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# Effect of pre-cooking times on enzymes, properties, and melanosis of Pacific white shrimp during refrigerated storage

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## Abstract

Pre-cooked shrimp have gained interest due to their attractive color and flavor. However, shrimp without sufficient pre-cooking have faced melanosis, particularly during the extended storage. This leads to the rejection by consumers and the market. Therefore, pre-cooking with appropriate time would be a means to tackle such a problem. The objective of this study was to investigate the effect of pre-cooking times on enzyme activities, properties, microstructure, and melanosis of Pacific white shrimp (*Litopenaeus vannamei*). Pacific white shrimp were subjected to pre-cooking at 80°C for various times (0 to 120 s). The residual activities of polyphenoloxidase (PPO) and protease decreased with increasing pre-cooking times ($p < 0.05$). Nevertheless, the increasing pre-cooking time resulted in more cooking loss. With cooking time above 60 s, a cooking loss of 9.85% was obtained ($p < 0.05$). Marked decreases in the relative PPO and protease activities were observed within the first 30 s of pre-cooking, and negligible activities were detected after 120 s. The microstructure study revealed that the muscle fibers of pre-cooked shrimps were less attached with concomitantly higher shrinkage of the sarcomere, compared with those of the raw counterpart. Shrimps pre-cooked with longer time showed a lower development of black spots as evidenced by a lower melanosis score throughout storage of 7 days at 4°C. Therefore, a pre-cooking time of 30 s at 80°C was sufficient to lower PPO and protease activities with the minimized cooking loss and melanosis during refrigerated storage.

**Keywords:** Pre-cooking time, Pacific white shrimp, Melanosis, Polyphenoloxidase, Protease, Cooking loss, Microstructure

## Background

Nowadays, Pacific white shrimp (*Litopenaeus vannamei*) is one of the shrimp species having a high-demand culture worldwide due to its appealing appearance, taste, flavor, and texture (Rattanasatheirn et al. 2008). Thailand is well known as the world's largest shrimp producer, manufacturer, and exporter. Thailand shrimps and their products have been known for their prime quality and delicacy (Rattanasatheirn et al. 2008). Shrimp, a highly perishable product, commonly have a limited shelf life associated with the development of melanosis formation and microbial spoilage (Gokuglu and Yerlikaya 2008). Melanosis is a natural post-mortem biochemical process in a crustacean caused by polyphenoloxidase

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(PPO). PPO catalyzes the oxidation of phenolic substrates to quinones, which undergo auto-oxidation and polymerization to form melanin, a high molecular weight dark pigment (Kim et al. 2000). Among shrimp products, the pre-cooked white shrimp become much more popular due to their desirable appearance to consumers, especially their reddish color (Manheem et al. 2012). Generally, pre-cooking is classified as a simple process with low cost, which can be used to extend the shelf life of some food products. Pre-cooking can also deactivate some endogenous enzymes associated with shrimp quality loss and destroy some microorganisms, thereby providing a product with good appearance and storage stability (Kim et al. 2000).

PPO has been reported as the major contributor for melanosis in Pacific white shrimp (Nirmal and Benjakul 2012). However, pre-cooking at high temperature was able to decrease the melanosis in shrimp (Manheem et al. 2012). Heating or pre-cooking at a higher temperature led to the loss in cooking yield. This was mainly associated with the thermal denaturation of the muscle protein in shrimp, particularly the myosin heavy chain (Benjakul et al. 2011). To produce pre-cooked shrimp with a long shelf life, sufficient pre-cooking is required to inactivate PPO, but it may cause the enhanced cooking loss and quality changes of pre-cooked shrimp. Nevertheless, there is little information regarding the effect of pre-cooking times on quality changes and melanosis of pre-cooked Pacific white shrimp during refrigerated storage. Therefore, the objectives of this study were to determine the enzyme activities, properties, and microstructure of pre-cooked Pacific white shrimp as affected by pre-cooking time and to monitor melanosis of pre-cooked shrimp during the refrigerated storage.

## Methods

### Chemicals

L-β-(3,4 dihydroxylphenyl)alanine (L-DOPA), L-tyrosine, Brij-35, glutaraldehyde, and casein were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonium sulfate, Tris(hydroxymethyl)aminomethane, sodium chloride, trichloroacetic acid (TCA), sodium dihydrogen phosphate, and absolute ethanol were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

### Sample collection and preparation

Pacific white shrimp (*L. vannamei*) with a size of 55 to 60 shrimp/kg were purchased from the dock in Songkhla province, Thailand. The shrimp were kept in ice with a shrimp/ice ratio of 1:2 (*w*/*w*) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h. Upon arrival, shrimps were washed with tap water and stored in ice until use (not more than 2 h).

For pre-cooking, whole shrimp (head-on) were placed in the stainless cooking pot filled with boiling water (100°C) using a shrimp/water ratio of 1:3 (*w*/*v*). The samples were pre-cooked until the core temperature of the second segment of the shrimp reached 80°C. The come-up time was approximately 3 min. Pre-cooked samples were suddenly transferred into the temperature-controlled water bath (Memmert, Schwabach, Germany) at 80°C and were held for 0, 15, 30, 60, and 120 s. To measure the core temperature, the thermocouple (Union, Kowloon, Hong Kong) was inserted into the middle of the second segment of abdomen. After heating for a designated time, the samples were cooled rapidly

in iced water for 1 min, and then the shrimp samples were drained for 5 min at 4°C. The samples referred to as 'pre-cooked shrimp' were subjected to analyses and subsequent storage.

## Determination of PPO activity

### *Preparation of crude PPO extract*

The pre-cooked cephalothoraxes of 20 shrimps were separated and powderized by grinding with liquid nitrogen in a Waring blender (AY46, Moulinex, Guangdong, China). To extract PPO, the powder from the cephalothoraxes with different pre-cooking times (50 g) was mixed with 150 mL of 0.05 M sodium phosphate buffer, pH 7.2, containing 1.0 M NaCl and 0.2% Brij 35 according to the method of Simpson et al. (1987) with a slight modification. The mixture was stirred continuously at 4°C for 30 min, followed by centrifugation at 8,000×*g* at 4°C for 30 min using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). Solid ammonium sulfate was added into the supernatant to obtain 40% saturation. The mixture was allowed to stand at 4°C for 30 min and then centrifuged at a speed of 12,500×*g* at 4°C for 30 min. The obtained pellet was collected and dispersed in a minimum volume of 0.05 M sodium phosphate buffer, pH 7.2. The pellet solution was dialyzed against 15 volumes of the same buffer at 4°C with three changes overnight. After dialysis, insoluble materials in the dialysate were removed by centrifugation at 3,000×*g* at 4°C for 30 min, and the supernatant was used as 'crude PPO extract'.

### *Measurement of PPO activity*

PPO activity was determined by monitoring the rate of dopachrome formation using L-DOPA as a substrate according to the method of Nirmal and Benjakul (2010) with a slight modification. Crude PPO extract (100 μL) was mixed with 100 μL of distilled water and 400 μL of 0.05 M sodium phosphate buffer (pH 6.0) pre-incubated at 45°C. To initiate the reaction, 600 μL of 15 mM L-DOPA was added into the assay mixture. The reaction was run for 3 min at 45°C, and the formation of dopachrome was monitored by measuring the absorbance at 475 nm ($A_{475}$) using a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan). One unit of PPO activity was defined as the enzyme causing an increase in $A_{475}$ by 0.001 per min. Enzyme and substrate blanks were prepared by excluding the substrate and enzyme, respectively, from the reaction mixture, and the distilled water was used instead. Remaining activity was expressed relative to that of the raw sample.

## Determination of protease activity

### *Preparation of crude protease extract*

Cephalothorax powder (20 g) was mixed with two volumes of 0.01 M sodium phosphate buffer (pH 7.6) (Brauer et al. 2003). The mixture was homogenized for 5 min using a homogenizer (IKA Labortechnik, Selangor, Malaysia) at a speed of 11,000 rpm. The homogenate was stirred for 30 min at 4°C, followed by centrifugation at 10,000×*g* for 30 min at 4°C using a refrigerated centrifuge. The supernatant was used as 'crude protease extract' (CPE).

### *Determination of protease activity*

Proteolytic activity of CPE was determined using casein as a substrate according to the method of An et al. (1994). To the pre-incubated reaction mixture containing 2 mg of

casein, 200 μL of distilled water, and 625 μL of reaction buffer (0.05 M Tris–HCl buffer, pH 8.0), CPE (200 μL) was added to initiate reaction. The mixture was incubated at 70°C (Manheem et al. 2012). After 10 min, the reaction was terminated by adding 200 μL of 50% (*w*/*v*) TCA. Unhydrolyzed protein substrate was allowed to precipitate for 15 min at 4°C and centrifuged at 8,500×*g* for 10 min. The oligopeptide content in the supernatant was determined by the Lowry method (Lowry et al. 1951) using tyrosine as a standard. One unit of activity was defined as that releasing 1 mmol of tyrosine/min. A blank was run in the same manner, except that the enzyme was added after addition of 50% TCA. Remaining activity was expressed relative to that of the raw sample.

### Determination of cooking yield and cooking loss

Cooking yield and cooking loss were measured by weighing the shrimp before and after pre-cooking. Shrimp were pre-cooked to obtain core temperatures of 80°C and the designated pre-cooking time, immediately cooled in iced water for 1 min, and drained at 4°C for 5 min. The pre-cooked shrimp were weighed. Cooking yield and cooking loss were calculated using the following equations:

$$\text{Cooking yield}(\%) = (B/A) \times 100$$
$$\text{Cooking loss } (\%) = ((A - B)/A) \times 100,$$

where $A$ is the weight before pre-cooking and $B$ is the weight after pre-cooking, followed by cooling in iced water.

### Determination of microstructure

Microstructures of raw shrimp and those pre-cooked for different times (0, 30, and 120 s) were analyzed as described by Jones and Mandigo (1982) with a slight modification. All samples were cut into a cube ($4 \times 4 \times 4$ mm$^3$) with a razor blade. The prepared samples were fixed with 0.2 M phosphate buffer, pH 7.2, containing 2.5% glutaraldehyde at room temperature for 2 h. All specimens were washed three times with deionized water for 15 min and dehydrated with serial concentrations of 25%, 50%, 75%, 95%, and 100% ethanol for 30 min each. All specimens were coated with 100% gold (Sputter coater SPI-Module, West Chester, PA, USA). The microstructures were visualized using a scanning electron microscope (Quanta 400, FEI, Eindhoven, the Netherlands).

### Determination of melanosis

To monitor melanosis in the samples, 14 shrimps were placed on a polystyrene tray ($8 \times 8$ in.$^2$) and covered with shrink film. Thereafter, the samples were stored at 4°C for 7 days. The samples were taken every day for melanosis assessment. Melanosis or blackening of pre-cooked shrimp was evaluated through visual inspection by ten trained panelists using a 10-point scoring test following the method of Montero et al. (2001) with some modifications. To prepare the references, shrimp stored at 4°C for 0, 2, 4, 6, 8, and 10 days were cooked to obtain the core temperature of 60°C for 1 min. Those samples were used as the references with melanosis scores of 0, 2, 4, 6, 8, and 10, respectively. Training was conducted for two sessions. Panelists were asked to give the melanosis score (0 to 10), where 0 = complete absence of black spots, 2 = slight (up to 20% of the shrimp's surface is affected), 4 = moderate (20% to 40% of the shrimp's surface is affected), 6 = notable (40% to 60% of the shrimp's surface is affected), 8 = severe (60% to 80% of the shrimp's

surface is affected), and 10 = extremely heavy (80% to 100% of the shrimp's surface is affected).

### Statistical analysis

The experiments were run in triplicate with three different lots of samples. Data were subjected to analysis of variance, and mean comparison was carried out using Duncan's multiple range test (Steel and Torrie 1980). Analysis was performed using the Statistical Package for Social Sciences (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA).

## Results and discussion

### Effect of pre-cooking time on PPO and protease activities of Pacific white shrimp

The effect of pre-cooking time on PPO and protease activities of Pacific white shrimp cephalothoraxes is shown in Figure 1. The activity of PPO in the cephalothoraxes (Figure 1a) obviously decreased after pre-cooking was performed for up to 30 s. The higher decrease in PPO activity was obtained as the pre-cooking time increased ($p < 0.05$). The result suggested that PPO was lost in activity due to the thermal denaturation, which depended not only on the core temperature but also on the pre-cooking time. Protein and enzyme are known to denature and become inactivated when they are exposed to

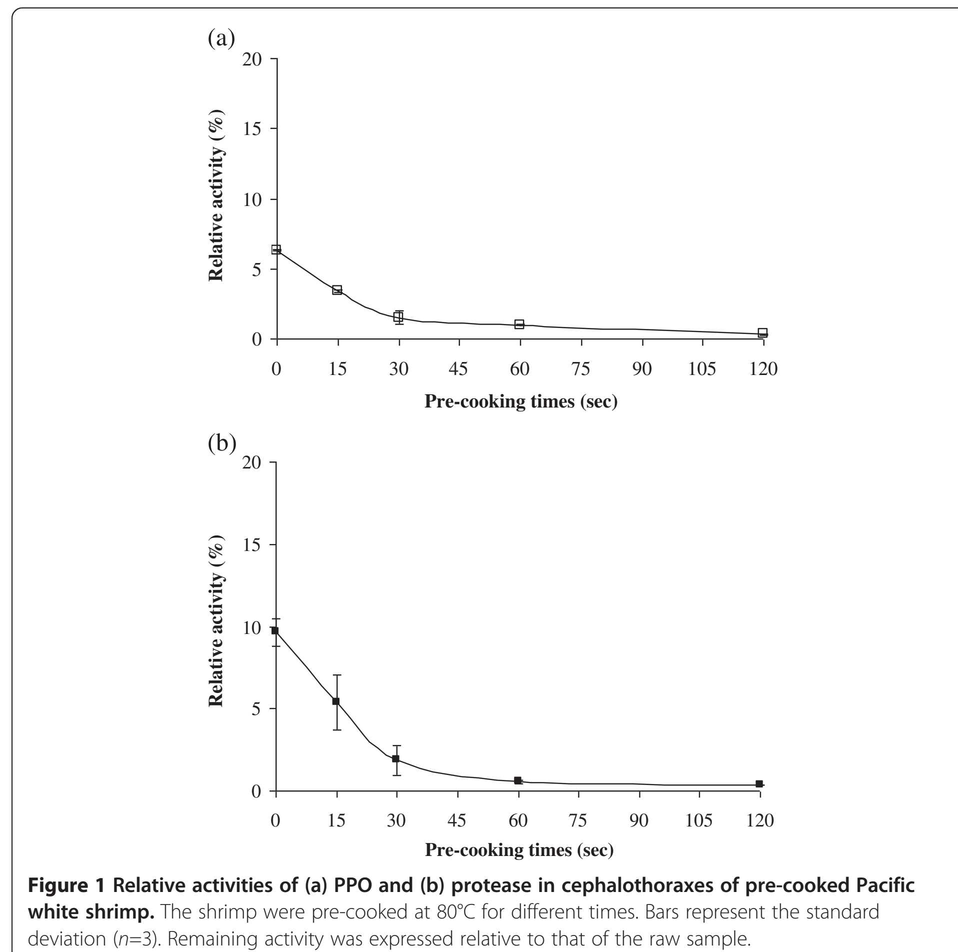


**Figure 1 Relative activities of (a) PPO and (b) protease in cephalothoraxes of pre-cooked Pacific white shrimp.** The shrimp were pre-cooked at 80°C for different times. Bars represent the standard deviation ($n$=3). Remaining activity was expressed relative to that of the raw sample.

biological surroundings such as temperature, mechanical forces, radiation, chemicals, and transition metals (Benjakul et al. 2011). Unfolding of the native structure to a random coiled structure of enzyme leads to the loss in catalytic activity (Manheem et al. 2012; Kim et al. 2000). PPO activity in shrimp generally depended on different factors such as species, method and season of capture, handling, molting cycle, sex, proteases, and storage condition (Bartolo and Birk 1998; Manheem et al. 2012).

Protease activity of pre-cooked Pacific white shrimp is depicted in Figure 1b. As the pre-cooking time increased, the continuous decreases in the proteolytic activity of shrimp cephalothoraxes were observed ($p < 0.05$). When pre-cooking time reached 30 s, the relative proteolytic activity was sharply decreased from 9.65% to 1.86%. The remaining proteases might be able to activate proPPO to PPO to some degree, thereby inducing melanosis in pre-cooked shrimp during storage (Manheem et al. 2012). It has been suggested that the activation of proPPO is regulated by serine proteases in the hemolymph (Zamorano et al. 2009). Serine and cysteine proteases were reported to be the major proteases in the Pacific white shrimp (Thepnuan 2007). The accessibility of proteolytic enzymes to proPPOs is another important factor affecting melanosis of shrimp (Manheem et al. 2012). Furthermore, proteolytic enzymes leaching from the digestive tract may contribute to proPPO activation during storage (Zamorano et al. 2009). In addition, Yan and Taylor (1991) reported that the activity of PPO from Norway lobster heads including the viscera increased after being activated by endogenous proteases.

### Effect of pre-cooking time on cooking yield and cooking loss of Pacific white shrimp

Cooking yield and cooking loss of pre-cooked Pacific white shrimp subjected to pre-cooking at 80°C for different times are shown in Figure 2a,b, respectively. Cooking loss of pre-cooked Pacific white shrimp increased when pre-cooking time increased ($p < 0.05$). With increasing pre-cooking time, the amount of water retained in the shrimp meat decreased with coincidental increases in fat and protein contents (Benjakul et al. 2008). Pre-cooking shrimp in boiling water at high temperature caused protein denaturation and coagulation, which in turn led to lower water-holding capacity (Niamnuy et al. 2007). The water was mainly lost as a result of heat-induced denaturation of proteins during cooking of the meat. As a result, less water was entrapped within the muscle structure (Aaslyng et al. 2003). The results suggest that the protein denaturation depended not only on the core temperature but also on the pre-cooking time. Pre-cooking times of 60 and 120 s resulted in the higher cooking loss with the lower cooking yield ($p < 0.05$). In addition, Erdogdu et al. (2004) reported that the yield of cooked meat under commercial conditions depends on the size and age of the shrimp. Therefore, pre-cooking at high temperature for a longer pre-cooking time directly affected the cooking loss and cooking yield of Pacific white shrimp. Even though high temperature with longer heating time could prevent melanosis effectively, it caused the detrimental effect on cooking yield (Manheem et al. 2012). Pre-cooking at 80°C with a time of 30 s did not cause the severe cooking loss, but it inactivated both PPO and proteases in cephalothoraxes to a high degree.

### Effect of pre-cooking time on microstructure of Pacific white shrimp

Microstructures of raw Pacific white shrimp and those pre-cooked for various times are illustrated in Figure 3. For the longitudinal section, raw meats had a well-organized

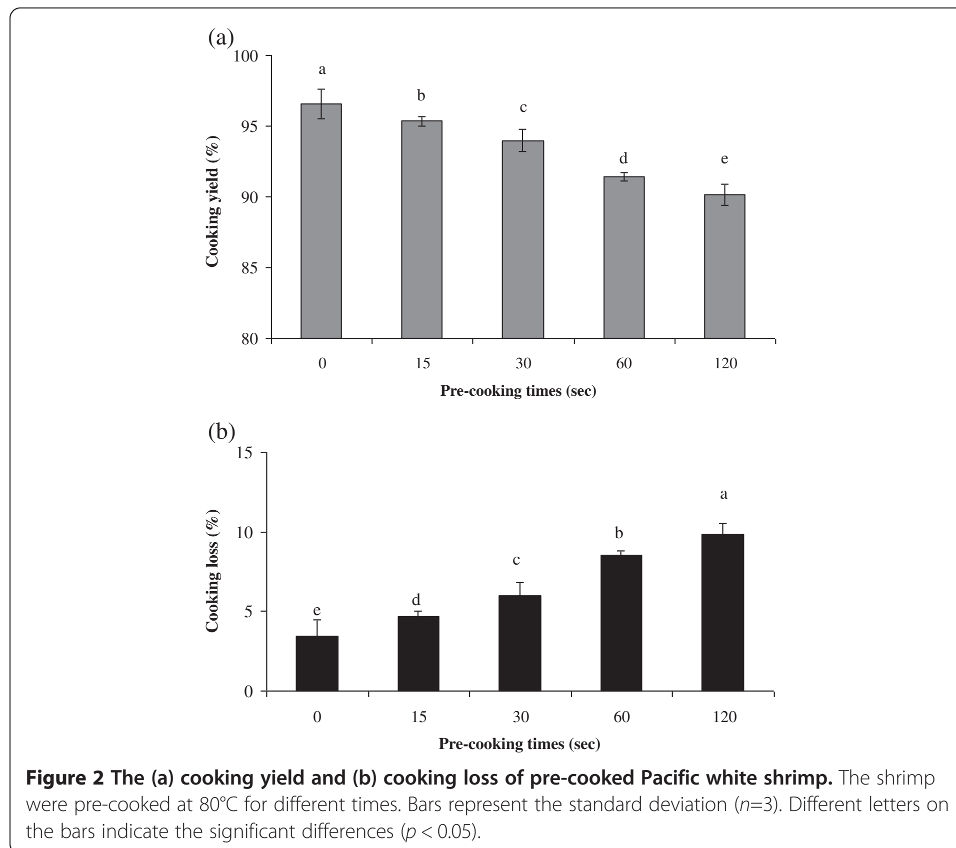


**Figure 2 The (a) cooking yield and (b) cooking loss of pre-cooked Pacific white shrimp.** The shrimp were pre-cooked at 80°C for different times. Bars represent the standard deviation ($n$=3). Different letters on the bars indicate the significant differences ($p < 0.05$).

structure of myofibrils with less gaps between fibrils (Figure 3a). Raw shrimp tended to have the denser structure, compared with the pre-cooked counterpart. The shrinkage of the sarcomere was obvious in pre-cooked shrimps. More pronounced changes in the microstructure of Pacific white shrimp meats were observed when pre-cooking time increased, especially when pre-cooking was longer than 30 s (Figure 3a). It was suggested that the heating process caused the shrinkage of the muscle protein of shrimp (Benjakul et al. 2008). Heat treatment increased firmness and the degree of shrinkage of black tiger shrimp and white shrimp (Benjakul et al. 2008). Denaturation and disintegration of the perimysium and endomysium collagen, together with the denaturation of myofibrils, might result in the shrinkage of muscle fibers with coincidental Z-line disintegration or disruption.

For the transverse sections, gaps between muscle fibers and bundles were visible in all shrimp samples, regardless of pre-cooking time (Figure 3b). The gaps might occur due to some degradation of muscle and endomysium connective tissue (Sotelo et al. 2004). Among all pre-cooked samples with different pre-cooking times, the higher shrinkage of muscle fibers was found in shrimp pre-cooked for 120 s. Collagen, around the muscle fibers (endomysium) and around the muscle bundles (perimysium), was postulated to convert to gelatin to a higher extent when being pre-cooked for a longer time (Niamnuy et al. 2008). When the proteins underwent thermal denaturation, the water was less imbibed or bound in their structure (Benjakul et al. 2008). The release of water from protein molecules might facilitate the muscle fiber to align closely, leading to the more compact muscle fibers. Nip and Moy (1988) reported the microstructural changes of the boiled meat of prawn (*Macrobranchium rosenbergii*).

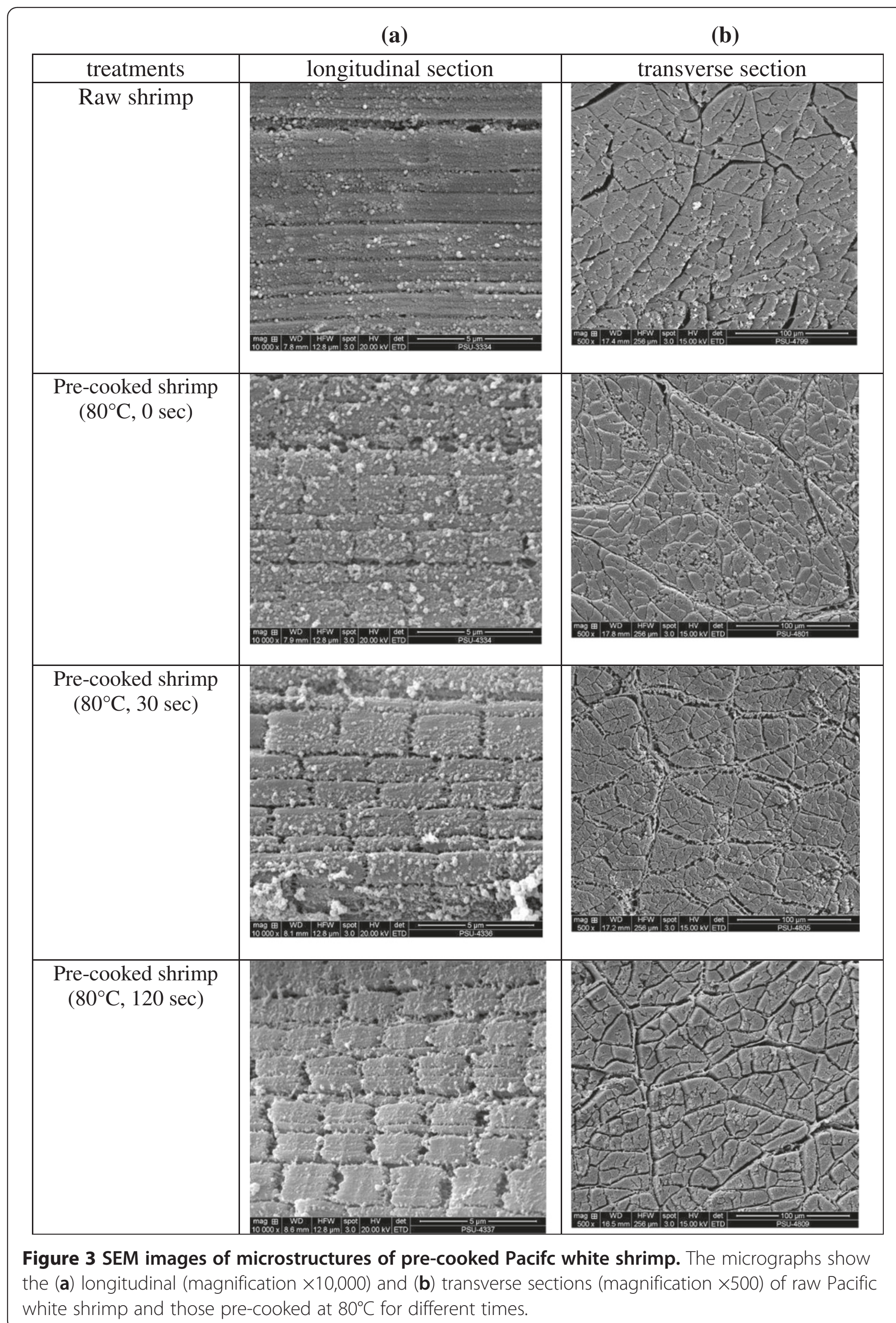


**Figure 3 SEM images of microstructures of pre-cooked Pacifc white shrimp.** The micrographs show the (**a**) longitudinal (magnification ×10,000) and (**b**) transverse sections (magnification ×500) of raw Pacific white shrimp and those pre-cooked at 80°C for different times.

### Effect of pre-cooking time on melanosis of Pacific white shrimp during refrigerated storage

The melanosis score of Pacific white shrimp pre-cooked for different times during 7 days of refrigerated storage is shown in Figure 4. At day 0, no melanosis was observed in all samples (score = 0). When the storage time increased, the melanosis score of all samples increased ($p < 0.05$). However, the sample with the pre-cooking time of 120 s had no melanosis within the first 2 days of storage. The result showed that melanosis was more retarded when the

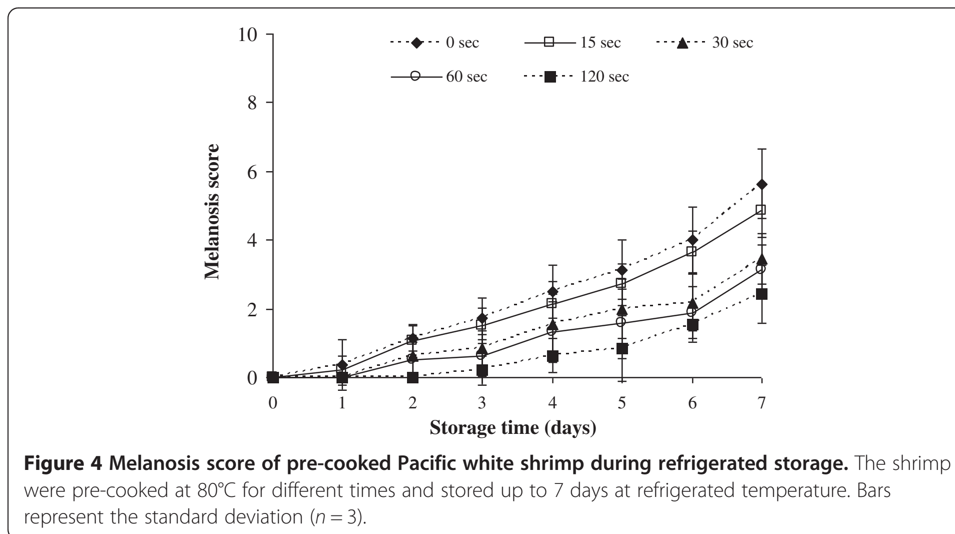


**Figure 4 Melanosis score of pre-cooked Pacific white shrimp during refrigerated storage.** The shrimp were pre-cooked at 80°C for different times and stored up to 7 days at refrigerated temperature. Bars represent the standard deviation ($n = 3$).

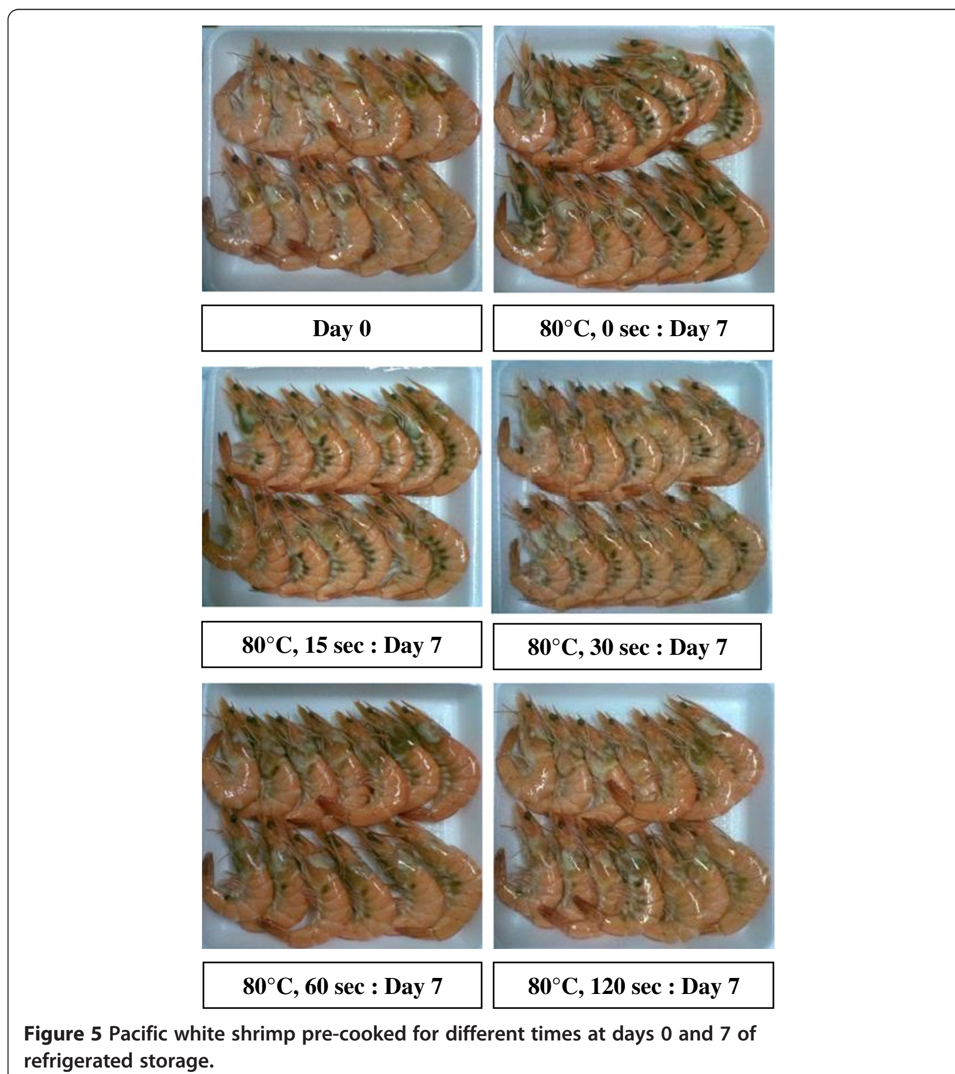


**Figure 5 Pacific white shrimp pre-cooked for different times at days 0 and 7 of refrigerated storage.**

pre-cooking time increased ($p < 0.05$). The result suggested that PPO was likely inactivated at a high temperature, particularly with longer pre-cooking time. Endogenous shrimp PPO catalyzes the initial step of black spot formation and remains active throughout post-harvest processing unless the shrimps are frozen or cooked (Gokuglu and Yerlikaya 2008).

Pre-cooked Pacific white shrimp with different pre-cooking times at days 0 and 7 of refrigerated storage are illustrated in Figure 5. No melanosis was observed in all samples at day 0, regardless of pre-cooking time. After 7 days of storage, marked melanosis was developed in pleopods, and the moderate melanosis took place in cephalothoraxes. This black discoloration was caused by endogenous PPO, which oxidizes phenols to quinones and polymerizes the colorless quinones to a black high molecular weight pigment (Montero et al. 2006). The highest melanosis was found in pre-cooked shrimp with the lowest pre-cooking time, whereas a lower melanosis was detectable in shrimp pre-cooked for a longer time. Slight melanosis occurred in samples with a pre-cooking time of 120 s. PPO, which played a role in melanosis, could be inactivated at high temperature for a longer time. Therefore, the rate of melanosis could be regulated by pre-cooking for an appropriate time.

## Conclusions

PPO, proteases, and melanosis of pre-cooked Pacific white shrimp were affected by pre-cooking time. PPO and proteases decreased with increasing pre-cooking time. Both enzymes were more retained when pre-cooking time was lower than 30 s. Pre-cooked shrimp had the increased cooking loss but lowered melanosis formation after 7 days of refrigerated storage when pre-cooking time increased. Furthermore, the microstructure of shrimp meat was affected by pre-cooking times. Therefore, the appropriate cooking temperature and time could be used to minimize the cooking loss and melanosis formation in pre-cooked shrimp.

#### Abbreviations

CPE: crude protease extract; L-DOPA: L-β-(3,4 dihydroxylphenyl)alanine; PPO: polyphenoloxidase; TCA: trichloroacetic acid.

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

SB formulated the hypothesis and designed the studies. KM performed the experiments and analyses. KM and SB wrote the paper. KK, NF, and WV gave advice for the experiments. All authors have approved the final version of the manuscript.

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