JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of Pepsin-Susceptible and Pepsin-Resistant Epitopes in Native and Heat-Treated Peanut Allergen Ara h 1

EVELIEN L. VAN BOXTEL,[†] STEF J. KOPPELMAN,[§] LAMBERTUS A. M. VAN DEN BROEK,[#] AND HARRY GRUPPEN*,[†]

Laboratory of Food Chemistry, Wageningen University, P.O. Box 8129, 6700 EV Wageningen, The Netherlands; TNO Quality of Life, P.O. Box 360, 3700 AJ Zeist, The Netherlands; and WUR-AFSG Biobased Products, P.O. Box 17, 6700 AA Wageningen, The Netherlands

This study was aimed at the determination of the pepsin-susceptible and pepsin-resistant epitopes in native and heat-treated Ara h 1, a major allergen from peanuts. Both the oligomeric structure and the trimeric structure of the allergen were investigated. Under the in vitro conditions applied, oligomeric Ara h 1, either unheated or preheated, was hydrolyzed by pepsin at a lower rate than trimeric Ara h 1. Peptides with relatively high molecular masses were shown to be able to bind IgE, whereas peptides with lower molecular masses (<2 kDa) did not. In these latter fractions, fragments of 15 previously published epitopes of mature Ara h 1 were identified. As a result, these epitopes are not likely responsible for the induction of systemic food allergic reactions to peanuts. Using sequential chymotrypsin digestion, the pepsin-resistant IgE-binding peptides were deduced to contain the previously identified intact epitopes EDWRRPSHQQ (amino acids 50–59) and PRKIRPEG (amino acids 60–67). The presence of four additional earlier published intact epitopes (covering amino acids 6–13, 14–21, 24–31, and 40–47) on the pepsin-resistant peptides could be neither deduced nor ruled out. The two deduced and four possible pepsin-resistant epitopes are all situated in the N-terminal part of Ara h 1, which does not show homology with other vicilin proteins. Consequently, this unique N-terminal part of Ara h 1 is proposed to be responsible for the allergen's ability to induce systemic allergic reactions.

KEYWORDS: Food allergy; Ara h 1; epitopes; IgE binding; mass spectrometry

INTRODUCTION

The resistance of the IgE-binding capacity of allergens toward digestion in the human gastrointestinal tract is considered to be a prerequisite for food allergens to cause (severe) systemic reactions in allergic individuals. This resistance toward digestion implies that allergens have to withstand the low pH and pepsin activity in the stomach in a way allowing either the intact allergen or peptides containing IgE epitopes to be preserved, so that when they reach the intestinal mucosa, they can be absorbed (*1*). At least two IgE epitopes on one protein or peptide are necessary to induce cross-linking of the IgE receptors, the latter being necessary for allergic symptoms to occur. Considering linear IgE binding epitopes to have a length of approximately 15 amino acids (*2*), peptides of about 30 amino acids, corresponding to approximately 3 kDa, in theory are able to elicit allergic responses.

For many allergenic proteins (linear) IgE-binding epitopes have been determined. However, for most allergens it is not known which of these epitopes remain intact after peptic digestion. It seems worthwile to determine pepsin-resistant epitopes, as these epitopes are likely to be responsible for the induction of systemic, often severe, food allergic reactions.

The digestibility of proteins can be affected by various factors, such as heating and chemical modifications. Heat-induced unfolding of proteins may, for example, increase the accessibility of the proteins for hydrolytic enzymes. Opposite to heat-induced unfolding, chemical modifications and heat-induced aggregation, the latter often occurring after protein unfolding, may decrease the accessibilities for digestive enzymes (*3*). Protein digestibility may also be influenced by other components present in the food matrix. For example, covalent interactions of phenolic compounds with soy glycinin enhanced the digestibility by pancreatin (*4*). Also, covalent interactions of egg lysozyme with various phenolic compounds resulted in an increased digestibility by trypsin, chymotrypsin, and pancreatin, although accompanied by a decreased digestibility by pepsin (*5*).

Peanut allergy is one of the most prevalent food allergies in adults. Allergic reactions to peanuts are usually persistent for

* Corresponding author (telephone +31 317483211; fax +31 317484893; e-mail Harry.Gruppen@wur.nl).
[†] Wageningen University.
[§] TNO Quality of Life.
[#] WUR-AFSG Biobased Products.

10.1021/jf072907n CCC: $40.75
Published on Web 02/26/2008

Table 1. Previously Identified Epitopes on the Amino Acid (a.a.) Sequence of Ara h 1 (P43238)

epitope	a.a. sequence	a.a. in sequence Ara h 1
1[a]	ERTRGRQP	6−13
2[a]	GDYDDDRR	14−21
3[a]	RREEGGRW	24−31
4[a]	EREEDWRQ	40−47
5[a]	EDWRRPSHQQ	50−59
6[a]	PRKIRPEG	60−67
7[a]	PGQFEDFF	211−218
8[a]	YLQEFSRN[c]	228−235
9[a]	FNAEFNEIRR	241−250
10[a]	QEERGQRR	261−268
11[a]	DITNPINLRE	309−318
12[a]	NNFGKLFEVK	325−334
13[a]	GNLELV	379−384
14[a]	RRYTARLKEG	414−423
15[a]	ELHLLGFGIN	441−450
16[a]	HRIFLAGDKD	455−464
17[a]	IDQIEKQAKD	467−476
18[a]	KDLAFPGSGE	475−484
19[a]	KESHFVSARP	494−503
20[a]	EKESPEKE	514−521
21[b]	NEGVIVKVSKEHVEELTKHAKSVSK	277−301

[a] Identified by Burks and co-workers (*7*). [b] Identified by Shreffler and co-workers (*9*). [c] E = G in amino acid sequence Ara h 1.

life and can cause life-threatening symptoms (*6*). Ara h 1 is one of the most important allergens in peanuts and is described as a 63 kDa glycosylated seed storage protein. On the mature polypeptide 21 epitopes have been identified (*7*–*9*) (**Table 1**).

Ara h 1 is classified as a member of the vicilin protein family, belonging to the cupin superfamily. The allergen has long been assumed to occur in peanuts as a trimer. However, it was recently reported that the allergen upon extraction from peanuts occurs as a large oligomer (*10*). The oligomerization of Ara h 1 is supposed to be caused by non-covalent interactions of proanthocyanidins with Ara h 1 trimers, which results in complexes containing multiple Ara h 1 trimers interlinked with proanthocyanidins (*11*).

Heating of trimeric Ara h 1 (85 °C, 60 min) causes an irreversible denaturation of the protein, which results in the formation of water-insoluble aggregates. The IgE binding of the allergen is reported not to be affected by heating (*12*). Upon pepsin digestion, unheated Ara h 1 was shown to be rapidly degraded into persisting peptides, which were still able to bind to IgE (*13*–*15*). Furthermore, digestion fragments were shown to have retained their ability to induce histamine release (*14*). No identification of the peptides has been established. So far, the effects of heat-induced denaturation and aggregation on the digestibility of Ara h 1 have not been described in the literature.

As epitopes remaining upon gastrointestinal digestion are likely to be responsible for the induction of systemic food allergic reactions, it would be relevant to identify the pepsin-resistant epitopes of major food allergens. Besides, as most foods are heat-processed before consumption, the effects of heating on the digestion and remaining IgE binding of food allergens are also important in this respect. The aim of the present study was, therefore, to determine the pepsin-susceptible and pepsin-resistant IgE epitopes of the mature major peanut allergen Ara h 1, either unheated or heated before pepsin digestion. Both the oligomeric structure of Ara h 1 (Ara h $1_{oligomers}$), which is considered to be representative for the structure of the allergen to be normally consumed by humans (*11*), and the purification-induced trimeric form of the allergen (Ara h $1_{trimers}$) were used as substrates.

MATERIALS AND METHODS

Materials. All chemicals were obtained from Merck (Darmstadt, Germany) or Sigma (Sigma-Aldrich, Inc., St. Louis, MO), unless stated otherwise. Peanuts of the Runner market-type were provided by Imko Nut Products (Doetinchem, The Netherlands) and stored at 4 °C until use. The oligomeric and trimeric forms of Ara h 1 were purified as described before (*11*) and denoted Ara h $1_{oligomers}$ and Ara h $1_{trimers}$, respectively. The purity of the proteins was estimated to be >95%, as estimated using a densitometric scan of an SDS-PAGE gel stained with Coomassie Brilliant Blue. Millipore water was used for all experiments (Millipore Corp., Bedford, MA). The ionic strength of Ara h 1 in water was arbitrarily set at 0 M.

Plasma from five patients with allergy for peanuts (obtained from Plasmalab International, Everett, WA) and serum from one patient allergic to peanuts were used in this study. CAP-FEIA levels specific for peanuts were between 45 and >100 kU L^{-1}. Part of the plasma and serum samples were mixed in equal volume ratios, denoted Plasmapool, and used for IgE dot-blotting experiments.

Concentration. Protein samples were concentrated at ambient temperature in a vacuum centrifuge (Thermo Electron Corp., Waltham, MA) until the desired volume.

Protein Quantification. The nitrogen content of samples was determined using the combustion (Dumas) method on an NA 2100 Nitrogen and Protein Analyzer (CE Instruments, Milan, Italy). The instructions of the supplier were followed, and methionine was used as a standard. A protein conversion factor of 6.03 for Ara h 1 was calculated from its amino acid sequence (www.expasy.org/sprot; accession no. P43238) and its content and composition of linked N-glycans (*16*).

Differential Scanning Calorimetry (DSC). DSC experiments were performed on a VP-DSC Microcalorimeter (MicroCal Inc., Northampton, MA). Thermograms were recorded from 20 to 120 °C with a heating rate of 1 °C min^{-1}. Experiments were conducted with protein samples in 15 mM sodium phosphate buffer, pH 6.2, with or without the addition of 180 mM NaCl (I = 0.2 and 0.02 M, respectively).

Heating Experiments. Ara h 1 (2.0–2.5 mg mL^{-1}) in 15 mM sodium phosphate buffer, pH 6.2, with or without the addition of 180 mM NaCl, was heated in a water bath at 75, 85, 95, and 100 °C for 10 min. After heating, samples were immediately cooled on ice. Samples that were subjected to pepsin digestion afterward were used directly, whereas for the other investigations samples were centrifuged at 22000*g* for 5 min at 4 °C, after which the supernatant was collected and used for further analysis.

In Vitro Digestion Experiments. The pH of 2.5 mg mL^{-1} heated (10 min, 100 °C) and unheated protein solutions in 15 mM sodium phosphate buffer, pH 6.2, was adjusted to 2.0 with 1 M HCl. Next, samples were mixed 1:1 (v/v) with 5 μg mL^{-1} pepsin (P6887, Sigma) in 30 mM NaCl, of which the pH was adjusted to 2.0 with 1 M HCl. Samples were incubated at 37 °C during different time intervals. The reaction was stopped by raising the pH to 7.0–7.2 with 1 M NaOH.

Size Exclusion Chromatography (SEC) under Non-denaturing Conditions. Protein samples (100 μL) were applied onto a Superose 6 10/300 column (GE Healthcare, Uppsala, Sweden). The column was equilibrated and eluted with 15 mM sodium phosphate buffer, pH 6.2, containing 180 mM NaCl (I = 0.2 M), at a flow rate of 0.5 mL min^{-1} using an Äkta Purifier system (GE Healthcare) operated at room temperature. The eluate was monitored at 280 nm.

SEC under Denaturing Conditions. Protein samples (100 μL) were mixed with 50 μL of acetonitrile (ACN), containing 2% (v/v) trifluoroacetic acid (TFA). After mixing at 1000 rpm for 1 h at ambient temperature in an Eppendorf mixer (Eppendorf AG, Hamburg, Germany), the samples were centrifuged (10 min, 22000*g*) and the supernatants (50 μL) were applied onto a Shodex Protein KW-802.5 column (8 × 300 mm, Showa Denko K.K., Kanagawa, Japan), using an Äkta purifier system (GE Healthcare). The column was equilibrated and eluted with 30% (v/v) aqueous acetonitrile containing 0.1% (v/v) TFA. The flow rate was 0.5 mL min^{-1}, and the absorbance of the eluate was measured at 220 nm. Fractions (0.2 mL) were collected.

Matrix-Assisted Laser Desorption/Ionization−Time-of-Flight Mass Spectrometry (MALDI-TOF MS). MALDI-TOF MS was

performed using an Ultraflex workstation (Bruker Daltonics, Bremen, Germany) operated in the positive mode. The instrument was controlled by Flexanalysis 2.2 software (Bruker Daltonics). The mass ranges used were *m/z* 500–4000, *m/z* 3000–7000, and *m/z* 3000–12000. The laser power was 23%. Protein samples were mixed on a MALDI-TOF plate in a ratio of 1:1 (v/v) with a 50 mg mL^{-1} solution of α-cyanohydroxycinnamic acid or a 10 mg mL^{-1} dihydroxybenzoic acid solution in 50% (v/v) acetonitrile and allowed to dry in the air. The mass spectrometer was calibrated with a mixture of peptides (Bruker Daltonics). At least 200 spectra were collected for each sample.

Reversed-Phase High Performance Liquid Chromatography–Mass Spectrometry (RP-HPLC-MS). ACN and formic acid (FA) were added to protein samples to final concentrations of 5% (v/v) and 0.1% (v/v), respectively. Afterward, samples (50 μL) were applied onto a Vydac C18 MS column (C18MS52; 250 × 2.1 mm; GraceVydac, Hesperia, CA), installed on a Spectra System HPLC (Thermo Separation Products, Fremont, CA), at a flow rate of 0.2 mL min^{-1}. The solvents used were (A) water containing 0.1% (v/v) FA and (B) ACN containing 0.085% (v/v) FA. The column was eluted for 10 min with 95% A and 5% B, followed by a linear gradient from 5 to 45% B until 80 min, a linear gradient from 45 to 95% B until 90 min, isocratic elution at 95% B for 5 min, and a linear gradient from 95 to 5% B in 1 min. Subsequently, the column was eluted at 5% B during 14 min. The UV absorbance at 214 nm was measured. Next to UV detection, mass detection was performed using an LCQ Deca XP MAX (Thermo Finnigan, San Jose, CA) with the use of electrospray ionization and detection in the positive mode. The capillary spray voltage was 4.7 kV, and the capillary temperature was 200 °C. The instrument was controlled by Xcalibur software version 1.3 (Thermo Finnigan). The scan range was set from *m/z* 400 to 2000. MS/MS functions were performed in data-dependent mode. The collision energy was 35%. Bioworks software, version 3.3 (Thermo Electron, San Jose, CA) was used for automatic sequencing and database search for the sequences in a database containing the sequence of Ara h 1 (Swissprot accession no. P43238). In the database search the possible oxidation of methionine was included. To discriminate between correct and incorrect peptide sequence assignments, the cross-correlation value (Xcorr) for each identified peptide was used as a criterion. For positive identification of the peptides an Xcorr threshold of 1.5 for single charged peptides, 2.0 for double charged peptides, and 2.5 for triple charged peptides was used (*17, 18*). Before every analysis, the instrument was tuned with a 1.67 μmol mL^{-1} solution of a peptide consisting of methionine−arginine−phenylalanine−alanine (MRFA).

IgE Dot-Blotting Experiments. For IgE dot-blotting protein samples in 30% (v/v) ACN and 0.1% (v/v) TFA (10 μL) were applied to poly(vinyl difluoride) (PVDF) sheets (Bio-Rad Laboratories Inc., Hercules, CA; article 162-0177). The sheets were air-dried at 30 °C. Subsequently, membranes were blocked with 3% (w/v) bovine serum albumin (BSA, Sigma, article A4503) in TBS buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl) for 1 h at room temperature and subsequently incubated overnight at room temperature with Plasmapool, 30 times diluted in washing buffer [TBS containing 0.5% (w/v) BSA and 0.1% (w/v) Tween-20]. After washing with washing buffer, phosphatase-labeled anti-IgE was added (500 times diluted in washing buffer; Sigma, article A3076), and the membranes were incubated for 2 h at room temperature. After washing, membranes were stained using 5-bromo-4-chloro-3-indolyl phosphate/Nitroblue Tetrazolium (BCIP/NBT) liquid substrate (Sigma, article B1911).

Chymotryptic Hydrolysis. Pooled SEC (under denaturing conditions) fractions of peptic hydrolysates were lyophilized. Next, 80 μL of 100 mM Tris-HCl buffer, pH 7.8, containing 10 mM $CaCl_2$ was added. Subsequently, 3–10 μL of an 0.05 μg mL^{-1} chymotrypsin (article 1418467, Roche Diagnostics GmbH, Penzberg, Germany; specifically hydrolyzes peptide bonds at the C-terminal of tyrosine, phenylalanine, tryptophan, leucine, methionine, alanine, aspartic acid, and glutamic acid) solution in 100 mM Tris-HCl buffer, pH 7.8, containing 10 mM $CaCl_2$ was added. Next, samples were incubated overnight at 25 °C while mixing. After incubation, ACN and FA were added to final concentrations of 5 and 0.1% (v/v), respectively and samples were analyzed using RP-HPLC-MS.

N-Terminal Sequencing. N-Terminal sequencing was performed by Eurosequence (Eurosequence BV, Groningen, The Netherlands). Peptide fractions were analyzed using N-terminal sequence analysis after they had been loaded on a prewashed and Biobrene-coated glass filter. N-Terminal sequence analysis was performed by Edman degradation (*19, 20*) with a Procise 494 (Applied Biosystems, Foster City, CA) automated sequencing system (*21*), equipped with a 140 C Microgradient System and a 758A absorbance detector, and using protocols, reagents, chemicals, and materials from Applied Biosystems.

RESULTS AND DISCUSSION

Heat Stability. The aim of the present study was to identify the pepsin-susceptible and pepsin-resistant epitopes of the mature major peanut allergen Ara h 1 and to investigate the effect of heating on the pepsin digestibility of this allergen. Both the oligomeric (Ara h $1_{oligomers}$) and trimeric structures (Ara h $1_{trimers}$) of the allergen were studied (*11*).

The heat stability of the two quaternary forms of Ara h 1 was investigated to compare their behaviors upon heating. The DSC experiments showed that the denaturation temperatures of Ara h $1_{oligomers}$ and Ara h $1_{trimers}$ were similar at ionic strengths of 0.02 and 0.2 M, respectively. At an ionic strength of 0.02 M both proteins had a denaturation temperature of 83.3 ± 0.1 °C, whereas at an ionic strength of 0.2 M Ara h $1_{oligomers}$ denatured at 86.4 ± 0.0 °C and Ara h $1_{trimers}$ at 86.3 ± 0.2 °C. The denaturation was found to be irreversible for both protein preparations, as upon reheating the protein samples, no transition could be observed anymore. The denaturation temperatures obtained were somewhat lower than the denaturation temperature of 87 °C for trimeric Ara h 1, at $I = 0.02$ M, which was reported before (*12*). However, the pH values differed between the present experiments (pH 6.2) and those described previously (pH 7.4) (*12*), which might (partly) explain the difference in denaturation temperatures observed.

When Ara h $1_{oligomers}$ and Ara h $1_{trimers}$ were heated at temperatures ranging from 75 to 95 °C at pH 6.2 and the supernatants were analyzed after centrifugation on SEC, it appeared that both protein preparations behaved similarly. The native protein peaks diminished when samples were heated at temperatures of 85 °C or higher. The protein concentration in the supernatant was also diminished in these samples (data not shown), indicating that Ara h 1 forms insoluble aggregates upon heating, regardless of the protein's quaternary structure prior to heating. Our results coincide with those described in the literature, as the formation of insoluble protein aggregates upon heating was earlier reported for trimeric Ara h 1 (*12*).

Altogether, from these results it can be concluded that the heat stability of Ara h $1_{oligomers}$ is not different from the heat stability of Ara h $1_{trimers}$, as both protein preparations irreversibly denatured at similar temperatures and formed insoluble aggregates upon heating.

Digestion. Unheated and heated Ara h $1_{oligomers}$ and Ara h $1_{trimers}$ were subjected to peptic digestion. Afterward, the proteins and their hydrolysates were analyzed with SEC under denaturing conditions. Under these conditions, all samples were solubilized. The chromatograms of unheated and heated protein preparations and their hydrolysates were comparable, and in **Figure 1** the chromatograms of the unheated proteins and their hydrolysates are given. As can be seen, with increasing incubation times with pepsin, the area of the parental protein peak diminished in both protein samples, whereas a number of peaks eluting at higher elution volumes appeared.

Overall, it was shown that the digestions of Ara h $1_{oligomers}$ and Ara h $1_{trimers}$ resulted in similar degradation patterns, that is, the formation of peptides of comparable sizes. At all

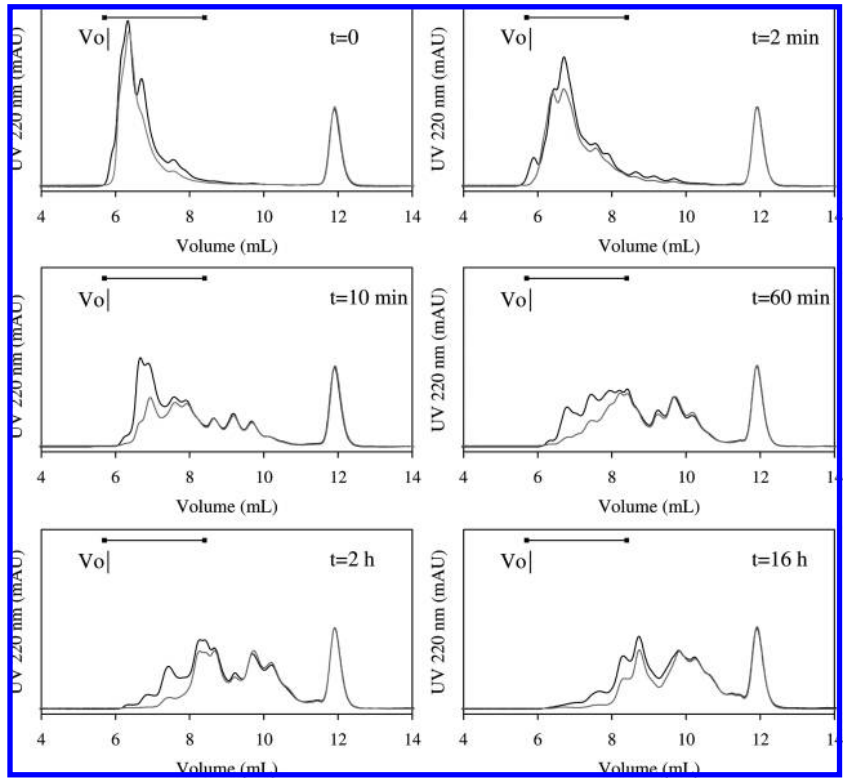

Figure 1. Size exclusion chromatograms (under denaturing conditions) of peptic hydrolysates of unheated Ara h $1_{oligomers}$ (black lines) and Ara h $1_{trimers}$ (gray lines). The horizontal black lines indicate the area at which at $t = 0$ all UV absorbance was measured. Indicated is the void volume of the column (Vo).

Table 2. Proportion (Percent) of Hydrolysates of Ara h $1_{oligomers}$ and Ara h $1_{trimers}$ Present as Relatively Large Molecular Mass Peptides[a]

pepsin incubation time (*t*)	Ara h $1_{oligomers}$	Ara h $1_{trimers}$
0	100	100
2 min	94	96
10 min	76	68
60 min	52	39
120 min	37	22
16 h	17	9

[a] Indicated is the proportion (percent) of the total UV absorption (at 220 nm) eluting between 5.7 and 8.4 mL in the chromatograms, as indicated in **Figure 1**.

incubation times, Ara h $1_{oligomers}$ exhibited higher peaks corresponding to peptides with relatively high molecular masses than Ara h $1_{trimers}$. The same was observed in the samples that were preheated before pepsin incubation. These results thus indicate that Ara h $1_{trimers}$ were more quickly hydrolyzed by pepsin compared with Ara h $1_{oligomers}$, regardless of preheating the protein preparations. To verify this, the relative amount of UV absorbance eluting between 5.7 and 8.4 mL (the area at which at $t = 0$ all UV absorption was detected) was calculated, and the results are given in **Table 2**. These results quantitatively underscored our observation: After 2 h of peptic digestion, which is an average gastric transit time (*22*) and which is generally used as the maximum time of pepsin digestion in in vitro tests (*14, 23, 24*), about 37% of the total UV absorbance of Ara h $1_{oligomers}$ corresponded to relatively high molecular mass peptides, whereas for Ara h $1_{trimers}$ this proportion was 22%.

IgE Binding after Digestion. Heated and unheated Ara h $1_{oligomers}$ and Ara h $1_{trimers}$ and their peptic hydrolysates were investigated for their IgE binding by dot-blotting. We chose to perform dot-blotting experiments, as the partial insolubility of the proteins and peptides after peptic hydrolysis under non-denaturing conditions was expected to hinder more quantitative techniques, which require these non-denaturing conditions. For dot-blotting the samples could be solubilized before being dried on the PVDF membrane, by adding ACN and TFA to 30 and 0.1% (v/v), respectively.

Although the IgE binding was the highest in the nondigested protein samples, IgE binding was detected in all hydrolyzed samples, even after 16 h of pepsin incubation (data not shown). These results coincide with literature data, stating that Ara h 1 is still IgE reactive upon peptic hydrolysis (*13–15*).

To discriminate which populations of the peptides remaining upon peptic digestion caused the IgE binding that was detected in the hydrolysates, samples after 16 h of peptic hydrolysis (considered to be an end point of peptic hydrolysis) were fractionated with SEC under denaturing conditions. In **Figure 2** the fractionation of peptic hydrolysates of unheated Ara h $1_{oligomers}$ is shown. As can be seen, a broad but distinctive distribution of peaks was obtained, and the first three peaks eluting from the column were denoted SEC peaks A, B, and C, respectively. The dot-blotting results of the SEC fractions of the four different Ara h 1 preparations (Ara h $1_{oligomers}$ and Ara h $1_{trimers}$, heated and unheated) were similar. As an example, the results for unheated Ara h $1_{oligomers}$ are given in **Figure 3**. It can be seen that the fractions corresponding to SEC peaks A (fractions 1–3) and B (fractions 4–6) exhibited the most intense color on dot-blot, indicating the presence of peptide(s) with intact epitope(s) in these peaks. Fractions under SEC peak C (fractions 7–10) exhibited a less intense color. Subsequent fractions (fractions 11–25) did not show any color on the dot-blot, implying that these fractions did not bind IgE and thus did not contain peptides bearing intact epitopes. The color observed on dot-blot in fractions under SEC peak C was highest in the first two fractions of this peak and diminished in subsequent fractions of the peak. Consequently, the IgE binding observed in SEC peak C was assumed to be caused by peptides

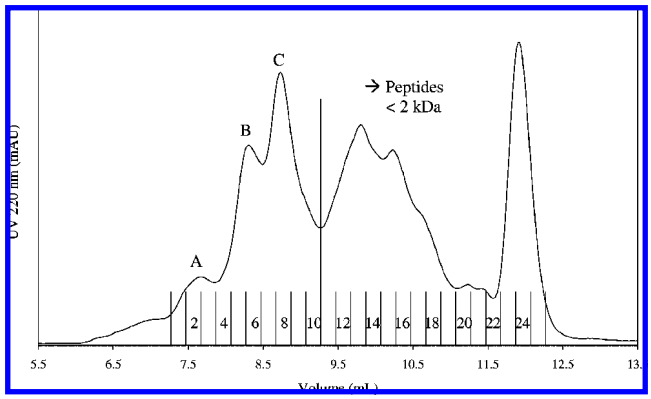

Figure 2. Size exclusion chromatogram (under denaturing conditions) of unheated Ara h $1_{oligomers}$ after peptic hydrolysis during 16 h: black lines, UV pattern at 220 nm; gray lines, collected fractions (1–25; even fraction numbers indicated).

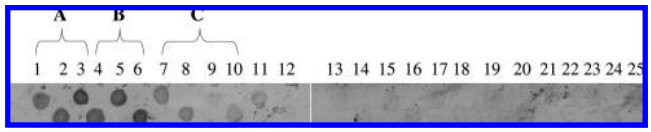

Figure 3. Dot-blot of fractions obtained from size exclusion chromatography, as indicated in **Figure 2**.

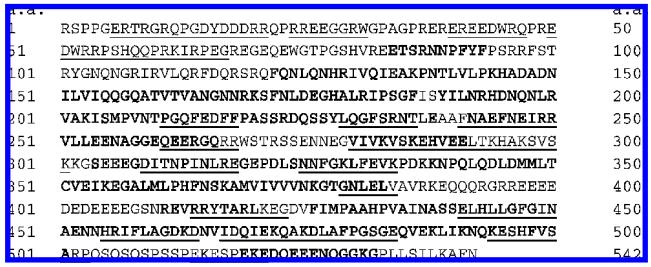

Figure 4. Correspondence of identified <2 kDa peptic peptides of unheated Ara h $1_{oligomers}$ with the amino acid sequence of Ara h 1 (P43238): amino acids (a.a.) in bold, coverage with identified peptides; underlined a.a., previously identified epitopes (*7, 9*).

eluting in the tail of SEC peak B, which overlapped with SEC peak C. As a result, SEC peak C was not assumed to represent intact epitopes other than those in SEC peak B.

Identification of Pepsin-Susceptible Epitopes. To investigate which of the epitopes described for Ara h 1 were susceptible to peptic digestion, non-IgE-binding fractions, containing peptide masses lower than 2 kDa (**Figure 2**, fractions 11–25) as determined with MALDI-TOF MS, were analyzed by RP-HPLC-MS. The peptides identified were compared with the amino acid sequence of Ara h 1. The results for the four Ara h 1 preparations were comparable, and the results for unheated Ara h $1_{oligomers}$ are shown in **Figure 4** and **Table 3**. As can be seen, approximately 63% coverage of the amino acid sequence of Ara h 1 was found. Only in the first part of the amino acid sequence of Ara h 1 (amino acids 1–83), no peptides were identified. The identified peptides included fragments of 15 of the 21 previously identified epitopes of Ara h 1 (*7, 9*). Two peptides were identified containing the complete epitopes 16 and 18 (**Table 1**). However, several other peptides were identified containing fragments of these epitopes, indicating that these epitopes are also susceptible to peptic hydrolysis.

Identification of Pepsin-Resistant Epitopes. For the identification of pepsin-resistant epitopes of Ara h 1, the IgE binding SEC peaks A and B were further investigated. SEC peak C was also further investigated, although, as was mentioned before, it was assumed that this peak did not contain intact epitopes other than those in SEC peak B. One preparation (heated Ara h $1_{oligomers}$) was investigated for the determination of pepsin-resistant epitopes.

Fractions present in SEC peaks A, B, and C were separately combined and denoted SEC peaks A, B, and C, respectively. MALDI-TOF MS analyses showed for SEC peak A two broad peaks between approximately 8 and 9 kDa, indicating the presence of two or a few peptides. MALDI-TOF MS analyses of SEC peaks B and C showed that these peaks contained several peptides with masses <6.5 and <5 kDa, respectively (data not shown). RP-HPLC analysis showed for SEC peak A a single peak in the chromatogram (**Figure 5**). For SEC peak B approximately 10 peaks were observed in the chromatogram (**Figure 5**) and for SEC peak C even more (data not shown). The presence of a single peak in SEC peak A indicated that the two or more peptides in this peak (as determined with MALDI-TOF MS) had a comparable hydrophobicity correlated with their sizes (*25*). Consequently, it seemed likely that the peptides in this peak consisted of largely the same amino acid sequence and differed only slightly in size, as measured by MALDI-TOF MS. The differences in size could be caused by the relative unspecific activity of pepsin, causing ragged N and/or C termini of the peptides.

Peptides in SEC peaks A−C were too large to be analyzed directly on RP-HPLC-MS. Therefore, to identify to which regions of Ara h 1 the IgE-binding peptides in these peaks corresponded, samples were hydrolyzed with chymotrypsin, and the resulting peptides were analyzed by RP-HPLC-MS afterward, an approach previously developed for soy glycinin (*26*).

In the chymotryptic digest of SEC peak A one peptide with amino acid sequence WRQPREDW was identified, corresponding to amino acids 45–52 of mature Ara h 1 (**Table 4**). Unfortunately, probably because of the relatively low protein concentration in the sample, no (expected) additional peptide(s) could be identified in this sample. The identified chymotryptic peptide corresponded to that part of the amino acid sequence of Ara h 1 from which no low molecular mass peptic peptides were identified (**Figure 4**). Theoretically, chymotrypsin is not able to cleave the amino acid sequence of Ara h 1 between amino acids 44 and 45, whereas pepsin is. Chymotrypsin is able to hydrolyze the amino acid sequence of Ara h 1 between amino acids 52 and 53. As a result, the peptide in SEC peak A was most likely C-terminally extended before being hydrolyzed by chymotrypsin. This was confirmed by N-terminal sequencing experiments, as the major N-terminal peptide sequence that was identified in SEC peak A consisted of amino acids WRQPRE (amino acids 45–50). Consequently, on the basis of our results, the major peptide(s) in SEC peak A start(s) at amino acid 45. Considering peptide masses of approximately 8–9 kDa and the preferred cleavage sites of pepsin (*27*), amino acid 115 could very well be the C-terminal of the largest peptide in this peak (resulting in a peptide mass of 8779.6 Da), whereas amino acid 111 could be the C terminus of the smaller peptide (resulting in a peptide mass of 8235.0 Da). On these peptides the previously identified intact epitopes with amino acid sequences EDWRRPSHQQ and PRKIRPEG, respectively (epitopes 5 and 6, **Table 1**), are situated. On the basis of our results, these epitopes thus seem to remain intact upon peptic digestion of Ara h 1 and likely caused the IgE binding that was detected in SEC peak A. As it concerns two epitopes on one peptide, this peptide might be able to induce clinically relevant symptoms. Minor signals that were observed upon N-terminal sequencing of SEC peak A pointed toward the amino acid sequence

Table 3. Identified Peptides in the Low Molecular Mass (<2 kDa) Peptic Hydrolysate Fractions of Ara h $1_{oligomers}$, Showing Coverage with the Amino Acid Sequence of Ara h 1 (P43238)

peptide a.a. sequence[a]	a.a.	epitopes[b]	peptide a.a. sequence[a]	a.a.	epitopes[b]
ETSRNNPFYF	84–93	no epitope	REGEPDLSNNF	317–327	f.m. e11, 12
FQNLQN	122–127	no epitope	REGEPDLSNNFGKL	317–330	f.m. e11, 12
FQNLQNHRIVQ	122–132	no epitope	LFEVKPDKKNPQLQD	330–344	f.m. e12
QNHRIVQIEAK	126–136	no epitope	FEVKPDKKNP	331–340	f.m. e12
IEAKPNT	133–139	no epitope	FEVKPDKKNPQL	331–342	f.m. e12
IEAKPNTLVL	133–142	no epitope	FEVKPDKKNPQLQD	331–344	f.m. e12
KPNTLVLPK	136–144	no epitope	FEVKPDKKNPQLQDL	331–345	f.m. e12
KPNTLVLPKHADADNIL	136–152	no epitope	FEVKPDKKNPQLQDLD	331–346	f.m. e12
LVLPKHADAD	140–149	no epitope	LQDLDMMLTCVEIKE	342–356	no epitope
LVLPKHADADNIL	140–152	no epitope	LQDLDMMLTCVEIKEG	342–357	no epitope
VLPKHADADNIL	141–152	no epitope	VEIKEGA	352–358	no epitope
LPKHADADNILVI	142–154	no epitope	VEIKEGALM	352–360	no epitope
VIQQGQA	153–159	no epitope	VEIKEGALMLPHF	352–364	no epitope
VANGNNRKSFNL	163–174	no epitope	IKEGALMLPHF	354–364	no epitope
VANGNNRKSFNLDEGHA	163–179	no epitope	GALMLPHF	357–364	no epitope
GNNRKSFNLDEGHA	166–179	no epitope	ALMLPHF	358–364	no epitope
NNRKSFNLDEGHA	167–179	no epitope	LMLPHF	359–364	no epitope
NLDEGHALRIPSGF	173–186	no epitope	NSKAMV	365–370	no epitope
LRIPSGF	180–186	no epitope	VIVVVNKGTGNLEL	370–383	f.m. e13
YILNRHDNQNL	189–199	no epitope	IVVVNKGTGNL	371–381	f.m. e13
ILNRHDNQNL	190–199	no epitope	IVVVNKGTGNLEL	371–383	f.m. e13
NRHDNQNL	192–199	no epitope	VVVNKGTGNLEL	372–383	f.m. e13
RVAKISM	200–206	no epitope	VNKGTGNLEL	374–383	f.m. e13
RVAKISMPVNTPGQF	200–214	f.m. e7	REVRRYTARL	411–420	f.m. e14
PVNTPGQF	207–214	f.m. e7	FIMPAAHPVAINASSEL	426–442	f.m. e15
EDFFPASSRDQSSY	215–228	f.m. e7, 8	LHLLGF	442–447	f.m. e15
EDFFPASSRDQSSYL	215–229	f.m. e7, 8	LHLLGFGIN	442–450	f.m. e15
EDFFPASSRDQSSYLQG	215–231	f.m. e7, 8	HLLGFGIN	443–450	f.m. e15
FPASSRDQSSYL	218–229	f.m. e7, 8	GINAENNHRIF	448–458	f.m. e15, 16
FPASSRDQSSYLQG	218–231	f.m e7, 8	GINAENNHRIFL	448–459	f.m. e15, 16
PASSRDQSSYL	219–229	f.m. e8	AENNHRIF	451–458	f.m. e16
FSRNTLE	232–238	f.m. e8	AENNHRIFL	451–459	f.m. e16
NAEFNE	242–247	f.m. e9	AENNHRIFLAG	451–461	f.m. e16
EIRRVLLEENAG	247–258	f.m. e9	AENNHRIFLAGDKD	451–464	**e16**
IRRVLL	248–253	f.m. e9	LAGDKDNVIDQ	459–469	f.m. e16, 17
LEENAGGEQEERGQ	253–266	f.m. e10	AGDKDNVIDQ	460–469	f.m. e16, 17
EENAGGEQEERGQ	254–266	f.m. e10	IEKQAKDLAF	470–479	f.m. e17, 18
VIVKVSKEHVEE	280–291	f.m. e21	IEKQAKDLAFPGSGEQ	470–485	**e18**, f.m. e17
IVKVSKEHVEE	281–291	f.m. e21	LAFPGSGE	477–484	f.m. e18
VKVSKEHVEE	282–291	f.m. e21	LAFPGSGEQ	477–485	f.m. e18
SEEEGDITNPINL	304–316	f.m. e11	LAFPGSGEQVEKL	477–489	f.m. e18
EGDITNPINL	307–316	f.m. e11	PGSGEQVEKL	480–489	f.m. e18
GDITNPINL	308–316	f.m. e11	VEKLIKNQKESHF	486–498	f.m. e19
DITNPIN	309–315	f.m. e11	IKNQKESHF	490–498	f.m. e19
DITNPINL	309–316	f.m. e11	IKNQKESHFVSA	491–501	f.m. e19
ITNPINL	310–316	f.m. e11	EKEDQEEENQGGKG	519–532	f.m. e20

[a] Underlined amino acids: coverage with previously identified epitopes (*7, 9*). [b] e7 = epitope 7 (**Table 1**). f.m. (fragment) indicates that the identified peptic peptide contained a fragment of the epitope mentioned.

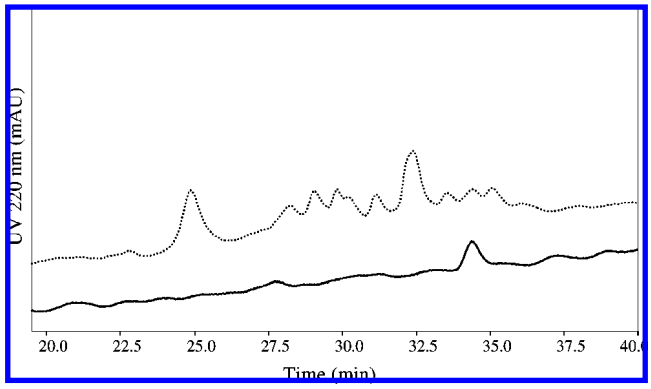

Figure 5. RP-HPLC chromatograms of SEC peak A (solid line) and SEC peak B (dashed line), as indicated in **Figure 2**.

WGTPG (amino acids 74–78). If present, peptides of approximately 8 and 9 kDa, starting with this N-terminal sequence, would not have contained intact epitopes (**Table 1**).

In the chymotryptic digest of SEC peak B, five peptides were identified (**Table 4**), of which four covered parts of the amino acid sequence of Ara h 1 between amino acids 46 and 91. Considering the theoretical cleavage sites of chymotrypsin (indicated in **Table 4**) and the maximum peptide mass of approximately 6.5 kDa that was detected with MALDI-TOF MS in this peak upon peptic digestion, possible peptic peptides of this peak could be deduced (**Table 4**). On these deduced peptic peptides the intact epitopes 1−6 (**Table 1**) were present, indicating that these epitopes could have been intact on the peptic peptides of this peak and caused the IgE binding observed to this peak.

The fifth chymotryptic peptide that was identified in SEC peak B (KVSKEHVEEL) could possibly be derived from a ∼6.5 kDa peptic peptide, covering amino acids 283–340. This theoretical peptide contains intact epitopes 11 and 12. As these two epitopes were proven to be pepsin-susceptible (**Table 3**), the presence of this peptic peptide in SEC peak B seems to be unlikely.

Table 4. Identified Chymotryptic Peptides in SEC Peaks A−C (**Figure 2**) Showing Coverage with the Amino Acid Sequence of Ara h 1 (P43238) and Their Deduced Peptic Peptides

			deduced N-terminally extended peptic peptides[c]			deduced C-terminally extended peptic peptides[c]	
identified peptide	a.a.[a]	N-terminal c.t. site[b]	a.a.	e	C-terminal c.t. site[b]	a.a.	e
peak A, max ~9 kDa							
WRQPREDW	45–52	−	*d*	*e*	+	45–115	5, 6[f]
peak B, max ~6.5 kDa							
RQPREDW	46–52	+	1–52	1–4	+	46–98	5, 6
GTPGSHVREETSRNNPF	75–91	−	*d*	*e*	+	75–128	*e*
VREETSRNNPF	81–91	+	40–91	4–6	+	81–132	*e*
SRNNPF	86–91	−	*d*	*e*	+	86–138	*e*
KVSKEHVEEL	283–292	−	*d*	*e*	+	283–340	11, 12
peak C, max ~5.0 kDa							
RQPREDW	46–52	+	13–52	2, 3, 4	+	46–86	5, 6
KIRPEGREGEQE	62–73	−	*d*	*e*	+	62–103	*e*
VREETSRNNPF	81–91	+	51–91	6	+	81–120	*e*
KVSKEHVEEL	283–292	−	*d*	*e*	+	283–326	11
NNEGVIVKVSKEHVEEL	276–292	−	*d*	*e*	+	276–320	11
NEGVIVKVSKEHVEEL	277–292	−	*d*	*e*	+	277–321	11
GVIVKVSKEHVEEL	279–292	−	*d*	*e*	+	279–323	11
VIVKVSKEHVEEL	280–292	−	*d*	*e*	+	280–324	11
VIVKVSKEHVEELTKH	280–295	−	*d*	*e*	+	280–324	11
IVKVSKEHVEEL	281–292	−	*d*	*e*	+	281–325	11
VKVSKEHVEEL	282–292	−	*d*	*e*	+	282–326	11
GSEEEGDITNPINL	303–316	−	*d*	*e*	+	303–346	11
LDMMLTCVEIK	345–355	−	*d*	*e*	−	[d]	*e*
IKNQKESHF	390–398	+	390–430	14	+	355–398	13

[a] a.a., coverage with amino acids of the sequence of Ara h 1 (P43238). [b] Indicated is whether the N-terminal and C-terminal ends of the identified chymotryptic peptides were theoretical chymotrypsin cleavage sites. +, cleavage sites for the used chymotrypsin preparation [C-terminal of phenylalanine (F), tyrosine (Y), tryptophan (W), methionine (M), leucine (L), alanine (A), aspartic acid (D), and glutamic acid (E)]; −, no cleavage site for the used chymotrypsin preparation. [c] Indicated is the coverage with amino acids of the sequence of Ara h 1 (a.a.) and the presence of intact epitopes (e) on the deduced peptic peptides, which were based on the theoretical chymotrypsin cleavage sites and the maximum peptides mass, as determined with MALDI-TOF MS. [d] No N- or C-terminally extended, deduced peptic peptide possible. [e] No intact epitopes present on deduced peptic peptides. [f] Intactness of epitopes was confirmed by the results from N-terminal sequencing.

In SEC peak C 14 chymotryptic derived peptides were identified (**Table 4**). Taking into account the maximum peptide mass in SEC peak C upon peptic digestion of approximately 5 kDa and the possible cleavage sites for chymotrypsin, the deduced possible peptic peptides in this peak could have contained intact epitopes 2−6, 11, 13, and 14 (**Table 1**). As was previously mentioned, IgE binding to SEC peak C was considered to be caused by coelution of peptides from SEC peak B. Consequently, the IgE binding in SEC peak C was assumed to be caused only by those epitopes that were also denoted to be possibly present in SEC peak B. As they were not denoted to be possibly intact in peptides in SEC peak B, the presence of intact epitopes 13 and 14 in SEC peak C was not likely. Moreover, as was previously mentioned, epitopes 11, 13, and 14 were earlier identified as pepsin-susceptible.

Conclusion. Altogether, under the in vitro conditions applied, 15 of the 21 previously identified epitopes of Ara h 1 could be identified as being pepsin-susceptible. Two epitopes were deduced as being pepsin-resistant, whereas for the four remaining epitopes, it could not be concluded whether they are pepsin-susceptible or pepsin-resistant, although there were indications for pepsin resistancy. According to Shin and co-workers (*28*), the majority of the IgE epitopes of Ara h 1 are clustered in two regions of the allergen monomers. In the trimeric model of Ara h 1, these regions are situated in the interfaces between the Ara h 1 monomers (*13*). It is interesting to note that the two deduced (5–6) and four possible pepsin-resistant (1–4) epitopes mentioned above are not situated in either one of these regions, but they are all situated on the N-terminal part of the allergen. This indicates the importance of this N-terminal part for the allergenic properties of Ara h 1. This N-terminal part of Ara h 1 has been reported not to occur in other (allergenic) vicilins, such as pea vicilin, lentil vicilin, and soy β-conglycinin (*29*), although vicilins with N-terminal extensions do occur. Some of these vicilins were previously denoted convicilins, but they in fact are to be considered as the α polypeptides of trimeric vicilin proteins (*30*). These larger α-polypeptides of vicilin proteins, like the α- and α'-polypeptides of soy β-conglycinin and the α-polypeptides of pea vicilin, do bear an N-terminal extension. However, these extensions do not share a high homology with the N-terminal extension of Ara h 1. Consequently, the unique N-terminal part of Ara h 1, which was shown to contain pepsin-resistant epitopes, might explain why allergies to peanuts often cause severe, systemic reactions compared to other legume foods.

ACKNOWLEDGMENT

We thank Edwin Bakx for assistance during the RP-HPLC-MS measurements.

LITERATURE CITED

(1) Astwood, J. D.; Leach, J. N.; Fuchs, R. L. Stability of food allergens to digestion in vitro. *Nat. Biotechnol.* **1996**, *14* (10), 1269–1273.

(2) Huby, R. D. J.; Dearman, R. J.; Kimber, I. Why are some proteins allergens? *Toxicol. Sci.* **2000**, *55* (2), 235–246.

(3) Maleki, S. J.; Chung, S. Y.; Champagne, E. T.; Raufman, J. P. The effects of roasting on the allergenic properties of peanut proteins. *J. Allergy Clin. Immunol.* **2000**, *106* (4), 763–768.

(4) Kroll, J.; Rawel, H. M.; Rohn, S.; Czajka, D. Interactions of glycinin with plant phenols—influence on chemical properties and

proteolytic degradation of the proteins. *Nahrung−Food* **2001**, *45* (6), 388–389.
(5) Rawel, H. M.; Kroll, J.; Rohn, S. Reactions of phenolic substances with lysozyme−physicochemical characterisation and proteolytic digestion of the derivatives. *Food Chem.* **2001**, *72* (1), 59–71.
(6) Sicherer, S. H.; Munoz-Furlong, A.; Burks, A. W.; Sampson, H. A. Prevalence of peanut and tree nut allergy in the US determined by a random digit dial telephone survey. *J. Allergy Clin. Immunol.* **1999**, *103* (4), 559–562.
(7) Burks, A. W.; Shin, D.; Cockrell, G.; Stanley, J. S.; Helm, R. M.; Bannon, G. A. Mapping and mutational analysis of the IgE-binding epitopes on Ara h 1, a legume vicilin protein and a major allergen in peanut hypersensitivity. *Eur. J. Biochem.* **1997**, *245* (2), 334–339.
(8) de Jong, E. C.; Zijverden, M. v.; Spanhaak, S.; Koppelman, S. J.; Pellegrom, H.; Penninks, A. H. Identification and partial characterization of multiple major allergens in peanut proteins. *Clin. Exp. Allergy* **1998**, *28* (6), 743–751.
(9) Shreffler, W. G.; Beyer, K.; Chu, T. H. T.; Burks, A. W.; Sampson, H. A. Microarray immunoassay: association of clinical history, in vitro IgE function, and heterogeneity of allergenic peanut epitopes. *J. Allergy Clin. Immunol.* **2004**, *113* (4), 776–782.
(10) van Boxtel, E. L.; Van Beers, M. M. C.; Koppelman, S. J.; Van den Broek, L. A. M.; Gruppen, H. Allergen Ara h 1 occurs in peanuts as a large oligomer rather than as a trimer. *J. Agric. Food Chem.* **2006**, *54* (19), 7180–7186.
(11) van Boxtel, E. L.; Van den Broek, L. A. M.; Koppelman, S. J.; Vincken, J. P.; Gruppen, H. Peanut allergen Ara h 1 interacts with proanthocyanidins into higher molecular weight complexes. *J. Agric. Food Chem.* **2007**, *55*, 8772–8778.
(12) Koppelman, S. J.; Bruijnzeel Koomen, C. A. F. M.; Hessing, M.; de Jongh, H. H. J. Heat-induced conformational changes in Ara h 1, a major peanut allergen, do not affect its allergenic properties. *J. Biol. Chem.* **1999**, *274* (8), 4770–4777.
(13) Maleki, S. J.; Kopper, R. A.; Shin, D. S.; Park, C.; Compadre, C. M.; Sampson, H.; Burks, A. W.; Bannon, G. A.; Park, C. W. Structure of the major peanut allergen Ara h 1 may protect IgE-binding epitopes from degradation. *J. Immunol.* **2000**, *164* (11), 5844–5849.
(14) Eiwegger, T.; Rigby, N.; Mondoulet, L.; Bernard, H.; Krauth, M. T.; Boehm, A.; Dehlink, E.; Valent, P.; Wal, J. M.; Mills, E. N. C.; Szepfalusi, Z. Gastro-duodenal digestion products of the major peanut allergen Ara h 1 retain an allergenic potential. *Clin. Exp. Allergy* **2006**, *36* (10), 1281–1288.
(15) van Boxtel, E. L.; van den Broek, L. A. M.; Koppelman, S. J.; Gruppen, H., Legumin allergens from peanuts and soybeans−effects of denaturation and aggregation on allergenicity. *Mol. Nutr. Food Res.* (in press).
(16) Kolarich, D.; Altmann, F. N-Glycan analysis by matrix-assisted laser desorption/ionization mass spectrometry of electrophoretically separated nonmammalian proteins: application to peanut allergen Ara h 1 and olive pollen allergen Ole e 1. *Anal. Biochem.* **2000**, *285* (1), 64.
(17) Link, A. J.; Eng, J.; Schieltz, D. M.; Carmack, E.; Mize, G. J.; Morris, D. R.; Garvik, B. M.; Yates, J. R. Direct analysis of protein complexes using mass spectrometry. *Nat. Biotechnol.* **1999**, *17* (7), 676–682.
(18) Storms, H. F.; van der Heijden, R.; Tjaden, U. R.; van der Greef, J. Optimization of protein identification from digests as analyzed by capillary isoelectric focusing-mass spectrometry. *J. Chromatogr., B* **2005**, *824* (1–2), 189–200.
(19) Ilse, D.; Edman, P. Formation of 3-phenyl-2-thiohydantoins from phenylthiocarbamyl amino acids. *Aust. J. Chem.* **1963**, *16* (3), 411–416.
(20) Edman, P. On the mechanism of the phenyl isothiocyanate degradation of peptides. *Acta Chem. Scand.* **1956**, *10* (5), 761–768.
(21) Hewick, R. M.; Hunkapiller, M. W.; Hood, L. E.; Dreyer, W. J. A gas−liquid solid-phase peptide and protein sequenator. *J. Biol. Chem.* **1981**, *256* (15), 7990–7997.
(22) Untersmayr, E.; Jensen-Jarolim, E. The effect of gastric digestion on food allergy. *Curr. Opin. Allergy Clin. Immunol.* **2006**, *6* (3), 214–219.
(23) Moreno, F. J.; Mellon, F. A.; Wickham, M. S. J.; Bottrill, A. R.; Mills, E. N. C. Stability of the major allergen Brazil nut 2S albumin (Ber e 1) to physiologically relevant in vitro gastrointestinal digestion. *FEBS J.* **2005**, *272* (2), 341–352.
(24) Fu, T. J.; Abbott, U. R.; Hatzos, C. Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid−a comparative study. *J. Agric. Food Chem.* **2002**, *50*, 7154–7160.
(25) van der Ven, C.; Gruppen, H.; de Bont, D. B. A.; Voragen, A. G. J. Reversed phase and size exclusion chromatography of milk protein hydrolysates: relation between elution from reversed phase column and apparent molecular weight distribution. *Int. Dairy J.* **2001**, *11* (1–2), 83–92.
(26) Kuipers, B. J. H.; Gruppen, H.; Bakx, E. J. Functional region identification in proteins by accumulative−quantitative peptide mapping using RP-HPLC-MS. *J. Agric. Food Chem.* **2007**, *55*, 9337−9344.
(27) Keil, B. *Specificity of Proteolysis*; Springer-Verlag: Berlin, Germany, 1992.
(28) Shin, D. S.; Compadre, C. M.; Maleki, S. J.; Kopper, R. A.; Sampson, H.; Huang, S. K.; Burks, A. W.; Bannon, G. A. Biochemical and structural analysis of the IgE binding sites on Ara h1, an abundant and highly allergenic peanut protein. *J. Biol. Chem.* **1998**, *273* (22), 13753–13759.
(29) Bown, D.; Ellis, T. H. N.; Gatehouse, J. A. The sequence of a gene encoding convicilin from pea (*Pisum sativum* L.) shows that convicilin differs from vicilin by an insertion near the N-terminus. *Biochem. J.* **1988**, *251* (3), 717–726.
(30) O'Kane, F. E.; Happe, R. P.; Vereijken, J. M.; Gruppen, H.; van Boekel, M. Characterization of pea vicilin. 1. Denoting convicilin as the α-subunit of the *Pisum* vicilin family. *J. Agric. Food Chem.* **2004**, *52*, 3141–3148.

Received for review October 1, 2007. Revised manuscript received January 2, 2008. Accepted January 4, 2008.

JF072907N