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Original Article

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

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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.

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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

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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 Timothy 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	5	more than 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.

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 Allergen 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 ≥ 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.

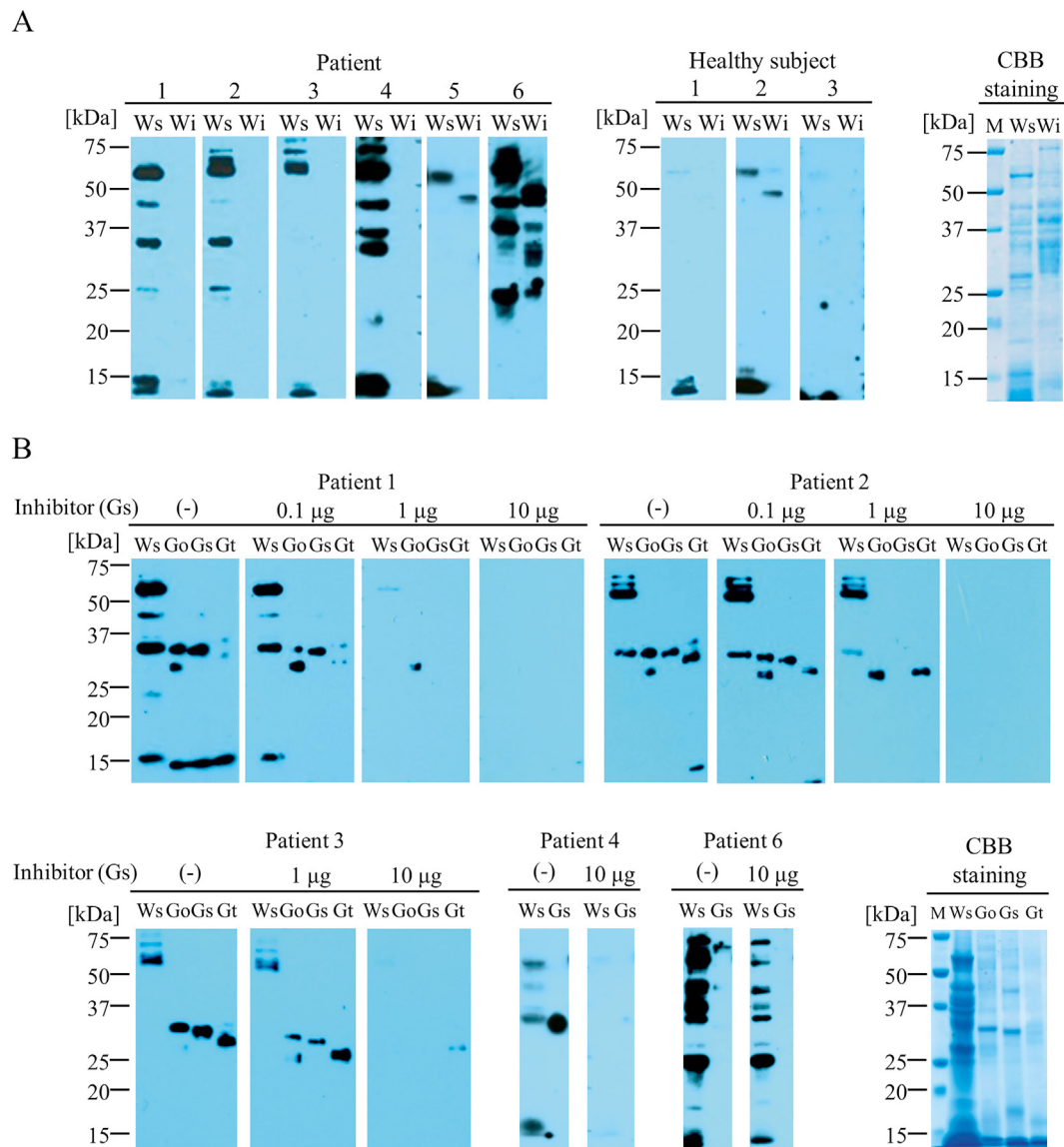


Fig. 1. IgE immunoblot analysis of wheat proteins and grass pollen proteins. **A**) Water-soluble wheat proteins (Ws) 10 μ g and water-insoluble wheat proteins (Wi) 10 μ g were immunoblotted with sera from six patients with GPWA and three healthy subjects. M, molecular weight marker. **B**) Patients'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

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 $\geq 95\%$ confidence six tryptic digested peptides using ProteinPilotTM. 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). ProteinPilotTM 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

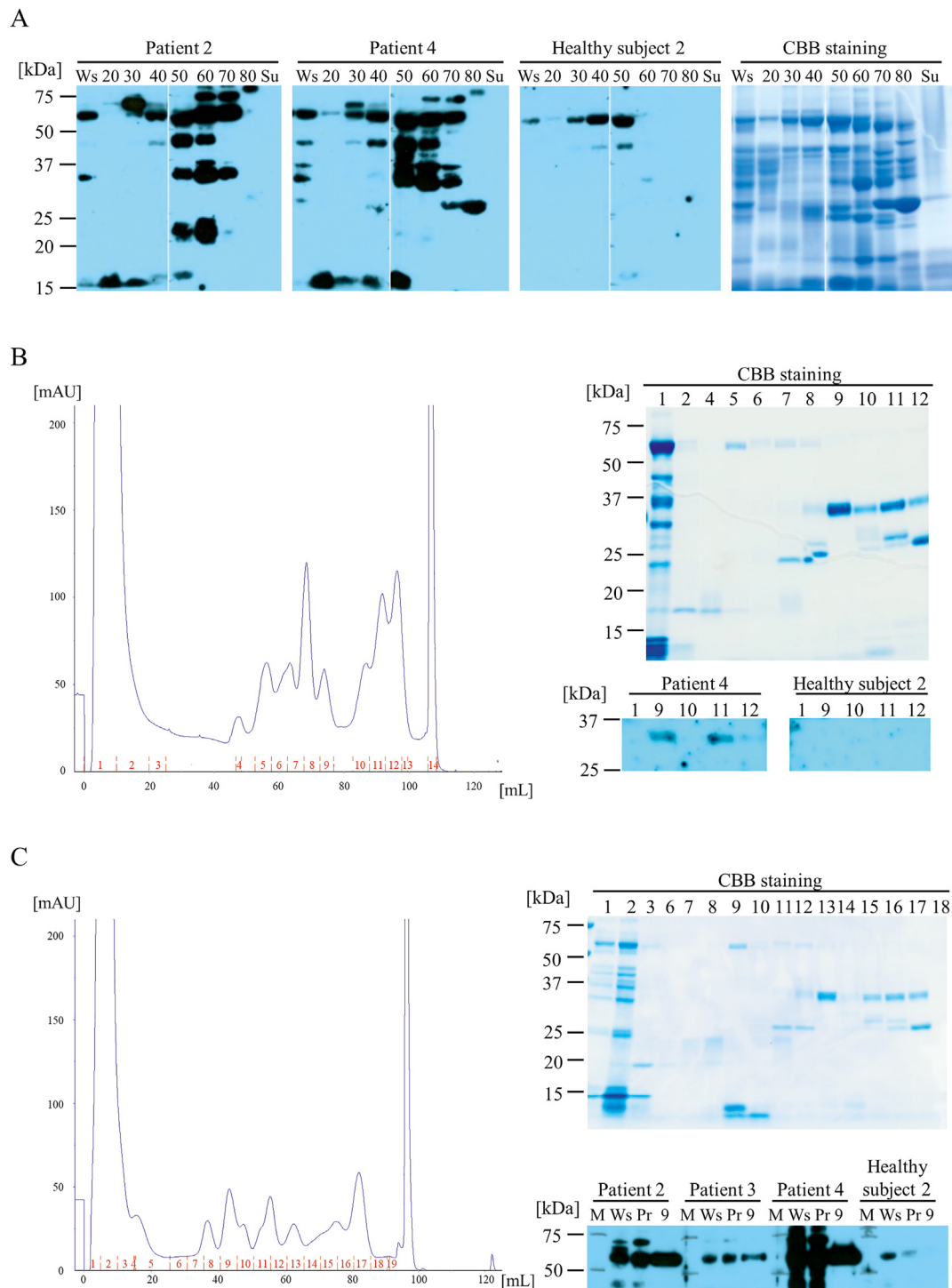


Fig. 2. Purification of 35-kDa and 60-kDa allergens in water-soluble wheat proteins. **A)** Water-soluble wheat proteins fractionated by ammonium sulfate precipitation from 20% to 80% were immunoblotted with patients' sera. Ws, water-soluble wheat proteins; 20, <20%; 30, 20–30%; 40, 30–40%; 50, 40–50%; 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,

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

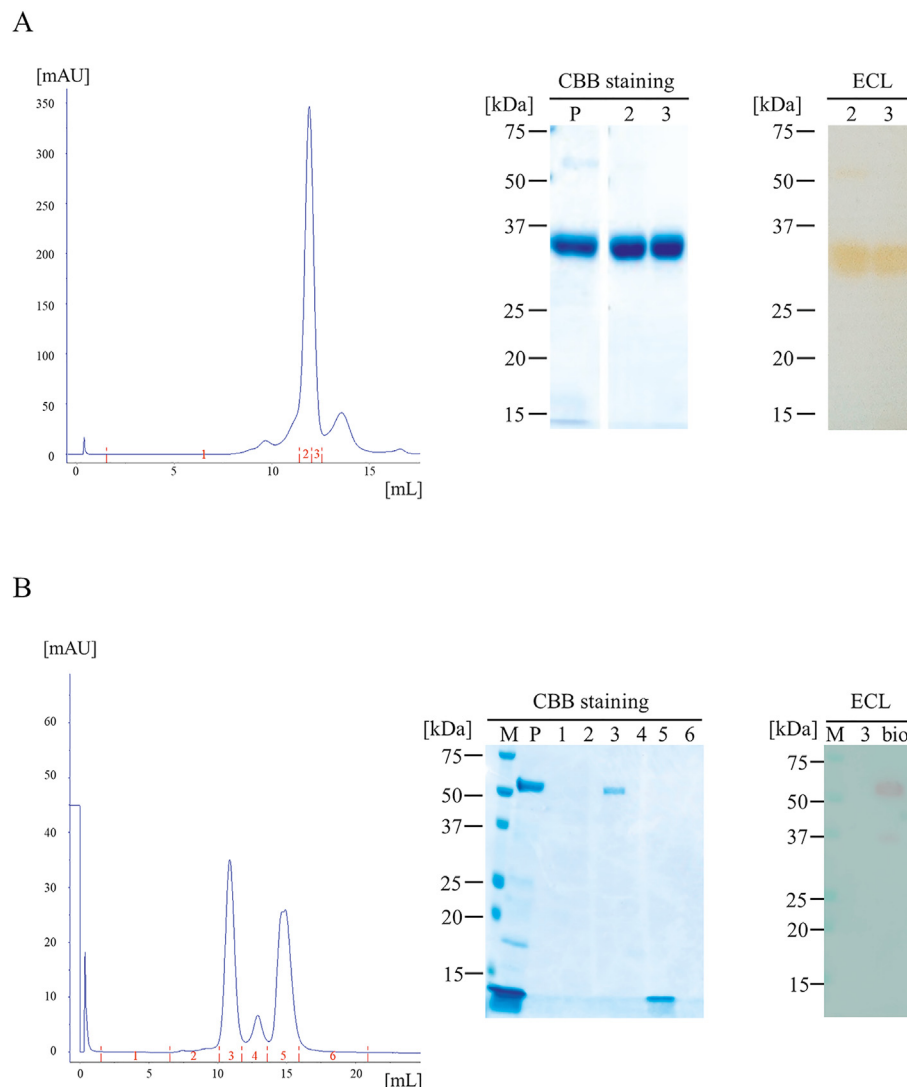


Fig. 3. Purification and biotinylation of wheat peroxidase-1 and beta-glucosidase for CAP-FEIA system. **A)** The peroxidase-1 containing cation-exchange fraction was further purified by gel-filtration chromatography after being biotinylated, and visualized using CBB staining and horseradish peroxidase (HRP)-conjugated streptavidin. P, peroxidase-1 preparation before gel-filtration; 2, fraction no. 2; 3, fraction no. 3 (2.5 μ g 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 μ g 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.

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 Poaceae 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.

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.

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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.

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Supplementary Methods

Preparation of wheat proteins

To fractionate the wheat proteins, 1,000 g of commercial-blend wheat flour (Nisshin Camellia[®]; Nisshin Flour Milling Inc., Kobe, Japan) was kneaded with 600 mL distilled water by hand for approximately 30 min and the dough was left to rest for 1 h. To extract water-soluble proteins, the dough was kneaded with 100 mL of fresh distilled water. This suspension was centrifuged at 22,200 *g* for 20 min at 4°C and the supernatant was collected. This procedure was repeated eight times and all supernatants were mixed as water-soluble proteins. After extraction of water-soluble proteins, 2 g of residual dough was mixed with 2% (w/v) sodium dodecyl sulfate solution containing 6% β-mercaptoethanol. After boiling at 95°C for 10 min, the mixture was centrifuged at 21,500 *g* for 15 min. The supernatant was collected as water-insoluble proteins. The concentrations of water-soluble and insoluble proteins were determined using DC[™] Protein Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and RC DC[™] Protein Assay Kit (Bio-Rad), respectively.

ω-5 Gliadin was purified from wheat flour as previously described¹⁴ and used as native ω-5 gliadin. HWP (Glupearl 19S[®]) was provided by Katayama Chemical Industries Co., Ltd., Osaka, Japan.

Immunoblot analysis

Samples were separated by SDS-PAGE using a 12.5% polyacrylamide gel. The fractionated proteins were transferred electrophoretically onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA, USA). The membrane was blocked with 5% skim milk in tris-buffered saline (pH 7.6) containing 0.1% Tween-20 (TBS-T) for 1 h followed by incubation with a 1:10 dilution of sera for 20 h at 26°C. After washing with TBS-T, the membrane was incubated with a 1:40,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-human IgE Abs (ϵ -chain) (GtxHu-002-EHRPX; ImmunoReagents, Inc., Raleigh, NC, USA) for 1 h at room temperature. After washing with TBS-T again, IgE-binding proteins were visualized on Super RX (FUJIFILM Co., Tokyo, Japan) using an Amersham ECL-Prime kit (GE Healthcare UK Ltd, Buckinghamshire, UK).

Purification of wheat allergens by cation-exchange chromatography

Water-soluble wheat proteins were stirred with 20% ammonium sulfate for 2 h at 4°C and centrifuged at 22,200 *g* for 20 min at 4°C to obtain a precipitate of 20% ammonium sulfate. The supernatant was collected and ammonium sulfate added to reach

to more 10% saturation. This precipitation procedure was repeated at 10% intervals up to 80% saturation. After dissolving with 50 mM HEPES-NaOH (pH 7.0), each precipitate was dialyzed in the same buffer using cellulose tubes.

The dialyzed solution containing proteins bound to serum IgE was filtered using a 0.45 μ m syringe filter. The filtered solution was fractionated by linear gradient with 50 mM HEPES-NaOH (pH 7.0) containing 0–9% NaCl, using a cation-exchange column (HiTrap SP HP 5 ml, GE Healthcare) and high performance-liquid chromatography (HPLC) (AKTA FPLC system; GE Healthcare).

Preparation of wheat allergens for mass spectrometry analysis

Water-soluble wheat proteins fractionated by ammonium sulfate precipitation and cation-exchange chromatography were separated by SDS-PAGE and visualized by Coomassie brilliant blue (CBB) staining. The IgE-binding proteins were extracted from the CBB-stained gel, subjected to consecutive reductive alkylation by dithiothreitol and iodoacetamide, and digested with trypsin at 37°C for 20 h. After cleaning with a MonoSpin[®] C18 (GL Sciences Inc., Tokyo, Japan), the digested peptides were crystallized with α -cyano-4-hydroxycinnamic acid on a stainless-steel plate.

Preparation of CAP-FEIA system

To obtain the 35-kDa allergen, the supernatant of the 40% ammonium sulfate precipitation was precipitated with 60% ammonium sulfate (40–60% ammonium sulfate precipitation). The fractionated proteins of the 40–60% ammonium sulfate precipitation were further fractionated by HPLC using a cation-exchange column (HiTrap SP HP 5 ml). The chromatography column was equilibrated mobile phase A and eluted with a linear gradient of 0–9% of mobile phase B over 30 min at a flow rate of 2.5 mL/min. Mobile phase A was 50 mM HEPES-NaOH (pH 7.0) and mobile phase B was 50 mM HEPES-NaOH (pH 7.0) containing 1 M NaCl. The eluted proteins were monitored at a wavelength of 280 nm.

To obtain the 60-kDa allergen, the supernatant of the 50% ammonium sulfate precipitation was precipitated with 70% ammonium sulfate (50–70% ammonium sulfate precipitation) and further fractionated by HPLC using a cation-exchange column. The chromatography column was equilibrated with mobile phase A and eluted with a linear gradient of 0–3.5% of mobile phase B over 12.5 min at a flow rate of 2.0 mL/min, using the same mobile phases used for the purification of the 35-kDa allergen.

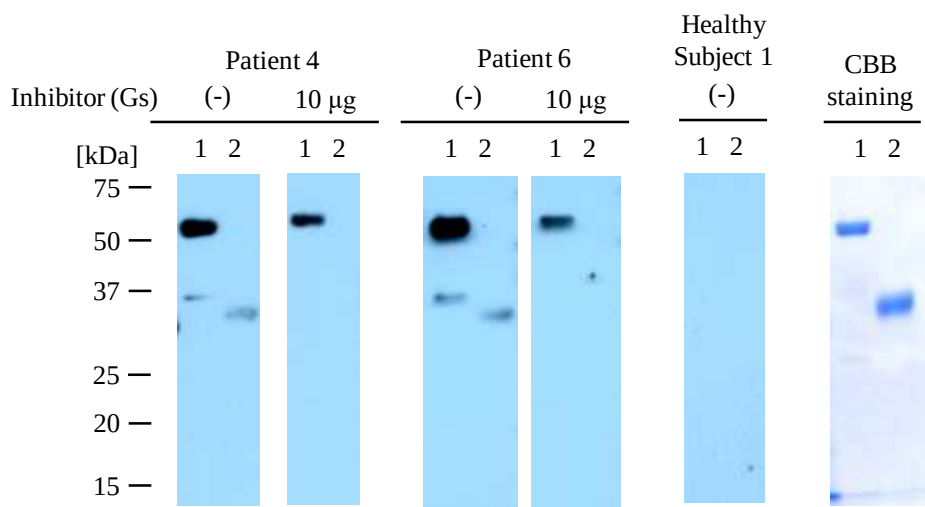
After identification of the target proteins by mass spectrometry, the solution containing wheat allergen was further fractionated by HPLC using a gel-filtration column

(Superdex 75 10/300 GL, GE Healthcare) with 50 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl as a mobile phase. The flow rates were set to 0.3 and 0.5 mL/min for the 35- and 60-kDa allergens, respectively.

The purified wheat allergens were biotinylated using Ez-linkTM sulfo-NHS-LC biotin (Thermo Fisher Scientific Ltd., Waltham, MA, USA). Briefly, wheat allergen solutions were dialyzed with phosphate-buffered saline using a HiTrap desalting column (GE Healthcare). Solutions containing 1 g of 35- or 60-kDa allergens were incubated with 0.14 or 0.089 mmol biotin for 30 min at room temperature, respectively. Unreacted biotin was then removed using a HiTrap desalting column. This biotinylation procedure was performed before gel-filtration chromatography for 35-kDa allergen and after gel-filtration chromatography for 60-kDa allergen.

To establish a CAP-FEIA system for wheat allergen, 2.5 µg of biotinylated wheat allergen was applied to the solid-phase streptavidin ImmunoCAPTM and incubated for 30 min at 37°C. The serum IgE values specific to wheat allergen were measured according to the manufacturers instructions'.

Supplementary Fig. 1



Supplementary Fig. 1

Inhibition assay using purified wheat beta-glucosidase and peroxidase-1

Biotinylated beta-glucosidase (lane 1, 1.5 µg) and peroxidase-1 (lane 2, 1.5 µg) were separated by SDS-PAGE. To evaluate IgE-cross reactivity, patients' sera were pre-incubated with sweet vernal grass pollen proteins (Gs). Total protein was stained with Coomassie brilliant blue (CBB). Inhibitor, sweet vernal grass pollen proteins (0, 10 µg).

Supplementary Table 1 The diagnostic criteria of wheat-dependent exercise-induced anaphylaxis (WDEIA)

-
- [1] Immediate-type allergic reactions, such as urticaria, occur after taking wheat products due to secondary factors[†] such as exercise.
 - [2] Oral wheat provocation test[‡] (wheat intake + exercise, aspirin + wheat intake or aspirin + wheat intake + exercise) induces immediate-type allergic reactions.
 - [3] Wheat protein (including ω -5 gliadin)-specific IgE is demonstrated in serum.
 - [4] Wheat protein prick test is positive.
-

Diagnose food-dependent exercise-induced anaphylaxis, when [1] and [2] are satisfied, or [1] is repeated more than once, and [3] or [4], or both are satisfied.

[†]Secondary factors include non-steroidal anti-inflammatory drugs and alcohol consumption.

[‡]Cited from reference: Matsuo H, Morimoto K, Akaki T, et al. Exercise and aspirin increase levels of circulating gliadin in patients with wheat-dependent exercise-induced anaphylaxis. *Clin Exp Allergy* 2005; **35**: 461–6.

Supplementary Table 2 Sensitization profile of the patients with GPWA on ImmunoCAP ISAC™

Patient	Bromelain	Timothy (Phl p)								Wheat (Tri a)		
	MUXF3 [†]	1	2	4	5	6	7	11	12	14	19	aATI
1	0.8 [‡]	45.8	36.5	1.9	54.1	5.2	33.9	33.5	2.0	0	0	0
2	0.4	26.8	0.2	11	30.9	10.5	0	9.1	0.3	5.4	0	0
3	0.1	51.1	0	6.3	64.5	4.1	0	0	0.2	0	0	0
4	0.5	7.0	0	1.1	15.6	0	0	0	0.3	0	0	0
5	0.1	37.8	0.9	0.2	0.3	0.2	31.4	16.2	0	0	0	0
6	0.3	0	0	0.6	0.3	0	0	0	0	0.2	0	0.3

[†]MUXF3, marker of cross-reactive carbohydrate determinants.

[‡]IgE value was expressed as ISU-E.

Supplementary Table 3 Identified peptides by MALDI-TOF mass spectrometry

Protein name (Molecular size)	Sequence	Modifications	Measured <i>m/z</i>
peroxidase-1 (35 kDa) Accession No. 300087071	GAVVSCADILALAAR	Carboxymethyl(C)@6	1486.742
	GLSFDFYR		1004.438
	LFPRPDPTINPPFLAR		1850.948
	DIGLAAGLLR		998.506
	TPNVFDNQYYVDLVNR	Deamidated(N)@3	1957.844
beta-glucosidase 4, partial (60 kDa) Accession No. 359828768	DFFEQFGVSMGK	Oxidation(M)@10	1407.491
	IVGAFADYAEFCFK	Carbamidomethyl(C)@12	1637.776
	NWFTFNEPR		1210.585
	TEPYIVTHNIILSHAAVQR		2233.197
	VVGYYFAWSLLDNFEWR		2001.985
	DFHIGWFLDPITNGR	Deamidated(N)@13	1788.883
	IGILLDFVWYEPHSNSNADQGAAQR	Deamidated(N)@15; Methyl(Q)@24	2816.326
	FGIAYVDFNTLK	Formyl@N-term	1415.728

The tryptic digested peptides were measured using MALDI-TOF mass spectrometry, and generated mass lists were searched against the wheat protein database (NCBI txid4565) obtained from protein data bank of the National Center for Biological Information using ProteinPilot™ software. MALDI-TOF, matrix-assisted laser desorption/ionization time of flight.

Supplementary Table 4 Results of peroxidase-1- and beta-glucosidase-specific IgE test and immunoblot analysis

Patient	ImmunoCAP™ (kU _A /L) †		Immunoblotting	
	Peroxidase-1	beta-glucosidase	35-kDa	60-kDa
1	1.17 (2)	1.53 (2)	(+)	(+)
2	0.169 (0)	0.500 (1)	(+)	(+)
3	< 0.1 (0)	0.113 (0)	(-)	(+)
4	1.69 (2)	3.27 (2)	(+)	(+)
5	< 0.1 (0)	< 0.1 (0)	(-)	(+)
6	7.78 (3)	5.31 (3)	(+)	(+)

†Specific IgE values (kU_A/L) were classified as follows; class 0 (negative), < 0.34; class 1 (positive), 0.35–0.69; class 2, 0.70–3.49; class 3, 3.50–17.49.

Supplementary Table 5 Comparison of serum specific IgE levels for wheat, grass pollens, peroxidase-1, and beta-glucosidase among 23 patients with grass pollen allergy

ImmunoCAP™ (kU _A /L)	GPWA (n = 6)	Grass pollen allergy [†] (n = 17)	<i>p</i> -value
Sweet vernal grass	35.5 (25.3–75.6) [‡]	6.54 (1.52–17.9)	0.013
Timothy grass	61.2 (35.7–95.1)	5.46 (2.65–23.5)	0.002
Wheat	1.29 (1.05–2.16)	0.10 (0.10–0.67)	0.010
Peroxidase-1	0.67 (0.12–1.56)	0.10 (0.10–0.30)	0.063
beta-glucosidase	1.02 (0.21–2.84)	0.10 (0.10–0.45)	0.032

[†]Grass pollen allergy without episode of wheat allergies.

[‡]Specific IgE values (ImmunoCAP™) in each allergy group indicate as median and interquartile range. Statistical analyses were performed using Wilcoxon rank-sum test in RStudio (version 1.1.453, RStudio, Inc., Boston, MA, USA). *P*-values < 0.05 were considered statistically significant.

Supplementary Table 6 Results of BLAST analysis between wheat peroxidase-1 and Poaceae tribe proteins

Origin	Accession no.	Amino acid sequences							
Wheat [†]	300087071	31	VARGLSFDY	RRTCPRAESI	VRGFVQDAVR	KDIGLAAGLL	RLHFHDCFVQ	GCDASVLLDG	SATGPGEQQA
Timothy	JAA00049.1	13	LSREFY	KASCPDAEKI	VAAVIEKKLK	EDPGTAAGLL	RLLFHDCFAN	GCDASILIDP	LSNQSAEKEA
Oat	AAC31550.1	29	FY	DTSCP KALAT	IKSGVAAAVS	SDRRMGASLL	RLHFHDCF—	GCDASVLLSG	N-----EQNA
Ryegrass	CAH55694.1	33	LSLEHY	AKTCPNVEHV	VRTEMECA—	-----	-----	GCDGSVLLDD	TATLIGEKQA
Wheat	300087071		PPNL-TLRPSA	FKAVNDIRDR	LERECRGAVV	SCADILALAA	RDSVVVSGGP	DYRVPLGRRD	SRRFATRQDV
Timothy	JAA00049.1		GPNI-SVR--G	FEVIDDIKKE	LEAKCP-KTV	SCADIVALGT	RDAVRISGGP	AYEVPTGRRD	S-LVSNREEA
Oat	AAC31550.1		APNAGSLR--G	FSVIDNIKTQ	VEAVCK-QTV	SCDDILAVAA	RDSVVALGGP	SWTVPLGRRD	STSATGNT--
Ryegrass	CAH55694.1		EQNV-N-SLKG	FELVDKIKEK	LEAECPG-TV	SCADLLAIAA	RDATVLVGGP	YWDVPVGRDL	SKE-ASDLA
Wheat	300087071		LSDL PAPS N	VPSLLALLRP	LGLDATDLVT	-ISGGHTIGQA	HCSSFEDRLF	PRPDPT--INP--P	FLARLKGTC P
Timothy	JAA00049.1		DNNLP GPDIP	IPKLTSEFLS	RGFTPEEMVV	LLAGGHSIGKV	RC-----IF	IEPDATP-MDP--G	YQASISKLCD
Oat	AAC31550.1		-GDL PAPTSS	LAQLQA AFSK	KNLDTTDMVA	-LSGAHTIGQA	QCKNFRSRIY	G-GDTN--INA--A	FATSLQANCP
Ryegrass	CAH55694.1		NKDIPTPQQG	LITLISK FWE	KGLDATDMVA	-LVGSHTIGFA	RCANFRERIY	GDFEMTSKSNPASAT	YLSKLKEICP
Wheat	300087071		----AKGTDRRTV-L	DVRTPNVFDN	QYYVDLVNRE	GLFVSDQDLF	TNDITRPIVE	RFARSQRDFF	EQFGVSMGKM
Timothy	JAA00049.1		----GPNRDTGFVNM	DEHNPNVIDS	SYFANVLAKK	MPLTVDRLLG	LDSKTPPIIK	NMLNKPNDFM	PTFAKAMEKL
Oat	AAC31550.1		QATGGSGDSSLAP-L	DTKTPNAFDN	SYNNLLSQK	GLLHSDQVLF	NNGTTDNTVR	NFASSASAFT	GAFTTAMIKM
Ryegrass	CAH55694.1		----LDGGDDNISAM	DSYTSSTFDN	AYFETLIKGE	GL 251			
Wheat	300087071		GQMRVRTSDL	GEVRRNC SA-R	NPGPAAADEL	QWPSFVQTIV	358		
Timothy	JAA00049.1		SVLKVITGKD	GEIRKSCSEFN	NPMP S 307				
Oat	AAC31550.1		GNISPLTG TQ	GQIRLSCSK-V	N 312				

[†]The name and calculated molecular weight of each protein is following: Wheat, peroxidase-1 (38.8 kDa); Timothy, uncharacterized protein, partial (40.0 kDa); Oat, peroxidase PXC2 precursor (32.3 kDa); Ryegrass, putative peroxidase (27.0 kDa). Amino acid residues which matched among all or three sequences were highlighted.

Supplementary Table 7 Results of BLAST analysis between wheat beta-glucosidase and Poeae tribe proteins

Origin	Accession no.	Amino acid sequences							
Wheat [†]	359828768	251	YQPHQKGRIG	ILLDFVWYEP	HSNSNADQGA	AQRARDFHIG	WFLDPITNGR	YPSSMLKIVG	NRLPGFSTNE
Timothy	JAA00456.1	1	G	ILLDFVWYEP	LTYNTEDEFA	AHRAREFTLG	WFMHPITYGH	YPETMQRLVA	DRLPNFTDEQ
Oat	CAA55196.1	297	YK-GDDGQIG	MVLDMAYEP	YGNNFLDQQA	QERAIDFHIG	WFLEPMVRGD	YPFSMRSLVG	DRLPFFTKSE
Ryegrass	AFA36600.1	1	QATQGGKIG	LTLLGWWYEP	GTQAPEDVAA	AARMNDFHIG	WYMHPLVHGD	YPPVMRKNVG	SRLPSFTAEE
Wheat	359828768		SRMVKGSIDY	VGINQYTSYY	MK--DPGAWNLT	PVSYQDD----WHV	GFVYERNQVP	IGPRANSDWL	YIVPWGMNKA
Timothy	JAA00456.1		TRLLQGSADI	VGVNHYTTY	AK--NHE--NLT	HMSYAND----WQV	QLVYERNQIP	IGKQGYSKWL	YVVPWGFYKA
Oat	CAA55196.1		QEKLVSSEYDF	VGINYYTSRF	AKHIDISPEFIP	KINTDDV----YSN	PEVNSNGIP	IGPDVGMFYI	YSYPKGLKNI
Ryegrass	AFA36600.1		LERVRGSFDY	VGFNHYIAVY	VK-ADLSKLDQN	LRDYMGDAAVKYDM	PFLNSKNQLL	FGLK--TDFA	TSTPWALKKM
Wheat	359828768		VTYVKERYGN	PTMILSENG---M	DQPGNVSIAD	GVHDTIRIRY	YRDYITELKK	AIDNGARVV-G	YFAWSLLDNF
Timothy	JAA00456.1		VMHVKDKYRN	PLMIIGENG---I	DQSGSDTLPH	ALYDKFRIDY	FDQYLHELKR	ATDDGARVT-G	YFAWSLLDNF
Oat	CAA55196.1		LLRMKEKYGN	PPIYITENGTA	DGWGNPPMTD	PLDDPLRIEY	LQQHMTAIKE	AIDLGRRTLGR	HFTWSLIDNF
Ryegrass	AFA36600.1		LEHLQVKYKN	PVVMIHENG	161				
Wheat	359828768		EWRLGYTARF	GIAYVDFNT-L	KRYPKDSALW	FKNMLSEKKR	S 501		
Timothy	JAA00456.1		EWRMGFTSKF	GIVYVDRKT-F	TRYPKDSTRW	FRKVIKNEE	238		
Oat	CAA55196.1		EWSLGYLSRF	GIVYIDRNDGC	KRIMKSAKW	LKEFNGATKK	552		

[†]The name and calculated molecular weight of each protein is following: Wheat, beta-glucosidase 4, partial (56.5 kDa); Timothy, uncharacterized protein, partial (28.3 kDa); Oat, beta-D-glucosidase (65.0 kDa); Ryegrass, beta-glucosidase 31, partial (20.5 kDa). Amino acid residues which matched among all or three sequences were highlighted.