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To cite this article: Yang Tian, Huan Rao, Wenhui Fu, Sha Tao & Wen-Tong Xue (2019) Effect of digestion on the immunoreactivity and proinflammatory properties of recombinant peanut allergen Ara h 1, Food and Agricultural Immunology, 30:1, 418-431, DOI: [10.1080/09540105.2019.1592123](https://doi.org/10.1080/09540105.2019.1592123)

To link to this article: <https://doi.org/10.1080/09540105.2019.1592123>



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Published online: 31 Mar 2019.



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Effect of digestion on the immunoreactivity and proinflammatory properties of recombinant peanut allergen Ara h 1

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ABSTRACT

Ara h 1 is one of the major allergens existed in peanut, peptides formed in the digestion process of Ara h 1 could induce allergic symptoms and cause local inflammatory. The mechanism of intestine epithelial immune has not been clarified yet. Here we explored the effect of digestion on the immunoreactivity and proinflammatory properties of recombinant Ara h 1 (rAra h 1). rAra h 1 was treated (or untreated) with heat, digested by pepsin. According to the results, in the early period of digestion, both native and heated rAra h 1 were degraded. Q-PCR and Western-blot results revealed that the NF- κ B pathway was involved in the cytokine secretion. The proinflammatory effect were less pronounced in the samples treated with heat, demonstrating that digestion, instead of thermal process, could modify the proteins and peptides, which could then play a significant role in the activation of Caco-2 cells via the NF- κ B pathway.

ARTICLE HISTORY

Received 3 February 2019
Accepted 28 February 2019

KEYWORDS

Digestion; peanut allergy; recombinant Ara h 1; immunoreactivity; proinflammatory properties

Introduction

Food allergy, defined as “an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food” (Boyce et al., 2010; Sicherer & Sampson, 2018), affect more than 10% of the population worldwide (Sicherer & Sampson, 2018). Peanut, among the food list which are responsible for the most series allergy, affecting around 2% of school-age children in the UK (Mills et al., 2007). The estimated prevalence of challenge-proven peanut allergy across Europe is approximately between 0.8% and 4.5% (Bunyavanich et al., 2014; Teodorowicz, Fiedorowicz, Kostyra, Wichers, & Kostyra, 2013). There are about 1.25 million patients allergic to peanuts in the US in 2017. Besides, the prevalence of peanut allergy in 1-year-olds in the US was

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tripled (from 1.7% to 5.2%) between the year 2001 and 2017 (Lieberman et al., 2018). Even traces of peanut can cause symptoms like urticarial, wheals, diarrhoea, and bronchospasm, those clinical disorders are usually severe and potentially fatal (Guillon, Bernard, Drumare, Hazebrouck, & Adel-Patient, 2016). As the leading cause of anaphylaxis and death due to food allergy, peanut allergy have imposed substantial psychosocial and economic burdens on patients and their families (Du Toit et al., 2015).

Ara h 1, one of the dominant allergens in the peanut kernel, could be recognized by serum IgE from >90% of peanut-sensitive patients (Blanc et al., 2011; Maleki et al., 2000). Previous studies have shown that the trimetric complex structure could help to protect the linear epitopes which located in the areas where the subunits connect with each other (Chruszcz et al., 2011). That might be the reason why the allergenic capacity could be preserved after the allergen digested by pepsin. Besides, it has been investigated that short digestion-resistant peptides (SDRPs; <10 kDa) of Ara h 1, which contain short continuous epitope sequences and possessed substantial allergenic potential (Prodic et al., 2018), could reach the gut mucosa, and thus affect the intestinal homeostasis (Teodorowicz et al., 2013).

The way human exposed to food allergens are majorly by food intake (Rao, Tian, Fu, & Xue, 2018). Apparently the gastrointestinal cell and immune cell, which distributed along the gastrointestinal tract, are participated in the intestine immune process. Besides, Intestine epithelial immune system is a complex self-adaptive system, which possesses the ability to prevent outside pathogens (Lai et al., 2016). Just like inflammation, the immune system also presents complex development with the secretion of cell factors related to immune function. NF- κ B, a kind of transcription factor, is a crucial regulator of inflammatory diseases and immune responses (Pan et al., 2017). It can regulate several processes like the development and survival of lymphocytes and lymphoid organs, and the control of immune responses and malignant transformation (Liu, Zhang, Joo, & Sun, 2017). Once activated by allergens or pathogens, NF- κ B would move into the nucleus, and cause the secretion of inflammatory cytokines such as IL-8, which could enhance the immune response subsequently. Thus, the NF- κ B signalling pathway may be involved in the intestine epithelial immune process.

To clarify the effect of how the intestinal homeostasis were influenced by Ara h 1, cytokine secretion measurement was utilized to analyse biological activity of allergen. It has been reported that local immune system would be influenced by intestinal epithelial cells for their cytokine secretion (Peterson & Artis, 2014; Rooks & Garrett, 2016). Besides, potent proinflammatory cytokines like IL-8 would induce the movement of macrophages and neutrophils from the circulation to the inflamed mucosa (Shin et al., 2015). It has been proved IL-8 is one of the key mediators in epithelial immune responses which influences several factors including activation, growth, differentiation, and migration of neutrophils, basophils and T cells (Teodorowicz et al., 2013). Furthermore, IL-8 secretion measurement has been used to monitor the intestine epithelial immune process (Teodorowicz et al., 2013).

Normally the Ara h 1 will degrade very quickly when digested by pepsin (Rao et al., 2018), but the allergen still have the ability to inhibit Caco-2 cell proliferation and stimulate IL-8 secretion (Teodorowicz et al., 2013). The contradiction between these two results have not been reasoned yet. In sum, we decided to explore the effect of digestion on the immunoreactivity and proinflammatory properties of recombinant Ara h 1 (rAra h 1),

and explore the mechanism of intestine epithelial immune. Our research would provide new understanding to the sensitization mechanism of peanut allergen and the guidance of hypoallergenic food processing.

Methods

Sample preparation

Recombinant Ara h 1 (rAra h 1) was prepared according to the methods reported by Tian, Rao, Tao, and Xue (2018). rAra h 1 dissolved in the elution buffer (50 mM Tris-Cl, 0.1 mM EDTA, 600 mM NaCl, 1 mM DTT, 300 mM imidazole, pH 8.4) was ultra-filtrated and concentrated to 1.0 mg/mL. Then the protein buffer boiled at 100°C for 20 min was marked as heat treated rAra h 1. The digestion process was performed according to Teodorowicz et al. (2013) and Rao et al. (2018). Simulated gastric fluid was prepared by using pepsin (3200–4500 units/mg protein, Sigma Aldrich, USA) and then added to the protein samples. The final concentration of the pepsin in each mixture is 2000 U/mL, the total volume of each mixture is 200 µL. 1 M HCl was used to adjust the pH of each sample to 3 to imitate the stomach environment. The hydrolysis was lasted for different times (1, 5, 30, 60 min) at 37°C with agitation and stopped by the addition of 1 M NaOH, which helped to increase the pH of samples to 7–8. Samples were subsequently chilled on ice.

SDS-PAGE

Protein samples were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on a mini-protean II gel apparatus (Bio-Rad, USA). Methods from Rao et al. (2018) were modified as follows: loading buffer (Solarbio Science & Technology Co., Ltd, Beijing, China) were added into each sample, which was then boiled and loaded (10 µg/lane) to a 8% acrylamide separating gel. The gels were dyed by Coomassie brilliant blue R250 (Sigma Aldrich, USA) for 40 min and bleached in destaining solution (1.31 M HAc, 1.23 M methanol) overnight. BIO-RAD GelDoc 2000 gel imaging system (Multi Gauge V3.1, Fujitsu, Japan) was used to visualize and analysis the pictures of electrophoresis results.

Western-blotting

According to methods reported by Chen et al.(2018) and Tian, Rao, Zhang, Tao, and Xue (2018), western blots was performed as follows: The concentration of protein samples were measured by BCA assay kit (Solarbio Science & Technology Co., Ltd, Beijing, China) according to the manufacturer's instructions. To analysis the immunoreactivity of digested rAra h 1, 10 µg protein were loaded to each well. For the verification of NF-κB signalling, 40 µg protein were loaded to each well. Protein from the unstained SDS-PAGE gel were immersed in transfer buffer (25 mM Tris-HCl, 125 mM glycine, 20% v/v Methanol) for 10 min. And then transferred to a nitrocellulose membrane using a MiniProtean III system (BioRad) at 300 mA for 80 min. After blocking for 1 h at room temperature, membranes was incubated overnight with the rabbit anti-Ara h 1 antibody, or optimal primary

antibody dilutions at 4°C. The membranes were incubated by goat anti-rabbit IgE-HRP was for 2 h at 37 °C in the following day. Bands were then visualized by using a chemiluminescence (ECL) detection system (Kangweishiji, Beijing, China) and detected using the ChemiDoc XRS imaging system (Bio-Rad, Richmond, CA). Image Lab software was utilized to quantify the band intensities. All experiments were performed in triplicate.

Caco-2 cell culture

Methods reported by Starkl (2011) were modified as follows: Human Caco-2 cells (purchased in Xiehe cell library, Shanghai, China) were between passages 20 and 40. For all experiments, cells were cultured in DMEM (Sigma Aldrich, USA), supplemented with 20% FBS (Invitrogen, France), at 37°C in a humidified atmosphere of 5% CO₂ in air.

Determination of IL-8 secretion

Caco-2 cells were plated at a density of 2.5×10^5 cells/mL in 96-well plates (CorningCostar, USA) and grown for 7 days (Teodorowicz et al., 2013). After washed by PBS for 2 times, cells were incubated in the presence of protein samples (digested by pepsin for 1 h) at a concentration of 25 µg/ml for 24 h, or incubated with Lipopolysaccharide (LPS) at a concentration of 1 µg/ml for 24 h. Every experiment was performed in triplicate. The concentration of IL-8 existed in culture medium was measured by a commercially available ELISA kit (Proteintech Group, Chicago, USA) according to the manufacturer's instructions.

Gene expression analysis

Caco-2 cells were planted in 6-wells plates at a density of 2.5×10^5 cells/mL and grown for 7 days, and then incubated with 25 µg/ml protein samples (digested by pepsin for 1 h) or buffer containing LPS (250 pg/ml final concentration). After 24 h of the incubation, cells were washed twice and harvested. RNA was isolated using the RNeasy Kit (Qiagen, USA) and transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (TransGen Biotech, Beijing, China). Real-time PCR was conducted on a 7900HT Real-Time PCR System using the Power SYBR Green PCR Master Mix (TransGen Biotech, Beijing, China). Primers (Table 1) were designed according to the data reported by Starkl (2011), and synthesized by Qingke Biotech Company (Beijing, China). Real-time PCR was conducted in triplicates. Δ CT values were calculated based on the expression of the house keeping gene β -actin. Mean values of three experiments are presented.

Extraction of nuclear and cytosolic protein fractions

Caco-2 cells were digested with trypsin, washed by PBS, and collected by centrifugation. Cell membrane protein and cytoplasmic protein extraction kit (Beyotime Biotechnology, Shanghai, China) was used to isolate the cytoplasmic and nuclear proteins. Experiments were performed at 4°C following the manufacturer's instructions, and the proteins were stored at -80°C until use.

Table 1. Primer Sequence of investigated genes.

Gene symbol	Full name	Orientation	Sequence 5'→3'
ccl 20	Chemokine (C-C motif) ligand 20	fwd rev	gcgaatcagaagcagcaagca tgtgtgaaagatgatagcattgatgtca
tnf	Tumour necrosis factor/TNFA	fwd rev	tgacaagcctgtagcccatgt acctgggagtagatgaggtaca
ikbkb	Nhibitor of kappa light polypeptide	fwd rev	agatccagatcatgagaaggctga gcagaccacagcagttctca
mmp10	Matrix metalloproteinase 10/	fwd rev	tcttttcagttaaagaacatggagact gaggaaataaattgggtcctgatgc
cxcl9	Chemokine (C-X-C motif) ligand 9	fwd rev	tgggagaacagggtcagccaa tgggtgaagtgtctcttatgtagt
nos 2a	Nitric oxide synthase 2, inducible/	fwd rev	ctacaacatcctggaggaagtgt gtcacattctgtcttggaacta
il-6	Interleukin 6	fwd rev	ccctgagaaggagacatgtaaca tcctattgaatccagattggaag
irf-1	Interferon regulatory factor 1	fwd rev	acctgaggacatcatgaagctcttg acagctaaagtctccatagacagag

Peptides identified by LC-MS/MS

Methods from Ayoub et al. (2015) were modified as follows: LC-MS/MS analysis were performed on an easy nLC 1000 system coupled with a Q-Exactive HF mass spectrometer (Thermo Fisher Scientific, Waltham, USA). After loaded to a C18 precolumn (3 mm, 0.10 × 20 mm), the peptides were separated by a C18 column (1.9 mm, 0.15 × 120 mm). Q-Exactive HF (Thermo Fisher Scientific, Waltham, USA) were subsequently used to identify the peptides.

Statistical analysis

The statistical analysis was performed using SPSS Software (v15.0, SPSS Inc., USA). Difference was considered statistically significant when *P*-values were less than 0.05. ELISA analysis was conducted using paired t-test (two tailed), gene expression results were analysed by one-way ANOVA test.

Results

The effect of digestion on the conformation and allergenicity of (heated) rAra h 1

According to our previous research, the molecular weight of recombinant Ara h 1 (monomer) is 110 kDa, which is bigger than the natural Ara h 1 (64.5 kDa). That is because of the addition of protein tags and restriction enzyme cutting site in the recombinant plasmid, which were then used to express recombinant Ara h 1 in *BL21 E.Coli* cells. The additional sequence in recombinant plasmid contains 388 amino acids with a total molecular weight of approximately 46.56 kDa, resulting in a higher molecular weight of the recombinant protein.

As seen in Figure 1(A), there are bands (110 kDa) appeared in the first two tracks but no bands observed in the other lanes, demonstrating that raw Ara h 1 cannot preserve its intact structure when the digestion process longer than 5 min. This result is consist with Rao et al.'s (2018) research, Ara h 1 would be degraded within 1 min when the digestion process starts.

According to the previous research, rAra h 1 is assumed to have a looser construction after treated with thermal process, which could be the reason why heated rAra h 1 were much easier to be digested by pepsin compared with raw rAra h 1. Our data demonstrated that whether heated or not, rAra h 1 cannot resist to the digestion by pepsin.

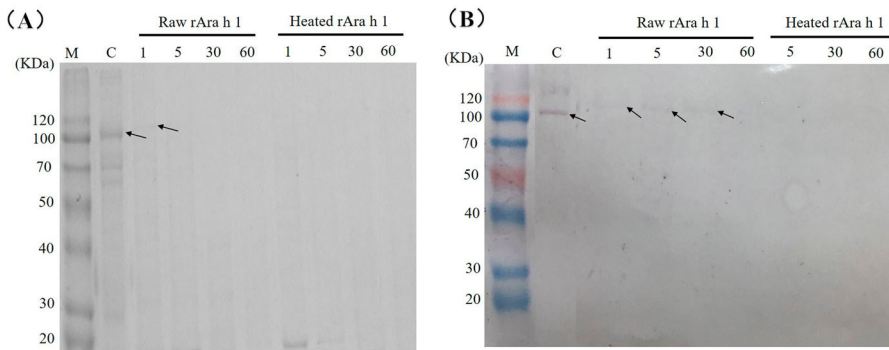


Figure 1. The effect of digestion on the conformation and allergenicity of (heated) rAra h 1. (A) Reducing SDS-PAGE analysis of digested (heated) rAra h 1. M: protein maker, C: raw rAra h 1 without digesting, 1–60 refer to samples digested for 1, 5, 30, and 60 min, respectively. (B) Detection of allergen contents and IgE binding efficiency by using Western-Blot. C: raw rAra h 1 without digesting, 1–60 refer to samples digested for 1, 5, 30, and 60 min, respectively.

The IgE binding efficiency of samples was evaluated by western-blot. For rAra h 1, bands could be observed in the first four tracks, indicating that the IgE binding capacity of the protein was still preserved after digested by pepsin for 30 min. However, the immunoreactivity was significantly decreased in the heated rAra h 1 samples for there is no bands could be found in the last three lanes of the Western-Blot pattern (Figure 1(B)). Destroyed epitopes during heat treatment could be a mechanism of reducing the allergenic nature of rAra h 1. It is worth noticing that proteins which was structurally destroyed by pepsin were proved to possess allergenicity, demonstrating that peptides formed in the digestion process are capable of causing severe allergic symptoms.

IL-8 secretion assay of Caco-2 cells treated by digested rAra h 1.

Lipopolysaccharide (LPS), a component of the outer gram negative bacterial cell wall, could induce inflammatory response and lead to the disturbance of the immune system function (Jing, Chunhua, & Shumin, 2015). Here we use LPS as the positive control to induce the secretion of IL-8 in Caco-2 cells.

As it shown in Figure 2, the content of IL-8 in cells treated with LPS (320.49 pg/mL) was 4.24 folds higher than which secreted by cells treated by cultural medium (61.73 pg/mL), demonstrating that the inflammation was significantly pronounced in the positive control. Besides, rAra h 1 and heated rAra h 1 did not have the ability to induce inflammation in Caco-2 cells, for the content of IL-8 in the two groups were both at a similar low level (approximately 70 pg/mL) when compared with the positive control.

However, proteins, whether heated or not, could significantly enhance the proinflammatory effect in human intestinal environment after digested by pepsin. Compared with rAra h 1, the IL-8 secretion was 5.15 folds higher in the cells treated with digested rAra h 1. Similarly, the content of IL-8 in cells treated with digested heated-rAra h 1 (434.57 pg/mL) was 5.67 folds of which in the heated rAra h 1 group. Compared with cells treated with digested rAra h 1, the content of IL-8 in the digested heated rAra h 1 was significantly higher than the positive group, indicating that heating before digesting, could enhance the proinflammatory capacity of rAra h 1 to a much higher extent.

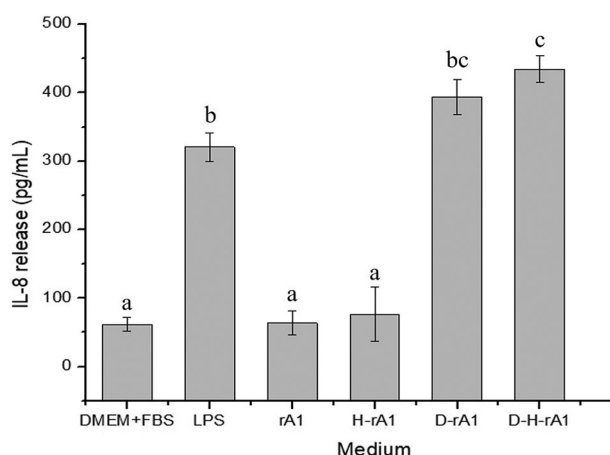


Figure 2. IL-8 secretion assay of Caco-2 cells treated with different protein samples. C: the content of IL-8 in the culture medium (DMEM) of Caco-2 cells, L: the content of IL-8 secreted by Caco-2 cells which treated with Lipopolysaccharide (the positive control), rA1: the content of IL-8 secreted by Caco-2 cells which treated with rAra h 1, H-rA1: the content of IL-8 secreted by Caco-2 cells which treated with heated rAra h 1, D-rA1: the content of IL-8 secreted by Caco-2 cells which treated with digested rAra h 1, D-H-rA1: the content of IL-8 secreted by Caco-2 cells which treated with digested-heated rAra h 1. Statistical analyses were performed using Students' test at 95% confidence.

The peptides of (Heated) rAra h 1 after digestion

Twenty typical peptides of the digested protein samples were presented in Table 2. After compared with the protein profile of Ara h 1, twelve linear epitopes were located. It turns out that digestion by pepsin cannot destroy all the lineal epitopes in Ara h 1. The pepsin cleavage is normally between leucine and phenylalanine, and that is why the peptides formed in the digestion process always started with L (leucine) or F (phenylalanine).

Table 2. The identification of peptides by using LC-MS.

	Sequence	Location in the native Ara h 1	Intensity
Peptides of digested rAra h 1	KDNVID QIEKQAKDL	547–561	3875600
	REG EQEW GTPGSHVR	152–166	3829800
	QSSYLQGF	309–316	3546000
	IVQIEAKPNTL	214–224	2997100
	LEENAGG EQEERGQRRW	337–353	2366800
	EDFFPASSRDQSS YLQGF SRNTL	299–321	1829700
	RDQSSYLQGF	307–316	1785000
	RNTLEAA FNAEF NEIRRVL	318–336	1401500
	AHPVAINASSELHLL	515–529	1109200
	ISMPVNT PGQFEDFF PASSRDQSSY	288–312	1009800
	EC DITNP INLRE	391–402	5447400
Peptides of digested heated-rAra h 1	KDLA FPGSGEQV	559–570	4323900
	HRIFLAGD KDNV	539–550	4208000
	IDQIEKQAKD	551–560	4178000
	NNFGKL FEVKPD	409–420	3506500
	NASSEL HLLGF GIN	521–534	3405800
	RRYTARL KEGDVF	498–510	3213900
	FIMPAAH PVAINASSEL HLLGF	510–531	2909500
	ISYILNRHDNQNL	271–283	1688600
	HRIVQIEAKPNTLVLPKHADADNIL	212–236	1628400

Note: The bold sequence is the linear epitopes existed in native Ara h 1.

Intestinal epithelial cells respond to rAra h 1 incubation with altered gene expression

According to previous studies (Starkl, 2011), thermal process could significantly enhance the binding efficiency between allergen and intestinal epithelial cells, and thus increase the immunogenic potential of the allergen (Chinthrajah, Hernandez, Boyd, Galli, & Nadeau, 2016). In order to verify the fact that if digestion could affect the sensitivity of rAra h 1, Caco-2 cells were incubated with rAra h 1, heated rAra h 1, digested rAra h 1, digested heated-rAra h 1 or buffer containing LPS. A set of representative genes involved in immune-signalling (NF- κ B, TLR, and JAK-STAT signalling (Figure 3) was investigated by real-time PCR.

As seen, incubation with different protein samples could stimulated the expression of ccl 20, thf, ikkbb (Figure 3(A)), which are the typical signalling components in NF- κ B. Apparently the digested samples were easier to cause the NF- κ B response when compared with the undigested ones, for the content of ccl 20 and tnf in S4 (digested rAra h 1 group) is significantly higher than which in S1 (LPS group). Besides, the secretion of ikkbb in S5 (digested heated-rAra h 1 group) is significantly higher than which in the control group, demonstrating that protein samples which treated by digestion (S4 group and S5 group, Figure 3(A)) could stimulate the NF- κ B pathway.

However, the investigation of genes related to JAK-STAT signalling, nitric oxide synthase (nos2a), was up-regulated by digested rAra h 1 (S4 group, Figure 3(B)). There is an up-regulation of the TLR signalling component il-6 after incubation with digested rAra h 1 (S4 group, Figure 3(C)). Compared with NF- κ B signalling, the alteration in gene expression was less pronounced. Our data indicate that Caco-2 human intestinal epithelial cells may directly and differentially respond to allergen contact at the genomic level, NF- κ B pathway might be stimulated by different forms of rAra h 1.

Digested (heat-treated) rAra h 1 activates the NF- κ B pathway in Caco-2 cells.

According to the results shown in Figure 4, stimulation with different forms of rAra h 1 could produce varying degree of response in NF- κ B pathway. Phosphor-IKK- α levels in

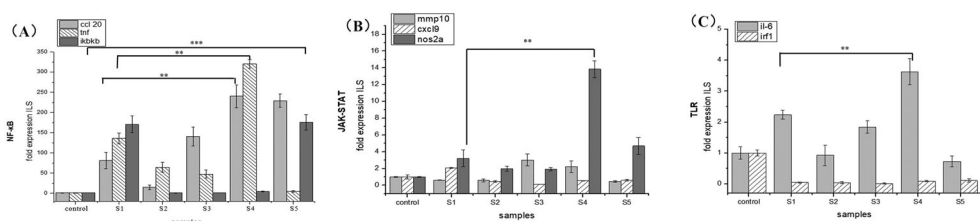


Figure 3. Intestinal epithelial cells respond to protein incubation with differential gene expression. The gene expression of representative signalling pathway components of (A) NF κ B -, (B) JAK-STAT-, and (C) TLR signalling were analysed by real-time PCR. In each picture, C represents for the control group; S1 represents for the positive control, which means the genes expression in Caco-2 cells treated with Lipopolysaccharide; S2 represents for the genes expression in Caco-2 cells treated with rAra h 1; S3 represents for the genes expression in Caco-2 cells treated with heated rAra h 1; S4 represents for the genes expression in Caco-2 cells treated with digested rAra h 1; S5 represents for the genes expression in Caco-2 cells treated with digested-heated rAra h 1. Values are presented as means $\pm$ SEM ($n = 3$). Analyses were performed using a one-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to untreated control.

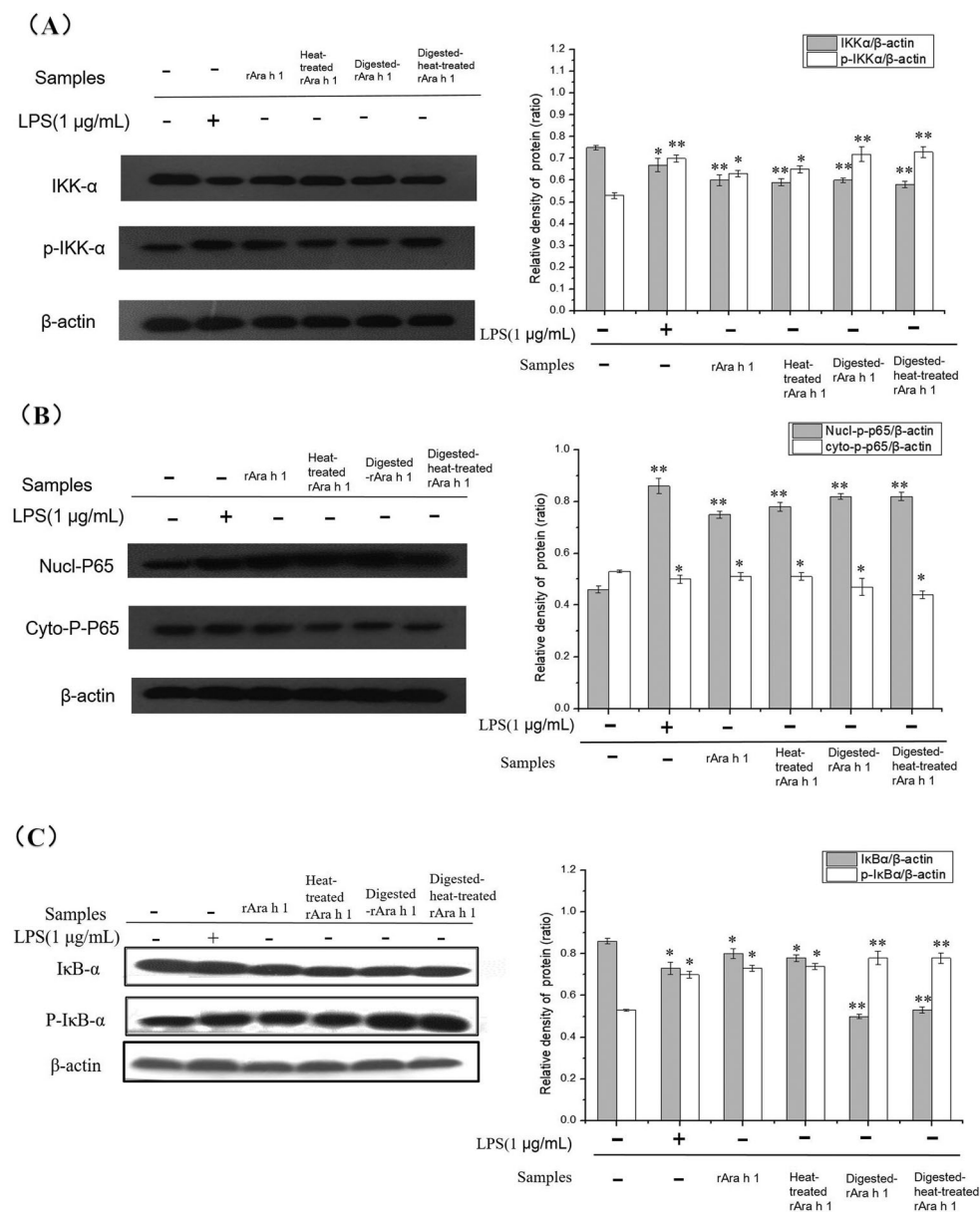


Figure 4. Digested (Heat-treated) rAra h 1 activates the NF-κB pathway in Caco-2 cells. Protein levels of IKKα (A), nucl-phospho-p65, cyto-phospho-p65 (B), and IκBα (C) were determined by Western blot analysis. The group in the first from left is the untreated group, which means the cells were not incubated with any protein samples or LPS. L represents for the positive control, which means the protein extracted from Caco-2 cells treated with Lipopolysaccharide; rAra h 1 represents for the protein extracted from in Caco-2 cells treated with rAra h 1; H-rAra h 1 represents for the protein extracted from Caco-2 cells treated with heated rAra h 1; D-rAra h 1 represents for the protein extracted from Caco-2 cells treated with digested rAra h 1; D-H-rA1 represents for the protein extracted from Caco-2 cells treated with digested-heated rAra h 1. Values are presented as means ± SEM ($n = 3$). Analyses were performed using a one-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the untreated group.

Caco-2 cells were increased more significantly when the cells were treated with digested samples, compared with which treated with rAra h 1 and heated rAra h 1 (Figure 4 (A)). Besides, in cells incubated with protein samples, nuclear levels of p65 were increased and cytoplasmic levels of p65 were reduced compared with the untreated group (Figure 4 (B)). Degradation of I κ B- α was observed in the cells incubated with protein samples. In the meantime, apparently the Phosphor-IKK- α levels were raised especially when the digested proteins were applied (Figure 4(C)). These results indicates that Caco-2 cells could be activated by digested protein samples via the NF- κ B pathway.

Discussion

According to our previous studies (Tian, Rao, Tao, et al., 2018), the immunoreactivity of Ara h 1 decreased significantly when the protein was heated for 20 min. Structural changes like reduced amount of α -helix, increased number of irregular coils, aggregation and degradation happened in the quaternary structure of allergens would contribute to the destruction of epitopes (Blanc et al., 2011; Rahaman, Vasiljevic, & Ramchandran, 2016; Vissers et al., 2011). Furthermore, there were low-molecular allergens identified in the cooking water, which demonstrate the possibility of migration of allergic peptides, from peanut kernels to the water (Rao et al., 2016). However, the digestibility, scavenger receptor recognition and IgE-binding capacity of heat-treated peanut allergens were poorly investigated. And the reason why digested Ara h 1 can still possess the ability to cause severe allergy have not be clarified yet. In our study, both native and heated rAra h 1 were used as subjects, and we explored the effect of digestion of rAra h 1 on the epimucosal response.

In comparison with Ara h 2/6 and Ara h 3, Ara h 1 were reported to be hydrolysed by pepsin in a more rapid way (Koppelman, Hefle, Taylor, & de Jong, 2010). Lower the concentration of pepsin could lead to a gradual degradation of Ara h 1. Peptides between 20 and 50 kDa would appear in the SDS-PAGE pattern when the ratio between pepsin and substrate is 0.1 U/ mg, peptides between 6–10 kDa are stable for 2–8 min in the digestion when the ratio between pepsin and substrate is 0.03 U/mg (Koppelman et al., 2010). To mimic the actual gastric environment, we set the ratio between pepsin and substrate to be 400 U/mg, which could be the reason of disappearance of small fragment of Ara h 1 in the SDS-PAGE pattern (Figure 1(A)). Besides, 8% acrylamide separating gel were used to capture the protein changes and peptides with a molecular lower than 15 kDa were hard to be observed in this gel.

IL-8 production assay were performed to evaluate the proinflammatory ability of different isoforms of rAra h 1. Results in Figure 2 confirmed that after digested by pepsin, rAra h 1 could trigger a local inflammation in gut mucosa what may be related to the high allergic potential of this molecule (Teodorowicz et al., 2013). It is worth noticing that peptides of digested rAra h 1 are different from that of heated one, which may because of the constructional alterations like degradation and aggregation happened during thermal processing (Blanc et al., 2011; Tian, Rao, Tao, et al., 2018).

Ara h 1 after digestion still holds linear epitopes (tab.2), which contribute to the allergic capacity of digested peptides. It has been estimated that different allergic potential were preserved by different kinds of epitopes (Ramesh et al., 2016). According to our previous research (unpublished data), epitopes located in the 393–402 of Ara h 1 could be

recognized by 60% of peanut allergic patients in China, while the reactivity of the epitopes located in the 498–508 of Ara h 1 was estimated to be 90%. As seen in tab 2, peptides were consist of different kinds of epitopes, which contribute to the proinflammatory potential and immunoreactivity of digested products. On the one hand, the stimulatory properties existed in the two samples hydrolysed by pepsin, illustrating that the fragments responsible for this effect were formed and protected in the digestion. On the other hand, it could be presumed that the small fragment formed in the digestion could adhere to the epithelial cells more easily, absorbed by the cells, and therefore start the cytokine release process. That may be the reason why digested peptides could stimulate the IL-8 release to a similar extent.

Compared with digested samples, the undigested proteins, including heated ones and native ones, were more likely to inhibit the secretion of IL-8. It has been speculated that bigger structures in allergens plays a crucial role in IL-8 inhibition (Hosoki, Itazawa, Boldogh, & Sur, 2016; Teodorowicz et al., 2013). According to the previous studies (Teodorowicz et al., 2013), allergens are normally possess cytotoxic properties against intestinal epithelia cells. From this point of view, the increase of IL-8 secretion by Caco-2 cells may be a cellular response to compensate for the anti-proliferative effects of this protein.

Previous studies have provide evidence that interaction of food allergens with the epithelial are normally the first step in ingestion, and could be crucial for the subsequent immune response. Different immune stimulatory factors of the gastro-intestinal tract like commensal bacteria were existed in the intestinal mucosa, in which cells could express a broad panel of surface receptor which participate in nutrient uptake and immune sensing (Starkl, 2011). Here we investigate the impact of digested allergens on the activation state of Caco-2 cells. As seen in Figure 3, gene expression analysis revealed that, after digested by pepsin, fractions of rAra h 1 (heated or not) could cause the inflammation in Caco-2 cells induced by the transcriptional up-regulation of genes involved in the NF- κ B pathway. Compared with the stimulation of LPS, Caco-2 cells exhibited higher expression of NF- κ B signalling related *ccl 20* and *thf*. Previous studies have shown that transcription factor NF- κ B which plays a critical role in inflammation and cancer development may be activated via the receptor for advanced glycation end products (RAGE)(Riehl, Németh, Angel, & Hess, 2009). It is presumed that advanced MRPs (Maillard reaction products), formed due to the glycation of Ara h 1, may exert their effect on Caco-2 cells through the RAGE which in indeed expressed by Caco-2 cells (Teodorowicz et al., 2013). According to the previous research (Rao et al., 2018), fragment formed in the ingestion process of crude peanut protein is speculated to be peptides with a low molecular weight of about 10–15 KDa. It is reasonable to presume that peptides could activate the NF- κ B signalling via other structures which can bind the surface of Caco-2 cells. However, this speculation requires further studies.

The activation of NF- κ B pathway could be induced by a wide variety of agents including protein, peptides, viruses, bacteria, inflammatory stimuli, cytokines, free radicals, carcinogens, tumour promoters and endotoxins (Piette, 2015). Peptides formed in the digestion still preserve epitopes, and possess abilities to cause inflammatory in the epithelial cells (Figure 2). It is reasonable to predict that those peptides could react with immune cells like basophilic granulocyte, cytokines like histamine and β -hexosaminidase would be released and cause serious allergic symptoms in human body. In our study,

peptides containing epitopes are the stimuli for the NF- κ B signalling. Compared with intact protein, small fragments formed in the digestion could be absorbed by epithelia cells easily and therefore activate the process. Once activated, the NF- κ B signalling would lead to the complete degradation of I κ B, and the translocation of NF- κ B to the nucleus (Serasanambati & Chilakapati, 2016). Approximately 400 genes, which involved in immune, growth and inflammation would then be regulated and expressed by NF- κ B signalling. It can be concluded that, for recombinant Ara h 1, peptides formed in the digestion were modified by pepsin and preserved the proinflammatory and immunoreactivity.

Conclusion

Digestion could induce serious degradation and reduced IgE binding capacity in rAra h 1 when compared with heat treatment. During the first five minutes of digestion, both native and heated rAra h 1 were degraded. However, peptides formed in the digestion were analysed to contain linear epitopes, and possess the ability to activate Caco-2 cells to produce proinflammatory cytokine IL-8 via the NF- κ B pathway. Compared with digested rAra h 1, the proinflammatory effect were less pronounced in the samples treated with heat, demonstrating that digestion, instead of thermal process, could modify the proteins and peptides and which could then play a significant role in the activation of Caco-2 cells.

Acknowledgements

This work was supported by the National Natural Science Foundation of China under Grant (number 31872904)

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the National Natural Science Foundation of China [grant number 31872904].

References

- Ayoub, D., Bertaccini, D., Diemer, H., Wagner-Rousset, E., Colas, O., Cianféroni, S., ... Schaeffer-Reiss, C. (2015). Characterization of the N-terminal heterogeneities of monoclonal antibodies using in-gel charge derivatization of α -Amines and LC-MS/MS. *Analytical Chemistry*, 87(7), 3784–3790. doi:10.1021/ac504427k
- Blanc, F., Vissers, Y. M., Adel-Patient, K., Rigby, N. M., Mackie, A. R., Gunning, A. P., ... Mills, E. N. C. (2011). Boiling peanut Ara h 1 results in the formation of aggregates with reduced allergenicity. *Molecular Nutrition & Food Research*, 55(12), 1887–1894. doi:10.1002/mnfr.201100251
- Boyce, J. A., Assa'ad, A., Burks, A. W., Jones, S. M., Sampson, H. A., Wood, R. A., ... Schwaninger, J. M. (2010). Guidelines for the diagnosis and management of food allergy in the United States: summary of the NIAID-sponsored expert panel report. *Journal of Allergy and Clinical Immunology*, 126(6), 1105–1118. doi:10.1016/j.jaci.2010.10.008

- Bunyavanich, S., Rifas-Shiman, S. L., Platts-Mills, T. A. E., Workman, L., Sordillo, J. E., Gillman, M. W., ... Litonjua, A. A. (2014). Peanut allergy prevalence among school-age children in a US cohort not selected for any disease. *Journal of Allergy and Clinical Immunology*, 134(3), 753–755. doi:10.1016/j.jaci.2014.05.050
- Chen, Q., Qi, C., Peng, G., Liu, Y., Zhang, X., & Meng, Z. (2018). Immune-enhancing effects of a polysaccharide PRG1-1 from *Russula griseocarnosa* on RAW264.7 macrophage cells via the MAPK and NF- κ B signalling pathways. *Food and Agricultural Immunology*, 29(1), 833–844. doi:10.1080/09540105.2018.1461198
- Chinthrajah, R. S., Hernandez, J. D., Boyd, S. D., Galli, S. J., & Nadeau, K. C. (2016). Molecular and cellular mechanisms of food allergy and food tolerance. *Journal of Allergy and Clinical Immunology*, 137(4), 984–997. doi:10.1016/j.jaci.2016.02.004
- Chruszcz, M., Maleki, S. J., Majorek, K. A., Demas, M., Bublin, M., Solberg, R., ... Minor, W. (2011). Structural and immunologic characterization of Ara h 1, a major peanut allergen. *Journal of Biological Chemistry*, 286(45), 39318–39327. doi:10.1074/jbc.M111.270132
- Du Toit, G., Roberts, G., Sayre, P. H., Bahnson, H. T., Radulovic, S., Santos, A. F., ... Lack, G. (2015). Randomized trial of peanut consumption in infants at risk for peanut allergy. *New England Journal of Medicine*, 372(9), 803–813. doi:10.1056/NEJMoa1414850
- Guillon, B., Bernard, H., Drumare, M.-F., Hazebrouck, S., & Adel-Patient, K. (2016). Heat processing of peanut seed enhances the sensitization potential of the major peanut allergen Ara h 6. *Molecular Nutrition & Food Research*, 60(12), 2722–2735. doi:10.1002/mnfr.201500923
- Hosoki, K., Itazawa, T., Boldogh, I., & Sur, S. (2016). Neutrophil recruitment by allergens contribute to allergic sensitization and allergic inflammation. *Current Opinion in Allergy and Clinical Immunology*, 16(1), 45–50. doi:10.1097/aci.0000000000000231
- Jing, W., Chunhua, M., & Shumin, W. (2015). Effects of acteoside on lipopolysaccharide-induced inflammation in acute lung injury via regulation of NF- κ B pathway in vivo and in vitro. *Toxicology and Applied Pharmacology*, 285(2), 128–135. doi:10.1016/j.taap.2015.04.004
- Koppelman, S. J., Hefle, S. L., Taylor, S. L., & de Jong, G. A. H. (2010). Digestion of peanut allergens Ara h 1. Ara h 2, Ara h 3, and Ara h 6: A comparative in vitro study and partial characterization of digestion-resistant peptides. *Molecular Nutrition & Food Research*, 54(12), 1711–1721. doi:10.1002/mnfr.201000011
- Lai, X., Pei, Q., Song, X., Zhou, X., Yin, Z., Jia, R., ... Jing, B. (2016). The enhancement of immune function and activation of NF- κ B by resveratrol-treatment in immunosuppressive mice. *International Immunopharmacology*, 33, 42–47. doi:10.1016/j.intimp.2016.01.028
- Lieberman, J., Sublett, J., Ali, Y., Haselkorn, T., Damle, V., Chidambaram, A., ... Mahr, T. (2018). Increased incidence and prevalence of peanut allergy in children and adolescents in the United States. *Annals of Allergy, Asthma & Immunology*, 121(5), S13. doi:10.1016/j.anai.2018.09.039
- Liu, T., Zhang, L., Joo, D., & Sun, S.-C. (2017). NF- κ B signaling in inflammation. *Signal Transduction and Targeted Therapy*, 2, 17023. doi:10.1038/sigtrans.2017.23
- Maleki, S. J., Kopper, R. A., Shin, D. S., Park, C. W., Compadre, C. M., Sampson, H., ... Bannon, G. A. (2000). Structure of the major peanut allergen Ara h 1 May protect IgE-binding epitopes from degradation. *The Journal of Immunology*, 164(11), 5844–5849. doi:10.4049/jimmunol.164.11.5844
- Mills, E. N. C., Mackie, A. R., Burney, P., Beyer, K., Frewer, L., Madsen, C., ... Van Ree, R. (2007). The prevalence, cost and basis of food allergy across Europe. *Allergy*, 62(7), 717–722. doi:10.1111/j.1398-9995.2007.01425.x
- Pan, G., Xie, Z., Huang, S., Tai, Y., Cai, Q., Jiang, W., ... Yuan, Y. (2017). Immune-enhancing effects of polysaccharides extracted from *Lilium lancifolium* Thunb. *International Immunopharmacology*, 52, 119–126. doi:10.1016/j.intimp.2017.08.030
- Peterson, L. W., & Artis, D. (2014). Intestinal epithelial cells: Regulators of barrier function and immune homeostasis. *Nature Reviews Immunology*, 14(3), 141–153. doi:10.1038/nri3608
- Piette, J. (2015). Signalling pathway activation by photodynamic therapy: NF- κ B at the crossroad between oncology and immunology. *Photochemical & Photobiological Sciences*, 14(8), 1510–1517. doi:10.1039/c4pp00465e

- Prodic, I., Stanic-Vucinic, D., Apostolovic, D., Mihailovic, J., Radibratovic, M., Radosavljevic, J., ... Cirkovic Velickovic, T. (2018). Influence of peanut matrix on stability of allergens in gastric-simulated digesta: 2S albumins are main contributors to the IgE reactivity of short digestion-resistant peptides. *Clinical & Experimental Allergy*, 48(6), 731–740. doi:10.1111/cea.13113
- Rahaman, T., Vasiljevic, T., & Ramchandran, L. (2016). Effect of processing on conformational changes of food proteins related to allergenicity. *Trends in Food Science & Technology*, 49, 24–34. doi:10.1016/j.tifs.2016.01.001
- Ramesh, M., Yuenyongviwat, A., Konstantinou, G. N., Lieberman, J., Pascal, M., Masilamani, M., & Sampson, H. A. (2016). Peanut T-cell epitope discovery: Ara h 1. *Journal of Allergy and Clinical Immunology*, 137(6), 1764–1771. e1764. doi:10.1016/j.jaci.2015.12.1327
- Rao, H., Tian, Y., Fu, W., & Xue, W. (2018). In vitro digestibility and immunoreactivity of thermally processed peanut. *Food and Agricultural Immunology*, 29(1), 989–1001. doi:10.1080/09540105.2018.1499710
- Rao, H., Tian, Y., Tao, S., Tang, J., Li, X., & Xue, W.-T. (2016). Key factors affecting the immunoreactivity of roasted and boiled peanuts: Temperature and water. *LWT - Food Science and Technology*, 72, 492–500. doi:10.1016/j.lwt.2016.05.014
- Riehl, A., Németh, J., Angel, P., & Hess, J. (2009). The receptor RAGE: Bridging inflammation and cancer. *Cell Communication and Signaling*, 7(1), 12. doi:10.1186/1478-811x-7-12
- Rooks, M. G., & Garrett, W. S. (2016). Gut microbiota, metabolites and host immunity. *Nature Reviews Immunology*, 16(6), 341–352. doi:10.1038/nri.2016.42
- Serasanambati, M., & Chilakapati, S. R. (2016). Function of nuclear factor kappa B (NF-κB) in human diseases – a review. *South Indian Journal of Biological Sciences*, 2(4), 368. doi:10.22205/sijbs/2016/v2/i4/103443
- Shin, H. S., Satsu, H., Bae, M.-J., Zhao, Z., Ogiwara, H., Totsuka, M., & Shimizu, M. (2015). Anti-inflammatory effect of chlorogenic acid on the IL-8 production in Caco-2 cells and the dextran sulphate sodium-induced colitis symptoms in C57BL/6 mice. *Food Chemistry*, 168, 167–175. doi:10.1016/j.foodchem.2014.06.100
- Sicherer, S. H., & Sampson, H. A. (2018). Food allergy: A review and update on epidemiology, pathogenesis, diagnosis, prevention, and management. *Journal of Allergy and Clinical Immunology*, 141(1), 41–58. doi:10.1016/j.jaci.2017.11.003
- Starkl, P. (2011). Heating affects structure, enterocyte adsorption and signalling, As well as immunogenicity of the peanut allergen Ara h 2. *The Open Allergy Journal*, 4(1), 24–34. doi:10.2174/1874838401104010024
- Teodorowicz, M., Fiedorowicz, E., Kostyra, H., Wichers, H., & Kostyra, E. (2013). Effect of maillard reaction on biochemical properties of peanut 7S globulin (Ara h 1) and its interaction with a human colon cancer cell line (Caco-2). *European Journal of Nutrition*, 52(8), 1927–1938.
- Tian, Y., Rao, H., Tao, S., & Xue, W.-T. (2018). Effect of boiling on the structure and immunoreactivity of recombinant peanut protein Ara h 1. *Food and Agricultural Immunology*, 29(1), 845–858. doi:10.1080/09540105.2018.1461812
- Tian, Y., Rao, H., Zhang, K., Tao, S., & Xue, W.-T. (2018). Effects of different thermal processing methods on the structure and allergenicity of peanut allergen Ara h 1. *Food Science & Nutrition*, 6(6), 1706–1714. doi:10.1002/fsn3.742
- Vissers, Y. M., Iwan, M., Adel-Patient, K., Stahl Skov, P., Rigby, N. M., Johnson, P. E., ... Wichers, H. J. (2011). Effect of roasting on the allergenicity of major peanut allergens Ara h 1 and Ara h 2/6: The necessity of degranulation assays. *Clinical & Experimental Allergy*, 41(11), 1631–1642. doi:10.1111/j.1365-2222.2011.03830.x