

Comparison of Physiological and in vitro Porcine Gastric Fluid Digestion

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Key Words

Peanut allergen Ara h 1 · Food allergy · Stomach pH · Gastric digestion · Simulated digestion conditions · Physiological digestion

Abstract

Background: In previous studies, the major peanut allergen Ara h 1 was digested in vitro using pepsin and porcine gastric fluid. The results suggested that in vivo gastric digestion of allergen protein can be modeled accurately by peptic hydrolysis in vitro. In the current investigation, studies were designed to follow the gastrointestinal (GI) digestion of peanut allergens under true physiological conditions. In vitro digestion with porcine gastric fluid was compared with actual physiological digestion of peanut allergens in the porcine digestive tract in vivo. **Methods:** Analysis of physiologic digestion was performed in piglets administered a 20-gram bolus of peanut meal followed by periodic sampling and analysis of GI contents. The pH was monitored, and digesta were analyzed by SDS-PAGE and immunoblot analysis. **Results:** Peanut meal initially neutralized stomach contents to a pH of approximately 7, which was subsequently acidified by HCl secretion within 30 min. Acidification to pH 2–4 resulted in active pepsin digestion of soluble protein in the stomach. Soluble intact protein/allergens were rapidly degraded to pepsin-resistant peptides in the stomach followed by hydrolysis of these fragments in the small intestine. Particulate material was evident in both the stomach and small intestine that could contribute to continued release of pea-

nut allergens Ara h 1, 2 and 3. **Conclusions:** Porcine gastric digestion of peanut proteins resembles true physiological digestion only under optimal physiologic conditions. Soluble proteins are rapidly digested and insoluble material continues to release IgE-reactive proteins throughout the GI tract. GI digestion of food allergens can play a prominent role when assessing allergens within the context of a food matrix or meal and during the sensitization phase of IgE-mediated allergy.

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Introduction

There is currently no reliable single or combined method to evaluate or predict the allergenicity of a protein [1]. Food allergens have generally been defined as heat stable and relatively resistant to proteolytic digestion [2]. Studies have been conducted comparing the digestibility of known food and non-food allergens with common non-food proteins using pepsin or a simulated gastric fluid and simulated intestinal fluid as described in the United States Pharmacopoeia [3]. With regard to potential allergenicity, it is generally thought that proteins susceptible to gastrointestinal (GI) digestion are inherently safer than those that are stable. Astwood et al. [2] proposed the validity of this concept for known allergens in peanuts, soybean, mustard, egg, and milk. Notable exceptions have been reported [4–7], and Fu et al. [8] found no clear relationship between pepsin digestibility mea-

sured in vitro and protein allergenicity. However, the authors concur with Taylor [9] who suggested that the study by Fu et al. [8] was not a fair assessment of protein digestibility as a predictor of the allergenic potential of a novel protein. Among the comments of Taylor [9], the most significant is the incredible pepsin to protein ratio of 10:1 that is highly unlikely in the abundance of the protein in the course of eating a meal. However, GI digestion criteria are still commonly used as indicators of potential allergenicity [10]. Partially hydrolyzed food proteins, or peptide fragments, that escape total hydrolysis in the lumen, would potentially be available for exposure to the GI-associated lymphoid tissues. This could result in the sensitization of food proteins with the production of allergen-specific IgE.

Recent studies indicate that nearly 4% of Americans have a food allergy and that the incidence of peanut allergy has doubled in young American children in the past 5 years [11]. Peanuts have received considerable attention because of the high frequency of anaphylactic reactions associated with their ingestion, largely as 'hidden' allergen sources. Peanut allergen levels as low as 0.1–2 mg have been established to cause mild, transitory subjective symptoms in allergic individuals [12]. In a specific oral provocation study [13], 25% of the peanut-allergic participants responded to less than 100 mg of peanut seed, a considerably lower dose than that needed for other food allergens to elicit a response.

The stability of proteins to in vitro digestion with pepsin is increasingly being used to identify and classify allergens. Schimek et al. [14] recently reported that simulated GI digestion of major birch pollen allergen Bet v 1-related food allergens rapidly destroyed their IgE-binding capacity. An in vitro pepsin digestion assay outlined by the FAO/WHO [15] and a multilaboratory evaluation have been used to assess the consistency and reproducibility of this common protocol for several proteins [16]. However, in vivo digestion can only be approximated using simulated in vitro digestion conditions. Many factors play a role in physiological digestion such as the recently reported interaction between protein allergens and lipids in model gastric emulsions [17].

In previous studies, porcine gastric fluid (PGF) was withdrawn from overnight-fasted anesthetized pigs and compared with pepsin digestion for proteolytic activity against purified Ara h 1 [18]. To determine the role of the food matrix, a more physiologically relevant model has been used to study allergen stability as a function of GI digestion. We chose to investigate the progressive digestion of peanut allergens in the swine digestive tract be-

cause of the prevalence of peanut allergy, the severity of the symptoms, and the fact that the allergy is considered to persist indefinitely [19–22]. The porcine system has been shown to be a valid approximation of the analogous systems in humans and has been used extensively to model human digestion [23–27]. We followed the digestion of peanut meal through the GI tract of overnight-fasted piglets measuring changes in pH as well as the extent of protein degradation as a function of time in the stomach and at various points along the intestinal tract. The progressive proteolysis of peanut protein allergens and an assessment of their allergenicity were determined by IgE-binding capacity.

Materials and Methods

Reagents

All reagents were of molecular biology grade purity.

Human Subjects and Experimental Animals

Human blood serum was collected from peanut-allergic volunteers in accordance with policies established by the University of Arkansas for Medical Sciences Institutional Review Board, and informed consent was obtained from each serum donor. Piglets were used in accordance with the guidelines described in the Arkansas Children's Hospital Research Institute Policy on Humane Utilization and Care of Laboratory Animals. All sedation, surgery and euthanasia were performed under the supervision of University of Arkansas for Medical Sciences veterinary personnel.

Chopped Peanut Meal

Peanut seeds (*Arachis hypogaea*, Leguminosae, Florunner cultivar) were finely chopped in an IKA Works, Inc. (Wilmington, N.C., USA) model A11 basic analytical mill. Twenty grams of chopped peanuts was suspended in 100 ml water to produce a peanut meal slurry that consisted of peanut particles and soluble peanut proteins. The peanut meal slurry was intended to represent the equivalent of masticated peanuts.

In vivo Digestion of Chopped Peanut Meal

Piglets (25 days old) were fasted 24 h and then given a 100-ml slurry of 20 g finely chopped peanut meal in water via a gastric tube. Aliquots (2 ml) of stomach contents were sampled through a surgically implanted gastric cannula at various points (0, 0.25, 0.50, 0.75, 1, 2, 3, 4, 5 and 6 h) after feeding. The piglet was then sacrificed, and intestinal contents at various distances (5, 10, 50, 70 and 90% length of the small intestine) from the stomach to the cecum were collected by dissection. All samples were immediately frozen following collection and measurement of pH.

Extended in vitro Digestion of Recovered Chopped Peanut Meal in Gastric Digesta

To confirm that the in vivo digestion of chopped peanut meal proteins was due to pepsin, a sample of stomach digesta was collected immediately after feeding a bolus of the peanut slurry. Aliquots of the digesta were immediately adjusted to a specific pH

value ranging from pH 7 to pH 1, and digestion was allowed to continue in vitro at 37°C for 45 min. Pepstatin was added to the pH-2.0 incubation mixture at time 0 and incubated for the 45-min time period to confirm pepsin activity. All incubation mixtures were then centrifuged, and the supernatants were analyzed by gel electrophoresis.

Gel Electrophoresis

Samples of digested peanut meal were mixed with electrophoresis sample buffer and electrophoresed on 4–12% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels in Tris-glycine buffer [28]. Equal volumes of each sample were loaded to illustrate differences or changes in protein concentrations. Proteins were visualized with Coomassie brilliant blue staining.

Western Immunoblots

Following electrophoresis, SDS-PAGE gels were electrophoretically transferred to a nitrocellulose membrane using an Invitrogen XCell blot module (Invitrogen Life Technologies, Carlsbad, Calif., USA) according to the manufacturer's instructions. The membrane was probed with pooled blood sera from 5 peanut-allergic individuals. The bound IgE was detected using ¹²⁵I-labeled goat anti-human IgE (DiaMed, Inc., Windham, Me., USA) followed by autoradiography on Kodak BioMax MS imaging film (Rochester, N.Y., USA).

Results

Changes in Digesta pH during Gastric and Intestinal Digestion

The results shown in figure 1 indicate that the highly acidic empty stomach is neutralized at time point 0 following the introduction of the chopped peanut slurry. Secretion of HCl into the stomach returns the pH to its original value of ≤ 2.0 within 0.5 to 0.75 h. A highly acidic pH is then maintained for 6–8 h prior to digesta passage into the small intestine. Digesta samples collected from various lengths of the small intestines at sacrifice of the animal show a gradual increase to pH 7.0.

In vivo Digestion of Soluble Peanut Proteins

Under conditions in which gastric digesta were collected at various time points, figure 2 shows the SDS-PAGE and IgE immunoblot results obtained from the collection time points in figure 1. Lane 3 shows the Coomassie-stained profile from a 10- μ l aliquot of the peanut meal slurry collected immediately following the gastric intubation of the peanut slurry. Although greatly diluted in the stomach, proteins stained between 28 and 62 kDa were still visible at 0.25 h (lane 4). Peanut proteins were digested to visible Coomassie blue-stained peptide fragments (≤ 14 kDa) in the porcine stomach once the pH

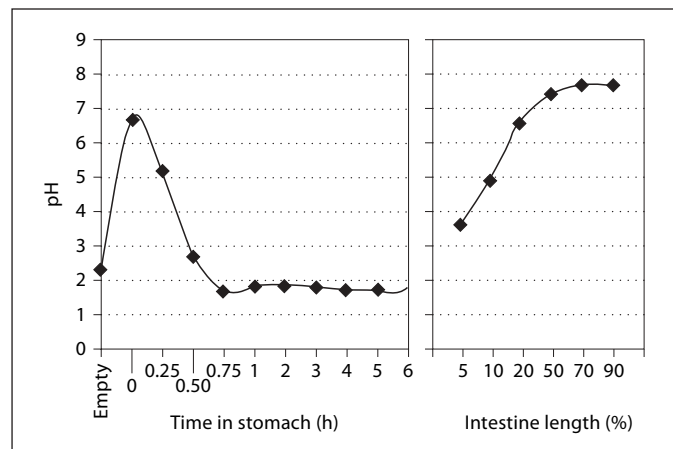


Fig. 1. Measured pH values (x-axis) of peanut digesta collected 0–6 h following a peanut meal and at 5, 10, 50, 70 and 90% of the total small intestine length 6 h after feeding (y-axis).

returned to acidic values (lanes 5–7, which represent 0.5, 0.75 and 1 h). Coomassie blue-stained protein fragments are undetectable beyond 1 h (lanes 8–12). The IgE immunoblot of an identical gel is shown in figure 2b. Soluble peanut proteins retain their IgE-binding potential while in the stomach for up to 4 h as peptides <28 kDa. Although intact soluble peanut protein is degraded relatively quickly in the stomach, pepsin-resistant peptide fragments that are IgE reactive persist. The IgE-binding capacity of collected digesta samples does not disappear prior to gastric contents emptying into the small intestine. We were able to detect both IgE-binding peptides and peptide fragments in the small intestine, suggesting that additional solubilized peanut proteins were being continuously released from the particulate digesta and had not simply been emptied from the stomach.

Release of Protein from Insoluble Peanut Material during in vivo Digestion

Centrifugation of the digesta samples produced insoluble peanut protein material that could be solubilized in 5% SDS. This solubilized material was electrophoresed and either Coomassie stained or immunoblotted as shown in figure 3. The Coomassie-stained gel pattern in figure 3a indicates that protein from particulate material collected from the porcine stomach can be quickly solubilized by treatment with 5% SDS. Coomassie blue-stained protein that had been released from insoluble material can be detected in samples. Figure 3b represents an IgE immunoblot of an identical gel. Although little pro-

Fig. 2. Soluble porcine gastric digesta contents. **a** Coomassie-stained SDS-PAGE profile. Lane 1 = Molecular weight standards; lane 2 = empty stomach fluid before feeding; lanes 3–17 = soluble proteins present in porcine gastric digesta collected following a peanut meal at 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5 and 6 h (lanes 3–12) and at 5, 10, 50, 70 and 90% of the total small intestine length 6 h after feeding (lanes 13–17). **b** IgE immunoblot probed with a serum IgE pool from 5 peanut-allergic individuals. Arrows indicate the major allergens Ara h 1 and isoforms of Ara h 2 (16 and 18 kDa).

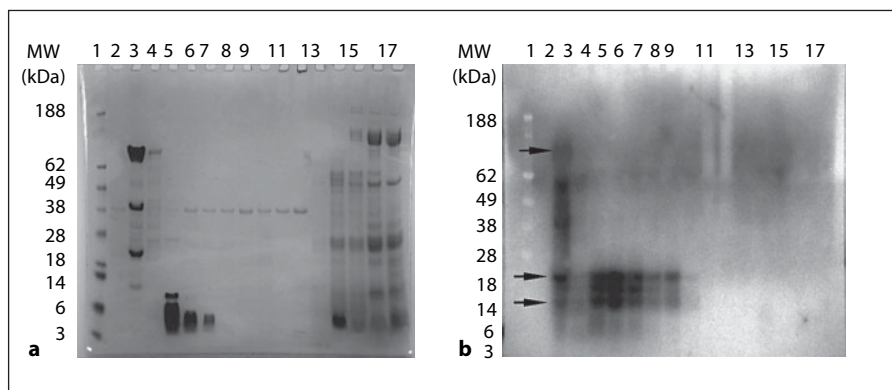
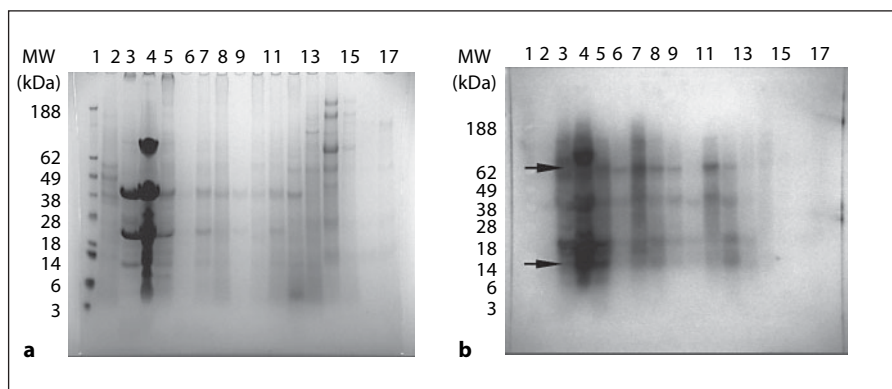


Fig. 3. Insoluble porcine gastric digesta. **a** Coomassie-stained SDS-PAGE profile. Lane 1 = Molecular weight standards; lane 2 = empty stomach fluid before feeding; lanes 3–17 = soluble proteins present in porcine gastric digesta collected following a peanut meal at 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5 and 6 h (lanes 3–12) and at 5, 10, 50, 70 and 90% of the total small intestine length 6 h after feeding (lanes 13–17). **b** IgE immunoblot probed with a serum IgE pool from 5 peanut-allergic individuals. Arrows indicate the major allergens Ara h 1 and isoforms of Ara h 2 (16 and 18 kDa).



tein can be detected by Coomassie blue staining, IgE-binding protein remains incorporated in the particulate peanut material through the stomach and first half of the intestinal tract. The results suggest that IgE-reactive protein could be continuously released from the insoluble particulate protein material as it traverses the GI tract. In both figures 2 and 3, allergen observed in the immunoblots include bands at 65 kDa corresponding to Ara h 1, several bands ranging from 25 to 45 kDa, likely representative of glycinin fragments, and two bands at 16 and 18 kDa corresponding to isoforms of Ara h 2.

Extended in vitro PGF Digestion of Gastric Digesta Recovered at Time 0 at Different pH Values

Gastric digesta recovered at time 0 by gastric intubation and immediately adjusted to various pH values are shown in figure 4. The pH decreases from pH 7 to pH 1 from lanes 3 through 9 showing proteolysis increases corresponding to lowered pH values. The proteolysis is due to the action of pepsin and is not a function of pH since the addition of pepstatin A, a specific pepsin inhibitor, to the pH-2.0 incubation mixture in lane 10 results in complete inhibition of the proteolysis.

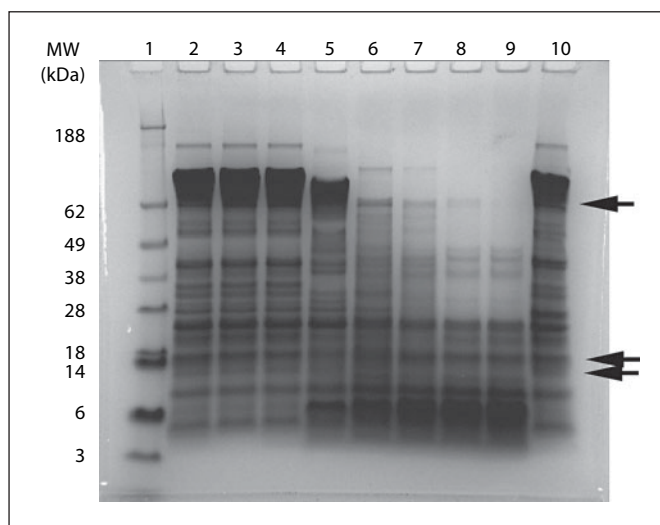


Fig. 4. Coomassie-stained SDS-PAGE profile. In vitro adjusted pH of gastric digesta collected immediately after feeding the peanut slurry adjusted to various pH values and allowed to continue digesting for 45 min. Lane 1 = Molecular weight standards; lane 2 = a reaction blank in which the digestion reaction was quenched immediately; lanes 3–9 = the adjusted pH decreasing from pH 7 to pH 1; lane 10 = a pH-2 incubation mixture with 1 μ M pepstatin, a pepsin inhibitor.

Discussion

In previous studies, the higher order structure of Ara h 1 and Ara h 2 was shown to be responsible for their resistance to enzymatic digestion [29]. In vitro PGF digestion of purified Ara h 1 paralleled that of PGF digestion of total peanut extract, suggesting that the appreciable protease activity taking place in PGF was due to pepsin. Although PGF pepsin concentrations in the pig and human have not been precisely compared, the porcine digestive system has been extensively used to model human digestion [23–27]. Results presented here demonstrate the digestibility of peanut proteins within a peanut matrix under normal physiological and in vitro digestion conditions.

Physiological gastric pH dramatically increased to pH 7 immediately following the introduction of the peanut slurry. The pH was restored within 30–45 min and maintained a very stable value of pH 1–2 as HCl is secreted by cells of the stomach lining. After passage through the pylorus, the digesta are neutralized to pH 7–8 by intestinal secretions during its passage through the first half of the small intestine.

Pepsin was present in the empty stomach and during the 6–8 h of sampling; however, significant peptic digestion of the introduced peanut proteins occurred only after the stomach pH dropped below pH 4. Proteolysis of the soluble peanut proteins took place rapidly, but small (3–6 kDa) pepsin-resistant fragments persisted that retained their IgE-binding capacity for up to 4 h. Gastric digesta emptied into the small intestine showed continued digestion by intestinal proteases. The persistence of IgE-binding protease-resistant peanut protein fragments under physiological conditions with peanut slurry was in complete agreement with our previously published results of in vitro digestions of purified peanut proteins using simulated gastric fluid and PGF [18]. Sequential digestion of Ara h 2 with pepsin, trypsin and chymotrypsin yielded a highly resistant 10-kDa fragment that also retained high IgE-binding capacity. Incubation of Ara h 1 with either pepsin or PGF resulted in virtually identical digestion fragment profiles with several low-molecular-weight fragments resistant to further digestion [18]. This report confirms that proteolysis by pepsin is the only appreciable protease activity taking place in PGF.

The effect of pH on gastric digestion was further demonstrated by the collection of gastric digesta collected immediately after the introduction of chopped peanuts into the porcine stomach. This digesta, neutralized to pH 7 by

the chopped peanut meal slurry, showed a predominantly intact peanut protein profile. Protein hydrolysis by the endogenous PGF pepsin was nonexistent at greater than pH 5, but increased to a maximum in digesta aliquots adjusted to pH 1–2. These results of stomach digesta contents reproduced previous results during progressive digestion of Ara h 1 and peanut extract with both purified pepsin and PGF under total in vitro conditions [18]. These results were consistent with a recent report that antacid medication inhibited digestion of dietary proteins [30]. Antacids can raise gastric pH to approximately 5 and maintain gastric pH at elevated levels for prolonged periods. At this pH, pepsin digestion is not relevant. In our study, we found an analogous situation in the first 30–45 min following a meal before gastric conditions returned to pH <2. Thus, prolonged neutralization of the acidic conditions in the stomach could lead to potential passage of soluble and dietary proteins into the lower digestive GI tract.

Our physiological results confirmed that IgE-binding peanut protein remains incorporated in the particulate material through the stomach and first half of the small intestine. This IgE-reactive protein is continuously released as the peanut meal passes through the GI tract. This continuous release could provide a source of allergen to the GI immune system. Considerable evidence suggests that both the form of an antigen as well as its route of delivery to the gut immune system will determine the type of immune response elicited [31]. While soluble dietary antigens transported by enterocytes are likely to induce oral tolerance, particulate antigens taken up through M cells of the follicle-associated epithelium stimulate mucosal and systemic immune responses. A recent study by Chambers et al. [32] demonstrated that protein bodies released by peanut cells during digestion are likely to favor the induction of immune responses rather than oral tolerance. Their data suggest that peanut protein body particles, due to their resistance to digestion and route of transport, may play a crucial role in the sensitization process. These data, combined with our current results, provide strong evidence that peanut allergens are being continually released and sampled by the GI immune system.

The results established in the porcine animal digestive system allow reasonable extrapolation to human GI digestion and continued exposure to pepsin-resistant peanut allergens. A finding that the roasting process reduces peanut protein solubility [33] will be used to compare the in vivo digestion of raw and processed peanut under similar physiological conditions in future studies. Reduced

solubility could increase the amount of surviving particulate material available for transport through the stomach and into the small intestine for antigen sampling in the GI mucosa.

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