

Letter to the Editor

Gly m 2S albumin is a major allergen with a high diagnostic value in soybean-allergic children

To the Editor:

Several soybean proteins have been suggested to have IgE reactivity but only 6 of them have so far been recognized by the WHO/IUIS Allergen Nomenclature Subcommittee.¹ Gly m 4, a pollen-related allergen, and the storage proteins Gly m 5 and Gly m 6 have been found to be associated with food allergy.^{2,3} The storage protein 2S albumin has not yet been recognized as an important allergen for soybean (Gly m 2S albumin), and its association with clinical symptoms is not clearly established.⁴ Biochemically, the 2S albumins are characterized by a conserved 3-dimensional structure, high stability to the gastrointestinal tract environment, and high resistance to thermal processing.⁵ The amino acid sequence identity between 2S albumins from different species is low, often less than 40%. Altogether these properties may explain the generally high diagnostic value of IgE measurements.⁶ In this study, the diagnostic value of Gly m 2S albumin was investigated and compared with Gly m 5 and Gly m 6, by analysis of IgE in sera from soybean-allergic children.

Sera from 55 soybean-sensitized Japanese children were collected (Table I). Nineteen were diagnosed with soybean allergy by positive oral food challenge (OFC) ($n = 16$) or a definitive history ($n = 3$) of urticaria within 1 hour after intake. The remaining 36 children were nonsymptomatic and were either diagnosed by negative OFC ($n = 17$) or a regular consumption in their daily life ($n = 19$). Ethical approvals were obtained through the institutional review boards at Sagamihara National Hospital and Aichi Children's Health and Medical Center. Among the symptomatic children, 16 had skin symptoms, 5 oral symptoms, 6 respiratory symptoms (coughing and wheeze), 1 gastrointestinal symptom (diarrhea), and 1 neurologic symptom (sleep). Intramuscular adrenaline injection was used in 1 case, which was considered as an anaphylaxis. No allergies to nuts were documented among the children in the study. OFCs were conducted in accordance with Japanese guidelines.⁷

Gly m 2S albumin was purified from soybeans by anion exchange chromatography followed by gel filtration. Purity and identity of Gly m 2S albumin were determined by SDS-PAGE, analytical gel filtration, matrix-assisted laser desorption/ionization mass spectrometry with time-of-flight ion separation (positive linear mode and peptide mass fingerprinting), and N-terminal protein sequencing (10 cycles). Experimental ImmunoCAP with Gly m 2S albumin was developed by Phadia AB (Uppsala, Sweden).⁸ Soybean, Gly m 5, and Gly m 6 are commercially available ImmunoCAP products. The detection limit of the test was 0.1 kU_A/L. The Methods are described more fully in this article's Online Repository at www.jacionline.org.

The recovery of pure Gly m 2S albumin was 1.1 mg/g of soybeans. Analytical gel filtration showed a homogenous dimer with a molecular size of 28 kDa (see Fig E1 in the Online Repository at www.jacionline.org). Matrix-assisted laser desorption/ionization mass spectrometry with time-of-flight ion separation revealed the mass 14 kDa (m/z) (Fig 1). Peptide mass fingerprinting and N-terminal sequencing showed agreement with UniProt accession no. P19594, and 80 amino acids of 120 (75%) were

TABLE I. Demographic, serologic, and clinical characterization of 55 soybean-sensitized Japanese children with or without symptoms

Patient characteristics	Symptomatic	Nonsymptomatic	P	
	(n = 19)	(n = 36)	value*	AUC†
Sex,	12/7	28/8		
male/female				
Age (y), median (range)	2.4 (0.7-9.8)	1.9 (0.6-10.3)		
Specific IgE to (kU _A /L),				
median (range)				
Soybean	23.7 (0.4-92.0)	4.1 (0.5-77.3)	.0258	0.69
2S albumin	6.8 (0.1-120.0)	0.5 (0.0-46.6)	.0024	0.75
Gly m 5	20.4 (0.1-58.3)	1.7 (0.1-81.9)	.0521	0.69
Gly m 6	15.5 (0.3-70.8)	2.5 (0.4-93.5)	.1016	0.64
Diagnosis of soybean				
allergy				
OFC	16	19		
History	3	17		
Symptoms				
Skin	16	—		
Oral	5	—		
Respiratory	6	—		
Gastrointestinal	1	—		
Anaphylaxis	1	—		

*Comparison between the study groups regarding IgE level to Gly m 2S albumin, soybean, Gly m 5, and Gly m 6 using the Mann-Whitney *U* test ($P < .05$ was considered significant).

†Area under the curve (AUC) from receiver operating characteristic (ROC) curve analysis comparing children in the symptomatic and nonsymptomatic groups.

identified (see Fig E2 in the Online Repository at www.jacionline.org). The N-terminal protein sequencing did not indicate the presence of any impurities. No evidence of the presence of carbohydrate chains was found. The allergenicity of Gly m 2S albumin was investigated by analysis of IgE antibody in sera (Table I). Seventeen out of 19 children with soybean allergy and 31 out of 36 nonsymptomatic children had IgE levels of more than 0.1 kU_A/L to Gly m 2S albumin. A significant differentiation between IgE levels in the symptomatic and the nonsymptomatic children was found ($P < .01$). Of the 2 sera with IgE levels of less than 0.1 kU_A/L in the symptomatic children, both had IgE to Gly m 5 and 6, and of the 5 sera with IgE levels of less than 0.1 kU_A/L in the nonsymptomatic children, all had IgE to Gly m 6 and 4 to Gly m 5. No significant differences in IgE levels between those who were OFC negative and history negative could be seen. Receiver operating characteristic analysis showed that Gly m 2S albumin had an area under the curve of 0.75, followed by soybean and Gly m 5 (0.69 for both) and Gly m 6 (0.64) (see Fig E3 in the Online Repository at www.jacionline.org). Receiver operating characteristic analysis by combining the 3 components, 2 or all 3, did not improve the area under the curve.

Gly m 2S albumin, Gly m 5, and Gly m 6 were found to be major allergens in the study group. Gly m 5 and Gly m 6 have in earlier studies been classified as major allergens in children and have also been correlated with severe symptoms from soybean.^{3,9} In this study, IgE to Gly m 2S albumin was significantly higher in the symptomatic group, while IgE to Gly m 5 and Gly m 6 was not, although a trend toward a significant differentiation was

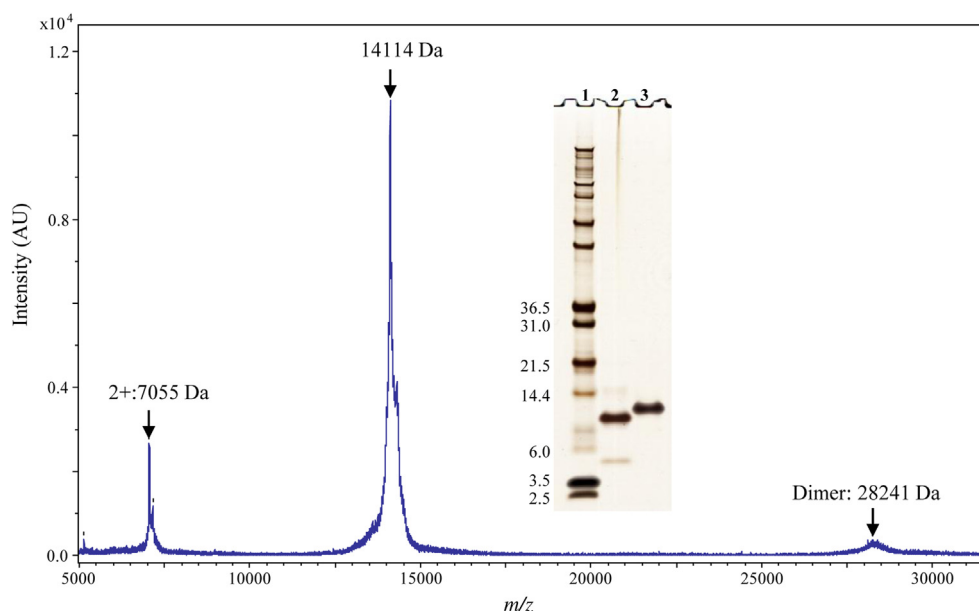


FIG 1. Gly m 2S albumin analyzed with MALDI-TOF MS in the positive linear mode showing a pure protein, and SDS-PAGE on NuPAGE 4% to 12% Bis-Tris Gel from Invitrogen (Carlsbad, Calif) under reduced conditions (lane 2, protein divided into 2 subunits 8 kDa and ~5 kDa) and nonreduced conditions (lane 3, 10 kDa), and molecular weight markers (lane 1). AU, Arbitrary unit; MALDI-TOF MS, matrix-assisted laser desorption/ionization mass spectrometry with time-of-flight ion separation.

seen. IgE reactivity to Gly m 2S albumin has been investigated earlier.⁴ Lin et al⁴ reported no IgE to 2S albumin in the soybean-allergic patients. This discrepancy between the study of Lin et al⁴ and this study may, for example, depend on the study population, geographical reasons, and different 2S albumin preparations, and is further discussed in the Online Repository at www.jacionline.org. Soybean allergy in adults and adolescents may be pollen-related, caused by IgE cross-reactivity between the PR-10 allergen component Bet v 1 from *Betulaceae* pollen and its homologue in soybean, Gly m 4.² The Gly m 2S albumin in this study has proved to be pure and homogeneous, and the identity correlates best with the reviewed 2S albumin from soybean with accession no. P19594. Proteins from the lipid transfer protein (LTP) group and the 2S albumin group generally have similar molecular weights, and it is therefore important to eliminate cross-contamination. Extensive efforts have been made to prove that LTP and other contaminating proteins are not present in Gly m 2S albumin. In addition, efforts to purify LTP from soybean have failed (data not shown). Others have shown that soybean LTP is present in the hull of the soybean, and it is therefore not considered a food allergen.¹

Soybean is 1 of 8 foods thought to cause more than 90% of food allergy reactions in children. Nevertheless, knowledge about specific soybean allergens associated with clinical symptoms is in large part uninvestigated. Earlier studies have shown that analysis of IgE to both Gly m 5 and Gly m 6 will facilitate the diagnosis of soybean allergy in children.^{3,9} In this study, we show that 2S albumin from soybean is a major allergen in Japanese children with soybean allergy. The biochemical features of 2S albumin allergens with generally low cross-reactivity between species may be advantageous in the differentiation between clinical soybean allergy and allergies caused by other legumes, for example, peanut. We put forward that analysis of IgE to Gly m 2S albumin will provide a high diagnostic

value and will even further facilitate the diagnosis of soybean allergy.

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REFERENCES

- Ballmer-Weber BK, Vieths S. Soy allergy in perspective. *Curr Opin Allergy Clin Immunol* 2008;8:270-5.
- Fukutomi Y, Sjölander S, Nakazawa T, Borres MP, Ishii T, Nakayama S, et al. Clinical relevance of IgE to recombinant Gly m 4 in the diagnosis of adult soybean allergy. *J Allergy Clin Immunol* 2012;129:860-3.
- Ito K, Sjölander S, Sato S, Movérare R, Tanaka A, Söderström L, et al. IgE to Gly m 5 and Gly m 6 is associated with severe allergic reactions to soybean in Japanese children. *J Allergy Clin Immunol* 2011;128:673-5.
- Lin JPR, Shewry PR, Archer DB, Beyer K, Niggemann B, Haas H, et al. The potential allergenicity of two 2S albumins from soybean (Glycine max): a protein microarray approach. *Int Arch Allergy Immunol* 2006;141:91-102.
- Moreno FJ, Clemente A. 2S Albumin storage proteins: what makes them food allergens? *Open Biochem J* 2008;2:16-28.

6. Ebisawa M, Movérare R, Sato S, Maruyama N, Borres MP, Komata T. Measurement of Ara h 1-, 2-, and 3-specific IgE antibodies is useful in diagnosis of peanut allergy in Japanese children. *Pediatr Allergy Immunol* 2012;23:573-81.
7. Ito K, Urisu A. Diagnosis of food allergy based on oral food challenge test. *Allergol Int* 2009;58:467-74.
8. Marknell DeWitt A, Niederberger V, Lehtonen P, Spitzauer S, Sperr WR, Valent P, et al. Molecular and immunological characterization of a novel timothy grass (*Phleum pratense*) pollen allergen, Phl p 11. *Clin Exp Allergy* 2002;32:1329-40.
9. Holzhauser T, Wacermann O, Ballmer-Weber BK, Bindslev-Jensen C, Scibilia J, Perono-Garoffo L, et al. Soybean (*Glycine max*) allergy in Europe: Gly m 5 (β -conglycinin) and Gly m 6 (glycinin) are potential diagnostic markers for severe allergic reactions to soy. *J Allergy Clin Immunol* 2009;123:452-8.

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METHODS

Food challenge

Oral soy challenges were conducted in accordance with Japanese guidelines by using an increasing amount of tofu (traditional soy paste containing 6.6% of soy protein) or boiled soybeans (16% protein). Increasing amount in 5 to 6 doses was given every 15 to 20 minutes, up to the cumulative dose of more than 3 g soy protein. Challenge results were considered as positive when obvious objective symptoms were observed. Negative challenge was confirmed after follow-up visits to ensure the absence of any symptoms after the ingestion of soy products.

Preparation of 2S albumin

Preparation of Gly m 2S albumin was performed from defatted and freeze-dried soybeans, *Glycine max* (Allergon, Ängelholm, Sweden). Extract of soybeans was applied to anion exchange chromatography (Q Sepharose HP; GE Healthcare, Uppsala, Sweden), and adsorbed material containing Gly m 2S albumin was eluted by a linear sodium chloride gradient. Further purification was performed by gel filtration on Superdex 75 pg (GE Healthcare). Fractions containing purified Gly m 2S albumin were pooled and concentrated by using an Amicon cell with regenerated cellulose (5 kDa) membrane filter (Millipore, Billerica, Mass). The protein concentration was determined by using the bicinchoninic acid (BCA) method (BCA protein assay kit, Thermo Scientific, Rockford, Ill). The prepared Gly m 2S albumin solution was frozen at -20°C until use.

Electrophoresis

SDS-PAGE was performed on NuPAGE 4% to 12% Bis-Tris Gel from Invitrogen (Carlsbad, Calif). Prepared Gly m 2S albumin was treated with NuPAGE lithium dodecyl sulfate sample buffer, and for the reducing condition dithiothreitol was added followed by alkylation with iodoacetamide, and heated at 97°C for 5 minutes. The gel was loaded with 0.5 μg Gly m 2S albumin (reduced and nonreduced). The molecular weight calibration of the gel was achieved by using Mark 12 unstained standard (Invitrogen). Electrophoresis was performed in 2-(N-morpholino) ethanesulfonic acid running buffer following the manufacturer's instruction. The gel was silver stained according to Blum et al^{E1} in a Hoefer Processor Plus (GE Healthcare).

Glycosylation analysis

The glycosylation status of 2S albumin was investigated by enzymatic deglycosylation of the protein with several glycosidases (PNGase F, Neuraminidase, GPase A, 3 *O*-glycosidases) followed by SDS-PAGE, where 5 and 10 μg of treated and untreated Gly m 2S albumin and 5 μg fetuin (positive control) were run on the gel under reducing and nonreducing conditions. The gel was stained by using a glycoprotein detection kit (Sigma-Aldrich, St Louis, Mo) based on the periodic acid-Schiff method.

Mass spectrometry analysis

Matrix-assisted laser desorption/ionization mass spectrometry with time-of-flight ion separation (MALDI-TOF MS) in the positive linear mode and peptide mass fingerprinting (PMF) were performed by using a Autoflex, II system (Bruker Daltonic, Bremen, Germany) from prepared Gly m 2S albumin in solution. For the PMF analysis, the Gly m 2S albumin solution was reduced with dithiothreitol and alkylated with iodoacetamide before it was digested with trypsin. Salt was removed by reversed phase chromatography on a C_{18} column (Millipore, Billerica, Mass). When the whole Gly m 2S albumin protein was analyzed in the linear mode, salt was removed directly on a C_{18} column.

Analytical gel filtration

The molecular weight of Gly m 2S albumin was estimated by using a calibrated Superdex 75 PC 3.2/30 column (GE Healthcare) on an Ettan LC system (GE Healthcare). Twenty microliters was applied to the column, and the Unicorn analysis module 5.0 (GE Healthcare) was used to estimate molecular size.

Amino acid sequencing

Five micrograms of Gly m 2S albumin was diluted in 1 mL 6 M guanidine-HCl, 0.1 M Tris, pH 8, and incubated with 5 μL 0.5 M dithiothreitol for 2 hours at 37°C . After cooling the solution to room temperature, 20 μL 0.5 M iodoacetamide was added and the solution was incubated in the dark for 15 minutes. N-terminal protein sequencing (10 cycles) was then performed by using the Edman degradation method in a G1000A sequencer (Hewlett-Packard, Palo Alto, Calif). The obtained sequence was compared with other known 2S albumins in UniProtKB^{E2} by using the BLAST function.

RESULTS

Purity of prepared Gly m 2S albumin

Analytical gel filtration of Gly m 2S albumin confirms that the preparation is homogeneous, but it also shows that it acts as a dimer with a molecular size of 28 kDa in the native state (Fig E1) in contrast to the analysis with MALDI-TOF MS in the positive linear mode, which reveals the mass determination, *m/z*, to be 14 kDa. The N-terminal protein sequencing was originally performed to obtain identity of the Gly m 2S albumin. By using the peak area from the internal standard, it was possible to calculate the mole content reflected by the obtained peak areas from the first 10 amino acids from the 2 subunits of Gly m 2S albumin. In this way, it could be shown that the total obtained amount mole corresponds to the amount mole of Gly m 2S albumin used in the analysis given an indirect proof of its purity.

Verification of the identity of Gly m 2S albumin

The identity of the prepared material was verified by N-terminal protein sequencing and PMF using MALDI-TOF MS. The N-terminal protein sequencing showed that the first 10 amino acids from the 2 subunits corresponded to 100% with 2S albumin (*Glycine max*) with accession no. P19594,^{E2} amino acid 22 to 31 and 82 to 91, respectively. Using PMF, peptides containing amino acids 24 to 50, 97 to 105, and 117 to 158, corresponding to accession no. P19594, could be shown, and together with the N-terminal sequences, a total identification of 80 amino acids of a total of 120 (75%) was obtained (Fig E2).

DISCUSSION

IgE reactivity to Gly m 2S albumin has been investigated earlier.^{E3} In the study by Lin et al,^{E3} none of the soybean-allergic patients including both children and adults from Europe was found to have IgE to 2S albumin. This discrepancy may depend on several factors, one being the different study group compositions. In the study by Lin et al,^{E3} the patients are a mixture of both children and adults, while the patients in this study are composed of children only. Soybean allergy in adults and adolescents has in several studies been shown to be pollen-related, caused by IgE cross-reactivity between the PR-10 allergen component Bet v 1 from Betulaceae pollen (birch) and its homologue in soybean, Gly m 4.^{E4-E6} The disagreement may also depend on geographic reasons, with different dietary habits regarding soybean products, and different prevalences regarding Betulaceae pollen-related allergies. Japan is one of the largest soybean-consuming populations in the world but has a low prevalence of Betulaceae pollen-related allergies, while the soybean consumption in Europe is low but with a high prevalence of Betulaceae pollen-related allergies, especially in central and northern parts. However, the reason may also be that different isoforms of 2S albumin have

been used in the 2 studies. In the study by Lin et al,^{E3} both native and recombinantly produced 2S albumin from soybean have been used for IgE measurements, while the Gly m 2S albumin used in this study was purified from soybeans.

REFERENCES

- E1. Blum H, Beier H, Gross HJ. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* 1987;8:93-9.
- E2. The UniProt Consortium. Reorganizing the protein space at the Universal Protein Resource (UniProt). *Nucleic Acids Res* 2012;40:D71-5. The UniProtKB database at <http://www.uniprot.org>.
- E3. Lin JPR, Shewry PR, Archer DB, Beyer K, Niggemann B, Haas H, et al. The potential allergenicity of two 2S albumins from soybean (*Glycine max*): a protein microarray approach. *Int Arch Allergy Immunol* 2006;141:91-102.
- E4. Mittag D, Vieths S, Vogel L, Becker WM, Rihs HP, Helbling A, et al. Soybean allergy in patients allergic to birch pollen: clinical investigation and molecular characterization of allergens. *J Allergy Clin Immunol* 2004;113:148-54.
- E5. Kosma P, Sjölander S, Landgren E, Borres MP, Hedin G. Severe reactions after the intake of soy drink in birch pollen-allergic children sensitized to Gly m 4. *Acta Paediatr* 2011;100:305-6.
- E6. Fukutomi Y, Sjölander S, Nakazawa T, Borres MP, Ishii T, Nakayama S, et al. Clinical relevance of IgE to recombinant Gly m 4 in the diagnosis of adult soybean allergy. *J Allergy Clin Immunol* 2012;129:860-3.

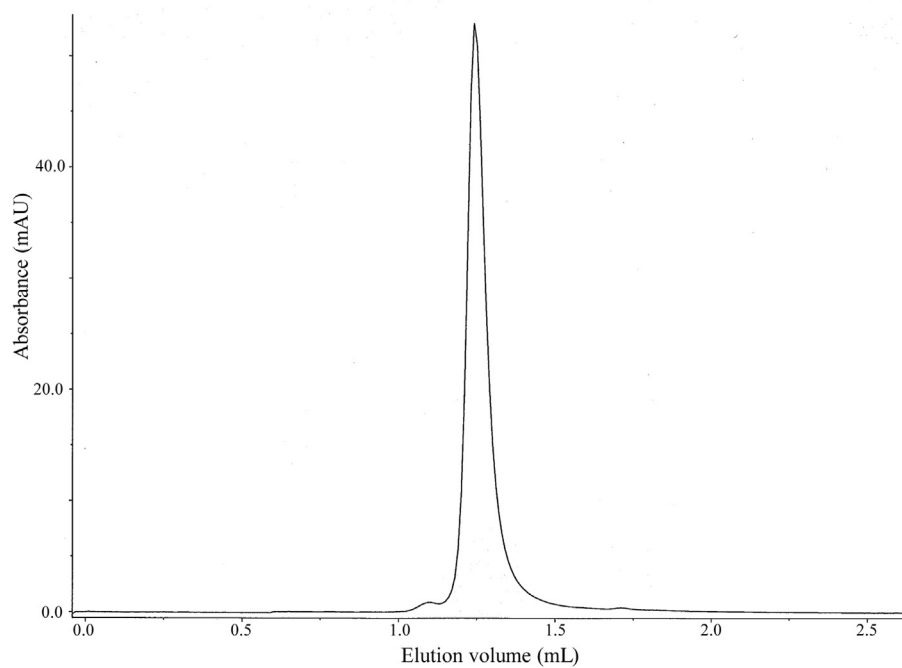


FIG E1. Analytical gel filtration of Gly m 2S albumin on Superdex 75 given the molecular size in native state. AU, Absorbance unit.

Short chain 22 30 40 50 60
SKWQHQQDSCRKQLQGVNLTPEKHIMEKIQGRGDDDDDDDD

Long chain 82 90 100 110 120
EGKDEDEEEEGHMQKCCTEMSELRSPKCQCKALQKIMENQSEELE
130 140 150 158
EKQKKKMEKELINLATMCRFGPMIQCDLSSDD

FIG E2. The short and long chains from the amino acid sequence of the soybean 2S albumin accession no. P19594. The *gray boxes* indicate identified amino acid residues from N-terminal sequencing and PMF of the prepared Gly m 2S albumin.

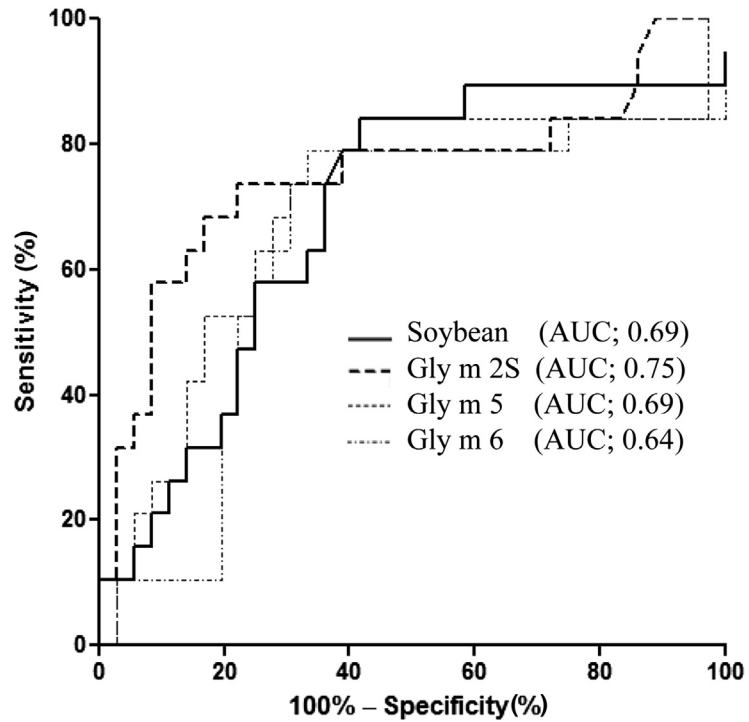


FIG E3. Comparison of ROC curves for soybean, Gly m 2S albumin (Gly m 2S), Gly m 5, and Gly m 6 in symptomatic versus nonsymptomatic children. *AUC*, Area under the curve; *ROC*, receiver operating characteristic.