



Peanut flour aggregation with polyphenolic extracts derived from peanut skin inhibits IgE binding capacity and attenuates RBL-2H3 cells degranulation via MAPK signaling pathway

Rishipal R. Bansode^a, Nathalie J. Plundrich^b, Priscilla D. Randolph^a, Mary Ann Lila^b, Leonard L. Williams^{a,*}

^a Center for Excellence in Post-Harvest Technologies, North Carolina Agricultural and Technical State University, North Carolina Research Campus, Kannapolis, North Carolina 28081, USA

^b Plants for Human Health Institute, Department of Food, Bioprocessing and Nutrition Sciences, North Carolina State University, North Carolina Research Campus, Kannapolis, North Carolina 28081, USA

ARTICLE INFO

Keywords:

Peanut skin
Peanut flour
Peanut allergy
Degranulation
Polyphenols
RBL-2H3 cells

ABSTRACT

This study investigates the anti-allergic properties of peanut skin polyphenols (PSP)-enriched peanut (PN) protein aggregates. PSP was blended with PN flour at concentrations of 5, 10, 15, 30, and 40% (w/w). Rat basophil leukemia cells (RBL-2H3) were sensitized with either anti-DNP-IgE or PN-allergic plasma followed by co-exposure to unmodified PN flour (control) or PSP-PN protein aggregates and Ca^{2+} ionophore, ionomycin. Immunoblotting and staining were performed to measure the IgE binding capacity of PSP-PN aggregates. Results showed that 30% PSP-PN aggregate significantly reduced β -hexosaminidase and histamine levels by 54.2% and 49.2%, respectively compared with control. Immunoblotting results revealed 40% PSP-PN aggregates significantly decreased IgE binding by 19%. The phosphorylation of p44/42 MAPK was significantly reduced while phosphorylation of p38 MAPK and SAPK/JNK increased upon PSP-PN protein aggregate exposure to the cells. Our results show that aggregation of PSP to PN proteins reduces allergic response by inhibiting Ca^{2+} -induced MAPK-dependent cell degranulation.

1. Introduction

Peanut allergy is considered one of the most severe food allergies because it can trigger life-threatening reactions (Anagnostou & Clark, 2014). As there is currently no cure for food allergies, the only intervention strategies include strict avoidance of allergens, early recognition and reaction management. This has a significant effect on the quality of life for sufferers and their families due to dietary and social restrictions (Anagnostou & Clark, 2014).

Because PN allergy presents high risks, there is intense interest in developing therapeutic strategies that could reduce the danger and severity of the allergic reaction to PN in sensitive patients. Various strategies have included enzymatic treatments, genetic modifications of PN allergens, or administration of polyphenolic compounds to patients to non-specifically counteract an allergic airway inflammation (Cabanillas et al., 2012; Fu et al., 2010; Nyanhanda, Gould, & Hurst, 2014).

Peanut allergy is considered an immunoglobulin E (IgE)-mediated hypersensitivity disorder. In an allergic individual, PN allergens bind to

high-affinity IgE receptors (FcεRI) on cells and basophils. This cross-linking of allergen-specific epitopes to IgE leads to cell degranulation and allergic response (Burks, 2008; Holowka & Baird, 1996; Lin & Sampson, 2009; Sicherer & Sampson, 2010; Trotter et al., 2001). Ara h 1, Ara h 2, Ara h 3, and Ara h 6 are considered major PN allergens (Plundrich et al., 2014; van Wijk, Hartgring, Koppelman, Pieters, & Knippels, 2004). Besides these allergens, at least 15 other PN proteins were found to bind IgE from sera of PN-allergic individuals (Clarke et al., 1998).

Previous studies have revealed that polyphenols have non-specific anti-allergic effects (Maeda-Yamamoto, 2013; Magrone & Jirillo, 2012). Among them, procyanidins have been shown to attenuate allergic biochemical markers including the reduction in serum IgE, histamine and suppressed IgE (FcεRI)-mediated cell activation *in vivo* (Aswar, Kandhare, Mohan, & Thakurdesai, 2015; Nakano et al., 2008). Procyanidin oligomers from cocoa have shown to stimulate both innate and early event adaptive immunity (Kenny, Keen, Schmitz, & Gershwin, 2007). Our own investigation has shown that cranberry and blueberry polyphenols, when aggregated with PN protein, decreased IgE binding

* Corresponding author.

E-mail address: llw@ncat.edu (L.L. Williams).

to PN allergens, histamine and β -hexosaminidase release in complementary *in vitro* experiments (Plundrich, Bansode, Foegeding, Williams, & Lila, 2017).

Peanut skins are a polyphenol-rich agricultural by-product with low economic value. Commercially, the skins are removed from blanched or roasted PN. They have a very distinct polyphenol composition compared to berry fruits and are rich in A-type procyanidin dimers, trimers and tetramers which are less water soluble (Yu, Ahmedna, Goktepe, & Dai, 2006). These procyanidin oligomers derived from PSP showed reduced inflammatory cytokine production and melanogenesis in cultured human melanoma HMV-II cells (Tatsuno et al., 2012). Tomochika et al., 2011, showed that PSP extract inhibited degranulation in rat basophilic leukemia (RBL-2H3) cells by suppressing downstream protein kinase C activation by Ca^{2+} influx.

However, as the bioavailability of polyphenols is significantly affected by food matrix and delivery methods, the potential of aggregating PSP with PN flour proteins to incorporate as colloidal food ingredients to reduce PN protein allergenicity has not been reported. Since we successfully demonstrated the viability of procyanidin-rich cranberry polyphenol-PN protein aggregates in alleviating PN allergy response *in vitro*, we hypothesized that PSP which are similarly high in procyanidins can potentially be used for polyphenol-PN protein aggregation.

Hence, this study tested the hypothesis that novel PSP-PN protein aggregates prepared by aggregating PSP extracts with PN proteins (in the form of commercial PN flour) could effectively produce hypoallergenic food-grade PN flour. Our specific aims for this study were to 1) formulate PSP-PN protein aggregate particles by aggregating PSP with edible PN proteins, 2) investigate the IgE binding capacity of the aggregate particles using semi-quantitative immunoblotting, and 3) analyze the mechanism of action of aggregate particles to inhibit cell degranulation *in vitro*.

2. Materials and methods

2.1. Peanut skin polyphenol-peanut (PSP-PN) protein aggregate preparation

Light roasted 12% fat PN flour containing $50\% \pm 2\%$ protein (*Arachis hypogaea* L.) and PN skins were provided by Golden Peanut and Tree Nuts (Alpharetta, GA, USA). Polyphenols from PN skins were extracted in a two-step extraction process. First, PN skins were extracted with distilled water (1:20; w/v) for 30 min at 80°C and the extract was subsequently freeze-dried. The freeze-dried extract was then further extracted (1:33; w/v) with 80% aqueous ethanol (v/v) overnight at 4°C and the ethanol was then evaporated using a rotary evaporator system (Büchi, Flawil, Switzerland). The total phenolic content found in the PN skin extract was determined by the Folin Ciocalteu method and expressed as Gallic acid equivalent (GAE) (Bansode, Randolph, Hurley, & Ahmedna, 2012; Zhang et al., 2006). The amount of PN skin extract (ml) and PN protein flour (g) required to generate dry, stable PSP-PN protein aggregates containing 5, 10, 15, 30, or 40% PSP (w/w) were added together and mixed under constant agitation for 15 min at room temperature. Mixtures were subsequently frozen and freeze-dried to form stable PSP-PN protein aggregates.

2.2. Protein distribution and semi-quantitative immunoblotting

Protein distribution in unmodified PN flour and PSP-PN protein aggregates was observed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (Plundrich et al., 2017). Briefly, samples from unmodified and modified PN flour aggregated with 5, 10, 15, 30, and 40% PSP extract were prepared in Laemmli sample loading buffer ($2\times$) and loaded onto a gel at a protein concentration of $5\text{ }\mu\text{g}$ (in $10\text{ }\mu\text{l}$) per well. The gel was electrophoresed at 200 V for 40 min, followed by Coomassie brilliant blue (CBB) staining. The stained gel was imaged using an imaging system.

Semi-quantitative immunoblotting was performed similarly except sample loading buffer was used without bromophenol blue (LI-COR, Lincoln, NE, USA). After electrophoresis, proteins in the gel were transferred to a PVDF membrane. The PVDF membrane was incubated overnight in pooled ($n = 7$) PN-allergic human plasma (primary antibody, 1:80 v/v) in PBS blocking buffer containing 0.2% Tween 20. Peanut-specific IgE levels ranged from 53 to $> 100\text{ kU L}^{-1}$ as determined via ImmunoCAP (Phadia, Uppsala, Sweden). Following incubation in primary antibody, the PVDF membrane was washed in PBS blocking buffer and incubated with a goat anti-human IgE (epsilon chain) secondary antibody for 1 h at room temperature. The membrane was washed four times for 5 min each with PBS-0.1% Tween 20 and three times with PBS and visualized using an Odyssey CLx infrared imaging system (LI-COR, Lincoln, NE, USA). The blot image was analyzed for quantitative analysis using LI-COR's Image Studio Lite software. Similarly, nitroblue tetrazolium (NBT) staining was performed on the electroblotted PVDF membrane after hydrating in 100% methanol as described earlier (Paz, Fluckiger, Boak, Kagan, & Gallop, 1991; Plundrich, Bansode, Foegeding, Williams, & Lila, 2017).

2.3. Cell culture preparation for degranulation assessment in RBL-2H3 cells

One hundred and fifty thousand RBL-2H3 cells were seeded into a 24-well plate and maintained in Eagle Minimum Essential Medium (EMEM) supplemented with 15% fetal bovine serum (FBS) and incubated overnight at 37°C , in a 5% CO_2 incubator. The cells were sensitized with $1.0\text{ }\mu\text{g ml}^{-1}$ of anti-DNP-IgE overnight. After sensitizing the cells with anti-DNP-IgE, aggregate matrices of PN flour containing PSP at various concentrations (0, 5, 10, 15, 30, and 40% PSP: PN flour ratio, w/w) were exposed to each well at a normalized PN protein exposure concentration of $100\text{ }\mu\text{g ml}^{-1}$. One microgram per milliliter of DNP-BSA and $1\text{ }\mu\text{M}$ ionomycin were also co-exposed to the cells for 3 h. In another experiment, plasma from PN-sensitive humans obtained from PlasmaLab International (Everett, WA, USA) was used to sensitize RBL-2H3 cells in EMEM containing 15% PN-sensitive plasma (pooled from each individual with IgE concentration of 53 kU L^{-1} and 71 kU L^{-1} and four individuals with IgE concentration $> 100\text{ kU L}^{-1}$) overnight. Cells were washed three times with PBS buffer solution and treatments were diluted using Tyrode buffer. Polyphenol (PSP)-PN protein aggregates (5, 10, 15, 30, and 40% polyphenol: PN flour ratio, w/w) were exposed at a concentration of $100\text{ }\mu\text{g ml}^{-1}$. PN protein was then incubated for 3 h at 37°C followed by with or without ionomycin at an exposure concentration of $1\text{ }\mu\text{M}$ for 30 min. The control wells received PN flour exposure without ionomycin treatment. RBL-2H3 cell supernatant was subsequently collected and stored at -20°C until further analysis.

2.4. Determination of the release of IgE levels using ELISA

Rat IgE levels were measured in the RBL-2H3 cells sensitized with human plasma obtained from peanut sensitive individuals and exposed to either PN protein (CTL) or PSP-PN aggregates using an ELISA procedure as per manufacturer's instructions (Life Diagnostics, Inc., West Chester, PA). Briefly, a 96-well plate was coated with anti-rat IgE followed by addition of standards or diluted supernatant samples into the wells and incubated for 45 min at room temperature with constant agitation (100–150 rpm). The plate was aspirated and washed 5 times with a wash solution and enzyme conjugate added and incubated for an additional 45 min at 100–150 rpm. Following washing the plate for 5 times, TMB reagent was added and incubated for 20 min at constant agitation between 100 and 150 rpm. The reaction was stopped using a stop solution and the microplate was read at a wavelength of 450 nm.

2.5. Determination of β -hexosaminidase release

Fifty microliters of supernatant collected from RBL-2H3 cell media

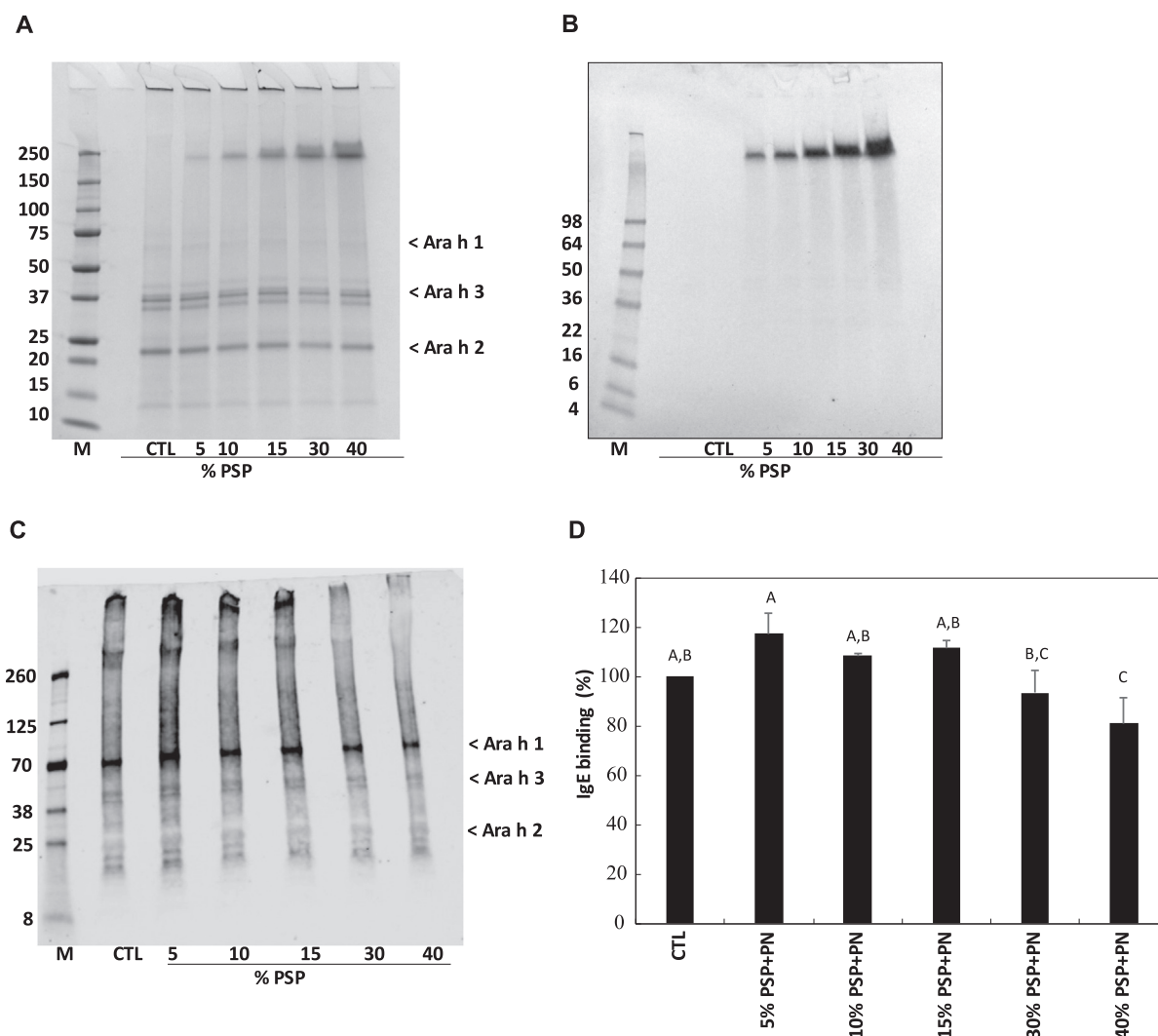


Fig. 1. SDS-PAGE of unmodified PN protein or peanut PN protein aggregates containing 5, 10, 15, 30, and 40% PSP and stained with Coomassie Brilliant Blue (A); staining of PSP-bound PN proteins by NBT (nitroblue tetrazolium) following SDS-PAGE and subsequent electrophoretic transfer to a PVDF membrane (B); representative corresponding Western blot for PSP-PN protein aggregates (C); semi-quantitative analysis of IgE binding capacity (based on Western blots) of PSP-PN protein aggregates (mean of five replicates with SE; values with different letters are significantly different at $p < 0.05$). M, molecular weight marker (kDa); Control (unmodified PN proteins). Tentative approximate locations for major peanut allergens are indicated. Grayscale was used for gels and membranes and contrast was optimized to improve visualization.

was transferred into each well of a black 96-well plate with 50 μ l of 1.3 mg ml⁻¹ β -hexosaminidase substrate (p-nitrophenyl N-acetyl- β -glucosamide in citrate buffer) in 0.05 M sodium acetate buffer (pH 4.4). Samples were mixed and incubated for 1 h at 37 °C. The reaction was stopped using 150 μ l of sodium carbonate. The β -Hexosaminidase release was quantified colorimetrically using a microplate reader at a wavelength of 450 nm using a Synergy 2 microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

2.6. Determination of histamine release

Twenty microliters of 1 M NaOH and 100 μ l of supernatant were transferred to a 96-well plate with 25 μ l of 1% phthalaldehyde in methanol. Samples were incubated at 37 °C in 5% CO₂ for 4 min. The reaction was stopped using 10 μ l of 3 M HCl and fluorescence measured at excitation (355 nm) and emission (460 nm) wavelengths using a Synergy 2 microplate reader (BioTek Instruments Inc., Winooski, VT, USA).

2.7. Immunoblotting of RBL-2H3 cell lysates to determine the effects of PSP-PN protein aggregates on MAPK signaling

RBL-2H3 cells were seeded in a six-well plate at a seeding density of 5×10^5 cells per well in EMEM containing 15% fetal bovine serum. The cells were incubated in a humidified incubator at 37 °C and 5% CO₂. The cells were then exposed to unmodified PN flour (control) or aggregates (5, 10, 15, 30, and 40% PSP: PN protein ratio, w/w). The exposure of the PN flour and aggregates to cells in the cell culture media were normalized to PN protein of 50 μ g ml⁻¹. The cells were exposed to either unmodified PN protein or PSP-PN protein aggregates for 3 h followed by addition of 1 μ M ionomycin for 10 min.

After the incubation of cells with PSP-PN protein aggregates, the cells were immediately washed three times with 1x PBS and lysed in Laemmli buffer (2x) containing 0.1 M DTT. The cell suspensions were boiled for 5 min and loaded onto 12% Tris-glycine gels (BioRad, Hercules, CA, USA) and electrophoresed for 3 h at a constant volt of 60 V. Separated proteins were subsequently transferred onto nitrocellulose membranes using a semi-dry electroblotting apparatus (Bio-Rad, Hercules, CA, USA). The blots were then blocked with 5% (w/

v) nonfat dry milk powder in Tris-buffered saline (TBS, pH 7.5) containing 0.1% Tween 20. The blots were subsequently incubated with phospho-p44/42 MAPK (Thr202/Tyr204), phospho-p38 MAPK (Thr180/Tyr182), phospho-SAPK/JNK (Thr183/Tyr185), total-p44/42 MAPK, and β -actin (Santa Cruz Biotechnologies, Inc., Dallas, TX, USA) primary antibodies in TBS containing 5% bovine serum albumin (BSA) overnight at 4 °C under constant agitation. Blots were washed three times with TBS and incubated in 5% (w/v) milk in TBS containing secondary anti-rabbit IgG, an HRP-linked antibody for 90 min. The blots were finally washed three times with TBS and enhanced chemiluminescent substrate (Thermo Fisher Scientific Inc., Waltham, MA, USA) was used to detect bands using a Fotodyne Gel imaging system (Fotodyne Inc., Hartland, WI, USA).

2.8. Statistical analysis

All experiments were conducted in triplicate unless mentioned otherwise to reduce the random error. The significant difference between the means was established by ANOVA and Duncan's multiple range tests. Data were analyzed using the JMP 11 software (SAS Inc., Cary, NC, USA).

3. Results

3.1. Protein distribution and semi-quantitative immunoblotting

Several major PN allergens from unmodified PN flour and PSP-PN protein aggregates were separated by SDS-PAGE and detected by CBB and are shown in Fig. 1 A. The CBB stain also revealed high molecular weight material above 250 kDa, which increased with an increase in polyphenol concentration. This was not observed for unmodified PN protein, which served as the control (CTL). High molecular weight protein material that was too big to enter the gel was observed for all samples (top of the gel). Staining of proteins with NBT indicated that the high molecular weight protein material, which was also stained by CBB, was modified in particular by polyphenols. The degree of NBT staining was polyphenol concentration-dependent (Fig. 1 B). As expected, PN proteins from unmodified PN flour did not react with NBT.

Fluorescence Western blotting revealed that major allergens, as well as unresolved protein material (smears in lanes) in unmodified PN flour and PSP-PN protein aggregates, were recognized by IgE from PN-allergic human plasma (Fig. 1 C). However, multiple replications of those Western blots indicated that the overall IgE binding decreased for the aggregates containing 40% PSP (decrease in fluorescence signal intensity). Semi-quantitative analysis of blots showed that 40% PSP in the PN protein aggregate was able to decrease IgE binding to PN proteins by 19%, and this was significant when compared to the control (unmodified PN proteins) (Fig. 1 D).

3.2. IgE release by sensitized RBL-2H3 cells exposed to PSP-PN protein aggregates or PSP extract

RBL-2H3 cells sensitized with peanut-sensitive individual's plasma showed a significant increase in IgE release. Complexation of peanut skin to peanut flour in an increasing proportion showed a dose-dependent reduction in the IgE levels in the supernatant media in which the RBL-2H3 cells were grown (Fig. 2). This reduction was significant ($p < 0.05$) at complexation of 15, 30 and 40% peanut skin to peanut flour complexation ratio. The IgE levels were restored to the basal levels with the peanut skin-peanut flour at 40% complexation ratio.

3.3. PSP-PN protein aggregates inhibit ionomycin-induced degranulation of RBL-2H3 cells

The addition of ionomycin significantly induced β -hexosaminidase

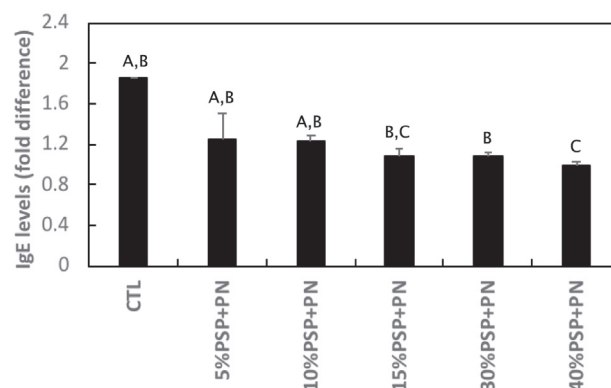


Fig. 2. Rat specific IgE levels in the supernatant following 3 h exposure of either PSP-PN protein aggregates (5–40% PSP) or unmodified PN protein (CTL) in RBL-2H3 cells that were sensitized with plasma collected from pooled peanut-sensitive individuals. Controls were cells which only received 1X PBS (Blank). Data shown are means of six replicates with SE; values with different letters are significantly different at $p < 0.05$. Exposure concentrations were standardized against peanut flour protein content and the data was normalized against cell death. PBS; phosphate buffered saline.

secretion in RBL-2H3 cells when compared to unstimulated cells. This induction of β -hexosaminidase was significantly suppressed by 5–30% PSP-PN protein aggregates in a dose-dependent manner as shown in Fig. 3 ($p < 0.05$). However, 40% PSP-PN protein aggregates showed a slight increase in β -hexosaminidase but the level was still significantly lower ($p < 0.05$) than the positive control (ionomycin). Histamine release showed a similar trend and was reduced for 5–30% PSP-PN protein aggregates and results are presented in Fig. 3.

3.4. PSP-PN protein aggregates inhibit PN-sensitive plasma-induced degranulation of RBL-2H3 cells

The effects of PSP-PN protein aggregates on the production of β -hexosaminidase and histamine were examined in RBL-2H3 cells stimulated in the presence of plasma from PN-sensitive individuals (Fig. 4). After the overnight exposure to PN-sensitive plasma (containing PN allergen-specific IgE), the cells were exposed to PSP-PN protein aggregates in the presence of ionomycin. The aggregation of PSP to PN protein significantly inhibited β -hexosaminidase and histamine release in a dose-dependent manner when compared to the unmodified PN flour and ionomycin treated group ($p < 0.05$).

3.5. Expression of phospho-p44/42 MAPK, phospho-p38 MAPK and phospho-SAPK/JNK

To further ascertain the mechanism of an ionomycin-induced reduction of an allergic response, we conducted Western blot analysis of RBL-2H3 cell lysates that were sensitized with plasma from PN-sensitive individuals. These sensitized cells were later incubated with varying concentrations of PSP-PN protein aggregates and ionomycin (Fig. 5). Our results showed that increasing the concentration of PSP in the PN protein aggregates decreased the phosphorylation of p44/42 MAPK and slightly increased the phosphorylation of p38 MAPK. Interestingly, the increase in the phosphorylation of SAPK/JNK was significant and peaked (> 20 -fold) between 15 and 40% PSP-PN protein aggregates.

4. Discussion

Potential IgE binding modifications upon PSP binding to PN proteins were investigated. PSP-PN protein aggregates were screened using SDS-PAGE, NBT staining and fluorescent Western blotting using PN-specific IgE from PN-allergic patient plasma (Fig. 1). Sodium dodecyl

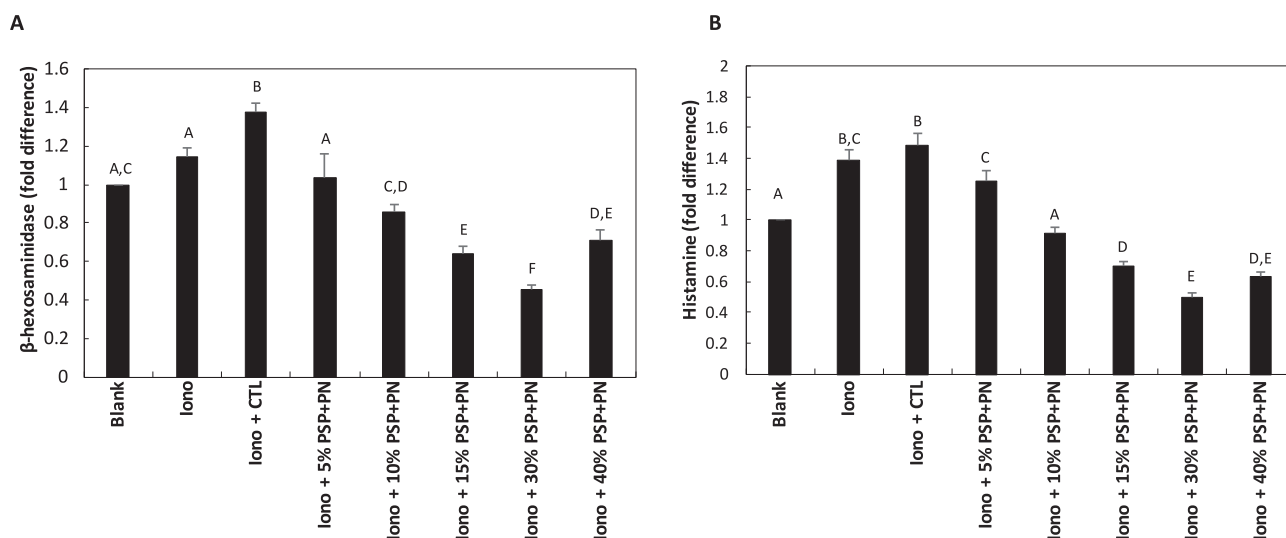


Fig. 3. β -hexosaminidase (A) and histamine (B) release following PSP-PN protein aggregates (5–40% PSP) or unmodified PN protein (CTL) co-exposure with DNP-BSA in RBL-2H3 cells sensitized with anti-DNP IgE and ionomycin followed by 1 μ M ionomycin with or without PSP-PN protein aggregates for 3 h. Controls were cells which only received 1X PBS (Blank) or ionomycin (Iono). Data shown are means of six replicates with SE; values with different letters are significantly different at $p < 0.05$. Exposure concentrations were standardized against peanut flour protein content and the data was normalized against cell death. PBS; phosphate buffered saline.

sulfate–polyacrylamide gel electrophoresis showed that polyphenol-binding to PN proteins resulted in PSP-PN protein aggregates with reduced electrophoretic mobility as can be seen by the upward movement of protein bands on the gel. High molecular weight material above 250 kDa, which did not enter the resolving gel was also observed (see the top of the gel). Since these PSP-PN protein aggregates were not disrupted by SDS (breaks non-covalent interactions) or β -mercaptoethanol (cleaves covalent disulfide bonds) the bonds formed between proteins and polyphenols must be partially covalent in nature. This has been reported in other studies focused on polyphenol binding with food proteins and enzymes (Rawel, Czajka, Rohn, & Kroll, 2002).

Nitroblue tetrazolium staining further indicated at least partial covalent polyphenol modification of PN proteins. Nitroblue tetrazolium

in the presence of glycine at alkaline pH allows for a redox-reaction with quinones (oxidized polyphenols that are bound to proteins). Results showed the stain specifically stained PN protein-quinone aggregates since unmodified PN proteins did not react with NBT. The high molecular weight protein material, which was also detected by CBB on a gel, did also react with NBT in a polyphenol concentration-dependent manner. Poorly resolved proteins (lane smears) that were stained by CBB were also stained by NBT. This was also observed and discussed in our recent study with cranberry and blueberry polyphenols (Plundrich et al., 2017).

Fluorescence Western blotting indicated that the high molecular weight protein material detected by CBB and NBT – particularly in the 40% polyphenol sample was modified by bound polyphenols to the

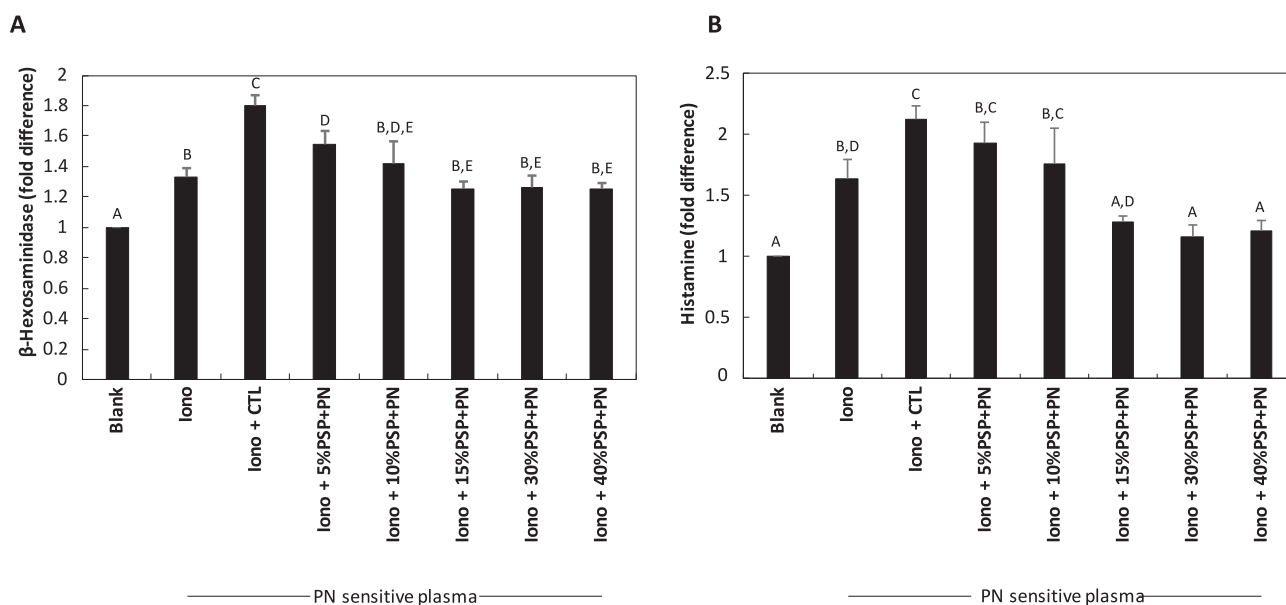


Fig. 4. β -hexosaminidase (A) and histamine (B) release following PSP-PN protein aggregates (5–40% PSP) or unmodified PN protein (CTL) exposure in RBL-2H3 cells pre-sensitized in presence of human plasma from peanut sensitive individuals overnight prior to treatment with polyphenol particles for 3 h. Controls were cells which only received 1X PBS (Blank) or 1 μ M ionomycin (Iono). Data shown are means of six replicates with SE; values with different letters are significantly different at $p < 0.05$. Exposure concentrations were standardized against peanut flour protein content and the data was normalized against cell death. PBS; phosphate buffered saline.

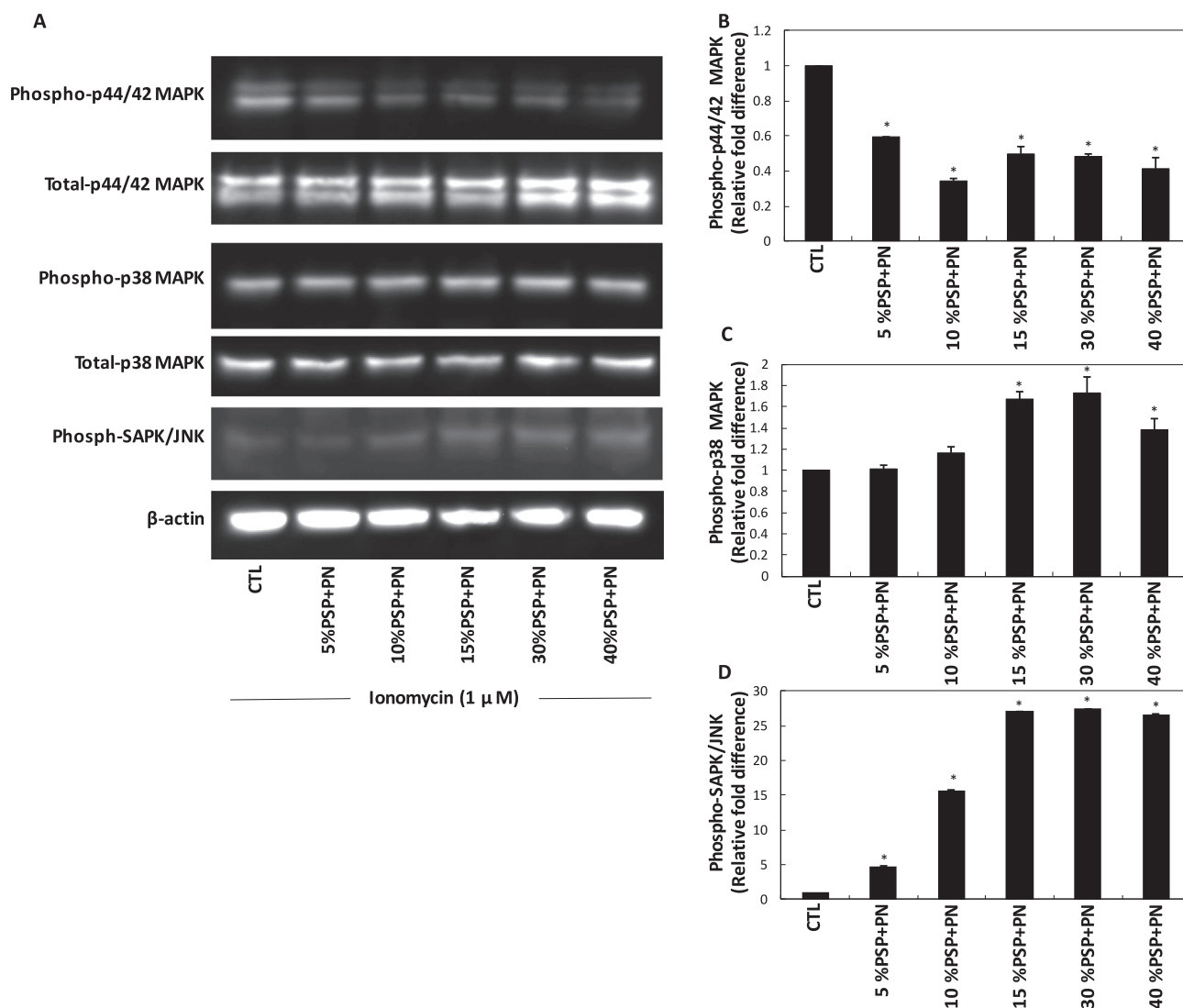


Fig. 5. Effect of PSP-PN protein aggregates (5–40% PSP) or unmodified PN protein (CTL) on ionomycin-mediated p44/42 MAPK and p-38 MAPK. RBL-2H3 cells were co-exposed with (A) unmodified PN protein (CTL) or PSP-PN protein aggregates (5–40% PSP) for 3 h followed by activation with 1 μ M ionomycin for 10 min. Whole cell lysates were separated by SDS-PAGE and immunoblots were probed with anti-phospho specific antibodies to p44/42 MAPK, p-38 MAPK, and SAPK/JNK. β -Actin served as a control (housekeeping gene). Quantitative expression of phospho-p44/42 MAPK (Thr202/Tyr204) (B); phospho-p38 MAPK (Thr180/Tyr182) (C); phospho-SAPK/JNK (Thr183/Tyr185) (D). *Significantly different from CTL at $p < 0.05$.

extent that IgE epitopes could at least partially not bind to PN-specific IgE antibodies (Fig. 1). Lack of antibody binding due to the absence of antigen was ruled out based on a total protein stain prior to immunoblotting (data not shown). Overall, total IgE binding to PN proteins was decreased significantly in the PSP-PN protein aggregates containing 40% PSP.

Our results suggest that PSP aggregation to PN protein potentially lowers allergenic response of PN flour by cross-linking with allergens. Previous studies have shown that food components can change the biological and immunological properties of food allergens by cross-linking thus mitigating their allergenicity (Mihajlovic et al., 2016). We have also demonstrated that the aggregation of cranberry and lowbush blueberry pomace polyphenols decreased IgE binding of PN flour (Plundrich et al., 2017). Similarly, polyphenol oxidase (PPO) and caffeic acid were also shown to reduce allergic properties of allergens, namely, Ara h 1 and Ara h 2 via crosslinking and IgE binding (Chung, Kato, & Champagne, 2005; Wu et al., 2016). Chung and Champagne (2004), also reported a 4-fold inhibition of IgE binding when polyphenol oxidase was added to PN extracts, resulting in the precipitation of PN allergens Ara h 1 and Ara h 2 (Chung & Champagne, 2004). They

also noted that the aggregation of these phenolics to Ara h 1 and Ara h 2 was irreversible.

We show here that PSP aggregation to PN protein not only reduced IgE release, but this reduction was attributed to the reduction in the ionomycin-stimulated degranulation in RBL-2H3 cells. The mitigation of an allergic response as measured by reduction of β -hexosaminidase and histamine release can be attributed to the blockage of active allergens caused by the aggregation of PSP with PN proteins. Earlier studies have shown that polyphenols can potentially reduce an allergic response when aggregated with PN proteins (Chung & Champagne, 2004). The effect observed due to PSP-PN protein aggregation in reducing ionomycin-induced degranulation shows that the activity of an anti-allergenicity mechanism depends on intracellular Ca^{2+} signaling.

Intracellular Ca^{2+} efflux is known to be responsible for allergenic reactions resulting in secretion of histamine, serotonin, heparin, proteases, and cytokines (Shakoory, Fitzgerald, Lee, Chi, & Krishnaswamy, 2004). Other studies have reported that allergens activate FC ϵ RI receptors on cell membranes while subsequently activating Ca^{2+} signaling. This increase in intracellular Ca^{2+} efflux results in a release of β -hexosaminidase and histamine, resulting in an anaphylactic response

in sensitive individuals (Baba et al., 2008). Earlier studies have shown that the Ca^{2+} /calmodulin pathway is responsible for the increase in cytosolic Ca^{2+} concentration in RBL-2H3 cells (Funaba, Ikeda, & Abe, 2003). Hanson and Ziegler (2002) showed that cell degranulation involved a signaling cascade dependent on lyn, syk, phospholipase C (PLC), protein kinase C (PKC) and intracellular mobilization (Hanson & Ziegler, 2002). The role of spleen tyrosine kinase Syk is also proposed as a signaling pathway involved in IgE-bound cell receptor-mediated inflammation (Siraganian, de Castro, Barbu, & Zhang, 2010). They also showed that granule release was dependent on ERK phosphorylation and this ERK activation was MEK independent and PKC dependent.

Our results suggest PSP-PN protein aggregates when exposed to ionomycin-induced RBL-2H3 cells inhibit the phosphorylation of p44/42 MAPK and slightly induced the phosphorylation of p-38 in a dose-dependent manner. These results suggest that polyphenol aggregation of PN proteins inhibits MAPK signaling by suppressing Ca^{2+} channeling either by inhibiting FCεRI activation or by inhibiting ionomycin-induced Ca^{2+} influx. This inhibition of Ca^{2+} channel signaling may partly be responsible for the inhibition of degranulation in RBL-2H3 cells.

Previous studies involving *Kaempferia parviflora* showed that the mechanism of inhibition of degranulation involved the inhibition of Ca^{2+} influx to the cells (Hersh, Hickam, Haynes, & McKibbin, 1991). Our study agrees with Hanson and Ziegler (2002), who showed that the cross-linking of IgE receptors on the RBL-2H3 cells using ionomycin involving a phosphorylation of MAPK is MEK independent and PKC dependent (Hanson & Ziegler, 2002). Their results showed that the inhibition of the Src kinase family, PKC, and MEK1/2 significantly inhibited an ionomycin-induced granule release, whereas the inhibition of p38 kinase did not have any effect. Contrastingly, our results showed that PSP-PN aggregates inhibited ionomycin-induced p42/44 phosphorylation and partially induced p38 and significantly induced SAPK/JNK phosphorylation. This is in agreement with literature since ionomycin is known to inhibit the activation of Ca^{2+} -induced p38/SAPK (Kreidweiss et al., 1999). In summary, our results showed that a PSP aggregation to PN protein decreased IgE binding capacity and reduced allergic response by mitigating β-hexosaminidase and histamine release in RBL-2H3 cells. Our results corroborate this finding and further demonstrate that peanut skin polyphenolic extracts when aggregated with peanut flour can reduce the Ca^{2+} -dependent degranulation in cells.

Acknowledgements

This research project was made possible by Grant No. 2015-38821-24370 from the US Department of Agriculture-National Institute of Food and Agriculture, United States. The statements made herein are solely the responsibility of the authors.

Conflict of interest

The authors declare that there is no conflict of interest regarding this paper.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.foodchem.2018.05.007>.

References

- Anagnostou, K., & Clark, A. (2014). Peanut immunotherapy. *Clinical and Translational Allergy*, 4, 30.
- Aswar, U. M., Kandhare, A. D., Mohan, V., & Thakurdesai, P. A. (2015). Anti-allergic effect of intranasal administration of type-A procyanidin polyphenols based standardized extract of cinnamon bark in ovalbumin sensitized BALB/c mice. *Phytotherapy Research*, 29(3), 423–433.
- Baba, Y., Nishida, K., Fujii, Y., Hirano, T., Hikida, M., & Kurosaki, T. (2008). Essential

- function for the calcium sensor STIM1 in mast cell activation and anaphylactic responses. *Nature Immunology*, 9(1), 81–88.
- Bansode, R. R., Randolph, P., Hurley, S., & Ahmedna, M. (2012). Evaluation of hypolipidemic effects of peanut skin-derived polyphenols in rats on Western-diet. *Food Chemistry*, 135(3), 1659–1666.
- Burks, A. W. (2008). Peanut allergy. *Lancet*, 371(9623), 1538–1546.
- Cabanillas, B., Pedrosa, M. M., Rodriguez, J., Muzquiz, M., Maleki, S. J., Cuadrado, C., ... Crespo, J. F. (2012). Influence of enzymatic hydrolysis on the allergenicity of roasted peanut protein extract. *International Archives of Allergy and Immunology*, 157(1), 41–50.
- Chung, S., & Champagne, E. T. (2004). Cross-linking of peanut allergens by polyphenol oxidase and its effect on peanut allergenicity. *Journal of Allergy and Clinical Immunology*, 113(2), S314–S315.
- Chung, S.-Y., Kato, Y., & Champagne, E. T. (2005). Polyphenol oxidase/cafeic acid may reduce the allergenic properties of peanut allergens. *Journal of the Science of Food and Agriculture*, 85(15), 2631–2637.
- Clarke, M. C., Kilburn, S. A., Hourihane, J. O., Dean, K. R., Warner, J. O., & Dean, T. P. (1998). Serological characteristics of peanut allergy. *Clinical & Experimental Allergy*, 28(10), 1251–1257.
- Fu, G., Zhong, Y., Li, C., Li, Y., Lin, X., Liao, B., ... Huang, S. (2010). Epigenetic regulation of peanut allergen gene Ara h 3 in developing embryos. *Planta*, 231(5), 1049–1060.
- Funaba, M., Ikeda, T., & Abe, M. (2003). Degranulation in RBL-2H3 cells: Regulation by calmodulin pathway. *Cell Biology International*, 27(10), 879–885.
- Hanson, D. A., & Ziegler, S. F. (2002). Regulation of ionomycin-mediated granule release from rat basophil leukemia cells. *Molecular Immunology*, 38(16–18), 1329–1335.
- Hersh, W., Hickam, D. H., Haynes, R. B., & McKibbin, K. A. (1991). Evaluation of SAPHIRE: An automated approach to indexing and retrieving medical literature. *Proceedings. Symposium on Computer Applications in Medical Care*, 808–812.
- Holowka, D., & Baird, B. (1996). Antigen-mediated IGE receptor aggregation and signaling: A window on cell surface structure and dynamics. *Annual Review of Biophysics and Biomolecular Structure*, 25, 79–112.
- Kenny, T. P., Keen, C. L., Schmitz, H. H., & Gershwin, M. E. (2007). Immune effects of cocoa procyanidin oligomers on peripheral blood mononuclear cells. *Experimental Biology and Medicine*, 232(2), 293–300.
- Kreidweiss, S., Ahlers, C., Nordheim, A., & Rühlmann, A. (1999). Ca^{2+} -induced p38/SAPK signalling inhibited by the immunosuppressant cyclosporin A in human peripheral blood mononuclear cells. *European Journal of Biochemistry*, 265, 1075–1084.
- Lin, J., & Sampson, H. A. (2009). The role of immunoglobulin E-binding epitopes in the characterization of food allergy. *Current Opinion in Allergy and Clinical Immunology*, 9(4), 357–363.
- Maeda-Yamamoto, M. (2013). Human clinical studies of tea polyphenols in allergy or life style-related diseases. *Current Pharmaceutical Design*, 19(34), 6148–6155.
- Magrone, T., & Jirillo, E. (2012). Influence of polyphenols on allergic immune reactions: Mechanisms of action. *The Proceedings of the Nutrition Society*, 71(2), 316–321.
- Mihajlovic, L., Radosavljevic, J., Nordlund, E., Krstic, M., Bohn, T., Smit, J., ... Cirkovic Velickovic, T. (2016). Peanut protein structure, polyphenol content and immune response to peanut proteins in vivo are modulated by laccase. *Food & Function*, 7(5), 2357–2366.
- Nakano, N., Nishiyama, C., Tokura, T., Nagasako-Akazome, Y., Ohtake, Y., Okumura, K., & Ogawa, H. (2008). Procyanidin C1 from apple extracts inhibits Fc epsilon RI-mediated mast cell activation. *International Archives of Allergy and Immunology*, 147(3), 213–221.
- Nyanhanda, T., Gould, E. M., & Hurst, R. D. (2014). Plant-derived foods for the attenuation of allergic airway inflammation. *Current Pharmaceutical Design*, 20(6), 869–878.
- Paz, M. A., Fluckiger, R., Boak, A., Kagan, H. M., & Gallop, P. M. (1991). Specific detection of quinoproteins by redox-cycling staining. *Journal of Biological Chemistry*, 266(2), 689–692.
- Plundrich, N. J., Bansode, R. R., Foegeding, E. A., Williams, L. L., & Lila, M. A. (2017). Protein-bound Vaccinium fruit polyphenols decrease IgE binding to peanut allergens and RBL-2H3 mast cell degranulation in vitro. *Food & Function*, 8(4), 1611–1621.
- Plundrich, N. J., Kulis, M., White, B. L., Grace, M. H., Guo, R., Burks, A. W., ... Lila, M. A. (2014). Novel strategy to create hypoallergenic peanut protein-polyphenol edible matrices for oral immunotherapy. *Journal of Agriculture and Food Chemistry*, 62(29), 7010–7021.
- Rawel, H. M., Czajka, D., Rohn, S., & Kroll, J. (2002). Interactions of different phenolic acids and flavonoids with soy proteins. *International Journal of Biological Macromolecules*, 30(3–4), 137–150.
- Shakoori, B., Fitzgerald, S. M., Lee, S. A., Chi, D. S., & Krishnaswamy, G. (2004). The role of human mast cell-derived cytokines in eosinophil biology. *Journal of Interferon and Cytokine Research*, 24(5), 271–281.
- Sicherer, S. H., & Sampson, H. A. (2010). Food allergy. *Journal of Allergy and Clinical Immunology*, 125(Suppl 2), S116–S125.
- Siraganian, R. P., de Castro, R. O., Barbu, E. A., & Zhang, J. (2010). Mast cell signaling: The role of protein tyrosine kinase Syk, its activation and screening methods for new pathway participants. *FEBS Letters*, 584(24), 4933–4940.
- Tatsuno, T., Jinno, M., Arima, Y., Kawabata, T., Hasegawa, T., Yahagi, N., ... Ohta, T. (2012). Anti-inflammatory and anti-melanogenic proanthocyanidin oligomers from peanut skin. *Biological & Pharmaceutical Bulletin*, 35(6), 909–916.
- Tomochika, K., Shimizu-Ibuka, A., Tamura, T., Mura, K., Abe, N., Onose, J., & Arai, S. (2011). Effects of peanut-skin procyanidin A1 on degranulation of RBL-2H3 cells. *Bioscience, Biotechnology, and Biochemistry*, 75(9), 1644–1648.
- Trotter, J. F., Everson, G. T., Bock, S. A., Wachs, M., Bak, T., & Kam, I. (2001). Transference of peanut allergy through liver transplantation. *Liver Transplantation*, 7(12), 1088–1089.
- van Wijk, F., Hartgring, S., Koppelman, S. J., Pieters, R., & Knippels, L. M. (2004). Mixed

- antibody and T cell responses to peanut and the peanut allergens Ara h 1, Ara h 2, Ara h 3 and Ara h 6 in an oral sensitization model. *Clinical & Experimental Allergy*, 34(9), 1422–1428.
- Wu, Z., Lian, J., Han, Y., Zhou, N., Li, X., Yang, A., ... Chen, H. (2016). Crosslinking of peanut allergen Ara h 2 by polyphenol oxidase: Digestibility and potential allergenicity assessment. *Journal of the Science of Food and Agriculture*, 96(10), 3567–3574.
- Yu, J., Ahmedna, M., Goktepe, I., & Dai, J. (2006). Peanut skin procyanidins: Composition and antioxidant activities as affected by processing. *Journal of Food Composition and Analysis*, 19(4), 364–371.
- Zhang, Q., Zhang, J., Shen, J., Silva, A., Dennis, D. A., & Barrow, C. J. (2006). A Simple 96-Well Microplate Method for Estimation of Total Polyphenol Content in Seaweeds. *Journal of Applied Phycology*, 18(3), 445–450.

Supplemental material

Method:

2.4. Cell viability of RBL-2H3 cells exposed to PSP-PN protein aggregates or PSP extracts

RBL-2H3 cells seeded into a 24 well plates at a seeding density of 1.5×10^5 cells per well were maintained in EMEM containing 15% FBS in a humidified incubator at 37 °C and 5% CO₂. The cells were serum starved in EMEM containing 1.5% FBS overnight. The cells were exposed to either unmodified PN flour or 5, 10, 15, 30 and 40% (w/w) PSP-PN protein aggregates at an exposure concentration equivalent to 50 $\mu\text{g mL}^{-1}$ PN protein for 3, 6, 12 and 24 hrs. Similarly, in another experiment, the amount of PSP extract in each aggregate sample was determined and added to the cells to independently determine the toxicity of polyphenols. The cell viability was measured using XTT assay (ATCC, Manassas, VA, USA).

Results:

Cell viability of RBL-2H3 cells exposed to PSP-PN protein aggregates or PSP extract

RBL-2H3 cells were exposed to 50 $\mu\text{g mL}^{-1}$ PN flour aggregated with 0, 5, 10, 15, 30, and 40% (w/w) of the PSP. In a similar manner, the total number of polyphenolics contained in each blend were determined and the respective amount added to RBL-2H3 cells to measure their cytotoxicity. The effects of PSP-PN protein aggregate on cell viability was tested using the cell counting kit-8 (CCK-8) assay (Supplemental Fig. 1). Results showed exposure of cells to aggregate matrices for 3, 6, 12 and 24 h lead to significant cell death in a dose-dependent and time-dependent manner ($p < 0.05$). The exposure of PSP extract alone was toxic to the cell even at low doses for at 3 hrs exposure time (Supplemental Fig. 1B).