Research Article

Expression and characterization of three important panallergens from hazelnut

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Several hazelnut allergens with different clinical relevance and crossreactive properties have been identified and characterized so far. The aim of this study was to develop protocols for producing relatively large amounts of three recombinant hazelnut allergens Cor a 1.04, Cor a 2, and Cor a 8 in a folded and immunologically active form. The availability of well-characterized, pure recombinant allergens will improve diagnostic *in vitro* tests for food allergy, by allowing a highly sensitive component resolved diagnosis. Depending on the individual hazelnut allergen, protocols for heterologous production – either as fusion or nonfusion protein – were developed to obtain homogenous protein batches. The resulting proteins were purified by a two-step FPLC method and their IgE antibody reactivity was verified. Identity was verified by N-terminal sequencing and MALDI-TOF-MS analysis. Their secondary and tertiary structure was controlled by circular dichroism (CD)-spectroscopy and NMR analysis. Decisions on the strategies for expression and purification of allergens on a large scale were made on a case by case basis: Preparation of rCor a 1.04 and rCor a 2 as fusion proteins in *E. coli* from inclusion bodies resulted in approximately 10 mg pure protein *per* liter whereas rCor a 8 expression in yeast as nonfusion protein yielded 30 mg/L.

Keywords: Cor a 1.04 / Cor a 2 / Food allergy / Hazelnut / Recombinant allergens

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1 Introduction

Hazelnut is a common cause of food-induced allergic reactions. In Europe, the prevalence of hazelnut (*Corylus avellana*) allergy is estimated to be between 0.1 and 0.5% [1, 2]. In the United States, tree nut allergy affects approximately 0.5% of the population [3, 4].

Differences exist in sensitization patterns between hazelnut allergic patients from Northern and Southern Europe. In Northern Europe allergy to hazelnuts is often found in patients with birch (*Betula verrucosa*) pollen allergy. These patients are primarily sensitized to birch pollen and frequently suffer from mild oral allergy syndrome (OAS) after ingestion of birch pollen related foods, including hazelnuts, caused by IgE crossreactivity between pollen and hazelnut proteins. In contrast, more severe and even life-threatening allergic reactions to hazelnuts were reported in patients from the Mediterranean area [5]. Hazelnut-allergic patients in the Mediterranean are frequently sensitized to nonspecific lipid-transfer proteins (nsLTPs), a member of class I food allergens, probably by primary sensitization after ingestion of food [6, 7]. Sensitization to hazelnut within this group of patients appears independent from allergy to birch pollen.

Up to now, several hazelnut allergens with different clinical relevance and crossreactive properties have been identified and characterized. Cor a 1.04, the major hazelnut allergen in Northern Europe, is a homolog of the major birch pollen allergen Bet v 1 and belongs to the family of pathogenesis-related plant proteins PR-10 [8, 9]. Interestingly, it appears that the epitopes of the hazelnut allergen Cor a 1.04 are less related to the hazel pollen allergen Cor a 1.01 than to Bet v 1 from birch pollen [9]. In 65 European patients

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Abbreviations: CD, circular dichroism; **IEC,** ion exchange chromatography; **IMAC,** immobilized metal affinity chromatography; **nsLTP,** nonspecific lipid-transfer protein; **SEC,** size exclusion chromatography

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with a positive double blind placebo controlled food challenge (DBPCFC) natural Cor a 1 was recognized by 100% of the hazelnut allergic patients [5]. Other studies found a prevalence of IgE-binding to recombinant Cor a 1.04 in the range of 98.5% [10] and natural Cor a 1 of 93% [11], respectively. Moreover, IgE reactivity to Cor a 1.04 was detected in 50% (7/14) of hazelnut allergic patients from the US [12] whereas only 1 out of 25 patients with adverse reactions to hazelnuts from the Mediterranean area showed IgE reactivity to this protein. So far, four different variants of the major hazelnut allergen have been cloned and expressed in recombinant form [13] of which the variant Cor a 1.0401 showed the highest IgE reactivity and biological activity. IgE-binding to Cor a 1 was abolished using roasted hazelnuts [11] and after gastrointestinal digestion no basophil activation was induced by Cor a 1.04 [14] indicating lability of the allergen in the gastrointestinal tract.

Cor a 2, the profilin from hazelnuts, is homologous to the birch pollen allergen Bet v 2, and has been cloned and described as a minor food allergen among hazelnut allergic patients (7/17) from Central Europe with concomitant birch pollen allergy [15, 16]. The large extent of crossreactivity among plant profilins is due to their high amino acid sequence identity (77–91%) and similar tertiary structures [17]. Like Cor a 1, the hazelnut profilin is not resistant to thermal [11] and protease treatment [18].

The hazelnut nsLTP has been identified as major allergen in the Mediterranean population and denominated as Cor a 8 [7]. Cor a 8 has a molar mass of 9.5 kDa and an p*I* of 9.3. LTPs are highly stable to protease and thermal treatment, presumably due to four α-helices forming a compact barrel-like structure, which is stabilized by 4 intermolecular disulfide bonds. This high stability is suggested to correlate with more severe and systemic reactions compared with other class II food allergens such as Bet v 1 homologs that are quickly digested in gastrointestinal fluids [19, 20].

In addition, Cor a 9 [12], an 11 S globulin-like seed storage protein, Cor a 11 [21], a 7 S vicilin-like protein and oleosin [22] were recently described as allergens or putative allergens from hazelnuts, but their clinical relevance across Europe is not yet known.

The main aim of this study was to produce the three most relevant hazelnut allergens as recombinant proteins in relatively high amounts (20–30 mg) and in a folded and immunologically active form. For physicochemical and clinical characterization a standard panel of methods was applied as defined in the Europrevall EU-project to provide structural and biochemical information in a standard manner for all the allergens.

2 Materials and methods

2.1 Recombinant Cor a 1 and rCor a 2 (*E. coli*)

2.1.1 Expression and purification of recombinant Cor a 1.04

The plasmid construct pET15b-Cor a 1.04 (GenBank® accession no. for Cor a 1.04: AF136945) [13] was used for expression and transformed in *E. coli* BL21(DE3) Star cells (Invitrogen, Groningen, Netherlands). Heterologous expression was performed in 2 L LB-medium containing 50 mg/L carbenicillin at 37°C using a bioreactor (MoBiTec, Göttingen, Germany). Protein synthesis was induced with 1 mM IPTG (isopropyl β-D-thiogalactoside) for 5 h at 37°C. Bacteria of two fermentations (4 L) were harvested by centrifugation (3000 × *g*, 20 min, 4°C) and stored at −80°C. The pellet was resuspended in 100 mL lysis buffer (50 mM NaH_2PO_4, 500 mM NaCl, and 2 mM imidazole, 6 M urea, pH 8) and subjected to three freeze–thaw cycles using liquid nitrogen. After centrifugation hexahistidine (His_6)-tagged rCor a 1.04 was purified by immobilized metal affinity chromatography (IMAC) and subsequent size exclusion chromatography (SEC). After subjection to IMAC, using Ni^{2+}-charged chelating agarose (Qiagen, Hilden, Germany), protein refolding was performed by stepwise decreasing the urea concentration on the column. Subsequently the target protein was eluted with increasing imidazole concentrations (20 mM–1 M). Fractions containing Cor a 1.04 were analyzed by SDS-PAGE and pooled. The second purification step was performed on a Superdex 75 prep grade 26/60 column (Amersham Biosciences, Uppsala, Sweden) in 20 mM MOPS, 0.5 M NaCl, pH 7.4. In a separate run, ovalbumin (43 kDa) and chymotrypsinogen A (25 kDa) were used as molecular weight standards. The purity of the final preparation was analyzed by SDS-PAGE and Cor a 1.04 containing fractions were concentrated using ultrafiltration units (Vivaspin 15r, MWCO 5 kDa, Sartorius, Göttingen, Germany). The protein content was determined using the BCA assay (Pierce, Cheshire, UK).

2.1.2 Expression and purification of Cor a 2

The plasmid construct of pET30a-Cor a 2 (GenBank® accession no. for Cor a 2: AF327622) [15] – encoding for a fusion protein containing a C-terminal His_6-Tag – was used for expression and transformed in *E. coli* BL21(DE3)-RIL cells (Stratagene, Amsterdam, Netherlands). The expression was performed in 4 L LB-medium containing 30 mg/L kanamycin at 37°C using a bioreactor (MoBiTec). Induction of protein synthesis and purification were performed according to the preparation of Cor a 1.04.

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2.2 Expression of rCor a 8 (*Pichia pastoris*)

2.2.1 Cloning and cDNA sequencing

For expression in *P. pastoris* the cDNA encoding Cor a 8 (GenBank® accession no. for Cor a 8: AF329829) was amplified by PCR (AmpliTaq-DNA polymerase, Applied Biosystems, Darmstadt, Germany) using the pET16b-Cor a 8 plasmid construct [7] as template. A sense primer (5′-CCG CTC GAG *AAA AGA GAG GCT GAA GCT TCC* CTG ACA TGC CCA CAG ATA AAA-3′) containing the *kex2 cleavage site* and an anti-sense primer (5′-GCT CTA GAA ACT TCA CGT TGT TGC AGT TGG TGG-3′) were designed for PCR (*Xho*I and *Xba*I restrictions sites are underlined). The sense primer allows the fusion of the coding region in frame with the sequence encoding the prepro-sequence of the *P. pastoris* alpha factor which is present in plasmid pPICZalphaA (Invitrogen). The alpha factor enables the entry of the target protein in the secretory pathway of yeast and is cleaved by yeast specific Kex2 endonucleases when released into the supernatant. Initially, Cor a 8 was cloned using the antisense primer without a stop codon, encoding for a fusion protein containing a c-myc-Tag and a (His_6)-Tag. In a final construct, a stop codon was inserted by site-directed mutagenesis (Quick-Change Site-Directed Mutagenesis Kit, Stratagene) to obtain the hazelnut LTP as nonfusion protein. The Cor a 8 PCR cDNA product was ligated into pPICZalphaA using *Xho*I and *Xba*I restriction sites. After transformation into TOP10 *E. coli* cells (Invitrogen) selected clones were subjected to DNA sequencing (MWG, Ebersberg, Germany) and the sequence was verified.

2.2.2 Expression and purification of Cor a 8

The plasmid construct pPICZalphaA-Cor a 8 was linearized with *Sac*I and used to transform X-33 *P. pastoris* cells (Invitrogen) by electroporation. Transformed cells were grown on yeast extract peptone dextrose sorbitol medium (YPDS) plates containing 100 µg/mL Zeocin at 30°C for 5–6 days. Positive (Zeocin-resistant clones) transformants were cultivated in 300 mL buffered glycerol-complex medium (BMGY) for 2 days at 30°C in a shaking incubator until the culture reaches an $OD_{600} = 2–3$. The preculture (300 mL) was used to inoculate 3.5 L BMGY in a BioFlow® 110 fermenter (New Brunswick Scientific, NJ, USA). Twenty-four hours fed-batch fermentation with glycerol was performed, followed by a brief carbon starvation and a subsequent switch to methanol as the carbon source. Methanol induced protein expression was continued for 5 days. The culture supernatant was separated from the cell culture by centrifugation (6000 × *g* for 20 min at 4°C) and stored at −20°C. A competitive ELISA was performed to quantify the amount of rCor a 8 in the supernatant of yeast culture. Briefly, purified rCor a 8 from *E. coli*, which was available from former studies, was coated on a 96 well plate (6 ng/mL) and used as standard competing with the rCor a 8 samples from the yeast supernatant for antibody binding. Rabbit-anti-Cor a 8 (provided by J. Lidholm, Phadia, Uppsala, Sweden) was used as primary and goat-anti-rabbit-IgG-HRP as secondary antibody.

For subsequent purification 1 L of supernatant was concentrated to 170 mL by ultrafiltration units (Vivaspin 20, MWCO 3 kDa) and dialyzed against starting buffer (50 mM NaAc/Ac, pH 5). After filtration through a 0.22 µm filter (Sartorius, Göttingen, Germany) the protein solution was applied to a 5 mL Mono S column (Amersham Biosciences). Bound proteins were eluted with a linear salt gradient (50 mM NaAc/Ac, 1 M NaCl, pH 5) at a flow rate of 1 mL/min. The second purification step was performed on a Superdex 75 prep grade 26/60 column (Amersham Biosciences) in 20 mM MOPS, 0.5 M NaCl, pH 7.4. Cor a 8 containing fractions were analyzed by SDS-PAGE and stored at −20°C. The protein content of the purified LTP was determined using the BCA assay (Pierce).

2.3 Patients' sera

To confirm the IgE antibody reactivity of the purified proteins we used two reference sera for each allergen. Preselected sera from hazelnut allergic patients with known IgE-binding profiles were taken from former studies [20, 21]. Patients 1 and 2 were sensitized to Cor a 1.04 (CAP™ $class_{Cor\ a\ 1.04} = 5$; CAP™ $class_{Cor\ a\ 1.04} = 4$), patient 3 was sensitized to all three hazelnut allergens (CAP™ $class_{Cor\ a\ 1.04} = 3$, CAP™ $class_{Cor\ a\ 2} = 3$, CAP™ $class_{Cor\ a\ 8} = 3$), patient 4 was sensitized to Cor a 1.04 (CAP™ $class_{Cor\ a\ 1.04} = 3$) and Cor a 2 (CAP™ $class_{Cor\ a\ 2} = 2$), and patient 5 to Cor a 1.04 (CAP™ $class_{Cor\ a\ 1.04} = 3$) and Cor a 8 (CAP™ $class_{Cor\ a\ 8} = 3$).

2.4 Electrophoresis and immunoblotting

Recombinant Cor a 1.04 and rCor a 2 were separated by SDS-PAGE (17.5%) [23] under reducing conditions. In contrast, rCor a 8 was treated under nonreducing conditions to achieve maximum IgE reactivity. Proteins were electroblotted (400 mA, 50 min) onto nitrocellulose membranes (0.2 µm). The membrane was blocked in Tris-buffered saline/0.3% Tween-20, incubated with 1:10 diluted patients' sera, followed by mouse-antihuman IgE coupled to alkaline phosphatase (1:750; Pharmingen, Hamburg, Germany) as secondary antibody. Bound antibodies were visualized with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as substrate in 0.1 M Tris-buffered saline, pH 9.5 according to the manufacturer's instructions (BioRad, Munich, Germany).

2.5 N-terminal amino acid sequencing

Purified recombinant hazelnut allergens were analyzed on a 492 Procise sequencer (Applied Biosystems, Foster City,

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CA, USA) in pulse-liquid mode by Edman degradation to determine the N-terminal partial sequence.

2.6 MALDI-TOF-MS analysis

Purified rCor a 1.04, rCor a 2, and rCor a 8 were separated by means of SDS-PAGE and stained with CBB for subsequent MS analysis. In gel digestion was performed according to Shevchenko *et al*. [24] with several modifications as published elsewhere (Albrecht *et al.*, *Mol. Nutr. Food Res.* 2008, this issue). The identity of rCor a 1.04, rCor a 2, and rCor a 8 was proven by comparing experimental peptide masses with masses that were obtained by *in silico* digestion of the amino acid sequence of the recombinant allergens. A maximum difference of 30 ppm was allowed for a match between experimental and theoretical data.

2.7 Circular dichroism (CD) spectroscopy

CD spectra of purified recombinant hazelnut allergens were recorded using 15.2 μM Cor a 1.04, 24.1 μM Cor a 2, and 30.4 μM Cor a 8 in 10 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.4) on a Jasco J-810 S spectropolarimeter (Jasco, Gross-Umstadt, Germany) with constant N_2 flushing at 20°C. The measurement was performed in a quartz glass cuvette (1 mm) with a step width of 1 nm and a band width of 1 nm. The spectral range was 180–260 nm at 50 nm/min. Ten scans were accumulated and buffer spectra were subtracted. The results are expressed as mean residue molar ellipticity $[\Theta]_{MRD}$, observed ellipticity θ (mdeg); *N*, number of amino residues; *d*, optical path length (cm); and *c*, protein concentration (mM):

$$[\Theta]_{MRD} = 100 \times \theta\ (\text{mdeg})/N\ (\text{number of amino acid residues}) \times c\ (\text{mM}) \times d\ (\text{cm})$$

2.8 NMR spectroscopy

Protein solutions of rCor a 1.04, rCor a 2, and rCor a 8 were prepared in 0.45 mL H_2O plus 0.05 mL of D_2O. The concentration of rCor a 2 was increased six-fold by centrifugation coupled with a centrifugal filter unit (Centricon®, Millipore). The final concentrations were 0.05 mM for rCor a 1.04, 0.23 mM for rCor a 2, and 0.15 mM for rCor a 8. Proteins were transferred into high-quality NMR tubes with Argon as head space gas. Two high-resolution NMR experiments were carried out, using a Bruker Avance 700 spectrometer operating at a proton resonance frequency of 700 MHz (11.7 Tesla), at 25°C. Both experiments differed in the method to manage the water signal. The pulse program of the zgpr experiment (spectra not reported) included a 1-D sequence with f1 presaturation to minimize the water signal. The zgesgp experiment included a 1-D sequence to have the water suppressed using excitation sculpting with gradients. For each experiment 1024 scans were programmed to analyze rCor a 1.04 and 256 scans for rCor a 2 and rCor a 8. Before and after the NMR experiments, the allergens were stored at −20°C.

3 Results

3.1 Expression and purification of recombinant hazelnut allergens Cor a 1.04 and Cor a 2 in *E. coli*

Plasmid constructs designed for cytoplasmic expression of His_6-tagged recombinant Cor a 1.04 and Cor a 2 were used for the production of each allergen on a scale of 30 mg. A two-step chromatographic procedure was devised for the purification of rCor a 1.04 and rCor a 2 from *E. coli* homogenate. From the soluble fraction after culture harvest and cell disruption the recombinant hazelnut allergens were purified by IMAC. A second step of preparative SEC using a Superdex 75 prep grade high load column was employed to remove high molecular compounds such as oligomers.

In Fig. 1, the elution profile of preparative SEC and the corresponding fractions are shown for the purification of rCor a 1.04 as an example. In addition to a prominent peak representing monomeric rCor a 1.04, small amounts of high molecular compounds are visible during SEC in the chromatogram, probably dimers and trimers of rCor a 1.04. Although the chromatogram showed a single peak corresponding to the 23 kDa target protein, during SDS-PAGE analysis under nonreducing conditions a high proportion of allergen oligomerization (44 and 65 kDa) was observed (exemplified for fraction 15, left) whereas under reducing conditions just a single 23 kDa Cor a 1.04 band appeared. The observations suggested the formation of intermolecular disulfide bridges during sample preparation for SDS-PAGE analysis which does not take place under reducing conditions. Heating seemed to be very relevant for oligomerization as an artifact since this effect was not observed under more physiological conditions of SEC. Only fractions containing Cor a 1.04 monomers (9–20) were pooled and SDS-PAGE analysis of the final product showed a purity of not less than 95% (Fig. 2, lane 1)

In the case of Cor a 2, some oligomers were separated during SEC similar to Cor a 1.04 and a contaminating 22 kDa protein – visible under reducing conditions (data not shown) – coeluted in the first fractions with the target protein using the Superdex 75 column. Therefore, only rCor a 2 containing fractions were pooled on the basis of the apparent separation in a Coomassie-stained gel. The final product showed a single band around 15 kDa and was to more than 95% pure (Fig. 2, lane 2). The yield of the purified rCor a 1.04 was 10–12.5 mg/L of culture of the production strain and 9 mg/L in the case of rCor a 2, the overall yield was 40–50 mg Cor a 1.04 (c = 1 mg/mL) and 36 mg Cor a 2 (c = 850 μg/mL). Expressions of rCor a 1.04 and rCor a 2 in *E. coli* were performed several times resulting in

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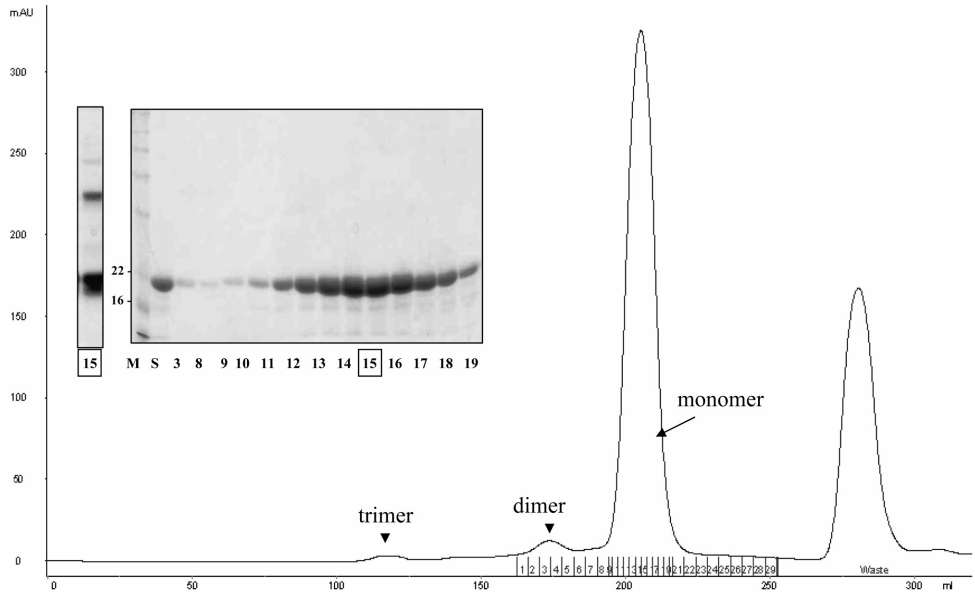

Figure 1. Purification of rCor a 1.04: SEC elution profile of pooled IMAC fractions (lane S, applied sample), recorded at 280 nm and the corresponding fractions analyzed by SDS-PAGE under reducing conditions (Coomassie staining). Fractions 9–20 were pooled. Fraction 15 was shown under nonreducing conditions on the left. Molecular weight standards, ovalbumin (45 kDa) and chymotrypsinogen A (25 kDa) eluted at 166 and 196 mL, respectively (data not shown).

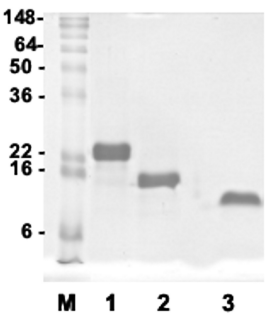

Figure 2. SDS-PAGE analysis of purified recombinant hazelnut allergens Cor a 1.04, Cor a 2, and Cor a 8 (Coomassie staining): lane 1, rCor a 1.04 (5 μg); lane 2, rCor a 2 (5 μg); lane 3, rCor a 8 (10 μg).

slightly different yields whereas the secondary structures (CD spectroscopy) of different batches were similar (data not shown).

3.2 Expression and purification of recombinant Cor a 8 in *P. pastoris*

For the expression of the LTP Cor a 8 a plasmid construct containing a *P. pastoris* pPICZalphaA vector was designed. After transformation into X-33 *P. pastoris* cells and a 5 days long methanol-induction phase, rCor a 8 was secreted as a nonfusion protein at high level of 80 mg/L into the culture medium as determined by a competitive ELISA. After a first step of ion exchange chromatography (IEC) an apparently pure protein product (~70%) free from oligomers was obtained. The second purification step (SEC) was required to separate rCor a 8 from remaining high molecular compounds, probably other yeast proteins having similar p*I*s. Figure 3 shows the elution profile of IEC (Mono S) and the corresponding fractions analyzed by SDS-PAGE under non-reducing conditions. The horizontal arrow indicates fractions (12–15) pooled for further purification by preparative SEC. The yield of pure (≥95%) Cor a 8 (Fig. 2, lane 3) derived from *P. pastoris* was 30 mg/L (37.5%) (c = 1.5 mg/mL). Expression of rCor a 8 was performed several times gaining similar yields and protein batches showed no differences in CD-spectroscopy analysis (data not shown).

3.3 Molecular characterization of recombinant hazelnut allergens

To verify the identity of the individual proteins after the purification process, SDS-PAGE analysis, N-terminal sequencing, and MS were performed. Their secondary and tertiary structure was controlled by CD spectroscopy and NMR analysis. Finally, the immune reactivity of the allergens was checked.

3.3.1 Verification of identity

Final products of rCor a 1.04, rCor a 2, and rCor a 8 were at least 95% pure and showed an apparent molecular mass of

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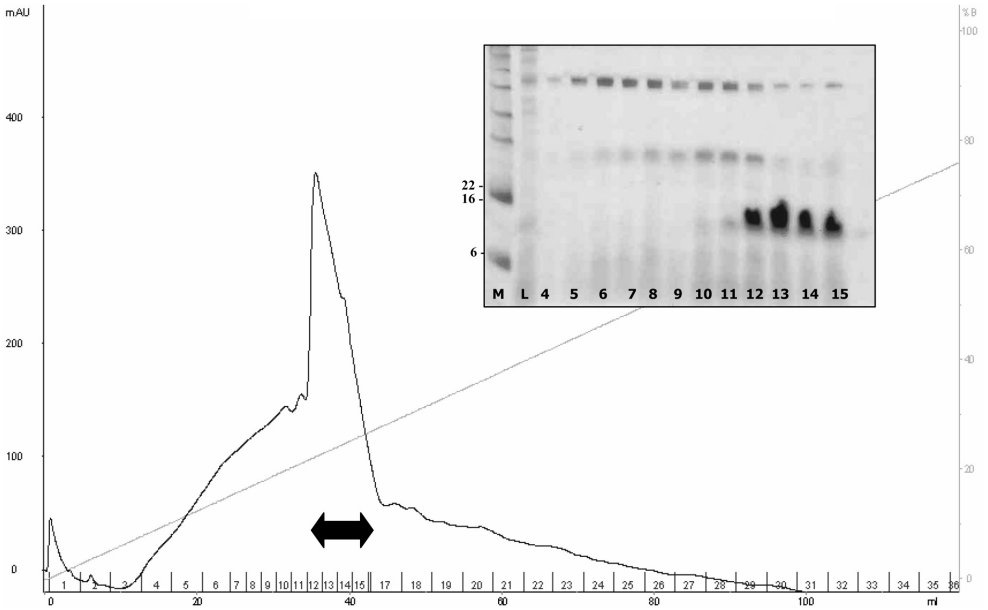

Figure 3. Purification of rCor a 8: IEC elution profile recorded at 280 nm (left *y*-axis) and a linear salt gradient (right *y*-axis) and the corresponding fractions analyzed by SDS-PAGE under nonreducing conditions (Coomassie staining). A horizontal arrow indicates fractions pooled for further purification.

Table 1. Summary of MS data for rCor a 1.04, rCor a 2, and rCor a 8

rCor a 1.04 Peptide position		Peptide position		rCor a 2 Peptide position		rCor a 8 Peptide position	
1–16	+	101–117	+	1–18	+	1–12	+
17–37	+	118–123	–	19–42	–	13–23	+
38–40	–	124–136	+	43–70	–	24–34	–
41–52	–	137–140	–	71–83	+	35–37	–
53–74	+	141–144	–	84–86	–	38–44	–
76–85	+	145–155	+	87–94	–	45–49	–
86–88	–	156–158	–	96–120	–	50–57	–
89–90	–	159–160	–	121–141	+	58–61	–
89–100	+	161–166	–	142–153	+	62–77	+
91–100	–	167–180	+			78–85	–
						86–96	–

Peptides theoretically obtained after trypsin digestion. A + indicates peptides which were detected by MALDI-TOF-MS.

23, 15, and 10 kDa in SDS-PAGE analysis (Fig. 2, lanes 1–3). This result was in agreement with the predicted theoretical masses of the individual proteins, also for rCor a 1.04 and rCor a 2 containing additional amino acids for the corresponding (His_6)-tags. The identity of the proteins was confirmed by amino acid sequencing of the N-terminal part and MALDI-TOF-MS analysis (Table 1). After in-gel tryptic digestion of rCor a 1.04 nine peptides were identified by MS, including the N-terminus with the (His_6)-tag and eight internal peptides (Fig. 4, Table 1). 76% of the expressed amino acid sequence of rCor a 1.04 was covered. In addition five amino acids were also detectable by N-terminal sequencing (GSSHH).

For rCor a 2, the C-terminal peptide inclusive of the (His6)-tag, the N-terminal peptide and two internal peptides were found by MS analysis after tryptic digestion. The amino acid sequence of the N-terminus was additionally confirmed by amino acid sequencing (Fig. 4, Table 1). The sequence coverage was 42%.

Eleven amino acids of the N-terminal part of rCor a 8 were clearly identified by N-terminal sequencing (Fig. 4). The first four amino acids of the sequence derived from the kex2-cleavage site of the alpha factor present in plasmid pPICZalphaA. MALDI-TOF-MS analysis revealed the N-terminal peptide and two internal peptides after tryptic digestion, covering 41% of the amino acid sequence. Post-translational modifications such as *N*-glycosylations were possible in the *P. pastoris* derived material. However, the only peptide of rCor a 8 that contains a potential *N*-glycosylation site was detected in its nonglycosylated form by MS analysis (peptide 13–23, Table 1).

3.3.2 Structural characterization by CD spectroscopy and NMR analysis

The secondary structures of rCor a 1.04, rCor a 2, and rCor a 8 were analyzed by CD spectroscopy. Recombinant Cor a 1.04 showed a broad minimum around 215 nm suggesting that the protein was folded and contained α-helical and β-sheet structures. In contrast, rCor a 8 processed two minima's at 208 and 222 nm representative for a purely α-helical folded protein. Only the hazelnut profilin Cor a 2 seemed to

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rCor a 1.0401 :

```
1    GSSHH HHHHS SGLVPRGSHM GVFCYEDEAT SVIPPARLFK 40
41   SFVLDADNLI PKVAPQHFTS AENLEGNGGP GTIKKITFAE 80
81   GNEFKYMKHK VEEIDHANFK YCYSIIEGGP LGHTLEKISY 120
121  EIKMAAAPHG GGSILKITSK YHTKGNASIN EEEIKAGKEK 160
161  AAGLFKAVEA YLLAHPDAYC
```

rCor a 2:

```
1    SWQTYGDEHL MCEIEGNRLA AAAIIGHDGS VWAQSSTFPQ 40
41   LKPEEITGVM NDFNEPGSLA PTGLYLGGTK YMVIQGEPGA 80
81   VIRGKKGPGG VTVKKTSQAL IIGIYDEPMT PGQCNMIVER 120
121  LGDYLIDQG LYADPNSSSVD KLAAALEHHH HHH
```

rCor a 8:

```
1    EAEASLTCPQ IKGNLTPCVL YLKNGGVLPP SCCKGVRAVN 40
41   DASRTTSDRQ SACNCLKDTA KGIAGLNPNL AAGLPGKCGV 80
81   NIPYKISPST NCNNVK
```

Figure 4. Amino acid sequences of rCor a 1.04, rCor a 2, and rCor a 8: peptides which were detected by MALDI-TOF-MS are underlined. Amino acids printed in bold letters have been confirmed by N-terminal sequencing, italics indicate trypsin cleavage sites.

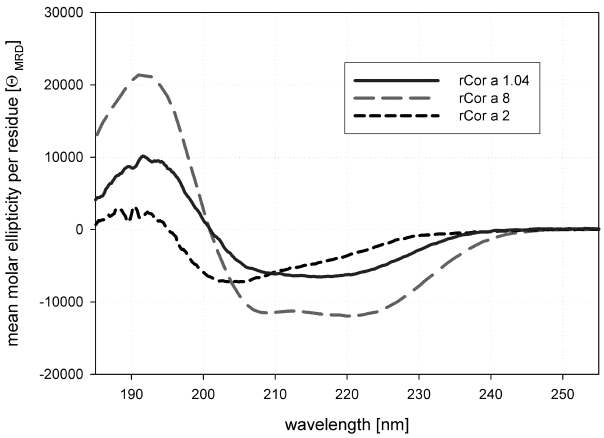

Figure 5. CD spectroscopy of rCor a 1.04, Cor a 2, and rCor a 8.

contain unfolded or "random coil" material, as suggested by a negative mean residue molar ellipticity $[\Theta]_{MRD}$ lower than 200 nm wavelength in the CD spectrum (Fig. 5).

The NMR spectra of rCor a 1.04 (Fig. 6a) showed many peaks within the whole range from −0.5 to 10 ppm. All peaks were well resolved either within the amide/aromatic zone (6–11 ppm) or within the aliphatic/alpha/beta zone (1–6 ppm). Moreover, dispersed signals of H-*alpha* around the water signal (4.5–5 ppm) were apparent and so are the dispersed methyl signals around 0 ppm. Spectra of this kind summarize all the characteristics of a well-defined tertiary structure and therefore the spectrum indicated that the prepared protein was folded. The spectra of rCor a 2 (Fig. 6b) and Cor a 8 (Fig. 6c) share the same characteristics but to a lesser extent. This was evident from either watching the spectra of each allergen separately, or visually comparing the spectra of the three proteins plotted together and properly scaled. The intervals from −0.6 to 1.1 ppm (Fig. 6d) and from 5 to 10 ppm (Fig. 6e) are especially informative. The spectra of the rCor a 2 and rCor a 8 preparations were slightly less extended on the side of the lowest frequencies (0 and negative ppm values). Nevertheless they show many properly resolved peaks. The same was observed in the amide/aromatic zone. For these reasons the presence of tertiary structure can be inferred for all the samples discussed here even if, eventually, to a different extent. The spectra of rCor a 2 are characterized by poor dispersion between 4.5 and 6.5 ppm and by fewer, broader peaks. This could be due to the absence of tertiary structure in one or more parts of the molecule in solution.

3.3.3 Immune reactivity

Immunoblot analyses were performed to examine the IgE antibody reactivity to the purified recombinant hazelnut allergens using preselected sera from hazelnut allergic patients with known IgE-binding profiles which were taken from former studies. In Fig. 7, immune reactivity to rCor a 1.04, rCor a 2, and rCor a 8 is obvious using sera from patients 1 to 5. The sensitization to the three different hazelnut allergens – known by ImmunoCAP analysis (see Section 2) – was confirmed by immunoblot analysis.

4 Discussion

The aim of the present study was the development of efficient methods for the production and purification of the most relevant hazelnut allergens on a 30 mg scale. Furthermore, it was planned to produce the recombinant allergens preferably in a folded and in a biologically active form. A structural comparison of the natural counterparts is advisable but the amount of purified allergens from natural food sources is often insufficient for structure analysis.

Recombinant Cor a 1.04 and rCor a 2 were produced in *E. coli* using the pET expression systems. Both allergens were expressed as Histidine-tag fusion proteins that allowed the simplified purification by IMAC. The purifications were performed under denaturing conditions from inclusion bodies to enhance the yield. Initial experiments using N-terminal or C-terminal tagged hazelnut allergens (rCor a 1.04, rCor a 2) showed no difference in IgE antibody reactivity in the CAP system (own unpublished data). After IMAC a second step of preparative SEC was employed to remove high molecular compounds such as aggregates.

Under these conditions the amount of Cor a 1.04 was upscaled to 10–12.5 mg/L. Small amounts of dimers and trimers of rCor a 1.04 visible in the chromatogram were separated by SEC to obtain pure monomeric Cor a 1.04. The molecular mass of purified rCor a 1.04 which was apparent as 23 kDa band in SDS-PAGE analysis was in agreement with the predicted theoretical mass of the protein containing additional amino acids for the N-terminal (His_6)-tag. Furthermore, Cor a 1.04 was identified by N-ter-

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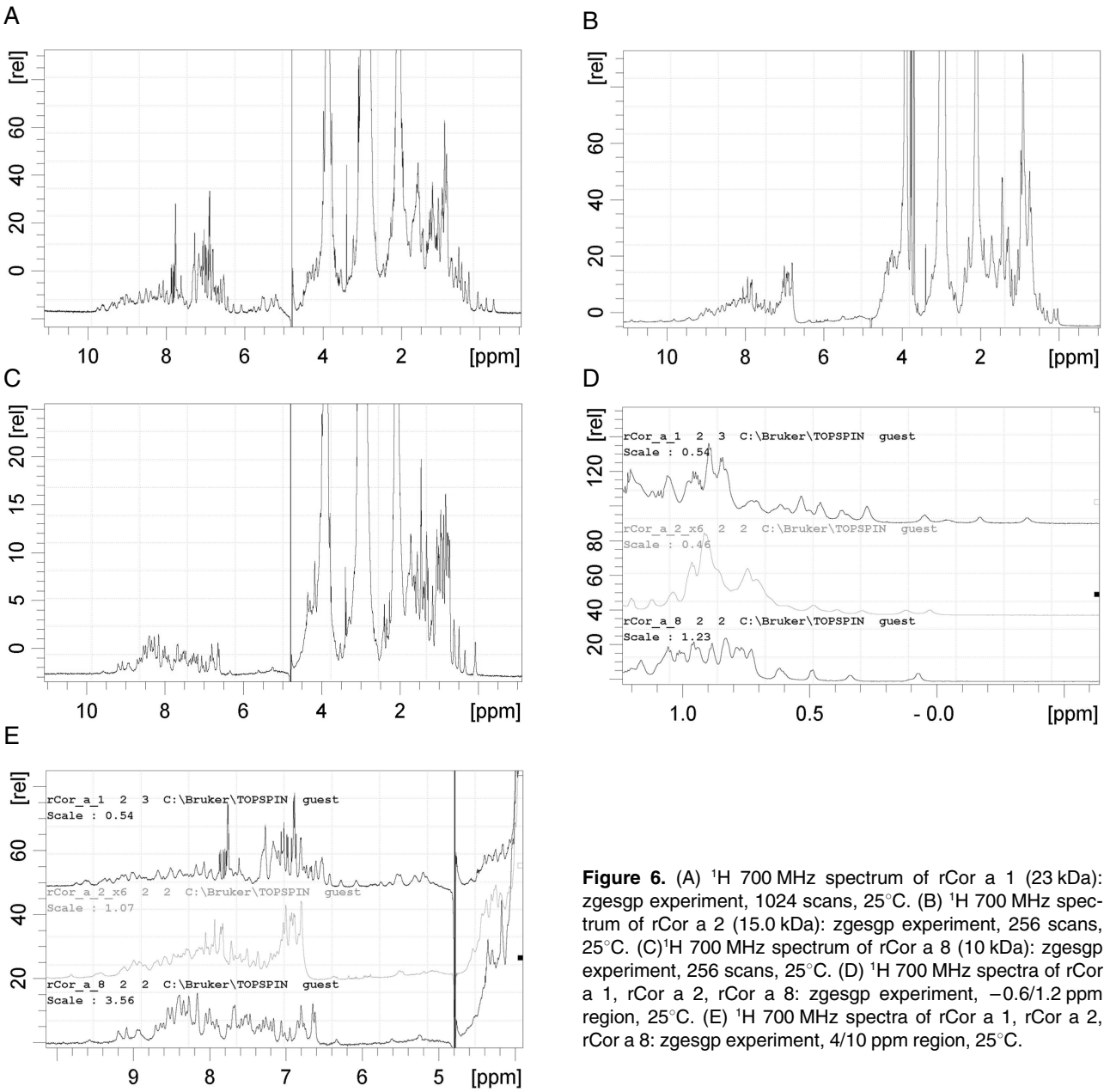

Figure 6. (A) ^{1}H 700 MHz spectrum of rCor a 1 (23 kDa): zgesgp experiment, 1024 scans, 25°C. (B) ^{1}H 700 MHz spectrum of rCor a 2 (15.0 kDa): zgesgp experiment, 256 scans, 25°C. (C)^{1}H 700 MHz spectrum of rCor a 8 (10 kDa): zgesgp experiment, 256 scans, 25°C. (D) ^{1}H 700 MHz spectra of rCor a 1, rCor a 2, rCor a 8: zgesgp experiment, −0.6/1.2 ppm region, 25°C. (E) ^{1}H 700 MHz spectra of rCor a 1, rCor a 2, rCor a 8: zgesgp experiment, 4/10 ppm region, 25°C.

minal sequencing and by peptide mass fingerprinting (sequence coverage: 76%). CD-spectroscopy and NMR-analysis showed that rCor a 1.04 adopted secondary and tertiary structures. CD-spectra revealed a protein with α-helical and β-sheet structures. The NMR spectra of Cor a 1.04 offer all attributes of a well defined tertiary structure. Finally, IgE-binding capacity was tested by immunoblotting using selected reference sera and demonstrated IgE antibody reactivity after the purification process. As IgE reactivity of Cor a 1.04 is due to conformational epitopes [14, 25, 26] we conclude that our purification strategy yielded in a folded and a biologically active protein, even though denaturing conditions were applied to obtain soluble material and in the first purification step.

In previous studies, approximately 6 mg hazelnut profilin Cor a 2 nonfusion protein had been purified from cell lysate obtained under native conditions from one liter culture by poly(L-proline) affinity chromatography (PLP) [16]. The plasmid construct of pET30a-Cor a 2 encoding for a fusion protein containing a C-terminal His_6-Tag allowed a simplified purification via IMAC and led to a slightly enhanced yield of purified Cor a 2 (9 mg/L) using denaturing conditions. To obtain monomeric rCor a 2, oligomers were separated by preparative SEC as final purification step. The molecular mass of the monomeric form apparent as single 15 kDa band in Coomassie-stained SDS-gel was consistent with the theoretical molecular weight of the fusion protein rCor a 2. The identification on amino

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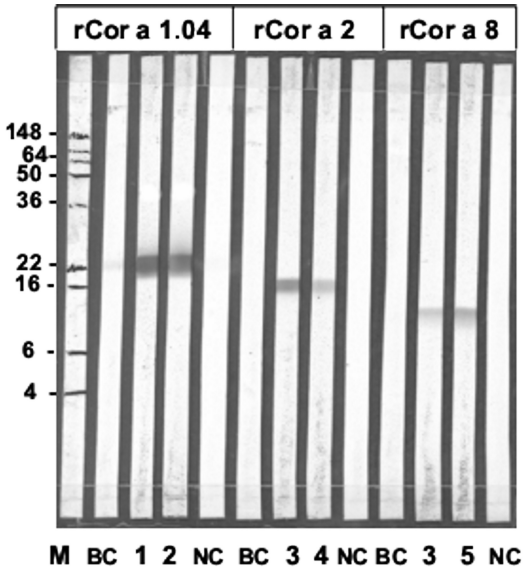

Figure 7. Immunoblotting of purified allergens: rCor a 1.04 (1 μg/cm), rCor a 2 (1 μg/cm), rCor a 8 (3 μg/cm) using different sera (BC, buffer control; NC, nonallergic patients; 1 – 5, hazelnut allergic patients used as reference sera).

acid basis with N-terminal sequencing and MALDI-TOF analysis (coverage rate: 42%) was successful. Secondary structure characterization by CD-spectroscopy of Cor a 2 suggested the presence of a portion of unfolded or "random coil" material in the final preparation. Furthermore, the NMR data indicated the absence of tertiary structure in one or more parts of the Cor a 2 molecule in solution suggesting that the refolding of the protein after removal of urea was not complete in this batch. In contrast, adopting the same strategy on the production of the celery profilin Api g 4 (Bublin, M. *et al., Mol. Nutr. Food Res.* 2008, DOI: 10.1002/mnfr.200700270, this issue) using identical conditions, no unfolded Api g 4 was observed in CD-spectroscopy analysis. Even within the same protein family, there were differences in protein refolding during the production process. Nevertheless, the final product showed IgE antibody reactivity in immunoblot analysis suggesting that the main portion of produced Cor a 2 was present in a biologically active form.

For the preparation of Cor a 8, the nsLTP of hazelnuts, an eukaryotic expression system such as *P. pastoris* was established. It was known from the literature that the expression of Cor a 8 (pET16b-Cor a 8) as hexahistidine-tagged fusion protein in *E. coli* and purification under native conditions led to low yields (1.5 mg/L) [7]. Furthermore, the correct formation of disulfide bonds is problematic in *E. coli* due to the reducing conditions of bacterial cytoplasma whereas the correct formation of disulfide bonds is favored in *P. pastoris* [27]. The compact 3-D structure defined by four specific disulfide bonds [28] is essential for the stability and IgE reactivity of the LTP family. Therefore, Cor a 8 was cloned and expressed as nonfusion protein using the yeast expression system (pPICZαA-Cor a 8), secreted at high level of 80 mg/L into the culture medium and purified by two step FPLC method (IEC, SEC) under native conditions. Eleven amino acids of the N-terminus of the final Cor a 8 preparation were identified by N-terminal sequencing whereas 41% of the amino acid sequence was covered by peptide mass fingerprinting. The single potential *N*-glycosylation site of rCor a 8 was unoccupied even though glycosylation is possible using the *P. pastoris* expression system. Post-translational glycosylation in plants was described neither for the nsLTP family including natural Cor a 8 so far. However MS-analysis of natural Pru p 3, the LTP from peach, did not reveal any glycosylation of the protein (unpublished data). The secondary structure of rCor a 8 processed two minima's at 208 and 222 nm representative for a pure α-helical folded protein. Moreover, NMR analysis showed that rCor a 8 adopted tertiary structures. Finally, the demonstrated IgE antibody reactivity of rCor a 8 is a further indication of the existence of a correct 3-D structure defined by four specific disulfide bonds. The recovery yield of pure folded and biologically active Cor a 8 derived from *P. pastoris* was 30 mg/L after the purification process.

In conclusion, hazelnuts (*C. avellana*) are a relevant food source and one of the most frequent causes of food allergy in Europe. Three relevant hazelnut allergens Cor a 1.04, Cor a 2, and Cor a 8 with different clinical relevance and crossreactive properties were produced as recombinant proteins in large amounts. Decisions on the expression and purification of allergens on a larger scale should be made on a case-by-case basis: preparation of rCor a 1.04 and rCor a 2 was performed as fusion proteins in *E. coli* from inclusion bodies whereas rCor a 8 was expressed in yeast and purified as nonfusion protein by common chromatographic methods.

Engineered allergens represent a pure and fully standardized allergenic source material which will improve diagnostic *in vitro* tests for hazelnut allergy, by allowing a highly sensitive component resolved diagnosis. Furthermore, pure allergens will be used for studying the impact of food processing, the food matrices, and digestion on their allergenicity under controlled conditions.

In the EuroPrevall project the patterns and prevalence of food allergy across Europe in infants, children, and adults will be studied. In collaboration with clinical partners the recombinant allergens described in this study will be used to determine sensitization profiles in hazelnut allergy in Europe. These profiles will then be correlated to different patterns of clinical reactivity. Furthermore, the potential of biological *in vitro* tests such as histamine release tests or basophil activation tests including assays performed with permanently growing cell lines will be evaluated using purified allergen reagents. Since a standard panel of methods was applied for physicochemical and clinical characterization of the allergens the same quality of material can be pro-

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duced repeatedly as the method can be used for confirming batch-to-batch consistency. This will allow laboratory scientists deeper insights in the quality of allergen material used and enhance the comparability of data obtained from different studies and in different laboratories.

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