Extraction and spray drying of Class II hydrophobin HFBI produced by *Trichoderma reesei*

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Abstract

Hydrophobins are considered the strongest surface-active proteins from microbial origin. The amphiphilic proteins are produced by filamentous fungi and exhibit unique physicochemical properties. Self-assembly into membranes at hydrophobic-hydrophilic interfaces illustrate their unique properties, leading to a broad range of applications, from foam stabilization to protein immobilization. Due to insufficient production quantities of Class II hydrophobins using native fungal strains and limited long-term storage stability of liquid formulations, commercial availability remains lacking. Within this research, the potential of trehalose for stabilization and preservation of hydrophobins during spray drying was studied. Therefore, the Class II hydrophobin HFBI, expressed by *Trichoderma reesei*, was produced by submerged cultures in fed-batch mode and extracted from the mycelium. Extracts purified with FPLC were subsequently spray dried. Residual activity of 93.62 ± 5.07 % was obtained with trehalose in a ratio of 2:1 (excipient:protein) to stabilize HFBI during spray drying, which demonstrates the huge potential of trehalose.

Keywords

Hydrophobin, *Trichoderma reesei*, Spray drying, Gushing activity

1. Introduction

Hydrophobins, produced by filamentous fungi, are relatively small (7-10 kDa) surface-active proteins which self-assemble at hydrophobic-hydrophilic interfaces [1]. The cysteine-rich proteins have a prominent role in the growth and reproduction of the fungi. Hydrophobins are secreted in the environment allowing growth of aerial hyphae by reducing the water surface tension and formation of hydrophobin coatings on hyphae and spores facilitates spore dispersal [2,3].

Based upon membrane solubility, membrane morphology and amino acid sequence similarities, hydrophobins are divided into two Classes, namely Class I and Class II hydrophobins [4–6]. Class I hydrophobins self-assemble into strong multilayer membranes and can only be resolubilized using strong acids (e.g. trifluoroacetic acid (TFA)). Class II hydrophobins however self-assemble into highly elastic monolayer membranes and are easily dissolved in 60 % ethanol or 2 % sodium dodecyl sulphate (SDS) [7–9]. The Class II hydrophobins HFB I and HFB II are both produced by *Trichoderma reesei* [3,10–15]. HFB I is expressed in media containing monosaccharides (e.g. glucose containing media) whereas HFB II is expressed in media containing lactose or complex polysaccharides (e.g. cellulose) [3]. Both hydrophobins show a high membrane elasticity ($0.5 - 0.7 \text{ Nm}^{-1}$) and are therefore very promising bio-active molecules for a wide range of applications [13,16].

Hydrophobins can be applied as stabilizers for foams and emulsions in both food and cosmetics industry [17–19], as well as encapsulating agents of flavour-active components, increasing their retention and flavour stability [1,20]. Moreover, their ability to coat surfaces with amphipathic membranes and thereby change the polarity of surfaces makes them also valuable in the field of biomaterials, medical equipment and biosensors [2,7]. However, commercial applications remain lacking due to insufficient production quantities while aqueous preparations are prone to loss of functionality and microbial degradation [8]. Techniques such as drying may however result in loss of affinity for apolar molecules [8].

In this research, the remarkable potential of trehalose for preserving hydrophobin activity during spray drying is addressed. In literature, it has been reported that excipients can enhance the stability of proteins during spray drying [21,22]. Carbohydrates (e.g. trehalose) and surfactants (e.g. polysorbate 80) can act as protein stabilizers, based on water replacement theory and preferential exclusion, respectively [23,24]. During the drying process, water molecules are removed from the protein surface disrupting the stabilizing effect of hydrogen bonds, causing protein denaturation or aggregation. Carbohydrates can interact with the proteins during the drying process, substituting water molecules as they are removed by dehydration. The formation of hydrogen bonds between the carbohydrates and the protein can result in restabilization of the protein structure [21,23]. Surfactants on the other hand occupy the air-water interface of the atomized droplets, preventing the proteins from unfolding at this interface [22,23].

In this study, the mycelium-bound hydrophobin HFBI was investigated. Prior to drying, HFBI was removed from the biomass by extraction with Tris(hydroxymethyl)aminomethane/HCl buffer (Tris/HCl buffer) containing 1 % SDS or ethanol. Different extraction techniques are reported in literature to remove HFBI from the fungal biomass. Askolin et al. (2001) reported the use of 100 mM Tris/HCl buffer of pH 9.0, containing 1 % SDS for extracting HFBI to be most effective compared to other detergents (Triton X-100, Tween 20) [25]. Other studies report the use of 60 % ethanol for extraction of HFBI [11]. Moreover, the presence of trehalose as excipient for preserving the hydrophobin activity during spray drying of HFBI was examined.

2. Material and methods

2.1. Bioproduction of Class II hydrophobin HFBI

For HFBI production, *Trichoderma reesei* MUCL 44908 (BCCM/MUCL Agro-Food & Environmental Fungal Collection, Belgium) was purchased and maintained on potato dextrose agar (PDA) (Merck, Germany) petri plates at 25 °C. The cultivation medium used contained:

40.0 g/L glucose (Tereos Syral, Belgium), 4.0 g/L peptone (Organo Technie, France), 1.0 g/L yeast extract (Organo Technie, France), 4.0 g/L KH_2PO_4 (Caldic, Belgium), 2.8 g/L $(\text{NH}_4)_2\text{SO}_4$ (Caldic, Belgium), 0.6 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Caldic, Belgium), 0.6 g/L CaCl_2 (Sigma-Aldrich, Belgium), 4.0 mg/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (Alfa Aesar, Germany), 3.2 mg/L $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (BDH Laboratory Supplies, England), 6.9 mg/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (Merck, Germany) and 10 mg/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (Merck, Germany). To adjust the pH to 4.75, 2 M HCl (Chem-Lab, Belgium) was used.

For inoculum preparation, 18 mL of sterile 8.5 % NaCl (VWR, Belgium) was transferred to a PDA plate, overgrown by *T. reesei*. The mycelium was scraped using an inoculum loop and 1 mL was pipetted into a tube containing 9 mL sterile 8.5 % NaCl. Subsequently, 1 mL of this spore suspension was pipetted to a culture tube containing 9 mL cultivation medium. This culture tube was incubated in a temperature-controlled orbital shaker (Certomat BS-1, B. Braun Biotech International, Germany) at 100 rpm and 30 °C for 15 hours. Then, the content of the culture tube was transferred to a culture flask containing 90 mL cultivation medium. This flask was aerated and incubated at 30 °C on a magnetic stirrer (250 rpm) (Mini MR Standard, IKA Werke GmbH, Germany) for 24 hours. After this incubation period, the inoculum was ready to be used for the bioproduction in a bioreactor of 5 L with a working volume of 1.5 L (UniVessel, Sartorius, Germany). The bioreactor containing 1.4 L cultivation medium was inoculated at 6.7 %. The bioreactor was automatically controlled for pH and temperature (Biostat B, Sartorius, Germany). The temperature during bioproductions was set at 30 °C and the pH at 4.75 (controlled by addition of 0.5 M HCl and 1 M NaOH (VWR, Belgium)). The agitation rate was set at 500 rpm and the aeration rate was kept constant at 1 vvm during the bioproduction. At several time points during the bioproduction, part of the broth was removed while new cultivation medium was added. More specifically, 4.0 L of the broth was removed in total while 5.0 L new cultivation medium was added in 6 intermediate steps. Furthermore, samples of the broth were taken at regular time intervals and the glucose and cell concentration in addition to the cell dry weight (CDW) were monitored during the bioproduction. The glucose concentration was determined using HPLC (UltiMate 3000, Thermo Fisher Scientific, Belgium)

with a 'Shodex sugar SH1011' column at a temperature of 60 °C and an RI detector (Refractomax 520, ERC, Germany) (mobile phase: 0.005 M H₂SO₄ with a flow rate of 0.60 mL/min). The cell concentration was determined using the plate count technique. Counting was performed on Plate Count Agar (Biokar Diagnostics, France), Sabouraud Dextrose Agar (Merck, Germany) and Oxytetracycline Glucose Yeast Extract agar. The CDW was determined by centrifuging (Awel centrifugation mf 108^R, France) the sample (4,000 rpm for 12 minutes) and washing twice the resulting pellet with 8.5 % NaCl. Between each washing step, the supernatant was removed by centrifuging at the same parameters as mentioned before. Finally, the pellet was dried in a drying oven (Heraeus, Germany) at 105 °C for 24 hours. The bioproduction was stopped after 140 hours.

2.2. Extraction of mycelium-bound proteins

After separation of the mycelium from the broth and washing of the mycelium as described before, the hydrophobins were extracted from 5.0 grams of wet mycelium with either 1 % SDS in 100 mM Tris/HCl buffer (pH 9) (Sigma-Aldrich, USA) or 60 % ethanol (>99 % purity, Chem-Lab, Belgium). Solvent to biomass ratios of 2:1, 3:1 and 4:1 (volume:weight) were applied. Extraction was carried out at 30 °C for 2 hours under continuous mixing at 100 rpm in a temperature-controlled shaker (Certomat BS-1, B. Braun Biotech International, Germany). The biomass was then separated from the extract by centrifugation (3,500 rpm for 15 min; Awel centrifugation mf 108^R, France). For extracts where 1 % SDS in 100 mM Tris/HCl buffer was used, SDS was first precipitated by addition of 2 M KCl (Merck, Germany) to the extract in a 0.4:1 ratio (KCl:extract). Water-insoluble potassium dodecyl sulphate was then removed by centrifugation (3,000 rpm for 10 min; Awel centrifugation mf 108^R, France). Total protein content of all extracts was determined using the bicinchoninic acid protein assay (BCA assay, Sigma-Aldrich, USA) and was expressed as the amount of proteins (mg) per g of biomass (dry weight).

For the drying experiments, a total amount of 176.8 g of wet mycelium with a CDW of 13.4 g was extracted using 1 % SDS in 100 mM Tris/HCl buffer with a solvent to biomass ratio of 4:1, resulting in a total extract volume of 803.0 mL.

2.3. Purification and characterisation of HFBI

2.3.1. Purification of HFBI with Reversed Phase Fast Protein Liquid Chromatography (RP-FPLC)

Purification of the extracts with RP-FPLC was carried out using a XK 26/40 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). The purification experiment was conducted five times, whereby for each experiment 100 mL of sample or 140 mg of total proteins were loaded on the column. Elution was carried out at a flow rate of 2 mL/min using a linear gradient of acetonitrile (ACN) (Sigma-Aldrich, USA) containing 0.1 % TFA (99.8 %, Acros Organics, Belgium) in demineralized water containing 0.1 % TFA (from 0 to 35 % ACN in 15 min and from 35 to 65 % ACN in 150 min). Protein elution was monitored with UV detection at 214 nm.

2.3.2. Modified Carlsberg gushing test

Fractions between 40 and 60 % ACN were screened by the modified Carlsberg gushing test to identify HFBI containing fractions. The gushing test was carried out based on the protocol outlined by Riveros et al. (2015) using carbonated water [11]. A 1 mL sample of each fraction was added to the bottles, after first removing the same volume of water. Shaking of the bottles was performed at 150 rpm during two days in a temperature-controlled shaker (Certomat BS-1, B. Braun Biotech International, Germany) at 25 °C.

2.3.3. Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Purity of gushing active fractions was determined using SDS-PAGE. Samples were prepared by adding 100 μ L Tricine sample buffer (Bio-Rad laboratories, Belgium), containing 5 % β -mercaptoethanol, to 100 μ L of gushing active fraction. Natural polypeptide SDS-PAGE standard (Bio-Rad laboratories, Belgium) was prepared in a 1:20 standard:sample buffer ratio. Samples and standards were loaded on a 16.5 % Criterion Tris-Tricine gel (Bio-Rad laboratories, Belgium). Electrophoresis was performed for 110 min with constant voltage of 125 V. Afterwards, coomassie blue staining (Coomassie Brilliant Blue G-250, Bio-Rad laboratories, Belgium) was used to visualize the separated proteins.

2.4. Spray drying process

Gushing active fractions (41.0 to 48.0 % ACN), purified using RP-FPLC, were mixed and 60 mL sample (protein content of 197 mg/L) was dried using a nano spray dryer (B-90, Buchi, Switzerland). The spray drying process was performed at a temperature of 70 °C. Air flow rate was set at 100 L/min with an internal pressure of 25 mbar. The feed was recirculated at 20 mL/min and a 5.5 μ m diameter spray cap was used for atomization. The drying process was stopped after 5.5 hours and the powder was collected. Moreover, the influence of an excipient (trehalose dihydrate, Carl Roth, Germany) on the drying process was evaluated. Therefore, trehalose dihydrate was added to the HFBI sample in a 2:1 excipient:protein ratio. The dried protein was resolubilized in demineralized water and the protein content was determined using BCA assay. Residual activity of spray dried HFBI was assayed with the modified Carlsberg gushing test.

3. Results and discussion

3.1. Bioproduction of HFBI

The bioproduction was effectuated using a submerged culture of *T. reesei* with glucose as carbon source (expression of the mycelium-bound HFBI [3]) for 140 hours. During the bioproduction, 4.0 L of the broth was removed in total while 5.0 L new cultivation medium was added in 6 intermediate steps. A cell concentration of 6 log (CFU/mL) was reached at the end of the bioproduction. Moreover, a total of 39 g dry biomass and 4.0 g mycelium-bound proteins were produced, corresponding to a yield of 0.10 g mycelium-bound proteins per g dry biomass (mycelium bound proteins extracted using 1 % SDS in 100 mM Tris/HCl buffer (pH 9) in a solvent to biomass ratio of 4:1).

3.2. Solvent screening for extraction of HFBI

As reported in literature, HFBI is a mycelium-bound hydrophobin and requires extraction prior to further purification [26]. The hydrophobin HFBI was extracted from the fungal biomass using two different extraction solvents, namely 1 % SDS in 100 mM Tris/HCl buffer (pH 9) and 60 % ethanol, and using several solvent to biomass ratios of 2:1, 3:1 and 4:1, respectively. The protein extraction capacity of the solvents was determined and expressed as the amount of proteins (mg) extracted per gram of dry biomass (Figure 1).

Extraction capacities of 72.8 ± 0.2 and 73.6 ± 1.2 mg/g dry biomass were obtained using 1 % SDS in 100 mM Tris/HCl buffer in the solvent:biomass ratios of 3:1 and 4:1, respectively. No significant difference could be noticed between these two ratios (95 % confidence interval). On the other hand, a significant decrease in extraction capacity was observed when 1 % SDS in 100 mM Tris/HCl buffer in a solvent:biomass ratio of 2:1 was used, presumably due to the limited amount of surfactant available to interact with the proteins.

Also, clear differences in protein extraction capacity between 1 % SDS in 100 mM Tris/HCl buffer and 60 % ethanol can be observed. The extraction capacity of 1 % SDS in 100 mM

Tris/HCl buffer was approximately 3.4 times higher than the extraction capacity of 60 % ethanol for all tested ratios. The increased protein extraction capacity of SDS could be derived from the stronger amphiphilic properties of SDS compared to ethanol. The strong interaction between either nonionic surfactants (e.g. Polysorbate 20) or anionic surfactants (e.g. SDS) and hydrophobins has been described by Tucker et al. (2014). For nonionic surfactants, the interaction is based on the combination of hydrophobic interactions between the surfactants alkyl chain and the hydrophobic patch of the hydrophobin as well as the hydrophilic interactions between the polar head of the surfactants and hydrophilic regions of the hydrophobin. When anionic surfactants are used, the interaction is based on electrostatic interactions between hydrophilic regions of the hydrophobin and the negatively charged surfactant [27]. This makes SDS more likely to extract the proteins from the fungal biomass compared to ethanol. As a result of this screening, 1 % SDS in 100 mM Tris/HCl buffer at pH 9, in a solvent to biomass ratio of 4:1, was selected for further extractions.

3.3. Reversed Phase Fast Protein Liquid Chromatography (RP-FPLC) purification of HFBI extracts

Based on the optimal extraction conditions, the protein extract of the end batch of the bioproduction was further purified with RP-FPLC (Figure 2). Protein elution was carried out using a linear gradient of ACN in demineralized water, containing 0.1 % TFA. Five purification runs were carried out. Each purification experiment yielded 70 mL purified HFBI solution, obtained after pooling the HFBI containing fractions. Fractions containing HFBI were identified using the modified Carlsberg gushing test. Gushing activity, ranging from 65 to 430 g/L expelled liquid, was detected in fractions eluting between 615 and 685 mL. The purity of the HFBI containing fractions was then assessed by SDS-PAGE (Figure 3). A clear band at 7.5 kDa could be observed for fractions that previously had been determined to be gushing active, confirming the presence of HFBI exhibiting a molecular weight of 7.532 kDa [15]. No protein

bands occurred on SDS-PAGE gels with a molecular weight range larger than 10 kDa (results not shown).

3.4. Spray drying of purified HFBI fractions

Finally, HFBI fractions purified with RP-FPLC were spray dried on a Buchi B-90 Nano Spray Dryer, with and without the addition of trehalose as excipient. The effect of the excipient on the residual activity of the hydrophobin was investigated and the activity is expressed as “quantity of liquid expelled by a gushing carbonated water”. Spray drying was chosen because of its suitability for heat-sensitive materials due to the cooling effect of the evaporating liquid [23]. Furthermore, the single step design facilitates the industrial scalability. First, gushing active and SDS-PAGE confirmed fractions containing HFBI (fractions eluted between 41.0 and 48.0 % ACN) were added together and the initial gushing activity of this mixture was determined using the modified Carlsberg gushing test. Subsequently, the HFBI solution, with and without trehalose (excipient:HFBI ratio of 2:1), was spray dried. The spray dried hydrophobin powders were then resuspended in demineralized water and the residual gushing activity after spray drying was assessed and compared to the initial gushing activity (Figure 4). A residual gushing activity of 7.7 % could be observed for the spray dried HFBI in the absence of trehalose. On the other hand, a residual gushing activity of 93.6 % was achieved when trehalose was present in the spray dried solution indicating the stabilizing effect of trehalose, a carbohydrate known for stabilizing proteins during dehydration [21,24]. The stabilizing effect of trehalose is based on the water replacement theory, where the carbohydrate substitutes the water molecules as they are removed from the protein surface during the drying process. Without this replacement, structural changes are allowed to happen and protein denaturation or aggregation takes place [21,23]. Control experiments using only trehalose suspended in demineralized water were gushing negative, indicating that trehalose does not promote the HFBI activity during gushing but acts as a protective agent.

The results demonstrate the outstanding potential of trehalose to maximally preserve the functionality of hydrophobins during spray drying. Further research will show whether the same tendency can be found for other Class II hydrophobins. In addition, long-term stability tests will be effectuated to gain insight in the stability of the functionality of the hydrophobin powders as a function of time.

4. Conclusions

Extraction of the mycelium-bound hydrophobin HFBI, produced by *T. reesei*, has been reported several times but no comparison between the used extraction protocols has been presented. Most promising extraction solvents and solvent to biomass ratios were compared, leading to the selection of 1 % SDS in 100 mM Tris/HCl buffer at pH 9 in a solvent to biomass ratio of 4:1 as most promising.

Furthermore, the influence of the presence of an excipient to maximally preserve the functionality of hydrophobins during spray drying was also studied. When trehalose was added to the HFBI solution during the drying process, a residual gushing activity of 93.6 % was observed compared to 7.7 % when no excipient was added. This demonstrates the stabilizing effect of trehalose during spray drying of the Class II HFBI being of primary importance for future application development and industrial valorization of hydrophobins.

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Figure legend

Figure 1. Extraction capacity (mean value $\pm$ SD) of 1 % SDS in 100 mM Tris/HCl buffer at pH 9 and 60 % ethanol in a solvent:biomass ratio of 2:1, 3:1 and 4:1 for the extraction of HFBI (n=2).

Figure 2. Purification of HFBI by Reversed Phase Fast Protein Liquid Chromatography, the full line represents the UV-absorbance at 214 nm, the dashed line represents the gradient of acetonitrile.

Figure 3. SDS-PAGE of gushing active, purified HFBI fractions. The left lane contains the polypeptide SDS-PAGE molecular weight standard. The right Lane contains a gushing active fraction (43-44 % ACN), obtained by RP-FPLC purification of HFBI extract.

Figure 4. Residual gushing activity (%) of HFBI, before and after spray drying (mean value $\pm$ SD, n=2). The mean value of the initial sample activity was set to 100 %.

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