Alpha-glucan acting enzymes in Aspergillus niger
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Chapter 4

Biochemical characterization of two GPI-anchored α-glucanotransferase enzymes from *Aspergillus niger* reveals a novel reaction specificity in subfamily GH13_1 of fungal α-amylases

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*Submitted*

**Abstract**

*Aspergillus niger* AgtA and AgtB are the first family GH13 α-(1,4) glucanotransferase enzymes described in fungi. Their main activity is disproportionation of maltopentaose or longer maltooligosaccharides substrates, including starch. Biochemical and phylogenetic characterization of AgtA and AgtB revealed clear differences with prokaryotic disproportionating enzymes. AgtA and AgtB are GPI-anchored to the cell wall, where they most likely play a role in cell wall α-glucan maintenance. They represent a novel reaction specificity in subfamily GH13_1, also comprising fungal extracellular α-amylases involved in starch degradation, and can be distinguished on the basis of specific sequence features.
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**Introduction**

Family 13 (GH13) is the largest of all sequence based glycoside hydrolase (GH) families, comprising at least 26 different reaction specificities (http://www.cazy.org) (Coutinho and Henrissat, 1999; Stam et al., 2006). A well-known member of this family is α-amylase, an enzyme hydrolyzing starch. Members of family GH13 have a common 3D structure and catalytic machinery. The active site of these enzymes is composed of four conserved amino acid regions containing the three catalytic residues and four additional highly conserved residues involved in substrate binding and stabilization of the transition state of the reaction (MacGregor et al., 2001). Based on amino acid sequence similarity, family GH13 was recently divided into 35 subfamilies, which correlated relatively well with the biochemical activities reported for enzymes in each subfamily (Stam et al., 2006). Analysis of the α-amylase type enzymes (subfamily GH13_1) encoded in the genomes of several *Aspergillus* species revealed that they could be categorized in two distinct groups. Apart from the extracellular α-amylases necessary for the hydrolysis of starch, all species harboured several cell wall, or membrane bound, GPI-anchored enzymes with clear similarity to α-amylases, but with aberrant conserved regions (Yuan et al., in preparation). Two of these enzymes, AgtA and AgtB from *A. niger*, were identified as α-glucanotransferases acting on starch and maltooligosaccharides (EC 2.4.1.25) (van der Kaaij et al., 2007). The phenotype of an agtA knockout strain, and the expression patterns of agtA and agtB in wild type *A. niger*, suggested that these enzymes play a role in synthesis and/or maintenance of cell wall α-glucans (van der Kaaij et al., 2007). Fungal cell wall α-glucan is generally composed of two types of polymers: α-(1,3) glucans with a small percentage of α-(1,4) glycosidic bonds, and nigeran with alternating α-(1,3) and α-(1,4) glycosidic bonds. The proposed role for AgtA/B and homologous fungal enzymes is further supported by the phenotype of a knockout strain for the homologous GPI-anchored protein Aah3p in *S. pombe*, which showed an aberrant cell shape and hypersensitivity to cell wall degrading enzymes (Morita et al., 2006).

Stam et al. (2006) expressed their concern with respect to the accuracy of sequence similarity-based annotations of Glycoside Hydrolase (GH) family enzymes in genome sequencing projects, especially in view of the “decreasing number of novel enzymatic characterization reports… reflecting the fact that the quest for increased impact factors renders journals reluctant to publish such characterizations”. This study provides a detailed characterization of the two novel subfamily GH13_1 AgtA/B enzymes from
A. niger. Biochemically, but not phylogenetically, they resemble prokaryotic disproportionating enzymes (e.g. amylomaltases, family GH77). We show that phylogenetic analysis, as performed by Stam et al., is not sufficient to distinguish between fungal α-amylases and Agt enzymes, but predicted GPI-anchoring and variations in the four conserved family GH13 regions clearly distinguish these two types of fungal enzymes.

**Materials and methods**

**Enzyme production and purification**

Production and purification of full length AgtA and AgtB were performed as described before (van der Kaaij et al., 2007). AgtA and AgtB were overexpressed in A. niger strain MGG029-ΔaamA (Weenink et al., 2006), which is a knockout for the genes encoding glucoamylase (glaA) and acid amylase (aamA) resulting in very low extracellular α-amylase activity. Both enzymes were purified from culture medium via anion exchange chromatography and stored in 50 mM NaAc pH 5.5 containing 1 mM CaCl$_2$ at -20 °C.

**General assay conditions**

All oligosaccharides were obtained from Sigma, except nigerotriose which was purchased from Dextra laboratories and nigerose (O-α-D-Glc-(1,3)-α-D-Glc) which was a kind gift from Nihon Shokuhin Kako Co. Ltd. (Shizuoka, Japan). The α-(1,3)-glucan isolated from A. nidulans was a gift from Dr. B. J. Zonneveld (Leiden University, Leiden, The Netherlands). Nigeran and α-(1,3)-glucan were dissolved in 1 M NaOH and the pH of a 1% solution was adjusted to pH 5.5 with HAc.

Quantitative measurements of glucanotransferase activity were performed with p-nitrophenyl-α-D-maltoheptaoside-(4,6)-O-benzylidine (PNP-G7) (Megazyme, County Wicklow, Ireland), a double blocked maltoheptaoside, as donor substrate (as adapted from Nakamura et al. (1994) and Van der Veen et al. (2000)). In the standard assay 100-500 ng AgtA or 400-1000 ng AgtB were incubated in a total reaction volume of 180 μl, containing 10 mM PNP-G7 as donor substrate, 40 mM acceptor substrate (maltose in standard reactions) and 1 mM CaCl$_2$ in 50 mM NaAc buffer. AgtA reactions were buffered at pH 5.5 and AgtB reactions at pH 4.8, unless indicated otherwise. To measure hydrolysis, reactions were performed without acceptor substrate. Samples of 30 μl were taken after 0, 5, 10, 15 and 20 min and were immediately transferred to preheated reaction tubes and incubated for 15 min (AgtA) or 5 min (AgtB) at 78 °C to deactivate the enzymes. After cooling on ice, 120 μl 50 mM NaAc buffer pH 4.5 containing 1 U A. niger α-glucosidase (Megazyme) was added to each sample and samples were incubated for 30 min at 37 °C to liberate p-nitrophenol (PNP) from the
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cleaved PNP-G7 product. Subsequently, 0.75 ml 1 M Na$_2$CO$_3$ was added to each sample and Abs$_{402}$ was measured. In each experiment autohydrolysis of PNP-G7 was always negligible. Reactions were performed at least in duplicate. Linear regression was applied on the data of each separate reaction and the slope of the trend line (with R$^2$ >0.99) was used as a measure for the reaction rate. 1 U of enzyme activity was defined as the amount of enzyme liberating 1 μMol of PNP per minute under the described reaction conditions. A calibration curve was made which indicated that the molar extinction coefficient of PNP under these conditions was 16.4 mM$^{-1}$cm$^{-1}$ at 402 nm. The experimental data were analysed using SigmaPlot version 9.0 (Systat Software).

**Temperature optimum**

Temperature optima were determined with 100 ng AgtA or 400 ng AgtB. Reactions with AgtA at 60 °C and AgtB at 48.5 °C and 53 °C were incubated 10 min (instead of 20 min), to prevent loss of activity due to heat inactivation.

**Kinetic analysis**

Kinetic analysis was performed at 45 °C for AgtA and 40 °C for AgtB, with 100 ng or 500 ng purified enzyme, respectively. For both enzymes, 11 different PNP-G7 concentrations were tested in the range of 0.5-18 mM (AgtA) or 0.5-22 mM (AgtB). The calculated molecular masses of the proteins were 58840 Da for AgtA and 57790 Da for AgtB, after removal of the C-terminal end for GPI-anchoring. The $K_m$ and $V_{max}$ values were determined via Lineweaver-Burk plots with R$^2$>99.

**Acceptor substrates**

AgtA (100 ng) or AgtB (500 ng) were incubated with different acceptor substrates (40 mM) at 37 °C and pH 5.5. Reaction rates were compared to the values observed with maltose, and corrected for autohydrolysis (release of PNP in the absence of acceptor substrate).

**Subsite modelling and acarbose inhibition**

Initial product formation was analysed using 800 ng AgtA or 1.5 μg AgtB in a total reaction volume of 66 μl containing 20 mM maltopentaose, maltohexaose or maltoheptaose. Samples of 12 μl were taken after 0, 5, 15, 30, 60 min of incubation and analysed by HPLC ( Dionex) (van der Kaaij et al., 2007). Inhibition by acarbose (0, 1 or 10 mM) was checked using 100 ng AgtA or 300 ng AgtB with 20 mM maltoheptaose in a total volume of 15 μl. Samples were taken after 60 min and analysed by thin layer chromatography as described before (van der Kaaij et al., 2007).
Sequence analysis
Alignment and tree building were performed with MEGA version 3.1 (Kumar et al., 1993). Sequence logos were made with WebLogo 2.8.2 (http://weblogo.berkeley.edu) (Crooks et al., 2004).

Fig. 1 Relative activity of AgtA and AgtB at different temperatures. For each enzyme, the total activity at the optimum temperature was set at 100%. This represented 90.5 U mg\(^{-1}\) for AgtA and 12.4 U mg\(^{-1}\) for AgtB.

Results and discussion
General enzyme characteristics
Purified \textit{A. niger} AgtA and AgtB, produced by homologous overexpression and ion exchange chromatography, were stable at 4 °C as well as -20 °C for at least several weeks. The optimum pH for the glucanotransferase reaction could not be measured quantitatively due to the instability of the PNP-G7 substrate at low pH, in combination with the high temperature needed for deactivation of the enzymes. Qualitative analyses previously demonstrated that AgtA was most active at pH 4.5-6 and AgtB at pH 4-5.5 (van der Kaaij et al., 2007), therefore we used pH 5.5 (AgtA) and pH 4.8 (AgtB) in subsequent quantitative assays. The relatively low pH optima of AgtA and AgtB are consistent with the acidifying properties of \textit{A. niger}, and similar values have been observed for other extracellular enzymes (Boel et al., 1990). The AgtA/B temperature
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Optima were determined using PNP-G7 as donor and maltose as acceptor substrate (Fig. 1). AgtA showed a relatively narrow temperature optimum between 45 – 50 °C, while AgtB showed a remarkably broad temperature optimum. Although the relative activity of both enzymes was similar between 47 °C and 53 °C, their stability was clearly different. AgtB lost activity rapidly at 50 °C and higher during the 20 min reaction period, while AgtA activity was stable at temperatures up to 55 °C (results not shown).

**Fig. 2** Relative activity of AgtA and AgtB with PNP-G7 as donor substrate and different acceptor substrates. For each enzyme, the activity with maltose as acceptor substrate was set at 100%, representing 75.5 U mg\(^{-1}\) for AgtA and 10.2 U mg\(^{-1}\) for AgtB.

**Kinetic analysis**

Michaelis-Menten type kinetics was observed for the α-glucanotransferase reactions of AgtA and AgtB with PNP-G7 and maltose. The values obtained for various kinetic parameters differed strongly between the two enzymes (Table 1). Notably, the \(V_{\text{max}}\) for AgtA was five times higher than for AgtB, and specific activities differed a factor 10.

This relatively low specific activity of AgtB compared to AgtA was also observed in reactions with maltooligosaccharides analysed by HPLC (results not shown). Hydrolysis of PNP-G7 was low but in a similar range for both enzymes (between 2 and 3.5 U mg\(^{-1}\)), and comparable to the starch hydrolysis rates (van der Kaaij *et al.*, 2007). The Michaelis-Menten kinetics of AgtA and AgtB with regard to donor substrate is
generally also observed for prokaryotic disproportionating enzymes. The $K_m$ value of AgtA/B for PNP-G7 as donor substrate is, however, 10 times higher than for bacterial cyclodextrin glucanotransferases (CGTases) (Leemhuis et al., 2003; Leemhuis et al., 2002), and actually more in the range of the $K_m$ for PNP-G7 in the hydrolysis reaction of related fungal $\alpha$-amylases, e.g. *A. niger* acid amylase (Boel et al., 1990). The $K_{cat}$ values of AgtA and AgtB are rather low compared to those of CGTases ($K_{cat}$ of disproportionation reaction around 1000 s$^{-1}$) (Leemhuis et al., 2003; Leemhuis et al., 2002), but similar to those reported for amylomaltases, members of family GH77 (e.g. of *Pyrobaculum aerophilum* IM2, 115 s$^{-1}$) (Kaper et al., 2005).

### Table 1 Kinetic parameters of AgtA and AgtB determined for the disproportionation reaction with PNP-G7 as donor substrate and maltose as acceptor substrate.

<table>
<thead>
<tr>
<th></th>
<th>AgtA</th>
<th>AgtB</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ (donor)</td>
<td>2.4 mM</td>
<td>4.9 mM</td>
</tr>
<tr>
<td>$V_{max}$</td>
<td>95 U mg$^{-1}$</td>
<td>18.4 U mg$^{-1}$</td>
</tr>
<tr>
<td>$K_{cat}$</td>
<td>334 s$^{-1}$</td>
<td>64 s$^{-1}$</td>
</tr>
<tr>
<td>$K_{cat}/K_m$</td>
<td>139 s$^{-1}$ mM$^{-1}$</td>
<td>13 s$^{-1}$ mM$^{-1}$</td>
</tr>
</tbody>
</table>

**Acceptor substrates and subsite modelling**

The relative efficiency of AgtA and AgtB towards different acceptor substrates, using PNP-G7 as donor substrate, was determined (Fig. 2). For both enzymes, the best acceptor substrate was maltotriose. Interestingly, nigerose and nigerotriose also were used as acceptor substrates, although not as efficiently as maltooligosaccharides. Maltopentaose and larger acceptor substrates were not tested because these may also act as donor substrates, thereby interfering with PNP-G7 as a quantifiable donor substrate. The polysaccharides nigeran and $\alpha$-(1,3)-glucan were not used as acceptor substrate (data not shown). Activities with glucose and isomaltose were very low, as opposed to amylomaltase enzymes that efficiently use glucose as acceptor substrate (Kaper et al., 2004).

In analogy to other family GH13 and GH77 enzymes, AgtA and AgtB most likely act by cleaving a donor substrate, covalently binding part of it to the amino acid acting as nucleophile, and the subsequent coupling of this donor substrate to the non-reducing end of an incoming acceptor molecule (see e.g. Uitdehaag et al. (1999)). We incubated AgtA and AgtB with different maltooligosaccharide substrates (G5-G7) and identified the initial products formed (Figs. 3 and 4).
Fig. 3 HPLC analysis of initial products formed by incubation of AgtA with maltohexaose (G6). Samples for analysis were taken after 0 min (A), 5 min (B), 15 min (C), 60 min (D) of incubation. The main products formed were identified as maltose (G2), maltotriose (G3), maltononaose (G9) and maltooligosaccharide composed of 12 anhydroglucose units (G12). Note the absence of glucose formation, even upon prolonged incubation.

On basis of the above-described steps in the glucanotransferase reaction, combined with information about the initial AgtA and AgtB products formed (Figs. 3, 4), we conclude that at least six sugar binding subsites, namely -3 to +3, are present in both enzymes. The presence of these six subsites is also demonstrated by the following additional observations:

1. Because maltotetraose and smaller maltooligosaccharides are relatively inefficient donor substrates, AgtA/B must possess more than 4 sugar binding subsites.
2. Glucose is not a major product (Fig. 3), nor is it used as acceptor substrate, indicating that at least 2 acceptor subsites (+1 and +2) are present. Interestingly, amylomaltase enzymes release glucose from their donor substrate, and may also use it as an acceptor substrate (see e.g. Terada et al. (1999)). This suggests that the +1 subsite is sufficient for acceptor substrate binding in amylomaltases, but not in AgtA/B. Differences in the active site of both
enzyme types also become apparent from the comparison of their conserved regions, containing many of the amino acids composing the active site (Fig. 6).

(3) The existence of a +3 acceptor binding site is indicated by the observation that maltotriose is a more efficient acceptor substrate than maltose (Fig. 2).

(4) Initial formation of maltose and G8 from maltopentaose clearly indicates the presence of a -3 subsite. In general, the production of specific initial reaction products suggests that AgtA/B perform a non-progressive type of reaction, which is similar to amylomaltases (see e.g. Terada et al. (1999)).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Main products</th>
<th>Substrate binding in subsites</th>
</tr>
</thead>
<tbody>
<tr>
<td>G5</td>
<td>G2 + G8</td>
<td>-4 O O O O Ø Ø</td>
</tr>
<tr>
<td>G6</td>
<td>G3 + G9</td>
<td>-4 O O O O Ø Ø</td>
</tr>
<tr>
<td>G7</td>
<td>G3 + G11</td>
<td>-4 O O O O Ø Ø</td>
</tr>
</tbody>
</table>

Fig. 4 Initial products formed by AgtA and AgtB from different maltooligosaccharides. AgtA/B were incubated with maltopentaose, maltohexaose or maltoheptaose and initial reaction products, which were the same for both enzymes, were identified by HPLC analysis (see Fig. 3). Indicated is the predicted binding of donor substrates in the different subsites leading to the formation of the observed initial products. Subsites indicated as described by Davies et al. (1997). O, glucose unit; Ø, reducing end.

**Phylogenetic analysis**

AgtA and AgtB are the first studied representatives of a group of homologous enzymes identified in genome sequences of many filamentous fungi (Yuan et al., in preparation). Phylogenetically, this group is closely related to fungal α-amylases in subfamily GH13_1, but not to any of the prokaryotic disproportionating enzymes, despite the α-glucanotransferase activity of AgtA/B described here (Table 2; Fig. 5). The predicted Agt homologues can be distinguished from the α-amylases by their C-terminal GPI-anchoring signal and, in most cases, the absence of the equivalent of His147 (conserved region I) and sometimes also His324 (conserved region IV) (numbering according to AgtA) (Fig. 6), two highly conserved amino acids in family GH13 enzymes, located in the -1 subsite. In Agt homologues, His147 is mostly replaced by rather small, generally hydrophilic amino acids such as Asp, Asn or Ser. Interestingly, all family GH77 amylomaltase enzymes also lack the His147 residue (Fig. 6) (Kaper et al., 2007). One of the few known GH13 enzymes lacking both histidine residues is acarviosyltransferase from Actinoplanes sp. strain SE50, the only known enzyme that can efficiently use the α-amylase inhibitor acarbose as a substrate for α-glucanotransferase activity (Hemker et
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AgtA and AgtB were only partly inhibited by 1 or 10 mM acarbose, which was not used as a substrate, suggesting that they are relatively insensitive for this inhibitor compared to other GH13 enzymes that generally have a Ki value for acarbose in the µmolar range (results not shown) (Kim et al., 1999). This acarbose insensitivity in AgtA/B may be based on the missing His residue(s), but this remains to be proven experimentally.

Fig. 5 Bootstrapped phylogenetic tree of A. niger AgtA/B/C and homologous proteins from A. fumigatus and S. pombe, predicted α-amylases from these fungi and several prokaryotic enzymes with disproportionation activity (listed in Table 2). The alignment and phylogenetic analysis were performed with MEGA version 3.1 using default settings. A bootstrapped tree was constructed with the neighbor-joining method using 500 replicates. The scale bar corresponds to a genetic distance of 0.2 substitution per position.
Fig. 6 Sequence logos of the four conserved sequence regions in (A) predicted GPI-anchored fungal α-glucanotransferase enzymes (based on 17 sequences), (B) predicted fungal extracellular α-amylases (17 sequences), and (C) family GH77 amylomaltase enzymes from bacteria and archaea (20 sequences). The first amino acid in each sequence logo is numbered according to AgtA (A), A. niger AamA (B) or E. coli MalQ (C).

Conclusions

The A. niger AgtA and AgtB enzymes, for the first time described in biochemical detail in this study, are representatives of a large group of predicted fungal GH13_1 enzymes. Both Agt enzymes perform the same α-(1,4) glucanotransferase type of reaction, but also display clearly different features. The enzymes possess some unique properties compared to previously described enzymes with α-glucanotransferase activity, all from prokaryotic origin. The characteristic properties of the AgtA/B enzymes still remain to be compared to those of their homologues from other ascomycete fungi. The precise in vivo reaction and physiological function of these enzymes is most likely in cell wall synthesis and/or maintenance, but this remains to be proven experimentally. In future work, their biochemical characteristics as studied here need to be related to the dynamics of the structure of fungal cell wall, adding to our as yet poor understanding of the process of α-glucan formation and maintenance.

Based on sequence similarity, AgtA/B and their homologues are classified into subfamily GH13_1, together with the fungal extracellular α-amylases (Stam et al., 2006). The activity of AgtA and AgtB has now been proven to be mainly of the glucanotransferase type, clearly different from that of the α-amylases, leading to a 'polyspecific' subfamily GH13_1. Other polyspecific subfamilies identified by Stam et al. generally displayed strongly related activities in which the variety was merely a matter of semantics or specificity of the applied assays. AgtA and AgtB as well as their homologues are not distinguishable from fungal α-amylases based on the phylogenetic analysis applied for the formation of subfamilies. They can, however, be distinguished
by their aberrant conserved regions and their predicted GPI-anchoring. These specific sequence characteristics should therefore be considered to improve the future annotation of fungal family GH13 enzymes.

Table 2 Database numbers of α-amylase and α-glucanotransferase protein sequences, used for phylogenetic analysis (see Fig. 5).

<table>
<thead>
<tr>
<th>Name</th>
<th>Genome or Swissprot nr.</th>
<th>Activity</th>
<th>Source</th>
<th>GH family</th>
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<td>Pyrobaculum aerophilum</td>
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<td>12</td>
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</table>

References: 1 (Boel et al., 1990); 2 (Korman et al., 1990); 3 this study; 4 (van der Kaaij et al., 2007); 5 (Nierman et al., 2005); 6 (Morita et al., 2006); 7 (Liebl et al., 1992); 8 (Lawson et al., 1994); 9 (Hemker et al., 2001); 10 (Takahashi et al., 1993); 11 (Pugsley and Dubreuil, 1988); 12 (Kaper et al., 2005).
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