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. [Thesis fully internal (DIV), University of Groningen]. [S.n.]. https://doi.org/10.1016/j.carres.2015.01.014

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Characterization of the starvation induced chitinase CfcA and alpha-1,3-glucanase AgnB of *Aspergillus niger*

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Submitted for publication
Abstract
The common saprophyte *Aspergillus niger* may experience carbon starvation in nature as well as during industrial fermentations. Starvation survival strategies, such as conidiation or the formation of exploratory hyphae, require energy and building blocks, which may be supplied by autolysis. Glycoside hydrolases are key effectors of autolytic degradation of fungal cell walls, but knowledge on their identity and functionality is still limited. We recently identified *agnB* and *cfcA* as two genes encoding carbohydrate active enzymes that had notably increased transcription during carbon starvation in *A. niger* (Chapter 2), (Nitsche *et al.*, 2012). Here we report the biochemical and functional characterization of these enzymes. AgnB is an α-1,3-glucanase that releases glucose from α-1,3-glucan substrates with a minimum degree of polymerization of 4. CfcA is a chitinase that releases dimers from the non-reducing end of chitin. These enzymes thus attack polymers that are found in the fungal cell wall and may have a role in autolytic fungal cell wall degradation in *A. niger*. Indeed, cell wall degradation during carbon starvation was reduced in the double deletion mutant ∆cfcA ∆agnB compared to the wild type strain. Furthermore, the cell walls of the carbon starved mycelium of the mutant contained a higher fraction of chitin or chitosan. The function of at least one of these enzymes, CfcA, therefore appears to be in the recycling of cell wall carbohydrates under carbon limiting conditions. CfcA thus may be a candidate effector for on demand cell lysis, which could be employed in industrial processes for recovery of intracellular products.
Introduction

The filamentous fungus *Aspergillus niger* secretes large amounts of proteins and metabolites, a capacity that is harnessed in industry for the production of enzymes and organic acids (Ward *et al.*, 2006). This common saprophyte may experience carbon starvation under natural and industrial growth conditions, which induces autophagy and autolysis: self-digestion accompanied by the expression of hydrolytic enzymes, fragmentation of hyphae and loss of biomass (Nitsche *et al.*, 2012; White *et al.*, 2002). These events may generate the energy and building blocks that are required for the initiation of survival strategies, such as conidiation or the formation of new exploratory hyphae. Autolytic phenomena may cause problems during industrial fermentations – such as damage to overexpressed proteins by proteases – but may also be taken advantage of, for example to release intracellular products (Emri *et al.*, 2008; White *et al.*, 2002). Understanding the processes initiated in response to carbon starvation is therefore key to understanding part of the fungal life cycle, and also may have commercial benefits.

The main effectors of autolytic degradation of fungal cell walls are glycoside hydrolases. As reviewed by (Emri *et al.*, 2008), autolytic events such as the production of hydrolytic enzymes in *A. nidulans* are repressed by glucose. Carbon starvation results in increased transcription of genes encoding proteases and glycoside hydrolases in both *A. niger* and *A. nidulans* (Nitsche *et al.*, 2012; Szilagyi *et al.*, 2013). In *A. nidulans*, the starvation induced chitinase ChiB and endo-β-1,3-glucanase EngA are important during autolysis, deletion of the corresponding genes results in a decrease of biomass loss and reduced pellet disintegration (Shin *et al.*, 2009; Szilagyi *et al.*, 2010; Yamazaki *et al.*, 2007). Interestingly, deletion of the homologous *A. fumigatus chiB1* gene - also encoding the main chitinase induced during starvation - does not result in a change in biomass loss during starvation (Jaques *et al.*, 2003): the physiological roles of these enzymes thus appear to be different. *A. fumigatus* ChiB1 hydrolyzes chitin and chitin oligosaccharides by cleaving mainly chitobiose from the non-reducing substrate end, and can also perform a transglycosylation reaction (Escott *et al.*, 1998; Jaques *et al.*, 2003; Lu *et al.*, 2009; Xia *et al.*, 2001). The *A. nidulans* ChiB appears to have similar activity (Erdei *et al.*, 2008).

The cell wall of Aspergilli contains - next to chitin and β-glucan – around 40 % alkaline soluble material (Maubon *et al.*, 2006), which mainly consists of α-1,3-linkage containing glucose polymers (α-1,3-glucan and α-1,3;1,4-glucan) (Johnston, 1965),
thus constituting an abundant potential carbohydrate source. Enzymes degrading α-1,3-glucan have been reported in several fungal species (Ait-Lahsen et al., 2001; Fuglsang et al., 2000; Sanz et al., 2005; Shalom et al., 2008; Wiater et al., 2001). The *Trichoderma harzianum* MutA enzyme has been characterized in most detail; it cleaves α-1,3-glucan substrates with an endo-mechanism and processively releases glucose (Grün et al., 2006). These α-1,3-glucanases enable cell separation and release of ascospores in the yeast *Schizosaccharomyces pombe* (Dekker et al., 2004; Dekker et al., 2007; Garcia et al., 2005) and are suggested to play a role in myco-parasitism in *Trichoderma* species (Ait-Lahsen et al., 2001; Sanz et al., 2005). An α-1,3-glucanase of *A. nidulans* hydrolyzes cell wall material during sexual reproduction, possibly to generate the energy needed for cleistothecium (fruiting body) production (Wei et al., 2001; Zonneveld, 1972b). In *A. niger*, which in contrast to *A. nidulans* has no known sexual cycle, the roles of α-1,3-glucanases are as yet unknown.

We recently used a systems biology approach to identify and investigate the regulation of carbon starvation induced glycoside hydrolases in *A. niger* (Chapter 2) (Nitsche et al., 2012). The putative α-1,3-glucanase AgnB (An07g08640) and the putative chitinase CfcA (An02g07020) were strongly induced in response to carbon starvation. The functions of these enzymes have not been described. In this study, we therefore have investigated the biochemical properties of the AgnB and CfcA enzymes, also aiming to elucidate their physiological roles during starvation in *A. niger*.

**Methods**

**Construction of *E. coli* expression vectors pBAD-MBP-23 and pET-15b-26.** Standard DNA manipulation techniques were used during cloning procedures (Sambrook et al., 1989) and the integrity of PCR products was confirmed by DNA nucleotide sequencing. RNA was obtained from batch cultures of *A. niger* grown under carbon limited conditions, and used to produce cDNA as described previously (Chapter 5). The *cfcA* (GenBank accession number NT_166519) coding region was amplified from this cDNA using primers Fw23 and Rev23 (Table 1) in a PCR with High Fidelity PCR enzyme mix (Fermentas) under the following conditions: 2 min at 94 °C, 30 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 90 s, followed by 7 min elongation. The isolated PCR product was cloned into vector pBAD-MBP (Heuts et al., 2007) using EcoRI and SalI restriction sites. The resulting vector PBAD-MBP-23
Encodes CfcA fused to an N-terminal maltose binding protein and a C-terminal 6xHis-tag (MBP-CfcA).

The coding region of *agnB* (GenBank accession number NT_166523) without the predicted signal peptide was amplified using primers Fw26 and Rev26 in a PCR under similar conditions as described for *cfcA* but with annealing performed at 55 °C and elongation for 120 s. The PCR product was cloned into pET-15b using the *Bam*HI and *Nde*I restriction sites. The resulting vector pET-15b-26 encodes AgnB fused to an N-terminal 6xHis-tag.

**Protein production and purification.** *E. coli* Top10 (Invitrogen) harbouring plasmid pBAD-MBP-23, encoding MBP-CfcA, was grown overnight and diluted 1:100 in Luria Broth (Sambrook *et al.*, 1989). Cultures were grown until an OD$_{600}$ of ~0.4 and protein expression was induced with 0.1 % (w/v) arabinose. After 4 h of growth, cells were harvested by centrifugation and dissolved in 20 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM EDTA. Cells were broken using sonication and cell free extracts were prepared by centrifugation. MBP-CfcA was purified using affinity chromatography with Amylose Resin (New England Biolabs) followed by anion exchange chromatography at pH 8.0 using a HiTrap Q Sepharose HP column (1 ml, GE Healthcare). Fractions containing the pure protein of interest were pooled and dialysed to 5 mM sodium acetate buffer pH 5.0.

*E. coli* BL21 DE3 (Invitrogen) harbouring pET-15b-26, encoding AgnB, was grown as described above. Protein expression was induced with 25 µM IPTG at an OD$_{600}$ of around 0.3. After 4 h of growth, cells were harvested and lysed with 20 ml B-PER Bacterial protein extraction reagent (Thermo Scientific) per aliquot of cells from 500 ml of culture, supplemented with lysozyme and DNAse. After 3 h at room temperature (RT), the suspension was centrifuged at $8 \cdot 10^3$ g for 10 min at 4 °C to collect insoluble material. The pellet was washed twice with 10x diluted B-PER and finally dissolved at RT in 8 M Urea supplemented with 1 mM DTT to unfold the AgnB protein. Refolding was achieved by dialysis to 10 mM Tris-HCl buffer pH 8.0, and after centrifugation to remove precipitated proteins, soluble AgnB was collected.

**Chitinase activity assays.** Chitinase activity was routinely determined as described before using GlcNAc$_2$-β-1,4-GlcNac-β-pNP (GlcNAc$_2$-pNP) as substrate (Chapter 5). Briefly, from incubations of MBP-CfcA with 0.5 mM (GlcNAc)$_2$-pNP at pH 6.0 and 45
°C, aliquots were taken in time and added to 1 M sodium carbonate. The increase in pH stops the reaction and allows quantitation of any released pNP groups at OD$_{405}$. The reaction rate was determined in triplo and product formation was linear in time under assay conditions. One unit of enzyme activity (U) was defined as the amount of enzymatic activity producing 1 µmol pNP min$^{-1}$ from 0.5 mM (GlcNAc)$_2$-pNP at 45 °C, pH 6.0.

To identify the pH optimum, release of pNP from (GlcNAc)$_2$-pNP was monitored in incubations performed at 45 °C and pH 3-6 in sodium citrate buffer or pH 6-8 in sodium phosphate buffer or pH 8-9 in Tris-HCl buffer. To detect the temperature optimum, the reaction rate on (GlcNAc)$_2$-pNP was determined in incubations performed at pH 6.0, with temperatures in the range of 25-75 °C using 5 °C increments. Temperature stability was monitored by incubating aliquots of enzyme (1.6 µg µl$^{-1}$) at pH 6.0, at 30-60 °C for 10, 30 or 60 min, followed by determination of the residual activity at pH 6.0 and 45 °C.

To determine the anomic form and identity of the hydrolysis products generated by MBP-CfcA, 1.7 mU of enzyme was incubated in 60 µl sodium acetate buffer of pH 6.0 with 0.5 mM (GlcNAc)$_5$ at pH 6.0, 45 °C for 1 min. Directly after incubation, an aliquot of 25 µl was analyzed using HPLC as described previously (Fukamizo et al., 2001), (Chapter 5).

To analyze the substrate range, 2.2 mU MBP-CfcA was incubated for 24 h in 200 µl with 20 mM sodium acetate buffer pH 5.0 and 0.1 % (w/v) A. niger cell walls, alkaline soluble cell wall fractions or alkaline insoluble cell wall fractions. Fungal cell walls and cell wall fractions were obtained as described before (Chapter 5). Activity on chitin was analyzed from incubations containing 2.2 mU MBP-CfcA per ml and 1 % (w/v) chitin, purified from shrimp shells (Sigma Aldrich). Activity on soluble chitin oligosaccharides was followed in time. Incubations were performed with 0.15 mU MBP-CfcA in 60 µl containing 1 mM substrate (GlcNAc)$_{2-6}$ (Megazyme), and 25 mM sodium phosphate buffer pH 6.0 at 45 °C. Aliquots of 5 µl were taken at 5 min intervals and heat inactivated in 245 µl ultra pure water. Product formation was assessed using HPAEC-PAD as described previously, using conditions that allow separation of chitin oligosaccharides in the range of GlcNAc to (GlcNAc)$_6$ (Chapter 6). To analyze the transglycosylation reaction, 1.5 mU MBP-CfcA was mixed with 1 mM (GlcNAc)$_2$-pNP in 60 µl 50 mM sodium acetate buffer pH 5.0, and incubated
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at 45 °C. Aliquots of 10 µl were taken and inactivated in 190 µl 10 mM hydrochloric acid. Products were analyzed using HPAEC-PAD as detailed above.

**Protoplastation assays.** The ability of MBP-CfcA to assist in releasing protoplasts from *A. niger* mycelium was tested by performing protoplastation as described (de Bekker *et al.*, 2009). An enzyme cocktail was used containing 5 mg ml⁻¹ lysing enzymes of *Trichoderma harzianum* (Sigma Aldrich), 460 U ml⁻¹ β-glucuronidase from *Helix pomatia* (Sigma Aldrich), and either 150 mU ml⁻¹ chitinase from *Streptomyces griseus* (Sigma Aldrich) or 80 mU MBP-CfcA ml⁻¹. *A. niger* was grown for 17 h at 30 °C at 160 rpm and 1.25 g mycelium (wet weight) was lysed in 10 ml volume for 2 h at 37 °C while shaking at 100 rpm. Protoplasts were harvested by filtration over Miracloth (Calbiochem) and washed with 40 ml STC (de Bekker *et al.*, 2009). After centrifugation, the top 30 - 35 ml liquid was removed and protoplasts were counted in the remaining volume. The stimulatory effect of MBP-CfcA on protoplast formation was tested statistically using a one-sided T-test.

**Substrates for α-1,3-glucan enzyme assays.** Nigeran from *Aspergillus japonicus* was obtained from Sigma Aldrich. Nigerose (Glc-α-1,3-Glc) and nigerotriose (Glc-α-1,3-Glc-α-1,3-Glc) were from Dextra Laboratories. *A. niger* cell walls were isolated as described previously (Chapter 4) and fractionated into an alkaline soluble part and alkaline insoluble part essentially as described (Fontaine *et al.*, 2000). Mutan isolated from *Streptococcus mutans* was used (Li *et al.*, 2012). The α-glucans EPS-180 (α-1,3/1,6-glucan) and EPS-ML1 (α-1,6-branched α-1,3-glucan), a gift of S. Kralj, Groningen, the Netherlands, were produced from sucrose using glucan-sucrase enzymes GTF180 and GTFML1 of *Lactobacillus reuteri* (Kralj *et al.*, 2004; van Leeuwen *et al.*, 2008). The α-1,3-glucan originating from fruiting bodies of *Polyporus betulinus* (Birch bracket) was a gift of Prof. J.R. Clamp, Bristol, UK (Grün, 2003). The alkaline soluble fraction of *A. nidulans* cell walls was a gift from B. J. Zonneveld, Leiden, the Netherlands.

Various α-1,3-glucan oligosaccharides were produced by partial acid hydrolysis of the alkaline soluble (AS) fraction of the *A. nidulans* cell wall, generally as described by (Grün *et al.*, 2006). In short, 10 portions of 100 mg AS were each incubated in 3 ml 98 % formic acid at 100 °C for 14 min, after which the formic acid was evaporated. The pellet was suspended in 2.5 ml 0.5 M TFA and heated for 15 min to 100 °C. The soluble fraction was collected, freeze dried and dissolved in 10 mM ammo-
nium bicarbonate. The hydrolysis products were fractionated on a Bio-Gel P-4 (fine) column (90 x 1 cm), and were eluted with 10 mM ammonium bicarbonate at a flow rate of 12 mL h⁻¹. Fractions (1.5 mL) were collected and analyzed by MALDI-TOF-MS. Selected fractions, for which the MALDI-TOF-MS mass spectra showed a major [M+Na]⁺ peak, indicating purity grades of >95%, were analyzed by 1D ¹H NMR.

**Matrix-assisted laser-desorption ionization time-of-flight mass spectrometry.** MALDI-TOF-MS experiments were performed on an Axima™ mass spectrometer (Shimadzu Kratos Inc., Manchester, UK) equipped with a nitrogen laser (337 nm, 3 ns pulse width). Positive-ion mode spectra were recorded using the reflector mode at a resolution of 5000 FWHM and delayed extraction (450 ns). The accelerating voltage was 19 kV with a grid voltage of 75.2%; the mirror voltage ratio was 1.12, and the acquisition mass range was 200–6000 Da. Samples were prepared by mixing on the target 0.5 μL sample solutions with 0.5 μL aqueous 10% 2,5-dihydroxybenzoic acid as matrix solution.

**NMR spectroscopy.** Resolution-enhanced one-dimensional 500-MHz ¹H NMR spectra were recorded in D₂O on a Bruker DRX-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University) or a Varian Inova Spectrometer (NMR Center, University of Groningen) at probe temperatures of 300 K. Prior to analysis, samples were exchanged twice in D₂O (99.9 atom % D, Cambridge Isotope Laboratories, Inc., Andover, MA) with intermediate lyophilization, and then dissolved in 0.6 mL D₂O. Suppression of the HOD signal was achieved by applying a WEFT pulse sequence. Chemical shifts (δ) are expressed in ppm by reference to internal acetone (δ 2.225 for ¹H).

**α-1,3 Glucanase activity assays.** To assess α-glucanase activity, 100 μl enzyme was incubated in 500 μl volume with 50 mM sodium acetate buffer pH 5.0, 2 mM sodium azide, 0.1 % (w/v) polymeric substrate or 0.15 μl oligosaccharide. After overnight incubation at 30 °C, insoluble material was removed by centrifugation. The formation of soluble reaction products was analyzed by HPAEC-PAD, using an ICS-3000 system (Dionex) equipped with a CarboPac PA-1 analytical column ( Dionex). Carbohydrate separation was achieved with a mobile phase of 0.1 M sodium hydroxide supplemented with 30 μM to 100 μM sodium acetate in a 30 min gradient. Detection was performed using a standard quadruple potential waveform (Rocklin et al., 1998).
Glucose determinations were performed using the GOPOD glucose detection assay (Megazyme), adapted to a microtiter plate format.

**Deletion of the *A. niger cfcA* and *agnB* genes.** To create an *A. niger cfcA* knockout strain, vector pDEST-23 was constructed. Primers Fw34 and Rev34 were used to amplify the 1057 bp region upstream of *cfcA* from genomic DNA of *A. niger* N402 (Bos et al., 1988) in a PCR under the following conditions: 2 min at 94 °C, 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 90 s, followed by 7 min elongation. The obtained product was introduced in vector pDONR P4-P1R of the MultiSite Gateway Three Fragment Vector construction kit (Invitrogen) according to the manufacturer’s specifications. Primers Fw30 and Rev30 were used to obtain a 1018 bp region downstream of *cfcA* by PCR as detailed above but with primer annealing at

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56 °C for 30 s. The obtained product was introduced in vector pDONR P2R-P3. Vector pDONR P4-P1R-34 and pDONR P2R-P3-30 were combined with pDONR221 containing the *A. oryzae* pyrG region (kindly provided by B. Nitsche, University of Leiden) and pDEST R4-R2 vector II, according to the manufacturer’s instructions, to create pDEST-23 which contains the *A. oryzae* pyrG region flanked by the up- and downstream region of *cfcA* (Fig. 1A). pDEST-23 was linearized with VspI and transformed to *A. niger* AB4.1, a pyrG derivative of N402 (van Hartingsveldt et al., 1987). Transformation was performed as described in detail by (Arentshorst et al., 2012). Genomic DNA of purified transformants was isolated using the E.Z.N.A Fungal DNA Kit (Omega Bio-tek). The absence of *cfcA* was tested by PCR using primers Fw34 and Rev29, annealing temperature of 58 °C and elongation time of 3.5 min. Genomic DNA of selected transformants was digested with *Bgl*II and *Xba*I or *Bgl*II and *Apa*I. Southern blotting was performed as described in the DIG applications manual for hybridization (Roche Applied Science), using a DIG labeled probe generated with the PCR DIG Probe Synthesis Kit (Roche Applied Science) and primers Fw34 and Rev34 using conditions as described above. The wild type genomic DNA is expected to give 2595 bp or 4231 bp fragments, respectively, and *cfcA* deletion is expected to yield 1725 bp or 2594 bp fragments, respectively (Fig. 1A). The strain carrying a *cfcA* gene deletion is referred to as strain Δ*cfcA*.

For the deletion of *agnB*, vector pDEST-50 was constructed using a strategy similar as described above. Primers Fw50 and Rev50 were used to amplify 982 bp of the upstream region of *agnB* in a PCR using an elongation time of 1.5 min and an annealing temperature of 58 °C. Likewise, primers Fw51 and Rev51 were used to amplify 1277 bp of the downstream region. Both fragments were inserted in vectors pDONR P4-P1R and pDONR P2R-P3. Vector pDONR221-P*pgdA*-hph-T*trpC*, containing the hygromycin resistance conferring *E. coli* hph under the *A. nidulans* *gpdA* promoter and flanked by the *A. nidulans* T*trpC* transcriptional terminator (kindly provided by B. Nitsche, University of Leiden) was combined with pDONR P4-P1R-50, pDONR P2R-P3-51 and pDEST R4-R2 vector II to create pDEST-50 (Fig. 1B). pDEST-50 was linearized with *Ssp*I and transformed to *A. niger* strain Δ*cfcA* as described by (Arentshorst et al., 2012). Transformants were tested for the absence of *agnB* by PCR using primers Fw26 and Rev26. Additionally the insertion of the deletion construct in the locus was tested using primers Fw53 (1.5 kbp upstream of *agnB* and Rev54, specific for *hph*, in a PCR with 4 min elongation and annealing temperature of 57 °C (Fig. 1B). Genomic DNA of selected transformants was
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Fig. 1. Schematic representation of the strategies followed for the construction of (A) strain \(\Delta cfcA\) and (B) strain \(\Delta cfcA\Delta agnB\). Sites for restriction enzymes BglII, Apal and PstI are indicated with B, A and P respectively. Dotted lines represent the probes used for Southern blotting. Integration of the deletion constructs was confirmed by Southern blotting, resulting in the displayed fragments for (C1, D1) the wild type strain N402, (C2) mutant \(\Delta cfcA\) and (D2) mutant \(\Delta cfcA\Delta agnB\).

Digested with PstI and subjected to analysis by Southern blotting as detailed above, using a DIG labelled probe generated using primers Fw51 and Rev51. A transformant with a single integration in the correct position in the genome was selected for further analysis and is referred to as strain \(\Delta cfcA\Delta agnB\). The wild type genomic DNA is expected to give 2366 bp fragments and an agnB deletion mutant is expected to yield 3463 bp fragments (Fig. 1B).

**Sensitivity of deletion mutants to disrupting compounds.** Sensitivity towards cell wall polymer binding dyes Calcofluor White and Congo Red was tested for deletion strains \(\Delta cfcA\) and \(\Delta cfcA\Delta agnB\) as described in detail by (Ram & Klis, 2006). Sensitivity towards SDS and sorbitol was determined similarly by supplementing agar plates with 0.005 and 0.01 % (w/v) of SDS or 0.6 M sorbitol.
Submerged liquid fermentation under carbon starvation. The effects of *cfcA* and *agnB* deletion were analyzed during carbon starvation using growth of *A. niger* strains N402 and Δ*cfcA*Δ*agnB* under controlled conditions in a fermentor. Production of spores and media composition were as described by (Nitsche *et al.*, 2012) except that glucose was used as carbon source. An Applikon fermentor of 15.7 L volume containing 7.7 L minimal medium was sterilized in situ. Separately sterilized glucose solution (60 g glucose monohydrate in 0.3 L) was added and the pH adjusted to 3.0. Duplo fermentations were performed, where each fermentor was inoculated with 4 · 10⁴ spores ml⁻¹. During germination, mixing was performed using the Applikon type P1000 motor at 150 rpm. After 5.5 h, stirring speed was increased to 500 rpm, and 1.6 L min⁻¹ aeration using a sparger was started. After 24 h of cultivation, anti-foam agent HODAG K-60K (Lambent Technologies) was added to a final concentration of 0.01 % (v/v). The pH was monitored using AppliSens pH sensor (Applikon) and maintained at pH 3 during germination and exponential growth using 5 % (w/v) sodium hydroxide. Upon carbon source depletion, pH control was terminated. Dissolved oxygen was monitored real-time using AppliSens dO2 sensor (Applikon). Gas from the fermentation was analyzed for oxygen and carbon dioxide levels using a Xentra 4100 Gas Purity Analyzer (Servomex).

Sampling and fermentation characteristics. Samples were drawn from the fermentor during exponential growth and for up to 133 h after carbon source depletion. Aliquots for microscopy and pellet analysis were directly frozen in liquid nitrogen. Samples for other types of analysis were vacuum-filtrated over a glass microfiber filter (Whatman, type GF/C) to separate fungal biomass and culture filtrate. The amount of biomass was determined in duplicate by determining the mass of filtrated mycelium that was dried to a stable weight using a Smart System 5 microwave moisture analyzer (CEM Corporation). Biomass values are given as mean dry mass per culture weight. The glucose concentration was quantified using GOPOD assay (Megazyme) in a microtiter plate format. Organic acids in the culture filtrate were identified by HPLC, kindly performed by Kerry BV (Almere, the Netherlands).

Analysis of proteins and enzymatic activities. The protein concentration of the culture filtrate was determined with a Bradford assay, using BSA as a standard (Bradford, 1976). Protease activity estimated by detecting hydrolysis of bovine hemoglobin (Sigma Aldrich), kindly performed by Kerry BV (Almere, the Netherlands), with one AHUT unit of protease activity defined as the amount of protein that re-
leased 1.10 µg ml⁻¹ tyrosine in 1 min at pH 3.0 and 40 °C. The activity of exo-acting glycoside hydrolases was determined with an end-point microtiter plate assay with 4-nitrophenol labeled carbohydrates as substrate (Chapter 2).

**Biomass fragmentation and cell wall changes.** Samples for microscopy were slowly defrosted on ice, and stained with lactophenol blue solution (Fluka). Microscopy was performed using a Zeiss Axioskop microscope with a Zeiss Achrostigmat 10x/0.25 objective. Images were acquired using a MicroMAX CCD camera (Princeton Instruments) and MegaVue software (MDS Analytical Technologies). Image analysis was performed using ImageJ (http://rsb.info.nih.gov/). Analysis of the carbohydrate monomer composition of the cell walls was performed by acid hydrolysis of isolated cell walls as described in detail previously (Chapter 4).

**Results**

**Production and purification of CfcA and AgnB.** To characterize the properties of the starvation induced putative α-1,3-glucanase AgnB and chitinase CfcA, both proteins were expressed heterologously in *E. coli*. CfcA was produced as a fusion protein, carrying an N-terminal maltose-binding protein (MBP). The MBP was used in a first purification step by amylose affinity chromatography, followed by anion-exchange chromatography to obtain pure MBP-CfcA. MBP-CfcA was produced with a yield of 8 ± 4 mg purified protein per liter culture and a specific activity of 0.27 ± 0.04 U mg⁻¹ on (GlcNAc)₂-pNP.

AgnB was produced in *E. coli* as insoluble protein aggregates that could be refolded to soluble and active protein using urea. However, the refolded protein was instable, resulting in protein precipitation during incubations. In attempts to produce stable AgnB, the protein was expressed as MBP-fusion protein in *E. coli* and as glycoprotein secreted by *P. pastoris*. Both alternative approaches yielded soluble but inactive proteins (results not shown). Therefore, AgnB characterization was performed using freshly prepared in *E. coli* produced and refolded protein.

**Reaction parameters of chitinase MBP-CfcA.** Determination of the optimum conditions for MBP-CfcA activity with (GlcNAc)₂-pNP showed that the enzyme is a chitinase that is highly active over a broad range of pH values, with an optimum pH around 5 and > 50% of activity between pH 3-7.5. The highest reaction rate was
observed at 65 °C. At 30 °C the activity of MBP-CfcA was reduced to 18 % of the maximum rate and at 45 °C it was reduced to 55 %. The temperature stability of MBP-CfcA was tested by incubating the protein at a set temperature for up to 1 h, and measuring the residual activity at 45 °C. Whereas the activity remained stable up to 50 °C for 1 h, at 60 °C it was reduced to 14 % of the original activity after 10 min of incubation.

**Chitinase MBP-CfcA releases (GlcNAc)₂ from the non-reducing substrate end.**

HPLC analysis, performed directly after incubation of MBP-CfcA with (GlcNAc)₅, showed that the main products are (GlcNAc)₂ and (GlcNAc)₃ (Fig 2). The (GlcNAc)₃ product displayed two partially overlapping peaks originating from the α and β anomer (Fukamizo & Hayashi, 1982), similar to the ~ 60:40 α/β anomer ratio during

![Figure 2](image_url)

**Fig. 2.** HPLC analysis of the reaction products of MBP-CfcA with (GlcNAc)₅ (A) and GlcNAc - (GlcNAc)₅ standard (B). Chitin oligosaccharides are indicated by their degree of polymerization (DP). The peaks originating from the α- and β-anomeric configuration of the reducing end are indicated by α and β, respectively.
Fig. 3. Reaction products of MBP-CfcA during incubation with (GlcNAc)_3 (A), (GlcNAc)_4 (B), (GlcNAc)_5 (C) (GlcNAc)_6 (D) and (GlcNAc)_2-pNP (E) as identified by HPAEC-PAD as GlcNAc (▲), (GlcNAc)_2 (∆), (GlcNAc)_3 (■), (GlcNAc)_4 (□), (GlcNAc)_5 (●) and (GlcNAc)_6 (○). In the chromatogram (E), the peaks were identified as (GlcNAc)_2-pNP (1), GlcNAc-pNP (2), (GlcNAc)_3 (3) GlcNAc (4) and (GlcNAc)_2 (5).
equilibrium, while (GlcNAc)$_2$ was found in the $\beta$ anomeric configuration only. CfcA belongs to glycoside hydrolase family 18 (Chapter 5). As chitinases from this family retain the $\beta$ anomeric configuration of their substrate (Terwisscha van Scheltinga et al., 1995), the (GlcNAc)$_2$ thus represents a newly produced reducing end, and is thus released from the non-reducing end of (GlcNAc)$_5$.

HPAEC-PAD was used to follow product formation from chitin oligosaccharides (GlcNAc)$_2$-(GlcNAc)$_6$ in time (Fig. 3). (GlcNAc)$_2$ was not hydrolyzed by MBP-CfcA (data not shown). (GlcNAc)$_3$ was cleaved to (GlcNAc)$_2$ and GlcNAc, although apparently rather inefficient (Fig. 3A). (GlcNAc)$_4$ was mainly split into (GlcNAc)$_2$ but also in GlcNAc and (GlcNAc)$_3$ (Fig. 3B). (GlcNAc)$_5$ was split into mainly (GlcNAc)$_2$ and (GlcNAc)$_3$, confirming the products detected by HPLC (Fig. 3C). Substrate (GlcNAc)$_6$ was mainly split into (GlcNAc)$_2$ and (GlcNAc)$_4$ but also into (GlcNAc)$_3$ (Fig. 3D). Taken together, the observed profiles indicate that MBP-CfcA releases mainly (GlcNAc)$_2$ but also some (GlcNAc)$_3$ from chitin oligosaccharides.

To investigate whether MBP-CfcA is able to catalyze a transglycosylation reaction, labeled chitin oligosaccharides were used as substrate. Incubation of MBP-CfcA with (GlcNAc)$_2$-pNP produced GlcNAc-pNP, GlcNAc, (GlcNAc)$_2$ and (GlcNAc)$_3$ (Fig. 3E). We conclude that (GlcNAc)$_3$ originated from a transglycosylation reaction catalyzed by MBP-CfcA.

**Chitinase MBP-CfcA hydrolyzes chitin and the *A. niger* cell wall.** Next to chitin oligosaccharides, MBP-CfcA hydrolyzed chitin: HPAEC-PAD analysis of incubation products showed MBP-CfcA produced mainly (GlcNAc)$_2$ (70-80 % of the total peak area) in addition to GlcNAc (20-30 %). Furthermore, the same products - with >90% (GlcNAc)$_2$ - were observed in incubations with complete *A. niger* cell walls, or the alkaline insoluble cell wall fraction, which consists of the $\beta$-1,3 glucan backbone with covalently linked polymers such as chitin (Fontaine et al., 2000). No products were released from the alkaline soluble fraction of the cell walls, which consists of $\alpha$-glucans (Johnston, 1965).

The ability of MBP-CfcA to hydrolyze the fungal cell wall was assessed by testing its capacity to assist in releasing protoplasts from *A. niger* mycelium. An *A. niger* standard protoplastation mixture, as described by (de Bekker et al., 2009) but without chitinase, released 0.13 (± 0.04) · $10^6$ protoplasts from 1.25 gram mycelium.
Addition of 150 mU *S. griseus* chitinase ml⁻¹ increased the number of protoplasts to 4.5 (± 0.8) · 10⁶, comparable to the amount quantified by (de Bekker et al., 2009). Addition of 80 mU MBP-CfcA ml⁻¹ (instead of the *S. griseus* chitinase) resulted in the production of 2.0 (± 0.9) · 10⁶ protoplasts, significantly (p ≤ 0.05) more than the mixture without chitinase. MBP-CfcA thus is clearly able to hydrolyze the *A. niger* fungal cell wall.

**AgnB is an α-1,3-glucanase.** Sequence analysis of AgnB indicated that the enzyme belongs to glycoside hydrolase family 71, and thus is a putative α-glucanase. HPAEC-PAD analysis of the incubation of refolded AgnB with insoluble α-1,3-glucan,

![Fig. 4](image)

*Fig. 4.* The HPAEC-PAD chromatograms of AgnB (red), or the negative (empty vector) control (blue) incubations with α-1,3-glucan (A), mutan (B), the cell wall of *A. niger* (C), α-1,3-oligosaccharides DP2-DP3 (D), DP3-DP4 (E) and DP4-DP5 (F). The black line shows the reference α-1,3-oligosaccharides DP1-DP6.
indicated that glucose – representing ≥ 85 % of the total peak area - was the main reaction product. In addition, an oligosaccharide with a degree of polymerization (DP) of 3 was detected (Fig. 4A). The production of glucose was confirmed using the glucose specific enzymatic GOPOD assay. Similarly, activity of AgnB was detected on the α-1,6-branched α-1,3-polymers mutan (Fig. 4B) and exo-polysaccharide EPS-ML1 (data not shown). A small amount of glucose was detected upon incubation with A. niger cell walls (Fig. 4C) and the alkaline soluble part of the cell wall. No activity was detected on nigeran, a glucan with alternating α-1,3 and α-1,4-linkages. In addition, no glucose was released from α-1,3/1,6-glucan exo-polysaccharide EPS-180.

To investigate the substrate and product specificity of AgnB in more detail, oligosaccharides were produced by acid hydrolysis from the alkaline soluble fraction of the A. nidulans cell wall. The oligosaccharides were separated based on size, and in selected fractions the glycosidic linkage type was shown by NMR to consist of mainly (≥ 95%) α-1,3-linkages (results not shown). HPAEC-PAD analysis of incubations of AgnB with the α-1,3-oligosaccharides with a DP of 2 and 3, showed that these oligosaccharides were not hydrolyzed (Fig. 4D). The DP4 oligosaccharide was hydrolyzed to glucose and DP3 (Fig. 4E). Similarly, incubation with DP5 resulted in the production of mainly glucose, DP3 as well as some (< 5 % of the total peak area) DP2 (Fig. 4F).

Deletion of A. niger cfcA and agnB. To investigate the physiological functions of cfcA and agnB, gene deletion strains were generated and analyzed. The gene cfcA was replaced with the pyrG selection marker in A. niger AB4.1. For one transformant, Southern blotting showed a signal corresponding to the 1725 bp fragment expected from BglII and XbaI digested DNA (results not shown). For this transformant, the signal obtained after digestion of the DNA with BglII and Apal corresponded to the expected fragment size of 2594 bp (Fig. 1A,C) This transformant thus carries a successful gene replacement and is referred to as A. niger strain ∆cfcA.

A ∆cfcA∆agnB double deletion strain was generated by replacing agnB in the ∆cfcA strain with a hygromycin resistance cassette. Southern blotting (Fig. 1B,D) using PstI digested DNA of transformants, resulted in the 3463 bp fragment expected upon successful gene replacement. One of these strains was selected for further analysis, and is referred to as A. niger strain ∆cfcA∆agnB.
Sensitivity to disrupting compounds. CfcA and AgnB hydrolyze the cell wall components chitin and α-1,3-glucan, respectively, therefore the sensitivity of these deletion mutants towards cell wall disrupting components was investigated. No differences were found in sensitivity towards cell wall disrupting dyes Calcofluor White and Congo Red, or SDS and sorbitol during growth on agar plates between the wild type strain N402 and strains ΔcfcA and ΔcfcAΔagnB (results not shown).

Submerged liquid fermentation under carbon starvation. As expression of cfcA and agnB is induced during carbon starvation in minimal medium (Chapter 2), the effects of their inactivation by gene deletion was analyzed during carbon starvation. Strain N402 and deletion mutant ΔcfcAΔagnB were grown under controlled conditions in fermentors. The growth curves were synchronized using the dissolved oxygen signal, which as observed before (Nitsche et al., 2012) showed a peak after the exponential growth phase at the point that glucose was depleted (defined as $t = 0$ h). For both strains, biomass accumulated to approximately 4.5 g dry weight per kg of culture broth (wet weight) at the end of the exponential growth phase,

![Graph](image_url)

**Fig. 5.** Biomass (filled symbols) and protein content of the culture filtrates (open symbols) of strain N402 (■) and strain ΔcfcAΔagnB (●). Graphs are synchronised at the time-point of carbon depletion, represented as $t = 0$ h.
and decreased in the 130 h after carbon depletion (Fig 5A). No major differences were found in the growth rate, glucose consumption or biomass decrease during starvation. During the exponential growth phase, sodium hydroxide was added to maintain a constant pH of 3. The level and rate of sodium hydroxide required to prevent medium acidification is related to the uptake of ammonium from the minimal medium (Hrdlicka et al., 2004) and thus an indicator for growth. The sodium hydroxide requirement varied between duplicate fermentations to a similar extent as between the strains. Upon release of pH control at t = 0 h, the culture pH increased to 4.5-5 and returned to 3-3.5 within the first 15 h of starvation for both strains. Subsequently, the culture pH gradually increased to 5.2-5.6. Pyruvic acid and fumaric acid accumulated to 17 and 1 mg L⁻¹ respectively during the exponential growth phase, but were no longer detected after 15 h of starvation, while oxalic acid was detected at levels up to 0.43 g L⁻¹ culture during the starvation phase. The culture filtrate changed from colorless and clear during exponential growth, to increasingly yellow-brownish during starvation.

Analysis of proteins and enzymatic activities. Protein in culture filtrates remained below 1 mg l⁻¹ during the exponential growth phase and under carbon starvation conditions steadily increased to 82 ± 2 and 61 ± 4 mg l⁻¹ for strains N402 and ∆cfcA∆agnB respectively. Extracellular protease activity increased during the first 40 h of starvation and subsequently remained stable to reach 69 ± 7 and 69 ± 4 AHUT ml⁻¹ for strains N402 and ∆cfcA∆agnB respectively at 130 h of starvation. The extracellular activity of exo-β-glucanases and exo-β-hexosaminidases, detected as pNP-β-Glc and pNP-β-GlcNAc hydrolyzing activity, increased from the start of starvation until 130 h, in a trend similar to the protein concentration. The specific activities at 130 h were similar for both strains, with 3.4 ± 0.3 and 3.6 ± 0.4 µmol pNP (mg protein)⁻¹ min⁻¹ released from pNP-β-GlcNAc for strains N402 and ∆cfcA∆agnB respectively, and 1.7 ± 0.1 µmol pNP (mg protein)⁻¹ min⁻¹ released from pNP-β-Glc for both strains.

Biomass fragmentation and cell wall changes. During exponential growth, the morphology of wild type strain N402 and the double deletion mutant ∆cfcA∆agnB was similar. Both grew mainly in pellets, clumps of mycelium. The morphology of the biomass changed in time. During exponential growth, microscopy showed healthy looking hyphae filled with cytoplasm. After 15 h of starvation (Fig 6A, C) empty compartments were visible in addition to the cytoplasm filled hyphae. Thin hyphae were
formed and from ~60 h of starvation onwards the formation of conidiophores and spores was detected. The degree of fragmentation and the amount of empty hyphae differed between the two strains. In N402, after 130 h (Fig 6B), spores, small pellet fragments and cytoplasm filled hyphal fragments were observed; only a low amount of empty hyphae was visible. In contrast, the mycelium of strain ΔcfcAΔagnB contained a large amount of empty hyphae (Fig. 6D) in addition to spores, pellet fragments and cytoplasm filled hyphae. Thus, deletion of cfcA and/or agnB results in preservation of cell wall structures during carbon starvation, likely through a reduction in the hydrolysis of chitin and/or α-glucan.

To monitor changes in the composition of the cell walls of the fungal mycelium, the carbohydrate monomer composition of cell walls was determined at five time points during growth and starvation. In the wild type strain (Fig. 7A), the amount of detected glucosamine – originating from N-acetyl-glucosamine or glucosamine liberated from chitin or chitosan – increased from the exponential growth until 60 h of starvation from 11.3 ± 0.3 to 14.8 ± 0.2 % of the total recovered cell wall carbohydrates. In strain ΔcfcAΔagnB (Fig. 7B), the cell wall composition was similar to that of N402 during the exponential phase. During starvation, the amount of glucosamine increased to 24.1 ± 0.3 %. The cell walls of strain ΔcfcAΔagnB thus contained more
chitin or chitosan during starvation compared to N402, and these differences increased during the starvation time.

**Discussion**
Filamentous fungi that experience carbon starvation recycle cell components such as cell walls to acquire energy and building blocks for starvation survival strategies. Glycoside hydrolases are key effectors in the autolytic degradation of fungal cell walls. In *A. niger*, the expression of chitinase *cfcA* and α-1,3-glucanase *agnB* genes is strongly induced in carbon starved submerged liquid cultures (Chapter 2). In this study we focused on the biochemical and physiological characterization of the enzymes encoded by these genes.

We show that CfcA, here expressed as MBP-fusion protein, is a chitinase that hydrolyzes chitin as well as chitin oligosaccharides with a minimum length of DP3. The enzyme releases mainly DP2 from the non-reducing end of its substrates, and is capable of performing a transglycosylation reaction with short oligosaccharides.

![Fig. 7. Carbohydrate monomer composition of the cell wall in % of recovered carbohydrates, of strain N402 (A) and strain ΔcfcAΔagnB (B), depicted as black (glucosamine), wide hatched pattern (glucose), small hatched pattern (galactose) and squared pattern (mannose) bars.](image)
This substrate-product specificity is similar to that of the CfcA homologs ChiB of A. nidulans and ChiB1 of A. fumigatus (Erdei et al., 2008; Jaques et al., 2003; Xia et al., 2001).

We show here that AgnB is an α-1,3-glucanase that hydrolyzes α-1,3-glucan and oligosaccharides with minimum length of DP4. Furthermore, we show that AgnB hydrolyzes α-1,3-glucan polymers that contain α-1,6-linkages such as EPS-ML1 (Kralj et al., 2004) and mutan (Li et al., 2012). However, no activity was observed on EPS-180, which may indicate that AgnB activity requires either consecutive α-1,3-linkages, terminal α-1,3-linkages or α-1,3-linked glucose units without 6-substitution, all of which are absent in GFT-180 (van Leeuwen et al., 2008).

AgnB produces glucose and a glucose trimer as the main reaction products, similar as described for α-1,3-glucanases from filamentous fungi for which the product identity and/or length was analyzed (Ait-Lahsen et al., 2001; Grün et al., 2006; Sanz et al., 2005; Zonneveld, 1972a). Since their reaction products consist mainly or only of glucose, these enzymes are often referred to as exo-glucanases. However, (Grün et al., 2006) and coworkers showed that these α-1,3-glucanases possess endo-acting activity with a polymer, since a substrate with chemically modified, inaccessible ends gave similar hydrolysis rates as the native substrate. They proposed that the fungal enzymes act as endo-glucanases at the first cleavage event, and then processively release glucose while moving towards the non-reducing end. The S. pombe α-1,3-glucanases Agn1 and Agn2 belong to the same glycoside hydrolase family 71; their activity on polysaccharides results in formation of oligosaccharides, indicating that these enzymes are true endo-glucanases (Dekker et al., 2004; Dekker et al., 2007). It is therefore likely that AgnB also has endo-acting activity. However, AgnB did not hydrolyze the tested oligosaccharides randomly at the first cleavage event (Fig. 4D-F); this would have resulted in accumulation of DP2. Possibly, the substrates tested were too small to occupy the binding sites in the enzyme active site such that random cleavage was allowed in the initial attack of the substrate.

The α-1,3-glucan substrate of AgnB is a cell wall polymer, suggesting that AgnB may be a cell wall modifying enzyme. The refolded AgnB was capable of releasing some glucose from the fungal cell wall material, but rather inefficiently. It thus remains to be confirmed that the physiological substrate of AgnB is the fungal cell wall. CfcA released carbohydrates from the Aspergillus cell wall, and assisted in the
release of protoplasts from A. niger mycelium; this enzyme thus is clearly capable of degrading chitin that is embedded in the fungal cell wall.

During carbon starvation, the submerged liquid fermentations of the wild type A. niger strain displayed characteristics that are classically associated with autolysis: the reduction and fragmentation of biomass, the expression of hydrolytic enzymes such as glycoside hydrolases and proteases, and the release of proteins or amino acids in the culture filtrate.

The $\Delta$cfcA$\Delta$agnB double deletion mutant had a phenotype similar to the wild type strain during exponential growth. During carbon starvation, differences in most autolytic parameters such as protein release, biomass loss or hydrolase secretion – if any – were subtle. More prominent are the differences in cell wall composition and degradation in hyphal fragments. Microscopy clearly showed decreased biomass fragmentation and reduced degradation of fungal cell walls in the mutant compared to the wild type. Analysis of the fungal cell wall by acid hydrolysis and subsequent quantification of the carbohydrate monomers, resulted in detection of relatively higher amounts of glucosamine in the cell walls of the double deletion mutant, as compared to the wild type, but only under starvation conditions. At this stage the cell walls of the deletion mutant contain relatively more (N-acetyl)-glucosamine, thus more chitin or chitosan than the wild type strain. This relative increase may be caused by hydrolysis of all cell wall components except chitin and supports a function for CfcA in hydrolysis of cell wall chitin during carbon starvation. Whether AgnB is involved in cell wall hydrolysis cannot be deduced from the cell wall composition. Our findings also show that a direct role for an enzyme in cell wall polysaccharide modification during autolysis does not necessarily result in measurable reduction in dry weight during carbon starvation.

Taken together, we show here that AgnB is an $\alpha$-1,3-glucanase, which hydrolyzes $\alpha$-1,3-glucan and its oligosaccharides to mainly glucose. This substrate is a fungal cell wall polymer, and the gene encoding this enzyme is strongly induced during carbon starvation. However, no clear data were obtained indicating that AgnB has a function in cell wall hydrolysis during carbon starvation. CfcA is a chitinase that releases mainly chitobiose from chitin, similar to its homologs in A. nidulans and A. fumigatus. Differences in fragmentation and cell wall composition between the wild type and the double deletion mutant $\Delta$cfcA$\Delta$agnB for the first time directly support a
clear role for CfcA in hydrolysis of cell wall chitin during carbon starvation in *A. niger*.

**Acknowledgements**

We thank A. van Wijk (University of Groningen, the Netherlands) for excellent technical assistance with analysis of the cell wall monomer composition. We thank Kerry BV (the Netherlands) for performing protease activity assays. We acknowledge B. M. Nitsche (University of Leiden, the Netherlands) for supplying vectors pDONR221-PgpdA-hpH-TtrpC and pDONR221-AOpyrG, and B. J. Zonneveld (University of Leiden, the Netherlands) for supplying the alkaline soluble fraction of *A. nidulans* cell walls.

**Reference List**


Fontaine, T., Simenel, C., Dubreucq, G., Adam, O., Delepierre, M., Lemoine, J., Vorgias,


Characterization of CfcA and AgnB


Wiater, A., Szczodrak, J. & Rogalski, J. (2001). Purification and characterization of an extra-


