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AFM and NMR imaging of squid Tropomyosin Tod p1 subjected to high hydrostatic pressure: evidence for relationships among topography, characteristic domain and allergenicity

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Abstract

The surface topography, characteristic domain and allergenicity of squid Tropomyosin Tod p1 (TMTp1) treated by single- and two-cycle high hydrostatic pressure (HHP) were analyzed. Atomic force microscopy (AFM) showed that HHP treatment led to a rougher surface of squid TMTp1; the two-cycle 600 MPa HHP treatment produced the largest effect, with a mean roughness, maximum height, skewness and kurtosis of 6.56 nm, 94.83 nm, 3.05 and 23.13, respectively. HHP treatment caused lower IgE and IgG-binding capacities, indicating significant reduction of the allergenicity ($p < 0.05$) due to variations in the AFM surface topography. The peaks of the main allergenic characteristics affected were 0.99, 1.16, 1.21, 1.79, 1.82, 2.84, 2.88 and 3.37 ppm (in initial ^{1}H nuclear magnetic resonance (NMR) spectra). HHP treatment changed the AFM surface topography and caused the migration or disappearance of related ^{1}H NMR peaks; these changes were significantly correlated ($p <0.05$) with the reductions in allergenicity observed.

Keywords: *seafood allergen; high hydrostatic pressure; Tropomyosin Tod p1; nuclear magnetic resonance; atomic force microscopy.*

1. Introduction

Seafood allergy is one of the most common, severe, and long lasting food allergies, and receives extensive attention from people with a predisposition to allergies[1]. Tropomyosin Tod p1 (TMTp1), a water soluble 38 kDa protein, is a major allergen which occurs extensively in crustacean and mollusc species[2]. Many attempts have been made to reduce the presence of allergens in various foods during processing[3-5]. High hydrostatic pressure (HHP), a valuable non-thermal food processing technology[6], can significantly reduce the activity of many allergens[7], such as β-lactoglobulin[8], and those found in soybean seeds[9] and almond milk[10]. In our previous study[11], single-cycle HHP treatments at 200, 400 or 600 MPa for 20 min decreased the allergenicity of squid TMTp1; the 400 and 600 MPa treatments were more effective than the 200 MPa treatment, according to indirect enzyme linked immune sorbent assays (ELISAs).

HHP affects the structure of proteins, shown by characterizing protein structures with circular dichroism spectra, as well as by analyzing the free sulfhydryl content and surface hydrophobicity index[8, 9, 11]. However, the precise nature of the internal motions within protein macromolecules remains a mystery and is not easy to describe accurately[12]. Highly sensitive and reliable methods to analyse squid TMTp1 are required to enable assessments of their allergenic properties.

Different proteins have different conformational properties which are important for the modification of their functional properties[13]. Many studies have been done in recent years for exploring the relationship between physical properties and biology functions, such as the research on Pre-T-cell receptor structure and interactions[14], the physical properties of supramolecular peptides[15], and antibacterial and/or antifouling property to the surface of peptides[16]. Atomic force microscopy (AFM) is the principal

way that complex chemical or biochemical changes and reactions can be clarified directly in many systems[17]. Surfaces with varying AFM surface topography indices have different functional properties[18]. For example, surface roughness, which directly corresponds to the sizes of proteins, could play an important role in defining different proteins' characteristics[19]. Altering the surface roughness influences the chemical reactivity of proteins[20], which affects properties like the sensitization response, *in vitro* osteoblast differentiation, and local factor production[21, 22]. HHP is likely to produce powerful percussive effects[23-25] that might have an impact on the morphology of the squid TMTp1 molecules in solution, and upon their adsorption onto the surface. Hence, AFM surface topography could be used to assess the effect of HHP treatments on squid TMTp1 and its associated allergenic properties.

Nuclear magnetic resonance (NMR) spectroscopy has become one of the most accepted methods for determining the structural properties of native and processed proteins[26]. NMR data, especially ^{1}H NMR spectra, provide information about the structure and dynamic properties of proteins, such as information on the positions, bonds and movements of specific atoms[27]. This information could be used to describe the changes of a processed protein in terms of its structural, chemical and dynamic properties. Previous research[27] illustrated that ^{1}H NMR spectra can be used to generate a fingerprint, give insights into the molecular folding of allergen proteins, and offers an independent method for assessing the structural properties of proteins. Clearly, NMR could be used to analyse changes in the characteristic regions of squid TMTp1 when it is HHP treated, as well as the positions, bonds and movements of characteristic peaks, which are related to the allergenic properties.

Compared with single-cycle HHP, multiple-cycle HHP may cause more significant structural damage to biomacromolecules[28], and could be more effective in

inactivating microorganisms, improving the food quality, and would lower the cost in comparison to a single-cycle of the same dwell time[29, 30]. Previously we found that two-cycle HHP treatments were more effective in controlling microbial growth and reducing the deterioration of squid[23]. However, there is limited information available to compare single- and two-cycle HHP treatments in respect of the nutritional, functional or other properties of seafood. Therefore, it is worthwhile to study the changes in allergenicity resulting from treating squid TMTp1 with both one- and two-cycle HHP.

The objectives of this study were to investigate the effect of one- and two-cycle HHP treatment on the allergenicity of squid TMTp1. To do this, the allergenic characteristic regions and peaks of ^{1}H NMR spectra of HHP treated squid TMTp1 were analyzed. AFM was then used to evaluate the changes of the surface topography of the HHP treated squid TMTp1. Finally, the relationships among the allergenic properties, AFM surface topography and NMR characteristic regions in the HHP treated squid TMTp1 samples were explored.

2. Materials and methods

2.1 Materials and sample preparation

Squid (*Todarodes pacificus*; 310 ± 25 g per whole squid) were obtained from the Chinese Academy of Fishery Sciences (Shanghai, China) and stored at −80°C until processing. The extraction, purification and identification of squid TMTp1 and the rabbit anti-squid TMTp1 polyclonal antibodies was carried out according to our previous methods[11]. All procedures concerning animals were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of Shanghai Jiao Tong University. The protocol was approved by the National Natural

Science Foundation Commission of China (Permit Number: 31271955) and the Committee on the Ethics of Animal Experiments of School of Agriculture and Biology, Shanghai Jiao Tong University. Individual human serum samples were collected from five patients (Xinhua Hospital, Shanghai, China) who were determined to have squid allergy based on the history and the objective manifestations after ingestion of squid. The pooled sera of two non-allergic individuals from the same hospital were used as a negative control. Also, written informed consent was obtained from each human before the human serum was collected.

The squid TMTp1 solution was diluted with 20mM Tris-HCl (pH 7.5) to create a final concentration of 1 mg/mL. Then, 20 ml samples of the diluted squid TMTp1 solution were individually packaged in polyamide/chlorinated polypropylene complex film bags (17 × 23 cm), the oxygen and water vapor permeabilities of which were 5.383×10^{-15} cm^3•cm/cm^2•s•Pa and 1.383×10^{-12} g•cm/cm^2•s•Pa, respectively. The bags were sealed for the HHP treatments.

2.2 High hydrostatic pressure (HHP) treatments

The experimental design comprised of a control, three single-cycle treatments and three two-cycle treatments, with three replicates of each treatment performed. For the control, the Squid TMTp1 samples were held at ambient pressure (0.1 MPa and ~25°C), with no HHP treatment. The three single-cycle treatments were carried out at 200, 400 or 600 MPa for 20 min, named as S-200, S-400 and S-600, respectively. For the three two-cycle treatments, two 10 min cycles at 200, 400 or 600 MPa were performed, named as T-200, T-400 and T-600, respectively. The treatments used in this study were based on our previous study[23].

The HHP treatments were carried out using an HHP device (HHP-750, Kefa High

Pressure Food Processing Inc., Baotou, China), for a total holding time of 20 min. Water was used as the pressure transmitting medium, and the temperature was maintained at 25 ± 1°C by a circulating water system. The pressure vessel (90 mm diameter × 320 mm height) had a volume of 2.5 L and a pressure range of 0–700 MPa. The rate for the treatments to come-up to the top pressure was set at approximately 200 MPa/min and the decompression time after the treatment was immediate (<4 s), in order to minimize adiabatic heating[29]. After HHP treatments, all the samples were freeze dried in a Freezone 2.5 L Triad system (Labconco Inc., Missouri, USA), and stored at −80°C until further analysis.

2.3 AFM analysis

The squid TMTp1 morphology was characterized using AFM (Vecco Metrology Group, Digital Instruments, Santa Barbara, CA, USA). A 2 μL droplet of the squid TMTp1 (25 μg/mL) was spread onto a freshly cleaved mica disk, which was adhered to a stainless plate using double-sided tape. The sample was air-dried for 60 min at ambient temperature. The sample surface topography was then measured using Multimode Nanoscope AFM. Imaging was performed in the tapping mode, with a cantilever resonant frequency of around 330 kHz and scan rate of 2.441 Hz. Three subsamples of every treatment sample were scanned three times each, under ambient atmospheric conditions. The original data was levelled, to remove tilt, by applying a numerical second-order correction, and the mean values of surface roughness (R_a), maximum height (R_{max}), skewness and kurtosis were determined using NanoRule software (Pacific Nanotechnology, Santa Barbara, CA, USA).

2.4 NMR analysis

^{1}H NMR analyses of the squid TMTp1 samples were carried out on an Avance

Bruker III HD 600 MHz NMR spectrometer (Bruker Biospin, Rheinstetten, Germany), equipped with a 5 mm TCl CryoProbe and maintained at 25°C. The solvent used was D_2O and a final sample concentration of 10 mg/mL and 500 µl solution was used for each analysis. Solvent pre-saturation was employed to suppress the water peak. The standard Carr-Purcell-Meiboom-Gill pulse sequence was used to record the spectra; the Carr-Purcell-Meiboom-Gill pre-saturation pulse sequence worked in the form of RD-90°-(t-180°-t)$_n$-ACQ, where RD is the relaxation delay of 2 s, 90° and 180° represent the RF pulses that trip the magnetization vector, t is the spin-echo delay of 400 ms, n represents the number of loops (80 in this experiment), and ACQ is the data acquisition period of 1.36 s. In this experiment, the data points were acquired with 128 transients and the time delay was 5 s. The NMR spectrum was imported into Chenomx NMR Suite 7.7 software (Chenomx, Inc., Alberta, Canada). Subsequently, the spectra were imported to AMIX (Bruker Biospin, Rheinstetten, Germany) and were all reduced to fixed integral regions (0.04 ppm) for further analysis. The normalization method was performed according to previous study[31], based on the general equation:

$$S_{new}(i) = \frac{S_{old}(i)}{\sum_j \left\{ \int_{j_j^l}^{j_j^u} \left(S(x)\right)^n dx \right\}^{1/n}} \times 100\%$$

where $S_{old}(i)$ and $S_{new}(i)$ are the intensities of the variable i (spectral feature) before and after normalization, respectively; j is an index of the spectral regions used for normalization; and j_j^l and j_j^u are the lower and upper borders of the spectral region j, for which the power n of the intensities $S(x)$ is integrated.

2.5 Indirect ELISAs

An indirect ELISA was performed to analyse the allergenic properties of the

squid TMTp1 samples by measuring their hydrolysates after *in vitro* digestion, according to our previous methods[11]. An indirect ELISA with human sera of five allergic patients (P^1–P^5) was performed. In addition indirect ELISAs with rabbit anti-squid TMTp1 polyclonal antibodies (R), with rabbit anti-squid TMTp1 polyclonal antibodies for hydrolysates after Simulated Gastric Fluid (SGF) digestion (R-SGF) and with rabbit anti-squid TMTp1 polyclonal antibodies for hydrolysates after Simulated Intestinal Fluid (SIF) digestion (R-SIF) were performed. An automated ELISA plate reader (Thermo Co., USA) was used to monitor the absorbance at 450 nm. Three replicate measurements were carried out.

2.6 Statistical analysis

Results are reported as mean ± standard deviation (SD). The statistical differences between treatments were assessed using analysis of variance (ANOVA), followed by Tukey's HSD post hoc test ($p < 0.05$) using SAS 9.2 software (SAS Institute Inc., Cary, NC, USA).

3. Results and discussion

3.1 AFM surface topography of HHP treated squid TMTp1

The AFM topographic images revealed the structural differences among the squid TMTp1 samples subjected to different HHP treatments. In the AFM image of the control squid TMTp1 samples (Fig. 1A), the particles were distributed relatively evenly. The initial AFM image of the control squid TMTp1 samples presented similar characteristics to human cardiac α-tropomyosin[32, 33]. The S-200 treatment caused significant changes of the surface topography in the squid TMTp1 samples (Fig. 1B), in which the appearance of the surface roughness was darkened. The S-400 treatment caused a further increase in the maximum height and there was no uniformity between

the surface topographies of the treated and control samples (Fig. 1C); the surface became even more rough and wrinkled when the pressure was increased to 600 MPa, and the AFM images produced from the S-600 treatment were entirely different to the control (Fig. 1D). This indicates that the higher pressures caused more changes to the surface topographical properties of the squid TMTp1 during the single-cycle treatments. Much more significant changes of the surface topography of the squid TMTp1 were observed with the two-cycle HHP treatments (Fig. 1E-G). All of the two-cycle HHP treatments led a much more rough and wrinkled surface topography in comparison to S-600.

To consider the AFM outputs further, the surface topography indices of R_a, R_{max}, skewness and kurtosis, of the different HHP treated squid TMTp1 samples were considered. The R_a and R_{max} are the most commonly used descriptors of surface roughness[34]. Skewness is a measure of symmetry of the statistical distribution, and when it is 0 there is an even distribution of peaks and troughs, of specific heights; a surface with larger peaks than troughs has a positive skewness, and vice versa[18]. Kurtosis is a measure of the spikiness of the statistical distribution and a normal distribution has a kurtosis equal to 3. If the kurtosis is < 3, it corresponds to a statistical distribution that is flatter than the normal distribution, and the opposite is true for distributions with a kurtosis > 3[18]. Surfaces with varying R_a, R_{max}, skewness or kurtosis values have different functional properties[20].

In this study, the control had an R_a of 0.37 nm, R_{max} of 15.20 nm, skewness of 7.10 and kurtosis of 91.51. In general, the HHP treated squid TMTp1 samples had significantly higher R_{max} values (28.74–94.83 nm; $p < 0.05$), higher R_a values (except for S-200; 0.84–6.56 nm; $p < 0.05$) and different values of skewness and kurtosis. It is interesting that the single-cycle HHP treatments gave rise to higher skewness values,

while the two-cycle HHP treatments led to lower values compared with the control. Similar results were obtained for the kurtosis values (apart from S-200, which was slightly lower). The T-600 treatment produced the biggest difference in the indices from the control, with a R_a of 6.56 nm, R_{max} of 94.83, skewness of 3.05 and kurtosis of 23.13. There were significant differences between the indices of the of the squid TMTp1 samples produced by the single- and two-cycle HHP treatments at the same pressure level.

Biopolymers like proteins show transformation of their native structure after HHP[35]. However, there is limited information available about the chemistry behind the effect of HHP on the changes of surface properties of proteins. HHP might impact the hydrogen bonds, ionic or hydrophobic interactions with the modification of functional characteristics, thus changing the protein structures[29, 35, 36]. When HHP was applied, the water molecules might been squeezed into the free spaces between the semi-crystalline and amorphous lamellae, with greater forces produced by the higher pressure treatments[35], indicating that the higher pressures caused more changes to these intermolecular changes (hydrogen bonds, ionic and hydrophobic interactions) of the squid TMTp1 during the single-cycle treatments. Much more significant intermolecular changes of the squid TMTp1 were observed with the two-cycle HHP treatments (Fig. 1E-G). Compared with the control, T-600 treatment produced the biggest intermolecular changes.

Surfaces with varying AFM surface topography indices have different functional properties[18]. Altering the surface roughness influenced the chemical reactivity of proteins[20], which affected protein properties. The hydrogen bonds, ionic and hydrophobic interactions of protein closely associated with allergenic properties, for example, hydrogen bonds help stabilization of allergenicity characteristics[37]. Hence,

these intermolecular and protein structure changes could potentially affect the allergenicity characteristics in squid TMTp1 samples.

3.2 NMR analysis of HHP treated squid TMTp1

The ^{1}H NMR spectra of the control and HHP treated squid TMTp1 show characteristic water resonance regions (4.70–5.02 ppm; Fig. 2), which are not analysed in this study. The sharp peak observed at 3.70 ppm in the ^{1}H NMR spectra of the squid TMTp1 samples is due to residual Tris buffer[38]. All of the squid TMTp1 samples showed multiple signals at approximately 0.85, 1.28, 1.88, 2.16, 2.68, 2.88, 3.27, and 3.51 ppm, amongst other chemical shifts (Fig. 2 and Table 2); the changes of the positions of the peaks within the 0.75–3.51 ppm range of the ^{1}H NMR spectra for the different HHP treated squid TMTp1 could be attributed to the protons of the protein's amino groups. The changes in the peaks between the different samples were attributed to the effect of the single- and two-cycle HHP treatments.

The NMR spectra (0.71–3.51 ppm) were allocated to 0.04 ppm integral regions and subjected to normalization analysis. The relative percentage of each integral region after normalization of ^{1}H NMR spectra for the squid TMTp1 after each HHP treatment is shown in Fig. 3; the regions of 1, 2, 58 and 59 represented 0.71–0.75, 0.75–0.80, 3.43–3.47 and 3.47–3.51 ppm in the ^{1}H NMR spectra, respectively (Supplementary Table 1). The HHP treatments clearly increased $P_{0.75–0.80}$ and $P_{0.80–0.84}$, but decreased $P_{2.95–3.00}$, compared with the control. $P_{0.75–0.80}$ increased from 0.0058% (control) to 0.0215% (T-600), but $P_{2.95–3.00}$ decreased significantly, from 0.0261% (control) to 0.0014% (T-600) (Fig. 3). The single- and two-cycle HHP treatments conducted at the same pressure level also produced significantly different results (Fig. 3).

These results indicated that HHP treatments modified the structure of the squid

TMTp1 by impacting some characteristic groups and controlling the allergenic properties, which was consistent with previously published studies[39, 40]. In general, the NMR spectroscopic analysis of the squid TMTp1 demonstrated that the HHP treatments changed some related NMR characteristic regions and resulted in variations of the protein's allergenic properties. The changes of protein residues, which were related to the allergenic properties, could be found by NMR data through information on the positions, bonds, movements of specific atoms and the normalization method[39, 40]. These residues, which were found in many allergens by NMR[39, 40], play an important role in IgE/G binding and allergenicity of these allergens by reacting with epitopes[42]. The epitopes of allergens are essential for an allergen and development of specific allergen immunotherapy[39, 40]. This demonstrated that the higher pressures and two-cycle caused more changes to these residues of squid TMTp1 during the HHP treatments. From NMR date (Supplementary Table 1 and Fig. 3), there were the biggest changes to these residues in T-600 treated squid TMTp1 sample. However, the exact mechanisms causing the changes in the samples should be further studied.

3.3 Allergenic properties

Indirect ELISAs were performed to study the allergenic properties of squid TMTp1 using the human sera of five allergic patients (IgE-binding capacity; IgE) or rabbit anti-squid TMTp1 polyclonal antibodies (IgG-binding capacity; IgG). Overall, the HHP treatments resulted in lower IgE and IgG values, representing a decrease in allergenicity, and the two-cycle HHP treatments (Table 3) caused significant decreases. Significant differences ($p < 0.05$) in the IgE values were observed between the control and two-cycle HHP treated samples; the higher the pressure of the HHP treatment the lower the IgE values. As such, the T-600 samples had the lowest IgE

values, of 0.87, 0.24, 0.25, 0.22 and 0.25 for the five human sera, respectively. The IgG (R) of the control squid TMTp1 samples was 1.66 which was in good agreement with our previous report[11]. The IgG (R) values clearly show that the two-cycle HHP treatments resulted in significantly lower binding capacities, compared with the control treatment (Table 3). The changes of the IgG of hydrolysates after *in vitro* digestibility (R-SGF and R-SIF) were subject to the variations of the control and HHP treated samples, and the control samples had the highest IgG (R-SIF) value (0.67). Previously, we reported the IgE and IgG of squid TMTp1 treated with HHP at 200, 400 or 600 MPa for 20 min[11]. The two-cycle HHP treatments showed significantly lower IgE and IgG values of the squid TMTp1 samples, compared to our previous study[11]. For example, the IgG (R) value of squid TMTp1 treated with a single-cycle of HHP at 200 MPa was 1.34, while the two-cycle treatment at the same pressure resulted in a significantly lower binding of 1.15.

Recent research has shown that the activity of phytoferritin (i.e., the iron release activity) is dramatically enhanced after HHP treatment[41], and the immunoreactivity of soybean seeds is reduced by HHP treatment at 300 MPa for 15 min, maintained at 40°C[9] . Also, HHP has been shown to induce structural unfolding, protein denaturation, and even dissociation of some proteins into subunits to change their functional properties[36, 42]. Meanwhile, multiple cycles of HHP have been shown to produce a more powerful percussive action and shear effect, resulting in a significant enhancement of the HHP effectiveness[23, 43, 44].

In this study, two-cycle HHP caused a more pronounced alteration of the protein structure of squid TMTp1, than single-cycle HHP (Fig. 1, 2 and 3) and these changes caused lower IgE and IgG values. These results indicate that HHP could decrease the allergenic properties of squid TMTp1, and that increasing the pressure level as well as

performing two-cycles of HHP creates more significant effects on controlling the allergenic properties.

3.4 Correlation among AFM surface topography, NMR characteristic domain and allergenicity of HHP treated squid TMTp1

Correlation analyses among the pressure level, cycle numbers, IgE, IgG and AFM surface topography of squid TMTp1 samples are presented in Supplementary Table S2. The correlations among the IgE and IgG values (i.e., the allergenic properties) are really high ($R \geq 0.79$, $p < 0.001$). The negative correlations between the pressure levels and the IgE values were all significant ($p < 0.001$), and there was also a significant negative correlation between pressure levels and the IgG values ($p < 0.01$). There were negative correlations between the cycle numbers and both skewness ($R = -0.80$, $p < 0.001$) and kurtosis ($R = -0.85$, $p < 0.001$), but the cycle numbers was positively correlated with R_a ($R = 0.90$, $p < 0.001$) and R_{max} ($R = 0.88$, $p < 0.001$). These results demonstrated that the pressure level and cycle number had significant correlation with the modification of the allergenic and AFM surface topography measures in squid TMTp1. In accordance with this, changes of the allergenic properties, caused by the HHP treatments, were significantly correlated to the AFM surface topography indices.

Correlation analyses between the NMR results, pressure level, cycle numbers, allergenic and AFM surface topography for the squid TMTp1 samples are presented in Supplementary Table S1. The relationships between the HHP pressure and NMR $P_{0.99–1.03}$, $P_{1.79–1.84}$, $P_{2.85–2.90}$, $P_{3.36–3.39}$ and $P_{3.40–3.43}$ indices were all significant ($p < 0.05$); the correlations of these NMR indices with the HHP mode were also significant ($p < 0.05$). This indicates that the HHP pressure and HHP mode significantly impacted upon some NMR characteristic regions of the squid TMTp1 samples. The

main ranges of the characteristic regions for the allergenic properties were 0.99–1.03, 1.19-1.24, 1.44-1.49, 1.79-1.84, 2.85-2.90, 3.36-3.39 and 3.40-3.43 ppm (Fig. 3). For example, there was a peak at 0.99 ppm, in the range of 0.99–1.03 ppm, for the squid TMTp1 sample of the control treatment, however, there was no peak in this range for the HHP treated samples (Table 2). The peak of 0.99 ppm in this study was the trimethylamine signal, a proton of $-CH_3$, and multiple of (t, 7)[45]. This means that HHP changed the $-CH_3$ group, which led to the modification of the allergenic properties (IgE and IgG values). The main allergenic characteristic peaks that were affected by HHP were 0.99, 1.16, 1.21, 1.79, 1.82, 2.84, 2.88 and 3.37 ppm, as observed in the control ^{1}H NMR spectra (Table 2). It is important to note that the HHP mode (cycle numbers) is highly relevant to the changes of the NMR data, which resulted in the variation of the allergenic properties and AFM surface topographies.

There were negative correlations between $P_{0.99–1.03}$ and both the R_a and R_{max} ($R \geq 0.76$, $p < 0.001$), as well as $P_{1.49–1.53}$ and both the R_a and R_{max} ($R \geq 0.74$, $p < 0.001$); however, $P_{3.32–3.36}$ and $P_{3.36–3.39}$ were positively correlated with R_a and R_{max} ($R \geq 0.84$, $p < 0.001$). The AFM indices of skewness (S) and kurtosis (K) contradicted the results, compared to the tendencies of variation in R_a and R_{max}. The ranges of the main characteristics for the AFM surface topography were 1.04–1.08, 1.49–1.53, 1.64–1.69, 1.74–1.79, 3.15–3.20, 3.24–3.27, 3.32–3.36 and 3.36–3.39 ppm. Further, the peaks of the main characteristics for the AFM surface topography that were affected by HHP were 1.06, 1.79, 3.23, 3.27 and 3.37 ppm (as identified in the control ^{1}H NMR spectra (Table 2). For example, there was a peak at 3.37 ppm, in the range of 3.32–3.39 ppm, for the control squid TMTp1 samples, however, there were peaks at 3.35 ppm in this range for all the HHP treated samples (Table 2). The peak at 3.37 ppm in this study was a characteristic signal of dimethyl ether, a proton of $-CH_3$, and

multiple of (s)[45]. This means that HHP induced this -CH_3 group migration and led to changes of the AFM surface topography (R_a, R_{max}, S and K indices).

The results of this study reveal that the changes (by migration or disappearance) of related NMR characteristic regions, caused by the HHP treatments, significantly impacted upon the allergenic and AFM surface topography of the squid TMTp1. The HHP mode significantly correlated ($p < 0.05$) with the variation of the NMR indices, which resulted in reducing the allergenicity and changing the structure of the squid TMTp1 samples.

4. Conclusion

HHP treatments resulted in the modification (by migration or disappearance) of related NMR characteristic regions in squid TMTp1 samples, which in turn caused a reduction in the allergenicity and change of AFM surface topography. The 400 MPa and 600 MPa single-cycle HHP treatments to squid TMTp1 caused significant increases in roughness and maximum height, and the surface topography was no longer uniform in comparison with the control. However, the single-cycle HHP treatments gave rise to higher skewness and kurtosis values, while two-cycle HHP led to lower values. In addition, the two-cycle HHP treatments significantly controlled the allergen (lower R values of 1.15–1.09).

The HHP treatments affected the squid TMTp1 by modifying some of the related NMR characteristic regions, apparent from the NMR spectroscopic analysis. The cycle numbers (mode) of HHP treatments significantly correlated ($p < 0.05$) with the NMR indices, which resulted in reductions in the allergenicity and change in the surface topography of the squid TMTp1 samples. The peaks of the main allergenic characteristics affected by HHP were 0.99, 1.16, 1.21, 1.79, 1.82, 2.84, 2.88, and 3.37 ppm (as identified in the control ^{1}H NMR spectra), and the peaks of the AFM

405 characteristics were 1.06, 1.79, 3.23, 3.27, and 3.37 ppm. The NMR spectra
406 demonstrated that HHP treatments affected the structural characteristics of the squid
407 TMTp1 samples by impacting the main characteristic regions; by increasing the
408 pressure level and performing two-cycles (instead of one), the HHP treatment was
409 more effective. This study provided meaningful information for the use of HHP as a
410 non-thermal, minimal processing technology to change some NMR characteristic
411 regions and control seafood allergens.
412

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505 **Figure captions**

506 **Fig. 1.** Atomic force microscopy topographic images of squid TMTp1 subjected to
507 different high hydrostatic pressure treatments.
508 A: untreated squid TMTp1; B-D: single-cycle HHP treated at 200, 400 or 600 MPa
509 for 20 min, respectively, and E-G: two 10-min cycles at 200, 400 or 600 MPa,
510 respectively.
511
512 **Fig. 2.** ^{1}H nuclear magnetic resonance spectra of different high hydrostatic pressure
513 treated squid TMTp1 samples in D_2O solution at 25°C.
514 S-200, S-400 or S-600: single-cycle HHP treated at 200, 400 or 600 MPa for 20 min,
515 respectively, and T-200, T-400 or T-600: two 10-min cycles at 200, 400 or 600 MPa,
516 respectively.
517
518 **Fig. 3.** The relative percentage of each integral region after normalization of ^{1}H NMR
519 spectra for different high hydrostatic pressure treated squid TMTp1 samples.
520 The main allergenicity characteristic domain of the HPP treated squid TMTp1 were:
521 A (number 7: range 0.99–1.03 ppm), B (11: 1.19–1.24 ppm), C (16: 1.44–1.49 ppm),
522 D (23: 1.79–1.84 ppm), E (45: 2.85–2.90 ppm) and F (56, 57: 3.36–3.43 ppm).

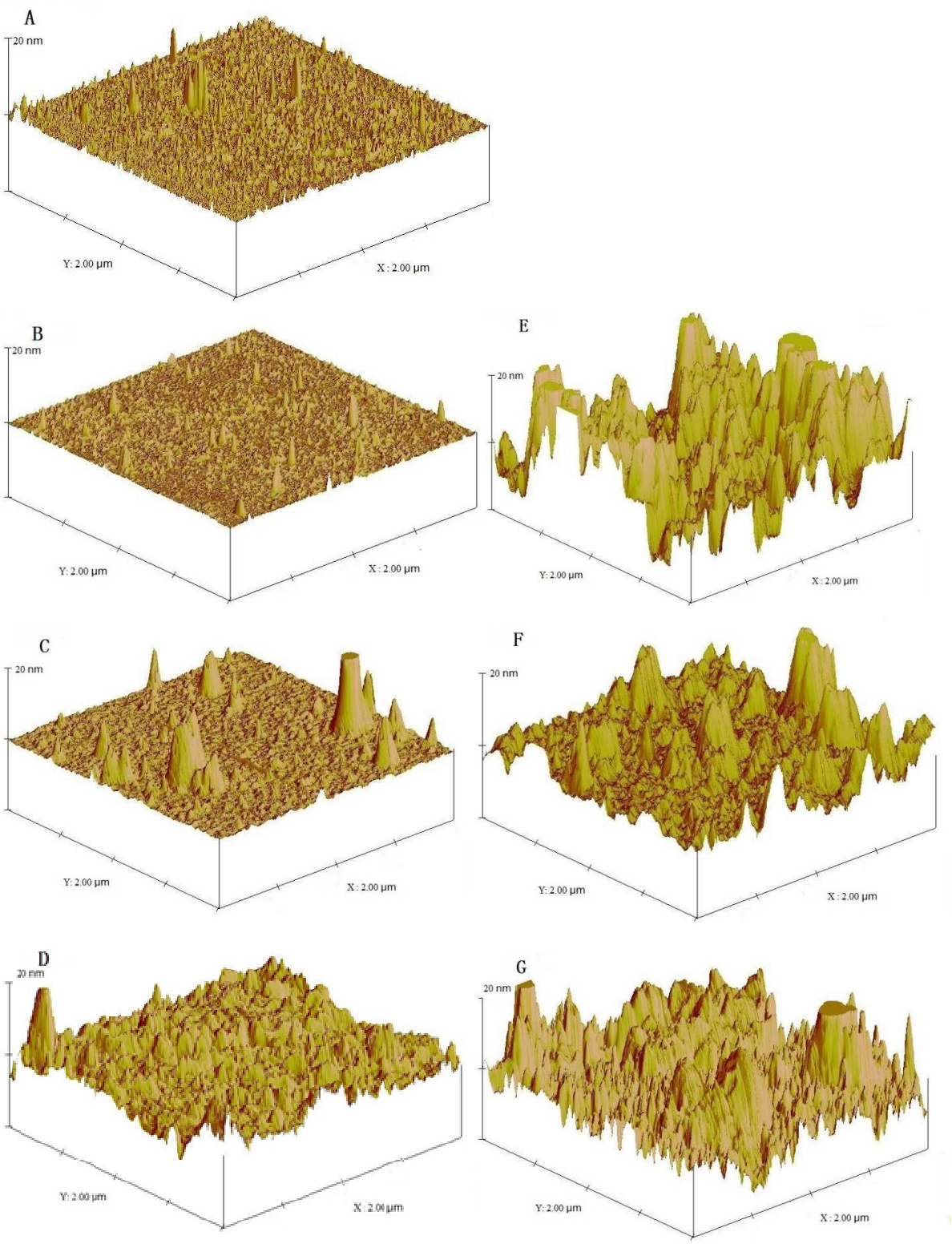

Fig. 1. Atomic force microscopy topographic images of squid TMTp1 subjected to different high hydrostatic pressure treatments.

A: untreated squid TMTp1; B-D: single-cycle HHP treated at 200, 400 or 600 MPa

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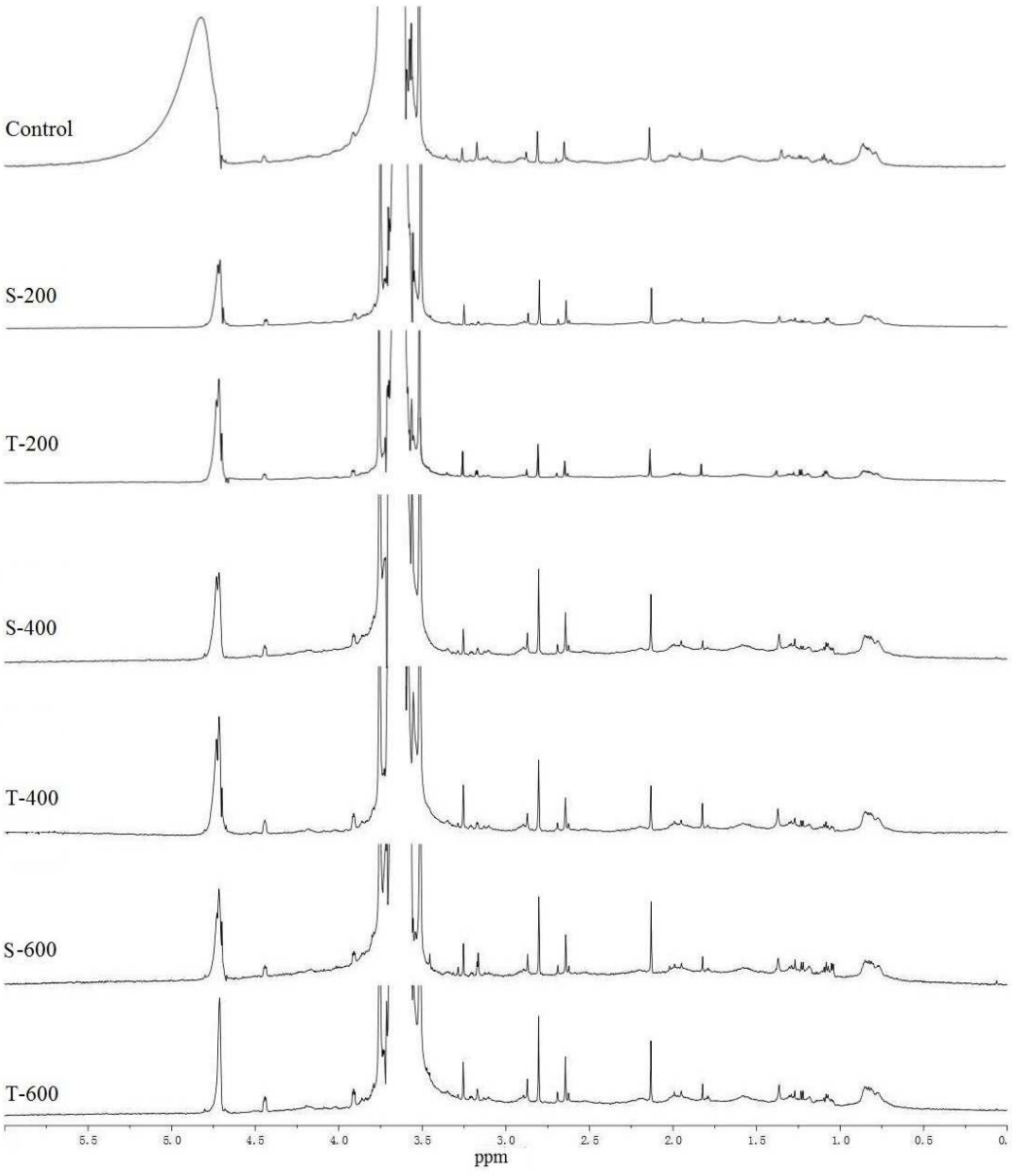

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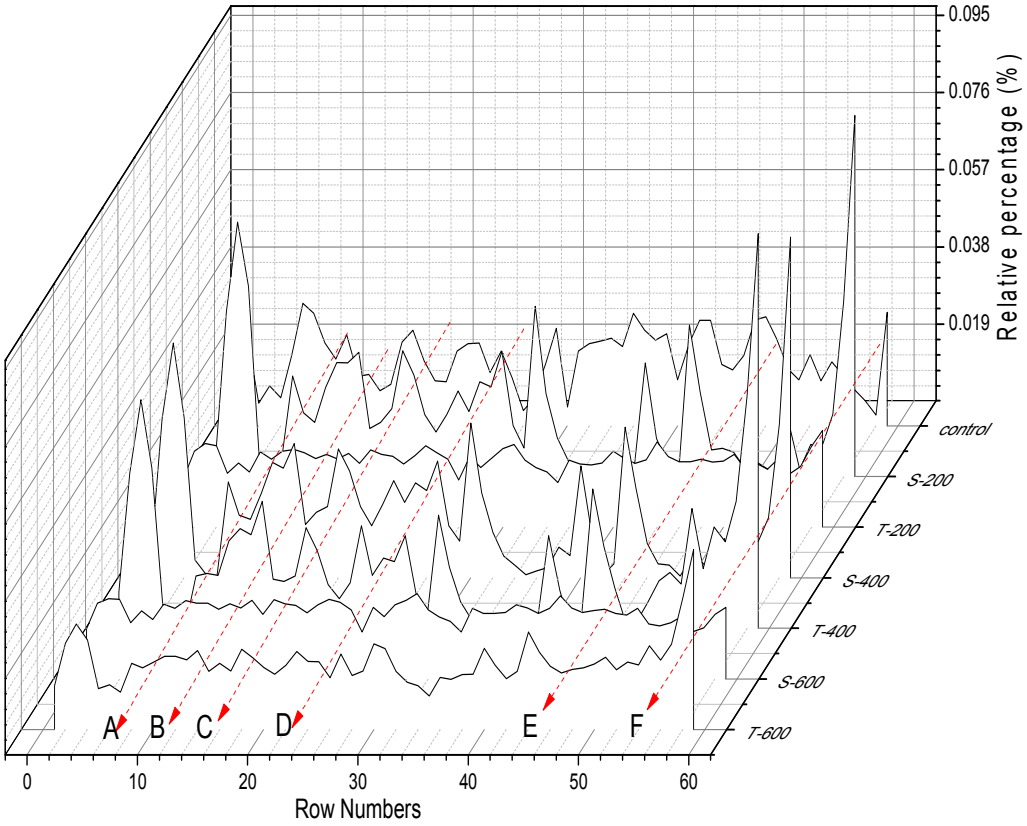

Fig. 3- The relative percentage of each integral region after normalization of ^{1}H NMR spectra for different HHP treated squid TMTp1.

The main allergenicity characteristic domain of the HPP treated squid TMTp1 were: A (number 7: range 0.99–1.03 ppm), B (11: 1.19–1.24 ppm), C (16: 1.44–1.49 ppm), D (23: 1.79–1.84 ppm), E (45: 2.85–2.90 ppm) and F (56, 57: 3.36–3.43 ppm).

544 **Table captions**

545 **Table 1**. Atomic force microscopy surface topography indices of the squid TMTp1

546 samples.

547 **Table 2**. Changes of the positions of peaks in the ^{1}H nuclear magnetic resonance

548 spectra of the squid TMTp1 samples.

549 **Table 3**. Results of the indirect ELISAs performed with the squid TMTp1 samples

550 against IgE and IgG.

551 **Table 1**. Atomic force microscopy surface topography indices of the squid TMTp1

552 samples.

553

Treatment group	Mode	Pressure (MPa)	AFM indices[#*]			
			Mean roughness (R_a, nm)	Max height (R_{max}, nm)	Skewness	Kurtosis
Control	-	0.1	0.37 ± 0.02^{f}	15.20 ± 0.50^{g}	7.10 ± 0.26^{b}	91.51 ± 0.32^{b}
S-200	Single	200	0.35 ± 0.02^{f}	28.74 ± 0.40^{f}	7.33 ± 0.36^{b}	85.58 ± 0.66^{c}
S-400	Single	400	0.84 ± 0.04^{e}	33.02 ± 0.26^{e}	8.58 ± 0.24^{a}	102.81 ± 0.53^{a}
S-600	Single	600	1.66 ± 0.01^{d}	29.31 ± 0.92^{d}	8.55 ± 0.55^{a}	100.06 ± 1.62^{a}
T-200	Two	200	5.60 ± 0.16^{c}	53.98 ± 0.96^{c}	2.67 ± 0.20^{c}	22.95 ± 0.49^{d}
T-400	Two	400	5.10 ± 0.04^{b}	85.29 ± 1.02^{b}	2.73 ± 0.56^{c}	24.31 ± 0.37^{d}
T-600	Two	600	6.56 ± 0.06^{a}	94.83 ± 0.89^{a}	3.05 ± 0.26^{c}	23.13 ± 0.79^{d}

554 [#] Values are expressed as average ± standard deviation (SD) (n = 3).

555 * Means in the same column with different lowercase letters (a–g) are significantly

556 different ($p < 0.05$).

557 **Table 2**. Changes of the positions of peaks in the ^{1}H nuclear magnetic resonance

558 spectra of the squid TMTp1 samples*.

	The position of peaks (ppm)						
	Control	S-200	S-400	S-600	T-200	T-400	T-600
1	3.51	3.51	3.51	3.51	3.51	3.51	3.51
2			3.46	3.47		3.46	3.47
3	3.37	3.35	3.35	3.35	3.35	3.35	3.35
4	3.29	3.28	3.28	3.29	3.29	3.28	3.29
5	3.27	3.26	3.25	3.26	3.25	3.25	3.26
6	3.23	3.23	3.21	3.21	3.21	3.21	3.21
7	3.17	3.17	3.17	3.17	3.17	3.17	3.17
8	3.14	3.13	3.13		3.13		
9		3.1	3.1	3.1	3.1	3.1	3.1
10	3.07	3.06					
11	3.00	3					
12	2.88	2.87	2.87	2.87	2.87	2.87	2.87
13	2.84						
14	2.81	2.8	2.8	2.8	2.8	2.8	2.8
15	2.78						
16	2.68	2.69	2.69	2.69	2.69	2.69	2.69
17	2.65	2.64	2.64	2.64	2.64	2.64	2.64
18	2.63	2.63	2.62	2.62	2.62	2.62	2.62
19	2.57	2.53	2.53	2.53	2.52	2.52	
20	2.39						
21	2.16	2.19	2.19	2.19	2.2	2.2	2.18
22	2.13	2.13	2.13	2.13	2.13	2.13	2.13
23	2.10						
24	1.98	1.99	1.99	1.99	1.99	1.99	1.99
25	1.88	1.95	1.95	1.95	1.95	1.95	1.95
26	1.82	1.82	1.82	1.82	1.82	1.82	1.82
27		1.79	1.79	1.79	1.79	1.79	1.79
28	1.70						
29		1.59	1.56	1.59	1.58	1.58	1.58
30	1.43						
31		1.37	1.37	1.37	1.37	1.37	1.37
32	1.34						
33	1.32						
34	1.28	1.29	1.29	1.29	1.29	1.29	1.29
35	1.26	1.27	1.27	1.27	1.27	1.27	1.27
36	1.23	1.23	1.23	1.23	1.23	1.23	1.23
37	1.21	1.22	1.22	1.22	1.22	1.22	1.22
38	1.16	1.18	1.18	1.19	1.19	1.19	1.18
39	1.09	1.1	1.09	1.1	1.1	1.09	1.1
40	1.08	1.08	1.08	1.08	1.08	1.08	1.08
41	1.06	1.07	1.07	1.07	1.07	1.07	1.07
42				1.05	1.05	1.05	1.05

43	0.99						
44	0.85	0.85	0.85	0.85	0.85	0.85	0.85
45	0.83	0.83	0.83	0.83	0.83	0.83	0.83
46	0.82	0.82	0.81	0.82	0.82	0.81	0.82
47		0.78	0.78	0.78	0.78	0.77	0.78

559

560 *S-200, S-400 and S-600 were the single-cycle high hydrostatic pressure (HHP)

561 treatments, maintained at 200, 400 and 600 MPa for 20 min, respectively. T-200,

562 T-400 and T-600 were the two-cycle HHP treatments comprised of two 10 min

563 cycles, at 200, 400 and 600 MPa, respectively.

564 **Table 3**. Results of the indirect ELISAs performed with the squid TMTp1 samples against IgE and IgG.

565

Treatment group	IgE binding*					IgG binding#		
	P^1	P^2	P^3	P^4	P^5	R	R-SGF	R-SIF
Control	1.67 ± 0.04^{a}	0.82 ± 0.01^{a}	0.74 ± 0.01^{a}	0.55 ± 0.06^{a}	0.47 ± 0.02^{a}	1.66 ± 0.03^{a}	1.37 ± 0.06^{a}	0.67 ± 0.03^{a}
T-200■	1.08 ± 0.07^{b}	0.28 ± 0.01^{b}	0.31 ± 0.02^{b}	0.24 ± 0.03^{b}	0.28 ± 0.01^{b}	1.15 ± 0.02^{bc}	0.90 ± 0.03^{bc}	0.41 ± 0.02^{b}
T-400	0.98 ± 0.05^{c}	0.26 ± 0.02^{bc}	0.28 ± 0.03^{bc}	0.23 ± 0.04^{b}	0.25 ± 0.01^{c}	1.12 ± 0.03^{cd}	0.86 ± 0.04^{cd}	0.42 ± 0.03^{b}
T-600	0.87 ± 0.05^{d}	0.24 ± 0.01^{c}	0.25 ± 0.02^{c}	0.22 ± 0.02^{b}	0.25 ± 0.01^{c}	1.09 ± 0.01^{d}	0.84 ± 0.02^{d}	0.37 ± 0.02^{c}

566

567 ■T-200, T-400 and T-600 are the two-cycle HHP treatments, comprised of two 10 min cycles, at 200, 400 and 600 MPa, respectively.

568 *P^1–P^5: indirect ELISAs performed with human sera of five allergic patients.

569 #R: indirect ELISA performed with rabbit anti-squid TMTp1 polyclonal antibodies. R-SGF: indirect ELISA performed with rabbit anti-squid

570 TMTp1 polyclonal antibodies for hydrolysates after Simulated Gastric Fluid digestion (SGF) digestion. R-SIF: indirect ELISA performed with

571 rabbit anti-squid TMTp1 polyclonal antibodies for hydrolysates after Simulated Intestinal Fluid (SIF) digestion.

572 [a–d]Values are expressed as mean ± standard deviation (SD) (n = 3); means in the same column with different lowercase letters (a–d) are

573 significantly different ($p < 0.05$).