

Evaluation of Allergenicity of Acid-Hydrolyzed Wheat Protein Using an in vitro Elicitation Test

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Key Words

Food allergy · Wheat gluten · Acid hydrolysis · FcεRI · Wheat-dependent exercise-induced anaphylaxis · Mast cell · IgE

Abstract

Background: We performed an in vitro elicitation test to determine the ability of different types of wheat-allergic patients' IgE to induce humanized mast cell activation after the addition of various time-treated acid-hydrolyzed wheat proteins (HWPs). **Methods:** The reactivity of heat- and various time-treated acid-hydrolyzed glutes (acid-HGs) and commercial acid-HWP (HWP1), using serum IgE from wheat allergy accompanied by skin and rhinoconjunctival sensitization to HWP1 in the facial soap, pediatric subjects with food allergy to native wheat, adult wheat-dependent exercise-induced anaphylaxis subjects, and nonatopic healthy subjects, was elucidated by dot blot and a luciferase assay-based in vitro elicitation test (EXILE test). **Results:** Serum from subjects sensitized with HWP1 reacted only to acid-HGs (acid-HGs treated for 0.5–3 or 6 h), but not native gluten, in the results of the dot blot. In contrast, sera from pediatric subjects sensitized with native wheat reacted to native gluten

more strongly and showed only slight reactions to 0.5- to 1-hour-treated acid-HGs. The results of the in vitro elicitation test showed that acid hydrolyzation of the gluten attenuated antigen-induced luciferase expression in a time-dependent manner for sera from native-wheat-sensitized pediatric subjects. On the other hand, in the sera from HWP1-sensitized subjects, acid hydrolyzation of the gluten for 0.5 h dramatically increased luciferase expression. **Conclusions:** Even after prolonged hydrolyzation, acid-HGs still retained the ability to activate mast cells in the case of HWP1-sensitized subjects.

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Introduction

An increasing number of studies have shown that hydrolyzed wheat protein (HWP) can induce immediate hypersensitivity through skin contact and/or food ingestion [1–8]. HWP is a common food and nonfood ingredient. For example, it can be used in cosmetics, shampoos, or hair conditioners. Lauriere et al. [1] showed that immediate contact urticaria can be triggered by HWP present in cosmetics. Moreover, some patients with such al-

lergies can also experience an immediate food allergy reaction when they eat foods containing HWP. In Japan, we reported 5 patients who were primarily sensitized to HWP present in facial soap and experienced wheat-dependent exercise-induced anaphylaxis (WDEIA) after the ingestion of normal wheat products [2]. After our report, more than 600 patients with such an allergy had been reported to date (June 10, 2012) from various institutes all around Japan. Importantly, the majority of the patients used the same facial soap (Cha no Shizuku®; Yuuka Co. Ltd., Fukuoka, Japan) containing acid-hydrolyzed wheat protein (acid-HWP).

Gluten is a salt-insoluble protein complex of wheat and exhibits viscoelastic properties. As for HWP, in most cases hydrolyzed gluten (HG) has been widely used in cosmetics. HG is a mixture of variously sized polypeptides. Their sizes depend on the condition of hydrolysis. The commercial acid-HWP contained in the facial soap (Glupearl 19S®; Katayama Chemical Industries Co. Ltd., Osaka, Japan; described as HWP1 in this article) was produced from gluten after partial hydrolysis with hydrogen chloride at 95°C for 40 min. Based on the SDS-PAGE pattern of HWP1, the molecular weight of a main band was 40–50 kDa.

In the present study, we focused on the allergenicity, as an ability to elicit mast cell activation, of acid-hydrolyzed gluten (acid-HG), which was generated by acid treatment under heated conditions, and intended to acquire new data about the reactivity of various time-treated acid-HGs and commercial acid-HWP (HWP1) using serum IgE from sensitized subjects as follows: sera from 10 subjects with wheat allergy accompanied by skin and rhinoconjunctival sensitization to HWP1 in the facial soap, 8 pediatric subjects with food allergy to native wheat, 9 adult subjects with WDEIA food allergy to native wheat, and 8 nonatopic healthy subjects.

Materials and Methods

HWP1 and Gluten

Glupearl 19S (HWP1) was supplied by Katayama Chemical Industries. Gluten was purchased from Sigma-Aldrich (catalog No. G5004).

Five hundred milligrams of HWP1 and gluten were suspended with 5 ml of 1 M Tris solution (pH 11.4) and left standing for more than 24 h at room temperature, and these suspensions were kept at 4°C and used as stock solutions.

Acid Hydrolysis of Gluten

One milligram of gluten was hydrolyzed in 1 ml of 0.1 N HCl at 100°C for 0.5, 1, 3, 6, 9, 12, 24, and 48 h. After a fixed time, the

acid solution was neutralized with 0.1 N NaOH. At a point of 0 h, gluten was added to the neutralized solution and not heated.

Each sample was separated in 15–25% acrylamide gel (DRC Co., Ltd., Tokyo, Japan) according to the Laemmli method; the gel was then stained with CBB.

Sera

Sera was collected from 10 subjects with wheat allergy accompanied by skin and rhinoconjunctival sensitization to HWP1 in the facial soap (HW1–10 in table 1), 8 pediatric subjects with food allergy to natural wheat (CW1–8), 9 adult subjects with exercise-induced anaphylaxis after ingestion of native wheat (AW1–9), and 8 nonatopic healthy persons (NA1–8). The presence of specific IgEs in the sera was determined using ImmunoCAP® (Phadia AB, Uppsala, Sweden). All of the patients with wheat allergy who had an obvious clinical history and/or a gluten-specific ImmunoCAP class of 3 or above were enrolled into this study. Informed consent was obtained from all patients and volunteers. Our study was approved by the Ethics Review Committee of the National Institute of Health Sciences.

Dot Blot

One microgram of nondenatured gluten or acid-HG was spotted onto a BA83 nitrocellulose membrane (Whatman™; GE Healthcare UK Ltd., Little Chalfont, UK). After being blocked with 0.1% casein/PBS, the membrane was incubated with patients' sera (5- to 10-fold diluted with 0.1% casein/PBS) overnight at 4°C. The membrane was then washed and gently shaken in 500-fold diluted HRP-linked anti-human IgE (Nordic Immunology, Tilburg, The Netherlands) solution for 90 min at room temperature. Binding of serum IgE and dotted samples was detected as HRP conjugates using color-developing Konica Immunostain (Konica Minolta, Tokyo, Japan).

In vitro Elicitation Test

An *in vitro* elicitation test was performed by assessing IgE crosslinking-induced luciferase expression (EXiLE) using human FcεRI genes- and nuclear factor of activated T-cell (NF-AT)-dependent luciferase reporter gene-introduced rat mast cells (RS-ATL8 cells), as previously described [9].

Briefly, RS-ATL8 cells (5×10^4 cells/50 µl/well) were sensitized with 1:100 diluted patients' sera overnight. After washing, the cells were stimulated with 100 ng/ml of acid-HGs suspended in 10% FCS-containing minimum essential medium at 37°C for 3 h in a 5% CO₂ incubator. Then, 50 µl of luciferase substrate solution containing cell lysis reagent (ONE-Glo, Promega Corp., Tokyo, Japan) was added to the cells, and chemiluminescence was measured using an EnVision multilabel plate reader (PerkinElmer, Waltham, Mass., USA). Luciferase expression levels were represented as the fold increase of light units compared with those of nonstimulated cells.

Statistical Analysis

The statistical significance of the differences between each period of the experimental groups was determined using the Steel test versus the NA group at individual time points. $p < 0.05$ was considered statistically significant.

Results

Binding of IgE from HWP- or Gluten-Sensitized Subjects to Various Time-Treated Acid-HGs

To determine the effect of the extent of the acid hydrolysis of gluten on allergenicity, gluten was treated with 0.1 N HCl at 100°C for various times up to 48 h. Figure 1a shows the CBB-stained pattern of the acid-HGs. The CBB-stained patterns changed to a smear-like pattern after acid treatment, and the molecular weight of the main protein bands gradually decreased from 40–70 kDa (0.5–1 h of treatment) to 6 kDa (9–12 h of treatment); after 24–48 h of treatment, CBB-stained bands larger than 6 kDa were no longer detected.

We then examined the binding of serum IgE to these acid-HGs. We performed immune dot blot tests using serum samples of subjects sensitized with HWP1 (HW1, 2), pediatric subjects with wheat allergy (CW1, 2), and an adult WDEIA subject (AW3). As shown in figure 1b, HW1 reacted only to acid-HGs [0.5- to 6-hour-treated acid-HGs and HWP1 (spot H)], while HW2 reacted to gluten and acid-HGs (0.5- to 3-hour-treated acid-HGs and HWP1). In contrast, CW1 and CW2 reacted to native gluten (acid-hydrolyzed for 0 h) more strongly and showed only slight reactions to 0.5- to 1-hour-treated acid-HGs. Minimal IgE binding was observed in the sample from the adult WDEIA subject (AW3).

We also examined the molecular sizes of IgE-binding proteins in acid-HGs and native gluten using Western blotting under denatured conditions (fig. 2). After 0.5–1 h of acid treatment, IgE immunoblotting with a serum sample from subject HW1 showed smears at around 40- to 70-kDa proteins, the molecular weight of which was relatively higher than that of main bands of native gluten on SDS-PAGE (around 30–50 kDa), producing the same binding results for the HWP1. However, after 3 h of acid treatment, the IgE-bindings to the 40- to 70-kDa proteins were time-dependently decreased. In contrast, a Western blot analysis using serum from a native-wheat-sensitized subject (CW1) showed clear binding to native gluten, and acid hydrolyzation decreased the binding in a time-dependent manner. On the other hand, the sample from the AW3 subject showed IgE binding to a 60-kDa protein, which corresponds to ω -5 gliadin (fig. 2c), although a weak signal was obtained using the dot blot test.

Elicitation Test of Various Time-Treated Acid-HGs Using Mast Cells Sensitized with Patients' Sera

We next performed an in vitro elicitation test to determine the ability of IgE from subjects sensitized with

Table 1. Information about sera from subjects with wheat-allergy in the study

Patient No.	Age, years	Gender	Total IgE IU/ml	Wheat-specific IgE, UA/ml	Gluten-specific IgE UA/ml	ω -5 gliadin-specific IgE UA/ml
HW1	42	F	4,050	23.4	31.4	2.06
HW2	22	F	412	9.23	12.4	<0.35
HW3	25	F	1,510	1.79	3.12	<0.35
HW4	55	F	123	4.38	6.15	<0.35
HW5	41	F	285	21.8	36.2	7.01
HW6	51	F	210	2.28	3.65	<0.35
HW7	45	F	3,170	14.6	24.2	<0.35
HW8	23	F	1,510	1.79	3.12	<0.35
HW9	38	F	485	1.93	1.94	<0.35
HW10	50	F	22.9	0.76	1.27	<0.35
CW1	2	M	1,050	19	50.4	1.83
CW2	3	M	512	ND	66.6	0.4
CW3	4	M	1,199	59	>100	6.38
CW4	1	F	191	17.6	ND	ND
CW5	4	M	226	15.9	20	0.44
CW6	7	M	ND	11.4	15.4	0.49
CW7	8	M	532.1	6.36	9.25	<0.35
CW8	4	F	ND	ND	ND	ND
AW1	20	M	283	2.48	3.09	22.8
AW2	16	F	2,120	4.84	7.7	24.9
AW3	62	M	1,480	1.04	3.74	11.1
AW4	56	M	ND	<0.35	0.7	4.3
AW5	49	M	182	4.3	14.2	30.4
AW6	38	M	2,160	1.16	6.63	13.5
AW7	42	M	43	<0.35	0.5	1
AW8	54	M	ND	<0.35	<0.35	10.7
AW9	69	M	1,990	9.92	11.3	26.4

ND = Not determined; HW1–10 = subjects with wheat allergy accompanied by skin and rhinoconjunctival sensitization to HWP1 in the facial soap; CW1–8 = pediatric subjects with food allergy to native wheat; AW1–9 = adult subjects with exercise-induced anaphylaxis after ingestion of native wheat.

HWP1 or native gluten to induce mast cell activation after the addition of acid-HGs. The humanized rat mast cell line RS-ATL8, which was established by introducing the NF-AT-responsive luciferase gene into human Fc ϵ RI-expressing RBL-SX38 cells, was used to measure IgE crosslinking-induced luciferase expression (EXiLE) [9]. A two-fold increase in luciferase expression relative to those without any stimulation is considered to be a clinically relevant cut-off level according to our very recent study [10], and this level is superimposed in figure 3.

As shown in figure 3, commercial acid-HWP (HWP1) induced a significant increase in luciferase expression in the HW and CW groups, but not in the AW group. Acid

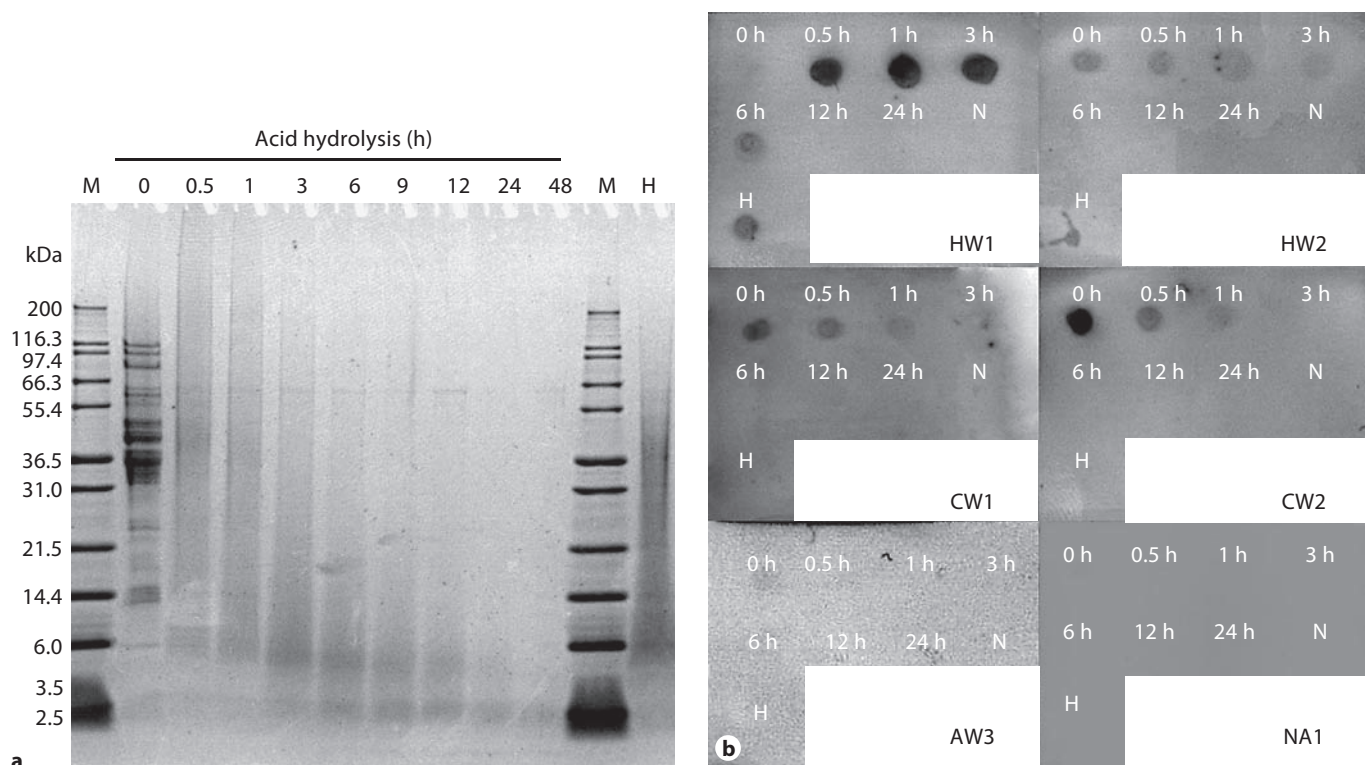


Fig. 1. Binding of IgE from subjects with wheat allergies to acid-HGs. **a** Coomassie brilliant blue-stained pattern of heat- and various time-treated acid-HGs on a 15–25% acrylamide gel. **b** Dot immunoblots of the gluten hydrolysates using sera from subjects sensitive to acid-HGs (HW1, 2), child wheat allergy (CW1, 2), adult WDEIA (AW3), and a nonatopic healthy person (NA1). M = Molecular weight marker; H = HWP1; N = PBS.

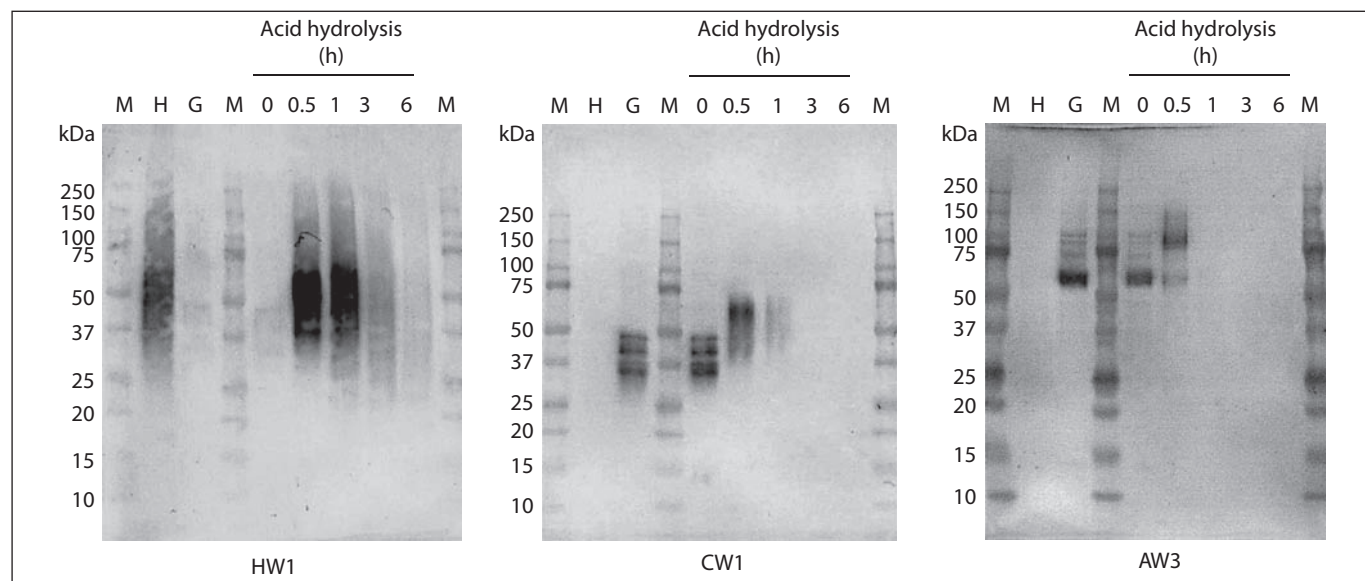
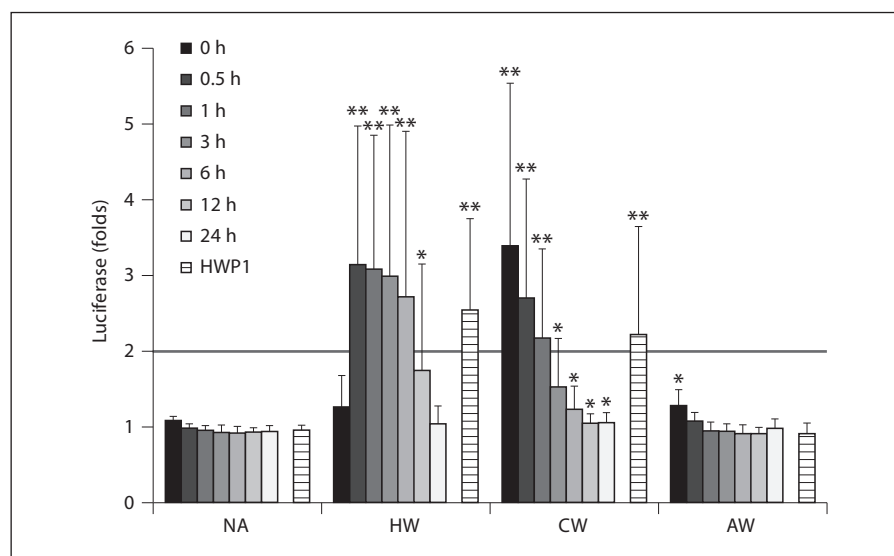


Fig. 2. Immunoblots of gluten hydrolysates using sera from subjects with wheat allergies. HWPs and gluten were separated on 10%–20% SDS-polyacrylamide gels and electronically transferred to nitrocellulose membranes, which were then incubated with serum diluted 5- (CW1, AW3) or 10-fold (HW-1). IgE-binding proteins were detected as HRP conjugates using color development. M = Molecular weight marker; H = HWP1; G = native gluten.

Fig. 3. In vitro elicitation (EXiLE) test using IgE in sera from subjects with wheat allergies. RS-ATL8 cells were sensitized with 1:100-diluted sera overnight and stimulated for 3 h with 100 ng/ml of acid-HGs or HWP1 suspended/dissolved in culture medium. Data are means $\pm$ SD (NA, n = 8; HW, n = 10; CW, n = 8, and AW, n = 9), shown as fold increases. The cut-off level (2-fold increase) is indicated by the gray line. * $p < 0.05$, ** $p < 0.01$ according to Steel's test vs. the NA group at a specific hydrolyzation period.



hydrolyzation of the gluten attenuated antigen-induced luciferase expression in a time-dependent manner for sera from native-wheat-sensitized pediatric subjects (CW4). On the other hand, in the sera samples from HW subjects, acid hydrolyzation of the gluten for 0.5 h dramatically increased luciferase expression, whereas native gluten had little effect on mast cell activation, suggesting the contribution of neo-epitopes. Even after prolonged hydrolyzation for 6 h, acid-HG still retained the ability to activate mast cells, with an activity level that was only slightly decreased. Contrary to the pediatric subjects, in the case of the adult WDEIA subject (AW), none of the HG, including HWP1, was capable of inducing mast cell activation.

Discussion

Although it is generally believed that hydrolysis reduces the allergenicity of allergenic protein, the number of studies that have shown that HWP induces immediate hypersensitivity via skin contact and/or food ingestion has increased [1–8]. The immunoreactivity of HWP has been reported by us and other groups [2, 6, 7]. Lauriere et al. [7] reported that the immunoblotting analysis of European patients' sera showed strong IgE binding to ω -1, ω -2, and γ -gliadin, but not to ω -5 gliadin. Bouchez-Mahiout et al. [6] reported that acid-HGs contained highly IgE-reacting high molecular weight entities and they have been suggested to have neo-epitopes which would be produced by rearrangements of hydrolyzed peptides. In the case of Japanese patients with HWP, their sera showed a low level of IgE-

positive binding to ω -5 gliadin, and it seems that they were sensitized by gliadins other than ω -5 (or glutenins), which can be clearly distinguished from conventional WDEIA [2]. However, the detailed allergenicity of HWP has not yet been examined. In our present study, the acid hydrolyzation of gluten has generated not only prolonged IgE binding activity but also an ability to elicit mast cell activation in cases of sera from HW subjects (fig. 1–3). In contrast, IgE from native gluten-sensitized pediatric subjects showed gradually decreased reactivities to gluten acid hydrolysates time dependently, suggesting that native gluten epitopes, which were recognized by these IgE, were labile by acid- and heat-treatment. Acid lability of native gluten epitopes is consistent with previous reports that wheat proteins which were extensively hydrolyzed by acid could not inhibit the binding ability between the IgE antibodies in the patient sera and wheat proteins [11]. These results also suggested that the neo-epitopes in acid hydrolysates are relatively stable after prolonged acid- and heat-treatment.

There have been some previous reports regarding assessments of the allergenicity of processed foods. Díaz-Perales et al. [12] reported that some pepsin digestion-resistant peptides (MW, 1,400–5,100 Da) could bind to a patient's IgE, inducing allergic symptoms. Takagi et al. [13] also kinetically analyzed the digestion of ovomucoid in simulated gastric fluid and showed that in 21% of the examined patients, the capacity of their IgE to bind to the small 4.5-kDa fragment was retained. Furthermore, another reference reported an increase in allergenicity after the transglutaminase-mediated crosslinking of ω -5 gliadin [14]. In the case of HWP1, acid hydrolyzation seems

to have induced the physicochemical changes in gluten proteins after acid and heat treatment and some modifications to the amino acid molecules and protein aggregates. These changes may lead to an increase in the allergenicity of gluten. The remaining allergenicity of the 6-hour-treated acid-HG, as shown in figure 3, can be explained by the trace amount of persistent protein aggregates with a high molecular weight or by acid treatment-resistant peptide fragments with a lower molecular weight.

The results of the in vitro elicitation test (fig. 3) were consistent with those of the dot blot analysis (fig. 1b), suggesting that the antigen-specific IgE that bound to the hydrolyzed proteins with a lower molecular weight in the dot blot analysis might have the ability to crosslink to FcεRI on mast cells in the HW subjects. On the other hand, the AW3 subject had negative results for both the dot blot analysis (fig. 1b) and the EXiLE test (fig. 3), although strong IgE binding to ω-5 gliadin was observed in the Western blotting analysis (fig. 2c). This discrepancy can be explained by the difference in IgE accessibility to the antigen under denatured (Western blot) and nondenatured (dot blot and EXiLE) conditions. In addition, in previous studies describing allergenicity assess-

ments of processed foods [12–14], ELISA and skin prick tests were mainly used. However, ELISA only provides binding information for IgE and the allergen, and skin prick tests are followed by a slight risk of anaphylaxis and sensitization. In this context, the EXiLE test may be a useful method for assessing the allergenicity of processed foods depending on IgE crosslinking-induced mast cell activation without any risk of anaphylaxis. Furthermore, we could confirm that the EXiLE system is a sensitive and convenient method to detect functional epitopes. The basophil activation test seems to be useful for similar purposes; however, this ex vivo test requires whole blood specimens which cannot be preserved for long periods.

In conclusion, this study showed production of neo-epitopes (and/or new arrangements of hidden epitopes) that are stable after prolonged acid and heat treatment. How such epitopes were generated by acid hydrolysis remains to be determined.

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