

# Fungal Hydrophobins as Predictors of the Gushing Activity of Malt

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## ABSTRACT

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Fungal infection of barley and malt, particularly by strains of the genus *Fusarium*, is known to be a direct cause of beer gushing. We have shown previously that small fungal proteins, hydrophobins, isolated from strains of the genera *Fusarium*, *Nigrospora* and *Trichoderma* act as gushing factors in beer. A hydrophobin concentration as low as 0.003 ppm was sufficient to induce gushing. The gushing-inducing abilities of the isolated hydrophobins varied probably due to their structural differences. The hydrophobins did not affect beer foam stability. A correlation was observed between the hydrophobin level analyzed by the hydrophobin ELISA developed and the gushing potential of malt. The risk of gushing was found to increase with hydrophobin concentrations above 250 µg/g malt. The levels of hydrophobin and the *Fusarium* mycotoxin deoxynivalenol (DON) in malts were not correlated which indicated that the formation of those two fungal metabolites may not be linked. Furthermore, we did not observe a correlation between the DON content and the gushing potential of the malt studied. Our observations suggest that the accuracy of predicting gushing could be improved by measuring the amount of the actual gushing factors, hydrophobins, in barley or malt.

**Key words:** Beer gushing, ELISA, hydrophobin, prediction of gushing risk in malt.

## INTRODUCTION

Gushing is a phenomenon in which beer spontaneously, without agitation, vigorously over foams out from the package immediately on opening<sup>10</sup>. Two types of gushing exist in beer<sup>3,6</sup>. Primary gushing is induced by fungal metabolites, so-called gushing factors, which are present in malt or in other cereal raw materials of beer. Non-malt related gushing, i.e. secondary gushing, may occur if beer contains e.g. haze, impurities from bottles, metal ions, calcium oxalate crystals, cleaning agent residues or excess of gas. Primary gushing is commonly caused by *Fusarium* fungi but other genera such as *Aspergillus*, *Nigrospora*, *Penicillium* and *Stemphylium* are also reported to induce gushing<sup>4</sup>. Gushing factors produced by fungi have been studied for decades. As shown in Table I, they have been reported to be polypeptides or peptide-containing substances. Very small amounts of these substances, quantities

in the ppm range or lower, have been reported to induce gushing of beer. Aastrup *et al.*<sup>2</sup> observed that addition of proteolytic enzymes to gushing-inducing malt extract significantly reduced gushing tendency, suggesting that the gushing-inducing factors present in malt were proteins or polypeptides. Gushing factors are assumed to be surface active molecules which stabilize carbon dioxide bubbles in beer by forming a layer around the microbubbles<sup>7,26</sup>. This layer may prevent breakdown of the bubbles, leading to overfoaming.

Gushing negatively affects the image of beer, incurring economic losses for breweries and maltsters. An increasing percentage of European malt samples analysed during the past five years have shown gushing tendency<sup>1</sup>. In the USA severe epidemics of *Fusarium* Head Blight in barley have occurred during the last decade, which have increased the risk of gushing<sup>28,31</sup>. Currently the gushing potential of barley and malt can be predicted by quantifying the presence of *Fusarium* fungi or their antigens<sup>8,22,32</sup>. The principle weakness inherent to these methods lies in the fact that they do not directly detect the actual gushing-inducing factors. Some studies have also shown that the actual *Fusarium* level of barley or malt is a poor predictor of gushing propensity<sup>23,28</sup>. The gushing test described by Vaag *et al.*<sup>33</sup> and modified for barley by Aastrup<sup>1</sup>, relies on an aqueous extract of barley or malt being added to bottled beer so that the gushing tendency of beer can be measured after three days of shaking. However, the test is labor-intensive and time-consuming, and is impractical for screening large numbers of samples.

Our recent studies indicated that small fungal proteins called hydrophobins act as the gushing factors of beer<sup>13,18</sup>. Hydrophobins are highly surface active, moderately hydrophobic proteins produced by filamentous fungi<sup>34</sup>. A characteristic feature of these proteins is their eight conserved cysteine residues forming four disulphide bridges in the molecule made up of 100 ± 25 amino acids. Under some conditions hydrophobins form aggregates. Based on sequence comparison, hydrophobins are divided into two different classes, I and II<sup>34</sup>. Hydrophobins self-assemble at their hydrophilic-hydrophobic interfaces to form amphipathic membranes<sup>35</sup>. This property allows hydrophobins to fulfill a broad spectrum of functions in fungal growth and development. Hydrophobins are present in fungal cell walls, where they are involved in the formation of structures of mycelium and spores<sup>34</sup>. Fungi secrete hydrophobins into their surroundings, where the proteins can decrease the surface tension of water or change the nature of a surface from hydrophilic to hydrophobic or *vice versa*<sup>36</sup>. These properties are useful when the fungus penetrates

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the air-water interface or attaches to hydrophobic host surfaces like cuticular waxes of plant tissues. The aim of this study was to isolate and characterize hydrophobins from gushing active fungi and to demonstrate that these hydrophobins are able to induce gushing in beer. The main goal of our work was to develop a test for detection of gushing potential of barley and malt by analysing the hydrophobin levels in samples.

## MATERIALS AND METHODS

### Fungal strains, media and cultivation

*Fusarium poae* VTT D-82182 (D182), *Nigrospora* sp. VTT D-79122 (D122) and *Trichoderma reesei* VTT D-74075 (D75) obtained from the VTT Culture Collection (Helsinki, Finland) were used. The *Fusarium* strain was cultivated in Czapek-Dox Broth (Difco Laboratories, Detroit, USA) or Potato Dextrose Broth (Difco Laboratories), the *Nigrospora* strain in Potato Dextrose Broth (Difco Laboratories) and the *Trichoderma* strain in *Trichoderma* minimal medium<sup>27</sup> buffered to pH 6. All strains were cultivated in shake flasks for at least 7 days at room temperature. The mycelium was separated from culture medium by filtration through GF/B glass fiber filter (Whatman International Ltd., Maidstone, UK), washed with water and frozen at  $-20^{\circ}\text{C}$ .

### Purification of hydrophobins

Hydrophobins were isolated as described by Nakari-Setälä *et al.*<sup>25</sup> using the sequential extraction of mycelium or by bubbling air through culture medium in which the fungal strains had been grown and then collecting the foam produced. The protein samples were further purified by preparative reversed phase high performance liquid chromatography (RP-HPLC) on a Vydac C4 column using the Äkta Explorer system (Pharmacia Biotech, Sweden). Elution was performed with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Fractions eluted with 40–50% of acetonitrile were collected.

### Protein analyses

Hydrophobins were detected using SDS-PAGE performed with 17.5% or 20% gels<sup>21</sup> using the 2050 Midget Electrophoresis System (Pharmacia LKB Biotechnology, Sweden) or PhastSystem (Pharmacia LKB Biotechnology), followed by visualization of the proteins by silver staining (Silver Stain Kit, Bio-Rad Laboratories, Hercules, USA) or immunoblotting. Polyclonal antibodies against the hydrophobins of *T. reesei* D75 and *F. poae* D182 were raised in rabbits. Immunisation took place four times within three months using the Freund's adjuvant. The hy-

drophobin antibodies together with goat anti-rabbit IgG (H+L)-alkaline phosphatase (AP) conjugate (Bio-Rad Laboratories) were used for the immunoblot analysis. The blot was developed using BCIP/NPT Colour Development Substrate (Promega, USA). Protein concentrations of the purified hydrophobin samples were determined using BC Assay Protein Determination Kit (Uptima, France) or BCA Protein Assay Reagent Kit (Pierce, Rockford, USA). In addition, a concentration of *T. reesei* hydrophobin samples was determined from the HPLC-chromatogram by using known amounts of the purified hydrophobins HFBI<sup>25</sup> or HFBII<sup>24</sup> as a standard. Partial N-terminal amino acid sequences of the purified proteins were determined by degradative Edman chemistry in the Protein Chemistry Laboratory of the Institute of Biotechnology, Finland<sup>14</sup>.

### ESI-MS analysis

The hydrophobins molecular weights were determined using the ESI-MS (electrospray-mass spectrometry) technique. RP-HPLC purified hydrophobin samples were diluted 1:5 in 0.1% formic acid in 50% acetonitrile and introduced into a Micromass Quattro Micro triple quadrupole mass spectrometer (Manchester, UK) and analyzed by continuous-flow injection using a syringe pump. The sample flow rate was 5  $\mu\text{L}/\text{min}$ . The electrospray ion source was operated at a capillary voltage of 3.00 kV and cone voltage of 20 V. Source and desolvation temperatures were  $80^{\circ}\text{C}$  and  $130^{\circ}\text{C}$ , respectively. Desolvation gas flow was 500 L/h and cone gas flow 20 L/h. The scan range was 400 to 2000  $m/z$ . Data was acquired and processed with MassLynx 3.5 software (Waters, Milford, USA).

### Beer foam stability

The effect of hydrophobins on beer foam stability was studied by adding 0.1, 1 and 10  $\mu\text{g}$  of the RP-HPLC purified HFBI<sup>25</sup> and HFBII<sup>24</sup> hydrophobins from *T. reesei* D75 into 0.33 L of bottled beer. The beer bottles were inverted once and the foam stability was measured using the NIBEM foam stability apparatus Model B (Haffmans BV Venlo, Holland) according to the manufacturer's instructions. The NIBEM apparatus recorded the collapse times in seconds required by foam to fall down 10, 20 and 30 mm.

### Hydrophobin ELISA

A competitive ELISA (Enzyme Linked Immunosorbent Assay) was developed for detection of hydrophobins in barley and malt. Kernels were ground with a Universal Laboratory Disc Mill DLFU (Bühler-Miag GmbH, Braunschweig, Germany) and 5 g of the fine flour was extracted with PBS buffer (10 mM sodium phosphate pH 7.3, 150 mM sodium chloride) in the proportion of 1:10. After centrifugation the supernatant was transferred to a clean

**Table I.** Properties of the gushing factors produced by different fungi: historical background 1963–1980.

Isolated from	Chemical structure	Molecular weight	Concentration needed	
			for gushing	Reference
<i>Nigrospora</i> sp.	Polypeptide	16.5 kDa	0.05 ppm	4, 16
<i>Stemphylium</i> sp.	Peptidoglycan	nd	4 ppm	4
<i>Fusarium graminearum</i>	Hexapeptide	nd	0.4 ppm	4, personal communication
<i>Penicillium crysogenum</i>	Cyclic tetrapeptide	nd	0.3 ppm	17
Northern European Malt	Peptide-like	10 kDa	0.5 ppm	15

nd: not determined.

tube and antibodies against the hydrophobin of *F. poae* D182 were added. After incubation the sample-antibody mixture was transferred into triplicate wells of immunoplates (Nunc-Immuno Modules, MaxiSorp polystyrene strips, Nunc, Rochester, USA) coated with a hydrophobin extract of *F. poae* D182. Goat anti-rabbit IgG (H+L)-alkaline phosphatase (AP) conjugate (Bio-Rad Laboratories) was used as a secondary antibody. p-Nitrophenyl phosphate tablets (Sigma, St. Louis, USA) in diethanolamine-MgCl<sub>2</sub> buffer (Oy Reagentia Ltd, Toivala, Finland) were used for AP detection. After incubation for 30 min at room temperature, the absorbance was read at 405 nm using a Multiskan Ex microtitre plate reader (Labsystems, Helsinki, Finland). Because of the nature of the competitive ELISA, a lower absorbance value corresponded to a higher amount of hydrophobins in the sample.

### Gushing potential

The gushing test was carried out according to the method of Vaag *et al.*<sup>33</sup> using a horizontal rotating shaker (50 rpm)<sup>11</sup> in order to analyze the gushing potential of malt. In this method, an aqueous extract of ground malt was added to bottled beer and the pasteurized bottles were shaken for three days. After shaking the bottles were kept still for 10 min, inverted three times and opened after 30 sec. The amount of gushing was determined from the change in weight of the bottle. The gushing-inducing ability of hydrophobins was studied by adding the purified hydrophobins into beer bottles and shaking the bottles as described above. The test was performed in duplicate except with the hydrophobin of *F. poae*.

### Deoxynivalenol analysis

The deoxynivalenol content of barley and malt was analysed using the EZ-Quant High Sensitivity Deoxynivalenol (DON) Test Kit (Diagnostix, Mississauga, Canada) or the EZ-Quant DON Test Kit (Diagnostix) according to the manufacturer's instructions.

## RESULTS AND DISCUSSION

### Isolation and characterization of hydrophobins

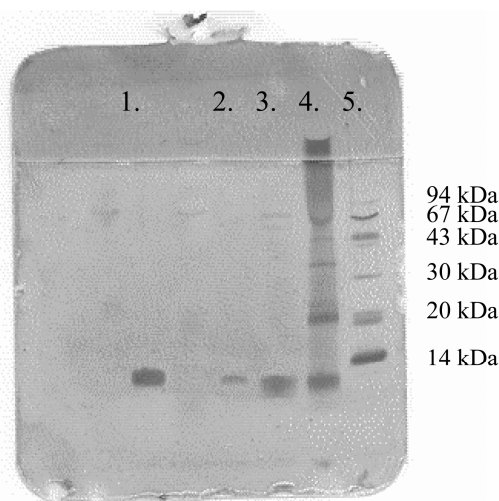
Hydrophobins were isolated from *F. poae* D182, *Nigrospora* sp. D122 and *T. reesei* D75 by bubbling the culture broths with air and collecting hydrophobin enriched foam or by sequential extraction of mycelium. The hydrophobins were subsequently purified by separation of these extracts by preparative RP-HPLC. The purified hydrophobin samples were analysed by SDS-PAGE which indicated that molecular weights of the protein bands were below 14 kDa (Fig. 1). The protein bands of *F. poae* (isolated by bubbling from Czapek-Dox Broth) and *Nigrospora* sp. (isolated by sequential extraction of mycelium cultivated in Potato Dextrose Broth) were slightly larger than that of *T. reesei*. Nakari-Setälä *et al.*<sup>25</sup> reported a predicted molecular weight of 7.5 kDa for the hydrophobin HFBI of *T. reesei*. When the RP-HPLC purified foam samples of *F. poae* and *Nigrospora* sp. were analysed by ESI-MS, both proteins had a molecular weight of approximately 8.5 kDa, which was consistent with their SDS-PAGE migration.

Partial N-terminal amino acid sequences of the RP-HPLC purified proteins of *F. poae* and *Nigrospora* sp. are presented in Fig. 2. When sequence data were compared to the consensus sequence of hydrophobins<sup>34</sup>, the typical conserved cysteine pattern of hydrophobins was found in the sequences of the *F. poae* and *Nigrospora* sp. proteins. Kitabatake and Amaha<sup>16</sup> reported that the gushing-inducing factor of *Nigrospora* (NFG) had a molecular weight of 16.5 kDa, consisted of 166 amino acid residues and had approximately 16 cysteine residues. If NGF is assumed to be in a dimeric form, then the number of amino acid and cysteine residues of the monomeric protein corresponds to those of hydrophobins.

In immunoblot analysis the polyclonal antibodies raised against the hydrophobin from *F. poae* D182 reacted only with its own antigen and not with the hydrophobins from *T. reesei* D75 and *Nigrospora* sp. D122 (data not shown). Correspondingly, the polyclonal antibodies raised against the hydrophobins from *T. reesei* D75 did not react with the hydrophobins of *F. poae* D182 or *Nigrospora* sp. D122 (data not shown). These results suggested that the three hydrophobins differed from each other to such an extent that they could not be detected with the same polyclonal antibodies. However, gushing of beer is considered to be most commonly caused by *Fusarium*<sup>11,28</sup>, which justifies the use of the *F. poae* hydrophobin antibodies in the ELISA test for prediction of the gushing propensity of barley and malt.

### Gushing and foam stability effects of hydrophobins

RP-HPLC purified hydrophobin fractions from *F. poae*, *Nigrospora* sp. and *T. reesei* were added to bottled beer and the bottles were rocked according to the gushing test protocol. Addition of an amount as low as 1 µg of *T. reesei* hydrophobins into bottled beer (0.33 L), corre-



**Fig. 1.** 20% SDS-PAGE of the hydrophobin samples isolated from mycelium or culture medium of the following fungi: *F. poae* D182 (1), *Nigrospora* sp. D122 (2) and *T. reesei* D75 (purified 3 and unpurified 4). The molecular weight markers are shown on the right (5). Total protein detected by silver staining.

Consensus cysteine spacing of hydrophobins:	
X <sub>2-38</sub> - C - X <sub>5-9</sub> - C - C - X <sub>11-39</sub> - C - X <sub>8-23</sub> - C - X <sub>5-9</sub> - C - C - X <sub>6-18</sub> - C - X <sub>2-13</sub>	
<i>F. poae</i> D182:	
TPPGYGGGGGGSGSNFDA C PGALYSQTQ CC SAGVGDIVDV...	
X <sub>18</sub> - C - X <sub>9</sub> - C-C ....	
<i>Nigrospora</i> sp. D122:	
TNDQPATGFVA C ANNGVLFSAPN CC ATDVLGLADLD C TPPKVP TSPXDFQ...	
X <sub>11</sub> - C - X <sub>11</sub> - C-C - X <sub>11</sub> - C ...	
where	C = cysteine, X = any other amino acid

**Fig. 2.** Partial N-terminal amino acid sequences of hydrophobins of *F. poae* D182 and *Nigrospora* sp. D122. Cysteine spacing in the hydrophobins of *F. poae* D182 and *Nigrospora* sp. D122 are compared to the consensus cysteine spacing found for fungal hydrophobins according to Wessels<sup>34</sup>.

sponding to a concentration of 0.003 ppm, was sufficient to cause gushing (Table II). A tenfold higher amount of the *Nigrospora* hydrophobin (0.03 ppm) was needed to induce gushing. Gushing activity of the hydrophobin from *F. poae* was the weakest at inducing gushing as 0.1 ppm. The concentrations of the *T. reesei*, *F. poae* and *Nigrospora* sp. hydrophobins needed to induce gushing were of the same order of magnitude as those of the gushing factors reported by other investigators and presented in Table I. Moreover, in a previous investigation we reported that the isolated hydrophobins could also induce gushing in mineral water, although the gushing was more pronounced in beer than in mineral water<sup>18</sup>.

The *T. reesei* hydrophobins, HFB I and HFB II, did not affect beer foam stability as determined by the NIBEM apparatus (data not shown), although 10 µg of the hydrophobin samples in beer was sufficient to induce gushing even when the bottles were not shaken but only gently in-

verted once. These results indicate that the properties of hydrophobins enable them to induce gushing rather than to stabilize foam in beer. According to current knowledge hydrophobic proteins in beer, such as Lipid Transfer Protein, increase beer foamability, but other components, such as bitter substances and polysaccharides, increase foam stability by cross-linking with the proteins<sup>5</sup>. It can be assumed that the hydrophobins from *T. reesei* did not favour the formation of cross-linkages.

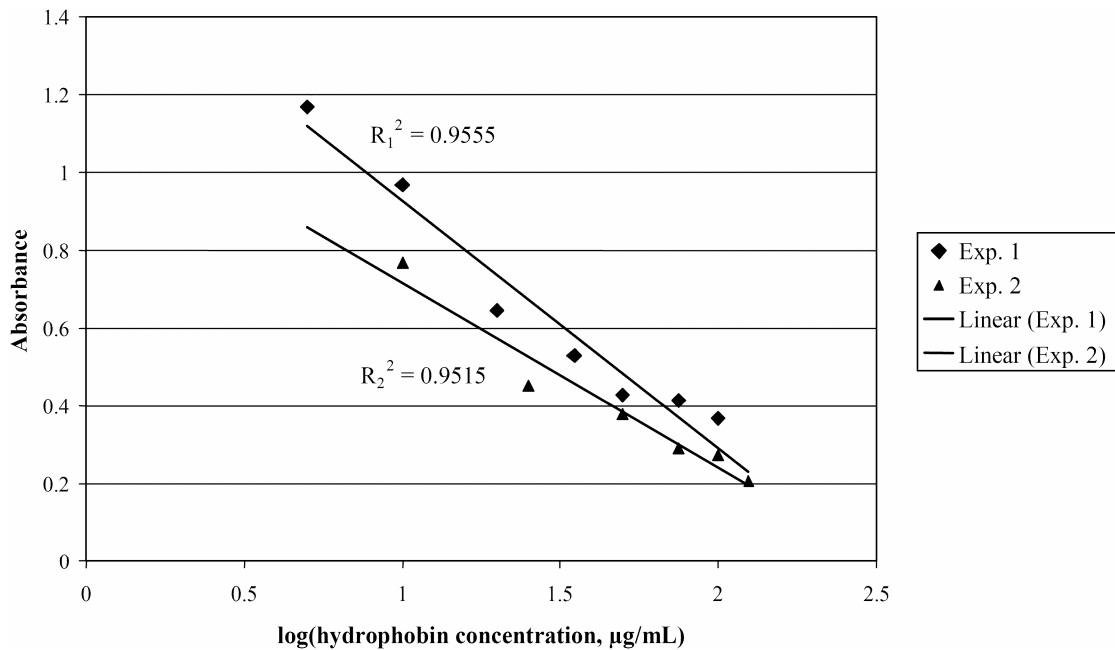
#### Validation of the hydrophobin ELISA test

Standards with known hydrophobin concentrations ranging from 5 to 200 µg/mL were prepared by diluting the RP-HPLC purified hydrophobin sample of *F. poae* with an aqueous extract of a gushing negative malt. Two sets of standards were analysed with the hydrophobin ELISA developed. A linear correlation ( $r^2 \approx 0.95$ ) was found between the logarithm of hydrophobin concentrations below 100 µg/mL and the results of the hydrophobin ELISA (A 405 nm) (Fig. 3). The differences between the absorbance values of the two standard sets are probably due to inaccuracy of the protein concentration analysis of the RP-HPLC purified hydrophobin samples. In addition, because of their hydrophobic nature, some of the hydrophobins may have adhered to the walls of tubes and pipette tips, which could have led to errors in hydrophobin concentrations particularly in the standards with low hydrophobin content. The hydrophobin ELISA could not distinguish between hydrophobin concentrations higher than 100 µg/mL, which means that the assay can be used to estimate hydrophobin levels below 1000 µg/g barley or malt without sample dilution.

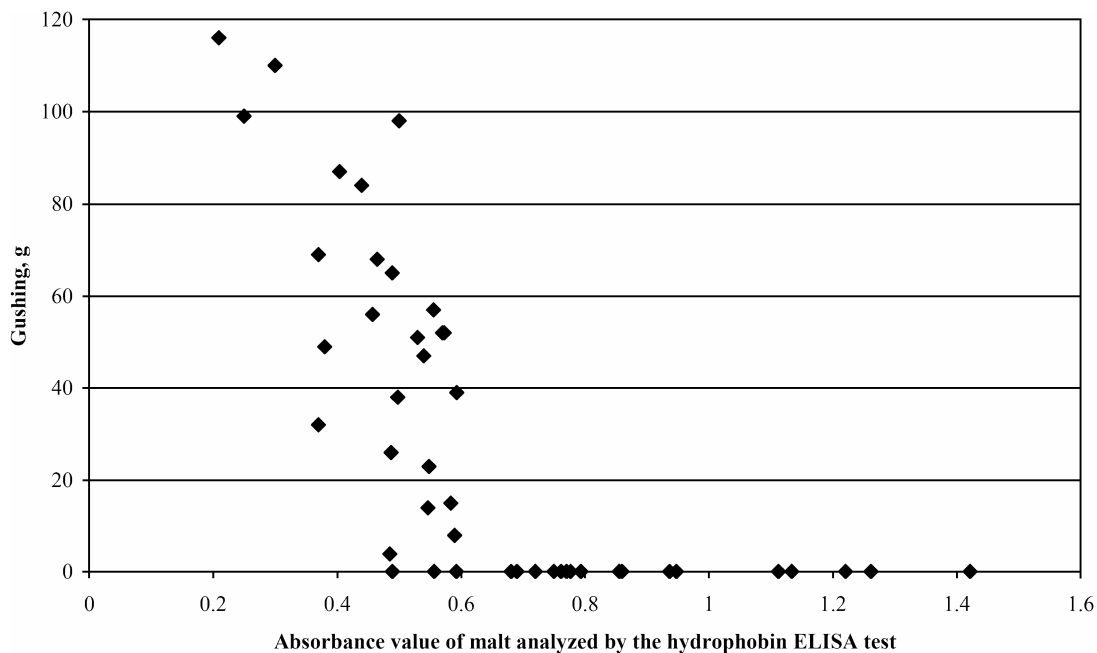
**Table II.** Gushing of beer induced by RP-HPLC purified hydrophobin fractions of *T. reesei* D75, *F. poae* D182 and *Nigrospora* sp. D122. Two different hydrophobins of *T. reesei*, HFB I and HFB II, were tested.

Amount of hydrophobin µg/0.33 L beer	Amount of beer gushed (g)			
	<i>T. reesei</i> HFB I	<i>T. reesei</i> HFB II	<i>F. poae</i>	<i>Nigrospora</i> sp.
0.01	0	0	nd	nd
0.1	0	0	nd	nd
1	10	12	0	0
10	189	192	0	66
45	nd	nd	27	183
60	nd	nd	80	nd

nd = not determined.



**Fig. 3.** Logarithmic concentrations of the hydrophobin standards of *F. poae* ( $\mu\text{g/mL}$ ) diluted in a malt extract versus the results of the hydrophobin ELISA ( $A_{405 \text{ nm}}$ ). Two different sets of standards (Exp. 1: 5, 10, 20, 35, 50, 75, 100, 150 and 175  $\mu\text{g/mL}$ , and Exp. 2: 10, 25, 50, 75, 100, 125, 150 and 200  $\mu\text{g/mL}$ ) were tested in duplicate. The results of the two highest standard concentrations are excluded from the standard curve because they were outside the linear range. The standard protein concentration experiments were the result of separated determinations of different hydrophobin preparations of *F. poae*.



**Fig. 4.** Absorbance values of the malt samples analyzed by the hydrophobin ELISA versus gushing potentials of the malts ( $n = 44$ ). In the competitive ELISA, low levels of absorbance reflect high levels of hydrophobin in the sample.

### Hydrophobin levels in barley and malt samples compared to gushing potential and deoxynivalenol content of malt

The hydrophobin ELISA developed was used to assess the levels of hydrophobin in barley and malt samples infected both naturally and artificially with *Fusarium* fungi.

The results of the hydrophobin ELISA were compared to the results of the gushing test. A correlation was found between the hydrophobin level and the gushing potential of malt; the risk of gushing was observed to be increased if the absorbance value of the malt in the hydrophobin ELISA was below 0.6, corresponding to a hydrophobin concentration of ca. 250  $\mu\text{g/g}$  malt (Fig. 4). All the malt



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