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1 **Proteomic and microscopic approaches in understanding mechanisms of shell-**
2 **loosening of shrimp (*Pandalus borealis*) induced by high pressure and protease**

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22 **Abstract**

23 Microstructure, protein profile, relationship between shrimp shell-loosening and protein, and
24 mechanisms of shell-loosening after high pressure (HP) and enzyme treatment were investigated. The
25 shell-loosening gap induced by an endoprotease with broad specificity (Endocut-03L) was much higher
26 (53 μm) than that induced by HP at 100 MPa (HP100, 12 μm), followed by an endoprotease with high
27 specificity (8 μm), and HP at 600 MPa (5 μm). The extent of protein changes in epidermis and shell
28 corresponded with the shell-loosening gap. Thus, the gap could be predicted by the fold change of
29 protein spots by a PLS model. Shell-loosening due to HP100 and Endocut-03L was mainly caused by
30 physical and enzymatic degradation of high molecular-weight proteins in shell and epidermis and
31 subsequent loss of degradation products, disrupting the structure of muscle-shell connection. However,
32 HP100 was less effective than Endocut-03L due to its stabilizing effect on the shell collagen, lowering
33 its shell-loosening effect.

34 **Keywords:** shrimp; peeling; shell loosening; 2D gel electrophoresis; microscopy; protein

1. Introduction

Peeling is an important step in shrimp processing. The process can affect the yield, sensory, organoleptic and nutritional quality of final shrimp products (Dang et al., 2018a). It is difficult to peel freshly caught shrimp since the shell attaches tightly to the meat. Therefore, loosening the shell from the meat is needed before peeling. The most commonly used shell-loosening methods include empirical maturation of shrimp on ice or in brine for a few days. However, such a long maturation could cause degradation in quality and risk of microbial growth in final products. To overcome the problem, enzymes were used as an alternative method, primarily speeding up the shell-loosening process by enzymatic hydrolysis in a short time, consequently reducing the degradation rate of quality while satisfactory shell-loosening was obtained (Dang et al., 2018b). Also, high pressure was reported to enhance peelability, tail integrity, drip loss, and color of white shrimp (Yang et al., 2010). However, the mechanisms of shell-loosening induced by the two techniques have not been understood.

Two-dimensional gel electrophoresis and microscopy are commonly used to investigate molecular mechanisms. Two-dimensional gel electrophoresis is a powerful technique to study the protein changes based on isoelectric point (first dimension) and molecular weight (second dimension) (Lopez, 2007). The technique has been used to understand mechanisms of protein-based process such as molting in which the living shrimp must loosen its shell from meat, shed the shell off, take up water or air to expand the new shell and quickly harden it for defense and movement (Cariolou & Flytzanis, 1994; Chang, 1985; Chang, 1995; Freeman & Costlow, 1979). Shell (cuticle) and epidermis are two of many parts where biochemical and physiological changes take place during molting (Chang, 1995). By studying the changes of cuticular and epidermal proteins, Cariolou and Flytzanis (1994) provided a better understanding of molecular occurrence during stages of shrimp (*Penaeus vannamei*) molting,

and concluded that modulation of cuticle-epidermis proteins took place during the process. Microscopy is also an efficient technique to study the mechanism of cuticle-loosening of fruit induced by different peeling methods (Floros, 1990; Floros & Chinnan, 1988; Li, Pan, Atungulu, Wood, & McHugh, 2014), or recently of shrimp induced by ultrasound-enzyme treatment (Dang et al., 2018c). Examinations of the microstructural behavior of cuticle under different treatments form the basis of understanding of those mechanisms.

The objective of this study was first to investigate the changes at microscopic and molecular level of epidermis and shell of shrimp following enzymatic and pressure treatments, second to find the relationship between the protein changes and microscopic shell-loosening gap, and finally to understand the mechanisms of shell-loosening induced by the two technologies.

2. Materials and methods

2.1. Shrimps and enzymes

Cold water shrimp (*P. borealis*), batch 1701500008 caught and packed 04/08/2015 and batch 1610610011 caught and packed 10/09/2016, were provided by Royal Greenland A/S (Svenstrup, Aalborg, Denmark). The shrimps were 160-200 pcs/kg in size, individually frozen, packed in carton packages and stored at -21 °C. Fine table salt (NaCl) was obtained from a local supermarket (Copenhagen, Denmark). Endocut-03L (Endo3) and Tail21 enzyme preparations were obtained from Tailorzyme A/S (Søborg, Denmark) and stored at 4-5 °C until use. Endocut-03L (Endo3) is a food-grade endoprotease with broad specificity (60 Units/g) produced from bacteria *Bacillus clausii*, and Tail21 is a food-grade endoprotease with high specificity (65 U/mL) produced from fungi *Rhizomucor miehei*, as stated by the manufacturer.

2.2. High pressure treatment

Frozen shrimp were thawed in tap water for 20 minutes, and packed into a plastic bottle fully filled with distilled water. The packed sample was subjected to a research high pressure food unit (Food Processing Press QFP-6 Avure Technologies AB, Vesterås, Sweden), using 5% sodium benzoate as pressure transmitting medium. Two pressure levels (100 and 600 MPa, coded HP100 and HP600 respectively) were used with a holding time of 1.75 min at 12 °C. After the treatment, the samples were chilled using ice packing for further analyses within the same day.

2.3. Enzyme treatment

The setup of enzymatic maturation of shrimp was elaborately described in our previous study (Dang et al., 2018b). Briefly, frozen shrimp were thawed in tap water for 20 minutes. Enzyme solution (Endo3 or Tail21) was prepared during the thawing time, *i.e.*, adding 0.5% enzyme to 2% NaCl solution in a beaker. A polyvinyl chloride rack that has three legs and 1-cm diameter holes on the surface was placed into the beaker. Thawed shrimp were horizontally aligned onto the rack, completely soaked into the enzyme solution which was magnetically stirred at 250 rpm at 5 °C for 20 h.

2.4. Histology and transmission electron microscopy (TEM)

Three shrimp per treatment were analyzed. For histology, the entire first abdominal segment was fixed 24 h in Karnovskys' fixative (5% glutaraldehyde, 4% paraformaldehyde, 0.1 M sodium cacodylate buffer at pH 7.3). The tissue was then further trimmed into smaller pieces containing the shell and a few mm of the meat and placed in fresh fixative for 1 h. After wash in buffer and demineralized water, samples were dehydrated in a graded ethanol series, infiltrated in melted Paraplast (paraffin) at 60 °C and embedded in Paraplast. Ten sections of 10 µm were dissected using Leica EM-

99 UC7 microtome (Leica Microsystem, Austria) with a glass knife. De-paraffination was done with
100 Histochoice clearing agent (Sigma-Aldrich) and a graded series of ethanol.

101 The slides were stained with Periodic acid Schiff's (PAS) and examined using a Nikon
102 microscope (Nikon Eclipse 80i, Nikon, Japan) at the objective of 100x immersion oil lens and
103 photographed with a connected digital camera (Canon EOS 1200D, Japan). Images were captured
104 within well-preserved areas and 50-80 measurements of the distance between epidermis and
105 endocuticle (shell-loosening gap) were performed for each treatment using Image J software.

106 For TEM, 2x1-mm specimen of the first abdominal segment was dissected and immediately fixed
107 for 20 h in Karnovsky's fixative (as above), and subsequently washed in cacodylate buffer and water.
108 Samples were post-fixed in 1% osmium tetroxide (with 0.1 M cacodylate buffer) for 4 h at 4 °C. After
109 washing in buffer and water, the samples were dehydrated in a graded acetone series, infiltrated with
110 three different ratios of Spurr's resin to acetone and embedded in Spurr's resin within flat molds. The
111 resin was polymerized in an oven at 60 °C for 8 h. Ultrathin sections (40 nm thick) were cut with a
112 diamond knife using a Reichert-Jung/LKB Supernova ultramicrotome and collected on pioloform-
113 coated copper grids. Sections were contrasted with 1% uranyl acetate and lead citrate (2.7% in 3.5%
114 sodium citrate) and examined in a Philips Model CM100 TEM microscope (Philips Electron Optics,
115 Eindhoven, Netherlands) equipped with high quality Morada G2 CCD camera (Olympus-SIS, Münster,
116 Germany) at acceleration voltage of 100 kV. The TEM image was photographed at magnification of
117 1100x.

118 Final image processing, cropping and mounting of the images were done with Adobe (San Jose,
119 CA, US) Photoshop CS2 and Illustrator CS2.

120 **2.5. 2D gel electrophoresis**

121 **2.5.1. Sample preparation for 2DE**

122 High pressure treatments were conducted on shrimp from batch 1701500008, and enzyme
123 treatments were conducted on shrimp from batch 1610610011. Due to seasonal difference between the
124 two batches, two controls were used for comparison, i.e. raw shrimp (RawHP) compared with pressure-
125 treated shrimp (HP100 and HP600), and raw shrimp (RawE) compared with enzyme-treated shrimp
126 (Endo3 and Tail21).

127 *Shell samples*

128 Shells from 10 shrimp were collected, and each shell was cut off a 100-mg piece from the dorsal
129 side resulting in a total of 1-g shell sample used for 2DE. Four sample replicates corresponding with
130 four gels were performed for Endo3 and Tail21, whereas duplicates were performed for HP100 and
131 HP600. The wet shell samples were ground in liquid nitrogen to obtain shell powder.

132 *Epidermis samples*

133 After peeling shells off, the epidermis from 10 shrimp were collected by carefully peeling off the
134 epidermal layer from the meat part on a frozen ice pack. A total of 92-mg epidermis sample was used
135 for 2DE. Four sample replicates corresponding with four gels were performed for Endo3 and Tail21,
136 whereas duplicates were performed for HP100 and HP600.

137 **2.5.2. Protein extraction and determination**

138 The epidermis (92 mg) and shell (1 g) samples were homogenized (Polytron PT 1200,
139 Kinematica, Littau, Switzerland) in 1 mL of buffer (8 M urea, 2 M thiourea, 50 mM dithiothreitol
140 (DTT), 1.5% CHAPS detergent, 2% Pharmalyte carrier ampholytes 3-10, 100 mM Tris base) three
141 times for 30 s with a pause on ice for 15 s. The homogenate was centrifuged at 20,000 x g for 15 min at

142 20 °C. The supernatant was collected as sample extract and stored at –80 °C until further analyses
143 (protein determination and 2DE performances). Protein concentrations were determined with
144 Peterson's simplification (Peterson, 1977) of the Lowry method (Lowry, Rosebrough, Farr, & Randall,
145 1951).

146 **2.5.3. Isoelectric focusing (the first dimension)**

147 Extracts from the shell and epidermis were defrosted at room temperature and the volumn
148 containing 1285 µg protein was adjusted to a total volume of 350 µL with buffer A (50 mM Tris base,
149 pH 7.4, 1 mM EDTA), followed by precipitating in 100 µL of trichloroacetic acid (TCA, 65% w/v) on
150 ice for 30 min. The samples were then centrifuged at 15,000 x g for 15 min at 4 °C. The pellet was
151 collected and washed with 1 mL of 90% acetone followed by centrifugation at 15,000 x g for 5 min at 4
152 °C. The supernatant was discarded and the pellet was air-dried at room temperature for 60 min. The
153 pellet was then dissolved in 450 µL of a solution containing 8 M urea, 2 M thiourea, 50 mM
154 dithiothreitol (DTT), 1.5% CHAPS, 2% Pharmalyte 3-10, 100 mM Tris base, 0.1% sodium dodecyl
155 sulfate (SDS) and a little Orange G as dye. A volume of 350 µL of the solution containing 1000 µg
156 protein was loaded to 1-D Immobiline Drystrip (18 cm, pH 4-7, GE Healthcare, Sweden) for reswelling
157 overnight. The strips were placed in a Multiphor II apparatus (GE Healthcare) at 20 °C for performing
158 the first dimension IEF. The separation was performed using the following step voltage focusing
159 protocol: 4 h at 100 V, 1 h at 500 V, 13 h at 3500 V. At the end of focusing, the IEF reached 74.2 kVh.
160 The 1-D strips were stored at –80 °C until separation in the second dimension.

161 **2.5.4. SDS-PAGE (the second dimension)**

162 After thawing, the 1-D strips were equilibrated in 10 mL of a equilibration buffer (6 M urea, 50
163 mM Tris-HCl, pH 8.8, 30% (v/v) glycerol, and 2% (w/v) SDS) with 1% (w/v) DTT for 20 min,

164 followed by alkylation in 10 mL of the equilibration buffer with 4.5% (w/v) iodoacetamide for 20 min.
165 The equilibrated 1-D strips were placed on top of acrylamide gels (12%) and sealed with 0.5% (w/v)
166 agarose, 25 mM Tris base, 192 mM glycine, and 0.1% SDS. The gels were run on a Hoefer DALT
167 system (Amersham Biosciences) at 15 °C with a maximum of 40 mA per gel. The gels were then fixed
168 in 250 mL of 40% (v/v) ethanol and 10% (v/v) acetic acid for 1 h, followed by incubating in 250 mL of
169 30% (v/v) ethanol and 2% (v/v) phosphoric acid for 30 min, 250 mL of 2% (v/v) phosphoric acid for 20
170 min, and 250 mL of 18% (v/v) ethanol, 2% (v/v) phosphoric acid and 15% (v/v) ammonium sulfate for
171 30 min. Colloidal Coomassie Blue solution (2% w/v) was used to stain the gels for 24 h. The stained
172 gels were imaged using a charge-coupled device (CCD) camera (Camilla, Raytest, Straubenhardt,
173 Germany).

174 **2.5.5. Image analysis**

175 2D gel images were analyzed using Progenesis SameSpots (version 4.5, Nonlinear Dynamics,
176 Newcastle, UK). The built-in functions of the software allowed aligning the gels, matching, quantifying
177 the spots, and normalizing the spot volumes. Fold change was calculated as the ratio of the average
178 normalized abundances (volume) corresponding to the two samples of interest. pI was calculated by
179 directly referring the spot position in the pH 4-7 area. Molecular weight (MW) was calculated based on
180 reference markers with known MW in the range 14.4 - 200 kDa. T-test ($p < 0.05$) was applied to
181 evaluate differential protein change levels between treated gels and control gels.

182 **2.6. Data analysis**

183 ANOVA and post-hoc Student-Newman-Keuls test were performed by the software SAS 9.4
184 (SAS ® Institute Inc., Cary, New York). Nonparametric Mood's median test was used to compare two
185 molecular-weight medians of increased spots and decreased spots in the same treatment and was

performed by the software OriginPro 2017 (OriginLab Corp., Northampton, MA, USA). Principle component analysis (PCA, correlation matrix) was performed by the software OriginPro 2017. Partial least square (PLS) regression was conducted by the *pls* package for R (Mevik & Wehrens, 2007). Leave-one-out cross validation (LOO) was used to validate the model and select the number of latent factors (components). Pearson's correlation was used to obtain the relationship between 2DE protein parameters and microscopic shell-loosening gap. 2DE protein parameters included fold (fold = the highest value between volume of treated spot and volume of untreated spot divided by the least value between volume of treated spot and volume of untreated spot), molecular weight (MW, experimental molecular weight obtained by SameSpots software), pI (experimental pI obtained by SameSpots software), volume change (Δ Volume = the volume of the treated spot subtract – the volume of the untreated spot), and shell-loosening gap change (Δ Gap = the shell-loosening gap of the treated shrimp – the shell-loosening gap of the untreated shrimp)

3. Results and discussion

3.1. Microstructure changes of shrimp integument

The native microstructure of shell, epidermis and meat of the shrimp is shown in **Figure 1A**. The thickness of the abdominal shell of mature *P. borealis* was ~34 μ m on average, and within the range of the shell thickness of shrimp *Metapenaeus spp* which was 30-65 μ m (Dall, 1965). The shell consisted of four layers: epicuticle (~2.6 μ m), exocuticle (~18.2 μ m), endocuticle (~8.3 μ m), and membranous layer (~4.3 μ m). In comparison with cuticle metrics of black tiger shrimp *P. monodon* (Xu et al., 2013), *P. borealis* had 0.6- μ m thinner epicuticle, 15.3- μ m thinner exocuticle, 7.8- μ m thinner endocuticle, and

no measures of membranous layer were reported for *P. monodon*. The epidermis was about 2.5 μm thick, however, its thickness was highly varied throughout parts of the body.

The effects of high pressure and enzyme treatments on shrimp integument histology are shown in **Figure 1B-G**. In both raw shrimp (**Figure 1B&E**), the connection between the shell and the epidermis was mostly continuous, occasionally having a small gap in between, named shell-loosening gap. The loosening gap in raw shrimp could be caused by frozen storage. It is noteworthy that the individually frozen raw shrimp were stored at $-21\text{ }^{\circ}\text{C}$ for seven months since trawl-caught date. The freezing could cause protein denaturation (Bhobe & Pai, 1986), autolysis (Bhobe & Pai, 1986), growth of ice crystals (Szczesniak, 1998), and therefore potentially disrupt some connective proteins between the shell and the meat. High pressure at 100 MPa enlarged the loosening gap to $12.2\text{ }\mu\text{m}$ ($\pm 11.7\text{ }\mu\text{m}$) compared to $2.7\text{ }\mu\text{m}$ ($\pm 4.4\text{ }\mu\text{m}$) in raw shrimp (**Figure 1H**). Increasing the pressure level to 600 MPa tightened back the loosening gap to $4.9\text{ }\mu\text{m}$ ($\pm 6.2\text{ }\mu\text{m}$). The observations suggest that relative mild physical pressure can break down the muscle-shell connection, leaving a relatively large loosening gap in between, while too high pressure will, on the contrary, bridge the gap and the shell becomes closer to the meat. However, the loosening gap markedly increased for Endo3-treated shrimp, being $53\text{ }\mu\text{m}$ ($\pm 28.9\text{ }\mu\text{m}$) wide, and corresponded with the high peelability obtained in our previous study (Dang et al., 2018b). Endocuticle of Endo3-treated shell was stained less intensely than other samples and some parts of epidermal layer toward the shell disappeared, indicating that Endo3 hydrolyzed not only muscle-shell connection, but also partially epidermis and shell. Indeed, the extractable protein content of shell and epidermis after Endo3 treatment were much lower than raw shell and epidermis, *i.e.*, 0.6% and 1.5% for Endo3-treated shell and epidermis, respectively, as opposed to 10.9% and 7.9% for raw shell and epidermis, respectively (**Table 1S**). Tail21 is also an endoprotease, but its function on shell-loosening

was not as high as Endo3, evidenced by 7.8 μm ($\pm$ 5.7 μm) wide loosening gap. Noticeably, Tail21 seemed to attack the shell prior to the muscle-shell connection since the shell reacted weakly with PAS stain, shown by less magenta than other samples (**Figure 1G**). It was possible that chitin and/or protein which account for 17-20% and 33-40%, respectively, of the shell (Rodde, Einbu, & Varum, 2008), was highly impacted by this enzyme. Based on the extractable protein content and the possible presence of chitinase, it was further assumed that Tail21 attacked chitin more intensively than protein in the shell. Particularly, as PAS stains both chitin and protein in magenta color, a decrease of either chitin content or protein content is accompanied by the decreased intensity in color displayed. If the largest decreased magenta in Tail21-treated shell was due to the protein decrease, its expected protein content would have been lowest compared to other shells. However, **Table 1S** showed that its extractable protein content was not lowest, but indeed higher than other shells and only lower than raw shell (RawE). This indicates that the markedly decreased magenta in Tail21-treated shell was more associated with the chitin decrease caused by chitin hydrolysis of chitinase. The possible presence of chitinase in Tail21 may be due to the insufficient purification of the commercial fungal protease.

3.2. Protein changes

The epidermal and cuticular proteins extracted from control shrimp and shrimp treated with high pressure and enzyme were separated by 2DE, and the abundance (normalized volume) of all protein spots detected in each treatment were analyzed by principle component analysis (PCA) as shown in **Figure 2**. An average of 961 spots was detected in epidermis gels, forming 961 variables in epidermis PCA model, whereas 777 spots were found in shell gels, forming 777 variables in shell PCA model. Overall, Endo3 caused the most considerable changes in protein abundance since Endo3-treated epidermis and shell were grouped separately from other samples. High pressure at 600 MPa (HP600)

250 affected the abundance of epidermal proteins more than cuticular (shell) proteins. Epidermal and
251 cuticular proteins from Tail 21 were less affected than Endo3, and proteins from HP100 were less
252 affected than from HP600. Enzyme treatments (Endo3 and Tail21) changed proteins differently from
253 high pressure treatments (HP100 and HP600), particularly enzyme samples grouped separately from
254 high pressure samples on PC 3 of epidermis plot and on PC 1 of shell plot.

255 Further analysis for the protein spots was conducted by comparing their abundance induced by
256 each treatment with the corresponding control group (expressed as fold change) and together with
257 obtaining MW and pI of the spots. **Figure 3** shows the distribution of significantly increased and
258 decreased protein spots with respect to MW, pI, and fold change. As seen, enzymatic treatments
259 resulted in more protein changes than high pressure treatments, shown by higher quantity of
260 significantly increased (black) and decreased (red) spots in both epidermis and shell. Endo3 resulted in
261 the highest number of significantly differential spots in both epidermis and shell, followed by Tail21.
262 Higher level of pressure (600 MPa) resulted in higher number of significantly differential spots than
263 lower level (100 MPa). Most increased spots of Endo3-treated epidermis were found in the lower MW
264 range (<50 kDa) while decreased spots had higher MW. Increased and decreased spots of Endo3-
265 treated shell had a similar pattern of distribution on the MW axis as spots of Endo3-treated epidermis.
266 Generally, protein spots (including significantly differential and non-significantly differential) were
267 distributed throughout the analyzed pI range from 4 to 7. Noticeably, a majority of increased proteins
268 from Tail21 epidermis were more acidic than decreased proteins. The fold change of differential
269 protein spots in epidermis and shell was highest for Endo3 treatment (ranged 1.4-15 fold), followed by
270 Tail21 (ranged 1.2-8 fold), high pressure at 600 MPa (ranged 1.2-3 fold) and high pressure at 100 MPa

271 (ranged 1.2-2 fold). This reveals that enzymes degraded proteins and peptides and produced peptides
272 more intensively in terms of abundance compared to high pressure treatments.

273 Further analyses for quantitative assessment of protein spots differentially present after the
274 treatments were conducted and shown in **Figure 4**. For high pressure treatment, changes in the
275 epidermal protein profile were more evident at the high pressure level (600 MPa, HP600) than at the
276 low pressure level (100 MPa, HP100), indicated by higher number of decreased and increased spots
277 (**Figure 4A**). Epidermal cells presented an immediate but subtle response to low-level pressurization,
278 characterized by 2 increased and 7 decreased protein spots upon 100-MPa pressurization. Only two out
279 of these protein spots were also altered by 600-MPa pressurization, evidenced by 2 overlapped spots
280 between HP100 and HP600 (**Figure 4E**). Therefore, the vast majority of the proteins affected by high
281 pressure only changed at a specific pressure level.

282 Epidermal proteins were more vulnerable to enzyme attack than to pressurization as higher
283 number of increased and decreased spots were observed, *i.e.*, 236 increase and 238 decrease after Endo3
284 maturation, and 97 increase and 91 decrease after Tail21 maturation (**Figure 4B**). Comparing Endo3
285 and Tail21, the former was more influential on the protein changes (474 vs 188 differential proteins,
286 respectively), and 45% (85 overlapped spots) of total differential spots by Tail21 also changed by
287 Endo3 (**Figure 4F**). This finding indicates that although the enzymes differed in specificity, they acted
288 on the same proteins to some extent. A common pattern seen for both enzymes was that the number of
289 increased spots was comparable to the number of decreased spots in each treatment, and median MW
290 of increased spots were significantly different from the decreased spots. The median MW of decreased
291 spots was higher than the increased spots in Endo3 gels while the opposite pattern was seen in Tail21
292 gels (**Figure 4J**). This suggests that large peptides (~65 kDa) were favorably degraded by Endo3

293 whereas smaller peptides (~37 kDa) were favorably degraded by Tail21. In comparison with Tail21, a
294 large number (236) of small peptides (~30 kDa, median MW of increased spots, **Figure 4J**) introduced
295 by Endo3 treatment, were probably products of enzymatic hydrolysis of large peptides. This finding
296 indicates that Endo3 was more powerful than Tail21 in protein hydrolysis.

297 For shell, generally, increasing level of pressure resulted in an increase in number of decreased
298 spots (**Figure 4C**) with low MW (**Figure 4K**) and a decrease in number of increased spots with high
299 MW. There were no differential spots identical between the two pressure levels. The effect of enzymes
300 on shell proteins was, in general, similar to the way they affected epidermis protein with regard to
301 pattern of differential spots, pattern of overlapped spots over total differential spots, and pattern of MW
302 distribution. The only difference between the effect of enzymes on epidermis and their effect on shell
303 was that the number of decreased spots was markedly higher than number of increased spots in Endo3-
304 treated shell. MW of decreased spots was significantly higher than that of increased spots, suggesting a
305 degradation of high MW proteins into smaller proteins taking place during Endo3 treatment. It is
306 noteworthy that a spot defined as decreased spot after treatment could be either having abundance
307 (volume) reduced or lost after treatment. In case of Endo3 treatment, evidences from extractable
308 protein content (0.6% for Endo3-treated shell vs 10.9% for raw shell) and from spot invisibility on
309 Endo3-treated shell gel confirmed that loss of numerous shell proteins occurred during Endo3
310 maturation. No identical spots among the four treatments for both epidermis and shell were found
311 (**Figure 4M&N**), indicating that neither epidermal nor cuticular proteins were consistently altered
312 throughout all four treatments due to their different nature of effect. However, five identical spots in
313 each epidermis set and shell set were equally altered between the two most efficient shell-loosening

314 treatments (Endo3 and HP100, **Figure 1H**), suggesting that the physical (high pressure) and enzymatic
315 methods could share some common features of shell-loosening.

316 At present, identity of the differential proteins described above was not in the scope of this study.
317 However, future identification of those proteins will allow us to obtain their specific structure and
318 function in the shell-loosening process.

319 **3.3. Correlation between shell-loosening gap and protein changes**

320 Pearson's correlation between the parameters of epidermal and shell protein spots and the shell-
321 loosening gap is shown in **Figure 5A-B**. Only fold change of protein spots in both epidermis and shell
322 was significantly ($p < 0.001$) positive correlated to the shell-loosening gap. The correlation indicates that
323 changes in protein spots could strongly describe the shell-loosening status of shrimp. Protein spots with
324 large fold change (volume change after treatment) were more likely associated with higher shell-
325 loosening gap. Therefore, the shell-loosening gap could be predicted by the fold change of protein
326 spots based on the PLS model, as shown in **Figure 5C-D** for epidermis and shell, respectively. As
327 shown, the predictive abilities of fold change in the epidermis group and shell group were 0.98 and
328 0.83, respectively, showing a promising approach for exploiting the microscopic shell-loosening gaps
329 in treated shrimps. Hence, future work can rely on the fold change of protein spots from 2DE gels,
330 either on epidermis or on shell, to predict the microscopic shell-loosening gap of cold-water shrimp.
331 This will help reduce the immense work of histology in comprehending the shell-loosening gap at
332 microscopic level.

3.4. Proposed mechanisms of shell-loosening induced by high pressure and enzyme

In theory, for high pressure treatment, decreased spots could result from degradation of high MW protein to smaller MW peptide, loss of protein, and stabilization of collagen structure (Potekhin, Senin, Abdurakhmanov, & Tiktopulo, 2009). Increased spots could result from the products of degradation of high MW protein, degradation of protein with MW > 200 kDa, charge change of protein, introduction of enzyme from other organs of shrimp, and interaction of protein with protein/chitin. Normally, degradation of high MW proteins to smaller MW proteins would lead to higher number of increased spots than the number of decreased spots and lower MW of increased spots than MW of decreased spots. However, intensive degradation could produce too small proteins (<14 kDa) out of the electrophoretic MW range (14.4 - 200 kDa range) and they would, therefore, not appear in the gel. In such situation, the number of increased spots would not necessarily be higher than the number of decreased spots. For HP100 epidermis, the median MW of increased spots was higher than MW of decreased spots, suggesting that increased spots were resulted from degradation of proteins within and outside the gel MW range. The loss of protein (8.4% HP100 vs 10.1% RawHP, **Table 1S**) could contribute to the higher number of decreased spots compared to the number of increased spots. For HP100 shell, the median MW of increased spots was significantly higher than the MW of decreased spots, suggesting that the increased spots might not result from degradation of decreased spots present in the gel, but from degradation of proteins with a MW above the gel range (>200 kDa). No loss of protein was seen after HP100 treatment (5.7% HP100 vs 5.6% RawHP, **Table 1S**), therefore decreased spots could not be from loss. The significantly decreased spots with very low volume or zero volume in HP100 shell could be attributed to the pressure stabilization of collagen structure. When collagen was stabilized, number of subunits (spots) produced from reducing agents during extraction was lower than number of subunits produced in native collagen (Ricardblum & Ville, 1989). The absence or very low

356 volume of proteins from this reduction of stabilized proteins resulted in decreased spots. We hereby
357 propose the mechanism of high-pressure induced shell-loosening as shown in **Figure 6A**. Under a mild
358 level of high pressure, i.e. 100 MPa for 1.75 min, physical degradation of protein, loss of some proteins
359 take place in the epidermis while physical degradation and stabilization of collagen protein structure
360 take place in the shell. Physical degradation of high MW shell-loosening-linked proteins caused by
361 pressure and some products of degradation in epidermis are lost during high pressure or subsequent
362 chilling in ice water. The degradation and loss of proteins interrupt the structure of muscle-shell
363 connection which is made of mainly proteins and chitin, and thereby loosen the connection. However,
364 the stabilization of collagen structure in shell may not facilitate the shell-loosening but may prevent the
365 gap expansion. Likely due to this prevention, HP100 was not as effective as Endo3 in the enlargement
366 of the gap.

367 For Endo3 enzyme treatment, decreased spots and increased spots could originate by the same
368 reasons as in high pressure, except for stabilization of collagen structure in terms of decreased spots
369 and interaction of protein with protein/chitin in terms of increased spots. Both epidermis and shell
370 followed the same patterns of protein changes but to different extent, *i.e.*, the number and median MW
371 of increased spots were lower than those of decreased spots, suggesting that increased spots mainly
372 resulted from degradation of higher MW decreased spots. The substantial loss of proteins as the
373 products of degradation seen in **Table 1S** (1.5% Endo3 epidermis vs 7.9% RawE epidermis, 0.6%
374 Endo3 shell vs 10.9% RawE shell) could be responsible for the lower number of increased spots
375 compared to decreased spots. A mechanism of enzymatic shell-loosening is suggested and shown in
376 **Figure 6B**. Under enzymatic maturation, particularly with Endo3, shrimp loosen their shell due to
377 intensely enzymatic degradation and subsequent loss of products of degradation taking place in both

378 shell and epidermis at different levels of intensity. Enzymes (both added and intrinsic) hydrolyze high
379 MW shell-loosening-linked proteins, produce low MW peptides, and some of the peptides is released to
380 the maturation solution causing loss of materials in the muscle-shell connection.

381 **4. Conclusion**

382 High pressure (100 and 600 MPa) and enzyme (proteases Endocut-03L and Tail21) had profound
383 effects on the microstructure and protein of epidermis and shell of cold-water shrimp and these changes
384 were associated with shell-loosening. Microscopic images revealed that both techniques enlarged the
385 gap between shell and epidermis to different extent, with the enzyme Endocut-03L being the most
386 effective, followed by HP100, Tail21, and HP600. The 2DE proteomic map of epidermis and shell
387 treated by the two techniques showed different patterns of protein changes between the two techniques
388 and between the two conditions within the same technique. Endo3 was the most effective technique
389 resulting in numerous changes in the protein profiles of both epidermis and shell. Fold change of
390 protein spots was strongly correlated to the microscopic shell-loosening gap, and thereby the gap could
391 be predicted by the fold change using a PLS model. Based on the obtained results, the mechanisms for
392 pressure and enzyme induced shell-loosening were proposed. High pressure disrupted some shell-
393 loosening-linked proteins in epidermis and shell and caused some loss of resulting small proteins,
394 hence disabled their connective function in the muscle-shell. However, high pressure also stabilized the
395 collagen protein structure to some extent, preventing the shell-loosening gap from being enlarged.
396 Enzyme intensively hydrolyzed proteins in both epidermis and shell, and many products of the
397 hydrolysis were lost during the enzymatic maturation, resulting in loss of materials in the muscle-shell
398 connection and leaving a wider gap between the shell and the meat.

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408 **Conflict of interest**

409 The authors declare that they have no conflict of interest.

410 **References**

- 411 Bhobe, A. M., & Pai, J. S. (1986). Study of the Properties of Frozen Shrimps. *Journal of Food Science*
412 *and Technology-Mysore*, 23(3), 143-148.
- 413 Cariolou, M. A., & Flytzanis, C. N. (1994). Differential Expression of Cuticle-Epidermis Proteins in
414 the Shrimp *Penaeus-Vannamei* During Molting. *Comparative Biochemistry and Physiology B-*
415 *Biochemistry & Molecular Biology*, 108(3), 367-373.
- 416 Chang, E. S. (1985). Hormonal Control of Molting in Decapod Crustacea. *American Zoologist*, 25(1),
417 179-185.
- 418 Chang, E. S. (1995). Physiological and Biochemical Changes During the Molt Cycle in Decapod
419 Crustaceans: An Overview. *Journal of Experimental Marine Biology and Ecology*, 193(1-2), 1-
420 14.
- 421 Dall, W. (1965). Studies on the Physiology of a Shrimp, *Metapenaeus* Sp. (Crustacea: Decapoda:
422 Penaeidae). Iii. Composition and Stucture of the Integument. *Australian Journal of Marine and*
423 *Freshwater Research*, 16(1), 13-24.
- 424 Dang, T. T., Gringer, N., Jessen, F., Olsen, K., Bøknæs, N., Nielsen, P. L., & Orlie, V. (2018a).
425 Emerging and Potential Technologies for Facilitating Shrimp Peeling: A Review. *Innovative*
426 *Food Science & Emerging Technologies*, 45, 228-240.
- 427 Dang, T. T., Gringer, N., Jessen, F., Olsen, K., Bøknæs, N., Nielsen, P. L., & Orlie, V. (2018b).
428 Enzyme-Assisted Peeling of Cold Water Shrimps (*Pandalus Borealis*). *Innovative Food Science*
429 *& Emerging Technologies*, 47, 127-135.

430 Dang, T. T., Gringer, N., Jessen, F., Olsen, K., Bøknæs, N., Nielsen, P. L., & Orlie, V. (2018c).
 431 Facilitating Shrimp (*Pandalus Borealis*) Peeling by Power Ultrasound and Proteolytic Enzyme.
 432 *Innovative Food Science & Emerging Technologies*, 47, 525-534.

433 Floros, J. D. (1990). Diffusion Phenomena During Chemical (Naoh) Peeling of Tomatoes. *Journal of*
 434 *Food Science*, 55(2), 552-553.

435 Floros, J. D., & Chinnan, M. S. (1988). Microstructural Changes During Steam Peeling of Fruits and
 436 Vegetables. *Journal of Food Science*, 53(3), 849-853.

437 Freeman, J. A., & Costlow, J. D. (1979). Hormonal Control of Apolysis in Barnacle Mantle Tissue
 438 Epidermis, in Vitro. *Journal of Experimental Zoology*, 210, 333-345.

439 Li, X., Pan, Z. L., Atungulu, G. G., Wood, D., & McHugh, T. (2014). Peeling Mechanism of Tomato
 440 under Infrared Heating: Peel Loosening and Cracking. *Journal of Food Engineering*, 128, 79-
 441 87.

442 Lopez, J. L. (2007). Two-Dimensional Electrophoresis in Proteome Expression Analysis. *Journal of*
 443 *Chromatography B-Analytical Technologies in the Biomedical and Life Sciences*, 849(1-2),
 444 190-202.

445 Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein Measurement with the
 446 Folin Phenol Reagent. *Journal of Biological Chemistry*, 193(1), 265-275.

447 Mevik, B. H., & Wehrens, R. (2007). The Pls Package: Principal Component and Partial Least Squares
 448 Regression in R. *Journal of Statistical Software*, 18(2), 1-23.

449 Peterson, G. L. (1977). A Simplification of the Protein Assay Method of Lowry Et Al. Which Is More
 450 Generally Applicable. *Analytical Biochemistry*, 83(2), 346-356.

451 Potekhin, S. A., Senin, A. A., Abdurakhmanov, N. N., & Tiktopulo, E. I. (2009). High Pressure
452 Stabilization of Collagen Structure. *Biochimica Et Biophysica Acta-Proteins and Proteomics*,
453 1794(8), 1151-1158.

454 Ricardblum, S., & Ville, G. (1989). Collagen Cross-Linking. *International Journal of Biochemistry*,
455 21(11), 1185-1189.

456 Rodde, R. H., Einbu, A., & Varum, K. M. (2008). A Seasonal Study of the Chemical Composition and
457 Chitin Quality of Shrimp Shells Obtained from Northern Shrimp (*Pandalus Borealis*).
458 *Carbohydrate Polymers*, 71(3), 388-393.

459 Szczesniak, A. S. (1998). Effect of Storage on Texture. In I. A. Taub & R. P. Singh (Eds.), *Food*
460 *Storage Stability*. Boca Raton, FL: CRC Press

461 Xu, Y., Bajaj, M., Schneider, R., Grage, S. L., Ulrich, A. S., Winter, J., & Gallert, C. (2013).
462 Transformation of the Matrix Structure of Shrimp Shells During Bacterial Deproteination and
463 Demineralization. *Microbial Cell Factories*, 12(1), 1-12.

464 Yang, H., Li, J., Yu, Y., Zheng, W., Zhu, S., & Hu, Y. (2010). *High Pressure Shelling of Shrimp and*
465 *Quality Improvement*. Paper presented at the American Society of Agricultural and Biological
466 Engineers Annual International Meeting 2010, ASABE 2010.

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Figure 1. Microstructural changes of muscle-shell attachment and microscopic shell-loosening gaps after high pressure and enzyme treatment. (A) TEM native microstructure of raw shrimp; Epi = epicuticle, Exo = exocuticle, Endo = endocuticle, Mem = membranous layer, and Ed = epidermis. Scale bar = 10 μm. (B) raw shrimp from high pressure batch; (C) shrimp treated with high pressure at 100 MPa; (D) shrimp treated with high pressure at 600 MPa, (E) raw shrimp for enzyme batch, (F) shrimp treated with Endo3, and (G) shrimp treated with Tail21. Arrow head indicates the gap between shell

475 and epidermis. Scale bar = 25 μm . (H) microscopic shell-loosening gaps of raw shrimp and treated
476 shrimp. Mean column + SD bar of 50-80 measurements.

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479 **Figure 2.** PCA score plot for the abundance of all epidermis protein spots (961) and shell protein spots
480 (777). RawHP, untreated shrimp for high pressure batch; HP100, shrimp treated with high pressure at
481 100 MPa; HP600, shrimp treated with high pressure at 600 MPa; RawE, untreated shrimp for enzyme
482 batch; Endo3, shrimp treated with Endo3 enzyme; Tail21, shrimp treated with Tail21 enzyme.

Figure 3. Distribution of increased and decreased protein spots versus molecular weight, pI, fold change. In the scatter plots, increased protein spots are shown in black, decreased protein spots are

485 shown in red, and non-significant spots are shown in blue. The inset image is corresponding
486 representative 2DE gel. HP100, shrimp treated with high pressure at 100 MPa; HP600, shrimp treated
487 with high pressure at 600 MPa; Endo3, shrimp treated with Endo3 enzyme; Tail21, shrimp treated with
488 Tail21 enzyme.

Figure 4. Protein spots differentially present at different treatments. (A-D) Number of (■) increased and (●) decreased protein spots of treated sample compared to untreated sample. (E-H) Venn diagram showing overlaps of protein spots differentially present between two treatments. (I-L) Box plot showing molecular weight of increased and decreased spots, with Tukey whiskers for minimum and maximum data, and a line for the median. ** $p < 0.01$ and *** $p < 0.001$ by Mood median test. (M-N) Venn diagram showing overlaps of protein spots differentially present among four treatments. HP100, shrimp treated with high pressure at 100 MPa; HP600, shrimp treated with high pressure at 600 MPa; Endo3, shrimp treated with Endo3 enzyme; Tail21, shrimp treated with Tail21 enzyme.

Figure 5. Relationship between 2DE and microscopic shell-loosening gap of shrimp. A) correlation between parameters of epidermal protein spots (volume change [ΔVolume], fold [Fold], experimental pI [pI], and experimental molecular weight [MW]) and microscopic shell-loosening gap change [ΔGap]; B) correlation among parameters of shell protein spots and microscopic shell-loosening gap difference; C) PLS predicted-observed values of $\log_{10}$ of microscopic shell-loosening gap based on fold change of epidermal proteins; and D) PLS predicted-observed values of $\log_{10}$ of microscopic shell-loosening gap based on fold change of shell proteins. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. In the bottom of the correlogram, the legend color shows the correlation coefficients and the corresponding colors. Positive correlations are displayed in blue color gradient and negative correlations in red color gradient. Color intensity and the size of the circle are proportional to the correlation coefficients. In PLS models, predictions were plotted against the known reference values of shell-loosening gap for each sample, $n\text{comp}$ is the number of select components/factors.

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Figure 6. Proposed mechanisms of shell-loosening induced by high pressure 100 MPa (A) and Endo3 proteolytic enzyme (B).

512 **Supplementary material**

513 **Table 1S.** Soluble protein content of epidermis and shell of raw, HP- and enzyme-treated shrimp

Sample	Epidermis (%)	Shell (%)
RawHP	10.1 ± 0.9 ^a	5.6 ± 0.6 ^b
HP100	8.4 ± 2.5 ^{ab}	5.7 ± 0.2 ^b
HP600	10.1 ± 2.1 ^a	5.4 ± 0.3 ^b
RawE	7.9 ± 0.9 ^{ab}	10.9 ± 1.3 ^a
Endo3	1.5 ± 0.4 ^c	0.6 ± 0.8 ^c
Tail21	6.6 ± 1.0 ^b	7.9 ± 1.7 ^b

514 Values in the same column with different letters are significantly different at p<0.05, based on Student-
515 Newman-Keuls test. RawHP, untreated shrimp for high pressure batch; HP100, shrimp treated with
516 high pressure at 100 MPa; HP600, shrimp treated with high pressure at 600 MPa; RawE, untreated
517 shrimp for enzyme batch; Endo3, shrimp treated with Endo3 enzyme; Tail21, shrimp treated with
518 Tail21 enzyme.