Ph.D. Thesis

EFFECT OF BLEEDING ON THE QUALITY OF AMBERJACK (*Seriola dumerili*) AND RED SEA BREAM (*Pagrus major*) MUSCLE TISSUES DURING ICED STORAGE AND DETECTION OF CATHEPSIN L IN RED CELL MEMBRANES OF FISH BLOOD

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Dedication

This work is dedicated to my wife, Carol.

For the inspiration, patience, family care, commitment, sacrifice and, the gifts of love and life. For all the things I never get to thank you. I owe you and the kids.

"A hundred times everyday, I remind myself that my inner and outer life is based on the labour of others". Albert Einsten (1879-1955).

List of abbreviations

$AgNO_3$:	Silver nitrate
AMP:	Adenosine monophosphate
ATP:	Adenosine triphosphate
$CaCl_2$:	Calcium chloride
CBB:	Coomassie brilliant blue
$CdCl_2$:	Cadmium chloride
$COCl_2$:	Carbon oxychloride
$CuCl_2$:	Copper (II) chloride
DMA:	Dimethylamine
DTNB:	5, 5'-dithiobis-(2-nitrobenzoic acid)
EDTA:	Ethylene diamine tetraacetic acid
$FeCl_3$:	Iron (III) chloride
$HgCl_2$:	Mercury (II) chloride
HPLC:	High performance liquid chromatography
Hx:	Hypoxanthine
HxR:	Inosine
IMP:	Inosine monophosphate
KCl:	Potassium chloride
K_2CO_3:	Potassium carbonate
$MgSO_4$:	Magnesium sulphate
*p*CMB:	*p*-Chloromercuribenzoate
PMSF:	Phenylmethylsulfonyl fluoride
SDS-PAGE:	Sodium dodecyl sulphate polyacrylamide gel

	electrophoresis
$SnCl_2$:	Tin (II) chloride
TLCK:	Tosyl-L-lysine chloromethyl ketone
TMA:	Trimethylamine
TMAO:	Trimethylamine oxide
VBN:	Volatile basic nitrogen.
$Zn(CH_3COO)_2$:	Zinc acetate
Z-Arg-Arg-MCA:	Benzyloxycarbonyl-L-arginyl-L-arginyl-7-amino-4-methylcoumarine
Z-Phe-Arg-MCA:	Benzyloxycarbonyl- L-phenylalanyl- L-arginine 4-methylcoumaryl-7- amide

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Chapter I. Introduction

The global increase in human population creates demand for more food and other necessary resources to sustain life. For ages, fish has been used for food mainly as an important source of animal protein and an element of trade. Recognition of the importance of the fisheries sector in providing food security, employment, foreign exchange balance, growth domestic product and poverty reduction, is the first step in appreciating the growing interest in research and sustainability of the resource.

Improvements in technology and communication, and the sustained demand for fish have shifted the sector from traditional subsistence fishing to full-scale industrial processing and export marketing. As a result, global employment in the sector is estimated to be 35 million people (FAO, 2009) and collected revenues, USD 55.2 million in fisheries export earnings. Further estimates show that 75% of the total global fish production was used for direct human consumption. Global per capita fish consumption increased from 9 Kg in 1961 to 16.7 Kg in 2006. Total consumption rose from 13 million to 27 million metric tonnes of fish during the same period. The contribution of fish to the total animal protein also rose from 13.7% in 1961 to 15% in 2006.

The increasing demand for fish and fishery products as an alternative source of animal protein can, in part, be explained by its

nutritional value. Fish is known to contain all the essential amino acids and is only comparable to milk and eggs. In humans, essential amino acids cannot be manufactured by the body and must be supplied in the diet. Their absence in the diet may result into deficiency syndromes. Fish contains the essential amino acids histidine, isoleucine, leucin, lysine, methionine (cystine), phenylanine (tyrosine), threonine, tryptophan and valine (Huss, 1993; Shang-gui *et al.*, 2004). Presence of varying amounts of minerals and vitamins required by the human body has been reported in fish. They include sodium, potassium, calcium, magnesium, phosphorus and vitamins A, B_1 (thiamine), B_2 (riboflavin), B_6, D, niacin and pantothenic acid (Murray & Butt, 1969). Their presence in the diet and thus human body facilitates proper growth, defence against disease and proper functioning of body systems.

With increasing demand for fish, there is pressure on the resource and cases of over-exploitation and depletion of some fish species have been witnessed world wide. There is need to close the gap between supply and demand while ensuring sustainability of the fisheries resources. Aquaculture research and production as an option to capture fisheries yields an effective alternative source of food for the home and market. Different fish species can now be effectively cultured in both marine and fresh water environments. In 2006, total aquaculture production was 51.7 million tonnes of fish, representing 35.7% of the total global fish production (FAO, 2009). While ensuring total control over resources and ready-to-harvest food, aquaculture

also limits the uncertainties, risks and dangers associated with fishing in deep waters, rough seas and oceans. There is increased safety for both humans and fishing equipment, and a reduction in bycatches.

Because of the perishable nature of fish and fishery products, not all the fish that is harvested reaches the dinner table. A big percentage of the fish is ruined or lost along the production chain. Global post-harvest fish losses are estimated at 10% (Esser, 1991) and 25% (FAO, 1996) of the total catch. In Africa, owing to inadequate or lack of appropriate infrastructure for transport and preservation, inadequate or relevant technologies and high tropical temperatures, such losses have been estimated at 20-25% and sometimes can be as high as 50% (Ward, 1998). Post-harvest losses reduce the amount of fish available for food and in the markets, while the price for available fish becomes unaffordable by the poor (Johnson & Esser, 2000). Much of the fish goes for the export markets where it fetches premium prices. The local population has to do with poor and low quality fish sometimes rejected by the processing industries. Quite often the reasons for rejecting such fish are related to size, wholesomeness, freshness and quality. Such fish may find other uses for example in the feed production industry but the prices are comparatively much lower. This translates into an economic loss to the fisherman.

The high nutritional value of fish is a safety guarantee against malnutrition and associated illnesses. In 1997-1999 an estimated 815 million people were reported to be malnourished world wide (FAO, 2002). Preservation, prevention of wastage and proper utilization of

the fish therefore while ensuring adequate food and reliable nutrients would improve health and lives of millions of people. This is in line with the objectives of the code of conduct for responsible fisheries (FAO, 1998). The need to develop technologies for preservation and conservation of fish and fishery products is thus a means to ensure food security while limiting financial and other losses that may be associated with post-harvest food losses. A strong and healthy population is a requisite for economic growth and development.

Various traditional methods of smoking, drying (by the sun or dehydrator) salting and more improved methods of canning, use of chemical preservatives, ionizing radiation, refrigeration and modified vacuum packaging have been used to preserve fish and reduce wastage. However, most of the preservation methods are limited to industrial level use that requires experience and technical know-how. The cost of setting up facilities for such preservation is sometimes so prohibitive that the facilities are not readily available to the common fisherman and fish consumers.

The high demand for fresh fish is built on the consumer perception that something new and fresh is always better. Fresh fish remains the most highly priced product form and while further processing involves additional costs, it does not result into increased product prices or profits. Freshness is often associated with food quality, safety and wholesomeness. In fish, it is closely linked with a natural fish muscle flavour, pleasant fish odour, colour and texture.

Consumer determination of freshness and quality in the open market is mainly based on sensory aspects of the fish product. In the open market, it is not uncommon to see consumers checking the odour or texture of a food product by sniffing at it and poking it with a finger. After capture and death, fish will lose its natural characteristics throughout the handling, processing, marketing and storage processes. Fresh fish is most prone to spoilage and the resulting degradation changes its sensory properties as a food product rendering it unsuitable for human consumption. The smell from spoiled fish is often very repulsive and eating such a product may cause stomach upsets and sickness.

The fish muscle consists of about 16-21% proteins and 0.2-25% lipids (Borresen, 1995a) which are important elements with regard to freshness and quality when the fish dies. The proteins consist of myofibrillar proteins including actin and myosin and water-soluble proteins that include enzymes which differ with species. About two thirds of the total proteins are essential in swimming and movement of the fish and these include actin and myosin. The fish muscle consists of the dark and ordinary muscles and their proportions vary with fish species relative to swimming activity. Actively swimming fish have a higher content of the dark muscle compared to less active fish (Love, 1974). Degradation of these muscle proteins, lipid oxidation as well as the relative proportions of the muscle types are important issues in post-mortem fish freshness and quality.

Nielsen (1995) reviewed sensory changes that take place after the fish dies which mainly affect appearance and texture. Hamada-Sato *et al.*, (2005) gave an account of the changes that occur in the muscles after a fish dies. All fish, after death, go through various changes that can be summarized as follows;

Catching → Rigor mortis →Dissolution of rigor → Autolysis → Spoilage.

Lack of oxygen circulation to the tissues after the fish dies sets into motion anaerobic glycolysis with a resultant accumulation of lactic acid in the muscle. Consequently, the pH drops from neutral pH 7.0 to around 5.0 which activates ATP-ase and the degradation of adenosine triphosphate (ATP) in the muscle (Fig. 1). However this drop in pH triggers an inhibitory feedback mechanism to the enzymes involved in anaerobic glycolysis and no more lactic acid is formed.

Rigor mortis has been directly evaluated based on changes in the physical or mechanical properties such as rigidity or rigor index (Bito *et al.*, 1984), shear strength (Montero & Boderias, 1990) or isometric muscle strength (Nakayama *et al.*, 1992). Indirect methods include measurement of pH and products of autolysis during rigor mortis as it is associated with the breakdown of ATP. The ATP in the fish muscle serves as a source of high energy required for muscle contraction in the living fish and as a muscle plasticizer (Huss, 1995). During rigor mortis, the ATP depots will have been exhausted and so the muscles cannot contract. The muscles shrink, fish becomes very stiff and difficult to manipulate. Processing of the fish in rigor is quite

difficult and may lead to physical and quality losses of the raw material.

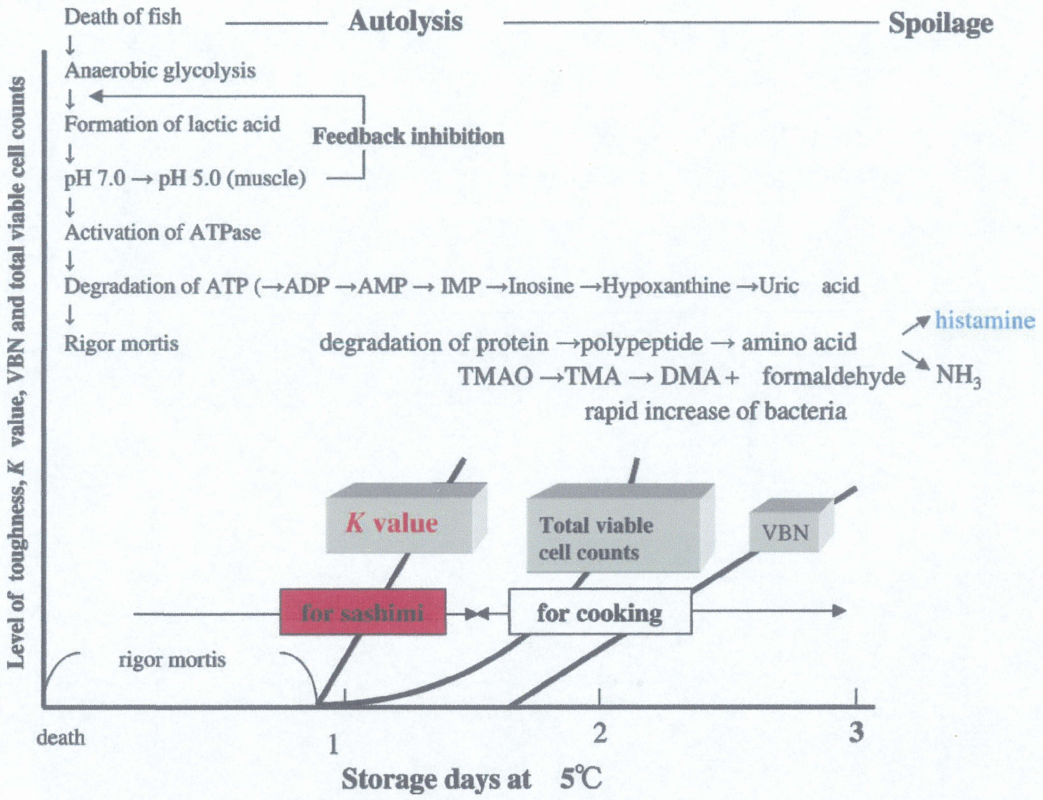

Fig. 1. Changes in the fish muscle after death (Watanabe, 1983, 1993).

The decomposition of ATP begins immediately after the fish dies but its content is kept constant due to synthesis from creatine phosphate and glycogen. Creatine phosphate, a fast energy source, may be immediately depleted by the handling process during harvesting or slaughter. Glycogen stores will also be depleted shortly afterwards, at a rate dependent on temperature, and the synthesis of ATP will stop. This, results into a decrease in ATP content and when the level reaches 1-2 μmol/g of tissue, the fish muscle enters into a state of rigor mortis (Iwamoto *et al.*, 1988). Dessolution of rigor mortis may be due to weakening of Z-disks of myofibrils (Seki & Tsuchiya,

1991), a degradation of the connective tissue (Seki & Watanabe, 1984; Ando *et al.*, 1993) or a weakening of the myosin-actin junction (Yamanoue & Takahashi, 1988). Dissolution of rigor allows for easy handling and processing of the fish raw material.

Even when ATP is degraded, the fish may still contain some glycogen depots. Cappeln *et al.*, (1999) observed synthesis of ATP in fish stored at 0°C whose source could be such retained glycogen depots. Rested fish have creatine phosphate at the time of death and may still be able to process ATP while stressed fish are likely to process their ATP from possible degradation of glycogen during storage (Nowlan & Dyer, 1969). In the fish processing industry, it is common practice to rest the fish in holding tanks before slaughter. When effectively done to allow rest without congestion, it might be an effective way to boost creatine phosphate levels and therefore production of ATP. When creatine and ATP are at the same concentration, ATP begins to decrease (Watabe *et al.*, 1991).

Fig. 2. shows the process of ATP and related compounds degradation and the various enzymes that facilitate the breakdown. ATPase is part of the myofibrillar fraction of the fish muscle proteins (Borresen, 1995b) and is responsible for ATP breakdown activated by calcium. Reduction of free muscle ATP results into interaction between actin and myosin thereby shortening the muscle to make it stiff and inextensible.

Without ATP, the cell cannot pump out Ca^{2+} or sequester it to the mitochondria or sarcoplasmic reticulum. The Ca^{2+} concentration

in the aqueous phase in the cell increases and activates many processes. Ca^{2+} can directly bind to, and activate many hydrolytic enzymes such as phospholipases, lipases and proteinases. The Ca^{2+} activated proteinases may be responsible for some of the tenderizing effects in postmortem muscle tissue. Ca^{2+} bind to proteins that activate enzymes, bind to enzymes such as proteases and activates them, binds the membranes and modifies surface properties, may weaken Z-disks and solubilise contractile proteins (Okuma *et al.*, 2002).

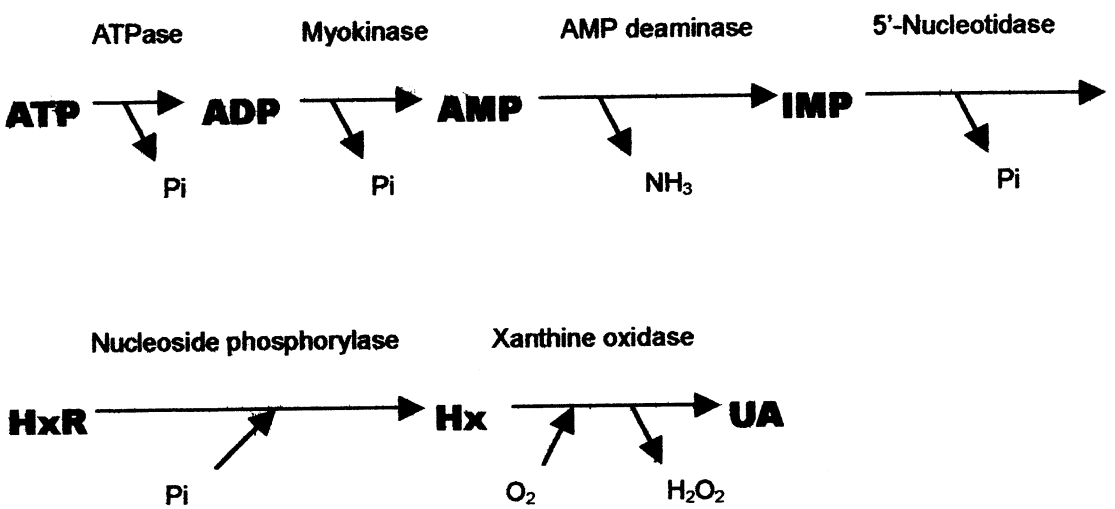

Fig. 2. Adenosine triphosphate degradation and the enzymes involved (Okuma & Watanabe, 2002).

Loss of ATP occurs when the tissue turns anaerobic preventing regeneration of ATP by mitochondria or when the pH drops or glycogen reserves are depleted preventing ATP regeneration by glycolysis. With the loss of ATP, membranes may undergo a number of

changes which affect their functioning. ATP provides the chemical energy for repairs when there is damage; without which energy the damage is irreversible. Damaging effects could include hydrolysis of phospholipids and proteins (Okuma & Watanabe, 2002). This in turn affects enzymatic reactions and modifies permeability properties of the membranes.

A biological system exists in some species of fish that produce DMA and formaldehyde from TMAO. Toughening of fish muscle during storage may be due to cross-linking of myofibrillar proteins from the formaldehyde produced. The changes in fish muscle contractible proteins by formaldehyde represent a potential cause for chemical modification of proteins leading to deterioration. The changes reflected in Fig. 1 and 2 have been correlated to eating quality of the fish as perceived through the senses in terms of appearance, odour, texture and taste. Changes involve a shift from fish with very fresh, sweet, sea-weedy and delicate taste; loss of characteristic odour and taste with a pleasant texture and no off-flavours; development of unpleasant-smelling substances and off-flavours with a soft and water or tough and dry texture before the fish becomes spoiled and putrid (Huss, 1995).

Fig. 3 shows the different phases, during iced storage, as determined from the eating quality index in cod, and the associated changes. It is clearly understood that as the storage time increases, eating quality deteriorates due to enzymes, autolysis and bacteria.

The changes associated with the different phases reflected in Fig. 3 can be used as a basis for the determination and estimation of fish freshness and quality.

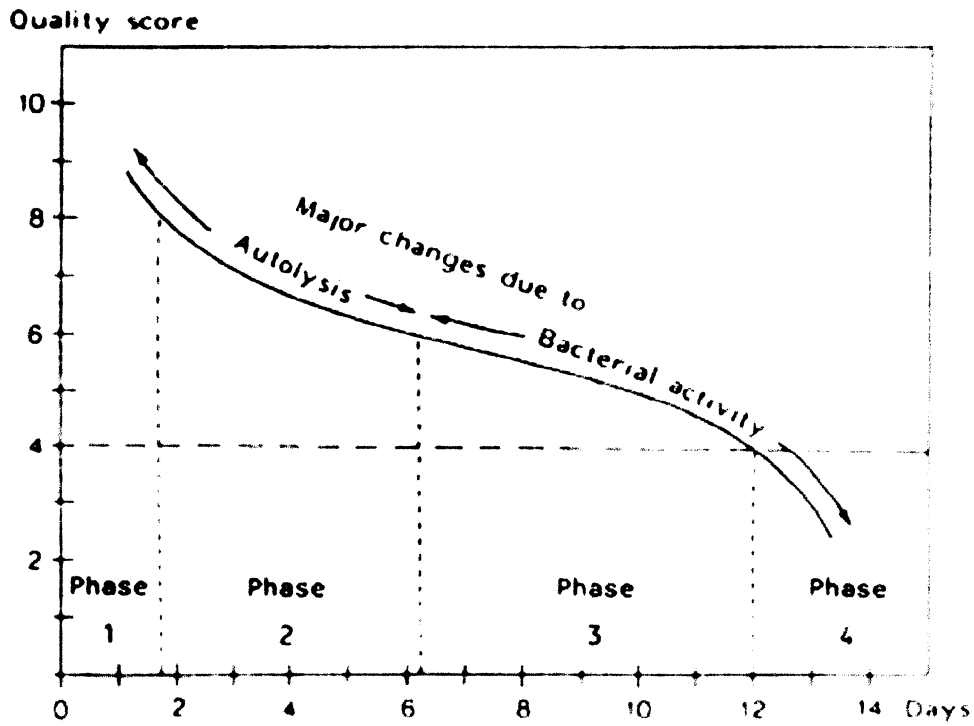

Fig. 3 Changes in eating quality of iced (0°C) cod (Huss, 1976).

Saito *et al.*, (1959), proposed the use of the *K*-value to evaluate freshness in fish defined as below:

$$K\% = \frac{[HxR] + [Hx]}{[ATP] + [ADP] + [AMP] + [IMP] + [HxR] + [Hx]} \times 100$$

The *K*-value has since attracted great attention and is widely used as an indicator of freshness (Okuma & Watanabe, 2002; Hamada-Sato *et al.*, 2005).

ATP degradation into adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (HxR) and hypoxanthine (Hx) in the fish muscle has been widely studied (Karube *et al*, 1984, Okuma *et al.*, 1992). ATP, ADP and AMP are rapidly degraded resulting into accumulation of HxR and Hx (Okuma & Watanabe, 2002). While IMP is reportedly known for the pleasant fresh flavour of meat (Howgate, 2005), Hx is responsible for the loss of flavour and creation of bitter off-tastes (Fletcher & Statham, 1988). Post-mortem accumulation of HxR or Hx reflects poor quality while a high level of the adenosine related compounds or IMP reflects better quality in the fish muscle (Manju *et al.*, 2007). The relative concentration of IMP, Hx and HxR will, in part, determine the quality of the fish muscle and their change with time will be reflected as well.

Microbial growth and metabolism may result into formation of amines, sulphides, alcohols, aldehydes, ketones and organic acids. These have unpleasant and unacceptable off-flavours and are a major cause of food spoilage (Gram, 1992; Dalgaard, 1995; Saloua *et al.*, 1996; Huss, 1988). Fermentation of carbohydrates and protein digestion by bacteria may result into production of foul-smelling products and putrefaction. Bacteria are responsible for the production of most of the volatile chemical compounds with unacceptable odours which develop in naturally spoiling fish (Shewan, 1962).

Low molecular weight water soluble nitrogen-containing compounds that are part of the non-protein-nitrogen fraction provide substrates for bacteria when fish loses immunity after death (Gram & Huss 1996; Huss, 1995). For example TMAO, a water-soluble nitrogen-containing compound of low molecular weight (Gram & Huss, 1996) is reduced by the spoilage bacteria to TMA during iced fish storage (Jorgensen *et al.*, 1997).

High concentrations of TMAO have been reported in marine fish (Oetjen and Karl, 1999) and some freshwater fish particularly Nile perch and Nile tilapia (Gram *et al.*, 1989) with 1-5% (dry weight) of TMAO reported in the fish muscle tissue (Anderson & Fellers, 1952). TMAO is an electron receptor in anaerobic respiration to produce TMA (Malle *et al.*, 1986) when specific spoilage bacteria utilize the Kreb's cycle (Huss, 1993). The specific bacteria responsible for TMAO reduction include *Vibrio* spp., *Alteromonas* spp., *P. putrefaciens*, aeromonads, *enterobacteriaceae*, *P. phosphoreum* and *S. putrefaciens*.

TMA is characterized by a fishy smell and in some species constitutes most of the total volatile base nitrogen (VBN) until spoilage. The VBN fraction includes ammonia, monoethylamine, DMA and TMA (Huss, 1995). VBN increases with increasing TMA content in the fish muscle and have been both used as spoilage indicators. The physical and chemical conditions of the fish may precipitate cellular enzyme activity. These may act independently or in association with bacterial degradation of the fish products.

Fish spoilage is a progressive proteolysis process of muscle tissue due to bacteria, chemical or enzymes as well as natural autolysis processes and the cause may vary in fresh/chilled and frozen products (Gill, 1992). Spoilage may range from practically normal to a condition of putrefaction. This will be in relation to the changes in composition and quantity of both degrading agents and the resultant products that are initiated immediately after fish death.

It is understood that the interaction between the components of the spoilage microflora, storage environment and physico-chemical properties of the fish will determine the extent of spoilage. The poikilothermic nature of fish, a high post-mortem pH of its flesh, large amounts of non-protein nitrogen coupled with specific and non-specific contamination favour fish spoilage. Lack of process control, poor conditions of hygiene, inadequate or lack of preservation facilities technology, high ambient temperatures and poor product handling may lead to product spoilage.

Exposure of fish and fishery products to high or different temperatures from the time of fish capture through distribution to the last consumer will result changes in freshness and quality of the product. Fish freshness and quality are known to be dependent on storage temperature and time. Storage of fish at higher temperatures usually results into a higher rate of freshness loss and spoilage than at lower temperatures. Where there has been no form of processing, frozen fish will last longer than ice-stored fish that lasts longer than fish stored without ice. After capture and death, the fish may not be

immediately iced especially in small-scale fisheries in developing countries. Reasons for delayed or lack of icing include lack of appropriate facilities for the production of ice, poor fish and ice holding equipment on board, location or remoteness of the fishing grounds and intended use of the fish captured.

I – 1. Fish killing methods and bleeding

Different methods have been used in the industry to kill fish after harvesting and different researchers have studied the effectiveness of the methods and effect on muscle quality. Air asphyxiation, ice asphyxia, percussive stunning, carbondioxide stunning, nitrogen stunning and severing vital organs and vessels have been used as fish killing methods. Willis *et al.*, (2006) studied and compared the effect of nitrogen stunning, air asphyxiation and percussive stunning in the rainbow trout. Roth *et al.*, (2002) studied the effect of carbondioxide stunning in Atlantic salmon. Acerete *et al.*, (2009) studied ice asphyxia and carbondioxide narcosis in the European seabass. Roth *et al.*, (2007) studied use of percussive blows to the head, electrical stunning and live exsanguinations with ice in turbot. In most of these studies, the fish were not bled before storage experiments.

Terayama & Yamanaka (2000) studied the effect of bleeding on skipjack quality. After 36 hours of iced storage, bled fish had high pH values, low metmyoglobin ratio and a higher a* value measured by

colour difference meter. Organoleptic assessment by consumers was high for bled samples based on colour and smell of the fish compared to the control.

Terayama *et al.*, (2002) studied the effect of instant killing and bleeding machine on skipjack and amberjack. They reported a high breaking force in the bled skipjack muscle compared to the control and in the bled amberjack muscle, until 24 hours-storage. Organoleptic assessment favoured bled samples compared to the control. It was concluded that sashimi firmness in spindle-formed fish decreases more slowly in bled fish than in the control.

Ando *et al.*, (1999) studied bleeding and postmortem fish muscle softening in the horse mackerel, yellow tail and stripped jack under chilled storage conditions over a 72 hours period. They reported a delay of the degradation of pericellular collagen fibres in bled yellow tail and horse mackerel as determined by transmission electron microscopy. A compression test showed slower weakening of the pericellular connective tissue. They concluded that bleeding may delay the degradation of collagen fibres and softening of the muscle in pelagic fish.

Mochizuki *et al.*, (1998) studied effects of bleeding on post-mortem changes in the muscle of horse mackerel during iced storage up to 7 days. They reported a slow rate of rigor mortis in bled samples compared to the control. Significant differences in rates of breaking strength and concentrations of ATP and creatine phosphate were observed 36 hours post-mortem. The *K*-value was found to be lower in

the bled group compared to the control between 3 and 7 days-storage. Conclusion was made that bleeding delays the progress of rigor mortis.

Erikson *et al.*, (1999) studied the contribution of bleeding to total handling stress during slaughter of Atlantic salmon by measuring high energy phosphates, creatine phosphate and ATP content. They reported that high energy phosphate levels increased as a result of bleeding and that the onset of rigor mortis and rigor strength was related to initial stress level and rate of ATP depletion. Their study showed that ante-mortem handling stress results into depletion of high energy phosphates and glycogen which results into rigor mortis to occur earlier than in rested fish. Bleeding therefore had a significant effect on freshness.

Giuffrida *et al.*, (2007) studied the influence of slaughtering methods on quality in the gilthead sea bream and rainbow trout killed by simple anoxia, electrical stunning and bleeding. The fish killed by bleeding had ATP/IMP ratios higher than those killed by anoxia but lower than electrically stunned fish in the rainbow trout. The storage conditions equally had significant effects with ATP/IMP ratios being higher in the gilthead sea bream stored in ice slurry than those stored in carbondioxide. However, the analysis was done for 48 hours and 72 hours for slaughter method effect and storage conditions respectively, not long enough to study the changes over a prolonged storage time period.

Mishima *et al.*, (2005) studied the influence of storage temperatures and killing procedures on post-mortem changes in the

horse mackerel muscle stored at 0, 5 and 10°C. They reported that temporal changes in ATP, IMP and lactic acid content were slowest in fish killed by spinal cod destruction and bleeding compared to fish killed instantly, through struggling and temperature shock. Analysis of the *K*-value showed slow temporal changes at lower storage temperatures and increased faster at higher temperatures. The fish killed by destruction of the spinal cod also showed a slow decrease in breaking strength compared to other groups. They concluded that killing fish by destruction of the spinal cod is an effective way of delaying post-mortem change in the horse mackerel muscle.

Fish contains blood equivalent to 1.5-6% of the total body weight (Smith, 1966). Fish bleeding is the process of removal of blood from the fish body immediately after fish death. This can be done by cutting one or two sets of gill arches (Roth *et al.*, 2005; Doyle, 1995) thereby severing the aorta to allow blood draining. It can also be performed by cutting off the tail which severs the caudal vein (Connell, 1995). While the above processes allow draining of blood through its vessels, some blood residues will remain in the heart, liver and other internal organs. Gutting which involves removal of all internal body organs therefore allows more bleeding effect (Roth *et al.*, 2005) when used in combination with severing the blood vessels.

It is estimated that about one quarter of economic losses associated with downgrading of smoked salmon is due to small hemorrhages or blood spots in the fish flesh (Michie, 2001). Bleeding

eliminates frequent blood spotting observed in smoked fish products (Roth *et al.*, 2005).

The colour tone of fish flesh is dependent on the amount of haemoglobin content in the muscles (Richards & Hultin, 2002). Oxidation of lipid and heame pigments is the main cause of development of undesirable odour and unpleasant colour during ice storage. Research shows that bleeding eliminates most of the heamoglobin from the fish muscle tissues and may limit oxidation (Sakai *et al.*, 2006; Sohn *et al.*, 2007) while immediate bleeding of fish after death delays rigor mortis (Mochizuki *et al.*, 1998).

In Japan, fish without bleeding treatment is not suitable for use as sashimi because of the undesirable colour and odour. Residual blood in fish muscle is one of the factors that lead to development of undesirable discolouration of the flesh and unpleasant flavour during iced storage (Sohn *et al.*, 2007). Consumers use colour and odour when evaluating freshness and quality of fish muscle (Sohn *et al.*, 2007) and bled fish has been rated highly compared to the control (Terayama & Yamanaka, 2000).

I – 2. Determination of fish muscle quality

Because fish freshness is described by a variety of definite properties of the fish, it is equally assessed by various indicators reflective of the properties in question. These properties are dependent on the different biological and processing factors that influence

changes in physical, chemical, biochemical and microbiological aspects of the fish post-mortem (Bremner & Sakaguchi, 2000; Huss, 1995; Botta, 1995). It is important that a method to be useful, it should be rapid, widely accepted and comparable to latest evaluation methods. Assessment of fish freshness and quality can be achieved through a number of sensory, biochemical and chemical, physical and microbiological methods.

Sensory methods involve evaluation of appearance, colour, odour, flavour and texture using the human senses. However, sensory analysis is subjective as it might reflect personal preferences and is often not documented. Sensory analysis requires trained personnel thereby becoming expensive. In the fish industry, the quality index method (QIM), structured scaling, triangle tests, ranking and profiling supported by statistics have been used by trained assessors to provide objective assessment of freshness in fish.

Biochemical and chemical methods have evolved where objective criteria and standards are required for the assessment of fish products. Various methods have been employed in the fish industry and include measurement of VBN, ammonia, TMA, DMA, biogenic amines, nucleotide catabolites, ethanol and oxidative rancidity. While some of the measured attributes are directly associated with freshness, the sum total or interaction of the various compounds affect the overall quality of the fish product.

Physical methods include measurement of the electrical properties of the fish skin or tissue, pH, redox potential (Eh) and

texture. These factors have been integrated into the industry and relevant technologies developed for automated measurements. Examples include development of the Torrymeter freshness grader and pH meters.

Microbiological methods include the estimation of the total number of viable bacteria, spoilage bacteria and spoilage reactions. The number of bacteria or specific bacteria and their reactions vary at different stages of storage and have been shown to correlate well with sensory analysis as well as biochemical/chemical tests.

Fish freshness and quality have traditionally been discussed and estimated on the basis of spoilage of the raw material or the product (Sorensen, 1992). The changes that take place within the fish muscle form the basis for the determination of freshness and quality. The methods are based on autolytical changes, development of microbial growth, and lipid oxidation among others. The use of sensory analysis, ATP degradation products and the *K*-value, TMA, VBN have been popular as results tend to address the subjective criterion often used by the consumer in determination of product freshness and quality through the senses of smell, taste and touch.

Various methods have been used for the determination and analysis of ATP and its breakdown products. These include capillary electrophoresis (Nguyen *et al.*, 1990), phosphorus-nuclear magnetic resonance (Chiba *et al.*, 1991), ion exchange chromatography (Saito *et al.*, 1959), electronic biosensors and immobilized enzyme strips (Karube *et al.*, 1984, Olafsdottir *et al.*, 2004) among others.

Various analysis techniques and instrumentation have been developed for the rapid and objective analysis of fish muscle freshness and quality. Vapour phase Fourier transfer infrared spectroscopy has been used for the determination of TMA in fish and cephalopods (Armenta *et al.*, 2006). A flow injection analysis has been developed for the determination of TMA and VBN (Ruiz-Capillas *et al.*, 2000). A flow system for fish freshness determination based on double multi-enzyme reactor electrodes (Okuma & Watanabe, 2002) has been in operation. Paquit *et al.*, (2006) suggested a volatile amine sensor to assess fish product freshness using an immobilized chemo-reactive dye formulation and LED-based colorimetry. Hammund *et al.*, (2001) described the use of semiconducting metal-oxide chemo-sensitive sensors for TMA analysis.

Mitsubayashi *et al.*, (2004) presented a trimethylamine biosensor with flavin-containing monooxygenase type 3 (FMO3) for fish freshness analysis. They reported that experiments with horse-mackerel showed good reproducibility and suggested it is effective and convenient for the detection of TMA in fish.

Adhoum *et al.*, (2003) studied use of flow injection potentiometric detection of TMA in seafood using tungsten oxide electrode. They reported TMA recoveries of 99-100% for fish extracts and results correlated well with the existing classical official method. They suggested the method is robust and requires use of limited reagents while it permits reproducible and sensitive determination of TMA.

Dyer (1945) proposed spectrophotometric determination of TMA using picric acid. The method involves volatilization of TMA by an alkali from the acid fish extract into a toluene phase to which picric acid has been added. This results in the formation of the picrate salt of TMA and the ionization of which is spectrophotometrically measured. Over the years, the method has been modified to improve efficiency and is the basis of the classical official method, AOAC Official method 971.14.

While some of the methods involve measurement of total nucleotides or both TMA and VBN, others measure only one particular compound. Because VBN contains ammonia, TMA and DMA, it can be estimated by measuring the concentrations of the individual components and summing them up. However, amines comprising of VBN contain one atom of nitrogen per molecule and it has become conventional to express VBN and constituent amines and ammonia on a nitrogen basis such as total volatile basic nitrogen.

VBN has been traditionally analysed using the micro-diffusion method (Fig. 4) according to Conway (1947). It has been popular due to the simplicity of the analytical procedure and low level of laboratory and human resources required for the measurement. The apparatus required for analysis is inexpensive and complete recovery of VBN can be achieved within 2 hours at room temperature or 1 hour at 37°C. However, the procedure requires precision and accuracy in titrating very minute volumes of solutions using microburettes.

The principle involves distillation of an alkaline muscle extract, and estimation of the collected free bases using standardized acid or alkali. Fish muscle contains nitrogen-containing substances other than those contributing to intrinsic VBN in the sample. These substances can decompose during the analysis, depending on the conditions, producing ammonia which contributes to the measured VBN content. This may lead to overestimation of VBN content in the fish muscle.

Conditions of low alkalinity and low temperature during distillation can result in no or negligible decomposition of the nitrogen-containing substances but the time for analysis increases and makes the procedure complex. High temperature and strong alkalinity favours decomposition of the nitrogen-containing substances and results into over-estimation of VBN content although it allows for simpler equipment to be used and the analysis procedure is fast.

Conway's micro-diffusion method ensures distillation at low temperature and atmospheric pressure and the result gives the true intrinsic VBN content in the sample as there is no concomitant decomposition of nitrogenous substances under the conditions of measurement.

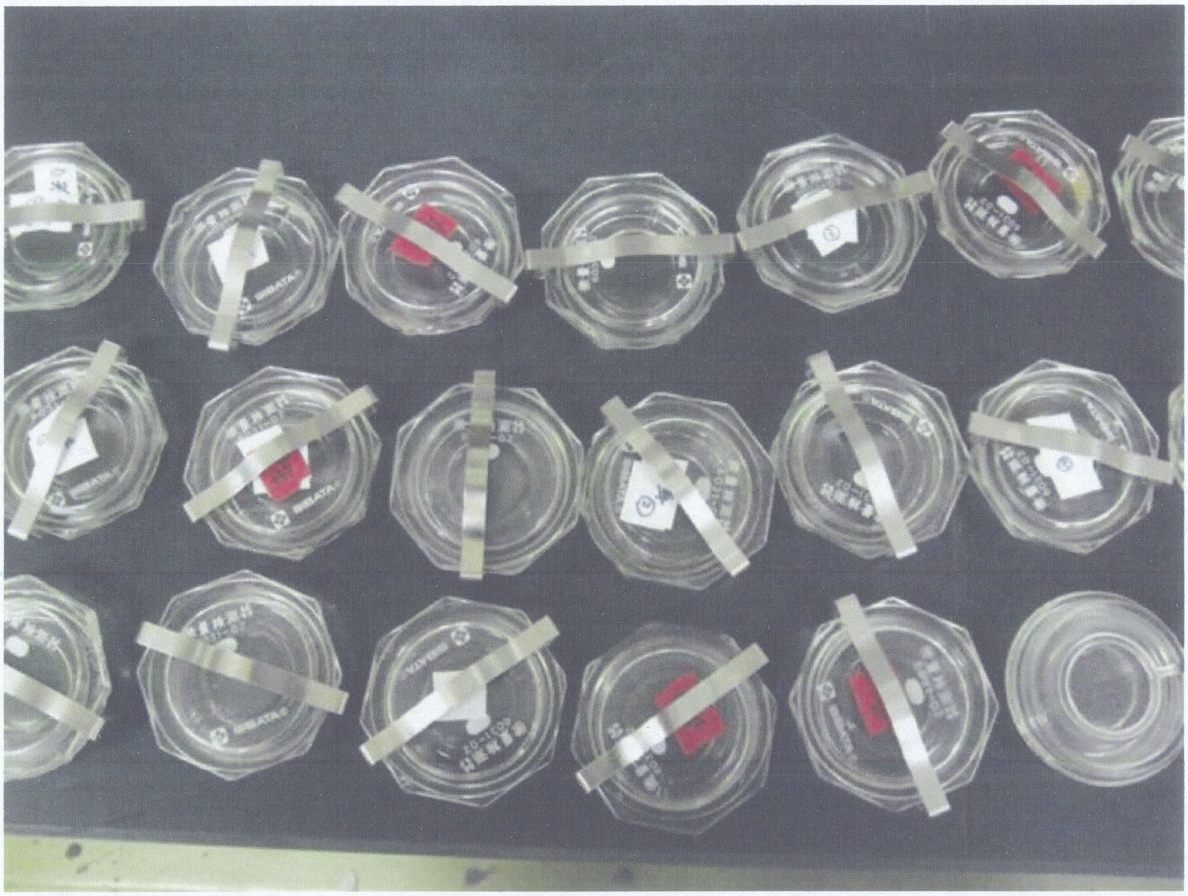

Fig. 4. The Conway micro-diffusion system for the analysis of VBN.

I –3. High performance liquid chromatography (HPLC)

In the case of related compounds like adenosine triphosphate degradation products and thus *K*-value measurements, measurement of only one compound at a time using a different methods is expensive, time consuming and prone to error. However, developments in technology have facilitated the analysis of the various compounds within a given sample in one complex analysis.

In the analysis of ATP and its degradation products, use of HPLC (Fig. 5) according to Ryder (1985) has resolved the need to analyse compounds separately therefore reducing errors and having results in one complex analytical procedure. Furthermore, the use of the same extract for several analyses results into saving sample quantity thereby reducing wastage.

The HPLC is the most commonly used tool for the analysis of nucleotides (Gill, 1992). This involves a separation technique that utilizes differences in distribution of compounds to a stationary and mobile phase. While the stationary phase designates a thin layer created on the surface of fine particles, the mobile phase designates the liquid flowing over the particles. Depending on solubility of the components in a sample in the phases and their molecular sizes, components move at different speeds over the stationary phase and are thereby separated from each other. The HPLC system uses

retention time as an index for qualitative determination and peak surface area for quantitative determination. Different compounds have different retention times depending on the column pressure, nature of the stationary phase, solvent composition and column temperature.

The retention time and concentration of the targeted compound are based on data obtained by analyzing a sample with known quantities of highly purified reference standards. The HPLC has been used to determine ATP and its breakdown products and the *K*-value in fish muscle.

Ryder (1985) studied the use of HPLC in the determination of ATP and its breakdown products in the fish muscle. He reported that ATP, ADP, AMP, IMP, HxR and Hx in neutralized perchloric acid extracts of fish muscle could be resolved in a single run by HPLC. The system showed recoveries of reference compounds added to the fish muscle perchloric acid extracts could be achieved to the level of 94-100%. Responses to repeated injections of mixed standards were highly reproducible and analysis time was reduced. It was determined to be an effective way of measuring the compounds and immediate calculation of the *K*-value.

Anderson & Murphy (1976) studied the use of HPLC with a micro-particulate reversed-phase column using phosphate buffers to separate compounds in biological extracts. Under isocratic conditions, all compounds could be eluted with reasonable resolution and retention time.

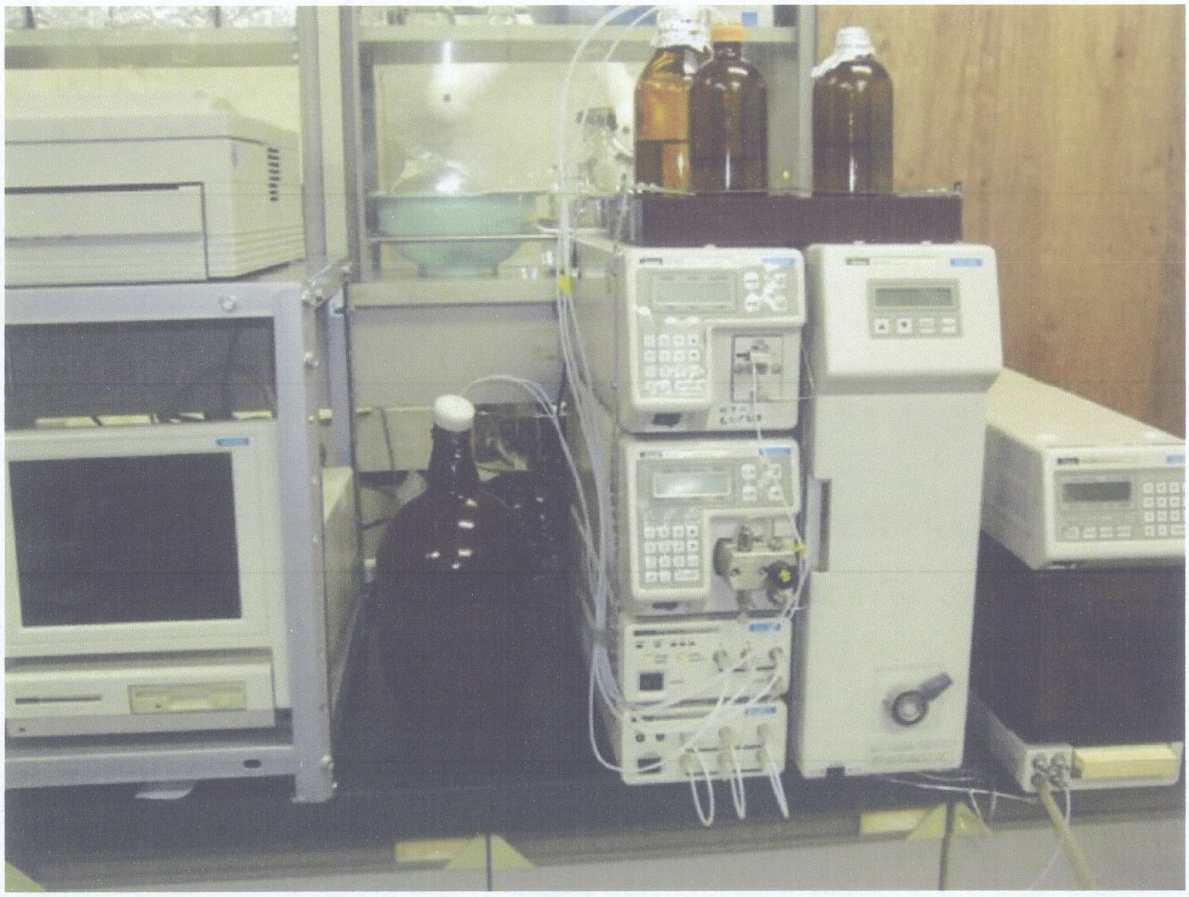

Fig. 5 The HPLC system for the analysis of ATP-related compounds.

Ozogul *et al.*, (2000) reported development of a modified HPLC method for the estimation of ATP and its related compounds that used phosphate buffers and acetonitrile in their study on herring. The gradient elution was reported to be expensive compared to isocratic systems but the system was found useful for the analysis of nucleotides. The system was found to have an advantage of reduced analysis time compared to other established systems.

The advantages of using the HPLC include its consistency, reliability and ability to analyse many samples.

I – 4. Cathepsin L and other cathepsins

In the fish muscle, the presence and role of endogenous enzymes, cathepsins (Aoki & Ueno, 1997; Aoki *et al.*, 1997; Aoki *et al.*, 2000; Ashie *et al.*, 1996; Huss, 1995), have been reported, and their post-mortem degradation of the fish muscle proteins has been studied. Cathepsins have been associated with post-mortem muscle softening and therefore change in muscle texture (Ando *et al.*, 1999; Aoki *et al.*, 2000).

In general, cathepsins are a class of globular lysosomal proteinases most of which contain an active-site cysteine residue. Cathepsins are synthesised as pre-procathepsins and processed to mature forms with targeting into lysosomes and then to initial degradation products. They are synthesized as larger glycosylated

precursor forms before processing into the native enzymes (Katunuma, 1989; Turk *et al.*, 1989). Lysosomal enzymes are synthesized as high molecular weight precursor molecules and subsequently converted to the mature enzyme by limited proteolysis post-transitionally (Nishimura *et al.*, 1989).

Mammalian cellular proteinases play an important role in cellular protein catabolism and extensive but controlled protein proteolysis. During intracellular protein degradation, proteins are packed and fused with lysosomes within which they are digested by intralysosomal cysteine proteases (Katunuma, 1989).

Cathepsins are involved in major degradative pathways of the post-mortem aging process. Although they are acid proteases located in the lysosomes, they may be liberated into both the cytoplasm and the intracellular spaces as a consequence of lysosomal disruption occurring after cell death due to a pH fall (Duston, 1993). Cathepsins are inactive in living tissue because they are not in contact with their substrates.

The role of cathepsins in fish post-mortem proteolysis has received little attention compared to other mammals. Proteolysis plays a critical role in post-mortem aging of muscle resulting in tenderization. In mammalian muscles, this is beneficial to meat quality but in fish, it results into deterioration (Aranishi *et al.*, 1997).

One of the mammalian cystein proteinase, cathepsin L belongs to the papain superfamily and is mainly involved in the degradation of autophagocytosed proteins (Turk *et al.*, 1989). Basic enzyme

characteristics common among the various samples of cathepsin L include: the range of their molecular masses from 23,000 to 30,000; two polypeptide chains linked by a disulfide bridge; a high endopeptidase activity; potent ability to act on the fluorescence ability of Z-Phe-Arg-MCA and a variety of proteins (Aranishi, 1999). As well as mammalian cathepsin L, fish cathepsin L has a strong proteolytic activity for several proteins including fish myofibrillar components, suggesting its participation in intracellular and extracellular protein catabolism in the fish muscle.

A remarkable hydrolytic activity towards Z-Phe-Arg-MCA has been observed in crude extracts from carp hepatopancreas during purification of cathepsin B and H (Aranishi *et al.*, 1992, 1997). The fish cathepsin L therefore seems to be contained at higher levels and carry out tissue degradation more successfully in internal organs than in ordinary muscle.

Various studies on cathepsin L have showed that TLCK and chymostatin (serine protease inhibitors) partially inhibit activity of the cathepsin. E-64, antipain and leupeptin (cystein protease inhibitors), DTNB and *p*CMB (sulfhydryl specific reagents) markedly or fully inactivate the enzyme. In the absence of EDTA, the enzyme is potentially inactivated by metal compounds $AgNO_3$, $CdCl_2$, $CuCl_2$, $FeCl_3$, $HgCl_2$, and $Zn(CH_3COO)_2$ and partially by $COCl_2$ and $SnCl_2$. Maximum activation of the enzyme is achieved by a combination mixture of EDTA and cystein showing that EDTA enhances enzyme activity only in the presence of cystein (Aranishi *et al.*, 1997).

Cathepsin L has strong endopeptidase activity compared to other cathepsins and is capable of digesting several physiological substrates such as casein, collagen and elastin (Barret & Kirschke, 1981). It is responsible for the degradation of matrix proteins, inhibition of normal antigen processing and promotion of proliferation processing. It is active at pH 3.0-6.5 and irreversibly inactivated at pH > 7 (Turk *et al.*, 1989).

Activation of procathepsin L catalysed autocatalytically and whole tissue extracts show that cathepsin L is partially complexed with endogenous inhibitors. Separation from the enzyme-inhibitor complex requires autolysis at acidic pH followed by fractionation with ammonium sulfate. It can be separated from other lysosomal cystein proteinases by most cation exchange chromatography media to which the enzyme shows an anomalous high affinity (Turk *et al.*, 1989).

Studies on cathepsin L in the fish muscle have been conducted on mackerel (Aoki *et al.*, 1997; Aoki & Ueno, 1997), chum salmon (Yamashita & Konagaya, 1990; 1991; 1992), carp (Aranishi *et al.*, 1997), seabass (Delbarre-Ladrat *et al.*, 2004; Cheret *et al.*, 2007), cod (Sovik & Rustad, 2006) and Atlantic salmon (Hultmann & Rustad, 2004) among others.

Knowledge about the enzyme involved in fish freshness and quality deterioration processes and variations according to species is necessary in order to work out appropriate handling and storage procedures and for evaluating the suitability of the products for different uses.

This study analyses the various products of ATP degradation and measurement of the *K*-value to assess the effect of bleeding treatment on muscle quality during iced storage. A comparison is made between bled fish samples and unbled fish samples for the entire iced storage period.

Because spoilage marks a different phase in muscle freshness and quality change, TMA and VBN were used to establish the stage at which these changes take place and trends in spoilage following fish bleeding and iced storage.

For any fish or fishery product to be popular, it must meet the customer/consumer's expectations on muscle quality. A pleasant taste and smell, absence of substances harmful or injurious to health and life; and should be able to respond positively to preservation treatments during storage.

This study examines the trends in both red-flesh fish (amberjack) and white-flesh fish (red sea bream) which are important species in both capture fisheries and aquaculture and in the diet especially as sashimi products. Amberjack (*Seriola dumerili*) called 'Kampachi' and red sea bream (*Pagrus major*) called "Madai" in Japanese are rare and prized fish species for which reason effort has been put into research and culture to increase production. In Japan, both fish species can prepared in different forms as 'Sashimi' or stuff for 'Sushi', grilled (Yakimono or Aburi), simmered (Nitsuke/Nitsuki), ("meshi") meuniere and fried making it a delicacy.

Results of this study will help in the accumulation of knowledge on muscle quality during iced storage, benefits of bleeding to fish freshness, and cathepsins involved in fish. This is useful for processors and fish product consumers.

Chapter II. Effect of bleeding on the quality of cultured amberjack muscle

II - 1. Materials and Methods

II - 1 - 1. Fish samples and bleeding method

Live cultured amberjack *Seriola dumerili* (mean weight 3.7 kg) was obtained after harvest from Mie Gyoren (The Federation of Mie Prefectural Fisheries Association), Owase, Japan. The fish were randomly assigned a treatment group (bled or unbled) in equal numbers (n=5). Bleeding was done by cutting the aorta and spinal cord. The fish was then held in the air with the head facing downwards. Blood drained out of the aorta through the mouth (Fig. 24). The fish was then placed in iced water to wash off the blood before taking 0-day samples. Unbled fish samples were sacrificed by a quick and incisive blow to the cranium resulting into immediate death.

For each fish, 0-day storage samples were immediately collected in sample bags and frozen on dry ice for transportation to the laboratory. The unbeheaded and ungutted fish specimens were coded, placed in polythene bags and transported on ice to the laboratory. These were stored on ice in Styrofoam boxes in a cold room maintained at a temperature below 4°C. Ordinary muscles were drawn from the dorsal site of the specimens along the lateral line. Sampling was done after 1, 3, 5, 7, 10 and 15 days of iced storage. The sampled

muscle was surgically and asceptically collected from the fish and the skin was removed. On each sampling day, standard operating procedures for hygienic handling of fish were observed to maintain wholesomeness. The samples were regularly re-iced on the sampling days and to maintain low temperatures.

II - 1 - 2. Analytical methods

II – 1 – 2 – 1. ATP- related compounds

Sample extraction and analysis of the ATP-related compounds was according to Ryder (1985). Two grams of the fish muscle was homogenized (Physcotron homogenizer, Niti-on, Tokyo, Japan) with 4 ml chilled 10% perchloric acid and centrifuged (Himac, CF7D2, Hitachi, Japan) at 3,000 x g for 3 minutes. The supernatant was collected and the residue further centrifuged, with agitation, twice, each time with 4 ml of chilled 5% perchloric acid. The residue was discarded.

The collected supernatant was neutralized to pH 6.4 with 10 N KOH followed by 1 N KOH. The neutralized supernatant was centrifuged at 3,000 x g for 3 minutes. The resulting supernatant was collected in a sample bottle and the residue further centrifuged at 3,000 x g for 3 minutes, twice after agitation. The supernatant was filled up to 25 ml with Milli-Q water and the stock sample was stored at -20°C until analysis.

Analysis of ATP-related compounds was done using the HPLC (Ryder, 1985). The HPLC system (Jasco, Japan) was fitted with an auto-sampler, 980-PU pump, UV-970 Detector and a YMC-pack ODS-A column (250 X 4.6 mm I.D, S-5μ, 120A). A 12 mM citric acid buffer including 18 mM 2-diethylaminoethanol was used as a mobile phase. A flow rate (0.5 ml/min), column temperature (40°C), detector sensitivity (0.056 AUFS) and injection volume (20 μl) were set for the analysis.

All samples were filtered prior to analysis (PTFE 0.20 μm filter unit, ADVANTEC, Japan). The mobile phase was also filtered using membrane filters (PTFE, 0.2 μm, 47 mm, ADVANTEC, Japan).

The resulting chromatogram was automatically recorded and printed out by a computerized programm (Jasco LCSS-905). Peak identification and quantification was determined by comparison to ATP, ADP, AMP, IMP, HxR and Hx standards of determined concentrations and corresponding retention times. All chemicals used were HPLC grade (Wako Pure Chemical Industries, Japan). Samples were analysed in duplicates and the average values calculated for all the samples.

II - 1 - 2 - 2. Trimethylamine (TMA) assay

TMA was determined using the picric acid method (Dyer, 1945). Two grams of fish muscle was homogenized with 6 ml distilled water for 30 minutes. 2 ml of 20% trichloroacetic acid (TCA) was added and

further homogenized for 10 minutes. The homogenate was filtered using filter paper (110 mm diameter, Advantec Toyo, Japan). The filtrate was then filled up to 10 ml with 20% TCA and stored at -20°C until TMA analysis.

To 2 ml of the TCA extract in a test tube, 0.5 ml of 10% formalin, 5 ml toluene and 1.5 ml of 25% potassium hydroxide solution was added. The mixture was vortexed for 40 seconds resulting into an upper and lower layer. The upper layer was collected into fresh dry test tubes and 0.25 g anhydrous sodium sulfate was added. To 2.5 ml of this solution, 2.5 ml of 0.02% picric acid was added to form the sample solution.

Absorbance of the sample was then determined at 410 nm using a spectrophotometer (Hitachi, U-1100). The values were plotted on a graph and the value of TMA content determined from a TMA standard curve.

II – 1 – 2 – 3. Volatile Basic Nitrogen (VBN) assay

VBN was determined using the micro-diffusion method (Conway, 1947). Two grams of the fish muscle was homogenized with 6 ml distilled water for 30 minutes. 2 ml of 20% TCA was added to the homogenate and further homogenized for 10 minutes. The homogenate was filtered using filter paper (110 mm diameter, Advantec Toyo, Japan). The filtrate was then filled up to 10 ml with 20% TCA and stored at -20°C until VBN analysis.

Boric acid solution (1 ml) was added to the inner chamber of the Conway micro-diffusion unit. In the outer chamber of the unit, 1 ml of the TCA extract and 1 ml of saturated K_2CO_3 was added diametrically. The unit was tightly covered with its lid and gently swirled to allow mixing of only the TCA extract and K_2CO_3 in the outer chamber. Care was taken to avoid mixing with the boric acid solution in the inner chamber. The unit was then incubated at 37°C for 80 minutes.

VBN was determined by titration with 1/50N HCl and calculated from the formula as below;

$$VBN\ (mg\ \%) = 0.28*(x-b)*f*100/0.21$$

x = titre volume (ml),

f = factor of 1/50N HCl

b = blank.

II - 2. Results

II – 2 – 1. ATP-related compounds

II - 2 - 1 - 1. Adenosine triphosphate (ATP)

Samples collected immediately after fish death revealed ATP presence in the fish muscle. Amberjack muscle contained an average ATP content of 0.23 μmol/g muscle on the first day of sampling. No ATP was identified in subsequent sampling showing that the compound was degraded to amounts below detection levels.

II - 2 - 1 - 2. Adenosine diphosphate (ADP)

Samples of the amberjack muscle immediately after death contained ADP in the fish muscle that continuously decreased in subsequent iced storage at different days. In the bled samples, the ADP content decreased from an average 0.16 μmol/g muscle on 0-day storage to 0.05 μmol/g muscle on 15 days-storage (Fig. 6). In the unbled samples, the ADP content decreased from an average 0.17 μmol/g to 0.04 μmol/g muscle during the same period. However there was no significant difference between the bled and unbled samples. The ADP content in the muscle was lower than both AMP and IMP content throughout the storage time.

II - 2 - 1 - 3. Adenosine monophosphate (AMP)

The amberjack muscle contained low amounts of AMP in the samples collected immediately after death. However, there was a general increase in AMP content in the fish muscle in both bled and unbled samples throughout the storage period (Fig. 7). In the bled muscle, AMP content increased from an average 0.24 μmol/g muscle on 0-day storage to 1.62 μmol/g muscle on 15 days-storage. In the unbled samples, the AMP content increased from an average 0.22 μmol/g to 1.46 μmol/g muscle during the same period. No differences existed between the bled and unbled samples until 3 days-storage. Bled muscles had a significantly higher AMP content ($p \leq 0.05$)

between 7-15 days-storage. The AMP in the amberjack muscle was higher than the ADP content but lower than IMP throughout the storage period.

II - 2 - 1 - 4. Inosine monophosphate (IMP)

Samples of freshly caught amberjack muscle contained a significantly high amount of IMP. There was a general decrease in both bled and unbled samples throughout the iced storage period (Figure 8). In the bled samples, the IMP content in the muscle decreased from an average 8.16 μmol/g muscle on 0 day-storage to 3.02 μmol/g muscle on 15 days-storage. In the unbled samples, it decreased from an average 7.41 μmol/g to 2.50 μmol/g during the same period. On 1 day-storage, bled samples had a significantly higher IMP content ($p \leq 0.05$) compared to unbled samples throughout the storage period. Compared to other ATP-related phosphate compounds, IMP content in the amberjack muscle was higher than both ADP and AMP during the storage period.

II - 2 - 1 - 5. Inosine (HxR)

HxR was observed in samples of the amberjack muscle collected immediately after death. HxR content in the muscle generally increased with storage time in both bled and unbled samples (Fig. 9). In the bled samples, HxR content increased from 0.99 μmol/g muscle

on 0-day storage to 3.45 μmol/g muscle on 15 days-storage. In the unbled samples, the HxR content increased from 0.99 μmol/g to 3.11 μmol/g during the same period. The HxR content was generally higher in bled samples than in the unbled samples. Bled samples had a significantly higher HxR content ($p \leq 0.05$) on 10 days-storage onwards during the storage time.

II - 2 - 1 - 6. Hypoxanthine (Hx)

Samples of the amberjack muscle immediately after death did not contain Hx. However, on 1 day-storage, Hx was detected and quantified throughout the storage period. There was a general increase of Hx content in the muscle throughout storage in both bled and unbled samples (Fig. 10). In bled samples, the Hx content increased from 0.90 μmol/g muscle on 1-day storage to 2.11 μmol/g muscle on 15 days-storage. In unbled samples, the Hx content increased from 1.01 μmol/g to 2.43 μmol/g muscle during the same period. After 3 days-storage, the unbled amber jack muscle contained a significantly high Hx content ($p \leq 0.05$) compared to the bled samples.

II - 2 - 1 - 7. *K*-value

An average *K*-value of 10% was observed in the amberjack muscle immediately after death. This value generally increased

throughout storage in both bled and unbled samples. The *K*-value increased to 54.2% in bled and 58.12% in the unbled samples on 15 days-storage (Fig. 11). Although the *K*-value was generally higher in the unbled than in the bled samples throughout storage, the difference became significant ($p \leq 0.05$) on 3 days-storage until the end of the storage period.

II – 2 – 2. Trimethylamine (TMA)

The TMA content in amberjack muscle samples collected immediately after death remained at a very low concentration. However, the value increased throughout the storage period (Fig. 12). In bled samples, the TMA content increased from 0.24 mg/100 g (0 day-storage) to 3.39 mg/100 g (15 days-storage). In the unbled samples, the TMA content increased from 0.33 mg/100 g to 3.7 mg/100 g muscle during the same period. Bled samples were not significantly different from unbled samples until 10 days-storage.

II – 2 – 3. Volatile basic nitrogen (VBN)

There was a general increase in the VBN content in both bled and unbled amberjack muscle (Fig. 13). The VBN content in the bled samples increased from 10.25 mg/100 g (0 day-storage) to 20.3 mg/100 g (15 days-storage). In the unbled samples, it increased from 10.7 mg/100 g to 23.85 mg/100 g during the same period. There was

a notable slow increase in the VBN content in both bled and unbled samples between 1-5 days-storage period and a further increase on 7 days-storage. Significant differences ($p \leq 0.05$) between bled and unbled samples were observed on 7 days-storage.

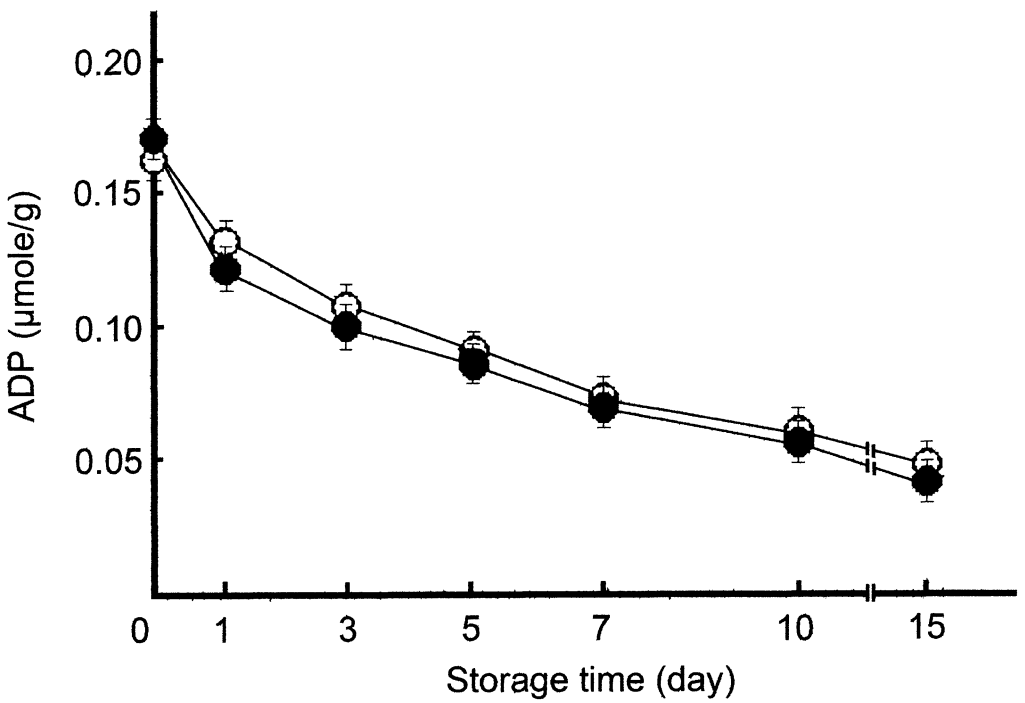

Fig. 6. Changes in ADP content in the amberjack muscle during iced storage.

(○) represents bled samples and (●) unbled samples

(mean values and standard deviation).

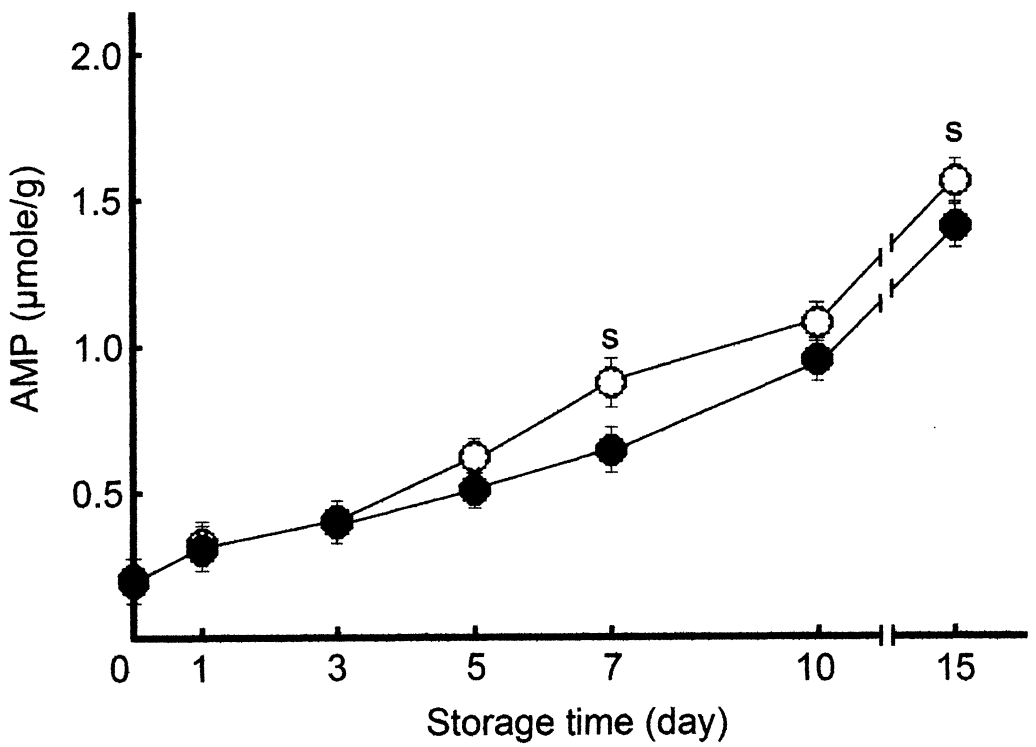

Fig. 7 Changes in AMP content in the amberjack muscle during iced storage.

(○) represents bled samples and (●) unbled samples

(mean values and standard deviation) and

S: significant differences.

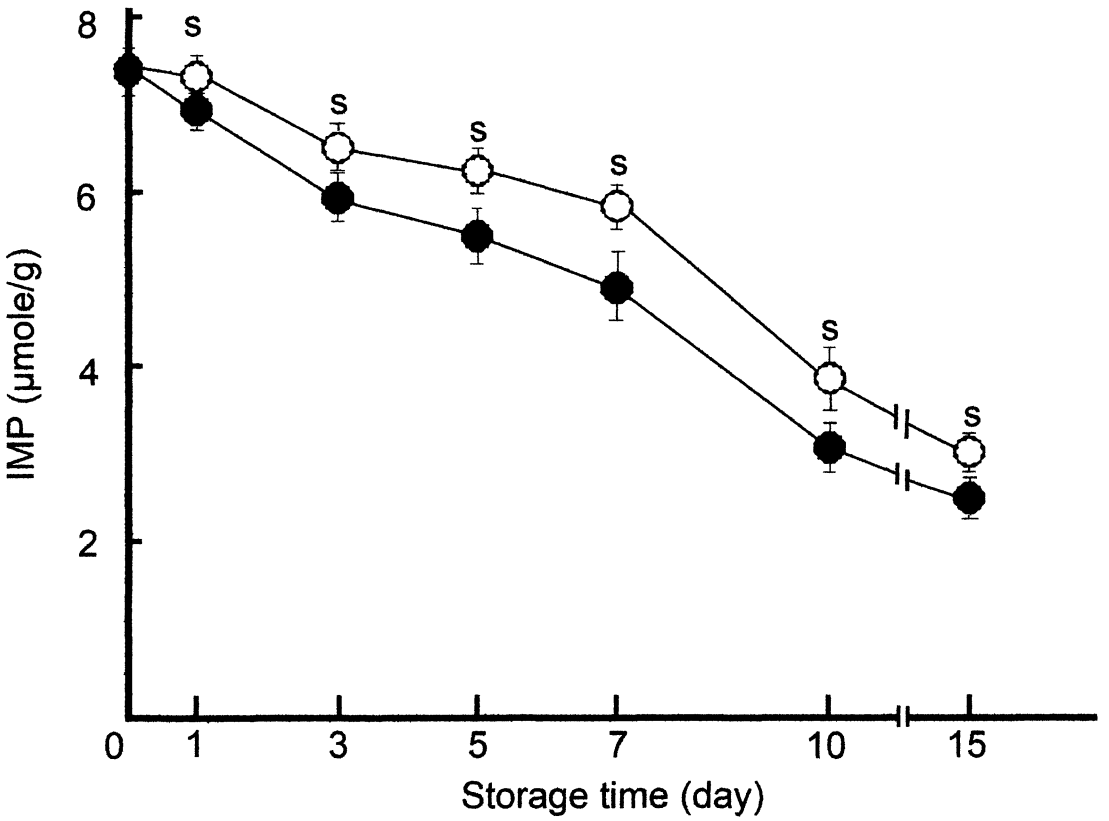

Fig. 8. Changes in IMP content in the amberjack muscle during iced storage.

(○) represents bled samples and (●) unbled samples (mean values and standard deviation).

S: significant differences.

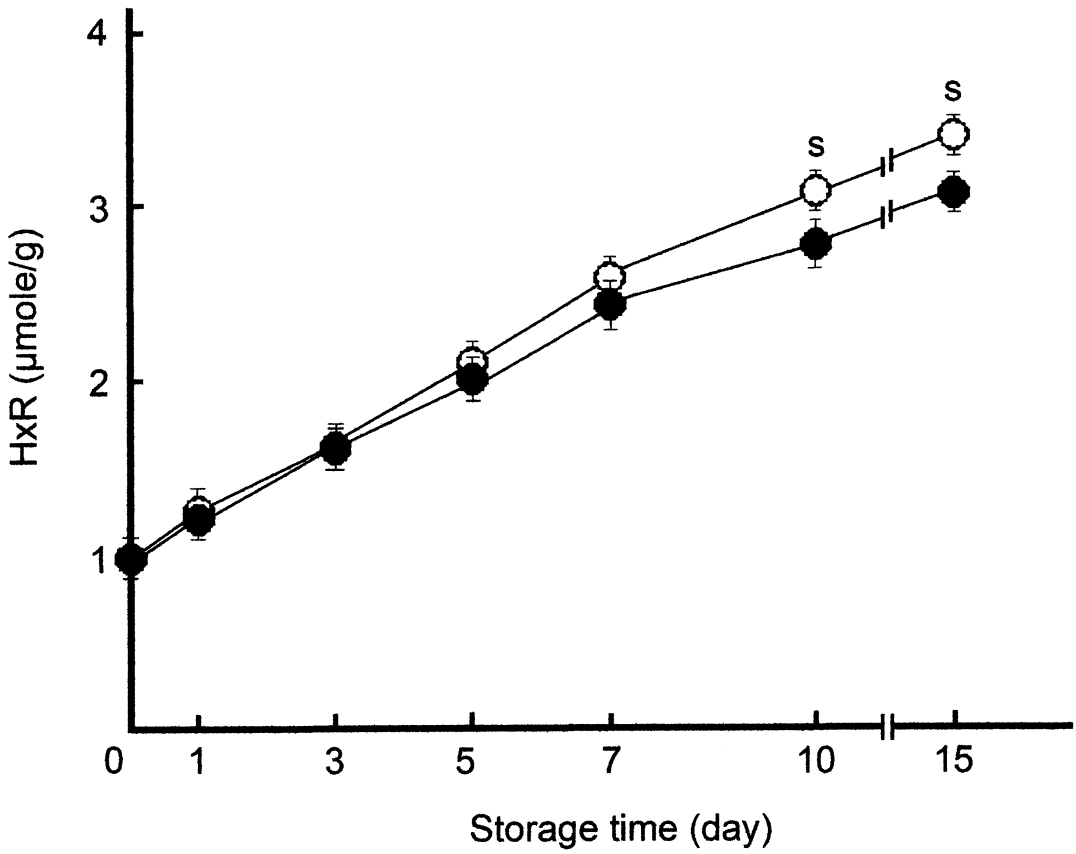

Fig. 9. Changes in HxR content in the amberjack muscle during iced storage.

(○) represents bled samples and (●) unbled samples (mean values and standard deviation).

S: significant differences.

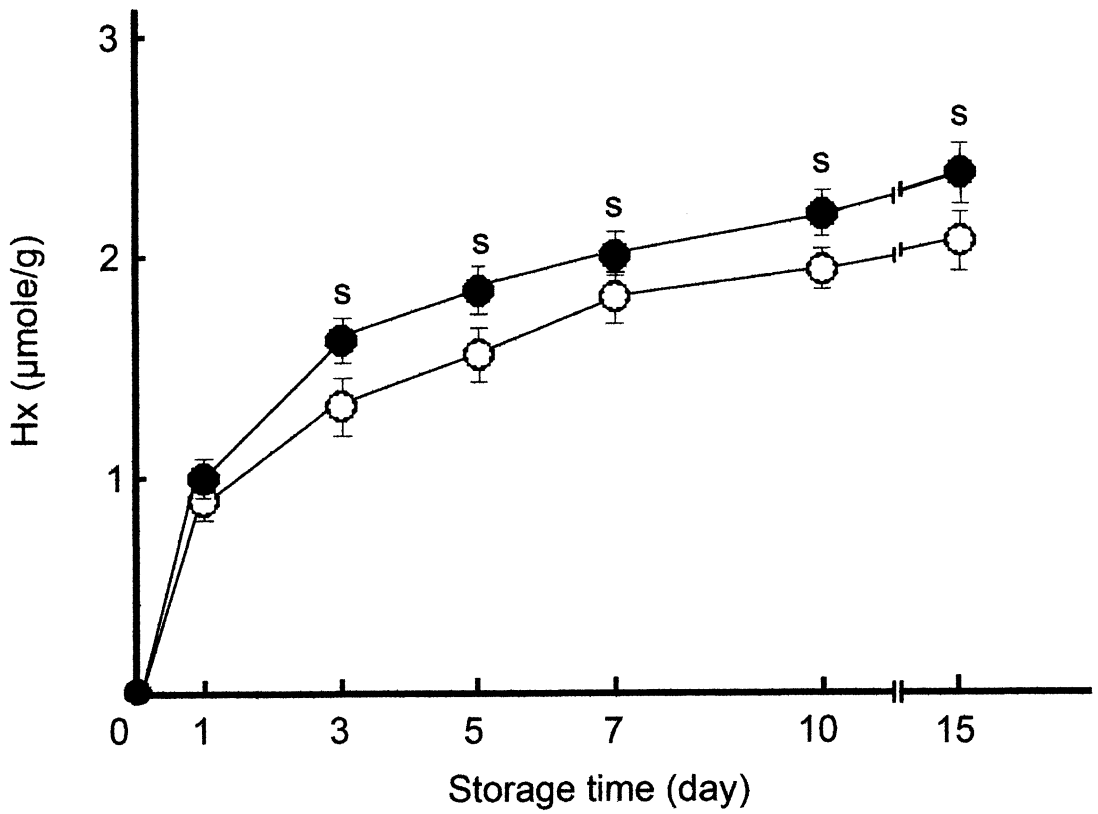

Fig. 10. Changes in Hx content in the amberjack muscle during iced storage.

(○) represents bled samples and (●) unbled samples (mean values and standard deviation).

S: significant differences.

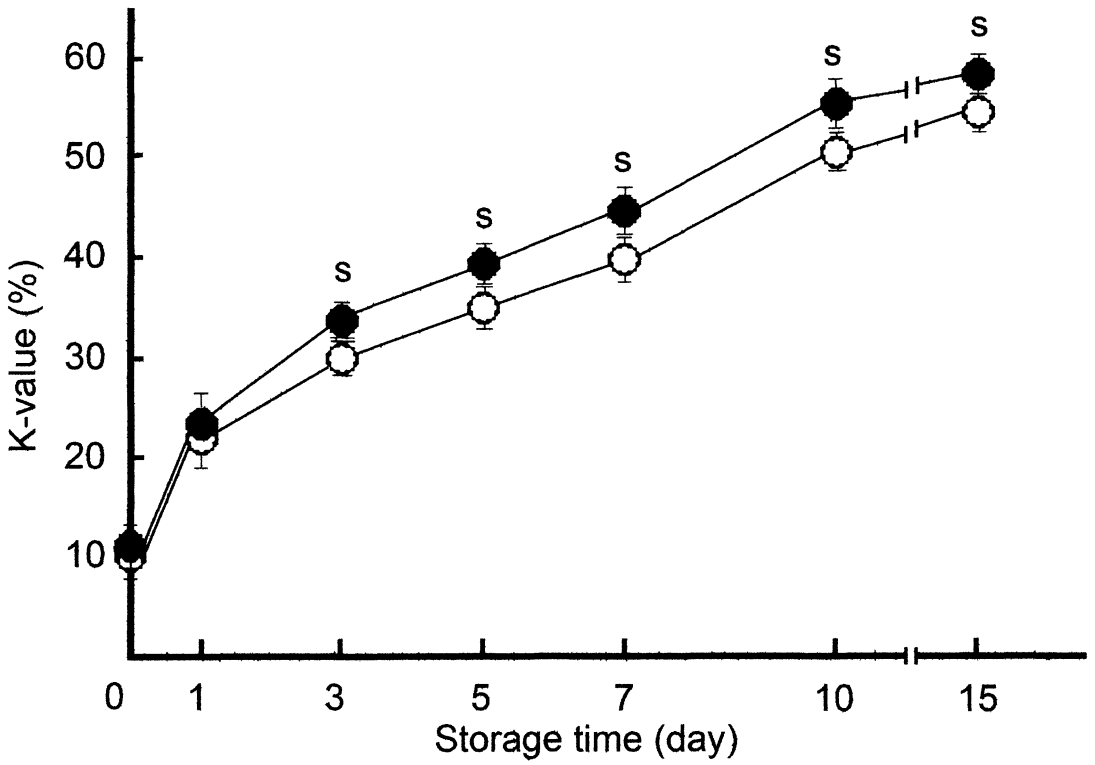

Fig. 11. Changes in the *K*-value in the amberjack muscle during iced storage.

(○) represents bled samples and (●) unbled samples (mean values and standard deviation).

S: significant differences.

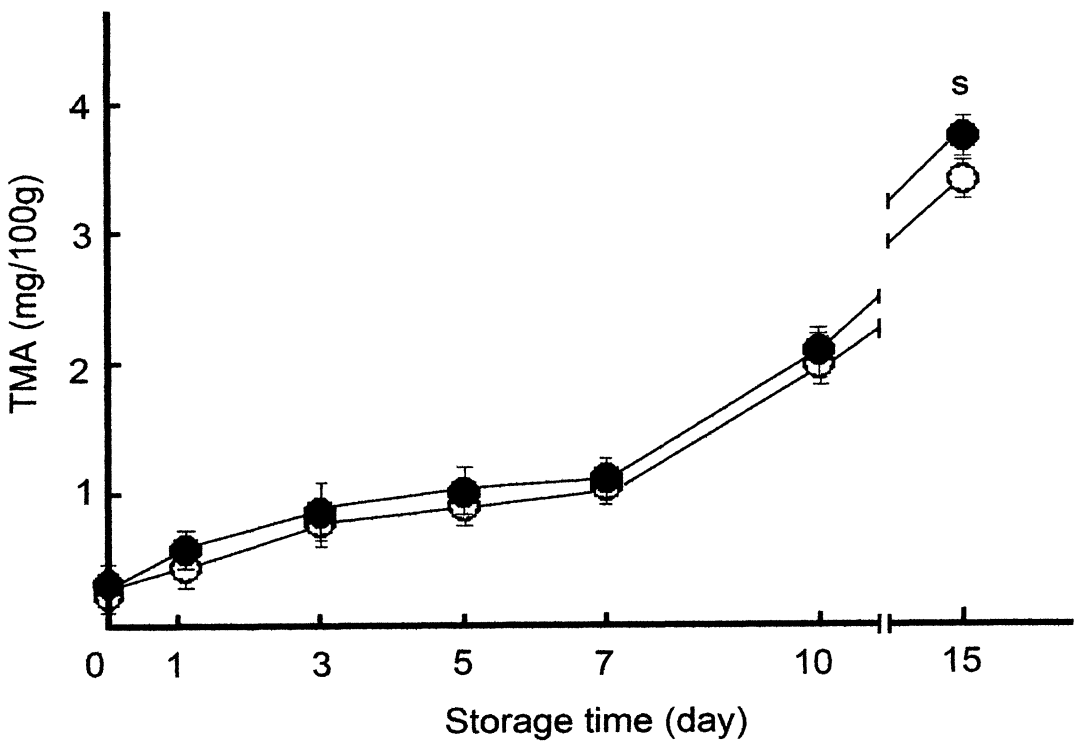

Fig. 12. Changes in TMA content in the amberjack muscle during iced storage.

(○) represents bled samples and (●) unbled samples (mean values and standard deviation).

S: significant differences.

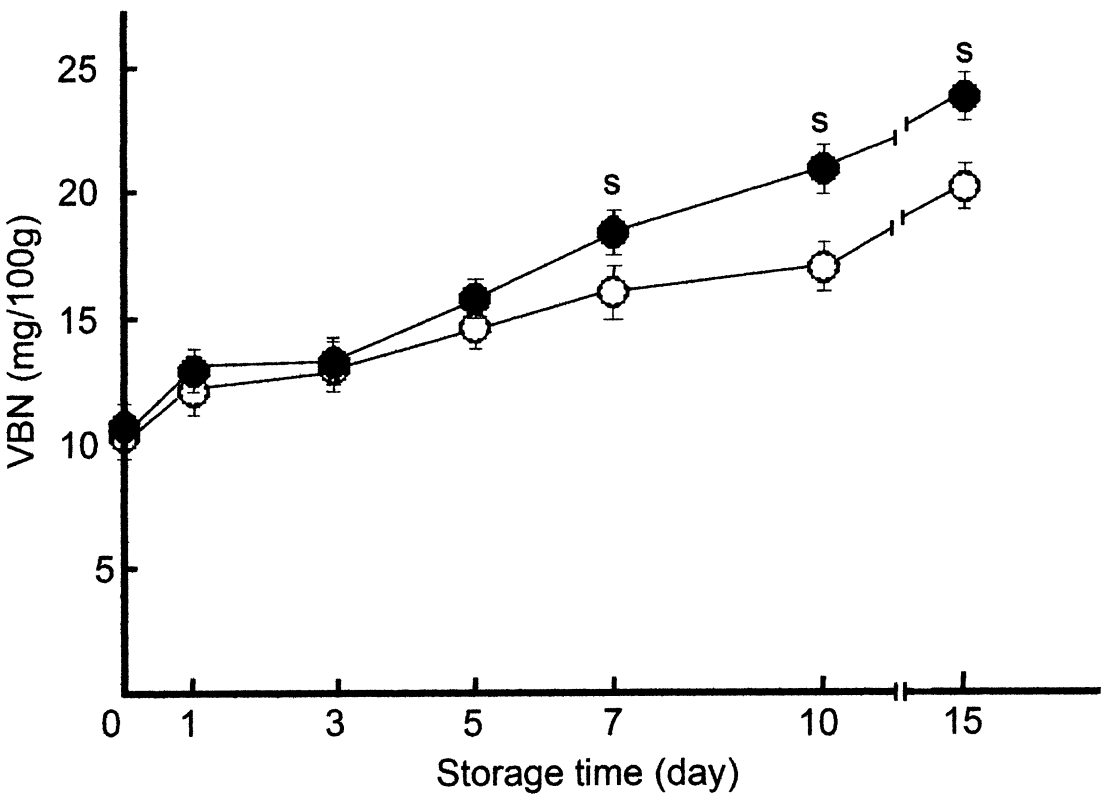

Fig. 13. Changes in VBN content in the amberjack muscle during iced storage.

(○) represents bled samples and (●) unbled samples (mean values and standard deviation).

S: significant differences.

II - 3. Discussion

Nucleotides are the second most abundantly occurring nitrogen-containing extracts of fish next to free amino acids (Borresen, 1995a). The pattern and rate of nucleotide degradation is thought to be species-specific and nucleotide analyses may be useful in pelagics, redfish, salmon, squid and of little value for the estimation of lean fish freshness.

ATP and its breakdown products are the most useful and reliable indicators of fish freshness (Karube *et al.*, 1984). Ehira & Uchiyama (1986) contend that the determination of ATP breakdown suits evaluation of fish freshness when high demands for fish freshness are required especially for special products like sashimi and sushi in Japan.

In this study, ATP, ADP and AMP content in the amberjack muscle on 0-day storage was very low. The complete degradation of ATP after 1 day-storage and subsequent low amounts of ADP and AMP show that much of these compounds were rapidly degraded to IMP which was in high concentration.

ATP dephosphorylation is fast after fish death and during chill storage and may occur during freezing and thawing of the fish muscle (Kennish & Kramer, 1987). While some of the decomposition might have taken place during capture and slaughter, decomposition might have also taken place during subsequent sample transportation on ice and processing before analysis.

The time lag between fish capture/slaughter and sample processing for analysis could have been long enough to allow decomposition. Transportation of the samples on dry ice meant that the samples were frozen and involved defrosting at 4°C while processing for analysis. This change in temperature over time could have equally affected ATP content and decomposition.

Low amounts of ATP, ADP and AMP have also been observed in other studies on the gilthead sea bream (Grigorakis *et al.*, 2002), skipjack (Sakai *et al.*, 2006), sardine (Ozogul *et al.*, 2007), herring (Ozogul *et al.*, 2000) and the rainbow trout (Ozogul & Ozogul, 2000). Studies on bream, flounder and seabass, showed that ATP was completely decomposed within 24 hours after death (Karube *et al.*, 1984). ATP, ADP and AMP content below a concentration of 0.05 μmol/g have also been reported in chub mackerel *Scomber japonicus* (Kuda *et al.*, 2007).

The ATP content that may be present in the fish muscle immediately after fish death may vary depending on the fish species, initial conditions of the fish, capture and slaughter methods, time-temperature relationships, handling and processing procedures.

IMP contributes to the fresh flavour of meat (Howgate, 2005) and IMP level is related to flavour intensity and acceptability (Bremner *et al.*, 1988). Conversion of ATP to IMP is thought to be complete within 1 day and presumed to be totally autolytic (Jones, 1965; Hiltz *et al.*, 1972). Autolysis is initiated when the muscles lose respiratory capability and the membranes breakdown (Bremner & Hallet, 1989).

IMP increases sharply between 5-24 hours after fish death and then gradually decreases.

Hattuala *et al.*, (1993) proposed IMP content of about 5 µmol/g (dry weight) as criteria for good quality fish. Based on this standard, results from this study show that the amberjack was of good quality up to 7 days-storage. In both bled and unbled amberjack in this study, results show that IMP was the dominant product of ATP degradation.

Erikson *et al.*, (1997) found IMP as the dominant product of ATP degradation in Atlantic salmon and an IMP content of 12 µmol/g (dry weight) on 13 days-iced storage. Howgate (2006) explains that IMP content in the fish muscle on 0 day-storage ranges from 2.75 µmol/g to 16.6 µmol/g with the median being 6.83 µmol/g. Alasalvar *et al.*, (2001) reported IMP content of 8 µmol/g in the gilthead sea bream on 1 day-storage that decreased steadily to 3.5 µmol/g after 23 days of iced storage.

Mazorra *et al.*, (2000) observed IMP content of 7 µmol/g on 0-day storage and 0.245 µmol/g after 24 days of iced storage in the black skipjack muscle. Castillo-Yanez *et al.*, (2007) observed IMP content of 11.3 µmol/g in the experimental samples in Sierra fish and 10.6 µmol/g in the consumer samples on 0 day-storage. These values decreased to 4.2 µmol/g and 4.5 µmol/g in the experimental samples and consumer samples respectively after 15 days of iced storage. Ryder *et al.*, (1984) observed IMP content of 10.3 µmol/g on 0-day storage and 1.1 µmol/g on 23 days-iced storage in New Zealand jack mackerel.

Grigorakis *et al.*, (2003) noted a slow decomposition of IMP from 8.21 μmol/g on the first day to 3.26 μmol/g after 15 days of storage in the gilthead sea bream stored on ice. Hamada-sato *et al.*, (2005) studied the skipjack mackerel and noted a rapid increase in IMP content within 24 hours after death. The rate of IMP decomposition is related to phosphatase or 5'-nucleotidase in the fish muscle (Ehira *et al.*, 1974; Ehira & Uchiyama, 1986).

Since bled amberjack muscle had a higher value of IMP content than unbled muscle, it can be assumed that the concentration of enzymes that may be present in the blood and responsible for IMP breakdown is reduced by bleeding. Consequently this affects the rate of IMP degradation during iced storage.

HxR and Hx are formed from the breakdown of IMP. Some fish are known to produce more HxR than Hx while others produce more Hx than HxR (Ehira & Uchiyama, 1986). While the conversion of IMP to HxR is facilitated by 5'-nucleotidase with liberation of inorganic phosphate, nucleoside phosphorylase facilitates the conversion of HxR to Hx and requires inorganic phosphate.

Post-mortem accumulation of HxR and Hx reflects poor quality of the fish muscle (Gill, 1992) and increases with storage time. Accumulation of HxR and Hx in the muscle is thought to be related to both autolytic and microbial action (Gill, 1992). HxR accumulation in the fish muscle tissue reflects the initial phase of autolytic deterioration as well as bacterial spoilage (Woyewoda *et al.*, 1986).

The HxR and Hx content in the fish muscle increases when IMP content begins to decrease (Hamada-Sato *et al.*, 2005) and Hx has been solely used as a freshness indicator (Ehira & Uchiyama, 1973). Hx accumulation is reported to be involved in progressive loss of desirable fish flavour leading to off-tastes (Fletcher & Statham, 1988; Ozogul *et al.*, 2000).

Hx content of 8-9 μmol/g was reported in Atlantic salmon muscle after 13 days of iced storage (Erikson *et al.*, 1997). In the gilthead sea bream stored on ice, Grigorakis *et al.*, 2003 reported a slow increase in Hx content from 0.06 μmol/g muscle to 0.49 μmol/g muscle during winter and a change from 0.02 μmol/g muscle to 0.27 μmol/g muscle after 15 days-storage during summer. HxR increased from 0.02 μmol/g to 1.15 μmol/g during winter and from 0.0 μmol/g to 1.41 μmol/g during summer.

Mazorra-Manzano *et al.*, (2000) reported HxR and Hx content of 0.17 μmol/g muscle and 0.52 μmol/g muscle respectively in very fresh black skipjack. Kuda *et al.*, (2007) reported HxR and Hx content of 1.16 μmol/g and 0.04 μmol/g muscle respectively, on 0-day storage in chub mackerel. After storage at 4°C, the HxR content increased to 4.0 μmol/g before decreasing to 1.2 μmol/g after 7 days-storage while Hx increased to 7.2 μmol/g muscle.

The total ammount of ATP-related compounds is usually constant and the ratio (%) of the amounts of HxR and Hx to the total amount of the ATP-related compounds defines the *K*-value. Use of the *K*-value is especially more appropriate to determine freshness in the

early stages after fish death (Hamada-Sato *et al.*, 2005). It is important when autolytic changes immediately after fish death, before microbial spoilage, are solely responsible for degradation (Manju *et al.*, 2007). It is also very useful when the use of TMA content is not possible due to low or lack of TMAO in some fish species (Nikkila, 1951; Hebard *et al.*, 1982).

The *K*-value of very fresh fish suitable for raw consumption should be less than 20% (Hoshi *et al.*, 1991) and there exists a good relationship between the *K*-value and muscle changes after instant fish death (Hamada-Sato *et al.*, 2005). *K*-value limits of 20% (Ehira & Uchiyama, 1986); 30% (Williams *et al.*, 1993; Chiou *et al.*, 1995) have been proposed for good quality fish of different species. The larger the *K*-value, the poorer the eating quality (Manju *et al.*, 2007). A *K*-value of 80% has been suggested as the upper limit of acceptability (Hattuala *et al.*, 1993; Lin & Morrissey, 1994).

For salmon muscle, an upper limit of 70% to 80% has been suggested and is only useful in the first 7 days-storage (Erikson *et al.*, 1997). However, in the study of sole, while sensory rejection was established after 22 days-iced storage, the *K*-value was less than 40% at the point of rejection (Tejada *et al.*, 2007). In trout muscle, sensory rejection was attained after 12 days-iced storage and the *K*-value at the point of rejection was 73% (Ozogul *et al.*, 2002).

For sardines, Pacheco-Aguilar *et al.* (2000) observed a *K*-value of 10.1% in very fresh samples and 50.7% after 15 days-iced storage. Kuda *et al.* (2007) observed an initial *K*-value of 9.9% in mackerel

muscle which increased to 65% on 7 days-storage at 4°C. Mazorra-Manzano *et al.*, (1998) noted an increase in *K*-value from 19.5% on 2 days-storage to 70.6% on 18 days-iced storage in the black skip jack muscle.

The *K*-value is reported to vary between and within fish species. Flathead sole was reported to attain a *K*-value of 80% within 24 hours (Green *et al.*, 1990) while Pacific cod attained a *K*-value of 100% within 2 days-storage but was still organoleptically acceptable after 10 days-storage on ice (Luong *et al.*, 1992). Turbot becomes unacceptable after 19 days-storage (Aubourg *et al.*, 2005; Ozogul *et al.*, 2006), seabass; 16-19 days-storage (Castro *et al.*, 2006) and trout; 12 days-storage (Ozogul *et al.*, 2002) with a *K*-value more than 70%. Results in this study therefore suggest that iced storage of whole amberjack maintains its quality beyond 15 days-storage.

Unbled amberjack muscle had a higher *K*-value compared to the bled samples. It has been reported that the *K*-value is greatly influenced by the rate of IMP decomposition (Olafsdottir, 1997; Arvanitoyannis *et al.*, 2005). Since unbled samples contained a lower IMP content, it affected the *K*-value. The higher the *K*-value, the more the ATP degradation has gone towards the final products (HxR and Hx), and the increase in *K*-value may reflect directly on storage time.

The use of TMA content is based on its characteristic odour that is associated with spoiling fish (Ringø *et al.*, 1984). It is formed from

bacterial reduction of TMAO present in the fish muscle (Malle *et al.*, 1986) but can also be formed from intrinsic enzyme activity when bacterial growth is inhibited (Ringø *et al.*, 1984; Sadok *et al.*, 1996). Though widely recommended and used, TMA has been reported to be only useful for the evaluation of advanced spoilage rather than loss of freshness (Ashie *et al.*, 1996).

Results in this study showed TMA content less than 0.2 mg/100g of amberjack muscle samples on 0 day-storage. Freshly caught fish contains a TMA content of less than 1 mg/100 g muscle (El Marrakchi *et al.*, 1990) and 1.5 mg/100 g muscle for cold water fishes. The European Union (EU) markets regulations on fish and fishery products use a TMA content of 12 mg/100 g muscle as the limit of acceptability for good quality fish. A TMA content of 10-15 mg/100 g muscle have as the upper limit of acceptability for good quality fish has been proposed for most fish species (Connell, 1975; Huss, 1988).

In the Nile perch, the TMA content in the muscle increased from 0.0 mgN/100 g muscle in very fresh samples to 3-5 mgN/100 g muscle during iced storage and 16 mgN/100 g muscle in samples stored at ambient temperatures after 17 hours (Gram *et al.*, 1989). This signifies the effect of storage temperature on the rate of TMA production.

Nakashima & Nishida (2001), studied different fishes using capillary electrophoresis and reported that TMA was not detected in some of the fish species such as sardine, skipjack and red sea bream

on 0-day storage. Chouliara *et al.,* (2005) studied the effect of irradiation on the sea bream *Sparus aurata* and reported TMA values of 6.3 mg/100 g - 7.9 mg/100 g muscle on 49 days-storage, using the picric acid method. Pacheco-Aguilar *et al.*, (2000) reported final TMA values of 1.35-1.62 mg/100 g muscle in sardine. Papadopoulos *et al.*, (2003) reported final TMA values of 0.07 mg/100 g muscle in the seabass. Baixas-Nogueras et al., (2003) reported less than 1 mg/100 g in fresh hake muscle.

In this study, the increase in TMA content was very slow suggesting its production was limited. However, after 15 days-storage unbled fish had a higher TMA content than bled fish. This difference may be attributed to post-harvest handling of the fish including the slaughter method used (Oehlenschläger, 1992, 1997).

Presence of TMAO in the fish muscle is the primary factor in determining TMA production. Presence and concentration of the enzymes and bacteria responsible for the reduction of TMAO is also of great significance. In a natural production and processing setting, enzymes are present within the fish muscle. However, handling and production/processing conditions may enhance growth and multiplication of the specific spoilage bacteria within the fish muscle or more bacteria may be introduced through unhygienic practices.

The initial loads of the spoilage bacteria and their growth vary with biological factors such as season, fishing grounds, species and post rigor pH. The maximum amount of TMA that can be formed may be dependent on the growth of the relevant micro-organisms rather

than availability of the TMAO substrate. While TMA presence and ammount will vary from species to species, sensitivity and accuracy of the analytical method used and product handling may influence the results.

A VBN content of 25-35 mg/100 g muscle is the acceptable limit for sardines (Ababourch *et al.*, 1996) and 30-40 mg/100 g muscle for cold-water fishes (Connell, 1975). Results of this study showed VBN content below the 30-40mg/100 g muscle standard for cold water fishes after 15 days of storage.

VBN is made up of up to 75% ammonia and other volatile amines (including TMA) from the deamination of ATP-related compounds and amino acids facilitated by bacterial enzymes (Gill, 1990; Huss, 1995; Oehlenschläger, 1997). TMAO content, existence of specific spoilage bacteria, storage temperature as well as post-harvest handling may influence ammonia, TMA and other volatile amines production and so the VBN content (Oehlenschläger, 1992; 1997; Papadopoulos *et al.*, 2003; Castro *et al.*, 2006).

The low values of VBN content may be a reflection of the low TMA content in the amberjack muscle during the storage period. The increase noted after 1 week of iced storage may be due to the production of ammonia within the fish muscle from the deamination of ATP-related compounds and amino acids.

Any factors that limit ammonia or TMA production in the fish muscle will limit VBN content in the muscle. Grigorakis *et al.*,

(2003) studied seasonal patterns of spoilage in ice-stored gilthead sea bream. They noted a slightly lower VBN content in winter fish compared to summer fish. Castillo-Yanez *et al.,* (2006) studied freshness loss in sierra fish stored on ice. They reported that production of VBN followed a similar pattern to that of TMA. In this study, both TMA and VBN content in the amberjack muscle showed increases after 7 days-iced storage showing a similar trend.

In this study, results showed that bled amberjack muscle had a lower VBN content compared to unbled muscle. The differences are noticeable at the point of spoilage indicating that the two different treatment groups spoil at different rates. It can be assumed that bleeding and removal of blood from the fish limits the amount of nutrients that are available for bacterial growth and metabolism. As a consequence, there are limited products of bacterial metabolism that contribute to the total VBN.

It has been argued however, that because of the variation within and between fish species, VBN may not be a very good indicator of product freshness in some species (Ababouch *et al.*, 1996; Rodriguez *et al.*, 1999., Tejada & Huidobro, 2002) but can provide a rough estimate of raw material quality (Oehlenschläger, 1997). This is true in the sense that VBN in part monitors the effects of bacterial action on the fish muscle by monitoring changes in the spoilage phase. Since TMA forms the bulk of the

VBN content and is mainly produced through bacterial action, VBN has more to do with spoilage than freshness.

Ehira & Uchiyama (1974) studied the rates of freshness in cod and sea bream and used the Conway's method to measure TMA and VBN content in the fish muscle. They reported that measurement of TMA may be superior to VBN because unlike VBN, TMA is not influenced by the amount of adenine nucleotides present in the muscle. However, this assumption is only true in those fish species that contain considerable amounts of TMAO and therefore produce TMA. On the other hand, many fresh water fish do not contain TMAO or produce TMA and thus cannot use this criterion for evaluation of spoilage.

One of the issues that arise during the analysis of TMA is the interference from ammonia, DMA and other volatile compounds. It has been suggested that use of formaldehyde, 25% KOH and analysis at low temperatures leads to efficient extraction and reproducible results of TMA measurement. On the other hand, the use of formaldehyde is rather controversial as it raises health and safety issues.

II - 4. Summary

Fish bleeding is an important method for maintenance of amberjack muscle quality during iced storage. Changes in IMP content in bled fish muscle are very slow and a high IMP content is maintained during storage. This maintains the fish muscle flavour

and appeal. Bleeding maintains fish muscle freshness as measured by the *K*-value and is an important attribute in mentainance of quality in the amberjack muscle.

The TMA content in the amberjack muscle is very low even after prolonged storage and not a very useful index for the determination of fish quality. Measurement of VBN in the amberjack muscle seems to be a better index than TMA. The fact that both indices appear to increase after one week-storage may help to determine when the fish begins to spoil and general indicators of spoilage.

Chapter III. Effect of bleeding on the quality of cultured red sea bream muscle

III - 1. Materials and Methods

III - 1 - 1. Fish samples and bleeding method

Live red sea bream *Pagrus major* (mean weight 1.4 Kg) was obtained after harvest from Mie Gyoren (The Federation of Mie Prefectural Fisheries Association), Owase, Japan.

Fish bleeding, sample storage and treatment was as described in II – 1. Sampling was done after 1, 3, 5, 8, 10 and 15 days of iced storage. Samples that were not analysed immediately were stored at -80°C until analysis.

III - 1 - 2. Analytical methods

III – 1 – 2 – 1. ATP-related compounds

Sample preparation and analysis was according to the procedures described in II – 1 – 2 – 1.

III – 1 – 2 – 2. Trimethylamine (TMA) assay

Trimethylamine (TMA) was determined using the picric acid method (Dyer, 1945) as described in II – 1 – 2 – 2.

III – 1 – 2 – 3. Volatile Basic Nitrogen (VBN) assay

Volatile basic nitrogen (VBN) was determined using the micro-diffusion method (Conway, 1947) as described in III – 1 – 2 – 3.

III - 2. Results

III – 2 – 1. ATP-related compounds

III - 2 - 1 - 1. Adenosine triphosphate (ATP)

Samples collected immediately after fish death contained low quantities of ATP in both bled and unbled samples. The average ATP content in 0 day-storage samples was 3.10 μmol/g. However, no ATP was observed in samples collected at 1 day-storage and subsequent days of iced storage in both treatment groups.

III - 2 - 1 - 2. Adenosine diphosphate (ADP)

Samples collected at 0 day-storage had an average ADP content of 0.46 μmol/g muscle. The ADP content decreased rapidly to 0.11 μmol/g muscle on 1 day-storage. This low ADP value remained constant only showing slight variations during subsequent iced storage. There was no significant difference in ADP content between bled and unbled fish samples (Fig. 14).

III - 2 - 1 - 3. Adenosine monophosphate (AMP)

AMP content in the red sea bream muscle decreased rapidly to less than half the initial value on 1 day-storage. In bled samples, the AMP content decreased from an initial 0.26 μmol/g on 0-day storage to 0.04 μmol/g muscle on 15 days-storage. In the unbled samples, it decreased from 0.28 μmol/g to 0.06 μmol/g muscle during the same storage period. There was a significant difference in AMP content ($p \leq 0.05$) between bled and unbled samples on 3- and 5 days-storage (Fig. 15). The AMP content in the fish muscle decreased throughout iced storage.

III - 2 - 1 - 4. Inosine monophosphate (IMP)

The IMP content in the red sea bream muscle increased from the initial 3.68 μmol/g on 0-day storage to 4.81 μmol/g and 4.78 μmol/g muscle in bled and unbled samples respectively on 1 day-storage. This was then followed by a decrease to 3.17 μmol/g and 2.81 μmol/g muscle in bled and unbled samples respectively on 15 days-storage (Fig. 16).

Samples collected immediately after fish death showed considerable high amounts of IMP compared to other ATP-related compounds. The IMP content in bled samples was significantly higher ($p \leq 0.05$) in bled samples compared to unbled samples on 5 days-

storage. The values remained relatively higher compared to other ATP-related compounds throughout the storage period Fig.16).

III - 2 - 1 - 5. Inosine (HxR)

The HxR content in the red sea bream muscle showed increasing trends until after 10 days-storage before showing a decrease on 15 days-storage. In bled samples, the HxR content increased from an initial 0.68 μmol/g on 0-day storage to 2.90 μmol/g muscle on 10 days-storage before decreasing to 2.32 μmol/g muscle on 15 days- storage. In the unbled samples, it increased from the initial 0.70 μmol/g to 2.96 μmol/g muscle before decreasing to 2.28 μmol/g muscle during the same period. Generally, unbled samples had a higher HxR content compared to bled samples but these differences were only significant on 8 day-storage (Fig. 17).

III - 2 - 1 - 6. Hypoxanthine (Hx)

There was no Hx observed in samples collected immediately after fish death in the red sea bream muscle. After 1 day of iced storage, the muscle had an average Hx content of 0.04 μmol/g which increased to 0.84 μmol/g (bled) and 0.98 μmol/g (unbled) on 15 days-storage. After 3 days-storage, unbled samples had a higher hypoxanthine content compared to bled samples (Fig. 18). However,

these differences were significant ($p \leq 0.05$) on 5 days-storage and subsequent days of iced storage.

III - 2 - 1 - 7. *K*-value

Bled samples had an initial *K*-value of 5.8% on 0-day storage which increased to 31.7% on 10 days-storage. In the unbled samples the *K*-value increased from 5.8% to 34.2% on 10 days-storage. Generally, unbled samples had a higher *K*-value compared to bled samples throughout the storage period (Fig. 19). However, the differences in *K*-value between bled and unbled samples were significant ($p \leq 0.05$) on 5, 8 and 10 days of iced storage.

III – 2 – 2. Trimethylamine (TMA)

Generally, the red sea bream contained very low ammounts of TMA throughout the storage period. The TMA content increased from the initial average of 0.20 mg/100 g muscle on 0-day storage to 0.42 mg/100 g (bled) and 0.60 mg/100 g (unbled) on 15 days-storage (Fig. 20). During the first 5 days-storage, there were no significant changes in TMA content in both bled and unbled samples. After 8 days-storage, unbled samples had a significantly higher TMA ($p \leq 0.05$) content compared to the bled samples.

III – 2 – 3. Volatile Basic Nitrogen (VBN)

VBN content in the red sea bream muscle increased with storage time (Fig. 21). It increased from an average 16.15 mg/100 g muscle at 0-day storage to 20.90 mg/100 g (bled) and 24.75 mg/100 g (unbled) on 15 days-storage (Fig. 19). There was no significant difference between bled and unbled samples between 0 and 5 days-storage. Unbled samples had a significantly higher VBN content ($p \leq 0.05$) compared to bled samples after 8 days of iced storage.

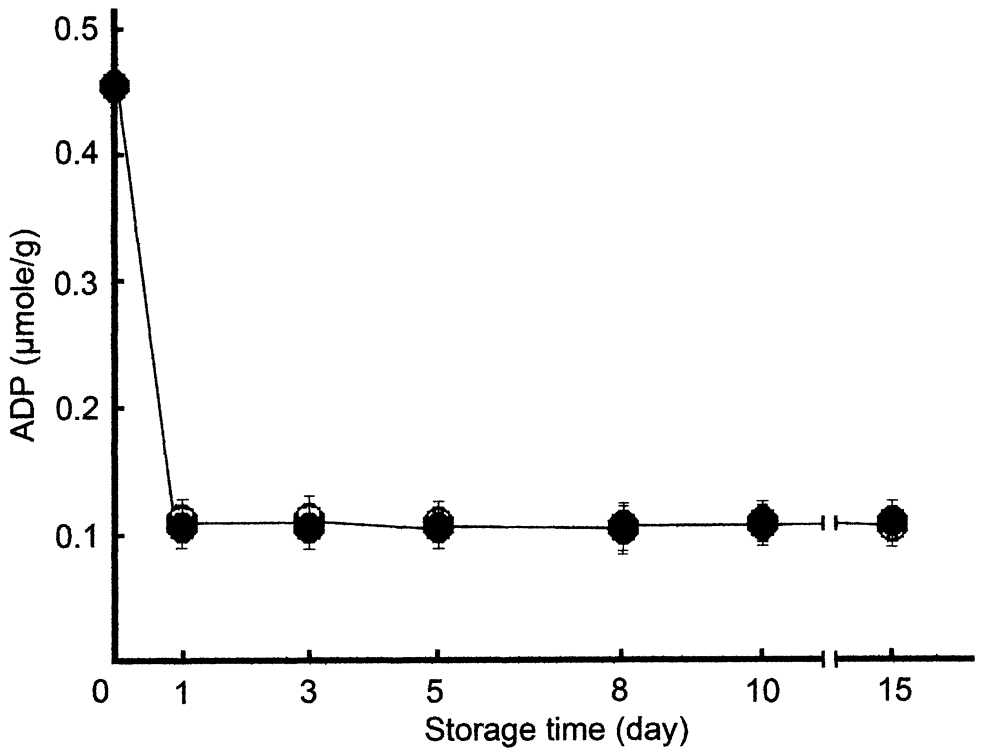

Fig. 14. Changes in ADP content in the red sea bream muscle during iced storage.

(○) represents bled and (●) unbled samples

(mean values and standard deviation).

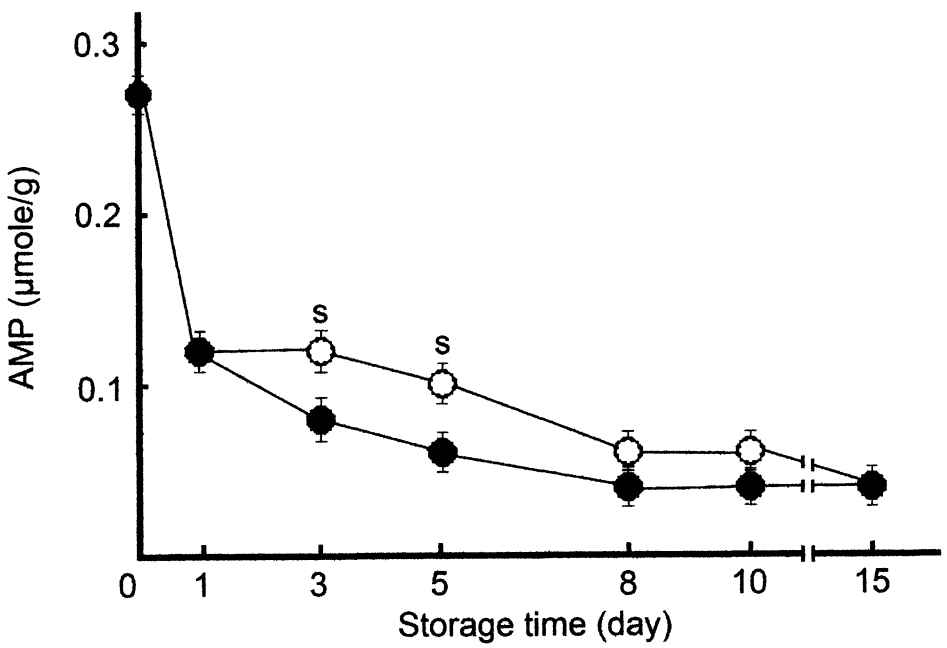

Fig. 15. Changes in AMP content in the red sea bream muscle during iced storage.

(○) represents bled and (●) unbled samples

(mean values and standard deviation).

S: significant differences.

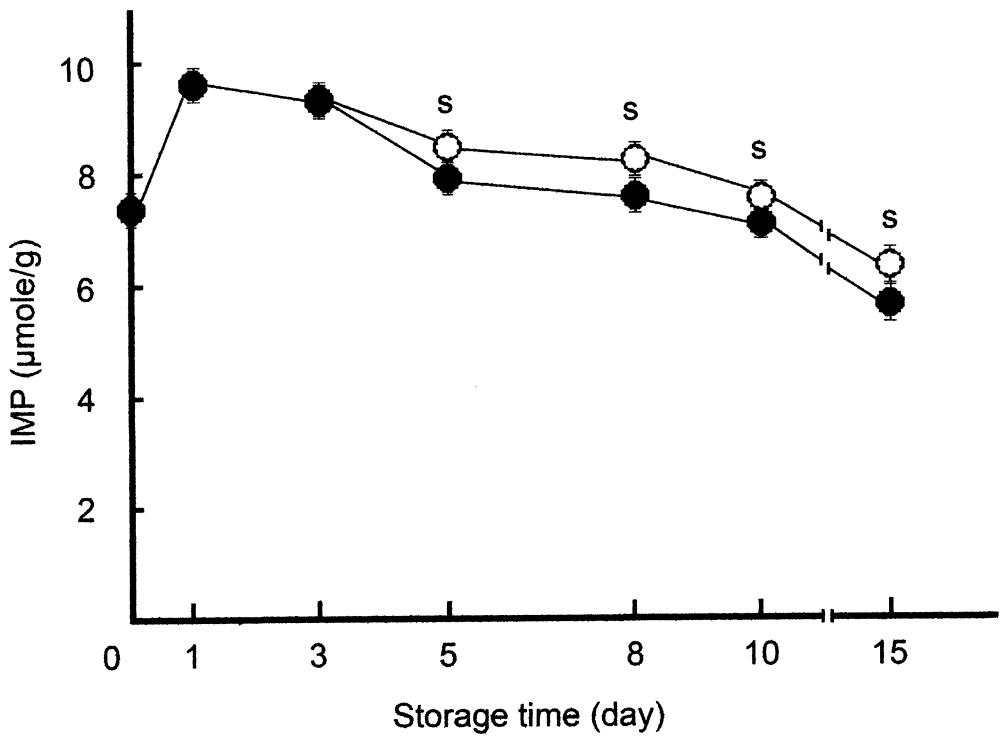

Fig. 16. Changes in IMP content in the red sea bream muscle during iced storage.

(O) represents bled and (●) unbled samples

(mean values and standard deviation).

S: significant differences.

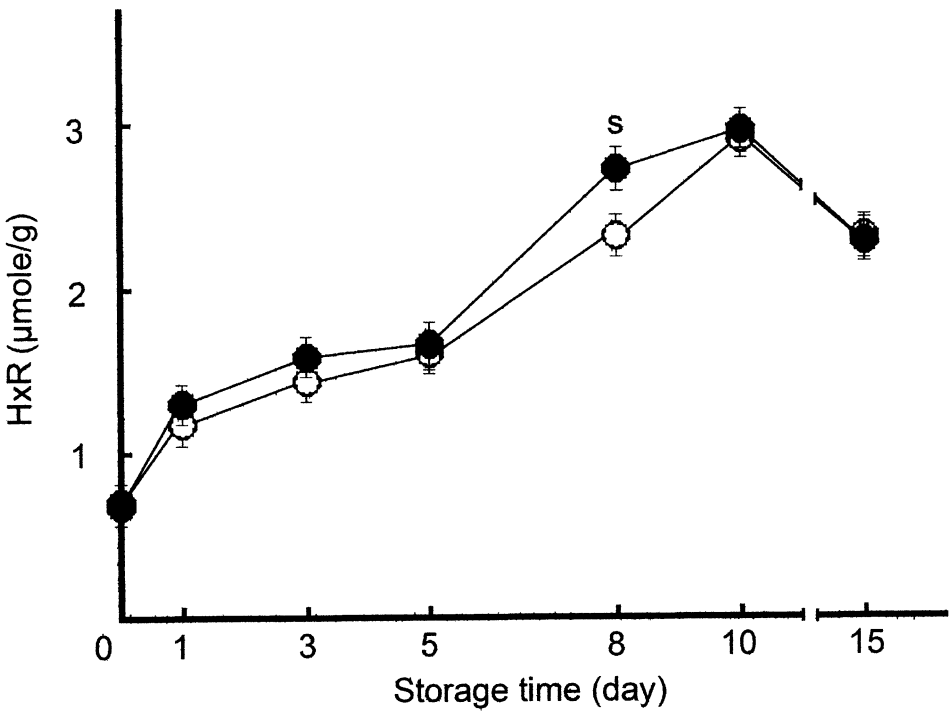

Fig. 17. Changes in HxR content in the sea bream muscle during iced storage.

(○) represents bled and (●) unbled samples

(mean values and standard deviation).

S: significant differences.

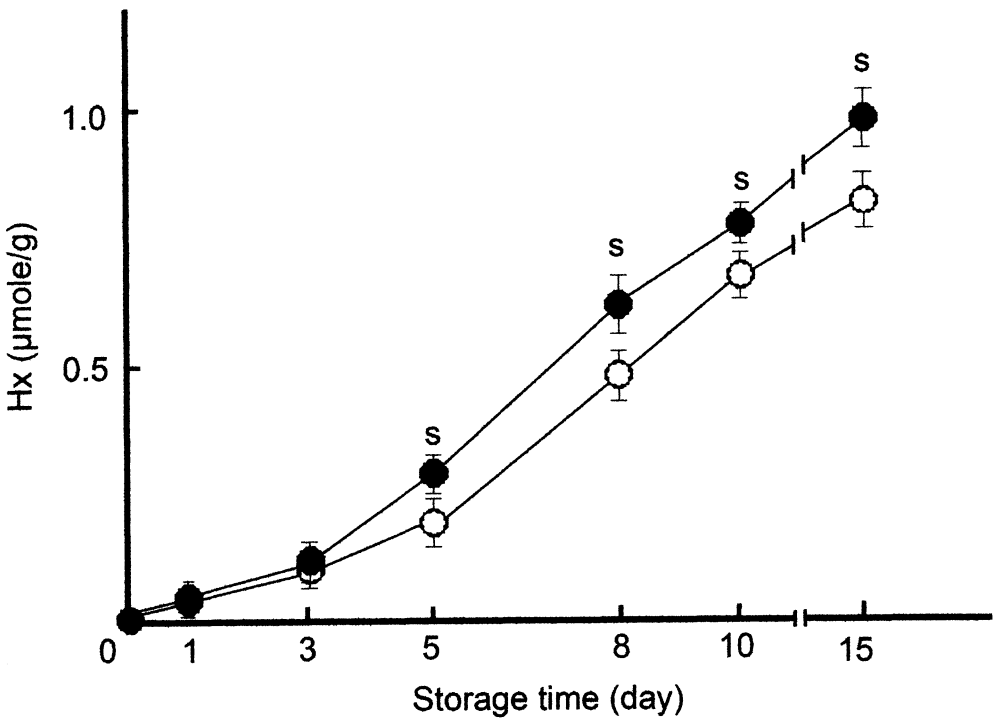

Fig. 18. Changes in Hx content in the red sea bream muscle during iced storage.

(○) represents bled and (●) unbled samples

(mean values and standard deviation).

S: significant differences.

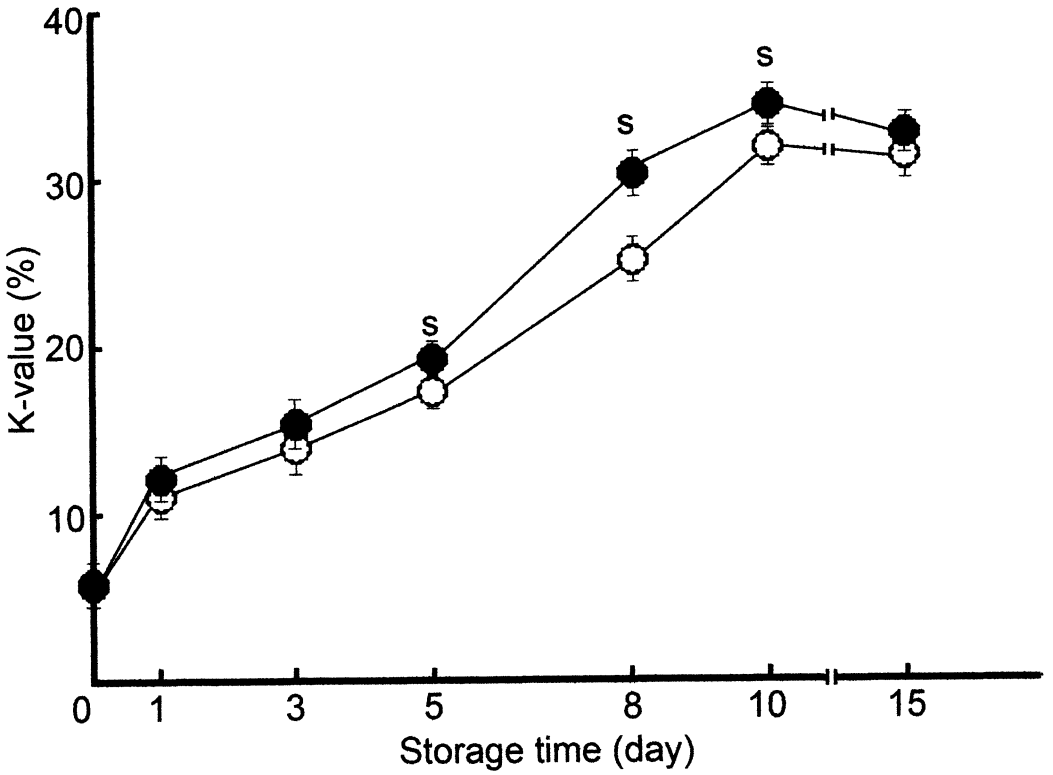

Fig. 19. Changes in the *K*-value in the red sea bream muscle during iced storage.

(○) represents bled and (●) unbled samples

(mean values and standard deviation).

S: significant differences.

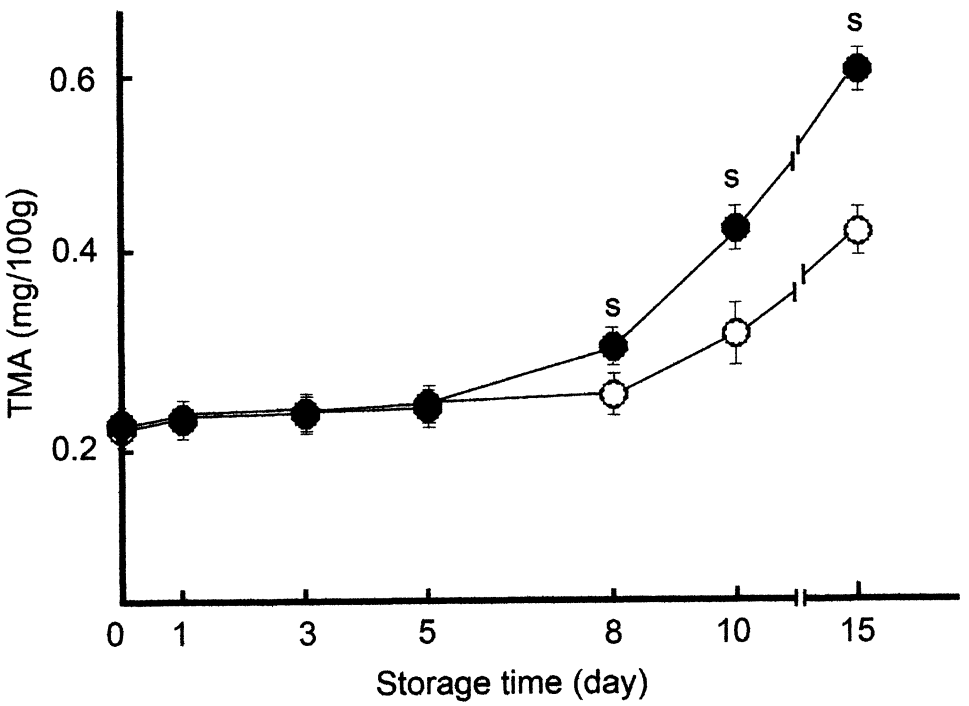

Fig. 20. Changes in TMA content in the red sea bream muscle during iced storage.

(○) represents bled and (●) unbled samples

(mean values and standard deviation).

S: significant differences.

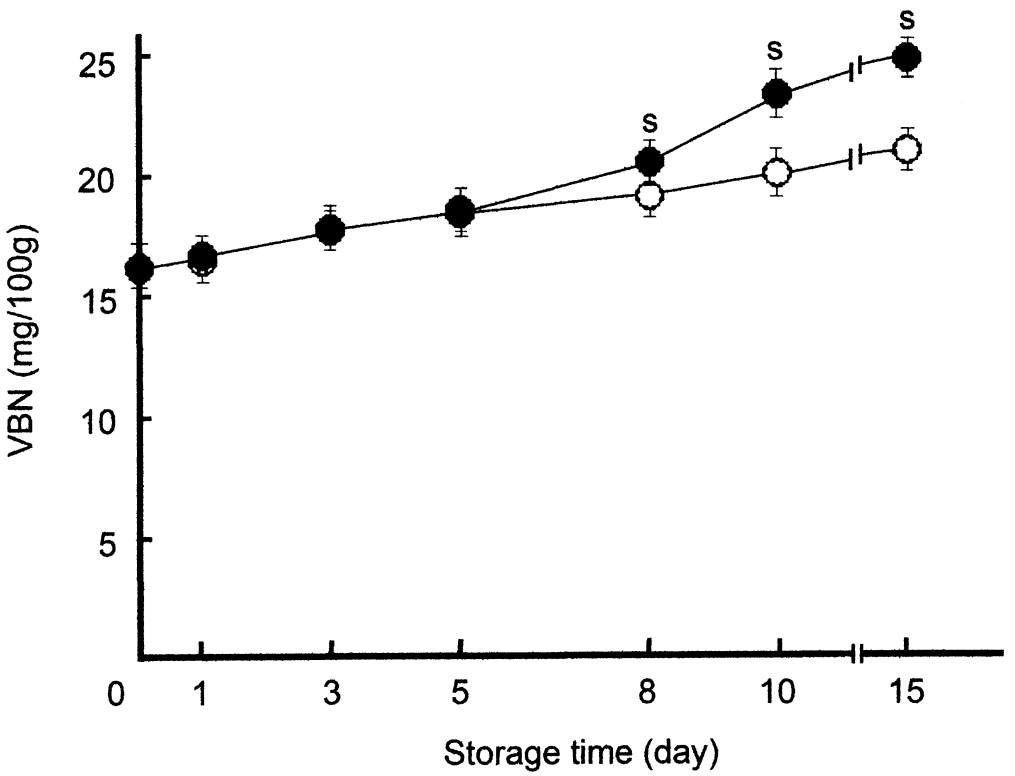

Fig. 21. Changes in VBN content in the red sea bream muscle during iced storage.

(○) represents bled samples and (●) unbled samples

(mean values and standard deviation).

S: significant differences.

III - 3. Discussion

During chilled storage, biochemical changes such as change in protein and lipid fractions, formation of volatile or biogenic amines and Hx take place. This leads to deterioration in sensory quality, loss of nutritional value and negative modifications of fish muscle physical properties (Olafsdottir *et al.*, 1997). Decisions on whether to accept or reject a fishery product can be based on physico-chemical, microbiological or sensory evaluation (Arvanitoyannis *et al.*, 2005).

Freshness and quality are important criteria on which decisions about acceptance of fish as raw materials or products are based. Objective methods for the determination of fish quality are not only important for the seller in grading the fish product but also the buyer who will pay appropriate prices relative to product quality. Determination of the degree of ATP breakdown suits evaluation of fish freshness when high demands for fish freshness are required especially for special products like sashimi and sushi (Ehira & Uchiyama, 1986), traditional Japanese delicacies. The red sea bream is one of the top species used for such special products in Japan.

It was noted that ATP content in the red sea bream muscle disappeared after 1 day of iced storage while ADP and AMP were in very low amounts. Much of the ATP is rapidly converted to at least AMP and sometimes further to IMP during the capture/harvest process and associated struggling. As a result, most fresh fish muscle samples contain low ATP content (Howgate, 2006).

The dephosphorylation of ATP is fast after fish death and during chill storage, and may occur during freezing and thawing of the fish muscle (Kennish & Kramer, 1987). The red sea bream muscle samples were transported frozen on ice and involved thawing/defrosting at 4°C before analysis.

Mazorra-Manzano *et al.*, (2000) studied the skipjack muscle and reported that at 0°C storage, ATP, ADP and AMP decreased very rapidly while IMP content increased sharply within 24 hours. Kuda *et al.*, (2007) reported levels of ATP, ADP and AMP below 0.05 μmol/g in the chub mackerel muscle.

Castillo-Yanez *et al.*, (2007) studied sierra fish and reported barely detectable amounts of ATP, ADP and AMP with a composite figure below 0.2 μmol/g, 12 hours from capture. They contend that these compounds are catabolised during the struggle associated with capture and during the elapsed time from capture to analysis.

Olsen *et al.*, (2006) studied the quality of the Atlantic salmon and noted that the fish is exposed to stress such as handling, transport and during transfer from cages at the fish farms to the slaughter houses. The red sea bream in this study was obtained from a holding cage after capture from the farm. Stress during capture, transport, crowding associated with holding cages and capture from the cage before slaughter could ultimately affect the amount of adenosine compounds in the fish muscle. During struggle, the creatine phosphate and glycogen reserves in the fish muscle may be

exhausted. This will ultimately affect the rate of degradation and amount of ATP, ADP and AMP in the muscle.

Increasing levels in HxR and Hx content accompanied by a decrease in ATP, ADP, AMP and IMP have been reported in several species of fish. Willis *et al.,* (2006) reported ATP degradation and the accumulation of IMP and small amounts of ADP and AMP intermediates in the muscle of rainbow trout killed using different stunning methods. They reported Hx levels below 0.3 μmol/g which is consistent with results obtained for the red sea bream in this study. They concluded that the degree of ATP degradation to IMP varied with the fish stunning methods.

The degradation of IMP in the red sea bream muscle was considerably slow and substantial amounts remained until the end of the storage period. Kuda *et al.,* (2007) studied the chub mackerel and reported 10.9 μmol/g of IMP, 0.04 μmol/g of Hx, 1.16 μmol/g of HxR on 0 day-storage showing a high IMP content compared to other compounds. After storage at 4°C, HxR increased to 4.0 μmol/g before decreasing to 1.2 μmol/g. Although the study was conducted at a higher temperature (4°C), a similar trend of increase and then a decrease was noted in the red sea bream muscle during iced storage in this study.

The decrease in IMP content is due to its breakdown to form HxR and is catalysed by the enzyme 5'-nucleotidase (Okuma & Watanabe, 2002). The decrease in IMP content is therefore followed by an increase in HxR content. The accumulated HxR further breaks

down to Hx which is catalysed by the enzyme nucleoside phosphorylase.

As the degradation of IMP continues together with the formation of HxR, it seems probable that at a certain stage, more of the HxR will be converted to Hx than can be formed from IMP breakdown. When the rate of HxR breakdown proceeds faster than its formation from IMP, the amount of HxR will decrease. In the red sea bream muscle, this was observed after 10 days-storage.

Results in this study show IMP as the major product of ATP degradation in the red sea bream muscle. However, the rate of degradation of IMP in the red sea bream muscle tissue was slow. The slow degradation is probably due to the fact that the red sea bream is a white muscle fish or rather a reflection of the content of the white muscle compared to other muscle types.

Yada *et al.,* (2000) reported that the speed of degradation of IMP in carp was slower in the white muscle compared to red and pink muscle. Mazorra-Manzano *et al.,* (2000) studied the black skip jack muscle and reported 7 μmol/g of IMP on 0 day-storage and 0.24 μmol/g on 24 days-storage, a linear increase in Hx content and a constant level of HxR. These studies show consistency with the amount and rate of degradation of IMP relative to accumulation of HxR and Hx in the red sea bream muscle in this study.

In the red sea bream muscle, IMP content was at its maximum on 1 day- and not on 0 day-storage. IMP content will first increase and then start decreasing (Murray *et al.,* 1985). When ATP is still present,

IMP may not be at its maximum especially on 0 day-storage (Howgate, 2006). The amount of ATP present in the red sea bream muscle on 0 day-storage was probably the limiting factor to IMP reaching its peak.

Levels of IMP have been associated with flavour intensity and acceptability (Bremmer *et al.,* 1988), and a pleasant flavour in meat (Howgate, 2005). High IMP content in the fish muscle therefore implies better flavour and appeal of the fish to the consumers. Post-mortem accumulation of Hx and HxR in the fish muscle may be a reflection of poor quality while presence of HxR or any of the adenosine compounds reflects relatively high quality (Gill, 1992). Based on this, bled red sea bream muscle showing a higher IMP content, lower HxR and Hx content than unbled muscle is judged to have better quality.

While conversion of ATP to IMP is presumed to be totally autolytic, subsequent accumulation of HxR and Hx is thought to be related to both autolytic and microbial action (Jones, 1965; Hiltz *et al.,* 1972). However, microbial activity may be absent or limited immediately after death and in the early stages of iced storage. In this case, accumulation of these compounds will probably be limited to autolytic action. In later stages however, when microbial activity begins, the amount and rate of accumulation of HxR and Hx may be higher.

It can be assumed that there were more nutrients in form of blood for microbial growth in unbled fish muscle and the amount and rate of accumulation of HxR and Hx was higher owing to enhanced microbial growth and metabolism.

In the red sea bream muscle, HxR production was more dominant than Hx production. HxR has also been reported to be dominant in the Atlantic salmon (Erikson *et al.,* 1997). While Hx alone can be used as a freshness indicator (Ehira & Uchiyama, 1973), its accumulation in the fish tissue reflects the initial phase of autolytic deterioration as well as bacterial spoilage (Woyewoda *et al.,* 1986). Hx accumulation is involved in progressive loss of desirable fish flavour leading to off-tastes (Fletcher & Statham, 1988). A variation is believed to exist between fish species; some species produce more HxR than Hx while others produce more Hx than HxR (Ehira & Uchiyama, 1986).

It appears that the decomposition of the red sea bream muscle results into production of more HxR than Hx. Given a lower HxR and Hx content in the bled samples compared to unbled samples, and their reported influence on flavour and taste, bled samples can be assumed to have better flavour and taste.

Gill (1992) argues that because the pattern and rate of nucleotide degradation is species-specific, nucleotide analysis may be of limited use in certain species. They are most useful for the analysis of pelagics, red fish, salmon and squid and of little value for the estimation of lean fish. However, such patterns and trends in nucleotide degradation in various fish species can only be established through careful studies that are relevant to everyday fishing, preservation and processing conditions. Many fish species are yet to be studied and usefulness of nucleotide degradation on muscle quality still remains unknown.

The *K*-value has been described as a very useful index for the determination of freshness in the fish muscle (Ozogul *et al.,* 2002). It is extensively used in Japan (Ehira & Uchiyama, 1986) and is widely acceptable because of the agreement between the rise in *K*-value and progressive loss of fish freshness. Its use is usually more appropriate to determine fish freshness in the early stages of storage (Hamada-Sato *et al.,* 2005). On the other hand, the term remains technical and may not be clearly understood by an average fisherman, fish consumer or trader. Understanding the usefulness of the *K*-value is more appropriate for the more technical industry, the market and government regulatory bodies in the fish industry.

Establishment of standards as well as critical limits is one of the important factors in quality control in the food industry. The *K*-value of fresh fish suitable for raw consumption should be less than 20% (Hoshi *et al.,* 1997). *K*-value limits of 20% (Ehira & Uchiyama, 1986) or 30% (Williams *et al.,* 1993., Chiou *et al.,* 1995) have been proposed for very good quality fish. In salmon, a *K*-value of 70-80% has been proposed for good quality salmon and less than 40% for excellent quality (Erikson *et al.,* 1997). This sets the reference standards upon which the freshness of various fish species can be judged.

In the red sea bream muscle, the initial *K*-value was below 20% after 5 days-storage and still below 40% after 15 days-storage. Sakaguchi & Koike (1992) reported a *K*-value below 20% on 6 days-iced storage in the sea bream muscle. Pacheco-Aguilar *et al.,* (2000) reported a *K*-value of 21.7% on 5 days-storage in sardine muscle.

Kuda *et al.,* (2007) reported an initial *K*-value of 9.8% that increased to 65% on 7 days-storage at 4°C. The differences in the *K*-value are a reflection of the differences associated with species, handling as well as analytical procedures and methods.

The *K*-value is greatly influenced by the rate of decomposition of IMP and this is related to the enzyme phosphatase or 5'-nucleotidase in the muscle (Ehira *et al.,* 1974; Ehira & Uchiyama, 1986). IMP being the major determinant of the *K*-value means that if it is depleted (non-existent) in the muscle, the *K*-value reaches a maximum and will not increase anymore. Also, since IMP degradation is dependent on the enzyme, conditions that favour enzyme activity will lead to rapid degradation of IMP and vice versa. Consequently the rate of increase of the *K*-value will be affected accordingly.

The *K*-value varies from species to species (Luong *et al.,* 1982; Green *et al.,* 1990). A *K*-value of 80% has been proposed as the upper limit for good quality fish (Hattuala *et al.,* 1993). A *K*-value of 25-35% was reported in the gilthead sea bream (Grigorakis *et al.,* 2003) after 15 days-iced storage. Ozogul *et al.,* (2002) studied the rainbow trout stored on ice and reported a *K*-value of 73% after 12 days-storage when the fish was unacceptable based on sensory analysis. Tejada *et al.,* (2007) studied the sole, and sensory rejection was after 22 days-iced storage with a *K*-value below 40%. In the Atlantic salmon, the *K*-value was reported to vary between 40-80% after 7 days-storage (Erikson *et al.,* 1997).

After 10 days-iced storage, the *K*-value in the red sea bream muscle seemed to level off, with no further increase. A similar observation was made by Erikson *et al.,* (1997) in the Atlantic salmon muscle. They reported that *K*-values in salmonids tend to increase sharply before leveling off after 7 days-storage. In the red sea bream muscle, this leveling off after 10 days-storage seems to coincide with a sudden drop in IMP and HxR content. This probably signifies the dependence of the *K*-value on IMP and HxR content. Because IMP production was dominant over both HxR and Hx production, its content as a denominator in the *K*-value equation will have significant effects. Equally important in the *K*-value equation will the HxR content in the muscle.

Yada *et al.,* (2000) in their study on carp reported an increased rate of *K*-value in the red muscle tissue compared to the white muscle tissue. This corresponded to a faster speed of IMP degradation in the red muscle. If IMP degradation results in the production of mainly HxR, the two compounds will be the major determinants of the *K*-value. The rates of degradation of IMP and subsequent accumulation or further degradation of HxR in the fish muscle are critical aspects in the *K*-value determination. Equally important is the distribution (ratio) of the red muscle tissue within the fish species compared to the white muscle, relative to sampling. If during the sampling, more of a particular muscle fibre is collected over the other, different rates of the *K*-value may be obtained.

In the red sea bream muscle in this study, unbled fish had significantly higher *K*-values than the bled fish. In the rainbow trout, Willis *et al.,* (2006) reported that the extent of ATP breakdown to the various compounds varied with the killing method. Manju *et al.,* 2007 studied pearlspot and pomfret and reported a correlation between the *K*-value and sensory rejection. They noted that the higher the *K*-value, the poorer the eating quality. In this respect, in the red sea bream, unbled fish muscle with higher *K*-values would therefore deemed to be of poor quality compared to the bled fish.

Spoiling fish has a foul odour sometimes referred to as a "fishy" smell which has been associated with TMA. The formation of TMA in fish has been reported to be entirely by spoilage bacteria and the TMA content in fish can be associated with bacterial growth and metabolism (Parkin *et al.,* 1981). When bacterial growth is inhibited, TMA production is achieved through intrinsic enzyme activity (Pedrossa-Ringesten, 1990). The quantitative level of TMA in fish is considered a major index of quality of marine fish (Connell, 1975).

Whereas almost all marine fish contain TMAO, a precursor for TMA production, some fish species especially fresh-water fish do not contain TMAO. Exceptions to this have been reported in the Nile perch (*Lates niloticus*) and Nile tilapia (*Oreochromis niloticus*) which contain considerable amounts of TMAO and are capable of producing TMA. In the Nile perch, a TMAO content of 25-35 mg/100 g muscle was reported in 3 Kg fish and 11 mg/100 g in 0.5-1.0 Kg fish. Flat and pelagic fish have been reported to have the lowest amount of TMAO

while cod has a greater amount and the highest is found in elasmobranchs with up to 250 mg/100 g muscle (Huss, 1988).

In the red sea bream muscle, TMA content remained below 0.7 mg/100 g muscle in both bled and unbled samples. In the seabass, Papadopoulos *et al.,* (2003) reported an initial TMA content of 0-0.07 mg/100 g muscle in very fresh samples that increased to 4.40 mg/100 g muscle on 16 days-iced storage. In sierra fish, Castillo-Yanez *et al.,* (2007) reported an initial TMA content of 0.25 mg/100 g muscle that increased to 0.76 mg/100 g muscle on 15 days-iced storage in experimental fish samples. A TMA content of 0.48 mg/100 g muscle was found in consumer samples and increased to 1.27 mg/100 g muscle during the same storage period.

Pacheko-Aguilar *et al.,* (2000) studied seasonal variations in TMA content in sardines stored at 0°C. Samples collected in spring showed an increase in TMA content from 0.33 mg/100 g to 1.35 mg/100 g muscle on 15 days-storage. Winter samples had an increase in TMA content from 0.81 mg/100 g to 1.62 mg/100 g muscle during the same storage period.

The above studies demonstrate that TMA content in the fish muscle varies from species to species. They also show variation with regard to the season when the fish is harvested or captured and to a certain extent the geographical area where the fish is harvested. Some bacteria tend to be in a bloom at certain times of the year and different areas may contain different varieties of bacteria including spoilage bacteria from pollution or otherwise. It is important to note

that, in general, initial TMA content in the fish muscle was below 1.0 mg/100 g.

The TMA content in absolutely fresh fish muscle is on average estimated to be 2 mg/100 g but may vary from 1 mg/100 g to 4 mg/100 g, wet weight (Oehlenschläger, 1997). Ehira & Uchiyama (1987) reported that fish is of medium quality at TMA levels of 1.4 mg/100 g. Huss *et al.,* (1988) reported less than 1.5 mg/100 g of TMA for good quality cold water fish and 10-15 mg/100 g as the limit of acceptability. El Marrakchi *et al.,* (1990) proposed less than 1 mg/100 g muscle as 1st grade, 1-3 mg/100 g as 2nd grade, and 3-5 mg/100 g as being of intermediate freshness. Ryder *et al.,* (1984) reported a TMA content of 2 mg/100 g in mackerel muscle on 12 days-iced storage. In sardines stored on ice, a TMA content of 4.8 mg/100 g was reported on 9 days-storage and 10.8 mg/100 g on 18 days-iced storage (El Marrakchi *et al.,* 1990).

This shows that the TMA content in the fish muscle never reaches the lower limit of 5 mg/100 g in some species and yet can be well above 10 mg/100 g in other species. This can be explained by various factors associated with the fish muscle. The physiological conditions of the fish that ultimately affect growth and metabolism of the spoilage bacteria, the initial load and content of bacteria on the fish, the storage conditions, handling practices, source or origin of the fish as well as the TMAO content in the muscle will all affect TMA production.

A high TMAO content, a high initial load of spoilage bacteria, contamination of the fish during handling and storage by introduction of new or more bacteria, high storage temperatures and capture of fish from contaminated areas will all favour TMAO reduction and thus increased TMA production.

In this study, the TMA content in the red sea bream muscle shows no reasonable increase between 0 and 8 days-storage. Oehlenschläger (1997) also reported that TMA remains constant until the onset of microbial action around 10 days-storage and then increases continuously until spoilage. Because of its universality, TMA can be used as an indicator of the onset of spoilage and for the different stages of spoilage. In the red sea bream muscle, a significant increase was noted after 10 days-iced storage.

In fish muscle, volatile bases result from the degradation of proteins and non-protein nitrogenous compounds (Connell, 1975) and VBN is widely used as indicator of fish deterioration (Olafsdottir, 1997). The VBN content in freshly caught fish is about 5-20 mg/100 g muscle and 30-35 mg/100 g muscle is the limit of acceptability (Huss, 1988). It is generally below 20 mg/100 g but may vary between 10-25 mg/100 g depending on the species and fishing ground (Oehlenschläger, 1997). The proposed maximum acceptable limit for VBN in fish is 25-35 mg/100 g for sardines (Ababouch *et al.,* 1996) and 30-40 mg/100 g for cold water fish (Connell, 1975). In this study, the maximum amount of VBN produced was below 30 mg/100 g on 15 days-storage in both bled and bled fish muscle samples.

Different results on VBN content have been reported for different species of fish. Gram *et al.,* (1989) reported an initial VBN content of 9-11 mg/100 g in very fresh Nile perch that increased to 30 mg/100 g muscle at ambient temperature and 14-24 mg/100 g on 17 h- iced storage. Papadopoulos *et al.,* (2003) studied cultured seabass and reported VBN content of 25.71 mg/100 g on 1 day-storage that increased to 27.72 mg/100 g on 16 days-iced storage in ungutted samples. The values increased from 27.22 mg/100 g on 1 day-storage to 35.95 mg/100 g in the gutted samples during the same period. Ozogul *et al.,* (2006) studied chilled storage of wild turbot and reported a VBN content of 12.1 mg/100 g on 0 day-storage which increased to 31.1 mg/100 g on 19 days-storage. Mazorra-Manzano *et al.,* (2000) studied the black skipjack stored on ice and reported an increase in VBN content from 25.5 mg/100 g on 0 day-storage to 34.3 mg/100 g on 24 days-storage. Baixas-Nogueras *et al.,* (2003) studied the Mediterranean hake and reported an increase in VBN value from 14 mg/100 g on 0 day-iced storage to 40 mg/100 g on 18 days-storage. Results of the initial VBN content in the red sea bream muscle in this study are consistent with, and within range of content level from other studies on marine fish.

VBN increase is thought to be due to increase in ammonia liberated from the deamination of nitrogenous compounds (Sakaguchi *et al.,* 1980). Ammonia represents a major proportion of the volatile amines being a product from several enzymatic processes of spoilage (Gill, 1990). Ammonia is present in marine fish muscle at an average

concentration of 10 mg ammonia-N/100 g, wet weight but may vary between 7 mg/100 g and 15 mg/100 g (Oehlenschläger, 1997). This amount of ammonia adds up to the total volatile nitrogen observed in the red sea bream muscle.

Reduction of TMAO by bacterial enzymes to produce TMA will also result into increased VBN content in the fish muscle (Parkin *et al.*, 1981). It is assumed that any conditions that favour degradation of the muscle will result into elevated levels of VBN. Increased deamination of muscle proteins and non-protein nitrogenous compounds, increased levels of specific spoilage bacteria that reduce TMAO, a favourable temperature and pH for microbial growth and multiplication will all add up to the amount of VBN produced in the muscle. The content of TMAO in the fish muscle is believed to vary between species and within species. The depth at which the fish lives is one of the determinants of TMAO content in the muscle; the deeper the fish lives, the higher the TMAO content. Pelagic species have a lower TMAO content while red fish and elasmobranchs have a higher TMAO content and may be attributed to the enzyme TMAOase demethylase (Oehlenschläger, 1997). The rate of degradation of TMAO can be a function of the amount of the enzyme and substrate present in the fish muscle.

The rate of VBN increase is slowed down when the fish is iced immediately after capture (Karungi *et al.*, 2004). In their study on the Nile perch, VBN content increased faster in iced fish samples after 3 hours and 6 hours compared to fish iced immediately. Similar results

were obtained by Gram *et al.*, (1989) who compared the VBN content in Nile perch stored on ice and at ambient temperatures. These studies show that the rate of VBN production in the fish muscle is dependent on storage conditions such as the holding temperature. The higher the temperature, the faster is the rate of VBN accumulation and the lower the temperature, the slower the rate of accumulation.

Because of differences among trials and with different fish species, VBN is thought not to be a good universal quality indicator with a specific set of criteria and standards applicable to all species (Ababouch *et al.*, 1996). VBN is not homogenous throughout the fish muscle and a decrease may be observed especially during the first week of iced storage due to leakage of ammonia in melting ice. While it is of limited use in small fatty pelagic fish, it can be used as a rough quality check for incoming raw material and as a good spoilage indicator.

In this study, the differences between bled and unbled samples with respect to all the measured indices may be a reflection of the role of blood and its various components on overall fish quality. While blood may contain various enzymes and a medium of transport, it also contains various nutrients and substrates that can be used by micro-organisms and affect product quality. Removal of the blood therefore reduces the amount of the various degradation agents that may be present in the blood and the amount of substrate available for the growth and multiplication of spoilage organisms. As a result, the

unbled fish muscle will lose its quality at different rates compared to bled fish. The rate and differences will be dependent on how much blood the fish contains and is removed, the effectiveness of the blood removal process, and the intrinsic conditions of the fish and product handling.

III - 4. Summary

ATP, ADP and AMP content in the red sea bream muscle decreased very fast during iced storage. Bled red sea bream muscle had a higher IMP content compared to unbled fish which may be a major attribute towards fish taste. The red sea bream muscle accumulated more HxR than Hx during storage. Differences between bled and unbled samples in the TMA and VBN content appeared after 7 days-iced storage. Although the TMA content remains low during storage, VBN accumulation is significant.

Chapter IV. Detection of cathepsin L in red cell membranes from fish blood

IV - 1. Materials and Methods

Live amberjack *Seriola dumerili* (average weight 3.7 kg) and red sea bream *Pagrus major* (average weight 1.4 kg) were obtained from Mie Gyoren, Owase, Japan. Live carp *Cyprinus carpio* was purchased from a local fish market (Tsu, Japan) to serve as a control for the detection of cathepsin L.

Z-Arg-Arg-MCA and Z-Phe-Arg-MCA were purchased from the Peptide Institute Inc. (Osaka, Japan). The anti-human cathepsin L antibody (Carbiochem) was purchased from EMD Bioscience Inc. (La Jolla, CA, USA) and the secondary antibody (goat anti-rabbit IgG-HRP) was purchased from Cosmo Bio Co. Ltd. (Tokyo, Japan). The Blot (Clear Blot Membrane-P) was purchased from ATTO Corp. (Tokyo, Japan). All other reagents were of analytical grade.

IV - 1 - 1. Fish samples and method of collecting blood

Live amberjack and red sea bream were killed by quickly incising the aorta and were allowed to bleed out. The blood was collected through the mouth, and diluted 1:1 with fish Ringer (145

mM NaCl, 5 mM $CaCl_2$, 1 mM $MgSO_4$, 4 mM KCl, 10 mM Hepes, and 5 mM glucose, pH 7.9) (Michel & Rudloff, 1989). The carp were anaesthetized with ethyl 3-aminobenzoate methanesulfonate (MS-222). The carp blood was collected from the dorsal aorta by inserting a heparinized syringe through the mouth. The collected blood was kept on ice before preparation of red cell membranes.

IV - 1 - 2. Preparation of blood red cell membranes

The erythrocyte membranes were prepared using a modified method based on the description of Aoki *et al.,* (1996). The diluted blood with fish Ringer was placed on Ficoll-Paque PLUS (GE Healthcare, Sweden). The mixture was then centrifuged at 400 × g for 40 minutes and the red cells were collected. All further procedures were carried out at 4°C. The red cells were washed three times with fish Ringer then hemolyzed by dilution in a 1:10-15 mixture of ice-cold 5 mM Tris-HCl (pH 7.6) containing 5 mM $CaCl_2$ and 0.15 mM phenylmethylsulfonyl fluoride (PMSF). The suspension was kept on ice for 5 minutes, then centrifuged at 40,000 × g for 20 minutes. The upper precipitate layer was collected and suspended in ice-cold 5 mM Tris-HCl (pH 7.6) containing 0.15 mM PMSF, followed by centrifugation at 40,000 × g for 20 minutes. The precipitate was suspended in a two-fold dilution of buffer A (75 mM Tris, 12.5 mM $MgCl_2$, and 15 mM EDTA, pH 7.5) (Michel & Rudloff, 1989) containing 5 mM $CaCl_2$ and 0.15 mM PMSF. The suspension was then

homogenized with a tight-fitting Dounce homogenizer (10 strokes) and centrifuged at 40,000 × g for 20 minutes. The resulting membrane pellet was re-suspended in buffer A and homogenized (20 strokes). The membrane suspension was then placed on a sugar cushion (40% sucrose, 10 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.5) and centrifuged at 700 × g for 15 minutes in a swing-out rotor. The overlay and inter-phase fractions were collected and centrifuged at 40,000 × g for 20 minutes. The membrane pellets were re-suspended in buffer B (20 mM Tris-HCl, 2 mM EDTA, pH 7.5) (Michel & Rudloff, 1989) and homogenized (10 strokes). The final blood red cell membrane preparation was stored at -20°C.

IV - 1 - 3. Assay of cathepsins L and B

Cathepsin activity was measured following the method of Barret (1980) modified by Aoki *et al.*, (2002). Hydrolysis of Z-Phe-Arg-MCA represented cathepsins (B + L) activity whereas hydrolysis of Z-Arg-Arg-MCA represented the activity of cathepsin B. The substrate stock solution (1 mM Z-Phe-Arg-MCA or 1 mM Z-Arg-Arg-MCA in dimethyl sulfoxide) was diluted to 0.2 mM with distilled water before use. The reaction mixture consisted of 0.5 ml of 0.4 M phosphate buffer (pH 6.2) containing 4 mM EDTA and 8 mM dithiothreitol, 0.1 ml of the blood red cell membrane preparation, 0.9 ml of 0.1% Brij 35, and 0.5 ml of the 0.2 mM substrate solution. The mixture was incubated at 37°C for 15 minutes and the reaction was stopped by the addition of 2

ml of 0.1 M sodium monochloroacetate. One unit of cathepsin activity was defined as the amount required to hydrolyze 1 μmol of aminomethylcoumarin per minute at 37°C.

IV - 1 - 4. Protein concentration

Protein concentration was determined following the method of Lowry *et al.*, (1951) using bovine serum albumin as the standard.

IV - 1 - 5. Sodium dodecyl sulfate - polyacrylamide gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed using 9.0% separating gel and 3.5% stacking gel slabs. A 30 mA current was applied to each slab at room temperature (Leammli, 1970). The protein sample buffer was prepared as described previously (Aoki *et al.*, 1996). The proteins were stained with Coomassie brilliant blue (CBB) R-250. Established procedures for the electroblotting (Hirano & Watanabe, 1990) and immunoblotting (Towbin *et al.*, 1979; Dominingo & Marco, 1989) were followed. Cathepsin L antibody was used in the immunoblot assay.

IV – 2. Results

The activity of cathepsins L and B in amberjack, carp and red sea bream blood red cell membranes was measured (Table 1). The Z-Phe-Arg-MCA hydrolyzing activity was significantly higher in amberjack blood red cell membranes than in the carp. The Z-Arg-Arg-MCA hydrolyzing activity was undetectable in the carp, and was very low in the amberjack compared to the Z-Phe-Arg-MCA hydrolyzing activity.

Table 1. Enzyme activity of fish red cell membranes against synthetic substrates.

Specific activity (units/mg) / Substrate	Carp red cell membranes	Amberjack red cell membranes	Red sea bream red cell membranes
Z-Phe-Arg-MCA	0.66	5.37 (100%)	0.30 (100%)
Z-Arg-Arg-MCA	n.d.	0.69 (12.8%)	0.27 (90.3%)

n.d., Not detected.

Because the Z-Arg-Arg-MCA hydrolyzing activity was relevant to the activity of cathepsin B, these results suggest that cathepsin B existed in amberjack at low amounts, while carp did not contain

cathepsin B in their blood red cell membranes. The Z-Phe-Arg-MCA hydrolyzing activity was detected in the red sea bream, and was lower than in the carp. Cathepsin B also hydrolyzed Z-Phe-Arg-MCA, so this activity was detected as the total activity of cathepsins B and L in the red sea bream. Table 1 indicates cathepsin L activity in the red sea bream blood red cell membranes which was 0.03 units/mg membrane protein and 4.68 units/mg in the amberjack.

IV - 2 - 1. Detection of cathepsin L in the carp red cell membrane preparation

The blood red cell membrane preparation of carp yielded one major diffuse band and several minor bands on the SDS-polyacrylamide gels under CBB staining (Fig. 22 and 23, lane 4). The major diffuse band had a higher molecular weight and was apparent, and corresponded to the human band-3 protein, (Aoki *et al.*, 1996; Barret, 1980). The band 3 protein is a major glycoprotein in the human blood red cell membrane. The molecular mass of the carp band-3 protein was estimated to be 105 kDa, based on a comparison with molecular mass standards. This is the same molecular mass of the human band-3 protein. Two bands appeared on the immunoblot using anti-cathepsin L antibody. These corresponded to 75 kDa and 70 kDa proteins in the carp blood red cell membrane preparation (Fig. 22 and 23, lane 5).

IV - 2 - 2. Detection of cathepsin L in the amberjack blood red cell membrane preparation

The blood red cell membrane preparation of amberjack yielded one major diffuse band and several minor bands on the SDS-polyacrylamide gels under CBB staining (Fig. 22, lane 2). The major diffuse band with a higher molecular mass was considered to correspond to the human band-3 protein. However the molecular mass of the amberjack was 150 kDa which is higher than that of the human band-3 protein (105 kDa).

Two bands appeared on the immunoblot using anti-cathepsin L antibody (Fig. 22, lane 3). These corresponded to 120 kDa and 85 kDa proteins in the amberjack red cell membrane preparation.

IV - 2- 3. Detection of cathepsin L in the red sea bream blood red cell membrane preparation

The blood red cell membrane preparation of red sea bream yielded one diffuse band and several bands on the SDS-polyacrylamide gels under CBB staining (Fig. 23, lane 2). The diffuse band had a higher molecular weight and was considered to correspond to the human band-3 protein, and the molecular mass of the red sea bream band-3 protein estimated to be 150 kDa as the same as the amberjack band-3 protein.

One band appeared on the immunoblot using anti-cathepsin L antibody (Fig. 23, lane 5). This corresponded to 30 kDa protein in the red sea bream red cell membrane preparation.

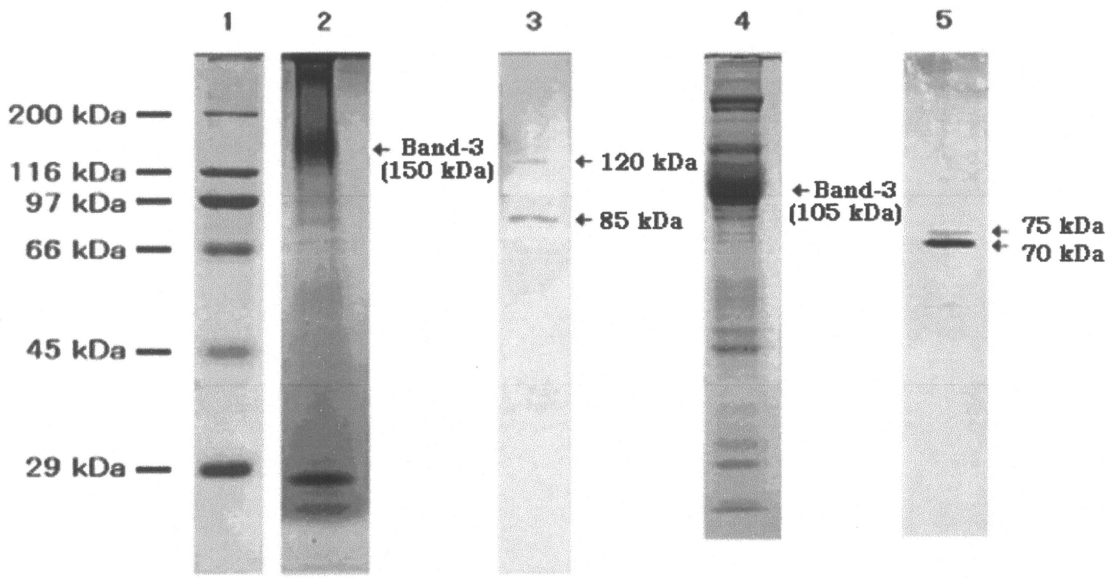

Fig. 22. Detection of cathepsin L in the blood red cell membranes of amberjack and carp.

Lane 1, molecular mass standards: porcine myosin (200 kDa), β-galactosidase(116 kDa), phosphorylase b (97 kDa), bovine albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa); lane 2, 3, amberjack blood red cell membrane preparation; lane 4, 5, carp blood red cell membrane preparation; lane 1, 2, 4, the protein bands were stained with Coomassie blue; lane 3, 5, after blotting onto PVDF membrane, the reaction was done with the antibody to a human cathepsin L.

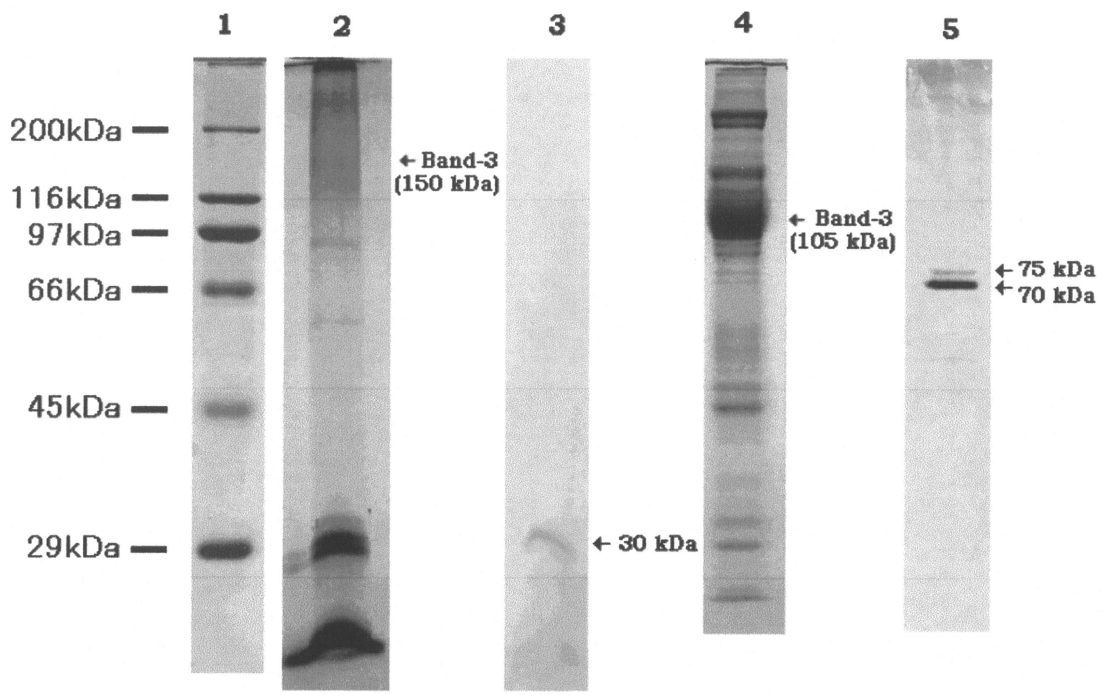

Fig. 23. Detection of cathepsin L in the blood red cell membranes of red sea bream and carp.

Lane 1, molecular mass standards: porcine myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa); lanes 2, 3, red sea bream blood red cell membrane preparation; lanes 4, 5, carp blood red cell membrane preparation; lanes 1, 2,4, the protein bands were stained with Coomassie blue; lanes 3, 5, after blotting onto PVDF membrane, the reaction was done with the antibody to a human cathepsin L.

IV - 3. Discussion

Ando *et al.*, (1999) suggested the possibility that the blood of marine red-flesh fish contains a protease that has collagenolytic activity and causes post-mortem softening of the muscle tissue. Accordingly the protease hydrolyzes collagen at weak acidic conditions; both chum salmon cathepsin L and rat liver cathepsin L hydrolyze collagen (Yamashita & Konagaya, 1991; Kirschke *et al.*, 1982). Lysosomal cathepsin L is also the primary cause of post-mortem autolysis in chum salmon (Yamashita & Konagaya, 1990) and mackerel (Aoki *et al.*, 1997)

The activity of cathepsin L was detected in the carp, amberjack and red sea bream blood red cell membrane preparations. Cathepsin L activity was significantly higher in amberjack blood red cell membranes than in the carp and the red sea bream. These results indicated that the blood of amberjack contained high amounts of cathepsin L as marine red-flesh fish compared to fresh water fish and marine white-flesh fish species.

Analysis on SDS-PAGE showed a diffuse band that corresponded to the band-3 protein but with a higher molecular mass. Band-3 exists as the cytoplasmic domain of the erythrocyte membrane and represents a binding site for a variety of other proteins of the erythrocyte membrane and the cytoplasm including several enzymes (Ruckmann & Schubert, 2002). Band 3, the major integral membrane

protein of mammalian red blood cells, has a molecular mass of 95 kDa and consists of two major domains (Lux *et al.*, 1989).

The NH_2-terminal of 400 amino-acids forms a water soluble, highly elongated cytoplasmic tail that serves as the attachment site for the binding of membrane skeletal and other cytoplasmic proteins (Low, 1986). The remainder of the protein is a 55 kDa hydrophobic membrane domain responsible for catalyzing anion exchange. However, Michel & Rudloff (1989) reported band-3 protein to have a molecular mass of 116 kDa. Using molecular mass standards for comparison, Aoki *et al.*, (1996) estimated the molecular mass of carp band-3 protein to be 105 kDa and the rainbow trout band-3 protein to be 116 kDa. This means that the molecular mass of band-protein varies from species to species between 95 kDa to 116 kDa. The interaction between the band-3 protein and the state of oxygenation of hemoglobin affect various physiological and functional characteristics of the erythrocyte such as glycolysis, ion fluxes and cell deformity (Russo *et al.*, 2008).

Yamashita & Konagaya (1992) reported a cathepsin L-inhibitor complex in the white muscle of chum salmon and latent form cathepsin L with a molecular mass of 50 kDa. The complex was separated into 3 bands on SDS-PAGE corresponding to three forms; pre-form cathepsin L, cathepsin L and the complex.

Aranishi *et al.*, (1997) reported a single protein band on native-PAGE with a molecular mass of 30 kDa which after reduction

migrated as two protein bands corresponding to molecular masses of 30 kDa and 24 kDa on SDS-PAGE.

The cathepsins are synthesized as inactive precursors and are activated by proteolytic removal of the N-terminal polypeptide. In vitro, this can be through activation by other proteases such as pepsin or by autocatalytic activation at acidic pH (Turk *et al.*, 2001).

In the carp and amberjack blood red cell membrane preparations, there were two bands on the immunoblots using anti-cathepsin L antibody. These corresponded to 75 and 70 kDa proteins in the carp preparation and, 120 kDa and 85 kDa proteins in the amberjack. The mature form of cathepsin L typically has a much lower molecular mass. For example, the molecular mass of mature form cathepsin L is 30 kDa in chum salmon (Yamashita & Konagaya, 1991) and 27 kDa in mackerel (Aoki *et al.*, 1997). Conversely, cathepsin L precursors tend to have a higher molecular weight. The precursor of human cathepsin L is 42 kDa, whereas the mature forms are 34 kDa and 26 kDa (Smith *et al.*, 1989). A complex form of cathepsin L (50 kDa) is composed of two forms of cathepsin L and an endogenous inhibitor in chum salmon muscle (Yamashita & Konagaya, 1992). A latent form of cathepsin L, with a higher molecular weight (69 kDa), also exists in lysosomes of mackerel muscle (Aoki *et al.*, 1997).

The estimated molecular masses of cathepsin L in the carp and amberjack blood red cell membrane preparations were higher than that of mature form cathepsin L. This suggests that the carp and amberjack blood red cell membranes contain a cathepsin L precursor.

In the red sea bream blood red cell membrane preparation, one band appeared on the immunoblot using anti-cathepsin L antibody. This corresponded to 30 kDa proteins in the red sea bream preparation. This suggests that the red sea bream blood red cell membranes contain the mature form of cathepsin L.

Inappropriate action by the cathepsins is controlled by cystatins (stefins, cystatins and kininogens) which are the endogenous lysosomal cystein protease inhibitors (Turk *et al.*, 2001). However, disturbance of the normal balance of enzymatic activity may lead to pathological conditions (Kirsche *et al.*, 1995). The pH, temperature and ionic strength around the lysosomes affect release of the cathepsins. Presence or absence of the natural inhibitors will equally determine what happens in the cell. Although the activity of cathepsin L may decrease with the amount of ageing, which may be attributed to changes in muscle pH, it does not totally preclude them from being active. In the mackerel muscle, Aoki & Ueno (1997) observed a decrease in the activity of cathepsin L to ca. 30% after 7 days ageing at 0°C.

Cathepsins L and L-like have a high potential for post-mortem muscle tenderization. Cathepsin L has been found in macrophage-like phagocytes between muscle fibres of mature salmon white muscle during spawning and is highly correlated to muscle softening (Yamashita & Konagaya, 1990, 1991). Immediately after death, when microbial activity is limited, cathepsins may play a major role in

muscle degradation, and may be a precursor to loss of fish muscle freshness and lead to spoilage.

In the previous chapters, the effect of bleeding on the quality of amberjack and red sea bream muscles was evaluated, and the muscle of amberjack (marine red-flesh fish) was more affected than the red sea bream (marine white-flesh fish) muscle. The red blood cells are gradually hemolyzed after death and this process progresses during storage and post-mortem autolysis. From the results in this study, it is suggested that this process releases cathepsin L from blood red cell membranes and would affect the breakdown and quality of fish muscle tissues.

IV - 4. Summary

In both the amberjack and red sea bream, blood red cell membrane preparations under CBB yielded one major diffuse band that corresponded to the human band-3 protein but with a higher molecular mass than the human band-3 protein. The molecular mass was estimated to be 150 kDa based on comparison with molecular standards. Immunoblotting using anti-human cathepsin L antibody yielded one band in the red sea bream with a molecular mass of 30 kDa and two bands in amberjack with molecular masses of 120 kDa and 85 kDa. The blood red cell membrane of the amberjack contains a cathepsin L precursor while that of the red sea bream contains a mature form of cathepsin L. However, the blood red cell membrane

cathepsin L activity was higher in the amberjack compared to the red sea bream. Given the activities and role of cathepsin L in fish post-mortem autolysis, freshness and quality of the fish muscle will be greatly affected when the conditions are favourable for cathepsin activity.

Chapter V. Synopsis

The effect of bleeding on fish muscle freshness and spoilage in cultured amberjack and red sea bream during iced storage and the existence of cathepsin L in fish blood was investigated. Cathepsin L plays a major role in post-mortem fish muscle autolysis and protein degradation leading to loss of freshness and quality.

ATP-related compounds and the *K*-value were used as freshness indices and measured using HPLC. TMA was measured using Dyer's picric acid method while VBN was measured using Conway's micro-diffusion method. Both TMA and VBN content were used as indicators of spoilage. Fish muscle samples for the determination of freshness and spoilage were collected on 0, 1, 3, 5, 7 or 8, 10, 15 days-storage. Fish blood was collected immediately after killing the amberjack and red sea bream and from live carp's dorsal aorta. Existence of cathepsin L was investigated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting using human anti-(cathepsin L) antibody.

The levels of ATP, ADP and AMP in the fish muscle rapidly decreased throughout iced storage and only a small amount of ADP and AMP was observed in subsequent sampling. IMP was the main degradation product in both the amberjack and red sea bream muscle. IMP content decreased from 7.36 μmol/g on the first day of sampling to 6.32 μmol/g in bled samples and 5.60 μmol/g in the unbled samples in the red sea bream. In the amberjack muscle, the values

varied from 7.78 μmol/g on the first day of sampling to 3.02 μmol/g in bled samples and 2.50 μmol/g in unbled samples on 15 days-iced storage. Its content remained higher than any other ATP-related compounds throughout the storage period. In both amberjack and red sea bream muscles, there was no Hx observed in the muscle on 0 day-storage. Subsequent sampling showed a higher Hx content in the unbled samples than in the bled samples which increased throughout storage.

HxR content in both species increased throughout storage and differences between bled and the unbled samples only became noticeable after 7 days-storage. In the amberjack muscle, the *K*-value increased from an average 10% on 0 day-storage to 54% in bled samples, and 58% in the unbled samples on 15 days-storage. Red sea bream muscle had an average *K*-value of 5.8% on 0 day-storage and this increased to 31.2% in bled samples and 32.6% in unbled samples on 15 days-storage.

The average TMA content was low in both amberjack and red sea bream muscles and remained below 1 mg/100 g on 15 days-storage. Lower values of TMA were observed in bled samples compared to the unbled samples on 7- and 8 days-storage in both amberjack and red sea bream muscles respectively.

VBN content in the amberjack muscle increased from an average value of 10.1 mg/100 g on 0-day storage to average 20.5 mg/100 g in bled and 24 mg/100 g in the unbled samples on 15 days-storage. Bled samples had significantly lower VBN values for the entire

storage period from 7 days-storage compared to the unbled samples. In the red sea bream muscle, VBN content increased from an average of 15.1 mg/100 g on 0 day-storage to 20.1 mg/100 g muscle in bled samples and 25 mg/100 g muscle in the unbled samples on 15 days-storage. Bled samples had significantly lower VBN content compared to the unbled samples after 8 days-storage.

Both amberjack and red sea bream blood red cell membrane preparations yielded one major diffuse band on SDS-PAGE gels under Coomassie brilliant blue staining that corresponded to the human band-3 protein but had a higher molecular mass (150 kDa) than the human band-3 protein (105 kDa). In the amberjack blood red cell membrane preparations, two bands appeared on the immunoblot using anti-cathepsin L antibody that corresponded to 120 kDa and 85 kDa proteins. However, only one band appeared on the immunoblot and corresponded to 30 kDa protein in the red sea bream blood red cell membrane preparation. The amberjack blood contained an active precursor form of cathepsin L while red sea bream blood contained a mature form of cathepsin L.

These results suggest that fish blood contains cathepsin L that may be a cause of post-mortem muscle autolysis. Fish bleeding results into low *K*-values and VBN content and thus may help preserve freshness and quality.

Fig. 24. Collection of blood (red sea bream).

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