

ORIGINAL ARTICLE

Allergens

Gly m 5/Gly m 8 fusion component as a potential novel candidate molecule for diagnosing soya bean allergy in Japanese children

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Summary

Background: Soya bean is a major food allergen in children. Component-resolved diagnostics has improved the accuracy of diagnosing immunoglobulin E (IgE)-mediated food allergies.

Objective: We aimed to develop a novel component for the diagnosis of soya bean allergy using recombinant technology.

Methods: Japanese paediatric patients with suspected soya bean allergy (n = 91) were included, and symptomatic (n = 40) and asymptomatic (n = 51) cases were divided through oral food challenge testing. Specific IgE (slgE) antibodies to each recombinant allergen component were analysed by enzyme-linked immunosorbent assay, and the diagnostic performances of the components were assessed by area under the receiver operating characteristic curves (AUC).

Results: Among the recombinant components, slgE antibody levels to Gly m 8 showed the highest AUC (0.706). A combination of Gly m 8 and α' subunit of Gly m 5, improved the diagnostic performance of the single components. Moreover, the N-terminal extension region of α' subunit of Gly m 5, which has low cross-reactivity among the vicilins, showed higher diagnostic performance (AUC 0.695) than the full-length α' subunit of Gly m 5 (AUC 0.613). Based on these findings, we developed a fusion protein of Gly m 8 plus the extension region of α' subunit of Gly m 5; this fusion protein was very efficient for diagnosing soya bean allergy (AUC 0.801).

Conclusion: A fusion protein of Gly m 8 and the extension region of α' subunit of Gly m 5 could potentially diagnose soya bean allergy in paediatric patients. Fusion proteins may be useful for producing novel allergen components with improved diagnostic value.

KEYWORDS

component-resolved diagnostics, epitope, molecular allergology, soya bean

1 | INTRODUCTION

An accurate diagnosis of food allergies is necessary to ensure that individuals avoid foods that could trigger allergic reactions. Specific immunoglobulin E (sIgE) antibody testing to allergen components, termed component-resolved diagnostics (CRD), has been used for diagnosing food allergies in routine practice.¹⁻⁴ As the prevalence of food allergies has been increasing in recent years, CRD has become an important diagnostic tool as it has improved the accuracy of diagnosing IgE-mediated food allergies.¹⁻⁴ Knowing the allergen components linked to allergic symptoms in oral food challenge (OFC) tests helps improve diagnosis, and identifying a patient's specific IgE antibody profile to allergen components might help discriminate different allergic phenotypes.

Soya bean is one of the most common food allergens, with a prevalence of 0.4% in young children in the United States (US).⁵ Allergic reactions to soya bean have been observed after exposure to processed soya bean (tofu) and soy milk. In Japan, soya bean is the tenth most common food allergen causing anaphylaxis.⁶ The prevalence of soya bean allergy might be higher in Japan than in the US and Europe.^{6,7} Soya bean allergy is primarily mediated through absorption via the gastrointestinal tract but is also caused by cross-reactivity with pollen allergens; symptoms range from mild oral allergy to severe reactions.⁸⁻¹⁰ Diagnosing a soya bean allergy in children is complicated due to a high rate of false positives in sIgE antibody testing of crude extract. A discrepancy between results of sIgE antibody to soya bean proteins and clinical symptoms after ingestion of soya bean has been reported.^{11,12}

Eight soya bean proteins have been registered in the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Subcommittee. The primary sensitizers for allergy are thought to be the seed storage proteins Gly m 5 (vicilin), Gly m 6 (legumin), and Gly m 8 (2S albumin).^{7,13-16} Gly m 5 and Gly m 6 are composed of several subunits (Gly m 5: α subunit Gly m 5.0101, α' subunit Gly m 5.0201, and β subunit Gly m 5.0301; Gly m 6: A1aB1b subunit Gly m 6.0101, A2B1a subunit Gly m 6.0201, A1bB2 subunit Gly m 6.0301, A5A4B3 subunit Gly m 6.0401, and A3B4 subunit Gly m 6.0501). A measurement of sIgE antibody levels to Gly m 8 was a better tool for distinguishing between symptomatic and non-symptomatic children for soya bean, although it still results in misclassification at a relatively high rate.¹⁴⁻¹⁶ Gly m 4 (pathogenesis-related class 10, Bet v 1 related homologue) is a risk factor for severe oral allergy syndrome and systemic reactions to soya bean in patients sensitized to Bet v 1, a major allergen of birch pollen.^{10,17} Gly m 1 and Gly m 2 have been associated with asthma outbreaks in Spain.¹⁸ Gly m 3 (profilin), a homologue of Bet v 2, was identified through determining sIgE antibody levels in individuals with food allergies in Germany.¹⁹

Recombinant technology allows for the routine preparation of allergen components, even if the amount in crude extract is low, and the design of novel allergen components. In this study, we analysed sIgE antibody levels to allergen components in paediatric patients suspected of soya bean allergy and compared their diagnostic

performances. Based on these results, we designed a fusion protein with the aim of producing a novel allergen component with high diagnostic performance.

2 | METHODS

2.1 | Subjects

We consecutively enrolled 91 children sensitized to soya bean who were referred to the Sagamihara National Hospital and Aichi Children's Health and Medical Center.

Upon study entry between 2005 and 2012, an OFC was performed openly to identify their symptoms after ingestion of soya bean. OFC was performed with processed soya bean (tofu) in all children according to the Japanese Pediatric Guidelines for Food Allergies and American Academy of Allergy, Asthma & Immunology-European Academy of Allergy and Clinical Immunology PRACTALL consensus report.^{20,21} The OFC was considered positive when objective symptoms were observed. Negative OFC results were defined as the absence of any symptoms after ingestion of the maximum amount (100 g) of tofu (5-7 g of soya bean protein/100 g tofu).

Ethical approval was obtained from the institutional review boards of the Sagamihara National Hospital and Aichi Children's Health and Medical Center. Written informed consent was obtained from all children and/or their parents prior to study entry. This study has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.2 | Preparation of recombinant common soya bean allergen components

Designations of the allergen components that were included in this study are shown in Table 1. The nucleotides coding Gly m 2 and Gly m 7 protein sequences were synthesized, while cDNAs for all other soya bean allergen components were prepared using RT-PCR. The amplified Gly m 1, Gly m 2, Gly m 3, Gly m 4, Gly m 8, Gly m KTI, and Gly m BBI fragments were then inserted into the pGEX expression vector (GE Healthcare Life Sciences, Little Chalfont, UK), and the Gly m 7 fragment was inserted into the pET52 expression vector (Merck Millipore, Billerica, MA, USA). The resulting expression plasmids were transformed into BL21(DE3) (Merck Millipore) or ShuffleT7 (New England Biolabs Inc., Ipswich, MA, US) competent *Escherichia coli*. After expression, recombinant Gly m 1, Gly m 2, Gly m 3, Gly m 4, Gly m 8, Gly m KTI, and Gly m BBI proteins were purified using the Glutathione Sepharose 4B (GE Healthcare Life Sciences) and a Superdex 200 pg gel filtration column (GE Healthcare Life Sciences). Glutathione S-transferase (GST) fusion proteins were then cleaved using PreScission Protease (GE Healthcare Life Science), and the GST was removed via subsequent gel filtration column chromatography. Recombinant Gly m 7 was purified using the Ni Sepharose FastFlow (GE Healthcare Life Sciences) and a Superdex 200 pg gel filtration column (GE Healthcare Life Sciences).

TABLE 1 Soya bean allergen components in the study

Component name	Isoallergen no.	Biochemical name	UniProt no.
Gly m 1	Gly m 1.0101	Hydrophobic protein	Q9S8F3, Q9S7Z9
Gly m 2	Gly m 2.0101	Defensin	C6T0M2 ^a
Gly m 3	Gly m 3.0101	Profilin	O65809
Gly m 4	Gly m 4.0101	Pathogenesis-related protein	P26987
Gly m 5- α	Gly m 5.0101	Vicilin	O22120
Gly m 5- α'	Gly m 5.0201	Vicilin	Q9FZP9
Gly m 5- β	Gly m 5.0301	Vicilin	P25974
Gly m 6-A1aB1b	Gly m 6.0101	Legumin	P04776
Gly m 6-A3B4	Gly m 6.0501	Legumin	Q7GC77
Gly m 7	Gly m 7.0101	Seed biotinylated protein	C6K8D1
Gly m 8	Gly m 8.0101	2S albumin	P19594
Gly m KTI		Trypsin inhibitor	P01070
Gly m BBI		Protease inhibitor	I1L3Q3
Gly m Bd 30k		Thiol protease-like protein	O64458

KTI, Kunitz-type trypsin inhibitor; BBI, Bowman-Birk protease inhibitor.

^aThe number was assumed from the short sequences of Gly m 2 reported previously in the reference no. 18.

To construct Gly m 5- α , Gly m 5- α' , and Gly m 5- β expression plasmids, a histidine tag was introduced at the Gly m 5 C-terminus in the previously prepared plasmids.²² A Gly m 6-A1aB1b expression plasmid was constructed using the Champion pET101 Directional TOPO Expression kit (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. These recombinant proteins were then purified using the Ni Sepharose FastFlow (GE Healthcare Life Sciences) and a HiLoad 16/600 Superdex 200 pg column (GE Healthcare Life Sciences). Gly m 6-A3B4 was prepared as previously described.²³

Recombinant Gly m Bd 30k was prepared using the BaculoDirect Baculovirus Expression system (Thermo Fisher Scientific), according to the manufacturer's instructions. The amplified Gly m Bd 30k fragment was restriction digested, and ligated into the pENTR4 entry vector (Thermo Fisher Scientific), and the entry clone was recombined with a BaculoDirect linear DNA using LR Clonase II Enzyme Mix (Thermo Fisher Scientific). Sf9 cells were transfected with the recombinant baculovirus DNA using Cellfectin (Thermo Fisher Scientific). After culture, Gly m Bd 30k was purified from the media using the Ni Sepharose FastFlow (GE Healthcare Life Sciences) and a HiLoad 16/600 Superdex 200 pg column (GE Healthcare Life Sciences).

2.3 | Preparation of the Gly m 5- α' extension region and Gly m 8 plus Gly m 5- α' extension region fusion protein

To construct an expression plasmid for the N-terminal extension region of the α' subunit of Gly m 5 (Gly m 5- α'), a fragment was

amplified using the expression plasmid for Gly m 5- α' as a template, and then ligated into the cloning sites of the pET52 vector (Merck Millipore). Nucleotides for a fusion protein of Gly m 8 plus the Gly m 5- α' extension region (Gly m 5- α' EXT/Gly m 8) were synthesized and ligated into the pGEX vector (GE Healthcare Life Sciences). The recombinant proteins were expressed in *Escherichia coli* and purified using both Ni Sepharose FF (GE Healthcare Life Sciences) and a HiLoad 16/600 Superdex 200 pg column (GE Healthcare Life Sciences). Glutathione S-transferase (GST) of the fusion protein was cleaved using the PreScission Protease (GE Healthcare Life Sciences) and removed during gel filtration column chromatography.

2.4 | Protein analysis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using TGXTM (12% or Any kDTM) gels (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proteins were stained with Coomassie Brilliant Blue R-250.

For mass spectrometry, proteins were alkylated and digested with mass spectrometry grade trypsin (Thermo Fisher Scientific). Mass spectrometric analysis of the digested proteins was performed using the LC-MS/MS QTOF system G6520 (Agilent Technologies, Santa Clara, CA, USA) that was equipped with an HPLC-Chip Cube system G4240 (Agilent Technologies) or an Orbitrap Elite (Thermo Fisher Scientific). Mass spectra were processed and analysed using the Mascot Server (Matrix Science, London, UK).

For N-terminal amino acid sequences, proteins blotted on a polyvinylidene difluoride membrane (GE Healthcare Life Sciences) were visualized with Ponceau-S and protein bands of interest were excised. N-terminal amino acid sequences were determined with a PPSQ-31A (Shimadzu, Kyoto, Japan) or Procise 491 cLC (Thermo Fisher Scientific) sequencer.

2.5 | Enzyme-linked immunosorbent assay for the measurement of specific immunoglobulin E antibody levels

The levels of specific IgE to soya bean components were measured using a fluorescent enzyme-linked immunosorbent assay (ELISA). ELISA plates were coated overnight with proteins in 0.05 M carbonate buffer (pH 9.6) at 4°C, washed with PBS containing 0.05% (v/v) Tween 20 (PBS-T), and blocked with 1% (w/v) BSA in PBS-T. After washing the plates, serum samples diluted 1:10 in blocking buffer were added and incubated for 3 hours at room temperature (RT). After three washes, a β -galactosidase-conjugated anti-human IgE antibody (Thermo Fisher Diagnostics, Uppsala, Sweden) diluted 1:10 in blocking buffer was added. After 2 hours incubation at RT, the plates were washed three times and the substrate (0.2 mmol/L 4-methylumbelliferyl β -D-galactopyranoside) was added. Fluorescence was measured with a microplate reader at 355 nm excitation and 460 nm emission. A pool of serum samples with known specific IgE levels for soya bean (from ImmunoCAP analysis) were used to establish a standard curve and the results were expressed as ELISA-based kilo-units per litre (kU_E/L).

2.6 | ImmunoCAP assay for the measurement of total and specific immunoglobulin E antibody levels

Total IgE and sIgE antibody levels to soya bean in patient sera were measured using an ImmunoCAP (Thermo Fisher Diagnostics). An experimental Gly m 5- α' EXT/Gly m 8 ImmunoCAP assay was developed through biotinylation of Gly m 5- α' EXT/Gly m 8 using EZ-link sulfo-NHS-LC-biotin (Thermo Scientific) immobilized to Gly m 5- α' EXT/Gly m 8 via streptavidin.

2.7 | Statistical analysis

Statistical analysis was performed using SPSS, version 24.0 (IBM Japan, Ltd. Tokyo, Japan). The Mann-Whitney *U*-test and Fisher's exact test (both two-tailed) were used to compare continuous and categorical data between symptomatic and asymptomatic groups. Pearson correlations were used to calculate the associations between the logarithm of sIgE antibody levels to the allergens. Diagnostic values were assessed with the area under the curve (AUC), using the receiver operating characteristic (ROC) curve analysis.

3 | RESULTS

3.1 | Patient characteristics

Ninety-one Japanese children suspected of having a soya bean allergy were enrolled in the study (Table 2), whereas 40 children were diagnosed with a soya bean allergy based on positive OFC results, 51 were asymptomatic, as defined by negative OFC results. Among the symptomatic children, skin symptoms were most commonly observed (32 patients, 80%) during OFCs followed by respiratory (18 patients, 45%), gastrointestinal (14 patients, 35%), cardiovascular (1 patient, 3%), and neurological symptoms (1 patient, 3%). An intramuscular adrenaline injection was administered to one child.

TABLE 2 Clinical characteristics of the patients

Characteristics	Symptomatic group	Asymptomatic group	P value
Patient			
Number of patients	40	51	
Sex, no of boys (%)	26 (65.0%)	39 (76.5%)	0.251
Age (y), median (range)	3.5 (0.8-9.7)	2.5 (0.5-8.8)	0.042
Total IgE (kU/L)	48-10,822	15-29,579	0.946
Specific IgE (kU _A /L) for soya bean	0.43-101	0.35-101	0.146
Allergic complications, no (%)			
Atopic dermatitis	34 (85.0%)	44 (86.3%)	1.000
Bronchial asthma	7 (17.5%)	12 (23.5%)	0.606
Other food allergies	28 (70.0%)	28 (54.9%)	0.193
OFC total dose (g), median (range)	33.5 (2-100)	100 (6-100)	

IgE, immunoglobulin E; OFC, oral food challenge.

3.2 | Diagnostic performances of recombinant soya bean allergen components

We analysed the frequencies of sensitization to and diagnostic performances of 14 allergen components in symptomatic and asymptomatic patients (Figure 1A and Table 3).

Patients in the symptomatic group were sensitized to an average of seven of the 14 allergen components tested in this study. The sensitization frequencies to the two Gly m 5 subunits (Gly m 5- α , 92.5%; Gly m 5- α' , 85.0%) and the two Gly m 6 subunits (Gly m 6-A1aB1b, 92.5%; Gly m 6-A3B4, 90.0%) were higher than those to the other allergen components. We observed significant correlations in sIgE antibody levels between the subunits (Gly m 5- α and Gly m 5- α' : $r_{\log} = 0.890$; Gly m 6-A1aB1b and Gly m 6-A3B4: $r_{\log} = 0.829$). Sensitization frequency of Gly m 8 (60.0%) was low compared with those of the two Gly m 5 subunits and the two Gly m 6 subunits. Sensitization frequencies to Gly m 3 and Gly m 4, which are related to pollen-food syndromes, were 42.5% and 17.5%, respectively. sIgE antibody levels for Gly m 3 and Gly m 4 exhibited a moderate correlation ($r_{\log} = 0.558$).

The diagnostic performances of the soya bean allergen components were calculated by comparing sIgE antibody levels between symptomatic and asymptomatic patients (Table 4). The AUC calculated from the ROC analysis was highest for Gly m 8 (AUC 0.706, 95% confidence interval [CI] 0.597-0.815; *P* value, 0.001), owing to its relatively high specificity (82.4%), followed by those of the two Gly m 5 subunits (Gly m 5- α : AUC 0.607; Gly m 5- α' : AUC 0.613). The AUC values of the other allergen components ranged from 0.460 to 0.574. The combination of sIgE antibody levels to Gly m 5- α' and Gly m 8 improve their diagnostic value, with an AUC of 0.727 (95% CI 0.624-0.831). Further, we calculated relative sIgE values (a ratio of sIgE/total IgE), as some of the children seem to have extraordinarily high total IgE values (Figure 1B and Table 5). The data from the relative sIgE values confirm the tendency in those from sIgE values.

These data on serum IgE binding to the crude extract and individual components clearly indicate that soya bean sensitization is fairly individual without easily identification of major allergenic components with both high diagnostic value and high sensitization frequency.

3.3 | Diagnostic performance of Gly m 5- α' extension region

Gly m 5- α and Gly m 5- α' contain N-terminal extension regions (of 125 and 141 residues, respectively) in addition to the core regions (of 420-440 residues); these are common to all three subunits (Gly m 5- α , Gly m 5- α' , and Gly m 5- β).²² The extension regions have a low sequence similarity among the vicilin proteins, suggesting a low cross-reactivity when compared to the proteins' full sequences.

We prepared a recombinant protein of the extension region of Gly m 5- α' (Gly m 5- α' EXT) and analysed its sensitization frequencies and diagnostic performance (Figure 2). sIgE antibody levels to

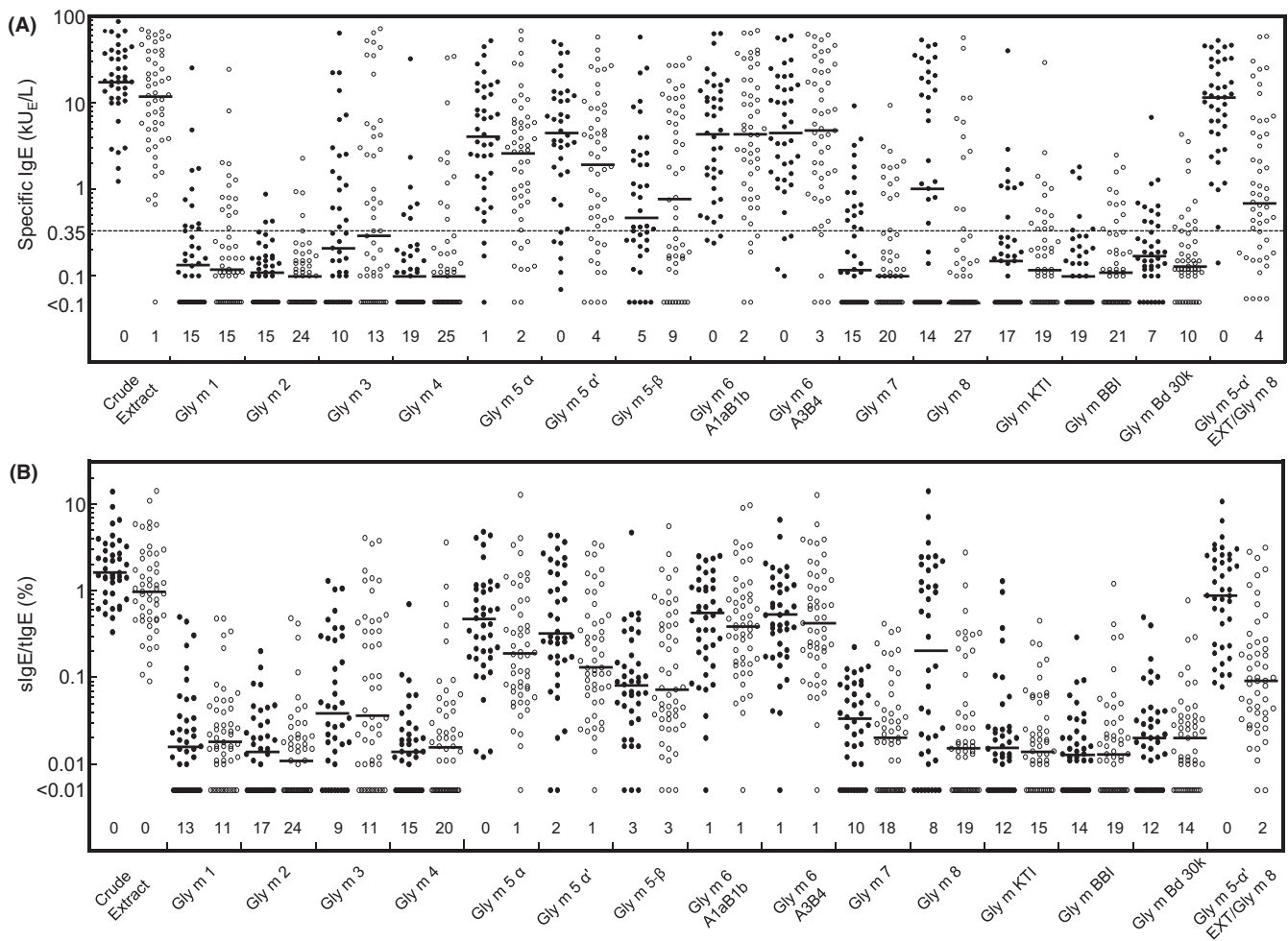


FIGURE 1 sIgE levels and relative sIgE to crude extract and individual soy allergen components. (A) sIgE levels in the symptomatic and asymptomatic groups are indicated as closed and open circles, respectively. Black and dashed lines indicate median values of the respective groups and cut-off levels (≥ 0.35 kU_E/L), respectively. Numbers of patients with <0.1 kU_E/L of sIgE to crude extract and individual soy allergen components are shown. (B) sIgE/total IgE ratios (relative sIgE) in the symptomatic and asymptomatic groups are indicated as closed and open circles, respectively. Black lines indicate median values of the respective groups. Numbers of patients with $<0.01\%$ of relative sIgE are shown

Gly m 5- α' EXT ranged from 0.1 kU_E/L to 35.5 kU_E/L, with a median of 4.0 kU_E/L; its sensitization frequency was 80.0% in the symptomatic group. ROC curve comparison between symptomatic and asymptomatic study participants showed that Gly m 5- α' EXT had a higher AUC (0.695 for sIgE, 95% CI, 0.583-0.806; 0.723 for relative IgE, 0.615-0.832) than that of Gly m 5- α' (0.613 for sIgE, 95% CI 0.497-0.729; 0.647 for relative IgE, 95% CI, 0.531-0.762). The sensitivity and specificity of Gly m 5- α' EXT were 72.5% and 68.6% for sIgE (77.5% and 64.7% for relative IgE), respectively; both were higher than those of Gly m 5- α' (sensitivity for sIgE, 67.5%, specificity for sIgE, 56.9%; sensitivity for relative IgE, 77.5%, specificity for relative IgE, 54.9%).

3.4 | Diagnostic performance of a fusion protein of Gly m 8 plus Gly m 5- α' extension region

To improve the accuracy of diagnosing soya bean allergy through CRD, we designed the fusion protein Gly m 5- α' EXT/Gly m 8 and

evaluated its diagnostic performance (Figure 2). The AUC value of the ROC analysis calculated from sIgE values was higher for Gly m 5- α' EXT/Gly m 8 (AUC 0.801, 95% CI 0.709-0.894) than for Gly m 8 (AUC 0.706, 95% CI 0.597-0.815) (Figure 2C). The sensitivity and specificity of Gly m 5- α' EXT/Gly m 8 were 85.0% and 68.6%, respectively. Those from the relative sIgE values indicate similar tendency (Figure 2D). These findings indicate that the Gly m 5- α' EXT/Gly m 8 fusion protein had a better diagnostic performance than commonly used allergen components.

4 | DISCUSSION

Our study, to the best of our knowledge, is the first report on sIgE antibody levels for all soy-containing allergen components registered in the WHO/IUIS Allergen Nomenclature Subcommittee.

A purity, folding, and integrity of proteins of allergen components contributed towards IgE binding capacity. All recombinant

TABLE 3 Specific immunoglobulin E antibody levels to the soya bean allergen components and frequencies of sensitization (cut-off, ≥ 0.35 kU_E/L)

Component	Symptomatic group (n = 40)		Asymptomatic group (n = 51)	
	Concentration (kU _E /L), median (range)	Frequency of sensitization, no. (%)	Concentration (kU _E /L), median (range)	Frequency of sensitization, no. (%)
Crude extract	18.1 (1.2-86.9)	40 (100)	11.5 (0.1-70.3)	50 (98.0)
Gly m 1	0.1 (<0.1-25.3)	11 (27.5)	0.1 (<0.1-24.4)	15 (29.4)
Gly m 2	0.1 (<0.1-0.9)	3 (7.5)	0.1 (<0.1-2.3)	4 (7.8)
Gly m 3	0.2 (<0.1-64.0)	17 (42.5)	0.3 (<0.1-71.6)	23 (45.1)
Gly m 4	0.1 (<0.1-32.3)	7 (17.5)	0.1 (<0.1-34.4)	11 (21.6)
Gly m 5- α	4.1 (<0.1-52.3)	37 (92.5)	2.7 (<0.1-68.4)	41 (80.4)
Gly m 5- α'	4.5 (0.1-51.1)	34 (85.0)	1.8 (<0.1-57.6)	39 (76.4)
Gly m 5- β	0.5 (<0.1-57.7)	24 (60.0)	0.7 (<0.1-32.9)	29 (56.9)
Gly m 6-A1bB1b	4.7 (0.2-63.4)	37 (92.5)	4.3 (<0.1-68.8)	46 (90.2)
Gly m 6-A3B4	4.6 (0.1-59.8)	36 (90.0)	4.7 (<0.1-62.3)	46 (90.2)
Gly m 7	0.1 (<0.1-9.3)	16 (40.0)	0.1 (<0.1-9.4)	13 (25.5)
Gly m 8	1.1 (<0.1-56.1)	24 (60.0)	0.1 (<0.1-56.8)	12 (23.5)
Gly m KTI	0.2 (<0.1-40.1)	9 (22.5)	0.1 (<0.1-29.3)	14 (27.5)
Gly m BBI	0.1 (<0.1-1.8)	5 (12.5)	0.1 (<0.1-2.5)	11 (21.6)
Gly m Bd 30k	0.2 (<0.1-6.8)	11 (27.5)	0.1 (<0.1-4.3)	11 (21.6)

TABLE 4 Diagnostic values of soya bean allergen components, calculated from sIgE

	Crude extract	Gly m 1	Gly m 2	Gly m 3	Gly m 4	Gly m 5- α	Gly m 5- α'	Gly m 5- β
AUC	0.609	0.463	0.548	0.463	0.469	0.607	0.613	0.485
95% CI	0.492-0.725	0.342-0.584	0.428-0.668	0.343-0.582	0.349-0.590	0.490-0.724	0.497-0.729	0.364-0.605
P value	0.076	0.545	0.434	0.540	0.613	0.080	0.065	0.801
Optimal cut-off value (kU _E /L)	9.8	0.1	0.1	0.1	0.2	6.5	3.0	0.3
Sensitivity (%)	85.0	52.5	82.5	62.5	37.5	45.0	67.5	75.0
Specificity (%)	45.1	51.0	27.5	41.2	66.7	78.4	56.9	41.2
	Gly m 6-A1aB1b	Gly m 6-A3B4	Gly m 7	Gly m 8	Gly m KTI	Gly m BBI	Gly m Bd 30k	
AUC	0.489	0.492	0.538	0.706	0.468	0.460	0.574	
95% CI	0.369-0.609	0.373-0.611	0.418-0.658	0.597-0.815	0.343-0.592	0.339-0.580	0.454-0.693	
P value	0.860	0.898	0.532	0.001	0.597	0.508	0.228	
Optimal cut-off value (kU _E /L)	7.1	0.9	0.3	0.7	1.0	0.1	0.2	
Sensitivity (%)	47.5	87.5	40.0	57.5	20.0	45.0	55.0	
Specificity (%)	60.8	23.5	74.5	82.4	92.2	60.8	62.7	

AUC, area under the curve; CI, confidence interval.

allergen components in the study were expressed in their soluble forms. The purified allergen components were checked by SDS-PAGE. During purification, all allergen components showed a single peak in the gel filtration chromatography. This suggests that all allergen components seem to be folded. However, circular dichroism spectra measurement may be needed to confirm their secondary structures in a prospective study.

The allergen components useful for diagnosing a soya bean allergy have been assessed in previous studies. In 55 Japanese and 41 US children suspected having a soya bean allergy, Gly m 8 had a

higher diagnostic value (AUCs 0.75 and 0.82 in children in Japan and in the US, respectively) than Gly m 5 and Gly m 6.^{14,16} Gly m 8 was also reported to have a high diagnostic value in 46 adult patients with suspected soya bean allergy in the Netherlands,¹⁵ although the AUC of Gly m 8 was comparable to the results of a skin prick test and sIgE antibody levels to crude extract. In our study, Gly m 8 had the highest AUC value (0.706) among the allergen components we examined. Native Gly m 8 used in the previous studies¹⁴⁻¹⁶ is processed by protease in soya bean seeds, resulting in the two subunits linked by disulphide bonds.²⁴ Conversely, recombinant Gly m 8 in

TABLE 5 Diagnostic values of soya bean allergen components, calculated from relative sIgE

	Crude extract	Gly m 1	Gly m 2	Gly m 3	Gly m 4	Gly m 5- α	Gly m 5- α'	Gly m 5- β
AUC	0.654	0.460	0.532	0.461	0.466	0.632	0.647	0.476
95% CI	0.542-0.767	0.337-0.582	0.407-0.656	0.342-0.580	0.345-0.588	0.516-0.748	0.531-0.762	0.357-0.596
P value	0.012	0.512	0.606	0.528	0.584	0.031	0.017	0.701
Sensitivity (%)	72.5	90.0	80.0	87.5	85.0	82.5	77.5	72.5
Specificity (%)	62.7	21.6	47.1	21.6	39.2	47.1	54.9	43.1
	Gly m 6-A1aB1b	Gly m 6-A3B4	Gly m 7	Gly m 8	Gly m KTI	Gly m BBI	Gly m Bd 30k	
AUC	0.516	0.513	0.553	0.685	0.486	0.481	0.532	
95% CI	0.395-0.637	0.393-0.632	0.431-0.675	0.567-0.802	0.364-0.608	0.359-0.603	0.411-0.652	
P value	0.798	0.835	0.388	0.003	0.820	0.752	0.606	
Sensitivity (%)	35.0	67.5	47.5	45.0	85.0	87.5	95.0	
Specificity (%)	76.5	47.1	72.5	94.1	29.4	37.3	27.5	

AUC, area under the curve; CI, confidence interval.

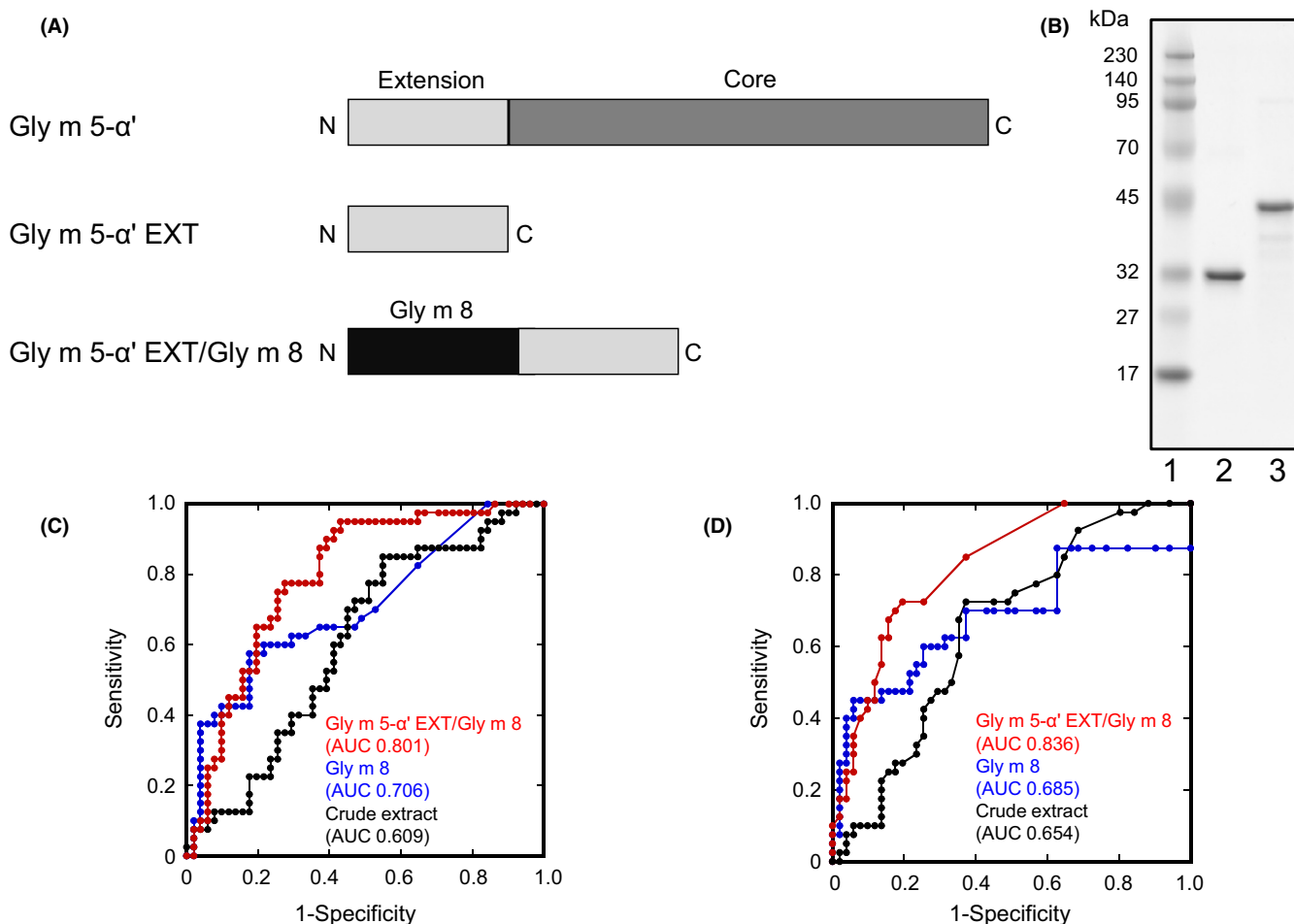


FIGURE 2 Fusion protein of Gly m 8 plus the Gly m 5- α' extension region. (A) Schematic representations of the structures of Gly m 5- α' , Gly m 5- α' EXT, and Gly m 5- α' EXT/Gly m 8. (B) Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified Gly m 5- α' EXT and the Gly m 5- α' EXT/Gly m 8 fusion protein. Lane 1: molecular weight markers; lane 2: purified Gly m 5- α' EXT; lane 3: purified Gly m 5- α' EXT/Gly m 8. (C) Receiver operating characteristic curves for specific IgE antibody levels to the fusion protein of Gly m 8 and Gly m 5- α' extension region. Results are based on enzyme-linked immunosorbent assay (ELISA) results of sera from symptomatic and asymptomatic study participants. Red, blue, and black curves represent receiver operating curves of Gly m 5- α' EXT/Gly m 8, Gly m 8, and crude extract, respectively. (D) Receiver operating characteristic curves for relative sIgE to the fusion protein of Gly m 8 and Gly m 5- α' extension region. Red, blue, and black curves represent receiver operating curves of Gly m 5- α' EXT/Gly m 8, Gly m 8, and crude extract, respectively

the present study was not processed. The structural difference of Gly m 8 between native and recombinant proteins might explain a difference of diagnostic performance.

In the present study, Gly m 5- α' showed the second-best diagnostic performance (AUC of 0.613 for sIgE, and 0.647 for relative sIgE). Furthermore, Gly m 5- α' EXT (AUC of 0.695 for sIgE, and 0.723 for relative sIgE) showed a better diagnostic performance than full-length Gly m 5- α' . This indicates that cross-reactivity affects the diagnostic performance, as the extension regions of the vicilin proteins have a lower level of similarity than that of the core regions. We designed the Gly m 5- α' EXT/Gly m 8 fusion protein, which showed a high diagnostic value (AUC of 0.801 for sIgE, and 0.836 for relative sIgE) (Figure 2). These data suggest that the development of a fusion allergen component with a high diagnostic performance might be a promising approach in molecular allergology.

Potential limitations are present in our study. OFCs rather than double-blind placebo-controlled food challenges were used. Therefore, only objective symptoms were used to define a positive OFC result. The validity and sufficiency of using open challenges have previously been confirmed.²⁵ Another limitation is that the ELISA measurement instead of ImmunoCAP test was used to quantify the sIgE levels in sera. We reported that the clinical performance calculated from both methods were very similar using sesame allergen components.²⁶ In addition to it, we measured sIgE antibodies in sera of patients positive for soya bean ($n = 32$) by an experimental Gly m 5- α' EXT/Gly m 8 ImmunoCAP test and the ELISA assay. Based on these, a good correlation for the sIgE values calculated from both methods was confirmed ($r = 0.987$, Figure S1). Furthermore, there is a limitation in the diagnostic performance of Gly m 5- α' EXT/Gly m 8. The Gly m 5- α' EXT/Gly m 8 had increased sensitivity in comparison with Gly m 8 but at the same time had significantly decreased specificity in comparison with Gly m 8. The optimal cut-off level to discriminate between the symptomatic and asymptomatic groups might be determined in future multi-centre studies.

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AUTHOR CONTRIBUTIONS

N. Maruyama, S. Sato, K. Ito, and M. Ebisawa were involved in study concept and design, analysis, preparation of the article, and gave final approval of the version to be published. C. Cabanos and A. Tanaka were involved in analysis, interpretation of data, critical revision of the article, and final approval of the version to be published.

CONFLICT OF INTEREST

M. Ebisawa and K. Ito have received research support from Health and Labor Sciences Research Grants for Research on Allergic Diseases and Immunology from the Ministry of Health, Labour, and Welfare, Japan (201414009A). N. Maruyama, M. Ebisawa, and K. Ito have received research support from the Practical Research Project for Allergic Disease and Immunology of the Japan Agency for Medical Research and Development, AMED (16ek0410019 h0002). N. Maruyama has received research support from "The Salt Science Research Foundation, No 1444." M. Ebisawa is a member of the DBV Technologies Scientific Advisory Board. M. Ebisawa received lecture fees from Pfizer and Siemens. A. Tanaka is an employee of Thermo Fisher Diagnostics. The rest of the authors declare that they have no relevant conflict of interests related to this work.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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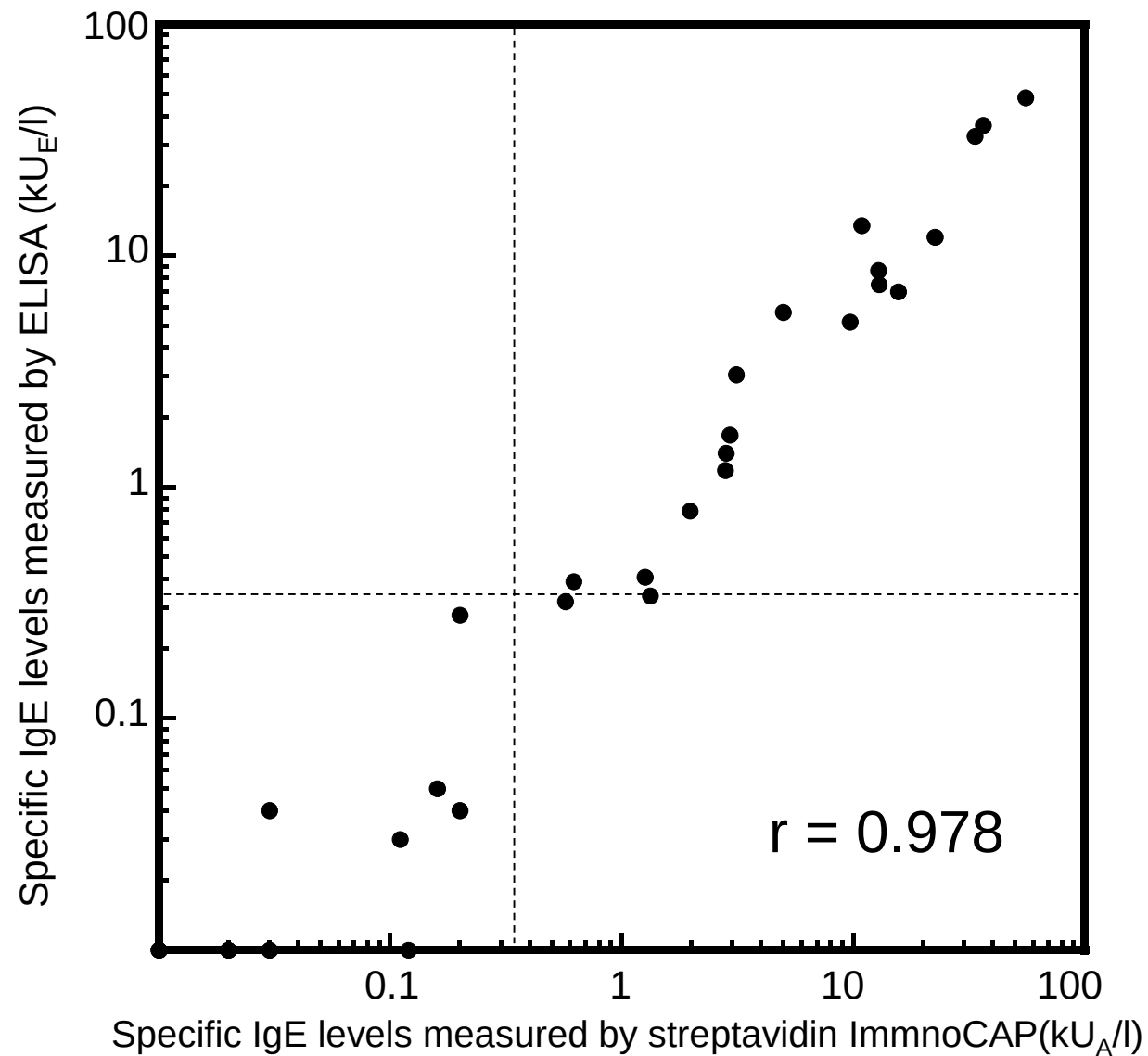


Figure S1

Correlation of specific IgE to a fusion protein calculated from the ELISA method and streptavidin ImmunoCAP