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Partially Folded Forms of Barley Lipid Transfer Protein Are More Surface Active[†]

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ABSTRACT: Thermal and chemical modification of protein structures is known to affect their interfacial activity. We have looked in detail at the effect of heating on the structure and subsequently the surface properties of LTP1, a nonspecific lipid transfer protein from barley, both in the presence and in the absence of its lipid adduct. CD and NMR spectroscopy showed that some of the protein molecules refold back to the native state structure after being heated to 100 °C for 2 h. However, for a proportion of the molecules, the structure of the protein was irreversibly unfolded which resulted in an increase in surface activity irrespective of the presence of the lipid adduct. These molecules show an increase in surface activity, which is normally associated with an increase in molecular flexibility and surface hydrophobicity and is a property that has been shown to be highly sensitive to structural changes. This explains why thermal and chemical modification of LTP1 is important in optimizing the surface properties of the protein that are essential in diverse applications from biosensors to beer foam.

The nonspecific lipid transfer proteins (nsLTPs)[1] are 9 kDa plant proteins that belong to the prolamin superfamily, which comprises 2S albumins, nonspecific lipid transfer proteins, and cereal α-amylase/trypsin inhibitors (*1*). These proteins have similar three-dimensional folds that are rich in α-helices and share a conserved pattern of cysteine residues with characteristic Cys-Cys and Cys-X-Cys motifs. The nsLTP family is widely distributed throughout the plant kingdom. It comprises basic polypeptides (pI 8.5−10) with 90−95 amino acid residues. The eight conserved cysteine residues that form the network of four disulfide bridges are essential for the maintenance of the nsLTP fold and therefore the lipid binding properties of these proteins. In barley LTP1, these form four disulfide bonds, Cys3−Cys50, Cys13−Cys27, Cys28−Cys73, and Cys48−Cys87. Three-dimensional structures have been determined for a number of nsLTPs, including barley, and show that the four intrachain disulfide bonds stabilize a four-α-helix bundle structure with a central lipid-binding pocket, reflecting its ability to transfer lipids between membranes in vitro (*2*). A post-translationally modified form of barley LTP1, LTP1b, has been identified in which a lipidlike adduct is attached to the protein via the side chain of Asp7. There is some debate about the precise nature of the adducts, with *cis*-7-heptadecenoic acid proposed in one study (*3*), while others have it as α-ketol 9-hydroxy-10-oxo-12(*Z*)-octadecenoic acid (*4*). The presence of the lipid adduct, which lies in the hydrophobic cavity, increased the flexibility of the molecule (*5*). Initially, nsLTPs were defined on the basis of their ability to transfer phospholipids from lipsomes to mitochondria in vitro, but the in vivo role of nsLTPs in plants has been extensively debated, although their role in plant defense mechanisms against phytopathogens seems now to be well-established (*6*) and some nsLTPs have been designated as belonging to PR (pathogenesis-related) group 14, and/or being involved in the transport of cutin and suberin monomers to the site of waxy cuticle synthesis (*7*).

nsLTPs are remarkably thermostable, only going through a phase transition at temperatures around 100 °C, a property conserved in nsLTPs from a diverse range of plant species. Previous work on the thermal denaturation of LTP1 from rice has shown that it unfolds and refolds via predominant species of partially structured intermediates (*8*). Native nsLTPs, while surface active, do not form a viscoelastic interface and hence are poor emulsifiers (*9*). Processing-induced changes in conformation appear to be important for optimal interfacial activity of these proteins in food systems. This may explain why barley nsLTP is the major foam-promoting protein in beer (*10*). During the brewing process, barley grain is allowed to germinate (malting), which begins to liberate sugars within the grain, followed by a heating or kilning process which is thought to induce glycation of the protein. Further in the process, the malted barley extract (wort) is subjected to a boiling process that lasts typically 1−2 h, known as wort boiling. This has been shown to produce unfolded forms. These unfolded and glycated forms are thought to be the entities that have enhanced foam promoting properties in beer (*11*, *12*). Previous work on the effect of heating barley LTP1 has shown that where limited unfolding is induced the surface hydrophobicity is significantly increased (*13*−*15*) and the foaming properties are enhanced. However, if the heating and

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[1]Abbreviations: NMR, nuclear magnetic resonance; TOCSY, total correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; COSY, $^{1}H-^{1}H$ correlation spectroscopy; ROESY, rotational frame nuclear Overhauser effect spectroscopy; CD, circular dichroism; FTIR, Fourier transform infrared; SEC, size exclusion chromatography; LC−MS, liquid chromatography−mass spectrometry; nsLTP, nonspecific lipid transfer protein.

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the unfolding induce aggregation, the foaming properties are poor (*16*). In the work described here, we have examined in detail the effect of heating on the structure and subsequently the surface properties of LTP1 from barley both in the presence and in the absence of the lipid adduct.

EXPERIMENTAL PROCEDURES

The following chemicals were obtained from the companies listed: hydrochloric acid, sodium hydroxide, and deuterium chloride from the Aldrich Chemical Co. (Milwaukee, WI); deuterium oxide (>99.9%) from Apollo Scientific Ltd. (Cheshire, U.K.); sodium deuteroxide (>99%) from Sigma Chemical Corp. (St. Louis, MO); and dioxan from BDH Chemicals Ltd. (Poole, U.K.).

All water used was analytical grade deionized high-purity water, prepared by a reverse osmosis and ion-exchange Milli-Q system.

Extraction and Purification of LTP1 and LTP1b from Barley. Barley flour (800 g, Cultivar Optic, provided by Brewing Research International, Nutfield, U.K.) was mixed with 2 L of pure water and gently stirred at room temperature for 5 h. The mixture was centrifuged at 4000*g* for 20 min, and then the supernatant was centrifuged at 12000*g* for 20 min. Proteins were precipitated with 1 kg of $(NH_4)_2SO_4$ (95% saturation at 1 °C). The precipitation was suspended in 400 mL of 20 mM MES buffer (pH 5.6) and dialyzed with a 3500 cutoff membrane tube against 20 mM MES buffer (pH 5.6).

LTP1b was purified using two ion-exchange chromatography steps followed by gel filtration. The first step used a Biocad Sprint perfusion chromatography system (Applied Biosystems Inc.) in which 250 mg per run was loaded onto a column (2.6 cm × 18 cm) of SP Sepharose Fast Flow (GE Healthcare), followed by dialysis against 20 mM MES buffer (pH 5.6) and rechromatography of 10 mg per run on a column (0.46 cm × 10 cm) of Poros HS 20 (Applied Biosystems Inc.). The final stage of purification was conducted using an AKTA FPLC Basic system (GE Healthcare) using a Sephadex 75 preparative grade gel filtration column (1.6 cm × 60 cm) (GE Healthcare) loaded with 10 mg/run, equilibrated and eluted with 50 mM sodium phosphate and 150 mM sodium chloride buffer (pH 7.0). The fractions containing LTP1b were pooled and dialyzed with a 3500 cutoff membrane against water. After the samples were freeze-dried, 150 mg of LTP1b was obtained. A small amount of LTP1 was present in the sample. Further purification was attempted with hydrophobic interaction chromatography and HPLC but was unsuccessful.

Isolation of LTP1 is similar to that described above for LTP1b, but the barley flour was defatted first with a chloroform/methanol mixture (2:1, v/v) before extraction with water. This method was used because the conversion of LTP1 to LTP1b occurs during the aqueous extraction of the bran to solubilize the protein. Thus, extraction of the bran with a chloroform/methanol mixture prevents the conversion from occurring, either by removal of the majority of the lipid precursor of the adduct, by inactivation of the enzyme responsible for the conversion, or by a combination of the two mechanisms.

Identification of LTP1b was confirmed by comparison with previous work (*3*, *4*), indicating the identification of the modification in our sample being the same as the one in the literature. Analysis of our protein by LC−MS gave a mass for LTP1 of 9683 Da (this is 9691 Da when corrected for four disulfide bonds). This matches EXPASY ref A8YPK3 with a molecular mass of post-translationally modified protein (LTP1) of 9694.9 Da. For LTP1b, our MS-determined molecular mass was 9978 Da (this is 9986 Da when corrected for four disulfide bonds) which is in close agreement with the literature value of 9983 Da (*11*).

Heating of LTP1 and LTP1b. An LTP1 or LTP1b solution (0.1 mM) was prepared in 0.1 M citrate buffer (pH 5). Samples (1 mL) were put into a HPLC vial and gently blown with argon for a few minutes, and the vial was sealed and heated in a hot block to the desired temperature for various times of ≤2 h. The samples were then left at room temperature for 0.5 h to cool and were then stored at 4 °C overnight before structure and surface property analysis.

Size Exclusion Chromatography (*SEC*). Size exclusion chromatography was performed on a Hiload 16/60 Superdex 75 preparative column attached to an AKTA FPLC Basic system. The column was eluted with 50 mM phosphate buffer (pH 7) containing 150 mM NaCl and the eluate monitored for protein by UV absorption at 220 nm at 1 mL/min. The following molecular mass markers were used: thyroglobulin (bovine) (670 kDa), γ-globulin (bovine) (158 kDa), ovalbumin (chicken) (44 kDa), myoglobin (horse) (17 kDa), aprotinin (bovine) (6.5 kDa), and vitamin B_{12} (1.35 kDa).

HPLC. HPLC was performed with an AKTA FPLC Basic system using a reverse phase Jupiter C4, 300 Å, 5 μm, 250 mm × 4.6 mm column. Eluate was monitored by UV absorption at 220 nm. Solvent A was water containing 0.1% (v/v) trifluororic acid, and solvent B was 90% (v/v) acetonitrile with 0.1% (v/v) trifluororic acid, with a gradient from 20 to 40% solvent B over 60 min.

Surface Property Test. The surface tension (γ) was measured using an FTA200 pulsating drop tensiometer (First Ten Ångstroms, Portsmouth, VA). The technique measures the surface tension using the pendant drop technique, in which an image of a liquid droplet hanging from the tip of a syringe is captured and the shape of the drop (determined by its density and the surface tension) is analyzed using a derivation of the Young−Laplace equation (equation of capillarity) to give the surface tension. The text refers to surface pressure, which is defined as the surface tension of the buffer minus the surface tension of the sample. The initial drop volume was 12 μL, and the syringe volume was 100 μL, fitted with a Teflon-coated, flat-ended tip 0.94 mm in diameter. All measurements were taken at room temperature (~20 °C).

Electrophoresis. SDS−PAGE was performed with a Nupage 12% Bis-Tris precast mini gel from Invitrogen Ltd. (Paisley, U.K.), run with Nupage MES running buffer. DTT was used as a reducing reagent for reduced samples. The samples were diluted 1:4 with Nupage sample buffer, and 4 μg was loaded onto the gel. The gel was stained with SimplyBlue SafeStain from Invitrogen. The following molecular mass markers from the Mark 12 standard (LC5677) also from Invitrogen Ltd. were used: myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase B (97.4 kDa), BSA (66.3 kDa), glutamic dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), aprotinin (6.0 kDa), insulin B chain (3.5 kDa), and insulin A chain (2.5 kDa).

Circular Dichroism (*CD*) *Measurements*. Far-UV (190−260 nm) and near-UV (250−350 nm) CD spectra were recorded at 20 °C using a J-710 CD spectra polarimeter (Jasco Ltd.). Protein (10 μM) in 10 mM citrate buffer (pH 5) and a 0.5 mm cell were used for far-UV experiments; 0.1 mM protein in 0.1 M citrate buffer (pH 5) and a 10 mm cell were used for near-UV

experiments. Spectra were collected as the average of four accumulations at a rate of 100 nm/min, with a time constant of 2 s, a resolution of 0.5 nm, and a sensitivity of ±100 mdeg.

Fluorescence Spectroscopy. Fluorescence measurements were performed at 20 °C with an LS 55 luminescence spectrometer (Perkin-Elmer, Wellesley, MA) using a quartz cuvette with a path length of 1.0 cm. Samples contained 0.5 μM protein in 0.5 mM citrate buffer (pH 5). The fluorescence spectra were collected in 300−400 nm range with excitation at 280 nm using excitation and emission slits at 5 nm. The scan speed was 100 nm/min.

LC−MS. Mass spectra were recorded using a Micromass Quattro II triple-quadrupole mass spectrometer (Micromass, Manchester, U.K.) coupled to a Jasco PU-1585 triple-pump HPLC system equipped with an AS-1559 cooled autoinjector, a CO-1560 column oven, and a UV-1575 UV detector [Jasco(UK) Ltd., Great Dunmow, U.K.]. A reverse phase column (Jupiter C4, 300 Å, 5 μm, 250 mm × 4.6 mm) was used. Mass spectra were recorded in positive ion electrospray mode using a Micromass Z-spray ion source.

NMR. One-dimensional (1D) NMR experiments were performed at pH 5 and 25 °C on LTP1 and LTP1b, and on the same samples after heat treatment; 3 mM samples of LTP1 and LTP1b in Eppendorf tubes were sealed with Nescofilm (Bando Chemical Industries Ltd.) and heated on a dry block for 2 h at 100 °C, before being allowed to cool to 25 °C. The NMR samples were prepared in a 95% H_2O/5% ^2H_2O mixture in Shigemi tubes. NMR experiments were conducted on home-built spectrometers at the Department of Biochemistry, University of Oxford, at 500, 600, or 750 MHz. A sweep width of 6024.0 Hz was used at 500 MHz and scaled accordingly for higher fields. For LTP1b, NOESY spectra with mixing times of 50, 100, and 200 ms were recorded at pH 4 and 37 °C. NOESY spectra with mixing times of 200 ms were also recorded at pH 3 and 26 °C and at pH 5 and 25 °C. For LTP1, 200 ms mixing time NOESY spectra were recorded at pH 4 and 37 °C and at pH 5 and 25 °C. TOCSY spectra with a mixing time of 74 ms were recorded for LTP1b at pH 3 and 26 °C, pH 4 and 37 °C, and pH 5 and 25 °C. TOCSY spectra for LTP1 were recorded at pH 4 and 37 °C and at pH 5 and 25 °C. The NMR spectrum of LTP1b was assigned, and assignments for LTP1 at pH 5 and 25 °C were obtained by comparison with published assignments at pH 4 and 37 °C.

PFG NMR. NMR diffusion measurements were conducted at 25 °C using the PG-SLED sequence (*17*) for LTP1 and LTP1b samples before and after they were heated at 100 °C for 2 h at pH 5. Five microliters of 10% 1,4-dioxane was added to each sample as an internal standard (*17*, *18*). A total of 20 spectra were collected with the gradient strengths varying linearly from 5 to 100%. The diffusion coefficients for the protein and the dioxane in the solution were determined as described previously (*17*, *18*). The ratio of the diffusion coefficients of the dioxane and the protein (D_{diox}/D_{BLA}) and the known hydrodynamic radius of dioxane (R_H^{diox} = 2.16 Å) are used to calculate the hydrodynamic radius of the protein: $R_H^{BLA} = (D_{diox}/D_{BLA})R_H^{diox}$. The errors for the measured values are estimated on the basis of repeat measurements with each sample.

RESULTS

Effect of Heating on the Surface Activity of LTP1. Initially, the effect of the lipid adduct on heat-induced changes in LTP surface properties was investigated. Solutions of both LTP1 and LTP1b were heated to 100 °C for 2 h, and the surface activity of 10 μM solutions was assessed by measuring surface pressure at the buffer−air interface for a period of 15 min, as shown in Figure 1. Before being heated, the LTP1b showed greater surface activity, and this may be due to its increased flexibility as shown previously (*5*). After being heated, both LTP1 and LTP1b showed increased surface activity as revealed by the higher values of surface pressure reached after 15 min, 3 mN/m in each case.

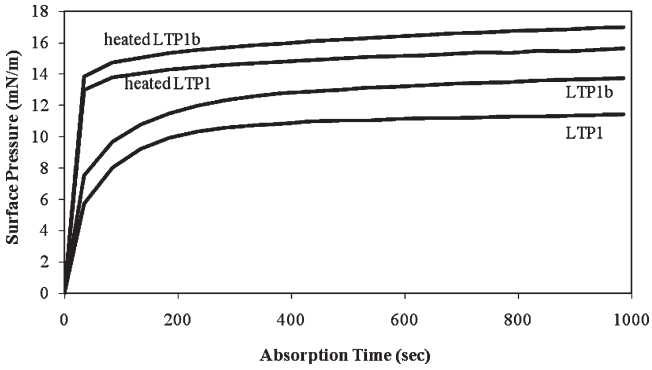

FIGURE 1: Surface pressure as a function of adsorption time for native and heated (100 °C for 2 h) LTP1 and LTP1b. Solutions contained 10 μM protein in 10 mM citrate buffer (pH 5).

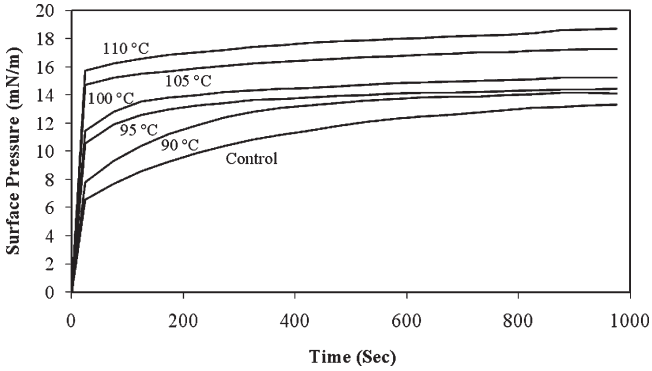

FIGURE 2: Surface pressure of LTP1b as a function of time for samples heated to different temperatures for 2 h.

To examine these phenomena in more detail, we heated solutions of LTP1b to various temperatures ranging from 90 to 110 °C for 2 h. The surface activity of the solutions was then assessed, and the results are given in Figure 2. Data for the surface pressure of LTP1b as a function of heating temperature showed that the surface activity of the heated LTP1b increased with the heating temperature. This is not inconsistent with the result for the thermal stability of LTP1 by Lindorff-Larsen and Winther (*12*) suggesting that the thermal transition was above 100 °C.

Effect of Heating on Barley nsLTP Secondary Structure and Aggregation. To understand the extent to which thermally induced changes in protein structure may determine the changes in surface properties, we assessed the effect of heating on the secondary and tertiary structure of barley LTP1 and LTP1b using CD spectroscopy. The near-UV CD spectrum of a protein is largely determined by its tertiary structure; those of both LTP1 and LTP1b (Figure 3a) had two minima at around 265 and 293 nm, although the latter minimum was much reduced in LTP1b. After being heated for 120 min at 100 °C, both proteins gave spectra that were very similar to that of the unheated protein apart from the region between 270 and 300 nm which can be attributed to tyrosine residues, LTP1 lacking tryptophan or

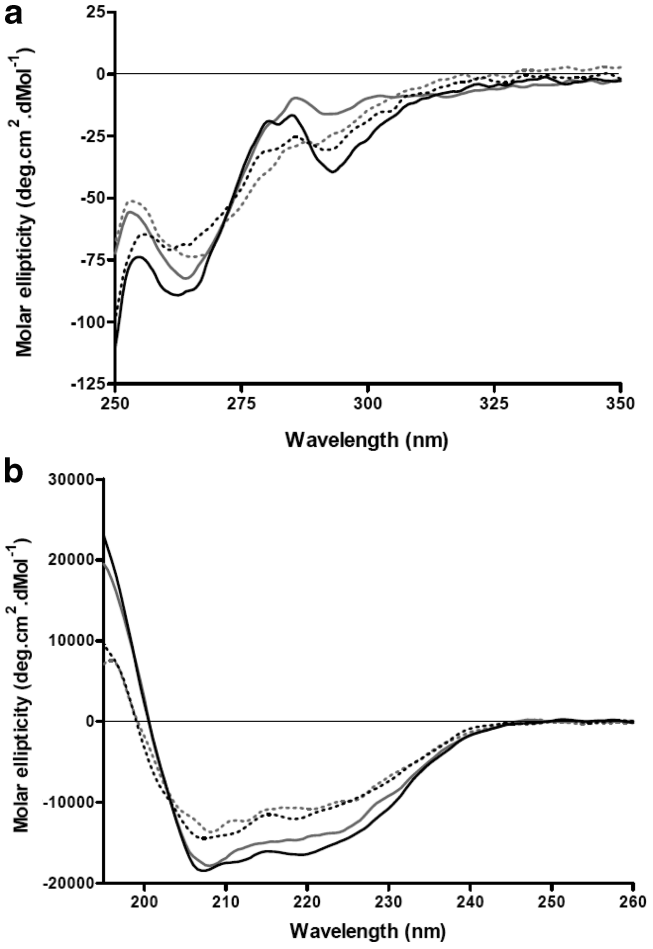

FIGURE 3: (a) Near-UV CD spectra of native (solid lines) and heated (dashed lines) LTP1 (black) and LTP1b (gray). (b) Far-UV CD spectra of native (solid lines) and heated (dashed lines) LTP1 (black) and LTP1b (gray).

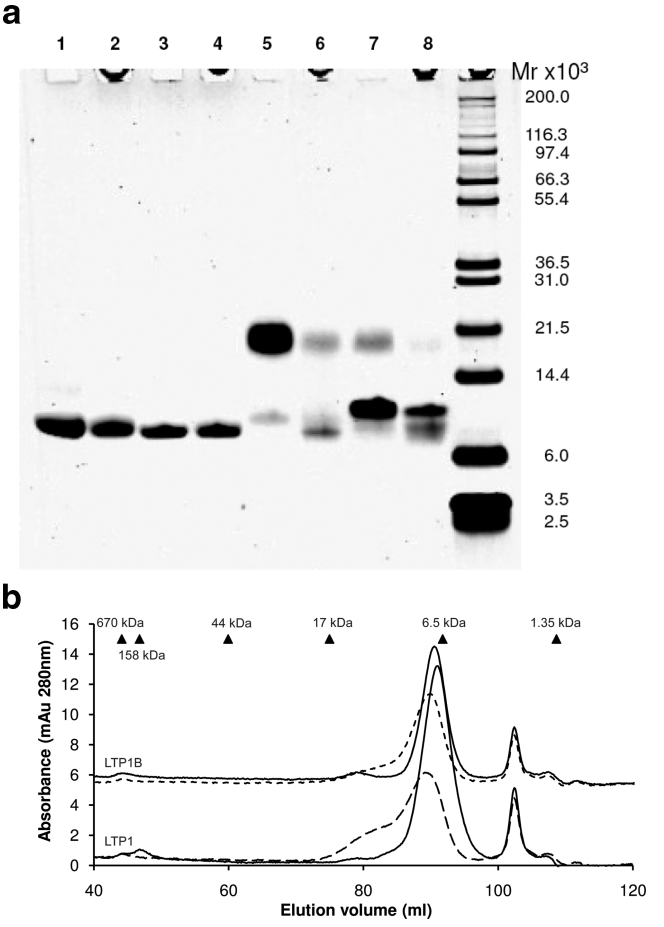

FIGURE 4: (a) SDS−PAGE of LTPs under reduced and nonreduced conditions: tracks 1 and 5, native LTP1; tracks 2 and 6, LTP1 heated at 100 °C for 2 h; tracks 3 and 7, native LTP1b; tracks 4 and 8, LTP1b heated at 100 °C for 2 h; tracks 1−4, reduced; tracks 5−8, nonreduced. (b) Size exclusion chromatogram of native (solid lines) and heated (dashed lines) (100 °C for 2 h) LTP1 (bottom lines) and LTP1b (top lines).

phenylalanine residues. Thus, the LTP1b spectrum had a larger minimum at 293 nm after heating, while that of LTP1 was reduced in intensity. These data show that a proportion of the LTP1 molecules within the sample lost some tertiary structure upon heating, but either the change or the proportion undergoing change was less significant than the small change induced by binding of the ligand in LTP1b. The far-UV CD spectra (Figure 3b), which are determined by the secondary structure of the protein, were typical for α-helical structures with double minima at around 220 and 208 nm for all samples. However, the ellipticity of the heated LTPs was decreased, indicating either that both LTP1 and LTP1b had irreversibly lost approximately 25% of their helical structures after heating or a change in the disposition of the α-helices. The heat-induced decrease in ellipticity for both proteins was comparable to the differences that have been seen by others after heating to 96 °C (*15*) and similar to a glycated fraction of LTP1 and LTP1b purified from boiled wort (*13*).

The changes in secondary and tertiary structure were accompanied by changes in LTP1 mobility following SDS−PAGE analysis under nonreducing conditions, which were lost on reduction (Figure 4a). Following reduction, both native and heated LTPs had nearly identical apparent masses, with LTP1b running with a slightly faster mobility (M_r) of 9900 kDa than LTP1, which had an M_r of 10170 kDa. However, LTP1 ran anomalously without reduction with an M_r of 21000 kDa (Figure 4a, track 5), presumably because of the protein's ability to bind lipids and form unusual complex structures (*19*). This form was less evident in LTP1b, running instead as a species with an M_r of 12460 kDa (Figure 4a, track 7). The anomalous mobility has also been seen in other LTPs, such as that from maize (*20*). The difference in behavior of LTP1 and LTP1b may be attributed to the presence of the lipid adduct in LTP1b, which is located in the LTP1 cavity (*5*) and probably disrupts interactions with SDS. Upon being heated, the higher-M_r forms of both LTP1 and LTP1b were greatly reduced in abundance (Figure 4a, tracks 6 and 8, respectively) but are accompanied by the appearance of lower-M_r forms of 9640 and 9930 kDa. These data indicate that heating altered the shape and surface properties, affecting the interactions of LTP1 and LTP1b with surfactants such as SDS. Similarly, the apparent molecular mass determined by gel-permeation chromatography was altered, with a shift in M_r from 6900 kDa for the native protein to 7250 kDa for the heated protein. This was accompanied by the appearance of a broad shoulder representing an ensemble of species running with M_r values of 9930−16490 kDa (Figure 4b). Similar changes were found for LTP1b (data not shown). This suggests that the hydrodynamic properties of the LTP were altered by heating with the formation of an ensemble of slightly different forms as a result of an expansion of the protein structure relative to that

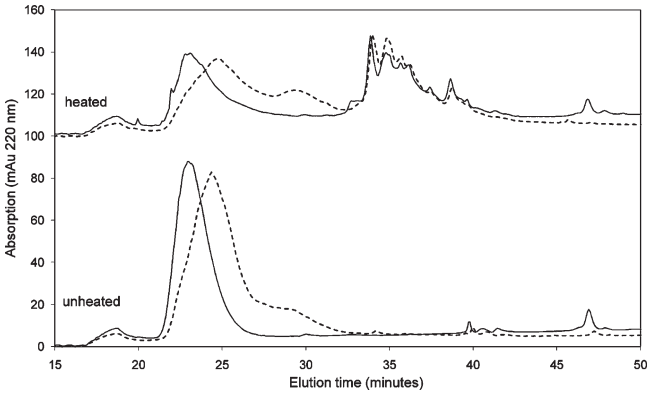

FIGURE 5: Reverse phase HPLC of native and heated (100 °C for 2 h) LTP1 (—) and LTP1b (···).

found in the native form, and these may have induced an increase in surface hydrophobicity caused by the partial unfolding of the protein after heating. To test this hypothesis, reverse phase HPLC was undertaken (Figure 5). This showed that there was a cluster of peaks from the heated LTP1 that eluted later than the nativelike protein peak, which indicates that a proportion of the denatured forms was more hydrophobic than the native form. The heated sample comprised ~50% of these denatured forms and is similar to changes reported by others (*15*). The heated LTP1b had HPLC and SEC profiles similar to those of heated LTP1 (data not shown).

NMR Analysis of Heated LTP. Low-resolution CD spectroscopic analysis of unfolded states was supported by 1D NMR analysis of LTP1 and LTP1b before and after they were heated for 2 h at 100 °C (Figure 6, aromatic and methyl regions of 1D NMR spectra). While the heated protein samples gave spectra showing resonances with considerable chemical shift dispersion, typical of a native folded protein, it was not possible to fully superimpose spectra of heat-denatured and native LTP1 and LTP1b. Thus, heating resulted in some irreversible changes, particularly evident in the aromatic/NH region of the spectra. This conclusion is supported by the fact that the chemical shift dispersion of the large methyl envelope (1−0.5 ppm) was slightly smaller (more obviously for LTP1b) and the the level of fine structure reduced in both the heated samples compared with the native proteins. These spectral data indicate both heated LTP1 and heated LTP1b contain some proportion of protein in a non-native conformation and are probably unfolded to some extent.

Subsequently, two-dimensional (2D) TOCSY and NOESY analysis of the samples before and after they had been heated were undertaken (Figure 7). A few peaks corresponding to residues adjacent to ionizable residues show small differences in chemical shift, which may be ascribed to small changes in pH during sample handling. Inspection of the spectra of both LTP1 and LTP1b after heating showed that the majority of peaks could be overlaid on those in the spectra of the native proteins. This shows that a proportion of the protein molecules refold correctly into the native state, even after prolonged heating. However, there was also evidence of unfolded proteins in the LTP1b samples since most of the low-intensity peaks in the unheated sample were no longer visible in the heat-treated sample, indicating that the proportion of protein retaining the native fold was smaller than in the case of LTP1.

Some additional peaks with low chemical shift dispersion are also clearly visible, in the center of the TOCSY spectral region (close to the S40 HB and S88 HB peaks) of both heated LTP1

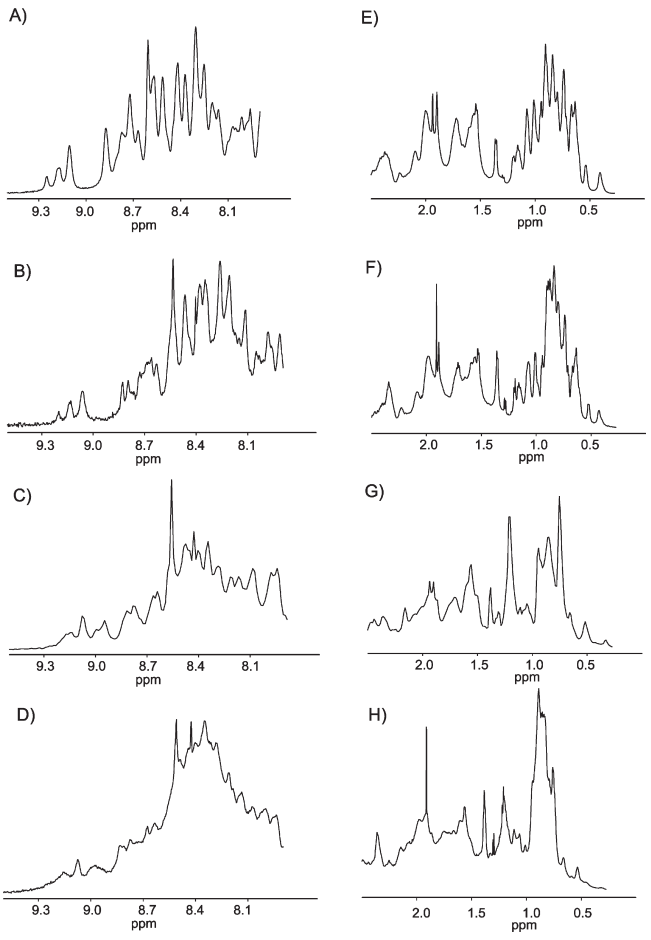

FIGURE 6: 1D NMR spectra recorded at 25 °C and pH 5 for (A and E) LTP1 before being heated, (B and F) LTP1 after being heated for 2 h at 100 °C, (C and G) LTP1b before being heated, and (D and H) LTP1b after being heated for 2 h at 100 °C. The figures in the left column (A−D) show the downfield region (aromatic and NH) and the figures in the right column (E−H) the upfield (methyl) region.

(Figure 7) and LTP1b (data not shown), certain of which appear to be fairly broad, suggestive of partially unfolded protein. In addition, several very sharp new peaks could be seen, some of which were common to both heated LTP1 and LTP1b, although more pronounced in the latter. These may be ascribed to a peptide formed by hydrolysis of the parent protein(s). As there are not many such strong peaks, it is possible to speculate that the peptide is fairly short. Attempts to identify the peptide using ROESY spectroscopy were unsuccessful. N-Terminal sequencing data identified a very small amount (~1%) of a peptide corresponding to T80−I85, which was detected in the heated samples of both proteins. This may be formed as a result of initial hydrolysis of the protein chain at Asp86 followed by further proteolysis. Such hydrolyses of the Asp−X bond of disulfide-bonded proteins following heating have been documented, at low pH (*21*, *22*). It is not clear if the C-terminal region following this region (i.e., D86-C87-S88-R89-I90-Y91) exists as another cleaved peptide or whether it is still connected to the main polypeptide chain by the C48−C87 S−S bond. However, it is unfolded, giving strong unfolded peaks seen in spectra of both proteins.

The hydrodynamic radii for unheated LTP1, unheated LTP1b, heated LTP1, and heated LTP1b were determined at pH 5 and 25 °C (Table 1) using PFG diffusion experiments. These experimentally determined values were compared with those predicted for both native and highly unfolded states of a protein calculated

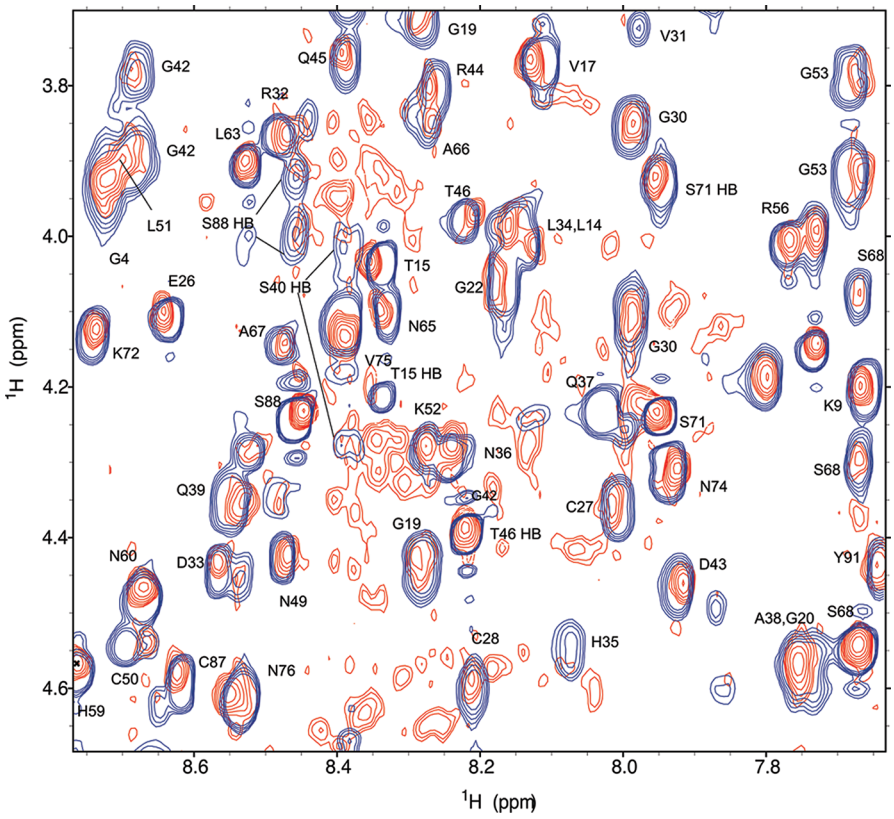

FIGURE 7: 2D TOCSY spectrum at pH 5 and 25 °C of unheated LTP1 (blue) overlaid with the spectrum of the protein heated to 2 h at 100 °C (red). The peaks are labeled for LTP1 (assignments taken from ref *2* at pH 4 and 37 °C and transferred to our experimental conditions by collecting TOCSY spectra at intermediate temperature and pH values).

Table 1: Effective Hydrodynamic Radii for Unheated LTP1 and LTP1b and for Samples of LTP1 and LTP1b Heated at 100 °C for 2 h

	hydrodynamic radius (Å)	
protein	experimentally determined	calculated[a]
LTP1	17.05 ± 0.5	17.94
LTP1b	17.00 ± 0.7	
LTP1 (heated)	17.86 ± 0.7	–
LTP1b (heated)	18.07 ± 0.7	–
unfolded LTP1	–	29.43

[a]Calculated with the empirical equations given in ref *18*.

from the number of residues in the polypeptide chain (*18*). For a protein with 91 residues (such as LTP1 and LTP1b), this gives an expected hydrodynamic radius of 17.94 Å in the native state, and 29.43 Å in the unfolded state. The hydrodynamic radius observed experimentally for native LTP1 and LTP1b is comparable with the empirical estimate detailed above, and also with other proteins of comparable size. For example, ubiquitin (76 residues) has a hydrodynamic radius of 17.03 Å (*23*) and cytochrome *c* (104 residues) a hydrodynamic radius of 18.8 Å (*24*). A small increase in hydrodynamic radii was seen in LTP1 and LTP1b when they were heated for 2 h at 100 °C but was within the experimental error for determining the hydrodynamic radii. The dominant resonances in the heated 2D NMR spectra are those of the nativelike protein rather than unfolded states. The PFG NMR method is therefore probing the effective hydrodynamic radius of mainly nativelike molecules. The increase in effective R_H seen for both heated LTP1 and LTP1b probably reflects contributions from a small proportion of partially unfolded molecules with larger R_H values.

DISCUSSION

We have undertaken a molecular study of the effect of heating on the structure and surface properties of native LTP and of the molecule modified by a lipid adduct, LTP1b. There are differences in surface activity between LTP1 and LTP1b, with the latter showing a higher surface activity (*5*). This is likely to be due to the increased molecular flexibility caused by the attached lipid moiety in the hydrophobic cavity changing the internal stresses in the molecule. Heating produces a similar increase in surface properties for both molecules, with significant increases in surface activity only being observed in samples that have been heated to temperatures above 90 °C. This matches the fact that the phase transition occurs at temperatures in excess of 100 °C (*12*). Surface activity is governed by a number of factors, with surface hydrophobicity and molecular flexibility both being important. The similarity in the increase in surface activity due to heating of both LTP1 and LTP1b suggests that the increase is due to partial unfolding of both proteins causing an increase in surface hydrophobicity, which is unaffected by the presence of the lipid adduct on LTP1b. Reverse phase HPLC also showed that a proportion of the heated proteins had an increased surface hydrophobicity, although the SEC also showed the presence of an ensemble of folded states with different hydrodynamic radii. With regard to surface activity, the presence of aggregates or molecules with increased hydrodynamic radii works counter to the effect of increased surface hydrophobicity in that aggregation reduces the surface hydrophobicity of the molecules and the increased size of the adsorbing moiety reduces the rate of diffusion of the protein to the interface. This suggests that the unfolding due to heating was quite significant, and the chromatography results indicate that only a proportion of the protein

was left in this significantly unfolded state after heating. Additionally, measurements of near-UV CD showed only minor changes in the apparent tertiary structure, although since CD is largely sensitive to changes in α-helical structure and only one (Y16) of the tyrosine residues in LTP1 is located in an α-helix it is not a sensitive indicator of subtle changes in structure and not of changes local to the more flexible C-terminal (N74−Y91) end of the molecule. Thus, we may surmise that the majority of the surface activity changes seen as a result of heating, and possibly of lipid adduct binding, are generated by structural changes that are confined to one segment of the protein structure, such as the C-terminal region. This region is the only region that has shown evidence of hydrolysis during processing (*11*) and very possibly for the same reasons of increased flexibility.

Like the chromatography data, the NMR data show that some nativelike protein was present, and despite the prolonged heating, some proportion of the protein refolds correctly. The proportion of the protein that correctly refolded may be smaller for LTP1 than for LTP1b. The NMR spectrum of both LTP1 and LTP1b after heating also shows more of the LTP1b than the LTP1 remaining in the native form. These results could be explained by a greater proportion of LTP1b being unfolded compared to the native LTP1 (due to more extensive hydrolysis and/or disulfide interchange), and therefore altering the relative proportions of native to denatured LTP1 and LTP1b. In some molecules, the lipidlike modification may also have been cleaved from the LTP1b N-terminal region during the heating process.

Rice LTP1 (CAG28937), which is 38% identical in sequence with barley LTP1 (P0597), including all the cystines residues, has been shown to unfold and refold via predominant species of partially structured intermediates containing non-native disulfide bonds in a study using disulfide scrambling experiments (*8*). Most interestingly with regard to the results reported in this paper are the disulfide scrambling experiments performed at high temperatures (55, 65, and 75 °C for up to 60 min) in the presence of 2-mercaptoethanol (*8*). Scrambled species of LTP1 were identified, with four such species being predominant. Two of the species (termed X-LTP1-a and X-LTP1-b) were extensively unfolded with ∼10% helical content and predominated under strongly denaturing conditions. The other two (X-LTP1-c and X-LTP1-d) contained 30% of the native helical structure and predominated under moderate denaturing conditions, and both contained the native 48−87 disulfide bond. On the basis of the fact that disulfide shuffling was seen previously in bovine α-lactalbumin (*25*) under similar conditions and also seen in LTP1 from rice (*8*), one proposed mechanism for the enhanced surface properties after heating could be that the loss of the Cys3−Cys50 and Cys28−Cys73 bonds would be likely to cause an increase in molecular flexibility such as that inferred from the increase in surface activity.

In this work, we have shown that even after being extensively heated there was a proportion of the protein that retained its native fold. Thus, while the majority of the protein unfolded after heating, showing increased flexibility and consequently enhanced surface properties, there was a significant amount that was relatively unaffected. This work highlights the intrinsic stability of the LTP1 structure and shows why this protein is important in stabilizing beer foam as it is one of the few to survive the long boiling times required in the beer brewing process. The structural stability may also be an important factor in defining why this protein family is important in food allergy. While the amount of native fold LTP1 in beer is likely to be rather minimal, cases of allergic reactions have been reported (*26*), supporting the idea that LTP1 is extremely thermostable and that heating produces an ensemble of structures ranging from the near native to those that are sufficiently unfolded to be able to stabilize the foam.

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