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Allergens in raw and roasted hazelnuts (*Corylus avellana*) and their cross-reactivity to pollen

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Abstract Hazelnuts (HNs), widely distributed in sweets, provoke one of the most frequent pollen-associated food allergies. In this study HN allergens were investigated and immunologically characterized, focussing on their heat stability and cross-reactivity with known allergenic structures. Therefore, 27 sera from HN-allergic patients and 28 sera from children with positive CAP classes for HN and birch pollen were submitted to immunoblot and immunoblot inhibition. The major HN allergen was found to present a molecular mass of about 17–18 kDa, and to share IgE epitopes with Bet v 1, the major birch pollen allergen. This allergen was recognized by IgE of 93% of our HN-allergic patients and by 79% of sensitized children. A 48 kDa glycoprotein was identified as a minor HN allergen with cross-reactive carbohydrate determinants. The major *N*-glycan species was determined by matrix-assisted laser desorption mass spectrometry to be Man₃XylGlcNAc₂. IgE binding to this protein was detected in sera of 41% of the allergic patients and 61% of the children. Partial *N*-terminal sequencing demonstrated similarity to legume storage proteins. The IgE reactivity of this structure was partially resistant to heating. The rat basophil leukaemia cell mediator release assay was used for estimation of cross-sensitiza-

tion between HN and birch pollen, confirming the finding that HNs contain a heat-resistant allergenicity without cross-reactivity to birch pollen allergens.

Keywords Hazelnut allergens · Cross-reactivity · Cross-reactive carbohydrate determinants · Heat-stable allergens · Rat basophil leukaemia cell assay

Introduction

Hazel (*Corylus avellana*) is known to cause inhalant allergies induced by sensitization with hazel pollen [1, 2] as well as provoking food allergy after HN ingestion [3–5]. Allergy to HN is a very common food allergy accompanied with symptoms such as oral allergy syndrome, urticaria, angioedema and respiratory symptoms [4–6]. Rarely, anaphylactic reactions have been reported [3, 7, 8]. Raw and roasted hazelnuts are used in many processed foods which are sometimes inadequately labelled for the allergic consumer [9]. Small amounts of these processed foods could signify a considerable danger for highly sensitized persons, because of the presence of hidden and occasionally heat-stable allergens [10, 11].

Normally, HN allergy is associated with tree pollen allergy [1, 12–23]. For pollen allergens, especially the major allergens of trees from the order Fagales (alder, birch, hazel, hornbeam), an immunological cross-reactivity has been reported [13, 24–26]. Evidence for cross-reactivity between birch pollen allergens (Bet v 1, Bet v 2) and allergens from vegetables, fruits and nuts was obtained by identifying allergen structures that share similar IgE binding sites [27–29]. HN allergens immunologically similar to the hazel pollen allergens Cor a 1 and hazel pollen profilin have been described by Hirschwehr et al. [22]. Immunological similarity or cross-reactivity between HN allergens and allergens from other foods have been described in detail for

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Apiaceae (carrot, celery, fennel) [12], brazil nut [30, 31], kiwi [32, 33], peanut [31], poppyseed [32, 33], rye flour [32, 33], and sesame [32, 33].

Various structures have been identified that may be responsible for the appearance of pollen associated food allergy: (a) the existence of common structures on major allergens from pollens and foods, e. g. allergens related to the 17 kDa major birch pollen allergen Bet v 1, (b) the presence of pan-allergens ubiquitously distributed in plants, animals and microorganisms, namely proteins of the profilin family with molecular masses between 12 kDa and 15 kDa, and (c) glycoproteins with cross-reactive carbohydrate determinants (CCDs) of molecular masses between 40 kDa and 70 kDa [34–38]. Whereas Bet v 1-related allergens and profilins are a homogeneous groups of allergens, the CCDs are present on numerous allergens, mostly heat-resistant [39] and of high molecular mass with carbohydrate side chains as epitopes or parts of epitopes recognized by allergen-specific IgE [40, 41]. As key features for IgE binding α -1,3 fucose and β -1,2 xylose were identified. The present study was focussed on the investigation of allergenic structures in HNs, taking into consideration those resistant to heat treatment. In the tested sera of patients with HN allergy, IgE reacting with known allergenic structures and IgE to heat resistant structures was found.

Material and Methods

Chemicals. If not otherwise stated, all chemicals were of analytical grade.

Patients' sera, rabbit antisera. The first group of human sera was from 27 patients with allergy to HN, selected from the in-house collection of the Paul-Ehrlich-Institut. Detailed characteristics of 12 representative patients from this group are listed in Table 1. Their sera were used for a comparative immunoblot study with extracts from raw and roasted hazelnuts.

Three of these sera, PEI 12, PEI 17, and PEI 77, were investigated with different immunoblot inhibition systems (raw allergens, recombinant allergens, carbohydrate structures). Non-allergic control sera PEI 58 and PEI 74 were from the in-house collection. The second group of sera was from children and adolescents aged between 2 years and 17 years, participating in the allergy surveillance of the pediatric hospital of the Humboldt University, Berlin. These sera were positive in the CAP assay to HN and birch pollen (>0.35 kU/l; Pharmacia CAP-System, Pharmacia, Uppsala, Sweden). For immunological characterization of proteins in HN extract, rabbit serum with antibodies directed against HN-corylin (Riedel-de Haën, Seelze, FRG) were applied. Normal rabbit serum (Riedel-de Haën) was used as control.

Determination of specific IgE. Specific IgE was determined by the semi-quantitative enzyme immunoassay "Allergopharma specific IgE ELISA (RV)" (Allergopharma, Reinbek, Germany). The allergen disks were prepared using extracts from raw and roasted HNs coupled to cyanogen-bromide-activated paper disks (Hycor, Kassel, Germany) according to a method published by Ceska and Lundkvist [42]. The results were expressed as enzyme allersorbent test (EAST) classes: class 0: <0.35 U/ml; class 1: <0.7 U/ml; class 2: <3.5 U/ml; class 3: <17.5 U/ml and class

4: ≥ 17.5 U/ml. These arbitrary units correspond to the PR Unit/ml of Phadebas RAST (Pharmacia, Uppsala, Sweden). All determinations were run in duplicate.

Further determination of IgE specific to raw hazelnut was done using commercial fluorescence enzymeimmunoassay (FEIA) Pharmacia CAP System RAST FEIA (Pharmacia & Upjohn, Uppsala, Sweden). Test results were expressed in CAP-classes: Class 0: <0.35 U/ml; class 1: <0.7 U/ml; class 2: <3.5 U/ml; class 3: <17.5 U/ml; class 4: <50 U/ml; class 5: <100 U/ml and class 6: ≥ 100 U/ml. WHO-IgE standard based calibrators were used for determination of specific IgE antibodies and results were expressed in U/ml.

Allergen extracts, recombinant allergens, and inhibition reagents. Six varieties of HN (*Corylus avellana*), Tonda Giffoni, Mortarella, Nocciole Ordu (NO), Piemonte, San Giovanni and Tonda Romana were provided by Dr. G. Malgarini (Sorematec, Arlon-Schoppach, Belgium). A further unknown variety was purchased in a local food store. Approximately 30 g of HNs were frozen with liquid nitrogen and ground four times for about 20 s with an analytical mill (IKA M20, IKA Labortechnik, Staufen, Germany). Subsequently, 1 g of the HN powder was extracted with 20 ml phosphate buffered saline (PBS, pH 7.4; 10 mM $\text{NaH}_2\text{PO}_4 \times 2\text{H}_2\text{O}$, 70 mM $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$, 150 mM NaCl) for 4 h at 4°C. After consecutive filtration with cellulose acetate filters with pore diameters of 0.45 μm and 0.2 μm (Sartorius, Göttingen, Germany), the filtrates were dialysed overnight against distilled water. Determination of the protein content according to a modified Bradford method [43] using Coomassie Plus Protein Assay (Pierce, Rochford, Ill., USA) was performed as described previously [10, 11]. The extract was freeze-dried.

For the EAST inhibition experiments birch pollen extract (BPE) (*Betula verrucosa*; Mast Diagnostika, Reinfeld, Germany) and olive tree pollen extract (OTPE) (*Olea europaea*; Allergopharma, Reinbek, Germany) were used. The recombinant allergens rBet v 1 and rBet v 2 were obtained from Biomay, Linz, Austria. The inhibitor reagents bromelain-glycopeptide, or $\text{Man}\alpha 1\text{--}6[\text{Xyl}\beta 1\text{--}2]\text{Man}\beta 1\text{--}4\text{GlcNAc}\beta 1\text{--}4[\text{Fuc}\alpha 1\text{--}3]\text{GlcNAc}\text{--Asn}\text{--Glu}\text{--Ser}\text{--Ser}_{0.5}$; (M0XF³), and fibrin-glycopeptide or $\text{Man}\alpha 1\text{--}6[\text{Man}\alpha 1\text{--}3][\text{Xyl}\beta 1\text{--}2]\text{Man}\beta 1\text{--}4\text{GlcNAc}\beta 1\text{--}4[\text{Fuc}\alpha 1\text{--}3]\text{GlcNAc}\text{--Gly}\text{--Glu}\text{--Asn}$ (MM) were prepared as described previously [41] and their compositions were analyzed by matrix-assisted laser desorption mass spectrometry (data not shown). Inactive horseradish peroxidase (peroxidase-CM) was prepared by S-alkylation with iodoacetic acid using standard procedures. The deglycosylation of peroxidase was performed by Dr. K. Fötisch in our lab with the GlycoFree, deglycosylation kit K-500 (Oxford Glyco Systems, Abingdon, UK). Ovalbumin was purchased from Sigma-Aldrich.

SDS-PAGE analysis. Vertical SDS-PAGE was performed according to the method of Lämmli [44] and modified as previously described by Vieths et al. [45] and Müller et al. [46]. The protein amounts applied on the gels for silver stain detection were 16 μg /cm, whereas the gels for immunoblotting were loaded with 20 μg /cm HN protein. For detection of molecular masses a commercial mix of six markers with molecular weights of 14.4, 20.1, 30.0, 43.0, 67.0 and 94.0 kDa (LMW Electrophoresis Calibration Kit, Pharmacia Biotech, Freiburg, Germany) was used.

Silver staining. The SDS gels for analysis of protein patterns were stained with a modified silver stain method according to Swain et al. [47]. The gels were equilibrated in fixation solution [30% (w/v) ethanol, 10% (w/v) acetic acid] for 3 h. After fixation, the gels were soaked for 30–120 min in the incubation solution [30% (w/v) ethanol, 0.5% (v/v) glutaraldehyde, 0.5 M sodium acetate, 0.02 M sodium thiosulphate] and subsequently washed three times for 20 min with distilled water. After washing, the gels were gently agitated for 30–60 min in a freshly prepared solution of silver nitrate [0.1% (w/v) silver nitrate, 0.02% (v/v) formaldehyde] followed by staining in developing solution [2.5% (w/v) sodium carbonate, 0.01% (v/v) formaldehyde; freshly prepared] un-

til bands became visible. The process was stopped by rinsing gels for 20 min in stopping solution (50 mM EDTA), and finally gels were washed in distilled water and photographed.

Immunoblot and immunoblot inhibition. The proteins of the SDS gels for western blots were transferred from the gels onto nitrocellulose (NC) membranes (0.2 µm, Schleicher und Schüll, Dassel, Germany) by semi-dry electroblotting as previously described [45]. The NC membranes were cut lengthwise into strips of 3–4 mm and incubated overnight with 1 ml of the dilutions of human sera, human sera and inhibitors or rabbit antisera, respectively. The incubation buffer was tris buffered saline (TBS) [100 mM tris(hydroxymethyl)aminomethane (TRIS), 100 mM NaCl, 2.5 mM MgCl₂ × 6H₂O adjusted with HCl to pH 7.4, with 0.1% BSA, and 0.05% (v/v) Tween 20]. Exact dilutions are given in the figure legends.

The detection of human IgE antibodies bound to immobilized allergens was done with alkaline phosphatase (AP)-labelled mouse anti-human IgE (1:1000, 4 h; Pharmingen, Hamburg, Germany) and nitroblue tetrazolium / 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Biorad, Munich, Germany) as the substrate.

Detection of immunoreactive HN proteins bound to IgG from rabbit antisera was done by subsequent incubations of biotin labelled goat anti-rabbit IgG (Dako, Hamburg, Germany) and AP-labelled streptavidin (Medac, Hamburg, Germany).

N-glycan analysis. HN extract was separated by SDS-PAGE using a Biorad Mini-Protein II cell. The 48 kDa band corresponding to about 6 µg of protein (by comparison with protein mass marker proteins) was excised, S-alkylated, digested with sequencing grade trypsin (Roche) and extracted as described [48].

N-glycans were released by a peptide, N-glycosidase A (Roche, Basle, Switzerland) and purified using cation and anion exchange resins and reversed phase silica gel. Matrix-assisted laser desorption mass spectrometry was performed with a Dynamo (ThermoBioAnalysis, Santa Fé, USA) using a mixture of 2,5-dihydroxybenzoic acid, 2-hydroxy-5-methoxybenzoic acid and arabinosazone as the matrix. Details of this procedure will be published elsewhere (D. Kolarich, F. Altmann; in press). The masses obtained were interpreted according to the current knowledge of N-glycosylation in plants [49].

Partial N-terminal sequence analysis. Protein from raw HN (50 µg/cm) was separated by SDS-PAGE and subsequently semi-dry blotted to PVDF protein sequencing membranes (0.2 µm, BIORAD, Hercules, Calif., USA) previously equilibrated for 5 min in methanol. The membrane was stained with 0.025% coomassie brilliant blue dissolved in 40% methanol. After destaining with 50% methanol solution and drying, a protein band near 48 kDa was cut off the membrane with a scalpel. N-terminal sequence analysis was done according to the method of Matsudaira [50], as described previously [51].

RBL cell mediator release assay. A murine in vitro model of the type I allergic reaction was applied to estimate the biological activity of the HN allergen preparations [52, 53]. The assay is based on a culture of permanently growing RBL cells, which represent functional analogues of mucosa mast cells and can be passively sensitized with murine and rat IgE, but not with human IgE. Specific IgE was raised in BALB/c or BP2 mice by five injections of raw HN extract containing 10 µg protein or BPE containing 0.1 µg of the major allergen Bet v 1, respectively. The extracts were adsorbed to 5 mg Al(OH)₃ in 0.5 ml PBS and injected at intervals of 2 weeks. Final bleeding was performed 7 days after the last immunization. Sera of at least five responding mice with β-hexosaminidase release >20% were pooled.

The RBL cell assay was performed as previously described [52, 53]. Results were expressed as a percentage of the total release after subtraction of the spontaneous release [52, 53].

Table 1 Patients' histories and detailed data of 12 investigated patients' sera. *n.d.* not determined, ? not known, *E* EAST

General information			Symptoms	RAST, CAP, EAST							
Serum Code	Sex/date of birth	Total IgE kU/l	Hazelnut ^a	Allergy to pollen ^b	Allergy to further foods ^c	rBet v 1	rBet v 2	Hazelnut (CAP)	Birch pollen (CAP)	Hazelnut, raw (EAST)	Hazelnut, roasted (EAST)
Bo 70	m / 1958	2840	ts	b,g	a,al,p	5	0	3	5	3	0
Bo 126	f / 1967	2250	mi,ts	?	a,al,an,p,cu	6	0	3	6	3	0
Bo 129	f / 1971	2240	mi,ti	b,g,m	a,p,ch	n.d.	n.d.	4	E 3	4	0
PEI 2	f / 1951	154	mi,ti	b,m	a,ca,ch,ki,p,pe,pn,po	5	0	3	5	3	0
PEI 12	f / 1950	2620	mi,ti	b,g	cu	5	0	3	n.d.	3	2
PEI 17	f / 1955	n.d.	mi,ls	b,g,m	a,pe,po,rf	6	0	3	6	4	0
PEI 29	m / 1942	804	mi	b	ki,mi,sh	1	1	6	3 ^d	4	4
PEI 41	f / 1946	n.d.	as	b,g,m	a,ch,cu,cw,fi,p,pa,pn,po,pr,wn	3	0	3	4	2	2
PEI 62	f / 1966	n.d.	ms,ts	b,g,m	a,al,ap,ch,ki,ml,p,pe,pl,pm,po,pr,wn,mn	4	0	3	6	3	0
PEI 63	f / 1969	n.d.	ms,ls	b,g	a,al,ap,ba,ch,ki,p,pe,po	4	0	3	4	3	0
PEI 65	f / 1970	n.d.	as,mi,ls	b,g,m	a,ch,pn,po,wf	5	0	4	5	4	4
PEI 77	m / –	n.d.	ls,si	b,g,m	a,bn,cn,pi,po,pn	4	0	3	4	3	1

^a *an* Anaphylaxis, *as* asthma, *ls* lip swelling, *mi* mouth itching, *ms* mouth swelling, *si* skin itching, *ts* throat swelling

^b *Birch*, *g* grasses, *m* mugwort

^c *a* Apple, *al* almond, *an* anise, *ap* apricot, *ba* banana, *bn* brasil nut, *ca* carrot, *ch* cherry, *cu* curry, *cw* caraway, *fi* fish, *ki* kiwi, *cn* cashew nut, *me* melon, *mi* milk, *mn* macademia nut, *p* pear, *pa* paprika, *pe* peach, *pl* pineapple, *pl* parsley, *pm* plum, *pn* peanut, *po* potato, *pr* pepper, *rf* rye flour, *sh* shrimps, *wf* wheat flour, *wn* walnut

^d Tree pollen mix

Results and discussion

Protein pattern of extracts from the analysed HN varieties

The extracts of seven different HN varieties, Tonda Giffoni, Tonda Romana, Piemonte, San Giovanni, Nocciolo Ordno, Mortarella and a commercially distributed unknown variety were compared in their protein pattern using SDS-PAGE and silver staining (Fig. 1, lanes 1–9). Applying this analytical method, about 40–50 protein bands in the molecular mass range 14–94 kDa were visible. Protein clusters at 21–22 kDa, 24–28 kDa, 30 kDa, 38–40 kDa and 44–45 kDa were observed, being similar in all HN varieties. The extract of HN variety NO roasted at 140 °C for 20, 30 or 40 min, respectively, presented a reduced number of protein bands compared with extract from raw NO (Fig. 1, lanes A,B,C,D). Only five broad protein bands in molecular mass ranges of 21, 24, 27, 30, and 44 kDa were observed.

Immunoblotting with rabbit antiserum

Immunodetection after SDS-PAGE and semi-dry blotting was used for immunological characterization of the components from raw and roasted HN. On a blot of raw HN (var. Piemonte) rabbit anti-HN corylin serum detected many protein bands in the molecular mass range 14 to 67 kDa (Fig. 2). With blotted extract from roasted HN, the same antiserum bound to five areas at molecular masses between 14 kDa and 45 kDa. Specific bands at 17, 30, 48, and 65 kDa detected in blotted ex-

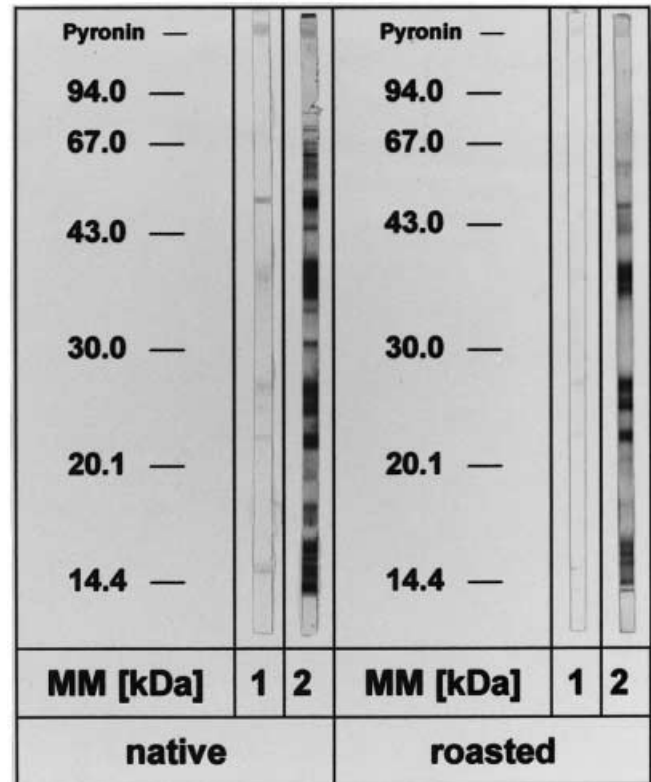


Fig. 2 Immunoblot of extracts from raw and roasted HN (var. Piemonte), respectively with rabbit anti-HN corylin serum. 1 Control with stabilized rabbit normal serum (1:2000), 2 rabbit anti-HN corylin serum (1:2000)

tract from raw HN were not detected in the blot of roasted HN extract (Fig. 2).

Screening of patients' sera

Subsequently, a screening of sera from patients with allergic symptoms after HN ingestion was carried out with membrane strips from both extracts, raw and roasted HN variety Piemonte (Fig. 3a, b). Detailed data of the patients are listed in Table 1. Of the 12 sera tested, 11 reacted with a 17–18 kDa protein in the extract from raw HN (Fig. 3a). This band completely disappeared in the extract from roasted HN (Fig. 3b). Furthermore 6 of the 12 sera bound to a structure in the molecular mass region of 48 kDa of the extract from raw HN (Fig. 3a, lanes 1, 2, 3, 4, 5, 9). Four of these six sera had IgE specific to roasted HN proteins in concentrations >0.35 U/ml (Table 1) and detected many bands in a high molecular mass range between 36 kDa and 90 kDa (Fig. 3a, lanes 2, 4, 5, 9). The same four sera had antibodies directed to a band of 43 kDa in the extract from roasted HN (Fig. 3b, lanes 2, 4, 5, 9). The two sera PEI 12 and PEI 65, positive to roasted HN with EAST classes 2 and 4 respectively (Table 1), reacted with proteins in the low molecular mass region

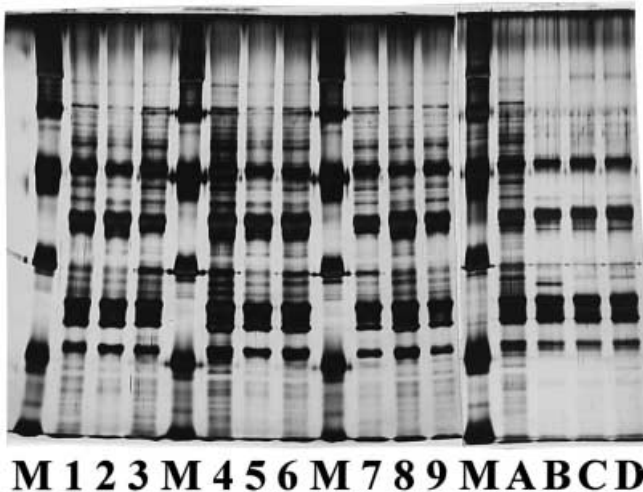


Fig. 1 SDS-PAGE, silverstain from HN extracts (16 µg/cm). M molecular mass marker (14.4, 20.1, 30, 43, 67, and 94 kDa), 1 Tonda Giffoni, 2 unknown variety, 3 Mortarella, 4 Nocciolo Ordu (NO), 5 unknown variety, 6 Piemonte, 7 San Giovanni, 8 Piemonte, 9 Tonda Romana, A raw NO, B NO, after 20 min at 140 °C, C NO, after 30 min at 140 °C, D NO, after 40 min at 140 °C

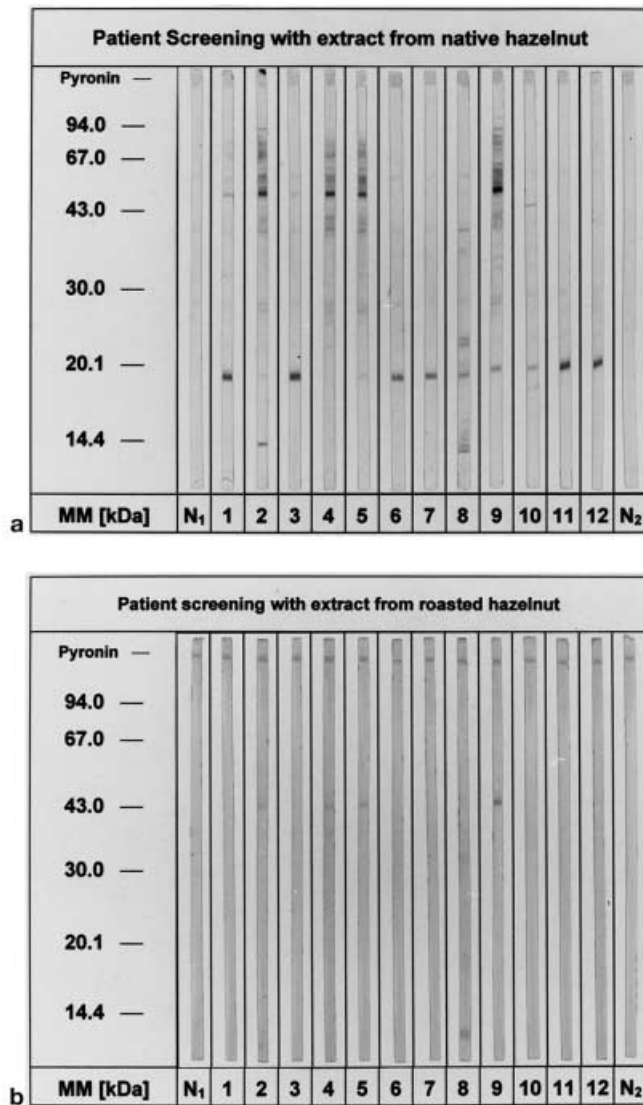


Fig. 3a, b Screening of patients sera with extracts from **a** raw and **b** roasted HN. N₁ non allergic control (PEI 58), 1 PEI 2, 2 PEI 12, 3 PEI 17, 4 PEI 29, 5 PEI 41, 6 PEI 62, 7 PEI 63, 8 PEI 65, 9 PEI 77, 10 Bo 70, 11 Bo 126, 12 Bo 129, N₂ non allergic control (PEI 74)

of 12–14 kDa (Fig. 3a, lanes 2, 8). These proteins appeared to be partially resistant to the roasting procedure (Fig. 3b, lanes 2, 8). Besides this, further bands were visible on the immunoblots. A more detailed profile of the allergens detected in extract from raw HN by sera of 27 patients with allergy to HN, including the 12 sera of Fig. 3 is shown in Fig. 4a.

The second group of sera analysed were from the allergy surveillance of the pediatric hospital of the Humboldt University, Berlin. These 28 sera were selected by the criteria of a positive CAP-test to HN and to birch pollen, independent of the presence of allergic symptoms to HN. The allergen recognition profile with extracts from raw HN Piemonte by these children's sera is summarized in Fig. 4b.

Immunoblot inhibition of selected sera with specific inhibitors

The sera PEI 12, PEI 17, and PEI 77 were used for specific inhibition experiments to further characterize the HN allergens. Therefore, the three sera were used in inhibition tests with blotted extract from raw HN, with and without addition of specific inhibitors: rBet v 1, rBet v 2, BPE, OTPE, and ovalbumin as a control for unspecific reactions (Fig. 5a–c). The inhibition control with extract from raw HN revealed the complete inhibition of IgE reactions in all sera (not shown).

IgE from the serum PEI 17 bound exclusively to allergens of a molecular mass of 17–18 kDa. This reaction was inhibited by BPE and rBet v 1 and not inhibited by rBet v 2 and OTPE (Fig. 5a).

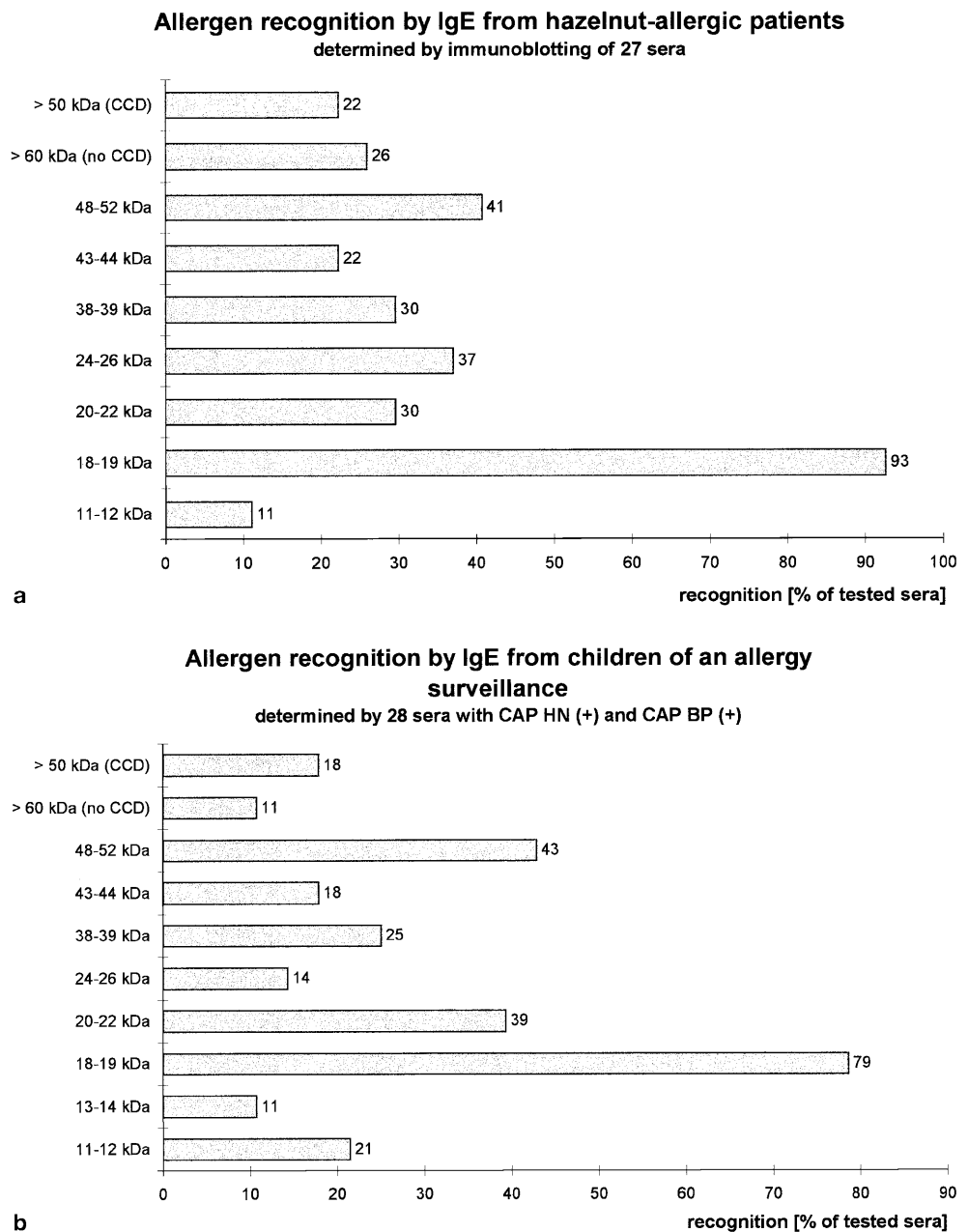
Specific IgE antibodies of serum PEI 12 reacted with a 12–14 kDa band and with other protein bands at higher molecular masses with a major reactivity at 48 kDa. Whereas the latter reaction was inhibited by BPE and OTPE, the antibodies to the 14 kDa band did not react to any of the inhibitors (Fig. 5b).

The IgE of the serum PEI 77 reacted with bands in the high molecular mass range (>41 kDa, major reactivity at 48 kDa). These reactions were inhibited similarly as described for the high molecular bands in the blot of serum PEI 12 by BPE and OTPE (Fig. 5c, lanes 5, 6). This broad cross-reactivity suggested that the inhibited band contained ubiquitous carbohydrate epitopes. To prove this hypothesis, the two sera PEI 12 and PEI 77 presenting IgE reactivity in the high molecular mass region were used for inhibition assays with inactivated peroxidase-CM (a glycoprotein with >6 N-glycans containing α -1,3-fucose and β -1,2-xylose, deglycosylated peroxidase-CM, and the purified MM-glycan and M0XF³ (Fig. 6a, b). M0XF³ is a known IgE epitope in about 25% of celery-allergic patients (Jankiewicz et al. [39], Fötisch et al. [40]). The glycopeptide preparations contained no measurable amounts of proteins, as determined by SDS-PAGE and silver staining (not shown) M0XF³ as well as peroxidase-CM presented complete inhibition of the high molecular protein bands including the 48 kDa band (Fig. 6a, b, lanes 1, 4), indicating the presence of glycans containing the fucose and xylose on these HN allergens. The deglycosylated peroxidase showed only a weak inhibition of these protein bands (Fig. 6a, b, lane 5). The protein band at 14 kDa detected by PEI 12 was not inhibited by any of the applied inhibitors for CCDs (Fig. 6a).

Characterization of a 48 kDa IgE-binding protein from raw HN extract

Partial N-terminal sequencing of the 48 kDa protein band from raw HN extract, recognized by 6 of the 12 screened sera, revealed the following amino acid sequence in one letter code: SEESY-GEEQD?NPYVFS. A BLAST search [54] identified

Fig. 4 a Distribution of the allergens of extract from raw HN detected by 27 sera of patients with allergy to HN. **b** Distribution of the allergens of extract from raw HN detected by 28 sera of patients with positive CAP to HN and birch pollen



11 of 18 amino acids analysed (61%) with the protein P54, a legume storage protein of the vicilin family from pea (*Pisum sativum*) (Acc. No. Y11207 [59]). P54 is composed of 483 amino acids, has a calculated molecular mass of 54 kDa and one N-glycosylation site. The match with the *N*-terminus of the 48 kDa HN protein was found between amino acid positions 78 and 95 from the *N*-terminus of P54. Analysis of the N-glycans of the 48 kDa allergen from HNs by mass spectrometry revealed MMX ($\text{Man}_3\text{XylGlcNAc}_2$) to be the major structure present with only a minute amount of M0XF³ (Table 2, Fig. 7).

RBL cell mediator release stimulated by extracts from raw and roasted HN

The capacity of a HN extract (variety: Piemonte) to trigger a specific mediator release was tested by the RBL cell assay. Our interest in this study was focussed on the cross-reactivity between HN and birch pollen allergens in triggering the release from RBL-2H3 cells and in detecting heat-resistant allergens in HN extracts. Therefore, cells were passively sensitized with pooled murine antisera raised against HN extract or BPE, respectively. The mediator release of the cells, sensitized with murine anti-HN IgE (1:100) ranged between 10% and 25% for both, raw and roasted HN (Fig. 8a). By

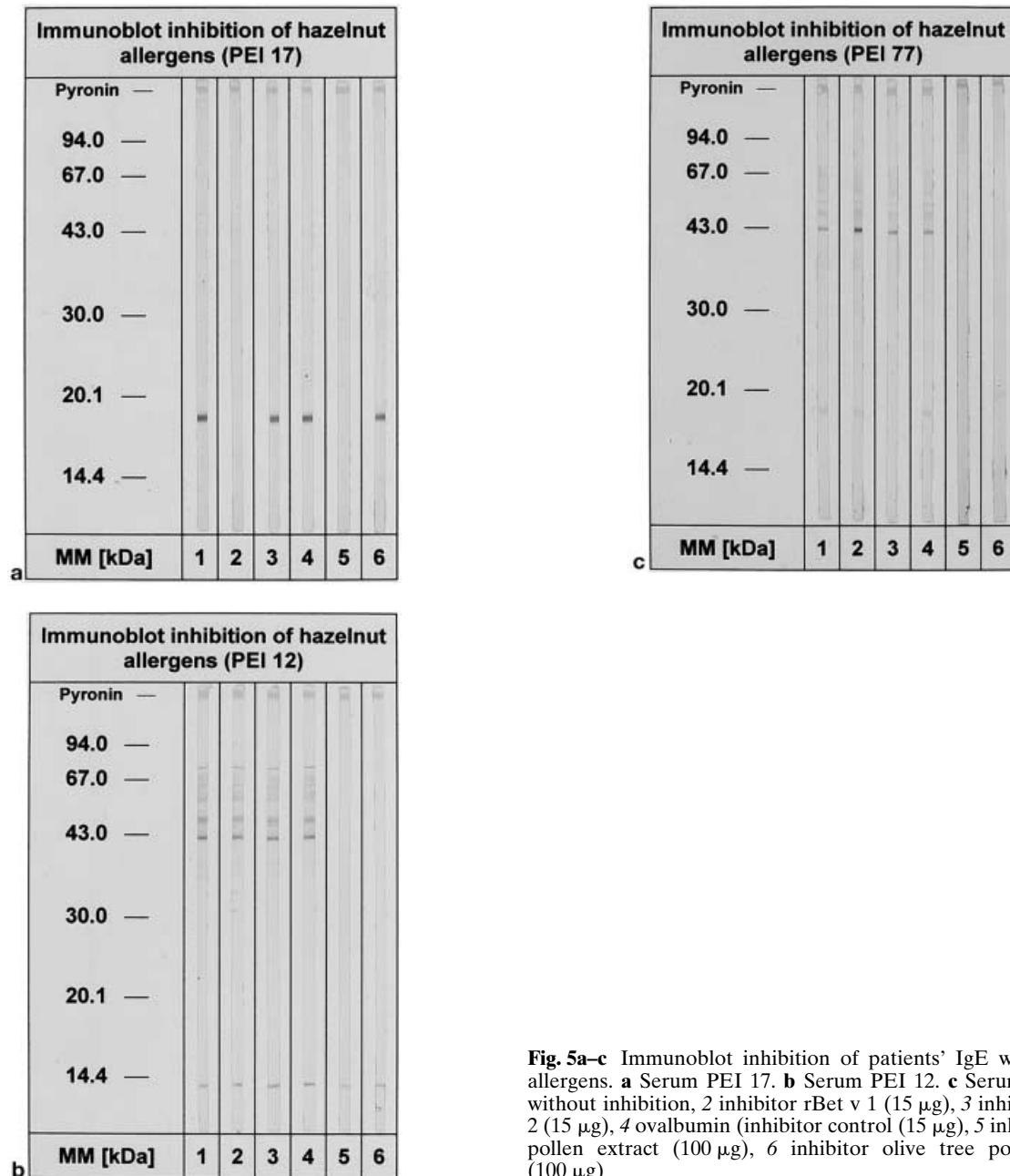


Fig. 5a–c Immunoblot inhibition of patients' IgE with selected allergens. **a** Serum PEI 17. **b** Serum PEI 12. **c** Serum PEI 77. 1 without inhibition, 2 inhibitor rBet v 1 (15 μ g), 3 inhibitor rBet v 2 (15 μ g), 4 ovalbumin (inhibitor control (15 μ g), 5 inhibitor birch pollen extract (100 μ g), 6 inhibitor olive tree pollen extract (100 μ g)

contrast, the RBL cells sensitized with anti-birch pollen IgE (1:100) did not present a release after stimulation with extracts from roasted HN, whereas raw HN extracts provoked β -hexosaminidase release of up to 25% at concentrations of 10–100 μ g/ml HN protein (Fig. 8b). The positive control (standard BPE) triggered a maximal release of 50% (not shown).

Allergen extracts from different varieties of HN presented similar protein profiles, a result corresponding to reports on four varieties of celery and four varieties of peanut [55]. HN subjected to a roasting procedure (140 $^{\circ}$ C, 40 min) revealed a less distinct protein pattern in SDS-PAGE of the aqueous extract, when compared to extract from raw HN. With rabbit antiserum raised

against HN corylin, immunological reactivity of proteins from both raw and roasted HN was proven.

For certain patients, the potential allergenicity of HN products is not reduced by roasting the nuts, due to a number of heat-resistant proteins with allergenic reactivity found in extracts from roasted HN, a phenomenon previously described for peanut [10].

The screening of patients allergic to HN showed that IgE to known pollen related allergenic structures, (a) the Bet v 1 related HN allergen Cor a 1.0401 [22, 56] and (b) CCDs was found in the sera of the patients studied. Ninety-three percent of sera from adults with a history of HN allergy reacted with the Bet v 1 related HN major allergen, and 79% of children's sera, positive

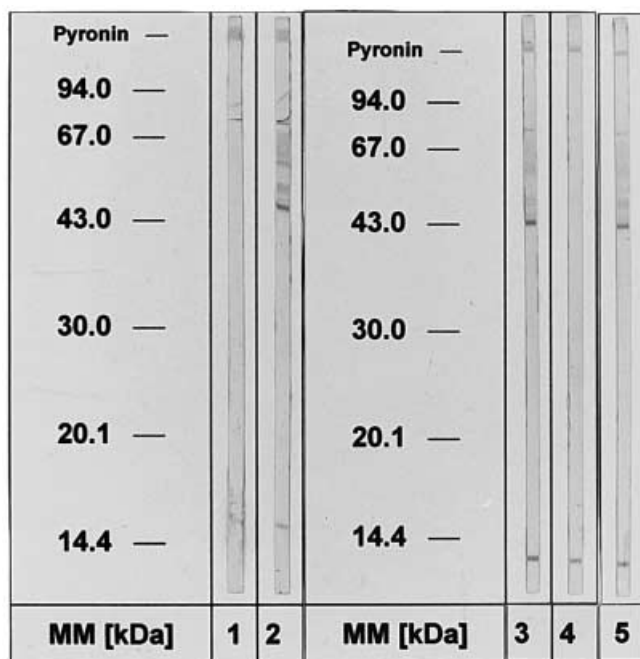


Fig. 6 a Immunoblot inhibition of the serum PEI 12 with cross reactive carbohydrate determinants CCDs. 1 inhibitor M0XF³ (10 µg), 2 inhibitor MM (10 µg), 3 without inhibition, 4 inhibitor peroxidase, carboxymethylated (15 µg), 5 inhibitor peroxidase, deglycosylated (15 µg). **b** Immunoblot inhibition of the serum PEI 77 with CCD, 1 inhibitor M0XF³ (10 µg), 2 inhibitor MM (10 µg), 3 without inhibition, 4 inhibitor peroxidase, carboxymethylated (15 µg), 5 inhibitor peroxidase, deglycosylated (15 µg)

in CAP to HN and birch pollen, contained IgE to this allergen. Blot with extract of roasted HN as well as the RBL cell mediator release assay with anti-birch pollen IgE clearly indicated that this major HN allergen is

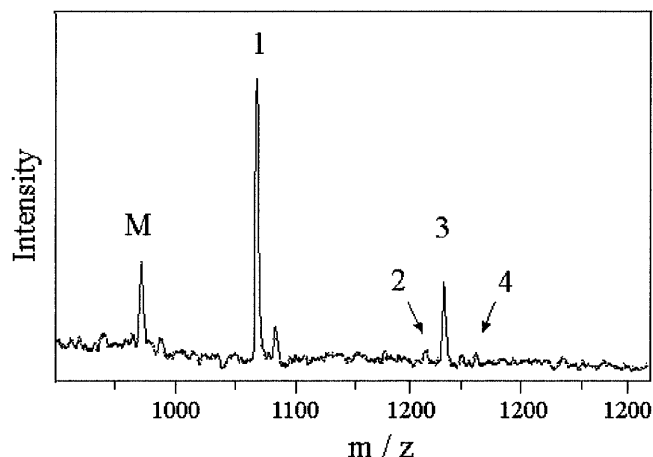


Fig. 7 Analysis of the N-glycans of the 48 kDa HN protein by matrix-assisted laser desorption mass spectrometry. The oligosaccharides were prepared as described in the Materials and methods section. N-glycans were detected mainly as sodium adducts (peaks 1 to 4). A peak at 927.2 amu (*M*) is a matrix impurity. Masses and deduced compositions of N-glycans are given in Table 2

Table 2 The N-glycans of the 48 kDa glycoprotein from HNs: The interpretation of the oligosaccharide masses obtained by matrix-assisted laser desorption mass spectrometry (Fig. 7) was done according to current knowledge of structures and biosynthesis of N-glycans in plants [41, 48, 49]. The values refer to average masses of [M+Na]⁺ ions

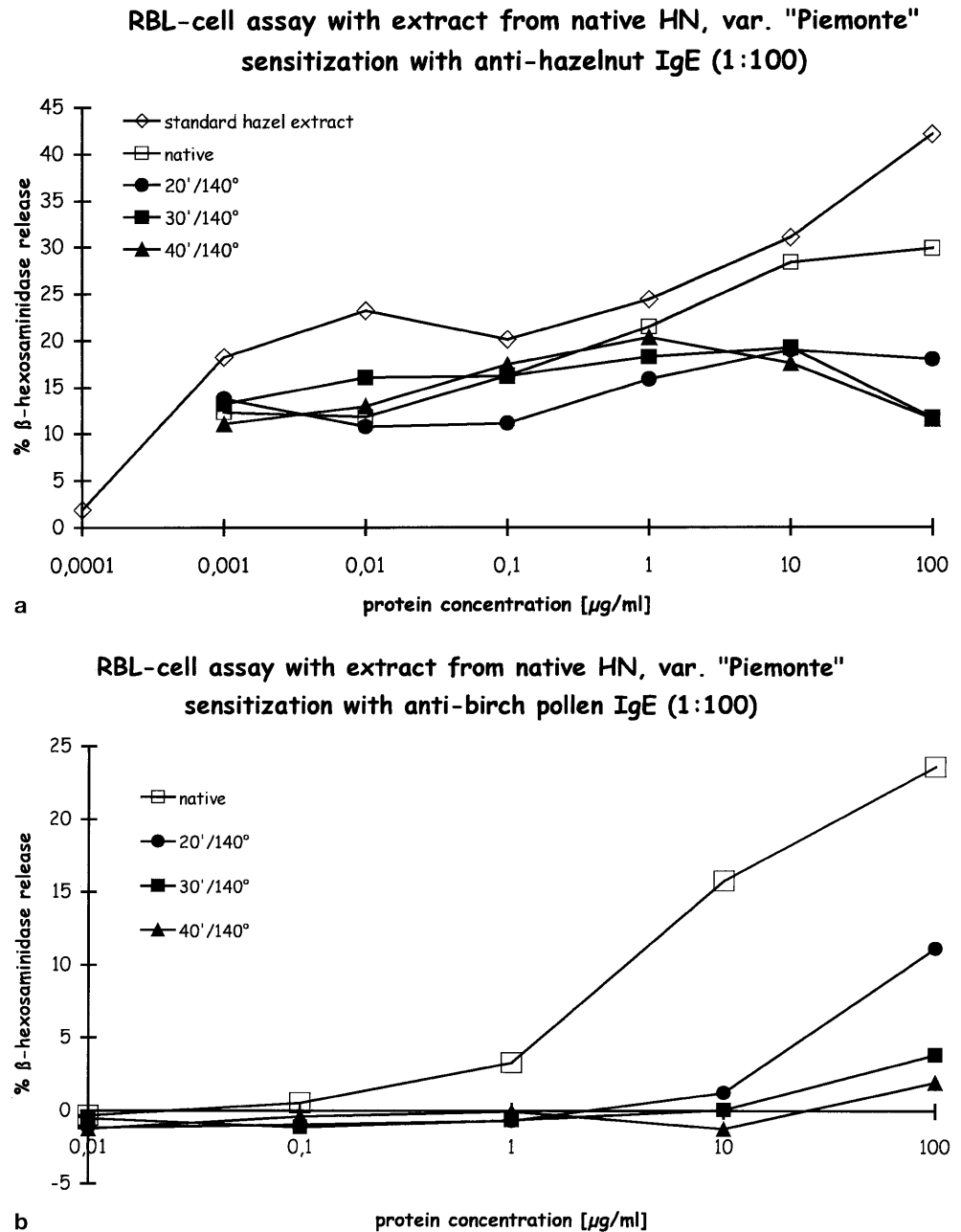
Experimental mass (amu)	Calculated mass (amu)	Deduced composition (%)	Relative amount
1066.1	1065.9	Man ₃ XylGlcNAc ₂	74
1212.3	1212.1	Man ₃ XylFucGlcNAc ₂	3
1228.2	1228.1	Man ₄ XylGlcNAc ₂	21
1258.4	1258.1	Man ₅ GlcNAc ₂	2

heat-labile. Bands between 46 kDa and 52 kDa were recognized by 41% of the patients with known HN allergy and by 61% of the sera from HN-sensitized children. As shown by immunoblot, four of six sera with IgE binding to this structure reacted with a 43 kDa band in the extract of roasted HN, suggesting that a heat-stable allergenic structure is recognized by IgE from these patients.

Normally, patients with HN allergy are also allergic to tree pollen, as a result of sensitization to cross-reactive Bet v 1-related major allergens, or to heat-stable, high molecular mass CCDs of these pollens. Jankiewicz et al. described similar types of cross-reactivity for raw and heated celery roots [19, 57]. Hence, we suspected CCDs to be the heat-stable allergenic component in HN extract.

Further inhibition studies done with modified peroxidase, and the glycopeptides MM and M0XF³ purified from fibrin and bromelain, respectively, revealed that

Fig. 8a, b Rat basophil leukaemia cell test with extracts from HN (var. Piemonte), sensitization with **a** anti-HN IgE (1:100) and **b** anti-birch pollen IgE (1:100)



IgE specific to high molecular mass bands including a dominant 48 kDa band of both tested sera (PEI 12, PEI 77) were inhibited by carboxymethylated peroxidase and M0XF3-glycopeptide, whereas IgE to the low molecular mass, heat-resistant protein, detected by serum PEI 12 was not inhibited. Deglycosylated peroxidase and MM-glycopeptide did not react with IgE of PEI 12 and PEI 77, indicating the specificity of the assay and the involvement of xylose and, possibly, fucose in the epitope structure. Interestingly, analysis of the N-glycan by mass spectroscopy revealed that oligosaccharides containing β -1,2 xylose but no fucose are the major constituents of the carbohydrate moiety. Hence, the carbohydrate epitope involved appears to be different

from other plant glycans [41]. Since four of six sera with IgE against the 48 kDa protein reacted with extract of roasted HN, we conclude that the bands at 48 kDa carry carbohydrate epitopes and may contribute to the heat-resistant allergenicity of HN.

Partial N-terminal sequencing of the 48 kDa protein and comparison with protein sequence databases showed 61% identity with a partial sequence of the pea protein P54 (Acc. No. Y11207 [59]) belonging to the superfamily of legume storage proteins (vicilin, legumin) (Dr. M.I. Rodrigo, Valencia, Spain, personal communication). Some of these proteins are known for their heat-resistant glycan structures as well as for their allergenic reactivity. For example the major peanut al-

lergen, Ara h 1 is known to be a glycosylated vicilin [58].

The 43 kDa band in the extract from roasted HN detected by four sera with sensitivity to the 48 kDa band and other CCD-containing proteins in raw HN extract may result from protein alteration of the 48 kDa band during heat treatment.

The RBL cell mediator release assay which was developed in our laboratory for quality control of allergenic extracts was applied to estimate cross-sensitization of HN and birch pollen in this in vitro model. RBL cells passively sensitized with mouse anti-HN IgE released β -hexosaminidase in similar amounts after challenge with extract from raw and roasted HN, respectively. By contrast, the indicator enzyme was released by the cells passively sensitized with anti-birch pollen IgE only after challenge with extract from raw HN at protein concentrations between 10 μ g/ml and 100 μ g/ml.

These results suggest that HN may also contain a pollen-independent, heat-resistant allergenic potential. Since the patients sera PEI 12 and PEI 65 showed IgE binding to a 12 kDa allergen in HN which was not inhibited by pre-incubation with BPE, this protein is a candidate to explain the mediator release results obtained with murine anti-HN IgE.

Further work is in progress to investigate the relevance of this new allergenic structure in HN and its resistance to thermal processing in more detail.

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