Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 108 (2008) 571–581
Food Chemistry
www.elsevier.com/locate/foodchem

Immunoreactivity reduction of soybean meal by fermentation, effect on amino acid composition and antigenicity of commercial soy products

Y.-S. Song [a], J. Frias [b], C. Martinez-Villaluenga [b], C. Vidal-Valdeverde [b], E. Gonzalez de Mejia [a,*]

[a] *Department of Food Science and Human Nutrition, University of Illinois Urbana-Champaign, 228 ERML, MC-051, 1201 W Gregory Drive, Urbana, IL 61801, United States*
[b] *Instituto de Fermentaciones Industriales (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain*

Received 10 August 2007; received in revised form 3 November 2007; accepted 8 November 2007

Abstract

Food allergy has become a public health problem that continues to challenge both the consumer and the food industry. The objectives of this study were to evaluate the reduction of immunoreactivity by natural and induced fermentation of soybean meal (SBM) with *Lactobacillus plantarum*, *Bifidobacterium lactis*, *Saccharomyces cereviseae*, and to assess the effect on amino acid concentration. Immunoreactivity of commercially available fermented soybean products and ingredients was also evaluated. ELISA and western blot were used to measure IgE immunoreactivity using plasma from soy sensitive individuals. Commercial soy products included tempeh, miso and yogurt. Fermented SBM showed reduced immunoreactivity to human plasma, particularly if proteins were <20 kDa. *S. cereviseae* and naturally fermented SBM showed the highest reduction in IgE immunoreactivity, up to 89% and 88%, respectively, against human pooled plasma. When SBM was subjected to fermentation with different microorganisms, most of the total amino acids increased significantly ($p < 0.05$) and only few of them suffered a decrease depending on the type of fermentation. All commercial soy containing products tested showed very low immunoreactivity. Thus, fermentation can decrease soy immunoreactivity and can be optimized to develop nutritious hypoallergenic soy products. However, the clinical relevance of these findings needs to be determined by human challenge studies.

Keywords: Soybean; *Glycine max*, protien, food allergy; IgE immunoreactivity, fermentation, microbial proteolysis, hypoallergenic foods, antigenicity

1. Introduction

Allergenic conditions affect millions of people worldwide (Goodwin, 2004; Sampson, 1997), and it is believed that one in 25 Americans is susceptible to food allergies (Sicherer, Munoz-Furlong, & Sampson, 2004). Today, more than 15% of the general population believes that they may be allergic to some food components (Burks & Ballmer-Weber, 2006).

Food allergens are complex mixtures of potentially immunoreactive proteins. Small regions in the allergenic proteins, called epitopes, provoke the IgE mediated allergenic response (Taylor & Hefle, 2001). The allergen generates an initial IgE antibody response followed by a secondary IgE antibody response, which triggers an allergic reaction (Babu, Arshad, & Holgate, 2001). Food proteins bind to the allergen-specific IgE molecules residing in the mast cells and basophils, causing them to release inflammatory mediators (Beyer & Teuber, 2004).

Soybean is one of the most important grains of the legume family accounting for 20% of the US production (USDA, 2007). Its acceptance has increased due to the low-cost and high nutritional quality in relation to other traditional protein sources (Friedman & Brandon, 2001). The estimated prevalence of soybean allergies is about 0.5% of the total US population (Sicherer & Sampson, 2006). The Food Allergen Labeling and Consumer

* Corresponding author. Tel.: +1 217 244 3196; fax: +1 217 265 0925.
E-mail address: edemejia@uiuc.edu (E.G. de Mejia).

0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.11.013

Protection Act of 2004 (P.L. 108-282) (FALCPA, 2004) includes soy in its definition of the "big 8" which comprises those foods that cause the most allergenic reactions (Cordle, 2004; Kabourek & Taylor, 2003; Taylor & Helfe, 2000). The recent applications in the labeling regulations make imperative the identification of a processing technology able to eliminate the allergens from soybean protein containing products.

There have been 33 immunoglobulin E (IgE) binding allergenic proteins identified in soybean (FDA, 2004). According to Wilson, Blaschek, and de Mejia (2005) soybean allergens comprise proteins with molecular masses from 7.0 to 71 kDa. However, only a few of these 33 proteins are responsible for a majority (~90%) of the allergenic responses, making it important to identify these key proteins.

The estimated allergy threshold level is usually low and a very small amount of the allergen may be enough to trigger an allergenic reaction (Poms, Anklam, & Kuhn, 2004). The reported threshold for soybean allergen ranges from 0.0013 to 500 mg of soy protein (Bindslev-Jensen, Briggs, & Osterballe, 2002; FDA, 2005; Fiocchi et al., 2003; L'Hocine, Boye & Munyana, 2007). Therefore, the reduction or elimination of immunoreactivity in soy ingredients or products may be important for soy allergic individuals.

Several studies have reported the degradation of soybean allergens (Gly m Bd 30K) during fermentation by microbial proteolytic enzymes such as in soy sauce, miso, soybean ingredients and feed grade soybean meals improving their nutritional and functional properties (Hong, Lee, & Kim, 2004; Ito, Kato, & Matsuda, 2005; Kobayashi, 2005; Ogawa, Samoto, & Takahashi, 2000; Yamanihi, Huang, Tsuji, Bando, & Ogawa, 1995). Thus, the use of fermentation to reduce or eliminate allergenicity of soy products represents an interesting opportunity to produce hypoallergenic food products. The objectives of this study were to evaluate the reduction of immunoreactivity by natural and induced fermentation of soybean meal (SBM) with *Lactobacillus plantarum, Bifidobacterium lactis, Saccharomyces cereviseae* and to assess the effect of fermentation on amino acid concentration. Immunoreactivity of commercially available fermented soybean products and ingredients was also evaluated.

2. Materials and methods

2.1. Chemicals

Trizma-hydrochloride, 2-mercaptoethanol (2-ME), sodium chloride, sodium carbonate, ammonium sulfate, phosphate buffered saline tween (PBST) buffer pH 7.4, bovine serum albumin (BSA), tween-20, Tris buffered saline tablets, para-nitrophenyl phosphate (PNPP), deoxycholate, and Corning™ Sterile syringe filter pore size 0.2 μm were obtained from Sigma–Aldrich™ (Saint Louis, MO, USA). SDS–PAGE was performed with Phastsystem (Amersham-Pharmacia Inc., Piscataway, NJ). Reagents used for SDS–PAGE included Laemmli blue dye, running buffer with TG/SDS, 8–25% precast polyacrylamide gradient mini-gels (Amersham), buffer strips for Phastsystem mini-gels (Amersham), Coommassie Blue bio-safe dye, and prestained SDS–PAGE molecular weight broad-range standards were purchased from Biorad (Hercules, CA, USA). Immunoblot PVDF membranes (for protein blotting 0.2 μm) 7 × 8.4 cm were purchased from Biorad™. Bradford assay was conducted using Biorad™ Bradford reagent. Other reagents, such as analytical grade NaOH, H_2SO_4, and HCl, used occasionally to adjust buffers and to stop the color substrate reaction, were purchased from Sigma Chemical (Saint Louis, MO, USA).

2.2. Samples

Defatted soybean meal (SBM) containing 48% protein (*Glycine max* (L.) Merill) was purchased from Solae Co. (Gibson, IL).

Commercial brands of tempeh, miso, and yogurt were purchased in local markets in Champaign, Illinois and analyze without further preparation. Fermented soy ingredient I and fermented soy protein concentrate ingredient II and enzymatically hydrolyzed soybean ingredients (soy flour, protein concentrate, protein isolate and peptides) were provided by SAI International, Co. (Chicago, IL, USA).

2.3. Soybean fermentation

2.3.1. Preparation of cultures

L. plantarum CECT 748 (ATCC 14917) was purchased from the Spanish Type Culture Collection (CECT), *B. lactis* Bb12 was obtained from Probio-Tec, Hansen, DenMark and *S. cereviseae* IFI 87 from to the collection of the Spanish Institute of Industrial Fermentations (Madrid, Spain). Stock cultures of *L. plantarum* and *B. lactis* were grown and maintained on MRS agar (Difco Laboratories, Detroit, MI, USA) for 18 h at 30 °C. The cells were washed twice in sterile saline solution and inoculated to give a final inoculation of 1×10^7 cfu/ml. *S. cereviseae* was grown in YPD broth (Difco) for 72 h at 37 °C, and then washed twice in sterile saline solution and inoculated to give a final inoculation of 1×10^7 cfu/ml.

2.3.2. Liquid state fermentations

Four small-scale liquid fermentations were performed in SBM, including natural fermentation (NF, carried out with the only microorganisms naturally present in soybean seeds), and three different induced fermentations with each of the microorganisms mentioned above in Section 2.3.1.

Suspensions of SBM in sterile distilled water (200 g/l, w/v) were prepared and were allowed to ferment either spontaneously (no microorganisms added) or with 10% v/v of the suspensions of *L. plantarum*, *B. lactis*, or *S. cereviseae*. The fermentation processes were carried out in a 2 μl stirred fermentor (Infors AG, Switzerland) for 48 h at 37 °C, as in Doblado et al. (2003). Start of the fermentation

was considered when the complete suspension was stirred and the temperature controlled (10–40 min after the preparation of suspension). Samples were collected at times 0 and 48 h for pH and microbiological analyses. Fermented samples were freeze-dried for further analysis.

2.3.3. Microbiological analysis

The fermentation process was monitored by withdrawing samples at times 0 and 48 h using plate counts to determine changes in viable cells. *L. plantarum* and *B. lactis* were counted on MRS agar (Difco) plates after incubation in 5% CO_2 atmosphere at 37 °C for 72 h. Yeast cultures were counted on YPD agar plates after incubation aerobically at 30 °C for 48 h.

2.4. Protein chemistry

2.4.1. Protein extraction from soybean

Protein extraction was performed by the procedure described by De Mejia, Vasconez, De Lumen, and Nelson (2004). It consisted in placing 0.05 g of the lyophilized fermented soybean powder and 1 ml of the extracting buffer (0.05 M Tris–HCl buffer, pH 8.2) in an Eppendorf tube. After the extract was mixed, the samples were placed in an ultrasonic bath (Bransonic model 2510, Branson Ultrasonic Corporation, Danbury, CT) for 70 min, mixing again every 10 min to avoid settlement. The temperature of the water bath was controlled at 40 °C by using a recirculating bath (Endocal model RTE-9, Neslab Instruments, Portsmouth, NH). At least three independent extractions were performed for each sample. Following extraction, the samples were centrifuged at 20,000*g* for 30 min at 4 °C in an Eppendorf centrifuge (model 5417R, Brinkmann Instruments, Westbury, NY) and the obtained supernatant transferred to a new Eppendorf tube. This material was then diluted (1:2, v/v) with extracting buffer for ELISA analysis.

All samples of commercial fermented products and ingredients were also extracted, except for yogurt, which was diluted 1:10 in distilled water and 3.75 ml of this suspension was mixed with extraction buffer (7.25 ml) (Franck, Moneret-Vautrin, Dousset, Kanny, & Nabet, 2002).

2.4.2. Total soluble protein quantification

Soluble protein was quantified by the DC™ protein assay kit (Bio-Rad Laboratories, Hercules, CA) for microplates. The reaction fundamentals are based on the Lowry (Folin) assay and a micro assay procedure consisting on adding 25 μl of reagent A, followed by 5 μl of either protein extract or BSA for the construction of an standard curve in the range 10–1600 μg/ml ($y = 0.186x + 0.006$, $R^2 = 0.99$). Once the protein extract was added, immediate mixing was required. Then, 200 μl of reagent B were added followed by 15 min incubation at room temperature. Microplates were read at 690 nm. The total soluble protein concentrations of the soybean extracts were quantified based on the standard curve.

2.4.3. Analysis of total amino acids by HPLC

Determination of total amino acids was carried out by acid hydrolysis, derivatization and HPLC quantification using the method described by Martínez-Villaluenga, Gulewicz, Frias, Gulewicz, and Vidal-Valverde (2007). In brief, 200 μl (0.2 mmol/ml) of DL-norleucine (Sigma, Chem Co.) were added to 50 mg of sample as internal standard. Protein hydrolysis was carried out with 6 M HCl for 21 h at 110 °C in a vacuum closed vial. Hydrolysates were dried under vacuum and rinsed twice with water. PITC (phenylisothiocyanate 99%, Aldrich) was used for amino acid derivatization.

The chromatographic system consisted of an Alliance Separation Module 2695 (Waters, Milford, CT, USA), a Photodiode Array detector 996 at 254 nm (Waters, Milford, CT, USA) and a personal computer running the Empower 2 for Microsoft Windows chromatographic software (Waters). The sample (20 μl) was injected into a C_{18} reversed phase Alltima 250 × 4.6 mm i.d., 5 μm size column (Alltech) equipped with a guard column (Alltech) at 43 °C. The linear gradient system with buffer A (0.1 M ammonium acetate, pH 6.5) and buffer B (0.1 M ammonium acetate/acetonitrile/methanol: 44/46/10; v/v/v) at pH 6.5, allowed separation of the amino acids in 65 min.

2.4.4. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

Electrophoresis was carried out in a Phastsystem Kit (Amersham-Pharmacia Inc., Piscataway, NJ) with an 8–25% polyacrilaminde gradient gel. Extracted samples were centrifuged (20,000*g*) to eliminate any precipitate that might have formed during freezing or thawing. The supernatant (20 μl) was added to 20 μl of Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) with 5% β-mercaptoethanol (Sigma, MO, USA). Samples were boiled for 5 min by using the Laemmli buffer system. After the samples and molecular weight standard had cooled down to room temperature, 1.5 μg of soybean protein per well was loaded into PhastSystem ready gels. A broad-range protein molecular weight Precision Plus Protein standards (Biorad) 10, 15, 20, 25, 37, 50, 75, 100, 150, 250 kDa were used. The gels were run at 55 mA constant for 20 min (60–125 V), fixed for 20 min in methanol/acetic acid/water (5:5:2, v/v/v), and stained with Coomassie Brilliant G-250 for 1 h. Then, gels were destained with two washes of 5 min with acetic acid/water (40%) and finally, washed once again with deonized water. They were read in a Kodak Image Station 440 CF, where the respective molecular weights and band intensities were recorded for the different soy protein samples.

2.4.5. Western blot procedures with human plasma

The characteristics of human plasma (PlasmaLab International, Everret, WA) used are described in Table 1. Plasmas A–D correspond to donors with soybean allergies. Plasmas E–F correspond to allergic non-atopic subjects. Plasma G was a WHO pooled IgE plasma standard

Table 1
Characteristics of human plasma from six soybean-sensitive donors[a]

Plasma code	Age (years)	Gender	Soybean specific IgE by InmunoCAP analysis (kUA/l)[a]
A	36	m	97.5
B	36	m	71.6
C	42	f	34.2
D	48	f	11.3
E	63	m	8.0
F	59	m	< 0.35
G	WHO Standard 75/502 pooled plasma IgE[b]		5000

[a] Donors were selected on the basis of clinical allergy to soybean and *in vitro* IgE evaluation. A–D, donors reporting soybean allergies; E–F, non-atopic subjects. The presence of soybean-specific IgE was measured by inmunoCAP analysis (PlasmaLab International, Everett, WA).

[b] Standard 75/502 pooled plasma IgE obtained from the World Health Organization. Professor J. Pepys, Brompton Hospital, London collected lyophilized pooled sera from nine patients suffering with allergic disorders. This WHO standard was used only for comparison with a pooled sample made by combining plasmas A–F.

75/502 obtained from the World Health Organization (London, UK) used only for comparison purposes with a prepared pooled plasma. Specific IgE levels were measured by the ImmunoCAP IgE method. The pooled plasma sample, for ELISA and western blot analyses, was prepared by combining equal volumes of human plasma samples A, B, C, D, E and F.

Unstained gels were soaked in 20 ml of blotting buffer pH 8.3, which consisted of 10% (v/v) methanol, 25 mM Tris base, 192 mM glycine, for 3 min. A western blot sandwich was assembled by placing a sponge, a filter, the gel, the Immun-Blot PVDF membrane, another filter and sponge, avoiding the formation of bubbles. The transfer was run at 25 mA constant for 20 min (20 V, 1 W). After the transfer was completed, the membrane was then saturated by incubation in 5% non-fat dry milk (NFDM) in 0.01% Tween in TBS (TTBS) buffer for 1 h, followed by overnight incubation at 4 °C in a 1:5 dilution of the soybean positive plasma from donors (PlasmaLab International, Everret, WA) in 3% BSA, 0.1% Tween TBS buffer. The membrane was washed three times in TTBS and then incubated for 1 h at room temperature in a 1:1000 dilution goat anti-human IgE alkaline phosphatase (Bethyl Lab, Inc., Montgomery, TX, USA) prepared in 3% NFDM in TTBS buffer. The membrane was washed three times for 5 min with 0.01% TTBS and prepared for detection using an Immun-Star Chemiluminescence Alkaline Phosphatase substrate solution (Bio-rad Laboratories, Hercules, CA, USA) following manufacturer's recommendations. After adding the substrate to the blot, the intensity of the bands was read in a Kodak Image Station 440 CF (Kodak, USA) with a total exposure time of 2 min and 8 captures.

2.4.6. IgE immunoreactivity by ELISA

An enzyme-linked immunosorbent assay (ELISA) was used to study the soy IgE immunoreactivity using plasma from donors clinically tested to suffer from soybean allergies. For ELISA, 100 µl of the soy protein extracts (antigen) were applied first onto the Maxisorp™ immunoassay plate at various dilutions in PBS, in triplicate and kept overnight at 4 °C. The unoccupied space on the 96-well immunoassay microplate was blocked for 1 h at room temperature with 300 µl per well of a solution containing 5% BSA, 1% Tween-20, 1 tablet of TBS in 15 ml of deionized water. Between each step in the procedure, the plates were washed four times in PBST buffer at pH 7.4 using a Biotek® Instruments Elx50 autostrip washer (Winooksi, VA). A 100 µl per well of human plasma A (1:5 in 1% BSA), with the highest specific IgE (97.5 kUA/l), and the pooled plasma (1:5 in 1% BSA) (Table 1) were applied onto the Maxisorp™ immunoassay plate, incubated for 1 h at 37 °C and washed again. A buffered based was used as a negative control. Then, a 100 µl per well of goat-anti-human IgE alkaline phosphatase diluted in antibody buffer (1:1000 in 1% BSA) (Bethyl Labs Inc., Texas, USA) was added, and allowed to incubate in the plate for 1 h at room temperature and washed to eliminate any remaining unbound antibody. Bound secondary antibody was detected by addition of 100 µl per well of color reagent *p*-nitrophenyl phosphate (PNPP) for 45 min at room temperature. The reaction was stopped by addition of 3 N NaOH. The plate was read with an automatic reader Elx80810 ultra microplate reader (Biotek® Instruments; Winooksi, VA) at 405 nm using K.C. Junior™ computer program, exported and analyzed in Microsoft Excel™. The concentration of the immunoreactive proteins, in each sample, was based on triplicate analysis and multiple dilutions.

2.5. Statistical analysis

Protein and immunoreactivity were analyzed with SAS program using ANOVA and means were generated and adjusted with LSD's post-test. Probability $p < 0.05$ indicated statistically significant differences. Amino acid data were expressed as the mean of three determinations and were subjected to multi-factor analysis of variance with use of the least significance difference test with the Statgraphic 4.0 Program for Windows (Statistical Graphics Corporation, Rockville, MD).

3. Results and discussion

3.1. pH and microbial counts during fermentation

After 24 h, both, natural and induced fermentation with *L. plantarum* lowered the pH of the medium more sharply than fermentation inoculated with *B. lactis* or *S. cereviseae* (from 6.4 at time 0 to 4.7, 4.3, 5.6 and 6.2, respectively). After 48 h of fermentation, pH reached values of 4.5, 4.5, 4.7 and 5.2, respectively. Microbiological monitoring of fermented samples inoculated with *L. plantarum* or *B. lactis* revealed that lactic acid bacteria were predominant, indicating that the inoculated strains were able to dominate soybean fermentation. For naturally fermented soybean,

the decrease on the pH is characteristic for lactic acid microorganism, as it was visualized in the plate counts. The microorganisms involved in the natural fermentation are essentially surface microflora and, during the fermentation process the lactic acid-producing bacteria grow rapidly lowering the pH and inhibiting the growth of other competing or pathogens microorganisms (Deshpande et al., 2000; Doblado et al., 2003; Frias et al., 1996; Gibbs, 1987). When SBM was fermented with *S. cereviseae* the visual microbial counts revealed the growth of such yeast, indicating the ability of this strain to dominate the fermentation. *S. cereviseae* is able to produce mainly ethanol, and CO_2, and minor organic acid from hexoses (Bisson, Coons, Kruckeberg, & Lewis, 1993) and, therefore, the lowering on pH is rather minor.

3.2. Immunological reactivity of fermented SBM

Natural or induced fermentation reduced significantly the immunoreactivity of SBM. Table 2 presents the efficacy of yeast (*S. cereviseae*) and bacteria (*L. plantarum* and *B. lactis*) to reduce immunoreactivity of SBM. Both, *S cereviseae* and *B. lactis* reduced in 77% the immune response, when using 97.5 kUA/l human plasma and 89% and 86%, respectively, when the pooled human plasma was used. Natural fermentation reduced immunoreactivity in 80% and 88% when using plasma 97.5 kUA/l and the pooled sample, respectively.

The observed practicality and effectiveness of natural fermentation in reducing immunoreactivity was outstanding. It consisted in a simple treatment of soaking the (non-sterile) SBM in distilled water and allowing only microorganisms naturally present on the seeds to undergo the fermentation process. Several microorganisms were detected in the natural fermentation of SBM with strong presence of *Lactobacillus* species. This natural process showed similar degrees of reduction as *L. plantarum* (87%) and *B. lactis* (86%).

A remarkable reduction in the extractable protein concentration of the fermented samples was detected under the conditions used (0.05 M Tris–HCl buffer, pH 8.2) (Table 2). The extracted soluble protein from fermented soybean products ranged from 83.1 to 55.9 mg protein/g product, four times lower than the extractable protein of the original non-fermented SBM (215 mg protein/g product). This could be due to the fact that microorganisms and enzymes involved in the fermentation process can easily hydrolyze soluble proteins. Consequently, new peptides were formed and the conditions used to extract original proteins were probably not adequate for extracting the new peptides.

Fig. 1 shows the electrophoretic patterns and western blots of the tested samples. Fig. 1A presents the protein profile characteristic of non-fermented and fermented soybean products. Lane 1 presents the protein profile of a partially fermented commercial product (fermented soy ingredient I). Even though this product has been fermented, the protein profile mimics the profile shown by SBM (lane 2). The electrophoretic profile of SBM (lane 2) presents high intensity bands of β-conglycinin subunits 68 kDa and 48 kDa; P34 fraction; glycinin acid (33 kDa) and basic (22 kDa) subunits; trypsin inhibitor (20 kDa) and Gly m 1 (7 kDa). The presence of Gly m 1 or hull protein can be attributed to an inefficient cleaning during the milling process of ingredients, allowing the inclusion of some hull contaminants. The typical protein profile of SBM can be compared to the reduced intensity of immunodominant proteins after yeast and bacterial fermentation (lanes 4–7). A similar pattern for the non-fermented sample was observed previously by Lee et al. (2007). If peptides were grouped as large (>70 kDa), medium (20–60 kDa) and small (<10 kDa), SBM contained considerable larger and medium size peptides than fermented soybean products. The amount of smaller size peptides in fermented soybean products was considerably greater and the immunoreactivity was reduced.

Among the four fermented soybean samples, the electrophoretic pattern of *S. cerevisiae* (lane 4) was the experimental fermented SBM with less intense P34 fraction and generated a weaker response against all human plasma samples (Fig. 1B).

S. cerevisiae is mainly used for bread making and beer brewing where fermentable sugars provide suitable substrates. Infant weaning foods from fermented blends of cereal and soybean have been developed (Sanni, Onilude, &

Table 2
Effect of liquid fermentation of defatted soybean meal (SBM) on immune response of human plasma (97.5 kUA/l) and pooled human plasma by ELISA[A]

Microorganism	Extracted protein (mg protein/g product)	$OD_{405\,nm}$/g product[B]	Reduction (%)[B,C]	$OD_{405\,nm}$/g product[D]	Reduction (%)[C,D]
Non-fermented soybean meal	215.2 ± 17.4^{a}	19.2 ± 0.3^{a}	0.0 ± 0.0^{d}	11.9 ± 0.6^{a}	0.0 ± 0.0^{c}
Natural fermentation	$63.3 \pm 5.5^{b,c}$	3.9 ± 0.2^{c}	79.5 ± 0.8^{a}	1.5 ± 0.1^{b}	$87.5 \pm 0.1^{a,b}$
Induced fermentation					
Saccharomyces cereviseae	83.1 ± 6.3^{b}	4.4 ± 0.1^{b}	77.2 ± 0.2^{c}	1.3 ± 0.0^{b}	88.7 ± 0.8^{a}
Bifidobacterium lactis	55.9 ± 5.8^{c}	4.4 ± 0.2^{b}	77.2 ± 0.2^{c}	1.6 ± 0.0^{b}	86.3 ± 0.4^{b}
Lactobacillus plantarum	$56.2 \pm 0.3^{b,c}$	$4.2 \pm 0.1^{b,c}$	78.0 ± 0.2^{b}	1.6 ± 0.2^{b}	86.9 ± 0.8^{b}

[A] Different superscript, per column, means statistical differences ($p < 0.05$, $n = 3$).
[B] Human plasma (97.5 kUA/l) from soy sensitive individuals.
[C] Reduction calculated based on gram of product.
[D] Pooled human plasma from soy sensitive individuals.

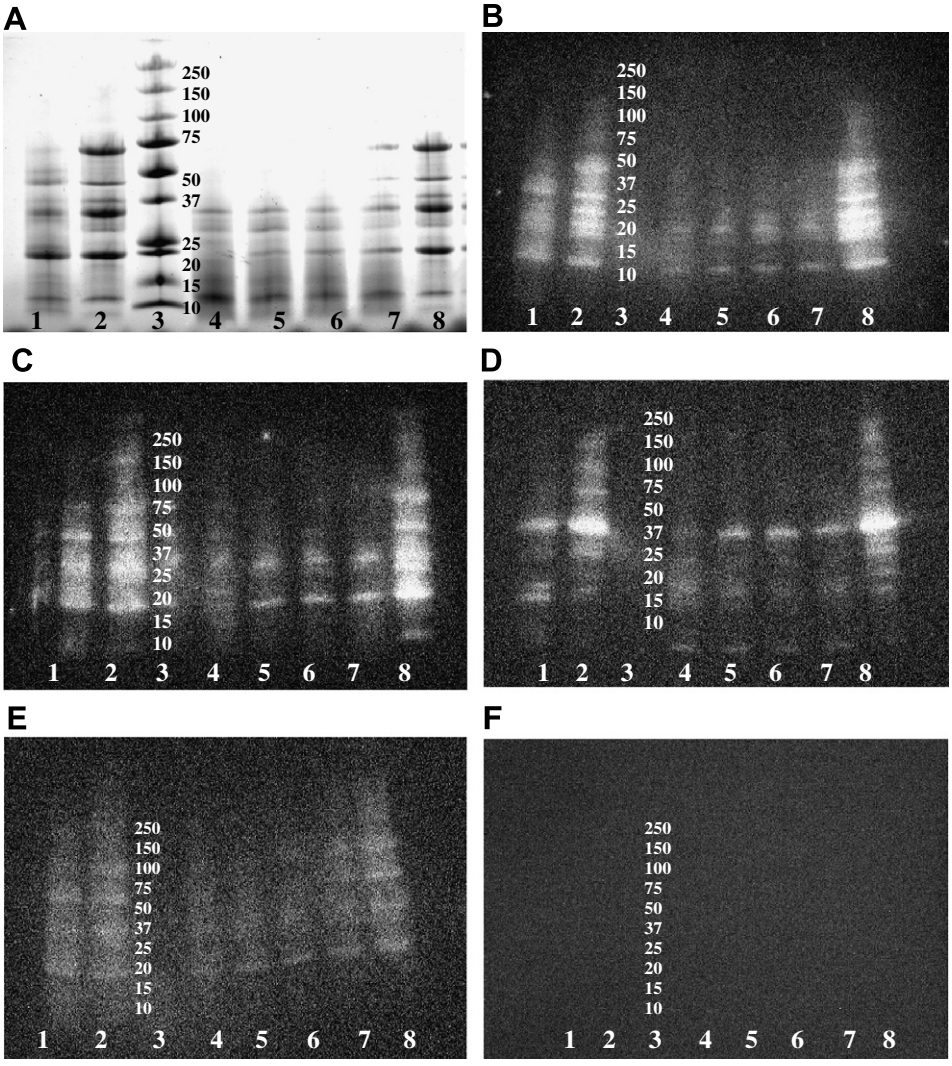

Fig. 1. A. SDS–PAGE of SBM before and after fermentation. B. Immunoblot of the same samples as A exposed to human plasma (97.5 kUA/l) and detected by chemiluminescence, C. Immunoblot of the same samples as A using pool of six human plasmas, D. Immunoblot of the same samples as A using human plasma 71.6 kUA/l, E. Immunoblot of the same samples as A using human plasma 34.6 kUA/l, F. Immunoblot of the same samples as A using human plasma 11.6 kUA/l.: 1 = Fermented ingredient I; 2 = defatted soybean meal 48% (SBM); 3 = MW Precision Plus Protein standard: 10, 15, 20, 25, 37, 50, 75, 100, 150, 250 kDa; 4 = Fermented SBM with *Saccharomyces cerevisiae*; 5 = Fermented SBM with *Bifidobacteria lactis*; 6 = Fermented SBM with *Lactobacillus plantarum*, 7 = Natural fermentation of SBM; 8 = SBM (non-fermented).

Ibidapo, 1999). The fermentation with *S. cereviseae* suggests the potential of exploring the residual enzymes from malting and brewing industries for the development of a high quality and inexpensive hypoallergenic protein hydrolysate with functional benefits.

Soy fermented with *L. plantarum* (Fig. 1B, lane 6) and natural fermentation (Fig. 1B, lane 7) showed smaller size and less intense immunoreactive peptides below 30 kDa. Lactic acid bacteria as *L. plantarum* has been used for legume fermentation and nutritional improvement (Doblado, Frias, Muñoz, & Vidal-Valverde, 2005), as well as to improve the antioxidant properties (Pyo, Lee, & Lee, 2005).

Bacterial fermentations (*B. lactis, L. plantarum*) generated similar protein patterns as natural fermentation and their immunoreactivity by western blots against human plasma showed a less intense P34 fraction, acid (33 kDa) and basic (22 kDa) glycinin subunits.

B. lactis has been used to induce soybean milk fermentation (Kamaly, 1997). Lee, Lee, Cho, Oh, and Ryu (2004) used a combination of three microorganisms (*Lactococcus lactis*, *Aspergillus oryzae* and *Bacillus subtilis*) to ferment boiled soybean resulting in amino acids and polypeptides of less than 10 kDa. These degraded proteins were tested against a serum of a soybean-sensitive patient and no antigenicity was detected. These authors suggested that proteolysis could destroy some epitopes, particularly conformational epitopes, but it could also expose linear buried epitopes within the three-dimensional native structure and/or located in hydrophobic domains of the protein upon hydrolysis (Lee et al., 2004; L'Hocine and Boye, 2007).

The results of immunoreactivity with different human plasmas showed a great deal of variability. The highest intensity plasma 97.5 kUA/l showed intense immunoreactive bands (Fig. 1B). The pooled plasma (composed by

six individual plasma samples) (Fig. 1C) showed a slightly more intense representative profile of immune reactivity. Moreover, plasma 71.6 kUA/l showed a generally weak immune-recognition with most immunodominant proteins in soybean (Fig. 1D). Because 11.6 kUA/l plasma (Fig. 1F), as well as <0.35 kUA/l (data not shown) revealed no visible results, the detection limit of the western blot assay suggests the need of plasma with potency more than 11.6 kUA/l when a protein level of 1.5 μg is applied.

The IgE results of the pooled plasma (composed by six individual plasma samples) used in this study perfectly agreed with the WHO pooled plasma G (data not shown). Therefore, because of wide individual variation, exclusive range of sensitization and different levels of exposure dependent upon nutritional habits, the utilization of the freeze-dried pool 75/502 standard (WHO) presented in Table 1, could be an alternative pooled plasma for *in vitro* assays.

Absolutely no immune reactivity was detected when plasma 0.35 kUA/l was used by ELISA or western blot assays; in agreement with Van Ree, Vieths, and Poulsen (2006).

Because the soybean products were tested against only few soybean-sensitive human plasmas, it is difficult to generalize that these products are safe for consumption by a larger population of soybean allergenic individuals. Consequently some peptides, even present in trace amounts, may be a major harmful residual allergen for some sensitized individuals. The results of this study suggest, however, that the inclusion of fermented SBM as ingredients into human and animal diets will reduce the level of allergenic proteins. In addition, fermented SBM products documented in this study can be used as an excellent protein source.

The reduction in immunoreactivity attained in this study ranged from 77% to 89% depending on the type of fermentation and sensitivity of the plasma used. These levels of reduction may be important for some allergic individuals depending on their sensitivity threshold and on the inclusion rate of the soy protein in the finished product consumed (L'Hocine and Boye, 2007). However, the clinical relevance of these findings in a practical setting still needs to be determined by human challenge studies.

3.3. Composition of total amino acids of fermented SBM products

Table 3 presents the protein concentration (nitrogen × 6.25) and amino acid profile of soybean ingredients. Unfermented SBM revealed a protein level of 47% dry matter in accordance to levels reported in the literature, 45.1–52.6% (Hong et al., 2004). The fermentation process brought about a significant increase in total protein, from 47% to 50% when SBM was naturally fermented. SBM fermented with *S. cerevisae* increased its protein level to 58%, the highest effect of all tested microorganisms. These different results in protein concentration can be due to the microorganism load during processing. With regards to levels of non-essential amino acids, unfermented SBM presented a large amount of glutamic acid followed by aspartic acid, arginine, alanine, glycine and serine, and proline. Among the essential amino acids, leucine presented the highest amount (2.3%), followed by lysine, isoleucine, valine, threonine, tyrosine, phenylalanine and histidine. In lower amounts were cysteine and methionine (0.54% and 0.48%, respectively). These results are similar to those reported by Hong et al., 2004 but slightly lower than those reported by Karr-Lilienthal, Grieshop, Merchen, Mahan, and Fahey (2004) in soybean meals.

Table 3
Total amino acid composition of defatted soybean meal (SBM) before and after fermentation (expressed as grams of amino acids per 100 g of dry matter)[a]

	Unfermented Soybean meal	Fermented soybean meal			
		Natural	*L. plantarum*	*B. lactis*	*S. cerevisae*
Protein (%)	47.08^{a}	50.16^{b}	52.08^{c}	52.14^{c}	58.08^{d}
Non essential amino acids					
Glu	6.66^{b}	8.69^{d}	7.48^{c}	6.91^{b}	5.27^{a}
Asp	4.29^{b}	5.92^{d}	5.31^{c}	4.04^{b}	2.06^{a}
Arg	2.13^{a}	2.32^{a}	2.32^{a}	2.08^{a}	4.94^{b}
Ala	2.02^{a}	2.69^{c}	2.47^{bc}	2.70^{c}	2.25^{ab}
Gly	1.69^{a}	1.90^{c}	1.74^{ab}	1.85^{bc}	2.41^{d}
Ser	1.67^{a}	2.30^{e}	2.18^{d}	1.85^{b}	2.06^{c}
Pro	1.60^{ab}	1.78^{c}	1.67^{bc}	1.53^{a}	2.14^{d}
Essential amino acids					
Leu	2.29^{b}	2.56^{c}	2.42^{bc}	2.37^{b}	2.01^{a}
Lys	1.77^{b}	2.31^{d}	2.08^{c}	1.95^{bc}	0.91^{a}
Ile	1.71^{a}	1.91^{b}	1.77^{a}	1.76^{a}	1.73^{a}
Val	1.52^{a}	1.76^{c}	1.62^{ab}	1.63^{b}	1.86^{d}
Thr	1.44^{ab}	1.66^{b}	2.10^{c}	1.47^{ab}	1.28^{a}
Tyr	1.35^{a}	1.94^{c}	1.62^{b}	1.68^{b}	2.79^{d}
Phe	1.30^{a}	1.56^{bc}	1.47^{b}	2.09^{d}	1.72^{c}
His	0.99^{ab}	1.12^{b}	0.97^{a}	0.99^{ab}	1.73^{c}
Cys	0.54^{c}	0.48^{c}	0.37^{b}	0.23^{a}	0.84^{d}
Met	0.48^{b}	0.52^{b}	0.49^{b}	0.41^{a}	0.47^{b}

[a] Data are the mean of 3 independent results. Different superscripts in the same row means statistical differences ($p < 0.05$).

When SBM was subjected to fermentation with different microorganisms, most of the amino acids increased significantly ($p < 0.05$) and only few of them suffered a decrease depending on the type of fermentation. Focusing our attention on the limiting amino acids, methionine levels did not change significantly ($p < 0.05$) under natural fermentation or when fermented with *L. plantarum* or *S. cerevisae*; while *B. lactis* caused a reduction of 15%. Cysteine, however, decreased in naturally fermented SBM or under *B. lactis* or *L. plantarum* fermentation but underwent a sharp rise from 0.54% to 0.84% after fermentation with *S. cerevisae*. Taking into consideration the limiting essential amino acids, the fermentation of SBM with *S. cerevisae* should be recommended since although methionine content was not significantly changed, cysteine showed a sharp increase (56%, $p < 0.05$). Dakwa, Sakyi-Dawson, Diako, Annan, and Amoa-Awua (2005) reported similar results than those presented here after fermenting dehulled, boiled or roasted and sun dried soybeans. According to Hong et al. (2004),

fermentation of SBM treated with *A. oryzae* strain did not affect the essential amino acid concentration but increased the concentration of glycine, glutamic and aspartic acids. Raumbault (1998) showed the effectiveness of fungal fermentation on nutritional quality and decrease in some anti-nutritional compounds (phytate and trypsin inhibitors) contained in the crude soybean. Similarly, bacterial enzymatic proteolysis have shown enhanced bioavailability of protein, fat, and increased availability of free amino acids and short chain fatty acids (Parvez, Malik, Kang, & Kim, 2006). The inclusion of novel fermented soybean ingredients with higher protein value and low anti-nutritional factors will help *in vivo* efficacy of the fermented hypoallergenic soybean ingredients.

3.4. Immunoreactivity of commercial fermented and hydrolyzed soybean ingredients

Fig. 2A shows the SDS–PAGE of the protein profile of commercially available fermented and hydrolyzed soybean ingredients. The fermented soy ingredient I (lane 1) showed an intense P34, glycinin and β-conglycinin fractions, eliminating peptides above 75 kDa. Lane 2 presents a non-fermented SBM showing the main bands of soybean protein: β-conglycinin α (68 kDa) and β (48 kDa) subunits, Gly m Bd 30 k or P34, glycinin acid (33 kDa) and basic (22 kDa) subunits. The fermented soybean protein concentrate ingredient II (lane 3) presented only peptides below 20 kDa in size. Hydrolyzed soy flour (lane 4) showed proteins <2 kDa while the rest of the enzymatically hydrolyzed soy ingredients (lane 6–8) showed proteins <20 kDa. The results suggest that depending on the fermentation treatment or the degree of hydrolysis, their protein profile varied. Fig. 2B shows the immunoreactivity against human pooled plasma of the same commercially fermented and hydrolyzed products. The results of the western blot analysis were in accordance with the results obtained by ELISA. Non-fermented soybean sample reflected a strong immunoreactivity towards the main soybean protein bands. The fermented ingredient I showed a slightly weaker immune response than the conventional soybean product. Absolutely no immune-reactivity was observed for the fermented soy protein concentrate ingredient II or the hydrolyzed soybean ingredient even with the most potent human plasma. Maldonado, Gil, Narbona, & Molina, 1998 have found that acid hydrolyzed proteins are extensively hydrolyzed (>80%) and unlikely to elicit allergenic reactions. The same authors suggest that heat treatments affect the conformational epitopes, while enzymatic hydrolysis destroys the sequential IgE-binding epitopes.

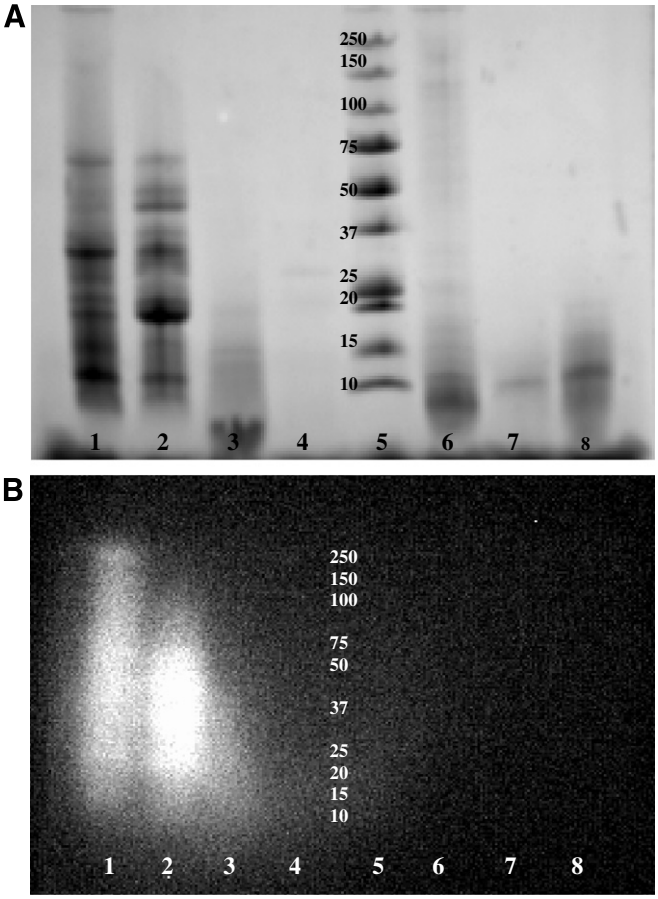

Fig. 2. A. SDS–PAGE of soybean protein extracts of various fermented or enzymatically hydrolyzed commercially available soybean ingredients. B. Western blot of the same samples as A, exposed to human pooled plasma. 1 = Fermented ingredient I; 2 = Non-fermented SBM; 3 = Fermented soy protein concentrate ingredient II; 4 = Hydrolyzed soy flour; 5 = MW Precision Plus Protein standard: 10, 15, 20, 25, 37, 50, 75, 100, 150, 250 kDa; 6 = Hydrolyzed protein concentrate; 7 = Hydrolyzed protein isolate; 8 = Hydrolyzed concentrated peptides.

Table 4 shows the protein and fat concentrations as indicated on the labels of commercially available fermented products. Moisture content, extracted protein, and $OD_{405\,nm}$ values, used as indicator of IgE immunoreactivity as measured by ELISA, are also included. Tempeh and miso products contained average soluble protein concentrations of 81.5 ± 25.0 mg/g and 70.1 ± 7.8 mg/g, respectively. This represents 7–8 times higher values than average soluble protein concentrations in yogurt products (9.7 ± 4.0 mg/g). Yogurt presented the lowest $OD_{405\,nm}$/g product (0.02 ± 0.0) followed by miso (0.5 ± 0.0) and tempeh (0.7 ± 0.1). In comparison to unfermented soybean meal (Table 2) all of these fermented products presented a drastic reduction in IgE immunoreactivity as evaluated by the $OD_{405\,nm}$ values. These levels of reactivity are also lower than the results obtained with natural and induced fermentation of soybean meal. The most common yogurt cultures used were *L. bulgaricus*, *S. thermophilus, L. acidophilus, B. bifidum, L. casei, L. rhamnosus and L. lactis.* Organisms other than lactic acid bacteria currently used in probiotic preparation include bacteria (*Bacillus* sp.), yeasts (*S. cerevisiae*) and fungi (*A. oryzae*) (Parvez et al., 2006).

Moreover, tempeh products are composed of cooked soybean that was fermented by the mold *Rhizopus oryzae*. These results agree with Franck et al. (2002) that confirmed all technologies applied to soybean-based products induce

Table 4
Protein, fat, moisture and immune response of fermented commercial soybean products

Commercial fermented soybean products	mg protein/g product[a]	mg fat/g product[a]	Moisture (%) $X \pm SD$	Extracted protein (mg protein/g product)	$OD_{405\,nm}$/g product[b]
Tempeh					
Tempeh "5 grain"	157.1	71.4	55.5 ± 3.7	63.9 ± 0.1	0.8 ± 0.3
Tempeh "organic soy"	210.5	105.3	52.7 ± 0.7	99.2 ± 3.7	0.7 ± 0.2
Miso					
Jaeresik	111.1	55.6	17.1 ± 0.2	58.1 ± 0.2	0.6 ± 0.0
Kong Duen Jang	133.3	33.3	24.5 ± 4.0	66.4 ± 1.9	0.5 ± 0.1
Shin Shu Aka (white)	111.1	55.6	33.6 ± 1.8	73.5 ± 0.1	0.7 ± 0.4
Shin Shu Aka (red)	111.1	55.6	35.9 ± 0.8	75.5 ± 3.9	0.4 ± 0.0
Gung Chung	133.3	133.3	14.3 ± 0.8	76.9 ± 2.0	0.5 ± 0.2
Yogurt					
Yogurt plain	22.0	50.0	85.4 ± 1.9	4.9 ± 0.2	0.02 ± 0.0
Yogurt vanilla	23.5	14.7	79.8 ± 0.5	9.5 ± 0.1	0.02 ± 0.0
Mango Live smoothie	29.2	179.2	80.3 ± 0.1	6.6 ± 0.1	0.02 ± 0.0
Raspberry Live smoothie	29.2	179.2	82.8 ± 0.6	7.9 ± 0.0	0.02 ± 0.0
Plain yogurt	35.3	20.6	87.3 ± 3.2	10.4 ± 0.1	0.03 ± 0.0
SoYogurt vanilla	35.3	20.6	76.6 ± 1.7	11.7 ± 0.4	0.03 ± 0.0
O'Soy yogurt vanilla	41.2	11.8	78.0 ± 0.5	17.0 ± 0.2	0.03 ± 0.0

Results from three independent ELISA measurements using human plasma code A (98.7 kUA/l).
[a] Values were taken from the product's label.
[b] Moisture concentrations were determined from three independent measurements.

remarkable variations in the protein profile and allergenicity.

ELISA and western blots results confirmed the hypoantigenic properties of the hydrolyzed and fermented commercially available soybean ingredients. During the fermentation process, epitopes of immunodominant proteins in soybean may be altered, becoming less reactive and reduce IgE binding potential. The removal of IgE epitopes, unfolding and subsequent microbial proteolysis of proteins, offers the possibility of introducing neo-epitope sites, generating new peptides and decreasing their antigenicity.

Fermentation has been used for centuries as means of improving and keeping quality of foods. Microorganisms, by virtue of their metabolic activities, contribute to the development of sensory, shelf-life and nutritional qualities. Fermented soybean can be used as functional ingredient with high protein digestibility and a good source of probiotics. There is a great deal of potential in the development of soy probiotics with low allergenicity. It is believed that probiotics have the potential to enhance endogenous barrier mechanisms of the gut and alleviate intestinal inflammation, providing a useful role for treating food allergy (MacFarlane & Cummings, 2002).

The increase in the amount of small peptides during fermentation is attributed to partial digestion of large peptides in SBM by proteases secreted by different microorganisms. Microbial hydrolysis of soy protein can change the structure of the allergens and consequently interfere with the antigen–antibody complex.

In this study, we confirmed that fermentation hydrolyzes proteins into smaller peptides, changing the amino acid composition and reduces immunoreactivity. Depending on the degree and type of microbial hydrolysis, fermentation could potentially decrease or even eliminate allergenic reactions. SBM fermented by bacteria or yeast can be excellent sources of proteins for inclusion in human and animal diets.

A clinical multi-center study on food allergy to soy in Europe, including double-blind, placebo-controlled food challenges and determination of threshold levels for clinical reactivity to soy predict that 1% of patients with soy allergy would react subjectively and objectively with 0.21 and 37.2 mg of soy protein, respectively (Ballmer-Weber et al., 2007). They also found that the pattern of IgE reactivity against proteins with molecular weights of between approximately 10 and 70 kDa was highly individual among the patients and did not correlate with the severity of symptoms. Therefore, specific IgE quantification results must be interpreted with appropriate caution. The concentration of specific IgE cannot be used as an absolute diagnostic parameter but rather as a tool to assess risk of allergic reaction. Despite the limitations, quantitative measurement of specific IgE is likely to become an increasingly important parameter in the clinical investigation of food allergy (Asero et al., 2007).

4. Conclusions

Either natural or induced fermentation significantly reduced IgE immunoreactivity up to 89% in soybean meal. Furthermore, fermentation increased significantly the levels of total protein and amino acids. Natural fermentation and *S. cereviseae* presented unique total amino acid patterns, increasing most of the essential amino acids of the final fermented SBM. All commercial soy containing products tested showed a very low immunoreactivity. Yogurt products presented the lowest antigenic activity followed

by miso and tempeh. Hydrolyzed and fermented soy ingredients showed negligible reactivity, if proteins were <20 kDa. However, the clinical relevance of these findings needs to be determined by human challenge studies. Studies on the optimization of the effect of fermentation and hydrolysis on the reduction of immune response are ongoing in our laboratory, which may lead to the development of hypoallergenic soy foods.

Acknowledgements

This work was partly supported by the Comision Española Interministerial de Ciencia y Tecnología (CICYT) with project number AGL2004-0886/ALI and USDA-Future Foods Initiative. We wish to thank Dean Andrea Bohn for her support to Y.S. Song as part of the University of Illinois, College of ACES, graduate study abroad program.

References

Asero, R., Ballmer-Weber, B. K., Beber, K., Conti, A., Dubakiene, R., Fernandez-Rivas, M., et al. (2007). IgE-mediated food allergy diagnosis: Current status and new perspectives. *Molecular Nutrition and Food Research, 51*, 135–147.

Babu, K. S., Arshad, S. H., & Holgate, S. T. (2001). Anti-IgE treatment: An update. *Allergy, 56*, 1121–1128.

Ballmer-Weber, B. K., Holzhauser, T., Scibilia, J., Mittag, D., Zisa, G., Ortolani, C., et al. (2007). Clinical characteristics of soybean allergy in Europe: A double-blind, placebo-controlled food challenge study. *Journal of Allergy and Clinical Immunology, 119*, 1489–1496.

Beyer, K., & Teuber, S. (2004). The mechanism of food allergy: What do we know today? *Current Opinions in Allergy and Clinical Immunology, 4*, 197–199.

Bindslev-Jensen, C., Briggs, D., & Osterballe, M. (2002). Can we determine a threshold level for allergenic foods by statistical analysis of published data in the literature? *Allergy, 57*, 741–746.

Bisson, L. F., Coons, D. M., Kruckeberg, A. L., & Lewis, D. A. (1993). Yeast sugar transporters. *Critical Reviews in Biochemistry and Molecular Biology, 28*, 259–308.

Burks, W., & Ballmer-Weber, B. (2006). Review: Food allergy. *Molecular Nutrition and Food Research, 50*, 595–603.

Cordle, C. T. (2004). Soy protein allergy: Incidence and relative severity. *American Society for Nutritional Sciences, 134*, 1213S–1239S.

Dakwa, S., Sakyi-Dawson, E., Diako, C., Annan, N. T., & Amoa-Awua, W. K. (2005). Effect of boiling and roasting on the fermentation of soybeans into dawadawa (soy-dawadawa). *International Journal of Food Microbiology, 104*, 69–82.

De Mejia, E. G., Vasconez, M., De Lumen, B. O., & Nelson, R. (2004). Lunasin concentration in different soybean genotypes, commercial soy protein, and isoflavone products. *Journal of Agricultural and Food Chemistry, 52*, 5882–5887.

Deshpande, S. S., Salunkhe, D. K., Oyewole, O. B., Azam-Ali, S., Battcock, M., & Bressani, R. (2000). *Fermented Grain Legumes. Seeds and Nuts. A global Perspective*. Rome, Italy: F.A.O.

Doblado, R., Frias, J., Muñoz, R., & Vidal-Valverde, C. (2005). Effect of processing on the antioxidant vitamins and antioxidant capacity of *Vigna sinensis* var. Carillla. *Journal of Agricultural and Food Chemistry, 53*, 1215–1222.

Doblado, R., Zielinski, H., Piskula, M., Kozlowska, H., Muñoz, R., Frias, J., et al. (2003). Fermentation of *Vigna sinensis* var carilla flours by natural microflora and Lactobacillus species. *Journal of Food Protection, 66*, 2313–2320.

Fiocchi, A., Travaini, M., D'Auria, E., Banderali, G., Bernardo, L., & Riva, E. (2003). Tolerance to a rice hydrolysate formula in children allergic to cow's milk and soy. *Clinical and Experimental Allergy, 33*, 1576–1580.

Food and Drug Administration (FDA). (2005). Approaches to establish thresholds for major food allergens and for gluten in food. Available from http://cfsan.fda.gov/~dms/alrgn.html#table-ii-1.

Food and Drug Administration (FDA). (2004). Food Allergen Labelling and Consumer Protection (FALCP) Act of 2004. Available from http://www.cfsan.fda.gov/~acrobat/alrgact.pdf.

Franck, P., Moneret-Vautrin, D. A., Dousset, B., Kanny, G., & Nabet, P. (2002). The allergenicity of soybean-based products is modified by food technologies. *International Archives of Allergy and Immunology, 128*, 212–219.

Frias, J., Vidal-Valverde, C., Kozlowska, H., Tabera, J., Honke, J., & Hedley, C. L. (1996). Natural fermentation of lentils influence of time, on the kinetics of monosaccharides, disaccharide and alpha-galactoside. *Journal of Agricultural and Food Chemistry, 44*, 579–584.

Friedman, M., & Brandon, D. L. (2001). Nutritional and health benefits of soy proteins. *Journal of Agricultural and Food Chemistry, 49*, 1069–1086.

Gibbs, P. A. (1987). Novel uses for lactic acid fermentation in food preservation. *Journal of Applied Bacteriology Symposium, Suppl. 16*, 51S–58S.

Goodwin, P. R. (2004). Food allergen detection methods: A coordinated approach. *Journal of AOAC International, 87*, 1383–1390.

Hong, K. J., Lee, C. H., & Kim, S. W. (2004). *Aspergillus oryzae* GB-107 fermentation improves nutritional quality of food soybean and feed soybean meals. *Journal of Medical Food, 7*, 430–435.

Ito, M., Kato, T., & Matsuda, T. (2005). Rice allergenic proteins, 14–16 kDa albumin and α-globulin, remain insoluble in rice grains recovered from rice miso (rice containing fermented soybean paste). *Bioscience Biotechnology and Biochemistry, 69*, 1137–1144.

Kabourek, J. L., & Taylor, S. L. (2003). Soy foods and allergies: Separating fact from fiction. *The Soy Connection, 11*, 1–6.

Kamaly, K. M. (1997). Bifidobacteria fermentation of soybean milk. *Food Research International, 30*, 675–682.

Karr-Lilienthal, L. K., Grieshop, C. M., Merchen, N. R., Mahan, D. C., & Fahey, G. C. Jr., (2004). Chemical composition of soybeans and soybean meals from five leading soy bean-producing countries. *Journal of Agricultural and Food Chemistry, 52*, 6193–6199.

Kobayashi, M. (2005). Immunological functions of soy sauce: Hypoallergenicity and antiallergenic activity of soy sauce. *Journal of Bioscience and Bioengineering, 100*, 144–151.

L'Hocine, L., & Boye, J. I. (2007). Allergenicity of soybean: new developments in identification of allergenic proteins, cross-reactivities and hypoallergenization technologies. *Critical Reviews in Food Science and Nutrition, 47*, 127–143.

L'Hocine, L., Boye, J. I., & Munyana, C. (2007). Detection and quantification of soy allergens in foods: Study of two commercial enzyme-linked immunosorbent assays. *Journal of Food Science, 72*, 145–153.

Lee, U.-W., Keum, E.-H., Lee, S.-J., Sung, D.-E., Chung, D.-H., Lee, S.-I., et al. (2007). Allergenicity of proteolytic hydrolysates on the soybean 11S globulin. *Journal of Food Science, 72*, 168–172.

Lee, J. O., Lee, S. I., Cho, S. H., Oh, C. K., & Ryu, C. H. (2004). A new technique to produce hypoallergenic soybean proteins using three different fermenting microorganisms. *Journal of Allergy and Clinical Immunology, 113*, S239.

MacFarlane, G. T., & Cummings, J. H. (2002). Probiotics, infection and immunity. *Current Opinion Infectious Diseases, 15*, 501–506.

Maldonado, J., Gil, A., Narbona, E., & Molina, J. (1998). Special formula in infant nutrition: A review. *Early Human Development, Suppl. 53*, S23–S32.

Martínez-Villaluenga, C., Gulewicz, P., Frias, J., Gulewicz, K., & Vidal-Valverde, C. (2007). Assessment of protein fractions of three cultivars of *Pisum sativum* L.: Effect of germination. *European Food Research and Technology*. doi:10.1007/s 00217-007-0678-9.

Ogawa, T., Samoto, M., & Takahashi, K. (2000). Soybean allergens and hypoallergenic soybean products. *Journal of Nutritional Science and Vitaminology, 46*, 271–279.

Parvez, S., Malik, K. A., Kang, S.-Ah., & Kim, H.-Y. (2006). Probiotics and their fermented food products are beneficial for health. *Journal of Applied Microbiology, 100*, 1171–1185.

Poms, R. E., Anklam, E., & Kuhn, M. (2004). Polymerase chain reaction techniques for food allergen detection. *Journal of AOAC International, 87*, 1391–1397.

Pyo, Y. E., Lee, T. C., & Lee, Y. C. (2005). Effect of lactic acid fermentation on enrichment of antioxidant properties and bioactive isoflavones in soybean. *Journal of Food Science, 70*, S215–S220.

Raumbault, M. (1998). General and microbiological aspects of solid substrate fermentation. *Journal of Biotechnology, 1*, 174–187.

Sampson, H. A. (1997). Food allergy. *Journal of the American Medical Association, 278*, 1888–1894.

Sanni, A. I., Onilude, A. A., & Ibidapo, O. O. T. (1999). Biochemical composition of infant weaning food fabricated from fermented blends of cereal and soybean. *Food Chemistry, 65*, 35–39.

Sicherer, S. H., Munoz-Furlong, A., & Sampson, H. (2004). Prevalence of seafood allergy in the United States determined by a random telephone survey. *Journal of Allergy and Clinical Immunology, 10*, 159–165.

Sicherer, S. H., & Sampson, H. A. (2006). Food allergy. *Journal of Allergy and Clinical Immunology, 117*, S470–S475.

Taylor, S. L., & Hefle, S. L. (2001). Food allergies and other food sensitivities. *Food Technology, 55*, 68–83.

Taylor, S. L., & Helfe, S. L. (2000). Hidden triggers of adverse reactions to foods. *Canadian Journal of Allergy and Clinical Immunology, 5*, 106–110.

United States Department of Agriculture. (2007). Soybean industry statistics. Available from http://www.ers.usda.gov/News/soybeancoverage.htm.

Van Ree, R., Vieths, S., & Poulsen, L. K. (2006). Allergen-specific IgE testing in the diagnosis of food allergy and the event of a positive match in the bioinformatics search. *Molecular Nutrition and Food Research, 50*, 645–654.

Wilson, S., Blaschek, K., & de Mejia, E. G. (2005). Allergenic proteins in soybean: Processing and reduction of P34 allergenicity. *Nutrition Reviews, 63*, 47–58.

Yamanihi, R., Huang, T., Tsuji, H., Bando, N., & Ogawa, T. (1995). Reduction of the soybean allergenicity by the fermentation with *Bacillus natto*. *Food Science and Technology International, 1*, 14–17.