Minireview:

Are Hydrophobins and/or Non-Specific Lipid Transfer Proteins Responsible for Gushing in Beer? New Hypotheses on the Chemical Nature of Gushing Inducing Factors

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Gushing of beer is characterised by the fact that immediately after opening a bottle a great number of fine bubbles are created throughout the volume of beer and ascend quickly under foam formation, which flows out of the bottle. This infuriating gushing phenomenon has been, and still is, a problem of world-wide importance to the brewing industry.

It is generally assumed that the causes of malt-derived gushing are due to the use of "weathered" barley or wheat and the growth of moulds in the field, during storage and malting. We now develop a hypothesis connecting several lines of evidence from different laboratories. These results indicate that the fungal hydrophobins, hydrophobic components of conidiospores or aerial mycelia, are gushing-inducing factors. Furthermore, increased formation of ns-LTPs (non-specific lipid transfer proteins), synthesised in grains as response to fungal infection, and their modification during the brewing process may be responsible for malt-derived gushing.

Introduction

Regarding the causes of gushing, the research group of Carlsberg (Gjertsen *et al.*, 1963; Gjertsen, 1967) proposed to divide them into two types: "primary gushing" which occurs periodically and appears to be related to the quality of malt, and "secondary gushing" which is due to faults during beer production or to the incorrect treatments of packaged beer.

As a result of the continued efforts to clarify the cause of gushing in beer for the past 50 years it has been shown that the primary gushing is most probably caused by mould growth, either before threshing (field fungi like *Fusarium, Alternaria, Stemphylium, Cladosporium*), during storage (storage fungi like *Aspergillus, Penicillium* or *Rhizopus*) or during malting (Gjertsen *et al.*, 1965; Amaha *et al.*, 1973; Gyllang and Martinson, 1976; Haikara, 1980; Weideneder, 1992; Niessen, 1993; Zepf, 1998).

Prentice and Sloey (1960) and Sloey and Prentice (1962) clearly showed, that especially the *Fusaria*

cause changes in the analytical values of malt samples, such as increase in α-amylase activity, soluble nitrogen, extract and gushing. Gjertsen *et al.* (1965) confirmed in experimental malting tests that inoculation of several *Fusarium* cultures onto barley grains during stepping could yield gushing malts. The addition to the mash of mycelial extracts or culture filtrate of *Fusarium* failed to show gushing in beer. The authors concluded that gushing is not caused by the *Fusarium* itself but as result of an interaction between *Fusarium* and the germinating grain. Several research groups tried to isolate and to characterise substances from culture filtrates of different moulds, but also from malt, mash, wort and gushing beer, which could induce malt-derived gushing (see below), but up to now, the exact chemical nature and the physical and chemical mechanisms of these substances are unknown.

In this communication new hypotheses on the molecular identity of factors responsible for the primary gushing based on published data will be presented.

The "hydrophobin story"

In the 70th Amaha, Kitabatake and co-workers succeeded in the isolation and characterisation of a gushing-inducing substance, produced by *Nigrospora* sp. no 207 in a liquid culture medium (Amaha *et al.,* 1973; Kitabatake and Amaha, 1974, 1977). The authors showed that the "*Nigrospora* gushing factor" (NGF) must be a hydrophobic polypeptide with a molecular weight of 16.5 kDa and an isoelectric point of 4.0. NGF is water soluble, heat stable, surface active, and resistant to digestion by proteases. Amino acid analyses revealed that NGF is lacking the four residues methionine, histidine, tyrosine and tryptophan, but containing 16 cysteine residues forming 8 intramolecular disulfide bonds.

According to its molecular and chemical properties it is most likely that NGF is identical to a hydrophobin dimer (Table 1). Hydrophobins, which were discovered in the 90th, are small secreted fungal proteins with molecular weights between 7 and 20 kDa (Wessels *et al.*, 1991). They are among the most surface-active molecules and self-assemble to an amphiphilic film at any hydrophobic-hydrophilic interface. These films are insoluble and exhibit a typical ultrastructure of a mosaic of interwoven rodlets (Kershaw and Talbot, 1998; Wösten and de Vocht, 2000).

Hydrophobin encoated rodlet layers are common features of aerial structures including mushroom caps, spore surfaces and aerial hyphae. They have been shown to be important in many morphogenetic processes, including sporulation, fruit body development and infection structure forma-

tion. Hydrophobins are therefore likely to be ubiquitous in filamentous fungi (Wessels, 1997).

The water soluble monomers contain 8 cysteine residues forming 4 intramolecular disulfide bonds in a particular manner within their amino acid sequence. The aromatic amino acids tyrosine and tryptophan are always lacking, methionine and histidine could likewise be absent. Besides this structural similarities hydrophobins and the Japanese gushing factor are secreted in submerged liquid cultures without an infection of barley or wheat grains.

In view of the above mentioned data we make the following assertion: NGF is most likely identical to a hydrophobin dimer. According to their strong surface activity hydrophobins are able to induce gushing in beer. Furthermore, the rodlet layers with their typical rough surfaces may serve as condensation nuclei for the release of CO_2-bubbles. As components of conidiospores or aerial mycelia from field- as well as storage-fungi the entry of hydrophobins *via* contaminated grains into beer is easy to imagine.

The role of "ns-LTPs"

The Japanese succeeded in the isolation of a second gushing-inducing factor, which they extracted from wort (Kitabatake, 1978). The malt sample used to prepare the wort was a commercial lot product in the northern Europe, which was found to be gushing active. Unfortunately there are no data, whether the malt was contaminated or not and if, what kind of fungi were present.

The isolated gushing factor was found to be mainly composed of polypeptides (57%) accompanied with some portions of glucose (17%). The substance is acidic in nature and the molecular weight was estimated to be approximate 10 kDa. The amino acid composition was different from that of NGF and is characterised by extremely low levels of basic amino acid residues and comparatively higher amounts of acidic residues. The gushing factor is water soluble, heat stable, surface active and resistant to various enzymes such as proteases and carbohydrases; only Pronase was able to inactivate the wort factor.

Weideneder (1992) extracted gushing-inducing substances (GIS) from wheat malt which was strongly contaminated with *Fusarium culmorum*.

Table 1. Structural and chemical properties of the *Nigrospora*-derived gushing factor and a hydrophobin dimer in comparison.

Nigrospora-derived gushing factor or hydrophobin dimer
■ Fungal polypeptide
■ Water soluble
■ Heat stable
■ Resistant against proteases
■ Surface-active
■ MW: 16.5 kDa or 14–40 kDa
■ 8 intramolecular disulfide bonds
■ Lack of tryptophan and tyrosine
■ Lack of methionine and histidine
■ Secretion into cultur media
■ No infection process necessary for production

These GIS were shown to be water soluble, heat stable and surface active. They were polypeptides with molecular weights of about 10 kDa and extreme acidic isoelectric points between 1.8 and 3.5. In his comprehensive work Weideneder investigated the molecular weight distribution of proteins extracted from control (uninfected) wheat malt (Fig. 1, lane a) and from wheat grains infected by *Fusarium* (Fig. 1, lane b). The SDS-polyacrylamide gel electrophoretic pattern of the infected malt-extract gave a broad band in the region of 10 kDa in comparison to the control, meaning a large quantity of protein. By preparative isoelectric focusing Weideneder (1992) split up the protein extract from contaminated wheat grains in 30 subfractions depending on pH (Fig. 2). After dialysis against water every subfraction was tested to be gushing active by addition of two ml of the dialysate to 500 ml bottled non-gushing beer. The gushing activity of the bottled beer was then measured by the method described in detail by Weideneder (1992) and estimated with the volumes (ml) overflowed from the opened bottle. As shown in Fig. 2 only the subfractions with pH values below 4.0 exhibited a gushing-inducing potential. Further gel electrophoretic analyses of the protein-composition of these acidic subfractions revealed that not a single protein but quite a number of proteins with different isoelectric points were contained in every subfraction (Fig. 3). Weideneder did not comment or discuss these findings and he did not

Fig. 2. Protein content, pH values and gushing activities of subfractions from a protein extract derived from wheat kernels, infected by *Fusarium*. The gushing activities of the subfractions were tested by addition of 2 ml of the corresponding dialysates to 500 ml bottled non gushing beer and were estimated with the volumes (ml) overflowed from the opened bottles. Experimental conditions and preparation of the protein-extract and the subfractions respectively were as described by Weideneder (1992).

determine the molecular masses of these proteins. Remembering the molecular weight distribution of the unfractionated protein extract (Fig. 1, lane b), we conclude that they could possibly be about 10 kDa.

Small polypeptides exhibiting properties like the Japanese wort component or the gushing-inducing substances from Weideneder are to be found in
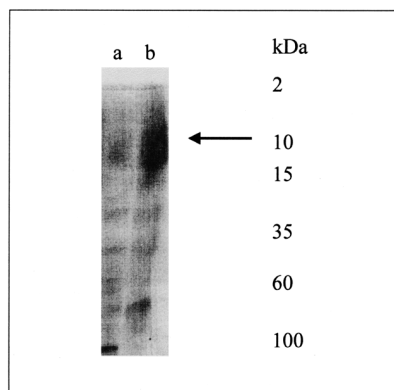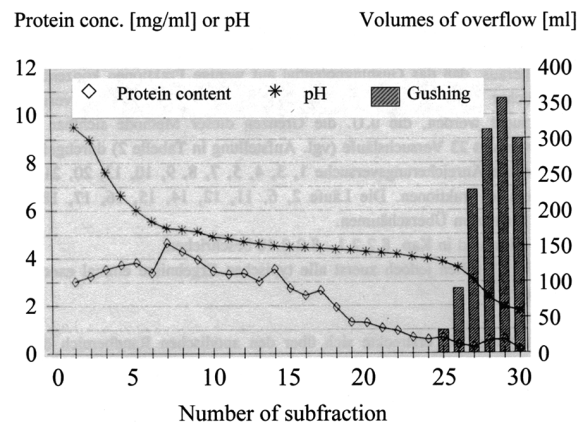

Fig. 1. SDS-polyacrylamide gel electrophoretic pattern of protein extracts derived from uncontaminated wheat grains (lane a) and with *Fusarium* contaminated wheat kernels (lane b). Experimental conditions and preparation of protein extracts were as described by Weideneder (1992).
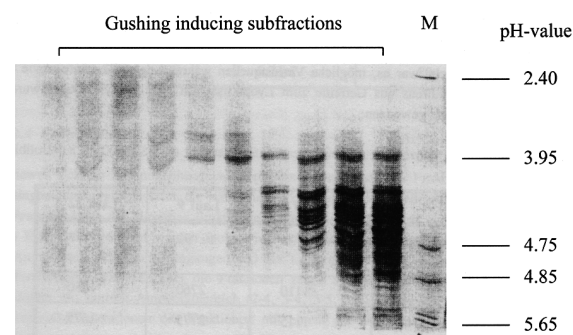

Fig. 3. SDS-polyacrylamide gel electrophoretic pattern of proteins contained in the acidic subfractions shown in Fig. 2. The proteins of every subfraction were seperated with the aid of isoelectric focusing (IEF). Experimental conditions were as described by Weideneder (1992). M: IEF-Marker. The original illustration was not anymore available. This Fig. was scanned what explains the poor quality. The same holds for Fig. 1.

the multigenic family of non-specific lipid transfer proteins (ns-LTPs). ns-LTPs are ubiquitous lipid binding proteins in plants. They have been comprehensively reviewed by Yamada (1992) or Kader (1996). Two main groups of ns-LTPs, ns-LTP1 and ns-LTP2 have been identified with molecular masses of about 10 and 7 kDa, respectively.

ns-LTP1 are synthesised with a N-terminal signal peptide, which indicates that they follow the secretory pathway, in agreement with their localisation in extracellular layers, i.e. cell walls or cutin. Furthermore, ns-LTP1 are abundant soluble proteins of the aleurone layers from grain endosperms!

Determination of the three-dimensional structures of ns-LTP1 has shown that these proteins share a common folding, stabilised by four disulfide bonds, and are characterised by a four helix bundle covered by a long C-terminal arm with no regular secondary structure. The loose folding of the hydrophobic side chains in the protein core can produce a cavity, which is able to bind different types of monoacylated and diacylated lipid molecules. Such cavities have been seen in barley, wheat, maize and rice ns-LTP1 (Heinemann *et al.*, 1996; Gincel *et al.*, 1994; Shin *et al.*, 1995; Chavolin *et al.*, 1999; Gomar *et al.*, 1996; Lee *et al.*, 1998).

ns-LTP1 is water soluble, heat stable and surface-active adsorbing at air-water interfaces (Subirade *et al.*, 1995).

The isoelectric point of ns-LTP1-proteins range between 8.8 and 10, depending on the plant source. The basic nature of ns-LTP1 is in contrast to the acidic nature of the Japanese wort component and the gushing-inducing substances from Weideneder, but this inconsistence will be addressed later.

As already mentioned ns-LTP1 can facilitate the transfer of a broad range of lipids from donor to acceptor membranes – *in vitro*! The true *in vivo* function remains to be established. It was suggested that they are involved in the formation of cutin layers (Sterk *et al.*, 1991). The observation of antimicrobial activities of ns-LTP1 *in vitro* led to speculation of a possible role in the defence of plants against pathogens (García-Olmedo *et al.*, 1995). Furthermore, several LTP genes in barley are upregulated in response to both the infection by various strains of fungal pathogens as well as abiotic environmental factors such as extreme temperatures and salt or drought stresses (García-Olmedo *et al.*, 1995).

However, in brewing technology the formation and stability of foam are important criteria concerning beer quality. Sørensen and co-workers (1993) reported that barley LTP1 is involved in beer foam formation. LTP1 purified from beer created a good beer foam with high potential in a foam assay. In contrast LTP1 purified from barley displayed only poor foaming properties. The authors concluded that LTP1 is modified during the malting and brewing processes, whereas the modified LTP1 species is the most important one for the beer foam potential.

Jégou and co-workers (2000) investigated the chemical modifications of barley LTP1 that occur during the malting and brewing process, by comparing the proteins isolated from seeds and beer. In one protein fraction containing barley LTP1 the authors found a major compound with a molecular mass of 9689 Da and a minor peak with a molecular mass of 9525 Da with the aid of electrospray mass spectrometry technology (Fig. 4a). The former is identical with the theoretical mass of barley LTP1, the latter corresponds to LTP1 having lost the C-terminal tyrosine residue *via* carboxypeptidase activity in seeds.

The mass spectrum of the LTP1-fraction from beer showed multiple peaks, whereas the first two peaks were identical with the barley LTP1 peaks (Fig. 4b). The masses of the other peaks corresponded to adducts of one or several units with a mass of about 162 Da. Jégou *et al.* (2000) showed that these adducts correspond to glucose units that were covalently bound upon the protein *via* Maillard-reaction.

The Maillard-reaction is a well known non-enzymatic reaction which can form covalent linkages between reducing sugars and protein-free amino groups and is preferred by high-temperature treatments during the brewing processes.

Besides glycosylation the authors observed cleavage of disulfide bonds. In case of reduction of all four disulfide bonds the protein becomes totally unfolded. Glycosylation increases the solubility, whereas unfolding leads to an exposition of hydrophobic amino acid residues which are normally involved in the formation of the hydrophobic cavity. Both processes increase the amphiphilicity of modified LTP1 coming along with a better effi-

a LTP1-fraction from
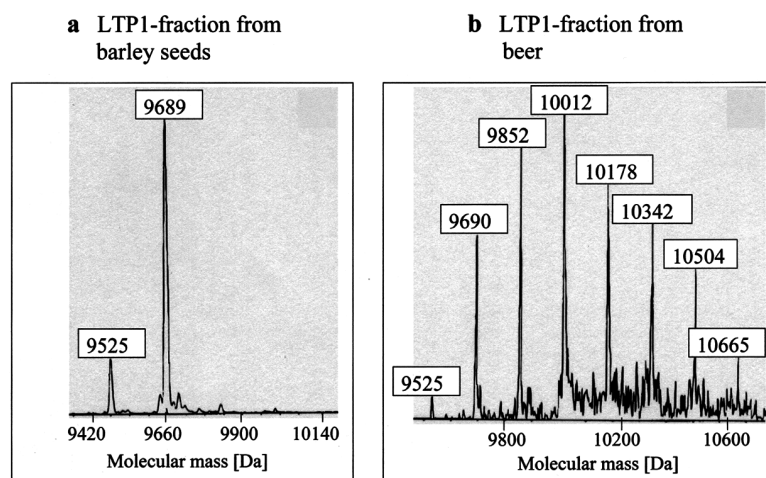barley seeds

b LTP1-fraction from
beer

Fig. 4. Deconvoluted and reconstructed electrospray mass spectra from multicharged ions spectra of LTP1 from barley seeds (a) and from beer (b). Experimental conditions were as described by Jégou *et al.* (2000).

ciency to form foam in comparison with native barley LTP1.

Regarding to the Maillard-reaction reducing sugars react with free amino acid groups of proteins as mentioned above. Available groups are the ε-NH$_2$ of the side chain of lysine residues and the guanidino amino group of arginine residues. This is a very important point, because lysine as well as arginine are basic amino acids, which are together with the histidine residues responsible for the basic nature of ns-LTP1. It was shown, that under oxidative conditions histidine could convert to aspartic acid and proline to glutamic acid (Brnjas-Kraljevic *et al.*, 1991).

With regard to the amino acid composition of non modified barley LTP1 (Table 2) the protein contains eleven basic and seven acidic amino acid residues. Modifications *via* glycosylation and oxi-

Table 2. Amino acid composition of barley lipid transfer protein 1 and kind of modification.

Amino acid	Amount of *basic*/**acidic** residues	Modification
Lysine	*4*	4 → neutralisation *via* Maillard-reaction
Arginine	*5*	5 → neutralisation *via* Maillard-reaction
Histidine	*2*	2 → oxidative conversion to aspartic acid
Glutamic acid	**2**	**2**
Aspartic acid	**5**	**5**
Proline	6	6 → oxidative conversion to glutamic acid
Threonine	3	
Serine	8	
Glycine	9	
Alanine	4	
Valine	6	
Leucine	6	
Isoleucine	6	
Methionine	1	
Cysteine	8	
Tyrosine	3	
Asparagine	9	
Glutamine	4	
Total	*11 basic*/**7 acidic** residues	*no basic* residues/**15 acidic** residues

dation reveal a partial or total loss of basic residues and an accumulation of negatively charged residues. These modifications must be accompanied with a shift of the isoelectric point from basic to acidic. One can imagine, that depending on mode and degree of modification quite a number of LTP1-species could be generated with molecular masses among 10 kDa and multiple acidic isolectric points. Remembering the gushing-inducing substances of Weideneder (Fig. 3) we assert, that the proteins he found in the acidic, gushing inducing subfractions must be modified LTP1-species with acidic isoeloctric points.

Remembering the chemical properties of the Japanese wort component (MG: 10 kDa; acidic IP; composition: amino acids (57%) and glucose (17%); water soluble; heat stable and surface-active) it seems that these gushing factor is likewise identical with modified LTP1.

Concluding we make a second assertion: The Japanese wort component as well as the gushing-inducing substances of Weideneder are most likely modified ns-LTP1-species.

Modified ns-LTP1-species are normal and wanted ingredients of beer, because they mainly contribute to foam formation as mentioned above. We assert, that gushing occurs whenever the content of modified ns-LTP1-species exceeds a threshold value. Up to now two hypothetical reasons for an oversupply of modified ns-LTP1 would be conceivable: During growth and ripening and also during harvesting and storage barley or wheat grains are more or less contaminated with a considerable variety of microorganisms. In response to the infection the LTP1-genes in embryos are upregulated resulting in an increased level of defensive LTP1-protein compared to uninfected grains. The synthesis of LTP1 in response to pathogen infestation could occur in the field, where especially *Fusarium* could exhibit strong LTP1-inducing potentials due to growing inside the kernels. But also during storage and germination induction of LTP1 could continue or just set on in response to storage fungi or other microorganisms. An interesting point is that primary gushing was observed sometimes although no (visible) contamination of the harvest (especially barley) could be determined (private information). May be in this special cases the activity of storage microflora was sufficient to exceed the tolerable LTP1-levels in the finished beers.

The second possibility in causing elevated LTP1-levels in beer rests on metabolic activities of microorganisms populate the grains resulting in the release of cell wall bound ns-LTP1, which normally remains in the draff.

Indirect evidence for this second possibility has been offered by Zepf (1998). He infected sterilised draff as relevant but on nutrient poor medium with multiple *Fusarium* strains to investigate the potential of these fungi in producing a gushing active factor.

With the knowledge about the acidic nature of gushing-inducing substances he analysed the pH values of worts prepared from the infected draffs and surprisingly he found not a decrease but an increase in pH in most of the samples (Fig. 5). Zepf had no idea or explanation for these findings and there was no further working on this topic.

We assert, that the increase of pH was due to the release of basic compounds either originating from the fungi themselves or from the nutrient medium as a consequence of metabolic activity during fungal growth. In view of the results of Gjertsen *et al.* (1965), mentioned above and Weideneder (Fig. 1) and regarding the fact, that native LTP1 is basic in nature, we assert, that the increase in pH is due to a release of non-modified LTP1 – a side effect of destructive metabolic processes caused by *Fusarium*.
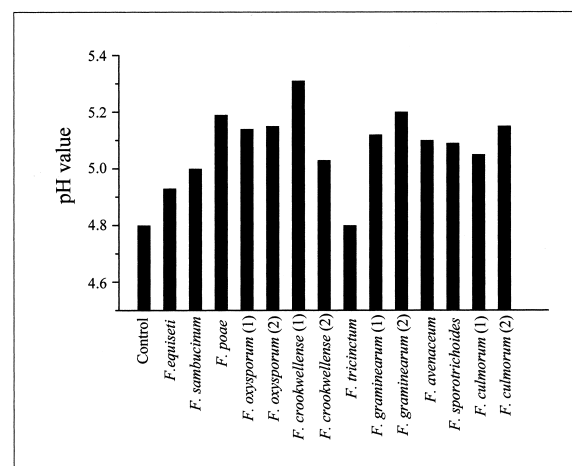

Fig. 5. pH-values of wort samples which were prepared from draff, infected with different *Fusarium* strains. Uncontaminated draff served as control. Experimental conditions were as described by Zepf (1998).

Conclusions

The chemical nature of already described malt-derived gushing inducing factors has been elucidated. By studying early publications from the 60th and 70th, which demonstrated the close connection between mould growth on grains and primary gushing, and very recently published data concerning biochemical and molecular properties of surface-active proteins, we conclude that the two groups of small, cysteine-rich, water soluble, heat stable and surface-active polypeptides, the fungal hydrophobins and the plant-derived ns-LTPs, are responsible for malt-derived gushing in beer.

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