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Isolation of recombinant antibody fragments (scFv) by phage display technology for detection of almond allergens in food products

Silvia de la Cruz [a, 1], Carolina Cubillos-Zapata [b, 1], Inés María López-Calleja [a], Satyabrata Ghosh [a], Marcos Alcocer [c], Isabel González [a], Rosario Martín [a], Teresa García [a, *]

[a] Departamento de Nutrición, Bromatología y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad Complutense de Madrid, 28040, Madrid, Spain
[b] Tumor Immunology Lab/Innate Immunity Group, IdiPAZ, Hospital La Paz, 28046, Madrid, Spain
[c] Department of Nutritional Sciences, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, LE12 5RD, UK

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ABSTRACT

Tree nut allergies are considered an important health issue in developed countries. To comply with the regulations on food labeling, reliable allergen detection methods are required. In this work we isolated almond-specific recombinant antibody fragments (scFv) from a commercial phage display library bypassing the use of live animals, hence being consistent with the latest policies on animal welfare. To this end an iterative selection procedure employing the Tomlinson I phage display library and a crude almond protein extract was carried out. Two different almond-specific scFv (named PD1F6 and PD2C9) were isolated after two rounds of biopanning, and an indirect phage ELISA was implemented to detect the presence of almond protein in foodstuffs. The isolated scFvs demonstrated to be highly specific and allowed detection of 40 ng mL^{-1} and 100 ng mL^{-1} of raw and roasted almond protein, respectively. The practical detection limit of the assay in almond spiked food products was 0.1 mg g^{-1} (110–120 ppm). The developed indirect phage ELISA was validated by analysis of 92 commercial food products, showing good correlation with the results obtained by a previously developed real-time PCR method for the detection of almond in foodstuffs. The selected phage clones can be affinity maturated to improve their sensitivity and genetically engineered to be employed in different assay formats.

1. Introduction

Almond (*Prunus dulcis*, family Rosaceae) is a tree nut used as ingredient in a wide variety of food products due to its desirable organoleptic attributes, claimed health benefits, and prebiotic properties (Jenkins, Hu, Tapsell, Josse, & Kendall, 2008; Kamil & Chen, 2012; Kendall, Josse, Esfahani, & Jenkins, 2010; Mandalari, Nueno-Palop, Bisignano, Wickham, & Narbad, 2008). However, almond allergens can trigger adverse immune responses in sensitized individuals, including severe anaphylactic shock reactions (Costa, Mafra, Carrapatoso, & Oliveira, 2012; Teuber, Comstock, Sathe, & Roux, 2003). To prevent health-threatening situations, authorities strongly recommend allergic consumers the total avoidance of the offending food. However, unintended exposure to almond-containing products can happen because of mislabeling of food products, cross-contamination during food processing, or undeclared presence of almond in a ingredient. In order to minimize the accidental ingestion of allergenic foods, labels must provide accurate information concerning the list of ingredients. Several countries have enacted labeling guidelines to safeguard allergic individuals (European Parliament and Council, 2011; Regulations Amending the Food and Drug Regulations, 2011). Nevertheless, enforcement of these regulations depend on the availability of sensitive and specific analytical methods that would permit to verify the accuracy of labels.

Current methods for almond detection include mainly DNA-based and immunochemical techniques. Food labeling regulations do not require the detection of allergenic proteins but the allergenic ingredient itself. According to this, DNA represents a more stable

Abbreviations: BCA, bicinchoninic acid; BSA, bovine serum albumin; CDR, complementary determining regions; ELISA, enzyme-linked immunosorbent assay; FR, framework regions; LOD, limit of detection; OD, optical density; PBS, phosphate buffered saline; SD, standard deviation; scFv, single chain variable fragment; VH, variable heavy; Vκ, variable kappa light.

* Corresponding author. Tel.: +34 913943747.
E-mail address: tgarcia@vet.ucm.es (T. García).

[1] These authors contributed equally to this work.

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target molecule than proteins, and it is not influenced by geographical or seasonal variations that affect protein composition (Poms, Klein, & Anklam, 2004). However, despite its high sensitivity, the use of DNA analysis in allergen detection is controversial, since it does not directly detect the allergen or any specific protein (Lau, 2014; Poms et al., 2004). Immunochemical techniques are based on the interaction between an antibody and an antigen, which can be an allergen or a marker protein. The enzyme-linked immunosorbent assay (ELISA) is the most widely used technique for routine screening of allergens in foods, and up-to-date ELISA assays have been developed to detect almond proteins (Acosta, Roux, Teuber, & Sathe, 1999; Ben Rejeb, Abbott, Davies, Cléroux, & Delahaut, 2005; Hlywka, Hefle, & Taylor, 2000; Su et al., 2013). Although assays may differ in format, all of them have in common the use of antibodies raised in animals.

Phage display technology provides an alternative to traditional antibody production, allowing the generation of large quantities of affinity probes with a well defined and constant amino acid sequence. Moreover, it permits to bypass of the use of live animals, hence being consistent with the latest policies on animal welfare (European Parliament and Council, 2010). Phage display technology employs libraries of recombinant bacteriophages that expose on their surface functional antibody binding sites, and it allows the isolation of recombinant antibodies with the desirable binding affinity against the antigen by an iterative selection procedure (Clementi et al., 2012). Due to its versatility, phage display technology has multiple applications (Bratkovič, 2010). Regarding food science, phage display technology has been successfully applied to monitor the presence of *Salmonella typhimurium* in food (Sorokulova et al., 2005) or to detect spores of *Clostridium tyrobutyricum* in milk (Lavilla et al., 2010). For it does not depend on animal immunization, it can be used to obtain specific antibody fragments against toxic or allergenic compounds. Using this technology, Lauer, Ottleben, Jacobsen, and Reinard (2005) isolated a scFv against fumonisin B1, a highly toxic mycotoxin found in corn based products, and Garet, Cabado, Vieites, and González-Fernández (2010) reported the isolation of four scFv to detect palytoxin, a marine biotoxin, in shellfish. Phage display technology has also been used to isolate specific scFv that allow the detection of *Cryptosporidium parvum* sporozoites in water and foodstuff (Boulter-Bitzer, Lee, & Trevors, 2009).

The aim of this work was to isolate specific recombinant antibodies against almond proteins employing a phage-display library of single chain variable antibody fragments (scFv), and to develop a phage-ELISA to detect almond proteins in commercial food products using the isolated affinity probes.

2. Materials and methods

2.1. Materials and chemicals

Tomlinson I library, M13KO7 helper phage, and *Escherichia coli* TG1 strain (K12 Δ *(lac-proAB) supE thi hsdD5/F′ traD36 proA⁺B lacI^q lacZΔM15*) were obtained from Source BioScience (Nottingham, UK). The library has a size of 1.47×10^8 pfu mL^{-1}, and is based on a single human framework for VH (V3-23/DP-47 and JH4b) and Vk (O12/O2/DPK9 and Jk1), which encodes the most common human canonical structure. ScFv is displayed fused with the terminal phage gene III protein, and it is cloned in the ampicilin resistant phagemid vector pIT2.

Almonds "Marcona" cultivar and other tree nuts were acquired from local retailers in Madrid (Spain). After being shelled, they were stored at −20 °C until further use. A total of 13 different almond varieties (Table 1) kindly provided by the IRTA (Institut de Recerca i Tecnologia Agroalimentàries, Tarragona, Spain) and the CEBAS-CSIC (Centro de Edafología y Biología Aplicada del Segura-Consejo Superior de Investigaciones Científicas, Murcia, Spain), were analyzed to check the specificity of the assay. Animal and plant species —including representative members of the Rosaceae family— employed in specificity assays (Table 2) were purchased in different local markets in Madrid. Finally, a total of 92 commercial food products were purchased from different retail markets and delicatessen stores (Spain).

Table 1
Phage-ELISA results (OD 450 nm) obtained for different almond kernel cultivars with scFvs from clones PD1F6 and PD2C9.

Prunus dulcis cultivars	PD1F6	PD2C9
Almond "Marcona"	4.05 ± 0.05	3.96 ± 0.06
Almond "Bitter"	3.69 ± 0.19	3.63 ± 0.16
Almond "Guara"	4.37 ± 0.20	4.55 ± 0.03
Almond "Glorieta"	4.15 ± 0.21	4.13 ± 0.25
Almond "Ferragnes"	3.68 ± 0.14	3.53 ± 0.06
Almond "Vairo"	4.39 ± 0.17	4.38 ± 0.16
Almond "Francoli"	4.12 ± 0.12	4.03 ± 0.15
Almond "Masbovera"	3.33 ± 0.10	3.36 ± 0.17
Almond "Belona"	4.45 ± 0.10	4.28 ± 0.06
Almond "Marinada"	3.64 ± 0.19	3.57 ± 0.20
Almond "Tarraco"	4.42 ± 0.14	4.41 ± 0.24
Almond "Constanti"	4.07 ± 0.08	4.00 ± 0.09
Almond "Soleta"	4.49 ± 0.10	4.49 ± 0.07
Almond "Marta"	4.25 ± 0.17	4.22 ± 0.22

HRP/anti-M13 monoclonal mouse antibody was purchased from GE Healthcare (München, Germany). Tryptone, yeast extract and European bacteriological agar were purchased from Laboratorios Conda (Madrid, Spain). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA).

PBS composition is 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4. The protein extraction buffer consisted of 0.035 M phosphate solution containing 1 M NaCl, pH 7.5. 2xTY broth is 16 g L^{-1} tryptone, 10 g L^{-1} yeast extract and 5 g L^{-1} NaCl. TYE agar is 15 g L^{-1} bacto-agar, 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract and 8 g L^{-1} NaCl.

2.2. Preparation of protein extracts

All samples (Tables 1 and 2) were ground using a mortar and pestle, and stored in screw-capped vials at −20 °C. Protein extracts were prepared by adding 200 mg of sample to 1800 μL of protein extraction buffer. After shaking for 10 min at room temperature in a vertical rotator (HulaMixer Sample Mixer, Life Technologies, Carlsbad, CA) to extract soluble proteins, the slurry was centrifuged at 10,000 *g* for 10 min at 4 °C, and the supernatant was filtered through a 0.45 μm syringe filter (Sartorius, Göttingen, Germany). Bicinchoninic acid (BCA) assay (Thermo Fisher Scientific Inc., IL, USA) was employed to determine protein concentration. Protein extracts were stored at −20 °C until further use.

2.3. Preparation of binary mixtures

To evaluate the sensitivity of the assay, binary mixtures of almond Marcona in wheat flour (100–0.01 mg g^{-1}) were prepared using a food processor (Thermomix, Vorwerk, Germany). Concentration of 100 mg g^{-1} was prepared by adding 50 g of ground almonds to 450 g of wheat flour. Then, 50 g of the former mixture was added to 450 g of wheat flour (10 mg g^{-1}). Remaining concentrations were made in a similar way. Additional mixtures of 50, 25, 5, and 0.5 mg g^{-1} were prepared by mixing 250 g of wheat flour with 250 g of the mixtures containing 100, 50, 10 and 1 mg g^{-1}