

Application of high pressure processing to improve digestibility, reduce allergenicity, and avoid protein oxidation in cod (*Gadus morhua*)

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ABSTRACT

In this study, comprehensive analysis was performed to evaluate the impact of high hydrostatic pressure (HHP) and traditional thermal processing methods (baking and steaming) on cod proteins. Results showed that HHP, but not baking or steaming, was able to increase the content of soluble protein nitrogen (1.42-fold), compared with control. Total peptide contents of HHP-treated samples were also significantly higher than baked and steamed ones. In addition, protein oxidation was greatly increased after baking (1.56-fold) and steaming (1.97-fold), whereas HHP did not exhibit any appreciable effect. Furthermore, the allergenicity of cod was significantly reduced after HHP as reflected by the attenuated IgE and IgG-binding capacities (67–84% relative to control), while baking and steaming resulted in higher allergenicity. This study strongly supports the potential of HHP for reducing allergenicity, avoiding protein oxidation, and improving digestibility of cod and other protein-rich foods susceptible to quality deterioration during thermal processing.

1. Introduction

Cod is well-known for its high quality protein, which is a rich source of amino acids and functional peptides (Broekman et al., 2011). Numerous studies have reported that cod and similar seafood species have balanced amino acid profiles, high antioxidant and anti-inflammatory nutrient contents, and structurally diverse bioactive components, with an array of potential physiological functions in humans and applications as natural food additives (Kim & Wijesekara, 2010; Ngo et al., 2011). For example, bioactive peptides (gastrin/CCK- and CGRP-like peptides) possessing antioxidative properties have been obtained from cod by protein hydrolysis and these peptides have been incorporated in many functional food products (Slizyte et al., 2009).

Despite its nutritional value and potential health benefits, allergy to cod has been identified to be one of the most common, severe, and long-lasting seafood allergies. Symptoms of cod allergy range from angioedema, gastrointestinal distress and rhinitis to asthma and anaphylaxis (Sophia et al., 2012). The 12.3-kDa parvalbumin with two calcium-binding sites corresponding to EF-hand motifs is the major allergen in cod. Furthermore, many new allergens have been characterized in cod over the past two decades (Sophia et al., 2012). Food processing technologies are known to contribute to chemical changes of the amino acid residues that result in protein modification (Pelitli, Janiak, Amarowicz,

& Alasalvar, 2017). Unfortunately, most traditional processing methods have failed to attenuate the allergenicity of seafood proteins, including cod allergens (Galland et al., 1998). In the meantime, high hydrostatic pressure (HHP), a non-thermal food processing technology, has been shown to be highly effective in reducing the allergenicity of many foods, such as squid (Zhang, Deng, & Zhao, 2017), soybean seeds (Penas, Gomez, Frias, Baeza, & Vidal, 2011) and almond milk (Dhakal et al., 2014). Our previous studies have also demonstrated that HHP is an effective method for controlling microbial growth, modifying seafood protein structure, and reducing the allergenicity of seafood proteins (Zhang, Dai, Deng, & Zhao, 2015; Zhang, Dai, Deng, & Zhao, 2016; Zhang et al., 2017; Zhang, Wang, Bi, Cheng, & Chen, 2019). However, there is limited information regarding the different effects of traditional food processing methods and HHP on protein quality and allergenicity of cod and other types of seafood.

Analytical measurement of proteins provides a solid basis for evaluating nutritional properties and protein quality of food products and the human diet (Rand, Pellett, & Young, 2003). Meanwhile, the different types of food processing can cause chemical changes in proteins and release bioactive peptides and/or free amino acids, which can result in the modification of structure and/or functional properties of proteins (Chang, Alasalvar, Bolling, & Shahidi, 2016; Korhonen, Pihlanto, Rantamaki, & Tupasela, 1998). For example, changes of

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soluble protein nitrogen (SPN) and *in vitro* digestibility are commonly used indicators of changes in solubility and digestibility of seafood proteins (Zhang et al., 2016). Changes in the total peptide content can be used to determine the extent of protein destruction and peptide release in food during processing. In addition, changes in free sulfhydryl content, carbonyls, and surface hydrophobicity are widely used to evaluate structural changes in food proteins. For instance, the amount of carbonyl groups is usually used to evaluate oxidative deterioration of proteins during processing (Armenteros, Morcuende, Ventanas, & Estévez, 2016). The availability of these well-established indicators allows comprehensive analysis of the impact of various processing techniques on the quality of food products.

The present study aimed to investigate the respective impact of baking, steaming and HHP on protein quality (electrophoresis, protein content, and nitrogen content) and allergenicity (indirect enzyme-linked immunosorbent assay (ELISA), using sera from allergic human subjects or rabbit anti-cod protein polyclonal antibodies) of cod. Furthermore, changes in the physiochemical properties of cod proteins after the above treatments were also analyzed by measuring their *in vitro* digestibility, peptide content, carbonyl content, free sulfhydryl content, and surface hydrophobicity index.

2. Materials and methods

2.1. Materials and sample preparation

Cod (*Gadus morhua*, 550 ± 30 g per whole cod), a common and economically important species of marine fish (Girgih et al., 2015), was obtained from a supermarket (Jingshen seafood market, Beijing, China) in January 2018. After thawing, cleaning, and dissecting the cod. Slabs of cod muscle (120 ± 15 g, length, width, and thickness were 7.5 ± 0.5 cm, 10.5 ± 1.0 cm, and 2.0 ± 0.2 cm, respectively) were subjected to the different treatments as described below.

2.2. Treatments

The cod slabs were baked, steamed or treated with HHP. Cod samples maintained at ~25 °C for 20 min were used as the control. Baking was performed in a baking oven (T3-L326, Midea, Midea Holding Co. Ltd, Guangdong, China) at 100 °C for 20 min. Steaming was performed at 100 °C for 20 min, and HHP treatment was performed at 200 MPa/min at 25 °C for 20 min using the HHP system (HHP-750, Kefa High Pressure Food Processing Inc., Baotou, China). Water in the circulating system also served as the pressure-transmitting medium during HHP treatment.

2.3. Protein content of cod

Protein content (PC) of cod samples was determined according to the standard method of the Association of Official Analytical Chemists (AOAC, 1995). Experiments were performed in triplicate. And PC of the cod samples were calculated as follows (AOAC, 1995):

$$PC(\%) = \frac{(V_s - V_0) \times a \times 0.014}{M} \times 6.25 \times 100\%$$

where PC is expressed in wt% (g protein/100 g cod muscle), *a* is the concentration of hydrochloric acid (0.1012 mol/L), *M* is the weight of the cod sample (g), *V_s* and *V₀* are the titration volumes of cod samples and the blank (mL), respectively.

2.4. Nitrogen content of cod

Soluble protein nitrogen (SPN) content, non-protein nitrogen (NPN) content, and total nitrogen (TN) content of the cod samples were determined according to a previously described method (Zhao et al., 2008). All cod samples were freeze-dried using a Freezone 2.51 Triad

system (Labconco Corp., Kansas City, MO, USA) and stored at −80 °C before nitrogen analysis. In brief, for SPN, cod muscle (~3 g) was extracted with buffer (30 mL, 50 mM PBS, 0.1% PMSF, pH 6.0) by homogenization at 15,000 × g for 1 min (Z236 K, HERMLE Labortechnik GmbH, Wehingen, Germany). The homogenate was centrifuged at 12,000 × g for 45 min, and the supernatant was collected for Kjeldahl determination. The SPN results were expressed in g/100 g dried cod. For NPN, cod muscle (~3 g) was extracted with buffer (30 mL, 50 mM PBS, 0.1% PMSF, 10% TCA, pH 6.0) by homogenization at 15,000 × g for 1 min. The homogenate was then centrifuged at 12,000 × g for 15 min and the supernatant was obtained for Kjeldahl determination. The NPN results were expressed in g/100 g dried cod. For TN, cod muscle (~3 g) was extracted with water (30 mL) by homogenization at 15,000 × g for 1 min, centrifuged at 12,000 × g for 45 min, and then 10% TCA (30 mL) was added. After that, the solution was centrifuged at 12,000 × g for 15 min for Kjeldahl determination. The TN results were expressed in g/100 g dried cod. Each analysis was performed in triplicate.

2.5. *In vitro* digestibility of cod

In vitro digestibility was measured under simulated gastrointestinal conditions (Kaur, Rutherford, Moughan, Drummond, & Boland, 2010). For *in vitro* gastric digestion (IVGD), 2.3 g cod samples were dispersed in 0.1 M HCl (17 mL) at 37 °C for 30 min. Next, pepsin solution (2.5 mL, enzyme-to-substrate ratio (w/w) = 1:100, 2.5 mg of pepsin per 1.0 mL of 0.1 M HCl) was added to simulate gastric digestion at pH = 1.9. After reacting for 60 min, the pH was adjusted to 8.0 by adding 1.0 M NaOH to inactivate the pepsin. IVGD was calculated as follows (Kaur et al., 2010):

$$IVGD(\%) = \frac{N_2}{N_1} \times 100\%$$

where *N₁* and *N₂* are the total protein nitrogen content of the cod sample and the protein nitrogen content of the supernatant after gastric digestion, respectively.

For *in vitro* pancreatic digestion (IVPD), the samples after IVGD were added to pH 8.0 pancreatin solution (2.5 mL, enzyme-to-substrate ratio (w/w) = 1:30, 2.5 mg of pancreatin per 1.0 mL of 0.1 M pH 8.0 PBS). After IVPD for 2 h, 10% trichloroacetic acid (5 mL) was added. After that, the solution was centrifuged at 8000 × g for 5 min. Then the supernatant was obtained for the Kjeldahl determination. Three replications were performed. IVPD was calculated as follows (Kaur et al., 2010):

$$IVPD(\%) = \frac{N_3}{N_1} \times 100\%$$

where *N₁* and *N₃* are the total protein nitrogen content of the cod sample and the protein nitrogen content of the supernatant after pancreatic digestion, respectively.

2.6. Protein extraction from cod

Protein of cod samples were extracted based on the methods of Arnesen and Gildberg (2006) and Oppen-Berntsen, Helvik, and Walther (1990), with some modifications. Briefly, cod samples (1.5 g) from different treatments were immersed in buffer solution (30 mL; 0.1 M PBS, 1 mM EDTA, and 1 mM Tris; pH 6.0) and homogenized at 15,000 × g for 1 min. The homogenates were then mixed at 100 × g for 120 min and centrifuged at 12,000 × g for 45 min. The resulting supernatant was analyzed to determine the total soluble protein content of the cod samples.

2.7. Electrophoresis of cod proteins

Protein extract of the cod samples was analyzed using sodium

Table 1

Changes in protein content, soluble protein nitrogen, non-protein nitrogen, total nitrogen, and *in-vitro* digestibility of baked, steamed and high hydrostatic pressure (HHP)-treated cod samples.

	Protein content (PC, %, g/100 g wet cod)	Soluble protein nitrogen (SPN, %, g/100 g dry cod)	Non-protein nitrogen (NPN, %, g/100 g dry cod)	Total nitrogen (TN, %, g/100 g dry cod)	<i>In-vitro</i> gastric digestibility (IVGD, %)	<i>In-vitro</i> pancreatic digestibility (IVPD, %)
Control	11.62 ± 0.90 ^C	5.27 ± 0.16 ^B	0.69 ± 0.03 ^A	13.39 ± 0.89 ^A	34.47 ± 2.01 ^C	56.24 ± 1.82 ^C
Baked	15.84 ± 0.48 ^A	5.12 ± 0.18 ^B	0.70 ± 0.03 ^A	12.32 ± 0.52 ^A	44.09 ± 3.84 ^B	63.86 ± 1.52 ^B
Steamed	13.07 ± 0.36 ^B	5.63 ± 0.47 ^B	0.70 ± 0.09 ^A	12.73 ± 1.00 ^A	44.25 ± 2.58 ^B	65.47 ± 1.55 ^B
HHP	11.78 ± 1.03 ^{BC}	7.49 ± 0.29 ^A	0.72 ± 0.06 ^A	13.37 ± 0.50 ^A	53.46 ± 1.93 ^A	73.29 ± 3.02 ^A

^{A-C}Values are expressed as mean ± standard deviation (SD) (n = 3); means in the same column with different letters are significantly different ($P < 0.05$).

dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as described previously (Jin et al., 2015; Zhang et al., 2017). Polyacrylamide slab gels formed from 10% acrylamide solution were used. HiMark Pre-Stained Protein Standard (LC5699, Thermo Scientific, Massachusetts, USA) was used as the marker for MW determination of the protein bands.

2.8. Protein carbonyl content of cod proteins

Protein carbonyl content (PCC) was measured to analyze protein oxidation (Armenteros et al., 2016). Briefly, protein from cod samples was precipitated using cold 10% TCA and homogenized at 800 × g for 5 min (Z236 K, HERMLE Labortechnik GmbH, Wehingen, Germany). One pellet was treated with 2 M HCl (1 mL, protein concentration measurement) and another was treated with an equal volume of 0.2% (w/v) dinitrophenylhydrazine (DNPH) in 2 M HCl (carbonyl content measurement). Both samples were incubated for 1 h at room temperature, precipitated using 10% TCA, and washed twice with ethanol/ethyl acetate (1 mL, 1:1, v/v) to remove DNPH. The pellets were then dissolved in 20 mM sodium phosphate buffer containing 6 M guanidine HCl (1.5 mL, pH 6.5), stirred, and centrifuged at 620 × g for 2 min to remove insoluble fragments. The amount of carbonyls was expressed as nmol of carbonyl per mg of protein using an absorption coefficient of 21 nm⁻¹·cm⁻¹ at 370 nm for protein hydrazones.

2.9. Allergenicity analysis of cod proteins

Indirect ELISA with the sera from five allergic human subjects (P₁–P₅) was performed to determine the allergenicity of the cod protein extract samples using previous method (Jin et al., 2015; Zhang et al., 2015; Zhang et al., 2017). All experiments were performed in accordance with the requirements of the Guide for the Care and Use of Laboratory Animals and Humans of Peking University (PKU). Furthermore, indirect ELISA was also performed with rabbit anti-cod protein polyclonal antibodies (R) to determine allergenicity of cod protein hydrolysates after simulated gastric fluid digestion (R_{SGF}) and simulated intestinal fluid digestion (R_{SIF}), respectively. Three replicate measurements were performed.

2.10. Total peptide content of cod proteins

Total peptide content (TPC) was determined using a trinitrobenzene sulfonic acid method (Lee, Kim, & Lee, 2018; Wiśniewski & Rakus, 2014). Experiments were performed in triplicate.

2.11. Free sulfhydryl content of cod proteins

Free sulfhydryl content (FSC) was determined according to a previously reported method with modifications (Lee et al., 2016). Measurements were performed in triplicate. FSC was calculated as follows (Lee et al., 2016):

$$\text{FSC}(\mu\text{mol/gprotein}) = \frac{A_{412}}{C} \times 73.53 \times D$$

where FSC is expressed in μmol/g cod protein, A₄₁₂ is the absorbance of the sample, C is the protein concentration (mg/mL) in the tested sample, and D is the dilution coefficient (3.02).

2.12. Surface hydrophobicity index of cod proteins

Surface hydrophobicity index (SHI) of cod protein was determined using ANS as a fluorescence probe (Sun et al., 2015). The initial slope of the fluorescence intensity vs. protein concentration plot was used as the SHI. Experiments were performed in triplicate.

2.13. Statistical analysis

Results are reported as mean ± standard deviation (SD). 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. Protein nitrogen content and *in vitro* digestibility of baked, steamed and HHP-treated cod

Table 1 shows the protein nitrogen content, *in-vitro* gastric digestibility (IVGD), and *in-vitro* pancreatic digestibility (IVPD) of baked, steamed and HHP-treated cod samples. The protein content (PC) in raw wet cod muscle was 11.62%, while samples subjected to baking and steaming treatments had higher PCs (15.84% and 13.07%, respectively). The increase in PC after the thermal treatments was likely due to loss of moisture during heating (Costa et al., 2015). In contrast, HHP did not significantly affect the PC of the cod samples, which was in agreement with a previous report on squid (Zhang et al., 2016). The initial soluble protein nitrogen (SPN), non-protein nitrogen (NPN) and total nitrogen (TN) values of cod samples were 5.27%, 0.69%, and 13.39%, respectively. None of the three treatments caused any significant change in the NPN and TN contents. HHP, but not baking or steaming, was able to significantly ($P < 0.05$) increase the SPN content of the cod samples when compared with the control. This was in agreement with previous studies demonstrating improved solubility of squid proteins (Zhang et al., 2016) and enhanced dispersion stability of soybean protein isolate after HHP treatment (Manassero, Vaudagna, Añón, & Speroni, 2015). HHP might produce powerful percussive action and shear effects that result in denaturation of the cod proteins and release of free amino acids and peptides, which improved solubility (Zhang et al., 2015).

As presented in Table 1, the initial IVGD and IVPD values of fresh (control) cod were 34.47% and 56.24%, respectively. Overall, the IVGD and IVPD values of all treated cod samples were higher than those of the control sample, indicating improvement in protein digestibility after the treatments. Of note, the IVGD and IVPD of the cod samples after HHP treatment were significantly better than those after baking

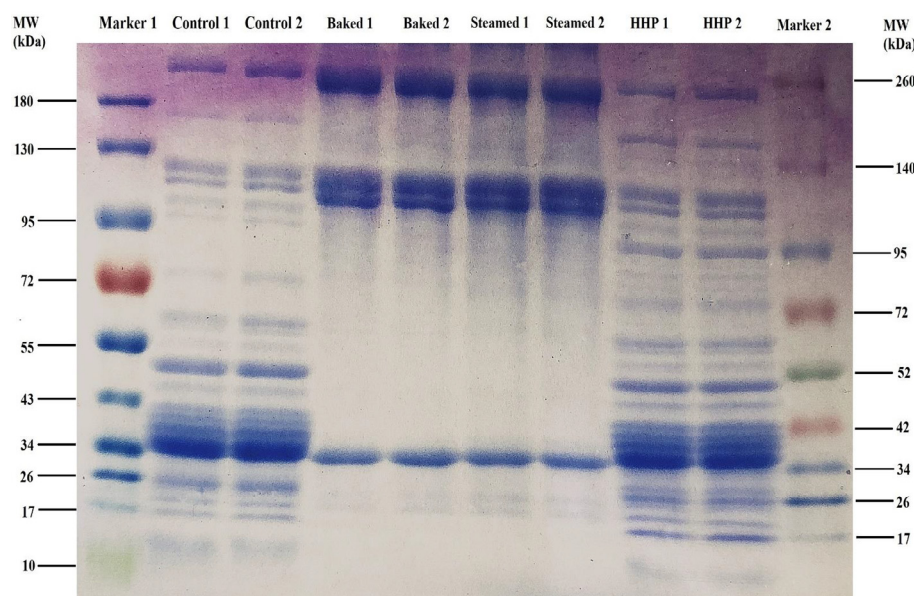


Fig. 1. Electrophoresis (SDS-PAGE) of cod samples after subjecting to baking, steaming and high hydrostatic pressure treatment, respectively.

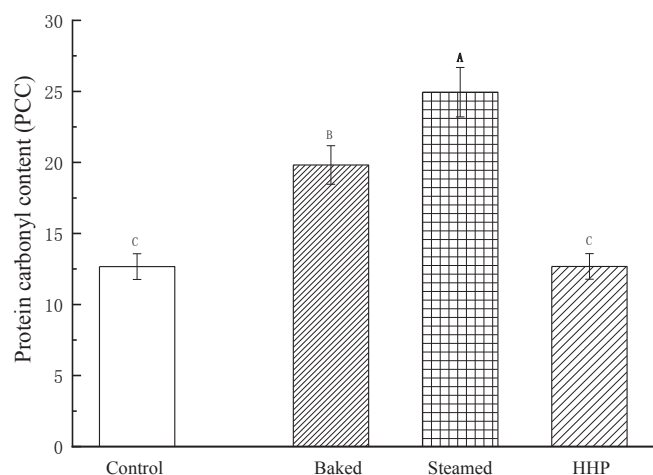


Fig. 2. Protein carbonyl content (PCC, nmol carbonyls/mg protein) of baked, steamed and high hydrostatic pressure treated cod samples ($n = 3$).

and steaming. These data suggest that HHP is a better way than baking and steaming to improve protein digestibility (nutritional value) of cod. The increase in protein digestibility after HHP, baking and steaming might be due to changes in the secondary and tertiary structures (Lee et al., 2016) of cod proteins, which could in turn affect protein solubility and enzyme accessibility. Nonetheless, the underlying

mechanisms of the changes brought about by these processing methods are not fully understood and require further study.

3.2. Electrophoresis of cod protein extracts

Fig. 1 shows the SDS-PAGE data of raw, baked, steamed and HHP-treated cod protein samples. The control samples were proteins of cod muscle and were composed of 250, 172, 122, 75, 50, 40, 38, 34, 30, 26, and 17 kDa bands. In general, baking and steaming caused much more dramatic changes in cod proteins when compared with HHP treatment. However, HHP-treated cod protein samples exhibited no significant difference in SDS-PAGE compared with the fresh samples, except for the bands at 95 (newly formed in HHP-treated cod samples) and 26 kDa.

For the baked and steamed cod samples, the increase in band intensity of protein with MW of 112 and 250 kDa was obvious for the protein crosslinking during the thermal process. In addition, changes in the bands of around 38 kDa, which correspond to tropomyosin (Jin et al., 2015), suggest that baking and steaming caused severe damage to tropomyosin in cod. This was evident from the disappearance of the 75, 50, 40, 38, 30, 26, and 17 kDa bands and the tremendous increase in the intensities of the 122 and 250 kDa bands in the baked and steamed cod samples compared with the control. This might be due to the protein crosslinking during the thermal process. For HHP-treated cod samples, the formation of the 95-kDa protein bands the might be due to crosslinking among proteins and peptides. A previous study proved that HHP

Table 2

Results of the indirect ELISAs performed with cod proteins against IgG and IgE.

	IgG binding capacity			IgE binding capacity				
	R [#]	R _{SGF}	R _{SIF}	P ₁ [*]	P ₂	P ₃	P ₄	P ₅
Control	2.56 ± 0.16 ^B	1.92 ± 0.08 ^B	1.63 ± 0.06 ^B	1.30 ± 0.05 ^B	1.16 ± 0.12 ^B	0.92 ± 0.05 ^{BC}	0.69 ± 0.04 ^B	0.56 ± 0.03 ^B
Baked	2.43 ± 0.19 ^B	2.13 ± 0.20 ^B	1.87 ± 0.06 ^A	1.38 ± 0.11 ^B	1.49 ± 0.05 ^A	1.14 ± 0.06 ^{AB}	0.87 ± 0.04 ^A	0.80 ± 0.04 ^A
Steamed	2.78 ± 0.09 ^A	2.42 ± 0.09 ^A	1.98 ± 0.04 ^A	1.54 ± 0.05 ^A	1.56 ± 0.07 ^A	1.19 ± 0.20 ^A	0.95 ± 0.07 ^A	0.80 ± 0.03 ^A
HHP	1.83 ± 0.18 ^C	1.51 ± 0.10 ^C	1.09 ± 0.11 ^C	0.97 ± 0.05 ^C	0.98 ± 0.06 ^C	0.71 ± 0.09 ^C	0.53 ± 0.04 ^C	0.38 ± 0.03 ^C

^{A-C}Values are expressed as mean ± standard deviation (SD) ($n = 3$); means in the same column with different letters (A–C) are significantly different ($P < 0.05$).

[#] R: indirect ELISA performed with rabbit anti-cod protein polyclonal antibodies; R_{SGF}: indirect ELISA performed with rabbit anti-cod protein polyclonal antibodies for hydrolysates after simulated gastric fluid (SGF) digestion; R_{SIF}: indirect ELISA performed with rabbit anti-cod protein polyclonal antibodies for hydrolysates after simulated intestinal fluid (SIF) digestion.

^{*} P₁–P₅: indirect ELISA performed with human sera from five allergic patients.

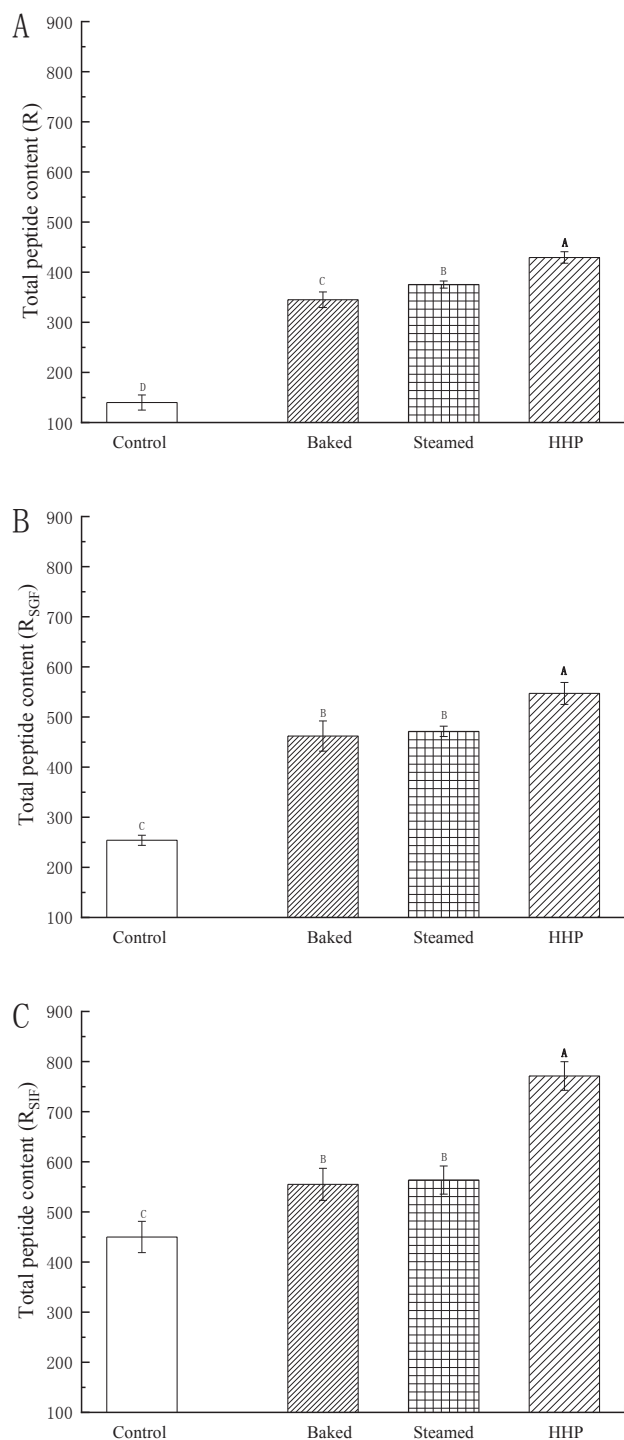


Fig. 3. Total peptide content (mg/L) of baked, steamed and high hydrostatic pressure treated cod samples ($n = 3$). A: cod protein samples (R); B: hydrolysates samples after simulated gastric fluid digestion (R_{SGF}); C: hydrolysates samples after simulated intestinal fluid digestion (R_{SIF}).

treatment could enhance proteolytic degradation to release peptides (Quiros, Chichon, Recio, & Lopez, 2007), although these degraded protein fragments or peptides were too weak for gel resolution and lastly lost in the buffer, resulting in them being invisible in SDS-PAGE (Zhang et al., 2017). Hence, the lack of other appreciable changes in the pattern of protein bands could be due to the degraded protein fragments or released peptides after HHP treatment being too weak for SDS-PAGE determination.

3.3. Effect of baking, steaming and HHP treatment on protein carbonyl contents of cod

Protein carbonyl content (PCC) is an index for analyzing protein oxidation in high-protein foods (Armenteros et al., 2016). Protein oxidation can be induced directly by reactive oxygen species (either free radical or non-free radical) and reactive nitrogen species (RNS), or indirectly by secondary products of oxidative stress (Soladoye, Juárez, Aalhus, Shand, & Estévez, 2015). Protein oxidation in foods is among the major degradative processes responsible for losses in food quality and has been considered as a risk factor for human health (Kanner, 2007). In the present study, PCC was evaluated by the dinitrophenylhydrazine (DNPH) spectrophotometric method and the results are shown in Fig. 2. It was found that PCCs of the steamed and baked samples were 1.40- and 1.72-fold higher, respectively than that of the control. The baked samples had the highest value of 22.96 nmol carbonyls/mg protein. On the other hand, there was no significant difference between the PCC of the HHP-treated and control samples, suggesting that HHP treatment did not cause protein oxidation in cod. Protein oxidation caused by baking and steaming might contribute to the formation of harmful volatile compounds and other reaction products, such as genotoxic heterocyclic aromatic amines (Zhao et al., 2017). Furthermore, protein oxidation may also adversely affect the color, texture, nutritive value, and even safety of foodstuffs, especially high-protein foods (Gibis, 2016). These data clearly support the superior advantage of HHP treatment to avoid protein oxidation in cod over the other two methods.

3.4. Allergenic properties of baked, steamed and HHP-treated cod proteins

One of the major concerns about the consumption of cod, a highly nutritious protein-rich seafood, is allergy in sensitive individuals. Given the fact that the allergic symptoms could range from gastrointestinal distress to even anaphylaxis, research to characterize the allergens and to develop strategies to attenuate or eliminate them remains intensive. Thus far, a number of cod allergens have been characterized (Galland et al., 1998; Sophia et al., 2012; Van, Hordvik, Endresen, & Elsayed, 2003), which facilitated the assessment of the various methods to attenuate cod allergenicity in our study. Indirect ELISAs using human sera (IgE-binding capacity; IgE) from five allergic human subjects and rabbit anti-cod protein polyclonal antibodies (IgG-binding capacity; IgG) were performed. As shown in Table 2, the baked and steamed samples had higher IgG and IgE values than the control samples, representing an increase in allergenicity, while HHP treatment caused a significant ($P < 0.05$) decrease in IgG and IgE values compared with the control, representing a decrease of cod allergenicity. HHP treatment resulted in the lowest IgE values (0.97, 0.98, 0.71, 0.53, and 0.38) for the five human serum samples, and the lowest IgG values (1.83, 1.51 and 1.09) for the rabbit anti-cod protein polyclonal antibodies. These results strongly demonstrate that HHP treatment can effectively weaken the allergenicity of cod.

The increase in allergenicity of cod after baking and steaming was probably caused by the destruction of microstructures, changes in protein conformation (Zhang et al., 2015), and/or damage of proteins during the heating processes (Fig. 1). In contrast, HHP treatment might also modify the conformation and/or structure of cod proteins (Jin et al., 2015; Zhang et al., 2017), but in a way which reduced the allergenic potency of the purported allergens. HHP treatment has been shown to decrease the immune reactivity of soybean seeds (Penas et al., 2011). Previously, we reported that the allergenicity of squid was significantly decreased after HHP treatment (Jin et al., 2015; Zhang et al., 2015; Zhang et al., 2017), which resulted in protein degradation and cross-linking among proteins and peptides. Furthermore, the degraded protein fragments and/or released peptides might compromise the antibody binding sites, thus reducing IgE and IgG values (Quiros et al., 2007). Alternatively, it is possible that different processing methods

Table 3

Free sulfhydryl content (FSC, $\mu\text{mol/g}$ protein) and surface hydrophobicity index (SHI) of cod protein from baked, steamed and high hydrostatic pressure (HHP)-treated samples.

	FSC			SHI		
	R	R _{SGF}	R _{SIF}	R	R _{SGF}	R _{SIF}
Control	34.70 $\pm$ 1.52 ^A	39.66 $\pm$ 0.91 ^A	42.79 $\pm$ 0.49 ^A	465.25 $\pm$ 16.56 ^B	483.40 $\pm$ 13.27 ^C	505.15 $\pm$ 19.81 ^C
Baked	31.70 $\pm$ 1.10 ^B	36.31 $\pm$ 1.30 ^B	38.93 $\pm$ 0.50 ^B	474.74 $\pm$ 17.93 ^B	513.52 $\pm$ 24.57 ^B	540.17 $\pm$ 15.77 ^B
Steamed	28.83 $\pm$ 1.10 ^C	34.58 $\pm$ 1.15 ^B	35.25 $\pm$ 0.25 ^C	478.76 $\pm$ 17.17 ^B	522.15 $\pm$ 28.04 ^B	544.17 $\pm$ 24.64 ^B
HHP	27.41 $\pm$ 2.08 ^C	35.24 $\pm$ 2.22 ^B	36.58 $\pm$ 2.01 ^C	503.67 $\pm$ 18.23 ^A	565.44 $\pm$ 18.87 ^A	612.35 $\pm$ 12.01 ^A

^{A–D}Values are expressed as mean $\pm$ standard deviation (SD) (n = 3); means in the same column with different letters are significantly different ($P < 0.05$).

[#]R: performed with cod protein samples; R_{SGF}: performed with hydrolysates after simulated gastric fluid (SGF) digestion; R_{SIF}: performed with hydrolysates after simulated intestinal fluid (SIF) digestion.

tend to affect different types of proteins in foods, and HHP, but not traditional thermal methods, is capable of exerting a significant damaging effect on the allergens.

3.5. Peptide content of baked, steamed and HHP-treated cod proteins

Cod is famous for its high content of functional peptides. Some of these peptides have been reported to exhibit beneficial biological activities, such as antimicrobial, antithrombotic, antioxidant and anti-inflammatory activities. The total peptide content (TPC) of the cod protein samples subjected to different treatments are shown in Fig. 3. All the three processing methods increased the TPC of cod proteins significantly from an initial 140 mg/L (control) to 345 mg/L (baked), 375 mg/L (steamed), and 429 mg/L (HHP-treated) after processing. Similar trends were also observed after IVGD and IVPD. HHP-treated samples consistently demonstrated the highest TPC values of 429, 547, and 771 for cod protein samples (R), hydrolysates after the simulated gastric fluid digestion (R_{SGF}), and hydrolysates after the simulated intestinal fluid digestion (R_{SIF}), respectively. The results herein demonstrate that the three processing methods (especially HHP) can improve peptide release from cod proteins, with the effects being more obvious after *in-vitro* digestion.

Very often, many proteins in foods are converted into the peptides and amino acids during processing (Nakajima, Nozaki, Ishihara, Ishikawa, & Tsuji, 2005). These changes, especially the generation of peptides, can modulate the functional properties and nutritional value of the food products concerned. In the present study, HHP treatment gave rise to the highest TPC values, which were $\sim$ 2–3-fold higher than the TPC of the control samples and were also significantly higher than the steamed and baked samples. Of note, SDS-PAGE analysis (Section 3.2) only showed a minor difference in the band patterns (95 kDa and 26 kDa bands) between the HHP-treated and control samples. The discrepancy between the SDS-PAGE and TPC assays was likely due to the peptides released from HHP treatment being too weak for determination by SDS-PAGE. In fact, several studies have demonstrated that HHP treatment can release peptides and enhance proteolytic degradation (Cheret, Delbarre, Lamballerie, & Verrez, 2005; Quiros et al., 2007), and these degraded protein fragments or released peptides are often too weak and small for electrophoretic resolution, resulting in their loss in the buffer during SDS-PAGE (Zhang et al., 2017). For example, small peptides, including YAEERYPII, FRADHPFL, and RADHPFL, can be released from ovalbumin by HHP (Quiros et al., 2007).

3.6. Free sulfhydryl content and surface hydrophobicity index of baked, steamed and HHP-treated cod proteins

Free sulfhydryl content (FSC) and surface hydrophobicity index (SHI) were explored to analyze changes in baked, steamed and HHP-treated cod proteins. SHI was evaluated using an extrinsic fluorescent sensor ANS. ANS specifically binds to sterically accessible hydrophobic moieties of soluble protein molecules and displays greater affinity for

aromatic amino acids. SHI is known to be positively correlated with protein denaturation and gel strength. The sulfhydryl groups of proteins are usually buried in the tertiary structure of the native molecule, with FSC being a characteristic indicator of the tertiary structure (Hoffmann & Mil, 1997). The FSC and SHI results of the cod samples from different treatments are summarized in Table 3.

The initial FSC and SHI values of the cod protein samples were 34.70 $\mu\text{mol/g}$ protein and 465.25, respectively. All the three treatment methods significantly decreased FSC and increased SHI of the cod samples, with HHP demonstrating the most significant impact. This observation indicates that HHP is more efficient in releasing peptides from cod proteins, which is in agreement with the result of the TPC analysis discussed in former section. Similar trends were also observed for the IVGD and IVPD samples, although the differences among the three treatment methods were bigger than for the protein samples described above. The changes in FSC after baking, steaming or HHP treatment might arise from the disruption of preexisting disulfide bridges and/or random aggregation of the unfolded polypeptide chains through nonspecific interactions. For baked and steamed samples, the increase in SHI should result from the exposure of hydrophobic groups to the surface of thermally unfolded proteins (Chihi, Messian, Sok, & Saurel, 2016). For HHP-treated samples, the changes in SHI were likely caused by pressurization processes (decompression and compressions) and the pressure itself (Zhang et al., 2015; Zhang et al., 2017). HHP treatment has been shown to affect secondary and tertiary protein structures, and variations in allergen secondary structure might play an important role in modulating the allergenicity of squid (*Todarodes pacificus*) (Zhang et al., 2015; Zhang et al., 2017) and shrimp (*Litopenaeus vannamei*) (Long, Yang, Wang, Hu, & Chen, 2015).

Taken together the parameters pertinent to the physicochemical and functional properties of cod proteins, the significantly different effects between thermal and HHP treatments suggest that heating (baking and steaming) are more detrimental to protein quality, enzyme activity, and other intrinsic properties, compared with HHP. The capability of HHP to decrease FSC, increase SHI and release peptides from cod proteins might be part of the underlying mechanism which contributes to its effectiveness in reducing allergenicity of cod.

4. Conclusion

In conclusion, protein quality, *in vitro* digestibility, carbonyl content, allergenicity, peptide content and surface hydrophobicity index have been investigated to analyze the different effects of baking, steaming and HHP treatment on cod samples. Baked, steamed and HHP-treated samples had higher IVGD, IVPD, and SHI values, and lower FSC values, compared with the control sample. However, baking and steaming increased protein oxidation and allergenicity in cod samples, while HHP treatment improved these negative property indicators. Compared with the control, HHP treatment increased the SPN, IVGD, IVPD, SHI, and peptide content values by 1.42-, 1.55-, 1.30-, 1.08-, and 3.07-fold, respectively, and significantly reduced the IgE/G binding

capacities. This study provides valuable information supporting the application of HHP treatment to improve the nutritional value and reduce the allergenicity of cod samples. Future studies should characterize the allergen peptides in cod that are affected by HHP and the mechanism involved.

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Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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