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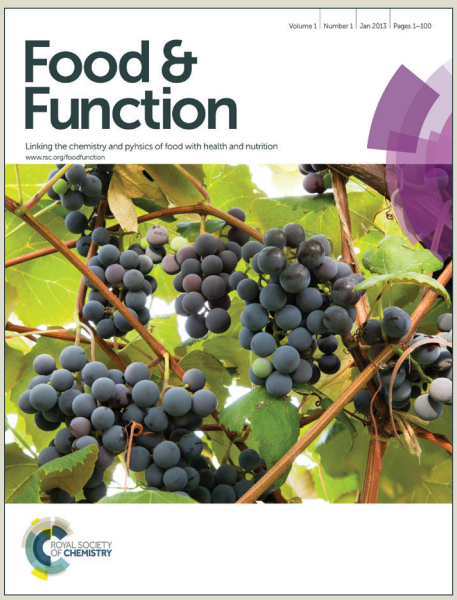

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Stability and Immunogenicity of Hypoallergenic Peanut Protein-Polyphenol Complexes during In Vitro Pepsin Digestion

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Abstract

Allergenic peanut proteins are relatively resistant to digestion, and if digested, metabolized peptides tend to remain large and immunoreactive, triggering allergic reactions in sensitive individuals. In this study, the stability of hypoallergenic peanut protein-polyphenol complexes was evaluated during simulated *in vitro* gastric digestion. When digested with pepsin, the basic subunit of the peanut allergen Ara h 3 was more rapidly hydrolyzed in peanut protein-cranberry or green tea polyphenol complexes compared to uncomplexed peanut flour. Ara h 2 was also hydrolyzed more quickly in the peanut protein-cranberry polyphenol complex than in uncomplexed peanut flour. Peptides from peanut protein-cranberry polyphenol complexes and peanut protein-green tea polyphenol complexes were substantially less immunoreactive (based on their capacity to bind to peanut-specific IgE from patient plasma) compared to peptides from uncomplexed peanut flour. These results suggest that peanut protein-polyphenol complexes may be less immunoreactive passing through the digestive tract *in vivo,* contributing to their attenuated allergenicity.

1. Introduction

The prevalence of peanut allergy in North America is increasing and is currently estimated to occur in about 1% of infants or children and about 0.6% of adults.[1] Peanut components that trigger the allergic reaction are primarily storage proteins found in the edible seed. Peanut allergy is considered a type I hypersensitivity and is mediated by immunoglobulin E (IgE). Upon peanut consumption by a peanut-allergic individual, certain parts of the allergenic proteins, known as epitopes, bind and cross-link peanut-specific IgE antibodies located on mast cell and basophil surfaces. This results in a cascade of reactions that trigger mast cells and basophils to degranulate and to release immunological mediators (such as histamine) responsible for local and/or systemic allergic symptoms.[2]

Of the peanut proteins, Ara h 1, Ara h 2, Ara h 3, and Ara h 6 are the major allergens.[3] A characteristic which peanut allergens (or food allergens) have generally in common (and which is recognized as a means to distinguish between potentially allergenic and non-allergenic proteins) is their relative resistance to digestion. Allergenic proteins maintain their epitopes within acidic conditions found in the gastric system (stomach) and are resistant to proteolytic hydrolysis (digestion) by various enzymes in the gastrointestinal tract compared to non-allergenic proteins.[4] While hydrolysis was observed during the pepsin-mediated digestion of a protein extract from roasted peanuts, the resultant peptides remained highly allergenic (IgE binding properties were not affected).[5,6] The structure of some peanut allergens may naturally resist digestion, and/or allergenic proteins can be rendered even less digestible by processing, allowing them to remain intact for a longer period than non-allergenic proteins in the gastrointestinal tract. If subjected to prolonged and extensive digestion conditions (*i.e.* high pepsin concentrations), these allergenic proteins decompose into (relatively large) peptide

fragments that remain immunoreactive (intact and accessible IgE binding epitopes), triggering allergic reactions in peanut-sensitive individuals.[7,8]

Polyphenols found in fruits, vegetables and other edible plant sources provide health-promoting and disease preventative benefits mainly attributable to their antioxidant and anti-inflammatory properties.[9] Polyphenols also hold promise as natural allergy-alleviating agents since they may have modulating effects on different biological pathways, and immune cell functions in an allergic immune response.[10] Dietary polyphenols can also alter immunoreactivity via their significant binding capacity to proteins, which can result in the creation of soluble and insoluble protein-polyphenol complexes. Proteins and polyphenols can interact either through reversible non-covalent forces, such as hydrogen bonding, hydrophobic interactions[11,12], or through irreversible covalent bonds.[13] These interactions can change structural, functional and nutritional properties of both proteins and polyphenols. Changes in secondary and tertiary protein structure, altered protein solubility and enzymatic digestibility, and a loss of some amino acids may occur due to protein-polyphenol interactions.[14]

In a recent study, we described an approach to reduce allergenicity of light roasted 12% fat peanut flour; the current ingredient used for peanut-specific oral immunotherapy (a strategy that involves the administration of small doses of peanut flour, in a clinical setting, to induce clinical tolerance to peanut allergens[15]). Polyphenolic plant compounds were complexed with the proteins and the peanut protein-polyphenol complexes showed substantially reduced allergenicity based on complementary assays.[16] There was reduced peanut-specific IgE binding in Western blots, particularly when cranberry, cinnamon or green tea polyphenols were used to create the peanut protein-polyphenol complexes. Additionally, a protein-cranberry polyphenol complex reduced basophil degranulation. The protein-cranberry polyphenol complex also

triggered less mast cell degranulation, a marker for allergic reactions, compared to uncomplexed peanut flour when used to orally challenge peanut-allergic mice *in vivo*.[16]

In the present work, selected peanut protein-polyphenol complexes were subjected to simulated gastric digestion (*in vitro*) in an effort to elucidate possible *in vivo* mechanisms for reduced allergenicity. We hypothesized that certain peanut protein-polyphenol complexes would modulate gastric digestion of peanut-allergenic proteins (compared to unmodified peanut allergens) and render digestive peptides less allergenic when screened for peanut-specific IgE binding capacity.

2. Materials and methods

2.1. Materials and reagents

Procyanidin A2 was obtained from Chromadex (Irvine, CA, USA). Procyanidin trimer [epi-(4β → 6, 2β → O→7)-epi- (4β → 8, 2β → O→7)-cat] and tetramer [epi-(4β → 8, 2β → O→7)-epi-(4α → 6)-epi-(4β → 8, 2β → O→7)-cat] were obtained from Planta Analytica (Danbury, CT, USA). Catechin**,** pepsin from porcine gastric mucosa (3,802 U mg protein^{-1}, 92% purity) and urea were purchased from Sigma-Aldrich (St. Louis, MO, USA). Biotinylated goat IgG-anti-human-IgE was obtained from Kirkegaard & Perry Laboratory, Inc (Gaithersburg, MD, USA). NeutrAvidin HRP (horseradish peroxidase) and Super Signal West Pico Chemiluminescent Substrate were purchased from Thermo Scientific (Rockford, IL, USA). Bovine serum albumin (BSA), Novex 16% Tricine gels, Tricine SDS running buffer (10×), NuPAGE reducing agent (10×), Novex Tricine SDS sample buffer (2×), SeeBlue Plus2 Pre-stained Protein Standard, SimplyBlue SafeStain, MagicMark XP Western Protein Standard, and iBlot Transfer Stacks (PVDF) were purchased from Life Technologies (Grand Island, NY,

USA). Polyclonal rabbit anti-Ara h 1 and polyclonal rabbit anti-Ara h 2 sera were obtained from Indoor Biotechnologies, Inc (Charlottesville, VA, USA) and goat anti-rabbit IgG-HRP was purchased from SouthernBiotech (Birmingham, AL, USA).

2.2. Peanut protein-polyphenol complex formation

Cranberry (*Vaccinium macrocarpon* Ait.) juice concentrate (50 ºBrix) was provided by Ocean Spray (Lakeville-Middleboro, MA, USA), cinnamon stick powder (*Cinnamomum burmannii* Blume, A grade, 3% oil) was purchased from Frontier Co-op (Norway, IA, USA) and organic green tea leaves (*Camellia sinensis* (L.) Kuntze) from Sri Lanka were provided by QTrade Teas & Herbs (Cerritos, CA, USA). Light roasted 12% fat peanut (PN) flour (*Arachis hypogaea* L.) containing 50% ± 2% protein was obtained from Golden Peanut Co. LLC (Alpharetta, GA, USA). Cinnamon powder and green tea leaves extracts as well as cranberry juice were prepared and subsequently used to create the peanut protein- cinnamon, green tea, or cranberry polyphenol complexes as described previously.[16] Briefly, PN flour was combined with (1:1) diluted cranberry juice concentrate or extracts (30 g L^{-1}), mixed for 15 min at room temperature to allow polyphenols to complex with PN proteins, and centrifuged for 20 min at 3,434 x *g*. The supernatant was decanted and the pellet was freeze-dried to yield the protein-polyphenol complex. A peanut protein blank was also prepared by using the same complexing process with PN flour and water rather than a polyphenol solution.

2.3. Protein quantification in protein-polyphenol complexes

Percent total nitrogen in protein-polyphenol complexes, peanut protein blank and uncomplexed PN flour were determined using a 2400 CHN Elemental Analyzer (Perkin Elmer, Norwalk, CT, USA) and converted to %protein (n=2, conversion factor 5.46 for peanut).

2.4. Protein precipitation capacity

The capacity of liquids to precipitate PN proteins was investigated by complexing PN flour with juice or extracts and measuring the protein content in the soluble portion. For this, PN flour (30 g L^{-1}) was added to cranberry juice, cinnamon powder extract or green tea leaves extract, mixed, and the dispersion centrifuged (20 min at 6,064 x *g*) as previously described.[16] A peanut protein blank (PN flour complexed with water only) was also prepared and evaluated. Soluble protein in the supernatants after centrifugation was determined using the EZQ protein quantitation kit and ovalbumin as a reference standard (Life Technologies, Grand Island, NY, USA).

2.5. Protein-polyphenol interactions

To investigate non-covalent PN protein-polyphenol interactions in the protein-polyphenol complexes, protein-polyphenol complexes or PN flour were dispersed in urea solutions and assayed for soluble protein. The amount of protein-polyphenol complex or PN flour required to provide equivalent protein content (50 mg), was used. Samples were dispersed in 1, 2, 4, 6, 8 and 10 M aqueous urea solutions. Dispersions (1 mL) were vortexed for 5 min and sonicated for 10 min and subsequently centrifuged for 10 min at 13,793 x *g*. Soluble protein, in the supernatants, was determined using the EZQ assay.

159 ### *2.6. Dispersibility of protein-polyphenol complexes*

160 The dispersibility of PN protein-polyphenol complexes was compared to uncomplexed
161 PN flour under acidic conditions using simulated gastric fluid (SGF). SGF was prepared
162 according to the United States Pharmacopeia with minor changes.[17] A solution of 0.2% NaCl
163 (w/v) in deionized water was prepared and adjusted to a pH of 2.0 using 6 N or 1 N HCl. The
164 amount of protein-polyphenol complex or PN flour required to provide equivalent protein
165 content (5, 10, 25, 50 and 100 mg) was used. Ten milliliter dispersions of protein-polyphenol
166 complexes or PN flour in SGF (w/v) were prepared and the pH subsequently adjusted to 2. The
167 dispersions were stirred, centrifuged (20 min at 3,434 x *g*) and analyzed for soluble protein using
168 the EZQ assay.
169

170 ### *2.7. Digestibility and IgE binding capacity of protein-polyphenol complexes*
171 ### *during simulated gastric digestion*

172 A simulated gastric pepsin digestion assay was used to investigate the enzymatic
173 hydrolysis (digestion) of PN proteins in protein-polyphenol complexes compared to native
174 proteins in uncomplexed PN flour. A dispersion of a protein-polyphenol complex or PN flour
175 was prepared in SGF resulting in 55.5 mg total protein in a total volume of 40 mL each.
176 Dispersions were adjusted to pH 2, stirred on a stir plate for 5 min, and then a 4 mL aliquot from
177 each of the dispersions was added to separate 15 mL centrifuge tubes (controls, no pepsin
178 added). Remaining dispersions (36 mL, 50 mg protein) were placed into a 37 °C water bath and
179 pre-warmed for 15 min while a 0.2 mg mL^{-1} pepsin solution (14 U mg $protein^{-1}$) in SGF (w/v)
180 was prepared and also pre-warmed (for only 5−10 min to prevent a possible loss of enzyme
181 activity due to autodigestion). Four milliliters of pepsin solution were added to each tube

containing the dispersions. Four milliliter digestive aliquots were taken after 0.5, 1, 2, 4, 8, 16, 30 and 60 min, added to separate 15 mL centrifuge tubes, and immediately subjected to a 90 °C water bath for 15 min to inactivate pepsin. Each control (a 4 mL aliquot of each dispersion without pepsin) underwent the same heat treatment. Throughout the digestion period, samples were inverted several times to ensure proper mixing of dispersion and enzyme. Pepsin-inactivated digested samples and controls were centrifuged for 20 min at 7,921 x *g*, transferred to 2 mL centrifuge tubes and centrifuged a second time for 5 min at 11,750 x *g* to remove insoluble particles. The resulting supernatants were used for further analysis. SDS-PAGE and Western blotting were performed following the method of Plundrich *et al.*[16] to evaluate digested samples and controls for protein distribution and IgE binding capacity. Blots were incubated in 1) pooled plasma from ten peanut-allergic individuals obtained from PlasmaLab International (Everett, WA, USA), 2) rabbit anti-Ara h 1 sera (1:100,000; v/v) or 3) rabbit anti-Ara h 2 sera (1:20,000; v/v). Biotinylated goat IgG-anti-human-IgE + NeutrAvidin HRP conjugate (1:10,000; v/v) or goat anti-rabbit IgG-HRP (1:4,000; v/v) were used to bind plasma or polyclonal antibodies, respectively.

In addition, digested samples were screened for simple or higher polymerized procyanidins using HPLC. Samples were prepared in triplicate for analysis to evaluate consistency of HPLC chromatograms. Samples were filtered through 0.2 µm syringe filters and dissolved 1:1 (v/v) in deionized water prior to HPLC analysis. Proanthocyanidins (PAC) were analyzed as previously described.[16]

2.8. Statistical analysis

All experiments were replicated three times if not stated otherwise, and analysis of variance (ANOVA) was performed using JMP 11.0 (SAS, Cary, NC, USA). When significant ($p < 0.05$), means were separated using Student's t test.

3. Results

3.1. Protein quantification in protein-polyphenol complexes

Concentrations of total protein (%) in uncomplexed PN flour or PN protein-polyphenol complexes were determined. The protein content in uncomplexed PN flour was 51.3%, which is comparable with the supplier's product specification (50% ± 2%). Protein concentrations remained comparable in cinnamon (56.3%) and green tea (47.7%) protein-polyphenol complexes, but were significantly reduced in the protein-cranberry polyphenol complex (32.0%).

3.2. Protein precipitation capacity

The highest concentration of soluble protein, which corresponds to the proteins that remained dispersed after the complexation process, (0.85 mg mL^{-1} or 5.52% of the original protein content), was found in the supernatant of the peanut protein blank (formed after mixing PN flour with water only). Significantly less PN protein was solubilized and lost to the supernatant when the PN flour was complexed with cranberry juice (0.19 mg mL^{-1} or 1.98% of the original protein content), cinnamon powder extract (0.15 mg mL^{-1} or 0.89% of the original protein content), or green tea leaves extract (0.01 mg mL^{-1} or 0.07% of the original protein content).

3.3. Evaluation of protein-polyphenol interactions

Generally, uncomplexed PN flour yielded the highest amount of solubilized PN protein across all urea concentrations tested (Fig. 1). Proteins from PN flour were 100% solubilized (50 mg mL^{-1}) when 8 M and 10 M urea solutions were used. Proteins in protein-polyphenol complexes were less affected by urea. In fact, even high urea concentrations did not lead to a disruption of all protein-polyphenol interactions (and/or protein denaturation) since significantly less proteins remained dispersed after centrifugation. Interestingly, the protein content in the protein-cinnamon polyphenol complex was very similar to that of PN flour (56.3% and 51.3% respectively), yet proteins in the protein-cinnamon polyphenol complex were significantly less affected by low to relatively high urea concentrations (Fig. 1).

3.4. Protein-polyphenol complex dispersibility in simulated gastric fluid

Stability of proteins in protein-polyphenol complexes or PN flour in SGF was evaluated on the basis of degree of dispersibility of a normalized protein content in a sample of PN protein-polyphenol complex or PN flour (Fig. 2). Proteins from PN flour and protein-cinnamon polyphenol complex were highly dispersible across all treatments in a dose-dependent manner (Fig. 2). Proteins from the protein-green tea polyphenol complex and the protein-cranberry polyphenol complex were significantly less soluble in SGF. PN flour complexed with cranberry juice rendered PN proteins least soluble. In fact, the amounts of soluble protein from protein-cranberry polyphenol complex were too close to the detection limit of the assay (close to zero), thus values were not displayed in Fig. 2. No protein appeared to be soluble or was below detection limits across all samples tested when protein content was normalized to 5 mg. Furthermore, while for all protein-polyphenol complexes and PN flour, up to 100 mg protein was used, proteins were only partially soluble across all samples. The maximum yield of soluble

250 protein, 6.67 mg mL^{-1} (equal to 66.7% of original protein content) was observed from PN flour
251 (Fig. 2). Proteins in protein-cranberry polyphenol complex and protein-green tea polyphenol
252 complex were significantly less soluble than proteins in PN flour or the protein-cinnamon
253 polyphenol complex.
254

255 ***3.5. Digestibility and IgE binding capacity of protein-polyphenol complexes***
256 ***during simulated gastric digestion***

257 The digestibility of proteins was evaluated by monitoring the disappearance of intact
258 protein bands on SDS-PAGE while a potential release of complexed polyphenols was tracked by
259 measuring their concentration in digestive samples. Proteins from protein-polyphenol complexes
260 and PN flour were rapidly digested into smaller molecular weight fragments (Fig. 3 A).
261 However, the enzymatic hydrolysis of some PN allergens into peptides generally occurred more
262 rapidly in protein-polyphenol complexes compared to allergenic proteins in PN flour. As
263 expected, Ara h 1, which appears as a 64 kDa band under reducing conditions[8], was quickly
264 digested in all samples tested after 0.5 min. This also applied to Ara h 3 acidic subunits (at 42
265 kDa and 45 kDa)[18]; however, the Ara h 3 basic subunit (at 25 kDa)[18] was only rapidly digested in
266 protein-cranberry or protein-green tea polyphenol complexes (Fig. 3 A and C). Ara h 2 and Ara h
267 6 (15 kDa)[19] in both uncomplexed PN flour and protein-polyphenol complexes were highly
268 resistant to enzymatic hydrolysis (Fig. 3 A). Ara h 2, consisting of two isomers with 16.7 kDa
269 and 18 kDa[20], remained intact after 2 min in PN flour. Only after 30 min was Ara h 2 entirely
270 digested into smaller peptides. In the protein-cinnamon polyphenol complex, Ara h 2 appeared
271 partially intact even after 30 min. The higher molecular weight isomer appeared to disappear
272 after 8 min in the protein-cranberry polyphenol complex while the lower molecular weight

isomer was digested after 30 min. Ara h 2 appeared blurry in the protein-green tea polyphenol complex sample after only 0.5 min, making it impossible to evaluate the appearance and disappearance of the two allergen isomers (Fig. 3 A and C). Ara h 6 was not affected by pepsin hydrolysis until after 60 min in the protein-cranberry polyphenol complex while the allergen in PN flour, protein-cinnamon polyphenol complex and protein-green tea polyphenol complex was decomposed into smaller peptides after 16 min. In addition, digestive peptides at ~10 and ~4 kDa were seen, that likely corresponded to pepsin-resistant fragments of Ara h 2 and Ara h 6.[8,21] More conclusive interpretations of resulting digestive peptides cannot be made on the basis of a mixture of various allergenic and non-allergenic PN proteins present in our samples. In summary, based on pepsin hydrolysis, digestion of allergens occurred more rapidly in protein-cranberry and protein-green tea polyphenol complexes compared to protein-cinnamon polyphenol complex and uncomplexed PN flour.

Gel electrophoresis showed the disappearance of the intact antigen and production of peptides while IgE binding predicted antigenicity (Fig. 3 B). Peanut-specific IgE levels in the pooled plasma ranged from 42 to > 100 kU L^{-1} as determined via ImmunoCAP (Phadia, Uppsula, Sweden) (Table 1). Studies have shown that even extensive digestion of PN allergens by gastrointestinal enzymes can result in the survival or formation of peptide fragments that remain immunoreactive (intact and accessible IgE binding epitopes).[21,22] In the present study, allergenic protein bands which were no longer visible on the coomassie-stained gel in some cases still bound IgE on Western blots. Ara h 1 disappeared after 0.5 min of pepsin hydrolysis in all samples tested. Western blots using rabbit anti-Ara h 1 sera showed similar immunorecognition patterns for cinnamon, green tea and uncomplexed PN flour digestive peptides, but the protein-cranberry polyphenol digestive peptides showed reduced IgG recognition. (Fig. 4 A). IgE

296 binding to Ara h 1 epitopes in samples could not be evaluated since it was impossible to
297 determine which digestive peptides (and respective bands recognized by IgE on Western blots)
298 belonged to Ara h 1. Ara h 2 retained its IgE binding capacity across all samples; however,
299 compared to PN flour, it was far more pronounced in the protein-cinnamon polyphenol complex
300 (even at 60 min), while no IgE binding was observed in digestive samples of the protein-
301 cranberry polyphenol complex after 60 min and after 30 min in digestive aliquots from the
302 protein-green tea polyphenol complex (Fig. 3 B). Results from Western blots using rabbit anti-
303 Ara h 2 sera revealed that rabbit IgG antibodies recognized the same peptides as did human IgE
304 antibodies (Fig. 4 B). Cranberry and green tea protein-polyphenol digestive peptides showed
305 reduced recognition of IgG binding epitopes, compared to protein-cinnamon polyphenol or
306 uncomplexed PN flour digestive peptides. In addition, IgE binding was observed in the
307 molecular weight region of Ara h 3 in PN flour, protein-cranberry polyphenol complex and
308 protein-cinnamon polyphenol complex digestive samples. No IgE binding to Ara h 3, however,
309 was seen in protein-green tea polyphenol complex digestive samples. In summary, digestive
310 samples from protein-cranberry polyphenol complexes and particularly protein-green tea
311 polyphenol complexes showed less IgE binding compared with PN flour. Interestingly, fewer
312 total procyanidins were released from protein-polyphenol complexes than from uncomplexed PN
313 flour during the course of digestion (Table 2).
314

315 ## 4. Discussion

316 Our previous study showed that peanut protein-polyphenol complexes created by binding
317 polyphenolic plant compounds to peanut proteins were substantially less allergenic based on
318 complementary *in vitro* and *in vivo* experiments.[16] In the present work, select peanut protein-

polyphenol complexes (derived from cranberry, cinnamon and green tea) were subjected to an *in vitro* simulated gastric pepsin digestion in an effort to elucidate possible *in vivo* mechanisms for reduced allergenicity.

Total protein concentrations in PN protein-polyphenol complexes and uncomplexed PN flour were determined. The potential of aqueous juice or extracts to precipitate or solubilize PN proteins during the complexation process (compared to water alone, for the protein blank) was investigated to determine if a loss of soluble proteins occurred for the complex. While some proteins were extracted (solubilized) from PN flour during complexation with aqueous juice or extracts, there was much less protein solubilization and more protein precipitation than when water alone was used. These results are likely due to a) various fruit or plant compounds already present in juice or extracts, likely making it more difficult for proteins to go into solution without oversaturation and b) known protein precipitating properties of plant polyphenols.[23] Since the complexation process did not lead to a significant loss of proteins into solution, it is likely that the observed reduced protein content in the protein-cranberry polyphenol complex was a result of sorbed plant compounds (such as polyphenols or sugars) which diluted the concentration of proteins present. A study by Grace *et al.*[24] reported similar findings working with cranberry-fortified pea or soy protein isolate matrices. On the other hand, proteins originally present in cinnamon powder extract[25] and/or a loss of sugars and other compounds found in PN flour (solubilized into the liquid phase during the complexation process) are possible reasons for the increased protein content in the protein-cinnamon polyphenol complex compared to PN flour. Hence, based on these findings, further experiments with these protein-polyphenol complexes were normalized to equivalent protein content.

Protein-polyphenol interactions in protein-polyphenol complexes were further evaluated. Since complexation of PN proteins with polyphenols from juice or extracts was performed at an acidic pH, non-covalent forces were thought to be prevalent. Accordingly, urea was used to attempt to disrupt protein-polyphenol interactions, and soluble protein was subsequently measured. Urea is an organic compound with both polar and non-polar properties. It has been suggested that urea functions by hydrophobic interactions with non-polar protein residues, as well as hydrogen bonding to the protein backbone (and to water molecules in the water hydrogen bond network), to weaken protein secondary, tertiary, and quaternary structures. Hence, protein stability is decreased ultimately resulting in protein denaturation (unfolding) and increased solubility in urea solutions.[26] In fact, high concentrations of urea (8 M and 10 M) solubilized 100% of the proteins present in uncomplexed PN flour indicating that all proteins were dispersed into particles small enough to remain dispersed after centrifugation. However, urea had little effect on protein-polyphenol complexes suggesting that protein-polyphenol complexes were protected from protein solubilization and were too large to remain dispersed after centrifugation. It is not clear why soluble protein from the protein-cranberry polyphenol complex appeared to initially increase with increased urea concentration and then decreased. This observation warrants further investigation. PN flour or protein-cranberry, protein-cinnamon, and protein-green tea polyphenol complexes were prepared in 10.0%, 18.3%, 9.43% and 11.1% dispersions (w/v), respectively, each containing 50 mg protein, thus it is unlikely that a decrease in protein solubility in complexes was due to oversaturation in urea solutions used. Instead, this evidence suggests that it was difficult for urea to solubilize the proteins once they were complexed with polyphenols. Similarly, when Oh *et al.*[27] investigated interactions in protein-tannin complexes, they found that 6 M urea had no effect on protein-tannin complex dissociation. Our results

indicate that PN protein-polyphenol interactions are partially hydrophobic in nature, however, additional experiments are needed to verify this and other possible non-covalent mechanisms (such as hydrogen bonding). In addition, unique features of both proteins and polyphenols (e.g. type or size) as well as other parameters (e.g. pH or ionic strength) all affect protein-polyphenol interactions. Further studies with both crude and isolated proteins and polyphenols are needed to determine disassociation as well as relative binding affinities and stoichiometry of protein-polyphenol complexes.

Since allergenic proteins are resistant to harsh conditions such as the highly acidic environment present in the stomach, the stability of PN protein-polyphenol complexes compared to uncomplexed PN flour (*i.e.*, solubility capacity of PN proteins) in SGF (pH 2) was investigated. The observed low solubility (66.7% of original protein content) of proteins from roasted PN flour was not surprising, since it has been shown that progressive roasting can lead to decreased protein solubility compared to raw peanuts.[28,29] However, even less proteins were dispersible in SGF from protein-cranberry -or protein-green tea polyphenol complexes than from uncomplexed PN flour, which showed that these complexes are highly stable in SGF at a physiological pH. However, the solvent volume of SGF (10 mL) which was chosen to create dispersions without under -or oversaturation, may have limited the reaction. Effects of various solvent volumes (resembling volume of gastric fluid in stomach) warrant further investigation.

An *in vitro* simulated gastric pepsin digestion assay was used to mimic *in vivo* digestion under acidic conditions and at a physiological temperature, and to investigate IgE binding capacity of digestive products. In contrast to previous studies that have typically used isolated PN allergens for *in vitro* digestion, we used whole PN protein-polyphenol complexes or uncomplexed PN flour in this work, which more closely mimics human consumption. While all

major allergens could be identified by SDS-PAGE, Ara h 2 appeared blurry in the protein-green tea polyphenol complex over the course of digestion (Fig. 3 A and C). It has been suggested that protein modifications such as glycosylation or complexation with polyphenols may result in diffuse appearance of protein bands.[30,31] Alvarez observed that honey protein bands appeared diffuse on SDS-PAGE, however, after polyphenol removal, clear bands appeared, indicating that honey proteins naturally associate with polyphenols.[31]

Furthermore, based on pepsin hydrolysis, digestion of allergens occurred more rapidly in protein-cranberry- and protein-green tea polyphenol complexes and their digestive peptides showed less IgE binding and reduced IgG binding (to Ara h 2 epitopes) compared with uncomplexed PN flour (Fig. 3 A-C and Fig. 4 B). While IgG antibodies (and likely low levels of Ig isotopes other than IgG) against Ara h 1 or Ara h 2 – which are recognized by up to 100% of peanut-allergic individuals[32] -were produced in rabbits as opposed to humans, these anti-sera were generally able to recognize the same peptides as did human IgE from plasma of peanut-allergic individuals. Similar findings were made by Mouécoucou *et al.* [33] performing *in vitro* digestion and allergenicity experiments using peanut allergens in the presence of polysaccharides, which suggests that findings made with antibodies raised in rabbits potentially also apply to humans. Protein-cranberry- and protein-green tea polyphenol complexes are less allergenic based on Western blotting when solely dissolved in lithium dodecyl sulfate buffer and screened for peanut-specific IgE binding.[16] These results may be linked to the increased stability or protein-polyphenol interactions in protein-cranberry and protein-green tea polyphenol complexes as described earlier (Fig. 1 and 2). On the other hand, while the protein-cinnamon polyphenol complex appeared to be a promising candidate based on previous Western blot results[16], IgE binding capacity was retained when this complex was hydrolyzed into peptides by

pepsin *in vitro*. In addition, pepsin-digested samples from protein-polyphenol complexes and uncomplexed PN flour were screened for procyanidins with various degrees of polymerization. Procyanidins released during hydrolysis can be contributed by both the plant polyphenol source used to create the complexes (*e.g.* cranberry juice, cinnamon extract) and from the PN flour itself (Table 2). Roasted PN flour contains numerous phenolic compounds, and detected procyanidins likely arose from peanut skin residuals which adhered during the industrial blanching process prior to PN flour production.[34] In summary, fewer total procyanidins were released from protein-polyphenol complexes than from uncomplexed PN flour. This is not surprising since diffused protein bands and band smearing indicated protein modification by complexed polyphenols. Accelerated and altered hydrolysis of certain allergens in some protein-polyphenol complexes compared to PN flour could possibly be due to enhanced pepsin activity by certain polyphenols present in digestive preparations. For example, a study by Tagliazucchi *et al.*[35] showed that phenolic compounds such as catechin, quercetin, epigallocatechin-3-gallate but also phenolic-rich beverages such green tea were able to affect V_{max} of pepsin by increasing its initial velocity when denatured hemoglobin was degraded. Another study, working with PN extract from raw peanuts revealed that a catechin-enriched polyphenol green tea extract promoted pepsin activity and resulted in a more rapid digestion of major PN allergens Ara h 1 and Ara h 2.[36] In general, polyphenols are able to bind to enzymes (proteins), potentially changing their conformation and activity and rendering them either more active or impaired.[35,37]

While all three plant donors tested in this study are rich in polyphenolics, they significantly differ in types and concentrations of phenolic compounds present. Cinnamon stick powder is rich in procyanidin monomers ((+)-catechin and (-)epicatechin) as well as procyanidin dimers, trimers, tetramers and oligomers (mainly A-type but also B-type structures).[38-40]

Unfermented (un-oxidized) green teas, on the other hand, contain high levels of flavanol monomers (flavan-3-ols), in particular (–)-epigallocatechin gallate and (–)-epigallocatechin.[41,42] Small amounts of procyanidins (dimers and trimers) were also detected.[43] Cranberry contains flavonols (mainly in glycosidic form), anthocyanins, proanthocyanidins, and various phenolic acids.[42] Cranberry is particularly rich in procyanidin dimers and higher polymerized procyanidins (oligomers and polymers) that are predominately A-type linked.[44] In this study, no direct connection between polyphenol types present in plant sources used and observed findings can be made. Further experiments need to be performed to determine which polyphenolic compounds (present in cranberry juice and green tea extract) are involved and how they are producing less allergenic peptides.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed general information about how or to what extent PN proteins present in protein-polyphenol complexes or PN flour were broken down by pepsin. Trypsin and chymotrypsin were not evaluated in this study, but are, together with pepsin, all important enzymes that could influence the digestibility and allergenicity of tested PN protein-polyphenol complexes *in vivo*. However, our previous study using either a protein-cranberry polyphenol complex or uncomplexed PN flour to orally challenge PN-allergic mice showed that the protein-cranberry polyphenol complex triggered significantly less mast cell degranulation (reduced cross-linking capacity) *in vivo* compared to uncomplexed PN flour, indicating an *in vivo* effect.

5. Conclusion

In our previous *in vitro* assessment of intact peanut allergens, cranberry, cinnamon and green tea polyphenols were able to reduce IgE binding capacity by epitope masking and/or

456 modification.[16] However, in the present study, when cranberry, cinnamon or green tea protein-
457 polyphenol complexes were subjected to an *in vitro* pepsin hydrolysis, only the digestive
458 peptides from PN protein-cranberry and PN protein-green tea polyphenol complexes exhibited
459 reduced IgE binding. Further screening for IgG binding capacity revealed that peptides resulting
460 from the digestion of PN protein-cranberry polyphenol complexes showed reduced recognition
461 by rabbit anti-Ara h 1 and rabbit anti-Ara h 2 antibodies whereas peptides from PN protein-green
462 tea polyphenol complexes revealed a decreased recognition by rabbit anti-Ara h 2 but not rabbit
463 anti-Ara h 1 antibodies. Cinnamon polyphenols, on the other hand, had no effect on PN allergen
464 epitope digestion by pepsin as was shown by similar immunorecognition patterns by both, IgE
465 and IgG antibodies, compared to uncomplexed PN allergens. Collectively, our results suggest
466 that PN protein-cranberry- and PN protein-green tea polyphenol complexes may be less
467 immunoreactive passing through the digestive tract *in vivo* as shown by decreased IgE binding
468 capacity of pepsin digestive peptides *in vitro*.
469

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476

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Figure captions

Fig. 1. Protein concentration in dispersions of peanut (PN) protein-polyphenol complexes and PN flour in urea. Samples were normalized for protein (50 mg). Data shown are means plus standard deviation. Values within each treatment with different letters are significantly different at $p < 0.05$.

Fig. 2. Protein concentration in dispersions of peanut (PN) protein-polyphenol complexes and PN flour in simulated gastric fluid at pH 2. Samples were normalized to protein (5, 10, 25, 50 or 100 mg). Data shown are means plus standard deviation. Values within each treatment with different letters are significantly different at $p < 0.05$.

Fig. 3. Simulated gastric pepsin digestion of peanut (PN) protein-polyphenol complexes and PN flour and their respective controls (M; protein standard marker, CTL; protein-polyphenol complexes or PN flour in simulated gastric fluid at pH 2 and no pepsin added) at time points 0.5, 1, 2, 4, 8, 16, 30 and 60 min: (A) SDS-PAGE under reducing conditions; (B) associated Western blots; (C) comparison of digestive patterns of Ara h 3 and Ara h 2 (SDS-PAGE). Approximate locations for peanut allergens are shown. Gray scale was used and contrast was optimized for SDS-PAGE. Exposure time of 153 sec was chosen across all blots to optimize the visualization for relatively faint MW bands of interest.

Fig. 4. Simulated gastric pepsin digestion of peanut (PN) protein-polyphenol complexes and PN flour and their respective controls (M; protein standard marker, CTL; protein-polyphenol complexes or PN flour in simulated gastric fluid at pH 2 and no pepsin added) at time points 0.5,

1, 2, 4, 8, 16, 30 and 60 min: (A) Western blot using polyclonal rabbit anti-Ara h 1 sera; (B) Western blot using polyclonal rabbit anti-Ara h 2 sera. Exposure time of 31.3 sec was chosen across all blots and the contrast was adjusted for some blots to optimize the visualization for relatively faint MW bands of interest.

Table 1 Donor Peanut-Allergic History

Donor	Age	Sex	CAP (kU L^{-1})
1	22	M	>100
2	20	F	>100
3	22	M	>100
4	21	F	>100
5	19	M	>100
6	18	M	53
7	24	F	54
8	23	F	42
9	33	F	79
10	29	M	79

Table 2 Individual Procyanidins from Juice or Extracts Sorbed to Peanut (PN) Matrices and Stability of Protein-Polyphenol Complexes during Simulated Gastric Pepsin Digestion Evaluated by Free Procyanidins (μg mL^{-1}) in Digestive Aliquots Taken at Different Time Points.[a]

		pre pepsin digestion[b]		post pepsin digestion[b]							
source	**procyanidin[c]**	**sorbed to matrix[d]**	**control[e]**	**0.5 min**	**1 min**	**2 min**	**4 min**	**8 min**	**16 min**	**30 min**	**60 min**
PN flour	DP1	NA[f]	ND[g]	ND	ND	ND	ND	ND	ND	ND	ND
	DP2	NA	44	43	43	42	41	43	43	44	42
	DP3	NA	ND	ND	ND	ND	ND	ND	ND	ND	ND
	DP≥4	NA	ND	564	560	570	555	579	572	625	604
	polymers	NA	1491	1583	1732	1910	1877	1877	2055	2348	2373
	total	NA	1535	2190	2335	2522	2473	2499	2670	3017	3019
protein-cranberry polyphenol complex	DP1	22	ND	ND	ND	ND	ND	ND	ND	ND	ND
	DP2	99	59	58	57	57	61	57	57	59	60
	DP3	23	55	59	58	58	63	59	60	58	65
	DP≥4	6	496 c	521 ab	519 ab	519 b	533 a	521 ab	525 ab	530 ab	530 ab
	polymers	434	890 f	949 f	1078 e	1093 e	1289 d	1270 d	1459 c	1616 b	1851 a
	total	584	1500	1587	1712	1727	1946	1907	2101	2263	2506
protein-cinnamon polyphenol complex	DP1	0	ND	ND	ND	ND	ND	ND	ND	ND	ND
	DP2	0	ND	ND	ND	ND	ND	ND	ND	ND	ND
	DP3	0	ND	ND	ND	ND	ND	ND	ND	ND	ND
	DP≥4	122	532 c	ND	848 a	704 b	704 b	741 b	690 b	672 b	681 b
	polymers	154	1153	1771	1574	1401	1417	1673	1960	1643	1989
	total	276	1685	1771	2422	2105	2121	2414	2650	2315	2670
protein-green tea polyphenol complex	DP1	81	44 c	59 ab	56 ab	54 b	60 ab	61 ab	54 b	59 ab	65 a
	DP2	1	49 d	52 c	53 bc	53 c	52 c	55 ab	52 c	54 bc	56 a
	DP3	4	42	41	45	44	44	42	43	47	47
	DP≥4	10	485 f	513 de	506 ef	530 cd	536 c	537 c	564 b	584 b	617 a
	polymers	220	1330 e	1471 de	1430 de	1477 cde	1552 bcd	1663 bc	1740 b	2038 a	2184 a
	total	316	1950	2136	2090	2158	2244	2358	2453	2782	2969

a) Values within each row with different letters are significantly different at $p < 0.05$.
b) Free polyphenols in samples pre digestion and post digestion were measured by HPLC using external standard curves and expressed as μg procyanidin per mL solvent.
c) DP, degree of polymerization; DP1, monomers; DP2, dimers; DP3, trimers; DP≥4, tetramers and oligomers.
d) Concentration of procyanidins in supernatant (left after complexation with peanut flour) subtracted from procyanidin concentrations in original juice or extracts (before complexation) in μg mL^{-1}.
e) Control, protein-polyphenol complex in simulated gastric fluid (pH 2) only (time point 0 min, before pepsin was added).
f) NA, not applicable
g) ND, not detectable

Fig. 1.

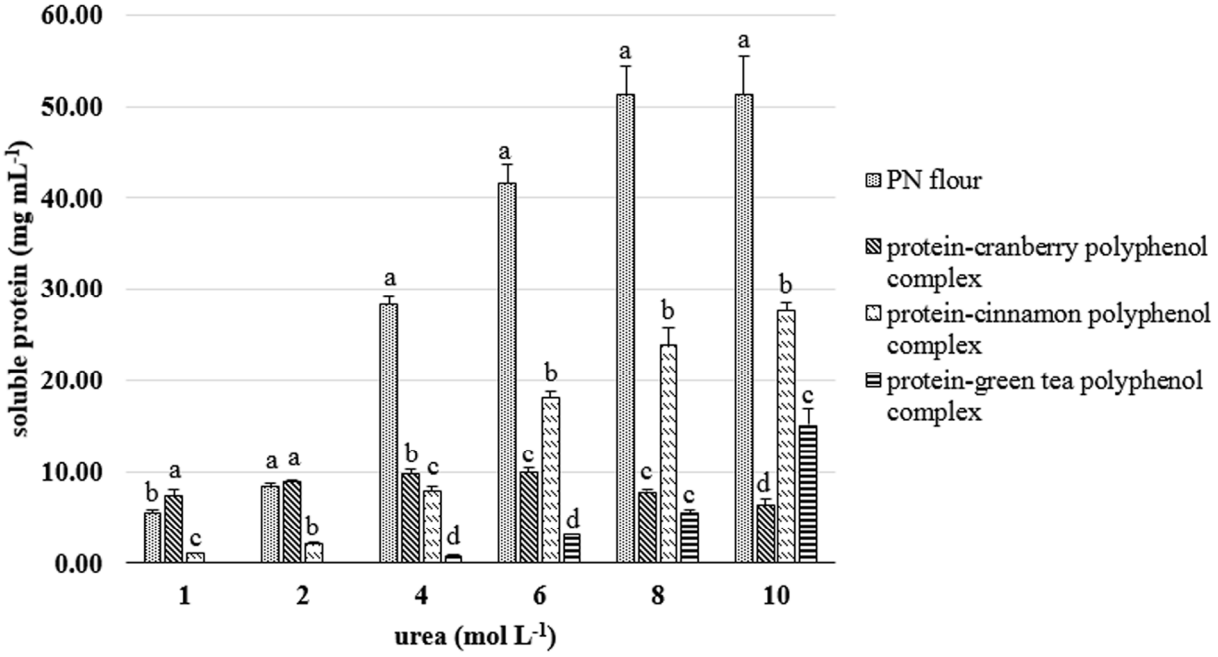

Fig. 2.

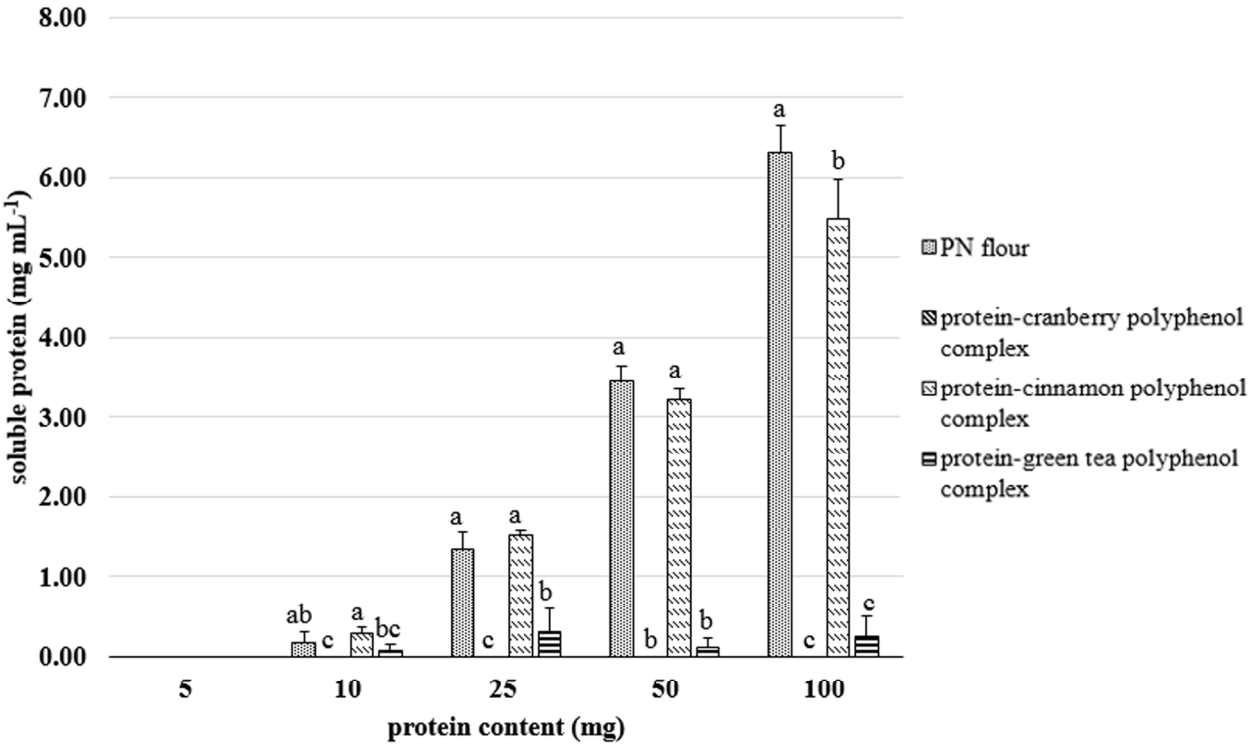

Fig. 3.

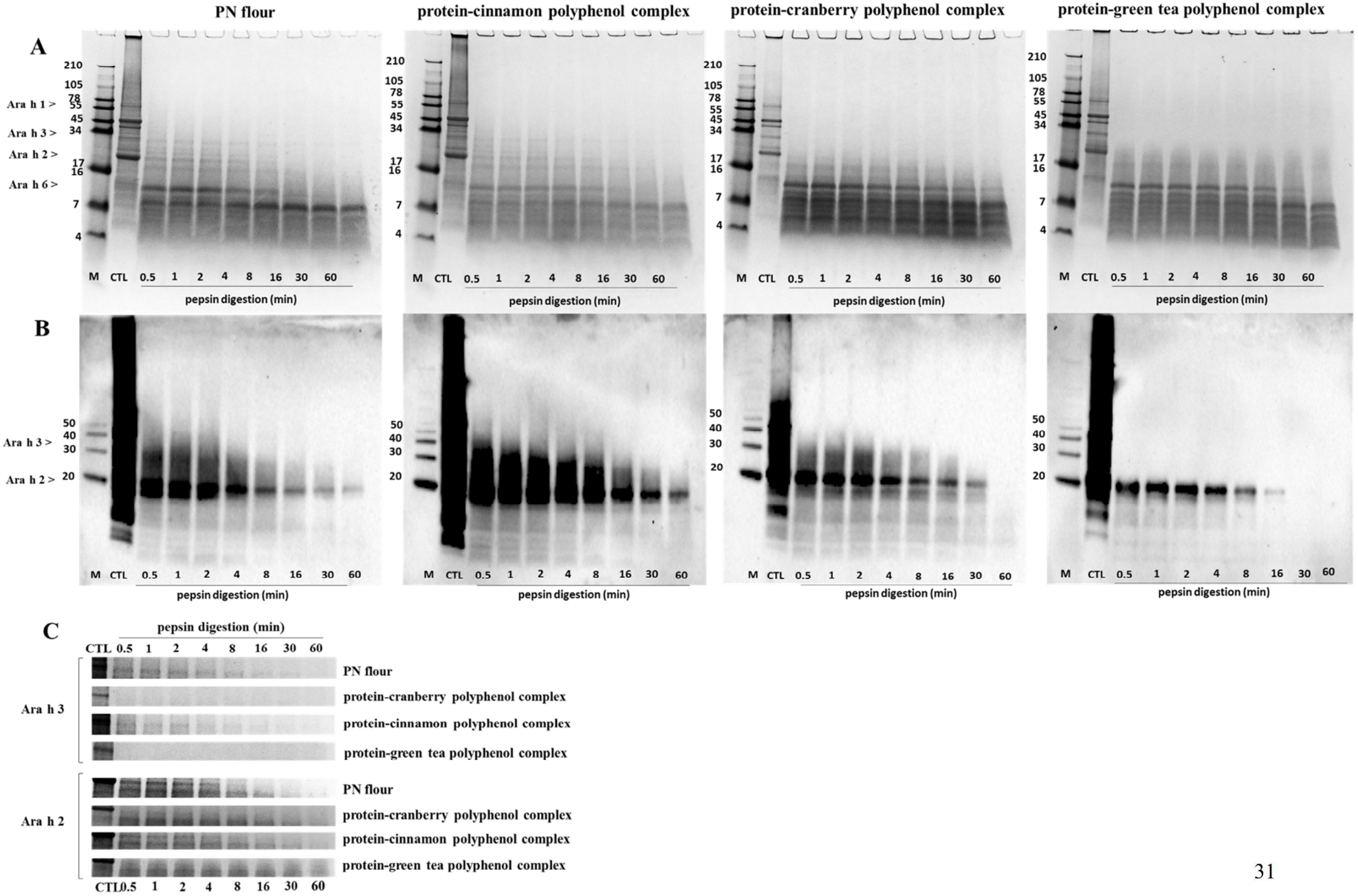

Fig. 4.