

Evaluation of the cross-reactivity of antigens in Glupearl 19S and other hydrolysed wheat proteins in cosmetics

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Summary

Background. In Japan, over 2000 users of a facial soap containing Glupearl 19S (GP19S), a hydrolysed wheat protein (HWP), developed immediate-type systemic wheat allergy (HWP-IWA), and ~70% of them developed associated contact urticaria.

Objectives. We investigated whether HWP-IWA patients cross-react with other HWPs, and analysed HWP antigenic characteristics.

Methods. We used 10 types of HWP that are commercially available as cosmetic ingredients, and 16 subjects with HWP-IWA. We performed an enzyme-linked immunosorbent assay (ELISA) to evaluate the reactivity to each HWP, and western blotting to evaluate the characteristics of the antigens by using HWP-IWA patients' serum IgE antibodies. We also performed prick tests with the HWPs.

Results. The patients reacted to four other HWPs in addition to GP19S, according to ELISA, and this was confirmed by strong reactions in the prick tests to the same four types of HWP. Smears of antigens with molecular weights ranging from the high range to the low range were seen on western blotting with the four HWPs that showed strong reactions in the ELISA and prick tests.

Conclusions. HWP-IWA patients cross-react with other HWPs. The antigens that they cross-reacted to had a molecular weight distribution similar to that of GP19S present in the HWPs.

Key words: anaphylaxis; antigenicity; contact urticaria; cosmetics; cross-reactivity; Glupearl 19S; hydrolysed wheat protein; immediate-type wheat allergy.

In Japan, over 2000 users of a facial soap containing a specific type of hydrolysed wheat protein (HWP) have developed *de novo* immediate-type wheat allergy (HWP-IWA), and ~70% of patients have developed

contact urticaria; this has become a social issue. The facial soap concerned was called Cha no Shizuku ('Drop of Tea'), and contained a type of HWP called Glupearl 19S (GP19S) (1, 2). HWP-IWA, in many ways, resembles conventional wheat-dependent exercise-induced anaphylaxis (CO-WDEIA), but these conditions differ in terms of the presence of specific IgE antibodies against GP19S (3). Discontinuing the use of this soap has been shown to reduce the levels of GP19S-specific IgE antibodies and alleviate symptoms. Thus, avoiding contact with the antigen in the soap is believed to be important for a good prognosis. HWP is a widely used raw ingredient in cosmetics, and exists in numerous other forms, apart from GP19S.

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The question of whether other types of HWP contain antigens that can cause HWP-IWA needs to be urgently addressed.

Although there have been a few reported cases from Europe and the United States of immediate-type wheat allergy to HWP in cosmetics, there have been no previous report of large numbers of adverse events such as those caused by GP19S (4–7). HWP is used not only in cosmetics but also as a food additive, and Denery-Papini et al. reported cases of allergy to HWP in food (8). In the case of Cha no Shizuku soap, many patients were asymptomatic when they used the soap itself, and symptoms only developed after ingestion of food that contained wheat. For allergies with this type of mechanism, it can be difficult to identify the cause before large numbers of patients become affected (9). Although no widespread adverse effects of HWPs other than GP19S have been reported, their re-evaluation is an important task.

Materials and Methods

Hydrolysed wheat proteins

Samples of 10 different types of HWP, including GP19S, were supplied by their manufacturers or by the Japan Cosmetic Industry Association, together with data on matters such as their method of decomposition and average molecular weight (Table 1). HWP-1 is a raw ingredient made by a Japanese company, and was used for a short time as a substitute raw ingredient in Cha no Shizuku soap instead of GP19S. HWP-2, HWP-3, HWP-4 and HWP-5 are raw ingredients made by a German company, and HWP-6, HWP-7, HWP-8 and HWP-9 are made by a British company.

Subjects

The HWP-IWA group (n = 16) consisted of patients diagnosed with immediate-type wheat allergy induced

Table 1. Properties of hydrolysed wheat proteins (HWPs)

Sample	Hydrolysis method	Average molecular weight	State
GP19S	Acid	55 000	Powder
HWP-1	Alkali and enzyme	Several hundred	Powder
HWP-2	Enzyme	80 000	Powder
HWP-3	Enzyme	500	Solution
HWP-4	Enzyme	2500–5000	Solution
HWP-5	Enzyme	2500–5000	Solution
HWP-6	Enzyme	3000	Powder
HWP-7	Enzyme	1000	Solution
HWP-8	Enzyme	3500	Solution
HWP-9	Alkali	100 000	Solution

by GP19S according to the diagnostic criteria established by the Special Committee for the Safety of Protein Hydrolysates in Cosmetics of the Japanese Society of Allergology; the CO-WDEIA group (n = 5) consisted of patients with CO-WDEIA; and the healthy control group (n = 5) consisted of individuals without wheat allergy. The presence of wheat-specific, gluten-specific and ω -5 gliadin-specific IgE was determined with ImmunoCAP (Phadia, Uppsala, Sweden), and GP19S-specific IgE was measured with an enzyme-linked immunosorbent assay (ELISA) (3) (Table 2). This study was approved by the Ethics Committee of Fujita Health University (No. 11-210). Venous blood samples were collected with patients' informed consent.

Enzyme-linked immunosorbent assay

GP19S, HWP-1, HWP-2 and HWP-6 were dissolved in 1% phosphate-buffered saline (PBS) and centrifuged, and the supernatants were used as samples. For HWP-3, HWP-4, HWP-5, HWP-7, and HWP-8, the original solutions were used as samples, and for HWP-9, the original solution was diluted 1:1 with water for use as the sample. Next, 100 μ l

Table 2. Specific IgE antibody titres for each patient

ID	Specific IgE			
	GP19S Units	Wheat UA/ml	Gluten UA/ml	ω -5 gliadin UA/ml
CO-WDEIA 1	<3.0	2.21	10.9	9.28
CO-WDEIA 2	<3.0	1.19	2.37	4.58
CO-WDEIA 3	<3.0	1.18	3.85	15.9
CO-WDEIA 4	<3.0	1.51	5.01	15.1
CO-WDEIA 5	<3.0	1.07	3.55	5.25
HWP-IWA 1	8.7	<0.34	0.56	<0.34
HWP-IWA 2	10.8	0.35	0.73	<0.34
HWP-IWA 3	12.6	<0.34	<0.34	<0.34
HWP-IWA 4	20.5	1.70	2.53	<0.34
HWP-IWA 5	28.3	0.67	1.41	<0.34
HWP-IWA 6	28.3	1.27	1.84	<0.34
HWP-IWA 7	35.1	0.49	1.11	<0.34
HWP-IWA 8	35.6	0.68	0.81	<0.34
HWP-IWA 9	39.0	1.08	1.53	<0.34
HWP-IWA 10	60.4	3.49	10.0	<0.34
HWP-IWA 11	71.3	2.21	1.94	<0.34
HWP-IWA 12	>100*	4.52	7.16	<0.34
HWP-IWA 13	>100*	3.36	8.33	<0.34
HWP-IWA 14	>100*	6.24	11.8	<0.34
HWP-IWA 15	>100*	3.0	5.45	0.49
HWP-IWA 16	>100*	2.28	5.37	0.71

*The upper limit for this test method is 100 units.

CO-WDEIA, conventional wheat-dependent exercise-induced anaphylaxis; GP19S, Glupearl 19S; HWP-IWA, immediate-type systemic wheat allergy to hydrolysed wheat protein.

of sample were added to each well of a Nunc MaxiSorp® flat-bottomed 96-well plate (Thermo Fisher Scientific Inc., Waltham, MA, USA), and the plate was sealed and left overnight at 4°C. The plate was blocked with 1% skimmed milk/PBS with 0.1% Tween-20 (PBS-T) for 1 h at room temperature, after which 100 µl of the subjects' serum (healthy, n = 5; CO-WDEIA, n = 5; HWP-IWA, n = 10) diluted to 20% in 1% skimmed milk/PBS-T was added to the wells, and a further incubation was performed for 1 h at room temperature. The plate was then washed with 1% skimmed milk/PBS-T. A total of 100 µl of 0.1 µg/ml anti-human IgE-horseradish peroxidase (HRP) conjugate (KPL, Gaithersburg, MD, USA) in 1% skimmed milk/PBS-T was added to the wells, and the plate was incubated for 1 h at room temperature. The plate was washed, and the colorimetric reaction was developed by adding 1-Step™ Ultra TMB-ELISA (Thermo Fisher Scientific) and incubating for 15 min at room temperature. The reaction was stopped by adding 2 M H₂SO₄. Absorbance was measured with a VersaMax® multi-plate optical densitometer (Molecular Devices, LLC, Sunnyvale, CA, USA), at a wavelength of 450 nm.

Prick test

HWPs were diluted with sterile physiological saline (PS). One drop of the diluted solution was applied to the forearm skin, which was then pierced with a lancet (Prick Lancetter, Ewo Care AB, Gislaved, Sweden). Excess liquid was removed with a paper towel, and the test site was examined after 15 min. The short and long diameters (mm) of wheals were measured.

Western blotting

HWPs were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [Novex NuPAGE 4–12% gels, with MOPS buffer (Life Technologies, Carlsbad, CA, USA)]. SDS-PAGE gels were blotted onto poly(vinylidene difluoride) membranes (Immobilon-FL PVDF; Millipore, Billerica, MA, USA). Membranes were blocked with 3% skimmed milk/PBS-T for 1 h at room temperature. After sera had been mixed in each group (healthy group, n = 4; CO-WDEIA group, n = 4, nos. 1–4; HWP-IWA group, n = 4, nos. 11–14), the membranes were incubated with a total of 10% of the mixed sera diluted in 3% skimmed milk/PBS-T for 1 h at room temperature. The membranes were washed with 3% skimmed milk/PBS-T, and then incubated with 0.1 µg/ml anti-human IgE-HRP conjugate (KPL) in 3% skimmed milk/PBS-T for 1 h at room temperature. The membranes were washed, and the chemiluminescence

reaction was developed with ECL™ Plus Western Blotting Detection Reagents (GE Healthcare, Little Chalfont, UK), and the fluorescent images were captured with Typhoon™ FLA 9500 (GE Healthcare).

Results

Enzyme-linked immunosorbent assay

Healthy individuals' and CO-WDEIA patients' IgE antibodies hardly reacted to any type of HWP. The reactions of IgE antibodies from patients with HWP-IWA to different types of HWP varied; there was little reaction to HWP-1, HWP-2, HWP-3, HWP-4, and HWP-5, but strong reactions to HWP-6, HWP-7, HWP-8, and HWP-9. These patients reacted significantly more strongly to HWP-6, HWP-7 and HWP-8 than CO-WDEIA patients, and significantly more strongly to HWP-9 than healthy individuals and CO-WDEIA patients (Fig. 1). Regarding the reactivity of HWP-IWA patients' IgE antibodies to the HWPs, each patient's sample reacted differently to the different types of HWPs, with patient HWP-IWA no. 8 reacting weakly to all HWPs, and patient HWP-IWA no. 13 reacting to HWP-2 and HWP-4. Overall, the reactions to HWP-6, HWP-7, HWP-8 and HWP-9 were as strong as those to GP19S (Fig. 2).

Prick test

Patient CO-WDEIA no. 1 developed a slight wheal in response to GP19S and HWP-9, but this was equivalent to the response to PS, and no HWP-induced wheal formation was observed. The HWP-IWA patients all developed wheals in response to HWP-6, HWP-8, and HWP-9, and all but patient HWP-IWA no. 10 also reacted to HWP-7 with the formation of a wheal. Patients HWP-IWA no. 12 and HWP-IWA no. 14 developed wheals in response to all of the HWPs, but the diameters of the wheals in response to HWP-6, HWP-7, HWP-8 and HWP-9 were larger than those in response to HWP-1, HWP-2, HWP-3, HWP-4, and HWP-5. The 2 patients reacted strongly to HWP-9, almost as strongly as to GP19S (Table 3).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

No proteins were detectable for HWP-1, HWP-6, and HWP-8. The main protein detected in HWP-2 had a molecular weight of ~10 000, and the main proteins detected in HWP-3, HWP-4 and HWP-5 had molecular weights of ~37 000. A protein smear in the low molecular weight region was observed for HWP-7. Proteins with molecular weights spanning from the low range to the

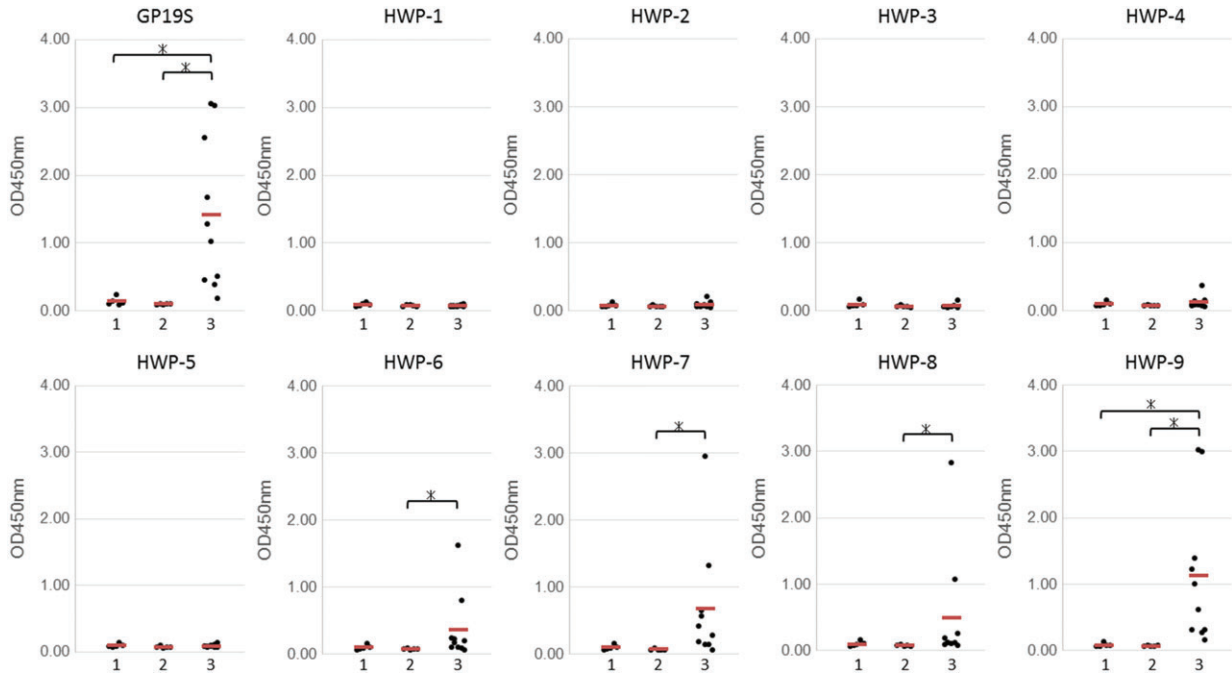


Fig. 1. IgE antibody reactions with each type of hydrolysed wheat protein (HWP) evaluated with enzyme-linked immunosorbent assay. Samples from 5 healthy individuals, conventional wheat-dependent exercise-induced anaphylaxis (CO-WDEIA) patients 1–5, and immediate-type systemic wheat allergy to HWP (HWP-IWA) patients 1–10 were used. The statistical significance of the differences between each group was determined with the Steel–Dwass test. A p -value of <0.05 was considered to be statistically significant. 1, healthy controls; 2, CO-WDEIA group; 3, HWP-IWA group. —, average; * $p < 0.05$. GP19S, Glupearl 19S; OD, optical density.

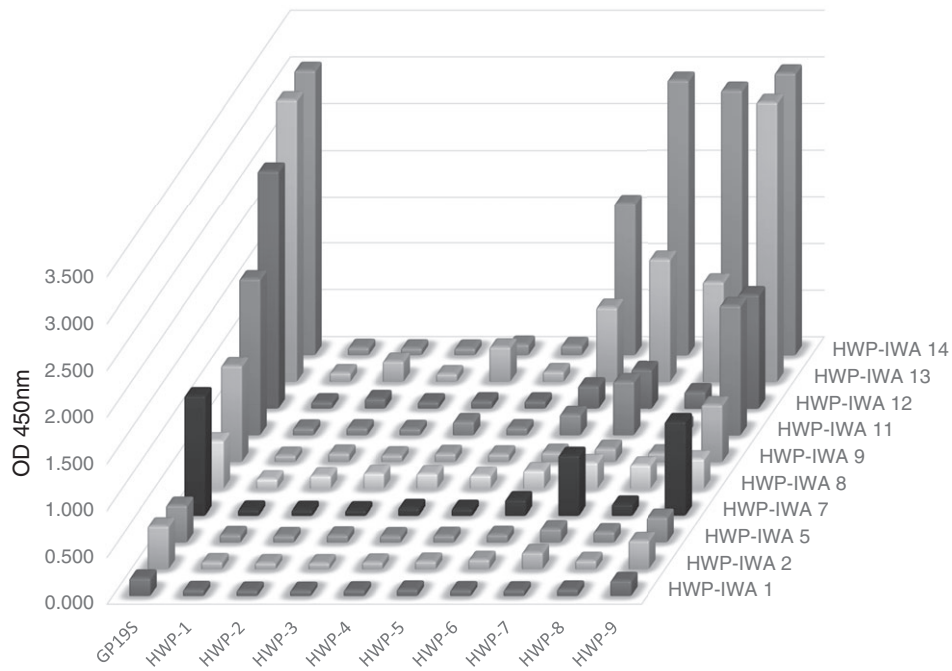


Fig. 2. Comparison of the reactions of IgE antibodies from patients with immediate-type systemic wheat allergy to hydrolysed wheat protein (HWP) (HWP-IWA) to each type of HWP. OD, optical density.

Table 3. Prick test results in those patients who reacted to at least one solution.

ID	Prick test														Histamine								
	GP19S		HWP-1		HWP-2		HWP-3		HWP-4		HWP-5		HWP-6			HWP-7		HWP-8		HWP-9			
	0.01%	0.1%	0.01%	0.1%	0.01%	0.1%	0.01%	0.1%	0.01%	0.1%	0.01%	0.1%	0.01%	0.1%		0.01%	0.1%	0.01%	0.1%	0.01%	0.1%		
CO-WDEIA 1	1 × 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1 × 1	1 × 1	1 × 1	5 × 5
HWP-IWA 3	3 × 3	0	2 × 2	0	0	0	0	0	0	0	0	3 × 3	5 × 3	2 × 2	2 × 3	2 × 2	3 × 3	2 × 2	3 × 3	5 × 4	4 × 4	0	5 × 5
HWP-IWA 4	ND	5 × 5	ND	0	ND	0	ND	0	ND	0	1 × 1	ND	2 × 2	2 × 2	ND	1 × 1	ND	2 × 2	2 × 2	ND	2 × 2	0	5 × 5
HWP-IWA 6	3 × 3	0	0	0	0	0	0	0	0	0	0	0	1 × 1	0	1 × 1	0	1 × 1	0	1 × 1	3 × 3	4 × 3	0	5 × 5
HWP-IWA 10	5 × 5	ND	ND	0	ND	0	ND	0	ND	0	ND	0	ND	3 × 3	ND	0	ND	3 × 3	ND	3 × 3	2 × 2	0	5 × 5
HWP-IWA 12	5 × 6	6 × 4	3 × 3	3 × 3	2 × 2	2 × 3	2 × 2	2 × 2	2 × 2	2 × 2	2 × 2	2 × 2	4 × 4	5 × 5	4 × 4	6 × 6	4 × 4	5 × 4	5 × 5	10 × 6	0	5 × 5	5 × 5
HWP-IWA 14	5 × 5	11 × 8	3 × 3	3 × 3	2 × 2	2 × 2	2 × 2	2 × 2	1 × 1	1 × 1	1 × 1	5 × 5	8 × 8	4 × 4	4 × 4	6 × 6	5 × 5	4 × 4	6 × 6	5 × 5	8 × 8	0	4 × 4
HWP-IWA 15	4 × 4	6 × 6	ND	0	ND	0	ND	0	ND	0	ND	3 × 3	3 × 3	ND	3 × 3	ND	3 × 3	ND	3 × 3	ND	3 × 3	0	4 × 4
HWP-IWA 16	4 × 4	ND	ND	1 × 1	ND	0	ND	0	ND	0	ND	1 × 1	ND	2 × 3	ND	2 × 2	2 × 2	2 × 2	2 × 2	ND	5 × 3	0	5 × 4

CO-WDEIA, conventional wheat-dependent exercise-induced anaphylaxis; GP19S, Glutpearl 19S; HWP, hydrolysed wheat protein; HWP-IWA, immediate-type systemic wheat allergy to hydrolysed wheat protein; ND, not done; PS, physiological saline. Prick test results are shown as long diameter × short diameter of the wheal.

high range were observed for HWP-9, which was similar to what was found for GP19S (Fig. 3a).

Western blotting

Neither healthy individuals nor CO-WDEIA patients had any IgE antibodies that bound to any HWP. HWP-IWA patients varied in their reactivity, depending on the type of HWP. Nothing was detected with HWP-1. Protein bands with molecular weights of ~37 000 were weakly detected for HWP-2, HWP-3, HWP-4, and HWP-5. Proteins with molecular weights spanning from the low range to the high range were observed for HWP-6, HWP-7, HWP-8, and HWP-9, and intense bands at ~30 000 and ~40 000 were detected for HWP-7. The pattern for HWP-9 resembled that for GP19S, with a strong reaction. For all of the HWPs, the proteins that reacted with IgE antibodies had molecular weights of >10 000 (Fig. 3b).

Discussion

In Japan, over 2000 users of Cha no Shizuku facial soap containing GP19S, a type of HWP, have developed HWA-IWA, and this has become a social issue. As discontinuing the use of this soap has been shown to reduce the level of GP19S-specific IgE antibodies and alleviate symptoms (9), avoiding contact with this antigen is believed to be important for a good prognosis, but it is unclear whether HWP contains antigens that can cause HWP-IWA. In this study, we investigated the risk of allergy to other HWPs from the perspective of cross-reactivity with GP19S.

Ten types of HWP including GP19S that are actually used as raw ingredients for cosmetics were subjected to analysis. In terms of the characteristics of HWPs as described by the manufacturer or other sources, the difference between GP19S and other HWPs is acid hydrolysis during production rather than differences in average molecular weight or state. We carried out *in vitro* analysis of the antigenicity of these HWPs by using serum IgE antibodies in an ELISA, and *in vivo* antigenic analysis by means of a prick test. The *in vivo* and *in vitro* results were similar, with HWP-6, HWP-7, HWP-8 and HWP-9 eliciting strong reactions in both. The reactions to HWP-9 were particularly strong, and were approximately the same strength as the reactions to GP19S (Fig. 1, Table 3).

Regarding the ELISA results for each HWP-IWA patient, there was an overall trend for a stronger reaction to other HWPs – mainly HWP-6, HWP-7, HWP-8, and HWP-9 – for those who reacted more strongly to GP19S. Although this tendency was also visible in the prick test

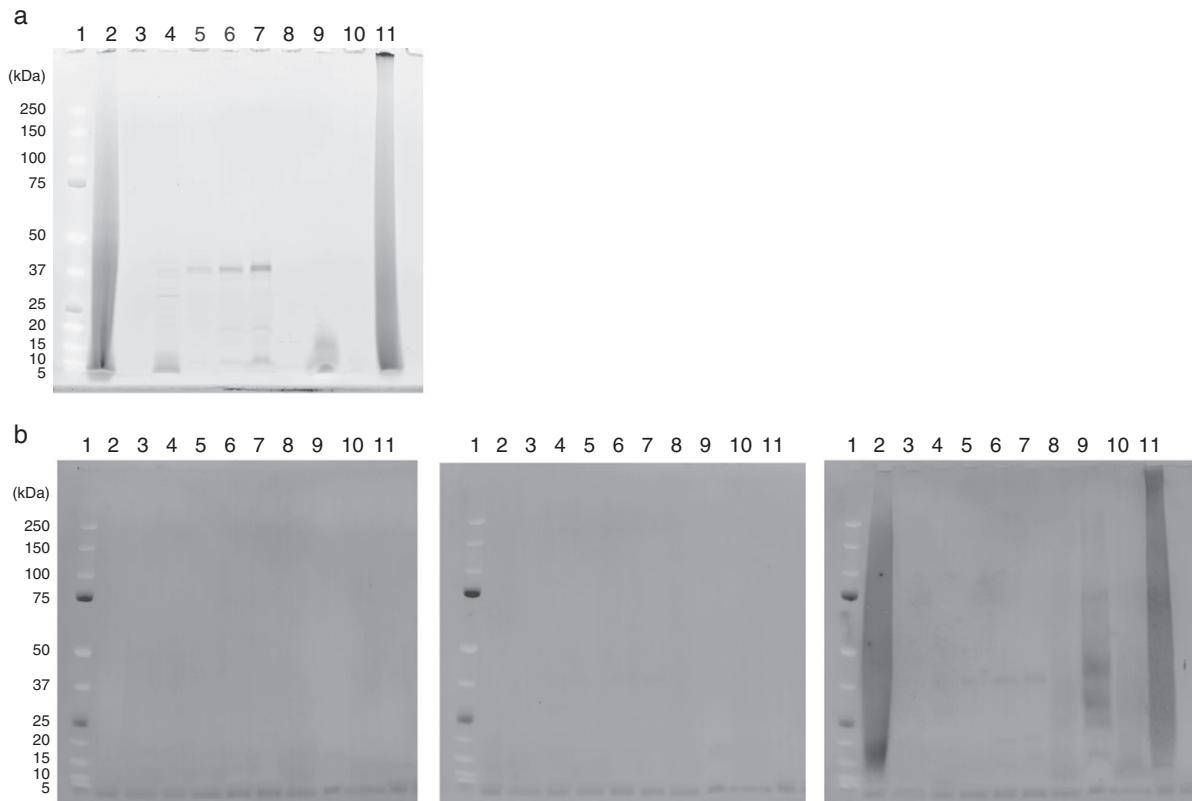


Fig. 3. (a) Fluorescence staining of each hydrolysed wheat protein (HWP) subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. (b) Western blotting with serum IgE antibodies. Left: healthy individuals. Centre: patients with conventional wheat-dependent exercise-induced anaphylaxis. Right: patients with immediate-type systemic wheat allergy to HWP. Lane 1: protein marker. Lane 2: Glupearl 19S. Lane 3: HWP-1. Lane 4: HWP-2. Lane 5: HWP-3. Lane 6: HWP-4. Lane 7: HWP-5. Lane 8: HWP-6. Lane 9: HWP-7. Lane 10: HWP-8. Lane 11: HWP-9.

results, each patient has individual characteristics, which suggests that a degree of variation exists between patients in the epitope sequences that recognize IgE antibodies (Fig. 2). A comparison of the ELISA and prick test results showed that several patients reacted strongly to HWPs other than HWP-6, HWP-7, HWP-8, and HWP-9. For example, patients HWP-IWA no. 12 and HWP-IWA no. 14 developed wheals in reaction to HWP-1, HWP-2, HWP-3, HWP-4 and HWP-5 in the prick test, despite showing almost no reaction to them in the ELISA (Fig. 2, Table 3). This might be because the ELISA only evaluated proteins that adhere to the plate, whereas the prick test is a reflection of the antigenicity of proteins that have entered the skin. From the viewpoint of immediate-type allergies, it is also possible that the ELISA only evaluated the binding of IgE antibodies and antigens, whereas the prick test reactions may be elicited by mechanisms other than that mediated by IgE antibodies.

Although both the *in vivo* and *in vitro* tests yielded strong reactions to HWP-6, HWP-7, HWP-8, and HWP-9,

these did not have anything in common in terms of characteristics such as method of hydrolysis and average molecular weight (Table 1). HWPs are made from wheat, and CO-WDEIA patients are believed to possess IgE antibodies that react specifically to wheat, gluten or ω -5 gliadin, but none of the CO-WDEIA patients in this study showed a specific reaction to HWP (Table 2, Fig. 1). This suggests that significant changes in amino acid sequences that bring about major changes in antigenicity may occur in the process of manufacturing HWPs. Laurière et al. found that antigenicity was generated by decomposing gluten with acid or enzymes (5). Nakamura et al. reported that acid–heat treatment of gluten also generated antigenicity (10). The HWPs that showed high antigenicity – HWP-6, HWP-7, HWP-8, and HWP-9 – were all manufactured in the United Kingdom, and the provision of more detailed information on matters such as their raw materials and manufacturing processes might reveal some of the characteristics contributing to their antigenicity.

To analyse the antigens in the HWPs, we performed western blotting with serum IgE antibodies, and found that these IgE antibodies reacted with proteins in HWP-6, HWP-7, HWP-8, and HWP-9, resulting in a smear that was distributed from the low to the high molecular weight range. All of these HWPs had shown high antigenicity in the ELISA and prick test. The results for HWP-9 closely resembled those for GP19S, reflecting the results of the ELISA and prick test. For all of the HWPs, the proteins that reacted with IgE antibodies extended into the high molecular weight region above 10 000, suggesting that it might be possible to reduce their antigenicity by subjecting them to sufficient decomposition. However, it was difficult to predict their antigenicity on the basis of the average molecular weight data supplied by the manufacturers. The amount of proteins in HWP-6, HWP-7 and HWP-8 that bound to IgE antibodies was too low to be detectable by SDS-PAGE fluorescent staining, and their average molecular weight may not be accurate (Fig. 3a, b).

Our results have shown that patients who developed allergy to GP19S also showed cross-reactivity to some other HWPs. This suggests that other HWPs might contain the epitope sequences in GP19S. Those HWPs that showed cross-reactivity contained protein antigens

with molecular weights spanning from the low to the high molecular weight range, and it might be possible to reduce their antigenicity by subjecting them to sufficient decomposition. Although this study has shown that HWPs might cross-react in patients with immediate-type wheat allergy who are sensitized to GP19S, it does not show that these HWPs possess a similar capacity for sensitization. No actual adverse effects of HWPs other than GP19S in large numbers of patients have been reported, and the risk of sensitization may be low. The risk will be further examined by the use of animal tests such as those carried out by Nakamura et al. (11). Although there have been several reports that the deamidation reaction is involved in the antigenicity of HWPs (8, 12, 13), no study has yet definitely identified the epitope of GP19S. Whether other HWPs also contain similar epitopes is an important topic for future research. The present study has shown that there are some HWPs actually used in cosmetics that cross-react with GP19S, which has caused adverse effects in a large number of people in Japan.

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