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Phylogenetic analysis and substrate specificity of GH2 β-mannosidases from *Aspergillus* species

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ABSTRACT

Phylogenetic analysis of glycoside hydrolase family 2 including *Aspergillus* sequences and characterised β-mannosidases from other organisms, clusters putative *Aspergillus* β-mannosidases in two distinct clades (A and B). *Aspergillus* species have at least one paralog in each of the two clades. It appears that clade A members are extracellular and clade B members intracellular. Substrate specificity analysis of MndA of *Aspergillus niger* (clade A) and MndB of *Aspergillus nidulans* (clade B) show that MndB, in contrast to MndA, does not hydrolyse polymeric mannan and has probably evolved to hydrolyse small unbranched β-mannosides like mannobiose. A 3D-model of MndB provides further insight.

1. Introduction

β-Mannosidases (E.C. 3.2.1.25) hydrolyse terminal non-reducing mannose residues from β-mannosidic substrates of various complexity and length. β-Mannoside structures are present in plants, notably in storage polysaccharides such as galactomannanan, and galactoglucomannan, the major soft wood hemicellulose.

β-Mannosidases have been reported from both bacteria [1,2] and fungi [3–6]. They are also present in plants and animals, including mammals [7–9] and are mainly classified into glycoside hydrolase family 2 (GH2), a diverse family with several exo-glycosidase specificities. Some characterised β-mannosidases are also classified into GH1 and GH5 [10,11]. β-Mannosidases have been characterised from various environments. For instance, the soil bacterium *Cellulomonas fimi* produces an intracellular GH2 β-mannosidase (CfMan2A) [2], while the human gut bacterium *Bacteroides thetaiotamicron* secretes a GH2 β-mannosidase (BtMan2A) [12]. BtMan2A is also hitherto the only high-resolution GH2 β-mannosidase crystal structure, showing an active site pocket with a −1 subsite essential for recognition of terminal mannosyl residue [12]. A few extracellular fungal GH2 β-mannosidases have been reported [3,5,6]. In addition, human and bovine lysosomal β-mannosidases also belong to GH2 [9,13].

Given the fact that β-mannosidases are widely distributed and β-mannoside structures occur in various types of substrates, these enzymes may have evolved to hydrolyse different substrates in quite different cellular locations and environments. β-Mannosidase activity is widely assigned based on the hydrolytic activity on artificial colorimetric substrates such as *p*-nitrophenyl-β-mannopyranoside (*p*NP-β-Man) [1,2]. Such analysis, however, does not give any indication about differences in fine-tuned substrate specificity, for example, the effect of substrate degree of polymerization or substitutions carried by mannosyl moieties.

Several sequences in the *Aspergillus* genome database have been bioinformatically predicted as β-mannosidases, but only few gene products have been experimentally shown to have this activity [6,14,15]. The GH2 β-mannosidase MndA from *Aspergillus niger* is the only cloned β-mannosidase from Aspergilli which has been characterised on natural substrates [3]. The β-mannosidase MndB (AN3368) [16] from *Aspergillus nidulans* was identified using *p*NP-β-Man screening in a dedicated global shotgun gene expression effort [15].

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In light of potential diverse substrate preferences of GH2 β-mannosidases, there is a need for systematic investigations (such as phylogenetic analysis) elaborating also on the structure–function relation and substrate specificity for this group of enzymes. These aspects are addressed in this study which includes comparison of GH2 β-mannosidases, with focus on *Aspergillus* candidates. The choice of the organisms was based on availability of several sequenced *Aspergillus* genomes, the biotechnological importance of enzymes from Aspergilli and the initial observation of the occurrence of putative GH2 paralogous β-mannosidase sequences in several species [16]. The main aim of this study was to address the potential differences between these homologs from *Aspergillus* and compare to other characterised β-mannosidases.

2. Materials and methods

2.1. Bioinformatic analysis

Two sequences, MndA (Gene ID CAB63902.1) and MndB (AN3368/ABF50864.1), associated with β-mannosidase activity were used as queries for BLASTP search [17] to identify homologs in the *Aspergillus* genome database [18] (http://www.aspergillusgenome.org/). The identified putative *Aspergillus* β-mannosidase orthologs and potential paralogs along with all cloned and characterised GH2 β-mannosidases from other organisms were used for phylogenetic analysis and bioinformatic analysis, including signal peptide and secretion analysis (see Supplementary methods).

2.2. Substrates

Mannobiose (M_2), mannotriose (M_3), mannotetraose (M_4), mannopentaose (M_5), mannohexaose (M_6), 6^1-α-D-galactosyl-mannobiose (GM_2), 6^1-α-D-Galactosyl-mannotriose (GM_3), 6^3 6^4-α-D-Galactosyl-mannopenatose (G_2M_5) and ivory nut mannan (INM) were purchased from Megazyme international (Bray, Ireland). *p*-Nitrophenyl-β-mannopyranoside (*p*NP-β-Man), *p*-nitrophenyl-β-galactopyranoside (*p*NP-β-Gal), *p*-nitrophenyl-β-glucopyranoside (*p*NP-β-Gluc), *p*-nitrophenyl-β-xylopyranoside (*p*NP-β-Xyl) were purchased from Sigma (Steinheim, Germany).

2.3. Enzyme preparation

Recombinant MndA was expressed and purified as described previously [14]. *Pichia pastoris* X-33 tranformants harboring MndB gene from *A. nidulans* FGSC A4 cloned in pPICZα vector was obtained from the fungal genomics stock centre (School of Biological Sciences, Kansas City, University of Missouri, MO). The expression and purification of MndB is detailed in Supplementary methods.

2.4. Basic characterisation of MndB

The standard β-mannosidase assay was performed as described previously [3] using 1 mM of *p*NP-β-Man and incubation for 10 min at 30 °C and pH 7. For specificity analysis *p*NP-β-Gal, *p*NP-β-Gluc and *p*NP-β-Xyl were used in equivalent conditions. Optimal pH was determined by the standard activity assay using buffers between pH 2 and 9. The buffers used were 50 mM citrate buffer for pH 2–5, 50 mM phosphate buffer for pH 5–7 and 50 mM Tris–HCl buffer for pH 7–9. Temperature optimum was determined at pH 6 by the standard β-mannosidase assay at 23, 30, 37 and 50 °C. Temperature and pH stability of the enzyme was analysed as residual activity after 3–24 h of incubation. K_M and k_{cat} values for MndB using *p*NP-β-Man (0.1–5 mM) were determined by a continuous assay, monitoring absorbance at 405 nm for 5 min. Incubations were done in 50 mM Tris–HCl buffer pH 7 at 30 °C. Kinetic parameters were obtained by fitting the rate of the reaction and substrate concentration in a Michaelis–Menten equation.

2.5. k_{cat}/K_M analysis for MndA and MndB

Reactions of 1 ml were set up with 750 nM of MndB and 30 μM M_2 or M_3 in 20 mM Tris–HCl buffer pH 7 at 30 °C. MndA (7.2 nM) was incubated with 30 μM of M_2 or M_3 in 50 mM sodium acetate buffer pH 4.5 at 37 °C. Aliquots of 150 μl were taken at a regular time interval for 5 h and the reactions were stopped by boiling for 5 min. The decrease in substrate concentration was analysed with high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Dionex, Sunnyvale, USA) as described previously [3]. All the incubations were done in duplicate. k_{cat}/K_M was calculated according to the Matsui equation by plotting Ln (S_0/S_t) as a function of time (t), were S_0 is the initial substrate concentration at time zero, and S_t is the substrate concentration at time t [19]. Data was analyzed using Graph Pad Prism (Graph Pad Software Inc., San Diego, CA).

2.6. Hydrolysis experiments with different β-mannosidic substrates

Each of the galactosyl substituted manno-oligosaccharides GM_2, GM_3, and G_2M_5 (5 mM) was incubated with 2.5 nkat/ml (1.88 μM) of MndB in 50 mM Tris–HCl at pH 7 and 30 °C, in a total reaction volume of 100 μl. GM_2 (5 mM) was also incubated with 11 nkat/ml (8.2 μM) of MndB. Samples (5 μl) were analysed by thin layer chromatography (TLC) [20]. INM was washed five times with distilled water. 2.5 mg/ml of INM was incubated with 2.5 nkat/ml of enzyme, MndB (1.88 μM), MndA (23.8 nM) or CfMan2A (40 nM), in 20 mM Tris–HCl buffer pH 7 at 30 °C for MndB and CfMan2A (Megazyme, Bray, Ireland), 20 mM acetate buffer, pH 4.5 at 37 °C for MndA. The reaction was followed for 24 h and release of mannose was quantified on a PA10 column with HPAEC-PAD.

2.7. Homology modeling

The chain B in the PDB structure of BtMan2A (PDB ID: 2JE8, 29% identity and 36% positives compared with MndB) at a resolution of 1.7 Å was used as a template to build a 3D model of MndB in swiss model work space (http://swissmodel.expasy.org/). QMEAN scoring for model quality estimation indicated a reliable MndB model, with Z score of −1.28 and 0.647 as Q Mean score. 91.4% of the amino acids were in allowed regions and 98% in favored regions of the Ramachandran plot as analysed by Molprobity [21]. All the analysis and molecular representations were rendered in PyMOL (The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, and LLC). The identity between BtMan2A and MndA (23%) was not sufficient to make a reliable model.

3. Results

3.1. Bioinformatic analysis

MndA of *A. niger* and MndB of *A. nidulans* are two distantly related GH2 protein sequences associated with β-mannosidase activity and share only 21% identity at protein sequence level. A BLASTP search with these two sequences in the *Aspergillus* genome database resulted in identification of at least two putative β-mannosidase homologs from each of the *Aspergillus* species. The phylogenetic analysis with all cloned and characterised β-mannosidases clustered the majority of the putative Aspergilli β-mannosidase sequences into two different clades; clade A and clade B (Fig. 1). However, four of the predicted β-mannosidase sequences

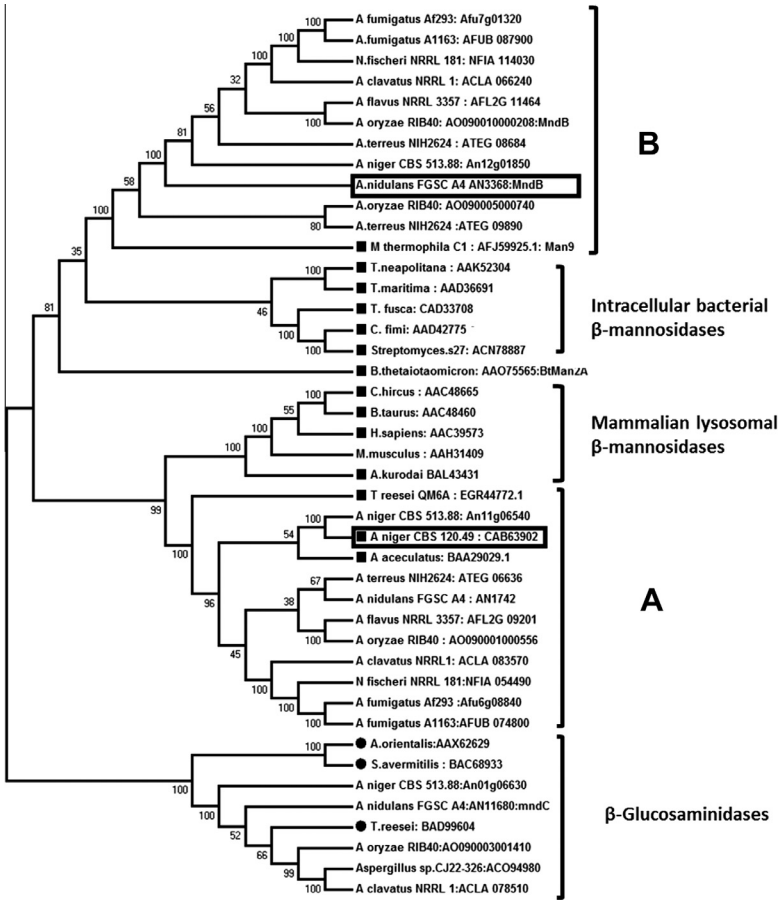

Fig. 1. Phylogenetic analysis of GH2 β-mannosidases: phylogenetic tree of β-mannosidase homologs from *Aspergillus* species and characterised β-mannosidases from GH2. The out group is fungal β-glucosaminidases. Systemic names from *Aspergillus* genome database are used for uncharacterised orthologs of *Aspergillus* species. Genbank ID is used for all characterised β-mannosidases. Clades A and B, referred to in the text, are marked. Aspergilli β-mannosidases for which function was investigated in this study are boxed. Previously characterised β-mannosidases are marked by fullbox (■), characterised β-glucosaminidases in the out group are marked as full circles (●).

from the *Aspergillus* genome database clustered in the out group used (containing characterised β-glucosaminidases). These sequences, with at least 75% sequence identity to characterised out group enzymes are more likely to be β-glucosaminidases rather than β-mannosidases (Fig. 1). Clade A *Aspergillus* homologs are more related to the mammalian lysosomal β-mannosidases than to clade B. Clade B homologs, in turn, are clearly distinct from clade A, but are more related to intracellular bacterial β-mannosidases. The identity within each of these clades (A and B) is greater than 70%. Interestingly, both clades harbour at least one β-mannosidase homolog from each of the *Aspergillus* species. Analysis of the presence of secretory signal peptide indicated that none of the *Aspergillus* β-mannosidases from clade B have a signal peptide and in addition they have a value lower than or similar to the threshold value (0.5) for the possibility of non-classical secretion. The clade A sequences have a signal peptide or, in four cases, predicted to be secreted by a non-classical pathway. MndA from clade A that has been previously characterised and known to be an extracellular β-mannosidase [3], and MndB from clade B for which merely β-mannosidase activity has been displayed were chosen for comparative functional analysis, and was thus expressed and purified (see Supplemental information).

3.2. Properties of MndB

MndB was shown to be a specific β-mannosidase, as far analysed by hydrolysing *p*NP-β-Man, but not *p*NP-β-Gal, *p*NP-β-Gluc or *p*NP-β-Xyl. MndB showed activity at pH 5–8 with optimum at pH 6 (Fig. 2A). The temperature optimum was 37 °C when assayed for 10 min at pH 6 (Fig. 2B). However, the stability was highest at pH 7 (Fig. 2C). When incubated for 24 h at pH 7, MndB remained stable at 4 °C (100% residual activity) and showed some activity loss at 30 °C (89% residual activity), while the loss was greater at 37 °C (68% residual activity) (Fig. 2D). Thus, as far analysed the optimal operational condition is pH 7.0 and 30 °C. Comparison of the activity of MndB with MndA showed that MndB has a lower k_{cat} of 79.2 min^{-1} but a similar K_M of 0.22 mM on *p*NP-β-Man (Table 1).

3.3. Hydrolysis of β-mannosidic substrates

The hydrolysis profile of MndB on M_2, M_3 and galactosyl substituted manno-oligosaccharides GM_2, GM_3 and G_2M_5 was analysed. MndB released mannose from both M_2 and M_3. Interestingly, MndB released only the terminal non-reducing mannose residue from GM_3 (Fig. 3, lane 6), whereas, no mannose release was detected from action on GM_2 (Fig. 3, lane 4), even with five times higher enzyme concentration compared to the GM_3 incubation. These results were also consistent with the observation that no mannose release was detected from G_2M_5 (Fig. 3, lane 8). This contrasts MndA, which previously has been shown to release mannose from GM_2 and G_2M_5 ([2], Table 1). MndB, MndA and the known intracellular β-mannosidase from *C. fimi* (CfMan2A) [2] were incubated with INM and analysed for mannose release. MndA released 11% mannose in 24 h, while no mannose release was detected for MndB (Table 1) and CfMan2A.

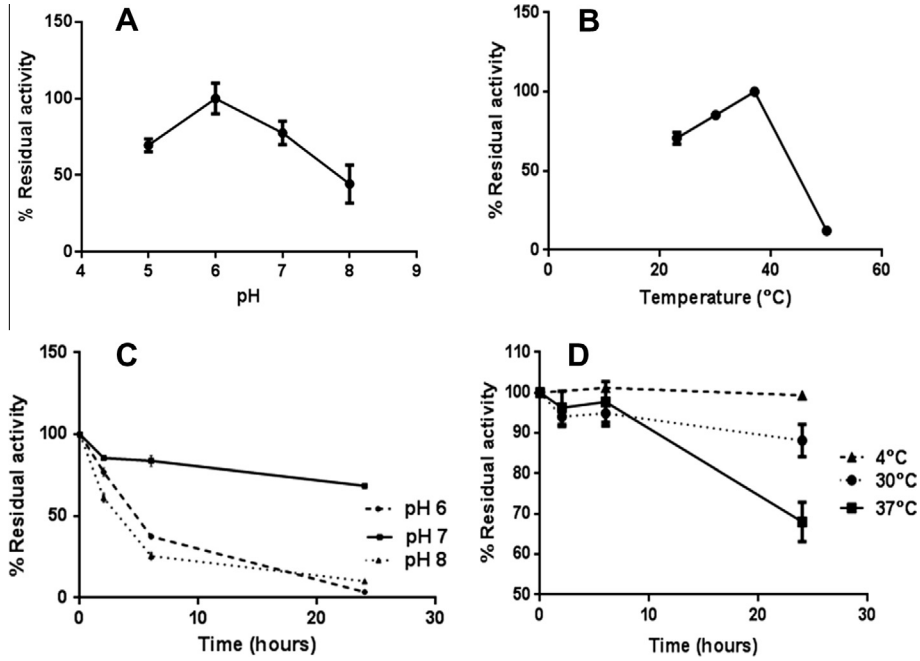

Fig. 2. Characterisation of MndB: effect of temperature and pH on β-mannosidase activity and stability. The results of two incubations at each pH and time point are shown. (A) pH optima: hydrolysis of *p*NP-β-Man for 10 min at pH 2–9. (B) Temperature optima: hydrolysis of *p*NP-β-Man for 10 min at pH 6 (C) pH stability: MndB incubated at pH 6–8 at 37 °C for 3–24 h. (D) Temperature stability: MndB incubated at 4 °C, 30 °C and 37 °C in pH 7 for 3–24 h.

Table 1
Properties of MndB, MndA and BtMan2A on various β-mannosides kinetic constants k_{cat}, K_M and k_{cat}/K_M on *p*NP-β-Man. k_{cat}/K_M values for M_2 and M_3 with standard deviation in parentheses.

	Substrate	MndA[#]	MndB[ø]	BtMan2A[*]
k_{cat} (min^{-1})	*p*NP-β-Man	4048	79.2 (1.7)	7689 (289)
K_M (mM)		0.46	0.22 (0.034)	0.19 (0.021)
k_{cat}/K_M (min^{-1}/M)		1.3×10^7	3.6×10^5 (5×10^4)	4.0×10^7 (1.4×10^6)
k_{cat}/K_M (min^{-1}/M)	M_2	4.45×10^5 (2.1×10^3)	1.45×10^3 (0.34×10^2)	6.0×10^4 (3.9×10^2)
k_{cat}/K_M (min^{-1}/M)	M_3	7.8×10^5 (2.4×10^3)	9.5×10^2 (0.2×10^2)	1.3×10^6 (1.8×10^3)
	INM	+	–	+
	GM_3	+	+	+
	GM_2	+	–	+
	G_2M_5	+	–	–

"+" Indicates activity and release of mannose. "–" Indicates no detectable release of mannose.
ø Kinetic constants and properties determined in this study.
Kinetic constants for manno-oligosaccharides determined in this study and kinetic constants for *p*NP-β-Man from [3].
* Data from [12].

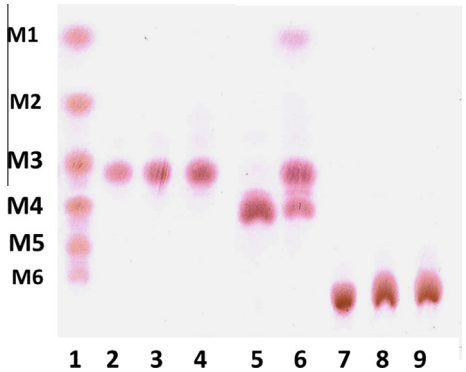

Fig. 3. TLC Analysis of hydrolysis of galactose substituted manno-oligosacharides by MndB: M_1–M_6 (lane 1); GM_2 standard (lane 2); GM_2 –0 h (lane 3), 24 h (lane 4); GM_3 –0 h (lane 5), 24 h (lane 6); G_2M_5 0 h (lane 7), 24 h (lane 8). MndB was incubated with 5 mM substrate in a total volume of 100 μl in all reactions.

3.4. k_{cat}/K_M of MndA and MndB on M_2 and M_3

M_2 and M_3 were both hydrolysed by MndB and MndA. However, MndA was 300–800 times more efficient with manno-oligosaccharides as substrates compared to MndB (Table 1). MndA hydrolysed M_3 with 1.8-fold higher catalytic efficiency (k_{cat}/K_M) compared to M_2. In contrast, MndB was more efficient (1.5-fold) on M_2 than on M_3.

3.5. Homology model

Superimposition of the active sites of MndB and BtMan2A suggests a conserved –1 subsite essential for mannosyl binding and catalysis with the predicted nucleophile (Glu 432) and acid/base (Glu 528) overlayed with the template structure (Fig. 4). However, differences are apparent in the predicted aglycone binding site (+1) of MndB. Tyr and Trp residues, typically involved in stacking saccharide substrate interactions in GH active site subsites [22] are lacking in the MndB predicted +1 subsite (Fig. 4). A less

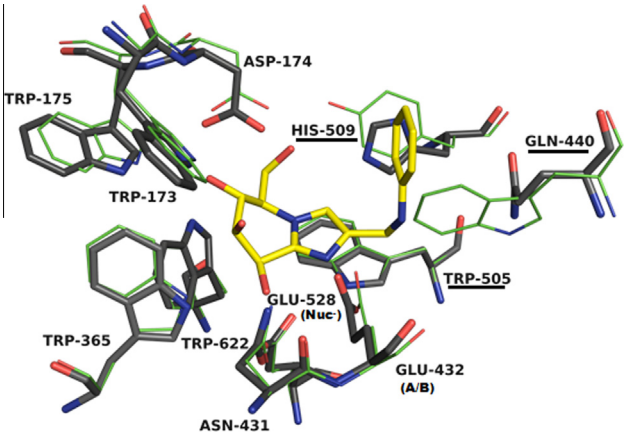

Fig. 4. Overlay of the active site pocket of MndB with BtMan2A in complex with inhibitor mannoimidazole (PDB 2VOT) [26]. The amino acids predicted to form the active site pocket in MndB (BtMan2A) are: Trp-173 (198), Asp-174 (199), Trp-175 (200), Asn-431 (461), Glu-432 (462), Trp-505 (533), Glu-528 (555), Trp-622 (645), Gln-440 (Trp 470) and His 509 (Tyr-537). The underlined amino acids are involved in formation of predicted +1 subsite. The unmarked residues form the −1 subsite (conserved in all clades A and B sequences in the alignment used for phylogenetic analysis), where predicted catalytic acid base (A/B) Glu-432 and nucleophile (Nuc^-) Glu528 are marked. The inhibitor (yellow) and MndB amino acids are shown in stick representation with carbon colored gray, oxygen colored red and nitrogen colored blue, whereas BtMan2A active site pocket residues are shown as line representation.

hydrophobic +1 subsite and a solvent accessible His 509 at the entrance of the active site pocket of MndB, compared to a Tyr (Tyr537) in BtMan2A [12] may contribute to the overall lower catalytic efficiency (Table 1). Residues involved in the −1 subsite formation and the surface exposed His residue are conserved in all the clade B sequences as judged by the multiple alignment used for phylogenetic analysis.

4. Discussion

Parallel phylogenetic and functional analysis of exo-glycosidase homologs from the same GH family with the same basic activity is performed in this study for GH2 β-mannosidases. They hydrolyse β-mannosidic linkages, however as found in this study, their substrate preferences and functional role may vary. Our study gives insight into the differences in the fine-tuned substrate specificity of these enzymes and provides knowledge to use these enzymes as bioanalytical and biotechnical tools, e.g., in plant cell wall studies.

Initial sequence analysis indicated the presence of multiple homologous β-mannosidases in *Aspergillus* species [16]. Phylogenetic analysis clustered *Aspergillus* β-mannosidase homologs into two different clades: clade A and clade B, respectively (Fig. 1). Clade A proteins are likely secreted. This is also consistent with the fact that the cloned and characterised MndA (clade A), originally was purified from the growth medium of an *A. niger* culture [3]. Clade B proteins appear to be intracellular. A member from this clade (β-mannosidase, MndB) is characterised in this study. Gene expression studies on different manno-configured substrates indicate that both Aspergilli members of clade A and clade B could be involved in the mannan utilisation system in *Aspergillus*. MndA is shown to be induced by galactomannans, whereas expression of AO09001000208, an ortholog of MndB from *Aspergillus oryzae* clustering in clade B, is induced by mannobiose mediated by ManR, a transcriptional regulator of β-mannan utilisation [23]. Comparative functional analysis of two representative enzymes from each of the clades, MndA (clade A) and MndB (clade B), showed different kinetic properties and substrate preferences in terms of finer substrate specificity. The difference in pH activity range of MndA (2.5–5) [3] and MndB (5–8) is in accordance with an acidic extracellular environment and intracellular pH of 6–7 [24], respectively.

For the few GH2 β-mannosidases which have been characterised on natural substrates differences are logical when considering their predicted cellular locations. The k_{cat}/K_M analysis on manno-oligosaccharides for both MndB and MndA suggests that MndA is overall a much more efficient enzyme with k_{cat}/K_M values comparable to the extracellular β-mannosidase BtMan2A (Table 1). It is also more efficient on mannotriose compared to mannobiose as a substrate while MndB prefers mannobiose over mannotriose (Table 1). Considering MndB to be intracellular, it is logical that MndB has no activity against polymeric mannan, similar to the intracellular CfMan2A as described in this study, while the extracellular β-mannosidases (MndA and BtMan2A) release mannose from polymeric mannan (Table 1). Recently, a β-mannosidase purified from *Myceliopthora thermophila* which we classify into the clade B (Fig. 1) was shown to have no activity on polysaccharides [25]. Furthermore, a β-mannosidase characterised from culture filtrate of *Trichoderma reesei* that releases mannose from galactomannan [4] is classified into clade A in our phylogenetic analysis. These observations support our view that clade A enzymes, but not clade B enzymes are active on polysaccharides. Additionally, in contrast to MndA, MndB is severely restricted by galactosyl substitutions at the +1 subsite. The 3D model of MndB was constructed using BtMan2A as template. BtMan2A is an extracellular β-mannosidase with similar properties to that of MndA. The model suggests a less hydrophobic +1 subsite of MndB compared to BtMan2A, which may contribute to the overall lower catalytic efficiency of MndB, which appears to be more pronounced with longer substrates resulting in no hydrolysis when using polymeric substrates like ivory nut mannan.

5. Conclusions

Our study indicates that β-mannosidase homologs in *Aspergillus* have evolved via two different lines, resulting in clades A and B, where enzymes belonging to clade A have evolved to be extracellular, taking part in the hydrolysis of plant mannan polymers and acting on manno-oligosaccharides, e.g., produced by β-mannanase catalysed endo-hydrolysis [16]. In line with this, clade B enzymes on the other hand, may have evolved to be intracellular and being specialised to hydrolyse smaller unsubsituted β-mannosides such as mannobiose. Our study thus provides insight into enzyme evolution and differences in fine-tuned substrate preferences of enzyme paralogs. It therefore not only serves as a reference for future studies of GH2 β-mannosidases, but also for more detailed studies on enzyme paralogs in other enzyme families.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.08.029.

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