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Effect of Simulated Gastric and Intestinal Digestion on Temporal Stability and Immunoreactivity of Peanut, Almond, and Pine Nut Protein Allergens

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ABSTRACT: Current models of digestibility utilize pepsin stability to assess the safety of allergenic versus nonallergenic food proteins. Dietary protein digestion in vivo, however, requires acid denaturation and protease cleavage by pepsin, trypsin, and/or chymotrypsin. The ability of this approach to identify food protein stability in the mammalian gut may be limited. We determined the temporal stability and immunoreactivity of almond, pine nut, and peanut allergenic proteins under simulated physiologic gastric and intestinal digestive conditions in vitro. Gel electrophoresis and immunoblot analyses were used to determine protein stability and immunoreactivity, respectively. Peanut, almond, and pine nut proteins were pepsin- and pancreatin-stable and immunoreactive for up to 1 h after initiation of digestion. Moreover, successive acid denaturation and pepsin and pancreatin cleavage were necessary to hydrolyze these allergenic proteins and reduce their IgG- and IgE-binding capacity, which suggests that digestibility models must be improved for more accurate safety assessment of food allergens.

KEYWORDS: *food allergens, tree nuts, peanuts, digestibility, immunoreactivity*

INTRODUCTION

Allergenic food proteins are stable to digestion, a common feature of many food allergens.[1,2] Food protein that is resistant to proteolytic digestion and acid denaturation has an increased probability of reaching the intestinal mucosa where absorption can occur. The longer these allergenic food proteins remain intact within the mammalian gastrointestinal tract, the more likely they are to trigger an immune response.[3] Thus, the ability of food allergens to reach the jejunal mucosa is a prerequisite for allergenicity. Protein is partially digested within the gastric mucosa by acidic denaturation and the proteolytic action of pepsin. Proteolytic fragments of allergenic proteins are emptied into the duodenum and mixed with pancreatic secretions of trypsin and chymotrypsin, catalyzing the final steps of digestion, which yields free amino acids and peptides readily available for absorption within the jejuna. Digestive-stable proteolytic fragments of allergenic food proteins, however, can be absorbed within the jejuna, potentially eliciting an immunologic response.

Pepsin digestion does not reduce the IgE immunoreactivity of milk protein β-lactoglobulin[4] or egg protein ovomucoid,[5] and fragments of these allergenic proteins remain immunoreactive. Peanut allergen Ara h 2, recognized by more than 90% of peanut-allergic patients, is resistant to pepsin, trypsin, and chymotrypsin cleavage due to the protection provided by the secondary and tertiary protein structure.[6] The digestive stability and immunoreactivity of most allergenic food proteins and/or their proteolytic fragments have yet to be determined. Most current models of digestion have exclusively investigated the stability of food proteins to pepsin, whereas complete protein digestion also requires the proteolytic action of trypsin and/or chymotrypsin. Protein digestion is also dependent on gut motility and transit time through the alimentary tract, which determines the temporal exposure of dietary proteins to digestive enzymes and conditions. In the present study, we aimed to determine the temporal digestion of pepsin and trypsin of commonly consumed tree nuts (almonds and pine nuts) and peanuts and to determine the immunoreactivity of these proteins and/or their proteolytic fragments. Recently, dietary inclusion of pine nuts and almonds has become widespread because their heart-healthy nutritional attributes have led to increased consumption.[7,8] Peanut and tree nut allergies affect approximately 1.1% of the United States adult general population, or about 3 million Americans, representing a significant health concern.[9] Peanut allergy has increased during recent decades and now affects 1−2% of young children.[10] Improved understanding of the digestive products of food allergens will facilitate the development of more sensitive and effective antibodies to detect food allergens, establish limits of detection for method development, establish threshold levels of sensitization, and help to determine the allergenicity of novel food proteins.

MATERIALS AND METHODS

Sample Preparation. Prepackaged peanuts, almonds, and pine nuts were purchased from local grocery stores. All nuts were ground with a mortar and pestle, and 100 g of the ground nut was mixed and dissolved in hexane and filtered through Whatman paper. This process was repeated three times and the sample filtrate was collected and allowed to air-dry overnight. Several different 100 g samples were dissolved in hexane and filtered once through a glass funnel lined with Whatman paper, positioned over a glass beaker. The collected filtrate from each different 100 g sample was pooled and stored at −20 °C.

Protein Extraction. Total proteins were extracted from defatted flours (flour to buffer ratio 1:10 w/v) in 20 mM Tris-HCl buffer, pH 8.1, supplemented with 1% HALT protease inhibitor (catalog no. 78425, Thermo Scientific, Rockford, IL). Samples were vortexed and

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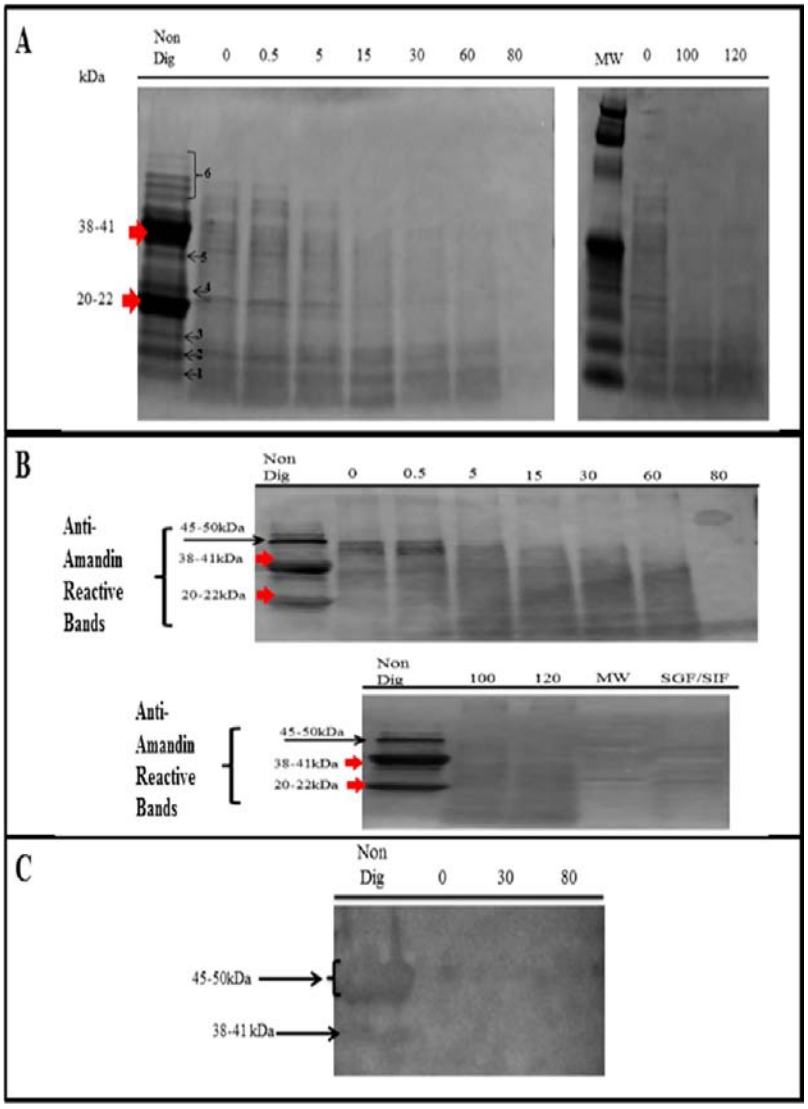

Figure 1. Temporal pepsin digestion and immunoreactivity of total proteins extracted from almonds. (A) Proteins (40 μg) were separated by SDS–PAGE under reducing conditions and stained with Coomassie brilliant blue. Lanes: MW = molecular weight marker; Non-Dig = nondigested almond proteins; 0, 0.5, 5, 15, 30, 60, 80, 100, and 120 = time (minutes) of pepsin digestion. Thick arrows indicate the two major polypeptides of 20–22 and 38–41 kDa. Minor polypeptides are present at (1) 12.5, (2) 15, (3) 17.5, (4) 25, (5) 35, and (6) 43–50 kDa. (B) Electrophoretically separated almond proteins were transferred to a nitrocellulose membrane and immunoblotted with rabbit amandin antibodies (1:1000) to determine immunoreactivity. In lane SGF/SIF, almond proteins were consecutively subjected to acid denaturation and 60 min of pepsin digestion followed by 30 min of pancreatin digestion under simulated physiologic conditions. Anti-amandin immunoreactive polypeptides are shown by arrows at 20–22, 38–41, and 45–50 kDa. (C) Electrophoretically separated almond proteins were transferred to a nitrocellulose membrane and immunoblotted with antibodies of an almond allergic patient (1:2) to determine immunoreactivity after 0, 30, and 80 min of pepsin digestion. Immunoreactive polypeptides are shown by arrows at 38–41 and 45–50 kDa.

centrifuged for 10 min at 4 °C and the supernatants were used for analysis. Total soluble protein was determined by use of a bicinchoninic acid (BCA) protein assay kit (catalog no. 23227) purchased from Thermo Scientific. Protein extracts were aliquoted and stored at −20 °C.

Reagents. Pepsin, 2546 units/mg activity (catalog no. P7012), and pancreatin, 26 296 inits/mg activity (catalog no. P1750), were purchased from Sigma Chemical Co. (St. Louis, MO) in the highest available purity. The gels and running buffer for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) Mini-Protean TGX (catalog no. 456-1034) were purchased from Bio-Rad (Hercules, CA), and reducing sample buffer (catalog no. 39000) was purchased from Thermo Scientific. Western blots were performed by use of the Trans-Blot Turbo mini system (catalog no. 170-4155) and nitrocellulose transfer packs (catalog no. 170-4158) purchased from Bio-Rad. Immunoreactive digestive protein fragments and undigested proteins were detected by use of rabbit allergen-specific (peanut, almond, or pine nut) primary antibodies raised in-house, donkey anti-rabbit IgG-horseradish peroxidase (HRP) secondary antibodies (catalog no. sc-2313) from Santa Cruz Biotechnology (Santa Cruz, CA), and chromogenic substrates chloronaphthol and diaminobenzidine (CN/DAB, catalog no. 34000) from ThermoScientific. Allergen-specific rabbit primary antibodies were produced via a protocol that was approved by the Institutional Animal Care and Use Committee.

Immunoreactive digestive protein fragments and undigested proteins were detected by use of allergen-specific primary antibodies collected from allergic and nonallergic human donors or purchased commercially from Plasma Lab International (Everett, WA), goat anti-human IgE-HRP secondary antibodies (catalog no. 074-1004) from KPL (Gaithersburg, MD), and CN/DAB from ThermoScientific.

Simulated Gastric Fluid Digestion Assay. The protocols of Astwood et al.[11] were followed with some modifications. Simulated

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gastric fluid (SGF) was prepared as described in the U.S. Pharmacopeia:[12] 1 mg/mL solution comprised 2.546 units of pepsin/μL in 0.03 mol/L NaCl (pH 1.2). SGF was aliquoted (500 μL) into a 5.0-mL glass tube for each time interval (0, 0.5, 5, 15, 30, 60, 80, 100, and 120 min) and incubated in a 37 °C water bath for 10 min. Test protein (1600 μg) was added to each tube to begin the temporal SGF digestion assay, and 75 μL of 1 N NaOH was added to each vial to stop the reaction. SGF digestive products were subsequently stored at −20 °C until analyzed.

Simulated Intestinal Fluid Digestion Assay. Simulated intestinal fluid (SIF) was prepared as described in the U.S. Pharmacopeia:[12] 10 mg/mL pancreatin (chymotrypsin, trypsin, amylase, and lipase) in 0.05 mol/L KH_2PO_4, (pH 7.5) containing 26 296 units of pancreatin/mL. SIF was aliquoted (500 μL) into a 5.0-mL glass tube for each time interval (0, 0.5, 5, 15, 30, 60, 80, 100, and 120 min) and incubated in a 37 °C water bath for 10 min. Test protein (1600 μg) was added to each tube to begin the temporal SIF digestion assay at an 8:1 ratio. An additional tube was prepared to determine the digestibility of test proteins to concurrent pepsin, trypsin, and chymotrypsin (SGF/SIF), in which 1600 μg of test protein was subjected to 60 min of pepsin digestion, followed by 30 min of pancreatin digestion at 37 °C. The reaction was immediately stopped by placing each tube in a boiling water bath for 10 min. All digestive products were subsequently stored at −20 °C until analyzed.

Production of Allergen-Specific Rabbit Antibodies. Rabbit antibodies were prepared by subcutaneous inoculation of adult New Zealand White rabbits (>2.0 kg). Prepared antigens were amandin, purified from defatted almond flour by reverse-phase HPLC, and peanut and pine nut proteins, extracted from defatted peanut and pine nut flour with phosphate-buffered saline, pH 8. Antigens were dialyzed overnight against normal saline and suspended to a final concentration of 1.0 mg/mL. One hundred micrograms of antigen was injected into multiple sites on the shaved back of the rabbit. The initial injection was antigen-suspended 1:1 in normal saline/complete Freund's adjuvant. Rabbits were inoculated 2−3 weeks later with antigen suspended 1:1 in normal saline/incomplete Freund's adjuvant. Two subsequent injections consisted of 100 μg of protein suspended in normal saline. For blood collection, animals were anesthetized with an intramuscular injection of ketamine/xylazine/acepromazine. Rabbits were euthanized by intravenous injection of potassium chloride. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC), and rabbits were housed in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) accredited facility.

SDS−PAGE Analysis and Immunoblotting. Digested or undigested test proteins (40 μg for immunoblots with rabbit primary antibodies or 80 μg for immunoblots with human primary antibodies) were loaded per lane on a 10% polyacrylamide Tris−glycine gel and electrophoretically separated under constant voltage with Tris/glycine/SDS buffer according to the manufacturer's instructions. Proteins were visualized by Coomassie brilliant blue staining and digitally imaged. To detect allergen-specific IgE-binding peptides, immunoblot analysis was performed with allergen-specific antibodies generated in rabbits in-house. SDS−PAGE-resolved proteins were transferred to nitrocellulose membranes electrophoretically according to the manufacturer's instructions. The membranes were then washed three times in Tris-buffered saline with 0.1% Tween (TBST) and subsequently blocked in 5% nonfat dry milk (NFDM) in TBST for 2 h at room temperature. The membranes were incubated at 4 °C overnight in 1:1000 dilution of rabbit allergen-specific antibodies in 5% NFDM in TBST or 1:2 dilution of human plasma from allergic donors in 5% NFDM in TBST. The membranes were washed three times in TBST and incubated in secondary antibodies (donkey anti-rabbit IgG-HRP dilution 1:1000 in 5% NFDM in TBST or goat anti-human IgE-HRP 1:10 dilution in 5% NFDM in TBST) at room temperature for 1 h. Biodetection was determined by utilizing chromogenic peroxidase substrate CN/DAB-based detection of HRP activity.

RESULTS AND DISCUSSION

Almond (*Prunus dulcis*). Tree nuts are widely consumed due to their pleasant taste and potentially "heart-protective" health benefits. In 2009, almonds ranked first in the global trade of tree nuts, followed by cashews, pistachios, and hazelnuts, with almonds being more widely consumed in Europe.[13] Hence, with increasing consumption, a growing number of individuals have become sensitized to tree nuts and peanuts,[14] and almond allergy is now the third most commonly reported tree nut allergy in the United States.[15] Some almond allergenic proteins have been identified and characterized by their biochemical function, but very few studies have examined the effects of temporal digestion under simulated physiologic conditions and the immunoreactivity of almond allergenic proteins, protein fragments, and digestive products.

Eight major allergic proteins—Pru du 1, Pru du 2, Pru du 2S albumin, Pru du 3, Pru du 4, Pru du 5, Pru du 6 (amandin), and Pru du −γ conglutin—have been identified in almonds. Amandin is a major water-soluble storage protein that dominates almond protein composition[16,17] and is an oligomeric protein comprising two major polypeptides with estimated molecular masses of 20−22 and 38−41 kDa, linked via disulfide bonds, and several additional minor polypeptides.[18,19] Electrophoretic analysis under reducing conditions of total extracted almond proteins clearly revealed the major 20−22 and 38−41 kDa polypeptides (thick arrows) and minor polypeptides (thin arrows): (1) 12.5, (2) 15, (3) 17.5, (4) 25, (5) 35, and (6) 43−50 kDa (Figure 1A). These six minor resolved polypeptides were of lesser quantitative importance based on Coomassie brilliant blue staining (Figure 1A). These proteins were completely digested at 80, 100, and 120 min of pepsin digestion, with proteolytic peptide fragments visualized after 0.5 min of pepsin digestion, being stable up to 60 min in vitro (Figure 1A). All minor (thin arrows) polypeptide fractions greater than 41 kDa were completely digested after 15 min of pepsin digestion under simulated physiologic conditions (Figure 1A).

To determine the immunoreactivity of almond digestive fragments and proteins, almond proteins digested with pepsin for increasing lengths of time were analyzed by immunoblotting with rabbit anti-amandin IgG antibodies. Anti-amandin IgG binding of three almond polypeptides of 20−22, 38−41, and 45−50 kDa was observed in the undigested total almond protein sample (Figure 1B). These peptides and their digested fragments remained immunoreactive to anti-amandin-specific rabbit antibodies from 0 to 60 min of pepsin digestion in vitro, with no amandin-specific immunoreactivity at the 80, 100, and 120 min time points (Figure 1B). Although denaturing SDS−PAGE analysis indicated substantial digestion of almond proteins after 60 min of pepsin digestion (Figure 1A), the digested protein fragments remained immunoreactive (Figure 1B), indicating that almond allergenic proteins/fragments remain immunoreactive even after 1 h of pepsin digestion. Conversely, when almond proteins were digested with pepsin (60 min) and then with pancreatin (30 min), they were nonreactive to rabbit anti-amandin IgG antibodies (Figure 1B, SGF/SIF lane).

Undigested almond polypeptides of 38−41 and 45−50 kDa were immunoreactive with plasma IgE antibodies from an almond allergic patient (Figure 1C), consistent with other studies demonstrating that almond proteins of 45 kDa[20] and 50 kDa[21] are immunoreactive with the sera of almond allergic

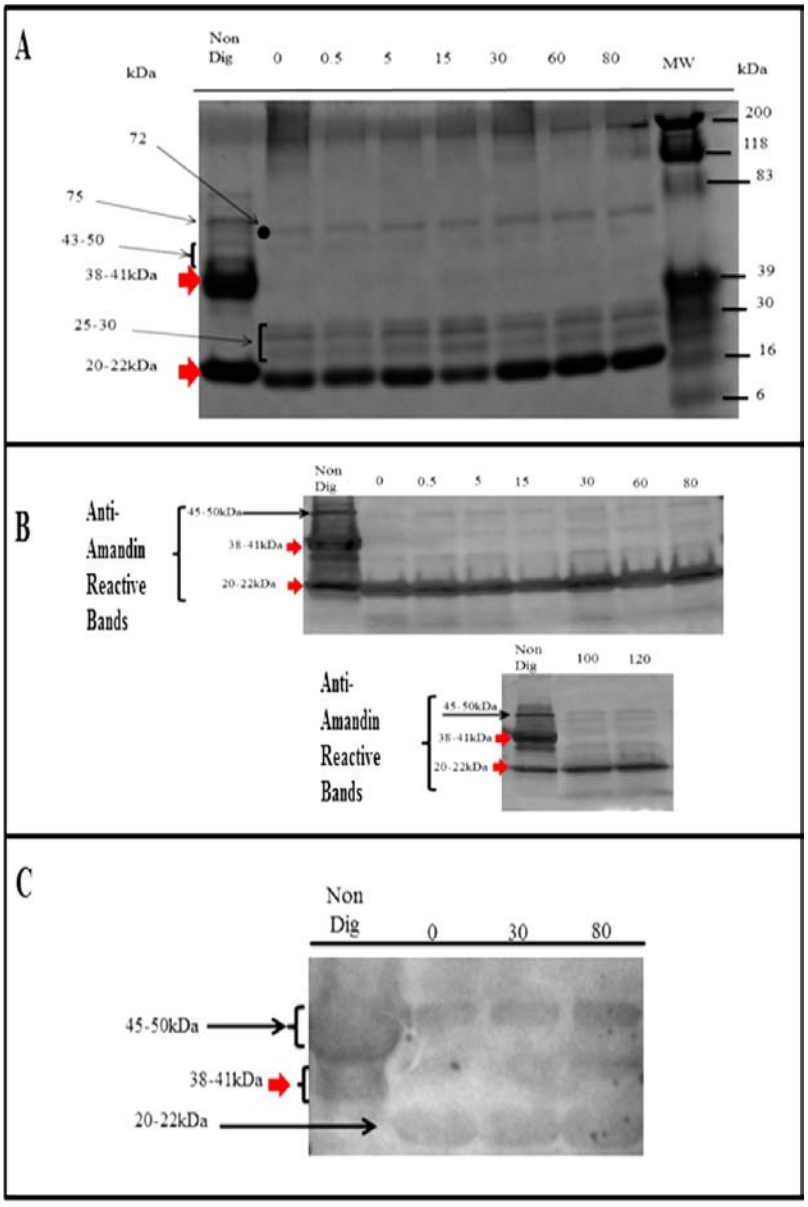

Figure 2. Temporal pancreatin digestion and immunoreactivity of total proteins extracted from almonds. (A) Nondigested or pepsin-digested proteins (40 μg) were separated by SDS−PAGE under reducing conditions and stained with Coomassie brilliant blue. Lanes: MW = molecular weight marker; Non-Dig = nondigested almond proteins; 0, 0.5, 5, 15, 30, 60, and 80 = time (minutes) of pancreatin digestion. Thick arrows indicate the two major polypeptides of 20−22 and 38−41 kDa. Minor polypeptides (thin arrows) are seen at 43−50 and 75 kDa. (B) Electrophoretically separated digested almond proteins were transferred to a nitrocellulose membrane and immunoblotted with a rabbit amandin antibody (1:1000) to determine immunoreactivity of almond digested proteins after 0, 0.5, 5, 15, 30, 60, 80, 100, and 120 min of pancreatin digestion. Anti-amandin immunoreactive polypeptides are marked with arrows at 20−22, 38−41, and 45−50 kDa. (C) Electrophoretically separated almond proteins were transferred to a nitrocellulose membrane and immunoblotted with the antibodies of an almond allergic patient (1:2) to determine immunoreactivity after 0, 30, and 80 min of pancreatin digestion. Immunoreactive polypeptides are shown by arrows at 20−22, 38−41, and 45−50 kDa.

human patients. These polypeptides were nonreactive after 0, 30, and 80 min of in vitro pepsin digestion based on immunoblot analysis (Figure 1C).

Amandin major protein at 20−22 kDa and protein bands 72 kDa (●) and 25−30 kDa (bracketed area) were very stable, even after 80 (Figure 2A), 100, and 120 min (data not shown) of pancreatin digestion in vitro, as shown by denaturing SDS−PAGE analysis. Protein bands between 25 and 30 kDa (Figure 2A, bracketed area) appeared only in the pancreatin-digested sample between 0 and 80 min, implying that these bands are the reduced and/or proteolytic products of other almond proteins since they were not observed in the undigested almond total protein sample (Figure 2A). Major amandin protein at 20−22 kDa was pancreatin-stable and highly immunoreactive (0−120 min) to rabbit amandin IgG, while protein bands at 38−41 and 45−50 kDa had faint immunoreactive responses between 0 and 120 min of pancreatin digestion (Figure 2B). Almond polypeptides of 38−41 and 45−50 kDa in the undigested and pancreatin-digested (0, 30, and 80 min) protein samples were IgE-reactive to plasma from an almond allergic patient as shown by immunoblotting analysis (Figure 2C), with polypeptide 20−22 kDa being immunoreactive after 0, 30, and 80 min of pancreatin digestion. In addition, undigested and digested

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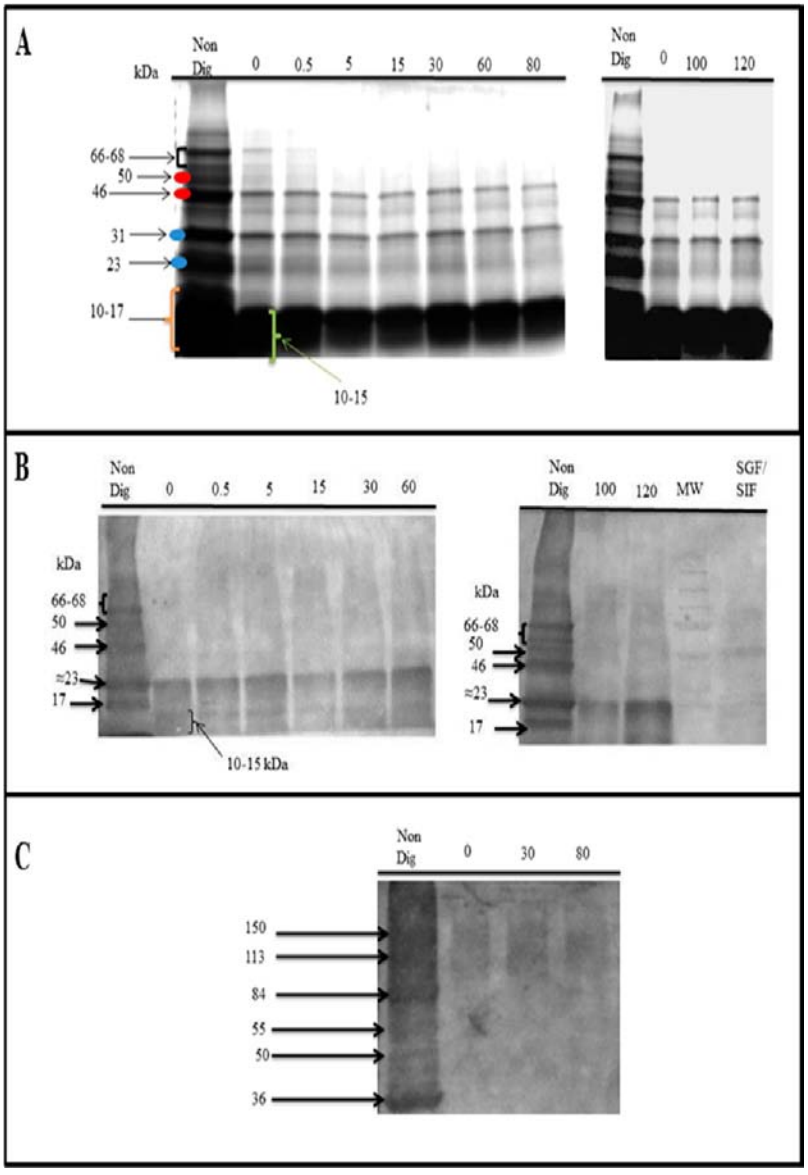

Figure 3. Temporal pepsin digestion and immunoreactivity of total proteins extracted from pine nut. (A) Nondigested or pepsin-digested proteins (40 μg) were separated by SDS−bAGE under reducing conditions and stained with Coomassie brilliant blue. Lanes: MW = molecular weight marker; Non-Dig = nondigested pine nut proteins; 0, 0.5, 5, 15, 30, 60, 80, 100, and 120 = time (minutes) of pepsin digestion. Major polypeptides: bracketed area, 66−68 kDa; red and blue circles, 50, 46, 31, and 23 kDa; and braced area, 10−17 kDa in the nondigested pine nut sample. (B) Electrophoretically separated digested and nondigested pine nut proteins were transferred to a nitrocellulose membrane and immunoblotted with a rabbit anti-pine nut antibody (1:1000) to determine the immunoreactivity of pine nut digested proteins after 0, 0.5, 5, 15, 30, 60, 100, and 120 min of pepsin digestion. Anti-pine nut immunoreactive polypeptides are shown by thin arrows 17, 23, 46, 50, and 66−68 kDa in the pine nut protein sample and 23 and 10−15 kDa in the pepsin-digested pine nut protein samples. In lane SGF/SIF, pine nut proteins were consecutively subjected to acid denaturation and 60 min of pepsin digestion followed by 30 min of pancreatin digestion under simulated physiologic conditions. (C) Electrophoretically separated pine nut proteins were transferred to a nitrocellulose membrane and immunoblotted with antibodies from a pine nut allergic patient (1:2) to determine immunoreactivity after 0, 30, and 80 min of pepsin digestion. Immunoreactive polypeptides are shown by arrows 150, 113, 84, 55, 49, and 36 kDa.

(pepsin, pancreatin) almond proteins were nonreactive to IgE antibodies in the plasma of a nonallergic (peanut and tree nut) donor patient or conjugated secondary antibodies, according to immunoblot analysis (data not shown).

Allergenic almond proteins were completely hydrolyzed and lost immunoreactivity after 80 min of pepsin digestion in vitro, whereas three to four almond allergenic proteins were stable to pancreatin and remained immunoreactive from 0 to 120 min under simulated physiologic conditions, demonstrating that pepsin was the most efficient protease for hydrolyzing almond proteins. Sathe[18] demonstrated that almond allergenic proteins are partially digested after 30 min of pepsin digestion versus chymotrypsin and/or trypsin digestion in vitro, whereas most nonallergenic food proteins are rapidly denatured and digested under common physiologic gastric conditions within 30 s.[11] The present study shows that allergenic almond proteins must remain in the gastric lumen under denaturing conditions for up to 80 min for complete hydrolysis to occur, without which these allergenic proteins have an increased probability of transiting to the jejunal mucosa for absorption.

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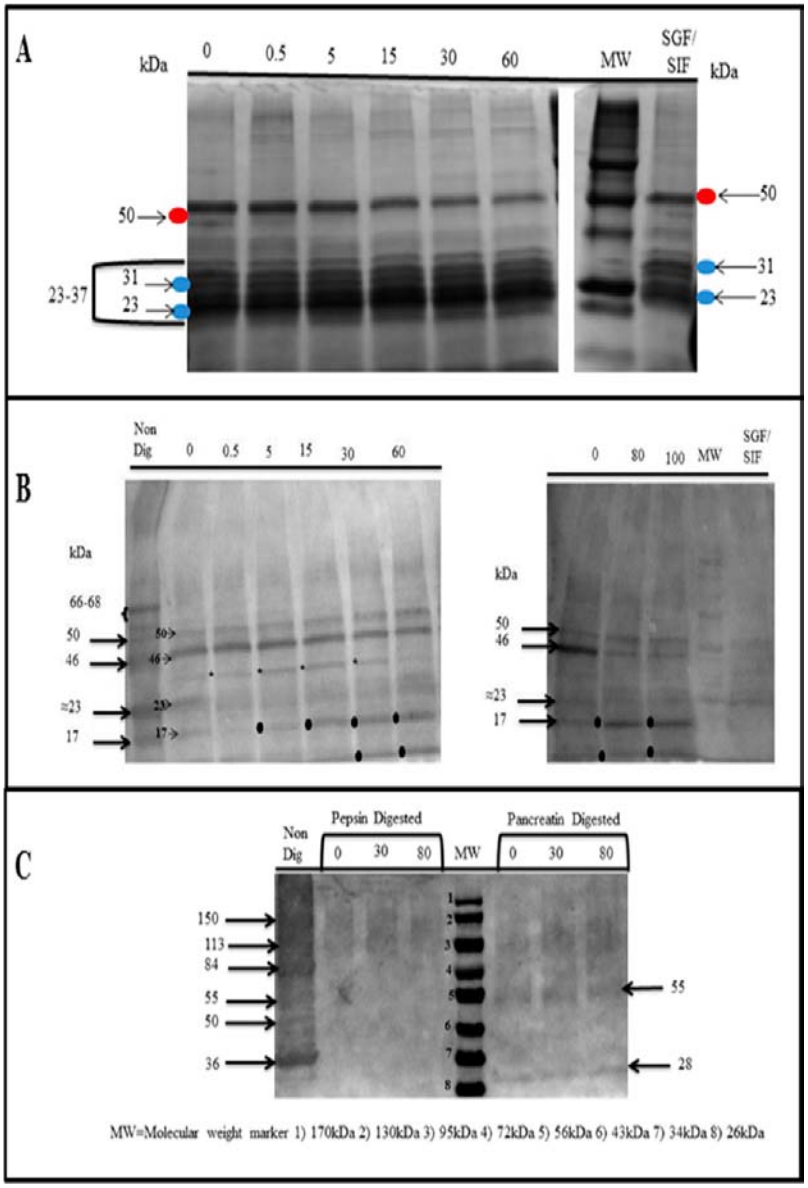

Figure 4. Temporal pancreatin digestion and immunoreactivity of total proteins extracted from pine nut. (A) Pine nut proteins (40 μg) were separated by SDS−PAGE under reducing conditions and stained with Coomassie brilliant blue. Lanes: MW = molecular weight marker; 0, 0.5, 5, 15, 30, and 60 = time (minutes) of pancreatin digestion. Major polypeptides: bracketed area, 23−37 kDa; red and blue circles, 50, 31, and 23 kDa in the digested pine nut sample. In lane SGF/SIF, pine nut proteins were consecutively subjected to acid denaturation and 60 min of pepsin digestion followed by 30 min of pancreatin digestion under simulated physiologic conditions. (B) Electrophoretically separated digested and nondigested pine nut proteins were transferred to a nitrocellulose membrane and immunoblotted with a rabbit anti-pine nut antibody (1:1000) to determine immunoreactivity of pine nut digested proteins after 0, 0.5, 5, 15, 30, 60, 80, and 100 min of pancreatin digestion. In lane SGF/SIF, pine nut proteins were consecutively subjected to acid denaturation and 60 min of pepsin digestion followed by 30 min of pancreatin digestion under simulated physiologic conditions. Anti-pine nut immunoreactive polypeptides are shown by thin arrows at 17, 23, 46, and 50 kDa and a braced area at 66−68 kDa in the nondigested pine nut protein sample. (*) Immunoreactive smaller protein bands (35 kDa) appear between 0 and 30 min. (●) Quantitative temporal increase in immunoreactivity of 17 and 10 kDa protein bands from 5 to 100 min. (C) Electrophoretically separated pine nut proteins were transferred to a nitrocellulose membrane and immunoblotted with antibodies from a pine nut allergic patient (1:2) to determine immunoreactivity after 0, 30, and 80 min of digestion. MW = Molecular mass markers: (1) 170, (2) 130, (3) 95, (4) 72, (5) 56, (6) 43, (7) 34, and (8) 26 kDa. Immunoreactive polypeptides are shown by arrows at 150, 113, 84, 55, 50, and 36 kDa for nondigested total proteins and at 55 and 28 kDa for pancreatin-digested pine nut proteins.

Pine Nut (*Pinus pinea*). Pine nuts are now widely consumed in the American diet (desserts, chocolate-covered pine nuts, pastries, oil, salads, and pesto sauce) due to their unique flavor and common use in Italian cooking. As with many other allergenic foods, pine nuts may be present in food as hidden ingredients, putting the sensitive allergic consumer at risk. Pine nut allergenic proteins have yet to be identified and biochemically characterized, with few published studies describing the allergenicity of pine nut proteins. In this study, we aimed to determine the temporal digestion and potential allergenicity of pine nut total proteins under simulated physiologic conditions in vitro.

Under reducing electrophoretic conditions, six visible protein bands were resolved: 10−17, 23, 31, 46, 50, and 66−68 kDa,

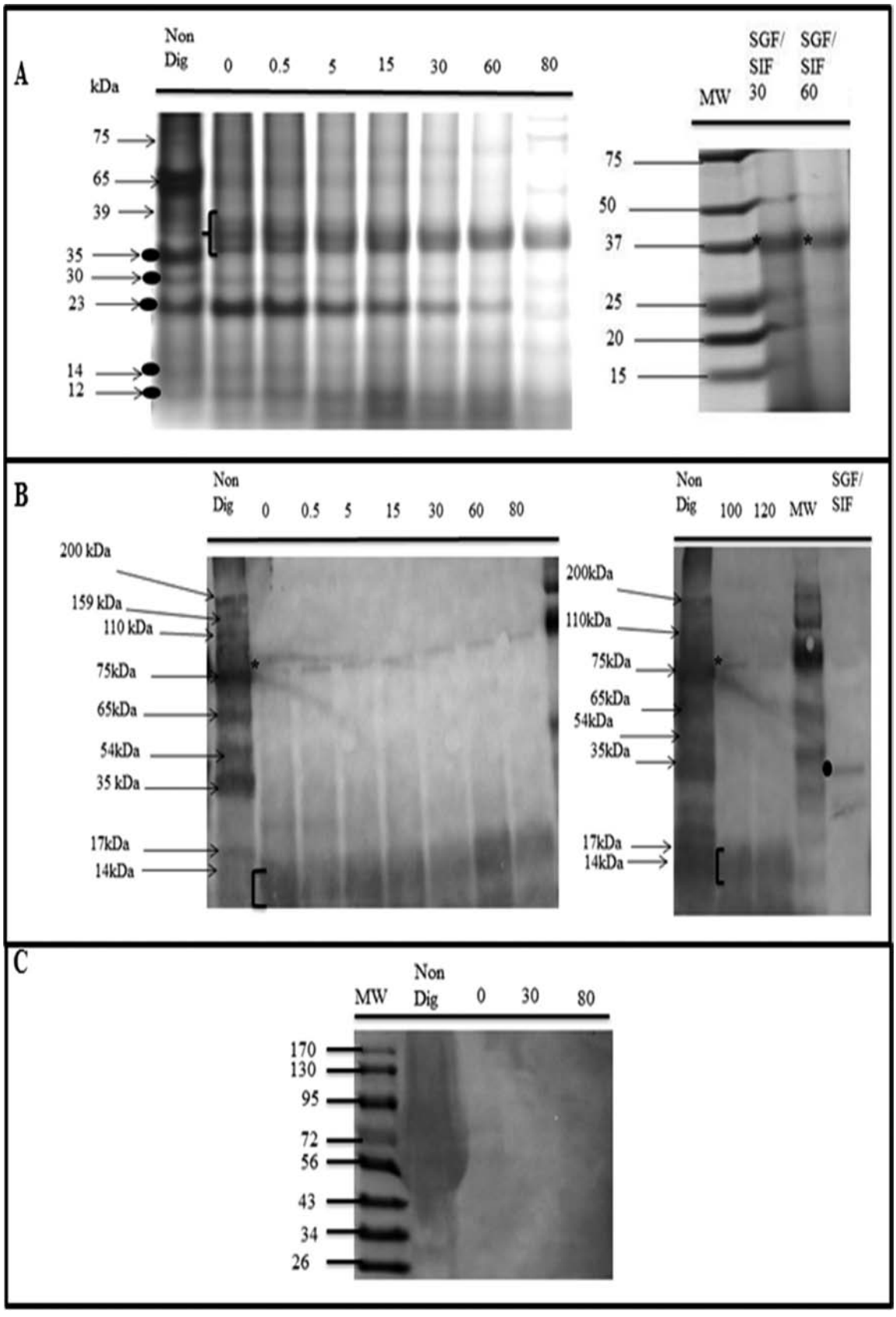

Figure 5. Temporal pepsin digestion and immunoreactivity of total proteins extracted from peanut. (A) Nondigested or pepsin-digested proteins (40 μg) were separated by SDS−PAGE under reducing conditions and stained with Coomassie brilliant blue. Lanes: MW = molecular weight marker; Non-Dig = nondigested peanut proteins; 0, 0.5, 5, 15, 30, 60, and 80 = time (minutes) of pepsin digestion. Major polypeptides: (●) 12, 14, 23, 30, and 35 kDa; thin arrows 39 and 75 kDa; thick arrow 65 kDa in the nondigested peanut sample. In lane SGF/SIF, peanut proteins were consecutively subjected to acid denaturation and 60 min of pepsin digestion followed by 30 or 60 min of pancreatin digestion under simulated physiologic

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Figure 5. continued

conditions. (*) 35 kDa protein band in SGF/SIF-digested samples. (B) Electrophoretically separated digested and nondigested peanut proteins were transferred to a nitrocellulose membrane and immunoblotted with a rabbit anti-peanut antibody (1:1000) to determine immunoreactivity of peanut digested proteins after 0, 0.5, 5, 15, 30, 60, 80, 100, and 120 min of pepsin digestion. In lane SGF/SIF, peanut proteins were consecutively subjected to acid denaturation and 60 min of pepsin digestion followed by 30 min of pancreatin digestion under simulated physiologic conditions, indicating that protein band 35 kDa (●) was immunoreactive. Peanut immunoreactive polypeptides are shown by the bracketed area at 12−14 kDa and (*) 75 kDa in the pepsin-digested peanut proteins. (C) Electrophoretically separated peanut proteins were transferred to a nitrocellulose membrane and immunoblotted with antibodies from peanut allergic patient (1:2) to determine immunoreactivity after 0, 30, and 80 min of digestion.

with the bands at 23, 31, 46, and 10−17 kDa being very prominent in the nondigested protein pine nut sample (Figure 3A). Similarly, Senna et al.[22] reported the electrophoretic resolution of several protein bands ranging from 8 to approximately 90 kDa, with marked bands at about 25 and 32 kDa and a very thick band ranging from 8 to 11 kDa in pine nut extracts. Protein bands of 46, 31, and 23 kDa were pepsin-stable for 0−120 min of digestion, whereas proteins bands at 66−68 and 50 kDa were completely hydrolyzed and the protein band at 10−17 kDa was partially hydrolyzed after 0 min under simulated physiologic gastric conditions (Figure 3A). Interestingly, a protein band corresponding to 10−17 kDa in the nondigested protein sample appeared to be partially hydrolyzed after 0−120 min of pepsin digestion and appeared as a 10−15 kDa protein band under reducing SDS−PAGE conditions (Figure 3A), suggesting that this protein is only partially hydrolyzed by pepsin.

Upon immunoblotting analysis, undigested pine nut proteins of 66−68, 50, 46, 23, and 17 kDa were immunoreactive with anti-pine nut rabbit IgG (Figure 3B). After pepsin digestion, however, only one protein band of 23 kDa was immunoreactive with anti-pine nut rabbit IgG, with a protein band of 10−15 kDa remaining immunoreactive between 0 and 120 min of pepsin digestion in vitro (Figure 3B). These immunoreactive bands were pepsin-stable pine nut proteins and were possibly the proteolytic protein fragments/products obtained from digestion (Figure 3B).

Only undigested pine nut proteins of 36, 50, 55, 84, 113, and 150 kDa were immunoreactive with antibodies in the plasma of a pine nut allergic patient donor on immunoblot analysis, while no reaction was detected after 0, 30, or 80 min of pepsin digestion in vitro (Figures 3C and 4C). Consistent with this finding, Senna et al.[22] reported strong IgE reactivity with pine nut proteins between 14 and 67 kDa by immunoblotting analysis using human sera from pine nut allergic human patients. Other studies reported IgE-reactive pine nut peptides of 50 and 66−68 kDa[23,24] and 17 kDa[25] with sera of pine nut allergic patients. Only pine nut protein bands at 55 and 28 kDa were immunoreactive (after 0, 30, and 80 min of pancreatin digestion) with the antibodies from a pine nut allergic patient donor (Figure 4C). In addition, undigested and digested (pepsin, pancreatin) pine nut proteins were nonreactive to IgE antibodies in the plasma of a nonallergic (peanut and tree nut) donor patient or conjugated secondary antibodies, according to immunoblot analysis (data not shown).

Electrophoretically separated total pine nut proteins of 23−37 and 50 kDa digested with pancreatin were stable between 0 and 60 min (Figure 4A). Moreover, pine nut protein bands of 50, 31, and 23 kDa were stable to consecutive protease treatment with pepsin (60 min) and pancreatin (30 min) as shown in Figure 4A (lane SGF/SIF). Pine nut total proteins of 50, 46, 23, and 17 kDa were immunoreactive with anti-pine nut rabbit IgG for 0−100 min in the pancreatin-digested samples, with protein band 23 kDa immunoreactive only at 0 and 0.5 min (Figure 4B). With increasing time intervals of pancreatin digestion, additional immunoreactive smaller protein bands (35 kDa) appeared between 0 and 30 min of digestion (Figure 4B, *), along with a quantitative temporal increase in immunoreactivity of 17- and 10-kDa protein bands from 5 to 100 min (Figure 4B, ●). All pine nut proteins were nonreactive to anti-pine nut rabbit IgG after consecutive pepsin and pancreatin digestion (Figures 3B and 4B, lanes SGF/SIF). While these fragments/products of pine nut proteins were stable to pepsin and pancreatin, the loss of immunoreactivity implies a loss of epitope binding, possibly due to denaturation and loss of secondary and/or tertiary structures.

Peanut (*Arachis hypogaea*). Peanuts are widely used to prepare a variety of packaged foods in the United States and are relied upon as a protein extender in developing countries. Peanut allergens Ara h 1, Ara h 2, and Ara h 3 have been well-characterized, cloned, and sequenced, with their IgE-binding and T-cell epitopes identified.[26,27] Ara h 1, a 64 kDa glycoprotein, is recognized by more than 95% of peanut allergic patients, with Ara h 1 being initially identified by IgE immunoblot analysis with pooled serum from peanut allergic patients.[26] Ara h 1 occurs naturally in trimeric form of approximately 185 kDa via noncovalent interactions.[28] Ara h 2 migrates as a doublet at approximately 20 kDa, consisting of two isoforms.[28] Ara h 3 is a post-translationally proteolytically processed protein of triplet at 42−45 kDa, band at 25 kDa, and less prominent bands at 12−18 kDa.[28] Current in vitro and in vivo studies have demonstrated that purified Ara h 2 triggers the release of histamine from basophils containing peanut-specific IgE, and reactivity in skin prick test with peanut allergic individuals was far more pronounced for Ara h 2 compared to Ara h 1.[28] Ara h 6 (15 kDa) IgE binding and histamine release from basophils has been found to be very similar to Ara h 2 activity.[29] The percentage of patients recognizing Ara h 3 is much lower than that of patients recognizing Ara h 1 and Ara h 2, and the ability of Ara h 3 to trigger histamine release is much lower than Ara h 2 and Ara h6 but in the range of Ara h 1.[30] These differences in allergenicity have been attributed to the random differences in human subjects and study populations.[28−30]

Several other minor peanut allergens, Ara h 4, 5, and 7−10, have been identified and characterized,[31−35] but their allergenic properties are uncertain. Due to the complexity of non-allergenic and allergenic proteins within crude peanut protein extracts, most digestibility studies have examined pepsin stability of purified peanut allergens Ara h 1, 2, 3, and 6.[28] Whereas the use of purified allergens reduces the complexity of data interpretation and identification, it does not parallel in vivo gastric/luminal digestion in which an individual consumes and is sensitized to a complex mixture of allergenic and non-allergenic peanut proteins in packaged foods. Therefore, we elected to utilize crude peanut protein extracts to parallel

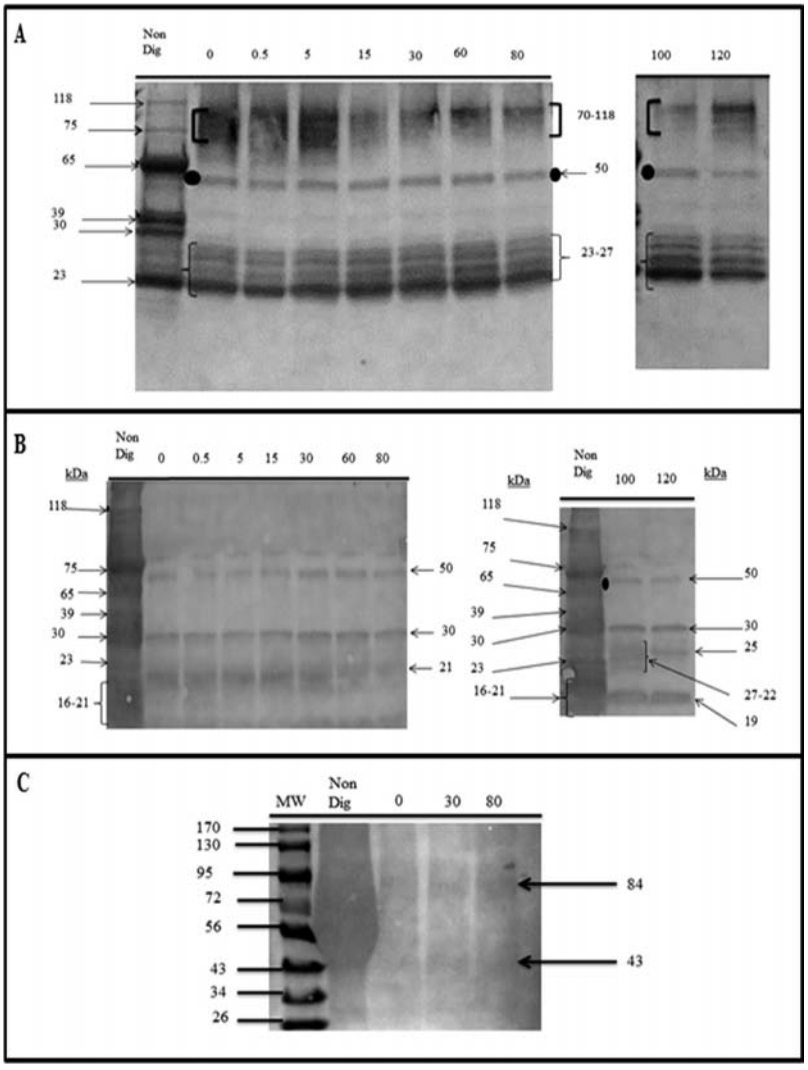

Figure 6. Temporal pancreatin digestion and immunoreactivity of total proteins extracted from peanut. (A) Nondigested or pancreatin-digested proteins (40 μg) were separated by SDS−PAGE under reducing conditions and stained with Coomassie brilliant blue. Lanes: MW = molecular weight marker; Non-Dig = nondigested peanut proteins; 0, 0.5, 5, 15, 30, 60, 80, 100, and 120 = time (minutes) of pancreatin digestion. Major polypeptides are shown by arrows at 23, 30, 39, 65, 75, and 118 kDa in the nondigested peanut sample. Bracketed area 70−118 kDa, (●) 50 kDa, and braced area 23−27 kDa were pancreatin-stable for 0−120 min. (B) Electrophoretically separated digested and nondigested peanut proteins were transferred to a nitrocellulose membrane and immunoblotted with a rabbit anti-peanut antibody (1:1000) to determine immunoreactivity of peanut digested proteins after 0, 0.5, 5, 15, 30, 60, 80, 100, and 120 min of pancreatin digestion. Anti-peanut immunoreactive polypeptides were seen at 16−21, 23, 30, 39, 65, 75, and 118 kDa in the nondigested peanut protein sample and at 75 kDa (●), and 30 and 21 kDa (thin arrows) in the pancreatin-digested peanut samples from 0 to 80 min. Additional protein bands of 19, 27−22, 25, and 50 kDa appear in the 100 and 120 min pancreatin-digested peanut protein samples. (C) Electrophoretically separated peanut proteins were transferred to a nitrocellulose membrane and immunoblotted with antibodies from peanut allergic patient (1:2) to determine immunoreactivity after 0, 30, and 80 min of digestion. Immunoreactive polypeptides are shown by arrows at 84 and 43 kDa after 0, 30, and 80 min of pancreatin digestion.

natural consumption and digestion of peanuts and peanut byproducts. Very few studies have examined the temporal effect of the digestive enzymes pepsin and pancreatin on crude peanut proteins and the immunoreactivity of these proteins and their digestive products under simulated physiologic conditions.

Upon reducing SDS−PAGE analysis, undigested crude peanut proteins appeared as multiple protein bands of apparent molecular masses of 75, 65, 39, 35, 30, 23, 14, and 12 kDa. Protein bands at 65, 35, and 23 kDa are prominent (Figure 5A), which parallels findings by Koppelman et al.[28] Protein bands of 23, 30, and 65 kDa were pepsin-stable for 0−30 min of digestion, and absent in the 60 and 80 min pepsin-digested samples, with decreasing visibility of the banding pattern indicative of temporal pepsin digestion (Figure 5A). In peanut samples digested with pepsin for 0−80 min, protein bands of 35−37 kDa (braced area) appeared, differing from the banding pattern seen in the undigested peanut total protein sample, and may be digestive products (Figure 5A). These banding patterns of pepsin-digested peanut total proteins were consistent at all time intervals between 0 and 120 min (data not shown). Similarly, only the peanut protein band at 35 kDa was stable to consecutive digestion with pepsin (60 min) and pancreatin (30 min) by SDS−PAGE analysis (Figure 5A) and was immunoreactive with rabbit anti-peanut IgG (Figure 5B).

With immunoblotting procedures, protein bands at 200, 159, 110, 75, 65, 54, 35, 17, and 14 kDa from the undigested total peanut protein were reactive to rabbit anti-peanut IgG, while only protein bands at 75 and 14 kDa were stable to pepsin

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digestion (0−120 min) in vitro and remained immunoreactive (Figure 5B). Bands corresponding to 12−14 kDa (bracketed area) were immunoreactive to rabbit peanut IgG from 0 to 120 min of pepsin digestion in vitro and may potentially be digestive products or Ara h 3 proteins (Figure 5B). In parallel, Vieths et al.[36] demonstrated that substantial proteolytic digestion with pepsin did not alter the IgE-binding properties of many peanut allergens. Peanut protein bands in the undigested sample between 170 and 50 kDa were immunoreactive to antibodies in the plasma of a peanut allergic donor patient, with very prominent bands appearing between 95 and 50 kDa (Figures 5C and 6C) and no reactivity at 0, 30, and 80 min after pepsin digestion (Figure 5C).

Electrophoretically separated pancreatin-digested peanut total protein bands of 70−118 kDa (bracket), 50 kDa (●), and 23−27 kDa (brace) were stable from 0 to 120 min of digestion in vitro under simulated physiologic conditions (Figure 6A). Pancreatin-digested peanut protein bands of 50, 30, and 21 kDa, however, were immunoreactive to rabbit peanut IgG from 0 to 60 min in vitro, with protein bands of 50 and 30 kDa remaining immunoreactive at 100 and 120 min of pancreatin digestion (Figure 6B). Interestingly, peanut protein bands of 25, 27−22, and 19 kDa were immunoreactive to rabbit anti-peanut IgG only in the 100 and 120 min pancreatin-digested samples and may be the products of pancreatin digestion (Figure 6B). Peanut protein bands in the undigested sample between 170 and 50 kDa were immunoreactive to antibodies in the plasma of peanut allergic donor patient (Figures 5C and 6C), with immunoreactive protein bands of 84 and 43 kDa present in the 0, 30, and 80 min pancreatin-digested samples (Figure 6C), suggesting that these proteins are pancreatin-stable and potentially allergenic. Also, in a negative control immunoblot, undigested and digested (pepsin, pancreatin) peanut proteins were nonreactive to IgE antibodies in the plasma of a nonallergic (peanut and tree nut) donor patient or conjugated secondary antibodies (data not shown).

Many food allergen proteins have strong intramolecular disulfide bonds, which makes them resistant to acid denaturation and may be important to their allergenicity.[37] Allergenicity of peanut allergen Ara h 2 is diminished after reduction of the intramolecular disulfide bonds, allowing rapid digestion by pepsin, chymotrypsin, or trypsin.[6] Ara h 1 is very resistant to protease digestion and denaturation due to structural protection as a stable trimer complex, allowing passage of Ara h 1 with several intact IgE binding epitopes across the small intestine, contributing to its overall allergenicity.[38] Conversely, Astwood et al.[11] reported that Ara h 1 loses immunoreactivity within 15 s of in vitro pepsin digestion, on the basis of a digestibility model and immunoblotting analysis. While this study demonstrates the stability and immunoreactivity of peanut protein bands of 65, 14, and 17 kDa, these bands cannot be positively identified as Ara h1 (65 kDa), Ara h3 (14 kDa), or Ara h2 (17 kDa), respectively, without protein sequencing. Additionally, variations in allergenicity and potency of peanut allergens (Ara h 1−10) between study subjects and human study populations makes interpretation and identification of immunoblotting results more complex. Nevertheless, studies that parallel in vivo digestion of orally consumed allergen-containing foods are necessary for the identification of nonallergenic and allergenic food proteins and their epitope binding and IgE reactivity. In future digestibility studies, we aim to sequence digestive-stable and immunoreactive proteins identified in this study for biochemical identification, sequence homology, and biochemical characteristics.

In this paper, we elected to use total pine nut, almond, and peanut proteins and not purified allergenic proteins in each of the temporal digestion assays to more closely parallel in vivo conditions, in which peanut, pine nuts, or almonds are orally consumed in the diet. However, this approach proves to be more challenging in the complexity of the data, consisting of a pool of various allergenic and nonallergenic proteins and digested protein fragments in comparison to analysis of the digestive stability of a purified allergenic protein. Nevertheless, this approach more closely parallels the pool of digested dietary proteins found in the mammalian gut and available for nutrient absorption after consumption of pine nut, peanut, or almond containing foods. Current digestibility models of food proteins exclusively utilize pepsin stability as the criterion for safety assessment and for identifying food proteins as allergenic. This approach may be somewhat limited in identifying food protein stability in vivo in the mammalian gut.

In summary, the findings of the present study indicate that some peanut, almond, and pine nut proteins and their fragments are pepsin-stable, pancreatin-stable, and immunoreactive for up to at least 1 h. Only successive protease treatment and denaturation either predominantly hydrolyzed these proteins and/or reduced their immunoreactivity. Thus, the overall contribution of this paper demonstrates that successive treatment with both proteases (pepsin with acid denaturation and pancreatin) either aided in protein digestion or reduced immunoreactivity (pine nut and peanut) of these proteins, indicating that use of both pepsin and pancreatin in a digestive model would serve as a better tool to assess the allergenicity of food proteins for safety assessement.

Although these models are representative of human digestion (gastric and intestinal), they do not predict protein half-life or protein stability in vivo. However, an improved digestion model for food allergens may enhance our understanding regarding the stability of these digestive products and the subsequent nutrient uptake of these digestive-stable and potentially allergenic food proteins in the mammalian gut.

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Notes

The authors declare no competing financial interest.

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