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Development of two ELISA formats to determine glycinin. Application to detect soy in model and commercial processed food

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ABSTRACT

Indirect competitive and sandwich ELISA formats were developed to determine glycinin and applied to detect soy in model and commercial processed food. Antiserum to glycinin was used in the competitive format and specific purified antibodies in the sandwich format. Results obtained showed that the sandwich format could detect as low levels as 0.005% and 0.05% of incurred soy protein isolate in sausages and bread, respectively; whereas indirect competitive format could only detect levels of 0.25% and 0.5% of soy protein in the same incurred foods. However, soy protein could not be detected in pâté using both formats even at the highest level assayed of 1%. Furthermore, the sandwich ELISA could detect glycinin in samples of UHT milk spiked with 0.05% of soy drink and in cookies spiked with 0.005% of soy seeds. The sandwich format could also discriminate between commercial foods with declared or not soy proteins or derivatives as ingredients, although glycinin could not be detected in some soy labelled foods. A prototype of the sandwich format was prepared and validated, obtaining adequate results in terms of recovery, precision and robustness. The results of this study indicate that the concentration of immunoreactive glycinin decreased with the intensity of heat processing applied to food. Furthermore, the antibody population used and the ELISA format applied influence greatly the determination of glycinin in processed food.

1. Introduction

Soybean (*Glycine max*) and its derivatives have been proved to be versatile ingredients due to their functional properties, nutritional and health benefits, and competitive price (Jideani, 2011). However, soybean is included in the group of the "*big eight*" foods that are believed to be responsible for 90% of all food allergies. The estimated prevalence of soy allergy range from 0.8% to 1.2% in children and from 0.3% to 0.4% in adults and it depends on local dietary habits and exposure to allergens (EFSA, 2014).

Soy allergy is of particular importance as the use of soy proteins is widely used in the food industry, and thus its incidence is expected to increase. In fact, soy proteins represent a particular insidious source of hidden allergens due to contamination during shipping, storage and processing, as well as to inadequate cleaning of processing equipment (L'Hocine & Boye, 2007).

Glycinin is the main protein of soy 11S fraction and one of the most abundant seed storage proteins, accounting for about 40% of total soy protein. Furthermore, glycinin, denoted as Gly m 6, is one of the major soy allergens (He et al., 2015; L'Hocine & Boye, 2007). Glycinin is a hexameric protein of about 320–360 kDa. Each subunit is composed by acidic and basic polypeptide chains of molecular weight of 31–45 kDa and 18–20 kDa, respectively, which are linked by disulphide bonds (Staswick, Hermodson, & Nielsen, 1981).

The best way to prevent an allergic reaction to a certain food is to avoid its ingestion. Accurate information on foodstuff labelling is thus needed. The current legislation in the European Union (Regulation EU 1169/2011) and United States (Food Allergen Labelling & Consumer Protection Act of 2004, FALCPA) includes a list of the most common allergenic foods that must be declared and highlighted in the label to alert the consumer of their presence in the product, soy being among them. Unfortunately, according to the French Allergo-Vigilance network, the accidents related to hidden allergens represent 8.6% of severe allergic reactions (AFSSA, 2008). This fact entails a threat to allergic consumers, as the estimated threshold level for common food allergy is usually low. To ensure protection of allergic consumers, development of

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sensitive and reliable methods to detect allergens in processed foods is needed to implement an allergen management plan in food industry and to enforce current legislation (Koppelman, Lakemond, Vlooswijk, & Hefle, 2004; Poms, Klein, & Anklam, 2004).

Enzyme-linked immunsorbent assays (ELISA) have been the preferred techniques to detect allergens because they show a high sensitivity and specificity. Additionally, they have some advantages like technical simplicity, the possibility to test a large number of samples and the use of relatively inexpensive equipment (Pedersen et al., 2008; Poms et al., 2004).

Several ELISA have been developed for the detection of soy in food. They are based on the determination of different individual target proteins, such as glycinin (Chen, Wang, Song, & Ma, 2014; Ma et al., 2010), β-conglycinin (Hei, Li, Ma, & He, 2012), soy trypsin inhibitors (Cucu, Devreese, Kerkaert, & Meulenaer, 2012) or the whole soy protein extract (Cucu et al., 2012; Koppelman et al., 2004; Sánchez, Pérez, Puyol, & Calvo, 2002). ELISA can be based on different formats like sandwich or competitive assays, and use different types of antibodies, monoclonal or polyclonal. ELISA techniques based on the determination of glycinin recently developed have been exclusively applied to determine this protein in soy from different origins and in several soy products (Chen et al., 2014; Ma et al., 2010). However, from our knowledge, the application of ELISA techniques based on the determination of glycinin to detect soy in processed foods has not been reported in a peer journal so far.

The effect of processing is an important aspect to consider when detecting allergens in food because it usually induces denaturation and aggregation of the target protein, which impairs its recognition by specific antibodies and reduces its extractability (Montserrat et al., 2015; de Luis et al., 2008). The most appropriate approach to determine the performance of ELISA assays should be based on the use of model processed foods, which are products incurred with allergenic ingredients and then processed. This approach would allow evaluating the actual effect of processing on the extraction and recovery of target protein, and detection efficiency (Cucu et al., 2012; Poms et al., 2004).

However, in spite of the growing interest in the study of the effect of thermal processing on the detection of allergenic food components, only few studies have been focused on the effect of processing on soy allergen detection in food matrixes (Cucu et al., 2012; Gomaa & Boye, 2013).

In this study, two ELISA formats (sandwich and indirect competitive) for glycinin determination were developed to detect soy. The performance of the assays was evaluated using three types of soy incurred foods elaborated with a mixture of two commercial soy protein isolates as ingredients. The sandwich format was single laboratory validated and used to survey commercial foods with declared or not soy proteins or derivatives in the label.

2. Material and methods

2.1. Materials

Soy protein isolates (SPI) were kindly provided by Ceylan (Zaragoza, Spain) and Mané (Barcelona, Spain). Raw soy seeds, food commodities and commercial products were purchased from local stores. Hitrap NHS-activated HP columns were supplied by GE Healthcare (Little Chalfont, UK) and the Lightning-Link (R) HRP Conjugation Kit by Innova Biosciences (Cambridge, UK).

2.2. Glycinin isolation

Glycinin was purified from raw soy seeds as previously described (Iwabuchi & Yamauchi, 1987; Liu et al., 2007). Proteins were extracted by stirring 10 g of defatted soybean flour with 150 mL of 0.03 M Tris-HCl, pH 8.5. The proteins that precipitated at pH 6.4 and 4 °C overnight were collected by centrifugation and subjected to salting out precipitation. The protein fraction precipitating between 51% and 66% ammonium sulfate saturation was collected by centrifugation, dialyzed and applied onto a Sepharose CL-6B column (1 × 85 cm). The purity of isolated glycinin was higher than 96%, as determined by SDS-PAGE and densitometry by using an EPSON EU-88 Image Scanner III (Long Beach, CA).

2.3. SDS-PAGE and western-blotting

SDS-PAGE was performed according to the method of Laemmli (1970) using polyacrylamide gradient gels (4–20%) and a Mini-PROTEAN Tetra Cell (Bio-Rad Laboratories, Hercules, CA). Gels were stained with Coomasie Brillant Blue R-250. Western-blotting was performed as described by Benfeldt, Larsen, Rasmussen, Andreasen, and Petersen (1995) using antiserum to glycinin.

2.4. Antibody production, purification and labelling

Antisera to glycinin were obtained by immunization into rabbits, which were kept on a soy-free diet, as previously described (Wehbi et al., 2005). All procedures were approved by the Ethic Committee for Animal Experiments from the University of Zaragoza (Project Licence PI48/10). The care and use of animals were performed as stated in the Spanish Policy for Animal Protection RD 53/2013, which meets the EU Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

Titer of antisera was determined using an indirect non-competitive ELISA assay. Specific antibodies to glycinin were purified using an immunosorbent of glycinin insolubilized in a HiTrap NHS-Activated HP column (1 mL). Purified antibodies were labelled with horseradish peroxidase (HRP) using a commercial kit.

2.5. Indirect competitive ELISA

Microtitration plates were coated with 120 μL per well of glycinin (5 μg/mL) in 0.05 M sodium carbonate buffer, pH 9.6 overnight at 4 °C. Wells were washed three times with distilled water and excess binding sites were blocked with 300 μL of 3% (w/v) ovoalbumin solution in 8 mM Na_2HPO_4, 3 mM KCl, 0.14 M NaCl, 1.5 mM KH_2PO_4 buffer, pH 7.4 (PBS) for 2 h at room temperature. Then, wells were washed three times with PBS and after drying, they were stored at 4 °C.

Before its use, wells were washed three times with 300 μL of PBS containing 0.5% Tween 20 (PBST). For the assay, 50 μL of standards or samples and 50 μL of an adequate dilution of the anti-glycinin antiserum were added to wells and incubated for 30 min at room temperature. After washing wells three times with PBST, they were incubated with 100 μL/well of goat IgG anti-rabbit IgG labelled with HRP (diluted 1/3000 in PBS) for 30 min at room temperature. Finally, after washing, 100 μL of tetramethylbenzidine (TMB) substrate were added to each well and after 30 min incubation at room temperature, the enzymatic reaction was stopped by adding 50 μL of 2 M H_2SO_4 and the absorbance was read at 450 nm. All samples and standards were analysed by duplicate.

2.6. Sandwich ELISA

For the sandwich ELISA, wells were coated using specific purified antibodies to glycinin (3 μg/mL) and blocked, as indicated above. For the assay, standards or samples (100 μL/well) were added and incubated for 30 min at room temperature. Next, plates were incubated with an appropriate dilution of anti-glycinin antibodies HRP-conjugated in PBS for 30 min at room temperature. After washing, the substrate was added and after 30 min of incubation, reaction was stopped and the absorbance was read at 450 nm. All samples and standards were analysed by duplicate.