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Preliminary Study for Tracing the Geographical Origin of Wheat Flour in Breads Using Stable Isotope Analysis of Wheat Proteins

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Abstract

To trace the geographical origin of wheat flour used in breads, we examined the stable carbon, nitrogen, and oxygen isotope ratios ($\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$) of wheat flour proteins. The presence or absence of auxiliary materials, such as yeast, butter, and non-fat dry milk powder, had little influence on the protein composition of wheat glutenin fractions extracted from breads. We determined the $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values of the wheat glutenin fractions from bread samples made from wheat flour obtained from Canada, USA, and Japan. The $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values of the glutenin fractions were positively correlated with those of wheat flour ($R = 0.985$, $p < 0.001$ in $\delta^{13}C$; $R = 0.989$, $p < 0.001$ in $\delta^{15}N$; $R = 0.884$, $p < 0.001$ in $\delta^{18}O$), and the $\delta^{13}C$ and $\delta^{15}N$ values of the wheat glutenin fractions in bread made from Japanese wheat flour were lower than those of the other samples. This shows that stable isotope analysis of wheat glutenin fractions is a potentially useful tool for tracing the geographical origin of wheat flour in breads.

Keywords Wheat glutenin · Proteomics · Stable isotope analysis · Food authenticity · Processed foods

Introduction

The adoption of labeling policies by an increasing number of countries for products of protected geographical origin indications has accompanied the globalization of the food trade. In the EU, to protect and/or promote the production of food and agricultural products from specific regions, there are a number of food-labeling policies, such as protected designation of origin (PDO), protected geographical indication (PGI), and country-of-origin labeling (COOL) (European Commission, Agriculture and Rural Development Quality policy 2020). In Japan, Food Labeling Laws require companies to provide information, such as the name of the food, ingredients, preservation method, country of origin, and name of the manufacturer and its address (Consumer Affairs Agency, Government of Japan 2017). These polices regulate the use of geographical names in food labels and ensure the use of various levels of traceability to document food origin and production processes. The Japanese Food Labeling Law, which has been in effect since April 2015, requires country of origin labeling (COOL) for ingredients used in the production of processed foods manufactured in Japan for 22 food groups and four food items. However, since September 2017, Japan requires COOL labeling for the main ingredient, by weight, in all domestically manufactured processed foods. Unfortunately, these policies cannot completely eliminate food fraud, which occurs worldwide, as packages can be incorrectly labeled, either accidentally or intentionally. Thus, various analytical methods must be developed to validate the authenticity of foods. The development of analytical methods for tracing the geographical origin of the ingredient in processed foods is therefore required.

Stable isotope and trace element analyses have been used to identify the cultivation region of food materials (Kelly et al. 2005, and Gonzalvez et al. 2009). However, the mineral composition of foods is significantly altered after processing, probably by elimination and/or dilution of the elements during the cooking process (Ersoy et al. 2006; Lomolinon et al. 2016). On the other hand, the stable isotopic composition is relatively less affected by the cooking process. For example, no significant difference in the carbon and nitrogen isotope ratios of meats was observed after boiling, baking, and steaming (Zhou et al. 2015). Wadood et al. (2019) reported on the use of carbon, nitrogen, hydrogen, and oxygen isotope

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ratios of wheat and its products, such as noodles and cooked noodles, to trace their geographical origin. No significant changes were observed in carbon, nitrogen, and oxygen isotope ratios during wheat-processing. Bostic et al. (2015) examined the effect of cooking processes on the $\delta^{13}C$ and $\delta^{15}N$ values of yeast buns and sugar cookies. There were no significant differences in the $\delta^{13}C$ and $\delta^{15}N$ values during baking and fermentation. These results suggest that cooking processes in wheat processed food do not affect the $\delta^{13}C$ and $\delta^{15}N$ values. Therefore, stable isotope analysis has been applied to trace the geographical origin of processed foods such as liquor (especially, wine) (Christoph et al. 2015), juice (Bat et al. 2016), tea (Liu et al. 2020), coffee, (Rodrigues et al. 2009), dairy products (Camin et al. 2012; Ehtesham et al. 2013), edible oils (Bontempo et al. 2019), meat products (Zhou et al. 2015), and wheat products (Wadood et al. 2019). However, it is very difficult to trace the geographical origin of multi-ingredient foods using bulk stable isotope analysis. Bostic et al. (2015) showed that there were significant differences between the $\delta^{13}C$ values of beet sugar cookies and cane sugar cookies. Since the effect of auxiliary materials should be removed, an analytical method for tracing the geographical origin of processed food should be designed to overcome the effects of both the cooking process and the presence of auxiliary materials.

In the present paper, we have focused on a specific protein fraction of the target raw materials to assess the geographic origin of the target materials in processed foods. As an initial attempt, we targeted the protein fractions of wheat flour in breads. Wheat is an important and widely consumed food crop and is used to produce a variety of food products, such as bread, noodles, and pasta. Bread generally comprises wheat, water, salts, sugar, yeast, and dry milk powder. Commercial bread also contains additives, some of which are non-nutritional, to improve the flavor, texture, color, shelf life, and ease of manufacturing. To determine the geographical origin of wheat flour in breads, other auxiliary materials must be removed. Wheat seeds contain proteins that account for 10–18% of the total dry matter (Belderok et al. 2000). According to their solubility, wheat proteins are classified into four fractions: water-soluble albumin, salt-soluble globulins, alcohol-soluble gliadins, and acid- and alkali-soluble glutenins. The glutenins belong to the glutelin of wheat. Albumins and globulins constitute 15–20% of the total wheat proteins (Salcedo et al. 2011). On the other hand, the gliadins and glutenins, recognized as the major wheat storage proteins (Belderok et al. 2000; Abdel-Aal et al. 1996), constitute ~ 75–85% of the total wheat seed proteins, with glutenins accounting for ~ 50% (Huebner and Wall 1976). The gliadins and glutenins are also present in gluten, which influences the qualities of food products made from wheat, such as bread and noodles. In this study, we extracted the protein fractions from wheat flours in bread and its products and made a qualitative comparison with some wheat cultivars using one-dimensional (1D) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). In addition, to trace the geographical origin of wheat in bread samples, we performed carbon, nitrogen, and oxygen isotope analyses of wheat protein fractions extracted from breads.

Materials and Methods

Baking of Bread Samples for Evaluating the Effect of the Presence or Absence of Auxiliary Materials Using the Electrophoresis Patterns

We collect one Japanese wheat flour (sample no. 1) and one Japanese rice flour from the market and prepared the eight bread samples (sample no. 2 to no. 9) using a straight method in the laboratory (Table 1). The dough (composed of 280 g flour, 210 g water, 3 g baking powder, 20 g butter, 12 g non-fat dry milk powder, 16 g sugar, and 5 g salt) was placed in a bread pan and was baked using a basic bread regular course consisting of kneading, rising, and baking (3 h) using a bread maker (BB-HS10-CB, Zojirushi Corporation, Japan). For comparison with or without auxiliary materials using the electrophoresis patterns of proteins in bread, we prepared 5 types of wheat breads with the following compositions: wheat flour, water, salt, sugar, and baking powder (sample no. 2); wheat flour, water, salt, sugar, baking powder, and yeast (sample no. 3); wheat flour, water, salt, sugar, baking powder, and non-fat dry milk powder (sample no. 4); wheat flour, water, salt, sugar, baking powder, and butter (sample no. 5); wheat flour, water, salt, sugar, baking powder, yeast, non-fat dry milk powder, and butter (sample no. 6). To compare the repeatability of baking breads in the laboratory, we prepared breads from wheat flour, water, salt, sugar, baking powder, yeast, non-fat dry milk powder, and butter twice more, on separate days (sample no. 7 and no. 8). We also prepared rice bread from rice flour, water, salt, sugar, baking powder, yeast, and non-fat dry milk powder (sample no. 9).

Baking of Bread Samples for Tracing the Geographical Origin of Wheat Flour in Bread Using Stable Isotope Analysis

We collected reliable 20 wheat flour samples from the NIPPON FLOUR MILLS CO., LTD. Of the 20 samples, 5 (sample no. 10 to no. 14) were from Canada (CA), 8 (flour sample no. 15 to no. 22) were from the USA (US), and 7 (flour sample no. 23 to no. 29) were from Japan (JP) (Table 1). We baked 20 bread samples (sample no. 30 to no.49) from water, salt, sugar, yeast, non-fat dry milk powder, shortening, and wheat flour using the sponge and dough method (Table 1). The sponge was made by mixing 70% of flour (200 g) with

Table 1 Sample information about ingredients, country of origin of flour, baking process, and analytical method

Sample no.	Ingredients	Country of origin of flour	Baking process	Analytical method
1	Wheat flour	Japan	Not baking	Electrophoresis patterns of albumin/globulin and glutenin fraction
2	Bread; wheat flour + water + salt + sugar + baking powder	Japan	Straight dough method in laboratory (see section "Baking of Bread Samples for Evaluating the Effect of the Presence or Absence of Auxiliary Materials Using the Electrophoresis Patterns")	
3	Bread; wheat flour + water + salt + sugar + baking powder + yeast			
4	Bread; wheat flour + water + salt + sugar + baking powder + milk powder			
5	Bread; wheat flour + water + salt + sugar + baking powder + butter			
6	Bread; wheat flour + water + salt + sugar + baking powder + yeast + milk powder + butter			
7	Bread; wheat flour + water + salt + sugar + baking powder + yeast + milk powder + butter			
8	Bread; wheat flour + water + salt + sugar + baking powder + yeast + milk powder + butter			
9	Bread; rice flour + water + salt + sugar + baking powder + yeast + milk powder + butter			
10 to 14	Wheat flour	Canada	Not baking	$\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values of whole samples and glutenin fractions
15 to 22	Wheat flour	The USA		
23 to 29	Wheat flour	Japan		
30 to 34	Bread; wheat flour (no. 10 to no. 14) + water + salt + sugar + yeast + milk powder + shortening	Canada	Sponge-dough method in flour milling company (see section "Baking of Bread Samples for Tracing the Geographical Origin of Wheat Flour in Bread Using Stable Isotope Analysis")	
35 to 42	Bread; wheat flour (no. 15 to no. 22) + water + salt + sugar + yeast + milk powder + shortening	The USA		
43 to 49	Bread; wheat flour (no. 23 to no. 29) + water + salt + sugar + yeast + milk powder + shortening	Japan		

40% water, 2.2% yeast, and 0.1% yeast food with a spiral mixer (SK200, SK MIXER CO., LTD.) for 2 min at low speed and 2 min at medium speed. The sponge was fermented for 4 h at 27 °C and 75% relative humidity. The sponge was mixed with 30% flour, 5% sugar, 2% salt, 2% skim milk powder, and an appropriate amount of water to make dough, which was then mixed with a spiral mixer for 2 min at low speed, 3 min at medium speed, and 7 min at high speed. The dough was combined with 5% of shortening and mixed for 2 min at low speed, 3 min at medium speed, and 7 min at high speed. The dough was fermented for 20 min at 27 °C and 75% relative humidity. The bread dough was divided into a mass of 230 g per piece and risen on the bench for 20 min at room temperature. The bread dough was rolled out of the dough using a molder (Widefine, Oshikiri Machinery Ltd.) and was fermented at 38 °C and 85% relative humidity. The dough was placed in a pullman loaf pan (120 mm × 260 mm × 120 mm) and baked in a preheated oven (LR-10-401, FUJISAWA-MARUZEN Co., Ltd.) at 210 °C for 30 min. The breads were freeze-dried (FDU-2200, EYELA) for 24 h. The dried samples were ground to a fine powder using a food processor (IFM-800, Iwatani Corporation, Japan).

Extraction of Wheat Protein Fractions

Wheat proteins were fractionated according to a slight modification of the sequential extraction procedure developed by Akagawa et al. (Akagawa et al. 2007). Briefly, 250 mg of wheat flour or powdered freeze-dried breads was mixed with 2 mL of 50 mM Tris-HCl buffer (pH 8.8) for 2 h at 4 °C by rotation with a rotator (RT-50, Taitec Corp., Saitama, Japan). After centrifugation at 20,000×*g* for 10 min at 4 °C, the supernatant was carefully separated from the pellet and collected as the albumin/globulin fraction. The obtained pellet was washed once with 4 mL of the albumin/globulin fraction extraction buffer for 1 h at 4 °C with constant mixing by rotation. After centrifuging at 20,000×*g* for 30 min at 4 °C, the pellet was stirred in 4 mL of 75% ethanol at 4 °C overnight with constant mixing by rotation. The resultant suspension was centrifuged at 20,000×*g* for 10 min at 4 °C, and the supernatant was collected as the gliadin fraction. The pellet was suspended in 1.25 mL of 50 mM Tris-HCl (pH 8.8) containing 1% SDS and 0.5% dithiothreitol (DTT) for 2.5 h at 4 °C with constant mixing by rotation. After centrifugation at 20,000×*g* for 30 min at 4 °C, the supernatant was collected as the glutenin fraction. The protein concentrations in the albumin/globulin and glutenin fractions were determined with a 660 nm Protein Assay Reagent Kit (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA), without or with the Ionic Detergent Compatibility Reagent (IDCR, Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA), respectively. Some of the protein solutions were stored as aliquots at − 80 °C until use, and the remaining solutions were powdered by freeze-drying for carbon, nitrogen, and oxygen isotope analyses to trace their geographical origin.

A 1.5-μg sample of each albumin/globulin protein fraction or a 10-μg sample of each glutenin protein fraction extracted from wheat flours or bread was mixed with an equal volume of 2x Laemmli sample buffer (#161-0737, Bio-Rad Laboratories, Inc., Hercules, CA, USA), denatured at 95 °C for 5 min, and separated by SDS-PAGE on a 10–17.5% gradient gel (DRC Co., Ltd., Tokyo, Japan) under reducing conditions (Laemmli 1970). Coomassie brilliant blue (CBB) staining (Quick CBB, #299-50101, Wako Co., Ltd., Tokyo, Japan) was used for detecting proteins after electrophoretic separation on the polyacrylamide gels. The stained gel was scanned with a SAYACA imager (DRC Co., Ltd., Tokyo, Japan); the scanned images were processed with Adobe Photoshop CS6 and stored as TIFF image files (Adobe Systems, San Jose, CA, USA).

To remove SDS from the protein fractions, the extracted glutenin fractions were precipitated by adding a 10-fold volume of pre-chilled acetone. After vortex mixing, the mixtures were incubated overnight at − 30 °C. After centrifugation at 10,000×*g* for 15 min at 4 °C, the obtained pellets were air-dried and used for carbon, nitrogen, and oxygen isotope analyses.

Stable Isotope Analysis of Wheat Flour and Wheat Protein Fractions Extracted from Bread Samples

For carbon and nitrogen isotope analysis, freeze-dried fine wheat flour and their breads were weighed around 5.0 mg into a tin capsule (5.0 × 9.0 mm). The air-dried protein fractions were weighed around 0.3 mg into a tin capsule (5.0 × 9.0 mm). Then, each sample was analyzed by elemental analyzer/isotope ratio mass spectrometry (EA/IRMS) using an IsoPrime 100 (Isoprime Ltd) interfaced with an Elementar vario Pyro cube (Elementar Analysensysteme GmbH) to determine the carbon and nitrogen isotope ratios. The measured $\delta^{13}C$ and $\delta^{15}N$ values were normalized by using isotope-known 5 amino acid standards (histidine: $\delta^{15}N = -7.6‰$ and $\delta^{13}C = -10.1‰$, L-alanine: $\delta^{15}N = -1.06‰$ and $\delta^{13}C = -19.94‰$, glycine: $\delta^{15}N = +1.2‰$ and $\delta^{13}C = -30.5‰$, L-alanine: $\delta^{15}N = +10.1‰$ and $\delta^{13}C = -19.6‰$, and L-alanine: $\delta^{15}N = +20.0‰$ and $\delta^{13}C = -19.6‰$) purchased from Shoko Science Co., Ltd., Japan. These standards were calibrated by Dual-inlet method using international standards IAEA-N-1, IAEA-N-2, and NBS 19-limestone (NIST RM #8544). The 5 working standards were determined every 12 samples to confirm the reproducibility of the measurements. The standard deviation of three replicates was smaller than ± 0.2‰ in $\delta^{13}C$ and ± 0.3‰ in $\delta^{15}N$, respectively.

For oxygen isotope analysis, around 1.0 mg of freeze-dried fine wheat flour and their breads, and air-dried protein

Table 2 Protein contents of albumin/globulin and glutenin fractions extracted from wheat flour or breads

Sample no.		1	2	3	4	5	6	7	8	9
Protein fractions	Albumin/globulin (mg)	2.09 ± 0.08	0.62 ± 0.03	0.38 ± 0.03	1.75 ± 0.10	0.66 ± 0.04	1.29 ± 0.04	1.11 ± 0.03	1.13 ± 0.06	0.76 ± 0.05
	Glutenin (mg)	6.83 ± 1.11	7.03 ± 1.88	6.00 ± 1.57	6.80 ± 1.67	6.28 ± 1.58	7.43 ± 2.14	6.32 ± 1.42	6.95 ± 1.73	4.38 ± 1.23

1: wheat flour without baking; 2: bread containing wheat flour, water, salt, sugar, and baking powder; 3: bread containing wheat flour, water, salt, sugar, baking powder, and yeast; 4: bread containing wheat flour, water, salt, sugar, baking powder, and non-fat dry milk powder; 5: bread containing wheat flour, water, salt, sugar, baking powder, and butter; 6, 7, and 8: bread containing wheat flour, water, salt, sugar, baking powder, yeast, non-fat dry milk powder, and butter, baked on different days; 9: bread containing rice flour, water, salt, sugar, baking powder, yeast, non-fat dry milk powder, and butter. The values are expressed as mean ± standard deviation for three independent extractions

fractions were weighed into a silver capsule (3.3 × 5.0 mm). Then, oxygen isotope analysis was carried out using thermal conversion EA/IRMS (TCEA/IRMS) by an IsoPrime 100 (Isoprime Ltd) interfaced with an Elementar vario Pyro cube (Elementar Analysensysteme GmbH). The measured $\delta^{18}O$ values of rice samples were normalized by using isotope-known benzoic acid standards (+ 71.4‰, + 23.2‰) purchased from Indiana University, USA. Four working standards: dibenzo-24-crown-8 (− 15.7‰), dibenzo-18-crown-6 (+ 1.7‰), β-D-galactose pentaacetate (+ 12.7‰), and D-(+)-sucrose octaacetate (+ 26.8‰) were determined every 12 samples to confirm the reproducibility of the measurements. The standard deviation of three replicates was smaller than ± 0.5‰ in $\delta^{18}O$ values.

The isotopic composition was reported in the δ notation:

$$\delta = \left(R_{\text{sample}}/R_{\text{standard}} - 1\right) \quad (1)$$

where R sample is the isotope ratio (i.e., $^{13}C/^{12}C$, $^{15}N/^{14}N$, and $^{18}O/^{16}O$) of the sample, and R standard is the isotope ratio of the international standards: for carbon: Vienna Peedee Belemnite (VPDB); for nitrogen: air; and for oxygen: Vienna Standard Mean Ocean Water (VSMOW). Isotope values were given per mil (‰).

Statistical Analyses

A paired t test was performed to test the significance of the differences between the $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values of the whole samples and protein fractions in wheat flour and their breads before and after the cooking processes. Correlations between the $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values of wheat flour and protein fractions were tested independently using the Pearson product–moment correlation coefficient. One-way analysis of variance (ANOVA) was performed to test the significance of the differences between the $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values of the protein fractions obtained from three countries (CA, US, and JP). For all tests, $p < 0.05$ indicated the statistical significance. All analyses were conducted using IBM SPSS Statistics 23.0 (IBM).

Results and Discussion

Effects of Auxiliary Materials on the Electrophoresis Patterns of Proteins in Bread

Wheat flours and breads prepared with or without auxiliary materials were used in the experiments to analyze wheat proteins. Approximately 0.38–2.09 mg of albumin/globulin proteins and 4.38–7.43 mg of glutenin protein were obtained from 250 mg of wheat flour and breads (Table 2). The protein contents of the glutenin fractions obtained in this study were

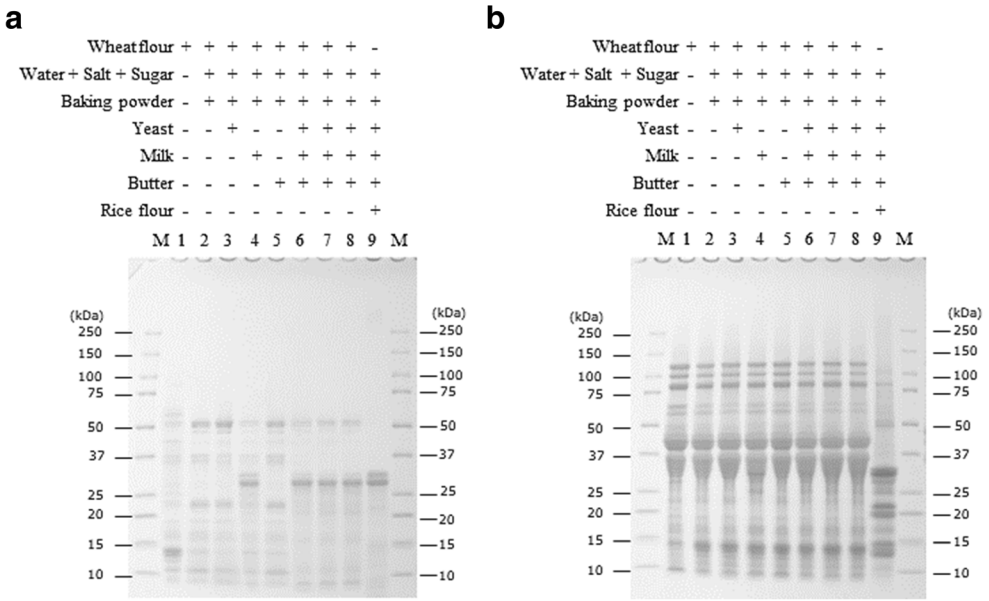

Fig. 1 SDS-PAGE analysis of wheat protein fractions from wheat flour and breads. **a** Albumin/globulin fractions. **b** Glutenin fraction; M, protein weight marker; sample no. 1: wheat flour without baking; sample no. 2: bread containing wheat flour, water, salt, sugar, and baking powder; sample no. 3: bread containing wheat flour, water, salt, sugar, baking powder, and yeast; sample no. 4: bread containing wheat flour, water, salt, sugar, baking powder, and non-fat dry milk powder; sample no. 5: bread containing wheat flour, water, salt, sugar, baking powder, and butter; sample nos. 6, 7, and 8: bread containing wheat flour, water, salt, sugar, baking powder, yeast, non-fat dry milk powder, and butter, baked on different days; sample no. 9: bread containing rice flour, water, salt, sugar, baking powder, yeast, non-fat dry milk powder, and butter

consistent with those found in previous reports (Belderok et al. 2000; Abdel-Aal et al. 1996), whereas the protein contents of the albumin/globulin fractions were lower than previously reported (Salcedo et al. 2011). The lower protein content in the albumin/globulin fractions may be related to the length of the extraction time for the albumin/globulin fractions.

In order to compare the protein components in the protein fractions extracted from wheat flours and breads, the protein fractions were subjected to 1D SDS-PAGE, and the gels were detected by CBB staining. The results showed that the electrophoretic pattern of albumin/globulin proteins extracted from wheat flour (Fig. 1a, lane 1) was different from that of the proteins extracted from wheat breads (Fig. 1a, lanes 2–8). On the other hand, there was little difference in the electrophoretic patterns of glutenin proteins extracted from wheat flours and breads (Fig. 1b, lanes 1–8). These results suggest that the glutenin fraction is minimally affected by the processing carried out during bread making.

The results of 1D SDS-PAGE also revealed that the albumin/globulin protein fractions had different electrophoretic patterns depending on the type of auxiliary material added (Fig. 1a, lanes 2–9). In contrast, the glutenin protein fractions showed similar electrophoretic patterns, regardless of the presence of auxiliary materials in the bread (Fig. 1b). These results suggest that the glutenin fractions were not affected by the addition of auxiliary materials as they were mainly composed

Table 3 The $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values of whole samples and glutenin fractions in wheat flour (sample no. 10 to no. 29) and their breads (sample no. 30 to no. 49)

Sample no.	Flour or bread	Country of origin of wheat flour	Whole samples							Glutenin fractions					
				$\delta^{13}C$ (‰)		$\delta^{15}N$ (‰)		$\delta^{18}O$ (‰)		$\delta^{13}C$ (‰)		$\delta^{15}N$ (‰)		$\delta^{18}O$ (‰)	
			n	Average	1σ	Average	1σ	Average	1σ	Average	1σ	Average	1σ	Average	1σ
10 to 14	Wheat flour	Canada	5	−24.2	0.5	3.2	0.3	24.9	0.7	−25.2	0.4	4.0	0.3	17.5	0.7
15 to 22		The USA	8	−24.7	0.6	3.0	0.5	26.8	0.7	−25.3	0.5	4.0	0.5	19.2	0.8
23 to 29		Japan	7	−27.5	0.3	0.4	0.9	23.3	0.3	−27.3	0.4	1.2	1.0	16.9	0.4
30 to 34	Bread	Canada	5	−23.2	0.8	3.3	0.1	25.2	0.9	−24.9	0.4	3.8	0.2	17.6	0.9
35 to 42		The USA	8	−24.4	0.5	2.8	0.4	26.9	0.7	−25.0	0.5	3.7	0.5	18.9	0.6
43 to 49		Japan	7	−26.4	0.5	0.6	1.0	23.5	0.4	−27.2	0.4	1.2	0.9	17.2	0.8

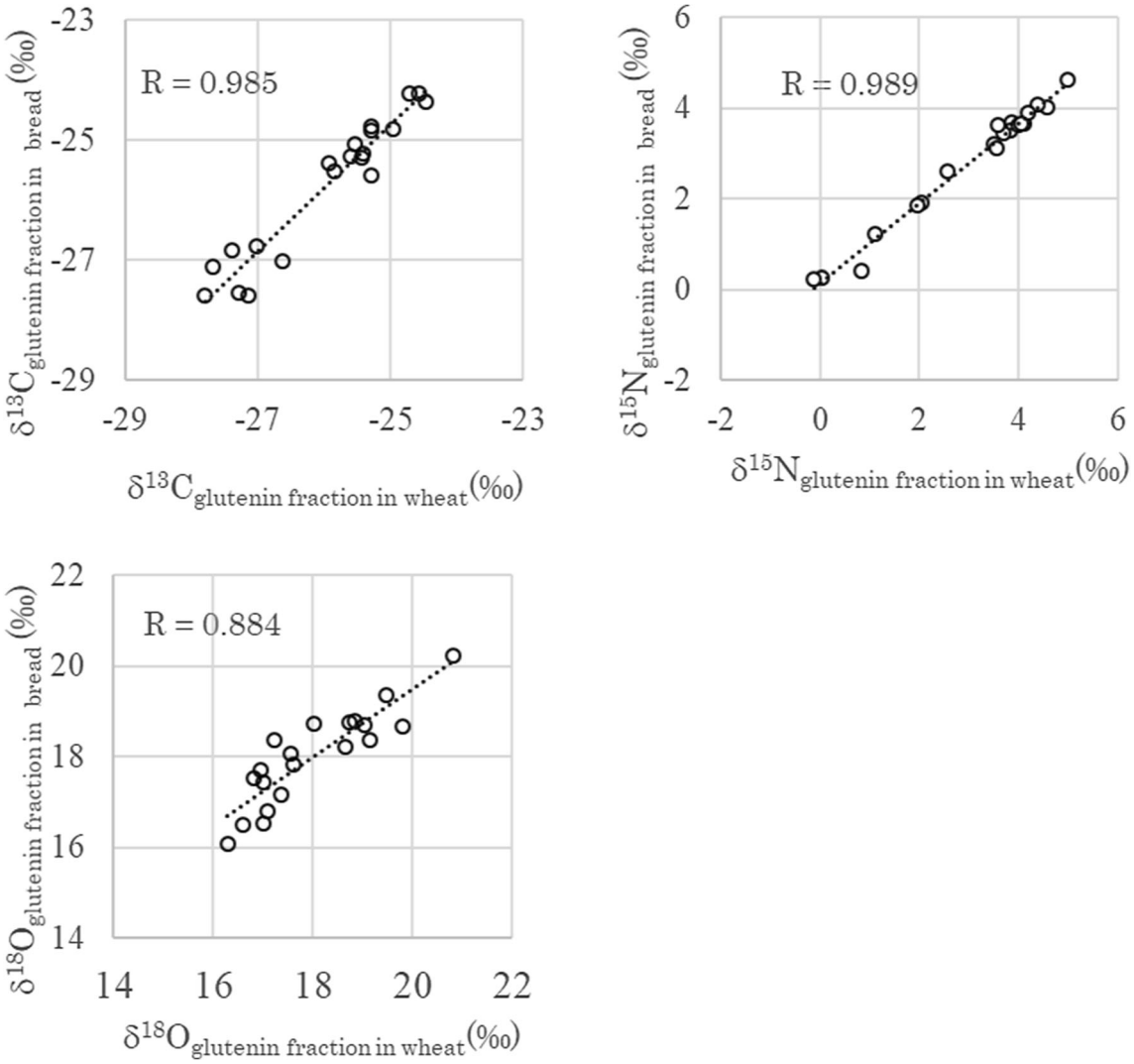

Fig. 2 Relationship between the $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values of wheat flour (sample no. 10 to no. 29) and glutenin fractions obtained from bread samples (sample no. 30 to no. 49)

of wheat proteins, and not proteins extracted from the auxiliary materials.

$\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ Values of Whole Samples and Glutenin Fractions in Wheat Flour and Their Breads

The mean and 1 σ standard deviation of the $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values of the whole wheat flour, whole bread, and glutenin fractions in wheat flour and breads are shown in Table 3. There was no significant difference in the $\delta^{15}N$ and $\delta^{18}O$ values between whole wheat flour and their whole bread ($p = 0.50$ to 0.67 in the $\delta^{15}N$ values, and $p = 0.36$ to 0.97 in the $\delta^{18}O$ values). The $\delta^{13}C$ values between whole wheat flour and their whole bread were significantly different in Canadian and Japanese samples ($p < 0.05$). In general, commercial breads contain wheat flour, water, salt, sugar, yeast, and non-fat dry milk powder. The $\delta^{13}C$ values of sugar, yeast, and non-fat dry milk powder have natural variations. Bostic et al. (2015) showed that the carbon isotope ratios of cane sugar cookies were significantly higher than those of beet sugar cookies. These results suggest that the $\delta^{13}C$ values of auxiliary materials, such as sugar in processed wheat-based food, affect the $\delta^{13}C$ values of whole breads.

On the other hand, the $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values of the glutenin fractions in wheat flour and their breads were not significantly different ($p = 0.15$. to 0.98) (Fig. 2). The $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values of the glutenin fractions in breads were positively correlated with those in wheat flour ($R = 0.985$, $p < 0.001$ in $\delta^{13}C$, $R = 0.989$, $p < 0.001$ in $\delta^{15}N$, $R = 0.884$, $p < 0.001$ in $\delta^{18}O$). These results suggest that the glutenin fractions were mainly composed of wheat proteins, rather than proteins extracted from auxiliary materials, and did not affect the addition of auxiliary materials. Thus, the $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values of the glutenin fractions could be a potential tool for tracing the geographical origin of wheat flour in breads.

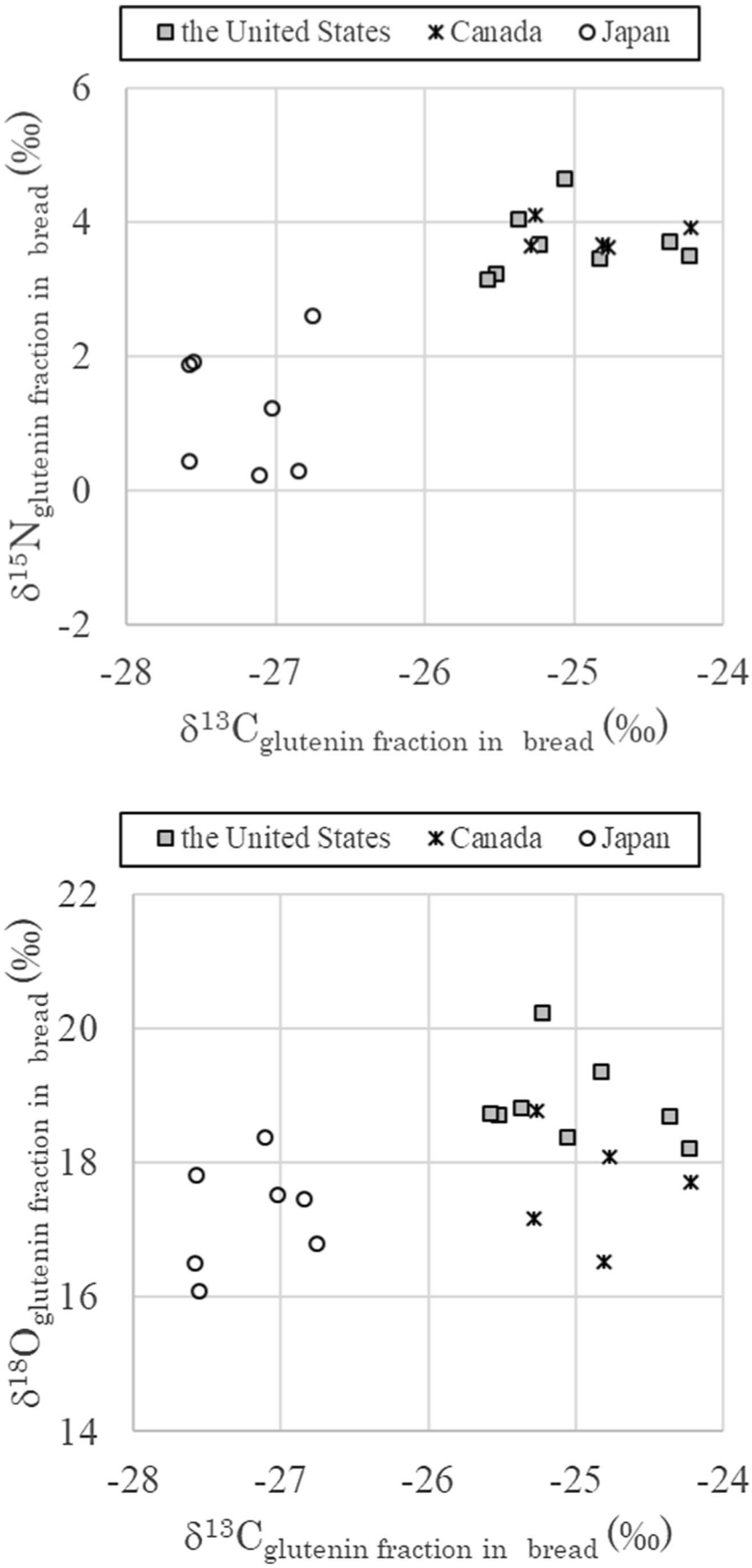

Fig. 3 Distributions of the $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values of glutenin fractions from bread samples (sample no. 30 to no. 49) prepared using AU, CA, US, and JP wheat flours

$\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ Values of Glutenin Fractions in Breads Made from Canadian, US, and Japanese Wheat Flour

The distributions of the $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values of the glutenin fractions in wheat flour and breads made from Canadian, US, and Japanese wheat flours are shown in Fig. 3. The $\delta^{13}C$ values of the glutenin fractions in Japanese wheat breads were lower than those of Canadian and the US wheat breads ($p < 0.001$). In a previous study, Brescia et al. (2002) reported on the $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values of durum wheat semolina samples from Italy, Canada, Turkey, and Australia. Canadian (-24.01 ± 0.22‰) wheat samples had higher $\delta^{13}C$ values than Italian wheat samples (-26.00 ± 0.53‰). The $\delta^{13}C$ values in plants depend on fractionation during the diffusion of CO_2 into the leaf and subsequent photosynthetic metabolism and water-use efficiency, which suggests that carbon isotope discrimination is associated with well-watered conditions (Farquhar et al. 1989; Saurer et al. 1995). The main production states of wheat flour in Canada are the States of Saskatchewan, Alberta, and Manitoba, those in the US are North Dakota, Minnesota, Kansas, Oklahoma, and Texas while in Japan the primary wheat-producing region is Hokkaido Prefecture. Based on Meteorological statistical information from the Japan Meteorological Agency (Japan Meteorological Agency, Meteorological statistical information 2014), the average level of precipitation in the States of North Dakota, Minnesota, Kansas, Oklahoma, and Texas (550 to 900 mm/year) and in Saskatchewan, Alberta, and Manitoba (350 to 550 mm/year) is relatively lower than that of Hokkaido (1200 to 1450 mm/year). The large amount of precipitation in Japan would account for the decrease in the $\delta^{13}C$ values of wheat flour.

The $\delta^{15}N$ values of the glutenin fractions in Japanese wheat breads were lower than those of Canadian and the US wheat breads ($p < 0.001$). The nitrogen isotopic composition of plant materials primarily depends on soil nutrition (Kohl et al. 1973; Meints et al. 1975). Wheat is very sensitive to insufficient nitrogen and is very responsive to nitrogen fertilization. When soils in the fields originally contain very low levels of nitrogen, the yield and protein content of wheat flour will be low. To increase their yield and protein content, nitrogen fertilization is required. Generally, organic fertilizers, such as cow, chicken, and pig manure, have higher $\delta^{15}N$ values than chemical fertilizers. In a previous study on the variation of $\delta^{15}N$ values in plants supplied with chemical or organic fertilizers (cow, chicken, and pig manure), the former decreased and the latter increased the $\delta^{15}N$ values of plants (Suzuki and Nakashita 2013). Thus, the nitrogen isotope composition sheds light on the agricultural practices of the area in question.

The glutenin fractions in the US wheat breads had higher $\delta^{18}O$ values than those in Canadian and Japanese wheat breads ($p < 0.001$). The oxygen isotopic composition of plant materials primarily reflects the precipitation in the area, which depends on latitude and altitude. Based on the weighted annual $\delta^{18}O$ values in the Global Network of Isotopes in Precipitation (GNIP) Database from the International Atomic Energy Agency (IAEA) (Global Network of Isotopes in Precipitation : The GNIP Database 2015) and Katsuyama et al. (2015), the $\delta^{18}O$ value of precipitation is approximately -6 to -2‰, which is higher than that of Hokkaido (-13.7 to -9‰) and Canada (-17 to -11‰). Brescia et al. reported the $\delta^{18}O$ values of durum wheat semolina samples from Italy, Canada, Turkey, and Australia. The Canadian wheat samples exhibited relatively lower $\delta^{18}O$ values. Suzuki (2019) reported the $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values of Japanese wheat flours. The $\delta^{18}O$ values of Japanese wheat flours ranged from $+20.2$ to $+31.6$‰. Wheat flour from Hokkaido had the lowest $\delta^{18}O$ value ($+20.2$ to $+24.7$ ‰). Hokkaido is the most northern prefecture in Japan and has the lowest ambient water $\delta^{18}O$ value (-13.7 to -9‰). The agricultural output of Hokkaido accounts for over 65% of wheat flour in Japan. In particular, wheat flour for baking bread is produced in Hokkaido. In this study, we collected Japanese wheat flour from Hokkaido. Thus, $\delta^{18}O$ values could be particularly useful as a diagnostic tool for tracing the geographical origin of wheat.

Conclusions

The glutenin protein fractions showed similar electrophoretic patterns, regardless of the presence of auxiliary materials in the bread. This result showed that the glutenin fractions in breads were mainly composed of wheat proteins. The $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values of the glutenin fractions in bread were positively correlated with those in wheat flour. The $\delta^{13}C$ and $\delta^{15}N$ values of the glutenin fractions in Japanese wheat breads were significantly lower than those of Canadian and the US wheat breads. These results suggest that carbon, nitrogen, and oxygen isotope analysis of glutenin fractions is potentially useful for tracing the geographical origin of wheat in breads. However, in this study, there were not enough samples to establish an authentication database. In the future, a statistical investigation using a large number of samples should be carried out. In addition, annual variations in the $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values of wheat flour samples should be evaluated.

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Compliance with Ethical Standards

Conflict of Interest Yaeko Suzuki declares that there is no conflict of interest. Shotaro Murata declares that there is no conflict of interest. Tomoki Tanaka declares that there is no conflict of interest. Eiji Hirao declares that there is no conflict of interest. Koji Noguchi declares that

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Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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