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

Thomas Holzhauser, PhD,[a] Olga Wackermann, MSc,[a] Barbara K. Ballmer-Weber, MD,[b] Carsten Bindslev-Jensen, MD,[c] Joseph Scibilia, MD,[d] Lorenza Perono-Garoffo, PhD,[e] Shigeru Utsumi, PhD,[f] Lars K. Poulsen, PhD,[g] and Stefan Vieths, PhD[a] *Langen, Germany, Zurich, Switzerland, Odense and Copenhagen, Denmark, Milan, Italy, and Kyoto, Japan*

Background: Soybean is considered an important allergenic food, but published data on soybean allergens are controversial.
Objective: We sought to identify relevant soybean allergens and correlate the IgE-binding pattern to clinical characteristics in European patients with confirmed soy allergy.
Methods: IgE-reactive proteins were identified from a soybean cDNA expression library, purified from natural soybean source, or expressed in *Escherichia coli*. The IgE reactivity in 30 sera from subjects with a positive double-blind, placebo-controlled soybean challenge (n = 25) or a convincing history of anaphylaxis to soy (n = 5) was analyzed by ELISA or CAP-FEIA.
Results: All subunits of Gly m 5 (β-conglycinin) and Gly m 6 (glycinin) were IgE-reactive: 53% (16/30) of the study subjects had specific IgE to at least 1 major storage protein, 43% (13/30) to Gly m 5 , and 36% (11/30) to Gly m 6. Gly m 5 was IgE-reactive in 5 of 5 and Gly m 6 in 3 of 5 children. IgE-binding to Gly m 5 or Gly m 6 was found in 86% (6/7) subjects with anaphylaxis to soy and in 55% (6/11) of subjects with moderate but only 33% (4/12) of subjects with mild soy-related symptoms. The odds ratio ($P < .05$) for severe versus mild allergic reactions in subjects with specific IgE to Gly m 5 or Gly m6 was 12/1.
Conclusion: Sensitization to the soybean allergens Gly m 5 or Gly m 6 is potentially indicative for severe allergic reactions to soy. (J Allergy Clin Immunol 2009;123:452-8.)

Key words: *Soybean allergy, soybean allergens, β-conglycinin, glycinin, DBPCFC, anaphylaxis, cDNA expression library*

Soybean (Glycine max)–induced allergic symptoms may range from skin, gastrointestinal, or respiratory reactions to anaphylaxis.[1-3] Food allergy to soy has been described primarily in young children with atopic dermatitis,[4-6] but published data on the prevalence of soybean allergy in childhood are controversial. Moreover, the prevalence of soybean allergy in adults is still unknown.[7,8] Recently, we described the clinical characteristics of soybean allergy in 25 European adults and 5 children, in whom soy allergy was confirmed by a positive DBPCFC or according to a convincing history of anaphylaxis to soy.[3] Cumulative threshold doses in soybean DBPCFC were at least 1 order of magnitude higher than observed in peanut allergy,[9,10] but no correlation was found between the level of threshold doses and the severity of symptoms. In addition, the pattern of IgE reactivity determined by immunoblotting was highly individual and apparently did not correlate with the severity of symptoms.[3] To date, at least 16 IgE-binding soybean proteins have been described, of which several have been characterized in more detail: the soybean Kunitz trypsin inhibitor,[11-13] the thiol-protease Gly m Bd 30k that has been suggested as a major soybean allergen,[14-16] the α subunit of the major storage protein β-conglycinin (BC),[17] the acidic chain of the major storage protein glycinin (G) G1 subunit,[18] and the basic chain of the G2 subunit.[19] Apart from a few exceptions, such as the work of Helm et al,[15,16,19] most of the studies were based on subjects with atopic dermatitis whose food-related symptoms were not confirmed by DBPCFC. Moreover, IgE reactivity of the A5B3 glycinin was shown in children with cow's milk allergy but without confirmed soybean allergy.[20] Finally, the putative soybean allergen 2S albumin[13] showed no IgE-binding in 23 European subjects with soybean allergy.[21] Interestingly, only the soybean hull proteins Gly m 1 and Gly m 2 that are known as inhalant allergens in occupational or environmental soybean allergy as well as the birch pollen–related allergens Gly m 3 and Gly m 4 have been accepted officially as soybean allergens by the International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-Committee (http://www.allergen.org/Allergen.aspx). Unlike the legume peanut with at least 8 identified and officially accepted allergens, there is still a lack of knowledge on soybean allergens. The identification of relevant soybean allergens

From [a]the Division of Allergology, Paul-Ehrlich-Institut, Langen; [b]the Allergy Unit, Department of Dermatology, University Hospital, Zurich; [c]the Allergy Center, Odense University Hospital; [d]the Allergology and Clinical Immunology Unit, Niguarda Cá Granda Hospital, Department of Medicine, Milan; [e]the National Council of Research, Istituto di Science delle Produzioni Alimentari; [f]the Research Institute for Food Science, Kyoto University; and [g]the Allergy Clinic, National University Hospital, Copenhagen.

Supported by the 5th Framework Programme: Quality of Life and Management of Living Resources of the European Commission, QLK4-CT-2001-00301, and by the Swiss Federal Office for Education and Science, BBW01.0159-1.

Disclosure of potential conflict of interest: C. Bindslev-Jensen has served as an expert witness in a patient antihistamine injury case. L. K. Poulsen has served as an expert witness for Novozymes Ltd. S. Vieths is an associate at the Institute for Product Quality Berlin; has received honoraria from Phadia and the Food Allergy Resource and Research Program; has received research support from the European Union, the Germany Research Society, the Research Fund of the German Food Industry, Monsanto Co, and the European Directorate for the Quality of Medicine and Health Care; and has served as a member of the European Academy of Allergy and Clinical Immunology, the International Union of Immunological Diseases, the European Agency for the Evaluation of Medicinal Products, the European Pharmacopoeia Commission, the International Life Sciences Institute Health and Environmental Science Institute, the European Committee for Standardization, and Deutsche Gesellschaft fur Allergie und Klinische Immunologie. The rest of the authors have declared that they have no conflict of interest.

Received for publication June 4, 2008; revised September 10, 2008; accepted for publication September 19, 2008.

Available online November 11, 2008.

Reprint requests: Thomas Holzhauser, PhD, Division of Allergology, Paul-Ehrlich-Institut, Paul-Ehrlich-Strasse 51-59, D-63225 Langen, Germany. E-mail: holth@pei.de.

0091-6749/$36.00

doi:10.1016/j.jaci.2008.09.034

Abbreviations used
AP: Alkaline phosphatase
BC: β-Conglycinin, Gly m 5
DBPCFC: Double-blind placebo-controlled food challenge
G: Glycinin, Gly m 6
IUIS: International Union of Immunological Societies
OAS: Oral allergy syndrome
OR: Odds ratio
pfu: Plaque-forming unit

therefore remains an important task to achieve better understanding of the molecular mechanisms of soybean allergy with special regard to diagnosis, risk management, and future therapy. As an important step into this direction, we performed the identification of 2 soybean allergens, Gly m 5 (β-conglycinin) and Gly m 6 (glycinin), using serum samples of 30 subjects with soybean allergy.[3] The 2 soybean major storage proteins, β-conglycinin and glycinin, are 7 S and 11 S globulins and account for about 30% and 40% of the total seed proteins, respectively. In the vacuole, the mature form of β-conglycinin is accumulated into the densely packed state as trimers that are composed of 3 subunits, α (~67 kd), α′ (~71 kd), and β (~50 kd).[22] Glycinin is synthesized in the seeds during embryogenesis. It is a hexameric protein that is assembled by 5 different subunits, G1 (A1aB1b, 53.6 kd), G2 (A2B1a, 52.4 kd), G3 (A1bB2, 52.2 kd), G4 (A5A4B3, 61.2 kd), and G5 (A3B4, 55.4 kd). Each features at least 1 acidic (A) and basic (B) peptide chain that are linked by a disulfide bond.[23] Finally, the IgE reactivities to this complete panel of 8 subunits of the major storage proteins were correlated to the clinical characteristics of our patient group to investigate the potential use as biomarkers for severe allergic reactions to soybean.

METHODS

Patients

The patients were recruited in 3 European allergy centers—Zurich (Switzerland), Odense (Denmark), and Milan (Italy)—according to protocols approved by the Ethical Committees of the respective centers. The primary inclusion criterion was a positive food challenge (DBPCFC) with soy or a convincing history of anaphylactic reactions to soy. The clinical details of the study subjects have been published recently.[3] Challenges were discontinued after the dose leading to objective allergic symptoms or ingestion of the whole meal of 50 g soy (~26.5 mg soy protein). The lowest observed adverse effect levels for subjective and for objective allergic reactions were 5.3 mg and 240.6 mg of soy protein, respectively. The most relevant data necessary for interpretation of the *in vitro* results obtained in this study are summarized in Table I. To set uniform and comparable criteria for the depicted most severe symptom, symptoms were recorded as experienced under challenge. In addition, a convincing history of anaphylaxis, evaluated by a standardized case report form, was accepted. The severity of symptoms on soy ingestion was graded as follows: (1) mild: symptoms such as oral allergy syndrome (OAS), tightness of the throat, nausea, gastrointestinal pain, or dyspnea without monitored drop in FEV_1; (2) moderate: symptoms such as rhinitis, flush, urticaria, angioedema, diarrhea, or emesis; and (3) severe: drop of blood pressure, or life-threatening laryngeal edema.

SDS-PAGE and IgE immunoblot analysis

SDS-PAGE was performed under reducing conditions, and IgE immunoblot analysis was performed as recently described.[3] Soybean extract was subjected to SDS-PAGE at 20 μg/cm gel, purified natural BC was analyzed at 7.5 μg/cm with 2.5 μg/cm of each subunit (α, α′, and β), and recombinant proglycinin was analyzed at 10 μg/cm with each subunit (A1aB1b, A1bB2, A2B1a, A3B4, and A5A4B3) at 2 μg/cm. IgE reactivities were detected with horseradish peroxidase on polyclonal anti-IgE and visualized by chemiluminescence on x-ray–sensitive film. Alternatively, alkaline phosphatase (AP)–conjugated monoclonal anti-IgE (Pharmingen, San Diego, Calif; dilution 1:1000, 4 hours) and colorimetric staining with an AP-substrate solution (Bio-Rad Laboratories, Hercules, Calif) were applied. Silver staining of gels was performed according to Heukeshoven and Dernick.[24]

Establishment of a soybean cDNA expression library

Total RNA was isolated from freeze-dried almost ripened but slightly green soybean seeds as previously described by Hoff et al.[25] Five micrograms of poly(A) mRNA were purified from the isolated total RNA fraction by the Oligotex mRNA Midi Kit (Qiagen, Hilden, Germany). After reverse transcription and addition of *Eco*R I/*Hind* III linker arms, the cDNA was size-fractionated (Mini Column Fractionation Kit, Novagen, Madison, Wis) with fragments of as many as 3000 bases and a major portion of 1000 to 2000 bases in length. A cDNA expression library was established by using the λSCREEN-1 system (Novagen) according to the manufacturer's instructions (technical bulletin TB119 02/99). The most prominent cDNA fraction of approximately 300 to 2000 bases in length was directionally ligated into λSCREEN vector and *in vitro* packed. The primary library had a titer of 4.5×10^5 plaque-forming units per milliliter (pfu/mL; total library size, 2.25×10^5 pfu) and was amplified to 4×10^9 pfu/mL.

IgE screening of the soybean cDNA expression library

For immunoscreening, 2×10^4 pfu was plated on 90-mm plates, incubated at 37°C until plaques appeared (5-7 hours), and overlaid with nitrocellulose filters (Hybond C; GE Healthcare, Munich, Germany; 4°C; overnight). The nitrocellulose plate lifts were blocked in 50 mmol/L TRIS-buffered saline (pH 7.4) containing 1% gelatin and 0.1% Tween 20 and incubated with patient serum no. 28 (diluted 1:10, overnight). The bound IgE was visualized with anti–IgE-AP as described. Nonspecific binding was reduced by serum preincubation with equal volumes of suspension and lysate of BL21(DE3)pLysE-plating cells. IgE-reactive phage recombinants were screened by PCR with SP6 promoter and T7 terminator primers. The purified PCR products were sequenced.

Purification of natural BC subunits

Soybean extract was prepared in 10 mmol/L PBS (pH 7.4) as recently described.[3] Natural α, α′, and β subunits of BC were purified from soybean extract by preparative SDS-PAGE (model 491 Prep-Cell; Bio-Rad Laboratories, Munich, Germany) as described by Reese et al.[26] As much as 50 mg total protein from soybean extract was separated in the 28-mm internal diameter column with a 25-mm-high stacking gel (5% total acrylamide concentration; 1.5% cross-linker acrylamide concentration) and a 65-mm-high separation gel (7,5% T, 1.5% C). Fractions containing the purified single subunits were identified by IgE Western blotting, pooled, and dialyzed against 3-(N-morpholino)-propanesulfonic acid buffer (20 mmol/L 3-[N-morpholino]-propanesulfonic acid, 8 mmol/L sodium acetate, 1 mmol/L 2-[bis (carboxymethyl) amino] acetic acid, pH 7.0). The purity of the isolated BC subunits was checked by SDS-PAGE and silver staining, and purity and identity were verified by N-terminal sequencing and liquid chromatography tandem mass spectrometry.

N-terminal sequencing and LC-MS/MS

N-terminal sequencing analyses were performed on a Procise 492 protein sequencer (Applied Biosystems, Monza, Italy) after protein adsorption on ProSorb PVDF membranes (Applied Biosystems). For LC-MS/MS analyses, the spots cut from the gel were destained and the proteins trypsinized as described by Hellmann et al.[27] The tryptic mixtures were analyzed by LC-MS/MS as previously described.[28]