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Evaluating pH-Induced Gastrointestinal Aggregation of *Arachis hypogaea* 1 Fragments as Potential Components of Peanut Allergy

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Supporting Information

ABSTRACT: The seed storage glycoprotein *Arachis hypogaea* (Ara h) 1 is a major allergen found in peanuts. The biochemical resistance of food proteins to protease digestion contributes to their allergenicity. The rapid proteolysis of Ara h 1 under gastric conditions challenges this model. Biophysical and in vitro digestion experiments were carried out to identify how Ara h 1 epitopes might survive digestion, despite their facile degradation. The bicupin core of Ara h 1 can be unfolded at low pH and reversibly folded at higher pH. Additionally, peptide fragments from simulated gastric digestion predominantly form noncovalent aggregates when transferred to base. Disulfide cross-links within these aggregates occur as intermediates in relatively low amounts only at early times and play no role in shielding peptides from degradation. It is proposed that peptide fragments which survive gastric conditions form large aggregates in basic environments such as the small intestine, making epitopes available for triggering an allergic response.

KEYWORDS: *Arachis hypogaea 1, pH aggregation, digestion, allergy, peanut*

INTRODUCTION

Food allergies are a major public health challenge, affecting 6−8% of children under the age of four (www.niaid.nih.gov). The highest frequencies of sensitivities come from components in milk, eggs, fish, shellfish, soy, tree nuts, wheat, and peanuts. Nuts, in particular, are among the leading cause of severe or fatal allergic reactions. The primary strategy for managing food allergies is avoidance, but accidental exposure is difficult to prevent. Food sensitivities are frequently caused by a specific set of proteins. Understanding how the physical and chemical properties of these proteins pertain to sensitization and elicitation of allergies is important in the attempted development of effective therapies.

An unanswered question in the field of food allergy research is "Why do certain proteins elicit an IgE-mediated immune response, while other proteins are tolerated?" One compelling hypothesis is the existence of a link between digestibility and allergenicity. Proteolysis of proteins into peptide fragments smaller than 3−5 kDa can significantly reduce their ability to induce an immune response.[1−3] Astwood and colleagues demonstrated that nonallergens such as spinach ribulose bisphosphate carboxylase/oxygenase (rubisco), potato phosphofructokinase, and barley β-amylase were significantly degraded in vitro in simulated gastric conditions within 15 s. On the other hand, known food allergens such as soybean β-conglycinin and peanut *Arachis hypogaea* (Ara h) 2 were stable for an hour or longer.[4] However, several subsequent studies found poor or nonexistent correlations between protein digestibility and their classification as allergens or nonallergens.[5−8] This hypothesis continues to be actively debated.

To investigate the structural nature of allergens during digestion, we focus on a peanut vicilin, Ara h 1 (a seed storage protein), because it is a key immunodominant allergen recognized in >90% of peanut-sensitive individuals.[9] The vicilin proteins are primarily β-sheet proteins (Figure 1a). The β-sheets form a cup-shaped six-stranded β-barrel called the cupin. The Ara h 1 monomer (62 kDa glycoprotein) consists of two tandem cupin folds (bicupin), where three bicupins assemble to form a highly stable Ara h 1 homotrimer. The oligomerization of bicupins into higher order assemblies is hypothesized to be the mechanism for stabilization with a trimer-to-monomer dissociation at ∼50 °C followed by full denaturation at 85 °C.[10] In addition to interactions between bicupins, the homotrimer is stabilized by the coupling of small α-helical "handshake domains" that pack through hydrophobic interactions.[11] Deletion of these domains in a related species bean phaseolin was shown to completely disrupt trimerization.[12] Many key IgE binding epitopes are found in this region, occluded by monomer−monomer contacts.[13,14] Thus, oligomerization may play an important role in shielding these epitopes from proteolysis during gastric processing. Ara h 1 has been shown to form much larger oligomers, which may have further implications for its allergenicity.[15,16]

In this study, we examine the in vitro aggregation behavior of Ara h 1 in environments that simulate changing acidity levels that digesting foods experience in passing from the acidic stomach to the basic intestines. It has been shown in multiple studies that Ara h 1 is rapidly proteolyzed into smaller fragments when subjected to in vitro conditions simulating

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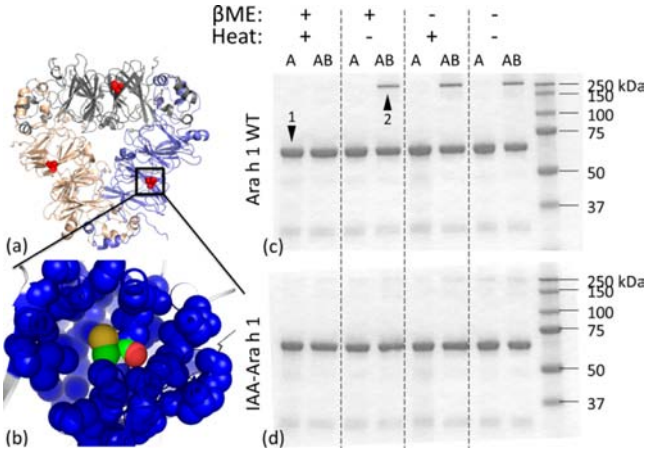

Figure 1. (a) Ara h 1 homology model after loop remodeling and molecular dynamics minimization techniques were used to generate missing regions in the crystal structure (PDB ID 3S7I [22]) from the Protein Data Bank. The homology structure has an alignment RMSD of 0.9 Å with the crystal structure. A single Cys 435 (colored as red, green, and yellow atoms) is shielded by surrounding residues (depicted as blue spheres) in the bicupin fold as shown in (b). SDS-PAGE at conditions ± βME and ± heat for Ara h 1 WT (c) and IAA-Ara h 1 (d) in acidic (A) and acidic-to-basic (AB) environment shows the migration of two distinct bands. Arrows highlight monomer (band 1), and an intermediate protein aggregate (band 2) that is abolished when cysteine residues are capped with IAA.

gastric fluid. Therefore, a mechanism whereby the tertiary and quaternary protein structure prevents proteolysis and preserves epitopes may not apply in the case of peanut allergy. Instead, it has been suggested that aggregation of protein fragments during digestion may preserve immunogenic components.[17] Aggregated peptides may be protected from complete digestion in the small intestine, allowing their absorption and leading to sensitization or elicition of IgE-mediated allergic reactions.[18,19] Food processing conditions such as dry roasting or the boiling of peanuts result in different conformational changes in Ara h 1, and both processes cause protein aggregation.[10,20] As the bicupin region of Ara h 1 is purportedly more stable against heat, this leaves the α-helices and unstructured domains of the protein more prone to unfolding and aggregation.[21] In relation to the digestion of Ara h 1, peptide fragments with sizes <2 kDa can aggregate to sizes of ~20 kDa, and these aggregates have sensitization capacity as shown in an animal model.[19] These studies by Bogh et al. are excellent as they provide insight into the survival of short peptides from the digestion process. In our study, we clarify the conditions under which such peptide fragments can survive via their potential to form aggregates. We also demonstrate that the bicupin core of Ara h 1 is not stable at low pH, and its ensuing unfolding is likely involved in rapid digestion of the protein.

MATERIALS AND METHODS

Purification of Ara h 1 from Peanuts. Peanuts of the Tifguard variety[23] were provided by the USDA-ARS Peanut Research Laboratory, Dawson, GA, USA. Mature Ara h 1 wild type (WT) was extracted and purified using the method of Maleki[13] with minor modification. A total of 70 g of ground peanuts was stirred in 200 mL of hexane for 2 h at 4 °C. The solid defatted peanut meal was air-dried overnight at room temperature and then stirred overnight at 4 °C in 500 mL of extraction buffer [50 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid, 200 mM sodium chloride (NaCl), 5 mM dithiothreitol (DTT), and 1 mM phenylmethanesulfonyl fluoride; pH 8.3]. The mixture was clarified by centrifugation (12000*g*, 30 min, 4 °C) and then subjected to sequential protein precipitations at 70 and 100% ammonium sulfate. The protein pellet was recovered by centrifugation, dissolved in approximately 20 mL of 50 mM Tris-HCl buffer at pH 8.3, and dialyzed against 50 mM Tris-HCl at pH 8.3 (four buffer exchanges of 1000 mL each every 12 h at 4 °C). The dialyzed solution of Ara h 1 WT was loaded on an anion exchange column (2.5 × 8.5 cm; MacroPrep High Q Support, Bio-Rad, Hercules, CA, USA), followed by sequential washing of the column and elution of protein fractions with increasing NaCl in 50 mM Tris-HCl buffer (200, 300, and 400 mM NaCl). Purified Ara h 1 was eluted from the column at 300 mM NaCl in 50 mM Tris-HCl, and the collected fractions were pooled to give a final volume of approximately 16 mL. The purified Ara h 1 solution was dialyzed against 20 mM sodium phosphate, pH 8, buffer (four buffer exchanges of 1000 mL each every 12 h at 4 °C) and then stored in aliquots at −20 °C for later use. This method produced Ara h 1 WT with a purity >95% as determined by high-performance liquid chromatography (refer to the Supporting Information). The molecular weight of Ara h 1 was confirmed by mass spectroscopy at the Biological Mass Spectroscopy Facility at Rutgers University, Piscataway, NJ, USA, and the mature form of Ara h 1was confirmed by N-terminal sequencing at The University of Texas Medical Branch, Biomolecular Resource Facility, Galveston, TX, USA.

Preparation of Iodoacetic Acid (IAA)−Ara h 1. Mature Ara h 1 WT was mixed with guanidine hydrochloride (GdHCl) and DTT dissolved in 50 mM Tris (pH adjusted to 8) to give final concentrations of 20.6 μM Ara h 1, 5.3 M GdHCl, and 20 mM DTT. The mixture was incubated for 30 min at 60 °C. Following the incubation, a stock solution of IAA (400 mM IAA in 50 mM Tris; pH adjusted to 7.8 with sodium hydroxide) was added to the mixture to give a final concentration 40 mM IAA, and the mixture was incubated for an additional 30 min at room temperature in the dark with gentle rocking. The mixture was then serially dialyzed against GdHCl (in decreasing concentrations: 2, 1, 0.5, 0.25, 0.1, and 0 M) in 20 mM sodium phosphate, pH 8. The efficiency of cysteine capping in IAA−Ara h 1 was estimated at ~98% by mass spectrometry (refer to the Supporting Information).

In Vitro Partial Digestion of Ara h 1 by Pepsin. Ara h 1 (WT or IAA-modified) was incubated in 0.2 M hydrochloric acid (HCl)−potassium chloride (KCl), pH 2, for 1 h, followed by the addition of pepsin A (Worthington Biochemical Corp., Lakewood, NJ, USA) to give 0.005 pepsin unit/μg Ara h 1. Aliquots of the mixture were inhibited with either pepstatin (3× dry weight pepsin; final pH ~2; acidic environment) or 0.2 M sodium bicarbonate (final pH ~8; acidic-to-basic environment) at time points of 0, 1, 2, 5, 10, 15, and 30 min. All steps were carried out at 4 °C.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE). Digestion sample aliquots were prepared under nonreducing conditions [no β-mercaptoethanol (βME) and no heat] by mixing with an equal volume of Laemelli sample buffer (Bio-Rad). In addition, nondigested samples (t = 0) were evaluated in the presence (+) or absence (−) of βME in Laemelli sample buffer and heat (95 °C for 5 min). All samples were run on a precast polyacrylamide gel (Any kD Mini-PROTEAN TGX, Bio-Rad) and stained with Coomassie blue (Life Technologies Corp., Grand Island, NY, USA).

Size Exclusion Chromatography (SEC) of Ara h 1 Digestion Products. Digestion samples were separated on an AKTA FPLC system (GE Healthcare Lifesciences) using a Superdex 200 10/300 GL column that was pre-equilibrated with either acidic (200 mM HCl−KCl, pH ~3) or basic (200 mM sodium phosphate, pH 8) buffer at 4 °C. A volume of 200 μL of digested Ara h 1 (0.5 mg/mL) was loaded onto the column, and the buffer running volume was set to 25 mL at a flow rate of 0.7 mL/min. Absorbance spectra were collected at 280 nm.

Circular Dichroism (CD) Spectra of Ara h 1 at Various pH Values. Ara h 1 (WT or IAA-modified) was mixed with the following buffers for 6 h minimum at room temperature: pH 1−2 (0.2 M HCl−KCl), pH 3−5 (0.2 M citrate−phosphate), and pH 6−8 (0.2 M sodium phosphate). CD wavelength scans were performed on an

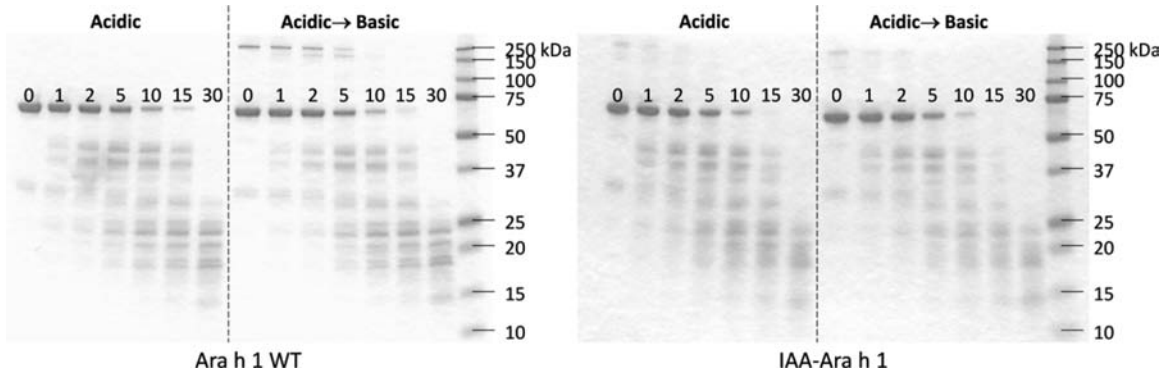

Figure 2. Nonreducing SDS-PAGE shows Ara h 1 dissociated into monomer (62 kDa) and the presence of larger aggregates (~250 kDa) formed in acidic-to-basic environment. Ara h 1 is digested at low pH at time points shown (in minutes), followed by pepsin inhibition with pepstatin (Acidic) or with sodium bicarbonate (acidic→basic). Refer to text for details.

AVIV model 420SF spectrophotometer (Aviv Biomedical, Lakewood, NJ, USA) from 190 to 260 nm (single scan, 10 s averaging) at 25 °C. Buffer blank subtraction was performed for each sample, and the molar residual ellipticity (MRE) was calculated by correcting for concentration, sequence length, and cell path length. Spectral baselines were zeroed at 250−260 nm where no signal was present. A high dynode voltage is indicative of unreliable data, which can occur at low wavelengths (<200 nm) for some of these buffers. CD data are presented for dynode voltages <650 V, which is within the capability of this machine.

RESULTS AND DISCUSSION

Aggregate Intermediates of Ara h 1 Are Formed in Transitioning from Acidic to Basic Environment. To characterize the effect of changing acidity on Ara h 1 WT, we treated the protein with two environments: (i) pH 2 to simulate stomach acidity and (ii) a change in acidity from pH 2 to a basicity of pH 8 to simulate solution expulsion from the stomach to the small intestine. The protein samples were analyzed by SDS-PAGE under conditions of ±βME and ±heat (Figure 1c). Under reducing conditions (+βME/+heat), Ara h 1 WT is unfolded by heat and any disulfide bonds (if present) are reduced by βME. Additionally, all hydrophobic interactions in the protein are disrupted by the presence of SDS. The characteristic strong band for Ara h 1 monomer at 62 kDa is shown in the figure. The weak band at ~30 kDa is a fragment of Ara h 1 that is typically difficult to remove during purification, and all other weak bands are protein impurities. Under reducing conditions, there is no difference between the two environments of acidic and acidic-to-basic. However, for nonreducing conditions (−βME/−heat), a relatively strong band at 250 kDa is observed for the acidic-to-basic environment. The existence of both protein bands implies that Ara h 1 WT, which is partially unfolded in acid, may form two subpopulations of aggregated species. The first, a relatively high amount of aggregates, is likely held together by hydrophobic interactions that can be disrupted by SDS (band at 62 kDa). The second is a subpopulation of lower amounts of aggregates that is likely cross-linked by disulfide bridges (band at 250 kDa). Further evidence for the aggregation states of the 62 and 250 kDa bands is given by SEC data (described later) and SDS-PAGE data on IAA-modified Ara h 1 (described below), respectively. N-Terminal sequencing revealed that both protein bands had RSPPGE (single-letter amino acid nomenclature), which is the starting sequence for mature Ara h 1, and mass spectroscopy analysis showed that Ara h 1 was the predominant protein in both bands. Finally, as the presence of the 250 kDa protein band at +βME/−heat and −βME/+heat is confounded, we cannot determine whether heat or covalent cross-linking involving cysteine is responsible for forming this intermediate aggregate.

To resolve the issue of whether the 250 kDa aggregate is due to heat or disulfide bridging, we irreversibly alkylated the sulfhydryl groups of all cysteine residues in the protein solution (Ara h 1 and impurities) using IAA. The IAA−Ara h 1 was then subjected to acidic and acidic-to-basic environments. The resulting SDS-PAGE demonstrates the absence of the 250 kDa band (Figure 1d). We conclude that an intermediate aggregate at 250 kDa is formed from covalent bonding between cysteine residues. Because each mature Ara h 1 monomer contains only one cysteine (Cys 435), protein dimerization is only possible among unfolded monomers or with cysteine-containing protein impurities in solution. It is possible that the band at 250 kDa represents a noncompact dimer that migrates slowly during electrophoresis.

Ara h 1 Is Partially Unfolded in Acidic Environment. Ara h 1 WT is known to exist as a stable trimer at pH 7−8 and may remain in this conformation at pH 2 for short times.[13,14] Therefore, we measured the secondary structure of Ara h 1 WT at room temperature under various pH values for times >6 h (Figure 3) and observed the following changes. At pH 6−8, the structure is similar to literature spectra of the native form,

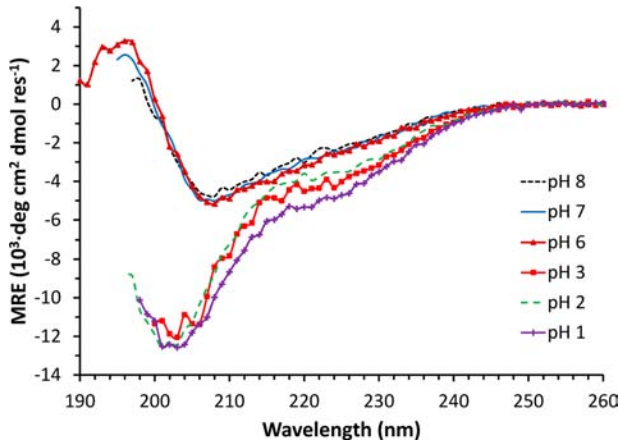

Figure 3. Native secondary structure of Ara h WT occurs at pH 6−8. Partial protein unfolding occurs in acidic environment (pH 1−3). No CD spectra were obtained for pH 4−5 due to precipitation of the protein.

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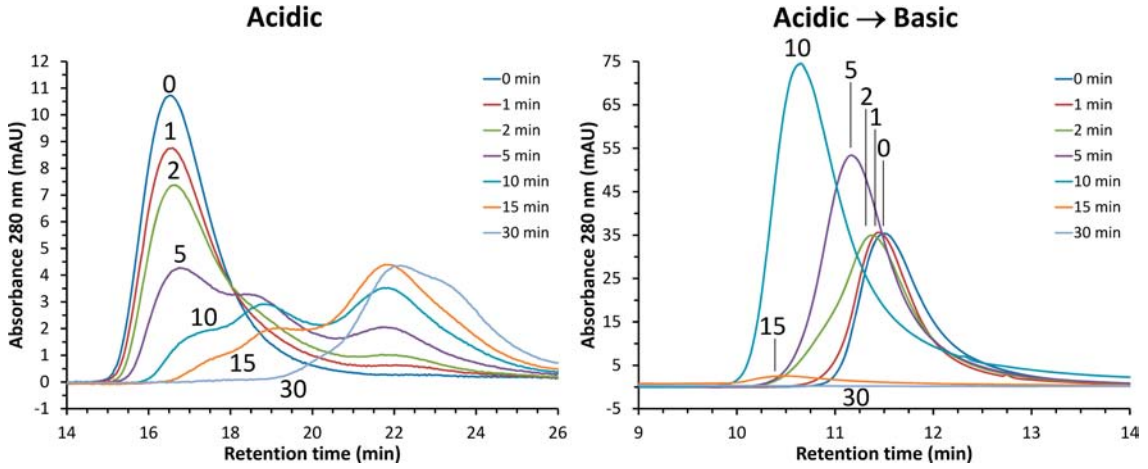

Figure 4. Elution profiles of Ara h 1 by SEC after various digestion times followed by acidic (left) and acidic-to-basic (right) treatment. Shorter retention times indicate larger aggregate formation in going from acid to base.

where the CD data manifest a main negative peak at 208 nm and slight negative shoulder peak at ∼219 nm.[10] Peak locations from our CD data were determined by multipeak curve fitting with Igor Pro software (Wavemetrics Inc., Lake Oswego, OR, USA). In contrast, Ara h 1 WT in an acidic environment at pH 1−3 shows partial unfolding of its secondary structure. A strong negative peak is manifested at 202 nm with a pronounced negative shoulder peak at 222 nm. These spectra are typical of proteins having a mixture of only α-helices and random coils. CD spectra deconvolution gave estimates of 33 ± 3% α-helix and 67 ± 3% random coil. The absence of β-sheet implies that acid disrupts the bicupin. No CD spectra were obtained for pH 4−5 due to protein precipitation that occurs near its isoelectric point (p*I* ∼4.5).

We also investigated whether the secondary structure of IAA−Ara h 1 was different from the WT. The structure of the cysteine-capped protein is perturbed relative to WT as shown by overlaying the two CD spectra (Figure S3, Supporting Information). Additionally, both proteins possess different retention times as measured by SEC in pH 8 buffer (no prior exposure to acid). The retention time for Ara h 1 WT homotrimer is ∼16.2 min. The retention time for IAA−Ara h 1 is ∼11.7 min, which indicates a much larger structure. Interestingly, this retention time is similar to that of aggregated Ara h 1 (compare to t = 0 in Figure 4 and Figure S3 in the Supporting Information). The partial unfoldings of WT and IAA-modified Ara h 1 in acid appear dissimilar (compare spectra in Figure 3 with Figure S4 in the Supporting Information). First, CD scans at pH 6 could not be obtained as protein precipitation prevented measurement, indicating that the p*I* of IAA−Ara h 1 may have shifted from 4.5 to a value of 5−6. Second, peak intensities of IAA−Ara h 1 were lower. Third, peak locations were different for IAA−Ara h 1, indicating unfolding at all pH values; there is a main negative peak at ∼203−205 nm and a slight negative shoulder peak at ∼222 nm. Despite these perturbations in structure, SEC measurements reveal that both proteins have comparable sizes in acidic and acidic-to-basic environments (compare t = 0 samples in Figure 4 versus Figure S5 in the Supporting Information). Any major structural differences in WT versus IAA-modified Ara h 1 may be abolished when the protein is acidified and perturbed when the solution is changed back to basic.

Characterization of Early Digestion Fragments of Ara h 1. The early stages of digestion of Ara h 1 (WT and IAA-modified) were examined under conditions that slowed the rate of proteolysis. These conditions included the use of low temperature (4 °C) and a low pepsin-to-Ara h 1 ratio of 0.005 unit pepsin/μg substrate protein that was not clinically relevant, but allowed a determination of how the protein was methodically digested into peptide fragments. In fact, even under these stringent conditions, Ara h 1 digestion remained relatively rapid. A typical ratio used by researchers for in vitro pepsin digestion of Ara h 1 is 10 units pepsin/μg substrate protein.[24]

The SDS-PAGE of partially digested Ara h 1 (WT and IAA-modified) demonstrates transient accumulation of peptide fragments at the different times of digestion (Figure 2). For example, the peptide band at ∼37 kDa reaches its peak intensity at 5−10 min and becomes undetectable by 30 min. There are minor differences in digestibility between WT and IAA-modified protein: (i) in acidic-to-basic environment, cross-linked aggregates at 250 kDa appear much weaker in intensity or are absent for IAA-modified protein compared to the WT; and (ii) the rate of digestion of IAA−Ara h 1 appears somewhat faster as the 62 kDa protein bands are difficult to visualize at 15 min compared to the WT. The digestion of Ara h 1 WT after 30 min (under these slow proteolytic conditions) destroyed any ability of the protein to form intermediate aggregates as evidenced by the nonexistence of the 250 kDa band at this time for acidic-to-basic environment. We conclude that these intermediate aggregates play no role under clinically relevant pepsin digestion.

Ara h 1 Digestion Fragments Form Aggregates in Transitioning from Acidic to Basic Environment. One disadvantage of using SDS-PAGE to analyze digestion products is that SDS disrupts higher order structures such as aggregates. SEC was therefore used to qualitatively determine the relative sizes of protein/peptide species present during digestion in acid, as well as the relative species sizes of aggregates formed with the change from acid to base. The digestion profiles of Ara h 1 WT under these two environments are shown in Figure 4. The retention time for undigested Ara h 1 WT in acid is ∼16.5 min, which is similar to the retention time of ∼16.2 min for the stable homotrimer at pH 8 with no previous acid exposure. The disappearance of this peak during digestion corresponds to the

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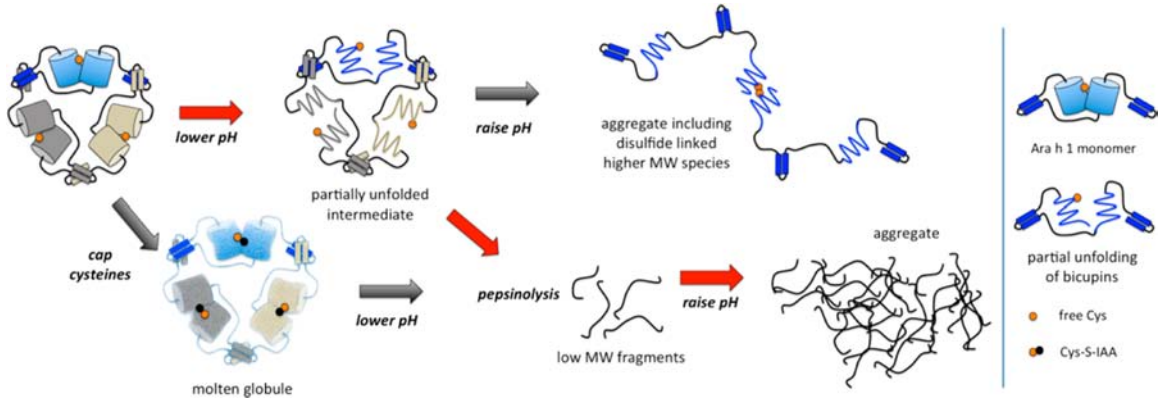

Figure 5. Model for Ara h 1 degradation. A summary of the changes in higher order structures of Ara h 1 under conditions of changing pH, digestion, and residue modification. Red arrows indicate path modeling natural digestion.

appearance and growth of peptide fragment peaks at longer retention times (Figure 4, left plot). Longer retention times indicate smaller peptide fragments, but it is difficult to determine which peaks correspond to the bands observed by SDS-PAGE (Figure 2) without analyzing fractions by mass spectroscopy. In contrast, Ara h 1 WT and its digestion products aggregate when the environment is changed from acidic to basic (Figure 4, right plot). The short retention time of 11.5 min for Ara h 1 WT (t = 0 min; Acidic→Basic) indicates very large structures relative to the homotrimer. In addition, the aggregate peaks shift to the left from 11.5 to 10.6 min, indicating continued growth. The increase in relative peak absorbance as they shift to the left may result from a change in the absorption coefficient of the fragmented peptides. The low absorbance at times >10 min is due to very large aggregates, >1300 kDa (which is the SEC column exclusion limit), becoming trapped on the column. This protein mass was able to be washed off the column with acidic buffer, indicating that the resolubilized aggregates are held together by noncovalent forces, such as hydrophobic interactions.

The retention times for IAA−Ara h 1 digestion products determined by SEC have minor differences compared to those of WT (Figure S5, Supporting Information). These results support the finding that disulfide bridging plays no role in aggregating digested peptides.

Model for Ara h 1 Digestion. Ara h 1 is a rapidly digestible protein yet it still has the capability to cause sensitization and IgE-mediated allergic reactions in humans. We investigated the structural changes of Ara h 1 in acid and found that the purportedly stable bicupin core is readily unfolded and can be refolded in base (with small perturbation in structure) to its tertiary structure with concomitant formation of soluble aggregates. This interesting behavior underlies an important observation: that partially digested peptide fragments can also aggregate in going from acid to base, forming even larger structures than intact aggregated protein. It is conceivable that the inner mass of such structures can be protected from further digestion in the small intestine. Digestion by trypsin and chymotrypsin in the small intestine is an obvious way for peptide fragments to escape the aggregation. Because the residence time for absorption is short (approximately 15 min), these proteolytic enzymes may only have time to loosen the aggregate and partially digest its constituents. Under these conditions, it may be likely that immunogenic peptide fragments can be absorbed by the Peyer's patches of the small intestine.

Structural insight into how peptide fragments aggregate during digestion is crucial for developing a molecular level understanding of food allergy. These experimental studies support a model where Ara h 1 becomes partially unfolded at low pH, leading to rapid pepsinolysis. Subsequent rising of the pH drives aggregation of the fragments (Figure 5). The partially unfolded state does not denature completely under acidic conditions in the absence of pepsin, and the protein can be refolded (with small perturbation in structure) by raising the pH. The presence of partial α-helical structure at low pH suggests the helical handshake domains are kept intact under these conditions. The bicupin β-sheet region is easily perturbed, as shown by chemical modification of Cys, which results in a molten globule-like state under neutral conditions, where secondary structure is maintained but the volume of the complex as assessed by SEC is increased.[25] Preventing disulfide-linked complexes from forming does not affect digestion, indicating that preventing cross-linking of this food protein is not a viable method for improving food safety, as has been found for other food-derived allergens.[26]

Several questions remain unanswered, including how to determine the extent of protection of these peptide fragments (i.e., what is the structure of the aggregates?), how to show that proteolytic enzymes of the small intestines can cause peptide escape from the aggregate leading to absorption by Peyer's patches, and whether short sequences in Ara h 1 can be engineered to act as aggregate disrupters. We can also examine a wide range of other allergenic proteins for their propensity to form aggregates following partial gastric digestion to ascertain whether the phenomenon of pH-induced gastrointestinal aggregation can be generalized.

■ ASSOCIATED CONTENT

Supporting Information

Determination of Ara h 1 WT purity by high-performance liquid chromatography; determination of IAA-capping efficiency of Ara h 1 by mass spectroscopy; reversibility of Ara h1 WT in acid; characteristics of Ara h 1 (WT and IAA-modified) (secondary structure by CD and estimated size by SEC); measurement of secondary structure of IAA−Ara h 1 at various pH values; measurement of IAA−Ara h 1 digestion fragments by SEC; standard protocol for LC-MSMS for protein identification. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

*β*ME, beta-mercaptoethanol; CD, circular dichroism; Cys, cysteine; DTT, dithiothreitol; GdHCl, guanidine hydrochloride; HCl, hydrochloric acid; IAA, iodoacetic acid; KCl, potassium chloride; kDa, kilodaltons; LC-MSMS, liquid chromatography−tandem mass spectroscopy; MRE, mean residual ellipicity; NaCl, sodium chloride; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SEC, size exclusion chromatography; Tris, tris(hydroxymethyl)-aminomethane; WT, wild type

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