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Novel Prediction Method of Beer Foam Stability Using Protein Z, Barley Dimeric α-Amylase Inhibitor-1 (BDAI-1) and Yeast Thioredoxin

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Foam stability is an important quality trait of beer. Our previous results of two-dimensional gel electrophoresis (2DE) analyses of beer proteins implied a relationship between barley dimeric α-amylase inhibitor-1 (BDAI-1) and beer foam stability as judged by the NIBEM-T analyzer. To develop a novel prediction method of beer foam stability under different conditions of barley cultivar and malt modification, multiple linear regression analysis was applied. The spot intensities of major beer proteins on 2DE gel were quantified and used as explanatory variables. The foam stabilities of 25 beer samples each brewed from malt with different malt modification in one of the three cultivars (cultivars A, B, and C) were explained by the spot intensities of BDAI-1 at the 5% significance level ($r = 0.421$). Furthermore, two other major protein spots (b0 and b5) were observed on the 2DE gels of Japanese commercial beer samples with different foam stability. Then, multiple regression for foam stability was calculated using these three spot intensities as explanatory variables. As a result, 72.1% of the beer foam stability in 25 beer samples was explained by a novel multiple regression equation calculated using spot b0 and BDAI-1 as positive explanatory variables and spot b5 as a negative variable. To verify the validity of the multiple regression equation and the explanatory variables, the beer foam stability in practical beer samples was analyzed. As a result, 81.5% of the beer foam stability in 10 Japanese commercial beer samples was also explained by using spot b0 and BDAI-1 as positive explanatory variables and spot b5 as a negative variable. Mass spectrometry analyses followed by database searches revealed that protein spots b0 and b5 were identified as protein Z originated from barley and thioredoxin originated from yeast, respectively. These results confirm that BDAI-1 and protein Z are foam-positive factors and identify yeast thioredoxin as a possible novel foam-negative factor.

KEYWORDS: Beer foam stability; BDAI-1; protein Z; thioredoxin; multiple regression analysis

INTRODUCTION

Beer foam quality is essentially defined by its stability, lacing, whiteness, intensity, strength, and creaminess (*1*). Among these factors, foam stability is an essential quality character. Previous investigators have studied various factors relating to foam stability and have concluded that factors such as iso-α-acids from hop (*2, 3*), proteins derived from barley malt (*4*−*6*), nonstarch polysaccharides (*7, 8*), and metal ions (*2*) contributed to foam stability. The key interaction is considered to be that between proteins and the hop iso-α-acids. On the other hand, proteinase A from yeast (*9*−*13*) and lipids (*14, 15*) have been considered as negative factors.

Among these factors, proteins such as protein Z, lipid transfer protein 1 (LTP-1), hordein, and barley dimeric α-amylase inhibitor-1 (BDAI-1) play important roles in determining foam stability. It has been suggested that the more hydrophobic a protein, the more foam positive it will be (*16*−*18*). LTP-1 is considered a positive factor for foam stability, because LTP-1 binds to lipids, which prevent beer foam stability (*19*−*22*). Evans et al. (*23*) quantitatively examined the relationship between beer foam stability and proteins, namely protein Z4, protein Z7, and LTP-1. They indicated that the level of beer

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foam stability was explained by the malt protein Z4 content and the wort β-glucan as explanatory variables in the stepwise multiple regression analysis (*23*). They applied multiple regression analysis only on a restricted number of proteins (protein Z4, protein Z7, and LTP-1). However, the two-dimensional gel electrophoresis technique used by Okada et al. (*24*) indicated that beer proteins should be more comprehensively investigated to reveal their relationship with beer foam stability.

Two-dimensional gel electrophoresis (2DE) followed by the protein identification using mass spectrometry enables us to analyze proteins comprehensively. Perrocheau et al. (*25*) isolated proteins in beer by 2DE and identified major protein spots by mass spectrometry analysis. Hao et al. (*26*) also examined beer proteins by SDS-PAGE, and many proteins were identified using mass spectrometry. However, they did not quantitatively indicate which proteins contributed to beer foam stability.

As reported previously (*24*), beer samples were brewed from malt of three cultivars (cultivars A, B, and C) with different malt modifications. We found that the beer foam stability in cultivar A did not change even when malt modification increased, whereas that of cultivars B and C decreased. No other malting barley cultivar with the foam properties of cultivar A had previously been reported. To investigate the property of foam stability in cultivar A, the beer proteins of three fractions were analyzed using 2DE, namely total beer proteins, salt-precipitated proteins, and the proteins concentrated from beer foam. If spot intensity of a protein in cultivar A was constant or increased as malt modification increased while those in cultivars B and C decreased, it was regarded the protein spot as a foam-promoting protein. As a consequence, several protein spots were categorized under the criterion of beer foam-promoting protein. These protein spots were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF-MS) followed by a peptide database search. As a result, all of these protein spots were identified as BDAI-1. It was suggested that BDAI-1 is a possible foam-promoting protein specific to cultivar A.

The purpose of this study was to develop a novel prediction method of beer foam stability including factors of barley cultivars and levels of malt modification. First, 25 beer samples were brewed from the malt made from three cultivars (cultivars A, B, and C) with different levels of modification. The spot intensities of BDAI-1 were quantified on two-dimensional gels, and the relationship between the foam stability and the spot intensity of BDAI-1 was examined. In order to explain the beer foam stability sufficiently, beer foam stability was analyzed by multiple linear regression analysis. To support the results, the beer foam stability of Japanese commercial beer samples was analyzed by the same method.

MATERIALS AND METHODS

Barley Samples. For brewing trials, North American malting barley cultivar A and Japanese malting barley cultivars B and C were used. Barley grain of cultivar A was harvested in Canada in 2000 (for first trial) and 2002 (for second trial), that of cultivar B was harvested in Japan in 2002, and that of cultivar C was harvested in Japan in 2000 (for first trial) and 2002 (for second trial), respectively.

Beer Samples. For beer foam stability and 2DE analyses, 25 beer samples shown in **Table 1** were used. The malts were made from 75 kg of barley grain in one of the three cultivars at different levels of ex-steep moisture (36−45%) according to a previous report (*24*). The worts were prepared at a 400 L pilot-scale plant according to a previous report (*24*) except for mashing in temperature. The mashing in temperature was set to 50 or 60 °C (**Table 1**). The fermentation conditions were previously described (*24*). Beer samples commercially available in Japan in 2004 were also used in the analyses of beer foam stability and 2DE. Among these samples, a beer made from only malt was defined as "all-malt beer", a beer made from malt and adjuncts (67% of malt, in total raw materials) was defined as "Japanese regular beer", and a low-malt beer (below 24% of malt, in total raw materials) was defined as "Happoshu", a special category of Japanese beer. The foam stability of each beer was assessed as a NIBEM value using the foam stability tester type NIBEM-T (Haffmans B. V., Venlo, Holland) according to the manufacturer's instructions.

Table 1. List of Beer Samples Brewed from Malt with Different Levels of Malt Modification from One of Three Cultivars (Cultivars A, B, and C)

		sample name	mashing in temp	NIBEM (s)	malt KI[a]
cultivar A	first trial[b]	A1-36[c]	50 °C	269	39.4
		A1-38		270	42.1
		A1-40		271	46.0
		A1-43		269	50.0
		A1-45		271	48.9
		A1-38 (60)[d]	60 °C	285	42.1
		A1-43 (60)		264	50.0
	second trial	A2-37	50 °C	270	41.7
		A2-39		267	46.9
		A2-42		266	48.8
		A2-43		265	50.2
cultivar B		B-37	50 °C	281	43.7
		B-39		264	43.6
		B-42		253	48.5
		B-43		234	49.1
cultivar C	first trial	C1-37	50 °C	251	47.3
		C1-40		240	49.4
		C1-42		240	49.8
		C1-44		223	54.0
		C1-37 (60)	60 °C	245	47.3
		C1-44 (60)		234	54.0
	second trial	C2-37 (60)	60 °C	262	43.3
		C2-40 (60)		258	47.1
		C2-42 (60)		260	50.1
		C2-43 (60)		243	51.6

[a] KI indicates Kolbach index (soluble nitrogen/total malt nitrogen × 100). [b] For cultivars A and C, two times of brewing trials were conducted. See Materials and Methods. [c] The number after the hyphen is the level of ex-steep moisture at malting. [d] (60) indicates 60 °C of mashing in temperature. No symbol indicates 50 °C of mashing in temperature.

Sample Preparation and Two-Dimensional Gel Electrophoresis (2DE). Three milliliters of completely degassed beer samples was applied to a PD-10 column (GE Healthcare Biosciences, Japan), and desalted proteins were eluted by 4 mL of distilled water. After the desalted proteins were lyophilized, 2DE analysis and silver staining were carried out according to our previous report (*24*).

Quantification of Protein Spot Intensity on 2DE Gel. Before 2DE, the total protein concentration of the sample solution was measured by the Bradford method using bovine serum albumin as a standard (*27*). After the two-dimensional gel was silver stained, spots observed on the gel were analyzed using the software package Image Master 2D Platinum (GE Healthcare Biosciences). To compare spot intensities between 2DE gels excluding the difference of the degree of staining of the 2DE gels, the calculation shown below was conducted. Protein spots on the gel were detected; subsequently, absolute values of intensity of all spots on the gel were examined using the software by volume as a unit. Then, prespot intensity, the percentage of each spot intensity (vol.) against all spots, was calculated by volume percent as a unit. The spot intensity was calculated by multiplying the protein concentration and the prespot intensity by dimensionless as a unit.

Mass Spectrometry Analysis. Mass spectrometry analysis using MALDI TOF-MS was carried out according to our previous report (*24*). Peptides detected were searched against a protein database on an in-house server equipped with MASCOT search engine (http://www.matrixscience.com/) (*28*). The parameters for the peptide mass finger printing (PMF) analysis are described as follows: database, National Center for Biotechnology Information (NCBI-nr); enzyme, trypsin; peptide mass tolerance, ± 20 ppm; minimum numbers of peptides to

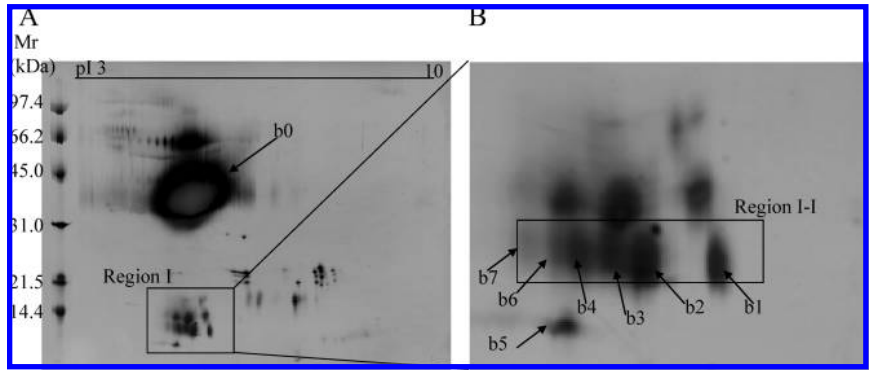

Figure 1. Two-dimensional gel electrophoresis (2DE) patterns of the beer proteins of A2-37 (**Table 1**): (**A**) whole gel image of 2DE, pI 3−10; (**B**) enlarged image of region I. Arrows indicate spot numbers.

match, 4; fixed modification, carbamidomethyl; variable modifications, oxidation of methionine, protein N-terminus acetylated, and peptide N-terminal Gln to pyro-Glu. When a protein was not identified by PMF analysis using trypsin as a proteinase, the sample was double digested by trypsin and lysil endopeptidase. Tris-HCl buffer (pH 8.0) containing lysil endopeptidase was added to decolorized sample gel prepared according to a previous report (*24*), and the gel was incubated for 3 h at 35 °C. Then, trypsin was added to this sample and incubated for 20 h at 35 °C. Subsequently, all the sample solutions were applied to liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS). The condition of LC-MS/MS analysis is described as follows: equipment, MAGIC 2002 (Michrom BioResources, Inc., Auburn, CA); column; Magic C18 (0.1 mm × 150 mm, Michrom BioResources, Inc., Auburn, CA); mobile phase, 2% acetonitrile + 0.1% formic acid and 90% acetonitrile + 0.1% formic acid; flow rate, 250−300 nL/min; mass spectrometer, Q-Tof2 (Micromass, U.K.); ionization method, Nanoflow-LC ESI; ionization mode, positive mode; electric potential of capillary, 1.8 kV; collision energy, 20−35 eV. In order to identify protein species, NCBI-nr was searched by resultant values of product ion from all precursor ions using MASCOT search engine (*28*).

Statistics Analysis. Statistical analysis was carried out using the software package S-PLUS ver. 6.1 (Insightful Corporation, Seattle, WA).

RESULTS

Quantification of Spot Intensity in the Region I-I (BDAI-1). In a previous report (*24*), protein spots in the region I-I (**Figure 1**) were found to be as foam-promoting proteins specific in cultivar A, which provided a desirable profile in beer foam stability. From the mass spectrometry analysis followed by a database search, all the spots in the region I-I were identified as belonging to the barley dimeric α-amylase inhibitor-1 (BDAI-1). In this study, 25 beer samples were brewed from the malt of three cultivars (cultivars A, B, and C) with different levels of modification (**Table 1**). The beer samples showed a wide range of foam stability (average: 258.2; standard deviation: 15.7), particularly those made from cultivars B and C that were responsive to malting conditions altering their modification. The proteins of 25 beer samples were analyzed using 2DE; subsequently, spot intensities in the region I-I (b1, b2, b3, b4, b6, and b7) were quantified (**Table 2**). The mean values of the spot intensity in b6 and b7 were lower than those of the other spots. To investigate the relationship between foam stability and BDAI-1, regression analysis was performed between the intensity of the spots in the region I-I and the NIBEM value (**Table 3**). The results showed significant correlation coefficients between the NIBEM value and the spot intensities for both b2 and b4. These results confirm that BDAI-1 is a foam-promoting protein as previously reported (*24*), although the values for the correlation coefficient were relatively low (**Table 3**). On the other hand, significant correlations were observed between the spot intensities in region I-I (**Table 3**). To avoid multicollinearity in the multiple regression analysis, only spot b2 was selected to represent these spots, since its spot intensity was the highest in region I-I (**Table 2**).

Table 2. Summary of the Spot Intensities of b1−b4, b6, and b7 of 25 Beer Samples Brewed from Malt from Cultivars A, B, and C, Malted to Different Levels of Malt Modification

spot intensity[a]	spot b1	spot b2	spot b3	spot b4	spot b6	spot b7
mean	29.1	61.3	25.6	36.8	11.2	10.9
standard deviation	35.5	60.3	44.6	42.9	32.0	30.3
maximum	130.9	214.2	160.8	148.2	149.3	134.2
minimum	0.0	11.4	0.0	0.0	0.0	0.0

[a] Dimensionless as a unit.

Table 3. Correlation Coefficients between Each Spot Intensity in Region I-I (Spot b1−b4, b6, and b7) and NIBEM Values for 25 Beer Samples

	spot b1	spot b2	spot b3	spot b4	spot b6	spot b7
spot b2	0.762[c]					
spot b3	0.767[c]	0.924[c]				
spot b4	0.680[c]	0.870[c]	0.815[c]			
spot b6	0.406[a]	0.615[b]	0.534[b]	0.671[c]		
spot b7	0.409[a]	0.600[b]	0.538[b]	0.724[c]	0.971[c]	
NIBEM	0.218	0.421[a]	0.388	0.410[a]	0.271	0.273

[a] $P < 0.05$. [b] $P < 0.01$. [c] $P < 0.001$.

2DE Patterns of Japanese Commercial Beer Samples. Analysis of the 2DE patterns of six Japanese commercial beer samples, the all-malt beers (beers AL-1 and AL-2), Japanese regular beers (beers R-1 and R-2) and the Happoshus (beers H-1 and H-2) were conducted (**Figure 2**). The spot intensities in the region I-I in beers AL-1, AL-2, R-1, R-2, and H-2 were very weak regardless of differences in the foam stability. These results suggest that another foam-related protein exists besides BDAI-1. In the all-malt beers (beers AL-1 and AL-2), a few protein spots such as a large, intensely staining spot(s) (spot b0) around an isoelectric point (pI) of 4−5 and a molecular mass (M_r) of 30−45 kDa and spot b5 at M_r of about 10 kDa were observed as major spots (**Figure 2**). As the spots that appeared on 2DE gels of the all-malt beers were limited, these spots were examined in detail. The spot intensities for b5 in the beers with lower NIBEM values (beers AL-2, R-2, and H-2) were denser than those in the beers with higher NIBEM values (beers AL-1, R-1, and H-1). These results suggest that the protein of spot b5 might be a candidate of foam-negative protein. On the other hand, it was assumed that protein spot b0 (**Figures 1** and **2**) was the foam-positive protein, protein Z (1, 2, 19, 23), due to mass spectrometric analysis previously reported by

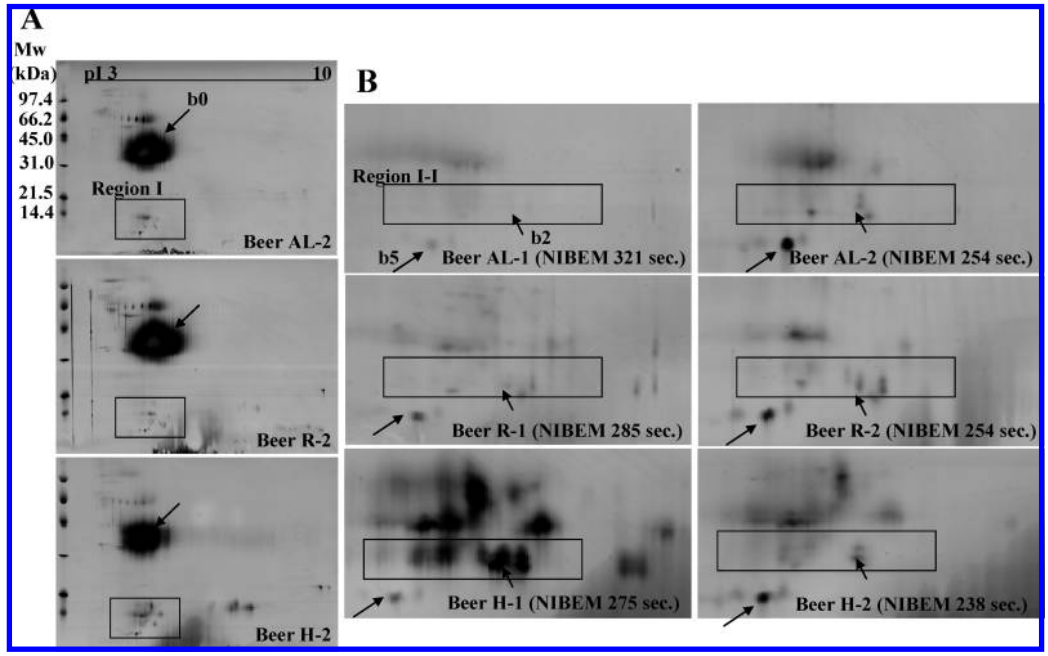

Figure 2. 2DE patterns of the all-malt beers (beers AL-1 and AL-2), Japanese regular beers with medium malt content (beers R-1 and R-2), and Happoshu beers that have low malt content (beers H-1 and H-2). The left figures (**A**) are whole patterns of beers AL-2, R-2, and H-2. The right figures (**B**) are enlarged images of region I (see **Figure 1**) of beers AL-1, AL-2, R-1, R-2, H-1, and H-2.

Table 4. Summary of the Spot Intensities for b0, b2, and b5 and NIBEM Value of 25 Beer Samples Each Brewed from Malt with Different Levels of Malt Modification of One of the Three Barley Cultivars (Cultivars A, B, and C)

	spot intensity[a]			
	spot b0	spot b2	spot b5	NIBEM (s)
mean	11193	61.3	31.8	258
standard deviation	1860	60.3	20.2	16
maximum	15434	214.2	75.7	285
minimum	8113	11.4	6.2	223

[a] Dimensionless as a unit.

Perrocheau et al. (*25*) and Hao et al. (*26*). Therefore, the NIBEM values were analyzed by multiple regression analysis using the protein spot intensities (spots b0, b2, and b5) as explanatory variables.

Multiple Linear Regression Analysis of the NIBEM Values of 25 Beer Samples. To investigate the relationship between foam stability and the beer proteins using multiple linear regression analysis, spot intensities of b0, b2, and b5 in 25 beer samples were quantified (**Table 4**). Multiple linear regression analysis of the NIBEM values in 25 beer samples were carried out using the spot intensities of b0, b2, and b5 as explanatory variables. As a result, the NIBEM values were substantially explained by three spot intensities (b0, b2, and b5) with a coefficient of multiple determination, $R^2 = 0.721$*** (adjusted) (*** indicates $P < 0.001$). Multiple regression expression is shown in eq 1:

$$\text{NIBEM (s)} = (0.00242^{*} \times \text{spot b0}) + (0.0838^{**} \times \text{spot b2}) + (-0.483^{***} \times \text{spot b5}) + 241.4 \quad (1)$$

where * indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$.

Actual measured NIBEM values and the calculated NIBEM values using eq 1 were plotted (**Figure 3**). As a result, a significant relationship between actual measured NIBEM values and the calculated NIBEM values was observed at the 0.1% significance level.

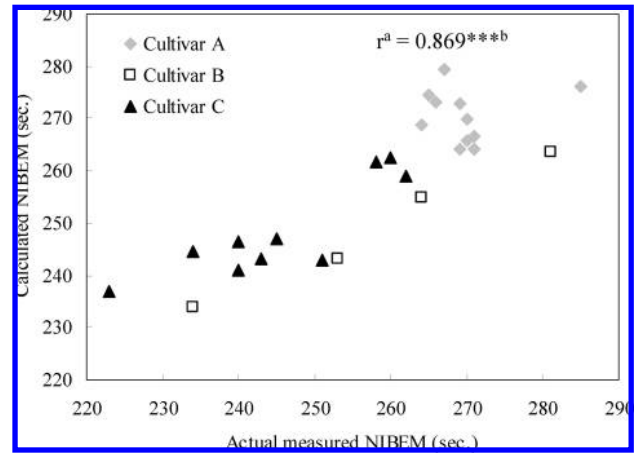

Figure 3. Multiple linear regression analysis of the NIBEM values of 25 beer samples brewed from malt from cultivars A, B, and C that were malted to different levels of malt modification (**Table 1**). Calculated NIBEM values were calculated using eq 1. [a] *r* indicates correlation coefficient; [b] *** indicates $P < 0.001$.

Table 5. Summary of the Spot Intensities for b0, b2, and b5 and NIBEM Value of 10 Japanese Commercial Beer Samples

	spot intensity[a]			
	spot b0	spot b2	spot b5	NIBEM (s)
mean	13638	32.9	22.9	276
standard deviation	3025	49.8	22.4	29
maximum	17610	165.0	71.3	321
minimum	6892	0.0	3.2	238

[a] Dimensionless as a unit.

Multiple Linear Regression Analysis of the NIBEM Values of Japanese Commercial Beer Samples. NIBEM value measurement and 2DE analyses of 10 Japanese commercial beer samples of the all-malt beers, Japanese regular beers, and Happoshu beers were carried out (**Table 5**). The NIBEM values and the spot intensities of b0, b2, and b5 in these samples also

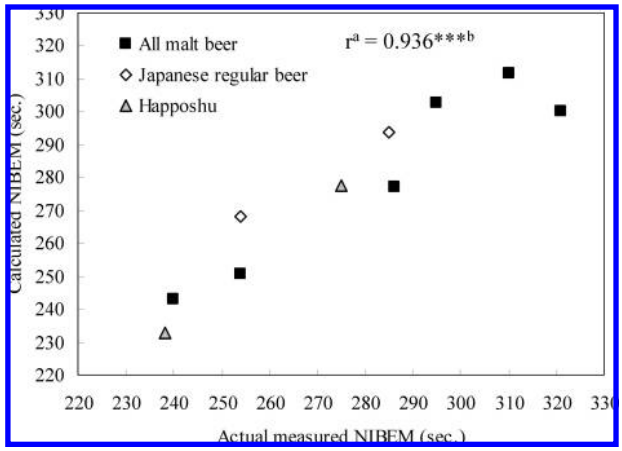

Figure 4. Multiple linear regression analysis of the NIBEM values of 10 Japanese commercial beer samples, the all-malt beers, Japanese regular beers (medium malt content), and Happoshu beers (low malt content). Calculated NIBEM values were calculated using eq 2. [a] *r* indicates correlation coefficient; [b] *** indicates $P < 0.001$.

Table 6. Standard Partial Regression Coefficients in Multiple Linear Regression Analysis of the Beer Foam Stability of 25 Beer Samples[a] and 10 Japanese Commercial Beer Samples

	spot b0	spot b2	spot b5
25 beer samples[a]	0.287[b]	0.323[c]	−0.621[d]
10 of Japanese commercial beer samples	0.778[c]	0.133	−0.851[c]

[a] The beer samples brewed from malt with different levels of malt modification in one of the three cultivars (cultivars A, B, and C) (**Table 1**). [b] $P < 0.05$. [c] $P < 0.01$. [d] $P < 0.001$.

showed a wide range of variation. Following the method above, multiple linear regression analysis of the NIBEM value was carried out by the spot intensities of b0, b2, and b5. As a result, the NIBEM value was significantly explained with a coefficient of multiple determination, $R^2 = 0.815$** (adjusted) (** indicates $P < 0.01$). Multiple regression expression is shown in eq 2:

$$\text{NIBEM value (s)} = (0.00739^{**} \times \text{spot b0}) + (0.0766 \times \text{spot b2}) + (-1.09^{**} \times \text{spot b5}) + 197.6 \quad (2)$$

where ** indicates $P < 0.01$.

Actual measured NIBEM values and the calculated NIBEM values using eq 2 were plotted (**Figure 4**). As a result, a significant relationship between actual measured NIBEM values and the calculated NIBEM values was also observed at the 0.1% significance level. Although the magnitude of the coefficients for the explanatory variables in eq 2 was different from those in eq 1, the positive or negative sign of explanatory variables corresponded. It was concluded that the NIBEM values of Japanese commercial beer samples, which included various beer types, could be explained by the spot intensities of b0, b2, and b5.

The standard partial regression coefficients in the multiple linear regression analysis are presented in **Table 6**. In both sample sets (25 experimental beers and 10 commercial beers), the standard partial regression coefficients for spot b5 were the highest among the three explanatory variables. The correlation coefficients between the spot intensities of b0, b2, and b5 and the NIEBM value of the 25 beer samples were 0.514**, 0.421*, and −0.762***, respectively (* indicates $P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$) (**Table 7**). Similar results were observed in 10 Japanese commercial beer samples (data not shown).

Table 7. Correlation Coefficients for the Relationship between the Spot Intensities of b0, b2, and b5 and NIBEM Values for 25 Beer Samples[a]

	spot b0	spot b2	spot b5
spot b2	0.051		
spot b5	−0.340	−0.135	
NIBEM	0.514[c]	0.421[b]	−0.762[d]

[a] The beer samples each brewed from malt with different levels of malt modification of one of the three cultivars (Cultivars A, B and C) (Table 1). [b] $P < 0.05$. [c] $P < 0.01$. [d] $P < 0.001$.

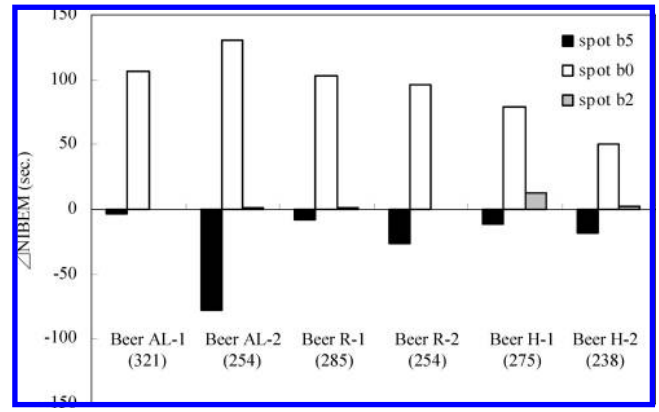

Figure 5. Contribution of each explanatory variable to the NIBEM value of the all-malt beers (beers AL-1 and AL-2), Japanese regular beers with medium malt content (beers R-1 and R-2), and the Happoshu beers with low malt content (beers H-1 and H-2). The spot intensities of b0, b2, and b5 and the corresponding partial regression coefficient in eq 2 were multiplied. These calculated values, ΔNIBEM values, show how each explanatory variable contributed to the NIBEM value of the beer. The number in the parentheses is the NIBEM value.

Mass Spectrometry Analysis. The above results show that the intensities of protein spots b0, b2, and b5 on 2DE gel are important as explanatory variables in explaining the beer foam stability. Protein spot b2 was identified as BDAI-1 in a previous report (*24*). To identify the protein species of spots b0 and b5, these protein spots were analyzed using MALDI TOF-MS or LC-MS/MS, and then the mass fragments patterns were searched on a protein fragment database. As a result, spots b0 and b5 were identified as protein Z type serpin (protein Z4 and protein Z7) (NCBI accession No. CAA66232 and CAA64599) from *Hordeum vulgare* and thioredoxin (y-TRX2) (NCBI accession No. NP_011725) from *Saccharomyces cerevisiae*, respectively.

Influences of Protein Z, BDAI-1, and y-TRX2 on Beer Foam Stability. For commercial beer samples, the spot intensities of b0, b2, and b5 and the corresponding partial regression coefficient in eq 2 were multiplied. These calculated values, ΔNIBEM values, show how each explanatory variable contributed to a higher or lower NIBEM value of the beer (**Figure 5**). When malt content was high, the contribution of spot b0 (protein Z) to the NIBEM value was also high, which is logical since protein Z was derived from barley malt. On the other hand, in Happoshu beer, H-1 with low malt content, the contribution of spot b2 (BDAI-1) to the NIBEM value was higher, which was opposite to the results of spot b0 (protein Z). In particular, the influence of spot b2 on the NIBEM value in all-malt beers was considerably small. Comparing the samples of same malt content, the contribution of intensity of spot b5 (y-TRX2) to the NIBEM value was low when the NIBEM value was high.

DISCUSSION

Twenty-five beer samples were brewed from malt of three cultivars (A, B, and C) that were malted to different levels of modification (**Table 1**). Analysis of these beers showed substantial differences in foam stability and 2DE patterns of foam-positive and foam-negative proteins for the beers. As reported previously (*24*), it was confirmed that BDAI-1 is a probable foam-promoting protein. The spot intensities in the region I-I (BDAI-1) were quantified, and then, the relationship between each spot in the region I-I and foam stability were examined (**Table 3**). However, the correlation coefficient with the NIBEM value although significant, was relatively low. The explanation may be that a high level of BDAI-1 in beer is a characteristic of beers brewed from the malt of cultivar A, as was examined in a previous paper (*24*). Therefore, the foam stability differences in barley cultivars resulted partly from the content of BDAI-1 of cultivar A.

The analysis of 25 beer samples and 10 commercial beer samples enabled the development of a novel multiple regression equation for the prediction of NIBEM values from the spot intensities of b0 (protein Z), b2 (BDAI-1), and b5 (y-TRX2) (eqs 1 and 2). As the beer foam stabilities of both sample sets (25 experimental beers and 10 commercial beers) were explained by levels of protein Z, BDAI-1, and y-TRX2, it is suggested that these three factors are important in explaining the practical beer foam stability. In eqs 1 and 2, the coefficients of spot b0 (protein Z) and b2 (BDAI-1) had positive values, while the coefficient of spot b5 (y-TRX2) had a negative value. These results suggest that protein Z and BDAI-1 are positive factors contributing to beer foam stability, while y-TRX2 is a negative factor. The novel attribution of yeast thioredoxin as a foam-negative protein has not been reported previously. The standard partial regression coefficients for spot b5 (y-TRX2) in both eqs 1 and 2 were the highest among the three explanatory variables (**Table 6**). Therefore, y-TRX2 is suggested as the most important factor on beer foam stability among these explanatory variables. As shown in **Table 7**, the correlation coefficients between the spot intensities of b0 (protein Z), b2 (BDAI-1), and b5 (y-TRX2) and NIEBM value were relatively low. These results indicate that the NIBEM values were best explained by a combination of all the three explanatory variables.

The spots for BDAI-1 were not observed in the 2DE patterns for the all-malt beer samples (beers AL-1 and AL-2) (**Figure 2**). As observed in a previous report (*24*), BDAI-1 in beer made from the malt of cultivar A was little changed by malting modification conditions. However, the spots for BDAI-1 in the beers made from cultivars B and C decreased or disappeared with higher malt modification. Therefore, these all-malt beers can potentially be brewed from malts without appreciable levels of BDAI-1. Conversely, as shown in **Figure 5**, the contribution of spot b2 (BDAI-1) to the foam stability was relatively high (ΔNIBEM = 12 s) in Happoshu beer H-1. This Happoshu sample was brewed from malt and adjuncts such as unmalted barley grain. The explanation for the substantial contribution of spot b2 (BDAI-1) in this beer is currently unknown, but it may be influenced by unmalted barley grain. Despite the strong correlations between the explanatory variables and foam stability, this evidence does not necessarily confirm the direct role of these proteins in foam stability. However, taken with other circumstantial evidence present in the literature (*1, 2*), it appears likely that these proteins are genuine foam-promoting proteins. Further research is required to define how these proteins improve foam stability and how they interact with other proteins and foam-promoting compounds such as hop iso-α-acids.

From the results of mass spectrometry analysis, spot b0 was identified as protein Z originated from *H. vulgare* L. Protein Z is a major protein in beer (*24*). As shown in **Figure 5**, the contribution of spot b0 (protein Z) was higher as the malt content increased. These results confirm previous reports that protein Z is important for beer foam stability (*1, 2*) especially in all-malt beer and Japanese regular beer. Evans et al. (*23*) observed that the malt protein Z4 content is an important factor whose level is significantly correlated with beer foam stability. However, as discussed above, the mechanism by which protein Z supports beer foam stability is yet to be determined. Protein Z has a function of serine proteinase inhibitor (serpin). The barley serpin family contains protein Z4 and protein Z7 forms, both of which were detected in beer (*23*). The mass spectrometry analysis conducted here confirms that protein spot b0 contains both protein Z4 and protein Z7. Evans et al. (*19*) examined the contents of protein Z4 and protein Z7 in barley grain and malt of various cultivars, and they revealed that there are varietal variations in protein Z4 and protein Z7 contents with protein Z4 comprising 80% of total protein Z content on average. In future research, the concentrations of each of protein Z4 and protein Z7 in beer by using each specific antibodies to investigate the relationship among beer foam stability and protein Z form will be examined.

Spot b5 was identified as thioredoxin (y-TRX2) from *Saccharomyces cerevisiae* by mass spectrometry analysis. The results suggest that yeast thioredoxin in beer is a negative factor with regards to beer foam stability (eqs 1 and 2). This is the first report showing the negative relationship between beer foam stability and yeast thioredoxin. Thioredoxin is a ubiquitous antioxidant and low molecular weight protein disulfide oxidoreductase (*29*). It has been suggested that yeast thioredoxin protects gastric mucosa due to its anti-inflammatory effect (*30*) and reduces the allergencity of allergens (*31*). While thioredoxin of *S. cerevisiae* has three isoforms, y-TRX1, y-TRX2, and y-TRX3 (*32, 33*), only y-TRX2 was identified by mass spectrometry analysis of spot b5 in this study. In some reports, it was demonstrated that y-TRX2 expression is upregulated in response to oxidative stress, even with the addition of chemical reagents such as hydrogen peroxide, *tert*-butyl hydroperoxide, and diamide (*34*−*36*). As shown in **Figure 2**, the spot intensities for b5 (y-TRX2) were extremely different between the beers whose NIBEM values were higher and lower. The reason for these differences is not known at present, but it might be derived from the difference in the level of oxidative stress to yeast cell during brewing. Perhaps this stress is also related not only to the upregulation of y-TRX2 but also the leakage of foam destroying yeast proteinase A (*9*−*13*). This study indicates that yeast thioredoxin is a useful marker related to potential beer foam stability and that the selection of brewing yeast strains contributing to low level of y-TRX2 may improve foam stability. In future studies, we plan to examine the relationship between proteinase A activity and concentration of yeast thioredoxin in beer.

In conclusion, the beer foam stability of 25 beer samples each brewed from malt from three cultivars (cultivars A, B, and C) with different levels of modification and 10 Japanese commercial beer samples were analyzed. Multiple linear regression analysis showed that NIBEM foam stability was significantly explained by the protein spot intensities of protein Z, BDAI-1, and y-TRX2 on 2DE gels. A novel foam negative factor, thioredoxin, from yeast was identified. However, the mechanism by which these proteins practically influence beer foam stability is yet to be defined. A clear understanding of these factors may enable

prediction of beer foam stability for quality control of beer brewing and indicate strategies for malting barley varietal improvement. As described above, it is suggested that LTP-1 is one of the foam-positive proteins (*19*−*22*). However, LTP-1 was not identified in this study as a foam-positive protein. The beer foam stability of the samples in this study was well explained without LTP-1, because LTP-1 concentrations in the beer samples might be similar. In future work, we will examine not only BDAI-1, protein Z, and y-TRX2, but also LTP-1 in beer.

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