

Allergenicity, trypsin inhibitor activity and nutritive quality of enzymatically modified soy proteins

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Abstract

Two ultrafiltered soy flour protein fractions were evaluated; the first was obtained by hydrolysis (0.5–3 kDa, F_{0.5–3}), and the second was an enzymatically methionine-enriched fraction (1–10 kDa, F_{1–10E}). Amino acid profiles, protein quality, allergenicity (against soy-sensitive infant sera) and trypsin inhibitor activity were determined. Fraction F_{1–10E} fulfilled amino acid requirements for infants, whereas the F_{0.5–3} fraction was methionine deficient. Both fractions were similar in net protein utilization, and F_{1–10E} digestibility was comparable with casein and higher ($P < 0.05$) than F_{0.5–3} or soy isolate. Allergenicity of SF was reduced to 21.5% with the hydrolysis in F_{1–10E} and it was not detected in F_{0.5–3}. Residual trypsin inhibitor activity with respect to soy flour was 8.1%, 3.3% and 1% for hydrolysate, F_{1–10E} and F_{0.5–3}, respectively. Both fractions presented high nutritive quality and reduced or null allergenicity. The trypsin inhibitor activity decreased along processing and could be a useful indicator for production of hypoallergenic proteins.

Keywords: *Allergenicity, nutritive-quality, enzymatically-modified, soy-protein*

Introduction

Food allergy is a common and increasing cause of illness in infants and children (Chandra 2002). The nutritional treatment as well as prevention for high-risk infants is total avoidance of cows' milk protein and substitution with 'hypoallergenic' formulas (Zeiger 2003). These formulas are prepared with hydrolyzed protein from whey (Giampietro et al. 2001), casein (Hoffman & Sampson 1997), collagen, or soy (Ogawa et al. 2000).

Protein hydrolyzates do not completely meet the sulfur amino acid requirements for infants suggested by the FAO/WHO (1991) and present a bitter taste (Muraro 2001). In order to reduce the bitterness (produced by extensive hydrolysis) and prevent anaphylactic reactions, molecular size is controlled by ultrafiltration (UF) after mild or medium hydrolysis. Additionally, hypoallergenic formulas are supplemented with free methionine to meet infant requirements, affecting the taste and aroma. This can also be done by enzymatic binding of methionine after the hydrolysis process (Kimura & Arai 1988; Hajós et al. 1996; Calderón de la Barca et al. 2000). Previously, we

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developed a simple process to modify soy protein obtaining an excellent amino acid pattern and nutritive quality for infants (De Regil & Calderón de la Barca 2004); however, it was not evaluated for allergenicity.

The allergenicity of soy proteins treated with proteases has been evaluated by detecting a major allergen, *Gly m Bd 30K*, as well as other minor ones (Ogawa et al. 1991; Yamanishi et al. 1996; Muraro 2001). The antigen *Gly m Bd 30K* is a labile large molecule that must be greatly affected by hydrolysis and/or UF. Therefore, the minor allergens must be used as indicators for producing hypoallergenic soy protein hydrolysates.

Trypsin inhibitors, in addition to their anti-physiological effects, are allergenic for a reduced number of patients (Ogawa et al. 1991). We propose that measurement of their activity can be used as a process indicator in the preparation of hypoallergenic soy proteins and its inactivation will correlate with the actual allergenicity of the protein. Therefore, the objectives of this study were to evaluate the allergenicity and nutritive quality of hydrolyzed and methionine-enriched soy protein fractions, and to evaluate the activity of trypsin inhibitors as a process indicator.

Methods

Materials

Defatted soybean flour (SF) was obtained from GAMESA, S.A. (Sonora, Mexico). Commercial soy protein isolate (Supro 500E) was obtained from The Solae, Co. (St Louis, MO, USA). High-performance liquid chromatography-grade reagents were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Other reagents were analytical grade obtained from Sigma Chemical Co. (St Louis, MO, USA).

Enzymatic modification of soy protein

Hydrolysis and synthesis. Hydrolysates were produced from defatted SF as previously described (Calderón de la Barca et al. 2000). Briefly, SF (4% w/v) was hydrolyzed using pancreatin (trypsin and chymotrypsin activities of 12.5×10^{-3} and 2.3×10^{-3} $\mu\text{g } p\text{-nitroanilide/min.mg}$) for 6 h at 50°C, pH 8. After enzyme inactivation (85°C, 30 min) the hydrolysate (SH) supernatant was recovered by centrifugation and then spray dried (APV Anhydro A/S; Søborg, Copenhagen, Denmark). Methionine was enzymatically bound to SH (20% w/v in water) using chymotrypsin and 0.076 g methionine-methyl ester per gram of SH, to obtain the methionine-enriched SH.

Ultrafiltration. SH and methionine-enriched SH were ultrafiltered (Amicon, Beverly, MA, USA) through 10 kDa and 1 or 3 kDa and 0.5 kDa cut-off membranes and were diafiltered. The collected fractions of 0.5–3 kDa ($F_{0.5-3}$) and 1–10 kDa (F_{1-10}), as well as the methionine-enriched fraction $F_{1-10}\text{E}$, were freeze dried (Labconco Liph-Lock 12; Kansas City, MI, USA) and were analyzed for composition and amino acids.

Protein characterization

Proximate and amino acid analyses. SF, soy isolate (SI), SH and fractions ($F_{0.5-3}$, F_{1-10} , $F_{1-10}\text{E}$) were analyzed for moisture, ash and protein (AOAC 1990). Protein content

in solution (Lowry et al. 1951) and sodium content (atomic absorption spectrophotometry) were determined. The amino acid content was determined after digestion (Vazquez et al. 1995), using a Varian 9010 chromatograph (high-performance liquid chromatography) equipped with a Fluorochrom II detector.

Protein quality evaluation. Six weaning Sprague–Dawley rats were randomly assigned per treatment and housed individually in metabolic cages (Nalgene) with food and water *ad libitum* for 10 days, and a 12 h light and dark cycle. All diets were isoenergetic with 10% protein, 8% corn oil, 1% vitamin premix, 5% mineral premix, 1% cellulose, 5% water and 69.68% starch+dextrose (AOAC, 1990; FAO/WHO, 1991). Protein sources were ANRC-Casein (Bioserv, Inc., NJ, USA), F_{1–10}E, F_{0.5–3} and soy isolate SUPRO 500E (SI). Diets with F_{0.5–3} and soy isolate were supplemented with 0.32% free L-methionine to reach the level of the F_{1–10}E diet. An extra nitrogen-free treatment was included. Two different bioassays (experiment 1, F_{0.5–3}; experiment 2, F_{1–10}E and SI) with their own casein diets were performed. Residual food and excretion were collected daily and recorded to estimate net protein utilization (NPU) and true digestibility (TD). Values were related (R) to the casein diet, and were expressed as R-NPU and R-TD. Protein utilization was estimated by the ratio $\Delta w/g$ protein.

Immunological assays

Patients and sera. Forty-three infants (≤ 2 years old) with a family history of atopy were studied. Thirty presented symptoms suggestive of food allergy and high total immunoglobulin (Ig) E. Parents signed an agreement approved by the institute's ethical committee. A family history of atopy, age of onset, allergic symptoms, feeding practices and ingested formulas and foodstuffs was registered by parent's interview. Blood samples were taken from 30 infants.

Detection of soy and cow's milk protein sensitive sera. A direct enzyme-linked immunosorbent assay (ELISA) was applied to detect IgE antibodies to soy and cow's milk protein (Fritsché & Bonzon 1990). Micro-plates were coated with 100 μ l soy or cow milk proteins (100 μ g/ml) in 0.1 M sodium bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Residual free binding sites were blocked with 100 μ l of 1% solution of fish gelatin, in dilution and washing buffer (0.1 M Tris–HCl, pH 7.4 + 0.05% Tween 20, 0.05% phenol red, 5 mM sodium azide) for 1 h. Plates were incubated with infant sera (1:40 dilution) overnight. Incubation with rabbit antibodies anti-human IgE-biotin (1:2000 dilution) was performed for 2 h and then 1 h incubation with horseradish peroxidase (HRP)-streptavidin (1:2000 dilution). Developing with 3,3'-5,5'-tetramethyl-bencidine was carried out and read at 450 nm (Micro-plate Reader, mod. 550; Bio-Rad Laboratories, Hercules, CA, USA). A pooled sera of healthy non-allergic ($n = 3$) infants was used as the control.

Allergenicity by direct and inhibition ELISA. The same protocol as described in the previous paragraph was used to test allergenicity of soy proteins and fractions. These were tested using six infants' sera with the highest IgE titles. Results were expressed as the percentage of remaining allergenicity: $(A_{sx}/A_{sf}) \times 100$ (Clemente et al. 1999),

where A_{sx} is the highest absorbance of each soy protein source tested and A_{sf} is the highest absorbance of SF obtained for the same serum. An inhibition ELISA for only two sera was carried out to verify the binding of the fraction $F_{0.5-3}$ to the microtiter plate. One hundred microliters of soy flour or fraction (200 $\mu\text{g/ml}$) were incubated with 200 μl each allergenic sera (1:20) for 2 h at room temperature. Residual unbound IgE antibodies were determined by direct ELISA, as already described, by adding 100 μl pre-incubated sera to the first line of the microtiter plates, which had previously been coated with soy protein (SF) or the soy protein fraction ($F_{0.5-3}$).

Antigenicity by direct ELISA. The antigenicity of soy flour, isolate, hydrolysate and fractions ($F_{0.5-3}$, F_{1-10} , $F_{1-10}\text{E}$) was measured by a direct ELISA assay similar to the former one for allergenicity, using rabbit anti-soy protein antibodies produced according to Jurd and Bøg-Hansen (1990) and purified on protein A. HRP-swine anti-rabbit antibodies (DAKO A/S, Denmark) were used as second antibodies.

Trypsin inhibitors

Activity. Trypsin inhibitor activity was assayed (method 71-10; AACC, 1976), using BA ρ A (benzoyl-DL-arginine-p-nitroanilide-HCl) as the substrate and bovine pancreatic trypsin (Sigma). Results were expressed as trypsin inhibitor units (TIU)/g protein and mg trypsin inhibitor/g protein.

Electrophoresis and immunodetection. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed (Laemmli 1970) in two 10–25% gradient gels. Gels were either stained with Coomassie brilliant blue G-250, or transferred to a nitrocellulose membrane. Immunodetection of Kunitz trypsin inhibitor (KTI) was performed using rabbit anti-KTI (1:500 in Tris buffer, pH 7.2) produced at the laboratory (Jurd & Bøg-Hansen 1990). Second antibodies were HRP-conjugated swine anti-rabbit antibodies (DAKO A/S) at 1:2000 dilution, and developing was carried out with diaminobenzidine (Sigma) using H_2O_2 as substrate.

Statistical analysis

Analysis of variance (GLM-ANOVA) and the Tukey–Kramer multiple comparison test (NCSS, 1996) were applied to data of the nutritional quality evaluation and amino acid composition. Significance was defined at $P < 0.05$.

Results and discussion

Enzymatic modification of soy protein

Hydrolysate (SH) presented a 23.5% degree of hydrolysis, 53.5% protein, 9.2% ash, 6.3% moisture, and sodium was not detected. The total protein recovered and the peptide mass distribution was the same as previously (Calderón de la Barca et al. 2000). After ultrafiltration, protein recovery was 18.6% for $F_{0.5-3}$, 21.3% for F_{1-10} and 23.3% for $F_{1-10}\text{E}$. The protein content of the fractions was 68%, which classifies them as concentrates (Liu 1997).

Table I. Comparison of suggested FAO/WHO (1991) patterns for infant amino acid requirements with the amino acid profiles of soy protein products assayed (g/100 g protein).^a

	Infant	SF	SI	F _{0.5-3}	F ₁₋₁₀	F _{1-10E}
Threonine	4.30	3.95 ^A	3.66 ^A	4.67 ^B	4.12 ^B	4.76 ^B
Phenylalanine + Tyrosine	7.20	9.92 ^A	8.78 ^A	10.95 ^B	7.10 ^C	7.02 ^C
Methionine + cysteine ^b	4.20	2.94 ^A	2.95 ^A	3.01 ^A	2.02 ^B	5.95 ^C
Valine	5.50	5.61 ^A	5.03 ^A	5.51 ^A	4.51 ^B	5.56 ^A
Isoleucine	4.60	5.31 ^A	4.78 ^B	6.26 ^C	5.10 ^{AB}	5.78 ^A
Leucine	9.30	6.99 ^A	8.01 ^B	8.17 ^B	8.39 ^B	8.15 ^B
Hystidine	2.60	2.64 ^A	2.34 ^A	2.47 ^A	2.07 ^B	2.60 ^A
Lysine	6.60	6.56 ^A	6.24 ^A	7.39 ^B	6.20 ^A	6.18 ^A
Chemical score	100	70	70	72	48	88

^aSoy flour (SF), soy isolate (SI), non-enriched ultrafiltrated soy fraction between 0.5 and 3 kDa (F_{0.5-3}), between 1 and 10 kDa (F₁₋₁₀), and enriched fraction between 1 and 10 kDa (F_{1-10E}). Data are medium values of three replicates. Means in a row with different uppercase superscript letters differ ($P < 0.05$).

^bTheoretical cysteine + cystine content of soy protein (Boza et al. 1999).

Amino acid pattern of the SH fraction between 0.5 and 3 kDa (F_{0.5-3}) was significantly ($P < 0.05$) improved with respect to SF (Table I). Except for methionine, the amino acid content was enough to fulfill infant requirements (FAO/WHO 1991). Enzymatic synthesis improved the chemical score of F_{1-10E} similarly to our previous report (De Regil & Calderón de la Barca 2004), with the best levels of methionine and Threonine (Table I).

Protein quality evaluation

No significant difference existed between both modified fraction-containing diets on NPU, as presented in Table II. The R-NPU value of F_{1-10E}, was not different from that of soy protein isolate (plus free methionine), and was rather better than that reported for its protein source, SF (Friedman & Brandon 2001). However, TD was higher for fraction F_{1-10E} than for F_{0.5-3} or soy isolate and comparable with the value for casein. Methionine enrichment of soy hydrolysates improve digestibility and

Table II. Bioassay parameters and protein quality indexes obtained in rats fed with experimental diets.^a

Diet ^b	Protein intake (g/day)	Weight gain (g/day)	Δ w/g protein	R- Δ w/g protein (%)	NPU ^c	R-NPU (%)	TD ^c	R-TD (%)
Experiment 1								
Casein	0.98	2.74	2.79	100 ^A	68.8	100 ^A	96.4	100 ^A
F _{0.5-3}	1.04	2.85	2.74	98.0 ^A	64.7	94.0 ^B	93.5	97.0 ^B
Experiment 2								
Casein	1.17	4.61	3.94	100 ^A	75.1	100 ^A	96.7	100 ^A
SI	1.04	3.55	3.41	86.6 ^B	73.3	97.6 ^{AB}	93.9	97.1 ^B
F _{1-10E}	1.11	3.65	3.29	83.5 ^B	71.1	94.7 ^B	97.2	100 ^A

^aResults expressed as the mean ($n=6$). Means in a row with different uppercase superscript letters differ ($P < 0.05$). ^bCasein ANRC, soy isolate (SI) SUPRO 500E (+ free methionine) and hydrolysate between 0.5 and 3 kDa (F_{0.5-3}) (+ free methionine), hydrolysate with bound methionine between 1 and 10 kDa (F_{1-10E}). ^cR, related to animals fed the reference diet (casein).

nutritional values with respect to the source protein (Kimura and Arai 1988; Hajós et al. 1996; Hussein et al. 2000). This could be due to a reduction of the antiphysiological factors and to an increase of the intestinal absorption.

The only indicator related to maintenance of animal grow was the weight gain per gram of protein consumed (Table II). $F_{0.5-3}$ appears to be more efficient to support rat growth than SI and F_{1-10} E. Because this parameter was assayed in a 10-day period, the high TD of F_{1-10} E could be more efficiently used in long-term feeding.

Immunological evaluation

Soy and cow's milk protein sensitive infants. Nine and seven of the 30 sera samples studied presented specific IgE to soy and cow's milk protein, respectively. The majority of the infants had been fed with soy-based formulas, a custom in the region of study (Calderón de la Barca et al. 1996). Soy sensitivity prevalence was rather high if it is considered that a minority of children with cow's milk allergy can also have soy allergy (Muraro 2001). Although it is considered that soy-based formulas are not recommendable for dietary management of clinical allergy to cow's milk or for routine management of colic (Wham 1992; American Academy of Pediatrics 1998), they are widely used for feeding infants with these symptoms.

In vitro allergenicity. Six of the nine soy-sensitive sera with the higher titles were used to determine soy protein allergenicity. Table III presents mean values of residual allergenicity measured as percentages of the absorbance ratio (sample/soy flour) of serum's IgE response. It was high against soy flour or isolate proteins, and decreased against the whole hydrolysate and medium size fractions (F_{1-10} and F_{1-10} E). None of the six soy-sensitive sera presented IgE antibodies against $F_{0.5-3}$. Inhibition ELISA demonstrated that the differences found between native and enzymatically modified soy proteins were not attributable to their ability to bind to the micro-plates, but to their IgE binding activity.

Approximately 15 soy proteins are allergenic (Ogawa et al. 1991, 2000). The major allergens (60, 30 and 28 kDa proteins) are easily hydrolyzed using proteolytic

Table III. Residual allergenicity, antigenicity and trypsin inhibitors' activity of soy proteins in products assayed.^a

Sample	Residual allergenicity (%)	Residual antigenicity (%)	Trypsin inhibitors (TIU/g protein)
SF	100	100	43,636
SI	88.07 ± 13.85	46.25	37,500
SH	28.73 ± 2.44	14.53	3,542
F_{1-10}	22.91 ± 1.89	0	1,813
F_{1-10} E	21.45 ± 1.83	0	1,459
$F_{0.5-3}$	0	0	467

^aResults expressed as the mean value of three replicates, except for allergenicity, which is presented as the mean ± standard deviation to reflect infant sera variability ($n = 6$). Soy flour (SF), soy isolate SUPRO 500E (SI), soy hydrolysate (SH), and modified fractions between 1 and 10 kDa non-enriched (F_{1-10}) and enriched with methionine (F_{1-10} E), and a non-enriched fraction between 0.5 and 3 kDa ($F_{0.5-3}$).

enzymes (Yamanishi et al. 1996). Minor allergens under 10 kDa have been identified (Ogawa et al. 2000) but not studied in relation to enzymatic hydrolysis. As expected, allergenicity decreased in the way the polypeptides on the fractions were smaller.

In vitro antigenicity. The anti-soy protein antibodies recognized epitopes strongly on soy flour and isolate, poorly in whole hydrolysate and none in the modified fractions (Table III).

Trypsin inhibitors

Activity and processing. Soy flour and soy isolate contained 43,636 and 37,500 TIU/g protein, respectively (Table III). Hydrolysis reduced 92% of the trypsin inhibitor activity of SF, while ultrafiltration reduced 96 and 99% in F₁₋₁₀ and F_{0.5-3}, respectively. Enzymatic modification did not affect significantly trypsin inhibitor activity. Immunodetection results (data not shown) were in agreement with these in Table III, although only the Kunitz inhibitor was detectable, while the Bowman–Birk, another trypsin inhibitor in soy, could be detected by activity.

Trypsin inhibitor activity has been evaluated after different technological processes such as heating and hydrolysis (Friedman & Brandon 2001; Vaintraub & Yattara 1995). UF causes further reduction on activity because of the trypsin inhibitors molecular weights (8 and 21 kDa) (Liu 1997). Maybe the negligible 1% residual activity is part of the technical error to determine trypsin inhibitors' activity (Friedman & Brandon 2001). According to Anderson and Wolf (1995), the trypsin inhibitors' content of infant formulas based on soy protein ranges from 2.2 to 15.5 mg/g protein (approximately from 4000 to 30,000 TIU/g protein). This is between two and 20 times higher than the content of our enzymatically modified fractions (Table III).

Trypsin inhibitors and allergenicity. Trypsin inhibitor activity is a better indicator of processing than antigenicity (Table III) to obtain hypoallergenic proteins. While antigenicity was completely lost after the first UF, trypsin inhibitor activity was still detectable until the end of the process when even allergenicity had disappeared. Allergenicity and trypsin inhibitor activity had a strong correlation (0.98), showing that the latter can be a useful indicator for processing. However, this association does not necessarily mean that it is a function of one to the other and *vice versa*.

In conclusion, modified soy protein fractions obtained in this study are potentially useful for hypoallergenic formulas because of their high nutritive value, low or null trypsin inhibitor activity, and hypoallergenicity and null immunogenicity. F_{0.5-3} could be used in formulas for treatment of cow's milk protein allergy and the F₁₋₁₀E with partially reduced allergenicity, used in allergy prevention formulas as recommended (Chandra 2002). Previously we have demonstrated a good sensorial acceptability for the F₁₋₁₀E fraction (De Regil & Calderón de la Barca 2004). Before application, the formulated products based on the studied soy protein fractions should be tested in children with proven cow's milk allergy, as recommended by the American Academy of Pediatrics (1998).

Acknowledgements

The authors thank Dr H. Stone for helping in the diagnosis of allergy, A. Ruiz Salazar, F. Vázquez, J.R. Valenzuela, A.V. Bolaños Villar and A.L. Frisby for technical assistance, and Dr G. Yepiz for reviewing the manuscript. The research was supported by the Mexican Council for Science and Technology (CONACyT, grants 3948P-B and 34237-B).

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