Identification of wheat sensitization using an in-house wheat extract in Coca-10% alcohol solution in children with wheat anaphylaxis

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Summary

Background: **Identification of wheat sensitization by a skin prick test (SPT) is essential for children with wheat-induced anaphylaxis, since oral food challenge can cause serious adverse effects. Wheat allergens are both water/salt and alcohol soluble. The preparation of wheat extract for SPT containing both water/salt and alcohol soluble allergen is needed.**

Objective: **To determine if a wheat extract using Coca's solution containing 10% alcohol (Coca-10% EtOH), prepared in-house, contians both water/salt and alcohol soluble allergens.**

Methods: **Serum of children with a history of anaphylaxis after wheat ingestion was used. Wheat flour was extracted in Coca-10% alcohol solution. An SPT with both commercial and in-house wheat extracts was performed as well as specific IgE (sIgE) for wheat and omega-5 gliadin. Direct and IgE inhibition immunoblots were performed to determine serum sIgE levels against water/salt as well as alcohol soluble (gliadins and glutenins) allergens in the extracts.**

Results: Six children with history of wheat anaphylaxis had positive SPT to both commercial and in-house extracts. They also had different levels of sIgE against wheat and omega-5 gliadin allergens. The results of direct immunoblotting showed all tested sera had sIgE bound to ~35 kDa wheat protein. Further IgE inhibition immunoblotting identified the ~35 kDa wheat protein as gliadin but not gluten allergen.

Conclusion: The in-house prepared Coca-10% EtOH solution could extract both water/salt and alcohol soluble allergens. The ~35 kDa gliadin appears to be a major wheat allergen among tested individuals. ***(Asian Pac J Allergy Immunol 2016;34:153-8)***

Keywords: *wheat allergy, diagnosis, wheat extract, wheat anaphylaxis, major allergen*

Introduction

Food allergy affects as many as 6% of young children.[1] Wheat is the most common cause of food-dependent exercise-induced anaphylaxis.[2-5] It is one of the most common causes of food-induced anaphylaxis observed worldwide, especially in Asia.[6]

The gold standard for diagnosing immediate wheat allergy is a double blinded placebo controlled food challenge.[7] However, this procedure has the risk of anaphylaxis occurring during the challenge process. The improved diagnostic modality assessing specific IgE (sIgE) to ω-5 gliadin is a more sensitive method.[8]

The protein components of wheat can be divided into two parts, i.e. water/salt-soluble albumins and globulins, and water/salt-insoluble gliadins and glutenins.[9] Among these proteins, gliadins and high molecular weight (HMW) glutenins have been reported to be the major allergens for IgE-mediated wheat allergy and wheat-dependent, exercise-induced anaphylaxis (WDEIA).[8,10]

Gliadins are major wheat allergens that can be dissolved in aqueous alcohol.[11] They can be separated by electrophoresis into alpha-, beta-, gamma- and omega-gliadins. The omega-5 (ω-5) gliadin has been identified as the major allergen in WDEIA.[12,13] Moreover, sIgE to ω-5 gliadin is highly predictive of immediate allergy to ingested wheat in

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children with increased levels correlating with positive oral wheat challenge results.[8,14]

In developing countries, testing of sIgE to ω-5 gliadin is not routinely accessible. The skin prick test (SPT) with wheat extract to diagnose immediate wheat allergy is essential, and a number of commercial wheat extracts are available for use in this test. However, the preparation of these commercial extracts, especially as an aqueous solution, may not be sufficient for the diagnosis because some major wheat allergens are alcohol-soluble proteins. Gliadins and glutenins are alcohol-soluble proteins and can be extracted by alcohol solutions containing a reducing agent. The two-step protocol using 50% iso-propanol, dithiothreitol (DTT) and ethanol can completely extract both gliadins and glutenins from wheat flour.[15] However, the process of extraction is complicated and the use of DTT for SPT in humans has not been proven to be safe. For this reason, we developed an in-house wheat allergen extract using Coca's solution containing 10% alcohol (Coca-10% EtOH) for SPT in children with wheat anaphylaxis. In this report, we investigated if the in-house wheat extract in Coca-10% EtOH contained both water/salt and alcohol soluble allergens.

Methods

Subjects

This study was approved by the Siriraj Institutional Review Board (si312/2009). Patients 1-15 years of age who came to the pediatric allergy clinic at Siriraj Hospital, Thailand, from 2009 to 2012, with a history of wheat anaphylaxis were recruited. Written informed consent from parents or guardians and the assent of children older than 7 years of age were obtained. Patients with underlying diseases such as cardiovascular (CVS), hepatobiliary, and renal diseases were excluded.

Alcohol-soluble wheat extract

Alcohol-soluble wheat extract was prepared by extracting whole wheat flour to the final concentration of 1:10 w/v in Coca's solution (29.8 mM $NaHCO_3$, 86 mM NaCl and 42.5 mM phenol) containing 10% v/v of absolute alcohol (Coca-10% EtOH) under in a laminar flow cabinet with sterile technique. The mixture of wheat flour and Coca-10% EtOH solution was magnetically stirred for 1 hour at room temperature. The solution was then it was centrifuged at 17,210 x g for 10 min before being sterile filtered through a 0.2 micron filter. The wheat extract was stored at 4°C and was used within a period of 3 months.

Skin prick testing

All of the subjects underwent SPT with commercial wheat extract (ALK, Abello, Sweden) and our alcohol-dissolved wheat extract. SPT was performed on the volar aspect of the forearm with a monodentate lancet. 1% histamine was used as a positive control and normal saline as a negative control. The results were considered positive if the wheal size had a mean diameter at least 3 mm larger than the negative control.

Specific IgE

All patients were tested for wheat and □-5 gliadin sIgE antibodies measured by ImmunoCAP (Phadia, Uppsala, Sweden; lower detection limit < 0.35 kAU/L) according to the manufacturer's recommendations.

Gliadin and glutenin extraction

Gliadin and glutenin extraction were performed using a modified extraction protocol.[15] Briefly, gliadins were extracted by mixing 200 mg wheat flour with 1 ml of 50% (v/v) aqueous iso-propanol with continuous mixing for 60 min at room temperature, followed by centrifugation at 17,210 x g for 1 min at room temperature. The supernatant is referred to as the first gliadin extract'. The second and third gliadin extracts were extracted from the pellet in 50% (v/v) aqueous iso-propanol using the same procedure. The pellet from the third extract was used for the glutenin extract by extracting the pellet in 50% (v/v) aqueous iso-propanol, 50 mM Tris–HCl (pH 7.5) containing 1% (w/v) DTT for 60 min at 60°C with mixing every 5 to 10 min, followed by centrifugation at 17,210 x g for 1 min at room temperature. The supernatant was kept as the glutenin extract.

Based on our preliminary test, iso-propanol, sodium dodecyl sulfate (SDS), and DTT inhibited sIgE binding to allergens; therefore, iso-propanol was eliminated by evaporation and both SDS and DTT were diluted by at least 1,000 fold in the inhibition of IgE immunoblot experiments. The preparation was performed as follows. Both gliadin and glutenin extracts were dried using a vacuum evaporator. Dried gliadin and glutenin were dissolved in 50 mM Tris-HCl pH 7.5 containing 0.25% SDS and incubated at 65°C with vigorous shaking. These two preparations were designated SDS dissolved gliadin and gluten. The concentration of dissolved proteins was determined by a bicinchoninic acid (BCA) protein assay.

Direct IgE immunoblot and IgE inhibition immunoblot

Based on the sIgE levels shown by the ImmunoCap results, the sera of patients and controls were diluted 1:30-1:60 in PBS containing 3% skimmed milk (buffer A) and used in the following tests.

For the direct IgE immunoblot, 1 µg of gliadin and glutenin extracts were loaded per well of 16% SDS-PAGE gel and separated. Separated proteins were electro-transferred onto a nitrocellulose membrane before the membrane was incubated in buffer A for 1 hour at room temperature. The membrane was washed with PBS containing 0.2% v/v Tween-20 (buffer B) before it was incubated overnight at 4ºC with the diluted sera of patients and controls in buffer A. The membrane was washed with buffer B and was incubated with 1:10,000 diluted horseradish peroxidase (HRP) conjugated mouse IgG anti-human IgE antibody (KPL, MD, USA) in buffer A for 1 h. After washing, the membrane was incubated with HRP substrate (Millipore, MA, USA) and the emitted signal was captured by x-ray film.

For the IgE inhibition immunoblot, the diluted sera of patients and controls were incubated with 100 µg of SDS dissolved gliadin and gluten overnight at 4ºC before centrifugation at 17,210 x g for 10 min at room temperature. The supernatant was incubated with the nitrocellulose membrane for 2 h at room temperature. The remaining steps of the inhibition immunoblot were the same as those of the direct immunoblot.

Results

Study population

Six subjects (three boys and three girls) with a history of wheat anaphylaxis were recruited. The characteristics of the subjects are shown in Table 1. The mean age of onset was 8 $\pm$ 2.4 months (range 4-11 months). The level of sIgE against wheat allergens ranged from 56-518 kAU/L and that of sIgE against ω-5 gliadin ranged from 7-38 kAU/L (Table 1).

Direct IgE immunoblot and IgE inhibition immunoblot

To determine the profile of IgE bound allergens in wheat extracted in Coca solution and in Coca-10% EtOH, the sera of six patients were tested. The results showed four patients (P1, P2, P4, P5) had a strong signal of sIgE bound to a ~35 kDa allergen (Figure 1). A strong signal of sIgE bound a ~20 kDa allergen was also observed in three patients (P1, P2, P5) (Figure 1). Interestingly, only P2 had a strong signal of sIgE bound to ~40 and ~17 kDa allergens (Figure 1). Moreover, a weak signal of sIgE bound to a >46 kDa allergen was observed in four patients (P1, P2, P4, P6) (Figure 1). Overall, Coca-10% EtOH provided higher sIgE binding signal than Coca solution.

To determine whether the sIgE-bound wheat allergens in our in-house extract were gliadin and glutenin allergens, the profile of sIgE bound allergens from wheat gliadin and glutenin extracts were examined in selected patients (P1-P4). The results of the immunoblot showed serum sIgE bound to multiple proteins with MW ranging from 25-46 kDa in both the gliadin and glutenin s compared to the sIgE bound ~35 kDa allergen in Coca-10% EtOH extract (Figure 2, lane 1-4). Interestingly, only one patient (P4) also had sIgE bound to high MW (80-160 kDa) gliadins and glutenins (Figure 2, lane 3,4). Further analysis was performed to determine whether gliadin and glutenin allergens were solubilized in Coca-10% EtOH extract. Serum IgE was pre-incubated with either SDS-dissolved gliadins or glutenins before IgE immunoblotting (Figure 2, lane 5-8). After pre-incubating IgE with SDS-dissolved gliadins, the results of the immunoblot showed that no bands of specific IgE bound allergens were observed in the Coca-10% EtOH extract (Figure 2, lane 5). However, two out of four patients (P1, P2) showed a low intensity of sIgE bound allergens in the 50%v/v iso-propanol extract (Figure 2, lane 6). Also, after IgE was pre-incubated with SDS-dissolved glutens, the immunoblot results showed that no bands of sIgE bound allergens were observed in the Coca-10% EtOH extract (Figure 2, lane 7). Interestingly, sIgE bound gliadins with a MW of 30-40 kDa were found in the 50%v/v iso-propanol extract in three patients (P1-P3) (Figure 2, lane 8).

Discussion

To our knowledge, this is the first study to demonstrate sIgE to both water/salt-soluble allergens (~20 kDa) and alcohol-soluble wheat allergens (30-46 kDa) in wheat-induced anaphylactic children. The level of sIgE against ω-5 gliadin of all tested sera was 1/6-1/52 of that of sIgE against wheat allergens, suggesting that the majority of sIgE may be specific to allergens other than ω-5 gliadin. As the results of direct immunoblot showed, the sera of all six children had sIgE bound to a ~35

Table 1. Patients' characteristics

Pt. No.	Sex	Age of onset (mo)	Symptom	other food allergy	SPT (mm)		sIgE(kAU/L)	
					com	In-house	wheat	gliadin
1	M	9	ANA	egg white	5.5	13	518	37.8
2	M	4	ANA	No	6.5	12.5	439	8.49
3	F	8	ANA	egg white	4.5	14	70.5	12.6
4	F	11	ANA	No	10	9.5	78.6	9.59
5	M	7	ANA	egg white, EY, CM, soy, peanut, seafood,	10	15	56	7.43
6	F	9	ANA	CM	4	4.5	81.5	6.55

Note: ANA = anaphylaxis, EY = egg yolk, CM = cow's milk, SPT = skin prick test, com=commercial

kDa protein in the alcohol-soluble extract, and three out of six children also had sIgE bound to a ~20 kDa water/salt-soluble allergen. Based on these results, the in-house wheat extract probably contains both water/salt and alcohol soluble allergens. Coca's solution has been proven to be safe for SPT and is used for extracting allergens from many sources.[18] This is also the first study to use Coca's solution mixed with EtOH at a final proportion of 10% to extract wheat allergens. As the results show, our in-house prepared solution was able to efficiently extract the major allergens of both water/salt soluble and alcohol soluble allergens. Our in-house wheat extract is also a cost-saving extract that should benefit local medical units to use in the diagnosis of wheat allergy. Because a bottle (500 ml) of Coca's solution costs $ 2.10, based on 30 baht per US $, 5 ml of in-house wheat extract would cost approximately $0.60, including materials and operation costs. However, 5 ml of imported commercialized Wheat Grain (ALK Abello, Sweden) extract costs $83. Therefore, the in-house extract is a cost-saving extract with good efficiency.

It appears that all tested sera had sIgE against a ~35 kDa alcohol-soluble protein, suggesting that gliadin might be a major wheat allergen. As also observed, a band of sIgE bound to ω-5 gliadin did not appear in the direct immunoblot. This is likely due to a low level of sIgE bound ω-5 gliadin in all tested sera based on sIgE determination (Table 1) and the high dilution of sera used in the direct immunoblot.

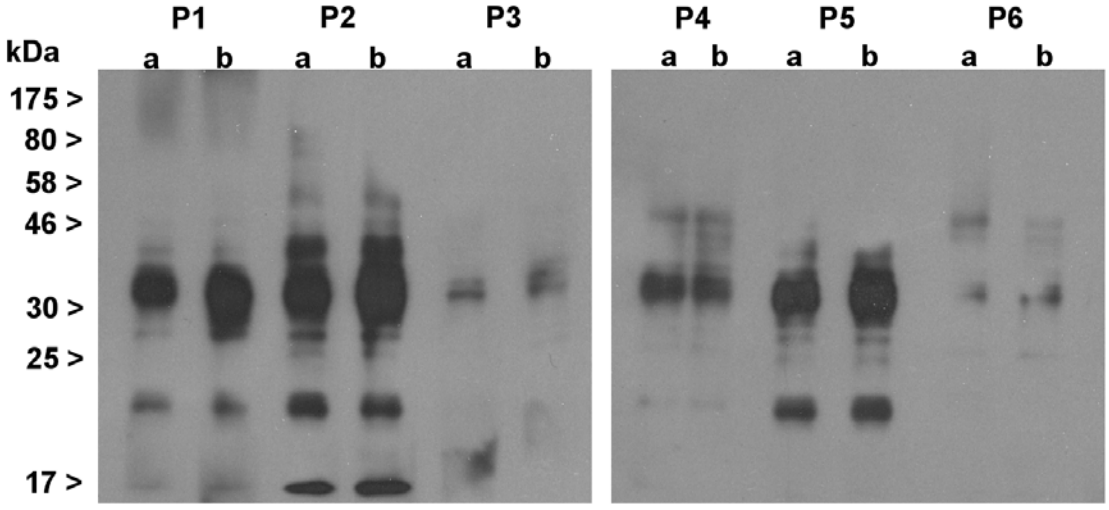

Figure 1. Direct immunoblot using serum specific IgE to wheat allergens from 6 patients (P1-P6).
Note: a = wheat extracted in Coca's solution, b = wheat extracted in Coca's solution with 10%v/v Ethanol. No signal of IgE bound proteins was observed with serum of non-atopic donors (data not shown).

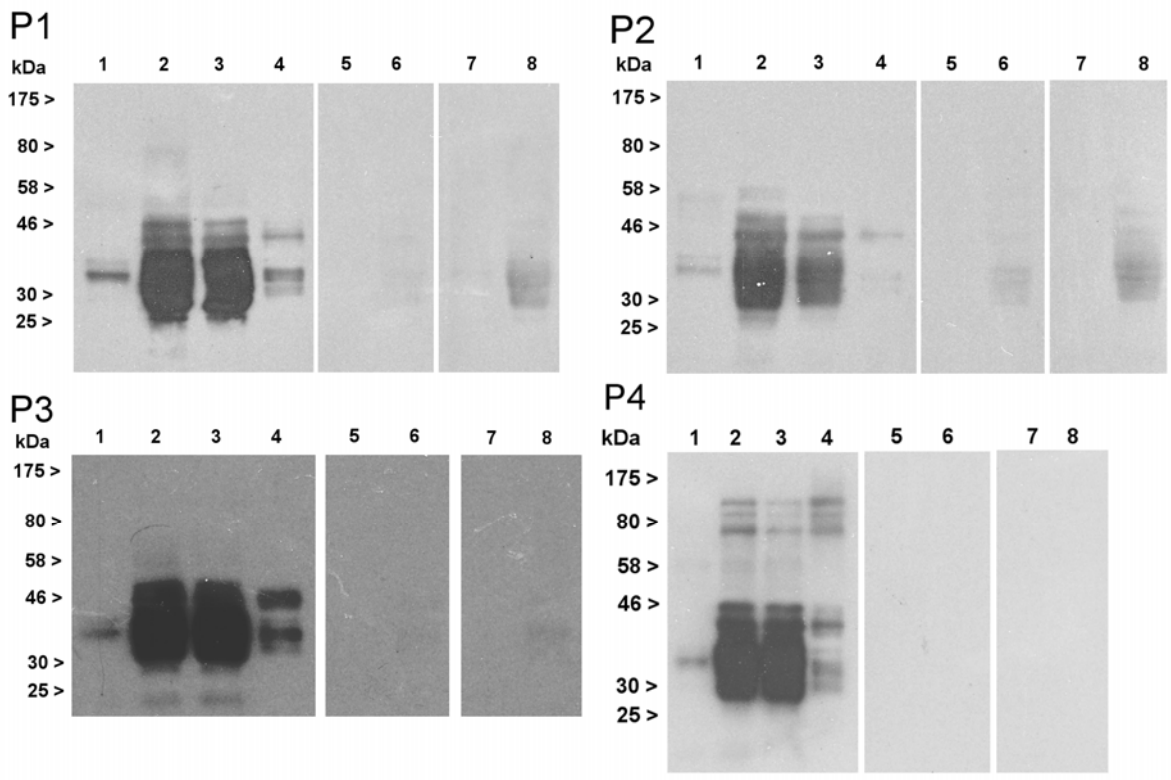

Figure 2. Direct and IgE inhibition immunoblot using serum specific IgE to wheat allergens from 4 patients (P1-P4). Direct IgE immunoblot were in lane 1-4; 1 = wheat extracted in 10%v/v Ethanol-Coca's solution, 2 = wheat extracted in 50%v/v Iso-propanol, 3 = SDS-dissolved gliadins, 4 = SDS-dissolved glutenins. Inhibitions of IgE immunoblot were in lane 5-8. In IgE inhibition, lane 5 and 7 were proteins from wheat extract in 10%v/v Ethanol-Coca's solution, lane 6 and 8 were proteins from 50%v/v Iso-propanol extract. Serum IgE were incubated with SDS-dissolved gliadins before incubated with proteins in lane 5,6. Serum IgE were incubated with SDS-dissolved glutens before incubated with proteins in lane 7,8.

Based on IgE inhibition immunoblot results, the ~35 kDa alcohol-soluble protein is likely gliadin rather than glutenin, since SDS-dissolved gliadins blocked nearly 90% of IgE binding in all four tested sera. In contrast, SDS-dissolved glutenins blocked 90% of IgE binding in two tested sera and blocked 50% of IgE binding in another two tested sera. Although further experiments are needed to identify this ~35 kDa alcohol-soluble protein, when compared with reported results of 2-D SDS-PAGE and tandem MS, this ~35 kDa has a similar mobility to that of α or β –gliadin.[19] Wheat α and β–gliadins have a MW of 30-46 kDa compared to ω5-gliadin with a MW of ~60 kDa when run on an SDS-PAGE gel.[19] Although it has previously been reported that ω5 -gliadin is a major wheat allergen for wheat-allergic Thai children,[14] it has also been reported that a high prevalence of IgE to α/β/γ –gliadins could be found together with that to ω5 –gliadins; thus, the inclusion of α/β/γ –gliadins in a diagnostic test could provide additional diagnostic value to ω5-gliadin IgE-negative patients.[20] Further IgE immunoblot experiments with a larger number of wheat allergic children are required to determine the prevalence of IgE against α or β gliadins in Thai patients.

Our direct IgE immunoblot results also show the three out of six sera had sIgE bound a ~20 kDa water/salt-soluble allergen. It appears that this is the first report of a major water/salt-soluble wheat allergen. Further determination of IgE binding profiles in many sera of wheat allergic children is needed to confirm this observation. Moreover, identification of this water/salt-soluble wheat allergen is needed as well.

The results of the IgE inhibition immunoblotting also showed a weak signal intensity of IgE bound allergens in the Coca-10% EtOH total wheat extract compared to that of IgE bound allergens in the gliadin or glutenin extracts on the same membrane

(Figureure 2, lane 1-4). This discrepancy may have occurred because a total wheat extract is usually comprised of a high amount of non-allergenic proteins and specific allergens, while in an alcohol extract, most of the water/salt soluble proteins are excluded, leaving gliadins mixed with specific allergens. Thus, one microgram of protein in the total wheat extract may have a lower amount of specific allergens compared to one microgram of protein in the gliadin extract. However, despite a weak signal intensity of IgE bound allergens shown by immunoblotting, it is still sufficient to give a positive SPT.

The gold standard for diagnosing wheat allergy is a double-blind placebo-controlled food challenge test. However, it is not practically used due to the risk of anaphylaxis. SPT and wheat sIgE have low sensitivity in diagnosing wheat allergy.[11,16,17] Recently, it has been reported that IgE to ω-5 gliadin is highly predictive of immediate allergy to ingested wheat in children[8,14] and can identify as many as 82% of wheat allergic patients.[20] Additionally, a wheat extract containing both water/salt and alcohol soluble proteins could detect cases with negative sIgE to ω-5 gliadin.

Taken together, using a modified formula, our Coca-10% EtOH wheat extract contains both water/salt and alcohol soluble allergens and contains different major wheat allergens to identify wheat sensitization in patients with wheat anaphylaxis. The extract is easily prepared and inexpensive.

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