

# Identifying the stability of a new wheat gliadin extract by protein analysis, skin tests and cell degranulation assay

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## Abstract

**Background:** The commercial wheat extract for skin prick test (SPT) provides less sensitivity to predict wheat allergy, compared to in-house gliadin extracts. SPT is a preferred method to study extract stability as it is the aim of developing extract. The role of cell degranulation assay, a functional assay with the same mechanism as SPT, is not widely used to determine extract stability.

**Objective:** To study the stability of in-house gliadin extracts stored at different periods, by using protein analysis, SPT and degranulation assay of humanized rat basophilic-leukemia (RBL-SX38) cells.

**Methods:** Patients with a history of wheat allergy and positive SPT to wheat, were recruited. The gliadin extracts stored for 1, 6, 9, and 12 months at 2–8°C were used in SDS-PAGE, SPT and cell degranulation assay. The cell degranulation was determined by  $\beta$ -hexosaminidase release.

**Results:** Forty children were recruited. The gliadin extract stored for 9 and 12 months provided lighter protein bands than 1 and 6 months. However, the wheal diameters from SPT using extracts stored at different periods, were not significantly different ( $p = 0.09$ ). There were also no significant differences of the  $\beta$ -hexosaminidase released using 0.1 and 1  $\mu\text{g}/\text{mL}$  of gliadin extracts stored at different periods ( $p > 0.05$ ). The 10  $\mu\text{g}/\text{mL}$  of gliadin extracts stored at longer periods, significantly stimulated higher  $\beta$ -hexosaminidase release ( $p = 0.01$ ). The extracts were sterile at all storage times.

**Conclusions:** To determine the stability of in-house gliadin extracts, SPT or cell degranulation assay provided additional information to SDS-PAGE. The extracts were stable for up to 12 months.

**Key words:** allergen extract, basophil degranulation, gliadin, skin test, stability, wheat allergy, wheat extract

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### Abbreviations:

MWD mean wheal diameter  
OFC oral food challenge  
OIT oral immunotherapy  
RBL-SX38 humanized rat basophilic-leukemia cells  
SD standard deviation  
SPT skin prick test

## Introduction

Wheat is one of the most common causes of food allergy worldwide. The prevalence of wheat allergy is 0.5–1.2% in general population and 0.5–4% in children.<sup>1–3</sup> The proportion of wheat allergy in children with food allergies varies significantly among countries ranging from 11–20%.<sup>4</sup> The IgE-mediated wheat allergy may involve skin, gastrointestinal, respiratory, or cardiovascular systems with varying severity from mild to life-threatening. In Thailand, the median age at onset of IgE-mediated wheat allergy is 7 months and 50% presented with anaphylaxis.<sup>5</sup> The resolution rate is 45% by 5 years of age.<sup>6</sup> The gold standard for diagnosing wheat allergy is oral food challenge (OFC) since there are some discrepancies between patient-reported reaction and OFC results.<sup>7</sup> However, the OFC is a time-consuming procedure and is associated with the risk of severe allergic reaction.<sup>8</sup>

Skin prick test (SPT) is a useful method to determine food sensitization and support the diagnosis of IgE-mediated food hypersensitivity with minimal risk of severe systemic reaction.<sup>9,10</sup> The accuracy of SPT for detecting allergic sensitization depends on several factors including the quality of allergen extract, prick/puncture devices and area of the body.<sup>11,12</sup>

Wheat grain mass consists of 70% carbohydrate starch and 10–18% of proteins, which mainly causing wheat-induced allergic disease. Depending on the solubility in different solvents, the wheat grain proteins are categorized into 4 main groups: albumins (15%), globulins (7%), gliadins (33%), and glutenins (45%). Albumins are water soluble while globulins are salt soluble. Gliadins are soluble in alcohol and glutenins are soluble in diluted acid and alkali.<sup>2</sup> The role of SPT in determining wheat allergy is complicated since the ideal wheat extracts should contain both salt/water-soluble and alcohol/diluted acid and alkali-soluble proteins.<sup>2,11,13</sup> Recently, the major wheat allergens reported to cause IgE-mediated wheat allergy, as well as anaphylaxis, are mostly salt/water insoluble proteins which are  $\omega$ -5-gliadin (Tri a 19) and high molecular weight glutenin (Tri a 26).<sup>14–16</sup> Using oral wheat challenge as a gold standard, our group recently reported that SPT with our in-house gliadin extract, showed the highest performance for the diagnosis of IgE-mediated wheat allergy compared to other in-house and commercial wheat extracts.<sup>17</sup> However, the stability of this new in-house gliadin extract has not been determined.

Stability studies should determine the retention of biological activity.<sup>18</sup> The ideal way to study stability of the in-house gliadin extract is SPT with this extract stored at different periods as the aim of developing this product is mainly for SPT. Although several other methods, such as Bradford assay, SDS-PAGE, IgE immunoblotting and two-site enzyme-linked immunosorbent assay (ELISA), were used by many studies, the results might not indicate allergic reaction as seen from that of SPT.<sup>18–23</sup> Advanced functional assays are being developed such as histamine release or T-cell activation assay.<sup>18</sup> Since SPT reactions are caused by degranulation of IgE-bound mast cells and basophils after allergens interacting,

using humanized rat basophilic-leukemic (RBL-SX38) cells to determine degranulation after adding allergen may confirm the results of SPT.

The objective of this study was to uncover the stability of a newly invented in-house gliadin extract stored at 2–8°C over a 12 month-period, by using protein analysis, SPT and degranulation assay of humanized rat basophilic-leukemia (RBL-SX38) cells.

## Methods

### Study design and subjects

This study was a cross-sectional study. The protocol was approved by human ethical committee the Siriraj Institutional Review Board (SiRB) of the Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand (COA no. Si 300/2021). Written informed consent from guardians, and assent from children older than 7 years of age, were obtained prior to inclusion in the study.

Patients with history of immediate allergic reaction and positive SPT to wheat who were followed at the Pediatric Allergy Clinic, Department of Pediatrics, Faculty of Medicine, Siriraj Hospital during 2020–2021, were recruited. Immediate allergic reaction to wheat was defined as any significant allergic symptoms, such as urticaria, angioedema, rhinoconjunctivitis, stridor, coughing, wheezing, collapse, tachycardia, hypotension, or anaphylaxis, developed within 2 hours of wheat ingestion. Anaphylaxis was diagnosed according to the National Institute of Allergy and Infectious Disease/Food Allergy and Anaphylaxis Network symposium criteria.<sup>24</sup> Children with underlying conditions, such as chronic renal disease, chronic liver disease, cardiovascular diseases, bleeding disorder, uncontrolled cutaneous lesions including atopic dermatitis with acute eczema, skin infection at testing area, uncontrolled chronic urticaria, were excluded. Demographic data and clinical characteristics were obtained from all patients. The diagnosis of other allergic diseases was performed by allergists.

### The preparation of wheat gliadin extract

The in-house gliadin extract was prepared as described in the Thai petty patent no 1903001819 and the previous report.<sup>17</sup> In brief, 1:10 W/V of durum wheat semolina flour was mixed and stirred in Tris-sodium-based solution (30 mM Tris, pH 8, 500 mM NaCl) for 2 hours at room temperature. The extract was centrifuged at  $17,210 \times g$  for 10–15 minutes. The pellet was dissolved in the Gliadin buffer (50 mM Tris-HCl, pH 6.8, 0.25% SDS, 10% glycerol). After stirred for 1 hour at room temperature, the extract was centrifuged at  $17,210 \times g$  for 10–15 minutes. The supernatant was filtered through a sterile 0.2-micrometer filter. The gliadin extract was determined protein concentration by bicinchoninic acid (BCA) protein assay kit (ThermoFisher, USA). Then, the gliadin extract was further diluted with the Gliadin buffer to a final concentration of 1 mg/mL and stored for 1, 2, 3, 6, 9, and 12 months at 2–8°C.

All chemical components of the solutions were certified safe for use in humans. The concentration of SDS was below the level which cause skin irritation (1–2%).<sup>25</sup> All stored gliadin extracts, were cultured for bacteria for sterility testing according to the United States Pharmacopeia standards, serviced by the Department of Microbiology, Siriraj hospital.

#### SDS-PAGE analysis of protein in the gliadin extracts

The protein concentration of the gliadin extract was measured when it was prepared. The concentration of the gliadin extract was 1 mg/mL. The 24 µL of extracts stored for 0, 1, 2, 3, 6, 9, and 12 months were resolved per well of 12% SDS-PAGE gel at constant current. Resolved proteins in SDS gel were stained in a solution containing Coomassie Brilliant Blue G250 for 1 hour before being washed in distilled water.

#### Skin testing

Participants were asked to discontinue antihistamine and systemic corticosteroids for at least 7 days prior to SPT. The in-house gliadin extracts which were opened and stored for 1, 6, 9, and 12 months were used for SPTs in all participants. Histamine (10 mg/ml) and normal saline were used as the positive and negative control, respectively. All SPTs were performed in a double-blinded manner of which both the patients and professional operators did not know the storage time of gliadin extracts.

Using the standard SPT method, the single-use metal lancet was passed through a drop of extract and pricked the epidermis on each patient's upper back before carefully wiping the extract away.<sup>26</sup> The results were recorded at 15 minutes after SPT. The diameters of the wheal and flare were measured by a digital caliper with a Scotch tape technique. The mean wheal diameter (MWD) was calculated from the sum of the perpendicular longest diameters across the wheal and divided by two. According to our previous study, the best cut-off MWD for gliadin extract was at least 2.5 mm larger than the negative control.<sup>17</sup> Each participant was observed for 60 minutes after SPT in the physician office.

#### Cell degranulation assay

Humanized rat basophilic-leukemia (RBL-SX38) cells, expressing the human high-affinity IgE receptor (Fcε-RI), were primed with patient's serum. The cell degranulation assay was determined from the released β-hexosaminidase by primed RBL-SX38 cells incubated with the gliadin extracts stored for 1, 6, 9, and 12 months.

Serum of 10 current wheat allergic patients, who were older than 10 years of age without history of receiving wheat oral immunotherapy, were collected and used in this assay. The RBL-SX38 cells were incubated overnight with 1/20 diluted patient's sera. All cells were washed with Siraganian buffer (25 mM PIPES, 119 mM NaCl, 5 mM KCl, 5.6 mM Glucose, 1 mM CaCl<sub>2</sub>, 0.4 mM MgCl<sub>2</sub>, 40 mM NaOH and 0.1% BSA, pH 7.4). Various concentrations (0.1, 1, and 10 µg/mL) of the gliadin extract stored for 1, 6,

9, and 12 months, as well as diluted goat anti-human IgE antibody (positive control), were added to the cells before incubated for 30 minutes at 37°C. The concentrations of gliadin extract were used by previous report.<sup>27</sup> These cells were prepared in Siraganian buffer. A negative control was cells incubated with Siraganian buffer without serum. Supernatants were collected before attached cells were lysed with 0.2% Triton X-100.

The released β-hexosaminidase in the medium and in the cell lysate were determined by adding the enzyme substrate solution, 100 mM citrate pH 4.5 containing 0.1 mM 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide, to both supernatants and cell lysate and incubated for 30 minutes at 37°C. After stopped the reaction by 0.25 M glycine, the fluorescence was measured by using 380-nm excitation and 440-nm emission. The degranulation levels were calculated from the following formula according to the previous report.<sup>28</sup>

% released β-hexosaminidase

$$= \frac{\text{supernatant} - \text{background}}{[(\text{supernatant} - \text{background}) + (\text{lysed cells} - \text{background})]} \times 100$$

Note: 'supernatant' is the released enzyme in the supernatant; 'lysed cells' is total enzyme activity within the cell; 'background' is the negative control.

#### Statistical analysis

Data analysis was performed using a computer base and SPSS statistical software (version 20.0). Categorical data were described as frequency and percentage. Continuous data were reported as mean and standard deviation (SD) for data with normal distribution, and as median and range for non-normally distributed data. MWD of SPT between each gliadin extract, stored at 2–8°C for 6, 9, 12 months compared with 1 month and % released β-hexosaminidase were analyzed using Wilcoxon signed-rank test and Paired-samples T-test for non-normally and normally distributed data, respectively. A comparison of MWD and % released β-hexosaminidase among all extracts was made using Friedman test. Results were considered statistically significant at a *p*-value of < 0.05.

#### Results

Forty children with history of immediate wheat allergy and positive SPT to wheat, were recruited. Demographic data and clinical characteristics of all participants are shown in **Table 1**. The median age of children was 6.04 years, and 50% were male. The median onset of wheat allergy was 10.50 months. Almost half of the patients had history of wheat-induced anaphylaxis. None of the patients had history of wheat dependent exercise induced anaphylaxis. Allergic rhinitis, asthma, atopic dermatitis, and other food allergies were found in 60%, 7.50%, 42.50%, and 50% of the participants, respectively. Sixty percent of the patients had family history of atopy. The most common symptom associated with wheat ingestion was cutaneous (100%), followed by respiratory (37%) and gastrointestinal (22.50%).

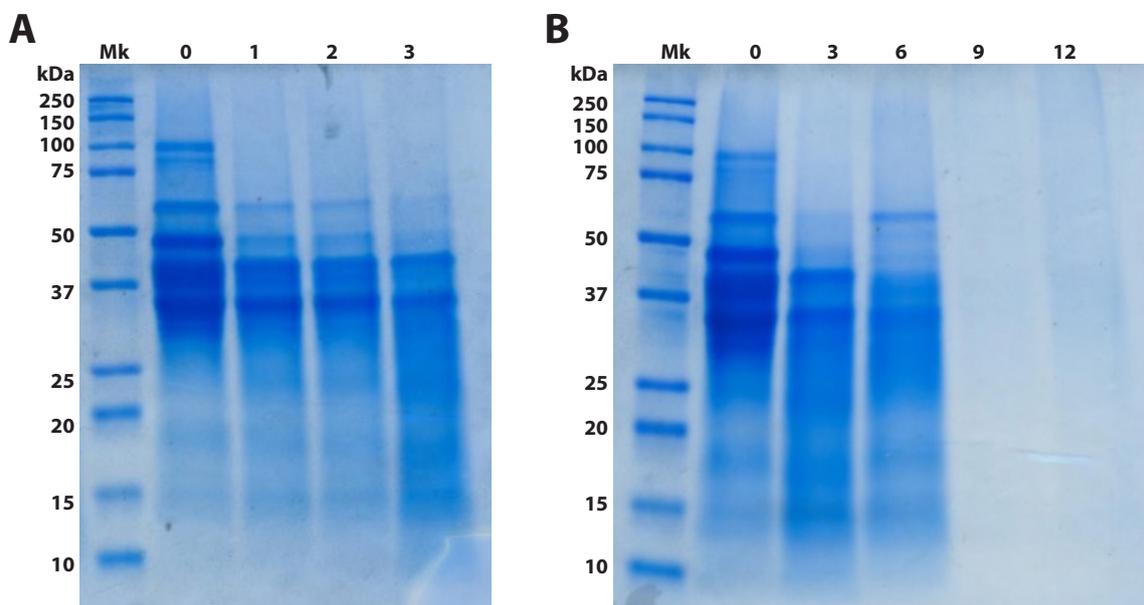
**Table 1. Demographic data and clinical characteristics of study participants.**

Patient characteristics	n = 40
Age (years), median (range)	6.04 (2–18.50)
Male gender, n (%)	20 (50)
Age at onset (months), median (range)	10.50 (2–144)
Severity of symptoms, n (%)	
Wheat allergy	21 (52.50)
Wheat anaphylaxis	19 (47.50)
Personal history of atopy, n (%)	
Allergic rhinitis	24 (60)
Asthma	3 (7.50)
Atopic dermatitis	17 (42.50)
Multiple food allergies	20 (50)
Positive family history of atopy, n (%)	24 (60)

The status of wheat allergy was current wheat allergy 27 (67.50%), wheat allergy on immunotherapy 7 (17.50%) and natural resolution of wheat allergy 6 (15%) of our patients. The results of SPT were not significantly different in these 3 subgroups (data not shown).

All gliadin extracts, stored for 1, 6, 9, and 12 months, were subjected for sterility testing for bacteria and the results revealed lack of bacterial growth. The protein profile of the extracts stored at different times was determined by SDS-PAGE (**Figure 1**). The protein profile of fresh extract showed multi-heavy stained bands mobilized between MW of 30–50 kDa and 3 light stained bands mobilized between MW of 55–100 kDa (lane 0, **Figure 1A, 1B**). The multiple high intensity bands became less intense as storage time increased. The 2 protein bands, 30–40 kDa, were observed during the first 3 storage months (**Figure 1A**). However, the 40 kDa protein band disappeared while the 30 kDa protein band smeared in the 6-month stored gliadin extract (**Figure 1B**). Among the 3 low intensity bands, both 80 and 100 kDa were disappeared in the 1-month storage while the 50–60 kDa band were seen until 6-month storage (**Figure 1B**). In contrast to those of short storage, the protein bands of the extracts stored for 9 and 12 months were hardly seen (**Figure 1B**).

The MWD of SPT using the gliadin extracts stored for 1, 6, 9, 12 months at 2–8°C are presented in **Figure 2**. The medians (range) of MWD of SPT using 1, 6, 9, 12 month-stored gliadin extracts, were 6.53 (0–26.50), 5.63 (0–26.90), 6.98 (0–27.35), 5.75 (0–29.35) mm, respectively ( $p = 0.09$ ). Compared to the 1 month-stored, there was no statistically significant difference of MWD using 6, 9, and 12 month-stored gliadin extracts ( $p = 0.25, 0.36, 0.09$ , respectively).



**Figure 1. SDS-PAGE analysis of the gliadin extracts of different storage time. A) the gliadin extracts stored for 0, 1, 2 and 3 months; B) the gliadin extracts stored for 0, 3, 6, 9 and 12 months.**

Note: Mk, molecular weight marker, (unit in kDa)

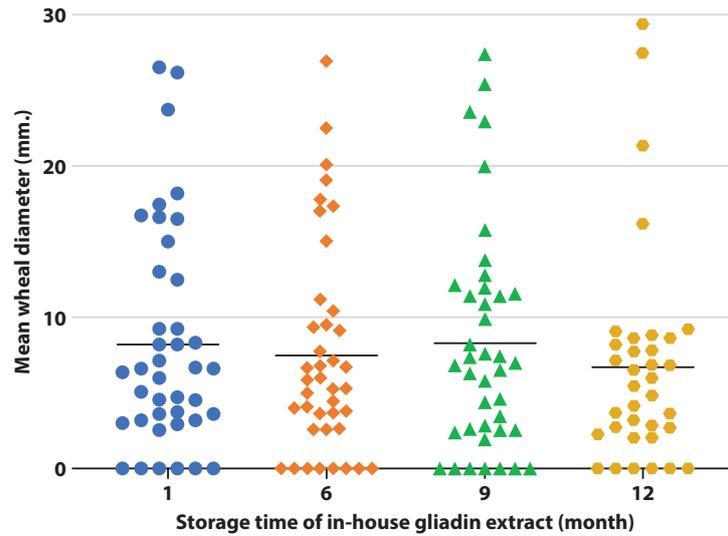


Figure 2. Comparisons of mean wheal diameter from skin prick test using the gliadin extracts of different storage time.

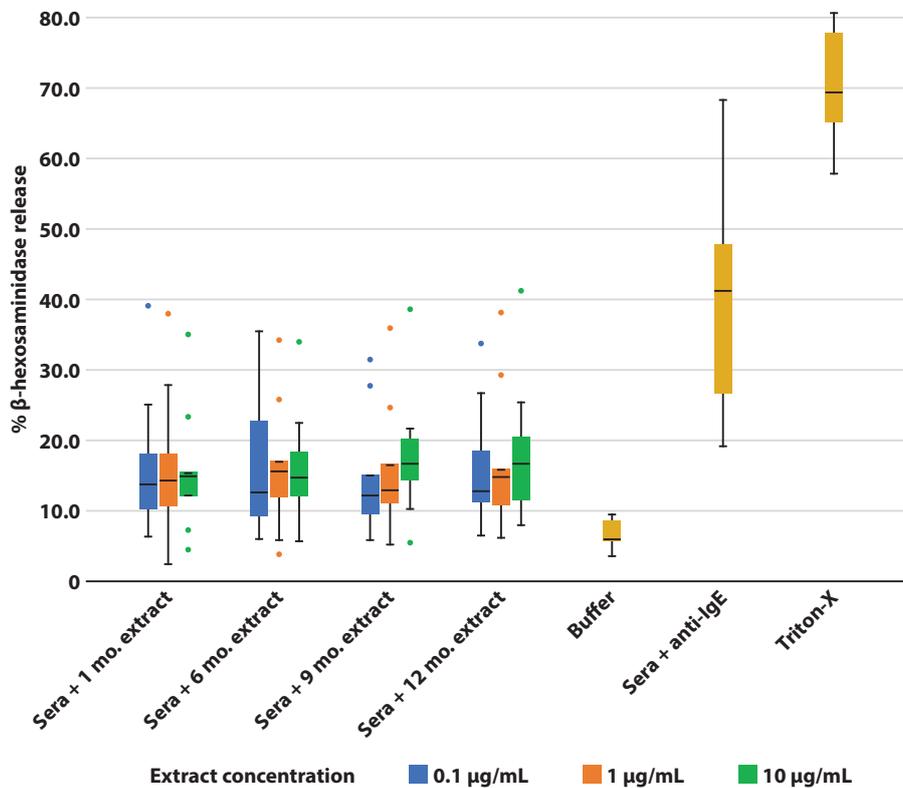


Figure 3. The degranulation of IgE-primed humanized RBL SX-38 cells incubated with the gliadin extract. Released of  $\beta$ -hexosaminidase from RBL SX-38 cells were determined by priming 1:20 diluted of 10 wheat-allergic serum before incubated with 0.1, 1.0 and 10  $\mu\text{g}/\text{mL}$  of the gliadin extract (stored for 1, 6, 9 and 12 months). The assay was performed in duplicate with serum of individual. Degranulation indicated as  $\beta$ -hexosaminidase release (%).

Note: box plot indicating the median with lines, the interquartile range with a box, the minimum and maximum values with whiskers, and outlier data points with extra circles

For cell degranulation assay, serum primed RBL-SX38 cells were incubated with the in-house gliadin extracts stored for 1, 6, 9, and 12 months. The primed RBL-SX38 cells released  $\beta$ -hexosaminidase after incubated with various concentration of the in-house gliadin extracts (**Figure 3**). The 0.1  $\mu\text{g}/\text{mL}$  in-house gliadin extracts, stored at 1, 6, 9, and 12 months, stimulated released  $\beta$ -hexosaminidase at 14.02% (6.56–39.22%), 12.87% (6.27–35.60%), 12.42% (6.12–31.59%), and 13.05% (6.73–33.86%), respectively ( $p > 0.05$ ). The 1.0  $\mu\text{g}/\text{mL}$  in-house gliadin extracts, stored at 1, 6, 9, and 12 months, stimulated released  $\beta$ -hexosaminidase at 14.57% (2.78–38.13%), 15.85% (4.18–34.41%), 13.13% (5.46–36.08%), and 15.06% (6.42–38.31%), respectively ( $p > 0.05$ ). The 10  $\mu\text{g}/\text{mL}$  in-house gliadin extracts, stored at 1, 6, 9, and 12 months, stimulated released  $\beta$ -hexosaminidase at 15.13% (4.84–35.19%), 14.93% (5.92–34.14%), 16.93% (5.69–38.68%), and 16.92% (8.23–41.30%), respectively ( $p = 0.01$ ). There is a trend that the 10  $\mu\text{g}/\text{mL}$  in-house gliadin extracts elicited higher amount of  $\beta$ -hexosaminidase release than the 0.1  $\mu\text{g}/\text{mL}$  and 1  $\mu\text{g}/\text{mL}$ , at 1, 9 and 12-month storage. However, the results were not significantly different ( $p > 0.05$ ).

## Discussion

Skin prick test to wheat is a standard tool to determine wheat sensitization. Wheat protein extract used in SPT plays a major role to predict IgE-mediated wheat allergy. However, commercial wheat extracts contain mainly salt/water soluble proteins while the wheat allergens causing allergic reaction including anaphylaxis, are mainly salt/water insoluble proteins.<sup>29–31</sup> Our previous studies revealed that the SPT using commercial wheat extracts did not offer a good performance compared with in-house wheat extracts.<sup>32</sup> Phisitbuntoon, et al.<sup>17</sup> compared the performance of SPT using commercial extract and 4 in-house wheat extracts (wheat-Coca-10% EtOH, wheat-salt, gliadin, and glutenin) in children with IgE-mediated wheat allergy. Of the 5 extracts, gliadin extract provided the best SPT performance with 84.2% sensitivity, 88.9% specificity, 94.1% positive predictive value, 72.7% negative predictive value, 7.59 positive likelihood ratio, 0.18 negative LR, and 85.7% accuracy. However, the stability of this newly invented gliadin extract has not been determined.

The stability of allergen extract can be determined by several methods. Although in-vivo skin test is a desired method, it may be challenging to enroll patients. Therefore, many in-vitro tests such as SDS-PAGE, Bradford assay, IgE immunoblotting, ELISA and ELISA inhibition have been used to study the stability of extracts.<sup>18–23</sup> The study on stability of cereal allergens in extract was limited. Varjonen et al, studied the stability of wheat extracts by SDS-PAGE and immunoblotting. They found that wheat proteins were stable for at least 21 months when stored at 4°C in aqueous solution containing 50% glycerol and 0.45% NaCl. In solution containing 0.4% phenol and 0.9% NaCl, degradation of many proteins was seen after 1 month.<sup>33</sup> It was known that glycerin provided longer expiration date.

Our study used Gliadin buffer (containing 10% glycerol) which extracted appropriate amount of gliadin and provided stability of extract for at least 12 months.

In this study, we recruited patients with history of immediate allergic reaction and positive SPT to wheat who were followed at the Pediatric Allergy Clinic. Our aim was to include all status of wheat allergic patients who required SPT in real life (need for sensitization evaluation) regardless of oral food challenge results or current wheat allergy treatment.

We used SDS-PAGE to visually assessment of stability of protein over time. However, SDS-PAGE patterns does not conclusively verify allergen potency.<sup>18</sup> Some allergen may be degraded but the IgE-binding fragment may be maintained. This phenomenon was seen in our previous report that although the commercial wheat extract showed almost no protein band in SDS-PAGE, it still elicited SPT reaction.<sup>17</sup> The SPT was chosen to assess stability and biological potency of the extracts as it reflected the functional assay. It was also the main purpose of using allergen extract in clinical practice. Moreover, the potency and stability of the gliadin extracts were also assessed by the RBL-SX38 cell degranulation assay, a cell-based functional assay to determine IgE-mediated allergic reactions of food allergens. The RBL-SX38 cell degranulation assay appears to be a much practical assay than basophil activation test since it requires only serum but not fresh mast cells or basophils of patients. This assay is useful in the study that patient recruitment is difficult such as in a multicenter study. To our knowledge, the study using the RBL-SX38 cell degranulation assay to determine the potency and stability of food allergen extracts is limited.

We found that the MWD of SPT using the in-house gliadin extracts stored for 6, 9, and 12 months at 2–8°C were not statistically different from the 1-month stored extract as a reference among wheat-sensitized participants. In addition, all of the used extracts were sterile through the storage period.

The humanized RBL-SX38 cells degranulation was observed after the IgE primed cells were incubated with tested extracts as released  $\beta$ -hexosaminidase was detected. The percentage of released  $\beta$ -hexosaminidase from the cells incubated with all tested extracts was mostly not significantly different except for the concentration of 10  $\mu\text{g}/\text{mL}$  in-house gliadin extracts groups, stored at 9, and 12 months, which surprisingly showed released  $\beta$ -hexosaminidase more than newly-prepared 1-month stored extract. It was presumed that the 9 and 12-month stored extracts might contain a high number of peptides containing IgE-binding epitopes. The IgE-binding epitope of omega-5 gliadin, a major wheat allergen, were small peptides contained 7 amino acids.<sup>34</sup> Due to a limited serum, a response to the extracts was determined at only 3 concentrations of extract. Therefore, factors affecting the dose-response curve such as the characteristic of antigen epitope for IgE binding could not be examined.<sup>35</sup>

In contrast to our SPT result and cell degranulation assay, the analysis results from SDS-PAGE showed that the 9 and 12-month stored extracts contained less proteins with MW ranging from 10 to > 100 kDa mobilized in the gel suggesting protein degradation. However, it is possible that peptides with MW < 10 kDa containing IgE epitopes might still remain in these extracts but not visible on the SDS-PAGE gel. As a result, the remaining peptides containing IgE epitopes in both 9 and 12-month stored extracts could elicit the wheal reaction and the released  $\beta$ -hexosaminidase similar to the newly prepared extracts. This result suggested that other functional assays such as SPT and cell degranulation assay were needed to provide additional information to SDS-PAGE analysis, to study the stability of wheat extracts. Our previous study also showed that the SDS-PAGE analysis of a commercial wheat extract demonstrated absent protein band. However, the commercial extract could elicit skin prick test reaction although the reaction was inferior to gliadin extract.<sup>17</sup>

In conclusion, the in-house gliadin extracts for determining wheat sensitization were stable and sterile at 2–8°C for up to 12 months. The gliadin extracts stored at different storage time provide similar SPT results and can mostly stimulate serum IgE primed RBL-38X cells releasing at similar percentage of  $\beta$ -hexosaminidase. These functional assays should be used, in addition to protein analysis to identify the stability of allergen extracts.

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## Conflict of interest

All authors declare no conflict of interest.

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