

Gliadin-specific IgE in wheat-dependent exercise-induced anaphylaxis

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ABSTRACT

Food-dependent exercise-induced anaphylaxis is a unique form of food allergy. Recent studies indicate that gliadin is a major allergen in patients with wheat-dependent exercise-induced anaphylaxis (WDEIA). We evaluated the value of serum gliadin-specific IgE in the diagnosis of WDEIA, as well as the characteristics and digestibilities of the IgE-binding components of gliadin. We analyzed 32 adult subjects sensitized to wheat; 26 were asymptomatic sensitizers and 6 had WDEIA. Wheat flour and gliadin-specific IgE levels were measured, and corresponding receiver operator characteristics (ROC) curves were determined. Patients with WDEIA had higher levels of gliadin-specific IgE than patients without WDEIA ($p = 0.003$). The area under the ROC curve for gliadin-specific IgE was 0.872, which suggested this assay could be used as a supplementary test for the diagnosis of WDEIA. IgE immunoblot analyses of reactions to wheat and gliadin extracts were compared both with and without simulated gastric fluid (SGF) and intestinal fluid incubation (SIF). All six WDEIA patients had high IgE binding signals to 50 kDa of gliadin extract on IgE immunoblots. This binding was suppressed by SGF, although minimal inhibition was noted with SIF incubation.

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Key words: Anaphylaxis, digestive enzyme, exercise, food allergy, food-dependent exercise-induced anaphylaxis, gliadin, IgE, ROC curve, wheat

Postprandial exercise-induced anaphylaxis, a condition whereby ingestion of food in connection with physical exercise induces symptoms of anaphylaxis, was first reported by Maulitz in 1979.¹ Since then, two subsets of food-dependent exercise-induced anaphylaxis (FDEIA) have been reported: the first involves a broad range of unspecified foods, whereas the second is dependent on specific foods. FDEIA is a unique allergic disease with important clinical significance; understanding FDEIA can potentially help elucidate the pathogenetic mechanisms of allergic diseases. The frequently reported cause of FDEIA in Europe and Asia is wheat.^{2–5} Recent findings surrounding the pathogenetic mechanisms of wheat-dependent exercise-induced anaphylaxis (WDEIA) indicate that the causative allergen is ω_5 -gliadin.^{3,6,7} Several factors such as exercise, aspirin, and alcohol can facilitate the absorption of the allergenically intact gliadin

from the gastrointestinal tract.^{8–10} However, diagnosis of FDEIA and the causative food is often difficult.

Double-blind placebo-controlled food challenge (DB-PCFC) is considered the gold standard for diagnosing food allergy.^{11,12} For the diagnosis of FDEIA, food and exercise challenges are administered by trained medical staff in appropriate facilities. The DBPCFC was not performed not only because of the exceptional difficulty associated with its use for the diagnosis of FDEIA, but also because of the special needs required for its performance in terms of specialized facilities, trained medical staff, time, cost, risk, and difficulties in blinding of the test.¹³ Tests indicative of clinical reactivity during these challenges include a skin-prick test (SPT) and the measurement of specific IgE levels.¹² In a prospective analysis of children and adolescents with atopic dermatitis and food allergy, discrete food-specific IgE concentrations were used to predict clinical reactivity to eggs, milk, peanuts, and fish with >95% certainty.¹⁴ Unfortunately, levels of specific IgE to wheat were too general, preventing a predictive value of 90% from being reached.¹⁵ In addition, 20% of patients with WDEIA were CAP-fluorescent enzyme immunoassay (Pharmacia Diagnostics, Uppsala, Sweden) negative to gluten.¹⁶

To improve on current diagnostic procedures, we analyzed the significance of serum-specific IgE antibodies to wheat and gliadin for the diagnosis of WDEIA. In addition, we characterized IgE-binding

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components using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and IgE immunoblots and examined their stability in the presence of digestive enzymes.

MATERIALS AND METHODS

Subjects

Six patients with WDEIA and recurrent episodes of anaphylaxis and 26 wheat-sensitized patients without wheat-associated allergy symptoms were enrolled. WDEIA patients were enrolled either by referral to the Allergy Clinic or through visitation to the Emergency Department of Ajou University Hospital in Suwon. All patients showed a history of exercise-induced anaphylaxis after consuming bread or noodles. Control subjects ($n = 26$) were chosen from the 1700 patients who visited the Allergy Clinic between January and December 2001 for various allergic symptoms. These patients had positive wheat SPT results, but none had FDEIA symptoms or wheat-associated allergies. Informed written consent was obtained from all patients. Serum from these patients was tested for specific IgE to wheat and gliadin by enzyme-linked immunosorbent assay (ELISA). ELISA inhibition tests, IgE immunoblotting, and digestibility tests were also performed.

Preparation of Allergen Extracts

Common inhalant allergens and histamines for SPTs were purchased (Allergopharma Joachim Ganzer KG, Reinbek, Germany). Thirty food allergens commonly consumed in Korea were prepared. Gliadin was purchased from Sigma (Sigma Co., St. Louis, MO). Food allergens were prepared according to a method described previously.¹⁷ Briefly, foodstuffs were homogenized in phosphate-buffered saline. The homogenate was kept at 4°C overnight, followed by centrifugation. The supernatant was then dialyzed in 4 L of PBS using a molecular weight cutoff of 6 kDa (Spectrum Medical Industries, Houston, TX). The supernatant was subsequently passed through a syringe filter (Nalgene, Rochester, NY) and lyophilized for ELISA, inhibition, immunoblotting, and digestibility assays. For SPTs, the extract was mixed with an equal amount of glycerin (Sigma Co.).

Skin-Prick Test

Routine SPTs were performed on the backs of patients using 50 common inhalant allergens and 30 food allergens. Histamine (1 mg/mL) and PBS were used as positive and negative controls. The results were assessed after 15 minutes. The wheal size produced by each antigen and histamine was expressed as a mean of the maximum diameter and the vertical length at the maximal length midportion. Skin reactivity was expressed as the ratio of allergen wheal size to histamine

(A/H).¹⁸ If the A/H ratio was between 0.1 and 1 and the erythema had a diameter <21 mm, it was assigned a score of 1+. For A/H ratios between 0.1 and 1 having erythema >21 mm, a score of 2+ was assigned. The following scores were assigned for the remaining A/H ratios: 3+ for 1–2, 4+ for 2–3, and 5+ for ratios >3. Positive responders were deemed to be those showing a response >2+ on the SPT.

Wheat and Exercise Challenge Test

Patients with a history of WDEIA underwent open food and exercise challenge tests in the Allergy Clinic. Subjects fasted for 6 hours before testing. The type and quantity of wheat product used to challenge each patient were determined based on their clinical history. The amount of wheat intake was 50–100 g. Treadmill exercise was performed 20–30 minutes after wheat ingestion using a standard Bruce protocol. All six patients with history of WDEIA showed more than one of the anaphylactic symptoms or signs, such as urticaria, wheeze, dyspnea, or hypotension, within 15–30 minutes of treadmill exercise.

ELISA for Wheat and Gliadin-Specific IgE Antibodies

ELISA was performed to determine the presence of specific IgE to wheat and gliadin as previously described.¹⁹ Microtiter plates (Costar, New York, NY) were coated with 100 μ L/well of extract and stored at 4°C for 24 hours. Each well was washed and blocked by 3% bovine serum albumin–phosphate buffered saline tween-20. Wells were then incubated for 3 hours at room temperature with either 50 μ L of patient or control serum. Biotin-labeled goat anti-human IgE antibody (Vector Co., Burlingame, CA) was then added and incubated for 1 hour. Wells were then incubated with streptavidin peroxidase (Sigma Co.). Colorimetric conversion was developed with B (3,3',5,5'-tetramethylbenzidine) substrate solution for 10 minutes at room temperature. The reaction was stopped and the absorbance was read at 450 nm using an automated microplate reader (Benchmark; Bio-Rad, Hercules, CA). All assays were performed in duplicate using three washes with PBST between each incubation stage. The amounts of specific antibodies were calculated from control curves made using serial dilutions of a positive control, using a spline fit (Microplate Manage; Bio-Rad) to express arbitrary units (A.U.).

ELISA Inhibition Test

Using serum from three patients, competitive ELISA inhibition assays were performed to determine the specificity of IgE binding to wheat and gliadin and confirm cross-allergenicity. The serum was preincubated with four concentrations (1–100 μ g/mL) of

Table 1 Characteristics of patients with wheat-dependent exercise-induced anaphylaxis

Subject	Sex/Age	A/H Ratio*	Specific IgE (A.U.)		Total IgE# (KUA/L)
			Wheat Flour	Gliadin	
1	M/53	3+	60	8.54	297
2	M/18	3+	20.5	110.77	158
3	M/39	3+	80	357.84	860
4	M/35	3+	10	0.49	62
5	M/58	3+	400.6	337.76	189
6	M/62	3+	10	1.0	312

*Ratio of wheal size of allergen to histamine; the allergen was wheat flour.

#Total IgE levels were quantified using the ImmunoCAP system (Phadia, Uppsala, Sweden).

wheat and gliadin overnight at 4°C, after the ELISA procedure. As a control, samples were preincubated with equivalent volumes of PBS (pH 7.5) in place of inhibitors. The inhibition percent of the specific IgE binding was expressed as

$$\text{inhibition\%} = 100 - \frac{\text{allergen} \cdot \text{absorbance}}{\text{control} \cdot \text{absorbance}} \times 100.$$

SDS-PAGE and IgE Immunoblotting

Wheat and gliadin were subjected to electrophoresis in 10% polyacrylamide separation gel with 4% stacking gel. Proteins from the gel were transferred onto a nitrocellulose membrane (Bio-Rad), which was incubated with the serum diluted at 1:5 vol/vol in PBST. Bound-specific IgE was detected using biotin-labeled goat anti-human IgE antibody (Sigma Co.) conjugated with streptavidin alkaline phosphatase, followed by 0.66% 4-nitroblue tetrazolium and 0.33% 5-bromo-4-chloro-3-indolyl-phosphate (Sigma Co.), used as a substrate.

Digestion of Crude and Preheated Extracts by Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF)

The digestibility of the extract in SGF and SIF was examined as described previously.^{20,21} Briefly, for the SGF digestibility test, 680 µg of protein was dissolved in 200 µL of prewarmed 100 mmol/L HCl, with 30 mmol/L NaCl containing 0.32% w/v pepsin A (Sigma Co.). Digestion was performed at 37°C with continuous shaking. Aliquots of the digest solution (20 µL) were collected at 0 and 30 s and 1, 10, 30, and 60 minutes. These aliquots were quickly mixed with 26 µL of a sample buffer containing 2% 2-mercaptoethanol and 4% SDS, together with 6.0 µL of Na₂CO₃ solution. The mixture was then boiled for 5 minutes and stored at -20°C until further analysis.

For the SIF digestibility test, 680 µg of protein was dissolved in 260 µL of prewarmed intestinal control

solution (0.05 M of KH₂SO₄, pH 6.8) containing 1.0% w/v of pancreatin (Pancreatin USP; Sigma Co.) and incubated at 37°C with continuous shaking. Aliquots of the digest solution (26 µL) were collected at 1 second; 1, 10, and 30 minutes; and 1 and 4 hours and quickly boiled for 5 minutes after mixing with 26 µL of sample buffer containing 2% 2-mercaptoethanol and 4% SDS. These pretreated digests were stored at -20°C until further analysis.

Statistics

Statistical analysis was performed with SPSS 12 (SPSS, Inc., Chicago, IL). The specific IgE levels were compared between these groups using Mann-Whitney *U* test. Values of *p* < 0.05 were considered significant. To examine the diagnostic value of the tests, receiver operator characteristics (ROC) curves were produced, and the area under the ROC curves (AUCs) were calculated.

RESULTS

Wheat and Gliadin-Specific IgE

The characteristics of the six WDEIA patients and their specific IgE results are summarized in Table 1. The levels of wheat-specific IgE ranged from 10.0 to 500.47 A.U., whereas gliadin-specific IgE levels ranged from 0.16 to 357.84 A.U. Patients with WDEIA had higher levels of gliadin-specific IgE than patients without WDEIA (*p* = 0.003; Fig. 1). Specific IgE levels to wheat showed no significant difference between groups (*p* = 0.621). The diagnostic value of the gliadin-specific IgE was tested using the AUC. The AUC for gliadin-specific IgE was 0.872 (95% confidence interval: 0.694 ~ 1.049; *p* = 0.005; Fig. 2). The equivalent AUC for wheat-specific IgE was 0.429 (figure not shown). A cutoff value of 0.93 A.U. yields the best sensitivity and specificity (83.3 and 84.6%, respectively). The corresponding positive and negative predictive values were 55.6 and 95.7%, respectively.

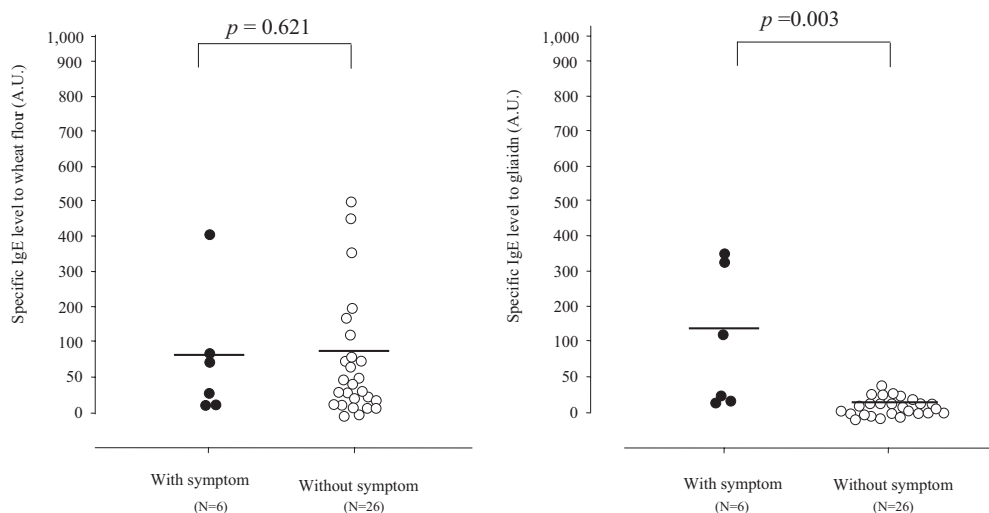


Figure 1. Specific IgE binding to wheat flour and gliadin determined using ELISA for symptomatic patients and asymptomatic sensitized patients.

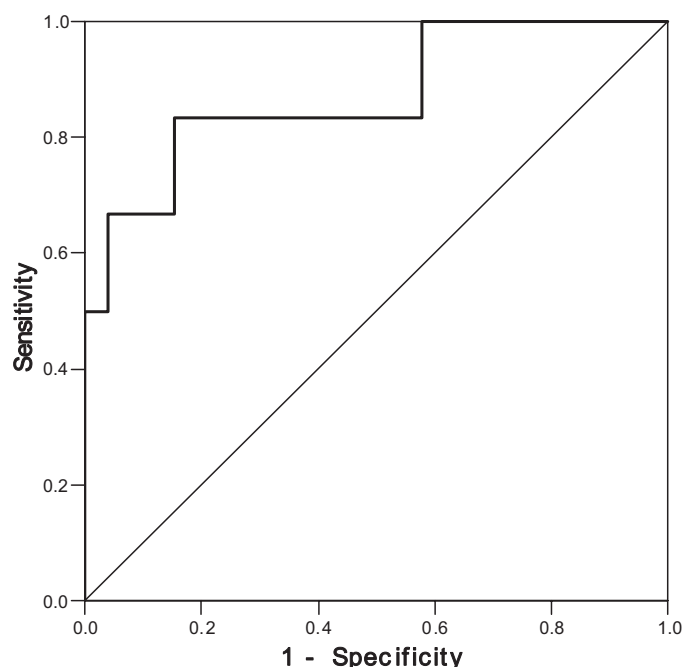


Figure 2. Receiver operator characteristics (ROC) curve for specific IgE to gliadin and wheat-dependent exercise-induced anaphylaxis (WDEIA). The area under the ROC curve (AUC) for gliadin-specific IgE was 0.872 (95% confidence interval: 0.694 ~ 1.049; $p = 0.005$).

Specific IgE Inhibition

Gliadin extracts inhibited 70–90% of IgE binding to gliadin in a dose-dependent manner as shown in the ELISA inhibition assay (Fig. 3). Moderate inhibition of IgE binding to gliadin was achieved when wheat flour extract was used as the inhibitor. Sixty to 70% inhibition to specific IgE binding to wheat was noted when wheat extract was used as the inhibitor. However, minimal inhibition of IgE binding to wheat was achieved with gliadin using serum from WDEIA patient 2. This result showed that wheat-specific IgE from patient 2 was quite specific to wheat while having no

reactivity to gliadin. The other specific IgE antibodies had some degree of cross-reactivity of unknown clinical significance between gliadin and wheat.

IgE Binding Signals to 50-kDa of Gliadin Extract on IgE Immunoblot

All six WDEIA patients had high IgE binding to the 50-kDa gliadin extract on the IgE immunoblot. In contrast, none had specific binding to wheat flour. The asymptomatic patients sensitized to wheat similarly had no specific IgE binding (Fig. 4).

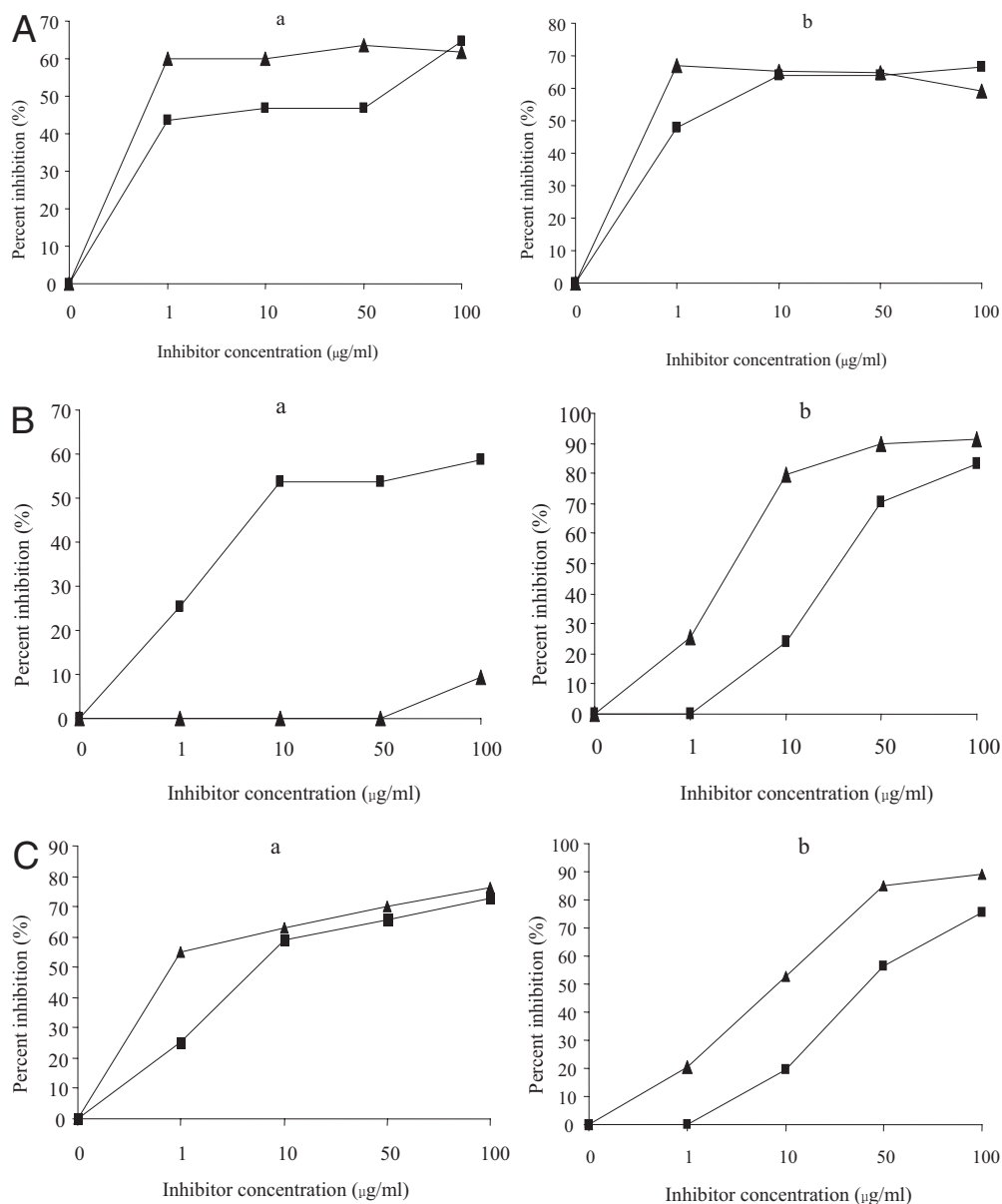


Figure 3. Specific IgE ELISA inhibition results for wheat flour (a) and gliadin (b) coated wells with serial additions of wheat flour (■) and gliadin (▲) extracts. (A) Symptomatic patient 1; (B) symptomatic patient 2; (C) symptomatic patient 3.

Effects of Digestive Enzymes on IgE-Binding Components

IgE immunoblotting using pooled sera showed that IgE binding to the 50-kDa component was substantially digested by SGF within 10 minutes of enzyme treatment (Fig. 5 A). Exposure to SIF revealed that IgE binding to the 50-kDa gliadin extract was minimally digested until 4 hours (Fig. 5 B).

DISCUSSION

Three clinical forms of IgE-mediated allergy to wheat are known: Bakers' asthma, IgE-mediated food allergy, and WDEIA. Bakers' asthma and rhinitis are caused by inhaling wheat flour, usually in an occupational set-

ting. Food allergy occurs after ingestion of wheat-containing foods. Subjects suffering from WDEIA show an allergic reaction after ingestion of wheat only in connection with physical exertion.

Wheat proteins are classified into four types: water/salt-soluble albumins, water/salt-soluble globulins, water/salt-insoluble gliadins, and glutenins.^{22,23} The water/salt-soluble albumins and globulins are mainly structural proteins and metabolically active enzymes. The water/salt-insoluble gliadins and the glutenins, collectively known as prolamins or gluten, are the major storage proteins in wheat grain. A relationship between protein solubility and the various clinical manifestations of wheat allergy has been suggested.^{6,24–26}

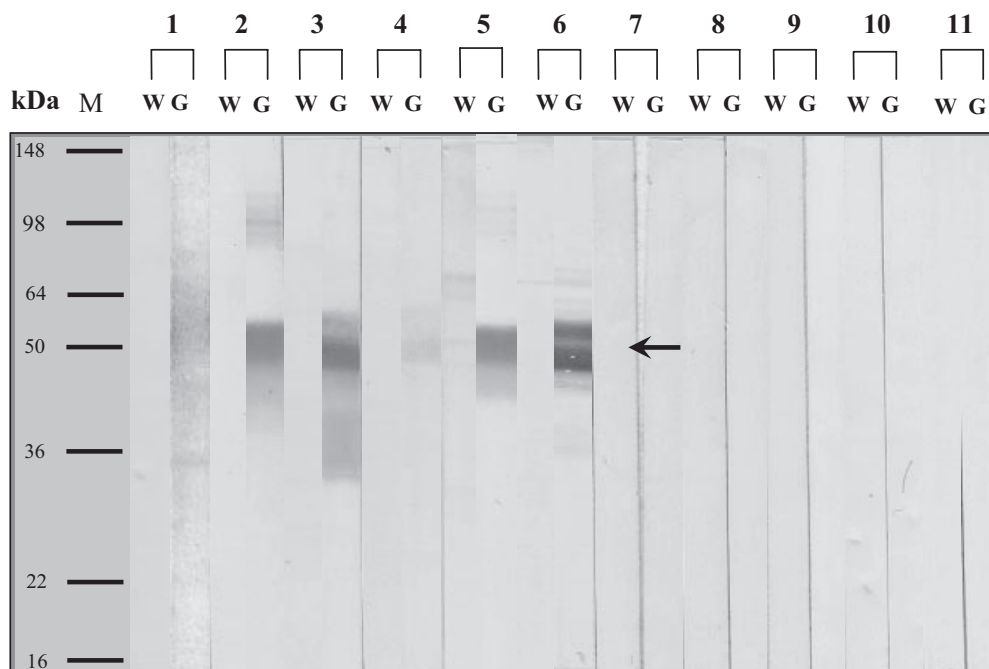


Figure 4. IgE binding component of wheat flour (W) and gliadin (G) in symptomatic subjects (1–6), asymptomatic sensitized subjects (7–9), normal control,¹⁰ and buffer control.¹¹ M, marker. Arrow indicates 50-kDa gliadin.

Water/salt-soluble proteins are resorbed in the respiratory tract through inhaling flour and may elicit IgE-mediated allergic reaction, whereas water/salt-insoluble proteins may be resistant to digestion during passage through the gastrointestinal tract and may be resorbed as structurally intact allergens to trigger allergic symptoms.

Gliadins, along with glutenins, are classified as cereal prolamin of wheat. Prolamins are the major endosperm storage proteins in all cereal grains, with the exception of oats and rice. Recent classification of the prolamins of Triticeae (wheat, barley, and rye) assigns these proteins into three broad groups: sulfur-rich (S-rich), sulfur-poor (S-poor), and high molecular weight prolamins.²⁷ Gliadins can be classified into α -, γ -, and ω -groups based on their electrophoretic mobility.²⁸ The ω -gliadins, known as a major allergen in WDEIA, belong to S-poor prolamins, whereas α - and γ -gliadins are S-rich prolamins.²⁷ In addition, ω -gliadins are subdivided into ω_1 -, ω_2 -, and ω_5 -gliadin components according to their electrophoretic mobility in acid PAGE and their N-terminal sequences. The ω_5 or fast ω -gliadin is suspected to be a major allergen in WDEIA.^{3,6,7} Recently, IgE-binding epitopes on gliadins for patients with WDEIA have been identified.^{7,16,29}

In the present study, we compared the serum-specific IgE levels to wheat and gliadin in WDEIA patients to controls. We also assessed the diagnostic values of these tests using AUCs. AUC is a measure of the overall performance of a diagnostic test and is interpreted as the value of the sensitivity for all possible values of

the specificity.³⁰ The closer AUC is to 1, the better the overall diagnostic performance of the test, and the practical lower limit for the AUC of a diagnostic test is 0.5. If the AUC is 0.5, the test is unable to distinguish between individuals with or without the chosen outcome. The AUC for gliadin-specific IgE was 0.872 and a cutoff value of 0.93 yields the best sensitivity and specificity (83.3 and 84.6%, respectively), and AUC for wheat-specific IgE was 0.429. According to our results, the gliadin-specific IgE had a discriminative power, whereas this was not the case for specific IgE to wheat in our study population. One of the possible limitations of our study was the small size of the WDEIA patient pool. The low prevalence of WDEIA³¹ makes it difficult to enroll enough patients for better statistic analysis. These findings suggested that circulating serum-specific IgE to gliadin can be used as a supplementary marker for the diagnosis of WDEIA. In the present study, the cutoff value was chosen based on the calculated sensitivity and specificity with our assay. Diagnostic cutoff values should be determined based on the incidence and clinical impact of the disease. The suggested cutoff point in this study should be reevaluated by future studies in terms of its clinical relevance.

We found that a 50-kDa protein in gliadin extract is likely the main gliadin allergen for patients with WDEIA. It is possible that this protein is the same allergen found by Palosuo *et al.*,^{3,6} who described a 65-kDa ω_5 -gliadin as a major allergen for subjects with WDEIA. The discordance in molecular weights of the identified proteins likely results from interstudy vari-

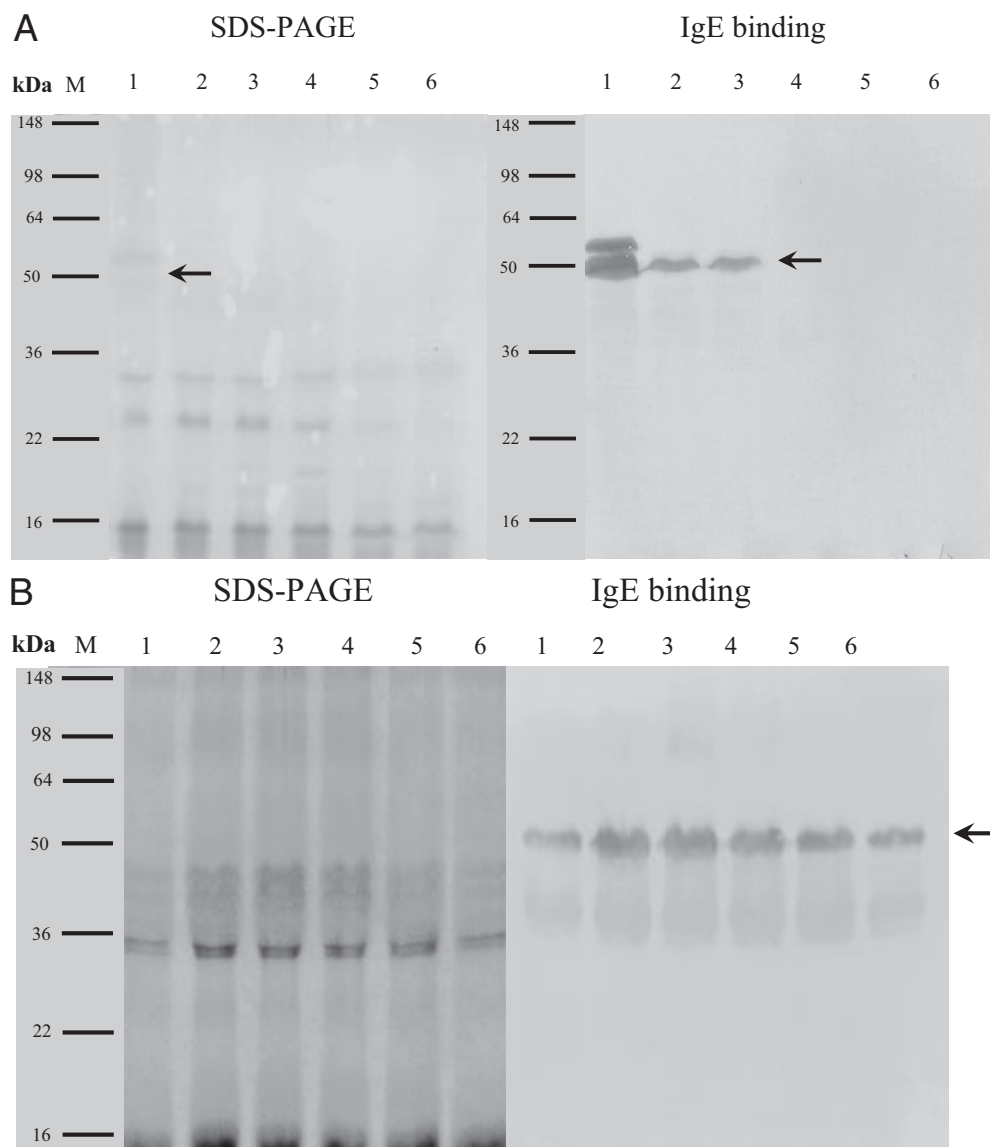


Figure 5. Effect of (A) simulated gastric fluid and (B) simulated intestinal fluid treatment on gliadin extracts using SDS-PAGE and IgE binding components in sera pooled from five patients (M, marker); (A) lanes 1–6: 0 and 30 seconds; 1, 10, and 30 minutes, respectively; (B) lanes 1–6: 0 seconds; 1, 10, and 30; and 1 and 4 hours, respectively.

ation. Our data on the stability of gliadin against digestion differed from that of Mittag *et al.*²⁵ in that we showed a relatively short digestion time in SGF and striking resistance to digestion in SIF. The first possible reason for these differences is the source of gliadin; our gliadin was commercially purchased, whereas Mittag *et al.* extracted gliadin from the whole wheat grain. The other possibility is the difference in the digestion method. Mittag *et al.* used commercial porcine stomach extract, whereas we prepared SGF using pepsin A. We further simulated intestinal digestion using pancreatin, whereas Mittag *et al.* incubated in SGF before adding pancreatin. Our results suggest that in conditions in which reduced gastric emptying time increases the passage of immunologically intact gliadins into the

small intestine, there is an increased risk of this allergen being absorbed into the blood stream.

Although DBPCFC still represents the gold standard for diagnosing food allergies, it is time-consuming, expensive, troublesome for the patient, and involves the risk of severe systemic allergic reactions. An *in vitro* test that could accurately diagnose food allergy, as well as WDEIA, would be of great value. Studies show that specific IgE concentrations can be used to predict clinical reactivity to eggs, milk, peanuts, and fish with >95% certainty.^{14,32} However, in the case of wheat, the positive predictive value remains below 75%.^{15,32} This poor capacity may be explained by the fact that wheat-induced allergies such as WDEIA are mainly caused by water-insoluble proteins such as gliadin, rather than

water/salt-soluble proteins, which are usually extracted and used in laboratory tests. Our data suggest that SPTs with wheat extract and serum levels of wheat-specific IgE have little value in the diagnosis of the WDEIA. However, we found that circulating gliadin-specific IgE can be used as a supplementary marker for the diagnosis of WDEIA.

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