Efficient Purification of Recombinant Proteins Using Hydrophobins as Tags in Surfactant-Based Two-Phase Systems†

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ABSTRACT: In this work we describe the new concept of using fungal hydrophobins as efficient tags for purification of recombinant fusion proteins by aqueous two-phase separation. Hydrophobins are a group of small surface-active proteins produced by filamentous fungi. Some characteristics of hydrophobins are that they are relatively small (approximately 100 amino acids), they contain eight disulfide-forming Cys residues in a conserved pattern, and they self-assemble on interfaces. The aqueous two-phase systems studied were based on nonionic surfactants that phase-separate at certain temperatures. We show that the use of hydrophobins as tags has many advantages such as high selectivity and good yield and is technically very simple to perform. Fusion proteins with target proteins of different molecular size were compared to the corresponding free proteins using a set of different surfactants. This gave an understanding on which factors influence the separation and what rationale should be used for optimization. This unusually strong and specific interaction between polymeric surfactants and a soluble protein shows promise for new developments in interfacing proteins and nonbiological materials for other applications as well.

For selective purification of proteins, affinity chromatography is one of the most efficient methods available. The selectivity of this method can be based on, for example, recognition of the target protein by specific antibodies or the use of various affinity tags fused to the target protein. Examples of such tags are, for instance, glutathione *S*-transferase or small peptides such as the His tag that selectively bind to purification columns (*1, 2*). If desired, the affinity tags can later be cleaved off from the target protein by specific proteases recognizing and cleaving at recognition sites that have been introduced to the fusion protein. Often affinity chromatography methods are only suited for analytical purposes and for purification of products of high value and are too expensive for large-scale products such as industrial enzymes. Furthermore, the methods are difficult to scale up for large-scale purification (*3*).

Liquid−liquid extraction in an aqueous two-phase system (ATPS)[1] can offer an efficient technique for protein purification even in large scale (*4*−*6*). Methods mainly based on poly(ethylene glycol) (PEG)−salt, PEG−dextran, and PEG−starch are used. Recently, also thermoseparating polymers and surfactants, which are especially suitable for protein purification, have been introduced. The partitioning of a protein to one of the phases may depend on its surface charge or hydrophobicity, but the driving forces are not always well understood. Obviously, the selectivity and overall efficiency of the purification depend on how much the particular protein of interest differs in these relevant properties as compared to the rest of the proteins in the mixture. To achieve selective protein purification in ATPS, small hydrophobic tags containing tryptophans have been fused to the protein to be purified (*7*). The production of these fusion molecules has however not been efficient, possible reasons being problems in secretion of the tagged proteins or proteolytic degradation of the tag.

We here describe a very efficient method for selective protein purification in ATPS which is based on the use of natural small hydrophobin purification tags in fusion proteins. These unique proteins are produced by filamentous fungi and form layers, for instance, on fungal cell walls or spore surfaces (*8*−*10*). Hydrophobins of different classes do not share much amino acid identity but have a characteristic pattern of eight Cys residues. Despite their name they are only moderately hydrophobic but are special in being amphiphilic and very surface active. The hydrophobins of interest to us are the class II (*11*) hydrophobins of *Trichoderma reesei.* The *T. reesei* hydrophobins that have been most extensively studied are HFBI (*12*) and HFBII (*13*). Both have a size of about 7.5 kDa. They are, however, expressed under different conditions, HFBII under cellulase-inducing conditions (such as cellulose or lactose as the carbon source)

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[1] Abbreviations: ATPS, aqueous two-phase system; PEG, poly(ethylene glycol); HPLC, high-performance liquid chromatography; CBD, cellulose-binding domain; EGI, endoglucanase I; EGIc, the truncated EGI consisting only of a core domain; HFBI, hydrophobin I; CBD_{CBHI}, CBD of the enzyme CBHI; CBD_{CBHII}, CBD of the enzyme CBHII.

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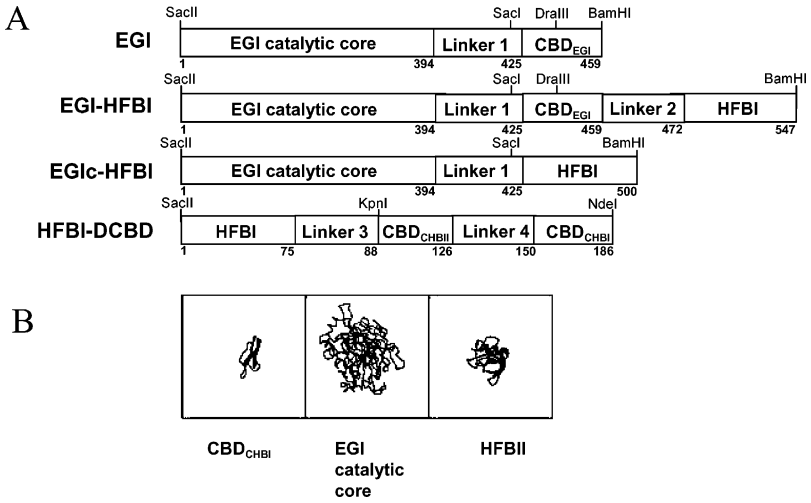

FIGURE 1: Proteins and different fusion proteins used in this study. (A) Schematic representation of EGI native endoglucanase I, CBD, cellulose-binding domain, DCBD double cellulose-binding domain, and the fusion proteins of these constructed with HFBI. (B) To support the discussion on the effect of protein size for the purification, the three-dimensional structures of the proteins are shown [structures drawn with the program Molscript (*40*)]. The structure of the closely related HFBII is used to show the size of HFBI. No structure has been determined for the linkers. HFBI has a mass of 7.5 kDa The two CBDs in the HFBI-DCBD fusion protein together with the linkers has a mass of 11 kDa. EGIc has a mass of about 42 kDa including the linker. EGIc, HFBII, and CBD_{CBHI} have the PDB (*41*) entries 1EG1, 1R2M, and 1CBH, respectively. The primary structures of the linkers are described in the Materials and Methods section.

and HFBI under cellulase-repressing conditions (such as glucose as the carbon source). The exact role of hydrophobins in fungal physiology is not known, but HFBII is found on spores and is likely to be involved in spore formation. Both proteins are very stable and show similar physicochemical properties. These properties are dominated by their surface activity (*14*, *15*), and as a consequence they adhere well to several types of interfaces (*16*). The first atomic resolution 3D structure of a hydrophobin to be solved was of HFBII (*17*). These hydrophobins can be produced in gram per liter levels, either as secreted into the culture medium or as adhered to the fungal biomass (*18*, *19*).

We demonstrate the usefulness of the approach for high levels of protein production and purification by using the filamentous fungus *T. reesei* (*20*). This host is widely used in industry for enzyme production. In optimized conditions, the fungus can secrete tens of grams per liter of a mixture of extracellular hydrolytic enzymes. This culture medium contains typically tens of different enzymes that due to their similar properties can be difficult to purify from each other. We demonstrate the purification of two fusion proteins of different size, consisting of a target protein and HFBI. The target proteins were (i) the cellulase endoglucanase I (EGI) and (ii) a combination of two small cellulose-binding domains from the cellobiohydrolases CBHI and CBHII, fused to the N- and C-termini of HFBI, respectively (Figure 1). Selective purification of the fusion proteins was achieved from a mixture of extracellular enzymes.

MATERIALS AND METHODS

Surfactants. The technical grade surfactant C12-18EO5 that has the commercial product name Agrimul NRE 1205 was obtained from Cognis Chemicals, Düsseldorf, Germany. The technical grade surfactant C11EO2 was obtained from Akzo Nobel, Stenungsund, Sweden. The highly purified homogeneous surfactants, C10EO2, C10EO3, C10EO4, and C10EO5, were purchased from Nikko Chemicals, Tokyo, Japan. All of the surfactants used were of linear polyoxyethylene fatty alcohol type. Their structures are abbreviated C*x*EO*y*, where *x* denotes the length of the fatty alcohol and *y* the number of polyoxyethylene units (*21*, *22*).

To determine the temperature dependency of the phase separation, 2% (w/w) mixtures of surfactants in water were made with all surfactants. Surfactants were mixed with water in a 10 mL glass test tube with volume indication. The tubes were placed in water baths with precise temperature control. After approximately 1 h, the volumes of the upper and lower phases were measured. The two phases were mixed again and incubated at a new temperature.

Fungal Strains. The *T. reesei* strains QM9414 (VTT-D-74075), Rut-C30 (VTT-D-86271), and Rut-C30 Δ*hfb2* (VTT-D-99676; (*18*)) were used as host strains. Hydrophobin fusion protein producing transformants of the strains constructed in this study are VTT-D-98693 (EGI-HFBI, QM9414), VTT-D-98691 (EGIc-HFBI, QM9414), VTT-D-99702 (EGIc-HFBI, Rut-C30), and VTT-D-99727 (HFBI-DCBD, Rut-C30 Δ*hfb2*).

DNA Constructs. The vectors pMQ103, pMQ113, and pTNS30, which are the final expression vectors for production of the EGI-HFBI, EGIc-HFBI, and HFBI-DCBD fusion proteins, respectively, were constructed as follows.

First, a PCR fragment was amplified with the primer pair 5′ TCGGGCACTACGTGCCAGTATAGCAACGACTACT ACTCGCAATGCCTTGTTCCGCGTGGCTCTAGTTCT-GGAACCGCACCAGGCGGCAGCAACGGCAACGGC 3′ (sense) and 5′ TCGTACGGATCCTCAAGCACCGACG-GCGGT 3′ (antisense) that consists of the mature HFBI (from Ser-23 to STOP codon) preceded by an artificial linker peptide VPRGSSSGTAPGG. The fragment was digested with *Dra*III and *Bam*HI and used to replace the internal *Dra*III and *Bam*HI fragment of the *egl1* cDNA in plasmid pPLE3 (*23*). The primer pair 5′ ACTACACGGAGAGCTC-GACGACTTCGAGCAGCCCGAGCTGCACGCAG*A*GC-AACGGCAACGGC 3′ (sense) and 5′ TCGTACGGATC-CTCAAGCACCGACGGCGGT 3′ (antisense) was used to amplify a fragment consisting of the mature form of HFBI

that was preceded by amino acids 410−425 of EGI (coding for the natural EGI linker). This fragment was cloned into pPLE3 (*23*) as a *Sac*I and *Bam*HI fragment by removing the CBD encoding sequences from the plasmid and by replacing them with HFBI. The resulting plasmids pMQ103 and pMQ113 carried the coding sequences for full-length EGI linked to HFBI via an engineered peptide linker and for EGIc linked to HFBI via the natural EGI linker, respectively, under the control of 2.2 kb of *cbh1* promoter and 0.7 kb of terminator sequences.

To construct the expression cassette for the HFBI-DCBD fusion, the primer pair 5′ GGAATTCCGCGGACTGCGCATCATGAAGTTCTTCGCCATCGCC 3′ (sense) and 5′ TGAATTCCATATGTTAGGTACCACCGGGGCCCATGCCGGTAGAAGTAGAAGCCCCGGGAGCACCGACGGCGGTCTGGCAC 3′ (antisense) was used to amplify the full-length HFBI followed by a Met-containing linker peptide PGASTSTGMGPGGT. The obtained PCR fragment was cloned into the pAMH110 (*24*) expression vector as a *Sac*II and *Kpn*I fragment followed by removal of interfering restriction enzyme cleavage sites from the vector polylinker by digestion with *Sac*I and *Bam*HI and blunt end ligation after T4 DNA polymerase treatment. In the last step, the primer pair 5′ TGAATTCGGTACCCAGGCTTGCTCAAGCGTC 3′ (sense) and 5′ TGAATTCCATATGTCACAGGCACTGAGAGTAGTA 3′ (antisense) was used to amplify the DCBD fragment using as a template an *E. coli* expression vector described in ref *25*. The amplified fragment was cloned downstream of the Met-containing linker as a *Kpn*I−*Nde*I fragment. The final expression plasmid, pTNS30, carried the coding region for the fusion protein consisting of HFBI and DCBD linked in frame via a Met-containing linker peptide. Expression of the fusion protein was regulated by the same *cbh1* transcriptional control sequences as for the other fusion proteins.

Fungal Transformations. T. reesei strain QM9414 was cotransformed essentially as described (*26*) using the plasmids pMQ103 and pMQ113 and the selection plasmid pToC202 containing the *amdS* gene of *Aspergillus nidulans* (*27*, *28*). The plasmid pTNS30 was transformed similarly except that the *T. reesei* strain Rut-C30 Δ*hfb2* was used as the host. Plasmid pMQ113 was also cotransformed into the strain Rut-C30 together with pARO21 (*29*) containing the *hph* gene of *Escherichia coli*. The transformants were selected and purified to uninuclear clones on the selective medium containing acetamide or hygromycin as described in ref *26*.

Transformants producing fusion proteins were screened by growing the transformants on minimal medium (*26*) containing either (i) 3−4% Solka flock cellulose (James River Corp., Berlin, NH), (ii) 3−4% whey and 1.5−2% complex grain-based nitrogen source (*30*), or (iii) 3% lactose and 0.2% peptone in shake flasks at 28 °C for 4 days. Culture supernatants were collected on the last day of the cultivation and analyzed for fusion protein expression by measuring endoglucanase activity using hydroxyethylcellulose (HEC) as a substrate (IUPAC 1987) and/or by Western blotting with anti-EGI and anti-HFBI antibodies.

Bioreactor Cultivations. Host strains and selected transformant strains were cultivated in a 10 L bioreactor on minimal medium (*31*) containing 40 g of Solka floc cellulose and 20 g of complex grain-derived nitrogen source (*30*) or 40 g of lactose, 4 g of peptone, and 1 g of yeast extract per liter at 28 °C for 4−5 days. The mycelia and culture media were separated by centrifugation after the runs, and the clarified culture filtrates were used further in the ATPS experiments.

Surfactant Extraction Using the Technical Grade C12-18EO5 and C11EO2. Typically 2% (w/v) of surfactant was added to samples of the culture supernatant. After careful mixing, the samples were allowed to settle. When the solution was kept at a temperature above the cloud point of the surfactant, the system phase-separated forming an upper surfactant phase and a lower aqueous phase. We used temperatures between 20 and 30 °C. The two phases were separated from each other using a separation funnel or by carefully drawing the lower, aqueous, phase off with a pipet.

The protein from the surfactant phase was recovered by adding isobutyl alcohol. When isobutyl alcohol was added to the separated C12-18EO5 surfactant phase, the mixture separated into two phases. The upper phase contained the surfactant and isobutyl alcohol, and the lower phase contained mainly the water that had been entrapped with the surfactant. The aqueous phase of this isobutyl alcohol recovery step also contained the hydrophobin fusion protein. In the case of C11EO2 the initial surfactant phase contained very little water. Therefore, water (or buffer) was added to the recovered surfactant phase prior to addition of isobutyl alcohol. Typically the volume of water that was added was equal to that of the surfactant.

Determination of Partition Coefficients. The concentration of the enzyme stock was determined by measuring the absorbance at 280 nm using $\epsilon = 59400$ for EGIc-HFBI and EGIc and $\epsilon = 70590$ for full-length EGI (HFBI has practically no effect on the extinction coefficient since it contains no Trp or Tyr residues). Dilutions were made to obtain enzyme solutions of about 50 μg/mL. The enzyme solution (980 mg) was added to a glass test tube containing 20 mg of surfactant in order to get a 2% (w/w) mixture of surfactant and enzyme solution. Enzyme solution and surfactant were mixed by vortexing. The tube was placed in a water bath at a fixed temperature. After separation had taken place (approximately 1 h), an aliquot of the lower phase was taken for analysis.

To determine the concentration EGIc(-HFBI) present after separation and sample preparation, an enzyme activity assay was used. Four hundred microliters of 0.05 mM 2-chloro-4-nitrophenyl β-D-cellobioside (CNP-G_2, Sigma) was added to Eppendorf tubes containing 100 μL of the lower phase from the sample separation. Enzymatic hydrolysis of CNP-G_2 yielded a yellow color that was measured. To stop the reaction, 500 μL of 1 M Na_2CO_3 was added after exactly 5 min. All of the samples were analyzed by adsorption at 600 nm using a microtiter plate absorbance reader. Controls included buffer only, protein without surfactant, and surfactant without protein. To determine the concentration of HFBI, DCBD, and HFBI-DCBD present after separation and sample preparation, the lower phase was analyzed using analytical HPLC. Standard curves using weighed amounts of pure, lyophilized protein were used for concentration determination. The column used was a 20 cm Vydac C4 (20 cm, i.d. 4.6 mm, Grace Vydac). A stepwise gradient was used running from 20% B buffer to 60% B buffer at 1 mL/min at the elution segment. The A buffer was 0.1% trifluoroacetic

acid in water, and the B buffer was 0.1% trifluoroacetic acid in acetonitrile.

The partition coefficient (K) was calculated as a measure of the distribution of protein between two immiscible phases. It is the ratio of the protein concentration in the upper phase to the concentration of protein in the lower phase; $K = (c_{upper}/c_{lower})$.

Purification of EGIc-HFBI on a Preparative Scale. For more detailed studies of EGIc-HFBI, a preparative scale purification was performed. Culture supernatant (500 mL) was extracted with 2% C12-18EO5 at 22 °C for 1 h. The surfactant phase was separated, and to this an equal volume of isobutyl alcohol was added. The resulting lower aqueous phase was desalted using Bio-Rad 10-DG (Bio-Rad) columns and 10 mM acetate buffer, pH 5.0. The desalted sample was loaded on a Resource Q (Amersham Pharmacia) column and eluted with a linear gradient of NaCl up to 0.2 M. The EGIc-HFBI was identified by specific enzymatic activity on CNP-G_2 and Western blots using polyclonal anti-HFBI and anti-EGI.

Cleavage of the Fusion Protein. HFBI can be removed from the fusion protein by cleaving the linker between HBFI and the target protein. In this work we achieved this using cyanogen bromide that uniquely cleaves at Met residues at low pH.

Approximately 0.25 mg of cyanogen bromide was dissolved in 50 μL of water. To this was added 10 μL of a 10 mg/mL solution of HFBI-DCBD, and HCl was added to a final concentration of 10 mM. After mixing, the tube was wrapped in aluminum foil and stored overnight at room temperature. The contents of the tube were then diluted with 1 mL of water and freeze-dried. The protein then was dissolved in 1 mL of 0.1% TFA in water, and part of the sample was analyzed using C4 HPLC as above. To separate the cleaved DCBD from the fusion protein and the free hydrophobin, 2% (w/w) C11EO2 was added to the rest of the sample. After separation, the sample was again analyzed using C4 HPLC.

RESULTS

Hydrophobin Fusion Proteins Are Efficiently Produced by T. reesei. Constructs for the expression of three different types of hydrophobin fusion proteins (Figure 1A) were made. These were expressed in three different strains of *T. reesei* under the strong *cbh1*-cellulase promoter. This promoter is regulated by the carbon source and allows high production of recombinant proteins on media containing, for example, plant materials, cellulose, or lactose (*32*). As target proteins, domains of native *T. reesei* cellulases were used. The cellulases typically consist of a catalytic domain that is linked to a cellulose-binding domain (CBD) through a linker region. Two of the target proteins were fused to the N-terminus of HFBI, namely, the wild-type EGI endoglucanase (giving EGI-HFBI) and the catalytic core domain of EGI (giving EGIc-HFBI). The third construct contained two cellulose-binding domains (CDB) linked to the C-terminus of HFBI (giving HFBI-DCBD). These CBDs were from the two *T. reesei* cellobiohydrolases, CBHII and CBHI. In the case of CBHII the CBD segment consisted of the 41 amino acid residues from the N-terminus of the enzyme, and in the case of CBHI the CBD segment consisted of the 57 C-terminal residues of the enzyme.

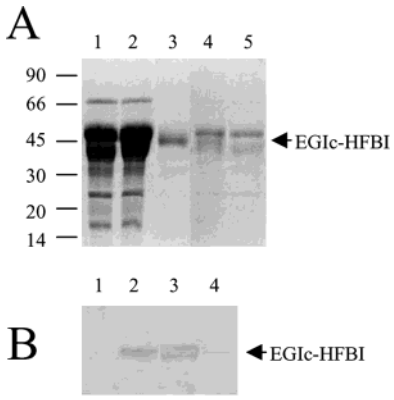

FIGURE 2: Purification of EGIc-HFBI from culture supernatant. (A) Lane 1 is the culture supernatant before extraction. Lane 2 is the lower phase (residue) of the extraction. Lane 3 is the surfactant phase. Because lanes 1 and 2 are overloaded, 1:10 dilutions were made of them (lanes 4 and 5). From the gel we can see that very little of the protein in the lower phase is extracted to the surfactant phase. Only one major band is seen in the surfactant phase. The size of this band corresponds to the EGIc-HFBI protein and is indicated by an arrow on the right-hand side of the gel. From the diluted samples it can be seen that there is a gap at the position of the extracted protein. (B) Western blot of a control strain not producing EGIc-HFBI (lane 1), supernatant before extraction (lane 2), the extracted protein (lane 3), and lower phase after extraction (lane 4). Detection was with anti-HFBI that does not react with any naturally occurring protein of the corresponding size in the supernatant of the parent strain. The gel is 10% homogeneous vertical SDS−PAGE. Molecular mass standards are indicated on the left-hand side of the gel and are in units of kDa.

T. reesei was shown to secrete catalytically active EGI-HFBI hydrophobin fusion proteins into the culture medium in the gram per liter level. The best QM9414-based production strains VTT-D-98693 (EGI-HFBI) and VTT-D-98691 (EGIc-HFBI) and the Rut-C30-based strains VTT-D-99702 (EGIc-HFBI) and VTT-D-99727 (HFBI-DCBD) were cultivated in a bioreactor on cellulose- and lactose-containing media as described in Materials and Methods. The amounts of EGI-HFBI and EGIc-HFBI produced by the transformants were estimated on the basis of endoglucanase activity measurements using hydroxyethylcellulose as a substrate (IUPAC, 1987). Production levels of the EGIc-HFBI fusion protein were 2−3-fold higher in comparison to the endogenous EGI produced by the host strains. It was estimated that 1.7 g/L EGIc-HFBI fusion is produced while 5−6 g of total protein/L was obtained. The production level of the HFBI-DCBD fusion protein was estimated to be 1.35 g/L on the basis of HPLC analysis. Although naturally most of HFBI hydrophobin is cell wall-bound, SDS−PAGE analysis indicated that the fusion proteins were among the most prominent bands in the culture supernatants (Figure 2, lane 1; Figure 3 lane 1).

Properties of Technical Grade Surfactants. In this study we initially used two technical grade surfactants: C12-18EO5 (Agrimul NRE 1205) and C11EO2 (Berol 532). The property that we were primarily interested in was the ability of nonionic surfactants to separate into two phases above a certain temperature, called the cloud point. For all surfactants used in this work the surfactant phase was the upper phase.

The main difference between the two surfactants, C12-18EO5 and C11EO2, is in their degree of hydrophobicity. In general terms this affects their properties in two ways: the temperature for phase separation (cloud point) and the

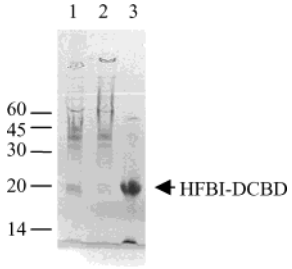

FIGURE 3: Purification of HFBI-DCBD by extraction with C11EO2. Lane 1: The culture supernatant of the strain producing HFBI-DCBD. Lane 2: The lower (residue) phase after extraction. Lane 3: The protein fraction after isobutyl alcohol back-extraction of the surfactant phase. In this fraction the only major bands are the HFBI-DCBD target protein and a smaller amount of free hydrophobin. The position of the target protein is indicated by an arrow on the right-hand side of the gel. The gel is a horizontal Phast 4−20% gradient SDS−PAGE. Molecular mass standards are indicated on the left-hand side of the gel and are in units of kDa.

volume of the resulting surfactant phase. For C12-18EO5 the phase separation occurred at 19 °C. Slightly above this temperature the volume of the surfactant phase was very large, comprising about 80% of the total volume. Increasing the temperature further to 22 °C rapidly reduced the surfactant phase volume to about 20% of the total volume. There was a second slight increase in surfactant volume at 30 °C. Increasing the temperature further did not essentially change the volume ratio until above 40 °C when no phase separation was any longer observed. Because of the large volume fluctuations between 19 and 21 °C, we analyzed the protein partitioning at 22 °C and at a higher temperature, namely, 30 °C. C11EO2, on the other hand, is more hydrophobic and formed a separate phase at the whole range studied from 9 to 40 °C. The C11EO2 phase was also more concentrated, comprising less than 10% of the total volume. All phase separations occurred readily and did not require centrifugation. Centrifugation at 5000*g* was tested and did not affect surfactant volumes or protein partitioning.

Extraction of EGIc-HFBI and EGI-HFBI from Culture Supernatant Using C12-18EO5. As an example of a typical experiment, the extraction of the EGIc-HFBI protein is shown in Figure 2. First 2% (w/v) C12-18EO5 was mixed with the culture supernatant, and the resulting mixture was allowed to settle (1 h, 21 °C). Two phases were formed, and aliquots from both the upper (surfactant) and the lower (aqueous) phases were taken and loaded on a SDS−PAGE gel. As shown in Figure 2A, a protein with a size corresponding to EGIc-HFBI migrated to the surfactant phase. Western blotting with anti-HFBI antibodies (Figure 2B) verified that the band was EGIc-HFBI. The extraction was very efficient, since the protein initially present in the culture supernatant was essentially missing from the aqueous phase after extraction. There is also a clear increase in the concentration of the fusion protein during the extraction.

Additionally, the partitioning of the EGI-HFBI fusion protein was compared to that of EGIc-HFBI using crude supernatants. Using C12-18EO5 at 30 °C in initial experiments both proteins showed similar apparent partitioning coefficients, 4 for EGIc-HFBI and 3 for EGI-HFBI, compared to 0.3 and 0.2 for the corresponding EGI and EGIc proteins, respectively. The values are lower than other reported elsewhere in this paper, but this is due to interfering activities of endogenous proteins in the culture supernatant that cause underestimation of coefficients. Nonetheless, it can be concluded that both fusion proteins partition similarly. EGIc-HFBI was chosen for further more detailed characterization.

Table 1: Partitioning Coefficients (*K*) and Yields for Purified Fusion Proteins and the Respective Target Proteins with Two Surfactants at 22 °C

	C12-18EO5		C11EO2	
protein	*K*-value	yield (%)	*K*-value	yield (%)
EGIc	0.5 ± 0.1	10 ± 2	0	0
EGIc-HFBI	25 ± 3	88 ± 8	0	0
DCBD	ND[a]	ND	0.7 ± 0.2	7 ± 3
HFBI-DCBD	13 ± 2	79 ± 8	139 ±15	84 ± 8

[a] ND = not determined.

Extraction of HFBI-DCBD from the Culture Supernatant Using C11EO2. Figure 3 shows selective purification of HFBI-DCBD protein from the culture supernatant. This experiment differs from the procedure described above (Figure 2) in containing an additional step where the HFBI-DCBD protein was recovered from the surfactant phase by adding isobutyl alcohol and 50 mM sodium acetate buffer, pH 5 (Figure 3, lane 3). The volume of buffer was equal to the volume of the surfactant phase (see section on protein recovery by isobutyl alcohol extraction). Isobutyl alcohol extraction from the surfactant phase prior to SDS−PAGE was necessary when using C11EO2, because loading this surfactant in SDS−PAGE gave poor results. When using C12-18EO5, SDS−PAGE could be run directly on samples from the surfactant phase.

The gel showed two proteins in the isobutyl alcohol extracted fraction. When the fraction was loaded on a C4 HPLC column, the proteins could be separated. The band migrating at close to the 20 kDa standard was identified as the HFBI-DCBD using anti-HFBI and anti-CBD_{CBHI} antibodies in Western blots. MALDI-TOF mass spectroscopy showed that the protein was heterogeneously glycosylated. The small protein migrating close to the front in SDS−PAGE was identified as HFBI using antibodies (see ref *33*). Wild-type HFBI should not have been produced under the applied (cellulase-inducing) growth conditions, so we conclude that some proteolytic cleavage of the fusion protein had likely occurred.

Partition Coefficients and Yields for EGIc-HFBI and HFBI-DCBD in C12-18EO5 and C11EO2. The SDS−PAGE analysis of the extractions indicated a very high partitioning of hydrophobin fusion proteins in the surfactant system. Measurement of exact partitioning coefficients (*K*-values) using culture supernatants was complicated by the fact that *T. reesei* also produces native EGI and other endoglucanases with similar enzymatic activities. These proteins cause background problems when attempting to determine the amount of EGIc-HFBI remaining in the lower phase by quantification by enzymatic activity. Therefore, experiments to determine *K*-values were performed using pure proteins. Quantification of EGIc and EGIc-HFB was done by their enzymatic activity. When needed, protein concentrations were obtained by comparing the activity of samples to enzyme of known concentration. For the HFBI-DCBD and DCBD, analytical HPLC was used to quantify the protein

concentration. The obtained *K*-values are shown in Table 1. As expected from the SDS−PAGE analysis the hydrophobin fusion proteins showed a significantly higher partitioning than the target proteins as such. The most surprising result was however that EGIc-HFBI did not partition by any measurable extent to C11EO2. On the other hand, extraction of HFBI-DCBD was more efficient into C11EO2 than C12-18EO5.

Recovery of Proteins from Surfactant. For calculating the overall purification yield of the fusion proteins, one must take into account both the surfactant extraction step and the subsequent isobutyl alcohol recovery step. The HFB fusion proteins could be recovered from the surfactant phase by adding isobutyl alcohol to it. The isobutyl alcohol addition resulted in a two-phase system where the lower phase was aqueous and the upper phase consisted of surfactant and isobutyl alcohol. The volume of the lower phase compared to that of the surfactant depended on how much water was entrapped in the surfactant phase in the first extraction. The quantity of water entrapped in C11EO2 was so low that water or buffer had to be added in the isobutyl alcohol recovery step for practical reasons. Therefore, it was not meaningful to calculate the overall volume reduction factors for the purification since it depended on practical rather than theoretical limitations. However, as can be seen from Figure 3 the volume reduction (concentrating effect of the purification) is significant. Recovery yields for HFBI-DCBD in this C11EO2 system were 92 ± 5%. The amount of isobutyl alcohol required for maximal recovery depended on the surfactant. For HFBI-DCBD a 2.5 times excess of isobutyl alcohol to that of the surfactant was sufficient using C11EO2. However, for C12-18EO5 five times more isobutyl alcohol than surfactant was required. The yields of EGIc-HFBI recovery from C12-18EO5 were 94 ± 10%. The measurements of EGIc-HFBI yields were complicated by the saturation of the water phase by isobutyl alcohol that disturbed the CNP-G_2 activity assay. This problem could partially be overcome by comparing the extracted samples to control samples in isobutyl alcohol saturated water or by desalting of the sample by size exclusion chromatography prior to activity measurements.

Cleavage of HFBI-DCBD and Recovery of the Separated Domains. The HFBI-DCBD fusion protein was designed to contain a single Met residue in the linker region between HFBI and CBD_{CBHII}. The feasibility of cleaving the linker between the different proteins was tested by specific hydrolysis by cyanogen bromide. This method was possible to use because the only Met residue in the fusion protein was in the linker region. Figure 4 shows how surfactant extraction can be used to specifically reextract the uncleaved fusion protein and the cleaved hydrophobin while leaving the cleaved target protein (DCBD) as the final product in the water phase.

Partitioning of Pure Proteins in Homogeneous Surfactants. For a more detailed study of how the surfactant structure affects the separation, a series of highly purified homogeneous surfactants was used. The four surfactants C10EO2, C10EO3, C10EO4, and C10EO5 were used. These experiments were performed with pure proteins. The phase separation of surfactants was studied in the range 9−66 °C. The partitioning of EGIc-HFBI, EGIc, and HFBI was studied up to 50 °C. The results are shown in Figure 5. Three parameters as functions of temperature are described in the figure; phase volume, yield, and partitioning coefficient. Two of these are independent, and the third is determined by the other two. Yields and volumes were measured and used to calculate the partitioning coefficients.

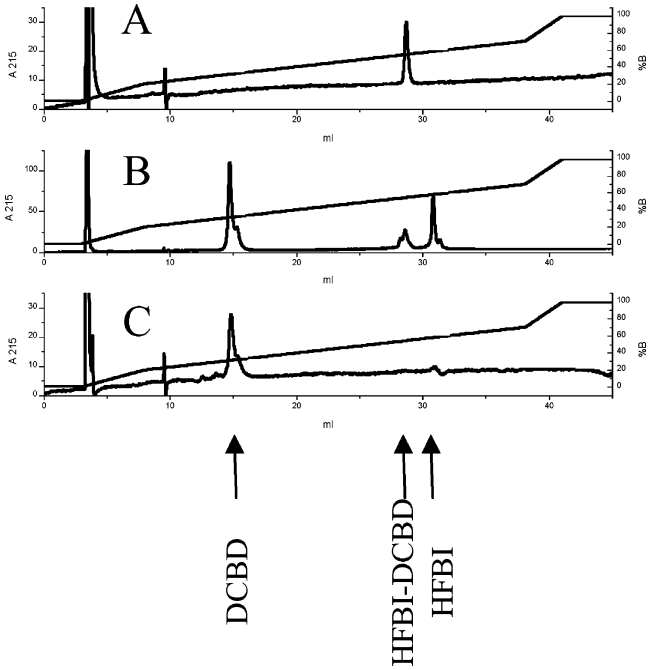

FIGURE 4: The low nonspecific extraction of proteins in the surfactant extraction makes it possible to selectively remove hydrophobin from a mixture. In (A) a chromatogram of the purified HFBI-DCBD is shown. In (B) the linker between the HFBI and DCBD domains had been cleaved by cyanogen bromide. The uncleaved fusion protein and the cleaved HFBI can be selectively removed by a second extraction step (C).

DISCUSSION

In this work we have shown how hydrophobins can be used as tags for the purification of recombinant fusion proteins by two-phase extraction with nonionic surfactants. As target proteins we chose two clearly different types of proteins and fused these to either the N- or C-terminus of the hydrophobin HFBI (Figure 1). One construct contained two small wedge-shaped binding domains (CBDs) from cellulases fused to each other by a linker, forming a so-called double cellulase-binding domain (DCBD) (*25*). The CBDs have masses of 3.7 and 4 kDa, giving the total mass of 11 kDa including the linker segments for the DCBD. In this construct the target protein was in the C-terminus and the hydrophobin in the N-terminus. In the second construct the enzyme EGIc was used. It is a relatively large (42 kDa), glycosylated globular core domain of a cellulase. In this construct the target protein was in the N-terminus and the hydrophobin in the C-terminus. In a third construct, EGI-HFBI, a variation of the EGIc-HFBI construct, was made using the full-length cellulase instead of only the core domain. After initial characterization that showed that EGIc-HFBI and EGI-HFBI functioned similarly, the more detailed studies were made with only EGIc-HFBI. Despite the complex structures, the fusion proteins were produced very well in *T. reesei*, giving production yields of over 1 g/L in bioreactor cultivations.

The overall ATPS purification scheme involved two steps: the first was the extraction of the fusion protein into

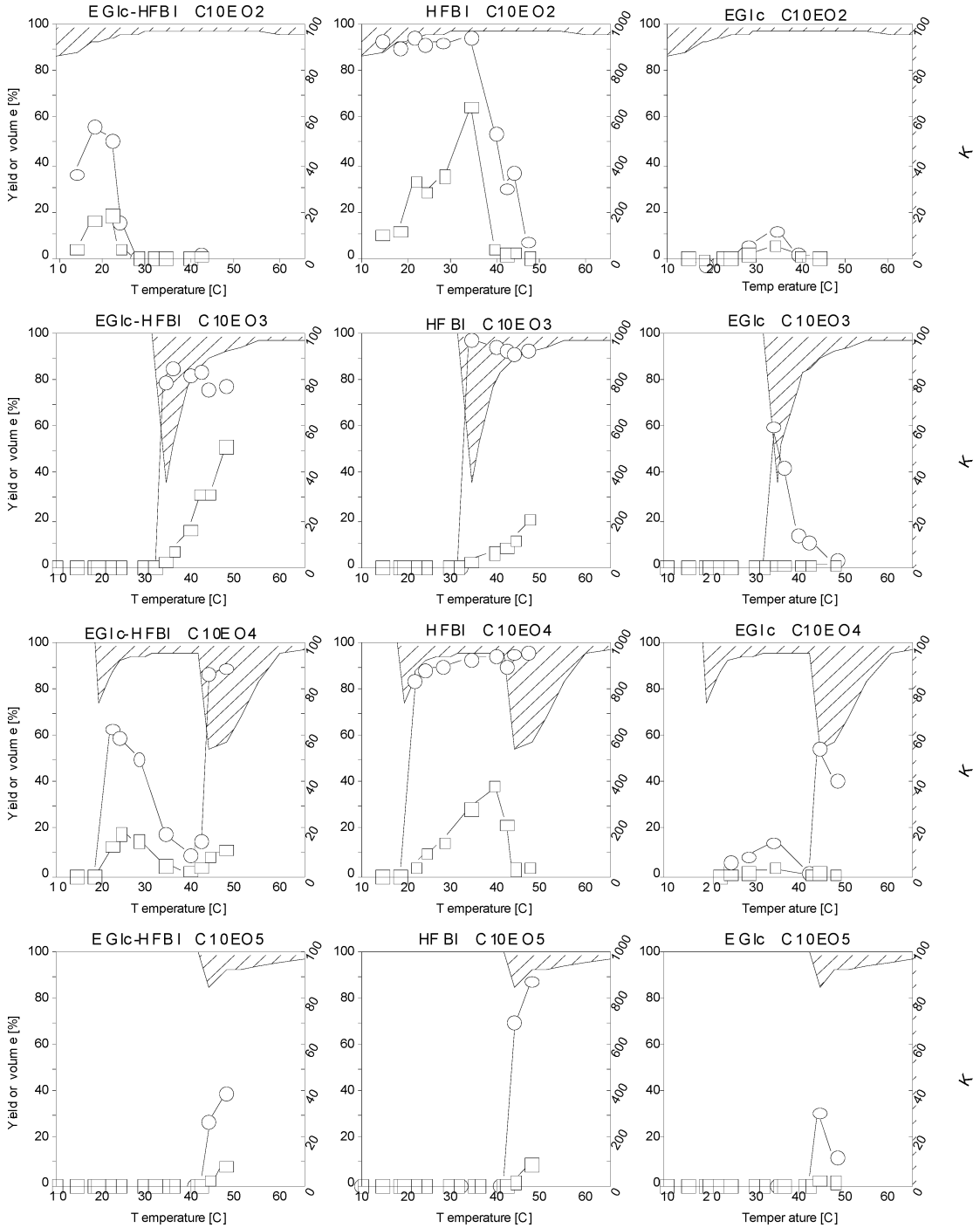

FIGURE 5: A more detailed understanding of surfactant−hydrophobin interactions is gained from studying phase volumes and partitioning of proteins at different temperatures using highly purified homogeneous surfactants. The volume of the surfactant phase is drawn as a shaded area in the upper part of the diagram with the unit on the left side as percent of total volume. Yield is shown as circles (○) on the left side, and the partitioning coefficient (*K*) is shown on the right side with square (□) symbols. Note that the *K*-values for HFBI are drawn with a different scale. The combination of protein (EGIc-HFBI, EGIc, or HFBI) and surfactant (C10EO2, C10EO3, C10EO4, C10EO5) is indicated above each plot.

the surfactant and separation of the surfactant phase. In the second step, the protein was recovered from the surfactant phase into buffer by addition of isobutyl alcohol. The main finding of this work is that the fusion proteins could be extracted with a very high yield and selectivity from culture supernatants that contained large amounts of other naturally secreted proteins (Figures 2 and 3). Recovery by isobutyl alcohol proceeded with good yields after optimization of the volume of isobutyl alcohol. The overall concentration effect (volume reduction) obtained depended on the amount of entrapped water in the first surfactant extraction. For the more hydrophobic surfactants the volume of entrapped water was so low that buffer had to be added for the recovery step. The surfactant extraction also provides a convenient way of removing the hydrophobin tag from the fusion protein after extraction, as demonstrated with the cyanogen bromide cleavage of the HFBI-DCBD fusion protein (Figure 4).

Role of Surfactant Structure. Of the two steps involved in the purification it was the initial surfactant extraction that required the most optimization. In the initial part of the study

we used two readily available, inexpensive technical grade surfactants, C11EO2 and C12-18EO4. These experiments showed surprisingly that the EGIc-HFBI fusion protein was not extracted by C11EO2 although it was efficiently extracted by C12-18EO4. On the other hand, HFBI-DCBD was efficiently extracted by both surfactants. This clearly suggested that there exists a relationship between the size of the fusion partner, the composition of the surfactant, and the partitioning of the proteins. It is likely that this relation depends on an excluded volume effect. A dense hydrophobic surfactant contains less water in the space between micellar or lamellar structures and, therefore, excludes bulky hydrophilic groups. The situation can be seen as analogous to size exclusion chromatography, where large molecules are excluded and thus elute first. The overall partitioning would then be a balance between the affinity between surfactant and hydrophobin on one hand and the size exclusion repulsion of the hydrophilic group on the other. Optimal balancing of these would give the best combination between concentration, purity, and yield.

Mass spectroscopy analysis of both C11EO2 and C12-18EO4 showed that both are complex mixtures containing variable chain lengths of both the hydrophobic and hydrophilic segments. Both surfactants also contained free polyethoxy chains. To get a better understanding of the relationship between partitioning of hydrophobins, fusion partners, and fusion proteins, we decided to test their partitioning on a range of different surfactants. For this purpose we used a series of highly purified homogeneous surfactants with an increasing length of the hydrophilic headgroups together with the fusion protein EGIc-HFBI and its constituent proteins HFBI and EGIc.

The series of homogeneous surfactants used was C10EO2, C10EO3, C10 EO4, and C10EO5. In this series the balance between the hydrophobic and hydrophilic groups shifts toward more hydrophilic. This property of a surfactant is described as the hydrophilic−lipophilic balance (HLB) in the literature (*21*). On the basis of the result with the technical grade surfactants it would be expected that HFBI would have the highest affinity toward the most hydrophobic surfactant while fusion protein would require a more hydrophilic surfactant. The C10EO3 surfactant describes how the separation of the fusion proteins depends on the surfactant properties (see Figure 5). The cloud point occurred at 30 °C, with an initially very large surfactant volume (C12-18EO5 showed a similar behavior in this respect). The partitioning of the isolated fusion partner EGIc was low, the *K*-value was about 1 at maximum. However, because the volume of the surfactant was about 70% at its maximum, even this partitioning caused a significant part of the EGIc to partition to the surfactant phase. When the temperature was increased, the partitioning remained low, and as the surfactant volume decreased, so did the yield. The partitioning of HFBI increased as the temperature increased. At the same time the surfactant volume decreased, but the yield remained high because these factors compensated each other. The behavior of the fusion protein was a combination of the properties of the two components. The partitioning of the fusion protein was much lower than that for HFBI but still significantly higher than for the target protein. In an optimization of the temperature, the most efficient separation of the nonspecific protein (exemplified by EGIc) and fusion protein occurred at the highest temperature in this particular case.

Next we examine how the partitioning of the different components changed as the hydrophilic−lipophilic balance of the surfactant was varied. C10EO2 was the most hydrophobic surfactant and showed phase separation already below the lowest temperature studied, 9 °C. As expected, the highest partitioning value for HFBI was also noted for this hydrophobic surfactant. The EGIc partitioning remained low, but the fusion protein showed some partitioning at low temperatures where the surfactant volume was the largest; i.e., the surfactant contained more water in its structure. Comparing C10E3 and C10E4, we noticed that C10E4 initially forms a second phase at a lower temperature despite its larger hydrophilic group. This complex behavior of a surfactant is called "pivotal phase behavior" and is not very well understood (see ref *22* for a more in-depth discussion). It is clear, however, that C10E4 undergoes changes in phase structure in the studied temperature range. Interestingly, the partitioning coefficient of HFBI follows this behavior. Surprisingly, the partitioning of the fusion protein into the high-temperature end (about 40 °C) of the first phase of C10E4 was very low but increased drastically as the structure of the phase changed above 43 °C. At this higher temperature, the phases of both the C10E3 and C10E4 behaved similarly. C10E5 started to form a phase at 41 °C that behaved very similarly to that of C10E3 starting at around 30 °C.

The surfactant series C10E2 to C10E5 was selected because it forms a long series with surfactants that have cloud points in the range 10−50 °C that could be practical for purification purposes. Good selectivity (high fusion partitioning, low EGIc partitioning) was achieved at the high temperature end of the surfactant phase C10E3 and the middle part of the lower temperature phase of C10E4. Increasing the temperature further in the C10E4 experiments would probably have resulted in a similar good selectivity as seen for the surfactant phase of C10E3. A phase diagram for C12-18EO5 has been prepared (*34*) and is similar to that of C10E4. We also noted a similar volume behavior with similar (pivotal phase behavior) minima and maxima for C12-18EO5 and C10E4 (data not shown). From the phase diagram we can see that the separation described for C12-18EO5 in this paper was performed at the middle part of the first phase. We conclude that separation behavior of the proteins seems to be dependent on the phase structure of the surfactant, but for practical purposes different optimal conditions may be found at different temperatures. Especially, it seems that robust separation temperatures could be achieved using conditions corresponding to the phase of C10E3. In practice, the thermal stability of the target protein may determine the optimal conditions for separation.

Despite, or perhaps due to, its heterogeneous character the technical grade C12-18EO5 worked very well for the purification of the hydrophobin fusion proteins. The method was robust and repeatable. For preparative purification of fusion proteins, surfactant extractions must be performed with technical grade surfactants because of the very high price of homogeneous surfactants. This seems to pose no problems, especially since the best yields were obtained with the technical grade surfactants. It is even possible that the

mixture including free polyoxyethylene and lipid alcohol chains may improve the separation.

Removal of Surfactant from the Extracted Protein by Isobutyl Alcohol Extraction. For the use of the surfactant extraction for preparative protein purification there must be a practical way of recovering the protein from the surfactant phase. Isobutyl alcohol extraction has been described previously as a method of recovery in similar purification schemes (*35*). For purifying wild-type hydrophobins, isobutyl alcohol proved to work well in a comparison to other similar compounds in our earlier work (*33*). In the current work, we found that the isobutyl alcohol extraction was very robust and gave good yields. We did not observe any denaturation of any of the proteins in the presence of isobutyl alcohol, but this may be a concern for other more labile target proteins. It has been observed previously that branched alcohols, like isobutyl alcohol, are less denaturing than unbranched ones. Denaturation of proteins by alcohols is also dependent on pH, salts, and temperature (*36*). Therefore, some target proteins may require optimization of these parameters in the surfactant removal step. Another alternative to surfactant removal is the use of polymers, such as ethylene oxide−propylene oxide copolymers, as described in ref *37*.

Protein Structure−Function Aspects of Partitioning. Hydrophobins are very special among soluble proteins in their partitioning into nonionic surfactants. High separation yields have previously been observed mostly for membrane proteins, such as cholesterol oxidase ($K = 10$) and bacteriorodopsin ($K = 51$) (*38*, *39*). The recently solved structure for HFBII gives some clues to how the separation might function (*17*). A striking structural feature of HFBII is that the structure is highly stabilized by four disulfide bonds. In fact, much of the protein's interior is made up of Cys residues. On the surface of the protein there is very clearly a hydrophobic patch of aliphatic side chains. The residues are highly conserved in hydrophobins belonging to the same class and do not include any aromatic side chains. As water-soluble proteins typically are thought of as having an internal hydrophobic core that stabilizes the structure, the hydrophobin can be thought of as if it had been turned inside out. It has a hydrophobic patch on the outside, and the core is instead stabilized by the disulfides. In aqueous solutions, hydrophobins apparently multimerize with their hydrophobic patches toward each other. In this way they shield their hydrophobic patches and become soluble. In our previous experiments we have shown that both HFBI and HFBII are soluble to at least 100 mg/mL (unpublished data). Energetically, the multimers are expected to be unstable, however. This is seen as a general surface activity of the hydrophobins. The strong partitioning of the hydrophobins with nonionic surfactants must depend on how the surfactant structure can lower the solubilization energy of the hydrophobin. Our hypothesis is that the tetramer (or other multimer) structure that forms in water is of intermediate energy. Hydrophobins will migrate to interfaces that can lower the solubilization energy of hydrophobins even further. It is interesting that surfactants can do this, while the isobutyl alcohol phase in the recovery step does not do this. The reason may be that the isobutyl alcohol does not have a phase structure that contains water and is thus not able to solubilize the hydrophilic part of the protein. Therefore, the hydrophilic part of the protein drives it back to the aqueous phase when isobutyl alcohol is added.

The described scheme for protein production fulfills most requirements for a good purification method. As long as the temperature of the mixture can be controlled within a few degrees, the method is very robust. The extraction offers a good volume reduction in the sample and high yield and is very selective. In practice, the only impurity is any free hydrophobin present in the culture supernatant. The problem of endogenous hydrophobin can be avoided by making strains where the hydrophobins have been knocked out (*18*). The technical grade surfactants are not typically used biochemical reagents but are, however, easily obtainable from suppliers. The cost of the surfactant for purifying 1 L of culture supernatant is less than $1.00 [based of the cost of Berol 532 (Akzo Nobel, personal communication)]. The ATPS method is also easily scalable. Experiments could be easily made in 100 μL scale in test tubes or at the scale of several liters without differences. If one chooses the surfactant and temperature carefully, the separation can be optimized for fusion proteins with different sized fusion partners. This is nonetheless not always required as, for example, C12-18EO5 functioned well as an all-around surfactant.

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