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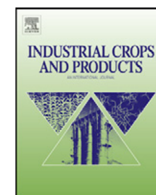

A novel method for hydrophobin extraction using CO_2 foam fractionation system

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ABSTRACT

Due to the exceptional properties and many potential applications of hydrophobins, special fungal proteins, it becomes necessary to develop a real scale procedure for their production and purification. In our previous study (Deckers et al., 2010) [CO_2–hydrophobin structures acting as nanobombs in beer, Brew. Sci. 63:54–61], the strong interaction of CO_2–hydrophobin was demonstrated. For the first time, in an approach to isolate hydrophobin HFBII from the growth media of *Trichoderma reesei*, a foam fractionation system using CO_2 as the sparging gas was investigated in this study. Using CO_2 foam fractionation, the concentration of HFBII was increased from 0.10 ± 0.02 mg/mL up to 0.57 ± 0.04 mg/mL. This was shown after a purification step by conventional liquid chromatography and identification of the goal protein using MALDI-TOF. The obtained molecular weight of the protein was 7.042 kDa which corresponds to the complete molecule of HFBII, minus the last aminoacid. Micro-spectrophotometry was used for quantification of purified HFBII. Moreover, different parameters of the foam fractionation system were optimized. The concentration of the protein after treatment by CO_2 followed by liquid chromatography was increased from 0.32 ± 0.02 to 0.44 ± 0.06 mg/mL when the flow rate of gas injection was changed in the range of 1–3 L/min. The highest amount of HFBII equal to 0.57 ± 0.04 mg/mL was obtained by the highest ratio of liquid height over the column height. Using the larger pore size frits causes increased protein absorption as well. The gushing potential of samples revealed that in contrast to the samples before CO_2 treatment, interestingly, no gushing was observed for the samples after treatment. The possibility that stable aggregates of HFBII molecules are formed as a consequence of their high concentration is discussed in this paper. By using DLS analysis of the overfoam, 100 nm particle size of CO_2 nanobubbles coated by HFBII was obtained. The final concentration of the protein was carried out using Amicon® ultracentrifuge device with the average recovery of 63.8 ± 8.2%.

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1. Introduction

Hydrophobins are a large family of cysteine rich proteins synthesized by fungi with a molecular mass of around 10 kDa (Cooper and Kennedy, 2010). They are secreted into liquid media by fungi or present on the surface of aerial mycelia (Stubner et al., 2010). They can assemble at a hydrophilic–hydrophobic interface forming an amphipathic film (Linder, 2009). A broad spectrum of functions in fungal growth and development is related to these proteins. Based on the self-assembly, hydrophobins are divided into two classes: I and II (Wessels, 1994). Molecular aggregates formed in these two classes are distinguished on the basis of solubility and morphology.

Thanks to their extraordinary properties, hydrophobins were suggested for a number of applications (Khalesi et al., 2012). Many of them involve the adsorption of hydrophobins to modify surfaces (Janssen et al., 2002). The self-assembly of hydrophobins (Wosten and Wessels, 1997) makes them interesting for using as stabilizers of emulsions (Wosten et al., 1994), foaming agents (Hektor and Scholtmeijer, 2005) and targets for the immobilization of other components (Linder, 2009). The application of hydrophobins in biosensor developments (Bilewicz et al., 2001), and in tissue engineering (Janssen et al., 2002) were studied as well. Therefore, developing methods to detect, isolate and purify these valuable proteins in real scale is interesting for industries, especially those which are looking for products in the area of nano-biotechnology.

One of the class II hydrophobins with exceptional characteristics, is HFBII. The structure of HFBII shows a rigid and amphiphilic molecule (Linder et al., 2005). This explains its surface activity and the formation of supra-molecular assemblies. It was demonstrated that HFBII could reduce the air/water surface tension down to 30 m Nm^{-1} (Cox et al., 2007). However, more knowledge is required to fundamentally understand the surface behavior of HFBII.

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Therefore, it becomes important to study the required parameters for production, isolation and purification stages at larger scale.

Foam fractionation of substances is an adsorptive bubble separation method for enriching diluted surface-active substances dissolved in water (Maruyama et al., 2006). This accessible technique has been studied in many fields, such as chemical/biochemical engineering, analytical chemistry, and wastewater treatment (Maruyama et al., 2007). In principle, the strong amphipathic nature of proteins and enzymes, with polar and non-polar groups, causes them to be preferentially adsorbed at the gas–liquid interface and foam fractionation can be used to separate and to concentrate such proteins (Aksay and Mazza, 2007). The adsorption at the gas–liquid interface in this system lowers the surface tension and enhances bubble formation. Moreover, the molecules form an elastic film around the bubbles increasing foam stability (Linke et al., 2005). The rich foamate is then collected. The enhanced protein concentration in the foam is due to the combined effect of interfacial adsorption and foam drainage, and it is proposed that adsorption occurs only while the bubbles rise in the liquid pool (Bhattacharjee et al., 1997). This technique is simple and has significant potential for lowering the high costs of protein recovery (Tseng et al., 2006).

The type and the flow rate of gas injection, the ratio of the column height to initial liquid height, the pore size of the sintered glass disk, the initial liquid volume as well as the initial surfactant concentration are essential parameters which may cause different influences on performance of foam fractionation. Sarachat et al. (2010) demonstrated that by increasing the air flow rate, biosurfactant recovery was increased significantly, but the enrichment ratio was reduced. Similarly, Chana et al. (2007) reported that both the foam volume and the recovery percentage of the proteins in wastewater samples increased exponentially when increasing the air flow rate, while the enrichment ratio was decreased. Likewise, high feed flow rate results in low enrichment factors as the higher mass of surface-active molecules stabilizes the foam, thus increasing the volume of collected foamate (Merz et al., 2011). Consequently, the stability of the foam decreases leading to liquid holdup reduction. Another important factor which can affect the separation process is pH. Obviously, the isoelectric point is the optimum condition to enhance the separation (Linke et al., 2007). Despite the recent advances in foam fractionation, delivering both high biosurfactant recovery and high enrichment ratio at the same air flow rate is still impossible. Therefore, depending on the objective, the parameters have to be chosen.

During this study, we developed a simple, inexpensive method to produce, concentrate and purify hydrophobin HFBII using a foam separation technique with CO_2 as sparging gas. This is based on the knowledge of the strong interaction of CO_2 with hydrophobins, which by the way causes gushing problem in beer (Deckers et al., 2010). Gaining a better understanding of the HFBII–CO_2 interaction in such a system is another objective.

2. Experimental

2.1. HFBII production

Hydrophobin HFBII was produced using *Trichoderma reesei* MUCL 44908 (purchased from BCCM-BCCM-/MUCL (Agro)Industrial Fungi and Yeast Collection company) in a medium with lactose as carbon source described by Bailey et al. (2002) (Table 1).

The pH of the medium was adjusted to 4.5–5 by using HCl and heat sterilized. For inoculation, mycelium was collected from surface cultures of *T. reesei* on petri dishes with the medium of Malt Extract Agar (MEA) and subjected into the test tubes containing fresh medium culture. These were used as the overnight culture. After 12 h, the overnight cultures were added into the fresh medium with the ratio of 1:3 for further growth. Fungal growth was initiated at 25 °C, in a 2-L working volume fermenter (KGW-type 7174) with stirring and temperature control. After 7 days the medium was centrifuged (Beckman CouLter^{TM}, 8000 g) at 6 °C for 25 min and the supernatant was directed to the foam fractionator.

Table 1
The amount of different components in 1-L medium culture.

Component	Amount (per L)
Lactose monohydrate	41.33 g
Peptone	4.00 g
Yeast extract	1.00 g
KH_2PO_4	4.00 g
$(NH_4)_2SO_4$	2.80 g
$MgSO_4·7H_2O$	0.60 g
$CaCl_2$	0.60 g
$CoCl_2·6H_2O$	4.00 mg
$MnSO_4·H_2O$	3.2 mg
$ZnSO_4·7H_2O$	6.9 mg
$FeSO_4·7H_2O$	10 mg

2.2. Design of the foam fractionation device and optimization procedure

The foam fractionator consisted of a glass column (ID = 52 mm) as shown in Fig. 1. For foaming, the CO_2 passed through a sintered glass disk. The collapsed foam was collected and stored for further analysis.

Gas flow rate, ratio of the liquid height over the column height as well as frits' pore size were optimized for the improvement of the CO_2 foam fractionation system. For gas flow rate, three different amounts of CO_2 flow were tested; 1, 2 and 3 L/min. In all cases, G_3 frit was used. The ratio of the liquid height over the column height was adjusted to 0.23. Three different ratios of the liquid height over the column height (0.13, 0.23, 0.33) and 3 different frit sizes (G_3,

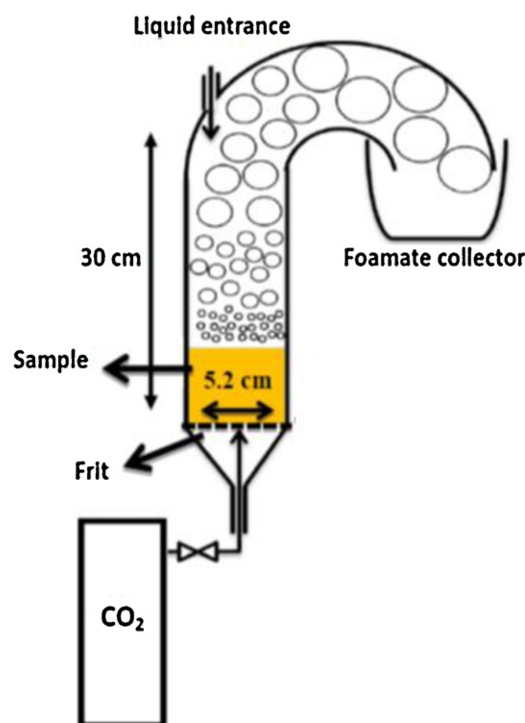

Fig. 1. Schematic diagram of CO_2 foam fractionation system.

G_4, G_5) were tested when the flow rate of CO_2 was adjusted to 3 L/min. The quantity of HFBII after chromatographic purification was compared in different conditions.

2.3. Dry matter analysis

The changes in dry matter before and after fractionation of the medium was determined by the weight change of 1 mL initial samples ($n = 3$) after drying at 105 °C for 18 h.

2.4. HFBII purification and identification

HFBII produced in the fermenter was purified by liquid chromatography using a 15RPC column (4.6 × 200 mm; GE Healthcare) (Deckers et al., 2011) and the eluted fractions were monitored by UV detection at 214 nm. Fractions were collected for further analyses.

To confirm the presence of hydrophobin HFBII in the chromatographic fractions, these were subjected to Matrix-Assisted Laser Desorption/Ionization-Time Of Flight (MALDI-TOF) using an Ultraflex II instrument model in linear mode (Deckers et al., 2011). Two microliters of HFBII-rich fractions were subjected to microspectrophotometry (NanoDrop ND-1000) at 280 nm wavelength for quantification (Luo et al., 2007).

2.5. Detection of gushing by HFBII and DLS analysis

The gushing test of samples was carried out according to the procedure suggested by Deckers et al. (2011). Two milliliters of 1-L sparkling water was removed and replaced with 2 mL of the sample taken either before foam fractionation (0.10 ± 0.02 mg HFBII/mL) or after foam fractionation (0.57 ± 0.04 mg HFBII/mL). The bottles were sealed and shaken for 3 days at 25 °C using a horizontal shaker (Edmund Buhler GmbH, SM-30, 150 rpm). After opening the bottles and gushing detection, the bottles were weighed to determine the lost weight. The carbon dioxide concentration of sparkling water was determined using the manometric method (Deckers et al., 2011). Overfoam after collapsing and degassing was also collected for chromatographic analysis and DLS analysis. Thereafter, 4.5 mL of gushed sample after degassing was placed into a measurement cell (4 optical sides) and the particle size was determined by Dynamic Light Scattering (DLS) with a 90Plus Particle Size Analyzer (Brookhaven Instruments Corporation). The details were described previously by Deckers et al. (2011).

2.6. Concentration

In order to increase the final concentration of HFBII, centrifugal filter devices created by styrene/butadiene (Amicon® Ultra-0.5) was used. In this case, 300 μL of the sample obtained by chromatography was transferred into the filter device and after spinning at 14,000 × g for 10 min followed by recovery at 1000 × g for 2 min, the residue in the collected tubes was collected. The percentages of concentrate recovery was obtained by the following equation:

$$\% \text{ concentrate recovery} = 100 \times \frac{W_c \times C_c}{W_0 \times C_0}$$

where W_c is total weight of concentrate before assay, C_c is concentrate concentration, W_0 is weight of fractions from chromatography, and C_0 is original starting fraction concentration.

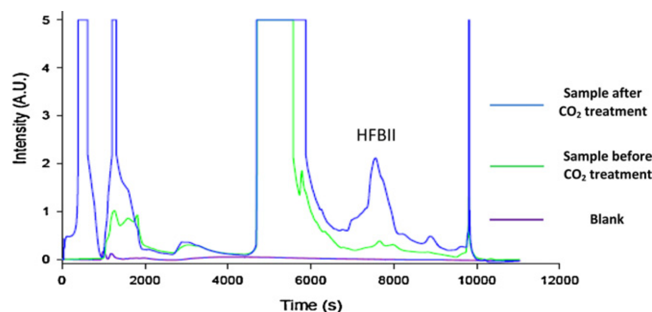

Fig. 2. Chromatograms of blank sample, sample before CO_2 treatment, and sample after CO_2 treatment.

3. Results and discussion

3.1. Foam fractionation of medium culture with CO_2 and dry matter changes

After 7 days, the pellet was discarded by centrifugation and the medium culture was directed to the foam fractionator. Without centrifuge step, the obtained HFBII shows more impurities, probably due to contamination with other ingredients. In our set up, centrifugation also avoids the blockage of tubes connecting the HFBII medium to the fractionator.

The percentage of dry matter in the medium was 5.75 ± 0.96% while in the foamate it was 7.62 ± 0.55%, hence the dry matter average was increased about 33%. It appears that CO_2 interacts mainly with molecules which exhibit a high degree of hydrophobicity, thereby, specifically enriching molecules with hydrophobic regions exposed to the medium such as hydrophobins. As an improvement over air, CO_2 binds more specifically with hydrophobic components, thus making the CO_2 foam fractionation system better tailored to purify hydrophobin than the previous system supplemented by air (Winterburn et al., 2011a,b).

The origin of this idea corresponds to the gushing phenomenon, an economic problem of breweries. Hydrophobins illustrate a strong affinity to CO_2 (Deckers et al., 2010; Shokribousjein et al., 2011) due to the presence of hydrophobic patch, and may result in primary gushing of beer. This phenomenon led us to consider that CO_2 might be a better and more specific gas for foam fractionation of biosurfactants than air, *especially* hydrophobic ones such as HBFII. Since CO_2 is less polar than air, HFBII would be attracted to CO_2 gas bubbles more efficiently than to air bubbles.

Liquid chromatography using a Reversed Phase Column (RPC) was used for detection and purification of extracted HFBII. In Fig. 2, the chromatographic profile is shown for a sample after foam fractionation with 10 times dilution. In the region of interest (fractions number 55–65) there is only one significant peak, starting with fraction 58 and ending with fraction 62. This peak, which appears after foam fractionation more obvious than before CO_2 treatment, confirms the protein enrichment generated by CO_2 foam fractionation.

3.2. HFBII identification by MALDI-TOF analysis

Though it was shown in chromatograms that HFBII was isolated from the fungal growth medium by foam fractionation with CO_2, for further confirmation, the chromatography fractions were submitted to the MALDI-TOF system (Fig. 3).

In comparison with other fractions, fractions 59 and 60 show only one peak without any important interference and with very small baseline noise. These two fractions seem to contain only one type of protein with a molecular weight of 7.042 kDa which was reported before by Neuhof et al. (2007). They reported that

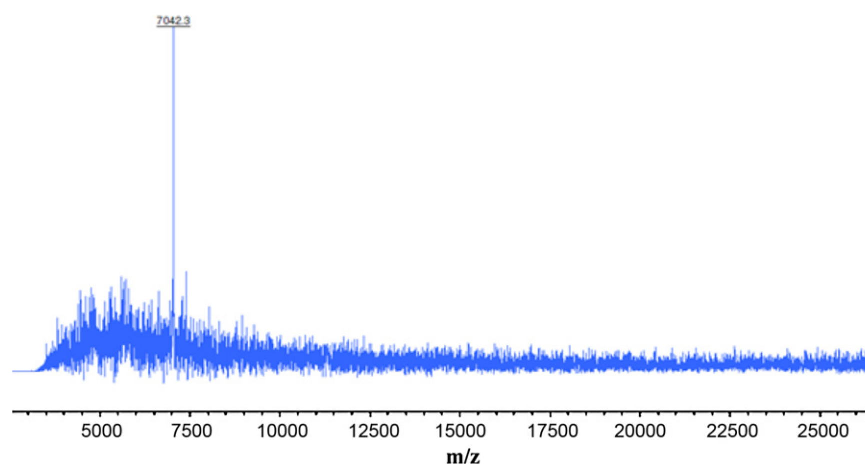

Fig. 3. MALDI-TOF spectrum of HFBII-rich sample, collected by chromatography.

when hydrophobin HFBII loses the last amino acid (phenylalanine), the molecular weight of this protein is reduced from 7.189 to 7.042. No reason for losing the phenylalanine was given, although it seems that this may occur during the last period of fungal growth. Phenylalanine is an essential amino acid and its limited synthesis during the final growth period may be compensated by some fungal peptidase activities releasing the amino acid from proteins in the medium. For comparison, ochratoxin A in the medium culture of some fungi is broken down back to phenylalanine and the rest of the molecule at the end of the growth phase in the same way (Astoreca et al., 2007).

The results of micro-spectrophotometer analysis reveal that the fractions before foam fractionation contain trace amounts of hydrophobin, which in the purest fraction was equal to 0.10 ± 0.02 mg/mL. For samples after foam fractionation, the amount of protein in fraction 60 increased up to 0.57 ± 0.04 mg/mL. By comparing the results of dry matter analysis, it can be concluded that though the dry matter was increased only 33%, which is not really a great enrichment, the amount of pure HFBII was increased more than 5 times. This comparison demonstrates that CO_2 can capture the hydrophobin HFBII more specific than other ingredients present in the medium culture and therefore, CO_2 foam fractionation is a convenient system to concentrate hydrophobin HFBII.

3.3. Optimization of foam fractionation

Different parameters of foam fractionation were tested in order to optimize the extraction step. The amount of protein in fractions obtained by chromatography was quantified using micro-spectrophotometer. The results are summarized in Table 2.

The results show that the amount of HFBII obtained after liquid chromatography was increased when the flow rate of CO_2 was increased as well. This result is the same as reported by Sarachat et al. (2010). In fact, an increase in the air flow rate enhances both the transportation of the bulk liquid into the foam and the biosurfactant adsorption due to increasing surface area of the air bubbles. By increasing the ratio of liquid height over the column height from 0.13 to 0.33, the amount of hydrophobin was increased as well. It seems that in the case of low initial volume, other proteins which show less affinity to CO_2 than goal protein might also be enriched and sometimes mask the goal protein. The amount of protein is also correlated to the frit pore size. The frit size is the main responsible for bubble size. The smaller the frit pores diameter, the smaller the bubble size, and the wetter the bubbles, thus, the lower the concentration of HFBII. In this experiment, it was observed that for the frit No. G_5, the efficiency of foaming is too low. For the frit No. G_4 the foams had much lower HFBII than when using frit No. G_3. When using the frit No. G_3, the bubbles were larger and contained less amount of water than the bubbles produced by frit No. G_4. Therefore, the concentration of surfactant is increased in drier bubbles. Consequently, the absorption of protein for frits with larger pore size diameter is significantly higher than that of the smaller frits.

3.4. Gushing test and DLS analysis

Hydrophobin–CO_2 interaction is the main responsible for primary gushing of carbonated beverages and is one of the problems in breweries. To estimate the gushing potential of hydrophobin HFBII produced in the culture medium of *T. reesei*, a gushing test was carried out as described in Section 2.5, using the sparkling water which contained 6.3 g/L CO_2 (determined by manometric method). The reason that sparkling water was used in the gushing test is its higher sensitivity than other carbonated beverages such as beer. In beer, new bubble interfaces are immediately covered by other surface active substances and the hydrophobin layers would be covered by substances which can reduce the gushing potential of samples. It was found that samples taken before foam fractionation caused gushing with an overfoaming in the range of 2.5–3%. The overfoam was collected and analysed by chromatography and the expected peak corresponding to hydrophobin HFBII was found (Fig. 4).

DLS analysis was carried out on overfoams as well (Fig. 5). Two main peaks were observed by this experiment. The first one was in the region of 100 nm, though the second one was around 1000 nm. This is exactly similar to the results reported by Deckers et al. (2011) who proved that CO_2–hydrophobin bubbles are stabilized at 100 nm diameter under atmospheric pressure.

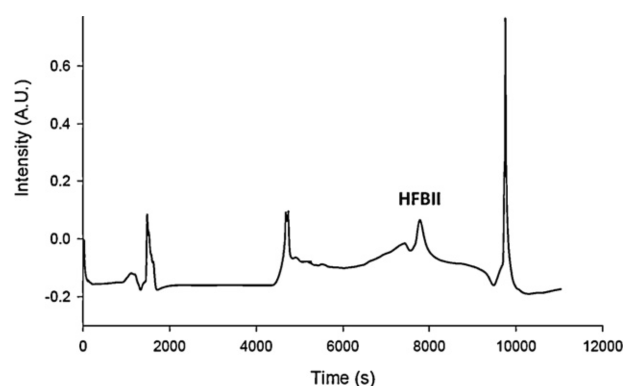

Fig. 4. The chromatogram of injected overfoam to liquid chromatography.

Table 2
The concentration of hydrophobin HFBII, before and after treating the sample by CO_2 in different conditions and after using amicon device.

Sample	Concentration (mg/mL) before using amicon	Concentration (mg/mL) after amicon	Amicon recovery (%)
Sample before treating	0.10 ± 0.02*	0.54	58
Type of frit – the ratio of height of liquid over the height of column–CO_2 flow rate			
G_3–0.13–3 L/min	0.40 ± 0.03	2.10	78
G_3–0.23–1 L/min	0.32 ± 0.02	3.76	76
G_3–0.23–2 L/min	0.41 ± 0.03	4.78	62
G_3–0.23–3 L/min	0.44 ± 0.06	5.74	54
G_3–0.33–3 L/min	0.57 ± 0.04	5.16	68
G_4–0.23–3 L/min	0.33 ± 0.03	4.28	60

*Each data point represents the mean and standard deviation of three independent experiments.

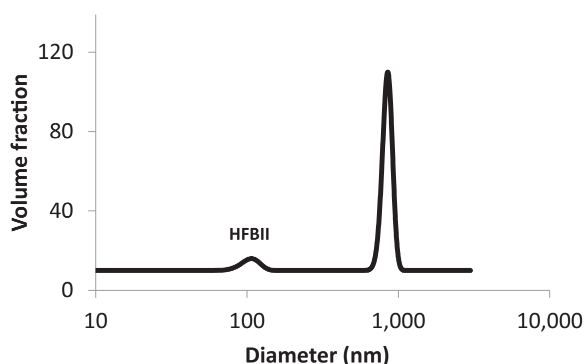

Fig. 5. The result of DLS for the overfoam.

Surprisingly, no gushing was detected for samples taken from the foamate. Normally, when the concentration of hydrophobin is high, it results in vigorous gushing. In the foamate however, the concentration of HFBII may be so high that it causes the formation of a stable polymer, which does no longer interact with CO_2. In other words, the binding together of HFBII molecules surpasses the binding with CO_2. Similar to the critical lowest concentration of hydrophobin required for gushing (i.e. 3 ppb) (Sarlin et al., 2005), there could be a critical highest concentration of hydrophobin too. Higher than this amount, hydrophobins start forming strong films and the hydrophobic amino acids involved in CO_2 binding are now involved in interactions with other hydrophobin molecules. Though more studies are needed to confirm this hypothesis, increasing the hydrophobin concentration higher than the described critical highest concentration might prevent gushing in carbonated beverages.

3.5. Concentration

Table 2 reveals the capability of the ultracentrifuge procedure in order to increase the concentration of protein in samples. The average recovery concentration was 63.8 ± 8.2%. Therefore, this device is convenient when high concentration of goal proteins in solvent is required. Since the filters are hydrophobic, it is proposed that for increasing the concentration of proteins at an industrial scale, designing hydrophobic membranes to capture the hydrophobic part of the protein would be useful.

4. Conclusion and prospective

Our results show that hydrophobin HFBII can be isolated from the growth medium of *T. reesei* by the CO_2 foam fractionation method. This was shown by column chromatographic analysis of the extract followed by the MALDI-TOF method for further determination. The results of MALDI-TOF analysis indicate that the HFBII isolated from the medium after 7 days of fungal growth has lost the last amino acid (phenylalanine) probably due to the biodegradation. For further improvement of the foam extraction method with CO_2, several parameters (i.e. frit porosity, CO_2 flow rate, ratio of the column height to initial liquid height) were optimized. The results show that by increasing the flow rate and the initial liquid volume the obtained protein using liquid chromatography is increased. The higher diameter pore size leads to improved protein extraction due to increased bubble size and therefore, improves the absorption procedure. A very intriguing result is the finding that the gushing inducing properties of hydrophobin are lost at a certain so-called "critical highest concentration". This could be explained by the formation of hydrophobin films and the consequent loss of hydrophobic patches in the protein involved in the binding of CO_2. This leads us to the question where the CO_2 binds to the protein. We suppose that when CO_2 is injected in a sample which contains HFBII, it is immediately directed to hydrophobic amino acids which have created β-strand structures. Multiple hydrophobins and CO_2 molecules interact with each other rather than a 1-on-1 interaction. Therefore, a complex method is required to modelize HFBII–CO_2 interaction which allows consideration of real experimental condition such as temperature, pressure and working pH. The authors propose that by modeling and fundamentally understanding the CO_2–hydrophobin interaction, new applications for HFBII might be found. This model is under investigation by our group.

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