

## CHAPTER 1

### INTRODUCTION AND LITERATURE REVIEW

#### 1.1 Introduction

Dark muscle fish species currently make up 40-50% of the total fish catch in the world (Hultin and Kelleher, 2000a). There is a great interest in using the large quantities of these low value fatty pelagic fish for human food, particularly for surimi production. However, problems faced with producing surimi from small pelagic species, such as sardine and mackerel is the high content of dark muscle associated with high content of lipid and myoglobin. Those contribute to the difficulties in making high-quality surimi (Chen, 2002; Ochiai *et al.*, 2001). Due to the limited fish resources, especially white muscle fish, dark muscle fish have been paid more attention as a potential alternative raw material, especially for surimi production (Wu *et al.*, 2000; Chen *et al.*, 1997; Kelleher *et al.*, 1994). So far, sardine and mackerel have been used for surimi production even to a small portion (Ochiai *et al.*, 2001).

Myoglobin is a globular heme protein localized in red muscle fibers. The concentration depends on species and age of animal, muscle type as well as the way the meat is treated (Livingston and Brown, 1981; Giddings, 1974). Myoglobin has been known to be a major contributor to the color of muscle, depending upon its derivatives. Since hemoglobin is lost rather easily during handling and storage, while myoglobin is retained by the muscle intracellular structure (Livingston and Brown, 1981), most color changes in meat are due to the reaction of myoglobin with other muscle components, especially myofibrillar proteins (Hanan and Shaklai, 1995). For surimi processing, myoglobin and hemoglobin play an essential role in the whiteness, which is one of factors determining the quality of surimi gel (Chen, 2002). Generally, both heme proteins in fresh fish can be removed during the washing process, leading to whiter in color. However, heme proteins become less soluble as the fish undergo deterioration. Denaturation or

oxidation of myoglobin may induce the adduction with muscle proteins. As a consequence, the surimi produced from the unfresh fish mostly faces with the discoloration, darker in color.

Therefore, the reduction of interaction between heme proteins and muscle should be a promising means to prevent the discoloration of surimi. Due to over-exploitation of fish resources, the fish fleet has to go farther and take a long time prior to unloading. During the handling and storage of fish, a number of biochemical, chemical and microbiological changes occur, depending on both intrinsic and extrinsic factors (Pacheco-Aguilar *et al.*, 2000). Conversion of oxymyoglobin to metmyoglobin is associated with the discoloration (O'Grady *et al.*, 2001; Faustman *et al.*, 1992). This phenomenon can be influenced by many factors, such as pH, temperature, ionic strength, oxygen consumption reaction, etc. (Renner and Labas, 1978). Metmyoglobin formation is positively correlated with lipid oxidation (Lee *et al.*, 2003a; Chan *et al.*, 1997). Furthermore, metmyoglobin formed the cross-linking with myosin in the presence of hydrogen peroxide (Hanan and Shaklai, 1995). Oxidation products, especially aldehyde can adduct to proteins or act as the cross-linker to various proteins including heme proteins (Alderton *et al.*, 2003; Lynch *et al.*, 2001; 2000). Since many factors are involved in the adduction of myoglobin and muscle proteins, a better understanding of those factors should be taken into consideration. Therefore, such an interaction can be minimized, which the removal of myoglobin from the fish muscle can be improved. As a consequence, the better quality of surimi from dark flesh fish can be obtained.

## **1.2 Literature reviews**

### **Meat pigments**

The pigments associated with the bright red color on the surface of meat include hemoglobin and myoglobin (Haard, 1992; Livingston and Brown, 1981). Myoglobin is the principal but not the whole source of color in meat. Additional contributors to the color of meat are the blood pigments, hemoglobin and cytochrome. The proportion of hemoglobin in the meat color depends mostly on the degree of bleeding out of the meat (Eder, 1996).

Myoglobin and hemoglobin have the same function and similar structures in different animal species but there are small differences in their molecular masses due to variations in their amino acid sequence (Ponce-Alquicira *et al.*, 2000). Pegg and Shahidi (1997); Pearson and Young (1989) reported that myoglobin had a molecular weight of about 16,800 daltons and contained only one heme group per molecule, in contrast to hemoglobin which contained four heme groups and had an approximate molecular weight of 67,200 daltons. There is, however, some variation in the molecular weights of myoglobin from different species, caused by the differences in amino acid composition (Pearson and Young, 1989).

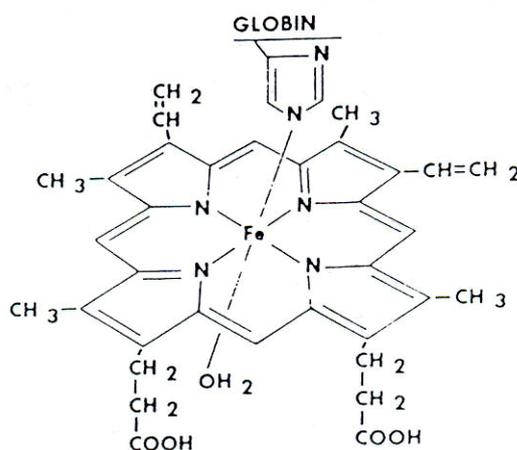
Hemoglobin is normally a tetramer of myoglobin-like monomers. Because of their similar extinction coefficients, the two pigments should contribute similarly to meat color (Haard, 1992). Normally, hemoglobin contributes less to appearance of seafood than myoglobin because it is lost rather easily during handling and storage, while myoglobin is retained by the intracellular structure (Livingston and Brown, 1981).

### **Physical and chemical properties of myoglobin**

Myoglobin is a globular protein that is made up of a single polypeptide chain, globin, consisting of 153 amino acids and a prosthetic heme group, an iron (II) protoporphyrin-IX complex (Hayashi *et al.*, 1998; Pegg and Shahidi, 1997) (Figure 1). It is extremely compact, and its dimensions are approximately  $45 \times 35 \times 25 \text{ \AA}^{\circ}$  (Pegg and Shahidi, 1997). In simplified terms, the globin is folded around the iron of the heme group in 8 right-handed  $\alpha$ -helical segments ranging in length from 7 to 24 residues separated by non-helical regions (Misumi *et al.*, 2002; Moczygemba *et al.*, 2000) (Figure 2). The helical regions make up approximately 70% of the molecule (Pearson and Young, 1989). The well-defined three dimensional structure of globin is stable over a fairly wide range of external conditions, but it can be disrupted by sufficiently drastic changes in physical or chemical environments. This process, known as denaturation, has important consequences regarding structural and functional aspects of the protein. The heme moiety is held in a cleft of the globin by a coordinate bond between the imidazole nitrogen of the proximal histidine residue and the ferrous ion, and by several nonpolar and hydrogen-bonding interactions at the porphyrin periphery (Dunn *et al.*, 1999; Pegg and Shahidi, 1997). This heme

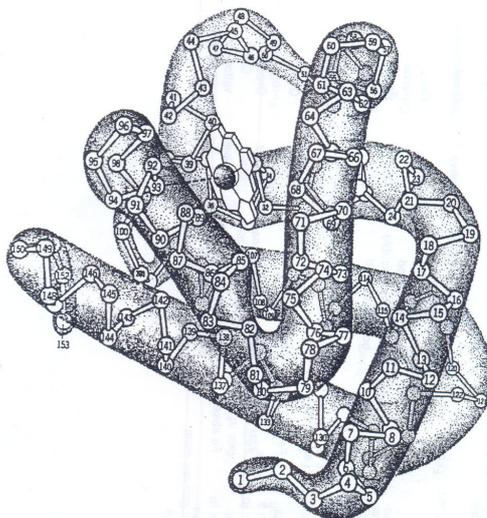
group gives myoglobin and its derivatives their distinctive color. It is also the principal site for meat curing as it relates to color development.

Heme molecule is an organometallic compound (Pegg and Shahidi, 1997). The organic portion consists of four pyrrole groups linked by methane bridges forming a tetrapyrrole ring. Four methyl, two vinyl, and two propionate side chains are attached to the ring, yielding the protoporphyrin-IX molecule (Figure 1). The iron atom is bonded to the four nitrogens in the center of a near-planar ring. Iron is coordinated in an octahedral environment such that it can further accept two ligands to the heme plane. These sites are occupied by an imidazole group of the histidine residue of globin and an atom possessing a free electron pair. These bonding sites are called the fifth and sixth coordination positions, respectively. The heme iron atom may exist in the ferrous (+2) or the ferric (+3) state, depending on the presence of reductants and oxidants in the medium (Livingston and Brown, 1981). Lacking a covalent complex, either state can coordinate water. The structure and chemistry of the iron atom are key to understanding the reactions and color changes that myoglobin undergoes (Livingston and Brown, 1981).



**Figure 1.** Chemical structure of myoglobin

Source: Pearson and Young (1989)



**Figure 2.** Tertiary structure of myoglobin

Source: Elbe and Schwartz (1996)

### **The chemistry of myoglobin color**

The bright red color of raw meat is mainly due to oxymyoglobin, resulting from myoglobin's great affinity for  $O_2$ . This color is recognized by consumer to be associated with freshness (Tang *et al.*, 2003; Giddings, 1977). Myoglobin reacts rapidly and reversibly with  $O_2$ . Consequently, the surface of comminuted meat blooms to a bright red color within minutes of exposure to air (Giddings, 1977; 1974). With time, the small layer of oxymyoglobin present on the surface of the meat propagates downward, but the depth to which  $O_2$  diffuses depends on several factors such as activity of oxygen-utilizing enzymes, temperature, pH, and external  $O_2$  pressure (Ledward, 1992a; 1992b).

In contrast, the interior tissue of meat is purple-red in color. This is the color of myoglobin, sometimes called deoxymyoglobin, and the color persists as long as reductants generated within the cells by available enzymes. When these substances are depleted, the heme iron is oxidized to the ferric state. The brown pigment formed, which is characteristic of the color of meat left standing for a time, is called metmyoglobin. It is generated by the removal of a superoxide anion, or its conjugate acid,  $HO_2$ , from the heme and its replacement by a water molecule gives a high spin ferric heme (Renerre and Labas, 1987). The ferric iron, unlike its

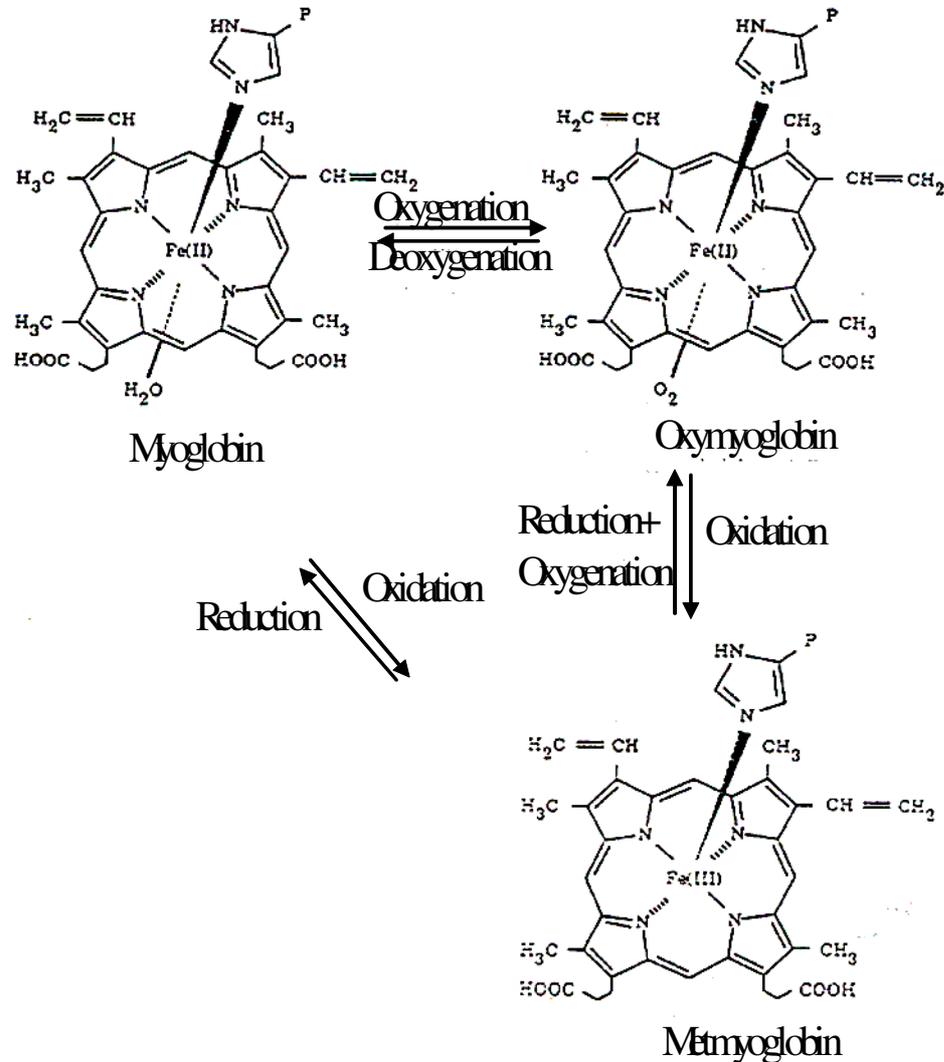
ferrous counterpart, has a high nuclear charge and does not engage in strong  $\pi$  bonding. Therefore, metmyoglobin is unable to form an oxygen adduct (Pegg and Shahidi, 1997). In raw meat, there is a dynamic cycle, in which three pigments including myoglobin, oxymyoglobin and metmyoglobin are constantly interconverted. When metmyoglobin is denatured by thermal processing, meat remains brown in color, but this denatured pigment can be oxidized further to form yellow, green or colorless porphyrin substances by bacterial action or photochemical oxidation. The relationship between raw meat pigments is illustrated in Figure 3.

### **Properties of myoglobin in food system**

Meat color is mainly a result of the concentration of meat pigments, the chemical state of these pigments, and physical characteristics such as fat deposition and diverse surface properties (Postnikova *et al.*, 1999). Dependent on species, breed, sex, age, type of muscle, training and nature of nutrition, the myoglobin content of meat can vary greatly (Postnikova *et al.*, 1999). A general rule states that the more intensively a muscle is used, the higher its myoglobin content is. The iron atom of the heme can exist in either the ferrous or the ferric state and can form complexes with certain ligands, all of which greatly affects the color of the meat. The color of fresh meat is defined by the relative amounts of three derivatives of myoglobin (deoxymyoglobin, oxymyoglobin and metmyoglobin).

The absorption spectra of these three myoglobin derivatives are shown in Figure 4. Hanan and Shaklai (1995) demonstrated that reduced myoglobin was the purple pigment of deep muscles and the meat under vacuum ( $\lambda_{\max} = 555$  nm). Upon exposure to air, myoglobin combined with oxygen to form the bright red oxymyoglobin ( $\lambda_{\max} = 542$  nm), which was synonymous with freshness and considered attractive by the consumer. However, contact of myoglobin with oxygen also leads to the formation of the oxidized form, metmyoglobin ( $\lambda_{\max} = 505$  and 635 nm), which is brown or gray and rather unattractive (Eder, 1996). These three forms of myoglobin are in a state of equilibrium in meat muscles and are constantly interconverted within a dynamic cycle. Giddings (1974) reported that the formation of the attractive oxymyoglobin was favored by a high oxygen tension ( $pO_2 > 4$  torr). The undesired oxidation

toward metmyoglobin was predominant when the oxygen tension was about 4 torr and an oxygen tension below 4 torr led to the predominant formation of deoxymyoglobin.



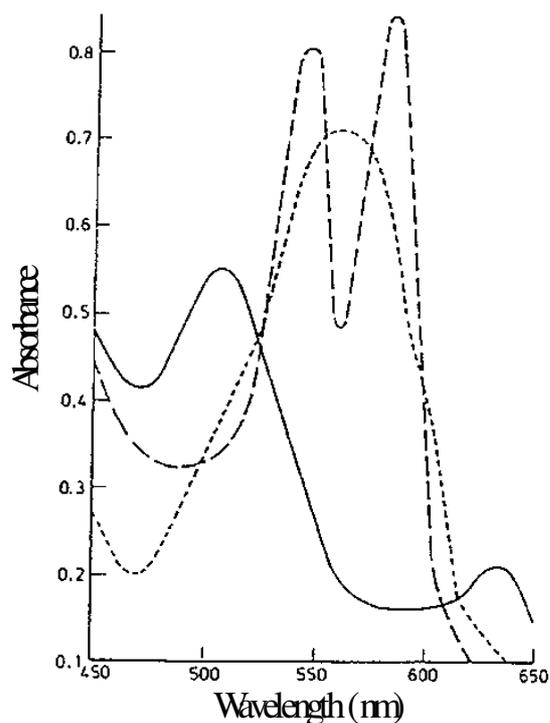
**Figure 3.** Interrelationship between pigments of raw meat

Source: Pegg and Shahidi (1997)

In fresh meat, reducing substances like  $NAD^+$  or  $FAD^+$  are endogenously produced, and they are responsible for the constant reduction of the brown-gray metmyoglobin or the purple myoglobin, which means the dynamic cycle can continue as long as sufficient oxygen is present (Eder, 1996). The heating of meat samples, however, causes considerable destruction of the iron-porphin complex and therefore mainly the denatured hemoproteins are present in cooked meats. The dark red color of cured meat is achieved by the reaction of myoglobin with nitrite

ions, a process which forms nitrosyl myoglobin. After heat denaturation, this pigment is converted to the more stable nitrosyl hemochrome, which is pink (Giddings, 1977).

The red color is regarded as an important parameter of meat and dark meat fish quality. It is well known that the consumer considers the bright red color of oxymyoglobin in fresh meat desirable, while the brown color of metmyoglobin is considered undesirable. Faustman *et al.* (1992) reported that the saturation of red color in meat was directly related to myoglobin concentration. The stability of myoglobin also affects the color of meat. This pigment is very susceptible to heat, acid denaturation, and autoxidation at freezing temperature (Chen, 2003; Chanthai *et al.*, 1998; Tajima and Shikama, 1987; Suzuki and Kisamori, 1984). In addition, the stability of myoglobin from different animals varies considerably (Cashon *et al.*, 1997).



**Figure 4.** Absorption spectra of oxymyoglobin (---), deoxymyoglobin (....) and metmyoglobin (—)

Source: Eder (1996)

### Fish myoglobin

In fish muscle, the red, white and intermediate fibers tend to be more distinctly segregated than they are in muscle from land animals. The dark muscle is normally concentrated along the side of the body and may represent 15-30% of the total muscle in migratory fish such as mackerel and 2-12% of less active fish (Haard, 1992). Muscle of yellowfin tuna contained myoglobin ranging from 37 to 128 mg% in light muscle and 530 to 2,440 mg% in dark muscle (Brown, 1961). The normal myoglobin content of veal, beef and old beef were 100-200, 400-1,000 and 1,600-2,000 mg%, respectively (Haard, 1992). Certain very active fish like tuna possessed deep-seated dark muscle, which was normally richer in myoglobin than superficial dark muscle. The myoglobin content of Atlantic cod dark muscle varied with fishing grounds (Love *et al.*, 1974). The intraspecific variation in myoglobin in the dark muscle of fish appears to be directly related to the amount of exercise by the animal (Haard, 1992).

#### **Characteristics of fish myoglobin**

Myoglobins from fish have amino acid compositions, which differ considerably from myoglobin of mammals (Brown, 1961). Some fish myoglobins contain a cysteine residue. The sequence analysis of yellowfin tuna myoglobin (146 residues) showed 79 to 85 amino acid substitutions when compared to the molecule from mammals, birds and shark (Haard, 1992). Residues 133-139, which are highly conserved in other myoglobins, are totally altered in tuna myoglobin. However, the oxygen binding property of tuna myoglobin is similar to that of other myoglobins. Suzuki *et al.* (1984) isolated native oxymyoglobins from the red muscle of sharks by chromatographic separation method. Kitahara *et al.* (1990) found that the isoelectric point of the bigeye tuna (*Thunnus obesus*) myoglobin was 7.6 for the oxy-form and 8.5 for the met-form. Fosmiri and Brown (1976) showed that the sedimentation velocity for the sperm whale myoglobin sample was 1.94S while the tuna myoglobin value was 1.76S. The denaturation behavior of fish myoglobin was studied by Fosmiri and Brown (1976), who found that 50% denaturation point was 5.3 M urea for the tuna myoglobin and 7.1 M urea for the whale myoglobin.

#### **Autoxidation of myoglobin**

The oxidation product of myoglobin, metmyoglobin, is responsible for discoloration of meat during storage. Metmyoglobin is also a prooxidant of lipid oxidation in raw fish more effectively than in raw turkey, chicken, pork, beef and lamb (Livingston *et al.*, 1981). In general, fish myoglobins are more readily oxidized than mammalian myoglobins (Haard, 1992). Discoloration of tuna during frozen storage is associated with the formation of metmyoglobin. The rate of metmyoglobin formation is greater at the meat surface and is mostly arrested at storage temperatures of  $-33^{\circ}\text{C}$  (Bito, 1968 cited by Haard, 1992). Benjakul and Bauer (2001) suggested that the freeze-thaw process caused damage of cell and heme protein, resulting in the release of prooxidants. At intermediate frozen storage temperatures between  $-5^{\circ}\text{C}$  and  $-15^{\circ}\text{C}$ , decreasing the storage temperature could result in an increased rate of autoxidation of tuna myoglobin and beef myoglobin (Brown *et al.*, 1969). Miki *et al.* (1984 cited by Haard, 1992) found that the apparent activation energy ( $E_a$ ) for the rate of metmyoglobin formation in skipjack tuna was 25.2 Kcal/mole at temperature above  $-2^{\circ}\text{C}$ , 90.5 Kcal/mole between  $-2^{\circ}\text{C}$  and  $-5^{\circ}\text{C}$ , and 18.3 Kcal/mole at temperature below  $-5^{\circ}\text{C}$ . The autoxidation of tuna myoglobin is very temperature sensitive. The  $Q_{10}$  value of the reaction between  $-2^{\circ}\text{C}$  and  $22^{\circ}\text{C}$  was about 5 (Brown *et al.*, 1969)

Fish myoglobins are at least 2.5 times more sensitive to autoxidation than mammalian myoglobins. Autoxidation becomes greater as temperature increases and pH decreases (Livingston *et al.*, 1981). The rate of tuna myoglobin autoxidation, like that of mammals, is inversely related to oxygen concentration. Atmospheres enriched in carbon dioxide are effective in delaying spoilage of fish; however  $\text{CO}_2$  promotes the oxidation of oxymyoglobin to metmyoglobin and hence discoloration (Haard, 1992).

### **Interaction between lipid oxidation products and myoglobin**

Heme pigments are able to catalyze lipid oxidation in meat (Han *et al.*, 1994). Generally, myoglobin is more effective than hemoglobin in acceleration of oxidation. Love (1983) reported the ability of heme compounds to catalyze lipid oxidation. High concentration of myoglobin and other heme compounds in red meats functioned as prooxidants in muscle tissue. It

has been generally assumed that lipid oxidation in meat is nonenzymatic, and hemoproteins, especially myoglobin, are the major catalysts of lipid oxidation (Love, 1983). Morey *et al.* (1973) found that  $H_2O_2$ , acting as an oxidizing agent, caused changes in the oxidation state of the iron in hemoprotein and formed red-brown in color. The interaction of  $H_2O_2$  with metmyoglobin led very rapidly to generation of an active species, which could initiate lipid peroxidation (Chan *et al.*, 1997; Kanner *et al.*, 1985). The myofibrillar proteins, particularly myosin, of many fish species may be altered by their interaction with different types of lipids or lipid oxidation products during frozen storage (Saeed *et al.*, 2002). This interaction caused the considerable changes in some functional properties of fish muscle. Metmyoglobin was found to induce myosin cross-linking in the presence of  $H_2O_2$  with the subsequent loss in ATPase activity (Hanan and Shaklai, 1995). Lynch *et al.* (2001) demonstrated that propional, pentanal, hexanal, and 4-hydroxynonenal (4-HNE) were the primary aldehydes formed during lipid oxidation. These aldehydes can form adducts with proteins and may have implications for protein stability and functionality.

### **Effect of myoglobin on lipid oxidation**

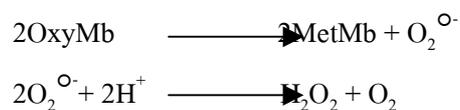
#### **Iron (II) myoglobin-induced lipid oxidation**

The physiologically active myoglobin species are the purple high-spin iron (II) myoglobin (deoxymyoglobin), which has the sixth coordination site of the heme iron vacant, and the bright cherry-red low-spin oxy-iron (II) myoglobin (oxymyoglobin), which bind a molecule of oxygen at the sixth coordination of the heme iron, due to their high affinity for oxygen (Baron and Andersen, 2002; Gorelik and Kanner, 2001; Faustman *et al.*, 1999). Like metmyoglobin, disturbance of the globin structure can result in binding of an unusual ligand at the sixth coordination of the heme iron and induce the formation of a low-spin iron (II) species, known as hemochromes. Hemochromes differ from hemichromes exclusively in the oxidation state of the iron center. The former is in its oxidation state II. Hemochrome, like hemichrome, can be found either reversible or irreversible (Baron and Andersen, 2002). Yin and Faustman (1993; 1994) and O'Grady *et al.* (2001) reported a high correlation between oxymyoglobin oxidation and lipid

oxidation both in microsomes and liposomes and believe that oxymyoglobin oxidation and lipid oxidation are coupled.

The prooxidative activity of deoxymyoglobin in biological system including muscle foods has not been investigated (Baron and Andersen, 2002). This is mainly due to the fact that deoxymyoglobin initiated lipid oxidation demands strictly anaerobic condition; to exclude oxymyoglobin initiated lipid oxidation and the subsequent propagation of lipid oxidation. However, Richards and Hultin (2000) suggested that deoxyhemoglobin was able to initiate lipid oxidation even at low lipid hydroperoxide concentrations.

The effect of oxymyoglobin and its oxidation products on lipid oxidation in fresh meat are important since the oxidation of oxymyoglobin results in production of two species necessary for a prooxidant complex, namely metmyoglobin and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) (Chan *et al.*, 1997). It has been proposed that superoxide anion ( $\text{O}_2^{\ominus}$ ) and  $\text{H}_2\text{O}_2$  are produced during oxidation of oxymyoglobin (Gotoh and Shikama, 1976):



When oxymyoglobin is oxidized to metmyoglobin,  $\text{H}_2\text{O}_2$  may be produced and would enhance the catalytic activity of metmyoglobin. This was in agreement with Yoshida *et al.* (1994) who reported that oxyhemoglobin needed to undergo oxidation to methemoglobin to induce liposome oxidation. Greene and Price (1975) also suggested that meat pigments, both  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  forms, could catalyze lipid oxidation, but this conversion to the  $\text{Fe}^{3+}$  state was necessary for rapid catalysis.

Superoxide anion by itself is not considered as an active catalyst of lipid oxidation, but can further react with  $\text{H}_2\text{O}_2$  and  $\text{Fe}^{3+}$  via the Fenton reaction to produce hydroxyl radical and facilitate lipid oxidation (Chan *et al.*, 1997). Hydrogen peroxide can react with metmyoglobin to form a prooxidative ferrylmyoglobin radical (Decker *et al.*, 1995). Harel and Kanner (1985) reported that  $\text{H}_2\text{O}_2$ -activated metmyoglobin caused rapid oxidation of poultry skeletal muscle microsomes.

Superoxide dismutase (SOD), which catalyzes the breakdown of superoxide anion to  $H_2O_2$ , accelerated autoxidation of oxymyoglobin *in vitro* (Gotoh and Shikama, 1976). SOD scavenged superoxide anion, thus driving the oxidation of oxymyoglobin to metmyoglobin. Anton *et al.* (1993) showed that SOD inhibited both oxymyoglobin and lipid oxidation in an oxymyoglobin-microsome model and suggested that superoxide anion was involved in catalyzing lipid oxidation.

Catalase, which catabolizes  $H_2O_2$  into  $H_2O$  and  $O_2$ , inhibited oxymyoglobin oxidation *in vitro*, suggesting that  $H_2O_2$  involved in the conversion of oxymyoglobin to metmyoglobin (Gotoh and Shikama, 1976). Removal of  $H_2O_2$  by catalase also prevented the formation of  $H_2O_2$ -activated metmyoglobin, and resulted in reduced lipid oxidation in microsome systems (Harel and Kanner, 1985; Anton *et al.*, 1993). Chan *et al.* (1997) reported the relative role of metmyoglobin and oxymyoglobin in lipid oxidation, and the potential involvement of  $H_2O_2$  and superoxide anion in oxymyoglobin-catalyzed lipid oxidation. Prooxidant effect of oxymyoglobin towards lipid oxidation was concentration-dependent (Chan *et al.*, 1997).

The addition of catalase into the oxymyoglobin-liposome system resulted in significantly decreased oxidation of oxymyoglobin and lipid, suggesting a role of  $H_2O_2$  in the interaction between oxymyoglobin and lipid. The addition of superoxide dismutase showed no effect, suggesting that superoxide anion was not directly involved in mediating the oxidation of oxymyoglobin and lipid. Therefore, the actual process of oxymyoglobin oxidation is catalyzed by lipid oxidation with  $H_2O_2$  as a major factor (Chan *et al.*, 1997).

### **Iron (III) myoglobin-induced lipid oxidation**

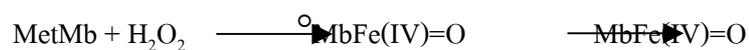
High-spin iron (III) myoglobin, commonly known as metmyoglobin, binds a molecule of water at the sixth coordination site of the heme iron (Pegg and Shahidi, 1997; Renner and Labas, 1987). Disturbance of the globin structure may alter the tertiary structure of the molecule and thereby exposing the heme iron to unusual ligands (e.g., the distal histidine in the heme cavity, exogenous amino acids as histidine and methionine, or a hydroxyl group). As a consequence, the corresponding low-spin iron (III) myoglobin species better known as hemichromes are formed (Baron and Andersen, 2002). Hemichrome formation is either reversible

(binding to the imidazole group of the distal histidine or hydroxyl ion) or irreversible (binding to the imidazole group of free histidine) depending on the type of ligand at the sixth coordination site of the iron and the extent of globin denaturation. Hemichrome formation from iron (III) myoglobin is the intermediate step in the heat denaturation of myoglobin in muscle foods (Baron and Andersen, 2002). Formation of metmyoglobin is highly correlated to the extent of lipid oxidation in muscle foods (Andersen and Skibsted, 1991). Baron *et al.* (1997) found that metmyoglobin is an effective prooxidant at acidic pH and in the presence of hydroperoxides due to different charge distribution on both the fatty acid and the heme protein. In contrast, at physiological pH and in the presence of lipids, metmyoglobin can undergo a rapid neutralization due to formation of the noncatalytic hemichrome pigment. However, further denaturation of the heme proteins due to a high lipophilic environment may result in heme release or further exposure of the heme group to the surrounding lipids, thereby inducing lipid peroxidation (Baron and Andersen, 2002).

Additionally, the lipid to heme protein ratio has been demonstrated to be an important factor affecting the prooxidative activity of heme proteins (Kendrick and Watts, 1969). At lower linoleate/heme protein ratios, heme proteins become ineffective initiators of lipid oxidation (Nakamura and Nishida, 1971). The mechanism responsible for the inhibition of lipid peroxidation at low linoleate/heme protein ratios was elucidated. Fatty acid anions bind reversibly to metmyoglobin, resulting in a spin transition to yield the low-spin metmyoglobin derivative, hemichrome, which was found not to be prooxidative (Baron *et al.*, 1998). At high linoleate-to-heme ratios, metmyoglobin immediately denatures and results in exposure or release of the heme group to the environment that instantly initiates hematin-induced lipid peroxidation in the system (Baron *et al.*, 2002). At high linoleate-to-heme ratios, lipid peroxidation induced by metmyoglobin or metmyoglobin/H<sub>2</sub>O<sub>2</sub> was accompanied by a nearly complete, or even total, disappearance of the soret absorption band, indicating the destruction of the heme protein during incubation. In contrast, incubation of ferrylmyoglobin with linoleate emulsions did not result in significant decrease in the characteristic soret absorption band, irrespective of the linoleate-to-heme ratios. The interaction between metmyoglobin and long chain free fatty acids at physiological pH results in the reversible formation of the low-spin iron (III) myoglobin species hemichrome (Baron *et al.*, 1998).

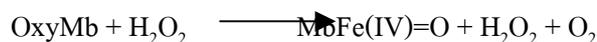
### Iron (IV) myoglobin-induced lipid oxidation

The reaction between hydrogen peroxide and metmyoglobin resulted in the formation of a red pigment, ferrylmyoglobin (Baron and Andersen, 2002). During this interaction, the production of free radicals was postulated to occur in the globin part of the heme protein. Kanner and Harel (1985) indicated that hydrogen peroxide activation of metmyoglobin (also called activated-myoglobin) was a necessary step in the conversion of metmyoglobin to a prooxidant. Further studies have shown that interaction between metmyoglobin and hydrogen peroxide is a complex mechanism, resulting in the generation of two distinct hypervalent myoglobin species, perferrylmyoglobin ( $^{\circ}\text{MbFe(IV)=O}$ ) and ferrylmyoglobin ( $\text{MbFe(IV)=O}$ ) (Davies, 1990; 1991):



The formation of these hypervalent myoglobin species proceeds via a direct transfer of two oxidation equivalents, from hydrogen peroxide to metmyoglobin, giving perferrylmyoglobin (Egawa *et al.*, 2000). One equivalent is located at the iron center forming an oxoferryl complex ( $\text{Fe(IV)=O}$ ), while the other equivalent is suggested to be rapidly transferred from the heme to an amino acid of the globin, giving a protein radical (Davies, 1990). Perferrylmyoglobin is a transient species with a very short half-life and autoreduces rapidly to the more stable ferrylmyoglobin (Baron and Andersen, 2002). Ferrylmyoglobin is a relatively stable species which is slowly reduced back to metmyoglobin at physiological pH but with an increasing rate at decreasing pH due to an acid-catalyzed process (Mikkelsen and Skibsted, 1995).

The iron (II) myoglobin species, deoxymyoglobin and oxymyoglobin, can likewise react with hydrogen peroxide, resulting in the formation of ferrylmyoglobin by direct two-electron oxidation of these iron (II) myoglobin species (Davies, 1991), as shown for oxymyoglobin in following equation, without formation of the transient perferrylmyoglobin:



### Role of perferrylmyoglobin in lipid oxidation

Perferrylmyoglobin can effectively transfer its radical to other proteins, which may subsequently induce lipid oxidation (Reeder and Wilson, 1998). The ability of perferrylmyoglobin to initiate lipid oxidation and to abstract an allylic hydrogen atom from fatty acids (LH) has been suggested by Kanner and Harel (1985):



### Role of ferrylmyoglobin in lipid oxidation

Ferrylmyoglobin has been reported to be responsible for the oxidation of a variety of substrates including proteins, ascorbic acids, tocopherols, glutathione,  $\beta$ -carotene and Trolox (Baron and Andersen, 2002). Under conditions similar to those found in muscle foods, ferrylmyoglobin has been shown to initiate lipid oxidation (Hogg *et al.*, 1994). However, under the conditions found in fresh meat (pH 5.5-5.8), ferrylmyoglobin autoreduces rapidly to metmyoglobin, and it can not be excluded that the observed prooxidative activity is a result of metmyoglobin initiated lipid oxidation. Nevertheless, under physiological conditions (pH 7.4), ferrylmyoglobin has also been shown to initiate lipid oxidation under conditions where metmyoglobin is not a prooxidant. These findings confirm the ability of ferrylmyoglobin to initiate lipid oxidation (Baron and Andersen, 2002). Under physiological conditions, ferrylmyoglobin is a strong prooxidant, able to abstract a hydrogen atom from fatty acids with subsequent stereospecific addition of oxygen (Rao *et al.*, 1994). The prooxidative activity of ferrylmyoglobin is independent of pH and of lipid concentration (Baron *et al.*, 2002). Under

physiological conditions and in the presence of fatty acids, ferrylmyoglobin is not converted to nonprooxidative hemichrome. Increasing the lipid/heme protein ratio did not affect the prooxidative activity of ferrylmyoglobin, nor did it result in any drastic heme protein denaturation, as observed for metmyoglobin (Baron *et al.*, 2002). Ferrylmyoglobin is expected to be an effective prooxidant under the conditions found in muscle food, as well as under physiological conditions. Its potential to oxidize lipids is dependent on hydrogen peroxide, lipid hydroperoxide production, the concentration of reducing agents and their compartmentalization in the muscle cells (Baron and Andersen, 2002).

### **Characteristics of dark muscle fish**

Dark muscle fish are often referred to as fatty fish. This is a reflection of their high lipid content. The presence of high lipid content has important implications in the storage, processing, stability and nutritional value of fish muscle (Hultin and Kelleher, 2000a). The differences between the dark and ordinary muscles relate to their functions. Ordinary muscle is considered an anaerobic organ, whose function is to provide energy quickly and intensively. Ordinary muscle tires easily and primarily uses glycogen as the energy source (Hultin and Kelleher, 2000a). Ordinary muscle fibers have a larger diameter, are poorly vascularized, lack myoglobin, have fewer and smaller mitochondria, have enzymes for anaerobic glycolysis, store glycogen and have little lipid (Kisia, 1996). The dark muscle is designed for long-term exercise and is used by migrating species that travel for great distances. Dark muscle relies on the oxidative metabolism of lipid as its principal source of energy. This is the reason for the high content of oil in the muscle (Hultin and Kelleher, 2000a). Dark muscle fibers are smaller and more uniform in diameter than ordinary muscle fibers. They have greater quantities of mitochondria, myoglobin, fats, glycogen and cytochromes and have a more abundant vascular supply. The fibers respire aerobically and have higher activities of respiratory and citric acid cycle enzymes (Kisia, 1996). Hashimoto *et al.* (1979) determined the protein compositions of the dark and the white from sardine (*Sardinops melanosticta*) muscle. The dark muscle contained 23-29% of sarcoplasmic protein, 62-66% of myofibrillar protein, 6-9% of alkali-soluble protein and

2-3% of stromal protein. The white muscle comprised 33-37% of sarcoplasmic protein, 59-61% of myofibrillar protein, 1-5% of alkali-soluble protein and 1-2% of stromal protein.

### **Lipids in dark muscle fish**

The polar lipids of the membrane systems in muscle have a higher content of the highly polyunsaturated fatty acids than do the neutral triacylglycerols. Whereas the high neutral lipid content of the fatty species, especially in the dark muscle, is related to the sustainable energy source required by these species, the highly unsaturated nature of the membrane lipids is necessary for the metabolic functional requirements of the membrane (Hultin and Kelleher, 2000a). Sarma *et al.* (1998) reported that the crude lipid content in sardine muscle (*Sardinella longiceps*) was 3.99%. Pacheco-Aguilar *et al.* (2000) found that the fat content in Monterey sardine muscle (*Sardinops sagax caerulea*) from the winter period was 8.4%, while the fat content of 1.1% was found in the spring period. The decrease in the lipid contents in muscle in spring could be related to its spawning season (Pacheco-Aguilar *et al.*, 2000).

Fatty acid composition is not the only difference between the neutral oils and the polar membrane lipids. Because the polar phospholipids of the membrane exist primarily as a bilayer, they have a very large surface area exposed to the aqueous phase of the cell. The effective concentration of a component in the nonwater phase of the cell is the surface area that is exposed to the aqueous phase. Based on an equal weight basis, the area of the polar phospholipids is approximately two orders of magnitude greater than that of the neutral triacylglycerols. In fish muscle, which contains 10% neutral lipid and 1% phospholipids, the phospholipids fraction would have 10 times more exposure to prooxidants in the aqueous phase than the triacylglycerols at the surface of the oil droplets (Hultin and Kelleher, 2000a). Bligh and Scott (1966) found that dark muscle of cod contained about three times as much total lipid as white muscle but the composition was quite similar. The most significant difference was that the dark muscle lipid contained more esterified cholesterol and less phosphatidyl choline. The dark and white tissue lipids contained 77.3 and 91.4 % phospholipids, respectively (Bligh and Scott, 1966). In addition to their greater surface area, membrane lipids are found in association with components that can accelerate their oxidation. Mitochondrial inner membrane processes most of the molecular

oxygen of the cell and reactive oxygen species may escape from its electron transport chain. Other membrane systems also have electron transport systems that, although they may not be as active as the mitochondrial inner membrane, can still produce reactive oxygen species. In addition, membrane components, such as cytochromes or nonheme iron proteins, can convert species like superoxyl radicals (or the protonated  $\text{HOO}^\ominus$ ) into more reactive species such as the hydroxyl radical. The juxtaposition of these membrane components and the highly unsaturated fatty acids encourage oxidation of the fatty acids (Hultin and Kelleher, 2000a). Another characteristic of the polar membrane lipids, which may affect their rate of oxidation, is the pH of their immediate environment. When the phospholipids bilayer forms in the membrane, the charged heads of the polar lipids are exposed to the aqueous phase. This produces a net negative charge at the surface of the membrane at neutral pH. The negatively charged surface could then attract hydrogen ions, thus producing a lower pH at the surface (Fromherz and Masters, 1974).

The conversion of superoxide ( $\text{O}_2^{\ominus}$ ) to more reactive  $\text{HOO}^\ominus$  would favor oxidative reactions. The  $\text{HOO}^\ominus$  also has the ability to penetrate into the hydrophobic lipid region of the bilayer, which would make it a more effective prooxidant. On the other hand, some prooxidant processes are less favored at low pH. The lower activity of the sarcoplasmic reticulum for reducing ferric iron to the reactive ferrous iron in the presence of NADH was found at pH values less than 6.8 (McDonal and Hultin, 1987).

### **Lipolysis and lipids oxidation in fish muscle**

The lipid components of fish muscle tissue postmortem are rapidly changed because fatty acids of fish lipids are much more unsaturated than those of mammals and birds and thus would be expected to undergo more rapid oxidation with associated development of off-odors and flavors (Foegeding *et al.*, 1996). In general, a larger proportion of fish tissue is marketed in the frozen state. Freezing can facilitate lipid oxidation, partly because of concentration effects. Thus, lipid oxidation is relatively more important in frozen muscle tissue than in fresh tissue. Pacheco-Aguilar *et al.* (2000) reported that the shelf-life of oily fish was limited by lipid oxidation. Peroxide values during storage at  $0^\circ\text{C}$  of sardine muscle was increased from 2.9-8.9 meq/kg lipid and reached final values of 15.0-26.1 meq/kg lipid at day 15.

Oxidation of lipids also occurs during postmortem storage of muscle tissue, especially in fish muscle. The extent of oxidation depends on the concentration of pro-oxidants, such as endogenous ferrous iron, and the fatty acid composition of the meat. Meats such as fish and poultry contain a high concentration of polyunsaturated fatty acids and are therefore more susceptible to oxidation (Pacheco-Aguilar *et al.*, 2000). Furthermore, the concentration of ferrous iron, and the ability of that iron to be active in the lipid oxidation reaction, will be a key factor causing differences among species and cuts of meat. In general, dark meats tend to have more reactive iron. Other constituents of meat can accelerate oxidation. Enzymatic and non-enzymatic reducing systems convert iron from the inactive ferric form to the active ferrous state, thereby promoting oxidation (Foegeding *et al.*, 1996).

Lipid in surimi are even more unstable if prooxidant, such as iron (from water pipes, machinery, or residual heme proteins), are present. The mincing and washing procedures generally incorporate a large amount of oxygen into the surimi, making lipid more prone to oxidation (Lanier, 2000). Lipid oxidation seems to be a distinct problem in surimi made from some dark-fleshed fish and particularly surimi from mammalian and avian muscle. In the latter, muscle lipid oxidation may be the primary factor for limiting storage life, causing the formation of disagreeable flavors and leading to the denaturation of proteins and decreased gelling ability through peroxide formation (Lanier, 2000).

In biological tissues such as fish muscle, other components such as proteins, amino acids, ascorbate, etc., can interact with these lipid-free radicals to terminate the reaction. The high concentration of non-lipid components which can interact with the lipid free radicals may be one reason why lipid oxidation occurs more slowly in muscle tissue than in isolated lipids (Hultin, 1992).

One important change that occurs in fish muscle lipids postmortem is hydrolysis of glycerol-fatty acid esters with release of free fatty acids. This is catalyzed by lipases and phospholipases (Pacheco-Aguilar *et al.*, 2000). In general, lipase activity is greater in dark muscle than in ordinary muscle of the same fish species (Foegeding *et al.*, 1996). Free fatty acids in fish tissues are expected to have a greater effect on contractile proteins since these proteins are less stable in fish muscle than those in warm-blooded animals (Foegeding *et al.*, 1996).

## Some factors responsible for lipid oxidation in fish muscle

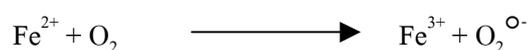
### Oxygen

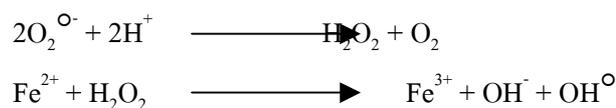
Although oxygen is a very strong oxidizing agent, molecular oxygen does not normally react with most cellular components because it has both kinetic and thermodynamic restrictions on its activity. Molecular oxygen is a double free radical because it has two unpaired electrons. The interaction of molecular oxygen with ground state molecules, such as unsaturated fatty acids, is spin forbidden. However, once the superoxide radical is formed, this spin restriction is removed (Skulachev, 1996). This one-electron reduction of molecular oxygen to superoxide is also unfavorable thermodynamically. All the other single electron reductions, however, as well as the two- and four-electron reductions of molecular oxygen to hydrogen peroxide and water are thermodynamically favorable (Skulachev, 1996).

Superoxide and the protonated neutral hydroperoxyl radical ( $\text{HOO}^\circ$ ), hydrogen peroxide, and the highly reactive hydroxyl radical can cause damage to cellular components. These reactive oxygen species are normally prevented from forming in biological systems by reducing molecular oxygen with enzymes, which bind the reactive intermediates. This binding results in the release of only relatively stable products, for example, water in the case of mitochondrial respiration (Hultin and Kelleher, 2000a). Approximately 80% of the oxygen used in aerobic cells is processed by mitochondria. Another source of superoxide is the autoxidation of oxyhemoglobin (Everse *et al.*, 1997) or oxymyoglobin (Kruger-Ohlsen and Skibsted, 1997). The superoxide produced can be readily converted to other reactive forms by interacting with transition metals such as iron (Hultin and Kelleher, 2000a).

### Ferrous iron

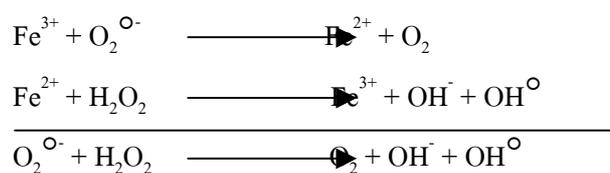
Ferrous iron can react with molecular oxygen to produce  $\text{O}_2^{\circ-}$  with concomitant oxidation to  $\text{Fe}^{3+}$ . Hydrogen peroxide, which may be produced by dismutation of  $\text{O}_2^{\circ-}$ , can react with  $\text{Fe}^{2+}$  to produce  $\text{OH}^\circ$ .





The last reaction is termed the Fenton reaction.

Ferric iron can be reduced by  $\text{O}_2^{\ominus}$  to  $\text{Fe}^{2+}$ , which can interact as described with  $\text{H}_2\text{O}_2$  to produce  $\text{OH}^\ominus$  via the iron-catalyzed Haber-Weiss reaction.



### Temperature

The temperature at which fish is stored is important. As with most chemical reactions, lipid oxidation rates increase with increasing temperature (Hultin and Kelleher, 2000a). Besides the general increase in the rate of lipid oxidation with increasing temperature, the relative contributions of various initiation mechanisms could be changed with temperature. As temperature increases, the contribution of non-enzymatic processes would become relatively more important than enzymic ones (Hultin, 1992). Saeed and Howell (2002) studied the effect of frozen storage on the lipids in Atlantic mackerel stored for up to 24 months at  $-20^\circ\text{C}$  and  $-30^\circ\text{C}$ . The result showed that the rate of lipid oxidation increased with increasing storage temperature and time.

### NaCl

NaCl has been shown to catalyze lipid oxidation in muscle tissue including fish (Nambudiry, 1980). Chloride ion can be converted to a radical via a mechanism as observed with myeloperoxidase (Hultin, 1992). It could then add directly to a double bond or abstract a

hydrogen (Kanner and Kinsella, 1983). Alternatively, the  $\text{Na}^+$  may replace iron from a cellular complex via an ion exchange reaction. The displaced iron may then participate in the initiation of lipid peroxidation (Hultin, 1992).

### **pH**

The different catalysts have different optimal pH values for their activity. In addition, some of reactants are more reactive in their protonated than their non-protonated state (Hultin, 1992). For example, even if the capacity of mitochondria and the sarcoplasmic reticulum to reduce  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  is optimal in the range of pH 6.5-7.0, the superoxide anion that would be produced from the reaction of  $\text{Fe}^{2+}$  with  $\text{O}_2$  would be less reactive than the protonated  $\text{HOO}^\circ$  formed at lower pH (Hultin, 1992).

### **Compositions and functionalities of muscle from dark-fleshed fish**

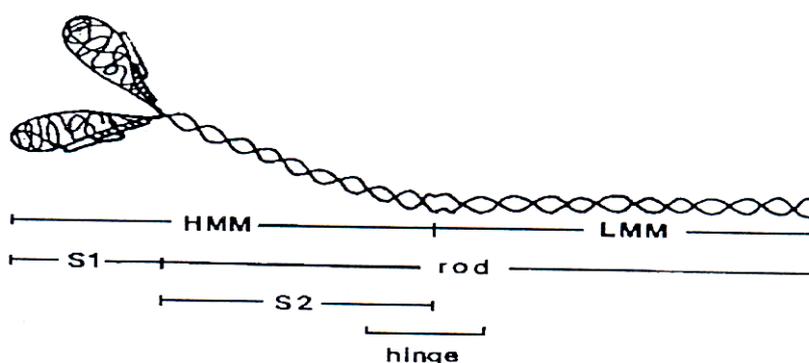
The proteins of muscle tissue are usually grouped into three categories on the basis of their solubility characteristics (Hultin and Kelleher, 2000b). One group is the sarcoplasmic proteins, which are soluble in water or solutions of dilute salt. Haard *et al.* (1994) suggested that the sarcoplasmic proteins from fish included myoglobin, enzymes and other albumins. Salt-soluble proteins are generally myofibrillar proteins. These are defined as proteins soluble in salt concentrations more than 0.3 M, with or without pH adjustment or the presence of components such as magnesium ion and ATP. Stromal proteins are not soluble in either of these extracting solutions. These proteins are primarily connective tissue proteins but also include denatured myofibrillar proteins and membrane proteins (Hultin and Kelleher, 2000a; 2000b).

Myofibrillar proteins can be extracted from the comminuted fish meat with neutral salt solutions of ionic strength above 0.15, usually ranging from 0.30-1.0 (Sikorski *et al.*, 1990). Changes in these proteins lead later to resolution of the stiffness while during long-term frozen storage they may cause toughening of the meat. The myofibrillar proteins are also mainly responsible for the water holding capacity of fish, for the characteristic texture of fish products, as

well as for the functional properties (Sikorski *et al.*, 1990). Hashimoto *et al.* (1979) reported that the dark muscle from sardine contained 62-66% of myofibrillar fraction.

Myosin is a large molecule (about 500,000 daltons) that contains six polypeptide subunits arranged into a native protein molecule with two globular heads attached to a long  $\alpha$ -helical rodlike tail  $\alpha$ -helical. The rod portion is responsible for the assembly of myosin into thick filaments, and the two globular heads contain both the enzymatic active site and the actin-binding region. Thus, myosin has the biochemical properties of both a globular and a fibrous protein (Pearson and Young, 1989). The myosin molecule is approximately 150 nm in length, with a diameter of approximately 8 nm in the globular region and around 1.5-2.0 nm in the  $\alpha$ -helical region. Myosin contains two identical heavy chains (approximately 220,000 daltons each) and two sets of light chains that range in size from 14,000 to 20,000 daltons, depending on species and muscle type. Approximately 50% of each heavy chain starting at the carboxy-terminal end folds together to form a coiled coil of  $\alpha$ -helices that make up the fibrous portion of the molecule. This is the part of the myosin molecule involved in assembly into thick filaments. The other 50% of the length of each heavy chain is associated with two light chains to form one of the two globular or head regions of the molecule. One of the light chains in each myosin head is required for enzymatic activity, and the other light chain in each globular head has a regulatory action. Myosin has an isoelectric point of 5.3, indicating that it is a negatively charged protein, which contains the substantial amounts of aspartic acid and glutamic acid. Myosin includes an overall  $\alpha$ -helical percentage of 56% (95% for the fibrous tail portion and about 30% for the globular regions), a sedimentation coefficient value of 6.4, and an intrinsic viscosity of 215 cm<sup>3</sup>/g (Pearson and Young, 1989). If solutions of myosin are treated with either trypsin or chymotrypsin, the two new species are generated. One of them is called light meromyosin (LMM) and the other is heavy meromyosin (HMM) (Watabe *et al.*, 1994). The HMM fragment contains all ATPase activity and actin-binding ability. The LMM portion is responsible for packing of myosin into the body of thick filaments. The treatment of HMM with papain results in formation of two additional fragments termed S1 and S2 (Watabe *et al.*, 1994). Both of S1 fragments contain the globular head region of myosin, and the S2 fragment contains the portion of the  $\alpha$ -helical region between the head and the LMM. Generation of HMM, LMM, rod, S1 and S2 proteolytic fragments of myosin are shown in Figure 5.

Actin is the second most abundant myofibrillar protein in the myofibrils of cardiac and skeletal muscle, composing approximately 20% of the total (Pearson and Young, 1989). It is a highly conserved protein that polymerizes into filaments that are required for mobility in a variety of contractile systems other than striated muscle. Mobility or movement in muscle cells is based to a large extent on the ability of myosin's head to bind to actin filaments concomitant with the hydrolysis of ATP. The molecular weight of G-actin is approximately 42,000 daltons. The diameter of each G-actin monomer is approximately 5.0 nm, and each thin filament in skeletal muscle contains approximately 400 molecules of actin. Accordingly, the molecular weight of an intact thin filament is approximately  $1.7 \times 10^7$ . Actin shows a sedimentation coefficient of 3.0, slightly less than 30%  $\alpha$ -helic, and an intrinsic viscosity of a perfect sphere of 3.3 (Pearson and Young, 1989).



**Figure 5.** Generation of HMM, LMM, rod, S1 and S2 proteolytic fragments of myosin

Source: Watabe *et al.* (1994)

The dark muscle of pelagic species has been reported to contain higher concentrations of sarcoplasmic proteins than the ordinary muscle. Haard *et al.* (1994) and Hashimoto *et al.* (1979) reported that the content of sarcoplasmic protein was generally higher in pelagic fish, such as sardine and mackerel than in white muscle fish. Sikorski *et al.* (1990) suggested that dark muscle, both superficial and deep-seated, contained more hemoglobin, myoglobin and cytochrome *c* than the ordinary muscles. Spinelli and Dassow (1982) and Hashimoto *et al.* (1979) also reported that myoglobin was dominant in dark muscle of fish.

Fish muscle proteins have several functionalities, particularly gelation. Fish meat, when ground with salt, forms a viscous sol. Upon heating, the sol turns into a viscoelastic gel. The rheological characteristics of the gel depend on the properties of myofibrillar proteins, which are affected by the species and freshness of the fish, as well as on the processing parameters, mainly protein concentration, pH, ionic strength, and temperature (Niwa, 1992). The rate of decline in the strength of gels, resulting from storage of the fish after catch, is species characteristic (Martinez, 1989).

The strengthening of the gel, as a result of cooking, is caused by disulfide and hydrophobic interactions involved (Roussel and Cheftel, 1990). It has been established that the heat gelation of myosin is based on an irreversible aggregation of the myosin heads, in which disulfide exchange is involved, and on thermal helix-coil transition of the tail part of the molecules which is followed by formation of a three-dimensional network, buttressed by noncovalent interactions (Samejima *et al.*, 1981; Ishioroshi *et al.*, 1981). Surimi prepared from mackerel whole muscle was darker and its gel strength was lowered more rapidly during frozen storage than surimi prepared from light muscle (Kelleher *et al.*, 1994).

### **Surimi from dark muscle fish**

Due to the higher lipid contents, less stable proteins, greater concentrations of heme proteins, higher proteolytic activities and higher concentrations of sarcoplasmic proteins of dark muscle, the difficulties in making high-quality surimi from raw material containing high contents of dark muscle are generally faced.

One of the principal reasons why dark muscle tissue is difficult to process into good surimi is its propensity to undergo oxidations. Good handling practices may not be able to eliminate this problem entirely because peroxides are present in the living animal (Nakamura *et al.*, 1998). Murakawa *et al.* (2003) reported that oxidized lipids can interact with proteins, causing denaturation, polymerization, changes in functional properties and bring about an adverse effect on the quality of surimi products. Although most of the depot fat is removed when fish are headed, gutted, and skinned, however, a small percentage of membrane phospholipids are present in fish muscle, which are difficult to remove by washing. These phospholipids are highly

unsaturated and often in contact with muscle heme iron and are therefore sensitive to deteriorate by oxidation. Such oxidation causes off-flavors and may hasten denaturation of the myofibrillar proteins (Lanier, 2000). The presence of sarcoplasmic proteins of dark muscle has often been cited as one of the reasons for the poorer gelation characteristics of dark muscle fish compared with light muscle (Haard *et al.*, 1994). Sarcoplasmic proteins bind to the myofibrillar proteins and thus interfere with the formation of gels. Hultin and Kelleher (2000a) and Haard *et al.* (1994) reported that small quantities of sarcoplasmic proteins had an adverse effect on the strength and deformability of myofibril protein gels. These proteins may be interfered with myosin cross-linking during gel matrix formation because they did not form gels and had poorer water holding capacity. The presence of sarcoplasmic proteins may change the rheological properties of the fish gels (Sikorski, 1994). Aldolase and glyceraldehydes-3-phosphate dehydrogenase were difficult to extract at ionic strengths lower than 0.1, as they were strongly bound to the myofibrils during the heat treatment, thus decreasing the strength of the gel (Sikorski, 1994). In addition, dark muscle also has a higher proteolytic activity than white muscle (Shimizu *et al.*, 1992). This can cause modori, the gel weakening that occurs if the gel is held too long at a temperature around 50-60°C (Sikorski, 1994).

The gel-forming ability of dark muscle fish meat has been known to be lower than that of ordinary muscle. This apparently resulted from the difference in the unfolding abilities of the myosin between the muscles. Lo *et al.* (1991) found that the head portions of the heavy chain of myosin from the dark and ordinary muscles did not differ significantly in the thermostability, but the ordinary muscle myosin had two thermal transition points, at 36 and 57°C, whereas that of the dark muscle had only one transition point, at 68°C. Ochiai *et al.* (2001) suggested that to prepare high-quality surimi and process it into a fish cake of higher gel strength and better whiteness, it was necessary to remove dark muscle as much as possible. Several techniques have been suggested to remove dark muscle (Spencer and Tung, 1994; Shimizu *et al.*, 1992). The location of the dark muscle along the lateral line near the skin makes deep skinning a feasible process for some species. Another method is to freeze the skin of a fillet onto a drum. The skin is then cut away, removing some dark meat and subcutaneous fat. As more dark muscle is removed, some light muscle is obtained but the yields decrease (Hultin and Kelleher, 2000a). Dark muscle also has greater mechanical strength than light muscle because of its higher content

of connective tissue (Hashimoto *et al.*, 1979). Therefore, much dark muscle can be removed during the refining process because it remains intact while the softer light muscle is pushed through the orifices. Water under pressure has also been used to remove the softer light meat while leaving the dark meat attached to the skin. This process requires much water (Hultin and Kelleher, 2000b). However, in the case of red-fleshed fish such as mackerel and sardine, abundant dark muscle is difficult to remove with a meat separator (Ochiai *et al.*, 2001). Hence, the washing process is necessary for color improvement of products prepared from whole muscle.

The color of surimi can be improved by increasing the washing cycle, washing time, and water quantity (Kim *et al.*, 1996). Long period washing would result in high hydration of mince and degradation of myofibrillar proteins, making the subsequent dehydration process more difficult and could lower the gel forming ability (Lin and Park, 1996). The traditional batch type or continuous rotary screen washing system is widely used but long washing time and large quantity of water required are serious problems. The color of dark-fleshed fish surimi could be improved by leaching of mince with hydroperoxide or sodium percarbonate, or by adding some fat/casein material to mask the color (Chen *et al.*, 1997). The pH of dark-fleshed fish mince decreased rapidly after slaughter, and the gel forming ability was inhibited once the pH dropped to around its isoelectric point (Chen *et al.*, 1996). In general, undesirable materials, such as lipid and TMAO, can be removed effectively during washing under acidic conditions (Eide *et al.*, 1982). An adjustment of pH of wash water with alkali reagents or addition of reductive materials to surimi would increase its viscoelasticity (Chen *et al.*, 1997).

### **Some washing processes used in dark surimi production**

#### **Alkali solution washing**

The addition of alkali in the surimi wash water produces a higher quality product than just using water (Shimizu *et al.*, 1992). Various concentrations of sodium bicarbonate may be added in one or more of the wash steps to increase the pH. Sodium chloride is also sometimes added. It has been suggested that gelation is improved after this type of washing process because the solubility of the sarcoplasmic proteins is increased, and there is a decreased rate of

denaturation as the muscle pH is increased (Shimizu *et al.*, 1992). This process also releases more of the fat than washing without alkali. Furthermore, the addition of salt aids in the removal of heme pigments (Hultin and Kelleher, 2000a). In an improved process, a mixture of sodium pyrophosphate and sodium bicarbonate was used to wash fish muscle, that had been homogenized into fine fragments, for 20-30 min under vacuum,. The pyrophosphate was added to dissociate actomyosin. The vacuum treatment and small particle size favor removal of impurities such as colored and odorous substances and fat. Because of the small size of the fragments, however, the protein has to be recovered with a decanter centrifuge rather than by a rotary sieve or screw press (Hultin and Kelleher, 2000a).

#### **Ozonized water washing**

Ozone ( $O_3$ ), a strong oxidation reagent capable of damaging the porphyrin of myoglobin or hemoglobin, was used successfully in the decoloration of swine hemoglobin (Chang *et al.*, 1996) and mackerel muscle (Lin and Chang, 1995; 1994). Chen *et al.* (1997) investigated the effect of ozonized water washing method on the improvement of color and gel forming ability of horse mackerel mince. The color of mince was improved within a shorter washing period (10-20 min). However, oxidation of the fish oil occurred during ozone treatment.

#### **Air-flotation washing**

Chen (2002) investigated the decoloration effects and gel-forming ability of horse mackerel mince by air-flotation washing. The lowest residual myoglobin content and best decoloration effect were found in the mince washed with air-flotation washing method. Air-flotation washing also effectively improved gel strength of heated surimi. The physical removal of myoglobin was suggested to be the main factor contributing to improved decoloration with air-flotation washing. Two reasons might account for the color improvement in the mince processed by air-flotation washing; one is the color lightening of myoglobin and the other is the flocculation of myoglobin by bubble flotation. Air-flotation washing also reduced the amount of washing

water required by around 50% when compared with cold water washing and alkali solution washing.

### **1.3 Objectives of study**

1. To study the chemical compositions and properties of sardine and mackerel muscles
2. To study the changes in color and pigment of sardine and mackerel muscle during iced storage
3. To study the lipid oxidation of sardine muscle during iced storage
4. To purify and characterize myoglobin from sardine muscle
5. To study some factors affecting the interaction between fish myoglobin and myofibrillar proteins

### **1.4 Scope of research**

This research was focused on the characterization of muscles of tropical dark-fleshed fish species commonly used for surimi production including sardine and mackerel. Different washing processes were used to remove the myoglobin from sardine and mackerel mince and the effects on gel forming ability were also elucidated. Changes in color, pigments and lipids of sardine muscle during iced storage were monitored. Due to the similarity among fish myoglobin, myoglobin purified from yellowfin tuna, myoglobin-rich species, was used for the study of myoglobin-myofibrillar protein interaction. Role of lipid oxidation products on the interaction between myoglobin and myofibrillar proteins was also studied. Furthermore, the impacts of some factors involving ionic strength, temperature, time and frozen storage on the binding of tuna myoglobin to fish myofibrillar proteins *in vitro* were elucidated using both temperate (bluefish) and tropical (sardine) fish. To study the effect of species-specific on myoglobin-myofibrillar proteins interaction, natural actomyosin or myosin from different fish comprising bluefish, yellowfin tuna and sardine were used in the model system. All experiments

in this research were expected to provide the beneficial information for the improvement of gel quality and color of surimi produced from dark-fleshed fish.