

Lipid emulsion enhances fish allergen parvalbumin's resistance to *in vitro* digestion and IgG/IgE binding capacity

Chen Luo^a, Yuman Guo^a, Zhenxing Li^{a,*}, Ishfaq Ahmed^a, Siddanakoppalu N. Pramod^b, Xiang Gao^c, Liangtao Lv^a, Hong Lin^a

^a College of Food Science and Engineering, Ocean University of China, No. 5, Yushan Road, Qingdao, Shandong Province 266003, PR China

^b Laboratory of Immunomodulation and Inflammation Biology, Department of Studies and Research in Biochemistry, Sahyadri Science College, Kuvempu University, Shimoga 577203, Karnataka, India

^c The Affiliated Hospital of Medical College Qingdao University, Qingdao, Shandong Province 266071, PR China

ARTICLE INFO

Keywords:

Parvalbumin
Simulated digestion
Lipid emulsion
Binding ability

ABSTRACT

This study was performed to determine Parvalbumin (PV), a well-known fish allergenic protein, digestion kinetics and immunoreactivity of digestion products with Immunoglobulin G/Immunoglobulin E recognition to understand its allergic potential with or without lipid emulsion process. PV was subjected to simulated gastrointestinal digestion in emulsified condition. Digestion kinetics of the protein was analysed by electrophoresis, IgG/IgE binding ability by immunoblotting and indirect ELISA. Lipid emulsion significantly ($p < 0.01$) reduced the degree of PV hydrolysis by 52.10% for gastric digestion. Immune fragments of gastric digestion were detectable for 90–120 min longer in emulsified condition showing resistance. Consequently, lipid emulsion decreased the digestive ability of PV in stomach, increasing resistance to gastrointestinal digestion by pepsin proteases. It also altered IgG/IgE binding ability of digestion products, thereby indicating that PV with lipid emulsion was resistant to digestion and possessed increased IgE binding ability resulting in higher risk of allergy among sensitized individuals.

1. Introduction

Food allergy refers to an abnormal or exaggerated reaction of the immune system caused by exposure of relatively harmless specific food proteins or dietary antigens. Closely related dietary proteins induce Immunoglobulin E (IgE) antibodies in the blood by stimulating specific cells of the immune system (Ahmed et al., 2018). Food allergy is a major public health problem, which affects around 5–8% of young children and 2–4% of adults. However, it depends on genetic factors, exposure to allergenic products early in life and also the dietary habits (Clark, Espinola, Rudders, Banerji, & Camargo, 2011; Sicherer & Sampson, 2010; Wai et al., 2014).

In human diet, fish is a valuable source of proteins, and is also known as a common cause inducing severe IgE-mediated immune reactions in sensitive populations. Patients become sensitized by allergen exposure via the gastrointestinal tract (GIT) during ingestion, which is the major route of sensitization. Fish allergens are supposed to sensitize through the GIT, although the specific mechanism by which food

allergens cause allergic reactions in human body is not yet clear. The resistance of a dietary protein to gastrointestinal digestion, especially pepsinolysis and proteolysis, is often regarded as one of the bases for determining the protein allergenicity (Lv, Lin, Li, Ahmed, & Chen, 2017; Moran, Tetteh, Goodman, & Underwood, 2014). Hence, it can be speculated that for a protein to sensitize our body, it must have a stable structure and characteristics that protect itself from the digestion by the GIT and impose resistance to low pH environment and hydrolysis by proteolytic enzymes. It is reported that, a sufficient dose of the allergen with an intact structure and immunologically active form is taken up by the intestinal epithelial cells, which facilitates allergic reactions (Bøgh & Madsen, 2016; Taylor, 2002; Taylor & Hefle, 2001). Therefore, resistance to digestion and retention time in the GIT for a protein are considerable factors for prediction of possible allergenicity as the digestive stability of proteins in GIT may pose risk to trigger allergic potential in sensitive subjects (Consolini et al., 2012).

To predict the allergenicity of novel food proteins, several *in vitro* simulated gastrointestinal digestion models have been developed over

Abbreviations: PV, parvalbumin; GIT, gastrointestinal tract; SGF, simulated gastric fluids; ESGF, emulsified simulated gastric digestion; SIF, simulated intestinal fluids; ESIF, emulsified simulated intestinal fluids

* Corresponding author.

E-mail address: lizhenxing@ouc.edu.cn (Z. Li).

<https://doi.org/10.1016/j.foodchem.2019.125333>

Received 18 February 2019; Received in revised form 17 July 2019; Accepted 6 August 2019

Available online 07 August 2019

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the past decades which can assess the stability and resistibility of potential allergens to GIT digestion. Food and Agriculture Organization of the United Nations (FAO), the World Health Organization (WHO) and Codex Alimentarius Commission (CAC) use *in vitro* simulated digestion as a means of allergen risk assessment. In 2001, FAO/WHO used the stability of *in vitro* digestion as an indicator of protein sensitization (Moreno, 2007). In 2003, CAC confirmed the stability to pepsin proteolysis as a key parameter for sensitization assessment of proteins (Ladics, 2008).

However, any alteration in factors related to food processing and treatment methods involved with protein prior to digestion may result in changes in protein hydrolysis pattern and efficiency by *in vitro* model, which in turn alters allergen potential and sensitivity (Foegeding & Davis, 2011). In this respect, interactions between allergens with other food components, particularly lipids during processing methods, have been largely neglected in the assessment of allergenicity (Breiteneder & Mills, 2005; Jiménez-Saiz, Ruiz-Henestrosa, López-Fandiño, & Molina, 2012). In the course of food processing and ingesting, the introduced lipids may interact with proteins to form emulsions and other structures in GIT given the mixing of the chyme in stomach and intestine. Proteins are adsorbed to the oil/water interface of the emulsion due to its amphiphilic nature, reducing surface tension and increasing stability of the emulsion (Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009). Above conditions may lead to changes to the conformation and proteolytic efficiency. Lipid can interact with proteins in a variety of ways, as proteins might bind to lipids by non-covalent bonding through hydrophobic and electrostatic interactions (Burnett et al., 2002). Schwager et al. (2017) reported that compared with defatted peanuts, non-defatted peanuts could enhance the immune response of heated peanut allergy in patients. Consolini et al. (2012) found that emulsion brought a protection to epitope of wheat α -amylase inhibitors. Again, high-fat food is reported to increase gastric retention time as compared with low-fat foods, which can have an impact on uptake of allergens and induce subsequent reactions in allergic patients (Mackie et al., 2012). Therefore, it is necessary to determine the extent to which lipid emulsion can affect digestive patterns and potential IgE binding ability of allergens during the digestive process, simultaneously the effect of emulsification must be considered when developing models for potential allergic assessments.

Fish and its products are one of the eight major allergenic food proposed by FAO of the United Nations (Leung et al., 2014), and parvalbumin (PV), an EF-hand (a helix-loop-helix structural domain or motif) calcium-binding protein with a molecular mass of 10–12 kDa is the major fish allergen (Kobayashi et al., 2016). Since PV comprised many different isoforms (Sharp & Lopata, 2014), PV purified from turbot was investigated as a prominent member.

The purpose of this study was to determine the changes in the digestion pattern and potential allergenicity properties of PV with or without emulsified conditions in the digestion process. The effects on digestion mode and efficiency were characterized by digestive stability and Immunoglobulin G (IgG)/IgE reactivity by immunoassays, which provide an insight into allergenic potential of the digestion products from intact and emulsified PV and address the possible sensitivity in susceptible populations.

2. Materials and methods

2.1. Materials and chemicals

2.1.1. Chemicals and reagents

Rabbit polyclonal antibody against PV was purchased from BGI Co. (Shenzhen, China). Pepsin (from porcine gastric mucosa), trypsin (from porcine pancreas), L- α -Phosphatidylcholine (from egg yolk) and 3,3',5,5'-tetramethylbenzidine (TMB) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). While, horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG, α -chymotrypsin (from bovine

pancreas), bovine serum albumin (BSA) and bile salt (Bovine) were obtained from Solarbio Co. (Beijing, China). Western blot chemiluminescent substrate and Inject Alum Adjuvant were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). HRP-labelled goat anti-mouse IgE was purchased from Southern Biotech Co. (Birmingham, AL, USA). N-hexadecane was obtained from Macklin Co. (Shanghai, China). All other reagents used were pure and of analytical grade unless otherwise stated.

2.1.2. Purification of parvalbumin from turbot

Turbot (*Scophthalmus maximus*) was purchased from the local fish market of Qingdao and the PV was purified in the Laboratory of Food Safety, Ocean University of China, as described earlier (Ma, Pavase, Li, & Lin, 2017). Briefly, the flesh of turbot was isolated with the extraction buffer (Tris 0.1 mol/L, Glycine 0.5 mmol/L, DTT 0.1 mmol/L) and the supernatant was dialyzed overnight and then boiled in the water bath for 15 min. PV was obtained by using the salt fractionation (60% and 100%, w: v) with ammonium sulphate.

2.1.3. Generation of allergic anti-PV IgE mouse serum

Mouse anti-PV IgE serum was generated in the same laboratory. Specific pathogen-free (SPF) female BALB/c mice, 6–8 weeks old, were purchased from Daren Fortune Animal Technology Co. (Qingdao, China). Mice were dosed intraperitoneally eight times on day 14, 21, 28, 36, 44, 49, 54, 58 with the mixture of 100 μ L PV solution (2 mg/mL) and 100 μ L Inject Alum Adjuvant (that is, 200 μ g PV/mouse). Blood was drawn on day 59. Blood samples taken from the orbital plexus were centrifuged at 6000 rpm/min for 10 min at 4 °C to obtain serum. Individual serum samples were stored at –80 °C until analysis. All experiments were performed following guidelines and experimental protocols in accordance with the Ethical Committee of Experimental Animal Care at Ocean University of China (No: SPXK20150001).

2.2. Preparation of simulated gastric and intestinal fluid for *in vitro* digestion

2.2.1. Preparation of simulated gastric fluid (SGF)/emulsified simulated gastric fluid (ESGF) and simulated intestinal fluid (SIF)/ emulsified simulated intestinal fluid (ESIF)

In vitro simulated gastric and intestinal digestion models have been previously described by Wu et al. (2015) and van Aken (2010) and were slightly modified based on *in vitro* digestion data as reported by Minekus et al. (2014).

SGF contained 35 mM NaCl, 6.64 mM phosphatidylcholine. ESGF contained 0.61 M n-hexadecane additionally compared with SGF, and was ultrasonicated (KQ5200B, Shumei Co. Ltd, Kunshan, China) for 1 min and oscillated (Genius 3, IKA Co., German) for 30 s (s) before the digestion, to form homogeneous and stable oil-in-water emulsions. SGF and ESGF were stored at 4 °C prior to *in vitro* digestion for a maximum of 4 h (h).

The final composition of SIF was 16 mM of bile salt, 50 mM of KH_2PO_4 . ESIF contained 0.61 M n-hexadecane additionally compared with SIF, and was ultrasonicated (KQ5200B, Shumei Co. Ltd, Kunshan, China) for 1 min and oscillated (Genius 3, IKA Co., German) for 30 s before the digestion, to form homogeneous and stable oil-in-water emulsions. SIF and ESIF were stored at 4 °C prior to *in vitro* digestion for a maximum of 4 h.

2.2.2. Determination of pepsin and trypsin activity in SGF/ESGF and SIF/ESIF

The activity of pepsin, trypsin and α -chymotrypsin was determined by the method of Lowry, Rosebrough, Farr, and Randall (1951) with slight modification. The enzyme activity is expressed in U, which is the amount of enzyme needed to catalyse the conversion of one micromole of substrate per minute (min) under the specified conditions of the assay method.

2.3. Gastric digestion of PV using pepsin in SGF/ESGF

PV was subjected to gastric digestion in SGF and ESGF at pH 1.2. PV was dissolved to 0.80 mg/mL in SGF/ESGF. 250 μ L PV solution was mixed with 50 μ L of pepsin containing SGF or ESGF at concentration of 2.8 μ g/mL. So, the ratio of enzyme to substrate is 0.0007:1 w:w (1.20 U/mg). The digestion was performed at 37 °C in an incubator with moderate agitation, and 25 μ L aliquots were withdrawn from the digestion mixture at 0, 1, 5, 15, 30, 60, 90 and 180 min for further analysis. The simulated gastric digestion was stopped by raising the pH to 7.5 using 10 μ L 0.2 M Na₂CO₃ solution and stored for analysis. All the experiments were repeated three times and no obvious differences were identified.

2.4. Intestinal digestion of PV using trypsin and chymotrypsin with SIF/ESIF

PV was dissolved to 0.80 mg/mL in SIF. 250 μ L PV solution was mixed with 50 μ L of trypsin and α -chymotrypsin containing SIF or ESIF at concentration of 106.7 μ g/mL and 33.4 μ g/mL respectively. So, the ratio of trypsin to protein is 0.0265:1 w:w (40 U/mg), the ratio of α -chymotrypsin is 0.0017:1 w:w (10 U/mg). The digestion was performed at 37 °C in an incubator with moderate agitation, and 25 μ L aliquots were withdrawn from the digestion mixture at 0, 1, 5, 15, 30, 60, 90, 150, and 180 min for further analysis. The SIF digestion was stopped by heating at 95 °C water bath for 5 min. All the experiments were repeated three times and no obvious differences were identified.

2.5. Digestive kinetics analysis by Tris-Tricine SDS-PAGE

Digestion products of PV at different times were analysed via Tris-Tricine-SDS-PAGE according to the method depicted by Yuan et al. (2018). The digested samples and bromophenol blue buffer (25% glycerol, 2% SDS, 14.4 mM β -mercaptoethanol, and 0.1% bromophenol blue in 1 M Tris-HCl, pH 6.8) were mixed at 3:1 ratio (v:v), respectively. After heating (100 °C for 7 min), the mixtures were loaded into each well of the Tris-Tricine-SDS-PAGE gel (20% resolving gel, 10% interlayer gel, 4% stacking gel). Similarly, the protein markers having 3.3–31 kDa were loaded in the electrophoresis gel in order to determine the molecular weight (Mw) of the digestion product of PV. Electrophoresis was carried out at 30 V for 1 h and 100 V for 6 h. After resolving the proteins, the gels were stained with 0.125% Coomassie brilliant blue G-250 (Bio-Rad, Richmond, CA, USA) and de-stained with decolouring solution (10% methanol, 10% acetic acid) to get the clear solution. Images were captured and analysed with gel imaging and analysis software, HP Scanjet G4050 (China Hewlett-Packard Co, Beijing, China).

2.6. Enzymatic efficiency and prediction of degree of hydrolysis to PV

Hydrolysis efficiencies of pepsin, trypsin and α -chymotrypsin are represented by the change of the degree of hydrolysis. The grey value (Band intensity) of PV (or single subunit) in simulated digestion for t min was expressed as A_t. The degree of hydrolysis (%) of the PV (or single subunit) at t min was defined as the ratio of the grey value of the sample at t min A_t to the grey value of the sample at 0 min A₀. Grey value of bands on SDS-PAGE gel is measured by software ImageJ (Version 1.51J8; Bethesda, MD, USA).

2.7. IgG/IgE binding capacity of raw, emulsified and digested PV analysis

2.7.1. Western blotting for IgG/IgE binding to PV

Western blotting was conducted to determine the IgG/IgE binding ability of the samples, according to the method described by Ahmed et al. (2018). Protein samples were separated with Tris-Tricine SDS-PAGE and electrically transferred onto polyvinylidene fluoride (PVDF) membrane by iBlot® 2 Gel Transfer Device (Life Technologies Co.,

Israel) for 7 min at room temperature, and the transfer efficiency was determined by pre-stained protein marker. The membrane was washed with iBind solution kit and it was incubated in iBind™ Western Device (Life Technologies Co., Israel) with rabbit antibodies (for IgG detection, diluted 1:1000) or mouse serum (for IgE detection, diluted 1:5) as the primary antibody. Later on, incubated with goat anti-rabbit IgG antibody (diluted 1:1000) or anti-mouse IgE antibody-HRP (diluted 1:1000) as the secondary antibody for 5 h at room temperature in total. After being washed with deionized water, the results were visualized with western blot chemiluminescent substrate. PVDF membranes were exposed for 10 s (for IgG-immunoblot) or 10 min (for IgE-immunoblot). Images were captured by Tanon 5200 Multi Chemiluminescent Imaging System (Tanon Science & Technology Co., Shanghai, China).

2.7.1.1. Indirect ELISA for IgG binding to PV. The IgG binding ability of original PV and digestion products of PV were further analyzed by indirect enzyme-linked immunosorbent assay (ELISA). Intact and digested PV samples were diluted to 30 μ g/mL in 50 mM sodium carbonate buffer (pH 9.6). The ELISA plate was coated with 100 μ L of diluted samples per well and incubated at 4 °C overnight. Then, the plate was rinsed with PBST in triplicate, followed by blocking using 1% BSA and 5% skimmed milk powder in PBST at 37 °C for 2.5 h to block the non-specific binding. Using rabbit polyclonal antibody (1:10000 dilution with PBS, v/v) against PV as first antibody and HRP-labelled goat polyclonal antibody (1:5000 dilution with PBS, v/v) anti-rabbit IgG as secondary antibody. The incubation time for the antibodies was set at 1.5 h, afterwards, the TMB was added followed by incubation for 20 min at 37 °C, and then 2 M sulfuric acid was added to terminate the reaction. The absorbance was recorded at 450 nm with a microplate reader (CMax Plus, Molecular Devices, LLC., San Jose, CA, USA).

The absorbance of PV in simulated digestion for t min was expressed as A_t. Binding ability (%) of the sample at t min was defined as the ratio of the absorbance of the sample at t min A_t to the absorbance of the sample at 0 min A₀.

2.8. Statistical analysis

Density analyses of the SDS-PAGE bands, Image J peak areas and IgG binding results were statistically processed with the help of SPSS for Windows (Version 19.0; Chicago, IL, USA). The degree of the hydrolysis and IgG binding ability were expressed as relative percentages. Both relative percentages of each sample were expressed as a mean $\pm$ standard error for n = 3. Significant differences were evaluated by one-way analysis of variance followed by post hoc multiple-comparison using Tukey's test. The difference was considered significant at p < 0.05, extremely significant at p < 0.01.

3. Results and discussions

3.1. Identification of digestive fluids and PV

The SDS-PAGE profiles of purified PV samples are shown in Fig. 1A. Two bands of the native and emulsified PV (Fig. 1A, lane 9 and 10) were found around the 11 kDa protein marker. As calculated from Tris-Tricine-SDS-PAGE, the large Mw subunit (α -subunit) was about 10.4 kDa, while the small Mw subunit (β -subunit) was about 8.4 kDa. The purified PV was observed with the same Mw as reported in Ma et al. (2017). The crude pepsin (lane 2), trypsin (lane 3) and α -chymotrypsin (lane 4), which were proteolytic enzymes used for PV digestion, had Mw in the range of 35–11 kDa and were not interfering in PV detection. The SGF/ESGF and SIF/ESIF (lane 5–8) had not indicated the presence of any proteins.

IgG/IgE binding ability of purified intact and emulsified PV was shown in Fig. 1B. Both the subunits recognized IgG antibodies with the same binding intensity, indicating them as immunologically active. However, the IgE binding abilities of the two subunits were different,

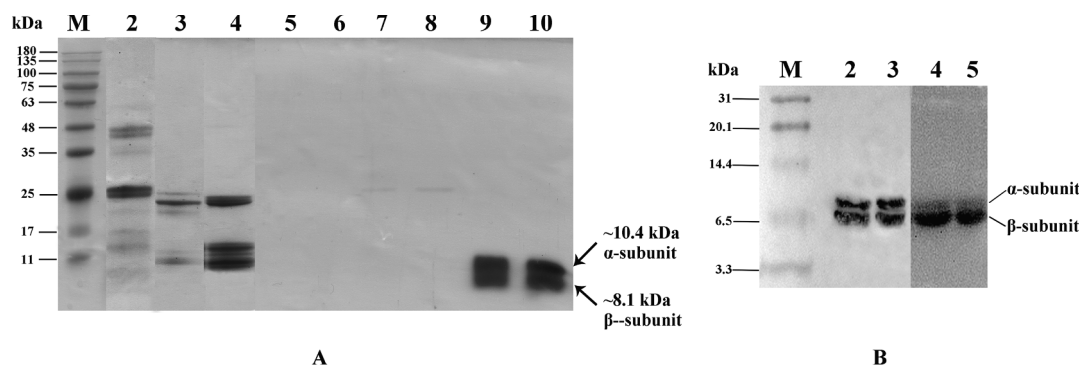


Fig. 1. Identification of digestive fluids and PV. SDS-PAGE. The protein content was 6 μ g for lane 2, 3, 4, 9 and 10. Lane M: protein marker; lane 2: pepsin; lane 3: trypsin; lane 4: α -chymotrypsin; lane 5: SGF; lane 6: ESGF; lane 7: SIF; lane 8: ESIF; lane 9: PV; lane 10: emulsified PV. (B) Western Blot. The protein content was 6 μ g for lane 2, 3, 4 and 5. Lane M: protein marker; lane 2: PV; lane 3: emulsified PV; lane 4: PV; lane 5: emulsified PV. (Lane 2, lane 3) IgG-immunoblot. (lane 4, lane 5) IgE-immunoblot. Mouse serum Nos. 1700 were used.

while the β -subunit had a stronger IgE binding ability compared with the α -subunit. This result has not been reported in any earlier studies, and the reason may be attributed to the variability of antigenic and allergic epitopes in the subunits of PV that makes high specificity for IgE antibodies to β -subunit. No considerable difference was observed between IgG/IgE binding ability of native and emulsified PV.

SDS-PAGE patterns of emulsified PV and digestive fluids had also been shown in Fig. 1A. There was no structural differences observed between PV and emulsified PV, and likewise, no discrepancy was observed in their IgG/IgE binding abilities (Fig. 1B). Unlike enzyme-aided crosslinking of proteins, which are induced by hydrophobic and electrostatic interactions, proteins might bind to lipid emulsion by weaker non-covalent bonding (Burnett et al., 2002). From the observations, it can be depicted that β -subunit has potential allergenic property that induced more IgE binding in PV sensitised subjects, and is the main cause for eliciting allergic symptoms.

3.2. SDS-PAGE analysis of digestion products of PV by pepsinolysis in SGF/ESGF and its IgG/IgE immunoreactivity

The SDS-PAGE profiles of digestion products of PV in SGF and ESGF were shown in Fig. 2. As it was reported in earlier studies that the lower detectable limit for allergenic peptides appears up to the Mw of approximately 3.5 kDa (Bøgh & Madsen, 2016). The structural changes in

the digestive fragments of PV with potential allergenicity were observed using Tris-Tricine-SDS-PAGE. Two subunit bands of PV had no obvious changes during 1 min of SGF digestion. When digested for 5 min, the staining ability of α -subunit reduced indicating initiation of digestion and β -subunit still resisted. At the same time, a new fragment band sized about 6.5 kDa appeared in the gel, and predicted to be originating from the α -subunit. Moreover, the new 6.5 kDa fragment disappeared at 90 min. The digestion of β -subunit started at 15 min, and was completely digested at 180 min, whereas the α -subunit disappeared at 30 min. As evident from the results, the β -subunit showed more resistance to simulated gastric digestion compared to the α -subunit.

IgG/IgE binding ability of the digested samples obtained from SGF pepsinolysis treatments was analysed by immunoblotting. Fig. 2 represented IgG/IgE binding ability of digested PV and newly formed peptides during gastric digestion (180 min). The IgG binding of PV was gradually decreased with the hydrolysis of both the subunits. α -Subunit was detected for 5 min, whereas β -subunit was persistent till 90 min with reduction in binding ability. These results indicated that β -subunit is structurally stable and retains most of the epitopes till 60 min and in case of α -subunit most of the epitopes were destroyed within 5 min upon digestion, inferring that anti-IgG antibody recognition and binding efficiency of PV partly depends on the integrity of the protein structure (De Jongh et al., 2013). IgE binding was weakly observed only for β -subunit of PV and was persistent with a mild reduction in intensity

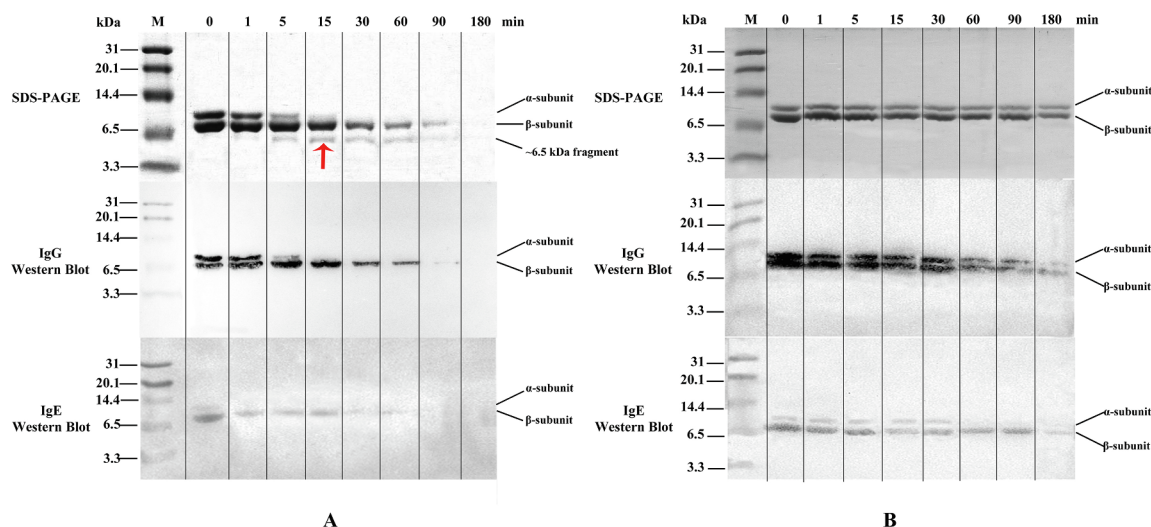


Fig. 2. Digestibility and IgG/IgE binding of PV in SGF/ESGF. (A) Digestibility and IgG/IgE binding of PV in SGF. (B) Digestibility and IgG/IgE binding of PV in ESGF. Mouse serum Nos. 1701 were used in IgE-Western Blot of A and B. Lane M: protein marker; lanes 2–9: allergen digested for 0, 1, 5, 15, 30, 60, 90, 180 min. The protein content was all 6 μ g for each lane.

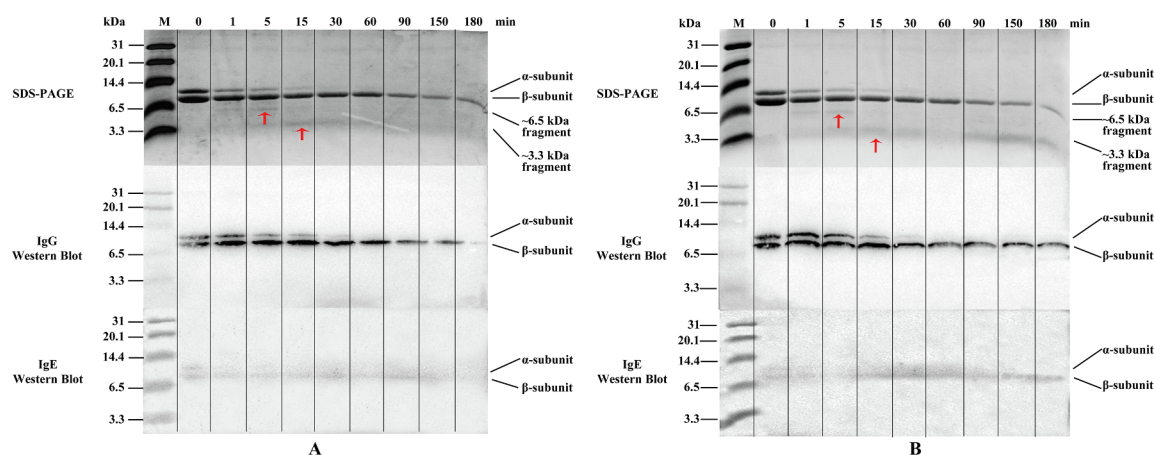


Fig. 3. Digestibility and IgG/IgE binding of PV in SIF/ESIF. (A) Digestibility and IgG/IgE binding of PV in SIF. (B) Digestibility and IgG/IgE binding of PV in ESIF. Mouse serum Nos. 1810, 1815 were used as a pooled serum in IgE-Western Blot of A and B. Lane M: protein marker; lanes 2–10: allergen digested for 0, 1, 5, 15, 30, 60, 90, 150, 180 min. The protein content was all 6 μ g for each lane.

till 60 min. On the other hand, α -subunit showed very weak binding at 0 min and was not detected in the later digestion stage. The results indicated that the high susceptibility to GIT digestion reduces IgE elicitation against α -subunit. Whereas, β -subunit retains intact structure and predicts the possibility of high risk in inducing allergic IgE response.

The PV digestion pattern and immunoreactivity in ESGF were shown in Fig. 2B. Addition of simulated lipid (n-hexadecane) resulted in high resistance of PV to simulated gastric digestion at the same pepsin/substrate ratio (U/w) used in SGF. The α -subunit of PV was marginally digested and both the original bands (α and β subunits) of PV were found to be visible even at 180 min without any reduction in the band intensity of β -subunit. No obvious newly generated digested fragments appeared in ESGF digestive pattern. It was observed that the interaction between lipid emulsion and PV at gastric pH is strong enough to retard the breakdown of the PV during gastric digestion, retaining intact subunits of PV for the whole length of digestion period. There are indications that lipid emulsion altered the digestive pattern of PV, similar to the result reported by Sancho et al. (2011). The digestion of rApi g 1 (homolog of major birch allergen Bet v 1) was slowed down in the presence of physiologically relevant levels of phosphatidylcholine (Sancho et al., 2011). Likewise, Angelina et al. (2016) found that in *in vitro* digestion, with phosphatidylglycerol enhanced the resistance of Sin a 2 and Ara h 1 to gastrointestinal digestion. The emulsification of proteins with lipid increased the resistance to gastric digestion, so lipid emulsion may make it easier for proteins to reach the intestinal epithelial cells and sensitive immune cells to pose risk of allergy.

Under emulsified condition, the PV showed IgG/IgE binding till 180 min, and binding ability was observed to be decreased with the progression of digestion time. Both the fragments showed similar binding ability for IgG in ESGF, whereas IgE binding of α -subunit was very weak and completely disappeared after 60 min, while β -subunit exhibited high intensity band and retained IgE binding till 180 min. Differences in IgG/IgE binding between PV and emulsified PV during simulated gastric digestion suggested that the presence of lipid might increase the possible resistibility to pepsinolysis and also kept the immune epitopes of PV. This might allow them to reach small intestine, wherein, the stable subunits can interact with immune cells to induce the allergic response. The lipid emulsion of PV increases its allergenic sensitivity as compared to the native PV and oil frying or addition of high lipids during fish processing and cooking methods will increase the risk of allergenicity in PV sensitized subjects.

3.3. SDS-PAGE analysis of digestion products of PV by proteolysis with pancreatic proteases in SIF/ESIF and its immunoreactivity

The SDS-PAGE profiles of digestion products of PV in SIF and ESIF having a Mw more than 3.3 kDa were shown in Fig. 3. In SIF treatment, the α -subunit of PV was digested completely within 30 min, while the β -subunit had not been digested for 180 min in simulated intestinal digestion, indicating stronger resistance to trypsin and α -chymotrypsin compared with the α -subunit. The newly digested fragments sized 6.5 kDa and 3.3 kDa were formed after 1 min and 5 min, and disappeared at 15 min and 60 min, respectively. Differences in the gastric and intestinal digestive patterns of PV were observed in our results, which were attributed to the specific cleavage of peptide bonds by pepsin, trypsin and α -chymotrypsin (Huang et al., 2010). PV showed higher resistance to intestinal digestion compared to gastric digestion considering the ratio of enzyme activity to the allergenic protein. It may depend on the variability of peptide recognition by the proteolytic enzymes and the denaturation process occurred due to physical conditions and nature of digestion mixture.

In contrast to ESGF, the digestion of PV in ESIF showed a remarkable difference with respect to the α -subunit of PV. It was digested completely after 30 min, while the β -subunit had not been digested even at 180 min. Similarly, the newly digested fragments sized 6.5 kDa were formed after 1 min, disappeared on the gel at 15 min. The newly digested fragments sized 3.3 kDa were formed after 5 min and retained and persisted until 180 min. However, as shown in SDS-PAGE (Fig. 3A and Fig. 3B), it can be concluded that there is no obvious difference between SIF and ESIF digestion pattern of PV in simulated intestinal digestion in contrast to the simulated gastric digestion of PV, which is in agreement with the previously reported work (Moreno, Mackie, & Mills, 2005). The appearance and disappearance of major fragment in SGF, ESGF, SIF and ESIF based on different time intervals and their IgG/IgE binding potentials are summarized in Table 1.

That's to say, the protective effect of lipid emulsion was lost in intestinal digestion, with the PV being rapidly broken down. This is probably because the bile salts in the intestinal digestion mix have mild detergent action on the intact protein and digestive fragments. Burnett et al. (2002) observed desorption of several protein allergens from model stomach emulsions with the addition of bile salts. Upon reaching the duodenum, the emulsion droplets are mixed with bile and pancreatic secretions, becoming highly negatively charged and are normally stabilized against further coalescence (van Aken, 2010). However, the β -subunit was stable and intact in both SIF/ESIF, indicating its potential to be proteolytic resistant and support its nature of IgE responder in immune sensitive populations.

Table.1

The appearance and disappearance of major fragment based on different time intervals and their IgG/IgE binding.

Fragments	Events	Time in the treatment			
		SGF	ESGF	SIF	ESIF
α-subunit	Completely digested	30	EXIST	30	30
	Lose IgG binding	15	EXIST	60	60
	Lose IgE binding	1	60	1	5
β-subunit	Completely digested	180	EXIST	EXIST	EXIST
	Lose IgG binding	90	EXIST	180	EXIST
	Lose IgE binding	90	EXIST	180	EXIST
~6.5 kDa fragment	Produced	5	NONE	1	1
	Completely digested	90	NONE	15	15
~3.3 kDa fragment	Produced	NONE	NONE	5	5
	Completely digested	NONE	NONE	60	EXIST

Notes: EXIST: visible on the gel or PVDF film until final time point.

NONE: no fragment produced in related treatment.

IgG/IgE binding ability was analysed for the digested samples collected from SIF and ESIF digestion by immunoblotting (Fig. 3). The IgG/IgE binding activity of PV gradually decreased with the hydrolysis of two subunits. Similar results were observed for both SIF and ESIF. Earlier studies reported that, during the intestinal digestion process, the emulsified conditions did not obviously change the digestion of protein by pancreatic proteases. In both SIF and ESIF, α-subunit showed IgG binding ability for 30 min and β-subunit persisted its binding till 180 min. Whereas, the IgE binding was very weak and the intensity of band appeared similar in both SIF/ESIF. The observations clearly inferred that the β-subunit is strong and retains its epitopes and is not subjected to proteolytic hydrolysis. The variabilities of IgG and IgE binding indicate the strength and nature of specific epitopes and their availabilities for recognition in the digestion products. The digestion fluid may alter the surface exposure of epitopes which may be responsible for differential binding strength of IgG and IgE for digestive fragments obtained during intestinal digestion.

Compared with intestinal digestion, lipid emulsion had a great influence on the digestibility of PV in gastric digestion, which resulted in a great difference in IgG/IgE binding of digestion product of gastric digestion under the presence or absence of simulated lipid. So, it is concluded that, lipid emulsion enhances the IgG/IgE binding by reducing the enzymatic efficiency during gastric digestion and retains more intact structure of PV in the digestion products.

3.4. Enzymatic hydrolysis and degree of hydrolysis of intact and emulsified PV in simulated gastric and intestinal systems

Enzymatic efficiency of pepsin and pancreatic proteases was shown by the degree of hydrolysis of PV or single subunit; the latter was calculated by the decrease of the grey scale of the bands of two subunits or single subunit. Percent variation of the hydrolysis of the PV was predicted in SGF/ESGF and SIF/ESIF.

3.4.1. Pepsinolysis efficiency and degree of hydrolysis of PV in SGF/ESGF

As shown in Fig. 4A, after 30 min of SGF treatment, more than 90% of the α-subunit had been digested, and it was considered completely digested in SDS-PAGE. At the same time, the degree of hydrolysis of β-subunit did not exceed 60%, and was clearly visible in SDS-PAGE pattern. As in insert Fig. 4A, the results showed 70.80%, 89.51%, and 98.56% digestion of PV after 30, 90 and 180 min, respectively, during SGF digestion. As the results described (section 3.2), the two subunits of PV had different resistance to pepsinolysis.

While the emulsification condition significantly ($p < 0.01$) changed the result of ESGF treatment, as shown in the picture insert A, only 19.37%, 26.42%, 46.46% of PV was digested after 30, 90 and 180 min respectively. After 180 min, the degree of hydrolysis of α-subunit or β-subunit under ESGF treatment was less than half as compared to SGF (Fig. 4A). However, lipid emulsion did not alter pepsinolysis induced stability and the diversity of the two subunits of PV: α-subunit is easier to be digested compared to the β-subunit.

3.4.2. Pancreatic protease efficiency and degree of hydrolysis of PV in SIF/ESIF

As shown in Fig. 4 insert B, the ratio of digested PV was 68.90%, 74.28% and 81.89% in SIF treatment and 63.00%, 74.15%, 80.89% in ESIF treatment after 30, 90 and 180 min, respectively. Stronger resistance of β-subunit to proteolysis by trypsin and α-chymotrypsin was observed, similar to the resistance to pepsinolysis exhibited by the β-subunit in simulated gastric digestion. In GIT, the β-subunit had higher resistance to proteolysis than the α-subunit, so it is suggested that compared with α-subunit, the risk associated to the β-subunit is probably higher.

In intestinal digestion, lipid emulsion didn't induce any strong protective effect to proteolysis of PV. As shown in Fig. 4B, the α-subunit or the β-subunit of PV had almost the same degree of hydrolysis at the same time, regardless of the lipid emulsion. No significant ($p > 0.05$) differences were observed in the effect of lipid emulsion to simulated intestinal digestion of PV.

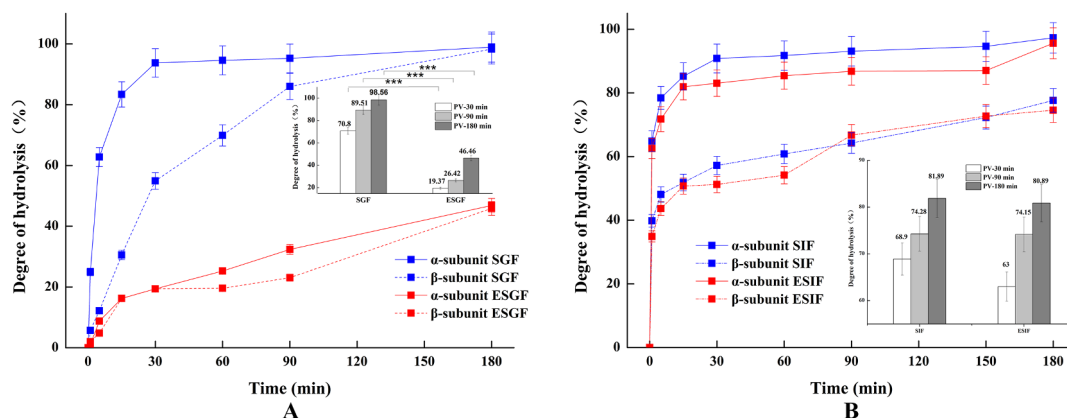


Fig. 4. Hydrolysis degree of PV in SGF/ESGF & SIF/ESIF. (A) Hydrolysis degree of α and β subunit of PV in SGF/ESGF. (Figure insert A) Hydrolysis degree of PV in SGF/ESGF in 30, 90 and 180 min. (B) Hydrolysis degree of α and β subunit of PV in SIF/ESIF. (Figure insert B) Hydrolysis degree of PV in SIF/ESIF in 30, 90 and 180 min. Degree of hydrolysis (%) of PV (or single subunit) at t min was defined as the ratio of grey value of the sample at time t to the grey value of the sample at time 0. The data was represented as the mean ± SD (n = 3). ** represented significance at $p < 0.1$, *** represented significance at $p < 0.05$ and **** represented significance at $p < 0.01$.

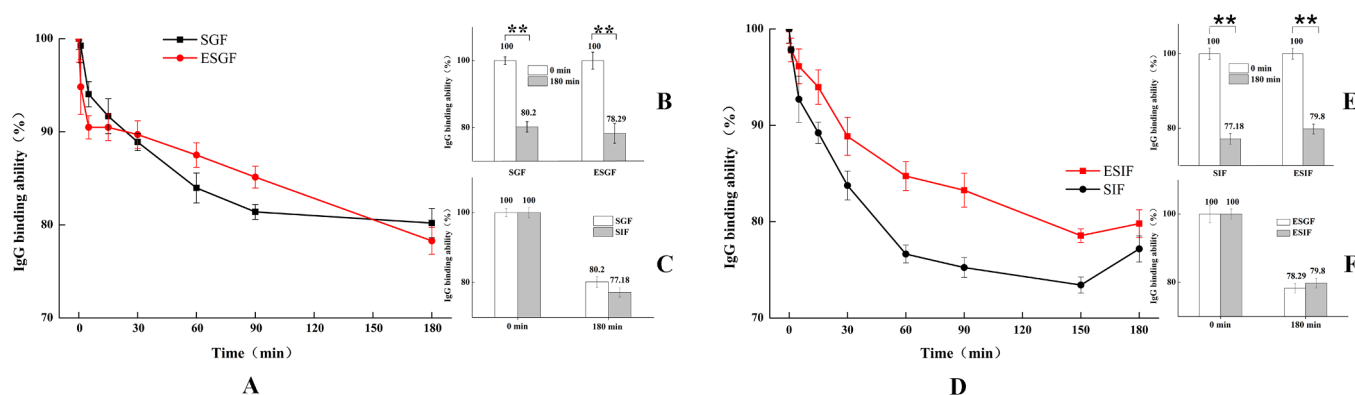


Fig. 5. IgG binding ability of digestion products in SGF/ESGF & SIF/ESI. (A, B) IgG binding ability of digestion products in SGF/ESGF. (D, E), IgG binding ability of digestion products in SIF/ESIF. (B, E) IgG binding ability of digestion products in 0 and 180 min. (C) IgG binding ability of digestion products in SGF/SIF in 0 and 180 min. (F) IgG binding ability of digestion products in ESGF/ESIF in 0 and 180 min. Binding ability (%) of the sample at t min was defined as the ratio of the absorbance of the sample at t min to the absorbance of the sample at 0 min. The data was represented as the mean $\pm$ SD (n = 3). “*” represented significance at $p < 0.1$, “**” represented significance at $p < 0.05$ and “***” represented significance at $p < 0.01$.

Hence, it was indicated that compared with intestinal digestion, lipid emulsion significantly ($p < 0.01$) reduced the enzymatic hydrolysis efficiency of digestive enzymes in SGF, thereby prolonging the presentation of PV in the digestive system. Thus, it increases the probability of immune system absorbing allergens in the gastrointestinal tract (Goodman et al., 2008; Korte, Bräcker, & Brockmeyer, 2017).

3.5. Extent of immune-reactivity of PV digestion product to predict their chance of epitope presentation

As described before, two bands of PV gradually disappeared in SDS-PAGE and immunoblot (Fig. 2 and Fig. 3) in the presence or absence of lipid emulsion during the digestion that led to the generation of digestive fragments. Although some digested fragments were not shown on SDS-PAGE and immunoblot clearly, they may still have potential IgG/IgE binding activity and retain allergenic properties. In order to explore the effect of lipid emulsion on specific IgG binding capacity of digestion products, which contained the main fragments and the invisible digestive fragments, indirect ELISA was performed to predict the presence of IgG reactive epitopes in digestion product.

3.5.1. Indirect ELISA to predict binding ability of digestion products in SGF/SIF

Fig. 5A and Fig. 5D showed the specific IgG binding ability of PV in gastric and intestinal digestion process. Under current physiological conditions, as shown in Fig. 5C, gastric and intestinal digestions can only reduce the IgG binding by 19.80% (180 min) and 22.82% (180 min) to PV digestion product, indicating that PV formed many small fragments during digestion, which appeared to retain IgG epitopes. This further confirmed the possibility of PV as an allergen and the potential of PV to elicit the allergic reaction following gastrointestinal digestion. Our findings are in agreement with Eiwegger et al. (2006) work, who observed that gastro-duodenal digestion fragments of Ara h 1 retained the T cell stimulatory and IgE-binding properties of the intact protein. It was also found that an increase in the IgG binding activity of PV after intestinal digestion for 150 min (Fig. 5D), regardless of lipid emulsion, which possibly be due to further destruction of the secondary or tertiary structure of the digested fragments and exposure of the epitopes. After gastric digestion for 180 min and intestinal digestion for 180 min, the degree of hydrolysis showed that 98.56% (Fig. 4 insert A) and 81.89% (Fig. 4 insert B) of PV was digested, however, the digested products had 80.20% and 77.18% IgG binding activity (Fig. 5C), indicating low Mw peptides (not shown on SDS-PAGE) still retained many antigenic determinants. Therefore,

gastrointestinal digestion can partly reduce but not eliminate the allergic activity of PV. It had been widely accepted that the lower limit for allergenicity of peptides is a Mw of approximately 3.5 kDa (Huby, Dearman, & Kimber, 2000; Lack et al., 2010), although digestive fragments sized more than 3.5 kDa disappeared in SGF for 180 min, the lower Mw peptides had retained 80.20% specific IgG binding activity.

3.5.2. Indirect ELISA to predict binding ability of digestion products in ESGF/ESIF

In gastric digestion, the IgG binding capacity of the digestion products observed in emulsified condition was higher than that in non-emulsified condition between 30 min and 150 min (Fig. 5A). Similarly, the IgG binding capacity of the digestion products in ESIF was higher than that in SIF during the whole intestinal digestion process (Fig. 5D). Especially, lipid emulsion significantly ($p < 0.05$) increased specific IgG binding ability of digestion products in both gastric digestion (3.74%) (Fig. 5A) and intestinal digestion (8.02%) (Fig. 5D) in 90 min. In SGF or ESGF treatment for 180 min, the IgG binding activity of the digestion products was almost the same and no significant ($p > 0.05$) difference was observed (Fig. 5B), so does the intestinal digestion (Fig. 5E). However, from the entire digestion process, it is confirmed that the digestive fragments produced in emulsified condition have higher IgG binding activity with higher antigenicity and potential allergenicity (Consolini et al., 2012; Moreno et al., 2005; Sancho et al., 2011).

However, it is noted that, our ELISA data only measured IgG binding, which may not be exactly equivalent to IgE allergenicity prediction. In order to further confirm the allergenic potential of PV digestion product obtained in emulsified condition, more reformed *in vitro* and *in vivo* experiments are needed. Those may include, IgE binding, basophil degranulation and mediator release assays and IgE induction in allergic mouse models, which can provide information and prove the basis of PV induced allergenicity.

4. Conclusions

This study suggested that the digestion process can lead to a reduction of intact PV content in the GIT and decreased the chance of presentation to trigger allergic immune response targeting the elicitation of specific IgE to PV. The two subunits of PV had different digestive abilities, and the β -subunit had higher resistance to proteolytic hydrolysis. Regardless of the emulsified conditions, the gastric and intestinal digestion products of PV exhibited high IgG binding abilities. The anti-digestibility of PV and the high IgG binding activity of the digestion products appear to be one of the characteristic features of allergen

compared to other non-allergenic proteins.

Lipid emulsion had significantly ($p < 0.01$) altered the digestive kinetics of PV in stomach, increasing the resistance to gastrointestinal digestion and IgG/IgE binding ability of digestion products. The protective effect of lipid emulsion in the gastric and intestinal digestion suggests that dietary lipid in the GIT may allow more proteins to reach the mucosal immune system in the small intestine. More clinical evidences are required to determine potential epitope changes in digestion products of PV in emulsified and non-emulsified condition to attribute its ability to induce clinical allergenicity. The results indicated that the effect of lipid emulsion must be considered when conducting safety assessment of PV during food processing and hydrolysed products to determine allergenicity.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to express their sincere thanks to National Natural Science Foundation of China (31871719) for supporting the research.

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