The role of autophagy during carbon starvation in Aspergillus niger

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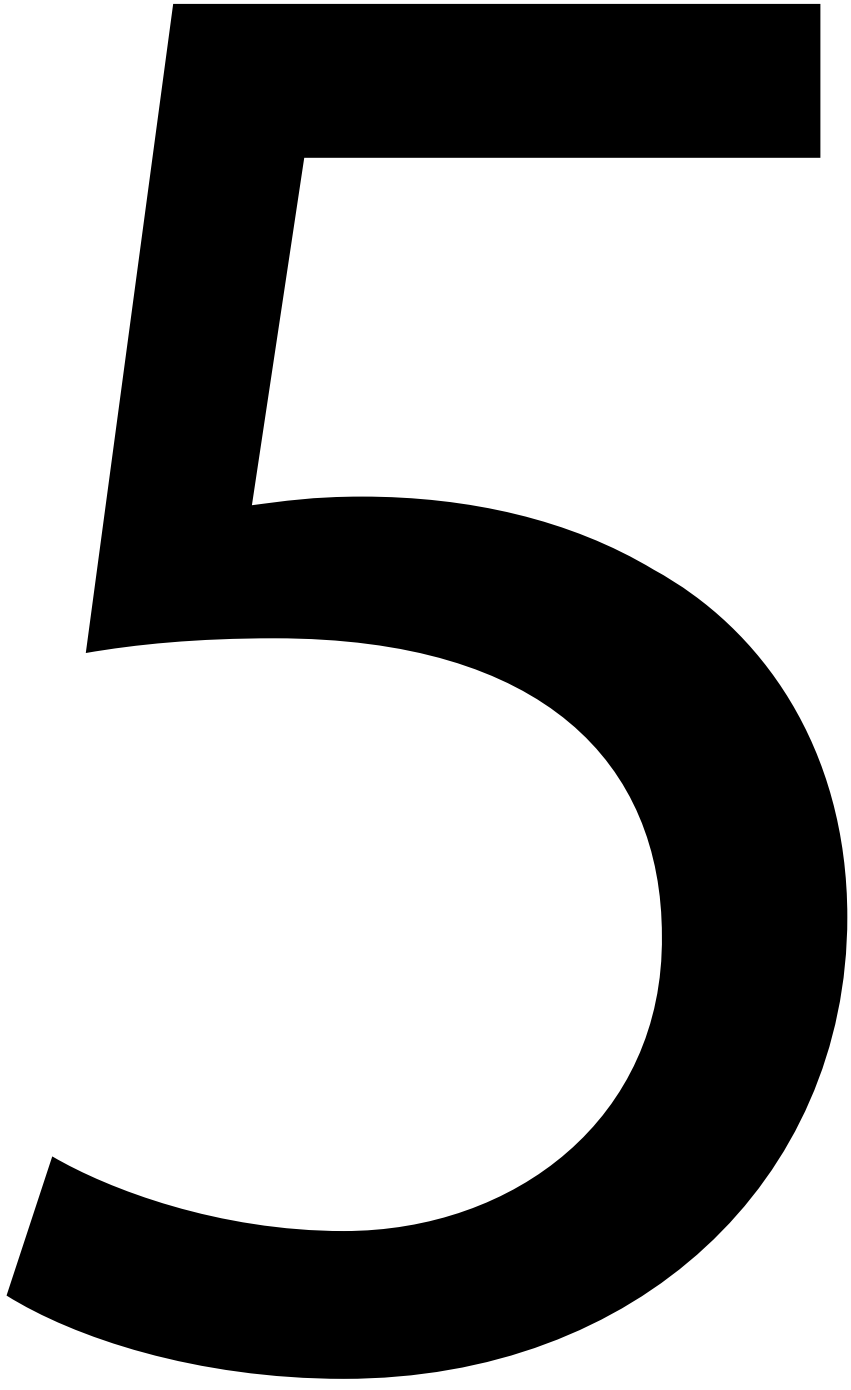

Chapter 5

The unconventional secretion of PepN is independent of a functional autophagy machinery in the filamentous fungus *Aspergillus niger*

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Abstract

During unconventional protein secretion (UPS), proteins do not pass through the classical endoplasmic reticulum (ER)-Golgi-dependent pathway, but are transported to the cell membrane via alternative routes. One type of UPS is dependent on several autophagy-related (Atg) proteins in yeast and mammalian cells, but mechanisms for unconventional secretion are largely unknown for filamentous fungi. In this study, we investigated whether the autophagy machinery is used for UPS in the filamentous fungus *Aspergillus niger*. An aspartic protease, which we called PepN, was identified as being likely to be secreted unconventionally, as this protein is highly abundant in culture filtrates during carbon starvation while it lacks a conventional N-terminal secretion sequence. We analyzed the presence of PepN in the culture filtrates of carbon starved wild-type, *atg1* and *atg8* deletion mutant strains by Western blot analysis and by secretome analysis using nanoLC-ESI-MS/MS (wild-type and *atg8* deletion mutant). Besides the presence of carbohydrate active enzymes and other types of proteases, PepN was abundantly found in culture filtrates of both wild-type and *atg* deletion strains, indicating that the secretion of PepN is independent of the autophagy machinery in *A. niger* and hence most likely occurs via a different mechanism.

Introduction

The vast majority of extracellular proteins is secreted via the classical secretory pathway, which involves vesicle-mediated transport from the endoplasmic reticulum (ER) through the Golgi apparatus to the cell membrane. An N-terminal signal peptide (leader sequence) is responsible for introducing these proteins into the secretion pathway. During their passage through the secretory pathway, secretory proteins are modified and processed to ensure that they are active and stable. These posttranslational modifications steps include the formation of disulphide bridges to ensure a proper tertiary structure of the protein, glycosylation on asparagine or serine and threonine residues, and further processing, e.g. by proteases.

During the last decades, several studies have reported on the presence of extracellular proteins lacking a typical signal peptide, being transported independently of the classical ER-Golgi route (Zhang and Schekman, 2013). This process is now generally known as unconventional protein secretion (UPS), which collectively describes several distinct vesicular and non-vesicular transport pathways. One form of unconventional secretion involving vesicles is associated with the autophagy pathway, requiring autophagy-related (Atg) proteins (Ponpuak *et al.*, 2015). Classically, autophagy is a constitutively active, catabolic process mediating the transport of cytoplasmic components within double membrane vesicles to vacuoles for degradation and recycling. It is strongly induced by starvation conditions as a mechanism to help cells cope with nutrient stress. Besides its degradative function, autophagy is also shown to be involved in unconventional protein secretion as autophagy-related proteins are present in compartments for UPS (CUPS) (Bruns *et al.*, 2011) and the unconventional secretion of different cargo proteins is dependent on several components of the autophagy machinery in yeast and mammalian cells (Duran *et al.*, 2010; Manjithaya *et al.*, 2010; Dupont *et al.*, 2011). Since genes encoding candidates for unconventionally secreted proteins such as PepN, ChiB and hemolysin are expressed during late exponential or stationary phase in *A. niger* (Braaksma *et al.*, 2010; Nitsche *et al.*, 2012; van Munster *et al.*, 2015b), which coincides with the induction of autophagy-related genes (Nitsche *et al.*, 2012), the autophagy process is a likely candidate to be involved in the unconventional secretion of these proteins. PepN has been previously described as PepAb (Wang *et al.*, 2008). As the gene name does not fulfill the gene nomenclature guidelines, we have renamed the gene *pepN*.

In this study we investigated whether the autophagy machinery is involved in the secretion of PepN in *A. niger*. The PepN protease was identified as an extracellular protein likely to be secreted via the unconventional secretion pathway and hence used as a target protein to show its presence in the secretome of Δ*atg1* and Δ*atg8 A. niger* mutants. Atg1 and Atg8 are the orthologs of the Atg1 and Atg8 proteins of *Saccharomyces cerevisiae* (Nitsche *et al.*,

2013). Proteome and Western analyses showed that PepN was abundantly present in culture filtrates of carbon starved cultures of both wild-type and *atg* deletion mutants, indicating that the secretion of PepN is not dependent on a functional autophagy machinery in *A. niger*.

Materials and Methods

Strains, culture conditions and molecular techniques

A. niger strains used in this study are listed in Table 1. Strains were cultivated in standard minimal medium (MM) or complete medium (CM) (Arentshorst *et al.*, 2012). For plate growth, the medium was solidified by the addition of 1.5% agar. Transformation plates were supplemented with 200 µg ml^{-1} hygromycin and 500 µg ml^{-1} caffeine and hygromycin-resistant transformants were subsequently purified on plates containing 100 µg ml^{-1} hygromycin. Transformation of *A. niger* and genomic DNA extraction were performed as described by Arentshorst *et al.* (2012). Southern blot analysis was conducted as described by Sambrook and Russell (2001), using [α-32P]dATP-labeled probes synthesized with the DecaLabel DNA labeling kit (Thermo Scientific).

Generation of an *A. niger pepN* deletion mutant

The *A. niger pepN* gene (An01g00370) was deleted by replacing its open reading frame (ORF) with the hygromycin resistance cassette using the split marker approach as was described in detail by Arentshorst *et al.* (2015b). Approximately 700 bp flanking regions of the *pepN* ORF were PCR amplified from genomic DNA of the N402 strain using primer pairs as listed in Table 2. The hygromycin cassette was PCR amplified from the plasmid pAN7.1 (Punt *et al.*, 1987) using primers hygP6for and hygP7rev (Table 2). Subsequently, 5' and 3' split marker fragments were obtained in two separate fusion PCR amplifications using the respective flank and the hygromycin cassette PCR products as a template and primer pairs according to Table 2. The split marker fragments were transformed to *A. niger* strain MA234.1 and homologous integration was confirmed by Southern analysis (Fig. S1). Strain MA234.1 (*ku70*$^{-}$) was obtained by transformation of strain MA169.4 (*ku70*$^{-}$, *pyrG*$^{-}$) with a 3.8 kb *Xba*I fragment containing the *A. niger pyrG* gene, resulting in the full restoration of the *pyrG* locus in strain MA234.1.

Proteolytic activity

Proteolytic activity was assayed on pre-acidified (pH 3.0) MM plates supplemented with 0.3% skim milk and solidified with 1.5% agarose. Halo sizes were compared after seven days of growth at 30°C. Fluorometric analysis was performed at pH 2.8 using the P-CHECK protease detection kit (Jena Bioscience) according to manufacturer's instructions.

Table 1 | A. niger strains used in this study

Strain	Genotype	Reference
N402	*cspA1* derivative of ATCC9029	Bos *et al.* (1988)
AB1.13	*cspA1*, *prtT*$^-$	Punt *et al.* (2008)
MA234.1	*kusA*::DR-*AmdS*-DR	This study
AW45.1	Δ*pepN*::*hyg* in MA234.1	This study

Table 2 | Primers used in this study

Primer	Sequence (5′ – 3′)	Target	Reference
PepN_P1F	AAGATGGAGT CAGAGCCACC C	5′ flank *pepN* and 5′ split marker fragment	This study
PepN_P2R	CAATTCCAGC AGCGGCTTGA TGGAGGATAA TTGATCAAAG	5′ flank *pepN*	This study
PepN_P3F	ACACGGCACA ATTATCCATC GACGAGTGGC TGAATCTATC ATG	3′ flank *pepN*	This study
PepN_P4R	AAGCCCGTCA GCTCCTTGA	3′ flank *pepN* and 3′ split marker fragment	This study
hygP6for	AAGCCGCTGCTGGAATTGGGCTCTGAGGTGCAGTGGAT	hygromycin cassette	Arentshorst *et al.* (2015b)
hygP7rev	CGATGGATAATTGTGCCGTGTTGGGTGTTACGGAGCATTCA	hygromycin cassette	Arentshorst *et al.* (2015b)
hygP9r	GGCGTCGGTTTCCACTATC	5′ split marker fragment	Arentshorst *et al.* (2015b)
hygP8f	AAAGTTCGACAGCGTCTCC	3′ split marker fragment	Arentshorst *et al.* (2015b)

Secretome analysis

For protein identification, 1 mL of culture filtrate was freeze dried and analyzed using nanoLC-ESI-MS/MS, which was performed by Proteome Factory (Berlin, Germany). Proteins were identified using MS/MS ion search of the Mascot search engine (Matrix Science, London, England) and the non-redundant protein database (National Center for Biotechnology Information, Bethesda, USA). Signal peptide predictions were taken from Braaksma *et al.* (2010) where available or by using the SignalP 4.0 algorithm (Petersen *et al.*, 2011).

Western blot analysis

Anti-PepN antibodies used for Western blot analysis were developed in rabbits by Davids Biotechnologie (Regensburg, Germany) against two different synthetic peptides (Fig. S2) and purified by affinity purification. Extracellular protein samples from bioreactor cultures were obtained by vacuum filtration of the culture broth through glass microfiber filter (Whatmann). Shake flask cultures were filtered through miracloth. Filtrate samples were

mixed with 4x SDS protein sample buffer (8% SDS, 40% glycerol, 20% beta-mercaptoethanol, 0.08% bromophenol blue and 240 mM Tris-HCl pH 6.8), heated for 5 minutes at 95°C and loaded on 9% SDS polyacrylamide gels. Proteins were either stained with the SYPRO® Ruby protein gel stain (Life Technologies) or blotted to a PVDF membrane through semi-dry electrophoretic transfer. The membranes were blocked for 1 hour with 5% non-fat dry milk in PBST (PBS, 0.05% (v/v) Tween-20), incubated with the anti-PepN antibody (1:50) for 1 hour and subsequently incubated with the goat anti-rabbit horseradish peroxidase secondary antibody conjugate (1:20000) (Bio-Rad). Immunoreactive bands were detected using peroxide and luminol/enhancer reagents (Bio-Rad).

Results

PepN is likely to be secreted via an unconventional secretion mechanism

The PepN protein was used as a target protein to study unconventional secretion of proteins in *A. niger.* PepN is a pepsin-like aspartic protease which is abundantly present in the culture filtrate of carbon starved cultures as determined by proteomics studies (Braaksma *et al.*, 2010; Nitsche *et al.*, 2012; Krijgsheld *et al.*, 2012). In contrast to other secreted proteases, it is predicted not to have a typical leader sequence (Wang *et al.*, 2008; Braaksma *et al.*, 2010), indicating that this protein might not pass through the classical secretory pathway, but is secreted by unconventional means. Comparison of the protein sequence of PepN with the secreted aspartic protease PepA in different *Aspergillus* species further showed that PepN misses the conserved pro-peptide which is present in PepA orthologs (Fig. S3). It should be noted that from the available proteome data no peptide sequences could be identified from the first 100 amino acids of PepN (Fig. S2). Although this might be explained by the distribution of arginine and lysine residues in this part of the sequence rendering two very large and several very small peptides upon digestion, it precludes formal proof of secretion of the predicted full length PepN protein. Considering the C-terminal end of the sequence, peptides could be identified resolving the correct annotation of the PepN protein out of two available sequences (NCBI accession: XP_001388485). The protein sequence of PepN contains four putative N-glycosylation sites, but none of these are conserved in other PepN orthologs (Fig. S3), suggesting that these sites in PepN are not glycosylated *in vivo* (Park and Zhang, 2011). This was supported by proteome analyses, which identified two putative glycosylation sites as non-glycosylated residues in the identified peptides (Fig. S2) and by the observed activity of a PepN antibody developed against an epitope containing one of the sites (see below).

The deletion of *pepN* in *A. niger* does not affect proteolytic activity

An *A. niger* Δ*pepN* mutant was generated by replacing the open reading frame with the hygromycin resistance cassette using the split marker approach (Arentshorst *et al.*,

2015b). Homologous integration was confirmed by Southern analysis (Fig. S1). Wild-type and mutant strains were subsequently tested for proteolytic activity by growing them on casein-containing medium for seven days. The size of the clear zone (halo) which is being formed upon degradation of casein correlates to the protease production in the respective strain (Sekine *et al.*, 1969). The results show that halo sizes were comparable between wild-type and Δ*pepN* strains, indicating that strains defective for PepN are not largely affected in total proteolytic activity (Fig. 1A), although Wang *et al.* (2008) showed that deletion of the *pepN* gene affected secreted levels of a heterologous laccase. In agreement with the plate assays, fluorometric analysis of total protease activities of culture filtrates harvested at different time points showed no difference between wild-type and Δ*pepN* mutants (Fig. 1B). To analyze whether expression of the *pepN* gene is controlled by the regulator PrtT (Punt *et al.*, 2008) which regulates the major secreted protease genes *pepA* and *pepB* (van den Hombergh *et al.*, 1997b), transcriptome data of the *prtT* mutant strain AB1.13 (Mattern *et al.*, 1992; Punt *et al.*, 2008) were analyzed for *pepN* expression. In contrast to the very low expression levels for *pepA* and *pepB*, *pepN* expression was still highly abundant (Fig. 1C), indicating that expression of *pepN* is not regulated by PrtT. Whereas PrtT-regulated proteases are responsible for the majority of the protease activity in *A. niger*, PepN activity could be involved in extracellular proteolysis of specific substrates.

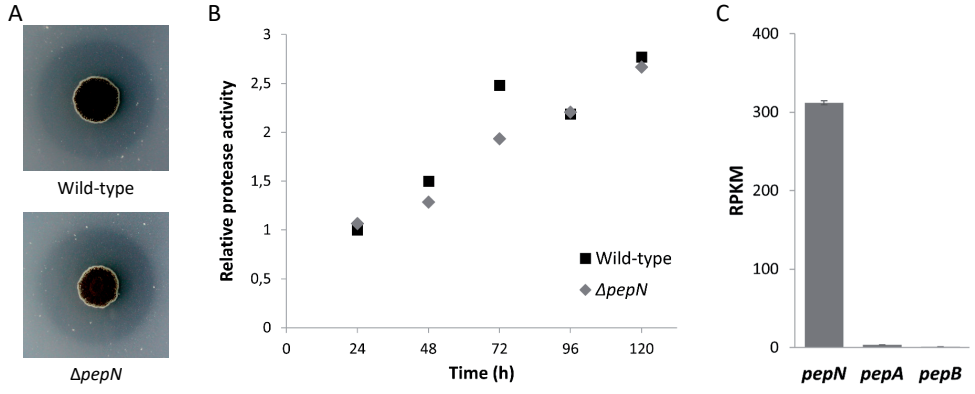

Figure 1 | Deletion of *pepN* in *A. niger* does not affect proteolytic activity. A) Spores from wild-type and Δ*pepN* strains were point inoculated on pre-acidified skim milk plates (pH 3.0) and grown for seven days at 30°C before halo sizes were compared. B) Wild-type and Δ*pepN* strains were grown in CM at 30°C and supernatants were collected at the indicated time points. Proteolytic activities were normalized using wild-type (24h) as a reference. C) Expression of genes encoding selected proteases (PepA, PepB and PepN) in the *prtT*- mutant strain AB1.13. RNA for RNAseq analysis was isolated from shake flask cultures grown for 48 hours in Medium 12 as described by Li *et al.* (2012). Only low RPKM (reads per kilobase per million) values were found for *pepA* and *pepB* protease-encoding genes, whereas higher expression was observed for *pepN*. Error bars indicate standard deviation from two biological replicates.

Proteome analysis of the *A. niger* Δ*atg8* mutant during carbon starvation shows the presence of PepN in culture filtrate

To investigate whether the secretion of PepN is dependent on a functional autophagy machinery, secretomes of wild-type and Δ*atg8* mutant strains were determined and analyzed. The bioreactor cultivations of the wild-type and Δ*atg8* mutant were performed and described previously (Nitsche *et al.*, 2013). For this study, proteins were precipitated from culture filtrates of wild-type and Δ*atg8* bioreactor cultures after 20 hours of carbon starvation, digested with trypsin, and analyzed by mass spectrometry, which revealed that PepN was abundantly present in both wild-type and Δ*atg8* secretomes (Table 3). In agreement with the observed increase in activity of extracellular hydrolases during carbon starvation (White *et al.*, 2002; Nitsche *et al.*, 2012), other proteins that were identified include several proteases and carbohydrate active enzymes, together with some cell wall proteins. The profile of identified proteins showed only minor differences between wild-type and Δ*atg8* secretomes, with some proteins being present in wild-type but not in Δ*atg8* and vice versa. Interestingly, PepN was the only identified protein lacking a predicted signal peptide.

Autophagy-independent secretion of PepN is confirmed by Western analysis of Δ*atg1* and Δ*atg8* culture filtrates.

The presence of PepN in culture filtrates of both wild-type and Δ*atg8* strains indicates that the secretion of PepN is not dependent on a functional Atg8 protein. In order to confirm this result and to test other mutants on their ability to secrete PepN into the culture medium, Western blots were performed using PepN specific antibodies. Two individual peptide antibodies were produced (Fig. S2), which were first tested for their specificity to PepN using culture filtrates of the wild-type and the Δ*pepN* mutant, collected from shake flask cultures during carbon starvation. Protein staining following gel electrophoresis showed a comparable protein profile between wild-type and Δ*pepN* samples (data not shown). Furthermore, both of the antibodies showed activity against the PepN protein (data not shown), and the antibody against the epitope closest to the C-terminal was chosen to be used for further experiments. It showed a clear band in wild-type samples between 40 kDa and 50 kDa, which corresponds to the expected size of the non-glycosylated protein (47 kDa) (Fig. 2A). As expected, this band was not observed in Δ*pepN* culture filtrates. It should be noted that the antibody showed cross-reactivity towards a slightly larger protein, but this cross reactivity was also found in proteins from the culture fluid of the *pepN* mutant (Fig. 2 and data not shown). The detection of this cross-reactive protein was variable between different experiments. The antibody was subsequently used to investigate whether PepN was also present in bioreactor culture filtrates of the autophagy mutants Δ*atg1* and Δ*atg8* (Nitsche *et al.*, 2013). In agreement with the results from the secretome analysis, PepN could be detected on Western blot in culture filtrates of the wild-type and the Δ*atg8* mutant after 20 hours of carbon starvation (day 1, Fig. 2B). Additionally, PepN was not present during the exponential growth phase, but was found on all other carbon starvation time points tested,

Table 3 | Secretome data

			Protein abundance[a]		
Identifier	**Gene**	**(Predicted) function**	**N402**	**Δ*atg8***	**SP[b]**
Proteases/peptidases					
An01g00370	*pepN*	Aspartic protease	+++	++++	No SP
An01g00530	*pepB*	Acidic protease	+	-	SP
An01g01750		Sedolisin protease	+	+[c]	SP
An02g04690		Serine-type carboxypeptidase	+	+[c]	SP
An03g05200	*protF*	Carboxypeptidase	+	+	SP
An06g00190		Sedolisin protease	+	+[c]	SP
An08g04490	*protA*	Lysosomal carboxypeptidase	++	-	SP
An08g04640	*protB*	Sedolisin protease	+	-	SP
An14g04710	*pepA*	Aspartic protease	++++	++++	SP
An18g01320		Pepsin protease	+[c]	+	SP
ATCC 53364		Aspartic protease	-	+	SP
Carbohydrate active enzymes					
An01g11010	*crhD*	Putative chitin-β-glucanosyltransferase	+	++	SP
An01g12450	*exsG*	Exo-1,3-β-glucanase	+	+++	SP
An01g12550	*msdS*	1,2-α-mannosidase	-	++	SP
An03g05290	*bgtB*	Glucan endo-1,3-β-glucosidase	++	+	SP
An03g06550	*glaA*	Glucan 1,4-α-glucosidase	++++	++++	SP
An09g00670	*gelD*	1,3-β-glucanosyltransferase	+	+[c]	SP
An11g03340	*aamA*	acid α-amylase	++	+++	SP
An15g02300	*abfB*	α-arabinofuranosidase	++	++	SP
An16g03330		GPI-anchored cell wall protein	-	+	SP
An16g06800	*eglB*	Endo-1,4-β-glucanase	+	+[c]	SP
ATCC 53033		1,3-β-glucanosyltransferase	+	++	SP
Cell wall proteins					
An04g01230	*ecmA*	Putative cell wall organization protein	+	++	SP
An06g00160		Hypothetical protein	++	+++	SP
An08g09420		Antigenic cell wall galactomannoprotein	-	+	SP
An14g01820	*phiA*	Cell wall protein	+	-	SP
An14g02100	*cwpA*	GPI-anchored cell wall mannoprotein	+	++	SP
Other					
An02g13750		Glutaminase A	+	++	SP
An07g08400		Strong similarity to rAsp f 4	-	+	SP
An16g01880	*lipanI*	Lysophospholipase	++	+[c]	SP
ATCC 55058		Hypothetical protein	+[c]	+	SP

[a] Protein abundance as roughly determined from biological duplicate experiments as described by Ishihama *et al.* (2005): (++++) > 10%; (+++) > 4%; (++) > 2%; (+) > 0%; (-) not detected.
[b] Signal peptide prediction (Braaksma *et al.*, 2010; Petersen *et al.*, 2011).
[c] Result based on detection in only one of the duplicates.

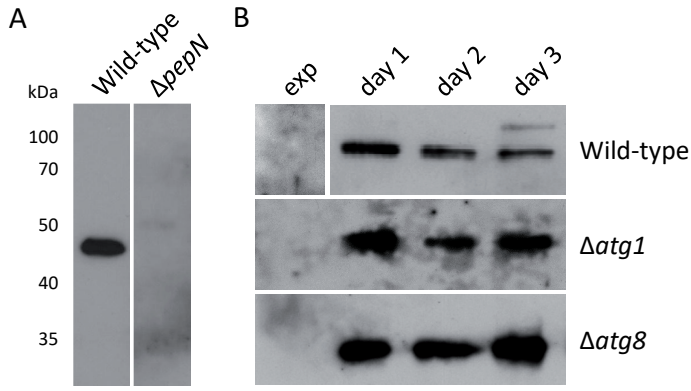

Figure 2 | Western blot analysis on the presence of the PepN protein. A) Culture filtrate samples were collected from wild-type and Δ*pepN* shake flask cultures after 120h of growth at 30°C in CM. The protein samples were separated by gel electrophoresis and immunodetected with an anti-PepN antibody. B) Culture filtrates were collected from wild-type, Δ*atg1* and Δ*atg8* bioreactor cultures (Nitsche *et al.*, 2013) during the exponential growth phase (exp) and during carbon starvation as indicated (day 1 – day 3). Equal amounts of culture fluid (15 µL) were loaded in each lane to be separated by gel electrophoresis and immunodetected with an anti-PepN antibody. In some cases, a second band was observed above the PepN band, which is probably the result of cross-reactivity of the antibody with another protein in the sample.

both in wild-type, Δ*atg1* and Δ*atg8* culture filtrates. This indicates that the secretion of PepN is induced by carbon starvation conditions, which has been reported before (Braaksma *et al.*, 2010; Nitsche *et al.*, 2012), but neither *atg1* nor *atg8* is required for the efficient secretion of PepN into the culture broth.

Discussion

Improving our understanding of the mechanisms for UPS in filamentous fungi is of great interest, because the use of these pathways for the production of heterologous proteins could result in highly improved production yields and activities as proteins exported via unconventional secretion most likely do not undergo unwanted posttranslational modifications. One of the major bottlenecks for the production of heterologous proteins in filamentous fungi arises with the strict protein quality control in the ER, which recognizes misfolded proteins and targets them for degradation via the ER-associated degradation pathway (Ruggiano *et al.*, 2014). Unwanted modification processes also include glycosylation and processing by Golgi-localized proteases, which might affect the activity of the secreted proteins (Schmidt, 2004; Nevalainen and Peterson, 2014). To circumvent the posttranslational modification of extracellular proteins during their passage through the classical secretory pathway, the use of UPS pathways is a promising alternative. This

principle has already been successfully applied in *Ustilago maydis*, in which heterologous proteins were secreted unconventionally by fusing them to the N-terminus of Cts1 (Stock *et al.*, 2012). However, in contrary to other eukaryotic organisms, the mechanisms by which proteins are exported via UPS pathways are largely unknown for filamentous fungi. To gain more insights into the process of UPS in *A. niger*, we investigated whether autophagy is an involved mechanism by analyzing secretomes of *atg* deletion mutants for the presence of the PepN protein.

An increasing number of proteins have been shown to be secreted in an unconventional manner. As such, we identified the PepN protein to be exported most likely without transiting through the conventional secretory pathway. An alternative explanation for the presence of PepN in the culture filtrate could be the occurrence of cell lysis. However, in previous studies (Braaksma *et al.*, 2010; Nitsche *et al.*, 2012), and in this present study, PepN was found to be abundantly present in the culture fluid while the contribution of cytosolic proteins in these studies was found to be very limited. Currently, we can formally not exclude that PepN is a cytosolic protein that is present in the culture filtrate after cell lysis and that detection of PepN is possible because it is resistant against proteolytic degradation while other cytosolic proteins are quickly degraded. However, we find this scenario less probable also given the fact that unconventional secretion of other proteins, e.g. Cts1 in *Ustilago maydis* has been shown to take place (Stock *et al.*, 2012). The structure of the PepN protein sequence is different from that of the secreted protease PepA, as it lacks the conserved pro-peptide sequence and a typical secretion peptide (Fig. S3). BLASTp analysis showed that PepN orthologs are found in other *Aspergillus* species, as well as in *Trichoderma* and *Penicillium* species, also lack a predicted signal peptide sequence (see Fig. S3). The absence of a predicted signal peptide provides evidence for the PepN protein to be secreted via an unconventional pathway. Furthermore, no indications were found for posttranslational processing events on PepN, as putative glycosylation sites were determined to be non-glycosylated *in vivo* (Fig. S2), while the size of the protein on Western blot corresponded well to the expected full-length protein size (Fig. 2A).

The deletion of *pepN* in a heterologous laccase producing Δ*pepA* background increased the total extracellular laccase activity compared to the parental strain (Wang *et al.*, 2008), indicating that eliminating the proteolytic activity deduced by PepN could improve production yields. Proteolytic activity was also decreased upon the deletion of *pepA* or *pepB* in *A. niger* (Mattern *et al.*, 1992; van den Hombergh *et al.*, 1997b). However, the single deletion of *pepN* performed in this present study did not have an effect on total proteolytic activity compared to wild-type (Fig. 1), which might indicate that its activity is highly substrate specific as was observed for other aspartic proteases such as chymosin (Kageyama, 2002) or could only be observed in combination with the deletion of other highly expressed protease genes like *pepA* or *pepB*, or under specific growth conditions.

Autophagy has been associated to UPS based on studies in yeast and mammalian cells showing that the unconventional secretion of several proteins is dependent on elements of the autophagy machinery (Ponpuak *et al.*, 2015). The unconventional secretion of Acyl-CoA binding protein (Acb1) in yeast requires several autophagy-related genes, including *atg1* and *atg8* (Duran *et al.*, 2010; Manjithaya *et al.*, 2010). Similarly, in mammalian cells it was shown that the unconventional secretion of the cytokine IL-1β is promoted by the induction of autophagy in an Atg5-dependent manner (Dupont *et al.*, 2011). By using both proteome (mass spectrometrical) and immunoblotting analyses, we were able to show that PepN was present in secretomes of wild-type, Δ*atg1* and Δ*atg8* mutants (Table 3; Fig. 2B), indicating that both Atg1 and Atg8 are not required for the secretion of PepN in *A. niger*. Since these autophagy proteins are considered to have important roles in the initiation of autophagy and the formation of autophagosomal vesicles, respectively (Inoue and Klionsky, 2010; Bartoszewska and Kiel, 2011), and were shown to be essential for autophagy in *A. niger* (Nitsche *et al.*, 2013), it was concluded that the secretion of PepN is independent of the autophagy machinery. However, it cannot be excluded that autophagy is involved in the unconventional secretion of proteins other than PepN, although this might be dependent on specific culture conditions as PepN was the only protein identified during this study lacking a predicted signal sequence. An obvious candidate gene with a possible function in non-classical secretion in *A. niger* is the GRASP (Golgi-reassembly and stocking protein) homolog. GRASP has been shown to be required for the unconventional secretion of Acb1 in *Dictyostelium discoideum* and *Pichia pastoris* (Kinseth *et al.*, 2007; Manjithaya *et al.*, 2010). A BLASTp search identified a candidate GRASP protein in *A. niger* (An11g05650) and its possible role in PepN secretion could be addressed in future experiments. It should be noticed however that subsequent studies also showed that Acb1 secretion required, in addition to GRASP, autophagy proteins (Duran *et al.*, 2010; Manjithaya *et al.*, 2010). Although our study does not exclude the possibility of GRASP involvement, the mechanism of unconventional secretion of PepN in *A. niger* is likely to be different from that of the Acb1 protein as autophagy is dispensable for PepN secretion. Further studies are required in order to increase our understanding of mechanisms for UPS as well as to identify genes that are involved in these processes in *A. niger.*

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