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Impacts of freeze-thawing on melanosis and quality change of pre-cooked Pacific white shrimp

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Abstract

Polyphenoloxidase (PPO), protease activities, melanosis and exudate loss of pre-cooked Pacific white shrimp (*Litopenaeus vannamei*) subjected to multiple freeze-thaw cycles (1, 3 and 5 cycles) using different thawing methods were studied. PPO and protease activities increased as freeze-thaw cycles increased ($p < 0.05$). Thawing using running tap water resulted in the higher increases in both PPO and protease activities of samples, compared with thawing at 4°C when freeze-thawing cycles were higher than 1 and 3 cycles, respectively. Exudate loss increased as freeze-thawing cycles increased ($p < 0.05$), but thawing methods had no impact on the formation of exudates. During refrigerated storage at 4°C, pre-cooked Pacific white shrimp had the continuous increases in melanosis. Generally, melanosis was more pronounced when subjected to repeated freeze-thawing ($p < 0.05$). Samples thawed with running tap water showed the higher melanosis than those thawed at 4˚C, especially with 3 and 5 freeze-thaw cycles and extended storage time ($p < 0.05$). Therefore, freeze-thawing had a detrimental effect on quality of pre-cooked Pacific white shrimp during refrigerated storage.

Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is an economically important crustacean in Thailand (Donato *et al*., 2005). Nowadays, Pacific white shrimp is the most important shrimp for aquaculture, replacing *Penaeus monodon* and *P. chinensis* (FAO, 2009). Furthermore, Pacific white shrimp has the highest value of all internationally traded fishery products (FAO-FIPS, 2010). Generally, marine species are very perishable and their quality and freshness rapidly decrease during post-mortem handling and storage. Microbial spoilage and biochemical reaction, especially melanosis (blackening) in crustacean, have been known to limit their shelf-life (Aubourg *et al*., 2007). Melanosis reduces the market value, leading to the considerable financial loss of crustacean (Kim *et al*., 2000). It is triggered by a biochemical mechanism, caused by PPO, which induces the oxidation of phenols to quinones. This is followed by non-enzymatic polymerization of the quinones, giving rise to pigment of high molecular weight and very dark or black coloring (Montero *et al*., 2001). PPO from some crustacean has been known to be activated by proteases (Martínez-Álvarez *et al*., 2005). Recently, Manheem *et al.* (2012) found that PPO from Pacific white shrimp was activated by trypsin from hepatopancreas.

Frozen storage is an important preservation method for seafood product. It can effectively retard quality changes of shrimp and shrimp products. After shipping and distribution of frozen products, thawing is a common means to be implemented prior to the display of the products. The repeated freeze-thawing is therefore a practice in retail shop, restaurant or home (Boonsumrej *et al*., 2007). This process can enhance melanosis of shrimp products after thawing and subsequent distribution under the refrigerated condition in retail shop. Melanosis initially takes place at carapace and spreads to other portions, leading to quality losses. So far, a little information about the impacts of freeze-thawing on melanosis and quality change of pre-cooked Pacific white shrimp has been reported. Thus, the objective of this work was to evaluate the effect of freeze-thaw cycle and thawing methods on PPO and protease activities, exudate as well as melanosis of pre-cooked Pacific white shrimp during extended refrigerated storage.

Materials and Methods

Chemicals

L-β-(3, 4 dihydroxylphenyl) alanine (L-DOPA), *L*-tyrosine, Brij-35 and casein were purchased from

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Sigma-Aldrich (St. Louis, MO, U.S.A.). Ammonium sulphate, Tris (hydroxymethyl) aminomethane, sodium chloride and trichloroacetic acid (TCA) were obtained from Merck (Darmstadt, Germany). All chemicals used were of analytical grade.

Sample collection and preparation

Pacific white shrimp (*Litopenaeus vannamei*) with the size of 55-60 shrimps/ 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 used (not more than 2 h).

Whole Pacific white shrimp (head-on) were pre-cooked by submerging the samples in boiling water (100°C) until the core temperature of the second segment of abdomen reached 80°C. Thereafter, the heating at 80°C was maintained for 30 sec. To measure the core temperature, the thermo-couple (Union, Kowloon, Hong Kong) was inserted into the middle of the second segment of abdomen. After heating for 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 were referred to as 'pre-cooked shrimp'.

Freeze-thawing of pre-cooked Pacific white shrimp

Whole pre-cooked shrimp were packed in polyethylene bags (20 shrimps per bag), heat-sealed and frozen at -20°C using an air-blast freezer (Patkol Co. Ltd., Bangkok, Thailand) for 48 h. These frozen shrimp were thawed using two different methods: (1) leaving in a refrigerator (4˚C) for 6 h (Nirmal and Benjakul., 2010) and (2) using running tap water (27-28˚C) for 1 h . The core temperature of the second segment of shrimp after thawing was approximately 0-2°C. Thawed samples were again frozen for 48 h. Freeze-thawing was repeated up to 3 and 5 cycles. Pre-cooked sample without freeze-thawing was used as the control. All samples obtained were subjected to measurement of PPO and protease activities, exudate loss and melanosis.

Determination of PPO activity

The pre-cooked cephalothoraxes of twenty shrimps subjected to different thawing methods with various freeze-thaw cycles (0, 1, 3 and 5 cycles) were separated and powderized by grinding with liquid nitrogen in a waring blender (AY46, Moulinex, Guangdong, China). To extract PPO, the powder from cephalothoraxes with different treatments (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 8,000xg at 4°C for 30 min using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). Solid ammonium sulphate 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 12,500xg 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 3,000xg at 4°C for 30 min and the supernatant was used as 'crude PPO extract'.

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 were 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 the absorbance by 0.001/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.

Determination of protease activity

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 10,000xg for 30 min at 4°C using a refrigerated centrifuge. The supernatant was used as 'crude protease extract' (CPE).

Proteolytic activity of CPE was determined using casein as a substrate according to the method of An et al. (1994). To the preincubated 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 70ºC (Manheem *et al*., 2012). After 10 min, the reaction was terminated by adding 200 µL of 50% (w/v) trichloroacetic acid (TCA). Unhydrolyzed protein substrate was allowed to precipitate for 15 min at 4°C and centrifuged at 8,500xg for 10 min. The oligopeptide content in 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 mmole of tyrosine/min. A blank was run in the same manner, except CPE was added after addition of 50% TCA.

Determination of exudate losses

Exudate losses were measured by weighing the shrimp before and after freeze-thawing for different cycles.

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

where : A = weight of pre-cooked shrimp before freeze-thawing

B = weight of pre-cooked shrimp after freeze-thawing

Determination of melanosis

To monitor melanosis in the samples, pre-cooked Pacific white shrimp subjected to different freeze-thaw cycles with various thawing methods (14 shrimps) were placed on a polystyrene tray (8×8 inches) 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 of pre-cooked shrimp was evaluated through visual inspection by ten trained panelists using 10-point scoring test following the method of Montero *et al.* (2001) with some modifications. Panelists were asked to give the melanosis score (0-10), where 0 = completely absent of black spots; 2 = slight (up to 20% of shrimp's surface affected); 4 = moderate (20% to 40% of shrimp's surface affected); 6 = notable (40% to 60% of shrimp's surface affected); 8 = severe (60% to 80% of shrimp's surface affected); 10 = extremely heavy (80% to 100% of shrimp's surface affected).

Statistical analysis

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

Results and discussion

Effect of multiple freeze-thaw cycles and different thawing methods on PPO and protease activities of pre-cooked Pacific white shrimp

The effect of different freeze-thaw cycles and thawing methods on PPO and protease activities of pre-cooked Pacific white shrimp cephalothoraxes is shown in Figure1. No differences in PPO activity were observed between the samples without freeze-thawing (zero freeze-thaw cycle) and those subjected to 1 freeze-thaw cycle, regardless of thawing methods used ($p > 0.05$) (Figure 1a). Thereafter, PPO activity of pre-cooked Pacific white shrimp increased when the freeze-thaw cycles increased ($p < 0.05$). The result suggests that freeze-thawing might activate PPO activity to some degree. During repeated freezing and thawing, tissues were injured and several cells were disrupted. Thus, the inactive form of PPO (proPPO) stored in hemocytes, the digestive gland and chromatophores were easily released and activated (Díaz-Tenorio *et al*., 2007). For the same freeze-thaw cycle, there was no difference in PPO activity between samples subjected to both thawing methods when freeze-thawing cycles were 0 and 1 ($p > 0.05$). Nevertheless, PPO activity of samples thawed using the running tap water was higher than that of samples thawed at 4°C, when freeze-thaw cycles were 3 and 5 ($p < 0.05$). Thawing by running tap water caused the increase in PPO acitivity since this thawing method was associated with more rapid increase in temperature environment (Nirmal and Benjakul, 2010). As a consequence, the tissues were more disrupted, thereby liberating more proPPO. The resulted was in agreement with Nirmal and Benjakul (2010) who found that the frozen Pacific white shrimp thawed at 4°C showed the lower PPO activity when compared to those subjected to other thawing methods (thawing using running tap water and thawing at room temperature). Díaz-Tenorio *et al.* (2007) recommended cryogenic freezing and thawing at 4°C of white leg shrimp (*Litopenaeus vannamei*) for marketing purpose.

The protease activity of pre-cooked Pacific white shrimp, as affected by freeze-thawing cycles and thawing method, showed similar trend to PPO activity. The proteolytic activity of crude protease extract from the cephalothoraxes of pre-cooked shrimp was slightly increased when freeze-thaw cycles were more than 1 cycles ($p < 0.05$). Crustaceans such as shrimp, crayfish and American lobster, etc. contain highly active proteases including trypsin and chymotrypsin in hepatopancrease (Simpson, 2000). Proteases are one of the main factors determining PPO activation

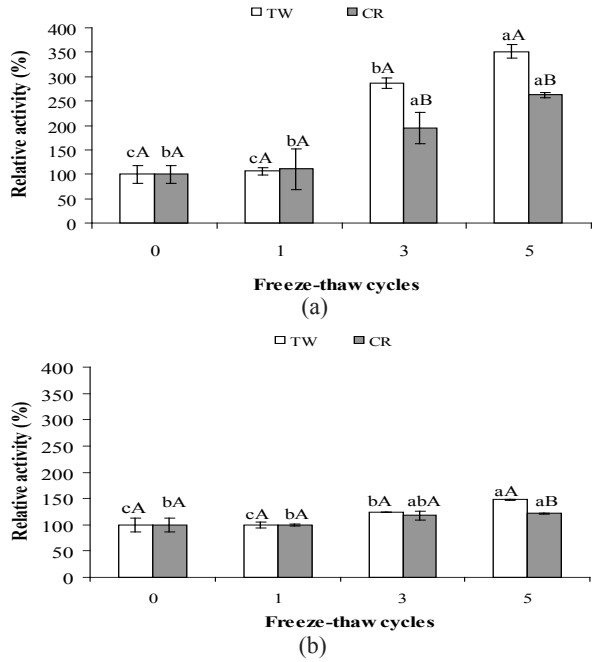

Figure 1. Relative activities of PPO (a) and protease (b) of pre-cooked Pacific white shrimp subjected to different freeze-thawing cycles using different thawing methods. Different lowercase letters on the bars within the same thawing method indicate the significant differences ($p < 0.05$). Different uppercase letters on the bars within the same freeze-thaw cycle indicate the significant differences ($p < 0.05$). Bars represent the standard deviation ($n = 3$).

(Nirmal and Benjakul, 2010). Muhlia-Almazán and García-Carreño (2002) reported that trypsin and chymotrypsin are the most abundant proteolytic enzymes in the midgut gland of shrimp. Recently, Sriket *et al*. (2011) reported that trypsin-like protease was the dominant enzyme in hepatopancreas of freshwater prawn. During freezing, the ice crystals will damage the cell tissue. This allows endogenous protease to release after cell disruption, particularly during thawing (Ashie and Simpson, 1996). Proteases might be able to activate PPO to some degree, thereby inducing melanosis in pre-cooked shrimp during storage. Arthopod PPO are present as zymogens (pro-PPOs) that are activated by a serine protease (Manheem *et al*., 2012). When comparing protease activity in pre-cooked shrimp subjected to different thawing methods, samples thawed using the running tap water showed the higher protease activity than those thawed at 4ºC when 5 freeze-thaw cycles were implemented ($p < 0.05$). No differences in activity were noticeable between both thawing methods as the freeze-thawing cycles were lower than 5 ($p > 0.05$).

Effect of multiple freeze-thaw cycles and different thawing methods on exudate loss of pre-cooked Pacific white shrimp

Figure 2 shows the exudate loss of pre-cooked Pacific white shrimp with multiple freeze-thaw cycles and different thawing methods. An increase in number of freeze–thaw cycles resulted in the increase in exudate loss ($p < 0.05$) as shown in Figure 2. When the number of freeze-thaw cycles increased, the muscle fiber and cells were disrupted and lacked of continuity to a higher extent. Furthermore, the spacing between muscle fibers increased (Boonsumrej *et al.*, 2007). Repeated melting during thawing and reformation of ice crystals during freezing in multiple freeze–thaw situations was detrimental to muscle tissues by causing mechanical damage to cell membranes and the loss of water holding capacity (Srinivasan *et al.*, 1997). However, at the same freeze-thaw cycle, no differences in exudate loss were observed between the pre-cooked shrimp thawed at 4°C and using running tap water ($p > 0.05$). After being thawed, the water frozen out was not reabsorbed during thawing, leading to a high exudates formed (Boonsumrej *et al*., 2007). In addition, Erdogdu *et al.* (2004) also reported that protein aggregation was induced by heating and internal forces caused by repeated freeze-thawing cycle, thereby inducing connective tissue shrinkage. Sriket *et al*. (2007) reported that protein denaturation and disruption of endomysium induced by freeze–thawing, possibly resulted in a less compact structure. Moreover, Díaz-Tenorio *et al*. (2007) reported that freezing promotes protein aggregation and water loss after thawing, yielding a stiffer and harder product. Therefore, freeze–thawing showed a detrimental effect on pre-cooked Pacific white shrimp, as manifested by the increased exudate loss.

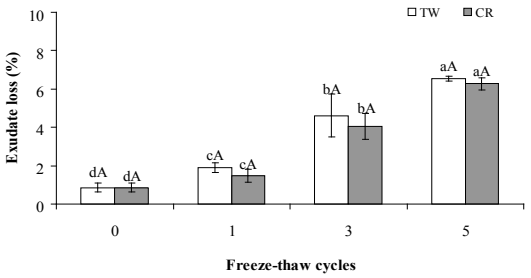

Figure 2. Exudate loss of pre-cooked Pacific white shrimp subjected to different freeze-thawing cycles using different thawing methods. Different lowercase letters on the bars within the same thawing method indicate the significant differences ($p < 0.05$). Different uppercase letters on the bars within the same freeze-thaw cycle indicate the significant differences ($p < 0.05$). Bars represent the standard deviation ($n = 3$).

Effect of multiple freeze-thaw cycles and different thawing methods on melanosis of pre-cooked Pacific white shrimp

Melanosis score of pre-cooked Pacific white shrimp subjected to multiple freeze-thaw cycles and different thawing methods during 7 days of refrigerated storage is shown in Figure 3. At day 0,

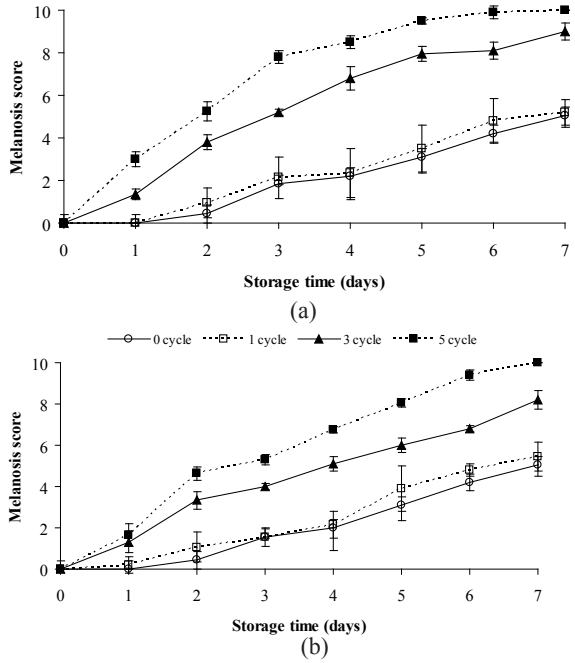

Figure 3. Melanosis score of pre-cooked Pacific white shrimp subjected to different freeze-thawing cycles using different thawing methods during 7 days of refrigerated storage. (a): thawing using running tap water, (b): thawing at 4˚C.

no melanosis was observed in all samples (score = 0). Melanosis score of pre-cooked Pacific white shrimp increased as the storage time increased ($p < 0.05$). At the same storage time, the melanosis was more enhanced as the number of freeze-thaw cycles increased ($p < 0.05$). Nevertheless, the pre-cooked shrimp subjected to 1 cycle of freeze-thawing had no difference in melanosis score, compared with those without freeze-thaw cycles throughout the storage of 7 days ($p > 0.05$). It was noted that the increase in melanosis was more notable in shrimp thawed using the running tap water than that subjected to thawing at 4°C, especially with 3 and 5 freeze-thaw cycles ($p < 0.05$). Such a difference in melanosis score was obviously observed during 2-5 days of storage. However, at the last day of storage (day 7), there was no difference in melanosis score ($p > 0.05$). Rapid development of the black spots was observed when the frozen kuruma prawns were stored after thawing (Adachi *et al.*, 2001). Nirmal and Benjakul (2010) found that the melanosis score of fresh Pacific white shrimp increased as the number of freeze-thaw cycles increased. Melanosis was more pronounced in Pacific white shrimp thawed at room temperature or using tap water than that found in samples subjected to thawing at 4°C (Nirmal and Benjakul, 2010). At day 7, pre-cooked shrimp with multiple freeze-thaw cycles (3 and 5 cycles) had melanosis score of 8-10, whereas those with 1 freeze-thaw cycle and without freeze-thawing had the score of 5-6. The increasing rate of melanosis in samples subjected to increasing freeze-thawing cycles was coincidental with increasing PPO and protease activities (Figure 1) ($p < 0.05$). Melanosis in crustaceans is induced by endogenous PPO or 1,2 benzenediol; oxygen oxidoreductase (EC 1.10.3.1) (Díaz-Tenorio *et al.*, 2007). This is followed by non-enzymatic polymerization of the quinones, yielding the brown or dark pigment, melanin (Montero *et al.*, 2001). With enhanced protease activity in sample subjected to multiple freeze-thawing, PPO could be activated to a higher extent. PPO from some crustacean has been known to be activated by proteases (Martínez-Álvarez *et al.*, 2005). During freezing and thawing, the remaining inactive PPO could be released and activated (Yang *et al.*, 1993). In the presence of substrates and oxygen, melanosis was developed more rapidly (Díaz-Tenorio *et al.*, 2007). Therefore, melanosis of pre-cooked Pacific white shrimp was governed by freeze-thawing as well as the storage time.

Figure 4 illustrates freshly prepared pre-cooked Pacific white shrimp and those subjected to different freeze-thawing cycles using different thawing methods after 7 days of refrigerated storage. At day 0, no melanosis was found in the all samples. After 7 days of storage, melanosis was developed increasingly in cephalothoraxes as well as pleopods. Melanosis was more pronounced in pre-cooked shrimp with the higher freeze-thawing cycles. The result confirmed that freezing and thawing process accelerated melanosis in pre-cooked Pacific white shrimp.

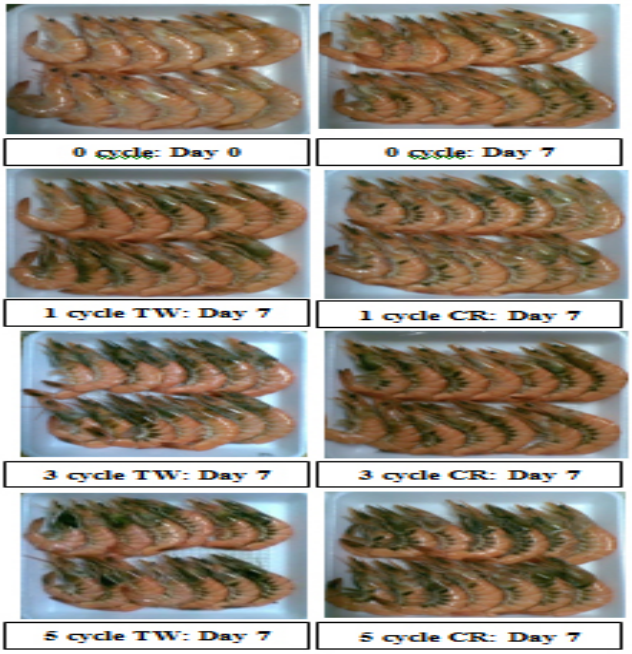

Figure 4. Freshly prepared pre-cooked Pacific white shrimp and those subjected to different freeze-thawing cycles using different thawing methods after 7 days of refrigerated storage. TW: thawing using running tap water, CR: thawing at 4˚C.

Conclusions

PPO, protease and melanosis as well as exudate loss of pre-cooked Pacific white shrimp were enhanced by the multiple freeze-thaw cycles. Thawing using running tap water showed more negative effect on quality of pre-cooked shrimp than thawing at 4˚C. Freeze-thawing therefore induced melanosis, more likely mediated by the increased PPO and protease activities. Thus, the repeated freeze-thawing must be avoided in order to lower the melanosis in pre-cooked shrimp.

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