Fungal hydrophobins in the barley-to-beer chain

Produced by filamentous fungi. A characteristic feature of growth and development is the nature of a surface from hydrophilic to hydrophobic. By self-assembly, hydrophobins are able to stabilise air bubbles and oil droplets in amphipathic membranes. This property allows hydrophobins to fulfil a broad spectrum of functions in fungal and plant membranes. Hydrophobins are small surface-active proteins produced by species of Fusarium, Gibberella, and other fungi.

**ABSTRACT**

Fungal hydrophobins have been shown to induce gushing of beer. In order to study the occurrence and fate of hydrophobins throughout the brewing process, barley samples non-inoculated and artificially inoculated with Fusarium graminearum and Gibberella zeae were collected during the growing period as well as during various stages of the malting process. In addition, naturally infected malt was brewed in pilot scale and the beer filtered. The results showed that fungi produced hydrophobins that accumulated in the wort and some loss of hydrophobins during brewing. The presence and gushing of hydrophobins was confirmed by the hydrophobin ELISA. Our recent studies revealed that fungal hydrophobins are able to stabilise air bubbles and oil droplets in amphipathic membranes. This property allows hydrophobins to stabilise air bubbles and oil droplets in amphipathic membranes. This property allows hydrophobins to stabilise air bubbles and oil droplets in amphipathic membranes. This property allows hydrophobins to stabilise air bubbles and oil droplets in amphipathic membranes. This property allows hydrophobins to stabilise air bubbles and oil droplets in amphipathic membranes.

**Key words:** Brewing, hydrophobins, malting.
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ABSTRACT

Fungal hydrophobins have been shown to induce gushing of beer. In order to study the occurrence and fate of hydrophobins at different stages of the production chain of beer, barley samples artificially infected in the field with Fusarium culmorum, F. graminearum and F. poae were collected during the growing period as well as during various stages of the malting process. In addition, naturally infected malt was brewed in pilot scale and samples were collected throughout the process. The samples were assayed for hydrophobin content using an ELISA method. The results showed that fungi produced hydrophobins that accumulated during barley grain development in the field, but that production was more pronounced during malting. Prolonged storage of barley tended to reduce the ability of fungi to produce hydrophobins in malting. Studies on the fate of hydrophobins during the brewing process revealed that mashing released hydrophobins from the malt into the wort. Some loss of hydrophobins occurred throughout the brewing process with spent grains, cold break (wort boiling) and surplus yeast. In addition, the beer filtration step reduced hydrophobin levels. Despite the substantial loss of hydrophobins during brewing, the level was high enough to induce the gushing detected in the final beer.

Key words: Brewing, Fusarium, hydrophobins, malting.

INTRODUCTION

Hydrophobins are small surface-active proteins produced by filamentous fungi1–3,18. A characteristic feature of these proteins is their eight cysteine residues, whose sequence position is conserved. Hydrophobins self-assemble at their hydrophilic-hydrophobic interfaces to form amphipathic membranes19. This property allows hydrophobins to fulfills a broad spectrum of functions in fungal growth and development20. By self-assembly, hydrophobins are able to stabilise air bubbles and oil droplets in water, decrease the surface tension of water, and change the nature of a surface from hydrophilic to hydrophobic and vice versa19. Our recent studies revealed that fungal hydrophobins induced undesirable beer gushing6,7. Gushing is a phenomenon in which beer spontaneously, without agitation, vigorously overfoams out from the container immediately on opening. Fungal infection of barley and malt, in particular by species of Fusarium, is known to cause beer gushing3,5,8,13. However, the distribution and fate of fungal gushing factors, hydrophobins, in the beer production chain is still largely unknown. We have developed an enzyme linked immunosorbent assay (ELISA) for hydrophobins and showed that the hydrophobin ELISA can be used for prediction of the gushing risk of malt12. In addition, the genus Fusarium contains toxigenic species which produce mycotoxins, such as deoxynivalenol (DON), during barley grain development in the field and during malting8,11,14. A high portion of DON present in malt has been shown to survive the brewing process and end up in the beer14. Although the presence of hydrophobins is not completely predictive of the presence of mycotoxins8,12 in some ways their presence and gushing is a consumer warning of the more sinister quality concerns with gushing beer.

The aim of this investigation was to study the occurrence and fate of hydrophobins at different stages of the beer production chain by analyzing the hydrophobin levels of samples taken throughout the barley-to-beer chain with the hydrophobin ELISA.

MATERIALS AND METHODS

Barley and malt samples

Barley samples non-inoculated and artificially inoculated in the field with Fusarium culmorum VTT D-80148, F. graminearum (teleom. Gibberella zeae) VTT D-95470 and F. poae VTT D-82182 as previously described by Pekkarinen et al.9 were studied. The gushing positive and negative malt samples for lab-scale mashing studies were purchased from Carlsberg Research Laboratory, Denmark. For pilot scale brewing studies, a heavily Fusarium infected malt (six-rowed malting variety Robust, crop year 2002) was kindly provided by Dr. Paul Schwarz, North Dakota State University, USA.

Laboratory scale malting

Barley samples (1 kg) were steeped separately for two days at 12°C to a moisture content of 46% and germinated for 6 days at 14°C in a Seeger laboratory malting unit (Seeger GmbH, Germany). The moisture contents of the samples were determined daily and if necessary, distilled
water was added to adjust the moisture content to 45%. Kilning was carried out in 21 h with a stepwise temperature increase up to 85°C in a forced-air Seeger laboratory kiln (Seeger GmbH, Germany). Samples for the hydrophobin analysis were taken after steeping, germination and kilning. Moist samples were freeze-dried prior to the analysis.

**Laboratory scale mashing**

Laboratory scale high gravity mashing was performed using the gushing positive and negative malt samples purchased from Carlsberg Research Laboratory, Denmark. The former sample had repeatedly induced vigorous beer gushing in the gushing test. Ground malt, 50 g, was mixed with 200 ml of preheated water (50–52°C) containing 100 ppm CaCl₂ and 0.75 mM H₂SO₄. The sample mixture was then transferred to the mashing bath (Bender & Hobein, Munich, Germany) and the following mashing procedure was performed with a temperature increase rate of 2°C/min: 48°C/30 min – 63°C/30 min – 72°C/30 min – 80°C/10 min. After mashing the hot mash was filtered through a GF/A glass fiber filter (Whatman International Ltd., Maidstone, UK) and the wort was boiled for 45 min. The boiled wort was left to stand in the cold overnight and the formed cold break was removed by centrifugation. The amounts of hydrophobins in malt grist, spent grains, cold break and wort were analyzed using the hydrophobin ELISA. The hydrophobins continued throughout the growing period (Fig. 2), which made them easily distinguishable in the field. These malts also induced the most vigorous gushing in the field trials at approximately 3, 5, 7 weeks and from the field II trials also 9 weeks after inoculation (n = 8). Standard deviations of the hydrophobin level were studied by adding purified hydrophobins of *F. poae* to distilled water. Distilled water was used instead of beer because beer naturally contains high amounts of many proteins which can interfere with the determination of the low hydrophobin contents of the samples. Two different hydrophobin concentrations, approximately 5 µg/mL and 15 µg/mL, were examined. Water-hydrophobin samples, 30–50 mL, were filtered through Seitz K150 filter sheets (SEITZ-FILTER-WERKE GmbH, Bad Kreuznach, Germany) in the laboratory scale beer filtration unit (Sartorius, Goettingen, Germany) with a pressure of 1–1.4 bar. The hydrophobin concentrations in water before and after filtration were determined in duplicate with the BC Assay Protein Determination Kit (Upptima, France) according to the manufacturer’s instructions.

**Hydrophobin analysis**

Competitive ELISA (Enzyme Linked ImmunoSorbent Assay) developed for detection of hydrophobins in barley and malt was used to determine the hydrophobin levels in the samples collected from the field or during malting and brewing. Briefly, 5 g of the ground sample was extracted with PBS buffer (10 mM sodium phosphate pH 7.3, 150 mM sodium chloride) in the proportion of 1:10. Liquid samples, such as wort and beer, were tested directly without the extraction. The extract was centrifuged and the supernatant was transferred to a clean tube, and antibodies against the hydrophobin of *F. poae* were added. After incubation the sample-antibody mixture was transferred to at least three replicate wells of an immunoplate (Nunc-Immuo Modules, MaxiSorp polystyrene strips, Nunc, Rochester, USA) coated with a hydrophobin extract of *F. poae*. Goat anti-rabbit IgG (H + L)–alkaline phosphatase (AP) detection. The absorbance was read at 405 nm using a MultiSkan Ex microtiter plate reader ( Labsystems, Helsinki, Finland) and the mean of the absorbance values of the replicate wells was calculated. Because of the nature of the competitive ELISA, a lower absorbance value corresponds to a higher amount of hydrophobins in the sample. The hydrophobin level is expressed as the inverse of the mean absorbance value (1/Abs). A standard deviation was calculated using the Microsoft Office Excel 2003 program. A standard curve generated from purified hydrophobins of *F. poae* was used to approximate the hydrophobin content in the samples collected and analysed by the hydrophobin ELISA.

**Gushing potential**

Gushing potential of malt was determined according to the gushing test described by Vaag et al. using a horizontally rotating shaker (50 rpm). In this test, an aqueous
Extract of ground malt was added to bottled beers 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.

RESULTS AND DISCUSSION

Formation of hydrophobins during the growing period of barley

Fungi, especially Fusarium species, are known to be able to produce gushing factors during the growing period of barley\(^4\). In order to study the formation of hydrophobins during the growing period, head samples of the non-inoculated and two F. culmorum inoculated barleys were collected from two fields approximately 3, 5, 7 and 9 weeks after inoculation. The weather conditions during the field trials favored the growth of Fusarium spp., resulting in high infection rates in the head samples, and in the harvested barleys 78–100% of the kernels were contaminated with Fusarium spp\(^5\). Hydrophobin levels in the freeze-dried head samples were determined in duplicate using the hydrophobin ELISA. The results revealed that hydrophobins were present in the F. culmorum infected heads three weeks after inoculation and the formation of hydrophobins continued throughout the growing period (Fig. 1). Differences between the infection rates of the individual heads randomly collected from the 10 m\(^2\) test blocks could cause fluctuations in the results of the sensitive hydrophobin ELISA. This could explain the reduction in hydrophobin level of the F. culmorum inoculated sample I taken 7 weeks after inoculation, compared to the hydrophobin level of the sample taken 2 weeks earlier.

Hydrophobins in the malting process

Fusarium fungi are able to proliferate and produce gushing factors during the malting process\(^4\). The results of the hydrophobin ELISA of the two non-inoculated and the three Fusarium-inoculated barley samples collected during the lab-scale malting process revealed that hydrophobins were also produced during malting, especially during the steeping and germination steps (Fig. 2). Lower hydrophobin levels were detected in the final malt after removal of the rootlets. A comparison between the estimated amounts of hydrophobins detected in barley and in malt revealed that over tenfold higher amounts of hydrophobins were found in malt, compared to those in the corresponding barley (data not shown). The highest hydrophobin levels were detected in the malts produced from inoculated barleys inoculated with F. culmorum and F. graminearum (Fig. 2). These malts also induced the most vigorous gushing in the gushing test (data not shown).

Relatively high initial hydrophobin levels were detected in barleys inoculated with F. culmorum and F. graminearum (Fig. 2), which made them easily distinguishable from the non-gushing barley samples based on the results of the hydrophobin ELISA. However, the formation of hydrophobins during malting complicates the use of the hydrophobin analysis to predict the gushing potential directly from barley with a low initial hydrophobin level, as in the case of the F. poae inoculated samples.
(Fig. 2). A short incubation of moist barley in order to activate the hydrophobin production prior to the hydrophobin analysis could help to overcome this problem.

Samples of the two non-inoculated barley lots I and II, from the field trials were malted two or three times during the storage period of 17 months at 15°C. The hydrophobin level and the gushing potential of the malts were determined after each malting. The results showed reduction in hydrophobin formation as well as in the gushing potential as a result of prolonged storage (Table I). These findings are consistent with the results reported by Munar and Seibee, who also observed a substantial loss of gushing activity of hydrophobin in barley stored for 5–6 months, the hydrophobin level of the malt Ia produced from barley I was three times higher than that of the malt IIa produced from barley II (Table I). This observation revealed the significant effect of malt on the hydrophobin formation if the barley lot is contaminated with viable gushing-active Fusarium species, as in the case of the non-inoculated barley II.

**Hydrophobins in mashing**

Hydrophobins must be released from malt in the mashing process, survive the process conditions and end up in the wort in order to be able to induce gushing in beer. In order to study the behavior of hydrophobins during mashing, we mashed both gushing positive and negative malt samples on a laboratory scale and determined the hydrophobin levels of the corresponding wort, freeze-dried spent grains, freeze-dried cold break and wort. The results of the hydrophobin ELISA indicated that hydrophobins were partly extracted into the wort during mashing, although hydrophobins were also detected in spent grains and cold break (Fig. 3). When the estimated extractable hydrophobin content of malt was calculated and compared with the hydrophobin content of the corresponding wort (data not shown), a substantial loss of hydrophobins was observed to occur during mashing; at most 20% of the hydrophobins present in malt grist was found in wort. In this study we mashed the malt samples according to one standard mashing procedure. Changes in mashing procedure, such as in a proteolytic stand or in agitation speed, could affect the hydrophobin levels that end up in the wort.

**Purified hydrophobins in wort boiling and beer filtration**

The results of the gushing test of the boiled and unboiled hydrophobin + wort and hydrophobin + water samples are shown in Table II. The gushing inducing potential of the two different hydrophobins was determined after each malting. The results showed reduction in hydrophobin formation as well as in the gushing potential as a result of prolonged storage (Table I). These findings are consistent with the results reported by Munar and Seibee, who also observed a substantial loss of gushing activity of hydrophobin in barley stored for 5–6 months, the hydrophobin level of the malt Ia produced from barley I was three times higher than that of the malt IIa produced from barley II (Table I). This observation revealed the significant effect of malt on the hydrophobin formation if the barley lot is contaminated with viable gushing-active Fusarium species, as in the case of the non-inoculated barley II.

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of the boiled hydrophobin + wort samples was observed to be lower than that of the corresponding unboiled samples, suggesting that some of the hydrophobins were precipitated or inactivated during wort boiling. Boiling alone could not destroy or inactivate hydrophobins, because the gushing inducing potential of the boiled hydrophobin (50 µg) + water samples was comparable to that of the unboiled hydrophobin (50 µg) + wort samples (Table II). The results indicate that wort contains substances which interact with hydrophobins in wort boiling, causing a reduction in the gushing activity of hydrophobins.

The effect of beer filtration on hydrophobin levels was also studied by filtering the hydrophobin-water mixtures through the beer filtration unit. The results of the filtration studies suggested that part of the hydrophobins can be removed by filtration (data not shown). Based on the properties of hydrophobins, it can be assumed that hydrophobins adhere to the filter sheets or that they form aggregates large enough to be retained by the filters. However, the level of reduction was dependent on the initial hydrophobin concentration of the sample; with a hydrophobin concentration of 15 µg/mL, 60% of the hydrophobins were lost during the filtration, compared to a loss of only 15% with a hydrophobin concentration of 5 µg/mL. Robinson et al. observed that the filter sheets removed proteins until saturated, after which the protein contents of the filtrated beers were at the same level as that of the beers before filtration. The saturation of filter sheets could also occur with hydrophobins decreasing the removing effect of filtration.

**Hydrophobins in the pilot-scale brewing process**

Hydrophobin levels were studied throughout the brewing process by brewing the gushing negative and positive malts on a 10-liter scale. As can be seen from Fig. 4, the beer brewed from the gushing positive malt contained a higher amount of hydrophobins than the beer brewed from the gushing negative malt. The former beer overfoamed when shaken according to the gushing test, indicating that a hydrophobin level high enough to induce gushing of beer was first extracted from the malt and then survived the brewing process. As in the mashing studies (Fig. 3), some of the hydrophobins were removed with spent grains and cold break (wort boiling), but also with surplus yeast (data not shown). In addition, the beer filtration step reduced the hydrophobin levels (Fig. 4).

The changes in the relative levels of hydrophobins during brewing were calculated based on the approximation of the total hydrophobin content of the gushing positive malt and the corresponding brewing liquors (Fig. 5). The

![Fig. 3. Hydrophobin levels in aqueous extracts of malt grist, spent grains and cold break, and in wort of the gushing negative and positive malt samples mashed on a laboratory scale. Hydrophobin levels were analyzed using the hydrophobin ELISA (n = 3). Standard deviation bars are included.](image-url)

Table II. The effect of boiling on the gushing inducing ability of *T. reesei* HFBII hydrophobin boiled in wort or in water. The test was performed in duplicate.

<table>
<thead>
<tr>
<th>HFBII addition</th>
<th>Boiled</th>
<th>Unboiled</th>
</tr>
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<tbody>
<tr>
<td>0 µg in wort</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10 µg in wort</td>
<td>38</td>
<td>188</td>
</tr>
<tr>
<td>50 µg in wort</td>
<td>149</td>
<td>199</td>
</tr>
<tr>
<td>100 µg in wort</td>
<td>173</td>
<td>—</td>
</tr>
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---: not determined.
calculation revealed that most of the hydrophobins originating from the gushing positive malt were removed with the spent grains (up to 70%). In addition, wort boiling, fermentation and beer filtration steps reduced the hydrophobin content to such an extent that only approximately 10% of the original hydrophobin content of the gushing positive malt was present in the finished beer. These results are in accordance with the results obtained in the

Fig. 4. The effects of wort boiling and beer filtration on the hydrophobin levels of brewing liquors produced from the gushing negative and positive malts. Hydrophobin levels were analyzed using the hydrophobin ELISA (n = 4). Standard deviation bars are included.

Fig. 5. Relative levels of hydrophobins in the pilot-scale brewing process. The calculated amounts of hydrophobins in brewing liquors were compared to the calculated amount of hydrophobins in gushing positive malt used for brewing.
laboratory scale mashing studies, as well as with the results obtained in the boiling and filtration studies of the purified hydrophobins.

CONCLUSIONS

*Fusarium* fungi were able to produce hydrophobins in the field during the growing period of barley, as well as during the malting process, especially during the steeping and germination steps. Due to the decreased viability of *Fusarium* spp, the capability of fungi to produce hydrophobins was reduced during prolonged storage of barley. Hydrophobins were extracted during mashing and a portion survived the brewing process, ending up in the final beer where they induced gushing when present in sufficiently high levels. Hydrophobins were removed from the brewing process mainly with spent grains, but also with cold break and surplus yeast. In addition, the beer filtration step reduced the hydrophobin level of the final beer. The estimation of hydrophobin content, based on the hydrophobin ELISA results of the samples collected during the pilot-scale brewing study, showed that only about 10% of the hydrophobin present in the gushing positive malt was found in the final beer.

Our studies revealed that the amount of hydrophobins can increase during malting, but decrease during brewing. Malting and brewing processes vary between different malt houses and breweries, which may significantly influence the production and the fate of hydrophobins during the beer production chain. Further large scale studies are required to investigate the extent of the effects of commercial malting and brewing processes on the hydrophobin content migrating in the beer production chain.

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REFERENCES


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