



IN VITRO AND ANIMAL STUDIES

Effect of extrusion processing on immune activation properties of hazelnut protein in a mouse model

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ABSTRACT

Although food processing can alter food allergenicity, the impact of extrusion processing on *in vivo* hazelnut allergenicity is unknown. Here, we tested the hypothesis that extrusion processing will alter the immune activation properties of hazelnut protein (HNP) in mice. Soluble extrusion-processed HNP (EHNP) was prepared and evaluated for immune response using an established transdermal sensitization mouse model. Mice were sensitized with identical amounts of EHNP versus raw HNP. After confirming systemic IgE, IgG1 and IgG2a antibody responses, oral hypersensitivity reaction was quantified by hypothermia shock response (HSR). Mechanism was studied by measuring mucosal mast cell (MMC) degranulation. Compared to raw HNP, the EHNP elicited slower but similar IgE antibody (Ab) response, lower IgG1 but higher IgG2a Ab response. The EHNP exhibited significantly lower oral HSR as well as MMC degranulation capacity. These results demonstrate that the extrusion technology can be used to produce soluble HNP with altered immune activation properties.

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Introduction

Food allergies such as nut allergy represent an undesirable immune response characterized by specific IgE antibody response to food proteins that lead to a potentially lethal disease (Boyce et al. 2011; Sicherer & Sampson 2014). Recent reports suggest that the prevalence of food allergies is ~8% among children and 5% among adults (Gupta et al. 2011; Sicherer & Sampson 2014). In addition, nearly 40% of children with food allergies experience severe life-threatening anaphylactic reactions most often linked to tree nuts or peanuts (Gupta et al. 2011). Furthermore, both the prevalence and the severity of food allergies are growing at an alarming rate with an economic burden of ~\$24 billion/year in USA alone (Branum & Lukacs 2009; Boyce et al. 2011; Gupta et al. 2013; Sicherer & Sampson 2014).

Hazelnut, also known as filbert, is considered as a major allergenic tree nut by the regulatory agencies in the USA, Canada, the European Union and Australia (Osborne et al. 2011; Gendel & Zhu 2013;

Nwaru et al. 2014). There is currently no effective method to prevent hazelnut anaphylaxis other than complete avoidance of exposure to hazelnut. It is generally thought that similar to other nut allergies, hazelnut allergy is also a persistent type of food allergy with lower potential to outgrow (Gonipeta et al. 2010; Sicherer & Sampson 2014). Consequently, once sensitized during childhood, the patients have the challenging and often frustrating life-experience of the need to avoid them at all the time.

Previous studies demonstrate that food-processing technologies may be potentially used to alter (increase or reduce) allergenicity of food proteins (Sathe et al. 2009; Masthoff et al. 2013). This is possible because changes in protein structure induced by food processing could potentially alter (increase or decrease) the ability of food proteins to bind to IgE antibody and trigger mast cell activation leading to clinical reactions (Besler et al. 2001). Although significant efforts are underway to evaluate the effect of processing on various food allergens including hazelnut with most studies using *in vitro* methods such as IgE binding assays and

basophil degranulation assay (Müller et al. 2000; Wigotzki et al. 2000; Besler et al. 2001; Pastorello et al. 2002; Sathe & Sharma 2009; López et al. 2012). However, very little is known about the effect of extrusion food processing on *in vivo* immune system activating properties of tree nut proteins in general and the effect of extrusion processing on *in vivo* immune system activation properties of hazelnut is unknown—a critical gap in our knowledge and focus of this study.

We previously reported a novel adjuvant-free mouse model of hazelnut allergy and characterized this model extensively (Birmingham et al. 2005, 2007; Parvataneni et al. 2009; Gonipeta et al. 2013, 2015a, 2015b). Notably, this model exhibits many features similar to life-threatening human nut allergy including the following: (i) this model does not use adjuvants such as alum or cholera toxin or *Staphylococcus* enterotoxins, but uses a transdermal exposure (TDE) method to induce sensitization followed by elicitation of clinical reactions upon oral challenge to simulate the near-fatal human anaphylaxis (Gonipeta et al. 2015a, 2015b); (ii) nut allergy in this model is controlled by non-MHC genetics with a major role for IL-4 (Parvataneni et al. 2009); (iii) nut allergy in this model remains persistent for long periods despite withdrawal of allergen exposure (Gonipeta et al. 2010); and (iv) the model exhibits robust systemic IgE antibody response and mucosal mast cell (MMC) degranulation responses (Birmingham et al. 2007; Gonipeta et al. 2015a, 2015b). Thus, this model can serve as a valuable tool to evaluate the effect of food processing methods on *in vivo* immune activation properties of nut proteins (such as hazelnut) (Gonipeta et al. 2015a, 2015b).

Using this model, here, we tested the hypothesis that extrusion processing will alter the immune activation properties of soluble hazelnut protein (HNP) in mice. Our testing in this mouse model collectively demonstrates that the extrusion technology can be used to produce soluble HNP with altered immune system activation properties.

Methods

Preparation of defatted hazelnut flour and extrusion processing

Hazelnuts (HN) (Diamond Foods, Stockton, CA) were shelled, ground into flour and defatted using hexane following the standard methods optimized in the cereal chemistry laboratory at Michigan State University. The defatted HN flour was used in extrusion processing (APV Baker MPF-19/25 twin-screw extruder, Staffordshire, England). Hazelnut flour was extruded

using an optimized protocol. The screw configuration used was: 8D Twin, $7 \times 30^\circ$ FKP (forward kneading paddle), 4D Twin, $4 \times 60^\circ$ FKP, $4 \times 30^\circ$ RKP (reverse kneading paddle), 2D Twin, $6 \times 60^\circ$ FKP, $4 \times 30^\circ$ RKP, 1D Single, $7 \times 90^\circ$ P, 2D Single where 1 Diameter (D) equals 19 mm and 1 paddle (P) equals 0.25D. The temperature profile was 147–124–101–62–60 °C. HN flour was extruded at a screw speed of 150 rpm and feed rate of 16.42 g/min with feed moisture of 31.6%. The sample was collected over the course of 5 min at 140 °C, 20 psi and 14.7% torque. The extruded HN sample was stored at -70°C until protein extraction was conducted.

Soluble HNP extraction and quantification

Extruded HN and raw HN were ground and soluble protein was extracted with Tris-HCl (30 mM, pH 8.0). Soluble HNP extract was syringe filtered (MILLEX® – HA; 0.45 µm; EMD Millipore, Billerica, MA). Sterile samples were used throughout the study. Protein concentration was determined by repeated measurements of samples using the Lowry-Folin method using BSA as the standard (Lowry et al. 1951). Samples were tested at three dilutions in quadruplicates and the average from six data points was used to determine the protein concentration. Identical amount of soluble proteins were used in animal experiments.

Mice: Female BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at age six weeks. Mice were allowed to rest for one week prior to beginning experimentation. All experiments were conducted under the authorization of Michigan State University (MSU) Institutional Animal Care and Use Committee (IACUC). Mice were housed in irradiated (sterile) 100% PET cages (Innovive, San Diego, CA) with sterile aspen chip bedding, 4–5 mice per cage with a 12 h light/dark cycle. Mice diet was free from all nuts (8640 Teklad rodent diet, Harlan Laboratories, Indianapolis, IN).

Transdermal sensitization and oral elicitation of hypothermia shock response (HSR) and MMC degranulation response with HNPs

Sensitization of mice was conducted via TDE described previously (Birmingham et al. 2007; Gonipeta et al. 2015a, 2015b). Overall experimental design for animal experiment is illustrated in Figure 1(A). Briefly, hair was clipped from the back of mice one week after arrival and as needed throughout the experiments. Mice that received oral challenge (10 mg/mouse) had

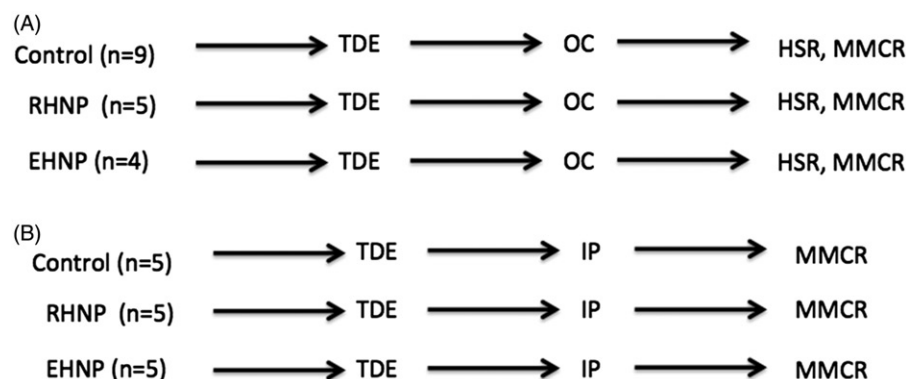


Figure 1. Overall experimental design used in the present study. (A) This shows the study design for analysis of hypersensitivity shock responses (HSR) (as measured by rectal thermometry) and mucosal mast cell response (MMCR) (as measured by elevation of plasma mMCP-1 levels) upon oral challenge (OC) in mice that were sensitized by a transdermal exposure (TDE) method described in the text. (B) This shows the study design for analysis of MMCR upon systemic (intraperitoneal) challenge in mice that had been sensitized by the transdermal exposure (TDE) method.

been sensitized with 1 mg/mouse/week for seven weeks of raw HNP (RHNP) or extrusion processed HNP (EHNP). Control group of mice received the vehicle (30 mM Tris-HCl buffer, 50% v/v Glycerin, 0.2% Phenol) alone without the protein. Blood samples (saphenous vein) were collected one week after arrival (prior to experimentation) and once every 15 days after beginning experimentation. Samples were collected in heparin-coated Microvette capillary tubes (Sarstedt, Nümbrecht, Germany) and centrifuged. Plasma was extracted and stored at -70°C .

Transdermal sensitization and systemic elicitation of MMC response with HNPs

Sensitization of mice was conducted via TDE as described previously (Birmingham et al. 2007; Gonipeta et al. 2015a, 2015b). Overall experimental design for animal experiment is illustrated in Figure 1(B). Blood samples (saphenous vein) were collected before and at 45 min after intraperitoneal injection (1 mg/mouse). Samples were analyzed for mMCP-1 levels with a minimum of two replicates.

Measurement of hazelnut specific IgE, IgG1 and IgG2a antibody levels

Hazelnut (HN) specific IgE, IgG1 and IgG2a antibody levels were measured using ultrasensitive indirect ELISA-based methods described previously (Birmingham et al. 2003). Detection was carried out with biotin rat anti-mouse IgE or anti-IgG1 or anti-IgG2a antibody (BD Pharmingen, San Diego, CA) as described previously (Birmingham et al. 2003). All samples were analyzed for a minimum of two to three replicates.

Quantitation of HSR by rectal thermometry

After seven weeks, sensitized mice were orally challenged with 10 mg of RHNP or EHNP. Control mice were challenged with vehicle alone. Rectal temperatures were taken prior to oral challenge, and at 10, 20 and 30 min post-oral challenge (Physitemp Thermalert TH-5; The Sea Ranch, CA) as described earlier (Birmingham et al. 2007; Gonipeta et al. 2015a, 2015b).

Quantitation of in vivo MMC degranulation

Blood plasma samples collected before and after allergen challenge were screened for mouse mast cell protease-1 (mMCP-1) using a sandwich ELISA (Mouse mMCP-1 ELISA Ready-SET-Go!® eBioscience, San Diego, CA) (Gonipeta et al. 2015a). The sensitivity of the assay was 120 pg/mL and quantification was performed using a standard curve generated on each plate with a range of 120–15,000 pg/mL. All samples were analyzed with a minimum of two replicates.

Statistical analysis

Differences in antibody responses, rectal thermometry data and plasma mMCP-1 protein levels between raw versus extruded HNP-sensitized/challenged mice were analyzed by Student's *t* test.

Results

Comparison of IgE, IgG1 and IgG2a antibody responses upon TDE to raw versus extruded HNPs in mice

Groups of adult female mice received TDE to RHNP or EHNP for seven weeks. Plasma samples collected

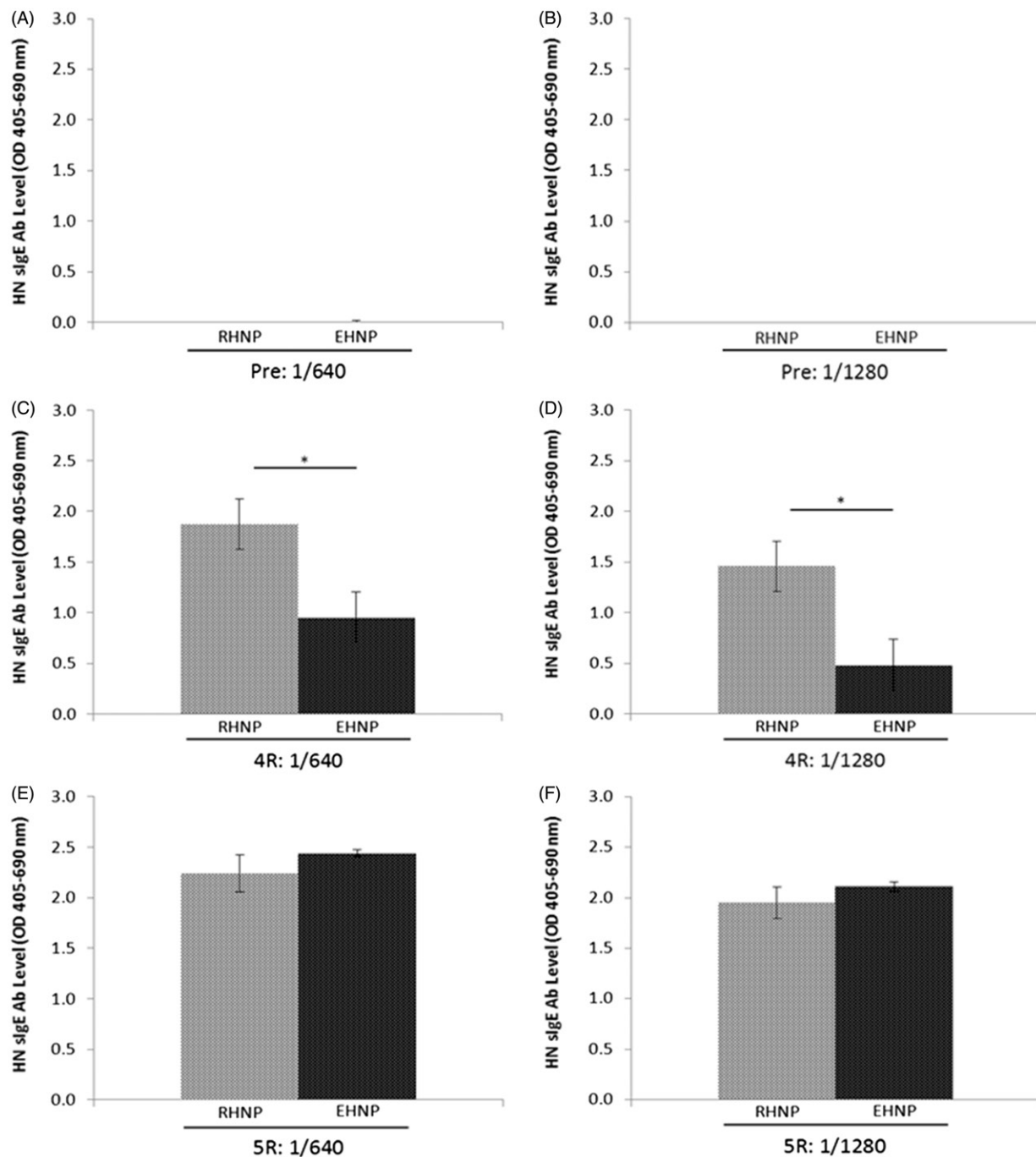


Figure 2. Extruded-hazelnut protein elicits slower, but similar IgE antibody response compared to the raw hazelnut protein extract. The y-axis shows hazelnut (HN)-specific IgE (sIgE) antibody (Ab) levels in BALB/c mice before (Pre) (A, B) and after four transdermal exposures (4R) (C, D) and five transdermal exposures (5R) (E, F) to raw versus extrusion processed hazelnut protein extract. The X-axis shows two plasma dilutions, 1/640 (A, C, E) and 1/1280 (B, D, F) used in ELISA analysis. Student's *t*-test: *indicates significant difference, $p < 0.05$.

prior to TDE (Pre), after four TDE (4R) and after five TDE (5R) were used in HNP specific (S) IgE, SIgG1 and SIgG2a antibody analysis. Samples were analyzed using two-fold serial dilutions and results were statistically analyzed using the data from the linear portion of the titration curves. As expected, none of the mice had SIgE or SIgG1 or SIgG2a antibodies in the pre-

exposure plasma sample (Figures 2–4(A,B)). Upon four TDE, EHNP elicited significantly lower SIgE responses compared to RHNP (Figure 2). However, after five TDE, SIgE antibody responses were comparable suggesting slower development of IgE antibody response to EHNP (Figure 2). The EHNP elicited significantly lower SIgG1 responses compared to RHNP (Figure 3).

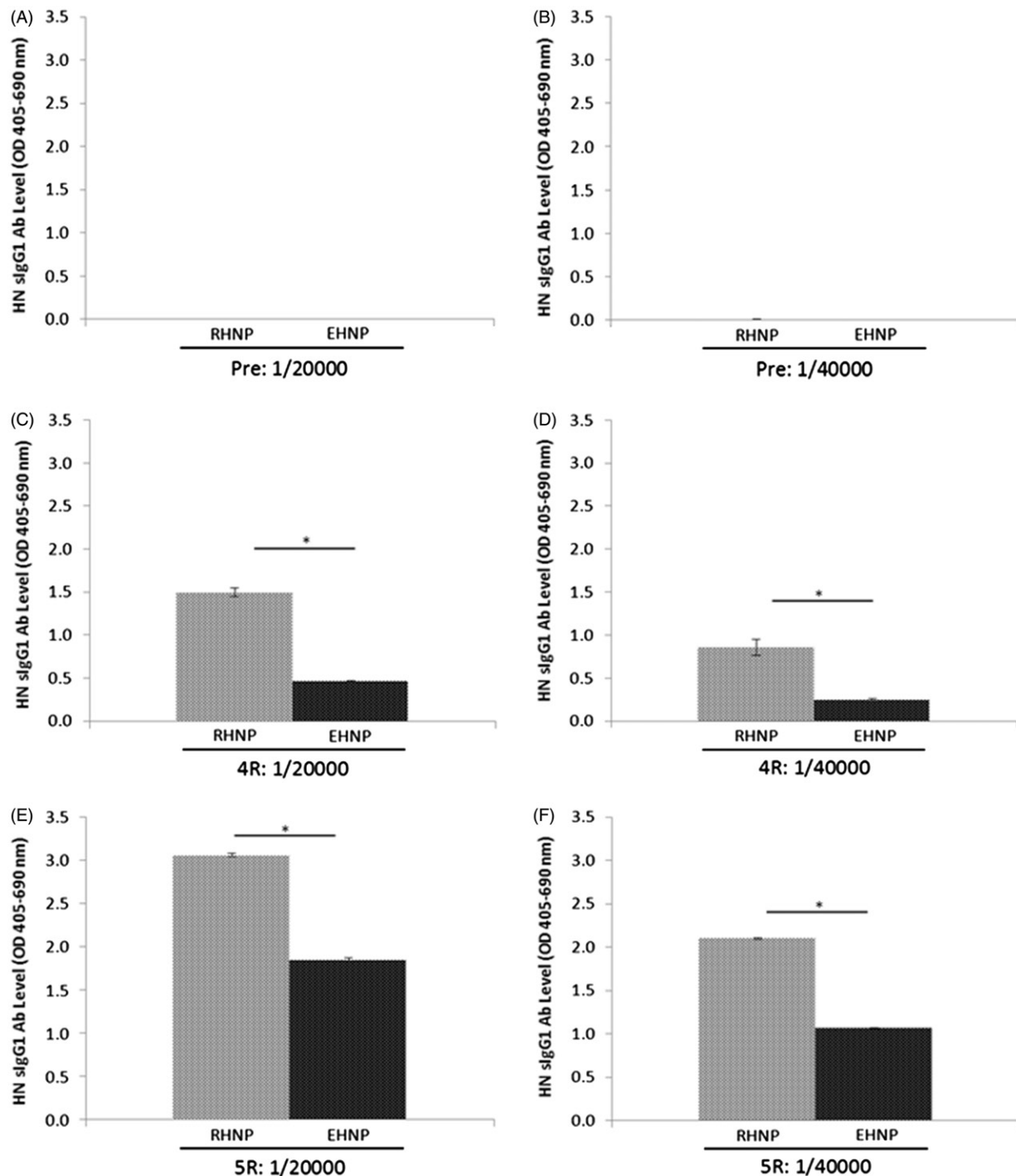


Figure 3. Extruded-hazelnut protein elicits significantly lower IgG1 antibody response compared to the raw hazelnut protein. The y-axis shows hazelnut (HN)-specific IgG1 (sIgG1) antibody (Ab) levels in BALB/c mice before (Pre) (A, B) and after four transdermal exposures (4R) (C, D) and five transdermal exposures (5R) (E, F) to raw versus extrusion processed hazelnut protein extract. The X-axis shows two plasma dilutions, 1/20,000 (A, C, E) and 1/40,000 (B, D, F) used in ELISA analysis. Student's *t*-test: *indicates significant difference, $p < 0.05$.

Although EHNP elicited slower IgG2a responses, the final responses were significantly higher compared to RHNP (Figure 4). The vehicle control group plasma samples were negative for SIgE, SIgG1 and SIgG2a antibodies (data not shown).

Extrusion processed HNP elicits significantly lower HSR upon oral administration

Mice were orally challenged to evaluate HSR to RHNP versus EHNP. Control mice ($n=9$) that received OC with vehicle did not elicit hypothermia response

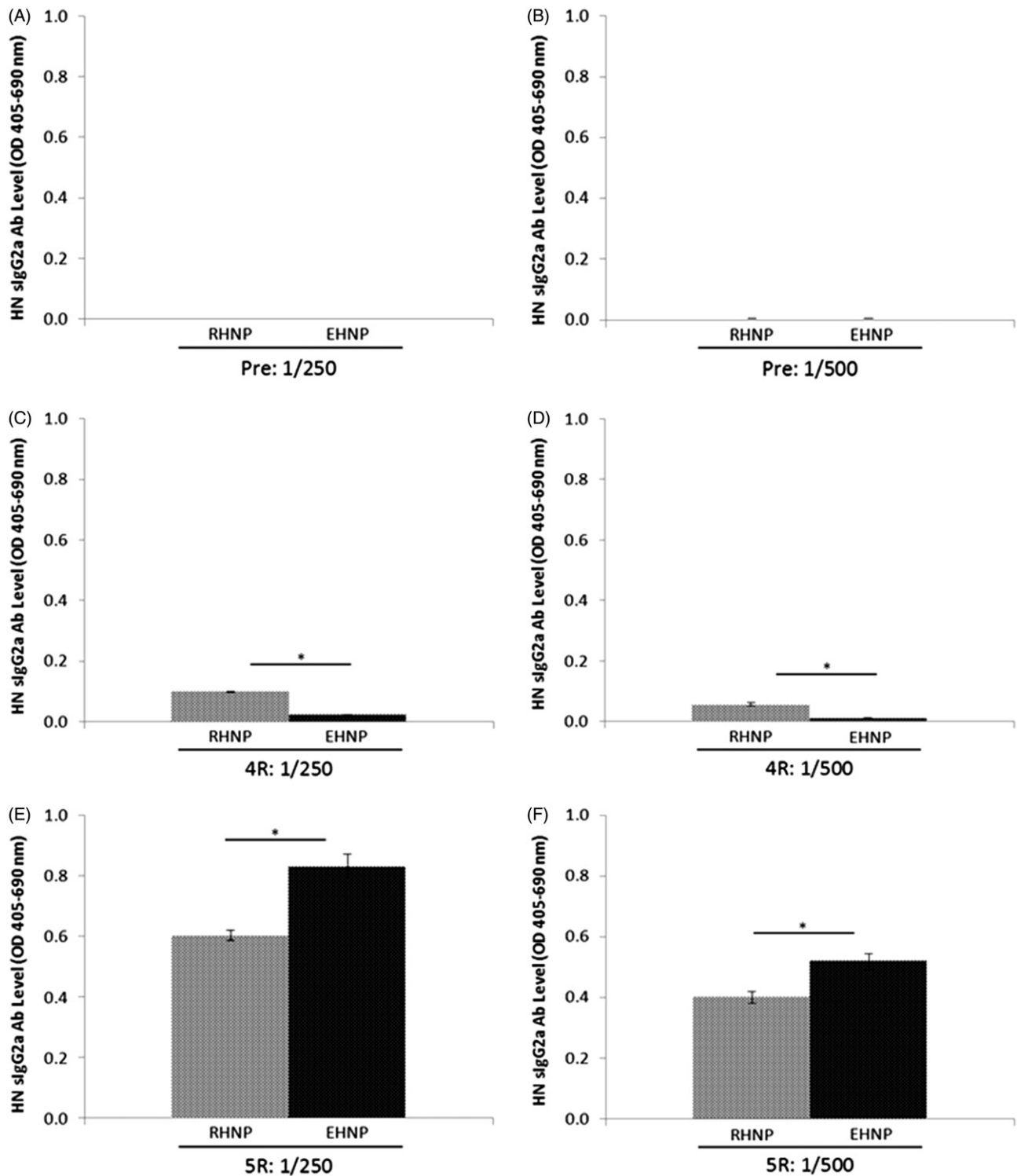


Figure 4. Extruded-hazelnut protein elicits significantly higher IgG2a antibody response compared to the raw hazelnut protein. The y-axis shows hazelnut (HN)-specific IgG2a (slgG2a) antibody (Ab) levels in BALB/c mice before (Pre) (A, B) and after four transdermal exposures (4R) (C, D) and five transdermal exposures (5R) (E, F) to raw versus extrusion processed hazelnut protein extract. The X-axis shows two plasma dilutions, 1/250 (A, C, E) and 1/500 (B, D, F) used in ELISA analysis. Student's *t*-test: *indicates significant difference, $p < 0.05$.

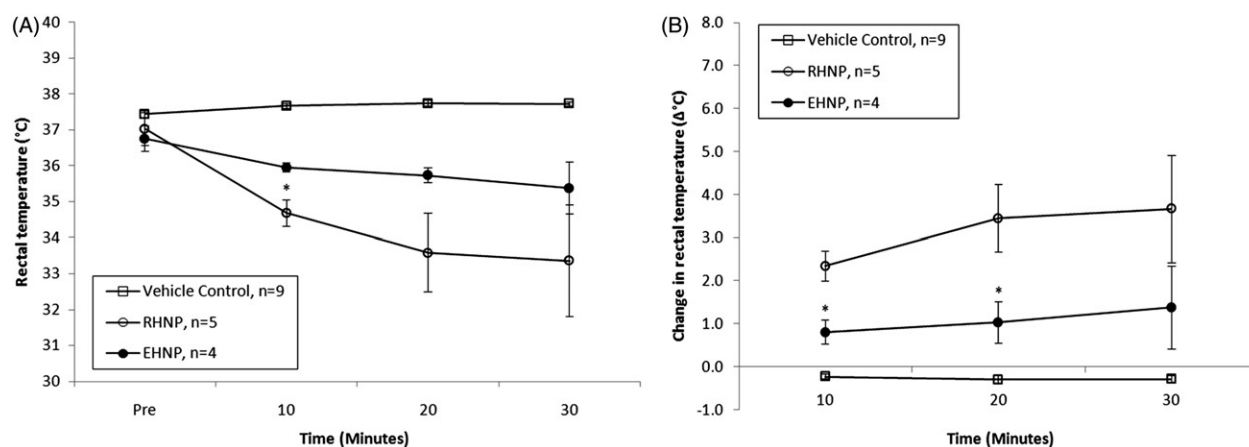


Figure 5. Extrusion processing significantly reduces the hypothermia shock inducing ability of hazelnut protein. Groups of mice were orally challenged with 10 mg of raw hazelnut protein (RHNP) versus extrusion processed hazelnut protein (EHNP) extracts or 100 μ L vehicle control to test for systemic shock reaction by rectal thermometry. (A) Actual rectal temperature; (B) change in temperature from pre to 30 min post oral feeding. *Student's *t*-test, $p < 0.05$.

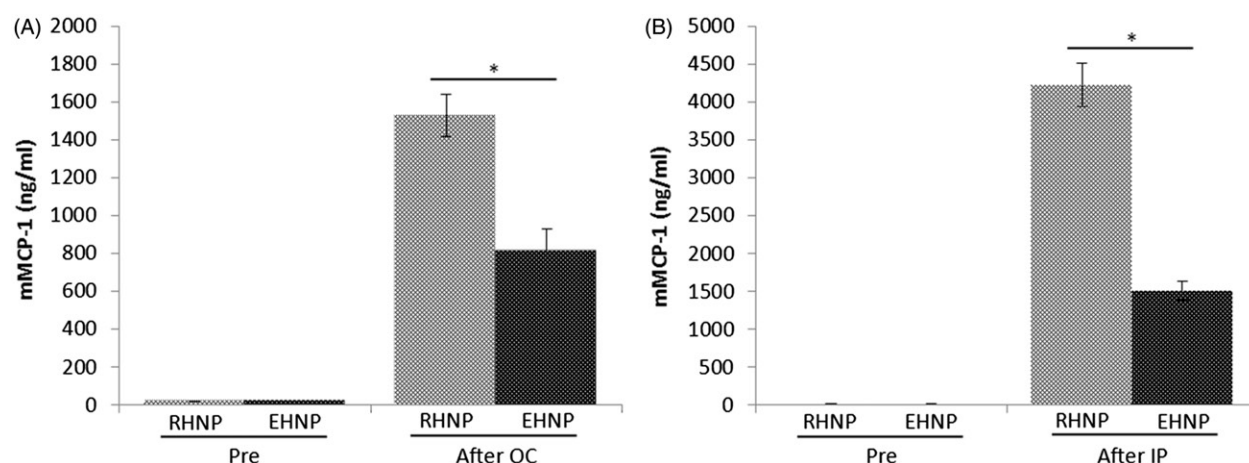


Figure 6. Extrusion-processed hazelnut protein elicits significantly reduced mucosal mast cell degranulation capacity. (A) Analysis of mucosal mast cell degranulation capacity upon oral challenge with raw versus extrusion-processed hazelnut protein. Figure shows mucosal mast cell protease (mMCP)-1 level in the blood before (Pre, left panel) and after (right panel) oral challenge (OC) induced by raw hazelnut protein (RHNP) versus extrusion-processed hazelnut protein (EHNP) extracts. (B) Analysis of mucosal mast cell degranulation responses upon intra-peritoneal (IP) challenge with raw versus extrusion-processed hazelnut protein. Figure shows mucosal mast cell protease (mMCP)-1 level in the blood before (Pre, left panel) and after (right panel) IP challenge (OC) induced by raw hazelnut protein (RHNP) versus extrusion-processed hazelnut protein (EHNP) extracts. The plasma samples were analyzed by ELISA method using a commercial mMCP-1 quantitation kit. Student's *t*-test: *indicates significant difference, $p < 0.05$.

(Figure 5(A)). The RHNP sensitized mice upon OC elicited significant HSR (Figure 5(A)). In contrast, EHNP sensitized mice elicited significantly lower HSR compared to the RHNP group (Figure 5(A)). Analysis of the change in rectal temperatures (Δ °C) also revealed significant reduction in oral HSR to EHNP (Figure 5(B)).

Extrusion processed HNP elicits significantly reduced MMC degranulation response

Mice were evaluated for MMC degranulation response after oral challenge by measuring blood levels of mouse

mast cell protease-1 (mMCP-1), a biomarker of IgE-mediated anaphylaxis (Khodoun et al. 2011). Plasma samples collected prior to (Pre) and 45 min after oral challenge (After OC) were screened. As evident, EHNP elicited significantly reduced mMCP-1 release into the blood upon oral challenge as compared to RHNP (Figure 6(A)). Additional experiments were conducted to confirm this by systemic challenge of mice via the intra-peritoneal route (Figure 1(B)). As evident, EHNP consistently elicited significantly lower MMC degranulation (Figure 6(B)) confirming that extruded-HNP exhibits substantially reduced capacity to cause MMC degranulation. There was no marked MMC response in the vehicle control group (data not shown).

Estimation of HSR inducing capacity and MMC degranulation capacity of extruded-HNP relative to the raw HNP

Using the data from HSR ($\Delta^\circ\text{C}$) percent reduction in HSR inducing capacity was calculated. On an average, extrusion processing caused a reduction of 71% in this capacity. Based on mMCP-1 data, we estimated that extrusion processing caused a reduction of 47% and 64% in MMC degranulation capacity of HNP in oral and systemic challenges, respectively.

Discussion

This research was conducted to evaluate the impact of extrusion processing on *in vivo* immune system activation properties of soluble HNP in a mouse model. A novel adjuvant-free mouse model of hazelnut allergy that we had characterized extensively was used for this purpose (Birmingham et al. 2007; Gonipeta et al. 2010, 2015a, 2015b). Here we tested the hypothesis that extrusion processing will alter the *in vivo* immune activation properties of HNP in mice. Our data collectively supports this hypothesis.

There are three significant and novel findings from this study: (i) soluble extrusion-processed HNP elicited slower, but similar IgE, lower IgG1 but significantly higher IgG2a antibody responses; (ii) extrusion processing significantly reduced the hypothermia shock inducing capacity of soluble HNP and (iii) extrusion processing also significantly reduced the MMC degranulation capacity of HNP.

A major strength of this study is that it is the first study to conduct *in vivo* evaluation of immune system activation properties of extrusion-processed HNP. We were uniquely positioned to do this work because we had previously established an adjuvant-free mouse model of food allergy—another strength of the study. Thus, our data provides the first *proof-of-principle* on the potential of using this model as testing tool to develop food processing methods to produce soluble food proteins with altered immune properties.

The second major strength of this study is that we also evaluated the capacity of extruded HNP to elicit not only IgE but also IgG1 and IgG2a antibody iso-types. The underlying reason is that where IgE antibody responses show sensitization capacity, the IgG1 antibody responses represent general immunogenicity of food proteins (Birmingham et al. 2002; Dearman & Kimber 2005). Although extrusion-processed HNP elicited lower IgE responses initially, continued exposure resulted in IgE levels comparable to the raw HNP suggesting slower kinetics of IgE antibody responses to

extruded-HNP. Our findings show that extruded HNP exhibits reduced immunogenicity as measured by IgG1 responses. In contrast to IgE that is dependent upon Th2 cytokine (IL-4) in mice, IgG2a antibody responses are dependent upon Th1 cytokine (IFN- γ) that is generally associated with anti-allergic immune responses (Mosmann & Coffman 1989; Yang et al. 1993; Gangur et al. 2003). We found that extruded HNP exhibits significantly higher IgG2a response, which may be considered as a desirable property for future development of hypoallergenic food proteins.

The third major strength of this study is evaluating the *oral* immune activation properties of extruded soluble HNP. Such studies in animal models have not been reported in the literature so far. Notably, potential of extrusion processing technology in altering food allergens has been limited to lupine and soybean with reduction in allergenicity (as defined by human IgE binding *in vitro*) was noted for soybean (Franck et al. 2002; Alvarez-Alvarez et al. 2005). However, *in vivo* oral immune activation properties of extruded soybean or lupine (e.g. in animal models) was not evaluated (Franck et al. 2002).

There are only two human studies evaluating the ability of processed HNP to elicit allergic reactions upon oral administration in humans. Hansen et al. (2003) reported that roasted HN (140°C), when administered to birch pollen-sensitized subjects, elicited reduced symptoms associated with oral allergy syndrome (OAS). Worm et al. (2009) reported that roasted HNP (144°C) when administered to birch-pollen or hazelnut-sensitized subjects, elicited reduced frequency of OAS symptoms, skin itching, erythema and rhino-conjunctivitis.

The fourth major strength of this study is that we examined the underlying mechanism to explain the reduction in hypothermia shock inducing capacity of extruded HNP. We used mMCP-1 as a specific biomarker of MMC degranulation because it identifies IgE but not IgG1 mediated oral anaphylaxis in mice (Khodoun et al. 2011). Thus, our findings show that reduced ability of extruded-HNP to cause HSR is linked to its reduced capacity to cause MMC degranulation.

One limitation of this study is that we have not addressed the molecular mechanisms of effect of extrusion processing on HNP molecular structure. However, based on studies conducted with other food proteins, there are three potential hypotheses to be tested: (i) extrusion-induced HNP denaturation and altered epitopes structure leading to lack of IgE signaling via mast cells; (ii) extrusion-induced HNP degradation leading to absence of oral reaction causing specific protein/s;

and (iii) extrusion-induced HNP modification such that the resultant protein epitopes may be more sensitive to inactivation in the gut (Besler et al. 2001; Sathe & Sharma 2009; Masthoff et al. 2013). Future efforts are expected to evaluate these hypotheses.

Another obvious limitation of this study is that this is an investigational pilot study conducted in a mouse model. Consequently, it is imperative that conclusions on allergenicity/anaphylactic potency of extrusion-processed HNP must be made only after further testing in larger experiments and in human hazelnut allergic subjects.

Conclusions

Our data collectively demonstrates the first *proof-of-principle* that extrusion food processing technology can be used to produce soluble HNP with altered immune system activation properties including reduced MMC degranulation capacity. This study also demonstrates for the first time the utility of an adjuvant-free mouse model as an *in vivo* assessment tool for developing innovative food-processing methods to produce food proteins with altered immune properties.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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