A and *rgh*A) [35] of pectin (see Fig. 2), respectively. The results of the northern blot analysis are presented in Fig. 3. It is clear, that the presence of galA in the medium immediately triggers the expression of all the genes, except *rgh*A (the type III profile). In the latter case, most probably other sugar constituents released from the pectin molecule later during the incubation or changes in the physiological conditions trigger the expression. The combination of galA and rha, which was found to induce the *A. aculeatus rgh*A [36], is not the right combination for the *A. niger rgh*A, since only a very low level of transcription can be detected 10 and 24 hours after the shift (see Fig. 3). The induction of the remaining genes is characterised by two typical profiles. *pel*A and *ply*A mRNA levels (the type I profile) are high during the first 10 hours on galA and the polymeric substrates and decrease or are absent at 24 hours. The presence of rha in the medium negatively affects the induction levels of both genes, notably *ply*A. The second profile (type II), characterised by a fluctuation of mRNA levels on galA during the time of the incubation, is typical for *pgx*, *pme* and *rgl*A. The *pgx* transcript is detectable already within half an hour in the presence of galA or in combination with rha, but is hardly visible on polygalacturonic acid, where the release of galA probably did not start yet (Fig. 4). This indicates, that galA is the inducer of this gene, as previously concluded for *A. tubingensis pgx* [37]. The *pgx* expression strongly decreases 6 hours after the shift and again increases after 10 hours on all media-containing galA. A similar pattern of expression in the presence of galA was previously found for the constitutively expressed *pga*A and *pga*B [14]. Taking into the account the *pel*A and *ply*A expression patterns, this phenomenon can not be explained as the bad adaptation of fungal metabolism to the utilisation of galA, as was proposed in the case of *pga*A and *pga*B. Rather it is feasible, that the expression of these genes reflects the intracellular concentration of galA. The HPAEC-PAD profiles of the culture media (Fig. 4) show that the initial uptake is rather slow (only 6.4 % from the initial concentration of galA is utilised within the first 6 hours after the shift) whereas between 10 and 24 hours galA is exhausted. To test this hypothesis it would require the measurement of the intracellular concentration of galA at different time points. The *rgl*A induction profile is similar to *pgx*.

However, it is possible that yet another regulatory factor, similar to that involved in the *rgh*A expression, affects the *rgl*A transcription on pectin 24 hours after the shift (see Fig. 3). The *pme* mRNA levels are lower compared to the other genes. It is possible that galA is not the only inducer of *pme*, since the 2 hours induction pattern shows a higher level of the *pme* mRNA on pectin compared to galA (see Fig. 3). The presence of rha in the medium has mainly a negative effect on the expression of the pectinase genes. At this moment we can not satisfactory explain this.

From Fig. 3 it is furthermore evident, that the transcription of all six genes is not induced in the presence of sucrose in the medium.

Figure 4. The HPAEC-PAD analysis of SM media of *A. niger* N400 collected at four time points after the transfer to galA and polygalacturonic acid. The analysis of the sugars was done as described previously [13]. The individual elution profiles are off-set for clarity.
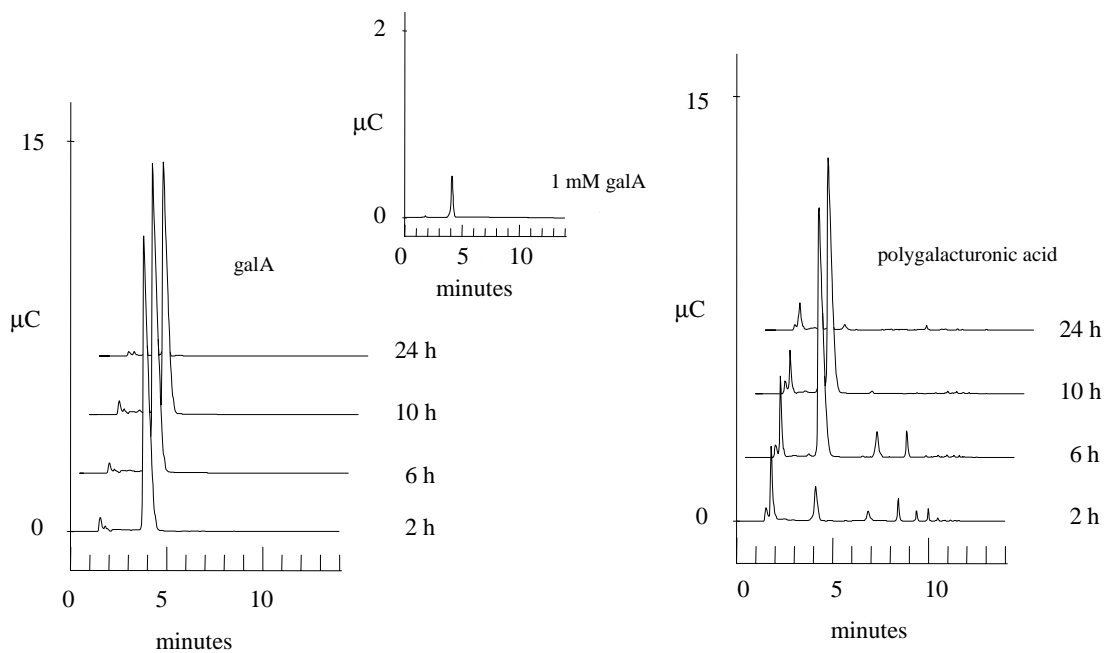
Detection of *pga*E mRNA. It was reported previously [13], that the *pga*E mRNA could not be detected neither by northern blot analysis nor by RT-PCR. However, further improvement of the RT-PCR conditions (see Materials and Methods) and using up to 10 µg of RNA for the first cDNA strand synthesis, led to the detection of the *pga*E transcript (data not shown) in the samples isolated from the mycelia 6 hours after the shift, containing any of the carbon sources tested above. This demonstrates, that *pga*E is expressed at an extremely low level under the conditions applied, and it can explain the failure of the detection of other *pga* transcripts, i.e.

*pga*II, *pga*C and *pga*D, by northern blot analysis as well. It is possible, that other factors than the carbon source are important for the induction of the various PGs (*cf.* the differences in the production of PGs in the SsF and the SmF).

Isolation of **A. niger** *pectinase regulatory mutants*.

Selection of *pgx-pyr*A and *pel*A-*pyr*A *A. niger* transformants. As mentioned before, the strategy to isolate *A. niger* xylanase regulatory mutants using the *pyr*A gene as the selection marker, led to the isolation of *xln*R [21], encoding the transcriptional activator of the *A. niger* xylanolytic genes. Therefore we selected two promoters, 939 bp in size for *pgx* and 1123 bp for *pel*A, representatives of the pectinase induction profiles II and I (Fig. 3), to construct the promoter-reporter gene plasmids pIM3815 and pIM3900 (Table 2). These plasmids were used to transform the *A. niger* NW219 *pyr*A auxotrophic mutant. The non-interference of D-sorbitol used as the osmotic stabiliser, when inducing the *pgx* and *pel*A promoters during the transformation, was checked by northern blot analysis (data not shown). The respective expression levels of *pel*A and *pgx* in the medium containing 1 % (w/v) galA or 5 % (w/v) D-sorbitol with 1 % (w/v) galA as the carbon source were comparable 2 hours after the shift, but were lower after 10 hours in the presence of D-sorbitol. However, D-sorbitol alone did not trigger the expression of the two genes. Although D-sorbitol had some negative effect on the transcription of *pgx* and *pel*A upon prolonged incubation, we considered these conditions suitable to obtain *A. niger* NW219 pIM3815 and pIM3900 transformants.

Figure 5. Schematic representation of the integration of pIM3815 in the *A. niger* PGX 1.5 transformant. The lines at the bottom of the figure represent the hybridising fragments after probing of the Southern blot of *Eco*RI restricted chromosomal DNA with the *pgx* promoter fragment and the *pyr*A fragment derived from pIM3815 and pGW635 (*cf.* Table 2).
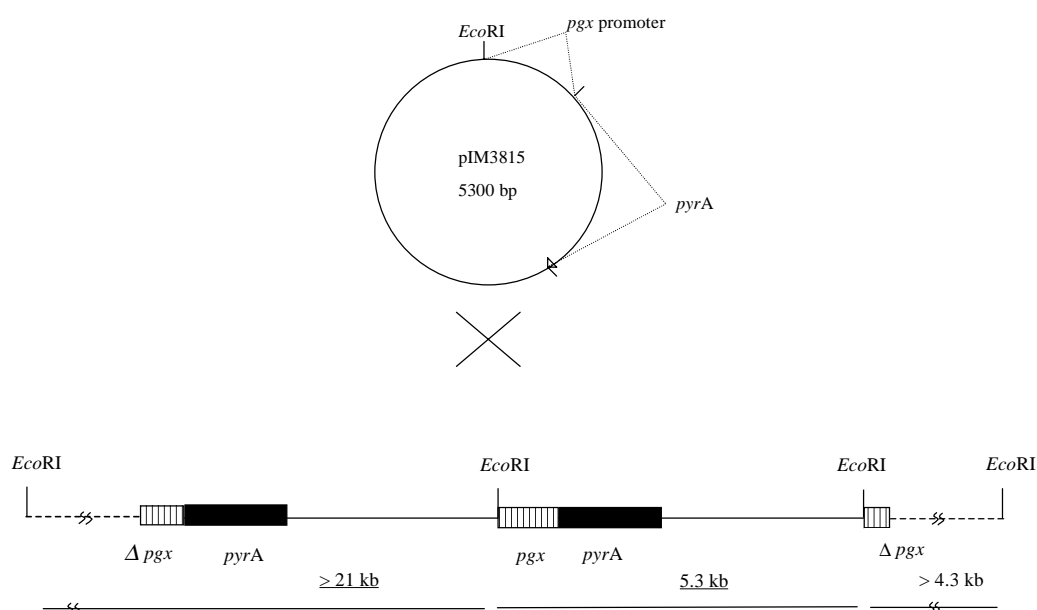
34 and 38 of the *pgx-pyr*A and *pel*A-*pyr*A *A. niger* transformants, respectively, were tested for the right phenotype (growth only in the presence of galacturonic acid, but not in the presence of glucose and in the absence of uridine in the media), prior to further molecular analysis. On the basis of Southern blot analysis (data not shown), strains *A. niger* NW219::pIM3815::1.5 (PGX 1.5) and NW219::pIM3900::2.4.8 (PELA 2.4.8) (Table 1) were selected. They contain ectopically integrated 2 and 3-4 copies of the *pgx-pyr*A and *pel*A-*pyr*A reporter construct respectively. Fig. 5 depicts the way of the tandem integration of pIM3815 plasmid in the *A. niger* PGX 1.5 strain, which occurred through the 5' end of the *pgx* promoter.

UV mutagenesis of the PGX 1.5 and PELA 2.4.8 *A. niger* transformants and phenotypical characterisation of potential pectinase regulatory mutants. To select for the pectinase regulatory mutants the conidiophores of the *A. niger* strains PGX 1.5 and PELA 2.4.8 were UV irradiated to a survival of approximately 30 %. After 4-5 days of incubation at 30 ^{o}C, colonies of *A. niger* mutants were detected. 84 and 112 of the potential *pgx* and *pel*A regulatory mutants respectively, were isolated. Upon testing their phenotype, only 29 and 31 mutants derived from the *A. niger* PGX 1.5 and PELA 2.4.8 strains respectively, were uridine auxotrophs. Several of those were selected and retested on SM plates containing 1 % (w/v) galA with and without uridine, 1 % (w/v) sugar beet pectin and 1 % (w/v) D-glucose with uridine. The results of the classification of the mutants based on their phenotype are presented in Table 3.

Table 3. **The classification the potential *A. niger pgx* and *pel*A regulatory mutants from the first UV mutagenesis experiment.**

category / phenotype	PGX 1.5	PELA 2.4.8
AI: uridine-dependent on galA, normal growth on pectin	3	12
AII: partly uridine-dependent on galA, normal growth on pectin	6	-
AIII: poor growth only on pectin	12	-
W: phenotype of the parental strain	5	7
M: morphological mutants	2	-
?: not assigned	-	2
total number of mutants scored:	28	21

It should be mentioned here, that the morphology of the PGX 1.5 and PELA 2.4.8 mutants was quite different. While the *pel*A mutants showed uniform and good growth, a considerable

number of the *pgx* mutants grew poorly (category AIII and the morphological mutants indicated in Table 3). The PGX 1.5 derived mutants had also more heterogeneous phenotypes. The category AI mutants, uridine dependent on galA, showed the expected phenotype and were therefore further analysed for the effect of these mutations on the endogeneous *pgx* and *pel*A expression. Since there is no plate-screening assay available, which discriminates easily among different pectinolytic activities, all the potential mutants were analysed by northern blot analysis.

Characterisation of the potential *pel*A regulatory mutants. Twelve potential *pel*A regulatory mutants (*cf.* Table 3) were pregrown on CMS with 1 % (w/v) D-glucose and uridine for 20 hours and subsequently transferred to SM with 1 % (w/v) galA and uridine. Samples were harvested 2 and 5 hours after the transfer. Figure 6A shows the northern analysis of the 2 h samples of the potential *pel*A regulatory mutants (A1-A12), the parental strain PELA 2.4.8 and the wild type *A. niger* N400.

Figure 6. Northern blot analysis of the potential *pel*A (panel A) and *pgx* (panel B) regulatory mutants obtained from the first UV mutagenesis experiment with the *A. niger* PELA 2.4.8 and PGX 1.5 transformants. The rRNA picture after staining (0.04 % (w/v) methylene blue in 0.5 M Na-acetate buffer, pH 5.2) of the northern blot is presented as the loading control. In panel B the time points of harvesting the mycelia after the shift are indicated in the middle.
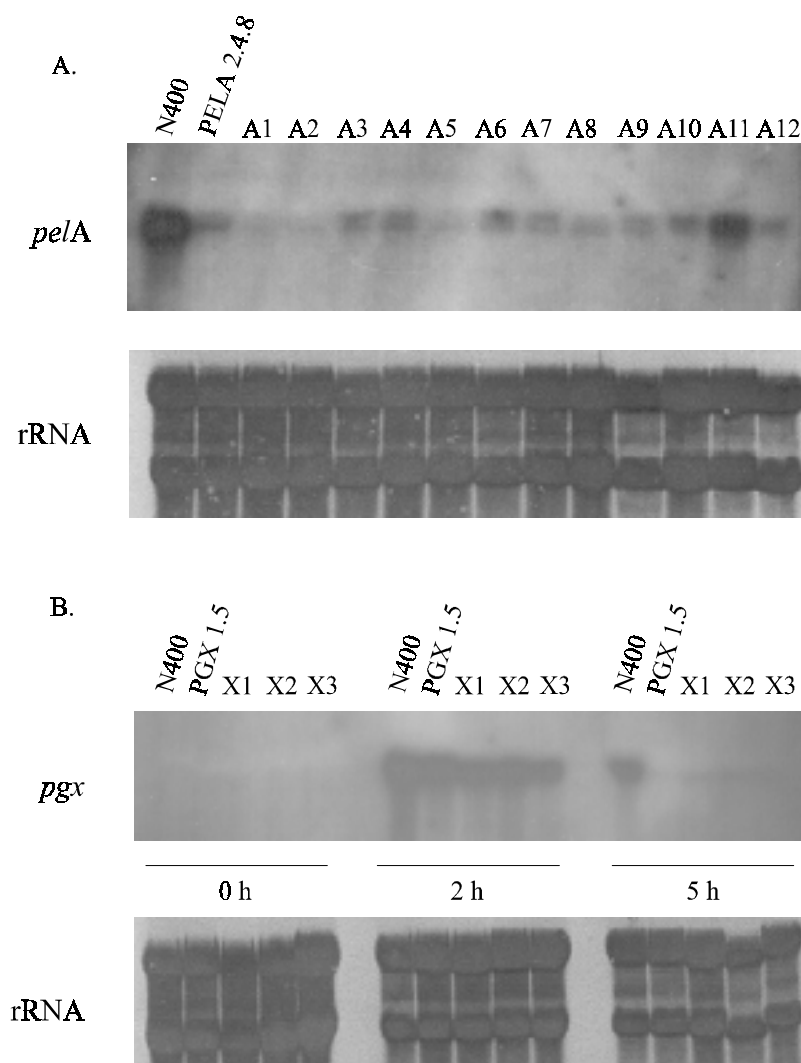
No *pel*A expression was observed after pregrowth on D-glucose (data not shown) and the 5 hour time point of the *pel*A expression profile was similar to that obtained at 2 hours (the lower signals of the mutants A1, A2 and A5 became clearly visible 5 hours after the transfer). The levels of the *pel*A mRNA of the mutants and the parental strain are similar and lower than observed in the wild type *A. niger* N400 (Fig. 6A). This indicates that the mutations did not affect the endogeneous *pel*A expression, but only the *pel*A-*pyr*A reporter plasmid. It is unlikely that 3-4 copies of the *pel*A-*pyr*A plasmid in the *A. niger* PELA 2.4.8 transformant were mutated all at once leading to a *pyr*A⁻ phenotype. Therefore this strain was checked for its stability. Plating of *A. niger* PELA 2.4.8 conidia on SM plates with 1 % (w/v) D-glucose without uridine resulted in many revertants and thus loss of the inducible transcription of *pyr*A. This demonstrates that the *A. niger* PELA 2.4.8 transformant is not stable. It is therefore possible, that the category AI *pel*A mutants have lost some copies of the *pel*A-*pyr*A plasmid, resulting in a phenotype expected for a potential *pel*A regulatory mutant.

The dramatic decrease of the *pel*A mRNA signal in the *A. niger* PELA 2.4.8 strain compared to the *A. niger* wild type N400, prompted us to further investigate the effect of the genetic background. Using the same RNA samples of the PELA 2.4.8 and N400 *A. niger* strains, the expression levels of *ply*A and *pgx* (induction profile I and II, in Fig. 3) were studied. This was done in order to determine, whether these pectinase-encoding genes were affected simultaneously with *pel*A. In the case of *ply*A, the transcript was detected only in the *A. niger* wild type but not in the *A. niger* PELA 2.4.8 strain and the derived mutants thereof (data not shown). In *A. niger* PELA 2.4.8 strains the *pgx* mRNA levels were comparable with the wild type *pgx* transcription after 2 hours but a bit lower after 5 hours (data not shown). This result confirmed that *A. niger* PELA 2.4.8 was affected in the expression of at least two pectinase-encoding genes (*pel*A and *ply*A, induction profile I) prior to UV mutagenesis. A possibility was that any of the original mutations of the host strain *A. niger* NW219 affected *pel*A expression. Therefore, we selected the wild type *A. niger* N400 and the derived mutants, *viz.* N402, NW219 and NW133 (Table 1), to compare the *pel*A expression. The mycelia were pregrown as described above and shifted to SM containing 1 % (w/v) galA. The samples were harvested 2, 6 and 10 hours after the shift. The northern blot analysis revealed that the *pel*A expression was lower in all the *A. niger* mutants when compared to the wild type (data not shown). Since *A. niger* NW219 and NW133 are derivatives of *A. niger* N402, the mutation affecting the *pel*A expression is already present in *A. niger* N402. According to Bos *et al.* [38], this strain contains two independent mutations, which were obtained after two rounds of UV mutagenesis of *A.*

niger N400. The phenotype of these mutations is characterised by a decrease of the conidiophore length (*csp*A1) and an increase in spore density on plates. It is therefore feasible, that during UV mutagenesis a locus important for the *pel*A expression was also affected. Currently we are investigating, whether there is a direct involvement of the *csp* loci in *pel*A expression.

Categories of potential *pgx* regulatory mutants. The 3 potential *pgx* regulatory mutants (X1-X3; Table 1 and 3) were analysed the same way as described above. The results of the northern blot analysis are presented in Figure 6B. While 2 hours after the shift the *pgx* expression is similar in all strains tested, the 5 hour mRNA profile shows lower levels of the *pgx* transcript for *A. niger* PGX 1.5 and the derived mutants than for *A. niger* N400. Because 2 hours after the transfer to galA p*gx* mRNA was present in the potential *pgx* regulatory mutants, we concluded, that these strains are affected only in the *pgx-pyr*A expression, thus the mutation is *cis*-acting. We can not satisfactory explain at this moment the decrease of the *pgx* mRNA levels in the 5 hour samples. It is possible, that *pgx* expression is also under the control of the same regulatory mechanism involved in the regulation of the *pel*A gene expression, which is affected by the *csp*A1 mutation in the host strain *A. niger* NW219, thus explaining the simultaneous decrease of the signal in the parental *A. niger* PGX 1.5 and the mutant strains. Because the number of the category AI *pgx* mutants was low and we did not find *pgx* regulatory mutants amongst those, the UV mutagenesis experiment with the *A. niger* PGX 1.5 strain was repeated. Two hundred mutants in total were isolated and tested for their phenotype on plates containing SM with 1 % (w/v) galA, 1 % (w/v) sugar beet pectin or 1 % (w/v) D-glucose with or without uridine. Only thirty-one mutants were uridine auxotrophs in the presence of galA. Sixteen strains were selected and re-tested and their classification according to their phenotype is listed in Table 4.

Table 4. **Classification of the potential *pgx* regulatory mutants derived from *A. niger* PGX 1.5 in the second UV mutagenesis experiment.**

category / phenotype	mutants
BI : growth on sugar beet pectin uridine-dependent; growth on galA $\pm$ uridine bad.	848.4/6/8/13/15
BII : growth on galA and sugar beet pectin uridine-dependent	848.2/5/9/10/11/12/14/16
W: phenotype of the parental strain	3

The phenotype of the BI category of mutants suggested, that the mutation affected galA catabolism or uptake. Therefore, the growth of these strains was compared with the parental strain *A. niger* PGX 1.5 on different carbon sources (D-glucose, D-galacturonate, D-gluconate, D-glucuronate, glycerol and L-rhamnose). From the five strains tested (see Table 4), the growth of 848.4 and 848.6 was reduced on galA and gluA compared to the parental strain. Furthermore, mutant 848.15 showed an even stronger phenotype, reduced growth on galA and no growth on gluA. This demonstrates that the mutations affected galacturonic and glucuronic acid catabolism. We are further analysing these strains with respect to uptake and intracellular metabolite profile after growth on galA and gluA.

The eight strains classified as BII showed the phenotype expected for the potential *pgx* regulatory mutants (*cf.* Table 4). To exclude the possibility, that the uridine-dependent phenotype on galA was not due to a mutation in the *pgx* regulatory pathway, these strains were further characterised genetically (Table 5).

Table 5. **Complementation test of the 8 potential *pgx* regulatory mutants (category BII)**. As a control the *A. niger* PGX 1.5 strain was used. The formation of heterokaryons was done according to the protocol described previously [49]. The transformation with pGW635 was performed to exlcude the possibility of a mutation in another *pyr* locus. -, no growth; +, growth. ? = some background growth was observed.

strain / mutant	growth on SM with 1% (w/v) galA, -uri	heterokaryon formation mutant x *A. niger* N888	pGW635 (*pyr*A) transformation
848.2	-	+	-
848.5	?	-	+
848.9	?	-	+
848.10	?	-	+
848.11	?	-	+
848.12	?	+	+
848.14	?	+	+
848.16	-	+	-
control	++	+	+

The formation of heterokaryons between the BII mutants made in *A. niger* PGX 1.5 (*csp*A1, *nic*A1, *leu*A1, *pyr*A6, *pgx-pyr*A), and *A. niger* N888 (*csp*A1, *pyr*A5, *phe*A1, *fwn*A) was tested in order to determine the basis of the mutation (dominant or recessive). If the mutant is affected in the *pgx* induction pathway, the mutation should be a recessive one, which is complemented in the diploid. In the case of a dominant mutation, this mutation has to affect the *pgx-pyr*A reporter construct. Four mutants were not able to form diploids in the cross with *A. niger* N888, *viz.*

strains 848.5, 848.9, 848.10 and 848.11 and therefore these mutations were classified as dominant.

Figure 7. Analysis of the genetic background of the potential *pgx* regulatory mutants obtained after the second UV mutagenesis experiment with the PGX1.5 transformant. The chromosomal DNA of the mutants (the numbers at the top, *viz.* Table 1) was digested with *Eco*RI and the Southern blots were hybridised with the *pyr*A (panel A) or the *pgx* promoter (panel B) radiolabeled fragments (for the probes see Table 2). At the left-hand side of both panels the identification of the hybridising fragments based on Fig. 5 is depicted.

Figures C-E represent the Southern blots of the dominant *pgx* mutants upon restriction of their chromosomal DNA with *Eco*RI and probing with different regions of the *pgx* promoter. The localisation of the DNA fragments within the *pgx* promoter used as probes is depicted below and the lettering corresponds to the patterns in Figure C, D and E, respectively.
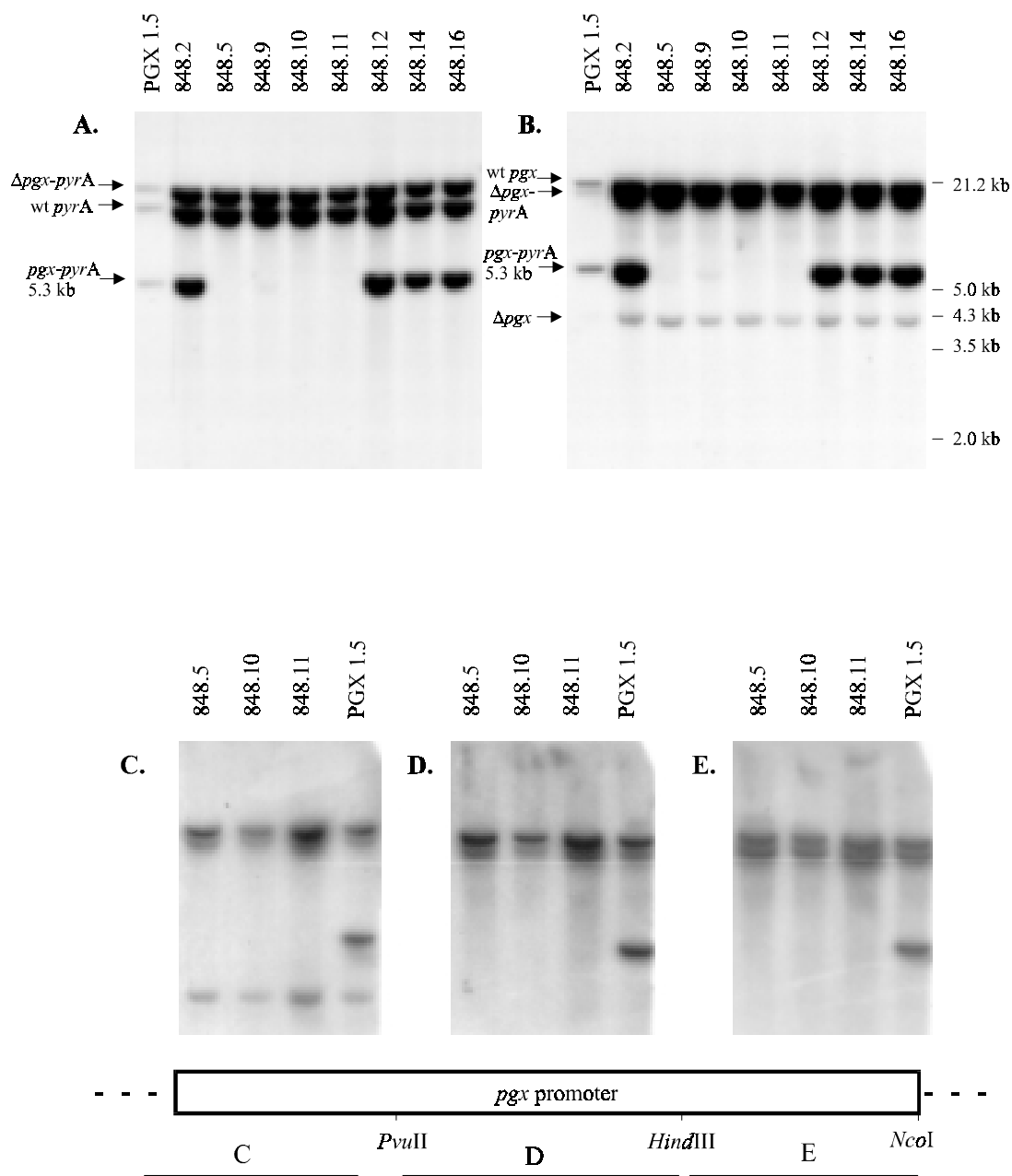
The four remaining mutants were characterised as recessive. The transformation with pGW635 (*pyr*A) was performed in order to demonstrate that no other *pyr* mutations, except *pyr*A, had been induced by UV mutagenesis (*viz. pyr*B, [39]). Upon transformation, two of the recessive mutants (*cf.* 848.2 and 848.16, Table 5) were unable to restore the uridine prototrophy; thus they were classified as potential *pyr*B mutants. Only the mutant strains 848.12 and 848.14 had the genetic background expected for potential *pgx* regulatory mutants.

To determine the nature of the mutations, first a Southern blot analysis was performed (Fig. 7A, B). The chromosomal DNA of the BII category *pgx* mutants and the parental strain *A. niger* PGX 1.5, was digested with *Eco*RI and hybridised with the 0.95-kb *Eco*RI/*Nco*I *pgx* promoter and the 0.4-kb *Hind*III *pyr*A fragment (Table 2). The picture clearly shows that in the case of three mutants, *viz.* 848.5, 848.10 and 848.11, the 5.3-kb *pgx-pyr*A hybridising band is missing.

Figure 8. The sequence of the *pgx* promoter. The nucleotide sequences of the DNA binding proteins are depicted: in bold the putative CREA 5` SYGGRG 3' binding sites [42], in bold and capitals the UAS *pga*II 5' TNATTGGT 3' like sequence and in italics the hexanucleotide sequence 5' CCCTGA 3' [12].

-939   ctgcagtcgagaagtctacctaaacaactcttgttcattgacaaggattgcgtaaagagcagcctgagcgacaatagattgtc -857

-856   ttggcattggtgagtaggctcatagagcatattatgagatgaagttgg **ctggagg**cgactccgacttatatatgtacatattcc -773

-772   cttataaattgcatcgacattcctctttgcttgccagtgttgtag**ACCAATGA**aaccattgagatcggcttatgtga*ccc* -693

PvuII

-692   *tga*aatggagatactgagactctttggtgactttctcacagtatgatgcttacttggttgaagctgagcagctgttggccttttaa -607

-606   atggcgtctttgtggtgatgtatattgtccgtgagtattgtaccacacggagtttgcactaacctgcacttaccgcaagttgcgg -522

-521   gaattggcctgcgcgacaatatcatctaaccgggatcaatgaataaccggataatgttttcattgaaaatgaagtcatgatgc -439

-438   agagaatgg**ctggggt**aagttccctgccaattccccatacacacgtttttcccccaatcggataatcgtcgccgacaagccc -357

-356   tcctatcacatcgtctctcgttgttc**ctccacg**caatc**ccccac**cacctcattctcccgcagctaactgtccccaagtgccct -273

HindIII

-272   gttgattacaggagcaagccacaagcttagcatag **ctccacc**gtcgtggtttgttcgctttgtttggttgatttcgttt **ccccgg** -188

-187   atatggagcacagcgaaatccccaacttgaaggatcaatggtgtggtcagtgtgggcaagact **gcggggg**agatgatgcct -108

-107   actcgatgcaatggatataaattccagaggcgaccgtcattcctttgcattgactttgttcaag **ctccgc**tcgtgaaaccggac -24

-23   ttgtcacctgtcaatctctcaca   -1

The 848.9 strain showed a weakly hybridising band using both probes and taking the genetic characterisation into the account (Table 5), this strain is probably not a pure isolate. Based on the known integration of pIM3815 in the genome (Fig. 5), the three mutants obviously lost the intact copy of the *pgx-pyr*A construct. The coincidence of the dominant character of the mutation in these strains and the loss of the intact copy of the *pgx-pyr*A construct suggests that the remaining 5' truncated *pgx-pyr*A fragment is not inducible in the presence of galA, thus causing the *pyr*A$^-$ phenotype. In order to identify the 5' displaced region of the pgx promoter, specific fragments of the *pgx* promoter were used in a Southern analysis of *Eco*RI digested chromosomal DNA of the selected mutants and compared with the parental strain (Fig. 7C-E). Based on the hybridisation patterns obtained one can conclude that less than 263 bp of the 5' region of the *pgx* promoter was displaced during the integration of pIM3815 in the genome. To identify whether this *pgx* promoter fragment, which is localised between -939 to -676 bp from the translation start point, contains some known DNA regulatory sequences, the entire *pgx* promoter (939 bp) was analysed (Fig. 8). Interestingly, this region contains the hexanucleotide sequence 5' CCCTGA 3' found already previously in other pectinolytic genes of *A. niger* [12-14], and also the truncated version of the UAS of *pga*II, *viz.* 5' TCATTGGT 3', responsible for high expression of PGII on pectin [12,20]. It is therefore very likely, that this *pgx* promoter region plays a crucial role in the induction of the gene by galA and should be taken as the start point for further promoter deletion analysis.

The two remaining potential *pgx* regulatory mutants carrying a recessive mutation, *viz.* strains 848.12 and 848.14, and the 848.9 mutant were examined by a northern blot analysis as described above. Figure 9 shows the *pgx* induction profiles in these strains.

Figure 9. The northern blot analysis of the potential *pgx* regulatory mutants carrying a recessive mutation. The mycelium was pregrown for 20 hours in SM medium containing 1.5 % (w/v) sucrose and transferred to SM with 1 % (w/v) galA. The rRNA picture after staining of the northern blot with methylene blue is presented as the loading control.
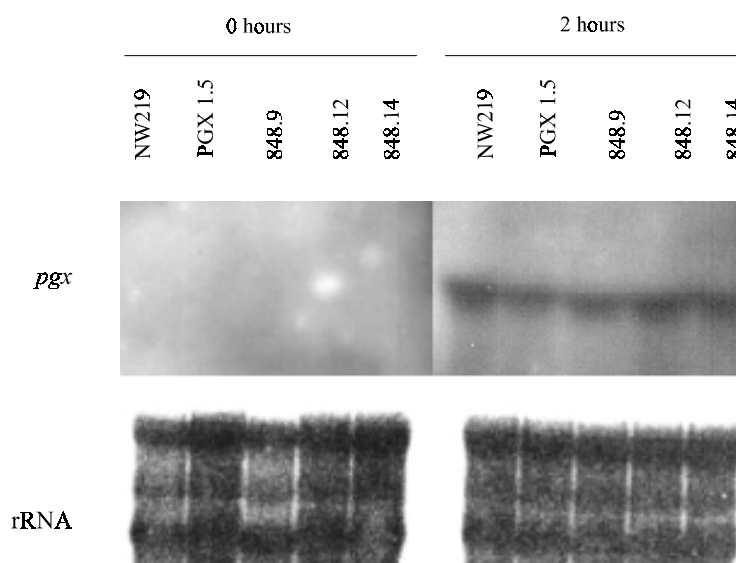
No effect on *pgx* expression was observed in the potential *pgx* regulatory mutants 2 hours after the transfer, although the expression was lower after 6 and 10 hours when compared to the wild type *A. niger* (data not shown), similarly as described before for the mutants obtained in the first UV mutagenesis. Therefore, it was concluded that these mutations are *cis*-acting only on the *pgx-pyr*A construct.

Although the approach to exploit the *pyr*A gene as the selection marker for the isolation of pectinase regulatory mutants did not succeed, the results allowed us to disclose some important elements of the *A. niger* pectinase induction mechanism. Based on the differences in expression of the *pel*A and *pgx* genes in the strains carrying the *csp*A1 mutation we can suggest that the expression of these genes is under the control of two different regulatory mechanisms. In the case of *pgx*, the induction is most probably mediated via a system, which responds to galA as the inducer. In the case of the *pel*A regulatory system (see General Discussion) another inducer is likely to be involved. The question remains, whether there is a direct link between the decrease of the *pel*A expression and the *csp*A1 mutation, which would indicate the involvement of a more general regulatory mechanism controling the expression of the *A. niger* lyase genes. Since the expression of the pectinase-encoding genes might be under the control of several regulatory systems as indicated by the induction profiles I-III, which act on different target sequences in these promoters, using specific promoter regions may facilitate the selection of mutants.

During the UV-mutagenesis of *A. niger* PGX 1.5 we also considered the possibility that the galA utilisation pathway (including the transport system and the intracellular enzymes) is under the control of the same regulator as the *pgx* expression itself. This would enable us to isolate regulatory mutants in this way. We used D-sorbitol in addition to galA in the selection plates. However, this experiment also did not give positive results (data not shown).

In conclusion, the experiment aiming at the isolation of the pectinase regulatory mutants, demonstrated the complexity of the pectinase regulatory mechanism of *A. niger* and indicated that for the successful continuation of the pectinase regulation studies it is necessary to extend our knowledge about the physiology of the fungus, including the catabolic pathway of galA.

*A deletion study of the **pelA** promoter using **goxC** as a reporter system and the use of the reporter system to identify inducers.*

For the *pel*A promoter deletion study *gox*C, encoding glucose oxidase from *A. niger* [30], was selected as the reporter gene. This reporter system was previously successfully used in the study of the xylanase-encoding gene, *xln*A, from *Aspergillus tubingensis* [40].

Although the results presented before showed that there is an effect of the *csp*A1 mutation on the *pel*A expression in *A. niger* NW133, the induction remained specific and therefore this strain was selected for the transformation of the *pel*A-*gox*C constructs.

The deletion analysis of the *pel*A promoter. Two plasmids carrying the *pel*A promoter - *gox*C reporter gene were constructed. pIM3820 (*viz.* Materials and Methods) and pIM3821 (Table 2) contain the 1.1 kb *Eco*RV/*Nsi*I and the 379 bp *Nco*I/*Nsi*I *pel*A promoter fragments, respectively (for the *pel*A sequence see [8]). In Fig. 10 the schematic representation of the *pel*A promoter regions in pIM3820 and pIM3821 and the localisation of the consensus DNA binding sequences of the known fungal regulatory proteins are depicted.

Figure 10. Schematic picture of the *pel*A promoter fragments fused with the *gox*C reporter. The position of the DNA binding sequences of C, CREA [42]; P, PacC, 5' GCCARG 3' [43]; X, XLN R, 5' GGCTAA(A) 3' [21] and the hexanucleotide sequence (H) of the *A. niger* pectinolytic genes [12] is depicted. The forward and reverse orientation of the DNA protein binding sequences in the *pel*A promoter is demonstrated by the up and down orientation of the lettering respectively.
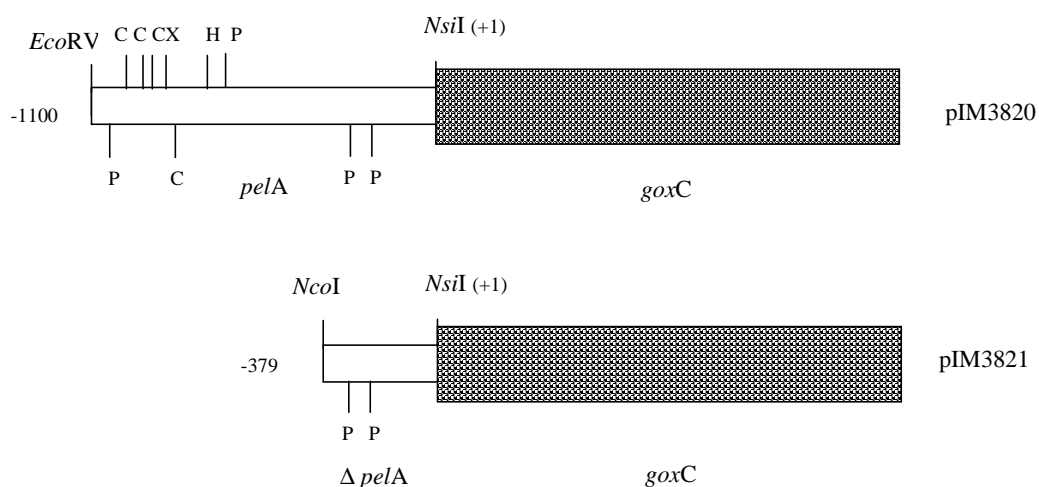
Upon transformation of the glucose oxidase-negative *A. niger* strain NW133 with pIM3820 and pIM3821, forty transformants were randomly chosen for each construct. These strains were tested for GOX expression in the presence of different carbon sources using a plate assay. While in the case of *A. niger* NW133::pIM3821 transformants only a very low GOX activity could be detected after prolongated incubation in the presence of galA (data not shown), in the *A. niger* NW133::pIM3820 transformants the GOX activity was detected quickly in the overlay assay. Based on the results of the Southern blot analysis of some of the selected transformants (Fig. 11A,B), it is obvious that the low GOX activity or the absence of it observed in the *A. niger* NW133::pIM3821 transformants is not due to the low copy number of the integrated plasmid.

Figure 11. Southern blot analysis of the pIM3820 (panel A) and pIM3821 (panel B) *A. niger* **NW133 transformants.** The chromosomal DNA of the selected transformants was digested with *Bam*HI and probed with the radiolabeled *pel*A-*gox*C *Eco*RI fragment (Table 2). The hybridising bands correspond to the fragments depicted at the left-hand side of the figure. *Bam*HI cuts once in both plasmids.
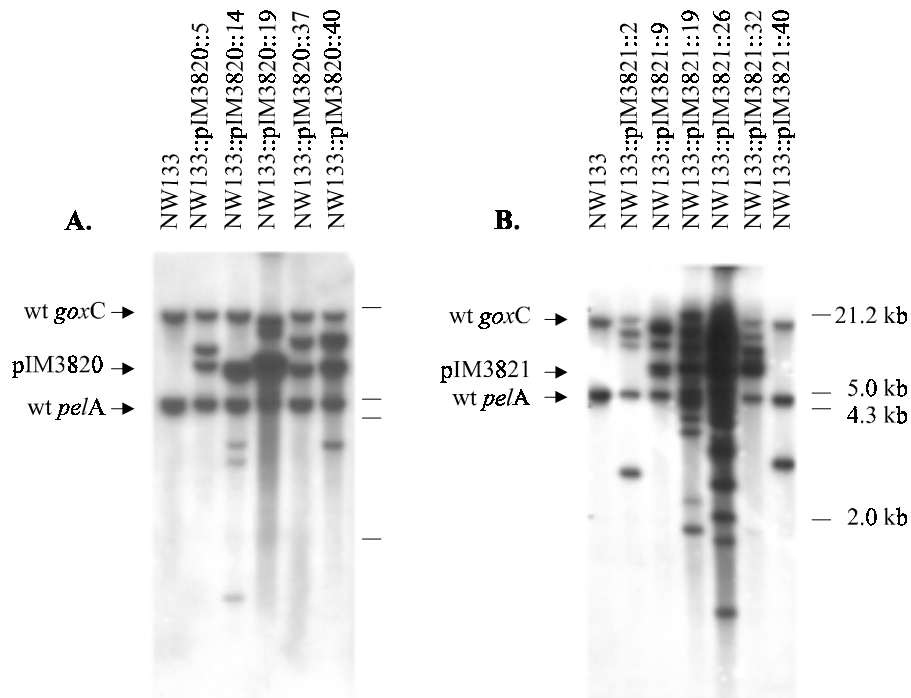
In Table 6 the comparison of the GOX activity of five selected *A. niger* NW133::pIM3820 transformants, based on the intensity of staining of the colonies, is presented. Induction was observed in all strains in the presence of galA. However, also the presence of other carbon

sources like D-sorbitol, D-xylose and D-glucose led to some extent to *gox*C expression under the control of the *pel*A promoter. The correlation between the copy number of pIM3820 and the GOX activity (Table 6) clearly demonstrates that the transformants containing more copies of pIM3820 have higher GOX activity, but the induction is less specific.

Table 6. The effect of the carbon source on the expression of the *gox*C reporter under the control of the *pel*A promoter in selected *A. niger* NW133::pIM3820 transformants using a plate assay.

strain / copy number	time [min]	1 % galA	1 % sorbitol	1 % glucose	1 % xylose
NW133::pIM3820::5	15	++	0	0	0
/ 2	45	++	+/-	+/-	+
NW133::pIM3820::14	15	+++	0	+/-	+/-
/ 3-4	45	+++	+/-	++	+
NW133::pIM3820::19	15	+++	+/-	+++	+++
/ 6-7 *	45	++++	++	++++	+++
NW133::pIM3820::37	15	+	0	+/-	0
/ 2-3	45	+	+/-	+	0
NW133::pIM3820::40	15	+++	+/-	++	+/-
/ 4-5	45	++++	+	+++	++
NW133 control	15	0	0	0	0
	45	0	0	0	0

0 = no activity, +/- = trace of activity, + = low activity, ++ = intermediate activity, +++ = high activity, ++++ = very high activity
* notice in Fig. 11, the integration of pIM3820 into the *gox*C locus

Based on these results we can conclude, that the 721 bp *pel*A promoter region (-1100 to - 379 from the translation start point) is important to obtain high expression of the gene in the presence of galA. However, the low level of GOX expression under the control of the 721 bp *pel*A promoter fragment, was observed also on other carbon sources, including D-glucose. This *pel*A promoter fragment is currently being further analysed.

Application of the GOX protoplast assay for the analysis of *pel*A inducers. The *A. niger* NW133::pIM3820::5 transformant was selected based on the genetic background (Fig. 11) and the GOX phenotype test (Table 6) for the development of a GOX protoplast assay. Our aim was to establish a method by which a number of different carbon sources can be screened for their *pel*A inducing properties in a quick and effective way.

The protoplasts of *A. niger* NW133::pIM3820::5 and the *A. niger* control strain NW219, which contains the intact *gox*C gene, were prepared as described in Materials and Methods. Two types of protoplast stabilising media were used containing either D-sorbitol or salt, in order to exclude the effect of buffer components on the *pel*A gene expression. The protoplasts were incubated with the carbon source of interest to induce the *de novo* synthesis of GOX under the control of the *pel*A promoter. Initially sucrose was used to grow the mycelia. However, no GOX expression was observed when protoplasts were derived from this mycelium and incubated with galA. When sucrose was replaced by sugar beet pectin, the protoplasts isolated from the mycelia were able to produce GOX activity in the presence of galA (see below). It is therefore very likely, that the specific transporter of galA is also inducible by a pectin constituent. The effect of different galA concentrations on the expression level of the *pel*A promoter is shown in Fig. 12.

Figure 12. The GOX protoplast assay. The increase of the GOX activity upon lysis of the *A. niger* NW133::pIM3820::5 protoplasts incubated for 3 hours at 30 $^\circ$C with 50 mM (●), 10 mM (■) or 5 mM (▲) galA is depicted as the increase in absorbance at 490 nm. The results were similar using the PROTO SM1 and PROTO SM2 buffers and all different treatments, C1-C3, did not show any GOX activity (see Materials and Methods).
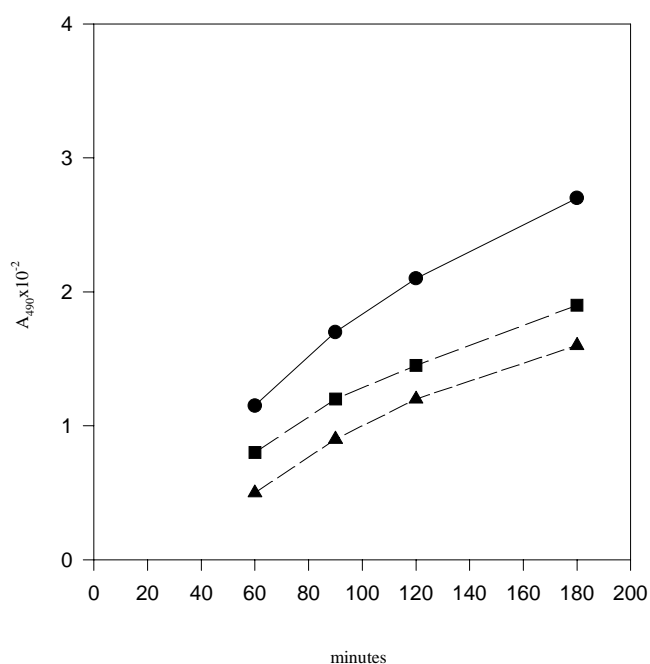
An increase of the GOX activity was observed during the time of the reaction and the highest GOX activity was obtained using 50 mM galA. In a second experiment digalacturonate, rha and xyl (5 mM and 50 mM) were tested in order to compare their inducing capacity with galA. While in the incubation mixture containing the dimer no GOX activity was observed, 50 mM L-rhamnose and 50 mM D-xylose were able to induce the *pel*A-*gox*C reporter up to 33.7 % and 36.8 % of the GOX activity measured with 50 mM galA as the inducer (data not shown). These

results again confirmed that besides galA also other sugars stimulate the transcription from the *pel*A promoter.

The GOX protoplast method using the *A. niger pel*A-*gox*C low copy number transformant proved to be a quick and reliable tool for screening inducers of pectin lyase A.

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CHAPTER 6

Evaluation of RFLP analysis for the classification of selected black aspergilli

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Abstract

Thirty *Aspergillus* strains representing different isolates of the "*A. niger* aggregate", *viz. A. awamori*, *A. phoenicis* and *A. foetidus*, and *A. carbonarius* strains were studied in order to explore the potential of a fast RFLP analysis to identify fungal strains. The patterns were compared with those characteristic for *A. niger*, *A. tubingensis*, *A. carbonarius*, *A. japonicus* and *A. aculeatus* represented by *A. niger* CBS 120.49, *A. tubingensis* NW 756, *A. carbonarius* CBS 111.26, *A. japonicus* CBS 114.51 and *A. aculeatus* CBS 101.43 and also with those of the type strains of *A. heteromorphus* CBS 117.55 and *A. ellipticus* CBS 707.79.

Sma I digested chromosomal DNA revealed characteristic rDNA patterns after ethidium bromide staining which were used in combination with hybridisation patterns of *Pst* I/*Sal* I double digested chromosomal DNA with well-defined probes. This allowed clear distinction of eight separate species within the *Aspergillus* section *Nigri* group. The probes used were a 0.9-kb fragment of the 28 S rDNA from *Agaricus bisporus*, an internal fragment of the *pki*A gene from *A. nidulans*, and the *pel*A gene from *A. niger*. The strains classified as *A. awamori*, *A. phoenicis*, and *A. foetidus* were shown to belong either to *A. niger, A. tubingensis* or a group representing isolates of *A. foetidus* varieties, among which *A. foetidus* var. *acidus* CBS 564.65 and *A. foetidus* var. *pallidus* CBS 565.65. Among the *A. carbonarius* isolates, the strain CBS 101.14 was found to be misclassified. This isolate was characterised as *A. japonicus*. For all other *A. carbonarius* strains examined, only the *pki*A RFLP pattern showed some intraspecific variations.

Introduction

The filamentous *Aspergillus* spp. are cosmopolitan micro-organisms. Their ability to produce metabolites as organic acids as well as extracellular enzymes makes them important tools for the food industry. Particularly important are representatives of the black aspergilli (*Aspergillus* sect. *Nigri*) *A. niger* Tiegh., *A. tubingensis* Mosseray, *A. japonicus* Saito, *A. aculeatus* Iizuka and *A. foetidus* Thom & Raper, products of which hold the GRAS status (generally regarded as safe) and which are therefore commonly used in fermentation processes.

Some of the aspergilli, however, have a negative impact on society as they can act as plant or human pathogens. It is therefore of utmost importance to clearly establish the taxonomical position of an *Aspergillus* strain prior to its application in food or feed processes. Methods to evaluate the taxonomical position of strains are important in connection to their industrial use and legal protection and, hence, should rely on unambiguous criteria.

Phenotypic features like colour, shape, size and ornamentation of conidia and the length of the conidiophore have been in the past and are still used for classification. On the basis of classical morphological features Raper & Fennell (1965) accepted twelve different species within the section *Nigri*. This classification was revised by Al-Musallam (1980) who divided the black aspergilli on the basis of her morphological studies into seven species, five of which were readily distinguished i.e. *A. carbonarius* (Bainier) Thom, *A. heteromorphus* A.C. Bat. & Maia, *A. ellipticus* Raper & Fennell emend. Al-Musallam, *A. helicothrix* Al-Musallam, later found to be only a morphological variant of *A. ellipticus* (Kusters-van Someren, Samson & Visser, 1991) and *A. japonicus*. At that point *A. aculeatus* was classified as a variety of *A. japonicus*. The remaining strains were considered to form the "*A. niger* aggregate" and were divided into two species *A. foetidus* and *A. niger*, the latter species consisting of six varieties and two formae.

However, the phenotype of a fungal strain can vary considerably, depending on growth conditions (Samson, 1994). Additional criteria, independent of growth conditions have therefore been applied, e.g. RFLP, RAPD, DNA fingerprinting and nucleotide sequencing to establish the identity of a strain. In our laboratory we have mainly focused on developing RFLP methods. The studies by Kusters-van Someren, Samson & Visser, 1991, employing such an RFLP analysis, demonstrated that the "*A. niger* aggregate" can be divided into two different species, *A. niger* and *A. tubingensis*. Furthermore, these authors showed that in spite of pronounced morphological features of the *A. carbonarius* species, like the large biseriate conidial heads and large verrucose to tuberculate conidia, sometimes the classification of an *A. carbonarius* strain is incorrect. They noticed that for the *A. carbonarius* strain CBS 127.49, the

Table 1. *Aspergillus* **strains used in RFLP analysis.** T- type strain, NT - neotype strain; *, T of *Sterigmatocystic acini-uvae* (the origin of *Sterigmatocystic* came from Cramer in 1859, who recognised a separate genus among aspergilli); **, probably T of *S. fusca*

Original name	Collection number	Source	Reference/ Isolated by
A. awamori	ATCC 22342	unknown	J. van Lanen
A. awamori	CBS 113.33	unknown	A. Blochwitz
A. awamori	CBS 121.48	unknown	R. Mosseray
A. awamori	CBS 115.52	Japan, kuro-koji	K. Sakaguchi
A. awamori	CBS 117.52	unknown	R. Ciferri
A. awamori	CBS 119.52	unknown	R. Ciferri
A. awamori	CBS 126.52	unknown	R. Ciferri
A. foetidus	CBS 103.14	unknown	A. Blochwitz
A. foetidus (T)	CBS 114.49	unknown	Yakoyama
A. foetidus (T) of *A. citricus*	CBS 618.78	unknown	C. Wehmer
A. foetidus var acidus	CBS 128.52	Japan	K. Sakaguchi
A. foetidus var acidus (T)	CBS 564.65	Japan	R. Nakazawa
A. foetidus var pallidus	CBS 114.52	unknown	R. Ciferri
A. foetidus var pallidus (T)	CBS 565.65	Japan	R. Nakazawa
A. foetidus var pallidus	CBS 619.78	unknown	R. Nakazawa
A. phoenicis	CBS 118.36	chemical, USA	L.B. Lockwood
A. phoenicis	CBS 114.37	unknown	Y.K. Shih
A. phoenicis (T) of *A. veletinus*	CBS 139.48	unknown	R. Mosseray
A. phoenicis	CBS 629.78	France	da Fonseca
A. phoenicis	ATCC 13156	whole shelled corn	A.E. Staley
A. phoenicis	CBS 126.49	Japan	Yakoyama
A. niger	CBS 120.49	unknown	Anthony
A. tubingensis	NW 756	unknown	M.A. Kusters-van Someren
A. aculeatus (T)	CBS 101.43	*Pterocarpus santalinus*	De Körver
A. japonicus (T)	CBS 114.51	unknown	K. Kominami
A. carbonarius (NT)	CBS 111.26	paper	A.F. Blakeslee
A. carbonarius	CBS 101.14	Unknown	A. Blochwitz
A. carbonarius	CBS 110.49	Air Indonesia	K.B. Boedijn
A. carbonarius	CBS 111.80	South Africa	E. N. Lawson
A. carbonarius	CBS 112.80	unknown	Herrick and May
A. carbonarius	CBS 113.80	*Theobroma cacao* Nigeria	J. A. Broadbent
*A. carbonarius**	CBS 114.29	Spain	A. Caballero
A. carbonarius	CBS 115.49	Air Indonesia	K. B. Bodeijn
A. carbonarius	CBS 127.49	*Coffee arabica* seed	M. Sainclivier
*Sterigmatocystis fusca***	CBS 420.64		F. da Fonseca
A. heteromorphus (T)	CBS 117.55	culture contaminant	A.C. Batista
A. ellipticus (T)	CBS 707.79	soil Costa Rica	Al-Musallam

RFLP patterns based on the ribosomal repeat unit, and the pectin lyase A encoding gene *pel*A were identical to *A. tubingensis* NW 756 (CBS 643.92). These patterns did not fit to the RFLP patterns of the neotype strain *A. carbonarius* CBS 111.26. The methods used showed to be powerful and quick. Varga *et al.* (1993, 1994) confirmed the division of the "*A. niger* aggregate" into two species analysing mitochondrial DNA. The latter authors introduced a third distinct group, represented by a limited number of Brazilian isolates. These studies again strengthened the power of RFLP based methods in *Aspergillus* taxonomy.

In this study we therefore extend the molecular analysis of the black aspergilli to a selection of *A. foetidus*, *A. awamori* Nakaz., *A. phoenicis* (Corda) Thom and *A. carbonarius* strains in order to obtain a comprehensive overview of these groups. Two *A. foetidus* strains including the type strain CBS 121.28 and the neotype strain of *A. awamori* CBS 557.65 were previously identified to belong to *A. niger* (Kusters-van Someren, Samson & Visser, 1991). The patterns of a much larger set of strains were now compared with those of *A. niger* CBS 120.49, *A. tubingensis* NW 756, and *A. carbonarius* CBS 111.26, which were previously characterised (Kusters-van Someren, Samson & Visser, 1991). The *A. carbonarius* isolate CBS 127.49 was also re-examined. The *A. japonicus* type strain CBS 114.51, and the *A. aculeatus* strain CBS 101.43 were included to allow comparison with the new selection of strains. Furthermore, type strains of *A. heteromorphus* and *A. ellipticus* were examined.

Materials and Methods

Strains and plasmids. Strains (Table 1) were obtained from the Centraal Bureau voor Schimmelcultures in Baarn, The Netherlands. *Aspergillus tubingensis* NW 756 is an industrial strain which belonged to the *A. niger* aggregate (Kusters-van Someren, Samson & Visser, 1991). Plasmids carrying the *pki*A gene of *A. nidulans* (de Graaff & Visser, 1988b), the *pel*A gene of *A. niger* (Harmsen, Kusters-van Someren & Visser, 1990) and the 0.9-kb *Eco*RI fragment of the 28 S rDNA was present at our own laboratory.

Growth conditions and isolation of chromosomal DNA. Cultures were grown by inoculating 10^6 spores.ml^{-1} in minimal medium (Pontecorvo *et al.*, 1953) using 1 % (w/v) of glucose as a carbon source and 0.85 % (w/v) yeast extract in 50 ml of medium in 250 ml flasks. The cultures were grown at 30 $^\circ$C in a rotary shaker (250 rpm). Mycelia were harvested by filtration after 20 h of culturing. DNA isolation was carried out as described by de Graaff, van den Broek & Visser (1988a).

DNA digestion and gel electrophoresis. Digestions of 5 µg of DNA were carried out overnight with 40 units of enzyme in 200 µl volume at the temperature and with buffers as recommended by the

manufacturer of the restriction endonucleases (Gibco BRL). Gel electrophoresis in 0.7 % (w/v) agarose gel was done at 1.5 V.cm^{-1}, for 16-20 h in TAE buffer, using a horizontal gel kit according to Sambrook, Fritsch & Maniatis (1989). Bacteriophage lambda DNA double digested with restriction enzymes *Hind* III and *Eco* RI was used as a size marker. The separated DNA was visualised by UV translumination.

Southern blotting and hybridisation. The DNA was transferred to a Hybond^{TM}-N membrane (Amersham) by vacuum blotting (VacuGene^{TM}XL unit, Pharmacia Biotech. AB) using a standard protocol for transfer of high molecular weight DNA as described in the instruction manual. The DNA was fixed to the membrane by UV cross-linking for 3 min. Probes were made according to Kusters-van Someren, Samson & Visser (1990).

The following probes were used: 1) the 0.8-kb internal *Sal* I fragment of the *A. nidulans pki*A gene encoding pyruvate kinase A; 2) the 0.9-kb *Eco* RI fragment comprising the 3' end of the 28 S ribosomal DNA of *Agaricus bisporus* ; 3) the 1.6-kb *Cla* I fragment containing the C-terminal part and some downstream sequence of the *A. niger pel*A gene encoding pectin lyase A. Probes were radioactively labelled by random priming (Feinberg & Volgestein, 1983).

Blots were prehybridised for 2 h according to Sambrook, Fritsch & Maniatis (1989) followed by hybridisation overnight at 60 $^{\circ}$C. The blots were washed twice for 30 min with 2 x SSC (0.3M NaCl, 0.03M tri-sodium citrate dihydrate; pH 7.0), 0.5 % (w/v) SDS at 60 $^{\circ}$C. After exposure of the Southern blots hybridised with either the *pki*A probe or the *pel*A probe, signals were stripped by rinsing the membranes for 1 min in 0.1 % (w/v) SDS at 100 $^{\circ}$C and the blots were subsequently used for hybridisation with the 28 S probe.

Results and Discussion

The strains selected from the CBS culture collection to be examined by RFLP analysis are listed in Table 1. They were classified before on the basis of morphological criteria. Except for the reference strains only four others (CBS 618.78, CBS 126.49, CBS 420.64 and CBS 127.49) were previously analysed by restriction analysis of chromosomal DNA (Kusters-van Someren, Samson & Visser, 1991).

The chromosomal DNA was digested with two restriction endonucleases - (*Pst* I and *Sal* I) - to perform the hybridisation experiments. This combination of restriction enzymes was chosen since this led to a more reliable interpretation of fragment lengths when compared to fragments resulting from single digests. The use of double digests, though, may lead to an increase in the complexity of the patterns. Furthermore, when observing variability in patterns, it is not always possible to indicate which restriction site is altered by mutation. Bearing this in mind we only use a pattern or the presence of identical fragments in patterns otherwise differing as a diagnostic feature for strain or species identification. The patterns observed for each individual probe are

listed in Table 2 for the *pki*A and the 28 S rDNA and in Table 3 for the *pel*A probe, respectively. We have also indicated the characteristic patterns of the various reference strains.

Table 2. RFLP patterns observed in *Pst*I /*Sal* I digests of chromosomal DNA of various black aspergilli using a 0.8-kb *Sal* I fragment of *A. nidulans pki*A and a 0.9-kb *Eco* RI fragment of *Agaricus bisporus* 28 S rDNA as probes. **A. tubingensis* strains usually have pattern B except for strain NW756 (for *pki*A) which shows polymorphism.

Reference strain	Fragment length (kb)	Pattern
***pki*A**		
A. carbonarius CBS 111.26	6.0	A I
	0.9	A II
	4.8	A III
A. niger CBS 120.49	3.4	B
A. aculeatus CBS 101.43	3.0	C
A. japonicus CBS 114.51		C
A. ellipticus CBS 707.79	2.8	D
A. heteromorphus CBS 117.55		D
A. tubingensis NW 756 *	2.8 & 0.65	E
28S rDNA		
A. carbonarius CBS 111.26	5 & < 0.3	A
A. tubingensis NW 756	5 & 0.9	B
A. niger CBS 120.49	5 & 1.5	C
A. foetidus var. *acidus* CBS 564.65	5 & 1.8	D
A. japonicus CBS 114.51	6 & 1.2	E
A. aculeatus CBS 101.43	6 & 1.6	F
A. ellipticus CBS 707.79	7.5 & < 0.5	G
A. heteromorphus CBS 117.55		G

The data clearly illustrate the diagnostic power of the *Pst* I/*Sal* I digests, particularly in combination with the 28 S rDNA probe and the *pel*A probe. All reference strains can be clearly distinguished but in addition some new patterns are seen which are associated with some of the new strains. Previously it was reported (Kusters-van Someren, Samson & Visser, 1991; Varga *et al.*, 1993) that digestion of chromosomal DNA with *Sma* I leads to clearly visible, diagnostic banding patterns of the highly repetitive rDNA unit upon staining with ethidium bromide. Therefore, this restriction enzyme was also used here. Five different patterns were found which

allow to discriminate between some of the reference strains, i.e. *A. carbonarius* (pattern A, 4.1 and 2.3 kb), *A. niger* (pattern B, 3.2, 2.3 and 1.8 kb), *A. tubingensis* (pattern C, 2.6, 1.9 and 1.8 kb), *A. japonicus* and *A. aculeatus* (pattern D, 1.9 and 1.8 kb) and *A. heteromorphus* and *A. ellipticus* (pattern E, 1.9, 1.8 and 1.6 kb).

Table 3. RFLP patterns observed in *Pst* I/*Sal* I digests of chromosomal DNA of various black aspergilli using a 1.6-kb *Cla* I fragment of the *A. niger pel*A gene as a probe. Fragment lengths are indicated in kb. * the species representatives have the following patterns:
B, *A. tubingensis* NW 756; E, *A. niger* CBS 120.49; G, *A. ellipticus* CBS 707.79; H, *A. aculeatus* CBS 101.43; I, *A. japonicus* CBS 114.51; J, *A. carbonarius* CBS 111.26; K, *A. heteromorphus* CBS 117.55.

Pattern*	A	B	C	D	E	F	G	H	I	J	K
		6.0									
		5.0									
			4.8	4.8							
	4.5				4.5	4.5					
							3.8				
								3.6			
									3.2		
										2.9	
			2.8								
									2.4		2.4
			2.2	2.2							
							2.0				
								1.8			
									1.7		
			1.5								
		1.2		1.2	1.2					1.2	
					1.0						
						0.9					
								0.8			
									0.6		

From the new strains tested representing *A. foetidus*, *A. awamori* and *A. phoenicis* isolates, no new patterns were seen. These strains either have the *A. tubingensis* or the *A. niger Sma* I pattern. The *A. carbonarius* neotype strain CBS 111.26 and the other *A. carbonarius* strains

tested, except for CBS 101.14 and CBS 127.49, have the same pattern A. CBS 101.14 has a pattern corresponding with the *A. japonicus* type strain and CBS 127.49 has the *A. tubingensis* pattern as observed previously by Kusters-van Someren, Samson & Visser (1991).

A final compilation of strains grouped according to their pattern similarities is presented in Table 4. This identifies *A. foetidus* as a separate group also represented by the type strains *A. foetidus* var. *acidus* Nakaz. Simo & Watan. (CBS 564.65) and *A. foetidus* var. *pallidus* Nakaz. Simo & Watan. *et al.* (CBS 565.65). This group does not include CBS 121.28, the type strain of *A. aureus = A. foetidus*, and CBS 114.49, designated as typical for *A. foetidus* by Thom & Raper (1945). Both strains fit in the *A. niger* group as found by Kusters-van Someren, Samson & Visser (1991). The two major divisions, *A. tubingensis* and *A. niger*, each have their unique pattern profile which is also present in the neotype strains of each group. There are some anomalies for each species, which will be discussed below. All the *A. carbonarius* strains, except CBS 101.14 and CBS 127.49, can be grouped together based on the *Sma* I, 28 S and *pel*A RFLP patterns. The *A. carbonarius* taxon, represented here by eight isolates, however, shows heterogeneity in the *pki*A locus (see Table 2, patterns AI-AIII). The pattern AII (0.9-kb) may represent the *A. carbonarius* equivalent of the *A. nidulans* 0.8-kb *Sal* I *pki*A internal fragment and the remaining patterns AI and AIII could be explained by an alternation of one of the *Sal* I restriction sites. The previous indications about the true identity of CBS 127.49 (*A. tubingensis*) and CBS 101.14 (*A. japonicus*) were again confirmed. The next major division, *A. aculeatus* and *A. japonicus*, included in this study as reference strains originate from a preliminary study of 23 strains by M. E. G. Suykerbuyk (unpublished results). This study uses a RFLP analysis not only based on a *Pst* I/*Sal* I digest but also on the combination *Xho* I/*Kpn* I which allows clear-cut identification of *A. aculeatus* and *A. japonicus* (see also Chapter 7). The two remaining strains *A. heteromorphus* and *A. ellipticus* were classified as two separate species by Al-Musallam (1980). Only the *pel*A probe discriminates between these strains but the use of other restriction enzyme combinations might be useful to separate these species more clearly. There is certainly also a need to include more isolates in this case.

As already mentioned the individual pattern profiles do not match perfectly in all cases. The strain *A. awamori* ATCC 22342 shows a 28 S rDNA pattern characteristic for the group near varieties of *A. foetidus* and a *pel*A pattern typical for *A. tubingensis* (*cf.* Table 2, 3). The *pel*A patterns of *A. foetidus* varieties (pattern C) and of *A. tubingensis* (pattern B) do not show any band equal in size. However, the 28 S rDNA patterns share the 5.0-kb band while it can be easily envisaged that the second hybridising band (1.8-kb and 0.9-kb, respectively) can originate

from one another by a single point mutation. Therefore this particular strain was considered to be *A. tubingensis*. Of the strains which belong to *A. niger*, *A. tubingensis* and 'A. *foetidus*' all but one showed the same pattern B with the *pki*A probe, a single 3.4 kb band. The *A. tubingensis* strain NW 756 shows two hybridising bands (2.8-kb and 0.65-kb) which add up to an approximate size of 3.4-kb suggesting this pattern arises from an additional restriction site.

A. phoenicis CBS 629.78 is another strain with an anomalous pattern profile, *pel*A pattern A versus pattern B. We cannot satisfactorily explain the relation between *pel*A pattern A and B (Table 3). Despite this difference in *pel*A pattern, *A. phoenicis* CBS 629.78 was assigned to be *A. tubingensis* based on the remaining three patterns for *Sma* I, *pki*A and 28 S that are diagnostic for this species.

Within the *A. niger* group two strains, *A. awamori* CBS 121.48 and *A. phoenicis* ATCC 13156, show deviating *pel*A patterns. Their origin is not entirely clear as the *pel*A probe may hybridise to another member of the *pel* gene family, namely *pel*D (Kusters-van Someren, Samson & Visser, 1991). The three identical patterns (*Sma* I, *pki*A and 28 S) for each strain are in favour of their placement within the *A. niger* group. Moreover, the relation of the deviating *pel*A patterns is demonstrated by the occurrence of the 1.2-kb band for *A. awamori* CBS 121.48 and the 4.5-kb band for *A. phoenicis* ATCC 13156, both bands also being present in *pel*A pattern E.

Under the present classification, 'A. *niger*' segregates into three taxa: *A. niger*, A. *tubingensis* and strains grouped around the varieties of *A. foetidus* detected by our molecular data but which cannot be recognised from morphological criteria. Al-Musallam (1980) distinguished *A. foetidus*, including var. *acidus* and *pallidus*, by the olivaceous brown conidial heads and smooth to finely roughened conidia. Kozakiewicz (1989) named *A. foetidus* and these varieties *A. citricus* (Wehmer) Mosseray based on a herbarium specimen, which she believed, represented *A. niger-A. citricus*. She distinguished *A. citricus* by SEM observation of the punctate excrescences of the conidial ornamentation.

Our studies show that both type (CBS 121.28; Kusters-van Someren, Samson & Visser, 1991) and typical strains (CBS 114.49) of *A. aureus* and *A. foetidus* are indistinguishable from *A. niger* which concludes the synonymy with *A. niger*. For the strains near the varieties of *A. foetidus*, another epithet should be chosen. Because of the new strategy in *Aspergillus* nomenclature to stabilise the conserved names (Pitt & Samson, 1993) a proposal for a new name must be discussed and approved by the IUMS International Commission of *Penicillium* and *Aspergillus* Systematics (ICPA).

Table 4. **Final grouping of black aspergilli strains.** * *Pst* I/*Sal* I 28S pattern E I contains 6.0-kb and1.4-kb hybridising bands, however the *Kpn* I/*Xho* I digests in combination with the 28S probe (M.E. G. Suykerbuyk, unpublished results) clearly group this strain into the *A. japonicus* taxa.

Group	Strain/name	CBS/ATCC	*Sma* I	*pki*A	28S	*pel*A
A. foetidus varieties	*A. awamori*	115.52	C	B	D	C
	A. awamori	119.52	C	B	D	C
	A. foetidus	103.14	C	B	D	C
	A. foetidus var. *acidus*	128.52	C	B	D	C
	A. foetidus var. *acidus* (T)	564.65	C	B	D	C
	A. foetidus var. *pallidus*	114.52	C	B	D	C
	A. foetidus var. *pallidus* (T)	565.65	C	B	D	C
	A. foetidus var. *pallidus*	619.78	C	B	D	C
	A. phoenicis	139.48	C	B	D	C
A. tubingensis	*A. awamori*	ATCC 22342	C	B	D	B
	A. awamori	126.52	C	B	B	B
	A. phoenicis	114.37	C	B	B	B
	A. tubingensis	NW 756	C	E	B	B
	A. carbonarius	127.49	C	B	B	B
	A. phoenicis	629.78	C	B	B	A
A. niger	*A. awamori*	113.33	B	B	C	E
	A. awamori	117.52	B	B	C	E
	A. foetidus	114.49	B	B	C	E
	A. foetidus	618.78	B	B	C	E
	A. phoenicis	118.36	B	B	C	E
	A. niger	120.49	B	B	C	E
	A. phoenicis	126.49	B	B	C	E
	A. awamori	121.48	B	B	C	F
	A. phoenicis	ATCC 13156	B	B	C	D
A. carbonarius	*A. carbonarius* (NT)	111.26	A	A I	A	J
	A. carbonarius	110.49	A	A I	A	J
	A. carbonarius	111.80	A	A II	A	J
	A. carbonarius	112.80	A	A III	A	J
	A. carbonarius	113.80	A	A III	A	J
	A. carbonarius	114.29	A	A II	A	J
	A. carbonarius	115.49	A	A I	A	J
	S. fusca	420.64	A	A III	A	J
A. aculeatus	*A. aculeatus*	101.43	D	C	F	H
A. japonicus	*A. japonicus* (T)	114.51	D	C	E	I
	A. carbonarius	101.14	D	C	E I*	I
A. heteromorphus	*A. heteromorphus* (T)	117.55	E	D	G	K
A. ellipticus	*A. ellipticus* (T)	707.79	E	D	G	G

Conclusions

Based on the double digests with the restriction enzymes and probes used in this study it is demonstrated that the biseriate black aspergilli can easily be separated into different species. We can discriminate *A. niger*, *A. tubingensis*, *A. carbonarius* and a group of strains of varieties of *A. foetidus*, whereas *A. heteromorphus* and *A. ellipticus* type strains, which seem to be closely related to each other, are distinct from the other biseriate black aspergilli. For strains belonging to either *A. aculeatus* or *A. japonicus* the restriction enzyme combination *Pst* I/*Sal* I is less well suited. For a fast screening of a new black *Aspergillus* isolate it is therefore recommended to use two combinations of restriction enzymes: *Pst* I/*Sal* I and *Xho* I/*Kpn* I (M. E. G. Suykerbuyk, unpublished results).

The method employed here can serve also as a basis to make a library of RFLP patterns for the non-black aspergilli that allows fast future identification of new isolates.

The division of the *A. niger* aggregate into two species, *A. niger* and *A. tubingensis*, was already previously reported by Kusters-van Someren, Samson & Visser (1991), Megnegneau, Debets & Hoekstra (1993) and Varga *et al.* (1993). It is shown by this study that strains classified by morphological criteria as either *A. foetidus* or other members of the *A. niger* aggregate (Thom & Raper, 1945; Al-Musallam, 1980) can now be separated into three species.

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CHAPTER 7

Combined molecular and biochemical approach identifies
Aspergillus japonicus and *Aspergillus aculeatus* as two species

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Abstract

Nine *Aspergillus japonicus* and ten *Aspergillus aculeatus* isolates have been examined using molecular and biochemical markers. The study included DNA sequencing of the ITS 1-5.8S-ITS 2 region, RFLP and secondary metabolite analyses and a comparison with reference strains representing other taxa of the black aspergilli.

The sequence data of the internal transcribed spacers (ITS 1 and ITS 2) and the 5.8S rRNA gene did not allow a clear distinction between *A. japonicus* and *A. aculeatus*. However, the phylogenetic analysis showed that these two taxa are more distantly related to the other species of the black aspergilli. *A. niger* pyruvate kinase (*pki*A), pectin lyase A (*pel*A) and *Agaricus bisporus* 28S rRNA genes, used as well-defined probes in the restriction fragment length polymorphism (RFLP) analysis, revealed a clear polymorphism between these two taxa. The *A. niger pki*A and *pel*A probes separated six strains into an *A. japonicus* group. Twelve isolates did constitute an *A. aculeatus* group, which showed much higher intra-specific variations using the *pel*A gene as a probe. The secondary metabolite profiles support the division of the isolates between the *A. japonicus* and *A. aculeatus* species. The strains classified as *A. japonicus* are characterized by the presence of indol alkaloids and a polar metabolite, while the *A. aculeatus* isolates produce neoxaline, okaramins, paraherquamide-like ompounds and secalonic acid.

A. aculeatus strain CBS 114.80 showed specific RFLP patterns for all loci examined. The secondary metabolite profiles also differed from those of *A. japonicus* and *A. aculeatus*. This strain is therefore likely to represent a third taxon.

Introduction

Aspergillus taxonomy dates back from 1926, when Thom and Church [36] for the first time systematically approached the classification of 350 isolates of this genus. The black aspergilli (The Black *Aspergilli*, *A. niger* and Allies), represented by 44 isolates, were separated based on the color of the spores or the metulae present or not. The uniseriate (i.e. metulae not present) isolates were further subdivided based on morphological criteria - the conidial shape and presence or absence of sclerotia. Mosseray (1934) separated the uniseriate black aspergilli into 4 species: *A. japonicus* Saito, *A. atro-violaceus* Mosseray, *A. atro-fuscus* Mosseray and *A. malvaceus* Mosseray [26], whereas Thom and Raper (1945) [37] classified the black aspergilli with only phialides into *A. luchuensis* group. The latter group was further subdivided, comprising the species *A. luchuensis* Inui, *A. nanus* Montagne and/or *A. subfuscus* Johan-Olsen, *A. japonicus* and *A. violaceus-fuscus* Gasperini. However, this classification was not always clear since occasionally biseriate heads could be detected in older colonies. Raper and Fennell (1965) published a description of 132 *Aspergillus* species, 55 of these were not previously described [31]. They reduced the number of the uniseriate black aspergilli and recognized *A. japonicus* and *A. aculeatus* Iizuka. Kozakiewicz (1989) using scanning electron microscopy (SEM) to examine five or more weeks old conidia, separated the black aspergilli into ten species and discriminated between the uniseriate species *A. atroviolaceus* (*A. aculeatus*) and *A. japonicus* [17].

As it is clear that the black aspergilli exhibit a wide range of variability in their morphological and physiological characteristics, the unambiguous identification of a new isolate requires molecular and biochemical identification techniques. The main effort concerning the group of the black aspergilli has been on analysis of the '*A. niger* aggregate' [20,24,29,39-41]. Using mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) RFLP`s and RAPD patterns, the strains of the '*A. niger* aggregate' [1] were proposed to be divided into 4 taxa: *A. niger*, *A. tubingensis* [20], *A. brasiliensis* [40] and strains grouped around the varieties of *A. foetidus* [29]. Similar studies including the examination of the isoenzyme patterns and carbon source utilization have been carried out with isolates belonging to *A. carbonarius* [15,28] and *A. japonicus* and *A. aculeatus* species [9]. In the latter publication the authors detected a high degree of mtDNA polymorphism among the *A. aculeatus* and *A. japonicus* isolates, which correlated with RAPD patterns. However, the molecular data also showed, that the only three *A. aculeatus* strains analyzed belong to one group.

DNA sequencing of the ITS 1 and ITS 2 regions has also been useful resulting for example in the characterization of closely related species of terverticillate penicillia [21,34]. Analysis of DNA sequence data from the large subunit rRNA gene of *Aspergillus* species, subgenus *Circumdati*, furthermore led to re-classification of this subgenus [30].

Secondary metabolites are suitable for chemotaxonomical purposes since they are produced typically by a single species or by closely related species. *A. aculeatus* has been reported to produce the secondary metabolites emodin, secalonic acid D and F [2,18], aculeasins [25,32], and okaramin A, B, H and I [13], while in *A. japonicus* festuclavine and cycloclavine [8], E-64 [10-11] and neoxaline [14,16] were identified.

In the present study we analyzed 19 *A. japonicus* and *A. aculeatus* strains by comparing sequences of the ITS 1-5.8S-ITS 2 region, RFLP's of nDNA and secondary metabolite profiles. The results were compared with data from well-characterized representatives of the other black aspergilli species (see Table 1).

MATERIALS AND METHODS

Strains and plasmids. The strains (Table 1) were obtained from the Centraalbureau voor Schimmelcultures in Baarn, The Netherlands. Plasmids carrying the pyruvate kinase-encoding *pki*A (pGW1100) gene of *Aspergillus niger* [4], the pectin lyase A-encoding *pel*A (pGW820) gene of *A. niger* [12], a 0.9 kb *Eco*RI fragment (pIM2131) of the 28S rRNA of *Agaricus bisporus* [33] as well as the whole rDNA unit (pIM2132) [P. J. Schaap, unpublished results], were propagated in *Escherichia coli* DH5α [43].

Isolation of genomic DNA, digestion and gel electrophoresis. Genomic DNA isolation, digestion using *Sma*I, *Xho*I / *Kpn*I and *Pst*I / *Sal*I restriction enzymes and gel electrophoresis were carried out essentially as described previously [29]. Instead of 0.7 % (mass/vol.) agarose gels, 0.8 % (mass/vol.) was used.

Southern blot and hybridization. The transfer of DNA from agarose gels was described previously [29]. Radioactive labelling of the probes was carried out according to Kusters-van Someren *et al.* [19].
To analyze for DNA polymorphisms the following probes were used for Southern blot hybridization: a 3.5-kb *Bam*HI / *Hind*III fragment of the *A. niger pki*A gene comprising the entire coding region and the 5' and 3' non-coding regions; a 10-kb *Bam*HI fragment carrying the whole rDNA repeat of *A. bisporus*; a 0.9-kb *Eco*RI fragment containing the 3` end of the 28S rRNA of *A. bisporus* and a downstream sequence; a 1.6-kb *Cla*I fragment of the *A. niger pel* A gene comprising part of the coding region and some adjacent 3' non-coding sequence (see Fig. 1A,B).

The hybridization and washing was carried out as described previously [29]. The blots carrying *Pst*I / *Sal*I digested chromosomal DNA were after probing with the *pel*A probe stripped at 100 ^{o}C in 0.1 % (mass/vol.) SDS solution, and subsequently used for reprobing with the 28S rRNA probe.

Table 1. The black aspergilli isolates used in the study. The sequences of the ITS 1 -5.8S- ITS 2 region were deposited under the EMBL accession no. listed. T, type strain; NT, neotype strain. * re-classified by Pařenicová *et al.* [28].

Original name	CBS numbers	EMBL accession no.	source	reference / depositor
A. japonicus	CBS 114.34	AJ279996	man skin	R. Nakazawa
A. atroviolaceus (T)	CBS 113.48	AJ279984	air Amsterdam	R. Mosseray
A. japonicus (T)	CBS 114.51	AJ279985	unknown	K. Kominami
A. japonicus	CBS 568.65	AJ279991	Panama soil	K. B. Raper
A. japonicus	CBS 620.78	AJ280002	New Guinea tent cloth	White
A. japonicus	CBS 312.80	AJ279989	unknown	
A. bruneoviolaceus (T)	CBS 621.78	AJ280003	Brasil	K.B. Raper
A. japonicus	CBS 522.89	AJ279990	air Amsterdam	E.S. van Reenen-Hoekstra
A. japonicus *	CBS 101.14	AJ279983	unknown	A. Blochwitz
A. luchuensis	CBS 101.43	AJ279995	*Pterocarpus santalinus*	Boxmeer
A. lucknowensis	CBS 119.49	AJ279986	*Lactuca sativa*	K.B. Boedijn & J.Reitsma
A. aculeatus	CBS 172.66	AJ279988	tropical soil	K.B. Raper
A. aculeatus	CBS 186.67	AJ279998	litter Zaire	G.L. Hennebert
A. aculeatus	CBS 610.78	AJ280001	tropical soil	A.F. Blakeslee
A. aculeatus	CBS 611.78	AJ279992	tropical soil	K.B. Raper
A. aculeatus	CBS 114.80	AJ280005	soil India	P.P. Tiwari
A. luchuensis	CBS 116.80	AJ279997	palm oil Nigeria	A. Onions
A. aculeatus	CBS 308.80	AJ279999	soil Nigeria	A. Al-Musallam
A. aculeatus	CBS 313.89	AJ280000	soil Fiji	J.C. Frisvad
A. niger	CBS 120.49	AJ280006	unknown	Anthony
A. tubingensis	CBS 643.92	AJ280008	unknown	M. Kusters-van Someren
A. carbonarius (NT)	CBS 111.26	AJ280011	paper	A.F. Blakeslee
A. heteromorphus (T)	CBS 117.55	AJ280013	culture contaminant	A. C. Batista
A. ellipticus (T)	CBS 707.79	AJ280014	soil, Costa Rica	A. Al-Musallam
A. foetidus var *acidus* (T)	CBS 564.65	AJ280009	Japan	R. Nakazawa
A. brasiliensis	IMI 381727	AJ280010	soil Pedreira São Paulo, Brasil	J. Varga

Figure 1. Restriction endonuclease map of: A) the *Bam*HI / *Hind*III fragment of the *A. niger pki*A gene, B) the *Sal*I fragment of the *A. niger pel*A gene, and C) the rDNA repeat from *A. niger* according to O'Connell *et al.* [27]. NTS = non-transcribed spacer, ETS = external transcribed spacer, ITS = internal transcribed spacer, IGS = intergenic spacer region (—), consists of NTS and ETS. Restriction endonuclease sites: S - *Sal*I, P - *Pst*I, E - *Eco*RI, X - *Xho*I, H - *Hind*III, K - *Kpn*I, B – *Bam*HI, C - *Cla*I.
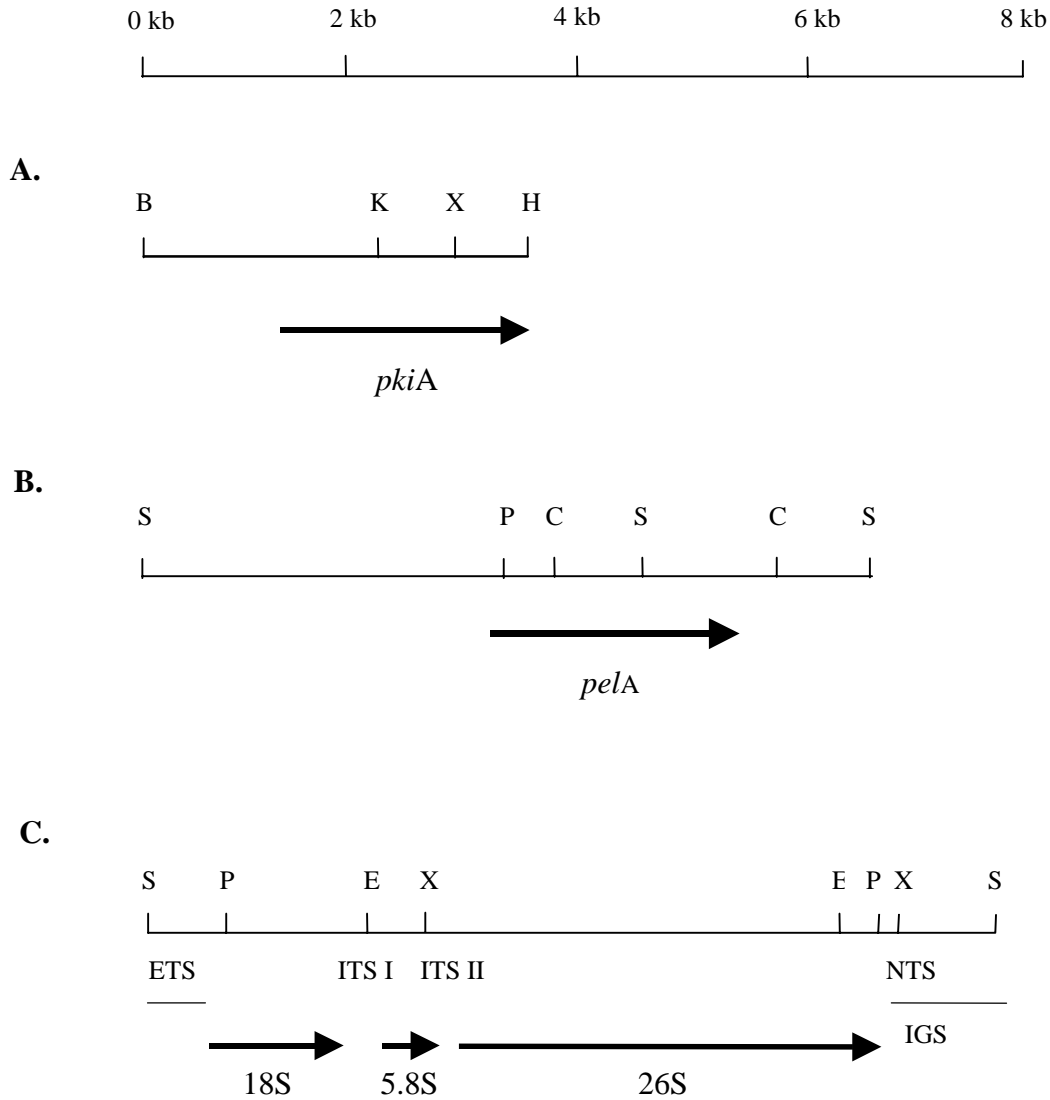
PCR amplification. The ribosomal ITS 1-5.8 S - ITS 2 region (approximately 600 bp) was amplified using the primers ITS4 and ITS5 [42]. The PCR amplification was performed in 50-µl reactions containing 5 µl of genomic DNA template (5-10 ng), 2.5 U of *AmpliTaq* DNA polymerase (PE Corporation, Norwalk, CT, USA), 1 µM each of primers ITS4 and ITS5, 200 µM each of dNTP, reaction mix (50 mM of KCl, 50 mM of Tris/HCl, pH 8.3, 0.1 mg/ml of bovine serum albumin, 3 mM of MgCl$_2$), 0.25 % (vol./vol.) Tween 20 and 10 % (vol./vol.) dimethylsulphoxide (DMSO). Amplification was performed in a GeneAmp PCR system 2400 (PE Corporation) with the following temperature profile: initial denaturation at 94 °C for 1 min, followed by 40 cycles of 15 sec at 94 °C, 1 min at 53 °C, and 1 min at 72 °C. Following amplification, clean-up of PCR products was performed using MicroSpin S-400 HR columns (Amersham Pharmacia Biotech., Uppsala, Sweden).

DNA sequencing and analysis. PCR products were sequenced using the primers ITS1, ITS2, ITS3 and ITS4 (for primer sequences see White *et al.* [42]), the Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia Biotech., Little Chalfort, U.K.) and an automated sequencer (A.L.F. express, Amersham Pharmacia Biotech., Uppsala) according to the manufacturers instructions. Sequences were generated from both strands and edited and initially aligned by using the CLUSTAL W Multiple Sequence Alignment Program, version 1.6 [38]. Manual corrections were included to improve the alignment using the MacClade 3.05 program [23]. The data matrix consisted of 541 aligned nucleotide characters, some of which were scored as deletions or unknowns in one or more taxa. Phylogenetic analysis was performed with the software PAUP 4.0b2 [35] using neighbor joining, maximum likelihood distances and bootstrapping as described previously [34].

Nucleotide sequence accession numbers. The ITS 1-5.8 S-ITS 2 sequences of the *A. japonicus* and *A. aculeatus* strains reported in this paper have been deposited at the EMBL Nucleotide Sequence Database under the accession no. AJ279983-AJ279986 and AJ279988-AJ279992 (strains belonging to sequence type I), AJ279995-AJ280003 (strains belonging to sequence type II), and AJ280005 (strain CBS 114.80), and are depicted in Table 1. The EMBL accession number for the other black aspergilli reported in this paper are *A. niger* CBS 120.49 (AJ280006), *A. tubingensis* CBS 127.49 (AJ280007), CBS 643.92 (AJ280008), *A. foetidus* var. *acidus* CBS 564.65 (AJ280009), *A. brasiliensis* IMI 381727 (AJ280010), *A. carbonarius* CBS 111.26 (AJ280011), *A. heteromorphus* 117.55 (AJ280013), and *A. ellipticus* CBS 707.79 (AJ280014). The following representatives of other species (with corresponding accession no.) were included in the study: *A. fumigatus* (AFO078889), *A. flavus* (AF138287), *Petromyces albertensis* (AJ005673), *P. muricatus* (AJ005674) and *Neosartorya fischeri* (U18355).

Isolation of secondary metabolites and their identification. The *Aspergillus* strains were cultured on the following media optimized for the production of secondary metabolites: Czapek yeast autolysate agar (CYA) and yeast extract sucrose agar (YES) [6]. All cultures were incubated for 10 days in darkness at 30 °C.

For metabolite analysis the content of each plate was extracted following the method described by Frisvad and Thrane [5] and analyzed by high performance liquid chromatography (HPLC) with diode array detection (DAD) [7]. The metabolites found were compared to a spectral UV library made from authentic standards run at the same conditions, and retention indices were compared with those of standards.

RESULTS AND DISCUSSION

Sequence analysis of the ITS 1 -5.8 S- ITS 2 region

The ITS 1-5.8S-ITS 2 sequences from the 19 *A. japonicus* and *A. aculeatus* strains differed in only three nucleotide positions, i.e. the sequenced region was extremely conserved within these taxa. Based on the ITS sequence differences, the 19 *A. japonicus* and *A. aculeatus* strains were divided into three distinct sequence types. Sequence type I comprised 9 strains, sequence type II contained 9 strains and the third sequence type consisted of only one strain, *A. aculeatus* CBS 114.80 (Table 5). All strains of the sequence type I differed from the

sequence type II strains in having a T instead of a C at position 211 and 229 respectively. Numbering refers to the ITS 1-5.8S- ITS 2 sequence of the sequence type I (*A. japonicus* group, Table 5), which starts at the 5' end of the conserved primer ITS 5 [42]. The ITS sequence of the *A. aculeatus* CBS 114.80 strain (type III) was found to be identical to the type I strains except for a single nucleotide (C instead of T) at position 189, unique to this strain. Except for three strains, *viz. A. japonicus* CBS 312.80, *A. lucknowensis* CBS 119.49 and *A. aculeatus* CBS 172.66, which have a type I sequence, but which on the basis of RFLP and secondary metabolite analysis (Tables 5 and 6) belong to *A. aculeatus*, the sequence type I strains represent mainly *A. japonicus* isolates. Sequence type II strains are not found in the *A. japonicus* taxon, but only in the *A. aculeatus* taxon. Since the sequenced region is so conserved, sequence data of the ITS 1-5.8S- ITS 2 region are indicative, but do not allow to assign an isolate with certainty to one of the uniseriate taxa. However, RFLP and secondary metabolite analyses do enable this (see below).

Comparison of the ITS 1-5.8S-ITS 2 sequences of the 19 *A. japonicus* and *A. aculeatus* strains with the corresponding sequences of the other black aspergilli (Table 1) revealed a significant number of differences. The highest degree of ITS sequence variability (approximately 18.5 %) was found between the *A. japonicus* / *A. aculeatus* strains on the one hand and the strains belonging to the '*A. niger* aggregate' on the other [1]. Phylogenetic relationships among the black aspergilli were inferred from neighbour-joining analysis of the aligned ITS sequences. The results of the phylogenetic analysis are shown in Fig. 2. The closely related species *A. fumigatus* and *Neosartorya fisheri* were designated as outgroups based on comparisons of 18S rRNA gene sequences [22]. *A. flavus* and two *Petromyces* species were included in the analysis based on comparisons of morphology and secondary metabolite profiles. The analysis showed that the *A. japonicus* group and the *A. aculeatus* CBS 114.80 isolate form a well-supported clade with the *A. aculeatus* group. This clade represents the uniseriate black aspergilli and was clearly separated from the biseriate black aspergilli, i.e. high bootstrap support (99 %) was found for the branch leading to the cluster including the '*A. niger* aggregate' strains, *A. carbonarius* and the *A. heteromorphus* / *A. ellipticus* clade.

RFLP analysis

*Sma*I **digestion and ribosomal DNA polymorphism.** Among fungi the rDNA repeat (*cf.* Fig. 1C) is present in 100 to 300 copies per haploid genome [3]. As previously reported [29], using a *Sma*I single digestion of nDNA, five clearly different rDNA banding patterns were established upon ethidium bromide staining and UV visualization of the agarose gels (*cf.*

Table 2). Here we used the entire 10-kb rDNA unit of *A. bisporus* in a Southern blot analysis of the *Sma*I digested nDNA to further characterize these patterns.

Figure 2. Neighbour-joining tree based on phylogenetic analysis of the ITS 1 -5.8S- ITS 2 rDNA sequences showing the relationships of the *A. japonicus* and *A. aculeatus* taxa to the other black aspergilli (*A. niger*, *A. tubingensis*, *A. foetidus* var. *acidus*, *A. brasiliensis*, *A. carbonarius*, *A. heteromorphus*, and *A. ellipticus*) and reference representatives of other species. Numbers at branching points are percentages of 1000 bootstrapped data sets supporting the specific internal branches. Bootstrap values below 50 % are not shown. For the phylogenetic analysis, the *A. japonicus* CBS 114.51 strain was selected as representative of sequence type I, *A. aculeatus* CBS 610.78 as representative of sequence type II and *A. aculeatus* CBS 114.80 is sequence type III. Reference species representatives: *A. flavus* (accession no. AF138287), *Petromyces albertensis* (accession no. AJ005673) and *Petromyces muricatus* (accession no. AJ005674). *Neosartorya fischeri* (accession no. U18355) and *A. fumigatus* (accession no. AFO078889) were chosen as outgroups. The bar indicates 10 % estimated sequence divergence.

154
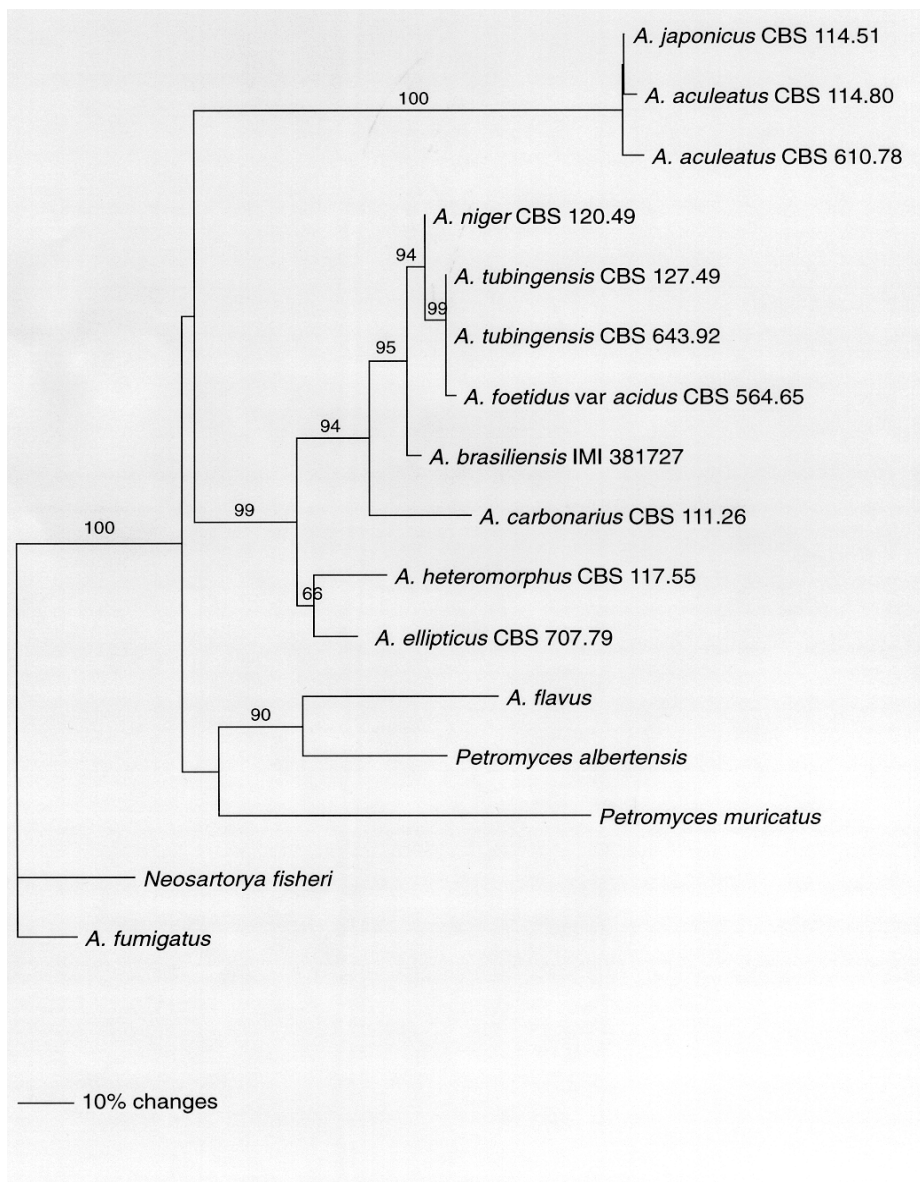
The results are shown in Table 2. The number of hybridizing bands using the rDNA repeat of the basidiomycete *A. bisporus* as a probe differs from those reported for the black aspergilli by Megnegneau *et al.* [24] and Hamari *et al.* [9]. We expect that this is due to the origin of the rDNA probe, since the two other groups used the *A. nidulans* rDNA repeat in their hybridization experiments.

A comparison of the visualized and hybridizing *Sma*I / rDNA bands allows us to discriminate between six different banding patterns (*cf.* Table 2). All the *A. japonicus* and *A. aculeatus* isolates have the same pattern (D). The *Sma*I generated rDNA-banding pattern of *A. brasiliensis* (Table 2; pattern F; [40]) is also clearly different from those already established [29].

Table 2. The banding patterns of the nuclear rDNA obtained after *Sma*I digestion, ethidium bromide staining and UV visualization and hybridization with the entire 10-kb rDNA unit of *Agaricus bisporus*. The bands, which gave a hybridization signal upon the Southern blot analysis are in bold. The sizes of the bands are in kb.

pattern	species representative	UV visualized and hybridizing bands
A	*A. carbonarius*	**4.1** & **2.3**
B	*A. niger*	**3.2**, 2.3 & **1.8**
C	*A. tubingensis* & *A. foetidus* var. *acidus*	**2.6**, 1.9 & **1.8**
D	*A. japonicus* & *A. aculeatus*	1.9, **1.8** & **0.9**
E	*A. ellipticus* & *A. heteromorphus*	1.9, **1.8** & **1.6**
F	*A. brasiliensis*	2.1, **2.1** & **1.8**

Analysis of the 26S rRNA locus. In order to determine the level of relationship between the *A. japonicus* and *A. aculeatus* isolates more closely, two other combinations of restriction enzymes for RFLP were included in the study of the large subunit rRNA gene. The 3´ part of the *A. bisporus* 28S rRNA gene including some downstream sequence of the non-transcribed spacer (NTS) region was used as a probe.

The sequence analysis of the ITS 1 -5.8 S- ITS 2 region and the RFLP of the rDNA repeat upon *Sma*I digestion of nDNA revealed very small or no differences among the *A. japonicus* and *A. aculeatus* isolates, respectively. However, using another combination of restriction enzymes, *viz. Xho*I / *Kpn*I and the 0.9-kb 28S rRNA *Eco*RI fragment as a probe, the *A.*

japonicus and *A. aculeatus* isolates were separated into two groups (*cf.* Table 5). Four patterns (A-D) were detected upon probing the restricted nDNA of the various black aspergilli, each containing a single hybridizing band of 7.5-kb, 7-kb, 5-kb or 4-kb (Fig. 3). Three *A. japonicus* isolates, one *A. atroviolaceus* and one *A. aculeatus* isolate showed pattern A. Also the CBS 101.14 strain, already previously identified as an *A. japonicus* isolate [28], was characterized by this pattern. Since the type strain *A. japonicus* CBS 114.51 also segregates here, we consider these strains to represent the *A. japonicus* taxon. The remaining twelve strains, *viz.* five *A. aculeatus*, three *A. japonicus*, two *A. luchuensis*, one *A. lucknowensis* and one *A. bruneo-violaceus*, exhibited the typical pattern B. These strains were grouped into the *A. aculeatus* taxon. Only one *A. aculeatus* isolate, CBS 114.80, showed a deviating pattern from the other *A. japonicus* and *A. aculeatus* strains, pattern C.

Figure 3. RFLP patterns of selected black aspergilli obtained after digestion of nDNA with *Kpn*I and *Xho*I and probing with the 0.9-kb 28S rRNA *Eco*RI fragment of *Agaricus bisporus*. On the left the size of the λ DNA fragments obtained after digestion with *Eco*RI and *Hind*III is indicated, on the right the patterns are depicted. The hybridizing bands from left to right correspond to the following isolates: *A. japonicus* CBS 114.51, *A. aculeatus* CBS 611.78, *A. luchuensis* CBS 101.43, *A. aculeatus* CBS 308.80, *A. bruneo-violaceus* CBS 621.78, *A. aculeatus* CBS 114.80, *A. niger* CBS 120.49, *A. tubingensis* CBS 643.92, *A. foetidus* var. *acidus* CBS 564.65, *A. brasiliensis* IMI 381727, *A. carbonarius* CBS 111.26, *A. heteromorphus* CBS 117.55 and *A. ellipticus* CBS 707.79.
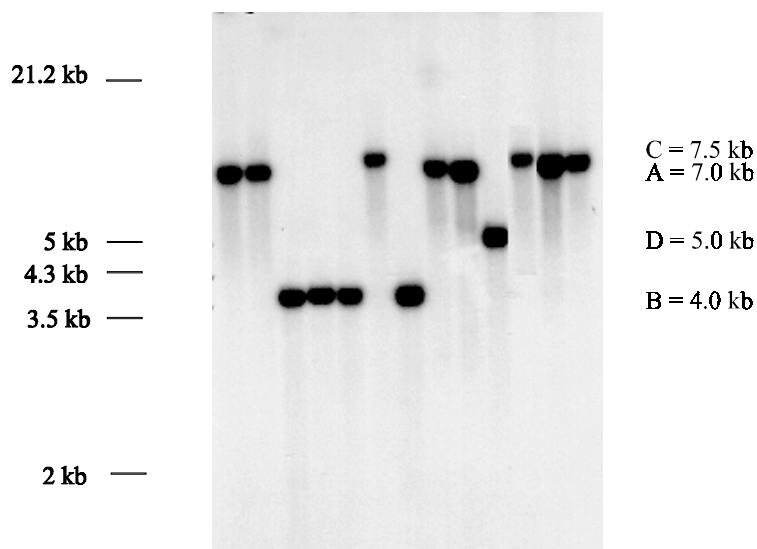
However, when the same 0.9-kb *Eco*RI 28S rRNA probe was used to examine the RFLP patterns upon *Pst*I / *Sal*I digestion of the nDNA, much more variation was detected in the 26S rRNA locus among all the *A. japonicus* and *A. aculeatus* isolates (see Fig. 4 and Table 5). The 6.0-kb hybridizing band, which was found in each of the eleven patterns typical for the *A.*

japonicus and *A. aculeatus* isolates (*viz.* patterns F-P), could easily distinguish these uniseriate taxa from the other black aspergilli (Fig. 4, patterns A-E). The small hybridizing bands detected in the different patterns reflect the DNA sequence variation in the NTS of the rDNA repeat as concluded from the restriction map of *A. niger* rDNA (Fig. 1C). Thus digestion by *Pst*I / *Sal*I particularly depicts the intra- group variations among the isolates of *A. japonicus* and *A. aculeatus*. It is obvious, that the NTS region of the *A. japonicus* and *A. aculeatus* isolates frequently underwent DNA rearrangements like the introduction of small insertions, deletions or nucleotide exchanges, which was demonstrated by small size variations in the patterns. The *A. aculeatus* strain CBS 114.80 shows only one hybridizing band of 8.0-kb, *viz.* pattern Q (*cf.* Fig. 4).

Figure 4. RFLP patterns of different black aspergilli (A-E) and of *A. japonicus* and *A. aculeatus* isolates (F-Q) upon digestion of nDNA with *Pst*I / *Sal*I and probing with the 0.9-kb 28S rRNA *Eco*RI fragment of *A. bisporus*. At the left hand side the size of the bands corresponding to the λ DNA digested with *Hind*III and *Eco*RI is depicted. At the right hand side the sizes of the small hybridizing bands of the *A. aculeatus* and *A. japonicus* banding patterns F-P are indicated. Below the picture the black aspergilli representative for the patterns A-P are listed.
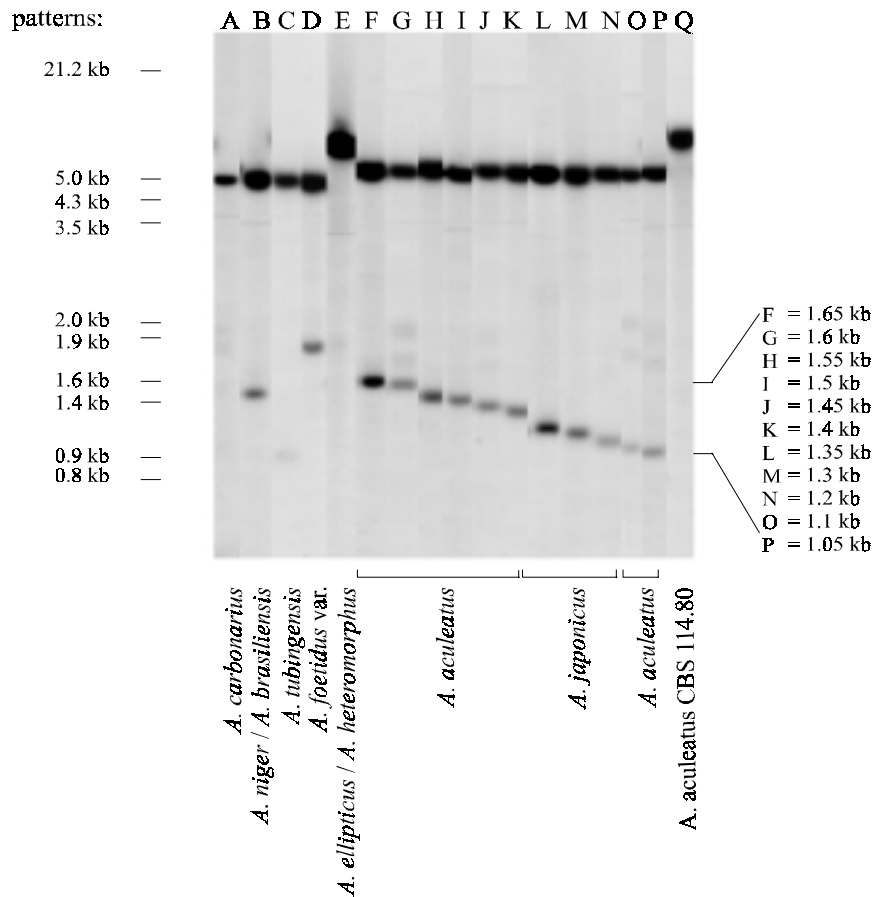
Analysis of the *pki*A locus. In order to examine the RFLP variability in a second conserved locus, the 3.5-kb *Bam*HI / *Hind*III fragment (Fig. 1A) containing the entire coding region of the *A. niger pki*A gene and the 5' and 3' non-coding sequences was used as a probe. The *A. japonicus* and *A. aculeatus* isolates showed distinct patterns from the other black aspergilli (*cf.* Table 3 and 5). A unique pattern F (5.3-kb, 4.8-kb and 0.5-kb, Table 5) was found for the isolates already separated based on the 26S rRNA RFLP's into the *A. japonicus* taxon. The RFLP patterns for the remaining uniseriate strains (Table 3) showed two (7.5-kb and 0.4-kb) out of three hybridizing bands to be identical. Since the 0.4-kb hybridizing fragment most probably corresponded to the internal conserved *Kpn*I / *Xho* I fragment (Fig. 1A) of the *pki*A gene equivalent, the differences among the patterns are due to changes in one of the restriction sites in the 5' or 3' non-coding regions. The *A. brasiliensis pki* pattern was also different, although it shared the 7.5-kb and 0.4-kb hybridizing bands. This strain showed clearly different banding profiles in all other loci examined. Again *A. aculeatus* CBS 114.80 was different from the *A. japonicus* and *A. aculeatus* taxa showing three hybridizing *pki* bands (6.7-kb, 1.2-kb and 0.5-kb; pattern K in Table 3).

Table 3. Patterns obtained after the Southern blot hybridization of the *Kpn*I / *Xho*I digested genomic DNA with the 3.5-kb *Bam*HI / *Hind*III *A. niger pki*A fragment. The sizes of the hybridizing bands are expressed in kb.

patterns	*pki*A *Kpn*I / *Xho*I
A	3.2, 1.8 & 0.4
B I	5.0, 2.5 & 0.5
B II	5.0, 2.5 & 0.4
C	5.5, 1.9 & 0.4
D	4.5, 1.7 & 0.4
E	7.5, 1.9 & 0.4
F	5.3, 4.8 & 0.5
G	7.5, 5.0 & 0.4
H	7.5, 2.3 & 0.4
I	18, 7.5 & 0.4
J	12.5, 7.5 & 0.4
K	6.7, 1.2 & 0.5

Examination of DNA polymorphism in the less conserved *pel* A locus. The final RFLP examined used the 1.6-kb *Cla*I fragment of the *A. niger pel*A gene in Southern blot hybridization of *Pst*I / *Sal*I digested nDNA. This restriction enzyme / probe combination

Table 4. RFLP patterns observed in *Pst*I / *Sal*I digests of the nDNA using 1.6-kb *Cla*I fragment of the *A. niger pel*A gene as a probe. Fragment lengths are indicated in kb. The fragments hybridizing strongly are underlined.

pattern	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
		6.0				6.7	**6.0**													
											5.3			**5.3**		**5.1**	**5.5**			
				5.0													**5.0**			
		4.5	4.8									**4.8**								
						4.7					**4.7**			4.7		**4.4**			**4.4**	
						3.8					3.8	3.6	**3.6**	3.8		3.6	3.6	3.7		
								3.2	**3.0**	**3.2**				**3.0**	**3.1**					
	2.9		**2.8**									2.8	2.8					**2.8**		**2.9**
					2.4		2.4	**2.4**	**2.4**						2.5				2.4	**2.5**
			2.2		2.2		**2.2**				**2.3**						**2.2**			
						2.0														
					1.8							1.8	**1.8**		**1.8**			**1.8**		**1.8**
						1.6		**1.7**	1.7											
										1.5										
			1.4		1.4			1.4	1.4											
							1.35													
											1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	**1.3**	
	1.2	1.2		**1.2**																
		1.0						1.0	1.0											
										0.8	**0.8**	**0.8**	**0.8**	**0.8**	**0.8**	0.8	**0.8**	**0.8**	**0.8**	**0.8**
					<0.6	0.6	0.6	0.6	0.6											

159

proved to be suitable in the past to determine inter- and intra- species variation within the 'A. niger aggregate' [20,29].

The pelA RFLP patterns obtained for the A. japonicus and A. aculeatus isolates have been compared with the patterns for the selected black aspergilli (Table 4). The A. japonicus and A. aculeatus isolates expressed together 13 different patterns. By looking at the RFLP's of the strains considered to belong to A. japonicus (cf. Table 4 and 5), the pelA results strongly support them to belong to one taxon. Five strains are characterized by pattern H. Moreover, they share five out of six hybridizing bands with pattern I, shown by A. aculeatus CBS 611.78. In the final grouping (Table 5) this strain certainly appears to be an A. japonicus isolate.

The remaining pelA patterns belong to the isolates classified as A. aculeatus. They all have a small 0.8-kb hybridizing band in common, including the pattern of A. aculeatus CBS 114.80. When compared to the restriction map of the A. niger pel A gene (Fig. 1B), the 0.8-kb hybridizing band might correspond to the pelA equivalent of A. aculeatus. The remaining hybridizing bands probably reflect DNA polymorphism in the 5´and 3´pelA non-coding region (for the localization of the A. niger ClaI pelA fragment used for the probe see Fig. 1B) and in other pectin lyase encoding genes present in the genome of different isolates, which also hybridize [19-20]. From the pelA RFLP's it is clear that of all the black aspergilli the A. aculeatus taxon shows the highest degree of variation in the pectin lyase-encoding genes.

Profiles of secondary metabolites. The results of the secondary metabolite analysis are presented in Table 6. The strains grouped into the A. japonicus taxon, all produce several unknown secondary metabolites. All strains except CBS 114.51 produce several polar metabolites with end absorption near 200 nm. One of these metabolites may prove to be thiol protease inhibitor E-64, reported previously for an A. japonicus strain by Hanada et al. [10-11]. This should be confirmed by comparison to an authentic standard. Three strains of A. japonicus produce festuclavine (Table 6) as reported for another strain from this species [8]. The remaining strains produce other indol alkaloids, but it could not be confirmed whether these metabolites include cycloclavin [8]. A. japonicus CBS 114.51 and CBS 568.65, A. japonicus var. japonicus CBS 522.89 and A. aculeatus CBS 611.78 all produce a secondary metabolite with a characteristic chromophore in common.

The strains in the A. aculeatus group are also producers of several unknown secondary metabolites, but they exhibit a typical secondary metabolite profile, which allows them to group together. They are characterized by the presence of nitrogen containing secondary

Table 5. Final grouping of the *A. japonicus* and *A. aculeatus* isolates after RFLP analysis of the nDNA and the ITS 1-5.8S- ITS 2 sequencing.

groups	strain	CBS no.	rDNA *SmaI*	*pkiA* *KpnI / XhoI*	26S *KpnI / XhoI*	26S *PstI / SalI*	*pelA* *PstI / SalI*	ITS 1-5.8S-ITS 2
Aspergillus japonicus	A. japonicus	568.65	D	F	A	L	H	I
	A. japonicus	522.89	D	F	A	L	H	I
	A. atroviolaceus	113.48	D	F	A	M	H	I
	A. japonicus	101.14	D	F	A	M	H	I
	A. japonicus	114.51	D	F	A	N	H	I
	A. aculeatus	611.78	D	F	A	N	I	I
	A. japonicus	114.34	D	G	B	I	L	II
	A. lucknowensis	119.49	D	H	B	I	M	I
	A. luchuensis	116.80	D	G	B	P	L	II
	A. luchuensis	101.43	D	H	B	I	M	II
	A. japonicus	620.78	D	G	B	H	J	II
Aspergillus aculeatus	A. japonicus	312.80	D	I	B	F	K	I
	A. aculeatus	172.66	D	J	B	H	N	I
	A. aculeatus	610.78	D	G	B	G	O	II
	A. aculeatus	186.67	D	G	B	J	P	II
	A. aculeatus	308.80	D	G	B	K	Q	II
	A. aculeatus	313.89	D	G	B	P	R	II
	A. bruneo-violaceus	621.78	D	G	B	O	T	II
?	A. aculeatus	114.80	D	K	C	Q	S	III
A. niger	A. niger	120.49	B	B1	B	B	B	
A. tubingensis	A. tubingensis	643.92	C	BII	A	C	D	
A. foetidus var.	A. foetidus var.acidus	564.65	C	BII	A	D	C	
A. brasiliensis	A. brasiliensis	IMI 381727	F	E	D	B	G	
A. carbonarius	A. carbonarius	111.26	A	A	C	A	A	
A. heteromorphus	A. heteromorphus	117.55	E	D	A	E	F	
A. ellipticus	A. ellipticus	707.79	E	C	A	E	E	

metabolites, *viz*. neoxaline, okaramins and paraherquamide-like compounds and by secalonic acid [2]. Three of the isolates, *viz*. *A. japonicus* CBS 620.78, *A. japonicus* CBS 312.80 and *A. aculeatus* CBS 172.66 do not produce any of the secondary metabolites containing nitrogen, but they are characterized by the presence of an unknown secondary metabolite with a characteristic chromophore produced in large quantities. In contrast to the report by Hirano *et al*. [14], neoxaline is a secondary metabolite produced by *A. aculeatus*, not by *A. japonicus*.

Table 6. Classification of the isolates of *Aspergillus aculeatus* and *A. japonicus* species based on the production of known secondary metabolites (all isolates produce several additional unknown secondary metabolites).

***A. japonicus* taxon:**	
A. japonicus CBS 101.14:	Unknown indol alkaloid[a]
A. atroviolaceus CBS 113.48:	Festuclavin
A. japonicus CBS 114.51:	Unknown indol alkaloid[a]
A. japonicus CBS 568.65:	No known secondary metabolites
A. aculeatus CBS 611.78:	Festuclavin
A. japonicus CBS 522.89:	Unknown indol alkoloid
***A. aculeatus* taxon:**	
A. bruneo-violaceus CBS 621.78:	Neoxaline, okaramins[b]
A. lucknowensis CBS 119.49:	Neoxaline, secalonic acid D, okaramins, paraherquamide like
A. luchuensis CBS 116.80:	Neoxaline, secalonic acid D, okaramins, paraherquamide like
A. luchuensis CBS 101.43:	Neoxaline, secalonic acid D, okaramins, paraherquamide like
A. japonicus CBS 620.78:	Secalonic acid D
A. japonicus CBS 312.80:	No known secondary metabolites
A. japonicus CBS 114.34:	No known secondary metabolites
A. aculeatus CBS 610.78:	Neoxaline, secalonic acid D, paraherquamide like
A. aculeatus CBS 186.67:	Neoxaline, secalonic acid D, paraherquamide like
A. aculeatus CBS 308.80:	Neoxaline, secalonic acid D
A. aculeatus CBS 313.89:	Secalonic acid D, paraherquamide like
New species:	
A. aculeatus CBS 114.80:	Secalonic acid D, okaramin A,B,H,I

[a] Cycloclavine uncertain
[b] The okaramins in *A. aculeatus* also appear to be okaramin A, B, H and I (see also Hayashie *et al*. [13]), but the new species represented by CBS 114.80 was a particularly good producer of these secondary metabolites.

The grouping of the strains based on the secondary metabolite analysis entirely corresponds with the division based upon the RFLP analysis.

A. aculeatus CBS 114.80 produces secondary metabolites in common with both *A. aculeatus* and *A. japonicus* but it also produces a series of secondary metabolites with unique chromophores. Taking all data into account, this strain most probably represents a new species.

By the present study we completed the nDNA RFLP analysis of the available isolates of the black aspergilli [28-29]. The combination of the restriction enzymes and probes, *viz. Sma*I, *Kpn*I / *Xho*I and *Pst*I / *Sal*I, and as probes the rDNA repeat, 28S rRNA, and the *pki*A and *pel*A genes respectively, allowed us to distinguish ten different taxa amongst black aspergilli (*cf.* Table 5). The *A. brasiliensis* IMI 381727 RFLP patterns are clearly distinct from those of *A. niger*, *A. tubingensis* and the representatives of the *A. foetidus* varieties, and thus support the conclusions of Varga *et al.* [40] to classify this isolate as a new species. Based on the sequence data of the ITS 1 -5.8S- ITS 2 region, the RFLP and secondary metabolite analyses, we were able to separate a third uniseriate taxon among the black aspergilli represented by the CBS 114.80 isolate.

Acknowledgements

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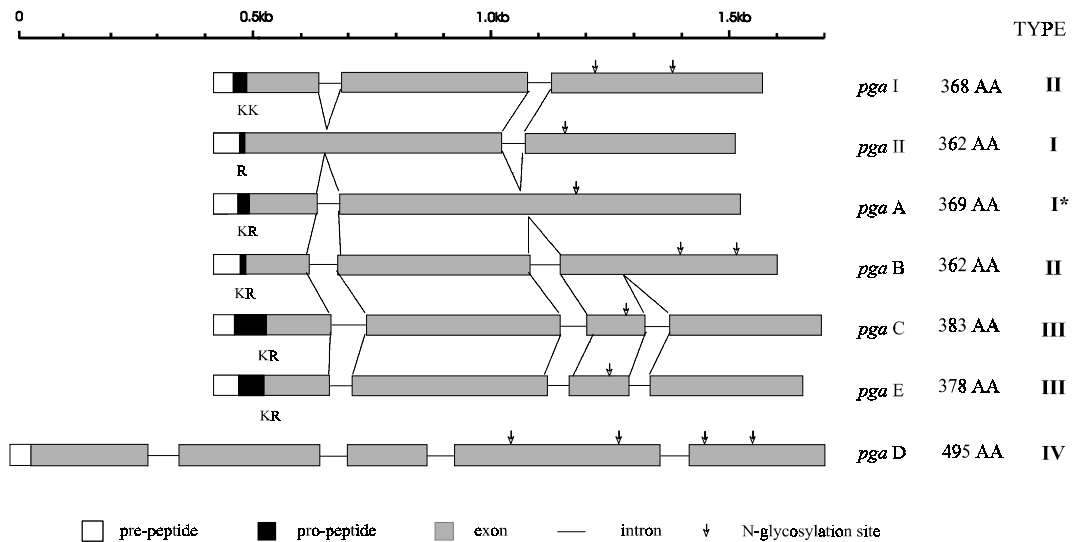
CHAPTER 8

General Discussion

General Discussion

Aspergillus and pectinases are the major topics of this study. Chapters 2 to 4 focus on the molecular and biochemical characterisation of four new *A. niger* endopolygalacturonases, in chapter 5 the expression of a large spectrum of pectinase-encoding genes of this fungus is studied. Chapters 6 and 7 describe the relationship of the species belonging to the black aspergilli and exploit one of the pectinase genes, *pel*A encoding pectin lyase A, as a probe to develop a RFLP-based species identification method.

In order to adapt to various environmental conditions *A. niger* produces a number of extracellular enzymes, such as proteases, cellulases, hemicellulases and pectinases able to degrade polymeric substrates, which generate the nutrition for the fungus. This saprophytic micro-organism is particularly well ´equipped´ with pectin-degrading enzymes, which are encoded by more than 20 genes, amongst which a family of pectin lyases and a family of endopolygalacturonases [1,2]. One of the questions addressed in our pectinase research is, why a relatively simple eukaryotic micro-organism like *A. niger* possesses multiple genes encoding specific pectinase activities. Is each of the enzymes necessary for the fungus to adapt to a new environment or are some of these pectinolytic activities redundant? The molecular and biochemical analysis of the four new members, *pga*A, *pga*B, *pga*D and *pga*E, of the endopolygalacturonase-encoding gene family described in Chapters 2 to 4, and the initial studies on the pectinase regulation (Chapter 5) together with data on polygalacturonases published during the course of this PhD. study now indeed confirm that *A. niger* adapts to the presence of pectin in a typical way.

The exon-intron organisation of the three new genes, *pga*A, *pga*B and *pga*E, shows structural similarity to the previously isolated *pga*I, *pga*II and *pga*C genes from *A. niger* (Fig. 1) and to those from other aspergilli (Chapter 1). The *A. niger pga* genes contain one to four introns ranging in size from 50 bp (*pga*E) to 75 bp (*pga*C). The length of the *pga*-encoded amino acid sequences fits well within the sizes (362-383 amino acids) described for the fungal PGs (Chapter 1). The exception is *pga*D (Fig. 1), which shows a different structural organisation compared to the other *A. niger pga* genes. Furthermore, the 495 amino acids long protein sequence of PGD exceeds by more than 100 residues the average length of the primary protein sequences reported for the fungal PGs, except for BCPG3 from *Botrytis cinerea* that consists of 514 amino acid residues [3].
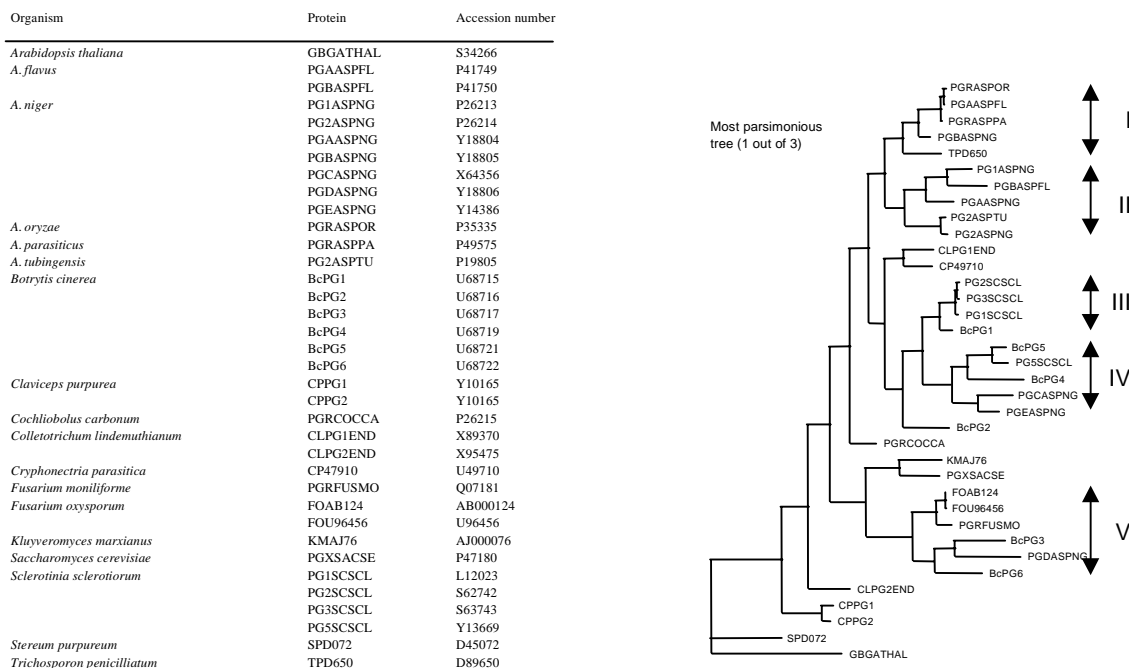
Fig. 1. **Alignment of the seven *A. niger pga* genes.** The exon/intron organisation is depicted, and the pre-propeptide and the possible N-glycosylation sites are marked. The amino acids preceeding the pro-peptide cleavage site are indicated below the sequence. On the right-hand side the number of amino acids of the corresponding protein sequence is shown. The *pga*D gene is aligned based on the sequence homology with the other *A. niger pga* genes. 'TYPE' corresponds to the number of introns in the *Aspergillus pga* gene as described in Chapter 1. I*; contains one intron but in the 5' end of the gene when compared to TYPE I. IV; represents a new TYPE of the *pga* gene containing four introns.

However, when compared to the endopolygalacturonase-encoding genes of bacterial and plant origin, which show much higher variability in the length of the encoded proteins (Chapter 1), it is possible that the PG-divergence in fungi has not been disclosed yet.

The increasing number of the endopolygalacturonase-encoding genes in the databases allowed the analysis of evolution of this class of pectinases. The analysis based on the protein sequence alignment of 35 related fungal and yeast PGs [3] showed the existence of five different monophyletic groups, each containing a minimum of three PGs originating from more than one fungal species. The seven *A. niger* PGs were separated into four monophyletic groups: I (PGB), II (PGI, PGII and PGA), IV (PGC and PGE) and V (PGD) (Fig. 2). The closely related monophyletic groups I and II contain all the PGs isolated so far from other aspergilli, *viz. A. oryzae*, *A. flavus*, *A. parasiticus* and *A. tubingensis*. The monophyletic groups IV and V, which among others contain PGC, PGE and PGD from *A. niger*, are more distantly related to the monophyletic groups I and II, and reveal a closer relationship with PGs from the phytopathogenic fungi *Sclerotinia sclerotiorum*, *B. cinerea* and *Fusarium* spp.

Fig. 2. Phylogenetic analysis of fungal PGs according to Wubben *et al*. [3]. The analysis was performed by using an optimal alignment generated from the PGs depicted in the table. The tree shows the most parsimonious tree and identifies the different monophyletic groups that were defined as a result of the analysis. The *Arabidopsis thaliana* PG was used as an outgroup to root the tree.

Organism	Protein	Accession number
Arabidopsis thaliana	GBGATHAL	S34266
A. flavus	PGAASPFL	P41749
	PGBASPFL	P41750
A. niger	PG1ASPNG	P26213
	PG2ASPNG	P26214
	PGAASPNG	Y18804
	PGBASPNG	Y18805
	PGCASPNG	X64356
	PGDASPNG	Y18806
	PGEASPNG	Y14386
A. oryzae	PGRASPOR	P35335
A. parasiticus	PGRASPPA	P49575
A. tubingensis	PG2ASPTU	P19805
Botrytis cinerea	BcPG1	U68715
	BcPG2	U68716
	BcPG3	U68717
	BcPG4	U68719
	BcPG5	U68721
	BcPG6	U68722
Claviceps purpurea	CPPG1	Y10165
	CPPG2	Y10165
Cochliobolus carbonum	PGRCOCCA	P26215
Colletotrichum lindemuthianum	CLPG1END	X89370
	CLPG2END	X95475
Cryphonectria parasitica	CP47910	U49710
Fusarium moniliforme	PGRFUSMO	Q07181
Fusarium oxysporum	FOAB124	AB000124
	FOU96456	U96456
Kluyveromyces marxianus	KMAJ76	AJ000076
Saccharomyces cerevisiae	PGXSACSE	P47180
Sclerotinia sclerotiorum	PG1SCSCL	L12023
	PG2SCSCL	S62742
	PG3SCSCL	S63743
	PG5SCSCL	Y13669
Stereum purpureum	SPD072	D45072
Trichosporon penicilliatum	TPD650	D89650

Based on the results of the phylogenetic analysis the authors suggested that the ancestor genes of each of the clusters existed prior to the species divergence. Thus, also in the other aspergilli the *pga*C, *pga*D and *pga*E homologues may be present.

The degree of the mutual amino acid similarity of the mature *A. niger* PGs is depicted in Table 1 and shows that apart from PGD the percentages of the amino acid similarities among the enzymes are high and range from 66.7 % for PGC-PGII to 85.0 % for PGE-PGC. The derived primary protein sequence of the *A. niger* PGs, schematically presented in Fig. 1, shows that all enzymes contain a pre-prosequence with the exception of PGD, which only has a pre-sequence. Whereas the size of the pre-peptide among the enzymes varies only by 5 amino acid residues, the length of the pro-sequence ranges from 6 to 24 amino acid residues (Table 1). The cleavage of the pro-peptide occurs after one (monobasic; Arg) or two (dibasic; Lys-Arg or Lys-Lys) basic amino acids (see Fig. 1). Recently Jalving *et al*. [4] demonstrated the presence of a kexin-like maturase in *A. niger*, the homologue of the Kex2 dibasic peptidase from yeast, which is able to cleave '*in vitro*' the substrate after a pair of two basic amino acids (Lys-Arg, Arg-Arg or Lys-Lys) or with a lower rate after one basic amino acid (Arg). This enzyme can be involved in the processing of PGs. Furthermore, the *A. niger* PGs

contain one to four possible N-glycosylation sites, which in some enzymes, i.e. the first N-glycosylation site in PGI, II, A, C and E, is found in a conserved position (Fig. 1).

Table 1. **Percentages of the amino acid similarity among the mature *A. niger* PGs as calculated using the Clustal method with PAM250 residue weight table**. The numbers in brackets behind the enzyme names in the right column correspond to the number of amino acids present in the pre-pro sequence. Bold indicates the highest and the lowest % of similarity.

PGI	PGII	PGA	PGB	PGC	PGD	PGE	
X	72.0	80.2	73.5	70.8	31.5	71.0	PGI (18-13)
	X	74.1	75.7	66.7	32.7	69.3	PGII (21-6)
		X	75.2	72.5	33.5	73.5	PGA (19-13)
			X	68.6	36.3	73.3	PGB (20-6)
				X	**31.2**	**85.0**	PGC (16-24)
					X	32.1	PGD (16)
						X	PGE (19-20)

To further analyse the *A. niger pga* genes, their expression was studied by northern blot analysis. The experiments were performed using fed-batch cultures containing a standardised minimal medium with various carbon sources. The mycelium was pregrown overnight in minimal medium containing an easy metabolisable carbon source like glucose or sucrose, and was subsequently transferred to the medium containing the carbon sources to be tested. Because polymeric substrates like pectin or polygalacturonic acid can not enter the cell, it is expected that their degradation product(s) or metabolites (Chapter 1) are the actual inducers of the pectinase system. Therefore, the monomeric constituents of the pectin backbone, D-galacturonic acid and L-rhamnose, were also used as carbon sources. Glucose or sucrose was also included to investigate whether the genes are under the control of carbon repression. The studies on the expression of the four new *A. niger pga* genes (Chapters 2 to 4) showed that they are differentially expressed. The *pga*A and *pga*B mRNAs were easily detected by northern blot analysis whereas for *pga*D and *pga*E mRNA could only be detected using RT-PCR. In the case of *pga*D, the transcript was detected only in the multicopy *A. niger pga*D transformant grown on pectin. The expression studies showed that *pga*A, *pga*B and *pga*E are constitutively expressed, their mRNA was detected in the mycelium grown in the presence of all carbon sources tested. The very low levels of the *pga*D and *pga*E expression prompted us to analyse *pga*I, *pga*II and *pga*C in the same way (Chapter 5). By western blot analysis, the production of PGI, PGII and PGC was previously detected by Bussink and co-workers [2,5-6] in culture filtrates of multicopy *A. niger* or *A. nidulans pga*I, *pga*II and *pga*C transformants

grown in medium containing 1 % (w/v) apple pectin and 1 % (w/v) sugar beet pulp as the carbon source. Moreover, PGI and PGII represent the majority of the endopolygalacturonase activity determined in the commercial *A. niger* pectinase preparation [7] and were called the 'major' *A. niger* endopolygalacturonases. Therefore both *pga*I and *pga*II mRNAs were expected to be present in high amounts. However, under the experimental conditions applied, using sugar beet pectin as a sole carbon source, only the *pga*I mRNA was present at a low level, whereas the *pga*II and *pga*C mRNAs could not be detected by northern blot analysis (Chapter 5). It is therefore possible that other factors such as pH or aeration (Chapter 1), in addition to the carbon source, affect the expression of *pga*II, *pga*C, *pga*D and *pga*E. It is also obvious that analysis of the enzyme production in *A. niger pga*-multicopy transformants does not provide an accurate view on the regulation of the expression of the corresponding gene (*cf. pga*II, [8]). The constitutively expressed *A. niger* pectinases, PGA and PGB, can play an important role in generating an inducer molecule and in triggering an induction cascade of the pectinolytic system. This view is further supported by the biochemical characterisation of the encoded enzymes (see below).

The phylogenetic analysis (Fig. 2) shows that the *pga*B gene from *A. niger* groups together with the endopolygalacturonase-encoding genes from the phytopathogenic fungi *A. flavus* and *A. parasiticus*. The expression of these genes was also detected in the presence of glucose and pectin in the medium [9,10]. The *pga*I gene from *A. niger* is closely related to *pec*B from *A. flavus* [9], both being specifically induced in the presence of pectin in the medium. The *pga*A gene groups with *pga*I in the monophyletic group II. In addition to a constitutive expression of this gene a positively acting regulatory mechanism operating via D-galacturonic acid or its metabolite(s) as an inducer may be involved (Chapter 3). From this comparison it became evident that the evolutionary relationship of the *Aspergillus pga* genes goes beyond the protein coding region of these genes and involves the regulatory mechanism as well.

The overproduction of individual *A. niger* PGs was made possible by using a *pki*A-*pga* fusion construct, in which the transcription of the gene is under the control of the strong glycolytic *pki*A (pyruvate kinase A) promoter. The growth conditions applied to an *A. niger* multicopy *pki*A-*pga* transformant, *viz.* glucose or fructose as a carbon source, repress the expression of the majority of other pectinases (Chapter 5).

The purification and biochemical characterisation of the new *A. niger* PGs allowed to compare all the members of the endopolygalacturonase-encoding gene family (Table 2). Similarly as found for PGI, PGII and PGC [11], also the new PGs have higher apparent

molecular masses than calculated from the deduced amino acid sequences. In the case of PGD it was shown, that this increase is partially caused by O-glycosylation of the enzyme (Chapter 4).

Table 2. **Summary of the physicochemical and biochemical properties of the *A. niger* PGs.** The molecular mass of the mature enzyme was calculated from the deduced amino acid sequence (cal.) or determined by SDS-PAGE calibrated with protein molecular markers (det.). pI corresponds to the calculated value. pH opt., optimum pH; $K_{m,app}$ and $V_{m,app}$, K_m and V_{max} determined for polygalacturonate or lemon pectin with 7 % DE (*); % DE, designates the preferred substrate (lemon pectin) with a certain degree of methylesterification (DE).

PG	Type	Mw [kDa] cal./det.	pI	$pH_{opt.}$	$K_{m,app}$ mg.ml^{-1}	$V_{max, app.}$ μkat.mg^{-1}	% DE	proces -sivity	subsites	Ref.
PGI	endo	34.944 / 58.0	3.9	4.2	<0.15	13.8	0	+	min. 7	[11]
PGII	endo	34.963 / 38.0	4.9	4.2	<0.15	36.5	0	-	min. 7	[11]
PGA	endo	35.497 / 54.7	3.4	4.0	<0.15	18	7-22	+	min. 7	Ch.3
PGB	endo	35.188 / 38.1	6.2	5.0	0.9*	14.1*	22	-	min. 7	Ch.3
PGC	endo	36.231 / 61.0	3.7	4.2	<0.15	0.415	0-7	+	min. 7	[11]
PGD	oligo?	50.788 / >100	4.1	4.2	0.2	1.57	0	+	4	Ch.4
PGE	endo	35.584 / 60.0	3.6	3.8	2.5	1.33	0-7	-	min. 5	Ch.2,[11]

All the *A. niger* PGs are active at acidic pH, in the range from 3.8 to 5.0, as reported for other fungal and yeast PGs (Chapter 1). However, these enzymes differ remarkably in their kinetic parameters using polygalacturonate as a substrate (Table 2). While $V_{max,app}$ of PGA and PGB are high and of the same order as determined for PGI and PGII, demonstrating that polygalacturonate is a good substrate for these enzymes, PGE and PGD show much lower activity on this substrate and are comparable with PGC in this respect. For PGE (Chapter 2) it was suggested that polygalacturonate is not the optimal substrate and that it might prefer an as yet unidentified specific part of the pectin molecule. The kinetic characterisation of PGD showed that this enzyme may represent an oligogalacturonase (Chapter 4). PGB was the only *A. niger* PG that showed an increase (3.7-fold) of the specific activity on polygalacturonate due to the increased ionic strength (i.e. 175 mM NaCl, Chapter 3). Similarly as found for PGI, PGII and PGC (Table 2), the $K_{m,app}$ value in the case of PGA could not precisely be determined due to an enzyme assay limitation, but was below 0.15 mg.ml^{-1}. Also PGD shows a high affinity for this polymeric substrate, whereas for PGE $K_{m,app}$ is much higher, *viz.* 2.5 mg.ml^{-1}. Although PGE and PGC are the most closely related *A. niger* PGs (Table 1 and 2) and both show low activity on polygalacturonate, they differ in their $K_{m,app}$ values by one order of magnitude, suggesting that the amino acid changes strongly affect the binding affinity of this substrate.

Generally endolytic enzymes either attack a polymer chain only once every encounter, the so-called single-attack enzymes, or they may attack the same polymer chain repeatedly during one encounter, the multiple-attack or processive enzymes. The product progression of single-attack enzymes typically shows the formation of long oligomers, which are gradually converted into smaller oligomers upon increased reaction time. In contrast, multiple-attack enzymes accumulate small oligomers or even monomers from the start of the reaction and the formation of longer oligomers is reduced. In Fig. 3 selected high performance anion exchange chromatograms of samples taken during polygalacturonate degradation by the *A. niger* PGs are shown. Each of the seven *A. niger* PGs degrades this polymeric substrate in a specific way as demonstrated by the accumulation of different oligomers during the course of the reaction. PGI, PGA, PGC and PGD are processive enzymes, whereas PGII, PGB and PGE show profiles typical for non-processive endolytic enzymes. The nature of the product formation on the polymeric substrate was addressed in kinetic studies using oligogalacturonates of a defined length as substrates. These studies showed that the differences among the enzymes are caused primarily by different binding affinities towards galacturonate at the individual subsites (Chapters 2-4; [11]), and also by a different number of subsites in the enzyme (Table 2). Using reduced oligogalacturonates it became evident that all PGs attack the substrate from the reducing end. Some of these enzymes like PGA and PGB prefer a partially methylesterified substrate over polygalacturonate (Table 2), whereas PGD can tolerate methylesterification to a relatively high degree. The molecular characterisation of the new endopolygalacturonase-encoding genes together with the biochemical studies of these enzymes suggests that PGA and PGB, the constitutively expressed endopolygalacturonases, which are together active over a broad pH range, i. e. 3.5 to 6.0 and which both prefer methylesterified pectin, could allow a quick adaptation of the fungus to pectin-containing substrates. The degradation products of pectin (D-galacturonic acid or its metabolites) generated by these enzymes or the subsequent intracellular activities would then trigger the induction of other pectinases (Chapter 5). Furthermore, the processive enzyme PGD, which is most probably a cell wall associated oligogalacturonase, would enable the fungus to quickly generate a low molecular weight inducer. For PGE as well as for PGC the actual substrate specificity remains to be established. However, the capability of these enzymes to hydrolyse reduced tetragalacturonate at the first glycosidic linkage from the reduced end, i.e. between galacturonic acid and the alditol, allows to speculate that another sugar moiety than galacturonic acid can be accommodated at this

Fig. 3. HPAEC profiles of polygalacturonate degradation by the *A. niger* PGs. On the left-hand side the profiles of the processive enzymes, PGI, PGC, PGA and PGD are shown, on the right-hand side the non-processive enzymes, PGII, PGB and PGE are depicted. G1-G5 correspond to the galacturonic acid oligomers with degree of polymerisation 1 to 5. IS means an internal standard used in the HPAEC analysis.
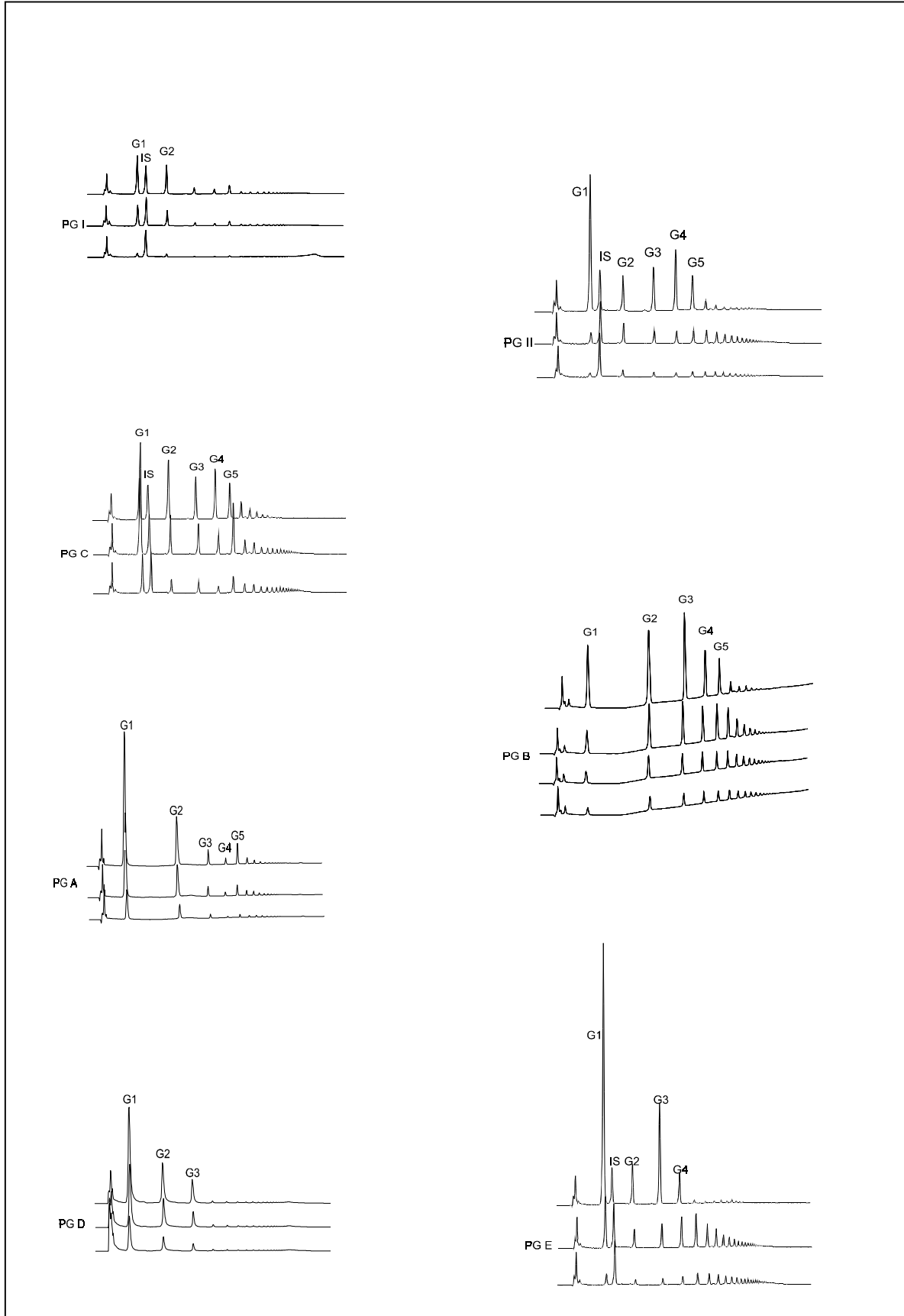
subsite. PGI and PGII of *A. niger* represent the true endopolygalacturonases, they both exhibit a high activity on polygalacturonate and PGII shows the highest sensitivity from all *A. niger* PGs towards methylesterification of the substrate [11].

The biochemical characterisation of the individual *A. niger* PGs and the information from the recently solved crystal structure of *A. niger* PGII [12] allows to extrapolate these data to the amino acid sequences and thus identify the amino acid residues responsible for catalysis, substrate binding [13], processivity [14] and substrate specificity. This opens new perspectives in the field of protein engineering.

In conclusion the characterisation of the four new *A. niger* endopolygalacturonases, PGA, PGB, PGD and PGE, shows that each of these enzymes can play a specific role for the fungus under '*in vivo*' conditions. Although overall they show a high degree of amino acid similarity, they differ in their biochemical properties and substrate specificity. Moreover, their expression is differentially regulated.

The isolation of a large number of pectinase-encoding genes from *A. niger* in our laboratory allowed to analyse for the first time their expression in a comprehensive way (Chapter 5). The main interest was to identify the molecular inducer(s) of the different pectinase genes encoding homogalacturonan- (*pme* - pectin methylesterase, *ply*A - pectate lyase, *pgx* - exopolygalacturonase, *pel*A - pectin lyase) and rhamnogalacturonan- (*rgh*A - rhamnogalacturonan hydrolase and *rgl*A - rhamnogalacturonan lyase) specific enzymes. Three types of induction profiles were found based on the 24-h expression profiles of the genes on polymeric (sugar beet pectin and polygalacturonic acid) and monomeric (D-galacturonic acid with and without L-rhamnose) sugars. D-galacturonic acid was identified as the inducer of the pectinase genes of the type I (*pel*A, *ply*A) and II (*pgx*, *pme*, *rgl*A) induction profile. The type I induction profile is characterised by an initially high level of expression followed by a gradual decrease of the gene expression with time on all media containing D-galacturonic acid. The type II profile is characterised by a fluctuation of the expression with time on all media containing D-galacturonic acid. It is therefore obvious that other factors affect the expression of these genes besides D-galacturonic acid, or that the actual inducer(s) of one of the systems is a degradation product of this sugar. The latter view is supported by the fact that in bacteria (*Erwinia* spp.), 2-keto-3-deoxygluconate, the intermediate of the D-galacturonic acid degradation pathway, was identified as the inducer of the pectinolytic system [15]. Additional

support for this has been obtained from the analysis of pectinase production in two D-galacturonic acid non-utilising mutants of *A. nidulans* isolated by Uitzetter *et al.* [16]. Western blot analysis of the culture fluid of these mutants grown on pectin using an *A. niger* PELA monoclonal antibody showed that *A. nidulans* WG262 did not produce the PELA homologue, whereas in *A. nidulans* WG222 the expression of PELA was enhanced, when compared to the wild type. The biochemical lesions of the mutations in these strains were identified and occurred in the catabolic pathway of D-galacturonic acid leading to the accumulation of L-galactonate (*A. nidulans* WG262) or the subsequent catabolic product, presumably 2-keto-3-deoxygalactonate (*A. nidulans* WG222) [17]. This indicated that the latter compound is the real inducer of PELA.

The type III induction profile is characterised by a high level of expression only in the presence of pectin after a 24 h incubation period. Thus suggests that other inducing molecule(s) than D-galacturonic acid or a change in physiological conditions does affect the expression. Although *rgl*A shows the type II induction profile, the transcription level of this gene has increased after 24 hours on pectin as well suggesting that in addition to the type II regulatory system the type III system (*rgh*A) is involved as well. The *rgh*A and *rgl*A genes encode endolytic enzymes active on the rhamnogalacturonan part of the pectin molecule. The function of RGLA 'in vivo' could be to split quickly the complex pectin molecule into the homogalacturonan and rhamnogalacturonan parts, thus providing substrates which are better accessible for the specific depolymerases. The degradation of the rhamnogalacturonan part could be accomplished later by RGHA. In *A. aculeatus* also exolytic enzymes active on the rhamnogalacturonan part of pectin were detected, *viz.* RG-galacturonohydrolase and RG-rhamnohydrolase [18,19]. In *A. niger* their homologues have not yet been discovered. These enzymes could play an important role in the induction cascade of those pectinase genes, which encode enzymes specific for the rhamnogalacturonan part of pectin by generating the appropriate low molecular weight inducer(s).

The expression of the selected pectinase genes was not detected in the presence of sucrose in the medium indicating that unlike some of the endopolygalacturonases these pectinase genes are under the control of carbon repression. The sucrose-mediated repression of pectinases is clearly visible in the HPAEC profiles of the culture media of *A. niger* grown on polygalacturonic acid with and without sucrose (Fig. 4). The degradation of polygalacturonate is delayed in the medium containing sucrose and polygalacturonic acid and the observed initial (6 h) degradation most probably corresponds to the activity of the constitutively

expressed PGA and PGB. Similar profiles were found in the case polygalacturonate was replaced by pectin (data not shown).

Fig. 4. **HPAEC profiles of culture filtrates of *A. niger* grown on 1 % (w/v) polygalacturonate (the left column) or 1 % (w/v) polygalacturonate with 1 % (w/v) sucrose (the right column)**. The samples were taken 2, 6 and 24 hours after the transfer of the overnight pregrown mycelia. galA; D-galacturonic acid.
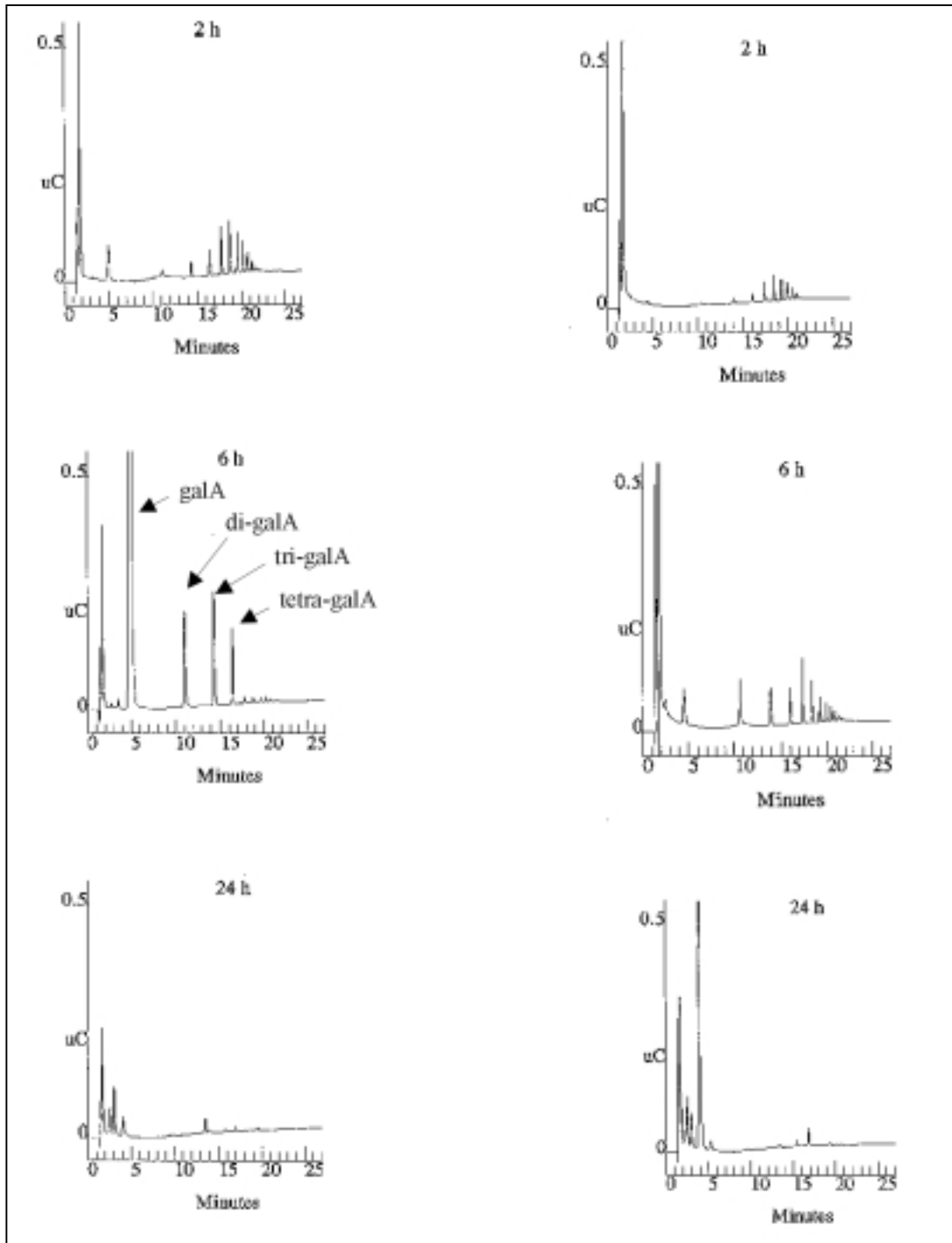
As the next step in the study of pectinase regulation we addressed the isolation of *A. niger* mutants affected in the expression of two pectinases, *pgx* and *pel*A. The methodology employed, i.e. selection of the mutants using *pyr*A as a reporter, previously led to the isolation of *xln*R encoding the positively acting regulator of the *A. niger* xylanolytic system [20]. However, this approach was not successful for the isolation of *A. niger* regulatory mutants for *pel*A and *pgx*. In the case of *pel*A this approach was hampered by a mutation, *csp*A1, present already in the *A. niger* NW219 strain used for the transformation of the *pel*A-*pyr*A construct. This mutation negatively affected the level of the *pel*A and also *ply*A expression, but did not influence the transcription level of *pgx*, thus further supporting the hypothesis of the presence of at least two different regulatory systems involved in the expression of the type I and II pectinase genes (see above). For *pgx* several categories of mutants were analysed, but none of them was affected in the expression of the endogenous gene. However, the genetic and molecular characterisation of one of the categories of the putative *pgx* regulatory mutants led to the identification of an approximately 260 bp promoter fragment responsible for the induction of *pgx* on D-galacturonic acid. This fragment contains the hexanucleotide 5' CCCTGA 3' sequence present in the other *A. niger* pectinase genes and also the sequence TGATTGGT similar to the *pga*II UAS (5' TCATTGGT 3'). The *pga*II promoter fragment, which contains the latter sequence, was found to be important for high expression of PGII on pectin [2,8]. The analysis of that particular class of mutants thus indirectly identified the *pgx* promoter fragment, localised approximately -676 bp to -939 bp from the translation start point of *pgx*, responsible for the high expresion of the gene in the presence of D-galacturonic acid.

Using a shorter promoter fragment can facilitate the isolation of the regulatory mutants using this methodology and therefore a *pel*A promoter deletion study was initiated (Chapter 5). The *gox*C gene encoding glucose oxidase from *A. niger* was exploited as a reporter system, which allowed a quick screening of the GOX activity using a plate assay. The 721-bp *pel*A promoter fragment localised at -1100 bp to -379 bp from the translation start point is important to obtain a high level of the GOX expression on D-galacturonic acid. This fragment contains the hexanucleotide sequence (see above) and also the XLNR, 5' GGCTA(A) 3', DNA-binding site [20]. A low copy number *A. niger pel*A-*gox*C transformant was exploited to screen the inducing properties of various sugars. Under the control of the *pel*A promoter GOX expression is, apart from being induced by D-galacturonic acid, also stimulated to a small extent by other sugars like D-xylose, D-sorbitol, D-glucose and L-rhamnose. Furthermore, using a GOX-protoplast assay it was shown that the transport of D-galacturonic acid is also

inducible. This is evidenced by the fact that the presence of glucose in the culture medium negatively affects the uptake of D-galacturonic acid. The uptake is delayed in the presence of a more easily metabolisable carbon source (Fig. 5).

Fig. 5. HPAEC profiles of the uptake of D-galacturonic acid (galA) by *A. niger* in the presence (the right-hand side) and the absence (the left-hand side) of glucose (glu) in the medium. The chromatograms at the bottom show the uptake of glu.
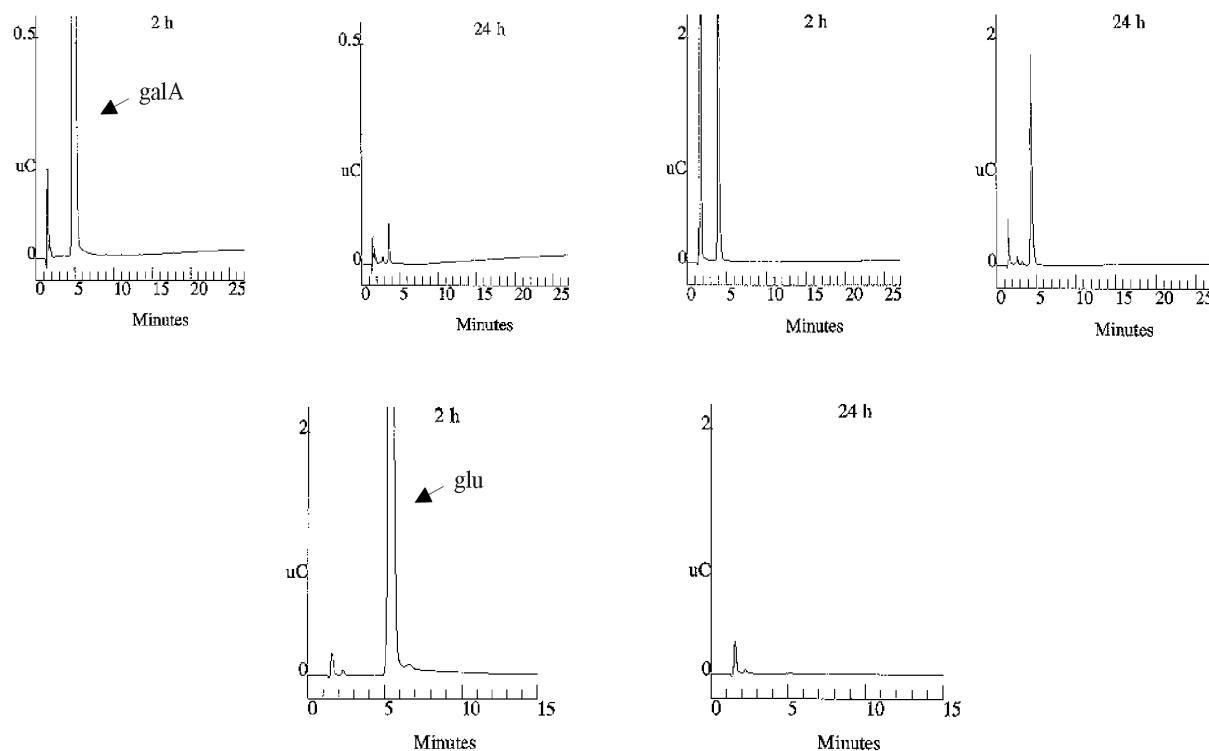

A. niger is an important industrial micro-organism. The huge potential of this fungus to produce many different extracellular enzymes, amongst which endopolygalacturonases, is also demonstrated in Chapters 2 to 4. However, new isolates of the black aspergilli must be unambiguously identified prior to their industrial exploitation. This is often a difficult task since many of the industrially important strains have altered morphology as a consequence of different rounds of mutagenesis to adapt the strain to the specific industrial needs. Our aim in Chapters 6 and 7 was to develop a quick and reliable method, which would be diagnostic for the individual species, which belong to the black aspergilli. The scheme for the identification of an unknown black *Aspergillus* isolate based on nuclear DNA (nDNA) restriction fragment length polymorphism (RFLP) analysis is shown in Fig. 6.

The combination of the restriction enzymes (*Sma*I, *Pst*I/*Sal*I and *Kpn*I/*Xho*I) and the loci examined (rDNA repeat, 26S rRNA, *pki*A and *pel*A) was suitable to reveal the differences

among the individual isolates on the level of the inter- species variations. For each of the species at least two out of five RFLP patterns are diagnostic (Fig. 6).

Fig. 6. Scheme for the identification of an unknown black *Aspergillus* isolate based on nDNA RFLP analysis. The pattern description is identical to that described in Chapter 7. On the left-hand side the combination of a probe and restriction enzymes is depicted. *; the crucial identification pattern of the species. #; this pattern in combination with the *Sma*I RFLP pattern identifies the species.
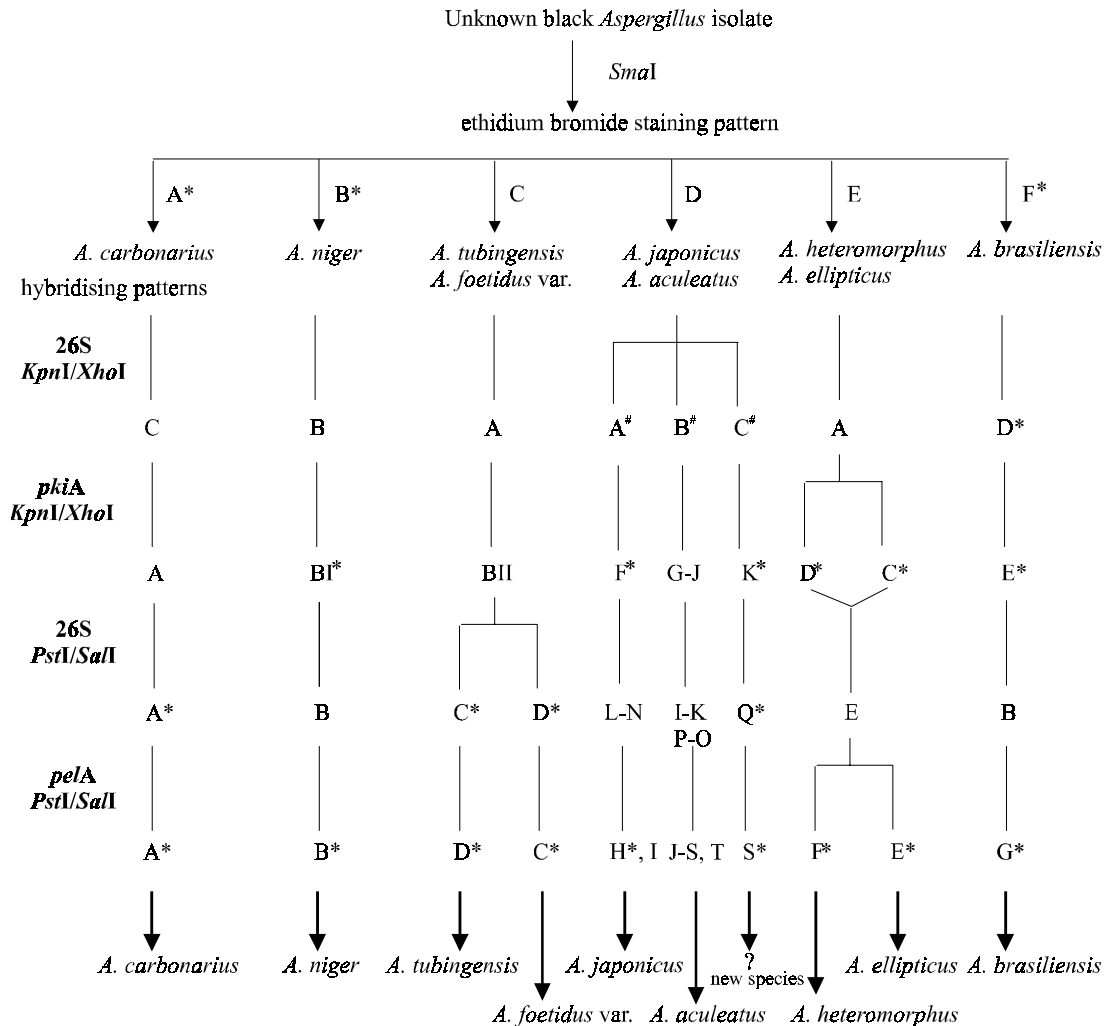
The extended analysis of the isolates belonging to the '*A. niger* aggregate' in Chapter 6 showed that *A. phoenicis*, *A. awamori* and *A. foetidus* isolates can now be classified as *A. niger* and *A. tubingensis* as previously established by Kusters-van Someren *et al.* [21], or as a new species represented by *A. foetidus* var. *pallidus* and *A. foetidus* var. *acidus* type strains. Furthermore, the existence of a new species closely related to *A. niger*, represented by *A. brasiliensis* isolates [22], was firmly confirmed by the ITS 1-5.8S- ITS 2 sequence data and by the specific

RFLP patterns (Chapter 7). The taxonomical classification of *A. aculeatus* and *A. japonicus* has been a matter of debate for many years (Chapter 1). These closely related uniseriate species of the black aspergilli can now clearly be distinguished using the molecular (RFLP, rDNA sequencing) and biochemical (secondary metabolite analysis) methods developed here. Although the sequences of the ITS 1-5.8S- ITS 2 region showed only small variations among the isolates examined, the RFLP-based division of these species is consistent with the secondary metabolite profiles typical for *A. aculeatus* and *A. japonicus* isolates, thus identifying these taxa.

Using molecular methods to establish reliable taxonomy tools for the black aspergilli, we can now distinguish nine species: *A. niger*, *A. tubingensis*, *A. foetidus* var., *A. carbonarius*, *A. heteromorphus*, *A. ellipticus*, *A. brasiliensis*, *A. japonicus* and *A. aculeatus*. Furthermore, *A. aculeatus* CBS 114.80 represents a third uniseriate species of the black aspergilli.

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SUMMARY

The microfilamentous fungus *Aspergillus niger* is an important industrial micro-organism. It is used mainly for the production of organic acids and extracellular enzymes such as proteases, cellulases, hemicellulases and pectinases. Pectinases are mainly exploited in food, feed and beverage industries. The 'products' of this species hold the GRAS (Generally Regarded As Safe) status.

Pectinases are enzymes which degrade pectin, a heteropolysaccharide found in the middle lamella and the primary cell wall of higher plants. The number of pectinase-encoding genes identified in this fungus most probably reflects the chemical and structural complexity of this polymer. Amongst more than twenty pectinase genes from *A. niger* isolated up to the present day two gene families encoding pectin lyases (PELs) and endopolygalacturonases (PGs) are found.

PGs are enzymes, which hydrolyse the α-[1$\rightarrow$4] glycosidic bond between two adjacent D-galacturonic acid residues in the main backbone of pectin called homogalacturonan. One of our questions in the beginning of the pectinase studies was, why a simple eukaryotic micro-organism such as *A. niger* contains multiple genes-encoding one specific pectinase activity. Are all of these genes necessary for the fungus to adapt to a new, pectin containing environment or are these activities redundant? The molecular and biochemical characterisation of four new *A. niger* PGs (PGA, PGB, PGD and PGE) presented here together with the data published on three other PGs (PGI, PGII and PGC) during the course of this study now indeed confirm that each of these enzymes can have a specific '*in vivo*' role for the fungus.

The previously identified PG-encoding genes, *pga*I, *pga*II and *pga*C, have been compared with four new *A. niger pga* genes isolated, *viz. pga*A, *pga*B, *pga*D and *pga*E. Except for *pga*D all these genes encode enzymes with a high mutual amino acid similarity (66.7 % - 85.0 %). They are closely related to PGs of other aspergilli present in the Databases. Expression studies revealed that *pga*A, *pga*B and *pga*E are constitutively expressed. The *pga*I mRNA was detected in the mycelium grown on pectin whereas the inducing conditions for *pga*II, *pga*C and *pga*D could not be established. This study thus showed that the members of the PG-encoding gene family are differentially expressed. The individual overproduction of these enzymes was made possible by using a strong *pki*A (pyruvate kinase) glycolytic promoter, which drives the expression of the particular *pga* gene under growth conditions, which represses the expression of the majority of other pectinases. The isolation of PGA, PGB, PGD

and PGE now allowed to compare the biochemical properties of all the members of the family. All enzymes are active in the acidic pH range (3.8 to 5.0), and they differ in their kinetic parameters ($K_{m,app}$ and $V_{max,app}$), when using polygalacturonate as a substrate. For PGA and PGB, the constitutively expressed PGs, the $V_{max,app}$ values are 16.5 μkat.mg^{-1} and 8.3 μkat.mg^{-1}, respectively, which are of the same order as determined for PGI and PGII. However, both PGA and PGB prefer a partially methylesterified pectin (pectin with a degree of esterification of 22 %) as a substrate. PGB is the only *A. niger* PG, which shows higher activity on polygalacturonate with increasing ionic strength. PGE shows much lower specific activity on polygalacturonate (0.5 μkat.mg^{-1}) and is comparable to PGC in this respect. It may therefore preferentially hydrolyse an as yet unidentified part of the pectin structure. For PGD it was tentatively concluded that it represents an oligogalacturonase. The analysis of the product progression using polygalacturonate as a substrate together with the kinetic studies on oligogalacturonates with defined length identified PGA and PGD as processive enzymes, whereas the PGB and PGE product progression profiles were typical for endolytic non-processive enzymes. All these enzymes attack the substrate from the reducing end.

The results of these studies have an impact in several areas: (i) in the pectin industry to design 'tailor-made' enzyme preparations, (ii) in carbohydrate research to elucidate the pectin fine structure using specific pectinases, and (iii) in biochemistry to understand the mechanism of pectin hydrolysis by PGs.

The study on the regulation of the expression of several pectinase genes encoding enzymes active on the homogalacturonan and rhamnogalacturonan parts of pectin revealed three typical induction profiles (type I-III). The type I and II induction is triggered by the presence of D-galacturonic acid or possibly an intermediate metabolite (type I) and was typically found for the gene-encoding enzymes active on homogalacturonan. The type III induction profile, which is characterised by a high expression of genes on pectin later during growth, has been found for *rgh*A and also in the case of *rgl*A expression. Both *rgh*A and *rgl*A encode enzymes active on the rhamnogalacturonan part of pectin. All the selected pectinase genes studied were repressed in the presence of sucrose. Thus in the process of the pectin degradation by *A. niger*, the constitutively expressed PGA and PGB and later the cell wall attached oligogalacturonase, PGD, generate D-galacturonic acid, which is the inducer of a set of other pectinase genes. The products of these genes can at a later stage of the pectin degradation release another inducer molecule, which might activate the type III regulatory system. It may be that other factors play a role in this induction process. In the next step we addressed the isolation of *A. niger*

mutants affected in the expression of the exopolygalacturonase (*pgx*) and pectin lyase A (*pel*A) genes. We exploited a methodology based on the *pyr*A gene as a reporter, which was successfully applied in the regulation study of the *A. niger* xylanolytic system and led to the isolation of XLNR, the positively acting regulator. This approach was not successful here, however it led to some partial achievements, which help to better understand the *A. niger* pectinase regulation. For *pel*A and *pgx*, the representatives of the type I and II induction profiles respectively, shorter promoter fragments were identified, which are responsible for the high induction of the genes in the presence of D-galacturonic acid. In the case of *pel*A this promoter fragment was also responsible for induction of expression in the presence of other sugars like L-rhamnose, D-xylose and D-glucose, although to a lower extent. Furthermore, in the course of this study evidence was found that pectin or its degradation products induce the D-galacturonic acid transport system.

In order to identify unknown isolates, which belong to the black aspergilli, a restriction fragment length polymorphism (RFLP) based identification method was developed, which allows, in a quick and reliable way, to establish the taxonomical position of an isolate. Molecular techniques such as DNA sequencing, RLFPs and RAPDs are also reliable to establish the taxonomical origin of industrial strains, which very often underwent a number of morphological changes in the process of 'domestication', which renders classic morphology criteria inapplicable. Thus, the combination of the restriction enzymes *Sma*I, *Pst*I/*Sal*I and *Kpn*I/*Xho*I and the 28 S, *pki*A and *pel*A probes provides the diagnostic RFLP patterns for each species of the black aspergilli, i.e. *A. niger*, *A. tubingensis*, *A. foetidus* var., *A. brasiliensis*, *A. carbonarius*, *A. heteromorphus*, *A. ellipticus*, *A. aculeatus* and *A. japonicus*.

SAMENVATTING

Aspergilli zijn saprofytische micro-filamenteuze schimmels. Deze micro-organismen worden uit verschillende bronnen van over de gehele wereld geïsoleerd, bijvoorbeeld uit de bodem, de lucht, van papier, palmolie, fruit enz. De schimmel past zich aan wisselende milieu omstandigheden aan door enzymen te produceren die in staat zijn om voedingsstoffen vrij te maken uit verschillende complexe substraten. Deze enzymen zijn voornamelijk proteases, cellulases, hemicellulases en pectinases.

Aspergillus niger behoort tot het genus *Aspergillus*, sectie *Nigri* (zwarte aspergilli) en wordt veel in de industrie toegepast door zijn grote potentie om onder andere pectinases te produceren. Daarnaast hebben de producten van deze schimmel de 'GRAS' status (Generally Regarded As Safe), waardoor deze mogen worden toegepast in de levensmiddelenindustrie.

Pectinases breken pectine af dat, naast cellulose, hemicellulose en eiwitten, voorkomt in de celwand van hogere planten. Pectine is een zeer complex polymeer wat wordt bevestigd door het feit dat de schimmel een groot aantal genen bevat die coderen voor verschillende pectinases. Tot nu toe zijn uit *A. niger* meer dan twintig pectinase-coderende genen geïsoleerd. Van de pectinases is slechts een klein deel gekarakteriseerd, in het bijzonder de enzymen behorend bij de gen-families van de polygalacturonases en van de pectine lyases.

Polygalacturonases zijn enzymen die de glycolytische binding tussen twee [1-4]α-D-galacturonzuur moleculen in de hoofdketen van pectine (homogalacturonan) hydrolyseren. Een van de vragen aan het begin van het pectinase onderzoek was, waarom een relatief eenvoudig micro-organisme als *A. niger* een zo groot aantal pectinase genen bevat, coderend voor enzymen met een ogenschijnlijk identieke katalytische werking. Een mogelijkheid is dat de schimmel dit grote aantal enzymen nodig heeft voor een snelle aanpassing aan veranderde milieu omstandigheden.

De moleculair biologische en biochemische karakterisering van vier nieuwe *A. niger* polygalacturonases (PGA, PGB, PGD en PGE), zoals beschreven in dit proefschrift, tezamen met de gedurende dit onderzoek gepubliceerde data voor drie andere polygalacturonases (PGI, PGII en PGC), tonen inderdaad aan dat elk enzym voor de schimmel een specifieke *'in vivo'* rol vervult.

Zes van de zeven *A. niger* polygalacturonases zijn sterk verwant maar vertonen duidelijk verschillende biochemische eigenschappen. Het onderzoek aan PGI en PGII leerde dat deze enzymen als polygalacturonases kunnen worden geclassificeerd vanwege de hoge specifieke activiteit op polygalacturonzuur en de geringe tolerantie van het substraat voor verestering

189

met methylgroepen. PGA en PGB daarentegen vertonen een hogere activiteit op een meer 'natuurlijk' substraat zoals pectine met een lage veresterings graad (22 %). Van alle *A. niger* polygalacturonases vertonen PGC en PGE een zeer lage specifieke activiteit op polygalacturonzuur. Op basis hiervan is voorgesteld dat deze twee enzymen inwerken op een nog niet opgehelderd structureel deel van het pectine molecuul. Op basis van de kinetische eigenschappen waaruit het aantal subsites werd afgeleid kon voor PGD voorgesteld worden dat dit een oligogalacturonase is. Ook is PGD mogelijk geassocieerd met de celwand van de schimmel vanwege de aanwezigheid van een lange N-terminale extensie die overeenkomst vertoont met sequenties die in andere eiwitten als membraanverankering fungeren.

Naast verschillen in substraat specificiteit tussen de verschillende *A. niger* polygalacturonases blijken ook de expressie patronen van de verschillende polygalacturonase genen onderling te verschillen.

De *pga*A, *pga*B en *pga*E genen komen constitutief tot expressie, terwijl het *pga*I gen specifiek wordt geïnduceerd bij groei op pectine. De condities waaronder de *pga*II, *pga*C en *pga*D genen tot expressie komen zijn nog niet vastgesteld.

De resultaten van het onderzoek zoals beschreven in dit proefschrift zijn van belang voor: (i) de pectine industrie, voor het ontwikkelen van producten met behulp van specifieke enzym preparaten, (ii) het koolhydraat onderzoek, voor de opheldering van de pectine fijnestructuur door gebruik te maken van zuivere specifieke pectinases, en (iii) het biochemisch onderzoek naar de relatie tussen structuur en functie van pectinases, voor het verkrijgen van meer inzicht in het reactiemechanisme van de hydrolyse van pectine door polygalacturonases.

Het onderzoek naar de regulatie van de gen-expressie van verschillende pectinases die inwerken op het homogalacturonan deel of het rhamnogalacturonan deel van het pectine molecuul heeft geresulteerd in het onderscheiden van drie typen inductie (type I, II en III).

Inductie van het type I en II wordt gekarakteriseerd door het feit dat de gen-expressie wordt geïnitieerd door de aanwezigheid van D-galacturonaat (type II) of een metaboliet ervan (type I). Type I en II inductie was kenmerkend voor de pectinase genen coderend voor enzymen betrokken bij de afbraak van het homogalacturonan deel van pectine.

Type III inductie wordt gekarakteriseerd door een hoge expressie op pectine in een later stadium van de groei. Type III inductie bleek te gelden voor het rhamnogalacturonan hydrolase gen (*rgh*A). De expressie van het rhamnogalacturonan lyase gen (*rgl*A) bleek gereguleerd volgens type II. Waneer inductie op D-galacturonaat wordt beschouwd maar heeft ook karakteristieken van type III op pectine.

De huidige inzichten hebben geresulteerd in de volgende werkhypothese: Het pectinase spectrum van *A. niger* wordt ten dele geïnduceerd door D-galacturonaat dat wordt gegenereerd door de constitutief tot expressie komende polygalacturonases A en B. De geïnduceerde pectinases, op hun beurt, zijn verantwoordelijk voor het vrijmaken van andere componenten uit het substraat die de rest van het pectinase spectrum induceren. Hierbij kunnen ook andere factoren zoals de aanwezigheid van andere nutriënten en de pH van het milieu een rol spelen. Van de genen coderend voor het exopolygalacturonase (*pgx*) en het pectine lyase A gen (*pel*A) representatief voor type II en type I inductie, respectievelijk, zijn korte stukjes van de promoter sequentie geïdentificeerd die verantwoordelijk zijn voor een hoge genexpressie in aanwezigheid van D-galacturonaat. Voor het *pel*A gen bleek bovendien dat de expressie in aanwezigheid van andere suikers zoals L-rhamnose, D-xylose and D-glucose ook gereguleerd wordt door dit specifieke promoter fragment. Verder is in dit onderzoek aangetoond dat de expressie van het D-galacturonaat transport eiwit wordt geïnduceerd door groei op pectine.

Voor het identificeren van onbekende zwarte aspergillus schimmels werd een methode ontwikkeld gebaseerd op Restrictie Fragment Lengte Polymorfisme (RFLP) analyse. Dit resulteerde in een snelle en betrouwbare methode om de taxonomische indeling van een isolaat vast te stellen. Gebleken is dat classificatie van industriële stammen niet mogelijk is op basis van klassieke morfologische criteria, daar deze stammen in het gehele traject van veredeling en aanpassing aan industriële toepassing vaak een aantal morfologische veranderingen hebben ondergaan. Succesvolle toepassing van moleculair biologische technieken zoals DNA-sequentie analyse, RFLP-analyse en Randomly Amplified Polymorphic DNA (RAPD) analyse voor het vaststellen van de taxonomische oorsprong van industriële stammen heeft de betrouwbaarheid van de methoden aangetoond. In dit proefschrift is een RFLP analyse methode beschreven waarbij, het schimmel DNA wordt gedigesteerd met de restrictie enzymen *Sma*I, *Pst*I/*Sal*I en *Kpn*I/*Xho*I. De DNA-probes voor hybridisatie met het gedigesteerde DNA zijn fragmenten van de 28S, pyruvaat kinase (*pki*A) en pectine lyase A (*pel*A) genen. Op basis van de RFLP patronen die onstonden met genoemde probes konden alle geanalyseerde species van de zwarte aspergilli geclassificeerd worden.

SHRNUTÍ

Aspergilli jsou plísně, které lze isolovat z různých zdrojů jakými jsou např. tropická půda, palmový olej, vzduch nebo ovoce. Nacházejí se na celém světě, patří mezi tzv. kosmopolitní mikroorganismy. Mezi nejznámější z plísní rodu *Aspergillus* patří druh *Aspergillus niger*, který má široké uplatnění v potravinářském průmyslu zejména díky schopnosti produkovat organické kyseliny a mimobuněčné enzymy, např. proteásy, celulásy, hemicelulásy a pektinásy. Pektinásy se aplikují v podobě enzymových směsí při produkci různých nápojů jako jsou mošty, džusy a nektary, ale nacházejí své uplatnění i v jiných oblastech jako např. při přípravě krmných směsí pro dobytek. Tyto enzymy specificky štěpí glykosidické vazby v hlavním polysacharidovém řetězci pektinu. Pektin je chemicky a strukturálně nejsložitějším polysacharidem nacházejícím se v primární buněčné stěně a střední lamele vyšších rostlin. Běžně je pektin spojen v našich myslích se schopností polymerizace, nebo-li tvoření gelu při přípravě ovocných marmelád a džemů.

Hlavním tématem této práce je studium plísní z rodu *Aspergillus*, sekce *Nigri* a pektinás, konkrétně endopolygalakturonás (PG), produkovaných průmyslově významným druhem *A. niger*.

Pektinásy se dají rozdělit do několika kategorií na základě kritérií jako jsou např. substrátová specificita nebo typ reakce, kterou katalyzují. Endopolygalakturonásy tvoří jednu z kategorií pektinás. Jsou to enzymy, které štěpí α-[1→4] glykosidickou vazbu náhodně mezi dvěma molekulami D-galakturonové kyseliny v řetězci, v tzv. homogalakturonové části pektinu. V pektinu, který je izolovaný z různých přírodních zdrojů jako cukrová řepa, jablka nebo citrusové plody, je většina jednotek D-galakturonové kyseliny methylesterifikovaná na karboxylové skupině. Proto před hydrolýzou homogalakturonanu endopolygalakturonásami tyto methylové skupiny musí být 'odstraněny' jiným enzymem, tzv. pektin methylesterásou (PME). Tři geny (*pga*I, *pga*II a *pga*C), kódující endopolygalakturonásy PGI, PGII a PGC, byli v minulosti isolované z *A. niger* N400 (= CBS 120.49). Na základě restrikční DNA analýsy se navíc zjistilo, že v genomu mohou být přítomny ještě další čtyři geny (*pga*A, *pga*B, *pga*D a *pga*E). Jedna z otázek položených na počátku tohoto výzkumu byla, proč primitivní eukaryotický mikroorganismus jako *A. niger* potřebuje až sedm PG? Liší se tyto enzymy významně ve svých biochemických vlastnostech, jsou produkované za různých růstových podmínek, anebo jde o pouhou duplikaci genů, a tudíž některé z těchto enzymů jsou nadbytečné? V kapitolách 2 až 4 je popsaná izolace nových genů kódujících endopolygalakturonásy, nadprodukce enzymů pomocí

expresní kazety obsahující silný cizorodý promoter, jejich izolace a biochemická charakterizace. Výsledky této a dalších prací ukázaly, že i když primární proteinová struktura těchto enzymů je velmi podobná, každý z nich má jiné biochemické vlastnosti a s velkou pravděpodobností tak plní i různou fysiologickou funkci. Tak např. PGA, PGB a PGE jsou produkovány do prostředí v přítomnosti různých růstových podmínek, t.j. různých testovaných cukrů, zatímco PGI je exprimována pouze v přítomnosti pektinu a podmínky produkce dalších enzymů, viz. PGII, PGC a PGD, se nepodařilo ustanovit (kapitola 5). pH optimum pro všechny PG je v kyselé oblasti (pH 3.8 až 5.0), největší rozdíly mezi těmito enzymy jsou v kinetických parametrech K_m a V_{max} naměřených pro polygalakturonát. Na základě těchto výsledků se předpokládá, že konstitutivně exprimované PGA a PGB mají funkci 'generátora' menších pektinových jednotek, t.j. monoméru, D-galakturonové kyseliny. Ty se pak dostanou dovnitř buněk *A. niger*, kde plní funkci induktora a umožní zahájit syntézu dalších enzymů, které se podílejí na štěpení pektinu, ale nejsou produkovány konstitutivně, t.j. za každých podmínek. PGE a PGC, protože hydrolyzují polygalakturonát velmi pomalu, mohou upřednostňovat jinou část molekuly pektinu, která ještě nebyla izolována. PGI a PGII jsou typickými endopolygalakturonásami a PGD nejpravděpodobněji představuje novou třídu polygalakturonás v plísních, tzv. oligogalakturonásy.

Výsledky tohoto studia mohou být aplikované v různých oblastech: 1) v průmyslu, při přípravě nových enzymových koktejlů, které by obsahovaly enzymy se známými substrátovými specificitami, 2) ve výzkumu chemické struktury pektinů a 3) v biochemii, kde přispívají k objasnění mechanismu hydrolýzy pektinu pomocí PG.

Další část této práce se zabývá regulací exprese různých pektinásových genů kódujících enzymy s homo- a rhamno- galakturonovou substrátovou specicitou. Na základě tohoto studia se zjistilo, že dané geny jsou pod kontrolou nejméně třech regulačních systémů (TYP I-III). Geny s indukčním profilem TYP I a II jsou exprimované v přítomnosti D-galakturonové kyseliny nebo nejpravděpodobněji jejím následným katabolickým produktem (TYP I) a kódují enzymy aktivní především při štěpení homogalakturonové části pektinu. TYP III je charakterizován expresí genů pouze v přítomnosti pektinu a to 24 hodin po zahájení indukce. Tento typ byl nalezen u genů *rgh*A a *rgl*A, kódující rhamnogalakturonásu A a rhamnogalakturonan lyásu A, jejichž substrátem je rhamnogalakturonan. Všechny studované pektinásy jsou reprimované v přítomnosti cukrů jako je glukóza nebo sacharóza.

Dalším naším cílem byla isolace kmenů *A. niger*, mutovaných v jednom z pektinásových regulačních systémů. K tomuto byla využita metoda, založená na *pyr*A genu jako reportéru,

kódujícím jeden z enzymů syntetické dráhy uridínu. Tímto způsobem byl v nedávné době v naší laboratoři izolován aktivátor xylanolytického systému v *A. niger*. Aplikace této metody při izolaci regulátoru exopolygalakturonásy (*pgx*, TYP II) a pektin lyásy A (*pel*A, TYP I), bohužel nebyla úspěšná. Výsledky tohoto výzkumu však přinesly dílčí úspěchy na poli regulace pektinás. V promótorových oblastech obou genů se izolovaly DNA úseky, které jsou nezbytné pro dosažení vysoké exprese *pgx* a *pel*A v přítomnosti D-galakturonové kyseliny. V případě *pel*A, tento úsek promótoru byl taktéž indukovatelný jinými cukry - D-xylósou, L-rhamnósou a D-glukósou, i když v nižší míře. Dále se v průběhu těchto studií zjistilo, že transportní systém pro D-galakturonovou kyselinu je taktéž indukovaný a to v přítomnosti pektinu nebo jeho následných štěpných produktů.

V průmyslu se často setkáváme s využíváním různých kmenů z rodu *Aspergillus*. Proto, aby se mohli aplikovat v biotechnologických procesech, jejich příslušnost k tzv. povoleným druhům, t.j. neprodukujícím toxiny, musí být jednoznačně stanovena. Protože tyto kmeny jsou často mutanti, kritéria, která se používají v klasické taxonomii, se nedají použít. V kapitolách 6 a 7 je popsaná metoda, která byla vyvinutá na základě RFLP ('Restriction Fragment Length Polymorphism'). Kombinace restrikčních endonukleás *Sma*I, *Pst*I/*Sal*I a *Xho*I/*Kpn*I a prób 28S rRNA (28S ribosomální gen), *pki*A (pyruvát kinása A) a *pel*A umožnily nalézt identifikační vzorky pro všechny druhy z rodu *Aspergillus*, sekce *Nigri*. Na základě těchto výsledků můžeme kmeny 'černých aspergilů' rozčlenit mezi následující druhy: *A. niger*, *A. tubingensis*, *A. foetidus* var., *A. brasiliensis*, *A. carbonarius*, *A. heteromorphus*, *A. ellipticus*, *A. aculeatus*, *A. japonicus* a nový druh representovaný kmenem *A. aculeatus* CBS 114.80.

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CURRICULUM VITAE

Lucie Pařenicová was born as her twin-brother Patrik in Ostrava (Czechoslovakia) on the 14-th of August 1971. Her interest in nature has been nourished since early childhood; she spent a lot of time in her grandparents house, which was surrounded by a big garden in the village Osíčko below the Valašské Beskydy mountains. Her early life 'career', attending of the primary school with sport specialisation in athletics and selection into the volleyball Training Centrum of Youth in Ostrava, would rather suggest, she would become a professional sportsman. However, after finishing the high school in June 1989, she decided for a study of Biochemistry at the Comenius University, Faculty of Natural Sciences, in Bratislava. Her first experience on the premises of the university was the 'velvet' revolution in November 1989, which, started by students, broke down the more than forty years lasting communist regime in Czechoslovakia. The pulsing atmosphere of changes accompanied her five years of the university study, which she finished with a title of 'Magister, Mgr.' (M.Sc.), with distinction, in July 1994. Her interest in a continuation in research as a PhD student, after finishing the university, was clear since the third year of her studies, when she started to contact different universities abroad and ask for a possible PhD fellowship. It was a matter of 'ŠTĚSTÍ' or 'GOOD LUCK' that she could come to Wageningen in November 1994 and start to work in the group of Dr. Jaap Visser at the section Molecular Genetics of Industrial Micro-organisms, who provided her with a fellowship for the whole PhD period. This would not have happened without the assistance of Dr. Peter Biely from the Slovak Academy of Sciences and Prof. J. Kolarov, the head of the Biochemistry Department of the Faculty of Natural Sciences UK in Bratislava, who helped her to find the contact. She joined the 'pectinase' group and participated for four years in an EC financed project entitled: 'Exploitation of microbial enzymes specificity in pectin modification and degradation to improve and develop enzymatic processes in the agroindustry (AIR2-CT941345)', and in the project dealing with molecular analysis of black aspergilli financed by the Solvay company. Her PhD study was accomplished by writing this book, in December 1999.

Epilogue

One page left empty in this book and it is the last page. I saved it (by coincidence ☺) for the epilogue or 'na-word' as the Dutch people would say. I can't escape from getting a bit melancholic by this 'coincidence' since soon the 'last page' will be added also to my life in Holland, and I will move on from a country of 'kaas en klompjes' to a country of 'pasta è víno'. I will exchange aspergilli for plants but I will stay faithful to my favourite topic, transcription regulation.

Surely, it has been a great experience for me both professionally and privately to live in Holland for more than 5 years. At first, I would like to thank Jaap, who made it possible for me to stay in Wageningen till the very last moment and to finalise my work. And……thanks for your trust, Jaap! Well, the life in the lab and outside would not be such fun without the 'pectin boys' Jac & Harry. I think under your coaching, Jac, I learned to get more self-confidence and to work independently, what I was not used to before. Thanks for your patience with my Czech English. Harry, if I could start my Ph.D. once again, I would definitely like to spend more time on the 'enzyme stuff'. There is a lot to learn from you! Other colleagues contributed to my work as well, special thanks to Peter Vo, Yvonne, Hetty, Leo, Henk, Noël, Marco, Ronald and Peter S. My precious memories for the lab 1023 will be also connected with Jos, Suzanne, Matthé, Adriaan, Eddy, Wilbert, Steph, Guy, Allard, Erwin, Line, Sandrine, Riekje, Detmer and…………CD (Claudia Dekker)!

MGIM hosted a lot of foreign researchers during my stay. Somehow, the foreigners tend to group together, which resulted in several special friendships for me. With my flat- and lab-mate Palomilla, with tempered Paloma (I won`t forget our dancing classes in 1023 and many nice memories outside the lab!), 'crazy' Laurence, Mojca B., James, Sylvie & François, Sailaja and Bjarne. Bjarne, thanks for your 'technical support' during writing this thesis! I hope to keep in touch with all of you!!!!

Wageningen is a special university city in Holland, it is rather small but there live a lot of foreign people. The national and international cultures mix in a quite peculiar way. I am happy I had the opportunity to meet and to make friendship with Dutch, but also with people from all around the world - Bulgaria, Spain, Italy, Portugal, Andorra, France, Serbia, Poland, England, U.S.A., Brasil, Mexico, Australia……. And my life would not be complete without sporting activities! I am glad I had the opportunity to join the Waho Dames 1 and Cito Zeist volleyball teams and enjoy the atmosphere of the 'sunny island', the Copacabana Fit Club!

A na úplný závěr pár slov mé rodině a přátelům doma. Děkuji za Vaši podporu hlavně během těch méně 'slunných chvil' v cizině a doufám, že Vaši důvěru ani v budoucnu nezklamu!

200(0)! The End!