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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

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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.

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, *Paldalus 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

*Corresponding author.
Email: *psriket@hotmail.com*
Tel: 66 45 352000; Fax: 66 45 352088

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 Werb, 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 metallo proteases (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 camtschaticus* (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 1. Classification of proteases

Type of protease	Active site	Optimal pH	Molecular mass (kDa)	Source
Serine protease				
- Trypsin	Serine	7-11	18-35	Klomklao (2008)
- Chymotrypsin				
Cystein protease				
- μ-Calpain			80	
- m-Calpain	Cystein	5-7.5	28	Larsen *et al.* (2004)
- Cathepsin B H L			20-37	
Aspartic protease				
- Pepsin			35-46	
- Cathepsin D	Aspartic acid	2-4	46-48	Hughes *et al.* (2003)
Metalloprotease	Zn^{2+}	6-8	69-74	Carmeli *et al.* (2004)

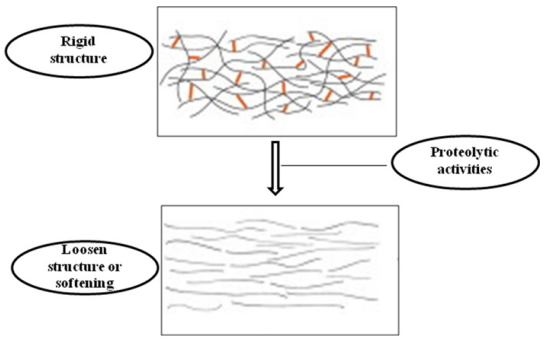

Figure 1. Model of muscle protein degradation by proteolytic cleavage

al., 2007; Sriket *et al.*, 2012b).

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 proteinase 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, endothiapepsin) (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, neprilysin, 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

Fish species	Quality deterioration	Enzymes involved	Sources
		Muscle proteases :	
Tilapia (*Oreochromis niloticus*)	Muscle softening	Serine and metalloproteases	Ishida *et al.* (2003)
Cod , Spotted wolffish and Atlantic salmon	Fillet softening and muscle gaping	Metalloproteases	Lødemel *et al.* (2004)
Silver carp (*Hypophthalmichthys molitrix*)	Muscle degradation	Myofibril-bound serine protease	Cao *et al.* (2004)
Atlantic salmon (*Salmo salar*)	Fillet softening	Cathepsin and collagenase-like enzyme	Hultmann and Rustad (2004)
Seabass (*Dicentrarchus labrax* L.)	White muscle deterioration	Calpain	Delbarre-Ladrat *et al.* (2004)
White Croaker (*Argyrosomus argentatus*)	Muscle degradation	Trypsin-like enzyme	Cao *et al.* (2005)
Seabass (*Dicentrarchus labrax* L.)	Postmortem softening of fish muscle	Cathesin B and L	Che´ret *et al.* (2007)
Dark muscle of common carp (*Cyprinus carpio*)	Muscle softening	Metalloproteases	Wu *et al.* (2008)
bream (*Pagrus major*)	Muscle softening, collagen degradation	Serine collagenolytic protease	Wu *et al.* (2010)
		Digestive proteases :	
White shrimp (*Penaeus vannamei*)	Mushy texture	Collagenolytic enzymes	Brauer *et al.* (2003)
Shrimp (*Penaeus orientalis*)	Mushy texture	Trypsin and collagenase-like enzyme from hepatopancreas	Oh *et al.* (2000)
Herring	Belly burst	Serine collagenolytic enzyme leaked from pyloric caeca	Felberg *et al.* (2010)
Freshwater prawn (*Macrobrachium rosenbergii*)	Muscle softening and mushy texture	Trypsin like collagenase form hepatopancreas	Sriket *et al.* (2012)

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 (Pornrat *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-Ladrat *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 collagenous protein 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

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

Method	Temperature	Application	Source
Liquid ice		Extension shelf-life of stored seabream	Huidobro *et al.* (2001)
Liquid ice		Extension shelf-life of stored pink shrimp (*Parapenaeus longirostris*)	Huidobro *et al.* (2002)
Slurry ice		Improvement of pink shrimp and fish qualities during storage	Medina *et al.* (2009)
Super-chilling	-1.5°C	Prevention of salmon fillet degradation	Bahuaud *et al.* (2008)
Super-chilling	-1.5°C	Suppression of yellow tail meat softening	Ando *et al.* (2007)
Super-chilling	-2°C	Freshness maintainance of Kuruma prawn meat	Ando *et al.* (2004)
Super-chilling		Extending shelf life of Cod fillet	Duun and Rustad (2007)
Super-chilling	-1.4°C and -3.6°C	Increasing shelf life of Atlantic salmon	Duun and Rustad (2008)
Super-chilling	0.1°C	Quality improvement of Salmon fillet	Hansen *et al.* (2009)
Super-chilling	-0.9°C	Extending shelf life of fresh Cod loins	Wang *et al.* (2008)

lower the aerobic plate count (APC) of Chinese shrimp (*Fenneropenaeus chinensis*) (Lu, 2009) and white shrimp (Thepnuan *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; Klomklao, 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 icing 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 was also reported (Huidobro *et al.*, 2002). Furthermore, Bahuaud *et al.* (2008) reported freeze damage during superchilling. The upper layer of the super-chilled fillets showed freeze damage as characterized by the formation of large intra- and extracellular ice crystals during super-chilling. Freeze damage due to super-chilling accelerated the amount of detachments between myofibres and increased the amount of myofibre breakages during storage time. Super-chilling accelerated the release of the proteolytic enzymes cathepsin B and L from the lysosomes, causing an acceleration of fish muscle degradation (Bahuaud *et al.*, 2008). Duun and Rustad (2008) found that myofibrillar proteins denatured more easily during super-chilled than during chilled storage both in salmon and cod fillets and the amount of free amino acids increased more rapidly due to exoproteolytic activity. Duun *et al.* (2007) also found a higher liquid loss in super-chilled samples, compared to ice chilled cod fillets.

The negative effect on texture of salmon (*Salmo salar*) (Gallart-Jornet *et al*., 2007; Hansen *et al*., 2009) and cod (*Gadus morhua*) (Wang *et al*., 2008) during super-chilling in combination with MAP has been reported. The negative effects such as loss in

Table 4. Application of protease inhibitors in fish and shellfish

Type of inhibitor	Function	Application	Source
Leupeptin	Binds at active site of cystein protease	To reduce tilapia muscle degradation	Ishida *et al.* (2003)
EDTA	A bivalent metal ionic chelator	To suppressed tenderization of flounder muscle	Kubota *et al.* (2001)
Soy cystatin	Cystein protease inhibitor	To inhibit the breakdown of myosin of ground arrowtooth flounder	Kang and Lanier (2005)
Soybean extract	Binds at active site of serine proteases	To retard the freshwater prawn muscle degradation	Sriket *et al.* (2012a)
Bambara groundnut extract	Trypsin inhibitor binds at active site of serine proteases	To reduce sardine muscle degradation	Kudre *et al.* (2012)
Beef plasma protein	Cystein protease inhibitor	To prevent the degradation of surimi gel	Benjakul *et al.* (2004)
Egg white	Trypsin-like protease inhbitor	To prevent surimi gel degradation	Benjakul *et al.* (2004)
Potato extract	Binds at active site of cystein and serine proteases	To increase gel strength of surimi	Benjakul *et al.* (2001) Feeney and Osuga (1988)

textural property, liquid loss and protein denaturation during super-chilling from those reports indicated that the iced storage and chilling process still need to be used to maintain the quality of fish and shellfish meat during distribution and storage. The combination with other methods including the use of food grade additive with the storage at low temperature (icing and chilling storage) is still needed to maximize shelf-life extension.

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 benzyloxycarbonyl-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 attentions in 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).

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 methods have been successfully applied to retard the protein degradation by endogenous proteases in many fish species including unicorn leatherjacket and bigeye snapper gelatins (Intarasirisawat *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 cystatin 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

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.

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