Chapter 1

Introduction

Proteolysis of fish muscle has generally been assumed to arise from the release of endogenous muscle proteinases, which are active at postmortem pH (Robbins *et al.*, 1979). This is a primary cause of quality losses during cold storage or handling (Haard *et al.*, 1994). The proteinases indigenous to skeletal muscle possibly include the Ca^{2+}-dependent proteases located in sarcoplasm and cathepsins located in lysosomes (Asghar and Henrickson, 1982). Ante-mortem muscle softening occurred in chum salmon during spawning migration due to degradation of myofibrillar proteins caused by lysosomal cysteine proteinases present in the muscle (Nomata *et al.*, 1985; Yamashita and Konagaya, 1990). Some fish species, e.g. pacific whiting has faced with the degradation caused by cathepsin L (Seymour *et al.*, 1994). The proteolytic enzyme was known to be responsible for tissue softening as well as gel weakening of surimi produced from this species (Benjakul *et al.*, 1997).

Proteolytic disintegration of surimi gels is characterized by high activity at temperatures above 50°C and by the rapid and severe degradation of myofibrillar proteins, particularly myosin (Jiang, 2000). Proteinases in fish muscle that cause the gel softening vary with species, but generally categorized into two major groups, cathepsins and heat-stable alkaline proteinases (Haard *et al.*, 1994; Jiang, 2000). Based on the extractability, it can be classified as sarcoplasmic and myofibril associated proteinases (Toyohara *et al.*, 1990a; Kinoshita *et al.*, 1990a). In general, sarcoplasmic proteinases can be removed to some extent during washing process (Benjakul *et al.*, 1996), while myofibril associated proteinases still remain in surimi, resulting in gel weakening (modori), particularly during thermal gelation. To alleviate such a problem, some food-grade proteinase inhibitors, such as beef

plasma protein, porcine plasma protein, egg white, etc. have been used to improve the surimi gel quality (Morrissey *et al.*, 1993; Benjakul *et al.,* 2001a).

Thailand is one of the most important surimi producing countries in the Southeast Asia with a total production of about 60,000 metric ton per year. An approximate 90% of products are exported to Japan and South Korea (Morrissey and Tan, 2000). The surimi production in Thailand is primarily based on threadfin bream (*Nemipterus* spp.) due to its high gel quality and white color. However, with the overexploitation of resources in the Gulf of Thailand, other species have become more economically important as a raw material for surimi production. Among those species, bigeye snapper is more common for surimi manufacture due to the high gel quality and availability. Additionally, it is not consumed directly due to its appearance and thick skin. Two species of bigeye snapper, *Priacanthus macracanthus* and *Priacanthus tayenus* are commonly caught from the Gulf of Thailand and Indian ocean. However, the former has been known to render the superior gel quality to the latter. Benjakul *et al.* (2001b) found that natural actomyosin from *P. tayenus* underwent higher aggregation via hydrophobic interaction and/or disulfide bonds than that from *P. macracanthus*. Furthermore, differences in other intrinsic factors, especially proteolytic activity, between two species are also hypothesized and the information obtained would help to improve gel quality of surimi from this species.

Literature review

1. Surimi and surimi production

Surimi is minced fish which has been extracted with water to remove strong flavors, pigments and nonfunctional proteins and subsequently dewatered to reduce the moisture content to approximately that of intact fish muscle (Suzuki, 1981). Surimi is commonly produced from white muscle fish. In Thailand, threadfin bream, bigeye snapper, lizardfish as well as croaker are economically important as a potential raw material for surimi production. Surimi production (Figure 1) starts with the separation of fish flesh from bones and skin (usually mechanically) followed by comminution. After the minced fish is washed with water, it becomes "raw surimi". The purpose of washing is the removal of water-soluble proteins, fat, connective tissue and undesirable muscle components such as blood and pigments (Suzuki, 1981). These fractions are thought to interfere with gel formation (Lee, 1986; Hultin, 1985; Rodger and Wilding, 1990). Washing also has the added function of concentrating the more desirable myofibrillar proteins (Kudo *et al.,* 1973; Lee, 1986), in particular myosin and actomyosin, which are the predominant proteins involved in gelation (Shimizu *et al.,* 1983; Akahane *et al.,* 1984). The use of fresh fish in the production of surimi is also essential since tissue autolysis by endogenous proteolytic enzymes during storage reduces the level of extractable actomyosin available for gelation (Makinodan *et al.,* 1980; Kim *et al.,* 1982). The raw surimi possesses enhanced gel-forming, water-holding, fat-binding, and other functional properties relative to minced fish (Okada, 1992). After elimination of excess water by means of centrifugal decantation, the raw surimi is mixed with cryoprotectants such as sugar or sugar alcohol in order to retard denaturation of the proteins when surimi is stored in the frozen state. Then it becomes "frozen surimi" (Okada, 1992).

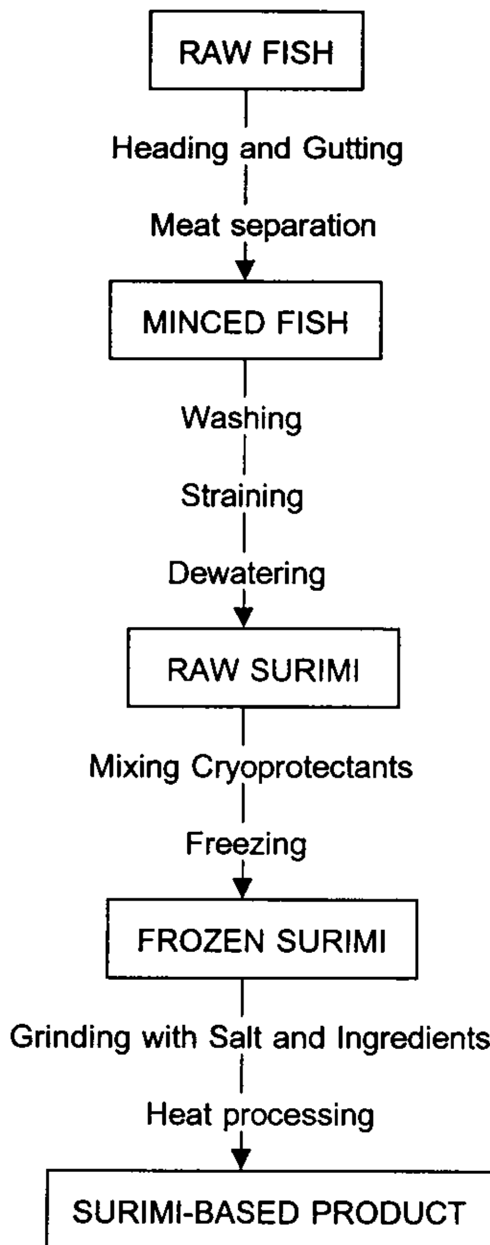

Figure 1 Production of surimi and surimi-based product
Source: Okada (1992)

Gel-forming ability is one of the most important attributes of surimi, which can be affected by both intrinsic and extrinsic factors including species, endogenous enzymes, additives as well as cooking procedure (Shimizu *et al.*, 1981; Niwa, 1992; Lee, 1986; Araki and Seki, 1993; Morrissey *et al.*, 1993).

Thermal gelation of fish muscle has been reported to occur in a three-step process including

1) Dissociation of myofibril structures by protein solubilization in the presence of salt.
2) Partial unfolding of protein structure caused by heat treatment.
3) Aggregation of unfolded protein via both covalent and noncovalent bonds to form a three-dimensional network (Stone and Stanley, 1992)

When fish mince sol (paste) containing 2-3% salt is heated to 50°C, a loose network (suwari) is formed from actomyosin and myosin molecules: This process is referred to as setting (Suzuki, 1981; Wu *et al.,* 1985). It is mediated by transglutaminase and considered as the reaction wherein a three-dimensional network is formed and subsequently acts as the back bone of the final gel (Niwa *et al.,* 1995). Setting is quite species dependent, occuring over a range of temperatures (up to 50°C) and to varying degrees, depending upon the type of fish employed (Taguchi *et al.*, 1978; Shimizu *et al.*, 1981; Hastings *et al.,* 1990).

As the temperature is increased over 45-50°C, suwari is partially disrupted to form a "broken net" structure (modori). Modori is associated with the action of endogenous heat-activated proteinases and/or the thermal behavior of myofibrillar proteins alone (Niwa, 1992). Once the temperature is increased above 65-70°C, the gel becomes ordered and non-transparent, this stage is referred to as kamaboko (Suzuki, 1981). In the final stage, the cohesiveness and elasticity of the gel is enhanced (Lanier *et al.,* 1982; Niwa *et al.,* 1983; Autio *et al.,* 1989; Akahane and Shimizu, 1990). Figure 2 shows changes in rheological properties of myosin and actomyosin during heating and delineates the three phases of texture formation mentioned previously.

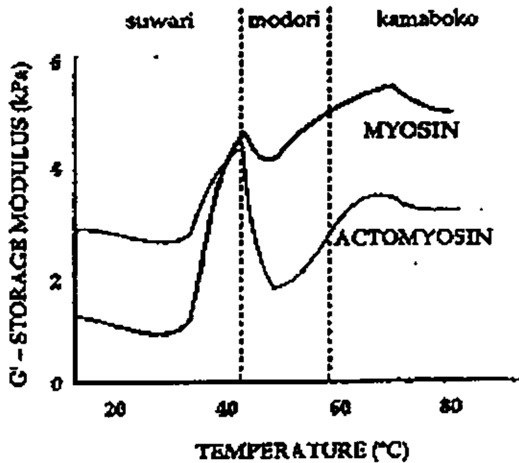

Figure 2 Changes in rheological properties of actomyosin and myosin during heating

Source: Adapted from Sano *et al.* (1988)

2. Proteolysis in fish muscle

The muscle of marine animals contain many proteinases that perform different metabolic functions in the living organisms. However, their action in postmortem affects the sensory quality and functional properties of seafood. Their activities are controlled by specific and endogenous inhibitors, activators, pH, and temperature of the environment. However, the proteolytic activity varies greatly among species (Wojtowicz and Odense, 1972) and with the harvesting season, gender maturation, spawning, and other variables (Toyohara *et al.,* 1991).

Proteinases exist in the muscle fiber and cytoplasm, and in the extracellular matrix of the connective tissue surrounding the cells of fish muscle (Makinodan *et al.*, 1963b; Toyohara *et al.*, 1990a,c). Most of proteinases are lysosomal and cytosolic enzymes, but some of them are in the sarcoplasm and associated with myofibrils or external to the cell in macrophages. After disintegration of tissue, the enzymes are found mainly in the sarcoplasmic protein fraction. However, the tissue enzymes may also

originate from other sources, such as those in the muscle of Pacific hake infected by myxosporidian parasites (Toyohara *et al.*, 1993).

Based on the postmortem pH of fish muscle, it is likely that muscle proteinase involves in postmortem degradation of muscle protein are active at pH 5-7. The ultimate pH of the muscle tissue of most fish drops from the physiological pH 6.2-6.6 at full rigor due to the accumulation of lactic acid and hydrogen ions. However, the pH can decrease to a final pH of 5.5, in the case of flatfish or increase to pH 7.0, resulting in a condition known as alkaline rigor, which is found in cod and some other fish species (Fraser *et al.*, 1961). There are two main groups of proteinase including lysosomal cathepsins and calpains that play important roles in the textural change of postmortem fish muscle (Wojtowicz and Odense, 1972).

The action of tissue proteinases can also contribute to product quality deterioration (Table 1). Post-harvest biochemical change caused by enndogenous enzyme, including proteinases, is the primary cause of quality loss in iced fish and also limits the efficacy of storage strategies, such as modified atmospheres, which target bacterial spoilage (Haard, 1992). Moreover, proteinases can be directly responsible for unusual textural defects in seafood, e.g. 'belly burst', 'gapping', and 'mushiness' of bony fish and 'tail meat' softening of crustacean species and thus may cause poor initial quality and fillet yields (Haard, 1994). Postmortem fish muscle is generally susceptible to proteolysis by endogenous proteinases, resulting in a texture often described as soft or mushy (Jiang, 2000).

Table 1 Importance of endogenous proteinases to quality deterioration of fishery products

Raw material	Quality deterioration	Enzyme(s) involved
	Holding or processing raw material	
Pelagic fish	Belly burst, flesh softening	Trypsin-like and other alkaline proteinases diffuse from pyloric ceca and intestine to cause degradation of myofibrillar and connective tissue proteins
Crustacean sp.	Abdomina muscle softening	Trypsin-like and collagenase enzymes difuse from hepatopancreases to cause 'tail meat' softening in products like shrimp and krill
Chum salmon	Whitish and very mushy texture	Cathepsins B and L are elevated in Chum salmon during spawning migration
Cod	Gapping, poor fillet yield	Collagenase(s) and multicatalytic proteinases may be involved with loss of muscle tissue integrity during post-harvest storage
	Thermoprocessed fish products	
Jellied fish	Extreme texture softening	Cysteine proteinases, etc. formed by myxosporidia contaminating muscle are very active during slow cooking
Fish	Loss of gel strength 'Modori'	Heat-stable alkaline proteinases and neutral proteinases (modori inducing) are active when the product temperature reaches 60-70°C
Scombroid fish	'Honeycomb'	Degradation of connective tissue appears to be associated with muscle alkaline proteinase(s) and pyloric ceca collagenase

Source: Haard (1994)

3. Softening (Modori) of surimi-based products

Temperature plays an important role in surimi gelation. In addition to its effects on the conformation of myofibrillar proteins, temperature can activate endogenous enzymes. Disintegration of surimi gels is characterized by high proteolytic activity at temperatures above 50°C and by the rapid and severe degradation of myofibrillar proteins, particularly myosin (Wasson *et al.*, 1992a,b). This disintegration has detrimental effects on surimi quality, which substantially lowers the gel strength and elasticity (Figure 3) (Boye and Lanier, 1988; Morrissey *et al.*, 1993; Jiang, 2000). Among numerous proteinases present in muscle, thermal stable endoproteolytic proteinases have more serious effect on texture due to their thermostability and ability to cleave the internal peptide bonds, while exopeptidase hydrolyzes terminal peptide bonds (Kirschke and Barrett, 1987).

The most active proteinases in fish muscle that can soften the surimi gels vary with species, but are generally categorized into two major groups: cathepsins (Toyohara *et al.*, 1993; Seymour *et al.*, 1994; Jiang *et al.*, 1996; 1997) and heat-stable alkaline proteinases (Makinodan *et al.*, 1984; 1985; Boye and Lanier, 1988; Wasson *et al.*, 1992a,b)

Kinoshita *et al.* (1990) characterized four distinct types of neutral proteinases, which degrade myosin heavy chain called 'modori-inducing proteinases', MIP. These include a sarcoplasmic-50°C-MIP which is easily extracted, optimally active at 50°C and insensitive to n-butanol; a sarcoplasmic-60°C-MIP, which is easily extracted, optimally active at 60°C and is sensitive to n-butanol; a myofibrillar-50°C-MIP which is tightly associated with myofibrils, optimal at 50°C and insensitive to n-butanol; and a myofibrillar 60°C-MIP, which is tightly associated with myofibrils, optimally active at 60°C and is sensitive to n-butanol. The myofibril associated MIPs

are not extracted from myofibrils, even in the presence of detergents (Toyohara *et al.*, 1990a, d).

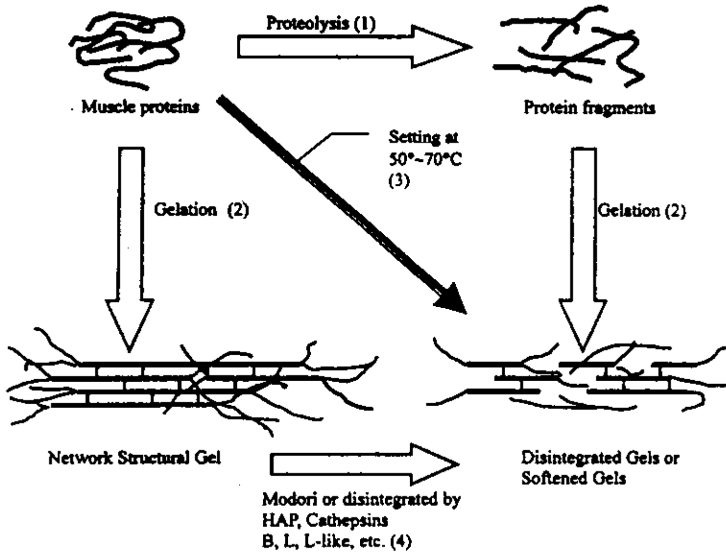

Figure 3 Proposed model of the gelation and disintegration of surimi gels. (1) proteolysis by calpains, cathepsins and other proteinases during storage; (2) gelation; (3) proteolysis by HAP, cathepsins B, L and L-like during setting at 50-70°C; (4) modori or disintegration by HAP, cathepsins, calpains and other proteinases if the formed network structure is not fixed by heating at 85-100°C

Source: Jiang (2000)

Twelve species of fish were classified into five groups based on MIP categories (Table 2). Species like threadfin bream, which contain only sarcoplasmic types of MIP, do not exhibit modori when the gel is prepared from surimi. On the other hand, fish like crucian carp or ovalfile fish, which exhibits extensive gel degradation cooking due to the presence of myofibril associated MIPs. Bleeding or aging fish prior to preparing a gel results in a weaker product and this has been related to the presence of serum inhibitors

of MIPs (Toyohara *et al.*, 1990b) and lowering of postmortem pH (Toyohara *et al.*, 1990c).

Cao *et al.* (1999) investigated the proteolysis of a myofibril-bound serine proteinase (MBP) from carp (*Cyprinus carpio*) on myofibrillar proteins and their gel formation ability. The optimum degradation temperature of MBP to myosin heavy chain in myofibril and kamaboko gel were 55°C and 60°C, respectively. Additionally, actin, α-actinin and tropomyosin were also hydrolyzed by this enzyme to different degrees. Therefore, MBP was the most probable proteinase causing the modori phenomenon.

Table 2 Distribution of four types of modori-inducing proteinase (MIP) among fish species

Fish species	MIP			
	Sp-50-MIP	Sp-60-MIP	Mf-50-MIP	Mf-60-MIP
Threadfin bream	×	×		
Mud dab		×		
Walleye pollack		×		
Red sea bream		×		
Rainbow trout		×		
Brown croaker		×		
Shortfin lizard fish		×	×*	×*
Nibe croaker		×		×
Tilapia		×		×
File fish			×	×
Pacific mackerel			×	×
Crucian carp			×	×

*showed modori-phenomenon partially

Source: Adaped from Kinoshita *et al.* (1990)

4. Classification of proteinases

Proteinases are classified according to their source (animal, plant, microbial), their catalytic action (endopeptidase or exopeptidase) and the nature of the catalytic site. In EC system for enzyme nomenclature, all proteinases (or peptide hydrolyses) belong to subclass 3.4, which is further divided into 3.4.11-19, the exopeptidases and 3.4.21-24, the endopeptidases or proteinases (Nissen, 1993). Endopeptidases cleave the polypeptide chain at particularly susceptible peptide bonds distributed along the chain, whereas exopeptidases hydrolyze one amino acid (or dipeptide, in the case of 3.4.14 and 15) or C terminus (carboxypeptidases) (Figure 4)

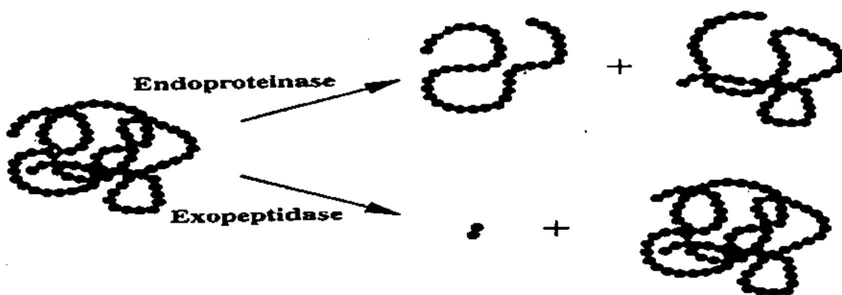

Figure 4 Action of endopeptidases and exopeptidases on protein structure
Source: An *et al.* (1996)

4.1 Endopeptidases

The four major classes of endopeptidases are serine proteinases (EC 3.4.21), cysteine proteinases (EC 3.4.22), aspartic proteinases (EC 3.4.23) and metalloproteinases (EC 3.4.24) (Table 3) (Nissen, 1993).

Serine, cysteine and aspartic proteinases have serine, cysteine and aspartic acid side chains, respectively, as a part of the catalytic site. Modification or blocking of this side chain usually leads to complete inactivation of the enzyme and is a standard way of determining the nature of

an unknown proteinase (Nissen, 1993). The serine proteinases have maximum activity at alkaline pH, while the closely related cysteine proteinases usually show maximum activity at more neutral pH values. The aspartic proteinases generally have maximum catalytic activity at acid pH. Among the digestive enzymes, the aspartic proteinase pepsin is secreted in the stomach and the serine proteinases trypsin and chymotrypsin are excreted in the duodenum, in accordance with pH values of the digestive tract (acid in the stomach and alkaline in the gut) (Nissen, 1993).

The metalloproteinases contain an essential metal atom, usually Zn and have optimum activity near neutral pH. Ca^{2+} generally stabilizes these enzymes and strong chelating agents, such as EDTA inhibit the activity (Nissen, 1993).

4.2 Exopeptidases

The aminopeptidases (EC 3.4.11) are ubiquitous, but less readily available as commercial products, since many of them are intracellular or brane bound. Carboxypeptidases are subdivided into serine carboxypeptidases (EC 3.4.16), metallocarboxypeptidases (EC 3.4.17) and cysteine carboxypeptidases (EC 3.4.18) according to the nature of the catalytic site (Nissen, 1993).

5. Muscle proteinases

Although proteolytic activity in skeletal muscle is generally much lower than in other tissues, it plays a significant role in protein turnover during growth and development of the live animal and in pathological conditions of the muscle. These muscle proteinases are found in the sarcoplasmic (or soluble) component of muscle tissues, in association with cellular organelles, connective tissues, myofibrils and in the interfiber space (Asghar and Bhatti, 1987; Haard, 1992; Haard *et al.*, 1994). The physicochemical/catalytic

properties and levels of these enzymes and other muscle proteins are influenced by a plethora of factors such as age, diet, exercise, habitat temperature, water depth (in the case of fishes and other aquatic animals) and other developmental and environmental factors (Greaser, 1986; Haard, 1990). Some of the proteinases which have been widely studied due to their influence on the quality of food products prepared from muscles include cathepsins, alkaline proteinases, Ca^{2+}-activated proteinases and metalloproteinases (Ashie and Simpson, 1997).

5.1 Cathepsins

The lysosomal apparatus is generally regarded as intracellular digestive system containing a large number of hydrolases, which act on phosphate and sulfate esters, carbohydrates, lipid and proteins. The proteinases found in this cellular organelle are generally referred to as cathepsins and have been detected in the muscles of various fish and mammalian species (Ashie and Simpson, 1997). Cathepsins are recognized as families of endopeptidases and/or exopeptidases. Many cathepsins have acidic pH optimum although some are most active at neutral pH. Cathepsin D is an aspartic proteinase while all others found in muscle thus far are serine or cysteine proteinase (Goll *et al.,* 1989). A list of cathepsins identified in muscle tissue and those known to be present in fish myosystems is summarized in Table 4. Although evidence now indicates that lysosomal proteinases play a minor role, *in vivo*, in initiating myofibrillar protein turnover (Goll *et al.,* 1989), a role of these enzymes in postmortem muscle protein degradation has been maintained for many years (Haard, 1994).

Table 3 Neutral and alkaline proteinases of fish muscles

	Enzyme	Optimum		Effect on muscle proteins
		pH*	temperature (°C)*	
Cysteine proteinases	Calcium-activated proteinases	6.9-7.5	30	Cleavage of myofibrillar proteins to TCA soluble fragments, degradation of cytoskeletal proteins
	Cathepsin L	5.0-5.6	40-50	Hydrolysis of most myofibrillar proteins, cleaving of telopeptidases from type I collagen
	Cathepsin B	5.7-6.0		Slight hydrolysis of myosin, actin, nebulin and troponin T
	Cathepsin C	6.0-6.5		
	Heat-activated cysteine proteinase	6.0-8.5	55-65	Hydrolysis of myosin
Serine proteinases	Heat-activated trypsin-like proteinases	6.2-8.0	50-60	Hydrolysis of myosin
	Multicatalytic proteinases	6.0-10.0	60-65	Hydrolysis of myosin
	Other trypsin-like proteinases	8.0-9.0	37-40	Hydrolysis of isolated myosin, disintegration of the cytoskeletal and contractile elements of intact myofibrils
Aspartyl proteinase	Cathepsin D	5.5	30	Hydrolysis of myosin
Metallo-proteinases	Neutral proteinase	7.2	40	
	Heat stable alkaline proteinases	7.0-8.0	50	Hydrolysis of type I collagen, gelatin and other cytoskeletal matrix proteins
	Myosinase I and II	7.0	40	Hydrolysis of myosin

*The range of data regards activity with different proteins and synthetic substrates

Source: Adapted from Kolodziejska and Sikorski (1996)

a) Cathepsin A

Cathepsin A (EC 3.4.16.1), now called carboxypeptidase A, is defined as an enzyme splitting carbobenzoxy-L-Glu-L-Tyr and is capable of splitting residues sequentially from the carboxyl terminus of peptides like glucagon. The exopeptidase has a pH optimum of 5-6 and is readily inactivated by heat and alkaline and normally has a molecular mass of 35 kDa. In mammals, the enzyme is believed to act synergistically with endopeptidases such as cathepsin D (Haard, 1994).

Table 4 Proteolytic enzymes associated with muscle lysosomes

Enzyme	Family	Activity	Fish
Cathepsin B_1 (cathepsin B)	Cysteine	Endopeptidase	Purified from muscle of several species; identified in many species
Cathepsin H (cathepsin B_3)	Cysteine	Endopeptidase	Identified in salmon muscle
Cathepsin L	Cysteine	Endopeptidase	Identified in salmon and mackerel muscle
Dipeptidyl peptidase I (cathepsin C)	Cysteine	Exopeptidase	Identified in muscle from several species
Cathepsin D	Aspartic	Endopeptidase	Purified and identified in muscle from many species
Carboxypeptidase A (cathepsins A and I)	Serine	Exopeptidase	Purified from several species and identified in muscle from many species
Cathepsin S	Cysteine	Endopeptidase	Tentatively identified in mackerel muscle

Source: Adapted from Goll *et al.* (1989)

Cathepsin A has been purified about 1700-fold to homogeneity from muscle (Toyohara *et al.*, 1982). The molecular mass was estimated to be 36 kDa by gel permeation chromatography and the pH optimum with synthetic substrates with 5.0. The purified enzyme did not hydrolyze any of the protein substrates tested and did not appear to contribute to the autolysis of carp muscle. Two cathepsin A-like proteinases (15.6 and 35.6 kDa) have been purified from milkfish muscle and had optimal activity with carbobenzoyl-L-Gly-L-Phe at pH 7.0 (Jiang *et al.*, 1990). Cathepsin A, partially purified from Atlantic cod muscle, hydrolyzed carbobenzoyl-α-Glutamyl-L-Tyr with an optimum of pH 5.0 (McLay, 1980). Cathepsin A-like activity has also been detected in muscle of tilapia, bombay duck, pomfret and shrimp by Sherekar *et al.* (1986) but was not detected in cod muscle (Siebert and Schmitt, 1965).

b) Cathepsin B

Cathepsin B (EC 3.4.22.1) was originally defined as an enzyme splitting benzoylarginine amide. It is now recognized that at least two enzymes have this activity including cathepsin B_1(B), a 27 kDa, endopeptidase with some exopeptidase activity and cathepsin B_2, a 50 kDa exopeptidase also called carboxypeptidase B. Cathepsin B_1 may occur in at least four isoenzyme forms (Keilova and Tomasek, 1973). Cathepsin B_3, now called cathepsin H, is a 25 kDa glycoprotein which is very heat stable. Although cathepsin B_1 is a major enzyme in skeletal muscle, it is reported to have relatively weak proteinase activity with myosin and other myofibrillar proteins (Kominami *et al.*, 1985). Okitani *et al.* (1988) reported that the myosin-degrading activity previously ascribed to cathepsin B was due to contamination with cathepsin L.

c) Cathepsin C

Cathepsin C (EC 3.4.4.9), also called dipeptidyl transferase and dipeptidyl-aminopeptidases, was first recognized as an enzyme which

deaminates Gly-Phe-NH_2. It is now established that this exopeptidase, an octomer of 25 kDa subunits, has an absolute Cl^- requirement for activity, and has a relatively broad specificity for splitting dipeptidyl residues from the amino terminus of polypeptide chains. Although cathepsin C actvity has been identified in muscle extracts from carp (Makinodan and Ikeda, 1971), true cod, Pacific whiting (Erickson *et al.*, 1983) and squid (Hameed and Haard, 1985; Lee and Pan, 1990), it apparently has not been purified from the muscle tissue of aquatic organisms.

d) Cathepsin D

Cathepsin D (EC 3.4.4.23) is an endopeptidase having pepsin-like activity with protein substrates but differs from pepsin in its activity with low molecular weight substrates (Haard, 1994). Cathepsin D is a glycoprotein, which normally has a molecular mass of about 40 kDa and optimal activity at about pH 3.5. Cathepsin D degraded actin and myosin at acidic pH values, i.e. below 6.0 (Okitani *et al.,* 1981). Invertebrates secrete cathepsin D in the hepatopancreas and appear to use the enzyme in digestion (Haard, 1994). Gildberg (1988) suggests an evolutionary progression of cathepsin D, fish pepsin I, fish pepsin II and mammalian pepsin. Since cathepsin D activity was substantially suppressed in postmortem pH and at low incubation temperatures, it was found to be less significant in fish as compared to cathepsin L or B. However, it is known to initiate protein hydrolysis and produce peptide fragments that can then further cleaved by other cathepsins (Huang and Tappel, 1971).

e) Cathepsin L

Cathepsin L (EC 3.4.22.15), the lysosomal cysteine proteinase isolated from different mammalian, avian, and fish tissues has similar properties, although the molecular weights of the various enzyme preparations range

from 20 to 55 kDa. The enzyme has high activity towards the synthetic substrate Z-Phe-Arg-MCA and attacks several proteins (Kolodziejska and Sikorski, 1996)

Cathepsin L was found in macrophage-like phagocytes between muscle fibers of the white muscles of mature salmon during migration (Yamashita and Konagata, 1991b). The extent of softening of such salmon is correlated to cathepsin L activity (Yamashita and Konagata, 1991a). The enzyme has a molecular weight of 30 kDa and isoelectric point of 5.2. The cathepsin L and cathepsin L-like enzyme purified from mackerel dorsal muscle are thiol-dependent proteinases and are inhibited by cystatin. Their molecular weights are 30 and 55 kDa and pH optimum for the hydrolysis of Z-Phe-Arg-MCA are 5.0 and 5.5, respectively (Jiang *et al.,* 1994a,b). Both proteinases lost activity rapidly at pH above the optimum value. The optimum temperatures are 50 and 40°C, respectively, but they are inactivated at 65-70°C

5.3 Calpains

Most recent attention to neutral proteinases in skeletal muscle has been directed to the Ca^{2+}-activated, neutral endopeptidases known as CANP and recently as calpains (EC 3.4.22.17) (Kolodziejska and Sikorski, 1996). CANP are ubiquitous in the sarcoplasm of muscles from terrestrial animals, fish and marine invertebrates. Two types of calpains have been isolated from muscles-calpain I or μ-calpain requiring only 5-50 μM Ca^{2+} for half-maximal activity and calpain II or *m*-calpain, which requires 0.15-1.0 mM Ca^{2+} (Kolodziejska and Sikorski, 1996). The concentration of Ca^{2+} necessary for activation of calpain I is similar to that prevailing in the sarcoplasm of living muscle. Postmortem concentration of free Ca^{2+} may increase up to about 0.1 mM due to the loss of calcium retaining ability of sarcoplasmic reticulum. Most calpains isolated from vertebrate muscles are composed of two subunits, 80

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kDa and 30 kDa (Kolodziejska and Sikorski, 1996). The molecular weight of calpain from tilapia muscle was first reported to be 80 kDa (Jiang *et al.,* 1991) and later 110 kDa, with subunits 80 and 28 kDa (Wang *et al.,* 1993). Calpains appear to be sarcoplasmic enzymes which release thick and thin filaments from the myofilament by degrading the z-disk. These enzymes are maximally active between pH 6.5 and 8.5 and have a cysteine active site (Haard, 1994). The calpains rapidly degrade several myofibril and cytoskeletal proteins including desmin, vimentin, gelsolin, spectrin, nebulin, vinculin and troponin T and more slowly degrade titin, troponin I, C-protein, tropomyosin and tubulin to large fragments rather than to small peptides and amino acids (Haard, 1994). Endogenous calpain inhibitor, called calpastatin, has also been extensively studied and is unique in its ability to inhibit calpain and not other cysteine proteinases (Haard, 1994). Calpastatin has been isolated from fish muscle (Toyohara *et al.,* 1983).

m-Calpain (calpain II) isolated from carp muscle exhibits maximum caseinolytic activity at pH 7.0 and 25 $^{\circ}$C (Taneda *et al.,* 1983). Calpain from tilapia muscle has optimal caseinolytic activity at pH 7.5 (Jiang *et al.,* 1991).

5.4 Alkaline proteinases

Muscle contains a family of high molecular mass, heat stable, alkaline proteinases composed of subunits ranging from about 20 to 35 kDa. These enzymes have been identified in mammalian muscle (Mayer *et al.*, 1974) but have been most extensively studied in fish muscle (Haard, 1994). Alkaline proteinases appear to be located in the muscle sarcoplasm, microsomal fraction, or are bound to myofibrils (Dahlmann and Reinauer, 1978; Makinodan *et al.*, 1982).

A unique feature of these enzymes is that they exhibit little or no catalytic activity unless assayed at a non-physiological high temperature (60-65°C) or are activated by protein denaturing agents such as urea, fatty acids

or detergent (Dahlmann *et al.*, 1985; Toyohara *et al.*, 1987). The property of heat activation has led researchers to study these proteinases as a cause of fish gel weakening during cooking of surimi products (Nishimoto *et al.*, 1987). Alkaline proteinase have also been implicated in postmortem changes in the cytoskeletal network of fish muscle (Busconi *et al.*, 1984; 1989a; 1989b).

Alkaline proteinase have pH and temperature optimum with casein substrate of 8.0 and 60-65°C, respectively (Haard, 1994). Enzymes from different sources of muscle normally have a molecular mass around 500-800 kDa but muscle alkaline proteinases have been reported with a range in size from 31 to 1363 kDa (Bracho and Haard, 1990). Degree of heat stability and the influence of Ca^{2+} on activity varied, depending upon species (Iwata *et al.*, 1974; Bracho and Haard, 1990). Serine and cysteine proteinase inhibitors have been found to exhibit the efficacy in preventing surimi gel weakening. However, its efficiency depends on cooking temperature and fish species (Toyohara *et al.*, 1990a,b,c,d).

5.5 Multicatalytic proteinases

A group of high molecular weight alkaline proteinases isolated from muscle have now been classified as multicatalytic proteinases (Dahlmann *et al.*, 1985). These enzymes have up to three catalytic sites which catalyze trypsin-like, chymotrypsin-like and endopeptidase reactions. The activity of these enzymes exhibit latency. They are similar to the general group of alkaline proteinases described above in exhibiting little activity unless activated by heat, detergent or lipid (Haard, 1994). This type of heat stable, multicatalytic proteinase has been isolated from a number of fish tissues including a white croaker skeletal muscle enzyme, 269 kDa (Folco *et al.*, 1988; 1989) and carp muscle enzyme, 570 kDa (Kinoshita *et al.*, 1990b). According to Kinoshita *et al.* (1990a), fish muscle multicatalytic proteinase has stronger endopeptidase activity than mammalian enzyme.

6. Tissue degradation by proteinases

a) Tissue degradation by cathepsins

Tissue degradation has been associated with cathepsin. Carp muscle cathepsin D was reported to be incapable of hydrolyzing myofibrillar proteins above pH 6.0 (Ashie and Simpson, 1997). Cathepsin D and other lysosomal proteinases were therefore ruled out as being involved in postmortem tissue degradation since their opimum pH generally falls below postmortem pH (Koohmaraie *et al.*, 1988b; Koohmaraie, 1994). However, Jiang *et al.* (1990b, 1992) reported fragmentation of fish myofibrils and Z-line caused by cathepsin D at 4°C, resulting in disappearance of α-actinin. Furthermore, fractions containing cathepsin D activity from winter flounder (*P. americanus*) and *T. mossambica* were also shown to retain reasonable levels of activity between pH 3.0 and 7.0 (Wasson, 1992). While cathepsin D showed various levels of activity at postmortem pH, its effect was enhanced by the presence of other cathepsins (Jiang *et al.*, 1990b; 1992). Yamashita and Konagaya (1992) suggested that cathepsin L is the main enzyme responsible for softening of the flesh even though its effects may be augmented by the activities of other catheptic enzymes like D and E. In Pacific whiting surimi and arrowtooth flounder muscle, the reduction of gel strength or softening of muscle tissue was attributed to degradation of myofibrillar components such as myosin, actin, β-tropomyosin/troponin T and collagen by cathepsin L which is maximally active at 55°C (An *et al.*, 1994b; Wasson *et al.*, 1992b). An *et al.* (1994b) also noted that while cathepsin L was the most active enzyme in surimi, cathepsin B was the predominant enzyme in the fillets and cathepsin H showed the least activity. This indicated that cathepsin L may be the principal proteinase contributing to texture deterioration during conventional slow cooking, while texture degradation at lower temperatures may be attributed to cathepsin B activity.

Aoki and Ureno (1997) studied the involvement of cathepsins B and L in the postmortem autolysis of mackerel muscle and concluded that mackerel cathepsin L hydrolyzed myosin, troponin T, troponin I and tropomyosin, while mackerel cathepsin B did not hydrolyze any myofibrils from mackerel white muscle. These results suggested that the main cause of postmortem degradation of mackerel muscle tissue was due to the activity of cathepsin L.

Ogata *et al.* (1998) showed that all myofibrillar components were partially degraded by carp cathepsin L at pH 6.5-7.0. Carp cathepsin L degrades not only carp myofibrillar components but also their resultant products between pH 5.0 and 7.0. It markedly acts on myosin heavy chain, α-actinin and troponin-T and –I. Carp cathepsin L likely contributes to postmortem muscle tenderization of carp fillet over an extensive pH range during storage (Ogata *et al.*, 1998). Nielsen and Nielsen (2001) reported that purified cathepsin D from herring muscle mainly degraded myosin, actin and tropomyosin in myofibrils at pH 4.23.

b) Tissue degradation by calpains

Calpain is another proteinase implicated in protein turnover or postmortem tissue proteolysis. The role of Ca^{2+} in muscle degradation was firstly reported by Davey and Gilbert (1969). They reported that EDTA inhibited the weakening and disappearance of Z-disks and speculated that EDTA probably acts by chelating Ca^{2+}. Koohmaraie *et al.* (1988b) also demonstrated that all postmortem changes were completed within 24 h when muscle slices were incubated in buffer solutions containing Ca^{2+}, but none of the postmortem changes occurred when EDTA/EGTA was included in the buffer instead of Ca^{2+}. Since neither EDTA/EGTA nor Ca^{2+} enhanced lysosomal enzyme (cathepsin B, H and L) activities, the cathepsins were completely ruled out. Other reasons for toning down the possible role of cathepsins in degradation of muscle tissue include the following: (1) at 10 mM

Ca^{2+} concentration, the activities of some of cathepsins are inhibited; (2) calpain are located in the cytosol primarily at the region of the Z-disk (66% on Z-disk; 20% in I-band; and 14% in A-band), while cathepsins are compartalized in the lysosomes and therefore do not have direct contact with the muscle proteins (Koohmaraie, 1994); (3) while it had been generally assumed that the lysosomes are ruptured under postmortem conditions releasing their enzymes into the cytosol, there is no experimental evidence to support this assumption (Koohmaraie, 1994; Asghar and Bhatti, 1987).

Tsuchiya and Seki (1991) studied the action of calpain on α-actinin within and isolated from carp myofibrils. It was found that calpain cleaved not only isolated α-actinin but also α-actinin within carp myofibrils.

c) Tissue degradation by alkaline proteinases

The role of alkaline proteinases in degradation of muscle tissue has therefore been investigated largely in fish tissue. It is now well known that surimi gel strength is predominantly due to the formation of a cross-linked network by myosin heavy chain during setting (Wasson, 1992). Proteolytic degradation of myosin heavy chain thererfore limits cross-link formation, which directly exhibit the detrimental effect on gel strength. Experiments with various fish species have demonstrated a causal link between alkaline proteinase and the reduced gel strength of value-added fish products like surimi which are processed at high temperatures (60-65°C) (Wasson, 1992). Makinodan *et al.* (1985) studied the autolytic activity of white croaker (*Sciena schlegeli*) muscle alkaline proteinase and elicited a strong correlation between temperature and gel strength. The autolytic activity at pH 6.8 was highest at 60°C and was reduced at both 50°C and 70°C. A decrease in gel strength was noted in the temperature range of highest autolytic activity. On the other hand, gel strength was increased in the temperature range where activity was low.

Cao *et al.* (2000) purified and characterized myofibril-bound serine proteinase (MBSP) from the skeletal muscle of lizardfish (*Saurida wanieso*). The optimum pH and temperature of the enzyme were 7 to 8 and 55°C, respectively. This enzyme degraded myosin heavy chain at 55-60°C, while α-actinin and actin were not at all hydrolyzed. Myofibril-associated serine proteinase (mekratin) was localized within sarcomeres, where it could readily cleave the contractile proteins such as troponin T, troponin I, myosin light chain 2 and titin (Levine *et al.*, 1999).

d) Tissue degradation by multicatalytic proteinases

Multicatalytic proteinase is believed to be the central cellular protein degradation system and functions differently from both lysosomal and calpain proteolytic systems. Multicatalytic proteinase are likely to be stable in postmortem muscle because it was found that multicatalytic activity of proteinase purified from bovine longissimus muscle was not affected by postmortem aging up to 14 days (Arbona and Koohmaraie, 1993). The multicatalytic proteinase from lobster is irreversibly activated by heating at 60° C and degrades most myofibrillar proteins with preferential hydrolysis of paramyosin, troponin-I, troponin-C and myosin α-light chain (Mykles, 1989). On the other hand, Koohmaraie (1992) reported that myofibrils were a very poor substrate for multicatalytic proteinase. Among myofibrillar proteins, only troponin-C and myosin light chain-2 and –3 were degraded by multicatalytic proteinase. Taylor *et al.* (1995) claimed that the multicatalytic proteinase caused significant changes in myofibrillar structure incubated at 37°C for 24 h into 50 mM Tris buffer pH 7.4, 100 mM KCl and 0.1% 2-mercaptoethanol. The multicatalytic proteinase caused the degradation of myosin heavy chain, α-actinin, actin, tropomyosin, troponin and myosin light chain (Matsuishi and Okitani, 1997). Additionally, completely loss of M-line and a partial loss of Z-

line structure were found when the purified multicatalytic proteinase from rabbit skeletal muscle was incubated with the myofibrils (Otsuka *et al.*, 1999).

7. Purification and characterization of proteinases from fish muscle

a) Purification and characterization of calpains

m-calpains from tilapia muscles was purified by Phenyl-Sepharose CL-4B, Sephacryl S-200, Q-Sepharose and Superose 12 HR column chromatographies (Wang *et al.*, 1993). Calpains from tilapia muscle had a molecular weight of 110,000 containing two subunits of 80,000 and 28,000 daltons. It had optimal temperature and pH of 30°C and 7.5, respectively. The proteinase was activated by dithiothreitol, glutathione and β-mercaptoethanol, and was inhibited by calpain inhibitor I, calpain inhibitor II, leupeptin, antipain, iodoacetic acid and p-(chloromercuri)benzoate, but not affected by pepstatin A and N-ethylmaleimide.

b) Purification and characterization of cysteine proteinases

Jiang *et al.* (1994) purified novel cysteine proteinase from mackerel (*Scomber australasicus*) muscle to electrophoretic homogeneity using Hiload DEAE-Sepharose, Hiload S Sepharose and FPLC Superdex 75 chromatographies. Purification fold of 24,534 with a yield of 1.0% was obtained. The molecular weight of purified proteinase was 99,000 daltons estimated by Superose 12 gel filtration. It hydrolyzed Z-Phe-Arg-MCA and Z-Arg-Arg-MCA but not Z-Arg-MCA and L-Arg-MCA. The optimal pH and temperature for the hydrolysis of Z-Arg-Arg-MCA were 6.0 and 35°C, respectively. The proteinase was stable at pH 5.5-6.0 but was unstable when the pH was higher than 7.0. This proteinase was activated by dithiothreitol, cysteine, glutathione and β-mercaptoethanol.

Purification of cathepsin B-like from white muscle of commom mackerel (*Scomber japonicus*) was carried out by ammonium sulfate fractionation and successive column chromatography (Aoki *et al.*, 1995). Puification fold of 2,510 with a yield of 7.6% was obtained. The purified enzyme hydrolyzed Z-Arg-Arg-MCA, but not Z-Phe-Arg-MCA. The molecular mass of the enzyme was estimated to be 55 kDa by gel filtration and have an optimum pH of 5.5. The enzyme was activated by sulfhydryl compounds such as dithiothreitol and cysteine. The enzyme was strongly inactivated by TLCK, TPCK, antipain, leupeptin and E-64.

Aoki *et al.* (1997) purified latent cysteine proteinase from the white muscle of commom mackerel (*Scomber japonicus*) by ammonium sulfate fractionation and successive column chromatography that obtained 4,700 puification fold with a yield of 4.90%. The molecular mass of the latent form was estimated to be 65 kDa by gel filtration and 69 kDa by SDS-PAGE. The optimum pH and temperature of purified enzyme were 5.3 and 37°C, respectively. The latent form enzyme was activated by acid treatment or pepsin treatment and was capable of hydrolyzing Z-Phe-Arg-MCA, hemoglobin and azocasein. Activity of active form enzyme was enhanced by sulfhydryl compounds, such as dithiothreitol and cysteine and was strongly inactivated by leupeptin and E-64.

Cathepsin L from carp hepatopancreas was purified by a method involving ammonium sulfate precipitation and a series of column chromatographies, in which the enzyme had an affinity toward Concanavalin A and Cibacron Blue F3GA (Aranishi *et al.*, 1997). Purification fold of 1,096 with a yield of 1% was observed. Its homogeneity was established by native-PAGE, but two protein bands corresponding to molecular masses of 30,000 daltons (single chain) and 24,000 daltons (heavy chain) migrated on SDS-PAGE. The enzyme exhibited a maximum activity for Z-Phe-Arg-MCA at pH 5.5-6.0 and 50°C. All tested cysteine proteinase inhibitor and TLCK and

chymostatin markedly inhibited its activity, whereas the other serine proteinase inhibitors and metalloproteinase inhibitor showed no inhibitory activity on the enzyme.

Capasso *et al.* (1999) purified cathepsin D from the liver of the Antarctic icefish (*Chionodraco hamatus*) by anion-exchange chromatography followed by affinity chromatography on concanavalin-A Sepharose. The purified enzyme showed a molecular mass of 40 kDa and displayed optimal activity at pH 3.0 with a synthetic chromogenic substrate, Pro-Pro-Thr-Ile-Phe*Nph-Arg-Leu.

c) Purification and characterization of serine proteinases

Ishida *et al.* (1995) purified two kinds (type-I and type-II) of neutral proteinases in salted muscle of anchovy, *Engraulis japonica* by ammonium fractination followed by Phenyl-Toyopearl 650M, SuperQ-Toyopearl 650M, Arg-Sepharose 4B and SuperQ-Toyopearl 650S, respectively. Molecular weights of type-I and type-II proteinases were estimated to be 25,000 and 37,000 daltons, respectively, on electrophoretic analysis. Optimum pH for type-I and type-II proteinase were 6.8 and 7.0-7.5, respectively. Highest activities were found at 45 and 50°C for type-I and type-II proteinases, respectively.

Purification of a novel myofibril-bound serine proteinse (MBP) from the ordinary muscle of the carp (*Cyprinus carprio*) was carried out by solubilizing from the myofibril fraction with acid treatment, followed by Ultrogel AcA 54 and Arginine-Sepharose 4B chromatography (Osatomi *et al.*, 1997). Purification fold of 3.94×10^{6} with a yield of 77% was obtained. The molecular weight of purified was 30 kDa by SDS-PAGE and gel filtration. The optimum pH and temperature of the enzyme were 8.0 and 55°C, respectively. The enzyme hydrolyzed Boc-Gln-Arg-Arg-MCA most rapidly and also

hydrolyzed the substrates for trypsin-type proteinase, but not chymotrypsin. Boc-Phe-Ser-Arg-MCA hydrolyzing activity of the purified enzyme was reduced by addition of NaCl.

Choi *et al.* (1999a) also purified two serine type proteinases (A and B) from Atlantic menhaden proteinses by Sephacryl S-200, CM-Sephadex C-5 and Sephacryl S-200, in which purification fold of 26.0 and 21.7 with a yield of 3.8 and 2.9 % was obtained, respectively. The molecular weight of enzyme A and B were 112,000 and 90,500 daltons, respectively. Optimum Z-Phe-Arg-NMec hydrolyzing activity was observed at pH 7.4 and 40 to 50°C for both proteinases A and B. Both proteinases were inhibited by 1 mM TLCK, 1 mM benzamidine, 1% egg white and 1% bovine plasma hydrolysate.

Alkaline proteinase from Atlantic menhaden muscle was purified by ammonium sulfate fractionation followed by DEAE-Sephacel and Sephacryl S-300, respectively (Choi *et al.*, 1999b). Two alkaline proteinases (A and B) were purified to 62.9 and 986.5 folds with a yield of 2.8 and 6.3%, respectively. The molecular weight of purified enzymes were 707,000 and 450,000 daltons, respectively. Both are probably serine type proteinases, and optimum caseinolytic activity was observed at pH 8.0 and 55°C.

Purified two anionic trypsins (trypsin A and trypsin B) from the hepatopancreases of carp were prepared (Cao *et al.*, 2000a). The purification procedures consisted of ammonium sulfate fractionation, and chromatographies on DEAE-Sephacel, Ultrogel AcA54 and Q-Sepharose. Trypsin A was purified to homogeneity with a molecular weight of approximately by 28 kDa, while trypsin B showed two close bands of 28.5 kDa and 28 kDa on SDS-PAGE, both under reducing and non-reducing condition. Trypsin A and B revealed optimum temperature of 40 and 45°C, respectively, and showed the optimum pH of 9.0 using Boc-Phe-Ser-Arg-MCA as substrate. Both enzymes were effectively inhibited by trypsin inhibitors.

d) Purification and characterization of multicatalytic proteinases

Multicatalytic proteinase from Atlantic salmon (*Salmo salar*) muscle was purified by DEAE-Sepharose and Mono Q chromatography (Stoknes and Rustad, 1995). Purification fold of 3,100 with a yield of 138% was obtained. The molecular weight was estimated to be about 600,000 daltons. The isoelectric point was 7.3. The proteinase was able to hydrolyze N-terminal-blocked 4-methyl-7-coumarylamide substrates for either trypsin- or chymotrypsin-like activity. It was also able to hydrolyze haemoglobin and myosin at temperatures of about 60°C. The trypsin-like activity was clearly inhibited by several serine proteinase inhibitors.

Objectives

1. To study the proteolysis of muscle from two species of bigeye snapper, *P. macracanthus* and *P. tayenus.*
2. To purify and characterize the proteinase from bigeye snapper, which had high proteolytic activity.