Evidence of the Glycation and Denaturation of LTP1 during the Malting and Brewing Process

Sandrine Jégou,[†,‡] Jean-Paul Douliez,*[,†] Daniel Mollé,[§] Patrick Boivin,[‡] and Didier Marion[†]

Unité de Biochimie et Technologie des Protéines, INRA, B.P. 71627, 44316 Nantes Cedex 03, France; Institut Français des Boissons et de la Brasserie Malterie, Pôle Technologique de Brabois, 7 rue du Bois-de-la-Champelle, B.P. 267, 54512 Vandoeuvre Cedex, France; and Unité de Recherches de Technologie Laitière, INRA, 35042 Rennes Cedex, France

The influence of malting and brewing processes on the chemical and structural modifications occurring on LTP1 was investigated by mass spectrometry and circular dichroism. Proteins were first purified from malt, and samples were collected at various steps of beer processing performed on two barley cultivars. The levels of LTP1 found in malt were not significantly different from the amounts in barley seed. However, in malt, both LTP1b, a post-translational form of LTP1, and a third isoform named LTP1c were isolated. Moreover, both of these proteins were found to be heterogeneously glycated but still exhibited an α-helix structure. Both glycated LTP1 and LTP1b were recovered during mashing. It was also shown that glycated LTP1 was unfolded during heat treatment of wort boiling, which is in agreement with the denatured form previously isolated from beer.

Keywords: *Barley; malt; brewing; lipid transfer protein; glycation; denaturation; foam*

INTRODUCTION

Beer is a complex mixture of surface-active compounds, which influence the presence of a stable and attractive head of foam in the consumer's glass. The balance and interactions between these constituents of diverse chemical structures, such as proteinaceous components, polysaccharides, and hop acids, determine the beer foam quality including quantity, stability, lacing, density, and viscosity (*1*). It is commonly accepted that beer polypeptides with molecular weights of >5 kDa display great foaming potential (*2*). These polypeptides can be discriminated by their hydrophobicity (*3*, *4*) or their viscosity (*5*, *6*).

Despite the heterogeneity of foam-promoting proteins, protein Z ($M_r \sim 43$ kDa) and the 9 kDa lipid transfer protein (LTP1), two barley albumins have been identified as the major proteins of beer and beer foams (*7*, *8*). Their resistance to extreme pH and to high temperatures and their protease-inhibiting properties may explain the survival of these proteins during the malting and brewing processes. Protein Z is a member of the serine protease inhibitor family (serpins) (*9*, *10*). LTP1, known as PAPI or probable amylase/protease inhibitor (*11*), has also been shown to inhibit cysteine endoproteases in green malt (*12*). However, an inhibitory activity was observed only after unfolding and proteolysis of barley LTP1 after heating (*13*).

Barley endosperm LTP1, abundantly expressed in aleurone layers (*14*, *15*), is a basic protein. It consists of 91 amino acid residues for an apparent molecular mass of 9694 Da (*11*). Recently, a second form of LTP1b has been isolated from barley seeds with a mass of 9983 Da and the same amino acid composition as LTP1 (*16*−*18*). It has been suggested that LTP1b resulted from a probable post-translational modification (*17*). The structure of the barley LTP1 is characterized by a four-helix bundle and a C-terminal arm that are stabilized by four disulfide bonds (*19*). Moreover, the presence of a hydrophobic cavity (*19*) provides a potential binding site for hydrophobic and amphiphilic lipids (*20*, *21*).

Although LTP1s are surface-active proteins (*22*), the native barley seed LTP1 displays poor foaming properties (*8*). This protein becomes a foam-promoting form only after unfolding during wort boiling. This LTP1 form concentrates in beer foams (*23*) to contribute widely to foam formation, whereas foam stability depends on protein Z (*8*, *24*). The structural and chemical modifications of the two proteins during the brewing process may explain their foaming potential in beer. In fact, protein Z displays different isoforms with acidic p*I* (*6*) further to its glycation during processing through the Maillard reaction (*9*). Recently, it has also been shown that beer LTP1 is glycated, probably by Maillard reaction that occurs on malting (*17*, *18*). Glycation might prevent protein from precipitation on unfolding during the wort boiling step. Both glycation and denaturation should increase the amphiphilicity of LTP1 polypeptides and then contribute to a better adsorption at air−water interfaces of beer foam. However, it is noteworthy that a significant part of the beer LTP1 is not denatured and could prevent beer foam destabilization by lipids.

The aim of the present work is to determine the effect of the brewing process on the glycation and unfolding of barley LTP1. The study was performed by characterizing the LTP1s purified from samples collected at different stages of the process using both mass spectrometry (MS) and circular dichroism (CD).

* Author to whom correspondence should be addressed [telephone (33) (0) 240 67 50 56; fax (33) (0) 240 67 50 25; e-mail douliez@nantes.inra.fr].

† Unité de Biochimie et Technologie des Protéines, INRA.

‡ Institut Français des Boissons et de la Brasserie Malterie.

§ Unité de Recherches de Technologie Laitière, INRA.

10.1021/jf010487a CCC: $20.00
Published on Web 09/11/2001

Table 1. Samplings Performed during the Malting and Brewing Processes for Purification of LTPs

variety	
Scarlett	Esterel
barley	barley
malt (M)	malt
spent grains (SP)	spent grains
sweet wort (SW)	sweet wort
boiled wort (BW)	boiled wort
green beer	ns[a]
finished beer	finished beer

[a] ns, no sampling.

MATERIALS AND METHODS

Preparation of Malt, Wort, and Beer Samples. Four kilograms of a spring barley cultivar (*Hordeum vulgare* cv. Scarlett) and 4 kg of a winter barley cultivar (*H. vulgare* cv. Esterel) were micromalted and microbrewed at the IFBM using standard procedures. Steeped barley was prepared from barley by three alternating steeping and air rest periods. Then, germination of grains was conducted for 5 days at an average of 16 °C, and green malts were kilned with the following successive air temperatures: 50, 64, and 80 °C. The mashing profile consisted of a 45 °C stand for 20 min followed by ramping to 64 °C and then to 74 °C. After lautering, worts were boiled for 90 min, then fermented, and stored for several days prior to filtration and bottling. For each variety, samplings were performed during the processes and are reported in Table 1.

Extraction of Soluble Proteins from Barley Seeds, Malts, and Spent Grains. Soluble proteins were extracted from 1 kg of whole barley or malt flour and from spent grains by a gentle stirring with 4 L of distilled water for 4 h at room temperature. After centrifugation, the soluble material was lyophilized for purification.

Purification of Lipid Transfer Proteins. Eight hundred milliliters of worts and beers, previously degassed, was dialyzed (dialysis tubing cutoff of 3.5 kDa) against deionized water to remove low molecular weight compounds. LTP1 from the different samples was purified as previously described (*17*). The purity of fractions was controlled by SDS-PAGE in the presence of 2-mercaptoethanol (*17*, *25*).

Mass Spectrometry. Protein molecular masses were measured using a Perkin-Elmer API III$^+$ (Sciex, Thornhill, Canada) triple-quadrupole mass spectrometer equipped with an atmospheric pressure ionization source [electrospray mass spectrometer (ES-MS)]. The sample analysis (1 mg/mL) was achieved either by infusion at 5 μL/min or by an on-line coupling between MS and reversed-phase high-performance liquid chromatography (RP-HPLC) (LC-MS). Elution was carried out on an RP-HPLC column (Symmetry C18, Waters, Milford, MA) at a flow rate of 0.25 mL/min (40 °C) with a split to the MS ionization source, which was set at a flow rate of 30 μL/min. Ion detection was performed in positive mode, and molecular masses were determined from charge *m*/*z* using Biomultiview 1.2 (software package Sciex).

Circular Dichroism Spectroscopy. The secondary structure of proteins was determined by CD in the far-ultraviolet (far-UV; from 190 to 250 nm). The measurements were performed at 25 °C on a CD6 Jobin-Yvon dichrograph. Proteins were solubilized in deionized water at a final concentration of 0.5 mg/mL. A quartz cell of 0.2 mm path length was used. Data were expressed as mean-residue ellipticity. Secondary structures were determined by using CONTIN software.

Amino Acid Sequencing. N-terminal amino acid sequencing was performed by Edman degradation on a model 477A gas-phase sequencer. The phenylthiohydantoin amino acids were analyzed on-line by RP-HPLC using a 120A analyzer (Applied Biosystems, Foster City, CA).

RESULTS

Characterization of LTP1s from Malt. *Purification of LTP1 from Malt and Molecular Mass Determination of Glycoconjugates.* After cation exchange and size exclusion chromatographies, the RP-HPLC pattern of Scarlett malt showed four main peaks, M1, M2, M3, and M4, eluted from 33 to 45% acetonitrile (Figure 1a). It must be remembered that from barley flour at this stage of the purification, only two peaks have been previously identified as LTP1 and LTP1b having molecular masses of 9689a and 9983 Da, respectively (*17*, *18*). The peak eluted at 40 min had an apparent molecular mass of ~30 kDa as shown by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) (not shown) and was not further considered. An identical RP-HPLC pattern was obtained for cv. Esterel (not shown).

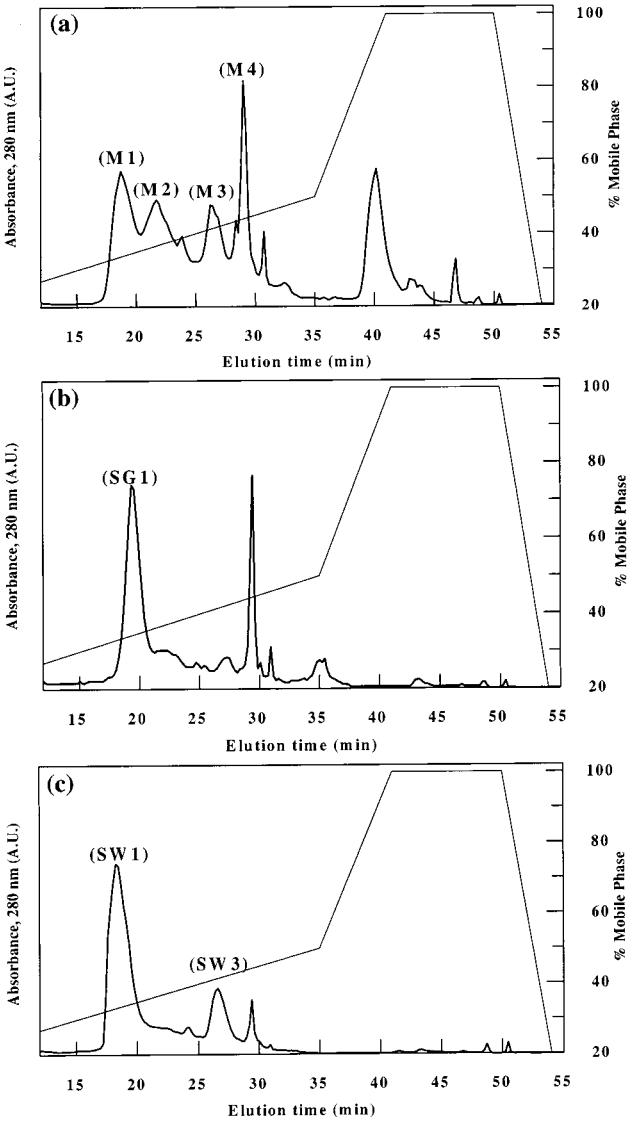

Figure 1. Semipreparative RP-HPLC patterns of the LTP1 purified from cv. Scarlett samples collected after kilning and at the end of mashing: (a) malt; (b) spent grains; (c) sweet wort. Soluble proteins were previously purified by cation exchange and size exclusion chromatographies. Peaks M (malt), SG (spent grain), and SW (sweet wort) represent fractions that were recovered for further analysis.

The deconvoluted and reconstructed ES-MS spectra of fractions M1, M2, and M3 showed multiple peaks with molecular masses in the range of 9000−11000 (Figure 2). For M1, the minor peak of mass 9688 Da and that at 9982 Da corresponded to barley LTP1 (*11*) and LTP1b having lost its C-terminal tyrosine residue (*18*), respectively. The peaks at higher masses, for which

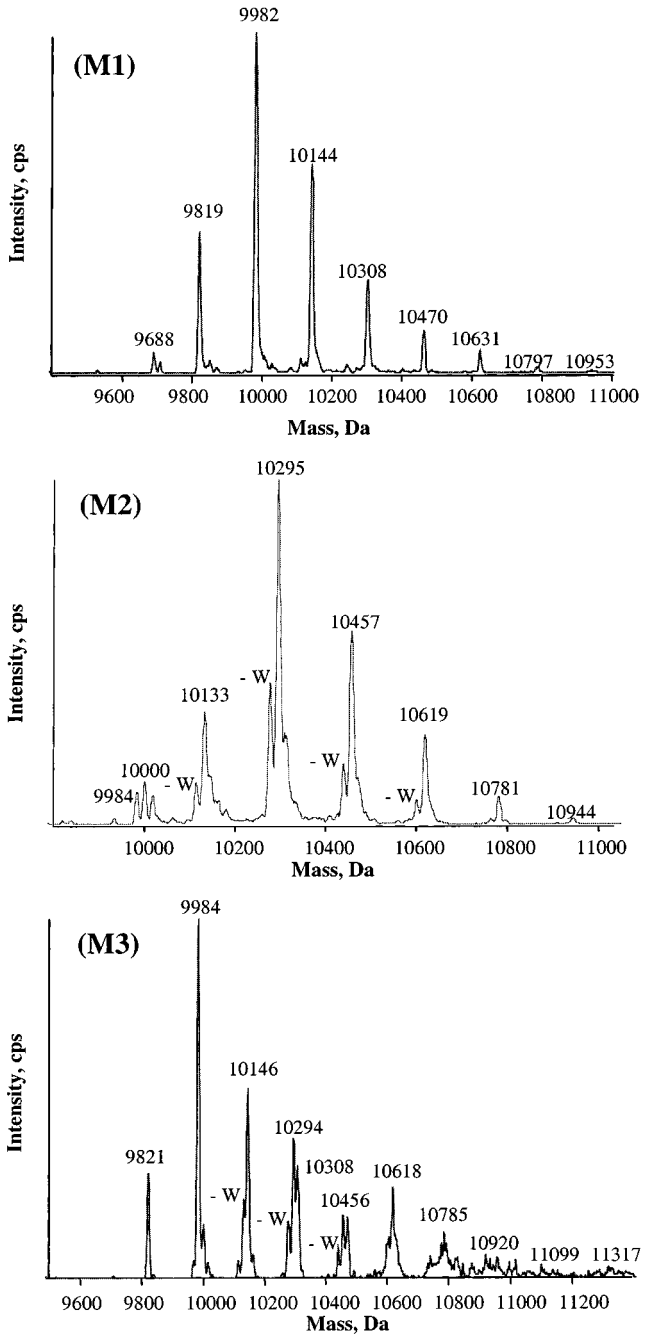

Figure 2. Effect of malting on the formation of glycoforms of LTP1s: deconvoluted and reconstructed electrospray mass spectra from multicharged ion spectra of fractions M1, M2, and M3 isolated from Scarlett malt after semipreparative RP-HPLC. "- W" represents Amadori products of LTP1 that have lost water molecules.

an increase of ~162 Da was observed, corresponded to LTP1b with covalently bound hexose moiety (*18*, *26*, *27*). Finding of glycoforms of LTP1b (Amadori compounds) in malt was in agreement with the presence of LTP1 glycated in beer (*18*).

In the same way, heterogeneous distribution of glycoforms was observed for fraction M2 with molecular masses in the range of 9984−10944 Da. The major peak exhibited a molecular mass of 10295 Da. This mass was detected in previous studies on barley seeds, cv. Plaisant (*28*), and during purification from wheat seeds (data not shown). This form was named LTP1c because the sequencing of the four first amino acids returned a sequence analogous to that of LTP1 and LTP1b. Moreover, the peak at 10133 Da corresponded to this form having lost its C-terminal tyrosine. This new form of LTP1 was also glycated as revealed by the peaks with increment of 162 Da. By comparison with fraction M1, each peak of fraction M2 displayed shoulders, with a lower mass of 18 Da compared to the main peak, indicating a loss of water molecules. This probably resulted from dehydration of LTP1c glycoforms to produce deoxydicarbonyl compounds as observed for egg lysozyme (*27*). The reconstructed electrospray mass spectrum of fraction M3 appeared to be more complex than that of fractions M1 and M2. LTP1b and LTP1c were found with their corresponding glycoforms in the ranges of 9821−10470 and 10132−11099 Da, respectively. The relative intensities of LTP1b and its glycoforms reflected probably the relative abundance of these species with regard to LTP1c forms. Finally, in the range of masses of 10400−11200 Da, fraction M3 displayed wide peaks and background noise that did not allow an accurate determination of masses. No mass was obtained for fraction M4.

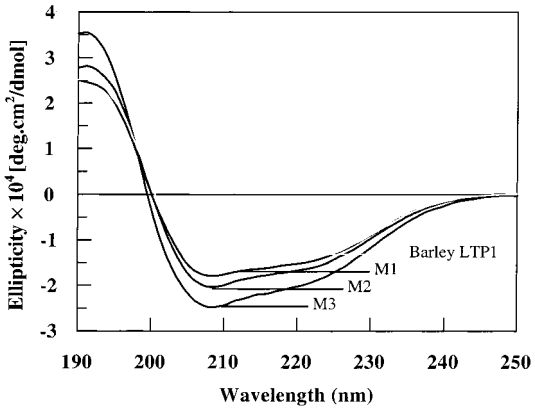

Figure 3. CD spectra of the glycated LTP1s purified from malt at a concentration of 0.5 mg/mL in ultrapure water. For comparison, that of the native barley LTP1 (structure obtained from Plaisant variety) is also reported.

Influence of Glycation on the Secondary Structure of Malt LTP1. We have shown in the previous section that LTP1 isoforms were glycated by Maillard reactions. The effect of these covalent linkages of carbohydrates on the protein secondary structure was evaluated by far-UV CD analysis. The CD spectrum (Figure 3) of native LTP1 purified from barley seeds exhibited two negative minima at 208 and 222 nm and a maximum at 190−195 nm, which were typical of α-helix structure. This secondary structure content is in agreement with the three-dimensional structure determined by Heinemann et al. (*19*). After malting, spectra of glycated LTP1s, corresponding to M1, M2m and M3 fractions, revealed only an increase in band intensity for all wavelengths of the far-UV CD without any significant shift of the peaks (Figure 3). The increase was much greater for fraction M3, which contained glycated LTP1b and LTP1c, than for fractions M1 and M2. Indeed, the α-helix content increased from 49% for barley LTP1 to 55% for M3.

Effect of Mashing and Wort Boiling on Glycated LTP1 Polypeptides. *Purification of LTP1 from Spent Grains, Worts, and Beers.* After cation exchange chromatography, the collected fraction containing LTP1 from spent grains was submitted directly to RP-HPLC and the chromatogram was compared to that of malt (Figure 1b). The pattern of spent grains provided a major wide

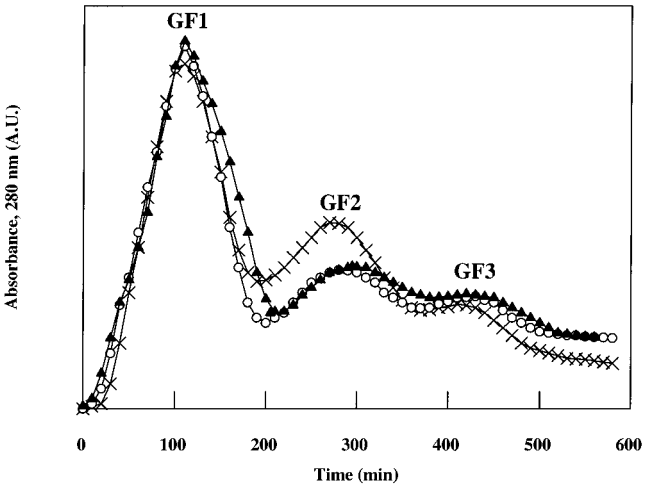

Figure 4. Chromatographic pattern on Sephadex G-50 size exclusion column of cv. Scarlett proteins from boiled wort (∗), green beer (○), and finished beer (▲).

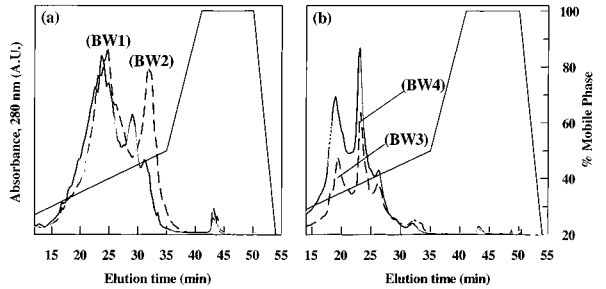

Figure 5. Semipreparative RP-HPLC pattern of the fractions GF2 (a) and GF3 (b) isolated by size exclusion chromatography from cv. Scarlett boiled wort (dashed line), green beer (solid line), and finished beer (gray line). Analyses were performed on fractions from boiled wort (BW).

peak eluted at ∼35% acetonitrile and was named SG1. For sweet wort, the RP-HPLC pattern revealed an intense broad peak denoted SW1 (Figure 1c) and eluted at a time analogous to that of fractions M1 and SG1. A second peak was collected at an elution time similar to that of fraction M3 and was named SW3.

Interestingly, size exclusion chromatography from boiled wort, green beer, and finished beer displayed identical patterns (Figure 4). Three fractions, GF1, GF2, and GF3, were obtained, which was in agreement with previous purification from a lager beer (*17*, *18*). Fraction GF1 was previously attributed to protein Z. SDS-PAGE revealed an apparent molecular mass in the 8−12 kDa range for the different fractions GF2 and GF3 (*18*). GF2 and GF3 fractions were submitted to RP-HPLC for further analysis. For fraction GF2 from wort and beers (Figure 5a), RP-HPLC patterns showed a broad peak eluting from 30 to 42% acetonitrile, which was followed by a second sharp peak around 45−50% acetonitrile. The two fractions were collected as BW1 and BW2 from boiled wort. In the same way, fraction GF3 provided identical RP-HPLC patterns for boiled wort, green beer, and finished beer (Figure 5b). Chromatograms displayed two major peaks eluted at 35 and 38%, respectively, and are referred to as BW3 and BW4.

Determination of Molecular Mass and Secondary Structure of Wort and Beer LTP1. To evaluate the influence of mashing and wort boiling on the LTP1, mass and CD measurements were performed. The mass spectrum from the sweet wort fraction (SW1) revealed numerous masses (Figure 6) corresponding to two

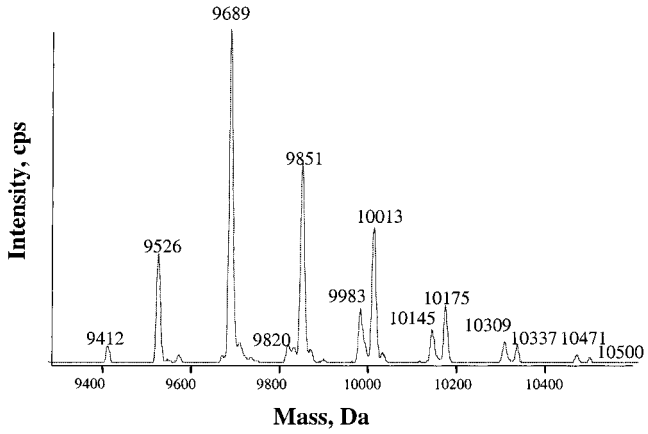

Figure 6. Effect of mashing on the LTPs: deconvoluted and reconstructed electrospray mass spectrum from multicharged ions spectra of fraction SW1 isolated by semipreparative RP-HPLC from cv. Scarlett brewing.

species in the ranges of 9412−10500 and 9820−10471 Da, respectively. As shown in malt, these multiple peaks suggested that hexose adducts of 162 Da were covalently bound to the proteins. The major peak had a molecular mass of 9689 Da corresponding to the mass of barley seed LTP1 (*8*), whereas the mass 9983 Da of LTP1b was also found. The masses 9526 and 9820 Da at 10133 Da corresponded to LTP1 and LTP1b having lost their C-terminal tyrosine, respectively. Finally, the peak at 9412 Da corresponded to LTP1 having lost both the C-terminal tyrosine and isoleucine residues. The relative intensities of LTP1 and the glycated forms revealed that these species were more abundant than the LTP1b one. It must be mentioned that an identical ES-MS spectrum was obtained from the collected fraction SG1 (not shown). Finally, sweet wort may contain LTP1c because the RP-HPLC pattern revealed a peak SW3 at an elution time equivalent to that of fraction M3.

Because similar chromatographic patterns were obtained from boiled wort, green beer, and finished beer, structural analyses were performed only on fractions collected from boiled wort. The reconstructed mass spectrum from fraction BW1 (Figure 7) displayed high background noise, but some masses could be determined. The *m*/*z* spectrum from BW2 (data not shown) was too complex to afford the calculation of masses. However, as for previous spectra, fraction BW1 exhibited a heterogeneous distribution of masses in the range of 9144−10933 Da corresponding to LTP1 with numerous adducts of 162 Da. The major peak with a mass of 10016 Da could correspond to a diglycated LTP1 form. In addition, the peaks depicted as LTP1-Y, LTP1-IY, LTP1-SRIY, and others detected at masses around 8459 Da suggested that the C-terminal residues were hydrolyzed. Compared to fraction SW1, the boiled wort mass spectrum BW3 (Figure 7) also revealed two clusters in the ranges of 9527−11113 and 9983−10661 Da, which were identified as glycated LTP1 and glycated LTP1b, respectively. In contrast with fraction SW1, the major peaks with masses of 10013 and 10175 Da were probably induced by a diglycation of LTP1 and LTP1b. The mass spectrum of fraction BW4 was not presented but displayed the same molecular mass of ∼7−8 kDa displayed by fraction B4 previously purified from beer (*18*).

The determination of the secondary structure of LTP1 fractions was also investigated and compared with the α-helix structure of the native barley LTP1. The far-

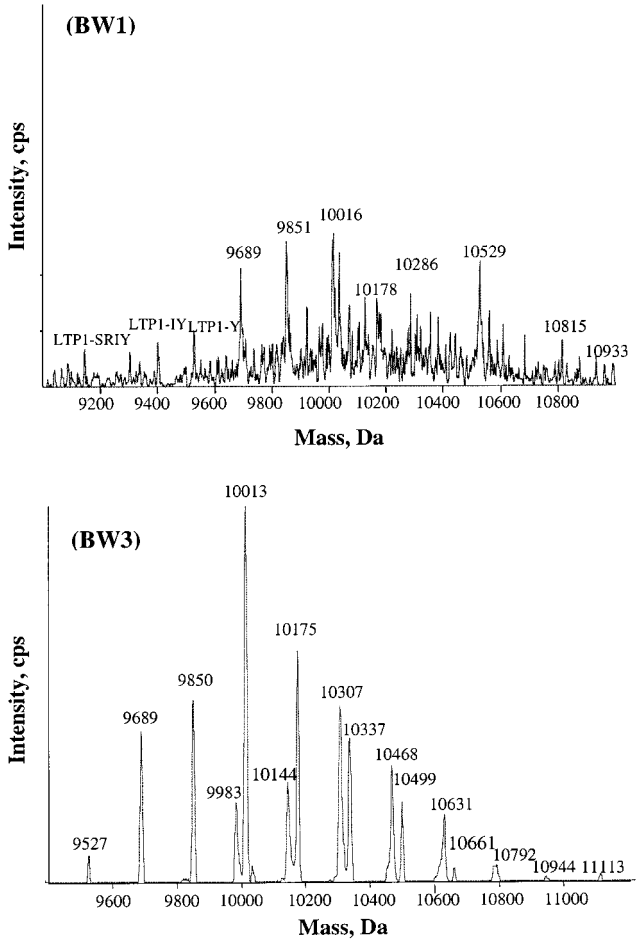

Figure 7. Eeffect of wort boiling on the LTPs: deconvoluted and reconstructed electrospray mass spectra from multicharged ions spectra of fractions BW1 and BW3 isolated by semipreparative RP-HPLC from cv. Scarlett brewing. In spectrum BW1, the three first peaks represent LTP1 forms that have lost C-terminal amino acids.

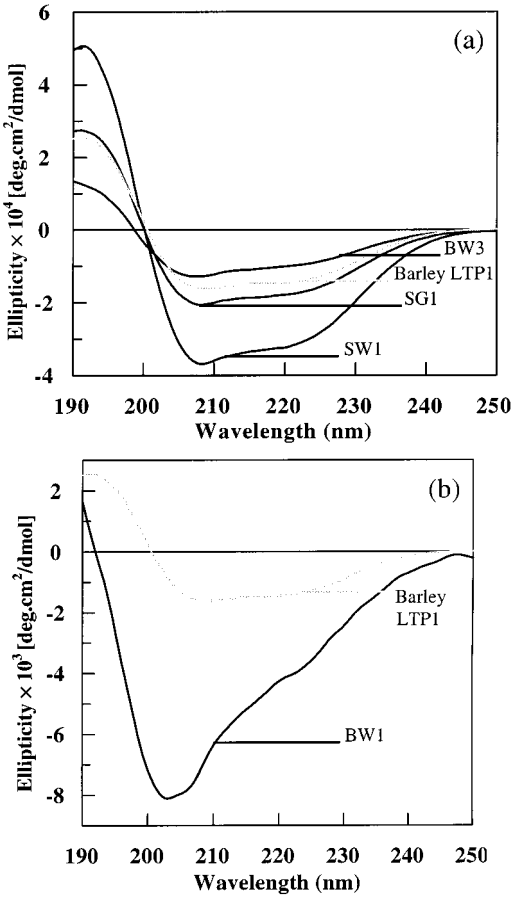

Figure 8. Study of the secondary structure of the LTP1s along mashing and wort boiling. CD spectra were obtained from LTP1 of (a) spent grains (SG1), sweet wort (SW1), and fraction of boiled wort (BW3) and (b) fraction of boiled wort (BW1). The protein concentration was 0.5 mg/mL in ultrapure water. Secondary structures were compared with the native α-helix structure of barley LTP1.

UV CD spectra of fractions SG1, SW1, and BW3 (Figure 8a) showed two minima at 208 and 222 nm and a maximum around 192 nm, which were characteristic of an α-helix content. However, SG1 and SW1 spectra exhibited an increase in the molecular ellipticity that was much greater for the sweet wort fraction. It revealed an increase in the α-helix content and a decrease in the random coil content. These changes could be due to grafting of carbohydrates on LTP1 and LTP1b. In the case of BW3, the band intensity decreased and then α-helix structure was probably affected. In contrast, the CD spectrum of BW1 (Figure 8b) from boiled wort displayed a strong negative band around 202 nm with a small shoulder between 220 and 230 nm, suggesting a complete loss of the α-helix structure and therefore an unfolding of the protein.

DISCUSSION

Changes in LTP1 Polypeptides during Malting Process. Whereas previous studies reported LTP1 and LTP1b isoforms isolated from barley seeds (*17*, *18*), the purification of malt proteins yielded only the identification of LTP1b and a new isoform, LTP1c. Both of these forms with their C-terminal tyrosine removed were also isolated. This is consistent with reports that the carboxypeptidases are present with high activities at all stages from barley to finished malt (*29*−*31*). The similarity of the N-terminal amino acid sequences between LTP1b and LTP1c, with LTP1, suggests that these isoforms correspond to a post-translational modification of barley LTP1. As shown for the LTP1 polypeptides from beer (*17*, *18*), masses of the two isoforms indicate that malt LTP1s are glycated probably by Maillard reactions. These reactions include the initial condensation reaction between proteins and reducing sugars to form Amadori intermediate rearrangement products or dehydration of the sugar moiety of intact Amadori compounds (*32*). Then the distribution of LTP1 glycoforms or Amadori compounds appears to be heterogeneous. The glycation can be explained by the abundance of glucose and maltose in barley malt (*33*), and these sugars may react with the four lysine residues of LTP1 that are potential sites of glycation. It must be mentioned that high temperature, hydration, or chemical composition during the malting process represent ideal conditions for the occurrence of Maillard reactions. This is consistent with the studies of Hodge (*32*), who described the main changes occurring during kilning steps as the browning or Maillard reaction. Moreover, intense kilning conditions have been shown to increase formation of protein−carbohydrate Maillard products (*1*). These results suggest that germination and kilning have a major influence on LTP1 modifications. In other words, the process improves the foaming properties of beer proteins.

The far-UV CD highlights that glycation does not modify the α-helix secondary structure of the various isoforms of LTP1 in malt. As previously suggested, the covalent binding of the carbohydrate moiety can stabilize the protein conformation through hydrogen bonds or van der Waals contacts and then contribute to thermal stability of proteins during malting (*36*, *37*). It was observed that proteins show denaturation resistance to heat treatment in the dry state (*38*) and that the addition of water favors protein unfolding. Only a slight increase in the α-helix content was noticed, showing that glycated proteins are more structured. However, this apparent increase in α-helix content could be due to an artifact because of the glycation of lysine residues. Indeed, lysine residues are located in α-helix regions, and their modification by sugars throughout the Maillard reaction may lead to local structural changes. Such modifications of structure have been already observed for the glycated legumin (*39*), with maltosyl derivatives of *β*-lactoglobulin (*40*) and by reaction of freeze-dried ovalbumin with glucose (*41*).

Changes in LTP1 Polypeptides during Brewing Process. During mashing, malt is milled and leads to the solubilization of compounds as proteins in sweet wort. In contrast to malt, both LTP1 and LTP1b were found together in spent grains and sweet wort. Because there is no protein synthesis during the mashing stage, we suggest that the hydrolysis of the post-translational adduct of LTP1b generates LTP1. Moreover, a heterogeneous distribution of LTP1 and LTP1b glycoforms was detected, which shows that glycation is not altered by the mashing process. The glycated isoforms of LTP1 were the major forms in spent grains and sweet wort, with a high level of the native LTP1. In contrast, Narziss et al. (*42*) observed that mashing at higher temperature increased the level of glycoproteins.

After boiling, the two glycated isoforms LTP1 and LTP1b were detected in wort despite the heat treatment. Only glycated LTP1s was present in fraction BW1, whereas fraction BW3 is a mixture of LTP1 and LTP1b glycoforms. In boiled wort, the LTP1 glycoforms modified by two adducts were present in higher levels than native LTP1s. Hejgaard et al. (*24*) suggested that the Maillard reaction was favored by high-temperature treatments during malting and brewing processes. In the same way, Roberts (*43*) showed that longer boiling steps are associated with increased levels of the amount of glycoprotein foam-active complexes in beer. This likely suggests that the Maillard reaction still occurs during the wort boiling to achieve the glycation of LTP1s. During the mashing stage of brewing starch is converted by amylases into maltose and glucose, which serve as a source of reducing sugars for Maillard reactions during wort boiling (*43*). These sugars may react with proteins either on the free lysine or by condensation with other sugars previously grafted on the LTP1 to form diketoamine proteins (*45*). The LTP1 glycoforms found in boiled wort were in agreement with the LTP1 polypeptides found in beer (*17*, *18*).

Determination of the secondary structure showed that the α-helix structure was maintained in isoforms of LTP1 from spent grains to sweet wort. As the malt glycated LTP1s, the helix content may have increased because of grafting of carbohydrates on LTP1 and LTP1b, leading to a more structured effect that reinforces the conformation. Upon heating in aqueous solution, boiling wort leads to an important unfolding of LTP1 of the BW1 fraction (Figure 8b), whereas the α-helix structure of the BW3 fraction was not disturbed. These different forms were found in beer previously (*17*, *18*). The present experiments confirm the denaturation of LTP1 polypeptides on wort boiling as suggested previously by Bech et al. (*23*). On the other hand, the LTP1 was found in beer as an unfolded form with all disulfide bonds cleaved (*18*). The disulfide bridges could be broken because of the reducing environment in wort or during the unfolding stage of heat denaturation as shown for *β*-lactoglobulin (*46*). Moreover, the denaturation would be affected by the pH of the thermal treatment. Indeed, Hermansson (*47*) showed that partial denaturation and heating of soy 7S and 11S led to increased unfolding of proteins if they were solubilized at a pH markedly away from their p*I*. The p*I* of barley seed LTP1 is ∼9, whereas beer pH is ∼4−5, so that the pH of beer may influence the LTP1 denaturation under heating. The disulfide bond rearrangement is limited also by acidic pH. Unfolding of the protein could enhance the glycation of LTP1 observed during wort boiling. Indeed, lysine residues of unfolded proteins are exposed outside and easily react to the reducing carbonyl group in sugars (*48*). Simultaneously, the carbohydrates bound on LTP1 polypeptides might protect the protein from precipitation that generally occurs upon heat treatment during boiling and fermentation (*49*) because of steric hindrance and high hydrophilicity of the glycated polypeptides.

CONCLUSION

Understanding food product properties and processing parameters is of great importance for food industries because this should afford manufacturers better control of product quality. In the case of LTP1, a foam-positive protein in beer, the study of the protein was investigated during malt and brewing process of Scarlett and Esterel cultivars. Similar results were obtained from the two analyzed cultivars. The major effect of the malting process on LTP1b glycation probably by Maillard reaction was proved. On the other hand, the unfolding of the LTP1 isoform was due to the heat treatment in aqueous solution during wort boiling. Identification of the stages of LTP1 modifications should now permit optimized conditions to be applied during brewing to improve beer foam quality. Finally, our data show that the cross-links induced between sugars and proteins are the first key step to produce foaming proteins. Therefore, the control of this glycation event during the malting process appears to be essential to optimize the foaming properties of beer.

ACKNOWLEDGMENT

We gratefully thank M. Malanda (IFBM) for providing micromalted and microbrewed samples for analysis.

LITERATURE CITED

(1) Bamforth, C. W. The foaming properties of beer. *J. Inst. Brew.* **1985**, *91*, 370−383.
(2) Asano, K.; Hashimoto, N. Isolation and characterization of foaming proteins in beer. *J. Am. Soc. Brew. Chem.* **1976**, *38*, 129−137.
(3) Onishi, A.; Proudlove, M. O. Isolation of beer foam polypeptides by hydrophobic interaction chromatography and their partial characterization. *J. Sci. Food Agric.* **1994**, *65*, 233−240.

(4) Slack, P. T.; Bamforth, C. W. The fractionation of polypeptides from barley and beer by hydrophobic interaction chromatography: the influence of their hydrophobicity on foam stability. *J. Inst. Brew.* **1983**, *89*, 397−401.
(5) Maeda, K.; Yokoi, S.; Kamada, K.; Kamimura, M. Foam stability and physicochemical properties of beer. *J. Am. Soc. Brew. Chem.* **1991**, *49*, 14−18.
(6) Yokoi, S.; Maeda, K.; Xiao, R.; Kamada, K.; Kamimura, M. Characterization of beer proteins responsible for the foam of beer. *Proc. Eur. Brew. Conv. (Zürich)* **1989**, 593−600.
(7) Kaersgaard, P.; Hejgaard, J. Antigenic beer macromolecules, an experimental survey of purification methods. *J. Inst. Brew.* **1979**, *85*, 103−111.
(8) Sørensen, S. B.; Bech, L. M.; Muldbjerg, M.; Beenfeldt, T.; Breddam, K. Barley lipid transfer protein 1 is involved in beer foam formation. *Master Brew. Assoc. Am. Tech. Q.* **1993**, *30*, 136−145.
(9) Hejgaard, J.; Kaersgaard, P. Purification and properties of the major antigenic beer protein of barley origin. *J. Inst. Brew.* **1983**, *89*, 402−410.
(10) Brandt, A.; Svendsen, I.; Hejgaard, J. A plant serpin gene. Structure, organization and expression of the gene encoding barley protein Z4. *Eur. J. Biochem.* **1990**, *194*, 499−505.
(11) Svensson, B.; Asano, K.; Jonassen, I.; Poulsen, F. M.; Mundy, J.; Svendsen, I. A 10 kD barley seed protein homologous with an α-amylase inhibitor from indian finger millet. *Carlsberg Res. Commun.* **1986**, *51*, 493−500.
(12) Jones, B. L.; Marinac, L. A. Purification, identification, and partial characterization of a barley protein that inhibits green malt endoproteinases. *J. Am. Soc. Brew. Chem.* **1997**, *55*, 58−64.
(13) Davy, A.; Svendsen, I.; Bech, L.; Simpson, D. J.; Cameron-Mills, V. LTP is not a cysteine endoproteinase inhibitor in barley. *J. Cereal Sci.* **1999**, *30*, 237−244.
(14) Mundy, J.; Rogers, J. S. Selective expression of a probable amylase/protease inhibitor in barley aleurone cells. Comparison to the barley amylase/subtilisin inhibitor. *Planta* **1986**, *169*, 51−63.
(15) Skriver, K.; Leah, R.; Müller-Uri, F.; Olsen, F. L.; Mundy, J. Structure and expression of the barley lipid transfer protein gene *Ltp1*. *Plant Mol. Biol.* **1992**, *18*, 585−589.
(16) Evans, D. E.; Hejgaard, J. The impact of malt derived proteins on beer foam quality. Part I. The effect of germination and kilning on the level of protein Z4, protein Z7 and LTP1. *J. Inst. Brew.* **1999**, *105*, 159−169.
(17) Jégou, S.; Douliez, J. P.; Mollé, D.; Boivin, P.; Marion, D. Biochemical and structural characterisation of beer LTP1. *Proc. Eur. Brew. Conv. (Cannes)* **1999**, 167−174.
(18) Jégou, S.; Douliez, J. P.; Mollé, D.; Boivin, P.; Marion, D. Purification and structural characterization of LTP1 polypeptides from beer. *J. Agric. Food Chem.* **2000**, *48*, 5023−5029.
(19) Heinemann, B.; Andersen, K. V.; Nielsen, P. R.; Bech, L. M.; Poulsen, F. M. Structure in solution of a four-helix lipid binding protein. *Protein Sci.* **1996**, *5*, 13−23.
(20) Lerche, M. H.; Kragelund, B. B.; Bech, I. M.; Poulsen, F. M. Barley lipid-transfer protein complexed with palmitoyl CoA: The structure reveals a hydrophobic binding site that can expand to fit both large and small lipid-like ligands. *Structure* **1997**, *5*, 291−306.
(21) Lerche, M. H.; Poulsen, F. M. Solution structure of barley lipid transfer protein complexed with palmitate. Two different binding modes of plamitate in the homologous maize and barley nonspecific lipid transfer proteins. *Protein Sci.* **1998**, *7*, 2490−2498.
(22) Subirade, M.; Salesse, C.; Marion, D.; Pézolet, M. Interaction of non specific wheat lipid transfer protein with phospholipid monolayers imaged by fluorescence microscopy and studied by infrared spectroscopy. *Biophys. J.* **1995**, *69*, 974−988.
(23) Bech, L. M.; Vaag, P.; Heinemann, B.; Breddam, K. Throughout the brewing process barley lipid transfer protein 1 (LTP1) is transformed into a more foam-promoting form. *Proc. Eur. Brew. Conv.* **1995**, 561−568.
(24) Hejgaard, J. Origin of a dominant beer protein. Immunochemical identity with a β-amylase-associated protein from barley. *J. Inst. Brew.* **1977**, *83*, 94−96.
(25) Laemmli, U. K. Cleavage of structural proteins during the assemblage of the head of bacteriophage T4. *Nature (London)* **1970**, *227*, 680−685.
(26) Nacka, F.; Chobert, J.-M.; Burova, T.; Leonil, J.; Haertlé, T. Induction of new physicochemical properties by the glycosylation of whey proteins. *J. Protein Chem.* **1998**, *17*, 495−503.
(27) Yeboah, F. K.; Alli, I.; Yaylayan, V. A.; Konishi, Y.; Stefanowicz, P. Monitoring glycation of lysozyme by electrospray ionization mass spectrometry. *J. Agric. Food Chem.* **2000**, *48*, 2766−2774.
(28) Jégou, S. Purification et caractérisation des protéines de transfert de lipides et des indolines de l'orge. DEA Université Bordeaux I, 1997.
(29) Breddam, K.; Sørensen, S. B.; Ottesen, M. Isolation of a carboxypeptidase from malted barley by affinity chromatography. *Carlsberg Res. Commun.* **1983**, *48*, 217−230.
(30) Breddam, K. Enzymatic properties of malt carboxypeptidases II in hydrolysis and aminolysis reactions. *Carlsberg Res. Commun.* **1985**, *50*, 309−323.
(31) Breddam, K.; Sørensen, S. B. Isolation of carboxypeptidases III from malted barley by affinity chromatography. *Carlsberg Res. Commun.* **1987**, *52*, 275−283.
(32) Hodge, J. E. Dehydrated food, chemistry of browning reactions in model systems. *J. Agric. Food Chem.* **1953**, *1*, 928−943.
(33) Allosio, N.; Quemener, B.; Bertrand, D.; Boivin, P. Application of high performance anion exchange chromatography to the study of carbohydrate changes in barley during malting. *J. Inst. Brew.* **2000**, *106*, 45−52.
(34) Townsend, A. A.; Nakai, S. Relationships between hydrophobicity and foaming characteristics of food proteins. *J. Food Sci.* **1983**, *48*, 588−594.
(35) Le Meste, M.; Colas, B.; Simatos, D.; Closs, B.; Courthaudon, J. L.; Lorient, D. Contribution of protein flexibility to the foaming properties of casein. *J. Food Sci.* **1990**, *55*, 1445−1447.
(36) Kato, A.; Nakamura, S.; Takasaki, H.; Maki, S. Novel functional properties of glycosylated lysozymes constructed by chemical and genetic modifications. In *Macromolecular Interactions in Food Technology*; ACS Symposium Series 650; American Chemical Society: Washington, DC, 1995; Chapter 19, pp 243−256.
(37) Gowda, D. C.; Petrella, E. C.; Raji, T. T.; Bredehorst, R.; Vogel, C. W. Immunoreactivity and function of oligosaccharides in cobra venom factor. *J. Immunol.* **1994**, *152*, 2977−2986.
(38) Morgan, F.; Vénien, A.; Bouhallab, S.; Mollé, D.; Léonil, J.; Peltre, G.; Levieux, D. Modification of bovine β-lactoglobulin by glycation in a powdered state or in an aqueous solution: immunochemical characterization. *J. Agric. Food Chem.* **1999**, *47*, 4543−4548.
(39) Caer, D.; Baniel, A.; Gueguen, J.; Colas, B. *In vitro* glycosylation of pea legumin. Effects on some functional properties. *Sci. Aliments* **1990**, *10*, 465−472.
(40) Waniska, R. D.; Kinsella, J. E. Enzymatic hydrolysis of maltosyl and glucosaminyl derivatives of β-lactoglobulin. *J. Agric. Food Chem.* **1984**, *32*, 1042−1044.
(41) Watanabe, K.; Sato, Y.; Kato, Y. Chemical and conformation changes of ovalbumin due to the Maillard reaction. *J. Food Process. Preserv.* **1980**, *3*, 263−274.

(42) Narziss, L.; Reicheneder, E.; Barth, D. Uber den beitrag von hochmolekularen protein fraktionen und glyoproteiden zum biershaum. *Brauwissenschaft* **1982**, *35*, 213−223.
(43) Roberts, R. T. Glycoproteins and beer foam. *Proc. Eur. Brew. Conv*. **1975**, *15*, 453−464.
(44) Moll, M.; Bazard, D.; Flayeux, R. Dosage des sucres fermentescibles dans le moût par chromatographie liquide à haute performance. *Bios* **1978**, *9*, 23−26.
(45) Karakus, M. Etude biochimique et technologique des réactions de brunissement au maltage. Thèse de Doctorat, Université Nancy I, 1975.
(46) Law, A. J. R.; Leaver, J. Effect of thermal denaturation of whey proteins in milk. *J. Agric. Food Chem*. **2000**, *48*, 672−679.
(47) Hermansson, A. M. Physico-chemical aspects of soy proteins structure formation. *J. Texture Stud*. **1978**, *9*, 33−58.
(48) Kato, A.; Mifuru, R.; Matsudomi, N.; Kobayashi, K. Functional casein-polysaccharide conjugates prepared by controlled heating. *Biosci., Biotechnol., Biochem*. **1992**, *31*, 567−571.
(49) Pierce, J. S. The role of positive and negative factors in head retention. *Proceedings of the 15th Convention*; The Institute of Brewing, Australian and New Zealand Section: 1978; pp 51−65.

Received for review April 12, 2001. Revised manuscript received July 12, 2001. Accepted July 17, 2001. This work was granted by Malteurs de France, Brasseurs de France, and the French Ministries of Research and of Agricultural and Fishing (CIFRE contract).

JF010487A