RESEARCH ARTICLE

Isolation, cloning, and characterization of the 2S albumin: A new allergen from hazelnut

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Scope: 2S albumins are the major allergens involved in severe food allergy to nuts, seeds, and legumes. We aimed to isolate, clone, and express 2S albumin from hazelnut and determine its allergenicity. **Methods:** 2S albumin from hazelnut extract was purified using size exclusion chromatography and RP-HPLC. After *N*-terminal sequencing, degenerated and poly-d(T) primers were used to clone the 2S albumin sequence from hazelnut cDNA. After expression in *Escherichia coli* and affinity purification, IgE reactivity was evaluated by Immunoblot/ImmunoCAP (inhibition) analyses using sera of nut-allergic patients. **Results:** *N*-terminal sequencing of a ~10 kDa peak from size exclusion chromatography/RP-HPLC gave two sequences highly homologous to pecan 2S albumin, an 11 amino acid (aa) *N*-terminal and a 10aa internal peptide. The obtained clone (441 bp) encoded a 147aa hazelnut 2S albumin consisting of a putative signal peptide (22 aa), a linker peptide (20 aa), and the mature protein sequence (105 aa). The latter was successfully expressed in *E. coli*. Both recombinant and natural 2S albumin demonstrated similar IgE reactivity in Immunoblot/ImmunoCAP (inhibition) analyses. **Conclusion:** We confirmed the postulated role of hazelnut 2S albumin as an allergen. The availability of recombinant molecules will allow establishing the importance of hazelnut 2S albumin for hazelnut allergy.

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1 Introduction

Legumes, tree nuts, and seeds such as peanut, hazelnut, walnut, sesame and mustard seeds are known to potentially induce severe food allergy. Several storage proteins have been identified as major allergens in these foods. These include, using peanut as an example, 7S (*e.g.* Ara h 1) and 11S (*e.g.* Ara h 3) globulins and 2S albumins (*e.g.* Ara h 2 and Ara h 6) [1–3]. In peanuts, 2S albumins have more impact on allergenicity than the globulins [4, 5]. 2S albumins are also considered major allergens in walnut, Brazil nut, oilseed rape, castor bean, and mustard seed (Moreno and Clemente [6], and references therein). 2S albumins are water-soluble seed storage proteins present in dicotyledonous plants, including legumes. They share a characteristic four helix, four disulfide bridges structure [5] with cereal α-amylase/trypsin inhibitors and nonspecific lipid transfer proteins (nsLTP) and are typically heterodimers, composed

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Abbreviations: aa, amino acid; **HRP,** horseradish peroxidase; **kUA/L,** kilounits of antibody *per* liter; **nsLTP,** non-specific lipid transfer protein; **RACE,** rapid amplification of cDNA ends

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of a large and small subunit joined by one or two disulfide bridges. These two subunits result from post-translational processing of the precursor protein at several sites, close to the *N*-terminus, internally, and at the *C*-terminus [7, 8]. Several 2S albumins show antimicrobial and antifungal properties [9, 10]. Due to their important nutritional role in human food and animal feeds, 2S albumins and the associated genes have been isolated from a number of plant species and characterized in great detail to facilitate protein manipulation and improvement [11–14]. Recombinant 2S albumin proteins from peanut [15, 16] and tree nuts (walnut and cashew) [17, 18] have been expressed in *Escherichia coli*.

Hazelnuts rank amongst the most important sources of food allergy, with a prevalence estimated to be between 0.1 and 4% [19]. Recent extensive surveys [20, 21] have confirmed the clinical importance of hazelnut using double blind, placebo-controlled food challenges. In addition, unpublished information from an ongoing multidisciplinary European framework study into the prevalence, cost, and basis of food allergy across Europe (EuroPrevall), supported that hazelnut allergy is the most common food allergy in the EU. Hazelnut allergy can vary between mild oral symptoms to potentially dangerous anaphylaxis [22]. Several hazelnut allergens have so far been identified and characterized [23]. Primary sensitization to pollen allergens, predominantly the birch pollen allergens Bet v 1 and Bet v 2 (profilin), frequently results in cross-reactivity to hazelnut Cor a 1 and Cor a 2, respectively [24]. These allergens usually only cause mild oral symptoms [25]. The nonpollen-related allergens that have so far been identified in hazelnut are the storage proteins Cor a 11 (7S globulin), Cor a 9 (11S globulin), and Cor a 8, a nsLTP [22]. Sensitization to some of these allergens has been demonstrated to be a risk factor for severe systemic reactions [22, 26]. They have also been described as allergens in young children that have not yet developed respiratory allergies [27]. Recently, also two oleosin isoforms (Cor a 12 and Cor a 13) were identified as allergens in hazelnut [28].

Because of its proven allergenicity in several legumes, nuts and seeds, 2S albumin has always been presumed to also be an allergen in hazelnut. However, to the best of our knowledge, only one study has reported the *N*-terminal sequence (14 aa) of an IgE-binding component from hazelnut with 50% identity to an internal stretch of a soybean 2S albumin precursor [29]. Additionally, one study reported the inhibition of IgE binding to almond 2S albumin by a hazelnut extract [30]. Furthermore, the apparent molecular mass of this putative hazelnut 2S albumin (32 kDa) on SDS-PAGE was quite different from the expected molecular mass between 10 and 20 kDa. Attempts to clone hazelnut 2S albumin using IgE screening of a hazelnut cDNA library [28] were also unsuccessful (own observations). The aim of this study was therefore to isolate natural 2S albumin from hazelnut, to clone the gene encoding this protein, and to determine the IgE-binding capacity.

2 Material and methods

2.1 Sera

Thirty-six sera from patients with moderate to severe clinical symptoms to nuts (sometimes specified as hazelnut) were recruited *via* the outpatient clinics of the Medical Centre Alkmaar and the University Medical Centre Utrecht (The Netherlands).

2.2 Hazelnut extract

Thirty grams of unskinned hazelnuts (purchased from a local store) were first ground in a Waring blender (Waring Commercial, Hartford, CT, USA) and then powdered under liquid nitrogen using a mortar and pestle. Four grams of the resulting hazelnut powder were added to 50 mL acetone and kept at -20°C for 2 h, followed by filtration in a Buchner funnel using 3 MM Whatmann filter paper. The retained hazelnut solid was then washed twice with 50 mL of ice-cold acetone and dried in a fume hood for 2 h. Three grams of the dried hazelnut powder were resuspended in water and gently agitated overnight at 4°C. After centrifugation (20 min, $10\,000 \times g$, 4°C) the supernatant was collected and filtered through 0.2 μm cellulose acetate filter (Sartorius Stedim Biotech).

2.3 Purification of 2S albumin from hazelnut extract

Ten milliliters of filtered extract were loaded on a Superdex 75 HR (1.6 cm × 60 cm) size exclusion column, attached to a Pharmacia P-1 fluid-phase LC pump and equilibrated with water. Elution was performed at a 0.75 mL/min flow rate and 4 mL fractions were collected. The fractions were pooled in four groups (Fig. 1A) and concentrated by freeze drying, Size exclusion fraction 3 (SE3) was resuspended in water and applied to a RP Grace Vydac C-18 semipreparative column coupled to a Gilson HPLC system and equilibrated in 95% buffer A (0.07% v/v TFA/water) and 5% buffer B (0.05% v/v TFA/acetonitrile). Proteins were eluted with a linear gradient (5–50%) at a flow rate of 2.5 mL/min.

2.4 *N*-terminal sequencing

Following SDS-PAGE, the proteins were blotted onto a polyvinylidene difluoride membrane using a 3-(cyclohexylamino)-1-propanesulfonic acid/methanol buffer system and stained with Coomassie R. Relevant bands were excised and subjected to *N*-terminal sequencing by Edman degradation (PNAC Facility, University of Cambridge, UK).

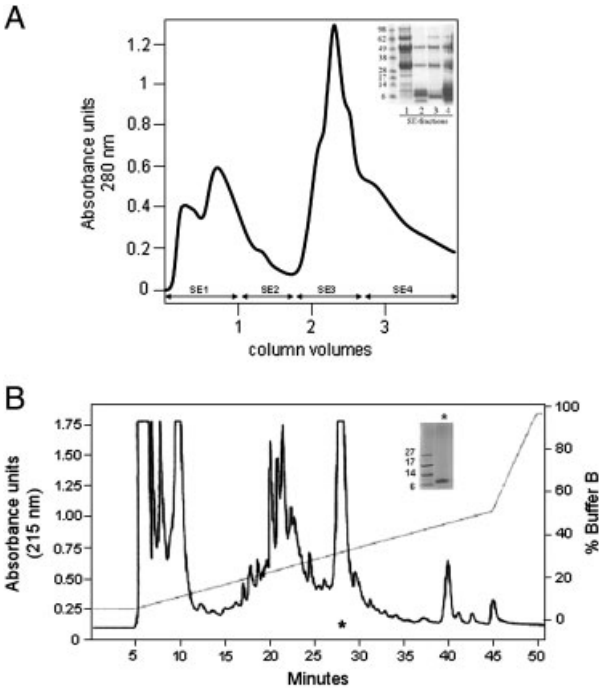

Figure 1. (A) Size exclusion chromatographic profile of the hazelnut extract. The four generated pools are indicated below, as well as their profile on SDS-PAGE (Coomassie-stained) gel. (B) RP-HPLC chromatogram of the SE3. The 2S albumin-containing peak is indicated by the asterisk (*). This fraction is shown on the Coomassie-stained Tricine gel, the purified fraction was used in CAP.

2.5 Isolation of the complete 2S albumin coding cDNA

RNA was isolated from hazelnuts (*cv* Tonda Gentile delle Langhe, Italy) according to the protocol of López-Gómez and Gomez-Lim [31] and then harvested and immediately stored at −80°C to preserve the RNA integrity. The RNA was reverse-transcribed using a poly(dT) anchored primer: RAP (5′-GGCCACGCGTCGACTAGTAC$(T)_{17}$-3′). The generated cDNA was amplified by means of PCR with a degenerate forward primer, pec2S (5′-CGAGGCGAGAGC-TGCCGGGARCAGGCNCAA-3′, R = A/G, N = A, C, G and T), based on the pecan 2S albumin gene (AY192569) and the *N*-terminal aa sequence data. The PCR products were cloned into pGEMT (Promega, Madison, WI, USA) and sequenced using the T7 and Sp6 primers (BaseClear Lab Services, Leiden, The Netherlands). After sequencing, the primers were designed to perform 5′ rapid amplification of cDNA ends (RACE)-PCR. Three hundred nanograms of the total RNA were used to generate cDNA according to the 5′ RACE System (Invitrogen, Carslsbad, CA, USA) with two gene-specific primers (GSP1, 5′-GTCTCCATAACCTCCCTC-3′ and GSP2, 5′-TTCCTCACCTCGCATCTC-3′). The purified products were cloned and sequenced as before. The putative signal peptide of the 2S albumin gene was predicted by web software (SignalP 3.0, www.cbs.dtu.dk/services/SignalP).

The cDNA of 2S albumin from hazelnut was amplified by using the *N*-terminal primer 2Sshort (5′-GGGAATTCCA-TATGCGAGGCGAGAGCTGCCGCGAGC-3′), containing an *Nde* I restriction site at the start codon, and the *C*-terminal primer 2Shazelnutrev (5′-CGCGGATCCCTAGAA-CCTTGCGGAGCG-3′), containing a *BamH*I restriction site. The resulting products were ligated in *Nde*I/*BamH*I digested pET11a vector (Merck KGaA, Darmstadt, Germany) and transformed into *E. coli* (DH5α). Selected clones were sequenced as before.

2.6 Expression and purification of recombinant 2S albumin

The cDNA was amplified using the *N*-terminal primer 2SHazelIII (5′-CCCAAGCTTTGCGAGGCGAGAGAGCT-GCCGCG-3′), containing a *Hind*III restriction site at the start-codon, and the *C*-terminal primer 2Shazelnutrev. The resulting products were ligated in *Hind*III/*BamH*I digested pPAL7 vector (Bio-Rad, Hercules, California, CA, USA). Recombinant 2S albumin expression was carried out with the Profinity eXact™ Protein Expression System (Bio-Rad) according to the manufacturers' instructions. To assess protein expression, samples ($t = 0$, 90 and 180 min) were centrifuged (5 min, 13 200 rpm) and pellets were sheared in 0.4 volumes of sample buffer (Invitrogen) using a syringe. To check for expression in inclusion bodies the cells were centrifuged (20 min, 6 000 rpm) and the pellet was resuspended in 0.1 volume lysis buffer (25 mM Tris/2mM EDTA, pH 7.6) containing 0.1 μg/mL lysozyme (Merck KGaA). After expression pellets were collected by centrifugation (10 min, 4600 rpm), resuspended in 0.1 volume of lysis/cell resuspension buffer (10 mM Tris/1 mM EDTA, pH 7.5, with acetic acid), sonicated (3 × 10 sec on a Branson Digital Sonifier, Model 250-D on ice) and centrifuged (10 min, 11 500 rpm, 4°C); the supernatant was retained and the pellet was resuspended in one volume of the same buffer. Recombinant 2S albumin was purified with the Profinity eXact™ Protein Purification System (Bio-Rad) according to the manufacturer's instructions, by using a five CV Bio-Scale Mini Cartridge attached to a syringe.

2.7 SDS-PAGE/Immunoblotting

Proteins were separated by SDS-PAGE (NuPAGE® 4–12%Bis-Tris gel, protein: 10 μg/cm) and Western blotting was performed by transferring the proteins semi-dry to nitrocellulose on a Novablot electrophoretic transfer apparatus, according to the protocol of the manufacturer (Invitrogen). After blocking with PBS/5% skim milk powder/0.02% Tween-20 for a minimum of 10 min, the blots were cut into strips and incubated overnight with 75 μL human serum in 3 mL of PBS/0.02% Tween-20/0.5% skim milk

powder. After washing 5 × (PBS/0.02% Tween-20), blots were incubated (4 h) with horseradish peroxidase (HRP)-labeled goat anti-human IgE (KPL, Gaithersburg, MD, USA) or IRDye800 custom-labeled mouse monoclonal anti-human IgE (Sanquin, Amsterdam, The Netherlands) and washed as before. Detection of HRP-label was performed with Enzymatic Chemiluminescence according to the protocol of the manufacturer (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Blots were exposed to X-ray film (Fuji Super RX, Fujifilm, Tokyo, Japan). The IRDye800-labeled proteins were detected using Odyssey V3.0 scanning software (Westburg, The Netherlands). For blot inhibition studies, ~5 μg inhibitor was added together with the patient serum. Incubation and detection were performed as above.

2.8 Biotinylation and CAP

Purified natural and recombinant 2S albumins were biotinylated using sulfosuccinimidyl-6-(biotinamido)hexanoate according to the manufacturers' instructions (Pierce, Rockford, IL, USA). Biotinylated (recombinant) 2S albumin fractions were coupled to a streptavidin-CAP (2.5 μg/CAP) and subsequently CAP analysis was performed using the UniCAP®100 according to the manufacturers' instructions (Phadia, Uppsala, Sweden). For CAP inhibition studies, the amount of serum was recalculated to ~6–8 kUA/L (kUA/L, kilounits of antibody *per* liter). Inhibitor was then added in the given concentration range to a final volume of 40 μL.

3 Results

3.1 Purification of 2S albumin from hazelnut

Hazelnut proteins were separated using a combination of size exclusion and RP chromatography. After the size exclusion step, two major peaks were obtained (Fig. 1A). The lower molecular weight peak (SE3) was concentrated and fractionated by RP-HPLC (Fig. 1B). Based on prior experience during purification of 2S albumin of peanut [1], a distinct peak eluting at 28 min (indicated by asterisk in Fig. 2) was predicted to be hazelnut 2S albumin. On SDS-PAGE, this peak migrated as a single band with an approximate molecular weight of 10 kDa (Fig. 1B). *N*-terminal sequencing of this peak provided support for its identification as hazelnut 2S albumin, based on two peptide sequences of 11 and 10aa length with 91 and 40% identity to pecan 2S albumin (Acc.No. AY192569.1). One sequence was almost identical to the *N*-terminus of the pecan 2S albumin, whereas the second sequence was located toward the *C*-terminus of the protein (aa 116–125, Fig. 2).

3.2 Cloning of the 2S albumin gene

The cDNA encoding the hazelnut 2S was amplified by PCR (using a degenerate primer based on the *N*-terminal sequence described above) and RACE protocols. The full-length clone is 441 bp and encodes a 147 aa, 17 kDa 2S albumin precursor (GenBank ID FJ358504, Fig. 2). The native protein is predicted to be 12.6 kDa, after post-translational clipping of an *N*-terminal and internal peptide as described for other 2S albumins. The clone has 62 and 66% identity (www.uniprot.org) to 2S albumins from walnut and pecan (Acc.Nos. AY102930.1 and AY192569.1, Fig. 2), respectively. Three isoforms were identified, differing at a single position (aa 79), being either a glutamine (Q) or a histidine (H) or a gap. This was confirmed in different clones, resulting from different PCR reactions. The hazelnut 2S albumin shared the typical cysteine signature reported for other 2S albumins.

3.3 Expression of recombinant hazelnut 2S albumin

The putative mature hazelnut 2S albumin sequence starting from the *N*-terminal sequence described above was

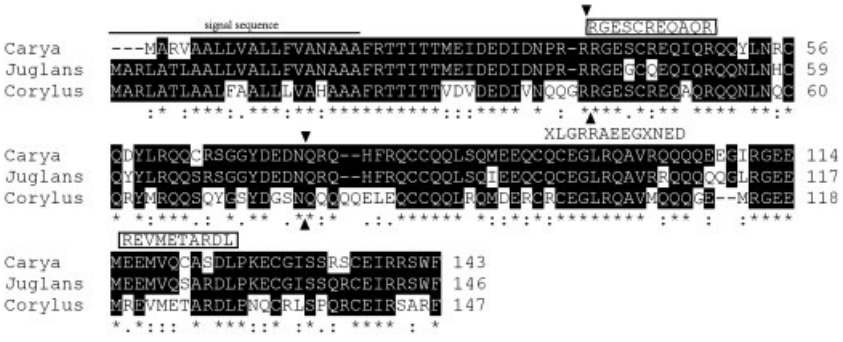

Figure 2. CLUSTAL 2.0.10 multiple sequence alignment of the cDNA-derived aa sequences of 2S albumins from *Carya illinoinensis* (pecan, AY192569), *Juglans nigra* (black walnut, AY102930.1), and *Corylus avellana* (hazelnut, FJ358504). Black background indicates sequence identity. The putative signal sequence of the hazelnut 2S is indicated as are the post-translational Asn cleavage sites beginning at the small and large subunits (▼). Boxed sequences indicate the sequences identified in this study, and the nonboxed sequence is the one identified previously by Pastorello *et al.* [29].

successfully expressed as a fusion protein and was detected both in the soluble fraction and in the inclusion bodies

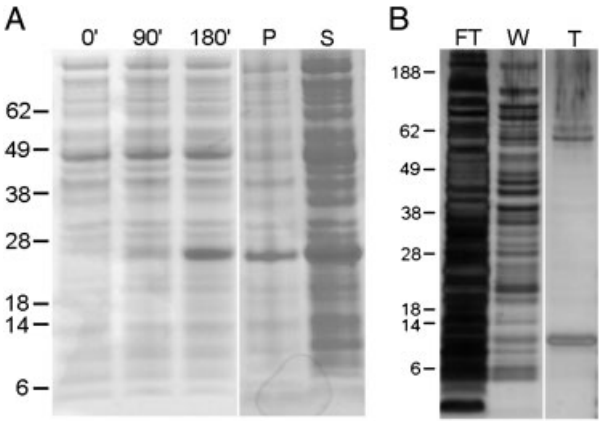

Figure 3. (A) Expression results of the recombinant 2S albumin clones. $t = 0$, 90 and 180 min are indicated, as well as the results after sonication (P: pellet, S: supernatant). (B) Silver staining of the affinity purification of fusion recombinant 2S albumin. FT: flow-through of the column; W: wash of the column; T: purified target protein. Marker sizes are indicated at the left-hand side.

(Fig. 3A). The Profinity eXact™ (Bio-Rad) protein purification system was used to obtain a pure fraction (Fig. 3B), which was used in the Immunoblot and ImmunoCAP tests. This system uses an immobilized protease that recognizes and binds to the tag, resulting in elution of the protein with the native *N*-terminus.

3.4 IgE reactivity: immunoCAP and Immunoblot analyses

A panel of patients ($n = 36$) was recruited based on a reported history of moderate to severe allergic reactions to nuts, sometimes specified as hazelnuts. The panel was tested in a CAP system against the recombinant 2S albumin, and 11 sera showed IgE-reactivity >0.35 kUA/L. The clinical characteristics of these patients are summarized in Table 1. All the patients also had CAP values >0.35 kUA/L to purified natural 2S albumin. Ten out of eleven patients were tested on immunoblot with both natural and recombinant 2S albumin, nine recognized both proteins and one (nr.10) did not show any recognition (Fig. 4). IgE reactivity to the natural 2S albumin, purified (Fig. 5A) or in a hazelnut

Table 1. Clinical characteristics of the sera used

Patient no.	Sex	DOB	Clinical history	SPT	CAP (kUA/L)-RAST (+/–)		
					Foods/pollen	2S[a)]	r2S
1	No data available					4.4	6.5
2	M	1986	Exercise-induced AN, pollen and food allergic (nuts including hazelnut)	ND	ha 5+, se, so, al, br, co 4+, to, pa 3+, p NEG	22.6	30.8
3	F	1996	AN/Angioedema on hazelnut/walnut	ha,wa 4+	(g)p, nm2 4+ nm1 3+, bu, pe 2+	15.2	16.1
4	M	1991	Pollen and food allergic (pea)nuts (including hazelnut), OAS	ha, nm1 3+ pe 2+ se, so NEG	ha, br, Cor a 1+ Cor a 8 NEG	7.2	7.5
5	F	1983	Pollen and food allergic. Peanut, hazelnut, sesame AN/ Angioedema/OAS	ha 3+	ha 6+	5.1	5.9
6	M	1972	Pollen and food allergic. Hazelnut, sesame. OAS/CU	ha 3	ha >100 kU/L	4.0	18.9
7	M	1934	Food allergic, eczema. Fish, hazelnut	ha NEG	ha 4+	5.3	1.0
8	M	1989	Asthmatic, oas/AN (hazelnut), smell of peanut butter	ha 3+ pe 2+	p, n 4+ ap, ha 3+	7.1	9.4
9	No data available					1.7	0.6
10	No data available					5.4	2.4
11	M	1986	AN (nuts, including hazelnut)	pe, nm1/2 4+, so, ha, br 3+, al, co 2+	No data available	7.2	3.3

DOB: date of birth, AN: Anaphylaxis, OAS: oral allergy syndrome, CU: chronic urticaria, SPT: skin prick test (2–4+: two to four times the histamine control). RAST: radio allergo-sorbent test (1–6+: measures for the amount of sIgE, ranging from >0.35->100 kUA/L). (g)p: (grass)pollen, nm1: nut mix 1 (pe, al, ha, br, co), nm2: nut mix 2 (pc, ca, pi, wa), n: nuts, ap: apple, ha: hazelnut, wa: walnut, pe: peanut, se: sesame, al: almond, br: brazil nut, ca: cashew, pc: pecan, pi: pistachio, co: coconut, bu: buckwheat, so: soybean, to: tomato, pa: paprika, ND: not done. NEG: Negative.
a) Purified hazelnut 2S as shown in Fig. 1B.

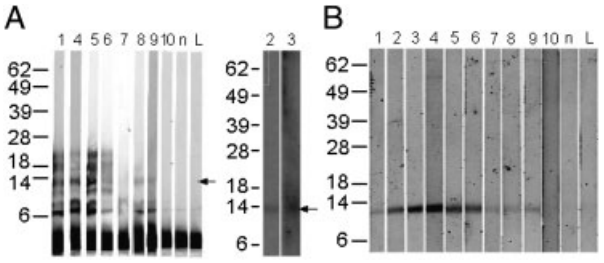

Figure 4. (A) Immunoblot results with the natural 2S albumin; n: negative serum control; L: Label control. Serum numbers are indicated above and the marker sizes on the left-hand side. Sera 2, 3 were detected with HRP-labeled anti-IgE and detected with ECL, all the other sera were detected with IRDye800-labeled anti-IgE. (B) Immunoblot results with the recombinant 2S albumin. Arrows indicate the 2S albumin band.

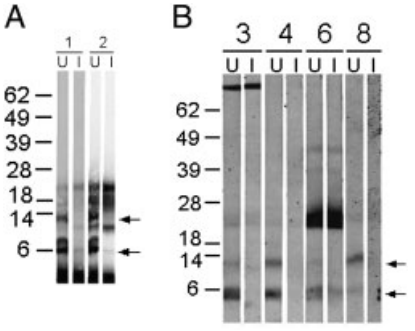

Figure 5. (A) Immunoblot of purified natural hazelnut 2S albumin inhibited with the recombinant. (B) Immunoblot of a total hazelnut protein extract inhibited with the recombinant hazelnut 2S albumin. Serum numbers are indicated above and marker sizes on the left-hand side; U: serum uninhibited; I: serum inhibited.

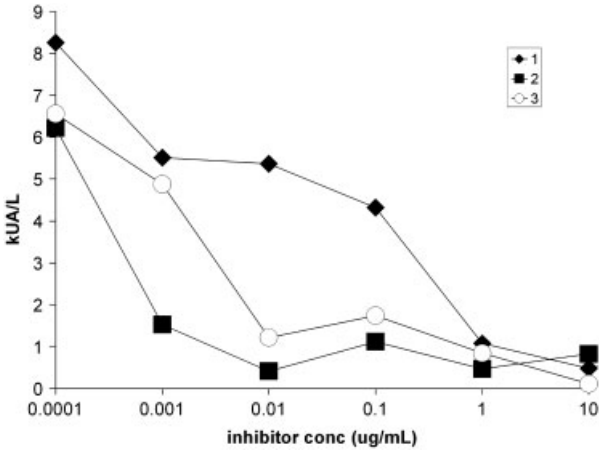

Figure 6. The ability of recombinant 2S albumin to inhibit IgE-reactivity to the natural 2S albumin coupled to ImmunoCAP was compared for three patients (symbols legend on the right-hand side). The curves show the values obtained at each concentration.

extract (present as 2 bands, the large and small subunit, Fig. 5B) could be inhibited by r2S in 6/9 sera, although in 1 serum (nr. 6) this inhibition was only partial. The other three sera showed very low to no signal, due to serum limitation and possibly due to a low concentration of natural 2S albumin in the hazelnut extract. IgE reactivity to natural 2S coupled to ImmunoCAP was fully inhibited (87, 98 and 99%, respectively) by r2S in the three completely inhibited sera (1–3) (Fig. 6).

According to the guidelines of the Allergen Nomenclature Committee the hazelnut 2S albumin was designated Cor a 14.0101.

4 Discussion

Although 2S albumins have been identified as major allergens in many allergenic nuts and seeds, there is only a single report of the possible identification of hazelnut 2S albumin as an allergen [29]. In this study, the isolation of natural 2S albumin from hazelnuts, and the subsequent cloning, sequencing, and expression of the corresponding gene in *E. coli* are reported. Both the purified natural and the expressed recombinant 2S albumin were shown to bind IgE of hazelnut-allergic patients, and immunoblot inhibition experiments indicated that the recombinant version had the potential to be used as a mimic of the natural allergen. The 147 aa long sequence of 2S albumin determined in this study shows only limited identity with that reported by Pastorello *et al.* [29]. The 14 aa *N*-terminal sequence from that study can be aligned to position 39 of the precursor molecule from the present study, resulting in only 4/14 identical positions. The *N*-terminus of our natural 2S starts at position 4 of the previously reported sequence [29]. Taking into account the limited sequence information available and the low degree of identity to our sequence, it is unclear whether the sequence reported by Pastorello *et al.* [29] does indeed represent a genuine hazelnut 2S albumin. In contrast, in this study the translated gene product displayed the conserved pattern of eight cysteine residues forming four disulfide bridges that is characteristic of 2S albumins and nsLTPs. The complete protein sequence also showed variable degrees of homology with several 2S albumins identified in other plant foods [11, 32, 33].

The full sequence obtained in this study contains a putative 22 aa signal sequence as predicted by the online SignalP 3.0 tool. The mature natural protein sequence starts 20aa further downstream from the signal peptide, suggesting that this linker sequence is post-translationally cleaved *C*-terminally of the Asn cleavage site (Fig. 2). Indeed, 2S albumins have been described as small globular proteins that undergo extensive post-translational proteolytic processing in the vacuoles of the plant cells, whereby the full-length precursor protein is usually cleaved into a large and a small subunit that stay associated through disulphide

bonds. During this post-translational processing, a signal peptide and short linker, and flanking sequences are excised, resulting in a smaller mature product [34]. *N*-terminal sequencing of our preparation also revealed a second peptide corresponding to an internal sequence, starting at position 120 of the precursor molecule or at position 79 of the mature protein.

The major challenge in the expression of properly folded 2S albumins is the formation of the four disulfide bridges. Previously, recombinant 2S albumins from various species have been successfully expressed in both the yeast *Pichia pastoris* [35–38] and *E. coli* [15–17, 39–43]. Three forms of hazelnut 2S albumin were cloned and expressed (not shown): the full-length ORF including signal sequence (ss) and linker sequence (''long''), the ORF without the putative ss (based on the mature walnut sequence, ''intermediate''), and the putative mature hazelnut sequence starting from the *N*-terminal sequence found in the purified natural protein (''short''). The long variant was detected exclusively in inclusion bodies, the intermediate and short variants were also found in the soluble fraction. All the three variants showed similar IgE/IgG binding.

The short version was chosen for further studies because of its mimic of mature natural 2S, as a fusion-tagged protein in *E. coli*. After affinity purification, the tag was cleaved off. Our experiments with a selected panel of nut-allergic patients indicate that the IgE-reactivity of the recombinant allergen is similar to the natural hazelnut 2S albumin. In addition, the recombinant 2S albumin is able to completely inhibit (87–99%) reactivity to the natural 2S albumin in the ImmunoCAP experiments. Currently, large-scale expression and purification are being performed to allow in-depth physicochemical and immunological characterization. These studies are integrated into the large European multicenter study on food allergies, EuroPrevall [44]. The EuroPrevall study has a serum collection [45] of over 600 well-characterized hazelnut-allergic patients from 12 European countries, of which almost 100 have been challenged by double blind, placebo-controlled food challenge. The availability of sera from these patients in combination with a panel of seven purified hazelnut allergens, two pollen allergens, and four extracts will allow us to set up an *in vitro* component-resolved diagnosis study to assess the geographical differences in symptom severity and sensitization profile to individually purified hazelnut allergens. Additionally, putative differences in allergen recognition by young *versus* older patients (primary sensitization by hazelnut/birch pollen) may be assessed. Additionally, this will allow us to establish the importance of 2S albumin for hazelnut-allergic patients, and to study the cross-reactivity to other nuts, legumes, and seeds.

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The authors have declared no conflict of interest.

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