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Role of ns-LTP1 in the Development of Primary Gushing

Our investigations were focussed on ns-LTP1 (non specific lipid transfer protein 1) as the main inductor of the primary gushing phenomenon. We asserted that gushing beer contains more ns-LTP1 than non-gushing beer and these overbalance is responsible for over foaming. Surprisingly, less to non ns-LTP1 was detectable in a set of gushing beers.

We showed that loss of ns-LTP1 depends on fungal infestation and heating procedure. ns-LTP1 degrading activity of heated culture filtrates of *Fusarium culmorum* and *Fusarium graminearum* support the assumption that heat stable extracellular proteinases secreted by the two Fusaria strains are responsible for protein degradation. Heating procedure also results in a destruction of naturally occurring proteinase inhibitors in wheat kernels. This is the condition precedent to an effective operation of the fungal proteases.

Furthermore our results support the conception that not ns-LTP1 itself, but rather glycated peptides generated during proteolytic fragmentation of modified ns-LTP1 species initiate gushing activity.

Descriptors: gushing, lipid tansfer protein 1, wheat, ELISA, *Fusaria*

1 Introduction

Gushing is a phenomenon of vigorous effervescence of beer. Upon the opening of a bottle a great number of fine bubbles are being formed spontaneously throughout the volume of beer, which ascend quickly under foam formation, and shoot out of the bottle. This infuriating gushing phenomenon has been and is still a problem of world-wide importance to the brewing industry.

The research group of *Carlsberg* [1, 2] divide gushing 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. In this paper primary gushing is discussed solely.

Prentice and *Sloey* [3] and Sloey and Prentice [4] 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. [5] confirmed in experimental malting tests that inoculation of several barley grains with *Fusarium* cultures during steeping 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 a result of an interaction between *Fusarium* and the germinating grain. Several research groups tried to isolate and characterise substances from culture filtrates of different moulds, but also from malt, mash, wort and gushing beer, which could induce malt-derived gushing [6-10]. But until now, the exact chemical nature and the physical and chemical mechanisms of these substances are unknown.

In 2002 we published new hypotheses on the chemical nature of gushing inducing factors [11]. Beside the fungal hydrophobines the group of non specific lipid transfer proteins (ns-LTPs) synthesised in grains as a response to fungal infection and their modification during the brewing process, was postulated to be responsible for malt-derived gushing.

ns-LTPs are ubiquitous lipid binding proteins in plants with a localization in extracellular layers, i. e. cell walls or cutin. Furthermore, ns-LTPs are abundant soluble proteins of the aleurone layers from grain endosperms. They have been comprehensively reviewed by *Yamada* [12] or *Kader* [13]. 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 is water soluble, heat stable and surface-active [14]. The isoelectric point of ns-LTP ranges between 8.8 and 10 depending on the plant source. The alkaline nature of ns-LTP1 is in contrast to the acidic nature of the Japanese wort component [6, 9] and the gushing inducing substances from *Weideneder* [10].

Sørensen and co-workers [15] reported that barley ns-LTP1 is involved in beer foam formation. ns-LTP1 purified from beer created a good beer foam with high potential in a foam assay. In contrast ns-LTP1 purified from barley displayed only poor foaming properties. The authors concluded that ns-LTP1 is modified during the malting and brewing processes, whereas the modified ns-LTP1 species is the most important one for the beer foam potential.

Jégou and co-workers [16] identified these modified ns-LTP1 species as sugar-adducts generated via Maillard-reaction.

The Maillard-reaction is a well known non enzymatic reaction which can form covalent linkages between reducing sugars and free amino groups of proteins and is preferred by high-temperature treatments during the brewing processes. Available amino 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 into aspartic acid and proline into glutamic acid [17].

With regard to the amino acid composition of non modified barley ns-LTP1 the protein contains eleven basic and seven acidic amino

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Tables and Figures see Appendix

acid residues. We conclude that modifications via glycation and oxidation reveal a partial or total loss of basic residues and an accumulation of negatively charged residues [11]. These modifications have to be accompanied by a shift of the isoelectric point from basic to acidic explaining the acidic nature of the historical found gushing inducing factors. One can imagine that depending on mode and degree of modification quite a number of ns-LTP1-species could be generated with molecular masses among 10 kDa and multiple acidic isoelectric points.

Modified ns-LTP1-species are natural and desired 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 one hypothetical reason for an oversupply of modified ns-LTP1 is 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 ns-LTP1-genes in embryos are upregulated resulting in an increased level of defensive ns-LTP1-protein compared to uninfected grains.

This is in agreement with the observation of antimicrobial activities of ns-LTP1 ***in vitro*** and the upregulation of several LTP genes in barley 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 [18].

The synthesis of ns-LTP1 in response to pathogen infestation could occur in the field, where especially the *Fusaria* could exhibit strong ns-LTP1-inducing potentials due to growing inside the kernels. But also during storage and germination induction of ns-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 crop (especially barley) could be determined (private information). Maybe in this special cases the activity of storage microflora was sufficient to exceed the tolerable ns-LTP1-levels in the finished beers.

This paper reports on wheat ns-LTP1-contents in gushing beers compared to non gushing wheat beers as well as in uninfected and infected grain, malt and wort. The data support the hypothesis that ns-LTP1 is causally involved in the development of primary gushing, but in an unexpected way.

2 Materials and methods

Reagents

All reagents were analytical grade, unless specified otherwise.

Preparation of ns-LTP1 antibody and quantification by PTA ELISA

ns-LTP1 isolation from wheat

150 g of wheat malt were ground (Ultra Centrifugal Mill Type ZM1 with conidur holes of 0.25 mm, F.K. Retsch GmbH & Co, Haan, Germany) and the proteins were extracted with ammoniumacetate-buffer (0.1 M NH_4Ac, 2 mM L-cysteine, 0.1 M EDTA, pH: 5.5 with pure acetic acid) overnight at 4 °C. After centrifugation (12.000xg for 30 min) the pellet was discarded and ammonium sulphate was added to the supernatant to a saturation of 60 %. After 4 h of stirring at room temperature the protein solution was centrifuged (12.000xg for 30 min), the pellet was discarded and the supernatant was saturated to 90 % and stirred over night at 4 °C. Resulting solution was centrifuged (12.000xg for 30 min) and solved in H_2O_{dist} and desalted with Econo-Pac 10 DG Colums from Bio-Rad (USA). The solution was lyophilised and resuspended in H_2O_{dist}. Determination of protein concentration and separation of proteins according to size occurred by Bradford (Bio-Rad Protein Assay, Cat-No. 500-0006) and SDS-PAGE. For the visualisation of the proteins the gel was blotted on PVDF (Polyvinylidene Difluoride) membran and stained with Coomassie Brilliant Blue R 250 (CBB R250, Bio-Rad, USA). The protein band with a molecular weight of 10 kDa was determined by amino acid analysis by “Prosequenz Bioanalytik” (Germany) and identified as ns-LTP1 from wheat. For the isolation of ns-LTP1 from wheat the 10 kDa bands were cut out of the SDS-PAGE-gel (stained with CBB R250), collected, lyophilised and pulverized.

Production of polyclonal antibodies

For the production of polyclonal antibodies a rabbit was immunised with the “ns-LTP1-powder”. After certain time intervals blood was taken from the rabbit to produce antiserum (12 x). To control the sensitivity of the antibodies the antisera were tested in an immunoblot assay (see below) to detect ns-LTP1 in wheat malt. The six antisera with the highest sensitivity were pooled and the antibodies were purified (protein A-sepharose column) to remove cellular constituents. After lyophilisation the antibodies were used in ELISA. The polyclonal antibody production has been carried out by the company “pabproductions”, Germany.

PTA ELISA

Quantification of ns-LTP1 concentrations in aqueous solutions were carried out by PTA ELISA (plate trapped antigen-enzyme linked immunosorbent assay). 96 well Maxisorp microtiter plates (Nunc, Denmark) were coated with different concentrations of the samples (=antigen) diluted in coating-buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, adjust to pH 9.6 with HCl) over night at room temperature. The antigen solution was discarded and the plates were washed three times with PBS (8.0 g NaCl, 0.2 g KH_2PO_4, 1.15 g Na_2HPO_4, 0.2 g KCl disolve in 900 ml H_2O_{bidist}, adjust pH to 7.4 with NaOH or HCl and make up to 1 l). To block the free binding-sites of the plates BSA (bovine serum albumin) diluted to 1 % with PBS were added to the wells for two hours at room temperature. After removal of the blocking solution the plates were washed three times with PBS-Tween 20 (0.05 % v/v Tween 20) and incubated with the primary antibody (rabbit-anti-LTP1-IgG) diluted in PBS (c=4 µg/ml) for two hours at room temperature. Alkaline phosphatase-labelled secondary antibody (Anti-Rabbit IgG, H&L (Goat) Alk. Phos. Conj., Calbiochem, USA, Cat-No. 401312,) diluted to 1:4000 with PBS was added to the wells after the primary antibody solution was discarded and the plates were washed. For quantification of the ns-LTP1 concentration the anti-rabbit-IgG-AP were removed, the plates were rinsed four times with PBS-Tween and the substrate solution (1 mg/ml 4- Nitrophenylphosphate·Na_2-salt (Serva, Germany, Cat-No. 30770) prepared in substrate buffer (97 ml Diethanolamine (Sigma-Aldrich) in 600 ml H_2O_{bidist}, adjust pH to 9.6 with HCl and make up to 1 l)) were added to each well. Enzymatic colour reaction was detected at a wavelength of 405 nm using a plate photometer (Titertek Plus MS 212, ICN, Germany).

Analyses of beer-proteins

1-SDS-PAGE

SDS-Polyacrylamide gel electrophoresis was performed according to Laemmli [19] using a Mini Protean II cell (Bio-Rad, USA) and 90 mm x 60 mm x 0.75 mm separating gels (T: 16.5 %, C: 2.6 %)

with stacking gel (T: 4%, C: 2.6 %). Samples were syntonized with loading buffer (2 x; 100 mM TrisHCl, pH 6.8, 12.5 % SDS (w/v), 25 % glycerine (87 %, v/v), 200 mM DTT (Dithiothreitol), 0.001 % Bromophenol blue (w/v)) to the desired concentrations.

2-D-SDS-PAGE

IEF (isoelectric focusing) was performed using Multiphor II system (Pharmacia, USA). 18 cm Immobiline DryStrips pH 3–10 NL (Amersham Biosience, USA) were rehydrated overnight at room temperature in 500 μl rehydration buffer (6.71 M Urea, 1.79 M Thiourea, 0.5 % w/v CHAPS, 0.2 % w/v DTT, 0.2 % w/v Pharmalyte 3-10) and 50 μg protein of each sample dissolved in 100 μl of CAPS lysis buffer (6.71 M Urea, 1.79 M Thiourea, 65 mM CAPS (2-Cyclohexylamino-1-propanesulfonic acid), 4 % w/v CHAPS (3-(3-Cholamidopropyl)-dimethylammonio-1-propansulfonat), 1 % w/v DTT, 0.5 % w/v Pharmalyte (ph 3-10, Amersham Pharmacia Biotech, USA) were cup-loaded on a IPG strip the next day. Protein focusing was achieved using the following IEF parameters: 150 V, step and hold, 150 Vh; 300 V, gradient, 900 Vh; 600 V, gradient, 600 Vh; 1000 V, gradient, 5 kVh; 3500 V, step and hold, 31.5 kVh, giving a total of 38.15 kVh. Strips were held at 100 V until removed and transferred immediately for the second-dimensional electrophoresis.

Prior to SDS-PAGE, the IPG strips were equilibrated for 15 min in 100 ml equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M Urea, 30 % w/v Glycerol, 2 % w/v SDS) containing 2 % w/v DTT and 15 min in 100 ml equilibration buffer containing 4 % w/v Iodoacetamide. The IPG strips were aligned on filter paper along one edge for 1 min to remove excess liquid before they were applied to vertical SDS gels. The equilibrated IPG strips were placed in close contact with the upper edge of the SDS gel. At the basic end of the IPG strips a sample application piece impregnated with 10 μl molecular weight marker (Precision Plus unstained, Bio-Rad, USA) was added and the strips were sealed with 2–3 ml of hot agarose (75 °C) solution containing 0.6 % w/v Agarose and 0.002 % w/v Bromophenol blue in electrode buffer (0.025 M Tris, 0.002 M SDS, 0.21 M Glycine).

SDS-PAGE was performed using 250 mm x 195 mm x 1 mm 15 % Polyacrylamide (30 %, 29:1) gels and run using a Hoefer DALT multiple slab gel unit (10 gels can run simultaneously). Settings for the protein separation were 50 mA (150 V) for 1 h followed by 80 mA (150 V) for 1 h and 165 mA (165 V) for 18 to 22 h at a constant 15 °C.

Immunoblotting

Sandwich immunoblotting was performed using Pharmacia LKB Nova Blot (semi dry). Prior to transfer of the proteins onto a nitrocellulose paper (0.2 μm, Pall Biosupport Membranes, USA) the gel (see 1-SDS-PAGE and 2-D-SDS-PAGE), the PVDF membrane (rinsed in Methanol for 1 min) and 4 pieces of filter paper were equilibrated in ice cold blotting buffer (10 mM CAPS, 10 % w/v Methanol, pH 11.0) for 20 min (gel) and 5 min, respectively. The electrotransfer of the proteins of 90 mm x 60 mm gels occurred at room temperature for 90 min at 0.8 mA/cm^2, the transfer of the proteins of 250 mm x 195 mm gels occurred at 2 °C for 12 h at 0.8 mA/cm^2.

After transfer the paper was washed with TBS (20 mM Tris HCl, 150 mM NaCl, pH 7.5) and transferred to TBS containing 1 % w/v Bovine serum albumin (blocking buffer) for 90 min at room temperature to block the free binding-sites. After another washing step (3 x TBST = TBS + 0.05 % v/v Tween 20) the immunoblot was incubated with rabbit anti-LTP1 primary antibodies diluted (1:2500) with blocking buffer for 60 min at room temperature. The rinsed paper (3 x TBST) was treated with secondary anti-rabbit antibodies conjugated with Alcaline phophatase diluted with blocking buffer (1:5000) for 1h at room temperature. 100 μl Nitro Blue tetrazolium chloride (NBT; 75 mg Nitro Blue tetrazolium chloride (Serva, Germany, Cat-No. 30550) in 1 ml N,N'-dimethylformamide (70 % v/v; Merck, Germany, Cat-No. 103053)) and 75 μl 5- Bromo -4-chloro-3-indolyl-phosphate·Na$_2$-salt (BCIP; 50 mg 5- Bromo -4-chloro-3-indolyl-phosphate·Na$_2$-salt (Serva, Germany, Cat-No. 15259) in 1 ml N,N-dimethylformamide 70 % v/v) + 1 ml deionised water) were added to 25 ml development solution (100 mM Tris HCl, 100 mM NaCl, 5 mM MgCl$_2$, pH 9.5). This solution was used as a substrate for the phosphatase reaction.

Protein preparation for immunochemical analyses and SDS-PAGE

Proteins of gushing and non-gushing beers (250 ml) were concentrated by fractionated salt precipitation carried out using a concentration of 90 % of ammonium sulphate $(NH_4)_2SO_4$. After stirring over night at 2°C the beer samples were centrifuged (12.000xg for 30 min) and the obtained pellets were redissolved in 30 ml distilled water, desalted by Econo-Pac 10 DG (Bio-Rad, USA) and stored at –20 °C until analysis.

For 1-SDS-PAGE and 2-D-SDS-PAGE the protein content of samples were determined by Bradford (Bio-Rad Protein Assay, Cat-No. 500-0006). Respectively, 1 μg (1-SDS-PAGE) and 50 μg (2-D-SDS-PAGE) of each sample were applied.

Determination of ns-LTP1 in non infested and infested aqueous extracts of wheat grain and malt as well as wort

Non infested wheat grain and malt were ground and the proteins were extracted with distilled water (100 g flour in 500 ml H_2O_{dist}). 100 ml of the aqueous extraction solutions were inoculated with mycelia of *Fusarium culmorum* and *F. graminearum,* respectively. After an incubation time of 12 days the solutions were filtrated and the filtrates were stored at –20 °C until analysis.

Wheat wort was inoculated with mycelia of either *Fusarium culmorum* or *Fusarium graminearum*. After an incubation time of 2 weeks growing mycelia were removed by filtration and the supernatants were stored at –20 °C until analysis.

Respectively, 100 ml of wheat wort were incubated in the presence of 2 ml of freeze-dried culture filtrates of *Fusarium culmorum* or *Fusarium graminearum*, resuspended in $H_2O_{dist.}$.

Culture filtrate preparation was done by inoculation of wheat wort (each 500 ml) with mycelia of the two *Fusaria* strains for 14 days at room temperature. After take out of the mycelia by filtration supernatants were boiled for 90 min and freeze-dried. The freeze-dried products were rehydrated with 10 ml $H_2O_{dist.}$.

ns-LTP1 contents of all samples were analysed using PTA ELISA.

Enzyme activities in non-gushing wheat beer

Ice cold non-gushing wheat beer (0.5 l bottles) was charged with 1 ml BSA-solution (3 mg/ml = control), 1 ml Trypsin (3 mg/ml, Sigma-Aldrich, Cat-No. T 1426) or 1 ml Proteinase K (3 mg/ml, Sigma-Aldrich, Cat-No. P 6556). After incubation at room temperature the bottles were opened after 10 days, gushing volumes were determined and the ns-LTP1 concentrations were analysed using PTA ELISA.

Gushing inducing potential of non infested and infested wort

500 ml of non infested wheat wort as well as wheat wort, infected with mycelium of *Fusarion culmorum*, were incubated for

14 days at room temperature. After filtration supernatants were boiled for 90 min and freeze-dried. The freeze-dried products were resuspended in 10 ml $H_2O_{dist.}$. Aliquots of 0.1, 0.5, 1 and 2 ml of the infected wort concentrate and 2 ml of non infected wort concentrate (control) were added to ice cold non-gushing plop-top wheat beer bottles (500 ml). After an incubation time of 2 h the over foaming volumes were determined and ns-LTP1 concentrations were analysed using PTA ELISA.

3 Results and discussion

In order to verify our hypothesis that gushing occurs anytime the content of modified ns-LTP1-species exceeds a threshold value, we compared protein profiles and ns-LTP1 contents of gushing and non gushing beers of the same brand.

In two dimensional SDS-PAGE non gushing beer (Fig. 1A) was characterised by a lot of different proteins, each indicated by a single stained spot. Particularly basic proteins and proteins smaller than 20 kDa seemed to lack in the over foaming beer (Fig. 1B).

Corresponding immunoblot analyses with a polyclonal antibody to wheat ns-LTP1 showed quite a number of protein spots in the non gushing beer (Fig. 1C) which differ to some extend in molecular weights, but mainly in isoelectric points. The differences in molecular weight were probably due to carboxypeptidase activity occurring already in seeds as reported by Jegou and co-workers [16]. Protein modifications during the malting and brewing processes account for differences in isoelectric points. Jegou et al. [16] showed that beer in contrast to seeds contains a set of glucose-ns-LTP1 adducts generated via Maillard-reaction. Regarding the reaction mechanism where reducing sugars react with available free amino acid groups of proteins, we speculated [11] that modification of ns-LTP1 via Maillard-reaction must be accompanied by a shift of the isoelectric point from alkaline to acid due to the loss of basic residues (ε-NH_2 of the side chain of lysine and guanidine amino group of arginine residues). The protein pattern shown in Figure 1C confirmes this assumption. Furthermore, Figure 1C demonstrates the capacity of the polyclonal antibody to bind to both alkaline (native) and acidic (modified) ns-LTP1 species.

Surprisingly less to non ns-LTP1 was detectable in the gushing beer (Fig. 1D). Further immunoblot analyses and quantification of ns-LTP1 using PTA-ELISA in gushing and non gushing beers of the same brand from different breweries support these finding. As shown in Figure 2 the over foaming types (indicated as AG, BG and CG) compared to the corresponding non gushing beers (indicated as A, B and C) show minimal ns-LTP1-contents. In the cases of AG and CG the concentrations were below 2 mg/500 ml as calculated in the antigen-enzyme linked immunosorbent assay and could not be visualised on the immunoblot.

Summarized, these results are contradictory to our hypothesis that guhing beer contains more ns-LTP1 than non-gushing beer and that this overbalance might be responsible for over foaming.

Further research were focused on the loss and relationships between loss, over foaming and infestation with *Fusarium* spp., known to be involved in malt-derived gushing.

Aqueous extracts of grounded non infested wheat grain and malt as well as hopped and for 90 min boiled wheat wort were inoculated with mycelia of *Fusarium culmorum* and *Fusarium graminearum*, respectively. After an incubation time of 14 days mycelia were removed by filtration and ns-LTP1 concentrations of the supernatants were measured by PTA ELISA. Control samples were not infected. As shown in Figure 3 no decline in ns-LTP1 contents were detectable in the infected grain (Fig. 3A) and malt (Fig. 3B) samples compared to uninfected controls. In contrast, significant reductions of the foam protein could be observed in the contaminated wort samples compared to the control (Fig. 3C). Interestingly, a decline in ns-LTP1 contained in the aqueous grain extract could be induced by boiling ahead of fungal infection. Control ns-LTP1 content was unaffected by heating procedure (Fig. 3D).

Supplementation of wort with heated culture filtrate of *Fusarium culmorum* or *Fusarium graminearum* was also able to cause a significant decrease of ns-LTP1 during a time scale of 4 weeks (Fig. 4).

We concluded that loss of ns-LTP1 depends on infestation and heating procedure. The activity of the heated culture filtrates strengthen the assumption that heat stable extracellular proteinases secreted by the two *Fusaria* strains are responsible for protein degradation.

To our knowledge little is known about the role of proteinases in the pathogenesis of cereal diseases. An alkaline proteinase from *Fusarium culmorum* was shown to contribute to the development of head blight [20]. The enzyme was characterised as a serine proteinase with a molecular mass of 28.7 kDa and was maximally active at pH 8.3-9.6 and 50°C, but was unstable under these conditions. Parts of the amino acid sequence were up to 82% homologous with those of several fungal subtilisins. *Hellweg* [21] isolated two subtilisin-like serine proteases from *Fusarium graminearum* culture medium with molecular weights of about 30 and 71 kDa, respectively. A putative role of the 30 kDa protease in the cleavage of structural proteins of the cell wall during infection of wheat was proposed. Recently it was shown [22] that growth of *Fusarium culmorum* on a medium containing thermostable proteins from potato tubers was accompanied by the production of proteinases exhibiting activity over a broad pH range (from 5 to 10). Proteinase activity was at the lowest value at pH 5.0 using azocasein as substrate. However, it should be noted that the enzyme activity at pH 5.0 may reflect the stability of *Fusarium culmorum* enzymes at acidic pH. Beer also exhibits pH values of about 5.0.

Furthermore, *Ievleva* and co-workers [22] showed that a near-complete inhibition of the enzymes was caused by proteinaceous proteinase inhibitors from potato tubers. One can imagine that similar proteinase inhibitors are present in wheat kernels. As shown in Figure 5 ns-LTP1 degradation in infested wort (Fig. 5B) was totally abolished by addition of non boiled aqueous wheat grain extract (Fig. 5C) indicating that inhibitory activity was destroyed by the heating procedure. Therefore ns-LTP1 decline only occurs in heat-treated samples as shown above.

ns-LTP1 degradation in non gushing wheat beer was initiated by adding the proteases Trypsin and Proteinase K (each 3 mg/500 ml). The control was supplemented with 3 mg of a non proteolytic protein (serum albumin). After incubation at room temperature the bottles were opened after 10 days, gushing volumes were determined and the ns-LTP1 concentrations were analysed using PTA ELISA. As shown in Table 1 ns-LTP1 content was strongly reduced in the presence of the proteases, whereas no changes were detectable in the control. Gushing activity occurred only in the Proteinase K treated samples where ns-LTP1 degradation was more pronounced compared to the trypsin treated beers.

Over supplementation of non gushing beer with ns-LTP1, using a concentrated wort-preparation, did not induce gushing activity. In contrast, addition of infected wort-preparations containing no detectable ns-LTP1 but degradation products provoked a strong concentration-dependent over foaming only after a short term incubation of 2 hours, although no ns-LTP1 decline occurred during this time scale (Table 2).

The results indicate that over foaming depends not directly on ns-LTP1 content in beer. Gushing activity but rather seems to be a function of the concentration of proteolytic degradation products of ns-LTP1. As mentioned above, beer contains a set of modified ns-LTP1 species generated via Maillard-reaction. One can imagine that degradation of modified ns-LTP1 therefore results in the production of a multitude of glycated peptides. Glycation increases the amphiphilicity of these fragments coming along with a better efficiency to form condensation nuclei for carbon dioxide in solution. If concentration peaks a particular threshold value gushing phenomenon occurs.

4 Conclusion

Our investigations were focussed on ns-LTP1 as the main inductor of the primary gushing phenomenon. We asserted that gushing beer contains more ns-LTP1 than non-gushing beer and these overbalance is responsible for over foaming. Surprisingly, less to non ns-LTP1 was detectable in a set of gushing beers.

We showed that loss of ns-LTP1 depends on fungal infestation and heating procedure. ns-LTP1 degrading activity of heated culture filtrates of *Fusarium culmorum* and *Fusarium graminearum* support the assumption that heat stable extracellular proteinases secreted by the two Fusaria strains are responsible for protein degradation. Heating procedure results in a destruction of naturally occurring proteinase inhibitors in wheat kernels. This is the condition precedent to an effective operation of the fungal proteases.

Furthermore our results support the conception that not ns-LTP1 itself, but rather glycated peptides generated during proteolytic fragmentation of modified ns-LTP1 species initiate gushing activity. Essential heating steps during the brewing procedure trigger just two deleterious processes regarding gushing development: generation of glycated ns-LTP1 via Maillard-reaction and inactivation of naturally occurring protease inhibitors. Both lead to accumulation of highly surface active peptides which are able to form condensation nuclei for carbon dioxide in solution.

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Appendix

Table 1 **Gushing volumes and ns-LTP1 contents of protease treated non gushing beers**

	Incubation time [days]	Gushing volumes [ml]	ns-LTP1 content [mg] per bottle
Control	0	0	72.3 ± 3.6
	10	0	72.9 ± 2.8
Trypsin treated	0	0	72.4 ± 3.2
	10	0	60.2 ± 4.5
Proteinase K treated	0	0	73.1 ± 3.1
	10	16.4 ± 4.8	51.4 ± 3.7

Standard deviations represent n=4 ; ns-LTP1 contents were determined by PTA ELISA

Table 2 **Gushing volumes and ns-LTP1 contents determined from beers treated with wort-concentrate**

Additive	added volumes [ml]	ns-LTP1 content of the additive [mg]	Gushing volumes [ml]	ns-LTP1 content [mg] per bottle
Infected wort-concentrate	0	0	0	72.5 ± 3.7
	0.1	0	13 ± 2.5	73.7 ± 2.4
	0.5	0	125 ± 18.5	71.6 ± 3.3
	1	0	175 ± 17	72.7 ± 3.6
	2	0	240 ± 10	73.9 ± 2.8
Non infected wort concentrate	2	15.5 ± 2.1	0	88.3 ± 3.1

Standard deviations represent n=4 ; ns-LTP1 contents were determined by PTA ELISA

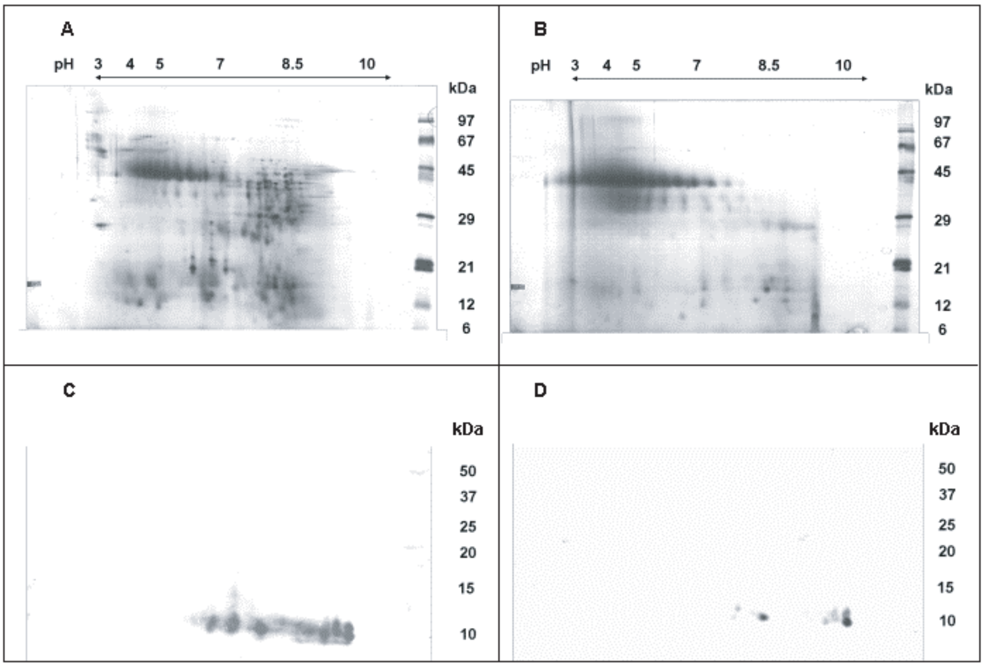
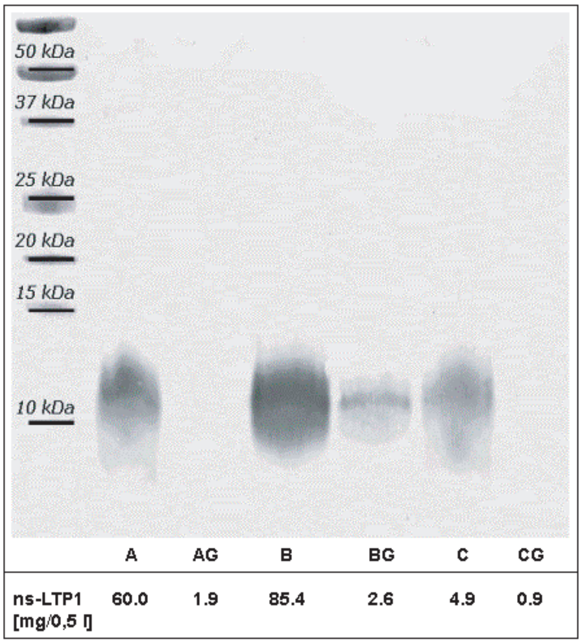


Figure 1 **Protein analyses of a non gushing beer compared to a gushing beer of the same brand**
Figure 1A and B: 2-D SDS PAGE of the non gushing beer and the gushing beer, respectively.
Figure 1C and D: corresponding immunoblotts probed with a polyclonal antibody to wheat ns-LTP1



	A	AG	B	BG	C	CG
ns-LTP1 [mg/0,5 l]	60.0	1.9	85.4	2.6	4.9	0.9

Figure 2 **Immuno-blot of six wheat beers and ns-LTP1 content calculated by PTA ELISA;**
Lane 1: molecular weight standard,
lane 2 to 7: proteins of wheat beers
(A, B and C non gushing beers; AG, BG and CG gushing beers)

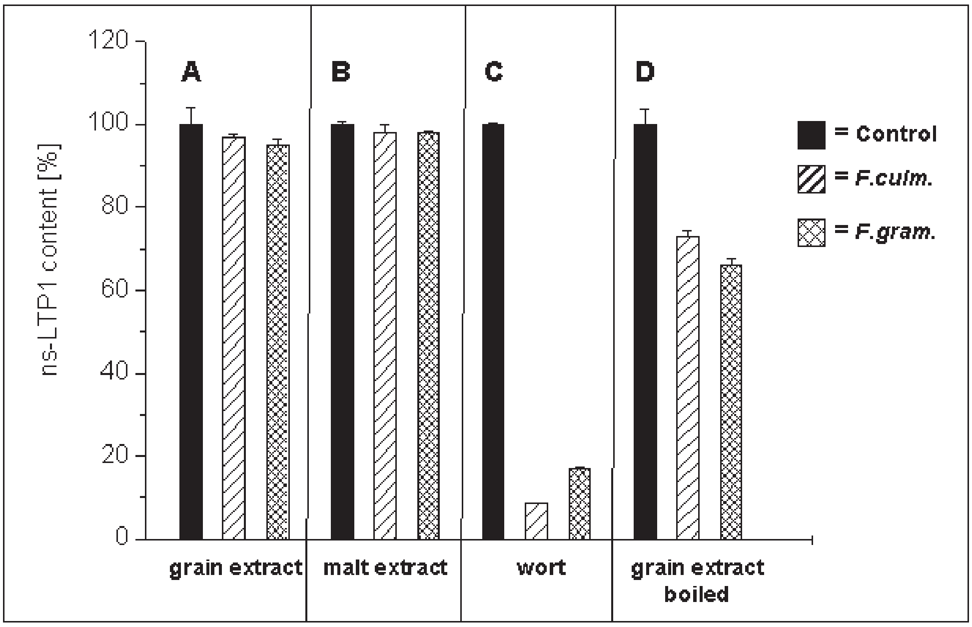


Figure 3 **Changes of ns-LTP1 contents in different samples by infection with *Fusarium culmorum* and *Fusarium graminearum*, respectively.**
Control samples were not infected. ns-LTP1 contents were determined by PTA ELISA: The results are given as percentage of the control which were set to 100 %.

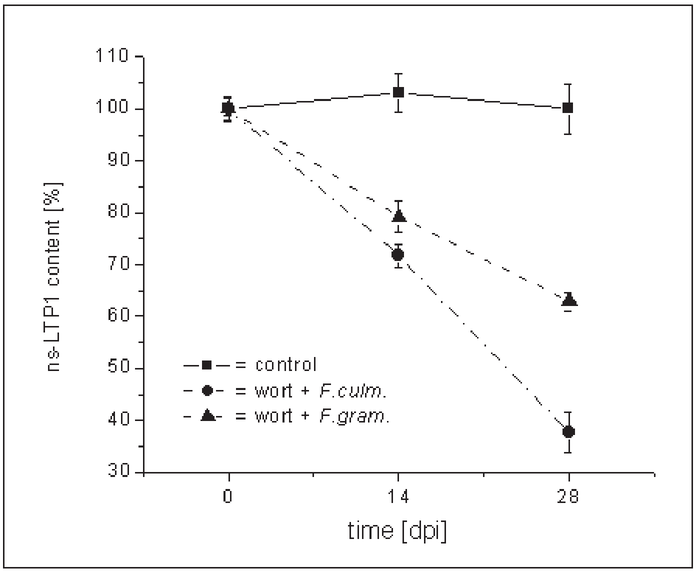


Figure 4 **ns-LTP1 content of wort inoculated with boiled culture filtrate of *Fusarium culmorum* and *Fusarium graminearum*, respectively.**
ns-LTP1 contents were determined by PTA ELISA.
The results are given as percentage of the control which were set to 100 %.

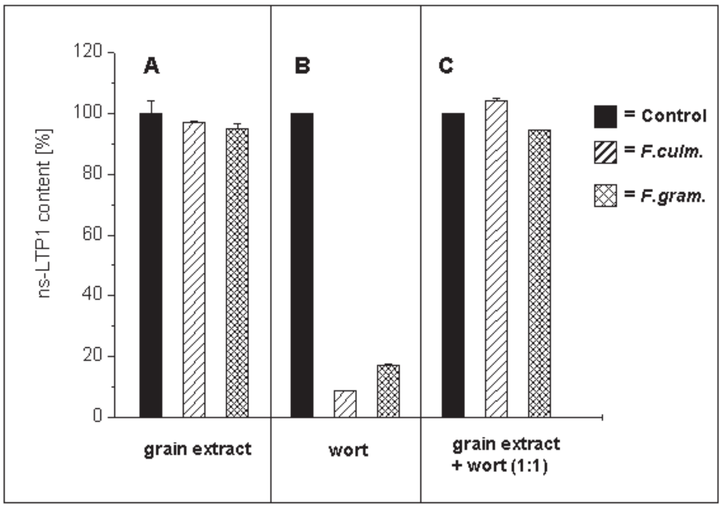


Figure 5 **Prevention of ns-LTP1 degradation in wort by addition of an aqueous grain extract**
ns-LTP1 contents were determined by PTA ELISA.
The results are given as percentage of the control which were set to 100 %.