Expression and engineering of hydrophobin genes
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Chapter 3: A sandwiched-culture technique for evaluation of heterologous protein production in a filamentous fungus

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Abstract

_Aspergillus niger_ is known for its efficient excretion machinery. However, problems have often arisen in obtaining high amounts of heterologous proteins in the culture medium. Here we present a quick method using sandwiched colonies to evaluate transgenic strains for secretion of heterologous proteins. Using this technique it was shown that low production levels of the ABH1 hydrophobin of _Agaricus bisporus_ in _A. niger_ are, at least partly, due to extracellular proteolytic degradation.

Introduction

Because of the characteristic self-assembly of class I hydrophobins into SDS-insoluble amphipathic membranes, interest has arisen in possible medical and industrial applications. This requires an efficient method for production of hydrophobins (Wessels, 1997). _Aspergillus niger_ is widely used for the commercial production of proteins because of its high capacity to secrete proteins (up to 30 g L\(^{-1}\)), its GRAS (Generally Regarded As Safe) status and the well developed large-scale fermentation processes, down-stream processing, mitotic crossing over and mutagenesis-screening methods (reviewed by Davies, 1991; Jeenes _et al._, 1991; van den Hondel _et al._, 1991; Gwynne and Devchand, 1992; MacKenzie _et al._, 1993; Archer _et al._, 1994; Davies, 1994; Verdoes _et al._, 1995; Gouka _et al._, 1997a; Archer and Peberdy, 1997). However, problems have often arisen in obtaining high amounts of heterologous proteins in the culture medium. Production of heterologous proteins can be limited at the transcriptional and the (post)-translational level, and several strategies have been developed to improve protein yields: (i) the introduction of a large number of gene copies, (ii) the use of strong fungal transcription regulatory sequences and efficient secretion signals, (iii) the construction and use of protease deficient host strains, (iv) development of an optimal production medium and (v) gene fusion strategies in which the gene of interest is fused at the 3' end of a gene encoding a well-secreted homologous protein (reviewed by Archer _et al._, 1994; 1997; Verdoes _et al._, 1995; Gouka _et al._, 1997b). Here we present a quick method using sandwiched colonies to evaluate transgenic strains for secretion of heterologous proteins. Using this sandwiched-culture technique we show that production of the ABH1 hydrophobin of the edible mushroom _Agaricus bisporus_ (de Groot _et al._, 1996; Lugones _et al._, 1996) in _A. niger_ is hampered by proteolytic degradation in the culture medium.

Materials and methods

**Strains and Plasmids**

Cloning was done in _E. coli_ JM83. To express _ABH1_ in _A. niger_, the plasmids pAN52-1NotI and pAN56-2 (van den Hondel _et al._, 1991) were used. _A. niger_ strain AB4.1, a _cspA1pyrG1_ derivative of strain ATTC 9029 (Bos _et al._, 1988), was used.
Growth conditions and media
For transformation, *A. niger* was grown at 30 °C and at 350 rpm for 24 h in complete medium (minimal medium [MM; Pontecorvo *et al.*, 1953] + 0.5% yeast extract and 0.1% casamino acids) enriched with 10 mM uridine. Selection of transformants was carried out on MM plates containing 100 µg ml⁻¹ hygromycin B (Roche, Germany). For analysis of proteins secreted into the medium and mRNA analysis, *A. niger* was grown in either 25 ml MM⁺ (MM in which the glucose concentration was increased 5 times and 3.5 g L⁻¹ of yeast extract was added) or MMdex (MM in which the glucose was replaced by dextrin) at 30 °C (125 rpm) for 3 days. Cultures were inoculated with 1-2·10⁶ spores ml⁻¹.

Construction of expression vectors and transformation of *A. niger*
For expression of *ABH1* in *A. niger*, a complete cDNA of *ABH1* (347 bp) was synthesized by PCR using oligonucleotide primers corresponding to sense nucleotides 1 to 18, introducing a *NcoI* site in the start codon, and to antisense nucleotides 322 to 339, supplemented with a *BamHI* site (Accession number *ABH1* sequence X92861; Lugones *et al.*, 1996). This fragment was cloned in expression vector pAN52-1NotI cut with *NcoI/BamHI*. This resulted in construct pA1AN, which contains the *ABH1* cDNA sequence in between the *gpd* promoter and *trpC* terminator of *A. nidulans*. To express *ABH1* as a fusion with the truncated glucoamylase gene (*GlaA*-G2), a 297 bp fragment of the *ABH1* cDNA sequence encoding the mature form of ABH1 was generated by PCR. One primer corresponded to sense nucleotides 69 to 87 and contained an additional *NarI* site and a spacer sequence encompassing a KEX2 site (cga atg gat aaa agg; RMDKR). The other primer corresponded to antisense nucleotides 322 to 339 and also contained an additional *NarI* site. This fragment was cloned behind the truncated *A. niger* glucoamylase gene (*glaA*-G2) in expression vector pAN56-2 cut with *NarI*, resulting in construct pKXA1AN. It encompasses the fusion of *glaA*-G2 and *ABH1*, separated by a KEX2 protease site, which is under regulation of the *glaA* promoter of *A. niger* and the *trpC* terminator of *A. nidulans*. The orientation and sequence of the *ABH1* fragment was confirmed by sequence analysis. To allow selection of transformants, a hygromycin B

*Figure 1.* Constructs used for transformation of *A. niger* AB4.1. (A) Construct pA1ANh, containing *ABH1* under regulation of the *gpd* promoter and *trpC* terminator of *A. nidulans*. (B) Construct pKXA1ANh, containing *ABH1* fused to a truncated *A. niger* glucoamylase gene (*glaA*-G2) separated by a KEX2 protease site and using the *trpC* terminator of *A. nidulans*.
resistance gene (Punt et al., 1987) was cloned as a \( \text{Not}I \) fragment in both expression vectors, resulting in constructs pA1ANh and pKXA1ANh, respectively (Figure 1). Co-transformation of a \( \text{pyrG} \) deficient \( \text{A. niger} \) strain (van Hartingsveld et al., 1987) with the expression vectors pA1ANh and pKXA1ANh and vector pAB4.1 containing the \( \text{pyrG} \) gene was carried out essentially as described by Punt and van den Hondel (1992). Transformants were obtained by selection for complementation of \( \text{pyrG} \) and growth on hygromycin B containing medium (100 \( \mu \)g ml\(^{-1} \)) and they were purified through conidia.

**Northern blot analysis**
RNA was isolated from mycelium that had been ground in liquid nitrogen using Trizol reagent (Gibco BRL, USA). It was separated on a 1% formaldehyde gel and blotted to Hybond-N\(^+\) membrane (Amersham, UK). The RNA was hybridized with a \( [^{32}\text{P}] \)-labelled DNA fragment of the coding sequence of \( \text{ABH1} \).

**Analysis of \( \text{ABH1} \) production in shaken cultures**
Mycelium was separated from the culture medium of shaken cultures using Miracloth (Calbiochem, USA). Total protein contained in the medium was precipitated with 25% TCA and washed with cold acetone. TFA was added to dissociate the \( \text{ABH1} \) hydrophobin. After removing the solvent with a stream of air, SDS-sample buffer (containing \( \beta \)-mercaptoethanol) was added and the samples were adjusted to pH 7 with concentrated ammonia, if necessary. SDS-PAGE was performed on 12.5% (w/v) polyacrylamide gels (Laemmli, 1970). The gels were stained with silver (Merril et al., 1981) or blotted onto a PVDF membrane for immunodetection with \( \text{ABH1} \)-antibodies (Lugones et al., 1996).

**The sandwiched-culture technique**
A perforated polycarbonate membrane (diameter 4.5 cm, pore size 0.1 \( \mu \)m; Poretics, USA) was put onto the surface of 10 ml of perfectly level solidified minimal medium (Wösten et al., 1991) containing 5% maltodextrin. The plate was heated to 70 °C and 1 ml of melted 1.25% agarose was poured over the surface. The temperature of the plate was brought to room temperature and 5 \( \mu \)l of spore suspension (\( 7 \times 10^7 \) spores ml\(^{-1} \)) was spotted at the center of the plate and allowed to soak into the agarose. A second perforated polycarbonate membrane was then put on top of the agarose layer. In all cases care was taken that no air bubbles were trapped. After 3 days of growth at 30 °C, the sandwiched culture was lifted from the agar, transferred to a PVDF membrane overlying fresh agar medium and incubated for 1 h at 30 °C. Secretion of \( \text{ABH1} \) was then monitored by immunodetection using \( \text{ABH1} \) antiserum.
Results

*A. niger* strain AB4.1 was transformed with constructs pA1ANh and pKXA1ANh (Figure 1). pA1ANh contains the coding sequence of *ABH1* placed under regulation of the *gpd* promoter of *A. nidulans*. pKXA1ANh encompasses the *ABH1* cDNA encoding the mature hydrophobin fused in frame with the 3' end of a truncated *A. niger* glucoamylase gene (*GlaA*-G2). This resulted in TAN1 and TAN2 transformants, respectively. After purification through conidia, 10 and 30 transformants, respectively, were screened for secretion of ABH1 by SDS-PAGE analysis of TCA precipitated protein of culture media of 2-day-old liquid shaken cultures. ABH1 was detected by silver staining and Western blot analysis in 70% of both types of transformants, but at low concentrations. No less than the equivalent of 5 ml of medium per lane was necessary in an SDS-polyacrylamide gel (PAGE) to obtain a clear ABH1 band. For comparison, 100 µl of medium is sufficient to visualize a similar amount of SC3 in *Schizophyllum commune*. Time course experiments showed the same low ABH1 protein level over a culture period of 5 days (not shown). Despite the low protein levels, Northern blot analysis showed clear *ABH1* mRNA accumulation in these transformants (not shown).

![Figure 2](image_url). Sandwiched-culture technique. **(A)** Schematic view of a sandwiched culture. **(B)** Immunodetection of secreted ABH1 on PVDF membranes underlying sandwiched colonies of a TAN1 transformant, TAN2 transformant and wild-type (WT), respectively, for 1 h.
To investigate whether ABH1 is degraded in the culture medium of *A. niger*, we used the so-called sandwiched-culture technique (Figure 2A) previously described (Wösten *et al.*, 1991; see M&M). A perforated polycarbonate membrane (pore size 0.1 µm) was put onto the surface of 10 ml solidified minimal medium containing 5% maltodextrin and 1 ml 1.25% agarose was poured over the surface. After solidifying, spores were inoculated on the center of the plate and a second perforated polycarbonate membrane was put on top of the agarose layer. After 3 days of growth, the sandwiched cultures of a wild-type strain (AB4.1) and of both types of transformants were transferred to PVDF membranes overlying fresh agar medium and incubated for 1 h. The immunosignals of ABH1 on the PVDF membranes (Figure 2B) showed predominant secretion at the edge of the colonies, indicating active secretion in the growing zone of the colony (as shown previously for glucoamylase [Wösten *et al.*, 1991]) and a higher ABH1 secretion in a TAN2 transformant compared to that of a TAN1 transformant. No reaction was detected in the wild-type.

Proteins are tightly bound to PVDF membranes, but a small part of ABH1 could be dissociated from the membrane by incubation with 100% trifluoroacetic acid (TFA) for 30 min at room temperature (the amount extracted was small because no decrease in the immuno-signal was detected on the PVDF membranes after extraction). After removal of TFA by a stream of nitrogen, the proteins were taken up in 1 ml SDS sample buffer, and 25 µl was subjected to SDS-PAGE and Western blot analysis (Figure 3, lane 1). ABH1 antibodies reacted with a protein band running at the 14-kDa position, representing monomeric ABH1 (Lugones *et al.*, 1996) successfully cleaved from glucoamylase at the KEX2 protease site. A 10-times dilution of the sample still gave a positive signal with anti-ABH1 antibodies (not shown). To analyze whether the enhanced excretion of ABH1 in sandwiched cultures compared to shaken liquid cultures was due to different culture conditions, a control experiment was carried out. A sandwiched TAN2 colony was grown for 3 days as described for the previous experiment, transferred to the surface of 10 ml of static liquid medium, and incubated for 24 h at 30 °C. The medium was harvested and a sample of 25 µl was subjected to SDS-PAGE. As in shaken cultures, no ABH1 was detected in static liquid medium. (3) ABH1, dissociated from PVDF as in (1) was not detectable after 24 h of incubation in a 3-day-old TAN2 culture medium. (4) ABH1, dissociated from PVDF as in (1) was intact after 24 h of incubation in water. (5) Partially increased stability of ABH1 was obtained when the pH and medium components were adjusted.
membrane, in the medium of a 3-day-old TAN2 transformant for 24 h at 30 °C, the protein could not be detected (Figure 3, lane 3), whereas its level was not reduced after incubation in H₂O (Figure 3, lane 4).

_A. niger_ strain AB1.18 (UV mutated in the _pepA_ gene encoding the protease aspergillopepsin A [Mattern _et al._, 1992]) was transformed with constructs pA1ANh and pKXA1ANh in an attempt to prevent proteolytic degradation of ABH1 in the culture medium. However, the use of this protease deficient strain did not yield more secreted ABH1 (data not shown). Van Noort _et al._ (1991) demonstrated a pH optimum for proteolytic activity in the culture medium of _A. niger_ AB4.1 at pH 4.0. We therefore analyzed the influence of pH on the proteolysis of ABH1 by increasing the initial pH from 6.0 to 7.0. In addition, the buffer capacity of the medium was increased by adding 4 times the amount of phosphate as indicated by Wösten _et al._ (1991) and adding 2% CaCO₃ to the medium. This resulted in a final pH of the medium of 6.1 instead of pH 3 to 4 after 24 h of growth of the TAN2 transformant. The adjustment of the pH and medium components only slightly increased the yield of ABH1 (Figure 3, lane 5).

Discussion

Because hydrophobins assemble at hydrophilic-hydrophobic interfaces and thereby change the hydrophobicity of a surface, these proteins are promising candidates for use in technical and medical applications. The ABH1 hydrophobin of the edible mushroom _A. bisporus_ coats the outer surface of the mushrooms (de Groot _et al._, 1996; Lugones _et al._, 1996) as well as the gas channels within the fruiting bodies (Lugones _et al._, 1999b). Therefore, ABH1 can be considered a food-grade hydrophobin. To increase production of the ABH1 hydrophobin of _A. bisporus_, we have initiated heterologous expression studies in _A. niger_. _A. niger_ was chosen for production of hydrophobins because it does not secrete hydrophobins in its culture medium (O.M.H. de Vries, unpublished; see Chapter 2), which will therefore not interfere with the purification of heterologously produced hydrophobins. Strain AB4.1 was transformed with constructs pA1ANh (TAN1 transformants) and pKXA1ANh (TAN2 transformants). pA1ANh contains the coding sequence of _ABH1_ under regulation of the _gpd_ promoter of _A. nidulans_. pKXA1ANh encompasses the _ABH1_ cDNA encoding the mature hydrophobin fused in frame with the 3’ end of a truncated _A. niger_ glucoamylase gene (GlaA-G2). Only low concentrations of ABH1 were detected in liquid shaken cultures of both types of transformants. This may be explained by (i) low expression at RNA and/or protein level, (ii) malfunction of translation and/or the secretory machinery, or (iii) instability of the protein in the culture medium. RNA analysis verified that the production of ABH1 was not hampered by a low expression level of the gene. To discriminate between the remaining possibilities, the sandwiched-culture technique (Wösten _et al._, 1991) was used. In this method a protein binding membrane (such as polyvinylidene difluoride [PVDF]) immobilizes proteins directly after secretion when placed under the colony. Specific antibodies allow detection of the newly secreted proteins. Immunosignals of ABH1 were highest in a TAN2
transformant indicating efficient secretion of ABH1. Only part of the protein could be extracted from the PVDF membranes with TFA. SDS-PAGE and Western blotting showed however that the amount of extracted ABH1 was considerably higher than the amount present in liquid shaken cultures. When a similar sandwiched TAN2 colony was transferred to the surface of static liquid medium, no ABH1 could be detected in the medium. This suggested that ABH1 is degraded in the medium, which is prevented by immobilizing the proteins (i.e. both ABH1 and proteases) on a PVDF membrane immediately after secretion. The degradation of ABH1 in the culture medium was confirmed by incubating the protein (extracted from the PVDF membranes with TFA) with the culture medium of A. niger.

The A. niger strain (AB4.1) used as a recipient for transformation experiments is known to contain extracellular proteases, most notably aspergillopepsin A and aspergillopepsin B, both belonging to the class of aspartyl proteases (Archer et al., 1992; Mattern et al., 1992). However the use of a protease deficient strain, AB1.18 (UV mutated in the pepA gene, encoding aspergillopepsin A [Mattern et al., 1992]), did not yield more secreted ABH1. Therefore, other proteases must be responsible for the degradation of ABH1. Van Noort et al. (1991) demonstrated a pH optimum for proteolytic activity of A. niger AB4.1 at pH 4.0. Myoglobin was efficiently degraded at pH 4.0, while no degradation was observed at pH 6.5 or higher. However, the amount of ABH1 only slightly increased when the pH of the medium was kept above 6.1.

The sandwiched-culture technique was also successfully used to detect the production of genetically engineered hydrophobins in S. commune, which in some cases could not be detected in the medium of liquid shaken cultures (Appendix to Chapter 6). This method therefore represents a rapid way to assess expression of a heterologous protein in filamentous fungi without interference by protein degradation.

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