Arabinase gene expression in *Aspergillus niger*: indications for coordinated regulation

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***Aspergillus niger* secretes three glycosylated glycosyl hydrolases which are involved in degradation of the plant cell wall polysaccharide L-arabinan: α -L-arabinofuranosidases (ABF) A and B, and endo-1,5- α -L-arabinase (ABN) A. The nucleotide sequence of the previously cloned gene encoding ABF A (*abfA*) from *A. niger* was determined. The coding region contains seven introns. Mature ABF A comprises 603 amino acids with a molecular mass of 65.4 kDa as deduced from the nucleotide sequence. The secreted enzyme is N-glycosylated. The primary structures of the three *A. niger* arabinases characterized lack similarity. Regulation of arabinase expression upon induction by sugar beet pulp and by L-arabitol was studied as a function of time. This was done in wild-type *A. niger* as well as in transformants carrying multiple copies of either one of the ABF-encoding genes. Each arabinase gene responded differently upon a mycelial transfer to L-arabitol-containing medium. Extra copies of *abfA* or *abfB* led to a decreased expression level of ABN A, though the repression elicited by *abfB* is stronger and more persistent than that effected by *abfA*. Multiple copies of both *abf* genes influence expression of the other ABF similarly, but to a far less pronounced degree than they affect ABN A synthesis. Four putative promoter elements, shared by all three arabinase genes, could be involved in coordination of L-arabinan degradation by *A. niger*.**

Keywords: *Aspergillus niger*, α -L-arabinofuranosidase, gene structure and regulation, endo-1,5- α -L-arabinase, promoter elements

INTRODUCTION

Enzymes degrading L-arabinan, a branched polymer largely made up of (1 $\rightarrow$ 5)-, (1 $\rightarrow$ 2)- and (1 $\rightarrow$ 3)- α -linked L-arabinofuranosyl residues, are commonly applied in structural studies of pectic and hemicellulosic plant cell wall heteropolysaccharides (e.g. Nishitani & Nevins, 1989; Sakai & Sakamoto, 1990; van de Vis *et al.*, 1991; Will & Dietrich, 1992; Lerouge *et al.*, 1993). The results of such studies indicate that the L-arabinan component is involved in covalent cross-linking of cell wall polysaccharides. This has led to renewed interest in arabinases, with respect to their potential use in agro-industrial

processes such as fruit juice technology, valorization of agricultural waste and bioconversion of plant biomass (cf. Whitaker, 1984; Voragen *et al.*, 1987).

The hyphal fungus *Aspergillus niger* secretes an endo-1,5- α -L-arabinase (ABN A) (EC 3.2.1.99) and two distinct α -L-arabinofuranosidases (ABF A and B) (EC 3.2.1.55) which are considered the main L-arabinan-degrading activities (Voragen *et al.*, 1987; Rombouts *et al.*, 1988; van der Veen *et al.*, 1991). These glycosylated enzymes act synergistically in degrading branched L-arabinan: the preferred substrate for ABN A is linear (debranched) (1 $\rightarrow$ 5)- α -L-arabinan, the backbone of the polymer, while the ABFs are typical glycosidases increasingly active against oligosaccharides of a lower degree of polymerization (Rombouts *et al.*, 1988; McCleary, 1991; Beldman *et al.*, 1993; Lerouge *et al.*, 1993). Both ABFs are also involved in arabinoxylan degradation as debranching hydrolases, removing α -linked L-arabinofuranosyl residues from wheat flour arabinoxylan fragments (Kor-

Abbreviations: ABF, α -L-arabinofuranosidase; ABN, endo-1,5- α -L-arabinase; CNBr, cyanogen bromide; PNP-A, *para*-nitrophenyl α -L-arabinofuranoside.

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melink *et al.*, 1993). Previously we studied the production of the *A. niger* arabinases using enzyme-specific antisera (van der Veen *et al.*, 1991, 1993). All three were found to be secreted when grown on plant cell wall fractions containing L-arabinan, e.g. sugar beet pulp. L-Arabitol, an intermediate of *A. niger* L-arabinose catabolism (Witteveen *et al.*, 1989), also mediates arabinase expression. Recently we cloned the three arabinase-encoding genes from *A. niger* and analysed the structure of two of these genes, *abnA* encoding ABN A and *abfB* encoding ABF B (Flipphi *et al.*, 1993a, b, c).

In this paper, arabinase expression induced by sugar beet pulp or L-arabitol has been studied as a function of time, both in wild-type *A. niger* and transformed *A. niger* strains containing multiple copies of either the *abfA* or the *abfB* gene. The nucleotide sequence of the *abfA* gene encoding ABF A and its structure are presented. Upon comparison of the three *A. niger* arabinase genes, common sequence elements were identified which might be involved in regulation of arabinase gene expression.

METHODS

Escherichia coli strains and plasmids. *E. coli* DH5 α was used to generate and propagate plasmids; *E. coli* JM101 was used to generate recombinant M13mp18/19 phages for sequencing. Plasmid vectors pEMBL (Dente & Cortese, 1987) and pGEM (Promega) were used in DNA subcloning. Plasmids pIM900, containing the *A. niger abfA* gene, and pC1X1, an *abfA* cDNA clone, were described previously (Flipphi *et al.*, 1993c). Plasmids pIM950 and pIM991 contain the *A. niger abnA* and *abfB* genes, respectively (Flipphi *et al.*, 1993a, b). Plasmid pSF5, kindly provided by Dr J. R. Kinghorn, (Plant Molecular Genetics Unit, University of St Andrews, Scotland, UK), carries the *A. nidulans* gene encoding γ -actin described by Fidel *et al.* (1988). General DNA manipulations were performed according to Sambrook *et al.* (1989).

Aspergillus strains and growth conditions. *A. niger* N402 (*cspA1*) originates from wild-type strain N400 (CBS 120.49) and is a morphological mutant with short conidiophores. Generation of *A. niger abfA* and *abfB* transformants was described previously (Flipphi *et al.*, 1993a, c). Growth conditions and media used in cultivation were as described by Flipphi *et al.* (1993c), using 6 g NaNO₃ l⁻¹ instead of NH₄Cl as the nitrogen source and 1% (w/v) of the carbon source indicated. Transfer experiments were performed according to Flipphi *et al.* (1993b). L-Arabitol was obtained from Sigma. Sugar beet pulp, a gift from CSM (Breda, The Netherlands), was ground in a Waring blender prior to use.

Sequence determination and analysis. DNA fragments were cloned both in plasmid vectors (pEMBL, pGEM) and in M13mp18/19. DNA sequences were determined by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) using the T7 Sequencing Kit from Pharmacia LKB according to the supplier's instructions. Single-stranded M13 DNA as well as alkali-denatured plasmid DNA were used as template; both general sequencing primers and gene-specific oligonucleotides were employed as primers. All nucleotide sequences were determined on both strands. Computer analysis was done using the PC/GENE program (IntelliGenetics) and the sequence analysis software package of the Genetics Computer Group. Cyanogen bromide (CNBr) cleavage of ABF A and

amino acid sequence determination were performed as described previously (Flipphi *et al.*, 1993b). The transcription initiation site was deduced upon primer extension mapping according to the method of Calzone *et al.* (1987) as described by Flipphi *et al.* (1993a). PolyA⁺ RNA, isolated from *A. niger abfB* transformant N593::pIM900-1 after 1 h of L-arabitol induction as described previously (Flipphi *et al.*, 1993c), was used as template. The 17-mer oligonucleotide which served as primer (5' TGTTGCC-GGCTGTCTGG 3') is complementary to nucleotides -16 to +1 of the determined *abfA* sequence, as shown in Fig. 2.

Enzymic deglycosylation. N-Linked carbohydrate moieties were removed from purified *A. niger* ABF A (van der Veen *et al.*, 1991) with peptide-N⁴-(N-acetyl- β -glucosaminyl) asparagine amidase (EC 3.5.1.52), which cleaves off all three types of N-linked carbohydrate oligomers (Hirani *et al.*, 1987; Tarentino & Plummer, 1987). N-Glycanase from Genzyme was used according to the manufacturer's instructions. Protein was analysed by SDS-PAGE using 10% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS (Laemmli, 1970). Protein test mixture 4 (Serva) provided molecular mass markers. The gels were stained with Coomassie brilliant blue R250.

Determination of arabinase activity in culture medium.

Medium samples were filtered over filter paper and centrifuged (3000 r.p.m., 15 min) to pellet debris. ABF was assayed by measuring hydrolysis of *para*-nitrophenyl α -L-arabinofuranoside (PNP-A) (Sigma) as described previously (van der Veen *et al.*, 1991). ABN activity was determined using ArabinaZyme tablets (Megazyme) according to the manufacturer's instructions.

Isolation of RNA and genomic DNA. Mycelium was harvested from liquid cultures, washed with saline and powdered as described previously (Flipphi *et al.*, 1993c). *A. niger* genomic DNA was isolated according to the method of de Graaff *et al.* (1988). Total RNA was isolated from the mycelial powder by guanidinium thiocyanate extraction (Chirgwin *et al.*, 1979) and subsequent centrifugation over a 5.7 M CsCl cushion overnight at 32000 r.p.m. (Glišin *et al.*, 1974). The quality of the RNA isolated was tested by native electrophoresis in 1% (w/v) agarose in the presence of 25 μ g ethidium bromide l⁻¹. The RNA concentration was calculated from A_{260} .

Slot blot analysis of RNA. Total RNA was denatured with glyoxal and dimethylsulfoxide according to McMaster & Carmichael (1977). Denatured RNA samples were diluted in 10 mM sodium phosphate buffer, pH 7.0. Either 5 or 1 μ g RNA was applied to each slot of a Hybri-Slot manifold (Gibco BRL), and was transferred to Hybond-N membrane (Amersham) using 10 mM sodium phosphate buffer, pH 7.0, as transfer buffer. The RNA was fixed to the nylon support by baking at 80 °C. Prior to hybridization glyoxal was removed as indicated by Sambrook *et al.* (1989). The slot blots were probed with ³²P-labelled DNA fragments specific for either one of the RNAs of interest using standard prehybridization and hybridization solutions containing 50% (v/v) formamide (Merck) and 6 $\times$ SSC at 42 °C, essentially according to Sambrook *et al.* (1989) – the hybridization solution, however, additionally contained 10% (w/v) dextran sulphate (Pharmacia LKB) and 0.1% (w/v) sodium pyrophosphate (Merck). Excess probe was removed by two sequential 20 min washing steps at 68 °C in 2 $\times$ SSC/0.1% (w/v) SDS/0.1% (w/v) sodium pyrophosphate. The *A. nidulans* γ -actin gene was used as a probe to quantify the amount of constitutively expressed γ -actin mRNA, which served as a reference. Hybridization with the heterologous actin probe was carried out at 37.1 °C and washing was done at 60 °C, using the same buffers as for the homologous probes.

RESULTS

Primary structure of the *abfA* gene

In a previous paper we reported the isolation of the *A. niger abfA* gene and proved its identity by overexpression of ABF A by *A. niger* and *A. nidulans* transformants in which additional copies of the *abfA* gene were introduced by co-transformation (Flipphi *et al.*, 1993c). Fig. 1 shows the part of plasmid pIM900, which carries the cloned *abfA* gene, from which the nucleotide sequence was determined. In addition, the N-terminal amino acid sequences of purified ABF A and of a peptide derived from ABF A upon CNBr treatment, were determined. The sequence XXLKVXTQGG (10 residues) was found for the N-terminus of ABF A, while for the N-terminus of the CNBr-generated peptide the sequence LQNPGQLQGTAPXLTA (15 residues) was determined. Residues which could not be assigned are indicated as X.

The amino acid sequence data enabled us to deduce the position of the coding region of the *abfA* gene within the determined DNA sequence, starting at position 1 and ending with a TAG stop codon situated at position 2237 (Fig. 2). The structural region contains several introns of which the two most 3'-positioned (introns F and G) were established by sequencing pC1X1, the cDNA clone used to isolate the *abfA* gene (Flipphi *et al.*, 1993c). Sequence analysis of a second cDNA clone (pC2X1 – approximately 1.5 kb cDNA insert), isolated from the same cDNA library from which pC1X1 originates, established introns B, C, D and E (results not shown). Intron A remains unestablished but its border sequences and the putative lariat formation site conform to the generally established consensus sequences proposed for introns of fungal genes (cf. Gurr *et al.*, 1987; Rambosek & Leach, 1987; Unkles, 1992). Moreover, it contains stop codons in all frames and removal of this 52 bp sequence brings the two determined amino acid sequences within the same reading frame (Fig. 2).

The two isolated cDNAs differ with respect to the length of the 3' non-coding sequence of the *abfA* transcript. The

ultimate 3' *abfA*-specific base of pC2X1 was found to correspond to the C at position 2371 (a non-coding sequence of 132 bp) and that of pC1X1 to the T at position 2419 (a non-coding sequence of 180 bp). This strongly suggests the presence of (at least) two sites for polyadenylation. Upon primer extension mapping using L-arabitol-induced polyA⁺ RNA from *A. niger abfA* transformant N593::pIM900-1 as template (results not shown), we could identify a single site for transcription initiation at position –39 relative to the start codon.

Deduced primary structure of ABF A

Removal of the seven intron sequences from the coding region of *abfA* leads to an open reading frame of 1884 bp coding for a protein of 628 amino acids (Fig. 2). Within the deduced sequence of the primary gene product the determined N-terminal sequence of mature extracellular ABF A is preceded by a 25 residue-long sequence which could serve as a signal peptide for secretion. Small neutral amino acid residues are present at positions –3 (alanine) and –1 (glycine) relative to the proposed signal processing site, as predicted using the method described by von Heijne (1986). Mature ABF A consists of 603 amino acids with a deduced molecular mass of 65.4 kDa and a theoretical isoelectric point of 3.7. These characteristics differ from those previously reported for the purified ABF A protein, where we found an apparent molecular mass of 83 kDa and an isoelectric point of 3.3 (van der Veen *et al.*, 1991). This discrepancy is likely to be due to carbohydrate moieties as ABF A is a glycoprotein (Voragen *et al.*, 1987). The amino acid sequence reveals the presence of 10 putative sites for N-linked glycosylation (Fig. 2). Removal of N-linked oligosaccharides from purified ABF A with N-glycanase resulted in a reduction of the apparent molecular mass of over 15 %, as determined upon SDS-PAGE (Fig. 3). The apparent molecular mass of the deglycosylated protein (68 kDa) is only slightly higher than the deduced molecular mass (65.4 kDa). Time course study of the deglycosylation (not shown) suggested that at least three N-linked oligosaccharides are present. One of these is highly resistant to enzymic hydrolysis, which leads to the minor band just above the main band present in lane 4 of Fig. 3.

Comparison of ABF A with other glycosyl hydrolases

The deduced amino acid sequence of ABF A does not show homology with the two other *A. niger* L-arabinan-degrading enzymes, ABF B and ABN A. ABF A and ABN A have one sequence EYGST/WTI/DT/-HT/GST in common, in ABF A present at position 132–143 and in ABN A at position 106–116 (Flipphi *et al.*, 1993b). No similarities were found upon comparison with three prokaryotic enzymes having ABF-like activities, i.e. two β -D-xylosidases exhibiting PNP-A-hydrolysing activity and an α -L-arabinofuranohydrolase only acting on oat spelt xylan (Kellett *et al.*, 1990; Utt *et al.*, 1991; Sakka *et al.*, 1993). Screening the major protein data libraries for similarity, however, resulted in the identification of several short, highly similar amino acid sequences (listed

Fig. 1. Restriction map of the 3958 bp fragment of pIM900 from which the DNA sequence is determined. The sites for *Pst*I, *Xho*I and *Kpn*I (cf. Flipphi *et al.*, 1993c) as well as for *Bgl*II, *Eco*RI and *Bam*HI are indicated. The coding region of the *abfA* gene is represented by a box, the shaded areas mark the introns. Underneath, the orientation and extent of the cDNA inserts from pC1X1 and pC2X1 are indicated. Upon sequence analysis, pC1X1 turned out to be a hybrid clone containing two fused cDNAs (two polyA stretches) of which only the 5' one (approximately 1.0 kb in length) is *abfA*-specific.

Fig. 2. The nucleotide sequence of the *A. niger* *abfA* gene and the deduced amino acid sequence of the gene product ABF A. The nucleotide sequence is numbered relative to the first base of the deduced start codon, the amino acid sequence is numbered relative to the N-terminal isoleucine residue of mature ABF A. The transcription initiation site is

in Table 1) shared by ABF A and two strongly related *Klebsiella* pullulanases (α -dextrin endo-1,6- α -glucosidase; EC 3.2.1.41) (Katsuragi *et al.*, 1987; Kornacker & Pugsley, 1989). Pullulanases belong to glycosyl hydrolase family 13, which contains only α -amylase-like hydrolases and transferases (Henrissat, 1991). Two of the sequences recognized overlap with homologous regions commonly found within α -amylases (Melasniemi *et al.*, 1990; Table 1). However, hydrophobic cluster analysis (cf. Gaboriaud *et al.*, 1987) could not verify the similarities indicated by primary structure alignment.

Study of arabinase expression in wild-type and transformant *A. niger* strains

As the three *A. niger* arabinases are involved in degradation of the same polysaccharide and their synthesis is simultaneously induced upon growth on polymeric substrates and L-arabitol (van der Veen *et al.*, 1991, 1993), regulation of expression of the corresponding genes could well be coordinated. We have studied arabinase expression in *A. niger* wild-type N402 and in transformant strains containing additional copies of either the *abfA* or the *abfB* gene. Several *abfA* and *abfB* transformants covering the available range from low to high copy numbers, as deduced from Southern blot analyses (results not shown), were selected.

The selected strains were grown submerged in liquid medium containing sugar beet pulp as the carbon source and 0.1% (w/v) yeast extract to initiate efficient germination of the inoculum. Medium samples were taken at 24, 36 and 48 h after inoculation, in which both the ABN activity and the PNP-A hydrolysing activity were measured (Table 2). This approach enabled us to monitor the effect of either *abf* gene on the expression of the ABN A-encoding gene (*abnA*) with time. The activity assay for ABN is highly specific, while the PNP-A hydrolysing activity represents the combined activities of ABF A and ABF B. In general, the levels of ABN activity produced by all *abf* transformants are progressively lower with increasing PNP-A hydrolysing activity. However, the reduction of the ABN A expression levels is rather correlated with the *abf* gene dosage than with increasing ABF activity. Transformant N593::pIM991-17 features a reduction intermediate between strains -135 and -121 while these three strains harbour similar amounts of additional *abfB* copies. N593::pIM991-17 is the most moderate overproducer of PNP-A hydrolysing activity, which could be due to abundant presence of non-functional *abfB* copies. This strongly suggests that extra *abf* copies titrate a common transcriptional activator. In

abfA transformants the repression never exceeds 50%, while the ABN A expression level is reduced to 20–40% in the *abfB* multiple copy strains. This indicates that distinct factors are titrated by the respective ABF-encoding genes. Notably, the wild-type ABN level remains constant while in all transformants except N593::pIM900-11, ABN expression increases with time.

To study the effect of additional *abf* gene copies on the expression of the individual ABFs, we investigated arabinase expression more directly at the level of transcription. The experiment was set up essentially as described previously (Flipphi *et al.*, 1993b; van der Veen *et al.*, 1993), performing mycelial transfer of sucrose-grown biomass to minimal medium containing the simple and defined substrate L-arabitol as sole carbon source. In such an experiment expression of all three arabinases is accomplished, and their secretion reaches steady state levels within 6 h of transfer, without the lag phase characteristic for induction on sugar beet pulp upon mycelial transfer (unpublished results). Mycelium was recovered at 1, 3 and 5 h after transfer. Biomass isolated 2 h after transfer to minimal medium containing D-glucose, was used as a control for proper induction. Slot blot analysis of total RNA (10 μ g) revealed the presence of small amounts of *abfA* and *abfB* mRNA in D-glucose transferred mycelium of all strains (data not shown). This suggests low constitutive expression of both ABF encoding genes on D-glucose.

Slot blot analysis of the time course of induction (Fig. 4) clearly shows that L-arabitol induces arabinase expression. The mRNA levels of the various transformant strains correlate with the overexpression of extracellular PNP-A hydrolysing activity (Table 2). The three genes studied respond differently on transfer to medium containing L-arabitol. For *abfA*, the expression levels were high 1 h after transfer and decreased with time. In contrast, *abnA* was hardly expressed after 1 h but the mRNA level sharply rose between 1 and 3 h after transfer. The *abfB* gene was clearly expressed after 1 h of induction and its expression level slowly increased with time. In all transformants, the presence of multiple copies of either *abf* gene led to a decrease of the expression level of *abnA* correlating with increasing gene dosage. This effect was most evident after 1 h but for the *abfB* transformants was also clear at both other time points. These data verify the activity measurements from the sugar beet pulp cultures, although repression of ABN A expression is far more persistent on sugar beet pulp. The slot blots (Fig. 4) suggest that extra *abfB* copies affect ABF A expression, although there is no clear relationship between the reduction of the expression level and the *abfB* gene

marked $\blacklozenge$. The 5' ultimate base of the cDNA insert from pC2X1 (position 486) is marked 5' $\blacktriangleright$; the 3' ultimate bases of the cDNA inserts from pC2X1 and pC1X1 are marked $\blacktriangleleft$ 3'. Putative promoter elements in the upstream non-coding sequence thought to effect transcription initiation are double underlined (TATA boxes/CT stretches). The CT-rich and GA-rich upstream sequences are marked by underlining. Various putative sequence elements are indicated by arrows either above or underneath the sequence, and marked with uppercase letters. A T-rich sequence found in the 3' non-coding sequence is underlined. Determined amino acid sequences are underlined. Putative N-glycosylation sites are indicated by double underlining.

Fig. 3. SDS-PAGE analysis of deglycosylation of ABF A. Deglycosylation of 2 µg ABF A was performed as described in Methods. Loading buffer was applied to stop the reaction and half the volume of each sample (20 µl) was directly applied on a 10% polyacrylamide gel. Lanes: 1, untreated ABF A (1 µg); 2, ABF A incubated in the absence of *N*-glycanase; 3, molecular mass markers (ovalbumin, 45 kDa; bovine serum albumin, 67 kDa; phosphorylase B, 92.5 kDa); 4, ABF A incubated in the presence of *N*-glycanase. The figure shows representative results for one of two independent experiments.

dosage. In case of ABF B in *abfA* transformants, effects similar to those found for ABN A were evident though far less pronounced. Expression of *abfB* was reduced in strains N593::pIM900-11 and -1, mainly at 1 h after transfer.

DISCUSSION

Previously we isolated and characterized two of the genes involved in L-arabinan degradation by *A. niger*, *abfB* and *abnA*. In this paper the structure of a third gene involved, *abfA*, and its gene product ABF A are presented. The deduced amino acid sequences from the three *A. niger* arabinases do not exhibit sequence similarity, although all act on (1 → 5)- α -glycosidic linkages between L-arabinofuranosyl residues. Primary structure alignments indicate that none of the arabinases is related to any of the prokaryotic glycosyl hydrolases known to exhibit PNP-A hydrolysing or L-arabinose releasing activities. It seems likely that the arabinases represent a novel class of glycosyl hydrolase families, reflecting a specific architecture fitting the furanosyl configuration of L-arabinose. Biochemically, a major difference between ABF A and the other two arabinases is that ABF A acts only on small oligomers; its activity was found to decrease progressively with increasing length of (1 → 5)- α -linked oligomers (Rombouts *et al.*, 1988). This coincides with the fact that ABF A is N-glycosylated while ABF B and ABN A are not. N-glycanase does not affect the apparent molecular masses of ABF B and ABN A (results not shown), indicating that these glycoproteins are O-glycosylated. Morosoli *et al.* (1992) suggested that N-glycosylation affects the substrate specificity as well as the kinetic properties of a xylanase from the yeast *Cryptococcus albidus*. Unglycosylated xylanase, produced intracellularly by transformed *E. coli*, exhibited reduced substrate affinity while the substrate specificity was shifted from oligomers towards polymers.

However, N-linked glycosyl moieties in fungal extracellular enzymes are generally considered to be involved in protection against host-specific proteases and in heat stability, rather than in activity (cf. Tanner & Lehle, 1987). One of the putative sites for N-glycosylation (Fig. 2) is located at position 12 of the determined N-terminal sequence of the internal CNBr-generated peptide. Attachment of a glycosyl group to the asparagine on that position could be the reason that we were unable to assign this residue upon Edman degradation of the CNBr-derived peptide. Enzymic deglycosylation not only reduces the apparent molecular mass, it also changes the appearance of ABF A upon SDS-PAGE from a broad diffuse band to a condensed sharp band (Fig. 3), indicating that the fuzzy appearance of purified (glycosylated) ABF A could well be a result of modifications of N-linked sugars.

Analysis of the 5' non-coding sequence of the *abfA* gene (Fig. 2) shows that the transcription initiation site (39 bp upstream of the start codon) is located 6 bases downstream of a 10 bp long CT stretch, an element involved in initiation of transcription of several highly expressed fungal genes (cf. Gurr *et al.*, 1987; Unkles, 1992). Further upstream two AT stretches TTAATTT (−91 to −85 relative to the start codon) and ATAAAA (−69 to −64) are present, located such that these sequences could operate as functional TATA-elements. Upstream of the most distal TATA-like element, *abfA* is highly structured. A CT-rich area of 104 bp (84.6% CT) is preceded by a 46 bp GA-rich area (82.6% GA) as reflected by the presence of a perfect 11 bp long dyad symmetry (denoted R in Fig. 2). The CT-rich area includes a complex 40 bp region devoid of Gs exhibiting extensive internal homology, i.e. there are five perfect 5 bp repeats (TCCAC) present. In Fig. 2 we indicated the internal homology by three 10 bp direct repeats of the sequence CT/ACCTCCACA/T (element A) together with a truncated 6 bp copy TCCACA. The most distal copy of this element partly overlaps the most proximal half of the dyad symmetry element R. Recent studies indicated that all three arabinase genes are subjected to carbon catabolite repression by D-glucose (van der Veen *et al.*, 1993). *abfA* contains several sequence elements (marked C in Fig. 2) perfectly matching the consensus sequence of the *cis*-acting element found in the *A. nidulans alcA* and *alcR* genes (G/CC/TGGGG), involved in D-glucose-mediated carbon catabolite repression of the ethanol utilization regulon by the DNA-binding repressor protein CREA (Kulmburg *et al.*, 1993). Several copies of this element are also present in *abnA* and *abfB* (Fig. 5).

The results indicate that regulation of arabinase synthesis exhibits coordinated features, which could be mediated through common *cis*-acting sequence elements. Comparing the sequences of the arabinase genes, several such elements were found (Fig. 5; see Fig. 2 for *abfA*). Element B (TCCCTGAA) is present as a single copy in each gene, though several truncated and complementary versions are present in all genes. The core of this element (CCCTGA) is found in all pectinolytic *A. niger* genes of which sequences are available (Bussink *et al.*, 1992). Element B

Table 1. Conserved amino acid sequence elements found in ABFA and two *Klebsiella* pullulanases by primary structure alignment

Protein*	Residue no.†	Amino acid sequence‡	Sequence element§
<i>A. niger</i> ABF A	209	DMKGSFLRF	Element c
<i>K. aerogenes</i> PUL	368	<u>DLKGAFYRY</u>	
<i>K. pneumoniae</i> PUL	358	<u>DLKGAFYRY</u>	
<i>A. niger</i> ABF A	270	DLGLVPVLGV	
<i>K. aerogenes</i> PUL	611	DLGMNVIMDV	
	491	IELLPVF DL	
<i>K. pneumoniae</i> PUL	601	DLGMNVIMDV	
	481	VELLPVF DL	
<i>A. niger</i> ABF A	302	IDDLVLNEL	
<i>K. aerogenes</i> PUL	532	VEEVLNQL	
<i>K. pneumoniae</i> PUL	531	VEEVLNQL	
<i>A. niger</i> ABF A	386	SMPEGSWVDYHDY	
<i>K. aerogenes</i> PUL	582	TVPEGSY	Element g
	689	<u>DGFRFDLMGY</u>	
	846	<u>YVSKHD</u>	
<i>K. pneumoniae</i> PUL	572	TVPEGSY	Element j
	679	<u>DGFRFDLMGY</u>	
	836	<u>YVSKHD</u>	
<i>A. niger</i> ABF A	427	RWEIDWP-NLKGS	
<i>K. aerogenes</i> PUL	732	RFEIASQINLKGT	
<i>K. pneumoniae</i> PUL	722	RFEIASQINLKGT	
<i>A. niger</i> ABF A	475	DLIGY	
<i>K. aerogenes</i> PUL	694	DLMGY	
<i>K. pneumoniae</i> PUL	684	DLMGY	
<i>A. niger</i> ABF A	589	TFSLPAWAVAVL	
<i>K. aerogenes</i> PUL	1072	SVTLPAWSVAVL	
<i>K. pneumoniae</i> PUL	1062	TVTLPWSVAVL	

* *K. aerogenes* pullulanase (PUL A) (Katsuragi *et al.*, 1987). *K. pneumoniae* pullulanase (PUL A) (Kornacker & Pugsley, 1990).

† The residue numbers apply to the N-terminal residue of the aligned sequences in the respective proteins.

‡ Identical amino acids are in bold face. Residues conserved amongst glycosyl hydrolases from family 13 are underlined.

§ Sequence elements described by Melasniemi *et al.* (1990).

bears similarity to a cAMP responsive element (UAS_{PDS}) in a yeast heat shock protein HSP70-encoding gene (TT/AAGGGAT), reported to mediate repression in the presence of fermentable carbon sources (Boorstein & Craig, 1990). A single copy of element D (T/CGACA/GTCA) is present in each gene and is highly similar to a cAMP responsive element (TGACGTCA) (CRE/ATF) found in mammalian genes, conferring both activating and repressing effects (e.g. Roesler *et al.*, 1988). Element A (CT/ACCTCCACA/T), the repeated element in the upstream region of *abfA* as discussed above, is found as a single copy in *abnA* and as a truncated copy (CACCTCCATT) in *abfB*. No functional analogues could be found for this element but it is also present in the upstream non-coding region of a number of totally unrelated fungal genes, e.g. amongst others, *Ascobolus*

immersus met2 (homoserine O-transacetylase) (Goyon *et al.*, 1988), *Neurospora crassa grg-1* (glucose repressible gene) (McNally & Free, 1988) and *Aspergillus awamori phy* (phytase) (Mullaney *et al.*, 1991) (data not shown). It seems likely that all the common sequence elements represent more general regulatory features. Whether these elements are actually involved in regulation of arabinase expression is under investigation.

Expression of arabinases in *A. niger* is inducible both by complex substrates containing L-arabinan and by L-arabitol. The two expression experiments described here were conducted under quite different experimental and physiological conditions. The different time scale and detection method chosen are a consequence of the totally distinct nature of the two compounds employed as

Table 2. PNP-A hydrolysing activities and ABN activities produced by *A. niger* wild-type and *abf*-transformed strains upon growth on sugar beet pulp

Transformed strains are ordered with increasing copy numbers as indicated by Southern blot analyses (not shown). Representative values from one of two independent experiments are given.

Time point (h)	Strain	Relative ABN activity*	Relative PNP-A activity†
24	N402	1.00	1.00
	N593::pIM900-4	0.80	1.85
	N593::pIM900-11	0.67	3.00
	N593::pIM900-1	0.53	6.83
	N593::pIM991-18	0.41	4.25
	N593::pIM991-135	0.28	5.15
	N593::pIM991-17	0.29	2.37
	N593::pIM991-121	0.34	7.61
36	N402	1.00	1.00
	N593::pIM900-4	0.87	1.87
	N593::pIM900-11	0.61	3.03
	N593::pIM900-1	0.51	6.43
	N593::pIM991-18	0.42	3.59
	N593::pIM991-135	0.30	4.97
	N593::pIM991-17	0.33	2.14
	N593::pIM991-121	0.35	6.82
48	N402	1.00	1.00
	N593::pIM900-4	0.92	1.88
	N593::pIM900-11	0.69	2.99
	N593::pIM900-1	0.58	6.31
	N593::pIM991-18	0.45	3.50
	N593::pIM991-135	0.33	4.65
	N593::pIM991-17	0.34	2.06
	N593::pIM991-121	0.36	6.35
	N593::pIM991-112	0.22	10.51

* ABN activity was determined in duplicate, as described in Methods assaying 50 µl of culture medium for 10 min. Activities are given relative to the wild-type level at each consecutive time point. The wild-type level produced by N402 is 0.241 Units ml⁻¹ (±0.004) at 24 h, 0.246 Units ml⁻¹ (±0.008) at 36 h and 0.238 Units ml⁻¹ (±0.008) at 48 h.

† PNP-A hydrolysing activity was determined in quadruplicate, as described in Methods assaying 10 µl of culture medium for 10 min. Activities are given relative to the wild-type level at each consecutive time point. The wild-type level produced by N402 is 0.241 Units ml⁻¹ (±0.015) at 24 h, 0.301 Units ml⁻¹ (±0.010) at 36 h and 0.342 Units ml⁻¹ (±0.013) at 48 h.

inducing agents. Nevertheless, we found clear indications that additional copies of either *abf* gene influence the expression of ABN A on sugar beet pulp and on L-arabitol similarly. In both experiments the influence of *abfB* is stronger than of *abfA*. However, even in the case of the highest *abfB* gene dosage (strain N593::pIM991-112), ABN A expression still reaches 20 % of the wild-type level

Fig. 4. Slot blot analysis of L-arabitol-induced arabinase expression by *A. niger* wild-type and *abf* transformed strains. Transformed strains are ordered with increasing copy numbers as indicated by Southern blot analyses (not shown). Each slot is loaded with 1 µg of total RNA. (a) Blot probed with a 2.0 kb *Pst*I fragment from pIM900, containing most of the coding area of the *abfA* gene (cf. Fig. 1). (b) Blot probed with a 2.8 kb *Pst*I fragment from pIM991, containing the complete coding area of the *abfB* gene (Flipphi *et al.*, 1993a). (c) Blot probed with the whole 3.1 kb *Hind*III insert from pIM950, carrying the *abnA* gene (Flipphi *et al.*, 1993b). (d) Blot probed with a 830 bp *Nco*I/*Kpn*I fragment from pSK5, containing mainly coding sequences from the heterologous *A. nidulans* γ -actin gene (Fidel *et al.*, 1988). Slots: 1–8, N402, N593::pIM900-4, –11, –1, N593::pIM991-18, –135, –121, –112 (mycelium isolated 1 h after transfer); 9–16, *ibid.* (isolated 3 h after transfer); 17–24, *ibid.* (isolated 5 h after transfer). These blots show representative results from one out of two independent experiments and should not be used to compare the expression levels of the three arabinase genes, as the specific activity of the probes used was not determined.

on sugar beet pulp (Table 2). The effect of extra *abfB* copies is also more persistent than that of extra *abfA* copies.

Extra copies of either *abf* gene seem to titrate distinct regulatory factors which could contribute to coordination of arabinase gene expression, especially with reference to ABN A. Another feature concerning ABN A expression was noticed previously (Flipphi *et al.*, 1993b). In *A. niger* transformants carrying multiple copies of the *abnA* gene grown on sugar beet pulp, overexpression of ABN A did not exceed a limit of five times that of the wild-type level.

Fig. 5. Schematic overview showing the localization and orientation of common putative sequence elements in the 5' regions (-900 to +300) relative to the translation start codon (ATG) of the *A. niger* arabinase genes (*abfA*, *abfB* and *abnA*). The four different elements (A, B, C, D) are represented by distinct arrows and the approximate distances from the respective translation start codon (ATG) are indicated, as for the major transcription initiation sites of the respective genes. Sequences similar to functional general eukaryotic promoter elements (CAAT box, TATA box and CT stretch) are represented by open boxes. Only those putative CREA sites (C) are given which are found in conjunction with either another CREA site or with any other of the indicated elements.

The PNP-A hydrolysing activity was not affected in these transformants (unpublished results). This limitation does not occur in heterologous *A. nidulans* multiple copy transformants nor in case of the two *abf* genes (Flipphi *et al.*, 1993a, c). Similar behaviour has been described in other fungal systems, e.g. cellobiohydrolase II (*cbh2* gene) of *Trichoderma reesei* and alcohol dehydrogenase I (*alcA* gene) of *A. nidulans* (Kubicek-Pranz *et al.*, 1991; Gwynne *et al.*, 1989). In the latter case this was due to limited expression of ALCR, the pathway-specific activator of the ethanol utilization regulon. In conclusion, the data presented suggest that at least three different activators, one titrated by multiple *abfA* copies, one titrated by multiple *abfB* copies and an *abnA*-specific one, are involved in ABN A expression. Moreover, arabinase expression seems to be co-regulated to some extent. The different behaviour of the three genes in wild-type and multiple copy transformants implies however that expression is basically governed by gene-specific factors, while coordinated control is restricted merely to modulation of the expression level.

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REFERENCES

- Beldman, G., Searle-van Leeuwen, M. J. F., de Ruiter, G. A., Siliha, H. A. & Voragen, A. G. J. (1993). Degradation of arabinans by arabinanases from *Aspergillus aculeatus* and *Aspergillus niger*. *Carbohydr Polym* **20**, 159–168.
- Boorstein, W. R. & Craig, E. A. (1990). Regulation of a yeast HSP70 gene by a cAMP responsive transcriptional control element. *EMBO J* **9**, 2543–2553.
- Bussink, H. J. D., Buxton, F. P., Fraaye, B. A., de Graaff, L. H. & Visser, J. (1992). The polygalacturonases of *Aspergillus niger* are encoded by a family of diverged genes. *Eur J Biochem* **208**, 83–90.
- Calzone, F. J., Britten, R. J. & Davidson, E. H. (1987). Mapping of gene transcripts by nuclease protection assays and cDNA primer extension. *Methods Enzymol* **152**, 611–632.
- Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294–5299.
- Dente, L. & Cortese, R. (1987). pEMBL: a new family of single-stranded plasmids for sequencing DNA. *Methods Enzymol* **155**, 111–119.
- Fidel, S., Doonan, J. H. & Morris, N. R. (1988). *Aspergillus nidulans* contains a single actin gene which has unique intron locations and encodes a γ -actin. *Gene* **70**, 283–293.
- Flipphi, M. J. A., van Heuvel, M., van der Veen, P., Visser, J. & de Graaff, L. H. (1993a). Cloning and characterization of the *abfB* gene coding for the major α -L-arabinofuranosidase (ABF B) of *Aspergillus niger*. *Curr Genet* **24**, 525–532.
- Flipphi, M. J. A., Panneman, P., van der Veen, P., Visser, J. & de Graaff, L. H. (1993b). Molecular cloning, expression and structure of the endo-1,5- α -L-arabinase gene of *Aspergillus niger*. *Appl Microbiol Biotechnol* **40**, 318–326.
- Flipphi, M. J. A., Visser, J., van der Veen, P. & de Graaff, L. H. (1993c). Cloning of the *Aspergillus niger* gene encoding α -L-arabinofuranosidase A. *Appl Microbiol Biotechnol* **39**, 335–340.
- Gaboriaud, C., Bissery, V., Benchetrit, T. & Mornon, J. P. (1987). Hydrophobic cluster analysis: an efficient new way to compare and analyse amino acid sequences. *FEBS Lett* **224**, 149–155.
- Glišin, V., Crkvenjakov, R. & Byus, C. (1974). Ribonucleic acid isolated by cesium chloride centrifugation. *Biochemistry* **13**, 2633–2637.
- Goyon, C., Faugeron, G. & Rossignol, J.-L. (1988). Molecular cloning and characterization of the *met2* gene from *Ascombolus immersus*. *Gene* **63**, 297–308.
- de Graaff, L., van den Broek, H. & Visser, J. (1988). Isolation and expression of the *Aspergillus nidulans* pyruvate kinase gene. *Curr Genet* **13**, 315–321.
- Gurr, S. J., Unkles, S. E. & Kinghorn, J. R. (1987). The structure and organization of nuclear genes of filamentous fungi. In *Gene Structure in Eukaryotic Microbes*. SGM Special Publication. Vol. 23, pp. 93–139. Edited by J. R. Kinghorn. Oxford: IRL Press.
- Gwynne, D. I., Buxton, F. P., Williams, S. A., Sills, A. M., Johnstone, J. A., Buch, J. K., Guo, Z.-M., Drake, D., Westphal, M. & Davies, R. W. (1989). Development of an expression system in *Aspergillus nidulans*. *Biochem Soc Trans* **17**, 338–340.
- von Heijne, G. (1986). A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res* **14**, 4683–4691.
- Henrissat, B. (1991). A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem J* **280**, 309–316.
- Hirani, S., Bernasconi, R. J. & Rasmussen, J. R. (1987). Use of peptide-N-glycosidase F to release asparagine-linked oligosaccharides for structural analysis. *Anal Biochem* **162**, 485–492.

- Katsuragi, N., Takizawa, N. & Murooka, Y. (1987). Entire nucleotide sequence of the pullulanase gene of *Klebsiella aerogenes* W70. *J Bacteriol* **160**, 2301–2306.
- Kellett, L. E., Poole, D. M., Ferreira, L. M. A., Durrant, A. J., Hazlewood, G. P. & Gilbert, H. J. (1990). Xylanase B and an arabinofuranosidase from *Pseudomonas fluorescens* subsp. *cellulosa* contain identical cellulose-binding domains and are encoded by adjacent genes. *Biochem J* **272**, 369–376.
- Kormelink, F. J. M., Gruppen, H. & Voragen, A. G. J. (1993). Mode of action of (1 → 4)- β -D-arabinoxylan arabinofuranohydrolase (AXH) and α -L-arabinofuranosidases on alkali-extractable wheat-flour arabinoxylan. *Carbohydr Res* **249**, 345–353.
- Kornacker, M. G. & Pugsley, A. P. (1989). Molecular characterization of *pulA* and its product, pullulanase, a secreted enzyme of *Klebsiella pneumoniae* UNF5023. *Mol Microbiol* **4**, 73–85.
- Kubicek-Pranz, E. M., Gruber, F. & Kubicek, C. P. (1991). Transformation of *Trichoderma reesei* with the cellobiohydrolase II gene as a means for obtaining strains with increased cellulase production and specific activity. *J Biotechnol* **20**, 83–94.
- Kulmburg, P., Mathieu, M., Dowzer, C., Kelly, J. & Felenbok, B. (1993). Specific binding sites in the *alcR* and *alcA* promoters of the ethanol regulon for the CREA repressor mediating carbon catabolite repression in *Aspergillus nidulans*. *Mol Microbiol* **7**, 847–857.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lerouge, P., O'Neill, M. A., Darvill, A. G. & Albersheim, P. (1993). Structural characterization of endo-glycanase-generated oligoglycosyl side chains of rhamnogalacturonan I. *Carbohydr Res* **243**, 359–371.
- McCleary, B. V. (1991). Comparison of endolytic hydrolases that depolymerize 1,4- β -D-mannan, 1,5- α -L-arabinan and 1,4- β -D-galactan. In *Enzymes in Biomass Conversion*. ACS Symposium Series **460**, pp. 437–449. Edited by G. F. Leatham & M. E. Himmel. Washington DC: American Chemical Society.
- McMaster, G. K. & Carmichael, G. G. (1977). Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc Natl Acad Sci USA* **74**, 4835–4838.
- McNally, M. T. & Free, S. J. (1988). Isolation and characterization of a *Neurospora* glucose-repressible gene. *Curr Genet* **14**, 545–551.
- Melasniemi, H., Paloheimo, M. & Hemiö, L. (1990). Nucleotide sequence of the α -amylase-pullulanase gene from *Clostridium thermohydrosulfuricum*. *J Gen Microbiol* **136**, 447–454.
- Morosoli, R., Durand, S. & Moreau, A. (1992). Cloning and expression in *Escherichia coli* of a xylanase-encoding gene from the yeast *Cryptococcus albidus*. *Gene* **117**, 145–150.
- Mullaney, E. J., Gibson, D. M. & Ullah, A. H. (1991). Positive identification of a λ gt11 clone containing a region of fungal phytase gene by immunoprobe and sequence verification. *Appl Microbiol Biotechnol* **35**, 611–614.
- Nishitani, K. & Nevins, D. J. (1989). Enzymic analysis of feruloylated arabinoxylans (feraxan) derived from *Zea mays* cell walls. II. Fractionation and partial characterization of feraxan fragments dissociated by a *Bacillus subtilis* enzyme (feraxanase). *Plant Physiol* **91**, 242–248.
- Rambosek, J. & Leach, J. (1987). Recombinant DNA in filamentous fungi: progress and prospects. *CRC Crit Rev Biotechnol* **6**, 357–393.
- Roesler, W. J., Vanderbark, G. R. & Hanson, R. W. (1988). Cyclic AMP and the induction of eukaryotic gene transcription. *J Biol Chem* **263**, 9063–9066.
- Rombouts, F. M., Voragen, A. G. J., Searle-van Leeuwen, M. F., Geraerds, C. C. J. M., Schols, H. A. & Pilnik, W. (1988). The arabinanases of *Aspergillus niger* – purification and characterisation of two α -L-arabinofuranosidases and an endo-1,5- α -L-arabinanase. *Carbohydr Polym* **9**, 25–47.
- Sakai, T. & Sakamoto, T. (1990). Purification and some properties of a protopectin-solubilizing enzyme that has potent activity on sugar beet protopectin. *Agric Biol Chem* **54**, 879–889.
- Sakka, K., Yoshikawa, K., Kojima, Y., Karita, S.-I., Ohmiya, K. & Shimada, K. (1993). Nucleotide sequence of the *Clostridium stercorearium xylA* gene encoding a bifunctional protein with β -D-xylosidase and α -L-arabinofuranosidase activities, and properties of the translated product. *Biosci Biotech Biochem* **57**, 268–272.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Tanner, W. & Lehle, L. (1987). Protein glycosylation in yeast. *Biochim Biophys Acta* **906**, 81–99.
- Tarentino, A. L. & Plummer, T. H., Jr (1987). Peptide-N-(N-acetyl- β -glucosamidyl) asparagine amidase and endo- β -N-acetylglucosaminidase from *Flavobacterium meningosepticum*. *Methods Enzymol* **138**, 770–778.
- Unkles, S. E. (1992). Gene organization in industrial filamentous fungi. In *Applied Molecular Genetics of Filamentous Fungi*, 1st edn, pp. 28–53. Edited by J. R. Kinghorn & G. Turner. London: Blackie Academic & Professional.
- Utt, E. A., Eddy, C. K., Keshav, K. F. & Ingram, L. O. (1991). Sequencing and expression of the *Butyrivibrio fibrisolvens xylB* gene encoding a novel bifunctional protein with β -D-xylosidase and α -L-arabinofuranosidase activities. *Appl Environ Microbiol* **57**, 1227–1234.
- van der Veen, P., Flipphi, M. J. A., Voragen, A. G. J. & Visser, J. (1991). Induction, purification and characterisation of arabinases produced by *Aspergillus niger*. *Arch Microbiol* **157**, 23–28.
- van der Veen, P., Flipphi, M. J. A., Voragen, A. G. J. & Visser, J. (1993). Induction of extracellular arabinases on monomeric substrates in *Aspergillus niger*. *Arch Microbiol* **159**, 66–71.
- van de Vis, J. W., Searle-van Leeuwen, M. F., Siliha, H. A., Kormelink, F. J. M. & Voragen, A. G. J. (1991). Purification and characterization of endo-1,4- β -D-galactanases from *Aspergillus niger* and *Aspergillus aculeatus*: use in combination with arabinanases from *Aspergillus niger* in enzymatic conversion of potato arabinogalactan. *Carbohydr Polym* **16**, 167–187.
- Voragen, A. G. J., Rombouts, F. M., Searle-van Leeuwen, M. F., Schols, H. A. & Pilnik, W. (1987). The degradation of arabinans by endo-arabinanase and arabinofuranosidases purified from *Aspergillus niger*. *Food Hydrocoll* **1**, 423–437.
- Whitaker, J. R. (1984). Pectic substances, pectic enzymes and haze formation in fruit juices. *Enzyme Microb Technol* **6**, 425–432.
- Will, F. & Dietrich, H. (1992). Isolation, purification and characterization of neutral polysaccharides from extracted apple juices. *Carbohydr Polym* **18**, 109–117.
- Witteveen, C. F. B., Busink, R., van de Vondervoort, P., Dijkema, C., Swart, K. & Visser, J. (1989). L-Arabinose and D-xylose catabolism in *Aspergillus niger*. *J Gen Microbiol* **135**, 2163–2171.

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