Food Chemistry 345 (2021) 128831

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Comparison of digestibility and potential allergenicity of raw shrimp (*Litopenaeus vannamei*) extracts in static and dynamic digestion systems

Yuman Guo [a], Chen Luo [a], Lili Xu [a], Hong Lin [a], Zhenxing Li [a,*], Tushar Ramesh Pavase [a], Yeting Wu [a], Yan Chen [b]

[a] College of Food Science and Engineering, Ocean University of China, No.5, Yushan Road, Qingdao, Shandong Province 266003, PR China
[b] NHC Key Laboratory of Food Safety Risk Assessment, Chinese Academy of Medical Science Research Unit (No. 2019RU014), China National Center for Food Safety Risk Assessment, Beijing 100022, PR China

ARTICLE INFO

Keywords:
Shrimp proteins
Static digestion system
Dynamic digestion system
Digestibility
Potential allergenicity

ABSTRACT

In this work, a simplified dynamic digestion system was developed, and used for comparing the digestibility and potential allergenicity of raw shrimp extracts (RSE) in static and dynamic digestion systems. Protein hydrolysis was analyzed by electrophoresis, and the potential allergenicity was reflected in IgG/IgE binding ability and activation of basophils. In comparison with static digestion, protein hydrolysis indicated different kinetic behaviors, especially tropomyosin (TM) showed better digestion stability during dynamic digestion. The potential allergenicity of RSE exhibited different changing trends with digestion in the two systems. However, the apparent molecular weight (Mw) of immune fragments (>11 kDa) showed good approximation, and the IgE-binding fragment near 70 kDa revealed outstanding digestion stability than primordial protein in both systems. In conclusion, the dynamic conditions had a significant impact on the assessment of the persistence and potential allergenicity of digestion-resistant allergens, while the apparent Mw of IgG/IgE binding hydrolysate was not affected.

1. Introduction

Food allergy refers to an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food (Boyce et al., 2010). There is evidence that gastrointestinal (GI) digestion can affect the immunogenicity of dietary proteins in relation to both IgE- and non-IgE-mediated adverse immune reactions to food (Pekar, Ret & Untersmayr, 2018). Digestibility is an important parameter to be considered in weight-of-evidence approach to assess potential allergenicity risks (Antonio Fernandez, 2019). European food safety authority (EFSA), Food and Agriculture Organization of the United Nations (FAO), World Health Organization (WHO), and Codex Alimentarius Commission (CAC) carry out *in vitro* digestion as a significant step of risk assessment for allergenic and novel food proteins (Ladics, 2008; Pali-Schöll, Verhoeckx, Mafra, Bavaro, Clare Mills & Monaci, 2019).

Although it is currently accepted that a protein in the food matrix that is resistant to proteolytic digestion and acid denaturation, it has an increased probability of reaching the intestinal mucosa where absorption and sensitization can occur (Liu et al., 2019). However, the correlation between digestion stability and allergenicity of food proteins is still highly controversial. The uses of different *in vitro* digestion assay conditions were shown to significantly influence the interpretation of the susceptibility of the given food allergens to digestion (Bøgh & Madsen, 2016). Of course, it is not only reflected in the digestion condition, but also related to the characteristics of the food allergy elicitation and sensitization. If a labile protein is at high concentration, sufficient protein may survive proteolysis to cause systemic allergic symptoms. Therefore, previous studies suggested that digestion may abolish, decrease, have no effect, or even increase the allergenicity of food allergens (Bøgh & Madsen, 2016; Moreno, 2007). The best way of protein allergy risk evaluation is combining digestibility assays with the assessment of potential allergenicity.

Digestion system plays an important role in influencing the potential allergenicity assessment of food allergen. The suitable *in vitro* digestion systems might better elucidate the interaction between proteins/fragments and the GI tract/immune reaction (Antonio Fernandez, 2019). Static digestion systems are the classical choices commonly utilized for the assessment of protein potential allergenicity, since ethical

* Corresponding author at: Food Safety Lab., College of Food Sci. & Engineering, Ocean University of China, PR China.
E-mail address: lizhenxing@ouc.edu.cn (Z. Li).

https://doi.org/10.1016/j.foodchem.2020.128831
Received 25 June 2020; Received in revised form 10 November 2020; Accepted 3 December 2020
Available online 8 December 2020
0308-8146/© 2020 Elsevier Ltd. All rights reserved.

constraints limit *in vivo* studies. The static protocol elaborated by the INFOGEST network within the COST Action aims to offer standardized digestion preconditions based on physiologically inferred conditions (Minekus et al., 2014). Inter-laboratory experiments have shown its characteristics of outstanding intra- and inter-laboratory reproducibility, robustness, simplicity, relatively low cost (Brodkorb et al., 2019). Nevertheless, static models were limited to study the endpoint digestion products and constrain tracking of the kinetic behavior of proteins during digestion. *In vitro* dynamic digestion systems that allow pH regulation, flow of the food and injection in real time of digestive enzymes in the different compartments of the GI tract are much more physiologically relevant than static systems (Dupont et al., 2019). Several works have been carried out to compare the gradual hydrolysis results of milk proteins between static and dynamic systems (Egger et al., 2019; Ménard et al., 2014; Miralles, Del Barrio, Cueva, Recio, & Amigo, 2018). It is generally agreed that peptide patterns of milk protein digestion products at gastric and intestinal endpoints were similar, but the kinetic behavior of the peptide profile emphasizes the effect of the dynamic condition on protein digestibility (Egger et al., 2019). Noteworthy, so far less comparative information was available on the potential allergenicity assessment between different digestion systems. Thus, it is essential to expand relevant research to explore the impact of dynamic variables on the assessment of food allergen digestibility and potential allergenicity as pointed out by (Dupont & Mackie, 2015).

Shrimp is a frequent cause of severe allergic reactions world-wide. Current studies mainly utilize static digestion systems to assess the potential allergenicity of purified protein or multi-component proteins in shrimp, and investigate the effects of external processes on it, such as ultrasound and heating (Liu et al., 2019; Zhang, Zhang, Chen & Zhou, 2018). Compared with the static digestion process, the addition of dynamic digestion conditions may affect the interaction between multi-component proteins and the relative distribution of allergens, thus affecting the potential allergenicity of digestion products. Hence, it is necessary to explore the differences and similarities of potential allergenicity changes of shrimp proteins during static and dynamic digestions.

This study aimed at the comparison of digestibility and potential allergenicity of raw shrimp extracts (RSE) between the static INFOGEST digestion system and the dynamic digestion system. A simplified dynamic digestion system was established as the first step to help with the comparison work. Additionally, digestion stability analysis and immunoassays were performed to reveal the characteristics changes of the RSE samples collected during time-resolved *in vitro* digestion at different levels of hydrolysis. This study provides an insight to explore the impact of dynamic conditions on the enzymatic degradation and potential allergenicity changes of food allergen in the GI tract, thereby extending information about *in vitro* dynamic digestion systems for food allergens risk assessment.

2. Materials and methods

2.1. Chemicals and reagents

Pepsin (P7012) and porcine pancreatin (P7545) were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Bile salt (Bovine) and horseradish peroxidase (HRP)-labelled goat anti-mouse IgG were obtained from Solarbio Co. (Beijing, China). The Western-blotting chemiluminescent substrate and the Imject Alum Adjuvant were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Mouse anti-shrimp tropomyosin (TM) IgG was obtained from Beijing Protein Innovation Co., Ltd. (Beijing, China), which has been used for TM detecting previously (Yu, Wang, Li, Pramod, Zhang & Lin, 2019). HRP-labelled goat anti-human specific IgE was acquired from Sigma-Aldrich (St Louis, Mo, USA). Human myeloma IgE was purchased from Merck Millipore (Billerica, MA). KU812 cells were purchased from Procell Life Co. (Wuhan, China). ELISA kit for histamine was purchased from R&D Systems (Minneapolis, MN, USA). All other chemicals used in this research were analytical grade.

2.2. Human sera

All clinically relevant human sera from shrimp-allergic patients were obtained from the Affiliated Hospital of Qingdao University (Qingdao, China). The patients were all diagnosed with shrimp allergy based on their clinical history of immediate hypersensitivity reactions, such as urticaria, pruritus, and shock, after ingestion of shrimp. The serum IgE levels were assessed using the ImmunoCAP system (Phadia AB, Uppsala, Sweden), and all shrimp specific IgE levels were above 0.5 kUA/L. The protocol was approved by the local medical ethics committee (File No. QD-PF-20171126396) to fully protect the rights and interests of the human subjects. All sera were stored at − 80℃ before analysis.

2.3. Preparation of raw shrimp extracts (RSE)

Shrimp (*Litopeanaeus vannamei*) was purchased from the local market of Qingdao, China, and the raw shrimp extracts (RSE) was prepared in the Laboratory of Food Safety, Ocean University of China. Briefly, the flesh of shrimp was suspended in five volumes of ice-cold acetone using a homogenizer. The acetone powder was suspended in the extracting solution with pH adjusted to 7.40, which containing 0.01 mol/L phosphate-buffered saline (PBS), 0.5 mmol/L Sodium dodecyl sulfate (SDS), and 0.5 mmol/L Dithiothreitol (DTT) according to the method described previously (Lasekan & Nayak, 2016) with some modification. Then the supernatant was dialyzed with 0.01 mol/L PBS and subsequent suction filtration was carried out using 0.22 mm pore diameter membrane (Solarbio Co., Beijing, China). Dialysis and filtration of the protein solution were carried out at least four times and three hours for each dialysis, followed by freeze-drying and storage at − 80℃ until usage.

2.4. The in vitro dynamic digestion system

The dynamic digestion system (Fig. 1A and 1B) consists of two consecutive compartments simulating the stomach and small intestine. Both compartments were set on an electromagnetic heating plate, where the temperature can be adjusted during the digestion according to the optimized temperature requirements of the contents. The simulated gastric cavity and intestinal cavity were equipped with temperature and pH sensors respectively (Electrode type: E-201G, REX, Shanghai, China). The micro peristaltic pumps (Kamoer, Shanghai, China) were equipped at different canals to control the flow rate and time of the meal, simulated gastric fluids (SGF), simulated intestinal fluids (SIF), bile, HCl, Na_2CO_3, and drain, which were designed according to the *in vivo* digestion studies and the classical dynamic systems, such as DIDGI® (Ménard et al., 2014) and SHIME (Molly, Vande & Verstraete, 1993). The digestion conditions such as flow rate and time of different peristaltic pumps, pH carves were set before digestion according to the protocol. The digestion conditions would be regulated automatically via the inbuilt software at predetermined rates and time. The pH carves were controlled by secreting either 1.0 mol/mL HCl to decrease the pH in the simulated gastric cavity or 1.0 mol/mL Na_2CO_3 to neutralize the pH in the simulated intestinal cavity.

2.5. In vitro static digestion conditions

RSE was digested *in vitro* according to the harmonized protocol (Brodkorb et al., 2019). Briefly, RSE (6 mg protein per mL) was mixed with an equal volume of SGF containing pepsin (2000U/mL of digesta) and incubated for different time points (0, 1, 3, 5, 10, 20, 30, 60, 90, 120 min) of the gastric phase. For the intestinal phase, equal volume SIF containing pancreatin (100 U trypsin activity/mL of digesta) and bile (10 mmol/L in the total digesta) were added at the 0 min point of the gastric phase and incubated for different time points (0, 1, 3, 5, 10, 20,

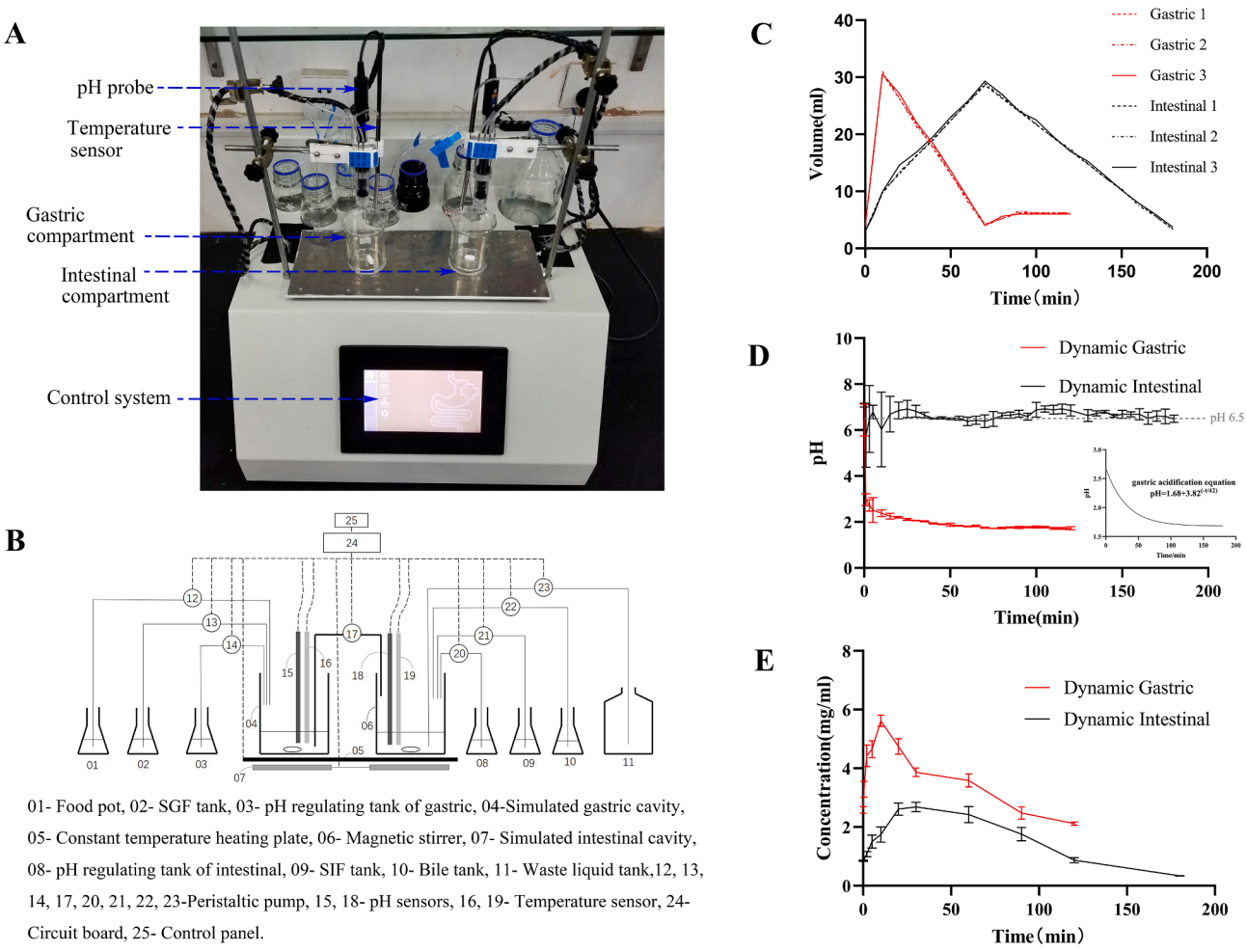


Fig. 1. The dynamic digestion system and the monitoring of the residual volume, pH, and protein concentration in simulated gastric and intestinal cavities. (A) Model appearance; (B) Framework map; (C) Residual volume; (D) pH; (E) Protein concentration. The red lines represent gastric curves, and the black lines represent intestinal curves. The gastric pH predetermined curve is $pH = 1.68 + 3.82^{(-t/42)}$ where "t" the time of delivery (min). Data are presented as mean ± SD (n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

30, 60, 90, 120 min). All of the incubation processes were performed at 37°C in a constant temperature incubator shaker and each time point was prepared with one separate tube. The enzyme activities and the bile concentration were measured according to the assay described in the harmonized protocol (Brodkorb et al., 2019). Pepsin reaction in samples collected during the gastric digestion was stopped by raising the pH to 7.0 using 0.2 mol/mL Na_2CO_3 solution and followed by immediate snap freezing in liquid nitrogen. Reactions in the intestinal phase were stopped by the addition of Pefabloc® (5 mM final concentration) prior to the snap freezing in liquid nitrogen.

2.6. *In vitro dynamic digestion conditions*

The dynamic *in vitro* digestion protocol was set up according to the parameters described previously (Kong & Singh, 2010; Ménard et al., 2014), and adjusted according to the volume of the sample to be digested. Parameters such as temperature, pH carve, time phase, SGF/SIF secretion rate, emptying rate, and quantity of enzymes during the dynamic gastro-intestinal digestion are provided in detail in the supplementary material. The pH curve in the simulated gastric cavity was: $pH = 1.68 + 3.82^{(-t/42)}$ where "t" the time of delivery, and the pH in the simulated intestinal cavity was maintained around 6.50 as described previously (Mans Minekusr, 1995). Meanwhile, the emptying carves of the simulated stomach and small intestine were simplified, linear emptying carves were used according to the relevant researches (Mulet-Cabero et al., 2020). Digestive enzymes were diluted in SGF/SIF with pH adjusted to 6.50 before digestion begins, while all the digestive enzymes were kept on ice throughout the experiment to avoid autolysis. Digesta was sampled after 0, 0.5, 2, 5, 10, 20, 30, 60, 90 and 120 min in the gastric phase, whereas for the intestinal phase sampled after 0, 2, 5, 10, 20, 30, 60, 90, 120 and 180 min, respectively. Samples were frozen in liquid nitrogen after the addition of enzyme activity inhibitors.

2.7. *SDS-PAGE and western-blotting analysis*

Digestion products were analyzed via SDS-PAGE as described by (Laemmli, 1970). RSE digestion samples were mixed with bromophenol loading buffer and proteins were loaded into SDS-PAGE gel composed of 12% polyacrylamide gels and 5% stacking gel. Electrophoresis was carried out at 80 V for 30 min at 120 V for 1 h. The gels were stained with Coomassie brilliant blue R-250 (Solarbio Co. Beijing, China). Images were captured by gel imager, SYSTEM GelDoc XR+ (Bio-Rad, Richmond, CA, USA).

Western-blotting was conducted to determine the IgG/IgE binding ability of the samples according to the previous method (Ahmed et al., 2018). Briefly, samples were separated by gels and electrically transferred onto polyvinylidene fluoride (PVDF) membrane by iBlot® 2 Gel Transfer Device (Life Technologies Co., Israel). Primary antibody (Mouse anti- shrimp TM IgG, 1:8000 dilution; or Human serum, 1:5 dilution) and secondary antibody (HRP-labelled goat anti-mouse IgG,

1:8000 dilution; or Goat anti-human IgE, 1:5000 dilution) were prepared with the ibind solution. The membrane was incubated in iBind™ Western Device (Life Technologies Co., Israel). Finally, the PVDF membrane was photographed by gel imager, SYSTEM GelDoc XR+ (Bio-Rad, Richmond, CA, USA).

2.8. Indirect enzyme linked immunosorbent assay (ELISA)

The IgG binding ability of the RSE digestion products was evaluated by indirect ELISA flowing the method described previously (Luo et al., 2020). Briefly, 5 μg proteins of RSE was placed overnight at 4 °C on a 96-well ELISA microplate. Mouse anti-shrimp TM IgG and the goat anti-mouse IgG were diluted 8000 times as the primary antibody and secondary antibody, respectively.

2.9. Mediator release assay of human basophil leukemia (KU812) cells

KU812 cells were cultured in accordance with the method reported by Zhu et al. (2019). The cells were placed in the complete Roswell Park Memorial Institute (RPMI) 1640 medium containing 100U/mL penicillin, 100 μg/mL streptomycin, and 15% fetal bovine serum (FBS) in a humidified incubator with 5% CO_2 at 37 °C. When the number of KU812 cells reached 2×10^6 cells per mL, human myeloma IgE (0.5 μg/mL) was added and then incubated for one week at 37 °C to stimulate the expression of IgE Fc receptors (FcεRI). The cells were then incubated in 10% human serum with 5% CO_2 at 37 °C for 12 h. Finally, KU812 cells were challenged with the RSE digestion products (500 μg/mL). The release of histamine from KU812 cells was measured with ELISA kit.

2.10. Statistical analyses

The results obtained were processed with the help of GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA) and expressed as the mean ± standard deviation (SD) of three independent experiments. Significant differences were calculated using a one-way analysis of variance (ANOVA). '*' represented significance at $p < 0.1$, '**' represented significance at $p < 0.05$, and '***' represented significance at $p < 0.01$.

3. Results and discussion

3.1. Monitoring of in vitro dynamic digestion system

The remaining volume, pH, and protein concentration of simulated gastric content and intestinal content monitored during dynamic digestion are represented in Fig. 1. The initial pH of the RSE formula was 6.50 and the pH regulation was accompanied by the whole process of digestion. After 10 min of ingestion and digestion, the simulated gastric cavity was mainly filled with food fluid. As shown in Fig. 1D, the pH changes monitored in both cavities did not fit the predetermined pH curve very well during the first 10 min, which was probably due to the rapid entry of food sample and the injection of SGF at the same time. Ménard et al. (2014) also reported a similar phenomenon that the pH electrode needs enough volume of fluid for reliable measurements. Good repeatability and stability of the pH changes were obtained after 10 min, and the gastric pH followed the equation of the gastric acidification curve well. At the endpoint of digestion, the gastric pH reached 1.76 and the intestinal pH was 6.71. Moreover, along with the ingestion, secretion, and GI transition, good reproducibility of remaining volume between experiments was observed in both phases (Fig. 1C), since the curves of the replicates were similar. The volume of HCl and sodium carbonate used to adjust the pH was relatively negligible in the whole phases of digestion. The volume of the contents increased in the first 10 min corresponding to the protein concentration increase in the gastric and intestinal phases with 0.32 and 0.11 mg/min, respectively. The gastric emptying and addition of gastric fluids were almost negligible during this short time. However, with the secretion of SGF and SIF, pepsin and pancreatin digestion played an important role in the decrease of protein concentration. The decrease rate of the protein concentration observed in the gastric and intestinal phases were about 0.04, and 0.016 mg/min. Multiple dynamic systems have also been developed previously and applied to simulate the digestion physiological processes, such as TNO gastric–small intestinal model (TIM; TNO, Netherlands Organisation for Applied Scientific Research) (Mitea, Havenaar, Drijfhout, Edens, Dekking & Koning, 2007), Human Gastric Simulator (HGS) (Ye, Cui, Carpenter, Prosser & Singh, 2019), etc. Compared with these models, the dynamic digestion conditions in our system such as food ingestion, SGF/SIF secretion, temperature, and pH curves were well simulated and could be adjusted flexibly, while its GI emptying carves were simplified as a semi-dynamic simulation (Mulet-Cabero et al., 2020).

3.2. Protein hydrolysis during static and dynamic in vitro digestions

The SDS-PAGE profiles of RSE digestion products in the static and dynamic systems were shown in Fig. 2. Bands corresponding to arginine kinase (AK, 40 kDa), tropomyosin (TM, 37 kDa), and sarcoplasmic calcium-binding protein (SCP, 20 kDa) were visible before digestion (Fig. 2E). The AK band was no longer visible at 3 min in the gastric phase and at 60 min in the intestinal phase of the static system (Fig. 2A and 2B), which indicated the important protein digestive function of the stomach in allergies. In the previous study, it was also revealed that the gastric bypass patients have an increased risk for developing allergies (Shakeri-Leidenmühler et al., 2015). AK was effectively hydrolyzed in the dynamic systems, and it was invisible throughout the whole phases (Fig. 2C and 2D). The TM band was invisible from 10 min onwards in both gastric and intestinal phases of the static system (Fig. 2A and 2B). Nevertheless, it was invisible at 60 min in both gastric and intestinal phases of the dynamic system (Fig. 2C and 2D). Ahmed et al. (2020) revealed that the intact TM was visible during the whole static GI digestion, while Gámez et al. (2015) mentioned that no TM was detected after 30 min when the pepsin activity was 10 U/μg. The uses of different *in vitro* digestion assay conditions were shown to have a significant influence on digestibility of food allergens (Bøgh & Madsen, 2016), thus affecting the digestion-resistant time of TM. The SCP band was visible in the whole gastric and intestinal phases of the static system (Fig. 2A and 2B), while it was no longer visible at 10 min in the gastric phase and at 60 min in the intestinal phase of the dynamic system (Fig. 2C and 2D). Compared with the static system, the relative grayscale of the SCP bands in the dynamic system were distinctly decreased, which can be visually observed (Fig. 2C and 2D). It can be concluded that SCP showed better digestion stability in the static system. Besides, the bands of digestion fragments (<11 kDa) were visible during the whole gastric phase and mainly distributed in the prophase (0 min-10 min) in the static system (Fig. 2A), while in the dynamic system, these were mainly distributed in the metaphase (2 min-30 min) of digestion (Fig. 2C). A decline over time was observed, more pronounced in the dynamic system samples than in the static ones.

The visible and invisible time points of these major bands in SDS-PAGE profiles were summarized and shown in Table 1. Comparing the digestion-resistant time, it could be found that the digestion stability of shrimp allergens (AK, TM, and SCP) showed a significant difference between the static and dynamic systems. In the dynamic digestion system, TM revealed longer digestion-resistant time than AK and SCP, which also reflected better digestion stability than that in the static digestion system. Accordingly, it can be concluded that dynamic digestion conditions had a significant impact on the digestion stability assessment of shrimp proteins. Liu et al. (2019) conducted the static GI digestion of raw shrimp extracts that SCP showed better digestion stability than TM, which was consistent with the digestion result in the static system as mentioned above. However, few studies considered the effect of dynamic digestion conditions on the digestive stability of shrimp proteins. As for other proteins, such as milk protein, the decline

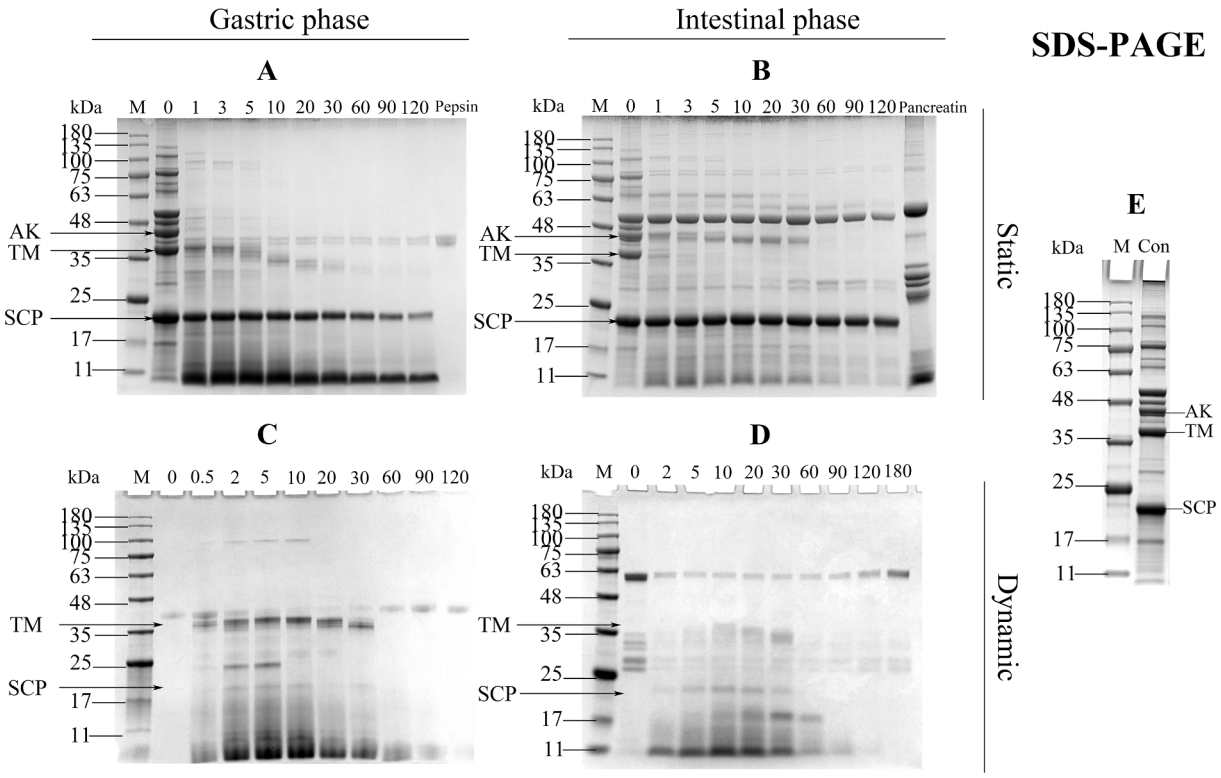


Fig. 2. SDS-PAGE analysis of RSE during static and dynamic *in vitro* digestions. (A) Gastric digestion in the static system; (B) Intestinal digestion in the static system; (C) Gastric digestion in the dynamic system; (D) Intestinal digestion in the dynamic system; (E) RSE without digestion; Lane M: protein marker; lanes 2–10: digestion time point; Pepsin and Pancreatin were shown in the right edges of A and B; 15 μg protein content for each band.

Table 1
The visible and invisible time points of major bands in SDS-PAGE results.

Band	System	Gastric		Intestinal	
		Visible	Invisible	Visible	Invisible
AK	Static	0	3	0	60
	Dynamic	NONE	/	NONE	/
TM	Static	0	10	0	10
	Dynamic	0.5	60	5	60
SCP	Static	0	EXIST	0	EXIST
	Dynamic	0.5	10	2	60
Fragment (<11 kDa)	Static	0	EXIST	0	120
	Dynamic	0.5	EXIST	2	120

[a]EXIST: visible until final time point. NONE: invisible in the whole phase.

of β-lactoglobulin had been reported to be more pronounced in the dynamic system samples than in the static system, which mainly due to gastric emptying and dilution with additional gastric fluids, in addition to the partial enzymatic degradation (Miralles et al., 2018). The existence of dynamic digestion conditions, such as GI emptying, secretion of SGF and SIF, and pH curves, made the proteins hydrolyzed in a more realistic digestion environment. One potential reason for the differences of protein digestion stability was that the combinations of amino acids, or modified amino acids that are encountered as pepsin processes the target proteins may alter the structure of pepsin at the active site, which means some proteins can in fact act as an inhibitor of digestion progress (Ofori-Anti, Ariyarathna, Chen, Lee, Pramod & Goodman, 2008). It is speculated that these dynamic conditions might contribute to the TM exerting its inhibitory effect, while facilitate the cleavage of exposed peptide bonds of other proteins by enzymes.

3.3. Specific IgG binding ability of RSE digestion products in static and dynamic systems

To obtain more information about the allergens during digestion, western-blotting with mouse anti-shrimp TM IgG was employed to observe the changing trends of specific IgG binding ability of RSE during static and dynamic digestions (Fig. 3A-3E). In comparison of the gastric phases in the two systems, the IgG-binding band of intact TM was almost invisible at 30 min in the static system (Fig. 3A), and at 60 min in the dynamic system (Fig. 3C). In the intestinal phases of two systems, it was almost invisible at 20 min in the static system (Fig. 3B), while in the dynamic system it was at 60 min (Fig. 3D). As expected, TM showed better digestion stability in the dynamic system, which was consistent with the digestive dynamic behavior of TM in SDS-PAGE. These results indicated that dynamic digestion conditions could promote intact pepsin-resistance proteins pass the gastric digestion to intestine smoothly, which may lead to longer persistence of its allergenicity and the enhancement of intestinal anaphylaxis, since the TM transported gut barrier in a dose-dependent manner (Kunimoto et al., 2011). In the previous study, TM in shrimp was regarded as the pepsin sensitive protein, and researchers explained that TM allergy was caused by these large stable proteolytic fragments generated during digestion that played an important role in sensitization via GI tract (Moreno, 2007). However, the sensitization of some intact protein seems to be underestimated during GI digestion in the static system. The application of dynamic digestion system may bring new reference to the interpretation of the relationship between digestive stability and allergenicity.

As for the IgG-binding bands of TM digestion products, TM was digested into fragments by pepsin and produced three main fragments with sizes of approximately 35, 20, and 15 kDa. In the intestinal phases, the fragments were near 35, 25, and 17 kDa, respectively (Fig. 3). These results were in accordance with the digestion results of the purified TM and shrimp proteins in previous studies (Liu et al., 2019; Lv et al., 2019),

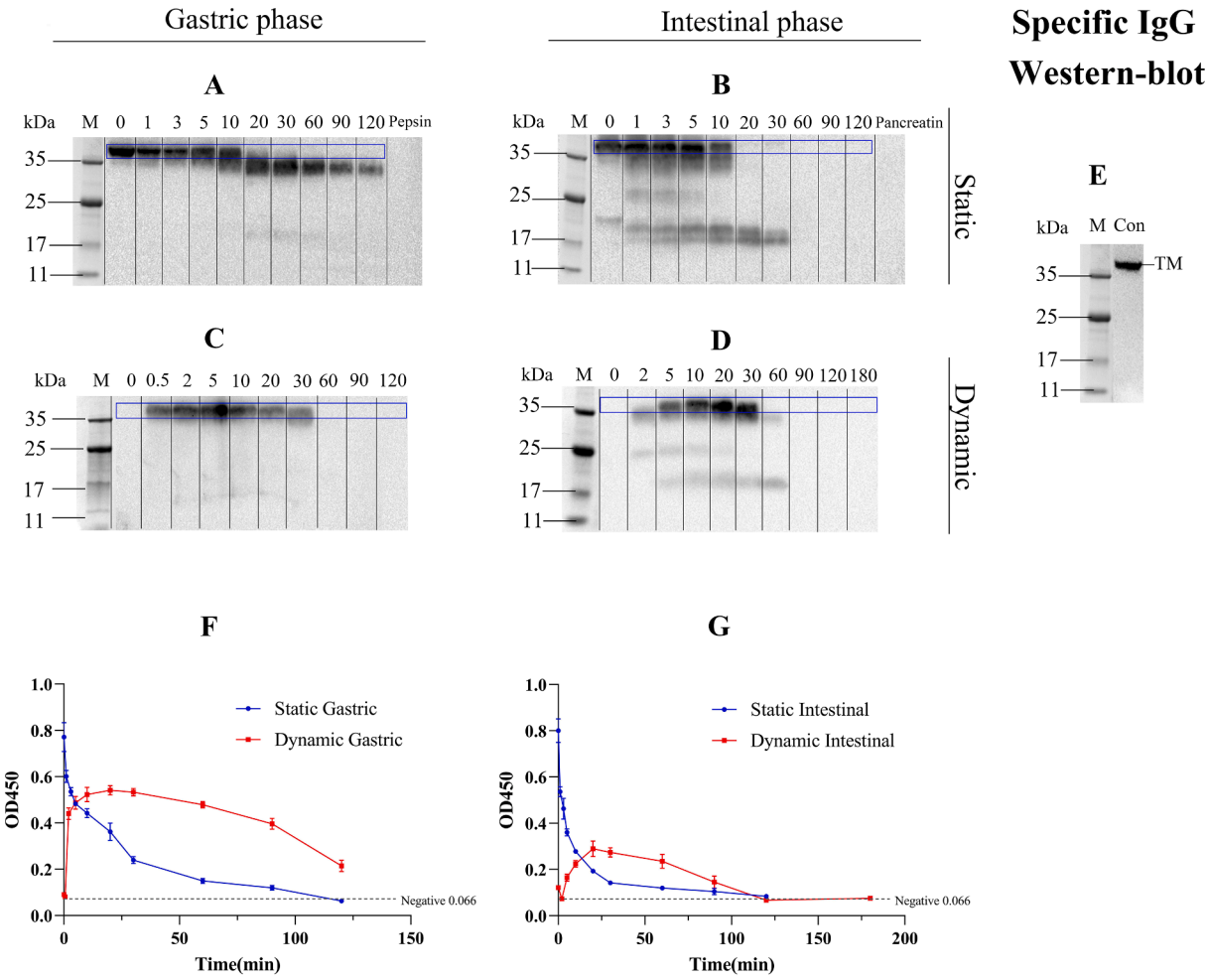


Fig. 3. Western-blotting and Indirect ELISA analyses for specific IgG-binding ability of RSE digestion products. (A) Western-blotting assay for gastric digestion products in the static system; (B) Western-blotting assay for intestinal digestion products in the static system; (C) Western-blotting assay for gastric digestion products in the dynamic system; (D) Western-blotting assay for intestinal digestion products in the dynamic system; (E) Western-blotting assay for RSE without digestion; (F) Indirect ELISA detection of IgG-binding ability in the gastric phases of the static system and dynamic system; (G) Indirect ELISA detection of IgG-binding ability in the intestinal phases of the static system and dynamic system; Lane M: protein marker. Pepsin and Pancreatin were shown in the right edges of A and B; First antibody: Mouse anti-shrimp TM IgG; Second antibody: goat anti-mouse IgG; Negative group: 0.2 mg/mL bovine serum albumin (BSA). Data are presented as mean ± SD (n = 3).

which meant that the apparent molecular weight (Mw) of TM digestion products was not affected by the presence of other proteins and dynamic digestion conditions. Although not exactly comparable with other dynamic digestion systems, Egger et al. (2019) reported that the GI digestive endpoints of static and dynamic systems showed a good approximation to the pig *in vivo* digestion. In short, TM showed different digestion kinetic behaviors and digestion stability in the static and dynamic systems, while the apparent Mw of its macromolecular fragments digestion product was not affected.

To further confirm the variations of specific IgG binding ability with RSE digestion products in the static and dynamic systems, indirect ELISA assay was performed. As shown in Fig. 3F and 3G, the IgG binding ability decreased gradually after GI digestions in the static system, while in the dynamic system, it increased in the first 20 min and then decreased with digestion. Different changing trends of specific IgG binding ability were observed during static and dynamic digestions. It is worth noting that, the specific IgG binding ability of digested RSE in the dynamic gastric phase was relatively higher than that of the static gastric phase from 10 min to 120 min (Fig. 3F). Meanwhile, in the intestinal phases of the two systems, the digestion products revealed higher IgG binding ability from 20 min to 90 min in the dynamic system (Fig. 3G). These phenomena indicated that static digestion system may lead to the underestimation of the potential allergenicity and allergy risk.

3.4. IgE binding ability of RSE digestion products in static and dynamic systems

Western-blotting analysis was carried out to analyze the IgE binding ability of digested samples obtained from the static and dynamic systems as shown in Fig. 4, and the serums of three shrimp-allergic patients were used respectively. Since the digested sample in our study was composed of complex proteins, using single patient serum would avoid the problem of unknown sources of IgE-binding bands. The western-blotting results in Fig. 4A-4E were treated with the IgE in serum No. 1. As it shown in the control group (Fig. 4E) and the 0 min of static digestion (Fig. 4A and 4B), the IgE-binding proteins in RSE that have been reported in previous works were myosin heavy chain (MHC, 200 kDa), and arginine kinase (AK, 40 kDa) (Chen et al., 2013; Huang et al., 2010). As soon as pepsin working, these bands were invisible and the new IgE-binding band around 70 kDa appeared (Fig. 4A-4D), which was quite possibly the degraded product of larger sized proteins titin (3000 kDa). Kamath et al. (2014) identified the titin fragment with 70 kDa Mw was the minor allergen of shrimp that was heat resistant and sensitizes through the GI.

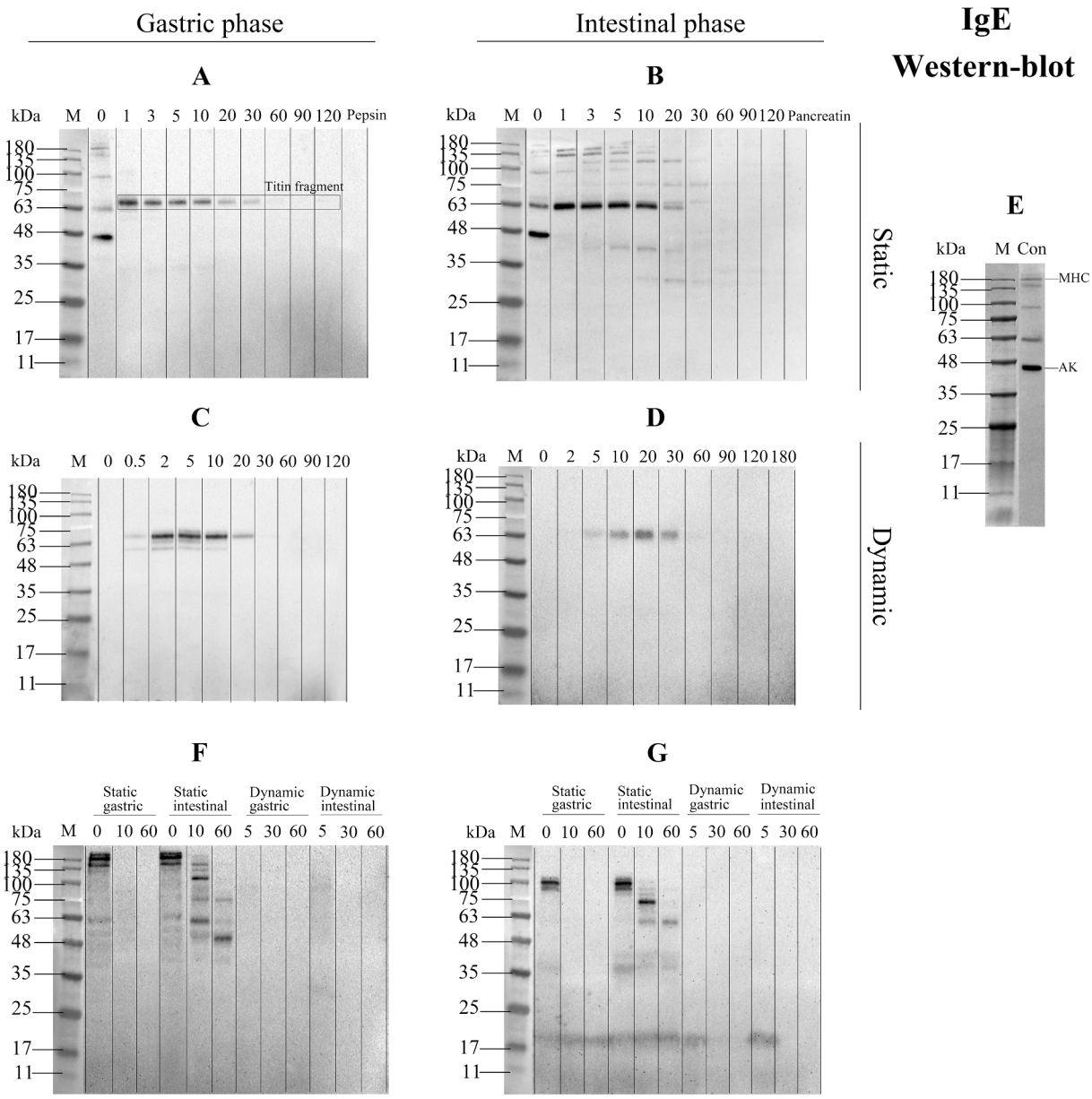


Fig. 4. Western-blotting analysis for IgE binding ability of RSE digestion products. (A) Gastric digestion in the static system; (B) Intestinal digestion in the static system; (C) Gastric digestion in the dynamic system; (D) Intestinal digestion in the dynamic system; (E) RSE without digestion; Serum No. 1 was used as the only source of IgE in A-E; (F) Western-blotting assay with IgE from Serum No. 2; (G) Western-blotting assay with IgE from Serum No. 3; Lane M: protein marker; Pepsin and Pancreatin were shown in the right edges of A and B; Second antibody: goat anti-human IgE.

Concerning the changing trends of IgE binding ability in the gastric phases, the titin fragment showed the strongest IgE binding ability at 1 min and decreased steadily until 120 min in the static system (Fig. 4A), while in the dynamic system it increased in the first 5 min and then decreased until 30 min (Fig. 4C), indicating a shorter potential allergenicity duration during dynamic gastric digestion. Furthermore, similar to the result of specific IgG binding ability, the apparent Mw of the IgE-binding bands in the gastric phases of two systems were approximate. The IgE-binding bands of intestinal digestion products were also mainly concentrated around 70 kDa (Fig. 4B and 4D) and the changing trends of IgE-binding ability were similar with their gastric phases. Noteworthy, it can be observed that in addition to the titin fragment around 70 kDa, a large number of IgE-binding bands existing in the entire intestinal phase of static system, while these were invisible in the dynamic system and the gastric phase of the static system. This phenomenon suggested that most of the IgE-binding products of serum No. 1 were extremely susceptible to pepsinolysis, which further emphasized the importance of the digestive function of stomach for food allergy patients (Pekar, Ret & Untersmayr, 2018). However, this kind of function may not be a protection, but may lead to a more persistent allergic reaction, since the titin fragment around 70 kDa that produced by enzyme hydrolysis revealed outstanding digestion stability and more persistent IgE reactivity than primordial protein in both systems. Antonio Fernandez (2019) also mentioned that food protein fragments which were stable to digestion, can be even more hazardous than the intact protein.

Overall, the IgE binding ability of RSE exhibited different changing trends during static and dynamic digestions, and the shrimp allergy patient of serum No. 1 was mainly allergic to the titin fragment around 70 kDa, although it was not apparent to be observed in SDS-PAGE profiles (Fig. 2). Besides, in order to confirm whether if the change of the IgE binding position before and after digestion was accidental,

another two serums were selected randomly and used for western-blotting assay with RSE digestion products (Fig. 5F and 5G). Similarly, the IgE-binding bands of No. 2 and No. 3 serums were mostly distributed in high Mw position before digestion, and the main allergenic components changed to hydrolytic fragments during digestion. This result illustrated that the digestion stability of food allergen was inadequate to be used as the prediction basis of potential sensitization. However, *in vitro* digestion can still be used as one of a few simple tools for evaluating the potential risk that a new dietary protein might become an important food allergen when there is not a substantial, clear history of consumption and allergenicity of the protein (Ofori-Anti, Ariyarathna, Chen, Lee, Pramod & Goodman, 2008).

3.5. Assessment of the activation of basophils by RSE digestion products in static and dynamic systems

Histamine release is a characteristic and critical function of basophils and it can cause a series of pathological effects on the human body which are directly related to the degree of the potential allergenicity of food allergen (Zhu et al., 2019). The histamine release from KU812 cells after sensitizing by the serum No. 4 and incubated with RSE digestion product is presented in Fig. 5. In the static system, the release of histamine was decreased along with the hydrolysis of RSE during gastric and intestinal digestion in general (Fig. 5A and 5B). Meanwhile, compared with the histamine release at 0 min, the release decreased by 53.16% after 120 min static gastric digestion (Fig. 5A) and 53.39% after 120 min static intestinal digestion (Fig. 5B). These results indicate that the potential allergenicity of RSE (6 mg/mL) decreased significantly ($p < 0.01$) after 120 min gastric or intestinal digestion in the static system.

However, along with the continuous ingestion of RSE, secretion of SGF, and gastric emptying, the histamine release reached the maximum at 20 min and reduced significantly ($p < 0.01$) at the endpoint of the gastric phase in the dynamic system (Fig. 5B). The protein concentration of simulated gastric content reached the maximum at 10 min during dynamic digestion (Food ingestion rate: 3.0 mL/min) (Fig. 1E). This phenomenon indicated that the number of allergen epitopes might continue to increase until 20 min and the hydrolyzed fragments in gastric digestion products have played an important role in stimulating basophil granulocyte degranulation. It had been widely accepted that the lower Mw limit for allergenicity of peptides is approximately 3.5 kDa (Huby, 2000), and the minimum peptide length able to activate T cells is nine amino acids (Naegeli et al., 2017). Compared with the gastric phases of the static system, the decreasing trend of histamine release in the dynamic system was faster (Fig. 5A), which may be mainly due to that the RSE ingestion stopped after 10 min, continuous supply of pepsin, and gastric emptying contributing to a sequential cleavage (Miralles et al., 2018).

The histamine release in the intestinal phase showed similar changing trend with the gastric phase in the dynamic system and there was no significant difference ($p > 0.05$) of histamine release between the endpoints and negative control group. Compared with the intestinal phases of the static system, the decreasing trend of histamine release in the dynamic system was relatively flat and slow, which may be mainly due to the slow entry speed of the samples to be digested in the intestinal phases of the dynamic system (Gastric emptying rate: 0.54 mL/min) (Fig. 1). This result also indicated that dynamic conditions may conduct the potential allergy risk preserving a relatively longer time during intestinal digestion. Overall, the differences in the activation of basophils by RSE digestion products reflected the potential allergenicity changing trends during static and dynamic digestions.

4. Conclusion

In this study, a simplified dynamic digestion system was established successfully, and it was used to compare with the static INFOGEST digestion system for the RSE digestibility and potential allergenicity assessment results. This dynamic digestion system allows food ingestion, SGF/SIF secretion, progressive adjustment of the pH and GI emptying at predetermined rates and time, and what's more a good reproducibility of remaining volume between experiments was observed. Our results demonstrated that the shrimp proteins hydrolysis showed different kinetic behaviors in the two systems, especially TM revealed better digestion stability than other allergens due to these dynamic digestion conditions. Besides, the potential allergenicity of RSE digestion products exhibited different changing trends between the static and dynamic systems, while the apparent Mw of IgG/IgE binding hydrolysate (>11 kDa) showed a good approximation. Nonetheless, dynamic digestion conditions had played an important role in influencing the digestion kinetic behaviors of digestion-resistant allergens, thus affecting the potential allergenicity assessment of shrimp proteins.

CRediT authorship contribution statement

Yuman Guo: Conceptualization, Methodology, Validation, Formal analysis, Writing - original draft. **Chen Luo:** Methodology, Software, Data curation. **Lili Xu:** Visualization. **Hong Lin:** Supervision. **Zhenxing Li:** Supervision, Project administration, Funding acquisition. **Tushar Ramesh Pavase:** Writing - review & editing. **Yeting Wu:** Investigation. **Yan Chen:** Resources.

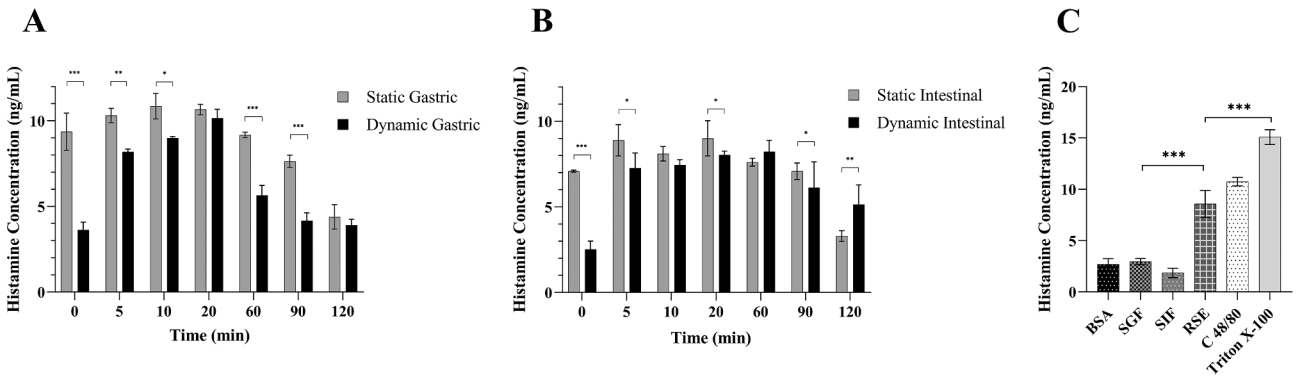


Fig. 5. Release of histamine in KU812 cells which was sensitized with patient serum No. 4 and induced by RSE digestion products. (A) Release of histamine in the gastric phases of the static system and dynamic system; (B) Release of histamine in the intestinal phases of the static system and dynamic system; (C) Control group: BSA (Negative), Compound 48/80 (C 48/80, Positive), SGF (Blank control of the gastric phase), SIF (Blank control of the intestinal phase), RSE (RSE without digestion), Triton X-100 (Total releasement). Data are presented as mean ± SD (n = 3). '*' represented significance at $p < 0.1$, '**' represented significance at $p < 0.05$, and '***' represented significance at $p < 0.01$.

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.

Acknowledgement

The authors would like to express their sincere thanks to National Key R&D Program of China (2019YFD0901704) for supporting the research and the support of CAMS Innovation Fund for Medical Science (CIFMS 2019-I2M-5-024).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.128831.

References

Ahmed, I., Lin, H., Xu, L., Li, S., Costa, J., Mafra, I., … Li, Z. (2020). Immunomodulatory Effect of Laccase/Caffeic Acid and Transglutaminase in Alleviating Shrimp Tropomyosin (Met e 1) Allergenicity. *Journal of Agricultural and Food Chemistry, 68* (29), 7765–7778. https://doi.org/10.1021/acs.jafc.0c02366.s001.

Ahmed, I., Lv, L., Lin, H., Li, Z., Ma, J., Guanzhi, C., Sun, L., & Xu, L. (2018). Effect of tyrosinase-aided crosslinking on the IgE binding potential and conformational structure of shrimp (*Metapenaeus ensis*) tropomyosin. *Food Chemistry, 248*, 287–295. https://doi.org/10.1016/j.foodchem.2017.12.071.

Antonio Fernandez, E. N. C. M. (2019). Safety Assessment of Immune-Mediated Adverse Reactions to Novel Food Proteins. *Trends in Biotechnology, 37*(8), 796–800. https://doi.org/10.1016/j.tibtech.2019.03.010.

Bøgh, K. L., & Madsen, C. B. (2016). Food Allergens: Is There a Correlation between Stability to Digestion and Allergenicity? *Critical Reviews in Food Science and Nutrition, 56*(9), 1545–1567. https://doi.org/10.1080/10408398.2013.779569.

Boyce, J. A., Ad, A. A., Burks, A. W., Jones, S. M., Sampson, H. A., Wood, R. A., … Fenton, M. J. (2010). Guidelines for the Diagnosis and Management of Food Allergy in the United States: Report of the NIAID-Sponsored Expert Panel. *Journal of Allergy and Clinical Immunology, 6*(126), S1–S58. https://doi.org/10.1016/j.jaci.2010.10.007.

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R., Carrière, F., Clemente, A., Corredig, M., Dupont, D., Dufour, C., Edwards, C., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A. R., Martins, C., Marze, S., McClements, D. J., Ménard, O., Minekus, M., Portmann, R., Santos, C. N., Souchon, I., Singh, R. P., Vegarud, G. E., Wickham, M. S. J., Weitschies, W., & Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols, 14*(4), 991–1014. https://doi.org/10.1038/s41596-018-0119-1.

Chen, H. L., Mao, H. Y., Cao, M. J., Cai, Q. F., Su, W. J., Zhang, Y. X., & Liu, G. M. (2013). Purification, physicochemical and immunological characterization of arginine kinase, an allergen of crayfish (*Procambarus clarkii*). *Food and Chemical Toxicology, 62*, 475–484. https://doi.org/10.1016/j.fct.2013.09.014.

Dupont, D., Alric, M., Blanquet-Diot, S., Bornhorst, G., Cueva, C., Deglaire, A., Denis, S., Ferrua, M., Havenaar, R., Lelieveld, J., Mackie, A. R., Marzorati, M., Menard, O., Minekus, M., Miralles, B., Recio, I., & Van den Abbeele, P. (2019). Can dynamic *in vitro* digestion systems mimic the physiological reality? *Critical Reviews in Food Science and Nutrition, 59*(10), 1546–1562. https://doi.org/10.1080/10408398.2017.1421900.

Dupont, D., & Mackie, A. R. (2015). Static and dynamic *in vitro* digestion models to study protein stability in the gastrointestinal tract. *Drug Discovery Today: Disease Models, 17–18*, 23–27. https://doi.org/10.1038/s41596-018-0119-1.

Egger, L., Ménard, O., Baumann, C., Duerr, D., Schlegel, P., Stoll, P., Vergères, G., Dupont, D., & Portmann, R. (2019). Digestion of milk proteins: Comparing static and dynamic *in vitro* digestion systems with in vivo data. *Food Research International, 118*, 32–39. https://doi.org/10.1016/j.foodres.2017.12.049.

Gámez, C., Zafra, M. P., Sanz, V., Mazzeo, C., Ibáñez, M. D., Sastre, J., & del Pozo, V. (2015). Simulated gastrointestinal digestion reduces the allergic reactivity of shrimp extract proteins and tropomyosin. *Food Chemistry, 173*, 475–481. https://doi.org/10.1016/j.foodchem.2014.10.063.

Huang, Y. Y., Liu, G. M., Cai, Q. F., Weng, W. Y., Maleki, S. J., Su, W. J., & Cao, M. J. (2010). Stability of major allergen tropomyosin and other food proteins of mud crab (Scylla serrata) by *in vitro* gastrointestinal digestion. *Food and Chemical Toxicology, 48*(5), 1196–1201. https://doi.org/10.1016/j.fct.2010.02.010.

Huby, R. D. D. R. (2000). Why are some proteins allergens? *Toxicological Sciences, 55*(2), 235–246. https://doi.org/10.1093/toxsci/55.2.235.

Kamath, S. D., Rahman, A. M. A., Voskamp, A., Komoda, T., Rolland, J. M., O'Hehir, R. E., & Lopata, A. L. (2014). Effect of heat processing on antibody reactivity to allergen variants and fragments of black tiger prawn: A comprehensive allergenomic approach. *Molecular Nutrition & Food Research, 58*(5), 1144–1155. https://doi.org/10.1002/mnfr.201300584.

Kong, F., & Singh, R. P. (2010). A Human Gastric Simulator (HGS) to Study Food Digestion in Human Stomach. *Journal of Food Science, 75*(9), E627–E635. https://doi.org/10.1111/j.1750-3841.2010.01856.x.

Kunimoto, A., Yokoro, M., Murota, K., Yamanishi, R., Suzuki-Yamamoto, T., Suzuki, M., … Kimoto, M. (2011). Gastrointestinal Digestion and Absorption of Pen j 1, a Major Allergen from Kuruma Prawn, Penaeus japonicus. *Bioscience, Biotechnology, and Biochemistry, 75*(7), 1249–1258. https://doi.org/10.1271/bbb.110021.

Ladics, G. S. (2008). Current codex guidelines for assessment of potential protein allergenicity. *Food and Chemical Toxicology, 46*(10), S20–S23. https://doi.org/10.1016/j.fct.2008.07.021.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680–685. https://doi.org/10.1016/j.fct.2008.07.021.

Lasekan, A. O., & Nayak, B. (2016). Effects of buffer additives and thermal processing methods on the solubility of shrimp (*Penaeus monodon*) proteins and the immunoreactivity of its major allergen. *Food Chemistry, 200*, 146–153. https://doi.org/10.1038/227680a0.

Liu, M., Liu, S., Han, T., Xia, F., Li, M., Weng, W., … Liu, G. (2019). Effects of thermal processing on digestion stability and immunoreactivity of the Litopenaeus vannamei matrix. *Food and Function, 10*(9), 5374–5385. https://doi.org/10.1016/j.foodchem.2016.01.015.

Luo, C., Guo, Y., Li, Z., Ahmed, I., Pramod, S. N., Gao, X., Lv, L., & Lin, H. (2020). Lipid emulsion enhances fish allergen parvalbumin's resistance to *in vitro* digestion and IgG/IgE binding capacity. *Food Chemistry, 302*, 125333. https://doi.org/10.1016/j.foodchem.2019.125333.

Lv, L., Lin, H., Li, Z., Nayak, B., Ahmed, I., Tian, S., Chen, G., Lin, H., & Zhao, J. (2019). Structural changes of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) treated shrimp tropomyosin decrease allergenicity. *Food Chemistry, 274*, 547–557. https://doi.org/10.1016/j.foodchem.2018.09.030.

Mans Minekusr, P. M. R. H. (1995). A multicompartmental dynamic computer-controlled model simulating the stomach and small intestine. *Atla-Alternatives to Laboratory Animals, 2*(23), 197–209.

Ménard, O., Cattenoz, T., Guillemin, H., Souchon, I., Deglaire, A., Dupont, D., & Picque, D. (2014). Validation of a new *in vitro* dynamic system to simulate infant digestion. *Food Chemistry, 145*, 1039–1045. https://doi.org/10.1016/j.foodchem.2013.09.036.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., … Brodkorb, A. (2014). A standardised static *in vitro* digestion method suitable for food – an international consensus. *Food and Function, 5*(6), 1113–1124. https://doi.org/10.1039/C3FO60702J.

Miralles, B., Del Barrio, R., Cueva, C., Recio, I., & Amigo, L. (2018). Dynamic gastric digestion of a commercial whey protein concentrate†: Dynamic digestion of whey proteins. *Journal of The Science of Food and Agriculture, 98*(5), 1873–1879. https://doi.org/10.1002/jsfa.8668.

Mitea, C., Havenaar, R., Drijfhout, J. W., Edens, L., Dekking, L., & Koning, F. (2007). Efficient degradation of gluten by a prolyl endoprotease in a gastrointestinal model: Implications for coeliac disease. *Gut, 57*(1), 25–32. https://doi.org/10.1136/gut.2006.111609.

Molly, K., Vande Woestyne, M., & Verstraete, W. (1993). Development of a 5-step multi-chamber reactor as a simulation of the human intestinal microbial ecosystem. *Appl Microbiol Biotechnol, 39*(2), 254–258. https://doi.org/10.1007/BF00228615.

Moreno, F. J. (2007). Gastrointestinal digestion of food allergens: Effect on their allergenicity. *Biomedicine & Pharmacotherapy, 61*(1), 50–60. https://doi.org/10.1016/j.biopha.2006.10.005.

Mulet-Cabero, A., Egger, L., Portmann, R., Ménard, O., Marze, S., Minekus, M., … Mackie, A. (2020). A standardised semi-dynamic *in vitro* digestion method suitable for food – an international consensus. *Food and Function, 11*(2), 1702–1720. https://doi.org/10.1039/C9FO01293A.

Naegeli, H., Birch, A. N., Casacuberta, J., De Schrijver, A., Gralak, M. A., Guerche, P., Jones, H., Manachini, B., Messéan, A., Nielsen, E. E., Nogué, F., Robaglia, C., Rostoks, N., Sweet, J., Tebbe, C., Visioli, F., Wal, J. M., Eigenmann, P., Epstein, M., Hoffmann Sommergruber, K., Koning, F., Lovik, M., Mills, C., Moreno, F. J., van Loveren, H., Selb, R., & Fernandez Dumont, A. (2017). Guidance on allergenicity assessment of genetically modified plants. *EFSA Journal,, 15*(6). https://doi.org/10.1039/C9FO01293A.

Ofori-Anti, A. O., Ariyarathna, H., Chen, L., Lee, H. L., Pramod, S. N., & Goodman, R. E. (2008). Establishing objective detection limits for the pepsin digestion assay used in the assessment of genetically modified foods. *Regulatory Toxicology and Pharmacology, 52*(2), 94–103. https://doi.org/10.1016/j.yrtph.2008.06.006.

Pali-Schöll, I., Verhoeckx, K., Mafra, I., Bavaro, S. L., Clare Mills, E. N., & Monaci, L. (2019). Allergenic and novel food proteins: State of the art and challenges in the allergenicity assessment. *Trends in Food Science & Technology, 84*, 45–48. https://doi.org/10.1016/j.tifs.2018.03.007.

Pekar, J., Ret, D., & Untersmayr, E. (2018). Stability of allergens. *Molecular Immunology, 100*, 14–20. https://doi.org/10.1016/j.molimm.2018.03.017.

Shakeri-Leidenmühler, S., Lukschal, A., Schultz, C., Bohdjalian, A., Langer, F., Birsan, T., … Untersmayr, E. (2015). Surgical Elimination of the Gastric Digestion by Roux-en-Y Gastric Bypass Impacts on Food Sensitisation—a Pilot Study. *Obesity Surgery, 25*(12), 2268–2275. https://doi.org/10.1007/s11695-015-1689-4.

Ye, A., Cui, J., Carpenter, E., Prosser, C., & Singh, H. (2019). Dynamic *in vitro* gastric digestion of infant formulae made with goat milk and cow milk: Influence of protein composition. *International Dairy Journal, 97*, 76–85. https://doi.org/10.1016/j.idairyj.2019.06.002.

Yu, Z., Wang, Y., Li, Z., Pramod, S. N., Zhang, L., & Lin, H. (2019). Development of ELISA Method for Detecting Crustacean Major Allergen Tropomyosin in Processed Food

Samples. *Food Analytical Methods, 12*(12), 2719–2729. https://doi.org/10.1007/s12161-019-01627-z.

Zhang, Z., Zhang, X., Chen, W., & Zhou, P. (2018). Conformation stability, *in vitro* digestibility and allergenicity of tropomyosin from shrimp (Exopalaemon modestus) as affected by high intensity ultrasound. *Food Chemistry, 245*, 997–1009. https://doi.org/10.1016/j.foodchem.2017.11.072.

Zhu, J., Deng, H., Yang, A., Wu, Z., Li, X., Tong, P., & Chen, H. (2019). Effect of microbial transglutaminase cross-linking on the quality characteristics and potential allergenicity of tofu. *Food and Function, 10*(9), 5485–5497. https://doi.org/10.1039/C9FO01118H.

Table 2. Characterization of sera from shrimp-allergic patients.

No.	Age (years)	Sex	Symptoms	Specific IgE (kUA/L) to Shrimp
1	27	F	Dr	9.18
2	40	M	U	1.59
3	45	F	S	1.74
4	12	M	U, P	0.96

[a] F, female; M, male; A, asthma; C, cheilitis; Dr, dermatitis; P, pruritus; U, urticaria; S, shock; A serum with specific IgE＞0.35 (kUA/L) is defined as positive.

Table 3. Gastrointestinal conditions for the in vitro dynamic digestion of RSE.

Project	Time	Condition
Volume ingested (mL)		30.0 mL
Ingestion rate (mL/min)		3.0 mL/min
Gastric conditions		
T/°C		37 °C
pH (acidification curve)		$pH = 1.68 + 3.82^{(-t/42)}$ (t: time after ingestion (min))
Fasted volume (mL)		4.0 mL SGF (2000 U/mL pepsin in gastric content)
Pepsin (porcine)	Flow rate	
	0-10 min	0.20 mL/min
	10 min-end gastric	0.10 mL/min
Gastric emptying	Emptying rate	0.54 mL/min
	Emptying time	70 min
Intestinal conditions		
T/°C		37 °C
pH		6.50
Fasted volume (ml)		2.0 mL bile solution (2%) + 1.0 mL pancreatin solution (4%)
2% Bile (bovine)	Flow rate	
	0-180 min	0.10 mL/min
4% Pancreatin	0-180 min	0.05 mL/min
Intestinal emptying	Emptying rate	0.37 mL/min
	Emptying time	170 min