Inactivation of *Kudoa septempunctata* in Olive Flounder Meat by Liquid Freezing

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*Kudoa septempunctata* in olive flounder meat was inactivated using 3 distinct freezing methods: liquid freezing for 5 min, air blast freezing at \(-30^\circ\)C for 5 h, and \(-80^\circ\)C for 1 h. The fracture curve of olive flounder meat subjected to liquid freezing resembled that of meat stored at 4°C, indicating that the structure of olive flounder muscle was well preserved. In contrast, air blast freezing induced the disappearance of the fracture point in the fracture curve, indicating that there was deterioration in the meat quality. Liquid freezing preserved the transparency of olive flounder meat to the same degree as that of meat stored at 4°C. However, air blast freezing induced meat cloudiness. These results indicate that liquid freezing can be used for *K. septempunctata* inactivation without affecting the meat quality.

**Key words**: Kudoa septempunctata / Parasite / Food-borne disease.

*Kudoa septempunctata* is a pathogen that causes food-borne diseases initiated by consumption of contaminated raw olive flounder (Kawai et al. 2012). *K. septempunctata* inactivation methods should be developed to prevent food-borne illnesses caused by this pathogen. It has been previously reported that heating the fish meat at 95°C for 10 min or freezing the meat at \(-80^\circ\)C overnight inactivated *K. septempunctata* (Kawai et al., 2012 : Ohnishi et al., 2013). Although both the heated and frozen pieces of olive flounder are edible, the meat texture deteriorates by heating and freezing treatments. In Japan, the commercial value of olive flounder depends on its food texture. Therefore, *K. septempunctata* inactivation by heating or freezing is unsuitable due to the resulting meat texture deterioration. The application of air blast freezing, which freezes the food in cold air, is commonly used. However, the ineffective heat exchange of the air blast freezing method causes ice crystal formation in food (Hisa, 2004). Ice crystal formation can damage the meat cells and cause drip exudation after thawing (Hisa, 2004). Therefore, freezing meat by using air blasting does not preserve the food quality.

Recent improvements in freezing technology, however, have offered new methods to effectively inactivate pathogens without compromising the meat quality. For example, the liquid freezing method uses an alcoholic liquid refrigerant that serves as an effective heat exchanger and enables rapid cooling without the development of cell-damaging ice crystals. It is said that ice crystal formation and drip loss in meat frozen by using liquid freezing is significantly lower than that of meat frozen by using the air blast freezing method. In addition, it is suggested that the food quality of meat treated with liquid freezing is comparable to that of unfrozen meat. In this study, we evaluated the effect of liquid freezing on *K. septempunctata* toxicity and the meat texture of olive flounder.

*Kudoa*-infected olive flounder was kindly provided by the National Research Institute of Aquaculture Fisheries Research Agency. The olive flounder skin was removed.
and the meat was cut into pieces that weighed 20 g each. Each piece was packed into a plastic bag and frozen by using an air blast freezer (MF-U338, SANYO Electric Co., Ltd, Osaka, Japan) at −30 °C, and an air blast freezer (UL-1386-5, Thermo Fisher Scientific Inc., MA) at −80 °C, or a liquid freezer (Tomin TL-1, Technican Co., Ltd, Kanagawa, Japan) at −30 °C for 1 min to 5 h. The frozen meat in plastic bags was thawed at room temperature. It has been reported that the thawing in ice water limited the quality change by freezing (Abe, 2011). Although this method may improve the quality of olive flounder meat after freezing, we thawed the pieces at room temperature in this study so as to evaluate the effects induced by freezing.

After thawing the samples, K. septempunctata spores were isolated from olive flounder meat, followed by K. septempunctata toxicity assessment using a previously reported method (Ohnishi, 2013). In brief, the transepithelial electrical resistance (TER) value of differentiated Caco-2 cells monolayer with or without spore inoculation was measured. The TER value of spore inoculated cells was measured after incubating the cells with the spores for 1 h. TER before inoculation of spores was represented as 100%. Values are represented as mean ± SD from three independent experiments.

Caco-2 cells inoculated with Kudoa spores, which were obtained from olive flounder and stored at 4 °C, showed 80% decrease in TER within 1 h (Figure 1). The decrease in TER is due to increased Caco-2 cell monolayer permeability, which indicates Kudoa spore toxicity to Caco-2 cells. Caco-2 cells inoculated with spores, which were frozen for 1 to 3 min by using a liquid freezer, also had a decreased TER value, indicating that the spore toxicity was retained (Figure 1). However, spores that were frozen for 5 min by using the liquid freezing method did not induce a decrease in TER value, indicating that the treatment eliminated Kudoa spore toxicity (Figure 1). The spores frozen at −80 °C for over 1 h by using an air blast freezer inhibited the decrease in TER value (Figure 1). The spores frozen at −30 °C for 5 h by using an air blast freezer did not completely inhibit the decrease in TER value (Figure 1).

Next, we compared the effects of various freezing methods on food appearance and texture. The olive flounder meat samples were frozen for the shortest time period required to significantly reduce the spore toxicity against Caco-2 cells (liquid freezer 5 min: air blast freezer at −80 °C 1 h: air blast freezer at −30 °C 5 h), followed by the evaluation of changes in meat appearance (Figure 2). The transparency of olive flounder meat frozen by using a liquid freezer was similar to that of the meat stored at 4 °C (Figure 2). The olive flounder meat frozen by using an air blast freezer at −80 °C and −30 °C lost its transparency and air blast freezing at −80 °C induced cloudiness (Figure 2).

The frozen meat was thawed, and changes in the meat texture was evaluated by application of a load on the fish meat in a vertical direction, followed by measurement of changes in stress and strain tolerance of food by using a creep meter (RE-33005S, Yamaden Co. Ltd., Tokyo, Japan) equipped with a shearing plunger (P-21, the width of the cutter is 56 mm) and 20 N load cell. The plunger was inserted into the sample to a depth of 90% of the sample thickness with 1 mm/s of compression velocity. The fracture curve was obtained by calculating the changes in stress and strain,
fracture point at 30% strain (Figure 3). This result indicated that the plunger of the creep meter had cut the surface layer of the meat. In addition, the olive flounder meat stored at 4°C showed fracture points at 38, 44, and 48% strain (Figure 3). These fracture points indicated that the plunger had cut the muscular bundles in the meat. The meat frozen by using the liquid freezer showed fracture characteristics that were similar to that of meat stored at 4°C, and clear fracture points were recognized at 32, 42, and 47% strain (Figure 3). These results indicated that the hardness and the structure of the muscle were relatively preserved by liquid freezing. In contrast, the first fracture point in meat frozen by using air blast freezing at −30°C and −80°C occurred at 50% and 39% of strain, respectively (Figure 3). Thus, the fracture point of the strain application was higher than that of meat maintained at 4°C and that of meat frozen by liquid freezing, indicating that the meat had lost its hardness and collapsed by the plunger load. The fracture points on the meat subjected to air blast freezing at −30°C and −80°C were less pronounced (Figure 3), indicating that the structure of muscle was destroyed by freezing.

These data demonstrated that liquid freezing inactivated K. septempunctata in a period of 5 min and preserved the quality of olive flounder meat. Although we have evaluated the effectiveness of liquid freezing under limited conditions, our results indicated that liquid freezing methods could be used to prevent K. septempunctata mediated food borne diseases.

In this study, we thawed olive flounder meat at room temperature. However, it has been reported that the thawing in ice water limits the change in quality caused by air-blast freezing. This method may improve the quality of olive flounder meat after freezing. This remains to be clarified.

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