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Article

Heat-Induced Changes in the Chemical Structure, Hydrophobicity, and Size Distribution of Free/Bound Lipid Transfer Protein 1 and Their Effects on Beer Foam

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ABSTRACT: During brewing processes, proteins such as lipid transfer protein 1 (LTP1) are exposed to high temperatures, which later affects the beer foam properties. To develop high-quality beer, it is therefore essential to understand the protein chemical modifications and structural alternations induced by the high temperatures and their impact on beer foam. This study characterizes heat-induced chemical modifications and changes in the molecular size distribution and structure of LTP1 and its lipid-bound isoform, LTP1b, using size-exclusion chromatography and reverse-phase chromatography/mass spectrometry. The results elucidate the relationships between these changes and foam properties. Specifically, heat treatment triggers lipid adduct dissociation from LTP1b, increases the concentration of deamidated hydrophobic LTP1 molecules, and causes deglycation. Both the dissociation of lipid adducts from LTP1b and the increase in deamidated hydrophobic LTP1 molecules result in lower quality foam. Thus, controlling brewing processes, especially in terms of heating conditions, can effectively retain LTP1b to enhance beer foam.

KEYWORDS: *beer, lipid transfer protein 1, foam, mass spectrometry, chemical modification*

INTRODUCTION

Beer is one of the most widely consumed alcoholic beverages worldwide, and the demand for high-quality beer continues to grow.[1,2] Notably, the foam is a key element that distinguishes high-quality beer.[3,4] Foam enhances the flavor and mouthfeel of beer while shaping consumer expectations through visual appeal.[5−8] Furthermore, beer foam acts as a barrier against direct oxygen exposure, thereby hindering oxidative deterioration.[9] Accordingly, foam is recognized as an important factor contributing to the overall quality of beer.

Proteins have a key role in foam stabilization.[8] Specifically, the adsorption of proteins at the air−liquid interface drives foam formation.[10] Additionally, intermolecular interactions among proteins create interfacial films and enhance interfacial viscoelasticity, thereby stabilizing the foam.[11−13] The four primary ingredients in beer are water, barley malt, hops, and yeast, and the proteins in beer are mainly derived from the barley malt.[14]

Beer is produced through a sequence of defined brewing steps, which influence the quality and attributes of the final beer.[15] The malted grains are first ground and mixed with warm water, producing sweet wort through enzymatic starch hydrolysis. The sweet wort is then boiled with hops to generate hopped wort. Finally, yeast is added to the hopped wort to initiate fermentation.[15] Most of the proteins derived from barley are lost during the brewing process, mainly during the high temperature (boiling) step. As a result, heat-stable proteins, including lipid transfer protein 1 (LTP1), are the main proteins in the final product, with a critical role in governing foam properties.[8,16]

LTP1 is a lipid-binding protein with a molecular weight (M_W) of 9686 Da and is abundant in the aleurone layer of the barley endosperm.[3,17,18] LTP1 comprises eight α-helices that are arranged to form a hydrophobic cavity where lipid can bind and be stabilized through four disulfide bridges, thus preventing its precipitation even after boiling.[16,19] During germination, LTP1 undergoes lipid adduction following the covalent attachment of the oxylipin, α-ketol 9-hydroxy-10-oxo-12(Z)-octadecenoic acid (M_W = 294 Da), resulting in the formation of its lipid-bound isoform, LTP1b (M_W = 9980 Da).[3,17,20−23] The C9 of this oxylipin forms a covalent bond with the oxygen atom of Asp-7 in LTP1, causing the oxylipin to adopt a branched structure.[23] The C10−C18 segment is embedded within the hydrophobic cavity of LTP1, whereas the C1−C8 segment is exposed on the protein surface, thus increasing its hydrophobicity. It has been reported that LTP1b exhibits higher interfacial activity than LTP1.[23−25]

Several studies have investigated the effect of heating during the brewing process on the physical properties of LTP1. One report indicated that the lipid adducts dissociate slowly during the heating treatment.[26] LTP1 and LTP1b also undergo Maillard reactions in the presence of reducing sugars during the mashing and boiling steps of the brewing process, resulting in glycated LTP1/LTP1b.[17,27−30] To our knowledge, the effects of glycation on LTP1/LTP1b foam properties have not been reported; however, glycation is known to affect the

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structural stability and foam properties of other proteins.[31−33] Considering that the brewing process includes multiple heat treatments, other post-translational modifications (PTMs), such as deamidation, might occur and affect foam properties. Moreover, the surface activities of LTP1/LTP1b can be altered due to the structural changes induced by a heat treatment at 100 °C.[25]

Although researchers have explored changes in PTMs and the structure of LTP1/LTP1b during heat treatments, reports have generally focused on changes in a single characteristic. Because multiple changes can be simultaneously induced by heating, a comprehensive analysis of LTP1/LTP1b PTMs and structures is desired. Moreover, the relationships between the changes in PTMs, structures, interfacial properties, and foam properties have not yet been elucidated. As a result, the effects of heat-induced changes remain unclear, thus precluding precise control over beer foam quality.

The present study aimed to elucidate these relationships involving beer foam properties by comprehensively analyzing the changes in LTP1 and LTP1b behaviors. LTP1 and LTP1b were purified from sweet wort and exposed to thermal stress (simulating the boiling step in the brewing process). The foam creation ability and stability of LTP1 and LTP1b solutions were assessed and compared before versus after applying the thermal stress. The PTMs and structural changes caused by the thermal stress were evaluated by liquid chromatography/mass spectrometry (LC−MS). In addition, LTP1 and LTP1b were analyzed in sweet wort, hopped wort, and beer to evaluate the impact of the brewing process on these proteins.

MATERIALS AND METHODS

Purification of LTP1 and LTP1b from Sweet Wort. Ammonium sulfate (Fujifilm Wako, Osaka, Japan) was added to the sweet wort to reach 60% saturation (390 g/L), and the mixture was stirred overnight at 4 °C. The precipitate was collected by centrifugation (8000 rpm, 10 min, 4 °C), redissolved in 20 mM acetate buffer (pH 4.5), and subjected to dialysis for desalting. Cation-exchange chromatography (CEX) was performed (Cytiva SP Sepharose Fast Flow, Marlborough, MA), with elution using 0.5 M NaCl (Fujifilm Wako) in the same buffer. The protein-rich middle fractions were collected as crude sweet wort protein fractions and buffer-exchanged with 20 mM phosphate buffer (pH 7.0).

To purify LTP1 and LTP1b, 2 M ammonium sulfate was added to the protein-rich fractions from the sweet wort to precipitate impurities, followed by centrifugation (10,000*g*, 10 min, 4 °C). The resulting supernatant was collected and filtered through a Millex-GV 0.22 μm membrane (Merck, Darmstadt, Germany). The supernatant was injected to an ÄKTA prime plus instrument (Cytiva). Hydrophobic interaction chromatography (HIC) was then performed using HiTrap Butyl HP columns (Cytiva). Gradient elution was performed using solvent A (50 mM acetate buffer containing 2 M ammonium sulfate, pH 5.3) and solvent B (50 mM acetate buffer, pH 5.3) with the following program: 0−22.5 min, 0% B; 22.5−50 min, linear increase to 36% B; 50−60 min, to 43% B; 60−62 min, to 44% B; 62−80 min, to 57% B; 80−83 min, to 58% B; 83−87 min, to 59% B; 87−120 min, to 62% B; 120−132 min, to 64% B; 132−137.5 min, hold at 100% B. The flow rate was set to 2.0 mL/min from 0 to 22.5 min, 1.0 mL/min from 22.5 to 132 min, and 5.0 mL/min from 132 to 137.5 min. The column temperature was maintained at 4 °C. Eluted fractions were collected and concentrated using Amicon Ultra centrifugal filters with a 3000 Da cutoff (Merck). Subsequently, 500 μL of the concentrate were injected into the ÄKTA prime plus (Cytiva) equipped with a Superdex 75 Increase 10/300 GL column (Cytiva) to isolate LTP1 and LTP1b. The separation was performed at a flow rate of 0.2 mL/min using 50 mM acetate buffer (pH 5.3) as the mobile phase, and the column temperature was maintained at 4 °C. The collected LTP1 and LTP1b fractions were concentrated using the Amicon device and further purified by a second size-exclusion chromatograph (SEC) to obtain LTP1 and LTP1b with different glycan profiles. The glycan profiles of LTP1 and LTP1b were adjusted by mixing low and high glycation fractions to enable comparability. The final concentrations of LTP1 and LTP1b were adjusted to 10 μM in 50 mM acetate buffer (pH 5.3; the typical pH of sweet wort).[34] Heat-treated samples were prepared by heating 200 μL of purified LTP1 or LTP1b at 100 °C for 90 min to simulate brewing conditions or for 180 min to simulate harsh conditions. The samples were then cooled and stored at 4 °C until analyses.

Crude Purification of Hopped Wort and Beer. Crude purification of hopped wort was carried out in the same way as sweet wort purification, i.e., via ammonium sulfate precipitation and CEX. For the crude purification of beer, the SP Sepharose Fast Flow instrument (Cytiva) was prewashed with distilled water and 20 mM acetate buffer (pH 4.5). Then, degassed beer (1 L) was mixed with 50 mL of the resin and gently stirred for 1 h to adsorb the proteins. After transferring the resin to a column, the proteins were eluted using 20 mM acetate buffer (pH 4.5) with 0.5 M NaCl (3 resin volumes). The protein-containing fraction was collected and adjusted to pH 7.0 with NaOH, and ammonium sulfate (1.8 M) was added for overnight salting-out at 4 °C. The resulting precipitate was redissolved and dialyzed in 20 mM phosphate buffer (pH 7.0).

Gel Electrophoresis of LTP1, LTP1b, and Crude Purified Sweet Wort. Purified LTP1 and LTP1b and sweet wort (6.5 μL each) were mixed with 1 μL of NuPAGE Sample Reducing Agent (10×) and 2.5 μL of NuPAGE LDS Sample Buffer (4×) (Thermo Fisher Scientific, Waltham, MA) and incubated at 70 °C for 10 min. Then, 10 μL of each sample and Mark12 Unstained Standard (Thermo Fisher Scientific) were loaded onto a NuPAGE 4−12% Bis-Tris Gel (1.0 mm × 12-well, Thermo Fisher Scientific). Electrophoresis was carried out using a Mini Gel Tank (Thermo Fisher Scientific) under a constant voltage of 200 V for 25 min in NuPAGE MES SDS Running Buffer (Thermo Fisher Scientific) with NuPAGE Antioxidant (Thermo Fisher Scientific). After electrophoresis, the gel was washed three times with distilled water for 5 min each with gentle agitation. The gel was then stained with SimplyBlue SafeStain (Thermo Fisher Scientific) for 1 h and destained overnight in water. Gel images were acquired using the iBright Imaging System (Thermo Fisher Scientific).

Dynamic Surface Tension. Dynamic surface tension was measured using the pendant drop method with a Theta Flex instrument (Biolin Scientific, Gothenburg, Sweden). Droplets with a volume of 5 μL were generated at a controlled rate of 1 μL/s to ensure consistent droplet formation, and their shape was monitored for 600 s to determine surface tension based on droplet curvature. Surface tension values were calculated every 2 s using OneAttension software (version 4.0.3) according to the Young−Laplace equation.

Foam Properties. An aliquot (15 mL) of each sample was transferred into a 50 mL graduated cylinder and foamed for 30 s by vigorous agitation at 11,400 rpm using a custom-built foaming device. Immediately after foaming, the total foam volume and the time required for complete foam collapse were recorded. The foam creation ability and foam collapse rate, which is an indicator of foam stability, were calculated using eqs 1 and 2, respectively

$$\text{foam creation ability (\%)} = \frac{V_0}{V_{\text{liquid}}} \times 100 \quad (1)$$

$$\text{foam collapse rate (mL/s)} = \frac{V_0}{t_{\text{collapse}}} \quad (2)$$

where V_0 is the foam volume (mL) immediately after foaming, V_{liquid} is the initial volume (mL) of the liquid before foaming, and t_{collapse} is the time (s) until the foam completely collapses. Each measurement was performed in triplicate. Statistical significance between LTP1 and LTP1b at each time point was evaluated using Welch's *t*-test, and comparisons across different heating times were performed using Tukey's multiple comparison test.

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Reverse-Phase High-Performance Liquid Chromatography/Mass Spectrometry. A Nexera X2 system (Shimadzu, Kyoto, Japan) connected to a maXis II ETD mass spectrometer (Bruker, Billerica, MA) was employed for reverse-phase high-performance liquid chromatography/mass spectrometry (RP-HPLC-MS) analysis. An aliquot (5 mL) of each sample was injected into the system and separated by an ACQUITY UPLC Protein BEH C4 column (300 Å, 1.7 μm, 2.1 mm × 100 mm; Waters, Milford, MA) at 40 °C with a flow rate of 0.2 mL/min using a gradient (from 10% to 34%) elution of 0.1% formic acid in acetonitrile (solvent B) against 0.1% formic acid in distilled water (solvent A). The ultraviolet absorbance of the solution at 280 nm was monitored. Data were analyzed using Bruker Compass HyStar (version 5.1.8.1, Bruker). Deconvolution was performed using the Maximum Entropy method with a mass range of 5000−15,000 Da, a data point spacing of 0.1 m/z, and an instrument resolving power of 40,000. Extracted ion chromatograms (XICs) were obtained using a ±0.05 m/z range centered on selected representative m/z values corresponding to target analytes. Isotope distribution simulations were performed by inputting representative molecular formulas and setting the full width at half-maximum (FWHM) to 0.26 Da. The root mean squared deviation (RMSD) was calculated based on the intensity differences between each isotopic peak in the simulated and experimental isotope distributions.

Size-Exclusion Chromatography. Samples, either unheated or heated for 90 or 180 min, were analyzed by SEC using a Vanquish UHPLC system (Thermo Fisher Scientific) equipped with a TSKgel UP-SW2000 column (4.6 mm inner diameter × 150 mm; Tosoh Bioscience, Tokyo, Japan). Isocratic elution was performed with a flow rate of 0.35 mL/min, a column temperature maintained at 25 °C for 20 min, and a mobile phase of 133 mM phosphate buffer containing 200 mM KCl and acetonitrile (95:5, v/v). A sample aliquot (5 μL) was injected for each run. The ultraviolet absorbance of the solution at 280 nm was monitored, and peak areas were calculated using the Chromeleon Chromatography Data System (version 7.3.2, Thermo Fisher Scientific).

Statistical Analysis. Data herein are presented as the mean ± standard deviation (SD). Statistical analysis of foam properties was performed using Welch's t-test and Tukey's multiple comparison test. A p-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Purification of LTP1 and LTP1b. These proteins were purified from sweet wort using HIC, followed by two rounds of SEC. In the HIC step, fractions that eluted at 62−83 min (F1) and 83−110 min (F2) were collected (Figure 1). To purify further these fractions, they were subjected to an initial SEC step, yielding fractions F1.1 (77−98 min; Figure S1a) and F2.1 (79−89 min; Figure S1b). Their purities were evaluated by SDS-PAGE. Two main bands were observed in the sweet wort sample at approximately 40 and 10 kDa, whereas the purified LTP1 and LTP1b samples each had a single band at approximately 10 kDa (Figure 2a), indicating high purity. The precise molecular weight of each fraction was analyzed by RP-HPLC-MS. A single peak was observed in the chromatogram, as detected by UV, for each fraction (Figure 2b), and the mass spectra showed that in addition to peaks corresponding to the theoretical masses of LTP1 (9686 Da) and LTP1b (9980 Da), other peaks were detected at increments of 162 Da, consistent with the glycation of LTP1 and LTP1b (Figure S1c,d). Interestingly, the proportion of glycated LTP1b (72.9%) was lower than that of glycated LTP1 (77.1%). This result implies that the lipid adduction might inhibit the glycation reaction due to steric hindrance. Indeed, Lys and Arg residues, which are potential glycation sites, are close to the lipid adduction site, i.e., Asp-7.[22] To compare the foam properties of LTP1 versus LTP1b with comparable glycation profiles, F1.1 and F2.1 were further fractionated based on their extent of glycation using SEC. Specifically, F1.1 was fractionated to F1.1a (79−84 min) and F1.1b (84−88 min), and F2.1 was fractionated to F2.1a (68−85 min) and F2.1b (85−89 min) (Figure S2a,b). The lower degrees of glycation of F1.1b and F2.1b relative to F1.1a and F2.1a, respectively, were confirmed by RP-HPLC-MS (Figure S2c,d), and F1.1b and F2.1b were used to evaluate the effects of glycation on foam properties. It was important to minimize the impact of glycation to clearly evaluate the impacts of the heat treatment and lipid adducts on foam properties. Therefore, selected proportions of two fractions (F1.1a and F1.1b; F2.1a and F2.1b) were combined to adjust the glycation profiles of LTP1 and LTP1b to the representative glycation profile of LTP1b in F2.1. The degrees of glycation were confirmed by RP-HPLC-MS (Figure 3a,b). The proportion of glycated LTP1 was 74.5%, and that of glycated LTP1b was 72.9%. After obtaining comparable levels of glycation, the samples were used for further experiments.

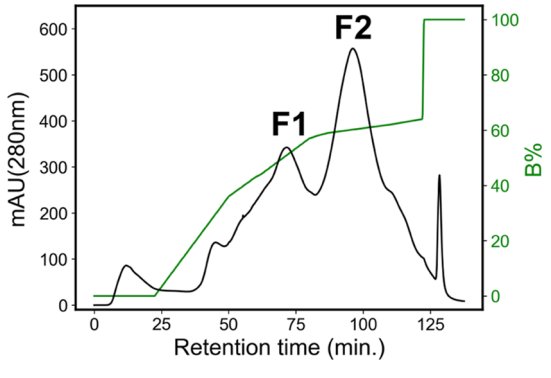

Figure 1. HIC purification of sweet wort. F1 and F2 were isolated and used in subsequent experiments.

Interfacial Activity and Foam Properties of LTP1 and LTP1b. To evaluate interfacial activity, the surface tension of LTP1 and LTP1b samples before and after heating were determined using dynamic surface tension measurements (Figure 4). Regardless of the heat treatment conditions, LTP1b had slightly lower surface tension than LTP1. Notably, oxylipin has been detected on LTP1b surfaces, potentially increasing interfacial activity and reducing surface tension.[24] Additionally, LTP1b exhibited significantly higher foam creation ability than LTP1 before heating (Figure 5a). The foam creation ability of the heated LTP1b solution decreased significantly, reaching a level comparable to that of the heated LTP1 solution. Interestingly, higher foam creation ability was observed after heating LTP1 in water.[35,36] This observation indicates that pH and ionic strength are important factors influencing the foam creation ability of LTP1, as suggested in previous reports using other proteins.[37,38] In general, the surface tension decreased after heating, whereas the foam creation ability did not increase appreciably. These observations are consistent with previous reports that showed a weak correlation between surface tension and foam creation ability. This was attributed to differences in the interfacial adsorption mechanisms, considering that surface tension is measured under static conditions, but foam formation occurs dynamically.[39,40] Further investigation is needed to clarify additional factors affecting the interfacial properties relevant to foam formation. These results highlight the importance of actual

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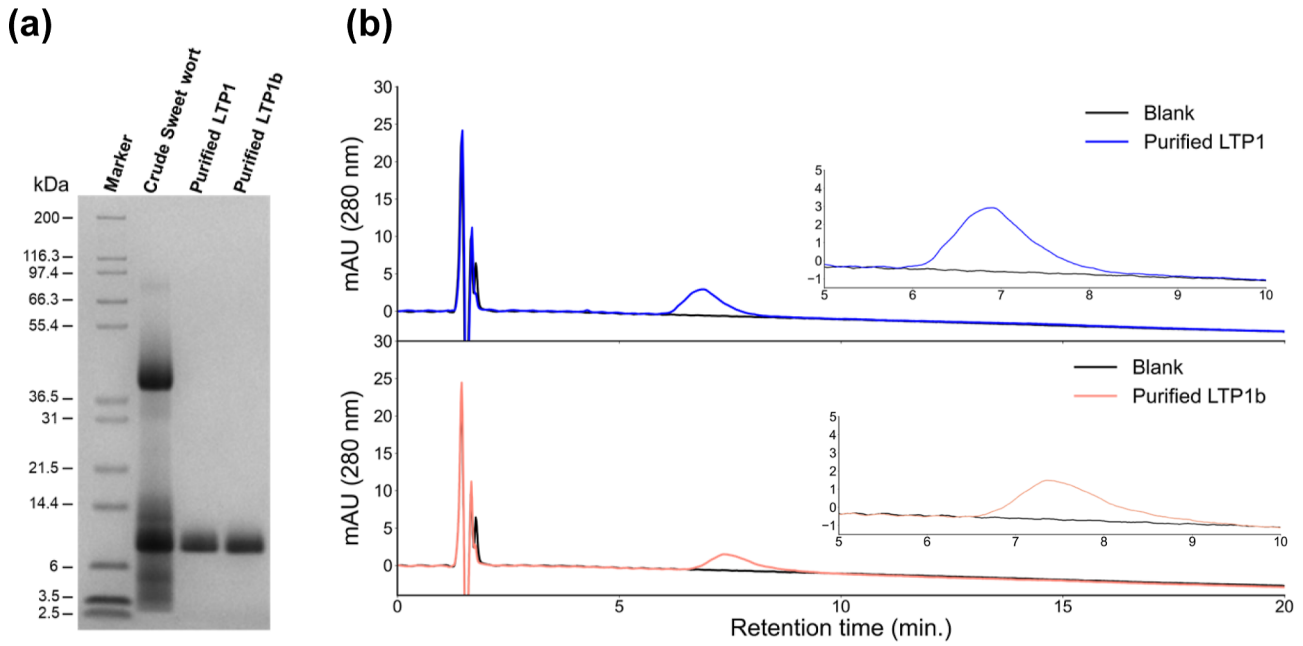

Figure 2. (a) SDS-PAGE analysis of sweet wort, purified LTP1, and purified LTP1b; (b) RP-HPLC chromatograms of purified LTP1 and purified LTP1b with expanded views of the main peaks.

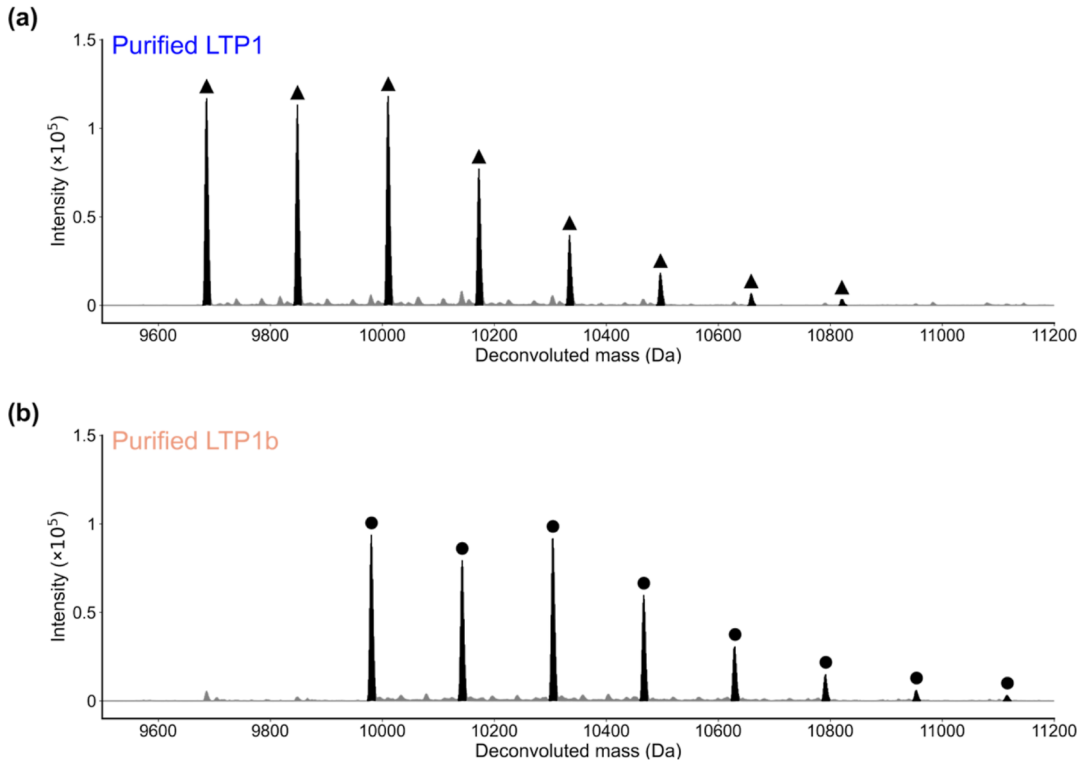

Figure 3. Representative deconvoluted mass spectra of (a) purified LTP1 and (b) purified LTP1b after glycation level adjustment. ▲ represents both nonglycated and glycated forms of LTP1. ● represents both nonglycated and glycated forms of LTP1b.

foaming tests, rather than interfacial activity measurements to evaluate foam formation.

LTP1b had a significantly lower foam collapse rate than LTP1 before heating, suggesting greater foam stability (Figure 5b). Proteins adsorb at the air−liquid interface, where they form viscoelastic interfacial films supported by intermolecular interactions.[11−13] Simulation studies have shown that LTP1b forms a denser interfacial layer than LTP1 at the air−liquid interface.[19] This indicates that LTP1b can form viscoelastic films at the air−liquid interface through stronger intermolecular interactions than LTP1, leading to higher foam stability. The foam stability of the LTP1b solution decreased significantly after 90 min of heating, whereas that of the LTP1 solution decreased appreciably only after 180 min. The following sections analyze the changes in the physicochemical

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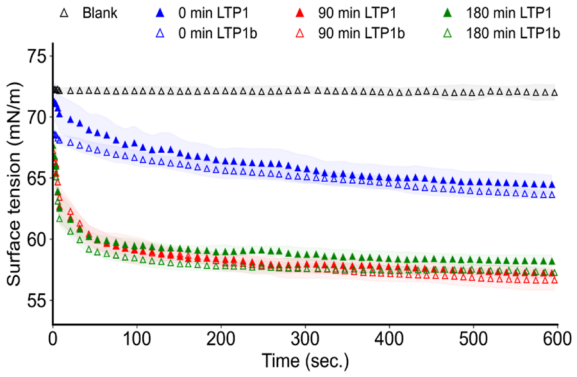

Figure 4. Surface tension of heat-treated LTP1 and LTP1b. Shaded areas represent standard deviations based on triplicate measurements at each time point.

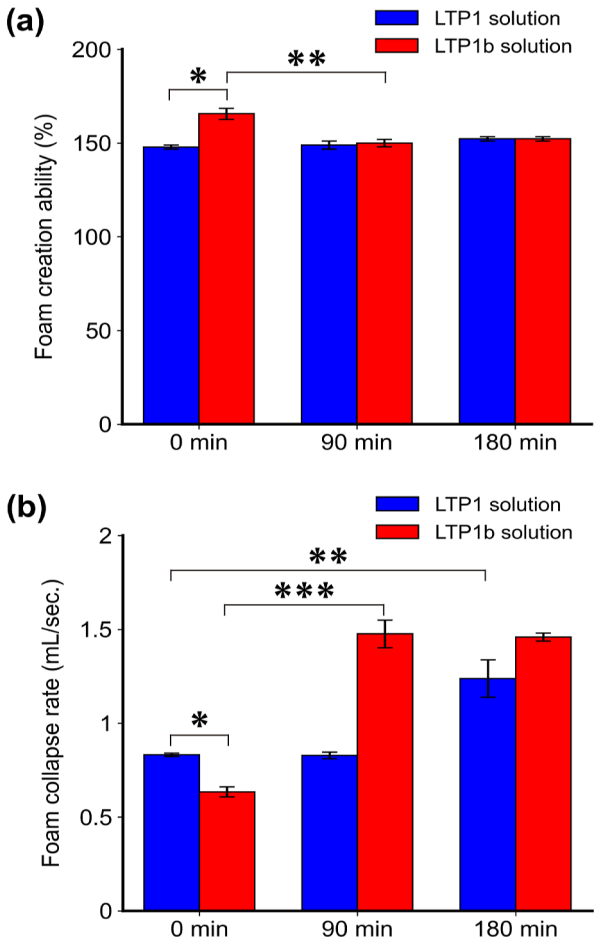

Figure 5. (a) Foam creation ability and (b) foam collapse rates of LTP1 and LTP1b solutions after 0, 90, and 180 min of heating. Asterisks indicate significant differences: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***).

properties of heated LTP1 and LTP1b and discuss the factors contributing to their reduced foam stabilities.

Heat-Induced Changes in Hydrophobicity, Lipid Adduction, and Glycation of LTP1 and LTP1b. Changes in the surface hydrophobicity and mass of LTP1 and LTP1b were examined by RP-HPLC-MS. As described above, a single peak (P1 and P1b) was detected in the chromatograms of purified LTP1 and LTP1b before heating (Figure 6a). The peak areas of P1 and P1b decreased after heating at 100 °C, and new peaks (P2 and P2b) emerged. The longer retention times of P2 and P2b relative to P1 and P1b indicate that proteins with higher surface hydrophobicity than LTP1 and LTP1b were generated by the heating process. The ratio of total peak areas (i.e., sum of P1 + P1b, and sum of P2 + P2b) remained essentially unchanged before versus after heating (Table S1), suggesting that the hydrophobic components were generated from LTP1 and LTP1b upon heating.

Each peak was characterized based on the corresponding mass spectrum. For the LTP1 sample, mass spectral peaks corresponding to LTP1 and glycated LTP1 were observed for both P1 and P2 (Figure 6b,c). The peak corresponding to LTP1 increased in intensity after heating LTP1b, indicating cleavage of ester bonds and release of a lipid adduct (Figures 6d and S3a,b). These results are consistent with previous research that showed that ester bond cleavage occurs within the temperature range 96−110 °C.[3,21,26] Notably, LTP1b and glycated LTP1b peaks were not detected in the hydrophobic peak, P2b (Figure 6e). This indicates that the dissociation of the lipid adduct might occur before the generation of hydrophobic components. In terms of the glycation profiles, the ratio of glycated LTP1/LTP1b peaks to nonglycated LTP1/LTP1b peaks decreased after heating. These observations indicate that the heat treatment caused deglycation, i.e., dissociation of sugars. Moreover, the P2 and P2b peaks had mass spectra with m/z peaks different from those of LTP1 by ±16−18 Da (Figure S4a,b). Given the high temperature condition (i.e., 100 °C), modifications such as dehydroalanine (DHA), 5-hydroxy-2-aminovaleric acid (5-HAVA), and α-aminoadipic semialdehyde (AAS) could occur.[41−43] Figure S4a,b reveals that the isotope distribution of LTP1 in P1 and P1b showed no appreciable change after the heat treatment, whereas that of LTP1 in P2 and P2b changed slightly, with the most significant peak shifting by +1 Da. An analogous 1 Da mass increase was also observed for glycated LTP1. Assuming that the observed 1 Da mass increase was caused by deamidation, we compared the simulated isotope distribution of deamidated LTP1 with the experimental data. The isotope distributions of LTP1 in P1 and P1b did not match those of simulated deamidated LTP1 (Figure S5a,b); however, those in P2 and P2b showed greater agreement with the deamidated simulation than with the nondeamidated simulation (Figure S5c,d). Thus, deamidation was observed only in the peaks with longer retention times (P2 and P2b). Deamidation is known to cause local structural changes, indicating that it may contribute to increased hydrophobicity and/or heat-induced structural alterations.[44,45]

Heat-Induced Changes in Purified LTP1 and LTP1b Size Distributions. SEC was used to evaluate the changes in protein size distribution following the heat treatment. In unheated samples, an intense peak was detected at 5.0−5.5 min, followed by small peaks (Figure 7a,b). The main peak was attributed to LTP1/LTP1b and their glycated analogs, and the small peaks were derived from buffer components (Figure S6). With increasing heating time, the peak broadened toward higher molecular weight for both LTP1 and LTP1b, suggesting that heating increased the proportion of protein with a larger hydrodynamic radius. In general, the hydrodynamic radii of proteins increase after denaturation. According to a previous report, LTP1b denaturation begins at approximately 100 °C.[26] Thus, one explanation for the peak broadening is that LTP1/LTP1b was denatured by heating at 100 °C. This hypothesis is

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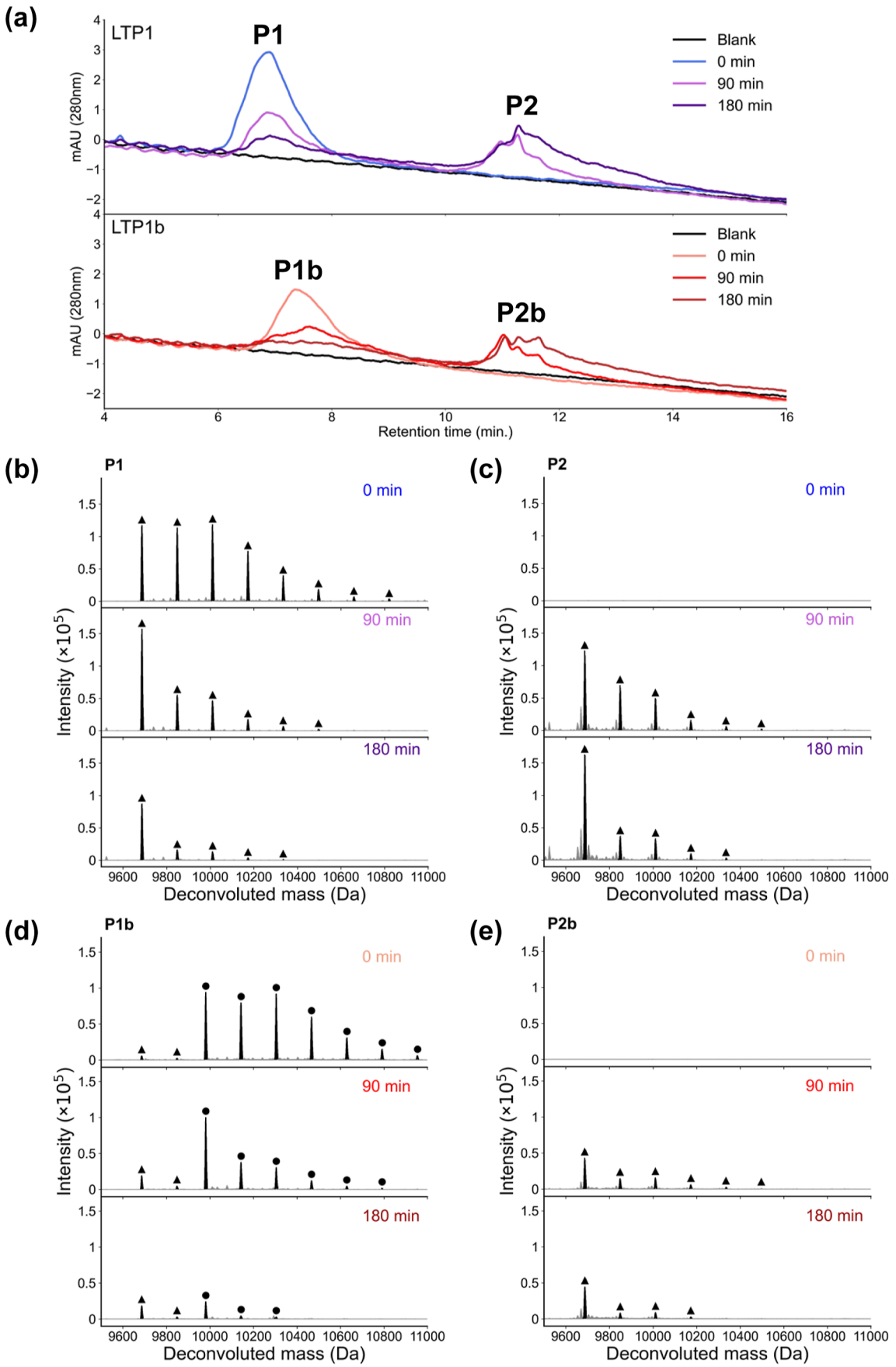

Figure 6. Representative RPLC-MS analysis of LTP1 and LTP1b before and after heating. (a) RPLC chromatograms; deconvoluted mass spectra of (b) P1, (c) P2, (d) P1b, and (e) P2b. ▲ represents both nonglycated and glycated forms of LTP1. ● represents both nonglycated and glycated forms of LTP1b.

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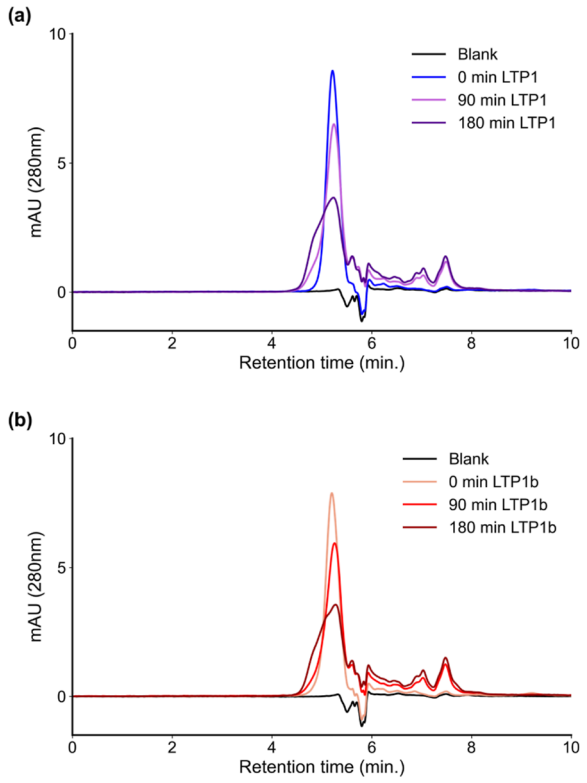

Figure 7. Representative SEC chromatograms of (a) purified LTP1 and (b) LTP1b before and after heating.

corroborated by the RP-HPLC result that showed a higher proportion of hydrophobic LTP1 molecules after heating because the hydrophobic region is typically folded inside the molecular structure but exposed upon denaturation. Another potential explanation is related to the generation of aggregates, which have larger hydrodynamic radii. Because these aggregates are more hydrophobic than the corresponding monomers, their presence is supported by RP-HPLC, similar to denatured proteins. Although the peaks corresponding to LTP1 and LTP1b were broadened, the retention times of the main peak apex were later after heating. This shift was likely due to the changes in hydrodynamic radius caused by the dissociation of the lipid adduct and/or deglycation.

Foam Properties of Heat-Induced LTP1/LTP1b Derivatives. RP-HPLC-MS and SEC revealed that the heat treatment generated highly hydrophobic LTP1 and LTP1b with limited glycation. The properties of the foam obtained from heat-treated derivatives were therefore evaluated. Because there was less glycation of LTP1 and LTP1b in F1.1b and F2.1b (vide supra) than in samples used for other analyses, the foam properties of F1.1b and F2.1b were examined to investigate the effect of glycation on foam properties (Figure 8a,b). There were no significant differences in the foam properties of LTP1 and LTP1b with low glycation relative to those of LTP1 and LTP1b with the representative glycation profile of LTP1. Previous studies using other proteins have demonstrated that glycation can alter foam properties, suggesting that the impact of glycation vary depending on the type of protein.

The foam collapse rates of LTP1 and LTP1b solutions increased significantly after heating (Figure 5b). These results indicate that heat-induced hydrophobic components, including denatured and/or aggregated LTP1 with deamidation, have

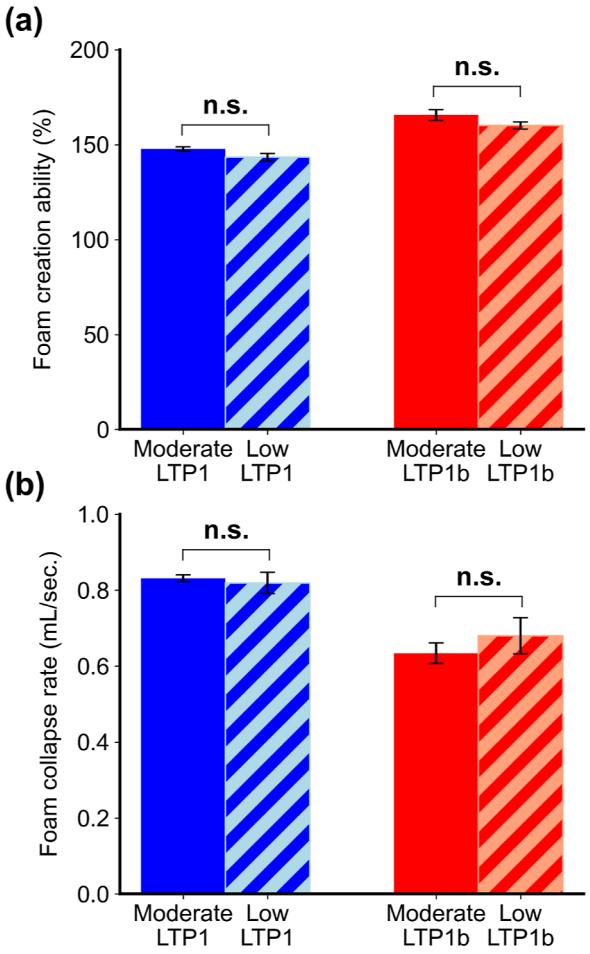

Figure 8. Comparison of (a) foam creation ability and (b) foam collapse rates of unheated LTP1 and LTP1b with different glycation profiles (moderate and low). The results for unheated LTP1 and LTP1b with moderate glycation ("0 min" in Figure 5) are presented here for comparison. "n.s." indicates no significant difference.

lower foam stability than unheated LTP1 and LTP1b. Despite the remarkable reduction in surface tension, indicating adsorption at the air−liquid interface, changes in intermolecular interactions may have hindered the formation of a network to stabilize the foam.[11−13] Hydrophobicity is considered to correlate with foam stability; however, such a correlation was not detected in this study. Indeed, a previous report suggested that hydrophobicity, surface charge, and structural flexibility all affect foam stability.[37] Thus, future work in our laboratory will aim to clarify the most relevant physical property and to investigate the combined effects of these properties on foam stability. The unheated LTP1b exhibited higher foam stability than LTP1; however, the foam stability of LTP1b after heating was lower than that of LTP1. Free fatty acids are known to destabilize foams, and it is therefore likely that the release of lipid adducts from LTP1b upon heating contributed to the observed reduction in foam stability for the heated LTP1b.[46] In this study, the LTP1b concentration was 10 μM; therefore, assuming that lipid adducts completely dissociated from the LTP1b, the resulting concentration of released fatty acids was estimated as 1400 μg/L. This is greater than the concentration threshold where negative effects on foam stability are observed.[3]

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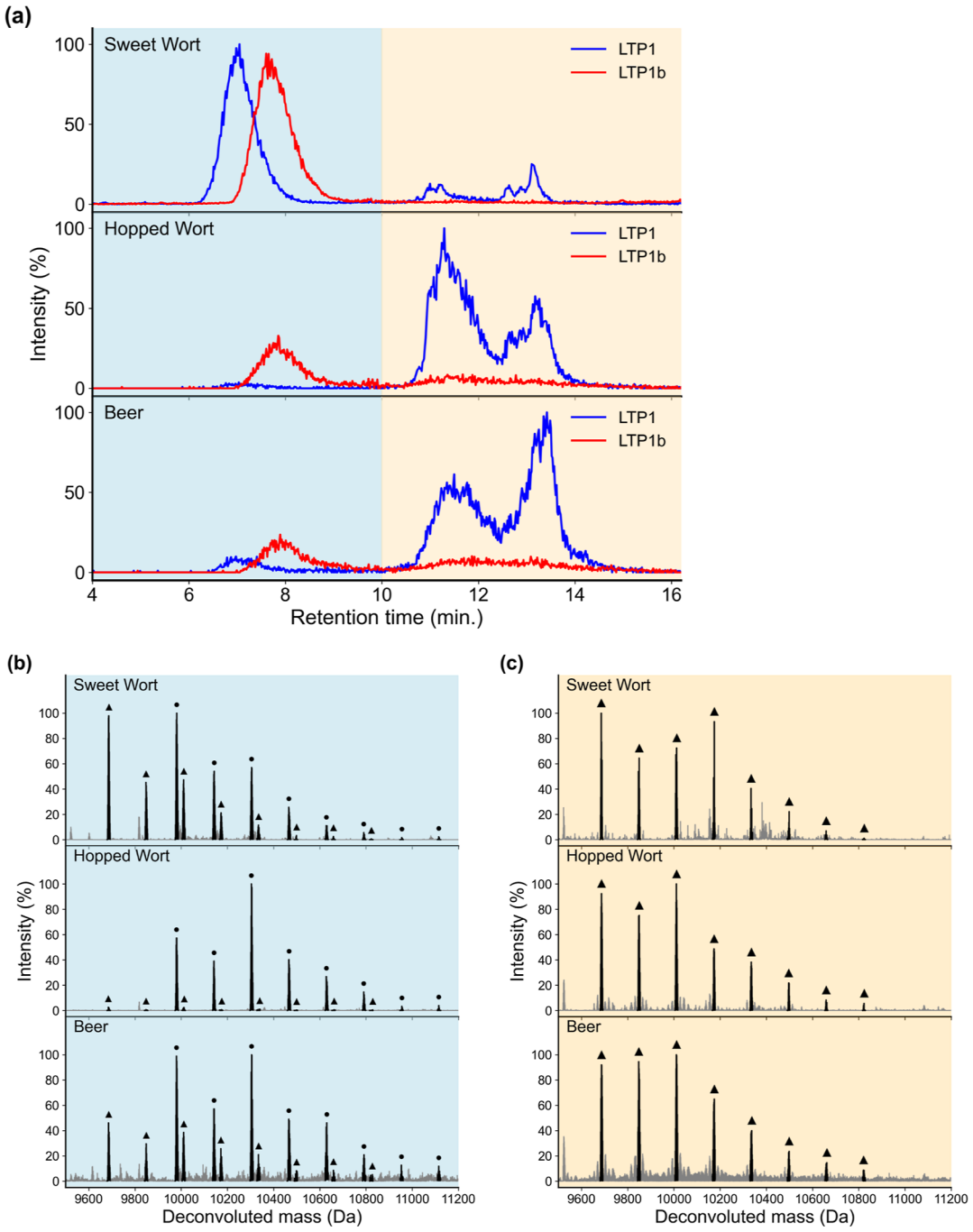

Figure 9. Representative XICs and mass spectra of LTP1 and LTP1b in sweet wort, hopped wort, and beer: (a) XICs and mass spectra of UV peaks detected at (b) 6−10 min and (c) 10−16 min for sweet wort, hopped wort, and beer. ▲ represents both nonglycated and glycated forms of LTP1. ● represents both nonglycated and glycated forms of LTP1b.

Brewing-Induced Changes in LTP1 and LTP1b. LTP1 and LTP1b in the sweet wort, hopped wort, and beer phases of the brewing process were analyzed by LC−MS following crude purification. XICs of nonglycated LTP1 and LTP1b are presented in Figure 9a. Consistent with the results from the heat-treated samples, peaks were eluted in hydrophilic (4−10 min) and hydrophobic regions (10−16 min). Minor fractions of hydrophobic components were observed in sweet wort, whereas hydrophobic components dominated in hopped wort and beer. These results suggest that the high-temperature conditions during the boiling step made LTP1 and LTP1b more hydrophobic. In this study, experiments were conducted under atmospheric pressure, although wort is exposed to higher pressures during brewing. Although the pressure required to induce protein structural changes is much higher than the typical pressures encountered during brewing,[47−49] there is a possibility that the pressure can affect protein structural changes by increasing the boiling point. Future studies considering pressure effects will be valuable to understand the changes in LTP1/LTP1b structures and foam properties under brewing conditions. Additionally, the absence of hydrophobic peaks containing the molecular weight of LTP1b indicated release of lipid adducts from LTP1b. The proportion of LTP1b decreased during both the fermentation

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and boiling stages, suggesting the dissociation of the lipid adduct from LTP1b could be triggered by high temperature and/or yeast-derived esterase activity.[50] Notably, two peaks were observed in the hydrophobic region, and the latter peak, which eluted at approximately 13 min, was not observed in heat-treated LTP1 or LTP1b solutions, indicating that other components contributed to their increased hydrophobicity. Notably, previous studies have shown that hop-derived iso-α-acids can interact with proteins to form hydrophobic complexes.[51,52] The relative peak area of the latter peak increased after the fermentation step, suggesting that yeast- and/or fermentation-derived components increased the hydrophobicity of LTP1. Because different beer styles vary in terms of the types and concentrations of constituents, it is essential to conduct style-specific evaluations.

Mass spectra of LTP1 in the hydrophilic and hydrophobic regions are shown in Figure 9b,c. Because there was no apparent difference in the mass spectra between the two hydrophobic peaks, mass spectra of the 10−16 min retention window were analyzed to investigate the chemical modifications within the hydrophobic peaks. In contrast to the heated LTP1 and LTP1b, the extent of glycation increased as the brewing process proceeded, except for hydrophobic LTP1 with three sugars. Because the sweet wort contains reducing sugars, the glycation reaction could proceed during boiling. Even if glycans are temporarily cleaved during heating (as in the purified samples), glycation may occur again in the presence of sugars.

The mass spectra of hopped wort and beer showed mass shifts of ±16−18 and +1 Da, likely attributable to deamidation (Figure S7). These modifications could be induced by the high temperature conditions, regardless of the presence of other components, such as barley-derived proteins or hop-derived iso-α-acids.

Based on the analysis of purified LTP1 and LTP1b, lipid adduct dissociation and more hydrophobic LTP1 molecules led to worse foam properties. Considering that sweet wort contains 10 nM LTP1,[3] the concentration of LTP1b was also assumed to be 10 nM because their peak areas were similar in sweet wort (Figure 9a). Under this assumption, approximately 3 μg/L of lipid adduct were estimated to be released following the heat treatment. Although this lipid adduct concentration is lower than the concentration reported to affect foam properties,[3,53,54] the potential negative impact of released lipid adducts on foam stability cannot be ruled out.[4,41,42] Furthermore, given that the foam properties of LTP1b were superior to those of LTP1, the dissociation of lipid adducts from LTP1b was considered to have an adverse effect on foam properties. Ongoing research in our lab is focused on examining the effects of hydrophobic LTP1 molecules (eluted at approximately 13 min in RP-HPLC) on foam properties.

In summary, we comprehensively analyzed heat-induced changes in lipid adducts, glycation, other PTMs, and the structures of LTP1 and LTP1b to reveal their effects on beer foam properties. Furthermore, we clarified the important aspects of LTP1/LTP1b (lipid adducts, structural changes) in terms of optimizing beer foam quality. Heat treatment increased the hydrophobicity of LTP1 molecules and induced PTMs, such as deglycation, lipid adduct dissociation, and deamidation. Before heating, LTP1b exhibited superior foam properties relative to LTP1; however, these properties decreased significantly after heating. This change was mainly due to increased hydrophobicity and lipid adduct dissociation. In addition to deglycation, the brewing process induces other chemical modifications and increases protein hydrophobicity. Both of these phenomena were similar to those observed in the heat-treated purified LTP1 and LTP1b samples. Analysis of samples taken directly from the brewing process suggested that interactions with hop- and/or yeast-derived compounds may promote the formation of more hydrophobic LTP1 molecules. Future studies can use peptide mapping analysis to identify chemical modifications and the involved amino acids. Meanwhile, Intact MS analysis using a high-resolution mass spectrometer is a powerful analytical tool. Our research group recently developed a method based on hydrogen/deuterium-exchange mass spectrometry to investigate the adsorption region of proteins to the air−liquid interface and to evaluate structural changes in the foam state.[55] These MS-based approaches are expected to provide further insights regarding the relationship between foam properties and changes in the chemical structures of proteins at a molecular level.

The chemical modifications and higher order structural changes in LTP1 and LTP1b are related to beer foam quality, making them useful indicators for developing brewing processes to obtain beer with high foam quality. Therefore, future efforts should focus on optimizing boiling conditions (e.g., pH, temperature, and duration) and analyzing the effects to determine the best brewing conditions for obtaining beer with high foam quality.

■ ASSOCIATED CONTENT

Data Availability Statement

Data will be made available on request.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.5c07895.

SEC purification of (a) F1 and (b) F2 and mass spectra of the collected fractions, (c) F1.1 and (d) F2.1 (Figure S1); SEC purification of (a) F1.1 and (b) F2.1 and mass spectra of the collected fractions, (c) F1.1b and (d) F2.1b (Figure S2); extracted ion chromatograms of nonglycated, monoglycated, and diglycated (a) LTP1 and (b) LTP1b in the heat-treated LTP1b sample (Figure S3); deconvoluted mass spectra of individual peaks at each heating time point in the mass range 9640−9720 Da: (a) LTP1 (P1 and P2); (b) LTP1b (P1b and P2b) (Figure S4); comparison of deconvoluted mass spectra for each peak at different heating time points, with isotope distribution simulations of native and deamidated LTP1: (a) P1, (b) P2, (c) P1b, and (d) P2b, which correspond to the peaks shown in Figure 6 (Figure S5); representative SEC chromatograms of the buffer-only control after heat treatment (Figure S6); deconvoluted mass spectra of LTP1 (9640−9720 Da) in sweet wort, hopped wort, and beer (Figure S7); ratio of total peak areas of LTP1 (P1 + P2) and LTP1b (P1b + P2b) at each heating time point. The total peak areas of LTP1 and LTP1b at 0 min were set to 100% (Table S1) (PDF)

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Notes

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■ ABBREVIATIONS

LTP1, lipid transfer protein 1
PTMs, post-translational modifications
LC-MS, liquid chromatography−mass spectrometer
CEX, cation exchange chromatography
HIC, hydrophobic interaction chromatography
XIC, extracted ion chromatograms
RMSD, root-mean-square deviation
SEC, size exclusion chromatography

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