Mini Review

Proteases in fish and shellfish: Role on muscle softening and prevention

*Sriket, C.

Program in Food Science and Technology, Faculty of Agriculture, Ubon Ratchathani Rajabhat University, Ubon Ratchathani, 34000, Thailand

Abstract

Textural quality of fish and shellfish is the most important factor because it limits consumer acceptance as well as market price. The muscle softening or mushiness of fish and shellfish during storage or distribution in ice is generally occurred. This phenomenon is considered as the muscle protein degradation due to the proteolytic activity. The presence of active proteases in muscle and digestive organ makes the flesh fish and shellfish prone to degrade especially during iced storage, since the digestive organ is not practically removed prior to storage. The digestive tracts have been known to have high proteolytic or collagenolytic enzymes. During storage of fish and shellfish, the intensive hydrolysis of myofibrillar and collagenous proteins by proteases can be observed. To lower the muscle degradation, different pre-treatment methods as well as protease inhibitors have been applied in the stored fish and shellfish. Thus, the knowledge gained can be then transferred to the seafood processors for the quality improvement of fish and shellfish, especially those with iced storage, leading to a full market value of fish species.

Keywords

Protease
Softening
Mushiness
Muscle degradation
Protease inhibitor
Prevention
Postmortem
Digestive enzyme

Introduction

Soft or mushy texture of fish and shellfish seems to be the most critical because it limits shelf-life, thereby impeding its marketing. During postmortem handling and storage, fish and shellfish proteins can be degraded by endogenous or microbial proteases (Shigemura et al., 2004; Sriket et al., 2011c). The autolysis of nucleotides as well as nitrogenous compounds becomes more intense after the prolonged storage, particularly with inappropriate conditions (Selvakumar et al., 2002; Aubourg et al., 2007).

Generally, fish and shellfish are locally distributed in ice, which renders them easily susceptible to a textural problem called “softening or mushiness”. This deterioration is usually influenced by the activity of digestive enzymes during iced storage which accordingly limits a shelf-life up to a week (Pornrat et al., 2007; Sriket et al., 2010). The development of mushiness in ice-chilled fish and shellfish has been described as the gradual and sequential degradation of muscle tissue, including the perimysium and endomysium connective tissue, as well as the proteins localized in Z-line and H-zones, caused by the action of digestive or hepatopancreatic enzymes (Figure 1) (Papadopoulos et al., 1989; Pornrat et al., 2007; Sriket et al., 2010). Fish and shellfish digestive organs such as hepatopancreas contain both peptidase and proteinase activities such as aminopeptidase, gelatinolytic proteases, trypsin and chymotrypsin, and collagenolytic proteases (Cao et al., 2000b; Aoki et al., 2003; Sriket et al., 2011a). Among those enzymes, collagenolytic enzyme has the pronounced impact on the softening of muscle (Brauer et al., 2003; Sriket et al., 2011c). Collagenases are defined as proteases capable of degrading the native triple helix of collagen under physiological conditions (Aoki et al., 2003). Serine proteases and collagenases have been purified from hepatopancreas of several fish and shellfish species, including carp (Cao et al., 2000b) shrimp, *Pandalus eous* (Aoki et al., 2003), white shrimp, *Penaeus vannamei* (Carlos Sainz et al., 2004), shrimp, *Pandalus borealis* (Hernandez-Cortes et al., 1997; Aoki et al., 2004a) and freshwater prawn, *Macrobrachium rosenbergii* (Sriket et al., 2012b).

However, indigenous proteases in muscle may also be involved in softening (Yoshida et al., 2009; Felberg et al., 2010; Sriket et al., 2011a). Therefore, the better understanding of the role of proteases, especially those with collagenolytic activity, in softening phenomenon would help the farmers or processors to prevent or retard the quality losses associated with those proteases during post-mortem handling or storage. As a consequence, the prime quality of fish and shellfish with high market value could be maintained and minimized the economical losses. Thus, this review article focuses on describing the softening phenomenon of fish and shellfish muscle as influenced by indigenous proteases which can help in farming strategies to retard the muscle softening.
caused by those enzymes.

**Proteolytic enzymes in fish and shellfish**

**Classification of proteases**

Enzymes that hydrolyse peptide bonds can be grossly grouped into two subclasses, exopeptidases and endopeptidases, depending on where the reaction takes place in the polypeptide substrate (Sternlicht and Web, 2001). Exopeptidases cleave peptide bonds at the amino or carboxyl ends of the polypeptide chain, whereas endopeptidases cleave internal peptide bonds (Sternlicht et al., 2001). Regardless of the source, proteases can be classified on the basis of their similarity to well-characterised proteases, such as trypsin-like, chymotrypsin-like, chymosin-like or cathepsin-like (Klomklao, 2008). They may also be classified on the basis of their sensitivity to pH, including acid, neutral or alkaline proteases. They are also often classified according to their substrate specificity, the response to inhibitors or by their mode of catalysis (Simpson, 2000).

The standard method of classification proposed by the Enzyme Commission (EC) of the International Union of Biochemists (IUB) is based on the mode of catalysis. This divides the proteolytic enzymes into four groups: serine, cysteine, aspartic and metalloproteases (Table 1). The name of each class is derived from a distinct catalytic group involved in the reaction (Rao et al., 1998).

**Serine proteases**

Serine or alkaline proteases are so-named because they have a “super-reactive” serine in the active site (Simpson, 2000). Two distinct families can be classified according to their structural homology to trypsin and subtilisin. The trypsin family is the largest enzyme found in both mammalian and bacterial members. Some common examples are the pancreatic digestive enzymes such as trypsin, chymotrypsin and elastase; as well as the blood-clotting enzymes such as thrombin, plasmin and many complement enzymes. In contrast, the subtilisin family is only found in bacteria (Hamilton et al., 2003).

Serine proteases are generally active at neutral and alkaline pH, with an optimal pH range of 7-11. Their molecular masses range between 18 and 35 kDa (Rao et al., 1998; Klomklao, 2008). The isoelectric points of serine proteases are generally between pH 4 and 6. Trypsins (EC 3.4.21.4), mainly members of a large family of serine proteases, specifically hydrolyze proteins and peptides at the carboxyl side of arginine and lysine residues (Klomklao et al., 2006). Trypsins play major roles in biological processes including digestion, activation of zymogens of chymotrypsin and other enzymes (Cao et al., 2000a). Trypsins from fish resemble mammalian trypsins with respect to their molecular mass (22-30 kDa), amino acid composition and sensitivity to inhibitors. Their optimal temperature for hydrolysis ranged from 35 to 65°C. Heat stable and/or activated serine proteases were also reported (Nalinanon et al., 2008; Ahmad et al., 2011b). These enzymes are synthesised as inactive zymogens or pro-enzymes which could be activated by proteolytic cleavage. Trypsin has been purified from many kinds of fish and shellfish (Aoki et al., 2003; Klomklao et al., 2010; Sriket et al., 2012b). Serine collagenases or trypsin-like proteinase were found in the intestines of Atlantic cod, Gadus morhua (Hernandez-Herrero et al., 2003), filefish (Kim et al., 2002) and the hepatopancreas of Northern shrimp, P. eous (Aoki et al., 2003), king crab, Paralithodes cantschaticus (Rudenskaya et al., 2004) and freshwater prawn, M. rosenbergii (Sriket et al., 2012b). Chymotrypsin was isolated from the hepatopancreas of Chinese shrimp, Fenneropenaeus chinensis (Shi et al., 2008) and viscera of Monterey sardine, Sardinops sagax caerulea (Castillo-Yanez et al., 2009).

Trypsin activity was dependent on fish species and pH values, where the neutrality or higher pHs were optimal for hydrolytic activity (Hultmann and Rustad, 2004). The fish and shellfish serine collagenolytic enzyme was relatively stable within the pH range of 6-11 (Aoki et al., 2003; Klomklao et

<table>
<thead>
<tr>
<th>Type of protease</th>
<th>Active site</th>
<th>Optimal pH</th>
<th>Molecular mass (kDa)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Trypsin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Chymotrypsin</td>
<td>Cystein</td>
<td>5-7.5</td>
<td>28</td>
<td>Larsen et al. (2006)</td>
</tr>
<tr>
<td>- p-Carboxypeptidase</td>
<td>Serine</td>
<td>80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- m-Carboxypeptidase</td>
<td>Cystein</td>
<td>28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Cathepsin B H L</td>
<td>Aspartic acid</td>
<td>2-4</td>
<td>46-48</td>
<td>Hughes et al. (2003)</td>
</tr>
<tr>
<td>- Cathepsin D</td>
<td>Aspartic acid</td>
<td>2-4</td>
<td>46-48</td>
<td>Hughes et al. (2003)</td>
</tr>
</tbody>
</table>

**Figure 1. Model of muscle protein degradation by proteolytic cleavage**

![Diagram of muscle protein degradation](image)
Cysteine proteases

This family includes several mammalian lysosomal cathepsins, the cytosolic calpains (calcium-activated) as well as several parasitic proteases. The most important cysteine proteases in mammals are cytoplasmic calpains and lysosomal cathepsins (Tetsumori, 2004). Calpains are cysteine proteases which need calcium ions for enzyme activation. Two types of calpain have been isolated which differ in their calcium requirement (µ-Calpain and m-Calpain) (Larsen et al., 2004). The pH optimum is neutral to weakly alkaline (pH ~ 7.5) (Maddock et al., 2005).

Calpain is primarily active within the first 24 hours postmortem (Camou et al., 2007). Bonnal et al. (2001) showed that dystrophin can be used as a pertinent indicator of the early proteolytic process as it is highly sensitive to calpain action. Dystrophin, a subsarcolemmal actin-binding protein (ABP) located in costameric structures, ensures a link between the actin cytoskeleton and the extracellular matrix through an association with a glycoprotein complex.

Lysosomal cysteine proteases, generally known as the cathepsins, play an important role in many physiological processes such as protein degradation (Turk et al., 2000). Cathepsins are mostly active at weakly acid pH values (pH 5). Among lysosomal enzymes, cathepsins B, C, H, L and S have been purified and characterised from fish and shellfish muscles and are the major proteases which participate in intracellular protein breakdown (Aoki et al., 2004b; Pangkey et al., 2000). Lysosomal membranes may lose their integrity under postmortem conditions, resulting in a release of catheptic enzymes into the sarcoplasm (Zeece and Katoh, 1989; Balti et al., 2010). Although the muscle cathepsins generally are most active at pH 3-4, some of them retain fairly high activity up to pH 7.0. The activity of several cathepsins is negligible at low temperature (Kolodziejska and Sikorski, 1997; Balti et al., 2010).

Cathepsins B, H and L activities of fish in spawning period were 3-7 times higher than those in feeding period, whilst the activities of metabolic enzymes decreased (Ashie and Simpson, 1997). In salmon muscle, the increased levels of cathepsins are considered to play an important role in the physiological changes occurring along with sexual maturation in spawning migration (Riley, 2005). Bahuaud et al. (2010) suggested that cathepsins B and L were the main enzymes responsible for softening of Atlantic salmon (Salmo salar L.) muscle. Cathepsin L is activated at high temperature. Cathepsin L was a predominant protease responsible for autolysis of arrowtooth flounder muscle at high temperatures (Visessanguan et al., 2001). In addition to hydrolyzing myofibrillar proteins, cathepsin L was reported to have high activity against various collagens. Thus, it is presumed to cause partial disintegration of the original extracellular matrix structure, which may play an important role in tissue softening of fish and shellfish.

Aspartic proteases

Aspartic proteases, commonly known as acidic proteases, depend on aspartic acid residues for their catalytic activity. Aspartic proteases are produced by a number of cells and tissues. Most of the aspartic proteases belong to the pepsin family. The pepsin family includes digestive enzymes such as pepsin and chymosin as well as lysosomal cathepsins D and processing enzymes such as rennin and certain fungal proteases (penicillopepsin, rhizopuspepsin, endotheiapepsin) (Hughes et al., 2003). These enzymes are active predominantly in the acidic range of pH 2-4.

Cathepsin D shows some activity in the lowest pH range prevailing postmortem in some fish. However, it is still uncertain whether it can be regarded as a very significant factor in softening of refrigerated fish of most species. Aoki (2000) detected cathepsin D in red or white muscle among 24 species, and no difference was found between red- and white-flesh fish, or freshwater fish. Wang et al. (2007) found the low activity of cathepsin D in three species including Atlantic herring (Clupea harengus L.), Atlantic salmon (Salmo salar L.) and wolffish (Anarhichas lupus L.).

Metalloproteases

The metalloproteases include enzymes from a variety of origins, such as collagenases from higher organisms, toxins from snake venoms, and thermolysin from bacteria. They contain a zinc atom which is catalytically active. In some cases, zinc may be replaced by another metal such as cobalt or nickel without loss of activity (Carmeli et al., 2004). The matrix metalloproteases (MMPs) are a family of zinc endopeptidases which are responsible for the degradation of collagen in extracellular fluids (Carmeli et al., 2004).

Many metalloproteases contain the sequence of His-Glu-Xaa-Xaa-His (HGXXH), which provides two histidine ligands for the zinc, whereas the third ligand is either a glutamic acid (thermolysin, nephrilysin, alanyl aminopeptidase) or a histidine (astacin) (Dauch et al., 1995; Kadonosono et al., 2007). The catalytic mechanism leads to the
Table 2. Endogenous proteases involved in muscle softening of fish and shellfish

<table>
<thead>
<tr>
<th>Fish species</th>
<th>Quality deterioration</th>
<th>Enzymes involved</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tilapia (Oreochromis niloticus)</td>
<td>Muscle softening</td>
<td>Serine and metalloproteases</td>
<td>Ishida et al., 2003</td>
</tr>
<tr>
<td>Cod, Spotted wolfish and Atlantic salmon (Hippoglossus stenolepis)</td>
<td>Fillet softening and muscle gaping</td>
<td>Metalloproteases</td>
<td>Lødemel et al., 2004</td>
</tr>
<tr>
<td>Silver carp (Hypophthalmichthys molitrix)</td>
<td>Muscle degradation</td>
<td>Myofibril-bound serine protease</td>
<td>Cao et al., 2004</td>
</tr>
<tr>
<td>Atlantic salmon (Oncorhynchus keta)</td>
<td>Fillet softening</td>
<td>Cathepsin and collagenase-like enzyme</td>
<td>Hultmann and Rustad, 2004</td>
</tr>
<tr>
<td>Seabass (Dentex dentex)</td>
<td>White muscle deterioration</td>
<td>Calpain</td>
<td>Delbarre-Ladrit et al., 2004</td>
</tr>
<tr>
<td>White Croaker (Argyrosomus argentatus)</td>
<td>Muscle degradation</td>
<td>Trypsin-like enzyme</td>
<td>Cao et al., 2005</td>
</tr>
<tr>
<td>Seabass (Dentex dentex)</td>
<td>Postmortem softening of fish muscle</td>
<td>Cathepsin Band L</td>
<td>Che et al., 2007</td>
</tr>
<tr>
<td>Atlantic salmon (Oncorhynchus keta)</td>
<td>Muscle softening</td>
<td>Metalloproteases</td>
<td>Wu et al., 2008</td>
</tr>
<tr>
<td>bream (Pagrus major)</td>
<td>Muscle softening, collagen degradation</td>
<td>Serine collagenolytic protease</td>
<td>Wu et al., 2010</td>
</tr>
</tbody>
</table>

**Digestive proteases:**

<table>
<thead>
<tr>
<th>Fish species</th>
<th>Quality deterioration</th>
<th>Enzymes involved</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>White shrimp (Penaeus vannamei)</td>
<td>Mushy texture</td>
<td>Collagenolytic enzymes</td>
<td>Brauer et al., 2005</td>
</tr>
<tr>
<td>Shrimp (Penaeus orientalis)</td>
<td>Mushy texture</td>
<td>Trypsin and collagenase-like enzyme from hepatopancreas</td>
<td>Oh et al., 2000</td>
</tr>
<tr>
<td>Herring</td>
<td>Belly burst</td>
<td>Serine collagenolytic enzyme leaked from pyloric caeca</td>
<td>Felberg et al., 2010</td>
</tr>
<tr>
<td>Freshwater prawn (Macrobrachium rosenbergii)</td>
<td>Muscle softening and mushy texture</td>
<td>Trypsin-like collagenase from hepatopancreas</td>
<td>Sriket et al., 2012</td>
</tr>
</tbody>
</table>

formation of a non-covalent tetrahedral intermediate after the attack of a zinc-bound water molecule on the carbonyl group of the scissile bond (Skiles et al., 2004). This intermediate is further decomposed by transfer of the glutamic acid proton to the leaving group (Graycar, 1999).

**Role of proteases in fish and shellfish muscle softening**

The biochemical change caused by endogenous enzyme, including proteases, is the primary cause of quality loss in fish and shellfish during iced storage (Brauer et al., 2003; Hultmann et al., 2007). Moreover, proteases can be directly responsible for unusual textural defects in seafood, e.g. ‘gaping’ and ‘mushiness’ of bony fish and ‘tail meat’ softening of crustacean (Pormrat et al., 2007; Gornik et al., 2009; Sriket et al., 2010). Postmortem fish and shellfish are generally susceptible to proteolysis by endogenous proteases, resulting in a soft or mushy texture (Jiang, 2000; Gornik et al., 2009; Sriket et al., 2011c). Proteases in muscle and from digestive tract of fish and shellfish associated with the muscle softening are shown in Table 2.

**Fish**

A softening phenomenon can take place within a day due to the collapse of Z-lines and fragmentation of myofibril (Shigemura et al., 2003; Delbarre-Ladrit et al., 2006). Softening of fish muscle during iced storage is also associated with the weakening of endomysium and the collapse of collagen fibrils (Shigemura et al., 2003; Shigemura et al., 2004). Disintegration of the pericellular connective tissue of fish muscle was histologically observed in Pacific bluefin tuna (Thunnus orientalis) muscle during storage (Nakamura et al., 2005). High collagen content resulted in a firm meat, indicating the relationship between collagen content and texture property (Jonsson et al., 2001; Kong et al., 2008).

Ice-chilled storage of fish had a gradual disintegration of collagens leading to separation of the muscle fibers, causing the softening of meat (Kubota et al., 2003). Disintegration of collagen type I and V is mainly responsible for the softening of fish muscle, presumably due to the action of autolytic collagenolytic enzymes (Kubota et al., 2003; Yoshida et al., 2009). Cleavage of these connective tissues by endogenous trypsin and chymotrypsin may lead to undesirable textural changes in fish. Mushiness of fish during ice storage was probably caused by the diffusion of digestive enzymes including trypsin and other proteolytic enzymes from autolyzed digestive tract (Ezquerra et al., 1997; Felberg et al., 2009).

These enzymes were presumably a major cause of “gaping” or breakdown of the myotome during long-term iced storage or short term storage at high temperature. For Atlantic cod, upon reaching 17°C, gaping is inevitable, presumably because of degradation of the connective tissue. Two proteases known to hydrolyze collagen of fish muscle include matrix metalloprotease (MMP) and serine protease (Kubota et al., 2003; Lødemel and Olsen, 2003). Heat-stable metalloproteases was identified in Pacific rockfish muscle (Bracho and Haard, 1995). Gelatinolytic proteases with properties similar to collagenase have been proposed to participate in the metabolism of collagens and in the post mortem degradation of fish muscle during cold storage in species like red sea bream (Yoshida et al., 2009; Wu et al., 2010). Collagenase activity was found in fish muscle tissues including skeletal muscle of mackerel, Scomber japonicus, Japanese flounder, Paralichthys olivaceus, rainbow trout and common carp (Saito et al., 2000; Park et al., 2002; Kubota et al., 2003;
Wu et al., 2008). The mackerel (Scomber japonicus) collagenase fraction was shown to be optimally active at pH 7.5 and 55°C (Park et al., 2002). The metalloproteases with molecular masses of 64, 67 and 75 kDa were found in dark muscle of common carp (Wu et al., 2008). The activity of these enzymes was highest at pH 7-9 and they were activated by calcium (Saito et al., 2000; Wu et al., 2008; Yoshida et al., 2009).

Shellfish

The initial deterioration of shellfish during iced storage is related with hydrolytic reactions catalyzed by endogenous enzymes, which produce nutrients, allowing bacteria proliferation (Hernandez-Herrero et al., 2003). Like other marine species, endogenous and bacterial enzymes are involved in the deterioration of crustacean and influence its shelf-life and wholesomeness during refrigerated storage and shipping (Pineiro et al., 2004; Aubourg et al., 2007; Múgica et al., 2008). During the storage, autolysis of cephalothorax, where hepatopancreas and other internal organs are located, could take place, thereby releasing the active proteases into the muscle (Sriket et al., 2011c). Hepatopancreas extracts from crustacean contain both peptidase and proteases, such as trypsin, chymotrypsin and collagenases capable of degrading the native triple helix of collagen under physiological conditions (Oh et al., 2000; Aoki et al., 2004a; Sriket et al., 2012b). Collagen molecules in the connective tissue generally undergo limited cleavage in the non-helical region by those enzymes (Yamashita et al., 1991; Klomklao et al., 2006; Sriket et al., 2011c). However, the degradation of collagen depends upon the source of collagen as well as on the types of protease. High collagen content resulted in a firm meat, indicating the relationship between collagen content and texture property (Jonsson et al., 2001; Kong et al., 2008).

The hydrolytic changes of collagen and of other extracellular matrix proteins are probably to some extent catalyzed by collagenolytic enzymes. Sriket et al. (2010) revealed that the degradation at the interface between the connective tissue of the myocommata and the muscle cell of freshwater prawn meat during iced storage caused the significant structural alterations within the muscle fiber. Among proteolytic enzymes, serine collagenase had the most impact on the softening of muscle (Brauer et al., 2003). During postmortem storage, the activity of a collagenolytic enzyme was detected in the muscle of white shrimp, Penaeus vannamei (Brauer et al., 2003) and freshwater prawn, M. rosenbergii (Sriket et al., 2011c). Ice-chilled storage of shellfish had a gradual disintegration of collagenous protein leading to separation of the muscle fibers, causing the softening of meat (Brauer et al., 2003; Sriket et al., 2010). Collagenase activity was found in shellfish muscle tissues of white shrimp and freshwater prawn (Brauer et al., 2003; Sriket et al., 2011c). Disintegration of collagen type I and V is mainly responsible for the softening of shellfish muscle, presumably due to the action of autolytic collagenolytic enzymes (Brauer et al., 2003; Sriket et al., 2011c). The relatively short shelf-life of iced-chilled shrimp associated with softening of tissue was due to the presence of collagenolytic enzymes (Brauer et al., 2003; Sriket et al., 2011c; Sriket et al., 2012b). Hepatopancreas is the important source of the collagenolytic proteases in shrimp species (Aoki et al., 2004a; Sriket et al., 2011a). Freshwater prawns stored in ice had a maximum shelf-life up to 6 days (Sriket et al., 2010; Begum et al., 2011). The short shelf-life of prawns may result from the degradation of protein structure by their endogenous enzymes (Sriket et al., 2011a). Denaturation and degradation mainly contribute to the loss of functional properties. Firmness is generally considered as the most crucial factor determining fish quality (Benjakul et al., 2003; Dileep et al., 2005; Sriket et al., 2010). Therefore, it is important for the fish processing industry to develop a storage method to maintain high quality and freshness of fish species.

Techniques for quality retention of fish and shellfish

Beheading and eviscerating

Pretreatment methods including beheading, eviscerating and/or gutting have been used to extend the shelf-life of fish and shellfish during storage. Beheading and evisceration could retard the muscle deterioration of bigeye snapper, Priacanthus tayenus and P. macracanthus (Benjakul et al., 2002) and lizardfish (Saurida tumbil) (Benjakul et al., 2003) during storage in ice. Gutting was reported as the means to extend the shelf-life of sea bass (Dicentrarchus labrax) (Papadopoulos et al., 2003) and sea bream (Sparus aurata) (Cakli et al., 2006) during iced storage. Thepnuan et al. (2008) reported that the decapitation of shrimp could lower protein degradation caused by digestive proteases of white shrimp, P. vannamei, kept under modified atmosphere packaging (MAP). Freshwater prawn samples with hepatopancreas removal showed the lower proteolytic activities than did sample without hepatopancreas removal during iced storage for 12 days (Sriket et al., 2011c). Additionally, the decapitation could
lower the aerobic plate count (APC) of Chinese shrimp (*Fenneropenaeus chinensis*) (Lu, 2009) and white shrimp (*Thepmuan et al., 2008*) stored under MAP. Furthermore, gutting resulted in a decrease of microbial load of seabream (*Sparus aurata*) (Tejada and Huidobro, 2002) and seabass (*Dicentrarchus labrax*) during iced storage (Paleologos et al., 2004).

At the beginning of storage in ice, endogenous enzymes are mainly involved in the gradual loss of fish and shellfish freshness. Thereafter, bacterial metabolism predominates and leads to final spoilage (Pacheco-Aguilar et al., 2000). The hepatopancreas of shrimp (Brauer et al., 2003; Aoki et al., 2004a; Sriket et al., 2012b), pyloric caeca and intestine of fish (Simpson, 2000; Kłomkla, 2008) are very rich in proteolytic and collagenolytic enzymes. The leakage of digestive enzymes also contributes to subsequent hydrolysis of fish and shellfish muscle proteins (Felberg et al., 2009; Sriket et al., 2011c). Therefore, pretreatment of fish and shellfish, including beheading, eviscerating and hepatopancreas removal prior to storage, could be another means to retard the deterioration caused by proteolysis.

**Icing and chilling**

Generally, fish meat softens rapidly during storage. The softening phenomenon indicates the deterioration of fish meat. The killing methods and storage conditions, affect the postmortem changes in fish (Shigemura et al., 2004; Bagni et al., 2007; Álvarez et al., 2009). Therefore, it is important to delay or prevent the progression of this phenomenon for maintaining fish freshness. Substantial portion of the fish and shellfish is still preserved by traditional chilling and icing. Different types of novel refrigeration systems have been widely used for the preservation of seafood products at subzero temperatures (-4 to 0°C) such as slurry ice or ozone-slurry ice combined refrigeration system (Campos et al., 2006; Álvarez et al., 2009; Pena et al., 2009) and the use of a cooling agent, e.g. dry ice (solid carbon dioxide) or a combination of dry ice and iced water (Jeyasekaran et al., 2004; Jeyasekaran et al., 2006).

Storage temperature (Table 3) can limit softening by decreasing protease activity (Ando et al., 2007). Super-chilling is one of the few promising techniques with the potential to preserve the prime quality of fresh fish. Super-chilling temperatures can be advantageous in maintaining food freshness and suppressing harmful microorganisms (Ando et al., 2004; Ando et al., 2005). Additionally, cold storage places a serious stress on living cells, resulting in generating of amino acids and sugars that could act as anti-freezing materials against cold temperatures. The shelf-life of various fish and shellfish can be extended by storage at subzero temperatures. This technique can be used for fish, where productive fishing grounds are so far from ports and consumers and the normal iced storage is insufficient for maintaining good quality products prior to being landed and sold (Dalgaard and Huss, 1997). However, some negative effects on quality have also been found in superchilled fish and shellfish. The disadvantages of slurry ice on fish quality including cloudy eyes and development of dull color was reported (Medina et al., 2009). Huidobro et al. (2001) reported that the cloudy eyes of seabream (*Sparus aurata*) stored in liquid ice (-2.2°C) significantly reduces the commercial value. The loss of characteristic bright colors and development of dull tones in the carapace of pink shrimp (*Parapenaeus longirostris*) stored in liquid ice significantly reduces the commercial value. The loss of characteristic bright colors and development of dull tones in the carapace of pink shrimp (*Parapenaeus longirostris*) stored in liquid ice significantly reduces the commercial value.

### Table 3. Application of icing and chilling methods in fish and shellfish

<table>
<thead>
<tr>
<th>Method</th>
<th>Temperature</th>
<th>Application</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liquid ice</td>
<td></td>
<td>Extension shelf-life of stored seabream</td>
<td>Huidobro et al. (2001)</td>
</tr>
<tr>
<td>Liquid ice</td>
<td></td>
<td>Extension shelf-life of stored pink shrimp (<em>Parapeneaus longirostris</em>)</td>
<td>Huidobro et al. (2002)</td>
</tr>
<tr>
<td>Sherry ice</td>
<td></td>
<td>Improvement of pink shrimp and fish qualities during storage</td>
<td>Medina et al. (2009)</td>
</tr>
<tr>
<td>Super-chilling</td>
<td>-1.5°C</td>
<td>Prevention of salmon filet degradation</td>
<td>Bahuaud et al. (2008)</td>
</tr>
<tr>
<td>Super-chilling</td>
<td>-1.5°C</td>
<td>Suppression of yellow tail meat softening</td>
<td>Ando et al. (2007)</td>
</tr>
<tr>
<td>Super-chilling</td>
<td>0°C</td>
<td>Freshness maintenance of Karuma prawn meat</td>
<td>Ando et al. (2004)</td>
</tr>
<tr>
<td>Super-chilling</td>
<td>-0.5°C</td>
<td>Extending shelf-life of fresh Cod fillets</td>
<td>Duun and Rustad (2007)</td>
</tr>
<tr>
<td>Super-chilling</td>
<td>-1.4°C and -1.0°C</td>
<td>Increasing shelf-life of Atlantic salmon</td>
<td>Duun and Rustad (2008)</td>
</tr>
<tr>
<td>Super-chilling</td>
<td>0.1°C</td>
<td>Quality improvement of Salmon fillet</td>
<td>Hansen et al. (2009)</td>
</tr>
<tr>
<td>Super-chilling</td>
<td>-0.9°C</td>
<td>Extending shelf-life of fresh Cod fillets</td>
<td>Wang et al. (2008)</td>
</tr>
</tbody>
</table>

**Note:**
- **Temperature ranges:**
  - 1.0°C to 1.5°C
  - 0.9°C to 1.4°C
  - 1.0°C to 1.5°C
  - 0.1°C to 0.9°C
  - 0.9°C to 1.0°C

**Source:**
- **Huidobro et al. (2001)**
- **Duun and Rustad (2007)**
- **Hansen et al. (2009)**
- **Ando et al. (2008)**
- **Medina et al. (2009)**
- **Wang et al. (2008)**
To reduce tilapia muscle degradation, Ishida et al. (2012a) applied a bivalent metal ionic chelator that binds at active site of cysteine protease and suppressed tenderness of flounder muscle. To retard the freshwater prawn muscle degradation, Kudre et al. (2012) reported that the injection of trypsin inhibition led to the reduction in sarine muscle degradation. To increase gel strength of surimi, Hopkins and Thompson (2001) reported that the injection of leupeptin and benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) during super-chilling from those reports indicated that the use of various protease inhibitors, especially cysteine protease inhibitors, was the most effective in preventing aging of meat. EDTA, a bivalent metal ionic chelator, and 1,10 phenanthroline, a specific inhibitor of metalloprotease, suppressed tenderization of flounder muscle (Kubota et al., 2001). Kang and Lanier (2005) reported that the addition of crude recombinant soy cystatin (CRSC) was able to greatly inhibit the breakdown of myosin of ground arrowtooth flounder, compared with water-soaked samples. In order to maintain the quality of fish and shellfish during storage, protease inhibitors, especially natural and/or food grade inhibitor, have been paid attention to maintaining muscle protein from proteolytic enzyme such as serine protease (Ayensa et al., 2002; Choi et al., 2005). The most commonly used food grade inhibitors used are dried beef plasma protein (BPP), egg white, milk whey and a white potato extract (Benjakul et al., 2004). In general, these protease inhibitors have three complex forming domains which react with trypsin-like and chymotrypsin-like enzymes independently (Feeney and Osuga, 1988). These additives exert various degrees of inhibition towards the proteases responsible for weak gelation of surimi. Although food grade protease inhibitors have been widely used, unwanted side effects have been noticed, including modified color and/or taste (Benjakul et al., 2001; Rawdkuen et al., 2007).

Protease inhibitors can be obtained from various plants. Some of them have been proved as effective in preventing fish and shellfish protein degradation (Ahmad and Benjakul, 2011a; Sriket et al., 2011b). Protease inhibitors in plant organ are proteins or peptides capable of inhibiting catalytic activities of proteolytic enzymes. These inhibitors form stable complexes with target proteases, blocking, altering or preventing the access to the enzyme active site. Among those, serine protease inhibitors are the most widely studied, and have been isolated from soybean and other leguminous seeds (Bhattacharyya et al., 2006; Sriket et al., 2011b).

Methods for applying the protease inhibitor have been reported to affect the uptake of those compounds. Previous reports revealed that soaking in solutions containing protease or trypsin inhibitors has been successfully applied to retard the protein degradation by endogenous proteases in many fish species including unicorn leatherjacket and bigeye snapper gelatin (Inarasirisawat et al., 2007; Nalinanon et al., 2008; Ahmad et al., 2011a). However, poor penetration of the inhibitors into the fillets by soaking method was reported by Kang and Lanier (2005). Some protease inhibitors have been injected in fish muscle to clarify the role of these enzymes in postmortem tenderization. Kubota et al. (2001) demonstrated the involvement of protease in the postmortem tenderization of fish muscle by injecting protease inhibitors into blood vessels in the caudal portion of live flounders. This method seemed not to exclude the effect of blood fluid, in which factors inducing muscle softening may exist. Bleeding is believed to reduce the muscle softening when fish are killed (Ando et al., 1999). Kang and Lanier (2005) successfully infused a recombinant crysytatin into arrowtooth flounder muscle chunks by injection to achieve reduction of proteolytic activity during cooking, resulting in firming of the meat. Sriket et al. (2012a) also reported that the injection of soybean and bambara groundnut extracts into the lower part of freshwater prawn carapace could retard the quality changes and maintain consumer acceptance of freshwater prawn meat during iced chilling storage.

### Table 4. Application of protease inhibitors in fish and shellfish

<table>
<thead>
<tr>
<th>Type of inhibitor</th>
<th>Function</th>
<th>Application</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leupeptin</td>
<td>Binds at active site of cysteine protease</td>
<td>To reduce tilapia muscle degradation</td>
<td>Ishida et al. (2003)</td>
</tr>
<tr>
<td>EDTA</td>
<td>A bivalent metal ionic chelator</td>
<td>To suppress tenderness of flounder muscle</td>
<td>Kubota et al. (2001)</td>
</tr>
<tr>
<td>Soy cystatin</td>
<td>Cystein protease inhibitor</td>
<td>To inhibit the breakdown of myosin of ground arrowtooth flounder</td>
<td>Kang and Lanier (2005)</td>
</tr>
<tr>
<td>Soybean extract</td>
<td>Binds at active site of serine proteases</td>
<td>To retard the freshwater prawn muscle degradation</td>
<td>Sriket et al. (2012a)</td>
</tr>
<tr>
<td>Bambara groundnut extract</td>
<td>Tryptin inhibitor binds at active site of serine proteases</td>
<td>To reduce sarine muscle degradation</td>
<td>Kudre et al. (2012)</td>
</tr>
<tr>
<td>Beef plasma protein</td>
<td>Cystein protease inhibitor</td>
<td>To prevent the degradation of surimi gel</td>
<td>Benjakul et al. (2004)</td>
</tr>
<tr>
<td>Egg white</td>
<td>Tryptin-like protease inhibitor</td>
<td>To prevent surimi gel degradation</td>
<td>Benjakul et al. (2004)</td>
</tr>
<tr>
<td>Potato extract</td>
<td>Binds at active site of cystein and serine proteases</td>
<td>To increase gel strength of surimi</td>
<td>Benjakul et al. (2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Feeney and Osuga (1988)</td>
</tr>
</tbody>
</table>

Use of protease inhibitors

Since autolysis causes the loss in quality of fish and shellfish, food grade protease inhibitors have been applied to lower the degradation and softening of meat (Table 4). Ishida et al. (2003) found that the reduction of breaking strength of stored tilapia was inhibited by the perfusion of leupeptin and benzylxoycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk). Hopkins and Thompson (2001) reported that the use of various proteases inhibitors, especially cysteine protease inhibitors, was the most effective in preventing ageing of meat. EDTA, a bivalent metal ionic chelator, and 1,10 phenanthroline, a specific inhibitor of metalloprotease, suppressed tenderization of flounder muscle (Kubota et al., 2001). Kang and Lanier (2005) reported that the addition of crude recombinant soy cystatin (CRSC) was able to greatly inhibit the breakdown of myosin of ground arrowtooth flounder, compared with water-soaked samples. In order to maintain the quality of fish and shellfish during storage, protease inhibitors, especially natural and/or food grade inhibitor, have been paid attention to protecting muscle protein from proteolytic enzyme such as serine protease (Ayensa et al., 2002; Choi et al., 2005).

The most commonly food grade inhibitors used are dried beef plasma protein (BPP), egg white, milk whey and a white potato extract (Benjakul et al., 2004). In general, these protease inhibitors have three complex forming domains which react with trypsin-like and chymotrypsin-like enzymes independently (Feeney and Osuga, 1988). These additives exert various degrees of inhibition towards the proteases responsible for weak gelation of surimi. Although food grade protease inhibitors have been widely used, unwanted side effects have been noticed, including modified color and/or taste (Benjakul et al., 2001; Rawdkuen et al., 2007).
storage. Furthermore, Carvajal-Rondanelli and Lanier (2010) reported that low molecular weight protease inhibitors such as cystatin can be effectively diffused into intact fish muscle cells to minimize proteolytic activity and meat softening.

Conclusion

Fish and shellfish muscle softening mainly caused by the intensive hydrolysis of muscle proteins by endogenous serine-like collagenolytic proteases during postmortem storage. The degradation of collagenous protein due to collagenolytic activity is a crucial problem. The use of natural serine or trypsin inhibitors is a better way to retard such a textural problem of fish species. Furthermore, there is a need to demonstrate the combination between low temperature storage and protease inhibitors injection.

References


Cakli, S., Kilinc, B., Cadun, A., Dincer, T. and Tolas, S. 2006. Effects of gutting and un gutting on microbiological, chemical, and sensory properties of aquacultured sea bream (Sparus aurata) and sea bass (Dicentrarchus labrax) stored in ice. Critical Reviews in Food Science and Nutrition 46(7): 519-527.


Kolodziejka, I. and Sikorski, Z. E. 1997. Neutral and alkaline muscle proteases of marine fish and


inhibitor containing fraction from chicken plasma on autolysis and gelation of Pacific whiting surimi. Food Hydrocolloids 21(7): 1209-1216.


