Carbohydrate-active enzymes that modify the cell wall of Aspergillus niger

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DOI: 10.1016/j.carres.2015.01.014

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Document Version
Publisher's PDF, also known as Version of record

Publication date: 2014

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
van Munster, J. (2014). Carbohydrate-active enzymes that modify the cell wall of Aspergillus niger: Biochemical properties and physiological functions during autolysis and differentiation [S.n.] DOI: 10.1016/j.carres.2015.01.014

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CHAPTER 5

Biochemical characterisation of Aspergillus niger CfcI, a glycoside hydrolase family 18 chitinase that releases monomers during substrate hydrolysis

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Microbiology 2012, vol 158, pag. 2168-2179
Abstract

The genome of the industrially important fungus *Aspergillus niger* encodes a large number of glycoside hydrolase family 18 members annotated as chitinases. We identified one of these putative chitinases, CfcI, as a representative of a distinct phylogenetic clade of homologous enzymes conserved in all sequenced *Aspergillus* species. Where the catalytic domain of more distantly related chitinases consists of a TIM barrel in which a small additional (α+β) domain is inserted, CfcI like proteins were found to have in addition a carbohydrate binding module (CBM18) that is inserted in the (α+β) domain next to the substrate binding cleft. This unusual domain structure, and sequence dissimilarity to previously characterised chitinases, suggests that CfcI has a novel activity or function different from chitinases investigated so far. Following its heterologous expression and purification, its biochemical characterization showed that CfcI displays optimal activity at pH 4 and 55 °C – 65 °C, and degrades chitin oligosaccharides by releasing N-acetyl-glucosamine from the reducing end, possibly via a processive mechanism. This is the first fungal family 18 exo-chitinase described that exclusively releases monomers. The *cfcI* expression profile suggests that its physiological function is important in processes that take place during late stages of the *Aspergillus* life cycle, such as sporulation.
Introduction

Aspergillus niger is a filamentous ascomycete of significant industrial importance. The fungus is the main producer of citric acid, and is used as a production host for both heterologously and homologously (over)expressed extracellular enzymes (Schuster et al., 2002). The genome sequences of two A. niger strains each revealed the presence of 16 genes encoding putative chitin degrading enzymes (Andersen et al., 2011; Pel et al., 2007). Chitinolytic enzymes are of major physiological importance in their fungal hosts. They are involved in degradation of chitin substrates (Lopez-Mondejar et al., 2009) and may become expressed during mycoparasitism as discussed by (Seidl, 2008). In addition, chitinases are suggested to modify chitin present as structural component in the fungal cell wall and thus play a role during morphological changes such as autolysis, hyphal branching or germination of conidia (Shin et al., 2009; Yamazaki et al., 2008).

The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) distinguishes two chitinolytic activities (IUBMB, 1992). The β-N-acetylhexosaminidases (EC 3.2.1.52) release N-acetyl-D-hexosamine residues from the non-reducing terminus of N-acetyl-β-D-hexosaminides such as chitin oligosaccharides. All fungal enzymes with this activity belong to family 20 of the sequence- and fold based Glycoside Hydrolases (GH) families as described in the CAZy database (Cantarel et al., 2009). Chitinases (EC 3.2.1.14) randomly hydrolyse β-(1-4) linkages between N-acetyl-β-D-glucosamines in chitin. Fungal chitinases belong to GH18. Meanwhile, chitinolytic enzymes have been identified with a substrate/product specificity that is not covered by this nomenclature. Chitobiosidase refers to chitinases releasing mainly or exclusively chitobiose, a common activity in family GH18. In addition, the terms endochitinase and exochitinase are generally applied to distinguish between enzymes that randomly cleave chitin substrates and enzymes releasing products from either end of their substrates, respectively (Seidl, 2008). In this paper the term exochitinase is used for enzymes releasing either monomers or chitobiose from the substrate end.

The genomes of filamentous Ascomycota usually contain 10 to 20 genes encoding GH18 enzymes (Karlsson & Stenlid, 2008; Seidl et al., 2005). GH18 chitinases use substrate assisted catalysis which involves the N-acetyl group of chitin, and retain the chitin β-anomeric configuration in their products (van Aalten et al., 2001). After recent revision of the phylogenetic groups harbouring fungal GH18 members, three
groups are distinguished (Karlsson & Stenlid, 2008; Seidl et al., 2005). Group B (previously class III) members display sequence similarity to plant chitinases. Their active site, on the top of an TIM barrel, forms an open groove (Rush et al., 2010; Terwisscha van Scheltinga et al., 1994). Chitinases with such an active site show endo-acting activity (Hoell et al., 2005; Horn et al., 2006; Terwisscha van Scheltinga et al., 1994). Group A enzymes (previously class V) have sequence similarity to bacterial chitinases. Their catalytic domain resembles that of group B, but an additional domain is inserted in the TIM barrel between β-sheet 7 and α-helix 7 (Perrakis et al., 1994). This 70-90 amino acid (α+β)domain forms part of the wall of the deep substrate binding cleft and influences substrate and product specificities (Li & Greene, 2010; Zees et al., 2009). Chitinases of group A have either exo- and/or processive activity (Fukamizo et al., 2001; Jaques et al., 2003). Group C members were identified recently, when the availability of complete genome sequences of filamentous Ascomycota revealed chitinase sequences distinct from, but with sequence similarity to, the known group A proteins (Karlsson & Stenlid, 2008; Seidl et al., 2005). The large proteins (up to 200 kDa) often contain multiple carbohydrate binding modules of family 18 (CBM18) and lysine motif modules (LysM) (Gruber et al., 2010). CBM18 modules consist of around 40 amino acids, organised in a structure of small α-helices, β-sheets and mostly coil regions, for which binding to chitin and chitin oligosaccharides has been shown (Boraston et al., 2004).

Analysis of expression conditions showed that CfcI of A. niger N402 is detected after the exponential growth phase, during nutrient limitation (Adav et al., 2010; Jorgensen et al., 2010; Lu et al., 2010; Martens-Uzunova & Schaap, 2009; van den Berg et al., 2010; Yuan et al., 2008). It therefore is an interesting candidate for a chitinase involved in sporulation or autolysis, the process where the aging mycelium degrades part of its own hyphae (White et al., 2002). Our first phylogenetic analysis of GH18 members in all eight sequenced Aspergillus species indicated that CfcI of A. niger N402 represents a conserved phylogenetic clade. Members of this clade show sequence similarity to both group A and C chitinases, and have a unique domain structure where a CBM18 is inserted in the catalytic domain. This paper provides the first description of the characterisation of an enzyme from this phylogenetic clade. Biochemical characterisation of the heterologously expressed and purified protein showed that CfcI catalyses the release of GlcNAc from the reducing end of chitin oligosaccharides. To our knowledge this is the first report of a fungal GH18 chitinase with this activity.
Methods

**Phylogenetic analysis.** *A. niger* CBS 513.88 (Pel et al., 2007) amino acid sequences containing Pfam motif PF00704 and catalytic residue motif PS01095 were used to perform Multispecies Blast against the *Aspergillus* Genome Database (AspGD, [http://www.aspergillusgenome.org](http://www.aspergillusgenome.org)) to identify all GH18 members in each genome (Arnaud et al., 2010). Sequences were aligned with M-Coffee (Moretti et al., 2007) to identify group members. Separate alignments were made for both B and A/C groups using Muscle (Edgar, 2004). Alignments were trimmed to the GH18 catalytic domain using BioEdit (Hall, 1999). Mega4 (Tamura et al., 2007) was used to generate bootstrapped (5000 replicates) Neighbour Joining phylogenetic trees, using the Poisson correction model with pairwise gap deletion. *A. niger* An09g06400 (group B) and An02g07020 (group A) were used as outgroup to root the NJ-trees of groups A and C and group B, respectively.

**Protein sequence analysis.** Conserved motifs were identified using InterProScan (Zdobnov & Apweiler, 2001) and ScanProsite (Gasteiger et al., 2003), signal peptides using SignalP (Bendtsen et al., 2004; Nielsen et al., 1997) and Glycosylphosphatidylinositol (GPI) anchors using Big PI fungal predictor (Eisenhaber et al., 2004). Sequence similarities were calculated with Mega4, using the p-distance model (Tamura et al., 2007).

**Construction of the *Escherichia coli* expression vector pBAD-MBP-21.** Standard DNA manipulation techniques were used (Sambrook et al., 1989) and DNA sequences obtained by PCR were confirmed through sequencing. RNA isolated from a batch culture of *A. niger* N402 grown for ~ 90 h in a 5 litre bioreactor (BioFlo 3000, New Brunswick Scientific) in minimal medium (Bennett et al., 1991) with 0.8% maltose, was kindly provided by B. Nitsche and A. Ram of the University of Leiden, the Netherlands. RNA was converted to cDNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Invitrogen) with the provided anchored-oligo(dT), primers. The coding region of *cfcI* was amplified using High Fidelity PCR enzyme mix (Fermentas) and gene specific primers Fw15 5’-GATAAGTCAATTATGAGCCT-GCAGTGCGTGGC-3’ (EcoRI site underlined) and Rev21 5’- GGAGGGTGAC-CAAGCTTTCAATGATGATGATGATGCTGGCAGCAACACCCCTC-3’ (HindIII site underlined, stop codon in bold and 6xhistidine tag in italics) under the following conditions: 2 min at 94 °C, 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 90 s,
followed by 7 min elongation. The amplified fragment was cloned into EcoRI/HindIII digested pBAD-MBP (Heuts et al., 2007), to produce pBAD-MBP-21 which encodes the 89.7 kDa Cfcl with a N-terminal Maltose Binding Protein (MBP) and C-terminal 6xHis-tag fusion.

Construction of *E. coli* expression vector pET-15b-25 and *Pichia pastoris* GS115 harbouring pPICZ α-A-21. To investigate whether the observed characteristics of the MBP-Cfcl fusion protein were dependent on the presence of MBP, or lack of glycosylation, Cfcl was produced both without the MBP fusion domain in *E. coli* and as secreted glycoprotein in *P. pastoris*. *cfcI* was amplified from pBAD-MBP-21 using Fw25 (5’-GATCGACATATGCGGTTCGCCATGTACGTTGATG-3’) (NdeI site underlined) and Rev25 (5’-GATCGAGGGATCCCTACTACCTGGGCAGCAACACC-3’) (BamHI site in italics, stop codon in bold). Ligation into NdeI/BamHI digested pET-15b resulted in pET-15b-25 that allowed expression of Cfcl with a N-terminal 6xHis-tag, and a predicted molecular mass of 47.3 kDa. To construct pPICZα-A-21 for Cfcl expression in *P. pastoris*, the *cfcI* coding region was obtained as EcoRI/SalI fragment from pBAD-MBP-21 and ligated into pPICZα-A. Construction of strain GS115 harbouring pPICZα-A-21 was performed using the EasySelect Pichia Expression Kit (Invitrogen) according to the manufacturer’s instructions.

**Protein expression and purification.** Precultures of *E. coli* TOP10 (Invitrogen) harbouring pBAD-MBP-21 were diluted 1:100 in Luria Broth (Sambrook et al., 1989). After growth at 30 °C until OD$_{600}$ ~ 0.4, expression was induced with 0.1 % (w/v) arabinose. After 4 h, cells were harvested by centrifugation, resuspended in 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 1 mM EDTA and broken by sonication. MBP-Cfcl was partly purified by affinity chromatography using Amylose Resin (New England Biolabs) and anion exchange chromatography at pH 8 using a HiTrap Q Sepharose HP column (1 ml, GE Healthcare). Progress in purification was followed by SDS-PAGE, and the purity in a representative final preparation was analysed by identification of proteins by LC-MS/MS, kindly performed by DSM Biotechnology Centre, Delft, the Netherlands. Protein concentrations were determined with Bradford reagent (Bradford, 1976), using BSA as a standard. Unless stated otherwise, all experiments were performed using the Cfcl-MBP fusion protein, referred to as Cfcl-MBP.

Cfcl without MBP was obtained using *E. coli* BL21 DE3 harbouring pET-15b-25, es-
essentially as described above. Cells were grown at 18 °C, induction was performed with 25 - 200 mM IPTG, and purification was performed using Nickel-NTA resin affinity chromatography. Cfci was obtained as secreted glycoprotein from *P. pastoris* strain GS115-pPICZα-A-21, grown for 48 h in buffered complex glycerol medium followed by 48 h growth in buffered complex methanol induction medium, using the EasySelect Pichia Expression Kit (Invitrogen) according to the manufacturer’s instructions. Cfci was partly purified by anion exchange chromatography. N-Glycosylation was verified by incubation of denatured Cfci with N-glycosidase F (New England Biolabs) and subsequent visualization by SDS-PAGE of the protein molecular mass reduction.

**Enzyme activity assays.** Chitinase activity was routinely measured by 4-nitrophenol group (pNP) release in incubations of 60 µl containing 50 mM sodium acetate buffer pH 5 buffer with 1 mM (GlcNAc)₃-pNP or 0.5 mM (GlcNAc)₂-pNP at 45 °C. Samples of 5 µl were taken at set intervals and the reaction was stopped by addition to 2.5 µl 1 M NaCO₃. The OD₄₀₅ was determined using a Nanodrop ND-1000 spectrophotometer. The initial rate (ΔOD₄₀₅ min⁻¹) is represented by the slope of the trendline produced by linear regression applied to each dataset. The protein concentration was adapted to obtain at least 5 data points. Measurements were performed in triplicate and expressed as mean ± SD. Data were converted to nmol pNP min⁻¹ mg⁻¹ protein using a reference 4-nitrophenol solution (Sigma Aldrich). One unit of enzyme activity (U) was defined as the amount of activity needed to release 1 µmol pNP min⁻¹ from 1 mM (GlcNAc)₃-pNP at 45 °C, pH 5.

**Determination of kinetic parameters.** Kinetic parameters were determined at 45 °C in 450 µl incubations containing 50 mM sodium acetate buffer pH 5 and 0.1 - 2.5 mM (GlcNAc)₂-pNP or 0.05 - 1.2 mM (GlcNAc)₃-pNP. Reactions were started by the addition of 9.3 mU or 2.5 mU Cfci-MBP respectively. At 1, 2, 3 and 4 min, 100 µl samples were inactivated in 100 µl 1 M NaCO₃. The initial rate was calculated as described above, based on at least three data points with measurements performed at least in duplicate. The production of pNP was linear in time under assay conditions and a maximum of 10% of the available substrate was used. Rates obtained from two independent experiments were fitted to the Michaelis-Menten equation using SigmaPlot 12.0. Resulting values are given as mean ± SE.

**Determination of reaction parameters.** The pH optimum was determined by as-
saying activity on \((\text{GlcNAc})_2\)-pNP and \((\text{GlcNAc})_3\)-pNP at pH 3 - 6 in 50 mM sodium citrate buffer and at pH 6 - 8 in 50 mM sodium phosphate buffer, at 45 °C. The temperature optimum was determined by measuring activity on \((\text{GlcNAc})_3\)-pNP in 50 mM sodium acetate buffer pH 5, at temperatures from 25 °C to 75 °C. Temperature stability was determined by incubating either 0.9 µg Cfcl-MBP µl⁻¹ or 0.11 µg Cfcl glycoprotein µl⁻¹ at 30 - 60 °C at pH 5, and assaying remaining activity with \((\text{GlcNAc})_3\)-pNP at 45 °C, pH 5.

**Determination of substrate range.** Cfcl-MBP (0.5-2 mU) was incubated with 10 mM - 1 mM of chitin oligosaccharide, with 0.01-0.1% (w/v) chitin or chitosan or fungal cell wall material (see below), in sodium acetate buffer pH 5, at 45 °C. Product formation was detected either by MALDI-TOF-MS, using 2,5-dihydroxybenzoic acid matrix, in an AXIMA Performance (Shimadzu Biotech), or with high performance anion exchange chromatography (HPAEC) as described below, or by release of pNP. Substrates \((\text{GlcNAc})_{(1-6)}\), \((\text{GlcN})_{(1-6)}\) and pNP-labelled \((\text{GlcNAc})_{(1-3)}\) were obtained from various suppliers (Carbosynth, Sigma Aldrich, Megazyme and Acros). Chitin and chitosan (³75% deacetylated), purified from shrimp shells, was from Sigma Aldrich. Cell walls were isolated from *A. niger* strain N402 (Bos *et al.*, 1988), grown for 48 h at 30 °C, 180 r.p.m., in minimal medium (Bennett *et al.*, 1991) supplemented with 0.5% (w/v) yeast extract and 0.1% (w/v) casamino acids. Mycelium was harvested by filtration over miracloth (Calbiochem), washed with ultra pure water and broken by sonication in 50 mM sodium acetate buffer pH 5. Cell walls were harvested by centrifugation (5900 g for 10 min), washed 3 times and boiled for 10 min to remove cell wall associated enzymatic activities. The cell wall was fractionated into an alkaline soluble and alkaline insoluble fraction essentially as described (Fontaine *et al.*, 2000).

**HPAEC-PAD analysis of reaction time course.** A quantitative HPAEC assay with pulsed amperometric detection was used to follow the reaction time course. Substrate hydrolysis and product formation by Cfcl-MBP on \((\text{GlcNAc})_{(3-6)}\) oligosaccharides was followed by sampling reactions of 60 µl containing ~ 1 mM substrate, 50 mM sodium acetate buffer pH 5 and 1.44 mU Cfcl-MBP activity at 45 °C. The reaction was terminated by dilution of the sample in 245 µl ultra pure water preheated to 90 °C. Analysis was performed on an ICS-3000 system equipped with a CarboPac PA-1 analytical and guard column (Dionex).
**Characterization of chitinase CfcI**

**Anomeric configuration of reaction products.** To identify the anomeric configuration of CfcI-MBP products, 2.9 mU CfcI-MBP was incubated for 2 min in 60 µl with 0.25 mM (GlcNAc)₄, 50 mM sodium acetate buffer pH 5, at 45 °C. Samples were analysed by HPLC immediately after incubation to prevent conversion between the anomeric forms. Volumes of 25 µl were injected on a TSK gel Amide 80 column (4.6 mm diameter, 5 µm particle size, Tosch), separating oligosaccharides by degree of polymerisation and anomeric configuration (Fukamizo et al., 2001). Carbohydrates were detected by ultraviolet absorption at 200 nm using a SpectraSYSTEM UV6000LP detector.

**CfcI reaction specificity.** To establish whether CfcI-MBP releases products from the reducing or non-reducing end, hydrolysis products of (GlcNAc)₂-pNP and (GlcNAc)₃-pNP were identified by HPAEC. Incubations contained 1 mM substrate, 25 mM sodium acetate buffer pH 5 and 1.44 mU CfcI-MBP in 60 µl. The reaction was stopped by adding 10 µl sample to 190 µl 10 mM HCl. In addition, degradation of a (GlcNAc)₅ alditol was analysed. 1 mg (GlcNAc)₅ was reduced (Hreggvidsson et al., 2011) and desalted using a Carbograph Ultra-Clean solid phase extraction column (Grace). Complete reduction was confirmed with MALDI-TOF-MS. 500 mM (GlcNAc)₅ alditol was incubated with 4 mU CfcI-MBP in 60 µl containing 10 mM sodium acetate pH 5 buffer for 40 min or 50 mM (GlcNAc)₅ alditol was incubated with 20 mU CfcI-MBP in 100 µl for 15 h. Reactions were stopped by heating and analysed by HPAEC.

**Results**

**Phylogenetic and sequence analysis.** To get insight in the diversity of *Aspergillus* chitinases and their phylogenetic relationship, 166 GH18 protein sequences were analysed originating from the 8 sequenced *Aspergillus* species (10 strains): *A. clavatus* NRRL 1, *A. flavus* NRRL 3557, *A. fumigatus* A1163, *A. fumigatus* Af293, *A. nidulans* FGSC A4, *A. niger* ATCC 1015, *A. niger* CBS 513.88, *A. oryzae* RIB40, *A. terreus* NIH2624 and *Neosartorya fischeri* NRRL 181. The sequences form three groups, groups A (previously class V), group B (previously class III) and group C. The average sequence identity within group A, B, and C is 42.7 %, 36.9 % and 40.0 % respectively. The overall sequence identity between group A and C is 25.1 %, between group A and B 17.7 %, and between group C and B 17.2 %. Group B and groups A / C together form two distinct groups which show too much sequence
dissimilarity to construct a reliable alignment. For these two groups, sequence alignments and phylogenetic trees were constructed separately. Each group consisted of several subgroups (Fig. 1) which were coherent with subgroups described before (Karlsson & Stenlid, 2008; Karlsson & Stenlid, 2009; Seidl et al., 2005).

In general, fungal sequences of group B are divided into five subgroups, of which only B-I, B-II and B-V contain *Aspergillus* sequences (Fig. 1a). For each analysed species, three to six proteins are found in group B. Characterised proteins are confined to subgroup B-I. *A. nidulans* ChiA (AN8241) is a GPI anchored enzyme localised to the cell wall at hyphal tips and sites where hyphae branch (Yamazaki et al., 2008). The crystal structure of *A. fumigatus* ChiA1 chitinase shows an open active site architecture, consistent with endo-chitinase activity (Rush et al., 2010). The catalytic domains (Pfam motif PF00704, AA residues 28 - 323) of the ChiA orthologs share 71.3 % identity. In subgroup B-II, a clade (indicated in Fig. 1a) is found that contains *A. oryzae* AO090020000231, *A. clavatus* ACLA_006770 and two proteins of *A. fumigates*, all of which have a C-terminal CBM19 (Pfam PF03427). Reannotation of the *A. flavus* and *N. fischeri* enzymes in this clade revealed that also these proteins contain a CBM19. Recently it was shown for the first time that family GH18 contains fungal endo-β-N-acetylglucosaminidases (EC 3.2.1.96), enzymes involved in protein deglycosylation (Hamaguchi et al., 2009; Stals et al., 2010). *Aspergillus* B-V members show homology to these *H. jecorina* and *F. velutipes* enzymes.

The number of group C chitinases ranges from 4 for *A. niger* CBS 513.88 to 12 for *A. terreus* NIH2624. Subdivision of these proteins in subgroups C-I and C-II based on their catalytic domain, coincides with the presence of a LysM module in some C-II proteins, which is in line with observations from others (Gruber et al., 2010).

![Fig. 1. Phylogenetic relationship of the 166 GH18 members present in the 8 sequenced genomes of *Aspergillus* species, based on the amino acid sequences of their catalytic domains, displayed as rooted Neighbour Joining tree. Branch support is indicated by bootstrap values. Sequences are labelled with locus/protein identifiers as found in AspGD and Genbank, followed by the protein name in parentheses if gene/protein features have been characterised. Protein models labelled with their model numbers only, indicated with an asterisk, belong to *A. niger* ATCC 1015. The scale bar corresponds to a distance of 0.1 amino acid substitutions per site. Subgroups are indicated by numbered bars using nomenclature described previously (Karlsson & Stenlid, 2008). Clades discussed in the text are marked with bars, discussed sequences are underlined. *A. niger* CBS 513.88 sequences are represented in bold. (a) Relationship of group B members. (b) Relationship of group A and C members.](image-url)
The genome of *A. clavatus* doesn’t encode subgroup C-II members. None of the putative chitinases in group C have been biochemically characterised. An14g07420 (group C-I), Gw1.4.291.1 (C-I), An12g05530 (C-II) and Gw1.7.232.1 (A-V) were found to be present in only one of the *A. niger* strains.

The *Aspergillus* group A sequences are confined to subgroups A-II, A-IV and A-V (Fig. 1b). Subgroup A-V enzymes generally consist of a catalytic domain with or without a secretion signal peptide. Subgroup A-V contains a clade of orthologous proteins, indicated in Fig. 1b, that includes ChiB of *A. nidulans*, a chitinase involved in autolysis (Yamazaki *et al.*, 2007). The catalytic domains of ChiB orthologs share 76.1 % sequence identity. The slightly more distantly related *A. fumigatus* ChiB1, shown in Fig. 1b, has been characterised as a chitobiosidase (Jaques *et al.*, 2003).

The position of subgroup A-II in the phylogenetic tree is ambiguous, subgroup members display similarity to both group A and group C. In our analysis the A-II subgroup is located closest to the C-I/C-II subgroups, but with a relatively low bootstrap value supporting this branch. Opinions vary whether the A-II group is most closely related to either the C-I/C-II subgroups (Karlsson & Stenlid, 2009) or to the A-IV/A-V subgroups (Alcazar-Fuoli *et al.*, 2011; Karlsson & Stenlid, 2008; Seidl *et al.*, 2005).

*A. niger* N402 CfcI is a representative member of the clade in subgroup A-II which is conserved in *Aspergillus* species. Proteins in this clade have a domain structure which is atypical for chitinases; a carbohydrate binding motif CBM18 (Pfam PF00187) is integrated in the (α-β)-domain in the C-terminal region of the catalytic core. This integration site suggests that spatially the CBM18 is located close to the substrate binding cleft. Two strains, *A. fumigatus* Af293 and *N. fischeri* NRRL 181, each harbour two sequences belonging to the clade. The *afu1g00310* gene has a stop codon located within CBM18, suggesting protein truncation. In addition to the CfcI clade, subgroup A-II contains AN0221, which lacks a CBM18 and has an ER-targeting signal. *A. nidulans* harbours the only member of this second clade among the *Aspergillus* species but homologs are present in other fungi (Karlsson & Stenlid, 2009).

Both the unusual domain organisation of CfcI and its orthologs, and their sequence dissimilarity to previously characterised chitinases, suggest that these enzymes may have an activity or function different from proteins investigated so far. To characterise CfcI, the protein was heterologously expressed and biochemically characterised.
Cloning and expression of CfcI. The coding region of cfcI, excluding the predicted secretion signal peptide, was amplified from cDNA. Initial tests to express CfcI resulted in insoluble protein. To facilitate folding of CfcI and thereby overproduction of soluble protein, the coding region was cloned into vector pBAD-MBP, allowing expression in E. coli of CfcI fused at the N-terminus to a Maltose Binding Protein (CfcI-MBP). Sequencing of the obtained coding region showed that the position and length of introns corresponds with the prediction of the recently updated gene model, GenBank accession number XM_001400452.2. CfcI-MBP was partly purified by MBP affinity chromatography and subsequent anion exchange chromatography, where the bulk of the CfcI-MBP protein eluted at 300 – 510 mM NaCl. The fusion protein contains a C-terminal His-tag, but since no binding to Ni-NTA resin could be achieved this tag could not be used for purification. The total yield of CfcI-MBP was 1.5 - 2.8 mg protein l⁻¹ culture, with an activity of 0.4-0.9 mU µg⁻¹. SDS-PAGE analysis showed CfcI-MBP was present as the main constituent of the purified protein sample, in addition to multiple bands of low intensity. LC-MS/MS analysis of the final sample of a representative purification revealed that the CfcI-MBP protein was free from contaminating proteins predicted to have interfering activities. In addition, CfcI was obtained as secreted glycoprotein from Pichia pastoris. N-Glycosylation was verified by incubation of CfcI with N-glycosidase F. Subsequent visualization by SDS-PAGE of the protein showed a reduction in molecular mass from ~ 61 - 71 kDa to ~ 47 kDa, which is the predicted mass for the non-glycosylated protein.

Reaction parameters. CfcI-MBP has a relatively acidic pH optimum (pH 4 - 5) and activity levels drop considerably around neutral pH (Fig. 2a). Relative activity on (GlcNAc)₃-pNP at pH 6 and 7 was higher than on (GlcNAc)₂-pNP. Activity at pH 3 was observed only on (GlcNAc)₃-pNP, and the enzyme was rapidly inactivated in these conditions. The maximum temperature for CfcI-MBP activity was found at 55 °C to 65 °C (Fig. 2b). Although CfcI-MBP has high activity at elevated temperatures, the enzyme was not stable under these conditions. Thermal stability assessment showed that after 30 min incubation at 40 °C or 50 °C, CfcI-MBP retained 75 ± 4 % or 34 ± 1 % of its activity, respectively. No residual activity was observed after incubation for 30 min at 60 °C. When repeating the experiments with a CfcI preparation obtained as heterologously expressed glycoprotein from P. pastoris, essentially the same pH and temperature dependencies were observed, although with higher activity at 60 °C and 65 °C. Also the thermostability of the glycosylated CfcI protein was clearly increased: after 30 min incubation at 40 °C or 50 °C, CfcI retained 99 ±
Fig. 2. Dependency of Cfcl-MBP activity on (a) pH, and (b) temperature. Activity was measured on (GlcNAc)_2-pNP (□) and (GlcNAc)_3-pNP (■). Values are given as percentages of the maximum activity and are the mean of three measurements ± standard deviation. The activity of Cfcl-MBP at pH 6 in sodium citrate buffer (value not depicted) is 89 ± 4 % of the activity in sodium phosphate buffer.

7 % or 91 ± 6 % of its activity, respectively. The K_m of Cfcl-MBP was determined with the two commercially available pNP substrates (GlcNAc)_2-pNP and (GlcNAc)_3-pNP giving a K_m of 2.0 ± 0.2 mM and 0.21 ± 0.02 mM respectively. These results indicate that the affinity of Cfcl-MBP for the longer substrate (GlcNAc)_3-pNP is approximately 10 times higher as for the short substrate (GlcNAc)_2-pNP.

**Substrate range.** Cfcl-MBP hydrolysed native chitin oligosaccharides (GlcNAc)_{3-6}, as well as (GlcNAc)_2-pNP and (GlcNAc)_3-pNP. The shorter GlcNAc-β-pNP, GlcNAc-α-pNP and (GlcNAc)_2 were not hydrolysed. Activity was observed on chitin, but this most likely resulted from degradation of the small fraction of soluble oligosaccharides present in the chitin preparation. After washing the polymer to remove such oligosaccharides, a strong decrease in product formation was observed. Incubation of 10 µg (3.2 U) Cfcl-MBP for up to 24 hr with cell walls of A. niger, or the cell wall alkaline insoluble fraction (containing chitin partly purified by alkaline extraction of surrounding glucans), did not result in detectable product formation.

**Reaction time course.** To gain insight into the reaction specificity of Cfcl-MBP, hydrolysis of chitin oligosaccharides was followed in time by quantification of substrates and products using HPAEC (Fig. 3). (GlcNAc)_3 was degraded into equimolar amounts of GlcNAc and (GlcNAc)_2. (GlcNAc)_4 was degraded to mainly GlcNAc and (GlcNAc)_2. (GlcNAc)_3 initially accumulated, but was re-utilised during the incuba-
Fig. 3. Time course of substrate hydrolysis by CfcI-MBP (a) (GlcNAc)$_3$, (b) (GlcNAc)$_4$, (c) (GlcNAc)$_5$, and (d) (GlcNAc)$_6$. Carbohydrates, identified as GlcNAc (▲), (GlcNAc)$_2$ (△), (GlcNAc)$_3$ (■), (GlcNAc)$_4$ (□), (GlcNAc)$_5$ (●) and (GlcNAc)$_6$ (○) are shown as quantified by HPAEC-PAD in a representative reaction.

tion, reaching final concentrations of ~ 0.2 mM. Comparable observations were made with substrates (GlcNAc)$_5$ and (GlcNAc)$_6$, the main reaction product GlcNAc reached concentrations of 1.1 mM and 1.2 mM, respectively. In addition, 160 - 250 µM (GlcNAc)$_3$ and (GlcNAc)$_2$ (an end product of the reaction) were produced, and maximal 45 µM of (GlcNAc)$_4$ and 40 µM (GlcNAc)$_5$ was detected. These results demonstrate that CfcI degrades chitin oligosaccharide substrates to produce predominantly GlcNAc. The final amount of GlcNAc detected in each incubation corresponds with the amount of monomers that would be generated by a strictly exo-acting enzyme that hydrolyses oligosaccharides by releasing GlcNAc from its termini until the length is decreased to (GlcNAc)$_2$. Similar results were obtained when repeating these experiments using CfcI without the MBP fusion, indicating that the MBP does not affect the course of the reaction. Also with glycosylated CfcI, obtained from heterologous expression in P. pastoris, no indications were found that glycosylation affected the reaction time course (data not shown).
Figure 4. HPLC analysis of the anomeric configuration of products from \((\text{GlcNAc})_4\) hydrolysis, as produced by Cfcl-MBP, depicted as a black line. For reference, peaks of the \(\alpha\) and \(\beta\) anomer of standards of GlcNAc, \((\text{GlcNac})_2\), \((\text{GlcNAC})_3\) and \((\text{GlcNAc})_4\) are shown in grey. The proposed underlying mechanism of action is shown schematically. The reducing end of the substrate is indicated in black and numbered arrows indicate consecutive hydrolysis events of which the products are shown.

**Anomeric configuration of reaction products and direction of substrate degradation.** The anomeric forms of the Cfcl-MBP products were determined by HPLC to establish whether Cfcl-MBP functions with retention or inversion of the \(\beta-(1-4)\) glycosidic linkage of the substrate. Quick, complete degradation of \((\text{GlcNAc})_4\) by Cfcl-MBP resulted in \((\text{GlcNAc})_2\) which was present as \(\beta\)-anomer \((\beta\text{-GlcNAC-(1-4)-}\beta\text{-GlcNAC})\), and GlcNAc which was present predominantly as \(\beta\)-anomer (Fig. 4). These results indicate that Cfcl-MBP retains the \(\beta\)-glycosidic linkage of its substrate during hydrolysis. The anomeric forms of the reaction products also identify their origin. \(\beta\)-GlcNAC-(1-4)-\(\beta\)-GlcNAC could not originate from the reducing end of the substrate. It is a product generated by two consecutive releases of GlcNAc from the reducing end. From each \((\text{GlcNAC})_4\) substrate molecule, the first cleavage results in \(\beta\)-GlcNAC-(1-4)-\(\beta\)-GlcNAC and GlcNAc in the equilibrium ratio of \(~60:40\) \(\alpha\)-anomer/\(\beta\)-anomer. Subsequent hydrolysis releases \(\beta\)-GlcNAC-(1-4)-\(\beta\)-GlcNAC and \(\beta\)-GlcNAC. Together the monomers have a \(~30:70\) \(\alpha\)-anomer/\(\beta\)-anomer ratio, as is observed (Fig. 4) as a result of Cfcl-MBP activity.

To further investigate the direction of the reaction, \((\text{GlcNAc})_2\)-pNP and \((\text{GlcNAC})_3\)-
Fig. 5. Products formed by CfcI-MBP during hydrolysis of labelled substrates (c) \((\text{GlcNAc})_2\)-pNP and (d) \((\text{GlcNAc})_3\)-pNP, separated and detected by HPAEC-PAD as described in the methods section. Identity of the peaks was determined by comparison to standards \((\text{GlcNAc})_{1-6}\) (a) and \((\text{GlcNAc})_{1-3}\)-pNP (b).
Characterization of chitinase CfcI

pNP hydrolysis was followed using HPAEC. (GlcNAc)$_2$-pNP hydrolysis exclusively resulted in the detection of (GlcNAc)$_2$ (Fig. 5c, note that unbound pNP can’t be detected using this method). Hydrolysis of (GlcNAc)$_3$-pNP yielded the detectable products (GlcNAc)$_3$, (GlcNAc)$_2$ and GlcNAc (Fig. 5d). These products are consistent with a reaction mechanism where the substrate is degraded from the reducing end, releasing the pNP group, followed by further degradation if possible. No products were observed that would be expected to arise from CfcI-MBP action on the non-reducing end, such as GlcNAc-pNP or (GlcNAc)$_2$-pNP. Correspondingly, reduction of (GlcNAc)$_5$, which changes the reducing end GlcNAc to an open ring structure, prevented degradation of the substrate by CfcI-MBP in conditions during which non-modified substrate was completely hydrolysed (data not shown). Only upon prolonged incubation with high amounts of CfcI-MBP, partial substrate hydrolysis was observed. Taken together, these results show CfcI-MBP acts through a hydrolytic activity that originates at the reducing end of chitin oligosaccharides.

Discussion

Although it is recognised that Ascomycetes have a large number of GH18 enzymes, the exact function of this is not yet understood (Karlsson & Stenlid, 2008; Seidl, 2008). Most likely functional and regulatory diversification took place during evolution, although recent gene duplications may have caused redundancy. Phylogenetic analysis of GH18 enzymes showed the existence of seven groups of proteins with direct orthologs in all sequenced Aspergillus species. Their conservation suggests these enzymes perform key physiological functions. So far characterised proteins show diverse functions and locations for representatives of each group (Hamaguchi et al., 2009; Jaques et al., 2003; Stals et al., 2010; Yamazaki et al., 2007; Yamazaki et al., 2008). CfcI represents one of the conserved groups of orthologs of which the activity was uncharacterised until now. The atypical domain organisation of CfcI suggested the protein may have an activity or function different from known chitinases.

CfcI, expressed as maltose binding protein fusion CfcI-MBP, is a chitinase with a mechanism of action that retains the β-(1,4) anomeric configuration found in its substrate, a common property of GH18 enzymes. However, the products formed by CfcI-MBP during substrate hydrolysis, and the direction of this reaction, are unusual. The time course analysis of hydrolysis of GlcNAc oligosaccharides revealed that CfcI releases monomers from its substrate. The molar amounts of formed products were consistent only with exo-chitinase activity. Experiments with CfcI-MBP acting
on both native and labelled substrates indicate this enzyme acts on the reducing end. The exo-acting enzymes of family GH18 usually release mainly \((\text{GlcNAc})_2\) from either the reducing end such as \(\text{ChiA}\) of \(\textit{Serratia marcescens}\), or from the non-reducing end, such as \(\textit{A. fumigates}\) ChiB and \(\textit{S. marcescens}\) ChiB (Horn \textit{et al.}, 2006; Jaques \textit{et al.}, 2003). The exclusive release of monomers is a property usually found in the GH20 family, but there the reaction starts at the non-reducing end. The activity of Cfcl is unique for fungal enzymes and uncommon in the GH18 family, although it is not biochemically unique. Controlled degradation of N-glycosylated proteins releases oligosaccharides with a terminal chitobiose, which can be hydrolysed by lysosomal GH18 enzymes in some higher eukaryotes (Balducci \textit{et al.}, 2008). The lysosomal enzyme from rat was shown to also be able of hydrolysing \((\text{GlcNAc})_2\) by releasing GlcNAc from the reducing end, but could not degrade GlcNAc-pNP (Aronson, Jr. \textit{et al.}, 1989). However, although biochemically similar, the protein location and pH activity profile suggest the function of these enzymes is not related to Cfcl function.

Following substrate hydrolysis in time showed that Cfcl-MBP produced mainly the reaction end products GlcNAc and \((\text{GlcNAc})_2\). The detected amount of intermediate products was low directly from the start of the reaction. Cfcl-MBP was found to have a higher affinity for the longer oligosaccharide, ruling out preferential hydrolysis of short intermediate products. This implies that the enzyme acts processively, without releasing the substrate after the initial attack of the glycosidic bound. Since chitin consists of alternately orientated GlcNAc residues, processive cleavage of the glycosidic bounds is thought to be possible only by advancing the substrate chain by two monomers before the next cleavage event. Indeed, processive chitinases often release a dimer from chitin and studies using the partly deacetylated chitosan show that this substrate is advanced by two sugar residues, resulting in products consisting mainly of an even number of sugar units (Eijsink \textit{et al.}, 2008). However, processive exochitinases analysed so far are all chitobiosidases, and the situation may be different for Cfcl. For Cfcl to be able to release monomers by a processive mechanism, the substrate chain should be moved in such a way through the active site that hydrolysis can take place after each GlcNAc. Possibly this could be achieved by releasing the substrate after cleavage from the active site, but holding on to it with the CBM18 and thus allow quick repeated binding and cleavage. The CBM18 in chitinases is usually clearly separated from the catalytic domain by a linker sequence. In Cfcl the CBM18 is embedded in the \((\alpha+\beta)\)-domain, which forms
part of the substrate binding cleft. This positions the CBM18 in close proximity to the substrate, enabling a direct function in substrate hydrolysis.

Taken together, our results show that the fungal enzyme CfcI is a representative of a phylogenetic clade conserved in *Aspergillus* species. CfcI is an exo-acting chitinase that releases monomers from the reducing end of chitin oligosaccharides, possibly using a processive mechanism. This activity is uncommon for GH18 members and so far unique among fungal GH18 enzymes. This suggests the function of CfcI might be different from currently characterised GH18 family members. Expression of CfcI has been identified by proteome and transcriptome studies during the late exponential or stationary growth phase and under nutrient limitation, however (with one exception) not during exponential growth (Adav *et al.*, 2010; Ferreira de Oliveira *et al.*, 2011; Jorgensen *et al.*, 2010; Lu *et al.*, 2010; Martens-Uzunova & Schaap, 2009; van den Berg *et al.*, 2010; Yuan *et al.*, 2008). These expression conditions indicate that the physiological function of CfcI is important in processes that take place during the late stages of the *Aspergillus* life cycle, such as autolysis or sporulation. Alternatively, during periods of nutrient shortage, CfcI could generate directly available sugars in the form of GlcNAc by hydrolysing chitin oligosaccharides. The exact in vivo physiological function of CfcI is currently under investigation.

**Acknowledgements**

We are grateful for the LC-MS/MS analysis performed by M. Akeroyd (DSM Biotechnology Centre, Delft, the Netherlands) and for RNA of *A. niger* provided by B. Nitsche and A. Ram (University of Leiden, the Netherlands). The genome sequences used were generated by the *Aspergillus* Comparative Sequencing Project, Broad Institute of Harvard and MIT (http://www.broadinstitute.org/), US department of Energy Joint Genome Institute (http://www.jgi.doe.gov/) and by J. Craig Venter Institute (http://www.jcvi.org/). We acknowledge SenterNovem for funding of IOP Genomics project IGE07008.

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