

Peanut protein allergens: Gastric digestion is carried out exclusively by pepsin

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Background: A major characteristic of many food allergens, including Ara h 1, a major peanut allergen, is their resistance to gastric digestion. One estimate of the allergenic potential of a possible protein allergen is its stability under simulated gastric conditions.

Objective: Because the rate and extent of digestion of allergenic proteins will affect the severity of any subsequent allergic response, it is important to correlate protein allergen digestion in simulated gastric fluid with that in actual gastric fluid.

Methods: A major peanut allergen, Ara h 1, was digested *in vitro* by using both pepsin and porcine gastric fluid. Several comparisons between the 2 sets of proteolytic conditions were assessed including pH optima and the effect of temperature, denaturants, and specific enzyme inhibitors.

Results: *In vitro* digestion of Ara h 1 with pepsin and porcine gastric fluid resulted in virtually identical hydrolysis patterns as observed on SDS-PAGE. The protease activity of both pepsin and gastric fluid were inhibited at high pH and in the presence of pepstatin. However, both remained active in 4 mol/L urea and at 60°C.

Conclusions: Protein digestion in the porcine stomach is carried out by pepsin. *In vivo* gastric digestion is modeled accurately by peptic hydrolysis. Digestion conditions *in vivo* are comparable to experimental conditions *in vitro* provided that the acidic nature of the stomach contents is optimal for characterization of the allergen under standard pepsin digestion conditions. Additional experimentation using crude food extracts, both in the presence and absence of a complete meal, is needed to elucidate the complete physiologic nature of food allergen digestion. (J Allergy Clin Immunol 2004;114:614-8.)

Key words: peanuts, food allergy, gastric digestion

Abbreviations used

MW: Molecular weight
PGF: Porcine gastric fluid
SGF: Simulated gastric fluid
SIF: Simulated intestinal fluid

When characteristics of known food allergens are examined, the single most prominent trait attributed to food allergens is protein stability. Food allergens in general, and peanut proteins in particular, tend to be soluble, low molecular weight (MW) glycoproteins with acidic isoelectric points. They are usually stable to heat and acidic conditions and relatively resistant to proteolytic digestion.¹⁻³ Therefore, the current predictive methods for the evaluation of protein allergenicity of food allergens include the detection of intact or residual antigenic fragments after artificial digestion procedures as determined by HPLC and SDS-PAGE/immunoblot analysis. Several food allergens have been shown to be more resistant than nonallergenic food proteins to proteolytic digestion with gastrointestinal tract enzymes or simulated gastric fluid (SGF).²

A general consensus seems to have been reached that proteins susceptible to gastrointestinal digestion are inherently safer than those that are stable with respect to allergenicity. In the analysis of digestibility of food allergens and non-food allergen proteins, Astwood et al² demonstrated the validity of this concept for known allergens within peanuts, soybean, mustard, egg, and milk relative to common non-food proteins by using an SGF as described in the United States Pharmacopoeia.⁴

To address the efficacy of digestibility for cross-reactive food allergens, Yagami et al⁵ extracted proteins from natural rubber latex and vegetable foods and treated them with both SGF and simulated intestinal fluid (SIF). Although most latex proteins were digested by SGF within 4 minutes, a 28-kd allergen and hevein (Hev b 6.02, 4.7 kd) were stable and not completely digested within 1 hour when analyzed in crude extracts. Most cross-reactive allergens from fruits were digested within 4 minutes. In contrast, potato proteins were stable in SGF with several bands (10, 20, 24 kd) clearly detectable even after 1 hour of SGF digestion.

Aalberse^{6,7} has established criteria to be considered when determining food protein allergenicity. Food proteins with both the ability to sensitize and to induce symptoms could be termed complete food allergens. Food

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proteins that can perorally induce allergic symptoms, even though they are digestible and cannot perorally sensitize individuals, could be termed incomplete food allergens. Notable exceptions of characterized food allergens to SGF stability include many pollen, fruit, and vegetable cross-reactive allergens. These include Bet v 1-related proteins and profilins that are heat labile and susceptible to enzyme digestion.^{1,8,9} However, the single most widely acknowledged trait of food allergens is protein stability. Although the stability of food allergens has been demonstrated by illustrating their ability to survive for extended periods of time, little is known about how these proteins resist digestion.

Because of the persistence and severity of the allergic reaction to peanuts, and the lack of effective treatment, an investigation of the molecular mechanisms of allergen stability and digestion is vital to understand the metabolic processing of these allergenic proteins in an attempt to mitigate the hypersensitivity reactions to these allergens. Data on allergen digestion with pepsin, *in vitro* digestion with SGF, or *in vivo* digestion studies are only meaningful if valid comparisons can be made between the various data. This is possible only if the digestive characteristics of actual gastric fluid are compared directly with those of purified pepsin, the primary component of SGF. Here we assess the digestive effect of porcine gastric fluid (PGF) on both purified Ara h 1 and peanut extract and compare these results directly with those obtained by using purified pepsin.

METHODS

Reagents

All reagents were of molecular biology grade purity. Pepsin (P-6887) was obtained from Sigma Chemical Company (St Louis, Mo).

PGF

PGF was collected from anesthetized pigs by endoscopy. The animals were fasted overnight before the removal of approximately 20 mL of gastric fluid. The fluid was centrifuged briefly to remove suspended material and stored at -20°C until used. PGF from individual piglets was used for experimentation.

Purified Ara h 1 protein

Peanut allergen Ara h 1 was purified by ammonium sulfate precipitation and cation exchange column chromatography as previously described.¹⁰

Peanut extract

Peanut seeds (*Arachis hypogaea* Leguminosae, Florunner cultivar) were ground in liquid nitrogen and then defatted 3 times in a Soxhlet extractor with diethyl ether. Peanut extract was produced by suspending the resulting dry defatted peanut flour prepared from raw or roasted (180°C for 10 minutes in a conventional oven) peanuts in TE buffer (65 mmol/L Tris, 1 mmol/L EDTA, 200 mmol/L NaCl, pH 8.3) and stirring for 30 minutes at room temperature. After centrifugation, the supernatant solution (crude peanut protein extract) was diluted to a concentration of 2 mg/mL measured by use of the bicinchoninic acid protein assay method and used for experiments.

Enzymatic digestion reactions

Digestions of purified Ara h 1 or peanut extract were carried out in 1.6-mL reaction mixtures by using either pepsin or PGF at 37°C in TE buffer pH 2.1 unless otherwise indicated. Ara h 1 was present at a final concentration of 0.5 mg/mL, and crude peanut extract was present at a final concentration of 1.0 mg/mL. Concentrations of pepsin or PGF were optimized experimentally and selected to produce comparable digestion over similar time periods to facilitate comparison. The digestion reactions were stopped by the addition of denaturing electrophoresis sample buffer and heating at 90°C for 10 minutes.

Gel electrophoresis

Samples were mixed with electrophoresis sample buffer containing SDS and electrophoresed on 4% to 20% gradient SDS-PAGE gels in Tris-glycine buffer.¹¹ Proteins were visualized with Coomassie brilliant blue staining.

RESULTS

Proteolytic activity of PGF in the digestion of peanut proteins

Peanut extract from both raw and roasted peanuts was mixed with various volumes of PGF. The resulting hydrolysis of the peanut proteins after a 15-minute incubation at 37°C is illustrated in Fig 1. Roasting reduces the solubility of peanut proteins and results in a lower yield of extractable protein. However, the protein that does remain soluble appears virtually identical to raw peanut extract when comparable amounts of total protein are compared. Even highly diluted samples of PGF are effective in the hydrolysis of total soluble peanut protein. Both the raw and roasted peanut proteins degrade readily and in an identical manner as followed by SDS-PAGE. The higher MW protein bands can be seen to decrease in intensity and collapse into several lower MW peptides that are highly resistant to further digestion. This rapid initial digestion of the intact peanut proteins into smaller resistant fragments by using purified Ara h 1 is described in a previous report from our group.¹⁰ The lower MW resistant peptides have been sequenced by N-terminal amino acid sequence analysis and shown to be fragments of the original higher MW proteins (data not shown). Lanes 1 and 8 represent control raw and roasted soluble peanut protein extract. Successive lanes indicate the effect of increasing amounts of PGF in the reaction mixture. The number of microliters of PGF used in 1.6-mL reaction mixtures containing fixed amounts of peanut extract are indicated below each lane. These volumes correspond to overall PGF dilutions in the final reaction mixture of 1:1600 in lanes 2 and 9, 1:800 in lanes 3 and 10, 1:400 in lanes 4 and 11, 1:200 in lanes 5 and 12, 1:100 in lanes 6 and 13, and 1:80 in lanes 7 and 14.

PGF contains a potent protease active only at acidic pH

The protease contained in PGF is active at pH 2.1, but not at neutral or basic pH. Various amounts of PGF were added to solutions of purified Ara h 1 buffered at pH 2.1. The mixtures were incubated at 37°C for 10 minutes. The reactions were quenched by the addition of electrophoresis

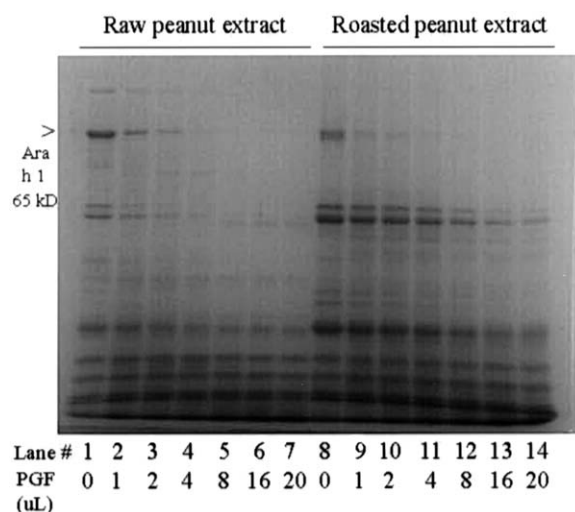


FIG 1. Digestion of raw and roasted peanut extract with porcine gastric fluid (PGF). Peanut extract from raw (lanes 1-7) and roasted (lanes 8-14) peanuts was treated with increasing volumes of PGF in reaction mixtures containing equal amounts of peanut extract. Controls (gel lanes 1 and 8); effect of increasing amounts of PGF in the reaction mixture (subsequent lanes).

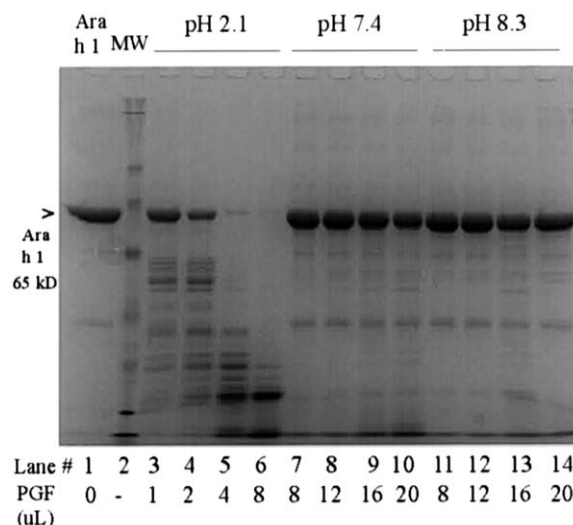


FIG 2. Digestion of Ara h 1 with PGF at different pH values. Reaction mixtures containing Ara h 1 at a fixed concentration were incubated for 10 minutes with the indicated volumes of PGF. The same reaction was performed at pH 2.1, 7.4, and 8.3. The relative proteolytic activity of the PGF at these 3 pH values can be compared on the Coomassie stained gel.

sample buffer containing SDS and electrophoresed on a 12% SDS-PAGE gel followed by Coomassie staining. The results are shown in Fig 2. Lane 1 contains only purified Ara h 1 protein. Lane 2 contains protein MW standards. Lanes 3 to 6 represent increasing amounts of PGF added to the Ara h 1. The number of microliters of PGF contained in a 1.6-mL reaction mixture is indicated below each lane. Lanes 7 to 10 and 11 to 14 represent corresponding reaction mixtures at pH 7.4 and 8.3. The small PGF volumes used for the reaction at pH 2.1 correspond to overall gastric fluid dilutions of 1:1600 in lane 3, 1:800 in lane 4, 1:400 in lane 5, and 1:200 in lane 6. Progressive hydrolysis of the Ara h 1 can be clearly seen. The volumes of PGF used in the reactions at pH 7.4 and 8.3 were larger. The corresponding overall dilutions of the original gastric fluid in these reactions were 1:200 in lanes 7 and 11, 1:133 in lanes 8 and 12, 1:100 in lanes 9 and 13, and 1:80 in lanes 10 and 14. Despite the fact that higher concentrations of gastric fluid were used in these reactions, there is no evidence of Ara h 1 hydrolysis at either neutral or basic pH.

Comparison of Ara h 1 digestion with pepsin and PGF

Hydrolysis of Ara h 1 with pepsin or PGF produced identical sets of peptide fragments. Parallel digestion reactions were carried out on 0.5 mg/mL Ara h 1 solutions at pH 2.1 for 15 minutes at 37°C. Various volumes of 100 µg/mL (2.86 µmol/L) pepsin or PGF were added to the Ara h 1 to produce reaction mixtures with a total volume of 1.6 mL. The reaction mixtures were quenched by the addition of electrophoresis sample buffer containing SDS and electrophoresed on a 12% SDS-PAGE gel. The resulting peptide fragments were visualized by Coomassie

staining. The resulting gel is shown in Fig 3. Lane 1 contains MW protein standards. Lanes 2 to 8 and 9 to 15 show the results of Ara h 1 digestion with the indicated volumes of 100 µg/mL pepsin or PGF, respectively. The pepsin concentrations in the final reaction mixtures in lanes 3 to 8 were 1.8, 3.6, 8.9, 18, 27, and 36 nM, respectively. The overall dilutions of the PGF in the final reaction mixtures in lanes 10 to 15 were 1:3200, 1:1600, 1:800, 1:320, 1:160, and 1:80, respectively. The resulting peptide fragments obtained on hydrolysis of Ara h 1 with pepsin (lanes 2-8) and PGF (lanes 9-15) are virtually identical. This indicates that the enzyme involved is specific for the same recognition sites, thus producing digestion fragments of the same size. It also shows that no enzyme activity other than pepsin is present in the PGF.

The active protease in PGF is pepsin

Characterization of the protease active in PGF identified it as pepsin. Several of the unique characteristics of pepsin were exploited to verify the identity of this active protease in PGF. Fig 4 shows the results of these experiments. Enzymatic digestion of purified Ara h 1 with commercial pepsin (lane 1) and PGF (lane 7) resulted in identical digestion patterns. The peptide fragments that were produced correspond exactly. The proteolysis in these lanes is obvious when compared with undigested control Ara h 1 in lanes 2 and 8. Pepsin retains its enzymatic activity in the presence of 4 mol/L urea. Digestion of Ara h 1 carried out in 4 mol/L urea by using pepsin (lane 3) and PGF (lane 9) indicates that both enzymes retained significant hydrolytic activity and produced comparable hydrolysis products. Pepsin is inactive at pH values greater than 6. Incubation of Ara h 1 with pepsin (lane 4)

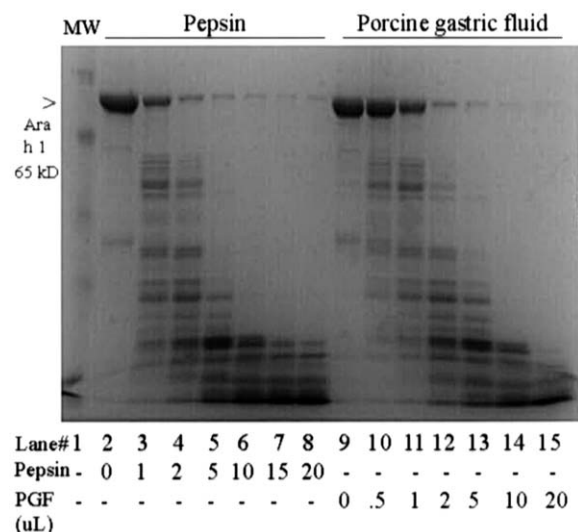


FIG 3. Comparison of Ara h 1 digestion with pepsin and PGF. Ara h 1 was incubated for 15 minutes with the indicated volumes of 100 µg/mL pepsin (lanes 2-8) or PGF (lanes 9-15). The reactions were then quenched and electrophoresed. The resulting peptide fragments were visualized by Coomassie staining.

and PGF (lane 10) at pH 8.3 resulted in no detectable proteolysis by either enzyme source. Pepsin is stable at temperatures up to 60°C. Ara h 1 digestion reactions at 60°C in the presence of pepsin (lane 5) and PGF (lane 11) indicate significant catalytic activity at this temperature. Incubation of Ara h 1 in the presence of pepstatin A, a highly specific pepsin inhibitor, with pepsin (lane 6) and PGF (lane 12) revealed that protease activity was completely inhibited in both reactions. There was no other protease activity detectable in the PGF when its pepsin was inactivated. Pepsin is an acid (aspartic) protease and is not affected by phenylmethylsulfonyl fluoride, a serine protease inhibitor. Neither pepsin nor PGF was inhibited by 1 mmol/L phenylmethylsulfonyl fluoride in similar experiments (data not shown). These results all indicate that the protease activity in PGF is caused by pepsin.

DISCUSSION

To assess food allergen stability (for which there is no standardized methodology to analyze the inherent ability of proteins to act as allergens), SGF and SIF digestion systems have been devised. Although relative gastric pepsin concentrations in the pig and human have not been precisely compared, the porcine system has been extensively used to model human digestion.¹²⁻¹⁵ Therefore, the results determined here should allow reasonable extrapolation to human gastric digestion.

Relative protein stability in SGF and SIF often correlates with allergenic activity. These criteria are by no means definitive proof of a protein's potential allergenicity; however, combined with other factors, they

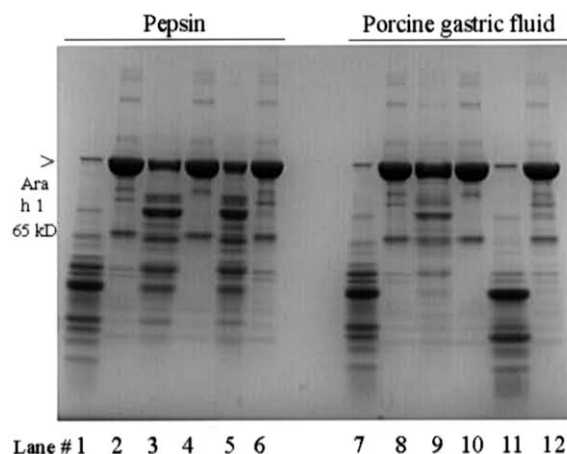


FIG 4. Pepsin is the exclusive protease in PGF. Incubation of Ara h 1 with pepsin and PGF (lanes 1 and 7) at pH 2.1. Control Ara h 1 in the absence of any hydrolytic enzymes (lanes 2 and 8). Digestion of Ara h 1 in 4 mol/L urea with pepsin and PGF (lanes 3 and 9). Ara h 1 digestion performed at pH 8.3 with pepsin and PGF (lanes 4 and 10). Incubation of Ara h 1 at 60°C with pepsin and PGF (lanes 5 and 11). Ara h 1 digestion performed in the presence of pepstatin A with pepsin and PGF (lanes 6 and 12).

contribute our best suggestion of allergenicity taking into consideration the plethora of proteins consumed.¹⁶ Only a few proteins (estimated at ~200) have been shown to be true allergens, and the majority meet these criteria. Continued surveillance and documentation will undoubtedly identify additional proteins as allergens; however, the ability to predict allergenicity of novel proteins must rely on the characteristics of known food allergens.

Previous studies from this laboratory have shown that higher order structures within Ara h 1 and Ara h 2 are largely responsible for their resistance to enzymatic digestion and therefore the persistence of their allergenicity.¹⁰ Ara h 1 forms stable homotrimers maintained by hydrophobic interactions between amino acids at the monomer-monomer contact points, the same regions where the majority of the 23 IgE binding epitopes are clustered.^{10,17} In the case of Ara h 2, resistance to proteolysis is imparted by the compact nature of this small protein enhanced by disulfide linkages between the molecule's 8 cysteine residues.¹¹ Exhaustive proteolysis of both Ara h 1 and Ara h 2 with digestive enzymes results in the survival of relatively large peptide fragments that still contain numerous potential enzyme cut sites and show a high degree of IgE binding capacity.^{10,11}

PGF is an extremely active protease, even when highly diluted. Even at dilutions of 1:1600, PGF shows significant digestion of both raw and roasted peanut protein extract in only 15 minutes. Because large dilutions of stomach fluid would be expected on the ingestion of a meal, this result is not unexpected.

Under optimal conditions, the rate of *in vitro* digestion of purified Ara h 1 with PGF parallels that of PGF digestion of total peanut protein extract. The prominent 65-kD Ara h 1 band in each figure can be seen to degrade at

comparable rates when equivalent amounts of PGF are used in the reaction mixture. In both cases, although the intact Ara h 1 protein is degraded relatively quickly, several lower MW peptides are resistant to further digestion. This was also shown to be true in the treatment of Ara h 2 with digestive tract enzymes.¹¹

The Ara h 1 hydrolysis patterns obtained on treatment with either pepsin or PGF are essentially identical. This is evidence that the single active protease present in PGF is pepsin. No other peptidases in PGF are degrading the peptide fragments produced by the pepsin. The extent of Ara h 1 hydrolysis produced by equal volumes of pepsin and PGF (as shown in Fig 3) is approximately equal. Because the amounts of Ara h 1 protein, the total reaction volumes, and the volumes of enzyme added were identical, the pepsin concentration in PGF can be approximated. Because equal volumes of both 100 µg/mL (2.86 µmol/L) pepsin and PGF produced the same extent of Ara h 1 hydrolysis, the pepsin concentration in the 2 solutions can be approximately equated. Therefore, the pepsin concentration in the stomach of a fasting pig can be estimated as approximately 100 µg/mL or 3 µmol/L.

Pepsin is the only significant component of PGF responsible for protein digestion. Figure 4 compares Ara h 1 digestion at pH 2.1 by using pepsin and PGF (lanes 1 and 7). Pepsin is one of the few digestive enzymes active at this pH, and both enzyme sources produce identical digestion patterns. Pepsin has the unique property of retaining its catalytic activity in 4 mol/L urea. Because PGF was active in 4 mol/L urea and produced an Ara h 1 digestion pattern identical to pepsin (lanes 3 and 9), it must be concluded that pepsin was the active component in PGF. A similar argument can be made with the parallel digestion reactions performed at 60°C with pepsin and PGF (lanes 5 and 11). Pepsin is uniquely resistant to denaturation at 60°C and shows high enzymatic activity in both pepsin and PGF. Finally, in lanes 6 and 12, Ara h 1 digestion was performed with both pepsin and PGF in the presence of pepstatin A, a highly specific pepsin inhibitor. The pepsin activity was completely inhibited in both reactions, and the PGF reaction produced no additional peptide fragments compared with the pepsin. This indicates that under these experimental conditions, no proteases other than pepsin were present in the PGF at any appreciable level. In this study, the only significant proteolytic enzyme active in PGF is pepsin.

Peanut proteins, both in purified form and as a crude peanut protein extract, when treated with either pepsin or actual PGF, resulted in the production of nearly identical sets of digestion products by both enzyme sources. We

show here that proteolysis by pepsin is the only appreciable protease activity taking place in gastric fluid. Simple peptic hydrolysis can be used to model *in vivo* digestion. However, additional enzyme digestion and antigen processing further along the gastrointestinal tract contribute significantly to the ultimate recognition of food proteins as allergens.

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