

Higher allergenicity of high molecular weight hydrolysed wheat protein in cosmetics for percutaneous sensitization

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Summary

Background. Wheat protein derivatives are used in a variety of products worldwide. Gluten is commercially used 'as is' or with modifications such as hydrolysis, which is carried out to overcome its insolubility. Several cases of contact urticaria following exposure to hydrolysed wheat protein (HWP) in cosmetics or of anaphylaxis caused by deamidated gluten in food or non-food products have been described.

Objectives. To evaluate the types of HWP that have higher allergenicity for percutaneous sensitization.

Methods. We enrolled 7 patients with wheat-dependent exercise-induced anaphylaxis who had been sensitized to HWP primarily through the percutaneous and/or the rhinoconjunctival route by using facial soap containing HWP. Reaction to wheat proteins was confirmed by IgE immunoblotting and basophil CD203c expression with six HWP variants.

Results. The IgE of all the patients reacted to HWPs composed of large polypeptide aggregates. High molecular weight (MW) HWPs were also found to induce significant enhancement of basophil CD203c expression.

Conclusions. HWPs composed of large polypeptide aggregates possibly induce sensitization to a greater degree than lower-MW HWPs. Basophil surface CD203c expression is useful for evaluating the allergenicity of HWPs.

Key words: cosmetics; hydrolysed wheat protein; percutaneous sensitization; wheat-dependent exercise-induced anaphylaxis.

Wheat-dependent exercise-induced anaphylaxis (WDEIA) is a special form of wheat allergy induced by wheat ingestion combined with physical exercise (1–3). Aspirin intake is another well-known trigger for allergic symptoms (4, 5). We identified wheat ω -5 gliadin as a major allergen in WDEIA patients, and found that a CAP system fluorescent enzyme immunoassay (CAP-FEIA) combined

with ω -5 gliadin improves diagnostic procedures (6). A new WDEIA subtype caused by hydrolysed wheat protein (HWP) has been recently reported (7–9). WDEIA patients who used facial soap containing HWP were found to be sensitized to HWP via the percutaneous and/or the rhinoconjunctival route (7–9). Patients with this WDEIA tested positive to HWP in the prick test and had serum HWP-specific IgE. Moreover, these patients showed characteristic facial angioedema distinct from those with conventional WDEIA (CO-WDEIA); they were designated as having HWP-sensitized WDEIA (HWP-WDEIA).

In order to evaluate the allergenicity of HWP, which could be inducing percutaneous and/or rhinoconjunctival sensitization, several HWPs with different sources

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Table 1. Clinical features of the patients with hydrolysed wheat protein (HWP)-sensitized wheat-dependent exercise-induced anaphylaxis (HWP-WDEIA)

Patient	Age (years)/sex	Atopy	Use of soap with HWP-A	Contact urticaria caused by HWP	Foods	Triggers	Symptoms
1	51/F	—	+	+	Spaghetti	Walking	A, U, S, NS, ND, P
2	49/F	—	+	+	Gyoza dumplings	Table tennis	A, U, S, NS, C, D, AP, V
3	52/F	—	+	+	Steamed bread	Jogging	A, U
4	44/F	—	+	+	Bread, udon noodles	Tennis	A, U, AP, anaphylactic shock
5	54/F	—	+	+	Udon noodles, gyoza dumplings	Walking	A, U, S, ND, AP, L
6	60/F	—	+	—	Udon noodles	Walking	A, U, S, C
7	35/F	—	+	+	Cracker	Internal use of analgesics	A, U, D

A, angioedema; AP, abdominal pain; C, conjunctivitis; D, dyspnoea; F, female; L, lacrimation; ND, nasal discharge; NS, nasal stuffiness; P, pharyngalgia; S, sneeze; U, urticaria; V, vomiting.

were analysed with IgE immunoblotting and a basophil CD203c expression test.

Methods

Patients

Seven female patients with HWP-WDEIA were enrolled in this study (Table 1). All patients had used the same brand of soap containing HWP-A. Six of these patients developed contact urticaria several months later, and subsequently had WDEIA. The predominant symptom of natural wheat ingestion-induced reactions was angioedema of the eyelids. Six of the 7 patients developed anaphylaxis, and 1 patient (patient 4) also developed anaphylactic shock. Urticaria following contact with soap preceded the food ingestion-induced reactions in all cases. None of the patients had atopic diathesis.

The patients tested positive in the prick tests with wheat (5/7), bread (5/7), 0.1% soap solution supplemented with HWP-A in saline (6/7), and 1 mg/ml HWP-A in saline (7/7) (Table 2). Five healthy controls were tested, and none of them reacted to any of the allergens in the prick test. Specific IgEs for wheat and gluten were detected by CAP-FEIA (Phadia, Uppsala, Sweden; detection range, 0.35–100 kU_A/l) in all HWP-WDEIA patients. Challenge tests included exercise, wheat ingestion, and aspirin intake. A combination of these challenges was performed for 3 of the 7 patients, as described previously (10). Aspirin and wheat challenge induced angioedema, urticaria and/or dyspnoea in 2 of these 3 patients. Patient 2 required a combination of aspirin, wheat and exercise challenge to induce symptoms.

This study was approved by the Ethics Committee of the Shimane University Faculty of Medicine (approval Nos. 469 and 703); all participants provided written informed consent.

Table 2. Results of immunological studies

Patient	Prick test				Specific IgE (kU _A /l)			Challenge test
	Wheat	Bread	Soap 0.1%	HWP-A, 1 mg/ml	Wheat	Gluten	ω -5 gliadin	
1	3+	3+	3+	3+	4.70	4.76	<0.34	NT
2	1+	1+	1+	2+	2.27	4.56	<0.34	+
3	—	—	3+	2+	0.46	0.65	<0.34	NT
4	2+	2+	2+	2+	9.26	15.8	1.16	+
5	2+	3+	3+	3+	5.17	7.37	<0.34	+
6	2+	2+	3+	2+	0.91	2.16	<0.34	NT
7	2+	2+	2+	2+	15.6	25.2	3.28	NT

HWP, hydrolysed wheat protein; NT, not tested.

Wheat: commercial wheat flour extract (1:20 wt/vol; Torii Pharmaceutical Co., Tokyo, Japan).

Bread: commercial bread (1:20 wt/vol; Torii Pharmaceutical Co.).

Soap 0.1%: 0.1% diluted solution of soap supplemented with HWP-F in saline.

Reactions were read at 15 min, and responses were compared with positive histamine controls (10 mg/ml): 1+, 25% of the area of the wheal induced by the positive histamine control; 2+, 50%; 3+, 100%; 4+, 200%.

HWP preparation

Six different HWPs (HWP-A, HWP-B, HWP-C, HWP-D, HWP-E, and HWP-F) were obtained from food or cosmetic manufacturers or directly from the producer. HWP-A and HWP-B were hydrolysed only under acidic conditions. The remaining HWPs were subjected to enzymatic and/or acidic hydrolysis.

Preparation of wheat proteins

ω -5 Gliadin was purified as described previously (11). Commercial blend wheat flour (Camellia®) was purchased from Nissin Flour Milling Inc., Kobe, Japan) for fractionation of wheat flour proteins. Wheat flour was mixed with 0.6 volumes of water with an arm kneader, and the dough was placed in water for 30 min. Water-soluble wheat proteins were extracted twice with two volumes of water with an arm kneader, and used for analysis. The residue was powdered with a tissue lyser (Qiagen, Tokyo, Japan) after lyophilization. The powdered water-insoluble wheat proteins were used for subsequent experiments.

Western blotting

All of the sera were analysed under the same conditions. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed with 12.5% polyacrylamide gels, according to the manufacturer's specifications (Atto Corp., Tokyo, Japan). Samples were solubilized in 2% sodium dodecyl sulfate containing 6% β -mercaptoethanol for 10 min at 95°C, and electrophoresed. The separated proteins were blotted under semi-dry conditions on poly(vinylidene difluoride) (PVDF) membranes (Millipore Corp., Billerica, MA, USA) (12). For IgE immunodetection, membranes were incubated overnight with 10 $\times$ diluted serum samples blocked with 5% skimmed milk in 10 mM Tris–HCl, pH 7.6, containing 150 mM NaCl and 0.1% Tween-20 (TBST). IgE fixation was detected with secondary goat anti-human IgE conjugated to horseradish peroxidase (Dako SA, Trappes, France) and the ECL-plus kit (GE Healthcare, Tokyo, Japan). Chemiluminescence was recorded on X-ray films (RX-U; Fujifilm, Tokyo, Japan) after few minutes of exposure.

Inhibition assays

To determine cross-reactivity of wheat proteins and HWP, 100 μ l of serum was incubated with serial dilutions of HWP-A (100 to 5 μ g/ml) at 37°C for 2 hr with constant stirring. The serum was then diluted 1/10 in 5% skimmed milk/TBST for immunodetection of IgEs by western blotting.

Basophil CD203c expression

An allergenicity kit (Beckman Coulter Inc., Brea, CA, USA) was used for quantification of basophil CD203c expression, as described previously (13). EDTA-containing whole blood samples of all patients were incubated with various concentrations of HWP-A or ω -5 gliadin for 15 min after a sufficient quantity of calcium solution to override the chelating capacity of EDTA had been added. The blood samples of patients were incubated with six types of 0.1 μ g/ml HWP for 15 min. Anti-IgE antibody (4 μ g/ml) and phosphate-buffered saline (PBS; pH 7.4) were used as the positive and negative controls, respectively. PC7-conjugated anti-CD3, fluorescein isothiocyanate-conjugated anti-CRTH2 and phycoerythrin-conjugated anti-CD203c antibodies were added during the reaction. The samples were analysed on a FACSCan (Cell Analyzer; Becton Dickinson, Franklin Lakes, NJ, USA). Basophils were detected according to forward–side scatter characteristics and CD3[–] and CRTH2⁺ expression. Upregulation of CD203c expression on basophils was determined with a threshold defined by the fluorescence of unstimulated cells (negative control), and expressed as percentage of CD203c (CD203c^{high}%). At least 500 basophils were analysed per assay.

Size-exclusion chromatography (SEC)

SEC was performed with 200 μ g of each HWP type loaded onto a TSK gel G2000 SWXL column (7.8 mm $\times$ 30 cm) (TOSOH Co., Tokyo, Japan) previously equilibrated with PBS at pH 7.0. A flow rate of 0.8 ml/min was used. Proteins were detected at 280 nm. Absorbance at 280 nm was expressed as absorbance unit (AU). The molecular weights (MWs) of the HWPs were estimated with a ladder of standard proteins.

Statistical analysis

Statistical differences were determined with Student's *t*-test.

Results

SDS–PAGE and western blotting

Coomassie blue staining of HWP-A subjected to SDS–PAGE showed smears characteristic of random degradation and peptide rearrangement (Fig. 1a). The smears spanned most of the separation of the gel, ranging from MW 0 to MW 250 000. In contrast, wheat proteins showed characteristic bands from MW 15 000 to MW 100 000.

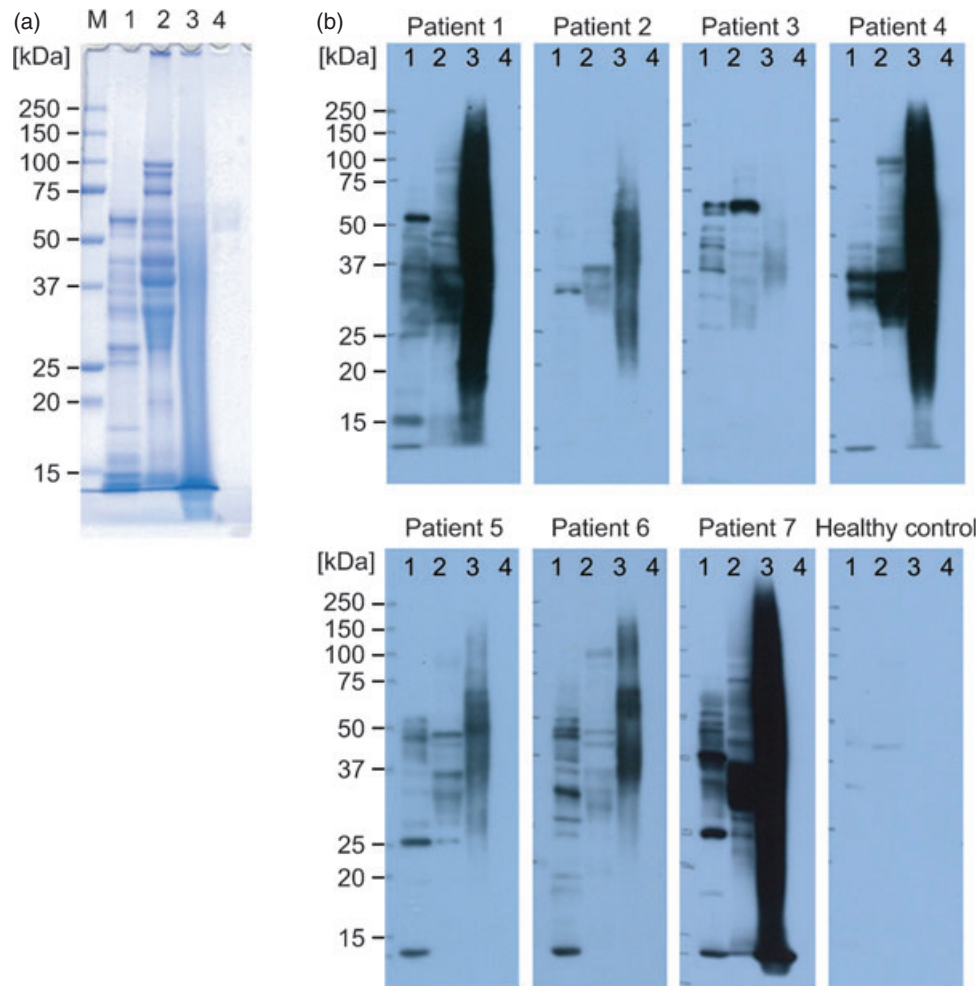


Fig. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting of wheat protein fractions and hydrolysed wheat protein (HWP)-A. (a) Gel stained with Coomassie Brilliant Blue. (b) IgE immunoblotting with the patients' sera. M, marker proteins. Lane 1: water-soluble wheat proteins. Lane 2: water-insoluble wheat proteins. Lane 3: HWP-A. Lane 4: purified ω -5 gliadin.

Western blotting showed smear patterns of IgEs of all patients reacting with HWP-A (Fig. 1b). The reaction intensity varied among the patients. The IgEs of all patients reacted with both water-soluble and water-insoluble wheat proteins, but not with ω -5 gliadin. In all patients, HWP-A inhibited the reaction of IgEs with wheat proteins in a dose-dependent manner after preincubation with the patients' sera (data not shown).

HWP induced basophil CD203c expression

HWP-A enhanced CD203c expression in a concentration-dependent manner in the HWP-WDEIA patients (Table 3). The maximum reaction exceeded 60% at 0.1 μ g/ml. No significant enhancement of CD203c was observed with

purified ω -5 gliadin. Patients 4 and 7 had ω -5 gliadin-specific IgE, but the reaction to purified ω -5 gliadin was negligible. To determine whether the basophil activation was mediated by IgE, we removed the cell-surface IgE in the leukocyte mixture by using lactic acid, as described previously (14). Basophil activation was abolished by this removal of cell-surface IgE (data not shown).

IgE and basophil activation in response to different HWPs

SDS–PAGE showed smear staining spanning the entire length of the gel for HWP-A (lane 6) and HWP-B (lane 1) (Fig. 2a). These values were considerably greater than the MWs of the native proteins, gliadins and glutenins, suggesting the presence of large polypeptide aggregates.

Table 3. CD203c expression (%) on basophils induced with hydrolysed wheat protein (HWP)-A and ω -5 gliadin

Patient	Negative control	Positive control	HWP-A (μ g/ml)			ω -5 gliadin (μ g/ml)		
			0.001	0.01	0.1	0.001	0.01	0.1
1	0.21	51.83	ND	18.26	52.07	ND	0.62	0
2	1.15	56.02	ND	63.13	63.72	ND	1.31	22.8
3	3.66	55.78	ND	78.31	60.83	ND	6.94	12.61
4	0	55.9	0.49	1.47	60.31	0.71	0.47	0.79
5	0.63	51.2	0.21	5.43	69.11	0	0.63	0.41
6	8.33	83.23	10.77	60.08	85.22	3.27	2.9	4.14
7	0	35.46	ND	2.6	61.9	0.67	0	1.00
Mean $\pm$ SEM	2.00 $\pm$ 1.16	55.63 $\pm$ 5.35*	3.82 $\pm$ 3.47	32.75 $\pm$ 12.53*	64.74 $\pm$ 3.91*	1.16 $\pm$ 0.72	1.84 $\pm$ 0.92	5.96 $\pm$ 3.27

ND, not determined; SEM, standard error of the mean.

* $p < 0.05$ with negative control.

HWP-D and HWP-E showed intense staining at the lower part of the gel, with several bands. HWP-C and HWP-F showed no remarkable staining. Patients' IgEs reacted to several protein hydrolysates. HWP-A and HWP-B showed MW 15 000–250 000 or higher smears. The large proteins at the top of the gradient gel represented the high end of the range. HWP-E showed several individualized bands in 3 of the 5 patients. HWP-C, HWP-D and HWP-F showed no reaction (Fig. 2b). All patients examined had almost identical reaction patterns.

HWP-A and HWP-B significantly enhanced basophil CD203c expression, to a similar extent as the HWP-A-supplemented soap solution used by the patients (Fig. 3). In contrast, other HWP preparations and the supplement-free soap solution did not significantly enhance CD203c expression.

SEC analysis

The SEC analysis showed dominant peaks of HWP-A and HWP-B at an elution volume corresponding to MWs

of approximately 669 000 and 158 000, respectively (Fig. 4). Peaks of HWP-D, HWP-E and HWP-F were observed at elution volumes corresponding to MWs between 13 700 and 6500, and the HWP-C peak was observed at <6500.

Discussion

We have already shown that the *in vitro* wheat protein-induced basophil activation test for quantifying CD203c expression aids in the diagnosis of HWP-WDEIA and CO-WDEIA, as CD203c expression clearly differentiates the sensitization conditions of both WDEIA subtypes in accordance with the results obtained with the prick test, serum allergen-specific IgE, and immunoblotting (9). Here, we successfully evaluated the allergenicity of six different HWP variants by IgE immunoblotting and surface CD203c expression on basophils. We devised a new procedure for detecting the allergenicity of HWP by measuring HWP-induced enhancement of CD203c expression on basophils.

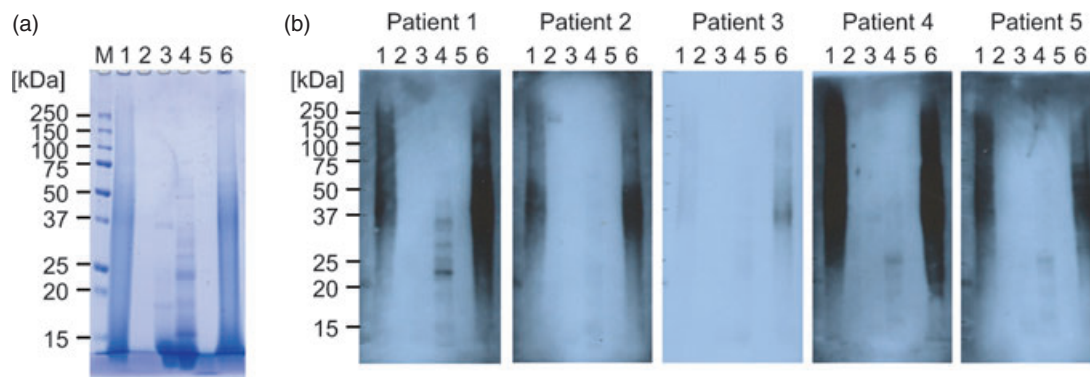


Fig. 2. Immunoblotting analyses. (a) Gel stained with Coomassie Brilliant Blue. (b) Immunoblotting of patients' sera. M, marker proteins. Lane 1: Hydrolysed wheat protein (HWP)-B. Lane 2: HWP-C. Lane 3: HWP-D. Lane 4: HWP-E. Lane 5: HWP-F. Lane 6: HWP-A.

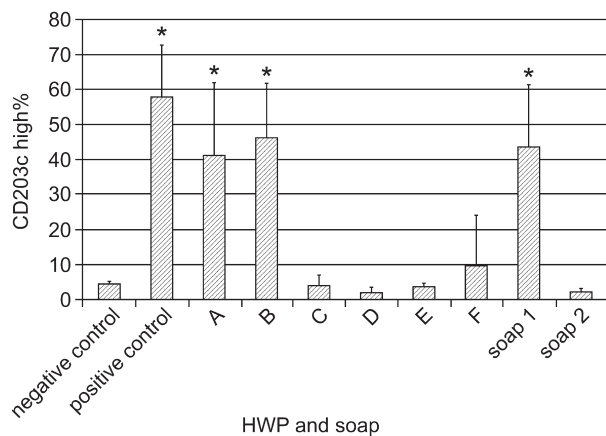


Fig. 3. Expression of CD203c on basophils induced with several hydrolysed wheat proteins (HWPs) and two soap solutions. (a) HWP-A. (b) HWP-B. (c) HWP-C. (d) HWP-D. (e) HWP-E. (f) HWP-F. Soap 1: soap containing HWP-A. Soap 2: soap containing no HWP. Each sample was added at 0.1 $\mu\text{g}/\text{ml}$. * $p < 0.05$ as compared with negative control.

Our results indicate that HWPs composed of large polypeptide aggregates induce sensitization to a greater degree than lower-MW HWPs, because HWP-A and HWP-B showed larger MWs than HWP-C, HWP-D, HWP-E, and HWP-F. HWPs are prepared either from insolubilized total flour proteins or gluten. In order to increase the solubility of gluten, acidic or enzymatic hydrolysis was performed. The procedure used and the degree of hydrolysis depend on the desired functionality and on the manufacturer. Large undefined aggregates of HWP-A and HWP-B produced by acid hydrolysis were observed as smears in SDS-PAGE (Fig. 2). Laurière et al. reported that IgE reacted mostly with large aggregates of HWP in patients with contact urticaria caused by cosmetics containing HWP (15). This is supported by a previous report (16) showing that artificial polymerization of ω -5 gliadin increases its direct reactivity with IgE in an immunosorbent assay and the prick test. It was also hypothesized that larger polymers have better IgE-bridging capacities.

CD203c is an ecto-enzyme belonging to a family of ectonucleotide pyrophosphatases and phosphodiesterases. It is expressed on the cell membranes of human peripheral basophils and mast cells, and cross-linking of the high-affinity IgE receptor upregulates CD203c expression on the cell membrane, indicating that the CD203c test is an excellent *in vitro* test for evaluating IgE sensitization and detecting allergens causing allergic symptoms. CD203c was previously proposed to be a useful marker in the diagnosis of paediatric allergies induced by wheat, hen eggs, and cow's milk (17, 18). In our previous study, we

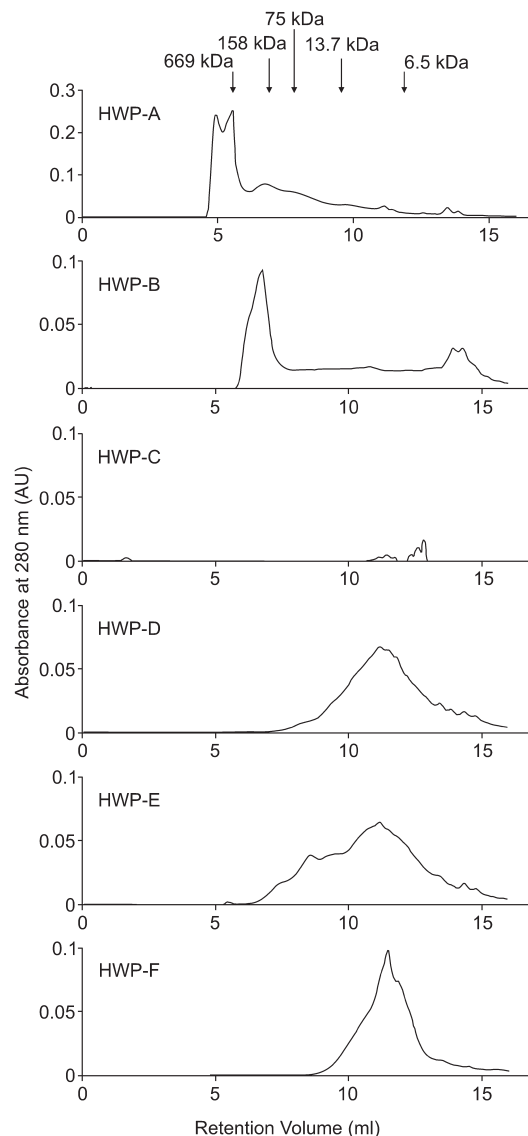


Fig. 4. Size-exclusion chromatography (SEC) profiles of the hydrolysed wheat proteins (HWPs).

showed that the *in vitro* wheat protein-induced basophil activation test for quantifying CD203c expression was very useful for diagnosing the subtypes of WDEIA, namely, HWP-WDEIA and CO-WDEIA, as the determination of CD203c expression clearly differentiated the sensitization conditions of both types of WDEIA in good accordance with the clinical conditions (9). In this study, native ω -5 gliadin did not induce upregulation of CD203c expression in patients with HWP-WDEIA, even in the case of the patient who had serum ω -5 gliadin-specific IgE as determined with the CAP-FEIA system. This indicated that IgE produced against HWP in patients with HWP-WDEIA

does not react to ω -5 gliadin, but to other undetermined protein components with specific epitopes. The present study extends the use of the CD203c test to evaluate the allergenicity of different kinds of protein such as HWP. The fact that the basophil activation test requires only small amounts of blood and allergen is another advantage of the test, because it is possible to use the test to simultaneously evaluate a series of allergens. To diagnose WDEIA, we typically perform an exercise/aspirin challenge test combined with wheat ingestion for patients who have episodes of anaphylaxis after wheat intake. However, the challenge test is unsafe for patients, because anaphylactic shock was sometimes elicited during the test. Therefore, an *in vitro* diagnostic method predicting the development of symptoms from wheat and exercise/aspirin challenge is necessary for patients with WDEIA.

All of our patients were considered to be sensitized to HWP-A via the percutaneous and/or rhinoconjunctival route, because they presented symptoms following use of the HWP-supplemented soap. The importance of percutaneous sensitization in the development of food allergies has already been well recognized in the case of latex allergies in which fruits and vegetables are the causative agents (19). Direct percutaneous sensitization by peanuts has recently been suggested by Fox et al. (20), who identified a dose–response relationship between environmental (non-oral) peanut exposure and the development of peanut allergies. It is possible that our patients have been sensitized by eating food containing HWP. However, almost all of our patients with HWP-WDEIA initially experienced contact urticaria after using HWP-A-supplemented soap, and later developed generalized symptoms upon ingestion of wheat products. In Japan, this soap was very popular, and more than 46 million soaps had been sold between March 2004 and September 2010. As a result, more than 1300 people who have used the same brand of soap have developed allergic symptoms after ingesting natural wheat proteins to date (21). Unfortunately, neither the manufacturer or the producer have ever noticed the danger of high-MW HWP, because they had little information about the protein itself. HWP has been widely used in cosmetics and foods, but only several cases of contact urticaria following exposure to HWP in cosmetics had been described before the outbreak in Japan (7–9, 15, 22–25). Among them, some patients developed generalized allergic symptoms upon ingestion of wheat products. This study provides evidence that HWP with high-MW components could selectively cause percutaneous sensitization when added as an ingredient in soaps or cosmetics. This remains to be determined with rodent sensitization tests.

For sensitization by the percutaneous and/or the rhinoconjunctival route, the molecule must penetrate the epithelium. Large molecules are not expected to cross the skin barrier unless the skin is damaged (26). Antigen exposure through inflamed skin or gastrointestinal mucosa might be involved in the establishment of allergy and tolerance (27). However, our patients did not have atopic dermatitis, which arises from impaired function of the skin barrier. In addition to the creation of new terminal amino-charged and carboxyl-charged groups, owing to the hydrolysis of peptide bonds, the presence of HWP in soap might facilitate its penetration into the epidermis, because soap contains surfactants that may destroy skin barriers.

The mechanism of IgE cross-reactivity in patients with HWP-WDEIA caused by natural wheat has not yet been clarified. Interestingly, native ω -5 gliadin did not enhance basophil CD203c expression in our patients, even in patients 4 and 7, who had serum ω -5 gliadin-specific IgE. This indicates that IgE produced against HWP-A in HWP-WDEIA patients does not react to ω -5 gliadin, but to other undetermined protein components with specific epitopes. This is in accordance with the previous observations of HWP-WDEIA patients with no or lower levels of ω -5 gliadin-specific IgE (7). It is suggested that acidic hydrolysis induces a conformational change in HWP, converting a glutamine to glutamic acid and an asparagine to aspartic acid (28), yielding new epitopes that differ from the epitopes of natural wheat proteins. As we discussed in the previous report (9), it is conceivable that humans do not have sufficient tolerance to HWPs that are not natural proteins, and are therefore easily sensitized to HWP. As wheat proteins contain repetitive amino acid structures rich in glutamine and proline, IgEs against HWP probably cross-react with natural wheat proteins. This is supported by the fact that preincubation of sera with HWP decreased binding of IgE to the natural wheat proteins.

In conclusion, HWPs composed of large polypeptide aggregates possibly induce sensitization to a greater degree than lower-MW HWPs. We devised a new procedure for detecting the allergenicity of HWP by measuring HWP-induced enhancement of surface CD203c expression on basophils. Because HWPs are used in a variety of products worldwide, physicians and industry should be aware of their allergenicity.

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