

Original article

Enhancement of hazelnut extract for IgE testing by recombinant allergen spiking

Background: Hazelnuts are a common cause of food allergic reactions. Most hazelnut allergic individuals in central and northern Europe are sensitized to Cor a 1, a member of the PR-10 protein family, while the lipid transfer protein Cor a 8 acts as a major allergen in the south of Europe. Other allergens, including profilin and seed storage proteins, may be important in subgroups of patients. Reliable detection of specific IgE in the clinical diagnosis of food allergy requires allergen reagents with a sufficient representation of all relevant allergen components. Some reported observations suggest that natural hazelnut extract may not be fully adequate in this respect.

Methods: The capacity of immobilized natural hazelnut extract to bind Cor a 1-, Cor a 2- and Cor a 8-specific IgE and IgG antibodies was investigated by serum adsorption and extract dilution experiments and by the use of allergen specific rabbit antisera. All measurements were performed with the ImmunoCAP assay platform.

Results: The experimental results revealed an incomplete capacity of immobilized hazelnut extract to capture IgE antibodies directed to the major allergen Cor a 1. Spiking of hazelnut extract with recombinant Cor a 1.04 prior to solid phase coupling gave rise to significantly enhanced IgE antibody binding from Cor a 1 reactive sera. The spiking did not negatively affect the measurement of IgE to extract components other than Cor a 1.

Conclusion: A hazelnut allergen reagent with enhanced IgE detection capacity can be generated by supplementing the natural food extract with recombinant Cor a 1.04.

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Hazelnuts are one of the most common causes of food allergic reactions in Europe. Symptoms upon ingestion are most often restricted to the oral cavity but may also be systemic and in rare cases life-threatening or even fatal (1, 2).

Several hazelnut allergens have been identified and characterized (3). Hazelnut allergic individuals from northern and central Europe are almost invariably sensitized to Cor a 1, a 17 kDa member of the PR-10 protein family of allergens related to the major birch pollen allergen Bet v 1 (4–8). In contrast, hazelnut allergic individuals from the Mediterranean region predominantly react to Cor a 8, a 9 kDa nonspecific lipid transfer protein related to peach and cherry allergens Pru p 3 and Pru av 3, respectively, and rarely to Cor a 1 (7). Profilin, designated Cor a 2 in hazelnut (4), appears to be of minor importance in hazelnut allergy regardless of geographical region (7, 9, 10).

Further hazelnut allergens reported include different seed storage proteins: a 2S albumin (7), the glycinin-like protein Cor a 9 (1) and the vicilin-like protein Cor a 11

(11). Reactivity to these allergens would arise independently of pollen sensitization and may be important in subgroups of patients but their clinical importance in wider populations of hazelnut allergic subjects remains to be established. The same is true for oleosin, an oil body associated protein which was recently reported as a hazelnut allergen (12).

Sensitization to Cor a 1 is believed to be caused mainly by cross-reaction to Bet v 1 following birch pollen sensitization. In this type of hazelnut allergy, symptoms upon ingestion are usually mild and restricted to the lips and oral cavity, referred to as the oral allergy syndrome (2, 7, 13–16). Local oral reactions to hazelnut are also common in individuals from the Mediterranean area, where Cor a 8 is a main culprit allergen, but individuals from this region more frequently also experience severe reactions to hazelnut, involving distal organs (7, 17, 18). Possible reasons for this asymmetry in reaction pattern include dose and stability of the allergen to which the patient reacts, affinity of IgE antibody binding to the food allergen, influence of pollen sensitization pattern and dietary habits.

Assessment of sensitization by allergen-specific IgE testing or skin prick testing (SPT) is a primary tool in routine clinical diagnosis of food allergy. To reliably detect sensitization, it is critical that the allergen reagent applied contains an adequate amount of all relevant allergen components. However, preparation of food allergen extracts with high and consistent quality is a difficult task. Several studies have shown great variation in allergen content between different manufacturers' extracts and, in some cases, severe shortage or lack of important components (19–25) which is likely to affect the diagnostic outcome. For hazelnut, several clinical studies have been reported and given quite different results regarding diagnostic sensitivity of testing with natural hazelnut extract (2, 26–28). While acceptable sensitivity was observed in some studies, a significant lack in sensitivity in other studies prompted us to undertake the work reported here.

In this study, we investigated the capacity of immobilized natural hazelnut extract to bind antibodies specific for Cor a 1, Cor a 2 and Cor a 8, and explored the possibility of enhancing its diagnostic sensitivity by supplementing the extract with recombinant Cor a 1.04.

Materials and methods

Hazelnut allergens and extract

rCor a 1.0401 (5), rCor a 2 (9) and rCor a 8 (18) were expressed as hexahistidine tagged proteins in *Escherichia coli* strain BL21 as described (29). The proteins were purified by immobilized metal ion affinity chromatography, followed by hydrophobic interaction chromatography on Phenyl Sepharose HP (rCor a 1.04) or size exclusion chromatography on Superdex 75 p.g. (rCor a 2 and rCor a 8) (GE Healthcare Life Sciences, Uppsala, Sweden). Natural hazelnut extract was prepared from ground and defatted hazelnuts (Allergon, Vålinge, Sweden), extracted in 0.1 M Na-phosphate pH 7.5 at a weight to volume ratio of 1 : 20. Following centrifugation, the extract was passed through a sandwich of 5 + 00H + 5 paper filters (Munktell Filter AB, Grycksbo, Sweden).

Human sera

For experiments with a technical purpose, hazelnut positive sera from an in-house collection were selected, based on their specific IgE reactivity pattern to rCor a 1.04, rCor a 2 and rCor a 8. For evaluation of clinical sensitivity, sera of hazelnut allergic subjects from Germany and Switzerland ($n = 50$) and Spain ($n = 10$) were used. Allergy to hazelnut in those subjects, who were included under ethical approval and informed consent in two previously reported studies (5, 18), was diagnosed on the basis of a convincing case history, positive SPT and positive open challenge or DBPCFC with raw hazelnuts. For negative control purposes, a pool of sera from nonatopic donors was used, spiked with 0, 1000 or 3000 kU/L of myeloma IgE.

IgE adsorption experiments

The capacity of natural hazelnut extract to bind Cor a 1-, Cor a 2- and Cor a 8-specific IgE antibodies was assessed by analysing

reduction of specific IgE concentrations in serum samples following adsorption to a detached assay solid phase carrying immobilized extract. The adsorption was performed by incubating 50 µl of serum with the solid phase for 40 min at room temperature and the sample was then recovered for analysis by centrifugation into a microcentrifuge tube. To assess the efficiency of the specific IgE adsorption procedure and obtain reference measurements for evaluation, serum samples were incubated in parallel with solid phases carrying pure rCor a 1.04, rCor a 2 and rCor a 8 at saturating density.

IgE inhibition experiments

The capacity of natural hazelnut extract to inhibit allergen-specific IgE antibody binding was assessed by measuring specific IgE to immobilized rCor a 1.04, rCor a 2 and rCor a 8 in pools of sera, with predominant reactivity to either allergen, preincubated with a dilution series of the extract. In parallel, the same serum pools were preincubated with soluble rCor a 1.04, rCor a 2 and rCor a 8 prior to IgE measurement.

Rabbit antisera

Polyclonal antisera against Cor a 1, Cor a 2 and Cor a 8 were raised at Agrisera AB (Vännäs, Sweden) by immunizing rabbits with the purified recombinant allergens. Specific antibody titres in serum samples were assessed by an experimental ImmunoCAP rabbit IgG assay. For an assessment of antibody binding to hazelnut extract and pure hazelnut allergens, the antisera were diluted 10^2 to 10^5 -fold to achieve signals within the linear part of the assay's response curve.

Allergen specific antibody measurements

Measurements of specific IgE binding were performed using the ImmunoCAP assay platform (Phadia, Uppsala, Sweden). Hazelnut allergen sensitization profiles of the human sera used were established using experimental ImmunoCAP tests incorporating purified rCor a 1.04, rCor a 2 and rCor a 8. Specific rabbit IgG antibody binding was analysed using an experimental ImmunoCAP rabbit IgG assay. Experimental ImmunoCAP tests were prepared as described (29).

Results

Allergen-specific antibody binding by immobilized hazelnut extract

The capacity of immobilized natural hazelnut extract to bind Cor a 1-, Cor a 2- and Cor a 8-specific antibodies was investigated by three complementary approaches. First, the ability of immobilized extract to deplete specific IgE antibodies from serum samples was assessed in a series of adsorption experiments. Nine human sera with known predominant IgE reactivity to Cor a 1, Cor a 2 and Cor a 8 were used, three for each of these allergens. Following incubation with the solid phase adsorbant carrying the immobilized extract, the remaining content of IgE reactive to rCor a 1.04, rCor a 2 or rCor a 8, respectively, in the effluent serum was measured and the data compared with the preadsorption concentrations.

The results of the adsorption experiments are shown in Fig. 1. In the cases of Cor a 1 and Cor a 2, the

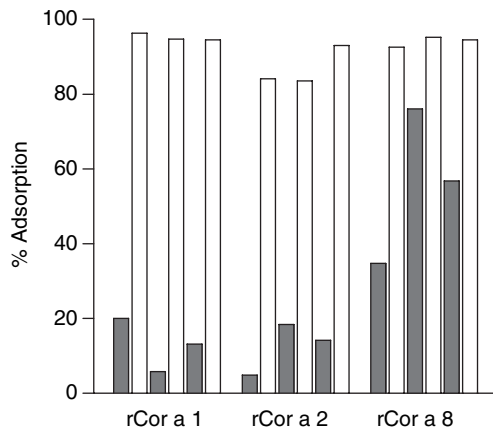


Figure 1. Adsorption of specific IgE antibodies to natural hazelnut extract (filled bars) and pure recombinant hazelnut allergen (open bars). For each of the three hazelnut allergens, three sera selected for predominant IgE reactivity were used. Specific IgE concentrations were measured before and after incubation with immobilized allergen and the adsorption results are expressed as percentage reduction.

measurements revealed that 80–95% of the specifically reactive IgE antibodies remained in the serum effluent, thus only 5–20% had been removed by the adsorption. In comparison, adsorption to pure rCor a 1.04 or rCor a 2 removed 83–96% of the specific IgE. For Cor a 8, 35–76% of the specific IgE was adsorbed by the hazelnut extract while adsorption to pure rCor a 8 allergen removed 93–95%.

In a second approach, the relative abundance of individual hazelnut allergens was assessed by measuring binding of Cor a 1-, Cor a 2- and Cor a 8-specific rabbit IgG antibodies to immobilized hazelnut extract. To eliminate the impact of differences in antibody titre, the results were normalized against measurements of binding to the corresponding recombinant hazelnut allergens, immobilized at saturating coupling density (Fig. 2). For

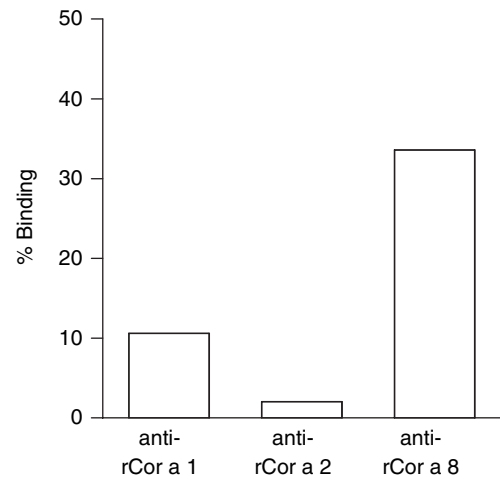


Figure 2. Binding of allergen-specific polyclonal rabbit IgG antibodies to natural hazelnut extract. Results are expressed as per cent of binding to tests carrying a saturating amount of purified rCor a 1.04, rCor a 2 and rCor a 8, respectively.

all three antisera, the binding was significantly higher to the pure allergen than to the natural hazelnut extract. The greatest difference, almost 50-fold, was found for the anti-Cor a 2 antiserum, followed by the anti-Cor a 1 antiserum which differed in binding by almost 10-fold. For Cor a 8, the rabbit antibody binding to hazelnut extract was about one-third of that to pure rCor a 8.

In a third approach, loss of IgE binding from selected human sera was monitored as a function of serial dilution of hazelnut extract prior to immobilization on the assay solid phase (Fig. 3). For four sera tested that were virtually monosensitized to Cor a 1, a 100-fold extract dilution caused the IgE binding to drop by up to 50%. At a 500-fold extract dilution, 84% and 93% of the IgE binding was lost for the two sera tested at this dilution, as compared with undiluted extract. Of six Cor a 2-reactive

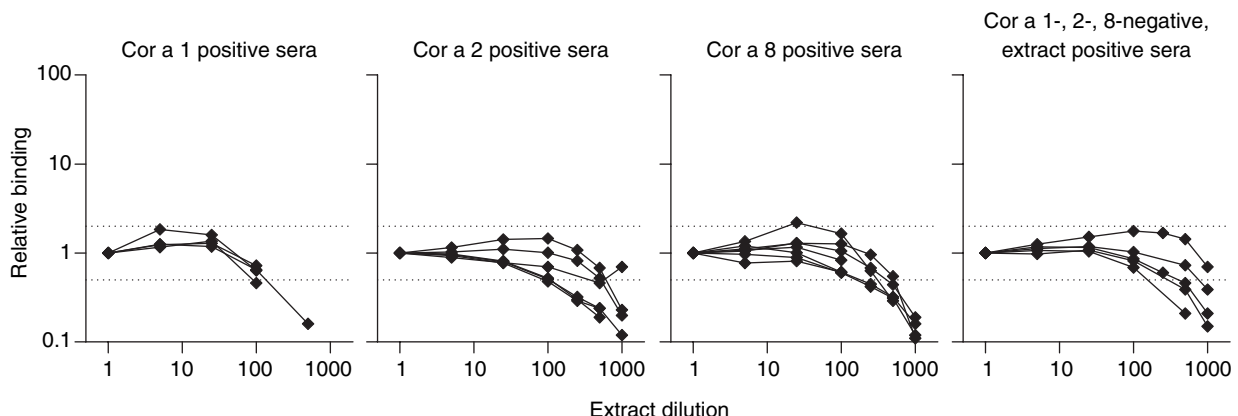


Figure 3. Differential loss of allergen activity upon extract dilution. Sera with predominant IgE reactivity to rCor a 1.04, rCor a 2 and rCor a 8 were used, as well as sera reactive to hazelnut components other than those three allergens. Results are expressed as relative IgE antibody binding, with binding to undiluted extract set to unity. Dotted lines indicate a 0.5 to 2-fold change in relative binding.

sera tested, all retained at least 50% IgE binding to 100-fold diluted extract and three retained approximately 50% binding at 500-fold dilution. The highest relative IgE binding capacity after extract dilution was observed for Cor a 8. At 100-fold dilution, all of seven sera tested retained at least 60% of the IgE binding and at 500-fold dilution, all of five sera tested retained at least 30% binding. Of five sera that were reactive to hazelnut extract but negative to rCor a 1.04, rCor a 2 and rCor a 8, all retained at least 70% IgE binding to 100-fold diluted extract and four retained at least 40% to a 500-fold dilution.

While the experiments described above were designed to determine specific allergen activity of immobilized natural extract, an attempt was also made to assess the concentration of those allergens in liquid extract, prior to immobilization. An IgE inhibition experiment was therefore performed, where the ability of the extract to prevent IgE binding to immobilized rCor a 1.04, rCor a 2 and rCor a 8 was studied, in relation to inhibition by each recombinant allergen itself. From the results with serial dilutions of inhibitor, the concentration of extract and pure allergen required to cause 50% inhibition was determined, and the concentrations of recombinant allergen equivalents in the natural extract were calculated. The concentrations of rCor a 1.04, rCor a 2 and rCor a 8 equivalents in undiluted extract, as determined by this procedure, were 12, 0.2 and 1 $\mu\text{g/mL}$, respectively.

Addition of rCor a 1.04 to natural hazelnut extract

Drawing from the finding of low Cor a 1 activity in immobilized hazelnut extract, experiments were performed to investigate whether this shortage could be functionally counteracted by simply adding recombinant Cor a 1.04 to the extract prior to immobilization onto the assay solid phase. Natural hazelnut extract was therefore supplemented with increasing amounts of rCor a 1.04 and changes in IgE binding capacity were measured using sera with known reactivity profiles (Fig. 4). The spiking was found to significantly enhance the IgE antibody binding from Cor a 1 reactive sera in a dose-related manner (Fig. 4a), without affecting IgE binding from hazelnut reactive sera that were Cor a 1 negative (Fig. 4b).

However, upon comparison to measurements with pure rCor a 1.04, it appeared that the spiked hazelnut extract did not suffice to bind all Cor a 1-reactive IgE antibodies, even at the highest level of rCor a 1.04 spiking (Fig. 4a). It was assumed that this might be as a result of competition for solid phase attachment sites by abundant endogenous components present in the extract. To examine this possibility, a dilution series of the hazelnut extract was prepared, to which the recombinant allergen was added prior to solid phase coupling. Analysis of IgE binding to tests prepared with the different extract dilutions revealed a significant further increase in IgE binding from Cor a 1.04 positive sera (Fig. 5a). Through the dilution series, no or

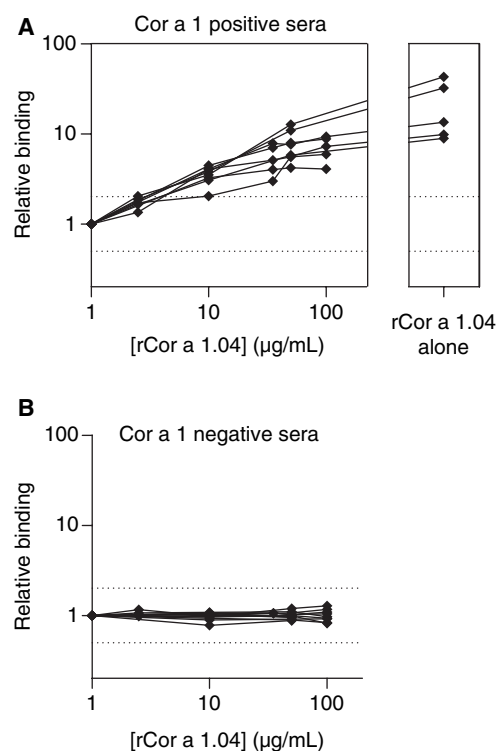


Figure 4. Effect on specific IgE antibody binding of rCor a 1.04 addition to hazelnut extract. (A) rCor a 1.04 positive sera, (B) rCor a 1.04 negative sera. Results are expressed as relative IgE antibody binding, with binding to unmodified extract set to unity. Relative binding to rCor a 1.04 alone is shown in the narrow graph. Dotted lines indicate a 0.5 to 2-fold change in relative binding.

minimal loss of IgE binding from Cor a 2- and Cor a 8-positive sera occurred (Fig. 5b). Similarly, a 100-fold dilution of the extract did not significantly affect the binding of IgE to extract components other than Cor a 1, Cor a 2 and Cor a 8 (Fig. 5b).

Evaluation of an improved reagent for the detection of hazelnut sensitization

Based on the investigations described above, an enhanced hazelnut allergen reagent was prepared by diluting the extract 25 times and adding rCor a 1.04 to a concentration of 20 $\mu\text{g/mL}$ prior to solid phase coupling. Binding of specific IgE to the new reagent was evaluated in comparison to unmodified extract using sera from 60 hazelnut allergic individuals (Fig. 6a) from Germany, Switzerland and Spain. Detectable specific IgE antibodies to rCor a 1, rCor a 2 and rCor a 8 were present in 96%, 36% and 10% of the central European sera ($n=50$) and in 0%, 0% and 80% of the Spanish sera ($n=10$) used. Four sera showed IgE binding to hazelnut extract which could not be accounted for by any of the three recombinant allergens tested.

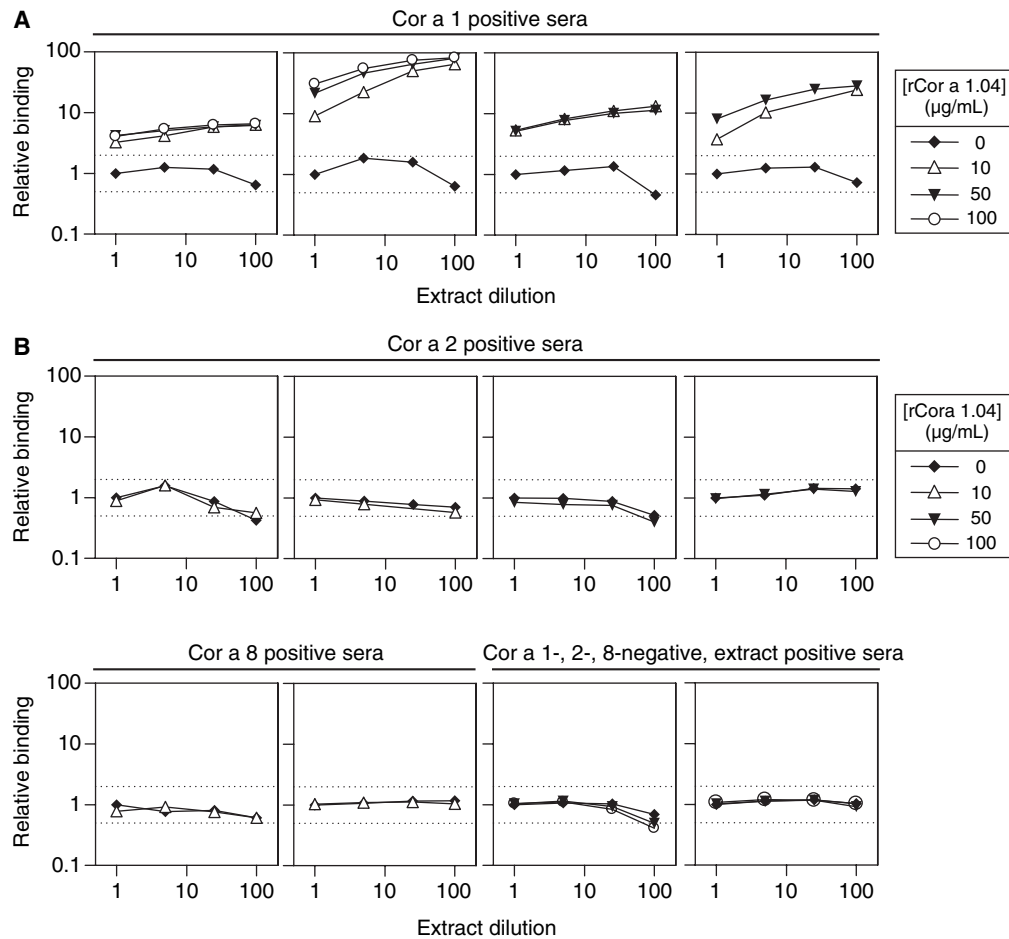


Figure 5. Effect of extract dilution on specific IgE antibody binding to extract supplemented with different amounts of rCor a 1.04. (A) Sera with predominant IgE reactivity to rCor a 1.04. (B) rCor a 1.04-negative sera with predominant reactivity to rCor a 2 or rCor a 8, or sera reactive to hazelnut components other than rCor a 1.04, rCor a 2 and rCor a 8. Results are expressed as relative IgE antibody binding, with binding to unmodified extract set to unity.

For almost all of the central European subjects, a dramatic increase in IgE binding to the modified extract was observed. The median increase of IgE binding was 6.8-fold in this population and a positive test result was obtained for all of eight sera which tested negative with the unmodified extract. Comparison of measurements with the modified extract and rCor a 1.04 alone (Fig. 6b) suggested a nearly quantitative binding of Cor a 1 reactive antibodies by the modified extract. For the Spanish subjects, who were all Cor a 1 negative (Fig. 6b), very similar IgE measurements were obtained with the modified and unmodified extract reagents (Fig. 6a), demonstrating that no loss of allergen activity relevant to these sera had occurred in the modified extract. In a control experiment for unspecific binding of IgE or detection conjugate, a pool of sera from nonatopic donors, spiked with up to 3000 kU/L of myeloma IgE, was tested on the modified hazelnut reagent (Fig. 6c). Even at the highest concentration of myeloma IgE, a response equivalent to only 0.06 kU/L was obtained, demonstrating an excellent assay specificity of the new reagent.

Discussion

Clinical diagnosis of food allergy relies in part on allergen extracts which allow reliable assessment of sensitization. However, the composition of natural allergen extracts is affected by a multitude of factors that are difficult to control, including differences and variation in abundance of individual allergens in the raw material, extraction efficiency and postextraction stability and susceptibility to protein modification activities. Such factors are known to affect the quality of certain food extracts, particularly those prepared from foods comprising fully hydrated and metabolically active tissues, passing through natural stages of maturation and ripening (30, 31).

In the case of hazelnut, questions have been raised regarding the content of Cor a 1 in commercially available extracts used for SPT (19, 20). Further, only 12 of 31 (39%) Dutch hazelnut allergic patients were reported to test positive with hazelnut ImmunoCAP (28), even if this in part was because of the use of a cut-off level of 0.7 kU_A/L. Eighteen of the 31 (58%) patients in that

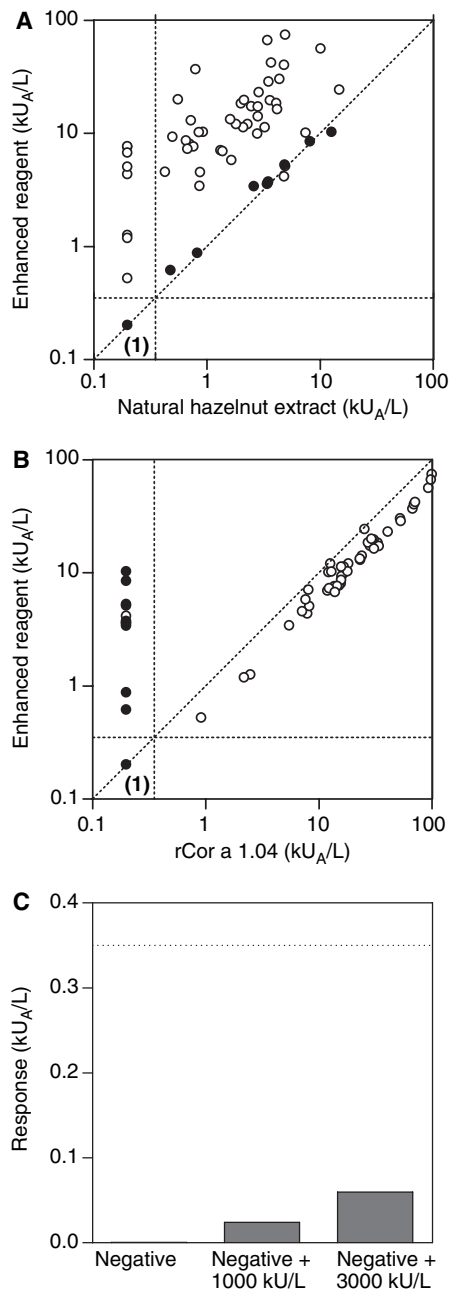


Figure 6. Evaluation of an enhanced hazelnut reagent with sera of hazelnut allergic subjects from central Europe ($n=50$, open symbols), Spain ($n=10$, filled symbols) and a negative control serum pool. Comparison of the enhanced reagent with (A) unmodified natural extract and (B) rCor a 1.04 alone. Results are expressed as kU_A/L of specific IgE. Dotted lines indicate a cut-off level of 0.35 kU_A/L (vertical and horizontal) or a 1 : 1 ratio of IgE binding (diagonal). For the purpose of clarity, IgE values below 0.35 kU_A/L are set to 0.2 kU_A/L in the graphs. In graph (C), a negative control serum pool spiked with 0, 1000 or 3000 kU/L of myeloma IgE was used to assess unspecific IgE binding by the enhanced hazelnut reagent.

study showed specific IgE levels above the conventional cut-off of 0.35 kU_A/L. In contrast, a sensitivity of 75%

($n=67$) of the same test was reported from a multicentre DBPCFC study on hazelnut allergy (2), despite the fact that the 0.7 kU_A/L cut-off level was also applied in this study.

In the work reported here, three approaches were taken to assess the relative antibody binding activity of Cor a 1, Cor a 2 and Cor a 8 in immobilized hazelnut extract: (i) analysis of differential IgE antibody adsorption by immobilized extract; (ii) analysis of allergen-specific rabbit IgG antibody binding to immobilized extract and (iii) monitoring of differential loss of specific IgE binding activities as a result of serial dilution of extract prior to immobilization.

Taken together, the experimental results indicated a scarcity of Cor a 1 and an abundance of Cor a 8 in natural hazelnut extract. Although the reason for the low level of Cor a 1 activity was not addressed in this work, the situation resembles that described for certain fruits, where rapid loss of PR-10 allergen activity upon extraction has been attributed to degrading or modifying enzyme activities or the release of endogenous phenolic compounds (25, 32–34). However, it is also possible that the observed shortage of Cor a 1 activity is in part related to an abundance of major storage proteins, competing with Cor a 1 for solid phase attachment sites. Yet another possible reason may be that Cor a 1 is inefficiently coupled to the solid phase or that its antibody binding function is negatively affected by that procedure. An indication that this may be the case comes from the assessment of allergen content in liquid hazelnut extract which showed a relatively high concentration of Cor a 1 in comparison with Cor a 2 and Cor a 8. Even if these measurements were performed using a comparative IgE inhibition approach rather than a validated quantitative assay, the results suggest that the differential allergen activity of immobilized extract observed is not simply a function of allergen concentration in the extract used.

It should be noted that the concentration estimates made in our study do not necessarily reflect allergen concentrations in the actual food as the solubility of a protein depends on its isoelectric point and hydrophobicity as well as the pH and salt conditions in the extraction buffer. PR-10 proteins, such as Cor a 1, and LTPs, such as Cor a 8, have very different biophysical properties, and factors of this nature may in part explain the unexpected differences in concentration estimates of Cor a 1 and Cor a 8 in liquid extract obtained in this study.

The scarcity of Cor a 1 activity in immobilized hazelnut extract made it of interest to examine whether this deficiency could be rectified by addition of recombinant Cor a 1.04 to the extract prior to solid phase coupling. Even though this would seem a straightforward adjustment to the extract composition, it could not be taken for granted that the recombinant allergen would remain intact and attach efficiently to the solid phase in the presence of competing extract substances or that it would not itself outcompete endogenous allergens to some extent. Evalu-

ation of an initial experiment, where increasing amounts of rCor a 1.04 were simply added to the extract prior to coupling to the solid phase, showed that progressively increased binding of Cor a 1 reactive IgE was achieved, but not to a level comparable with that obtained with rCor a 1.04 alone. To examine whether this might be a result of competition for attachment sites, rCor a 1.04 was added to different dilutions of hazelnut extract before immobilization to the solid phase. Evaluation was then performed using sera that were predominantly reactive to either Cor a 1, Cor a 2 or Cor a 8, as well as sera reactive to hazelnut components other than those three allergens, potentially including storage proteins. Satisfyingly, an almost quantitative binding of Cor a 1 reactive IgE antibodies was achieved at a 25-fold extract dilution and above, while very limited loss of binding of Cor a 2- and Cor a 8-reactive antibodies occurred.

Evaluation of an experimentally enhanced hazelnut reagent, using sera of subjects with diagnosed hazelnut allergy, demonstrated both an improved sensitivity in detection of hazelnut sensitization and a dramatic increase in binding capacity for Cor a 1-reactive IgE antibodies. Among the 50 central European subjects, all eight that tested negative with the unmodified extract tested positive

with the enhanced reagent, equivalent to a rise in sensitivity from 84% to 100% in the particular population studied. With respect to IgE quantitation, a median increase of 6.8 times with the enhanced reagent was observed. Considering the recent developments regarding the use of specific IgE quantitation for prediction of clinical reactivity to foods (35), we believe that this aspect of improved assay performance may also be of clinical importance.

For the Spanish subjects, all of whom were Cor a 1 negative, the unmodified and enhanced reagent compositions gave results that agreed within less than 20% in all cases. Thus, no negative effect of either the extract dilution or the rCor a 1.04 addition on the detection of hazelnut specific IgE antibodies in these sera was observed.

In conclusion, the present work demonstrates that a hazelnut allergen reagent with significantly improved diagnostic sensitivity and quantitative performance can be generated by supplementing natural extract with recombinant Cor a 1.04. This application exemplifies how recombinant allergens may be utilized to important clinical advantage already at a stage where not all relevant components in an allergen source are known or available in a pure form.

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