CHAPTER 1

INTRODUCTION

Seafoods, especially shrimps, have become economically important with new developments and expansions in production, processing and international marketing. However, shrimps are perishable, leading to the quality losses. The losses in quality may be associated with a number of enzymes in shrimps both in meat and cephalothorax. Proteases generally show negative effect on the quality of postharvest shrimp by inducing the deterioration and disintegration of muscle proteins during storage and processing (Jiang *et al*., 1991). Proteases are responsible for significant quality changes of fresh shrimp. Nip *et al.* (1988) reported that shelf-life of iced fresh water shrimp was not more than 3-4 days because of proteolytic activities. Among proteolytic enzymes, collagenase has the impact on the softening of muscle (Brauer *et al*., 2003). Collagenases are defined as proteases capable of degrading the native triple helix of collagen under physiological conditions. Serine collagenases have been purified from the hepatopancreas of several shrimp species, including *Pandalus eous* (Aoki *et al*., 2003), *Penaeus vannamei* (Van Wormhoudt *et al*., 1992) and *Penaeus monodon* (Lu *et al*., 1990).

Melanosis or blackening is another problem occurring in shrimps during postmortem storage (Ogawa *et al*., 1984; Simpson *et al*., 1987, 1988; Benner *et al*., 1994; Bartolo and Birk, 1998). Although the pigmentation seems to be harmless to consumers, it drastically reduces the product's market value (Montero *et al*., 2001a). This phenomenon is an enzymatic browning process induced by polyphenoloxidase (PPO). PPO induces the oxidation of phenolic substrates to quinones, which undergo autoxidation and polymerization to form melanin responsible for the black coloration (Wang *et al*., 1994). To reduce the melanosis in shrimp, some selected chemicals known as PPO inhibitors have been used including 4-Hexylresorcinol (Martínez-Alvarez *et al*., 2005a; Martínez-Alvarez *et al*., 2005b), kojic acid (Chen *et al*., 1991b), organic acid (ascorbic acid, citric acid and acetic acid) (Martínez-Alvarez *et al*., 2004), sodium benzoate, sulfiting agents (Gomez-Gilman *et al*., 2005), ethylenediaminetetraacetic acid (EDTA) and disodium dihydrogen pyrophosphate (Martínez-Alvarez *et al*., 2004), etc.

Raw shrimps have a very short shelf-life since the spoilage can take place rapidly. Freezing or the combined use for brining and chilling are common preservation methods for shelf-life extension of shrimp products (Dalgaard and Jorgensen, 2000). Among the technologies that ensure quality maintenance with minimum losses, modified atmosphere packaging (MAP) has become increasingly popular as a method of food preservation (Ashie *et al*., 1996). MAP in combination with low temperature can be stressed by their role to maintain the characteristics of fresh commodities and to extend the shelf-life. It is well known that the effect on microbial growth is attributed to CO_2 because this gas extends the lag phase and reduces the growth rate during the logarithmic phase (Church 1994 and Genigeorgis 1985). Apart from antimicrobial effect of CO_2 in MAP, the lowered O_2 could be an approach to reduce the blackening in shrimp. However, a little information about the shelf-life extension of black tiger and white shrimp by the combined technique has been reported. Thus, the objectives of this study were to characterize some enzymes associate with quality losses and to investigate the effect of modified atmosphere packaging in combination with melanosis inhibitor to prolong the shelf-life of both black tiger and white shrimp at refrigerated temperature.

Literature Review

1. Black tiger shrimp and white shrimp

Black tiger shrimps (*Penaeus monodon*) get their name from the dark stripes that encircle the shell of the black tiger. Black tiger shrimps are also known as giant tiger prawns, or tigers. This is one of the most popular varieties of shrimp on the Asian, European, and American markets. Since 1994 the production has decreased and it is now expected to remain steady or slightly increase. Most black tigers on the market are farmed in Asia, where rapid growth of shrimp farms has resulted in the loss of coastal wetlands and other environmental problems. Tiger shrimp can grow as long as 13 inches (33 cm) long. Giant tiger prawns are native to the Indian and western Pacific Ocean. They are also found from the east coast of Africa to Japan and Western Australia. The main sources of giant tiger prawns are Thailand, India, Indonesia and Vietnam. Giant tiger prawns sold in the U.S. market are primarily from Thailand, India and Indonesia (FAO, 2007).

The white shrimp (*Litopenaeus vannamei*) is the most important penaeid species cultivated in North and South America. Currently, *L. vannamei* is also an economically important crustacean species in Thailand (Saoud *et al*., 2003; Donato *et al*., 2005). White shrimps lay eggs and spawn in ponds naturally. Few white shrimp live as long as a year and the maximum lifespan are about two years. White shrimp are sold throughout the year. A white shrimp is currently sold in a variety of fresh or frozen product forms, including whole and tails (or headless), shell-on and peeled, round or split, and de-veined (Browdy, 1998). The white shrimp is easily recognizable by pale to whitish color with the tail edged in green. No groove presents along the base of either side of the rostrum or along the head. Antennae are significantly longer than body length, and may reach 2.5-3.0 times body length. Chromatophores in white shrimp are widely spaced, thus lending a lighter body color to this species than in either pink or brown shrimp. Overall body color is a bluish white, speckled with black, with pink-tinged sides. Pleopods are often marked with dark red, while the margins of the uropods of the tail are green along their margins (Williams 1984). White shrimp are usually found in waters with low salinity, organic rich, and muddy bottoms. White shrimp mature during their first year, and have a life span of around 2 years. They can grow up to 8 inches long (FAO, 2007).

2. Quality changes of shrimp during storage

The biological characteristics and the physiology of shrimp cause a high vulnerability and consequently a very short shelf life. Its shelf-life and wholesomeness during refrigerated storage and shipping is greatly influenced by both enzymatic and microbiological changes (Mendes *et al*., 2002). Shellfish undergo the deteriodation more rapidly than fish for a number of reasons. Firstly, they are smaller, and small fish spoil more rapidly than larger ones. Secondly and more importantly, the gut is usually not removed immediately after capture, hence postmortem autolytic changes will occur faster. A third reason is that the chemical composition of shellfish tissue is different and it contains a lot of non-protein nitrogenous compounds that encourage more rapid spoilage (Shamshad *et al*., 1990). Furthermore, shellfish are also prone to black spot or melanosis, a discoloration indicative of spoilage (Jiang *et al.,* 1988). Therefore, it is important for the shrimp processing industry to develop a storage method to maintain high quality and freshness of shrimp.

2.1 Degradation of shrimp muscle associated with proteinases

Like other marine species, endogenous and bacterial enzymes are involved in the deterioration of crustacean (Jiang *et al*., 1991). The endogenous proteases play an important role in protein degradation of posmortem fish muscle. The disintegration of the muscle structure was considered to be caused by proteases, such as calpain (Wang *et al*., 1992; Jiang *et al*., 1991) and cathepsin (Visessanguan *et al*., 2001; Gomez-Guillen and Batista, 1997). Proteases associated with the quality losses can be classified into 4 groups as follows:

a. Serine proteinases

The serine proteinases have been described as a group of endoproteinases with a serine residue in their catalytic site. This family of proteinases is characterized by the presence of a serine residue, together with an imidazole group and aspartyl carboxyl group in their catalytic sites (Simpson, 2000). The activity is inhibited by diisopropylphosphofluoridate (DFE), through reaction with the hydroxyl group of the active site serine residue (Simpson, 2000). The proteinases in serine subclass all have the same first three digits: EC 3.1.21. The three major serine proteinases purified and well characterized from the digestive glands of marine animals are trypsin, chymotrypsin, and elastase. Trypsin is assigned the code ED 3.4.21.4. Trypsin has a very narrow specificity for the peptide bonds on the carboxyl side of arginine and lysine. Chymotrypsin is assigned a code of EC 3.4.21.1 and it has a much broader specificity than trypsin. It cleaves peptide bonds involving amino acids with bulky side chains and nonpolar amino acids such as tyrosine, phenylalanine, tryptophane, and leucine (Simpson, 2000).

Crustaceans such as shrimp, crayfish and American lobster, contain highly active proteinases including trypsin and chymotrypsin, which are released from the hepatopancreas by postmortem processes autolyzing the meat. Trypsin-like proteinase was shown to be the most important enzyme involved in autolysis of krill tissue (Kawamura *et al*., 1981). Trypsin and chymotrypsin were found in brown shrimp (*Penaeus californiensis*) (Vega-Villasante *et al*., 1995) and other shrimp species (Jiang *et al*., 1991). Collagenases are the proteases capable of hydrolyzing collagen, resulting in the softened texture of fish and shellfish. Collagenases have been purified and characterized from the digestive glands of marine animals such as crab, lobster, and shrimp (Baranowski *et al*., 1984). Collagenases from marine animals resemble mammalian

collagenases in their action on native collagen. However, marine digestive collagenases differ from mammalian counterpart in exhibiting trypsinlike and chymotrypsinlike specificities (Rudenskaya, 2003). Collagenases from hepatopancreas of marine invertebrates are associated with the postharvest textural deterioration in these animals during storage (Nip *et al*., 1988). Proteases also contribute to melanosis by activating polyphenoloxidase (PPO). Thus, blackspot development in crustaceans is dependent on the proteolytic activation of PPO. Inhibition of the serine proteinases reduced phenolase activation, and therefore blackspot development (Ali *et al*., 1994).

b. Thiol/ Cysteine proteinases

The thiol or cysteine proteinases are a group of endoproteinases that have cysteine and histidine residues as the essential groups in their catalytic sites. These enzymes require the thiol (-SH) group furnished by the active site cysteine residue to be intact. Hence this group is named "thiol" or "cysteine" proteinases. The thiol proteinases are inhibited by heavy metal ions and their derivatives, as well as by alkylating agents and oxidizing agents (Mihalyi, 1978). The first three digits common to thiol proteinases are EC 3.4.22. An example of thiol proteinases from the digestive glands of marine animals is cathepsin B, which is designated as EC 3.4.22.1 (Simpson, 2000).

Cathepsin B plays important roles in the quality deterioration of fish products (Kolodziejska and Sikorski, 1996; Hernandez-Herrero *et al*., 2003). It is a lysosomal cysteine protease that hydrolyzes a wide range of proteins and has an important role in the hydrolysis of tissue proteins (Barrett and Kirschke, 1981). It is formed from a precursor that is latent in the tissues (Mort and Buttle, 1997) and is converted to the mature form by biological reactions, such as acidification (Mach *et al*., 1993), action of other proteolytic enzymes (Dalet-Fumeron *et al*., 1993; Kawabata *et al*., 1993) or autoactivation by cathepsin B itself (Rowan *et al*., 1992; Mach *et al*., 1993). Cathepsin B plays an important role in intracellular and extracellular protein catabolism together with cathepsins H and L; it is considered one of the main causes of post mortem degradation of muscle (Barrett, 1977; Ashie *et al*., 1996; Kolodziejska and Sikorski, 1996). Cathepsin B have been characterized from the digest glands of cod (*Gadus morhua*), saithe (*Pollachius virens*) and haddock (*Melanogrammus aeglefinus*) (Sovik and Rustad, 2006), and

from muscle in mackerel (*Scomber australasicus*, *Scomber japonicus*) (Jiang *et al*., 1994; Jiang *et al*., 1997) and Pacific whiting (*Merluccius productus*) (An *et al*., 1994). Moreover, cathepsin B from hepatopancreas (a digestive gland) of carp (*Cyprinus carpio*) was reported (Aranishi *et al*., 1997). Cathepsin H activity has also been demonstrated in muscle cells by several investigators (Kirschke *et al*., 1983). Cathepsin H has many similarities with cathepsin L which is also optimally active within a pH range of 5.0-6.0 (Ashie, 1997). Yamashita and Konagaya (1990a) found cathepsin L in chum salmon (*Oncorhynchus keta*) muscle. A proteinase responsible for tissue softening of Pacific whiting (*Merluccius productus*) was identified to be cathepsin L-like protease (Seymour *et al*., 1994). An *et al*. (1994) also noted that cathepsin L was the most active enzyme in surimi, whereas cathepsin B was the predominant enzyme in the fillets.

Thiol (cysteine) proteinases have been isolated from Norway lobster (*Nephrops norvegicus*) (Wang *et al*., 1992). Nevertheless, inhibition of the thiol proteinases had negligible effect on blacksport development (Wang *et al*., 1994).

c. Metallo proteinases

The metalloproteinases are hydrolytic enzymes whose activity depends on the presence of bound divalent cations. Chemical modification studies suggest that there may be at least one tyrosyl residue and one imidazole residue associated with the catalytic sites of metalloproteinases (Whitaker, 1994). The metalloproteinases are inhibited by chelating agents such as 1, 10-phenanthroline, EDTA, and sometimes by the simple process of dialysis. Most of the metalloproteinases known are exopeptidases. They all have a common first three digit as EC 3.4.24. The metalloproteinases have been characterized from marine animals (e.g., Pacific rockfish, crab, and squid mantle) but have not been found in the digestive glands except in the muscle tissue (Simpson, 2000).

Collagenases are metalloenzymes stimulated by Ca^{2+} ions and thiol reagent; depending on the source (Asghar and Bhatti, 1987). Collagenase activity was found in mammalian muscle (Venugopal and Bailey, 1978; Laakonen *et al*., 1970). Also, collagenolytic activity was found in fish muscle tissues of winter flounder (*Pseudopleuronectes americanus*) (Teruel and Simpson, 1995).

d. Aspartic proteases

The acid or aspartyl proteinases are a group of endopeptidases characterized by high activity and stability at acidic pH. They are referred to as "aspartyl" proteinases (or carboxyl proteinases) because their catalytic sites are composed of the carboxyl group of two aspartic acid acid residues (Whitaker, 1994). Based on the EC system, all the acid/aspartyl proteinases from marine animals have the first three digits in common as EC 3.4.23. Three common aspartyl proteinases that have been isolated and characterized from the stomach of marine animals are pepsin, chymosin, and gastricsin (Simpson, 2000).

Cathepsin D is the major lysosomal pepstatin-sensitive aspartic protease which participates in intracellular breakdown of tissue proteins (Chewale *et al*., 1985; Kirschke and Barrett, 1987). It may also be involved in post-mortem tenderness of fish muscle caused by fragmentation of myofibrils (Jiang *et al*., 1992a). Ueno *et al.* (1981, 1986) found higher cathepsin D activity in dark than in white muscle of carp and mackerel. Cathepsin D have been characterized from Pacific whiting (*Merluccius productus*) (Erickson *et al*., 1983), carp (*Cyprinus carpio*) (Makinodan and Ikeda, 1969), milkfish (*Chanos chanos*) (Jiang *et al*., 1993), tilapia (*Tilapia×Tilapia aurea*) (Jiang *et al*., 1992b), chum salmon (*Oncorhynchus keta*) (Yamashita and Konagaya, 1991), winter flounder (*Pseudopleuronectes americanus*) (Wasson *et al*., 1992) and the banded shrimp (*Penaeus japonicus*) and grass shrimp (*Penaeus monodon*) (Jiang *et al*., 1992a).

2.2 Characteristics of shrimp proteinases

Shrimp proteases have been reported to have different optimal temperatures, depending on species and anatomical locations. Proteases from grass shrimp (*Penaeus monodon*) digestive tract had the maximal activity at 55°C and 65°C (Jiang *et al*., 1991), whereas the optimal temperature of proteases from shrimp (*Penaeus monodon*) hepatopancreas was 60-70°C (Chukiatwattana, 2002). Collagenase from hepatopancreas of Northern shrimp (*Pandalus eous*) (Aoki *et a*l., 2004) and Antractic krill (*Euphausia superba*) (Turkiewicz *et al*., 1991) had the optimal temperature ranging from 40 to 45°C. Collagenolytic protease from greenshore crab digestive gland had the optimal temperature of 30°C (Roy *et al*., 1996).

For optimal pHs, proteases from different shrimps show the varying optimal pHs. The optimal pHs of proteases from grass shrimp digestive tracts were 7.0 and 8.0 (Jiang *et al*., 1991). Proteases from whole digestive tract of brown shrimp (*Penaeus californiensis*) had the optimal pH around 8.0 (Vega-Villasante *et al*., 1995). Optimal pHs of collagenase from shellfish e.g. the hepatopancreas of Northern shrimp (*Pandalus eous*) (Aoki *et al*., 2004), fiddler crab (*Uca pugilator*) (Eisen *et al*., 1973), and greenshore crab (*Carcinus maenas*) (Roy *et al*., 1996) were in the range of 6-7.

2.3 Deterioration of shrimps by microorganisms

Fish and shellfish are highly perishable and the quality deterioration is usually dominated by microbial activity. This deterioration is highly temperature dependent and can be reduced by low storage temperature. Shrimp is a very perishable product, and postmortem changes occur rapidly, compared with fish. The high content of free amino acids and other soluble non-nitrogenous substances, which partly contribute to the desirable, delicate sweet taste of shrimp, can also serve as easily digestible nutrients for microbial growth (Konosu and Yamaguchi, 1982). The production of protein breakdown products such as ammonia, indole, methanethiol, putrescine, trimethylamine and other off-odor compounds are caused by the growth of spoilage bacteria (Lakshmanan *et al*., 2002). Microorganisms are present on the external surfaces and in the gut and head of shrimp. Upon death, the microorganisms or the enzymes are free to invade or diffuse into the flesh where they react with the complex mixture of natural substances (Lee and Um, 1995). The bacteria causing the important chemical changes during fish spoilage in the presence of atmospheric O_2 include Gram-negative, psychrotrophic strains of *Pseudomonas*, *Achromabacter*, *Flavobacterium* and *Moraxellla* species (Erkan *et al*., 2007).

The quality deterioration of fresh shrimp is usually dominated by microbial activity. This deterioration is highly temperature dependent and can be reduced by low storage temperature. Leitao and Rios (2000) reported that freshwater prawns (*Macrobrachium rosembergii*) have a shelf-life around 10 days when stored at $0^{\circ}C$. The shelf-life is reduced to around 5 days when the storage temperature is increased to $5^{\circ}C$. According to the International Commission on Microbiological Specifications for Foods-ICMSF (ICMSF, 1986), fresh seafood show counts varying from log 3 up to log 7 CFU/ g with different conditions affecting the

contamination levels. Shamshad *et al*. (1990) reported that mean aerobic plate count of fresh shrimp (*Penaeus merguiensis*), initially 5 log CFU/g, increased with time and temperature to 9 log CFU/g at 35°C after 24 h. The total viable counts in shrimp (*Pandalus borealis*) increased from an initial level of 5 log CFU/g to 8 log CFU/g at the end of storage period in liquid ice at 1.5 °C (Zeng *et al*., 2005). The shelf-life depends on the numbers and types of microorganisms, mainly bacteria, initially present and their subsequent growth (Borch *et al*., 1996). H_2S producing bacteria which contributed 21% of the total flora of fresh prawn were identified as *Aeromonas*, *Shewanella*, *Enterobacter* and *Citrobacter*. These strains were capable of reducing TMAO and producing fishy H_2S off-odours (Lalitha and Surendran, 2006).

The bacterial flora changed during iced storage. The majority (65%) of the strains isolated after 1 week in ice were Gram-positive rods and cocci (*Bacillus*, *Streptococcus* and *Micrococcus*). After 2 weeks in ice, Gram-positive rods and cocci were shifted to Gram-negative bacteria constituted 75% of the total flora belonging to genera *Aeromonas*, *Shewanella*, *Moraxella* and *Pseudomonas*. Among Gram-positives, *Enterococcus*. *Micrococcus*, *Corynebacterium* and *Bacillus* were predominant. Motile Gram-negative rods (*Aeromonads, S. putrefaciens*, *Pseudomonas*), the most prolific organisms during the ice storage of fish were found at the end of storage (Lalitha and Surendran, 2006). Reilly *et al*. (1984) and Reilly and Dangla (1986) reported *S. putrefaciens* and *A. hydrophila* as the dominant spoilage organisms in pond reared *P. monodon* held at 0°C.

These spoilage organisms can be inhibited by packaging of products in an impermeable film under a CO_2-enriched atmosphere (Huss, 1971). Under these packaging conditions, the growth of common spoilage microorganisms is inhibited and microaerophilic strains of lactic acid bacteria become the dominant spoilage microorganisms. Inhibition of spoilage microorganisms is associated with the lower levels of chemical compounds, for example, trimethylamine (TMA), total volatile nitrogen (TVB-N), which are chemical indicators of microbial spoilage of fish and shellfish (Ashie *et al*., 1996).

2.4 Melanosis

Main attributes used for the assessment of shrimp quality are usually odor and appearance (Bremner, 1998). Melanosis or blackspot appears very frequently during the

postmortem storage of shrimp before the onset of spoilage. Melanosis is the formation of insoluble black pigments (melanins) in the internal shell surface due to enzymatic oxidation of phenolic precursors (Cobb, 1977). It is a natural mechanism caused by enzyme reactions that start as soon as the Penaeidae are taken from the water and come into contact with the oxygen of the atmosphere. After harvest and death, PPO systems are still active and can promote the development of black pigments around the shell and on the surface of the meat (Anonymous, 1998). However, blackspot varies with species, moulting cycle and harvesting and handling methods (Vinayakam and Nellaiappan, 1987; Marshall *et al*., 1988; Anonymous, 2000). The dark discoloration starts at the cephalothorax, usually within 2 to 4 day after catch, and extends through the abdomen, pereiopods, and tail during ice storage (Williams *et al*., 2003). The pigments themselves are not dangerous to human health, but their appearance is rejected by consumers. Refrigeration alone does not prevent, but only slows, blackspot development, because the enzyme remains active during refrigeration, storage on ice, and post-freeze thawing (Montero *et al*., 2004). PPO from different species exhibited different kinetic and thermodynamic properties which could in part explain the differences in susceptibility to melanosis (Marshall *et al*., 1988; Ferrer *et al*., 1989; Chen *et al*., 1991b).

Figure 1 provides an example of a visual scale for the progression of melanosis, while Table 1 shows the scale used to describe the progression of melanosis (black spot) on pink shrimp (*Penaeus dourarum*).

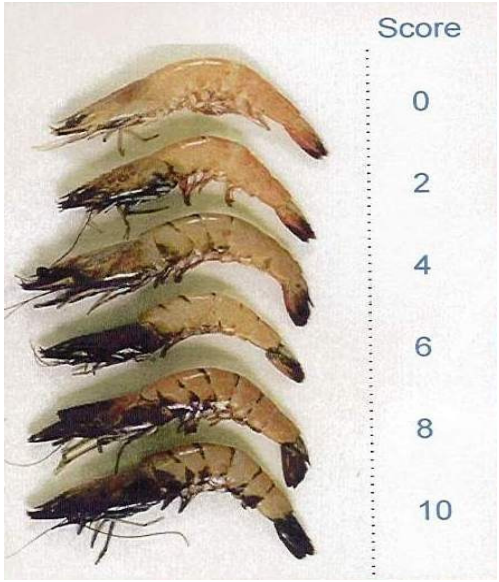

Figure 1 Melanosis progression scale of shrimp

Source: Otwell and Marshall (1986)

Table 1 Color scale used to describe the progression of melanosis (black spot) on shrimp

Melanosis Scale	Description
0	**Absent**
2	**Slight, noticeable on some shrimps**
4	**Slight, noticeable on most shrimps**
6	**Moderate, noticeable on most shrimps**
8	**Heavy, noticeable on most shrimps**
10	**Heavy, totally unacceptable**

Source: Otwell and Marshall (1986)

2.4.1 Polyphenol oxidase (PPO)

PPO catalyzes two basic reactions: hydroxylation to the *o*-position adjacent to an existing hydroxyl group of the phenolic substrate (monophenol oxidase activity), and oxidation of diphenol to *o*-benzoquinones (dipehnol oxidase activity). Both reactions utilize molecular oxygen

as a co-substrate. PPO is one of the metalloproteins with two copper atoms in the active site. A mechanism of the catalytic reactions of PPO is relatively well understood, in which PPO in met-type [Cu(II)Cu(II)] interacts with molecular oxygen to form PPO in oxy-type [Cu(II)Cu(II)O_2] being capable of catalyzing the reactions of mono- and diphenols (Pérez-Gilabent and García Carmona, 2000).

a. Monophenol oxidase

Monophenol oxidase catalyzes the hydroxylation of monophenols to *o*-diphenols (Figure 4). The enzyme is referred to as tyrosinase in animals, since L-tyrosine is the major monophenolic substrate (Whitaker, 1972). Tyrosinase activity is also used to describe monophenol and diphenol oxidases in plant systems, although L-tyrosine is probably not a major substrate for the enzyme in plant systems. Monophenol oxidase activity is generally overlooked in plants since the hydroxylation reaction is dramatically slower than the oxidation reaction required for quinone production and initiation of the browning reaction. Monophenol oxidase (tyrosinase) has been given somewhat more attention in insect and crustacean systems, owing to its physiological significance in conjunction with diphenolase activity, in hardening of the cuticle for sclerotization (Whitaker, 1972).

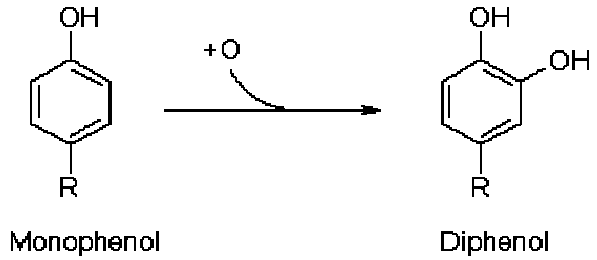

Figure 2 Monophenol oxidase pathway producing the diphenol

Source : Toussaint and Lerch (1987**)**

b. Diphenol oxidase

The oxidation of diphenolic substrates to quinones in the presence of oxygen is catalyzed by diphenol oxidase activity (Figure 5). Diphenol oxidases have received much

attention owing to their high catalytic rate and their association with the formation of quinones, which lead to production of the brown pigment, melanin.

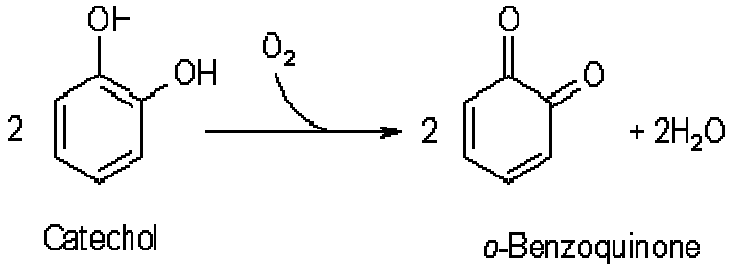

Figure 3 Diphenol oxidase pathway producing the quinones

Source : Toussaint and Lerch (1987)

PPO is localized in the carapace of the cephalothorax, in the caudal zone and in the cuticle of the abdomen, mainly where the cuticle segments are joined and where the cuticle is connected to the pleopods (Ogawa *et al*., 1984). In chilled shrimps, the melanosis reaction begins at the head and then spreads to the tail; the rate of spread of melanosis differs among the various species (Montero *et al*., 2001b).

The optimal temperature of PPO has been reported to be varied, depending on species and habitat temperature. PPO from carapace of shrimp (*Penaeus setiferus*) had the maximal activity at 45°C, whereas the optimal temperature of PPO from carapace of shrimp (*Penaeus japonicus*) cultured in Spain was 55°C (Simpson *et al*., 1988). The activity markedly decreased in very acidic and alkaline pH ranges. The unfolding of PPO molecules might occur at the extreme pHs owing to the increase in electrostatic repulsion, leading to the losses in their activity. Benjakul *et al*. (2005) reported that the optimal pH of PPO from the kuruma prawn cephalothorax was 6.5. The optimal pH of PPO from different crustaceans varied with the species. PPO from the carapace of shrimp (*Penaeus setiferus*) showed the maximal activity at pH 7.5 (Simpson *et al*., 1987). PPO from the prawn (*Penaeus japonicus*) had two pronounced peaks at pHs 5.0 and 8.0 (Montero *et al*., 2001a), whereas the optimal pH of black tiger prawn PPO was 6.0 (Rolle *et al*., 1991). Moreover, the optimal pH depends to a large extent on the physiological

pH, in which the enzyme activity occurs in nature. The optimal pH of PPO from the carapace of cephalothorax was 7.16, while it was 8.76 for PPO from abdominal cuticle.

2.4.2 Inhibition of melanosis

The use of melanosis or browning inhibitors in shellfish is restricted by considerations relevant to toxicity, wholesomeness, and effect on taste, flavor, texture, and cost. Melanosis inhibitors may be classified in accordance with their primary mode of action. Six categories of polyphenol oxidase inhibitors are applicable in the prevention of enzymatic browning (Table 2). These include (1) reducing agents; (2) acidulants; (3) chelating agents; (4) enzyme inhibitors (McEvily *et al*., 1992).

Table 2 Representative inhibitors of melanosis

Class	**Inhibitors**
Reducing agents	**sulfiting agents**
	ascorbic acid and analogs
	cysteine
	glutathione
Chelating agents	**phosphates**
	EDTA
	organic acids
Acidulants	**citric acid**
	phosphoric acid
Enzyme inhibitors	**aromatic carboxylic acids**
	aliphatic alcohol
	anions
	peptides
	substituted resorcinols

Source: Adapted from McEvily *et al*. (1992)

2.4.2.1 Reducing agents/Antioxidants

Reducing agents play a role in the prevention of enzymatic browning either by reducing *o*-quinones to colorless diphenols, or by reacting irreversibly with *o*-quinones to form stable colorless products (Marshall *et al*., 2000). Reducing compounds are very effective in the control of browning. Sulfiting agents are the most widely applied reagents for the control of browning in the food industry (Marshall *et al*., 2000).

a. Sulfiting agents

Sulfites are the most widely used inhibitors of enzymatic browning. Sulfiting agents include sulfur dioxide (SO) and several forms of inorganic sulfite that liberate SO_2 under the conditions of their use (Marshall *et al*., 2000). SO_2 and sulfite salts form sulphurous acid (H_2SO_3) and exist as a mixture of the ionic species, bisulfite (HSO_3^-) and sulfite (SO_3^{2-}) anions in aqueous solution. The predominant ionic species varies in accordance with pH, ionic environment, water activity, presence of non-electrolytes, and concentration of the medium in which they are dissolved. Increased concentrations of sulfite at pHs of less than 5 were observed to enhance the inhibition of PPO-catalyzed browning (Sayavedra-Soto and Montgomery, 1986).

Sulfites serve a multifunctional role in foods. They possess antimicrobial activity and inhibit both enzymatic and non-enzymatic browning reactions. Madero and Finne (1982) proposed that bisulfite exerted a competitive inhibitory effect on PPO by binding a sulfhydryl group at the active site of the enzyme. On the other hand Ferrer *et al.* (1989) proposed that PPO inhibition was due to the reaction of sulfites with intermediate quinones, resulting in the formation of sulfoquinones, which irreversibly inhibited PPO, causing complete inactivation.

Although sulfites are very effective in controlling browning, they are subject to regulatory restrictions owing to their potentially adverse effects on health. Many reports have described allergic reactions in humans, following the ingestion of sulfite-treated foods by hypersensitive asthmatics. The use of sulfiting agents in food processing is based on sulfur dioxide equivalence (Modderman, 1986). The Joint Expert Committee on Food Additives (JECFA) of the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) recommend an acceptable sulfite daily intake of 0.0-0.7 mg sulfur dioxide per kg of body

weight. Shrimp products having residual sulfite levels in excess of 100 ppm are considered adulterated, since these levels are considered unsafe (Federal Register, 1985). Since sulfiting agents can produce illness in sensitive people, alternative agents including acid compounds (citric acid), complexing agents (EDTA, boric acid), reducing agents (erythrobates, ascorbates, reducing sugar), ascorbic acid, cysteine, Everfresh2 or 4 hexylresorcinol, kojic acid, ficin and benzoic acid (Ogawa *et al*., 1983; Wagner and Finne, 1986; Taoukis *et al*., 1990; Applewhite *et al*., 1991; McEvily *et al*., 1991; Guandalini *et al*., 1998) have been used.

Sulfiting agents can block PPO activity, and provides some partial bleaching to maintain the preferred shrimp and lobster appearance (Anonymous, 1998). In addition, sulfites are commonly used as antimicrobial agents (Maldhavi *et al*., 1995) to prevent a possible bacterial growth that could occur in stored shrimps (Chinivasagam *et al*., 1998).

b. L-Ascorbic acid

Ascorbic acid is a moderately strong reducing compound, which is acidic in nature, forms neutral salts with bases, and is highly water-soluble. Ascorbic acid also acts as an oxygen scavenger for the removal of molecular oxygen in PPO reactions (Buta and Moline, 2001). PPO inhibition by ascorbic acid is attributed to the reduction of enzymatically formed *o*-quinones to their precursor diphenols (Walker, 1977). Ascorbic acid is however irreversibly oxidized to dehydroascorbic acid during the reduction process, thus allowing browning to occur upon its depletion (Figure 6). More stable forms of ascorbic acid derivatives, such as erythrobic acid, 2- and 3-phosphate derivatives of ascorbic acid, phosphinate esters of ascorbic acid, and ascorbyl-6-fatty acid esters of ascorbic acid, have however been developed to overcome these problems (Sapers and Hicks, 1989). Ascorbic acid esters release ascorbic acid upon hydrolysis by acid phosphatases (Liao and Seib, 1988).

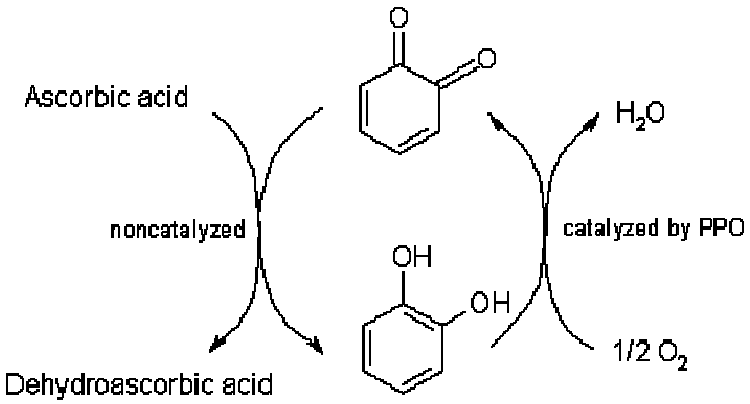

Figure 4 Mechanism of prevention of color formation by ascorbic acid

Source : Marshall *et al*. (2000)

Ascorbic acid causes a distinct yellow off-color, when used in the prevention of melanosis in shrimp (Otwell and Marshall, 1986). It is usually applied in conjunction with citric acid in order to maintain a more acidic pH level. In addition, it is also believed to have a chelating effect on the copper prosthetic group of polyphenol oxidase (Whitaker, 1972).

2.4.2.2 Acidulants

Ionizable groups of the protein structure of enzymes are affected by the pH of the food medium. These groups must be in the appropriate ionic form in order to maintain the conformation of the active site, bind substrates, or catalyze the enzymatic reaction (Segel, 1976). Changes in the ionization status of enzymes are generally reversible. Irreversible denaturation can however occur under conditions of extreme pH (Marshall *et al*., 2000). The stability of the substrate is also affected by changes in pH, since substrates can undergo chemical breakdown under extreme conditions of pH. Degraded substrates often behave as enzyme inhibitors, since they share the molecular features of the substrate (Tipton and Dixon, 1983).

Acidulants are generally applied in order to maintain the pH well below that required for optimum catalytic activity of an enzyme. Acidulants such as citric, malic, and phosphoric acids are capable of lowering the pH of a system, thus inactivating PPO (Richardson and Hyslop, 1985). Acidulants are often used in combination with other antibrowning agents.

Citric acid exerts its inhibitory effect on PPO by lowering the pH as well as by chelating the copper at the active site of the enzyme (Richardson and Hyslop, 1985).

2.4.2.3 Chelators

Enzymes generally possess metal ions at their active sites. Removal of these ions by chelating agents can therefore render enzymes inactive. Chelating agents complex with prooxidative agents, such as copper and iron ions, through an unshared pair of electrons in their molecular structures (McEvily *et al*., 1992). Chelators used in the food industry include sorbic acid, polycarboxylic acids (citric, malic, tartaric, oxalic, and succinic acids), polyphosphates (ATP and pyrophosphates), macromolecules (porphyrins, proteins), and EDTA. Other non-GRAS chelating agents which are capable of inhibiting polyphenol oxidase include cyanide, diethyldithiocarbonate, sodium azide and 2-mercaptobenzothiazole, carbon monoxide, mercaptobenzthiazol, dimercaptopropanol, and potassium methyl xanthate (Marshall *et al*., 2000).

a. EDTA

EDTA is a chelating agent permitted for use in the food industry as a chemical preservative. Calcium disodium EDTA (21 CFR 172.120) and disodium EDTA (21 CFR 172.135) have been approved for use as food additives by the United States Food and Drug Administration (Anon, 1992). Highly stable complexes are formed by the sequestering action of EDTA compounds on iron, copper, and calcium. Maximum chelating efficiency occurs at the higher pH values where carboxyl groups exist in a dissociated state (Dziezak, 1986). EDTA is generally used in combination with other chemical treatments for the prevention of enzymatic browning in foods.

b. Phosphates

Polyphosphates, sodium acid pyrophosphate, and metaphosphate are chelating agents, used as antibrowning agents for fresh-peeled fruits and vegetables at concentrations as low as 0.5 to 2 percent (final concentration in the dip solution) (McEvily *et al*., 1992). Sporix™, an acidic polyphosphate mixture (sodium acid pyrophosphate, citric acid,

ascorbic acid, and calcium chloride), has been observed to delay the onset of oxidation and enzymatic browning in fruits and vegetables (Gardner *et al*., 1991).

c. Kojic acid

Kojic acid (5-hydroxy-2-hydroxymethyl-4H-pyran-4-one) has potential applicability in the prevention of melanosis in both plant and seafood products. Kojic acid was shown to inhibit melanosis in pink shrimp (Applewhite *et al*., 1990). Tyrosinase inhibition by kojic acid was thought to be due to the ability of kojic acid to bind copper at the active site of the enzyme. Although kojic acid is a good inhibitor of PPO, its toxicity is of concern. Wei *et al*. (1991) reported weak mutagenic activity of kojic acid in a *Salmonella typhimurium* assay.

2.4.2.4 Hexylresorcinol

Hexylresorcinol (4-HR) has been used as an alternative to sulfites for prevention of melanosis of the shrimp (Lopez-Caballero *et al*., 2005). A solution containing 50 mg/L 4-HR was shown to prevent melanosis to the same degree as a 12.5 g/L sulfite solution (McEvily *et al.*, 1991) Sulfite, a reducing agent, reacts chemically with the black spot precursors, whilst 4-HR acts as a specific inhibitor of PPO (European Commission, 2003). Information involving the addition of this compound to different species of crustacean are available, showing divergences in the effective doses for melanosis prevention. McEvily *et al.* (1991) cited 50 mg/kg in brown shrimp (*Penaeus aztecus*) and in pink shrimp (*Penaeus duodarum*); Otwell *et al*. (1992) also cited 50 mg/kg in pink shrimp (*Penaeus duodarum*), whereas Guandalini *et al*. (1998) reported 100 mg/kg in deepwater pink (*Parapenaeus longirostris*). However, considerably higher concentration, as much as 5,000 mg/kg, was used in tiger shrimp (*Penaeus japonicus*) (Montero *et al*., 2001b). Guandalini *et al*. (1998) reported that the most effective concentration of 4-HR to inhibit or slow down melanosis in deepwater pink shrimp was 100 mg/kg within an optimum period of 7 days. Melanosis in tiger prawns (*Marsupenaeus japonicus*) was determined when different solutions involving 4-hexylresorcinol (0.1% and 0.05%) in combination with organic acids (citric, ascorbic, and acetic) and chelating agents (ethylenediaminetetraacetic acid (EDTA) and disodium dihydrogen pyrophosphate (PPi) were used. 4-hexylresorcinol could delay the appearance of melanosis during the 1st week of storage. A combination of 4-hexylresorcinol with

an acid showed the effect on melanosis similarly to that achieved by the use of 4-hexylresorcinol alone. However, the presence of chelating agents in combination with 4-hexylresorcinol and organic acids showed the most effective melanosis prevention and improved the appearance of shrimp (Martínez-Alvarez *et al*., 2005b). Montero *et al*. (2001b) used a combination of 4-hexylresorcinol with an acid (ascorbic acid or citric acid) as a melanosis inhibitor mixture in prawns (*Penaeus japonicus*) by dipping for 2 h. They found that the effect of each acid was intensified when combined with 4-hexylresorcinol. However, in deepwater pink shrimp, the above mentioned acids did not increase melanosis inhibition, but the appearance of the shrimp was much improved (Montero *et al*., 2004). These differing findings may be due to interspecies variations, to cyclical changes in physiological susceptibility, or to melanosis inhibitor concentration and the method of application employed (Montero *et al*., 2006).

3. Modified atmosphere packaging

Modified atmosphere packaging (MAP) is widely used as a supplement to ice or refrigeration to delay the spoilage and extend the shelf-life of fresh fishery products while maintaining a high-quality end product (Pedrosa-Menabrito and Regenstein, 1988; Reddy *et al*., 1995). MAP is defined as the enclosure of food products in gas-barrier materials, in which the gaseous environment changed (Reddy *et al*. 1992a). Gas packaging is simply an extension of the vacuum packaging technology. Food packaging under modified atmosphere employs different gases, such as CO_2, N_2, and O_2 (Wolfe, 1980). Several methods can be used to modify atmosphere such as gas packaging, vacuum packaging and atmosphere modifier. The use of MAP for fish and fish products has been reported by Stammen *et al.* (1990), Farber (1991), Reddy *et al*. (1992a), Davis and Slade (1995) and Gibson and Davis (1995).

Gas packaging

Gas packaging is simply an extension of vacuum packaging technology and involves the evacuation of air inside the packaging and replacing by a specific gas or a mixture of gases that differ from the air composition. Gas mixture is introduced into the packaging and no further control is carried out during storage (Wolfe, 1980). The three main commercially used

gases in modified atmosphere packaging are carbon dioxide (CO_2), nitrogen (N_2) and oxygen (O_2).

Carbon dioxide (CO_2) is soluble not only in water, but also in lipids. CO_2 is responsible for the bacteriostatic effect in modified atmospheres. Its general effects on microorganisms are an intensification of their latest growth stage and a decrease in the growth rate during the logarithmic stage (Farber, 1991). The bacteriostatic effect is influenced by the CO_2 concentration, the initial bacterial population, the storage temperature and the product being packaged (Reddy *et al*., 1992b). The concentration of CO_2 in the food is dependent on the water and fat content in the products. In food presenting high moisture and/or fat amounts, such as fish, beef and poultry, the excessive absorption of CO_2 may lead to a phenomenon known as "packaging collapse" (Parry, 1993). Increase in dripping is caused by the dissolution of gases on the muscles surface in atmospheres containing high CO_2 levels (>60%). The reduced pH leads to low protein water retention ability (Parry, 1993; Randell *et al.,* 1995). As a consequence, high CO_2 concentrations promote organoleptic changes and texture alterations in meat. N_2 can be used as an inert gas in smaller proportions than CO_2. O_2 can also be employed, providing fish without color alterations (Cann *et al*., 1983). According to Daniels *et al.* (1985), CO_2 influences bacterial cells as follows:

- Alterations in cell membranes functions, including effects on nutrients
- Direct inhibition of enzymes or decrease in enzymatic reactions
- Penetration in the bacterial membranes, leading to changes in the intra-cell pH
- Direct alterations in physico-chemical properties of proteins

Nitrogen (N_2) is an insipid and inert gas, and is mostly used as a filler gas in MAP, because of its low solubility in water and lipids. It is used for displacing the oxygen in the packaging, decreasing oxidative rancidity and inhibiting the growth of aerobic microorganisms (Farber, 1991). Due to its low solubility, it is used as a filling gas, preventing the possible packaging collapse caused by the accumulation of CO_2.

Oxygen (O_2) generally stimulates the growth of aerobic bacteria and may inhibit the growth of exclusively anaerobic bacteria, although anaerobic microorganisms show different sensitivity levels to oxygen (Farber, 1991). The presence of oxygen may cause oxidative rancidity problems in fish having high amounts of lipids, in which the formation of low molecular weight

aldehydes, ketones, alcohols and carboxylic acids can occur (Daniels *et al*., 1985), Thus, the use of O_2 in modified atmospheres is generally avoided with this kind of fish in order to minimize such adverse effects. The use of O_2 in modified atmosphere packaging for fish is supported by Davis and Slade (1995) who stated that the use of O_2 reduces the exudation in fish during storage.

Vacuum packaging

Vacuum packaging is one of methods commonly used to keep the foods. The product is placed inside a type of packaging presenting low permeability to oxygen. The air is exhausted and the packaging is sealed. The gaseous atmosphere of the vacuum packaging is reduced, but it is probably altered during storage. Thus it is considered as modified due to a 10 to 20% increase in the CO_2 amount produced by microbial activity (Lopez-Caballero *et al*., 2000). This CO_2 may inhibit the growth of undesirable microorganisms. This condition, that is low O_2 and elevated CO_2 levels, extends the shelf-life of fresh fish by inhibition the growth of aerobic spoilage microorganisms, particularly *Psuedomonas* spp. and *Aeromonas* spp. (Ashie *et al*., 1996).

Atmosphere modifier

Suitable atmosphere modifier may be used to provide the desired headspace atmosphere inside the package. The modifiers commercially available include O_2 absorber or scavengers, and ethanol generator (Ashie *et al*., 1996). Most of the technologies used in the manufacture of the modifier were originated in Japan and the materials have been used in that country for many years before their introduction in the world.

4. Used of MAP for shelf-life extension of fishery products

MAP can be used to retard ice or the spoilage of fresh fishery products (Sivertsvik *et al*., 2002). The achievable extension of shelf-life depends on species, fat content, initial microbial population, gas mixture, the ratio of gas volume to product volume, and most importantly, storage temperature (Sivertsvik *et al*., 2002). The gas composition used is dependent on the species (Church, 1998). The use of gas packaging, specifically elevated CO_2 levels, has been shown to inhibit normal spoilage bacteria including *Pseudomonas* spp., *Alteromonas* spp., *Shewanella* spp., *Moraxella* spp. and *Acinetobacter* spp. in fish as well as some pathogens

including *Staphylococcus aureus, Salmonella* spp., *Enterobacteriaceae* such as *Yersinia enterocolitica* and *Escherichia coli*. (Silliker and Wolfe, 1980; Reddy *et al*., 1992b; Kimura and Murakami, 1993). In general, the effectiveness of CO_2 inhibition is strongly related to storage temperature (Stammen *et al*., 1990). Reddy *et al*. (1995) studied shelf-life of MAP fresh tilapia fillets stored under refrigeration and temperature-abuse conditions. The fillets packaged in air had the shelf-life of 9-13 days at a storage temperature of 4°C, but decreased to 3-6 days at 6°C. Moreover, increased solubility of CO_2 at lower temperatures relatively increased the effect of MAP (Ogrydziak and Brown, 1982). From a number of studies, a longer shelf-life can be obtained for many fish (Table 3). MA packaged haddock had 2±4 days longer shelf-life when compared with those kept in air (Dhananjaya and Stroud, 1994). Stenstrom (1985) suggested 50% CO_2/ 50% O_2 as a suitable mixture for retail packages of cod. During storage of sliced hake packed in MAP, 60% CO_2/ 30% O_2/ 10% N_2 mixture was the most effective to preserve its quality. Pastoriza *et al*. (1996a) and Lopez- Galvez *et al*. (1998) described that gas mixtures containing CO_2 in combination with O_2 (20-60%) delayed microbial growth and some biochemical changes during storage of whole hake, both in controlled and modified atmospheres. Gas mixture of 40% CO_2/ 60% O_2 inhibited efficiently the microbial growth and maintained color appearance of tuna (Lopez-Galvez *et al*., 1995). Debevere and Boskou (1996) indicated that 60% CO_2/ 30% O_2/ 10% N_2 prevented the trimethylamine (TMA) production in cod kept under MAP. Different storage condition of fish products stored under MAP led to varying shell-life of different species (Table 3).

Table 3 Shelf-life of fresh fishery products packaged under MA, vacuum or air

Type of fishery product*	Storage temperature (°C)	Atmosphere $CO_2 : N_2 : O_2$	G/ P ratio	Shelf-life (days)
Herring (*C. harengus*) whole	0	60 : 40 : 0	ns	14
	0	Air	ns	12
Hybrid striped bass (*Morone*	2	60 : 34 : 6	ns	13
saxatilis×M. chrysops) strips	2	Air	ns	7
Mackeral (*Scombrus scombrus* L.) fillets	2	100 : 0 : 0	3	> 21
Rockfish (*Sebastes* spp.) fillets	1.7	80 : 20 : 0	-	13
		Air	-	6
Salmon, atlantic (*Salmo salar*) slices	2	100 : 0 : 0	1.5	18
	2	Air	1.5	8
Salmon, king (*Oncorhynchus*	4.4	60 : 15 : 25	ns	12
tshawytscha) fillets	4.4	Air	ns	6
	22.2	60 : 15 : 25	ns	2
	22.2	Air	ns	1
Salmon (ns) fillets	16	Air	ns	4
	16	75 : 25 : 0	ns	5-6
	16	Vacuum	-	3
	8	Air	ns	13-17
	8	75 : 25 : 0	ns	20-24
	8	Vacuum	-	> 6, < 10
	4	Air	ns	24-27
	4	75 : 25 : 0	ns	55-62
	4	Vacuum	-	34-38

Table 3 Shelf-life of fresh fishery products packaged under MA, vacuum or air (continued)

Type of fishery product*	Storage temperature (°C)	Atmosphere $CO_2 : N_2 : O_2$	G/ P ratio	Shelf-life (days)
Salmon (ns) steaks	0	60 : 40 : 0	3	12.9
	5	60 : 40 : 0	3	7.1
	10	60 : 40 : 0	3	3.4
	0	0	-	11.8
	5	Vacuum	-	8
	10	Air	-	3
Salmon (*S. salar*) fillets	2	60 : 40 : 0	1	17
	2	40 : 60 : 0	1	17
	2	Vacuum	-	17
	2	Air	-	11
Sardines (*Sardinops melanostictus*)	5	80 : 20 : 0	ns	13
	5	20 : 80 : 0	ns	6
	5	Air	ns	
Shrimp, spotted (*Pandalus platyceros*)	0	CA 100 : 0 : 0	ns	4
Whole Head on/off	0	Air	ns	4
	0	Air	ns	2
Snapper (*Chrysophrys auratus*) fillets	3	100 : 0 : 0	ns	6-8
	3	Vacuum	-	3
	3	Vacuum	-	6
	3	Air	-	3
Snapper (*C. auratus*) fillets	1	40 : 60 : 0	5	9
	1	Air	ns	9
	1	100 : 0 : 0	ns	18
	12	Vacuum	-	10
	12	0 : 100 : 0	Ns	13
	12	100 : 0 : 0	Ns	11
	8	Air	ns	6

Table 3 Shelf-life of fresh fishery products packaged under MA, vacuum or air (continued)

Type of fishery product*	Storage temperature (°C)	Atmosphere $CO_2 : N_2 : O_2$	G/ P ratio	Shelf-life (days)
	8	Vacuum	-	16
	8	0 : 100 : 0	ns	17
	8	100 : 0 : 0	ns	23
	8	90 : 8 : 2	ns	17
	8	65: 31 : 4	ns	16
	4	100 : 0 : 0	ns	40-53
Cod, blue (*Arapercis colias*) fillets	3	100 : 0 : 0	2	49
commercially smoked	3	Vacuum	2	14
	3	Air	2	14
	1.5	100 : 0 : 0	2	113
	1.5	Vacuum	2	35
	1.5	Air	2	28
Crayfish (*Pacifastacus leniusxulus*)	4	80 : 20 Air	ns	21
whole cooked	4	Air	ns	14
Flounder (*Limanda ferrugina*) fillets	26	Air	ns	2
	26	Vacuum	-	2
	26	0 : 100 : 0	ns	4
	26	100 : 0 : 0	ns	1
	12	Air	ns	5
	12	Vacuum	-	8
	12	0 : 100 : 0	ns	7
	12	100 : 0 : 0	ns	8
	8	Air	ns	5
	8	Vacuum	-	7
	8	0 : 100 : 0	ns	4

Table 3 Shelf-life of fresh fishery products packaged under MA, vacuum or air (continued)

Type of fishery product*	Storage temperature (°C)	Atmosphere $CO_2 : N_2 : O_2$	G/ P ratio	Shelf-life (days)
Haddock(*Melanogrammus aeglefinus*)	8	100 : 0 : 0	ns	10
whole	0	40 : 30 : 30	ns	10
	0	Air	ns	8
	5	40 : 30 : 30	ns	7
	5	Air	ns	7
	10	40 : 30 : 30	ns	4
	10	Air	ns	4
Haddock (*M. aeglefinus*) fillets	0	60 : 20 : 20	ns	14
	0	Air	ns	10
Hack (*Merluccius merluccius*) slices	2	50 : 45 : 5	2	14
	2	50 : 45 : 5	2	16
	2	Air	2	7-8
Herring, Baltic (ns) fillets	2	20 : 80 : 0	0.4	3
	2	20 : 80 : 0	1	3
	2	40 : 60 : 0	0.4	6
	2	40 : 60 : 0	1	8
	2	Vacuum	-	3
Herring (*Clupea harengus*) fillets	0	60 : 40 : 0	ns	14
	0	Air	ns	12

Source: Adapted from Sivertsvik *et al*. (2002)

ns = not stated in article.

G/P ratio = Volume of gas to volume of product (assuming density~1 kg L^{-1} for fish)

Nevertheless, MAP can affect the quality of the product, mainly owing to CO_2 dissolved in the muscle tissue, which is associated with increased carbonic acid (Pastoriza *et al*.,

1996b; Ashie *et al*, 1996). A greater loss of water-holding capacity of muscle protein occurs at lower pH values. Masniyom *et al*. (2002) reported that sea bass slices packaged in MAP had a high amount of exudate, especially when the storage time increased. Therefore, treatment of pyrophosphate (PP) prior to keeping the sea bass slices in MAP effectively retarded the loss of exudates and improved the physicochemical properties of the slices during storage at 4°C (Masniyom *et al*., 2004). Pastoriza *et al.* (1998) studied the effect of a mixture of gases (50% CO_2/45% N_2/5% O_2) combined with the sprinkling of sodium chloride on hake (*Merluccius merluccius*) slices. The sprinkling of sodium chloride showed better biochemical and microbiological inhibition, and decreased the sensory deterioration of MA packed hake slices. Exudation was also reduced and the shelf-life of the hake slices increased to 2 and 8 days, when packed under MA without and with sodium chloride solution treatment, respectively (Pastoriza *et al.*, 1998).

The use of high hygienic-sanitary quality raw materials represents an important factor for the successful use of modified atmosphere packaging. Besides initially using high quality raw materials, the use of good hygiene practices during fishing, the selection of the right packaging material and a good temperature control are also necessary (Stammen *et al.*, 1990).

5. Use of MAP for shelf-life extension of shrimps

Preservation of fresh shrimp have been carried out by chilling in ice (Shamshad *et al*., 1990 and Lakshmanan *et al*., 2002), in liquid ice (Huidobro *et al.*, 2002), modified ice chilling (Jiang and Lee, 1988), superchilling at 0°C ~ -4°C (Fatima *et al*., 1988), modified atmospheres packaging (Bak *et al*., 1999; Lopez-Caballero *et al*., 2000). Lannelongue *et al*. (1982), Layrisse and Matches (1984) reported that a shelf-life extension of shrimp was achieved using modified atmospheres containing different concentrations of CO_2 and O_2. Chen *et al.* (1992, 1993) observed the inhibition of PPO activity by CO_2. Similarly, a delay in nucleotide degradation during modified atmosphere storage was reported for Florida spiny lobster (*Panulirus argus*) (Chen *et al.*, 1993). Cann (1988) reported that crustacean and shellfish could be kept up to 30% longer at 0°C in MA (80% O_2/ 20% N_2) than the other types of packaging, and the onset of blackspot in shell on products is delayed. Some significant works dealing with the uses of MAP in shrimp have been reported: boiled whole and frozen shrimp under N_2 at -17°C (Bak *et al*.,

1999); cooked peeled and brined shrimps under 20% CO_2/ 80% N_2 (Dalgaard and Jorgensen, 2000); tail shrimps under 35-100% CO_2 in combination with 34-64% O_2 or 35-65% N_2 stored at 4°C (Lannelongue *et al*., 1982); head-on and head-off shrimps under 100% CO_2 modified atmospheres at 0±2°C (Layrisse and Matches, 1984). López-Caballero *et al*. (2002) studied the effect of two gas mixtures, involving 40% CO_2/ 30% O_2/ 30% N_2 and 45% CO_2 /5% O_2/ 50% N_2 on the quality of shrimp (*Parapenaeus longirostris*), MAP delayed the microbial growth compared to air-packed or iced stored shrimp. Furthermore, the formation of trimethylamine and total volatile nitrogen was retarded. However, the production of some biogenic amines seemed to be enhanced during the storage of MAP-shrimp**.**

The application of melanosis inhibitors in combination with MAP was also studied. Deepwater pink shrimp (*Parapenaeus longirostris*) kept under MAP (40% CO_2/ 30% O_2/ 30% N_2 and 45% CO_2 /5% O_2/ 50% N_2) in combination with sulfites (10 g/ 1 kg) had the shelf-life up to 9 days, compared with 4-7 days for sample stored in ice (Goncalves *et al*., 2003). Moreover, Martiez-Álvarez *et al*. (2004) reported that pink shrimp (*Parapenaeus longirostris*) treated with 4% sulfites in combination with a controlled atmosphere (53% CO_2 and 7% O_2) inhibited melanosis for 14 days, while melanosis was detected after 6 days in shrimp stored in ice. Shrimp (*Parapenaeus longirostris*) treated with 0.1% 4-hexylresorcinol and kept in a controlled atmosphere (53% CO_2 and 7% O_2) showed less melanosis than shrimp treated with 4-hexylresorcinol and kept in ice (Martiez-Álvarez *et al*. 2004). Nevertheless, McEvily *et al*. 1991; Otwell *et al*., 1992) found that the effectiveness varies, depending on the physiological condition and season of capture of the specimen, the type of crustacean and the way in which the melanosis inhibitor is applied.

Polyphosphates are used to increase water holding capacity, reduce drip and retard oxidative rancidity in fish product (Lin and Chuang, 2001). Though the shelf-life of shrimp can be extended by MAP, particularly under the high content of CO_2, drip is mostly produced. This problem causes unacceptability of shrimp. Therefore, a combination of phosphate and MAP would provide an effect means to improve the quality of shrimp. Phosphates are also used for improving the microbiological quality of muscle foods (Kim and Hearnsberger, 1994). Phosphates act as growth inhibitors of certain food spoilage microorganism, owing to their ability to chelate calcium, magnesium and iron needed for microbial growth (Dziezak, 1990). Kim *et al*.

(1995) reported that the combined monopotassium phosphate and sodium acetate effectively inhibited the growth of aerobic microorganism in catfish fillets during storage at $4^{\circ}C$. A combination of sodium acetate and monopotassium phosphate prolonged the shelf-life of catfish to 12 days at $4^{\circ}C$ by keeping counts below 10^{7} CFU/g. Possible method of combining preservatives with MAP is by dipping or soaking products in phosphate solution prior to the application of MAP. This method is particularly suitable for meats and seafood. Masniyom *et al*. (2005) treated seabass slices with 2% (w/v) pyrophosphate prior to MAP and found that the samples had the marked decrease in exudate. Pyrophosphate also exhibited the synergistic effect on the inhibition of some microorganisms.

Objectives

1. To characterize proteases, collagenase and polyphenol oxidase from black tiger shrimp and white shrimp
2. To study the effect of modified atmosphere packaging (MAP) with different conditions on quality of black tiger shrimp and white shrimp pretreated with and without melanosis inhibitors during storage at $4^{\circ}C$
3. To investigate the quality improvement of white shrimp stored under MAP using phosphate in combination with melanosis inhibitors during storage at $4^{\circ}C$