PROTEIN QUATERNARY STRUCTURE AND AGGREGATION IN RELATION TO ALLERGENICITY

EVELIEN L. VAN BOXTEL

Promotor:	**Prof. Dr. Ir. H. Gruppen** Persoonlijk hoogleraar bij de leerstoelgroep Levensmiddelenchemie
Co-promotoren:	**Dr. L.A.M. van den Broek** Onderzoeker bij WUR-AFSG Biobased products
	Dr. S.J. Koppelman Director R&D, HAL Allergy
Promotiecommissie:	**Prof. Dr. Ir. M.A.J.S. van Boekel** Wageningen Universiteit
	Prof. Dr. S.E. Kulling University of Potsdam, Germany
	Dr. E.N.C. Mills Institute for food research, Norwich, UK
	Prof. Dr. Ir. H.F.J. Savelkoul Wageningen Universiteit

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EVELIEN L. VAN BOXTEL

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ABSTRACT

In order to induce systemic food allergic reactions in humans, proteins after digestion in the human gastro-intestinal tract should still be able to bind IgE. The aim of the work presented in this thesis was to determine the effects of heating on the structure and digestibility of cupin and prolamin food allergens from peanuts, Brazil nuts and soybeans and to determine the effects of digestion on the IgE binding capacity of these allergens.
Representative allergen preparations were purified prior to the investigations. To this end, a large-scale purification method was developed for the purification of Brazil nut allergen Ber e 1, comprising its complete isoform pool. The denaturation temperature of the latter allergen at the common pH values of foods (5-7) appeared to be very high. As a result, this protein is assumed not to be denatured during common food processing and to be digested (mainly) in its native form. The native form of Ber e 1 is known to have a high stability towards peptic digestion, which likely explains the allergenicity of this protein.
The representative quaternary structure of vicilin allergen Ara h 1 from peanuts appeared to be an oligomeric structure, in which the protein is assumed to interact with proanthocyanidins via a specific distribution of proline residues on its surface. Heat-induced aggregation prior to peptic hydrolysis under *in vitro* conditions did not affect the digestibility of Ara h 1, whereas heat-induced aggregation of legumin allergens Ara h 3 from peanuts and glycinin from soybeans (slightly) decreased the digestion rates. Regardless of pre-heating, the IgE binding capacity of the latter allergens diminished fast during pepsin digestion. As a result, in terms of systemic food allergic reactions, legumin allergens from peanuts and soybeans might not be very important allergens.
Vicilin allergen Ara h 1 could still bind IgE after prolonged peptic digestion. Two pepsin-resistant epitopes were deduced and indications for four additional pepsin-resistant epitopes are presented. These epitopes are all situated on the unique N-terminal part of the allergen, which might explain why allergic reactions to peanuts are often severe, compared to other legume allergies.

Keywords: food allergy, IgE binding, digestion, peptides, heat-induced aggregation, Ber e 1, Ara h 1, Ara h 3, glycinin, β-conglycinin, protein purification, mass spectrometry

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GENERAL INTRODUCTION

FOOD ALLERGY

Adverse reactions to foods can be divided into reactions that are mediated and reactions that are not mediated by the immune system. Food intolerances like lactose intolerance are not mediated by the immune system, whereas food allergy is an immune-mediated disease. Adverse reactions to foods are becoming an increasingly important health issue. In particular, the incidence and prevalence of food allergies has grown over the past years (*1-3*) and is estimated to affect 1 to 4% of individuals (*4*). Food allergies may have profound effects on human well-being and in line with their increased occurrence there is an increase in the medical expenses and work absence due to food allergic complaints.
The immune response involved in food allergy is mediated by a specific type of antibody, immunoglobulin type E (IgE). The most important foods and food raw materials causing allergic reactions are cow's milk, eggs, soy, wheat, peanuts, tree nuts, fish, and shellfish. Food allergy is almost always caused by proteins. These are termed allergens and most allergenic foods contain several different allergens.
Allergens are normally named according to the Allergen Nomenclature Sub-committee (www.allergen.org), operating under the auspices of the International Union of Immunological Societies and the World Health Organization. They are denoted using a taxonomic classification. According to this classification, an allergen name comprises the first three letters of the taxonomic name of the genus of its botanical source, followed by the first letter of the species of this source, and an Arabic number. This number is assigned to an allergen in the chronological order of its identification in a species (*5*). For example, the first allergen identified in peanuts (*Arachis hypogaea*) has been denoted Ara h 1, while the second one has been denoted Ara h 2. Not all allergens identified have been named according to this system. For example, the protein glycinin from soybeans is known as an allergen (*6*), but the protein has not been given an official allergen name.
Allergens that are recognized by over 50% of a given substantial population are denoted *major* allergens. *Minor* allergens are recognized by less than 50% of a population.

DEVELOPMENT OF FOOD ALLERGY

Most food allergies are developed in the first one to two years of life. In the development of food allergy two phases can be distinguished. During these phases multiple immune responses occur. The most important ones are described below.
The first phase in the development of a food allergy is denoted the sensitization phase. During this phase people who are prone to develop an allergic reaction will activate $T_{helper,\ 2}$ cells upon encountering a food allergen. These $T_{helper,\ 2}$ cells secrete cytokines, which stimulate B cells to produce IgE antibodies. The IgE antibodies bind to IgE binding receptors (FcεR) on mast cells. Upon a subsequent exposure of the allergen (phase 2) an allergic reaction may occur via the binding of the allergen to two or more IgE antibodies, resulting in the cross-linking of the IgE binding receptors. The cross-linking of these receptors induces the mast cells to release several kinds of mediators, like histamine, which cause allergic symptoms (Figure 1). Food allergic symptoms usually occur after a few minutes till one hour after ingestion of an allergenic food. Severe reactions may also occur one to two hours after ingestion (*7*). Allergic reactions may include the dilatation of

blood vessels, the increase of vascular permeability, the contraction of smooth muscles, the damaging of local tissues and increased peristalsis. These features may result in symptoms like itches, urticaria, abdominal pain, nausea, and diarrhea. By far the most severe allergic reaction is anaphylaxis, which is characterized by edema in many tissues, together with a fall in blood pressure. This sudden fall in blood pressure, together with airway obstruction, may cause life-threatening situations (*8*). Peanut and tree nut allergy are known to be the most common causes of fatal and near-fatal anaphylaxis (*9-11*), rendering them very serious food allergies. Furthermore, most peanut and tree nut allergies are persistent for life (*12, 13*), while other allergies are often outgrown (*14*).

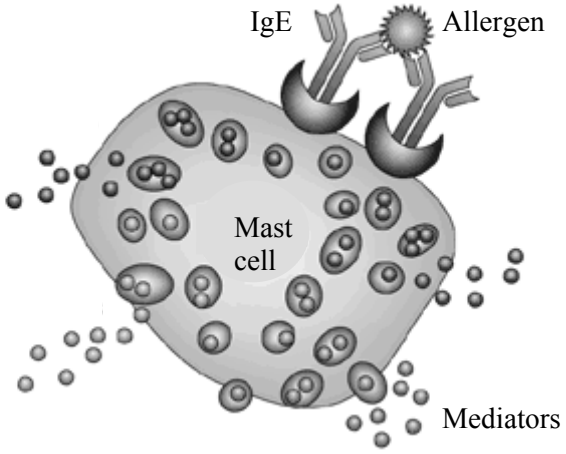


Figure 1: Cross-linking of IgE binding receptors causes the mast cell to release its mediators.

At this moment, an explanation for the increase in the prevalence of allergies in western countries is not known. It is known that genetics play a role (*8*), but also environmental factors and health status are factors that determine the susceptibility of a person to develop an allergy (*15*). The original function of IgE antibodies is to fight parasitic infections. However, in western countries, because of vaccinations and hygiene, parasitic infections are rare. Consequently, it has been suggested that due to the absence of environmental factors, IgE responses turn towards innocuous proteins (*16*). There have been studies supporting this theory (*17*). However, other studies showed opposite results (*18*). Probably there are several factors that may influence the development of allergies.

IgE BINDING

IgE antibodies raised towards a specific allergenic protein recognize parts of this allergen, to which they can bind to. These protein parts are denoted IgE epitopes. Besides IgE antibodies, T cells can also bind to allergens, via the binding to so-called T cell epitopes. As a result of binding to these latter epitopes, $T_{helper,\ 2}$ cells start the production of various cytokines, which stimulate the B cells to produce IgE. In this thesis, attention will only be given to IgE epitopes and the designation epitope therefore only applies to IgE epitopes.

IgE epitopes can be either linear or conformational. Linear epitopes are comprised of a single sequential part of the primary amino acid sequence of the protein. Conformational epitopes comprise multiple amino acid sequences, which are brought together spatially by the protein's three-dimensional structure (*19*). Conformational epitopes, as they are

dependent on a protein's three-dimensional structure, are less stable towards structural changes compared to linear epitopes. They can, for example, be disrupted during denaturation of the protein. Consequently, linear epitopes have been found to play the most important role as IgE binding sites in food allergies (*20*).
Many linear epitopes have been identified in allergens. Up till now, no common structural characteristics could be identified for these epitopes. Epitopes of some proteins have been subjected to mutational analysis in order to investigate the effects of amino acid substitutions on the IgE binding properties. For example, certain single amino acid changes in the immunodominant epitopes of *major* peanut allergens Ara h 1 and Ara h 3 caused a diminished or strongly decreased IgE binding of the epitopes. However, no position of the amino acid in the epitopes or consensus in the type of amino acid was found critical for IgE binding (*21, 22*).

CLASSES OF FOOD ALLERGY

The way in which allergens sensitize persons and the type of symptoms they cause may vary. When foods are consumed, their components can be absorbed into the blood and can come into contact with the immune system after they have reached the intestine. Upon absorption into the blood, these components can be distributed throughout the body. Via this gastro-intestinal route of absorption sensitization to allergens can occur. Allergens which sensitize via this route are believed to be able to cause (severe) systemic reactions involving multiple organs, as described above.
In order to be able to cause systemic allergic reactions after absorption in the intestine, proteins or protein fragments remaining after digestion should still be able to bind IgE and thus have intact epitopes. Consequently, the resistance of IgE binding capacity towards digestion in the gastro-intestinal tract is considered to be an important feature for food allergens (*23*).
In contrast to causing food allergic reactions as a result of sensitization via the gastro-intestinal tract, some allergic reactions to food proteins may be caused as a consequence of sensitization to inhalant allergens. Inhalant allergens, like pollen, sensitize via the respiratory tract. As several inhalant allergens show relatively high degrees of homology with some food proteins, a common phenomenon that is observed is that people with inhalant allergies also develop allergic reactions to food proteins. These allergic reactions are denoted pollen-IgE cross-reactions. For example: In apples, pears, and celery proteins occur, which are structurally homologous to the *major* birch pollen allergens Bet v 1 and Bet v 2. Due to this homology, IgE antibodies directed towards pollen allergens can also bind to these food proteins, resulting in allergic reactions like asthma and rhinitis (*7*) when consuming these foods (*24*). The symptoms caused by these cross-reactive allergies are usually mild and limited to the mouth area, as the passage through the gastro-intestinal tract usually results in the loss of IgE binding capacity of these pollen-related allergens. Consequently, these symptoms have been denoted the oral allergy syndrome (OAS).

Food allergic reactions as a result of sensitization via the gastro-intestinal tract are denoted **class 1** food allergic reactions. These reactions can induce serious systemic symptoms like anaphylaxis and are regarded as true food allergy. Pollen-related food allergy is denoted **class 2** food allergy. Class 1 and class 2 allergens can both be present in one food. For example, in peanuts several allergens (Ara h 1, 2, 3, 6, 7) have been identified, which have been related to class 1 food allergy (*25, 26*), whereas allergens Ara h 5 and Ara h 8 have been identified as being class 2 allergens (*27, 28*). In this study the focus is directed towards class 1 food allergy.

The diagnosis whether a food protein is an allergen is usually carried out via immunoblotting after SDS-PAGE. In this way, protein bands interacting with IgE from allergic people can be detected and identified (using mass spectrometry). Depending on the allergic symptoms of the (pool of) patient(s) used, the allergens can be designated class 1 or class 2 allergens. It should be noted that a representative pool of patients should be used, as a pool of patients with both class 1 and class 2 allergic symptoms to certain foods will not provide information on which allergens belong to which class of allergy. Besides, another aspect that has to be taken into account is that as a result of IgE cross-reactivity, proteins may be identified as being allergenic, while they exert no clinical reactions (*29*).
Several protein superfamilies have been related to class 1 and class 2 food allergy, respectively. As pools of patients do not always consist of persons with well-defined allergic symptoms, an allergen is often classified as a class 1 or a class 2 allergen, depending on its classification into an (allergenic) superfamily, rather than depending on the allergic symptoms it causes.

CLASSIFICATION OF FOOD ALLERGENS

Proteins can be classified according to the Structural Classification of Proteins (SCOP) system (scop.mrc-lmb.cam.ac.uk/scop). This classification is based on the presence of common major secondary structures in proteins. The proteins in this database are classified into folds, superfamilies, and families (*30*). The classification of proteins into folds is based on common major secondary structures in a common arrangement. Superfamilies consist of proteins which have a low amino acid sequence homology, but whose secondary structures and functional characteristics suggest a common evolutionary origin. The classification into families is based on amino acid sequence homology and evolutionary relationships between proteins. In general, proteins are classified into the same protein family when the amino acid sequence homology between protein is >30%. However, in some cases, due to similar functions and structures, proteins with lower amino acid sequence homologies are classified into the same family (*30*).
The majority of known class 1 food allergens belong to two superfamilies of plant proteins: the prolamin and the cupin superfamilies (Figure 2) (*31*). Proteins belonging to these superfamilies have long been classified according to their sedimentation coefficients into 2S, 7S, 11S, and 15S proteins. As these coefficients largely depend on the conditions used, nowadays the classification into superfamilies is generally used. Next to the

prolamin and cupin superfamilies, the papain superfamily has also been reported to represent class 1 food allergens (*32*).
Some homologous proteins within a superfamily contain conserved epitopes, which might explain the IgE cross-reactivity that is sometimes observed between proteins (*33*). Cross-reactivity occurs when IgE antibodies originally raised towards a particular allergen also bind to a similar protein from another source (*34*), which possibly causes an allergic reaction. In general, when >70% sequence identity is reached, IgE cross-reactivity can occur.

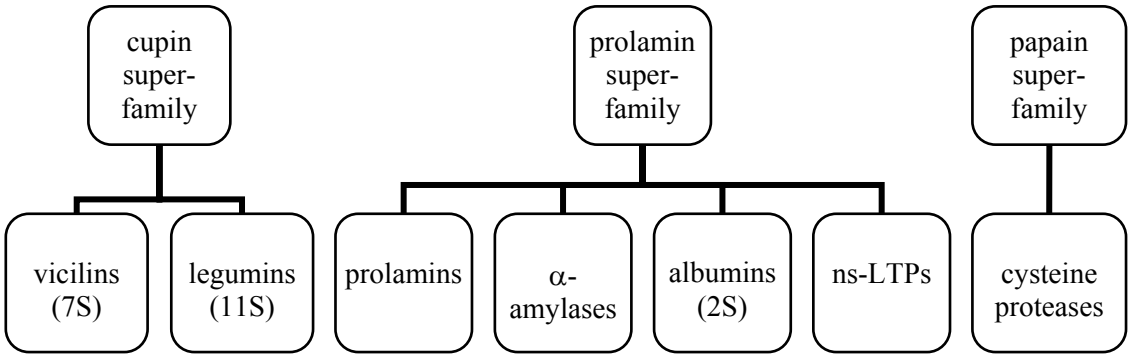


Figure 2: Classification of allergens, which have been related to class 1 food allergies into their superfamilies. ns-LTPs stands for non-specific lipid transfer proteins.

CUPIN SUPERFAMILY
Members of the cupin superfamily share a conserved domain, comprising a six-stranded β-barrel structure (Figure 3) (*35*). The two members of the cupin superfamily with known class 1 food allergenic capacity are the vicilin and legumin protein families. Both protein families generally are highly abundant in nuts and seeds, comprising up to 70% of seed proteins (*36*).
Vicilin allergens are for example Ara h 1 from peanuts (*25*), Jug r 2 from walnuts (*37*), and β-conglycinin from soybeans (*38*). Legumin allergens are for example Ara h 3 from peanuts (*22*) and glycinin from soybeans (*6, 39*).
Vicilin and legumin proteins share only 35-45% amino acid sequence identity, but they are similar in their three-dimensional structure, as both vicilins and legumins have similar N-terminal and C-terminal domains, comprising the cupin β-barrel structure (Figure 3) (*40*).

Vicilins have molecular masses of approximately 50 to 80 kDa. Vicilins are polypeptides which are often glycosylated (*41*) and which are reported to assemble in a trimeric structure with molecular masses ranging from approximately 150 to 190 kDa. Some vicilins undergo post-translational proteolytic processing, yielding various polypeptides next to the intact monomer. However, the hydrolyzed polypeptides are held together by non-covalent interactions, keeping the trimeric structure intact (*42*). The structural similarity of vicilins may cause IgE cross-reactivity between vicilin proteins of different species. For example, IgE cross-reactivity between peanut allergen Ara h 1 and pea vicilin (Pis s 1) (*43*), and between Ara h 1 and soybean β-conglycinin (*44*) has been described.

Legumin proteins are approximately 60 kDa in molecular mass and consist of a basic (approximately 20 kDa) and an acidic polypeptide (approximately 40 kDa), linked together by a disulfide bridge. Legumin proteins associate *in planta* into hexamers, resulting in a molecular mass of approximately 360 kDa (*45*). The quaternary structure of legumins after extraction is dependent on the ionic strength and pH. For example, soy glycinin at low ionic strength dissociates partly and reversibly into trimers (*46*).
For legumin proteins IgE cross-reactivity has also been reported. For example, two epitopes on the acidic polypeptide of one soybean glycinin isoform (G1) are homologous to two epitopes on peanut glycinin Ara h 3 (*39*) and one epitope on the basic polypeptide of another soybean glycinin isoform (G2) is highly conserved in Ara h 3 and in the glycinin isoform G1 (*33*).

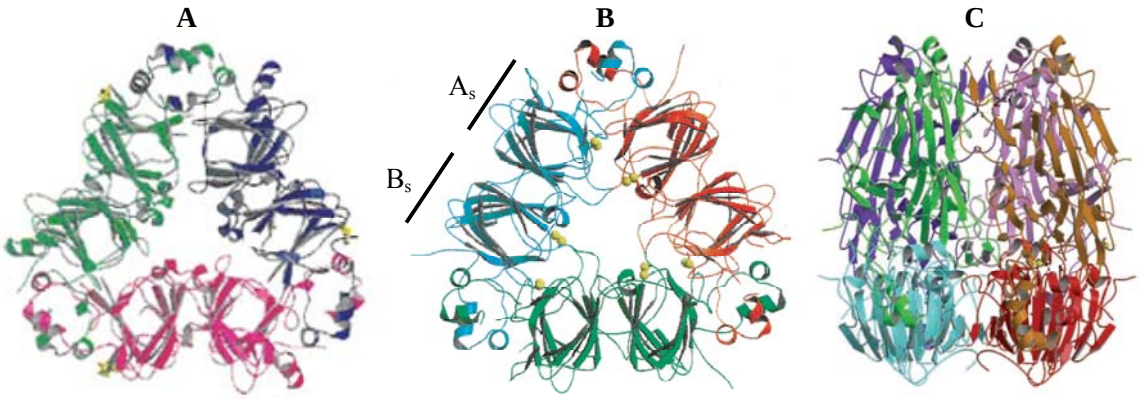


Figure 3: Ribbon presentation of aligned protein structures. A: soybean vicilin trimers (*47*); B: soybean legumin trimers (*48*); Indicated are the acidic (A_s) and basic (B_s) subunits of the protein; C: proposed model for soybean legumin hexamers, view from the side (*45*). The different colors in the Figures represent different protein polypeptides. With permission from Blackwell publishing, Elsevier, and PNAS USA, respectively.

PROLAMIN SUPERFAMILY

All members of the prolamin superfamily contain a conserved pattern of either six or eight cysteine residues within a sequence of approximately 100 amino acids. Most proteins have 4 or 5 disulphide bridges, of which two are conserved. The majority of the proteins belonging to the prolamin superfamily has molecular masses ranging between 7 and 16 kDa. Although members of the prolamin superfamily do not share a high sequence similarity, they do share a similar structure. All proteins contain four α-helices, which are stabilized by disulfide bonds (*32*).
Allergenic proteins belonging to the prolamin superfamily are subclassified into storage albumins, proline-rich storage prolamins, non-specific lipid transfer proteins (ns-LTPs), and α-amylase inhibitors. Storage albumins are reported to be *major* class 1 allergens in various seeds and nuts, like Jug r 1 in walnuts (*49*), Ses i 1 in sesame seeds (*50*), Ber e 1 in Brazil nuts (*51*), and Ara h 2 in peanuts (*26*). Prolamin allergens have been identified in wheat (*52, 53*). Ns-LTPs allergens have been detected in various fruits, like peach (*54*) and apple (*55*). Allergenic α-amylase inhibitors are known to be present in cereals like wheat (*56*) and rice (*57*).

Members of the prolamin superfamily have been identified as allergens both in foods and in pollen and are known to be able to sensitize both via the respiratory tract as well as the gastro-intestinal tract. As a result, proteins belonging to the prolamin superfamily have been related to both class 1 and class 2 food allergy (*56, 58-60*).

PAPAIN SUPERFAMILY

Members of the papain superfamily that have been related to food allergy are the cysteine proteases. Cysteine proteases generally are approximately 25 kDa (*61*). Cysteine proteases are known to represent pollen allergens, although there are indications that some cysteine proteases cause class 1 food allergy (*62*). Allergenic cysteine proteases in foods have been identified in fruits like for example kiwi fruit (*63*) and pineapple (*64*) and in soybeans (*65*). The cysteine protease in kiwi fruit does not show IgE cross-reactivity with pollen allergens, which implies that the allergen is a class 1 food allergen (*62*). This is subscribed by the fact that severe systemic reactions to kiwi are reported (*62, 66*). To our knowledge, the cysteine protease in kiwi fruit is the only cysteine protease related to class 1 food allergy.

The cysteine protease from soybeans, which is denoted Gly m Bd 30K, has been identified as a *major* allergen using serum from patients who suffer from atopic dermatitis and did not show severe systemic reactions to soybeans (*67*). People suffering from atopic dermatitis (a non-IgE mediated disease) also often suffer from pollen-related allergies (*68*). These results could imply that that Gly m Bd 30K is a class 2 allergen. Because of the uncertainty that cysteine proteases act as class 1 allergens no elaboration on these proteins is given later on in this chapter.

(PEA)NUT AND SOYBEAN ALLERGY

Peanut allergy and tree nut allergy are the most reported causes of serious food allergic symptoms (*9-11*). Peanuts are often used in industrial food products (*69*), causing high risks for peanut allergic patients to accidentally ingest hidden peanut allergens. Multiple peanut allergens have been identified and five of these allergens are classified into the prolamin or cupin superfamily (*32*). Brazil nuts are also used as ingredient in foods, like bakery products (*70*). Soybeans and soybean products are even more widely used as food ingredients, but allergenicity to soybeans has been less well studied compared to allergies to peanuts and tree nuts. Allergic reactions to soy have not been reported to be severe and / or life-threatening (*71*), except in one study (*72*). Multiple allergens belonging to the cupin or prolamin superfamily have been identified in soybeans, but some of them have not been named according to the allergen nomenclature system, as can be seen in Table 1.

Because of the widespread abundance of peanut, Brazil nut and soybean proteins in foods and / or the severity of the allergic reactions they cause, in this study we focused on *major* allergens of these foods. In Table 1 the known allergens from peanuts (*Arachis hypogaea*), Brazil nuts (*Bertholletia excelsa*), and soybeans (*Glycine max*) belonging to the cupin or prolamin superfamily are given. All peanut, soybean, and Brazil nut allergens classified into these major class 1 allergen superfamilies will be discussed below.

Table 1: The classification of identified allergens from peanuts, soybeans, and Brazil nuts into the cupin and prolamin superfamilies (*32, 67, 73*).

Protein classification	Peanut allergens*	Soybean allergens	Brazil nut allergens
Cupin superfamily			
Vicilins	Ara h 1 - *Conarachin*	*β-Conglycinin* *Gly m Bd 28 K*	
Legumins	Ara h 3 - *Arachin*	*Glycinin*	Ber e 2 - *Excelsin*
Prolamin superfamily			
Albumins	Ara h 2, 6, 7	*"Albumin"*	Ber e 1

*: the trivial names of the proteins, if known, are given in italic

ALLERGY TO VICILIN PROTEINS

ARA H 1 (PEANUT)

The proportion of peanut allergic individuals that recognizes Ara h 1 as an allergen varies from 65% to 90% (*25, 74, 75*), rendering the protein a *major* allergen. Two genes encoding Ara h 1 have been identified (*76*). Ara h 1 is a 63 kDa glycoprotein that is reported to occur in peanuts as a highly structured, stable trimer (*77-79*). The trimers are stabilized through hydrophobic interactions, but also through ionic interactions (*78*). During post-translational processing of Ara h 1 an N-terminal peptide of ~85 amino acids is cleaved off (*80*), rendering the 63 kDa protein. Throughout the complete amino acid backbone of the protein 24 linear epitopes have been mapped (*21, 81*) and 21 of them remain after post-translational processing (*80*) (Table 2).

β-CONGLYCININ (SOYBEANS)

The vicilin protein from soybeans has been well characterized. The protein consists of three polypeptides, which are denoted α, α', and β, having molecular masses of 63.5, 67.5, and 47.8 kDa, respectively (*82*). β-Conglycinin trimers consist of combinations of these polypeptides (βββ, ββα', ββα, βαα', βαα, ααα', and ααα). The trimeric protein, solubilized at low ionic strength (I < 0.2 M), reversibly associates into a hexameric form. At a high ionic strength (I > 0.5 M) the protein occurs as a trimer, while at intermediate ionic strengths both trimers and hexamers co-exist. Besides, at a very low ionic strength (I = 0.01 M), together with an associated form, a dissociated form of the protein may exist, caused by the reversible dissociation of the α polypeptide from the protein (*83*).

The α-polypeptide of β-conglycinin has been shown to be able to bind IgE, whereas the highly homologous α'-polypeptide is reported not to be able to bind IgE (*38*) (Table 2). The α-polypeptide of β-conglycinin was recognized by approximately 25% of a population of soybean-sensitive people. It should be noted that the population used for this particular study was a group of patients with atopic dermatitis (*38*). The results thus indicate that β-conglycinin is a *minor* allergen for people suffering from class 2 food allergy to soybeans. No studies have been performed aiming at determining the IgE binding to β-conglycinin using a population of persons with class 1 food allergies.

Nevertheless, as other vicilin proteins are described as class 1 allergens, β-conglycinin is also assumed to be a class 1 allergen (*32*).

GLY M BD 28K (SOYBEANS)

Besides β-conglycinin, in some soybean varieties a minor protein fraction occurs that has been related to vicilin proteins because it contains two cupin domains (*84*). The protein is denoted Gly m Bd 28K. It should be noted that most soybean varieties lack this protein (*85*). Upon post-translational processing, Gly m Bd 28K is splitted into two parts of approximately 23 and 28 kDa. Both polypeptides bind IgE from soybean allergic patients (*84*). The IgE binding to Gly m Bd 28K was studied using serum from patients suffering from atopic dermatitis (*86*), or from soybean allergic patients with no explanation of their allergic symptoms (*84*). Consequently, no conclusions have been drawn considering the route of sensitization of this protein. However, since the protein has homology with the class 1 vicilin proteins, it is likely that the protein is a class 1 allergen.

ALLERGY TO LEGUMIN PROTEINS

ARA H 3 (PEANUTS)

Peanut allergen Ara h 3 was first identified as a 14 kDa protein (*87*), but cloning of the gene encoding this protein revealed a protein of approximately 60 kDa, showing homology with legumin proteins (*22*). A peanut legumin protein was earlier identified as an allergen by Kleber-Janke and co-workers and denoted Ara h 4 (*75*). Ara h 3 and Ara h 4 are nowadays considered to be isoforms of the same allergen and usually denoted Ara h 3.

The acidic and basic polypeptides of Ara h 3 have molecular masses of approximately 45 kDa and 23 kDa, respectively. The acidic polypeptide of the protein is post-translationally processed at its C-terminal, yielding polypeptides with molecular masses of approximately 13 to 42 kDa (*88*). The protein at I = 0.2 M mainly (~90%) occurs as a hexamer, but also partly (~10%) as a trimer (*89*).

Ara h 3, depending on the populations studied, has been designated both as a *minor* and a *major* allergen, as it was recognized by serum IgE of approximately 44% and 53% of peanut allergic patient populations, respectively (*22, 75*). Furthermore, another study showed that Ara h 3 was the most important allergen in a group of peanut allergic children (*90*). For Ara h 3 four IgE binding epitopes have been identified, all situated on the acidic polypeptide of the allergen (*22*). IgE binding to the basic polypeptides of Ara h 3 has been reported (*89, 90*), which indicates the presence of more IgE binding epitopes than the four reported ones (Table 2).

GLYCININ (SOYBEANS)

The most abundant protein in soybeans is glycinin, belonging to the legumin protein family and comprising 25 to 35% of all proteins in soybeans (*91*). The structure of glycinin trimers and hexamers is given in Figure 4. Six glycinin genes have been identified, representing glycinin G1 to G5 (*92, 93*), and G7 (*94*). The molecular masses of

the basic polypeptides of glycinin are approximately 20 kDa, while those of the acidic polypeptides range from approximately 10 kDa to 40 kDa. Upon maturation, glycinin associates into a trimeric structure. After post-translational processing it associates *in planta* with another trimeric glycinin molecule to form a hexameric structure (*48*). This association occurs via face-to-face stacking of two trimeric complexes (*45*). Purified glycinin hexamers, when solubilized at a low ionic strength (I < 0.1 M), dissociate reversibly into trimers (*95*).
Glycinin is described as a *major* allergen (*6*). Initially, IgE binding to glycinin was assumed to occur at all different acidic and basic polypeptides of the protein (*96*). This was later narrowed down to the acidic and basic polypeptides of glycinin G1 and glycinin G2 (Table 2) (*6, 33, 97*).

BER E 2 (BRAZIL NUTS)
The legumin protein from Brazil nuts is denoted excelsin or Ber e 2. The molecular mass of the protein in its hexameric structure is approximately 300 kDa. Under non-reducing conditions the protein on SDS-PAGE shows bands ranging from approximately 11 to 51 kDa. Under reducing conditions the protein consists of several polypeptides with molecular masses ranging from approximately 11 to approximately 29 kDa (*98*).
Excelsin has been designated as a *minor* allergen (www.allergen.org), but no report has been published describing how this designation was performed. Indications for excelsin to act as an allergen have been described in one report (*73*), in which IgE binding to protein bands of molecular masses of approximately 34, 32, and 21 kDa was shown. These protein bands were considered to be polypeptides of excelsin (*73*), but no identification of the protein bands was performed. In another report about Brazil nut allergy IgE binding to protein bands ranging from approximately 18 to 45 kDa was reported. Again, no identification of the protein bands was performed (*99*).

ALLERGY TO ALBUMIN PROTEINS

ARA H 2 (PEANUTS)
Ara h 2 is composed of two polypeptides of approximately 17 kDa, which have essentially similar amino acid sequences (*26*). The largest polypeptide has an insertion of 12 amino acids, which causes the mass difference between the two protein isoforms (*100*). Ara h 2 proteins form four intermolecular disulfide bridges, which stabilize the structure of the protein to a large extent (*101*).
Ara h 2 is recognized by >90% of the peanut allergic populations studied and has been designated as a *major* allergen (*26, 102*). Ten epitopes have been identified on the smallest Ara h 2 isoform (denoted Ara h 2.01) (*103*). The largest isoform of the protein, denoted Ara h 2.02, bears an additional epitope (*100*).

ARA H 6 AND ARA H 7 (PEANUTS)
Besides Ara h 2, two other albumin proteins with allergenic activities have been identified in peanuts, which have been denoted Ara h 6 and Ara h 7. The molecular masses of the proteins are approximately 16 and 14 kDa, respectively. Both proteins are considered to

be *minor* allergens, being recognized by 38 and 43% of human peanut allergic sera, respectively (*75*). The amino acid sequences of Ara h 6 and Ara h 7 show a relatively high homology with the amino acid sequence of Ara h 2, but they are considered as being different allergens. For both allergens more than one isoform has been identified. Ara h 6 contains 5 possible disulfide bridges. Ara h 7 is likely to be a less stable protein than Ara h 2 and Ara h 6, as it contains only two disulfide bridges (*75*).

BER E 1 (BRAZIL NUTS)

Ber e 1 is a 12 kDa protein composed of two polypeptides with molecular masses of approximately 9 and 3 kDa, linked together by 4 disulfide bridges (*104*). Like Ara h 2, the structure of Ber e 1 is mainly stabilized by its disulfide bridges (*105*). Several isoforms of the protein have been identified (*104, 106, 107*).

Ber e 1 has been identified as the *major* allergen in Brazil nuts, with its large polypeptide being recognized by all sera of a population of Brazil nut allergic patients (*51*). One linear and one conformational epitope have been mapped on the large polypeptide of Ber e 1 (*108*).

“ALBUMIN” (SOYBEAN)

In soybeans the albumin protein has been related to food allergy. This protein has not been given a trivial name, nor has it been named according to the allergen nomenclature system. Therefore, in Tables 1 and 2 it is indicated with its family name between quotation marks. The albumin protein from soybeans consists of polypeptides of approximately 5 and 9 kDa, which are linked together by disulfide bridges. Two isoforms with IgE binding capacities have been identified. As soybean albumin was recognized by a minority of a population of patients with atopic dermatitis, the protein was designated as a *minor* allergen (*67*). It should be noted that a soybean allergic patient with a high level of IgE specific for soybeans was shown to bind IgE mainly towards a protein, which was identified as the large polypeptide of soybean albumin (*109*). These results suggest that soybean albumins, like other albumin proteins, could be class 1 food allergens.

Table 2: Overview of the number of epitopes on allergens of peanuts, soybeans, and Brazil nuts.

Source	Allergen	Desig-nation	Epitopes
Peanut			
	Ara h 1 (vicilin)	*Major*	21 after post-translational processing (*21, 81*)
	Ara h 2 (albumin)	*Major*	4 IgE binding regions (*81*) containing 10 epitopes (*103*)
	Ara h 3 (legumin)	*Major* / *minor*	4 on acidic polypeptide (*22*), IgE binding also reported to basic polypeptide (*89*)
	Ara h 6 (albumin)	*Minor*	No linear epitopes, indications for conformational epitopes (*110*)
	Ara h 7 (albumin)	*Minor*	[a]
Soybean			
	Glycinin (legumin)	*Major*	1 on acidic chain G1 (*97*), 11 on G2 (7 on acidic, 4 on basic chain) (*111*). Additional immunodominant region on acidic chain G2 (*33*)
	β-conglycinin (vicilin)	*Minor**	IgE binding region in α-polypeptide (*38*)
	Gly m Bd 28 K (vicilin-like)	*Minor**	1 epitope on C-terminal polypeptide and IgE binding to glycan moiety (*112*)
	"Albumin"	*Minor**	[a]
Brazil nut			
	Ber e 1 (albumin)	*Major*	One linear and one conformational epitope (*108*)
	Ber e 2 (legumin)	*Minor*	[a]

[a] = not known; * = allergen designation determined for patients with atopic dermatitis

DIGESTION OF FOOD ALLERGENS

As was previously mentioned, class 1 food allergens have to be stable to digestion in order to sensitize persons via the gastro-intestinal tract. Resistance of allergens to digestion implies that these allergens have to withstand the low pH and pepsin activity in the stomach in a way that epitopes are preserved, as when allergens reach the intestinal mucosa, they can be absorbed (*23*). Digestion by pepsin is the most important hydrolytic condition proteins have to withstand, but in the small intestine further hydrolysis can be induced by the action of trypsin. However, as absorption of peptides can also occur in the small intestine, there is no consensus on the time and importance of tryptic hydrolysis. As a result, many studies investigating the effects of digestion on food allergens only include pepsin hydrolysis (*23, 113-115*). Some studies have used trypsin incubation times varying from 15 minutes (*116*) to 2 hours of incubation with tryptic enzymes (*117*), either or not preceded by peptic digestion.

Pepsin cleaves protein bonds preferentially at the C-terminal or the N-terminal side of phenylalanine, tyrosine, tryptophan, and leucine amino acids (*118*). Other amino acids may also be hydrolyzed, although with a lower preference.

Several studies have been performed aiming at determining the *in vitro* peptic digestibility of food allergens, i.e. studying the time needed for the intact protein to be degraded into smaller peptides (*23, 119, 120*). Some of these studies indeed showed that, in comparison with non-allergenic proteins, allergenic proteins retain their intact form during relatively long periods of hydrolysis (*23, 120*). For example, Brazil nut allergen Ber e 1 is very stable to pepsin digestion, as approximately 25% of the protein remains intact upon prolonged pepsin digestion (*121*). However, from other studies studies it appeared that *major* food allergens (e.g. Ara h 1) were relatively quickly degraded into smaller peptides (*119*). In order to enable antibody cross-linking and mediator release from mast cells, peptides must contain at least two IgE epitopes. Considering that linear epitopes generally have lengths of approximately 15 amino acids long, peptides of 30 amino acids, which corresponds to approximately 3 kDa, in theory could elicit allergic reactions (*122*). Therefore, it is likely that intact allergens (whole proteins) after peptic digestion are not necessary to provoke allergic reactions. It rather seems necessary that peptides with sizes larger than 3 kDa, containing two or more intact epitopes, remain upon digestion. Consequently, in order to be able to draw solid conclusions about the ability of allergens to sensitize persons and to induce systemic allergic reactions via the gastro-intestinal tract, studies investigating the effects of gastro-intestinal digestion should be combined with immunological assays of the remaining hydrolysates (*123*), rather than on investigating the resistance to digestion alone.

Studies investigating the IgE binding capacity of allergens after pepsin digestion have shown the withstanding of IgE binding of food allergens upon gastric digestion (*116, 124, 125*), although some allergens are degraded into relatively small peptides (*116*). For example, digestion experiments with Ara h 1 showed a fast hydrolysis into multiple low molecular mass fragments, with molecular masses smaller than 6 kDa (as estimated by SDS-PAGE). Of these fragments, several could still bind IgE. Furthermore, the digestion products were still able to cross-link multiple IgE antibodies, as the release of mediators from mast cells could still be observed (*116*).
The effects of digestion on the IgE binding capacity of allergens from peanuts, soybeans, and Brazil nuts reported in literature are given in Table 3. As can be seen in this Table, only a few allergens from these sources have been investigated for their digestibility and concomitant IgE binding capacity. Different effects of digestion on the IgE binding capacity of allergens were reported, but all allergens investigated still showed IgE binding after peptic digestion.

Table 3: Overview of the effects of heating and digestion on the IgE binding of food allergens from peanuts, soybeans and Brazil nuts, belonging to the cupin and prolamin superfamily.

Allergen	Pepsin digestibility	IgE binding after digestion	Heat stability	IgE binding after heating	Digestibility after heating
Ara h 1	Fragments < 6 kDa formed (*116*)	Fragments able to bind IgE (*78, 116*)	Irreversible denaturation at 87 °C (*79*)	IgE binding is conserved upon heating (*79*)	[a]
Ara h 2	Stable ~10 kDa peptide formed (*101*)	~10 kDa peptide binds IgE (*101*)	Intra-molecular cross-links formed (*126*)	Less IgE binding after boiling and frying (*127*); increased IgE binding after Maillard reaction (*128*)	Increased stability to digestion after Maillard reaction (*126*)
Ara h 3	[a]	[a]	[a]	Less IgE binding after boiling and frying (*127*)	[a]
Ara h 6	Stable for 120 minutes (*129*)	[a]	[a]	IgE binding detected after Maillard reaction (*129*)	[a]
Ara h 7	[a]	[a]	[a]	[a]	[a]
Glycinin	Peptides < 23 kDa remaining (*130*)	~20 kDa peptide binds IgE (*130*)	Hexamers have a higher denaturation temperature than trimers (*131*)	IgE binding capacity is not affected by heating (*132*)	[a]
β-conglycinin	Peptides < 30 kDa remaining (*133*)	Decrease in IgE binding (*133*)	Denaturation > 75 °C (*134*)	[a]	[a]
Gly m Bd 28 K	[a]	[a]	[a]	[a]	[a]
Soy albumin	[a]	[a]	[a]	[a]	[a]
Ber e 1	Intact protein and ~6 kDa peptide remaining (*121*)	[a]	Denaturation pH 7 > 110 °C (*105*)	[a]	Similar to unheated (heating below Td*) (*121*)
Ber e 2	Peptides < 9 kDa remaining (*135*)	[a]	[a]	[a]	[a]

[a] = not known; *: Td = denaturation temperature

HEAT STABILITY OF FOOD ALLERGENS

EFFECTS OF HEATING ON THE IgE BINDING OF FOOD ALLERGENS

The peptide chain of native proteins (primary structure) is folded in a defined way, resulting in a secondary and a tertiary protein structure. These native proteins may associate and form multimeric proteins (quaternary structure) (*136*). The secondary, tertiary and quaternary structure of a protein is a result of various physical interactions, e.g. hydrophobic interactions, and disulfide bridges (*137*). A protein in its native state is thermodynamically most stable. Any change in a protein's environment, such as heat, pH, and ionic strength, affects the structure of a protein molecule. Small changes in structure, which do not drastically alter the molecular architecture of the protein, are usually regarded as conformational adaptabilities, whereas major changes in secondary, tertiary, and quaternary structures are regarded as denaturation.

Protein denaturation implies changes in secondary, tertiary, or quaternary protein structure, without the peptide bonds involved in the primary structure of a protein being disrupted. Depending on the protein and the conditions applied, various levels of denaturation can be obtained and denaturation may be confined to a region (domain) of a protein, or to the complete protein molecule (*138*). In general, during protein denaturation intermolecular and intramolecular bonds, which originally stabilized the native protein structure, are disrupted. As a result, hydrophobic amino acids become exposed to the solvent and the native protein structure unfolds and turns into a disordered one (*139*).

As was already mentioned, heating may result in protein denaturation. The temperature at which the molar concentration ratio of native and denatured protein is 1, is defined as the denaturation temperature (Td) of a protein. The denaturation temperature of proteins varies widely and is influenced by several conditions, like water activity, ionic strength, and pH. A low water activity, which is common in food products like nuts, generally stabilizes protein structures, resulting in a higher denaturation temperature compared with the same protein in an aqueous system (*140*).

Besides being caused by heat treatments, protein denaturation may also be caused by applying proteins to extreme pH values (pH values lower than 3 or higher than 10). Extreme pH values occur, for example, during digestion in the human stomach, where the pH is very low (pH 2.0). At such extreme pH values, unfolding as a result of ionization generally occurs (*136*).

The unfolded state of a protein, due to the exposure of hydrophobic regions, is an energetically unfavourable situation. Consequently, unfolded proteins usually associate to form aggregates, in order to reduce the exposure of hydrophobic groups to the aqueous solvent. Aggregation will occur when the attractive forces between protein molecules overcome the repulsive forces. Aggregation may eventually lead to the formation of insoluble protein aggregates and gel formation. During heat-induced aggregation, both non-covalent and covalent interactions (disulfide bridges) between proteins may occur (*141*).

Heat treatments may be applied to foods in order to obtain a prolonged shelf life, to inactivate anti-nutritional components, or to enhance functional properties of foods. As most foods are being processed before consumption, the stability of IgE binding to

(industrial) processing is considered a prerequisite for proteins to act as food allergens (*142*). A lot of food allergens are known to be rather stable to heating. The denaturation temperature of Brazil nut allergen Ber e 1 at neutral pH, for instance, exceeds 110 °C (*105*).
Heat-induced changes of proteins may affect the IgE binding properties of allergens. For example, protein unfolding may disrupt conformational epitopes. Heating may also increase the IgE binding capacity of proteins: Covalent modifications of proteins with other molecules induced by heating may introduce new IgE binding places (*143*). A chemical modification known to induce new IgE epitopes is the Maillard reaction (*126*).

A number of studies investigating the effects of heating on the IgE binding of foods have dealt with peanuts. Heat-induced denaturation of Ara h 1 was shown not to influence its affinity to bind IgE (*79*). However, roasting of peanuts induces an approximately 90-fold increase in IgE binding compared to raw peanuts (*126*), while boiling and frying cause a decrease in the IgE binding capacity of peanuts. The Maillard reaction, which occurs during roasting of peanuts, was shown to contribute to the increase in IgE binding in roasted peanuts (*127*). Other studies reporting on the effects of heating on the IgE binding of foods have, for example, shown that the IgE binding of milk and egg white decreases after heating (*144, 145*). The effects of heating on the IgE binding capacity of allergens from peanuts, soybeans and Brazil nuts reported in literature are given in Table 3. As can be seen in this Table, IgE binding to most allergens from these sources was detected upon heating. Depending on the allergen and heating conditions applied, the IgE binding to allergens after heating was either decreased or increased compared with the unheated allergens.

EFFECTS OF HEATING ON THE DIGESTIBILITY OF FOOD ALLERGENS
Most studies investigating the effects of processing on the allergenicity of proteins have solely dealt with the effects of processing on the IgE binding of still sequentially intact allergens, as described above. However, heating may also affect the digestibility of proteins. Heat-induced unfolding of proteins may, for example, increase the accessibility for hydrolytic enzymes in the gastro-intestinal tract. Opposite to heat-induced unfolding, heat-induced aggregation and chemical modifications may decrease the accessibility for digestive enzymes. For example, when peanut allergen Ara h 1 was subjected to the Maillard reaction, the IgE binding capacity of the allergen was not much altered compared to native Ara h 1. However, the allergen did become more stable to peptic digestion, probably as a consequence of intra-molecular cross-links formed (*126*).

As most foods are being heat-processed before consumption, the effect of heating on the digestibility of allergens is an important aspect, as well as the IgE binding of the hydrolysates of the heated allergens. The effects of heating on the digestibility of allergens from peanuts, soybeans, and Brazil nuts reported in literature are given in Table 3. As it can be seen in the Table, the digestibility of allergens after heat treatment has, to our knowledge, only been investigated for peanut allergen Ara h 2 and Brazil nut allergen Ber e 1. Moreover, it should be noted that the digestibility of Ber e 1 was investigated after heating the allergen at temperatures below its denaturation temperature (*121*). To

our knowledge, no data have been published on the effects of sequential heat-induced denaturation (and aggregation) and digestion on the IgE binding of the class 1 allergens from peanuts, soybeans, and Brazil nuts.

AIM AND SETUP OF THE THESIS

From the above it is clear that it is still largely unknown why certain proteins give rise to class 1 allergic reactions, whereas others do not. However, resistance of IgE binding capacity upon peptic digestion seems to be a prerequisite for a protein to exert systemic food allergic reactions. The digestibility of proteins is dependent on their structural characteristics. The aim of the work presented here was to understand the structural characteristics of *major* class 1 food allergens, belonging to the cupin and prolamin superfamily and to study the peptic digestibility of these allergens. Allergens from peanuts, soybeans, and Brazil nuts were studied. As digestibility is likely to be influenced by heat-processing, the effects of quaternary structure and heat-induced aggregation on pepsin hydrolysis were studied, as well as the IgE binding of the concomitant hydrolysates.
In chapter 2 the large-scale purification and characterization of the isoform pool of Ber e 1, using a novel purification technique, is described. In chapter 3 the heat stability of Ber e 1 is presented. Chapters 4 to 6 deal with homologous vicilin allergens from peanuts and soybeans. In chapter 4 and 5 the purification and characterization of native Ara h 1 from peanuts is described. The data presented in chapter 6 report the effects of heating and pepsin hydrolysis on the IgE binding of Ara h 1 and the determination of pepsin-resistant and pepsin-susceptible epitopes of this allergen. In chapter 7 the effects of heating and digestion on the IgE binding of legumin allergens from peanuts and soybeans are described. Finally, in chapter 8 a general discussion about the results from chapters 2-7 is given.

REFERENCES

1. Hourihane, J. O. Prevalence and severity of food allergy need for control. *Allergy* **1998,** 53, (S46), 84-88.
2. Cruz, N. V., Wilson, B. G., Fiocchi, A., Bahna, S. L. Survey of physicians' approach to food allergy: Part I. Prevalence and manifestations. *Annals of Allergy Asthma & Immunology* **2007,** 98, (1), A3-A3.
3. Devereux, G. The increase in the prevalence of asthma and allergy: food for thought. *Nature Reviews Immunology* **2006,** 6, (11), 869-874.
4. Mills, E. N. C., Mackie, A. R., Burney, P., Beyer, K., Frewer, L., Madsen, C., Botjes, E., Crevel, R. W. R., van Ree, R. The prevalence, cost and basis of food allergy across Europe. *Allergy* **2007,** 62, (7), 717-722.
5. King, T. P., Hoffman, D., Lowenstein, H., Marsh, D. G., Plattsmills, T. A. E., Thomas, W. Allergen nomenclature (reprinted from WHO bulletin, Vol 72, August 1994). *Allergy* **1995,** 50, (9), 765-774.

6. Helm, R. M., Cockrell, G., Connaughton, C., Sampson, H. A., Bannon, G. A., Beilinson, V., Livingstone, D., Nielsen, N. C., Burks, A. W. A soybean G2 glycinin allergen - 1. Identification and characterization. *International Archives of Allergy and Immunology* **2000,** 123, (3), 205-212.

7. Sicherer, S. H. Food allergy. *The Lancet* **2002,** 360, (9334), 701-710.

8. Abbas, A. K., Lichtman, A. H., *Basic immunology*. Saunders: Philadelphia, 2001.

9. Sampson, H. A., Mendelson, L., Rosen, J. P. Fatal and near-fatal anaphylactic reactions to food in children and adolescents. *New England Journal of Medicine* **1992,** 327, (6), 380-384.

10. Sicherer, S. H., Furlong, T. J., Munoz-Furlong, A., Burks, A. W., Sampson, H. A. A voluntary registry for peanut and tree nut allergy: Characteristics of the first 5149 registrants. *Journal of Allergy and Clinical Immunology* **2001,** 108, (1), 128-132.

11. 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. *Journal of Allergy and Clinical Immunology* **1999,** 103, (4), 559-562.

12. Bock, S. A., Atkins, F. M. The natural-history of peanut allergy. *Journal of Allergy and Clinical Immunology* **1989,** 83, (5), 900-904.

13. Dannaeus, A., Inganas, M. A follow-up-study of children with food allergy - clinical course in relation to serum IgE-antibody and IgG-antibody levels to milk, egg and fish. *Clinical Allergy* **1981,** 11, (6), 533-539.

14. Bock, S. A. Prospective appraisal of complaints of adverse reactions to foods in children during the 1st 3 years of life. *Pediatrics* **1987,** 79, (5), 683-688.

15. Kimber, I., Dearman, R. J. Food allergy: what are the issues? *Toxicology Letters* **2001,** 120, (1-3), 165-170.

16. Lynch, N. R., Goldblatt, J., Le Souef, P. N. Parasite infections and the risk of asthma and atopy. *Thorax* **1999,** 54, (8), 659-660.

17. Bodner, C. anderson, W. J., Reid, T. S., Godden, D. J. Childhood exposure to infection and risk of adult onset wheeze and atopy. *Thorax* **2000,** 55, (5), 383-387.

18. Paunio, M., Heinonen, O. P., Virtanen, M., Leinikki, P., Patja, A., Peltola, H. Measles history and atopic diseases - A population-based cross-sectional study. *Journal of the American Medical Association* **2000,** 283, (3), 343-346.

19. van Regenmortel, M. H. V., *Structure of antigens*. CRC Press: Boca Raton, 1992.

20. Bredehorst, R., David, K. What establishes a protein as an allergen? *Journal of Chromatography B* **2001,** 756, (1-2), 33-40.

21. 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. *European Journal of Biochemistry* **1997,** 245, (2), 334-339.

22. Rabjohn, P., Helm, E. M., Stanley, J. S., West, C. M., Sampson, H. A., Burks, A. W., Bannon, G. A. Molecular cloning and epitope analysis of the peanut allergen Ara h 3. *Journal of Clinical Investigation* **1999,** 103, (4), 535-542.

23. Astwood, J. D., Leach, J. N., Fuchs, R. L. Stability of food allergens to digestion *in vitro*. *Nature Biotechnology* **1996,** 14, (10), 1269-1273.

24. Ebner, C., Hirschwehr, R., Bauer, L., Breiteneder, H., Valenta, R., Ebner, H., Kraft, D., Scheiner, O. Identification of allergens in fruits and vegetables - IgE cross-reactivities with the important birch pollen allergens Bet-v-1 and Bet-v-2 (birch profilin). *Journal of Allergy and Clinical Immunology* **1995,** 95, (5), 962-969.

25. Burks, A. W., Williams, L. W., Helm, R. M., Connaughton, C., Cockrell, G., O'Brien, T. J. Identification of a major peanut allergen, Ara h 1, in patients with atopic dermatitis and

positive peanut challenge. *Journal of Allergy and Clinical Immunology* **1991,** 88, (2), 172-179.

26. Burks, A. W., Williams, L. W., Connaughton, C., Cockrell, G., O' Brien, T. J., Helm, R. M. Identification and characterization of a second major peanut allergen, Ara h II, with use of the sera of patients with atopic dermatitis and positive peanut challenge. *Journal of Allergy and Clinical Immunology* **1992,** 90, (1), 962-969.

27. Kleber-Janke, T., Crameri, R., Schreurer, S., Vieths, S., Becker, W. M. Patient-tailored cloning of allergens by phage display: peanut (*Arachis hypogaea*) profilin, a food allergen derived from a rare mRNA. *Journal of Chromatography B* **2001,** 756, (1-2), 295-305.

28. Mittag, D., Akkerdaas, J., Ballmer-Weber, B. K., Vogel, L., Wensing, M., Becker, W. M., Koppelman, S. J., Knulst, A. C., Helbling, A., S.L., H., Van Ree, R., Vieths, S. Ara h 8, a Bet v 1-homologous allergen from peanut, is a major allergen in patients with combined birch pollen and peanut allergy. *Journal of Allergy and Clinical Immunology* **2004,** 114, (6), 1410-1417.

29. Bernhisel-Broadbent, J., Taylor, S., Sampson, H. A. Cross-allergenicity in the legume botanical family in children with food hypersensitivity.2. laboratory correlates. *Journal of Allergy and Clinical Immunology* **1989,** 84, (5), 701-709.

30. Murzin, A. G., Brenner, S. E., Hubbard, T., Chothia, C. Scop - a structural classification of proteins database for the investigation of sequences and structures. *Journal of Molecular Biology* **1995,** 247, (4), 536-540.

31. Breiteneder, H., Radauer, C. A classification of plant food allergens. *Journal of Allergy and Clinical Immunology* **2004,** 113, (5), 821-830.

32. Mills, E. N. C., Jenkins, J. A. Structural, biological and evolutionary relationships of plant food allergens sensitizing via the gastrointestinal tract. *Critical Reviews in Food Science and Nutrition* **2004,** 44, (5), 379-407.

33. Xiang, P., Beardslee, T. A., Zeece, M. G., Markwell, J., Sarath, G. Identification and analysis of a conserved immunoglobulin E-binding epitope in soybean G1a and G2a and peanut Ara h 3 glycinins. *Archives of Biochemistry and Biophysics* **2002,** 408, (1), 51-57.

34. Aalberse, R. C. Structural biology of allergens. *Journal of Allergy and Clinical Immunology* **2000,** 106, (2), 228-238.

35. Dunwell, J. M. Cupins: A new superfamily of functionally diverse proteins that include germins and plant storage proteins. *Biotechnology & Genetic Engineering Reviews* **1998,** 15, (-), 1-32.

36. Bewley, J. D., Black, M., *Seeds, physiology of development and germination*. Plenum Press: New York, 1994.

37. Teuber, S. S., Jarvis, K. C., Dandekar, A. M., Peterson, W. R., Ansari, A. A. Identification and cloning of a complementary DNA encoding a vicilin-like proprotein, Jug r 2, from English walnut kernel (*Juglans regia*) a major food allergen. *Journal of Allergy and Clinical Immunology* **1999,** 104, (6), 1311-1320.

38. Ogawa, T., Bando, N., Tsuji, H., Nishikawa, K., Kitamura, K. Alpha-subunit of beta-conglycinin, an allergenic protein recognized by IgE antibodies of soybean-sensitive patients with atopic-dermatitis. *Bioscience Biotechnology and Biochemistry* **1995,** 59, (5), 831-833.

39. Beardslee, T. A., Zeece, M. G., Sarath, G., Markwell, J. P. Soybean glycinin G1 acidic chain shares IgE epitopes with peanut allergen Ara h 3. *International Archives of Allergy and Immunology* **2000,** 123, (4), 299-307.

40. Lawrence, M. C., Izard, T., Beuchat, M., Blagrove, R. J., Colman, P. M. Structure of phaseolin at 2.2 Angstrom resolution - implications for a common vicilin/legumin structure and the genetic-engineering of seed storage proteins. *Journal of Molecular Biology* **1994,** 238, (5), 748-776.

41. Mills, E. N. C., Jenkins, J. A., Marigheto, N., Belton, P. S., Gunning, A. P., Morris, V. J. Allergens of the cupin superfamily. *Biochemical Society Transactions* **2002,** 30, (6), 925-929.

42. Gatehouse, J. A., Croy, R. R. D., Morton, H., Tyler, M., Boulter, D. Characterization and subunit structures of the vicilin storage proteins of pea (*Pisum-Sativum*-L). *European Journal of Biochemistry* **1981,** 118, (3), 627-633.

43. Wensing, M., Knulst, A. C., Piersma, S., O'Kane, F., Knol, E. F., Koppelman, S. J. Patients with anaphylaxis to pea can have peanut allergy caused by cross-reactive IgE to vicilin (Ara h 1). *Journal of Allergy and Clinical Immunology* **2003,** 111, (2), 420-424.

44. Burks, A. W., Brooks, J. R., Sampson, H. A. Allergenicity of major component proteins of soybean determined by enzyme-linked assay (ELISA) and immunoblotting in children with atopic dermatitis and positive soy challenges. *Journal of Allergy and Clinical Immunology* **1988,** 81, (6), 1135-1142.

45. Adachi, M., Kanamori, J., Masuda, T., Yagasaki, K., Kitamura, K., Mikami, B., Utsumi, S. Crystal structure of soybean 11S globulin: Glycinin A3B4 homohexamer. *Proceedings of the National Academy of Sciences of the United States of America* **2003,** 100, (12), 7395-7400.

46. Wolf, W. J., Briggs, D. R. Studies on the cold-insoluble fraction of the water-extractable soybean proteins .2. factors influencing conformation changes in the 11S component. *Archives of Biochemistry and Biophysics* **1958,** 76, (2), 377-393.

47. Maruyama, N., Adachi, M., Takahashi, K., Yagasaki, K., Kohno, M., Takenaka, Y., Okuda, E., Nakagawa, S., Mikami, B., Utsumi, S. Crystal structures of recombinant and native soybean beta-conglycinin beta homotrimers. *European Journal of Biochemistry* **2001,** 268, (12), 3595-3604.

48. Adachi, M., Takenaka, Y., Gidamis, A. B., Mikami, B., Utsumi, S. Crystal structure of soybean proglycinin a1aB1b homotrimer. *Journal of Molecular Biology* **2001,** 305, (2), 291-305.

49. Teuber, S. S., Dandekar, A. M., Peterson, W. R., Sellers, C. L. Cloning and sequencing of a gene encoding a 2S albumin seed storage protein precursor from English walnut (*Juglans regia*), a major food allergen. *Journal of Allergy and Clinical Immunology* **1998,** 101, (6), 807-814.

50. Pastorello, E. A., Varin, E., Farioli, L., Pravettoni, V., Ortolani, C., Trambaioli, C., Fortunato, D., Giuffrida, M. G., Rivolta, F., Robino, A., Calamari, A. M., Lacava, L., Conti, A. The major allergen of sesame seeds (*Sesamum indicum*) is a 2S albumin. *Journal of Chromatography B* **2001,** 756, (1-2), 85-93.

51. Pastorello, E. A., Farioli, L., Pravettoni, V., Ispano, M., Conti, A., Ansaloni, R., Rotondo, F., Incorvaia, C., Bengtsson, A., Rivolta, F., Trambaioli, C., Previdi, M., Ortolani, C. Sensitization to the major allergen of Brazil nut is correlated with the clinical expression of allergy. *Journal of Allergy and Clinical Immunology* **1998,** 102, (6), 1021-1027.

52. Tanabe, S., Arai, S., Yanagihara, Y., Mita, H., Takahashi, K., Watanabe, M. A major wheat allergen has a Gln-Gln-Gln-Pro-Pro motif identified as an IgE-binding epitope. *Biochemical and Biophysical Research Communications* **1996,** 219, (2), 290-293.

53. Maruyama, N., Ichise, K., Katsube, T., Kishimoto, T., Kawase, S., Matsumura, Y., Takeuchi, Y., Sawada, T., Utsumi, S. Identification of major wheat allergens by means of the *Escherichia coli* expression system. *European Journal of Biochemistry* **1998,** 255, (3), 739-745.

54. Pastorello, E. A., Farioli, L., Pravettoni, V., Ortolani, C., Ispano, M., Monza, M., Baroglio, C., Scibola, E., Ansaloni, R., Incorvaia, C., Conti, A. The major allergen of peach (*Prunus persica*) is a lipid transfer protein. *Journal of Allergy and Clinical Immunology* **1999,** 103, (3), 520-526.

55. Pastorello, E. A., Pravettoni, V., Farioli, L., Ispano, M., Fortunato, D., Monza, M., Giuffrida, M. G., Rivolta, F., Scibola, E., Ansaloni, R., Incorvaia, C., Conti, A., Ortolani, C. Clinical

role of a lipid transfer protein that acts as a new apple-specific allergen. *Journal of Allergy and Clinical Immunology* **1999,** 104, (5), 1099-1106.

56. James, J. M., Sixbey, J. P., Helm, R. M., Bannon, G. A., Burks, A. W. Wheat alpha-amylase inhibitor: A second route of allergic sensitization. *Journal of Allergy and Clinical Immunology* **1997,** 99, (2), 239-244.

57. Nakase, M., Adachi, T., Urisu, A., Miyashita, T., Alvarez, A. M., Nagasaka, S., Aoki, N., Nakamura, R., Matsuda, T. Rice (*Oryza sativa* L) alpha-amylase inhibitors of 14-16 kDa are potential allergens and products of a multigene family. *Journal of Agricultural and Food Chemistry* **1996,** 44, (9), 2624-2628.

58. Breiteneder, H., Mills, E. N. C. Nonspecific lipid-transfer proteins in plant foods and pollens: an important allergen class. *Current Opinion in Allergy and Clinical Immunology* **2005,** 5, (3), 275-279.

59. Ordman, D. An outbreak of bronchial asthma in South-Africa, affecting more than 200 persons, caused by castor bean dust from an oil-processing factory. *International Archives of Allergy and Applied Immunology* **1955,** 7, (1), 10-24.

60. Battais, F., Courcoux, P., Popineau, Y., Kanny, G., Moneret-Vautrin, D. A., Denery-Papini, S. Food allergy to wheat: differences in immunoglobulin E-binding proteins as a function of age or symptoms. *Journal of Cereal Science* **2005,** 42, (1), 109-117.

61. Groves, M. R., Taylor, M. A. J., Scott, M., Cummings, N. J., Pickersgill, R. W., Jenkins, J. A. The prosequence of procaricain forms an alpha-helical domain that prevents access to the substrate-binding cleft. *Structure* **1996,** 4, (10), 1193-1203.

62. Pastorello, E. A., Pravettoni, V., Ispano, M., Farioli, L., Ansaloni, R., Rotondo, F., Incorvaia, C., Asman, I., Bengtsson, A., Ortolani, C. Identification of the allergenic components of kiwi fruit and evaluation of their cross-reactivity with timothy and birch pollens. *Journal of Allergy and Clinical Immunology* **1996,** 98, (3), 601-610.

63. Pastorello, E. A., Conti, A., Pravettoni, V., Farioli, L., Rivolta, F., Ansaloni, R., Ispano, M., Incorvaia, C., Giuffrida, M. G., Ortolani, C. Identification of actinidin as the major allergen of kiwi fruit. *Journal of Allergy and Clinical Immunology* **1998,** 101, (4), 531-537.

64. Baur, X. Studies on the specificity of human IgE-antibodies to the plant proteases papain and bromelain. *Clinical Allergy* **1979,** 9, (5), 451-457.

65. Ogawa, T., Tsuji, H., Bando, N., Kitamura, K., Zhu, Y. L., Hirano, H., Nishikawa, K. Identification of the soybean allergenic protein, Gly-M Bd 30k, with the soybean seed 34-kDa oil-body-associated protein. *Bioscience Biotechnology and Biochemistry* **1993,** 57, (6), 1030-1033.

66. Gall, H., Kalveram, K. J., Forck, G., Sterry, W. Kiwi fruit allergy - a new birch pollen-associated food allergy. *Journal of Allergy and Clinical Immunology* **1994,** 94, (1), 70-76.

67. Ogawa, T., Bando, N., Tsuji, H., Okajima, H., Nishikawa, K., Sasaoka, K. Investigation of the IgE-binding proteins in soybeans by immunoblotting with the sera of the soybean-sensitive patients with atopic-dermatitis. *Journal of Nutritional Science and Vitaminology* **1991,** 37, (6), 555-565.

68. Leung, D. Y. M. Atopic dermatitis: New insights and opportunities for therapeutic intervention. *Journal of Allergy and Clinical Immunology* **2000,** 105, (5), 860-876.

69. Ahmed, E. M., Young, C. T., Composition, nutrition and flavour of peanuts. In *Peanut Science and Technology*, Pattee, H. E.; Young, C. T., Eds. American Peanut Research and Education Society: Yoakum, 1982; pp 655-688.

70. Clemente, A., Chambers, S. J., Lodi, F., Nicoletti, C., Brett, G. M. Use of the indirect competitive ELISA for the detection of Brazil nut in food products. *Food Control* **2004,** 15, (1), 65-69.

71. Sicherer, S. H., Sampson, H. A., Burks, A. W. Peanut and soy allergy: a clinical and therapeutic dilemma. *Allergy* **2000,** 55, (6), 515-521.

72. Foucard, T., Yman, I. M. A study on severe food reactions in Sweden - is soy protein an underestimated cause of food anaphylaxis? *Allergy* **1999,** 54, (3), 261-265.

73. Bartolome, B., Mendez, J. D., Armentia, A., Vallverdu, A., Palacios, R. Allergens from Brazil nut: immunochemical characterization. *Allergology Immunopathology* **1997,** 25, (3), 135-144.

74. Clarke, M. C. A., Kilburn, S. A., Hourihane, J. O., Dean, K. R., Warner, J. O., Dean, T. P. Serological characteristics of peanut allergy. *Clinical and Experimental Allergy* **1998,** 28, (10), 1251-1257.

75. Kleber-Janke, T., Crameri, R., Appenzeller, U., Schlaak, M., Becker, W. M. Selective cloning of peanut allergens, including profilin and 2S albumins, by phage display technology. *International Archives of Allergy and Immunology* **1999,** 119, (4), 265-274.

76. Burks, A. W., Cockrell, G., Stanley, J. S., Helm, R. M., Bannon, G. A. Recombinant peanut allergen ara-h-1 expression and IgE binding in patients with peanut hypersensitivity. *Journal of Clinical Investigation* **1995,** 96, (4), 1715-1721.

77. 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. *Journal of Biological Chemistry* **1998,** 273, (22), 13753-13759.

78. 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. *Journal of Immunology* **2000,** 164, (11), 5844-5849.

79. 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. *Journal of Biological Chemistry* **1999,** 274, (8), 4770-4777.

80. de Jong, E. C., van Zijverden, M., Spanhaak, S., Koppelman, S. J., Pellegrom, H., Penninks, A. H. Identification and partial characterization of multiple major allergens in peanut proteins. *Clinical and Experimental Allergy* **1998,** 28, (6), 743-751.

81. 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. *Journal of Allergy and Clinical Immunology* **2004,** 113, (4), 776-782.

82. Brooks, J. R., Morr, C. V. Current aspects of soy protein fractionation and nomenclature. *Journal of the American Oil Chemists Society* **1985,** 62, (9), 1347-1354.

83. Thanh, V. H., Shibasaki, K. Major proteins of soybean seeds - reversible and irreversible dissociation of beta-conglycinin. *Journal of Agricultural and Food Chemistry* **1979,** 27, (4), 805-809.

84. Xiang, P., Haas, E. J., Zeece, M. G., Markwell, J., Sarath, G. C-terminal 23 kDa polypeptide of soybean Gly m Bd 28 K is a potential allergen. *Planta* **2004,** 220, (1), 56-63.

85. Ogawa, T., Samoto, M., Takahashi, K. Soybean allergens and hypoallergenic soybean products. *Journal of Nutritional Science and Vitaminology* **2000,** 46, (6), 271-279.

86. Tsuji, H., Bando, N., Hiemori, M., Yamanishi, R., Kimoto, M., Nishikawa, K., Ogawa, T. Purification and characterization of soybean allergen Gly m Bd 28K. *Bioscience Biotechnology and Biochemistry* **1997,** 61, (6), 942-947.

87. Eigenmann, P. A., Burks, A. W., Bannon, G. A., Sampson, H. A. Identification of unique peanut and soy allergens in sera adsorbed with cross-reacting antibodies. *Journal of Allergy and Clinical Immunology* **1996,** 98, (5), 969-978.

88. Piersma, S. R., Gaspari, M., Hefle, S. L., Koppelman, S. J. Proteolytic processing of the peanut allergen Ara h 3. *Molecular Nutrition & Food Research* **2005,** 49, (8), 744-755.

89. Koppelman, S. J., Knol, E. F., Vlooswijk, R. A. A., Wensing, M., Knulst, A. C., Hefle, S. L., Gruppen, H., Piersma, S. Peanut allergen Ara h 3: isolation from peanuts and biochemical characterization. *Allergy* **2003,** 58, (11), 1144-1151.

90. Restani, P., Ballabio, C., Corsini, E., Fiocchi, A., Isoardi, P., Magni, C., Poiesi, C., Terracciano, L., Duranti, M. Identification of the basic subunit of Ara h 3 as the major allergen in a group of children allergic to peanuts. *Annals of Allergy Asthma & Immunology* **2005,** 94, (2), 262-266.

91. Wolf, W. J., Babcock, G. E., Smith, A. K. Ultracentrifugal differences in soybean protein composition. *Nature* **1961,** 191, (479), 1395-1396.

92. Fischer, R. L., Goldberg, R. B. Structure and flanking regions of soybean seed protein genes. *Cell* **1982,** 29, (2), 651-660.

93. Scallon, B., Thanh, V. H., Floener, L. A., Nielsen, N. C. Identification and characterization of DNA clones encoding group-II glycinin subunits. *Theoretical and Applied Genetics* **1985,** 70, (5), 510-519.

94. Beilinson, V., Chen, Z., Shoemaker, R. C., Fischer, R. L., Goldberg, R. B., Nielsen, N. C. Genomic organization of glycinin genes in soybean. *Theoretical and Applied Genetics* **2002,** 104, (6-7), 1132-1140.

95. Lakemond, C. M. M., de Jongh, H. H. J., Hessing, M., Gruppen, H., Voragen, A. G. J. Soy glycinin: Influence of pH and ionic strength on solubility and molecular structure at ambient temperatures. *Journal of Agricultural and Food Chemistry* **2000,** 48, (6), 1985-1990.

96. Pedersen, H. S., Djurtoft, R. Antigenic and allergenic properties of acidic and basic peptide chains from glycinin. *Food and Agricultural Immunology* **1989,** 1, (2), 101-109.

97. Zeece, M. G., Beardslee, T. A., Markwell, J. P., Sarath, G. Identification of an IgE-binding region in soybean acidic glycinin G1. *Food and Agricultural Immunology* **1999,** 11, (1), 83-90.

98. Kamiya, N., Sakabe, K., Sakabe, N., Sasaki, K., Sakakibara, M., Noguchi, H. Structural properties of Brazil nut 11S globulin, excelsin. *Agricultural and Biological Chemistry* **1983,** 47, (9), 2091-2098.

99. Asero, R., Mistrello, G., Roncarolo, D., Amato, S. Allergy to minor allergens of Brazil nut. *Allergy* **2002,** 57, (11), 1080-1081.

100. Chatel, J. M., Bernard, H., Orson, F. M. Isolation and characterization of two complete Ara h 2 isoforms cDNA. *International Archives of Allergy and Immunology* **2003,** 131, (1), 14-18.

101. Sen, M., Kopper, R., Pons, L., Abraham, E. C., Burks, A. W., Bannon, G. A. Protein structure plays a critical role in peanut allergen stability and may determine immunodominant IgE-binding epitopes. *Journal of Immunology* **2002,** 169, (2), 882-887.

102. Burks, A. W., Cockrell, G., Connaughton, C., Karpas, A., Helm, R. M. Epitope specificity of the major peanut allergen, Ara-H-II. *Journal of Allergy and Clinical Immunology* **1995,** 95, (2), 607-611.

103. Stanley, J. S., King, N., Burks, A. W., Huang, S. K., Sampson, H., Cockrell, G., Helm, R. M., West, C. M., Bannon, G. A. Identification and mutational analysis of the immunodominant IgE binding epitopes of the major peanut allergen Ara h 2. *Archives of Biochemistry and Biophysics* **1997,** 342, (2), 244-253.

104. Altenbach, S. B., Pearson, K. W., Sun, S. S. M. Nucleotide sequences of cDNAs encoding two members of the Brazil nut methionine-rich 2S albumin gene family. *Plant Physiology* **1992,** 98, (4), 1520-1522.

105. Koppelman, S. J., Nieuwenhuizen, W. F., Gaspari, M., Knippels, L. M. J., Penninks, A. H., Knol, E. F., Hefle, S. L., de Jongh, H. H. J. Reversible denaturation of Brazil nut 2S albumin (Ber e1) and implication of structural destabilization on digestion by pepsin. *Journal of Agricultural and Food Chemistry* **2004,** 53, (1), 123-131.

106. Ampe, C., Van Damme, J., de Castro, L. A., Sampaio, M. J., Van Montagu, M., Vandekerckhove, J. The amino-acid sequence of the 2S sulphur-rich proteins from seeds of Brazil nut (*Bertholletia excelsa* H.B.K.). *European Journal of Biochemistry* **1986,** 159, (3), 597-604.

107. Gander, E. S., Holmstroem, K. O., de Paiva, G. R., de Castro, L. A. B., Carneiro, M., Grossi de Sa, M. F. Isolation, characterization and expression of a gene coding for a 2S albumin from *Bertholletia excelsa* (Brazil nut). *Plant Molecular Biology* **1991,** 16, (3), 437-448.

108. Alcocer, M. J. C., Murtagh, G. J., Wilson, P. B., Progias, P., Lin, J., Archer, D. B. The major human structural IgE epitope of the Brazil nut allergen Ber e 1: A chimaeric and protein microarray approach. *Journal of Molecular Biology* **2004,** 343, (3), 759-769.

109. Gu, X. L., Beardslee, T., Zeece, M., Sarath, G., Markwell, J. Identification of IgE-binding proteins in soy lecithin. *International Archives of Allergy and Immunology* **2001,** 126, (3), 218-225.

110. Suhr, M., Wicklein, D., Becker, W. M. Characterization of the peanut allergen Ara h 6: Isolation and mapping of IgE-binding epitopes. *Journal of Allergy and Clinical Immunology* **2002,** 109, (1), S286.

111. Helm, R. M., Cockrell, G., Connaughton, C., Sampson, H. A., Bannon, G. A., Beilinson, V., Nielsen, N. C., Burks, A. W. A soybean G2 glycinin allergen - 2. Epitope mapping and three-dimensional modeling. *International Archives of Allergy and Immunology* **2000,** 123, (3), 213-219.

112. Hiemori, M., Bando, N., Ogawa, T., Shimada, H., Tsuji, H., Yamanishi, R., Terao, J. Occurrence of IgE antibody-recognizing N-linked glycan moiety of a soybean allergen, Gly m Bd 28K. *International Archives of Allergy and Immunology* **2000,** 122, (4), 238-245.

113. Thomas, K., Aalbers, M., Bannon, G. A., Bartels, M., Dearman, R. J., Esdaile, D. J., Fu, T. J., Glatt, C. M., Hadfield, N., Hatzos, C., Hefle, S. L., Heylings, J. R., Goodman, R. E., Henry, B., Herouet, C., Holsapple, M., Ladics, G. S., Landry, T. D., MacIntosh, S. C., Rice, E. A., Privalle, L. S., Steiner, H. Y., Teshima, R., van Ree, R., Woolhiser, M., Zawodny, J. A multi-laboratory evaluation of a common *in vitro* pepsin digestion assay protocol used in assessing the safety of novel proteins. *Regulatory Toxicology and Pharmacology* **2004,** 39, (2), 87-98.

114. Akkerdaas, J. H., Wensing, M., Asero, R., Rivas, M. F., Knulst, A. C., Bolhaar, S., Hefle, S. L., Aalberse, R. C., van Ree, R. IgE binding to pepsin-digested food extracts. *International Archives of Allergy and Immunology* **2005,** 138, (3), 203-208.

115. Diaz-Perales, A., Blanco, C., Sanchez-Monge, R., Varela, J., Carrillo, T., Salcedo, G. Analysis of avocado allergen (Prs a 1) IgE-binding peptides generated by simulated gastric fluid digestion. *Journal of Allergy and Clinical Immunology* **2003,** 112, (5), 1002-1007.

116. 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. *Clinical and Experimental Allergy* **2006,** 36, (10), 1281-1288.

117. Anonymous, Simulated gastric fluid and simulated intestinal fluid, TS. In *The United States Pharmacopeia 23, The National Formulary 18*, The United States Pharmacopeial Convention inc.: Rockville, 1995; p 2053.

118. Keil, B., *Specificity of proteolysis*. Springer-Verlag: Berlin, 1992.

119. 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. *Journal of Agricultural and Food Chemistry* **2002,** 50, (24), 7154-7160.

120. Takagi, K., Teshima, R., Okunuki, H., Sawada, J. Comparative study of *in vitro* digestibility of food proteins and effect of preheating on the digestion. *Biological and Pharmaceutical Bulletin* **2003,** 26, (7), 969-973.

121. 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 Journal* **2005,** 272, (2), 341-352.

122. Huby, R. D. J., Dearman, R. J., Kimber, I. Why are some proteins allergens? *Toxicological Sciences* **2000,** 55, (2), 235-246.

123. Moreno, F. J. Gastrointestinal digestion of food allergens: Effect on their allergenicity. *Biomedicine & Pharmacotherapy* **2007,** 61, (1), 50-60.

124. Vassilopoulou, E., Rigby, N., Moreno, F. J., Zuidmeer, L., Akkerdaas, J., Tassios, I., Papadopoulos, N. G., Saxoni-Papageorgiou, P., van Ree, R., Mills, E. N. C. Effect of *in vitro* gastric and duodenal digestion on the allergenicity of grape lipid transfer protein. *Journal of Allergy and Clinical Immunology* **2006,** 118, (2), 473-480.

125. Takagi, K., Teshima, R., Okunuki, H., Itoh, S., Kawasaki, N., Kawanishi, T., Hayakawa, T., Kohno, Y. Kinetic analysis of pepsin digestion of chicken egg white ovomucoid and allergenic potential of pepsin fragments. *International Archives of Allergy and Immunology* **2005,** 136, (1), 23-32.

126. Maleki, S. J., Chung, S. Y., Champagne, E. T., Raufman, J. The effects of roasting on the allergenic properties of peanut proteins. *Journal of Allergy and Clinical Immunology* **2000,** 106, (4), 763-768.

127. Beyer, K., Morrow, E., Li, X. M., Bardina, L. B., Bannon, G. A., Burks, A. W., Sampson, H. A. Effects of cooking methods on peanut allergenicity. *Journal of Allergy and Clinical Immunology* **2001,** 107, (6), 1077-1081.

128. Gruber, P., Becker, W. M., Hofmann, T. 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. *Journal of Agricultural and Food Chemistry* **2005,** 53, (6), 2289-2296.

129. Suhr, M., Wicklein, D., Lepp, U., Becker, W. M. Isolation and characterization of natural Ara h 6: Evidence for a further peanut allergen with putative clinical relevance based on resistance to pepsin digestion and heat. *Molecular Nutrition & Food Research* **2004,** 48, (5), 390-399.

130. Lee, H. W., Keum, E. H., Lee, S. J., Sung, D. E., Chung, D. H., Lee, S. I., Oh, S. Allergenicity of proteolytic hydrolysates of the soybean 11S globulin. *Journal of Food Science* **2007,** 72, (3), C168-C172.

131. Lakemond, C. M. M., de Jongh, H. H. J., Hessing, M., Gruppen, H., Voragen, A. G. J. Heat denaturation of soy glycinin: Influence of pH and ionic strength on molecular structure. *Journal of Agricultural and Food Chemistry* **2000,** 48, (6), 1991-1995.

132. Burks, A. W., Williams, L. W., Thresher, W., Connaughton, C., Cockrell, G., Helm, R. M. Allergenicity of peanut and soybean extracts altered by chemical or thermal-denaturation in patients with atopic-dermatitis and positive food challenges. *Journal of Allergy and Clinical Immunology* **1992,** 90, (6), 889-897.

133. Keum, E. H., Leer, S. I., Oh, S. Effect of enzymatic hydrolysis of 7S globulin, a soybean protein, on its allergenicity and identification of its allergenic hydrolyzed fragments using SDS-PAGE. *Food Science and Biotechnology* **2006,** 15, (1), 128-132.

134. Mills, E. N. C., Huang, L., Noel, T. R., Gunning, A. P., Morris, V. J. Formation of thermally induced aggregates of the soya globulin beta-conglycinin. *Biochimica et Biophysica Acta-Protein Structure and Molecular Enzymology* **2001,** 1547, (2), 339-350.

135. Murtagh, G. J., Archer, D. B., Dumoulin, M., Ridout, S., Matthews, S., Arshad, S. H., Alcocer, M. J. C. *In vitro* stability and immunoreactivity of the native and recombinant plant food 2S albumins Ber e 1 and SFA-8. *Clinical and Experimental Allergy* **2003,** 33, (8), 1147-1152.

136. Creighton, T. E., *Proteins: Structures and molecular properties*. W.H. Freeman: New York, 1996.

137. Alber, T. Mutational effects on protein stability. *Annual Review of Biochemistry* **1989,** 58, 765-798.

138. Tanford, C., *Physical chemistry of macromolecules*. John Wiley and Sons: New York, 1968.

139. Boye, J. L., Ma, D.-Y., Harwalkar, V. R., Thermal denaturation and coagulation of proteins. In *Food proteins and their applications*, Damodaran, S.; Paraf, A., Eds. Marcel Dekker Inc.: New York, 1997; pp 25-26.

140. Gekko, K., Timasheff, S. N. Thermodynamic and kinetic examination of protein stabilization by glycerol. *Biochemistry* **1981,** 20, (16), 4677-4686.

141. Utsumi, S., Matsumura, Y., Mori, T., Structure-function relationships of soy proteins. In *Food proteins and their applications*, Damodaran, S.; Paraf, A., Eds. Marcel Dekker Inc.: New York, 1997; pp 257-291.

142. Besler, M., Steinhart, H., Paschke, A. Stability of food allergens and allergenicity of processed foods. *Journal of Chromatography B* **2001,** 756, (1-2), 207-228.

143. Davis, P. J., Williams, S. C. Protein modification by thermal processing. *Allergy* **1998,** 53, (S46), 102-105.

144. Gjesing, B., Osterballe, O., Schwartz, B., Wahn, U., Lowenstein, H. Allergen-specific IgE antibodies against antigenic components in cow milk and milk substitutes. *Allergy* **1986,** 41, (1), 51-56.

145. Anet, J., Back, J. F., Baker, R. S., Barnett, D., Burley, R. W., Howden, M. E. H. Allergens in the white and yolk of hens egg - a study of IgE binding by egg proteins. *International Archives of Allergy and Applied Immunology* **1985,** 77, (3), 364-371.

2

EXPANDED BED ADSORPTION AS A FAST TECHNIQUE FOR THE LARGE-SCALE PURIFICATION OF THE COMPLETE ISOFORM OF BER E 1, THE *MAJOR* ALLERGEN FROM BRAZIL NUTS

Based on: van Boxtel, E. L., van Koningsveld, G. A., Koppelman, S. J., van den Broek, L. A. M., Voragen, A. G. J., Gruppen, H. Expanded bed adsorption as a fast technique for the large-scale purification of the complete isoform pool of Ber e 1, the major allergen from Brazil nuts. *Molecular Nutrition & Food Research* **2006**, 50, (3), 275-281.

ABSTRACT

In this chapter, a new, fast, large-scale purification method for Ber e 1, the *major* allergen from Brazil nuts, using expanded bed adsorption (EBA) chromatography, is presented. Using EBA, crude extracts can be applied to a fluidized column, which allows the unhindered passage of particulate impurities, thereby avoiding time-consuming centrifugation or filtration steps. With this new purification method, 2.8 g of Ber e 1 was obtained from 85 g defatted Brazil nut meal, essentially within 1 day. Various structural as well as immunochemical characteristics of the purified protein were determined and compared to those of Ber e 1 purified using conventional chromatographic techniques. The complete pool of Ber e 1 isoforms was collected using EBA. The most abundant isoforms were observed to a have pI around 8 and heterogeneity was observed in both the large and the small subunit of the heterodimeric protein. Ber e 1 has a highly ordered secondary structure. No apparent differences in immune reactivity were observed between EBA purified Ber e 1 and conventionally purified Ber e 1, using IgE binding experiments. Thus, using EBA, Ber e 1 can be purified fast and on gram-scale, while having a purity equal to that of conventionally purified Ber e 1.

INTRODUCTION

IgE mediated allergic reactions to foods are nowadays considered as a serious public health problem. Moreover, it is expected that in the future the number of people suffering from food allergies will further increase (*1*). The most important foods causing allergic reactions are cow's milk, eggs, soy, wheat, peanuts, tree nuts, fish, and shellfish (*2*). At present, the only effective treatment for food allergic people is the avoidance of exposure to allergens (*3*), although a lot of research is focusing on the development of treatments for food allergy. Since it is still largely unknown why certain proteins give rise to food-related allergic reactions (*4*), there is a need for purified allergens that can be used to investigate their biochemical and immunochemical properties and to link this knowledge to their immunological activity *in vivo*. Next to that, purified (modified) allergenic proteins can be used for diagnosis and therapy.

Large quantities of purified allergens are for example needed for animal studies and clinical investigations. Also, studying the behaviour of allergens in a simulated food matrix during various food processing conditions, which may result in a better understanding of the changes in allergenicity during and as a result of processing, requires large amounts of purified allergens. However, purification of large quantities of allergens is often problematic. Problems that may occur are due to the presence of only low concentrations of the allergen in the source or the presence of other compounds in the food or ingredient matrix, such as lipids and phenolic compounds, that may hinder purification (*5*).

In order to overcome complications during purification, efforts have been made to produce recombinant allergens aimed to have the same properties as the native proteins (*6, 7*). Recombinant proteins may, however, differ in folding, degree of post-translational processing and glycosylation, from their natural counterparts (*6, 8, 9*). As a result, they

may show allergenic activities different from their natural forms (*5*) and are only suitable for a limited number of applications. Therefore, more efficient and more convenient methods to purify large amounts of native allergens remain desired.
One of the approaches for designing large-scale protein purification protocols is expanded bed adsorption (EBA). EBA is a chromatographic technique that was originally developed for the recovery of proteins from feed stocks containing cells and / or cell debris, in order to overcome the drawbacks caused by centrifugation / or (ultra)filtration steps (*10-12*). EBA enables direct adsorptive protein purification from crude extracts, in this way avoiding these clarification steps. With EBA, the bed of adsorbent particles is expanded because the mobile phase is applied in an upward direction with a relatively high flow rate. In this way, a distance between the particles is created, which enables an unhindered passage of particulate impurities during application of crude feed streams. Thus, the target protein adsorbs onto the column, while other proteins and particulates present in the feed stream pass through the expanded bed (*13*). The different EBA adsorbents comprise ion exchangers, affinity adsorbents and mixed mode ion exchangers. These mixed mode ligands enable a salt-tolerant binding, setting aside the need for dilution of extracts to a lower ionic strength. Furthermore, the high density of all adsorbents enables the application of undiluted, crude samples with a high density and / or viscosity.

Brazil nuts are the seeds of *Bertholletia excelsa* H.B.K., a tree that grows in the Amazon tropical forest. The nut kernels can be eaten raw and can be used as an ingredient in foods like bakery products (*14*). Allergic reactions in response to Brazil nut consumption are well-known and can be very severe and even life threatening. The *major* allergen from these nuts, Ber e 1, is a member of the 2S seed storage albumin group (*15, 16*). 2S albumins are products of multigene families and therefore various isoforms may exist. Also for Ber e 1 a number of isoforms have been identified (*17-19*). Various procedures for the purification of Ber e 1 have been described (*14, 15, 20-23*), but most of these methods consist of several time-consuming centrifugation and / or column chromatography steps, making them only suitable for the purification of small quantities of Ber e 1.

In view of the increasing demands for relatively large amounts of purified allergens, the aim of the presented study was to develop a fast method to purify gram quantities of an allergen, using EBA chromatography as a first capturing step. As an example, the allergen Ber e 1 from Brazil nuts was chosen as its present purification methods are rather time-consuming. The thus purified protein was physico-chemically characterized. Several protein characteristics were compared to those of Ber e 1 that had been purified using conventional chromatographic techniques and to the features of Ber e 1 described in literature.

MATERIALS AND METHODS

MATERIALS

All chemicals were obtained from Merck (Darmstadt, Germany), unless stated otherwise. Ber e 1 that had been purified using conventional chromatographic techniques was obtained from TNO Quality of Life (Zeist, The Netherlands) and was purified as described by Koppelman and co-workers (*23*). This protein was denoted Ber e $1_{conv.}$. Unshelled Brazil nuts were purchased from Imko Nut Products (Doetinchem, The Netherlands) and stored at 4 °C under vacuum until use.

BRAZIL NUT PROTEIN EXTRACTION

Brazil nuts were ground using a domestic type mechanical high-speed slicer (Kenwood Corp., Tokyo, Japan) and defatted with hexane using Soxhlet extraction. After drying at room temperature, the partially defatted meal was ground using a Waring blender (Waring Products Inc., New Hartford, CT, USA) and was subjected to a second defatting step. Hereafter, the defatted meal was dried at room temperature for 24 h and stored at 4 °C until use.

Defatted Brazil nut meal (100 g) was extracted by stirring in 20 mM sodium acetate buffer, pH 5.5, at a meal / solvent ratio of 1:20 (w/v), for 2 h at room temperature. These conditions were equal to those used during conventional purification of the protein (*23*). Subsequently, the extract was allowed to settle overnight at 4 °C. The supernatant was collected and sieved through a kitchen sieve and denoted Brazil nut extract.

PURIFICATION OF BER E 1 USING EBA

The Brazil nut protein extract (1.7 L) was applied directly onto a STREAMLINE Direct 24 expanded bed column (170 cm × 24 mm) containing STREAMLINE Direct CST-1, a mixed mode cation exchange adsorbent (GE Healthcare, Uppsala, Sweden).

The sedimented bed height of the STREAMLINE Direct column was 40 cm, which corresponds to a column volume of 175 mL. The bed was fluidized by applying an upward flow. The flow rate that was used during the whole purification was 48 mL min^{-1}. At this flow rate, the expanded bed height and volume were approximately 100 cm and 452 mL, respectively. The column was equilibrated with 10 fluidized column volumes of 20 mM sodium acetate buffer, pH 5.5, at 48 mL min^{-1} and loaded with 1.7 L Brazil nut extract, having a protein concentration of 5.0 mg mL^{-1} protein. The unbound material was washed out with 10 fluidized column volumes of 20 mM sodium acetate buffer, pH 5.5. The bound material was eluted with 4 fluidized column volumes of 20 mM sodium phosphate buffer, pH 8.2, containing 1 M NaCl, at a flow rate of 48 mL min^{-1}. Fractions exhibiting absorption at 280 nm were collected. During the course of the EBA purification, samples were collected at different stages (sample application, washing, and elution of the column).

The bound fraction of the STREAMLINE Direct column was further purified by applying it (180 mL per run) to a Superdex 30 column (58 cm × 10 cm, GE Healthcare), which was equilibrated and eluted with 50 mM sodium phosphate buffer, pH 7.0, at 40 mL min^{-1} on a Biopilot system (GE Healthcare). The eluate was monitored at 280 nm and fractions with a high absorbance were collected.

Size exclusion chromatography (SEC) fractions containing almost exclusively Ber e 1, as estimated by SDS-PAGE, were pooled and concentrated, using a cross-flow hollow fibre ultrafiltration membrane with a molecular weight cut-off of 1,000 (GE Healthcare). Next, the retentate was dialysed in 3,500 MWCO dialysis tubings (Spectrum laboratories Inc., Rancho Dominguez, CA, USA) against demineralized water. During dialysis the pH was kept at pH 5.0. Next, the sample was lyophilized and stored at –20 °C until use. The protein was denoted Ber e 1_{EBA}.

PROTEIN QUANTIFICATION

Protein concentrations were measured using the Bradford method (*24*). Bovine serum albumin (BSA, Sigma-Aldrich Inc., St. Louis, MO, USA) was used as a standard. All assays were performed at least in duplicate.

SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

SDS-PAGE was performed on a PhastSystem (GE Healthcare), according to the instructions of the manufacturer. High density Phastgels were used. Reducing conditions were obtained by adding β-mercaptoethanol to a concentration of 10 mM. Gels were stained according to the Coomassie Brilliant Blue procedure provided by the manufacturer. Polypeptide markers (2.5-16.9 kDa) from GE Healthcare were used. When protein concentrations in the samples were too low, samples were concentrated in advance with 3,000 MWCO centrifugal filters (Millipore, Billerica, MA, USA).

REDUCTION AND ALKYLATION OF BER E 1

The reduction and alkylation of Ber e 1 was performed as described previously (*23*) and was performed at room temperature. In short, 50 mg lyophilized protein was dissolved in 25 mL 6 M guanidinium chloride in 10 mM NH_4HCO_3 (pH 7.8) and heated to 56 °C. Dithiothreitol (DTT) was added to a concentration of 20 mM and the solution was stirred for 60 min. After cooling to room temperature, 2.5 mL 1 M iodoacetamide solution in 100 mM NH_4HCO_3 (pH 7.8) was added. To allow alkylation to occur, the stirred solution was placed for 90 min in the dark.

The alkylated protein solution was dialyzed against demineralized water using 2,000 MWCO dialysis tubing at 4 °C (Spectrum laboratories) and the reduced and alkylated (R+A) protein was subsequently lyophilized.

MATRIX-ASSISTED LASER DESORPTION / IONIZATION-TIME OF FLIGHT MASS SPECTROMETRY (MALDI-TOF MS)

MALDI-TOF MS analyses were performed with native and with reduced and alkylated (R+A) Ber e 1_{EBA}. Samples were mixed 1:10 (v/v) with 10 mg mL^{-1} matrix solution of α-cyano-hydroxycinnamic acid in 50% acetonitrile (v/v), containing 0.3% (v/v) TFA. MALDI-TOF MS experiments were performed on a Bruker Ultraflex-TOF (Bruker instruments, Bremen, Germany), operated in the linear positive ion mode. The mass spectrometer was externally calibrated with a mixture of proteins (mass range 5,734-16,952 Da, Bruker Daltonics, Leipzig, Germany).

CAPILLARY ISOELECTRIC FOCUSING

Isoelectric focusing of Ber e 1 preparations was performed on a Beckman Coulter P/ACE MDQ capillary electrophoresis system (Beckman Coulter Inc., CA, USA). The ProteomeLab capillary isoelectric focusing kit from pH 3 to 10 (Beckman Coulter Inc.) was used according to the instructions of the manufacturer. The markers used were ribonuclease A and β-lactoglobulin A, with pI's of 9.45 and 5.1, respectively. The absorbance was measured at 214 nm and analyses were performed in duplicate.

CIRCULAR DICHROISM (CD) SPECTROSCOPY

Far-UV CD measurements were performed with 0.25 mg mL^{-1} protein solutions in 5 mM sodium phosphate buffer, pH 7.0. Spectra were recorded on a Jasco-715 spectropolarimeter (Jasco Inc., Easton, MD, USA), thermostatted at 20 ºC, in the spectral range from 190 to 260 nm. A step resolution of 0.5 nm, a scan speed of 100 nm min^{-1}, a bandwidth of 1 nm and a response time of 0.125 sec were used. Ten scans were accumulated and averaged. Buffer spectra were subtracted from protein spectra. The secondary structure content was estimated using a spectral non-linear least square fitting procedure, using reference spectra, as described by de Jongh and co-workers (*25*).

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

IgE binding experiments were performed using the direct ELISA technique. The 96-well plates were coated with 100 μL of a 0-1 μg mL^{-1} Ber e 1 solution in phosphate-buffered saline, pH 7.4 (PBS) and subsequently blocked with 250 μL 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl (TBS) and 0.5% (w/v) BSA. After washing the wells three times with TBS containing 0.2% (w/v) Tween 20, 100 μL of 50 times diluted serum from a patient allergic to Brazil nuts was added. After incubation and washing, peroxidase labeled anti-IgE (100 μL, 2 times diluted; Diagnostic Products Coop., Los Angeles, CA, USA) was added and plates were incubated again. Subsequently, after washing, 100 μL of developing / coloring buffer solution was added to each well. This buffer solution was freshly prepared by mixing 30 mL 50 mM citric acid / 100 mM sodium phosphate buffer, pH 5.0, with 1 tablet of 1,2 phenylenediamine dihydrochloride (OPD·2HCl; Fluka Biochemika, Buchs, Switzerland) and 12 μL 30% (v/v) H_2O_2. The reaction was stopped by adding 50 μL 1 M sulfuric acid. Absorption at 490 nm was measured using a Bio-Rad Microplate Manager 4.01 (Bio-Rad Laboratories Inc., Hercules, CA, USA).

RESULTS AND DISCUSSION

PURIFICATION OF BER E 1

A crude, non-diluted Brazil nut protein extract was used to purify Ber e 1 using the EBA column. In order to determine the binding capacity of the column material for Brazil nut protein, a surplus of protein was applied to the column (8.5 g), i.e. an amount higher than the theoretical binding capacity of the column material (5.3 g BSA), and thus a break-through point of Brazil nut protein in the flow-through of the column was anticipated.

Approximately 1 L of extract had passed the adsorbent, corresponding to ~5.0 g of protein, when the protein concentration in the unbound fraction started to increase. In total, 3.1 g of the applied 8.5 g of protein remained unbound, whereas in the bound fraction 5.3 g of protein was present. The binding capacity for Brazil nut proteins was thus similar to the reported theoretical binding capacity for BSA. Furthermore, no clogging problems were observed during application of the extract to the EBA column.
SDS-PAGE of the samples collected showed that in the first samples of the unbound fraction no Ber e 1 was present, whereas proteins of other sizes were clearly visible. This indicates that under the conditions applied, Ber e 1 was tightly bound to the adsorbent. Subsequent samples contained increasing amounts of Ber e 1, which eventually became more abundant than the other proteins, clearly indicating that the break-through point for Ber e 1 had been reached. The bound fraction of the EBA column mainly contained Ber e 1, as only a few minor bands with sizes larger than Ber e 1 were visible on the gel (data not shown).
The bound fraction of the EBA column was further purified using SEC (Figure 1). The first peak in the chromatogram contained proteins with sizes larger than that of Ber e 1, as was estimated by SDS-PAGE and was discarded (data not shown). The second large peak in the chromatogram contained almost exclusively Ber e 1, as demonstrated by the presence of a single band of 12 kDa on the SDS-PAGE gel (Figure 2). Under reducing conditions two smaller bands became visible, which is in agreement with the fact that Ber e 1 consists of a heavy and light chain interlinked with S-S bridges (*19*). The purity of the protein was estimated to be >95%, as estimated using a densitometric scan of the SDS-PAGE gel stained with Coomassie Brilliant Blue.

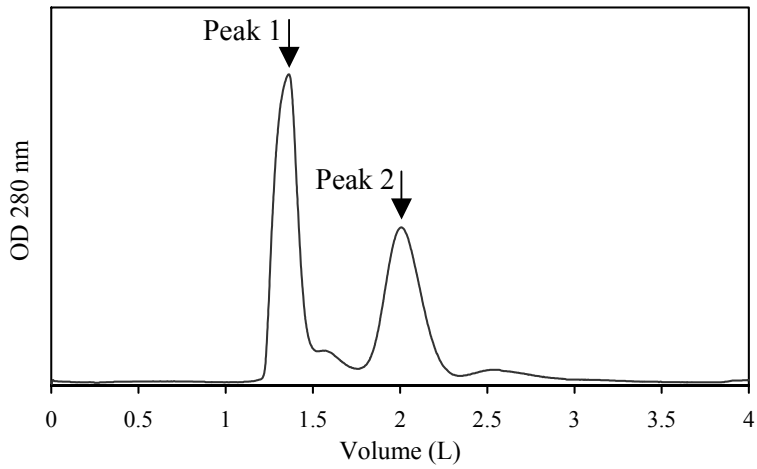


Figure 1: Size exclusion chromatogram of the EBA bound fraction on a Superdex 30 column.

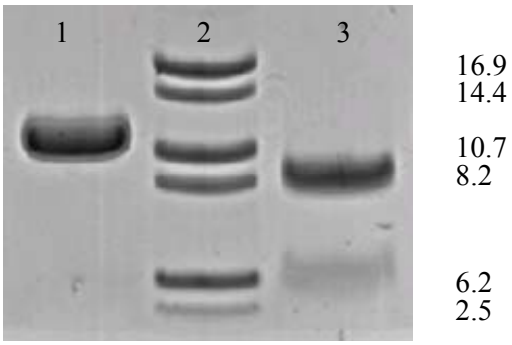
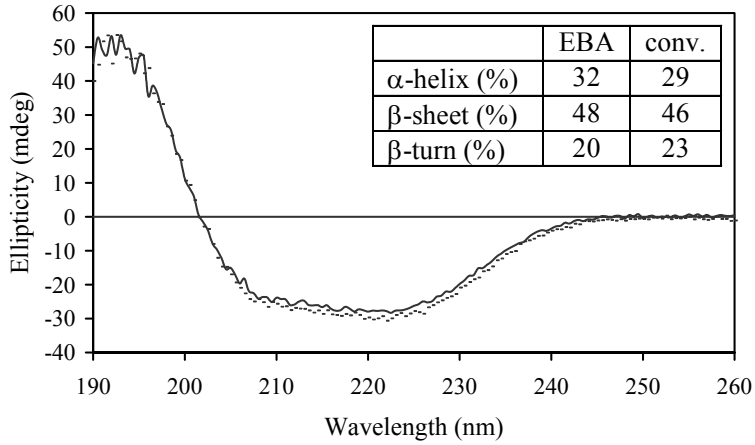


Figure 2: SDS-PAGE analysis of purified Ber e 1_{EBA}. Lane 1: Ber e 1_{EBA} (1 mg mL^{-1}) under non-reducing conditions; lane 2: low molecular weight marker (indicated right in kDa); lane 3: Ber e 1_{EBA} (1 mg mL^{-1}) under reducing conditions.

The complete Ber e 1 purification procedure, which consisted of capturing the protein using EBA and subsequent size exclusion chromatography, could be performed within 1 day and resulted in 2.8 g of essentially pure protein, starting from 85 g defatted Brazil nut meal. Compared to the purification of Ber e 1 using conventional chromatographic techniques, this new purification method thus results in an enormous gain in processing time, especially when large quantities of purified proteins are desired. This is mainly because time-consuming centrifugation steps are not required during the whole purification scheme. Moreover, as the EBA adsorbent used is a high density, mixed mode cation exchanger, which ensures salt-tolerant binding, extracts to be applied do not have to be diluted in advance to decrease their ionic strength and / or their viscosity. Altogether, the purification of Ber e 1 using EBA resulted in a 3 to 5 fold decrease in processing time when compared to purification using conventional chromatographic techniques (*14, 15, 20-23*), while the starting conditions were equal to those of the conventional method (*23*). Because of its generic properties, EBA chromatography can also be used for the large-scale purification of other food allergens.

CHARACTERISTICS OF BER E 1_{EBA} COMPARED WITH THOSE OF BER E $1_{CONV.}$

The characteristics of the Ber e 1 preparation purified with EBA (Ber e 1_{EBA}) were compared with those of a Ber e 1 preparation that was purified using conventional chromatographic techniques (Ber e $1_{conv.}$) (*23*). First, their structural characteristics were compared on a secondary folding level using far-UV CD spectroscopy. The spectra of the two preparations were almost identical, having a zero-crossing around 201 nm and two negative extremes around 222 and 208 nm (Figure 3). The spectra of Ber e 1 presented by Alcocer and co-workers (*6*) are also similar to those presented in this work. The spectra are indicative for proteins with a high α-helical and β-strand content. The higher absolute ellipticity at 222 nm, compared to the extreme at 208 nm, points towards a prevailing β-strand content. Spectral analysis, based on a non-linear least-squares fitting procedure, confirmed these observations: It was estimated that the proteins in both preparations had a similar secondary structure content, consisting of approximately 30% α-helix and approximately 47% β-strand.



	EBA	conv.
α-helix (%)	32	29
β-sheet (%)	48	46
β-turn (%)	20	23

Figure 3: Far-UV CD spectra of Ber e 1_{EBA} (solid line) and Ber e $1_{conv.}$ (dashed line). Inserted table: secondary structure estimates derived from spectra (EBA: Ber e 1_{EBA}; conv.: Ber e $1_{conv.}$).

An important characteristic of an allergen is its immunoreactivity. Testing the ability of the differently purified proteins for their IgE binding characteristics is a logical step for comparing the immunoreactivity of these preparations. Figure 4 shows that both Ber e 1 preparations are able to interact with IgE from a patient allergic to Brazil nuts. With increasing amounts of Ber e 1 added, the IgE binding increases, leading to saturation at approximately 50 ng. Both curves show a similar steep increase in the most sensitive domain of the coat quantity (between 0 and 20 ng), indicating similar IgE binding properties.

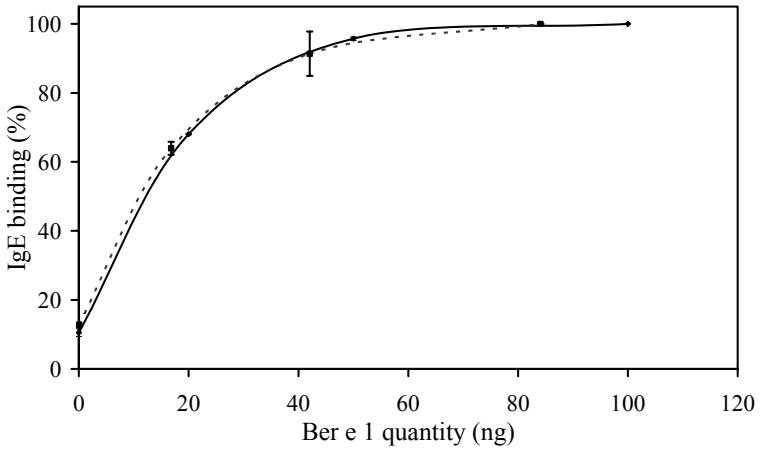


Figure 4: Relative IgE binding (%) of Ber e 1_{EBA} (solid line) and Ber e $1_{conv.}$ (dashed line) after direct ELISA IgE binding analysis. The vertical lines indicate the standarddeviations.

ISOFORM COMPOSITION OF BER E 1 POOL

Ber e 1 belongs to the multigeneous 2S albumins, which implies that different isoforms may be present. Several authors have already described the presence of various isoforms of Ber e 1 (*17-20*). MALDI-TOF MS analysis of Ber e 1_{EBA} showed several peaks around 12 kDa, suggesting the existence of various isoforms in our Ber e 1 preparation. The analysis of Ber e $1_{conv.}$ showed similar results. Analysis of reduced and alkylated Ber e 1_{EBA} showed various peaks around 3 and 9 kDa, indicating heterogeneity in both subunits (data not shown).

The large and small subunits of Ber e 1_{EBA} were separated using SEC. Subsequently, capillary isoelectric focusing measurements of the separated subunits showed various peaks for both alkylated subunits, denoting the presence of various isoforms in both subunits. These results point towards charge heterogeneity, which is a common characteristic of many seed proteins (*22*). The peaks of the alkylated large subunit were detected at pH values around pH 9.0, 7.8, 6.2 and 5.7, whereas the peaks of the alkylated small subunit of Ber e 1 were detected around pH 7.5, 5.7 and 4.5. Our results support the work of Moreno and co-workers (*20*), who found heterogeneity in both subunits of Ber e 1, rather than the work of Ampe and co-workers (*18*), who reported heterogeneity only in the large chain of the protein.
The results obtained from capillary isoelectric focusing measurements of native Ber e 1_{EBA} showed various peaks at pH values between 8.1 and 5.5, with the most abundant ones around pH 8 (data not shown). Moreno and co-workers (*20*) described the separation of Ber e 1 isoforms using chromatofocusing. They identified Ber e 1 isoforms with pI's ≥ pH 4.6. The pI's of the isoforms in our Ber e 1_{EBA} preparation are all ≥ 5.5, as was expected since Ber e 1 was purified using expanded bed cation exchange chromatography at pH 5.5. In general, purification protocols that include anion- or cation exchange chromatography techniques are prone to pI-selective fractionation. We therefore investigated if isoforms with pI's ≤ 5.5 were excluded by our extraction and / or EBA purification, by comparing the isoform distribution of the Ber e 1_{EBA} preparation with those of two Ber e 1 preparations that were extracted with water (Ber e $1_{Extr.\ H2O}$) and with sodium acetate buffer pH 5.5 (Ber e $1_{Extr.\ 5.5}$) and purified only by SEC. All preparations showed a similar isoform pattern, although the relative concentration of the different isoforms varied somewhat: In Ber e 1_{EBA} isoforms with higher pI's were more abundant, whereas in the two other protein preparations isoforms with lower pI's were more abundant. Nevertheless, it could be concluded that, although in somewhat different concentrations, all Ber e 1 isoforms were purified using our EBA purification method. Up till now, no data are available on possible differences in allergenicity between the various Ber e 1 isoforms. The large-scale EBA purification method offers the possibility to obtain sufficiently large quantities of a Ber e 1 isoform pool that can be a good starting point to further investigate the allergenicity of the various isoforms, including the less abundant ones.

In conclusion, the results show that EBA can be a fast and convenient method to purify allergens, as shown for Ber e 1. The biochemical and immunochemical parameters of Ber e 1 purified with this method were comparable to those of conventionally purified Ber e 1. The EBA purification method makes it possible to purify large amounts of Ber e 1 and to set up experiments to further elucidate the sensitizing potential and allergenicity of Ber e 1.

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REFERENCES

1. Burks, A. W., Sampson, H. A. Anaphylaxis and food allergy. *Clinical Reviews in Allergy and Immunology* **1999,** 17, (3), 339-360.
2. Hefle, S. L., Nordlee, J. A., Taylor, S. L. Allergenic foods. *Critical Reviews in Food Science and Nutrition* **1996,** 36, (Suppl. S), S69-S89.
3. Chatel, J. M., Bernard, H., Orson, F. M. Isolation and characterization of two complete Ara h 2 isoforms cDNA. *International Archives of Allergy and Immunology* **2003,** 131, (1), 14-18.
4. Bredehorst, R., David, K. What establishes a protein as an allergen? *Journal of Chromatography B* **2001,** 756, (1-2), 33-40.
5. Pastorello, E. A., Trambaioli, C. Isolation of food allergens. *Journal of Chromatography B* **2001,** 756, (1-2), 71-84.
6. Alcocer, M. J. C., Murtagh, G. J., Bailey, K., Dumoulin, M., Sarabia Meseguer, A., Parker, M. J., Archer, D. B. The disulphide mapping, folding and characterisation of recombinant Ber e 1, an allergenic protein and SFA8, two sulphur-rich 2S plant albumins. *Journal of Molecular Biology* **2002,** 324, (1), 165-175.
7. Rabjohn, P., Helm, E. M., Stanley, J. S., West, C. M., Sampson, H. A., Burks, A. W., Bannon, G. A. Molecular cloning and epitope analysis of the peanut allergen Ara h 3. *Journal of Clinical Investigation* **1999,** 103, (4), 535-542.
8. Murtagh, G. J., Archer, D. B., Dumoulin, M., Ridout, S., Matthews, S., Arshad, S. H., Alcocer, M. J. C. *In vitro* stability and immunoreactivity of the native and recombinant plant food 2S albumins Ber e 1 and SFA-8. *Clinical and Experimental Allergy* **2003,** 33, (8), 1147-1152.
9. Chapman, M. D., Smith, A. M., Vailes, L. D., Arruda, L. K., Dhanaraj, V., Pomes, A. Recombinant allergens for diagnosis and therapy of allergic disease. *Journal of Allergy and Clinical Immunology* **2000,** 106, (3), 409-418.
10. Hjorth, R. Expanded-bed adsorption in industrial bioprocessing: recent developments. *Trends in Biotechnology* **1997,** 15, (6), 230-235.
11. Chase, H. A., Draeger, N. M. Expanded-bed adsorption of proteins using ion-exchangers. *Separation Science and Technology* **1992,** 27, (14), 2021-2039.
12. Hansson, M., Stahl, S., Hjorth, R., Uhlen, M., Moks, T. Single-step recovery of a secreted recombinant protein by expanded bed adsorption. *Bio-Technology* **1994,** 12, (3), 285-288.
13. Fernandez Lahore, H. M., Geilenkirchen, S., Boldt, K., Nagel, A., Kula, M. R., Thommes, J. The influence of cell adsorbent interactions on protein adsorption in expanded beds. *Journal of Chromatography A* **2000,** 873, (2), 195-208.
14. Clemente, A., Chambers, S. J., Lodi, F., Nicoletti, C., Brett, G. M. Use of the indirect competitive ELISA for the detection of Brazil nut in food products. *Food Control* **2004,** 15, (1), 65-69.
15. Pastorello, E. A., Farioli, L., Pravettoni, V., Ispano, M., Conti, A., Ansaloni, R., Rotondo, F., Incorvaia, C., Bengtsson, A., Rivolta, F., Trambaioli, C., Previdi, M., Ortolani, C. Sensitization to the major allergen of Brazil nut is correlated with the clinical expression of allergy. *Journal of Allergy and Clinical Immunology* **1998,** 102, (6), 1021-1027.

16. Ewan, P. W. Clinical study of peanut and nut allergy in 62 consecutive patients: New features and associations. *British Medical Journal* **1996,** 312, (7038), 1074-1078.

17. Gander, E. S., Holmstroem, K. O., Paiva, G. R. d., Castro, L. A. B. d., Carneiro, M., Grossi de Sa, M. F. Isolation, characterization and expression of a gene coding for a 2S albumin from *Bertholletia excelsa* (Brazil nut). *Plant Molecular Biology* **1991,** 16, (3), 437-448.

18. Ampe, C., Van Damme, J., de Castro, L. A., Sampaio, M. J., Van Montagu, M., Vandekerckhove, J. The amino-acid sequence of the 2S sulphur-rich proteins from seeds of Brazil nut (*Bertholletia excelsa* H.B.K.). *European Journal of Biochemistry* **1986,** 159, (3), 597-604.

19. Altenbach, S. B., Pearson, K. W., Sun, S. S. M. Nucleotide sequences of cDNAs encoding two members of the Brazil nut methionine-rich 2S albumin gene family. *Plant Physiology* **1992,** 98, (4), 1520-1522.

20. Moreno, F. J., Jenkins, J. A., Mellon, F. A., Rigby, N. M., Robertson, J. A., Wellner, N., Mills, E. N. C. Mass spectrometry and structural characterization of 2S albumin isoforms from Brazil nuts (*Bertholletia excelsa*). *Biochimica et Biophysica Acta* **2004,** 1698, (2), 175-186.

21. Sun, S. S. M., Leung, F. W., Tomic, J. C. Brazil nut (*Bertholletia excelsa* H.B.K.) proteins: fractionation, composition and identification of a sulfur-rich protein. *Journal of Agricultural and Food Chemistry* **1987,** 35, (2), 232-235.

22. Sun, S. S. M., Altenbach, S. B., Leung, F. W. Properties, biosynthesis and processing of a sulfur-rich protein in Brazil nut (*Bertholletia excelsa* H.B.K.). *European Journal of Biochemistry* **1987,** 162, (3), 477-483.

23. Koppelman, S. J., Nieuwenhuizen, W. F., Gaspari, M., Knippels, L. M. J., Penninks, A. H., Knol, E. F., Hefle, S. L., Jongh, H. H. J. d. Reversible denaturation of Brazil nut 2S albumin (Ber e1) and implication of structural destabilization on digestion by pepsin. *Journal of Agricultural and Food Chemistry* **2004,** 53, (1), 123-131.

24. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **1976,** 72, (1-2), 248-254.

25. de Jongh, H. H., Goormaghtigh, E., Killian, J. A. Analysis of circular dichroism spectra of oriented protein-lipid complexes: toward a general application. *Biochemistry* **1994,** 33, (48), 14521-14528.

3

HEAT DENATURATION OF BRAZIL NUT ALLERGEN BER E 1 IN RELATION TO FOOD PROCESSING

Based on: van Boxtel, E. L., Koppelman, S. J., van den Broek, L. A. M., Gruppen, H. Heat denaturation of Brazil nut allergen Ber e 1 in relation to food processing. *Submitted*

ABSTRACT

Ber e 1, a *major* allergen from Brazil nuts, is very stable to peptic digestion under *in vitro* conditions. As heat-induced denaturation may affect protein digestibility, the denaturation behaviour of Ber e 1 was investigated at different conditions. The denaturation temperature of Ber e 1 varies from approximately 80 to 110 °C, depending on the pH. Upon heating above its denaturation temperature at pH 7.0, the protein partly forms insoluble aggregates and partly dissociates into its polypeptides, whereas heating the protein at pH 5.0 does neither induce aggregation, nor dissociation of the protein. The denaturation temperature of approximately 110 °C at pH values corresponding to the general pH values of foods (pH 5-7) is very high and is expected to be even higher in Brazil nuts itself. As a result, it is unlikely that heat processing causes the denaturation of all Ber e 1 present in food products. Consequently, the allergen is assumed to be consumed (mainly) in its native form, having a high stability towards pepsin digestion.

INTRODUCTION

Food allergens are considered to be able to exert systemic allergic reactions only after they have been absorbed through the intestinal mucosa (*1*). As a result, the resistance of the IgE binding capacity of an allergen towards digestion in the human gastro-intestinal tract is considered an important factor. Heat processing may affect the digestibility of a protein. Taking into account that most foods are being consumed after some kind of heat processing, the digestibility of pre-heated allergens is an important characteristic as well.
The most important foods and food raw materials causing allergic reactions are cow's milk, eggs, soy, wheat, peanuts, tree nuts, fish, and shellfish. Brazil nuts, belonging to this group of foods and food raw materials, are the seeds of *Bertholletia excelsa* H.B.K. The nuts can be eaten both raw and roasted (*2*) and are also used as an ingredient in foods like bakery products (*3*). Allergic reactions in response to Brazil nut consumption can be very severe and even life threatening (*4*). The most important allergen from these nuts, Ber e 1, is a member of the seed storage albumin group (*5*).
Seed storage albumins usually are products of multigene families. For Ber e 1, so far, 6 different isoforms have been identified (NCBI accession numbers gi/839533, gi/384327, gi/112754, gi/99609, gi/384326, and gi/81557) (*6*). Ber e 1 is synthesized as a 18 kDa polypeptide, which is post-translationally processed into a 12 kDa polypeptide. This polypeptide is subsequently processed into two polypeptides of approximately 9 and 3 kDa, linked together by 4 disulfide bridges (*7*). The protein contains 30-47% α-helices, depending on the isoform (chapter 2; *6, 8*). One immunodominant conformational epitope has been identified on the large polypeptide of the protein. The epitope corresponds to amino acids 26-63 of the large polypeptide of the protein (Swissprot accession number P04403), which comprises a helix-turn-helix conformation. When unfolded, this epitope binds at least four times less IgE compared with its folded couterpart. Besides the immunodominant conformational epitope, a linear epitope has been identified on the small polypeptide of Ber e 1, corresponding to amino acids 7 to 20 (QMQRQQMLSHCRMY) (*9*).

The denaturation temperature of Ber e 1 has been determined at pH 2.0 to be approximately 83 °C. The denaturation temperature of Ber e 1 at neutral pH is expected to be higher than 110 °C as no change in transition was observed upon heating till 110 °C (*10*). Ber e 1, when subjected to *in vitro* peptic hydrolysis, is cleaved into peptides ranging in molecular mass from <1 to approximately 6.5 kDa. The 6.5 kDa fragment contained the region in which the immunodominant conformational IgE epitope (*9*) of the allergen is situated (*11*). Upon reduction, this 6.5 kDa peptide dissociates into peptides smaller than approximately 3 kDa. It should be noted that after two hours of peptic digestion, which is considered as the average gastric transit time (*12*) and generally used as the maximum time of pepsin digestion in *in vitro* tests (chapters 6 and 7*; 11, 13-15*), approximately 25% of the protein was still intact (*11*). Consequently, it is assumed that the IgE binding capacity of Ber e 1 (partly) remains after peptic digestion (*11*).
Under the same conditions, peptic digestion of reduced and alkylated Ber e 1, compared to native Ber e 1, results in a fast degradation, with the complete disappearance of the intact polypeptides within 30 seconds (*10*). The structural stability of the native protein thus seems to protect the protein from peptic digestion.
The effects of heat-induced denaturation on the structure and digestibility of Ber e 1 are not known. Therefore, the aim of our research was to study the denaturation behaviour of Ber e 1 in more detail, in order to assess whether heat processing could induce changes in the digestibility of the protein and thereby changes in its IgE binding capacity after digestion.

MATERIALS AND METHODS

MATERIALS

All chemicals were obtained from Merck (Darmstadt, Germany) or Sigma (Sigma-Aldrich Inc., St. Louis, MO, USA), unless stated otherwise. Unshelled Brazil nuts were purchased from Imko Nut Products (Doetinchem, The Netherlands) and stored at 4 °C under vacuum until use. Ber e 1 was purified from defatted Brazil nuts as described in chapter 2 (*8*). The purity of the protein was estimated to be >95%, as estimated using a densitometric scan of bands at approximately 9 and 3 kDa on an SDS-PAGE gel, stained with Coomassie Brilliant Blue.

BRAZIL NUT PROTEIN EXTRACTION

Brazil nuts were ground using a domestic type mechanical high-speed slicer (Kenwood Corp., Tokyo, Japan) and defatted with hexane using Soxhlet extraction. After drying at room temperature, the partially defatted meal was ground using a Waring blender (Waring Products Inc., New Hartford, CT, USA) and was subjected to a second defatting step. Hereafter, the defatted meal was dried at room temperature for 24 h and stored at 4 °C until use. Defatted Brazil nut meal was extracted by stirring in water, at a meal / solvent ratio of 1:100 (w/v), for 1 h at room temperature. Afterwards, the extract was centrifuged (10 min, 22,000*g*, 4 °C). The supernatant was collected and used as Brazil nut extract.

PROTEIN QUANTIFICATION

Protein concentrations were measured using the Bradford method. Bovine serum albumin (BSA, Sigma) was used as a standard. All assays were performed at least in duplicate.

SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Ber e 1 samples were analyzed on a PhastSystem (GE Healthcare), according to the instructions of the manufacturer. High density Phastgels and polypeptide markers (2.5-16.9 kDa; GE Healthcare) were used. Reducing conditions were obtained by adding β-mercaptoethanol to a concentration of 10 mM and heating the samples for 5 min at 100 °C. Gels were stained according to the Coomassie Brilliant Blue procedure provided by the manufacturer.

DIFFERENTIAL SCANNING CALORIMETRY

DSC measurements were performed on a VP-DSC MicroCalorimeter (Microcal Inc., Northampton, MA, USA). Thermograms were recorded from 20 to 130 °C, using a heating rate of 1 °C min^{-1}. Brazil nut extract and purified Ber e 1 (2.0 mg mL^{-1}) were analyzed in water or in buffer of various pH's. At pH 2.0 and 3.0, the protein was solubilized in 10 mM sodium phosphate buffer and at pH 4.0 and 5.0 in 10 mM sodium acetate buffer. At pH 6.0 a 10 mM piperazine buffer and at pH 7.0 a 10 mM sodium phosphate buffer was used for solubilization of the protein. All samples were degassed prior to the experiments. Enthalpies were calculated using Origin software (Microcal Inc.), based on integration of the area of the transition. Analyses were performed in duplicate.

HEATING EXPERIMENTS

Ber e 1 solubilized (2.0-5.0 mg mL^{-1}) in 10 mM sodium phosphate buffer, pH 7.0, or 50 mM sodium acetate buffer, pH 5.0, was heated at 110 or 120 °C during various time intervals in kimax tubes. After heating, samples were cooled immediately on ice. Samples were centrifuged at 22,000*g* for 10 min at 4 °C, after which the supernatant was collected and used for analysis.

SIZE EXCLUSION CHROMATOGRAPHY (SEC)

Protein samples (0.1 and 4 mL) were applied onto a Superdex 75 XK 16/60 column or a Superdex 75 HR 10/30 column (GE Healthcare, Uppsala, Sweden). The columns were equilibrated and eluted with 10 mM sodium phosphate buffer, pH 7.0, at a flow rate of 1 mL min^{-1}, or 50 mM sodium acetate buffer, pH 5.0, at a flow rate of 0.5 mL min^{-1}, respectively, using an Äkta purifier system (GE Healthcare) operated at room temperature. Eluates were monitored at 280 and 214 nm and approprate fractions were collected.

REDUCTION AND ALKYLATION OF BER E 1

Lyophilized protein (50 mg) was dissolved in 25 mL 6 M guanidinium chloride in 10 mM ammonium hydrogen carbonate, pH 7.8 and heated to 56 °C. Dithiothreitol (DTT) was

added to a concentration of 20 mM and the solution was stirred for 60 min. After cooling to room temperature, 2.5 mL 1 M iodoacetamide solution in 100 mM ammonium hydrogen carbonate, pH 7.8, was added. To allow alkylation to occur, the stirred solution was placed for 90 min in the dark. Next, the alkylated protein solution was dialyzed against demineralised water using 2,000 MWCO dialysis tubing at 4 °C (Spectrum laboratories Inc., Rancho Dominguez, CA, USA) and the reduced and alkylated (R+A) protein was subsequently lyophilized.

RESULTS AND DISCUSSION

DENATURATION TEMPERATURE OF BER E 1

The stability of the IgE binding capacity of an allergen towards digestion is considered an important characteristic for food allergens. Unheated Ber e 1, the *major* allergen from Brazil nuts, is very stable to digestion. As heat-induced denaturation may influence the digestibility of food proteins, the denaturation behaviour of Ber e 1 was investigated.
Purified Ber e 1 solubilized in water showed a transition at approximately 110 °C. The denaturation temperature of purified Ber e 1 in the presence of a Brazil nut extract made in water (pH = ~7) turned out to be similar, as can be seen from Figure 1. In both samples a transition at approximately 110 °C was observed. The additional transition peak at approximately 72 °C in the sample containing Brazil nut extract likely represents the denaturation of other Brazil nut proteins, as this transition peak was also observed when analyzing only Brazil nut extract (data not shown). From these results it could be concluded that components present in Brazil nuts do not influence the denaturation temperature of Ber e 1 in an aqueous system.

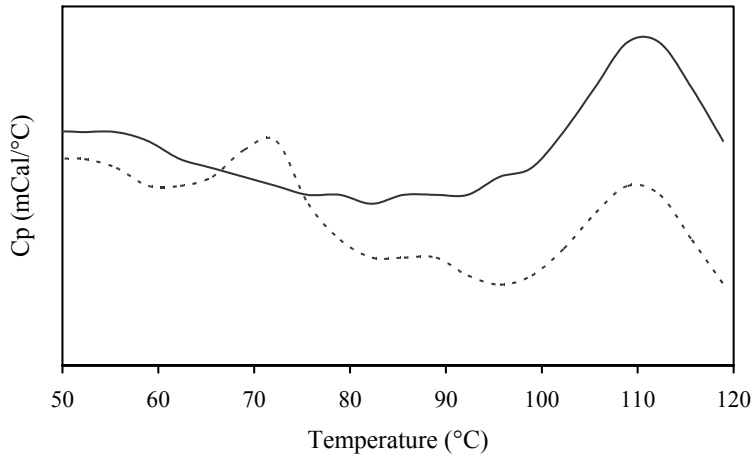

Figure 1: DSC thermograms of Ber e 1 solubilized in water (solid line) and Ber e 1 solubilized in Brazil nut extract made in water (dashed line).

DSC analysis (Figure 2 and Table 1) of purified Ber e 1 at various pH values showed that the denaturation temperature of Ber e 1 is pH-dependent. At a low pH (pH 2.0) the denaturation temperature is approximately 80 °C and increases with increasing pH till approximately 110 °C at pH 5.0. At pH values between pH 5.0 and pH 7.0 the denaturation temperature is stable around 110 °C. These results are in agreement with

those reported previously for solely pH 2.0 and 7.0 (*10*). The broader transitions observed at lower pH values indicate that at lower pH values, the cooperativity of unfolding is lower compared to the cooperativity of unfolding at higher pH values. The higher denaturation temperatures at pH values between 5.0 and 7.0 could be explained because globular proteins generally are most stable to denaturation at pH values close to their pI (*16*). Ber e 1 isoforms have pI values ranging from pH 5.5 to 8.1. In the protein preparation investigated, the most abundant isoforms had pI values around pH 8 *(*chapter 2; *8*). At pH 7.0 the enthalpy of unfolding was observed to be highest (Table 1), most likely because this pH value is closest to the most abundant pI value in this protein preparation (chapter 2; *8*).

The denaturation of Ber e 1 at low pH (pH 2.0) appeared to be partly reversible, as can be seen in Table 2. Upon reheating the protein, a transition was observed, although with a lower enthalpy. After a second reheating step again a transition could be observed. These results also correspond with those described in literature, as the denaturation of Ber e 1 at pH 2.0 was previously described to be partly irreversible (*10*). At pH 7.0 the denaturation of Ber e 1 appeared to be completely irreversible, as reheating the samples did not show a transition (no further data shown).

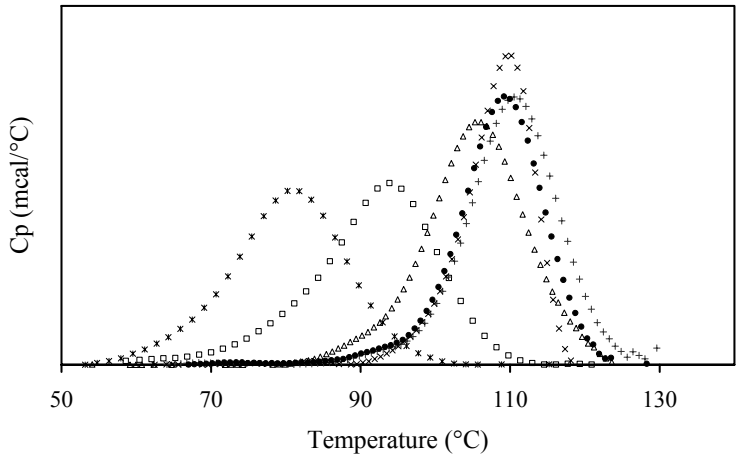

Figure 2: DSC thermograms of Ber e 1 solubilized in buffer with pH values ranging from 2.0 to 7.0. * pH 2.0; □ pH 3.0; Δ pH 4.0; + pH 5.0; • pH 6.0; × pH 7.0.

Table 1: Denaturation temperatures (Td) and calorimetric enthalpies of unfolding of Ber e 1 at various pH's, with standard deviations.

pH	Td (ºC)	ΔH (kJ mol^{-1})
2.0	81.1 ± 0.1	148 ± 5
3.0	94.3 ± 0.2	146 ± 3
4.0	105.7 ± 0.2	122 ± 1
5.0	110.3 ± 0.0	111 ± 3
6.0	109.1 ± 0.1	131 ± 4
7.0	109.9 ± 0.0	196 ± 8

Table 2: Calorimetric enthalpy (ΔH) of Ber e 1 during three subsequent heating experiments at pH 2.0.

Heating step	ΔH (J g^{-1})	ΔH (kJ mol^{-1})
1	12	148
2	11	133
3	6	73

DENATURATION AND AGGREGATION OF BER E 1

The pH of an aqueous Brazil nut extract was approximately 7, whereas the pH of foods generally ranges between pH 5 and pH 7. Therefore, pH values of 5.0 and 7.0 were used for further investigation of the denaturation behaviour of Ber e 1.

When Ber e 1 was heated at its denaturation temperature (20 min, 110 °C, pH 7.0), no precipitation occurred and on SEC, the protein eluted similarly to the native protein. These results indicate that no changes in the protein's structure had occurred upon heating. When the allergen was heated above its denaturation temperature (20 minutes, 120 °C, pH 7.0), the protein partly became insoluble. SEC analysis (Figure 3) of the supernatant after centrifugation showed that the soluble part of the protein had partly dissociated upon heating. Three peaks were present in the chromatogram of the heated protein sample, which were denoted SEC peaks 1, 2, and 3. SEC peak 1 eluted at the same elution volume as unheated Ber e 1, thus pointing towards this peak to represent intact Ber e 1. The other two peaks eluted at later elution volumes, indicating that these peaks contained proteins or peptides with lower molecular masses compared with intact Ber e 1.

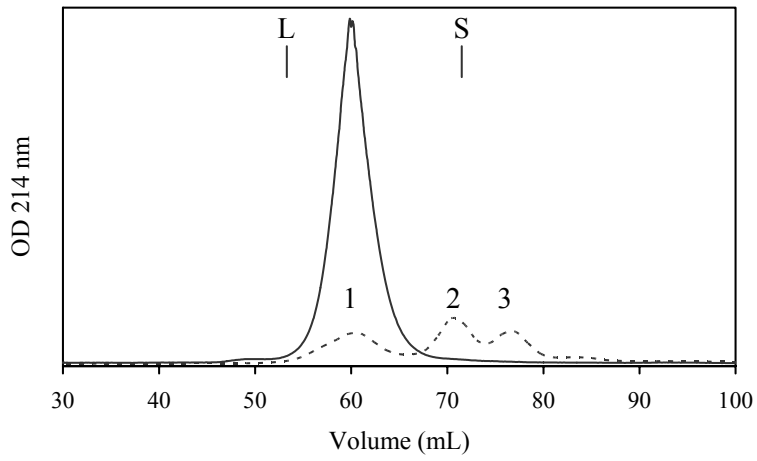

Figure 3: Size exclusion chromatograms of soluble Ber e 1 in buffer pH 7.0 before (solid line) and after (dashed line) heating at 120 °C for 20 minutes. Indicated are the elution volumes of the reduced and alkylated large (L) and small (S) polypeptides of the protein.

Fractions corresponding to the three SEC peaks were collected and analyzed on SDS-PAGE. Fractions under SEC peak 1 showed two protein bands on reducing SDS-PAGE, at approximately 3 and 9 kDa, thus pointing towards this peak to indeed represent intact Ber e 1. SDS-PAGE of the other two SEC peaks showed protein bands of approximately

3 kDa. This molecular mass coincides with the molecular mass of the small polypeptides of Ber e 1 (~3 kDa). Under non-reducing conditions, SEC peak 1 on SDS-PAGE showed a protein band at approximately 12 kDa, as well as a protein band at a molecular mass of approximately 9 kDa (data not shown). SEC peaks 2 and 3 on non-reducing SDS-PAGE showed similar results as the results obtained under reducing conditions (data not shown).
The results from non-reducing SDS-PAGE of SEC peak 1 indicated that next to intact Ber e 1, this (slightly asymmetric) SEC peak also represented the dissociated large (~9 kDa) polypeptides of the allergen. In order to verify this, reduced and alkylated Ber e 1 was applied to the same column, as can be seen in Figure 3. The reduced and alkylated large polypeptides (~9 kDa) of Ber e 1 eluted before the intact protein (the elution volume indicated with L in Figure 3), whereas the reduced and alkylated small polypeptides (~3 kDa) eluted at an elution volume of approximately 70 mL (the elution volume indicated with S in Figure 3). Considering a mass gain of 348.4 Da by alkylation of 6 cysteines, the reduced and alkylated large polypeptides were not expected to elute earlier than the parental protein on SEC. However, as SEC is dependent on hydrodynamic volume, the hydrodynamic volume of the reduced and alkylated large polypeptides of Ber e 1 could have caused them to behave differently from the compact, native structure of Ber e 1 (*17*). Apparently, the large polypeptides, which dissociated from the Ber e 1 complex upon heating, had a slightly more compact structure in comparison with the reduced and alkylated polypeptides, as they eluted later from the SEC column, at a comparable elution volume to that of intact Ber e 1 protein.
As already mentioned, SEC peaks 2 and 3 both showed single protein bands of approximately 3 kDa on SDS-PAGE. The elution volume of the first of the two peaks corresponded to the elution volume of the reduced and alkylated small polypeptides of Ber e 1, as can be seen in Figure 3. This peak thus likely represented the dissociated small Ber e 1 polypeptides. The presence of the third SEC peak, containing peptides with molecular masses of approximately 3 kDa, could not be explained.
Heating of Ber e 1 at pH 5.0 (20 minutes, 120 °C), in contrast to the results at pH 7.0, did not show any changes in the protein. No insolubility was observed upon heating and on SEC the protein eluted similar to the native protein (Figure 4). Also after prolonged heating (till 4 hours), no changes in the SEC elution profile between native protein and heated protein could be observed (data not shown). As Ber e 1 isoforms have iso-electric pH values of 5.5 and higher, with the most predominant isoforms having pI values of around 8 in the used protein preparation (chapter 2; *8*), at pH 5.0 all isoforms are expected to have a net positive charge. At pH 7.0, which is closer to the (main) pI of the protein preparation, the Ber e 1 isoform pool likely has a low net charge. These apparent differences in charge could have caused the differences in aggregation behaviour, as more charges are expected to induce more repulsion and thereby less aggregation and precipitation. Thus, although the denaturation temperatures of the protein at pH 5.0 and pH 7.0 are comparable and approximately 110 °C, the effects of heating on the aggregation behaviour of Ber e 1 differ greatly with the pH value.

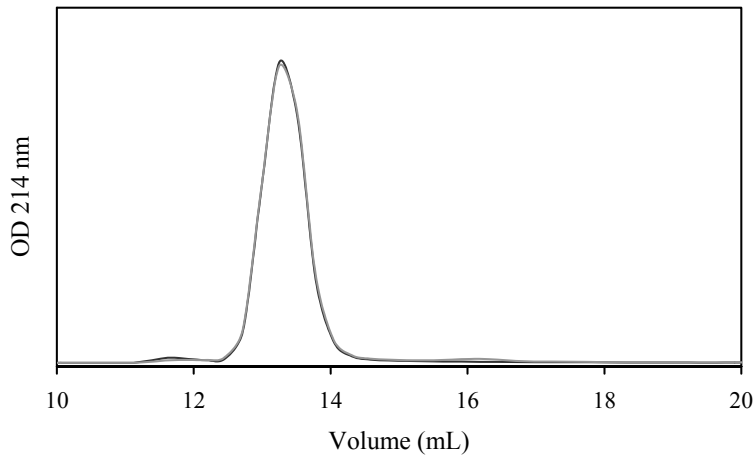

Figure 4: Size exclusion chromatograms of soluble Ber e 1 in buffer pH 5.0 before (black line) and after (gray line) heating at 120 °C for 20 minutes.

When Brazil nuts are used as an ingredient in for example cakes, the (crushed) nuts are usually mixed with the dough and distributed over the whole food product. The baking of such products generally implies heating temperatures ranging from 180 °C to 230 °C. Despite these relatively high temperatures, due to the water content in the crumb of these products, the temperature inside these crumbs will not exceed 100 °C. As a result, Ber e 1 situated only at the outside of a food product can possibly denature during heat processing. The majority of Ber e 1, however, will be present in the crumb of the products, where the lower temperature will not induce the protein to denature.

In conclusion, in aqueous solutions with pH values between 5.0 and 7.0, a temperature exceeding 110 °C is needed to denature Ber e 1. The denaturation temperature of Ber e 1 in Brazil nuts could, because of the low water content, be even higher than the temperature measured (*18*). When using common heat processing methods, the largest part of foods normally do not reach temperatures >100 °C. This implies that Ber e 1 will always be consumed (mainly) in its native structure, with its concomitant high stability to pepsin digestion (*11*). As a result, common heat processing techniques are not likely to affect the digestibility of Brazil nuts and its concomitant IgE binding capacity after digestion.

REFERENCES

1. Astwood, J. D., Leach, J. N., Fuchs, R. L. Stability of food allergens to digestion *in vitro*. *Nature Biotechnology* **1996,** 14, (10), 1269-1273.
2. Clark, J. P. Processing tree nuts. *Food Technology* **2002,** 56, (6), 121-122.
3. Clemente, A., Chambers, S. J., Lodi, F., Nicoletti, C., Brett, G. M. Use of the indirect competitive ELISA for the detection of Brazil nut in food products. *Food Control* **2004,** 15, (1), 65-69.
4. Ewan, P. W. Clinical study of peanut and nut allergy in 62 consecutive patients: New features and associations. *British Medical Journal* **1996,** 312, (7038), 1074-1078.
5. Pastorello, E. A., Farioli, L., Pravettoni, V., Ispano, M., Conti, A., Ansaloni, R., Rotondo, F., Incorvaia, C., Bengtsson, A., Rivolta, F., Trambaioli, C., Previdi, M., Ortolani, C.

Sensitization to the major allergen of Brazil nut is correlated with the clinical expression of allergy. *Journal of Allergy and Clinical Immunology* **1998,** 102, (6), 1021-1027.

6. Moreno, F. J., Jenkins, J. A., Mellon, F. A., Rigby, N. M., Robertson, J. A., Wellner, N., Mills, E. N. C. Mass spectrometry and structural characterization of 2S albumin isoforms from Brazil nuts (*Bertholletia excelsa*). *Biochimica et Biophysica Acta* **2004,** 1698, (2), 175-186.
7. Altenbach, S. B., Pearson, K. W., Sun, S. S. M. Nucleotide sequences of cDNAs encoding two members of the Brazil nut methionine-rich 2S albumin gene family. *Plant Physiology* **1992,** 98, (4), 1520-1522.
8. van Boxtel, E. L., van Koningsveld, G. A., Koppelman, S. J., van den Broek, L. A. M., Voragen, A. G. J., Gruppen, H. Expanded bed adsorption as a fast technique for the large-scale purification of the complete isoform pool of Ber e 1, the major allergen from Brazil nuts. *Molecular Nutrition & Food Research* **2006,** 50, (3), 275-281.
9. Alcocer, M. J. C., Murtagh, G. J., Wilson, P. B., Progias, P., Lin, J., Archer, D. B. The major human structural IgE epitope of the Brazil nut allergen Ber e 1: A chimaeric and protein microarray approach. *Journal of Molecular Biology* **2004,** 343, (3), 759-769.
10. Koppelman, S. J., Nieuwenhuizen, W. F., Gaspari, M., Knippels, L. M. J., Penninks, A. H., Knol, E. F., Hefle, S. L., Jongh, H. H. J. d. Reversible denaturation of Brazil nut 2S albumin (Ber e1) and implication of structural destabilization on digestion by pepsin. *Journal of Agricultural and Food Chemistry* **2004,** 53, (1), 123-131.
11. 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 Journal* **2005,** 272, (2), 341-352.
12. Untersmayr, E., Jensen-Jarolim, E. The effect of gastric digestion on food allergy. *Current Opinion in Allergy and Clinical Immunology* **2006,** 6, (3), 214-219.
13. 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. *Clinical and Experimental Allergy* **2006,** 36, (10), 1281-1288.
14. 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. *Journal of Agricultural and Food Chemistry* **2002,** 50, (24), 7154-7160.
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. *Molecular Nutrition and Food Research* in press.
16. Privalov, P. L., Khechina, N. N. Thermodynamic approach to problem of stabilization of globular protein structure - calorimetric study. *Journal of Molecular Biology* **1974,** 86, (3), 665-684.
17. Alcocer, M. J. C., Murtagh, G. J., Bailey, K., Dumoulin, M., Sarabia Meseguer, A., Parker, M. J., Archer, D. B. The disulphide mapping, folding and characterisation of recombinant Ber e 1, an allergenic protein and SFA8, two sulphur-rich 2S plant albumins. *Journal of Molecular Biology* **2002,** 324, (1), 165-175.
18. Gekko, K., Timasheff, S. N. Thermodynamic and kinetic examination of protein stabilization by glycerol. *Biochemistry* **1981,** 20, (16), 4677-4686.

4

ALLERGEN ARA H 1 OCCURS AS A LARGE OLIGOMER RATHER THAN AS A TRIMER UPON EXTRACTION FROM PEANUTS

Based on: 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. *Journal of Agricultural and Food Chemistry* **2006,** 54, (19), 7180-7186.

ABSTRACT

Ara h 1, a *major* peanut allergen, is known as a stable trimeric protein. Nevertheless, upon purification of native Ara h 1 from peanuts using only size exclusion chromatography, the allergen appeared to exist in an oligomeric structure, rather than as a trimeric structure. The oligomeric structure was independent of the salt concentration applied. Subjecting the allergen to anion exchange chromatography induced the allergen to dissociate into trimers. Ammonium sulfate precipitation did not bring about any structural changes, whereas exposing the allergen to hydrophobic interaction chromatography caused it to partly dissociate into trimers, with increasing amounts of trimers at higher ionic strengths. The (partial) dissociation into trimers led to a change in the tertiary structure of the monomeric subunits of the allergen, with the monomers in Ara h 1 oligomers having a more compact tertiary structure compared with the monomers in Ara h 1 trimers. As structural characteristics are important for a protein's allergenicity, this finding may imply a different allergenicity for Ara h 1 than previously described.

INTRODUCTION

Factors that are considered to be important for the allergenicity of food proteins are the ability of a protein to induce the production of and binding to IgE antibodies and their resistance to digestion in the human gastro-intestinal tract. These features largely depend on the structural characteristics of the protein. Accordingly, changes in these characteristics may result in changes in allergenicity, such as changes in epitope structure, epitope accessibility, and protein digestibility (*1, 2*). It is therefore important to investigate allergens in their natural form, as distinct differences in characteristics may result in inaccurate conclusions about their allergenic activity (*3*).

Peanut allergy is the third most prevalent food allergy (*4*) Ara h 1 is recognized by >90% of individuals sensitized to peanut, rendering it a *major* peanut allergen (*5*). The protein is described as a 63 kDa glycoprotein that is present in peanuts as a highly structured, stable trimer (*6-8*). The trimers are reported to be stabilized mostly by hydrophobic interactions and, to a lesser extent, by ionic interactions (*7*). Throughout the amino acid backbone of the protein 24 linear epitopes have been mapped (*9, 10*). Most IgE epitopes are clustered near the regions of the hydrophobic monomer-monomer contacts and, accordingly, upon formation of trimers ~40% of the residues within the epitopes are excluded from the surface of the protein. Thus, the formation of trimers is believed to protect Ara h 1 from protease digestion and denaturation, thereby promoting its allergenic properties (*7*).

Ara h 1 is classified into the 7S globulins, which are often denoted vicilins. Vicilins are seed storage proteins belonging to the cupin superfamily. Members of the cupin superfamily share a conserved domain comprising a six-stranded β-barrel structure (*11*). Together with the 11S globulins (legumins), the vicilins account for the majority of cupin allergens (*12*). Besides Ara h 1, vicilins that are known to have allergenic activity are, for example, vicilins from peas (*13*), walnuts (*14*), soybeans (*15*), and cashews (*16*). Because of the structural similarity of vicilins it is not surprising that cross-reactive IgE binding between Ara h 1 and other vicilins exists. Cross-reactivity between Ara h 1 and pea

vicilin (Pis s 1) (*13*) and between Ara h 1 and soybean β-conglycinin (*17*) has been described.
Vicilins are reported to be present as trimeric, often glycosylated, proteins ranging from ~150-190 kDa, with subunits from ~50 to 80 kDa (*12*). Although they share only 35-45% amino acid sequence identity with the 11S globulins, they are similar in their three-dimensional structure: both 11S and 7S globulins have similar N-terminal and C-terminal domains, comprising the cupin β-barrel structure (*18*). Some vicilins undergo post-translational proteolytic processing, yielding various polypeptides next to the intact monomer. However, all subunits are held together by non-covalent interactions, keeping the trimeric structure intact (*19*). During post-translational processing of Ara h 1 an N-terminal peptide of ~85 amino acids is cleaved off (*20*). Nevertheless, the remaining protein remains intact.
The 7S globulin β-conglycinin shows an association / dissociation behaviour, depending on pH and ionic strength: at high ionic strength (I >0.5) the protein occurs as a trimer, but at low ionic strength (I <0.2) the protein reversibly associates into hexamers. At intermediate ionic strengths the two forms coexist. Furthermore, at a very low ionic strength, together with an associated form (0.01 M), a dissociated form of the protein may exist, caused by the reversible dissociation of one of the subunits from the protein (*21*). This behaviour has never been reported for Ara h 1.
For the isolation of Ara h 1 several protocols have been described. Most of these methods include ion exchange chromatography (*5-8*), sometimes preceded by ammonium sulfate precipitation (*6, 7*). Recently, Boldt and co-workers (*22*) investigated a peanut extract for protein complexes, rather than looking at purified proteins. They described that Ara h 1 exists in peanuts as a complex with Ara h 3 isoallergens.
Although Ara h 1 in literature is always described to form stable trimers, after purification of the allergen using commonly used purification techniques, we found indications that at least part of the protein occurred in a higher oligomeric form. To further investigate this finding, the aim of our study was to purify native Ara h 1 and to investigate its possible association / dissociation behaviour.

MATERIALS AND METHODS

MATERIALS

All chemicals were obtained from Merck (Darmstadt, Germany), unless stated otherwise. Peanuts of the Runner market-type were generously provided by Imko Nut Products (Doetinchem, The Netherlands) and were stored at 4 °C until use.

PEANUT PROTEIN EXTRACTION

Peanuts were ground using a domestic type mechanical high-speed slicer (Kenwood Corp., Tokyo, Japan) and defatted with hexane using Soxhlet extraction. After drying at room temperature, the partially defatted meal was ground using a Waring blender (Waring Products Inc., New Hartford, CT, USA) and was subjected to a second defatting step. Next, the defatted meal was dried at room temperature for 24 h and stored at 4 °C

until further use. Defatted meal was extracted by stirring in 50 mM Tris-HCl buffer, pH 8.2, at a meal / solvent ratio of 1:10 (w/v) for 1 h at room temperature. Afterwards, the extract was filtered through a cheesecloth and subsequently centrifuged (25 min; 14,000*g*; 4 °C). The supernatant obtained was filtered over a 1.2 μm filter and denoted peanut extract.

SIZE EXCLUSION CHROMATOGRAPHY (SEC)

Protein samples were applied onto either a 320 mL Superdex 200 XK 26/60 column (5-13 mL applied per run) or a 25 mL Superose 6 10/30 column (100 μL applied per run; GE Healthcare, Uppsala, Sweden). The columns were equilibrated and eluted with 50 mM Tris-HCl buffer, pH 8.2, with or without 0.3 M NaCl, at a flow rate of 4.3 and 0.5 mL min^{-1}, respectively, using an Äkta purifier system (GE Healthcare). The Superose column was calibrated using a gel filtration calibration kit according to the instructions of the supplier (GE Healthcare). The eluate was monitored at 280 and 325 nm and appropriate fractions were collected.

ANION EXCHANGE CHROMATOGRAPHY (AEC)

A 25 mL Source Q XK 26/10 column was equilibrated with 50 mM Tris-HCl buffer, pH 8.2, using an Äkta explorer system (GE Healthcare) and loaded with sample at 10 mL min^{-1}. After washing, a 10 column volumes linear gradient from 0 to 0.5 M NaCl in 50 mM Tris-HCl buffer, pH 8.2, was applied at a flow rate of 10 mL min^{-1}. The eluate was monitored at 280 nm and appropriate fractions were collected.

HYDROPHOBIC INTERACTION CHROMATOGRAPHY (HIC)

Ammonium sulfate was added to protein samples to a final concentration of 3.3 M. Samples were stirred for 3 h and left overnight to settle. After centrifugation (15 min; 10,000*g*; 4 °C) the supernatant (which was denoted Ara h 1_{ASP}) was added to 15 mL Source 15 Phenyl material (GE Healthcare). The suspension was collected on a G3 glass filter and washed with 3.3 M ammonium sulfate in 50 mM Tris-HCl buffer, pH 8.2. The bound material was eluted with 20 mL water and the eluate was denoted Ara h 1_{HIC}.

DIALYSIS

Fractions were dialyzed in 10,000 MWCO slyde-a-lyzer mini dialysis units (Pierce, Rockford, IL, USA) against 50 mM Tris-HCl buffer, pH 8.2. Afterwards samples were stored at 4 °C until use or lyophilized and stored at -20 °C until use.

PROTEIN QUANTIFICATION

The nitrogen content of samples was determined using the combustion (Dumas) method on a NA 2100 Nitrogen and Protein Analyzer (CE Instruments, Milan, Italy). The instructions of the supplier were followed and methionine was used as a standard.

SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Protein samples were mixed 1:1 with sample buffer (1.4 mL distilled water, 2.0 mL 0.5 M Tris-HCl, pH 6.8, 2.0 mL 10% (w/v) SDS, 2.0 mL glycerol, and 0.4 mL 0.05% (w/v) bromophenol blue). Reducing conditions were obtained by adding β-mercaptoethanol to a final concentration of 10 mM and heating the samples for 5 min at 100 °C. Tris-HCl 10-20% linear gradient Ready Gels (Bio-Rad Laboratories Inc., Hercules, CA, USA) were used. To each lane 10 μL protein sample was applied and gels were run at 200 V. Gels were stained according to the Coomassie Brilliant Blue procedure provided by the manufacturer. Low molecular weight protein standards (GE Healthcare) were used according to the instructions of the manufacturer.

CIRCULAR DICHROISM (CD) SPECTROSCOPY

Far- and near-UV CD measurements were performed in the spectral range from 190 to 260 nm and from 250 to 350 nm, respectively. Protein solutions (0.25 mg mL^{-1} for far-UV CD and 0.5 mg mL^{-1} for near-UV CD) in 5 mM sodium phosphate buffer, pH 7.0, were analyzed. Spectra were recorded on a Jasco-715 spectropolarimeter (Jasco Inc., Easton, MD, USA), thermostatted at 20 ºC. A step resolution of 0.5 nm, a scan speed of 100 nm min^{-1}, a bandwidth of 1 nm, and a response time of 0.125 s were used. Ten scans were accumulated and averaged. Buffer spectra were subtracted from protein spectra. The secondary structure content from far-UV CD measurements was estimated using a spectral nonlinear least-squares fitting procedure, using reference spectra (*23*).

FLUORESCENCE SPECTROSCOPY

Tryptophan fluorescence spectra of 0.2 mg mL^{-1} protein solutions in 5 mM sodium phosphate buffer, pH 7.0, were recorded on a Perkin-Elmer luminescence spectrophotometer LS 50 B (Perkin-Elmer Corp., Boston, MA, USA) with a pulsed xenon source. Excitation was performed at 295 nm and emission spectra were recorded as the average of three spectra from 300 to 450 nm, using a scan speed of 100 nm min^{-1} and a resolution of 0.5 nm. Both the excitation and the emission slit were set at 2.5 nm. Spectra were corrected by subtracting the spectrum of a protein free sample obtained under identical conditions.

(IN-GEL) DIGESTION

Protein bands in SDS-PAGE gels were cut out manually and destained overnight in 200 μL 50% (v/v) methanol and 5% (v/v) acetic acid. After washing with 200 μL 50% (v/v) acetonitrile in 50 mM Tris-HCl buffer, pH 8, for 15 min at 37 °C, the gel pieces were dehydrated in 200 μL acetonitrile for 5 min at 37 °C. The acetonitrile was removed and the gel pieces were dried at ambient temperature in a vacuum centrifuge (Christ, Osterode, Germany). Next, samples were incubated with 30 μL 10 mM DTT in 100 mM Tris-HCl buffer, pH 8, for 45 min at 65 °C. The solvent was removed and subsequently 30 μL 100 mM iodoacetamide in 100 mM Tris-HCl buffer, pH 8, was added. Next, samples were incubated for 30 min in the dark at room temperature. The solvent was removed and the gel pieces were dehydrated for 5 min in 200 μL acetonitrile at room

temperature. The acetonitrile was removed and the gel pieces were dried in a vacuum centrifuge. Next, 30 μL of a 2 mg L^{-1} trypsin solution (Roche, Mannheim, Germany, article 11418025001) in 50 mM Tris-HCl buffer, pH 8, was added and samples were rehydrated on ice for 10 min. Afterwards, the excess of trypsin solution was removed and 50 mM Tris-HCl buffer, pH 8, was added until the gel pieces were completely covered. Digestion was performed overnight at 37 °C. The solution was collected and 30 μL 50 mM Tris-HCl buffer, pH 8, was added to the gel pieces. After 10 min at room temperature, this buffer solution was also collected and 30 μL 50% (v/v) acetonitrile and 5% (v/v) formic acid was added to the gel pieces. Samples were incubated for 10 min at room temperature before the solution was also collected. This step was repeated once. The collected solutions were combined and their volume was reduced to <20 μL in a vacuum centrifuge. Acetonitrile and formic acid were added to concentrations of 5% and 0.05% (v/v), respectively and samples were analyzed with nanospray liquid chromatography-mass spectrometry (LC-MS).

Direct digestion of protein samples was performed by adding 2 μL of a 0.5 mg mL^{-1} trypsin solution to 100 μL protein sample in 50 mM Tris-HCl buffer, pH 8.2 (~1 mg mL^{-1}). Samples were incubated overnight at 37 °C. The pH of the samples was adjusted to ~3 with 10% (v/v) formic acid and samples were analyzed with LC-MS.

NANOSPRAY LC-MS (NANO-LC-MS)

The LC apparatus consisting of an autosampler (Famos), a column switching device (Switchos), and a HPLC pump (Ultimate; all from LC Packings, Sunnyvale, CA, USA), controlled by Xcalibur v1.3 software, was connected to an LCQ Deca XP Max System with a nanospray (NSI) probe (Thermo Electron, Waltham, MA, USA). Trypsin-digested protein samples (1 μL) were applied onto a C18 PepMap μ-precolumn cartridge (5 μm, 100 Å, LC Packings) operating at a flow rate of 0.2 μL min^{-1} at room temperature. After 10 min, the guard column was switched online with a C18 PepMap capillary with a pore size of 300 Å (LC Packings). A linear gradient from 95% eluent A (0.05% (v/v) formic acid in water) and 5% eluent B (0.05% (v/v) formic acid in acetonitrile) to 55% A and 45% B in 45 min was applied, followed by a linear gradient from 55 to 5% A and from 45 to 95% B in 25 min. The LCQ was operated in the positive mode using a spray voltage of 3.5 kV. The capillary temperature was 200 °C and the capillary voltage was 49 V. Mass spectra were collected in a full mass scan, followed by an MS^2 and MS^3 scan of the highest peak in the spectrum.

MS results were analyzed using Bioworks software version 3.1 (Thermo Electron). The filter type was single threshold, xcorr. vs. charge state. For the analysis of the peptides, the Swissprot database (http://www.expasy.org/sprot/) was used containing 1) all annotated amino acid sequences, 2) all annotated peanut amino acid sequences, and 3) Ara h 1 amino acid sequence P43237 (*24*), respectively.

RESULTS AND DISCUSSION

PROTEIN PURIFICATION

The *major* peanut allergen Ara h 1 is described as a stable trimer (*6-8*) and is classified into the vicilin family, of which some members are known to show association / dissociation behaviour depending on the ionic strength and pH. Based on deviant observations during the isolation of Ara h 1, the aim of our research was to investigate the possible association and / or dissociation behaviour of Ara h 1. It was chosen to purify the allergen using only SEC at a low ionic strength, to ensure that no structural changes would be induced by the purification method used. Other processing steps such as freezing / thawing and lyophilization were also avoided during the purification.

When the peanut extract was applied to a preparative SEC column, the elution pattern given in Figure 1 was obtained. The first shoulder peak in the chromatogram eluted in the exclusion volume of the column. This shoulder peak was followed by two larger peaks, which were denoted SEC peak A and SEC peak B, respectively. The shoulder peak showed a relatively high absorption at 325 nm compared to the absorption at 280 nm, indicating the presence of other compounds instead of proteins. This was confirmed by SDS-PAGE: no protein bands were visible in the fractions collected under this peak (data not shown).

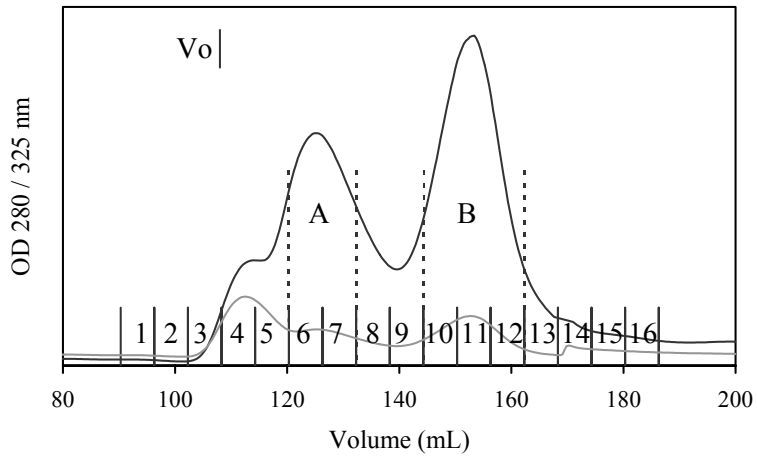

Figure 1: Size exclusion chromatogram of peanut extract on a Superdex 200 column in 50 mM Tris-HCl buffer, pH 8.2 (I = 0.02 M). Black line: OD at 280 nm; gray line: OD at 325 nm. Indicated is the void volume of the column (Vo), the fractions that were collected for SDS-PAGE (solid lines, fractions 1-16) and SEC peak A and B (dashed lines).

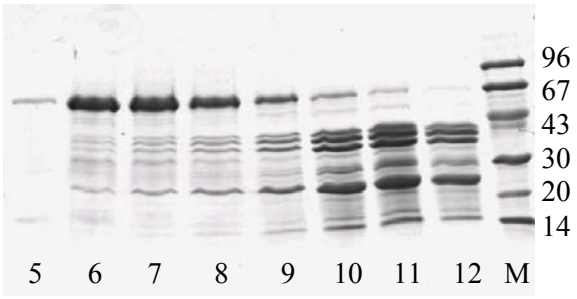

Figure 2: SDS-PAGE of fractions collected during SEC of peanut extract. The numbers represent the fraction numbers in Figure 1. M = low molecular weight marker, indicated right in kDa.

SDS-PAGE of fractions under SEC peaks A and B (Figure 2) showed that in fractions 5-8, representing SEC peak A, the predominant protein band corresponded to ~65 kDa. This protein band was earlier designated as Ara h 1 (*5*). Nano-LC-MS analysis of this protein band after in-gel digestion confirmed that it represented Ara h 1: a comparison of the amino acid sequences of the obtained peptides with a Swissprot-based peanut database showed the highest correlation by mass (~44%) with the deduced amino acid sequence of an Ara h 1 clone P43237 (*24*). The same result was obtained using the complete Swissprot database.
In the subsequent fractions after SEC peak A the Ara h 1 band clearly decreased. In all fractions also several other protein bands were visible, of which the relative intensity increased with the elution volume and which were predominantly present in fractions 10-12, corresponding to SEC peak B. The predominant bands corresponded to 40 and 23 kDa, showing a pattern typical for Ara h 3 (*25*). In-gel digestion was performed and the amino acid sequences of the peptides were compared with the peanut database. Protein bands that were analyzed were the two most predominant bands around 40 kDa (B1 at ~40 kDa and B2 at ~35 kDa) and the protein band of ~23 kDa (B3). B1 showed the highest correlation by mass (22%) with the deduced amino acid sequence of an arachin-encoding gene (Q5I6T2) (*26*), whereas for B2 the highest correlation by mass (~18%) was found with the amino acid sequence of a glycinin fragment (Q6IWG5). B3 showed 41% correlation by mass with the amino acid sequence of a cupin protein denoted Gly1 (Q9FZ11), which has been shown to be an Ara h 3 isoallergen (*22*). Results from direct digestion of SEC peak B resulted in 64% correlation by mass with the deduced amino acid sequence of an arachin gene (Q647H4) and 62% with a gene denoted Ara h 3 (Q8LKN1). These data confirmed that the analyzed protein bands from SEC peak B corresponded to the legumin Ara h 3, which is also called glycinin or arachin (*26*).
Our data thus demonstrated that Ara h 1 on SEC eluted prior to Ara h 3. As Ara h 1 is described as a trimeric protein of ~180 kDa (*6-8*) and Ara h 3 as a hexamer of ~400 kDa (*27*) this result was surprising. Because Ara h 1 eluted before Ara h 3 and shortly after the exclusion volume of the column (which has a separation range until ~600 kDa for proteins), the existence of Ara h 1 in a trimeric structure in peanuts seemed to be unlikely: it rather seemed that the allergen upon extraction exists in a higher oligomeric structure. SDS-PAGE analysis of fractions eluting later than SEC peak B did not reveal Ara h 1 in either trimeric or monomeric form. In those fractions only patterns comparable to albumin proteins were found (Ara h 2 and Ara h 6, data not shown). Peanut extract made from non-defatted peanuts showed similar results, thus excluding the possibility of Ara h 1 oligomerization induced by defatting (data not shown).
Next to the observation of Ara h 1 present as higher oligomers, on the basis of our results complex formation of Ara h 1 and Ara h 3, as described by Boldt and co-workers (*22*) seems to be implausible, as an identical SDS-PAGE pattern would then be expected in all fractions. Moreover, as two distinct peaks were recorded from SEC, Ara h 1 and Ara h 3 are likely to exist separately from each other.

To obtain pure Ara h 1 oligomers, SEC peak A was reapplied to the preparative SEC column. The chromatogram is given in Figure 3A. At an absorbance of 280 nm a

symmetric peak eluted from the column. The peak was fractionated into three parts, denoted A1-A3. The absorbance at 325 nm was relatively low. Nevertheless, the first part of the 280 nm peak showed a comparatively higher absorbance at 325 nm, which was most likely originating from the partially collected shoulder peak as observed in Figure 1. SDS-PAGE (Figure 3B) showed that Ara h 1 was predominantly present in fractions A2 and A3. Only a faint band at 65 kDa was detected in A1. Furthermore, the purity of Ara h 1 in A2 and A3 was >95%, as estimated using a densitometric scan of the SDS-PAGE gel stained with Coomassie Brilliant Blue.

Analytical SEC of fractions A1-A3 on a Superose 6 column with a theoretical separation range to 2,000 kDa for proteins was performed to investigate the purity of the fractions with SEC. It was anticipated that the non-protein shoulder peak, if present in any of the samples, would better be separated from Ara h 1 using this column. Hence, it would be possible to determine which of the samples A1-A3 contained this undesired impurity. The results are given in Figure 3C. Both fractions A1 and A2 showed a peak in the exclusion volume of the column and only A3 showed a symmetric Ara h 1 peak. From these results it was concluded that A3 contained pure Ara h 1 without undesired compounds. The fraction was renamed Ara h 1_{SEC} and used for further analysis. The apparent molecular mass of the purified Ara h 1 oligomer was estimated to be ~700 kDa, however, as SEC does not allow a precise determination of the molecular mass of a protein, no precise estimation of the number of monomers in the protein could be made.

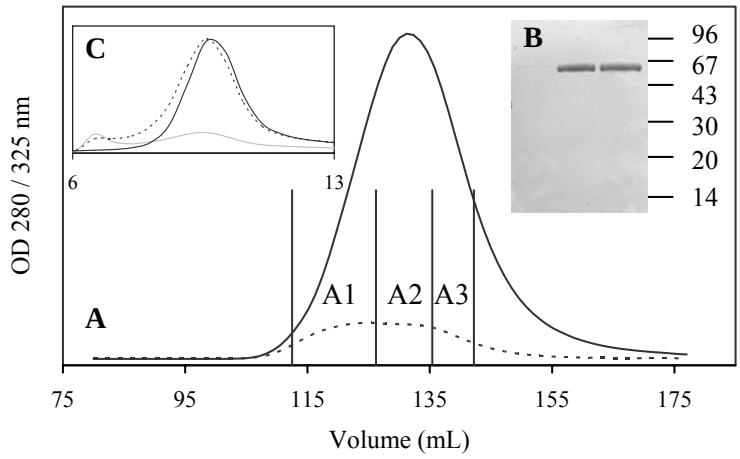

Figure 3: (A) Size exclusion chromatogram of SEC peak A on a Superdex 200 column in 50 mM Tris-HCl buffer, pH 8.2 (I = 0.02 M). Black line: OD at 280 nm; dashed line: OD at 325 nm. Indicated are the fractions (A1-A3) that were collected; (B) SDS-PAGE of fractions A1-A3. Low molecular weight markers are indicated at the right side in kDa; (C) Size exclusion chromatogram of fractions A1-A3 on a Superose 6 column in 50 mM Tris-HCl buffer, pH 8.2 (I = 0.02 M). Gray line: A1; dashed line: A2; black line: A3. The X and Y axes are equal to Figure 3A.

EFFECTS OF PURIFICATION METHODS ON ARA H 1 QUATERNARY STRUCTURE

Vicilins are described as trimeric proteins, but reversible association into hexamers is possible, depending on the ionic strength. For β-conglycinin, a lower ionic strength results in a higher amount of hexamers (*21*). The oligomeric structure of Ara h 1 was found to be independent of the ionic strength applied: the protein eluted as an oligomer

when incubated and eluted in buffers with ionic strengths of both 0.02 and 0.32 M. Also, after dialysis from 0.32 to 0.02 M and elution in 0.02 M, still only oligomers eluted from SEC (data not shown).
In literature Ara h 1 has always been described as a stable trimer of ~180 kDa (*7*, *8*), so our results are contradictory to those described earlier. The structure described for Ara h 1 was in all cases determined after purification of the protein using several other techniques besides SEC. Most of these purification methods included ammonium sulfate precipitation (*6*, *7*), followed by HIC and / or ion exchange chromatography (*5*-*8*). As all previously published results point toward trimeric Ara h 1 structures, one would suspect that these generally used purification steps might lead to (ir)reversible dissociation of the oligomeric allergen. To test this hypothesis, various purification techniques were investigated for their influence on the structure of Ara h 1. First, purified Ara h 1 oligomers were applied to an anion exchange column. Ara h 1 appeared to elute as a single peak around 26 mS cm^{-1}, corresponding to an ionic strength of ~0.32 M (data not shown). The elution of Ara h 1 at this conductivity corresponds with that reported in literature (*8*). The peak was collected and denoted Ara h 1_{AEC} and was analyzed for its quaternary structure with SEC, as can be seen in Figure 4.

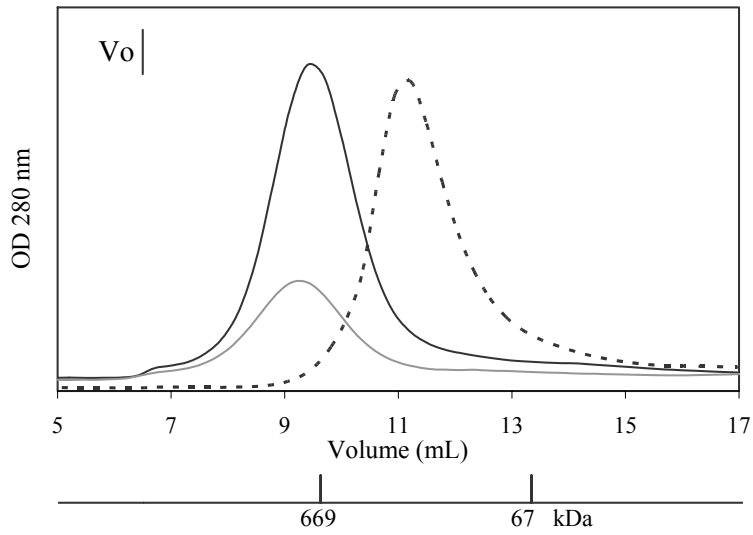

Figure 4: Size exclusion chromatogram of Ara h 1 on a Superose 6 column in 50 mM Tris-HCl buffer, pH 8.2 (I = 0.02 M). Black line: Ara h 1_{SEC}; dashed line: Ara h 1_{AEC}; gray line: Ara h 1_{ASP}. Indicated is the void volume (Vo) of the column.

It was shown that Ara h 1_{AEC} eluted as a single peak on SEC, corresponding to a molecular mass of ~200 kDa. This molecular mass coincides with a trimeric structure of Ara h 1. Thus, AEC led to the dissociation of Ara h 1 oligomers into trimers. SEC analysis at various ionic strengths confirmed the irreversible character of the dissociation: Ara h 1_{AEC} eluted as a trimer both at an ionic strength of 0.02 M (Figure 4) and 0.32 M (Figure 5). Also, after dialysis from 0.32 to 0.02 M, the protein only occurred in a trimeric form (data not shown). Accordingly, as it was shown before that adding salt to the protein solution did not induce changes in quaternary conformation, the interactions

with the anion exchange material probably led to the irreversible changes in the quaternary structure of Ara h 1.
The presence of a high ammonium sulfate concentration did not induce the association or dissociation of Ara h 1; the protein (Ara h 1_{ASP}) eluted as an oligomer on SEC (Figure 4). Thus, it was concluded that ammonium sulfate did not influence the quaternary structure of Ara h 1.
Furthermore, the effects of HIC on the allergen's structure were investigated. To this end, Ara h 1_{SEC} in 3.3 M ammonium sulfate was bound to and eluted from HIC material and denoted Ara h 1_{HIC}. In these samples another phenomenon was observed: at a low ionic strength (0.02 M) only oligomeric Ara h 1 was detected in Ara h 1_{HIC} (data not shown). What was striking, however, were the SEC results at a higher ionic strength (0.32 M): at this ionic strength part of the protein existed in an oligomeric form of ~700 kDa, whereas another part existed in a trimeric form of ~200 kDa (Figure 5). After dialysis back to the lower ionic strength, Ara h 1 was again solely present in an oligomeric form (data not shown). Hence, HIC seemed to induce a part of Ara h 1 to dissociate reversibly into trimers at higher ionic strengths.

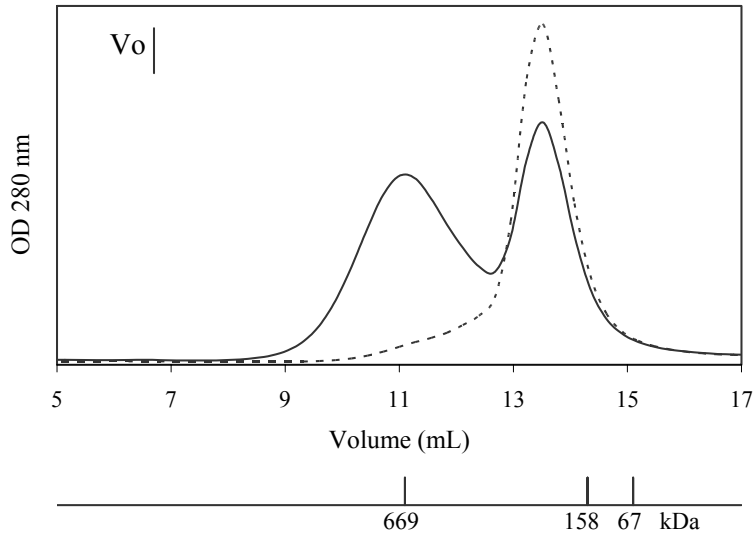

Figure 5: Size exclusion chromatogram of Ara h 1_{HIC} (solid line) and Ara h 1_{AEC} (dashed line) on a Superose 6 column in 50 mM Tris-HCl buffer, pH 8.2 containing 0.3 M NaCl (I = 0.32 M). Indicated is the void volume (Vo) of the column.

STRUCTURE ANALYSIS

Far- and near-UV CD analysis was performed with Ara h 1_{SEC}, Ara h 1_{HIC}, and Ara h 1_{AEC} to investigate possible differences in their secondary and tertiary structures. As the presence of Tris-HCl buffer disturbed the CD measurement at low wavelengths, Ara h 1 samples were dialyzed and lyophilized and hereafter dissolved in sodium phosphate buffer. SEC analysis before and after lyophilization showed no differences in the elution pattern of the samples (data not shown).
The CD results are given in Figure 6. The far-UV CD spectra of all three Ara h 1 samples were similar, having a zero-crossing around 201 nm and a negative extreme around 208 nm, indicative for proteins with a high α-helical content. The spectra were comparable to

those described by Koppelman and co-workers (*8*). With spectral analysis, based on a nonlinear least-squares fitting procedure, it was estimated that the proteins in all three preparations had a similar secondary structure content, consisting of 30 ± 3% α-helix, 14 ± 5% β-strand and 34 ± 5% β-turn. Thus, the changes in quaternary structure, induced by HIC and AEC, did not result in changes in the secondary structure of Ara h 1.

The near-UV CD spectra of the three Ara h 1 samples were, in contrast to the far-UV CD spectra, not similar, indicating differences in the surroundings of the aromatic amino acid residues. The intensity of the total spectrum of Ara h 1_{SEC} was higher compared to the intensities of the spectra of Ara h 1_{AEC} and Ara h 1_{HIC}. Furthermore, Ara h 1_{HIC} and Ara h 1_{AEC} showed similar spectra, except that Ara h 1_{HIC} showed a higher intensity in the 250-260 nm region.

Ara h 1_{SEC} showed a peak around 268 nm, a wavelength at which phenylalanine residues generally absorb (*28*). A lower intensity at this wavelength, which was observed for Ara h 1_{HIC} and Ara h 1_{AEC}, implies that the protein core, where the phenylalanine residues are most frequently located, is less compact. Ara h 1_{SEC} also showed a higher intensity in the 270-290 nm region, where tryptophan and tyrosine residues generally absorb (*28*). The lower intensity of Ara h 1_{HIC} and Ara h 1_{AEC} at these wavelengths indicates a lower mobility of the tyrosine and tryptophan residues.

Due to the fact that under the conditions used for CD measurements the proteins in Ara $h1_{AEC}$ were present as trimers, whereas the proteins in Ara $h1_{HIC}$ and Ara $h1_{SEC}$ were present as oligomers, the difference between Ara $h1_{HIC}$ and Ara $h1_{SEC}$ might be explained by the fact that Ara h 1_{HIC} oligomers are partly destabilized, as at a higher ionic strength they partially dissociate into trimers. Taking into account the quite similar near-UV CD patterns of Ara $h1_{HIC}$ and Ara $h1_{AEC}$, the destabilization induced by HIC probably largely corresponds to the destabilization of Ara h 1 induced by AEC.

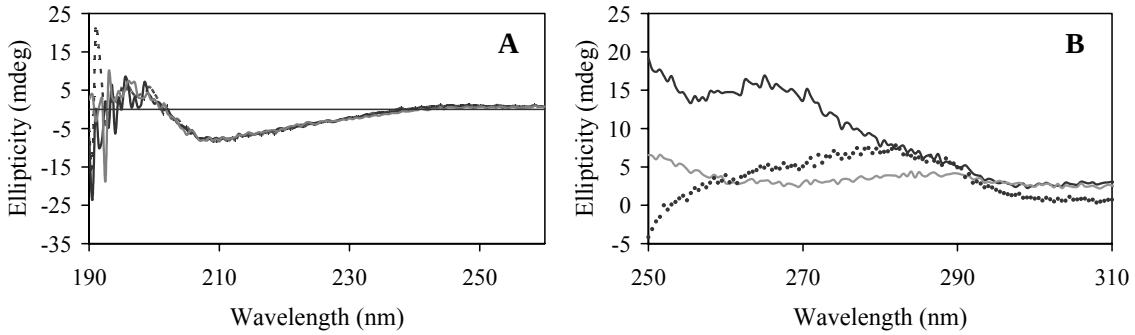

Figure 6: Far- (A) and near- (B) UV CD spectra of Ara h 1_{SEC} (black lines), Ara h 1_{HIC} (gray lines), and Ara h 1_{AEC} (dashed lines).

The intensity of the emission spectra obtained from tryptophan fluorescence spectroscopy measurements (Figure 7) was lowest for Ara h 1_{SEC} and highest for Ara h 1_{AEC}. Furthermore, a wavelength maximum around 348 nm for Ara h 1_{SEC}, around 350 nm for Ara h 1_{HIC} and around 351 nm for Ara h 1_{AEC} were measured. These emission wavelengths indicate that the tryptophan residues in all three protein preparations are in a nonpolar environment. An emission maximum at a higher wavelength, as observed for Ara h 1_{HIC} and Ara h 1_{AEC}, indicates that the environment of the tryptophan residues is

more polar compared to those of Ara h 1_{SEC}, the latter thus having either a more compact structure or a less exposed tryptophan environment. The increase in fluorescence intensity could be caused by a lower level of quenching, resulting from a lower degree of folding of the tryptophan environment. From these results, which were in agreement with the results from near-UV CD measurements, it could also be concluded that the tertiary structure of stable Ara h 1 oligomers (Ara h 1_{SEC}) is more compact than that of destabilized Ara h 1 oligomers (Ara h 1_{HIC}) and Ara h 1 trimers (Ara h 1_{AEC}): both Ara h 1_{HIC} and Ara h 1_{AEC} have a less compact tertiary structure then Ara h 1_{SEC}, with Ara h 1_{AEC}, consisting solely of trimers, having the least compact protein structure. Thus, subjecting Ara h 1 oligomers to HIC and inducing a reversible and partial dissociation of Ara h 1 oligomers seems to cause a similar but less extensive protein destabilization compared to subjecting the protein to AEC.

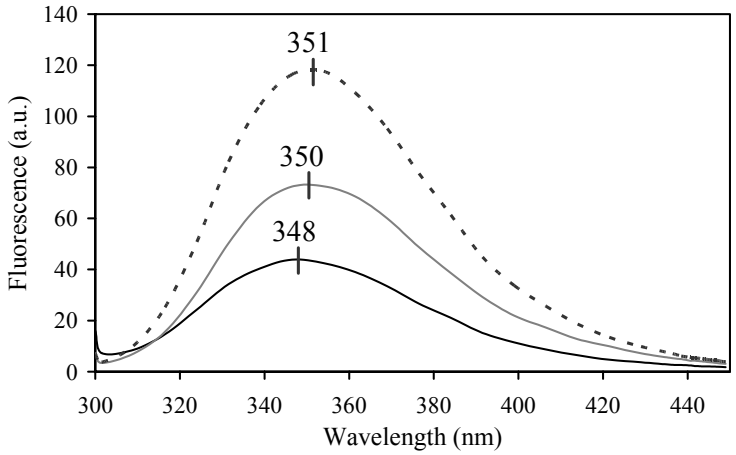

Figure 7: Tryptophan fluorescence spectra of Ara h 1_{SEC} (black line), Ara h 1_{HIC} (gray line), and Ara h 1_{AEC} (dashed line). The numbers indicate the wavelength maxima.

Table 1: Quaternary structure of Ara h 1 after various purification methods, at different ionic strengths.

Ionic strength	Purification method			
	SEC[1]	ASP[2]	HIC[3]	AEC[4]
0.02 M	oligomers	oligomers	oligomers	trimers
0.32 M	oligomers	oligomers	oligomers + trimers	trimers
0.32 → 0.02 M	oligomers	oligomers	oligomers	trimers

[1]: SEC = Size exclusion chromatography
[2]: ASP = Ammonium sulfate precipitation
[3]: HIC = Hydrophobic interaction chromatography
[4]: AEC = Anion exchange chromatography

On the basis of the results obtained, it can be stated that Ara h 1, a *major* allergen from peanuts, occurs as an oligomer instead of as a trimer upon extraction from peanuts, pointing towards the allergen to naturally occur in this oligomeric form. The oligomeric structure of the protein is independent of the ionic strength. Purification techniques have different effects on the allergen's quaternary structure, as summarized in Table 1: AEC

leads to irreversible dissociation of the allergen, whereas HIC induces a partial and reversible dissociation of oligomers into trimers at higher ionic strengths.
Both AEC and HIC have an influence on the allergen's tertiary structure and the (reversible) dissociation of Ara h 1 oligomers into trimers induces a less compact protein structure. As all characteristics of Ara h 1 have thus far been determined of the irreversibly dissociated trimeric protein, it could well be that inaccurate conclusions about the protein's characteristics have been drawn, including conclusions about the protein's allergenic capacity. Therefore, it is required to further investigate the differences in structural characteristics between oligomeric and trimeric Ara h 1 and relate them to the allergen's allergenic activity. Results of these investigations are described in chapters 5 and 6.

REFERENCES

1. Besler, M., Steinhart, H., Paschke, A. Stability of food allergens and allergenicity of processed foods. *Journal of Chromatography B* **2001,** 756, (1-2), 207-228.
2. Bredehorst, R., David, K. What establishes a protein as an allergen? *Journal of Chromatography B* **2001,** 756, (1-2), 33-40.
3. Pastorello, E. A., Trambaioli, C. Isolation of food allergens. *Journal of Chromatography B* **2001,** 756, (1-2), 71-84.
4. 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. *Journal of Allergy and Clinical Immunology* **1999,** 103, (4), 559-562.
5. Burks, A. W., Williams, L. W., Helm, E. M., Connaughton, C., Cockrell, G., O'Brien, T. Identification of a major peanut allergen, Ara h 1, in patients with atopic dermatitis and positive peanut challenge. *Journal of Allergy and Clinical Immunology* **1991,** 88, (2), 172-179.
6. 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. *Journal of Biological Chemistry* **1998,** 273, (22), 13753-13759.
7. 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. *Journal of Immunology* **2000,** 164, (11), 5844-5849.
8. Koppelman, S. J., Bruijnzeel Koomen, C. A. F. M., Hessing, M., Jongh, H. H. J. d. Heat-induced conformational changes in Ara h 1, a major peanut allergen, do not affect its allergenic properties. *Journal of Biological Chemistry* **1999,** 274, (8), 4770-4777.
9. 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. *European Journal of Biochemistry* **1997,** 245, (2), 334-339.
10. 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. *Journal of Allergy and Clinical Immunology* **2004,** 113, (4), 776-782.

11. Dunwell, J. M. Cupins: A new superfamily of functionally diverse proteins that include germins and plant storage proteins. *Biotechnology & Genetic Engineering Reviews* **1998,** 15, (-), 1-32.

12. Mills, E. N. C., Jenkins, J. A., Marigheto, N., Belton, P. S., Gunning, A. P., Morris, V. J. Allergens of the cupin superfamily. *Biochemical Society Transactions* **2002,** 30, (6), 925-929.

13. Wensing, M., Knulst, A. C., Piersma, S., O'Kane, F., Knol, E. F., Koppelman, S. J. Patients with anaphylaxis to pea can have peanut allergy caused by cross-reactive IgE to vicilin (Ara h 1). *Journal of Allergy and Clinical Immunology* **2003,** 111, (2), 420-424.

14. Teuber, S. S., Jarvis, K. C., Dandekar, A. M., Peterson, W. R., Ansari, A. A. Identification and cloning of a complementary DNA encoding a vicilin-like proprotein, Jug r 2, from English walnut kernel (*Juglans regia*) a major food allergen. *Journal of Allergy and Clinical Immunology* **1999,** 104, (6), 1311-1320.

15. Ogawa, T., Bando, N., Tsuji, H., Nishikawa, K., Kitamura, K. Alpha-subunit of beta-conglycinin, an allergenic protein recognized by IgE antibodies of soybean-sensitive patients with atopic-dermatitis. *Bioscience Biotechnology and Biochemistry* **1995,** 59, (5), 831-833.

16. Wang, F., Robotham, J. M., Teuber, S. S., Tawde, P., Sathe, S. K., Roux, K. H. Ana o 1, a cashew (*Anacardium occidental*) allergen of the vicilin seed storage protein family. *Journal of Allergy and Clinical Immunology* **2002,** 110, (1), 160-166.

17. Burks, A. W., Brooks, J. R., Sampson, H. A. Allergenicity of major component proteins of soybean determined by enzyme-linked assay (ELISA) and immunoblotting in children with atopic dermatitis and positive soy challenges. *Journal of Allergy and Clinical Immunology* **1988,** 81, (6), 1135-1142.

18. Lawrence, M. C., Izard, T., Beuchat, M., Blagrove, R. J., Colman, P. M. Structure of phaseolin at 2.2 Angstrom resolution - implications for a common vicilin/legumin structure and the genetic-engineering of seed storage proteins. *Journal of Molecular Biology* **1994,** 238, (5), 748-776.

19. Gatehouse, J. A., Croy, R. R. D., Morton, H., Tyler, M., Boulter, D. Characterization and subunit structures of the vicilin storage proteins of pea (*Pisum-Sativum*-L). *European Journal of Biochemistry* **1981,** 118, (3), 627-633.

20. 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. *Clinical and Experimental Allergy* **1998,** 28, (6), 743-751.

21. Thanh, V. H., Shibasaki, K. Major proteins of soybean seeds - reversible and irreversible dissociation of beta-conglycinin. *Journal of Agricultural and Food Chemistry* **1979,** 27, (4), 805-809.

22. Boldt, A., Fortunato, D., Conti, A., Petersen, A., Ballmer-Weber, B., Lepp, U., Reese, G., Becker, W. M. Analysis of the composition of an immunoglobulin E reactive high molecular weight protein complex of peanut extract containing Ara h 1 and Ara h 3/4. *Proteomics* **2005,** 5, (3), 675-686.

23. de Jongh, H. H., Goormaghtigh, E., Killian, J. A. Analysis of circular dichroism spectra of oriented protein-lipid complexes: Toward a general application. *Biochemistry* **1994,** 33, (48), 14521-14528.

24. Burks, A. W., Cockrell, G., Stanley, J. S., Helm, R. M., Bannon, G. A. Recombinant peanut allergen Ara-h-I expression and IgE binding in patients with peanut hypersensitivity. *Journal of Clinical Investigation* **1995,** 96, (4), 1715-1721.

25. Piersma, S. R., Gaspari, M., Hefle, S. L., Koppelman, S. J. Proteolytic processing of the peanut allergen Ara h 3. *Molecular Nutrition & Food Research* **2005,** 49, (8), 744-755.

26. Yan, Y. S., Lin, X. D., Zhang, Y. S., Wang, L., Wu, K. Q., Huang, S. Z. Isolation of peanut genes encoding arachins and conglutins by expressed sequence tags. *Plant Science* **2005,** 169, (2), 439-445.

27. Rabjohn, P., Helm, E. M., Stanley, J. S., West, C. M., Sampson, H. A., Burks, A. W., Bannon, G. A. Molecular cloning and epitope analysis of the peanut allergen Ara h 3. *Journal of Clinical Investigation* **1999,** 103, (4), 535-542.

28. Vuilleumier, S., Sancho, J., Loewenthal, R., Fersht, A. R. Circular-dichroism studies of barnase and its mutants - characterization of the contribution of aromatic side-chains. *Biochemistry* **1993,** 32, (39), 10303-10313.

5

PEANUT ALLERGEN ARA H 1 INTERACTS WITH PROANTHOCYANIDINS INTO OLIGOMERIC COMPLEXES

Based on: 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. *Journal of Agricultural and Food Chemistry* **2007**, 55, (21), 8772-8778.

ABSTRACT

Mildly extracted peanut allergen Ara h 1 was previously reported to occur as an oligomeric complex. In this chapter we describe that the protein in this oligomeric complex interacts noncovalently with phenolic compounds of the proanthocyanidin type. These interactions are being disrupted during anion exchange chromatography, resulting in the dissociation of the oligomeric Ara h 1 complex into protein trimers. By use of the known three-dimensional structure of β-conglycinin, a soy protein homologous to Ara h 1, proline-rich regions were observed *in silico* on both faces of its trimeric structure, which are conserved in Ara h 1. These proline-rich regions could explain the binding of proanthocyanidins to Ara h 1 and the formation of multiple Ara h 1 trimer complexes. This was supported by the observation that the addition of peanut proanthocyanidins to trimeric Ara h 1 and to β-conglycinin resulted in the formation of soluble oligomeric protein complexes. The structurally related legumin proteins do not contain such proline-rich regions on both sides of the protein and proanthocyanidins were shown to have a lower affinity for legumin proteins from peanuts and soybeans (peanut allergen Ara h 3 and soy glycinin, respectively). Ara h 1 present as the oligomeric complex is assumed to be the representative form of the allergen in which it is consumed by humans.

INTRODUCTION

Resistance to digestion in the human gastro-intestinal tract as well as stability upon cooking and industrial processing are properties considered to be prerequisites for food allergens. These properties depend to a large extent on the structural characteristics of the allergenic protein. In addition, these characteristics can be influenced by other components present in food. For example, phenolic components are known to be able to associate with proteins, resulting in conjugates affecting both the solubility (*1*) and digestibility (*2*) of the proteins.

Peanut allergy is the most prevalent food allergy in older children, adolescents, and adults. In addition, allergic reactions to peanuts are usually persistent for life and can cause life-threatening situations. In recent years much research has been devoted to identify and characterize the allergens present in peanuts. One of the *major* allergens in peanuts, Ara h 1, is described as a 63 kDa glycosylated seed storage protein (*3*). The gene encoding for Ara h 1 has high homology (60-65%) with those of the vicilin seed storage protein family (*4*). Members of this family are all described to be trimeric proteins (*5*), either or not reversibly associating into hexamers at low ionic strength (*6*). Ara h 1 has been assumed to occur as a trimer (*7*), but, as is described in chapter 4, the allergen was recently reported (*8*) to occur as a larger oligomer upon extraction from peanuts. It was observed that irreversible dissociation of the oligomers into trimers is induced by applying the allergen to anion exchange chromatography (AEC) (chapter 4; *8*), a commonly used purification technique.

The occurrence of Ara h 1 in a stable oligomeric structure is not consistent with other data described in the literature for Ara h 1 and for other vicilin proteins. Therefore, it was hypothesized that other compounds are present in the oligomeric protein complex,

causing the complexation of the trimeric protein. This complexation occurs via noncovalent interactions, the latter ones being disrupted during anion exchange chromatography. Phenolic compounds are able to interact noncovalently with proteins, which may result in complexation (*9*, *10*). Phenolic compounds are described to be present in peanuts. The predominant monomeric phenolic compound in peanuts is *p*-coumaric acid (*11*). Besides, oligomeric flavan-3-ols, also known as proanthocyanidins or condensed tannins, are reported to be present in (roasted) peanuts (*12*) and in peanut skins (*13*), with degrees of polymerization varying between 1 and 5 (*12*).

Noncovalent interactions between proteins and monomeric phenolic compounds require relatively high molar ratios of phenolic compounds to proteins (>100) in order to affect the functional properties of the proteins (*14*). The average content of phenolic compounds in raw peanuts is 47 mg kg^{-1} (*11*), next to 24 to 29% (w/w) protein, with Ara h 1 representing 12 to 16% thereof (*15*). Thus, per mole of Ara h 1 there would be a maximum of only 0.5 mol *p*-coumaric acid available per Ara h 1 molecule and this ratio would be too low to affect the functional properties of Ara h 1 (*14*). Proanthocyanidins, on the other hand, can affect the functional properties of proteins at much lower phenolic compound to protein ratios. Besides, proanthocyanidins, because of their larger sizes, are able to interact with more than one protein site, which may result in the formation of cross-links between proteins, likely resulting in aggregation (*16*). Interactions between proanthocyanidins and proteins have been reported to be noncovalent and larger proanthocyanidins normally have a higher ability to bind to proteins than smaller ones (*17*).

In this study we aimed to further characterize the previously identified oligomeric structure of Ara h 1 (chapter 4; *8*) and investigated if proanthocyanidins are responsible for the oligomerization of the allergen.

MATERIALS AND METHODS

MATERIALS

All chemicals were obtained from Merck (Darmstadt, Germany) or Sigma (Sigma-Aldrich Inc., St. Louis, MO, USA), unless stated otherwise. Peanuts of the Runner market-type and peanut skins were provided by Imko Nut Products (Doetinchem, The Netherlands) and were stored at 4 °C until use. Soy β-conglycinin and glycinin were purified from Hyland soybeans as described by Kuipers and co-workers (*18*). Millipore water was used for all experiments (Millipore Corp, Bedford, MA, USA).

ARA H 1 PURIFICATION

Ground peanuts were defatted with hexane using Soxhlet extraction. Defatted and nondefatted ground peanuts were extracted (10 g 100 mL^{-1} or 10 g 20 mL^{-1}, respectively) for 1 h under continuous stirring, in 15 mM sodium phosphate buffer, pH 6.2, containing 0.3% (w/v) sodium metabisulfite. Afterwards, the extracts were sieved through cheese-cloth and centrifuged (25 min; 14,000*g*; 4 °C). The supernatants obtained were filtered over a 1.2 μm filter and applied onto a Superdex 200 column (10 cm × 52 cm; 150 mL of

extract applied per run; GE Healthcare, Uppsala, Sweden). The column was equilibrated and eluted with 15 mM sodium phosphate buffer, pH 6.2, at a flow rate of 40 mL min^{-1} using an Äkta Explorer system (GE Healthcare) operated at room temperature. Eluates were monitored at 280 and 325 nm and appropriate fractions were collected and reapplied onto the same column. Again, fractions containing pure Ara h 1, as analyzed by SDS-PAGE and analytical size exclusion chromatography, were pooled and stored at -20 °C until use. The Ara h 1 preparation purified from defatted peanuts with skins was denoted Ara h $1_{oligomers}$ and the one purified from nondefatted peanuts without skins was denoted Ara h $1_{oligomers, NS}$.
Ara h 1 trimers were induced from Ara h $1_{oligomers}$ using anion exchange chromatography, as described in chapter 4 (*8*) and denoted Ara h $1_{trimers}$.

ARA H 3 PURIFICATION
For the purification of Ara h 3, defatted peanuts were extracted (10 g 100 mL^{-1}) for 1 h at room temperature under continuous stirring, in 50 mM Tris-HCl buffer, pH 8.2. Afterwards, the extract was sieved through cheese-cloth and centrifuged (25 min; 14,000*g*; 4 °C). The supernatant obtained was filtered over a 1.2 μm filter and subsequently applied onto a 320 mL Superdex 200 XK 26/60 column (13 mL applied per run; GE Healthcare). The column was equilibrated and eluted with 50 mM Tris-HCl buffer, pH 8.2, at a flow rate of 4.3 mL min^{-1}, using an Äkta Purifier system (GE Healthcare) operated at room temperature. Eluates were monitored at 280 nm and fractions containing Ara h 3, as analyzed by SDS-PAGE, were collected, pooled and applied onto a 1.2 L Source Q Fineline column, using an Äkta explorer system. The column was equilibrated with 50 mM Tris-HCl buffer, pH 8.2 and the flow rate used was 40 mL min^{-1}. After sample application and washing, a 10 column volumes linear gradient from 0.1 to 1 M NaCl in 50 mM Tris-HCl buffer, pH 8.2, was applied. The eluate was monitored at 280 nm and samples containing pure Ara h 3, as analyzed by SDS-PAGE, were collected and pooled. The pooled fractions were dialyzed in 10,000 MWCO dialysis tubings (Medicell Int. Ltd., London, UK) against water. Afterwards, samples were lyophilized and stored at –20 °C until use.

ANALYTICAL SIZE EXCLUSION CHROMATOGRAPHY (SEC)
Analytical SEC was performed on a Superdex 200 10/300 column (0.1 mL of sample applied per run, GE Healthcare). The column was equilibrated and eluted with 15 mM sodium phosphate buffer, pH 6.2, at a flow rate of 0.5 mL min^{-1}, using an Äkta purifier system operated at room temperature. The eluate was monitored at 280 and 325 nm.

SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)
Protein samples were analyzed using on a Mini-PROTEAN II system (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the instructions of the supplier. Reducing conditions were obtained by adding β-mercaptoethanol to a final concentration of 10 mM and heating the samples for 5 min at 100 °C. Tris-HCl 10-20% linear gradient Ready Gels (Bio-Rad) were used. Gels were stained according to the Coomassie Brilliant

Blue procedure provided by the manufacturer. Low molecular weight protein standards (GE Healthcare) were used according to the instructions of the manufacturer.

PROTEIN QUANTIFICATION

The nitrogen content of samples was determined using the combustion (Dumas) method on a 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 the part of the amino acid sequence remaining after post-translational processing (*19*) (accession number P43238) and its content and composition of linked *N*-glycans (*20*).

PEANUT PROANTHOCYANIDIN PURIFICATION

Peanut skins were defatted with hexane using Soxhlet extraction. Defatted peanut skins (60 g) were extracted three times with 1 L of 20% (v/v) aqueous methanol. Afterwards, the methanol was evaporated in a rotating vacuum evaporator and samples were lyophilized. Next, samples were dissolved in water and proanthocyanidins were extracted three times with ethyl acetate at a ratio of water to ethyl acetate of 1:1 (v/v). The ethyl acetate fractions were collected and combined. The ethyl acetate was evaporated and the remaining material was dissolved in a mixture of acetone, hexane, and ethanol (volume ratio 7:3:2). Samples of 10 mL (700 mg) were applied onto a preparative Inertsil column (30 × 250 mm, GL Sciences Inc., Tokyo, Japan), operated at a flow rate of 27.22 mL min^{-1}. The eluents used were (A) hexane and (B) acetone. A linear gradient from 40% to 60% B was executed in 30 min, followed by 20 min of eluting with 60% B and a linear gradient from 60% to 75% B in 20 min. UV spectra were recorded from 210 to 300 nm. Fractions containing proanthocyanidin pentamers and higher molecular weight oligomers, as determined by analytical normal phase chromatography with mass detection (masses equal to or higher than 1,434 Da), were collected, pooled, evaporated, and lyophilized. Analytical normal phase chromatography was performed on a Thermo Spectra system (Thermo Separations Products Inc., San Jose, CA, USA). Samples (dissolved in methanol) were injected onto a Luna Silica column (4.6 × 250 mm; Phenomenex Inc., Torrance, CA, USA) operated at room temperature. The eluents used were (A) methanol, (B) dichloromethane, and (C) 50% (v/v) acetic acid. The flow rate was 1.0 mL min^{-1} and detection was performed at 280 nm. A gradient from 82% A, 14% B, and 4% C to 61% A, 35% B, and 4% C was followed in 50 min. Mass spectra were recorded with an electrospray ionization mass spectrometer (LCQ Classic; Thermo Finnigan, San Jose, CA, USA), with detection in the negative mode. The capillary spray voltage was 5 kV and the capillary temperature was 270 °C.

BATCH-WISE ANION EXCHANGE CHROMATOGRAPHY (AEC)

Source Q material (0.75 mL; GE Healthcare) was washed with water, followed by 50 mM Tris-HCl buffer, pH 8.2 (washing buffer). Next, 5 mL of Ara h 1 solution (2 mg mL^{-1}) in 15 mM sodium phosphate buffer, pH 6.2, of which the pH was adjusted to 8.2 with 1 M NaOH, was added to the column material. Next, the column material was washed twice with 0.75 mL of washing buffer. Subsequently, the column material was eluted twice with

0.75 mL of 50 mM Tris-HCl buffer, pH 8.2, containing 0.5 M NaCl, and twice with 0.75 mL of 50 mM Tris-HCl buffer, pH 8.2, containing 1 M NaCl. Finally, the column material was washed twice with washing buffer and the remaining column material was air-dried. All buffers were removed from the anion exchange material after centrifugation (22,000*g*; 5 min; 20 °C).

PROANTHOCYANIDIN QUANTIFICATION

Samples were analyzed using the HCl-butanol assay (*21*). Samples were mixed with 0.1-0.5 mL of butanol, containing 5% (v/v) HCl and incubated in a water bath at 95 °C for 2 h. After incubation, the color of the samples was visually analyzed or samples were centrifuged (22,000*g*, 5 min; 4 °C) and the absorbance of the supernatant was measured at 550 nm (BioTek Iinstruments Inc., Winooski, VT, USA). A calibration curve was made using pentameric proanthocyanidins that were purified as described above.

SEQUENCE ANALYSIS

The amino acid sequences of Ara h 1 (accession number P43238, amino acids 85-626) and Ara h 3 (accession number O82580, amino acids 1-507) were aligned with the amino acid sequence of the β subunit of β-conglycinin (accession number P25974, amino acids 26-439) and glycinin G1 (accession number P04776, subunit A1a, B2, amino acids 1-495) from soybean, respectively, using the program MegAlign (DNAStar, Inc., Madison, WI, USA). A Clustal W alignment was performed. The weight table PAM 250 was used and the parameters gap length and gap penalty were set at 0.2 and 10, respectively. Next, the proline residues of Ara h 1 and Ara h 3 were projected onto the X-ray three-dimensional structure of soybean β-conglycinin (PDB code 1IPJ) and soybean glycinin (PDB code 1FXZ), respectively, using the program Deepview (www.expasy.org/spdbv/).

RECONSTITUTION OF PROTEIN-PROANTHOCYANIDIN MIXTURES

Solutions of pentameric peanut proanthocyanidins (purified as described above, using a molar mass of 1,434 for calculation) in 100 mM sodium phosphate buffer, pH 8.0, were added to separately weighted amounts (5 mg) of Ara h $1_{trimers}$, Ara h 3, soybean β-conglycinin, soybean glycinin and mixtures of two of these proteins, resulting in samples with molar ratios of proanthocyanidins to proteins varying from 3:1(:1) to 10:1(:1). Samples were mixed head-over-tail overnight at room temperature. Afterwards, samples were centrifuged (22.000*g*, 5 min; 4 °C) and the supernatants were analyzed for their nitrogen content (Dumas) and their molecular size distribution. For the latter, aliquots (100 μL) were analyzed on a Superdex 200 10/300 column, equilibrated and eluted with 100 mM sodium phosphate buffer, pH 8.0, at a flow rate of 0.7 mL min^{-1}, using an Äkta purifier system operated at room temperature. The column was calibrated using a high molecular weight calibration kit (GE Healthcare) and eluates were monitored at 280 nm.

RESULTS AND DISCUSSION

ARA H 1-PROANTHOCYANIDIN INTERACTIONS

The recently reported occurrence of Ara h 1 in an oligomeric structure after purification using mild extraction techniques (chapter 4; *8*) is contrary to previous observations, stating that Ara h 1 occurs as trimers (*7*). As proanthocyanidins are able to interact with proteins at relatively low molar ratios, possibly resulting in the formation of complexes (*16*), we investigated if proanthocyanidins were present in the oligomeric Ara h 1 protein complex. The results showed that per milligram of Ara h $1_{oligomers}$ 0.060 ± 0.008 mg of proanthocyanidins was present, while in Ara h $1_{trimers}$ no proanthocyanidins ($<5.0 \times 10^{-5}$ mg of proanthocyanidins per mg protein) were detected. Considering a molecular mass of flavan-3-ols of ~300 Da and monomeric Ara h 1 being ~67 kDa, this would eventuate in a molar ratio of ~14 mol of flavan-3-ols to 1 mol of monomeric protein, or ~3 mol of pentameric proanthocyanidins to 1 mol of monomeric protein, the pentamers being the proanthocyanidins with the highest degree of polymerization detected in peanuts (*12*).

As Ara h $1_{oligomers}$ eluted as a single peak from a SEC column upon detection at 280 nm (chapter 4; *8*), it can be stated that the proanthocyanidins and proteins are associated. The absence of proanthocyanidins in Ara h $1_{trimers}$ indicates that AEC, used in the purification procedure of Ara h $1_{trimers}$, causes a disruption of the protein-proanthocyanidin interactions. This disruption would subsequently result in the separation of the proanthocyanidins from Ara h 1 and the dissociation of the oligomeric Ara h 1 complex into a trimeric one.

EFFECT OF AEC ON ARA H 1-PROANTHOCYANIDIN INTERACTIONS

The binding of Ara h $1_{oligomers}$ to an AEC column did not result in an increase in the absorbance at 280 nm in the flow-through. Also, only a single peak eluted from the column during the salt gradient applied, corresponding to trimeric Ara h 1 (chapter 4; *8*). It thus seemed that the proanthocyanidins present in the Ara h $1_{oligomers}$ preparation bound to the AEC column and were not released during the salt gradient applied. A batchwise AEC experiment was performed to investigate whether the proanthocyanidins linked to Ara h 1 indeed bound to the column material. The results showed that none of the fractions collected (nonbound, wash, eluates) except the starting material, appeared to contain proanthocyanidins when analyzed with the HCl-butanol assay (data not shown). However, when the washed anion exchange material after application of Ara h 1 oligomers was analyzed, the column material turned pink (Figure 1). This indicates the presence of proanthocyanidins. Thus, the proanthocyanidins that were previously linked to Ara h 1 bound to the AEC column material, resulting in the dissociation of the oligomeric Ara h 1 complex into a trimeric one (chapter 4; *8*).

ORIGIN OF ARA H 1-PROANTHOCYANIDIN INTERACTIONS

The Ara h $1_{oligomers}$ were purified from peanuts with skins. As peanut skins are reported to contain more proanthocyanidins than peanuts themselves (*13*), the proanthocyanidins from peanut skins could have caused the oligomerization of Ara h 1 during extraction. Nevertheless, extracts of peanuts without skins on SEC showed the same results as extracts of peanuts with skins when analyzed on SEC, with Ara h 1 eluting at the same

volume, corresponding to an oligomeric structure (data not shown). Besides, as is described in chapter 4, Ara h 1 also occurs solely as oligomers in peanut extract made from nondefatted peanuts (*8*).

Although oligomerization was not induced by the proanthocyanidins in peanut skins during extraction, we could not determine whether the Ara h 1-proanthocyanidin interactions are induced during protein extraction, by enabling contact between the protein and the proanthocyanidins, or if these complexes already exist in the peanut itself. Flavan-3-ols are described to be synthesized in the cytosol, but they are accumulated in cell vacuoles, where they undergo condensation (*22*). Proteins are also accumulated in vacuoles. However, they are reported to be stored in protein storage vacuoles, while all other compounds are described to be stored in so-called vegetative vacuoles (*23*). This indicates that Ara h 1 and proanthocyanidins in peanuts occur separately from each other. However, since peanuts are either processed or masticated before consumption, thereby enabling contact between proanthocyanidins and Ara h 1, the oligomeric structure of Ara h 1 seems representative for the structure of Ara h 1, which is normally consumed by humans. The pH used for the protein extraction (6.2) resembles the pH of human saliva, which ranges between 6 and 7 (*24*). Although the time of mastication generally is short, we assume that complexation (if it had not already occurred) may take place, as interactions between proteins and salivary proline-rich proteins are also assumed to occur in the time span that food resides in the oral cavity (*25*). Consequently, we consider the oligomeric structure of Ara h 1 as the structure of the allergen in which it is generally consumed by humans.

TENTATIVE MECHANISM OF ARA H 1-PROANTHOCYANIDIN INTERACTIONS

The molecular mass of Ara h $1_{oligomers}$ was earlier estimated to be ~700 kDa (chapter 4; *8*) using SEC. This mass suggests a complex bearing multiple trimeric Ara h 1 proteins, most probably four. Although most proanthocyanidin-protein interactions result in precipitated complexes (*1*), the Ara h 1-proanthocyanidin interactions thus result in soluble complexes. In peanuts, the allergen Ara h 3 is also present. This protein belongs to the hexameric legumin family, which is structurally related to the vicilin family (*26*). Upon extraction at pH 8.2 and I = 0.02 M, this protein occurs solely in a hexameric structure (chapter 4; *8*), indicating that this protein is not able to form larger complexes because of interactions with proanthocyanidins.

The ability of proteins to form complexes with proanthocyanidins varies considerably and proanthocyanidins predominantly form complexes with proline-rich proteins (*25*). Hydrophobic interactions have been described to be the major driving force for the interaction between various small polyphenols, for example, epigallocatechin gallate (EGCG) and proteins (*1, 27, 28*). However, hydrogen bonding has been reported to be the main driving force for proanthocyanidin-protein interactions (*1, 29*). On the basis of these results, the interaction of peanut proanthocyanidins with Ara h 1 is also likely to be mainly driven by hydrogen bonding.

After post-translational processing, monomeric Ara h 1 contains 31 proline residues (*19*). To explain the apparent affinity of proanthocyanidins for Ara h 1, we looked at the three-dimensional structure of β-conglycinin, a homologous protein from soybeans (containing

21-38 proline residues, depending on the monomer subunit type), as the three-dimensional structure of Ara h 1 has not been determined. However, molecular modeling studies with Ara h 1 have shown that its three-dimensional structure is highly similar to that of β-conglycinin (*30*).

The three-dimensional structure of β-conglycinin has been determined for the trimer built up of three β subunits, each containing 21 proline residues. Alignment of the amino acid sequence of Ara h 1 with that of this β-conglycinin subunit (Table 1) reveals that the first 80 N-terminal amino acids of Ara h 1, containing 11 proline residues, are not covered. The other two subunits of β-conglycinin (α and α') also have an N-terminal extension, containing 14 and 17 proline residues, respectively. However, the three-dimensional structures of the N-terminal extensions of these subunits of β-conglycinin have not been determined.

In the three-dimensional structure of β-conglycinin, which is depicted in Figure 2, most of the proline residues are located on the surface of the molecule. Many of the surface-located prolines are centered around the hole in the middle of the trimeric complex. These proline-rich regions in the middle of the trimeric complex are present on both faces of the protein (indicated as A and B in Figure 2) and are highly conserved in Ara h 1 (Table 1). On face A, four conserved residues are present per monomer, thus 12 residues in total and on face B two conserved residues per monomer are present, thus six residues in total. The presence of these proline-rich regions on Ara h 1 might explain the binding of proanthocyanidins to the allergen. Moreover, the occurrence of proline-rich regions on both faces of the trimer likely explains the oligomerization of Ara h 1 into soluble complexes bearing several Ara h 1 molecules, as proanthocyanidins may act as a linker between the Ara h 1 trimers. Soy β-conglycinin in that case is also expected to be able to interact with proanthocyanidins and to form soluble higher molecular weight complexes. Yet, the structure of β-conglycinin, a protein that has been purified and characterized several times without anion exchange chromatography (*18, 31, 32*), has never been reported to occur as larger complexes or to interact with proanthocyanidins. The most obvious reason for this would be that soybeans do not to contain proanthocyanidins (*12*). However, if proanthocyanidins would be present in samples containing β-conglycinin, the protein would be expected to interact with these compounds in a similar manner as Ara h 1.

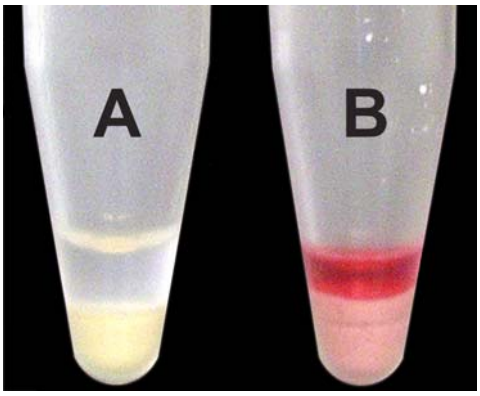

Figure 1: Source Q anion exchange material applied to the HCl-butanol assay, before (A) and after (B) binding of Ara h 1 oligomers and elution of the protein with 1 M NaCl.

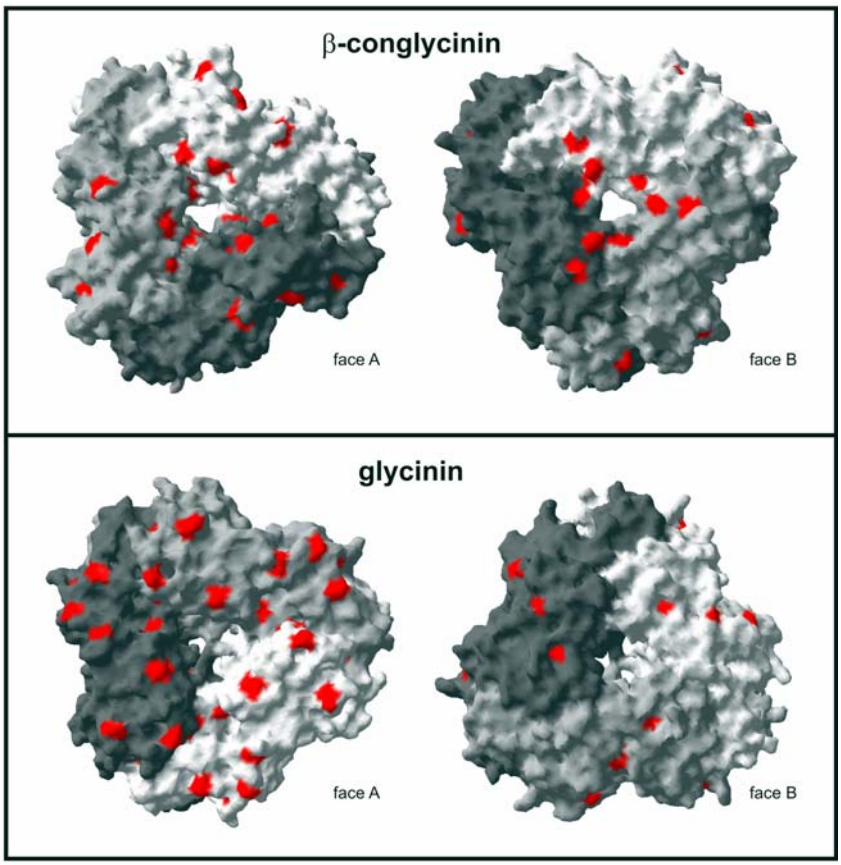

Figure 2: The three-dimensional structures of soybean β-conglycinin and glycinin. Prolines are indicated in red and monomeric subunits are indicated in white, gray, and dark gray.

Table 1: Alignment data with respect to proline of (the β-subunit of) soy β-conglycinin and Ara h 1 after posttranslational processing*.

	β-conglycinin	Ara h 1
prolines in amino acid sequence	21	31
conserved prolines in amino acid sequence	-	16
prolines in 3D structure	17	16
conserved prolines in 3D structure	-	14
prolines on surface 3D structure	12	10
conserved prolines on surface 3D structure	-	9
prolines in proline-rich region on face A	4	4
prolines in proline-rich region on face B	3	2

*: Face A and B are indicated in Figure 2.

TENTATIVE MECHANISM OF ARA H 3-PROANTHOCYANIDIN INTERACTIONS

Large oligomeric complexes were previously not detected for peanut allergen Ara h 3 (containing 28 prolines) present in the same extract as oligomeric Ara h 1 (chapter 4; *8*). To this end, we investigated this protein and looked at the three-dimensional structure of the homologous soy glycinin (containing 37 proline residues), also because the three-dimensional structure of Ara h 3 is not known. The alignment data are given in Table 2 and the three-dimensional structure of glycinin is shown in Figure 2. The three-dimensional structure of glycinin does not cover the complete amino acid sequence of the protein: four disordered regions (varying in lengths from 5 to 48 residues) are not covered in the three-dimensional protein structure. In Table 2 their proline contents are given, but no further investigation was performed into these sequences.

Most of the prolines on the surface of glycinin are conserved in Ara h 3. Furthermore, these conserved prolines are situated mostly on one face of the protein (indicated as face A in Figure 2). This face of the legumin protein is the hydrophobic part of the protein and interacts in the plant with the same side of another trimeric molecule, to form a hexameric structure (*33*). Thus, the prolines present on that face of Ara h 3 will not be available for interaction with proanthocyanidins as they will be shielded in the hexameric protein structure. The other face of the trimeric molecule (face B) contains four proline residues, of which three are conserved in Ara h 3. These proline residues are not situated in close proximity of each other and consequently do not comprise a proline-rich region. These data indicate that proanthocyanidins will not have a high affinity for Ara h 3 and glycinin. As a result, higher molecular weight complexes, due to cross-linking of Ara h 3 by proanthocyanidins, are not likely to occur. This hypothesis is in agreement with our earlier reported results, describing that Ara h 3 upon extraction from peanuts at pH 8.2, I = 0.02, has a lower molecular weight compared to Ara h 1 oligomers and solely occurs in a hexameric structure (chapter 4; *8*).

Table 2: Alignment data with respect to proline of soy glycinin and Ara h 3*.

	glycinin	Ara h 3
prolines in amino acid sequence	37	28
conserved prolines in amino acid sequence	-	20
prolines in 3D structure	20	18
conserved prolines in 3D structure	-	17
prolines on surface 3D structure	15	13
conserved prolines on surface 3D structure	-	12
prolines on face A	12	10
prolines on face B	4	3
prolines in disordered region 90-109	4	0
prolines in disordered region 179-197	3	3
prolines in disordered region 249-296	0	5

*: Face A and B are indicated in Figure 2

INDUCTION OF INTERACTIONS BETWEEN PROANTHOCYANIDINS AND VICILIN AND LEGUMIN PROTEINS

In order to verify our hypotheses concerning the affinity and interaction of proanthocyanidins with vicilin and legumin type proteins, we investigated whether the addition of proanthocyanidins to these proteins would result in an interaction, either or not eventuating in soluble oligomeric complexes. To this end, proanthocyanidins were added to Ara h $1_{trimers}$, β-conglycinin, Ara h 3, glycinin, and combinations of these proteins. Subsequently, the effects of these additions on the size and solubility of the proteins were analyzed. The results for Ara h $1_{trimers}$ and Ara h 3 are given in Figure 3 and the proportions of soluble protein at the conditions applied are given in Table 3. The Ara h $1_{trimers}$ preparation clearly increased in size with increasing concentrations of proanthocyanidins added, as the protein peak eluted after shorter elution volumes with increasing proanthocyanidin concentrations, thus corresponding to higher molecular weight complexes. The proportion of soluble protein in all Ara h 1 samples did not change much, indicating that almost exclusively soluble complexes were formed. The fact that an absolute increase in peak height was observed with increasing proanthocyanidin concentrations also points to the proanthocyanidins being bound to the protein, as proanthocyanidins contribute to the absorbance at 280 nm.

At pentameric proanthocyanidin to protein ratios higher than those calculated for Ara h $1_{oligomers}$ (3:1) soluble complexes with masses higher than 700 kDa were detected, the latter being the estimated mass of Ara h $1_{oligomers}$ (chapter 4; *8*). Furthermore, at every ratio tested, it appeared that Ara h 1-proanthocyanidin complexes with only a relatively small size distribution were obtained. Our data thus indicate that in the peanut itself the concentration of proanthocyanidins available for interaction with Ara h 1 is limiting, thereby constraining the size of the soluble complex to ~700 kDa.

The addition of proanthocyanidins to β-conglycinin showed results similar to those for Ara h 1: With increasing concentrations of proanthocyanidins soluble larger complexes were formed (data not shown). In the sample with the highest concentration of proanthocyanidins, the proportion (%) of soluble protein was somewhat lower compared to that of the other samples. However, it could very well be that the small amount (12%

w/w) of glycinin present in the sample (*18*) was responsible for this insolubilization. Altogether, it was concluded that interactions of proanthocyanidins with β-conglycinin and Ara h $1_{trimers}$ result in the formation of larger (mostly) soluble complexes, which can likely be explained by the distribution of prolines on the surface of these proteins. Batchwise AEC of (the soluble part of) Ara h 1 and β-conglycinin with proanthocyanidin to protein ratios 10:1 showed that the interactions between the proteins and proanthocyanidins were disrupted, resulting in the binding of proanthocyanidins to the column material (data not shown).

Samples of Ara h 3 with increasing proanthocyanidin concentrations showed that the total area corresponding to Ara h 3 (mainly hexamers, but also few trimers) decreased with increasing proanthocyanidin concentrations (Figure 4). In line with these results a decrease in protein solubility with increasing proanthocyanidin concentrations was observed (Table 3). Similar results were obtained for soy glycinin. The interactions of proanthocyanidins with glycinin and Ara h 3 thus resulted in the formation of insoluble protein-proanthocyanidin complexes, implying that an interaction between Ara h 3 and proanthocyanidins did occur. Next to prolines, other amino acids are able to interact with proanthocyanidins, although with a lower affinity (*28*). These interactions could have caused the precipitation of the legumin proteins.

Table 3: Proportion (%) of soluble protein (at pH 8.0, I = 0.3 M), with standard deviations, of protein samples with increasing molar ratios of proanthocyanidins to proteins (PA:P)

ratio PA:P	Ara h $1_{trimers}$	Ara h 3	β-conglycinin	glycinin
0:1	100 ± 3	100 ± 2	100 ± 4	100 ± 2
3:1	100 ± 2	38 ± 1	103 ± 3	92 ± 4
7:1	101 ± 1	32 ± 2	123 ± 3	32 ± 2
10:1	97 ± 3	15 ± 3	62 ± 0	2 ± 3

In Figure 3 and Table 4 the results from combinations of proteins with added proanthocyanidins are given. When proanthocyanidins were added to a combination of Ara h 1 and Ara h 3, with increasing proanthocyanidin concentrations the peak corresponding to Ara h 1 decreased and larger soluble complexes were formed, comparable to the results for only Ara h 1 with proanthocyanidins. The content of soluble protein did not decrease much. Also, at the highest proanthocyanidin concentration added, still Ara h 3 peaks (hexamers and trimers) were visible. Proanthocyanidins thus have a higher affinity toward Ara h 1 and form soluble complexes, rather than toward Ara h 3 and precipitate this protein. The same was found for combinations of β-conglycinin and glycinin with added proanthocyanidins (data not shown). Apparently, the affinity of proanthocyanidins for vicilin type proteins is higher than that for legumin type proteins, which is in agreement with the above-mentioned hypotheses.

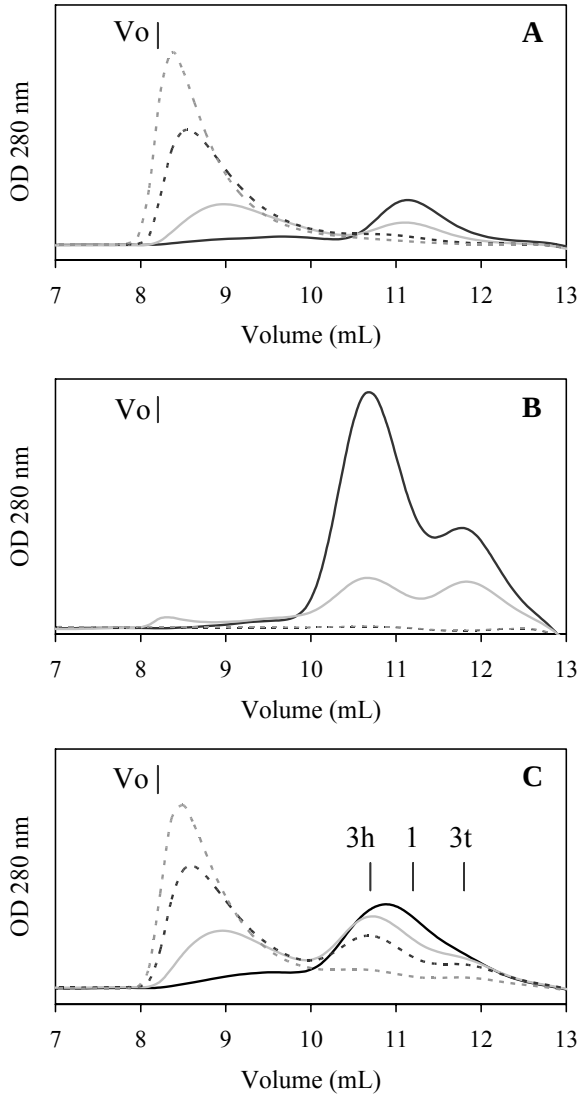

Figure 3: Size exclusion chromatograms of (A) Ara h $1_{trimers}$, (B) Ara h 3, and (C) Ara h $1_{trimers}$ and Ara h 3 with added peanut proanthocyanidins, on a Superdex 200 column. Molar ratios of proanthocyanidins to proteins (to proteins) in samples: 0:1(:1) (black lines); 3:1(:1) (gray lines); 7:1(:1) (black lines, dashed) 10:1(:1) (gray lines, dashed). Indicated is the void volume of the column (Vo) and the elution volume of Ara h 3 hexamers (3h), Ara h 3 trimers (3t), and Ara h 1 trimers (1).

Table 4: Proportion (%) of soluble protein (at pH 8.0, I = 0.3 M), with standard deviations, of protein samples with increasing molar ratios of proanthocyanidins to proteins to proteins (PA:P:P).

ratio PA:P:P	Ara h $1_{trimers}$ / Ara h 3	β-conglycinin / glycinin
0:1:1	100 ± 9	100 ± 7
3:1:1	117 ± 0	114 ± 3
7:1:1	94 ± 2	88 ± 3
10:1:1	75 ± 2	66 ± 2

In conclusion, in this study we have shown that Ara h 1 upon extraction from peanuts occurs as an oligomeric complex bearing noncovalent interactions with proanthocyanidins. The distribution of proline residues over the surface of vicilin proteins, to which Ara h 1 belongs, likely explains the propensity of proanthocyanidins to interact with vicilin proteins and form soluble high-molecular weight complexes. The oligomeric structure of such mildly extracted (pH 6.2, I = 0.02) Ara h 1 is considered to be representative for the structure of Ara h 1 in which it is consumed by humans, as consumption of peanuts always occurs after processing and / or mastication. As the interaction of proanthocyanidins with proteins may alter protein characteristics, like digestibility (*2*), the latter one being an important characteristic for allergenic proteins, it would be interesting to investigate those characteristics of Ara h 1 which are important for its allergenic activity (like digestion and heat stability) and compare them to trimeric Ara h 1. The outcomes of these experiments are described in chapter 6.

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REFERENCES

1. Hagerman, A. E., Rice, M. E., Ritchard, N. T. Mechanisms of protein precipitation for two tannins, pentagalloyl glucose and epicatechin$_{16}$ (4 → 8) catechin (procyanidin). *Journal of Agricultural and Food Chemistry* **1998,** 46, (7), 2590-2595.
2. 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.
3. Burks, A. W., Williams, L. W., Helm, R. M., Connaughton, C., Cockrell, G., O'Brien, T. Identification of a major peanut allergen, Ara h 1, in patients with atopic dermatitis and positive peanut challenge. *Journal of Allergy and Clinical Immunology* **1991,** 88, (2), 172-179.
4. Burks, A. W., Cockrell, G., Stanley, J. S., Helm, R. M., Bannon, G. A. Recombinant peanut allergen Ara h 1 expression and IgE binding in patients with peanut hypersensitivity. *Journal of Clinical Investigation* **1995,** 96, (4), 1715-1721.
5. Mills, E. N. C., Jenkins, J. A., Marigheto, N., Belton, P. S., Gunning, A. P., Morris, V. J. Allergens of the cupin superfamily. *Biochemical Society Transactions* **2002,** (6), 30, 925-929.
6. Thanh, V. H., Shibasaki, K. Major proteins of soybean seeds - reversible and irreversible dissociation of beta-conglycinin. *Journal of Agricultural and Food Chemistry* **1979,** 27, (4), 805-809.
7. 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. *Journal of Immunology* **2000,** 164, (11), 5844-5849.
8. 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. *Journal of Agricultural and Food Chemistry* **2006,** 54, (19), 7180-7186.

9. Hagerman, A. E., Tannin-protein interactions. In *Phenolic compounds in food and their effects on health 1. Analysis, occurence and chemistry*, Ho, C. T.; Lee, C. Y.; Huang, M. T., Eds. American Chemical Society: Washington, DC., 1992.

10. Hagerman, A. E., Robbins, C. T. Implications of soluble tannin-protein complexes for tannin analysis and plant defense-mechanisms. *Journal of Chemical Ecology* **1987,** 13, (5), 1243-1259.

11. Talcott, S. T., Passeretti, S., Duncan, C. E., Gorbet, D. W. Polyphenolic content and sensory properties of normal and high oleic acid peanuts. *Food Chemistry* **2005,** 90, (3), 379-388.

12. Gu, L. W., Kelm, M. A., Hammerstone, J. F., Beecher, G., Holden, J., Haytowitz, D., Prior, R. L. Screening of foods containing proanthocyanidins and their structural characterization using LC-MS/MS and thiolytic degradation. *Journal of Agricultural and Food Chemistry* **2003,** 51, (25), 7513-7521.

13. Karchesy, J. J., Hemingway, R. W. Condensed tannins - (4β–>8;2β–>*O*–>7)-linked procyanidins in *arachis-hypogaea* L. *Journal of Agricultural and Food Chemistry* **1986,** 34, (6), 966-970.

14. Prigent, S. V. E., Gruppen, H., Visser, A., van Koningsveld, G. A., de Jong, G. A. H., Voragen, A. G. J. Effects of non-covalent interactions with 5-O-caffeoylquinic acid (chlorogenic acid) on the heat denaturation and solubility of globular proteins. *Journal of Agricultural and Food Chemistry* **2003,** 51, (17), 5088-5095.

15. Koppelman, S. J., Vlooswijk, R. A. A., Knol, E. F., Bruijnzeel-Koomen, C. A., Van Reijsen, F. C., Knippels, L. M., Hessing, M. Quantification of major peanut allergens Ara h 1 and Ara h 2 in peanut varieties Runner, Spanish, Virginia and Valencia, bred in different parts of the world. *Allergy* **2001,** 56, (2), 132-137.

16. Prigent, S. V. E. Interactions of phenolic compounds with globular proteins and their effects on food-related functional properties. Ph.D. thesis, Wageningen University, Wageningen, The Netherlands, 2005.

17. Haslam, E. Natural polyphenols (vegetable tannins) as drugs: Possible modes of action. *Journal of Natural Products* **1996,** 59, (2), 205-215.

18. Kuipers, B. J. H., van Koningsveld, G. A., Alting, A. C., Driehuis, F., Voragen, A. G. J., Gruppen, H. Opposite contributions of glycinin- and beta-conglycinin-derived peptides to the aggregation behavior of soy protein isolate hydrolysates. *Food Biophysics* **2006,** 1, (4), 178-188.

19. Wichers, H. J., De Beijer, T., Savelkoul, H. F. J., van Amerongen, A. The major peanut allergen Ara h 1 and its cleaved-off N-terminal peptide; Possible implications for peanut allergen detection. *Journal of Agricultural and Food Chemistry* **2004,** 52, (15), 4903-4907.

20. 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. *Analytical Biochemistry* **2000,** 285, (1), 64.

21. Porter, L. J., Hrstich, L. N., Chan, B. G. The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin. *Phytochemistry* **1986,** 25, (1), 223-230.

22. Mol, J., Grotewold, E., Koes, R. How genes paint flowers and seeds. *Trends in Plant Science* **1998,** 3, (6), 212-217.

23. Vitale, A., Hinz, G. Sorting of proteins to storage vacuoles: how many mechanisms? *Trends in Plant Science* **2005,** 10, (7), 316-323.

24. Humphrey, S. P., Williamson, R. T. A review of saliva: Normal composition, flow and function. *Journal of Prosthetic Dentistry* **2001,** 85, (2), 162-169.

25. Hagerman, A. E., Butler, L. G. The specificity of proanthocyanidin-protein interactions. *Journal of Biological Chemistry* **1981,** 256, (9), 4494-4497.

26. Lawrence, M. C., Izard, T., Beuchat, M., Blagrove, R. J., Colman, P. M. Structure of phaseolin at 2.2 Angstrom resolution - Implications for a common vicilin/legumin structure and the genetic-engineering of seed storage proteins. *Journal of Molecular Biology* **1994,** 238, (5), 748-776.

27. Murray, N. J., Williamson, M. P., Lilley, T. H., Haslam, E. Study of the interaction between salivary proline-rich proteins and a polyphenol by 1H-NMR spectroscopy. *European Journal of Biochemistry* **1994,** 219, (3), 923-935.

28. Charlton, A. J., Baxter, N. J., Khan, M. L., Moir, A. J. G., Haslam, E., Davies, A. P., Williamson, M. P. Polyphenol/peptide binding and precipitation. *Journal of Agricultural and Food Chemistry* **2002,** 50, (6), 1593-1601.

29. Simon, C., Barathieu, K., Laguerre, M., Schmitter, J. M., Fouquet, E., Pianet, I., Dufourc, E. J. Three-dimensional structure and dynamics of wine tannin-saliva protein complexes. A multitechnique approach. *Biochemistry* **2003,** 42, (35), 10385-10395.

30. Barre, A., Borges, J. P., Rouge, P. Molecular modelling of the major peanut allergen Ara h 1 and other homotrimeric allergens of the cupin superfamily: a structural basis for their IgE-binding cross-reactivity. *Biochimie* **2005,** 87, (6), 499-506.

31. Thanh, V. H., Shibasaki, K. Major proteins of soybean seeds - straightforward fractionation and their characterization. *Journal of Agricultural and Food Chemistry* **1976,** 24, (6), 1117-1121.

32. Lakemond, C. M. M., de Jongh, H. H. J., Hessing, M., Gruppen, H., Voragen, A. G. J. Soy glycinin: Influence of pH and ionic strength on solubility and molecular structure at ambient temperatures. *Journal of Agricultural and Food Chemistry* **2000,** 48, (6), 1985-1990.

33. Adachi, M., Takenaka, Y., Gidamis, A. B., Mikami, B., Utsumi, S. Crystal structure of soybean proglycinin alaB1b homotrimer. *Journal of Molecular Biology* **2001,** 305, (2), 291-305.

6

DETERMINATION OF PEPSIN-SUSCEPTIBLE AND PEPSIN-RESISTANT EPITOPES IN NATIVE AND HEAT-TREATED PEANUT ALLERGEN ARA H 1

Based on: van Boxtel, E. L., van den Broek, L. A. M., Koppelman, S. J., Gruppen, H. Determination of pepsin-susceptible and pepsin-resistant epitopes of native and heat-treated peanut allergen Ara h 1. *Submitted*

ABSTRACT

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 pre-heated, 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 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 neither be 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.

INTRODUCTION

The resistance of the IgE binding capacity of allergens towards digestion in the human gastro-intestinal tract is considered a prerequisite for food allergens to cause (severe) systemic reactions in allergic individuals. This resistance towards digestion implies that allergens have to withstand the low pH and pepsin activity in the stomach in a way that either the intact allergen or peptides containing IgE epitopes are preserved, as 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 long (*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 responsible for the induction of systemic, often severe, food allergic reactions.

The digestibility of proteins can be affected by various factors, like 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 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).

Table 1: Previously identified epitopes on the amino acid sequence of Ara h 1 (P43238).

Epitope number	**Amino acid sequence**	**Amino acids 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*	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*)

*: E = G in amino acid sequence Ara h 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 (chapter 4; *10*). As is described in chapter 5, the oligomerization of Ara h 1 is supposed to be caused by noncovalent 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 (chapter 7; *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 literature.
As epitopes remaining upon gastro-intestinal 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 being 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 *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 (chapter 5; *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, USA), 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 (chapter 5; *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, USA).
Plasma from 5 patients with allergy for peanuts (obtained from Plasmalab International, Everett, WA, USA) and serum from 1 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 ratios, denoted Plasmapool and used for IgE dotblotting experiments.

CONCENTRATION

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

PROTEIN QUANTIFICATION

The nitrogen content of samples was determined using the combustion (Dumas) method on a 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 number 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, USA). Thermograms were recorded from 20 to 120 °C with a heating rate of 1 °C per minute. 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 M 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 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 afterwards were used directly, whereas for the other investigations samples were centrifuged at 22,000*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 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 acetonitrile (ACN), containing 2% (v/v) trifluoroacetic acid (TFA). After mixing at 1,000 r.p.m. for 1 h at ambient temperature in an Eppendorf mixer (Eppendorf AG, Hamburg, Germany), the samples were centrifuged (10 min, 22,000*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 / IONISATION-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 500-4,000, m/z 3,000-7,000, and m/z 3,000-12,000. 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-cyano-hydroxycinnamic 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 until a final concentration of 5% (v/v) and 0.1% (v/v), respectively. Afterwards, samples (50 μL) were applied onto a Vydac C18 MS column (C18MS52; 250 × 2.1 mm; GraceVydac, Hesperia, CA, USA), installed on a Spectra System HPLC (Thermo Separation Products, Fremont, CA, USA), 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 till 80 min, a linear gradient from 45 to 95% B till 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 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, USA) 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, USA) was used for automatic sequencing and database search for the sequences in a database containing the sequence of Ara h 1 (Swissprot accession number 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 treshold 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 DOTBLOTTING EXPERIMENTS

For IgE dotblotting protein samples in 30% (v/v) ACN, 0.1% (v/v) TFA (10 μL) were applied to polyvinyldifluoride (PVDF) sheets (Bio-rad Laboratories Inc., Hercules, CA, USA, 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 BCIP/NBT liquid substrate (Sigma, article B1911).

CHYMOTRYPTIC HYDROLYSIS

Pooled SEC (under denaturing conditions) fractions of peptic hydrolysates were lyophilized. Next, 80 µL 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) solution in 100 mM Tris-HCl buffer, pH 7.8, containing 10 mM $CaCl_2$ was added and 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 loading them on a pre-washed 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, USA) 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 heat stability of the two quaternary forms of Ara h 1 was investigated in order to compare their behaviour upon heating. The DSC experiments showed that the denaturation temperatures of Ara h $1_{oligomers}$ and Ara h $1_{trimers}$ were similar at an ionic strength of 0.02 M and of 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. 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 likely explains the difference in denaturation temperatures observed. When heating Ara h $1_{oligomers}$ and Ara h $1_{trimers}$ at temperatures ranging from 75 to 95 °C at pH 6.2 and analyzing the supernatants 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 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 $1_{trimers}$.

DIGESTION
Unheated and heated Ara h $1_{oligomers}$ and Ara h $1_{trimers}$ were subjected to peptic digestion. Afterwards, 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 digestion of Ara h $1_{oligomers}$ and Ara h $1_{trimers}$ resulted in a similar degradation pattern, i.e. the formation of peptides of comparable sizes. At all 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 pre-heated before pepsin incubation. These results thus indicate that Ara h $1_{trimers}$ were faster hydrolyzed by pepsin compared with Ara h $1_{oligomers}$, regardless of pre-heating the protein preparations. In order 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 underlined our observation: After 2 hours 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 (chapter 7; *14, 15, 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%.

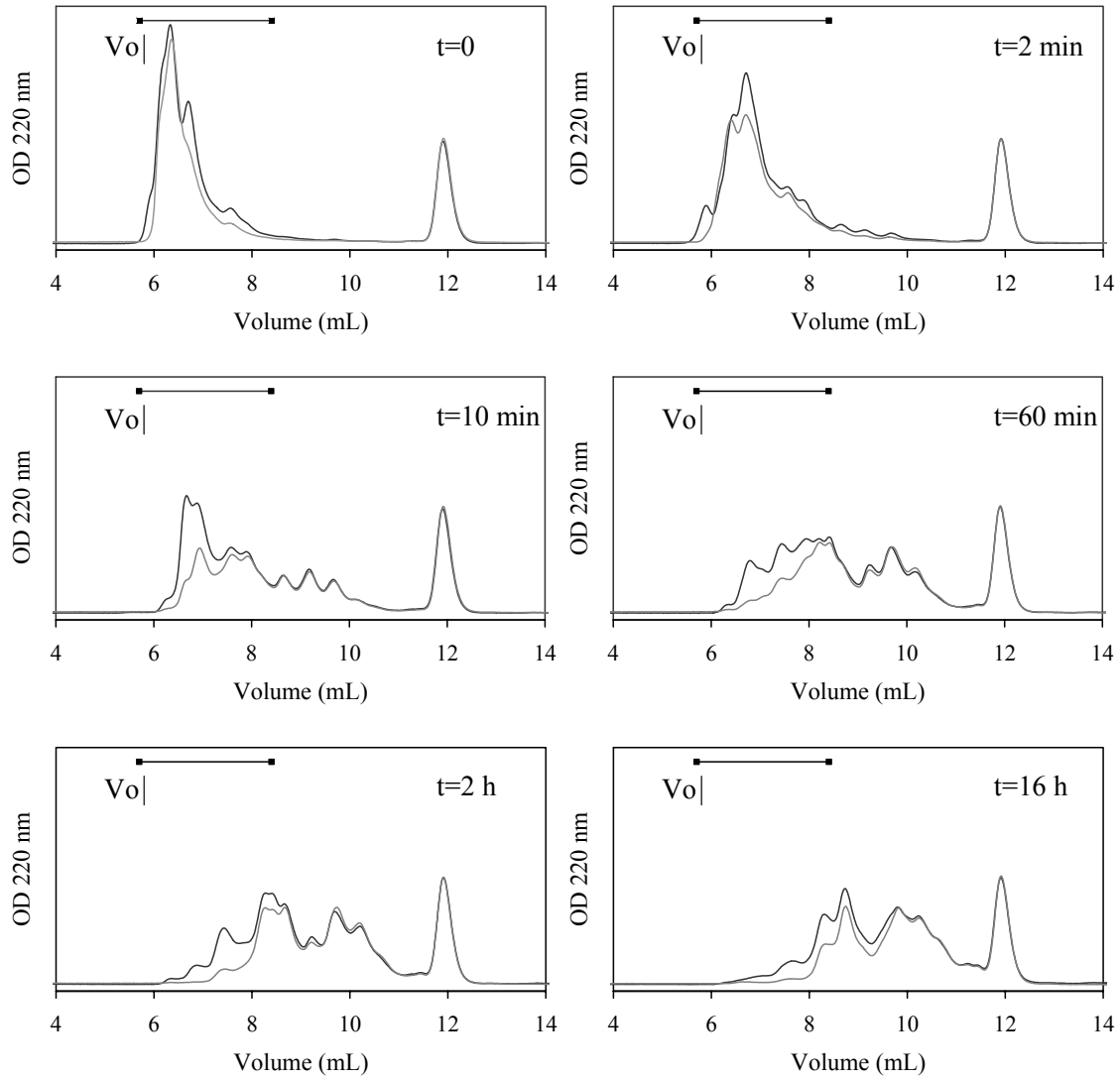

Figure 1: Size exclusion chromatograms (under denaturing conditions) of peptic hydrolysates of unheated Ara h $1_{oligomers}$ (black lines) and Ara h $1_{trimers}$ (gray lines). The horizontal black lines indicate the area at which at t=0 all UV absorbance was measured. Indicated is the void volume of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

Table 2: Proportion (%) of hydrolysates of Ara h $1_{oligomers}$ and Ara h $1_{trimers}$ present as relatively large molecular mass peptides*.

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

* indicated is the proportion (%) of the total absorption (at 220 nm) eluting between 5.7 and 8.4 mL in the chromatograms, as indicated in Figure 1

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 dotblotting. It was chosen to perform dotblotting 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 dotblotting the samples could be solubilized before being dried on the PVDF membrane, by adding ACN and TFA till 30 and 0.1% (v/v), respectively.

Although the IgE binding was the highest in the non-digested 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 (chapter 7; *13-15*).

In order 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 as an endpoint 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 peak A, B, and C, respectively. The dotblotting 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 dotblot, 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 dotblot, implying that these fractions did not bind IgE and thus did not contain peptides bearing intact epitopes. The color observed on dotblot 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 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.

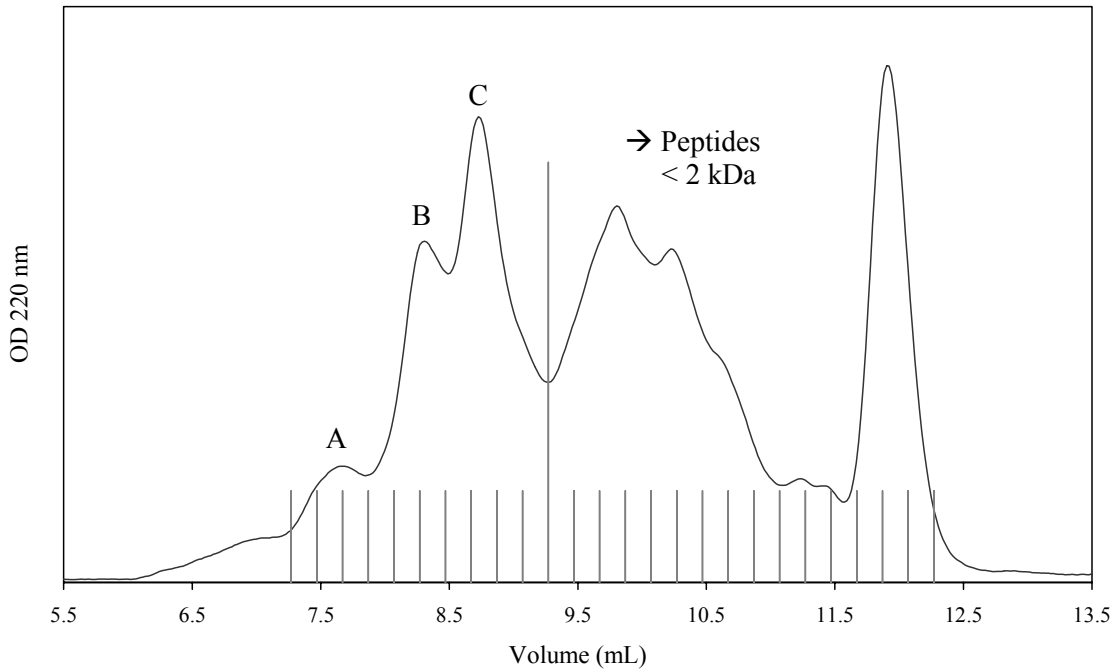

Figure 2: Size exclusion chromatogram (under denaturing conditions) of unheated Ara h $1_{oligomers}$ after peptic hydrolysis during 16 h. Black lines: OD at 220 nm, gray lines: collected fractions (1-25; even fraction numbers indicated). Indicated is the void volume of the column (Vo).

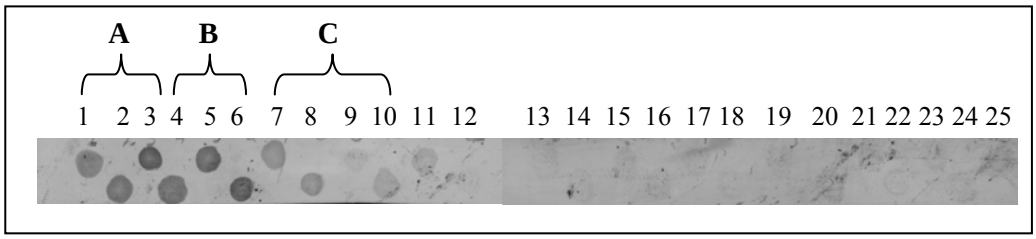

Figure 3: Dotblot of fractions obtained from size exclusion chromatography, as indicated in Figure 2.

IDENTIFICATION OF PEPSIN-SUSCEPTIBLE EPITOPES

In order 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 (data not shown), 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.

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*).

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.**	**epitopes****	**Peptide a.a. sequence***	**a.a.**	**epitopes****
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

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

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 peak A, B, and C, respectively. MALDI-TOF MS analyses showed for SEC peak A two broad peaks between approximately 8 and 9 kDa (data not shown), indicating the presence of two or a few peptides. MALDI-TOF MS analyses (data not shown) of SEC peaks B and C showed that these peaks contained several peptides with masses <6.5 kDa and <5 kDa, respectively. RP-HPLC analysis showed for SEC peak A one 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 to their sizes (*25*). Consequently, it seemed likely that the peptides in this peak consisted of largely the same amino acid sequence and only differed 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.

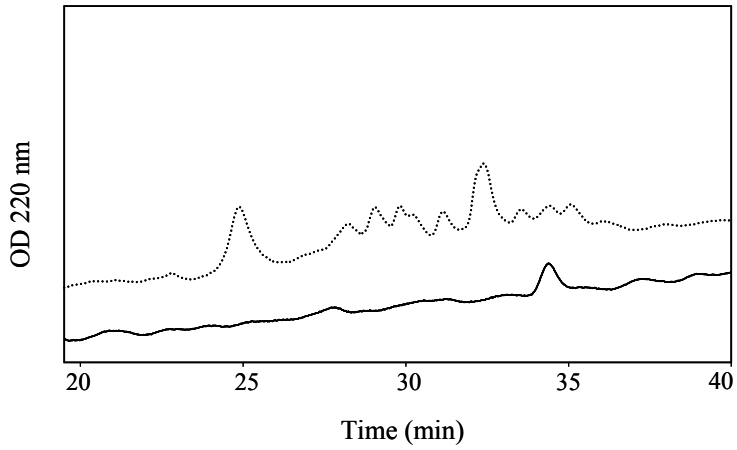

Figure 5: RP-HPLC chromatograms of SEC peak A (solid line) and SEC peak B (dashed line), as indicated in Figure 2.

Peptides in SEC peaks A, B, and C were too large to be analyzed directly on RP-HPLC-MS. Therefore, in order 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 afterwards, 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, based on 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 8,779.6 Da), while amino acid 111 could be the C-terminal of the smaller peptide (resulting in a peptide mass of 8,235.0 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. Based on 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 towards the amino acid sequence 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).

Table 4: Identified chymotryptic peptides in SEC peaks A, B, and C (Figure 2) showing coverage with the amino acid sequence of Ara h 1 (P43238) and their deduced peptic peptides.

Identified peptides	**a.a.***	**N-term. c.t. site****	**Deduced N-terminally extended peptic peptides**		**C-term. c.t. site****	**Deduced C-terminally extended peptic peptides**	
			a.a.	**e *****		**a.a.**	**e *****
Peak A, max ~9 kDa:							
WRQPREDW	45-52	-	[a]	[b]	+	45-115	5, 6[c]
Peak B, max ~6.5 kDa:							
RQPREDW	46-52	+	1-52	1-4	+	46-98	5, 6
GTPGSHVREETSRNNPF	75-91	-	[a]	[b]	+	75-128	[b]
VREETSRNNPF	81-91	+	40-91	4-6	+	81-132	[b]
SRNNPF	86-91	-	[a]	[b]	+	86-138	[b]
KVSKEHVEEL	283-292	-	[a]	[b]	+	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	-	[a]	[b]	-	[a]	[b]
VREETSRNNPF	81-91	+	51-91	6	+	81-120:	[b]
KVSKEHVEEL	283-292	-	[a]	[b]	+	283-326	11
NNEGVIVKVSKEHVEEL	276-292	-	[a]	[b]	+	276-320	11
NEGVIVKVSKEHVEEL	277-292	-	[a]	[b]	+	277-321	11
GVIVKVSKEHVEEL	279-292	-	[a]	[b]	+	279-323	11
VIVKVSKEHVEEL	280-292	-	[a]	[b]	+	280-324	11
VIVKVSKEHVEELTKH	280-295	-	[a]	[b]	+	280-324	11
IVKVSKEHVEEL	281-292	-	[a]	[b]	+	281-325	11
VKVSKEHVEEL	282-292	-	[a]	[b]	+	282-326	11
GSEEEGDITNPINL	303-316	-	[a]	[b]	+	303-346	11
LDMMLTCVEIK	345-355	-	[a]	[b]	-	[a]	[b]
IKNQKESHF	390-398	+	390-430	14	+	355-398	13

*: a.a.: Coverage with amino acids of the sequence of Ara h 1 (P43238)

**: Indicated is whether the N-terminal and C-terminal ends of the identified chymotryptic peptides were theoretical chymotrypsin cleavage sites. +: theoretical chymotrypsin cleavage site (C-terminal of phenylalanine (F), tyrosine (Y), thryptophan (W), methionine (M), and leucine (L)); -: no theoretical chymotrypsin cleavage site

***: 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. [a]: no N- or C-terminally extended, deduced peptic peptide possible; [b]: no intact epitopes present on deduced peptic peptides

[c]: intactness of epitopes was confirmed by the results from N-terminal sequencing

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 to 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 unlikely.

In SEC peak C fourteen 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, 3, 4, 5, 6, 11, 13 and 14 (Table 1). As was previously mentioned, IgE binding to SEC peak C was considered to be caused by co-elution of peptides from SEC peak B. Consequently, the IgE binding in SEC peak C was assumed to be caused only by those epitopes which were also denoted to be possibly present 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.

Altogether, 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 neither 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, like 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 (data not shown). 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.

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REFERENCES

1. Astwood, J. D., Leach, J. N., Fuchs, R. L. Stability of food allergens to digestion *in vitro*. *Nature Biotechnology* **1996,** 14, (10), 1269-1273.
2. Huby, R. D. J., Dearman, R. J., Kimber, I. Why are some proteins allergens? *Toxicological Sciences* **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. *Journal of Allergy and Clinical Immunology* **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 Chemistry* **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. *Journal of Allergy and Clinical Immunology* **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. *European Journal of Biochemistry* **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. *Clinical and Experimental 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. *Journal of Allergy and Clinical Immunology* **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. *Journal of Agricultural and Food Chemistry* **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. *Journal of Agricultural and Food Chemistry* **2007**, 55, (21), 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. *Journal of Biological Chemistry* **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. *Journal of Immunology* **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. *Clinical and Experimental 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. *Molecular Nutrition and Food Research* 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. *Analytical Biochemistry* **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. *Nature Biotechnology* **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. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **2005,** 824, (1-2), 189-200.
19. Ilse, D., Edman, P. Formation of 3-phenyl-2-thiohydantoins from phenylthiocarbamyl amino acids. *Australian Journal of Chemistry* **1963,** 16, (3), 411-416.
20. Edman, P. On the mechanism of the phenyl isothiocyanate degradation of peptides. *Acta Chemica Scandinavica* **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. *Journal of Biological Chemistry* **1981,** 256, (15), 7990-7997.
22. Untersmayr, E., Jensen-Jarolim, E. The effect of gastric digestion on food allergy. *Current Opinion in Allergy and Clinical Immunology* **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 Journal* **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. *Journal of Agricultural and Food Chemistry* **2002,** 50, (24), 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. *International Dairy Journal* **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. *Journal of Agricultural and Food Chemistry* **2007,** accepted.
27. Keil, B., *Specificity of proteolysis*. Springer-Verlag: Berlin, 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. *Journal of Biological Chemistry* **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. *Biochemical Journal* **1988,** 251, (3), 717-726.
30. O'Kane, F. E., Happe, R. P., Vereijken, J. M., Gruppen, H., van Boekel, M. A. J. S. Characterization of pea vicilin. 1. Denoting convicilin as the alpha-subunit of the *Pisum* vicilin family. *Journal of Agricultural and Food Chemistry* **2004,** 52, (10), 3141-3148.

7

LEGUMIN ALLERGENS FROM PEANUTS AND SOYBEANS - EFFECTS OF HEATING AND DIGESTION ON IgE BINDING

Based on: 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. *Molecular Nutrition and Food Research* in press.

ABSTRACT

Legumin proteins Ara h 3 from peanuts and glycinin from soybeans are increasingly described as important allergens. The stability of an allergen's IgE binding capacity towards heating and digestion is considered an important characteristic for food allergens. We investigated the effects of heating and digestion on the IgE binding of Ara h 3 and glycinin. Both proteins are relatively stable to denaturation, having denaturation temperatures ranging from 70 to 92 °C, depending on their quaternary structure and the ionic strength applied. Aggregates were formed upon heating, which were partly soluble for glycinin. Heating slightly decreased the pepsin digestion rate of both allergens. However, under the *in vitro* conditions applied, heating did not affect the IgE binding capacity of the hydrolysates, as after only 10 min of hydrolysis no IgE binding could be detected any more in all samples. Peanut allergen Ara h 1, when digested under equal conditions, still showed IgE binding after 2 hours of hydrolysis. Our results indicate that the IgE binding capacity of legumin allergens from peanuts and soybeans does not withstand peptic digestion. Consequently, these allergens are likely unable to sensitize via the gastro-intestinal tract and cause systemic food allergic reactions. In view of these systemic food allergic reactions, these proteins might thus be less important allergens than was previously assumed.

INTRODUCTION

Soybeans and peanuts belong to the eight most significant allergenic foods. Peanut allergy has been well studied and often causes acute and severe reactions. The prevalence and characteristics of soybean allergy have been less well studied, but allergy to soybeans is assumed not to cause severe reactions (*1*). Besides, not that many peanut allergic patients also suffer from clinically relevant allergies to soybeans (*2, 3*).

The most abundant protein in soybeans is glycinin, which belongs to the legumin protein family and comprises 25 to 35% of all proteins in soybeans (*4*). Glycinin is described as a *major* allergen (*5*). The homologous legumin protein in peanuts is denoted Ara h 3 and is highly abundant (*6*). Ara h 3 has been designated both a *major* and a *minor* allergen, depending on the study population, as it was recognized by serum IgE of approximately 44%, 53%, and 77% of peanut allergic patient populations, respectively (*7-9*).

Legumin proteins are approximately 60 kDa in molecular mass and consist of a basic (approximately 20 kDa) and an acidic polypeptide (approximately 40 kDa), linked together by a disulfide bridge. The acidic polypeptide of Ara h 3, in contrast to soy glycinin, is extensively post-translationally processed, yielding peptides with molecular masses between 13 and 45 kDa (*6*). Legumin proteins associate *in planta* into hexamers, resulting in a molecular mass of ~360 kDa (*10*). However, the quaternary structure of legumins after extraction is dependent on the ionic strength. At an intermediate ionic strength (I = 0.2 M) Ara h 3 is partially present as trimers (*6*), while glycinin shows the same behaviour at a lower ionic strength (I = 0.03 M). The dissociation of glycinin induces an increase of non-structured protein at secondary and tertiary level (*11*). For Ara h 3 the effects of dissociation on the secondary and tertiary structure are not known.

For glycinin six genes have been identified, representing glycinin G1 to G5, and G7. For Ara h 3 also several genes are known (Swissprot accession numbers O82580, Q9SQH7, Q647H4, Q8LKN1, and Q6IWG5). IgE binding to glycinin has been reported to occur to both the acidic (*12-14*) and the basic (*5*) polypeptides of glycinin. For Ara h 3 four IgE binding epitopes have been identified, all situated on the acidic polypeptide of the allergen (*7*). In addition, IgE binding to the basic polypeptide of Ara h 3 has been reported (*6, 14*), indicating the presence of additional IgE binding epitopes.
Although clinically relevant cross-reactivity between peanuts and soybeans is not very common, *in vitro* IgE cross-reactivity between soybeans and peanuts has been reported several times (*2, 15*). This could be caused by the sequence homology between soybean and peanut proteins. For example, two epitopes on the acidic chain of glycinin G1 are homologous to two epitopes on Ara h 3 (*13*) and one epitope on the basic polypeptide of glycinin G2 is highly conserved in both Ara h 3 and glycinin G1 (*14*).
Food allergens are believed to only be able to sensitize and exert systemic allergic reactions after passing through the gastro-intestinal tract, as they can be absorbed when they have reached the intestinal mucosa. Thus, in order to cause systemic symptoms, protein fragments remaining after pepsin digestion should still be able to bind IgE. Therefore, a factor that is considered to be important for the allergenicity of food proteins is the resistance of their IgE binding capacity upon digestion in the gastro-intestinal tract. Another factor is the stability upon cooking and industrial processing, as most foods are being consumed after some kind of processing. Accordingly, information about the effects of heating and digestion on the IgE binding of food allergens is necessary to determine the allergenic potential of these proteins. Several food allergens have, therefore, been investigated for their stability to heat and digestion. For example, Ara h 1, one of the *major* allergens from peanuts, is still able to bind IgE after digestion (*16*).
The IgE binding capacity of soy glycinin is reported not to be affected by heating (*17*), while sequential hydrolysis by pepsin and chymotrypsin is reported to result in peptides of 20 kDa and smaller, with a 20 kDa peptide still being able to bind IgE (*18*). The effects of heating and digestion on the IgE binding capacity of Ara h 3 have not been investigated yet. Our aim was to investigate these properties of Ara h 3 and soy glycinin and to compare the behaviour of both allergens. As digestibility is dependent on the pepsin concentration and activity used, we also subjected unheated peanut allergen Ara h 1, belonging to the homologous vicilin protein family, to the same pepsin concentrations, in order to validate our results. The results provide insights into the contribution of Ara h 3 and glycinin to the systemic allergic symptoms of peanut allergy and soybean allergy, respectively.

MATERIALS AND METHODS

MATERIALS

All chemicals were obtained from Merck (Darmstadt, Germany) or Sigma (Sigma-Aldrich Inc., St. Louis, MO, USA), unless stated otherwise. Peanuts of the Runner market-type were generously provided by Imko Nut Products (Doetinchem, The

Netherlands) and were stored at 4 °C until use. Glycinin was purified from Hyland soybeans as described by Kuipers and co-workers (*19*). (Oligomeric) Ara h 1 was purified from peanuts as described in chapter 4 (*20*), with the exception that extraction was performed at pH 6.2. The ionic strengths of Ara h 1, Ara h 3 and glycinin (~4 $\times 10^{-8}$ – ~8 $\times 10^{-8}$ M) in distilled water were arbitrary set at 0 M.
Plasma from 5 patients with allergy for peanuts (purchased from Plasmalab International, Everett, WA, USA) and serum from 1 patient allergic to peanuts were used in this study. Clinical characteristics consisted of a questionnaire filled in by the patients. All patients indicated that they suffered from peanut allergy. Besides, two patients clearly indicated that they suffered from allergic reactions upon eating soybean products (with symptoms like sore thoat, itchy mouth, stomach and intestinal upset). CAP-FEIA levels specific for peanuts were between 45 and >100 kU L^{-1} and for soybeans between 3 and 75 kU L^{-1}. Part of the plasma and serum samples were mixed in equal ratios, denoted Plasmapool and used for IgE immunoblotting and dotblotting experiments.

ARA H 3 PURIFICATION

For the purification of Ara h 3, defatted peanuts were extracted in 50 mM Tris-HCl buffer, pH 8.2, at a meal solvent ratio of 1:10 (w/v), during one h at room temperature under continuous stirring. Afterwards, the extract was sieved through cheese-cloth and subsequently centrifuged (25 min; 14,000*g*; 4 °C). The supernatant obtained was filtered over a 1.2 μm filter and subsequently applied (13 mL applied per run) onto a 320 mL Superdex 200 XK 26/60 column (GE Healthcare, Uppsala, Sweden). The column was equilibrated and eluted with 50 mM Tris-HCl buffer, pH 8.2, at a flow rate of 4.3 mL min^{-1}, using an Äkta Purifier system (GE Healthcare) operated at room temperature. The eluate was monitored at 280 and 325 nm and fractions containing Ara h 3, as analyzed by SDS-PAGE, were collected and applied onto a 1.2 L Source Q Fineline column, using an Äkta Explorer system (GE Healthcare). The column was equilibrated with 50 mM Tris-HCl buffer, pH 8.2, and the flow rate used was 40 mL min^{-1}. After sample application and washing, a 10 column volumes linear gradient from 0.1 to 1 M NaCl in 50 mM Tris-HCl buffer, pH 8.2, was applied. The eluate was monitored at 280 nm and samples containing pure Ara h 3, as analyzed with SDS-PAGE, were collected and pooled. The pooled fractions were dialysed in 10,000 MWCO dialysis tubings (Medicell Int. Ltd., London, UK) against distilled water. Afterwards, samples were lyophilized and stored at -20 °C until use.

SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Protein samples were analyzed using a mini-protean II system (Bio-rad laboratories Inc., Hercules, CA, USA) according to the instructions of the supplier. Reducing conditions were obtained by adding β-mercaptoethanol to a final concentration of 10 mM and heating the samples for 5 min at 100 °C. Tris-Tricine 16.5% Ready Gels (Bio-Rad) were used. Gels were stained according to the Coomassie Brilliant Blue procedure provided by the manufacturer. A polypeptide and a prestained protein molecular weight marker (article 161-0326 and 161-0373, respectively; Bio-Rad) were used for calibration.

PROTEIN QUANTIFICATION

The nitrogen content of samples was determined using the combustion (Dumas) method on a NA 2100 Nitrogen and Protein Analyzer (CE instruments, Milan, Italy) using methionine as a standard. Protein conversion factors of 5.27 for Ara h 3 and 5.57 for soy glycinin were calculated from their amino acid sequences (Swissprot accession number O82580 for Ara h 3 and accession numbers P04776, P04405, P11828, P02858 and P04347 for glycinin).

HEATING EXPERIMENTS

Ara h 3 and glycinin solubilized (2.5 mg mL^{-1}) in 35 mM sodium phosphate buffer, pH 7.6, containing 0.1 M NaCl (I = 0.2 M), were heated at 100 °C for 10 min. After heating, samples were cooled immediately on ice. Samples that were subjected to pepsin digestion afterwards were used directly, while for other investigations, samples were centrifuged at 22,000*g* for 5 min at 4 °C, after which the supernatant was collected.

DIFFERENTIAL SCANNING CALORIMETRY (DSC)

DSC experiments were performed on a VP-DSC Microcalorimeter (MicroCal Inc., Northampton, MA, USA). Thermograms were recorded from 20 to 120 °C with a heating rate of 1 °C per minute. Experiments were conducted with protein samples solubilized (5 mg mL^{-1}) in 35 mM sodium phosphate buffer, pH 7.6, (I = 0.03) with or without the addition of 0.1 M NaCl (I = 0.2 M).

SIZE EXCLUSION CHROMATOGRAPHY (SEC) UNDER NON-REDUCING AND NON-DENATURING CONDITIONS

Protein samples (100 µL) were applied onto a Superose 6 10/300 column (GE Healthcare). The column was equilibrated and eluted with 10 mM sodium phosphate buffer, pH 7.6, with or without the addition of 0.1 M NaCl, at a flow rate of 0.5 mL min^{-1}, using an Äkta Purifier system operated at room temperature. The eluate was monitored at 280 nm.

SEC UNDER REDUCING AND DENATURING CONDITIONS

Guanidinium chloride (6 M) was added to protein samples. Next, samples were continuously stirred for 1 h at ambient temperature. Subsequently, dithiothreitol (DTT) was added to a final concentration of 50 mM and samples were continously stirred for 1 h at room temperature. Afterwards, acetonitrile (ACN) and trifluoroacetic acid (TFA) were added to a final concentration of 30% (v/v) ACN and 0.1% (v/v) TFA. After stirring for 1 h at room temperature, samples were centrifuged (10 min, 22,000*g*, 20 °C). 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. 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 eluates was measured at 220 nm.

IN VITRO DIGESTION EXPERIMENTS

The pH of 2.5 mg mL^{-1} protein solutions in 35 mM sodium phosphate buffer pH 7.6, containing 0.1 M NaCl (I= 0.2 M), was adjusted to 2.0 with 2 M HCl. Next, samples were mixed 1:1 with 5 μg mL^{-1} pepsin (Sigma, article P6887) 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 2 M NaOH.

IgE IMMUNOBLOTTING AND DOTBLOTTING

For IgE immunoblotting proteins separated after SDS-PAGE were transferred to polyvinyldifluoride (PVDF) sheets (Bio-Rad, article 162-0177) using standard techniques. Samples for dotblotting (10 μL) were applied to the same PVDF sheets. Afterwards, the sheets were air-dried at 30 °C. Membranes were blocked with 3% (w/v) BSA 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 30 times diluted Plasmapool. After washing with washing buffer (TBS containing 0.5% (w/v) BSA and 0.1% (w/v) Tween-20) 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 BCIP/NBT liquid substrate (Sigma, article B1911).

RESULTS AND DISCUSSION

Legumin proteins from soybeans and peanuts are increasingly reported as important food allergens (*5, 7-9*). The withstanding of the IgE binding capacity of food allergens after (heating and) digestion appears to be a prerequisite for allergens to exert systemic food allergic reactions. We therefore investigated the effects of heating and pepsin digestion on the stability and IgE binding of Ara h 3 and glycinin, the major seed proteins from peanuts and soybeans, respectively.

HEAT STABILITY AND QUATERNARY STRUCTURE OF LEGUMIN PROTEINS

Both Ara h 3 and glycinin were subjected to DSC analysis in order to determine the transition temperatures of these proteins. The data are given in Table 1. At an ionic strength of 0.2 M for glycinin a single transition was observed with a transition temperature of approximately 85 °C. This transition temperature corresponds well with the earlier reported transition temperature of 86 °C for glycinin under the same conditions (*21*). For Ara h 3 two transitions were observed with temperatures of approximately 77 and 92 °C. At a low ionic strength (I = 0.03 M) Ara h 3 also showed two transitions with transition temperatures comparable to those at I = 0.2 M. For glycinin at a low ionic strength a peak with a maximum around 78 °C was observed with a shoulder peak with a maximum around 70 °C, also pointing towards two transitions. All transitions were irreversible, as in second heating scans no transitions were observed (no further data shown).

Table 1: Transition temperatures (°C) of Ara h 3 and glycinin trimers and hexamers at pH 7.6, with standarddeviations, at different ionic strengths.

	Ara h 3		**Glycinin**	
Ionic strength (I)	Transition of trimers	Transition of hexamers	Transition of trimers	Transition of hexamers
0.03	78.3 ± 0.2	90.7 ± 0.5	~70.5[a]	77.7 ± 0.1
0.2	76.6 ± 0.1	92.3 ± 0.1	-	85.2 ± 0.4

[a]: no standarddeviation could be calculated, because the transition peak was a shoulder peak
-: no transition of trimers was detected

The two transitions observed for Ara h 3 and glycinin point towards the proteins being present in two different forms, with different transition temperatures. It has been reported that purified Ara h 3 occurs mostly as hexamers, but also partly as trimers at I = 0.2 M (*6*), while glycinin under these conditions occurs solely as hexamers (*11*). SEC analysis confirmed this, as can be seen in Figure 1. Ara h 3 at I = 0.2 M occurs partly as trimers and partly as hexamers, while glycinin occurs mainly in its hexameric form and partly as aggregates with a high molecular mass, the latter probably being induced during lyophilization (*6*). SEC analysis at a lower ionic strength caused a shift in the elution volumes of legumin hexamers and legumin trimers as a result of differences in the ionic strength. Taking this into account, it was clearly observed that at a low ionic strength (I = 0.03 M) relatively more trimeric Ara h 3 was present compared to the high ionic strength. Besides, at a low ionic strength glycinin also partly occurred in a trimeric form, which corresponds with literature data (*11*). Thus, the transitions observed for both proteins are consistent with their dissociation behaviour. Trimeric glycinin denatures at a lower temperature than hexameric glycinin (*21*). Considering the homology between Ara h 3 and glycinin it seems obvious that the lowest denaturation temperature measured for Ara h 3 is the denaturation temperature of the trimeric form of the protein, while the highest transition temperature is the denaturation temperature of the protein in its hexameric form.

From these results it can be concluded that Ara h 3 differs from glycinin in that at higher ionic strengths this protein also partly occurs as trimers. Besides, Ara h 3 hexamers and trimers denature at higher temperatures than glycinin hexamers and trimers, indicating that Ara h 3 is more stable to heat processing than glycinin.

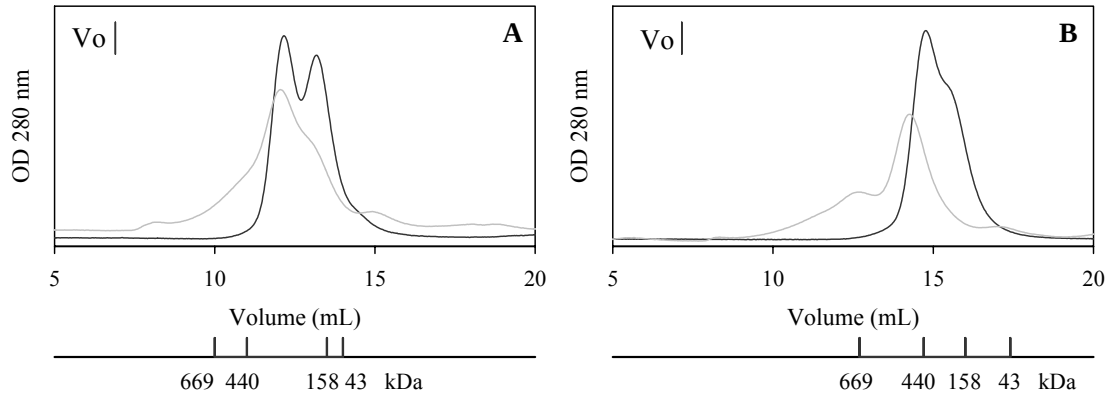

Figure 1: Size exclusion chromatograms of Ara h 3 (black lines) and glycinin (gray lines) at (A) I = 0.03 M and (B) I = 0.2 M. Indicated is the void volume of the column (Vo).

Upon subjecting Ara h 3 and glycinin to heating above their transition temperatures and analyzing the soluble part on SEC (Figure 2) it appeared that Ara h 3 solely formed insoluble aggregates, as no peaks were observed in the chromatogram. Dumas analysis confirmed the absence of protein in the supernatant. The heated glycinin showed a peak eluting just after the void volume of the column, which indicates the formation of soluble aggregates. Furthermore, a peak eluting after elution volumes higher than 15 mL was observed, which indicates the presence of dissociated polypeptides. These observations agree with the work of Mori and co-workers (*22*), who describe the formation of soluble aggregates upon heating and the dissociation of polypeptides from the glycinin complex, with the basic polypeptides precipitating upon heating. In our experiment, part of the protein had indeed become insoluble after heating: Dumas analyses showed that 45% of the protein turned insoluble. This insolubility is likely mainly caused by the insolubilization of the basic polypeptides upon heating (*22*).
Thus, despite their homology, Ara h 3 and glycinin differ in their aggregation behaviour, as glycinin under the conditions applied formed (partly) soluble aggregates, while Ara h 3 only formed insoluble precipitates. Furthermore, the dissociation behaviour of glycinin upon heating was not observed for Ara h 3.

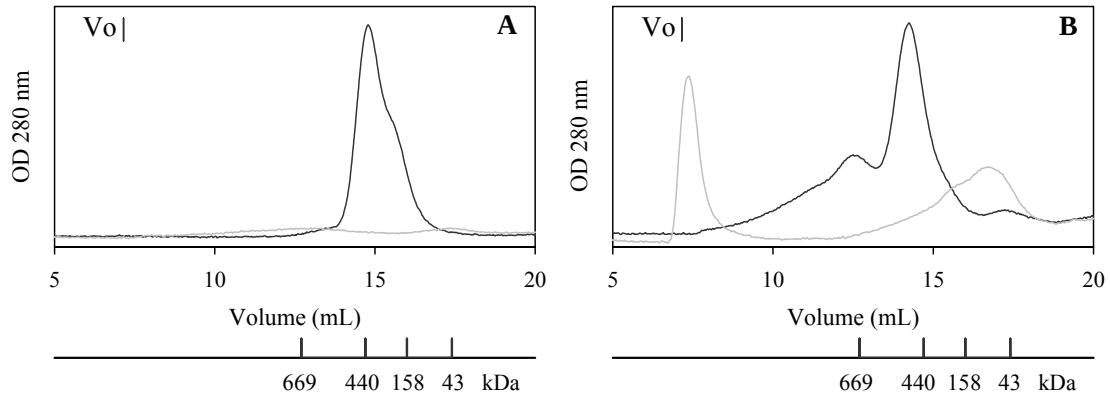

Figure 2: Size exclusion chromatograms of (A) Ara h 3 and (B) glycinin, before (black lines) and after (gray lines) heating at 100 °C at 0.2 M. Indicated is the void volume of the column (Vo).

DIGESTION

An important characteristic of food allergens is their stability to digestion, i.e. the withstanding of IgE binding after pepsin digestion. Pepsin digestibility is often studied for food allergens under stomach conditions (pH 2.0 and 37 °C). The ratios of pepsin to protein concentration that are commonly used in nutritional studies, as well as in studies concerning the effects of proteolytic digestion on protein allergenicity, generally range between 0.1-0.001 (w/w) (*23*). We investigated the digestibility of Ara h 3 and glycinin before and after heating and used a pepsin to protein concentration ratio of 0.002 (w/w). Peanut allergen Ara h 1 was digested under the same conditions, in order to verify if, under the conditions applied, this allergen would still be IgE reactive after digestion, as is described in chapter 6 and in literature (*16*).

The parental proteins and the hydrolysates obtained upon peptic digestion were analyzed on SEC under denaturing and reducing conditions, as shown in Figures 3, 4, and 5. As it can be seen, at t=0 the unheated and heated Ara h 3 and glycinin preparations showed comparable elution patterns, with two large peaks eluting directly after the void volume of the column. The peaks are comparable to the ones corresponding to the basic and acidic polypeptides of glycinin when analyzed on a similar column (*24*). The small peak eluting at approximately 7.5 mL probably represents degradation products or smaller parts of the protein remaining after post-translational processing (*6*).

After only 10 minutes of pepsin hydrolysis for Ara h 3 almost all protein had been degraded into peptides. The SEC pattern after 120 minutes of hydrolysis, which is an average gastric transit time (*25*), was similar to the SEC pattern after 60 minutes of hydrolysis, indicating that the peptides remaining after 60 minutes of hydrolysis were persistent.

After 10 minutes of hydrolysis heated Ara h 3 contained a larger proportion of relatively high molecular mass peptides than unheated Ara h 3. These results indicate that heating of Ara h 3 decreases the rate of pepsin hydrolysis. However, after hydrolysis times of 20 minutes and longer, no differences in degradation patterns could be observed any more (no further data shown), indicating that this effect was minor.

For glycinin after 10 minutes of hydrolysis peptides with higher molecular masses were detected than for Ara h 3. After 10 minutes of hydrolysis different elution patterns were also observed for heated and unheated glycinin, with the pattern for heated glycinin showing relatively more high molecular mass peptides compared with the pattern for unheated glycinin. Thus, for glycinin it was also shown that heating decreased the rate of pepsin hydrolysis, as was also observed for Ara h 3. Moreover, for glycinin differences in peptide sizes were not only observed after 10 minutes of hydrolysis, but until up to 60 minutes of hydrolysis (no further data shown). This indicates that the effect of heating on the hydrolysis rate of pepsin is more pronounced for glycinin than for Ara h 3.

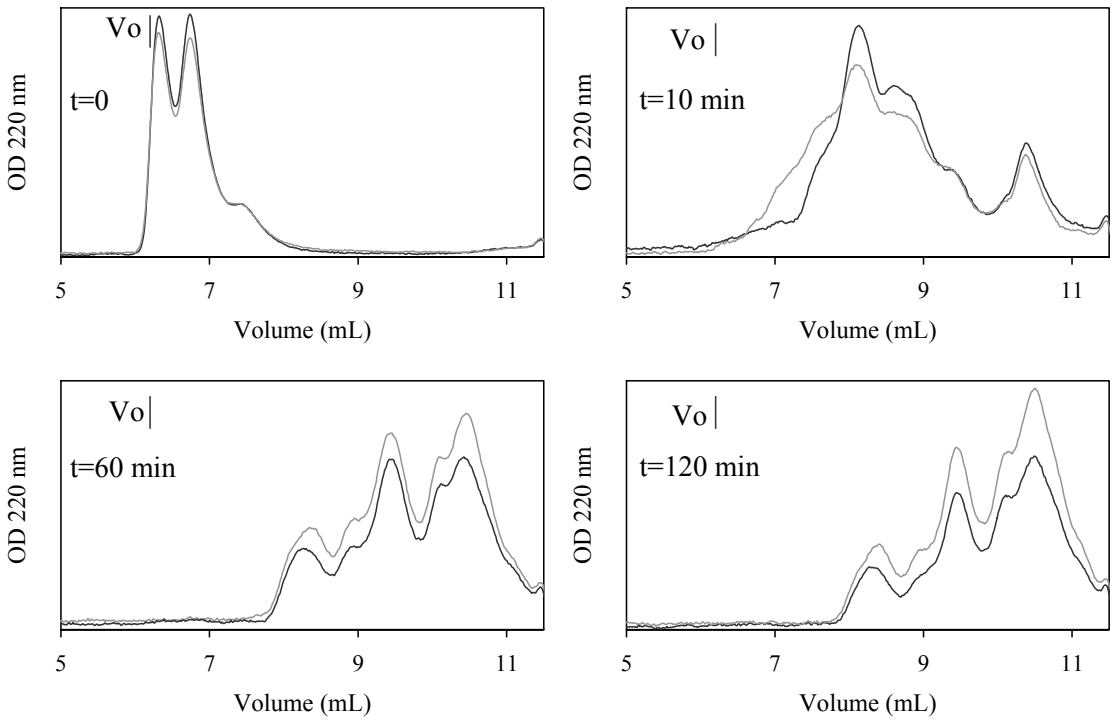

Figure 3: Size exclusion chromatograms (under reducing and denaturing conditions) of Ara h 3 samples after pepsin hydrolysis during different time intervals. Black lines: unheated Ara h 3; gray lines: heated Ara h 3. Indicated is the void volume (Vo) of the column.

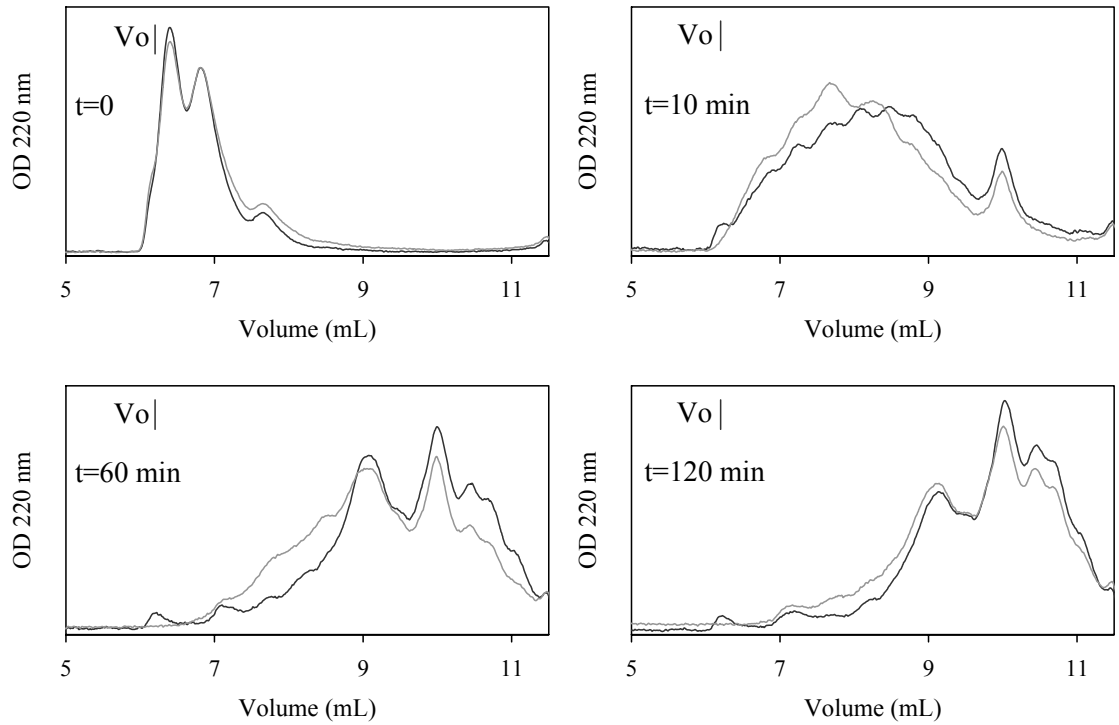

Figure 4: SEC chromatograms (under reducing and denaturing conditions) of glycinin samples after pepsin hydrolysis during different time intervals. Black lines: unheated glycinin; gray lines: heated glycinin. Indicated is the void volume (Vo) of the column.

The digestion of the reference material, unheated Ara h 1, resulted in a slower degradation of the protein than the digestion of Ara h 3 and glycinin. The degradation pattern obtained was comparable to the pattern displayed in chapter 6. After 10 minutes of hydrolysis a relatively large peak corresponding to the intact protein was still present. After prolonged (> 10 minutes) digestion this peak further decreased and peptides of lower molecular masses were formed. These peptides, in comparison with the peptides formed upon digestion of Ara h 3 and glycinin, had relatively high molecular masses, as they were approximately 28 kDa or lower. The digestion of Ara h 1 thus appeared to be slower than the digestion of Ara h 3 and glycinin. Besides, at similar conditions, the digestion of Ara h 1 resulted in peptides with higher molecular masses than the peptides obtained after hydrolysis of Ara h 3 and glycinin. The peptic digestion of Ara h 1 as described by Eiwegger and co-workers (*26*) resulted in a faster degradation of native Ara h 1 into peptides. However, the activity of pepsin they used (approximately 162 units per mg protein) was much higher than the pepsin activity that was used in the present study (approximately 6.5 units per mg protein).

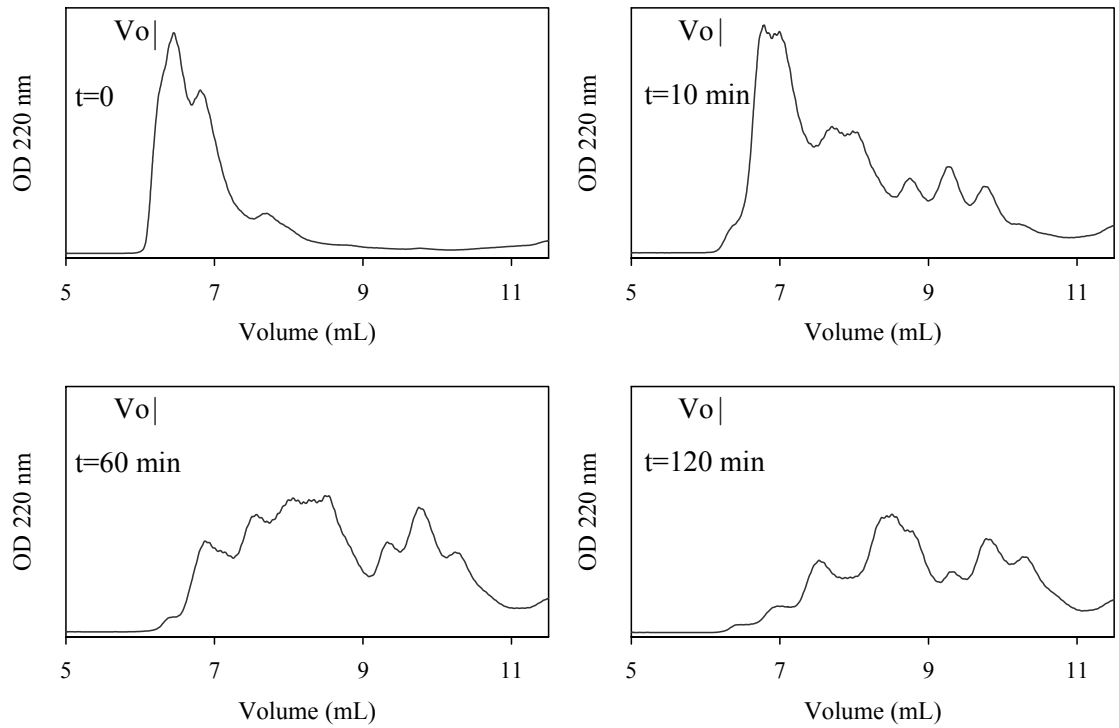

Figure 5: Size exclusion chromatograms chromatograms (under reducing and denaturing conditions) of unheated Ara h 1 samples after pepsin hydrolysis during different time intervals. Indicated is the void volume (Vo) of the column.

IgE BINDING

Ara h 3 and glycinin and their hydrolysates were investigated for their IgE binding by immunoblotting under reducing and denaturing conditions. The results for the unheated and heated parental proteins appeared to be similar, indicating that heating of Ara h 3 and glycinin did not cause changes in the IgE binding capacity of the allergens. Moreover, the results for the hydrolyzed heated and unheated samples were also comparable. Therefore, in Figure 6 only the results for the heated samples are shown. As it can be seen, samples before subjecting to stomach conditions and samples being subjected to stomach conditions without pepsin incubation (t=0) clearly showed IgE binding. As similar patterns were observed, it can be concluded that the acidic pH of the stomach per se does not have an influence on the IgE binding capacity of the legumin proteins. The IgE binding to both proteins was observed to both types of polypeptides, with the acidic polypeptide showing the most intense color, indicating the highest amount of IgE bound.

All Ara h 3 and glycinin samples being subjected to *in vitro* peptic hydrolysis did not to bind IgE, i.e. a color similar to non-specific binding was observed. This was confirmed by dotblot experiments (no further data shown). In contrast to this, all hydrolysates of Ara h 1, when analyzed by dotblot, clearly showed IgE binding (no further data shown). Thus, according to these results, the IgE binding capacities of the legumin proteins Ara h 3 and glycinin are pepsin-labile. Although heating (slightly) decreased the pepsin digestion rate of both proteins, heating did not affect the IgE binding capacity of the hydrolysates. These outcomes could imply that legumin allergens Ara h 3 and glycinin are not able to cause systemic food allergic reactions *in vivo*.

The fast decline of IgE binding during pepsin hydrolysis is not common for known food allergens. As was shown in the present study, peanut allergen Ara h 1 was still able to bind IgE after pepsin digestion using equal pepsin concentrations. These results are in agreement with those reported in chapter 6 and in literature (*16*). In addition, next to Ara h 1 peanut allergen Ara h 2 is also known to still be able to bind IgE after pepsin digestion (*27*). These allergens, therefore, are more likely to cause systemic peanut allergic reactions than Ara h 3. In soybeans several other allergens have been identified. The digestibility of these allergens is not known. However, the absence of severe systemic allergic reactions to soybeans coincide with our results on the rapid hydrolysis and loss of IgE binding of glycinin.

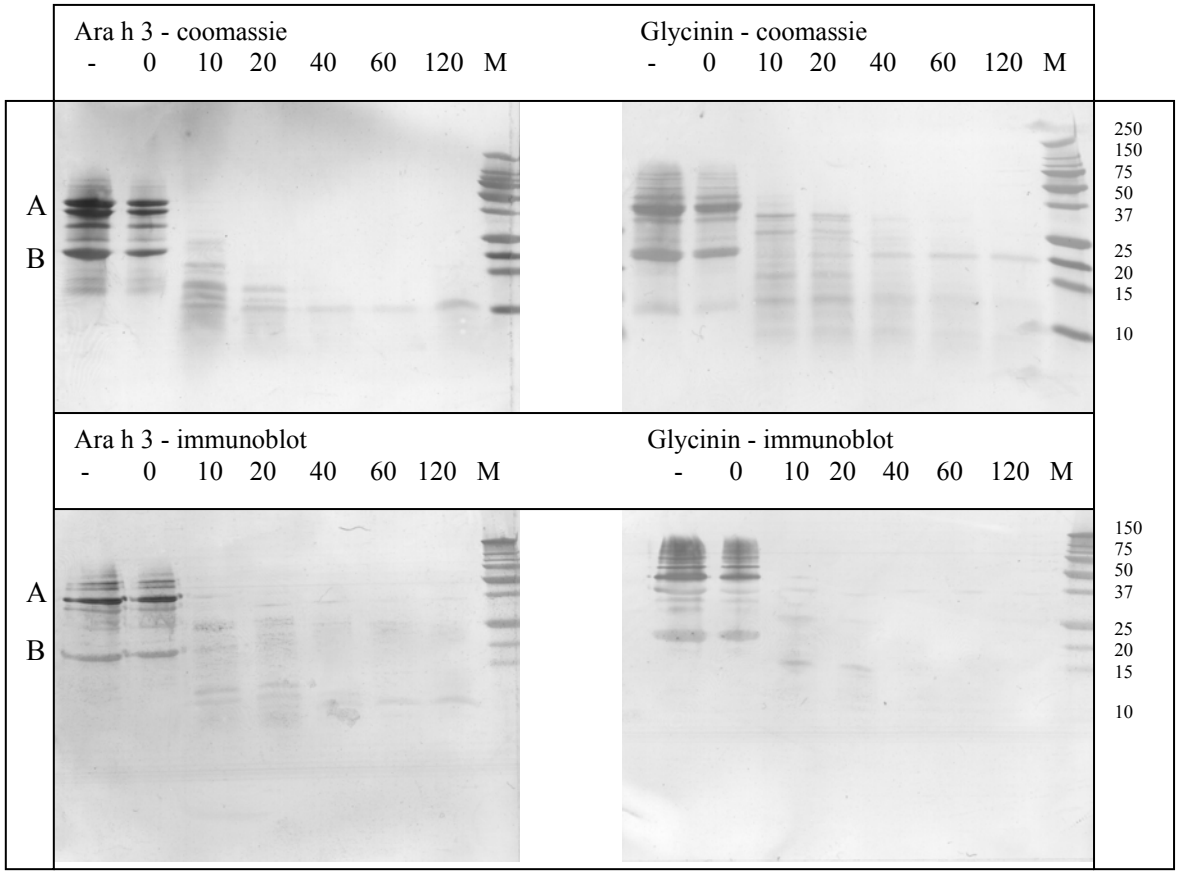

Figure 6: SDS-PAGE and IgE immunoblots of Ara h 3 and glycinin. Indicated is the time of incubation of the samples in minutes (- represents samples not subjected to stomach conditions). M = molecular weight marker, indicated right in kDa. Acidic (A) and basic (B) polypeptides are indicated left.

In conclusion, in this study we have shown that the major seed storage proteins from peanuts and soybeans, legumin proteins Ara h 3 and glycinin, do not maintain their IgE binding properties during digestion by pepsin under the *in vitro* conditions applied. Heating the proteins prior to digestion only increased the pepsin stability of the proteins to a limited extent, but this did not have effects on the IgE binding of the remaining peptides. As the preservation of IgE binding capacity upon digestion is considered a prerequisite for a protein to exert systemic food allergic reactions, in view of these

systemic reactions, these legumin allergens could be not such important food allergens as was previously assumed.

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REFERENCES

1. Sicherer, S. H., Sampson, H. A., Burks, A. W. Peanut and soy allergy: a clinical and therapeutic dilemma. *Allergy* **2000,** 55, (6), 515-521.
2. Bernhisel-Broadbent, J., Taylor, S., Sampson, H. A. Cross-allergenicity in the legume botanical family in children with food hypersensitivity. 2. laboratory correlates. *Journal of Allergy and Clinical Immunology* **1989,** 84, (5), 701-709.
3. Peeters, K., Koppelman, S., Penninks, A., Lebens, A., Bruijnzeel-Koomen, C., Hefle, S., Taylor, S., Hoffen, E., Knulst, A. Clinical relevance of sensitisation to lupine in peanut-sensitized adults. *Allergy* **2007,** 62, (S83), 25.
4. Wolf, W. J., Babcock, G. E., Smith, A. K. Ultracentrifugal differences in soybean protein composition. *Nature* **1961,** 191, (479), 1395-1396.
5. Helm, R. M., Cockrell, G., Connaughton, C., Sampson, H. A., Bannon, G. A., Beilinson, V., Livingstone, D., Nielsen, N. C., Burks, A. W. A soybean G2 glycinin allergen - 1. Identification and characterization. *International Archives of Allergy and Immunology* **2000,** 123, (3), 205-212.
6. Koppelman, S. J., Knol, E. F., Vlooswijk, R. A. A., Wensing, M., Knulst, A. C., Hefle, S. L., Gruppen, H., Piersma, S. Peanut allergen Ara h 3: isolation from peanuts and biochemical characterization. *Allergy* **2003,** 58, (11), 1144-1151.
7. Rabjohn, P., Helm, E. M., Stanley, J. S., West, C. M., Sampson, H. A., Burks, A. W., Bannon, G. A. Molecular cloning and epitope analysis of the peanut allergen Ara h 3. *Journal of Clinical Investigation* **1999,** 103, (4), 535-542.
8. Kleber-Janke, T., Crameri, R., Appenzeller, U., Schlaak, M., Becker, W. M. Selective cloning of peanut allergens, including profilin and 2S albumins, by phage display technology. *International Archives of Allergy and Immunology* **1999,** 119, (1-2), 265-274.
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. *Journal of Allergy and Clinical Immunology* **2004,** 113, (4), 776-782.
10. Adachi, M., Kanamori, J., Masuda, T., Yagasaki, K., Kitamura, K., Mikami, B., Utsumi, S. Crystal structure of soybean 11S globulin: Glycinin A3B4 homohexamer. *Proceedings of the National Academy of Sciences of the United States of America* **2003,** 100, (12), 7395-7400.
11. Lakemond, C. M. M., de Jongh, H. H. J., Hessing, M., Gruppen, H., Voragen, A. G. J. Soy glycinin: Influence of pH and ionic strength on solubility and molecular structure at ambient temperatures. *Journal of Agricultural and Food Chemistry* **2000,** 48, (6), 1985-1990.
12. Zeece, M. G., Beardslee, T. A., Markwell, J. P., Sarath, G. Identification of an IgE-binding region in soybean acidic glycinin G1. *Food and Agricultural Immunology* **1999,** 11, (1), 83-90.

13. Beardslee, T. A., Zeece, M. G., Sarath, G., Markwell, J. P. Soybean glycinin G1 acidic chain shares IgE epitopes with peanut allergen Ara h 3. *International Archives of Allergy and Immunology* **2000,** 123, (4), 299-307.

14. Xiang, P., Beardslee, T. A., Zeece, M. G., Markwell, J., Sarath, G. Identification and analysis of a conserved immunoglobulin E-binding epitope in soybean G1a and G2a and peanut Ara h 3 glycinins. *Archives of Biochemistry and Biophysics* **2002,** 408, (1), 51-57.

15. Eigenmann, P. A., Burks, A. W., Bannon, G. A., Sampson, H. A. Identification of unique peanut and soy allergens in sera adsorbed with cross-reacting antibodies. *Journal of Allergy and Clinical Immunology* **1996,** 98, (5), 969-978.

16. 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. *Journal of Immunology* **2000,** 164, (11), 5844-5849.

17. Burks, A. W., Williams, L. W., Thresher, W., Connaughton, C., Cockrell, G., Helm, R. M. Allergenicity of peanut and soybean extracts altered by chemical or thermal-denaturation in patients with atopic-dermatitis and positive food challenges. *Journal of Allergy and Clinical Immunology* **1992,** 90, (6), 889-897.

18. Lee, H. W., Keum, E. H., Lee, S. J., Sung, D. E., Chung, D. H., Lee, S. I., Oh, S. Allergenicity of proteolytic hydrolysates of the soybean 11S globulin. *Journal of Food Science* **2007,** 72, (3), C168-C172.

19. Kuipers, B. J. H., van Koningsveld, G. A., Alting, A. C., Driehuis, F., Voragen, A. G. J., Gruppen, H. Opposite contributions of glycinin- and beta-conglycinin-derived peptides to the aggregation behavior of soy protein isolate hydrolysates. *Food Biophysics* **2006,** 1, (4), 178-188.

20. 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. *Journal of Agricultural and Food Chemistry* **2006,** 54, (19), 7180-7186.

21. Lakemond, C. M. M., de Jongh, H. H. J., Hessing, M., Gruppen, H., Voragen, A. G. J. Heat denaturation of soy glycinin: Influence of pH and ionic strength on molecular structure. *Journal of Agricultural and Food Chemistry* **2000,** 48, (6), 1991-1995.

22. Mori, T., Nakamura, T., Utsumi, S. Gelation mechanism of soybean 11S-globulin - Formation of soluble aggregates as transient intermediates. *Journal of Food Science* **1982,** 47, (1), 26-30.

23. Fu, T. J., Abbott, U. R., Hatzos, C. Rebuttal on digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid - A comparative study. *Journal of Agricultural and Food Chemistry* **2003,** 51, (17), 5185-5187.

24. Kuipers, B. J. H., van Koningsveld, G. A., Alting, A. C., Driehuis, F., Gruppen, H., Voragen, A. G. J. Enzymatic hydrolysis as a means of expanding the cold gelation conditions of soy proteins. *Journal of Agricultural and Food Chemistry* **2005,** 53, (4), 1031-1038.

25. Untersmayr, E., Jensen-Jarolim, E. The effect of gastric digestion on food allergy. *Current Opinion in Allergy and Clinical Immunology* **2006,** 6, (3), 214-219.

26. 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. *Clinical and Experimental Allergy* **2006,** 36, (10), 1281-1288.

27. Sen, M., Kopper, R., Pons, L., Abraham, E. C., Burks, A. W., Bannon, G. A. Protein structure plays a critical role in peanut allergen stability and may determine immunodominant IgE-binding epitopes. *Journal of immunology* **2002,** 169, (2), 882-887.

8

GENERAL DISCUSSION

This study was aimed to investigate the effects of heating on the structure and digestibility of class 1 food allergens and to investigate the effects of digestion on the IgE binding capacity of these allergens. Cupin and prolamin allergens from peanuts, Brazil nuts, and soybeans were studied. It was chosen to investigate allergens from these sources because of the widespread abundance of these products in foods (peanuts and soybeans) and / or the severity of the allergic reactions they may cause in humans (peanuts and Brazil nuts).

REPRESENTATIVE ALLERGEN PREPARATIONS

In order to be able to investigate structural characteristics of an allergen, including its digestibility, the allergen has to be available in a purified form that coincides with the form of the allergen as it is present in food raw materials or as it is present in foods. When the allergen consists of multiple isoforms, a representative isoform pool should be isolated. The structure in which a protein occurs in its original matrix, i.e. the agricultural raw material, can be defined as its native state. However, the structure of a protein can only be well studied after extraction. It is, therefore, arbitrarily assumed that the structure of a protein upon extraction using conditions which, in protein biochemistry are considered as non-denaturing and non-reducing, should be denoted as its native structure.
As purification of native allergens is often problematic and time-consuming, recombinant allergens are often produced and used for structure investigations (*1*, *2*). However, care should be taken in this respect. In the first place, recombinant proteins may differ in folding and degree of post-translational processing from their natural counterparts. As a result, recombinant proteins may show different allergenic activities compared to their natural forms (*3*). Secondly, using recombinant techniques usually not all isoforms of a protein will be expressed, whereas different protein isoforms may display different allergenic properties (*4*). Because of these drawbacks of recombinant proteins, in this study it was aimed to purify allergenic proteins from their respective natural sources and use them for the investigations. It was attempted not to induce structural changes of the allergens during purification, in order to obtain representative allergen preparations.
From the results described in chapter 2 the conclusion that could be drawn is that new, innovative chromatographic techniques, like expanded bed adsorption (EBA), enable the fast and effective purification of relatively large amounts of allergens.
It should be noted that when using chromatography for purification of (allergenic) proteins, it must be ensured that the complete isoform pool of the allergen is remained and that no structural changes in the protein are induced by the purification method used. As was shown in chapter 4, purification techniques like ion exchange chromatography (IEC) may disrupt non-covalent interactions between proteins and other constituents, possibly resulting in a change in quaternary structure. As shown in chapter 2, in the case of Ber e 1, the complete isoform pool of this allergen could be purified using EBA. Besides, the structure of Ber e 1 was not affected during EBA purification. These results thus indicate that for Ber e 1, IEC can be used to obtain a representative allergen preparation. Also, the purification of legumin allergen Ara h 3 using IEC, which is

described in chapter 7, did not bring about changes in the quaternary structure of this protein, as determined with size exclusion chromatography (SEC).
In contrast to the results obtained for Ber e 1 and Ara h 3, from this study it can be concluded that IEC induces structural changes in peanut allergen Ara h 1. Upon purification of this allergen using only SEC at a low ionic strength, the allergen occured in a different quaternary structure than upon purification using IEC (chapter 4). The allergen obtained upon extraction occurred as a larger complex than the previously described trimeric structure (*5-7*) and interacted non-covalently with proanthocyanidins, as is described in chapter 5. Obviously, without an investigation of the quaternary structure of the protein both before and after IEC, this phenomenon would have been overlooked.

The oligomeric Ara h 1-proanthocyanidin complex was observed after extraction of the protein. It could not be determined whether Ara h 1 already occurred in peanuts as a complex with proanthocyanidins, or whether the proanthocyanidin-Ara h 1 interactions were induced upon extraction. Flavan-3-ols, the constituents of proanthocyanidins, are described to be synthesized in the cytosol and to be accumulated in vegetative vacuoles, in which they undergo condensation (*8*). Plant storage proteins are reported to be stored in another type of storage vacuoles, denoted protein storage vacuoles (*9*). This indicates that in peanuts, Ara h 1 and proanthocyanidins occur separately from each other. However, peanuts are often processed before consumption, thereby enabling contact between proanthocyanidins and Ara h 1 as a result of crushing and / or grinding. Moreover, during mastication, contact between the two constituents is also possible. Interactions between proteins and proanthocyanidins are generally considered to occur within a relatively short time span. For example, salivary proteins are assumed to interact with proanthocyanidins from foods in the time span that food resides in the oral cavity (*10, 11*). Consequently, we assume that complexation of Ara h 1 and proanthocyanidins, if it had not already occurred before, takes place during processing and / or mastication. As a result, we consider the oligomeric structure of Ara h 1 as the structure of the allergen in which it is generally ingested by humans (chapter 5).
The observation that Ara h 1 upon interacting with proanthocyanidins formed soluble complexes is not consistent with literature data, as interactions of proanthocyanidins with proteins are described to generally form insoluble complexes (*12, 13*). Probably, the three-dimensional structure of Ara h 1 and other vicilin proteins likely enables proanthocyandins to link vicilin trimers, resulting in soluble complexes (chapter 5).

Considering the importance of using purification techniques, which do not change the structural properties and isoform composition of an allergen, from this study it can be concluded that the pH used for protein extraction should be selected carefully (chapter 2). For the first purification (chapter 4) of Ara h 1, an extraction buffer with pH 8.2 was used, as extraction of Ara h 1 at this pH or at comparable pH values had been previously used by others for the purification of this allergen (*6, 7, 14*). As shown in chapter 4, this purification resulted in an oligomeric Ara h 1 preparation (Ara h $1_{\text{oligomers, 8.2}}$). During subsequent purifications (chapters 5, 6 and 7) an extraction buffer with pH 6.2 was used, which also resulted in Ara h 1 occuring in an oligomeric form (Ara h $1_{\text{oligomers, 6.2}}$). It was

deliberately chosen to use a lower pH value for protein extraction, as at pH values above pH 7 phenolic compounds may oxidize and consequently polymerize with other phenolic compounds or proteins (*15*). Moreover, foods and food products in general do not have pH values higher than 7 and as such, Ara h 1 in food-like situations will not be frequently subjected to pH values higher than 7. Consequently, the purification of Ara h 1 at pH 8.2, although carried out in several publications (*6, 7, 14*), is not desirable. An additional advantage of the purification of Ara h 1 at pH 6.2 was that Ara h 3, which on SEC elutes after Ara h 1 and therefore partly co-elutes with Ara h 1 (chapter 4), is hardly extractable at this pH. As a result, the purification of Ara h 1 at lower pH was more efficient.
Ara h $1_{oligomers,\ 6.2}$ was shown to be more slowly hydrolyzed by pepsin than the trimeric form of Ara h 1 (chapter 6), although the differences is hydrolysis rates were not very pronounced. However, when Ara h $1_{oligomers,\ 8.2}$ were subjected to peptic digestion, other results were obtained. SEC analysis showed that upon prolonged digestion of unheated and pre-heated Ara h $1_{oligomers,\ 8.2}$, a considerable high molecular mass protein peak still existed (Figure 1). Under the same conditions, prolonged hydrolysis of unheated and pre-heated Ara h $1_{oligomers,\ 6.2}$ resulted in comparable SEC chromatograms as those described in chapters 6 and 7, with several peaks corresponding to peptides with relatively low molecular masses. These results thus showed that the digestibility of Ara h $1_{oligomers,\ 8.2}$ was much lower compared to the digestibility of Ara h $1_{oligomers,\ 6.2}$.

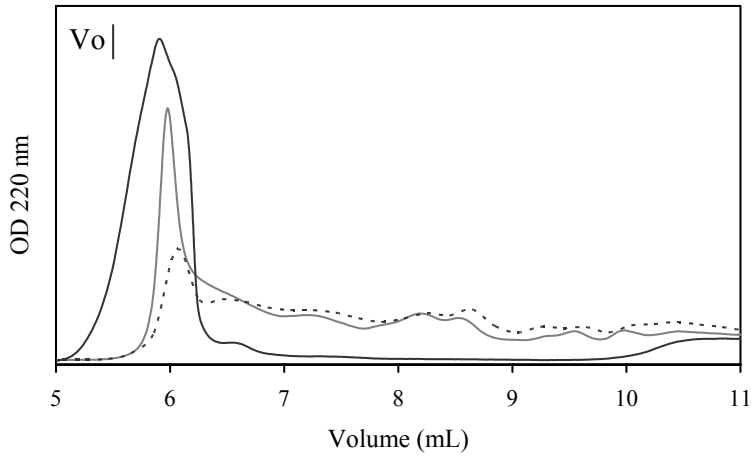

Figure 1: Size exclusion chromatogram (under denaturing conditions) of unheated Ara h $1_{oligomers,\ 8.2}$ before (black lines) and after hydrolysis with pepsin during 10 (gray lines) and 60 minutes (dashed lines). The protein to pepsin ratio (w/w) that was used was 1: 0.013. Indicated is the void volume of the column (Vo).

Ara h 1 in both oligomeric preparations (Ara h $1_{oligomers,\ 8.2}$ and Ara h $1_{oligomers,\ 6.2}$), was digested by pepsin at a lower rate, compared to the purification-induced trimeric structure of the protein. The proanthocyanidins present in the oligomeric Ara h 1 complexes could have caused these differences, for example by decreasing the accessibility of the allergen for pepsin. It should be noted that changes in pH may affect the affinity of proanthocyanidins to interact with proteins (*16*). Consequently, it remained unknown whether the proanthocyanidins in Ara h $1_{oligomers}$ at the pH of peptic digestion (pH 2.0) were still linked to Ara h 1 molecules and thus, if Ara h 1 at low pH still occurred as

larger complexes. If the proanthocyanidins were not linked to Ara h 1 at pH 2.0, the latter components could still have directly interacted with the pepsin and thereby inhibiting its activity (*17, 18*). Of course, one must bear in mind that the digestion studies we performed were *in vitro* tests. Consequently, proanthocyanidin-protein interactions *in vivo* can have other effects on the digestibility.
The lower digestibility of Ara h $1_{oligomers,\ 8.2}$ compared to Ara h $1_{oligomers,\ 6.2}$ points towards differences in the structure and / or amount of proanthocyanidins interacting with Ara h 1 between the two protein preparations. It could very well be that during extraction at a higher pH, oxidation of proanthocyanidins was induced, possibly resulting in polymerization of these latter compounds (*19*). In general, the affinity of proanthocyanidins to interact with proteins increases with an increasing degree of polymerization, until a maximum degree of polymerization when steric hindrance limits the interaction (*20*). Clearly, no covalent interactions between the proanthocyanidins and Ara h 1 were formed at both pH's of extraction, as from both extracts Ara h $1_{trimers}$ could be induced upon applying the oligomeric protein preparations to IEC (chapter 4 and 5).

In line with the differences in peptic digestibility between Ara h $1_{oligomers,\ 8.2}$ and Ara h $1_{oligomers,\ 6.2}$, differences in the behaviour upon heating were observed between the two protein preparations, as can be seen from Figure 2. In all heated Ara h 1 preparations no native peak was observed. In heated samples of Ara h $1_{oligomers,\ 8.2}$ a peak eluted in the void volume of the column, which indicates the presence of soluble aggregates. In the chromatograms of heated, centrifuged, Ara h $1_{oligomers,\ 6.2}$ no peaks were visible, which points at the formation of insoluble aggregates upon heating. These results thus indicate that Ara h $1_{oligomers,\ 8.2}$ form soluble aggregates upon heating, whereas Ara h $1_{oligomers,\ 6.2}$ and Ara h $1_{trimers}$ formed insoluble aggregates (chapter 6).
The formation of soluble aggregates upon heating rather than the formation of insoluble aggregates also points towards differences in the structure and / or amount of proanthocyanidins interacting with Ara h 1, caused by the different extraction pH's. The previously mentioned possible polymerization of proanthocyanidins in Ara h $1_{oligomers,\ 8.2}$ could explain this difference, as in Ara h $1_{oligomers,\ 8.2}$, as a result of polymerization, the steric hindrance between complexes could be higher than in Ara h $1_{oligomers,\ 6.2}$, causing the differences in the aggregation behaviour.
As oligomeric Ara h 1 upon purification at pH 6.2 is the most relevant form of this allergen, in the remaining part of this chapter the designation Ara h $1_{oligomers}$ refers to oligomeric Ara h 1, purified at a pH of 6.2.

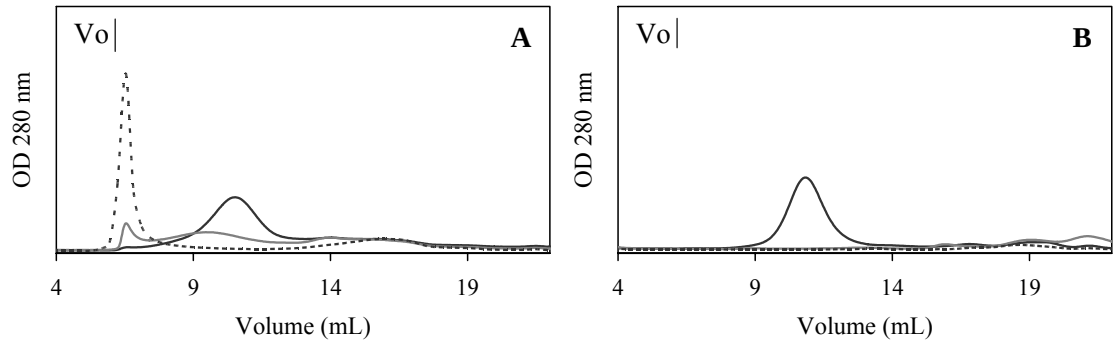

Figure 2: Size exclusion chromatograms (under non-reducing conditions) of (A) Ara h $1_{oligomers, 8.2}$ and (B) Ara h $1_{oligomers, 6.2}$ after centrifugation. Black lines: unheated protein preparations; gray lines: protein preparations heated at 85 °C; dashed lines: protein preparations heated at 95 °C.

Altogether, from the results described above the importance of the use of relevant purification methods for allergenic proteins can be deduced. This refers to IEC and pH, as it was shown that these characteristics may influence an allergens' isoform distribution and structural characteristics.

IgE BINDING PROPERTIES OF BER E 2

Allergen Ber e 1 has been identified as the sole *major* allergen in Brazil nuts, being recognized by 100% of a Brazil nut allergic population (*21*). The legumin protein from these nuts, excelsin, has been related to allergic reactions in two reports (*22, 23*) has been designated as a *minor* allergen, Ber e 2.

The plasma and serum from Brazil nut allergic individuals were analyzed on immunoblot as a preliminary screening for the ELISA results described in chapter 2. Surprisingly, all six plasma and serum showed IgE binding to multiple protein bands from Brazil nut extract. When serum from a person not allergic to Brazil nuts was used, no protein bands exhibited color upon immunoblotting (data not shown). These results indicated that the allergic patients, next to Ber e 1, all recognized several other Brazil nut proteins as being (potentially) allergenic.

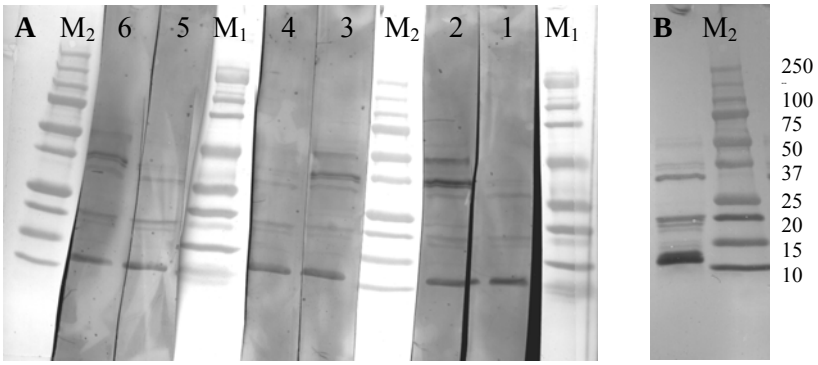

Figure 3: Immunoblot (A) and SDS-PAGE (B) of Brazil nut extract under reducing conditions. Numbers indicate plasma / serum samples. M_1, M_2, and M_3 indicate the molecular weight markers used. The molecular mass of marker M_2 is indicated on the right, in kDa.

As can be seen in Figure 3, serum and plasma of all patients exhibited IgE binding on an immunoblot (under reducing conditions) to a protein band of approximately 12 kDa, hypothesized to be Ber e 1 (chapter 2). Under non-reducing conditions, this band was also visible. Apparently, under the conditions applied, Ber e 1 did not (completely) dissociate into its subunits. Besides IgE binding to the protein band of approximately 12 kDa, all patients reacted towards multiple protein bands, ranging from approximately 20 to 55 kDa (Figure 3).

In order to identify the nature of the IgE binding protein bands, several IgE binding protein bands, as indicated in Figure 4, were applied to in-gel tryptic digestion. Afterwards, the peptides obtained were analyzed using liquid chromatography-mass spectrometry (LC-MS) using the method described in chapter 4 and compared with a database of known amino acid sequences of Brazil nut proteins (P04403, Q6Y8A2, O98880, and Q6Y8A3). The results are given in Table 1. As expected, the ~12 kDa band showed a relatively high correlation with the amino acid sequence of Ber e 1, confirming that this protein band represented this allergen. All other protein bands showing coverage with proteins from Brazil nuts exhibited the highest coverage with the amino sequence of Ber e 2 (legumin). These results thus indicated that Ber e 2 did bind IgE from all Brazil nut allergic persons investigated. As under non-reducing conditions four protein bands could be assigned to Ber e 2, our results point towards Ber e 2 being extensively post-translationally processed. Some legumin proteins from other sources are known to be extensively post-translationally processed as well, like for example Ara h 3 from peanuts (*24*).

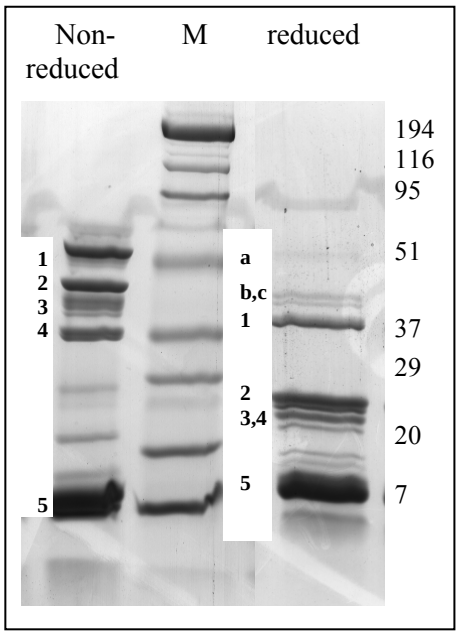

Figure 4: SDS-PAGE of non-reduced and reduced Brazil nut extract. Indicated are the bands that were used for in-gel digestion. M indicates the molecular weight marker, indicated on the right in kDa.

Table 1: Brazil nut protein bands and the highest coverage by mass (%) of their peptides after in-gel digestion with Brazil nut amino acid sequences.

band no.*	estimated molecular mass on SDS-PAGE (kDa)	coverage with amino acid sequence of	% coverage
1nr**	55	legumin	38
2nr	46	legumin	11
3nr	42	legumin	35
4nr	38	legumin	38
5nr	12	Ber e 1	32
1r	38	legumin	24
2r	24	legumin	15
3r	22	legumin	2
4r	21	legumin	5
5r	12	Ber e 1	48
a	55	-	-
b	42	-	-
c	41	-	-

*: protein bands are indicated in Figure 4

**: nr: non-reduced samples; r: reduced samples

-: no peptides were identified, most likely as a result of a too low peptide concentration

The results obtained showed that Ber e 2 was recognized by all serum and plasma of Brazil nut allergic patients used. As a result, it is tempting to conclude that Ber e 2 is a more important allergen than was previously assumed. However, in order to conclude this, it should first be ruled out that the IgE binding we detected was a result of clinical non-significant cross-reactivity, a phenomenon that is more often described to occur between legumin allergens from various sources (*25*). Moreover, the pools of serum and plasma from allergic patients used for this study comprised sera and plasma of 6 allergic persons. No specification of the clinical symptoms of these patients was available. We aimed to use these pools as analytical tools to investigate the IgE binding properties of proteins and how these characteristics change upon heating and digestion, rather than to draw conclusions about the relative importance of allergens in terms of allergen recognition and IgE binding quantification. As a result, our results for Ber e 2 are likely not representative for an allergic population, Consequently, it is recommended to investigate the IgE binding characteristics of a larger group of Brazil nut allergic people, while knowing their allergic symptoms, in order to be able to draw firm conclusions about the allergenicity of Ber e 2.

AGGREGATION, DIGESTION AND IgE BINDING OF CUPIN ALLERGENS

Most foods are being heat-processed before consumption. Peanuts, for example, are often boiled or roasted. Heating can induce various changes in protein structure. During heat-induced denaturation proteins unfold and often subsequently form large aggregates afterwards (*26*). The accessibility of proteins by pepsin can be affected as a result of

aggregation. For example, the formation of disulfide bridges, which may occur upon heating, may protect parts of a protein (aggregate) from rapid pepsin hydrolysis.
Based on the results described in chapters 6 and 7, a conclusion that could be drawn from this study is that the effect of heat-induced aggregation on the digestibility of cupin allergens is protein-dependent. As was shown in chapter 6, heat-induced aggregation of a purified vicilin allergen (Ara h 1) did not influence its digestibility by pepsin, whereas the digestibility of heat-induced aggregates of purified legumin allergens (Ara h 3 and glycinin) was (slightly) decreased compared to their unheated counterparts (chapter 7).
In chapter 6 it is described that Ara h 1, both in its trimeric and in its oligomeric structure, forms insoluble aggregates upon heating. The latter aggregates were shown not to contain disulfide bridges, as SEC analyses in the absence and presence of a reducing agent were comparable (data not shown). The low pH that was used for peptic hydrolysis (pH 2.0) mimicked the low pH of the human stomach. At such low pH values proteins may denature as a result of ionization (*27*). Besides, as was already mentioned, changes in pH may also affect the affinity of proanthocyanidins to interact with proteins (*16*). Unfortunately, with the SEC columns available, it was not possible to study the effects of pH changes on the quaternary structure of the heated and unheated protein preparations. At such low pH values, as a result of aspecific interactions, no reproducible results could be obtained by SEC. It should be noted, however, that when the pH of heat-treated Ara h $1_{trimers}$ and Ara h $1_{oligomers}$ was adjusted to pH 2.0, the previously turbid suspensions became clear, indicating that the aggregates had been solubilized upon lowering the pH (no further data shown). These results indicated that the heat-induced aggregates of Ara h 1 dissociated upon decreasing the pH. Apparently, after this dissociation the remaining (unfolded) protein structures were similarly susceptible to pepsin compared with those in non-heated Ara h 1, as the peptic digestibility of the unheated and pre-heated protein preparations was comparable.

In contrast to the results obtained for the vicilin allergen Ara h 1, heat-induced aggregation of legumin allergens from peanuts and soybeans did affect the digestibility of these latter proteins (chapter 7). Upon heating, the hydrolysis rate of glycinin was decreased to a larger extent than the hydrolysis rate of Ara h 3. Using SEC, differences in hydrolysis patterns between heat-treated and unheated proteins were observed until 60 minutes of hydrolysis of glycinin and until only 10 minutes of hydrolysis of Ara h 3.
For Ara h 3 only insoluble aggregates were observed upon heating, whereas for glycinin the heat-induced aggregation yielded both soluble and insoluble aggregates. The insoluble protein aggregates of both preparations could not be solubilized without using a reducing agent, indicating that inter-molecular disulfide bridges had been formed upon heating. These results are in agreement with those reported earlier (*28*), stating that the insoluble aggregates of glycinin contained inter-molecular disulfide bridges. The formation of disulfide bridges could have decreased the pepsin accessibility of the heated legumin proteins, compared with the unheated proteins. However, as the pepsin digestibility of both Ara h 3 and glycinin was not affected to a large extent by heating, it seemed likely that the heat-induced aggregates at pH 2.0 dissociated to a relatively large extent. This

corresponds with literature, as low pH values have been reported to dissociate peptide aggregates (*29*).

An additional conclusion that could be drawn from this study was that the IgE binding capacity of cupin allergens after pepsin digestion is protein-dependent. This can be seen in Table 2, in which an overview on the effects of heating and digestion on the IgE binding capacity of the allergens investigated is given. Under the same conditions, the IgE binding capacity of legumin allergens Ara h 3 and glycinin was diminished already after 10 minutes of peptic digestion, regardless of pre-heating the proteins. On the contrary, unheated and pre-heated vicilin Ara h 1 still was IgE reactive after 16 hours of peptic digestion (chapters 6 and 7). Our results suggest that the legumin allergens investigated are not able to induce systemic food allergic reactions (class 1 food allergy), in contrast to earlier made assumptions (*30*). Instead, these allergens might be (partly) responsible for the oral allergy syndrome (OAS) that is described for peanuts (*31*) and soybeans (*32*), respectively. It was previously published (*32*) that in a group of soybean-allergic patients, all showing OAS, approximately 63% on immunoblotting showed IgE binding to protein bands in the range of 8 to 50 kDa. Protein bands of approximately 18, 20, and 35 kDa, which were IgE reactive, could have corresponded to the acidic and basic polypeptides of soy glycinin. These results subscribe our hypothesis, stating that legumin allergens from soybeans and peanuts do not contribute to the systemic food allergic reactions that are observed to these foods.

Table 2: Overview on the effects of heating and digestion of the IgE binding on the studied food allergens from peanuts, soybeans, and Brazil nuts.

Investigated characteristics	**Ara h 1***	**Ara h 3**	**Glycinin**
Pepsin digestibility	Multiple fragments < 9 kDa formed	Multiple fragments formed	Multiple fragments formed
IgE binding after peptic digestion	Likely caused by epitopes 5 and 6, possibly also by epitopes 1-4	IgE binding diminishes within 10 minutes of digestion	IgE binding diminishes within 10 minutes of digestion
Heat stability	Irreversible denaturation at 83 °C, formation of insoluble aggregates	Td hexamers ~92 °C; trimers ~77 °C; insoluble aggregates formed upon heating**	Td ~85 °C (I = 0.2 M), soluble and insoluble aggregates formed upon heating
Digestibility after heat-induced aggregation	Similar to unheated Ara h 1	Slightly decreased compared with unheated Ara h 3 (differences till 10 minutes of digestion)	Slightly decreased compared with unheated glycinin (differences till 60 minutes of digestion)
IgE binding after heat-induced aggregation and digestion	Similar to unheated Ara h 1 upon digestion	Similar to unheated Ara h 3	Similar to unheated glycinin

*: results count for Ara h $1_{oligomers}$ as well as Ara h $1_{trimers}$
**: Td = denaturation temperature

PEPSIN-RESISTANT EPITOPES OF ARA H 1

As was previously mentioned, from this study it could be concluded that the vicilin preparation from peanuts (Ara h 1) remained IgE reactive upon peptic digestion (chapters 6 and 7). These results agree with those described in literature (*6, 33*). Pepsin-resistant peptides with maximum masses of ~9 kDa were identified in this study. It has been previously reported that only fragments <6 kDa remain upon peptic digestion of Ara h 1 (*33*). However, these latter data were based on SDS-PAGE results, whereas the results from this study were based on MALDI-TOF MS data, a technique that gives far more reliable results than SDS-PAGE in this context.
Another conclusion that could be drawn from the results of the present study is that of the 21 known epitopes of Ara h 1 (*34, 35*), 15 could be cleaved by pepsin under the *in vitro* conditions applied. Consequently, these latter epitopes are not likely to be responsible for the induction of systemic food allergic reactions to peanuts. No peptic peptides corresponding to the part of the Ara h 1 protein sequence between amino acids 1 and 83 were identified in the peptic hydrolysate of Ara h 1. These results indicated that this part of the protein remained (largely) intact upon peptic digestion. On this part of the amino acid sequence six previously identified epitopes are situated (*34*). N-terminal sequencing

analysis, together with chymotryptic hydrolysis of the relatively large IgE binding peptides remaining upon peptic digestion of Ara h 1, deduced two of these six epitopes (amino acid sequences 50-59, EDWRRPSHQQ and amino acids 60-67, PRKIRPEG) to remain intact upon peptic hydrolysis.

No pepsin or chymotrypsin derived peptides were identified between amino acids 1 and 45 of the amino acid sequence of Ara h 1. Consequently, of the remaining four previously identified epitopes (amino acids 6-13, 14-21, 24-31 and 40-47, respectively) it could not be concluded whether they could remain intact upon digestion, or whether they were fragmentated as a result of pepsin activity. Amino acids 1-45 could have been completely degraded by pepsin and / or chymotrypsin. It should be noted, however, that the fact that no coverage was found in this region could also have been caused by deviating amino acid sequence in this region of Ara h 1, or by a deviating N-terminus of the allergenic protein.

```
  1  R S P P G E R T R G R Q P G D Y D D D R R Q P R R E E G G R W G P A G P R E R E  Peanut
  1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  Soybean
  1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  Lentil
  1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  Pea

 41  R E E D W R Q P R E D W R R P S H Q Q P R K I R P E G R E G E Q E W G T P G S H  Peanut
  1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  Soybean
  1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  Lentil
  1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  Pea

 81  V R E E T S R N N P F Y F P S - R R F S T R Y G N Q N G R I R V L Q R F D Q R S  Peanut
  1  V R E D E - - N N P F Y F R S S N S F Q T L F E N Q N V R I R L L Q R F N K R S  Soybean
  1  S R S D Q - - E N P F I F K S - N R F Q T I Y E N E N G H I R L L Q K F D K R S  Lentil
  1  - R S D P - - Q N P F I F K S - N K F Q T L F E N E N G H I R L L Q K F D Q R S  Pea
```

Figure 5: Amino acid alignment of the N-terminal part of the amino acid sequences of vicilin proteins from peanut (P43238), soybeans (P25974), lentil (Q84UI0), and pea (P13918). Indicated in gray boxes are the two deduced and four possible pepsin-resistant epitopes of Ara h 1, situated on its N-terminal part.

```
  1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - - R S P P G  peanut
  1  - - - - - - - - - - - - - - - - - - - - - - - - - - - - N Y D E G S E T R V P G  pea
  1  M A T T I K S R F P L L L L L G I I F L A F V C V A Y A N D D E G S E P R V T G  lentil
  1  - - - - - - - - - - - - - - - - - - - - - - - - V E K E E C E E G E I P R P R P  soy a
  1  - - - - - - - - - - - - - - - - - - - - - - - V E E E E E C E E G Q I P R P R P  soy a'

  6  - - E R - T R G R Q P - G D Y D D D R - - - - - R - - - - - - - Q P R R E E G G  peanut
 13  - - Q R - E R G R Q E - G E K E E K R - - - - H G E W R P S Y E K E E H E E E K  pea
 41  - - Q R - E R G R Q E - G E K A E Q S R E Q S P G Q W R P S H G K E E D E E E K  lentil
 17  R P Q H P E R E P Q Q P G E K E E D E D E Q P R P I P F P R P - Q P R Q E E E H  soy a
 18  - - Q H P E R E R Q Q H G E K E E D E G E Q P R P F P F P R P R Q P H Q E E E H  soy a'

 30  - - - - - - - - - - - - - - - - - - - - R W G P A G P R E R E R E E D - - - - -  peanut
 45  Q K Y R Y Q R E K K E Q K E V Q P G R E R W E R E E D E E Q V E E E - - - - - W  pea
 77  - - - - - - - E - - - Q K E A Q S G R E K W E R K E D E E K V V E E E E - G E W  lentil
 56  - - - - - - - E Q R E E Q E W P R K E E K R G E K G S E E E D E D E D E - - - -  soy a
 56  - - - - - - - E Q K E E H E W H R K E E K H G G K G S E E E Q D E R E H P R P H  soy a'

 45  - - - - W R Q P R E D W R R P S H Q Q P R K I R - - - - - - - - - - - P E G R E  peanut
 80  R G S Q R R E D P E E R A R L R H R E E R T K R - - - - - - - - - D R R H Q R E  pea
106  R G S Q R H G D P E E R S R Q R H R E E K T K R Q V E E Q T E E K D R R Y Q H E  lentil
 85  - - - - - E Q D E R Q F P F P R P P H Q K E E R N E E E - - - D E D E E Q Q R E  soy a
 89  Q P H Q K E E E K H E W Q H K Q E K H Q G K E S E E E E E D Q D E D E E Q D K E  soy a'

 70  G E Q E W G T P G S H V R E E T S R N N P F Y F P S R R F S T R Y G N Q N G R I  peanut
111  G E E E E R S S - - - - - E S Q E H R N P F L F K S N K F L T L F E N E N G H I  pea
146  G K E E E T S S - - - - - E S Q E R R N P F L F K S N K F L T L F E N E N G H I  lentil
117  S E E S E D S E - - - - L R R H K N K N P F L F G S N R F E T L F K N Q Y G R I  soy a
129  S Q E S E G S E S Q R E P R R H K N K N P F H F N S K R F Q T L F K N Q Y G H V  soy a'
```

Figure 6: Amino acid alignment of the N-terminal part of (con)vicilin proteins from peanut (P43238), soybeans (P11827 (a’) and P13916 (a)), lentil (Q9M3X8), and pea (P13915).

The two deduced and four possible pepsin-resistant epitopes are situated on the N-terminal extension of mature Ara h 1, which is not present in many other (allergenic) vicilin proteins (*36*). It can be seen in Figure 5 that the N-terminal part of Ara h 1, comprising amino acids 1-80, is not present in the other vicilin proteins that were aligned (using the method described in chapter 5). It should be noted, however, that vicilin proteins with N-terminal extensions do occur (*37*). Some of these vicilins were previously denoted convicilins, but they in fact are to be considered as the α-polypeptides of trimeric vicilin proteins (*38*). Shorter vicilin polypeptides are generally denoted β-polypeptides (*39*). α-Polypeptides of vicilins have been identified in for example green peas (*38*), lentils, and soybeans (*39*). Alignment of the N-terminal part of known amino acid sequences of α-polypeptides from these sources with that of the amino acid sequence of Ara h 1 (Figure 6) showed that these extensions do not share high homology with the N-terminal extension of Ara h 1. More important, the 6 epitopes on the N-terminal part of Ara h 1 are not preserved in the N-terminal parts of the α-polypeptides displayed in Figure 6. These results thus indicate that the (possible) pepsin-resistant epitopes of Ara h 1 are unique for this vicilin. It might even be that the unique N-terminal extension of this protein likely explains why allergies to peanuts often cause severe, systemic reactions, compared to other legume allergies.

In line with the vicilin composition in soybeans, lentils, and peas, β-polypeptides could be part of peanut vicilin proteins as well. If so, these β-polypeptides would likely lack the

thus identified pepsin-resistant epitopes. It is recommended to investigate this and to investigate if breeding techniques could possibly be applied in order to obtain peanut variants lacking the α-polypeptide of Ara h 1, without disturbing the storage capacity of the peanuts. As in soybeans β-conglycinin trimers consisting only of β-polypeptides exist (*40*), this approach seems worthwile to be investigated.

REFERENCES

1. Alcocer, M. J. C., Murtagh, G. J., Bailey, K., Dumoulin, M., Sarabia Meseguer, A., Parker, M. J., Archer, D. B. The disulphide mapping, folding and characterisation of recombinant Ber e 1, an allergenic protein and SFA8, two sulphur-rich 2S plant albumins. *Journal of Molecular Biology* **2002,** 324, (1), 165-175.
2. Rabjohn, P., Helm, E. M., Stanley, J. S., West, C. M., Sampson, H. A., Burks, A. W., Bannon, G. A. Molecular cloning and epitope analysis of the peanut allergen Ara h 3. *Journal of Clinical Investigation* **1999,** 103, (4), 535-542.
3. Chapman, M. D., Smith, A. M., Vailes, L. D., Arruda, L. K., Dhanaraj, V., Pomes, A. Recombinant allergens for diagnosis and therapy of allergic disease. *Journal of Allergy and Clinical Immunology* **2000,** 106, (3), 409-418.
4. Chatel, J. M., Bernard, H., Orson, F. M. Isolation and characterization of two complete Ara h 2 isoforms cDNA. *International Archives of Allergy and Immunology* **2003,** 131, (1), 14-18.
5. 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. *Journal of Biological Chemistry* **1998,** 273, (22), 13753-13759.
6. 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. *Journal of Immunology* **2000,** 164, (11), 5844-5849.
7. 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. *Journal of Biological Chemistry* **1999,** 274, (8), 4770-4777.
8. Mol, J., Grotewold, E., Koes, R. How genes paint flowers and seeds. *Trends in Plant Science* **1998,** 3, (6), 212-217.
9. Vitale, A., Hinz, G. Sorting of proteins to storage vacuoles: how many mechanisms? *Trends in Plant Science* **2005,** 10, (7), 316-323.
10. Mehansho, H., Clements, S., Sheares, B. T., Smith, S., Carlson, D. M. Induction of proline-rich glycoprotein-synthesis in mouse salivary-glands by isoproterenol and by tannins. *Journal of Biological Chemistry* **1985,** 260, (7), 4418-4423.
11. Mehansho, H., Hagerman, A., Clements, S., Butler, L., Rogler, J., Carlson, D. M. Modulation of proline-rich protein-biosynthesis in rat parotid-glands by sorghums with high tannin levels. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences* **1983,** 80, (13), 3948-3952.
12. Hagerman, A. E., Rice, M. E., Ritchard, N. T. Mechanisms of protein precipitation for two tannins, pentagalloyl glucose and epicatechin$_{16}$ (4 -> 8) catechin (procyanidin). *Journal of Agricultural and Food Chemistry* **1998,** 46, (7), 2590-2595.
13. Charlton, A. J., Baxter, N. J., Khan, M. L., Moir, A. J. G., Haslam, E., Davies, A. P., Williamson, M. P. Polyphenol/peptide binding and precipitation. *Journal of Agricultural and Food Chemistry* **2002,** 50, (6), 1593-1601.

14. Wichers, H. J., De Beijer, T., Savelkoul, H. F. J., van Amerongen, A. The major peanut allergen Ara h 1 and its cleaved-off N-terminal peptide; Possible implications for peanut allergen detection. *Journal of Agricultural and Food Chemistry* **2004,** 52, (15), 4903-4907.

15. McEvily, A. J., Iyengar, R., Otwell, W. S. Inhibition of enzymatic browning in foods and beverages. *Critical Reviews in Food Science and Nutrition* **1992,** 32, (3), 253-273.

16. Prigent, S. V. E. Interactions of phenolic compounds with globular proteins and their effects on food-related functional properties. Ph.D. thesis, Wageningen University, Wageningen, The Netherlands, 2005.

17. Hussein, L., Abbas, H. Nitrogen-balance studies among boys fed combinations of faba beans and wheat differing in polyphenolic contents. *Nutrition Reports International* **1985,** 31, (1), 67-81.

18. Santos-Buelga, C., Scalbert, A. Proanthocyanidins and tannin-like compounds - nature, occurrence, dietary intake and effects on nutrition and health. *Journal of the Science of Food and Agriculture* **2000,** 80, (7), 1094-1117.

19. Hernandez, I., Alegre, L., Munne-Bosch, S. Enhanced oxidation of flavan-3-ols and proanthocyanidin accumulation in water-stressed tea plants. *Phytochemistry* **2006,** 67, (11), 1120-1126.

20. de Freitas, V., Mateus, N. Nephelometric study of salivary protein-tannin aggregates. *Journal of the Science of Food and Agriculture* **2002,** 82, (1), 113-119.

21. Pastorello, E. A., Farioli, L., Pravettoni, V., Ispano, M., Conti, A., Ansaloni, R., Rotondo, F., Incorvaia, C., Bengtsson, A., Rivolta, F., Trambaioli, C., Previdi, M., Ortolani, C. Sensitization to the major allergen of Brazil nut is correlated with the clinical expression of allergy. *Journal of Allergy and Clinical Immunology* **1998,** 102, (6), 1021-1027.

22. Bartolome, B., Mendez, J. D., Armentia, A., Vallverdu, A., Palacios, R. Allergens from Brazil nut: immunochemical characterization. *Allergology Immunopathology* **1997,** 25, (3), 135-144.

23. Asero, R., Mistrello, G., Roncarolo, D., Amato, S. Allergy to minor allergens of Brazil nut. *Allergy* **2002,** 57, (11), 1080-1081.

24. Koppelman, S. J., Knol, E. F., Vlooswijk, R. A. A., Wensing, M., Knulst, A. C., Hefle, S. L., Gruppen, H., Piersma, S. Peanut allergen Ara h 3: isolation from peanuts and biochemical characterization. *Allergy* **2003,** 58, (11), 1144-1151.

25. Bernhisel-Broadbent, J., Taylor, S., Sampson, H. A. Cross-allergenicity in the legume botanical family in children with food hypersensitivity.2. laboratory correlates. *Journal of Allergy and Clinical Immunology* **1989,** 84, (5), 701-709.

26. Boye, J. L., Ma, D.-Y., Harwalkar, V. R., Thermal denaturation and coagulation of proteins. In *Food proteins and their applications*, Damodaran, S.; Paraf, A., Eds. Marcel Dekker Inc.: New York, 1997; pp 25-26.

27. Creighton, T. E., *Proteins: Structures and molecular properties*. W.H. Freeman: New York, 1996.

28. Lakemond, C. M. M., de Jongh, H. H. J., Hessing, M., Gruppen, H., Voragen, A. G. J. Heat denaturation of soy glycinin: Influence of pH and ionic strength on molecular structure. *Journal of Agricultural and Food Chemistry* **2000,** 48, (6), 1991-1995.

29. Creusot, N., Gruppen, H., van Koningsveld, G. A., de Kruif, C. G., Voragen, A. G. J. Peptide-peptide and protein-peptide interactions in mixtures of whey protein isolate and whey protein isolate hydrolysates. *International Dairy Journal* **2006,** 16, (8), 840-849.

30. Mills, E. N. C., Jenkins, J. A. Structural, biological and evolutionary relationships of plant food allergens sensitizing via the gastrointestinal tract. *Critical Reviews in Food Science and Nutrition* **2004,** 44, (5), 379-407.

31. Mittag, D., Akkerdaas, J., Ballmer-Weber, B. K., Vogel, L., Wensing, M., Becker, W. M., Koppelman, S. J., Knulst, A. C., Helbling, A., S.L., H., Van Ree, R., Vieths, S. Ara h 8, a Bet

v 1-homologous allergen from peanut, is a major allergen in patients with combined birch pollen and peanut allergy. *Journal of Allergy and Clinical Immunology* **2004,** 114, (6), 1410-1417.

32. Mittag, D., Vieths, S., Vogel, L., Becker, W. M., Rihs, H. P., Helbling, A., Wuthrich, B., Ballmer-Weber, B. K. Soybean allergy in patients allergic to birch pollen: Clinical investigation and molecular characterization of allergens. *Journal of Allergy and Clinical Immunology* **2004,** 113, (1), 148-154.

33. 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. *Clinical and Experimental Allergy* **2006,** 36, (10), 1281-1288.

34. 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. *European Journal of Biochemistry* **1997,** 245, (2), 334-339.

35. 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. *Journal of Allergy and Clinical Immunology* **2004,** 113, (4), 776-782.

36. Barre, A., Borges, J. P., Rouge, P. Molecular modelling of the major peanut allergen Ara h 1 and other homotrimeric allergens of the cupin superfamily: a structural basis for their IgE-binding cross-reactivity. *Biochimie* **2005,** 87, (6), 499-506.

37. 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. *Biochemical Journal* **1988,** 251, (3), 717-726.

38. O'Kane, F. E., Happe, R. P., Vereijken, J. M., Gruppen, H., van Boekel, M. A. J. S. Characterization of pea vicilin. 1. Denoting convicilin as the alpha-subunit of the *Pisum* vicilin family. *Journal of Agricultural and Food Chemistry* **2004,** 52, (10), 3141-3148.

39. Brooks, J. R., Morr, C. V. Current aspects of soy protein fractionation and nomenclature. *Journal of the American Oil Chemists Society* **1985,** 62, (9), 1347-1354.

40. Sykes, G. E., Gayler, K. R. Detection and characterization of a new beta-conglycinin from soybean seeds. *Archives of Biochemistry and Biophysics* **1981,** 210, (2), 525-530.

SUMMARY

In order to induce systemic food allergic reactions in humans, IgE epitopes on allergens are required to remain intact upon digestion. The resistance of IgE binding capacity upon digestion is, therefore, considered as a prerequisite for proteins to cause systemic food allergic reactions. In this study it was aimed to determine the effect of heating on the structure and digestibility of class 1 food allergens and to determine the effect of digestion on the IgE binding capacity of these allergens. Cupin and prolamin allergens from peanuts, Brazil nuts, and soybeans were studied, because of their widespread abundance in foods (peanuts and soybeans) and / or the severity of the allergenic reactions they may cause in allergic persons (peanuts and Brazil nuts).

In chapter 1 a general introduction is presented, dealing with the definition of food allergy and the classification of food allergens based on their route of sensitization. Besides, the available data on cupin and prolamin allergens from peanuts, Brazil nuts and soybeans are discussed, with focus on heating and digestion and their effects on the IgE binding of these allergens. Moreover, the aim of this study is explained.

In chapter 2 a new, fast, large-scale purification method for Ber e 1, the major allergen from Brazil nuts, using expanded bed adsorption (EBA) chromatography, is presented. Using EBA, crude extracts can be applied to a fluidized column, which allows the unhindered passage of particulate impurities, thereby avoiding time-consuming centrifugation or filtration steps. With this newly developed purification method, gram quantities of Ber e 1 could be obtained from defatted Brazil nut meal, essentially within 1 day. Various structural as well as immunochemical characteristics of the purified protein were determined and were shown to be comparable to those of Ber e 1 purified using conventional chromatographic techniques. Moreover, it was shown that with the newly developed method, the complete pool of Ber e 1 isoforms was collected. The most abundant isoforms were observed to a have pI around 8 and heterogeneity was observed in both the large and the small subunit of the allergen.

Ber e 1 is known to be rather stable to peptic digestion. As heat-induced denaturation may affect protein digestibility, in chapter 3 the denaturation behaviour of Ber e 1 at different conditions is presented. It was shown that the denaturation temperature of Ber e 1 is high and varies from approximately 80 to 110 °C, depending on the pH. Upon heating above its denaturation temperature at pH 5.0, the protein on size exclusion chromatography (SEC) showed an elution behaviour similar to that of the unheated protein. Upon heating above the denaturation temperature at pH 7.0, Ber e 1 partly formed insoluble aggregates

and partly dissociated into its constituting polypeptides. The denaturation temperature of approximately 110 °C at pH values corresponding to the general pH values of foods (pH 5-7) is very high and is expected to be even higher in Brazil nuts themselves. As a result, it is unlikely that common heat processing causes the denaturation of all Ber e 1 present in food products. Consequently, the allergen is assumed to be consumed (mainly) in its native form, having a high stability towards peptic digestion. As a result, the investigation of the effects of heat denaturation on the digestibility and IgE binding capacity of Ber e 1 was considered not to be of prime relevance to the aim of this thesis.

In chapter 4 the quaternary structure of major peanut allergen Ara h 1 upon extraction is elucidated. Vicilin allergen Ara h 1 was previously described to occur as a stable trimeric protein. However, upon purification of native Ara h 1 from peanuts using only the mild purification technique size exclusion chromatography, the allergen appeared to exist in a larger oligomeric structure rather than in a trimeric structure. Subjecting the allergen to anion exchange chromatography, a technique that is commonly used for the purification of Ara h 1, induced the allergen to dissociate into trimers. Ammonium sulfate precipitation did not bring about any structural changes, whereas exposing the allergen to hydrophobic interaction chromatography caused it to partly dissociate into trimers, with increasing amounts of trimers at higher ionic strengths. The (partial) dissociation of Ara h 1 oligomers into trimers led to a change in the tertiary structure of the monomeric subunits of the allergen, with the monomers in Ara h 1 oligomers having a more compact tertiary structure compared with the monomers in Ara h 1 trimers.

In chapter 5 it is presented that Ara h 1 in its oligomeric complex interacts noncovalently with phenolic compounds of the proanthocyanidin type. These interactions are being disrupted during anion exchange chromatography, as this results in the dissociation of the oligomeric Ara h 1 complex into protein trimers and the binding of the proanthocyanidins to the column material. Oligomeric Ara h 1 is assumed to be the representative form of the allergen in which it is consumed by humans. By use of the known three-dimensional structure of β-conglycinin, a soy protein homologous to Ara h 1, proline-rich regions were observed *in silico* on both faces of the trimeric structure of this protein, which are conserved in Ara h 1. These proline-rich regions could explain the binding of proanthocyanidins to Ara h 1 and the formation of multiple Ara h 1 trimer complexes. This hypothesis was supported by the observation that the addition of peanut proanthocyanidins to trimeric Ara h 1 and to β-conglycinin resulted in the formation of soluble oligomeric protein complexes. The structurally related legumin proteins do not contain such proline-rich regions on both sides of the protein and proanthocyanidins were shown to have a lower affinity for legumin proteins from peanuts and soybeans (peanut allergen Ara h 3 and soy glycinin, respectively) than for the vicilin proteins from these sources.

As structural characteristics are important for a protein's allergenicity, the oligomeric structure of Ara h 1 was hypothesized to have a different allergenicity than trimeric Ara h 1. The study described in chapter 6 was, therefore, aimed at the investigation of the digestibility of heated and unheated Ara h 1 oligomers and Ara h 1 trimers. Moreover, the pepsin-susceptible and -resistant epitopes of Ara h 1 were determined. Oligomeric Ara h 1, either unheated or pre-heated, was hydrolyzed by pepsin at a lower rate than trimeric

Ara h 1 (either unheated or pre-heated), resulting in higher amounts of peptides with relatively high molecular masses. These relatively high molecular mass peptides were shown to be able to bind IgE, whereas peptides with lower molecular masses (<2 kDa) did not bind IgE. In these latter fractions, fragments of 15 previously published epitopes of Ara h 1 were identified. As a result, these epitopes are not likely to be responsible for the induction of systemic food allergic reactions to peanuts. 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 neither be confirmed, 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 shows no homology with other vicilin proteins. Consequently, this unique N-terminal part of Ara h 1 could very well be responsible for the allergen's ability to induce systemic allergic reactions.

In chapter 7 the effects of heating and digestion on the IgE binding of legumin proteins from soybeans (Ara h 3) and peanuts (glycinin) is described. Both allergens are relatively stable to denaturation, having denaturation temperatures ranging from 70 to 92 °C, depending on their quaternary structure and the ionic strength applied. Aggregates were formed upon heating, which were partly soluble for glycinin. Pre-heating the protein preparations slightly decreased the pepsin digestion rate of both allergens. However, heating did not affect the IgE binding capacity of the hydrolysates, as after only 10 minutes of hydrolysis no IgE binding could be detected any more in all samples. Peanut allergen Ara h 1, when digested under equal conditions, still showed IgE binding after 2 hours of hydrolysis, as was also shown in chapter 6. Our results indicate that the IgE binding capacity of legumin allergens from peanuts and soybeans does not withstand peptic digestion. Consequently, these allergens are likely unable to cause systemic food allergy symptoms. In view of these systemic food allergic reactions, these proteins might thus be less important allergens than was previously assumed.

Chapter 8 comprises a general discussion on the main results obtained in this study. Attention is especially paid to the importance of using relevant purification techniques in order to obtain representative allergen preparations. Besides, the different effects of heating and digestion on the IgE binding of cupin allergens are discussed in more detail. In addition, attention is paid to the (possible) pepsin-resistant epitopes of Ara h 1 and their presence on the unique N-terminal part of this allergen. In this respect, the amino acid sequence of Ara h 1 is compared to amino acid sequences of vicilin proteins from other sources.

SAMENVATTING

Om systemische voedselallergische reacties te kunnen veroorzaken moeten IgE-epitopen op allergene eiwitten intact blijven tijdens vertering. Het behoud van de IgE-bindingscapaciteit tijdens vertering wordt daarom beschouwd als een vereiste voor een eiwit om systemische allergische reacties te kunnen veroorzaken. Het doel van dit onderzoek was het bepalen van het effect van verhitten op de structuur en verteerbaarheid van klasse 1 voedselallergenen, en het bepalen van het effect van vertering op de IgE-bindingscapaciteit van deze allergenen. Allergene eiwitten uit pinda's, paranoten en sojabonen, behorende tot de cupine en prolamine superfamilies, werden bestudeerd. Er werd gekozen voor deze producten omdat deze veelvuldig gebruikt worden als ingredient in levensmiddelen (pinda's en sojabonen) en / of omdat ze zeer ernstige voedselallergische reacties kunnen veroorzaken (pinda's en paranoten).

In hoofdstuk 1 wordt een algemene inleiding gegeven over de definitie van voedselallergie en de klassificatie van voedselallergenen op basis van hun manier van sensitizeren. Daarnaast worden de bestaande gegevens over cupine en prolamine allergenen uit pinda's, paranoten en sojabonen beschreven. Hierbij ligt de nadruk op gegevens over verhitting en vertering en de effecten hiervan op de IgE-binding van deze allergenen. Daarnaast wordt in dit hoofdstuk het doel van dit onderzoek nader uitgelegd.

In hoofdstuk 2 wordt een nieuwe methode beschreven om Ber e 1, een belangrijk allergeen uit paranoten, op grote schaal te zuiveren. Hierbij wordt gebruik gemaakt van een bepaalde chromatografische techniek, expanded bed adsorption (EBA) genoemd. Als een constante vloeistofstroom wordt toegepast kan het EBA materiaal zich in een kolom verspreiden, wat resulteert in in een uitgezet bed waarop ruwe extracten kunnen worden opgebracht. Ongewenste deeltjes in deze extracten kunnen het bed ongehinderd passeren en zo worden verwijderd. Door het gebruik van EBA kunnen tijdrovende centrifugatie- en filtratiestappen worden voorkomen en met de nieuw ontwikkelde zuiveringsmethode kon Ber e 1 binnen een dag op gram-schaal worden gezuiverd uit ontvet paranootmeel. Verschillende structurele en immunochemische eigenschappen van het gezuiverde eiwit werden bepaald en het bleek dat deze eigenschappen vergelijkbaar waren met die van een Ber e 1 monster dat was gezuiverd met behulp van conventionele chromatografische technieken. Daarnaast bleek dat alle isovormen van Ber e 1 met de nieuw ontwikkelde methode werden gezuiverd. De meest voorkomende isovormen hadden pI waarden rond de 8 en heterogeniteit werd gevonden in zowel de grote als in de kleine polypeptide van het allergeen.

Het is bekend dat Ber e 1 behoorlijk stabiel is tegen vertering door pepsine. Omdat denaturatie als gevolg van hitte de verteerbaarheid van een eiwit kan beïnvloeden, wordt

in hoofdstuk 3 het denaturatiegedrag van Ber e 1 onder verschillende omstandigheden beschreven. De denaturatietemperatuur van Ber e 1 bleek hoog te zijn en varieert van ongeveer 80 tot 110 °C, afhankelijk van de pH. Na verhitten boven de denaturatietemperatuur bij pH 5.0 liet het eiwit met gel permeatie chromatografie een elutiegedrag zien dat gelijk was aan dat van het onverhitte eiwit. Tijdens verhitten boven de denaturatietemperatuur bij pH 7.0 vormde Ber e 1 gedeeltelijk onoplosbare aggregaten en viel gedeeltelijk uit elkaar in de polypeptiden waaruit het is opgebouwd. De denaturatietemperatuur van ongeveer 110 °C bij pH waarden die overeenkomen met de normale pH waarden van levensmiddelen (pH 5-7) is erg hoog. In paranoten kan de denaturatietemperatuur van Ber e 1 zelfs nog hoger zijn. Daarom is het onwaarschijnlijk dat een reguliere hittebehandeling de denaturatie van al het Ber e 1 aanwezig in levensmiddelen veroorzaakt. Dientengevolge wordt verwacht dat dit allergeen (voornamelijk) in zijn natieve vorm wordt geconsumeerd, welke een hoge stabiliteit heeft tegen vertering door pepsine. Het bestuderen van het effect van hittedenaturatie op de verteerbaarheid en IgE-bindingscapaciteit van Ber e 1 werd daarom als weinig relevant beschouwd voor het doel van dit promotieonderzoek.

In hoofdstuk 4 wordt de quaternaire structuur van het belangrijke pinda-allergeen Ara h 1 beschreven zoals het na extractie voorkomt. Eerder werd beschreven dat het viciline-eiwit Ara h 1 in een stabiele, trimere structuur voorkomt. Na zuivering van natief Ara h 1 uit pinda's, gebruik makende van alleen de milde zuiveringstechniek gel permeatie chromatografie, bleek echter dat het allergeen in een grotere, oligomere structuur voorkwam. Wanneer het allergeen werd onderworpen aan anionenwisseling chromatografie, een techniek die normaal gesproken gebruikt wordt voor de zuivering van Ara h 1, leidde dit tot de dissociatie van het oligomere complex in trimeren. Precipitatie met ammonium sulfaat zorgde niet voor veranderingen in de quaternaire structuur van Ara h 1. Het toepassen van chromatografie gebaseerd op hydrofobe interacties zorgde voor een gedeeltelijke dissociatie van Ara h 1 oligomeren naar trimeren, met grotere hoeveelheden trimeren bij een hogere zoutsterkte. De (gedeeltelijke) dissociatie van Ara h 1 oligomeren naar trimeren veroorzaakt een verandering in de tertiaire structuur van de monomere polypeptiden van het eiwit, omdat de monomeren in Ara h 1 oligomeren een compactere tertiaire structuur hebben dan de monomeren in Ara h 1 trimeren.

In hoofdstuk 5 wordt beschreven dat Ara h 1 in het oligomere complex niet-covalente interacties heeft met fenolische componenten van het proanthocyanidine type. Deze interacties worden verbroken tijdens anionenwisselingschromatografie. Deze techniek veroorzaakt de dissociatie van Ara h 1 oligomeren in trimeren en de binding van de proanthocyanidinen aan het kolommateriaal. Ara h 1 oligomeren worden beschouwd als de representatieve vorm van het allergeen zoals het wordt geconsumeerd door mensen.

In de drie-dimensionale structuur van β-conglycinine, een soja-eiwit dat homoloog is aan Ara h 1, werden proline-rijke regio's *in silico* geïdentificeerd aan twee kanten van de trimere structuur van dit eiwit. Deze proline-rijke regio's zijn ook aanwezig in Ara h 1 en zouden de binding van proanthocyanidinen aan Ara h 1 en de vorming van complexen die meerdere Ara h 1 trimeren bevatten, kunnen verklaren. Deze hypothese werd onderschreven door het feit dat de toevoeging van pinda-proanthocyanidinen aan Ara h 1

trimeren en aan β-conglycinine leidde tot de vorming van oplosbare, oligomere complexen. De structureel gerelateerde legumine-eiwitten hebben niet zulke proline-rijke regio's aan beide kanten van het eiwit. Proanthocyanidinen bleken ook een lagere affiniteit te hebben voor legumine-eiwitten uit pinda's en sojabonen (Ara h 3 uit pinda's en glycinine uit soja) dan voor viciline-eiwitten uit deze grondstoffen.
Omdat structurele eigenschappen belangrijk zijn voor de allergeniciteit van een eiwit zouden de oligomere en de trimere vorm van Ara h 1 kunnen verschillen in allergeniciteit. Daarom was het doel van het onderzoek beschreven in hoofdstuk 6 het bestuderen van de verteerbaarheid van verhitte en onverhitte Ara h 1 oligomeren en trimeren. Daarnaast was het doel om de pepsine-gevoelige en pepsine-resistente epitopen van Ara h 1 te bepalen. Ara h 1 oligomeren, zowel verhit als onverhit, werden langzamer gehydrolyzeerd door pepsine dan Ara h 1 trimeren (verhit en onverhit), wat resulteerde in grotere hoeveelheden peptiden met relatief hoge molecuulmassa's. Deze peptiden met relatief hoge molecuulmassa's konden IgE binden, terwijl peptiden met lagere molecuulmassa's (<2 kDa) geen IgE konden binden. In deze laatstgenoemde fracties werden fragmenten van 15 eerder gepubliceerde epitopen geïdentificeerd. Dientengevolge is het onwaarschijnlijk dat deze 15 epitopen verantwoordelijk zijn voor het veroorzaken van systemische allergische reacties tegen pinda's. Het kon worden herleid dat de IgE-bindende peptiden de eerder gepubliceerde intacte epitopen EDWRRPSHQQ (aminozuren 50-59) and PRKIRPEG (aminozuren 60-67) bevatten. De aanwezigheid van vier extra eerder gepubliceerde epitopen (overeenkomend met aminozuren 6-13, 14-21, 24-31 en 40-47) op de IgE-bindende, pepsine-resistente peptiden, kon niet worden bevestigd, noch uitgesloten. De twee herleide en vier mogelijke pepsine-resistente epitopen zijn allemaal gesitueerd op het N-terminale deel van Ara h 1, dat geen homologie vertoont met andere viciline-eiwitten. Het zou daarom heel goed kunnen dat het unieke N-terminale deel van Ara h 1 verantwoordelijk is voor de capaciteit van dit allergeen om systemische allergische reacties te veroorzaken.
In hoofdstuk 7 worden de effecten van verhitting en vertering op de IgE-binding van legumine-allergenen uit pinda's (Ara h 3) en sojabonen (glycinine) beschreven. Beide allergenen zijn relatief stabiel tegen denaturatie en hebben denaturatietemperaturen tussen de 70 en 92 °C, afhankelijk van de quaternaire structuur van de eiwitten en de zoutsterkte. Aggregaten werden gevormd tijdens verhitten, en deze waren in het geval van glycinine gedeeltelijk oplosbaar. Verhitting voorafgaand aan vertering zorgde voor een lichte afname van de afbraaksnelheid door pepsine, maar verhitting voorafgaand aan vertering had geen effect op de IgE-bindingscapaciteit van de hydrolyzaten, omdat al na 10 minuten verteren geen IgE binding meer kon worden gedetecteerd in alle monsters. Wanneer pinda-allergeen Ara h 1 onder gelijke omstandigheden werd verteerd werd er nog IgE-binding gedetecteerd na twee uur incubatie. Dit wordt ook beschreven in hoofdstuk 6. Deze resultaten duiden aan dat de IgE-bindingscapaciteit van legumine-allergenen uit pinda's en sojabonen niet bestand is tegen vertering door pepsine. Als gevolg hiervan lijken deze allergenen niet in staat te zijn om systemische voedselallergische symptomen te veroorzaken. Wat betreft deze systemische reacties zouden deze eiwitten dus minder belangrijk kunnen zijn dan eerder werd aangenomen.

Hoofdstuk 8 bevat een algemene discussie over de belangrijkste resultaten die werden verkregen tijdens dit onderzoek. Aandacht wordt besteed aan het belang van het gebruik van relevante zuiveringstechnieken om representatieve allergeen-monsters te verkrijgen. Daarnaast worden de verschillende effecten van verhitten en verteren op de IgE-binding van cupine allergenen gedetailleerd bediscussieerd. Ook wordt aandacht besteed aan de (mogelijke) pepsine-resistente epitopen van Ara h 1 en hun aanwezigheid op het N-terminale gedeelte van dit allergeen. Wat dit laatste betreft wordt de aminozuursequentie van Ara h 1 vergeleken met aminozuursequenties van viciline-eiwitten uit andere producten.

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Evelien

CURRICULUM VITAE

Evelien Louise van Eijk-van Boxtel werd geboren op 19 september 1980 in Arcen en Velden. In 1998 behaalde ze haar VWO diploma aan het Collegium Marianum in Venlo. Daarna begon ze aan de opleiding Levensmiddelentechnologie aan de Wageningen Universiteit en volgde de afstudeerrichting Levensmiddelenchemie. Ze voerde afstudeeronderzoeken uit bij de leerstoelgroep Levensmiddelenchemie en bij de leerstoelgroep Productontwikkeling en Kwaliteitskunde. Haar afstudeerstage deed ze bij Unilever Research in Vlaardingen. In september 2003 studeerde ze af, waarna ze in oktober 2003 als promovendus in dienst trad bij de leerstoelgroep Levensmiddelenchemie van de Wageningen Universiteit. Hier voerde ze het in dit proefschrift beschreven werk uit. Vanaf november 2007 is Evelien werkzaam als senior nutrition scientist bij Mars Nederland in Veghel.

LIST OF PUBLICATIONS

van den Broek, L. A. M., van Boxtel. E. L., Kievit, R. P., Verhoef, R., Beldman, G., Voragen, A. G. J. Physico-chemical and transglucosylation properties of recombinant phosphorylase from *Bifidobacterium adolescentis* DSM20083. *Applied Microbiology and Biotechnology* **2004**, 65, (2), 219-227.

van Boxtel, E. L., van Koningsveld, G. A., Koppelman, S. J., van den Broek, L. A. M., Voragen, A. G. J., Gruppen, H. Expanded bed adsorption as a fast technique for the large-scale purification of the complete isoform pool of Ber e 1, the major allergen from Brazil nuts. *Molecular Nutrition & Food Research* **2006**, 50, (3), 275-281.

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. *Journal of Agricultural and Food Chemistry* **2006,** 54, (19), 7180-7186.

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. *Journal of Agricultural and Food Chemistry* **2007**, 55, (21), 8772-8778.

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. *Molecular Nutrition and Food Research* in press.

van Boxtel, E. L., van den Broek, L. A. M., Koppelman, S. J., Gruppen, H. Determination of pepsin-susceptible and pepsin-resistant epitopes of native and heat-treated peanut allergen Ara h 1. *Submitted*

van Boxtel, E. L., Koppelman, S. J., van den Broek, L. A. M., Gruppen, H. Heat denaturation of Brazil nut allergen Ber e 1 in relation to food processing. *Submitted*

OVERVIEW OF COMPLETED PhD TRAINING ACTIVITIES

DISCIPLINE SPECIFIC ACTIVITIES
Industrial proteins, VLAG, Wageningen, 2003
Protein engineering, VLAG, Wageningen, 2004
Food related allergies and intolerances, WUR, Wageningen, 2004
European food allergy congres, EAACI, Budapest, 2004
Conference Allergy Matters!, WUR, Wageningen, 2004
Symposium Towards sustainable protein supply chains, WUR, Wageningen, 2004
Food Chemistry PhD trip, Japan, 2004
Ecophysiology of the gastro-intestinal tract, VLAG & WIAS, Wageningen, 2005
Expanded bed adsorption chromatography, Upfront chromatography a/s, Copenhagen, 2005
Summer school food & health, Paris, 2005
Food Chemistry PhD trip, Belgium, France, England, 2006

GENERAL COURSES
VLAG PhD week, Bilthoven, 2004
Scientific writing, Wageningen, 2004
Introductie probleemgestuurd onderwijs voor tutoren, Wageningen, 2004
Time planning and project management, Wageningen, 2005

ADDITIONAL ACTIVITIES
Preparation of the PhD research proposal, 2003
Food chemistry seminars, 2003-2007
Food chemistry colloquia, 2003-2007