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Mutation Analysis of Barley Malt Protein Z4 and Protein Z7 on Beer Foam Stability

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ABSTRACT: Beer foam stability is an important characteristic. It has been suggested that isoforms of protein Z, that is, protein Z4 and protein Z7, contribute to beer foam stability. We investigated the relationship between beer foam stability and protein Z4 and protein Z7 using their deficient mutants. As a protein Z4-deficient mutant, cv. Pirkka was used. Protein Z7 deficiency was screened in 1564 barley accessions in the world collection of Okayama University, Japan. The barley samples from normal, protein Z4-deficient, protein Z7-deficient, and double-deficient were genotyped in F_2 populations and then pooled based on the DNA marker genotypes of protein Z4 and protein Z7. For a brewing trial, F_5 pooled subpopulations were used. After malting and brewing, the foam stability was determined, and the results showed that the levels of foam stability in the four samples were comparable. Two-dimensional gel electrophoresis was used to investigate the proteome in these beer samples. The results showed that low molecular weight proteins, including lipid transfer protein (LTP2), in the deficient mutants were higher than those in the normal sample. Our results suggest that the contribution of protein Z4 and protein Z7 to beer foam stability was not greater than that of other beer proteins.

KEYWORDS: *beer foam stability, protein Z4, protein Z7, two-dimensional gel electrophoresis*

INTRODUCTION

Beer foam quality is an important trait for brewers and consumers. Foam quality is characterized by stability, quantity, lacing, whiteness, creaminess, density, viscosity, and strength.[1] Among these, stability is the most important characteristic and can be affected by proteins,[2,3] hop iso-α-acids,[4] nonstarch polysaccharides,[5] polyphenols,[6] and metal ions.[7] Especially, protein factors such as protein Z,[7−10] lipid transfer protein-1 (LTP1),[11,12] and barley dimeric α-amylase inhibitor-1[9,13] have been extensively analyzed for their contribution to foam stability.

Protein Z is a barley serine protease inhibitor (serpin) in the family of isoforms that includes Z4, Z7, and Zx. Among these, protein Z4 is a major protein among the protein Z family in beer. Beer proteome analysis enabled the efficient detection of protein Z4, protein Z7,[14,15] and even a trace amount of protein Zx.[16] Significant variation has been shown to exist in the ratio of protein Z4 to protein Z7 in barley varieties.[17,18] It was reported that protein Z4 showed positive correlations[5,18] and protein Z7 showed negative correlation[18] with beer foam stability. Douma et al.[19] and Maeda et al.[10] suggested that protein Z4 possesses the highest surface viscosity and elasticity among beer proteins. However, when protein Z was removed from beer by immunoaffinity treatment, the foam stability was reduced by only 10%.[20] Gibson et al.[21] brewed beers from cv. Pirkka (deficient mutant for protein Z4) and cv. Chariot (normal type for protein Z4) and observed little difference in foam stability between these beers. In this pioneering research, they used two cultivars that were clearly different in protein Z4 content. However, the other factors affecting beer foam stability might not be the same for the two cultivars, and the results were not enough to exclude the contribution of protein Z4 on beer foam stability. Although a number of previous reports tried to estimate the contribution of the protein Z family in beer, it has not yet been clearly elucidated.

To estimate the roles of protein Z4 and protein Z7, this study used a more genetically complete design of mutant materials for these proteins. As a protein Z4-deficient mutant, cv. Pirkka was used. A protein Z7-deficient mutant was newly discovered after screening the world barley germplasm collection. These mutant lines were crossed, and lines were developed for the analysis of mutant genotypes. Beer was brewed from these lines to investigate the relationship between beer foam stability and protein Z4 and protein Z7.

MATERIALS AND METHODS

Plant Material. As a source of protein Z4-deficient mutant, barley cultivar Pirkka was used. A protein Z7-deficient mutant was newly screened from 1564 barley accessions collected from various areas of the world (Japan, Korean Peninsula, China, Nepal, Southwest Asia, Turkey, Europe, North Africa, Ethiopia, and North America). These were preserved at the Institute of Plant Science and Resources, Okayama University, Japan. A cross was made between mutant genotypes of protein Z4 and protein Z7. To select for the protein

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Z4-deficient genotype in the segregating population, a polymerase chian reaction (PCR)-based DNA marker (Z4-D DNA marker) with the primer set (5′-GTGACCAGCTCGTCGCCATACTCGGAG-3′, 5′-TGCTCTACCCATGAGTTCACTTGACC-3′) was used. A DNA fragment was amplified in a homozygous of normal protein Z4 allele or heterozygous but not in a homozygous of mutant allele. To monitor the PCR conditions, a β-amylase gene was also amplified using the primer set (5′-ATCATCCATAGCCAGCATCCACAATGGAGG-3′, 5′-CACTCACGATGAATTCTCCGATGCCTGGGA-3′) together with the Z4-D DNA marker. The genomic DNA was isolated from the barley leaf blade according to the method of Iimure et al.[18] PCR was performed as follows: 1 μL of DNA solution, 0.5 μL of 10 mM primer solution, 5 μL of Premix Taq Ex Taq Version 2.0 (TaKaRa Bio Inc., Japan), and 3 μL of sterilized water was mixed, and PCR was then carried out using a thermal cycler, PTC-200 (MJ Research, Inc., United States) with the following program: 31 cycles at 94 °C for 1 min, 60 °C for 2 min, and 72 °C for 3 min, and 1 cycle at 72 °C for 7 min. To select the protein Z7-deficient genotype, a cleaved amplified polymorphic sequence (CAPS) marker (Z7-D DNA marker) on the upstream region from the translation initiation codon of the protein Z7 gene as described in Iimure et al.[18] was used. Because the Z4-D DNA marker could not discriminate between protein Z4 heterozygotes or normal homozygotes, a protein Z4 normal homozygote could not be selected from F_2 individuals of ((((Haruna Nijo/Pirkka)/Haruna Nijo)/Haruna Nijo)/Haruna Nijo)/OUU120 using the Z4-D DNA marker. Therefore, two crosses were made to develop the normal and mutant genotypes for a brewing trial, namely: the control, normal in both protein Z4 and protein Z7; Z4-D, protein Z4-deficient; Z7-D, protein Z7-deficient; and Z4-D/Z7-D, protein Z4-deficient/protein Z7-deficient (Table 1). The genotypes of protein Z4 and protein Z7 of the F_2 generation were determined using Z4-D and Z7-D DNA markers. To adjust the genetic background, F_2 individuals were selected to pool homozygous mutant genotypes according to the DNA marker genotypes of protein Z4 and protein Z7 (Table 1). After generation advancement, F_5 pooled subpopulations were grown in Gunma Prefecture, Japan, in 2010 and were used for the brewing trial.

Table 1. Sample List for the Brewing Trial[a]

sample name	cross	protein Z4	protein Z7	number of lines in F_2 generation
control	HarunaNijo/OUU120	normal	normal	78
Z4-D	((((Haruna Nijo/Pirkka)/Haruna Nijo)/Haruna Nijo)/Haruna Nijo)/OUU120	deficient	normal	21
Z7-D	HarunaNijo/OUU120	normal	deficient	49
Z4-D/Z7-D	((((Haruna Nijo/Pirkka)/Haruna Nijo)/Haruna Nijo)/Haruna Nijo)/OUU120	deficient	deficient	18

[a]The total number of F_2 individuals of Haruna Nijo/OUU120 and ((((Haruna Nijo/Pirkka)/Haruna Nijo)/Haruna Nijo)/Haruna Nijo)/OUU120 F_2 was 267 and 294, respectively. The Z4-D DNA marker was used to select for the protein Z4-deficient genotype in BC_3F_2 individuals of ((((Haruna Nijo/Pirkka)/Haruna Nijo)/Haruna Nijo)/Haruna Nijo). This line was used to make a cross with the protein Z7-deficient mutant OUU120. The brewing trial was conducted using F_5 pooled subpopulations.

Western Blot Analysis. Barley or malt proteins were isolated from 50 mg of milled grain in 1 mL of phosphate-buffered saline (PBS) with 0.28% dithiothreitol (Wako, Japan). Total proteins were isolated overnight at 4 °C with mixing. After the protein concentration was determined by the Bradford method using bovine serum albumin as a standard,[22] 10 μg of proteins was separated by 12.5% (w/v) sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). Subsequently, the separated proteins were transferred to an Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore Corp., United States) by electroblotting. The primary antibodies used were purified rabbit antirecombinant protein Z4 polyclonal antibody or rabbit antiprotein Z7 polyclonal antibody, described in Iimure et al.[18] The antibody was diluted 1 in 1000 (v/v). Goat antirabbit IgG-AP (Santa Cruz Biotechnology, CA) diluted 1 in 1000 (v/v) was used as a secondary antibody. Detection was performed in a mixture of nitroblue tetrazolium (Roche Diagnostics, Germany) and 5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics).

Micromalting and Brewing. A 3 kg barley sample (>2.5 mm screen) was malted using a Phoenix micromalting system (Phoenix Biosystems, Australia). Steeping comprised two cycles of 5 h immersion and 7 h air rest at 15 °C, with up to 43.5% of ex-steep moisture. Germination was controlled for 144 h at 15 °C. Kilning was programmed for 13.5 h at 55 °C, 8 h at 65 °C, 3.5 h at 75 °C, and 4 h at 83.5 °C. Malt characteristics were analyzed according to the standard methods of the European Brewery Convention.[23] Mashing and fermentation were carried out according to Okada et al.[13] In brief, the wort was prepared using the following program: 50 °C for 30 min, 65 °C for 60 min, and 75 °C for 3 min using only malt. After the addition of hops, the wort was boiled. After 15.0×10^6 cells/mL of lager yeast (brewery collected) was added to the wort (12° Plato), fermentation was conducted using a 2.5 L European Brewery Convention (EBC) tube at 13 °C for 4 or 5 days. Maturation was controlled at 1−7 °C for approximately 30 days. Carbonation was conducted using a carbonation tank (laboratory equipped) at 2 °C for 3 days to achieve the appropriate CO_2 content. The foam stability was determined using a foam stability tester type NIBEM-T (Haffmans B. V., Venlo, Holland) according to the manufacturer's instructions.

Determination of Total Protein and the Content of Protein Z4 and Protein Z7. The beer total protein content was determined by the Bradford method using bovine serum albumin as a standard.[22] The contents of protein Z4 and protein Z7 were determined by enzyme-linked immunosorbent assay (ELISA) using each specific antibody according to the method of Iimure et al.[18] In brief, for quantification of protein Z4, a semiquantitative direct adsorption ELISA was performed using purified rabbit antirecombinant protein Z4 polyclonal antibody, which did not cross-react with protein Z7 (data not shown), as the primary antibody and goat antirabbit IgG-AP (Santa Cruz Biotechnology) as the secondary antibody. Each beer sample was analyzed in triplicate. The color development reaction was carried out by the addition of 150 μL of coloring solution [1 mg/mL disodium *p*-nitrophenyl phosphate hexahydrate (Wako) in 10% diethanolamine]. After color development, the reaction was stopped by the addition of 3 M sodium hydroxide, and the absorbance was then measured at 405 nm by a VARIOSKAN microtiter plate reader (Thermo Electron Corp., Japan). To determine the protein Z7 content in the barley and beer, quantitative ELISA was performed according to the method of Iimure et al.[18] The primary and secondary antibodies used were rabbit antiprotein Z7 polyclonal antibody and rabbit antiprotein Z7 polyclonal antibody conjugated with biotin specific to barley and beer protein Z7, respectively, which was provided by Dr. E. Evans, Tasmania University, Australia. To screen protein Z7-deficient mutants from the barley collection, two grains from each accession were crushed with a hammer, and barley protein was extracted using 1 mL of PBS with 0.28% dithiothreitol overnight at 4 °C with mixing. After the protein concentration was determined by the Bradford method using bovine serum albumin as a standard,[22] ELISA was performed with protein Z7. All of the barley grains from the 1564 accessions were screened without replication. Of these, 40 accessions were estimated to have a low protein Z7 content. The second screening was performed with three replications to validate lower protein Z7 accessions. To determine the protein Z7 content in F_2 seeds derived from a cross between Haruna Nijo and OUU120, barley grain was halved using a knife. The half with the embryo was seeded. The other half without the embryo was crushed using a hammer to determine the protein Z7 content. One milliliter of PBS with 0.28% dithiothreitol was added to the crushed grain, and protein was extracted overnight at 4 °C with mixing. After the protein concentration was determined using the Bradford method with bovine serum albumin as a standard,[22] three replications of ELISA were

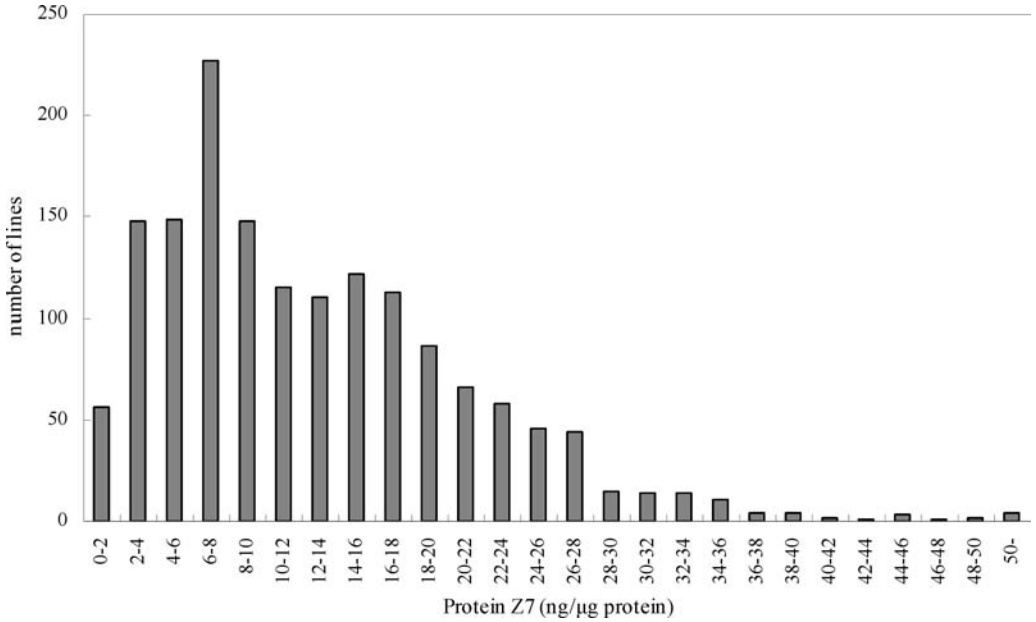

Figure 1. Distribution of barley grain protein Z7 content in the 1564 barley accessions collected from around the world.

performed with the protein Z7. The protein content was calculated by ng/μg protein to correct for the extraction efficiency of the barley grain.

Two-Dimensional Gel Electrophoresis (2DE) Analysis and Protein Identification. Two-dimensional gel electrophoresis (2DE) analysis was carried out according to Okada et al.[13] In brief, a beer sample was desalted using a PD-10 column (GE Healthcare Biosciences, Japan) and then lyophilized. The lyophilized protein was dissolved in 8 M urea (Wako, Japan) + 2% 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonic acid (CHAPS) (Dojindo Laboratories, Japan) solution containing 0.28% dithiothreitol (Wako). Subsequently, 100 μg of protein sample was applied to IPG dry strips, pI 3−10, 18 cm (GE Healthcare Biosciences), and isoelectric focusing was then carried out, followed by SDS-PAGE using a Multiphor II system (GE Healthcare Biosciences). Before SDS-PAGE, the IPG strip was treated 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). The gel was stained using a PlusOne Silver Staining Kit, Protein (GE Healthcare Biosciences). For mass spectrometry analysis, protein spots were stained using a Silver Stain MS Kit (Wako). Mass spectrometry analysis was performed as follows. Selected protein spots were first excised from the 2DE gel. To remove SDS, salt, and silver stain, gel pieces were washed with 50% acetonitrile. Subsequently, the protein spots were digested with trypsin (Promega, United States) solution in 50 mM ammonium-bicarbonate buffer, pH 8.7, for 8−10 h at 37 °C. Samples were analyzed using a 4700 Proteomics Analyzer with TOF (time-of-flight)/TOF ion optics (Applied Biosystems, United States). Before sample analysis, calibration was performed by calibration standard. Subsequently, both MS and MS/MS data were acquired using the instrument default calibration. Sequence tag searches were performed with the program MASCOT (http://www.matrixscience.com).

RESULTS

Screening for Protein Z7-Deficient Mutants and Development of Mutant Lines. To screen protein Z7-deficient mutants, the protein Z7 content in barley grain of 1564 accessions was determined by analysis of protein Z7 ELISA (Figure 1). The average, maximum, and minimum protein Z7 content was 12.78, 56.88, and 0.54 ng/μg protein, respectively. The accession showing the minimum protein Z7 content was OUU120. Even after the second ELISA screening of protein Z7 with three replications, OUU120 showed the lowest (0.48 ng/μg protein) protein Z7 content. Iimure et al.[18] reported that the protein Z7 content in 23 malting barleys cultivated in 2000, 2004, and 2008 ranged from 5.38 to 41.16 ng/μg protein. Therefore, a protein Z7 content of 0.48 ng/μg protein in OUU120 was considered the deficiency level for protein Z7. OUU120 is two-rowed barley that originated from France.

Two crosses were made to develop the control and mutant lines for a brewing trial (Table 1). For the Haruna Nijo × OUU120 cross, 267 F_2 individuals were analyzed for grain protein Z7 content and protein Z7 DNA marker genotypes. The average protein Z7 content in the Z7-D type was significantly lower than that in the Z7-Hi type at the 1% level (Figure 2). Consolidated samples of 78 individuals with Z7-Hi

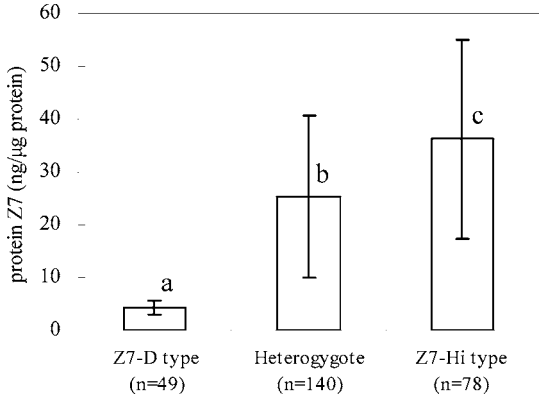

Figure 2. Average protein Z7 content in the protein Z7 homozygote (Z7-D and Z7-Hi) and heterozygote. Two hundred sixty-seven F_2 individuals derived from the cross between Haruna Nijo and OUU120 were used for the analysis. The bars indicate standard deviations. The different letters on the bars indicate significance at the 1% level.

and 49 individuals with Z7-D were defined as the control and Z7-D, respectively. Haruna Nijo/Pirkka BC_3F_2 and OUU120

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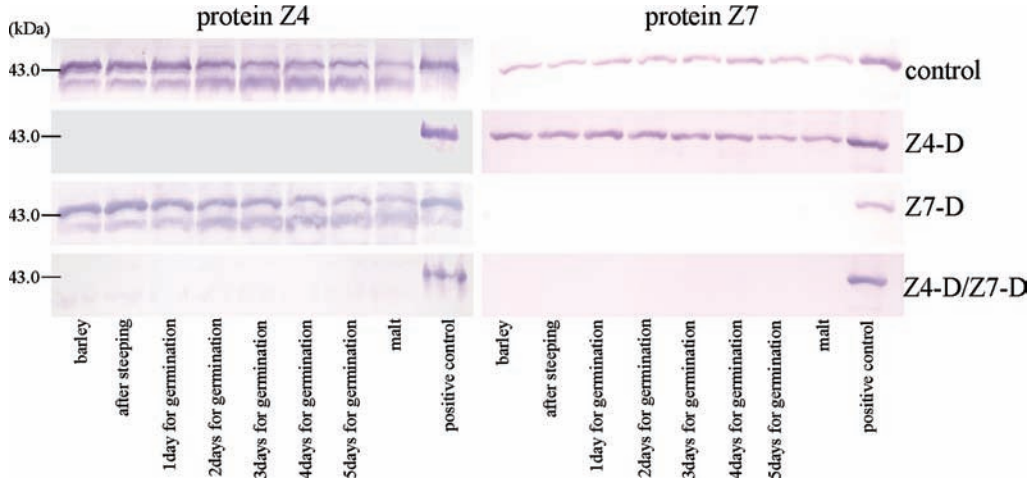

Figure 3. Western blot analyses for the F_5 subpopulation seed samples of the control and the mutants during malting using antiprotein Z4 and antiprotein Z7 antibodies. The positive control was barley grain from cv. Haruna Nijo.

were crossed to develop Z4-D and Z4-D/Z7-D (Table 1). The Z4-D and Z7-D DNA markers were applied to genotype 294 F_2 individuals. Because the Z7-D DNA marker was efficient for selecting protein Z7 deficiency as shown in Figure 2, ELISA performed with protein Z7 was not carried out for these F_2. The numbers of double homozygous F_2 individuals for Z4-D vs Z7-Hi and Z4-D vs Z7-D were 21 and 18, respectively (Table 1). Pooled progenies of these samples (F_5) were defined as the Z4-D and Z4-D/Z7-D subpopulation, respectively, and were used for the brewing trial.

Malt Characteristics for the Control and Mutant Lines. The control and mutant seed samples were micromalted. Western blot analyses were used to detect protein Z4 and protein Z7 during malting of the control and the mutants using antiprotein Z4 and protein Z7 antibodies (Figure 3). No band representing protein Z4 was observed by Western blot for Z4-D and Z4-D/Z7-D. Likewise, no band representing protein Z7 was observed for Z7-D and Z4-D/Z7-D. These results confirmed that Z4-D and Z4-D/Z7-D were deficient in protein Z4, and Z7-D and Z4-D/Z7-D were deficient in protein Z7. In the protein Z4 analysis, the intensity of the upper band (approximately 43 kDa) gradually decreased, and that of the lower band (approximately 39 kDa) increased both in the control and in Z7-D during malting. On the other hand, no obvious change was observed in the intensity of the protein Z7 band.

Malt characteristics in the control and mutant F_5 subpopulations were analyzed (Table 2). Total nitrogen in Z4-D, Z7-D, and Z4-D/Z7-D was higher (1.490−1.542%) than the control (1.429%). Soluble nitrogen in Z4-D and Z4-D/7-D (0.648−0.654) was slightly higher than in the control and Z7-D (0.593). The wort β-glucan content in Z4-D was slightly higher than that in the other samples. However, no clear difference was observed in viscosity. The other characteristics were at comparable levels among the samples.

Beer Characteristics of the Control and Mutant Lines. The beer samples were brewed from the control and mutants of the F_5 subpopulations. The NIBEM values, which define foam stability, were at comparable levels in all four samples (278−286 s) (Table 2). The contents of beer protein Z4 and protein Z7 were analyzed (Table 2). In Z4-D and Z4-D/Z7-D, the protein Z4 content was not at a detectable level. In Z7-D and Z4-D/Z7-D, the beer protein Z7 content was only 14−16% as compared to the control and Z4-D. Although the contents of protein Z4 and protein Z7 were drastically different between the samples, the beer total nitrogen in the samples was a comparable level (63−70 mg/100 mL), and no significant difference was observed in the total protein concentration

Table 2. Characteristics of the Malt and Beer in the Control and the Mutants from Grain Samples of the F_5 Subpopulations

characteristics	control	Z4-D	Z7-D	Z4-D/Z7-D
	malt			
malt moisture (%)	4.2	4.0	4.2	4.4
wort clarity	2	1	2	1
color (°EBC)	3.8	3.4	3.6	3.2
boiled wort color (°EBC)	4.8	4.9	5.1	5.4
extract (%, db)	81.9	82.1	81.0	81.1
soluble nitrogen (%)	0.593	0.654	0.593	0.648
total nitrogen (%)	1.429	1.521	1.490	1.542
Kolbach index	41.5	43.0	39.8	42.0
apparent attenuation limit (%)	82.6	82.1	82.5	83.0
diastatic power (WK)	205	230	248	262
viscosity (mP s)	1.56	1.57	1.54	1.52
friability (%)	89.2	83.2	86.0	83.0
wort β-glucan(mg/L)	50	98	54	42
	beer			
original gravity (%)	10.96	10.95	10.96	10.96
final extract (%)	3.27	3.22	3.33	3.34
apparent extract (%)	1.29	1.25	1.37	1.37
apparent attenuation limit (%)	88.1	88.5	87.0	87.2
alcohol (w/w %)	4.22	4.18	4.17	4.18
pH	4.76	4.71	4.75	4.81
color (°EBC)	6.0	6.4	6.3	6.4
bitter unit (mg/L)	26.6	25.9	25.3	24.5
polyphenol (mg/L)	162	127	162	166
total nitrogen (mg/100 mL)	64	70	63	70
free amino nitrogen (mg/L)	102	114	94	114
total protein concentration (μg/mL)	255	241	278	235
protein Z4 (μg/mL)	48.0	ND[a]	56.4	ND[a]
protein Z7 (μg/mL)	49.9	56.1	8.2	7.7
NIBEM value (s)	281	286	280	278

[a]ND indicates not detectable.

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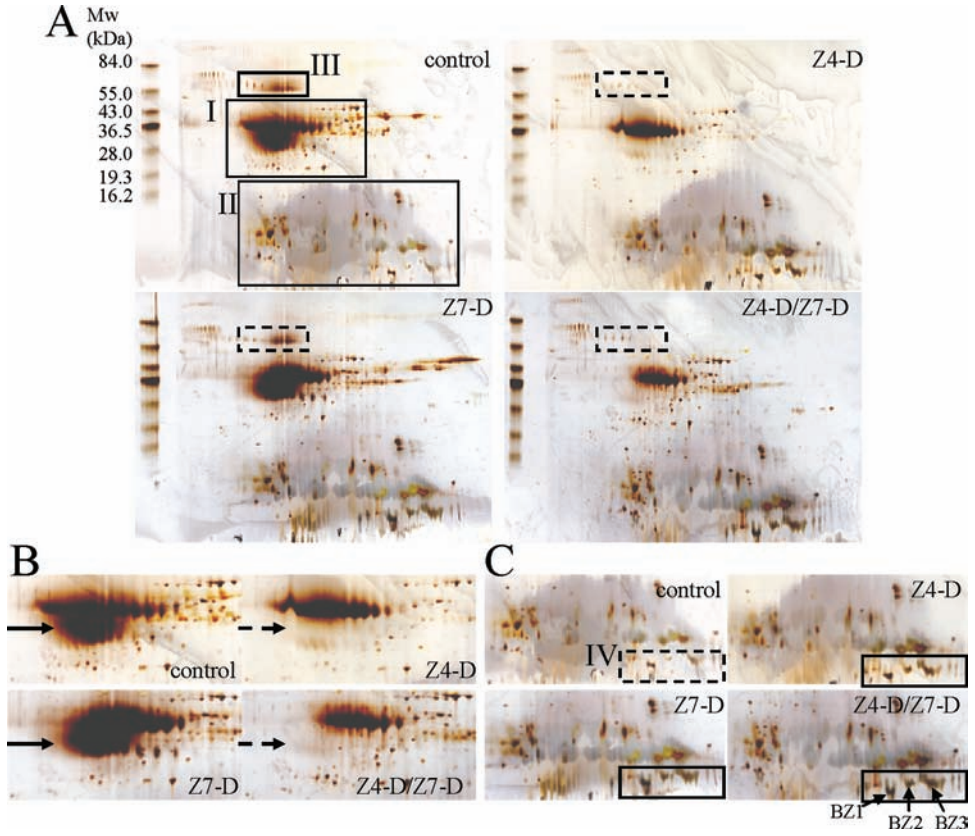

Figure 4. Two-dimensional gel electrophoresis (2DE) analysis of the beer samples brewed from the malt of the control and the mutants from the F_5 subpopulations. (A) Whole images of 2DE, (B) enlarged images of region I, and (C) enlarged images of region II.

(235−278 μg/mL) by one-way analysis of variance, as was determined by the Bradford method (Table 2). Other beer characteristics, including free amino nitrogen (FAN) and beer bitter units, showed no difference between the samples (Table 2).

2DE Analysis of the Beer Samples. 2DE analysis was performed to analyze the proteome of the beer samples (Figure 4). The huge spots in region I (approximately 40 kDa) contained protein Z4 and protein Z7 according to the beer proteome map.[15] The intensities of the 40 kDa spots were lower in Z4-D and Z4-D/Z7-D as compared to the control (Figure 4B). The 40 kDa spot was still stained in Z4-D/Z7-D. According to the results of protein Z4 and Z7 ELISA, protein Z4 was not detected, but a trace amount of protein Z7 was detected in Z4-D/Z7-D (Table 2). Therefore, the 40 kDa spot in Z4-D/Z7-D should be protein Z7. In the control and Z7-D (barley lines with protein Z4), the region located below the main spot of 40 kDa (arrows in Figure 4B) was stained. Conversely, this region was not stained in Z4-D and Z4-D/Z7-D (barley lines without protein Z4). These results suggested that the stained region was protein Z4. Similarly, several spots were observed in region III in the control and Z7-D but not in Z4-D and Z4-D/Z7-D (Figure 4A). Actually, the spots in region III were identified as protein Z4 according to the beer proteome map.[15] These results suggested that various modifications occurred during the malting and brewing processes in protein Z4 as compared to protein Z7. The intensities of the spots in region IV for Z4-D, Z7-D, and Z4-D/Z7-D were higher than the intensity in the control (Figure 4C). Proteins were identified in the spots of BZ-1, -2, and -3 by MALDI TOF/TOF MS and database search. BZ-3 was identified as nonspecific lipid transfer protein (LTP2) (accession no. gil 128377), but BZ-1 and BZ-2 were not identified. These results suggested that the content of the low molecular weight proteins, including LTP2 in the beers of Z4-D, Z7-D, and Z4-D/Z7-D, was higher than that of the control. No obvious difference was observed in the other protein spots.

DISCUSSION

The protein Z4-deficient mutant Pirrka was identified, and its foam characteristics for beer have already been reported by Gibson et al.[21] However, information about the protein Z7-deficient mutant has been missing until this report. The protein Z7-deficient mutant enabled us to develop a double mutation genotype in protein Z4 and protein Z7 and to investigate the role of protein Z4 and protein Z7 in beer foam stability. As shown in Figure 2, the average protein Z7 content in the Z7-D type grain was significantly lower than that in Z7-Hi type, suggesting that the protein Z7-deficient characteristic was strongly regulated by the protein Z7 gene or its adjacent region.

As shown in Table 2, malt total nitrogen in the mutants was higher than that in the control. The expression levels of the other proteins might increase by decreasing the expression levels of protein Z4 and/or protein Z7 in the mutants. The genetic locations of protein Z4 and protein Z7 were on chromosomes 4H and 5H, respectively.[24,25] However, QTLs of the malt total nitrogen were detected on chromosomes 2H and 7H in the cross between Mikamo Golden, which is a Japanese malting barley variety having Haruna Nijo as a parent and Harrington.[26] On the basis of the results of this study and previous studies, the reason for the increase in the total malt protein in the mutants might not be the genetic linkage of

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protein Z4 and protein Z7 with malt total nitrogen loci. However, a dynamic change in protein expression levels during seed maturation was caused by protein-deficient mutation. In addition, the total nitrogen and total protein contents in beer were comparable to levels in the control and mutants (Table 2). Conversely, on the basis of the results of ELISA and 2DE analysis shown in Table 2 and Figure 4, the protein Z4 and protein Z7 contents decreased remarkably in each mutant. Because protein Z4 and protein Z7 are major proteins in beer, the beer total nitrogen and total protein contents were expected to decrease in the mutants. Unexpectedly, the intensity of the spots in the low molecular weight region IV (Figure 4C) (below approximately 8 kDa, containing LTP2) was higher in the mutants than in the control. These results suggest that these low molecular weight proteins might compensate for decreased protein Z4 and protein Z7 in the mutants, causing the beer total nitrogen and total protein content to be comparable in the samples. In general, a deficiency in one type of protein may cause changes in the expression levels of other proteins. For example, it was reported that the B hordein content was reduced by 75% in a B hordein-deficient mutant, Risø 56, while C hordein increased up to 2-fold.[27]

Little difference was observed in the foam stability between the control and the mutants (Table 2). This may indicate that protein Z4 and protein Z7 are not the main factors controlling beer foam stability. Gibson et al.[21] also indicated that there was little difference in foam stability between the beers from the malt of Pirkka (protein Z4-deficient) and Chariot (normal) in a 50 L pilot brewing trial. Hollemans and Tonies[20] reported that even removal of 40 kDa proteins from beer by an immunoaffinity column resulted in only an approximate 10% decrease in beer foam stability. Our results support the findings of these reports. However, several reports show that the quantity of beer protein Z4 (positive) and protein Z7 (negative) was correlated with beer foam stability.[5,18] As mentioned above, a decrease in the expression level of protein Z4 and/or protein Z7 might affect the expression levels of other proteins, resulting in protein profiles that have been changed between the samples (Figure 4). Considering all of these results, there may be a pseudocorrelation between the interactions that govern protein Z4, protein Z7, and beer foam stability. Thus, it is presently unknown what factor(s) directly contribute to beer foam stability and correlate with protein Z4 and protein Z7.

Curioni et al. showed that glycated protein Z improved foam stability.[28] In this study, possible modifications of protein Z4 such as glycation and partial digestion were observed by Western blot and 2DE analyses (Figures 3 and 4). However, the foam stability of the control and protein Z4-deficient was comparable (Table 2). Therefore, it might be that modification of protein Z dose not significantly affect beer foam stability.

It has been suggested that protein Z4 may act as a sacrificial protease inhibitor because it was substantially cleaved by malt proteases in its reactive site loop for other proteins such as hordein.[8,29] Dahl et al.[29] postulated that protein Z4 as a protease inhibitor might act as an indirect foam protein. In this study, the molecular weight of protein Z4 decreased during the malting process according to the results of the Western blot analysis (Figure 3), suggesting that protein Z4 was modified in its reactive site loop during malting. However, the levels of foam stability were not different among Z4-D, Z4-D/Z7-D, and the control (Table 2). Therefore, the protease inhibitor in protein Z4 may not act as a foam-positive protein.

On the basis of the results in Table 2, the mutants were not suitable to detect the relationship between protein Z4, protein Z7, and foam stability. Since Iimure et al.[18] identified a significant relationship between these proteins and foam stability, it may occur only in normal cultivars but not in deficient mutants of protein Z4 and protein Z7. The grain protein content was higher in the mutants than in the control (Table 2). The studies of Evans et al.[17] and Giese and Hejgaard[30] showed that there was a trend for the higher levels of protein Z4 and protein Z7 to increase the grain protein content. In the current study, the relationship between grain protein content and protein Z4 and protein Z7 was unclear, which might be exceptional in case of protein deficiency as well as the relationship between protein Z (Z4 and Z7) and foam stability. This may result in an inefficiency of DNA markers for deficient mutants of protein Z4 and protein Z7, when used for screening barley lines with potentially higher foam stability as described in Iimure et al.[18] Since Iimure et al.[18] validated the effectiveness of the DNA marker system only through established cultivars, further brewing analysis using the progeny of a cross between nondeficient types would be necessary to confirm the use of DNA markers for selection in breeding.

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Notes

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