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Silmária Celestino Costa Santos^a, Ana Raquel Araújo da Silva^b, Maria Erivalda Farias de Aragao^a, Marcelo Róseo de Oliveira^{a,b}, Francisco Malcides Pereira Lucena^a, Lia Magalhães de Almeida^a, Jheyne Ariana Queiroz^a, Paulo Fernando Machado Paredes^a & Maria Izabel Florindo Guedes^{a,b}

^a Laboratory of Human Biochemistry, State University of Ceará, Av. Parajana, 1700, 60740-000, Fortaleza, CE, Brazil

^b Post-Graduate Program of Biotechnology, State University of Ceará, Av. Parajana, 1700, 60740-000, Fortaleza, CE, Brazil

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Humoral immune response in mice immunised by oral and subcutaneous routes with Isosoy proteins

Silmária Celestino Costa Santos^a, Ana Raquel Araújo da Silva^b,
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Francisco Malcides Pereira Lucena^a, Lia Magalhães de Almeida^a,
Jheyne Ariana Queiroz^a, Paulo Fernando Machado Paredes^a and
Maria Izabel Florindo Guedes^{a,b*}

^aLaboratory of Human Biochemistry, State University of Ceará, Av. Parajana, 1700, 60740-000 Fortaleza, CE, Brazil; ^bPost-Graduate Program of Biotechnology, State University of Ceará, Av. Parajana, 1700, 60740-000 Fortaleza, CE, Brazil

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This study compares the allergenic potential of Isosoy[®], a soy germ commercialised as isoflavone for dietary supplements, with crude soy extract (CSE) and purified isoflavone. Specific antibodies (IgG, IgE and IgG1) generated in Swiss mice by oral and subcutaneous route with Isosoy[®] also recognised proteins of crude soy extract, but no reaction against purified isoflavone, like clearly demonstrated by ELISA, Western blot and immunoprecipitation assay. These results are in agreement with the same protein profile between Isosoy[®] and CSE observed by polyacrylamide gel electrophoresis (SDS-PAGE). The immunoprecipitation assay demonstrates that Isosoy[®] boiling cannot inactivate the interaction with specific antibodies generated by oral route with unboiled Isosoy[®]. Results suggest that Isosoy[®] could lead to allergic reactions even after heat treatment.

Keywords: Isosoy; purified isoflavone; allergens

1. Introduction

Soy is one of the main legumes consumed in tropical countries due to its high nutritive value, because soybean seed has a high protein content, and the chemical characteristics of soy mean that it can be classified as a functional food (Potter, 1998). Thus, soy is classified among the food products whose alleged health benefits have been widely reported by the media in recent years (Behrens, Villanueva, & Silva, 2007).

Soy can therefore be used for preventive and therapeutic purposes, which is mainly due to the properties of its isoflavone component (Hasler, 1998). Soy isoflavones are known as phytoestrogens because they have an estrogenic effect and have demonstrated through experimental and clinical data that they represent a promising alternative in the prevention and/or treatment of several hormone-dependent illnesses, including cancer, cardiovascular diseases, osteoporosis and

*Corresponding author. Email: florinfg@terra.com.br

climacteric syndrome (Behrens, Roig, & Da Silva, 2001; Cassidy, Bingham, & Setchell, 1994).

Many commercialised products based on soy are currently available, such as flours, cookies, extracts and capsules enriched with Isosoy powder. In this context, dietary supplements are launched on the market without restrictions because they are not characterised as pharmacological products but rather as foods. However, there is a need to study the immunological response induced by these products, given the possibility that they may trigger allergic reactions. Vickery, Scurlock, Jones and Burks (2011) reported food allergy, which is hypothesised to result from a defect in oral tolerance, is a common, serious and growing problem in developed countries.

In this context, the objective of this study was to evaluate the humoral immune response of animals immunised, by oral and subcutaneous routes, with Isosoy which is freely commercialised throughout Brazil, to determine its possible allergenic effect.

2. Materials and methods

2.1. Antigens

The antigens in this study consisted of Isosoy (Isosoy®) and purified isoflavone food supplements sold in pharmacies in Brazil, and a crude protein extract (CSE) obtained from soybean seeds provided by Brazilian Agricultural Research Corporation–EMBRAPA.

2.2. Animals

Swiss mice (*Mus musculus*), aged approximately 42 days (20.0–25.0 g), were used in this study. All of the animals were provided by the Federal University of Ceará, Brazil. They were maintained in closed colonies at the Laboratory of Human Biochemistry at the State University of Ceará, Brazil. Food and water were provided on a daily basis. The methodology in this work was approved by the Ethics Committee for animal use at the State University of Ceará.

2.3. Preparation of crude soy extract (CSE)

The crude soy extract (CSE) was obtained from soybean seeds provided by EMBRAPA. The seeds were homogenised in a blender with a 0.1 M NaCl solution in the proportion of 1:1 (m/v). The extracts obtained were filtered through a double layer of gauze and then stored at –20°C in 0.5 mL aliquots. The protein content was determined by the Bradford (1976) dye-binding method.

2.4. Immunisation by subcutaneous and oral routes

Groups of six Swiss mice (females) were immunised by oral route with doses equivalent to 10 µg of Isosoy® proteins on 10 consecutive days according to the method described by Guedes and Pereira (2005). Another group containing the same number of animals was immunised through subcutaneous route with 46 µg of the same proteins (Guedes et al., 2004, with minor modifications). The animals received boosters 21 and 35 days after the start of the immunisation. The animals

were bled by the retro-orbital plexus before immunisation (pre-immune sample) and on days 7, 14, 21, 28, 35 and 42 after the beginning of the immunisation procedure. The blood samples collected were maintained at room temperature for one hour for clot retraction. The samples were then centrifuged at $3000 \times g$ for 10 min and the supernatant (serum) placed in Eppendorf tubes[®], labelled and stored in a freezer (-4°C).

2.5. Electrophoresis (SDS-PAGE)

The proteins of the CSE, Isosoy[®] and purified isoflavone were submitted 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 sample is 5 μg per lane.

2.6. Enzyme-linked immunosorbent assay (ELISA)

The specific antibodies for soy antigens, obtained from mice immunised with Isosoy[®] by subcutaneous and oral route, were submitted to the indirect ELISA test modified by Guedes et al. (2002). For this test, the plates (96-POLYSORP-NUNC-IMMUNO PLATE BATCH 016181) were sensitised with Isosoy[®] protein (2 μg of protein/well), CSE (2 μg of protein/well) or purified isoflavone (2 μg of protein/well). The antigens were diluted in phosphate buffered saline (PBS) (NaCl , KH_2PO_4 , $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and KCl). The plates were incubated overnight at 4°C , and then washed with PBS-Tween (0.05%) and blocked for 2 h at 37°C with PBS containing 5% skim milk powder (Molico[®]). After the plates had been washed, the antibodies (one procedure for each type of immunisation) diluted in blocking buffer (1:10 and 1:1.280) were added, and the plates were again incubated overnight at 4°C . The plates were washed and a combination of the peroxidase-conjugated immunoglobulins anti-IgE, anti-IgG and anti-IgG1, obtained from the mice, was added to each one, in a dilution of 1:1000, and incubated for 2 h at 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 μL of 2.5 N H_2SO_4 , and the intensity of the reaction was evaluated spectrophotometrically at a wavelength of 492 nm in an ELISA microplate reader.

2.7. Western blot technique

To carry out the Western blot test, the proteins of the CSE, Isosoy[®] and purified isoflavone were submitted SDS-PAGE as previously described and then transferred to nitrocellulose membranes with a pore size of 0.2 μm in a semi-dry electrotransfer apparatus, the transfer solution being composed of 48 mM Tris buffer, pH 7.2, with 39 mM of glycine added, 0.0375% SDS (m/v) and 20% methanol (v/v), according to Lin, Hsu and Hsu (1990). After the transfer the membranes were incubated in PBS buffer with 5% of skim milk powder (Molico[®]) for approximately 18 h at 8°C , to block the free active sites of the membrane.

After the incubation the membranes were washed four times for 15 min with a PBS-Molico[®] buffer solution (5%) under light shaking. The washed membranes were immersed in PBS buffer containing antibodies obtained from the subcutaneous

immunisation at a dilution of 1:100 and incubated for two hours. The membranes were then submitted to four washings of 15 min each in PBS-Molico[®] (5%) and transferred to a petri dish containing the secondary antibodies (anti-IgG, anti-IgA and anti-IgE of the mice, peroxidase-conjugated) for two hours under light shaking at room temperature. The membranes were then again submitted to four washing of 15 min in PBS-Molico[®] and placed in a developing solution, with a substrate for peroxidase, composed of 0.1 g/mL diaminobenzidine and 0.4 g/mL nickel chloride (NiCl₂) in 100 mM Tris-HCl buffer pH 7.2, with the addition of 12.5 µL of hydrogen peroxide (H₂O₂) 30V, maintained in the dark at room temperature for approximately 30 min, when the reaction was stopped with the washing of the membrane with distilled water.

2.8. Immunoblotting with immunoprecipitation

The immunoprecipitation was carried out by mixing 50 µL of a suspension of antigens with an equal volume of the antibodies obtained from the mice immunised with Isosoy[®] protein by oral route. The mixture was incubated at 4°C overnight. The mixture was then submitted to centrifugation at 9800 × g, for 10 min. After the centrifugation, the precipitate was washed with distilled water and resuspended in 20 mM Tris-HCl buffer pH 7.5, containing 1% of Triton X-100 (v/v) and 0.5 M NaCl and centrifuged at 9800 × g for 5 min. The new precipitate was submitted to the same treatment. To eliminate the Triton, the precipitate was washed three times with 10 mM phosphate buffer pH 7.2 containing 0.1% of SDS (m/v). This precipitate was submitted SDS-PAGE, after which a transfer was performed as described in the previous section. The immunoblots were developed using peroxidase-conjugated anti-IgG1, anti-IgA and anti-IgE as secondary antibodies.

2.9. Statistical analysis

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 GraphPad Prism 3.0. The significance level was set at $p < 0.05$.

3. Results

3.1. Electrophoresis-SDS-PAGE

The electrophoresis profile, obtained by SDS-PAGE, of the CSE revealed the migration of seven protein fractions, four of which were most evident with molecular masses estimated at approximately 96, 67, 47 and 30 kDa (Figure 1). A similar profile can be observed in relation to the Isosoy[®] protein, with the four protein fractions corresponding to those of the CSE, that with a molecular weight of 30 kDa being the most expressive.

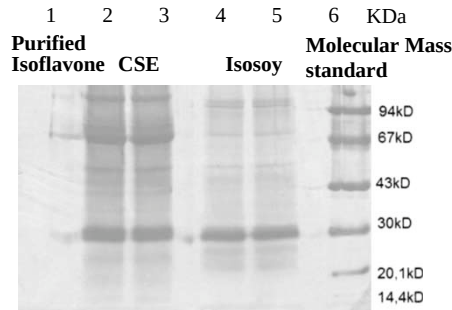


Figure 1. Electrophoretic profile of soy protein subunits obtained by 12% SDS-PAGE. Line 1: Purified isoflavone; Lines 2 and 3: CSE; Lines 4 and 5: Isosoy[®]; Line 6: Molecular Mass standard.

3.2. Immunological response

3.2.1. ELISA

The immunological responses of mice immunised by subcutaneous route with 46 μ g of Isosoy[®] proteins in the absence of adjuvant and by oral route with 10 μ g of the same antigen were evaluated using the indirect ELISA method to detect immunoglobulins type IgG, IgG1 and IgE. Figure 2 shows that the Isosoy[®] proteins, when administered by the subcutaneous route, induced the synthesis of type IgG immunoglobulins, which have specific reactivity against the Isosoy[®] proteins and CSE proteins, but only a slight response was detected when the plate was sensitised using purified isoflavone. There was no significant difference between the primary and secondary responses for both types of immunisation.

When the secondary antibodies anti-IgG1 and anti-IgE were used, it was observed that the Isosoy[®] proteins administered by oral route induced the synthesis of polyclonal antibodies which specifically recognised the Isosoy[®] proteins and CSE

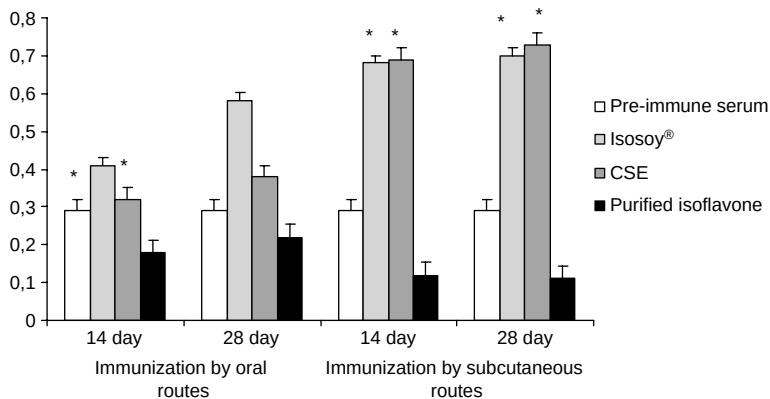


Figure 2. Production of IgG antibodies in mice immunised with Isosoy[®] by oral (10 μ g of protein) and subcutaneous (46 μ g of protein) routes detected through ELISA, in plates sensitised with pre-immune serum, Isosoy[®], CSE or purified isoflavone. The antibodies were obtained before and after booster (14 and 28 days after immunisation, respectively).

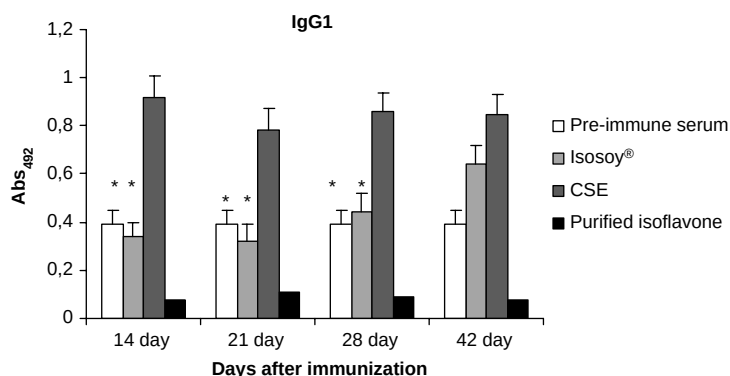


Figure 3. Production of IgG1 antibodies in mice immunised with Isosoy® by oral route (10 µg of protein) detected through ELISA, in plates sensitised with pre-immune serum, Isosoy®, CSE or purified isoflavone.

(Figures 3 and 4). On the other hand, there was no significant specific reaction when the plate was sensitised with purified isoflavone.

3.2.2. Western blot technique

In the Western blot test, using the polyclonal antibodies obtained by subcutaneous route, it was demonstrated that the Isosoy® proteins induced the formation of type IgG immunoglobulins, which specifically recognised the same protein fractions of the CSE and Isosoy® (Figure 5).

3.2.3. Immunoprecipitation and Immunoblotting

The method of immunoblotting with immunoprecipitation for the detection of IgA, IgE and IgG1, using the antibodies obtained by oral route, showed a stronger reaction when anti-IgG1 was used compared with anti-IgE from the mice, both

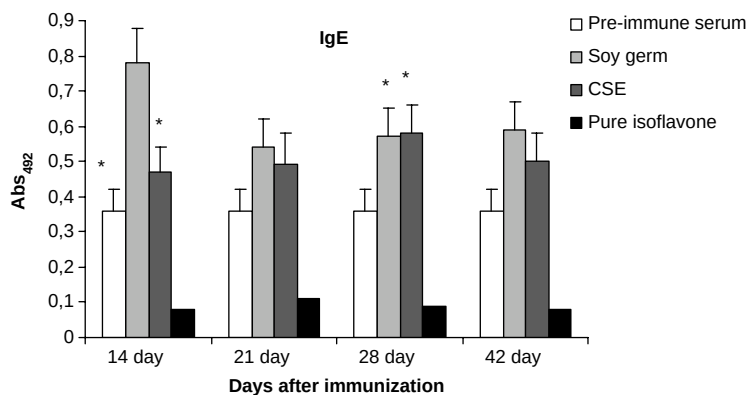


Figure 4. Production of IgE antibodies in mice immunised with Isosoy® oral route (10 µg of protein) detected through ELISA, in plates sensitised with pre-immune serum, Isosoy®, CSE or purified isoflavone.

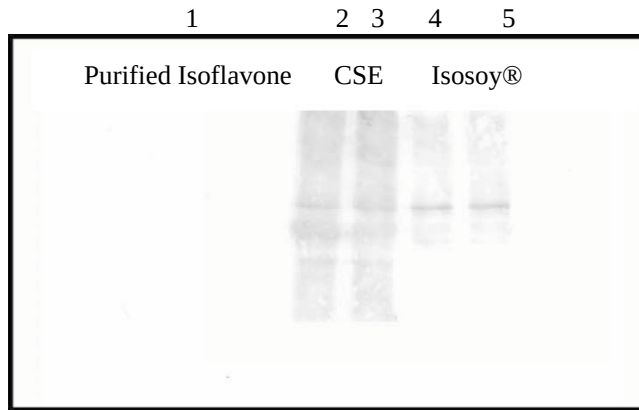


Figure 5. Western blotting showing the specificity of antibodies of mice immunised via subcutaneous route with 46 µg of proteins of Isosoy[®]. Line 1: Purified isoflavone; Lines 2 and 3: CSE; Lines 4 and 5: Isosoy[®].

peroxidase-conjugated (Figure 6). It can also be observed in Figure 6 that the type IgG1 antibodies are in the native form while those of type IgE were denatured and only the heavy chains were detected. The presence of IgA was not detected.

4. Discussion and conclusion

The electrophoretic profile obtained by SDS-PAGE for Isosoy[®] was very similar to that of the CSE, mainly with respect to the high concentration of the 30 kDa fraction (Figure 1). This suggests that the Isosoy[®], sold in pharmacies in Brazil as Isoflavone, does not differ from CSE. On the other hand, the product purified isoflavone did not show expressive protein bands.

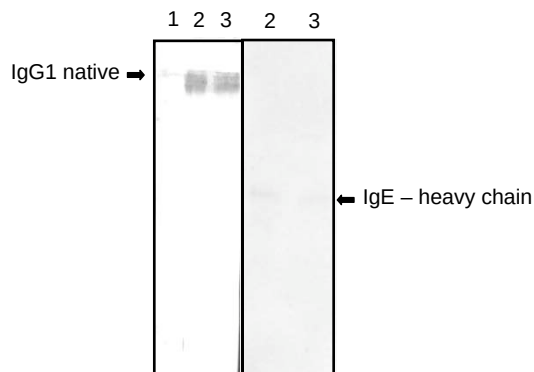


Figure 6. Immunoblotting after immunoprecipitation showing the specificity of IgG1 and IgE antibodies in mice immunised via oral route with 10 µg of proteins of soy germ. Line 1: Purified isoflavone; Line 2: CSE; Line 3: Isosoy[®]. For the detection of IgE, the sample was heated in a bain-marie, and for the detection of IgG1 the sample was only dissolved in buffer and placed on the SDS-PAGE gel.

However, soy has been cited as one of the main food items which trigger allergic reactions. Several soy allergens have been found to contain sub-units with molecular weights of 20 and 50–60 kDa (Herian, Taylor, & Bush, 1990). Also, the major protein fractions of soy proteins, 2S, 7S and 11S, show allergenic activity, the 2S fraction reportedly being the most allergenic in the soy protein composition (Shibasaki, Suzuki, Tajima, Nemoto, & Kuroume, 1980). Ogawa et al. (1991) identified a soy allergen with a molecular weight of approximately 34 kDa.

The immunological response of mice immunised with Isosoy® by subcutaneous and oral route was evaluated using ELISA, Western blotting and immunoblotting with immunoprecipitation tests, using the serum samples obtained from the mice immunised with Isosoy®.

It was observed from the indirect ELISA test that the Isosoy® proteins induced the synthesis of type IgG immunoglobulins, which showed specific reactivity against Isosoy® proteins and CSE proteins, for both the subcutaneous and oral routes. No reaction against purified isoflavone was observed as demonstrated in the ELISA test (Figure 2). It was observed that both routes led to the synthesis of type IgG immunoglobulins, however, the subcutaneous route showed higher antibody titers. The results show that the Isosoy® proteins induced the synthesis of specific IgG1 and IgE immunoglobulins, showing primary and secondary responses, for immunisation by oral route (Figures 3 and 4). On the other hand, no difference was observed in relation to the primary and secondary responses, for immunisation by oral and subcutaneous routes, where it can be seen that the response remained stable even after the booster at 21 days after the beginning of the immunisation. This result is of great interest considering that the administration of antigens by oral route generally leads to tolerance rather than the induction of antibodies (Weiner et al., 1994). Similar results have been observed in mice immunised with proteins of *Lablab purpureus* (Guedes & Pereira, 2005).

In the Western blot test, using peroxidase-conjugated anti-IgG1, anti-IgA and anti-IgE of the mice, it was demonstrated that Isosoy® proteins induced the formation of type IgG1 immunoglobulins, which specifically recognised some of the protein fractions of the CSE and Isosoy®. A strong reaction with the 30 kDa protein fraction was observed, suggesting greater immunogenicity (Figure 5). On the other hand, no reaction was observed against purified isoflavone. When the test was carried out to detect IgE and IgA, no reaction was observed (data not shown).

In the analysis using immunoblotting with immunoprecipitation for the detection of IgA, IgE and IgG1, a strong reaction could be observed with IgG1 and a weak reaction with IgE (Figure 6) for the oral administration. However, no presence of IgA was detected. The absence of IgA in the peripheral response does not discard the possibility of a mucosal immunological response because immunisation by oral route can induce the synthesis of specific IgA and IgG, both local and peripheral (Finkelman et al., 2004).

The Isosoy® proteins induced a low production of IgE by oral route. However, this does not necessarily mean that other products with soy proteins available on the market are free from the possible induction of an allergic reaction, because soy is included among the foods which frequently induce allergic responses in humans (Food and Agriculture Organization of the United Nations, 1995). In fact, soy proteins are considered to be powerful allergens when compared with some other foods which induce allergic reactions (Cordle, 2004).

The presence of immunoglobulins IgG and IgG1 with the absence of IgA observed in this study is notable since, according to Finkelman et al. (2004) the quantities of IgA and IgG antibodies are able to neutralise the antigen before it can bind to the IgE on the surface of the mastocytes and cause the symptoms characteristic of an allergic reaction. Thus, an allergic reaction is dependent not only on high concentrations of IgE isotype antibodies, but also on the absence or low concentrations of the IgG and IgA isotypes. However, the involvement of some isotypes of the IgG classes, particularly IgG1 and IgG2a, in allergic processes has been previously verified (Je-Ruei, Sheng-Yao, ming-Ju, Pei-Ying, & Chin-Wen, 2006). Thus, these results indicate that Isosoy[®] commercialised as isoflavone in pharmacies throughout Brazil may cause allergic reactions, suggesting that other functional foods derived from soy may also induce allergy.

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