Expression and engineering of hydrophobin genes
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Chapter 2: Heterologous expression of hydrophobin genes in *Aspergillus niger* and *Trichoderma reesei*

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Abstract

Filamentous fungi secrete small (± 100 aa), moderately hydrophobic proteins called hydrophobins. Upon contact with a hydrophilic-hydrophobic interface they self-assemble into a highly insoluble, amphipathic membrane. This enables hydrophobins to change the nature of a surface, a property that could be useful in technical and medical applications. To improve production levels of the SC3 hydrophobin of Schizophyllum commune, its gene was heterologously expressed in Aspergillus niger and Trichoderma reesei. SC3 was expressed in A. niger behind the gpd promoter of Aspergillus nidulans or as a fusion behind the G2 form of A. niger glucoamylase. Despite the fact that mRNA accumulated at relatively high levels, protein levels in the medium were less than 1% compared to that in S. commune. Levels of SC3 did not increase in strains with decreased protease activity in the culture medium. These data indicate that problems with production of the SC3 hydrophobin in A. niger occur intracellularly at the post-transcriptional level.

In contrast to A. niger, levels of SC3 in the medium of T. reesei were at least similar to that found in S. commune when SC3 was expressed behind the HFBI promoter in medium containing glucose or when SC3 was expressed behind the HFBII promoter grown in lactose containing medium.

Introduction

Hydrophobins are small (± 100 aa) secreted proteins that fulfil a broad spectrum of functions in fungal development (Wessels 1996; 1997; Wösten and Wessels, 1997). They are characterized by the presence of eight cysteine residues in conserved spacing and by similar hydropathy patterns (Wessels, 1994; 1997; Wösten and Wessels, 1997). The SC3 hydrophobin of S. commune is the best-characterized hydrophobin but other class I hydrophobins have similar properties. Upon contact with hydrophilic-hydrophobic interfaces SC3 monomers self-assemble into a highly insoluble, 10 nm thick, amphipathic membrane (Wösten et al., 1993; 1994a; 1995). By self-assembly hydrophobins change the nature of a surface. Hydrophobic solids (e.g. Teflon) can be made hydrophilic by submerging them in an aqueous solution of monomeric hydrophobin. Similarly, hydrophobins in aqueous solution assemble around oil droplets, thus stabilizing the suspension (Wösten et al., 1994a; 1995; Lugones et al., 1996; 1998; 1999b). On the other hand, hydrophobins can make hydrophilic surfaces, such as glass or paper, hydrophobic by assembly at the interface between the hydrophilic surface and the air (Wösten et al., 1993; Lugones et al., 1996; 1998; 1999b). The hydrophobin layer can strongly attach to these solid supports.

The properties of hydrophobins make them interesting candidates for use in technical and medical applications (Wessels, 1997). For instance, they could be used to increase biocompatibility of medical implants, to prevent microbial adhesion to e.g. catheter surfaces
or to attach cells or proteins to hydrophobic surfaces, as in biosensors. Hydrophobins can be considered safe in food applications because they are normally ingested by humans upon consumption of mushrooms and fungus-fermented foods. A requirement for successful application of hydrophobins is that these proteins can be produced in large quantities (i.e. grams per liter), although for some applications the quantities needed may be small (1 mg of SC3 is sufficient to coat 1 m² of Teflon [Wösten et al., 1994a]). S. commune naturally produces about 60 mg L⁻¹ SC3 in the medium. A. niger and T. reesei are widely used for the commercial production of heterologous proteins because of their high capacity to secrete proteins (up to 30 g L⁻¹), their 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; Keränen and Pentillä, 1995; Gouka et al., 1997a; Archer and Peberdy, 1997).

Here, A. niger and T. reesei were examined as hosts for production of SC3. Low levels of SC3 were obtained in the culture medium when SC3 was expressed in A. niger, most probably due to a limitation occurring intracellularly at the post-transcriptional level. Production levels of SC3 were at least similar to that in S. commune when SC3 was expressed behind the HFBI or HFBII promoter and using glucose or lactose as a carbon source, respectively.

Materials and Methods

Strains and Plasmids
Cloning was done in E. coli JM83. To express SC3 in A. niger, the plasmids pAN52-1NotI and pAN56-2 (van den Hondel et al., 1991) were used. The T. reesei expression vectors were constructed using plasmid pUC20. A. niger strains AB4.1, AB1.13 and D15#26 were used. A. niger strain AB4.1 is a cspA1pyrG1 derivative of strain ATTC 9029 (Bos et al., 1988). A. niger strain AB1.13 is a UV-derivative of strain AB4.1, which is deficient in extracellular proteases (van Hartingsveld et al., 1987; Mattern et al., 1992). Strain D15#26 is a derivative of strain AB1.13 containing a mutation, which leads to a reduced acidification of the culture medium (Mattern et al., 1992). T. reesei strain Rut-C30 (ATTC 56765 [Montenecourt and Eveleigh, 1979]) 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 (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 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 1-2·10^6 spores ml^{-1}.

_T. reesei_ was grown at 28 °C under a daylight lamp on Potato dextrose (PD) plates (Difco). Selection of transformants was carried out on MM plates (Pentillä _et al._, 1987) containing 100 µg ml^{-1} hygromycin B (Roche, Germany) and 1 M sorbitol. For screening of transformants, mRNA analysis and analysis of proteins in the culture medium, strains were grown for 3-4 days in MM with 3% glucose or lactose as carbon source. Cultures were inoculated with 10^6 spores ml^{-1}. The glucose concentration in the culture medium was monitored daily with Trinder reagent (Sigma, U.S.A.) and was kept above 1% by the addition of extra glucose, if necessary.

**Construction of expression vectors**

For expression of _SC3_ in _A. niger_, a complete cDNA of _SC3_ (424 bp) was synthesized by PCR using oligonucleotide primers corresponding to sense nucleotides 1 to 12, introducing a _NcoI_ site in the startcodon, and antisense nucleotides 392 to 411, containing an additional _BamHI_ site behind the stopcodon (Accession number _SC3_ sequence M32329). This fragment was cloned in expression vector pAN52-1NotI cut with _NcoI_/_BamHI_. This resulted in construct pS3AN, which contains the _SC3_ cDNA sequence in between the _gpd_ promoter and _trpC_ terminator of _A. nidulans_. To express _SC3_ as a fusion with the truncated glucoamylase gene (_GlaA-G2_), a 363 bp fragment encoding the mature form of _SC3_ was generated by PCR. One primer corresponded to sense nucleotides 76 to 99 and contained a _NarI_ site and a spacer sequence encompassing a KEX2 site (cga atg gat aaa agg; RMDKR). The other primer corresponded to antisense nucleotides 392 to 411 and also contained an additional _NarI_ site. The 363 bp fragment was cloned behind the truncated _A. niger_ glucoamylase gene (_glaA-G2_) in expression vector pAN56-2 cut with _NarI_, resulting in construct pKXS3AN. It encompasses the fusion between _glaA-G2_ and _SC3_, which is under regulation of the _glaA_ promoter of _A. niger_ and the _trpC_ terminator of _A. nidulans_. The orientation and sequence of the _SC3_ fragments were confirmed by sequence analysis. To allow selection of transformants, a _hygromycin B resistance_ gene (Punt _et al._, 1987) was cloned as a _NotI_ fragment in both expression vectors, resulting in constructs pS3ANh (Figure 1A) and pKXS3ANh (Figure 1B), respectively.

For expression of _SC3_ in _T. reesei_, the _SC3_ coding sequence (see above) was cloned in pUC20 cut with _NcoI/BamHI_, resulting in plasmid pS3TR. A 929 bp fragment of the _HFBI_ terminator region was amplified by PCR using oligonucleotide primers corresponding to sense nucleotides 1951 to 1970, containing a _SacI_ site, and antisense nucleotides 2849 to 2867, containing a _SalI_ site (Accession number _HFBI_ gene Z68124). The _SacI_ site of this _HFBI_ terminator fragment was made blunt using the Klenow fragment of DNA polymerase and was cloned in pS3TR cut with _BamHI/SalI_, of which the _BamHI_ site was made blunt. This resulted in plasmid pS3TR1. The promoter regions of the _HFBI_ and _HFBII_ genes were amplified by PCR using oligonucleotide primers corresponding to sense nucleotides 142 to 161 of _HFBI_ or sense nucleotides 13 to 32 of _HFBII_, both containing a _HindIII_ site and
antisense nucleotides 1501 to 1520 of HFBI or antisense nucleotides 1169 to 1188 of HFBII (Accession number HFBII gene Y11894). In both cases a NcoI site was introduced at the 3’ end. The 1384 bp HFBI promoter fragment and the 1181 bp HFBII promoter fragment were then cloned in pS3TR1 cut with HindIII/NcoI, resulting in plasmids pS3TR1.1 (Figure 1C) and pS3TR1.2 (Figure 1D), respectively.

**Figure 1.** Constructs used for transformation of A. niger and T. reesei. (A) Construct pS3ANh, containing SC3 under regulation of the gpd promoter and trpC terminator of A. nidulans. (B) Construct pKXS3ANh, containing SC3 fused to a truncated A. niger glucoamylase gene (glaA-G2) separated by a KEX2 protease site and using the trpC terminator of A. nidulans. (C) Construct pS3TR1.1, containing SC3 under regulation of the HFBI promoter and terminator of T. reesei. (D) Construct pS3TR1.2, containing SC3 under regulation of the HFBII promoter and HFBI terminator of T. reesei.

**Transformation of A. niger and T. reesei**

Co-transformation of a pyrG deficient A. niger strain (van Hartingsveld et al., 1987) with the expression vectors pS3ANh and pKXS3ANh and vector pAB4.1 containing the pyrG gene was carried out essentially as described by Punt and van den Hondel (1992). Transformants that were selected by complementing pyrG and growth on hygromycin B containing medium (100 µg ml\(^{-1}\)) were purified through conidia. Co-transformation of T. reesei strain Rut-C30 with the expression vectors pS3TR1.1 and pS3TR1.2 and vector pARO21, containing the hygromycin B resistance cassette, was carried out essentially as described by Pentillä et al. (1987). Transformants were purified through conidia after three rounds of selection on hygromycin B containing medium (100 µg ml\(^{-1}\)).
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 [32P]-labelled DNA fragment of the coding sequence of SC3. To compare expression levels of the SC3 gene or the fusion gene to that of the endogenous glucoamylase gene, RNA of A. niger was hybridized with a 300 bp NarI/BssHII fragment of vector pAN56-2 encompassing part of the truncated A. niger glucoamylase gene (glaA-G2). To compare SC3 expression levels to that of the endogenous HFBI and HFBII genes, respectively, RNA of T. reesei strains was hybridized with a 1384 bp HindIII/NcoI HFBI promoter fragment or a 1182 bp HindIII/NcoI HFBII promoter fragment, containing the 5' untranslated sequences of HFBI and HFBII, respectively. Re-hybridizing membranes with a S. commune 18S rRNA probe was carried out to quantify amounts of RNA loaded on the gel.

Southern blot analysis
DNA was isolated from mycelium, ground in liquid nitrogen, using the Isoplant DNA Extraction Kit (Eurogentec, Belgium). DNA was digested with Ncol or HindIII, separated on a 0.8% agarose gel, alkali denatured and blotted to Hybond-N+ membrane (Amersham, U.K.). The DNA was hybridized with a [32P]-labelled DNA fragment encompassing the coding sequence of the SC3 gene.

Analysis of SC3 production in culture media and purification of SC3
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, washed with cold acetone, dried, treated with TFA and dissolved in SDS-sample buffer for analysis by SDS-PAGE. Purification of SC3 was performed essentially as described (Wösten et al., 1993; Wessels, 1997). SC3 was assembled by vortexing the culture medium for 2 min at room temperature. After removal of air bubbles at reduced pressure, the assembled SC3 was spun down (30 min, 10000 g) and freeze dried. The material was treated with TFA and after removing the solvent with a stream of nitrogen gas taken up in 10 ml water. After removing insoluble material by centrifugation at 5000 g for 30 min, ethanol was added to a final concentration of 60% (v/v). Precipitates were removed by centrifugation, followed by addition of 2 volumes of 0.3 M NH4Ac in pure ethanol to the supernatant. SC3 was collected by centrifugation and after washing with 100% ethanol taken up in water. SC3 was re-assembled by electrobubbling (Lugones et al., 1998) and freeze dried.

Protein analysis
After treatment with TFA samples were taken up in SDS-sample buffer containing β-mercaptoethanol and adjusted to pH 6.8 with concentrated ammonia, if necessary. SDS-PAGE was performed on 12.5% (w/v) polyacrylamide gels (Laemmli, 1970). The gels were fixed in 10% TCA for 1 h and stained with colloidal coomassie brilliant blue (G-250) for 16 h.
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(Neuhof et al., 1988) or blotted onto PVDF for immunodetection with SC3-antibodies (Wösten et al., 1994b).

Results

Cloning of a complete cDNA of SC3
To allow heterologous expression of SC3 in A. niger, a complete coding sequence of SC3 was amplified. Its sequence (Figure 2) deviated from the sequence published previously (Schuren and Wessels, 1990). At the 5' end the cDNA contained 37 nucleotides positioned in the first intron as proposed by Schuren and Wessels (1990). Moreover, 4 nucleotides were missing in the coding sequence positioned immediately in front of this intron. It thus appears that the 143 bp intron as proposed by Schuren and Wessels (1990) is in fact a 37 bp exon flanked by two introns of 53 and 57 bp. The 5' splice site of the 53 bp intron (intron 1) is situated 4 nucleotides upstream of the site proposed before. Both small introns contain 5', 3' and internal splicing sequences, which are in agreement with the consensus sequences of basidiomycetous (Schuren, 1992) and ascomycetous genes (Gurr et al., 1987). The SC3 gene (sequence updated under accession number M32329) thus encodes a protein of 136 (instead of 125) amino acids, of which 112 are contained in the mature form after cleavage of the signal sequence. Residue Tyr32 (Schuren and Wessels, 1990) is absent and is replaced by 12 amino acids (T P P V T T V T V T T). SC3 does not contain putative N-glycosylation sites (NXS or NXT) but 14 O-glycosylation sites (Thr) are predicted (de Vocht et al., 1998; Chapter 6) that would link the 16-22 mannose residues of SC3 (de Vocht et al., 1998).

Analysis of SC3 production in A. niger
A. niger strain AB4.1 was transformed with constructs pS3ANh and pKXS3ANh. pS3ANh contains the coding sequence of SC3 placed under regulation of the gpd promoter of A. nidulans. pKXS3ANh encompasses the SC3 cDNA encoding the mature hydrophobin fused in frame with the 3' end of a truncated A. niger glucoamylase gene (GlaA-G2), separated by a KEX2 site. After purification through conidia, transformants were screened for secretion of SC3 by SDS-PAGE analysis of TCA precipitated protein of culture media of 3-day-old liquid shaken cultures. 7 out of 15 A. niger strains containing construct pS3ANh revealed a protein band with an apparent molecular weight of 26 kDa, which was absent in the medium of an untransformed strain. This band reacted with antibodies raised against SC3 (Figure 3, lane 1). SC3 isolated from S. commune migrates at 24 kDa, suggesting that SC3 expressed by A. niger is overglycosylated. Maximal amounts of SC3 were observed after 5 days of growth, coinciding with glucose depletion in the medium. Southern blot analysis of 10 randomly chosen transformants showed that in all cases the SC3 sequence had integrated in the genome, with a copy number varying between 1 and 14. Of the A. niger strains transformed with construct pKXS3ANh 50 were selected and screened for SC3 production via slot-blot analysis of protein contained in the culture medium of 2-day-old microtiter cultures. Of these strains, 5 transformants showed a faint reaction with the SC3 antiserum. These strains were
purified through conidia and production of SC3 was confirmed by immunodetection on PVDF membranes, which were placed underneath 2-day-old sandwiched cultures (Wösten et al., 1991; Chapter 3). 2 transformants showing the highest immuno-signal were grown in liquid shaken cultures and the culture medium was analyzed by SDS-PAGE and Western blotting. The culture medium of both transformants showed a protein band running at the 26 kDa position, which reacted with the SC3 antiserum (data not shown). This showed that SC3 in the medium was correctly processed from the glucoamylase G2-SC3 fusion protein.

Maximal amounts of SC3 were observed after 2 days of growth using dextrin as a carbon source.

**Figure 2.** The corrected nucleotide sequence of the SC3 gene and its deduced amino acid sequence. Nucleotides are numbered with reference to the transcription start point (Schuren and Wessels, 1990). The coding sequence is in upper case letters. Putative internal intron splicing sequences are underlined. The additional 37 bp sequence in the coding sequence is indicated in bold and upper case. The 4 nucleotides absent in the formerly published coding sequence are indicated in bold lower-case. Note that a previously deduced amino acid Tyr is missing. The nucleotide sequence is updated in databases under accession number M32329.
However, the amount of SC3 produced by both types of transformants was less than 1% compared to the amount secreted by S. commune.

Heterologous proteins produced in A. niger are often degraded in the medium. Therefore, A. niger strains AB1.13 and D15#26 were transformed with constructs pS3ANh and pKXS3ANh. Strain AB1.13 is a UV-derivative of strain AB4.1 that is deficient for both aspergillopepsin A and B and its derivative D15#26 acidifies the culture medium to a lesser extent (van Hartingsveld et al., 1987; Mattern et al., 1992). SDS-PAGE analysis showed that the amounts of SC3 were not increased when these strains instead of strain AB4.1 were used as hosts for expressing SC3 using pS3ANh and pKXS3ANh.

The SC3 mRNA level in the SC3 producing A. niger strains AB4.1, AB1.13 and D15#26 was analyzed to investigate whether the low level of SC3 in the culture medium corresponds to low levels of SC3 mRNA. Northern blot analysis showed that in all types of transformants SC3 mRNA accumulated to a level 20-40% of the amount in wild-type S. commune (Figure 4). From this we conclude that the low level of SC3 in the medium can only to a small degree be explained by expression of the gene and/or RNA stability.

**Analysis of SC3 production in T. reesei**

Constructs pS3TR1.1 and pS3TR1.2 were introduced into T. reesei strain Rut-C30 using co-transformation with vector pARO21. pS3TR1.1 and pS3TR1.2 encompass the SC3 coding sequence under regulation of the HFBI and HFBII promoter, respectively. pARO21 encompasses the hygromycin B resistance cassette. After three rounds of selection on hygromycin B containing medium, 34 (pS3TR1.1) and 19 (pS3TR1.2) transformants were selected and screened for SC3 production via slot-blot analysis of the culture medium of 4 day old microtiter cultures. Of these strains 8 and 9 transformants, respectively, showed a reaction with the SC3 antiserum. These strains were purified through conidia and production of SC3 was re-confirmed by slot-blot analysis of the culture media of two independent single spore colonies of each transformant grown in microtiter plate cultures. Of both types of transformants 10 conidia-derived strains were selected and analyzed by Western blot analysis of TCA precipitated protein of the culture media of 4 day old microtiter cultures, using antibodies raised against SC3. Of 10 transformants each, expressing SC3 behind the HFBI or HFBII promoter, 6 and 3, respectively, showed a clear immuno-positive band at 24 kDa (Figure 3, lanes 2 and 3), which is the same apparent molecular weight as that of SC3.
produced by \textit{S. commune} (Figure 3, lane 4). Southern blot analysis of 3 immuno-positive strains each showed that in all cases the SC3 sequence had integrated in the genome, the copy number varying between 1 and 9. Of each type of transformants 1 strain was selected and grown in liquid shaken cultures for analysis of SC3 production and mRNA accumulation under more optimal conditions (i.e. a strain expressing SC3 behind the HFBII promoter with a copy number of 6). The culture medium of both transformants showed a protein band running at the 24 kDa position, which reacted with the SC3 antiserum. Maximal amounts of SC3 were

\begin{figure}
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\includegraphics[width=\textwidth]{figure4}
\caption{Northern blot analysis of RNA isolated from \textit{A. niger} strains expressing SC3. The RNA was hybridized with (A) a probe encompassing 400 bp of the 3' part of the \textit{A. niger} glucoamylase gene and with (B) a SC3 cDNA probe. (1-3) \textit{A. niger} strain AB1.13 transformed with the glucoamylase-SC3 fusion construct, (4) \textit{A. niger} strain AB1.13 serving as a control. (5-7) Strain AB1.13 transformed with SC3 under regulation of the \textit{A. nidulans} gpd promoter, (8) \textit{A. niger} strain AB1.13 serving as a control. (9-10) \textit{A. niger} strain AB4.1 transformed with the glucoamylase-SC3 fusion construct, (11) strain AB4.1 serving as a control. (12-13) Strain AB4.1 transformed with SC3 under regulation of the \textit{gpd} promoter. (14-16) \textit{A. niger} strain D15#26 transformed with the glucoamylase-SC3 fusion construct, (17) strain D15#26 serving as a control. (18-20) Strain D15#26 transformed with SC3 under regulation of the \textit{gpd} promoter, (21) strain D15#26 serving as a control. (22) RNA of \textit{S. commune} strain 4-39.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure5}
\caption{Northern blot analysis of RNA isolated from \textit{T. reesei} strains expressing SC3. (A) The RNA was hybridized with a probe encompassing SC3 cDNA and (B) a probe containing the HFBI promoter and the 5' untranslated sequence (lanes 1-7) or the HFBII promoter (lanes 8-14). Lanes 1-5 contain RNA from \textit{T. reesei} expressing SC3 behind the HFBI promoter during 3 to 7 days of growth on glucose containing medium. Lane 6 contains RNA from wild-type \textit{T. reesei} after 7 days of growth on glucose containing medium. Lanes 8-12 contain RNA from \textit{T. reesei} expressing SC3 behind the HFBII promoter during 3 to 7 days of growth on lactose containing medium. Lane 13 contains RNA from wild-type \textit{T. reesei} after 7 days of growth on lactose containing medium. Lanes 7 and 14 contain RNA of \textit{S. commune} wild-type strain 4-39 serving as control.}
\end{figure}
observed after 7 days of growth when SC3 was expressed behind the HFBI or HFBII promoter, using glucose or lactose as carbon source, respectively (Figure 3, lanes 2 and 3). The amount of SC3 produced in the culture medium was at least similar to the amount secreted by S. commune. Northern blot analysis showed that in both cases SC3 mRNA accumulated to a maximum level similar to that formed in wild-type S. commune (Figure 5).

T. reesei secretes various proteins in its culture medium, including the class II hydrophobins HFBI and HFBII (Nakari-Setälä et al., 1996; 1997). Therefore, a purification procedure was carried out to examine whether SC3 could be purified from these proteins. For this, a 7-day-old culture of a T. reesei strain expressing SC3 behind the HFBII promoter was used. The purification method was essentially the same as described for SC3 produced by S. commune (Wösten et al., 1993; Wessels, 1997; Chapter 6), except that SC3 in the culture medium was assembled by vortexing for 2 min, followed by evacuation of air and centrifugation. SDS-PAGE analysis showed that this procedure separated SC3 from the other proteins in the culture medium (Figure 6) with a yield of 13 mg L⁻¹. This is similar to the amount purified from the medium of the S. commune ∆SC15 strain which secretes twice the amount of SC3 compared to the wild-type S. commune strain (O.M.H. de Vries, L.G. Lugones and R. Rink, personal communication).

**Discussion**

**Production of SC3 in A. niger**

To increase production of the SC3 hydrophobin of S. commune, A. niger and T. reesei were examined as hosts. A. niger was chosen because it does not secrete hydrophobins in its culture medium (O.M.H. de Vries, unpublished). The known hydrophobins RodA and DewA and Hyp1 of two related species, A. nidulans and A. fumigatus, respectively, are produced in phialides but not by submerged hyphae (Stringer et al., 1991; Stringer and Timberlake, 1995; Parta et al., 1994; Thau et al., 1994). Endogenously produced hydrophobins of A. niger will therefore not interfere with the purification from the medium of heterologously produced proteins.
hydrophobins. To allow heterologous production of SC3 in A. niger, the coding sequence of SC3 was placed under regulation of the gpd promoter and trpC terminator of A. nidulans (vector pS3ANh). Levels of SC3 in the culture medium of A. niger transformants were less than 1% compared to that produced in S. commune. Because Northern blot analysis showed that SC3 mRNA accumulated in A. niger to a level which was 20-40% of that in S. commune we conclude that production of SC3 in A. niger is mainly limited at the post-transcriptional level. A strategy to overcome limitations at the early stage of the secretory pathway is fusion of the gene of interest to the 3’ end of a highly expressed homologous gene. This approach increased the yield of various proteins secreted in the culture medium (Ward et al., 1990; Contreras et al., 1991; Roberts et al., 1992; Broekhuijsen et al., 1993; Jeenes et al., 1993; Nyyssönen and Keränen, 1995; Ward et al., 1995). It has been suggested that the homologous protein serves as a carrier improving translocation of the protein into the endoplasmic reticulum (ER) allowing proper folding, thereby protecting the heterologous protein from degradation. Therefore, the coding sequence of the mature form of SC3 was cloned behind the coding sequence of the G2 form of glucoamylase of A. niger, separated by a KEX2 protease site that allows cleavage of the fusion protein in the ER (vector pKXS3ANh). However, production levels did not increase indicating that the limitation of SC3 production occurs at another stage in the secretion pathway or occurs in the medium by the action of proteases. The main proteases secreted by A. niger are Aspergillopepsin A and B (Mattern et al., 1992). Aspergillopepsin A shows a broad specificity towards proteins and cuts after hydrophobic amino acids or lysine (http://www.chem.qmw.ac.uk/iubmb/enzyme/EC34/3423.html#2318). Aspergillopepsin B has been shown to cleave at the Asn3-Gln, Glu13-Ala and the Tyr26-Thr positions in the β-chain of insulin (http://www.chem.qmw.ac.uk/iubmb/enzyme/EC34/3423.html#2318). Because Asn65 of SC3 is followed by a Gln residue and the broad specificity of aspergillopepsin A, SC3 may be susceptible to these proteases. Therefore, the protease deficient A. niger strain AB1.13 was transformed with either vector pS3ANh or vector pKXS3ANh. AB1.13 is a UV-derivative of the wild-type strain AB4.1 and is deficient in both aspergillopepsin A and B, showing a residual protease activity of 1-2% compared to that in the parental strain (Mattern et al., 1992). The expression constructs were also introduced in strain D15#26, which is a UV-derivative of strain AB1.13 (Mattern et al., 1992) that acidifies the culture medium to a lesser extent than the parental strain (pH6 and pH4, respectively). Because the proteolytic activity has a pH optimum at pH 4.0 (van Noort et al., 1991), the residual protease activity of strain AB1.13 is likely to be inhibited in strain D15#26. However, production levels of SC3 in the culture media of both strains were similar to that in the wild-type strains, indicating that the low levels of SC3 are not caused by proteolytic degradation in the culture medium. This idea is supported by the observation that SC3 produced by S. commune is not degraded when it is added to the culture medium of A. niger (L.G. Lugones and H.A.B. Wösten, personal communication).

SC3 produced by S. commune runs at the 24 kDa position in SDS-PAGE, while deglycosylated SC3 has an apparent molecular weight of 14 kDa (O.M.H. de Vries, unpublished). The fact that SC3 produced by A. niger runs at the 26 kDa position indicates
that *A. niger* modifies SC3 in a different way. This is in agreement with the observation that the SC3 antiserum, which is mainly directed to the mannose residues of this hydrophobin, is less reactive to SC3 produced by *A. niger* than to SC3 produced by *S. commune*. To avoid problems with glycosylation, the non-glycosylated hydrophobin ABH3 of *Agaricus bisporus* was expressed in *A. niger* (K. Scholtmeijer, unpublished). This hydrophobin is found in the culture medium and is therefore, like SC3, expected to be relatively stable. Indeed, ABH3 was shown not to be degraded in the culture medium of *A. niger* (L.G. Lugones and H.A.B. Wösten, unpublished). However, production levels of ABH3 were similar or even less compared to that of SC3 in *A. niger*. From this we can conclude that the problems with production of hydrophobins in *A. niger* occur intracellularly and at the post-transcriptional level but it is not yet clear what exactly hampers secretion.

**SC3 production in T. reesei**

In contrast to *A. niger*, *T. reesei* secretes high amounts of the class II hydrophobins HFBI and HFBII in its culture medium (Nakari-Setälä *et al.*, 1996; 1997) and might therefore be a better host to produce hydrophobins in submerged cultures. SC3 was expressed in *T. reesei* under regulation of the HFBI and HFBII promoters (vectors pS3TR1.1 and pS3TR1.2, respectively). Levels of SC3 in the culture medium of *T. reesei* transformants expressing SC3 behind the HFBI or HFBII promoter, using glucose or lactose as carbon source, respectively, were at least similar to that produced in *S. commune*. Northern blot analysis showed that in these *T. reesei* strains SC3 mRNA accumulated to a level similar to that in *S. commune*. These results indicate that post-transcriptional limitations in production of hydrophobins, as noted in *A. niger*, do not occur in *T. reesei*.

The levels of SC3 produced by *T. reesei* and the fact that SC3 could be purified from the culture medium are promising. SC3 production may be further increased by (i) introduction of more copies of the expression vector, (ii) targeting of the expression vector to the cellobiohydrolase I (CBHI) locus, (iii) mutagenesis and screening methods or by (iv) growing the strains under more optimal conditions (e.g. in a fermentor). Furthermore, (v) levels of SC3 may be increased by expressing SC3 as a fusion to the highly expressed cellobiohydrolase I (CBHI) or endoglucanase I (EGI) genes (Nakari-Setälä, personal communication).

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