



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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ARTICLE INFO

Article history:

Received 22 January 2009

Received in revised form 13 March 2009

Accepted 13 March 2009

Available online 25 March 2009

Keywords:

Recombinant allergen

Jug r 1

IgE-binding epitopes

Pepsin cleavage

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 Fc ϵ RI-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.

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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).

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'-ATGGA-GATCGACGAGGACAT-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'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGTGGAAGTTCTGTTC-CAGGGGCCATG-3') and 3'-end (5'-GGGGACCACTTGTACAA-GAAAGCTGGGTCTTA-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 MgCl₂, 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 dialysis 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 acid 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 Multiprep 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 trifluoroacetic 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 Jug 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 shaken 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 bromochlorolindolophosphate (BCIP) substrate for alkaline phosphatase (Promega). The membrane was washed three times with deionized water and dried for scanning. The membrane can be used repeatedly after a regeneration step

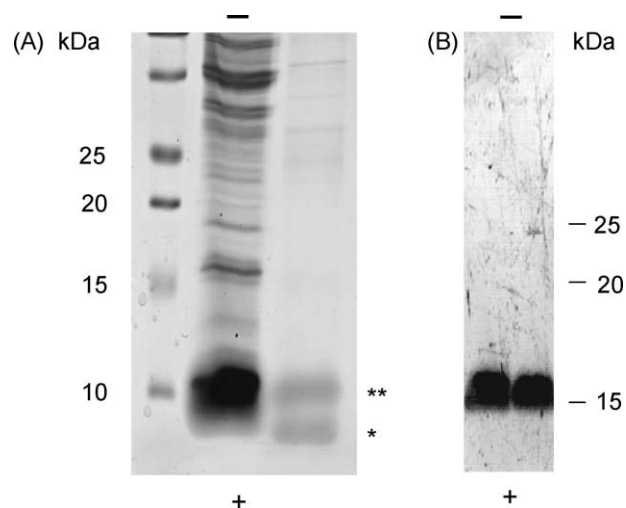


Fig. 1. SDS-PAGE. (A) Separation of seed protein extract (central line) and purified nJug r 1 (right line) on Coomassie blue stained 15% polyacrylamide gel. The heavy (**) and light (*) subunits are indicated. Protein markers of known Mr migrated on the left line. (B) Separation of purified rJug r 1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

consisting of 1–3 washes in dimethylformamide for 10 min each, three washes in deionized water, three washes in 8 M urea containing 1% (w/v) SDS and 1% (w/v) β -mercaptoethanol and three subsequent washes in a mixture of C_2H_5OH – CH_3COOH – H_2O (50:10:40, v/v/v).

Twelve-mer peptides corresponding to the core region of the IgE-binding epitope #2 (41GYDEDNQRRHQ51) and epitope #3 (70RQVRRRQQQQQG81) identified in Jug r 1, were synthesized by

solid-phase Synthesis (GenScript Corp., Piscataway, New Jersey, USA) and checked for reactivity towards IgE by ELISA in inhibition experiments. Twelve-mer peptides were chosen as probes to be sure they cover the entire amino acid sequence of the predicted IgE-binding epitopes.

The capacity of synthetic peptides to inhibit the IgE–Jug r 1 interaction was checked by ELISA. The wells of a flat bottomed standard plate (Limbro) were filled with 50 μ L of 10 mM PBS (pH

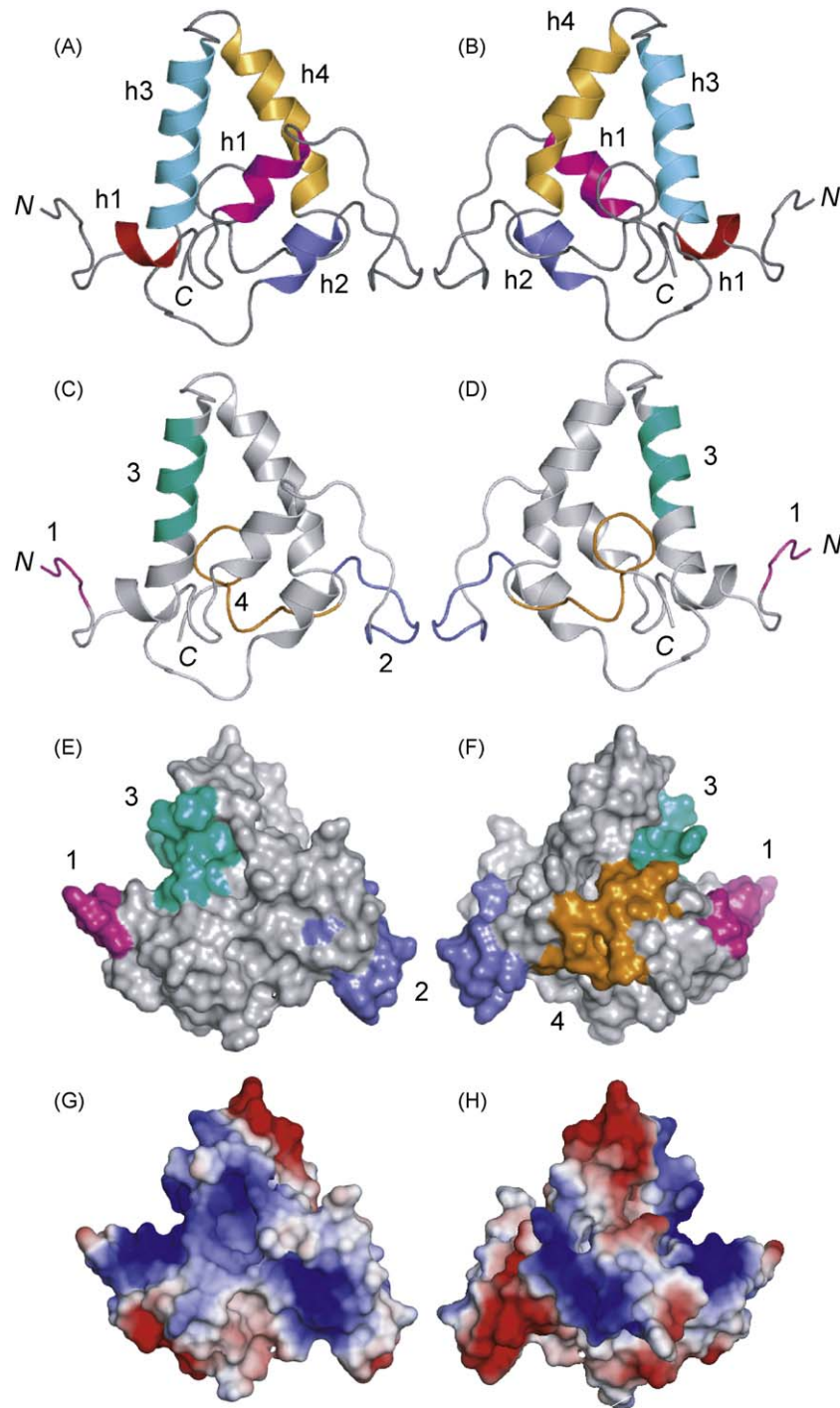


Fig. 2. Structure and IgE-binding epitopes of Jug r 1. (A and B) Ribbon diagram of both faces of the modeled Jug r 1. Helices h1–4 are colored red, violet, cyan and orange, respectively. Loops and coil regions are colored gray. Disulfide bridges are not represented. N and C correspond to the N- and C-terminus of the polypeptide (nJug r 1 is represented as a single polypeptide chain but consists in fact of two light and heavy chains covalently linked by two interchain disulfide bonds). (C and D) Localization of epitope #1 (pink), #2 (violet), #3 (green) and #4 (orange) on the ribbon diagram of Jug r 1. (E and F) Exposition of epitopes #1 (pink), #2 (violet), #3 (green) and #4 (orange) on the molecular surface of Jug r 1. (G and H) Mapping of the electrostatic potentials on the molecular surface of Jug r 1. Electronegatively and electropositively charged regions are colored red and blue, respectively. Neutral regions are white. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

7.5) containing $1 \mu\text{g mL}^{-1}$ of nJug r 1. The plates were incubated overnight at 4°C and washed three times with PBS (pH 7.5). $100 \mu\text{L}$ of PBS (pH 7.5) containing 0.1% Tween 20 (v/v) and 1% BSA (w/v) (PBSTB) were added and the plates were incubated at room temperature for 2 h. $50 \mu\text{L}$ of 1:30 diluted patient serum in PBSTB (pH 7.5) previously incubated with 1 mM or 2 mM of synthetic peptide were added and the plates were incubated for 1 h 30 min at room temperature under constant stirring. After three washes with PBSTB, $50 \mu\text{L}$ of 1:500 diluted mouse monoclonal anti-human IgE coupled to alkaline phosphatase (Sigma) were added and, after incubation at room temperature for 1 h, three washes were performed with PBSTB. $100 \mu\text{L}$ of alkaline phosphatase substrate (Sigma) was added and after incubation for 45 min in the dark, 50 mL of 3 M NaOH were added to stop the color reaction. The absorbance at 405 nm was recorded on a Titertek Multiscan spectrophotometer. Each value is the mean of three separate experiments. Appropriate blank and substrate controls were performed under the same conditions.

2.5. Degranulation of human FcεRI-expressing cells

Checking of nJug r 1 and rJug r 1 for *in vitro* degranulation assay was performed on transfected rat basophilic leukaemia cells expressing the human FcεRI α-subunit (RBL-2H3E5.D12.8) [29] cultured as a monolayer in DMEM-Glutamax medium (Gibco BRL) containing 10% (v/v) foetal calf serum (FCS), 100 U/mL penicillin, 100 U/mL streptomycin, 1 mg/mL G-418 (Gibco) and 20 mM Hepes (pH 7.3), after adding IgE and the corresponding allergens.

After a 72 h culture in the absence of G-418, adherent cells were washed twice in FCS-free medium, treated with trypsin-EDTA (Gibco), and incubated for 5 min at 37°C in a humidified atmosphere containing 5% CO_2 . Cells were then collected, washed and resuspended for counting using a Malassez counting chamber. Cells were replated at 2×10^4 cells/well ($200 \mu\text{L}$) in a standard 96 well plate in G-418-free medium containing human IgE (patient sera diluted 1/50). After an incubation of 48 h at 37°C in a humidified atmosphere at 5% CO_2 , the supernatant was discarded and the cells were washed with the G-418-free medium, a 1:1 mixture of Tyrode- D_2O (pH 7.4) (Sigma), and then flooded by $100 \mu\text{L}$ of a 1:1 mixture of Tyrode- D_2O (Sigma) containing increasing concentrations of the allergen to be assayed (from 5 ng/mL to 2000 ng/mL). After an incubation of 45 min at 37°C and centrifugation at $150 \times g$ for 5 min, $50 \mu\text{L}$ of supernatant from each well was used for immediate measurement of β-hexosaminidase activity. Cell samples were similarly incubated

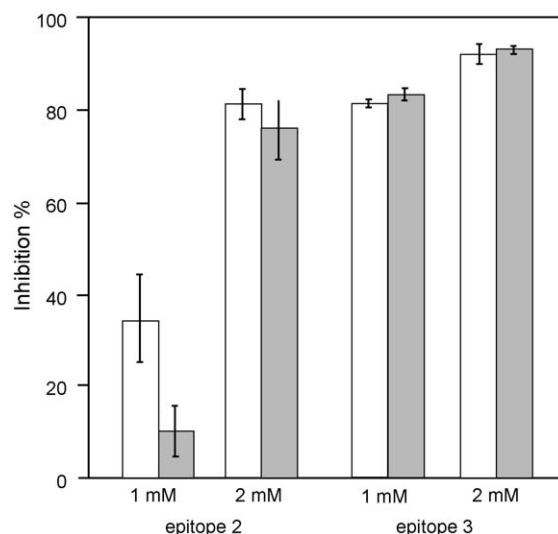


Fig. 4. Inhibition experiments. Inhibition by synthetic peptides corresponding to epitopes 2 and 3 used at 1 mM and 2 mM concentrations of the interaction (expressed as %) of IgE with immobilized nJug r 1 (white) and rJug r 1 (grey). Results are the means of three ELISA measurements.

with Tyrode- D_2O buffer free of patient serum for measuring the spontaneous release of β-hexosaminidase. Samples for measuring the β-hexosaminidase content of cells were treated with $3 \mu\text{L}$ of 10% (v/v) Triton X-100 for 3 min before centrifugation. Controls

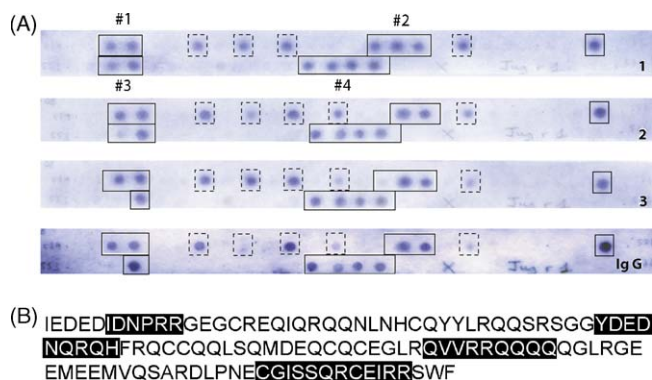


Fig. 3. Epitope mapping. (A) Examples of SPOT results obtained with nJug r 1 using IgE from three allergic patients (1–3) or rabbit polyclonal IgG (IgG) as the 1st antibody probe. Labelled spots corresponding to sequential epitopic regions #1, #2, #3 and #4 are black boxed. Spots restricted to a single peptide are dotted boxed. (B) IgE-binding epitopic stretches deduced from the IgE-reacting spots according to their exposition on the molecular surface of the Jug r 1 model are indicated in black boxed white letters along the amino acid sequence of Jug r 1.

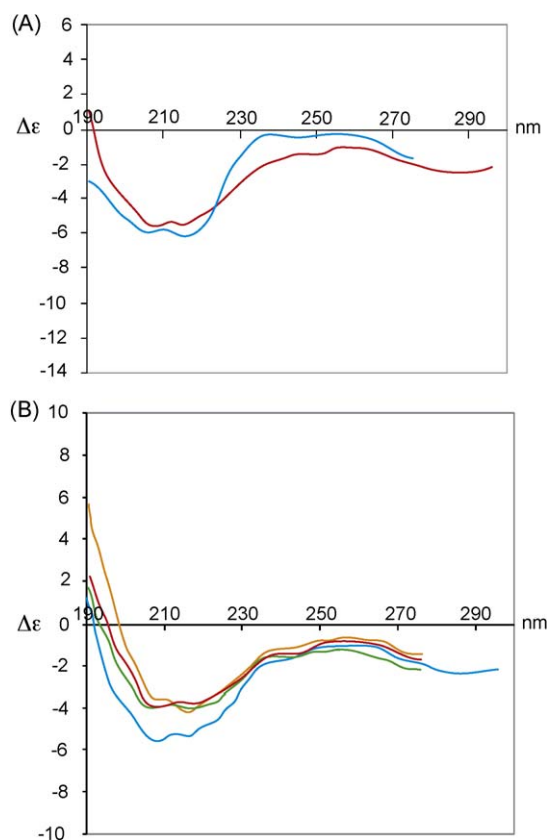


Fig. 5. Circular dichroism spectra of Jug r 1. (A) CD spectra of nJug r 1 (red) and rJug r 1 (blue) recorded in the far-UV region (190–260 nm). nJug r 1 and rJug r 1 were used at a concentration of 0.3 mg mL^{-1} and 0.1 mg mL^{-1} in 10 mM Tris buffer (pH 7.5), respectively. (B) CD spectra of nJug r 1 recorded at 20°C (blue) and after warming at 37°C (green), 60°C (red) and 90°C (orange) and subsequent cooling at 20°C . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

were performed with both sera from non-allergic individuals and BSA.

The 50 μL samples corresponding to the tested cell samples were distributed in a 96 well plate and 50 μL *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide at 1.3 mg mL^{-1} in 0.05 M citrate buffer (pH 4.5) were added. After incubation at 37 °C for 90 min, the enzymatic reaction was stopped by adding 150 μL of 200 mM glycine buffer (pH 10.7) and optical density at 410 nm was estimated. Results are expressed as the percentage of β -hexosaminidase released from the IgE-stimulated cells in the presence of nJug r 1 or rJug r 1 after correction for spontaneous release from unstimulated cells. Values are the means of triplicate cell incubations.

2.6. Molecular modeling

Multiple amino acid sequence alignments were carried out with CLUSTAL-X [33] and displayed with ESPript [8] using the Risler's homology matrix for structurally homologous residues [17]. The HCA (hydrophobic cluster analysis) [5] was performed to delineate the conserved secondary structural features (stretches of α -helix) along the amino acid sequence of Jug r 1 by comparison with the Castor bean (*Ricinus communis*) 2S albumin allergen Ric c 3 [14] used as a model. HCA plots were generated using the HCA plot server (<http://bioserv.rpbs.jussieu.fr>).

Homology modeling of Jug r 1 (access. AAB41308.1) was carried out on a silicon graphics O2 R10000 workstation, using the programs InsightII, Homology and Discover3 (Accelrys, San Diego, CA, USA) from the atomic coordinates of the castor bean allergen Ric c 3 [14] (RCSB protein data bank code 1PSY). The percentages of both identity ($\sim 35\%$) and similarity ($\sim 75\%$) Ric c 3 shares with Jug r 1 allowed us to build rather accurate three-dimensional models using the RMN coordinates of the Castor bean 2S albumin as a template. The extended N-terminal region, which is extremely

desordered in the 20 RMN models of Ric c 3, was omitted in the model building. Extra-loops occurring in Jug r 1 were tentatively modeled using the best geometrical fitting with the core domain as a criterion for choosing among the different loop conformations available in the PDB. Steric conflicts were corrected during the model building procedure using the rotamer library [16] and the search algorithm of Homology [11] to maintain proper side-chain orientation. The geometry of loop regions was corrected using the refine option of TurboFrodo [20]. An energy minimization of the final models was carried out by 100–150 cycles of steepest descent using the Polack–Ribiere algorithm of Discover3. The program TurboFrodo was run to draw the Ramachandran plot and to perform the superposition of the models with the template protein. Assessment of the geometric quality of the three-dimensional models was performed with PROCHECK [9]. About 80% of the residues of Jug r 1 were correctly assigned in the best allowed regions of the Ramachandran plot, the remaining residues being located in the generously allowed regions of the plot except for residues Glu8 and Glu57 which occur in the non-allowed region (result not shown). Using ANOLEA [12] to evaluate the model, only seven residues over 110 (vs. 6 over 93 for Ric c 3 (1PSY code) used as a template) exhibited an energy over the threshold value.

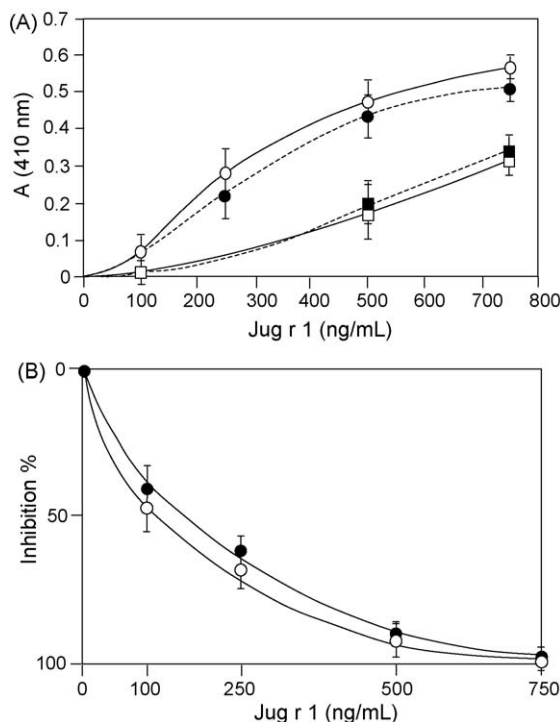


Fig. 6. ELISA measurements. (A) ELISA measurement of the interaction of increasing concentrations (ng/mL) of nJug r 1 (●), and rJug r 1 (○), with IgE from patient sera. Interaction of nJug r 1 (□), and rJug r 1 (■), with rabbit polyclonal IgG was also compared. (B) Inhibition (%) of the interaction of rJug r 1 with IgE by increasing concentrations ($\mu\text{g mL}^{-1}$) of nJug r 1 (●), or rJug r 1 (○).

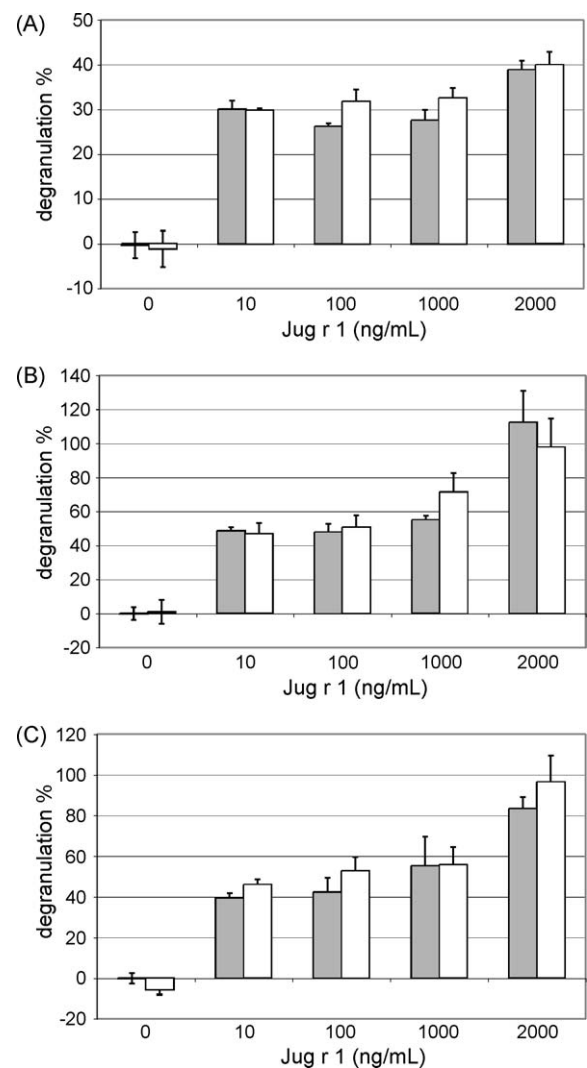


Fig. 7. Degranulation experiments. Degranulation, expressed as percentage (%), of transformed rat basophil leukaemia cells expressing the Fc ϵ RI α -subunit previously treated with IgE from three allergic patients (A, B, C) in the presence of increasing concentrations of nJug r 1 (grey) and rJug r 1 (white). Note that some degranulation occurs even in the absence of native or recombinant allergen.

Cartoons were drawn with PyMOL (<http://www.pymol.sourceforge.org>).

Electrostatic potentials were calculated and displayed with GRASP [13]. The solvent probe radius used for molecular surfaces was 1.4 Å and a standard 2.0 Å-stern layer was used to exclude ions from the molecular surface [6]. The inner and outer dielectric constants applied to the protein and the solvent were respectively fixed at 4.0 and 80.0 and the calculations were performed keeping a salt concentration of 0.145 M.

The overall conformation of the previously identified sequential IgE-binding epitopes along the amino acid sequence of Jug r 1 was calculated and displayed on the molecular surface of the allergen with PyMOL.

2.7. Resistance of Jug r 1 to thermal and proteolytic denaturation in vitro

Native Jug r 1 was dissolved at a concentration of 0.3 mg mL⁻¹ in 10 mM Tris buffer (pH 7.5) and warmed at 37 °C, 60 °C and 90 °C, respectively. Acquisition of CD spectra for each temperature was performed at 20 °C, after a cooling of 15 min, to check for a possible secondary structure change resulting from the heat denaturation of the allergen.

For pepsin hydrolysis, nJug r 1 was dissolved in prewarmed 0.1 M HCl with 30 mmol/L NaCl (pH 1.5) containing pepsin (Worthington) at a ratio pepsin/nJug r 1 of 10:1 (27000 U pepsin by mg of nJug r 1) and 1:1 (2700 U pepsin by mg of nJug r 1), respectively. Digestion was performed at 37 °C for 90 min. Aliquots corresponding to 2 µg of Jug r 1 were taken at 0 min, 0.5 min, 1 min, 2 min, 5 min, 10 min, 20 min, 30 min, 60 min and 90 min of incubation and analyzed. The enzymatic reaction was stopped by raising the pH to 8 with 1 µl of 1 M NaOH.

For trypsin/chymotrypsin hydrolysis, nJug r 1 was dissolved in prewarmed 25 mmol/L ammonium bicarbonate (pH 8) in the presence of immobilized trypsin and chymotrypsin (EnzyBeads Magnet AGRO BIO, France). Digestion was performed at 37 °C for 90 min at a ratio trypsin/chymotrypsin/nJug r 1 of 400 U:4 U:1 mg and 200 U:2 U:1 mg, respectively. Aliquots corresponding to 2 µg

of nJug r 1 were taken at 0 min, 0.5 min, 1 min, 2 min, 5 min, 10 min, 20 min, 30 min, 60 min and 90 min and analyzed.

Aliquot samples from both protease digestions were separated by SDS PAGE and after electrotransfer to nitrocellulose membranes, an immunodetection was carried out using rabbit polyclonal IgG raised against rJug r 1 as a 1st antibody. The IgG-binding capacity of nJug r 1 in aliquot samples was measured by ELISA in inhibition experiments.

3. Results

3.1. Isolation and structure of the native Jug r 1 allergen

The procedure used for the isolation of native nJug r 1 yielded a single protein fraction dissociated in the two constituent small (3.5 kDa) and large (8 kDa) subunits in SDS-PAGE (Fig. 1A). The MALDI-TOF measurements of the nJug r 1 preparation yielded two major ions of 3437 Da and 8009 Da, corresponding to the light and heavy subunits, respectively. The native walnut 2S albumin nJug r 1 consists of two polypeptide chains of 3.5 kDa (light chain) and 8 kDa (heavy chain), respectively, associated by two interchain disulfide bonds and non-covalent bonds, resulting from the processing of a single chain precursor (14 kDa). In addition to the two interchain disulfide bonds, two intrachain disulfide bonds occur in the heavy chain of nJug r 1. These conserved eight cysteine residues participating to the disulfide bridges confer a tightly globular packing to the allergen. Like for other seed storage 2S albumins, the three-dimensional model built for nJug r 1 consists of four α-helices (h1–h4) connected by loops to coil regions (Fig. 2A). In fact, helix h1 consists of two short α-helical stretches 8CREQI12 and 19NHYYL23 separated by an extended extra-loop region of six residues. This region corresponds to the light chain of Jug r whereas the remaining three α-helices h2–h4 belong to the heavy chain (Fig. 2A).

3.2. Mapping of IgE-binding epitopes

Epitope mapping using the three IgE-containing sera from walnut allergic patients identified four IgE-binding epitopic regions

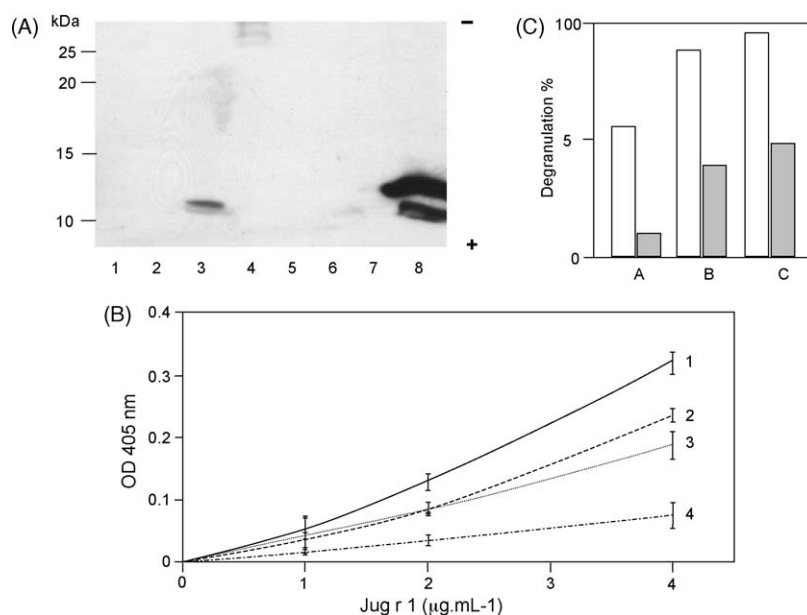


Fig. 8. IgE-binding cross-reactivity. (A) Immunoblot analysis of total soluble proteins from peanut (1), soybean (2), sesame (3), hazelnut (4), cashew nut (5), Pecan nut (6), Brazil nut (7) and nJug r 1 (8) showing that the rabbit anti-Jug r 1 polyclonal IgG only recognize nJug r 1 and sesame 2S albumin but do not react with other protein fractions. (B) ELISA measurements showing the inhibition by increasing concentrations of sesame seed extract (25 µg mL⁻¹ for curve 2; 100 µg mL⁻¹ for curve 3; 200 µg mL⁻¹ for curve 4) of the binding of Jug r 1 to anti-Jug r 1 polyclonal IgG. Curve 1 corresponds to the binding measured in the absence of sesame extract.

along the amino acid sequence of Jug r 1. They correspond to stretches 6IDNPRR11 (epitope #1 of six residues), 42YDEDNQQRH50 (epitope #2 of 9 residues), 72QVRRRQQQ80 (epitope #3 of 9 residues), and 102CGISSQRCEIRR113 (epitope #4 of 12 residues), respectively (Fig. 3A,B). All these epitopic regions contain charged residues and coincide with nicely exposed electropositively (epitopes #1, #3 and #4) and electronegatively (epitope #2) charged areas on the molecular surface of the allergen (Fig. 3E–H). Other additional single spots were systematically labelled in epitope mapping experiments (Fig. 3A) that correspond to smaller IgE-binding epitopic stretches 18EQI20, 24QNL26, 30QYY32 and 54CCQ56. However, some of these regions (24QNL26, 30QYY32) are poorly exposed on the molecular surface of Jug r 1 (result not shown) and most probably poorly account for the allergenicity of the protein. Other regions are located in the vicinity of epitopes #1 (18EQI20) and #2 (54CCQ56) and could participate in more extended discontinuous epitopes recognized by IgE from allergic patients. Very similar restricted epitopic regions were identified using rabbit polyclonal IgG instead of IgE from patient sera as a primary antibody source in epitope mapping experiments (Fig. 3A). Synthetic 12-mer peptides corresponding to epitopes #2 (GYDEDNQQRH) AND #3 (RQVRRRQQQQG) significantly inhibited the interaction of both nJug r 1 and rJug r 1 with IgE from allergic patient sera (Fig. 4), that confirms the IgE-binding capacity of the epitopic regions identified by the SPOT technique.

3.3. Structure and activity of the recombinant allergen

The rJug r 1 allergen expressed in *E. coli* readily differs from its nJug r 1 counterpart by a longer and unprocessed single polypeptide, that confers a higher mass (14,248 Da as measured by MALDI-TOF) to the recombinant protein (Fig. 1B). The lack in *E. coli* of processing enzymes most probably accounts for the absence of cleavage in the allergen precursor. The correct α -helical fold of rJug r 1 was assessed on the CD spectra showing two minima around 208 nm and 222 nm representative for a purely α -helical folded proteins for both nJug r 1 and rJug r 1 (Fig. 5A). In spite of these discrepancies, both nJug r 1 and rJug r 1 exhibit a very similar IgE-binding reactivity as shown from ELISA measurements (Fig. 6A). Very similar results were obtained using rabbit polyclonal anti-rJug r 1 IgG instead of IgE (Fig. 6A). In addition, a pre-incubation of the IgE-containing patient sera in the presence of increasing amounts of nJug r 1 significantly inhibited and finally abolished the interaction of rJug r 1 with the IgE and, similarly, the interaction of rJug r 1 with IgE was so efficiently inhibited by a pre-incubation in the presence of rJug r 1 (Fig. 6B). Finally, no significant difference occurred between rJug r 1 and nJug r 1 in their ability to trigger the *in vitro* degranulation of a Fc ϵ R1-expressing rat basophilic leukaemia cell line as estimated by the β -hexosaminidase release from IgE-stimulated cells previously treated by the allergens (Fig. 7).

3.4. IgE-binding cross-reactivity

Checking for possible IgE-binding cross-reactivity with other seed extracts containing 2S albumin allergens in Western blotting experiments, yielded a single positive result with sesame (*Sesamum indicum*) seeds (Fig. 8A). No cross-reaction could be detected with seed extracts from peanut (*Arachis hypogaea*), soybean (*Glycine max*), cashew nut (*Anacardium occidentale*), pecan nut (*Carya illinoensis*), Brazil nut (*Bertholletia excelsia*) and chestnut (*Corylus avellana*). Upon fractionation of the sesame seed extract in the presence of increasing concentrations of solid ammonium sulfate, the protein fraction precipitating at 80% ammonium sulfate saturation, which contains the 2S albumin Ses i 1 (access.) and Ses i 2 (access.) allergens, was confirmed as the sole IgE-binding reactive fraction in

Western blot experiments. Moreover, this sesame protein fraction significantly inhibited the binding of Jug r 1 to the corresponding IgE as measured by ELISA inhibition experiments (Fig. 8B). Epitope #3, which shares 66% of identity and 100% of similarity with the homologous region of Ses i 1 (QAVRQQQE in Ses i 1 instead of QVRRRQQQ in Jug r 1), most probably accounts for the IgE-binding cross-reactivity. This IgE-binding cross-reactivity is likely to be clinically relevant since a pre-treatment of Fc ϵ R1-expressing rat basophilic leukaemia cell line by a sesame seed extract resulted in some degranulation (Fig. 8C).

3.5. Resistance to heat denaturation and proteolysis of the allergen

As a seed storage protein, nJug r offers a moderate resistance to hydrolysis by pepsin *in vitro* at 37 °C and pH 1.5 (Fig. 9A,B). After a 1 h incubation period, intact heavy chain is no more detectable using IgE from allergic patients as a probe. Immuno-reactive peptides resulting from the proteolysis of the core region of nJug r 1 are not detected most probably because they are too small (8 and 12 residues for those containing epitope #2 and epitope #3, respectively, as predicted from the Peptide Cutter analysis and surface analysis showing the pepsin cleavage sites sufficiently exposed to be attacked by the enzyme (Fig. 10) to be retained on

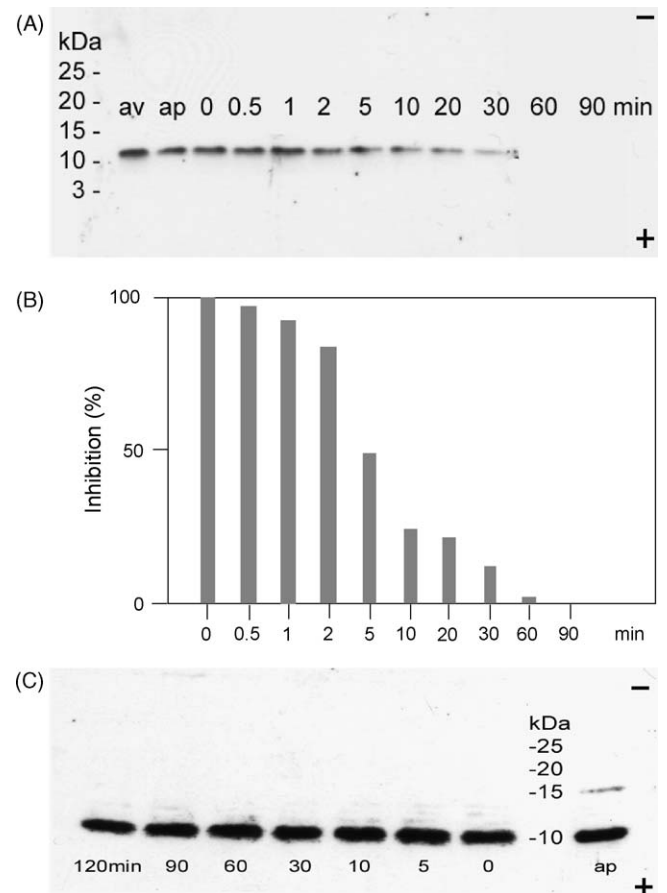


Fig. 9. Resistance to proteolysis. (A) Western blot of the IgE-reacting peptides resulting from the progressive *in vitro* digestion of nJug r 1 in the presence of pepsin at pH 1.5. The progressively vanishing IgE-labelled fraction corresponds to the heavy chain (11 kDa) of nJug r 1. (B) ELISA measurement of the inhibition, expressed as percentage (%), of the interaction of nJug r 1 with IgE from allergic patients by peptides obtained after *in vitro* incubation periods (up to 90 min) of nJug r 1 in the presence of pepsin at pH 1.5. (C) Western blot of the IgE-reacting peptides resulting from the progressive *in vitro* digestion of nJug r 1 in the presence of trypsin/chymotrypsin at pH 8.0. The unattacked IgE-reactive peptide corresponds to the heavy chain (11 kDa) of nJug r 1.

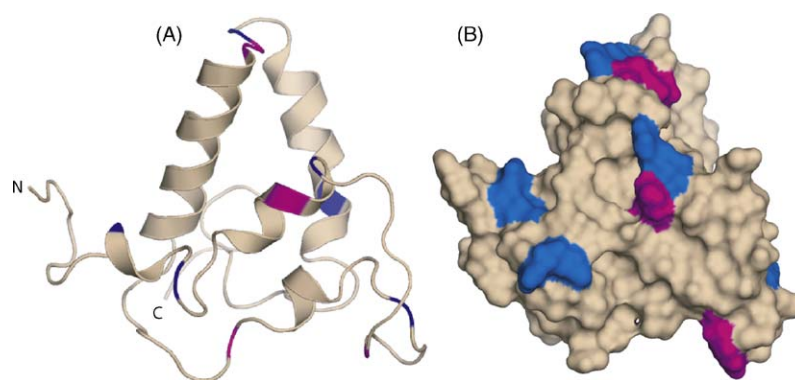


Fig. 10. Proteolytic sites. Exposed sites susceptible to pepsin (colored magenta) and trypsin (colored blue) on the ribbon diagram (A) and the molecular surface molecular surface (B) of Jug r 1. Other buried cleavage sites that, most probably, remain uncleaved upon proteolytic attack, are not represented. N and C indicate the N- and C-terminal ends of the polypeptide. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

the polyacrylamide gel used for the SDS-PAGE. Conversely, nJug r 1 resists to the attack of trypsin and chymotrypsin since the heavy chain is readily detected after 2 h of incubation at 37 °C and pH 8 (Fig. 9C). This resistance to trypsin/chymotrypsin is rather puzzling with respect to the occurrence of well exposed trypsin cleavage sites on the molecular surface of Jug r 1 (Fig. 10). In addition, nJug r 1 exhibits a good resistance to heat denaturation as shown from its capacity to retain its α -helical fold after heating at 90 °C and subsequent cooling to 20 °C (Fig. 5B).

4. Discussion and conclusion

Four main sequential IgE-binding epitopic regions were identified on the molecular surface of the walnut 2S albumin Jug r 1 allergen together with four other regions of more restricted area. Epitope #3 72QVVRQQQ80 (net charge 2) was identified in a region adjacent to the main IgE-binding epitope 81QGLRGEE-MEEMV92 (net charge -3) previously identified along the amino acid sequence of Jug r 1 by a similar SPOT technique using a set of overlapping 13-mer peptides with an offset of three residues [18]. Even though both sequences are well exposed on the surface of Jug r 1, they do not overlap. This discrepancy could result, at least in part, from the use in the present work of overlapping 15-mer peptides corresponding to the complete sequence (light + heavy chain) of Jug r 1 instead of the 13-mer peptides corresponding to the sole heavy chain of Jug r 1 used in the previous work. Accordingly, none of the checked immobilized peptides used in both investigations exhibited a similar amino acid sequence. In addition, another source of discrepancy could depend on the patient sera used for revealing the IgE-binding peptides.

The recombinant rJug r 1 allergen expressed in *E. coli* consists of an uncleaved single chain precursor which readily differs, in this respect, from the native allergen which consists of two covalently linked light and heavy chains resulting from the *in planta* processing of the single chain precursor. However, in spite of the absence of processing, the rJug r 1 precursor exhibits an overall fold and a disulfide bonding pattern virtually identical to that found in the native nJug r 1 allergen, as shown from the superposition of their CD spectra (Fig. 5A). In agreement with these structural similarities, both nJug r 1 and rJug r 1 exhibit a highly similar reactivity towards IgE as measured in ELISA experiments (Fig. 6) and *in vitro* degranulation of a Fc ϵ RI-expressing rat basophilic leukaemia cells (Fig. 7). In addition, the interaction of nJug r 1 and rJug r 1 with IgE from allergic patients was similarly inhibited by two synthetic 12-mer peptides corresponding to epitopes #2 and #3 identified on the molecular surface of Jug r 1 (Fig. 4). According to this virtually identical IgE-binding reactivity, rJug r 1 thus appears as a particularly useful tool

for replacing nJug r 1 in the component-resolved diagnosis of walnut allergy.

Like many other 2S albumin allergens, native Jug r 1 readily resists to the trypsin/chymotrypsin attack at basic pH (8.0) but progressively loses its allergenicity in the presence of pepsin at acidic pH (1.3) *in vitro*. This pepsin susceptibility is consistent with the occurrence of well exposed pepsin cleavage sites on the molecular surface of nJug r 1 (Fig. 10). However, the occurrence of similarly exposed trypsin cleavage sites on Jug r 1 makes puzzling the apparent lack of susceptibility recorded in both trypsin and chymotrypsin cleavage experiments (Fig. 9C). Except for the basic pH (8.0), other conditions that usually occur during the digestion in the small intestine, e.g., nJug r 1 embedded in food and previously hydrolyzed by pepsin in the stomach, are far from being respected in the *in vitro* trypsin/chymotrypsin cleavage experiments. The lack of proteolysis of nJug r 1 in the presence of trypsin/chymotrypsin could result, at least in part, from an unadapted technique that too crudely mimics the physiological conditions necessary for an efficient intestinal digestion.

Acknowledgements

The financial support of CNRS (C.S., R.C., A.B., P.R.) and the Société Française d'Allergologie et d'Immunologie Clinique is gratefully acknowledged. The excellent scientific advice of Dr. D. Aldon on the Gateway technology is acknowledged. The authors thank C. Nguyen for expert technical assistance in Spot synthesis. Many thanks to Dr. S. Mécheri (Institut Pasteur) for providing us with the rat basophil leukaemia cells expressing the human Fc ϵ RI α -chain. Thank you to H. Benoist for allowing us to use the technical facilities of INSERM Unité 858 (Toulouse).

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