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Regulation of Autolysis in *Aspergillus nidulans*

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Abstract In terms of cell physiology, autolysis is the centerpiece of carbon-starving fungal cultures. In the filamentous fungus model organism *Aspergillus nidulans*, the last step of carbon-starvation-triggered autolysis was the degradation of the cell wall of empty hyphae, and this process was independent of concomitantly progressing cell death at the level of regulation. Autolysis-related proteinase and chitinase activities were induced via FluG signaling, which initiates sporulation and inhibits vegetative growth in surface cultures of *A. nidulans.* Extracellular hydrolase production was also subjected to carbon repression, which was only partly dependent on CreA, the main carbon catabolite repressor in this fungus. These data support the view that one of the main functions of autolysis is supplying nutrients for sporulation, when no other sources of nutrients are available. The divergent regulation of cell death and cell wall degradation provides the fungus with the option to keep dead hyphae intact to help surviving cells to absorb biomaterials from dead neighboring cells before these are released into the extracellular space. The industrial significance of these observations is also discussed in this paper.

Keywords *Aspergillus nidulans* · Autolysis · Apoptosis · Chitinase · Proteinase · chiB · prtA · creA · fluG

Introduction

Autolysis can be defined as a natural process of self-digestion of aged hyphal cultures, occurring as a result of hydrolase activity, causing vacuolization and disruption of organelle and cell wall structures [1]. Professor Brian McNeil and his coworkers were the first to present data on that autolysis is an energy-dependent process [2], and later other researchers supported their view [3–6]. Nowadays, the autolysis of filamentous fungi is not considered

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as simple cell necrosis any longer, instead it is now thought to be an active and well-regulated process, which helps these microorganisms to survive in a continuously changing, hostile environment. The emerging regulatory elements of autolysis provide us with good opportunities to control and manipulate this process for several practical proposes. In the fermentation industry, induction of autolysis can accelerate the release of certain intracellular metabolites [1, 7] and enhance the production of numerous industrially important hydrolases including proteinases, chitinases, and glucanases [1] and other enzymes [8]. Inhibition of autolysis can also help us to lengthen the idiophase of industrial cultivations, can maintain pelleted cell morphology as well as can hinder or even block proteinase production and, hence, proteolytic degradation of valuable protein products [1, 4]. Moreover, a deeper understanding of the underlying mechanism of fungal autolysis may also be exploited in future antifungal drug research [9–11] and the development of transgenic plants with antifungal activity [12].

In this paper, we summarize our observations on the nature and regulation of autolysis induced by carbon starvation in *Aspergillus nidulans*. We are also aiming at the discussion of some potential industrial applications of these results.

Detection and Regulation of Autolysis in Carbon-starving *Aspergillus nidulans* Cultures

Autolysis of filamentous fungi can be characterized by several markers including decreasing viability, declination of dry cell mass, vacuolization, formation of empty hyphae, fragmentation of hyphae, decreasing pellet diameter, production of numerous intracellular and extracellular hydrolases (e.g., proteinase, chitinase, glucanase, RNase, DNase), and release of ammonia [1, 2, 4, 6, 13]. Most of these markers are not exclusively specific for the autolytic process, e.g., a decreasing viability can be the consequence of another age-related physiological process, autophagy, which also relies on hydrolases and results in vacuolization [14, 15], and the utilization of exogenous substrates (e.g., proteins) as energy sources also requires extracellular hydrolases (e.g., proteinases) and may liberate ammonia. In our experiments, we chose dry cell mass declination, hyphal fragmentation (together with the disintegration of pellets quantified by shrinking pellet diameters), and the production of extracellular chitinase to characterize autolytic phenotypes [6, 16–21]. All the selected markers are connected to cell wall degradation because cell wall represents high percentage of total dry cell mass of fungal cultures, and its breakage and effective disassembly need extracellular hydrolases including autolysis-related chitinase activity [22–24]. In stirred submerged cultures, shearing forces may profoundly influence the fragmentation of hyphae and disintegration of pellets but hyphae normally break at segments, which have already been weakened by progressing vacuolation [2, 25, 26].

In *A. nidulans*, a series of mutants with either nonautolytic or hyperautolytic phenotypes are available, which provides us with an excellent tool to study the physiology and regulation of carbon-starvation-related autolysis in filamentous fungi. For example, the loss-of-function *fluG* (*fluG1*) mutant showed a nonautolytic phenotype with no significant fragmentation of hyphae and autolytic loss of biomass, produced low extracellular chitinase activity, and preserved its pelleted morphology at least for a week after glucose depletion in submerged cultures [17]. The *fluG1* mutation also blocked proteinase production [17]. The *fluG* gene encodes a cytoplasmically localized protein with homology to the prokaryotic glutamine synthetase I [27–29], and the hypothesized small diffusible product of FluG activity is thought to initiate conidiogenesis and inhibit vegetative growth via the activation

of the conidiation-specific Flb proteins [30]. Mutants of the FluG signaling pathway [31], including loss-of-function *sfgA*, *flbB–D*, *brlA*, and *abaA* mutations also affected the appearance of autolytic markers; however, these changes were not so clear-cut and unidirectional as in the case of the *fluG1* mutant [17].

In contrast to *fluG1*, the *creA* null mutant showed a hyperautolytic phenotype with high extracellular chitinase and proteinase activities, and was characterized with significantly increased dry cell mass declinations and fragmentation of hyphae in comparison to control strains [19]. The product of the *creA* gene is the major carbon catabolite repressor transcriptional factor in *A. nidulans* [32] with a finger domain similar to that of the *Saccharomyces cerevisiae* carbon regulatory protein Mig1p [33]. CreA is a negatively acting repressor and there is evidence that CreA plays a regulatory role under both carbon catabolite repressing and derepressing conditions [34, 35]. It is interesting to note that the partial loss-of-function *creA204* mutation only had a minor effect on autolysis, and mutations in the other carbon catabolite regulators creB (an ubiquitin-processing protease; tested mutant: *creB15*; [36]) and creC (a WD40 repeat protein; tested mutant: *creC27*; [36]) did not affect significantly the autolytic cell wall degradation (data not shown). It is worth mentioning that glucose negatively influenced the autolytic cell wall degradation even in the *creA* null mutant by repressing FluG-dependent signaling [17].

These observations demonstrate that autolytic cell wall degradation and conidiation are tightly interconnected at the level of regulation and are under strong carbon repression. This is highly reasonable because one of the main functions of autolysis is to supply nutrients for sporulation, when no other energy and carbon sources are available [30, 37, 38]. Degradation of cell wall, which is one of the main constituents of dead vegetative cells, lets the culture develop conidia, which are not only propagating but also resting cell forms and, this way, helps the fungus to survive carbon starvation. When conidiation is blocked in starving submerged cultures autolysates may maintain yeast-like surviving morphological forms instead of giving rise to conidia [4–6, 23, 24, 39].

ChiB and PrtA are Involved in Autolytic Cell Wall Degradation in *A. nidulans*

Disassembly of cell wall biopolymers requires the cooperation of numerous hydrolytic enzymes but only few of them have been identified and characterized so far in *A. nidulans*.

Several literature data demonstrate that the ChiB, an extracellular chitinase, is involved in the autolytic cell wall degradation, namely, (1) the expression of the *chiB* gene was induced during autolysis [17, 19, 40], (2) more than 90% of the extracellular chitinase activity measured in autolysing cultures was attributed to ChiB [40], and (3) the *ΔchiB* mutant showed decreased autolytic cell wall degradation and produced low extracellular chitinase activity during carbon starvation [41]. As shown in Table 1 and Fig. 2, FluG-dependent signaling was important in the induction of ChiB production by carbon depletion because the inactivation of the FluG pathway resulted in a markedly decreased and delayed chitinase production [17]. Glucose and the glucose antimetabolite 2-deoxy-D-glucose profoundly repressed ChiB production via both CreA-dependent and CreA-independent pathways by repressing *brlA* in the latter case [19]. The transcriptional factor BrlA is an important early regulator of conidiogenesis, which is an upstream element in FluG/Flb-mediated signaling [30]. Other transcriptional factors that may affect chitinase production are the developmental regulator AbaA, which is activated by BrlA during the middle stages of conidiophore development [30, 42] and SfgA, a suppressor of conidiogenesis, the repressive effect of which is removed by FluG [31] (Table 1, Fig. 2).

Table 1 Chitinase production in carbon-starving *A. nidulans* cultures.

Culture condition/mutation[a]	Phenotype	Reference or relative activity[b]
Addition of glucose or 2-deoxy-D-glucose to FGSC 26 (*biA1 veA1*) reference strain or to *creA* null mutant	Decreased *chiB* transcription and ChiB production	[19]
Addition of colloidal chitin to FGSC 26 (*biA1 veA1*) reference strain	Increased *chiB* transcription	[40]
fluG1	Reduced and delayed chitinase production	[17]
ΔsfgA	Increased chitinase production	This study (141±12%)
ΔbrlA	Reduced and delayed chitinase production	[17]
abaA14	Reduced and delayed chitinase production	This study (47±5%)
wetA6	Similar to wild type	This study (96±9%)
ΔcreA	Increased (five times higher) chitinase production	[19]
creA204	Increased chitinase production	This study (134±15%)
creB15	Similar to wild type	This study (111±12%)
creC27	Similar to wild type	This study (103±12%)

[a] GanB and FadA signaling pathways had no significant effect on chitinase production [20, 21].

[b] Relative specific activities are shown in parenthesis where no reference is given. Specific activities (chitinase activity/dry cell mass [6, 19]) calculated in glucose- or chitin-supplemented cultures or in cultures of mutants were factored with those recorded in *A. nidulans* FGSC 26 (*biA1 veA1*) control cultures. Means±SD calculated from four to five independent experiments are presented.

As published by Pusztahelyi et al. [40], reactive oxygen species, which normally accumulate in carbon-starving cultures of filamentous fungi [6], did not influence the transcription of *chiB*. On the other hand, *N*-acetyl-D-glucosamine, chitooligomers, and even colloidal chitin induced the expression of *chiB* and, hence, low quantities of these saccharides, which are liberated from the cell wall in late-exponential and stationary growth phases, may contribute to the age-dependent upregulation of *chiB* and the induction of other chitin-metabolizing enzymes like the *N*-acetyl-β-D-glucosaminidase encoded by *nagA* [40]. Because ChiB equally splits chitin in exponential and autolytic growth phase mycelia, this hydrolase may also participate in the digestion and utilization of endogenous chitin during carbon starvation in addition to promoting the autolytic breakage of vacuolated hyphae [40, 43].

Although we did not find any clear-cut connection between autolytic cell wall degradation and proteinase production in carbon-starving *A. nidulans* cultures [20, 21], the elimination of the *prtA* gene coding for an extracellular proteinase [44] resulted in a statistically significant decrease in the declination of dry cell mass and delay in the disorganization of pellets (Fig. 1) during the early autolytic phase of cultivation (25–75 h; [6]). Considering that the *ΔprtA* mutation did not influence the autolytic chitinase production of the fungus at all (Fig. 1), PrtA itself was likely involved in autolytic cell wall degradation. In older cultures, we could not detect significant differences either in the proteinase production, in the DCM declination, or in the average pellet diameter. As the *ΔprtA* mutant still produced considerably high extracellular proteinase activities, and this mutation did not block the progression of autolysis, the involvement of other proteinases in autolytic cell wall degradation seems to be likely.

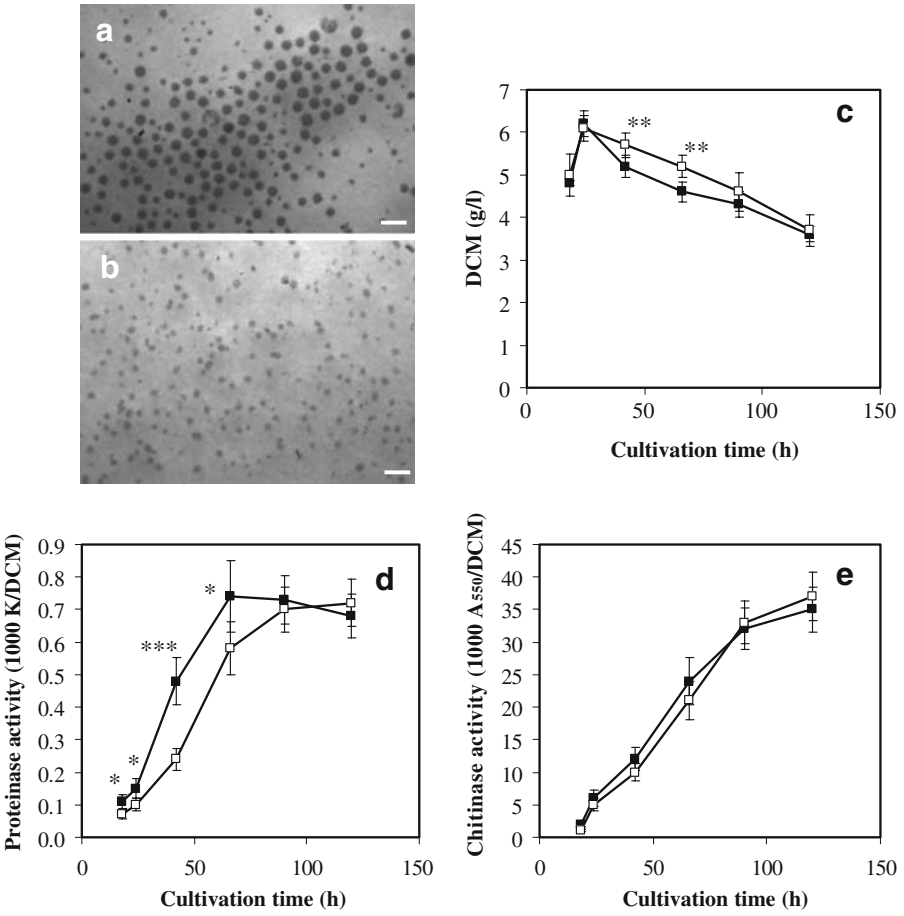

Fig. 1 Autolytic phenotype of the *A. nidulans* MK189 (*yA1 pabaA1*; *argB2*; *prtAΔ::argB1*; *open squares*) mutant in comparison to the MK191 (*yA1 pabaA1*; *argB2 [argB1]*; *closed squares*) strain as a control. **a**–**b** Comparison of pellet diameters observed at 75 h cultivation time (bars=5 mm). The average pellet diameter of the *ΔprtA* mutant (2.8±0.2, n=50; **a**) was significantly higher (P<0.05) than that of the control (1.2±0.2, n=50; **b**) as calculated by Student's t test. **c**–**e** Changes in the dry cell mass (*DCM*; **c**), and the specific extracellular proteinase (**d**) and chitinase (**e**) productions during autolysis. DCM, proteinase, and chitinase activities were measured according to Emri et al. [17], and mean±SD values calculated from four independent experiments are presented. Statistically significant differences determined by the Student's t test are marked with asterisks: *P<0.05, **P<0.01, ***P<0.001

As demonstrated in Table 2 and similar to the extracellular proteinase production of *Botrytis cinerea* [45], FadA- and GanB-dependent heterotrimeric G protein signalings [20, 21, 30, 46–48] were important in the regulation of proteinase production by *A. nidulans*, and the expression of *prtA* was also under GanB/RgsA control [21]. It is important to note that all subunits of the FadA/SfaD/GpgA (representing G_α, G_β, and G_γ subunits, respectively) G protein complex, which mediates signals supporting vegetative growth [48–50], hindered proteinase production meanwhile, paradoxically, both GanB G_α subunit and RgsA, a negative regulator of GanB-dependent signaling [47], were needed in the production of wild-type level extracellular proteinase activities (Table 2). One explanation for this complex regulation pattern could be that the GanB G_α subunit interacts with the

Table 2 Proteinase production in carbon-starving *A. nidulans* cultures.

Culture condition/mutation	Phenotype	Reference or relative activity[a]
Addition of glucose/2-deoxy glucose to FGSC 26 (*biA1 veA1*) reference strain or to *creA* null mutant	Decreased proteinase production	[19]
fluG1	No significant proteinase production	[17]
ΔsfgA	Increased proteinase production	This study (132±15%)
ΔbrlA	Slightly increased proteinase production	[17]
abaA14	Similar to wild type	This study (93±10%)
wetA6	Similar to wild type	This study (103±10%)
ΔcreA	Increased (20 times higher) chitinase production	[19]
creA204	Increased proteinase production	This study (124±11%)
creB15	Similar to wild type	This study (107±10%)
creC27	Similar to wild type	This study (109±10%)
ΔfadA	Increased proteinase production	This study (153±16%)
$fadA^{G203R}$	Delayed and increased proteinase production	[20]
ΔflbA	Early proteinase production	[20]
ΔganB	Reduced proteinase production	[21]
ΔrgsA	Reduced proteinase production	[21]
ΔsfaD	Increased proteinase production	This study (157±17%)
ΔgpgA	Increased proteinase production	This study (196±21%)

[a] Relative specific activities are shown in parenthesis where no reference is given. Specific activities (proteinase activity/dry cell mass [6, 19]) calculated in glucose-supplemented cultures or in cultures of mutants were factored with those recorded in *A. nidulans* FGSC 26 (*biA1 veA1*) control cultures. Means±SD calculated from four to five independent experiments are presented.

same SfaD and GpgA G_β and G_γ subunits as FadA and, hence, the stabilization of the inactive GanB(GDP)/SfaD/GpgA complex by RgsA [48] may result in an overall positive effect on protease production by hindering the release of the negative effector SfaD and GpgA subunits. As FluG blocks the FadA-dependent vegetative growth signal by activating FlbA, a negative regulator of G protein signaling, and also removes the repressive effect of SfgA on asexual development [30, 31, 48], it also stimulates proteinase production meanwhile SfgA affects it negatively (Table 2, Fig. 2).

Extracellular proteinase production was under a strong carbon metabolite control as well (Table 2, Fig. 2, [19]). Similar to *chiB*, proteinase production and the expression of *prtA* were repressed by glucose and 2-deoxy-D-glucose via both CreA-dependent and CreA-independent regulatory pathways [19]. In CreA-independent carbon regulation, proteinase production was most likely controlled via FadA-dependent signaling [19]. As *A. nidulans* proteinases are also subjected to nitrogen, sulfur, and phosphor repressions [44, 51, 52] their primary role could be to provide hyphae with nutrients when vegetative growth is restricted by any kind of nutrient limitation. Obviously, the first possibility for the fungus under these circumstances is to accelerate the mobilization of intracellular peptide (e.g., glutathione) and protein reserves [6, 20, 21], meanwhile the autolytic digestion of the proteinous constituents of the cell wall remains a second choice. In accordance with this assumption, the production of different autolysis-related proteinases was sequential in

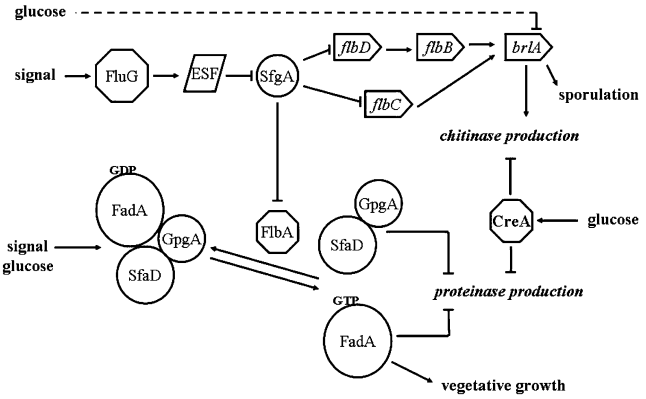

Fig. 2 A possible regulatory network of extracellular chitinase and proteinase production in *A. nidulans*

starving *P. chrysogenum* cultures allowing the organism to mobilize endogenous reserves at first and to initiate gross culture degradation later [13, 53].

The degradation of glutathione reserves is under a strict dual FadA/FlbA and GanB/RgsA control in *A. nidulans* [20, 21] because it may cause a severe glutathione/glutathione disulfide redox imbalance leading to the accumulation of reactive oxygen species and, consequently, to the onset of genomic cell death programs [6, 54].

Cell Death and Autolysis

It is interesting to note that neither *fluG1* nor *creA* null mutations had a significant effect on the accumulation of reactive oxygen species, appearance of apoptotic markers, and the decrease in viability observable under carbon starvation [18, 19]. These observations suggested that cell death and autolytic cell wall degradation were regulated independently in *A. nidulans*. In their earlier work, McIntyre et al. [2] also found that autolysis and decreasing metabolic activity are distinct phenomena in *P. chrysogenum*. Some more recent experimental data published by Leiter et al. [55] and Marx et al. [56] indicated that FadA-dependent heterotrimeric G protein signaling was involved in the initiation of apoptotic cell death in *A. nidulans*. Moreover, the generation of elevated levels of reactive oxygen species—elicitors of programmed cell death—was likely modulated via GanB/RgsA heterotrimeric G protein mediated signaling [21, 47].

One interpretation of these observations is that autolysis is sensu stricto the degradation of dead segments of filaments, which may succeed or may progress concomitantly with but independently of cell death. Nevertheless, programmed cell death is a common event in filamentous fungi under carbon starvation [3, 18, 19], and the mobilizable biomaterials of dead or dying cells flow easily into adjacent surviving cells leaving empty hyphal segments behind, which may undergo lysis later. Therefore, another option is to define this complex physiological process including both cell death (either apoptotic, autophagic, or even necrotic) and the age-dependent multilevel and sequential decomposition of cellular biopolymers, e.g., those of organelles, the cytosol, and cell wall, as autolysis sensu lato. The authors prefer the second definition because autolytic events including cell death—although regulated in different ways—are inseparable and are delicately balanced in wild-type strains. The regulation of the biosynthesis of cell-wall-degrading autolytic enzymes (chitinases, proteases, and glucanases) is especially sophisticated because either early release

or overexpression of these enzymes may threat the integrity of surviving cells. The independent but tightly coupled regulation of cell death and autolytic cell wall degradation may provide the fungus with another advantage to keep dead hyphae intact to avoid any early and uncontrolled collapse of conidia-bearing aerial hyphae in surface cultures and help surviving hyphal segments to take up and incorporate biomaterials coming from adjacent dead cells before these valuable building blocks are released into the extracellular space.

Industrial Significance

A deeper understanding of the underlying molecular mechanism and regulation of autolysis sensu lato can provide us with powerful tools in the metabolic engineering of the morphology of idiophasic cultures of filamentous fungi [5, 57]. When the maintenance of pelleted macromorphology is beneficial to lengthen the production phase, to minimize the energy uptake of stirring in fermenters, and/or to facilitate downstream processing, e.g., filtration, steps [1] mutations in the *fluG*, *brlA*, *chiB*, or *prtA* genes or in their homologues can be considered (Fig. 1; [17]). Moreover, elimination of *fluG* also results in a mutant strain with minute protease production (Table 2), which represents a highly advantageous background for the development of high-yield heterologous protein expression systems. It is important to note that these mutations had only minor effects on microbial growth and, at least in the case of *ΔchiB* and *ΔprtA*, conidiogenesis was not affected either [17].

It is important to note that the hypothetical small-molecular-mass diffusible product of the FluG protein (ESF), which activates the Flb/BrlA sporulation cascade [30] and initiates autolysis as well [17], has remained yet to be identified. This metabolite, if stable enough, would be an invaluable tool in the initiation of controlled gross autolysis of industrial-scale cultures, e.g., to facilitate the release of cell-bound or cell-absorbed metabolites [58], and in the stimulation of the conidiogenesis of weakly sporulating or nonsporulating industrial strains of filamentous fungi.

The elimination of the carbon catabolite repressor *creA* yielded remarkably high quantities of extracellular hydrolases, e.g., proteases and chitinases in *A. nidulans* (Tables 1 and 2; [19]). It is remarkable that a *cre1*-defected *Trichoderma reesei* mutant was also shown to be a hyperproducer of cellulolytic enzymes [59] and its xylanase production was inducible to a higher degree than that observed in the wild-type strain [60]. Because hydrolase production and autolysis were regulated via both CreA-dependent and CreA-independent pathways, fine-tuning and cooptimization of microbial growth, hydrolase production, and autolysis were possible in *A. nidulans* [19].

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