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Stability of Barley and Malt Lipid Transfer Protein 1 (LTP1) toward Heating and Reducing Agents: Relationships with the Brewing Process

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Barley lipid transfer protein (LTP1) is a heat-stable and protease-resistant albumin that concentrates in beer, where it participates in the formation and stability of beer foam. Whereas the barley LTP1 does not display any foaming properties, the corresponding beer protein is surface-active. Such an improvement is related to glycation by Maillard reactions on malting, acylation on mashing, and structural unfolding on brewing. The structural stability of purified barley and glycated malt LTP1 toward heating has been analyzed. Whatever the modification, lipid adduction or glycation, barley LTP1s are highly stable proteins that resisted temperatures up to 100 °C. Unfolding of LTP1 occurred only when heating was conducted in the presence of a reducing agent. In the presence of sodium sulfite, the lipid-adducted barley and malt LTP1 displayed higher heat stability than the nonadducted protein. Glycation had no or weak effect on heat-induced unfolding. Finally, it was shown that unfolding occurred on wort boiling before fermentation and that the reducing conditions are provided by malt extract.

KEYWORDS: Lipid transfer protein 1; protein unfolding; malting; brewing

INTRODUCTION

Lipid transfer proteins (LTPs) are ubiquitous plant lipid binding proteins that were originally identified by their ability to catalyze the transfer of lipids between membranes in vitro. LTPs are involved in responses toward biotic and abiotic stress, but their specific role is still unknown (*1*). LTP1 is an abundant soluble protein of the aleurone layers from barley endosperm (*2*). It accounts for ∼5% of soluble barley proteins. Barley LTP1 is characterized by a p*I* of ∼9 and consists of 91 amino acid residues for a molecular mass of 9689 Da. As other plant LTP1s, its compact structure comprises four α-helices and a C-terminal arm stabilized by four disulfide bonds (*3*). Moreover, the protein has a small hydrophobic cavity allowing the binding of different types of lipids (*4*, *5*). The pattern of cysteine residues constitutes a genuine signature common to all plant LTPs (*6*).

A modified form of LTP1, named hereinafter LTP1b, was highlighted in barley, with a mass of 9983 Da and a primary structure similar to that of LTP1. LTP1b corresponded to LTP1 with a covalently linked lipid-like molecule of 294 Da that was suggested to be a new post-translational modification (*7*, *8*). In malt another adducted LTP1, named LTP1c, was observed corresponding to a protein with two adducts of 294 and 312 Da (*9*).

During the malting and brewing processes, LTP1 becomes a surface-active protein that concentrates in beer foam (*10*). In fact, the native barley seed nonspecific LTP1 (nsLTP1) displays poor foaming properties and becomes a foam-promoting agent only after unfolding on wort boiling (*11*). Besides, it has been shown that beer nsLTP1 is glycated on malting (*9*). Glycation is due to Maillard reactions that occur on kilning and could hinder protein precipitation on unfolding that occurs during wort boiling. In addition, most of the LTP1 in beer and malt is adducted with the lipid-like molecule, although some hydrolysis of the adduct−protein bond occurred on brewing. Altogether, glycation, lipid adduction, and unfolding should increase the amphiphilic character of LTP1 polypeptides and contribute to a better adsorption at air−water interfaces (*9*). In addition, LTP1 is a protease inhibitor (*12*). This activity is critical in the malt quality and the brewing process and may be affected by glycation lipid adduction and unfolding of LTP1s.

Unfolding is essential for the expression of surface activity of LTP1 (*10*). Previous studies have observed thermal unfolding of barley LTP1 without considering glycation and acylation (*13*). In this context, we have no information on the effect of glycation and lipid adduction on the unfolding of the protein during the

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heat treatments occurring on brewing. In addition, the brewing process steps at which unfolding occurs were not identified. Therefore, we have followed the unfolding status of LTP1 along the brewing process and determined in vitro the physicochemical conditions needed for heat-induced unfolding. Because LTP is stabilized by disulfide bonds, reducing conditions induce unfolding (*14*). As a consequence, the effect of reducing conditions, provided by malt extract, on LTP1 unfolding was evaluated. Our study shows that reducing conditions and heating act synergistically on the achievement of LTP1 unfolding.

MATERIALS AND METHODS

Preparation of Wort and Beer Samples. Malt from barley (*Hordeum vulgare* cv. Scarlett) was brewed at the Institut Français des Boissons de la Brasserie Malterie (IFBM) using standard procedures. The mashing profile consisted of a 45 °C stand for 20 min followed by a ramping to 64 °C, a stand for 20 min, another ramping to 76 °C, and a stand for 30 min. After separation, worts were boiled (100 °C) for 90 min, then fermented, and stored several days prior to filtration and bottling. Samples were collected throughout boiling (0, 30, 60, and 90 min) and fermentation (beginning, middle, and end) stages.

Extraction and Purification of Barley, Malt, Wort, and Beer LTP1s. Soluble proteins were extracted from barley or malt flour by a gentle stirring with deionized water. After being centrifuged, the soluble material was freeze-dried for purification. Before freeze-drying, wort and beer samples were dialyzed (dialysis tubing cutoff 3.5 kDa) against deionized water. The lyophilized wort and beer samples were directly submitted to size exclusion chromatography, whereas lyophilized barley and malt extracts were first submitted to cation exchange chromatography.

The lyophilized barley or malt extract was solubilized in 20 mM ammonium acetate, pH 6, and loaded on a cation exchange Streamline SP column (Pharmacia). Proteins were eluted with a linear gradient from 0 to 700 mM NaCl in 20 mM ammonium acetate, pH 6. The collected fractions were analyzed by SDS-PAGE in the presence of 2-mercaptoethanol according to the Laemmli procedure (*15*) using a 10 cm acrylamide (15%) separating gel and a stacking gel. The fractions containing proteins of molecular mass around 10 kDa were pooled, dialyzed against deionized water, and freeze-dried. The dry material was solubilized in 20 mM ammonium acetate, pH 6, and loaded on a Sephadex G50 column (Pharmacia) equilibrated with ammonium acetate buffer. The elution was carried out at a flow rate of 2 mL/min, and the eluted fractions were analyzed by SDS-PAGE. The fractions containing proteins below 10 kDa were pooled, dialyzed against deionized water, freeze-dried, and fractionated by semipreparative reversed-phase HPLC using a column packed with C18 bonded silica Nucleosyl 5 μm 300 Å (Macherey-Nagel, Düren, Germany). Elution was performed at 50 °C, at a flow rate of 3 mL/min, with a linear gradient from 20 to 50% solvent B (acetonitrile, trifluoroacetic acid 0.04%) for 30 min, followed by a rapid increase at 100% solvent B. The proteins were detected by absorbance at 280 nm. The collected fractions were lyophilized after dilution with deionized water.

Mass Spectrometry. Protein purity and molecular masses were assessed with an ion trap mass spectrometer equipped with an electrospray ionization source at atmospheric pressure (LCQ Advantage mass spectrometer; ThermoFinnigan, San Jose, CA). The instrument operated in positive ion mode. Proteins, dissolved in 50% acetonitrile containing 0.5% of formic acid, were continuously infused at a flow rate of 5 μL/min. Measurements were recorded over the m/z range of 500−2000.

Circular Dichroism (CD) Spectroscopy. The secondary structure of proteins was determined by CD in the far UV (from 180 to 250 nm). Measurements were performed at 25 °C on a CD6 Jobin-Yvon dichrograph. Proteins were solubilized in deionized water at a final concentration of 1 mg/mL. A quartz cell of 0.2 cm path length was used. Data were expressed as main-residue ellipticity.

For measuring the temperature dependence of LTP1 secondary structure, solutions containing 1 mg/mL of proteins in deionized water were thermostated to temperatures between 25 and 100 °C with a 15 min step every 10 °C by coupling the cell holder to an external water bath. Temperature was checked with a temperature probe in the quartz cell. To study the role of reducing conditions, freeze-dried LTP1 was solubilized at room temperature in deionized water containing 10 mM sodium bisulfite ($Na_2O_5S_2$).

Detection of Protein-free Thiol Groups in 2D Electrophoresis. Barley and malt were milled in liquid nitrogen, and 1 g of flour was extracted in 10 mL of deionized water for 2 h at room temperature. Extracts were boiled at 100 °C for 30 min, filtered, and freeze-dried. Beers were provided by IFBM. They were degassed and freeze-dried. Freeze-dried protein extracts were dissolved in 50 mM Tris-HCl, pH 7.5, for 30 min at room temperature. Then, 10 mM dibromobimane (D1379, Molecular Probes) in acetonitrile was added to each sample. Samples were incubated for 20 min in the dark, and they were dialyzed against deionized water to remove the excess of dibromobimane. The proteins were precipitated by cold acetone and were dissolved in 8 M urea, 2% (w/v) CHAPS, 2% (v/v) IPG buffer, pH 3−10 (Amersham Pharmacia Biotech, Uppsala, Sweden), 0.01% bromophenol blue, and 20 mM DTT. Isoelectric focusing was performed using a Pharmacia Biotech IPGphor electrophoresis system (Amersham Pharmacia Biotech). Prior to the second dimension, the IPG strips were equilibrated for 10 min in equilibration buffer [50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.01% bromophenol blue] containing 10 mg/mL DTT, followed by 10 min in equilibration buffer containing 25 mg/mL iodoacetamide. The second dimension was run on 15% acrylamide gels on a vertical system. Gels were fixed in 12% trichloroacetic acid. The labeled fluorescent proteins were visualized under UV light, and silver staining (*16*) was performed to observe total proteins.

RESULTS AND DISCUSSION

LTP1 Unfolding from Barley to Beer. LTP1s were purified after cation exchange, size exclusion chromatography, and reversed-phase HPLC from barley, malt, wort (before and on boiling), and on fermentation. Their purity was checked by SDS-PAGE and mass spectrometry. As previously observed, three isoforms of LTP1s were obtained: LTP1 (9689 Da), LTP1b (9983 Da), and LTP1c (10295 Da) (*9*, *17*). More precisely, different LTP1s were recovered during the purification process: LTP1, LTP1b, and LTP1c from barley, LTP1b and LTP1c from malt, and LTP1 and LTP1b from wort and beer. Moreover, in the case of the malt, wort, and beer LTP1s, an increase of 162 Da was observed (**Figure 1**), which corresponded to LTP1s with covalently bound hexoses (*9*, *17*, *18*). The proteins were glycated through Maillard reactions that occur on malting (*9*). These reactions include the initial condensation reaction between proteins (amine groups from N-terminal and lysine residues) and reducing sugars to form Amadori intermediate rearrangement products (*19*). LTP1b was the main fraction of barley and malt LTP1s, whereas some LTP1 could be observed in wort and beer samples. Malt, wort, and beer LTP1s were all glycated. As expected, glycation induced by Maillard reaction was heat stable more than the ester linkage of the lipid like adduct of the barley LTP1 (*8*).

Secondary structures of LTP1s were determined by far-UV CD spectrometry. As previously observed, the CD spectra of the three isoforms of barley LTP1 exhibited two negative minima at 208 and 222 nm and a maximum at 190−195 nm, typical of the α-helix structure of these plant proteins (*3*). Lipid adduction did not induce a significant modification of the secondary structure of LTP1. As a consequence, unfolding of all the forms of LTP1s was observed through the measurement of the ellipticity at 220 nm. The data were normalized by considering relative ellipticity to the corresponding unmodified and purified barley LTP1 (**Figure 2**). A 50% decrease of relative ellipticity was observed at the end of mashing when the temperature increased to 76 °C. After 90 min of boiling, a 75%

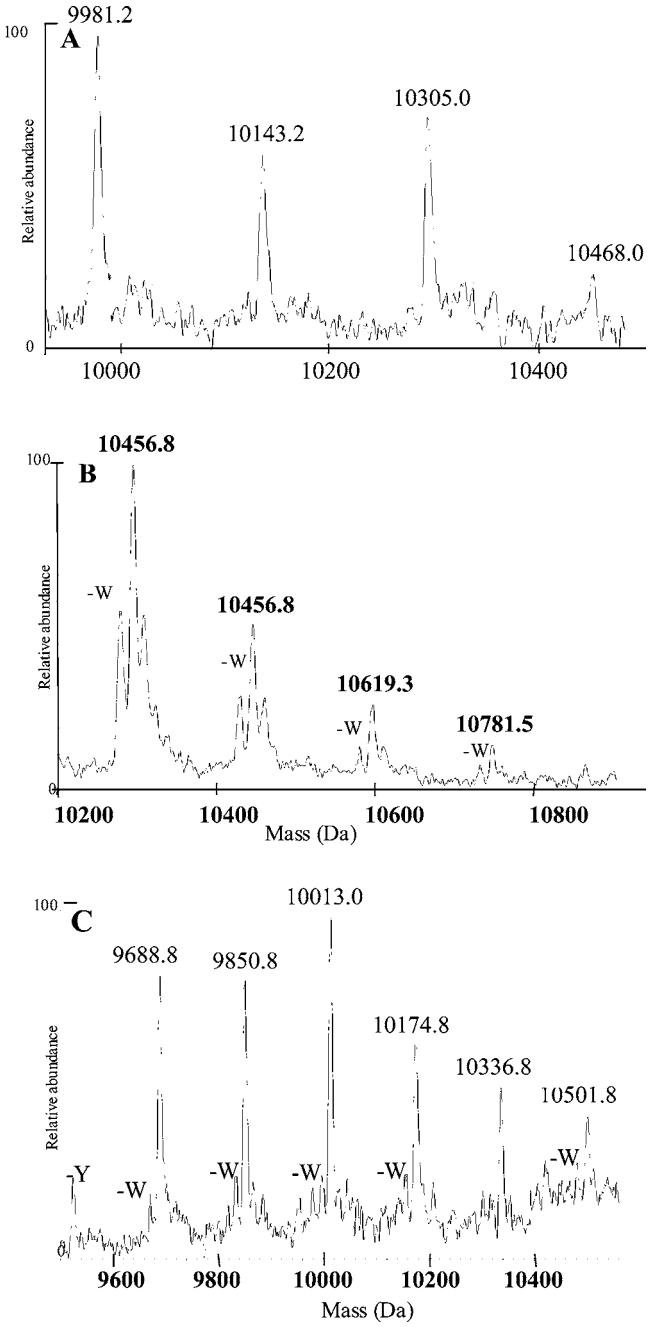

Figure 1. Glycoforms of LTP1s: deconvoluted electrospray mass spectra from multicharged ion spectra of purified (**A**) glycated LTPb isolated from malt and beer, (**B**) glycated LTPc isolated from malt, and (**C**) glycated LTP1 isolated from beer. "Y" represents LTP that has lost C-terminal tyrosine. "W" represents Amadori compounds that have lost water molecules.

decrease of relative ellipticity at 220 nm was observed, which corresponded to the complete denaturation of barley LTP1 as previously observed (*9*). The proteins remained unfolded from the beginning of fermentation to beer (**Figure 2**). Fermentation does not induce any structural modification of the LTP1s.

Therefore, unfolding of LTP1 starts on mashing and is achieved on boiling before fermentation. Unfolding of LTP1 through heat treatments contributes to improve the surface-active properties of LTP1. As a consequence, identification of the stage of LTP1 unfolding should permit optimized conditions to be applied during brewing to improve beer foam quality.

Role of Glycation and Lipid Adduction on the Stability of LTP1s toward Temperature and Reducing Conditions.

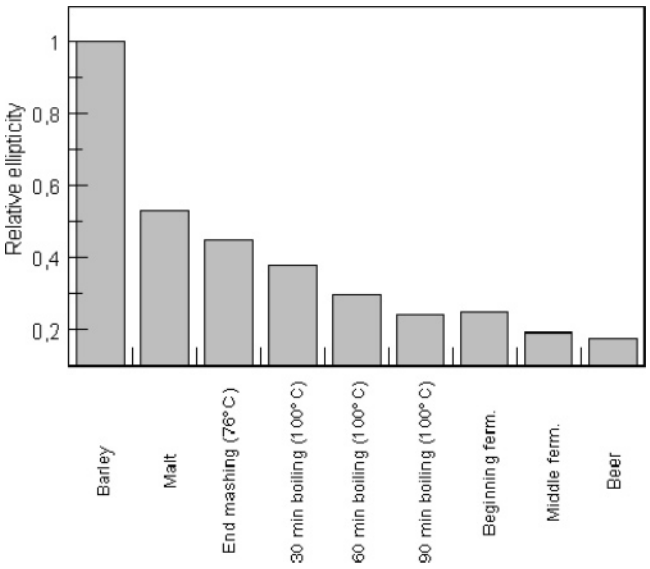

Figure 2. Relative ellipticity at 220 nm of LTP1s extracted from barley, malt, during wort boiling fermentation and beer.

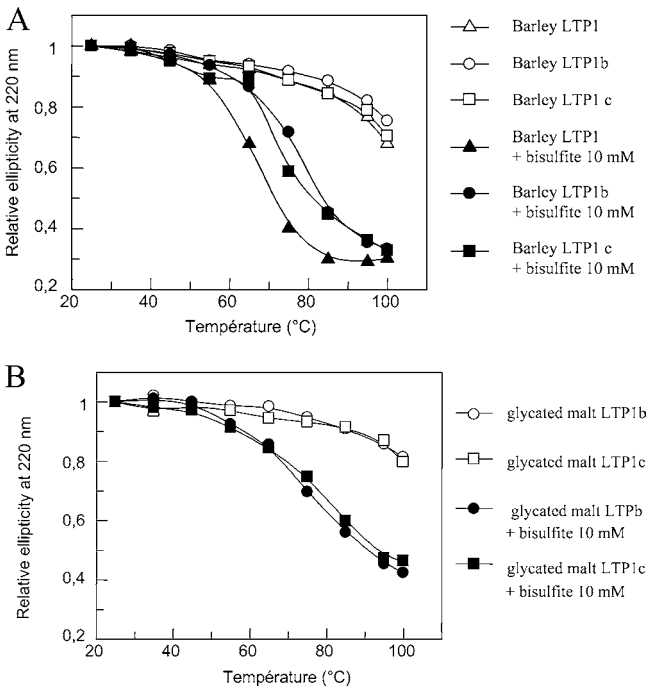

Figure 3. Heat-induced unfolding of barley LTP1, LTP1b, and LTP1c (**A**) and malt LTP1b and LTP1c (**B**). Relative ellipiticity was measured at 220 nm.

As models, purified LTP1, LTPb, and LTPc isolated from barley and malt were heated from 25 to 100 °C. The effect of heating in the presence or absence of sulfite, a reducing agent, on the secondary structure of purified barley LTP1, LTP1b, and LTP1c (**Figure 3A**) and malt LTP1b and LTP1c (**Figure 3B**) was followed by CD. For the three forms of barley LTP1 and the two forms of malt LTP1, the relative ellipticity at 220 nm decreased ~20% when the temperature increased from 20 to 100 °C. The initial ellipticity can be recovered by cooling the protein solutions to 25 °C (**Figure 4**). Therefore, in the absence of sulfite, and under the conditions used in this experiment, heating induced slight changes of LTP1 structure that are completely reversible.

In contrast, for all LTP1 forms, a cooperative decrease of relative ellipticity is observed on heating in the presence of

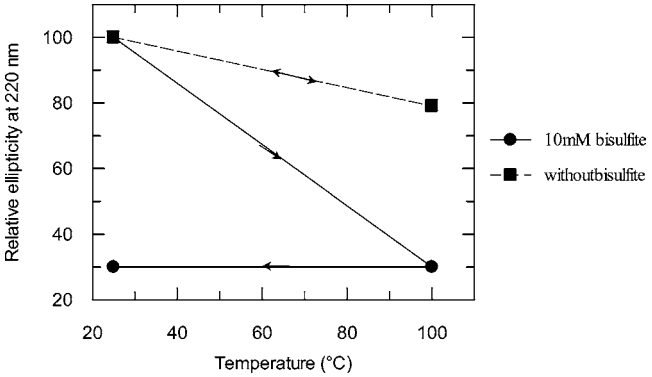

Figure 4. Reversibility of barley LTP1 unfolding measured by relative ellipticity at 220 nm in the absence (- - -) or presence (—) of 10 mM bisulfite. Initial circular dichroism spectra were first obtained at 25 °C, then at 100 °C, and finally at 25 °C.

sulfite (**Figure 3**). The cooperative curves are typical of an unfolding process. After heating to 100 °C, cooling to 25 °C did not allow recovery of the initial ellipticity (**Figure 4**). LTP1 unfolding was therefore nonreversible in the presence of sulfite. From these curves a midtransition temperature could be determined that was considered to be a measurement of unfolding temperature. Whereas barley LTP1 was unfolded at 65 °C, barley LTP1b and LTP1c were unfolded at 80 °C. Similar to barley LTP1b and LTP1c, malt LTP1b and LTP1c were unfolded at ∼80 °C in the presence of sulfite. These experiments showed that, in the presence of sulfite, lipid adduction increased significantly the thermal stability of LTP1, whereas glycation has no or only a slight effect on the structural stability. Therefore, whatever LTP1 modification, lipid adduction or glycation, heating and reducing environment act synergistically on LTP unfolding.

Labeling of Proteins with Free Thiol Groups. Reduction of disulfide bonds was surveyed by 2D electrophoresis using the labeling of free thiol groups of water-soluble heat-stable proteins of barley, malt, and beer. The free cysteines were detected using a fluorescent probe, dibromobimane. Corresponding to the location of LTP1 spots, a fluorescent signal was visualized on malt and beer gels, but not on barley gels (**Figure 5**). LTP1s are known to contain only disulfide bonds (*6*), in agreement with the absence of free cysteine labeling on the corresponding spots of barley gel. Similarly, detection of free cysteine in the corresponding LTP1 spots of beer gels agrees with previous chemical analysis showing the presence of free cysteine in beer LTP1 polypeptides (*17*) (**Figure 5C**). The presence of free cysteine in malt was surprising because, in the experiments described above, a reduction of disulfide bonds would necessarily induce unfolding on heating even in the absence of sulfite. For these experiments malt LTP1s were extracted and purified at room temperature in the absence of reducing agent so that reoxidation of cysteine can occur. On the contrary, reoxidation cannot occur in 2D electrophoresis because the protein sulfhydryls were alkylated just after boiling of malt protein extracts. Therefore, the reducing conditions essential for LTP1 unfolding are already provided by malt extracts in agreement with the observation that unfolded LTP1s are already observed on mashing.

In conclusion, temperature and reducing conditions can act synergistically on the unfolding of LTP1s whatever their chemical modifications, that is, glycation or lipid adduction. Although the thermal and physicochemical conditions used in the CD experiments do not represent the conditions of the brewing process, there is a good agreement with the unfolding

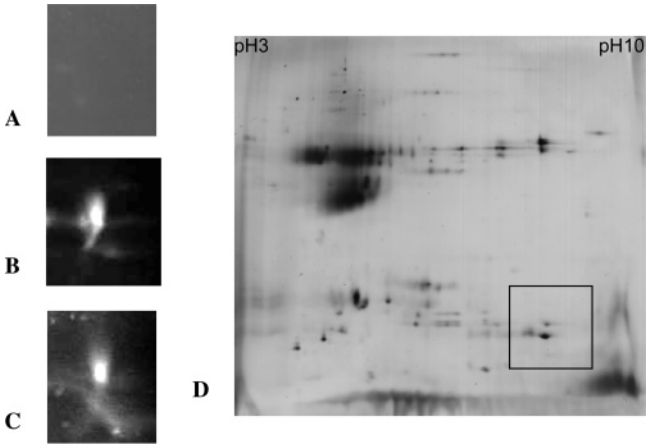

Figure 5. Dibromobimane labeling of barley, malt, and beer proteins. Protein sulfhydryls were alkylated with dibromobimane, and water-soluble heat-stable proteins were separated by 2D electrophoresis in the p*I* range from 3 to 10. Fluorescence spot zone corresponds to (**A**) barley, (**B**) malt, and (**C**) beer. (**D**) LTP1. Malt 2D gel silver-stained. Box indicates the LTP1 zone.

of LTP1 on wort boiling presented in this work. In the absence of reducing conditions, LTP1s are heat stable as previously observed (*20*, *21*). Adduction of LTP1 with lipid-like adduct increases the unfolding temperature, whereas glycation has a weak or no effect. The significant increase of unfolding temperature is, however, not sufficient with regard to the boiling temperature (100 °C) used before fermentation. This strengthens previous results showing that wort boiling temperature is critical in determining the final beer LTP1 content and foaming properties (*13*).

In a previous study Sorensen et al. (*10*) showed that beer LTP1 displays more foam-promoting properties than the corresponding protein isolated from barley. They showed later that this functional maturation was associated with structural changes of LTP1 (*11*). In the presence of free fatty acids, Nierop et al. (*13*) showed that native barley LTP1 improves the foam properties of beer, whereas unfolded barley LTP1 does not. They suggested that LTP1 could prevent beer foam from destabilization by lipids by binding free fatty acids. Glycation, lipid adduction, and unfolding observed on malting and brewing, in this work and in previous studies (*9*, *17*), should contribute to the transformation of barley LTP1 from a poor foaming to a foam-promoting protein. In this regard, it was previouly observed that unfolded and glycated beer LTP1s have good foaming properties in a lipid-free aqueous medium. In the same conditions, it was also shown that lipid adduction improved the foaming properties of both native and unfolded LTP1s (*22*). Therefore, considering that, in beer, all LTP1s are glycated and a major part of these glycated proteins contain a lipid adduct, it would be essential to study the impact of these unfolded forms on the foaming properties of beer in the presence and absence of lipids.

Sulfite was chosen as a reducing agent in this study because it is widely used as an antioxidant in foods and is also produced by yeast (*23*). Because reducing conditions are provided by malt extracts, before fermentation, sulfite is probably not involved in the unfolding of LTP1 during the brewing process. In plant cells, glutathione is the predominant nonprotein thiol and functions primarily as a reducing agent to maintain SH groups in a reduced state (*24*). Glutathione may occur endogenously in malt flour in the free reduced glutathione (GSH) and free oxidized glutathione disulfide (GSSG) forms as well as in the

form of protein−glutathione mixed disulfides (PSSG). The redox status of the various forms of glutathione in grain can undergo considerable changes in response to exogenous factors, for example, temperature (*25*). GSH is the major water-soluble intracellular antioxidant that resists heat treatment, although its content decreases 50% in barley (*26*).

Studies by Buchanan and collaborators have shown that there may be another system within cereal grains whereby the reduction states of various proteins in the grain are controlled by the presence of a system composed of NADPH, thioredoxin, and the NADP−thioredoxin reductase (*27*, *28*). Thioredoxins are small (12 kDa), ubiquitous proteins with protein disulfide reductase activity. The cysteines in the consensus active site sequence of thioredoxins form a disulfide bond. Electron donors, via thioredoxin reductases, reduce the disulfide bond in thioredoxins to the catalytically active dithiol form (*29*). Thioredoxins in the reduced form reduce disulfide bonds in target proteins and can thereby modulate the activity of proteins involved in a variety of cellular processes. Thioredoxin was found to reduce specifically the intramolecular disulfide bonds of a number of proteins in seeds (*30*). Among these proteins, LTP1 was identified as a target of thioredoxin (*31*). Therefore, further studies on the origin of the different oxido-reducing partners of LTP1 would be necessary to improve malting and brewing processes.

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