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Fungal α-arabinofuranosidases of glycosyl hydrolase families 51 and 54 show a dual arabinofuranosyl- and galactofuranosyl-hydrolyzing activity

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Abstract

Aspergillus niger possesses a galactofuranosidase activity, however, the corresponding enzyme or gene encoding this enzyme has never been identified. As evidence is mounting that enzymes exist with affinity for both arabinofuranose and galactofuranose, we investigated the possibility that α-L-arabinofuranosidases, encoded by the *abfA* and *abfB* genes, are responsible for the galactofuranosidase activity of *A. niger*. Characterization of the recombinant AbfA and AbfB proteins revealed that both enzymes do not only hydrolyze *p*-nitrophenyl-α-L-arabinofuranoside (pNp-α-Ara*f*) but are also capable of hydrolyzing *p*-nitrophenyl-β-D-galactofuranoside (pNp-β-Gal*f*). Molecular modeling of the AbfB protein with pNp-β-Gal*f* confirmed the possibility for AbfB to interact with this substrate, similarly as with pNp-α-Ara*f*. We also show that galactomannan, a cell wall compound of *A. niger*, containing β-linked terminal and internal galactofuranosyl moieties, can be degraded by an enzyme activity that is present in the supernatant of inulin-grown *A. niger*. Interestingly, purified AbfA and AbfB did not show this hydrolyzing activity toward *A. niger* galactomannan. In summary, our studies demonstrate that AbfA and AbfB, α-L-arabinofuranosidases from different families, both contain a galactofuranose (Gal*f*)-hydrolyzing activity. In addition, our data support the presence of a Gal*f*-hydrolase activity expressed by *A. niger* that is capable of degrading fungal galactomannan.

Keywords: AbfA; AbfB; *Aspergillus niger*; galactofuranose; galactofuranosidase; glycosyl hydrolase.

Introduction

Fungi secrete various enzymes to degrade complex molecules in their surroundings in order to provide them with the essential building blocks of life. *Aspergillus* spp. are saprophytic and secrete many different glycosyl hydrolases (GHs), which catalyze the breakdown of polysaccharides. The availability of genome sequences for aspergilli has allowed a detailed mining for GHs (Martens-Uzunova et al., 2006; Pel et al., 2007; Coutinho et al., 2009). For some particular GH activities, however, the corresponding gene and protein have never been identified. An example of such an unattributed activity is the enzymatic degradation of Gal*f*-containing glycoconjugates. This activity has been described for *Aspergillus niger* (Cousin et al., 1989; Wallis et al., 2001), for several other filamentous fungi (Daley and Strobel, 1983; Cousin et al., 1989; Reyes et al., 1992; Van Bruggen-Van Der Lugt et al., 1992), and for a prokaryote, *Bacillus* sp. (Ramli et al., 1995). No protein or gene responsible for this activity has been identified in any of these cases.

It was recently shown that thiodisaccharides with either Gal*f* or arabinofuranose (Ara*f*) as terminal units inhibit the activity of an exo-galactofuranosidase from *Penicillium fellutanum*, a close relative of *Aspergillus* (Repetto et al., 2009). In addition, an α-L-arabinofuranosidase (AbfD3) was found in *Thermobacillus xylanilyticus*, which displays transglycosylation activity and is able to use pNp-β-Gal*f* as the sugar donor (Remond et al., 2005). These data demonstrate that enzymes exist with affinity for both Ara*f* and Gal*f* and suggest the possibility that one or more arabinofuranosidases in *A. niger* play a role in the degradation or synthesis of Gal*f*-containing glycoconjugates. The filamentous fungus *A. niger* secretes two α-L-arabinofuranosidases, AbfA and AbfB (Flipphi et al., 1993a,b). It has been described that AbfA, belonging to GH family 51, hydrolyzes terminal α-linked Ara*f* from small substrates, such as pNp-α-Ara*f* and short-chain arabino-oligosaccharides. AbfB, belonging to GH family 54, has a similar activity on these substrates but is also able to hydrolyze polymeric substrates such as branched arabinan and arabinoxylan (Rombouts et al., 1988).

Here, we describe the cloning of the *abfA* and *abfB* genes of *A. niger* in *Pichia pastoris* and prove the capacity of the

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recombinant AbfA and AbfB proteins to hydrolyze pNp-β-Gal*f* in addition to pNp-α-Ara*f*. Molecular modeling of AbfB, using the crystal structure of AbfB from *A. kawachii* (Miyanaga et al., 2004), supports that both the pNp-β-Gal*f* and the pNp-α-Ara*f* substrates may fit within the active site of the enzyme. However, purified *P. pastoris* expressed AbfA and AbfB do not have the capacity to release Gal*f* from the biologically relevant polysaccharide galactomannan. Finally, we demonstrate that supernatant from both wild-type *A. niger* and Δ*abfA* mutant grown on inulin has a hydrolyzing activity that releases Gal*f* from the biologically relevant polysaccharide galactomannan, suggesting the presence of a dedicated β-galactofuranosidase expressed by *A. niger* N402 that has yet to be identified.

Results

α-Arabinofuranosidases from GH families 51 and 54 possess both α-arabinofuranosidase and β-galactofuranosidase activity

To investigate a putative Gal*f*-hydrolyzing activity of the α-arabinofuranosidases AbfA and AbfB, cDNAs coding for the *A. niger* genes *abfA* and *abfb* were amplified and cloned into pGAPZαB and expressed in *P. pastoris*. This resulted in the secretion of His-tagged AbfA and AbfB into the growth medium, from which the recombinant proteins were subsequently isolated and purified (Supplementary Figure S1). AbfA and AbfB were analyzed for their glycosyl-hydrolyzing capacity using a panel of pNp-sugar substrates. Two substrates from this panel were hydrolyzed by both enzymes, pNp-α-Ara*f* and pNp-β-Gal*f* (Figure 1), indicating a dual hydrolyzing activity of AbfA and AbfB. We did not find any hydrolysis

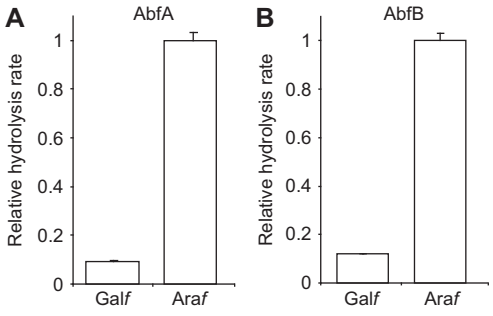

Figure 1 GH activity of AbfA and AbfB toward different pNp substrates.
(A) pNp-β-Gal*f* (Gal*f*) and pNp-α-Ara*f* (Ara*f*) (2 mM) were incubated for 3 h with 1 μg purified AbfA. Liberation of freed pNp molecules was measured at OD 405 nm. The average values of three samples were normalized for hydrolysis of pNp-α-Ara*f* (0.3 nmol/μg/min) and depicted as relative hydrolysis rate. Error bars depict normalized standard deviations. (B) pNp-β-Gal*f* (Gal*f*) and pNp-α-Ara*f* (Ara*f*) (2 mM) were incubated for 1 h with 0.1 μg purified AbfB. Liberation of free pNp molecules was measured at OD 405 nm. The average values of three samples were normalized for hydrolysis of pNp-α-Ara*f* (9.2 nmol/μg/min) and depicted as relative hydrolysis rate. Error bars depict normalized standard deviations.

of pNp-β-D-xyloside, pNp-*N*-acetyl-β-D-glucosaminide, pNp-β-L-arabinopyranoside, pNp-β-D-galactopyranoside, pNp-α-D-galactopyranoside, pNp-α-D-mannoside, pNp-β-D-mannoside, pNp-α-D-glucoside, pNp-β-D-glucoside, pNp-α-L-fucoside, or pNp-α-D-*N*-acetylneuraminic acid by these enzymes (data not shown). These data showed that α-arabinofuranosidases from different GH families possess a distinctive β-Gal*f*-hydrolyzing activity. To exclude any possible effects of differential glycosylation on the activity of these hydrolases, we also expressed a His-tagged AbfA in *A. niger*. This protein displayed similar GH activity as the His-tagged AbfA expressed in *P. pastoris* (data not shown).

Characterization of the β-galactofuranosidase activity of AbfA and AbfB

A comparative kinetic study was performed to characterize both the α-arabinofuranosidase and the α-galactofuranosidase activity of AbfA and AbfB. Michaelis-Menten diagrams can be found in Supplementary Figure S2. The K_m of the activity toward pNp-α-Ara*f* was an order of magnitude lower (23-fold for AbfA and 26-fold for AbfB) than the activity toward pNp-β-Gal*f* for both enzymes (Table 1), whereas the V_{max} was higher for pNp-α-Ara*f* than for pNp-β-Gal*f* for both enzymes. Thus, both enzymes display a higher hydrolyzing activity toward pNp-α-Ara*f* than to pNp-β-Gal*f*. AbfB seems to have a lower affinity (higher K_m) but a higher efficiency (higher V_{max}) for both substrates compared with AbfA (Table 1). To characterize both enzymes in more detail, the influence of pH (Figure 2) and temperature (Figure 3) on their activity toward both substrates was examined. The optimum for either glycosyl-hydrolyzing activity of both enzymes appears to be slightly above pH 3. At increasing temperatures, a higher activity of AbfA toward pNp-β-Gal*f* was measured, whereas the hydrolyzing activity toward pNp-α-Ara*f* was quite stable across a wide temperature range (Figure 3A). In contrast to AbfA, the β-galactofuranosidase activity of AbfB did not decrease at lower temperatures. Both the α-arabinofuranosidase and the β-galactofuranosidase activities of AbfB were optimal between 30°C and 35°C (Figure 3B).

3D modeling of AbfB with its proposed substrates

The availability of the crystal structure of the *A. kawachii* AbfB protein (Miyanaga et al., 2004) allowed the modeling of both the pNp-α-Ara*f* and pNp-β-Gal*f* into the corresponding substrate binding site. As both the N- and C-terminus (blue) are far away from the catalytic pocket or the glycosylation site (light blue), the addition of the tag is not expected to affect the catalytic domain or the glycosylation of the AbfB protein (Figure 4A). The *A. niger* AbfB protein is 97% identical to the amino acid sequence of the *A. kawachii* AbfB protein and the catalytic residues involved in catalysis and substrate binding residues (Cys176, Cys177, Met195, Trp206, Asp219, Glu221, Asn222, Leu224, and Asp297) are conserved. In *A. kawachii* AbfB, the carboxyl groups of Glu221 and Asp297 are located on either site of

Table 1 Apparent kinetic parameters for GH activities of AbfA and AbfB.

Substrate	K_m (mM)		V_{max} (nmol/μg/min)	
	AbfA	AbfB	AbfA	AbfB
pNp-α-Ara*f*	0.7	1.1	1.0	64
pNp-β-Gal*f*	17	28	0.3	42

The apparent kinetic parameters K_m and V_{max} were obtained by fitting the data for hydrolysis of these substrates at variable concentrations to the Michaelis-Menten equation using nonlinear regression analysis (SPSS 15.0 program; SPSS).

the anomeric C1 of α-L-Ara*f* indicating that they form the catalytic subunit (Miyanaga et al., 2004). For the α-L-Ara*f* substrate, three H-bonds are postulated, which is confirmed by our model as the co-crystallized substrate displays three H-bonds of 2.88, 2.92, and 3.14 Å length (Figure 4B). Both substrates that are hydrolyzed by AbfB, pNp-α-Ara*f* and pNp-β-Gal*f*, were fitted into the modeled *A. niger* AbfB protein structure. As shown in Figure 4C (pNp-α-Ara*f*) and Figure 4D (pNp-β-Gal*f*), both substrates fit into the pocket at the catalytic site. The residues Asp297 and Glu221 are located on either side of the anomeric C1 of both substrates, indicating they are most likely responsible for catalysis, whereas Gly296 and Asn223 seem mainly needed for substrate coordination. These pNp substrates form two H-bonds each in the manual docking, with lengths of the H-bonds being 2.59 and 2.80 Å for pNp-Gal*f* and 2.49 and 2.51 Å for pNp-Ara*f*. As both substrates fit into the substrate pocket in proximity to the catalytic residues, the modeling supports the biochemical data that AbfB has hydrolyzing activity toward both pNp-α-Ara*f* and pNp-β-Gal*f*.

Glycosyl-hydrolyzing activity of *A. niger* strains grown on glucose and inulin

A. niger has been shown previously to secrete a β-galactofuranosidase when grown on inulin (Wallis et al., 2001). To investigate whether AbfA and/or AbfB may be responsible for the hydrolysis of pNp-β-Gal*f* under these conditions, the hydrolyzing activity toward different substrates of *A. niger* strain N402, grown on glucose and inulin, respectively, was determined. Culture filtrates were collected and subjected to GH assays using a panel of substrates. *A. niger* grown on inulin showed a 2.8-fold higher hydrolyzing activity toward pNp-β-Gal*f* than toward pNp-α-Ara*f*, whereas by contrast, growth on glucose resulted in a 4.7-fold higher hydrolyzing activity toward pNp-α-Ara*f* than to pNp-β-Gal*f* (Figure 5). If AbfA or AbfB would be responsible for the Gal*f*-hydrolyzing activity of *A. niger* grown under these conditions, we would expect to see a similar rise in capacity to hydrolyze pNp-α-Ara*f* compared with activity toward pNp-β-Gal*f*, resulting in a ratio of both activities comparable to the results shown for the activity of AbfA and AbfB in Figure 1. Therefore, these results suggest that *A. niger* expresses another Gal*f*-hydrolyzing activity, in addition to AbfA or AbfB. Interestingly, although outside the scope of this study, we observed a huge increase in hydrolysis activity toward pNp-β-glucose and pNp-β-*N*-acetylglucosamine when *A. niger* was grown on inulin (Figure 5).

To provide further evidence for the possibility that AbfA is not responsible for the β-galactofuranosidase activity, an *abfA* deletion strain (Δ*abfA*) was constructed and verified by Southern blot analysis (Supplementary Figure S3). The Δ*abfA* mutant showed a phenotype similar to wild-type strain N402 when grown on glucose, inulin, maltose, or xylose (data not shown). The culture filtrate of the Δ*abfA* mutant grown on inulin was tested for hydrolysis of pNp-α-Ara*f* and pNp-β-Gal*f*. The Δ*abfA* mutant displayed a 5.3-fold higher activity toward pNp-β-Gal*f*

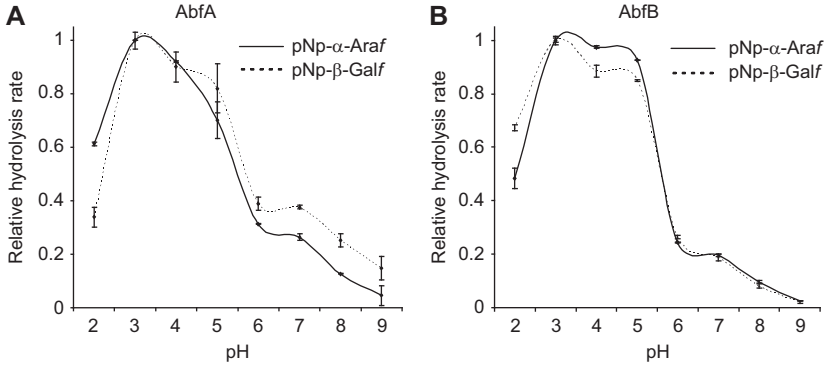

Figure 2 pH dependence of AbfA- and AbfB-hydrolyzing activities on pNp-α-Ara*f* and pNp-β-Gal*f*.
(A) Purified AbfA was incubated in duplicate with 0.5 mM pNp-α-Ara*f* (solid lines; 0.5 μg enzyme) and pNp-β-Gal*f* (dotted lines; 0.5 μg enzyme) at 37°C at the indicated pH. (B) Purified AbfB was incubated in duplicate with 0.5 mM pNp-α-Ara*f* (solid lines; 10 ng enzyme) and pNp-β-Gal*f* (dotted lines; 1 μg enzyme) at 37°C at the indicated pH. Absolute hydrolysis of both substrates was calculated from liberated pNp molecules measured at OD405. These data were normalized against the highest measured value for each substrate (for AbfA, 0.6 nmol pNp-α-Ara*f*/μg/min and 9 pmol pNp-β-Gal*f*/μg/min; for AbfB, 9.7 nmol pNp-α-Ara*f*/μg/min and 3.3 nmol pNp-β-Gal*f*/μg/min) and depicted as relative hydrolysis rate with error bars representing normalized SEM.

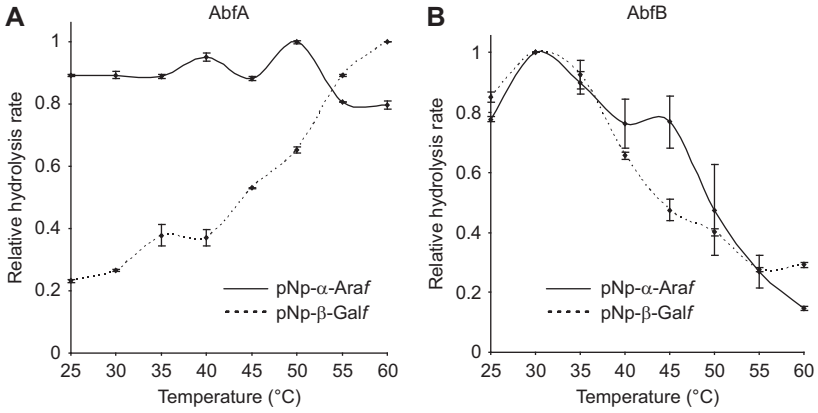

Figure 3 Temperature dependence of AbfA- and AbfB-hydrolyzing activities on pNp-α-Ara*f* and pNp-β-Gal*f*.
(A) Purified AbfA was incubated in duplicate with 0.5 mM pNp-α-Ara*f* (solid lines, 150 ng enzyme) and pNp-β-Gal*f* (dotted lines, 1.5 µg enzyme) at pH 4.5 at the indicated temperatures. (B) Purified AbfB was incubated in duplicate with 0.5 mM pNp-α-Ara*f* (solid lines, 8 ng enzyme) and pNp-β-Gal*f* (dotted lines, 40 ng enzyme) at pH 4.5 at the indicated temperatures. Absolute hydrolysis of both substrates was calculated from liberated pNp molecules measured at OD405. The average values were normalized against the highest measured value for each substrate (for AbfA, 0.2 nmol pNp-α-Ara*f*/µg/min and 20 pmol pNp-β-Gal*f*/µg/min; for AbfB, 6.4 nmol pNp-α-Ara*f*/µg/min and 0.5 nmol pNp-β-Gal*f*/µg/min) and depicted as relative hydrolysis rate. Error bars indicate normalized SEM.

than to pNp-α-Ara*f* (data not shown), indicating that AbfA is not essential for galactofuranosidase activity in *Aspergillus*.

Galactomannan is hydrolyzed by supernatant of inulin-grown *A. niger*, but not by AbfA or AbfB

We next tested the ability of recombinant AbfA and AbfB to hydrolyze Gal*f* moieties in galactomannan, a polysaccharide present in the cell wall of *A. niger*. Galactomannan was isolated from wild-type strain N402 and incubated with medium of inulin-grown N402, purified AbfA, or purified AbfB. Resulting samples were subsequently analyzed for remaining galactomannan in a sandwich ELISA using the anti-galactofuranose antibody EB-A2. Both AbfA and AbfB were unable to hydrolyze galactomannan (Figure 6A). In contrast, enzymes in the medium of inulin-grown *A. niger* were able to hydrolyze Gal*f* residues from galactomannan. The breakdown of galactomannan by supernatant of *A. niger* was greatly diminished when it was heat-inactivated prior to incubation with galactomannan, indicating that the enzymatic activity was destroyed by this

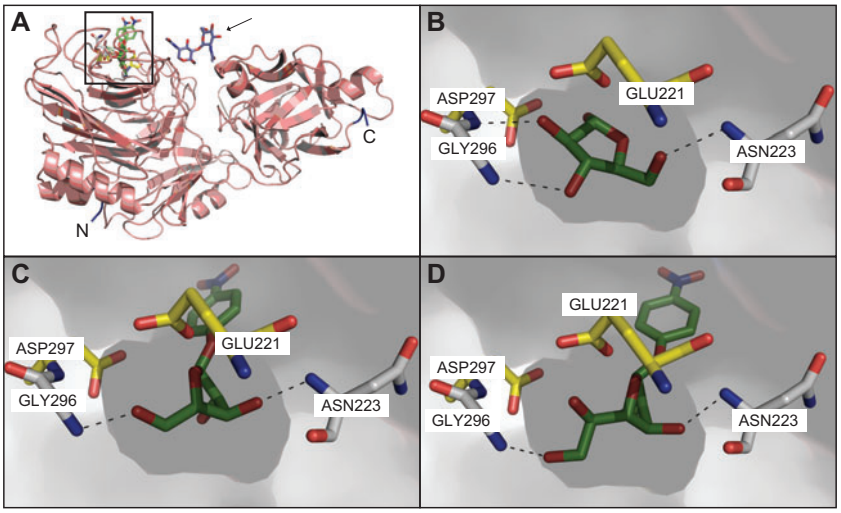

Figure 4 Manual 3D modeling of *A. niger* AbfB protein with three substrates.
A 3D model of AbfB was constructed with three manual substrate dockings. (A) Overview of the complete structure of AbfB. Two *N*-acetylglucosamines shown to be present on ASN202 are indicated by an arrow and the N- and C-termini are indicated by an N and C, respectively. The boxed area left of the glycosylation is the catalytic site that is shown in more detail in the other three panels. (B) A docking model of the catalytic site with the substrate α-L-Ara*f*. (C) A docking model of the catalytic site with the substrate pNp-α-L-Ara*f*. (D) A docking model of the catalytic site with the substrate pNp-β-D-Gal*f*. The amino acids in the catalytic site are indicated. ASP297 and GLU221 are important for catalysis, whereas GLY296 and ASN223 are important for substrate coordination. The substrates are depicted in the middle of each panel with the pNp-moiety sticking out to the back, when present. The proposed H-bonds are represented by dotted lines.

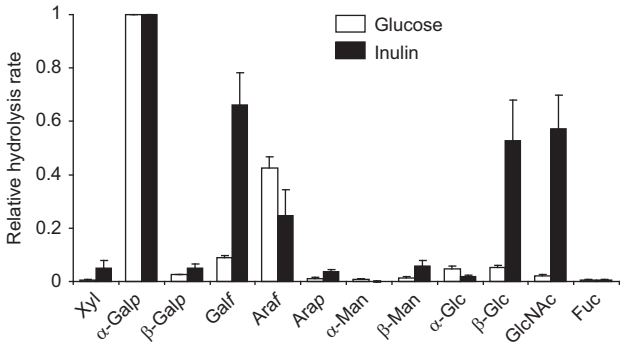

Figure 5 Galactofuranosidase activity increases when *A. niger* is grown on inulin.
The indicated pNp substrates (0.5 mM) were incubated in triplicate for 2 h at 37°C with 5 µl supernatant collected from wild-type *A. niger* strain N402 grown on glucose (white bars) or inulin (black bars). Liberated pNp molecules were measured at OD 405 nm. Values were normalized against the highest measured value (1.3 µmol pNp-α-Gal*p*/ml/h for glucose-grown and 2.2 µmol pNp-α-Gal*p*/ml/h for inulin-grown) and depicted as relative hydrolysis rate. Error bars depict standard deviation. Xyl=pNp-β-D-xyloside; α-Gal*p*=pNp-α-D-galactopyranoside; β-Gal*p*=pNp-β-D-galactopyranoside; Gal*f*=pNp-β-D-galactofuranoside; Ara*f*=pNp-α-L-arabinofuranoside; Ara*p*=pNp-β-L-arabinopyranoside; α-Man=pNp-α-D-mannoside; β-Man=pNp-β-D-mannoside; α-Glc=pNp-α-D-glucoside; β-Glc=pNp-β-D-glucoside; Fuc=pNp-α-L-fucoside; GlcNAc=pNp-*N*-acetyl-β-D-glucosaminide.

treatment (Figure 6B). Supernatant of an inulin-grown Δ*abfA* mutant was also tested for its capacity to hydrolyze galactomannan. Clearly, the galactomannan is also hydrolyzed by an activity within this supernatant (Figure 6B), indicating that other enzymes than AbfA are responsible for this activity in *A. niger*. To our knowledge, this is the first report of enzymatic breakdown of *A. niger* galactomannan by its own enzymes.

Discussion

In this study, we prove for the first time that fungal α-arabinofuranosidases belonging to GH51 (AbfA) and GH54 (AbfB) also have galactofuranosidase activity. Although the α-arabinofuranosidase activity of AbfA has been described and characterized in several *Aspergillus* spp. including *A. niger*, *A. oryzae*, *A. kawachii*, and *A. awamori* (Rombouts et al., 1988; Flipphi et al., 1993a,b; Koseki et al., 2003; Matsumura et al., 2004), the pNp-β-Gal*f* substrate was never included as a potential substrate. The recent observation that the α-arabinofuranosidase from *P. fellutanum* was inhibited by thiodisaccharides with either Gal*f* or Ara*f* as terminal units (Repetto et al., 2009) suggested that the enzyme has affinity for both Gal*f* and Ara*f* residues. Representative members of GH51 and GH54, *A. niger* AbfA and *A. niger* AbfB, respectively, were expressed in *P. pastoris*, which does not secrete GHs with activities that could interfere with these studies, and their enzyme activity was determined. For all these proteins, we detected a dual activity on pNp-β-Gal*f* besides pNp-α-Ara*f*. The enzymes were only active against the furanose form of the sugars and not active against the pyranose forms in agreement with earlier studies (Rombouts et al., 1988; Flipphi et al., 1993a,b; Koseki et al., 2003; Matsumura et al., 2004). In our analysis, we consistently found that that the activity of the enzymes toward pNp-α-Ara*f* was higher compared with the activity toward pNp-β-Gal*f*. Interestingly, we found that AbfA had different temperature optima for both substrates and a different shape of the temperature curve. We do not have an explanation for this phenomenon. Earlier studies on α-L-arabinofuranosidases showed temperature optima between 50°C and 60°C (Rombouts et al., 1988; Kaneko et al., 1998; De Ioannes et al., 2000; Matsuo et al., 2000; Koseki et al., 2003), but we could not find any reports on different tempera-

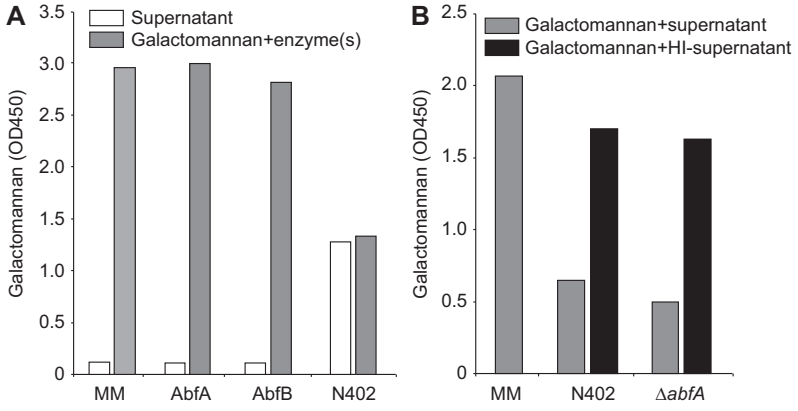

Figure 6 Galactomannan is hydrolyzed by supernatant of inulin-grown *A. niger*, but not by AbfA or AbfB.
(A) Minimal medium (MM), purified AbfA (1.1 µg), purified AbfB (2.1 µg), or 10.5 µl supernatant of inulin-grown wild-type *A. niger* strain N402 was incubated with 7.5 ng (gray bars) or without (white bars) galactomannan for 20 h at 37°C at pH 4.5. (B) Supernatant of inulin-grown N402 and Δ*AbfA* mutant was diluted 1:20 and heat-inactivated (HI-supernatant, black bars) or not (gray bars). Subsequently, 20 µl of these treated supernatants were incubated with 5 ng galactomannan for 24 h at 37°C at pH 4.5. Samples were analyzed for remaining galactomannan after incubation in an ELISA sandwich using antibody EB-A2 at an OD of 450 nm. These are typical experiments that have been performed at least three times independently.

ture optima for different substrates of the same enzyme, like we see for AbfA.

The availability of a crystal structure of the AbfB protein from *A. kawacchi*, allowed the 3D modeling of the *A. niger* AbfB protein with both the pNp-α-Ara*f* and the pNp-β-Gal*f* as substrates. The modeling strongly supports the possibility that both substrates fit in a similar way in the catalytic binding pocket of the enzyme, allowing a similar mechanism of hydrolysis of the substrate. Interestingly, pNp-Gal*f* seems to fit less well into the substrate cavity than pNp-Ara*f*, causing steric limitations that might cause less efficient H-bonding that is required for the substrate coordination in the catalytic action.

To investigate whether α-arabinofuranosidases could be responsible for the β-galactofuranosidase activity observed by Wallis et al. (2001), we cultured *A. niger* strain N402 in growth medium with inulin as a carbon source, and we could confirm the presence of a high β-galactofuranosidase activity. Interestingly, the relative hydrolysis activity of the culture filtrate was higher toward pNp-β-Gal*f* than to pNp-α-Ara*f* (Figure 5), in contrast to the ratio shown for purified AbfA and AbfB. We observed a shift in the ratio between the hydrolyzing activity toward pNp-β-Gal*f* and the activity toward pNp-α-Ara*f* in the medium of *A. niger* grown on inulin when compared with *A. niger* grown on glucose (Figure 5). This indicates that another enzyme with a preference for pNp-Gal*f* likely exists. To prove that the AbfA protein is not responsible for the β-galactofuranosidase activity in the culture filtrate of inulin-grown cells, we deleted the *abfA* gene by constructing a knockout strain. The knockout strain showed a similar β-galactofuranosidase activity as the wild-type strain, indicating that AbfA is not essential for the observed β-galactofuranosidase activity in *A. niger*.

In addition, we showed that the culture filtrate of inulin-grown N402 cells contains an enzyme activity that can degrade cell wall galactomannan (Figure 6). As can be concluded from Figure 5, the culture filtrate contained no activity against pNp-α-mannose but a high activity against pNp-β-Gal*f*, suggesting that the breakdown of the galactomannan is due to the presence of a β-galactofuranosidase. Interestingly, recombinant AbfA and AbfB did not show β-galactofuranosidase activity toward galactomannan isolated from the cell wall of *A. niger*, indicating that both AbfA and AbfB are not responsible for this specific β-galactofuranosidase activity. However, we cannot exclude that these enzymes are capable of degrading Gal*f*-containing lipids or proteins, which contain other glycosidic linkages (Tefsen et al., 2011).

To attribute a putative biological role for the dedicated Gal*f*-hydrolase is challenging. On the one hand, Gal*f* has never been found in plant polysaccharides, which argues against the possibility that these organisms need such a Gal*f*-hydrolase to provide them with Gal*f* residues destined as an energy source from plant debris. Ara*f* residues, on the other hand, are abundantly present in the plant polysaccharides hemicellulose and pectin. The genes encoding AbfA and AbfB are strongly induced by hemicellulose and pectin, suggesting that the enzymes are primarily involved in the hydrolysis of Ara*f* residues present in plant cell walls (Martens-Uzunova et al., 2006). The increase of β-galactofuranosidase activity upon carbon starvation has led others to suggest that the β-galactofuranosidase is involved in the recycling of fungal cell wall material during starvation (Mennink-Kersten et al., 2006) as Gal*f* residues are present in the galactomannan fractions and on glycoproteins present in the cell wall (Tefsen et al., 2011). Genome-wide expression analysis in *A. niger* maltose-starved mycelium, a condition that has been reported to induce β-galactofuranosidase activity (Wallis et al., 2001), did not result in the induction of *abfA* and *abfB* (B. Nitsche and A. Ram, unpublished results). This lack of induction of *abfA* and *abfB* during starvation supports our conclusions that these enzymes are not responsible for the β-galactofuranosidase activity displayed by *A. niger*.

The genome of *A. niger* contains three other genes encoding arabinofuranosidases, AbfC (An08g01710), AbfD (An09g00880), AbfE (Martens-Uzunova and Schaap, 2009), and an arabinoxylan arabinofuranohydrolase, AxhA (An03g00960) (Gielkens et al., 1997) that is also able to hydrolyze Ara*f* residues. Similar to AbfA and AbfB, the genes encoding AbfC, AbfD, and AxhA are not induced upon starvation. Expression data are not available for the *abfE* gene, as this gene is not present on the Affymetrix microarray (Martens-Uzunova and Schaap, 2009), but our preliminary data in which the *A. niger* AbfE protein is expressed in *P. pastoris* indicated that AbfE also has a higher activity toward pNp-α-Ara*f* compared with pNp-β-Gal*f*. Altogether, this suggests that the β-galactofuranosidase activity in starved cultures is not encoded by the known members of the *abf* gene families.

In summary, in this study, we demonstrated that AbfA and AbfB, α-L-arabinofuranosidases from GH families 51 and 54, both contain a Gal*f*-hydrolyzing activity and we have characterized these activities. Molecular modeling supported the experimental data. Furthermore, we demonstrated the presence of an enzyme activity expressed by *A. niger* that is capable of degrading fungal galactomannan.

Materials and methods

Strains and media

E. coli DH5α was grown in Luria broth and was used as cloning host. *Pichia pastoris* X33 (Invitrogen) was grown in yeast extract/peptone/dextrose (YPD) medium. Ampicillin (10 μg/ml) and Zeocine (20 and 100 μg/ml) were added when necessary. *A. niger* strains N402 and Δ*abfA* were grown for 7 days at 28°C on minimal *Aspergillus* medium (MAM), while shaking at 180 rpm (Bennett and Lasure, 1991) containing 1% inulin as a carbon source. The *abfA* deletion strain was constructed in MA169.4, a *ku70*, *pyrG* derivative of N402 (Carvalho et al., 2010).

Cloning of AbfA and AbfB

The *abfA* and *abfB* coding sequences were amplified from a cDNA library of *A. niger* constructed from RNA obtained after grown on xylose (kind gift from P.A. Vankuyk) using Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes, Woburn, MA, USA), and

Table 2 Primers used in this study.

Primer name	Sequence (5′–3′)[a]
5-Flank forward[b]	ggg aca act ttg tat aga aaa gtt gTC TTT TGA GAA GAC TGA AAT
5-Flank reverse[b]	ggg gac tgc ttt ttt gta caa act tgG TTG CCG GCT GTC TGG AGG A
3-Flank forward[c]	ggg gac agc ttt ctt gta caa agt ggC GTT GAT TGG GGC GAG CTC G
3-Flank reverse[c]	ggg gac aac ttt gta taa taa agt tgG GCC GGG TGG GGG TAT TTC T
HygroB forward[d]	ggg gac aag ttt gta caa aaa agc agg ctA GGA TTT CGG CAC GGC TAC
HygroB reverse[d]	ggg gac cac ttt gta caa gaa agc tgg gtT GTG GAG TGG GCG CTT ACA C
AbfA forward[e]	GTT CAC GTG ATC TCC TTG AAG GTC TCC ACC[f]
AbfA reverse[e]	CGT CTA GAA AGT TCG CCG CCA GGA CAG CCA C[g]
AbfB forward[h]	GTT CAC GTG GGC CCC TGT GAC ATC TAC GAA G[f]
AbfB reverse[h]	CGT CTA GAA ACG AAG CAA ACG CCG TCT CAA TC[g]
AbfA-*Nhe*I_F[i]	CCT AGC TAG CAT GGT GGC CTT TTC AGC TCT[j]
AbfA_*BsrG*I_R[i]	TTG CTG TAC AGT TCG CCG CCA GGA CAG CCA[k]

[a]Recombination sites used for Multisite Gateway recombination are in small letters.
[b]For amplification of the 5′-flanking region of *abfA*.
[c]For amplification of the 3′-flanking region of *abfA*.
[d]For amplification of the hygromycin marker.
[e]For amplification of the *abfA* gene (expression in *P. pastoris*)
[f]*Pml*I site underlined.
[g]*Xba*I site underlined.
[h]For amplification of the *abfB* gene (expression in *P. pastoris*).
[i]For amplification of the *abfA* gene (expression in *A. niger*).
[j]*Nhe*I site underlined, [k]*BsrG*I site underlined.

the primers are indicated in Table 2. The primers to amplify both genes were selected in such a way that the predicted signal sequences of AbfA and AbfB were not included. The PCR products were ligated in pJET1.2 (CloneJET™ PCR Cloning Kit Fermentas) and sequenced. Subsequently, a *Pml*I-*Xba*I fragment was cloned into *P. pastoris* expression vector pGAPZαB, introducing the coding sequences in frame with the N-terminal α factor signal sequence and the C-terminal myc epitope and polyhistidine tag (Invitrogen, Carlsbad, CA, USA). This vector contains the constitutive glyceraldehyde-3-phosphate dehydrogenase (GAP) to drive expression and the *Saccharomyces cerevisiae* α-factor signal sequence for translocation into the ER. The constructs were transformed by electroporation to *P. pastoris* X33 (Invitrogen), and zeocin-resistant colonies (100 μg/ml) were selected for further analysis. For the expression and purification of a His-tagged AbfA protein in *A. niger*, the *abfA* gene of *A. niger* was PCR amplified with primers listed in Table 2 using *A. niger* genomic DNA. The fragment was cloned in pJET1.2 and excised with *Nhe*I and *Bsr*GI. The fragment containing the *abfA* gene was subsequently cloned in *Nhe*I and *Bsr*GI digested pARAn19 (Roth and Dersch, 2010) to give pARAn19-AbfA. The final expression plasmid was sequenced.

Expression and purification of AbfA and AbfB

To determine levels of protein production, *P. pastoris* transformants were grown at 30°C for 72 h in YPD at 250 rpm. Each day, 1 ml of the supernatant was collected. The culture filtrates were analyzed by dot-blots on nitrocellulose membrane (Schleicher & Schuell) using anti-His (C-term)-HRP antibody (Invitrogen). Transformants producing high amounts of His-tagged protein were grown in 50 ml YPD in a 300-ml flask in an orbital shaker (250 rpm) at 30°C for 48 h. The cultures were centrifuged (5 min, 1500 rpm), and the supernatant was mixed with 10 mM imidazole (final concentration 5 mM) before loading at an equilibrated HisPur Ni-NTA 3 ml spin column (Pierce Biotechnology, Rockford, IL, USA). After three washing steps with 25 mM imidazole wash buffer, the enzyme was eluted with 250 mM imidazole. The purification was carried out at 4°C. Enzyme purity was estimated by SDS-PAGE (Supplementary Figure S1). To remove imidazole, the enzyme fractions were dialyzed with cellulose dialyzing tube (MWCO is 2 kDa) (Spectrum Laboratories, Rancho Dominquez, CA, USA) against distilled water at 4°C. Protein concentration was 0.1 mg/ml for AbfA and 0.2 mg/ml for AbfB, measured with a BCA assay (Pierce). The pARAn19-AbfA plasmid was transformed to *A. niger* strain AB1.13 (Mattern et al., 1992) and a strain containing three copies of the plasmid (JP18.1) was selected to purify AbfA-His. JP18.1 was grown for 5 days in 25 ml MAM at 30°C. The AbfA-His-tagged protein was purified from *A. niger* as described above for His-tagged AbfA *P. pichia*.

GH assays and enzyme kinetics

GH activity was assayed routinely as follows, unless indicated otherwise. Purified enzyme or medium from *A. niger* was incubated in 100 mM sodium acetate buffer (pH 4.5) with 0.5 mM pNp-conjugated glycoside (Sigma) in an end-volume of 60 μl in a flat-bottom polystyrene 96-wells plate (Greiner) at 37°C. The reaction was terminated by adding 240 μl of 250 mM NaOH, and absorbance was measured at 405 nm. The amount of liberated pNp was calculated using 4-nitrophenol as a standard and was used to determine the hydrolase activity of the pNp substrate in nanomoles per microgram per minute (nmol/μg/min). The experiments to determine the influence of temperature on the activity were performed in Eppendorf tubes in heatblocks.

For kinetic studies, different concentrations of substrate were incubated for 1 h at 37°C. The apparent kinetic parameters V_{max} and K_m were obtained by fitting the data to the Michaelis-Menten equation using nonlinear regression analysis (SPSS 15 program; SPSS, Chicago, IL, USA). Hydrolyzing enzymes in the supernatant were diluted 1:20 and heat-inactivated by incubation at 99°C for 10 min for galactomannan breakdown experiments.

Isolation of galactomannan

Cell walls were isolated from *A. niger* strain N402 after growth in complete medium (CM) (Carvalho et al., 2010) for 24 h at 30°C at 250 rpm. Spores (1×10^9) were inoculated in 1 l of CM in a 2-l Erlenmeyer flask. The mycelia were isolated by filtering over Myracloth and yielded around 16 g (wet weight). The mycelia were grinded in liquid nitrogen using a pestle and mortar, and the broken mycelia were washed three times with 1 M NaCl and three times with MilliQ at 4°C by centrifugation (3600 rpm, 10 min). Successful breakage of the mycelia was confirmed by microscopy. Isolated cell walls were lyophilized over night yielding 2.1 g of cell walls (dry weight). The galactomannan fraction was isolated according to Bardalaye and Nordin (1977). The yield of purified galactomannan was 3.0 mg/g of freeze dried cell walls. The purity of the galactomannan fraction was confirmed by HPAEC monosaccharide analysis on a PA10 column (Dionex) (Salvador et al., 2000) after TFA hydrolysis (data not shown).

Sandwich ELISA to monitor hydrolysis of galactomannan

Supernatant of *A. niger* strains, purified AbfA, purified AbfB, or minimal medium was incubated with or without 5 to 7.5 ng galactomannan for 20 to 24 h at 37°C at pH 4.5 in a volume of 20 to 40 µl. After addition of water to a final volume of 50 µl, these samples were added to microtiter plate wells containing coated antibody EB-A2 (recognizing Gal*f* moieties on galactomannan; Stynen et al., 1995) and 50 µl EB-A2 conjugated to HRP (Platelia Aspergillus EIA kit; BioRad). The wells were incubated for 90 min at 37°C and subsequently washed five times. After incubation for 30 min at ambient temperature with 200 µl TMB detection mixture, the coloring reaction was stopped by addition of 100 µl 1.5 N H_2SO_4. Remaining Gal*f* on galactomannan was measured at an OD of 450 nm.

AbfB modeling and substrate docking

The suggested sequence of the processed *A. niger* AbfB protein was submitted to the SWISS-MODEL protein structure homology modeling server (Peitsch, 1995; Arnold et al., 2006; Kiefer et al., 2009), specifying the crystal structure of *A. kawachii* IFO4308 AbfB (PDB entry 1WD4) as template (Martens-Uzunova et al., 2006). The sequence homology between the template and the submitted sequence is 98.3%. Three-dimensional *.mol structure files of the substrates where obtained from the ChemSpider database (http://www.chemspider.com/). The corresponding ChemSpider IDs are 133468 for pNp-α-L-Ara*f* and 113251 for pNp-β-D-Gal*f*, respectively. The substrates were docked manually into the substrate pocket, which is occupied by the α-L-Ara*f* molecule 1AHR in the protein-substrate PDB structure 1WD4. For molecular modeling and generation of the video frames, the PyMOL Molecular Graphics System (version 1.3, Schrödinger, LLC, version 0.99rc6) was used.

Construction of an *A. niger* Δ*abfA* mutant

The 5′- and 3′-flanking regions of AbfA (An01g00330) were amplified by PCR using the primers listed in Table 2. The resulting fragments were cloned into two destination Gateway vectors. A 3.1-kb hygromycin (HygroB) cassette was amplified by PCR using HygroB primers (Table 2) and pAN7.1 (Z32698.1) as template DNA and subsequently cloned in a third Gateway vector. Using Multisite Gateway recombination, the three fragments were combined to create a vector containing an *abfA* deletion cassette (designated pJH8.1). This vector was linearized with *Dra*I and then transformed to the MA169.4 strain as described (Meyer et al., 2010). Hygromycin-resistant clones were analyzed by Southern blot (see Supplementary Figure S3).

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