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Proteomic Analysis of Wheat Flour Allergens

MITSUGU AKAGAWA,*,† TRI HANDOYO,‡ TAKESHI ISHII,§ SHIGENORI KUMAZAWA,§ NAOFUMI MORITA,† AND KYOZO SUYAMA||

Department of Biological Chemistry, Division of Applied Life Science, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-1 Gakuen-chyo, Naka-ku, Sakai 599-8531, Japan, Department of Agronomy, Faculty of Agriculture, University of Jember, Jl. Kalimantan III/23 Jember 68121, East Java, Indonesia, School of Food and Nutritional Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka 422-8526, Japan, and Department of Sports Dietetics, Sendai University, Shibata-machi, Shibata-gun, Miyagi 981-1693, Japan

Wheat can cause severe IgE-mediated systematic reactions, but knowledge on relevant wheat allergens at the molecular level is scanty. The aim of the present study was to achieve a more detailed and comprehensive characterization of the wheat allergens involved in food allergy to wheat using proteomic strategies, referred to as "allergenomics". Whole flour proteins were separated by two-dimensional gel electrophoresis with isoelectric focusing and lithium dodecyl sulfate−polyacrylamide gel electrophoresis. Then, IgE-binding proteins were detected by immunoblotting with sera of patients with a food allergy to wheat. After tryptic digestion, the peptides of IgE-binding proteins were analyzed by matrix-assisted laser desorption ionization tandem time-of-flight mass spectrometry. In this study, we identified four previously reported wheat allergens or their sequentially homologous proteins [serpin, α-amylase inhibitor, γ-gliadin, and low molecular weight (LMW) glutenin] by a database search. As a result of the high resolution of two-dimensional gel electrophoresis, nine subunits of LMW glutenins were identified as the most predominant IgE-binding antigens. The two-dimensional allergen map can be beneficial in many ways. It could be used, for example, for precise diagnosis of wheat-allergic patients and assessment of wheat allergens in food. Additionally, we compared allergenomics to conventional biochemical methods and evaluated the usefulness of a proteomic strategy for identifying putative allergens to wheat allergy.

KEYWORDS: Allergen; allergenomics; food allergy; gliadin; glutenin; IgE; mass spectrometry; proteomics; wheat; wheat allergy

INTRODUCTION

Wheat (*Triticum aestivum* L.) is the most consumed crop in the world, but it can be responsible for IgE-mediated food allergy. Currently, the number of patients suffering from food allergy is increasing, and wheat allergy is particularly considered to be a serious problem globally (*1*). Symptoms of wheat allergy are typically generalized urticaria, atopic eczema/dermatitis syndrome (AEDS), and more severe reactions such as wheat-dependent exercise-induced anaphylaxis (WDEIA) (*2*).

On the basis of different solubility, wheat proteins can be classified into three fractions: (i) water/salt-soluble albumins and globulins, (ii) ethanol-soluble gliadins, and (iii) glutenins soluble only after treatment with detergents and reducing reagents (*3*). Wheat proteins in the water/salt-soluble fraction, such as α-amylase inhibitor, peroxidase, glycerinaldehyde-3-phosphate dehydrogenase, serpin, and triosephosphate isomerase, have been considered to be major allergens in patients with bakers' asthma, a typical occupational allergic disease (*4*−*7*). Sandiford et al. reported that α- and ω-gliadins are the allergens associated with bakers' asthma (*8*). Furthermore, α-, β-, γ-, and ω-gliadins, in addition to low molecular weight (LMW) glutenin, were characterized as the major allergens for patients with wheat allergies by some researchers (*3*, *9*−*15*). These observations indicate that a variety of wheat proteins are allergenic. Therefore, more specific information on their nature and distribution is needed for the prevention and diagnosis of wheat allergy.

Recently, proteomic strategies have been applied for the identification of allergenic proteins, and the new strategy is now referred to as "allergenomics" (*16*). In allergenomics, total proteins in allergen sources are solubilized with a strong nonionic detergent and urea and effectively resolved with two-dimensional gel electrophoresis. Subsequently, IgE-reactive proteins are detected by IgE immunoblotting using allergic patients' sera. Then, candidate allergens can be easily identified

* To whom correspondence should be addressed. Tel: +81-72-254-9460. Fax: +81-72-254-9921. E-mail: akagawa@biochem.osakafu-u.ac.jp.
† Osaka Prefecture University.
‡ University of Jember.
§ University of Shizuoka.
|| Sendai University.

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Table 1. Characteristic of Wheat-Allergic Subjects

patient	age (year)	sex	RIST (IU/mL)	RAST (IU/mL)	major symptom
1	4	M	92	6.8	urticaria
2	6	M	220	2.5	eczema, ear damage
3	19	F	12000	5.4	urticaria
4	6	M	502	13.5	respiratory diseases
5	6	F	390	ND[a]	atopic dermatitis
6	10	M	3100	7.0	urticaria
7	5	F	5300	820.0	urticaria

[a] ND, not detected.

by their site-specific degradation and the subsequent mass spectrometric analysis of the fragmented peptides and database search. Recently, as huge sequence data of proteins and genes are accumulated in databases, it has become easy to analyze candidates. To date, however, comprehensive and integrated analysis of total proteins in wheat flour by allergenomics has not been fully demonstrated. The previous researches in wheat allergy were limited to the analysis of each fraction of wheat proteins obtained by different solubilities (*3*−*8*, *11*). Furthermore, there are few studies that characterize IgE-binding proteins using allergic patients' sera by mass spectrometric analysis.

The aim of the present study was to characterize more comprehensively wheat allergens involved in food allergy to wheat by allergenomics. Additionally, we compared allergenomics to conventional biochemical methods and evaluated the usefulness of a proteomic strategy for identifying putative allergens to wheat allergy.

MATERIALS AND METHODS

Materials. Three Japanese wheat cultivars of flour, Norin No. 61, Kitanokaori, and Haruyokoi, were obtained from Miyake Flour Milling Co., Ltd. (Osaka, Japan). Cameria, commercial blend wheat flour, was purchased from Nisshin Flour Milling Inc. (Kobe, Japan). The Australian Standard White (ASW) flour, which is a wheat brand composed of several Australian varieties, was from Miyake Flour Milling Co., Ltd. No. 1 Canada Western Red Spring Wheat (1CW) flour prepared in 2003 was provided by Miyake Flour Milling Co., Ltd. The 1CW class is composed of high-protein hard red spring varieties. Acetonitrile (high-performance liquid chromatography grade), urea, trifluoroacetic acid (TFA), and dithiothreitol (DTT) were purchased from Wako Pure Chemical Industries (Tokyo, Japan). Polyvinylpyrrolidone was obtained from Sigma (St. Louis, MO). All other reagents were purchased from Nacalai Tesque (Kyoto, Japan) unless otherwise described.

Subjects. Seven serum samples with significant radioimmunosorbent test (RIST) or radioallergosorbent test (RAST) scores against wheat proteins were selected from sera of patients with known clinical histories of food allergies against wheat. The scores of RIST and RAST were determined using a Lumiward immunoassay system kit (Shionogi Pharmaceutical, Osaka, Japan). One control serum was obtained from a healthy adult volunteer who had no clinical history of allergic diseases. Informed consent was obtained from all donors before collecting their blood. **Table 1** shows the scores of RIST and RAST of patients' sera.

Extraction of Wheat Proteins. To prepare total protein extract for sodium dodecyl sulfate (SDS)−polyacrylamide gel electrophoresis (PAGE) and two-dimensional electrophoresis, we homogenized wheat flour (25 mg) in 1 mL of an urea extraction medium composed of 8 M urea, 40 mM Tris, and 4% 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate (CHAPS). The mixtures were sonicated by ultrasonication (30 s, six times) on an ice bath and centrifuged at 10000*g* for 20 min at 4 °C. The supernatants were collected and used as total protein extract.

Wheat proteins were fractionated according to the sequential procedure of Weiss et al. with some modifications. (*4*). Albumins/globulins were extracted from 500 mg of wheat flour with 10 mL of 50 mM Tris-HCl buffer (pH 8.8) for 1 h at 4 °C with constant stirring. After centrifugation at 20000*g* for 20 min at 4 °C, the supernatant was collected. The pellet was washed three times with 5 mL of the same buffer. Then, the residual precipitate was stirred in 10 mL of 75% ethanol for 1 h at room temperature. The suspension was centrifuged at 20000*g* for 20 min at 4 °C, and the supernatant was used as a gliadin fraction. The pellet was washed three times with 5 mL of 75% ethanol. Glutenins were extracted from the pellet with 10 mL of 50 mM Tris-HCl (pH 8.8) containing 1% SDS and 0.5% DTT by stirring for 2 h at 4 °C. After centrifugation at 20000*g* for 20 min at 4 °C, the supernatant was collected. Each fraction was diluted 1:1 with the urea extraction medium. The concentrations of protein were estimated using the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL) with BSA standards (Pierce).

Protein Separation by Conventional SDS-PAGE. Conventional SDS-PAGE was performed according to the method of Laemmli (*17*). Samples were treated with Laemmli sample buffer for 5 min at 100 °C and then run on 12.5% polyacrylamide slab gels. After electrophoresis, the gels were stained with Coomassie brilliant blue (CBB) R-250 in 25% methanol and 10% acetic acid at room temperature for 90 min and were destained in 10% acetic acid without methanol or used for immunoblotting.

Protein Separation by Two-Dimensional Gel Electrophoresis. Before two-dimensional gel electrophoresis, the total protein extract was desalted and put into a solution composed of 8 M urea and 4% CHAPS using MicroSpin G-25 Columns (Amersham Biosciences, United Kingdom). Then, 150 μL of total protein extract (50 μg of protein) was mixed with 6 μL of 0.5 M DTT, 1 μL of pH 3−10 Zoom carrier ampholyte (Invitrogen), and 3 μL of 0.1% bromophenol blue. Isoelectric focusing (IEF) was carried out using a Zoom IPGRunner system (Invitrogen, Carlsbad, CA) and an immobilized pH gradient (IPG) strip, Zoom strip, pH 3−10 NL (Invitrogen) according to the manufacturer's instructions. A Zoom strip was hydrated overnight with each sample at room temperature. IEF was performed at 200 V for 20 min, 450 V for 15 min, 750 V for 15 min, and 2000 V for 30 min. After IEF, the strips were equilibrated in 1 × NuPAGE lithium dodecyl sulfate (LDS) sample buffer (Invitrogen) containing 50 mM DTT at room temperature for 15 min with shaking and then for 15 min in 1 × NuPAGE LDS sample buffer containing 250 mM iodoacetamide. The equilibrated strip was applied to a NuPAGE 4−16% polyacrylamide gel (Invitrogen) and electrophoresed. After electrophoresis, the gels were stained with CBB R-250 as described above or used for immunoblotting.

Immunoblot Analysis. The immunoblot analysis was optimized by preliminary experiments. After electrophoresis, the proteins were electrotransferred onto a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences, Germany) at 2 mA/cm^2 for 60 min using a Bio-Rad Trans-Blot semidry electroblot system (Bio-Rad Laboratories, Hercules, CA) and a Tris-glycine transfer buffer system (25 mM Tris, 192 mM glycine, 0.1% SDS, and 10% MeOH). The membrane was washed three times with Tris-buffered saline (25 mM Tris-HCl, 137 mM NaCl, and 2.68 mM KCl, pH 7.4) containing 0.05% Tween 20 (TBS-T) and then blocked with 1% polyvinylpyrrolidone in TBS-T for 4 h at room temperature. After it was washed three times with TBS-T, the membrane was incubated overnight with subjects' or mixed patients' sera diluted 1:50 with TBS-T at room temperature. The mixed patients' sera were prepared by mixing equal volumes of sera from all seven patients. The membranes were washed three times with TBS-T and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-human IgE antibody (American Qualex, San Clemente, CA) at a dilution of 1:2000 for 2 h at room temperature. Blots were then washed three times in TBS-T before visualization. Antibody complexes captured by the immobilized target protein are detected by enhanced chemiluminescence (ECL) detection system (Amersham Biosciences, United Kingdom); in the presence of H_2O_2, HRP converted luminol to an excited intermediate dianion that emitted blue light (428 nm) on return to ground state.

Protein Identification. Gel pieces were washed in water containing 10 mM ammonium bicarbonate buffer (pH 8.0) and 50% methanol for 1 h, dehydrated in acetonitrile, and dried in a speed vac for 30 min. Samples were proteolyzed with 100−500 ng of sequence grade modified trypsin (Promega, Madison, WI) in 3−5 μL of 50 mM Tris-HCl buffer

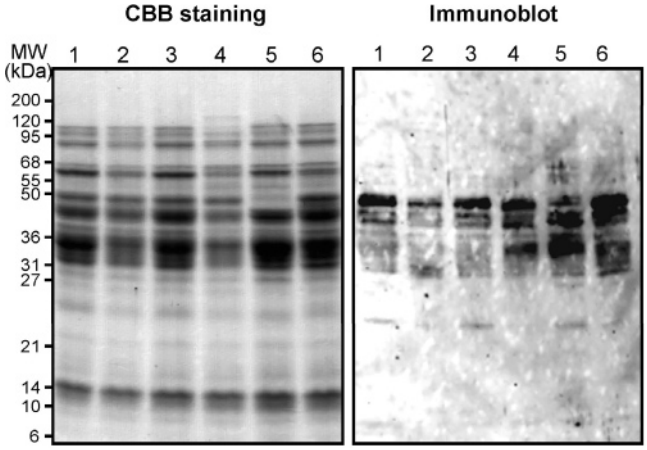

Figure 1. SDS-PAGE and IgE-immunoblotting analysis of total flour proteins. Total flour proteins extracted from six classes of wheat flour were separated by SDS-PAGE and then stained with CBB R-250 (left). Proteins shown in the left panel were analyzed by IgE-immunoblotting with the mixed sera of wheat-allergic patients (right). Lanes: 1, 1CW; 2, ASW; 3, Cameria; 4, Norin No. 61; 5, Kitanokaori; and 6, Haruyokoi.

(pH 8.8) overnight at 37 °C. The supernatant was collected, and peptides were further extracted with water containing 0.1% TFA, 50% acetonitrile containing 0.1% TFA, and acetonitrile. Peptide extracts were vacuum-dried and resuspended in water containing 0.1% TFA.

Peptide mass fingerprints were generated with an UltraFLEX matrix-assisted laser desorption ionization tandem time-of-flight (MALDI-TOF/TOF) mass spectrometer (Bruker Daltonics Japan, Tokyo, Japan). After desalted and concentrated using Zip tip μ-C18 (Millipore, Billerica, MA), a few microliters of the sample was mixed with an equal volume of a saturated solution of α-cyano-4-hydroxycinnamic acid (Bruker Daltonics) in 50% acetonitrile containing 0.1% TFA; 1 μL of the mixture was deposited on the MALDI-TOF/TOF mass spectrometry target. Proteins were identified with the Mascot (Matrix Science, United Kingdom) searching algorithms using the nonredundant database. Probability-based MOWSE scores were estimated by comparison of search results against the estimated random match population and were reported as $\sim 10 \times \text{Log } 10 (p)$, where p is the absolute probability. Scores greater than 65 were considered significant, meaning that for scores higher than 65 the probability that the match was a random event was lower than 0.05.

RESULTS

Analysis of IgE-Interactive Proteins in Wheat Flour. Because of the large difference in the solubility of individual wheat flour proteins, previous studies (*3*−*8*, *11*) on wheat allergy were confined to the analysis of solvent-fractionated wheat proteins. In this study, to establish a comprehensive allergen map of wheat flour proteins, urea extraction buffer (8 M urea, 40 mM Tris, and 4% CHAPS) was used to solubilize all of the flour proteins. The combination of urea and CHAPS is efficient for extraction of highly insoluble proteins and is most frequently used for proteomic sample preparations (*18*). As expected, after homogenization and sonication in the urea extraction buffer, the flour was almost completely solubilized, thus enabling the comprehensive analysis of wheat allergens. We evaluated the allergenic proteins in the total protein extract from wheat flour by conventional methods.

Figure 1, left) shows SDS-PAGE patterns of total protein extracts from six classes of wheat flour: 1CW, Cameria, Kitanokaori, and Haruyokoi are widely used in baking bread, while ASW and Norin No. 61 are preferred by many noodle manufacturers. About 25 bands were detected in all classes of flour with some differences in protein patterns. Allergenic

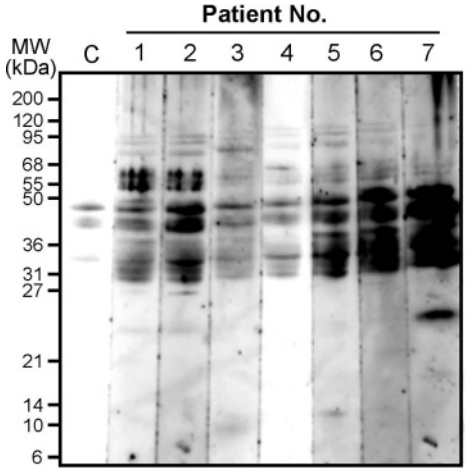

Figure 2. IgE reactivity of individual sera from patients with wheat allergy against total flour proteins. Total flour proteins extracted from 1CW were separated by SDS-PAGE. After electrophoresis, the separated proteins were transferred onto a membrane and then immunostained with each serum of wheat-allergic patients. The numbers of patients refer to corresponding numbers in **Table 1**. Lane C is a control lane with the serum of a nonallergic control subject.

proteins in total protein extracts were characterized by IgE immunoblotting with the mixed sera from all seven patients with wheat allergy. As shown in **Figure 1** (right), major IgE-reactive proteins were detected between 30 and 50 kDa in all wheat classes of flour. The distribution of IgE-binding proteins was apparently different in each flour, and the pattern was intrinsically compatible with the protein patterns.

IgE Reactivity of Each Wheat-Allergic Patients' Sera to Total Flour Proteins. The reactivity of IgE antibodies from individual wheat-allergic patients to total flour proteins extracted from 1CW, which is commonly used in Japan for blending for breads and noodles, was examined by immunoblotting. In this study, a total of seven patients with food allergy against wheat were tested. Clinical data of the patients are summarized in **Table 1**. The patients were three females and four males ranging in age from 4 to 19 years (mean, 8 years). The total and allergic IgE in patients' sera were assessed by RIST and RAST, respectively (*19*), and all patients except for patient 5 had positive wheat RAST. As shown in **Figure 2**, all patients had IgE antibodies directed against wheat flour proteins between 27 and 50 kDa, and there was a high degree of homology of IgE-binding patterns with different immunostaining intensities. Some individuals showed IgE reactivity to about 25, 55, and 60 kDa wheat proteins. Only a few positive bands with very low avidity were observed in the immunoblotting with the serum of the nonallergic control subject.

Analysis of Sequential Protein Extracts from Wheat Flour. To further evaluate the IgE-reactive proteins, we separated wheat flour proteins into albumin/globulin, gliadin, and glutenin fractions by sequential extraction. The compositions of sequential extracts were analyzed after SDS-PAGE and CBB staining (**Figure 3**, left). Numerous components between 10 and 95 kDa could be observed in the albumin/globulin extract, with major bands visible around 14, 27, and 60 kDa. Six major protein bands between 30 and 50 kDa could be observed in the gliadin fraction. The glutenin fraction contained a larger amount of proteins between 14 and 120 kDa with major bands visible around 40, 45, 50, 68, and 95 kDa than other fractions. The total protein extract with urea extraction buffer was noted to perfectly recover all proteins in each sequential fraction. **Figure**

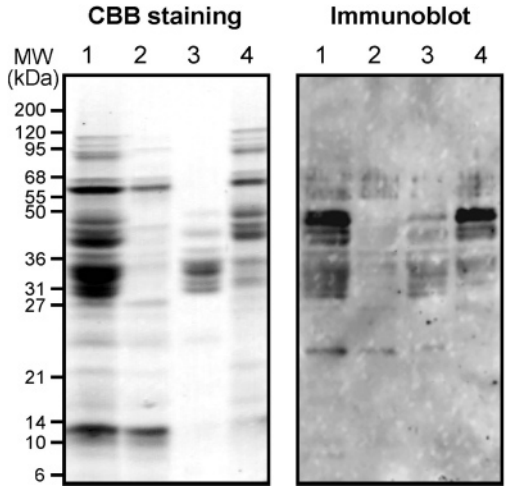

Figure 3. SDS-PAGE and IgE-immunoblotting analysis of sequential protein extracts from 1CW flour. Each extract was separated by SDS-PAGE and then stained with CBB (left). Proteins shown in the left panel were analyzed by IgE immunoblotting with the mixed sera of wheat-allergic patients (right). Lanes: 1, total protein extract; 2, albumin/globulin extract; 3, gliadin extract; and 4, glutenin extract.

3 (right) shows reactivity of IgE antibodies to each sequential extract. IgE-interacted proteins were widely distributed in all fractions, suggesting that a variety of wheat proteins are allergenic. The major IgE-reactive bands between 27 and 50 kDa were observed in the gliadin and glutenin fractions.

Two-Dimensional Electrophoresis and IgE Immunoblotting of Wheat Flour Proteins. Total proteins extracted from wheat flour were separated by two-dimensional gel electrophoresis with IEF and LDS-PAGE, and then, putative allergens were comprehensively evaluated. **Figure 4A** shows a typical two-dimensional map of the total protein extract from 1CW. More than 200 spots, each with a different isoelectric point or molecular weight, were detected. This resolving power is far superior to the conventional SDS-PAGE or IEF where proteins are separated one-dimensionally. **Figure 4B** shows the results of immunoblot with IgE antibodies from patients' sera. We detected 23 spots of proteins interacting with IgE antibodies because of the high resolving power of two-dimensional gel electrophoresis. However, three spots (5, 8, and 9) were nonspecifically detected by IgE immunoblotting both with the mixed patients' sera and with the serum of the nonallergic control subject (**Figure 4B**,**C**), indicating that these are not allergens.

Identification of IgE-Binding Proteins by MALDI-TOF/TOF. The spots of flour proteins that interacted with IgE antibodies were cut out from the two-dimensional gel. After tryptic digestion, we analyzed the resultant tryptic peptides with a MALDI-TOF/TOF instrument. On the basis of a peptide mass map of the tryptic peptides and MS/MS spectra of the intense

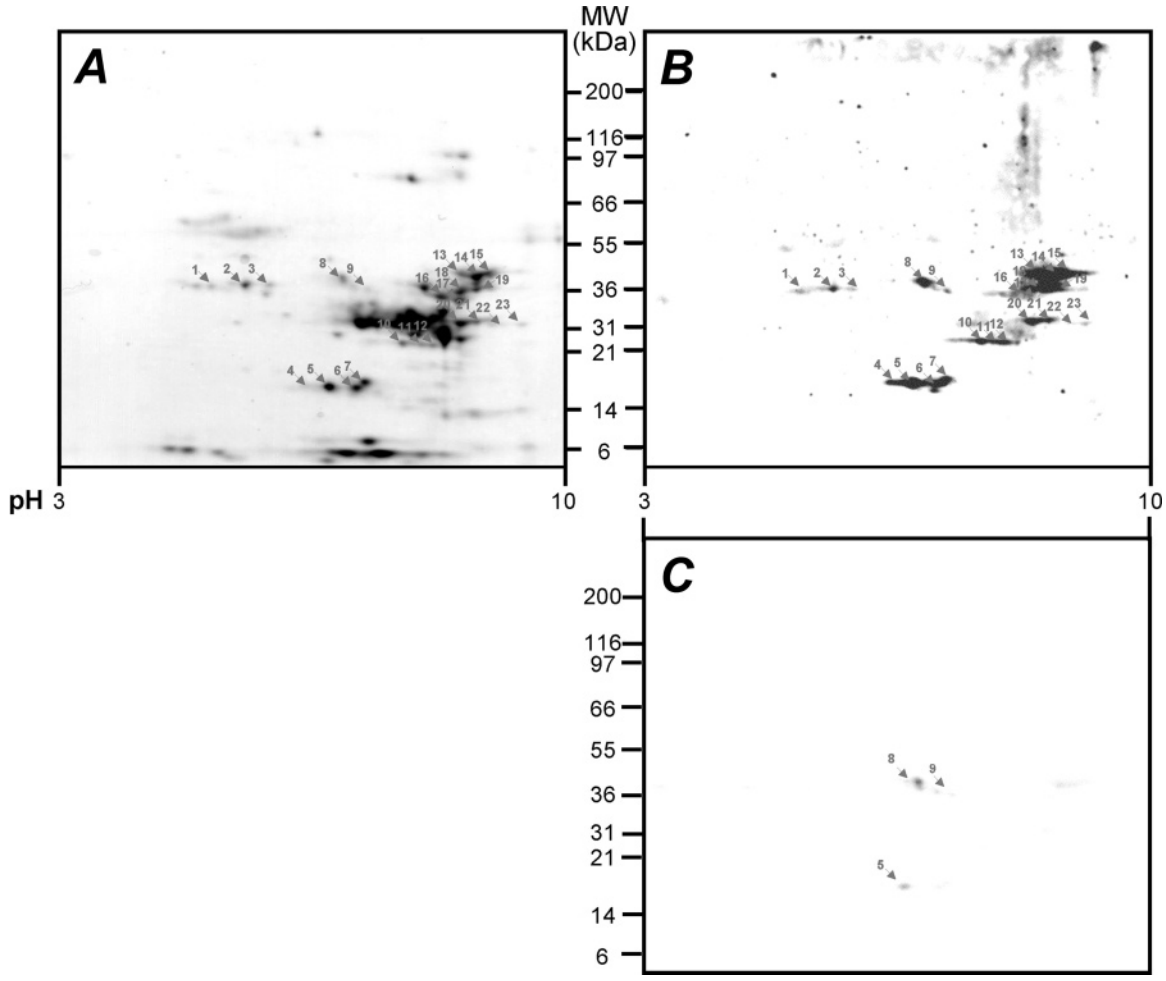

Figure 4. Two-dimensional gel electrophoresis and IgE immunoblotting of total flour proteins. Total proteins extracted from 1CW flour were two-dimensionally separated and then visualized by CBB (**A**). Proteins shown in panel **A** were analyzed by IgE immunoblotting with the mixed sera of wheat-allergic patients (**B**) or with the serum of the nonallergic control subject (**C**). IgE-interactive spots with the same position in the two-dimensional electrophoresis and the IgE immunoblotting are represented by arrows and numbers.

peptide ions, the proteins were identified by a database search with a PMF mode of Mascot. As a result of the high resolution of two-dimensional gel electrophoresis and the marked sensitivity of MALDI-TOF/TOF mass spectrometry, we identified four previously reported wheat allergens or their sequentially homologous proteins (serpin, α-amylase inhibitor, γ-gliadin, and LMW glutenin) without isolating each protein (**Table 2**).

DISCUSSION

Extensive analysis of allergenic proteins is generally time-consuming and labor-intensive. Accordingly, a rapid and easy procedure for allergen identification is required. In this study, we evaluated the usefulness of a proteomic approach for analyzing putative wheat allergens (allergenomics). Wheat proteins were first separated two-dimensionally according to their isoelectric point and molecular weight under denaturing conditions (**Figure 4**). Historically, the presence of a large number of subunits in the wheat proteome has hindered the research. In the present study, however, we could detect slight structural differences of isoallergens as distinct spots on the two-dimensional gel and subsequent IgE immunoblot (**Figure 4**). Moreover, we succeeded in extraction of total wheat flour proteins and recovered the albumin/globulin, gliadin, and glutenin fractions completely in the extraction (**Figure 3**, left). Thus, the complete solubilization of wheat flour proteins enabled us to comprehensively analyze all of the wheat flour proteins and detect predominant IgE-binding proteins in wheat flour. Furthermore, we could identify a number of IgE-binding proteins using MALDI-TOF/TOF mass spectrometry. Edman degradation-based N-terminal sequencing of a purified protein has so far been the first choice for IgE-reactive protein identification, but this technique is time consuming and requires a relatively large amount of purified sample. By combining with high-resolution two-dimensional gel electrophoresis, the MS/MS analytical approach permitted fast and comprehensive identification of IgE-interactive wheat proteins.

In agreement with previously published reports, we identified serpin, α-amylase inhibitor, γ-gliadin, LMW glutenin, and their sequentially homologous proteins as major IgE-binding proteins by the allergenomic approach. The α-amylase inhibitor protein family was previously shown to comprise major allergens in the albumin/globulin fraction responsible for bakers' asthma and to be involved in food allergy to wheat (*6*, *7*, *20*). Serpin, a serine proteinase inhibitor, has also been identified as a putative allergen for bakers' asthma in the albumin/globulin fraction (*6*). Although α-, β-, γ-, and ω-gliadins were characterized as the major allergens for patients with wheat allergies by some researchers (*3*, *10*−*15*, *21*), in this study, we identified only γ-gliadin subunits as major IgE-reactive proteins. Gliadins are monomeric proteins and account for about 30% of wheat grain proteins; they are classified into α-, β-, γ-, and ω-gliadins according to their sequences and electrophoretic mobility in acid-PAGE; the molecular masses of α-, β-, and γ-gliadins range from 30 to 40 kDa; those of ω-gliadins range from 40 to 50 kDa (*3*, *22*). While several cDNA sequences are available in databases, the reverse is not true for mature gliadins, whose structural analysis is hindered by the high number of wheat phenotypes, each containing at least 25 closely related gliadin forms (*23*). Recently, about 30 closely related gliadins have been identified by a proteomic study (*24*). In the present research, we could identify three IgE-binding isoforms of γ-gliadins with small differences in pI. Our results were also in good agreement with those from other studies on food allergy to wheat regarding IgE reactivity with LMW glutenin subunits (*3*, *25*−*27*). Moreover, a pentapeptide repeat motif, Gln-Gln-Gln-Pro-Pro, has been identified as a major IgE-binding epitope in LMW glutenins (*26*, *27*). Glutenins account for about 35% of total proteins and correspond to polymeric proteins linked by intermolecular disulfide bridges; they are composed of high molecular weight (HMW) subunits (apparent molecular masses in SDS-PAGE range from 80 to 120 kDa) and of LMW subunits; LMW glutenins are further divided into three subunit groups: the B group (42−52 kDa), the C group (30−40 kDa), which includes subunits related to αβ- and γ-gliadins, and the D group (60−75 kDa), which includes ω-gliadin-like subunits (*3*, *22*). At the molecular level, 20 members of the complex LMW glutenin family have been cloned and sequenced (*28*, *29*). The difficulty to resolve LMW glutenins by SDS-PAGE and the presence of a large number of subunits have hindered the research progress. The advantages of allergenomics in analyzing these troublesome allergens are demonstrated. In the present study, we could clearly identify nine subunits of LMW glutenins differing in pI and molecular mass as the most predominant IgE-binding antigens. Although HMW glutenins have been reported as putative wheat allergens in food allergy (*3*, *13*), we could not detect the corresponding bands (80−120 kDa) of HMW glutenins by IgE immunoblotting (**Figures 2**−**4**).

The results that we obtained by allergenomics were slightly different from those reported previously. We could not identify some previously reported allergens such as HMW glutenins and α-, β-, and ω-gliadins. However, such a discrepancy may occur due to differences in the wheat cultivar, patient population, and methods used. In this study, we analyzed allergenic proteins in six classes of wheat flour and observed a significantly different distribution of IgE-binding proteins with the flour by immunoblotting (**Figure 1**), presumably because of the diversity in the antigen contents. Another possible explanation may be that the allergens extracted from different flours differ slightly in amino acid composition and/or post-translational modifications and, therefore, exhibit different epitopes. Furthermore, we analyzed IgE reactivity of seven patients' sera with food allergy to wheat. Recently, ω-gliadins have been reported to be the responsible allergen in patients suffering from wheat-induced anaphylaxis (*11*−*14*). Because the symptoms of the subjects in this study are not anaphylaxis (**Table 1**), it may be reasonable that ω-gliadins were not detected as positive spots in **Figure 4**. Although immunostaining intensity was different among patients, all sera were commonly positive against wheat proteins between 27 and 50 kDa (**Figure 2**). Considering the results from allergenomic analysis, the IgE-binding proteins between 27 and 50 kDa could correspond to serpin and LMW glutenins, suggesting that these proteins are predominant wheat allergens for food allergy. However, some sera from subjects recognized components at about 25, 55, and 60 kDa, indicating that there are some interindividual variations in the allergic response to wheat proteins. On the other hand, the difference in the method used may also be responsible for the differences in the identified allergens: (i) The detection system was not sensitive enough to detect minor allergens. (ii) The structure of the proteins was altered by *S*-carbamidomethylation with the DTT/iodoacetamide treatment, and a considerable amount of the antibody binding sites (the conformational epitopes) has disappeared; these polypeptides are therefore poorly recognized by the antibodies. Other plausible reasons may be accounted for by the drawback of two-dimensional electrophoresis: (i) The proteins were not focusing in the IPG strip, due to insufficient denaturation or solubilization. (ii) The proteins were not migrating from the strip into second dimension gel, due to aggregation, precipita-

Table 2. Identification of IgE-Binding Proteins by MALDI-TOF/TOF of Tryptic Peptides[a]

spot	protein name	GI no.	no. of peptides matched	sequence coverage (%)	identified sequence		peptide identified by MS/MS
1	serpin (*T. aestivum*)	1885346	7	32	LSIAHQTR	11–18	
					VAFANGVFVDASLQLKPSFQELAVCK	99–124	
					VTTGLIK	152–158	
					LSAEPEFLEQHIPR	261–274	
					GLGLLLPFGAEADLSEMVDSPMAQNLYISSIFHK (Oxi-M)	301–334	
					QAPPPSVLDFIVDHPFLFLIR	358–378	
					EDTSGVVLFIGHVVNPLLSS	379–398	
2	serpin (*T. aestivum*)	1885350	16	53	LSIAHQTR	11–18	
					LASTISSNPK	23–32	
					SAASNAAFSPVSLHSALSLLAAGAGSATR	33–61	
					VAFANGVFVDASLQLKPSFQELAVCK	99–124	
					AAEVTTQVNSWVEKVTSGR	138–156	
					NILPSGSVDNTTKLVLANALYFK	159–181	
					LVLANALYFK	172–181	
					VLKLPYK	228–234	
					QGGDNRQFSMYILLPEAPGGLSSLAEK	235–261	
					QGGDNRQFSMYILLPEAPGGLSSLAEK (Oxi-M)	235–261	
					QFSMYILLPEAPGGLSSLAEK	241–261	
					QFSMYILLPEAPGGLSSLAEK (Oxi-M)	241–261	
					LSAEPDFLER	262–271	
					CLGLQLPFSDEADFSEMVDSPMPQGLR	302–328	
					CLGLQLPFSDEADFSEMVDSPMPQGLR (Oxi-M)	302–328	
					VSSVFHQAFVEVNEQGTEAAASTAIK	329–354	
3	serpin (*T. aestivum*)	5734504	8	32	LSIAHQTR	11–18	
					SAASNAAFSPVSLHVALSLLAAGAGSATR	33–61	
					VQTPFMSSMDDQYLSSSDGLK (Oxi-M)	195–226	
					QFSMYILLPEAPGGLSNLAEK	240–260	
					LSAEPDFLER	261–270	
					CLGLQLPFSNEADFSEMVDSPMAHGLR (Oxi-M)	301–327	
					VSSVFHQAFVEVNEQGTEAAASTAIK	328–353	
					EDISGVVLFMGHVVNPLLSS (Oxi-M)	379–398	
4	0.19 dimeric α-amylase inhibitor (*T. aestivum*)	54778513	4	47	LQCNGSQVPEAVVRDCCQQLANISEWCR (CAM-C)	26–53	
					DCCQQLANISEWCRCDALYNMLDSMYK (Oxi-M)	40–66	
					CDALYNMLDSMYKEHGAQEGQAGTGAFPR (CAM-C)	54–82	
					EHGAQEGQAGTGAFPRCR (CAM-C)	67–84	
5	nonspecific						
6	dimeric α-amylase inhibitor (*T. aestivum*)	146214683	5	65	LQCNGSQVPEAVVRDCCQQLANISEWCR	26–53	
					DCCQQLANISEWCRCDALYNMLDSMYK	40–66	
					CDALYNMLDSMYKEHGAQEGQAGTGAFPR (Oxi-M)	54–82	
					EHGAQEGQAGTGAFPRCR	67–84	
					LTAASITAVCKLPIIIDASGGR	90–111	
7	dimeric α-amylase inhibitor (*T. aestivum*)	146214683	5	65	LQCNGSQVPEAVVRDCCQQLANISEWCR	26–53	
					DCCQQLANISEWCRCDALYNMLDSMYK	40–66	
					CDALYNMLDSMYKEHGAQEGQAGTGAFPR (Oxi-M)	54–82	
					EHGAQEGQAGTGAFPRCR	67–84	
					LTAASITAVCKLPIIIDASGGR	90–111	
8	nonspecific						
9	nonspecific						
10	γ-gliadin (*T. aestivum*)	7548844	2	26	NFLLQQCNHVSLVSSLVSIILPR	74–96	○
					TLPTMCNVYVPPDCSTINIPYANIDAGIGGQ (CAM-C)	173–203	
11	γ-gliadin (*T. aestivum*)	7548844	2	26	NFLLQQCNHVSLVSSLVSIILPR	74–96	○
					TLPTMCNVYVPPDCSTINIPYANIDAGIGGQ (CAM-C)	173–203	
12	γ-gliadin (*T. aestivum*)	7548844	2	26	NFLLQQCNHVSLVSSLVSIILPR	74–96	○
					TLPTMCNVYVPPDCSTINIPYANIDAGIGGQ (CAM-C)	173–203	
13	LMW glutenin subunit (*T. aestivum*)	44885908	5	18	VFLQQQCSPVAMPQSLAR	237–254	
					VFLQQQCSPVAMPQSLAR (Oxi-M)	237–254	
					SQMLQQSSCHVMQQQCCQQLPQIPQQSR (Oxi-M)	255–282	
					TLPTMCNVNVSLYR (Oxi-M)	363–376	
					VPFGVGTGVGGY	381–392	
14	LMW glutenin subunit (*T. aestivum*)	44885908	5	18	VFLQQQCSPVAMPQSLAR	237–254	
					VFLQQQCSPVAMPQSLAR (Oxi-M)	237–254	
					SQMLQQSSCHVMQQQCCQQLPQIPQQSR (Oxi-M)	255–282	
					TLPTMCNVNVSLYR (Oxi-M)	363–376	
					VPFGVGTGVGGY	381–392	

Table 2. Continued

spot	protein name	GI no.	no. of peptides matched	sequence coverage (%)	identified sequence		peptide identified by MS/MS
15	LMW glutenin subunit (*T. aestivum*)	44885908	5	18	VFLQQQCSPVAMPQSLAR	237–254	
					VFLQQQCSPVAMPQSLAR (Oxi-M)	237–254	
					SQMLQQSSCHVMQQQCCQQLPQIPQQSR (Oxi-M)	255–282	
					TLPTMCNVNVSLYR (Oxi-M)	363–376	
					VPFGVGTGVGGY	381–392	
16	not identified						
17	not identified						
18	LMW glutenin subunit (*T. aestivum*)	109240248	5	21	VFLQQQCSPVAMPQSLAR (Oxi-M)	188–205	
					SQMLQQSSCHVMQQQCCQQLPQIPQQSR (Oxi-M)	206–233	
					YEAIR	234–238	
					AIIYSIVLQEQQQVR	239–253	
					VNVPLYR	321–327	
19	LMW glutenin subunit (*T. aestivum*)	109240248	5	21	VFLQQQCSPVAMPQSLAR (Oxi-M)	188–205	
					SQMLQQSSCHVMQQQCCQQLPQIPQQSR (Oxi-M)	206–233	
					YEAIR	234–238	
					AIIYSIVLQEQQQVR	239–253	
					VNVPLYR	321–327	
20	s-type LMW glutenin (*T. aestivum*)	47607142	4	17	VFLQQQCSPVAMPQSLAR (Oxi-M, CAM-C)	99–116	
					YEAIR	145–149	
					AIIYSIVLQEQQQVR	150–164	
					VNVPLYR	232–238	
21	s-type LMW glutenin (*T. aestivum*)	47607142	4	17	VFLQQQCSPVAMPQSLAR (Oxi-M, CAM-C)	99–116	
					YEAIR	145–149	
					AIIYSIVLQEQQQVR	150–164	
					VNVPLYR	232–238	
22	LMW glutenin storage protein (*T. aestivum*)	1857652	3	21	VFLQQQCSPVAMPQHLAR (CAM-C)	133–150	
					GTFLQPHQIARLEVMTSIALR	248–268	
					TLPTMCSVNVPLYSSITSAPLGVGSR (CAM-C)	269–294	
23	LMW glutenin storage protein (*T. aestivum*)	1857652	3	21	VFLQQQCSPVAMPQHLAR (Oxi-M)	133–150	
					GTFLQPHQIARLEVMTSIALR	248–268	
					TLPTMCSVNVPLYSSITSAPLGVGSR (Oxi-C)	269–294	

[a] Spots that were excised from the gel shown in **Figure 4** were identified by tryptic digestion and MALDI-TOF/TOF.

tion, or associations. Further investigations have to be performed to evaluate these problems in more detail.

In conclusion, we evaluated the usefulness of allergenomics for the analysis of wheat allergens. After a two-dimensional gel electrophoresis and subsequent IgE immunoblotting, we identified 18 wheat allergens containing isoallergens using MALDI-TOF/TOF mass spectrometry. The two-dimensional allergen map can be beneficial in many ways. It could be used, for example, for precise diagnosis of wheat-allergic patients and assessment of wheat allergens in food. In the future, the allergenomic technique must be generally valid.

ABBREVIATIONS USED

1CW, No. 1 Canada Western Red Spring Wheat; AEDS, atopic eczema/dermatitis syndrome; ASW, Australian Standard White; CBB, Coomassie brilliant blue; CHAPS, 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate; DTT, dithiothreitol; ECL, enhanced chemiluminescence; HMW, high molecular weight; HRP, horseradish peroxidase; IEF, isoelectric focusing; IPG, immobilized pH gradient; LDS, lithium dodecyl sulfate; LMW, low molecular weight; MALDI-TOF/TOF, matrix-assisted laser desorption ionization tandem time-of-flight; PAGE, polyacrylamide gel electrophoresis; RAST, radioallergosorbent test; RIST, radioimmunosorbent test; SDS, sodium dodecyl sulfate; TBS-T, Tris-buffered saline containing 0.05% Tween 20; TFA, trifluoroacetic acid; WDEIA, wheat-dependent exercise-induced anaphylaxis.

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