Improved extraction and purification of the hydrophobin HFBI

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Keywords

Class II hydrophobin; HFBI; *Trichoderma reesei;* extraction; Fast Protein Liquid Chromatography; Cold Induced Phase Separation

Abbreviations

ACN: Acetonitrile; **CIPS:** Cold Induced Phase Separation; **HFBs:** Hydrophobins; **RP-FPLC:** Reversed Phase Fast Protein Liquid Chromatography; **RP-HPLC:** Reversed Phase High Performance Liquid Chromatography; **SDS:** Sodium dodecyl sulphate; **TFA:** Trifluoroacetic acid

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Abstract

Hydrophobins (HFBs) are a group of highly functional, low molecular weight proteins with the ability to self-assemble at hydrophobic-hydrophilic interfaces. The surface active, cysteine-rich proteins are found in filamentous fungi such as *Trichoderma reesei*. In the present study multiple extraction solvents and conditions were screened for the mycelium bound hydrophobin HFBI and the effects on the total amount of extracted proteins, HFBI recovery and HFBI gushing activity were investigated to gain a more thorough scientific insight on the extraction efficiency and selectivity. Results indicated the enhanced selectivity for HFBI extraction from the fungal biomass using 60% ethanol compared to solutions containing 1% sodium dodecyl sulphate (SDS). Complementing the higher selectivity, HFBI recovery was increased from 6.9 ± 0.6 mg HFBI (1% SDS) to 9.4 ± 0.4 mg HFBI per gram dry fungal biomass for extracts containing 60% ethanol. Furthermore, subsequent to HPLC purification, Cold Induced Phase Separation (CIPS) of acetonitrile-water systems was investigated at different pH levels. CIPS at pH 2.0 was found to effectively remove the majority of sorbicillinoid pigments from the purified HFBI fraction. The improved method resulted in a recovery of 85.4% of the extracted HFBI after final purification.

Introduction

Hydrophobins (HFBs) are a family of relatively small (7- 10 kDa) but highly functional, surface active proteins [1]. These cysteine-rich proteins are found in filamentous fungi and are associated with the growth and development of the fungal hyphae. HFBs are known to self-assemble into amphipathic membranes at hydrophobic-hydrophilic interfaces, successfully lowering the water surface tension, allowing growth of aerial hyphae while coating of fungal spores aids in spore dispersal and reproduction of the fungi [1,2].

Based on morphological and functional properties of the self-assembled membranes and homogeneity in the amino acid sequences, two Classes of HFBs, namely Class I and Class II HFBs are considered. Class I HFBs self-assemble into rigid multilayer membranes and are only soluble in strong acids such as trifluoroacetic acid (TFA), whereas Class II HFB-membranes show remarkable elasticity and are easily dissolved in detergent solutions such as 2% sodium dodecyl sulphate (SDS) or 60% ethanol [1,3,4]. The remarkable elastic properties of Class II HFBs provide a high added value for applications in food industry as emulsion and foam stabilizers [5–8] whereas the self-assembling properties also enable coating and encapsulation of drug nanoparticles increasing solubility, uptake and drug release, relevant for pharmaceutical and medical applications [9,10]. On the other hand, Class II HFBs are known for causing primary gushing in carbonated beverages such as beer [11].

The Class II HFBs, HFBI and HFBII produced by *Trichoderma reesei* have been characterized extensively [12–16]. Both HFBs exhibit remarkable properties and although being produced by the same organism, they are secreted in different ways [12]. HFBI is expressed as a mycelium-bound HFB using glucose as a carbon source whereas HFBII is secreted in the culture medium during production using lactose or complex polysaccharides (e.g. cellulose) [17]. Being bound to the fungal cell wall, purification of HFBI requires a solvent extraction before further purification of the protein. A number of different extraction protocols for the isolation of HFBI from the fungal mycelium are found in literature. Askolin et al. (2001) reported the extraction of HFBI using 1% SDS. Extraction at different pH levels was reported whereby a solvent pH of pH 9.0 was selected as the optimal value, although pH values above pH 7.0 appeared to cause degradation of the HFB through N-terminal asparagine deamidation. Riveros et al. (2015) on the other hand reported the extraction of HFBI and the mycelium-bound HFB2-a2, produced by *T. harzianum,* using 60% ethanol, a solvent known to easily dissolve Class II HFB membranes and aggregates [4,12]. Both 60% ethanol and detergent solutions containing 1% SDS are suitable for HFBI extraction. However, the optimal solvent extraction method remains unclear due to the lack of comparable data and variances in the applied methods. An

optimal extraction with high selectivity for the target protein is required to minimize costs and maximize efficiency for subsequent downstream processing. Therefore, a detailed study of the extraction procedure for isolation of HFBI is reported. The high protein extraction capacity of 1% SDS solutions was already reported previously [18]. However, this paper reports a more in depth characterization of the extracts, providing new insights in the extraction capacity of the aforementioned solvents and their effect on the HFBs properties. These new insights and collected data allowed a better comparison of the previously reported extraction methods, resulting in an improved extraction procedure. The present contribution compares multiple solutions, containing 60 – 80% ethanol or 1% SDS for extraction of the mycelium-bound HFBI and extraction parameters such as time and solvent:biomass ratio are varied. The isolation of HFBI was investigated with regard to other proteins affiliated with the fungal biomass as well as other contaminants associated with the production of HFBI using the native strain *T. reesei* (e.g. sorbicillinoid pigments) while also the effect on HFBI functionality (gushing activity) was examined. The most selective extraction solvent was selected and extraction parameters were optimized to further enhance the HFBI recovery. Moreover, Reversed Phase Fast Protein Liquid Chromatography as well as Cold Induced Phase Separation (CIPS) at different pH levels were investigated in view of improving HFBI purity.

Materials and methods

Bioproduction of Class II hydrophobin HFBI

Production of HFBI was performed using a native strain of *Trichoderma reesei.* The strain MUCL 44908 (BCCM/MUCL Agro-Food & Environmental Fungal Collection, Belgium) was purchased and maintained on potato dextrose agar (Merck, Germany) at 25 °C. Inoculum and cultivation medium was prepared as previously described [18]. The strain was cultivated in fed-batch mode using a 5 l bioreactor (BIOFLO 3000, New Brunswick). Bioproduction was started at a working volume of 2.5 l and after 30 hours of cultivation, the feed containing 40% glucose was initiated. The feed was added at a rate of 0.25 $ml.min^{-1}$ for 90 hours. During cultivation, temperature and pH were automatically controlled. Temperature was set at 30 °C and pH was kept at 4.75 using 2.6 M NH_4OH (Brenntag, Belgium) and 0.5 M H_2SO_4 (Acros Organics, Belgium). Agitation was set at 400 rpm and aeration was kept constant at 2.5 $l.min^{-1}$. The cell concentration during production was determined as previously described [18]. The bioproduction was stopped after 120 hours.

Extraction of HFBI and mycelium-bound proteins

The mycelium was separated from the culture medium by filtration (Whatman 114V filters, GE Healthcare, Belgium) and washed twice with 0.85% NaCl (Merck, Germany). In total 560 g biomass (wet weight) was collected. Water content of the leaked-out mycelium was determined at 83% by drying a small sample of the biomass at 105 °C for 24 hours and determining the weight loss of the sample. Extraction of the biomass was carried out with either 60% or 80% ethanol (>99% purity, Chem-Lab, Belgium), both at pH 6.8, 1% SDS in 100 mM Tris(hydroxymethyl) aminomethane/HCl buffer (pH 9.0) or 1% SDS in 100 mM sodium phosphate buffer (pH 6.8) (Sigma-Aldrich, USA). 5 grams of leaked-out mycelium was submerged in either 10 or 20 ml solvent and extracted in a temperature-controlled laboratory shaker (125 rpm) at 30 °C (Certomat BS-1, B. Braun Biotech International, Germany). Dependent on the specified experimental conditions, the extraction was carried out for 2, 4 or 2x 2 hours with renewal of the extraction solvent. After extraction the biomass was separated from the extract by centrifugation (4000 rpm for 10 min; mf 108^{R}, Awel centrifugation, France). For extracts containing 1% SDS, SDS was removed by addition of 2M KCl (Merck, Germany) in a 0.1:1 ratio (KCl:extract). Potassium dodecyl sulphate was precipitated overnight at 4 °C and removed by centrifugation (3000 rpm for 10 min; mf 108^{R}, Awel centrifugation, France).

Bicinchoninic acid protein assay kit (BCA assay kit)

Total protein content of all extracts was determined using the bicinchoninic acid protein assay kit (BCA assay kit, Sigma-Aldrich, USA) using the instructions provided with the kit. All samples were mixed with the reagent and incubated for 2 hours at room temperature. After incubation, absorbance was measured at 562 nm and protein concentration was calculated using a standard curve of Bovine Serum Albumin (Merck, Germany). The total amount of proteins in a sample was calculated and expressed as mg proteins per g of biomass used for the extraction (dry weight).

Purification and characterization of HFBI

Purification of HFBI, Reversed Phase Fast Protein Liquid Chromatography (RP-FPLC)

Purification of the extract with RP-FPLC was carried out using a XK 26 column (Amersham Pharmacia, Sweden) packed with Amberchrom CG300M resin (DOW chemical, France) on an ÄKTA FPLC (Amersham Pharmacia, Sweden) chromatographic system, equipped with a Frac-900 fraction collector (Amersham Pharmacia, Sweden). Samples of 500 ml were loaded on the column. Elution was carried out at a flow rate of 10 ml.min^{-1} using a linear 35 to 55% water-acetonitrile (ACN) (Sigma-Aldrich, USA) gradient containing 0.1% TFA (99.8%, Acros Organics, Belgium). Protein elution

was monitored with UV detection at 214 nm. Fractions of 10.0 ml, collected between 37 and 50% ACN, were analyzed with Reversed Phase High Performance Liquid Chromatography (RP-HPLC) and HFBI containing fractions were pooled. The concentration of HFBI was determined using RP-HPLC.

Cold Induced Phase Separation (CIPS)

A phase separation can be induced in acetonitrile-water mixtures by cooling the samples to -20 °C, yielding both an acetonitrile-rich and water-rich phase. This separation method was used to remove the majority of acetonitrile from the purified HFBI. Furthermore, remaining sorbicillinoid pigments showed a great affinity for the acetonitrile-rich phase. Pooled fractions containing HFBI were stored overnight at -20 °C. After phase separation occurred, the upper ACN-rich phase containing most of the pigment (sorbicillinoids) was removed and HFBI concentration in the water-rich phase was determined using RP-HPLC.

To investigate the effect of pH on CIPS, samples were prepared by dissolving lyophilized HFBI (still containing the pigment) in 100 mM HCl/KCl buffer (pH 2.0), 150 mM citrate/phosphate buffer (pH 5.0), 100 mM phosphate buffer (pH 7.0) or 100 mM Tris(hydroxymethyl) aminomethane/HCL buffer (pH 9.0) (Sigma-Aldrich, USA). ACN was added in a 1:1 ratio to obtain a 50% ACN-water mixture. Samples were stored overnight at -20 °C. After phase separation occurred, the volume, HFBI concentration and pigment intensity of both phases was measured. Separation efficiency ($V_{\text{ACN-rich phase}}/V_{\text{water-rich phase}}$) was calculated as well as the partitioning coefficients ($C_{\text{ACN-rich phase}}/C_{\text{water-rich phase}}$) for HFBI and pigments. Finally HFBI enrichment ($C_{\text{water-rich phase}}/C_{\textit{ACN-water mixture}}$) was determined for all samples.

Quantification of HFBI, Reversed Phase High Performance Liquid Chromatography (RP-HPLC)

HFBI concentration was determined before and after purification using RP-HPLC (Alliance 2695 separation module, Waters, USA) equipped with a Hichrom Vydac C4 column (250x4.6 mm) using a 20 to 70% water-ACN gradient (Sigma-Aldrich, USA) containing 0.1% TFA (99.8%, Acros Organics, Belgium) at a flow rate of 1 $ml.min^{-1}$. Column temperature was set at 30 °C and UV detection was performed at 214 nm (2487 dual λ absorbance detector, Waters, USA). A calibration curve of HFBI was made using a sample of purified HFBI, kindly provided by VTT Technical Research Centre of Finland. The HFBI sample was used for quantification purposes only.

Pigment intensity

Pigment intensity was determined spectrophotometrically both after extraction and purification of HFBI. Each sample was diluted tenfold and 200 µl was added to the wells of a 96 well suspension culture plate in threefold (Cellstar, Greiner Bio-One, Germany). Absorbance was measured at 370

nm using a Spectramax 340pc384 spectrophotometer (molecular devices, USA) and was used as a measure for the pigment intensity.

Modified Carlsberg gushing test

Gushing activity of HFBI was determined using the protocol outlined by Riveros et al. (2015) with slight modifications. Samples were diluted to 85 $\mu g.ml^{-1}$ HFBI and 1 ml diluted sample was added to 1 l of sparkling water. Bottles were placed horizontal on a laboratory shaker (150 rpm, room temperature) and shaken for 48 hours. After 48 hours, the bottles were opened following the procedure stated by Riveros et al. (2015) and the amount of expulsed liquid was used to calculate the gushing activity as a percentage of the initial volume.

Statistical analysis

Experiments were carried out in triplicate unless stated differently. One-way analysis of variance (ANOVA) and statistical differences ($p < 0.05$) between samples were analyzed using IBM SPSS 25 statistical software.

Results

Effect of extraction solvent and pH: Detergent vs. Organic solvent

After bioproduction, the fungal biomass was separated and proteins were extracted in accordance with the conditions stated in the materials and methods section. Multiple extraction conditions were applied to investigate their effect on the extraction efficiency and HFBI selectivity. First, the type of extraction solvent was varied. The leaked-out biomass (5 grams) was submerged in 10 ml solvent (solvent:biomass ratio 2:1) and extraction took place for 2 hours at 30 °C. The total protein and HFBI content in the extracts was determined, the results are shown in Figure 1A. Subsequently, extraction parameters such as extraction time and solvent:biomass ratio were varied.

As can be seen in Figure 1A, the total amount of proteins extracted from 1 gram of dry biomass ranges from 38.7 ± 2.0 mg at condition A and 38.0 ± 2.6 mg at condition B to 71.7 ± 1.8 mg at condition C and 67.8 ± 3.4 mg at condition D, indicating a significant increase in total extracted proteins when using 1% SDS (conditions C and D) compared to 60 or 80% ethanol (conditions A and B), following our previously reported results [18]. However, when looking specifically at hydrophobins, the amount of HFBI extracted from 1 gram of dry biomass is 9.4 ± 0.4 mg, 9.0 ± 0.4 mg, 6.9 ± 0.6 mg and 6.2 ± 0.2 mg for conditions A, B, C and D, respectively, showing a significantly higher amount of extracted HFBI with ethanol as the extraction solvent. Figure 1A also shows the amount of HFBI

expressed as a fraction of the total amount of extracted proteins, resulting in 24.4 ± 0.5% and 23.6 ± 1.0% for conditions A and B, respectively and 9.6 ± 0.7% and 9.2 ± 0.5% for conditions C and D, respectively. When using either 60 or 80% ethanol as extraction solvent, the amount of HFBI in the extract is significantly higher when compared to the extracts using 1% SDS solutions, while extracting a lower amount of non-HFB proteins, thus indicating a higher selectivity for HFBI when applying ethanol as extraction solvent.

No significant difference in the total amount of extracted proteins or HFBI could be noticed between conditions A and B, nor between conditions C and D. The results in Figure 1A indicate that the amount of ethanol, nor the pH of the solution (in the used ranges) affects the protein or HFBI extraction.

In addition to the amount of total proteins and HFBI extracted from the biomass, the pigment intensity of the obtained extracts was measured. The production of HFBI using a native strain of *T. reesei* is accompanied by the production of sorbicillinoids, giving a typical yellow color to the culture broth and fungal biomass [20]. Consequently, this yellow pigment is also found in the protein extracts and acts as an additional contaminant. Therefore, the relative amount of pigment in the extracts was measured and is given in Table 1. The surface activity of HFBI was analyzed by means of the modified Carlsberg gushing test, of which the results also are given in Table 1.

The extract obtained using 80% ethanol contained significantly more sorbicillinoids compared to the extracts obtained with 1% SDS solutions. Also, although not significant, an increased pigment intensity was noticed in the extract containing 80% ethanol compared to 60% ethanol.

An overall gushing activity between 39 and 52% was obtained for all samples, showing the great surface activity of HFBI. No significant difference in gushing activity was obtained for HFBI in extracts A, C and D. However, a decreased gushing activity was noted for HFBI in extract B compared to extract A.

Effect of extraction time

As previous results indicated, 60% ethanol increased the amount of HFBI in the extract by 36% and showed improved selectivity compared to 1% SDS. 60% ethanol was therefore chosen as extraction solvent. Besides the effect of the extraction solvent, it was investigated whether altering other extraction conditions such as extraction time could further increase HFBI recovery and purity. The biomass was extracted in a solvent:biomass ratio of 2:1 for either 2 hours (A), two subsequent periods of 2 hours with renewal of the extraction solvent after 2 hours (AA') or one uninterrupted

period of 4 hours, without renewing the solvent (E). The results of the BCA protein analysis and HFBI quantification are given in Figure 1B and the relative pigment intensity of the extracts is presented in Table 2.

As previously described, extraction of the biomass for 2 hours using 60% ethanol (Figure 1B, (A)) yielded 38.7 ± 2.0 mg proteins, 9.4 ± 0.4 mg of which was HFBI. Increasing the extraction time from 2 to 4 hours (Figure 1B, (E)) augmented the total amount of extracted proteins to 44.7 ± 4.4 mg and the amount of HFBI extracted from 1 g dry biomass to 10.4 ± 1.0 mg while also slightly increasing the relative pigment intensity (Table 2, (A) and (E)). However, the observed increases were not significant. On the other hand, a 4 hour extraction with exchange of the solvent after the first 2 hours (Figure 1B, (AA')) increased the amount of extracted proteins to 52.5 ± 2.4 mg, 11.9 ± 0.4 mg of which was HFBI. The increase was not significant compared to extraction E but it was significant compared to extraction A, thus indicating that a two-fold extraction increased the total amount of HFBI in the final extract. Also, a clear but not significant raise in relative pigment intensity was seen for extraction AA' when compared to extractions A and E. The percentage of HFBI in the total amount of extracted proteins was 24.4 ± 0.5%, 22.8 ± 0.5% and 23.2 ± 0.5% for extracts A, AA' and E, respectively and did not differ significantly. This indicated that the high selectivity of 60% ethanol solutions for HFBI remained constant and was independent of the extraction time.

Effect of solvent:biomass ratio

A third parameter, the solvent:biomass ratio, was varied as previously described in the materials and methods section and the effect on the extraction of HFBI was investigated. Figure 1C shows the effect on protein and HFBI extraction for a 2 hour and two sequential 2 hour extractions with renewal of the extraction solvent (60% ethanol) using a solvent:biomass ratio of 2:1 (Figure 1C, (F) and (FF'), respectively) compared to a 2 hour and two sequential 2 hour extractions with renewal of the extraction solvent using a solvent:biomass ratio of 4:1 (Figure 1C, (G) and (GG'), respectively). The relative pigment intensity of the extracts is also given in Table 2.

Extraction conditions F and FF' correspond to the extraction conditions A and AA'. A different notation is however used due to the use of biomass from a second fed-batch bioproduction. The solvent:biomass ratio 4:1 was not investigated for a 4 hour extraction without solvent renewal (corresponding to extraction E) as previous results indicated no significant improvements.

A solvent:biomass ratio of 2:1 yielded 10.7 ± 0.5 mg HFBI in the first extraction step (Figure 1C, (F)), and increased to 13.3 ± 0.3 mg HFBI after the second extraction step (Figure 1C, (FF')). The amount

of total extracted proteins increased from 41.4 ± 1.0 mg to 56.9 ± 0.9 mg after the second extraction step. Both the increase of HFBI (+24.2%) and total extracted proteins (+37.4%) corresponded to the increases seen for extractions A and AA' (+26.5 and +35.6%, respectively), indicating that the use of biomass from a different fed-batch production did not alter the results. Increasing the solvent:biomass ratio to 4:1 (Figure 1C, (G)) resulted in an extraction of 12.9 ± 0.9 mg HFBI after the first extraction step which further increased to 14.8 ± 1.0 mg after the second step (Figure 1C, (GG')). The amount of total extracted proteins increased from 54.7 ± 1.2 mg to 69.2 ± 1.5 mg after the second extraction step. The percentage of HFBI in the total amount of extracted proteins was calculated on 25.9 ± 1.0%, 23.4 ± 0.9%, 23.5 ± 1.2% and 21.3 ± 1.0% for conditions F, FF', G and GG', respectively. The only significant difference was found between conditions F and GG'. Comparing conditions FF' and GG', the total amount of HFBI extracted, nor the percentage of HFBI in the total amount of extracted proteins differed significantly, indicating an equally selective extraction. When comparing the relative pigment intensity of the extracts however, a significantly greater intensity was observed for extracts with a solvent:biomass ratio of 4:1 (as seen in Table 2) indicating a larger amount of secondary contaminants in the extracts (G and GG'). Also, when comparing extractions F and FF' a significant increase in relative pigment intensity was observed, which was less pronounced for extractions A and AA'. Thus, including a solvent renewal during the extraction tends to also augment the extraction of secondary contaminants (sorbicillinoids).

Purification of the HFBI extract and effect of pH on CIPS

As described in the materials and methods section, the extract was further purified using RP-FPLC, successfully separating HFBI from other proteins present in the extract. However, the sorbicillinoids in the extract partially bound to the RP-resin and co-eluted with HFBI (40 to 50% ACN), contaminating the HFBI fraction. Following RP-FPLC, Cold Induced Phase Separation (CIPS) was applied to remove the majority of ACN from the purified HFBI solution. During phase separation, the majority of the pigment migrated to the ACN-rich phase. However, no complete removal from the water-rich phase could be obtained. Therefore, CIPS was further investigated in an attempt to increase the pigment removal. CIPS was applied on four HFBI-ACN-buffer mixtures with pH values of 2.0, 5.0, 7.0 and 9.0 as described in section 2.4.2. The pH of the HFBI fraction after RP-FPLC purification was pH 1.9, due to the presence of 0.1% TFA in the eluent. The separation efficiency, HFBI-enrichment as well as partitioning coefficients of HFBI and pigment are given in Table 3. Altering the pH of the system had no significant effect on the separation efficiency or HFBI enrichment. However, the pigment partitioning coefficient increased significantly with decreasing pH, from 0.34 ± 0.03 at pH 9.0 to 3.66 ± 0.29 at pH 2.0. Remarkably, at a pH value of 9.0 the pigment

almost completely remained in the water-rich phase, resulting in a partitioning coefficient lower than 1. No HFBI could be detected in the ACN-rich phase, which indicates that this step has little influence on the total HFBI recovery. As a result of the removal of the ACN-rich phase, the HFBI concentration in the water-rich phase increased 1.43- to 1.46-fold. CIPS was included in the downstream process for purification of HFBI because of the high HFBI recovery, removal of the pigment and HFBI enrichment of the water-rich phase.

The optimized process was applied to the remaining biomass (472.0 g, 80.24 g dry weight), extracting a total of 771.9 mg HFBI. An overview of the optimal downstream process for HFBI is given in Figure 2. The optimal conditions for each step are presented as well as HFBI yield, HFBI recovery and HFBI enrichment. Subsequent purification using RP-FPLC yielded 662.7 mg HFBI, or a process recovery of 85.8%, increasing the HFBI concentration 2.8-fold. CIPS was then applied on the pooled fractions eluted between 40 and 50% ACN (pH 1.9, -20 °C), removing the majority of ACN and remaining pigments from the purified HFBI solution. 99% of HFBI remained in the water-rich phase, giving a final yield of 659.8 mg HFBI. ACN removal further increased the HFBI concentration to 3.2 times the concentration found in the extract.

Discussion

Based on the results of the BCA and RP-HPLC analysis of the obtained extracts, ethanol clearly displays a significantly greater selectivity for the extraction of HFBI when compared to the anionic surfactant SDS. The enhanced selectivity can be explained both by the high solubility of Class II hydrophobin membranes and aggregates in ethanol phases [1,12] as well as the less selective nature of SDS. Ethanol reduces the polarity of the solution, making it easier to solubilize apolar hydrophobic proteins such as HFBI, whereas less hydrophobic proteins tend to precipitate by electrostatic aggregation in apolar solvents [21]. On the other hand, the surfactant SDS is widely used for the solubilisation and extraction of membrane-bound and membrane-embedded proteins and might therefore be less ideal for the extraction of a specific protein [22,23]. The data shown in Figure 1A indicate a significantly greater amount of proteins is extracted from the fungal biomass using SDS confirming the solubilization of most proteins affiliated with the membrane and therefore decreasing the selectivity for HFBI. On the other hand, extracts obtained through ethanol extraction contained a slightly higher amount of sorbicillinoids. These yellow colored components are associated with the growth of *T. reesei* and can be seen as secondary contaminants in the extract, aside from non-hydrophobin proteins [20,24]. Askolin et al. (2001) previously reported the great

difficulty to completely remove the pigments during HFBI purification. Limiting the amount of sorbicillinoids in the extract is thus important for subsequent downstream processing.

Apart from contaminants in the obtained extracts, the effect of the extraction solvent on the HFBI functionality was also investigated. More specifically, the gushing activity of HFBI was measured (see Table 1). The gushing activity of HFBI in the tested extracts was similar. A gushing activity of 52% indicates that 520 ml of the initial 1000 ml sparkling water was expulsed upon opening of the bottle, caused by only 85 µg of HFBI. This shows the great surface activity of these proteins.

Based on the selectivity for HFBI compared to other proteins present in the extract, 60% ethanol was selected as solvent for the extraction of HFBI from the fungal biomass in subsequent extraction experiments, varying the extraction time and solvent:biomass ratio. The obtained results showed that the total HFBI recovery could not be enhanced by increasing the extraction time alone. However, including a second extraction of the biomass after removal of the first extract did significantly increase the amount of HFBI recovered from the biomass. The increasing concentration of HFBI in the extract could limit the diffusion of HFBI from the biomass to the solvent which might be avoided by renewing the solvent during the extraction. Therefore, a two-step 2 hour extraction is suggested. Increasing the solvent:biomass ratio to 4:1 resulted in an equally effective extraction with regard to the total amount of HFBI and HFBI selectivity. On the other hand, the HFBI concentration in the extract was lower due to the greater extract volume, which implies a more time consuming subsequent downstream processing while the extracts also contained a greater number of secondary contaminants.

Although the HFBI specific selectivity was higher, the ethanol extracts also contained a greater number of secondary contaminants (sorbicillinoids). Purification of the extract by means of RP-FPLC removed the largest part of the pigment, but no complete removal could be obtained (data not shown). However, application of CIPS to remove the majority of acetonitrile from the purified HFBI solution also resulted in pigment migration to the ACN-rich phase. Incubation of an acetonitrile-water mixture at -16 or -20 °C results in a phase separation between acetonitrile and water as described by Shao et al. (2017). Starting from a 50% ACN-water mixture, the upper phase contained 71.7% acetonitrile on a molar basis and is therefore referred to as the ACN-rich phase, while the lower water-rich phase still contained about 13.6 % acetonitrile after phase separation [19]. Under the conditions after RP-FPLC purification (40-50% ACN, pH 1.9), the pigment migrated almost completely to the upper ACN-rich phase. The cause of this migration could be ascribed to the molecular structure of the pigment, comprising of a complex mixture of several sorbicillinoid

molecules having a highly oxygenated mono-, bi- or tricyclic framework [20,25]. The highly cyclic molecules remain water soluble but clearly show a higher affinity for organic phases, in this case for acetonitrile. In literature it was found that solvent-water systems using other organic solvents such as acetone, 2-propanol or methanol do not undergo the same phase separation [19]. Therefore, to improve the pigment removal using CIPS, the pH value of the current ACN-water mixture was altered from pH 2.0 to pH 9.0. Higher pH values were avoided as HFBI is less stable at alkaline pH values [16]. As seen in Table 3, the pH had little effect on the separation efficiency and no effect at all on HFBI partitioning. On the other hand, the pigment partitioning coefficient showed a greater pigment migration to the ACN-rich phase at acidic pH (pH 2.0) indicating that the obtained conditions after RP-HPLC can remain unaltered for a good pigment removal. At more alkaline pH (pH 9.0) almost no pigment migration to the ACN-rich phase was observed. Tris(hydroxymethyl) aminomethane, used to prepare the pH 9.0 buffer, could probably create a more suitable aqueous environment for the sorbicillinoids, stabilizing them through hydrogen bonding. Comparing the separation efficiency of the samples to values reported in literature, the obtained values were on the lower side. Shao et al. (2017) reported an approximate separation efficiency of 0.583 for CIPS of a 50% ACN-water mixture kept at -16 °C for 12h. A different method to induce phase separation in ACN-water mixtures is the addition of kosmotropic salts, easily inducing the phase separation at 4 °C [26]. High separation efficiencies up to 1.00 were reported by Gu and Shih (2004) using K_2HPO_4 and up to 0.54 using NaCl or KCl (salt mole fraction of 0.014). However, adding salts to the HFBI solution to improve the separation efficiency would require a final buffer exchange step to again remove the additives. Increasing the separation efficiency may thus increase the HFBI enrichment in the water-rich phase, on the other hand the additional buffer exchange could decrease final HFBI recovery and yield.

Comparing the proposed downstream process to the process reported in literature, important improvements were observed in this study. Askolin et al. (2001) extracted HFBI using 1% SDS at pH 9.0 followed by SDS precipitation with KCl. Chromatographic purification was done by means of Hydrophobic Interaction Chromatography. As reported here, 60% ethanol improves both the concentration of HFBI as well as the purity of HFBI in the extract compared to 1% SDS at pH 9.0. Furthermore, Askolin et al. (2001) reported a HFBI recovery of 50% for the Hydrophobic Interaction Chromatography purification, while RP-FPLC results in a HFBI recovery of 85.8%.

Conclusion

An adapted and optimal downstream process for extraction and purification of HFBI is proposed, including Cold Induced Phase Separation for removal of sorbicillinoids from the purified solution. From the obtained results it can be concluded that two sequential 2 hour extractions using 60% ethanol in a solvent:biomass ratio of 2:1 resulted in the highest recovery of the mycelium-bound hydrophobin HFBI. Subsequent purification using RP-FPLC effectively removed non target proteins while application of CIPS at pH 2.0 removed the majority of pigments, further increasing the purity of HFBI. Further research should be focused on the stability of HFBI in aqueous solutions.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflict-of-interest statement

The authors declare no commercial or financial conflict of interest

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Tables

Table 1 Pigment intensity of the extracts measured at 370 nm (n=3) and gushing activity (n=2) of HFBI extracted using different extraction solvents. All extractions were done in a solvent:biomass ratio of 2:1 for 2 hours. All data are presented as the mean ± SD. Means with different superscript lowercase letters in the same column indicate a significant difference ($p < 0.05$)

Extraction conditions	Pigment intensity (-)	Gushing activity (%)
60% Ethanol, pH 6.8 (A)	0.485 ± 0.07^{ab}	51.9 ± 0.2^{a}
80% Ethanol, pH 6.8 (B)	0.700 ± 0.09^{a}	38.7 ± 2.5^{b}
1% SDS, pH 9.0 (C)	0.367 ± 0.05^{bc}	46.0 ± 0.8^{ab}
1% SDS, pH 6.8 (D)	0.249 ± 0.01^{c}	46.6 ± 0.5^{ab}

Table 2 Relative pigment intensity of the extracts measured at 370 nm. All extractions were done using 60% ethanol in a solvent:biomass ratio of 2:1 unless stated differently. For comparison, the pigment intensity was normalized to account for the diluting effect of the different extract volumes (extracts AA',FF', G and GG'), resulting in the relative pigment intensity. All data are presented as the mean ± SD (n=3). Means with different superscript lowercase letters in the same column indicate a significant difference ($p < 0.05$)

Extraction conditions	Relative pigment intensity (-)
2 h (A)	0.254 ± 0.04^{a}
2x 2 h (AA')	0.370 ± 0.05^{a}
4 h (E)	0.282 ± 0.04^{a}
2 h (F)	0.321 ± 0.01^{a}
2x 2 h (FF')	0.455 ± 0.01^{b}
2 h, S:B ratio 4:1 (G)	0.537 ± 0.02^{c}
2x 2 h, S:B ratio 4:1 (GG')	0.633 ± 0.01^{d}

Table 3 Effect of solution pH on the separation efficiency, partitioning coefficients of pigments and HFBI and HFBI enrichment during Cold Induced Phase Separation. All data are presented as the mean ± SD (n=3). Means with different superscript lowercase letters in the same column indicate significant differences ($p < 0.05$)

	separation efficiency	partitioning coefficient		HFBI enrichment
	($V_{ACN\text{-}rich}/V_{water\text{-}rich}$)	pigments	HFBI	
pH 2.0	0.313 ± 0.004[a]	3.66 ± 0.29[a]	0.00 ± 0.00*	1.46 ± 0.06[a]
pH 5.0	0.349 ± 0.024[a]	1.97 ± 0.36[b]	0.00 ± 0.00*	1.46 ± 0.09[a]
pH 7.0	0.278 ± 0.045[a]	2.09 ± 0.28[b]	0.00 ± 0.00*	1.46 ± 0.13[a]
pH 9.0	0.291 ± 0.061[a]	0.34 ± 0.03[c]	0.00 ± 0.00*	1.43 ± 0.16[a]
RP-FPLC fraction (pH 1.9)	*0.167*	*6.86*	*0.05*	*1.22*

*No HFBI was detected in the acetonitrile-rich phase

Figure legends

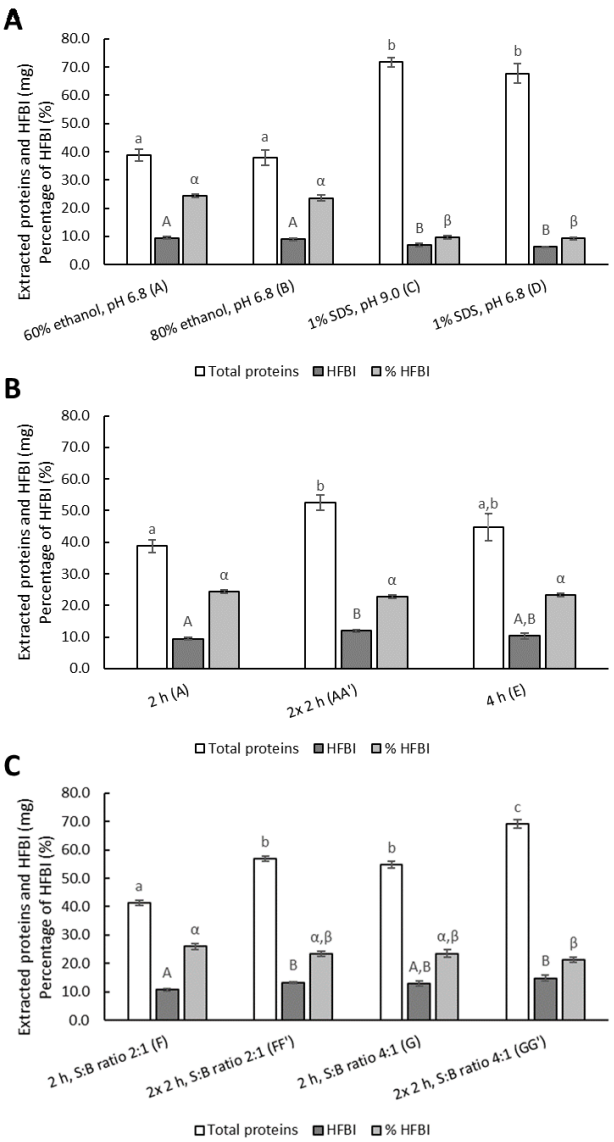

Figure 1 Total proteins (white bars) and HFBI (dark grey bars) extracted from 1 gram of dry fungal biomass using different extraction conditions and solvents (mean ± SD, n=3). (A) Extractions were carried out for 2 hours with a solvent:biomass ratio of 2:1. (B) Extractions were carried out using 60% ethanol, a solvent:biomass ratio of 2:1 was applied for all samples. (C) Extractions were carried out using 60% ethanol, S:B ratio represents solvent:biomass ratio. Light grey bars indicate the percentage of HFBI in the total extracted proteins. Lower case letters are used to statistically compare total proteins, capital letters compare HFBI content and Greek letters compare the percentage of HFBI. Means with different lower case, capital or Greek letters indicate a significant difference ($p < 0.05$)

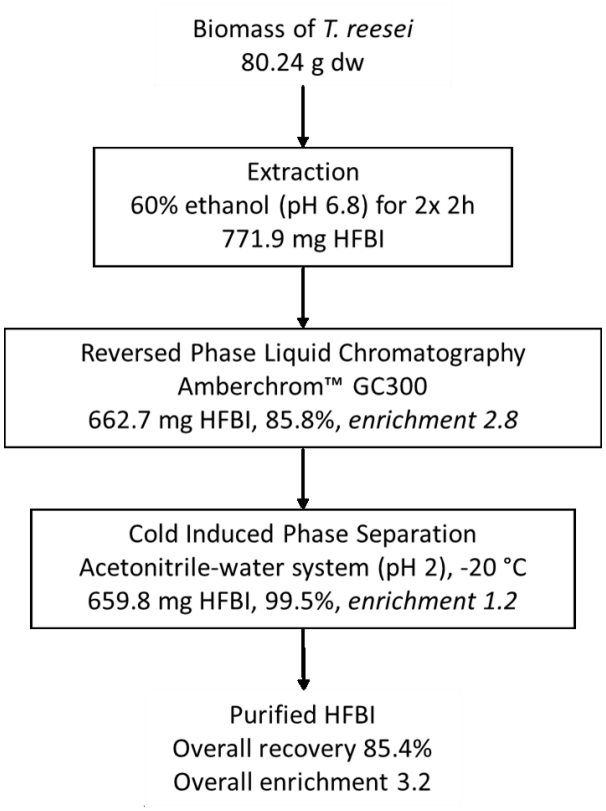

Figure 2 Overview of the improved downstream process for extraction and purification of HFBI. Optimal conditions, HFBI yield (mg), HFBI recovery (%) and HFBI enrichment are given at each step

In this work, the extraction of the mycelium bound hydrophobin HFBI was performed using solvents containing Ethanol or Sodium Dodecyl Sulphate (SDS) in different concentrations and at different pH. HFBI extracted with 60% ethanol was purified using Reversed Phase Fast Protein Liquid Chromatography followed by Cold Induced Phase Separation at pH 2. The improved method results in a straightforward downstream process with a high HFBI recovery, necessary for the development of new applications.