Research Article

The purification and characterisation of allergenic hazelnut seed proteins

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A lipid transfer protein (LTP, Cor a 8) together with the 11S (Cor a 9) and 7S seed storage globulins (Cor a 11) are major food allergens present in hazelnut. Methods are described for their purification and characterisation using in-gel tryptic digestion mass spectrometry to confirm their identities and circular dichroism and Fourier-transform infrared spectroscopies to demonstrate that they are authentically folded. Preliminary immunochemical studies have also confirmed that the purified preparations retain their immunological properties in terms of immunoglobulin E binding, determined by immunoblotting using serum from hazelnut allergic patients. These preparations form a basis for development of improved methods of diagnosis of food allergy based on the concept of component-resolved diagnosis.

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1 Introduction

Hazel (*Corylus avellana*) is a shrub, which is native to Europe and Asia. It is a member of the family *Betulaceae* that also contains a number of widespread trees: birches (*Betula* spp.), alders (*Alnus* spp.) and hornbeam (*Carpinus* spp.). The nuts may be eaten fresh (as cobnuts in the UK) or after drying to improve storage. They may also be roasted and crushed or ground into a paste for producing praline and chocolate products. Their characteristic flavour and texture means that they are widely used to produce baked products and confectionery.

Allergy to hazelnut is one of the more widespread tree nut allergies, with allergic reactions ranging from mild symptoms confined to the oral cavity, known as the oral allergy syndrome (OAS), and rhinoconjunctivitis, to more severe reactions including angio-oedema and anaphylactic shock [1–4]. At least five protein types appear to be involved in allergic reactions to hazelnuts in food products. One type of protein is associated with sensitization to the birch pollen allergen Bet v 1. In view of the close botanical relationship between birch and hazelnut it is not surprising that hazel pollen carries a major allergen (Cor a 1) related to Bet v 1 of birch. A related protein also occurs in hazelnuts, which can also lead to reactions on dietary exposure to hazelnut [5–7]. The potency of this allergen is reduced by roasting and generally causes the relatively mild symptoms associated with OAS. A second hazelnut allergen, Cor a 2 is a member of the profilin family of allergens members of which are found in both pollen and seed [5, 8].

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Abbreviations: **AR**, analar; **BCIP**, 5-bromo-4-chloro-3-indolyl phosphate; **CD**, circular dichroism; **FT-IR**, Fourier-transformed infrared spectroscopy; **IgE**, immunoglobulin E; **LTP**, lipid transfer protein; **NBT**, nitroblue tetrazolium; **nsLTP**, nonspecific lipid transfer protein; **OAS**, oral allergy syndrome; **RAST**, radioallergosorbent assay

The other three allergenic components all belong to seed protein families, which include allergenic proteins in other species. One of these is the nonspecific lipid transfer protein (LTP) Cor a 8 [4, 6], which belongs to the prolamin superfamily of proteins that are major food allergens in many plant food species [9 – 11]. This allergen is associated with a more severe form of allergy, which is most frequently found in the Mediterranean area although recent reports indicate that this type of allergy may now be emerging in the north of Europe [12]. It is associated with severe symptoms and may be associated with allergy to the peach LTP known as Pru p 3 [13]. Other types of allergy may involve sensitization to the seed storage globulins found in hazelnut including the 11S seed storage globulin Cor a 9 and the 7S globulin Cor a 11 [2, 14]. Related seed storage globulins are also major allergens in a number of other species [15].

The present paper describes the preparation and characterization of the LTP (Cor a 8) and globulin (Cor a 9, Cor a 11) allergens from hazelnuts while an accompanying paper reports the production of recombinant Cor a 1 and Cor a 2 [16]. Together, these form part of a project for development of a comprehensive library of allergens facilitating diagnosis in food allergy exploring the concept of component-resolved diagnosis [17].

2 Materials and methods

2.1 Materials and human serum samples

Shelled raw hazelnuts (produce of Turkey) were purchased from a local supplier. Nuts were ground in liquid nitrogen using a Waring Blendor (Fisher Scientific, Leicestershire, UK) and defatted by stirring in five volumes w/v of hexane for 1 h at room temperature. After drying, the flour was reground in an electric coffee grinder and the hexane extraction repeated.

A rabbit polyclonal antibody anti-rCor a 8 was kindly provided by Dr. Jonas Lindholm, (Phadia, Uppsala, Sweden). Goat anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate was obtained from Sigma-Aldrich, Dorset, UK. Protease inhibitor tablets were obtained from Roche Diagnostics, Sussex, UK. Gels, reagents and protocols for analytical SDS-PAGE were from Invitrogen, Renfrewshire, UK and for immunoblotting were from Bio-Rad, Hertfordshire, UK. All other chemicals were of AR grade unless specified and were obtained from Sigma-Aldrich, unless specified.

Human sera were from patients showing clear clinical symptoms associated with immunoglobulin E (IgE)-mediated allergies following consumption of hazelnuts, which ranged from OAS to systemic (anaphylactic) reactions. They were also sensitized to a total hazelnut protein extract, as determined by radioallergosorbent assay (RAST) (data not shown). Serum was taken with approval of the relevant local ethical committee. Cor a 9- and 11-specific IgE was monitored by immunoblotting using sera from 14 Cor a 9- and 11-sensitised patients from the Netherlands whilst Cor a 8-specific IgE was assessed using sera from Cor a 8-sensitised patients from Spain.

2.2 Purification of 7S globulin (Cor a 11)

Defatted hazelnut flour (10 g) was extracted twice by stirring for 2 h at 20°C with 50 mM Tris-HCl pH 7.0 containing 500 mM NaCl, 6.2 mM sodium azide and protease inhibitor (2 tablets/100 mL, Roche Complete Protease inhibitor tablet) (1:10 w/v). This higher salt concentration was employed to avoid the necessity of adding additional solid sodium chloride prior to application of the extract to the Con A Sepharose column. The extracts were centrifuged at 2000 × *g* for 10 min at 20°C and then pooled before being centrifuged at 34 000 × *g* for 40 min at 10°C. Aliquots (10 mL) of the resulting supernatant were then applied to a column (1.6 × 10 cm) of Con A Sepharose-4B (GE Healthcare, Buckinghamshire, UK) attached to a BioCad Sprint HPLC system (Applied Biosystems, Cheshire, UK) equilibrated with 20 mM Tris-HCl pH 7.5 containing 500 mM NaCl at a flow rate of 2 mL/min. After washing with the same buffer, bound protein was eluted by the addition of 0.4 M methyl mannopyranoside. Eluent was monitored for protein by following the absorbance at 220 and 280 nm. Protein-containing peak fractions were pooled and concentrated by ultrafiltration using an Amicon stirred cell equipped with a regenerated cellulose membrane with a 10-kDa exclusion limit (Millipore, Hertfordshire, UK). Aliquots (2 mL) were applied to a Superdex Preparative Grade S200 gel permeation column (GE Healthcare) attached to a BioCad Sprint HPLC system (Applied Biosystems) equilibrated and eluted with 5 mM sodium phosphate buffer pH 7.0, containing 200 mM NaCl. The eluent was monitored for protein by following the absorbance at 220 and 280 nm. Protein containing peak fractions were pooled, repeatedly dialysed in regenerated cellulose dialysis tubing with a 12-kDa exclusion limit at 4°C against 0.5 M ammonium bicarbonate before being freeze dried and stored at -20°C until required.

2.3 Purification of 11S globulin (Cor a 9)

Defatted hazelnut flour (5g) was extracted as described for Cor a 11 but using 50 mM Tris-HCl pH 7.0 (1:10 w/v) containing 200 mM NaCl, 6.2 mM sodium azide and protease inhibitor (1 tablet/50 mL, Roche Complete Protease inhibitor tablet) as the extraction buffer. After clarification by centrifugation, the extract was subjected to gel permeation chromatography on Superdex S200 as described for the final step in the purification of Cor a 11. Fractions containing Cor a 9 were concentrated using an Amicon stirred cell and then repeatedly dialysed in regenerated cellulose dialysis tubing with a 12-kDa exclusion limit at 4°C against

20 mM Tris-HCl pH 7.5 containing 500 mM NaCl. Whilst this preparation was free from Cor a 11, as indicated by SDS-PAGE but there were concerns that contamination below the limits of detection of SDS-PAGE might be present. Since Cor a 11 is not glycosylated it does not bind to Con A, any residual Cor a 11 in the partially purified protein could be removed by passage through a Con A column, the Cor a 11 eluting in the unbound fraction. Given low level of potential contamination of the Cor a 9 preparation by Cor a 11 the capacity of the Con A column to bind Cor a 11 was vastly in excess of that required for this final purification step. Thus, 25 mL aliquots of partially purified Cor a 11 were applied to a column (1.6 × 10 cm) of Con A Sepharose-4B (GE Healthcare) attached to a BioCad Sprint HPLC system (Applied Biosystems) equilibrated with 20 mM Tris-HCl pH 7.5 containing 500 mM NaCl and washed with equilibration buffer. The Cor a 9-containing fractions representing the unbound material were pooled before repeatedly dialysed in regenerated cellulose dialysis tubing with a 12-kDa exclusion limit at 4°C against 0.5 M ammonium bicarbonate before being freeze dried and stored at −20°C until required. The Con A column was then washed with equilibration buffer containing 0.4 M methyl mannopyranoside to remove bound Cor a 11 before re-use.

2.4 Purification of LTP (Cor a 8)

Defatted hazelnut flour (30 g) was extracted by stirring in 20 mM MES, pH 5.6 (1:13 w/v) containing 6.2 mM sodium azide, 3% (w/v) polyvinylpolypyrrolidone (PVPP, Sigma-Aldrich) and protease inhibitor (1 tablet/100 mL, Roche Complete Protease inhibitor tablet) for 1 h at 20°C . After filtration through a coarse glass sinter the extract was diluted with water (1:1, v/v). The pH was adjusted to 5.6 with 0.1 M HCl and the solution centrifuged (30 000 × *g*, 60 min, 20°C) and filtered by sequential passage through a graduated series of membrane filters (8, 3, 0.45 and 0.2 μm) using a water pump and Buchner flask equipped with a membrane filter holder (Sigma-Aldrich). The resulting filtrate was loaded onto a cation exchange column (2.6 × 15 cm) of SP Sepharose Fast Flow, (GE Healthcare) equilibrated with 20 mM MES, pH 5.6 attached to a BioCad Sprint HPLC system (Applied Biosystems) After washing with equilibration buffer to remove unbound protein, the bound protein was eluted with a salt gradient (0 to 1.0 M NaCl) in the same buffer. The eluent was monitored for protein by absorbance at 280 nm and protein-containing peak fractions were pooled.

Solid ammonium sulphate was added to the bound protein fraction to 3.0 M and centrifuged (30 000 × *g*, 1 h, 20°C) before applying the supernatant to a hydrophobic interaction column (1.6 × 18 cm) of Phenyl Sepharose, high substitution (GE Healthcare) attached to a BioCad Sprint HPLC system (Applied Biosystems), equilibrated in 15 mM MES pH 5.6, 3.0 M ammonium sulphate. After washing with equilibration buffer, Cor a 8 was eluted using a decreasing ammonium sulphate gradient (3.0 to 0.0 M in equilibration buffer over 180 mL in equilibration buffer at 3 mL/min. Fractions containing Cor a 8 were identified by immunoblotting with anti-rCor a 8, pooled and concentrated by ultrafiltration using a regenerated cellulose membrane with a 1-kDa exclusion limit (Millipore, Hertfordshire, UK), dialysed against six exchanges of distilled water (1 h,1 L) using 3.5-kDa cut-off dialysis tubing (Perbio Science, Northumberland, UK) and re-concentrated by ultrafiltration (1-kDa membrane).

Solid MES was added to a final concentration of 40 mM and the pH of the preparation adjusted to pH 5.6 with 0.1 M NaOH prior to applying 1 mL aliquots to a cation-exchange column (Poros HS20, 0.46 × 10 cm, Applied Biosystems), attached to a BioCad Sprint HPLC system (Applied Biosystems) equilibrated with 20 mM MES, pH 5.6. After washing with equilibration buffer to remove unbound protein, the bound protein was eluted using a 35 mL, 0 – 0.4 M NaCl gradient in equilibration buffer. Fractions containing Cor a 8 were again identified by immunoblotting, pooled and concentrated by ultrafiltration (1-kDa cut-off membrane). The Cor a 8 was finally subjected to gel filtration chromatography on a Superdex S75 preparative grade gel permeation column (1.6 × 60 cm; GE Healthcare), equilibrated and eluted in 25 mM sodium phosphate pH 7.0, 150 mM NaCl. The peak corresponding to Cor a 8 was concentrated by ultrafiltration (1-kDa cut-off membrane) and stored frozen in aliquots.

2.5 Analytical gel-filtration, SDS-PAGE and immunoblotting

The purified proteins were analysed on columns (1.6 × 60 cm) of either Superdex S200 preparative grade (Cor a 11 and Cor a 9) or Superdex S75 preparative grade (Cor a 8), equilibrated and eluted with 50 mM Tris/HCl, 150 mM NaCl pH 7.5, at a flow rate of 1.0 mL/min. The absorbance was monitored at 280 and 220 nm. The columns were calibrated with a set of gel filtration molecular weight standards (Bio-Rad, Hertfordshire, UK) and additionally with aprotinin (Sigma Aldrich) for the Superdex S75 column.

SDS-PAGE was performed on 4 – 12% gradient gels run for 35 min at 200 V in MES buffer, reduced gels were run by including 50 mM 1,4-dithioerythritol in the denaturing heating step. Reference markers with known molecular weights (Mark 12 unstained standard, Invitrogen) were run on the same gel. Gels were fixed in methanol:water:acetic acid (40:50:10 v/v) and stained using colloidal Coomassie G250 (Invitrogen). Gels were scanned using a GS800 calibrated densitometer and Quantity One software (Bio-Rad).

Gels for immunoblotting were run as above except the molecular weight standard was replaced by one containing pre-stained molecular weight marker proteins (SeeBlue

Plus2, Invitrogen). Immunoblotting was performed using two blotting systems:

(i) During the purification procedure the presence of Cor a 8 in Phenyl Sepharose column fractions was determined by semi-dry blotting of proteins from gels to NC membrane (0.2-μm pore, Trans-Blot transfer media, Bio-Rad) in 39 mM glycine, 48 mM Tris base, 0.0375% SDS, 20% methanol, pH 8.8 – 8.9 using a Trans-Blot SD, semi-dry transfer cell (Bio-Rad) at 15 V for 20 min. Membranes were blocked by incubation (1 h, 20°C) in 5% skimmed milk powder (Marvel, Premier Foods, Lincolnshire, UK) in PBST (137 mM NaCl, 0.01 M Na phosphate, 2.7 mM KCl, 0.05% v/v Tween 20, pH 7.4) followed by washing in PBST (3 × 5 min, 50 mL). The membrane was incubated using gentle shaking in a 1:10 000 × dilution of anti-Cor a 8 for 1 h at 18°C then washed in PBST (as above). The blot was then incubated with gentle shaking in a 1:1000 × dilution of goat anti-rabbit alkaline phosphatase conjugate for 3 h at 18°C and then washed in PBST (as above). Bound antibody was located by briefly washing the membrane in water (1 × 30 s, 100 mL) and then staining using 10 mL of a SIGMA *FAST*™ BCIP/NBT substrate tablet solution (1 tablet in 10 mL of water). The washing step is sufficient to remove any inhibitory effect of phosphate on the alkaline phosphatase.

(ii) The IgE-binding capacity of purified allergens was determined using immunoblotting using ~1 μg/track of protein followed by blotting using a Novablot electrophoretic transfer apparatus (Invitrogen, The Netherlands) according to the manufacturers protocol. Membranes were blocked with PBS/AT (PBS containing 10 mM EDTA, 0.3% w/v/ BSA and 0.1% v/v Tween 20). Strips (3-mm wide) were cut from the blots and incubated overnight with 2 mL of PBS/AT containing 75 μL of human allergic serum. After washing away unbound material, bound IgE was detected using I^{125}-radiolabelled sheep anti-human IgE (SH25-1p7, Sanquin Amsterdam, the Netherlands), followed by autoradiography using X-ray film (Eastman Kodak, Rochester, NY).

2.6 LC-ESI-MS

HPLC-MS of proteins was performed using a Jasco PU-1585 triple pump HPLC equipped with an AS-1559 cooled autoinjector, CO-1560 column oven and UV-1575 UV detector. 50 – 75 μL of protein solution (1 to 2 mg/mL) was injected onto an HPLC column 4.6 × 250 mm, Jupiter 5 μm, C4, 300 Å, Phenomenex) equipped with a guard cartridge (3 × 4 mm, wide-pore C4, "Security Guard", Phenomenex). Solvents were 0.1% w/v TFA in double-distilled water as solvent A and 0.085% w/v TFA in double-distilled water/ACN (10:90, v/v) as solvent B. Proteins were eluted by a linear gradient of solvent B from 0 to 100% in 15 min. The separation was carried out at 25°C. The 1 mL/min mobile phase flow exiting the HPLC column was split using an ASI 600 fixed ratio splitter valve (Presearch, Hitchin, UK) with approximately 20% being diverted to a Waters Quattro II triple quadrupole mass spectrometer. Spectra were obtained in positive ion electrospray mode using a Micromass Z-sprayTM ion source. The electrospray probe was operated at 3.46 kV and a cone voltage of 35 V. The source and desolvation temperatures were 120 and 300°C, respectively. The nitrogen nebulising and drying gas flow rate were optimised at 15 and 500 L/h, respectively. The mass range m/z 650 – 2200 was scanned every 5 s in continuum mode, with a 0.2-s interscan time.

MALDI-TOF analysis of in-gel tryptic cleavage products was also undertaken. Following SDS-PAGE analysis, the protein polypeptides were manually excised, and in-gel digested using a Progest Investigator automated in-gel digestion apparatus (Genomic Solutions, Cambridgeshire, UK). Prior to digestion samples were automatically reduced with DTT and alkylated with iodoacetamide. Trypsin solution (0.01 μg/μL in 10 mM ammonium bicarbonate of modified porcine trypsin, Promega, Madison, USA) was added to each sample. The digestion was performed for 3 h at 37°C and terminated by addition of 5 μL of 5% v/v formic acid and incubating for 20 min at room temperature. Tryptic peptides were analysed by MALDI-TOF-MS using a Reflex III mass spectrometer (Bruker) equipped with a SCOUT 384 ion source at the IFR-JIC joint proteomics centre. All spectra were acquired in a positive-ion reflector. Peptide profiles were searched (on the basis of mass) against the NCBI non-redundant protein database using the MASCOT programme from Matrix Science (http://www.matrixscience.com/) and Viridiplantae as the taxonomy in the search parameters. The probability of identification was shown by the MOWSE score [18].

2.7 Circular dichroism and FT-IR spectroscopy

Protein secondary structure was determined using a combination of circular dichroism (CD) and Fourier-transformed infrared spectroscopy (FT-IR). Far-ultraviolet (UV) CD spectra (270 – 190 nm) were recorded using a Jasco J-710 Spectropolarimeter (Jasco, Tokyo, Japan) with demountable 0.01- or 0.02-cm path length quartz cells (Hellma UK, Essex, UK). Protein was prepared in 10 mM Na phosphate buffer pH 7.6 by dialysing a solution against the measurement buffer (2 × 500 mL for 30 min) using a mini dialysis unit (3.5 kDa membrane, Perbio Science UK). Spectra represent the average of nine accumulations collected at 50 nm/min with a 2-s time constant, 0.5 nm resolution and sensitivity of ± 100 mdeg. Protein concentration was determined by measurement of the UV spectrum of the dialysed protein and using experimentally determined extinction coefficients at 280 nm. Molar CD (with respect to moles of amide bond) was calculated using CD Pro. Secondary structure was predicted using CDSSTR, ContinLL and Selcon3 and the predictions were averaged, as the predictions were similar.

FT-IR spectra were obtained by adding 128 scans at 2 cm^{-1} resolution on a Bio-Rad FTS 175C FT-IR spectrometer equipped with a Specac "Golden Gate" ATR module on a sample of protein that had been dialysed (12-kDa membrane) against 0.5 M ammonium bicarbonate and then freeze dried.

2.8 IgE-binding characteristics

The recognition of the Cor a 8 by human IgE was determined using RAST and immunoblotting. For RAST, serum (50 µL) was incubated overnight with 0.5 mg of Sepharose-coupled allergen in a final volume of 300 µL PBS, 0.3% w/v BSA, 0.1% v/v Tween-20 (PBS-AT). After washing away unbound serum components, radiolabeled sheep antibodies (Sanquin) directed to human IgE (in 500 µL PBS-AT containing 4.5% bovine and 0.5% sheep serum v/v) were added (approximately 20 000 cpm per test). After overnight incubation and washing, bound radioactivity was measured. Results were expressed as international units IgE per mL (IU/mL). IgE content was calculated using a standard curve obtained by RAST with a dilution series of a chimeric monoclonal IgE antibody against the major house dust mite allergen Der p 2 and Sepharose-coupled recombinant Der p 2.

For immunoblotting, proteins were separated by SDS-PAGE (NuPAGE 4–12% Bis-Tris gel, purified protein (approximately 1 µg/gel) according to the protocol of the manufacturer (Invitrogen). Immunoblotting was performed by transferring the proteins semi-dry to NC on a Novablot electrophoretic transfer apparatus, according to the protocol of the manufacturer (Invitrogen). Membranes were blocked with PBS-ATE (PBS-AT containing 10 mM EDTA). Blot strips were cut (3-mm wide) and incubated overnight with 2 mL PBS/AT and 75 µL patients' serum. After washing away unbound material, bound IgE antibodies were detected using ^{125}I-radiolabelled sheep anti-human IgE (SH25-1-p7, Sanquin), followed by autoradiography using to X-ray film (Eastman Kodak).

3 Results and discussion

3.1 Purification

3.1.1 7S globulin-Cor a 11

Cor a 11 is a glycosylated 7S storage globulin and therefore affinity chromatography on a column of Con A (a lectin, which binds sugar residues) was initially used, passing the crude preparation through a column of Con A Sepharose-4B, washing away unbound protein and eluting the bound Cor a 11 with buffer containing methyl mannopyranoside (Fig. 1a). Fractions identified as containing Cor a 11 by SDS-PAGE (indicated by double-headed arrow in Fig. 1a) were pooled, concentrated and separated by gel permeation chromatography on Superdex S200 (Fig. 1b), the fractions

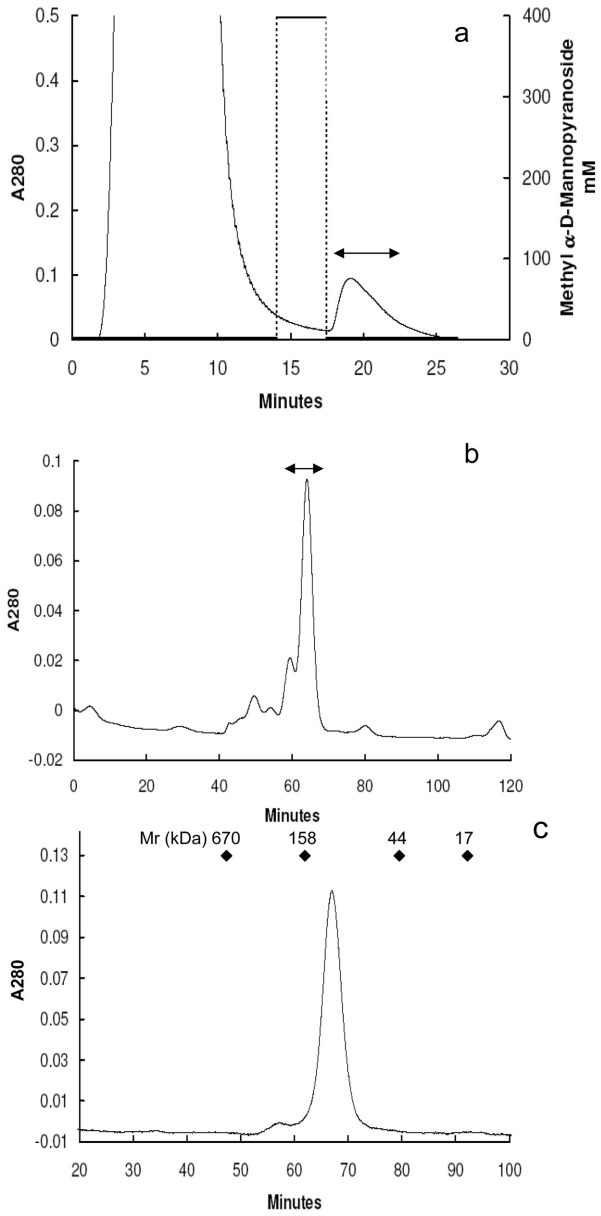

Figure 1. Purification of Cor a 11. (a) Affinity chromatography of crude hazelnut extract on a column of Con A Sepharose-4B. The dotted line corresponds to the methyl mannopyranoside gradient used to elute bound protein. The fractions indicated by the double-headed arrow were collected and pooled. (b) Gel filtration of the fractions from affinity chromatography on a column of Superdex S200. The fractions indicated by the double-headed arrow were collected and pooled. (c) Re-chromatography of the major Superdex S200 gel filtration peak fractions pooled on a column of Superdex S200. Molecular weight standards were bovine thyroglobulin (M_r 670 kDa), bovine gamma globulin (M_r 158 kDa), chicken ovalbumin (M_r 44 kDa) and horse myoglobin (M_r 17 kDa) indicated by filled diamonds.

containing the Cor a 11 (determined by SDS PAGE, indicated by double-headed arrow in Fig. 1b) being re-applied to the same column (Fig. 1c). The yield of purified protein

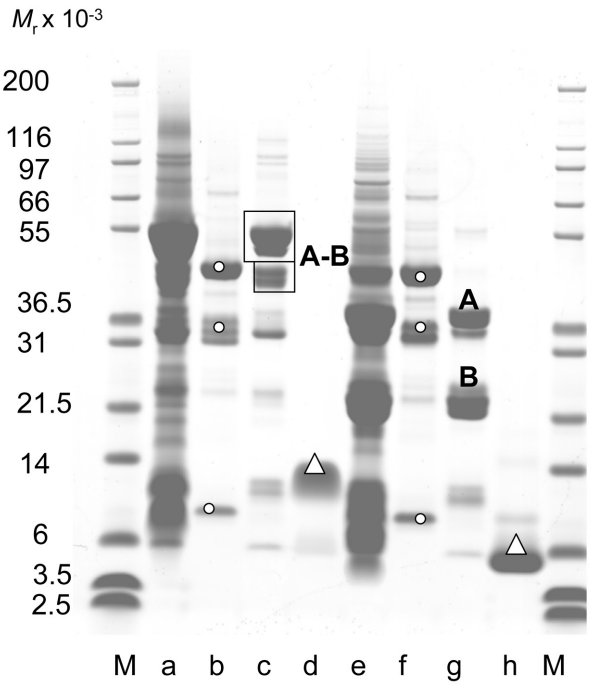

Figure 2. SDS-PAGE 4 – 12% gel of crude and purified hazelnut protein fractions. Tracks (a) and (e) are crude hazelnut extract; (b) and (f) are purified Cor a 11; (c) and (g) are Cor a 9 and (d) and (h) are Cor a 8. Samples in tracks (a – d) were separated without reduction while samples in (e – h) were reduced with dithioerythritol. Tracks marked M are protein markers of known molecular mass, shown as M_r on the left. Cor a 11 polypeptides marked with filled white circles; A – B – disulphide-linked acidic and basic subunits of Cor a 9 are boxed; A – reduced acidic subunit of Cor a 9; B- reduced basic subunit of Cor a 9. Cor a 8 polypeptides are marked by filled white triangles.

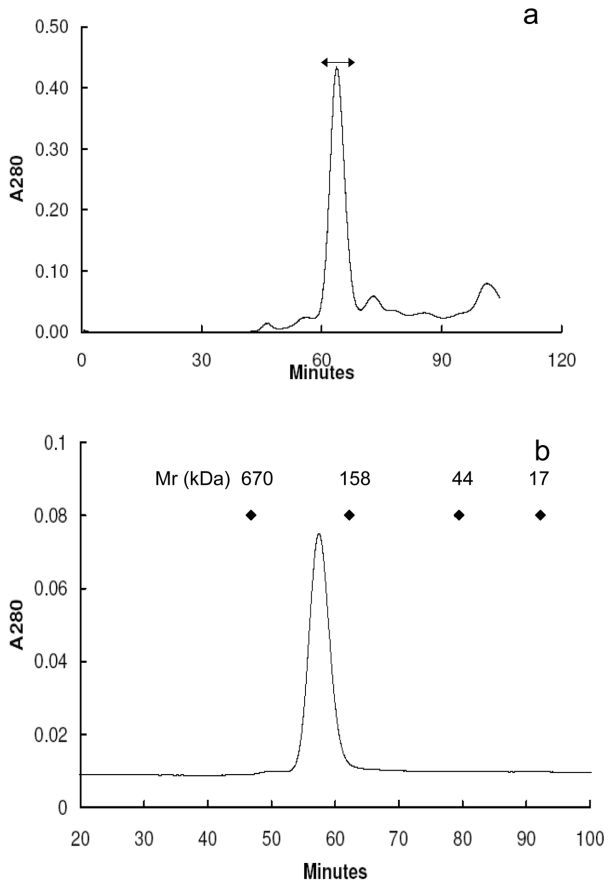

Figure 3. Purification of Cor a 9. (a) A crude extract of hazelnut was applied to a Superdex S200 column and the major peak fractions (indicated by the arrow) collected and pooled. (b) Re-chromatography of Cor a 9 on a column of Superdex S200, as described for Cor a 11 (see Fig. 1c) molecular weight markers being shown as filled diamonds.

was approximately 55 mg from 25 g of defatted seed meal. Analytical gel filtration chromatography (Fig. 1c) showed the main component of M_r 107 kDa, with a minor component of M_r 220 kDa. These are likely to correspond to the trimeric and hexameric forms of the protein, respectively, as found for other 7S seed storage globulins [20].

SDS-PAGE under non-reducing conditions showed three groups of polypeptides (marked with filled circles in Fig. 2, track b) which exhibited only small changes in mobility on reduction (Fig. 2, track f). MALDI-TOF analysis of in-gel tryptic digests of the main M_r 47-kDa polypeptide (Fig. 2, lanes b, f) gave a 70% sequence coverage corresponding to ExPasy Q8S4P9 with a MOWSE score of 278, scores of 65 generally being considered significant (p <0.05) (Table 1). It is probable that these larger polypeptides correspond to unprocessed subunits, the calculated mass of mature subunits encoded by sequence Q8S4P9 being 45 044 Da. The two smaller groups of polypeptides of M_r 31 – 36 kDa probably correspond to the products of subunits which have been proteolytically processed in the seed but which still assemble into trimeric structures. They were also identified as corresponding to ExPasy Q8S4P9 but with lower MOWSE scores of only 43 – 46. Similar mixtures of unprocessed and processed subunits have been characterized in other species, notably pea (reviewed in [19, 20]). Two polypeptides of M_r 23 and 9 kDa were not sufficiently abundant to give any identification by in-gel trypsin digestion. MS also provided evidence of polymorphism, giving a broad peak with molecular weights ranging from about 45.4 to 46.4 kDa. This may reflect variation in the primary structure and glycosylation of the unprocessed subunits. Only a mass corresponding to the more abundant, large subunit was detected.

3.1.2 11S globulin- Cor a 9

Cor a 9 is an 11S seed storage globulin and consequently is not glycosylated and was therefore purified by gel permeation chromatography on Superdex S200 (Fig. 3a) followed by passage through a column of Con A Sepharose-4B to remove contaminating Cor a 11 and analysis on Superdex

Table 1. Sequence coverage (bold underlined) obtained by MALDI-TOF analysis of purified Cor a 11, and Cor a 8

(a) Cor a 11 (ExPasy Q8S4P9)10

SSEESYGKEQ EENPYVFQDE HFESR**VKTEE GRVQVLENFT KR**SR**LLSGIE**
NFRLAILEAN PHTFISPAHF DAELVLFVAK GR**ATITMVR**E EK**RESFNVEH**
GDIIRIPAGT PVYMINRDEN EKLFIVK**ILQ PVSAPGHFEA FYGAGGEDPE**
SFYRAFSWEV LEAALKVRRE QLEKVFGEQS KGSIVKASRE KIR**ALSQHEE**
GPPRIWPFGG ESSGPINLLH KHPSQSNQFG RLYEAHPDDH KQLQDLDLMV
SFANITK**GSM AGPYYNSR**AT K**ISVVVEGEG FFEMACPHLS SSSGSYQK**IS
ARLRR**GVVFV APAGHPVAVI ASQNNNLQVL CFEVNAHGNS R**FPLAGK**GNV**
INEFERDAK**E LAFNLPSR**EV ERIFK**NQDQA FFFPGPNKQQ EEGGR**GGRAF
E

Underlined sequence represents N terminus identified by Pastorello *et al*. [6].

(b) Cor a 9 (ExPasy Q8W1C2)

MAKLILVSFS LCLLVLFNGC LGINVGLRRQ QQRYFGECNL DR**LNALEPTN**
RIEAEACQIE SWDHNDQQFQ CAGVAVIRR**T IEPNGLLLPQ YSNAPELIYI**
ERGR**GITGVL FPGCPETFED PQQQSQQGQR** QGQGQSQRSE QDRHQKIRHF
REGDIIALPA GVAHWCYNDG DSPVVTVSLL HTNNYANQLD ENPR**HFYLAG**
NPDDEHQRQG QQQFGQRRRQ QQHSHGEQGE QEQQGEGNNV FSGFDAEFLA
DAFNVDVDTA RRLQSNQDKR RNIVKVEGR**L QVVRPERSRQ EWER**QERQER
ESEQERERQR RQGGRGRDVN GFEETICSLR LRENICTR**SR ADIYTEQVGR**
INTVNSNTLP VLRWLQLSAE RGDLQR**EGLY VPHWNLNAHS VVYAIR**GRAR
VQVVDDNGNT VFDDELRQGQ VLTIPQNFAV AKRAESEGFE WVAFKTNDNA
QISPLAGRTS AIR**ALPDDVL ANAFQISR**EE ARRLKYNRQE TTLVRSSRSS
SERKRRSESE GRAEA

Underlined sequence represents N terminus identified by Pastorello *et al*. [6]. Shaded sequence represents the consensus cleavage site between the acidic and basic subunits.

(c) Cor a 8 (ExPasy Q9ATH2)

SLTCPQIKGN LTPCVLYLK**N GGVLPPSCCK** GVRAVNDASR TTSDRQSACN
CLKDTAK**GIA GLNPNLAAGL PGKCGVNIPY KISPSTNCNN VK**

Underlined sequence represents N terminus identified by Pastorello *et al*. [6].

S200 (Fig. 3b). This showed the purified 11S globulin to correspond to a M_r 234-kDa oligomer. SDS-PAGE analysis showed this oligomer comprised major polypeptides ranging from about 30 to 55 kDa (boxed and marked A–B in Fig. 2 track c), which were replaced by two discrete groups of polypeptides of about 21–25 kDa (marked B) and 31–35 kDa on reduction (marked A in Fig. 2 track g). The polypeptides in the unreduced preparation are assumed to correspond to subunits, which are reduced to give acidic chains (31–35 kDa) and basic chains (21–25 kDa). This is the typical 11S globulin subunit structure as described for many other species (reviewed in [19, 20]). In addition, tracks c and g also contain polypeptides of lower mass (M_r ~32 kDa, a doublet running at 10–12 KkDa and a single polypeptide of M_r 9 kDa) whose mobilities are not greatly affected by reduction and are presumably highly proteolytically processed subunits not containing disulphide bridges which are found in the M_r 234 kDa 11S globulin oligomer.

The identity of the protein was confirmed by MALDI-TOF analysis of in-gel tryptic digests of the polypeptide marked A-B from a non-reduced gel (Fig. 2, track c), which comprises the disulphide-bond linked acidic and basic subunits of Cor a 9. The protein was found to correspond to ExPasy Q8W1C2, having a significant a MOWSE score of 177 and 45% sequence coverage, peptides being identified, which corresponded to both the acidic and basic subunits (Table 1). Analysis of the reduced subunits (Fig. 2, track g marked A and B) also gave identifications for ExPasy Q8W1C2 with MOWSE scores ranging from 109–165 (data not shown). The N-terminal sequence for Cor a 9

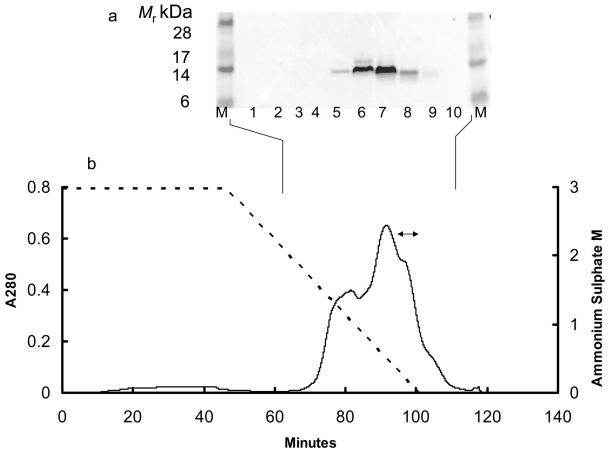

Figure 4. Preparation of crude Cor a 8. (a) Identification of Cor a 8-containing fractions by Western blotting using a rabbit polyclonal anti-rCor a 8 anti-serum. Lanes 1-10 correspond to fractions from hydrophobic interaction chromatography (see b). Lanes marked M correspond to molecular weight markers (see Section 2). (b) Separation of partially purified hazelnut extract by hydrophobic interaction chromatography (HIC) on a column of Phenyl Sepharose. Dotted line corresponds to the 3.0 – 0.0 M ammonium sulphate gradient. The fractions indicated by the double-headed arrow were collected and pooled.

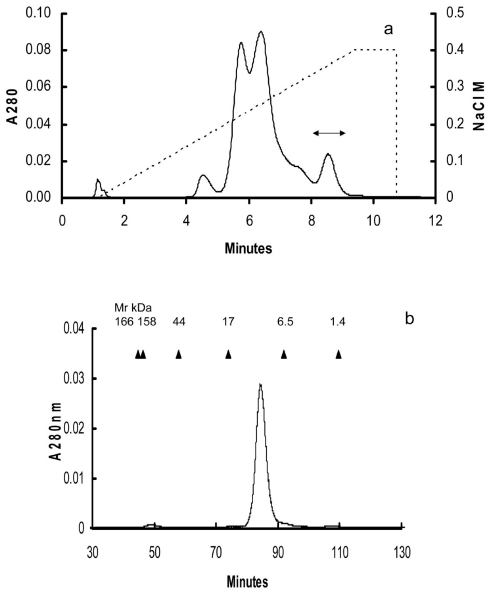

Figure 5. Preparation of highly purified Cor a 8. (a) Partially purified Cor a 8 from hydrophobic interaction chromatography (Fig. 3) was subjected to cation-exchange chromatography on a column of Poros HS20. Dotted lines denote a 0 – 0.4 M NaCl gradient. The fractions indicated by the double-headed arrow were collected and pooled. (b) Cor a 8 containing fractions from cation-exchange chromatography on a column of Superdex S75; the elution position of molecular weight standards are indicated by inverted filled triangles. Standards were bovine thyroglobulin (M_r 670 kDa), bovine gamma globulin (M_r 158 kDa), chicken ovalbumin (M_r 44 kDa), horse myoglobin (M_r 17 kDa), Aprotinin (M_r 6.5 kDa) and vitamin B12 (M_r 1.35 kDa).

identified by Pastorello and co-workers [9] is present in this accession but with a conservative substitution at the N terminus of a valine for isoleucine. Unfortunately, no peptides corresponding to this region were identified in the peptide mass fingerprint. A higher mass polypeptide of Mr 60 kDa was also identified as being an 11S globulin (Q8W1C2) with a MOWSE score of 121. Three minor of Mr 12, 20 and 6 kDa evident in Fig. 2 tracks c and g, which were unaffected by reduction were not identified with significant MOWSE scores by in-gel trypsin digestion.

3.1.3 LTP- Cor a 8

A fraction of proteins enriched in LTP and other proteins with high pI was prepared by cation-exchange chromatography. The LTP Cor a 8, was then purified from this fraction by hydrophobic interaction chromatography using Phenyl Sepharose (Fig. 4b), fractions containing the LTP being identified by immunoblotting using a rabbit polyclonal antibody against rCor a 8 (Fig. 4a). LTP containing fractions were pooled and then further purified by cation-exchange chromatography (Fig. 5a) and gel-permeation chromatography (Fig. 5b). SDS-PAGE under non-reducing conditions showed a single broad band of about 12 kDa, which was replaced by a sharper band of faster mobility on reduction (see filled triangles in Fig. 2 tracks d and h). Only traces of other low-molecular mass proteins were present. Cor a 8 was subjected to MALDI-TOF MS analysis, which tentatively confirmed its identity as an LTP from *Corylus avellana* (ExPASy Q9ATH2) with a 52% coverage and a MOWSE score of 58. This is just below the level of significance (Protein MASCOT scores greater than 65 are significant (p <0.05)). However, the small molecular mass of the protein means that it is then often difficult to generate sufficient peptides from trypsinolysis to obtain a firm identifica-

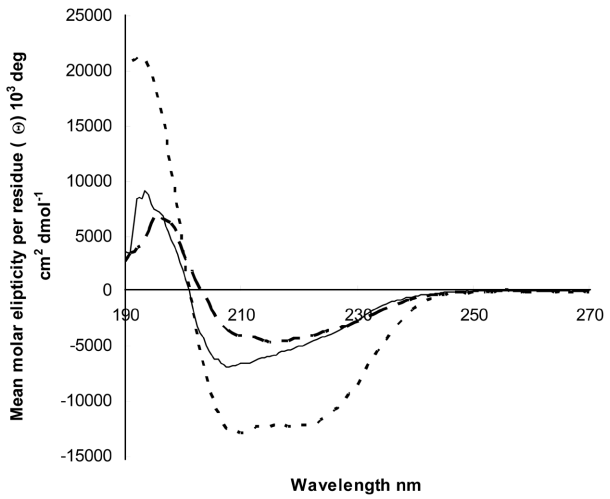

Figure 6. Far-UV circular dichroism (CD) spectroscopy of Cor a 8 (dotted line), Cor a 9 (dashed line) and Cor a 11 (solid line).

tion. Nevertheless, LC-MS analysis showed a major peak of 9466 Da (compared with a calculated value of 9468 Da based on the database accession ExPASy 9QATH2, with minor peaks at 9 580 and 9627 Da that could correspond to isoforms or impurities. Taken together with the fact that the protein is recognized by an antibody raised against rCor a 8, these data indicate that the protein is highly likely to correspond to hazelnut LTP.

RP-HPLC of the purified preparation showed a complex pattern, with a main peak at 29.5 min, a broader peak at 32–35 min and a sharp peak at 35.5 min. Changing the shape of gradient resulted in changes in the relative proportions of the 32–35-min and 35.5-min peaks, including the virtual elimination of the latter peak. This indicates that a ligand such as a lipid may be bound to the protein and released at a critical concentration of ACN. This is supported by ESI-MS analysis of three peaks, which showed that all contained major components of 9 466 Da (data not shown).

3.2 Spectroscopic analysis of secondary structure

An assessment was made of the folded state of purified allergens using CD spectroscopy (Fig. 6) and FT-IR spectroscopy (Fig. 7). The CD spectra of both Cor a 11 and Cor a 9 were characteristic of seed storage globulins, which are rich in β-sheet structures. Thus, the Cor a 11 spectrum gave a positive maximum at 192 nm and a negative minimum at 207 nm, the molar elipticity gradually increasing until reaching around zero at around 250 nm. While the intensity of the molar elipticity was slightly different, such a spectrum is entirely consistent with those previously reported for other 7S seed storage globulins such as β-conglycinin from soybean [21, 22]. The Cor a 9 spectrum showed subtle differences having a positive maximum at 195 nm, which is similar to that reported for the 11S globulin from soybean, glycinin [22] but had only a shallow negative minimum between 208–220 nm whereas glycinin has one negative minimum between 200–215 nm and a second between 220–230 nm. As might be anticipated, Cor a 8 gave a CD spectrum entirely consistent with the established α-helical structure of LTP, with a positive maximum at around 192 nm with an intense negative double minimum at 210 and 224 nm.

The FT-IR spectra (Fig. 7) essentially confirm the folded nature of the proteins, with Cor a 11 giving an amide II maximum at 1631 cm^{-1} with a shoulder at 1646 cm^{-1} and Cor a 9 giving a different peak shape with a maximum at 1637 cm^{-1} and a shoulder at 1656 cm^{-1} . The bands at around 1635 cm^{-1} are characteristic of β-sheet-rich proteins, including seed storage globulins, while those around 1650 cm^{-1} are attributed to α-helical or random structures, showing that these proteins comprise primarily β-sheet with some α-helical and more mobile "random" structures

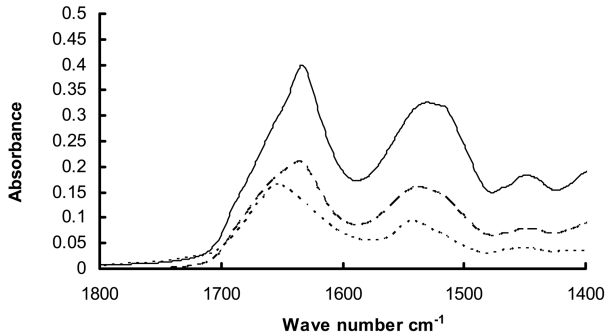

Figure 7. Fourier-transform infrared (FT-IR) spectroscopy of Cor a 8 (dotted line), Cor a 9 (dashed line) and Cor a 11(solid line).

[22]. The lack of a strong band at 1625 cm^{-1} which is normally ascribed to inter-molecular structure is characteristic of β-sheet structures formed as a consequence of aggregation [22] suggests that the purified allergens are in a native folded state. The spectrum of Cor a 9 is dominated by a peak maximum at 1656 cm^{-1}, consistent with this protein being almost entirely comprised of α-helical structures.

3.3 Immunological characterisation

The IgE-reactivity of Cor a 8, 9 and 11 was assessed using IgE immunoblotting. Cor a 9 and Cor a 11 were tested for their capacity to bind IgE using a panel of sera from 14 patients from the Netherlands (Figs. 8a amd b). IgE from 6 of these sera (patients 1, 3, 5, 7, 8, 10 and 13) bound to the large (acidic) subunit of Cor a 9, 4 of which showed strong and 2 weak IgE-binding reactions with the acidic subunit of Cor a 9. An Mr 48-kDa band present in the Cor a 11 preparation was also recognised by IgE from 7 sera (patients 3, 7, 8, 9, 10, 13). The IgE-binding reactions with Cor a 11 were generally weaker than with Cor a 9 and no binding to the small (basic) subunit of Cor a 9 was observed at all. Cor a 8 was specifically recognised on an immunoblot by serum IgE from 8 Spanish patients (Fig. 8c). Certain sera gave stronger reactions to low mass material running close to the dye front in both Cor a 9 which probably corresponds to a and Cor a 11, which may correspond to low-Mr polypeptides present in the purified proteins but for which no identification could be obtained by in-gel trypsin digestion (see above) but which were present in the intact oligomeric globulins. Further research will be required to characterise these minor polypeptides. Strong IgE binding to Cor a 8 was observed for sera from 5 patients (15, 19, 20, 21 and 22) with weak binding being observed with a further 3 sera (Patients 16–18). In summary, serum IgE from hazelnut allergic patients recognised discrete polypeptides from Cor a 8, 9 and 11 and no binding was evident in the preparations to other polypeptides confirming their purity.

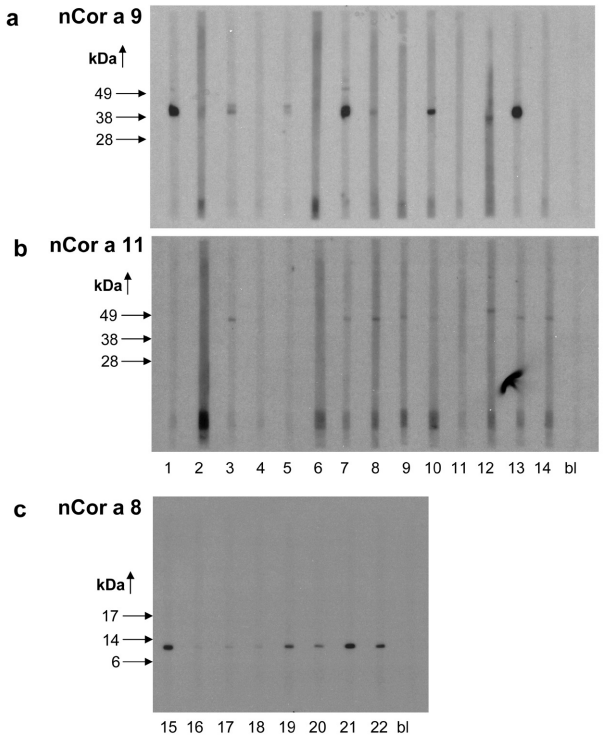

Figure 8. IgE-immunoblots of nCor a 11 (panel a), purified nCor a 9 (panel b) and purified nCor a 8 (panel c). After SDS-PAGE electrophoresis purified proteins were transferred to NC membranes and incubated with serum samples from hazelnut allergic patients from The Netherlands (lanes 1 – 14 panels a, b) and serum samples from Spanish patients (panel c lanes 15 – 22). Bound IgE antibodies were detected using ^{125}I-labelled anti IgE antibodies. Label controls are marked bl.

4 Concluding remarks

Methods have been developed to purify three major allergens from hazelnuts. These all belong to well-characterised families of seed proteins (LTP, 7S globulin, 11S globulin), which include well-characterised allergens in other foods (reviewed in [11, 13]). Spectroscopic analyses confirm that the proteins are correctly folded while immunological studies show that they retain their IgE-binding capacity. Sera from patients in the UK and the Netherlands reacted with either one or both of the globulin allergens, but in the case of Cor a 9 (the 11S globulin) reactions were only observed with the large acidic subunit, and not with the small basic subunit. A similar specificity was observed with sera to the related 11S globulin allergen (Ara h 3/4) of peanut [23]. Sera from Spain reacted exclusively with Cor a 8 in the purified Cor a 8 sample. No other IgE-binding proteins were detected by immunoblotting analysis, demonstrating the utility of the purified Cor a 8, together with Cor a 9 and 11, as tools to analyse for IgE specificity towards hazelnut allergens.

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