Allergology International xxx (xxxx) xxx

Contents lists available at ScienceDirect

Allergology International

journal homepage: http://www.elsevier.com/locate/alit

Original Article

Identification of peroxidase-1 and beta-glucosidase as cross-reactive wheat allergens in grass pollen-related wheat allergy

Ryohei Ogino [a], Yuko Chinuki [a], Tomoharu Yokooji [b], Daigo Takizawa [c], Hiroaki Matsuo [c], Eishin Morita [a, *]

[a] Department of Dermatology, Faculty of Medicine, Shimane University, Izumo, Japan
[b] Department of Frontier Science for Pharmacotherapy, Graduate School of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan
[c] Department of Pharmaceutical Services, Hiroshima University Hospital, Hiroshima, Japan

ARTICLE INFO

Article history:
Received 25 June 2020
Received in revised form
8 September 2020
Accepted 13 September 2020
Available online xxx

Keywords:
Allergens
Cross-reactivity
Food allergy
Grass
Wheat

Abbreviations:
aa, amino acid; BAT, basophil activation test; CBB, Coomassie brilliant blue; FEIA, fluorescent enzyme-immunoassay; GPWA, grass pollen-related wheat allergy; HRP, horseradish peroxidase; HWP, hydrolyzed wheat protein; WDEIA, wheat-dependent exercise-induced anaphylaxis

ABSTRACT

Background: Some patients with wheat-dependent exercise-induced anaphylaxis (WDEIA) or wheat allergy showed negative ω-5 gliadin-specific IgE test and high level of grass pollen-specific IgE. It was presumed that these patients developed allergic reaction upon cross-reaction of their IgE antibodies raised against grass pollen allergens to wheat allergens. This study aimed to clarify clinical characteristics and wheat allergens of this phenotype of WDEIA/wheat allergy, which were tentatively diagnosed as grass pollen-related wheat allergy (GPWA).
Methods: A total of six patients with GPWA were enrolled, and controls were 17 patients with grass pollen allergy but no episode of wheat allergy, and 29 patients with other wheat allergies: 18 with conventional WDEIA and 11 with hydrolyzed wheat protein allergy. Sensitization to wheat proteins was determined by basophil activation test (BAT). IgE-binding proteins in wheat flour were identified by immunoblotting followed by mass spectrometry. Wheat allergen-specific IgE tests were established by CAP-FEIA system.
Results: All the six patients with GPWA were sensitized to water-soluble wheat proteins in BAT and IgE-immunoblotting, and peroxidase-1 (35 kDa) and beta-glucosidase (60 kDa) were identified as specific IgE-binding wheat proteins. The binding of patient IgE to these proteins was inhibited by pre-incubation of patient sera with grass pollen. The peroxidase-1- and beta-glucosidase-specific IgE tests identified three and four of six patients with GPWA, respectively, but only two of 29 controls, indicating high specificity of these tests.
Conclusions: Peroxidase-1 and beta-glucosidase are specific wheat allergens for GPWA among grass pollen allergy and other types of wheat-induced food allergies.

Introduction

Wheat is a common foodgrain that elicits IgE-mediated allergies.[1,2] Wheat-induced food allergies are typically seen as immediate-type wheat allergy in young children and wheat-dependent exercise-induced anaphylaxis (WDEIA) in adolescents and adults.[2] The former wheat allergy commonly occurs in association with atopic dermatitis and has been partly sensitized percutaneously through eczematous skin, according to the dual-allergen-exposure-hypothesis proposed by Lack.[3] The latter WDEIA has been clarified as developed by a sensitization to ω-5 gliadin or high-molecular weight glutenin, both of which are components of water-insoluble wheat gluten proteins, possibly via the gastrointestinal tract.[4] In addition, we had an outbreak of WDEIA that occurred between 2009 and 2012 in Japan, which was caused possibly by mucocutaneous sensitization to hydrolyzed wheat protein (HWP) supplemented in a soap.[5,6] A nationwide survey revealed that more than 2000 individuals were affected by sensitization to HWP.[7] After withdrawal of the HWP-containing soap, more than 50% of the patients became remission at 60 months.[8] This second phenotype of WDEIA supports importance of the dual-allergen-exposure-hypothesis on the development of food allergies.

On the other hand, it is well known that sensitization to grass pollen allergens results in grass pollen allergy and the specific IgE

* Corresponding author. Department of Dermatology, Shimane University Faculty of Medicine, 89-1 Enya-cho, Izumo, Shimane 693-8501, Japan.
E-mail address: emorita@med.shimane-u.ac.jp (E. Morita).
Peer review under responsibility of Japanese Society of Allergology.

https://doi.org/10.1016/j.alit.2020.09.005

Please cite this article as: Ogino R et al., Identification of peroxidase-1 and beta-glucosidase as cross-reactive wheat allergens in grass pollen-related wheat allergy, Allergology International, https://doi.org/10.1016/j.alit.2020.09.005

against grass pollen allergens frequently causes cross-reaction to wheat allergens.[9] Constantin *et al.* have reported that profilin (nomenclated as Phl p 12 for timothy grass pollen profilin) is a predominant cross-reactive allergen between wheat flour and grass pollen due to common IgE epitopes, and 65% of patients with grass pollen allergy had positive results for wheat-specific IgE test (CAP-FEIA System, ImmunoCAP™, Thermo Fisher Diagnostics, Uppsala, Sweden), despite ingestion of wheat products without allergic reactions.[10] Nilsson *et al.* proposed MUXF3, a cross-reactive carbohydrate determinant, and profilin (Phl p 12) as cross-reactive allergens between timothy grass pollen and wheat upon CAP-FEIA System.[11] However, these allergens may be clinically irrelevant as most patients with timothy grass pollen allergy ingest wheat products without allergic reactions.

We experienced some adult patients with WDEIA/wheat allergy, who were sensitized to neither ω-5 gliadin nor HWP but to water-soluble wheat proteins. Interestingly, most of them were found strongly sensitized to grass pollens, such as timothy grass pollen and sweet vernal grass pollen. Thus, we presumed that these WDEIA/wheat allergy patients, who sensitized strongly to grass pollen allergens, developed an allergic reaction when they ingested wheat-containing foods upon cross-reaction of their IgE antibodies raised against grass pollen allergens to wheat allergens. This derives from the mechanism of pollen food allergy syndrome, which is caused by cross-reaction of pollen allergen-specific IgE to foods, such as fruits and vegetables, and typically seen in patients with birch pollen-related apple allergy.[12] However, wheat-mediated food allergy due to cross-reaction of grass pollen-specific IgE has been considered to be doubtful.

Major causative wheat allergens depend on the clinical types of wheat allergy. Water-insoluble proteins such as gliadins and glutenins have been identified as major allergens for WDEIA,[1,13–18] whereas water-soluble proteins, such as peroxidase, α-amylase/trypsin inhibitors, thiol reductase, and lipid transfer protein have been considered to be allergens in patients with baker's asthma, although its sensitization pattern varies among studies and countries.[1,19–22] Causative wheat allergens in immediate-type wheat allergy in young children are also variable depending on the investigation.[23–25]

In this study we aimed to clarify clinical characteristics and causative wheat allergens of the undetermined type of WDEIA/wheat allergy, which is not sensitized to ω-5 gliadin but strongly sensitized to grass pollen allergens.

Methods

Details of the experiments are given in Supplementary Methods.

Patients

The study included six adult or adolescent patients who had wheat-food allergy or fulfilled the diagnostic criteria for WDEIA (Supplementary Table 1), but showed negative ω-5 gliadin-specific IgE test and high level of sweet vernal grass pollen-specific IgE and timothy grass pollen-specific IgE. We tentatively diagnosed this third phenotype of WDEIA/wheat allergy as grass pollen-related wheat allergy (GPWA). These patients had no history of usage of a hydrolyzed wheat product-contained soap. The clinical features of the patients were shown in Table 1 and their sensitization profiles in Supplementary Table 2.

The serum allergen-specific IgE values were measured using ImmunoCAP™ and ImmunoCAP ISAC™ (Thermo Fisher Diagnostics). The controls included sera from three healthy subjects with no episodes of wheat allergies and negative wheat-specific

Table 1
Clinical features of the patients with GPWA.

Patient	Age	Gender[†]	Complications of allergic disease[‡]	Occupation	Episode of wheat allergy	Frequency	Season of episodes	Prick test[§]	ImmunoCAP™ (kU_A/L)					Diagnosis[¶]	Effect of wheat elimination diet
									Wheat	Gluten	ω-5 gliadin	Sweet vernal grass	Timothy grass		
1	32	M	Pollinosis	Office worker	1: generalized urticaria after ingesting udon noodle and acetaminophen/salicylamide, 2: urticaria after ingesting udon noodle	2	1, 2: November	wheat (−)	1.38	0.97	<0.1	116	183	WDEIA	Improved
2	38	F	AD, Pollinosis	Nursing teacher	1: urticaria, abdominal pain, diarrhea, and shock after ingesting pasta, 2: urticaria after ingesting pasta, 3: urticaria after ingesting gratin	3	1: January, 2: June, 3: December	wheat (−)	1.2	0.33	<0.1	29.4	35.9	Wheat allergy	Unknown
3	55	F	Pollinosis	Cook	1: facial edema, general erythema, and shock when walking after ingesting tenpura, 2: facial edema and throat pain when walking after ingesting bread	2	1: July, 2: August	wheat (−)	1.0	0.48	<0.1	41.6	86.8	WDEIA	Improved
4	65	M	Pollinosis	Worker	1: urticaria after ingesting bread, 2–5: facial edema, throat pain, rhinorrhea when ingesting ramen noodle after jogging, 6: facial edema when walking after ingesting ramen noodle	6	1–5: unknown, 6: March	wheat (−)	2.42	1.35	<0.1	23.9	35.6	WDEIA	Improved
5	14	M	AD, Pollinosis	Student	1–4: lip swelling and urticaria after exercise and lunch, 5: lip swelling during running after ingesting croquette, 6: urticaria after ingesting pancake	6	1–3: unknown, 4: March, 5: November, 6: December	wheat (+)	0.5	0.23	<0.1	86.9	97.8	WDEIA	Improved
6	67	M	EoE, BA	Office worker	1: vomiting and diarrhea after ingesting ramen noodle, 2–: urticaria and shock after meal with alcohol	more than 5	1: January, 2–: through the year	not done	15.1	6.94	0.1	6.16	14.1	Wheat allergy	Improved

[†] Gender; M, male; F, female.
[‡] Complications; AD, atopic dermatitis; EoE, eosinophilic esophagitis; BA, bronchial asthma.
[§] Skin prick test was performed with wheat extract (Torii Pharmaceutical Co., Ltd.).
[¶] Diagnosis; WDEIA, wheat-dependent exercise-induced anaphylaxis.

Please cite this article as: Ogino R et al., Identification of peroxidase-1 and beta-glucosidase as cross-reactive wheat allergens in grass pollen-related wheat allergy, Allergology International, https://doi.org/10.1016/j.alit.2020.09.005

IgE tests, 17 patients with grass pollen allergy but no previous episode of wheat allergy, 18 patients with conventional WDEIA based on the diagnostic criteria for WDEIA (Supplementary Table 1), and 11 patients with HWP allergy that fulfilled the diagnostic criteria for immediate wheat allergy to hydrolysed wheat product contained in facial soap and other products.[7] The sera were stored at −30 °C until use. This study was approved by the Ethics Committee of the Shimane University Faculty of Medicine (approval no. 1570). The study was explained and written informed consent was obtained from all patients.

Wheat and grass pollen proteins

Both water-soluble and insoluble wheat proteins were fractionated from commercial-blend wheat flour (Nisshin Camellia®; Nisshin Flour Milling Inc., Kobe, Japan) and three grass pollen proteins (sweet vernal grass, orchard grass, and timothy grass) were purchased from ITEA, Tokyo, Japan.

Immunoblot analysis

Samples were separated by SDS-PAGE using a 12.5% polyacrylamide gel and transferred electrophoretically onto Immobilon-P membrane (Millipore, Billerica, MA, USA). After blocked with 5% skim milk in tris-buffered saline (pH 7.6) containing 0.1% Tween-20, the membrane was incubated with a 1:10 dilution of sera for 20 h at 26 °C. To detect the IgE-binding proteins, we used a 1:40,000 dilution of horseradish peroxidase-conjugated goat anti-human IgE Abs (ImmunoReagents, Raleigh, NC, USA) and an Amersham ECL-Prime kit (GE Healthcare, Buckinghamshire, UK). For the inhibition study, sera were pre-incubated with sweet vernal grass pollen proteins (0, 0.1, 1, and 10 μg) for 1 h at 37 °C.

Basophil activation test

An allergen-induced CD203c expression-based basophil activation test (BAT) for fractionated wheat proteins was performed using an Allergenicity Kit® (Beckman Coulter, Brea, CA, USA) as previously described.[26]

Purification of wheat allergens by cation-exchange chromatography

To purify the allergens recognized by serum IgE from the patients with GPWA, water-soluble wheat proteins were first fractionated by precipitation with ammonium sulfate. The precipitates were dissolved with 50 mM HEPES-NaOH (pH 7.0) and dialyzed in the same buffer. After confirmation of the IgE-reactivity by immunoblot analysis, the dialyzed solution containing IgE-reactive proteins was further fractionated by AKTA FPLC system (GE Healthcare) using HiTrap SP HP 5 ml column (GE Healthcare). Each fraction was immunoblotted with serum IgE from the patients with GPWA.

Identification of wheat allergens by mass spectrometry

The IgE-binding proteins were extracted from the Coomassie brilliant blue (CBB)-stained gel. The extracted gels were subjected to tryptic digestion, and the digested peptides were crystallized with α-cyano-4-hydroxycinnamic acid on a stainless-steel plate. Mass spectra of peptides were obtained using TOF/TOF™ 5800 system (AB SCIEX, Framingham, MA, USA). The generated mass lists were searched against the wheat protein database (NCBI txid4565) from the National Center for Biological Information using the database search software ProteinPilot™ (ver. 4.5; AB SCIEX).

Preparation of CAP-FEIA system

To prepare the wheat allergens for the CAP-FEIA system, adequate quantities of 35-kDa and 60-kDa allergens were purified by ammonium sulfate precipitation, cation-exchange chromatography, and gel-filtration chromatography.

The purified allergens were biotinylated using Ez-link™ sulfo−NHS−LC biotin (Thermo Fisher Scientific, Waltham, MA, USA) to establish a CAP-FEIA system for wheat allergen, and applied to the solid-phase streptavidin ImmunoCAP™. The serum IgE values specific to wheat allergen were measured according to the manufacturer's instructions'. Specific IgE values $\geq$ 0.35 kU_A/L were determined to be positive.

Results

BAT and IgE immunoblot analysis with wheat proteins

The highest basophil activation was induced by the PBS-soluble fraction of wheat proteins in most of the patients with GPWA, when basophils from the patients' peripheral blood were stimulated with several fractions of wheat proteins (Table 2). More than 10% of basophils expressed CD203c molecules with the PBS-soluble fraction in all six patients, suggesting that cross-reactive allergens to grass pollen allergens commonly have water-soluble characteristics.

Immunoblotting showed that all six patients with GPWA reacted to the water-soluble wheat protein fraction; two of them additionally reacted to wheat-insoluble proteins, supporting that the major culprit wheat allergens were water-soluble wheat proteins (Fig. 1A). A 60-kDa band was observed in the water-soluble wheat proteins of all six patients and weakly in one of three healthy subjects (Healthy subject 2). A 35-kDa band was observed in three patients (Patients 1, 2, and 4). None of the patients reacted to HWP or ω-5 gliadin (data not shown).

To evaluate the IgE-cross-reactivity between grass pollen and wheat proteins, immunoblot inhibition assay using sweet vernal pollen as an inhibitor was performed for five patients with GPWA (Patients 1–4, and 6). IgE from the sera commonly bound to a 35-kDa protein in orchard and sweet vernal grass pollen and 37-kDa and 33-kDa proteins in timothy grass pollen in addition to water-

Table 2
Results of basophil activation test of the patients with GPWA.

Patient	Basophil activation (CD203c high%)[†]					
	Control		Wheat			
	Negative	Positive	PBS	Ethanol	Alkali	ω-5
1	4.41	94.6	73.1	9.31	10.5	6.12
2	1.36	82.7	37.2	0.84	1.07	0.95
3	2.59	60.4	11.7	6.17	3.44	2.57
4	2.46	86.9	40.7	0	33.7	3.00
5	3.21	78.2	53.4	3.70	4.09	4.73
6	3.15	54.9	50.0	51.7	19.3	4.55

[†] Basophile activation was presented as expression of CD203c on basophils induced by each fractionated wheat protein; PBS, phosphate-buffered saline soluble fraction; Ethanol, 70% ethanol soluble fraction (gliadins); Alkali, 2% Na_2CO_3 with 0.1 N NaOH soluble fraction (glutenins); ω-5, native ω-5 gliadin.

Please cite this article as: Ogino R et al., Identification of peroxidase-1 and beta-glucosidase as cross-reactive wheat allergens in grass pollen-related wheat allergy, Allergology International, https://doi.org/10.1016/j.alit.2020.09.005

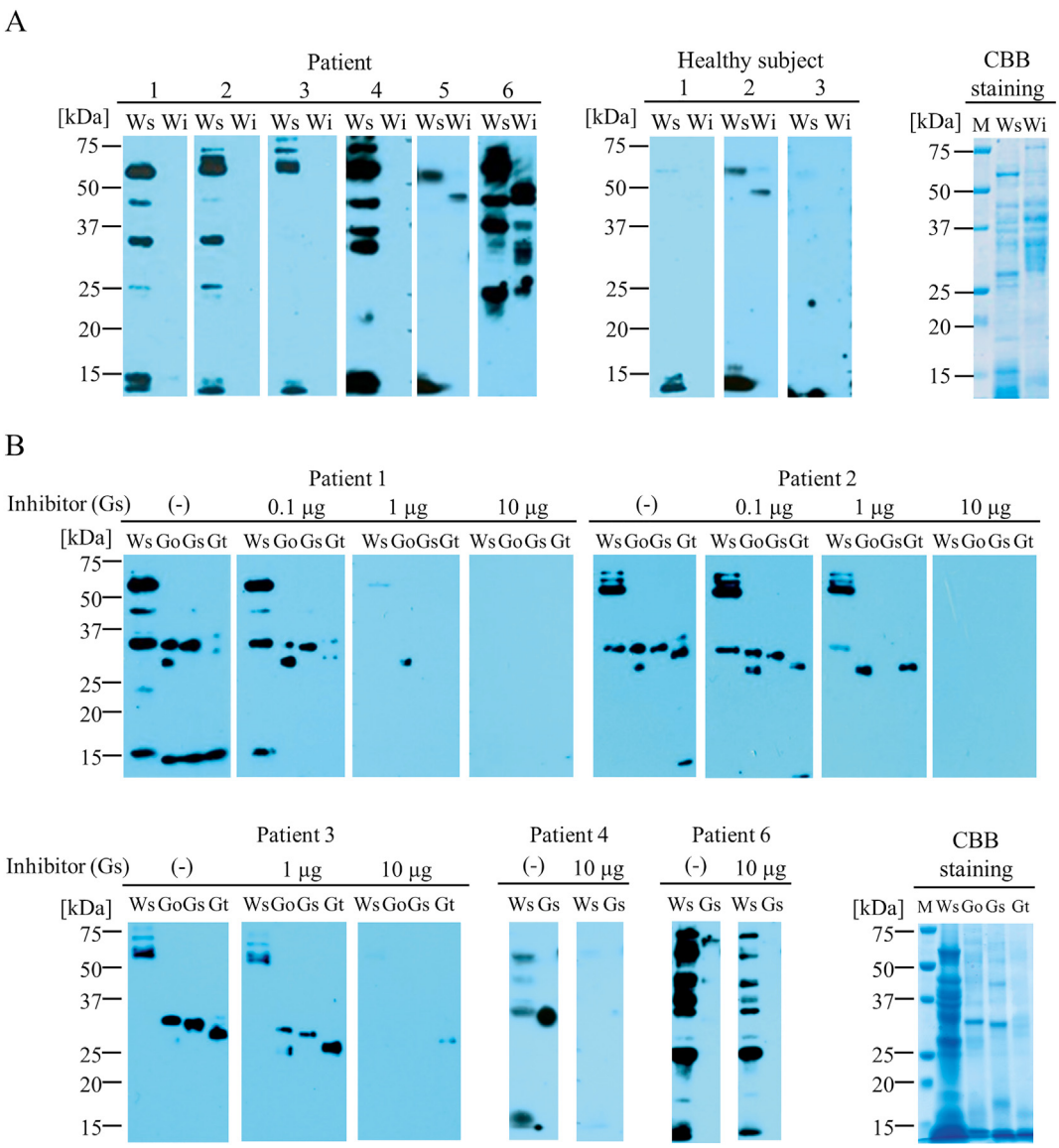

Fig. 1. IgE immunoblot analysis of wheat proteins and grass pollen proteins. **A)** Water-soluble wheat proteins (Ws) 10 μg and water-insoluble wheat proteins (Wi) 10 μg were immunoblotted with sera from six patients with GPWA and three healthy subjects. M, molecular weight marker. **B)** Patients' sera were pre-incubated with sweet vernal grass pollen proteins (0–10 μg), and applied to immunoblotting for Ws 20 μg, orchard grass pollen (Go) 0.05 μg, sweet vernal grass pollen (Gs) 0.05 μg, and timothy grass pollen (Gt) 0.05 μg.

soluble wheat proteins (Fig. 1B). Pre-incubation of patients' sera with sweet vernal grass pollen proteins inhibited the IgE-binding to 60-kDa and 35-kDa wheat proteins and three grass pollen proteins in a concentration-dependent manner (Fig. 1B). These results suggested that IgE from the patients with GPWA predominantly cross-reacted with 60-kDa and 35-kDa wheat proteins.

Purification and identification of 35-kDa and 60-kDa allergens in water-soluble wheat proteins

To identify the IgE-binding wheat allergens, water-soluble wheat proteins were fractionated by consecutive ammonium sulfate precipitation at 10% intervals. IgE-binding to 35-kDa bands were observed in the precipitate with 40–70% ammonium sulfate by immunoblotting using sera of patient 2 and 4 (Fig. 2A, lanes 50–70). From this result, fractionated proteins in 40–60% ammonium sulfate precipitation were further fractionated by cation-exchange chromatography (Fig. 2B). Each fraction was collected and presence of IgE-binding proteins was examined by immunoblot using patients' sera. After identifying the IgE-binding 35-kDa allergen in fraction no. 9 (Fig. 2B), we identified the 35-kDa allergen as peroxidase-1 (accession no. 300087071) with ≥95% confidence six tryptic digested peptides using ProteinPilot™. These peptides accounted for 21.5% of the amino acid (aa) sequence of peroxidase-1 (77/358 aa, Supplementary Table 3).

The 60-kDa proteins bound to the patients' IgE were found in the precipitates from 30 to 70% ammonium sulfate (Fig. 2A, lanes 30–70). ProteinPilot™ analysis of the 60-kDa protein precipitated in 40–50% ammonium sulfate (Fig. 2A, lane 50) revealed beta-amylase (accession no. 32400764). As the 60-kDa band was also seen in healthy subject 2, the binding was considered non-specific. However, the 60-kDa protein (Fig. 2A, lanes 60 and 70) reacted only to the patients' sera but not to that of healthy subject 2.

We further fractionated the proteins in the 50–70% ammonium sulfate precipitate (Fig. 2A, lanes 60 and 70) by cation-exchange chromatography (Fig. 2C). Each fraction was collected, and the presence of IgE-binding 60-kDa protein was confirmed by immunoblot using patients' sera. Specific binding of patients' sera IgE was

Please cite this article as: Ogino R et al., Identification of peroxidase-1 and beta-glucosidase as cross-reactive wheat allergens in grass pollen-related wheat allergy, Allergology International, https://doi.org/10.1016/j.alit.2020.09.005

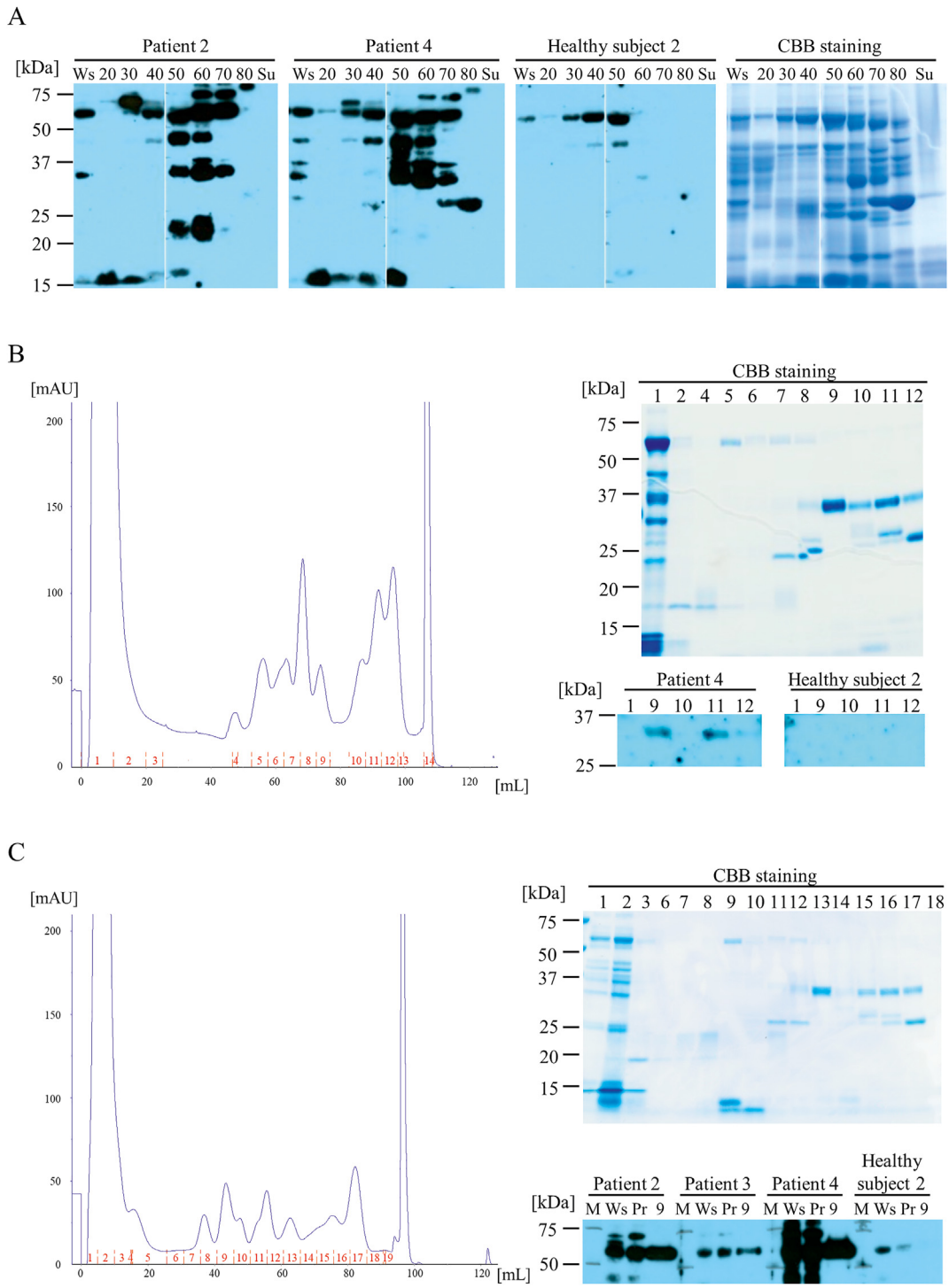

Fig. 2. Purification of 35-kDa and 60-kDa allergens in water-soluble wheat proteins. **A)** Water-soluble wheat proteins fractionated by ammonium sulfate precipitation from 20% to 80% were immunoblotted with patients' sera. Ws, water-soluble wheat proteins; 20, <20%; 30, 20–30%; 40, 30–40%; 50, 40–50%; 60, 50–60%; 70, 60–70%; 80, 70–80%; and Su, supernatant at 80% ammonium sulfate solution (20 μg each lane). **B)** The precipitate with 40–60% ammonium sulfate was fractionated by cation-exchange chromatography to purify the 35-kDa allergen. Fractions containing 35-kDa proteins were immunoblotted (2.5 μg each lane). **C)** The precipitate with 50–70% ammonium sulfate was fractionated by cation-exchange chromatography to purify the 60-kDa allergen. Water-soluble wheat proteins (Ws), precipitant of 50–70% ammonium sulfate (Pr) and fraction no. 9 containing 60-kDa protein (9) was immunoblotted (2.5 μg each lane). M, molecular weight marker.

detected in fraction no. 9 of the chromatography (Fig. 2C). ProteinPilot™ analysis revealed the 60-kDa protein in fraction no. 9 as beta-glucosidase (accession no. 359828768), with seven tryptic digested peptide that accounted for 22.2% of the aa sequence of beta-glucosidase (111/501 aa, Supplementary Table 3).

Evaluation of the CAP-FEIA system for peroxidase-1 and beta-glucosidase

To evaluate the usefulness of measuring peroxidase-1- and beta-glucosidase-specific IgE to identify patients with GPWA,

Please cite this article as: Ogino R et al., Identification of peroxidase-1 and beta-glucosidase as cross-reactive wheat allergens in grass pollen-related wheat allergy, Allergology International, https://doi.org/10.1016/j.alit.2020.09.005

peroxidase-1 (Fig. 2B, fraction no. 9) and beta-glucosidase (Fig. 2C, fraction no. 9) were further purified by gel-filtration chromatography and biotinylated (Fig. 3). Furthermore, we confirmed that the band for fractionated peroxidase-1 and beta-glucosidase specifically disappeared on pre-incubation of patients' sera with sweet vernal grass pollen proteins (Supplementary Fig. 1).

Upon establishment of the CAP-FEIA systems, serum IgE specific to peroxidase-1 was positively detected in three of six (Patients 1, 4, and 6) patients with GPWA (class 2: 2, class 3: 1) and serum IgE specific to beta-glucosidase was positively detected in four of six (Patients 1, 2, 4, and 6) patients with GPWA (class 1: 1, class 2: 2, class 3: 1) (Supplementary Table 4). Additionally, three of the 17 patients with grass pollen allergy had IgE positive to peroxidase-1 (class 1: 1, class 2 $\leq$: 2, positivity rate 17.6%), while five of these 17 patients had IgE positive to beta-glucosidase (class 1: 3, class 2 $\leq$: 2, positivity rate 29.4%). Of the 18 conventional WDEIA patients, only two showed positive results in the peroxidase-1-specific IgE (class 1: 2, positivity rate 11.8%) and beta-glucosidase-specific IgE (class 1: 1, class 2 $\leq$: 1, positivity rate 11.8%) tests, respectively. All patients with HWP allergy were negative with these tests (positivity rates 0.0%). Table 3 summarizes the results of allergen-specific IgE tests.

Discussion

In this study we provided some evidence that subjects who have been sensitized strongly to grass pollen allergens possibly cause immediate-type allergic reaction to wheat allergens on ingestion of wheat-containing foods due to cross-reaction of grass-pollen allergen-specific IgE to wheat allergens. In addition, we demonstrated that IgE in patients with GPWA reacted to peroxidase-1 and beta-glucosidase as specific allergens for GPWA from water-soluble wheat proteins. Sensitivity of peroxidase-1- and beta-glucosidase-specific IgE tests to identify GPWA was 50% and 67%, respectively (Table 4), suggesting that causative allergen of GPWA is not single. However, specificity of peroxidase-1- and beta-glucosidase-specific IgE tests to identify GPWA was 82% and 71%, respectively, in 23 patients with grass pollen allergy; six GPWA and 17 grass pollen allergy without episode of wheat allergy (Table 4), suggesting that

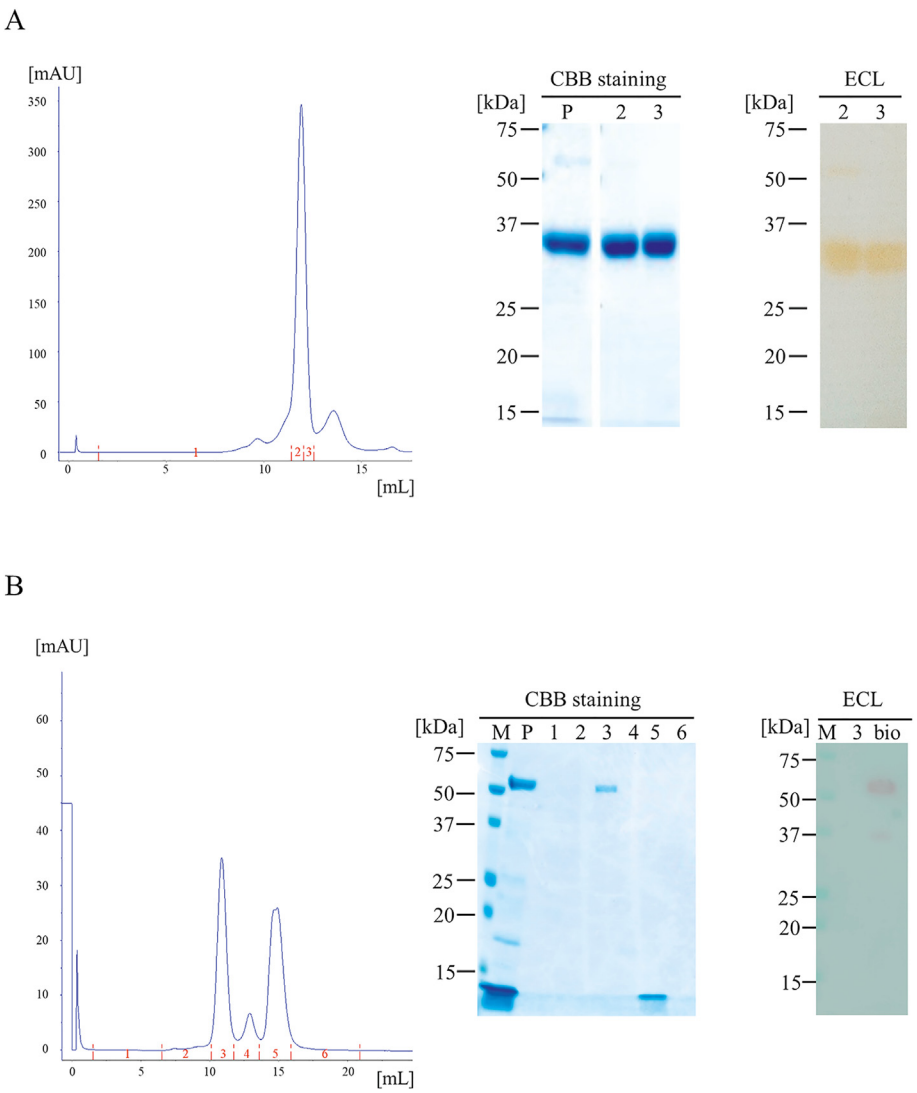

Fig. 3. Purification and biotinylation of wheat peroxidase-1 and beta-glucosidase for CAP-FEIA system. **A)** The peroxidase-1 containing cation-exchange fraction was further purified by gel-filtration chromatography after being biotinylated, and visualized using CBB staining and horseradish peroxidase (HRP)-conjugated streptavidin. P, peroxidase-1 preparation before gel-filtration; 2, fraction no. 2; 3, fraction no. 3 (2.5 μg each lane). Fraction no. 3 was used for CAP-FEIA system. **B)** The beta-glucosidase containing cation-exchange fraction was further purified by gel-filtration chromatography. The fractions were separated by SDS-PAGE (2.5 μg each lane); P, beta-glucosidase preparation before gel-filtration; each lane number corresponds to each fraction of gel-filtration. Fraction no. 3 was biotinylated and used for CAP-FEIA system; bio, biotinylated fraction no. 3.

Please cite this article as: Ogino R et al., Identification of peroxidase-1 and beta-glucosidase as cross-reactive wheat allergens in grass pollen-related wheat allergy, Allergology International, https://doi.org/10.1016/j.alit.2020.09.005

Table 3
Results of allergen-specific IgE tests for peroxidase-1, beta-glucosidase, wheat proteins, and grass pollens.

Patients group	ImmunoCAP™ class[†]	Peroxidase-1	beta-glucosidase	Wheat	Gluten	ω-5 gliadin	Sweet vernal grass	Timothy grass
Grass pollen-related wheat allergy (n = 6)	0	3 (50.0)[‡]	2 (33.3)	0 (0.0)	2 (33.3)	6 (100)	0 (0.0)	0 (0.0)
	1	0 (0.0)	1 (16.7)	1 (16.7)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)
	≥2	3 (50.0)	3 (50.0)	5 (83.3)	3 (50.0)	0 (0.0)	6 (100)	6 (100)
Grass pollen allergy[§] (n = 17)	0	14 (82.3)	12 (70.6)	11 (64.7)	13 (76.5)	17 (100)	0 (0.0)	1 (5.9)
	1	1 (5.9)	3 (17.6)	1 (5.9)	3 (17.6)	0 (0.0)	3 (17.6)	0 (0.0)
	≥2	2 (11.8)	2 (11.8)	5 (29.4)	1 (5.9)	0 (0.0)	14 (82.4)	16 (94.1)
Conventional WDEIA (n = 18)	0	16 (88.9)	16 (88.9)	9 (50.0)	6 (33.3)	3 (16.7)	11 (61.1)	9 (50.0)
	1	2 (11.1)	1 (5.6)	4 (22.2)	3 (16.7)	1 (5.6)	1 (5.6)	2 (11.1)
	≥2	0 (0.0)	1 (5.6)	5 (27.8)	9 (50.0)	14 (77.8)	6 (33.3)	7 (38.9)
HWP-allergy (n = 11)	0	11 (100)	11 (100)	7 (63.6)	7 (63.6)	11 (100)	8 (72.7)	7 (63.6)
	1	0 (0.0)	0 (0.0)	1 (9.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	≥2	0 (0.0)	0 (0.0)	3 (27.3)	4 (36.4)	0 (0.0)	3 (27.3)	4 (36.4)

[†] ImmunoCAP™ (kU_A/L) were classified as follows; class 0 (negative), <0.34; class 1 (positive), 0.35–0.69; ≥ class 2, ≥0.70.
[‡] Percentage within each patient group.
[§] Grass pollen allergy without episode of wheat allergies. WDEIA, wheat-dependent exercise-induced anaphylaxis; HWP, hydrolyzed wheat protein.

sensitization to these allergens is a risk factor to develop wheat-induced food allergy. On the other hand, specificity of peroxidase-1- and beta-glucosidase-specific IgE tests to identify GPWA in 35 patients with wheat-induced allergy; six GPWA, 18 conventional WDEIA, and 11 HWP allergy, was 93% and 93%, respectively (Table 4), suggesting that sensitization to these allergens are specific to GPWA.

Sánchez-Monge *et al.* first identified peroxidase-1 as an allergen for baker's asthma, reporting that six of 10 patients were sensitized to peroxidase-1 by dot-blot analysis.[19] Pastorello *et al.* reported that 11 of 22 patients with wheat allergy (18 adults and 4 children) reacted positively to peroxidase-1 by skin prick test, and that IgE binding to the 30–38 kDa proteins, including peroxidase-1, was inhibited by pre-incubation of patients' sera with grass pollen extract.[24] Our present results are compatible with those of these previous reports. Patient 2 showed negative result for the peroxidase-1-specific IgE test, although the serum IgE from the patient strongly bound to a 35-kDa protein in immunoblotting (Fig. 1, Supplementary Table 4). These results suggest that the sensitivity of our IgE test can be improved by optimizing biotinylation and/or allergen concentrations for the CAP-FEIA system. It is unlikely that peroxidase enzyme activity affected the immunoblot analysis because the 35-kDa band was not visualized with sera from healthy subjects (Fig. 1, 2, and Supplementary Fig. 1). In contrast, Sander *et al.* reported that none of 40 German bakers were sensitized to peroxidase as well as 10 subjects with mild asthma and hay fever.[20] These observations may indicate a diversity in sensitization to wheat allergens depending on their sensitization route, inhalation route or mucocutaneous route.

To the best of our knowledge, beta-glucosidase was newly identified as a cross-reactive allergen of IgE against grass pollen, which was previously reported as a 57-kDa component of wheat.[27] Although the IgE of all six patients bound the 60-kDa protein in water-soluble wheat proteins by immunoblotting (Fig. 1A), the sensitivity of beta-glucosidase-specific IgE test was only 67% and the test showed negative results for two patients (Patients 3 and 5, Supplementary Table 4). One possible explanation is that the IgE of the two patients recognized beta-amylase located also in the 60-kDa band (Fig. 2A, lane 50). According to previous reports,[24,27,28] beta-amylase might also be a good candidate allergen for GPWA.

The ImmunoCAP™ showed that five of six sera from GPWA patients reacted more strongly to grass pollens than to wheat proteins (Table 1) and these patients were strongly sensitized to grass pollens compared to the 17 patients with grass pollen allergies without episodes of wheat allergies (Supplementary Table 5). Thus, these patients were likely to be sensitized with grass pollen initially and allergic symptoms could be elicited by cross-reactivity to ingested wheat proteins, as suggested by Matricardi *et al.*[29] However, limited sample size of grass pollen allergic controls was one of the limitations of this study.

We suspected that Phl p 1 is a major sensitizing grass pollen allergen in patients with GPWA due to several reasons. First, several studies reported that Phl p 1 is a major allergen in grass pollen.[11,30] Second, most of our patients (five out of our six patients except patient 6) were strongly sensitized to Phl p 1, as shown in Supplementary Table 2. Third, immunoblot analysis showed that IgE in the patients' sera strongly bound 35-kDa proteins of grass pollens (Fig. 1B); the band was identified as a Group I pollen allergen, beta-expansin, which belongs to same allergen group as Phl p 1 by our mass spectrometry analysis (data not shown). However, the basic local alignment search tool analysis showed that the amino acid sequences of wheat peroxidase-1 and beta-glucosidase shared only 3% and 7% identities with timothy grass Phl p 1, respectively.

Two possible hypotheses may explain the mechanism of cross-reaction of grass pollen IgE to wheat protein allergen. First, patient IgE specific to grass pollen proteins except for Phl p 1 cross-react with ingested wheat allergens; peroxidase-1, beta-glucosidase, and possibly beta-amylase. On the basic local alignment search tool analysis using the Poaceae family Poeae tribe protein database (NCBI txid147387), we found that primary structure of wheat peroxidase-1 had significant similarity to that of timothy grass uncharacterized protein (accession no. JAA00049.1) with 37.7% amino acid homology (Supplementary Table 6). Additionally, significant similarities were found with those of oat grass (*Avena sativa*) peroxidase PXC2 precursor (accession no. AAC31550.1) and perennial ryegrass (*Lolium perenne*) putative peroxidase (accession no. CAH55694.1) with 44.6% and 38.9% amino acid homologies, respectively (Supplementary Table 6). In the same way, primary structure of beta-glucosidase had significant similarity to that of

Table 4
Sensitivity and specificity of allergen-specific IgE tests for wheat, peroxidase-1, and beta-glucosidase to identify GPWA among grass pollen allergy and wheat-induced food allergy.

ImmunoCAP™[†]	In 23 patients with grass pollen allergy[‡]		In 35 patients with wheat-induced food allergy[§]	
	Sensitivity	Specificity	Sensitivity	Specificity
Wheat	100% (6/6)	65% (11/17)	100% (6/6)	55% (16/29)
Peroxidase-1	50% (3/6)	82% (14/17)	50% (3/6)	93% (27/29)
beta-glucosidase	67% (4/6)	71% (12/17)	67% (4/6)	93% (27/29)

[†] ≥ 0.35 kU_A/L was considered as positive.
[‡] Six GPWA and 17 grass pollen allergy without episode of wheat allergy.
[§] Six GPWA, 18 conventional WDEIA, and 11 HWP allergy.

Please cite this article as: Ogino R et al., Identification of peroxidase-1 and beta-glucosidase as cross-reactive wheat allergens in grass pollen-related wheat allergy, Allergology International, https://doi.org/10.1016/j.alit.2020.09.005

timothy grass uncharacterized protein (accession no. JAA00456.1) with 54.5% amino acid homology (Supplementary Table 7). Significant similarities were also found with those of oat grass beta-D-glucosidase (accession no. AAC55196.1) and perennial ryegrass beta-glucosidase 31 (accession no. AFA36600.1) with 45.8% and 36.8% amino acid homologies, respectively (Supplementary Table 7). These results support the idea that IgE raised against grass pollen proteins, such as peroxidase or beta-glucosidase family, cross-reacts to wheat peroxidase-1 or beta-glucosidase in patients with GPWA. Our second speculation that a glycan moiety in peroxidase-1 is the IgE-binding epitope in patients with GPWA is supported by the fact that all three peroxidase-1 positive patients with GPWA were positive for MUXF3 by ImmunoCAP ISAC™ test (Supplementary Table 2). However, several reports have shown that IgE specific to plant glycan were irrelevant to clinical symptoms in patients with glycan-specific IgE.[11,31,32] Further studies are necessary to determine the IgE-binding epitopes of peroxidase-1.

In conclusion, specific IgE to grass pollen proteins dominantly cross-reacted with water-soluble wheat proteins that cause wheat allergies. Peroxidase-1 and beta-glucosidase are candidates of the cross-reactive wheat allergens recognized by the serum IgE of patients with GPWA. A CAP-FEIA system with peroxidase-1 and beta-glucosidase reduced the false-positive detection rate of wheat-specific IgE test in identifying GPWA in patients with grass pollen allergies, indicating the potential usefulness of these tests for the diagnosis of GPWA.

Acknowledgements

The authors thank Mrs. Kiyoe Ueda and Ms. Natsuko Asakura for technical assistance. The authors thank Center for Community-based Healthcare Research and Education, Shimane University for kindly introduce of hay fever patients. This work was supported in part by AMED Grant Number JP17ek0410020 and JSPS KAKENHI (Grant Number 16K09920).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.alit.2020.09.005.

Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

All authors contributed to the manuscript. RO, YC and EM designed research. RO and DT performed research and analyzed data. TY, HM and EM supervised experiments. YC and EM evaluated the clinical findings, collect serum, and made diagnosis. RO, TY, HM and EM wrote and edited the manuscript. All authors read and approved the final manuscript.

References

1. Tatham AS, Shewry PR. Allergens to wheat and related cereals. *Clin Exp Allergy* 2008;**38**:1712–26.
2. Ebisawa W, Ito K, Fujisawa T; On behalf of Committee for Japanese Pediatric Guideline for Food Allergy, The Japanese Society of Pediatric Allergy and Clinical Immunology, The Japanese Society of Allergology. Japanese guidelines for food allergy 2020. *Allergol Int* 2020;**69**:370–86.
3. Lack G. Epidemiologic risks for food allergy. *J Allergy Clin Immunol* 2008;**121**:1331–6.
4. Morita E, Matsuo H, Chinuki Y, Takahashi H, Dahlström J, Tanaka A. Food-dependent exercise-induced anaphylaxis -importance of omega-5 gliadin and HMW-glutenin as causative antigens for wheat-dependent exercise-induced anaphylaxis-. *Allergol Int* 2009;**58**:493–8.
5. Fukutomi Y, Itagaki Y, Taniguchi M, Saito A, Yasueda H, Nakazawa T, et al. Rhinoconjunctival sensitization to hydrolyzed wheat protein in facial soap can induce wheat-dependent exercise-induced anaphylaxis. *J Allergy Clin Immunol* 2011;**127**:531–3.
6. Chinuki Y, Morita E. Wheat-dependent exercise-induced anaphylaxis sensitized with hydrolyzed wheat protein in soap. *Allergol Int* 2012;**61**:529–37.
7. Yagami A, Aihara M, Ikezawa Z, Hide M, Kishikawa R, Morita E, et al. Outbreak of immediate-type hydrolyzed wheat protein allergy due to a facial soap in Japan. *J Allergy Clin Immunol* 2017;**140**:879–81.
8. Hiragun M, Ishii K, Yanase Y, Hiragun T, Hide M. Remission rate of patients with wheat allergy sensitized to hydrolyzed wheat protein in facial soap. *Allergol Int* 2016;**65**:109–11.
9. Jones SM, Magnolfi CF, Cooke SK, Sampson HA. Immunologic cross-reactivity among cereal grains and grasses in children with food hypersensitivity. *J Allergy Clin Immunol* 1995;**96**:341–51.
10. Constantin C, Quirce S, Poorafshar M, Touraev A, Niggemann B, Mari A, et al. Micro-arrayed wheat seed and grass pollen allergens for component-resolved diagnosis. *Allergy* 2009;**64**:1030–7.
11. Nilsson N, Nilsson C, Ekoff H, Wieser-Pahr S, Borres MP, Valenta R, et al. Grass-allergic children frequently show asymptomatic low-level IgE co-sensitization and cross-reactivity to wheat. *Int Arch Allergy Immunol* 2018;**177**:135–44.
12. Werfel T, Asero R, Ballmer-Weber BK, Beyer K, Enrique E, Knulst AC, et al. Position paper of the EAACI: food allergy due to immunological cross-reactions with common inhalant allergens. *Allergy* 2015;**70**:1079–90.
13. Palosuo K, Alenius H, Varjonen E, Koivuluhta M, Mikkola J, Keskinen H, et al. A novel wheat gliadin as a cause of exercise-induced anaphylaxis. *J Allergy Clin Immunol* 1999;**103**:912–7.
14. Morita E, Matsuo H, Mihara S, Morimoto K, Savage AW, Tatham AS. Fast omega-gliadin is a major allergen in wheat-dependent exercise-induced anaphylaxis. *J Dermatol Sci* 2003;**33**:99–104.
15. Matsuo H, Morita E, Tatham AS, Morimoto K, Horikawa T, Osuna H, et al. Identification of the IgE-binding epitope in omega-5 gliadin, a major allergen in wheat-dependent exercise-induced anaphylaxis. *J Biol Chem* 2004;**279**:12135–40.
16. Morita E, Kunie K, Matsuo H. Food-dependent exercise-induced anaphylaxis. *J Dermatol Sci* 2007;**47**:109–17.
17. Matsuo H, Dahlström J, Tanaka A, Kohno K, Takahashi H, Furumura M, et al. Sensitivity and specificity of recombinant omega-5 gliadin-specific IgE measurement for the diagnosis of wheat-dependent exercise-induced anaphylaxis. *Allergy* 2008;**63**:233–6.
18. Morita E, Chinuki Y, Takahashi H. Recent advances of in vitro tests for the diagnosis of food-dependent exercise-induced anaphylaxis. *J Dermatol Sci* 2013;**71**:155–9.
19. Sánchez-Monge R, García-Casado G, López-Otín C, Armentia A, Salcedo G. Wheat flour peroxidase is a prominent allergen associated with baker's asthma. *Clin Exp Allergy* 1997;**27**:1130–7.
20. Sander I, Rozynek P, Rihs HP, van Kampen V, Chew FT, Lee WS, et al. Multiple wheat flour allergens and cross-reactive carbohydrate determinants bind IgE in baker's asthma. *Allergy* 2011;**66**:1208–15.
21. Salcedo G, Quirce S, Diaz-Perales A. Wheat allergens associated with Baker's asthma. *J Investig Allergol Clin Immunol* 2011;**21**:81–92.
22. Sander I, Rihs HP, Doekes G, Quirce S, Krop E, Rozynek P, et al. Component-resolved diagnosis of baker's allergy based on specific IgE to recombinant wheat flour proteins. *J Allergy Clin Immunol* 2015;**135**:1529–37.
23. Palosuo K, Varjonen E, Kekki OM, Klemola T, Kalkkinen N, Alenius H, et al. Wheat omega-5 gliadin is a major allergen in children with immediate allergy to ingested wheat. *J Allergy Clin Immunol* 2001;**108**:634–8.
24. Pastorello EA, Farioli L, Conti A, Pravettoni V, Bonomi S, Iametti S, et al. Wheat IgE-mediated food allergy in European patients: alpha-amylase inhibitors, lipid transfer proteins and low-molecular-weight glutenins. Allergenic molecules recognized by double-blind, placebo-controlled food challenge. *Int Arch Allergy Immunol* 2007;**144**:10–22.
25. Venter C, Maslin K, Arshad SH, Patil V, Grundy J, Glasbey G, et al. Very low prevalence of IgE mediated wheat allergy and high levels of cross-sensitisation between grass and wheat in a UK birth cohort. *Clin Transl Allergy* 2016;**6**:22.
26. Tokuda R, Nagao M, Hiraguchi Y, Hosoki K, Matsuda T, Kouno K, et al. Antigen-induced expression of CD203c on basophils predicts IgE-mediated wheat allergy. *Allergol Int* 2009;**58**:193–9.
27. Sotkovský P, Sklenář J, Halada P, Cinová J, Setinová I, Kainarová A, et al. A new approach to the isolation and characterization of wheat flour allergens. *Clin Exp Allergy* 2011;**41**:1031–43.
28. Hofer G, Wieser S, Bogdos MK, Gattinger P, Nakamura R, Ebisawa M, et al. Three-dimensional structure of the wheat β-amylase Tri a 17, a clinically relevant food allergen. *Allergy* 2019;**74**:1009–13.
29. Matricardi PM, Bockelbrink A, Beyer K, Keil T, Niggemann B, Grüber C, et al. Primary versus secondary immunoglobulin E sensitization to soy and wheat in the Multi-Centre Allergy Study cohort. *Clin Exp Allergy* 2008;**38**:493–5.
30. Sekerkova A, Polackova M, Striz I. Detection of Phl p 1, Phl p 5, Phl p 7 and Phl p 12 specific IgE antibodies in the sera of children and adult patients allergic to Phleum pollen. *Allergol Int* 2012;**61**:339–46.
31. Mari A. IgE to cross-reactive carbohydrate determinants: analysis of the distribution and appraisal of the in vivo and in vitro reactivity. *Int Arch Allergy Immunol* 2002;**129**:286–95.
32. Mari A, Ooievaar-de Heer P, Scala E, Giani M, Pirrotta L, Zuidmeer L, et al. Evaluation by double-blind placebo-controlled oral challenge of the clinical relevance of IgE antibodies against plant glycans. *Allergy* 2008;**63**:891–6.