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Expression of Jug r 1, the 2S albumin allergen from walnut (*Juglans regia*), as a correctly folded and functional recombinant protein

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ABSTRACT

Jug r 1, the 2S albumin allergen from walnut, was isolated from ripe nuts as a native allergen and expressed in *Escherichia coli* using the Gateway® technology as a recombinant allergen. The recombinant Jug r 1 (15 kDa) differs from the native allergen by the absence of cleavage of the polypeptide chain in two covalently associated light (3.5 kDa) and heavy (8 kDa) chains. Recombinant rJug r 1 adopts the canonical α-helical fold of plant 2S albumins as checked on CD spectra. Four IgE-binding epitopic stretches were identified along the amino acid sequence of Jug r 1 and localized on the molecular surface of the modeled allergen. Both native and recombinant allergens exhibit similar IgE-binding activity and similarly trigger the degranulation of a FcεRI-expressing rat basophilic leukaemia cell line previously treated by IgE-containing sera. Native Jug r 1 resists to heat denaturation and to the proteolytic attack of trypsin and chymotrypsin but is readily hydrolyzed in the presence of pepsin at acidic pH after 1 h of incubation at 37 °C *in vitro*. Recombinant Jug r 1 could be used for a component-resolved diagnosis of food-allergy.

1. Introduction

Allergy to walnut (*Juglans regia*) is a commonly reported tree nut allergy responsible for severe anaphylactic reactions in both paediatric and adult populations [23,25]. Its high prevalence, estimated around 0.7% in the total population in USA [24], associated to the widespread consumption of walnut directly from the shell or as an ingredient in a variety of processed foods or pastries, make this tree nut an especially harmful food allergen. Accordingly, the European labelling law for foodstuffs containing life-threatening food allergens has included walnut in the list of potential allergens to be declared on the package, whatever the quantity [3]. Different allergenic proteins have been identified as major allergens in walnut, they correspond to a 2S albumin (Jug r 1) [30], a 7S vicilin-like globulin (Jug r 2) [31], a LTP (Jug r 3) [15], and a 11S legumin-like globulin (Jug r 4) [32,35]. Additionally, a profilin (Jug r 5) has also been shown to occur in walnut as a cross-reacting pan-allergen [34]. More attention has been paid to Jug r 1 [18], which belongs to the largely distributed family of inherently allergenic 2S albumins. These small-sized protein allergens occur in large amounts in many edible tree nuts as seed storage proteins [21] and participate in the IgE-binding cross-reactivity (cross-allergenicity?) commonly observed among tree nuts [1,2,7,19]. Here we report on the production of a correctly folded and fully functional recombinant Jug r 1 (rJug r 1) in *Escherichia coli* that could be used for a reliable component-resolved diagnosis of walnut allergy and other associated tree nut allergies.

2. Materials and methods

2.1. Isolation of native Jug r 1 and antibodies (IgE and IgG)

Native Jug r 1 (nJug r 1) was purified from mature walnuts by affinity chromatography through a column of immobilized anti-rJug r 1 IgG. Usually, 20 g of seed were finely ground in mortar and pestle and suspended in 200 ml of 20 mM Tris-buffered saline (TBS) containing 2 mM EDTA (pH 7.5). The slurry was stirred overnight at 4 °C and then centrifuged 20 min at 30,000 × *g*. The clear supernatant was poured in SpectraPor dialysis tubing (mol. wt, cut-off 3500 Da) and dialyzed against distilled water for 72 h at 4 °C. After centrifugation at 30,000 × *g* for 20 min at 4 °C, the supernatant was stored at −80 °C until used. Protein extract was loaded onto a 1 ml HiTrap NHS column (GE Healthcare) previously

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Abbreviations: HCA, hydrophobic cluster analysis; Jug r 1, *Juglans regia* allergen 1 (2S albumin); Ses i 1/Ses i 2, Sesamum indicum allergens 1 and 2 (2S albumins).

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linked to rabbit polyclonal IgG anti-rJug r 1 that have been purified by filtering rabbit immunsera through a Hitrap protein A column (GE Healthcare). The retained nJug r 1 was eluted with 100 mM glycine buffer, 500 mM NaCl (pH 2.7) and checked for purity by SDS-PAGE.

Polyclonal antibodies against rJug r 1 were raised in rabbits in the presence of Freund's complete adjuvant (Calbiochem). The allergen (100 μg) freshly dissolved in saline (400 μL) and emulsified with adjuvant (400 μL) were repeatedly injected subcutaneously (200 μL) at 10-day intervals during four months. Every three weeks, blood was collected and checked for polyclonal antibodies by ELISA. After blood clotting at room temperature and at 4 °C for 4 h and 12 h, respectively, the immune sera were collected by centrifugation, filtered through a 0.2 μm membrane and stored at −80 °C until used.

Blood samples were drawn after informed consent of three patients allergic to walnut as checked by positive skin prick tests and total IgE dosage. Patient sera were used for both Western blotting, ELISA measurements and degranulation assay of rat basophil leukaemia cells expressing the human FcεRI α-subunit. Control sera were obtained from individuals with no history of walnut allergy.

2.2. Analytical methods

The protein content of the Jug r 1 samples was estimated using the bicinchonic acid kit reagent (Pierce) [26] with bovine serum albumin (BSA) as a standard.

The purity of the Jug r 1 preparations was checked by SDS-PAGE in 15% polyacrylamide gels using Tris-glycine as trailing ion [28] and staining with Coomassie blue and silver nitrate. Coomassie blue stained bands were digested with trypsin in the gel and mass mapped by MALDI-TOF analysis as previously described [22]. The software protein prospector was used for the identification of the protein using the NCBI non-redundant database.

Far-UV circular dichroism (CD) spectra of nJug r 1 and rJug r 1 were recorded at room temperature with a spectropolarimeter Jobin & Yvon CD6 spectrophotometer (Division instrumentations S.A.). The light path for all measurements was 1 mm. A concentration of 0.3 mg mL^{-1} and 0.1 mg mL^{-1} in 10 mM Tris buffer (pH 7.5) was used for nJug r 1 and rJug r 1 samples, respectively. The proportion of α-helical segments was estimated from the CD spectra [27].

2.3. Cloning and expression of rJug r 1 in E. coli

Total RNA was extracted from the peel of mature seeds using the Qiagen extraction kit in the presence of sarkosyl as detergent. The reverse transcription was performed from 1 ng of total RNA previously treated at 70 °C for 3 min and the resulting cDNA was amplified by PCR using the oligonucleotide 5′-primer 5′-ATGGAGATCGACGAGGACAT-3′ and the oligonucleotide 3′-primer 3′-CTAGAACCAGCTTCTGCGAA-5′ for the reverse direction. Two additional PCR steps have been performed for introducing the recombination sites at B1 and at B2 (underlined) at both 5′- (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGTGGAAGTTCTGTTCCAGGGGCCCATG-3′) and 3′-end (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTTA-3′) of the cDNA. The PCR product was inserted in a pDONR201 vector to obtain the "pEntry" construct used to transform DH5α *E. coli* cells, and the DNA from several Kan^r clones were sequenced. Insertion of the cDNA into a pTH19 vector (Invitrogen) used as a "destination vector" was performed through a second recombination step in DH5α cells to select the Amp^r "expression clones" producing Jug r 1 in *E. coli* BL21-DE3 cells. The selected transformants were cultured in LB medium and 0.1 mg mL^{-1} ampicilline at 37 °C, and diluted to obtain a 0.1 OD at 600 nm. Induction was obtained by adding 1 mM IPTG to the culture during the exponential phase of growth (0.5–0.7 OD at 600 nm) and cells were harvested 2 h 30 min later. The cell pellets obtained by centrifugation were solubilized in 20 mM Phosphate buffered saline, 45 mM imidazole (pH 7.4) containing 10 mM $MgCl_2$, 3 μg mL^{-1} of DNAse and protease inhibitor (Roche). The rJug r 1 protein was then purified on a Ni-Sephadex column (GE Healthcare) and eluted with 500 mM NaCl. rJug r 1 was further purified on a Heparin Hitrap column (GE Healthcare) and, after an extensive dialyzis against distilled water, rJug r 1 was lyophilized.

2.4. Epitope mapping

Overlapping 15-mer peptides, frameshifted by three residues, corresponding to the entire amino a cid sequences of Jug r 1 and Ses i 1 were synthesized using the SPOT technique [4] as described previously [10] except for the use of the Multipep automatic Spot synthesizer (Intavis). Briefly, peptides were assembled using the Fmoc chemistry on a cellulose membrane carrying an amino polyethylene glycol moiety. The C-terminal residue of each peptide was coupled to the activated membrane. After Fmoc deprotection, the next amino acids were sequentially added. After completing the synthesis, side-chain protecting groups were removed by a trifluoracetic acid treatment leaving the linkage of the peptides to the membrane intact. Overlapping 15-mer peptides were synthesized as probes to minimize truncation in detecting longer IgE-binding peptide stretches occurring along the amino acid sequence of Ju g r 1.

The membrane was soaked overnight in 20 mL of TBS containing 2 mL blocking buffer (Roche) and 1 g sucrose (pH 7.0), and then washed three times with TBS containing 0.1% (v/v) tween (TBSTw). A 1:5 (v/v) diluted pool of patient sera was added in the presence of an anti-protease cocktail (Roche) and the membrane was incubated in a moist chamber for 2 h. After three washes with TBSTw (pH 7.0) the membrane was shaked in a 1:4000 dilution of mouse monoclonal anti-human IgE labelled with alkaline phosphatase (Sigma) for 1 h. After three washes with TBSTw (pH 7.0), the interacting peptide spots were stained for 30 min by adding the bromochlorylindolophosphate (BCIP) substrate for alkaline phosphatase (Promega). The membrane can be used repeatedly after a regeneration step

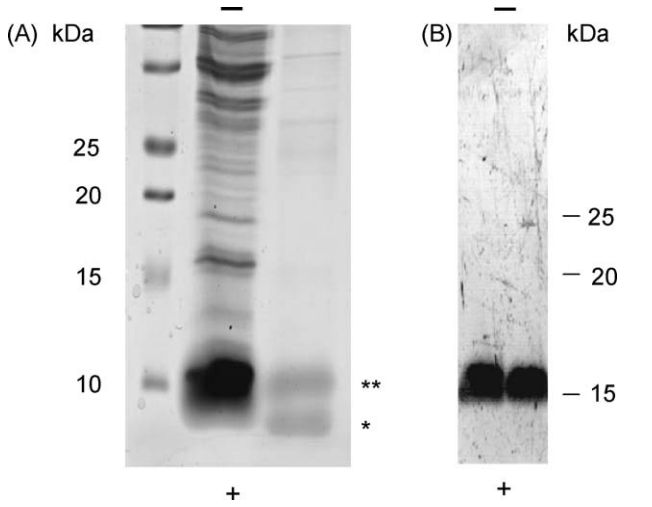

Fig. 1. SDS-PAGE. (A) Separation of seed protein extract (central line) and purified nJug r 1 (right line) on Coomassie blue stained 15% polyacrylamide gel. The heavy (**) and light (*) subunits are indicated. Protein markers of known *M*r migrated on the left line. (B) Separation of purified rJug r 1 on silver stained 15% polyacrylamide gel.