DOI 10.1002/mnfr.200700394 Mol. Nutr. Food Res. 2008, *52*, 1486 – 1496

Research Article

Soybean allergen detection methods – A comparison study

Mona H. Pedersen[1,2], Thomas Holzhauser[3], Caroline Bisson[4], Amedeo Conti[4], Louise B. Jensen[1], Per S. Skov[1], Carsten Bindslev-Jensen[5], Ditte S. Brinch[2] and Lars K. Poulsen[1]

[1] Laboratory of Medical Allergology, Copenhagen University Hospital, Denmark
[2] Novozymes A/S, Bagsvaerd, Denmark
[3] Division of Allergology, Paul-Ehrlich-Institut, Langen, Germany
[4] CNR-ISPA, Torino, Italy
[5] Allergy Center, Odense University Hospital, Denmark

Soybean containing products are widely consumed, thus reliable methods for detection of soy in foods are needed in order to make appropriate risk assessment studies to adequately protect soy allergic patients. Six methods were compared using eight food products with a declared content of soy: a direct sandwich ELISA based on polyclonal rabbit antibody (ab) to raw soy flakes, a commercial and an in-house competitive ELISA both based on ab to denatured, 'renatured' soy protein, an enzyme-allergosorbent test (EAST) inhibition based on two sera from soy allergic patients, histamine release (HR) using basophils passively sensitized with patient serum and a PCR method detecting soy DNA. Eight food products were selected as model foods to test the performance of the methods. There was an overall good agreement between the methods in terms of ranks of soy content but not the quantity. The sandwich ELISA aimed at native soy proteins had the lowest detection limit of 0.05 ppm, but only identified soy in 5/8 products, and generally in lower amounts compared to other methods. The competitive ELISA had a higher detection limit of 21 ppm, but seemed more successful in detecting processed soy. Only HR, EAST inhibition and PCR detected soy in all eight products. In spite of a general good correlation in terms of ranks of soy content, more than a single method may be necessary to confirm the presence of soy in foods.

Keywords: Allergen detection method / EAST / ELISA / Histamine release / PCR

Received: September 30, 2007; revised: January 17, 2008; accepted: February 1, 2008

1 Introduction

Avoidance diet is the only recommendation soy- and other food allergic patients can rely upon to avoid an allergic reaction. However, strict avoidance is highly dependent on meticulous risk assessments and risk management, *e.g.* labelling of food products. On the risk management side new food labelling legislations has recently come into effect in both the United States (Food Allergen Labelling & Consumer Protection Act (FALCPA)) and in the European Union (Directive 2003/89/EC of the European Parliament and of the Council amending Directive 2000/13/EC). These new rules demand the labelling of foods with ingredients from a defined list of the most common allergenic foods including soybean. Food authorities in the US and EU have not assigned a lower level of soy protein in the foods to be labelled, but are generally concerned with soy protein intentionally added as an ingredient only and not for instance soy allergen present as a result of a crosscontact contamination. Ideally, risk management should be based on risk assessments of food products suspected of containing allergenic protein no matter of the mechanism (ingredient or contamination) by which it is incorporated into the products.

Risk assessments can be made from looking at a combination of potential *exposure* and potential *effect*. The effect can be determined from clinical threshold studies from which dose–response relations are determined. Lately, a large multicentre study by Ballmer-Weber *et al.* 2007 [1] has made the

Correspondence: Professor Lars K. Poulsen, Allergy Clinic, 7551, National University Hospital, Blegdamsvej 9, 2100 Copenhagen Ø, Denmark
E-mail: lkpallgy@mail.dk
Fax: +45-35-45-75-81

Abbreviations: ab, antibody; **EAST,** enzyme-allergosorbent test; **HR,** histamine release; **HRP,** horse radish peroxidase; **LOAEL,** lowest observed adverse effect level; **NHS,** nonatopic healthy subjects; **OPD,** *o*-phenylenediamine; **SF-DR,** soy flakes denatured and 'renatured'; **SF-PBS,** soy flakes extracted with PBS; **TBS,** tris buffered saline

attempt to establish the clinical threshold dose for soybean in soybean allergic patients. The study included 30 patients between 1 and 69 years of age (mean 26.4). The no observed adverse effect level found in this study was 2 mg soy flour. For practical reasons the exposure is the concentration of allergen in a given product times the expected amount consumed of the particular product in a normal size meal. This can be a preknown amount of added soy protein as an ingredient or a potential crosscontamination.

A few detection methods for soybean allergen have been developed and published [2–4]. For some of these the detection limits are too high for the use in allergy risk analysis. Other methods determine single proteins and may not be representative of soy allergens as a whole, since different forms of processing may influence components differently. Koppelman *et al.* [5] reported the development of a soybean specific ELISA based on antibodies to an extract of whole soybean using an extraction buffer with very high pH. The ELISA had a detection limit of 4 ppm soybean, which should be sufficient to protect soy allergic individuals according to the authors.

There are few studies [6] comparing fundamentally different soy detection methods applied to a varied selection of soy-containing products. Since risk assessments are highly dependent on reliable detection methods, we aimed at comparing different approaches to identify soybean in foods with different levels of soy content and processing. We set up six soy detection assays and tested all six assays on eight food products bought at a local super market that were all labelled with 'soybean' or 'soybean protein' in the ingredients list. The detection methods were: (i) sandwich ELISA based on polyclonal rabbit antibodies to an extract of soy flake, (ii) commercial ELISA kit based on ab to denatured refolded protein, (iii) in-house competitive ELISA based on commercially available rabbit antisoy ab also raised to denatured refolded protein, (iv) enzyme-allergosorbent tests (EAST) inhibition based on sera from soy allergic patients, (v) histamine release (HR) using passively sensitized basophils with IgE from a soy allergic patient and finally (vi) a PCR method that detects soybean DNA.

2 Materials and methods

2.1 Soy standard material

Two standard extracts of soy protein were made for this study using the same raw material but different extraction procedures. The first extract (soy flakes extracted with PBS; SF-PBS) containing predominantly native soy proteins were extracted from defatted soy white flakes (Central Soya European Proteins A/S, Aarhus, Denmark) at a ratio of 1:20 w/v in PBS at pH 7.4 and 4°C over night. After centrifugation at $20\,000 \times g$ for approximately 15 min, the supernatant was filtered through a 1.2 μm cellulose acetate filter (Schleicher and Schuell, Germany). The protein content was determined to 7.8 mg/mL by amino acid analysis at the Technical University of Denmark (DTU), and kept in aliquots at −20°C.

A second extract (soy flakes denatured and 'renatured'; SF-DR) containing predominantly heat-denatured soy protein was made from the same soy white flakes using a 'denaturing/renaturing' procedure. This extraction procedure was inspired by the recommendations of a commercial soy detection kit (Tepnel BioSystems). Soy white flakes were homogenized using a Yellow Line DI 18 Basic homogenizer (IKA, Staufen, Germany) in 0.05 M tris-(hydroxymethyl)aminomethane (Tris)-HCl at pH 8.6 and room temperature at a ratio of 1:5 w/w. The homogenate was then diluted with a denaturing buffer containing 13 M urea, 20 mM DTT and 0.05 M Tris-HCl at a ratio of 1:4 v/v and incubated for 1 h at 100°C in a shaking water bath. The soy proteins were subsequently renatured by diluting 1:10 v/v with a renaturing buffer containing 55 mM NaCl, 7.4 mM L-cystine and 20 mM NaOH at pH 9.0 and at 50°C. The extract was allowed to cool to room temperature, before it was filtered and stored at −20°C. Due to the high molar urea buffer, SF-DR could not be analysed by amino acid analysis but was expected to have a protein content of approx. 2.5 mg/mL assuming 100% soy flake protein extractability.

2.2 Food samples

A selection of legumes, tree nuts and cereals were extracted for specificity testing of the soy ELISAs. Raw materials were extracted by the SF-DR method or extracted 1:20 w/v in a buffer composed of 8 mM Tris, 25 mM *N*-[tris(hydroxymethyl)methyl]glycine (Tricine) and 2 mM calcium lactate at pH 8.6. Samples were blended in the extraction buffer with a kitchen rod blender (Braun, Germany), and further homogenized using the Yellow Line DI 18 Basic homogenizer. The homogenates were extracted at room temperature at constant stirring for 3 h, and centrifuged twice at $1300 \times g$ for 10 min, and twice at $18\,000 \times g$ for 10 min for further clarification. Eight food products were selected as model foods to test the performance of six soy specific detection methods. The products included soy milk, tofu, dairy free ice cream (with tofu), Chinese spring rolls and a selection of meat products. All were from a local grocery store and had either soybean (products 1, 2 and 8) or 'soy protein' (products 3, 4, 5 and 7) declared as an ingredient. One product had soybean declared as a subingredient under the ingredient 'Chinese soy' (product 6). The eight products were extracted by the two methods described above for the soy standard extracts, SF-PBS and SF-DR.

2.3 Polyclonal antibodies to PBS-extracted soy flakes

Polyclonal antibodies to SF-PBS was made by immunizing a F1-generation of rabbits, where the parent generation had

been kept on a soybean free and peanut free diet from mating and the offspring all life. The rabbits were immunized subcutaneously once with SF-PBS (0.31 mg protein) along with Titermax® adjuvant (TiterMax USA, Los Angeles, CA, USA), and boosted once the same way after 30 days with an equal amount of protein but this time without the adjuvant. Blood samples were drawn at weeks 8, 10 and 12 after the first antigen injection and serum fractions were pooled. The IgG fraction was isolated on a protein G column according to instructions from the manufacturer (Amersham Biosciences, Uppsala, Sweden), and a portion of the rabbit antibodies to native soy was biotinylated for later use in ELISA using *N*-hydroxysuccinimido-biotin also according to the instructions of the supplier (Sigma, St. Louis, MO, USA).

2.4 Human serum samples

Serum was obtained from one patient (serum no. 1) from the Allergy Clinic of the National University Hospital of Copenhagen, Denmark. Another (serum no. 2) came from the serum collection of the Paul Ehrlich Institute, Langen, Germany. Inclusion criteria in this study were in both cases a convincing history of anaphylactic reactions after ingestion of soybean. Specific IgE was quantified in both sera by the Immunocap® system (Phadia, Uppsala, Sweden); serum no. 1 had more than 100 kU/L to soybean and 2.94 kU/L to peanut, and serum no. 2 had 52.5 kU/L to soybean and more than a 100 kU/L to peanut. Both sera tested negative for birch pollen specific IgE. More sera were screened in the HR and EAST methods (described further down), but the signals obtained were insufficient to use in these assays.

2.5 Test of rabbit antibodies for allergen reactivity

A Maxisorp™ microtitre plate (Nunc, Roskilde, Denmark) was coated over night at 4°C with 100 µL goat antihuman IgE (Chemicon International, Temecula, CA, USA) diluted to 1 µg/mL in PBS. Microtitre plates were washed three times with PBS containing 0.1% Tween-20 (PBS-T) between all incubation steps unless otherwise stated. The wells were blocked for 1 h with PBS-T. Serum no. 1 was diluted 1:50 in PBS-T and 100 µL incubated in each well for 2 h at 37°C. A serum pool from nonatopic healthy subjects (NHS, $n = 120$) was included as negative control. The plate was incubated with SF-PBS diluted to 1 µg/mL in PBS-T for 2 h at 37°C. Serial dilutions were made in parallel of the in-house rabbit antibodies to native soy protein, and a rabbit antibody (ab) raised to renatured soy, that had been treated with hot 'urea solution' according to the manufacturer (Sigma), and 100 µL of each dilution were added to the plate wells to incubate over night at 4°C. Purified IgG from a naïve rabbit was included as negative control. Horse radish peroxidase (HRP) labelled goat antirabbit IgG (Santa Cruz Biotechnologies, Santa Cruz, CA, USA) was diluted to 40 ng/mL in PBS-T and 100 µL was added to each well to incubate for another 2 h at 37°C. The plates were developed by incubating with 100 µL/well *o*-phenylenediamine (OPD)-substrate, and the colour reaction stopped by adding 150 µL/well 0.5 M sulphuric acid. Optical densities (OD) were read at 490 nm (reference 630 nm) using a microplate-reader (El_x808_{IU}, Ultra Microplate reader, Biotek Instruments, Vinooski, VT, USA). All experiments were performed in duplicate.

2.6 Characterization of rabbit antibodies and human serum IgE by Western blotting

SDS-PAGE and Western blotting were performed according to the methods of Laemmli [7] and Hansen *et al*. [8]. Briefly, soy flake proteins (SF-PBS) were electrophoresed in a 1 mm acrylamide gel with a 16% separation gel and a 3.6% stacking gel. The samples were initially reduced by 5 min of boiling in a reducing buffer containing mercaptoethanol and SDS. One hundred and twenty microlitres of soy protein extract with a total protein concentration of 10 µg/mL was added to a 46 mm long well. Rainbow marker (High range, Amersham Biosciences, Buckinghamshire, UK) was added to one well of the SDS-PAGE gel. The proteins were blotted on to a PVDF membrane (Millipore, Bedford, MA, USA) for 1.5 h ($I = 1.0$ mA/cm^2). The membrane was then blocked with tris buffered saline (TBS) pH 7.6 containing 5.0% BSA for 2 h at room temperature, and cut into strips before incubating over night at 4°C with 2 µg/mL rabbit IgG (in-house ab to native soy protein and commercial ab to renatured soy protein (Sigma)) or human serum samples (serum no. 1 and 2) diluted 1:10 in TBS with 0.5% BSA. After another wash the strips were incubated with either goat antirabbit IgG labelled with HRP (Santa Cruz Biotechnologies) diluted 1:120 000 or rabbit antihuman IgE labelled with HRP (Dako, Glostrup, Denmark) diluted 1:20 000 in PBS with 0.5% BSA. ECL Western blotting detection reagent (Amersham, Little Chalfont, Buckinghamshire, UK) was used as a substrate for HRP. Hyperfilms (Amersham) were then exposed to the blots at various times to visualize ab binding.

2.7 Characterization of rabbit antibodies to soy flake by 2-D Western blotting

Fifty micrograms of lyophilized extract of native soy proteins (SF-PBS) was resuspended in 125 µL of Destreak Hydration Solution (Amersham Biosciences) and focused by using 7 cm ReadyStrip (Amersham), pH 3 – 11 NL, on a Protean IEF Cell (BioRad Laboratories, Hercules, CA, USA). The voltage was increased, following a rapid linear slope to 8000 V, focusing was continued until 10 000 V · h were reached. Prior to SDS-PAGE, the strips were equilibrated for 15 min each in NuPAGE sample buffer (Invitrogen, Carlsbad, CA, USA) containing solution I: 50 mM

DTT and solution II: 65 mM iodoacetamide. SDS-PAGE was performed by using NuPAGE Gradient ZOOM gels 4–12% (Invitrogen), using MOPS-SDS running buffer at 200 V. Proteins were either stained with Coomassie Colloidal Blue or transferred to 0.2 μm nitrocellulose membranes (Sigma) on a semiwet blot module (Invitrogen) according to the manufacturer's instructions. Membranes were blocked with block buffer (TBS pH 7.4 containing 0.3% Tween-20) and incubated 2 h and 30 min with rabbit antinative soy antiserum, diluted 1:1500 in incubation buffer (TBS, 0.03% Tween-20, 0.1% BSA). Bound IgG antibodies were detected by incubating the membrane with an alkaline phosphatase conjugated monoclonal mouse antirabbit gamma chain ab (Clone RG 96, Sigma) diluted 1:2500 in incubation buffer for 1 h. Visualization was done using BCIP/NBT solution (BioRad).

Protein spots that were found being IgE reactive in previous immunoblotting experiments were excised from 2-D maps, in-gel digested with trypsin (modified porcine trypsin, sequencing grade; Promega, Madison, WI, USA) as described elsewhere [9] and subjected to MALDI-TOF analysis on a Bruker Reflex III MALDI-TOF spectrometer (Bremen, Germany). Protein identification was done using the following on-line databases: ProFound (http://prowl.rockefeller.edu/profound_bin/WebProFound.exe) and MASCOT (http://www.matrixscience.com).

2.8 Soy detection by direct sandwich ELISA

Microtitre plates (Nunc) were coated over night at 4°C with 100 μL/well rabbit IgG to native soy diluted to 1.3 μg/mL in PBS. After three cycles of washing with PBS-T, the wells were blocked for 1 h with PBS-T with 0.1% gelatine and washed again. Samples and standard (SF-PBS) were diluted in PBS-T containing 30% of a wheat flour extract (flour extracted 1:10 w/v in PBS-T for 30 min at room temperature), and 100 μL were added to the wells of the plate and incubated for 2 h at 37°C. Biotinylated rabbit antinative soy was diluted to 1.6 μg/mL in an undiluted wheat flour extract and 1% normal rabbit serum (Gibco, Invitrogen) was added. This solution was added to the plates after they had been washed, 100 μL to each well to incubate for another 2 h at 37°C. Finally, the plates were incubated with enzyme-labelled avidin (Sigma) for 30 min, and developed with OPD-substrate as described above. The lower LOD for the standard curve was defined as blank plus three times the SD obtained from the blank double determination ($OD_{blank} + 3 \times \sigma_{OD\text{-}blank}$).

2.9 Soy detection by a commercial ELISA kit

A commercially available competitive soy specific ELISA (Soya Protein Assay Kit, Tepnel Biosystems, Stamford, CT, USA) was used as instructed by the manufacturer, which involved extracting all samples by the denaturing/renaturing procedure as described above for the SF-DR extract. The kit is designed for soy detection in meat products in the low percentage range as a means of quality control (Kit LOD = 700 ppm protein or 1400 ppm whole soy flake).

2.10 Soy detection by competitive ELISA

A competitive ELISA was established using the denatured/renatured (SF-DR) extract as solid phase protein and soy protein standard, and a rabbit ab to renatured-soy as primary ab (Sigma). Microtitre plates (Nunc) were coated over night at 4°C with 100 μL SF-DR diluted in PBS to 3 μg/mL soy protein. After three cycles of washing with PBS-T, the wells were blocked for 1 h with PBS-T and washed again. Several dilutions of standards and samples along with a negative control were preincubated over night at 4°C with 5 μg/mL denatured-soy specific rabbit IgG. One hundred microlitres of each of the preincubated samples and standards were added to the microtitre plate wells and incubated at 37 C for 2 h followed by three cycles of washing. Finally, the plates were incubated with 100 μL/well HRP-labelled goat antirabbit IgG (Santa Cruz Biotechnologies) and developed as described before.

2.11 Soy detection by EAST inhibition

EAST was performed by coating microtitre plates over night at 4°C with 100 μL/well of 2 μg/mL SF-PBS diluted in PBS. Plates were washed three times in between all incubation steps with PBS-T. The coated wells were blocked with PBS-T including 2% v/v rabbit serum for 1 h at room temperature on an orbital shaker. Appropriate dilutions of samples and the standard soy extract (SF-PBS) were mixed with serum nos. 1 or 2 in PBS-T with 2% normal rabbit serum and preincubated over night at 4°C on an orbital shaker. One hundred microlitres of the preincubated samples and standards were added in duplicate to the blocked microtitre plates and incubated for 3 h at 37°C. Finally, the plates were incubated with HRP-labelled rabbit antihuman IgE (Dako) diluted in PBS-T with 2% rabbit serum at a concentration of 1.3 μg/mL IgG at 37°C for 2 h, followed by incubation with OPD-substrate. Finally, the plates were developed and read as described before. Standard inhibition curves were made using dilutions of SF-PBS, and an inhibition between 15 and 85% was considered positive.

2.12 Soy detection by basophil HR

Human basophils were obtained from buffy coats from the hospital blood bank. The donor cells were screened for HR activity to a mix of selected pollen allergen extracts and a mix of food allergen extracts, and only included when negative to the most common allergenic foods including soybean and peanut. The histamine releasing ability of donor cells was confirmed by incubation with rabbit antihuman

IgE. The leukocyte fraction in the buffy coat was purified using Lymphoprep™. The native IgE was stripped from the surface of the donor basophils and they were subsequently sensitized with undiluted human serum as described by Skov [10]. A negative control was included using basophils sensitized with NHS. Human IL-3 was added to the passively sensitized basophils, before the cells were incubated with various dilutions of soy standard extract (SF-PBS) or sample extracts to induce HR. The amount of released histamine from the cells was quantified using the glass microfibre method (RefLab, Copenhagen, Denmark), and the amount of released histamine was expressed as a percentage of total histamine content of nonchallenged cells. A result was considered positive if the relative HR exceeded 15%, as the negative control cells released between minus 10% and plus 14% histamine.

2.13 Soy detection by real-time PCR

Deoxyribonucleic acid (DNA) extraction and purification was done with a commercial kit (SureFood Prep Plant X, Congen Biotechnologie, Berlin, Germany) according to the manufacturer's instructions. Each sample was extracted twice. DNA from soybean was extracted and serially diluted and used as a positive control. Soybean specific primers (forward: 5′-tcc acc ccc atc cac att t-3′; reverse: 5′-ggc ata gaa ggt gaa gtt gaa gga-3′) and a fluorogenic probe (5′-aac cgg tag cgt tgc cag ctt cg 3′, modifications: 5′-6-FAM, 3′-TAMRA) for sequence verification of the amplicon were identical to that described for the soybean lectin reference gene according to the official Swiss method for the detection of genetically modified Roundup Ready Soybean (Method 2.1.7, Chapter 52B, Schweizerisches Lebensmittelbuch SLMB, July 2000). PCR was performed in 200 µL PCR tubes with 50 µL mastermix containing 5 µL of DNA from either sample or soybean standard. The soy-specific master mix consisted of 1X GeneAmp® 10X PCR Gold Buffer (Applied Biosystems, Darmstadt, Germany), each 200 µM dATP, dCTP, and dGTP, 400 µM dUTP, 5 mM $MgCl_2$ (Applied Biosystems), 300 nM of each Primer, 200 nM of the amplicon-specific probe, 0.5 U of AmpErase® Uracil *N*-glycosylase (Applied Biosystems), and 1.25 U of Platinum® Taq DNA Polymerase (Invitrogen, Karlsruhe, Germany). For each sample, two independent DNA extracts were analysed. Additionally, from each sample, one DNA extract was spiked with soybean DNA (diluted 1:1000 in ddH_2O) as a control for PCR inhibitors. In parallel, a tenfold serial dilution of soybean DNA ($1-10^{-5}$) was used for generation of an external standard curve for semiquantitative evaluation of the amount of amplifiable soybean DNA in the study samples. Purity of the soy-specific master mix was checked with two PCR reactions consisting only of the PCR master mix (without DNA). The PCR was performed in a real-time PCR cycler (Stratagene Mx 3005P) according to the following reaction conditions: 2 min at 50°C following 1 min at 95°C with subsequent 40 cycles of each 30 s at 95°C and 30 s at 60°C. Fluorescence readings for FAM were recorded at the end of each cycle. Samples were defined as positive, if both DNA extracts were positive. Negative results were true negatives if no PCR inhibition occurred in the corresponding inhibition control.

3 Results

3.1 Detection of native soy proteins by direct sandwich ELISA

Our initial aim was to develop an assay for the detection of native saline-soluble soy proteins as native allergens were thought to be most relevant in terms of IgE-recognition and thus possible clinical reactions. Rabbit antibodies were raised against an extract of soy flakes (SF-PBS) in rabbits that had been kept on a soybean free and peanut free diet in two generations in order to avoid oral tolerance. A direct sandwich ELISA was established using SF-PBS as soy protein standard. It was possible to extract about 32% of the total protein content of soy flakes with PBS, assuming that soy flakes contain about 50% protein. The direct sandwich ELISA had a detection range between 0.4 and 300 ng/mL soy protein. The calculated LOD in a given food sample using this assay was 0.05 ppm whole soy flake taken into account a 1:20 dilution factor during food sample extraction, and a protein extractability of 32%. The sandwich ELISA was validated for specificity using extracts of a selection of legumes, tree nuts and cereals commonly used in chocolates and baked goods. The assay was found highly specific with crossreactivity reactions only in the low ppm range, besides a positive signal seen in relation to an extract of corn flour corresponding to a 0.17% (1677 ppm) whole soy flake reactivity (Table 1). Because of the high sensitivity and little crossreactivity, the direct sandwich ELISA was deemed as potentially useful and the rabbit ab was further characterized.

The rabbit ab raised to SF-PBS and the commercial rabbit ab to denatured/renatured soy protein was compared in terms of their reactivity to IgE-binding proteins. Serum IgE from a highly soy allergic individual (serum no. 1) was incubated with SF-PBS followed by incubation with serial dilutions of the two rabbit antibodies. The rabbit ab to native soy demonstrated at least a 50-fold higher titre than the rabbit ab to denatured/renatured soy protein (Fig. 1).

The rabbit antibodies raised against the saline extract of soy flakes, SF-PBS, were further investigated for their soy protein reaction patterns in 2-D Western blotting technique. The whole protein map of SF-PBS was stained with Coomassie colloidal blue in one gel (Fig. 2a). Simultaneously, SF-PBS was separated in another 2D-gel and subsequently immunostained with the rabbit antiserum against SF-PBS after semidry blotting onto a nitrocellulose membrane (Fig. 2b). Specific immunoreactivities of the ab were assigned to

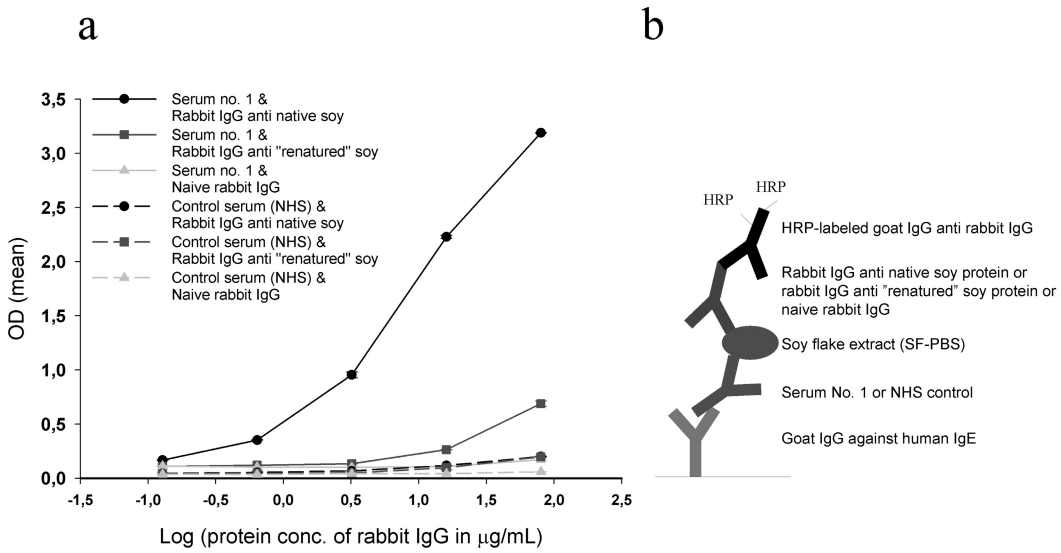

Figure 1. (a) Reactivity of two different rabbit IgG antibodies to native soy allergens. A rabbit ab raised to native soy proteins (circles, full line) and a rabbit ab raised to renatured soy protein (squares, full line) was compared in an ELISA set-up where native soy allergens where caught using serum from a soy allergic individual (serum no. 1). A serum pool of NHS was used as negative control. IgG purified from a naive rabbit was included as negative control for the rabbit antibodies. (b) Assay set-up.

Table 1. Specificity study on soy ELISAs

Raw material	Crossreactivity (ppm)	
	Sandwich ELISA	Competitive ELISA
Peanut	3.2	<21
White beans	Not quantified	<21
Green pea	<0.05	<21
Red lentils	1.1	<21
Chick pea	2.9	<21
Kidney bean	Not quantified	<21
Almond	0.5	<21
Hazel nut	<0.05	<21
Walnut	1.1	1400
Sesame	<0.05	<21
Wheat	2.7	<21
Barley	4.2	<21
Oat	0.6	<21
Rice	2.4	<21
Rye	4.4	<21
Corn flour	1677	N.D.

The sandwich ELISA is based on antibodies to saline extractable soy proteins (SF-PBS), and the in-house competitive ELISA is based on rabbit antibodies to renatured soy protein. 'Not quantified' means, that the read-outs from the dilutions of the product were not parallel with the read-outs from the dilutions of soy extract. N.D. means 'Not Done'. The results are expressed in ppm whole soy flake reactivity.

the SF-PBS soy flake map and the corresponding soy proteins excised from the stained gel (Fig. 2a), trypsin digested, and analysed in MALDI-MS. Peptide mass fingerprints were aligned with data base entries for identification of soybean-allergens. The rabbit antibodies showed specificity for all three subunits of the soybean major storage protein β-conglycinin. Also, the basic subunits of the major storage protein glycinin was detectable, however not its acidic subunits. Nonetheless, both major storage proteins from soybean, which account for more than 70% of total soybean protein (Besler, M., Helm, R. M., Ogawa, T., Allergen Data Collection Update: Soybean (Glycine max L.) – Internet Symposium on Food allergens, Volume 2, Supplement 3 (2000); http://www.food-allergens.de/symposium-vol1(2)/data/soy/soy-composition.htm.) were detected by the specific rabbit antibodies. Moreover, soybean Kunitz trypsin inhibitor, Gly m Bd 30 k, a sucrose binding protein, and agglutinin were detected.

The rabbit ab to SF-PBS was furthermore compared to the commercial rabbit ab to renatured proteins in a standard Western blot using SF-PBS for protein SDS-PAGE separation (Fig. 3). Both rabbit antibodies had multiple reactivities to many bands, especially in the higher molecular weight (MW) area above 40 – 100 kDa, but also smaller proteins at 38, 33 and 28 kDa. The rabbit ab to native soy furthermore had a strong recognition of a protein at 19 kDa, which may be the Kunitz trypsin inhibitor, whereas the ab to renatured soy had weak recognition of proteins at 23 and 20 kDa. The two sera from soy allergic individuals were also included in the blot to study reactivity patterns to the extract of raw soy flakes (Fig. 3). Both sera had IgE to at least five different proteins, but the patterns were not the same. Patient no. 1 reacted most strongly to proteins at 66 and 19 kDa and had weaker bands at 42, 33, and 28 kDa. Patient no. 2 had strong bands at 66, 42 and 33 kDa and weak reactivities to bands at 38 and 28 kDa. Unfortunately, some unspecific binding in the area 46 – 57 kDa (lane 6, Fig. 3) occurred, so no conclusion could be drawn from this region.

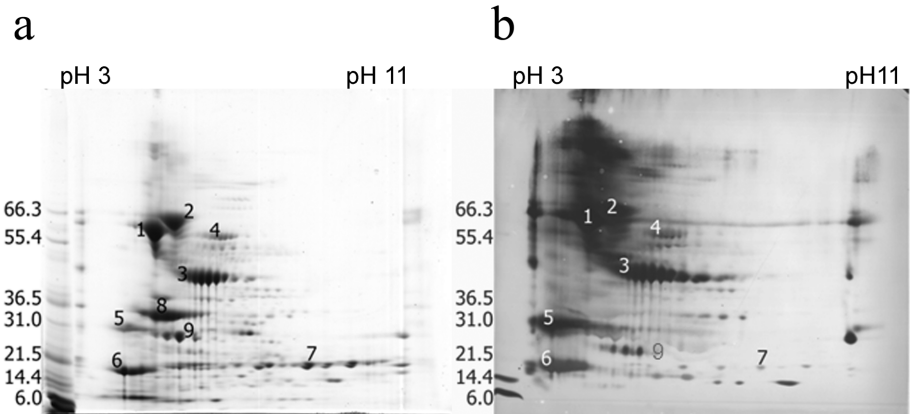

Figure 2. (a) 2-DE of saline soy flake extract SF-PBS stained with Coomassie colloidal blue and (b) 2-DE of SF-PBS detected with rabbit antibodies to the same extract. The proteins in (a) were subjected to in gel digestion and identified by MS. The overlap between the gel and the blot allowed the identification of the proteins which the rabbit antibodies attached to: 1, β-conglycinin, α-chain; 2, β-conglycinin, α′-chain; 3, β-conglycinin, β-chain; 4, sucrose binding protein; 5, Gly m Bd30k; 6, Kunitz trypsin inhibitor; 7, glycinin basic subunit; 8, glycinin acidic subunit; 9, agglutinin.

3.2 Competitive ELISA for the detection of renatured soy proteins

Although, the above described sandwich ELISA was shown to be both highly specific and had a very low detection limit, preliminary experiments on food products indicated problems detecting processed soy protein. Consequently, we aimed at developing another soy specific assay based on the rabbit ab raised to denatured/renatured soy protein. The calculated LOD in a given food sample using the in-house competitive ELISA was 21 ppm when a 1:200 dilution factor during food sample extraction had been included. The assay was furthermore found highly specific when tested against extracts of legumes, tree nuts and cereals, as only walnut gave a positive signal at the level of 1400 ppm (Table 1). A commercial soy kit was furthermore tested for crossreactivity to peanut, walnut and rye with results below the detection limit (1400 ppm soy) (not shown).

3.3 EAST inhibition

The two human sera from soy allergic patients that had been included in this study both showed a strong positive response in direct EAST to SF-PBS, which allowed analysis in EAST inhibition. Standard inhibition curves were made for both sera, and detection limits obtained for the two standard curves, EAST inhibition 1 (serum no. 1) and EAST inhibition 2 (serum no. 2) were 0.8 and 12 ppm soy flake, respectively.

3.4 Basophil HR

Two serum samples from patients with clinically documented soy allergy were tested in an HR assay for reactivity to SF-PBS. Donor cells were stripped of their native IgE, and subsequently passively sensitized with IgE from each

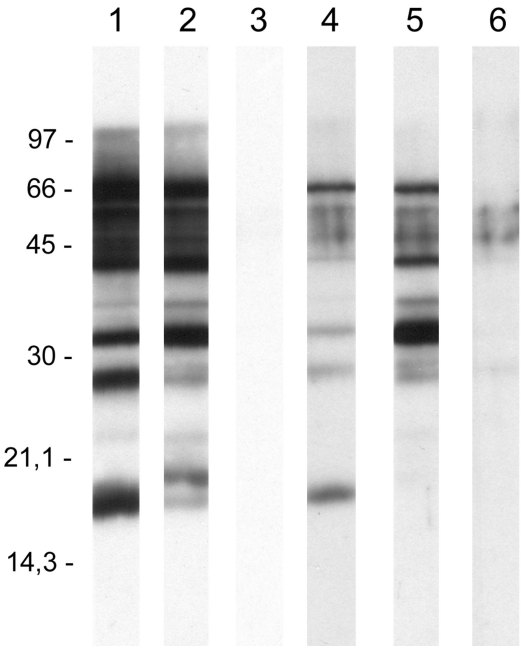

Figure 3. Western blot comparing rabbit ab reactivity and human IgE reactivity to an extract of soy flake protein. Protein in all lanes was a saline extract of soy flakes (SF-PBS). The primary antibodies were in lane 1, rabbit IgG to native soy protein (SF-PBS); 2, rabbit IgG to renatured soy protein; 3, buffer control; 4, human serum from a soy allergic individual (serum no. 1); 5, human serum from a soy allergic individual (serum no. 2); 6, human serum pool from nonatopic healthy subjects. Both rabbit IgG were diluted to a concentration of 2 μg/mL and sera were diluted 1:10.

of the soy allergic patients following incubation with soy standard extract. Both sera gave positive reactions, but serum no. 2 only to a relatively high protein concentration (in the μg/mL range). However, serum no.1 had a positive reaction as low as 1.4 ng/mL and a steep, straight standard

Table 2. Detection of soy in eight different food products using different detection methods

Food	Sandwich ELISA		Commercial ELISA kit		Competitive ELISA		EAST Inhibition serum 1		EAST Inhibition serum 2		Histamine Release serum 1		PCR
	1	*R*	2	*R*	3	*R*	4–1	*R*	4–2	*R*	5	*R*	6
1 Tofu	<0.05	1	240 000	8	250 000	8	200	2	170 000[a]	6	3800	5	Positive
2 Milk free ice cream	2000	7	32 000	6	36 000	5	3000	7	>1 000 000[a]	8	4600	6	Positive
3 Sausage 1	<0.05	1	26 000	5	33 000	4	1000	6	32 000	5	2 600	4	Positive
4 Sausage 2	0.6	4	20 000	3	25 000	3	700	4	30 000	3	4800	7	Positive
5 Sandwich meat	12	5	22 000	4	46 000	6	800	5	31 000	4	1 900	3	Positive
6 Spring roll	<0.05	1	<1400	1	<21	1	<0.8	1	100	1	4	1	Positive
7 Sausage 3	32	6	<1400	1	10 000	2	400	3	5000	2	1300	2	Positive[b]
8 Soy milk	15 000[a]	8	60 000	7	49 000	7	120 000	8	>1 000 000[a]	7	30 000	8	Positive
Assay detection limit	0.05 ppm		1400 ppm		21 ppm		0.8 ppm		12 ppm		~0.2 ppm		~10 ppm

All food products were purchased at a local grocery store, and had soybeans or soy protein declared as an ingredient. The results were expressed as ppm ‚whole soy flake' and ranked (*R*) from 1 to 8 in terms of the measured level of soy, 8 being the products with the highest content. Results obtained from the commercial ELISA kit was extrapolated from soy protein to ‚soy flake' to make direct comparison between assays possible (soy flake = 2 × soy protein). All detection limits takes protein extraction yield and dilution factor during extraction procedure into account.

a) Nonparallelism to standard curve observed during detection giving a less reliable result.

b) Positive at the level of LOD.

Table 3. Overview of soy detection methods

Method	LOD in whole soy flake	Detection range in protein conc.	Detects	Specificity	Limitations
Sandwich ELISA	0.05 ppm	0.4 – 300 ng/mL	Native soy proteins, saline extractable	Very good	None
Commercial ELISA kit	1400 ppm	3.5 – 70 mg/mL	Denatured/renatured soy proteins, urea/DTT extractable	Largely unknown	Costly
Competitive ELISA	20 ppm	50 – 8000 ng/mL	Denatured/renatured soy proteins, urea/DTT extractable	Very good	None (dependent on commercially available ab)
EAST inhibition 1	0.8 ppm	6 – 1700 ng/mL	Native soy allergens, saline extractable	Unknown	Dependent on soy allergic serum availability
EAST inhibition 2	12 ppm	0.1 – 200 mg/mL	Native soy allergens, saline extractable	Unknown	Dependent on soy allergic serum availability
HR 1	~0.2 ppm	1 – 140 ng/mL	Native soy allergens, saline extractable	Unknown	Dependent on soy allergic serum availability
PCR	~10 ppm	none	Soy lectin DNA	Very good	Does not detect potentially allergenic protein, but DNA

The detection limit of each assay was calculated as the lowest detectable amount of soy in the reference material (whole soy flake) taking both protein extractability and dilution factor during sample extraction into consideration.

curve was obtained (not shown). A positive result was defined to be at or above 15% HR, giving this assay an approximate detection limit of 0.2 ppm in a given food sample when using serum no. 1. Cells passively sensitized with serum no. 1 released no histamine when incubated with an extract of raw peanut.

3.5 Soy specific real-time PCR

Assuming that undiluted soybean DNA corresponded to 100% soybean, a 1:100 000 dilution simulates 0.001% (10 mg/kg, 10 ppm) soybean in a sample. The standard curve had an ideal slope of −3.382 (ΔCt over Δlog of dilution) that resulted in an overall PCR efficiency of 97.6%. The linearity was good ($R^2 = 0.97$). In theory, 1 copy of genomic soybean DNA was detectable at a threshold cycle (Ct) of 38. The undiluted standard was detected at 20.6 Ct, the 1:100 000 diluted standard at 37.0 Ct.

3.6 Soy detection in foods – Method comparison

Eight food products were selected as model foods and tested in the six soy specific detection methods (Table 2). The results obtained from the methods direct sandwich ELISA, EAST inhibition, HR and Competitive ELISA were all based on several dilutions of the product extract in order to have the average of several positives within detection range of the method (see method overview in Table 3).

Dilution curves of the product extracts were parallel with the standard curve in most cases, suggesting little or no interference from the food matrix during assay detection (not shown). Exceptions were product 8 (soy milk) in the sandwich ELISA and products 1, 2 and 8 in EAST inhibition 2, where some nonparallelity could be observed. No matrix-interference during detection was experienced in the methods Competitive ELISA, HR and EAST inhibition 1 for these particular eight products.

The results were ranked from 1 to 8 in terms of the measured level of soy within each method, 8 being the products with the highest content (Table 2). There was an overall good agreement between the methods in terms of ranks, as products 1, 2 and 8 had a higher content than for example products 6 and 7, with product 7 (spring rolls) being negative in four of the methods. However, the measured soy levels given by each of these assays were not in correlation with each other, allowing only semiquantitative estimations of the soy content.

The soy sandwich ELISA aimed at native soy proteins was able to detect soy in 5/8 products, and generally detected a much lower amount compared to other methods. The results using the commercial soy kit was in very good correlations with the in-house competitive ELISA assay in terms of ranks, but the kit seemed to detect about twice as much soy as the in-house assay. Only HR using serum no. 1 and EAST inhibition using serum no. 2 were able to detect soy protein in all eight products, however EAST inhibition using serum no. 2 measured unrealistically high levels of soybean especially for samples 2 and 8 with more than 100% soy. Generally, the measurements using EAST inhibition with serum from two different soy patients came up with results that did not correlate well. The soybean specific real-time PCR detected all tested products positive for soybean. However, a quantitative ranking was not possible because of the qualitative nature of the applied method.

4 Discussion

In this study we aimed at developing sensitive soy detection assays and compared them using common food items as model samples. The first method was a direct sandwich ELISA based on polyclonal rabbit antibodies raised to a saline extract of soy white flakes. The ELISA had a very low LOD of 0.05 ppm soy flake when both protein extractability and dilution factor during sample extraction had been included in the calculation (the lower limit on the standard curve was 0.4 ng/mL soy protein). The rabbit antibodies were furthermore shown to be highly specific for soy as crossreactivity to a wide range of legumes, tree nuts and cereals was negligible. The high sensitivity and specificity may be explained by the fact that immunization was done in a F1-generation of rabbits, where the parent generation had been kept on a soybean free and peanut free diet from mating and the offspring all life. The rabbit antibodies to native soy proteins was further characterized in a 2-D blot and shown to bind a large number of saline extractable proteins and a number of known soy allergens. Soy protein allergenicity has been extensively reviewed in a recent publication by L'Hocine and Boye [11]. A standard Western blot also showed that this ab could bind all proteins with MWs similar to proteins recognized by IgE from two soy allergic patients. The standard extract used in the direct sandwich ELISA was made with PBS, hence we extracted eight model foods using the same buffer and extraction conditions to eliminate any difference in extractability except what may be caused by the food matrices. Dilution curves of the eight tested products except soy milk (sample 8) were parallel with the standard curve, which is a prerequisite when the aim is to make quantitative determinations. However, only five of the eight products with declared soy content were positive according to the sandwich ELISA. This is very likely because antibodies raised to predominantly native soy proteins have difficulty recognizing soy proteins once they have been processed by various treatments like heat, which is the case with products 3 – 8 and fermentation which is the case with products 1 – 2. Perhaps the antibodies are directed towards conformational epitopes that are degraded as the proteins unfold and denature, or towards linear epitopes that may be chemically altered during processing. However, if epitope degradation/alteration is the case, it is surprising that sample dilution curves are parallel with standard dilutions of native proteins. Another reason could be that heat denaturation decreases soy protein extractability in PBS, but this is less likely, because the same extracts were tested in the methods basophil HR and EAST inhibition with much higher detection ability (Table 2).

As a consequence of the false negative results of the sandwich ELISA we developed a new competitive ELISA using a commercially available ab, and a different extraction procedure using first harsh denaturing and reducing conditions with boiling, saturated urea and DTT, followed by cooling and dilution in an oxidizing environment, a saline buffer with cystine. This extraction procedure was adopted from a commercial ELISA kit aimed at soy detection in meat products, which unfortunately had a detection limit insufficient for allergen detection in foods. Presumably, this procedure is able to solubilize all soy proteins by urea denaturation and disulphide bridge breakage, and then reforming new scrambled disulphide bonds creating modified versions of the original molecules. These renatured proteins are then able to stay soluble in a saline environment in which rabbit antibodies can coexist without loosing binding capacity, as this is necessary in methods like ELISA. The in-house competitive ELISA and the commercial kit yielded fairly similar results when applied to eight food products, but the detection limit of the two assays were quite far apart as they were 1400 and 21 ppm whole soy flake, respectively. This difference in LODs may be

explained by a number of factors, like concentration of ab reagents, a difference in ab quality or a difference en standard soy protein, but we speculate that the main reason is the use of much longer incubation periods in the in house competitive ELISA, which was 2 h compared to 10 min in the kit. The competitive ELISA was highly specific when tested against other legumes, tree nuts and cereals, whereas the kit lacked information on specificity of the antibodies in the specifications. The ab to renatured soy used in the in house competitive ELISA showed multiple reactivity in a standard Western blot (Fig. 3), but in an ELISA setup the ab seemed less reactive in strength to allergens recognized by IgE from a soy allergic individual in comparison to the antibodies to native soy protein that was used in the direct sandwich ELISA (Fig. 1). When testing the eight food samples in the soy detection kit, six of the products tested positive, and the in-house competitive ELISA found soy in all but one of the products (sample 6, Chinese spring rolls).

Two different detection methods based on human IgE from soy allergic individuals were also included in this study for comparison to rabbit ab based assays. Generally, these IgE based assays were able to identify soy in all the model samples except sample 6 in EAST inhibition using serum no. 1. Hence, IgE based assays may be a better choice for tracing processed soy protein than rabbit antibodies raised to saline soluble native soy protein. These assays, especially HR from human basophils, are also more biologically relevant in terms of allergenicity since only allergenic proteins are being detected contrasting to proteins in general. However, IgE based assays are known to have issues with specificity as IgE crossreactions between legume plant species is well known [12 – 14]. Basophil HR and EAST inhibition assays may also be a lot more difficult to standardize compared to rabbit IgG based methods because the measured outcome will depend largely on the individual serum used and also on the donor basophils [15]. Since the basophil HR method is a biological test a larger variance is to be expected, making the method less quantitative.

In addition, we consider it to be extremely rare to come across such a sensitive serum as serum no. 1 used in this study with the ability to detect soybean protein as low as 0.2 ppm in basophil HR. This serum is likely to be from a patient primarily sensitized to soybean as her level of specific IgE to soybean in more than 30 times higher than for peanut as seen from ImmunoCap results. Furthermore, we demonstrated in a Western blot experiment an IgE- binding to a protein at approximately 19 kDa (Fig. 3). This is consistent with the results of Herian *et al.* [16], who demonstrated in an immunoblotting experiment that a group of soy allergic patients who tolerated peanuts had a strong IgE binding to a protein at approximately 20 kDa. This binding was not observed in a group of soy allergic patients with a concomitant peanut allergy. Unfortunately, IgE from allergic individuals is a limited resource and cannot be applied in routine analysis.

With a soy-specific real-time PCR, the presence of soy was confirmed in all eight different food commodities even though soy protein as an ingredient does not picture whole soybean seed, all model products contained enough amplifiable DNA. The advantage of a PCR detection assay is the good sensitivity combined with a high specificity. The drawback is the lack of relevance in terms of allergenic protein since the method is based on soy DNA, which may not exist in amounts proportional to the soy protein content in a given food sample.

We are aware that proper validation of an assay's applicability on food samples should be investigated using recovery calculations from samples spiked with known amounts of soy protein. However, this would be an overwhelming task as such an experiment would have to include all or almost all the matrices soy may be found in combined with soy in all the forms and degrees it may be processed in, which was far beyond the scope of this project.

This study has emphasized the difficulties of detecting soy protein in processed foods, since it was not possible to make accurate quantifications, because different methods gave results that were not in good correlation with each other. Nevertheless, looking at ranks of soy content for each assay the correlation between methods was generally good (Table 2). The determination of an exact concentration with absolute certainty is difficult, and generally immunochemical detection methods applied to complex and/or processed food products generate a more qualitative than quantitative result.

Risk assessments of potential exposure are best performed using at least two different assays to confirm the presence of soy protein in processed food. One method could be an assay like our competitive ELISA with the ability to detect processed soy protein in various food matrices used in combination with a denaturing/renaturing extraction procedure. This would provide us with a detection limit of 21 ppm soybean (11 ppm soy protein), which is thought likely to be sufficiently low to make safe risk assessments. A precise clinical threshold has not yet been established for soybean, but in a recent large multicentre study by Ballmer-Weber *et al.* 2007 [1], the lowest observed adverse effect level (LOAEL) was found to be 10 mg soy flour (5.3 mg soy protein) for subjective symptoms, which corresponds to a level of 100 ppm soy in a meal of 100 g. This amount was the LOAEL in the most sensitive of 23 patients that were diagnosed with soybean allergy by a double blind placebo controlled food challenge (DBPCFC) method [17]. The LOAEL for objective symptoms found in the same study was much higher, namely 454 mg soy flour or 0.45% soybean in a meal of 100 g. The competitive ELISA method could be supplemented with a more biologically relevant assay like basophil HR using IgE from an individual with a confirmed severe soy allergy, depending on serum availability and the ability to overcome the difficulties regarding standardization and quantification as outlined above.

This study was funded by Novozymes A/S, the Danish Ministry of Science, Technology and Innovation, and the FAREDAT project (European Commission, 5th Framework program, QLK4-CT-2001-00301).

The authors have declared no conflict of interest.

5 References

[1] Ballmer-Weber, B. K., Holzhauser, T., Scibilia, J., Mittag, D., *et al*., Clinical characteristics of soybean allergy in Europe: A double-blind, placebo-controlled food challenge study, *J. Allergy Clin. Immunol*. 2007, *119*, 1489 – 1496.

[2] Mittag, D., Vieths, S., Vogel, L., Becker, W. M., *et al*., Soybean allergy in patients allergic to birch pollen: clinical investigation and molecular characterization of allergens, *J. Allergy Clin. Immunol*. 2004, *113*, 148 – 154.

[3] Bando, N., Tsuji, H., Hiemori, M., Yoshizumi, K., *et al*., Quantitative analysis of Gly m Bd 28K in soybean products by a sandwich enzyme-linked immunosorbent assay, *J. Nutr. Sci. Vitaminol. (Tokyo)* 1998, *44*, 655 – 664.

[4] Tsuji, H., Okada, N., Yamanishi, R., Bando, N., *et al*., Measurement of Gly m Bd 30K, a major soybean allergen, in soybean products by a sandwich enzyme-linked immunosorbent assay, *Biosci. Biotechnol. Biochem*. 1995, *59*, 150 – 151.

[5] Koppelman, S. J., Lakemond, C. M., Vlooswijk, R., Hefle, S. L., Detection of soy proteins in processed foods: literature overview and new experimental work, *J. AOAC Int*. 2004, *87*, 1398 – 1407.

[6] Hefle, S. L., Lambrecht, D. M., Nordlee, J. A., Soy sauce retains allergenicity through the fermentation/production process, *J. Allergy Clin. Immunol*. 2005, *115 (Suppl. 1)*, S32, Abstract.

[7] Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* 1970, *227*, 680 – 685.

[8] Hansen, T. K., Bindslev-Jensen, C., Skov, P. S., Poulsen, L. K., Codfish allergy in adults: IgE cross-reactivity among fish species, *Ann. Allergy Asthma Immunol*. 1997, *78*, 187 – 194.

[9] Hellman, U., Wernstedt, C., Gonez, J., Heldin, C. H., Improvement of an "In-Gel" digestion procedure for the micropreparation of internal protein fragments for amino acid sequencing, *Anal. Biochem*. 1995, *224*, 451 – 455.

[10] Skov, P. S., Pelck, I., Ebbesen, F., Poulsen, L. K., Hypersensitivity to the diphtheria component in the Di-Te-Pol vaccine. A type I allergic reaction demonstrated by basophil histamine release, *Pediatr. Allergy Immunol.* 1997, *8*, 156 – 158.

[11] L'Hocine, L., Boye, J. I., Allergenicity of soybean: new developments in identification of allergenic proteins, cross-reactivities and hypoallergenization technologies, *Crit. Rev. Food Sci. Nutr*. 2007, *47*, 127 – 143.

[12] Bernhisel-Broadbent, J., Sampson, H. A., Cross-allergenicity in the legume botanical family in children with food hypersensitivity, *J. Allergy Clin. Immunol*. 1989, *83*, 435 – 440.

[13] Bernhisel-Broadbent, J., Taylor, S., Sampson, H. A., Cross-allergenicity in the legume botanical family in children with food hypersensitivity. II. Laboratory correlates, *J. Allergy Clin. Immunol*. 1989, *84*, 701 – 709.

[14] Barnett, D., Bonham, B., Howden, M. E., Allergenic cross-reactions among legume foods – an in vitro study, *J. Allergy Clin. Immunol.* 1987, *79*, 433 – 438.

[15] Poulsen, L. K., In vivo and in vitro techniques to determine the biological activity of food allergens, *J. Chromatogr. B: Biomed. Sci. Appl*. 2001, *756*, 41 – 55.

[16] Herian, A. M., Taylor, S. L., Bush, R. K., Identification of soybean allergens by immunoblotting with sera from soy-allergic adults, *Int. Arch. Allergy Appl. Immunol*. 1990, *92*, 193 – 198.

[17] Bindslev-Jensen, C., Ballmer-Weber, B. K., Bengtsson, U., Blanco, C., *et al*., Standardization of food challenges in patients with immediate reactions to foods – position paper from the European Academy of Allergology and Clinical Immunology, *Allergy* 2004, *59*, 690 – 697.

