JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Impact of Different Wort Boiling Temperatures on the Beer Foam Stabilizing Properties of Lipid Transfer Protein 1

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Beer consumers demand satisfactory and consistent foam stability; thus, it is a high priority for brewers. Beer foam is stabilized by the interaction between certain beer proteins, including lipid transfer protein 1 (LTP1), and isomerized hop α-acids, but destabilized by lipids. In this study it was shown that the wort boiling temperature during the brewing process was critical in determining the final beer LTP1 content and conformation. LTP1 levels during brewing were measured by an LTP1 ELISA, using antinative barley LTP1 polyclonal antibodies. It was observed that the higher wort boiling temperatures (∼102 °C), resulting from low altitude at sea level, reduced the final beer LTP1 level to 2−3 μg/mL, whereas the lower wort boiling temperatures (∼96 °C), resulting from higher altitudes (1800 m), produced LTP1 levels between 17 and 35 μg/mL. Low levels of LTP1 in combination with elevated levels of free fatty acids (FFA) resulted in poor foam stability, whereas beer produced with low levels of LTP1 and FFA had satisfactory foam stability. Previous studies indicated the need for LTP1 denaturing to improve its foam stabilizing properties. However, the results presented here show that LTP1 denaturation reduces its ability to act as a binding protein for foam-damaging FFA. These investigations suggest that wort boiling temperature is an important factor in determining the level and conformation of LTP1, thereby favoring satisfactory beer foam stability.

KEYWORDS: Brewing; wort boiling; beer; foam quality; lipid transfer protein

INTRODUCTION

Product consistency is an important goal for brewers, particularly when national brands are brewed across multiple production sites. Beer foam, in both its appearance and stability, is an important aspect of overall beer quality and establishes the consumer's taste expectation of the product (*1*, *2*). The brewing process can be summarized as follows: (1) mashing, whereby ground malt (germinated and dried barley) is mixed with water and heated through a predetermined temperature profile, allowing various enzymes to complete the breakdown of the endosperm reserves begun in malting; (2) lautering, which is the separation of insoluble fraction from the hot mash to produce sweet wort; (3) wort boiling, during which wort is boiled in a "kettle" to inactivate enzymes, remove undesirable flavor components, sterilize the wort, isomerize hop α-acids, and precipitate haze-forming proteins and polyphenols; (4) fermentation, which is the conversion by yeast of wort sugars and nutrients into alcohol and flavor components; (5) maturation, when the temperature is decreased (2 °C) to allow final settlement of yeast, haze components, and removal of undesirable flavors by secondary fermentation; and (6) filtration and packaging, the former producing a bright, clear beer for sterile, anaerobic packaging into containers for dispensing (*3*).

Beer foam stability is dependent on the interaction of a number of components, including hop iso-α-acids and beer proteins/polypeptides (*4*). More specifically, to create the optimum balance for a good foam, foam favorable factors or foam positives such as hop acid, protein, metal ion, gas composition (ratio of nitrogen to carbon dioxide), and gas level generally improve foam when increased. Factors unfavorable to foam, or foam negative, such as lipids, basic amino acids, ethanol, yeast protease activity, and excessive malt modification, improve foam stability when decreased.

An area of much interest in beer foam research has been foam positive proteins (*4*). The key malt-derived foam proteins are lipid transfer protein (LTP1), protein Z4, and the members of the hordein barley storage protein family. LTP1 is associated with foam formation and stability, the latter only when in conjunction with other proteins. Both LTP1 and Z4 are tolerant of high temperatures and resistant to proteolysis (*5*−*7*), which contribute to their resilience and survival through the brewing process. LTP1 has been demonstrated to be foam promoting

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10.1021/jf035125c CCC: $27.50
Published on Web 04/16/2004

only in its heat-denatured form (*8*−*10*). It appears, however, that there is a limit to the extent to which heat denaturation is desirable. Narziss (*11*) observed that increasing the counterpressure on the wort in an external boiler system created mean boiling temperatures between 103 and 110 °C, which accelerated protein coagulation, wort dimethyl sulfide stripping, and isomerization of hop α-acids, thus reducing boiling time by 30−40%. However, a deterioration of foam was observed relative to boiling temperatures below 103 °C, as well as loss of body and flavor balance; therefore, a temperature no greater than 103−104 °C was recommended.

Of the potential beer components deemed to be negative to foam, most attention has been directed toward lipids (*12*, *13*). Lipids, derived from raw materials (malt or hops) or yeast, are believed to be detrimental to foam as they disrupt the continuity of the foam bubble film (*14*). Numerous other materials, for example, some detergents, have also been reported to exert a negative effect on foam, but detergent contamination of beer usually only results from poor brewery practice and hygiene (*15*). An estimation of the balance of foam positive and negative factors can be made by measuring the head retention of beer that has been serially diluted (*13*). In the case of lipids, their impact on foam may be determined by challenging beers with free fatty acids (FFA). These types of analyses test for "foam robustness" (*16*, *17*).

Although lipids introduced into beer have an initial deleterious impact on foam stability, the foam stability will recover either fully or partially if the beer is allowed to rest for 24 h (*13*). This is possible due to binding of the lipids, and it has been observed that certain unidentified beer proteins with lipid-binding activity reduce lipid-induced destabilization in beer containing undesirably high levels of lipids (*18*−*20*). The identity of these is unknown, but one potential lipid-binding protein shown to be present in malt is the hordoinoline/puroindoline group of proteins. However, Evans and Sheehan (*4*) have shown that these do not survive the brewing process and, consequently, are unlikely to contribute to a beer's lipid-binding potential. Although native LTP1 has the ability to bind certain lipids (*21*), a lipid-scavenging function in beer has not yet been attributed to the heat-denatured form.

Monitoring specific foam proteins such as LTP1 by enzyme-linked immunosorbant assay (ELISA) on the basis of antibody recognition is well established as a very sensitive and specific method (*22*, *23*). It follows that LTP1 may also reflect the behavior of other foam positive proteins in beer, and so by monitoring this protein it may act as a marker for all foam positive protein material (*23*). As a caution, Bech et al. (*8*) observed that some anti-LTP1 antibodies had a limited immunoreactivity to denatured LTP1 that resulted during kettle boiling. In this study the beer dilution, foam robustness, and LTP1 ELISA tests have been applied to investigate the impact of wort boiling on foam stability undertaken in both pilot brewery and commercial brewery scale trials. The integrity of the LTP1 before and after heat treatment was investigated using two-dimensional (2D) gel electrophoresis, high-performance liquid chromatography (HPLC), electrospray mass spectrometry (ESMS), and circular dichroism (CD) spectrophotometry.

MATERIALS AND METHODS

Pilot Brewery Trials. Trials were done in a 40 L brewlength BAM (Bavarian Apparatus and Machinery, Freising, Germany) microbrewery equipped with 32 L fermenters, a Pall (NY) Kieselguhr candle filter, and a Krones (Neutraubling, Germany) single-head filler packaging system. A Miag (Langerringer, Germany) dry roller mill was used for malt.

Milled malt was mashed at a 3:1 (v/w) ratio of water to malt. The mash was acidified with lactic acid to pH 5.2, and the mashing program was 63 °C for 1 h, 72 °C for 20 min, and 76 °C for 5 min with a ramp rate of 1 °C/min. The mash was lautered, and wort with a gravity of 14 °P (degrees plato, where 1 °P is 1 g of sucrose per 100 g of solution, measured by using an Anton Paar beer analyzer, Anton Paar, Graz, Austria) was transferred to the kettle. Wort was boiled (≈625 mmHg, <98 °C) for 1 h and immediately cooled to 11 °C; a gravity of 15.5 °P was achieved. Brewery-collected stationary phase yeast was added (pitched) to the wort at a final cell count of 20×10^6 cells/mL. Fermentation was maintained at 11 °C for 8 days until the end of primary fermentation when the yeast had flocculated, successive measurements of gravity (°P) had stabilized, and diacetyl [measured by using an Agilent gas chromatography diacetyl analyzer (Hewlett-Packard, Waldbron, Germany)] had reduced to <50 μg/mL. The primary fermented beer was then chilled to 2 °C (maturation) and left for 6 days to allow a further reduction in diacetyl to 20 μg/L by secondary fermentation. This was followed by dilution with carbonated water to 5% alcohol (v/v), filtration using a Kieselguhr candle filter, and packaging into bottles using a single-head filler packaging system.

Foam Analysis. The foam stability was analyzed using a Haffmans (Zeist, The Netherlands) Nibem foam stability tester ISD-01 according to the method of Klopper (*24*). The Nibem value was expressed in seconds and represents the time taken for artificially induced foam to collapse 30 mm.

A mini foam shake test was developed to permit direct and rapid comparison of foam. In this mini foam shake test, 40 mL of beer was dispensed into 100 mL glass measuring cylinders. Five different beer samples (samples 1−5) were dispensed in triplicate, and all of the cylinders were sealed with Parafilm (Pechiney Plastic Packaging, Boscobel, WI). Each set of five cylinders (samples 1−5) was shaken at the same time, vigorously up and down 10 times, after which the cylinders were set on the counter, the Parafilm was pierced, and a timer was set for 15 min. After 15 min, the foam was evaluated visually and the cylinders were arranged from best to worst. A rating of 5 to 1 was given, where the best was 5 and the worst was 1. When the foam was the same in two or more cases, the same rating was given. The relative trends are more important than the actual foam levels. The triplicate sets should show the same trends for reliable data.

LTP1 Preparation. LTP1 was purified as previously reported (*22*) from cv. Schooner barley grain. The purified LTP1 was stored in 50 mM acetate buffer, pH 5.0, 10 mM dithiothreitol (DTT), and 0.2 M NaCl, and for ESMS analysis, sodium was removed by ultrafiltration into analytical purity water (prepared by filtering through a MilliQ filtration system, Millipore Corp., Bedford, MA) using an Amicon concentrator (Millipore Corp.) with a YM3 (3 kDa cutoff) Amicon membrane.

LTP1 ELISA. LTP1 was determined by quantitative double-sandwich ELISA using polyclonal LTP1 antibodies and purified barley LTP1 as described previously (*22*) with a few minor changes. For LTP1 analysis of wort and beer samples a 2000-fold dilution was found to be suitable. All dilutions were done in duplicate, and each dilution was tested in duplicate, resulting in four analyses per sample. The coefficient of variance of the LTP1 analysis was <10% among the four values. The absorbance at 415 nm was determined using a Bio-Rad (Richmond, CA) model 450 microplate reader.

LTP1 was also measured using a quantitative double-sandwich ELISA using antibeer foam LTP1 and antinative LTP1 monoclonal antibodies as described by Lusk et al. (*23*).

FFA Analysis. FFA were methylated prior to gas chromatographic (GC) separation and analysis according to the method of Venter et al. (*25*). Although all of the individual FFA were quantified, only the total content of C10−C18:3 fatty acids was reported.

Laboratory Scale Heating of LTP1 and Unboiled Wort Samples to Different Temperatures. A Carlo Erba 6000 Vega series gas chromatographic oven (Milano, Italy) with a fan and digital temperature control (±0.1 °C) was used to heat up to 2 mL of LTP1 dissolved in distilled water or 2 mL of unboiled wort in 6 mL thick-walled glass tubes, sealed with a pressure-resistant screw cap and a Teflon seal. The tubes were placed in the preheated GC oven in a glass beaker for

60 min. Samples were heated to 96, 105, or 110 °C. Because the tubes were sealed, no loss of moisture occurred during the heating.

Spiking Beer for Foam Evaluation. Commercially produced beers were screened, and a sample with a low LTP1 content (<5 μg/mL) was selected. Six hundred milliliters of beer was spiked to a final concentration of 0.5 μg/mL with heptadecanoic acid (FFA) dissolved in a minimal volume of absolute ethanol. The spiked beer was divided into 4 × 150 mL subsamples and then spiked with purified LTP1 samples 1−4 to give a final concentration of 20 μg/mL: (1) native LTP1, (2) 96 °C heat-treated LTP1, (3) 105 °C heat-treated LTP1, and (4) the equivalent volume of water (LTP1 solvent) to serve as a control. A further 150 mL aliquot of beer containing no FFA was spiked with the equivalent amount of ethanol (used to dissolve the FFA) and water (used to spike the LTP1) to serve as the second control (sample 5). All of the samples (1−5) were then degassed by gentle overnight stirring at 4 °C. The foam of these treated beer samples was compared by the mini foam shake test.

Gel Electrophoresis of LTP1 and Wort Samples. *(a) One-Dimensional Gel Electrophoresis.* Native LTP1, unboiled wort, heat-treated LTP1, and wort samples were centrifuged at 800*g* for 5 min. The supernatant and the pellets were collected and subjected to SDS-PAGE. Sample buffer [Tris-HCl (0.05 M, pH 6.8) containing 4% sodium dodecyl sulfate (SDS, w/v), 5 M urea, 20% glycerol (v/v), and 143 mM β-mercaptoethanol] and 1% bromophenol blue were added to the samples, which were then mixed and boiled for 3 min before loading and electrophoresis. The method was performed according to that of Laemmli (*26*) using 1 mm separating gels (15%) and stacking gels (5%) on a Mini Protein III electrophoresis unit (Bio-Rad). Silver staining for total protein was performed using a silver stain plus kit (Bio-Rad). A GS-700 imaging densitometer (Bio-Rad) was used to scan the gels, and Multi-Analyst software (Bio-Rad) was used to calculate the band intensities.

(b) Two-Dimensional Gel Electrophoresis. For 2D gel electrophoresis, LTP1 samples were first diluted (1:2.5) in 8 M urea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.5% immobilized pH gradient gel (IPG) buffer, pH 3−10 (Amersham Biosciences, Sydney, Australia), 0.01% bromophenol blue (as a electrophorsis migration marker), and 18 mM DTT. The sample mixtures were then subjected to isoelectric focusing (IEF) on pH 3−10 18 cm IPG (immobilized pH gradient gel) strips (Amersham Biosciences). The gel strips were rehydrated beforehand in the dilution solvent for 10−12 h at 20 °C according to the manufacturer's instructions. The loaded strips were then developed on an IPGphor isoelectric focusing system (Amersham Biosciences) for 500 Vh at 500 V, for 1000 Vh at 1000 V, and for 32000 Vh at 8000 V, voltages being limited by a maximum current of 50 μA per sample strip. Focused IPG strips were equilibrated with 10 mL of 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 65 mM DTT for 15−30 min at room temperature. Each strip was then placed on top of the second-dimension reducing SDS-PAGE gel (*26*) (1.5 mm thick, 23.5 cm wide and ∼18 cm high, without stacking gel layer,). SeeBlue molecular weight markers (Novex, San Diego, CA), spotted onto a piece of filter paper (HomeBrand, Adelaide, Australia), were also placed on the gel. A 1% agarose overlay was then applied to secure the IPG strip and the molecular weight markers. The gels were then electrophoresed in a Hoefer DALT system (Amersham Biosciences) at constant current (67 mA per gel), limited by voltage (1000 V maximum) and power (100 W maximum) at 25 °C (controlled by Multitemp III, Pharmacia/Amersham), typically over 5−6 h. Once the bromophenol blue dye had migrated to the bottom of the gel, the gel was then stained for total protein with a Bio-Rad silver stain kit.

ESMS. ESMS, using a Micromass triple-quadrupole mass spectrometer fitted with an electrospray source, was employed to investigate the native and heat-treated LTP1 samples. Ten microliters of the sample solution (±2 μg/mL protein in water containing 1% formic acid) was introduced into the ESI-MS using a Rheodyne injector valve. The carrier solvent was acetonitrile/water (50:50, v/v) delivered at a flow rate of 20 μL/min throughout each analysis. A capillary voltage of 3.5 kV was applied, with the source temperature at 80 °C. The cone voltage was 60 V and the skimmer lens offset 5 V. Data acquisition was in the positive mode, scanning the first analyzer (MS1) from *m*/*z* 500 to 2000 at a scan rate of 300 amu/s. Representative scans were produced by combining the scans across the elution peak and subtracting the background. The molecular masses of the protein contained in the samples from the multiprotonated spectra were determined either using the MaxEnt algorithm (supplied with instrument software) or by manual calculation.

Table 1. Foam Stability of Beer Produced at the Pilot Brewery Scale (40 L) Using Samples Taken at Different Stages during the Brewing Process from Two Commercial Breweries, A and B

materials into pilot brewery	ex-brewery A (Nibem, s)	ex-brewery B (Nibem, s)
unprocessed raw materials	265	260
green wort	263	266
boiled wort	212	258
end of ferment beer	220	263

Reverse Phase High-Performance Liquid Chromatography (RP-HPLC). The purified barley LTP1 was further separated by RP-HPLC. Separations were achieved using a 2.1 × 250 mm Vydac C18 protein column (Vydac Separations Group, Hesperia, CA) with a linear gradient created over 30 min starting with 95% eluent A (0.05% trifluoroacetic acid in acetonitrile) and ending with 70% eluent B (0.04% trifluoroacetic acid in acetonitrile) at a flow rate of 0.2 mL min^{-1}.

CD. Solutions of the native and heat-treated LTP1 were prepared in 10 mM phosphate-buffered saline (pH 7.4). Analysis was undertaken at room temperature (25 °C) in a quartz cell (path length of 1 mm), on a J-810 spectropolarimeter (Jasco, Tokyo, Japan). CD spectra were measured, from 260 to 197 nm, with 0.2 nm bandwidth at a scanning speed of 100 nm min^{-1}. All spectra presented are the average of 10 scans. Results are expressed in molar ellipticity (θ), and the averaged CD spectra of the buffer were subtracted from that of the averaged sample spectra.

RESULTS AND DISCUSSION

Temperature-Dependent Foam Stability and LTP1 Levels. Historically it has been observed that two similar commercial South African based breweries (A and B) produce lager style beer (same brand profile) with habitually low (A) and high (B) Nibem foam stabilities, despite using comparable raw materials and production processes. To understand the reason for this difference, samples of unprocessed raw materials, sweet (unboiled) wort, boiled wort, and end of ferment beer were obtained from the breweries and were onward processed to final in-package beer at the same microbrewery. Analysis of the resultant beers indicated that there was a marked difference in the final product foams when material had been boiled in brewery A (**Table 1**). This implies that brewhouse practice during wort boiling had a substantial impact on the Nibem foam stability of the resultant beer.

As noted earlier, foam-promoting proteins and isomerized hop iso-α-acids are a fundamental basis of beer foam stability (*4*). As the level of beer iso-α-acids for both breweries was identical, it was suspected that foam-promoting proteins were being differentially destroyed during wort boiling. To test this hypothesis, the level of LTP1, a known foam-promoting protein, was measured in commercial brews by an ELISA that had previously been developed to detect native barley LTP1 (*22*). The results showed that LTP1, as measured, decreased during wort boiling (**Figure 1**). No further reduction in LTP1 level were observed as the wort was onward processed into final in-package beer (data not shown). More specifically, the commercial brewery A product equates to microbrewery "brewery A boiled wort" beer (low LTP1 and low Nibem foam values), whereas that of commercial brewery B equates to "microbrewery boiled wort" beer (high LTP1 and high Nibem foam values).

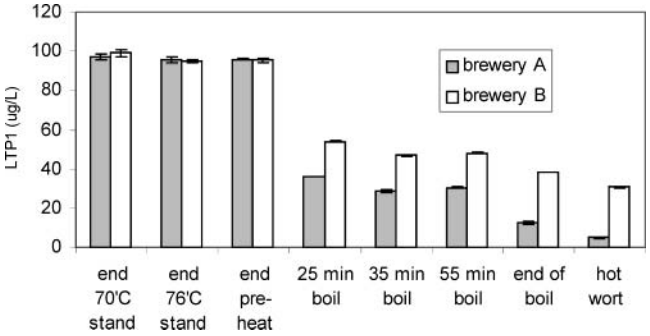

Figure 1. Brews from breweries A and B were monitored, using LTP1 ELISA, from mashtun to wort cooler. Four brews were monitored at each brewery. Despite inevitable changes in raw materials during commercial scale trials, all brews displayed similar trends; one brew from each brewery is represented here. Error bars represent SD of LTP1 analysis.

Table 2. Comparison of Typical Wort-Boiling and Beer Foam Characteristics of Three Different Breweries, Including the Relative Free Fatty Acid (FFA) Levels Typically Found in Their Beers

	brewery		
	A	B	C
location	coastal	inland	coastal
altitude (m)	~200	~1600	~150
boil temp (°C)	~102	~96	~102
LTP1 (μg/mL)	2–3	17–35	2–3
FFA (mg/mL)	2.84	1.12	0.70
foam	low	high	high

A further comparison of the two commercial brewhouses indicated possible differences in plant and brewing process (**Table 2**). The results, depicted in **Table 2**, show that a key difference between the two breweries was the boiling temperature. Brewery B, like the pilot brewery, is located at high altitude (1800 m above sea level), whereas brewery A is located at low altitude (sea level). This difference in altitude and therefore atmospheric pressure accounts for the difference in boiling temperature (*27*). Therefore, the higher levels of LTP1 in the beer produced by brewery B and in the pilot brewery are due to lower boiling temperature, compared to that of brewery A, where the lower LTP1 levels are due to high wort boiling temperature. This also suggested that the reduced beer foam stability of beer produced by brewery A was due to lower LTP1 levels (**Figure 1** and **Table 2**). Testing the products of five other breweries representing a mix of low- and high-altitude locations gave similar results. However, the results from one brewery (C) contradicted this pattern as the level of LTP1 in the beer was similar to that of brewery A beer (low due to high boiling temperature at sea level), yet the foam stability was as high as that found for brewery B's product (**Table 2**). Further investigation revealed that this contradiction can be explained by a second factor, the beer content of FFA, which are known foam destabilizing factors (*4*). It has been established that beer contains proteins that bind lipids (*18*−*20*), so negating the lipid foam-destabilizing effect. It follows that as native LTP1 can bind lipids (*21*), LTP1 could be a lipid-binding protein in beer, although it has yet to be demonstrated that thermally modified LTP1 retains its lipid-binding capacity. Consideration of both the level of LTP1 and the level of FFA explains the relative beer foam stabilities of beers from breweries A−C. It is proposed that in the case of brewery C, LTP1 levels are not as critical because the FFA levels are sufficiently low, so that good foam is maintained despite the relatively low level of LTP1 detected.

Table 3. Assessment of Foam Stability with Respect to Challenge by Lipids[a]

replicate	control, no FFA	control + FFA	LTP native + FFA	LTP 96 °C + FFA	LTP 105 °C + FFA
1	5	1	4	3	3
2	5	2	4	4	2
3	5	1	4	3	2

[a] Minicylinder foam shake test results are expressed as a relative foam rating, where 5 is best and 1 is the worst foam. FFA (0.5 μg/mL) and LTP1 (20 μg/mL) were doped into low LTP1 beer (3 μg/mL), and the samples were stored overnight prior to assessment.

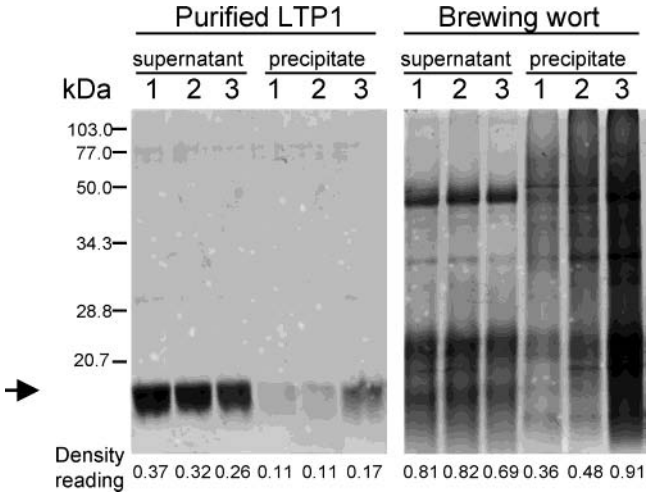

Figure 2. Silver-stained 15% SDS-PAGE of native LTP1 and unboiled wort (lanes 1) and LTP1 and wort, heat-treated for 60 min at 96 °C (lanes 2) and 105 °C (lanes 3). Each group of samples was loaded in the same relative proportions to allow comparison. Arrow indicates LTP1 band.

To establish that LTP1 is an important lipid-binding protein, purified native barley LTP1 was heat treated under laboratory conditions at 96 and 105 °C for 60 min. Although this is not a direct representation of commercial wort boiling, it did permit the preparation of reproducible samples under defined and controlled conditions and the subsequent use of the samples in the investigation of the impact of temperature, independent of brewery kettle design. These conditions simulated the wort boiling effects on LTP1 in breweries B and A, respectively. The temperature range used is also comparable to that reported in the literature (*28*, *29*). Narziss (*11*) also reports that boiling temperatures of 103−110 °C were observed to be detrimental to beer foam, body, and flavor. The temperature of the boil was accordingly reduced to 103−104 °C to minimize these potential problems.

The native and heat-treated LTP1 samples were spiked (20 μg/mL) into low-LTP1 (2−3 μg/mL) degassed beer containing 0.5 μg/mL FFA. After storage overnight, to allow time for the components in the beer to interact, beer foam stability was evaluated visually using a mini foam shake test (**Table 3**). FFA addition destabilizes the beer foam, but the inclusion of LTP1 enables a partial recovery of the beer foam stability. Comparison of the relative extent of foam recovery indicates that the native LTP1 is more effective at binding FFA than LTP1 thermally treated at 96 °C, which in turn was more effective than that treated at 105 °C (**Table 3**). Interestingly, the different LTP1 species did not visibly enhance the foam formation/stability when FFA were not introduced, suggesting that in these South African lager beers the LTP1 role as a lipid-binding protein is more important than in foam formation/stability. This and the

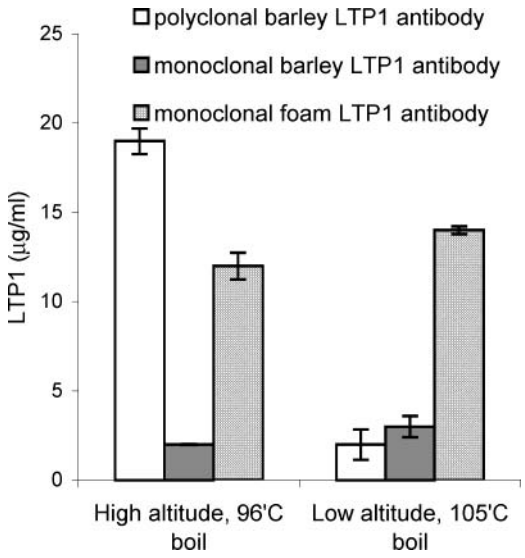

Figure 3. Comparison of LTP1 detection with anti-LTP1 polyclonal and monoclonal antibodies in beer samples obtained from two breweries that differ in altitude and therefore boiling temperature. Error bars represent standard deviation.

observation that the native LTP1 is the more effective lipid-binding protein than thermally denatured LTP1 contradict previous reports that thermally modified LTP1 improves foam stability (*8*).

Temperature-Dependent LTP1 Detection. The lower LTP1 levels detected at higher boiling temperatures may be due to the loss of the protein by precipitation or a limitation in the ability of the ELISA to detect the denatured LTP1. To resolve this question, an experiment was designed to investigate the loss of LTP1 due to protein precipitation during wort boiling and the limitations of the ELISA technique in the detection of LTP1. In the ELISA evaluation the effectiveness of different antibodies was evaluated. Purified native barley and heat-treated (96 and 105 °C) LTP1 samples were centrifuged, and the supernatant was separated from any pellet material. The same procedure was repeated on sweet (unboiled) wort, 96 °C heat-treated wort, and 105 °C heat-treated wort. The LTP1 samples and wort material were then run on SDS-PAGE, and the gel was silver stained for total protein (**Figure 2**). The total density of each protein band was quantified, and the relative values are shown (**Figure 2**). This experiment showed that although at 105 °C more LTP1 and wort protein precipitated compared to the native and 96 °C heat-treated samples, LTP1 was still present in all of the supernatants. The wort supernatants and precipitates also showed that as the amount of heating increased, so did protein precipitation, including the protein bands in the LTP1 region of the gel. Therefore, precipitation only partly explains the loss of LTP1 at higher boiling temperatures.

The binding of the anti-LTP1 antibodies was then investigated more rigorously. The antibody used for LTP1 ELISA so far in the study was a polyclonal antibody raised against native barley LTP1. This ELISA was reported to detect LTP1 in barley, malt, wort, and beer (*22*). It was compared to ELISAs developed using monoclonal antibodies raised against native barley LTP1 and foam LTP1 (*23*). Beer from brewery A (high boiling temperature and poor foam) and beer from brewery B (low boiling temperature and good foam) were analyzed using all three antibodies in ELISAs (**Figure 3**). Substantially more LTP1 was detected in beer from brewery B with the polyclonal antinative barley LTP1 antibody ELISA. The level of LTP1 detected with the monoclonal antinative LTP1 ELISA was low for both breweries. However, this result suggested that residual native barley LTP1 was present in these and other boiled samples (wort or beer, data not shown). In contrast, the monoclonal anti-foam LTP1 ELISA was able to detect similar LTP1 levels in both beers. The experiment suggests that the polyclonal antinative barley LTP1 antibody ELISA is as limited as the monoclonal antinative barley LTP in its ability to detect LTP1 that have been exposed to temperatures from 100 to 105 °C. The monoclonal antifoam LTP1 ELISA detected LTP1 exposed to boiling temperatures >100 °C, but not LTP1 exposed to

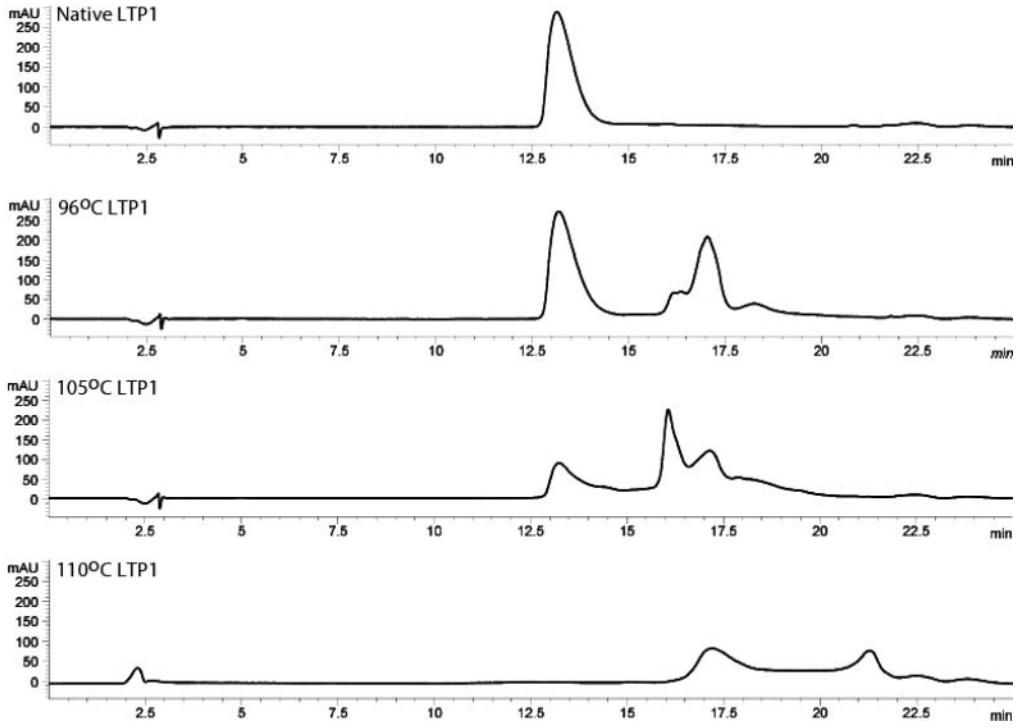

Figure 4. RP-HPLC traces of purified barley native LTP1 and LTP1 heat-treated at 96, 105, and 110 °C.

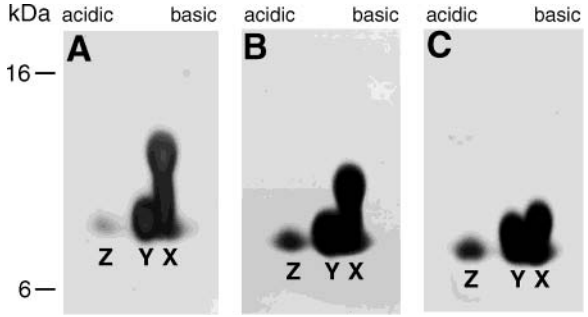

Figure 5. Portion of 2D electorphoresis gels (p*I* 3–10, 15% acylamide), stained for total protein with silver, showing the modification of purified LTP1 (1 mg/mL) protein by heating at (**A**) untreated (native), (**B**) 96 °C, and (**C**) 105 °C for 60 min. X, Y, and Z are labels for the protein spots.

temperatures >110 °C. The further loss of detection by the monoclonal antifoam LTP1 antibody at 110 °C coincides with the observed beer foam loss reported by Narziss (*11*). Although conformational changes in LTP1 are likely to occur below 100 °C, the loss of detection is most probably associated, apart from precipitation loss LTP1, with further conformational changes occurring above 100 °C. Correspondingly, it is also apparent from the foam stability assessments reported in **Tables 1** and **2** that higher boiling temperatures (>100 °C) and the possible conformational changes associated with this can be deleterious to foam stability, especially when FFAs are present.

Temperature-Induced LTP1 Conformational Changes. The variable detection of heated LTP1 in the three ELISAs indicates that wort boiling generates a complex series of LTP1 forms that modify the immunogenic epitopes, which in turn influence antibody binding. In wort this could result from the progressive reduction and rearrangement of the four disulfide bridges and heterogeneous Malliard glycosylation (*30*, *31*). A further level of complication is added by the observation that there are two major forms of LTP1 with molecular masses of ~9683 and ~9983 (*9*, *22*, *31*). The second, higher mass protein has recently been shown to be the result of an unusual post-translational modification resulting from the covalent linkage of Asp7 with a C17 putative fatty acid (*32*).

To simplify the study of LTP1 conformational changes or denaturation, the effect of heating purified native barley LTP1 to 96, 105, and 110 °C was investigated. RP-HPLC of native and heated LTP1 showed the diminution of the parent native LTP1 peak and the emergence of further peaks with progressively longer retention times (**Figure 4**). The longer HPLC retention times indicated an increase in LTP1 hydrophobicity with heat treatment. Increased hydrophobicity of proteins has long been regarded as being a characteristic beneficial to foam promotion (*33*) and was consistent with the observation that heat-denatured LTP1 (presumably ≤ 100 °C) was substantially more foam promoting (*8*). In addition, the total peak areas of the 105 °C sample and particularly the 110 °C LTP1 sample were reduced, which was consistent with the heat precipitation trend described earlier for LTP1.

Comparison of the 2D electrophoretic separation of native LTP1 with that heated to 96 and 105 °C showed changes in composition and apparent molecular weight (**Figure 5**). The native LTP1 used has previously been shown to be pure by electrophoretic and mass spectrophoretic examination (*22*). The native LTP1 contains two major protein spots, X and Y. It is likely that the Y spot is the unmodified LTP1 protein as the conjugation of the aspartic acid residue with the C17 fatty acid would be expected to reduce the acidity of the protein. Heating appears to have progressively decreased the apparent molecular weight of all of the putative LTP1 spots and resulted in the enhancement of the density of a third spot (S), which is more acidic than the original spots. The most obvious change is the progressive reduction in apparent molecular weight of the X spot. In contrast, the Y spot remains relatively consistent in intensity and apparent mass.

ESMS has been employed very successfully to probe conformational changes in proteins (*34*−*38*). Therefore, to explain the HPLC and 2D gel electrophoresis results, ESMS analyses were done on purified native LTP1, and this LTP1 preparation

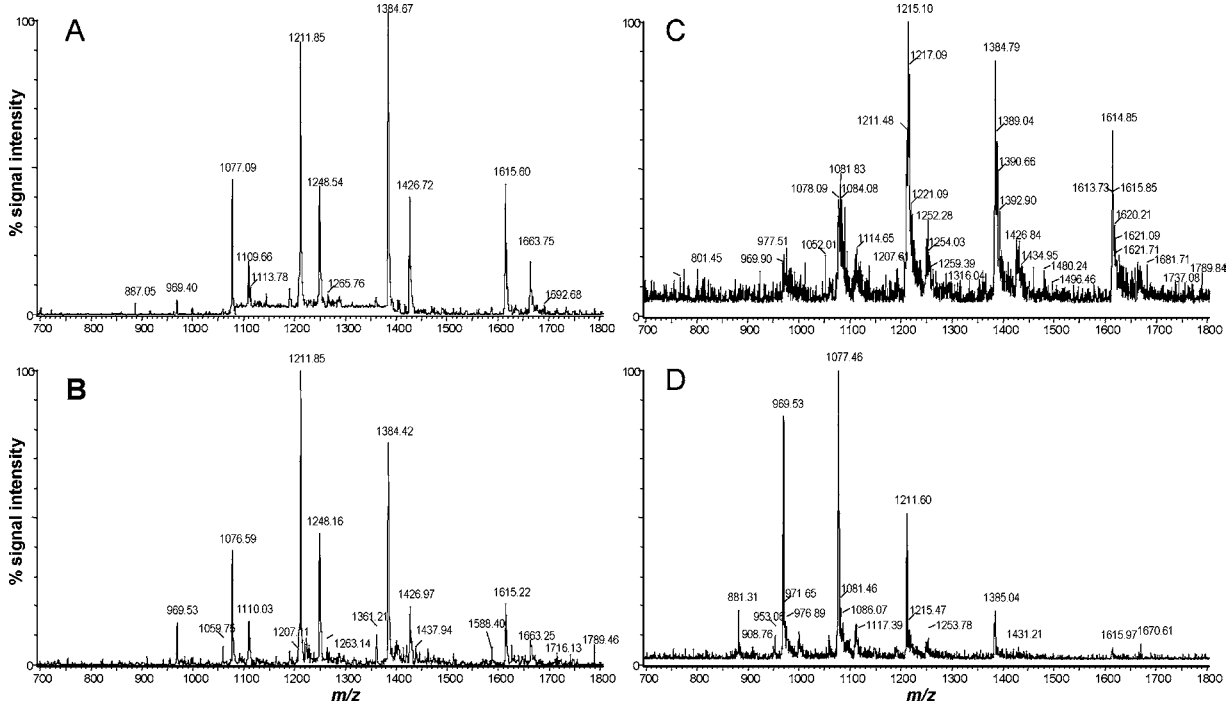

Figure 6. Multiprotonated ESMS spectra for LTP1 native (**A**) and LTP1 heated to 96 °C (**B**) and 105 °C (**C**, **D**). During analysis of 105 °C LTP two distinct peaks with a high ion total current were detected after injection, P1 (**C**) and a delayed P2 (**D**).

Table 4. ESMS Determination of the Integrity and Molecular Mass(es) of the Barley LTP1 in the Native and Heat-Treated Samples[a]

sample	M_r of major protein species	predicted identity
native LTP1	**9686** (9686.96 expected)	LTP1a (LTP1)
	9980 [9981.96 expected (*18*)]	LTP1b (modified LTP1)
96 °C heat-treated LTP1	9526	loss of C-terminal tripeptide unit (RIY) from
	9684	LTP1b
	9978	LTP1a
		LTP1b
105 °C heat-treated LTP1	P1: [b] **9688**	LTP1a with reduced disulfide bridge
	9715	LTP1a with oxidized Cys residue(s)
	9728	LTP1a–CH_3CN adduct or unidentified breakdown product
	9980–10200	LTP1b and unknown components
	P2:[b] 9521	loss of C-terminal tripeptide unit (RIY) from LTP1b
	9686	LTP1a, denatured
	9986	LTP1b, denatured, reduced
110 °C heat-treated LTP1	P1:[c] 9622	unknown breakdown products
	9683	LTP1a, denatured
	9784	LTP1a with sulfinic (Cys-SO_2H) and sulfonic (Cys-SO_3H) forms**
	P2:[c] 9622	breakdown product of oxidized LTP1a
	9686	LTP1a, denatured
	9736[d]	LTP1a with oxidized Cys residues (see above**)
	9963[d]	dehydrated LTP1b
	8300–8700[d]	unknown breakdown products

[a] The barley LTP1 was purified as described in text. M_r values were calculated manually, and only major components with a minimum of three following multiprotonated species detected were considered. M_r values in bold correlate expected LTP1 M_r values. [b] See **Figure 8** and text for explanation. [c] See text for explanation. [d] MaxEnt calculations.

was subjected to 96, 105, and 110 °C. Most of the calculated M_r values were 1−2 Da lower than expected, which could be the result inherent inaccuracy of the instrument and methods of calculation. The ESMS results indicated that the proteins in the untreated LTP1 samples were intact and of high purity (**Figure 6A** and **Table 4**), which corroborated the HPLC results. The native LTP1 sample contained two proteins, denoted LTP1a (the major protein) and LTP1b, with M_r values of 9686 and 9980, respectively. LTP1b has the same M_r as the modified LTP1 described by Lindorff-Larsen et al. (*32*).

The 96 °C heat-treated LTP1 sample contained the same two proteins LTP1a and LTP1b, but also one smaller product with M_r of 9526 (**Table 4**). This minor product could be due to the loss of a C-terminal tripeptide unit (Arg-Ile-Tyr) from LTP1b. This degraded protein may thus be the protein spot Z observed in the 2D gel electrophoresis, as the loss of an Arg residue will indeed cause the protein to have a lower p*I*. The existence of this minor protein in the 96 °C heat-treated LTP1 sample, however, does not explain the second large peak that appeared in the HPLC (**Figure 4**). The differences in the HPLC profiles of the native and the 96 °C heat-treated LTP1 could therefore only be attributed to conformational changes. Close inspection of the 96 °C heat-treated LTP1 ESMS multiprotonated spectrum revealed a slight shift of the spectrum to more charged molecular species, where the molecular ion with the highest signal intensity has a charge of +9, whereas this species in the native spectrum carried a +8 charge (compare panels **A** and **B** of **Figure 6**). This meant that some denaturation at 96 °C did occur in order to accommodate extra positive charges in the protein structure. Results from CD (**Figure 7**, see details later) also indicated some denaturation and loss of secondary structure in the 96 °C LTP1.

The 105 °C LTP1 sample also contained LTP1a and LTP1b, but they were more degraded, including a product with M_r 9526, and oxidized protein products were detected (**Table 4** and **Figure 6C**,**D**). Also, a curious chromatographic event occurred in the silanized capillary with which the sample is introduced into the ESMS sample chamber. The sample separated into two peaks (P1 and P2) with high ion current of which the first smaller P1 contained a protein with a multiprotonated spectrum and highest intensity molecular ion similar to that of the 96 °C sample. However, the calculated M_r from this spectrum was ±2Da higher than expected, indicating that one of the disulfide bonds may be reduced. The P2 peak had LTP1 that was more denatured, as the spectrum was totally shifted toward a higher charge (lower *m*/*z* region), and the molecular ion with highest intensity carried a +10 charge, which is near the optimum charge density of this size of protein (*34*−*38*). This result again corroborated the HPLC result, where a new later eluting peak appeared. The degradation was limited to the possible loss of the C-terminal RIY tripeptide from LTP1b and oxidized Cys residue(s).

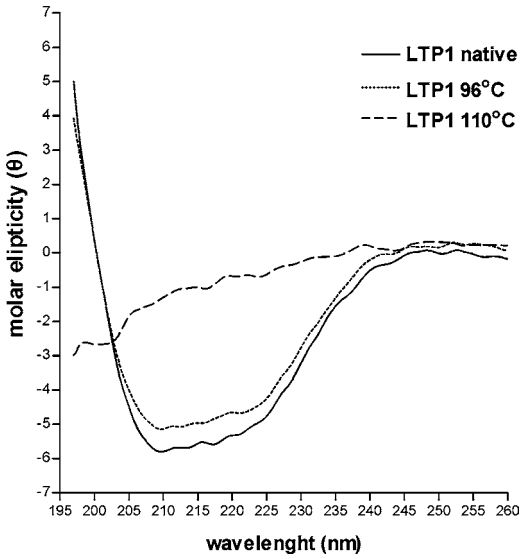

Figure 7. Average CD spectra of native LTP1 and heat-treated LTP1. The CD spectrum of the PBS buffer was subtracted from the sample spectra.

Table 5. Hypothesized Changes in Protein Conformation, Foam Activity, Lipid-Binding Capacity, and Antibody Recognition of LTP1 as It Is Exposed to Increasing Temperatures

Temperature	76°C End of mashing	~96-100°C high altitude boil	~102-106°C low altitude boil	>110°C extreme boil
Schematic representation				
Foam activity	×	✓	✓✓	?
Anti-LTP1 Pab[1] recognition	✓	✓	×	×
Anti-LTP1 Mab[2] recognition	×	×	✓	×

[1] Pab = antinative barley LTP1 polyclonal antibody. [2] Mab = antifoam LTP1 monoclonal antibody.

Results from the ESMS analysis of the 110 °C sample corroborated the HPLC results, and it was clear that a major part of this sample was highly degraded and oxidized (**Table 4**). We observed the same chromatographic behavior during the ESMS and the major protein species in P1 related to a small amount of slightly denatured (according to charge distribution) and slightly altered (±4 Da lower M_r than expected), a possible breakdown product of oxidized LTP1a (M_r 9622.0) and a species with M_r 9784.4, which could be LTP1a with sulfinic (Cys-SO_2H) and sulfonic (Cys-SO_3H) modification of some of its Cys residues. P2 also contained denatured LTP1a, the product with M_r 9622.4, a species with M_r 9736, which again could be LTP1a with oxidized Cys residues and a dehydrated LTP1b (M_r 9962.9). There was also a major group of unidentified breakdown products, compounds with molecular masses ranging from 8300 to 8700.

The influence of heat treatment on the secondary structure of LTP1, consisting of four long α-helical stretches (*39*), was evaluated by far-UV CD analysis. The CD spectra (**Figure 6**) for native and 96 °C LTP1 show similar maxima at >197 nm and minima at 208 and 220 nm, which is typical of the α-helix secondary structure observed by Jégou et al. (*30*). However, the 96 °C LTP1 sample contained 10−15% less secondary structure than the native LTP1, which corresponds to the denaturation predicted by ESMS. In contrast, heating to 110 °C substantially reduces the band intensity at 208 and 220 nm, creating minima at 195 and 203 nm, which is indicative of a random structure and confirms that heating has effectively disrupted the α-helix secondary structure.

Conclusions. Wort boiling temperature was shown at a pilot brewery scale to be critical for beer foam stability. This finding was confirmed at the commercial brewery scale by monitoring the foam protein LTP1 through the brewing process at different breweries. Brewery A with habitually poor foam had less LTP1 in the boiled wort and in the final beer than brewery B, with consistently good foam. The brewhouse boiling temperatures were highlighted as the only really significant difference in the brewhouse processes. Brewery B with the good foam had a lower boiling temperature due to its location at a relatively high altitude.

The role of LTP1 in foam is most important as a lipid-binding protein in the context of South African lager style beers. It was observed that brewery C, with a high boiling temperature and thus low beer LTP1 levels, could still maintain a good foam if the level of FFA in the beer was low. Therefore, LTP1 appears to be essential for foam stability when lipids are present. Using visual foam evaluation and the spiking into beer of purified LTP1, it was shown that as LTP1 is heated, its lipid-binding capacity decreases. Native LTP1 was observed to be the most active as a lipid binder. It has previously been reported that native LTP1 binds lipids (*21*). Here we provide strong circumstantial evidence that LTP1 is an important lipid-binding protein in beer and that heating during wort boiling results in conformational changes that reduce its ability to bind lipids.

Optimization of wort boiling temperature is essential for improved foam stability. The investigations show that conformational changes occurring within the temperature range of 96−110 °C have a critical impact on LTP1 solubility, its ability to promote foam formation, and most importantly its lipid-binding role. Results from HPLC, 2D gel electrophoresis, ESMS, and CD analyses show that heating of LTP1 disrupts the secondary structure to presumably expose hydrophobic residues, which make the protein less soluble resulting in a proportion precipitating out of solution but allowing it to become more concentrated in beer foam (*8*). However, more denaturation and degradation were detected in the 105 °C samples, and degradation was more pronounced in the 110 °C sample than the moderate denaturation in the 96 °C sample. It would thus appear that limited denaturation (up to an as yet to be defined extent) is good for foam stability, but beyond that foam stability is lost. This suggests that there is an optimal degree of LTP1 denaturation that must be attained during boiling to ensure good foam stability.

The hypothesis on the LTP1 conformational changes, function, and a summary of these results is represented diagram-

matically in **Table 5**. As the temperature increases, LTP1 undergoes conformational changes, which also affect antibody and lipid binding, and as unfolding continues, a point is reached when eventually little or no secondary structure remains. However, it has been observed that the foam promotion ability of LTP1 improves as hydrophobic areas are exposed (*8*). Ultimately, as the temperature increases further, none of the antibodies interact with the protein due to lack of intact epitopes for recognition, and it may no longer be foam active or be able to bind lipids. Effectively within the 96−105 °C temperature range it is proposed that there is an optimal boiling temperature giving a balance between the dual role of LTP1 in foam, namely, as a generator of foam (*9*, *10*) and as a lipid-binding protein.

ACKNOWLEDGMENT

We thank the directors of the South African Breweries Ltd. for their permission to publish these results. We also thank Dr. Lance Lusk (Miller Brewing Co., USA) for assistance with the monoclonal antibody LTP1 ELISA analysis. We are grateful to colleagues at the technical and production departments (South African Breweries Ltd.) for valuable assistance and to Jelle Lahnstein (University of Adelaide) for excellent assistance with 2D electrophoresis and RP-HPLC. Finally, we thank Dr. Thinus van der Merwe for assistance with the ESMS analyses.

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Received for review October 2, 2003. Revised manuscript received February 12, 2004. Accepted March 10, 2004. D.E.E. acknowledges the Grains Research and Development Corp. for financial support.

JF035125C