JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Influence of the Maillard Reaction on the Allergenicity of rAra h 2, a Recombinant Major Allergen from Peanut (*Arachis hypogaea*), Its Major Epitopes, and Peanut Agglutinin

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The influence of thermal processing and nonenzymatic browning reactions on the IgE-binding activity of rAra h 2 was studied and compared to findings recently reported for the allergen's natural counterpart. ELISA experiments as well as inhibition assays revealed that thermal treatment of rAra h 2 in the presence of reactive carbohydrates and carbohydrate breakdown products induces a strong increase of the IgE-binding activity, thus collaborating with the data reported for the natural protein isolated from peanuts. To localize the Ara h 2 sequences responsible for the formation of highly IgE-affine glycation sites, model peptides have been synthesized mimicking sequences which contain possible targets for glycation as well as the immunodominant epitopes. Immunological evaluation of these peptides heated in the absence or presence of reducing sugars and carbonyls, respectively, revealed that neither the two lysine residues of Ara h 2 nor its N-terminus are involved in the formation of IgE-affine structures by Maillard reaction. Also, the cysteine-containing major epitope 3 (aa 27−36) was found to lose its IgE-binding capacity upon heating. By contrast, the overlapping major epitopes 6 and 7, which do not contain any lysine or arginine moieties, showed a distinct higher level of IgE binding when subjected to Maillard reaction, thus giving the first evidence that nonbasic amino acids might be accessible for nonenzymatic glycation reactions and that these posttranslational modifications might induce increased IgE binding of the glycated Ara h 2. Analogous experiments were performed with peanut agglutinin, considered in the literature as a minor allergen. ELISA experiments revealed that the majority of tested sera samples from peanut-sensitive patients showed a high level of IgE binding to the lectin even after heat treatment. In contradiction to published data, nonenzymatic browning reactions seem to deteriorate the IgE affinity of the lectin.

KEYWORDS: Maillard reaction; food allergy; peanut agglutinin; Ara h 2

INTRODUCTION

Peanut allergy is known to cause severe and sometimes fatal reactions. Due to the widespread use of peanut products and the lack of therapies, this lifelong existing health threat not only represents a restriction of quality of life but is also considered as a severe and challenging problem for the industry with regard to food safety.

In past years the seven allergenic proteins or glycoproteins Ara h 1 (*1*−*4*), Ara h 2 (*4*−*6*), Ara h 3 (*7*), Ara h 4 (*4*), Ara h 5 (*4*), Ara h 6 (*4*), and Ara h 7 (*4*) have been isolated from peanut, and their structures have been successfully characterized. Among these allergens proteins Ara h 1 and Ara h 2 are considered as the dominant allergens because they are recognized by serum IgE from over 90% of patients with peanut hypersensitivity. Recently, the major linear IgE-binding epitopes of Ara h 2 were mapped using overlapping synthetic peptides (*6*). Ten segments have been identified as IgE-binding sites, among which the sequences covering aa 27−36 (epitope 3), aa 57−66 (epitope 6), and aa 65−74 (epitope 7) of the Ara h 2 molecule were recognized by all tested patients' sera (**Figure 1**).

Although the native food allergens such as, e.g., Ara h 1−Ara h 7 have been intensively studied, little is known so far about the influence of food processing on the structure and IgE-binding activity of these proteins. Some plant proteins such as the allergens from celery, cherry, and apple are considered to lose their allergenic activity upon food processing. This is well reflected by the fact that thermally processed fruit and vegetable products often show non- or less severe effects toward patients than the corresponding fresh plant material. Recent investiga-

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10.1021/jf048398w CCC: $30.25
Published on Web 02/15/2005

1	11	21	31
LTILVALALF	LLAAHASARQ	QWELQG**DRRC**	**QSQLER**ANLR
41	51	61	71
PCEQHLMQKI	QRDED**SYERD**	**PYSPSQDPYS**	**PSPY**DRRGAG
81	91	101	111
SSQHQERCCN	ELNEFENNQR	CMCEALQQIM	ENQSDRLQGR
121	131	141	151
QQEQQFKREL	RNLPQQCGLR	APQRCDLDVE	SGGRDRY

Figure 1. Sequence of Ara h 2 derived from an Ara h 2 cDNA-clone (6). Dominant epitopes are in bold.

tions demonstrated that the apple allergen Mal d 1 and the cherry allergen Pru av 1 become rapidly inactivated upon heat processing of the fresh fruit (*8*). By contrast, the allergenicity of semipurified allergens such as, e.g., the major celery allergen Api g 1 or the apple allergen Mal d 1 as well as the recombinant cherry allergen rPru av 1 are not or only slightly affected by heat treatment (*8*, *9*). These observations suggest interactions of the allergenic proteins with other fruit ingredients, thus leading to inactivation of the allergen. For example, the nonenzymatic browning reaction, the so-called Maillard reaction, between the cherry allergen rPru av 1 and reducing carbohydrates or carbohydrate breakdown products has been recently shown to strongly decrease the IgE-binding activity of the protein (*9*). In addition, the polyphenoloxidase-catalyzed enzymatic browning reaction between the allergen rPru av 1 and plant polyphenols has been demonstrated to deteriorate allergenicity of the protein as well (*9*).

In contrast to these so-called pollen-related allergens, roasting has been reported to increase the allergenicity of raw peanuts (*10*). For example, protein extracts of thermally treated peanuts have been shown to bind IgE antibodies from patients' sera at up to 90-fold higher levels than extracts obtained from the corresponding nontreated peanuts (*10*). In addition, inhibitory ELISA experiments revealed a significant increase in the IgE-binding activity of the purified major allergens Ara h 1 and Ara h 2 after thermal treatment in the presence of carbohydrates. Accompanying this process-induced change in bioactivity, formation of posttranslational protein modifications and cross links have been discussed on the basis of SDS−PAGE analysis. By means of Western blot analysis with anti-AGE antibodies, these modifications have been immunologically identified as advanced glycation end products (AGE) (*10*, *11*). These data suggest a correlation of the increased IgE-binding activity with the occurrence of AGEs in peanut proteins. Besides Ara h 1/2, peanut agglutinin, considered as a minor allergen (*12*), is reported to be converted into IgE-binding products when incubated with glucose or fructose at elevated temperatures (*13*).

To evaluate its suitability as a substitute of the native protein, the objective of the present investigation was to study whether recombinant rAra h 2 (*14*) features similar immunological changes upon Maillard-type reactions as reported for its natural counterpart (*10*). In addition, carbohydrate-induced changes in the IgE affinity of peanut agglutinin were reinvestigated. To localize the sequences responsible for the formation of highly IgE-binding glycation sites of Ara h 2, the influence of nonenzymatic browning on the IgE binding of synthetic epitope peptides was investigated next. To achieve this model peptides mimicking sequences which contain primary amino groups or arginine, known as primary Maillard targets, as well as the major epitopes 3, 6, and 7 of Ara h 2 were synthesized, and the IgE-binding activity of these peptides was evaluated prior to and after heat treatment in the absence or presence of reducing sugars and reactive carbohydrate breakdown products, respectively.

MATERIALS AND METHODS

Chemicals. The following chemicals were obtained from commercial sources: peanut lectin and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were from Sigma (Steinheim, Germany); D-ribose, glyoxal (40% solution in water), D,L-glyceraldehyde, and methylglyoxal were from Aldrich (Steinheim, Germany); maltose monohydrate, D-glucose, triisopropylsilane, *m*-chloroperbenzoic acid, and L-cysteine were from Merck (Darmstadt, Germany); *N*-acetyl-L-cysteine methylester (Ac-Cys-OMe), trifluoroacetic acid, D-fructose, and 1,2-ethanedithiol were from Fluka (Buchs, Switzerland); 2-sulfo-*N*-hydroxysuccinimide was from Pierce (Dallas, TX). Reagents for solid-phase peptide synthesis were obtained from Applied Biosystems (Weiterstadt, Germany); Fmoc-protected amino acids were from Novabiochem (Läufelfingen, Switzerland). Organic solvents were either freshly distilled or of peptide synthesis or HPLC grade. rAra h 2 was produced as described previously (*14*). The peptide ILDGDNLFP-KVAPQA covering the sequence aa 24−38 of the major birch pollen allergen Bet v 1 was a gift of Prof. Vieths (Paul-Ehrlich-Institut, Langen, Germany).

Sera. Patients sensitized to peanut (P1−P7) were selected according to case history, skin test reactivity, and positive CAP (Pharmacia, Uppsala, Sweden) tests. Sera were collected at the Medical Hospital Borstel after giving informed consent and used for detection of IgE binding to peanut proteins. Clinical data of sera P3 (JG3), P4 (HF4), P5 (JB5), P6 (SK26), and P7 (GK31) and IgE reactivity to peanut extract shown by Western blot were published recently (*15*). Sera from patients sensitive to sweet cherry (C1−C5) and from nonallergenic subjects (N1, N2) were obtained from the Paul-Ehrlich-Institut (Langen, Germany). A rabbit anti-rAra h 2 antiserum was obtained as described previously (*16*).

Solid-Phase Peptide Synthesis (SPPS). Peptides were accomplished automatically on a peptide synthesizer (Applied Biosystems 431) according to the standard Fmoc/O^{t}Bu technology (*17*) using Wang resin (1.13 mmol/g) and the following side-chain protecting groups: *tert*-butoxycarbonyl (Boc) for Lys and Trp, *tert*-butyl (^{t}Bu) for Ser and Thr, *tert*-butoxy (O^{t}Bu) for Asp and Glu, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg, trityl (Trt) for Asn, Cys, Gln, and His, and benzyl (Bzl) for Cys in dehydroalanine synthesis. Briefly, protected amino acids were coupled by activation with *N*,*N*′-dicyclohexylcarbodiimide/*N*-hydroxybenzotriazole using a 10-fold excess relative to the resin's loading, followed by piperidine-induced removal of the Fmoc group. Deprotection of the amino acid side chains and final cleavage from the resin was achieved by treatment with a mixture of trifluoroacetic acid/water/triisopropylsilane (95/2.5/2.5, v/v/v) or trifluoroacetic acid/water/1,2-ethanedithiole/triisopropylsilane (94/2.5/2.5/1, v/v/v/v) in the case of cysteine-containing peptides (10 mL/g resin, 90 min, rt). After filtration the cleavage reagents were removed in vacuo and the crude peptide was precipitated with ice-cooled *tert*-butylmethyl ether, dried in vacuo, dissolved in water, and lyophilized. The crude product was analyzed by LC-MS/MS, purified by means of RP-HPLC, and freeze-dried. LRPCEQHLMQKIQRDEDS (**I**): R_t (HPLC) 27.1 min; LC-MS (ESI-pos) *m*/*z* 1124.9 (17, $[M + Na + H]^{2+}$), 1113.9 (69, $[M + 2H]^{2+}$), 743.5 (100, $[M + 3H]^{3+}$). LQGRQQEQQF-KRELRNL (**II**): R_t (HPLC) 33.7 min; LC-MS (ESI-pos) *m*/*z* 1448.2 (12, $[2M + 3H]^{3+}$), 1086.5 (100, $[M + 2H]^{2+}$), 724.9 (14, $[M + 3H]^{3+}$). LTILVALALFLLAAHA-βAla-EEEEEE (**III**): R_t (HPLC) 62.7 min; LC-MS (ESI-pos) *m*/*z* 1663.9 (18, $[2M + 3H]^{3+}$), 1248.6 (100, $[M + 2H]^{2+}$), 832.7 (35, $[M + 3H]^{3+}$). RQQWELQGDRRCQSQL (**IV**): R_t (HPLC) 33.0 min; LC-MS (ESI-pos) *m*/*z* 1354.3 (16, $[2M + 3H]^{3+}$), 1016.3 (100, $[M + 2H]^{2+}$), 678.4 (37, $[M + 3H]^{3+}$). LQGDRRC-QSQLERAN (**V**): R_t (HPLC) 22.5 min; LC-MS (ESI-pos) *m*/*z* 1774.0 (31, $[M + H]^{+}$), 1183.2 (8, $[2M + 3H]^{3+}$), 887.8 (100, $[M + 2H]^{2+}$), 592.6 (9, $[M + 3H]^{3+}$).

(LQGDRRCQSQLERAN)$_2$ (VI). Crude **V** (8.5 mg, 5 μmol) was dissolved in ammonium acetate buffer (50 mM, pH 7.8, 5 mL), stirred overnight under air exposure, lyophilized, dissolved in water, and purified by means of RP-HPLC. R_t (HPLC): 26.5 min. LC-MS (ESI-pos): *m*/*z* 1775.1 (5, $[M + 2H]^{2+}$), 1183.0 (100, $[M + 3H]^{3+}$), 887.9 (35, $[M + 4H]^{4+}$), 710.7 (2, $[M + 5H]^{5+}$), 592.3 (3, $[M + 6H]^{6+}$).

LQGDRR-Cystine-QSQLERAN (VII). Crude **V** (17 mg, 10 μmol) and L-cysteine (12 mg, 100 μmol, 10 equiv) were dissolved in ammonium acetate buffer (50 mM, pH 7.8, 10 mL), stirred overnight under air exposure, lyophilized, dissolved in water, and purified by means of RP-HPLC. R_t (HPLC): 24.2 min. LC-MS (ESI-pos): *m*/*z*

1893.5 (12, $[M + H]^+$), 1262.3 (19, $[2M + 3H]^{3+}$), 947.2 (100, $[M + 2H]^{2+}$), 632.5 (22, $[M + 3H]^{3+}$).

LQGDRR-Cys(O_3H)-QSQLERAN (VIII). Formic acid (1.8 mL) was treated with H_2O_2 (30%, 200 μL) for 60 min; MeOH (200 μL) was added and cooled to −24 °C. Crude **V** (10 mg, 5.6 μmol) was dissolved in the mixture (500 μL), incubated for 150 min at −24 °C, lyophilized after addition of water (4 mL), resolved, and purified by means of RP-HPLC. R_t (HPLC): 24.1 min. LC-MS (ESI-pos): *m*/*z* 1822.6 (7, $[M + H]^+$), 1214.8 (11, $[2M + 3H]^{3+}$), 911.7 (100, $[M + 2H]^{2+}$), 608.7 (12, $[M + 3H]^{3+}$).

LQGDRR-Dha-QSQLERAN (IX). Boc-LQ(Trt)GD(O^{t}Bu)R(Pbf)R(Pbf)C(Bzl)-Q(Trt)S(^{t}Bu)Q(Trt)LE(O^{t}Bu)R(Pbf)A N(Trt)-Wang-resin was accomplished by means of standard SPPS protocols using the building block Fmoc-Cys(Bzl)-OH. The resin (400 mg, 85 μmol) was oxidized to the corresponding *S*-benzylcysteinesulfon resin using *m*-chloroperbenzoic acid (178 μmol, 2.1 equiv) in dichloromethane (DCM, 5 mL) for 3 h at 0 °C. After intensive washing with DCM and methanol, the dried resin (100 mg, 21 μmol) was treated with potassium *tert*-butylate (9 mg, 84 μmol, 4 equiv) in tetrahydrofuran (THF, 500 μL) for 120 min at room temperature, washed with THF and DCM, and dried. After final cleavage **IX** was purified by means of RP-HPLC. R_t (HPLC): 25.0 min. LC-MS (ESI-pos): *m*/*z* 1740.7 (11, $[M + H]^+$), 882.4 (25, $[M + H + Na]^{2+}$), 870.7 (100, $[M + 2H]^+$), 581.1 (28, $[M + 3H]^{3+}$).

LQGDRR-Lan(Ac)(OMe)-QSQLERAN (X). Boc-LQ(Trt)GD-(O^{t}Bu)R(Pbf) R(Pbf)-Dha-Q(Trt)S(^{t}Bu)Q(Trt)LE(O^{t}Bu)R(Pbf)AN(Trt)-Wang-resin (55 mg, 11 μmol), prepared as described above, was treated with Ac-Cys-OMe (6 mg, 33 μmol, 3 equiv) in THF (1 mL) for 48 h at room temperature, washed with THF and methanol, dried, cleaved, and purified by means of RP-HPLC. R_t (HPLC): 26.5 + 26.7 min (diastereomers). LC-MS (ESI-pos): *m*/*z* 1917.1 (3, $[M + H]^+$), 1278.5 (7, $[2M + 3H]^{3+}$), 959.3 (100, $[M + 2H]^{2+}$), 640.4 (43, $[M + 3H]^{3+}$).

RDEDSYERDPYSPSQ (XI). R_t (HPLC): 24.5 min. LC-MS (ESI-pos): *m*/*z* 1844.6 (9, $[M + H]^+$), 922.7 (100, $[M + 2H]^{2+}$).

PYSPSQDPYSPSPYD (XII). R_t (HPLC): 29.1 min. LC-MS (ESI-pos): *m*/*z* 1699.4 (100, $[M + H]^+$), 1306.4 (9, $[b_{12}]^+$), 1122.3 (11, $[b_{10}]^+$), 1035.3 (5, $[b_9]^+$), 850.5 (81, $[M + 2H]^{2+}$).

Model Experiments with Proteins/Peptides and Carbohydrates. Proteins and synthesized peptides were dissolved in a PBS buffer (pH 6.8) containing sodium phosphate (150 mmol/L) and sodium chloride (150 mmol/L) to give a final concentration of 1 mg/mL for peanut lectin as well as the peptides and a final concentration of 280 μg/mL for rAra h 2. An aliquot (150 μL) of that stock solution was mixed with a PBS solution (150 μL) of either maltose, glucose, fructose, ribose, glyceraldehyde, glyoxal, or methylglyoxal (100 mmol/L each) in a SafeLock Cap (500 μL, Eppendorf, Germany). After incubation at 100 °C for different time periods in an UNO-thermoblock (Biometra, Göttingen, Germany), the preparations were cooled in an ice bath. As control, either the protein or the carbohydrate component was displaced by PBS.

Enzyme-Linked Immunosorbent Assay (ELISA). Native as well as thermally treated peanut lectin and rAra h 2 were diluted in TBS buffer (pH 7.4) containing Tris (50 mmol/L) and sodium chloride (150 mmol/L) and coated onto Maxisorb mircotiter plates (Nunc, Wiesbaden, Germany; 50 μL/well overnight at 4 °C). After washing (3 × 300 μL TBS), plates were blocked using 1% BSA and 0.05% Tween 20 in TBS (300 μL, 1 h, rt), washed again (3 × 300 μL 0.05% Tween 20 in TBS), and incubated with sera (50 μL; 0.1% BSA and 0.05% Tween 20 in TBS overnight at 4 °C). All human sera were used in 1:10 dilution except P1 (1:100). The rabbit serum was diluted 1:200 000. After washing, human IgE specifically bound to the immobilized allergens was determined by a commercial enzyme allergosorbent test (EAST) using the calibration system of the manufacturer (Allergopharma, Reinbek, Germany). Bound rabbit immunoglobolines were visualized using anti-rabbit-Ig-biotin conjugate (Dako, Hamburg, Germany; 1:6000; 50 μL, 1 h, rt), streptavidine alkaline phosphatase conjugate (Medac, Wedel, Germany; 1:3000; 50 μL, 1 h, rt), and a PNPP test kit (Biorad, Munich, Germany). Generally, all experiments were carried out in duplicate. Inhibitory EAST assays using cyanogen bromide activated filter paper disks were performed as described previously (*9*). To achieve this aliquots of the nontreated rAra h 2 (50 ng) were coupled to the disk and dilution series of the thermally treated rAra h 2 preparations were used as inhibitors.

DotBlot Experiments. A 1 μL amount of a peptide solution (100 μg/mL in TBS) was spotted onto cyanogen bromide activated nitrocellulose stripes and dried for 30 min. After washing three times with 0.05% Tween 20 in TBS, stripes were blocked using 0.3% Tween 20 in TBS (2 × 15 min) and washed again. Incubations with sera and secondary antibodies were performed as described for ELISA above. After washing, specific antibody binding was visualized using a nitroblue tetrazolium kit (Biorad, Munich, Germany) according to the manufacturer's instructions. Colorization was stopped by transferring the stripes into water, followed by drying and documentation.

Peptide-ELISA. Aliquots of peptides incubations (200 μL) were cleaned by means of short RP cartridges (Waters, Eschborn, Germany) which were conditioned with acetonitrile (2 mL) and washed with water (10 mL) prior to use. Hydrophilic byproducts were removed by elution with water (1 mL); peptides were eluted with water/acetonitrile (4/1, 3 mL) or water/acetonitrile (1/1, 3 mL) in case of **III**, and the eluates were diluted to 5 mL with water. Peptides were coupled to CovaLink NH stripes (Nunc, Wiesbaden, Germany) according to the manufacturer's instructions. Briefly, diluted aliquots of peptide solutions (50 μL) containing 2-sulfo-*N*-hydroxysuccinimide (9.2 μg per μg of peptide) were activated using EDC (61.5 μg in 50 μL of water) and coupled for 120 min at room temperature. For example, in the case of peptide **V** the purified peptide solution was diluted to a concentration of 280 pmol in 25 μL, mixed with 2-sulfo-*N*-hydroxysuccinimide in water (25 μL), and activated with EDC to give a coated well containing approximately 280 pmol of peptide. After washing (3 × 300 μL water), plates were blocked and immediately used for ELISA as described above.

High-Performance Liquid Chromatography (HPLC). The HPLC apparatus (Bio-Tek, Eching, Germany) consisted of two pumps (type 522), a gradient mixer (M 800), a Rheodyne injector (100 μL loop), and a diode array detector (DAD type 540) monitoring the effluent at 210 nm. Preparative-scale separations (up to 2 mg samples) were performed on a stainless steel column (4.6 × 250 mm, flow rate 1.0 mL/min) packed with RP-18 (Nucleosil 100 C-18, 5 μm) as the stationary phase and a gradient of 1% (v/v) acetonitrile per min in 0.1% (v/v) aqueous trifluoroacetic acid as the liquid phase.

Liquid Chromatography/Mass Spectrometry (LC/MS). An analytical HPLC column (Nucleosil 100-5C18, Macherey and Nagel, Dürren, Germany) was coupled to an LCQ-MS (Finnigan MAT GmbH, Bremen, Germany) using electrospray ionization (ESI). After injection of the sample (2−10 μL), analysis was performed using a gradient of 1% (v/v) acetonitrile per minute in 0.1% (v/v) aqueous trifluoroacetic acid as the liquid phase.

RESULTS AND DISCUSSION

To increase knowledge on the influence of nonenzymatic browning reactions on the immunological activity of peanut proteins, natural peanut agglutinin and the recombinant form of the major allergen rAra h 2, respectively, were thermally treated in the absence and presence of carbohydrates and carbohydrate degradation products. The IgE-binding activities of the thermally treated proteins were then compared to those measured for the native, nonreacted counterparts.

Influence of Maillard Reaction on IgE-Binding Activity of Peanut Agglutinin. To determine the allergenic potential of the peanut lectin before and after Maillard reaction the IgE-binding activity of the native protein was investigated first by an ELISA assay. Single sera samples of seven persons allergenic to peanuts (P1−P7), five persons sensitive to sweet cherry (C1−C5), and two samples of nonallergenic subjects (N1, N2) were subjected to ELISA tests (**Figure 2**). Six out of seven patients allergenic to peanuts (P1, P3−P7) showed an increased level of IgE binding, whereas all patients with cherry allergy and the two nonallergenic subjects did not show any binding to the agglutinin (**Figure 2**). In contradiction to the data published in the literature (*12*), these results clearly indicate a specific

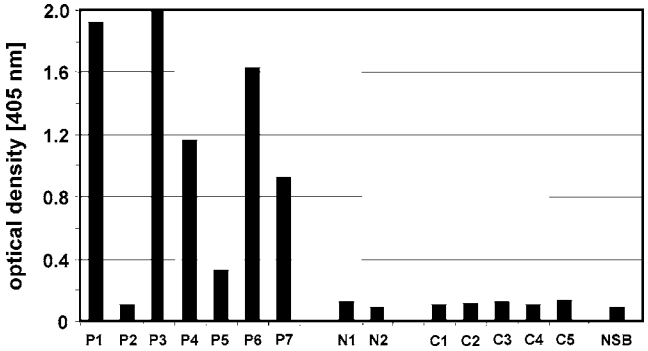

Figure 2. ELISA on specific IgE-binding activity of peanut lectin (250 ng/well) using sera from peanut-sensitive (P1–P7), nonallergenic (N1, N2), and cherry-sensitive subjects (C1–C5); NSB = nonspecific binding.

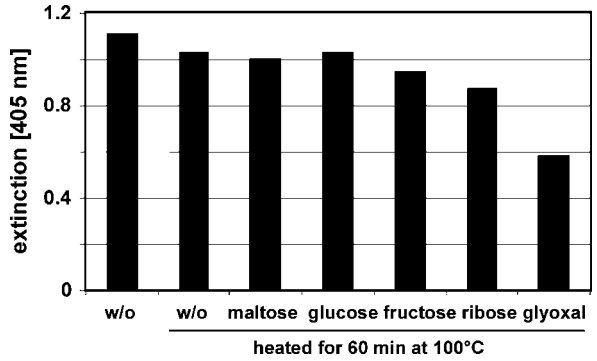

Figure 3. ELISA on the influence of carbohydrates and carbohydrate cleavage products on the IgE-binding activity of peanut lectin (125 ng/well) using pooled patients' sera.

interaction of peanut agglutinin with IgE antibodies from peanut-sensitive patients, thus suggesting this glycoprotein as a potential peanut allergen. Due to the fact that no recognition was observed in the case of sera from cherry-sensitive persons and nonallergenic subjects, nonspecific binding effects, for example, via the sugar affinity of lectins, seem to be very unlikely.

To gain insight into the influence of heat treatment and Maillard reaction on peanut agglutinin activity, the protein was thermally treated for 60 min at 100 °C in the absence and presence of the disaccharide maltose, the monosaccharides glucose, fructose, and ribose, or glyoxal, which is a well-known carbonyl compound formed during carbohydrate fragmentation (*18*). Equivalent amounts of treated and nontreated protein were then coated onto microtiter plates and incubated with pooled sera. ELISA experiments showed that heating in the absence of carbohydrates resulted in just a slight decrease of IgE binding compared to the nontreated agglutinin (control), thus demonstrating the thermal stability of the protein (**Figure 3**). Thermal treatment of the lectin in the presence of the disaccharide maltose as well as the aldohexose glucose showed only little effect on the IgE-binding capacity, whereas a significant loss of antibody binding was detected after incubation of the protein in the presence of the ketohexose fructose and the aldopentose ribose. However, the most dramatic changes have been observed when the protein was heated in the presence of the sugar-derived dicarbonyl glyoxal. These findings, which were further confirmed by competitive EAST assays (data not shown) as described recently (*9*), gave strong evidence that the allergenic activity of peanut agglutinin might be decreased by Maillard-type reactions.

Influence of Maillard Reaction on IgE-Binding Activity of rAra h 2. As earlier reported for its natural counterpart (*10*), the recombinant rAra h 2 was recently shown to have strong affinity toward antibodies from peanut-sensitive patients and a selective anti-rAra h 2 serum from rabbit. To evaluate the influence of Maillard reactions on the allergenic properties of rAra h 2, the recombinant protein was thermally treated in binary mixtures with maltose, glucose, fructose, ribose, glyoxal, and methylglyoxal for 90 min at 100 °C. The IgE-binding activity of the nontreated and the nonenzymatically glycated protein toward pooled patients' sera and the rabbit antiserum was analyzed by means of ELISA as described above. Independent from the serum samples used for this experiment, thermal treatment of rAra h 2 in the absence of any carbonyl compound resulted in a slight but significant higher IgE-binding activity of the protein (**Figure 4**). These findings might be explained by thermally induced changes in protein's three-dimensional structure, thus making linear epitopes better accessible for antibodies. Incubation of the protein in the presence of maltose and hexoses showed no effect on the IgE-binding activity compared to the heated protein, whereas reaction with the pentose ribose induced a partial increase of the IgE-binding ability. However, in particular, the carbohydrate breakdown products methylglyoxal and, even more pronounced, glyoxal caused a dramatic enhancement of the antibody-binding activity of rAra h2. These observations have been found in the case of human and rabbit sera to a comparable extent (**Figure 4**).

To further strengthen our findings, a competitive EAST assay using unmodified rAra h 2 coupled on cyanogen bromide activated filter paper disks and the glycated allergen samples as inhibitors supported these results. As shown in **Figure 5**,

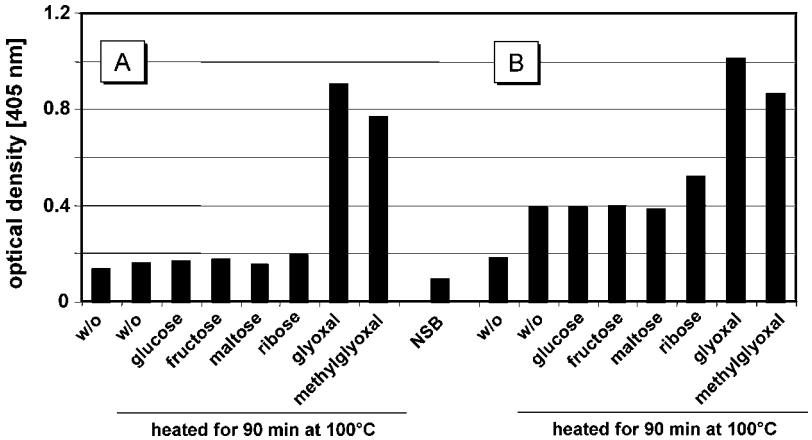

Figure 4. ELISA on the influence of carbohydrates and carbohydrate cleavage products on the IgE-binding activity of rAra h 2 (50 ng/well) using pooled patients' sera (A) and rabbit anti-rAra h 2 serum (B).

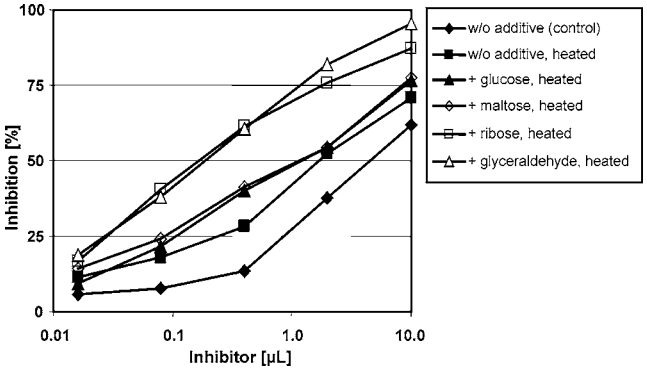

Figure 5. Inhibition assay on the influence of carbohydrates and carbohydrate cleavage products on the IgE-binding activity of rAra h 2 (50 ng of protein per disk; 1:200 dilution of serum P1).

rAra h 2 heated in the absence of any carbonyl revealed a higher inhibitory activity than the nontreated allergen. Incubation of rAra h 2 with glucose and maltose lead to a further increase of the allergen's IgE affinity as can been seen from smaller IC_{50} concentrations. Fitting well with the results of the ELISA experiments (**Figure 4**), the reaction of rAra h 2 with ribose resulted in glycoproteins showing a distinct higher level of IgE binding than glucose- or maltose-modified proteins. As an example of a highly reactive reducing carbohydrate, glyceraldehyde was found to convert rAra h 2 into exceedingly IgE-affine products too. Control experiments performed with sera obtained from nonallergenic subjects did not show any IgE binding at all, and also nonspecific binding was not detectable.

Taking all these data into account, it can be concluded that nonenzymatic browning reactions increase the IgE affinity of recombinant rAra h 2 as earlier shown for its native counterpart isolated from peanuts. Moreover, the sugar-induced enhancement of the antibody binding activity of rAra h 2 was detected not only with human patients' sera but also with rabbit anti-rAra h 2 serum.

Influence of the Maillard Reaction on IgE-Binding Activity of Individual Ara h 2 Sequences. Free primary amino groups within proteins are well-known targets for the attack of carbonyl compounds during Maillard-type reactions, leading to formation of stable glycation end products. Starting from the sequence of Ara h 2 published recently (**Figure 1**), it is very likely that the two lysine residues as well as the N-terminus may be key targets for nonenzymatic modifications with carbonyls. To investigate whether the IgE-binding activity of these Ara h 2 segments can be altered upon glycation, model peptides covering the sequences in question have been synthesized by means of solid-phase peptide synthesis (**Table 1**). As our preliminary experiments on Ara h 2 epitopes demonstrated that synthesized decapeptides did not show significant IgE-binding activity when subjected to immunological assays, we investigated the use of 15-meres for the following experiments. Indeed, these peptides showed pronounced IgE-binding activities; for example, a simple DotBlot assay performed with epitope sequences covalently coupled to cyanogen bromide activated nitrocellulose revealed a better recognition of the 15-meres of epitopes 6 and 7 than for the decapeptidic variants (**Figure 6**). Due to these results all peptides investigated in the following have been synthesized with a minimum length of 15 amino acids.

Using standard SPPS protocols the lysine-containing segments L^{39}RPCEQHLMQKIQRDEDS^{56} (**I**) and L^{116}QGRQQEQQFKRELRNL^{133} (**II**) have been obtained in good yields. There may be some uncertainty as to the nature of the Ara h 2 N-terminus. Though the published sequence starts with Leu^1, the part aa 1−18 is considered to represent a leader sequence, making Arg^{19} the N-terminal amino acid of natural Ara h 2. Due to the fact that the leader sequence is still present in the used recombinant protein it was decided to synthesize model peptides covering both the leader sequence aa 1−18 as well as the sequence aa 19−34.

Table 1. Sequences and Synthesis of Peptides I–XII

entry	amino acid sequence[a]	synthesis	purity of crude peptide [%]
I	LRPCEQHLMQKIQRDEDS	SPPS	69
II	LQGRQQEQQFKRELRNL	SPPS	89
III	LTILVALALFLLAAHA-βAla-EEEEEE	SPPS	80
IV	RQQWELQG***DRRCQSQL***	SPPS	53
V	LQG***DRR-Cys-QSQLER***AN	SPPS	68
VI	LQGDRR-Cys-QSQLERAN | LQGDRR-Cys-QSQLERAN	V + O_2	54[b]
VII	LQGDRR-Cystine-QSQLERAN	V + Cys + O_2	44[b]
VIII	LQGDRR-Cys(O_3H)-QSQLERAN	V + HCO(O_2)H	28[b]
IX	LQGDRR-Dha-QSQLERAN		9[b]
X	LQGDRR-Lan(Ac)(OMe)-QSQLERAN		15[b]
XI	RDEDS***YERDPYSPSQ***	SPPS	78
XII	***PYSPSQDPYSPSPY***D	SPPS	95

[a] Sequences of the dominant epitopes are marked in italics (*6*). [b] Total yield after purification.

Regarding the synthesis of the leader sequence, the hydrophobic nature of this sequence caused model peptides such as L^1TILVALALFLLAAHA^{16} and L^1TILVALALFLLAAHASAR^{19} to be insoluble in aqueous buffer, making them unusable for browning reactions and immunological evaluation. To overcome this problem we decided to attach a hydrophilic polyglutamic acid tag, which was considered to be indifferent toward browning reaction and immunological properties of the peptide, via a β-alanine spacer to the sequence. The peptide LTILVALALFLLAAHA-βAla-EEEEEE (**III**) was synthesized, purified by RP-HPLC, and found to be soluble in aqueous buffer. To model the Maillard reaction of the aa 19−34 region of Ara h 2, the 15-mer peptide R^{19}QQWELQGDRRCQSQL^{34} (**IV**) was synthesized by SPPS and purified by RP-HPLC.

To investigate the influence of the Maillard reaction on the IgE-binding activities of the peptides **I**−**IV**, each individual peptide was subjected to nonenzymatic browning reactions with different carbohydrates and α-dicarbonyles for 90 min at 100 °C. To remove the buffer salts, residual carbohydrate, and hydrophilic carbohydrate degradation products from the glycated peptides, the reaction mixtures were applied onto the top of RP18 cartridges and then rinsed with water. After eluting the glycated peptides with diluted acetonitrile, these were analyzed for their immunological activities by means of a special ELISA assay based on CovaLink NH stripes. To achieve this, nontreated (control) and glycated peptides were bound covalently to a linker grafted onto the wells surface. Thereafter, the IgE binding toward pooled patients' sera and single patient serum P1was measured. Being well in line with the recent finding that peptides **I**−**III** do not contain any or contain only small parts of sequences considered as minor Ara h 2 epitopes, no IgE-binding was observed for these molecules prior to or after reaction in the presence of carbohydrates (data not shown). Peptide **IV** showed a very weak IgE binding, which slightly decreased upon heating and became more decreased when incubated in the presence of carbohydrates (data not shown). The IgE binding detected may be based on the fact that peptide **IV** covers a

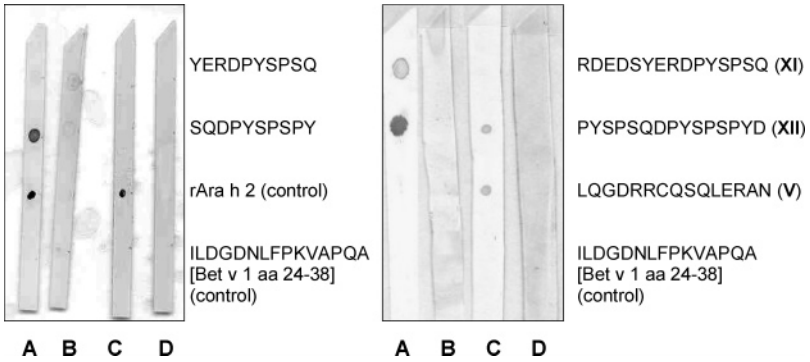

Figure 6. DotBlot assay of peptides covering the dominant epitopes synthesized as decamers (left) and pentadecamers (right, 100 ng each) compared to rAra h 2 and a non Ara h 2 peptide. Sera samples: anti-rAra h 2-rabbit (A, 1:20 000), rabbit prior to immunization (B, 1:20 000), peanut-sensitive P1 (C, 1:10), nonallergenic subject N1 (D, 1:10).

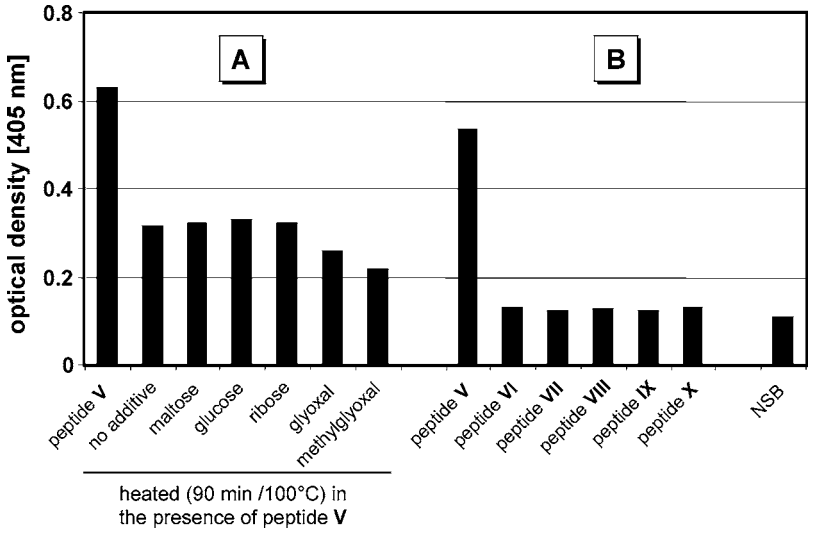

Figure 7. Peptide ELISA on specific IgE-binding activity of **V** (280 pmol/well) and the modified variants **VI**–**X** (280 pmol each): (A) pool serum, (B) serum P1.

sequence of eight amino acids belonging to the major epitope 3. In summary, carbohydrate-induced modifications of both the lysine residues and the N-terminus of either the leader sequence or the natural Ara h 2 molecule can be excluded from contributing to the formation of highly IgE-binding modifications of Ara h 2 during Maillard reaction.

Influence of the Maillard Reaction on the Major Epitopes of Ara h 2. Aimed at investigating the influence of Maillard-type modifications on the IgE-binding activity of the major Ara h 2 epitope 3, first, peptide L^{24}QGDRRCQSQLERAN^{38} (**V**) bearing the sequence of epitope 3 was synthesized and, then, heated in the absence or presence of carbohydrates for 90 min at 100 °C. After cooling, the IgE affinity of the nontreated (control), heated, as well as carbohydrate-modified peptide was analyzed using the ELISA assay developed above. As shown in **Figure 7**, peptide **V** was recognized by IgE from pooled patients' sera. Single heat treatment resulted in a drastic loss of IgE-binding activity, whereas the addition of carbohydrates prior to thermal treatment showed no additional effect. α-Dicarbonyl compounds led to a further decrease of IgE-binding capacity.

Taking a close look at the epitope's amino acid constitution, it is likely that the cysteine residue is affected by means of air oxidation during heating. In addition, formation of dehydroalanine residues and related derivatives such as, e.g., lanthionine are well-known thermal degradation products of protein-bound cysteine (**Figure 8**) (*19*). Those products might influence the level of IgE affinity of the epitope and could contribute to the

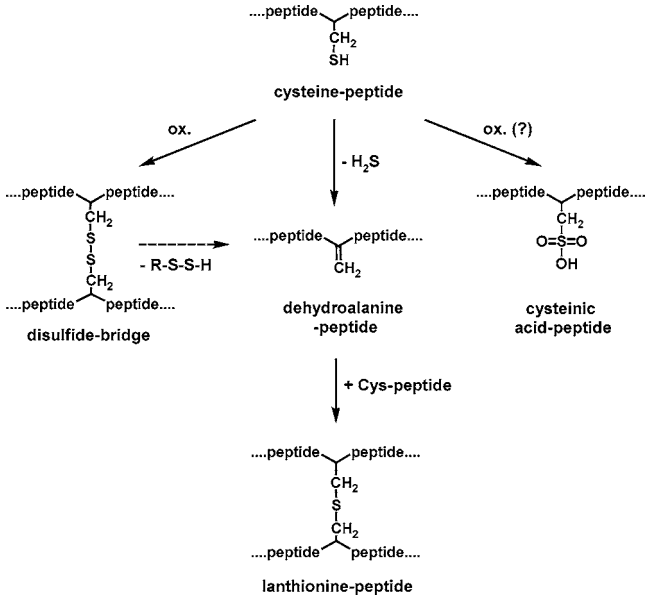

Figure 8. Structure of thermally generated degradation products formed from protein-bound cysteine.

observed effects. To verify this hypothesis some analogues of peptide **V** covering those modifications were synthesized (**Table 1**) and analyzed in their IgE-binding activities. Neither the disulfide-bridged dimer (**VI**) nor peptidic variations bearing

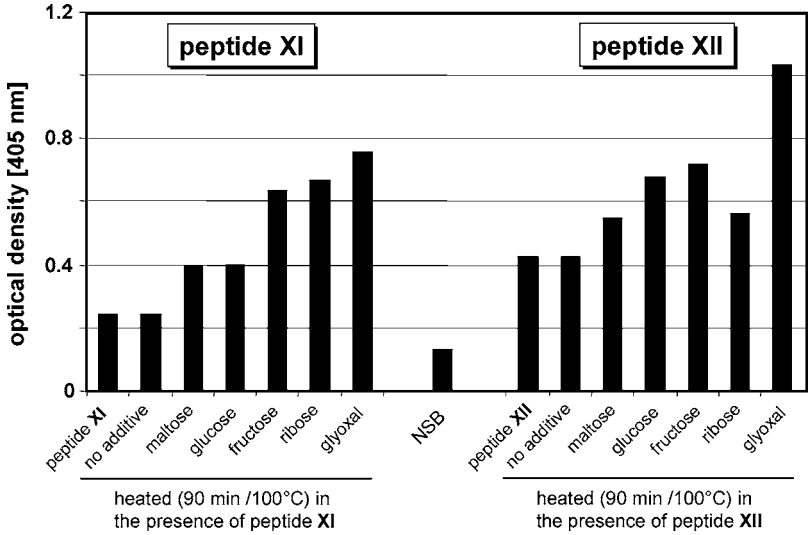

Figure 9. Peptide ELISA on the influence of carbohydrates and carbohydrate cleavage products on the IgE-binding activity of **XI** (270 pmol/well) and **XII** (300 pmol/well).

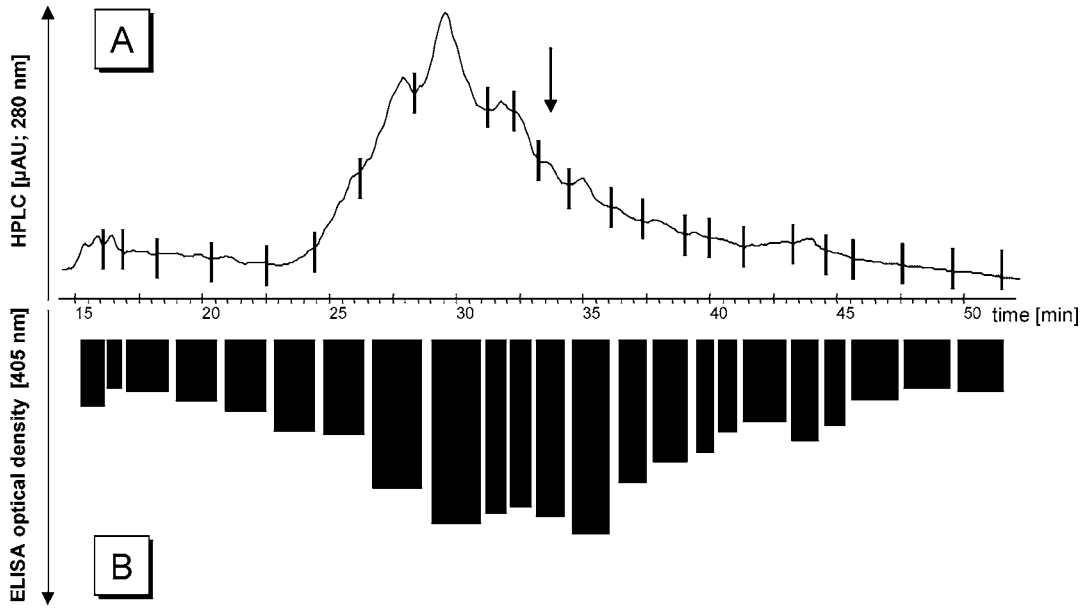

Figure 10. HPLC chromatogram (A) and IgE-binding activity (B) of isolated fractions of a heat-treated model incubation consisting of **XI** with glyoxal. Retention time of nonmodified peptide **XI** is marked with an arrow.

cystine (**VII**), cysteinic acid (**VIII**), dehydroalanine (**IX**), and lanthionine (**X**) instead of cysteine showed any IgE binding when subjected to ELISA equimolarly as seen in **Figure 7**. These results indicate that the unmodified Cys^{30} residue within epitope 3 is essential for IgE binding, which is in contrast to the results obtained recently from an alanine scan of the allergen's epitopes (*6*). Thus far, loss of IgE binding toward epitope 3 during heating under air exposure can be explained by formation of cysteine oxidation and degradation products. Furthermore, it is clearly demonstrated that epitope 3 is not involved in the formation of highly IgE-affine carbohydrate modifications of Ara h 2.

To further investigate the role of the Maillard reaction on other epitope structures, peptide R^{52}DEDSYERDPYSPSQ^{66} (**XI**), representing some part of epitope 5 and complete epitope 6 (of Ara h 2), and peptide P^{61}YSPSQDPYSPSPYD^{75} (**XII**), covering part of epitope 6 and entire epitope 7 of Ara h 2, have been synthesized, and their IgE-binding activities have been determined prior to and after heating in the presence of carbohydrates. Peptide **XI** showed a significantly higher level of IgE binding after incubation with carbohydrates and, even more pronounced, the carbohydrate breakdown product glyoxal for 90 min at 100 °C (**Figure 9**). As arginine moieties are well known as targets for Maillard modifications, the carbohydrates might be able to convert arginine residues into highly IgE-affine glycation sites. Surprisingly, the overlapping peptide P^{61}YSPSQDPYSPSPYD^{75} (**XII**) covering a part of epitope 6 and entire epitope 7 revealed the same effect (**Figure 9**). It is worth noting that **XII** does not contain any amino acid residue which is known to undergo Maillard-type glycation reactions such as, e.g., lysine and arginine. These data give strong evidence that in addition to these basic amino acids, other amino acids, which have been not yet considered as targets of the Maillard reaction, might be accessible for nonenzymatic glycation reactions and that these posttranslational modifications might induce increased IgE binding of the glycated Ara h 2.

Taking all the results into account, it can be summarized that overlapping epitopes 6 and 7 are candidate regions of the Ara h 2 sequence responsible for the carbohydrate-induced enhancement of IgE-binding activity of the protein. To gain first insight

into the reaction products formed upon reaction of the pentadecapeptide epitope with carbohydrate degradation products, the heated mixture of peptide **XI** and glyoxal was analyzed by RP-HPLC. As given in **Figure 10**, heating the binary mixture of peptide **XI** and glyoxal resulted in a complex "hump" of multiple reaction products, which could not be sufficiently separated. To gain at least some insight into the IgE-binding activities of such reaction products, the HPLC effluent was collected into 23 fractions, and after freeze-drying, their IgE-binding activities were determined by means of the ELISA assay developed. Several of the isolated fractions showed high levels of IgE binding when compared to nonmodified **XI** (**Figure 10**). Experiments to further narrow down the carbohydrate-accessible target amino acids in the sequence of the peptide **XI** are needed. Finally, it is interesting to note that all effects concerning the investigated peptides **I**−**XII** have been observed also when patient's sera were displaced by the rabbit anti rAra h 2 serum. Since the rabbit anti rAra h 2 serum is reactive to epitope 6 and 7, as shown by epitope mapping (*16*), this finding underlines that IgE reactivity is strengthened by Maillard products and not by sensitization to neoallergens.

ACKNOWLEDGMENT

We thank Prof. Dr. Stefan Vieths, Paul-Ehrlich-Institute, Department of Allergology, Langen, Germany, for supplying us with the peptide ILDGDNLFPKVAPQA and some sera samples.

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Received for review September 24, 2004. Accepted January 14, 2005. This work was supported by grants from the Deutsche Forschungsgemeinschaft (VI 165/2-3, Ho 2116/2-1, Ho 2116/2-3, Be 730/3-3,4).

JF048398W