Changes in Red Hake (Urophycis chuss) Muscle Induced by Different Freezing Strategies

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The effect of single and double freezing at −20°C on fresh and aged red hake with two different holding times of the thawed fillets between the first and second freezing treatments was determined. In addition, twice-frozen fresh red hake was treated with two antioxidation systems to assess their effectiveness in reducing lipid oxidation. Fresh, untreated red hake at 0 time was used for comparison. Significant dimethylamine (DMA) production occurred with all frozen samples, and the differences were not marked after 7-month storage. With a storage time of 3 months, however, there were differences between the treatments in both the fresh and aged samples with the once-frozen sample producing the least DMA, the twice-frozen sample with the 24-h holding time between freezings the most, and the twice-frozen sample with a 6-h holding time between freezings giving intermediate values. All samples showed a greater than 50%
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INTRODUCTION

For mass production of value-added fish products, the raw material, often in the form of headed and gutted fish, needs to be frozen at sea to arrest deleterious quality changes. The frozen raw material is then typically subjected to thawing, followed by filleting and freezing the fillets in blocks which are used to produce value-added fish portions. During frozen storage and in the freeze-thaw cycle, quality losses may occur. Post-mortem time prior to freezing, thawing speed and temperature, frozen storage temperature and time, and specific chemical pretreatments of the fish muscle are all factors that can have an impact on retarding or accelerating adverse quality changes. During freezing, frozen storage, and thawing, muscle proteins may denature, which could lead to water losses and, in turn, increased muscle toughness. Membrane disruptions may allow potent endogenous pro-oxidants to have access to lipids, especially the susceptible membrane lipids, causing undesirable flavor and color changes (Hultin, 1994). In addition, many of the whitefish species used to produce frozen portions have potent trimethylamine N-oxide (TMAO) dimethylases which convert TMAO in the fish to formaldehyde and dimethylamine (DMA) in equimolar concentrations. Formaldehyde has been hypothesized to act as a protein cross-linking agent forming extensive networks of proteins which leads to muscle toughening in frozen storage and water losses on thawing and cooking (Castell et al., 1973). Although the hypothesis that the cross-linking is accomplished through covalent bonds has been questioned (Connell, 1975; Owusu-Ansah and Hultin, 1987), there are clearly some changes in the protein molecules which cause them to lose their solubility and functionality.

The texture of red hake, a species with an active TMAO-demethylase activity, has been shown to deteriorate rapidly in frozen storage (Dyer and Hiltz, 1974). This has hampered its utilization. The objective of this study was, therefore, to investigate how the quality of fresh and aged red hake would be affected by different freezing strategies. Quality indicators under investigation were (1) the development of DMA as a indicator of formaldehyde

loss in protein solubility as determined by lithium chloride extraction after 3 months of storage, and the protein solubility declined further when samples were tested at 7 months. Samples with the best (once-frozen fresh) and worst (twice-frozen with 24-h holding time between freezings of 5-day aged muscle) treatments had the highest thiobarbituric acid-reactive substances (TBARS) values. Both antioxidant treatments were effective in reducing TBARS development.

KEYWORDS freezing fish, double freezing fish, fish, red hake (Urophycis chuss), quality changes in fish
formation, (2) protein solubility as an indicator of protein denaturation, and (3) formation of thiobarbituric acid-reactive substances (TBARS) as an indicator of the extent of lipid oxidation.

MATERIALS AND METHODS

Materials and Experimental Plan

The experimental design is outlined in Figure 1. Different treatments of twice-frozen fish were compared to once-frozen fish. Headed and gutted red hake was obtained fresh from day-boats from Point Judith, Rhode Island, USA. Part of the fish was placed in polyethylene bags and immediately frozen at −20°C on arrival at the laboratory while the other part of the fish was held on ice for 5 days to age the fish prior to freezing at −20°C in polyethylene bags. A portion of both the fish frozen on arrival and the fish aged for 5 days was held for 7 months at −20°C and was labeled as Once frozen and Aged-once frozen, respectively. These samples served as the experimental controls. The remaining fish were kept for 1 month at −20°C, followed by thawing at 6°C overnight and filleting and deep skinning the thawed fish. One portion of the fillets was then refrozen at −20°C after a holding time of 6 or 24 h at 6°C and was labeled as Twice frozen—6 h or Twice frozen—24 h, respectively. The aged fish was treated identically and was labeled as Aged twice frozen—6 h or Aged twice frozen—24 h. Another portion of the fillets was dipped in two different proprietary antioxidant solutions (5°C) for 2 min and then frozen after a holding time of 6 or 24 h at 6°C. The proprietary solutions were supplied by a local fish processor (contact corresponding author for further information). These portions were labeled as AO1 twice frozen—6 b or AO1 twice frozen—24 b, and AO2 twice frozen—6 b or AO2 twice frozen—24 b. Each set of experiments involved 6–8 fillets placed in sealed polyethylene bags and included some dark lateral muscle. The refrozen samples were kept at −20°C for 2 or 6 months and then subjected to the chemical tests described below. The total frozen storage time (including the 1 month at −20°C prior to thawing) was therefore 3 or 7 months. For the chemical analyses, the fillets were thawed at 6°C overnight. Samples were then randomly collected by slicing 1- to 2-cm strips across the muscle fibers thus collecting both red and white muscle. Fillets from fresh-headed and gutted red hake were also subjected to the same chemical tests.

Analysis for Dimethylamine (DMA)

DMA was quantified in samples taken from the thawed fillet to give an indication of the formation of formaldehyde during the different freezing and storage treatments. Formaldehyde reacts rapidly with cellular constituents
to give an indica-
ration, and

Figure 1 shows the experimental design for headed and gutted red hake. The fish was first frozen at -20°C for 10 lb, and then divided into three groups:

1. **-20°C (1 mo) 10 lb**
   - Thaw 6°C
   - Re-freeze 6h at -20°C
   - 2 mo 6 mo
     - "fresh, twice-frozen, long thaw"
     - Twice frozen - 6h

2. **-20°C (1 mo) 10 lb**
   - Thaw 6°C
   - Re-freeze 24h at -20°C
   - 2 mo 6 mo
     - "fresh, twice-frozen, long thaw"
     - Twice frozen - 24h

3. **-20°C (1 mo) 10 lb**
   - Thaw 6°C
   - Add AO's (soak)
   - AO blend 1
   - AO blend 2
   - 3 mo 7 mo
     - "Aged, once-frozen"

The fish was then separated into different treatments:

- **Re-freeze 6h at -20°C**
- **Re-freeze 24h at -20°C**
- **AO1 twice frozen - 6h**
- **AO1 twice frozen - 24h**
- **AO2 twice frozen - 6h**
- **AO2 twice frozen - 24h**

The fish was then stored at -20°C for 5 days and 20 lb, and then re-frozen at -20°C for 10 lb.
after it is formed. Thus, determination of formaldehyde is a less true estimate of formaldehyde production than is the determination of DMA. For the analysis, 50 g of red hake muscle was homogenized in 150 mL of 7% trichloroacetic acid (TCA) for 1 min, and the homogenate was filtered through Whatman no. 1 filter paper (Whatman Inc., Piscataway, NJ, USA). The filtrate was assayed for DMA according to Dyer and Mounsey (1945).

Muscle Protein Solubility/Extractability

A decrease of the muscle protein extractability in media of high salt has been used as a measure of a decrease in fish quality, especially for fish that have been frozen (Spinelli and Dassow, 1982). The solubility of both fresh hake muscle and muscle that had been subjected to different freezing strategies was assayed according to the lithium chloride extraction method of Kelleher and Hultin (1991). For this analysis, 40 g of red hake muscle was added to 760 mL of cold LiCl solution (4.2% LiCl, 20 mM lithium carbonate, pH 7.0) and homogenized using a Polytron for 1 min. An aliquot of this solution was transferred to a borosilicate glass tube and centrifuged at 2200 g for 30 min at 4°C. The supernatant was collected and analyzed for protein content employing the Lowry method (Lowry et al., 1951).

Measurement of Lipid Oxidation

To address potential development of lipid oxidation during frozen storage, thiobarbituric acid-reactive substances (TBARS) were measured using the method described by Lemon (1975) with some modifications. Two mL of the TCA extract (not including propyl gallate and EDTA) used to extract DMA were mixed with 2 mL of TBA reagent and heated for 40 min at 100°C. The solution was cooled on ice for 5 min, centrifuged for 15 min at 2200 g, and analyzed spectrophotometrically at 530 nm. Results are expressed as µmol malonaldehyde per kg muscle determined from a standard curve prepared using 1,1,3,3 tetraethoxypropane.

RESULTS AND DISCUSSION

This research was done to determine the effects of different freezing regimes on DMA production, protein solubility, and production of TBA-reactive substances (TBARS) at a typical commercial frozen storage temperature, ~20°C for a gadoid, red hake with a strong TMAO-demethylase activity. The results are shown in Figures 1, 2, and 3 for DMA, solubility, and TBARS, respectively. Initial values of fresh red hake are compared to treatments after total storage times of 3 and 7 months. The samples were either frozen once or...
Freezing Effects on Fish Muscle

FIGURE 2 DMA values of fresh red hake muscle and muscle stored for 3 and 7 months after being subjected to different freezing treatments, with or without antioxidants.

FIGURE 3 LiCl solubility of proteins from fresh red hake muscle and muscle stored for 3 and 7 months after being subjected to different freezing treatments, see Figure 2.

Frozen twice with varying periods of time in the thawed state. One group of red hake was frozen fresh while a second group of whole red hake was aged for 5 days before the treatments began. Another set of samples were freshly frozen but contained one of two antioxidant preparations which were added by dipping just before the second freezing.
At the end of the storage time, the amount of DMA that had been produced in the samples did not vary greatly with treatment but had increased compared to control ($p < 0.05$). The results ranged from approximately 0.74 to approximately 0.96 mmol DMA per 100 g of fish muscle. The once-frozen samples had slightly lower DMA contents after 7-months storage than the comparable twice-frozen samples. The rate at which the DMA formed, however, did vary with treatment. There was an increase in DMA production from the once-frozen to the twice-frozen with 6 h of thawed holding ($p < 0.05$) to the twice-frozen with 24 h of thawed holding for both the fresh and aged fish samples. It appears from the results that the processing conditions control the amount of DMA produced by red hake to a greater degree than does the post-mortem age of the muscle.

The effect of two commercial antioxidant mixtures in twice-frozen red hake muscle is also reported (Figure 2). The results were different from those obtained without the use of antioxidants. First, there were differences between the samples (all twice-frozen) as a function of post-mortem age. At the storage period of 3 months, the tissue with antioxidant mixture 1 was less prone to produce DMA than the sample with antioxidant mixture 2 ($p < 0.05$). In addition, the DMA content was less with the sample held thawed for 24 h versus the one held thawed for 6 h ($p < 0.05$). The samples containing antioxidant mixture 2 were equally susceptible, whether the thaw period was 6 or 24 h ($p > 0.05$). In addition, DMA production was high after both 3- and 7-months storage.

Superior extraction of red hake proteins by concentrated lithium chloride solutions have been demonstrated (Kelleher and Hultin, 1991). Extraction with lithium chloride proved to have little dependence on environmental factors such as temperature of extraction or foaming during mixing as the red hake proteins were less denatured than they were in the presence of the chlorides sodium or potassium (Kelleher and Hultin, 1991). The amount of soluble protein of all the frozen samples not treated with antioxidant varied roughly between 3.1 and 4.0 g protein per 100 g of muscle. The samples treated with the antioxidants seemed to have slightly lower concentrations. The major observation, however, was the sharp reduction in protein solubility after the first 3 months of storage compared to the fresh product ($p < 0.05$), which had about 8.3 g protein per 100 g muscle. Thus, over half of the original soluble protein became insoluble after 3-months storage, and the extractable protein of all samples decreased further over the next 4 months ($p < 0.05$). There did not appear to be any clear pattern of change in protein solubility that could be related to any specific treatment. The relatively low solubility of the fresh sample may have indicated that there was some damage to the muscle tissue before the treatments began. Typically, 12–13 g of soluble protein per 100 g of red hake muscle tissue have been reported (Kelleher and Hultin, 1991). The slight increase in the loss of protein solubility in the antioxidant-treated samples could be due to free radical-induced cross-linking.
had been pro-
duced, however, the
amount of TMAO pro-
duction, as measured by TBARS, was lower in the
once-frozen samples than in the fresh samples.
The samples were subjected to different freezing
and thawing treatments, and the effects of
antioxidants on the oxidative state of the protein
were assessed.

Cross-linking or the formation of disulfide bonds from protein sulphydryl groups. The presence of relatively stable free radicals produced from the antioxidants could be responsible for this.

Lipid oxidation as measured by TBARS (Figure 4) suggests that most of the formation of the TBA-reactive substances occurs in the first 3 months of storage, the only exceptions being the once-frozen sample and the sample that was first aged and then twice-frozen with a 24-h thawed storage period. The increase in TBARS between 3 and 7 months of the once-frozen sample could be related to the fact that some of the nucleotides, such as ATP and ADP, remained in the muscle tissue when it was frozen, and these compounds could be stimulatory to the pro-oxidant hemoglobin. A possibility for the same effect on the sample that was aged and twice-frozen with a 24-h period of thawed storage could be a longer time exposure to pro-oxidants in the unfrozen state where the pro-oxidants could diffuse more readily to the reactive lipids. These suggestions are highly speculative and require further investigation.

Sensory detection of rancidity in fish muscle tissues generally occurs at values > 10 μmol of TBARS per kg of tissue. All of the samples were below that level and so appear not to be seriously oxidized through the 7-month storage period. Nevertheless, there was a clear impact of the antioxidant treatments in reducing oxidation (p < 0.05).

The breakdown of TMAO to dimethylamine (DMA) is accelerated by tissue disruption; the greater extent of disruption, the more rapid the breakdown (Tokunaga, 1964; Parkin and Hultin, 1982). This is believed to be due to the

**FIGURE 4** Lipid oxidation of fresh red hake muscle and muscle stored for 3 and 7 months after being subjected to different freezing treatments with or without antioxidants as assessed by TBARS calculated as malonaldehyde.
mixing of the reactants of the system; i.e., the enzyme TMAO demethylase, the substrate TMAO, and perhaps, most importantly, the cofactors of the reaction. It is most likely that the primary set of cofactors involved are flavins (riboflavin, FMN, or FAD) and NADH (Phillippy and Hultin, 1993). These compounds are both present below their Km values, and thus any loss of these cofactors directly affects the rate of production of formaldehyde. The reaction is enhanced under anaerobic conditions and occurs in frozen tissue. The amount of DMA produced during the experiments in this report was such to indicate that there was probably some textural deterioration occurring (Parkin and Hultin, 1982). The appearance of the fillets had become grainy, and water could be easily squeezed out of the tissue, both of which are symptoms of textural deterioration.

The two samples of most interest for comparison were the once-frozen sample and the fresh sample that was frozen twice with the shorter (6 h) storage time. These probably represent two likely treatments that gadoid species might be expected to receive commercially. DMA production and loss of protein solubility were evident. Although it was not extreme, it was severe enough to warrant some caution. There was less DMA produced in the fresh, once-frozen sample than in the twice-frozen, 6-h thawed sample, but this difference was not evident in the lithium chloride solubility test of the proteins. It is possible that a difference in protein solubility in the two cases would have occurred if a salt that is more limiting on protein solubility, such as sodium chloride, had been used (Kelleher and Hultin, 1991). Sodium chloride is a less efficient extractant than lithium chloride, and it might be more sensitive to structural differences in the proteins. The storage temperature in these experiments was −20°C. The breakdown of TMAO is very sensitive to temperature, and −20°C is right at the point where the rate of formation of DMA is beginning to accelerate. In commercial operations, a constant temperature may not be possible in the distribution system, and even short periods of time at higher temperatures can lead to a much faster breakdown of the TMAO (Licciardello et al., 1981). It is possible that a somewhat higher temperature would put greater stress on the frozen fish muscle tissue, and greater differences would appear between the once-frozen and the twice-frozen, 6-h thawed samples. Studies on the comparison of single- and double-frozen samples should be conducted using exposures of the sample's to higher temperatures for significant periods of time.

REFERENCES


Dyer, W. J., an Environ. C.
Dyer, W. J., ar trimethyla
Hultin, H. O. cessing Ti
UK: Black
Kelleher, S. D. fish muscle
Lemon, D. W. Ke, P. J. a Oceans. p
Licciardello, J. Allsup, M frozen rec Developm Glouceste
Lowry, Q. H., measurem
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