JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

The Influence of Barley Malt Protein Modification on Beer Foam Stability and Their Relationship to the Barley Dimeric α-Amylase Inhibitor-I (BDAI-I) as a Possible Foam-Promoting Protein

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The foam stability of beer is one of the important key factors in evaluating the quality of beer. The purpose of this study was to investigate the relationship between the level of malt modification (degradation of protein, starch, and so on) and the beer foam stability. This was achieved by examining foam-promoting proteins using two-dimensional gel electrophoresis (2DE). We found that the foam stability of beer samples brewed from the barley malts of cultivars B and C decreased as the level of malt modification increased; however, the foam stability of cultivar A did not change. To identify the property providing the increased foam stability of cultivar A, we analyzed beer proteins using 2DE. We analyzed three fractions that could contain beer foam-promoting proteins, namely, beer whole proteins, salt-precipitated proteins, and the proteins concentrated from beer foam. As a result, we found that in cultivar A, some protein spots did not change in any of these three protein fractions even when the level of malt modification increased, although the corresponding protein spots in cultivars B and C decreased. We analyzed these protein spots by peptide mass finger printing using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. As a result, all of these spots were identified as barley dimeric α-amylase inhibitor-I (BDAI-I). These results suggest that BDAI-I is an important contributor to beer foam stability.

KEYWORDS: Beer foam stability; barley malt cultivar; malt modification; beer protein; two-dimensional gel electrophoresis

INTRODUCTION

Foam quality is one of the important characteristics in beer. The foam prevents the emanation of flavor and inhibits oxidation to prevent the direct contact of air and beer. Beer foam quality is characterized by its stability, lacing, whiteness, density, strength, and creaminess (*1*). Among these, foam stability has been intensively studied and reported (*2–6*). Some of the barley grain and barley malt proteins (*3, 4, 7*), iso-α-acids from hops (*2, 8*), and metal ions (*2*) have been considered as foam-positive factors. On the other hand, it has been suggested that yeast proteinase A (*9–12*) and lipids (*13, 14*) are foam-negative factors.

The level of malt modification is one of the important factors controlling foam stability. It is well-known that the malt Kolbach index (the index for the solubility of nitrogen components in barley malt) relates to malt modification. Malt modification (Kolbach index) is negatively correlated with foam stability (*1, 2*), because of the degradation of foam-positive proteins and loss of viscosity due to the degradation of nonstarch polysaccharides such as β-glucan and arabinoxylan in increased modification (*4, 15*); however, the impact of viscosity on foam is contentious (*16*). On the other hand, if the malt modification level is low, the malt extract is insufficient and the beer filtration efficiency is deteriorated (*17, 18*). So, it is well-known that an optimum malt modification level is needed for beer brewing.

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10.1021/jf0724926 CCC: $40.75
Published on Web 02/01/2008

Table 1. List of the Beer Samples Brewed from Single Barley Malt Sample

ex-steep moisture (%)		36−37	38−39	40−41	41−42	43−44	45−
cultivar A	first trial	A1-36[a] (39.4)[b]	A1-38 (42.1)	A1-40 (46.0)		A1-43 (50.0)	A1-45 (48.9)
	second trial	A2-37 (41.7)	A2-39 (46.9)		A2-42 (48.8)	A2-43 (50.2)	
cultivar B		B-37 (43.7)	B-39 (43.6)		B-42 (48.5)	B-43 (49.1)	
cultivar C		C-37 (47.3)		C-40 (49.4)	C-42 (49.8)	C-44 (54.0)	

[a] The number after the hyphen is the level of ex-steep moisture. [b] The number between the parentheses is the Kolbach index.

Among various proteins from barley grain and barley malt, there are many reports on protein Z (*3, 19–21*) and LTP-1 (*3, 7, 22–27*), which have been suggested as foam-promoting proteins. Evans et al. (*3*) measured the concentrations of proteins Z4, Z7, and LTP-1 in barley grains and barley malts and described that there were varietal variations in the concentrations of these proteins. They suggested that optimization of the malting process to maximize the concentrations of proteins Z4, Z7, and LTP-1 might improve beer foam quality (*3*). They also examined quantitative trait loci controlling the concentration of proteins Z4 and Z7. As a result, it was shown that the levels of these proteins were under simple genetic controls (*19*). On the other hand, Leiper et al. (*28*) reported that protein Z had no direct function in foaming. It has been assumed that LTP-1 contributes to beer foam stability since LTP-1 binds to lipids that prevent the stability of beer foam (*3, 7, 22–27*). LTP-1 has been well-studied, in terms of the relationship between the wort boiling temperature and the conformation of LTP-1 (*7*), the post-translational modification (*22, 25*), and the behavior of LTP-1 in the brewing process (*25*). Furthermore, some researchers reported that proteinase A derived from *Saccharomyces cerevisiae* degrades LTP-1 (*9, 10, 29, 30*). He et al. (*9*) demonstrated that the proteinase A activity was changed by the storage conditions of beer and that the foam stability decreased by the activity of proteinase A. Although the relationships between foam stability and foam formation and some proteins have been investigated, the contribution of proteins to foam stability and foam formation is still controversial.

Recently, the technique of protein identification by mass spectrometry has been advanced. Some researchers analyzed the proteins of barley grain, barley malt, and beer using two-dimensional gel electrophoresis (2DE) and identified the proteins by mass spectrometry (*7, 31–37*). Finnie et al. (*31*) separated the proteins of barley grain during grain maturation by 2DE and identified the major protein spots on 2DE gels. Various proteins from matured barley grain and malt have been identified (*32–36*). Hao et al. (*37*) identified proteins in beer. However, few researchers have shown how each identified protein influences beer foam stability by proteome analysis.

The purpose of this study was to investigate the relationship between the level of malt modification and the beer foam stability and to examine foam-promoting proteins using 2DE. First, we prepared barley malt samples made from barley grain of three varieties (North American cultivar A and Japanese cultivars B and C) at different levels of malt modification. Then, we brewed beer from each barley malt sample and analyzed the beer foam stability in each beer sample. Then, beer protein profiles by 2DE analysis were compared between cultivars, and between the levels of malt modification to identify possible factors controlling the level of the beer foam stability.

MATERIALS AND METHODS

Barley Samples. For the brewing trial, North American malting barley cultivar A and Japanese malting barley cultivars B and C were used. Cultivar A was harvested in Canada in 2000 and 2002, and cultivars B and C were harvested in Japan in 2002 and 2000, respectively.

Malting and Malt Quality Analysis. A 75 kg barley sample (>2.5 mm screen) was malted according to a previous report (*38*). Steeping was comprised of two cycles of 6 h immersion and 6 h air rest at 14 °C. The level of ex-steep moisture of each sample was controlled in the range of 35–45% (**Table 1**) to investigate the relationship between the level of malt modification and the beer foam stability. Germination lasted for 144 h at 14 °C to obtain the barley malt with homogeneous quality. The kilning program was as follows: 5.0 h at 55 °C, 5.5 h at 60 °C, 2.0 h at 65 °C, 1.5 h at 75 °C, and 4.0 h at 83 °C with a flow of 100% fresh air during kilning. Malt characteristics were analyzed according to the standard methods of the European Brewery Convention (*39*) for malt moisture, wort clarity, wort color, boiled wort color, malt extract (fine grind, 0.2 mm), malt total and soluble nitrogen, Kolbach index, apparent attenuation limit, diastatic power, viscosity, friability, and wort β-glucan.

Pilot-Scale Brewing. Beer samples were brewed from each barley malt according to the standard method of Product and Technology Development Center of Sapporo Breweries. In brief, the wort was prepared from barley malt (67%, w/w, of total raw materials), corn starch, rice, and hops at a 400 L pilot-scale plant. Each barley malt was mashed alone according to a program of 50 °C for 20 min, 65 °C for 40 min, and 73 °C for 3 min. After the wort was boiled for 90 min, the wort was diluted by hot water to a concentration of 10.9–11.1% of extract followed by cooling of the wort. The dissolved oxygen in cooled wort was adjusted to 10 ppm. The fermentation was started by adding 15.0×10^6 cells/mL of lager yeast (brewery collected) to the cooled wort. The fermentation was carried out at 10.5 °C for 8–10 days. The maturation was conducted at 8 °C for 8 days and then at 0 °C for 20–25 days. Filtration and bottling were done using the pilot-scale equipment that effectively excluded oxygen. Beer characteristics were analyzed according to the standard methods of the European Brewery Convention (*39*). The foam stability of each beer was scored as a NIBEM value using the foam stability tester type NIBEM-T (Haffmans B. V., Venlo, Holland) according to the manufacturer's instructions.

Sample Preparation for 2DE. Samples of beer whole proteins for 2DE were prepared as described below. Three milliliters of completely degassed beer samples was applied to the PD-10 column (GE Healthcare Biosciences, Japan), and desalted proteins were eluted by 4 mL of distilled water. After the protein concentration was measured by the Bradford method (*40*) using bovine serum albumin as a standard, this solution was lyophilized. The lyophilized protein fraction was used as beer whole proteins. Ammonium sulfate was added to 300 mL of degassed beer sample up to 25% saturation, and this solution was stirred for 1 h at room temperature. After centrifugation at 2000*g* for 30 min at 4 °C, the precipitate was washed twice with 25% saturated ammonium sulfate solution. This precipitate was defined as a 25% salt-precipitated fraction. To this supernatant, ammonium sulfate was added up to 35% saturation and stirred. Then, the precipitate was washed twice by 35% saturated ammonium sulfate solution. This precipitate was used as a 35% salt-precipitated fraction, and the supernatant was used as a nonprecipitated fraction. Finally, these precipitates were dissolved in 8 M urea (Wako, Japan) + 2% 3-[(3-cholamidopropyl)-dimethylammonio]propanesulfonic acid (CHAPS) (Dojindo Laboratories, Japan) solution containing 0.28% dithiothreitol (Wako). These solutions and supernatant were desalted using the PD-10 column, and these solutions were then lyophilized. Proteins concentrated from beer foam were prepared as described below. In a separating funnel, 600 mL of beer sample was sufficiently foamed. Then, the aqueous fraction (beer fraction) was removed to separate the foam fraction. To wash the foam fraction, 300 mL of distilled water was added to this foam fraction and sufficiently shaken in a separating funnel. The aqueous

Table 2. Quality Profiles of the Barley Malt and the Beer from Three Barley Cultivars (A−C)

	low modification				high modification			
ex-steep moisture (%)	A1-36[a] 35.7	A2-37 36.7	B-37 36.8	C-37 37.4	A1-43 42.8	A2-43 42.8	B-43 42.8	C-44 43.6
malt characteristics								
malt moisture (%)	5.4	5.1	5.4	4.2	4.5	4.7	4.4	4.5
wort clarity	1	1	2	1	1	1	1	1
color (EBC)	2.8	3.1	3.7	3.5	4.5	4.2	3.6	4.6
boiled wort color (°EBC)	5.7	5.6	6.6	7.2	9.8	7.7	6.8	8.4
extract (%, db)	82.3	81.9	84.7	82.4	82.3	82.6	84.4	82.1
soluble nitrogen (%)	0.727	0.725	0.760	0.776	0.887	0.875	0.838	0.849
total nitrogen (%)	1.847	1.738	1.741	1.639	1.774	1.742	1.707	1.571
Kolbach index	39.4	41.7	43.7	47.3	50.0	50.2	49.1	54.0
apparent attenuation limit (%)	83.1	82.0	81.7	82.4	85.7	84.3	82.9	84.4
diastatic power (°WK)	351	327	259	219	391	378	292	250
viscosity (mP s)	1.74	1.78	1.82	1.81	1.47	1.48	1.54	1.54
friability (%)	82.3	79.0	85.7	82.2	88.8	96.0	97.7	93.2
β-glucan (mg/L)	402	409	403	308	33	37	23	41
beer characteristics								
original gravity (%)	11.17	11.13	11.16	11.15	11.21	11.10	11.16	11.08
final extract (%)	3.52	3.60	3.33	3.48	3.32	3.32	3.33	3.25
final attenuation (%)	68.5	69.0	70.2	68.8	70.4	71.3	70.2	70.7
apparent extract (%)	1.70	1.83	1.47	1.67	1.46	1.50	1.47	1.40
apparent attenuation limit (%)	84.7	83.6	86.8	85.0	87.0	86.5	86.8	87.4
alcohol (w/w %)	3.93	3.87	4.02	3.94	4.05	3.99	4.02	4.02
pH	4.36	4.33	4.31	4.41	4.34	4.48	4.31	4.40
color (EBC)	3.7	3.9	4.5	4.7	5.7	5.4	4.5	5.8
bitter unit (mg/L)	23.6	23.7	22.4	23.0	22.9	23.1	22.4	21.4
polyphenol (mg/L)	128	160	168	179	137	164	168	186
free amino nitrogen (mg/L)	65	45	101	99	100	73	101	122
NIBEM (s)	269	270	234	251	269	265	234	223

[a] The number after the hyphen is the level of ex-steep moisture.

fraction was then removed. The foam fraction was washed twice. Finally, the foam fraction was eluted with 20 mL of distilled water and desalted using the PD-10 column and lyophilized. This lyophilized fraction was used as the proteins concentrated from beer foam.

2DE. The lyophilized protein sample was completely dissolved in 8 M urea + 2% CHAPS solution containing 0.28% dithiothreitol (swelling buffer). An appropriate volume of this solution containing 100 μg of protein sample, 6 μL of IPG buffer, pH 3–10 (GE Healthcare Biosciences), and 10 μL of 0.1% bromophenol blue was mixed, and swelling buffer was then added to a total volume of 300 μL. First-dimensional isoelectric focusing was carried out as described below. A total of 300 μL of this solution was applied to IPG dry strips, pI 3–10, 18 cm (GE Healthcare Biosciences), followed by focusing for 50 kVh using the Multiphor II system (GE Healthcare Biosciences). Prior to the second dimension, the IPG strips were equilibrated for 15 min in equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, and 0.01% bromophenol blue) containing 10 mg/mL dithiothreitol, followed by 15 min in equilibration buffer with 25 mg/mL iodoacetamide (Wako). Second-dimensional sodium dodecyl sulfate−polyacrylamide gel electrophoresis was carried out using a precast XL 12–14% gradient gel (GE Healthcare Biosciences) and the Multiphor II system. The gel was stained with the Silver Staining Kit, Protein (GE Healthcare Biosciences). For the mass spectrometry analysis, protein spots were stained by the Silver Stain MS Kit (Wako).

Mass Spectrometry Analysis. For mass spectrometry analysis, protein spots separated by 2DE were isolated from the gel and treated by the decoloring solution of the Silver Stain MS Kit (Wako). Tris-HCL buffer (pH 8.0) containing trypsin was added to the gel sample and incubated for 20 h at 35 °C. The sample was desalted using Zip-Tip (Nihon Millipore Ltd., Japan) and eluted by matrix solution. Then, this eluent was spotted to a target plate, air-dried, and analyzed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF-MS). MALDI TOF-MS analysis was carried out using Voyager-DE STR (Applied Biosystems, United States). The protein was identified by peptide mass fingerprinting (PMF) against the nonredundant amino acid database of the National Center for Biotechnology Information (NCBI-nr).

RESULTS AND DISCUSSION

Comparison of the Foam Stability of Beer Samples. In general, malt modification increases as the level of malt Kolbach index (mashing soluble protein/total malt protein × 100) increases. It is also well-known that beer foam stability generally decreases as the level of malt modification increases (*1, 2*). We investigated the beer foam stability of three barley malt cultivars with respect to different levels of malt modification (Kolbach index). To prepare the samples with different levels of malt modification, we malted barley grain of Canadian cultivar A and Japanese cultivars B and C at different levels of ex-steep moisture in a range of 36–45% (**Table 1**). It is well-known that the moisture content of barley grain during steeping has a substantial impact on malt modification. Subsequently, we brewed beer from each of the barley malt samples. **Table 2** shows the characteristics of malts with low and high malt modification and the beers brewed from them. In terms of malt quality, total nitrogen was lower in the sample of cultivar C than in the samples of cultivars A and B. So, at both low and high modification, the Kolbach index of cultivar C was higher than that of cultivars A and B. In terms of beer quality, bitter units that are positive for foam stability (*2, 8*) were not different between the beers. There were few differences in other parameters in each category, low and high modification, except for the NIBEM value. The fermentation characteristics (time course of residual extract and number of yeast cells in suspension) of these samples were not significantly different from each other (data not shown). The foam stability was assessed by the NIBEM method (**Figure 1**). NIBEM values decreased as the level of malt modification increased in the

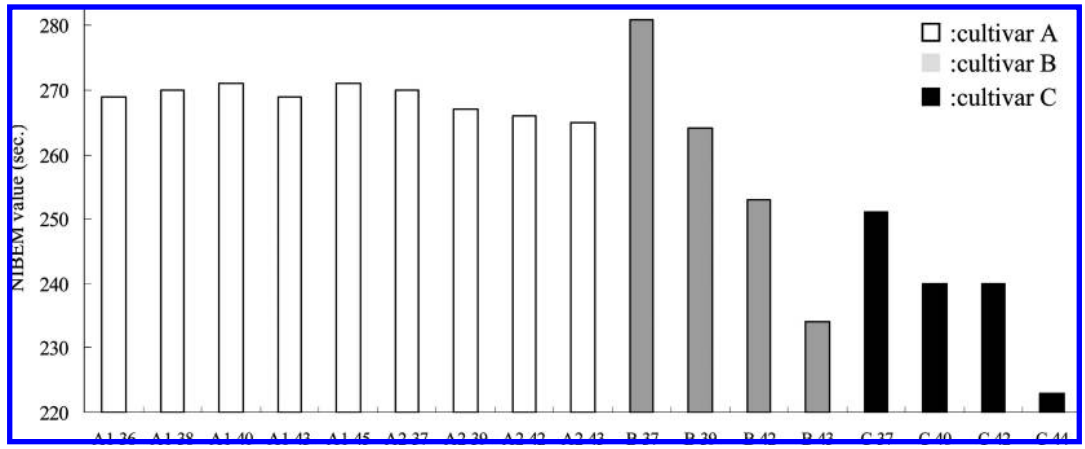

Figure 1. NIBEM value in the beers each brewed from the barley malt of cultivars A−C with different ex-steep moisture levels at malting. Data are means from two independent experiments.

Table 3. Criteria of Selection of Target Proteins (Foam Promoting Proteins) from 2DE[a]

	foam-positive	foam-negative
criteria no.	1	2
cultivar A	↑[b] or →	↓ or →
cultivars B and C	↓	↑

[a] We regarded the protein spots that satisfied criteria 1 or 2 in all of cultivars A−C as foam-promoting proteins. [b] Target protein (spot) is as follows: →, constant; ↑, increase; and ↓, decrease as malt modification (Kolbach index) increases.

beers of cultivar B (**Figure 1**). Similarly, the NIBEM values in beers from cultivar C decreased as the level of malt modification increased but more moderately than those in cultivar B. On the other hand, the NIBEM values of cultivar A did not decrease in two replications of the brewing trial even when the level of malt modification increased. This trend was clearly different from that with cultivars B and C. As described earlier, it is well-known that foam stability usually decreases as the level of malt modification increases, because foam-positive factors such as proteins and nonstarch polysaccharides are degraded at malting and mashing as the malt modification increases (*4, 15*). Although, the foam stability of cultivars B and C decreased with increasing malt modification, the foam stability of cultivar A did not change. This was an unexpected finding. A malting barley cultivar that has the properties of cultivar A has not been reported previously. Therefore, we further investigated these samples to identify the factors responsible for the unique foam stability in cultivar A.

Comparison of Beer Proteins Using 2DE. It has been reported that certain proteins are related to beer foam stability. In particular, the proteins of the hydrophobic fraction of beer and the proteins concentrated in beer foam were suggested to be positive factors for foam stability (*5, 41*). Then, we collected proteins from beer in three fractions. We designated each protein fraction as beer whole proteins, salt-precipitated proteins, and the proteins concentrated from beer foam. These fractions were analyzed by 2DE, and their distribution patterns were compared. Then, foam-promoting protein spots were evaluated according to the criteria shown in **Table 3**. We regarded the protein spots categorized as criteria 1 or 2 in all of the three fractions as foam-promoting proteins.

2DE Analysis of Beer Whole Proteins. The beer proteins of each beer brewed from barley malt of cultivars A−C with low and high malt modification were analyzed by 2DE (**Figure 2**). The distribution of protein spot patterns on the 2DE gels was compared between the low level of malt modification (ex-steep moisture, 36–37%) and the high level (ex-steep moisture, 43–44%) in each cultivar. In all of the 2DE gels, a large, intense staining spot(s) was observed at around an isoelectric point (pI) of 4–5 and a molecular mass (M_r) of 35–45 kDa. Presumably, this gel region includes protein Z, which has coincident characteristics with this region (*42*). Many spots were also observed below 30 kDa. We found cultivar A-specific protein spots in regions II and III and cultivar B-specific spots in region IV. However, the intensity of these spots decreased as the level of malt modification increased (**Figure 2**). These spots did not follow the criteria for foam-promoting proteins (**Table 3**). Enlarged images of region I were shown in **Figure 2b**. Among the spots in region I, the spots of regions I-I, I-II, and I-III were not greatly changed between different levels of malt modification in cultivar A (**Figure 2b**, A1-36 and A1-43). On the other hand, spots of regions I-I, I-II, and I-III in cultivar B were diluted or disappeared as the malt modification increased (**Figure 2b**, B-37 and B-43). In addition, the spots of regions I-I and I-III in cultivar C were diluted or disappeared, but the spots in region I-II were not greatly changed as malt modification increased. From these results, only the spots of regions I-I and I-III satisfied criterion 1 of foam-promoting proteins (**Tables 3** and **4**). It has been well-known that foam-positive proteins such as LTP-1 and protein Z survive in the beer due to their characteristics of being protease inhibitors and heat resistance (*3*). So, our results suggested that protein spots of regions I-I and I-III might be the candidate of foam-promoting proteins (**Table 4**) and have similar characteristics to LTP-1 and protein Z. On the other hand, low molecular weight proteins such as LTP-1, which is one of the foam-promoting proteins (*3, 7, 22–27*), might be missed in 2DE gels. Therefore, our results do not exclude the contributions of other foam-promoting proteins.

2DE Analysis of Salt-Precipitated Proteins in Beers. Some investigations suggested that one of the most important characteristics of foam-promoting proteins was that they were hydrophobic (*5, 41, 43, 44*). Therefore, we prepared fractions containing beer salt-precipitated proteins possibly containing hydrophobic proteins and applied them to 2DE. Then, the patterns of samples among barley cultivars with different levels of malt modification were compared. The 2DE patterns of the beer whole proteins, the 25% salt-precipitated fraction, the 35% salt-precipitated fraction, and the nonprecipitated fraction of A2-37 are shown in **Figure 3**. The massive spot(s) at a pI of around 4–5, M_r of about 40 kDa, mainly appeared in the nonprecipitated fraction among the above three fractions (**Figure 3**). The spots of regions II and III (containing cultivar A-specific protein spots)

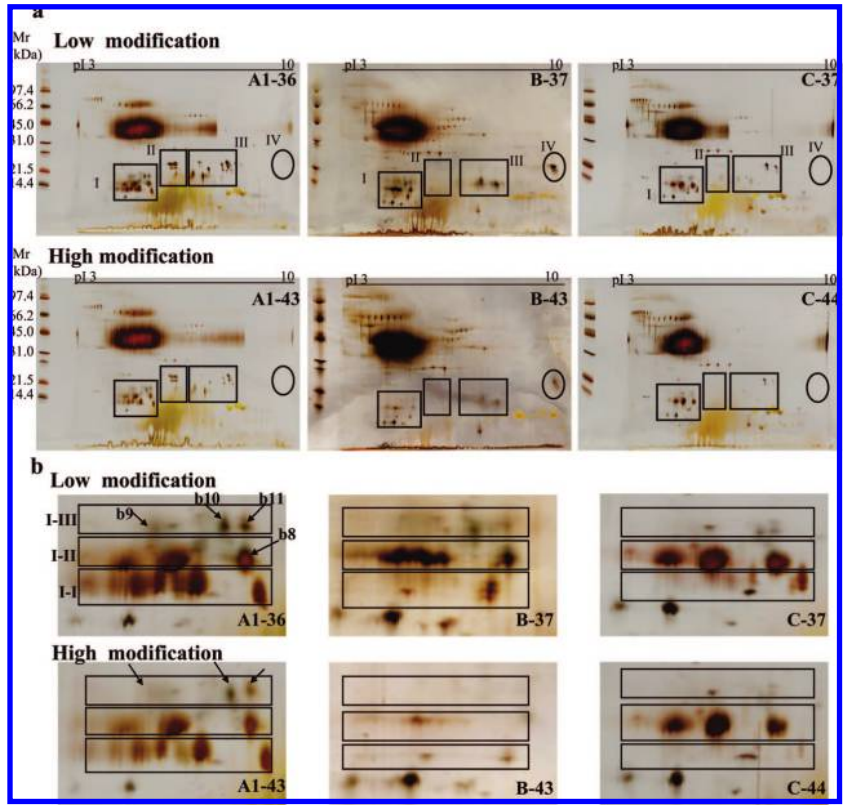

Figure 2. 2DE patterns of the beer proteins in cultivars A−C. A1-36, A1-43, B-37, B-43, C-37, and C-44 are sample names (**Table 1**). b8−b11 are spot numbers. The upper figures (**a**) are whole patterns of A1-36, A1-43, B-37, B-43, C-37, and C-44. The lower figures (**b**) are enlarged images of region I of A1-36, A1-43, B-37, B-43, C-37, and C-44.

Table 4. Candidate of Foam-Promoting Protein Selected by 2DE Analysis of Each Fraction

fraction name	candidate of foam-related protein
beer whole proteins	spots in regions I-I and I-III (spots b9, b10, and b11)[a]
25% salt-precipitated fraction	spots in region I-I spots b8 and b9
proteins concentrated from beer foam	spots in region I-I

[a] Spot names are shown in **Figures 2**, **4**, and **5**.

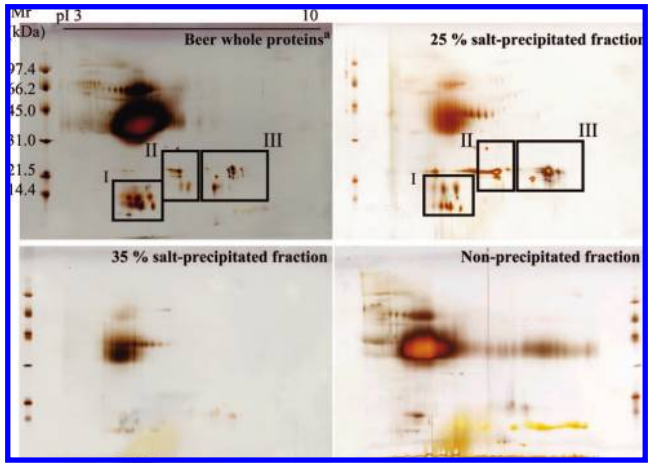

Figure 3. 2DE patterns of the beer whole proteins, 25% salt-precipitated fraction, 35% salt-precipitated fraction, and nonprecipitated fraction of beer sample A2-37. [a]Fraction names are described in the Materials and Methods.

appeared in the 25% salt-precipitated fraction (**Figure 3**), although the spot intensities decreased as the level of malt modification increased (data not shown). **Figure 4** shows

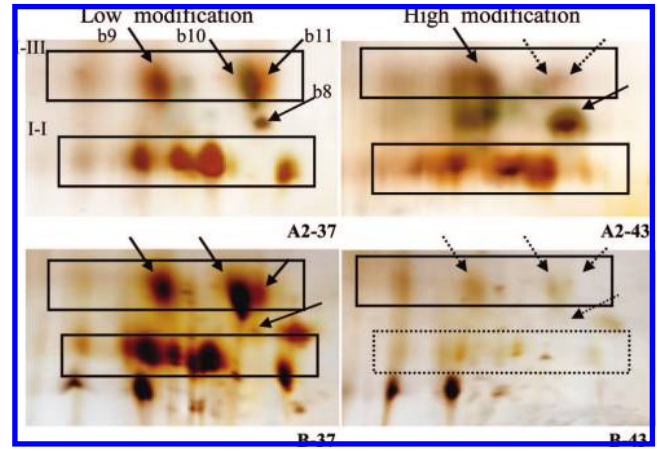

Figure 4. Enlarged images on region I of the 25% salt-precipitated fraction. A2-37, A2-43, B-37, and B-43 are sample names (**Table 1**). b8−b11 are spot numbers.

enlarged images on region I of the 25% salt-precipitated fraction in A2-37, A2-43, B-37, and B-43. Among the spots of region I, the spots in regions I-I and I-III and spot b8 appeared in the 25% salt-precipitated fraction, and the spots in region I-II except for spot b8 appeared in the nonprecipitated fraction (**Figures 3** and **4**). The spots of region I-I in cultivar A slightly decreased as malt modification increased, although these spot intensities in B-43 were considerably lower than those in B-37. Spot b8 disappeared in B-43, while this spot in cultivar A was little changed between A2-37 and A2-43. The spot intensity of b9 significantly decreased in cultivar B when malt modification increased, although it slightly decreased in cultivar A. On the other hand, in both cultivars A and B, the intensities of spots b10 and b11 decreased greatly with the increased level of malt

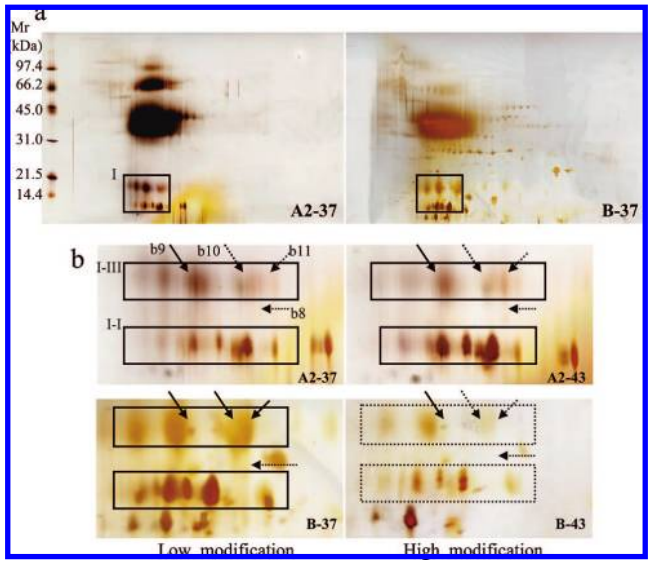

Figure 5. 2DE patterns of proteins concentrated from beer foam in cultivars A and B. A2-37, A2-43, B-37, and B-43 are sample names (**Table 1**). b8−b11 are spot numbers. The upper figures (**a**) are whole patterns of A2-37 and B-37. The lower figures (**b**) are enlarged images of region I of A2-37, A2-43, B-37, and B-43.

modification. From the results of salt-precipitated fractionation analyses, we assumed the spots of region I-I, spots b8 and b9, to be candidates for beer foam-promoting proteins (**Table 4**).

2DE Analysis of the Proteins Concentrated from Beer Foam. On the basis of the assumption that the proteins concentrated from beer foam are active in beer foam stability (*28, 37*), we analyzed this fraction by 2DE. **Figure 5** shows the 2DE patterns of these proteins in A2-37, A2-43, B-37, and B-43. This fraction mainly contained a large, intensively stained spot(s) at around M_r 40 kDa and the proteins of regions I-I and I-III (**Figure 5**). Spot b8, which was a candidate for foam-promoting protein from the results mentioned above, was not observed in any gels (**Figure 5**). In cultivar A, the proteins of region I-I were not changed even with the increased level of malt modification. However, these spots in cultivar B were not observed or significantly diluted with the increased level of malt modification. Spot b9 in cultivar A decreased as malt modification increased. Spots b10 and b11 were diluted in both high and low modification levels in cultivar A. From the analyses of proteins concentrated from beer foam, the protein spots of region I-I were estimated to be foam promoting (**Table 4**).

Mass Spectrometry Analysis of Spots of Region I-I. From the above 2DE results of the three fractions, only the spots of region I-I were categorized under criterion 1 of beer foam-promoting proteins (**Table 3**) in all three fractions (**Table 4**). Therefore, it is suggested that the protein spots of region I-I are probably foam-promoting proteins and might have the characteristics of resistance to malt modification, degradation of protein, and boiling. To identify the proteins in region I-I, each spot in region I-I was analyzed by PMF with MALDI TOF-MS. As such, all of the spots in region I-I were identified as barley dimeric α-amylase inhibitor (BDAI-I, NCBI accession CAA08836). Because these spots had the same molecular mass with different isoelectoric points, several forms of post-translational modification in BDAI-I such as glycosylation and phosphorization were assumed to occur. As shown in **Figures 3** and **4**, it is suggested that BDAI-I might be a comparatively hydrophobic protein. It has been known that the concentration of hydrophobic proteins might be related to beer foam stability (*5, 41, 43, 44*). So, it is also suggested that BDAI-I might be one of the important foam-promoting proteins because of its hydrophobicity. In addition, Gilbert et al. (*45*) suggested that wheat CM3, an α-amylase inhibitor subunit, is suitable as an emulsifier. Although the amino acid sequences of BDAI-I and CM3 are not similar (data not shown), hydrophobicity or high-dimension structures may be similar because the function of both proteins is close.

In conclusion, the foam stability of beer samples brewed from the barley malt of North American barley, cultivar A, was not changed even when the level of malt modification was substantially increased. To investigate the property of foam stability in cultivar A providing this desirable characteristic, beer proteins from cultivar A were analyzed by 2DE. As a result, BDAI-I was shown to resist modification and to be a possible foam-promoting protein. However, to demonstrate that BDAI-I is a foam-promoting protein, further purification and characterization are needed. Moreover, the foam stability difference in malt modification between barley malt cultivars may not be entirely explained by only BDAI-I. In future work, we will quantify the amount of BDAI-I in beer and attempt to investigate the relationship between BDAI-I and beer foam stability. We identified BDAI-I as a foam-promoting protein specific to cultivar A; however, our results do not exclude the contribution of other foam-promoting proteins such as LTP-1 and protein Z. Therefore, to improve/optimize beer foam stability, the levels of BDAI-I and other foam-promoting proteins will need to be controlled genetically within malting varieties and during the malting and brewing processes. The specific identification of the BDAI-I as a foam-promoting protein will enable the construction of a DNA marker system (*46, 47*) for marker-assisted selection of BDAI-I during barley breeding. Direct selection for BDAI-I should enable its content to be increased in such barley varieties to potentially improve the foam stability of beer made from them.

ACKNOWLEDGMENT

We appreciate all members of the Bioresources Research and Development Department, the Frontier Laboratories of Value Creation, and the Product and Technology Development Center, Sapporo Breweries, Ltd., for their continuous support.

LITERATURE CITED

(1) Bamforth, C. W. The foaming properties of beer. *J. Inst. Brew.* **1985**, *91*, 370–373.
(2) Evans, D. E.; Sheehan, M. C. Do not be fobbed off, substance of beer foam, a review. *J. Am. Soc. Brew. Chem.* **2002**, *60*, 47–57.
(3) Evans, D. E.; Hajgaard, 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, Z7 and LTP1. *J. Inst. Brew.* **1999**, *105*, 159–169.
(4) Evans, D. E.; Sheehan, M. C.; Stewart, D. C. The impact of malt derived proteins on beer foam quality part II: The influence of malt foam-positive proteins and non-starch polysaccharide on beer foam. *J. Inst. Brew.* **1999**, *105*, 171–177.
(5) Slack, P. T.; Bamforth, C. W. The fractionation of polypeptides from barley and beer by hydrophobic chromatography: The influence of their hydrophobicity on foam stability. *J. Inst. Brew.* **1983**, *89*, 397–401.
(6) Cooper, D. J.; Husband, F. A.; Mills, E. N.; Wilde, P. J. Role of beer lipid-binding proteins in preventing lipid destabilization of foam. *J. Agric. Food Chem.* **2002**, *50*, 7645–7650.
(7) Van Nierop, S. N. E.; Evans, D. E.; Axcell, B. C.; Cantrell, I. C.; Rautenbach, M. Impact of different wort boiling temperature on the beer foam stabilizing properties of lipid transfer protein 1. *J. Agric. Food Chem.* **2004**, *52*, 3120–3129.

(8) Simpson, W. J.; Hughes, P. S. Stablization of foams by hop-derived bitter acids: Chemical interactions in beer foam. *Cerev. Biotech.* **1993**, *19*, 39–44 .
(9) He, G. Q.; Wang, Z. Y.; Liu, Z. S.; Chen, Q. H.; Ruan, H.; Schwarz, P. B. Relationship of proteinase activity, foam proteins, and head retention in unpasteurized beer. *J. Am. Soc. Brew. Chem.* **2006**, *64*, 33–38.
(10) Leisegang, R.; Stahl, U. Degradation of a foam-promoting barley protein by a proteinase from brewing yeast. *J. Inst. Brew.* **2005**, *111*, 112–117.
(11) Kondo, H.; Shinbo, Y.; Fukui, N.; Nakatani, K.; Oda, K.; Amachi, T. Development of a novel and sensitive method for measurement of proteinase A in beer. *Eur. Brew. Conv. Congr. Proc., Brussels* **1995**, *25*, 669–676.
(12) Kondo, H.; Yomo, H.; Fukukubo, S.; Kawasaki, Y.; Nakatani, K. Advanced method for measuring Proteinase A in beer. *Proc. 25th Conven. Inst. Brew., Asia Pac. Sect., Perth* **1998**, *25*, 119–124.
(13) Kobayashi, N.; Segawa, S.; Umemoto, S.; Kuroda, H.; Kaneda, H.; Mitani, Y.; Watari, J.; Takashio, M. A new method for evaluating foam-damaging effect by free fatty acids. *J. Am. Soc. Brew. Chem.* **2002**, *60*, 37–41.
(14) Roberts, R. T.; Keeney, P. J.; Wainwright, T. The effects of lipids and related materials on beer foam. *J. Inst. Brew.* **1978**, *84*, 9–12.
(15) Evans, D. E.; Nischwitz, R.; Stewart, D. C.; Cole, N.; MacLeod, L. C. The influence of malt foam positive proteins and non-starch polysaccharides on beer foam quality. *Monogr. Eur. Brew. Conv.* **1998**, *27*, 114–128.
(16) Lusk, L. T.; Goldstein, H.; Ryder, D. Independent role of beer proteins, melanoidins and polysaccharides in foam formation. *J. Am. Soc. Brew. Chem.* **1995**, *53*, 93–103.
(17) Briggs, D. *Malting: Barley*; Chapman and Hall: London, 1978; pp 526–559.
(18) Stewart, D. C.; Hawthorne, D.; Evans, D. E. Cold sterile filtration: A small scale filtration test and investigation of membrane plugging. *J. Inst. Brew.* **1998**, *104*, 321–326.
(19) Evans, D. E.; Ratcliffe, M.; Jones, B. L.; Barr, A. R. Variation and genetic control of foam-positive proteins in Australian barley varieties. Aust. Barley Tech. Symposium Proceedings, Melbourne, 1999.
(20) Maeda, K.; Yokoi, S.; Kamada, K.; Kamimura, M. Foam stability and physicochemical properties of beer. *J. Am. Soc. Brew. Chem.* **1991**, *49*, 14–18.
(21) Evans, D. E.; Robinson, L. H.; Sheehan, M. C.; Tolhurst, R. L.; Hill, A.; Skerritt, J. S.; Barr, A. R. Application of immunological methods to differentiate between foam-positive and haze-active proteins originating from malt. *J. Am. Soc. Brew. Chem.* **2003**, *61*, 55–62.
(22) Jegou, S.; Douliez, J. P.; Molle, D.; Boivin, P.; Marion, D. Purification and structural characterization of LTP1 polypeptides from beer. *J. Agric. Food Chem.* **2000**, *48*, 5023–5029.
(23) Jones, B. L.; Marinac, L. A. Purification and partial characterization of a second cysteine proteinase inhibitor from ungerminated barley. *J. Agric. Food Chem.* **2000**, *48*, 257–264.
(24) Gorjanović, S.; Sužnjević, D.; Beljanski, M.; Ostojić, S.; Gorjanović, R.; Vrvić, M.; Hranisavljević, J. Effects of lipid-transfer protein from malting barley grain on brewers yeast fermentation. *J. Inst. Brew.* **2004**, *110*, 297–302.
(25) Perrocheau, L.; Bakan, B.; Bovin, P.; Marion, D. Stability of barley and malt lipid transfer protein 1 (LTP1) toward heating and reducing agents: Relationships with the brewing process. *J. Agric. Food Chem.* **2006**, *54*, 3108–3113.
(26) Gorjanović, S.; Sužnjević, D.; Beljanski, M.; Hranisavljević, J. Barley lipid-transfer protein as heavy metal scavenger. *Environ. Chem. Lett.* **2004**, *2*, 113–116.
(27) Clark, D. C.; Wilde, P. J.; Marion, D. The protection of beer foam against lipid-induced destabilization. *J. Inst. Brew.* **1994**, *100*, 23–25.
(28) Leiper, K. A.; Stewart, G. G.; McKeown, I. P. Beer polypeptides and silica gel. Part II. Polypeptides involved in foam formation. *J. Inst. Brew.* **2003**, *109*, 73–79.
(29) Wang, Z. Y.; He, G. Q.; Liu, Z. S.; Ruan, H.; Chen, Q. H.; Xiong, H. P. Purification of yeast proteinase A from fresh beer and its specificity on foam proteins. *Int. J. Food Sci. Technol.* **2005**, *40*, 835–840.
(30) Tanimizu, N.; Hayashi, R. Changes in vacuolar protease activities during synchronous culture of *Saccharomyces cerevisiae*. *Biosci., Biotechnol., Biochem.* **1996**, *60*, 1526–1527.
(31) Finnie, C.; Melchior, S.; Roepstorff, P.; Svenson, B. Proteome analysis of grain filling and seed maturation in barley. *Plant Physiol.* **2002**, *129*, 1308–1319.
(32) Finnie, C.; Bak-Jensen, K. S.; Laugensen, S.; Roepstorff, P.; Svensson, B. Differential appearance of isoforms and cultivar variation in protein temporal profiles revealed in the maturing barley grain proteome. *Plant Sci.* **2006**, *170*, 808–821.
(33) Østergaard, O.; Finnie, C.; Laugesen, S.; Roepstorff, P.; Svensson, B. Proteome analysis of barley seeds: Identification of major proteins from two-dimensional gels (p*I*4-7). *Proteomics* **2004**, *4*, 2437–2447.
(34) Kristoffersen, H. E.; Flengsrud, R. Separation and characterization of basic barley seed proteins. *Electrophoresis* **2000**, *21*, 3693–3700.
(35) Bak-Jensen, K. S.; Laugensen, S.; Roepstorff, P.; Svensson, B. Two-dimensional gel electrophoresis pattern (pH 6–11) and identification of water-soluble barley seed and malt proteins by mass spectrometry. *Proteomics* **2004**, *4*, 728–742.
(36) Østergaard, O.; Melchior, S.; Roepstorff, P.; Svensson, B. Initial proteome analysis of mature barley seeds and malt. *Proteomics* **2002**, *2*, 733–739.
(37) Hao, J.; Li, Q.; Dong, J.; Yu, J.; Gu, G.; Fan, W.; Chen, J. Identification of the major proteins in beer foam by mass spectrometry following sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Am. Soc. Brew. Chem.* **2006**, *64*, 166–174.
(38) Oogushi, K.; Barr, A. R.; Takahashi, S.; Takoi, K.; Ito, K. Lofty Nijo: A high quality malting barley variety released from an Australian-Japanese collaboration. *J. Inst. Brew.* **2002**, *108*, 13–18.
(39) EBC. Analytica, 4th ed.; European Brewery Convention; Brauerei und Getraenke Rundschau: Zurich, 1987.
(40) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.
(41) Yokoi, S.; Yamashita, K.; Kunitake, N.; Koshino, S. Hydrophobic beer proteins and their function in beer foam. *J. Am. Soc. Brew. Chem.* **1994**, *52*, 125–126.
(42) Kaersgaard, P.; Hejgaard, J. Antigenic beer macromolecules, an experimental survey of purification method. *J. Inst. Brew.* **1979**, *85*, 103–111.
(43) Onishi, A.; Proudlove, M. K.; Dickie, K.; Mills, E. N. C.; Kauffman, J. A.; Morgan, M. R. A. Monoclonal antibody probe for assessing beer foam stabilizing proteins. *J. Agric. Food Chem.* **1999**, *47*, 3044–3049.
(44) Ferreira, I. M.; Jorge, K.; Nogueira, I. C.; Silva, F.; Trugo, L. C. Effects of the combination of hydrophobic polypeptides, iso-alpha acids, and malt-oligosaccharides on beer foam stability. *J. Agric. Food Chem.* **2005**, *53*, 4976–4981.
(45) Gilbert, S. M.; Burnett, G. R.; Mills, E. N. C.; Belton, P. S.; Shewry, P. R.; Tatham, A. S. Identification of the wheat seed protein CM3 as a highly active emulsifier using novel functional screen. *J. Agric. Food Chem.* **2003**, *51*, 2019–2025.
(46) Kaneko, T.; Kihara, M.; Ito, K. Genetic analysis of β-amylase thermostablity to develop a DNA marker for malt fermentability improvement in barley *Hordeum vulgare*. *Plant Breed.* **2000**, *119*, 197–201.
(47) Kaneko, T.; Hirota, N.; Yokoi, S.; Kanatani, R.; Ito, K. Molecular marker for protein Z content in barley (*Hordeum vulgare* L.). *Breed. Sci.* **1999**, *49*, 69–74.

Received for review August 19, 2007. Revised manuscript received November 27, 2007. Accepted November 28, 2007. This study was supported by grants from CREST of Japan, Science and Technology Agency, Japan.

JF0724926