Food Chemistry 147 (2014) 106–110

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Development of an indirect competitive immunoassay for walnut protein component in food

Haiyan Wang [a,b], Gang Li [b], Yajun Wu [a], Fei Yuan [a], Ying Chen [a,*]

[a] Institute of Food Safety, Chinese Academy of Inspection and Quarantine, No. 3, Gaobeidian North Road, Chaoyang District, Beijing 100123, PR China
[b] Inner Mongolia Entry-Exit Inspection and Quarantine Bureau, No. 12, Erdos Street, Huhhot, Inner Mongolia 010020, PR China

ARTICLE INFO

Article history:
Received 10 June 2011
Received in revised form 1 September 2013
Accepted 4 September 2013
Available online 5 October 2013

Keywords:
Walnut
Allergen
Indirect competitive
ELISA

ABSTRACT

Among food allergens, walnut is a frequent cause of adverse food reactions in allergic patients. In this study, the walnut allergen protein 2S albumin precursor (*Jug r 1*) cDNA was synthesised and cloned into the pGEX-6P-1 expression vector. The recombinant plasmids were transformed into *Escherichia coli* (*E. coli*) BL21(DE3) pLys for expression of protein Jug r 1. Polyclonal antibodies were prepared against the expressed purified Jug r 1 protein. An indirect competitive enzyme-linked immunosorbent assay (ELISA) for the detection and quantification of walnut soluble proteins in processed foods was developed using the prepared polyclonal antibodies. The developed ELISA had a high specificity, walnut protein standard solution at 2.2 ng/mL [inhibition concentration (IC80) of the competitive test] was clearly identified by the ELISA. The mean recoveries ranged from 86% to 112%. The coefficient of variation (CV) for the 4 model foods was 6.4–8.7%.

1. Introduction

IgE-mediated type I hypersensitivity to foods affects approximately 1–3% of the adult population and up to 8% of the paediatric population (Zeiger, 1990). Allergies to tree nut (and peanut) generally begin in childhood, but unlike allergies to a number of other foods, persist throughout life (Crespo & Rodriguez, 2003; Ewan, 1996). However, it is likely, though not yet proven, that some patients with an early onset of milder forms of allergy will become tolerant of tree nuts after a period of avoidance, analogous to what has recently been shown for a minority of peanut-allergic patients (Sicherer, 2000). The seriousness of tree nut allergy is indicated by the fact that all the fatal allergic reactions to foods for individuals over the age of 6 reported to a US national registry have been caused by either peanuts or tree nuts (Bock, Munoz-Furlong, & Sampson, 2001).

In the US, allergic patients to walnut (34%) are more than allergic patients to any other tree nut (Sicherer, Furlong, & Munoz-Furlong, 2001). Of 54 paediatric tree nut-allergic subjects in one study, 26% were confirmed to be reactive to walnut seed protein extract (Sicherer, Burks, & Sampson, 1998). Although the black walnut species, *Juglans nigra* and *Juglans californica*, are fairly common in North American, they are not eaten. Consequently, most walnut-allergic patients have probably been sensitised to the commercially available and widely consumed English walnut (*Juglans regia*).

Several English walnut allergens have been identified. The first to be identified was a 2S albumin precursor, Jug r 1, with a molecular mass of 15–16 kD, the mature Jug r 1 allergen consists of a large subunit with a molecular mass about 10–12 kD and a small subunit with a molecular mass about 4–5 kD. The second allergen to be identified was a member to the vicilin family of seed storage proteins (a 7S globulin), Jug r 2, with a molecular mass of 47–48 kD (Teuber, Dandekar, & Peteson, 1998). The third and the fourth allergens were Jug r3 (Asero, Mistrello, & Roncarolo, 2000) and Jug r4 (Teuber, Peteson, & Uratsu, 2003), corresponding to PR-14(LTP) and legumin (11S), respectively. Among these walnuts allergens, Jug r 1 is a major allergen, its allergenicity is retained stably during heat processing (Teuber et al., 1998). Even small amounts of walnut can cause severe reactions, and thus strict management is required to minimise the potential harm. Currently, there are several published methods available for the detection of gene codifing for allergens and protein of walnuts in food products, including common PCR (Yano et al., 2007), real-time PCR (Brežńa, Hudecóva, & Kuchta, 2006) and ELISA (Hirotoshi, Yuki, & Haruki, 2008) methods. The PCR methods are specific, but ELISA method based on polyclonal antibodies generated against extracted allergen protein from walnut, it's a small subunit of about 4–5 kD of mature Jug r 1. Extraction of the Jug r 1 allergen protein is arduous and lacks of repetition, so a simple and reliable detection method for hidden walnut is necessary.

In the present study, the *Jug r 1* gene, codify for the walnut allergen protein Jug r 1 was synthesised and cloned into the pGEX-6P-1 expression vector. The recombinant plasmid was transformed into

* Corresponding author. Tel./fax: +86 (010) 8578 3587.
E-mail address: chenyingcaiq@163.com (Y. Chen).

0308-8146/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2013.09.013

Escherichia coli BL21(DE3) pLys for expression. Polyclonal antibodies were prepared after the expressed protein Jug r 1 being purified. An indirect competitive ELISA was developed for the detection and quantification of walnut soluble proteins in processed foods using the prepared polyclonal antibodies.

2. Materials and methods

2.1. Nut and other samples

All samples, including Walnut (*Juglans regia*), Hickory (*Carya cathayensis Sarg*), Pecan (*Carya illinoensis koch*), Pistachio nut (*Pistacia vera*), Cashew (*Artocarpus heterophyllus lam*), Peanut (*Arachis hypogae*), Brazil nut (*Bertholletia excelsa*), Hazelnut (*Corylus spp*), Almond (*Hodgsonia macrocarpa*), Korean Pine seed (*Pinus koraiensis siebet zucc*), Sunflower seeds (*Fructus helianthi annui*), Sesame (*Sesamum indicum*), Pumpkinseed (*Cucurbita pepo*), Rice (*Oryza sativa*), Corn (*Cornū*), Soy (*Glycine max*), Common wheat (*Triticum aestivum*) powder, Milk (*Lac vaccinum*), Beef (*Bov*), Mutton (*Mult*), Chicken (*Pullus*), Pork (*Porcus*) and Duck (*Anas domestica linnaeus*), were bought from local market. Excepting milk, for roasted samples, the samples were roasted at 120 °C for 15 min using an oven (MT10KC-AW, midea Croup, China)

2.2. Reagents and instrumentation

Taq DNA polymerase (5 U/μL), T4 DNA Ligase, Restriction enzyme *EcoR I* and *Xhol I*, MiniBEST plasmid purification kit ver.2.0 and Agarose gel DNA purification kit ver.2.0 were purchased from Takara Co. (China); pGEX-6p-1 plasmid, *E. coli* BL21 (DE3) Plys, MagneGST™ protein purification system, 3,30,5,50-Tetramethylbenzidene (TMB) Substrate solutions and 0.22 μm Nitrocellulose membranes were purchased from Promega Co.(USA); BCA protein assay kit was purchased from Novagen Co.(USA); Mini-vetical electrophoresis unit and Semi-dry Transfer cell were used in this study. Optical densities of the wells were measured with a microplate reader with KC3 software, a 450-nm sample filter was used for reading optical density.

2.3. Buffers

Sample buffer [pH6.8], consisted of 50 mM Tris, 10% glycerol, 2% sodium dodecylsulfate, 100 mM dithiothreitol, and 0.0025% bromophenol blue. Extraction buffer [pH 7.4], consisted of 1.0 M NaCl, 0.1 M NaH_2PO_4, 0.1% (v/v) Tween 20, 0.5% (v/v) Triton X-100. Blocking buffer consisted of PBS, 0.1% (v/v) Tween 20 and 5% skimmed milk (w/v). Dilution buffer consisted of 0.5% (w/v) skimmed milk and 0.1% (v/v) Tween 20 in PBS. Plate coating buffer [pH 9.6], consisted of 13 mM Na_2CO_3 and 35 mM $NaHCO_3$. Stop solution consisted of 2 M sulphuric acid.

2.4. Synthesisation of nucleotide sequence and cloning for Jug r 1

In order to express the allergen protein Jug r 1 of walnut, a open reading frame of *Jug r 1* nucleotide sequence (Genbank accession number u66866) was selected. For later ease in cloning and expression, the restriction enzyme *EcoR I* site and original codon ATG were added into the 5′-end of the nucleotide sequence, the restriction enzyme *Xhol I* site was added into the 3′-end of the nucleotide sequence. The whole cDNA sequence of 432 bp was synthesised, then the cDNA sequence was cloned into the pGEX-6P-1 plasmid expression vector, and the recombinant pGEX-6P-1 plasmid expression vector was identified by sequencing. The recombinant pGEX-6P-1 plasmid expression vector will result in the synthesis of foreign proteins as a fusion product.

2.5. Expression of fusion protein

In order to get the recombinant fusion protein Jug r 1(rJug r 1), the recombinant pGEX-6P-1 plasmids were transformed into protease-deficient *E. coli* BL21(DE3) Plys competent cells and grown in Luria–Bertani broth with ampicillin at 37 °C, cell growth was photometrically monitored to reach an optical density of 0.8 at 600 nm wavelength. Protein synthesis was induced by adding IPTG, IPTG was added to a final concentration of 1.0 mM, and incubation was continued at 37 °C. Subsequently, cells were harvested after 1 h, 2 h, 3 h, 4 h, 5 h, 6 h, 7 h, cells were centrifuged and resuspended in sample buffer. In the same way, the blank pGEX-6P-1 plasmids were also transformed into protease-deficient *E. coli* BL21(DE3) Plys as blank control, cells were centrifuged and resuspended in sample buffer after 6 h. Samples were boiled for 5 min in sample buffer, SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE) was carried out on a mini-vetical electrophoresis unit at a constant current of 20 mA and 15% SDS–polyacrylamide laemmli gel were used. Finally, the expressed proteins were analysed by coomassie brilliant blue staining.

2.6. Isolation and purification of fusion protein

Fusion proteins rJug r 1 was isolated and purified following the protocol by Frangioni and Neel (1993). Briefly, transformed protease-deficient *E. coli* BL21 (DE3) Plys was grown in Luria–Bertani broth with ampicillin at 37 °C until the optical density reached 0.6–0.9 at 600 nm. IPTG was added to a final concentration of 1.0 mM, and incubation was continued at 37 °C. After 6 h, cells were centrifuged and resuspended in STE buffer. Lysozyme was added to 100 μg/mL and incubated on ice for 15 min. Then dithiothreitol was added to a final concentration of 5 mM. The bacteria were lysed by the addition of *N*-laurylsarcosine to 1.5% and then sonicated for 1 min on ice. The lysate was centrifuged at 10,000*g* at 4 °C for 5 min. The supernatant was removed, Triton X-100 was added to 2% and vortexed, and the fusion proteins were isolated by the MagneGST™ Protein Purification System.

2.7. Production of polyclonal antibodies and western blot identification

The rJug r 1 protein solution set at 1 mg/mL was combined with Freund's complete and incomplete adjuvant for using immunisation of rabbits (Yeung, Newsome, & Abbott, 2000). Two rabbits were immunised against the rJug r 1 protein. Trial bleeds regularly monitored by ELISA for their titre and the lowest inhibition concentration corresponding to antibody-antigen binding. Then whole blood was collected, and the serum was separated. In order to get the antibodies to specific Jug r 1 protein, the GST Glutathione Sepharose 4B and Jug r 1 protein Glutathione Sepharose 4B were prepared, respectively, then polyclonal antibodies to rJug r 1 protein were passed through the two Purification column two times, respectively. To determine the reactivity of the antibody to jug r 1 with the expressed protein and the native peptides derived from the Jug r 1 precursor protein, western blot with the antibody to jug r 1 was performed. For western blot, after SDS–PAGE of samples, the protein were transferred to 0.22 μm nitrocellulose membranes 1.5 h at 0.68 mA/cm^2 by Semi-dry Transfer cell. The blots were blocked in blocking buffer for 2 h at room temperature(or overnight at 4 °C), and then incubated for 1 h at room temperature in the antibodies to Jug r 1 protein diluted 1:100 in dilution buffer. The next step, strips were washed for 10 min three times in washing buffer at room temperature and incubated with goat anti-rabbit IgG labelled with the HRP for 1 h at room temperature. Finally, the strips were washed three times with washing buffer at room temperature, every time for 10 min, then the TMB substrate solution was added onto strips and the reaction was stopped by the addition of PBS.