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Original research article

Multimeric recombinant antibody (scFv) for ELISA detection of allergenic walnut. An alternative to animal antibodies

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ABSTRACT

Walnuts are classified as an important allergenic ingredient that can cause severe reactions in sensitized individuals. To prevent unintended exposure to products containing walnut, food manufacturers have the responsibility to declare its presence in packaged foods. Immunochemical methods are widely used to detect walnut proteins. However, available immunoassays rely on the use of antibodies raised in animals. In this work, an affinity probe for walnut proteins has been isolated from the Tomlinson I library, and further engineered in *Pichia pastoris* to produce the *in vivo Juglans regia* Biotinylated Soluble Fragment-single chain and multimeric antibody (JrBSF-scFv). The multimeric scFv has been used to develop a direct enzyme-linked immunosorbent assay (ELISA), allowing detection of walnut in a food matrix with a limit of detection (LOD) of 1616 mg kg^{-1}. This is the first recombinant antibody available for detection of walnut proteins. The assay is specific, only cross-reacting to some extent (2.25%) to pecan, thus being useful as a screening tool for detection of walnut in raw or baked food matrices. Multimerization of the scFv with different avidin derivates could be of interest to improve sensitivity of the assay.

1. Introduction

Walnuts are amongst the most widely consumed of all commercially grown tree nuts in the world. Member of *Juglandaceae* family and seeds of *Juglans regia* L., walnuts are a highly nutritious food. The regular consumption of walnuts has been associated with decreased risk of cardiovascular disease, coronary heart disease and type II diabetes, while lessening aged related symptoms (Kris-Etherton, 2014; Rock et al., 2017). Accordingly, they are included as ingredient in many foodstuffs such as bakery products to enhance their nutrition value (Hayes et al., 2015; Mao et al., 2014; Wang et al., 2014). However, food-induced allergies are an emergent problem of public health. Among food allergens, walnut is classified as an important allergenic ingredient and frequent cause of adverse food reactions in allergic patients. Even small amounts of walnut can cause severe reactions in sensitized individuals, being a real problem of allergen management (Clark and Ewan, 2003). Food processing has the potential to alter walnut immunoreactivity due to modifications of specific epitopes in the walnut allergens. Nevertheless, boiling and roasting treatments do not affect the antigenicity of walnut proteins, while a slight decrease has been described after frying in vegetable oil at 191 °C for 1 min (Su et al., 2004). Only harsh conditions of pressure and temperature, like autoclaving at 2.8 atm (37 psi), 138 °C for 15 or 30 min, lead to the fragmentation of proteins accompanied by a reduction of the IgE binding (Cabanillas and Novak, 2017).

The walnut, within the group of tree nuts, is a product set by the European Union that causes allergy or intolerance. To protect consumers, and in accordance with Regulation 1169/2011, it is mandatory to indicate this ingredient when it is used in the manufacture or preparation of a food and still present in the finished product, even if in an altered form (The European Parliament and the Council of the European Union, 2011). Therefore, food manufactures have the responsibility to declare the presence of walnut on packaged foods even when trace residues may be present from the use of shared equipment or the adventitious contamination of ingredients (Niemann et al., 2009; Van Hengel, 2007).

There are several methods available for the detection of walnut allergens in food products. However, immunochemical assays such as enzyme-linked immunosorbent assay (ELISA) are by far the most widely used to detect and quantify walnut allergens or proteins, due to their direct assessment of the allergen or marker protein, low set-up cost, moderate running time and no special requirements for expertise

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knowledge (Costa et al., 2014). One of the drawbacks of available immunoassays for walnut is that they rely on the use of polyclonal or monoclonal antibodies raised in animals, while current trends in animal welfare (European Union, 2010) encourage avoiding the use of live animals when possible.

The phage display technology allows production of recombinant antibodies of defined specificity and constant amino acid sequence without animal immunization. This method uses libraries of recombinant bacteriophages that expose functional antibody binding sites in their surface, like the single-chain variable fragments (scFv). Isolation of phage-antibody fragments of the desired specificity is achieved by an iterative biopanning procedure with the immobilized antigen (Hoogenboom et al., 1998). The use of prokaryotic expression systems for production of antibody fragments can result in unstable proteins, leading to low scFv yields (Arbabi-ghahroudi et al., 2005; Miller et al., 2005). In this sense, the use of *Pichia pastoris* as alternative to *Escherichia coli*, provides appropriate post-translational modifications and is highly productive (Cregg et al., 2000).

In this work we describe the selection of a walnut-specific scFv from the synthetic Tomlinson I library, followed by the production and *in vivo* biotinylation of the scFv in *Pichia pastoris*. After tetramerization of the biotinylated probe with ExtrAvidin-peroxidase, a direct ELISA has been developed for detection of walnut protein in experimental food mixtures.

2. Material and methods

2.1. Materials and chemicals

The human scFv library Tomlinson I, M13 K07 helper phage and *Escherichia coli* TG1 strain (K12Δ (*lac-proAB) supE thi hsdD5/F' traD36 proA + B laclq lacZΔM15*) were obtained from Source BioScience (Nottingham, UK). The Tomlinson I library is constructed in the ampicillin resistant phagemid vector pIT2 (HIS myc tag) with a size of 1.47×10^8. This repertory is based on a single human VH framework (V3-23/D47 and JH4b), paired with a single Vk (O12/O2/DPK9 and JK1). The repertory has been designed to contain short complementarity-determining region 3 (CDR3) of the heavy chains while maintaining good antigen binding properties, and has been displayed as a fusion with the terminal phage gene III protein.

Walnuts, other tree nuts, heterologous products, and commercial food products were acquired from local retailers and delicatessen stores in Madrid (Spain).

Horseadish peroxidase/anti-M13 monoclonal mouse antibody was purchased from GE Healthcare (Little Chalfont, UK). Phosphate-buffered saline (PBS) composition is 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4. Milk phosphate-buffered saline (MPBS) contains 1% skimmed milk powder in PBS. Tris-buffered saline (TBS) composition is 0.05 M Tris-Cl and 150 mM NaCl, pH 7.6. TBST is TBS containing 0.05% Tween 20. The protein extraction buffer consisted of 0.035 M phosphate solution containing 1 M NaCl, pH 7.5. Tryptone, yeast extract and European Bacteriological agar were purchased from Laboratorios Conda (Madrid, Spain). 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.

Low salt Luria-Bertani (LB) agar is 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl, 15 g L^{-1} agar, pH 7.5. Buffered Glycerol-complex Medium (BMGY) is 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 100 mL of 100 mM potassium phosphate, pH 6.0, 100 mL 1.34% Yeast Nitrogen Base (YNB), 2 mL of 4×10^{-5}% biotin and 100 mL 1% glycerol. Buffered Methanol-complex Medium (BMMY) is BMGY but adding 100 mL 0.5% methanol instead of glycerol. Yeast Extract Peptone Dextrose Medium (YPD) is 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} dextrose and 20 g L^{-1} agar. Yeast Extract Peptone Dextrose Medium with Sorbitol (YPDS) is YPD with 1 M sorbitol.

Selection antibiotic Zeocin was purchased from Invitrogen (Carlsbad, CA, USA) and Blasticidin from InvivoGen (Toulouse, France).

E. coli XL1-Blue Chemically Competent Cells (Agilent Technologies, Santa Clara, CA, USA) were employed for the propagation of plasmids, and *P. pastoris* X-33 strain (Invitrogen) was used for scFv and biotin ligase (BirA) enzyme expression. *P. pastoris* expression vectors pPICZαB and pPIC6αA were purchased from Invitrogen. Restriction enzymes *PstI*, *NotI*, *XbaI* and *SacI*, calf intestinal alkaline phosphatase, T4 DNA ligase, and GoTaq DNA Flexi Polymerase were purchased from Promega (Madison, WI, USA). Plasmid purification kit (QIAGEN Plasmid Midi Kit), PCR product purification kit (QIAquick PCR Purification Kit) and gel extraction kit (QIAquick Gel Extraction Kit) were purchased from Qiagen (Hilden, Germany). HiTrap Protein L Column was purchased from GE Healthcare. Methanol was purchased from Fisher Scientific (Loughborough, UK). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Preparation of protein extracts

All food samples (5 g) were ground using an IKA A11 analytical mill (IKA®, Staufen, Germany), and stored in screw-capped vials at − 20 °C. The sample (200 mg) was mixed with 1200 µL of protein extraction buffer, and the mixture was shaken for 10 min at room temperature in a vertical rotator (HulaMixer Sample Mixer, Invitrogen) 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 mm syringe filter (Sartorius, Gottingen, 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. Selection of scFv against walnut by phage display

Preparation of the Tomlinson I phage display library for biopanning procedure was performed as described in the manufacturer's protocol. Following amplification of the library and poly-ethylene glycol (PEG)/NaCl phage precipitation, phages were tittered, and kept at 4 °C for short term storage or at −80 °C in 15% glycerol for longer term storage.

Polystyrene paddles and magnetic beads were alternately used for target immobilization to avoid the isolation of unspecific phages which would produce false-positive results. For the first and third rounds of selection, polystyrene paddles (Nunc, Denmark) with a surface area of 5.2 cm^2 were coated with 1 mL of 100 µg mL^{-1} walnut extract (positive screening) or pecan nut extract (negative screening) in PBS, and incubated overnight at 4 °C. Then, paddles were washed three times with PBS and blocked with 3% bovine serum albumin (BSA) at 37 °C for 1 h.

For the second round of selection, Dynabeads M-280 Tosylactivated (Invitrogen) were used to bind the target proteins following manufacturer's instructions. Briefly, 5 mg of Dynabeads were coated with 100 µg of walnut proteins (positive panning) in 0.1 M Na-phosphate buffer, pH 7.4, to a final volume of 150 µL and then, 100 µL of 3 M ammonium sulfate in Na-phosphate buffer was added. Coupling procedure was performed on a vertical rotator at 37 °C overnight. Next day, Dynabeads were blocked with 1 mL of 0.5% BSA in PBS for 1 h at 37 °C with rotation. The same procedure was performed with the Dynabeads used for negative panning, but employing a pecan nut protein extract as the ligand.

Three rounds of biopanning were performed for selection of walnut-specific phage-scFv, as previously described (Madrid et al., 2017) with the following modifications: approximately 10^{12} phage particles from Tomlinson I library were resuspended in 2 mL of 3% BSA in PBS and added to the pecan nut-coated polystyrene paddle. The mixture was incubated at 25 °C for 60 min on a rotator to capture phage-scFv recognizing pecan nut (negative panning). The supernatant containing unbound phage particles was added to the walnut coated paddle (positive panning) and incubated at 25 °C for 60 min with rotation, and for further 60 min without rotation. After positive panning, unbound