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Immunoreactivity and Amino Acid Content of Fermented Soybean Products

JUANA FRIAS,[†] YOUNG SOO SONG,[‡] CRISTINA MARTÍNEZ-VILLALUENGA,[†] ELVIRA GONZÁLEZ DE MEJIA,[‡] AND CONCEPCION VIDAL-VALVERDE*,[†]

Department of Food Science and Human Nutrition, University of Illinois at Urbana–Champaign, 228 ERML, MC-051, 1201 West Gregory Drive, Urbana, Illinois 61801, and Instituto de Fermentaciones Industriales (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain

Food allergy has become a public health problem that continues to challenge both the public and the food industry. The objective of this research was the detection and quantification of the major human allergenic soy proteins and to study the reduction in immunoreactivity and improvement of amino acid content after fermentation of soybean flour. Fermentation was carried out in the solid state of cracked seeds inoculated with *Aspergillus oryzae*, *Rhizopus oryzae*, and *Bacillus subtilis* and in the liquid state of milled soybean flours fermented naturally by microorganisms present only in the seeds or by inoculation with *Lactobacillus plantarum*. ELISA and Western blot were used to quantify IgE antibody response, and HPLC was used to identify and quantify total amino acids. *L. plantarum* fermented soy flour showed the highest reduction in IgE immunoreactivity (96–99%) depending upon the sensitivity of the plasma used. Among the solid fermented products, the lowest reduction in immunoreactivity was obtained when mold strains, *R. oryzae* and *A. oryzae*, were used (66 and 68%, respectively, for human plasma 97.5 kUA/L). Among the solid fermented products, those inoculated with *B. subtilis* yielded a 81 and 86% reduction in immunoreactivity against both human plasma 97.5 IgE kUA/L and human pooled plasma samples, respectively. When soybean was subjected to liquid fermentation, most of the total amino acids increased significantly ($p \leq 0.05$). In solid fermentation with *R. oryzae*, only Ala and Thr content improved. Fermentation can decrease soy immunoreactivity, and there is potential of developing nutritious hypoallergenic soy products.

KEYWORDS: Soybean; fermentation; immunoreactivity; IgE immunoreactivity; amino acids; hypoallergenic foods; antigenicity

INTRODUCTION

Food allergy is a relatively rare and violent reaction of the immune system toward food proteins. It is defined as an immunologically based adverse reaction in response to dietary antigens (*1*). Small regions in the allergenic proteins, called epitopes, provoke the immunoglobulin E (IgE)-mediated allergenic response (*2*). The allergen provokes an initial IgE antibody response followed by a secondary IgE antibody response, which signals an allergic reaction (*3, 4*). Food proteins bind to the allergen-specific IgE molecules residing in the mast cells and basophils, causing them to release inflammatory mediators, including histamine (*1*). Food allergy affects 3% of the adult population and up to 6–8% of infants. Allergenic conditions directly affect many millions of people worldwide (*5–7*). The estimated prevalence of soybean allergies is about 0.5% of the total U.S. population (*8–10*).

Soybean is important both from economical and nutritional standpoints. It is one of the most important grains of the legume family, and one of the main protein sources for livestock. Acceptance of soybean protein products has increased because of the low cost and high nutritional quality for human consumption and also as protein source for animal meals.

In the United States, the Food Allergen Labeling and Consumer Protection Act of 2004 (P.L. 108-282) (*11*) includes soy in its definition of the "big 8", which comprises those foods that cause the most allergenic reactions (*12–14*). Food allergens are complex mixtures of potentially immunoreactive proteins. Currently, there have been identified 33 IgE-binding allergenic proteins in soybean (*15*). According to Wilson et al. (*16*), soybean allergens comprise proteins with molecular masses from 7.0 to 71 kDa. A few of these 33 proteins are responsible for a majority (~90%) of the allergenic responses, making it important to identify and purify these key proteins.

Several studies have confirmed the degradation of soybean allergens during fermentation by microbial proteolytic enzymes in soy sauce, miso, soybean ingredients, and feed-grade soybean meals (*17–19*). Fermentation has the capacity to improve

* To whom correspondence should be addressed. Telephone: +34-915622900 Ext. 241. Fax: +34-915644853. E-mail: ificv12@ifi.csic.es.
[†] Instituto de Fermentaciones Industriales (CSIC).
[‡] University of Illinois at Urbana–Champaign.

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nutritional and functional properties compared to original products. Fermentation is an important area of research, which entails the ability to hydrolyze soy protein into smaller peptides. Soy sauce possesses microbial proteolytic enzymes able to degrade soybean proteins, including major soybean allergen (Gly m Bd 30K) into peptides and amino acids (*18*). Altering the structure of their epitopes can lower the immunoreactivity of soybean-containing products during processing. Moreover, the fermentation process affects the nutritional quality of legumes by improving the protein content and digestibility as a consequence of the partial degradation of complex stored proteins into more simple and soluble products (*20*).

The objectives of this study were the detection and quantification of soybean allergens, to produce hypoallergenic soy-containing ingredients by natural and induced fermentation with *Lactobacillus plantarum*, *Bacillus subtilis*, *Aspergillus oryzae*, and *Rhizopus oryzae* and to assess the type and level of amino acids produced during this process.

MATERIALS AND METHODS

Chemicals. Trizma-hydrochloride, 2-mercaptoethanol (2-ME), sodium chloride, sodium carbonate, ammonium sulfate, phosphate-buffered saline−Tween buffer (PBST) at pH 7.4, bovine serum albumin (BSA), Tween-20, Tris-buffered saline (TBS) tablets, *para*-nitrophenyl phosphate (PNPP), ethylenediaminetetraacetic acid (EDTA), deoxycholate, and Corning Sterile syringe filter pore size of 0.2 μm were obtained from Sigma-Aldrich (St. Louis, MO). Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS–PAGE) was performed with Phastsystem (Amersham-Pharmacia, Inc., NJ). Reagents used for SDS−PAGE included Laemmli blue dye, running buffer with TG/SDS, 12% precast polyacrylamide-gradient minigels (Amersham), buffer strips for Phastsystem minigels (Amersham), Coommassie Blue biosafe dye, and prestained SDS−PAGE molecular-weight broad-range standards, which were purchased from BioRad (Hercules, CA). The immunoblot PVDF membrane (for protein blotting, 0.2 μm) 7 × 8.4 cm was purchased from BioRad. The Bradford assay was conducted using a Biorad Bradford reagent. Other reagents, such as analytical-grade NaOH, H_2SO_4, and HCl, used occasionally to adjust buffers and stop the color substrate reaction, were purchased from Sigma Chemical (St. Louis, MO).

Biological Materials. Soybeans (*Glycine max* L. cv. *merit*) were provided by the Mang Fong Pacific Trading, S.A. Seeds were cleaned and stored in darkness in polyethylene containers at 4 °C until use. *A. oryzae* 2094T (ATCC 1011), *R. oryzae* CECT 2340 (ATCC 24563), *B. subtilis* CECT 39T (ATCC 6051), and *L. plantarum* CECT 748T (ATCC 14917) were purchased from the Spanish Type Culture Collection (CECT) and used as inocula, as in Fernández-Orozco et al. (*17*). Stock cultures were grown and maintained as follows: *A. oryzae* and *R. oryzae* were grown for 7 days on potato dextrose agar (Difco Laboratories, Detroit, MI) at 30 °C, and the spores were collected and washed twice in sterile saline solution and used as inocula. *B. subtilis* was grown aerobically in BHI broth (Difco Laboratories, Detroit, MI) for 18 h at 30 °C. The pelleted cells were washed twice in sterile saline solution and used as inocula. *L. plantarum* was grown in MRS broth (Difco Laboratories, Detroit, MI) for 18 h at 30 °C. The cells were washed twice in sterile saline solution (8.5% NaCl) and used as inocula.

The characteristics of human sera used in the immunological experiments, provided by PlasmaLab International (Everett, WA), are described in **Table 1**. A pooled plasma sample for Western blot analysis was prepared by combining equal volumes of human plasma samples A, B, C, D, E, and F.

Soybean Fermentation. Different fermentation processes of soybean were conducted as previously described (*21*).

Solid-state fermentations were carried out using cracked seeds suspended in sterile distilled water (1:2, w/v) for 16 h and subsequently autoclaved at 121 °C for 15 min. Further, *A. oryzae* (10^5 spores/g), *R. oryzae* (10^5 spores/g), or *B. subtilis* (10^5 CFU/g) starter cultures were inoculated at 5% (v/w) in cracked seeds. The microbial suspensions were aseptically distributed over Petri dishes containing 30 g of beans and placed in a climatic incubator (Memmert, Germany) at 30 °C and 90% relative humidity for 48 h. After fermentation, the fermented cracked seeds were autoclaved at 121 °C for 15 min and freeze-dried. Solid-state fermentations were performed in triplicate.

Table 1. Characteristics of Human Plasma Used in Immunological Experiments[a]

plasma number	age (years)	gender	soybean-specific IgE by inmunoCAP analysis (kUA/L)[a]
A	36	m	98.7, 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 and F, nonatopic 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, U.K., collected lyophilized pooled sera from nine donors, from patients suffering from allergic disorders.

Liquid-state fermentations were performed in either milled soybean flours (sieved at 0.25–0.50 mm) or in cracked soybeans that were suspended in sterile distilled water (1:5, w/v). They were then subjected to natural fermentation by means of the microorganisms naturally present on the surface of the seeds or were inoculated with 10% (v/v) of *L. plantarum* suspension (10^8 CFU/mL). Fermentation was carried out in a 2 L stirred fermentor (Infors AG, Switzerland) at 350 rpm for 48 h at 37 °C. After fermentation, the samples were autoclaved at 121 °C for 15 min and freeze-dried. Liquid-state fermentations were performed in duplicate. All fermentations were monitored to control the microbiological status as described in a previous work (*21*).

Protein Extraction from Soybean. The extraction procedure was performed by following the procedure described by De Mejia et al. (*22*) that consisted of placing 0.05 g of the lyophilized fermented soybean powder and 1 mL of the extracting buffer (0.05 M Tris-HCl buffer at 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 using a recirculation bath (Endocal model RTE-9, Neslab Instruments, Portsmouth, NH). At least three independent extractions were performed for each sample. After extraction, the samples were centrifuged at 20000*g* for 30 min at 4 °C in an Eppendorf centrifuge (model 5417R, Brinkmann Instruments, Westbury, NY) and the obtained supernatant was transferred to a new Eppendorf tube. This material was then diluted (1:2) with extracting buffer for enzyme-linked immunosorbent assay (ELISA) analysis.

Total Soluble Protein Quantification. Soluble protein was quantified by the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA) for the microplate system. The reaction fundamentals are based on the Lowry (Folin) assay, and a micro-assay procedure was developed by adding 25 μL of reagent A, followed by 5 μL of either protein extract or BSA for the standard curve performed in the range of 10−1600 μg/mL ($y = 0.186x + 0.006$, $R^2 = 0.996$). Once the protein extract was added, immediate mixing was required. Then, 200 μL of reagent B was added, followed by 15 min of incubation at room temperature. Microplates were read at 690 nm. The total soluble protein concentration of the soybean extracts was quantified on the basis of the standard curve. Soluble protein extracts low in protein were concentrated for SDS−PAGE. A Millipore stirred ultrafiltration cell (Model 8200, Millipore Corporation, Bedford, MA) was used with a Millipore ultrafiltration regenerated cellulose membrane (NMWL: 3000; Millipore Corporation, Bedford, MA).

High-Performance Liquid Chromatography (HPLC) Analysis of Total Amino Acids. Determination of total amino acids was carried

Table 2. Effect of Fermentation of Soybean (*G. max* L. cv. *merit*) Cracked Seeds and Flour on the Immune Response by ELISA[a]

G. max L. cv. *merit*	extracted protein (mg of protein/g of product)[b]	$OD_{405\ nm}$/g of product[c]	reduction[c,d] (%)	$OD_{405\ nm}$/g of product[e]	reduction[d,e] (%)
raw seed	320.0 ± 18.5 e	20.1 ± 0.4 g	0.0 ± 0.0 a	11.8 ± 0.4 g	0.0 ± 0.0 a
solid-state fermentation					
B. subtilis (cracked seed)	221.5 ± 9.9 d	3.8 ± 0.1 d	80.9 ± 0.7 d	1.6 ± 0.0 d	86.3 ± 0.3 d
R. oryzae (cracked seed)	92.0 ± 6.9 ab	6.9 ± 0.1 f	65.7 ± 0.5 b	3.0 ± 0.1 f	74.5 ± 0.1 b
A. oryzae (cracked seed)	82.3 ± 2.4 a	6.4 ± 0.1 e	68.3 ± 1.0 c	2.0 ± 0.0 e	82.9 ± 0.7 c
liquid-state fermentation					
natural fermentation (cracked seed)	109.3 ± 9.6 b	2.1 ± 0.1 c	89.6 ± 0.6 e	0.6 ± 0.1 c	95.3 ± 1.2 e
L. plantarum (cracked seed)	124.7 ± 6.3 c	1.5 ± 0.1 b	92.6 ± 0.6 f	0.4 ± 0.0 bc	97.0 ± 0.1 f
natural fermentation (flour)	99.9 ± 6.8 b	1.5 ± 0.2 b	92.4 ± 0.8 f	0.3 ± 0.0 bc	97.5 ± 0.1 f
L. plantarum (flour)	102.7 ± 7.6 b	0.7 ± 0.1 a	96.3 ± 0.5 g	0.1 ± 0.0 a	99.0 ± 0.0 g

[a] Columns with different letters indicate statistical differences ($p \leq 0.05$, $n = 3$). [b] Mean value ± standard deviation. Values are the means of three determinations. [c] Human plasma (97.5 kUA/L). [d] Reduction calculated on the basis of grams of fermented product. [e] Pooled human plasma (see the Materials and Methods).

out by acid hydrolysis, derivatization, and HPLC quantification using the method of Martínez-Villaluenga et al. (*23*). In brief, 200 μL (0.2 mmol/mL) of D,L-norleucine (Sigma Chem Co.) were added to 50 mg of sample as an 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. For amino acid derivatization, phenylisothiocyanate (PITC, 99%, Aldrich) was used. The chromatographic system consisted of an Alliance Separation Module 2695 (Waters, Milford, MA), a photodiode array detector 996 at 254 nm (Waters, Milford, MA), 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 at pH 6.5) and buffer B (0.1 M 44:46:10 ammonium acetate/acetonitrile/methanol, v/v/v) at pH 6.5 allowed for the separation of the amino acids in 65 min.

Parameters in Indirect ELISA. An indirect ELISA was performed to study the soy IgE immunoreactivity using plasma from donors clinically tested to suffer from soybean allergies. For indirect ELISA, 100 μL of the soy protein extracts were applied 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, and 1 tablet of TBS in 15 mL of deionized water. Between each step in the procedure, the plates were washed 4 times in PBST at pH 7.4 using a Biotek Instruments Elx50 auto-strip washer (Winooksi, VA). A total of 100 μL per well of the human plasmas (1:5 in 1% BSA) (**Table 1**) (Plasmalab Int., Everett, WA) was applied onto the Maxisorp immunoassay plate and incubated for 1 h at 37 °C and washed again. PBS buffer was used as a negative control. Then, a 100 μL per well of goat-antihuman IgE alkaline phosphatase diluted in antibody buffer (1:1000 in 1% BSA) (Bethyl Laboratories, Inc., TX) was added, allowed to incubate in the plate for 1 h at room temperature, and washed to eliminate any remaining unbound antibody. The bound secondary antibody was detected by the addition of 100 μL per well of color reagent PNPP for 45 min at room temperature. The reaction was stopped by the 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 the 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. The $OD_{405\ nm}$ values obtained were directly proportional to IgE concentrations. The percent reduction in immunoreactivity, in each fermented product, was calculated in comparison to the raw soybean meal as follows:

$$\frac{OD_{405\ nm}\text{/g of raw soybean} - OD_{405\ nm}\text{/g of fermented soybean}}{OD_{405\ nm}\text{/g of raw soybean}} \times 100$$

SDS−PAGE. Electrophoresis was carried out in a Phastsystem Kit (Amersham-Pharmacia, Inc., NJ) with an 8–25% polyacrylamide-gradient gel (Pharmacia). Extracted samples were centrifuged (20000*g*) to eliminate any precipitate that might have formed during freezing or thawing. The supernatant (20 μL, 3 mg/mL) was added to 20 μL of Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) with 5% β-mercaptoethanol (Sigma, MO). Samples were boiled for 5 min using the Laemmli buffer system. After the samples and molecular-weight standard had cooled down to room temperature, 3 μL of soybean protein extracts with an equivalent of 1.5 μg of protein (8 columns with a 1 μL loading volume application) were loaded into PhastSystem ready gels. A broad-range protein standard (Bio-Rad Kaledoiscope prestained) containing pure recombinant proteins of the following molecular weights: 250, 150, 100, 75, 50, 37, 25, 20, 15, and 10 kDa was used. Also, a polypeptide standard (BioRad) containing pure proteins of the following molecular weights: 1423, 3496, 6500, 14 400, 14 437, 16 950, 21 500, and 26 625 Da was used. The gels were run using the Phastsystem electrophoresis kit (Amersham-Pharmacia, Inc., NJ) at a 55 mA constant, and gels were run for 20 min (60–125 V). For electrophoresis, the gels were fixed for 20 min in methanol/acetic acid/water (5:5:2) 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. The different soy protein samples were read in a Kodak Image Station 440 CF, recording their respective molecular weights and band intensities.

Western Blot Procedures with Human Plasma. Unstained gels were soaked in 20 mL of blotting buffer at pH 8.3, which consisted of 10% (v/v) methanol, 25 mM Tris base, and 192 mM glycine, for 3 min. The membrane used was the Immun-Blot PVDF membrane. A Western blot sandwich was assembled by placing a sponge, a filter, and the gel, the PVDF membrane, another filter and sponge, avoiding the formation of bubbles. After the transfer was completed, the membrane was then saturated by incubation in 5% nonfat 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 soy-sensitive patients (PlasmaLab International, Everret, WA) in 3% BSA and TTBS buffer. The membrane was washed 3 times in TTBS and then incubated for 1 h at room temperature at 1:1000 dilution goat antihuman IgE alkaline phosphatase (Bethyl Laboratory, Inc., TX) prepared in 3% NFDM in TTBS buffer. The membrane was washed 3 times for 5 min with 0.01% TTBS and prepared for detection using an Immun-Star chemiluminescence alkaline phospatase substrate solution (BioRad Laboratories, Hercules, CA) following the recommendations of the manufacturer. After the substrate to the blot was added, the intensity of the bands was read in a Kodak Image Station 440 CF (Kodak, Rochester, NY) with a total exposure time of 2 min and 8 captures.

Statistical Analysis. Data were subjected to multifactor analysis of variation (ANOVA) using the least-squared difference test with the Statgraphic 4.0 for Windows (Statistical Graphics Corporation, Rockville, MD).

RESULTS AND DISCUSSION

Table 2 presents the concentration of the extracted protein and immunoreactivity values by indirect ELISA of fermented soybean ingredients (*G. max* L. cv. *merit*) that underwent different fermentation procedures, solid-state (cracked seeds) and liquid-state (flour and cracked seeds) fermentation.

The extractable protein concentration of raw soybean was 320 mg of protein/g of product, while the liquid fermented products, by natural and induced fermentation, using *L. plantarum*, were 99.9 and 102.7 mg of protein/g of product, respectively (**Table 2**). The extractable protein concentration was 3 times lower for liquid fermented products than raw seeds. This phenomenon has been previously observed in our laboratory (*23*). According to our own experience, the content of soluble protein decreases during legume fermentation. This could be due to the fact that microorganisms and enzymes involved in the fermentation process can easily hydrolyze soluble proteins. Consequently, new peptides are formed, and the conditions used to extract original proteins are not adequately extracting the new peptides.

Among the solid fermented products, the lowest reduction in immunoreactivity was obtained when using mold strains, *R. oryzae* and *A. oryzae* (66 and 68%, respectively). Molds grow at a lower rate, generating lower epitopes alteration, giving a higher immune response with human plasma (97.5 kUA/L).

Among the solid fermented cracked seeds, the highest soluble protein concentration (221.5 mg of protein/g of product) was obtained by *B. subtilis*. The fermented product with *B. subtilis* produced a reduction in immunoreactivity of 81% using highly sensitive human plasma (97.5 kUA/L) and 86% with pooled plasma.

The identity of the naturally present microorganisms in soybean was confirmed by the sampling of the final fermented extract in the deMan Rogosa Sharpe (MRS) medium under aerobic conditions, and similar bacterial growth as *L. plantarum* product was detected (*21*). From this control step, it can be deduced that the *Lactobacillus* species may be identified as one of the naturally present microorganisms in soybean seeds.

Fermented flour obtained by liquid fermentation with *Lactobacillus* sp. was able to further break down and use available proteins as nutrient sources. Its low immune response (99% reduction using pool plasma) might be related to modified epitopes with lower affinity or also to the complete transformation of the original epitopes into a less reactive site. Natural and *L. plantarum* fermented flour showed the highest reduction in immunoreactivity (92 and 96%, respectively) against human plasma (97.5 kUA/L), in comparison to liquid fermented cracked soybean (90 and 93% reduction, respectively). These results indicate that fermentation processes are affected by the particle size of the initial raw material.

The use of *in vitro* testing of immunoreactivity is relevant because it was performed using plasma from humans with declared allergy to soy. Plasmas with different degrees of immunoresponse were used, and a clear dose–response effect was observed. However, the concentration of specific IgE cannot be used as an absolute diagnostic parameter but rather as a tool to assess the 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 (*24*).

Several studies have explored various technologies, such as soybean preparations by mutation, salting out, or enzymatic degradation, and high pressure to enhance the reduction of soybean allergenic proteins (*25, 26*). Among these technological approaches, fermentation was chosen as a means to decrease residual antigenicity of soybean proteins. All fermentation technologies applied in this research to soybean-based products induced remarkable variation in the protein profile and immunogenicity. The goal was to produce hydrolysates with low immunoreactivity that could be used in special hypoallergenic

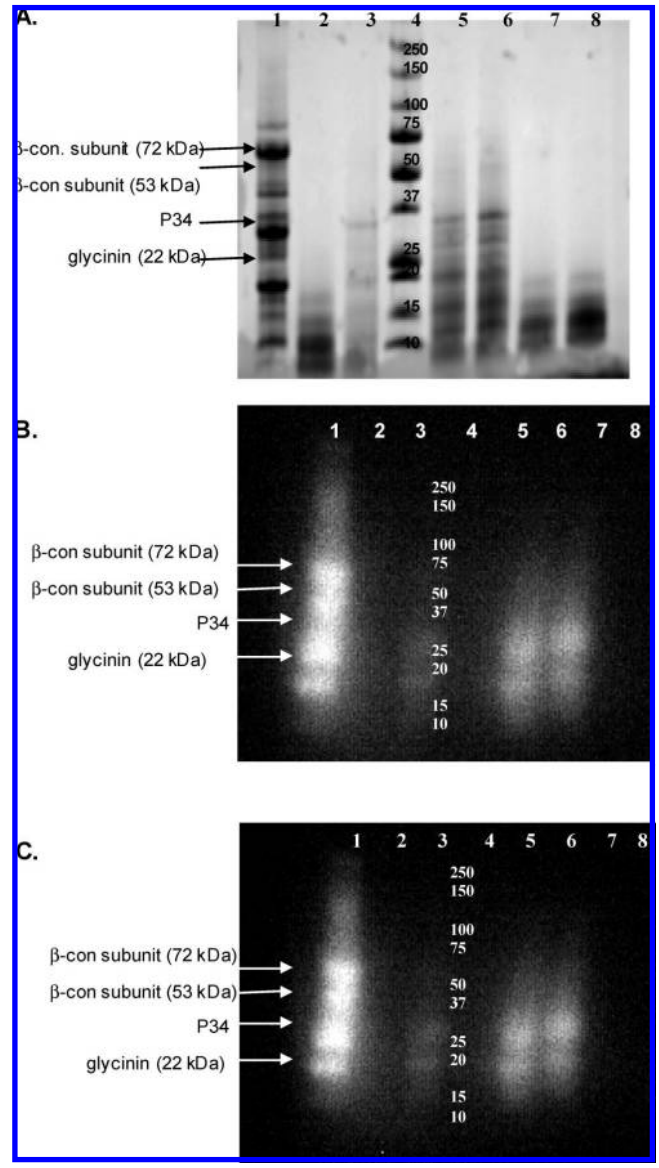

Figure 1. (**A**) SDS−PAGE profile of peptides in soybean (*G. max* L. cv. *merit*) before and after liquid and solid fermentation. (**B**) Immunoblot of the same samples as A using human plasma 97.5 kUA/L and detected by chemiluminescence. (**C**) Immunoblot of the same samples as A using pooled human plasma and detected by chemiluminescence. Lane 1, raw soybean seeds; lane 2, soybean flour *L. plantarum* fermentation; lane 3, soybean cracked *L. plantarum* fermentation; lane 4, molecular-weight marker Kaleidoscope prestained standard BioRad (Hercules, CA); lane 5, cracked soybean *A. oryzae* fermentation; lane 6, cracked soybean *R. oryzae* fermentation; lane 7, cracked soybean by *B. subtilis* fermentation; and lane 8, cracked soybean by natural fermentation. β-Conglycinin subunits (72 and 53 kDa), P34, and glycinin (22 kDa) are shown with arrows.

foods. The reduction in immunoreactivity attained in this study ranged from 66 to 99% depending upon the type of fermentation and sensitivity of the plasma used. These levels of reduction in immunoreactivity may be important for some allergic individuals depending upon their sensitivity threshold and the inclusion rate of the soy protein in the finished product consumed (*27*). However, the clinical relevance of these findings in a practical setting still needs to be determined by human challenge studies.

Figure 1A presents the electrophoretic profiles of raw and fermented soybean flour and cracked seeds. Parts **B** and **C** of **Figure 1** present the immunodominant proteins interacting with

human plasma 97.5 kUA/L and human pooled plasma samples, respectively. The nonfermented soybean was the sample presenting the highest complexity protein profile and plasma immunoreactivity toward α- (72 kDa) and β- (53 kDa) conglycinin subunits, P34 fraction, and glycinin basic (33 kDa) and acidic (22 kDa) subunits trypsin inhibitor (20 kDa). The complex protein profile of nonfermented soybean can be compared to the reduced intensity immunodominant proteins present in mold and bacterial fermentation products (lanes 2–8).

Liquid fermentation of flour with *L. plantarum* showed an electrophoretic profile with proteins below 15 kDa and a lower immune response, while liquid fermentation carried out with cracked soybean inoculated with this bacteria showed less intense bands below 40 kDa. These results are in accordance with previous immunoreactivity measured by indirect ELISA, confirming the efficacy of liquid fermentation.

The solid fermented *B. subtilis* product also showed low-range proteins below 20 kDa (**Figure 1A**), but no visible specific immunoreactivity was shown in Western blots (parts **B** and **C** of **Figure 1**).

In contrast, the two solid fermented products obtained from molds, *A. oryzae* and *R. oryzae*, showed middle-range proteins (<50 kDa). The immunoreactivity patterns presented by Western blots (**Figure 1B**) were in accordance with the indirect ELISA results (**Table 2**), showing more immunoreactive protein bands than bacteria fermented products. *R. oryzae* and *A. oryzae* were less effective, reducing the protein size below 40 kDa, and this was reflected in the interaction with plasma from allergic patients (parts **B** and **C** of **Figure 1**). These results suggest that mold fermented products undergo a slower growth of viable microorganisms, generating lower epitopes alteration and higher immunoreactivity than bacterial (solid) fermented products. Moreover, immunoreactivity toward P34 was still present in a medium intensity compared to the immunoreactivity of raw seeds. The glycinin basic (22 kDa) and acidic (33 kDa) subunits and β-conglycinin (53 kDa) were visible, but the α subunit (72 kDa) was not present.

Table 3 shows the amino acid profile of raw and fermented soybean products. Among nonessential amino acids (NEAA), Glu (7.30%) Asp (3.14%), and Arg (2.58%) were the major amino acids presented in raw soybean flour, while Leu (2.62%), Lys (1.94%), and Ile (1.76%) dominated among essential amino acids (EAA). Lower amounts were found of Met (0.57%), Cys (0.51%), and Thr (0.88%). The amino acid concentration exhibited in soybean flour resembles those reported by Hong et al. (*17*) for soybean seeds but was slightly lower than those reported by Karr-Lilienthal et al. (*28*) in different soybean and soybean meals from five leading soybean-producing countries.

Fermentation of soybean showed significant ($p \leq 0.05$) higher amounts of most of the NEAA, with the exception of solid fermentation of soybean with *R. oryzae*, in which only Ala increased significantly ($p \leq 0.05$). This increase trend was also observed for EAA in fermented soybean. Natural fermentation of soybean flour brought about rises in His, Val, Tyr, and Thr. *L. plantarum* fermentation of soybean flour led to increases in sulfur amino acids (Met plus Cys), Phe, Tyr, Lys, and Thr. Solid fermentation with *B. subtilis* and *A. oryzae* improved the content of most of EAA, while fermented soybean products with *R. oryzae* exhibited only significantly ($p \leq 0.05$) higher amounts for Thr. Similar results have been described in the literature, in which fermentation has improved the amino acid content of legumes, such as kidney beans (*29*), common beans (*30*), lentils and chickpea (*20*), and rice−soybean mixtures (*31*). Cereal fermentation also produces an increase in amino acid concentration. Chavan et al. (*32*) found that fermentation of sorghum, green gram, and a sorghum−green gram blend markedly increased their amino acid content. Mbithi-Mwikya et al. (*29*) reported increases in finger millet of 7.1% for Lys, and Khalil (*33*) observed a different effect of fermentation on the amino acid composition of mungbean depending upon the starter culture employed. Thus, probiotic strains, such as *Lactobacillus casei* and *Lactobacillus reuteri*, brought about the highest increases in amino acid concentration compared to *Lactobacillus helveticus*. According to Hong et al. (*17*), fermentation of soybean meal treated with *A. oryzae* strain did not affected the essential amino acids concentration but increased the concentrations of Gly, Glu, and Asp. This finding is only in partial agreement with the results of the present investigation, in which the content of most of EAA were improved. In addition, Hong et al. (*17*) showed that *A. oryzae* also presented a preference for specific amino acids rather than all amino acids.

Table 3. Total Amino Acid Composition of Raw and Fermented Soybean (g/100 g dm)[a]

	G. max cv. *merit*					
	raw	NF	LPF	BSF	AF	RF
NEAA						
Asp	3.14 a	4.19 b	4.91 c	4.97 c	5.35 c	3.12 a
Glu	7.30 a	8.37 bc	8.36 bc	9.49 d	8.58 c	7.78 ab
Ser	1.51 a	1.71 bc	1.86 cd	1.94 d	1.72 bc	1.61 ab
Gly	1.94 a	2.55 cd	2.44 bc	2.23 ab	2.77 d	2.08 a
Arg	2.58 a	2.98 a	2.64 a	2.71 a	2.71 a	2.44 a
Ala	1.90 a	2.38 bcd	2.52 cd	2.37 bc	2.28 b	2.55 d
Pro	1.94 a	2.35 c	2.17 ab	2.37 c	2.26 bc	2.04 a
EAA						
His	0.84 a	1.03 bc	0.94 ab	0.90 ab	1.13 c	0.96 ab
Val	1.67 a	1.84 b	1.66 a	1.87 b	1.89 b	1.71 a
Met	0.57 a	0.64 ab	0.69 bc	0.76 c	0.67 b	0.62 ab
Cys	0.51 a	0.53 ab	0.57 bc	0.61 c	0.57 bc	0.52 a
Ile	1.76 ab	1.88 abc	1.79 ab	1.91 bc	2.03 c	1.72 a
Leu	2.62 a	2.85 ab	2.73 ab	2.86 ab	2.99 b	2.88 ab
Phe	1.98 a	1.93 a	2.31 b	2.66 c	2.34 b	2.17 ab
Tyr	1.26 a	1.46 bc	1.51 cd	1.85 e	1.61 d	1.37 ab
Lys	1.94 a	2.26 ab	3.01 c	2.50 b	2.31 ab	2.30 ab
Thr	0.88 a	1.27 bc	1.42 c	0.94 a	1.27 bc	1.18 b

[a] Values are the means of three determinations. Rows with different letters indicate statistical differences ($p \leq 0.05$). NF, natural fermentation (flour); LPF, fermentation with *L. plantarum* (flour); BSF, fermentation with *B. subtilis* (cracked seeds); AF, fermentation with *A. oryzae* (cracked seeds); and RF, fermentation with *R. oryzae* (cracked seeds).

Increases in the concentration of certain amino acids could be attributed to microbial metabolism that takes place during fermentation of soy (*20, 33, 34*). As Doblado et al. (*35*) and Torres et al. (*36*) have shown, fermentation leads to a better nutritional value of legumes. In the processed seeds, nonnutritive compounds, such as α-galactosides, phytic acid, and trypsin inhibitors, are reduced while maintaining an acceptable amount of protein, starch, dietary fiber, available sugars, minerals, and vitamins.

In summary, the fermented cracked seeds and flour products showed low- and medium-range protein bands, and their immunoreactivity was affected by the type of microorganism used during the fermentation process. Specifically, mold proteolysis showed potentially weaker efficacy to eliminate immunoreactive proteins than bacterial proteolysis, which may be related to the slower growth rate of viable mold during the fermentation process. Because the same fermentation conditions were applied to all solid fermented products, the viable bacteria strain presented a faster growth rate and, consequently, resulted in a better efficacy in the breakdown and transformation of high-molecular-weight proteins and peptides.

B. subtilis, *L. plantarum*, and natural fermentation showed smaller size and less intense immunoreactive peptides below 30 kDa, yielding reductions greater than 80% in immunoreactivity for *B. subtilis* and greater than 90% in the case of *L. plantarum* and natural fermentation (**Table 2**).

The effectiveness and feasibility of the natural fermentation process is remarkable. It consisted of a simple treatment of soaking in water the soybean meal, allowing the microorganisms naturally present on the seeds to undergo fermentation (*21*). In this process, lactic acid bacteria were involved. However, the use of a starter culture has been introduced to control the fermentation process, accelerate the initial fermentation phase, and induce desirable changes. Lactic acid bacteria, as well as *L. plantarum*, has been used for legume fermentation, by which nutritional improvements (*37*) and antioxidant properties were achieved (*21, 35*).

In concolusion, fermented soybean products with significantly reduced immunoreactivity were successfully developed by induced fermentation. The highest performance was obtained by liquid fermentation of soybean flour. Furthermore, *L. plantarum* exhibited a better potential for developing reduced immunoreactive products compared to mold strains. Fermentation can reduce or eliminate antigenic soybean proteins and, at the same time, improve the nutritional value, increasing most of the EAA of the final fermented soybean meal. This approach can be a valuable tool for developing hypoallergenic soy food products.

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