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ABSTRACT

The soybean is widely used in food industry due to its nutritional and functional qualities, but some studies have pointed it as a potent food allergen, especially in children. The concentration of IgG, IgG1 and IgE immunoglobulins in mice supplemented orally with common and transgenic soy was analysed. Mice were divided into two groups (6 animals each): lyophilized conventional soy (LCS) and lyophilized transgenic soy (LTS). Both received 100 µg of extracts for 10 consecutive days. Animals before immunization (pre-immune) formed the control group. The serum samples of the animals were undergone enzyme-linked immunosorbent assay (ELISA). The results of the ELISA tests showed that LCS and LTS induced the synthesis of specific IgG1 and IgG classes immunoglobulins and induced the formation of IgE immunoglobulin when evaluated by western blotting test. These results evidence the allergenicity of soybean and individuals with some food hypersensitivity should avoid the consumption of this food.

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1. Introduction

Soybean (*Glycine max*) cultivation dates back more than 3000 years in China and other Asian countries, such as Japan and Korea (Yoshimitsu, Kiyota, Kajimura, & Yamano, 2019). It has been a staple in the diet of many Far Eastern countries for centuries, e.g. such as tofu, tempeh, and miso. These traditional soy foods are not very widely consumed in Western countries, but are sometimes used as an alternative to meat, which has made the consumption of food containing soy increase in the West in the last 50 years (Fehily, 2016).

Soybean crops have an economic impact of \$114 billion around the world represents the global soybean production for the year 2013–14. USA and Brazil are the leading producers of soybean in the world (He & Chen, 2013).

The consumption of soybean has been increasing over the past few years due to its positive effects on human health (He & Chen, 2013). Soybean seeds consists about 36.5% protein, 19.9% lipids, 30% carbohydrates and 9.3% dietary fibre. Soybean oil is the

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second largest vegetable oil produced in the world. It contains 15.6% total saturated fatty acids, 57.7% total polyunsaturated fatty acids and 22.8% total monosaturated fatty acids (USDA, 2013).

Soy proteins have been widely used to formulate foods with a goal of improving their nutritional and functional qualities due to the high protein level and well-balanced amino acid composition (Aijie, Shouwei, & Li, 2014). However, soy is known by its allergenic power in children, and can be serious for them (Sicherer & Sampson, 2009). Intake of soy-containing food can cause immunological symptoms in soy-allergic individuals, like urticaria, angioedema, rhinoconjunctivitis, asthma, gastrointestinal symptoms and anaphylactic reactions, or even death (Mansoor & Sharma, 2011).

Food allergy is a significant public health concern throughout the world affecting about 3–6% of the population (Sicherer, 2011). The only method currently available to avoid any allergic reaction is complete dietary exclusion of the offending foods. As food intolerances emerged considerably in the last years, science and food industry are searching for new technologies, including thermal and non-thermal technologies to control soybean allergy other than avoidance (Shriver & Yang, 2011; Verhoeckx et al., 2015).

Food allergy is defined as an immune-mediated adverse reaction to food. This definition includes IgE-mediated immediate hypersensitivity reactions, delayed non-IgE-mediated reactions, and disorders with contributions from both IgE-mediated and non-IgE-mediated immune pathways (Gupta et al., 2011).

Food specific antigen sensitization can occur when oral tolerance fails to develop (Ma, Wang, Nie, & Yin, 2017). This involves a TH2 skewed response, triggered by epithelial cell production of cytokines such as thymic stromal lymphopoietin (TSLP), IL-33, and IL-25. These cytokines skew the DC phenotype, promoting further TH2 cell expansion. TH2 lymphocytes secrete type 2 cytokines (IL-4, IL-5, and IL-13), promoting B cell production of IgE – the classic “allergy” antibody. It should be noted that sensitization does not equate to clinical reactivity, as only 30% to 40% of patients with food-specific IgE antibodies experience clinical symptoms upon ingestion of the food (Sampson & Ho, 1997). The presence of IgG antibody to specific foods has no proven role in clinical evaluation of food allergy, as it represents neither tolerance nor sensitivity (Sampson et al., 2014).

About 0–0.5% of the general population are influenced by allergies to soybean products (Katz et al., 2014). Gizzarelli et al. (2006) have demonstrated that both transgenic soybean and conventional soybean are able to develop allergic reactions of same intensity. According to Allergome database, at least 34 allergenic proteins have been identified in soybean, being IgE binding. These allergenic proteins include the Gly m Bd 30k, the α -subunit of β -conglycinin, and the acidic and basic chains of glycinin (Yang, Gao, Yang, Jun, & Chen, 2015).

Therefore, this study aimed to analyse the concentration of IgG, IgG1 and IgE immunoglobulins in mice supplemented orally with liophilized commom soy and Liophilized transgenic soy.

2. Materials and methods

2.1. Animals

This study used female BALB/c mice (*Mus musculus*), aged approximately seven to eight weeks (weighing 20.0–25.0 g). All the animals were provided by the University of Fortaleza

(UNIFOR), Brazil. They were maintained in closed colonies at the Laboratory of Biotechnology and Molecular Biology at the State University of Ceara, Brazil. Water and food were given *ad libitum* to the animals. The institutional ethics committee on the care and use of animals for experimentation approved the experimental protocols (08476492-9).

2.2. Antigen

The antigen in this study consisted of the lyophilized conventional soy (LCS) and lyophilized transgenic soy (LTS) extracted from soy seeds. LCS and LTS were provided by Brazilian Agricultural Research Corporation (EMBRAPA) at the Federal University of Ceara, Brazil.

2.3. Preparation of LCS and LTS

LCS and LTS was obtained from the grinding of the beans by solubilization of the proteins in phosphate-buffered saline buffer (PBS), with constant stirring for one hour, followed by centrifugation at 10,000 x g for 45 min at 4°C. The precipitate was discarded and the supernatant, called ET, was then filtered, lyophilized and stored in a clean vial at 4°C. Total proteins were quantified by the Bradford (1976) dye-binding method.

2.4. Immunization of mice

Initially, the blood of animals (n = 12) was collected and the values obtained represented pre-immune (PI) time. After that, these animals were divided into 2 groups (n = 6). They were immunized by oral route with doses equivalent of 100 µg of LCS and LTS diluted in distilled water and offered during 10 consecutive days. The animals received boosters 21 and 35 days after the start of the immunization. The animals were bled by the retro-orbital plexus on days 7, 14, 21, 28, 35 and 42 after beginning of the immunization procedure. The blood samples collected were maintained at a temperature of 37°C for 15 min for clot retraction. The samples were centrifuged 3000xg for 5 min and the supernatant (serum) was placed in Eppendorf tubes®, labelled and stored in a freezer (-4°C) (Pereira & Guedes, 2005).

2.5. Electrophoresis (SDS-PAGE)

LCS and LTS were submitted to gel electrophoresis (SDS-PAGE) according to the method described by Laemmli (1970) by using a 12% separation gel and a 4% stacking gel. The protein concentration of the sample was 10 µg per lane.

2.6. Enzyme-linked immunosorbent assay

The method employed for the analysis of the serum of the animals was the enzyme-linked immunosorbent assay (ELISA) indirect according to Clark and Adams (1977), in order to quantify the presence of antibodies. The test was applied to serum for the determination of isotypes of immunoglobulin IgG, IgG1 and IgE. To this end, the plates (U96 – PolySorp-

NUNC-IMMUNO PLATE BATCH 016181) were coated with 3 µg/well of each antigen and diluted in phosphate-buffered saline (PBS). After, the plates were incubated overnight at 4°C. On the next day, the plates were washed with PBS–Tween 20 (0.05%) and blocked for 2 h at 37°C with PBS containing 5% milk Molico® (PBS – Molico®). After the incubation and washing, 100 µl PBS was added into each well. Then 100 µl of serum was diluted in PBS present on the plate serial dilutions (1:10 to 1:1.280). The plates were incubated for 2 h at 37°C. Following another round of washes, to the conjugate was added immunoglobulin anti-mouse linked to peroxidase, in a dilution of 1:1000 for anti-IgE, anti-IgG and anti-IgG1 for 2 h at a temperature of 37°C. The reaction was revealed by the addition of orthophenylenediamine and the plate incubated for 20 min at 37°C. The reaction was interrupted by the addition of 20 mL of 2.5 N H₂SO₄, and the intensity of the reaction was evaluated spectrophotometrically at a wavelength of 492 nm in an ELISA microplate reader (Guedes, 1999).

2.7. Western blotting

LCS and LTS were used in the western blotting test. The antigen was subjected to polyacrylamide gel electrophoresis in 4–12% (SDS-PAGE) as described and transferred to a nitrocellulose membrane, (SIGMA®) with pores of 0.2 µm in an electro vessel transfer semi-dry with a transfer buffer composed of 39 mM glycine, 0.0375% SDS (w/v) and 20% methanol (v/v) in 48 mM Tris buffer pH 7.2, under conditions of constant amperage of 150 mA. After the transfer, the membrane was immersed in a dye solution Ponceau 0.5% (0.5 g colourant Ponceau, 1.0 ml of glacial acetic acid and 100 ml of Milli-Q water qs) for 10 min, followed by washing with distilled water to remove excess, when it was possible to visualize the protein in the membrane. Then the membrane was incubated with buffer PBS-Molico® and kept under refrigeration for 18 h to block the free active sites of the membrane. After blocking, the membrane was divided into longitudinal cuts into three strips. Each membrane strip was incubated with serum containing antibodies of immunized mice with LP at a dilution of 1:100 in PBS–Tween and incubated for 2 h. Then, each membrane strip was subjected to three washes of 15 min in PBS–Tween and transferred to a plate of petri containing secondary antibodies conjugated to peroxidase anti-mouse (Thermo Fisher Scientific®), diluted 1:1000, for 2 h under slow agitation at room temperature to detect the immunoglobulins (anti-IgG, anti-IgG1 and anti-IgE). The membranes were again subjected to four washes of 15 min in PBS–Tween, and placed in a revealing solution with substrate for peroxidase comprising diaminabenzidine (DAB) 0.1 g/ml and nickel chloride (NiCl₂) 0.4 g/ml in 100 mM Tris-HCl, pH 7.2 buffer with the addition of 12.5 µl of hydrogen peroxide (H₂O₂).

2.8. Definition of the cut-off and statistical analysis

In order to establish the cut-off (positivity limit), pre-immune serum was used as a control. All the experiments on the production of IgG, IgG1 and IgE were calculated as means ± standard deviation (SD). The one way analysis of variance (ANOVA) test was used to determine the statistical differences followed by Tukey's Multiple Comparison in Graph-Pad Prism 5.0. The significance level was set at $p < 0.05$.

3. Results

3.1. Electrophoresis

The electrophoretic profile extract used in SDS-PAGE with 10 µg / well of protein revealed the migration of eight protein fractions in both LCS and LTS, four were most evident, with molecular masses at approximately 86, 45, 37.5, 20.1 kDa (Figure 1).

3.2. ELISA

The production of immunoglobulin IgG and IgG1 of mice sensitized by oral route with 100 µl of LCS and LTS were evaluated by the ELISA method.

The results of the ELISA tests (Figure 2) showed that both proteins (LCS and LTS) induced the synthesis of specific IgG1 (Figure 2(A)) and IgG (Figure 2(B)) classes immunoglobulin.

3.3. Western blotting

It was used anti-mouse IgE bound to peroxidase in western blotting test and it was possible verified that the LCS and LTS induced the formation of IgE immunoglobulin which specifically recognized some protein fractions of the both proteins (Figure 3).

4. Discussion

Generally, the main allergenic compounds are protein structures. The results found in the present research are relevant because they show that the immunoglobulins tested are reactive in serum samples of mice immunized with LCS and LTS.

The electrophoretic profile in SDS-PAGE demonstrated that the LCS and LTS proteins have similar molecular weights, each one corresponding to 86, 45, 37.5, 20.1 kDa. This

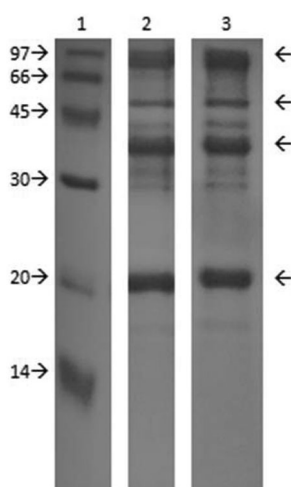


Figure 1. Electrophoretic profile of LCS and LTS extracts in SDS-PAGE 12%. Column 1: molecular weight marker; Column 2: LCS; Column 3: LTS.

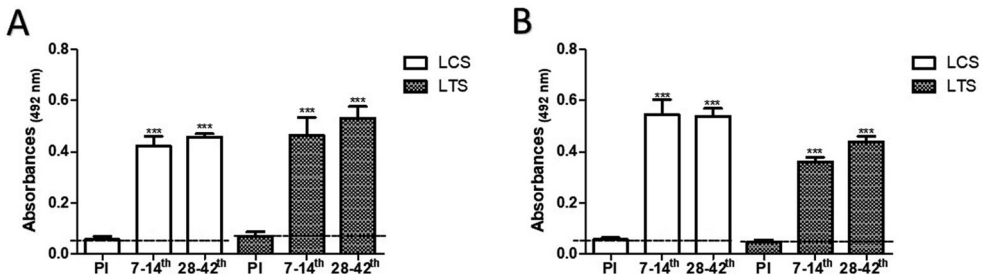


Figure 2. Production of immunoglobulins in mice sensitized orally with LCS and LTS during the experiment. PI= Pre-immune. In A (IgG1); B (IgG).

results corroborates with the dates reported by Yang et al. (2015) that found a similar profile of the soybean proteins. The same authors cited that the fractions 86, 45 corresponded to β -conglycine (7S) and the others fractions 35 and 20 (11S) corresponded to glycinin. This finding is relevant because β -conglycinin (7S) and glycinin (11S) are known to induce a variety of hypersensitivity reactions involving the skin, intestinal tract, and respiratory tract (Peng et al., 2018).



Figure 3. IgE specific antibodies against the LCS and LTS detected by immunoblotting. Column 1 (LCS); Column 2 (LTS).

The ELISA test of the samples used in the present study showed that both LCS and LTS were able to induce an increase in serum IgG and IgG1 concentration (by the ELISA method), compared to the data found in the PI ($p < 0,05$), and IgE (immunoblotting). These results corroborates with the dates reported by Yang et al. (2015) who found similar results evaluating the immunological response of Balb-c mice immunized with soybean proteins and germinated soybean proteins. They used the serum samples obtained from the mice sensitized, it was observed that the both soyprotein and germinated soybean proteins was able to induce, statistically ($p < 0,05$) when compared with animals of control group, the synthesis of IgG, IgG1 and IgE immunoglobulins by oral route.

Recent studies have shown that both IgE and IgG are involved in the allergic reaction in murine models. It has been reported that mice sensitized with soybean proteins by oral route could produce high levels of the cited immunoglobulins (Gizzarelli et al., 2006).

It was observed that IgG production (Figure 2) was higher for LCS when compared to LTS for all immunization days. Thus, we can see that the animals have immune memory cells for the antigens of both proteins, suggesting that the LCS has a greater capacity to stimulate this reaction (Sallusto, Lanzavecchia, Araki, & Ahmed, 2010).

After immunoblotting, an IgE reactive fraction was shown (Figure 3). The serum IgE dosage is not valid as a reliable food allergy diagnostic parameter, suggesting that a food challenge test is performed to confirm allergy (Soares-Weiser et al., 2014). This is due the insufficient sensitivity of the immunoassay and/or loss of minor allergens during extract preparation for the immunoassay (Gupta, Cox, Nowak-Węgrzyn, & Wang, 2018).

According to Pereira and Guedes (2005) and Oliveira et al. (2013), the allergic reactions from foods are characterized by specific IgE and IgG1 levels in serum and by activating gut mast cells.

According to Santos et al. (2012) in the western blotting test, using the polyclonal antibodies obtained by the subcutaneous sensitization, it was demonstrated that the Isosoy® proteins, present in a functional food similar to soybean, only induced the formation of type IgG immunoglobulins. Furthermore, the authors did not detect IgG1 and IgE in serum, diverging with the results found in the present study, suggesting that soy consumption should be cautious, due to its allergenic potential. Similarly, Silva et al. (2017) also identified the presence of IgG and did not detect IgG1 and IgE in the investigation of the humoral immune response in mice sensitized with phaseolamine.

5. Conclusion

We conclude that soy is capable of producing allergic reaction mediated by IgG1 and IgE antibodies. We also report that since the animals' diet was not soy free they already had a certain antigen tolerance, the presence of IgG proves the pre-sensitization. Both soybean were able to produce a greater immune response in the first days of immunization.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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