COMPARISON OF FREEZING AND THAWING TREATMENTS ON MUSCLE PROPERTIES OF WHITELEG SHRIMP (*LITOPENAEUS VANNAMEI*)

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ABSTRACT

Whiteleg shrimp (Litopenaeus vannamei) is traded as a frozen food. Changes in texture of thawed muscle are a negative economic factor. This study evaluated and compared two freezing methods, cryogenic and forced convection, and two thawing methods, at 4 and 25C by histological, textural and electrophoretical tests. Insignificant interaction was found between the two main effects for any of the evaluated texture parameters (shear force, fracturability, hardness and water-holding capacity). Significant differences ($P < 0.05$*) were found in muscle protein sarcoplasmic fraction. Interaction of the main effects was only observed when analyzing microstructure. In all treatments, a significant increase in melanosis was detected when compared with unfrozen specimens. Because texture was not significantly affected by either freezing method, for commercial purposes, we suggest forced convection freezing because it is cheaper. For research concerning cell and tissue structure, cryogenic freezing is recommended. Thawing at 4C is recommended for marketing purposes.*

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PRACTICAL APPLICATIONS

Whiteleg shrimp as a food is considered in the world market as a high-value commodity. It is a suitable animal for farming, because it can grow under varied conditions. Making the shrimp culture an important worldwide economic activity that contributes positively to the economies of countries that commercialize shrimp. That is possible because all steps in farming are considered; however, in most examples, postharvesting management is neglected, tampering previous investments and efforts. The present study is suited in helping the shrimp industry. Knowledge about the effect of freezing–thawing on muscle properties such as texture may help shrimp producers and retailers select conditions of preservation to keep texture properties of the product pleasing to the consumer.

INTRODUCTION

Meat from decapods, such as shrimp, lobster and crab are appreciated for their delicate taste. Export of these marine species significantly contributes to the economy of countries that trade them. In Mexico, whiteleg shrimp (*Litopenaeus vannamei*) is the main farmed and wild species. It is well documented that the freezing–thawing process affects the final muscle properties. For instance, the changes in edibility properties depend on many factors, such as freezing and thawing rates, as well as holding storage temperatures (Sikorski and Kolakowska 1990).

When aquatic organisms die, endogenous and microbial enzymes begin to degrade body structures, reducing freshness until ultimate deterioration appears (Sikorski *et al.* 1990; Haard 2002). To reduce the problem, muscle should be frozen, stored on ice or refrigerated. Freezing is more effective for preservation over long periods of time (Santos-Yap 1995), but protein denaturation and textural defects can occur depending on several factors, in addition to those previously mentioned, such as species and elapsed storage conditions between harvest and freezing (Matsumoto 1979). Besides, an appropriate thawing process is required to obtain a product similar to the unfrozen one. During the thawing operation, water may be reabsorbed by tissues and cells depending on ice crystal size and its localization in the tissue microstructure, thawing rate, biochemical and physiological state of the product before freezing, and water-holding capacity (WHC) in muscle before freezing (Pham and Mawson 1997). If the freeze–thaw protocol is ineffective, the final product can be rejected or at least lose some commercial value.

One of the biochemical processes affecting texture, flavor and color is protein denaturation (Xiong 1997). Marine animal muscles are more suscep-

tible to protein denaturation by freezing than mammalian muscle (Matsumoto 1979). Muscle proteins are the main contributors of texture characteristics, and their changes during freezing or thawing can be monitored directly by measuring changes in their surface hydrophobicity, amino acid modification, conformational stability, solubility and aggregation. Losses in functional properties of proteins are evaluated by monitoring functional attributes such as WHC, viscosity, gelation, emulsification, foaming and whipping (Xiong 1997). Texture modification can be evaluated using microscopy and texture measurements (Pan and Yeh 1993; Mallikarjunan and Hung 1997; Meinert *et al.* 1999).

A better knowledge of the effects of freezing–thawing treatments on texture of shrimp muscle would provide producers and processors a more efficient way to market their product. The main goal of this work was to evaluate and compare the effect of two freezing–thawing protocols on the muscle properties of headed whiteleg shrimp (*L. vannamei*). These data are essential for domestic fishermen and processors to achieve quality.

MATERIALS AND METHODS

Characteristics and Processing of Samples

The abdominal portion (14.5 ± 1.7 g) of farmed whiteleg shrimp *L. vannamei* (27.7 ± 2.8 g total weight) was processed by either of two freezing methods, cryogenic and forced convection, and either of two thawing procedures, adequate, 4C for 24 h and inadequate at 25C for 4 h. Specimens for the cryogenic freezing were immediately treated after harvesting from the pond. Shrimps were submerged one at a time in liquid nitrogen for 40 s, then transported in coolers that contained blocks of dry ice (CO_2). At the laboratory, specimens were randomly separated in groups of 20, packed in polyethylene plastic bags and stored at −80C. For the other freezing treatment, specimens receiving the forced convection treatment were covered with crushed ice for 5 h after harvest, and then packed under the previously mentioned conditions and stored at −20C inside a forced air convection freezer chamber. Samples were kept frozen for less than a week. Afterwards, the packed shrimps were thawed; the shell was manually removed to obtain the abdominal muscle for analyses.

Textural and WHC Analysis

Texture was evaluated on a universal testing machine (model 1132, series 125, Instron, Inc., Canton, MA). Shear force was evaluated using the Warner–Bratzer shearing device attached to the load cell (50-kg capacity) (Zhang and Mittal 1993). To mimic human bite, the shrimp abdomen was cut transversally

between the second and third somite; the cross-head speed was 10 cm/min. The first major force peak was recorded as the maximal shear force required to shear the muscle sample. Twenty shrimps were used in each treatment. Hardness and fracturability were measured, using texture profile analysis, according to Bourne (1978). Analytical samples were compressed to 75% of the original height (≈1.2 cm) using a double bite protocol. The first peak was recorded as fracturability and the last peak as hardness.

WHC was measured by centrifugation (Cheng *et al.* 1979). The fifth and sixth abdominal segments were cut longitudinally together for analysis; excess water was dried using a paper towel. Each segment was weighted (*Wi*), then placed in a centrifuge tube; samples were centrifuged at 28,000 × g at 4C for 30 min. Treated segments were removed with tweezers, dried using a paper towel, and final weight was recorded (*Wf*). WHC was calculated as $\% WHC = \{1 - [(Wi - Wf)/Wi]\} \times 100$.

Fractionation and Analysis of Muscle Proteins

Muscle proteins were extracted according to the solubility protocol outlined by Hashimoto *et al.* (1979). From the extraction procedure, three protein fractions were obtained: sarcoplasmic, myofibrillar and alkali soluble. One-gram shrimp muscle was homogenized with 10-mL phosphate buffer A (15.6-mM Na_2HPO_4, 3.5-mM KH_2PO_4, I = 0.05, pH 7.5). Extract was centrifuged at 5,000 × g at 4C for 15 min. The sediment was again mixed with 10-mL phosphate buffer A and centrifuged as mentioned before. Supernatants of both centrifugation steps, containing sarcoplasmic proteins, were mixed together. The sediment was mixed twice with 10-mL phosphate buffer B (15.6-mM Na_2HPO_4, 3.5-mM KH_2PO_4, 0.45-M KCl, I = 0.5, pH 7.5) and centrifuged as mentioned earlier. This supernatant contained the myofibril fraction. To extract alkali-soluble proteins, the second sediment was treated with 10-mL 0.1-M NaOH solution, stored at 4C for at least 24 h with slow agitation and then centrifuged as previously mentioned.

Protein concentration was quantified as described by Bradford (1976). Protein separation was carried out by gel electrophoresis (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) under reducing conditions, using a discontinuous gel (Laemmli 1970) with the separating gel containing 10% acrylamide. Electrophoresis was run constantly at 15 mA/gel in SE 250 Mighty Small II slab gel electrophoresis unit (Hoefer Scientific Instrument, San Francisco, CA). After electrophoresis, the gels were stained with Coomassie Brilliant Blue R solution (0.5% dye, 40% methanol, 7% acetic acid), and the excess of dye was removed with the same solution without the dye. The gels were scanned; protein bands in gels were analyzed with Kodak Digital Science 1D Image Analysis Software (East Kodak Co., Rochester, NY), and intensity was recorded.

Histology and Image Analysis

The second somite of thawed shrimps was cut and fixed in Davidson solution (Shaw and Battle 1957) then dehydrated, cleared and perfused with paraffin. Each paraffin-embedded specimen block was cut with a microtome into 3-μm thick sections and stained with hematoxylin–eosin. Samples were observed under optical microscopy (model BX50, Olympus Co., Tokyo, Japan). Microphotographs were taken at random with a digital camera CoolSNAP-Pro, (Media Cybernetics, Inc., Silver Spring, MD) connected to a personal computer.

An image analysis of each picture was made with Image-Pro Plus software version 4.5.19 (Media Cybernetics, Inc.). Blank areas, red-stained muscle tissue and purple-stained connective tissue were calculated. Results were expressed as percentages of total analyzed area.

Statistical Analysis

Data were analyzed using an analysis of variance (ANOVA) 2×2 factorial design (main effect one: freezing rate; main effect two: thawing rate; two levels each).When the interaction between the main effects was not significant, a one-way ANOVA and a multiple comparison test (least significant difference), when required, were applied. Untreated shrimp samples (control) were introduced to a newly statistical design, where data were analyzed by one-way ANOVA. Statistical significance was set at $\leq 5\%$. STATISTICA, version 5.5. (StatSoft Inc., Tulsa, OK), was used for analysis (StatSoft, Inc. 2000).

RESULTS AND DISCUSSION

Textural and Functional Properties

Food proteins change their structure during processing. Such changes define their final characteristics and determine if the method is adequate to the product and hence acceptable to consumers. For the evaluated texture parameters (shear force, fracturability, hardness and WHC), results indicate no significant interaction between the main factors: freezing and thawing methods ($P > 0.05$). Any combination of freezing–thawing was treated as an independent treatment. The treatments most significantly affected the texture attributes of shear force and hardness (Table 1). If not properly handled, freezing promotes protein aggregation and water loss after thawing, yielding a stiffer and harder product (Sikorski and Kolakowska 1990). Studies by Srinivasan *et al.* (1998) with freshwater prawn (*Machrobrachium rosenbergii*) showed that the shear force of raw frozen muscle was dependent on the presence of the shell.

TABLE 1.
EFFECTS OF FREEZING–THAWING ON SHEAR FORCE, HARDNESS, FRACTURABILITY AND WHC OF SHRIMP MUSCLE

Treatment (freezing–thawing)	Shear force (N)	Fracturability (N)	Hardness (N)	WHC (%)
NT	22.7 ± 4.0^{a}	45.8 ± 10.5^{ab}	83.1 ± 15.8^{a}	97.2 ± 0.5^{a}
CA	31.0 ± 6.7^{bc}	38.8 ± 10.9^{a}	92.8 ± 19.7^{ab}	96.8 ± 0.8^{a}
CI	28.2 ± 5.9^{b}	46.0 ± 17.6^{ab}	99.2 ± 16.2^{b}	96.9 ± 0.5^{a}
FA	32.8 ± 3.4^{c}	53.2 ± 7.1^{b}	102.1 ± 10.4^{b}	95.5 ± 0.7^{b}
FI	32.2 ± 4.4^{bc}	51.4 ± 9.6^{b}	99.2 ± 16.6^{b}	95.1 ± 0.5^{b}

Values within the same column with different letters are statistically different ($P < 0.05$). N, newton; WHC, water-holding capacity; NT, no treatment; C, cryogenic freezing; F, forced convection freezing; A, adequate thawing; I, inadequate thawing.

Shrimp frozen with the shell over a long period has significant changes in texture compared to shrimps frozen with no shell. Ezquerra Brauer *et al.* (2003) reported a significant impact of crushed ice storage on the shear force of raw shrimp muscle. In our study, the cryogenic method was the least damaging to overall texture characteristics of muscle product after thawing (Table 1).

Protein composition and conformation have significant effects on WHC. Water within protein structures takes one of two forms. One form is bound to protein and is not available as a solvent, and is highly dependent on the protein's physicochemical characteristics. The other form is trapped within the protein matrix and is affected by the muscle protein matrix structure (Barbut 1996). Experimental results also show that cryogenic freezing results in products with higher WHC ($P < 0.05$), indicating lower protein aggregation and fewer disrupted cells regardless of the thawing protocol. This agrees with previous studies on WHC for muscle of grass shrimp (*Penaeus monodon*) and tilapia (*Oreochromis* sp.) (Pan and Yeh 1993; Chen and Pan 1997). Using either liquid nitrogen or air blasting as the freezing alternatives, these researchers concluded that liquid nitrogen better maintains intact muscle and cell structures, and that shelf life of shrimp was longer than those where the air blast method was used. In our study, the freezing method rather than the thawing method affected shrimp texture.

One problem with cryogenic freezing is breakage in the dorsal region of the exoskeleton and outer muscle layer. Because fracturability is the force necessary to create the first significant break when analyzing texture (Bourne 2002), a rupture in the outer muscle layer reduces the force necessary to make a sample crumble, crack or shatter. For raw and cooked shrimp, low fracturability value is highly desired by consumers, because this characteristic is used as an indicator of a fresh, unfrozen product.

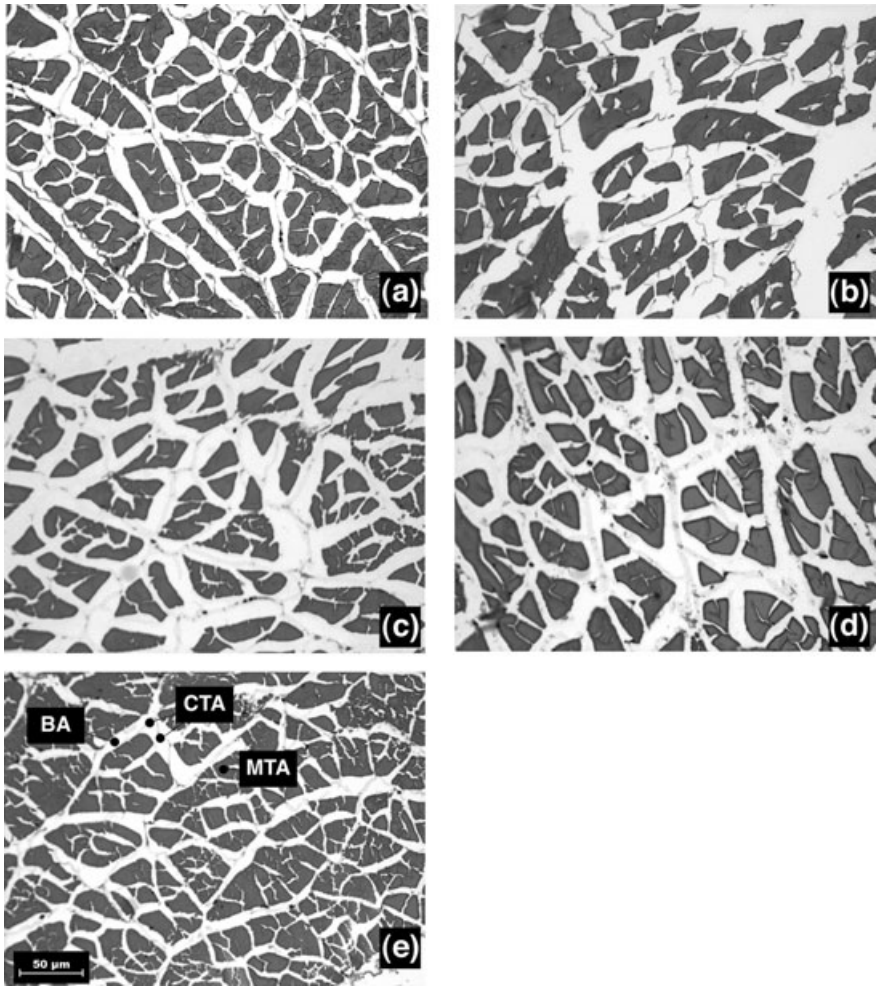

FIG. 1. CROSS SECTION OF SHRIMP MUSCLES STAINED WITH HEMATOXYLIN–EOSIN
Treatment of freezing–thawing: (a) cryogenic–adequate, (b) cryogenic–inadequate, (c) forced convection–adequate, (d) forced convection–inadequate and (e) no treatment.
BA, blank area; CTA, conjunctive tissue area; MTA, muscle tissue area.

Histological Analysis

The effect of treatments on the microscopic structure of muscle cross sections are shown in Fig. 1, where representative images of nonfrozen and treated shrimps are shown. Interaction of freezing and thawing on the amount of muscle tissue percentage and voids was significant ($P < 0.05$), but this was

TABLE 2.
EFFECT OF FREEZING–THAWING ON AREAS FROM MICROSCOPICAL STRUCTURE OF WHITELEG SHRIMP (*LITOPENAEUS VANNAMEI*)

Freezing treatment	Thawing treatment	Muscle tissue (%)	Blank space (%)	Connective tissue (%)
NT	NT	44.9 ± 9.9[a]	39.7 ± 10.7[a]	15.5 ± 4.8[a]
C	A	40.7 ± 7.6[a]	39.2 ± 9.6[a]	20.0 ± 5.9[a]
	I	45.5 ± 5.2[a]	34.9 ± 5.9[a]	19.7 ± 5.0[a]
F	A	45.3 ± 8.9[a]	35.4 ± 9.1[a]	19.0 ± 4.1[a]
	I	38.9 ± 6.3[a]	43.3 ± 6.2[a]	17.8 ± 3.8[a]

Values within the same column with different letters are statistically different ($P < 0.05$). Significant effect for interaction of both factors was found in muscle tissue and blank space ($P < 0.05$). NT, no treatment; C, cryogenic freezing; F, forced convection freezing; A, adequate thawing; I, inadequate thawing.

not the case for connective tissue. No differences were observed ($P > 0.05$) in histological samples of untreated and treated shrimp (Table 2). The insignificant histological differences may be a consequence of the short storage time of the treated specimens. Studies to determine the effect of prolonged storage in shrimp meat texture have not been completed.

Protein Analysis

The ratio of sarcoplasmic, myofibrillar and stroma proteins in muscle is related to muscle function in the living organism, such as walking, swimming and evasion (Matsumoto 1979). Slight effect among treatments was observed in the sarcoplasmic, myofibril and alkali-soluble proteins (Figs. 2–4).

Sarcoplasmic proteins are mostly hydrolases; this fraction includes proteases. When muscle is thawed, they are released and activated. They may hydrolyze peptide bond of major muscle proteins (myofibrillar fraction), thus affecting the tridimensional structure. If change in protein conformation is significant, then a modification in muscle texture is observed (Sikorski *et al.* 1990; Kijowski 2001). For the analysis of sarcoplasmic fraction, bands were grouped in five regions (Fig. 2). Comparing with no treated shrimp, in regions *a* and *b*, an increase in all bands was observed in cryogenic-inadequate (CI), forced-convection adequate (FA), and forced-convection inadequate (FI) samples, mainly in proteins above 220 kDa. In the same samples, regions *d* and *e* presented a reduction in band density. Such changes indicate that freezing causes denaturation and aggregation of proteins by the effect of ice formation that reduces water availability, increasing the ionic strength in the neighborhood. This may induce the formation of new covalent bonds (Shenouda 1980; Sikorski and Kolakowska 1990). Changes in *b–d* regions were observed in cryogenic-adequate (CA) samples.

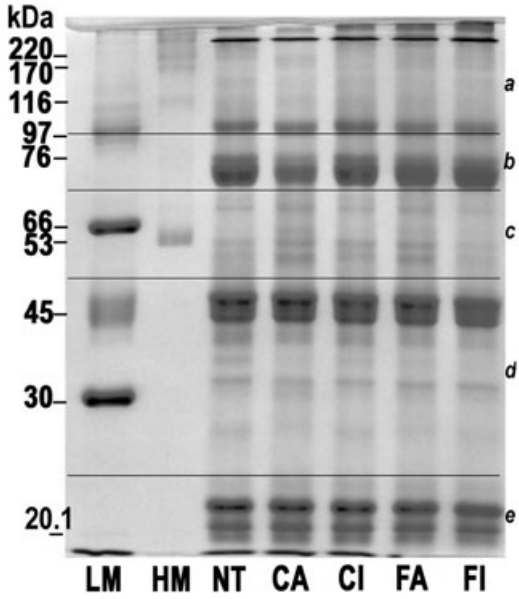

FIG. 2. EFFECT OF FREEZING AND THAWING ON SARCOPLASMIC PROTEIN FROM SHRIMP MUSCLE

a–e are regions selected to analyze protein bands. Standards in lane LM: phosphorylase b (97,000 Da), albumin (66,000 Da), ovoalbumin (45,000 Da), carbonic anhydrase (30,000 Da) and trypsin inhibitor (20,100 Da). Standards in lane HM: myosin (220,000 Da), α_2-macroglobulin (170,000 Da), β-galactosidase (116,000 Da), transferrin (76,000 Da) and glutamic dehydrogenase (53,000 Da). Standards in both lanes are molecular weight calibration kits for sodium dodecyl sulfate electrophoresis (Amersham Biosciences UK limited, Buckinghamshire, U.K.).
LM, low molecular weight marker; HM, high molecular weight marker; NT, not treated; F, forced convection freezing; C, cryogenic freezing; A, adequate thawing; I, inadequate thawing.

Electrophoregram of myofibrillar fraction (Fig. 3) does not allow a comparison among fractions; alkali-soluble fraction protein was used because it is similar to myofibrillar fraction (Hashimoto *et al.* 1979; Benjakul *et al.* 2002). In Fig. 4, main myofibrillar proteins are presented: myosin (light and heavy chains), paramyosin, actin and tropomyosin.

In spite of major changes in myofibrillar fraction that were expected because Kijowski (2001) and Matsumoto (1979) indicated that this fraction is susceptible to structural changes and aggregation when freezing and thawing, only paramyosin changed in FA. We speculate that some effects can be observed in longer periods of storage as asserted by French (1986), who reported that the effect of frozen storage on muscle protein is only observed in the myofibril fraction after 10 days at −20C. The results in our study suggest the need for testing longer storage periods.

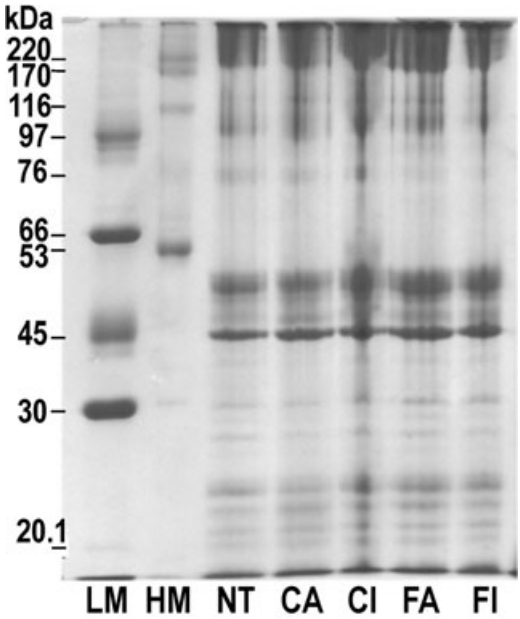

FIG. 3. EFFECT OF FREEZING AND THAWING ON MYOFIBRILLAR PROTEIN FROM SHRIMP MUSCLE
LM, low molecular weight marker; HM, high molecular weight marker; NT, not treated; F, forced convection freezing; C, cryogenic freezing; A, adequate thawing; I, inadequate thawing.

Melanosis

All frozen shrimp developed black spots during thawing, mainly in the gills and hepatopancreas (Fig. 5). This phenomenon is called melanosis or browning. This is a postmortem reaction in crustaceans caused by catalysis of the endogenous enzyme polyphenoloxidase (PPO) or 1,2 benzenediol;oxygen oxidoreductase (EC 1.10.3.1). Because flavor, odor, nutritional quality and safety characteristics are insignificantly changed by melanosis, only the appearance may affect acceptance (Kim *et al.* 2000). Previously, we observed black spots on shrimp during storage at 4C. Compared with those samples, freezing and thawing of recently caught organisms increased the rate of melanosis significantly, at a level similar to specimens stored only 2 or 3 days at 4C. It is likely that during freezing and thawing, the inactive form of PPO (proPPO) stored in hemocytes, the digestive gland (in R cells) and chromatophores (in muscle) (Yang *et al.* 1993) are easily released and activated, and in the presence of suitable substrates and oxygen, melanosis develops more rapidly.

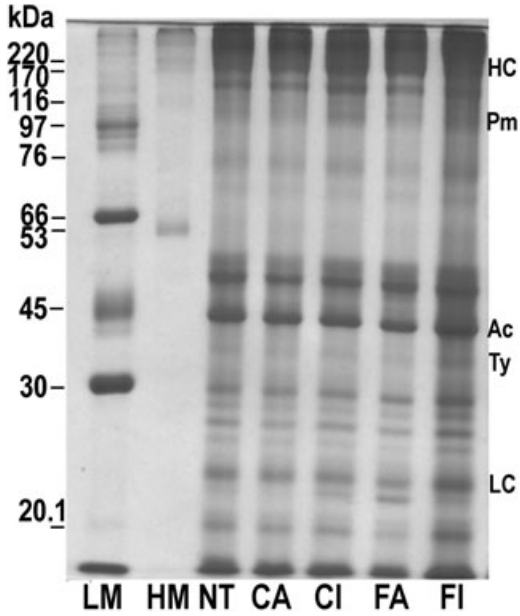

FIG. 4. EFFECT OF FREEZING AND THAWING ON ALKALI-SOLUBLE PROTEIN FROM SHRIMP MUSCLE

HC, heavy chain myosin; Pm, paramyosin; Ac, actin; Ty, tropomyosin; LC, light chain myosin; LM, low molecular weight marker; HM, high molecular weight marker; NT, not treated; F, forced convection freezing; C, cryogenic freezing; A, adequate thawing; I, inadequate thawing.

FIG. 5. MELANOSIS IN SHRIMP AFTER THE FREEZING–THAWING TREATMENT

CONCLUSIONS

Food safety and sensorial qualities are major concerns of consumers. For this reason, it is important to measure the impact of the preservation methods on desirable food characteristics. In this study, most of the measured variables showed no significant differences with interaction of freezing and thawing treatments. Compared to untreated specimens, textural variables of treated shrimp did not show significant differences. With these sets of results, we recommend forced convection freezing, but not cryogenic freezing, for commercial trade because forced convection freezing is less expensive. For research purposes concerning cell and tissue structure, cryogenic freezing is recommended. Slow thawing is recommended for retail trade. Melanosis occurs in thawed shrimps, which may affect acceptance by consumers. Normally, melanosis reactions can be easily delayed by chemical reagents, but new regulations in safety restrict their use. For this reason, the study of melanosis without chemical reagents is essential to consumer safety.

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