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Cloning of oleosin, a putative new hazelnut allergen, using a hazelnut cDNA library

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The clinical presentation of non-pollen related allergy to hazelnut can be severe and systemic. So far, only a limited number of non-pollen related hazelnut allergens have been identified and characterized. The aim of this study was to identify and clone new hazelnut allergens. A lambda ZAP cDNA library of hazelnut was constructed. The library was screened with serum of six hazelnut allergic patients displaying different IgE-binding patterns on hazelnut immunoblot. Rapid amplification of cDNA ends (RACE) protocols were applied to obtain full-length clones. Expression experiments were carried out in *Eschericchia coli.* Expression was monitored by SDS-PAGE, protein staining and immunoblotting. A hazelnut cDNA library was constructed. IgE screening resulted in the cloning of two isoforms of a novel putative hazelnut allergen. The clones were identified as oleosins, with theoretical molecular masses of 16.7 and 14.7 kDa and p*I* of 10.5 and 10.0, respectively. The isoforms demonstrated only 37% amino acid sequence identity but contained the typical hydrophobic stretch in the middle of the protein (53% identity) with the characteristic oleosin proline knot region (11/12 amino acids identical). Expression in *E. coli* of the longer isoform resulted in a clear band on SDS-PAGE. The expressed protein was recognized on an immunodot blot by IgE from serum that was used for screening the cDNA library. Hazelnut contains multiple isoforms of oleosin. IgE binding of a hazelnut-allergic patient to a recombinant version suggest that hazelnut oleosin is an allergen, as has been described for peanut and sesame.

Keywords: Oleosin / Hazelnut / Lambda ZAP cDNA library / Allergen / Diagnostic tests

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1 Introduction

Food allergic reactions after hazelnut ingestion are frequently observed [1, 2]. In areas where birch trees are endemic most hazelnut allergy in adolescents and adults is a result of primary sensitization to the major birch pollen allergen Bet v 1. IgE against Bet v 1 is cross reactive with the homologous allergen in hazelnut Cor a 1 [3]. Because of its pepsin susceptibility [4] and heat sensitive properties [5], symptoms induced by Cor a 1 are usually mild and restricted to the oral allergy syndrome [6]. Non-pollen related hazelnut allergy is observed in young children that have not (yet) developed inhalant allergies but also in areas without significant exposure to birch pollen like Spain. Non-pollen related hazelnut allergens more frequently induce severe systemic reactions. It is widely accepted that protease resistance is a requirement for allergens to be able to sensitize by the oral route and to cause systemic reactions [7]. A well-characterized example of such a food allergen is the nonspecific lipid transfer protein (LTP). This allergen has also been identified as an allergen in hazelnut and was designated Cor a 8 [8]. Sensitization to LTP has mainly been reported for patients from Mediterranean countries. It is believed that sensitization to LTP occurs by the oral route,

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Abbreviations: IPTG, isopropyl-β-D-thiogalactopyranoside; **LTP**, lipid transfer protein; **RACE**, rapid amplification of cDNA ends

and peach has been implicated as the primary sensitizer. Other non-pollen related allergens in hazelnut have recently been identified such as homologues of the major peanut allergens Ara h 1 (7S vicilin) and Ara h 3 (11S globulin), Cor a 11 and Cor a 9, respectively [9, 10]. Although a homologue of Ara h 2 and the major Brazil nut allergen Ber e 1, both 2S albumins, has been suggested to be an allergen in hazelnut as well, this putative allergen has not yet been isolated or cloned.

Despite the progress made in the identification of "true" hazelnut allergens, some patients with non-pollen related immediate allergic reactions to hazelnut are not detected by commonly used *in vitro* diagnostic tests (unpublished observations). Apparently some of the important hazelnut allergens are not well represented in hazelnut extracts used for diagnosis [10 – 12] and Luttkopf, D., Schocker, F., Scheurer, S., Haustein, D., Vieths, S., 1-12-2000. EMBL/GenBank/DDBJ/databases (www.ebi.al.uk/embl).

Recently it was demonstrated that the composition of commercially available skin prick test reagents is highly variable and that in some indeed the levels of important allergens like Cor a 8 are undetectable [13].

One of the intriguing new allergens that have recently been identified in peanut and sesame seeds is oleosin [14]. Oleosin has been proposed to stabilize lipid bodies during desiccation of seeds by preventing coalescence of the oil bodies. Oleosin probably interacts with both lipid and phospholipid moieties of oil bodies. Oleosins are characterized by a hydrophilic N-terminal amino acid stretch and a highly conserved hydrophobic central domain (~70 uninterrupted uncharged residues). The C-terminal flanking sequence consists of an amphipathic alpha helix, and is conserved among many oleosins [15 – 17]. The central domain is one of the longest hydrophobic domains that have yet been found in any naturally occurring protein [18]. The allergenic properties of oleosins have thus far not been characterized in full detail, but their localization in oil bodies might be a reason that they are underrepresented or denatured in most diagnostic extracts of nuts and seeds that are usually extensively defatted. The absence or presence of denatured (during defatting) oleosins might be involved in false-negative *in vitro* diagnosis of some hazelnut allergic patients. The aim of this study was to construct a cDNA library of hazelnut and clone non-pollen related allergens that might in the future be used to improve diagnostic tests for hazelnut allergy. Prime candidates were hazelnut 2S albumin [19] and oleosin [14].

2 Materials and methods

2.1 Sera

Serum was obtained from six patients with a convincing history of allergy to hazelnut (HZN1-6). Serum HZN1 was selected based on a negative birch pollen RAST and reactivity with unknown low molecular weight allergens (around 10 kDa) as was judged by immunoblot. Serum HZN2 was known to have cross-reactive IgE antibodies to profilin. However, the severity of reactions reported upon ingestion of many vegetable foods including hazelnut suggested that another hazelnut allergen might still be involved next to profilin (Cor a 2) which is generally believed to cause only mild symptoms. Serum HZN3 was selected because this contained specific IgE against LTP. Serum HZN 4 was selected because IgE from this serum against Ber e 1 (the major Brazil nut allergen, a 2S albumin, kindly provided by Dr. Marcos Alcocer) was cross-reactive with putative hazelnut 2S albumin in hazelnut extract as was demonstrated by RAST-inhibition. For sera HZN5 and HZN6 no data concerning recognition of specific allergens were available. As a negative control, a serum from a non-atopic donor (<0.3 IU/mL IgE) was used.

2.2 Hazelnut extract

Hazelnut polyvinylpolypyrrolidone (PVPP) extract was prepared as described previously, essentially following the protocol described by Bjorksten *et al*. [20]. In short, grounded hazelnut was extracted in a buffer containing PVPP and di-ethyldithiocarbamate (DIECA). These reagents prevent loss of allergenicity by inhibiting oxidative processes mediated by polyphenyloxidases upon disruption of the plant tissue. Polyphenols adsorb proteins and thereby prevent oxidative processes by steric hindrance. In particular, Bet v 1 related food allergens (*i.e.*, Cor a 1 in case of hazelnut [21]) have been shown to be lost due to these processes.

2.3 cDNA library construction, screening and sequencing

Total RNA was purified from hazelnuts essentially as described by Clendennen *et al*. [22]. cDNA was synthesized from poly(A)$^+$ RNA by the protocol described by the manufacturer (cDNA Synthesis Kit, ZAP-cDNA® Synthesis Kit and ZAP-cDNA® Gigapack® III Gold Cloning Kit). The cDNA library was constructed in lambda ZAP with a titer of 6×10^9 PFU/mL. Phages (3×10^4 PFU) were plated out with an *E. coli* XL1-Blue MRF' strain on a 150-mm Petri dish containing broth medium supplemented with 50 µg/mL of ampicillin. After 6 h of incubation at 37°C, the plate was overlaid with a Protran BA 85 (Schleicher & Schuell, Dassel, Germany) NC filter (previously saturated with 10 mM IPTG (isopropyl-β-D-thiogalactopyranoside)) to induce antigen expression. Human serum samples HZN1-6 (7 µL/cm^2 NC) were used to screen the cDNA library, non-atopic serum and buffer alone were used as negative con-

trols. Bound IgE was detected using ^{125}I-radiolabeled sSheep anti-human IgE in conjunction with autoradiography. Plaques with strong signals were collected. The pBluescript was excised from the Uni-ZAP® XR vector *in vivo* according to the manufacturer's description. Finally, clones were sequenced and compared in GenBank by basic local alignment search tool (BLAST).

2.4 5′ RACE protocol

One of the two clones for oleosin was truncated at the 5′ end. The full-length cDNA of this 16.7-kDa isoform of oleosin was obtained by 5′ rapid amplification of cDNA ends (Invitrogen, Carlsbad, CA, USA). First strand cDNA was synthesized from poly(A) RNA of *C. avellana* using a gene specific primer (SP1, 5′ CCCAATGACACTGCCAGCGAGCG 3′) according to the protocol of the manufacturer. The cDNA was tailed with a poly(A) sequence and subsequently amplified using a gene specific primer (SP2, 5′ GGCCGCGTTGTGGGTGGAC 3′) and the abridged anchor primer (AAP) included in the kit. Amplified cDNA was purified using the GFX purification kit (Amersham-Pharmacia-Biotech) and subsequently cloned in the pGEMT vector (Promega Benelux, Leiden, The Netherlands). Sequencing was performed by BaseClear Labservices (Leiden, The Netherlands) using the M13 forward and reverse primers. To finally obtain the full-length cDNA of the 16.7-kDa isoform a PCR was performed using a primer designed on the basis of the sequence information obtained from the 5′ RACE protocol (5′GGGTTTCATATGGCTGACCGTCCCCAACAGC 3′) and the reverse 5′ CGCGGATCCTCATGTTCTCCTTCCCTCC 3′. The amplified cDNA was purified as described above.

2.5 Expression of oleosin

cDNA coding for the 16.7-kDa isoform of oleosin was ligated into the pET19b expression vector (Novagen, Merck, kGaA, Darmstadt, Germany) using the *NdeI* and *BamHI* restriction sites. The sequence of the clones was confirmed using the T7 forward and reverse primers (BaseClear Labservices). After transformation of the pET-19b vector to *E. coli.* BL21 (DE3), the synthesis of hazelnut oleosin was induced by adding IPTG (final concentration 1 mM) to the culture medium at OD 600 = 0.6. Samples (500 μL) collected at different time intervals (t = 0, 35, 60, 120 and 180 s) were centrifuged for 3 min at 12 000 rpm. After removal of the supernatant, 200 μL sample buffer (50 μL NuPAGE LDS™ + 20 μL reducing agent NuPage™,) + 130 μL H_2O was added to the pellet. After shearing, 15-μL sample of each time point was analyzed for recombinant protein production by SDS/PAGE in conjunction with CBB staining. A BL21 (DE3) sample containing the vector without addition of IPTG was used as negative control.

2.6 Immunodotblot

Immunodotblot was performed using a Hybri · Dot Manifold equipment (Gibco BRL, Gaithersburg, MD, USA). Cell culture sample (500 μL) from t = 180 s was centrifuged for 3 s at 12 000 rpm.). The obtained cell pellet was resuspended in lysis buffer (200 μL PBS +20 μg lysozyme (Calbiochem, La Jolla, CA, USA)) and incubated for 20 s at 30°C. Undiluted culture supernatant (10 μL/well) was analyzed on the Hybri · Dot Manifold and incubated for 4 h with the pore size 0.45 μm NC membrane (Schleicher & Schuell, Dassel, Germany). Hazelnut PVPP extract (500 μg/mL, 10 μL/well) was used as positive control. After washing away unbound material with PBS/0.1% Tween20, the NC was blocked with PBS/0.3%BSA/0.1%Tween20 for 2 h and subsequently incubated with 0.5 mL serum HZN2 in 10 mL PBS/0.3%BSA/0.1%Tween20. After overnight incubation, unbound material was washed away and bound IgE was detected using ^{125}I-radiolabeled sheep anti-human IgE in combination with autoradiography.

2.7 SDS-PAGE/CBB staining

Samples from both the culture supernatant and cell lysates were analyzed for recombinant protein production by SDS-PAGE (under reducing conditions) on a Novex XCell II system using a 4 – 12% gel (Novex, San Diego, CA, USA), followed by CBB staining according the manufacturers instructions.

3 Results

3.1 Screening of the hazelnut cDNA library

Serum (HZN1-6) IgE-based screening of the hazelnut cDNA library produced several tens of clones for putative allergens, including clones coding for hazelnut vicilins [12]. Despite the use of a serum sample with specific IgE antibodies against a hazelnut allergen cross-reactive with Brazil nut 2S albumin (HZN1), no clones for hazelnut 2S albumin were detected. Similarly to serum HZN3 with IgE antibodies against Cor a 8 (LTP), no clones coding for this allergen were detected. After tertiary screening, specific binding of IgE was detected using serum HZN2 whereas radiolabel control (sheep anti-human IgE) and non-atopic were negative (Fig. 1).

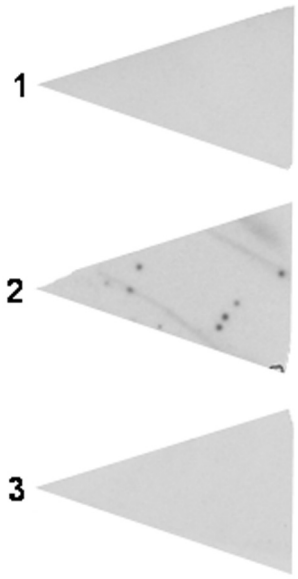

Figure 1. Tertiary screening of the second truncated clone oleosin (Q84T21). Parts cut from one NC filter were incubated with (1) ^{125}I-radiolabeled sheep anti-human IgE (label control), (2) serum HZN2 and (3) non-atopic serum. Bound IgE was detected with ^{125}I-radiolabeled sheep anti-human IgE in conjunction with autoradiography.

3.2 Cloning of hazelnut oleosin

Two clones coded for two related proteins that demonstrated homology with oleosins. The clone detected with serum HZN1 coded for a 14.7-kDa oleosin (primary accession number Q84T91). The second clone detected with serum HZN2 was 5′ truncated. The full-length clone obtained using the 5′ RACE protocol coded for a 16.7-kDa oleosin (Q84T21). Both clones demonstrated only 36% homology allowing a gap of 16 amino acids in the smaller isoform, starting from position 15 (Fig. 2).

In addition, the 14-kDa version was three amino acids shorter at the C terminus. Both isoforms of hazelnut oleosin shared the typical hydrophobic central part of the molecule

Table 1. Primary accession numbers, amino acid number and amino acid sequence identities form hazelnut oleosin (Q84T21 and Q84T91) and five other food oleosin are listed

Food oleosin	Accession number	Amino acid number	Q84T21 (seq.id.%)	Q84T91 (seq.id.%)
Hazelnut	Q84T21	159		32
Hazelnut	Q84T91	140	32	
Almond	Q43804	148	31	73
Carrot	Q43123	180	55	32
Maize	P13436	183	37	55
Peanut	Q6J1J8	176	48	33
Sesame	Q9XHP2	145	50	30

reported for other oleosins, including the characteristic proline knot region with 11/12 amino acids being identical. Overall homology with oleosins from other plants ranged from 31 up to 73% (Table 1, Fig. 3).

3.3 Expression of 16.7-kDa hazelnut oleosin

The longer isoform of hazelnut oleosin demonstrated the highest degree of homology with oleosins reported to be allergens, *i.e.*, peanut (48% homology) and sesame seed (50% homology). This isoform was therefore selected for expression experiments in *E. coli*. Expression was monitored by SDS-PAGE in conjunction with CBB staining. Time-dependent induction of a band with an apparent molecular mass between 16 and 17 kDa was observed (Fig. 4).

This molecular mass corresponds to the theoretical molecular mass of the larger oleosin isoform. This band was not observed in the negative control without IPTG. Immunoblotting with serum HZN2 did not result in a positive signal, possibly because IgE binding is sensitive to the denaturing and reducing conditions of SDS-PAGE. To limit denaturation, the expressed protein was subsequently subjected to dot blot analysis with serum HZN2. In this case, weak binding was demonstrated, confirming the IgE-binding properties of hazelnut oleosin (Fig. 5). No IgE binding to oleosin was observed when serum of the non-atopic control was tested.

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Q84T21      MADRPQQLQVHPQRGHGHYEGGIKNQRGGGPSAVKVMAVLAALPVGGTLLALAGLTLAGS 60
Q84T91      MAEHPRQLQDPAHQ----------------PRSHQVVKAATAATAGGSLLVPSGLILAGT 44
            **::*:***  .::                * : :*: . :* ..**:**. :** ***:

Q84T21      VIGLLVTSPLFIIFSPVLVPAAIVVGLAVASFLSSGALGLTGLSSLSWVLNYLRCASQSL 120
Q84T91      VIALTLATPLFVIFSPVLVPAVITVSLIIMGFLASGGFGVAAVTVLSWIYRYVTGRHPPG 104
            **.* :::***:*********.*.*.* : .**:**.:*::.:: ***: .*:     .

Q84T21     PREMDQAKRRMQDMAAFVGQKTREVGQEIQSRAQEGRRT 159
Q84T91     ADQLDHARMKLASKAREMKDRAEQFGQQHVTGSQGS--- 140
           . ::*:*: :: . *  : :::.:.**:  : :* .
```

Figure 2. Amino acid sequence alignment of two isoforms of hazelnut oleosin, Q84T21 and Q84T91. Consensus symbols below the alignment: "." semi conserved amino acid substitution, ":" conserved amino acid substitution, "*" identical amino acid. The characteristic conserved proline knot region for oleosins is depicted in bold.


```
Q84T21hazelnut      AVLAALPVGGTLLALAGLTLAGSVIGLLVTSPLFIIFSPVLVPAAIVVGLAVASFLSSGA 96
Q84T91hazelnut      KAATAATAGGSLLVPSGLILAGTVIALTLATPLFVIFSPVLVPAVITVSLIIMGFLASGG 81
Q43123carrot        AVVTLLPVGGTLLFLAGITLVGTLIGLAVATPLFLLFSPVLVPAALTIGLAVTGFLGSGA 100
Q6J1J8peanut        AVLVGVPTGGTLLLLSGLSLLGTIIGLAIATPVFIFFSPVIVPAVVTIGLAVTGILTAGA 111
Q43804almond        KAATAVTAGGSLLVLSGLVLAGTVIALTIATPLLVIFSPVLVPALITVALITMGFLTSGG 87
Q9XHP2sesameseed    KAATAVTAGGSLLVLSGLTLAGTVIALTIATPLLVIFSPVLVPAVITIFLLGAGFLASGG 86
P13436maize         KAATAATFGGSMLVLSGLILAGTVIALTVATPVLVIFSPVLVPAAIALALMAAGFVTSGG 101
                       .  . .. :*  :*: *  :::.* :::*::::***::*** : :     .:: :*.
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Figure 3. Sequence alignment of the hydrophobic part of seven different oleosins that showed the highest degree of amino acid conservation. The amino acid number of the last residue of each species is listed on the right. The characteristic conserved proline knot region for oleosins is depicted in bold.

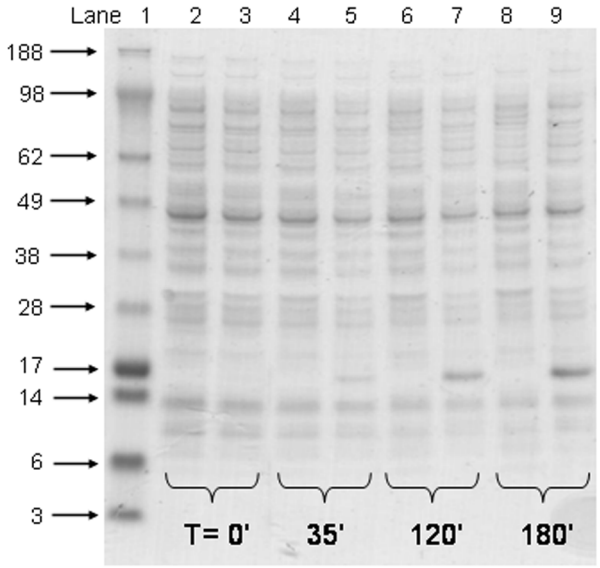

Figure 4. The expression of Q84T21 was monitored by SDS-PAGE followed by Coomassie staining. Lane 1: molecular weight standards. Lanes 2–9: even lanes: samples taken from un-induced cell culture at different time intervals. Odd lanes: samples from IPTG induced cell culture.

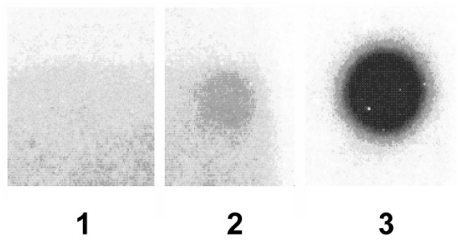

Figure 5. The expression of rQ84T21 in *E. coli.* BL21 (DE3) was monitored on immunodotblot. Cell lysate (not induced and induced) and hazelnut extract as a positive control were analyzed for IgE binding using human serum pf.200. Lane 1: not induced *E. coli.* BL21 (DE3) undiluted. Lane 2: IPTG-induced *E. coli*: BL21 (DE3) undiluted. Lane 3: Hazelnut extract 500 μg/mL.

4 Discussion

This study describes the screening of a hazelnut lambda ZAP cDNA library. The identification of two different isoforms of hazelnut oleosin, as putative allergens, was specifically of interest. Oleosins are alkaline proteins (p*I* ± 10) with molecular weights of 15 to 24 kDa [23]. They are proteins known to stabilize oil bodies in the cytoplasm [16]. Oleosins are embedded in the oil body with the central hydrophobic domain, whereas the hydrophilic alpha-helical N terminus and amphipathic alpha-helical C terminus are in contact with the aqueous cytoplasm [24]. The central hydrophobic domain is characterized by a so-called proline knot motif [25], which is conserved in many plant species. To prevent coalescence of oil droplets in plant cells, large amounts of oleosin are needed, thereby making it an abundant protein. Oil bodies contain triacylglycerol as energy source for germination and growth of seedlings. Association with oil bodies is the most likely explanation why the allergen has so far not been identified in hazelnut extracts as an allergen source. Preparation of extracts from foods like hazelnut containing high concentrations of fat are usually defatted, most likely resulting in denaturation or depletion of/for oleosin. *In vitro* diagnostics performed with such extracts will have poor sensitivity for the detection of specific IgE against oleosins.

The association of oleosins with oil bodies may also play a role in the protection of the allergen against rapid proteolysis in the upper gastro intestinal tract. Stability of food allergens is generally accepted as a factor distinguishing true food allergens (allergens able to sensitize and to induce systemic reactions) from labile cross reactive food allergens like the Bet v 1 homologue from hazelnut Cor a 1 [6].

Both isoforms of hazelnut oleosin demonstrated variable degrees of homology with several oleosins identified in other plant foods. In some of these plant foods, isoforms differing significantly in primary structure were identified. It is not yet clear which isoforms play a more important role as allergens. The first oleosin identified as food allergen was peanut oleosin by Pons *et al*. [14]. This group also produced IgE-binding recombinant peanut oleosin using the S*f*9-baculovirus expression system. In this study, expression experiments of the longer isoform of hazelnut oleosin followed by dot blot gave support for the identification of this protein as an allergen in hazelnut. The weak IgE binding on dot blot might be due to incorrect folding or aggregation caused by the relatively large hydrophobic stretch in

the middle of the protein. The importance of oleosins for non-pollen related hazelnut allergy, however, remains to be determined. Hazelnut LTP and 2S albumin were not picked up using the cDNA library. For LTP this is not surprising because serum samples used for screening were from Central/Northern Europe, where LTP sensitization has been reported to be rare [8, 19]. Although serum HZN4 with clear reactivity to 2S albumin from Brazil nut (Ber e 1) and cross reactivity to putative 2S albumin was used, we did not pick up this allergen. Incorrect folding by *E. coli* due to the presence of four disulfide bridges (common to LTP and 2S albumins) might explain this observation.

Finally, whether current diagnostics are indeed lacking sufficient quantities of the allergen due to defatting procedures is currently under investigation. Perhaps some false-negative *in vitro* results of patients with convincing immediate reactions to hazelnut can be explained by too low concentrations of oleosin. Currently, the expression of both isoforms of hazelnut is being optimized. Availability of well-folded recombinant oleosin will allow determining what the role of this allergen is in non-pollen related hazelnut allergy.

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