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Proteolytic degradation of fish flesh occurring at elevated temperatures is the primary limitation for the commercial utilization of arrowtooth flounder (ATF). Characterization of the autolytic activity of ATF muscle incubated at various pHs and temperatures indicated the involvement of heat-activated proteinases active at acidic and alkaline pHs. Further characterization of the proteinase extract from fish muscle indicated the proteinase was more active at acidic pH than at alkaline pH in hydrolysis of Z-Phe-Arg-NMec and all types of protein substrates tested. Based on molecular weight and hydrolytic properties, activity peak separated on size exclusion chromatography, or activity bands observed on activity-stained substrate gels were presumed to be cathepsin L or like. A muscle proteinase showing similar hydrolytic properties to a proteinase extract was purified to electrophoretic homogeneity and subsequently confirmed by kinetic studies to be cathepsin L. Therefore, the results clearly indicated that cathepsin L is primarily responsible for autolytic activity of ATF muscle and surimi at the elevated temperatures.

Gelation of fish myofibrillar proteins, mainly myosin, is an important process for surimi production. Elucidation of the gelation mechanism and the effect of proteolysis on myosin provide information regarding protein interactions that improve ATF product quality. Heat-induced changes in physicochemical properties of myosin, free of endogenous proteinases, indicated myosin gelation consisted of two processes, denaturation and aggregation. ATF myosin was shown to be extremely sensitive to heat, resulting in denaturation at a lower temperature than other fish myosins. Denaturation began at 25°C and was initiated by the unfolding of the α-helical region. Following denaturation was the exposure of the hydrophobic and sulfhydryl residues, which were subsequently involved in aggregation and the gelation process. Changes in dynamic properties indicated ATF myosin formed a gel in three different stages, as shown by the first increase in gel rigidity at 28°C, followed by a decrease at 35°C and a second increase at 42°C.

A model system using ATF myosin and papain was developed to investigate how proteolysis affects the heat-induced gelation of fish myosin. The addition of papain decreased the onset temperature and the rate at which G' developed during heating. DSC thermograms indicated papain significantly decreased the enthalpy required to induce myosin denaturation with no significant changes in the onset or the maximum temperature. Thermal denaturation kinetics indicated a decrease in both the activation energy of the denaturation process and the denaturation rate of myosin. Although myosin gels could be formed, structural disruption caused by proteolysis, i.e., reduction in molecular size and loss in structural domain, resulted in lowering of the gelling ability of myosin and rigidity of the formed gels.

Endogenous Proteinase and Myosin Gelation of Arrowtooth Flounder (*Atheresthes stomias*)

by

Wonnop Visessanguan

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I understand that my thesis will be become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Wonnop Visessanguan, Author

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CONTRIBUTION OF AUTHORS

Dr. Haejung An was involved in the design, analysis, and writing of each manuscript. Dr. Masahiro Ogawa assisted in data collection for the study. CD analyses were performed in the laboratory of Dr. Shuryo Nakai who also assisted in the interpretation of data.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

TABLE OF CONTENTS (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF FIGURES (Continued)

LIST OF TABLES

ENDOGENOUS PROTEINASE AND MYOSIN GELATION OF ARROWTOOTH FLOUNDER (*Atheresthes stomias*)

Chapter 1

INTRODUCTION AND LITERATURE REVIEW

I. Commercial utilization of arrowtooth flounder

Arrowtooth flounder, *Atheresthes stomias*, is the most abundant species and has increased significantly in both population and biomass in the Gulf of Alaska, the Pacific coast of the United States and Canada (Wilderbuer and Brown, 1992; Wilkins et al., 1998). However, arrowtooth flounder has experienced limited commercial utilization due to the common problem of muscle softening soon after capture and textural degradation during processing and normal cooking procedure (Greene and Babbitt, 1990). While the causes of fish flesh softening soon after capture have not been thoroughly investigated, it is believed to be related to a short rigor period of arrowtooth flounder (Anon, 1991). Textural degradation of fish flesh during cooking at elevated temperatures was found to be associated with endogenous heat-stable proteinase (Wasson, 1992a).

Arrowtooth flounder (*Atheresthes stomias*), also referred to as longjaw flounder, French sole, and turbot, is listed as a member of the flatfish family "*Plueronectidae*". Arrowtooth flounder is a large piscivorous flatfish found at the depths ranging from 101-200 m. (Martin and Clausen, 1995). It is usually caught in

bottom trawls. The U.S. Pacific Coast (Alaska, Washington, Oregon, and California) arrowtooth flounder landings were 36,296 tons in 1997 valued at 3.5 million dollars (Shapiro et al., 1998). Arrowtooth flounder larger than 53 cm are all females, and account for 85% of the market (Ricky, 1994). The average length of males was less than that of females in every month that market and survey samples were taken. Fish size and catch rates are highest in spring and summer. In winter, average length of harvested fish decreases by approximately 9.0 cm for males and 16.0 cm for females. Arrowtooth flounder spawn during fall-winter, beginning as early as September and completing by March (Ricky, 1994).

Arrowtooth flounder is under valued. In 1995, 9,000 mt of arrowtooth flounder was caught in the Berring Sea-Aleutian Islands, of which all but 500 mt was discarded. Average ex-vessel price was about $0.02 per pound. Because of its poor eating quality using a normal cooking procedure, arrowtooth flounder has been traditionally used as animal feed. Recent advances in food processing, however, have allowed the production of marketable quality surimi (Wasson et al., 1992b; Porter et al., 1993), fish powder, and other value-added products which may further stimulate development of the fishery for human consumption.

Surimi production

Similar to Pacific whiting (*Merluccius productus*), a soft-textured fish currently used for commercial surimi production, arrowtooth flounder has some desirable characteristics that are required for the production of surimi and analog

seafood products, such as, bland flavor, white color, and low lipid content. Surimi is an intermediate product for a variety of surimi seafoods, such as kamaboko, fish meat gel, crab and other shellfish analogs. It is produced by repeated washing of minced fish flesh and mixing with cryoprotectants to extend its frozen shelf-life. To overcome the problems caused by a heat-stable proteinase, which greatly reduce the strength of final gel products, Wasson et al. (1992b) demonstrated that the use of protease inhibitors make it possible to produce surimi from arrowtooth flounder flesh.

Fish powder

While past research has centered on removal or inactivation of the arrowtooth flounder protease, new processing methods have recently been developed to exploit an inherent characteristic of arrowtooth flounder in order to turn the fish into fish powder. Fish powder is fish flesh that has been deboned, refined, and dried to a fine powder, like flour. It can be used as a protein supplement for both human and livestock (Brown, 1997). To convert fish into powder in just 20 min, arrowtooth flounder are de-headed, gutted, and then ground up. Flesh is separated from skin and bone, and then cooked. A spinning centrifuge removes most of the oil and water from the cooked fish, which is then quickly dried. From this process a white powder consisting of about 96-97% protein is obtained. For human consumption, the powder can be used as reconstituted minced fish or mixed with other foods.

Protease extract

A crude protease extract prepared from arrowtooth flounder mince was demonstrated to be effective as a processing aid to recover muscle proteins from fishery by-products (Tschersich and Choudhury, 1998). When the enzyme was used with pollock mince, frames and skin, the frames were clean and free of adhering muscle tissue and the skin was broken down, showing a 54-64% mass transfer from a solid to liquid phase. Arrowtooth flounder muscle contains a high level of activity of endogenous heat-stable protease. Thus, arrowtooth flounder might be a good source of enzymes to prepare extracts directly from muscle or to recover activity from the wash water of the surimi process, since most enzymes originating from the muscle are washed out in the waste stream.

Proteolysis in fish muscle

Proteolysis of fish muscle during postmortem storage and processing has been associated with the endogenous muscle proteases, resulting in undesirable flavor and texture alterations. In living cells, muscle proteases play a significant role in regulating a variety of cellular processes in muscle cells during growth and development, as well as, a defense mechanism in pathological conditions (Asghar and Bhatti, 1987). The proteolytic activity of these enzymes is highly regulated to prevent inappropriate and uncontrolled degradation. Subcellular compartmentalization, controlling rates of synthesis and degradation, and the presence of endogenous activators or inhibitors are examples of those regulatory

mechanisms (Bond and Butler, 1987). However, after death, these mechanisms become questionable in terms of their effectiveness to prevent excessive proteolysis due to energy depletion and accumulation of certain waste products, especially lactate and hydrogen ions, which probably initiate protein degradation and denaturation.

Muscle proteases are found in the sarcoplasmic component of muscle tissues in association with cellular organelles, connective tissues, and myofibrils, and in the interfiber space (Haard et al., 1994). In general, these proteases can be classified into 2 main groups of exopeptidases and endopeptidases (Asgar and Bhatti, 1987). Exopeptidases require the presence of free amino- or carboxy-terminus to initiate hydrolytic action on the peptide bond, yielding free amino acids and small peptides (McDonald and Schwabe, 1977). Endopeptidases cleave peptide bonds internally in the peptides and usually cannot accommodate the charged amino- or carboxy-termini amino acids at the active site (Barrett and McDonald, 1986). Endopeptidase can be further classified into 4 groups as cysteine, serine, metallo, and aspartyl proteases according to the nature of the catalytic residues at the active sites (Bird and Carter, 1980). The catalytic residues can be determined by using specific protease inhibitors (Asghar and Bhatti, 1987).

A number of endogenous proteases have been investigated for postmortem softening of fish flesh and participate to a different extent in the degradation of myofibrillar proteins (Wasson, 1992b; An et al., 1996; Kolodziejska and Sikorski, 1996). Classification of a variety of proteinases that have been found to participate

in muscle protein degradation of fish is shown in Table 1. The implication and theproteolytic activity of these proteases vary depending on fish species, location in the muscle, the life cycle of the fish, the presence of endogenous activators and inhibitors, and pathological conditions (Ashie and Simpson, 1997). Their activity and relative contributions in the degradation process, however, may be determined by the biological changes related to postmortem and the conditions during fish storage and processing.

Based on the postmortem pH of fish muscle, it is likely that muscle proteases active at pH 5-7 are involved in postmortem degradation of muscle proteins. The ultimate pH of the muscle tissue of most fish drops from the physiological pH to 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). Among the endogenous endoproteinases, calcium-activated proteinases (CANP) or calpains and lysosomal cathepsins are found to play the most influential role in tissue softening of most fish in the postmortem stage. However, only cathepsin L (An et al., 1994; Yamashita and Konagaya, 1990a) and alkaline proteases (Wasson, 1992b) have been reported as active proteinases involved in the texture softening at the elevated temperatures normally employed during surimi processing. The proteases involved in the textural degradation are discussed in more detail below.

Table 1. Classification and contribution of endoproteinase on muscle protein degradation in various types of fish.

	Enzyme	Fish	Optimum		Effect of muscle proteins
			pH	T (°C)	
Cysteine proteases	Calcium-activated proteases	Tilapia (1), Carp (2)	6.9-7.5	30	Cleavage of myofibrillar proteins to TCA soluble fragments; degradation of cytoskeletal proteins
	Cathepsin B	Chum Salmon (3), carp (4), Grey mullet (5), Pacific whiting (6)	5.7-6.0	37	Slight hydrolysis of myosin , actin, nebulin, and troponin T
	Alkaline protease	Carp (7), Baracuda (8), White croaker (8), Atlantic croaker (9)	6.0-8.5	55-65	Hydrolysis of myosin
	Cathepsin L	Chum salmon (10) , Pacific whiting (11), Mackerel (12), Carp (13)	5.0-5.6	55	Hydrolysis of most myofibrillar proteins, cleaving type I collagen
Serine protease	Alkaline protease	Threadfin bream (14), White croaker (15)	6.2-8.0	50-60	Hydrolysis of myosin
	Multicatalytic proteinases	White croaker (16), Carp (17), Atlantic salmon (18)	6.0-10.0	60-65	Hydrolysis of myosin
	Trypsin-like proteases	Croaker (19), Hake (20)	8.0-9.0	37-40	Hydrolysis of isolated myosin, disintegration of the cytoskeletal and contractile elements of intact myofibrils
Aspartyl Protease	Cathepsin D	Carp (21), Flounder (22), Tilapia (23), Cod (24)	5.5	30	Hydrolysis of myosin heavy chain
Metallo protease	Collagenase	Pacific rockfish (25), Carp (26)	7.0-8.0	25-40	Hydrolysis of type I collagen, gelatin, and other cytoskeletal matrix proteins

1: Jiang et al. (1991); 2: Tsuchiya and Seki (1991); 3: Yamashita and Konagaya (1991a); 4: Hara et al. (1988); 5: Bonete et al. (1984); 6 An et al. (1994); 7: Iwata et al. (1974a); 8: Iwata et al. (1974b); 9: Lin and Lanier (1980); 10: Yamashita and Konagaya (1991); 11: Seymour et al. (1994); 12: Lee et al. (1993); 13: Ogata et al. (1998); 14: Kinoshita et al. (1992); 15: Busconi et al. (1984); 16: Folco et al. (1989); 17: Kinoshita et al. (1990b); 18: Stoknes and Rustard (1995); 19: Folco et al. (1988); 20: Martone et al. (1991); 21: Makinodan et al. (1983); 22: Wasson (1992b); 23: Doke et al. (1980); 24: McLay (1980); 25: Bracho and Haard (1990); 26: Makinodan et al. (1979)

Calcium-activated proteinases (CANP) or calpains

CANP are neutral proteinases that require Ca^{2+} ions for their activity and are ubiquitous in the sarcoplasm of muscle. Two forms of CANP have been reported, which are distinguished by their requirements of Ca^{2+} ions, 50-70 μM for μM-CANP and 1-5 mM for mM-CANP (Asghar and Bhatti, 1987). CANP have been isolated and characterized from the muscles of marine organisms, i.e., carp (Toyohara et al., 1983), lobster (Mykles and Skinner, 1986), and scallop (Maeda et al., 1992). Most calpains isolated from vertebrate muscles are composed of two subunits, 80 kDa and 30kDa. However, the molecular weight of these enzymes from invertebrates ranges from 59kDa to 310 kDa. CANP seems unique from other proteinases, in that it has no activity on small peptide substrates and requires fairly long peptides to express its activity. It is believed that CANP does not require any specific sequence of amino acid residues for cleaving the peptide bond (Asghar and Bhatti, 1987). Calpains are optimally active at neutral pH and at temperatures below 30°C. Consequently, calpains may have a significant impact on the postmortem changes of myofibrillar and cytoskeletal proteins in refrigerated fish after catch by making the protein fragments more prone for concerted attack by other proteases (Tappel, 1977).

Cathepsins

Cathepsins are mostly present in the lysosomes within the muscle fibre and in the extracellular matrix originating from macrophages and connective tissue cells

(Haard, 1994). In general, many cathepsins have acidic pH optima although some are most active at neutral pH (Haard et al., 1994). There are 15 to 20 types of lysosomal cathepsins reported (Bohley and Seglen, 1992). They can be broadly defined into 2 major groups based on the sensitivity to specific inhibitors as either lysosomal cysteine proteinase or lysosomal aspartic proteinase. Cathepsins B, H, L, and S are the major enzymes in the group of lysosomal cysteine proteinases (Kirschke et al., 1989). They are similar in several molecular properties; however, they can be distinguished by their ability to degrade small peptides (Barrett and Kirschke, 1981). Cathepsins B, H, and L have been found to cause softening of chum salmon (Yamashita and Konagaya, 1990b), tilapia (Sherekar et al., 1988) and mackerel (Jiang et al., 1994). In Pacific whiting, however, cathepsin L was found to be the most active enzyme in surimi, while cathepsin B was the predominant enzyme in the fillets, with cathepsin H being the least active (An et al., 1994). It is postulated that cathepsin L may be the principal proteinase contributing to texture deterioration during conventional slow cooking. Texture degradation at lower temperatures, however, may be attributed to cathepsin B activity. Cathepsin S was originally purified from bovine lymph nodes (Turksek et al., 1975) and bovine spleen. It is closely related to cathepsin L regarding molecular properties and substrate specificity, except that it can retain its hydrolytic activity in the alkaline pH range (pH 7-8) where cathepsin L is inactive (Brömme et al 1989). The contribution of cathepsin S in proteolysis of fish muscle has not yet been studied. Cathepsin D, a lysosomal aspartic proteinase, has been shown to be an important

proteinase for post-mortem degradation of mammalian and avian muscle tissues (Ashie and Simpson, 1997). Since cathepsin D activity was substantially suppressed in post-mortem pH and at low incubation temperatures, it was found to be less significant in fish as compared to cathepsins L or B. However, it is known to initiate protein hydrolysis and produce peptide fragments that can then be further broken by other cathepsins (Huang and Tappel, 1971).

Alkaline proteases

Alkaline proteases are present in muscle sarcoplasm, microsomal fraction, or are bound to myofibrils (Makinodan et al., 1982a; Dahlman and Reinauer, 1978 in Haard, 1994). Alkaline proteases in fish muscle are broadly defined on the bases of their optimal condition and sensitivity to specific inhibitors. They have been classified into 2 main types of serine and cysteine heat-activated proteases. Alkaline proteases isolated from fish muscle show wide variations in their characteristics. Most of them are oligomeric proteases with high molecular weights ranging from 560-920 kDa (Haard, 1990, Wasson 1992b), although an exception was found with an alkaline protease isolated by Lin and Lanier (1980) from Atlantic croaker. It was a much smaller protein with a molecular weight of 80-84 kDa. A unique characteristic of alkaline proteases is they exhibit little or no catalytic activity unless assayed at a non-physiologically high temperature or are activated by protein denaturing agents, such as, urea, fatty acids or detergents (Dahlmann et al., 1985; Toyohara et al., 1987). Alkaline proteases have been

implicated in post-mortem changes in the cytoskeletal networks of fish muscle (Busconi et al., 1984; 1989a; 1989b). The property of heat activation has led researcher to study alkaline proteases as a cause of fish gel weakening during cooking of surimi products.

Multicatalytic proteinase or proteasome

Multicatalytic proteinase, also known as proteasome, is a multisubunits proteinase that possesses at least five catalytic activities and is optimally active in slightly alkaline pH (Orlowski et al., 1993). Proteasome is found ubiquitously in eukaryotic organisms (Tanaka, 1992). It is located in the cytoplasm and nucleus of cells and is widely distributed in any tissue and organ. At least two different forms of proteasome have been found based on the sedimentation coefficient; 20S and 26S (Hilt and Wolf, 1996). Proteasome exhibits a novel topology of subunit arrangement and belongs to a new class of proteases that have threonine residue at the active site (Seemüller et al., 1995). In living cells, proteasome plays a regulatory role of several cellular processes that are essential for life (Hilt and Wolf, 1996). Proteasome is believed to be the central cellular protein degradation system and functions differently from both lysosomal and calpain proteolytic systems.

The role of proteasome on the postmortem transformation of fish muscle has not been studied. Proteasomes are likely to be stable in postmortem muscle because it was found that multicatalytic activity of the proteinase purified from

bovine longissimus muscle was not affected by postmortem aging up to 14 days (Arbona and Koohmaraie, 1993). With the limitation of ATP after animal death, 26S proteasome, which requires ATP for its activity, is possibly involved in protein degradation only at the early stage of death. However, after ATP is completely depleted, 26S proteasome has a tendency to dissociate into 20S proteasome, which can hydrolyze protein in an ATP-independent manner (Orlowski, 1990). The 20S proteasome was shown to completely and rapidly hydrolyze purified myofibrillar proteins from bovine muscle in an energy-independent manner (Robert et al., 1999). After 24 h of incubation of the 20S proteasome with bovine myofibrils, loss of myofibrillar proteins particularly from the rupture of Z-discs and I-bands was found. Complete loss of M-line and a partial loss of Z-line structure were found when the purified proteasome from rabbit skeletal muscle was incubated with the myofibrils (Otsuka et al., 1999). The participation of 20S proteasome in proteolysis is limited and depends largely on storage and processing conditions as a result of its latency, as shown by many studies done in different organisms. However, it has been found that enzymes can be activated by some chemicals that are not present physiologically, heat treatment (Coux et al., 1996; Mykle, 1997), or high hydrostatic pressures up to 100-150 MPa (Otsuka et al, 1999). Proteasome may play a role in textural degradation of muscle with other heat-stable proteinases.

Heat-induced gelation of fish myofibrillar proteins

Heat-induced gelation of surimi is a complex physicochemical process involving structural and functional changes of myofibrillar proteins. The formation of a myofribrillar network can be represented by three stages: (1) dissociation of myofibrillar structures, (2) thermal denaturation, and (3) aggregation reaction (Roussel and Cheftel, 1990). In the presence of salt, muscle fibers and proteins undergo major structural changes, leading to the solubilization of myosin, actin, and a number of other myofibrillar constituents (Parsons and Knight, 1990). Partial unfolding of the protein structure is accelerated by an increase in temperature (Table 1.2), resulting in the aggregation of unfolded regions between protein molecules to form a three-dimensional network.

Heat-induced gelation refers to the formation of protein networks which contribute not only to the elastic texture, but also to other functional properties that are important to simulate the appearance, flavor, and texture of the natural meat counterparts (Park et al., 1997). Formation of fish meat gel has been characterized, in respect to changes in three-dimensional structure by three stages of "suwari", "modori", and "kamaboko" as heating proceeds (Suzuki, 1981). "Suwari" or gel setting stage describes the formation of a loose network when fish mince paste containing 2- 3% salt is heated to 40-50°C. It is mediated by transglutaminase and considered as the reaction wherein a three-dimensional network is formed and subsequently acts as the backbone of the final gel (Niwa et al., 1995). "Modori" describes the partial disruption of the loose network as the temperature is increased

Table 1.2. Conformation changes occurring during thermal denaturation of actomyosin (Ziegler and Acton,1984)

Temperature (°C)	Protein (s) or segment involved	Description of events
30-35	Native tropomyosin	Thermally dissociated from the F-actin backbone
38	F-actin	Super helix dissociates into single chains
40-45	Myosin "Head" "Hinge"	Dissociates into light and heavy chains Possibly some conformational changes Helix to random coil transformation
45-50	Actin, myosin	Actin-myosin complex dissociates
50-55	Light meromyosin	Helix to coil transformation and rapid aggregation
>70	Actin	Major conformational changes in the G-actin monomer

further over 50-60°C. Modori is associated with the action of endogenous heat-activated proteinases and/or the thermal behavior of myofibrillar proteins alone (Niwa, 1992). "Kamaboko" or gel enhancement refers to the formation of ordered, strong and elastic gels once the temperature reaches above 65-70°C.

Myosin and its gel-forming ability

The ability of surimi to form an elastic gel is largely derived from myosin (Sano et al., 1988), which comprises 55-60% of the myofibrillar proteins (Harringtons and Rodgers, 1984). Myosin is a multidomain protein with two large

heavy chains (M_r 200,000) and four light chains (M_r 15,000-27,000) arranged into an asymmetrical molecule with two globular heads attached to a long α-helical rod-like tail (Privalov, 1982; Lopez-Lacomba et al. 1989). Since myosin is a relatively large molecule, various proteolytic subfragments of myosin have been prepared and used to study their structures and functions. The heavy chain can be cleaved by papain near the globular end of the rod to produce a myosin rod and subfragment-1 (S-1) or by trypsin at the hinge region to produce light meromyosin (LMM) and heavy meromyosin, which can be further cleaved to yield S-1 and subfragment-2 (S-2) (Fig. 1.1).

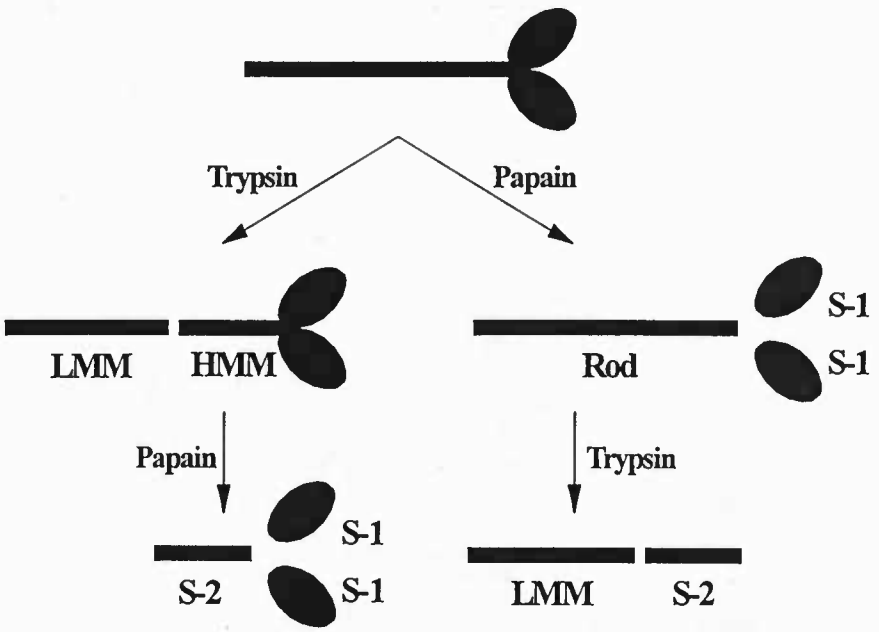

Fig. 1.1. Schematic diagram of a myosin and its subfragments (modified from Pearson and Young, 1989)

Myosin has been shown exclusively to possess the gelling ability, in contrast to other protein components. In surimi, myosin is present in a complex form with actin and other proteins, collectively called actomyosin, which could also form an elastic gel (Niwa, 1992). Since actin itself could not form a strong gel on heating, it was thought that the gelling characteristics of actomyosin are derived from the myosin portions. Binding with actin, however, has been shown to modify the gelling characteristic of myosin (Yasui et al., 1980). The addition of actin to myosin generally decreases the rigidity of the heat-induced gel (Culioli et al., 1993). However, an initial 2-3 fold increase in gel rigidity was observed when the myosin-to-G actin weight ratio was decreased from over 15 to 2.7, which is the optimal ratio of myosin to actin. Further addition of actin to the system decreased rigidity to the level expected for the dilution of myosin alone (Yasui et al., 1980)

Factors affecting gelling property of fish myosin

The ability of myosin to form a gel is affected by intrinsic factors, such as, species, muscle origin, and seasonality, as well as extrinsic factors, e.g., protein concentration, ionic strength, pH, and heating procedure. A particular functional property of protein is governed by a specific conformation. Consequently, any differences or alterations of protein structure in primary sequence, conformation, molecular size and shape, charge distribution, intra- and inter-molecular bonding of myosin may affect gelling ability to some degree.

Species

The gadoid species has a better gel-forming ability than the flatfish, although the distribution of myofibrillar and sarcoplasmic proteins is about the same for both groups (Shimizu, 1986). Since myosin heavy chain is the main subunit involved in gelation (Samejima et al., 1984), differences in composition and structure of myosin heavy chain among various fish species may be responsible for differences in cross-linking ability and the gelling properties of fish muscle under the same conditions. Species differences in the cross-linking of myosin heavy chain (MHC) is associated with the surface hydrophobicity displayed by the unfolded domains of the MHC and the temperature at which such domains unfold. (Wicker et al., 1986; Chan et al., 1992). It is postulated that the cross-linking ability of MHC may be localized at one or more discrete portions of the molecule and any structural/functional differences in these loci among different fish myosins might explain species differences in gel forming ability (Chan et al., 1993). In addition to hydrophobicity, myosin isolated from each fish species has its own thermal stability, which is related to physiological adaptation to the habitat environment (Davies et al., 1994). Discrepancies in thermal stability may affect how the proteins behave or interact with each other during heating, which directly relates to their ability to form a gel under a particular condition.

Muscle fiber types

In most fish, the myotomal musculature mainly consists of two homogeneous types of muscle fibers: white ordinary muscle and dark muscle running under the lateral line (Watabe and Hashimoto, 1980). It is generally accepted that the dark muscle is used for slow, continuous swimming and the white muscle for a quick burst (Bone, 1966). Myosin and myofibrillar proteins from white muscles generally exhibit a higher gel-forming ability than those from red muscles (Lefevre et al., 1998; Morita et al., 1987). Chemical differences between myosins from slow red and fast white fibres exist in classes of the light and heavy chains of the myosin molecule (Srakar et al., 1971; Lowey and Risby, 1981: Young and Davey, 1982). Myosin isolated from the white muscle have three light chain subunits with molecular weights of 26.5, 20, 17.5 kDa, respectively, while the myosin from dark muscle have only two lights chains with the molecular weights of 24 and 19 kDa (Watabe and Hashimoto, 1980; Lefevre et al., 1998). Differences in primary sequence of myosin isoforms may contribute to other properties such as solubility, surface hydrophobility (Ouali et al., 1988), and thermal stability (Lo et al., 1991; Watabe and Hashimoto, 1980).

Seasonal variation

Quality variation within a species is largely dependent on season. During and after spawning, before the feeding season begins, fish flesh loses gel-forming ability as a result of increased proteolytic activity, which leads to the proteolytic

breakdown of muscle tissue. Proteolysis decreases the myofibrillar protein level and increases moisture retention in the tissue (Okada and Tamoto, 1986). Changes in the actomyosin composition of mature hake were influenced by the metabolic state of the fish and associated with its reproductive cycles. Compared with resting and post-spawned fish, actomyosin from pre-spawned fish had a lower percentage of myosin. Changes in biochemical, physicochemical and functional properties of hake actomyosin during the reproductive cycle of fish have been reported (Roura et al., 1990). The loss of filamentous structure of actomyosin from pre-spawned hake was associated with a decrease in affinity between myosin and actin (Roura et al., 1992). Actomyosin from pre-spawned hake was more susceptible to denaturation than that from post-spawned samples as indicated by an increase in the rate of protein insolubilization or a decrease in viscosity and hydrodynamic properties (Montecchia et al, 1997).

Myosin concentration

The minimal protein concentration required to form a gel is highly dependent upon species, protein preparations, physicochemical conditions, and method of measurement (Lefèvre et al., 1998). Gill et al. (1992) found that the initial rate of aggregation of cod and herring myosin was concentration-dependent, increasing at higher myosin concentrations at all temperatures tested. A very weak gel was formed at a concentration of 10 g/L of myofibrils prepared from white muscle and 12 g/L from red muscle, but no gel was formed below these

concentrations. The increase in protein concentration from 10 to 20 or 25g/L induced an increase in gel rigidity and elasticity over the entire temperature range, while the transition temperature was unchanged (Lefèvre et al., 1998).

Ionic strength

Ionic strength determines the solubility or aggregation state of myosin and, thus, affects its heat-induced gelation (Boyer et al., 1996). Under high ionic strength, myosin depolymerizes and exists as monomers, while it notably exhibits the ability to polymerize into filaments under low ionic strength (Kaminer and Bell, 1966; Koretz, 1982; Huxley, 1983). The filamentogenesis of myosin occurs *in vivo* with the spontaneous association of molecules into thick filaments, which are the basis of both structure and function of the muscle cell. Most studies have found that myosin gels formed at low ionic strengths (0.1-0.3 M KCl) are of a higher rigidity than those produced at higher ionic strengths (>0.4 M KCl) (Ishioroshi et al., 1979; Egelandsdal et al., 1986; Hermansson et al., 1986). Myosin gels formed at low ionic strengths also have fine texture and are relatively translucent compared to a coarsely aggregated structure of myosin gels formed at high ionic strengths. The enhancement of hydrophobic interaction by the addition of salts has been well documented by Tanford (1980) and Nakai and Li-Chan (1988).

pH

The strength of surimi gel is dependent on the pH of the surimi paste. The optimum pH varies with fish species from which the surimi is prepared, as well as with formulation, since various types of ingredients interact with each other. When surimi was tested without added ingredients, the optimum pH was found to be between 6 and 7, with a minimum gel strength at pH 5. (Okada, 1986). By increasing the pH beyond 7, there was a gradual decrease in gel strength. At pH 6.1, the aggregation of minced rainbow trout occurs at lower temperatures and the final texture is firmer compared with gels formed at pH 7.4. The pH–dependency of gel formation is related to the dependency of the water-binding ability of myofibrillar protein on pH (Hamm, 1986). Since the formation of a stronger gel is obtained in a specific range of pHs above the isoelectric point, gel forming ability probably is influenced by charge distribution on myosin. As the pH approachs the pI of myosin, the negative and positive charges among protein molecule are about equal. Therefore protein molecules are strongly and quickly associated with each other, even without heating. At pH above the isoelectric point, the net charge of the protein becomes negative. The increased net charge offers more binding sites for water and balances the association and repulsion among the protein molecules, leading to the formation of an ordered protein-protein interaction.

Heating rate

Slower heating rates have been known to result in a higher modulus of rigidity (G'), as well as increased hardness, guminess and chewiness values and higher fluid losses during cooking. Lowering the heating rate favors the formation of different bonds between proteins. As a result, different types of gels can be formed (Lefèvre et al., 1998). Slow heating rate induced aggregation of both Pacific whiting and cod myosin to occur at a lower temperature because of prolonged time allowed for protein unfolding. Slow heating promotes aggregation of cod myosin and allows aggregates to form a more elastic gel. However in fish containing high protease, such as Pacific whiting, slow heating also activates proteinase, possibly associated with myosin, resulting in lowering gel elasticity (Yongsawatdikul and Park, 1998).

Others

In surimi gelation, the ability to form a myosin gel can be enhanced or hindered by the additives used in the formulation. The effect of polyphosphate on myofibrillar proteins has been controversial (Xiong, 1997). Pyrophosphate was found to increase the gel strength of myofibrils due to the combined effects on myosin extraction and the interaction with the myosin heads (Samejima et al., 1986). However, in a number of other studies, pyrophosphate treatment resulted in increased fluidity of the protein suspension and decreased myofibrillar protein gel strength (Robe and Xiong, 1993; Torley and Young, 1995). Sucrose and sorbitol

are used as cryoprotectants in surimi production to protect myofibrillar proteins from freeze denaturation. In addition, sorbitol can enhance the solubilization of myosin, which is important for the formation of elastic gel and increase the thermal stability of myofibrillar proteins (Konno et al.,1997). Protein additives, such as, bovine plasma protein (BPP), have also been widely used in surimi manufacture to maximize the gel strength (An et al., 1996). The main functions of BPP are believed to be proteinase inhibition by α_2-macroglobulin (α_2-M) and kininogens, and enhancement of gel network development by transglutaminase (Seymour et al., 1997). However, the effect of these protein additives on myosin gelation has not been studied. Pig plasma proteins (PPP) are effective in protecting myosin heavy chain from proteolytic degradation. Addition of pig plasma proteins has been shown to revert substantial decreases in the development rate and the magnitude of the gel modulus (G') of actomyosin gels with added proteinase, but not to the extent of the original rigidity of the gel (Visessanguan et al., 1999). PPP itself, however, was found to interfere with the formation of actomyosin gel. It was believed that some heat-stable protein components in PPP may interfere with cross-linking during matrix formation as they do not form gels.

Dynamic rheolgical testing

The dynamic rheological test or small-strain gel rigidity test has been widely used as an instrumental method to study heat-induced gelation of myofibrillar proteins (Hamann et al., 1990). The small-deformation mechanical

dynamic test is very useful for monitoring sol-gel transition and for characterizing the viscoelastic behaviour of gels in the linear region in which the amplitudes of the stress and strain are adjusted to sufficiently low values that stress is proportional to strain (Ross-Murphy, 1984). Dynamic measurements allow gelation to be monitored since the induced deformations are usually so small that their effect on the structure of the material being investigated is negligible. Rheological characteristics of the gel are described by three parameters, the storage modulus (G'), loss modulus (G"), and loss tangent (Tan δ, the ratio of G"/G'). When the gel is deformed by an imposed strain, part of the energy input is stored and recovered in each shear cycle due to the elastic response of the gel. Another part of the energy is dissipated as heat due to internal friction or viscous flow of the sample. Thus, G' and G" are measures of elasticity and viscosity of the gel samples, respectively, and Tan δ reflects the relative contribution of each to the overall rheological characteristics (Xiong, 1997).

Differences in the rheological patterns observed among myosin, actomyosin, and surimi studied under the same conditions (Sano et al., 1988) are largely attributed to the presence of other proteins in the system which probably interfere or modify the gelling properties of myosin. The changes in rheological properties of myosin and actomyosin during heating at a similar condition indicated that myosin produces more elastic gels than actomyosin and the gelation reflected three steps of texture formation previously described using fish mince (Sano et al., 1988). Although dynamic testing has not been shown to highly correlate with

sensory texture or rupture strength by other large strain rigidity tests (Hamann et al., 1990), this method is suitable to study the alteration of gelation properties caused by proteolysis and changes in processing conditions caused by the addition of protein additives (Liu and Xiong, 1997).

Research objectives

The overall objective of this research is to provide basic information about protein interactions in order to improve the quality of arrowtooth flounder products and to better utilize this low-valued fishery resources. The presence of a heatstable cysteine protease in arrowtooth flounder has been established. The interaction of the cysteine proteases with the myofibrillar proteins results in the softening of fish flesh and gel products. The identity and hydrolytic properties of the active enzyme, however, has not been reported. As a source of food protein, arrowtooth flounder has been utilized in commercial application through surimi production. Interaction of myofibrillar proteins, mainly myosin, is important for gel formation. Understanding how a gel is formed and how proteolysis affects myosin gelation would help manufacturers optimize the parameters involved in the processing and handling of fish in order to achieve high quality products.

The specific objectives were identified as follows:

1) Identify and study the hydrolytic properties of the proteinase present in the muscle of arrowtooth flounder with a focus on heat-activated proteinases.

2) Investigate the physico-chemical changes of arrowtooth flounder myosin during heating and its gelation mechanism.
3) Investigate how proteolysis affects the heat-induced gelation of fish myosin in the model system prepared with arrowtooth flounder myosin and papain.

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Chapter 2

HYDROLYTIC PROPERTIES OF HEAT-ACTIVATED PROTEINASE IN ARROWTOOTH FLOUNDER MUSCLE

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Abstract

Characterization of the autolytic profile of arrowtooth flounder muscle indicated the involvement of heat-activated proteinases active at both acidic and alkaline pHs. Further assay of the proteinase extract from fish muscle exhibited the maximum activity at 60°C against casein used as a substrate at both pH 5.5 and 8.0. The maximum activity shifted to lower temperatures by the addition of 2.5 and 4.0 M urea, but the maximum activity was reduced at pH 5.5, while increased at pH 8.0. The inhibition by E-64, a specific cysteine protease inhibitor, indicated the proteinase belongs to the cysteine proteinase class. The proteinase effectively hydrolyzed Z-Phe-Arg-NMec and all types of protein substrates tested, i.e., casein, bovine serum albumin, hemoglobin, and arrowtooth flounder myofibrils. However, the hydrolysis rate at pH 5.5 was twice as high as that at pH 8.0. Activity bands, observed in the range of 43-124 kDa on the activity stained-substrate gels, indicated the presence of several proteinases in fish muscle extract that are responsible for the proteolytic activity observed at pH 5.5 and 8.0. When proteins of fish extract were separated by HPLC size exclusion chromatography, only one proteolytic peak was observed. This fraction, observed at the retention time of 26 min, showed the previously observed effects of urea: the reduction of activity at pH 5.5 and its enhancement of activity at pH 8.0. Based on the molecular weights and substrate specificity, the results implied the presence of cathepsin L or likes as the major proteinase responsible for the autolysis of arrowtooth flounder muscle at elevated temperatures.

Introduction

Arrowtooth flounder, *Atherestes stomias*, is the most abundant species in the Gulf of Alaska along the Aleutian chain and the Bering Sea and found on the Pacific coast of the United States and Canada (Cullenberg,1994). Arrowtooth flounder has been underutilized due to the problem of muscle softening during processing and cooking, resulting from the endogenous protease activity in the muscle (Greene and Babbitt, 1990). A number of endogenous proteases have been investigated as enzymes contributing to postmortem softening of fish flesh, which also participate to a different extent in the degradation of myofibrillar proteins (Wasson, 1992; An et al., 1996; Kolodziejska and Sikorski, 1996). The action of these enzymes exerts detrimental effects on the sensory quality and functional properties of muscle foods (Asghar and Bhatti, 1987). Among them, heat-activated proteinases are of great concern for utilization of fish, since they are active at cooking temperatures and at neutral pH. In addition, they can damage fish textures and reduce the gel strength of surimi.

Proteolytic degradation of fish muscle proteins at elevated temperatures has been related to the presence of lysosomal cathepsins or alkaline proteinases. Many cathepsins have acidic pH optima, although some are most active at neutral pH (Haard et al., 1994). The cysteine-type cathepsins have a strong potential to be active at the postmortem muscle pH (5.5-6) and retain a significant level of activity up to pH 7 (Zeece et al.,1992). Cathepsins B, H, and L have been found to cause softening of chum salmon (Yamashita and Konagaya, 1990), tilapia (Sherekar et

al., 1988) and mackerel (Jiang et al., 1994). Cathepsin L was a major proteinase found to degrade the myofibrillar proteins in Pacific whiting (An et al., 1994). The gel-softening phenomenon or "modori" observed at 50-70°C was attributed to myosin hydrolysis by heat stable alkaline proteinases (Lin and Lanier, 1980; Boye and Lanier, 1988). Alkaline proteases isolated from fish muscle show wide variations in their characteristics. They are oligomeric protease with high molecular weights ranging from 560-920 kDa (Wasson 1992). However, an alkaline protease isolated by Lin and Lanier (1980) from Atlantic croaker was a much smaller protein with molecular weight of 80-84 kDa. These enzymes normally exhibit little or no catalytic activity unless assayed at a non-physiologically high temperature or are activated by protein denaturing agents like urea, fatty acids or detergents (Toyohara et al., 1987).

The presence of a heat stable cysteine protease in arrowtooth flounder was first reported by Greene and Babbitt (1990) and later partially characterized by Wasson et al. (1992). The partially purified enzyme was shown to have an approximate molecular weight of 32,000 daltons and an optimal condition at pH 6.0-7.0 and 55°C against casein. However, the identity of the active enzyme could not be clearly defined. Therefore, the objective of this study was to identify and study the hydrolytic properties of the major proteinase present in the muscle of arrowtooth flounder with a focus on the heat-activated proteinases involved in the autolysis of fish muscle at elevated temperature.

Material and methods

Chemicals

N-carbobenzoxy-phenyalanine-arginine 7-amido-4-methylcoumarin (Z-Phe-Arg-NMec), N-carbobenzoxy-arginine-arginine 7-amido-4-methylcoumarin (Z-Arg-Arg-NMec), L-arginine 7-amido-4-methylcoumarin (L-Arg-NMec), 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), pepstatin, leupeptin, phenanthrorine, ethylene diaminetetraacetic acid (EDTA), cytochrome C (horse heart), carbonic anhydrase, bovine serum albumin (BSA), hide powder azure, sweet potato amylase, blue dextran, Tris/base, 2-mercapthoethanol (ME), p-chloromercuribenzoate, aprotinin, and dithiothreitol were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium caseinate was obtained from US Biochemical Corp. (Cleveland, OH). Iodoacetic acid and phenylmethylsulfonyl fluoride (PMSF) were purchased from Calbiochem Co. (La Jolla, CA).

Samples

Arrowtooth flounder harvested off the Oregon Coast were obtained from a local processor as frozen fillets and kept frozen at -20°C until used.

Autolytic assays

Autolytic activity was measured by the modified method of Greene and Babbitt (1990). Frozen fish fillets were thawed and finely chopped. Three grams of chopped muscle were incubated for 30 min in a waterbath at temperatures specified

in the text. The autolytic reaction was stopped by adding 27 mL ice-cold 5% (w/v) trichloroacetic acid (TCA). The mixture was homogenized for 1 min using Polytron (Brinkmann Instruments, Westbury, NY) and kept on ice for 1 hr followed by centrifugation at 5,000 *xg* for 10 min to collect the TCA-soluble supernatant. Oligopeptide content was determined by Lowry assay (Lowry et al., 1951) and autolytic activity expressed as micromole of tyrosine released per minute (μmol Tyr/ min).

Preparation of fish extract

Sarcoplasmic fluid of fish muscle was prepared according to the method of Seymour et al. (1994). Frozen fish fillets were thawed, finely chopped, and then centrifuged at 5,000 *xg* for 30 min to collect the supernatant as an enzyme source.

Enzyme assay

Protease activity was assayed using casein as a substrate according to the method of An et al. (1994). Activity was determined by the TCA-Lowry assay. Fish extract (50-100 μL) was added to the preincubated reaction mixture of 2 mg of casein and 625 μL reaction buffer (McIlvaine's buffer pH 3-8 or 0.2 M Tris buffer pH 8-9). Distilled water was added to bring to final volume of 1.25 mL. The mixture was incubated at various pH and temperatures specified in the text for precisely 20 min. Enzymatic reaction was stopped by adding 200 μL of 50% (w/v) trichloroacetic acid (TCA). The reaction mixture was incubated at 4°C for 15 min

to precipitate unhydrolyzed proteins and the mixture was then centrifuged at 5,700 *xg* for 10 min to collect the supernatant. Activity was expressed as tyrosine equivalents in TCA supernatant, as measured by the Lowry assay (Lowry et al.,1951). One unit of activity (U) was defined as 1 nmole of tyrosine released per min (nmol Tyr/min). A blank was run in the same manner except the enzyme was added after the addition of TCA solution.

Effect of urea

Urea was dissolved in McIlvaine's buffers, pH 5.5 and 8.0, to a final concentration of 2.5 M and 4 M, respectively, to avoid possible pH changes in the reaction mixture. The enzyme activity was determined by TCA-Lowry assay as described above. Effect of urea was tested by changes in the caseinolytic activity of fish extract in the presence and absence of urea in the reaction mixture.

Effect of inhibitors

Fish extract was incubated with an equal volume of protease inhibitor stock solutions of E-64, sodium iodoacetate, leupeptin, sodium bisulfite, PMSF, soybean trypsin inhibitor, p-chloromercuribenzoate, aprotinin, pepstatin, 1,10-phenanthroline, dithiothreitol (DTT) and EDTA at the concentrations listed in the text for 15 min at room temperature. The residual activity was analyzed by TCA-Lowry assay using casein as a substrate.

Hydrolysis of synthetic substrates

The hydrolytic activity on various synthetic substrates including Z-Phe-Arg-NMec, Z-Arg-Arg-NMec, and L-Arg-NMec were determined according to the method of Yamashita and Konagaya (1990a) with slight modification. Fish extract, 20-25 μL, was diluted to 1000 μL with 0.1% Brij 35 and added with 500 μL of assay buffers (8 mM DTT McIlvaine's buffer, pH 5.5 or 8.0). To initiate the enzymatic reaction, 500 μL of 20 μM substrate solution was added to the mixture and then incubated at 55°C for 10 min. The reaction was stopped by adding 200 μL of 5 mM iodoacetic acid. The fluorescence intensity of aminomethylcoumarin was determined at the excitation wavelength, 370 nm, and emission wavelength, 460 nm, using a luminescence spectrophotometer (LS 50B, Perkin-Elmer Ltd., Beaconsfield, England). A unit of activity was expressed as 1nmole of methylcoumarin released/min.

Effect of neutral pH incubation

Fish extract was incubated at 37°C for 1, 2, 4, and 6 h with 10 volumes of 150 mM Tris-HCl, pH 7.5 containing 2 mM EDTA, 2 mM DTT, and 0.01% Triton X-100. The hydrolytic activity of preincubated fish extract was determined against Z-Phe-Arg-NMec according to the method previously described.

Hydrolysis of protein substrates

The protein substrates, i.e., BSA, acid-denatured bovine hemoglobin and purified arrowtooth flounder myofibrils (Kay et al., 1982), were tested as substrates in place of casein in the TCA-Lowry assay. One unit activity (U) is defined as nmole of tyrosine released per min per mg of protein (nmol Tyr/mg/min). Hydrolytic activity against azocasein was performed by TCA-azocasein method (An et al., 1994) and expressed as A450. Hide powder azure was tested by the method of Rinderknecht et al. (1968) with the slight modification of adding cold 50% (v/w) TCA solution to stop the reaction. Activity was determined spectrophotometrically at 595 nm and expressed as A595 compared with the absorbance of a blank.

Native discontinuous eletrophoresis and activity staining

Native discontinuous electrophoresis was carried out by leaving out the SDS and reducing agent from the standard Laemmli protocol (Laemmli, 1970) and stained for protease activity with the modified method of García-Carreño et al. (1993). Polyacrylamide gels were prepared for 5%, 7.5%, 10%, and 12.5% running gels with 4% stacking gels. Fish extract was mixed with the sample treatment buffer (0.125 M Tris-HCl, pH 6.8 containing 20% (v/v) glycerol) at 1:1 (v/v) ratio, and 18-36 μg of proteins were loaded on the gel. The proteins were subjected to electrophoresis at a constant voltage of 75 V by mini Protean II (Bio-Rad Laboratories Inc., Richmond, CA). After electrophoresis, gels were immersed in

100 mL of 2% (w/v) casein in 50 mM Tris buffer, pH 7.5 for 1 h at 0 °C to allow the substrate to penetrate into the gels at reduced enzyme activity. The gels were then immersed for 1 h in 2% casein (w/v) in 200 mM McIlvaine's buffer containing 8 mM DTT at pH 5.5 and 8.0, respectively. The gels were fixed and stained with 0.125% Coomassie blue R-250 in 50 % ethanol and 10% acetic acid and destained in 25% ethanol. Development of clear zones on blue background indicated proteolytic activity. The molecular weight of a protein under investigation was estimated by the modified method of Bryan (1977). Sigma native molecular weight standards including thyroglobulin (M_r 669,000), amylase (M_r 200,000), and carbonic anhydrase (M_r 29,000) were separated on 5%, 7.5%, 10%, and 12.5% acrylamide gels. Relative electrophoretic mobility (R_f) of the protein band was determined and the logarithm of R_f was plotted against gel concentration. The slope (K_r) was determined using linear regression and –log K_r values were plotted against log molecular weight of the standards.

Size exclusion HPLC

Size exclusion chromatography was performed on HPLC using Superose 12 HR 10/30 column (Pharmacia LKB Biotechnology,Uppsala, Sweden) connected with a Bio-Rad HPLC pump (Model 2700, Bio-Rad Laboratories, Inc., Hercules, CA) and a UV detector (Bio-Rad Model 1706). Fish extract, 200 µL, containing 5-10 mg protein was injected into the column after filtration through a 0.22 µM filter to remove large particles. The proteins were eluted isocratically with 50 mM

sodium phosphate buffer, pH 7.2 at a flow rate of 0.5 mL/min. Eluted proteins were monitored by absorbance at 280 nm and collected in 1.5 mL aliquots for further analyses. The caseinolytic activity of each fraction was determined at pH 5.5 and pH 8.0 in the presence and absence of 2.5 M urea.

The proteinase separated on SEC-HPLC was estimated for its molecular weight by plotting relative elution volume (Ve/Vo) against the logarithm of M_r of the protein standards. The elution volume (Ve) was measured for each protein standard and the proteinase, and the void volume (Vo) was estimated by the elution volume of blue dextran (M_r 2,000,000). The standards used included horse heart cytochrome C (M_r 12,400), carbonic anhydrase (M_r 29,000), BSA (M_r 66,000), and sweet potato amylase (M_r 200,000).

Protein determination

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

Results

Autolysis in arrowtooth flounder muscle

Autolytic activity of ATF muscle was characterized by the temperature and pH profiles, as shown in Figures 2.1 and 2.2, respectively. The temperature profile analyzed at the physiological pH of fish muscle indicated autolytic activity

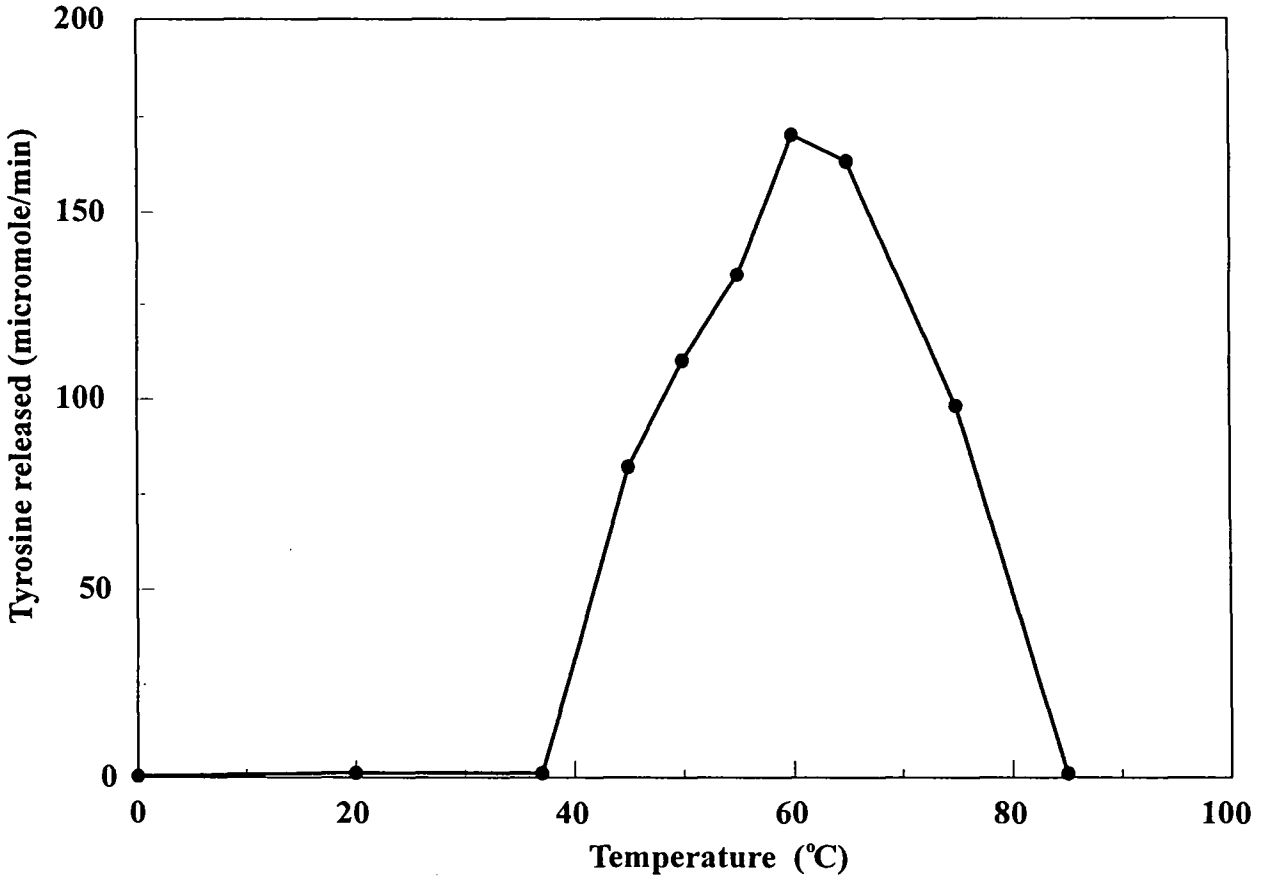

Fig. 2.1. Temperature profile of arrowtooth flounder autolytic activity. Autolytic activity was determined by incubating arrowtooth flounder mince at various temperatures. The TCA-soluble proteins were recovered and determined by Lowry assay and expressed as μmole of tyrosine released per mL (μmol Tyr/ min).

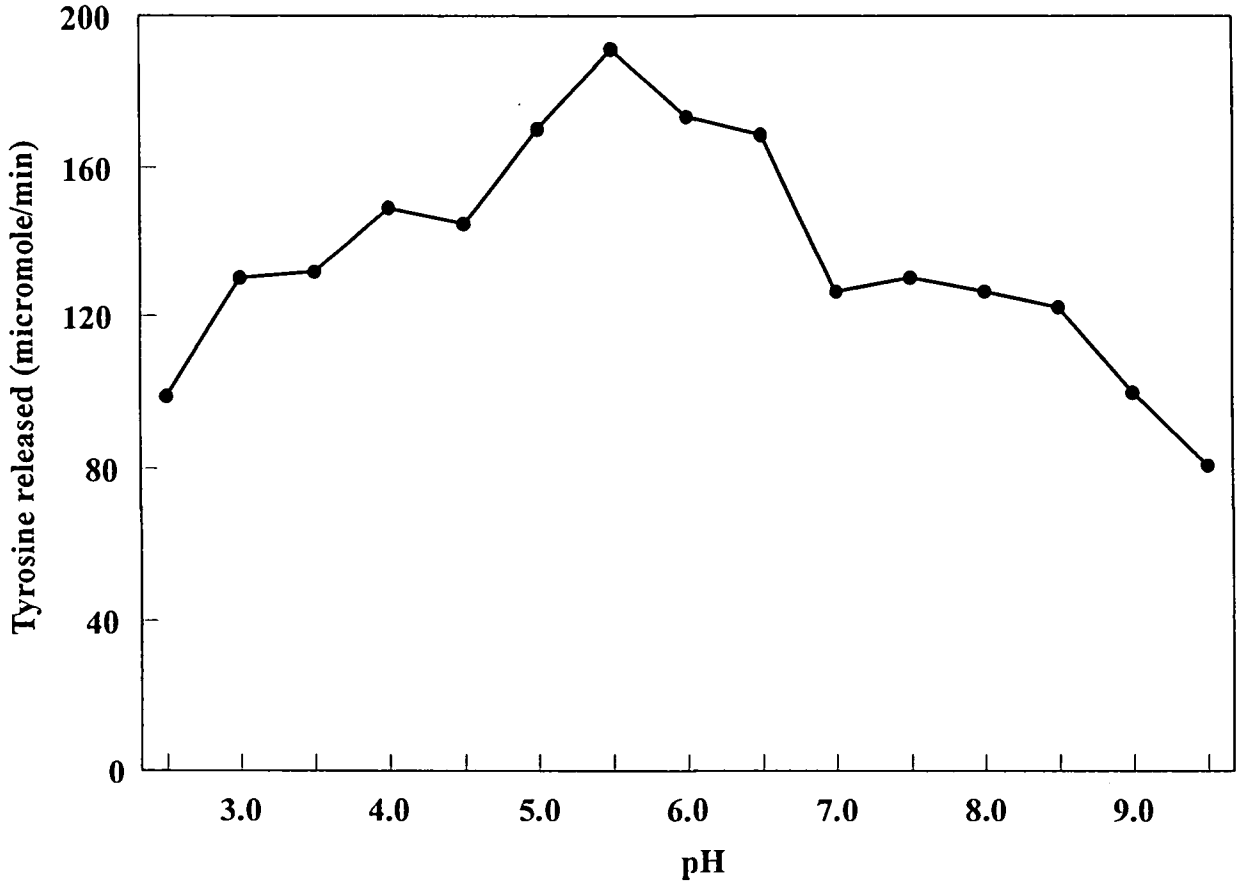

Fig. 2.2. pH profiles of arrowtooth flounder autolytic activity. Autolytic activity was determined by incubating arrowtooth flounder mince at 55°C or 60°C at various pH values. The TCA-soluble proteins were recovered and determined by Lowry assay. The activity was expressed by change in liberated Tyrosine (μmol Tyr/ min).

increased markedly from 37°C to the highest peak at 60°C before rapid inactivation at higher temperatures. The pH profile of autolytic activity analyzed at 60°C showed a major peak at pH 5.5 (Figure 2.2). Above pH 5.5, activity slightly decreased with an increase in pH, however, it showed a minor peak at pH 8.0 with approximately 60% of the activity observed at pH 5.5. Based on the temperature and pH optimum, autolysis of ATF muscle was thought to be mediated by heat-activated proteinases that are optimally active at slightly acidic and alkaline pHs. The results coincide with the post-mortem pH of fish muscle for flatfish, which is pH 5.5 (Eskin, 1990). This may explain the severe textural degradation observed with the fish after death and the undesirable paste-like texture occurring during cooking at elevated temperatures.

Effect of urea

The temperature profile of caseinolytic activity of fish extract was established at pH 5.5 and 8.0, respectively, where the autolytic activity peaks were found. Optimum temperature and the magnitude of activity were affected by the addition of urea. At pH 5.5, urea shifted the optimum temperatures down from 60°C to 45°C. The maximum activity was dependent on the urea concentration added. At temperatures up to 50°C, urea was generally found to increase the caseinolytic activity of fish extract, while at temperatures above 50°C, it reduced the activity slightly (Fig. 2.3a). At pH 8.0, urea not only shifted the activity curve toward the lower temperatures but also increased the activity at all temperatures

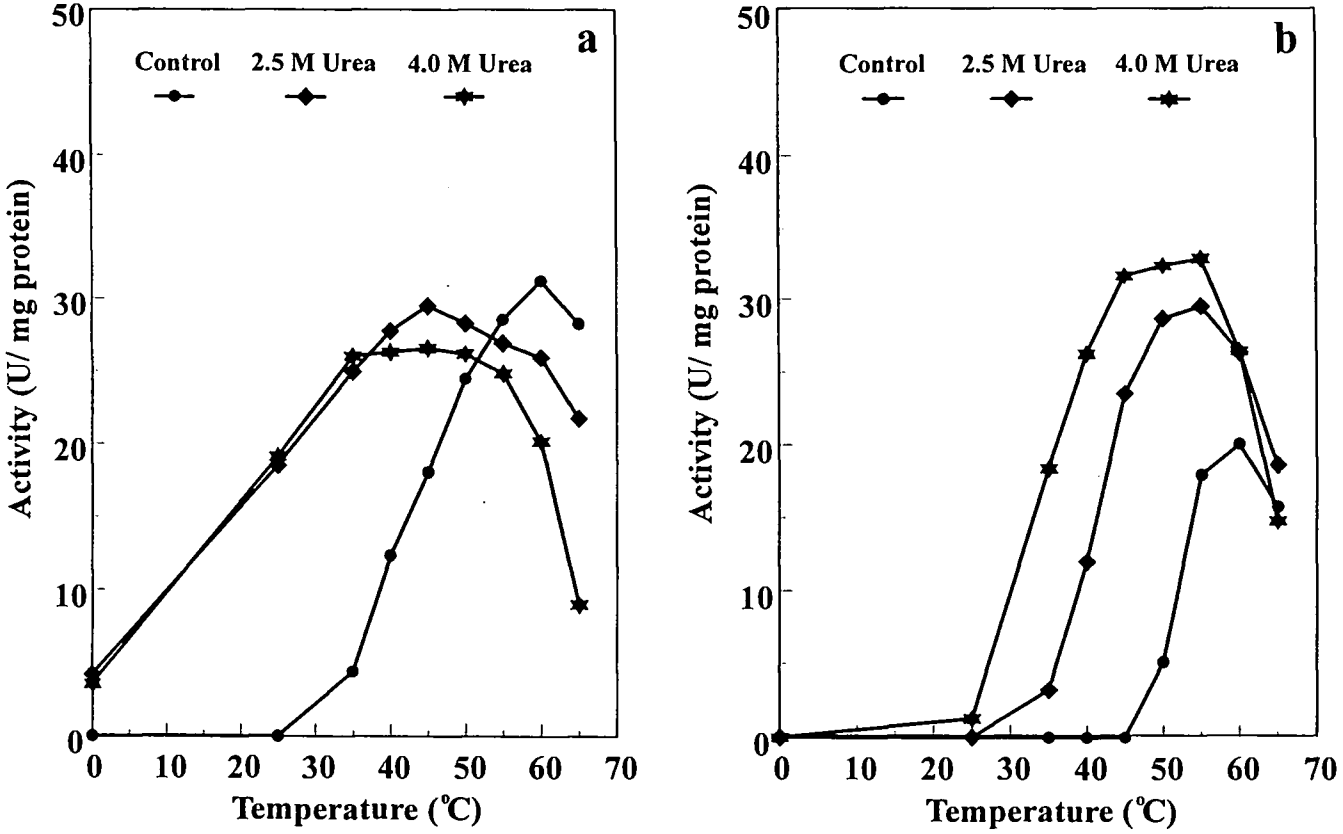

Fig. 2.3. Effect of urea on the temperature profile of caseinolytic activity of fish extract; (a) at pH 5.5 and (b) at pH 8.0. Urea was added in the activity assay buffer to the final concentrations of 2.5 and 4 M, respectively. The activity was determined by TCA-casein method and expressed as a μmole of tyrosine released per mg protein.

tested (Fig. 2.3b). Especially at temperatures of 45°C or below, where the caseinolytic activity of the control was rarely detected, the activity was induced approximately 20 to 30 folds in the presence of 2.5 and 4 M urea. However, the increase in activity was less significant at temperatures above 55°C.

Effect of proteinase inhibitors

All four types of inhibitors, i.e., serine, cysteine, aspartic acid and metallo-protease, were tested with ATF fish extract to determine the class of active proteinase at pH 5.5 and pH 8.0. Among the inhibitors tested the highest inhibitions, 90-92%, on both pH conditions were shown by E-64, a specific cysteine protease inhibitor (Table 2.1). Leupeptin and iodoacetate reported as both serine and cysteine protease inhibitors also showed the substantial inhibitory effect on the enzyme activity, but to a lesser extent, 74-90%. Specific inhibitors of serine proteinase (soybean trypsin inhibitor), aspartic proteinase (pepstatin) and metalloproteinase (dithiothreitol + EDTA) showed negligible inhibition of the activity. The results primarily indicate that the enzymes active at both pHs were cysteine proteinases, which is in agreement with the heat stable enzyme partially purified from arrowtooth flounder muscle by Wasson et al. (1992).

Table 2.1. Effect of proteinase inhibitors on proteinase in arrowtooth flounder extract

Inhibitors	Concentration	% Relative activity*	
		pH 5.5	pH 8.0
E-64	0.1 mM	8.0	10.7
Iodoacetate	1 mM	10.2	26.1
Leupeptin	1 mM	12.1	15.4
Sodium bisulfite	10 mM	47.9	93.5
PMSF	1 mM	73.7	55.9
1,10-phenanthroline	1 mM	83.2	87.9
Soybean trypsin inhibitor	0.1 g/L	92.0	99.7
PCMB	0.1 mM	93.2	103.5
Aprotinin	2 μg/ml	95.9	94.6
Pepstatin	1 mg/L	97.1	97.4
DTT+EDTA	2 mM each	98.7	101.5

*Averages of duplicate analyses representing residual activity analyzed using casein as a substrate for 20 min under the specified conditions.

Hydrolysis of synthetic and protein substrates

Hydrolytic activity of the fish extract was analyzed against the synthetic substrates specific for cathepsins B, H, and L which have been shown to be proteinases active in acidic pH ranges. To focus only on heat-activated proteinases active at pH 5.5 and 8.0, activity was measured at 55°C. Among the substrates tested, fish extract could hydrolyze only Z-Phe-Arg-NMec, a specific substrate commonly used to assay cathepsin L activity (Table 2.2). The higher rate on

Table 2.2. The hydrolytic activity of fish extract on various synthetic substrates

Assay	Activity (U/mg protein)*	
	pH 5.5	pH 8.0
Cathepsin L; Z-Phe-Arg-NMec	4.30 ± 0.02	1.89 ± 0.12
Cathepsin B; Z-Arg-Arg-NMec	ND	ND
Cathepsin H; L-Arg-NMec	ND	ND

*Averages of triplicates ± S.D.; ND: Non-detectable.

hydrolysis at pH 5.5 clearly indicated cathepsin L or cathepsin L-like are responsible for autolytic activity of arrowtooth flounder muscle in the acidic pH range. At pH 8.0, Z-Phe-Arg-NMec was also found to be the only substrates being hydrolyzed by fish extract, even though the rate was lower than that of pH 5.5. Since cathepsin L has been reported to be rapidly inactivated and irreversibly denatured at pH above 7, it was thought that activity detected was implicated by other proteinases with substrate specificity similar to cathepsin L. Among the cysteine proteinases capable of hydrolyzing Z-Phe-Arg-NMec, cathepsin S is found to be closely related to cathepsin L and can retain its activity at alkaline pH. Preincubation of the enzyme extract at pH 7.5 for 1, 2, 4, and 6 h significantly decreased hydrolytic activity of fish extract assayed at pH 5.5 against Z-Phe-Arg-NMec, but no significant effects were found when analyzed at pH 8.0 (Table 2.3). A neutral pH incubation was presumed to inactivate other lysosomal proteases with substrate specificity similar to cathepsin S, although it does not affect the activity

Table 2.3. Effect of preincubation at pH 7.5 on hydrolytic activity of fish extract at pH 5.5 and 8.0 using Z-Phe-Arg-NMec as a substrate

	Incubation Time (h)	Specific activity (U/ mg protein)	
		pH 5.5	pH 8.0
Fish extract	1	4.31 ± 0.02	1.90 ± 0.12
	2	4.18 ± 0.11	1.86 ± 0.04
	4	4.22 ± 0.14	1.82 ± 0.05
	6	4.24 ± 0.25	1.96 ± 0.02
Fish extract with a neutral incubation	1	4.06 ± 0.11*	1.91 ± 0.07
	2	3.86 ± 0.03*	1.82 ± 0.05
	4	3.72 ± 0.05*	1.87 ± 0.01
	6	3.48 ± 0.11*	1.82 ± 0.07

*Averages of triplicates ± S.D. * Values are significantly different ($p<0.05$) from those of fish extract incubated at 37°C for the same incubation time without preincubation at pH 7.5.

of cathepsin S (Kirschke et al., 1989). Preincubation of the enzyme extract at 40°C and pH 7.5 over a period of 60 min allowed determination of the activity of cathepsin S activity in the presence of cathepsins L and B using Z-Phe-Arg-NMec as a substrate. Thus, it can be presumed that cathepsin S may contribute to the activity detected at pH 8.0 and may also be involved in autolysis at alkaline pH.

In contrast to cathepsin classification, the alkaline protease is broadly defined on the basis of its optimal condition and its sensitivity to specific inhibitors. Due to no specific substrates for alkaline proteinases, hydrolytic activities of fish extract on various protein substrates were compared (Table 2.4). All types of protein substrates were hydrolyzed a higher rate at pH 5.5 than at pH 8.0. For both pH conditions, casein was the most preferred substrate. Myofibrils extracted from arrowtooth flounder muscle showed a hydrolysis rate comparable to that of casein, 86.3% and 91.6% at pH 5.5 and 8.0, respectively. The hydrolysis rate of acid-treated hemoglobin and bovine serum albumin were only 65-73% that of casein. Hydrolytic activity at pH 5.5 against azocasein was 3.4 fold that at pH 8.0. The lower hydrolysis rate observed at pH 8.0, compared to those of other substrates, implied that azocasein may not be a suitable substrate for determining activity of the proteinase active in alkaline pHs. Collagenolytic activity determined using hide powder covalently linked with Remazolbrilliant Blue (Rinderknecht et al., 1968) showed that the fish extract was more active at pH 5.5 than pH 8.0.

Activity-stained substrate gel electrophoresis

The proteinase activity in fish extract was identified by separation on native discontinuous substrate polyacrylamide gels followed by staining for proteolytic activity (Fig. 2.4.a). Activity stained-substrate gels of muscle extract incubated at pH 5.5 and pH 8.0 revealed a similar pattern of activity bands shown as clear zones on the dark background (Figs. 2.4.b and 2.4.c). The molecular weights of the bands

Table 2.4. Hydrolytic activity of arrowtooth flounder fish extract on various substrates

Substrates	Enzymatic activity	
	pH 5.5	pH 8.0
Casein[a]	21.51 ± 2.37	12.78 ± 3.90
ATF Myofibrils[a]	18.56 ± 1.46	11.71 ± 3.04
Acid-treated hemoglobin[a]	14.44 ± 2.18	9.26 ± 3.13
Bovine serum albumin[a]	13.88 ± 1.54	8.93 ± 3.30
Azocasein[b]	0.0310	0.0091
Hide powder azure[c]	0.0200	0.0067

[a] Averages of four replicates ± S.D. representing the nmole of tyrosine released per minute per mg protein of fish extract.

[b] Average of duplicate analyses representing an increase in absorbance at 450 nm (A450) per minute per 4.43 mg protein of fish extract.

[c] Average of duplicate analyses representing an increase in absorbance at 595 nm (A595) per minute per 1.15 mg protein of fish extract.

were estimated to be in the range of 43-124 kDa. The obtained results indicated that fish extract contained several proteinases active at pH 5.5 and 8.0 and the proteinases being involved in both conditions may be identical based on the activity-stained gels (Figs. 2.4.b and 2.4.c).

Separation of fish extract on SEC-HPLC

SEC-HPLC was employed as a method of choice, as it can separate proteins based on size in a non-denaturing condition and thus is capable of differentiating a high molecular weight alkaline protease from a lower molecular weight cathepsins

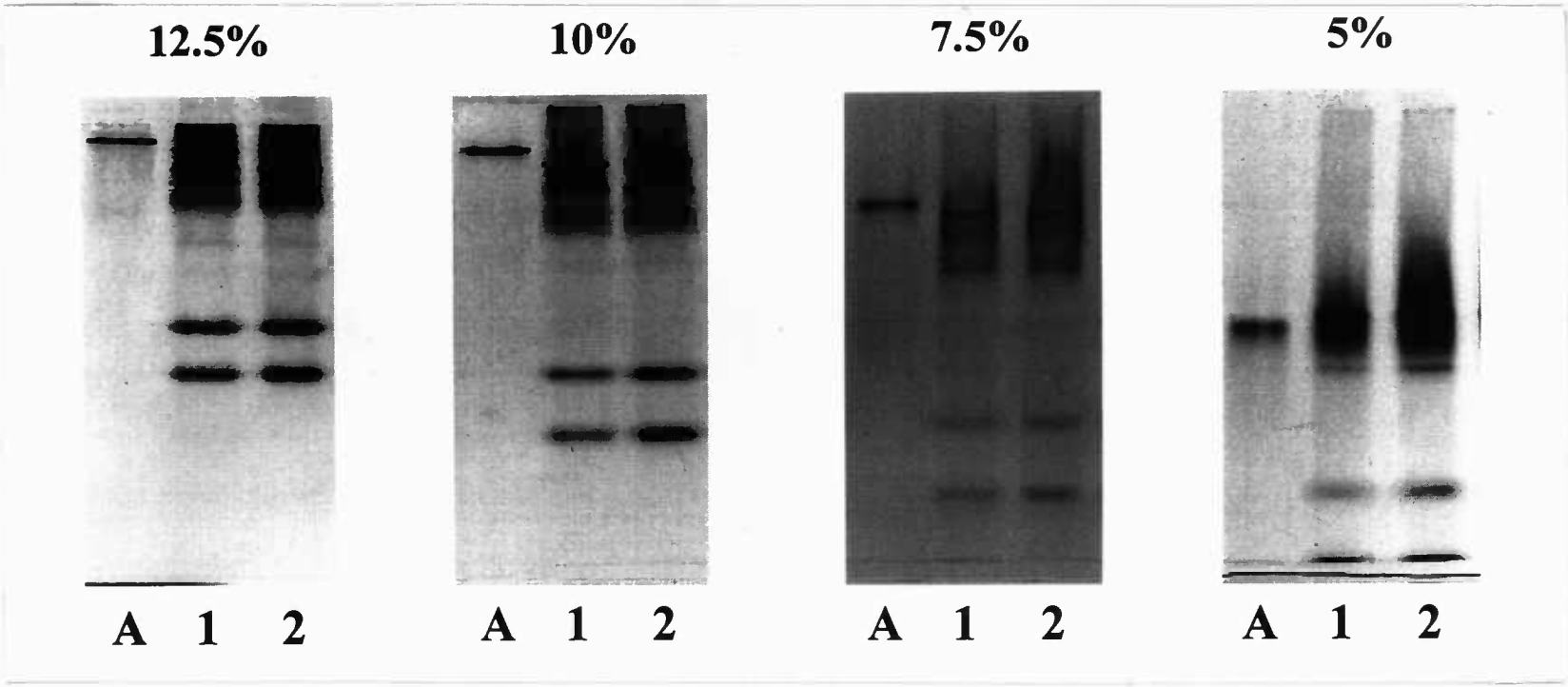

Fig. 2.4.a. Separation of fish muscle proteinases on native discontinuous gel electrophoresis followed by staining with Commasie blue R250. Lane A represents amylase used as a marker protein. Lane 1 and 2 were fish extract with 18 and 36 µg protein, respectively.

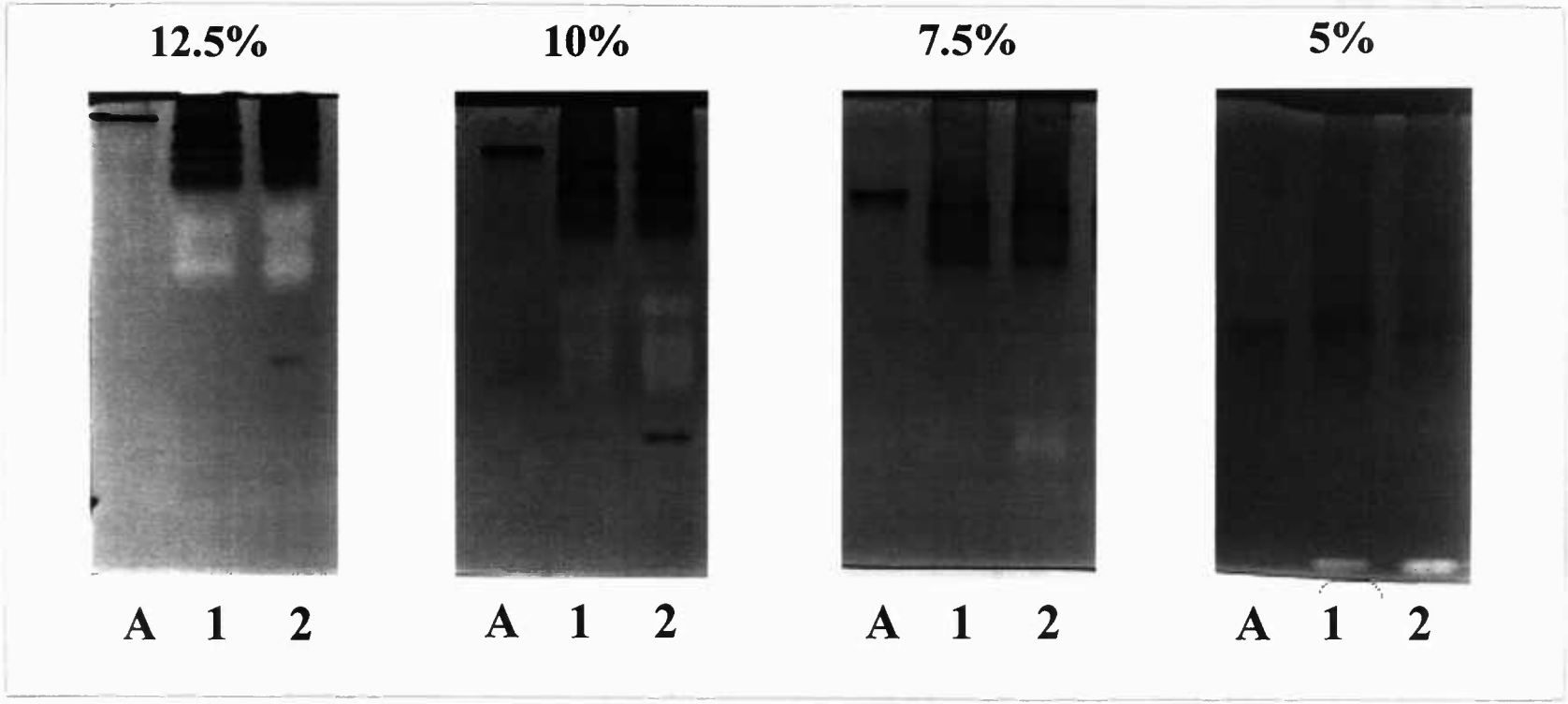

Fig. 2.4.b. Separation of fish muscle proteinases on native discontinuous gel electrophoresis followed by staining for proteolytic activity at pH 5.5, 55°C. Lane A represents amylase used as a marker protein. Lane 1 and 2 were fish extract with 18 and 36 μg protein, respectively.

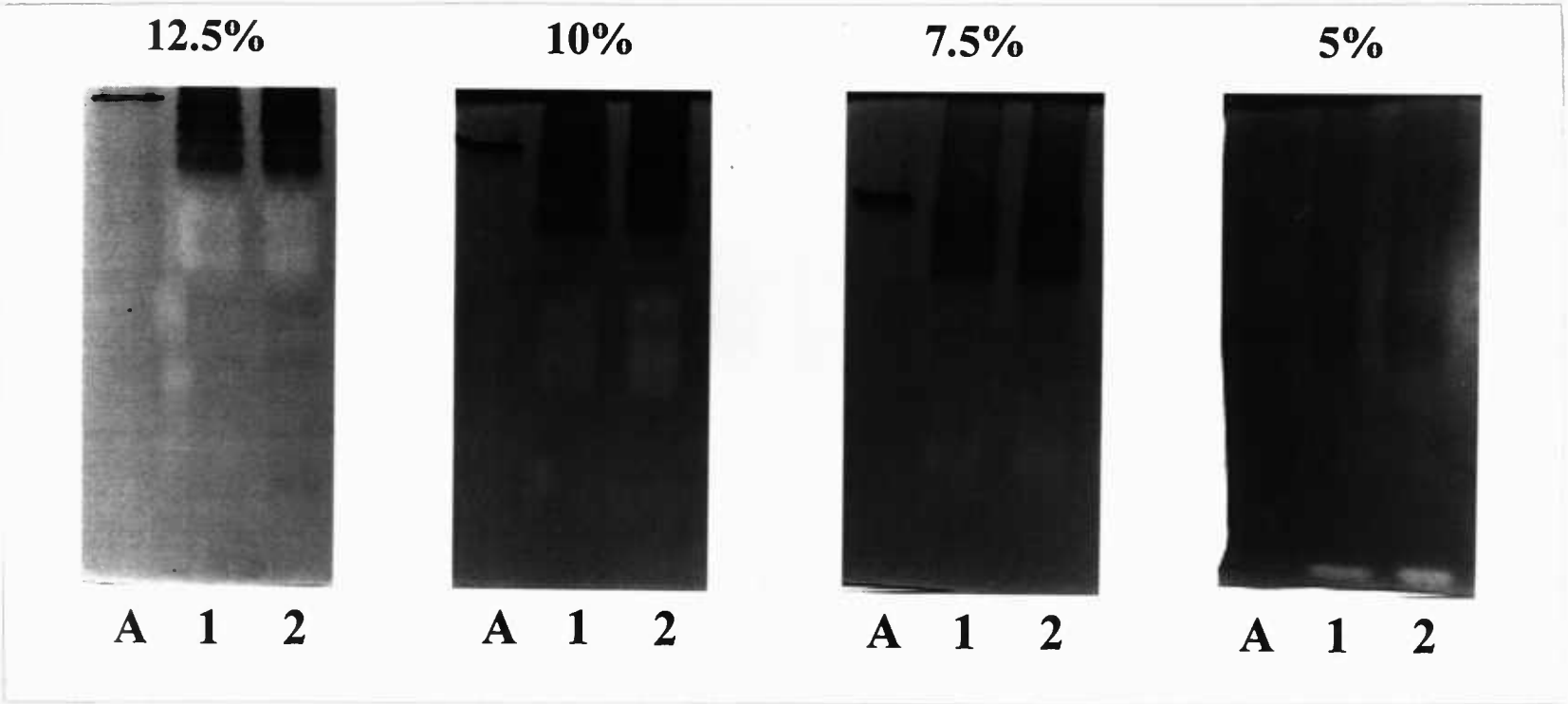

Fig. 2.4.c. Separation of fish muscle proteinases on native discontinuous gel electrophoresis followed by staining for proteolytic activity at pH pH 8.0, 60°C. Lane A represents amylase used as a marker protein. Lane 1 and 2 were fish extract with 18 and 36 μg protein, respectively.

L. When arrowtooth flounder fish extract was separated on SEC-HPLC (Fig. 2.5), only one proteolytic peak was observed. This peak, eluted at a retention time of 26 min, revealed the previously observed effects of urea; reduction of activity by the addition of urea at pH 5.5 (Fig. 2.6a) and enhancement at pH 8.0 (Fig. 2.6b). Its molecular weight was estimated to be M_r 39,800 on Superose 12 HR 10/30 (Fig. 2.7).

Discussion

Autolysis of arrowtooth flounder muscle at elevated temperatures is due to a heat- activated proteinase active at both acidic and alkaline pHs. The obtained results clearly indicated that cathepsin L or cathepsin L-like were the active proteinase in ATF fish extract and are primarily responsible for the autolysis of fish muscle at slightly acidic pH. Cathepsin L has been reported as a major proteinase in chum salmon (Yamashita and Konagaya, 1990) and Pacific whiting muscle (Seymour et al.,1994). Cathepsin L degrades proteins at least ten times faster than the other cysteine proteinases, including the cathepsins B and H (Bohley and Seglen,1992) and is very active in degrading myofibrillar proteins. The fact that the molecular activity of cathepsin L with myosin is 10 times greater than that of cathepsin B (Bird and Carter, 1980) may explain the complete myosin degradation during autolysis of arrowtooth flounder muscle at 60°C. In Pacific whiting, high protease activity and textural degradation are associated with the infection of myxosporean parasites (Kudo et al., 1988). However, no clear relationship between

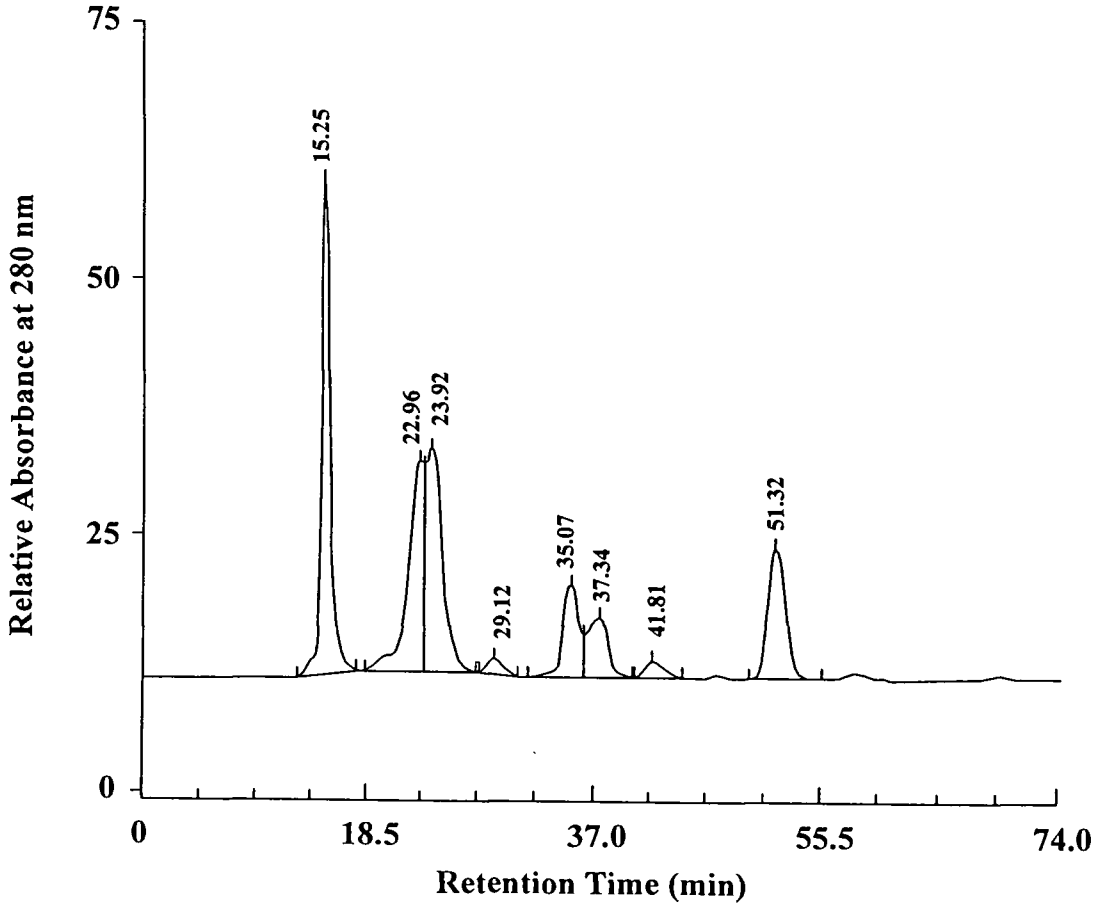

Fig. 2.5. Elution prolfile of fish extract. Fish extract proteins were applied on the Superose 12 HR 10/30 and eluted with 50 mM sodium phosphate buffer, pH 7.2 at the flow rate of 0.5 mL/min. The eluted proteins were monitored by absorbance at 280 nm.

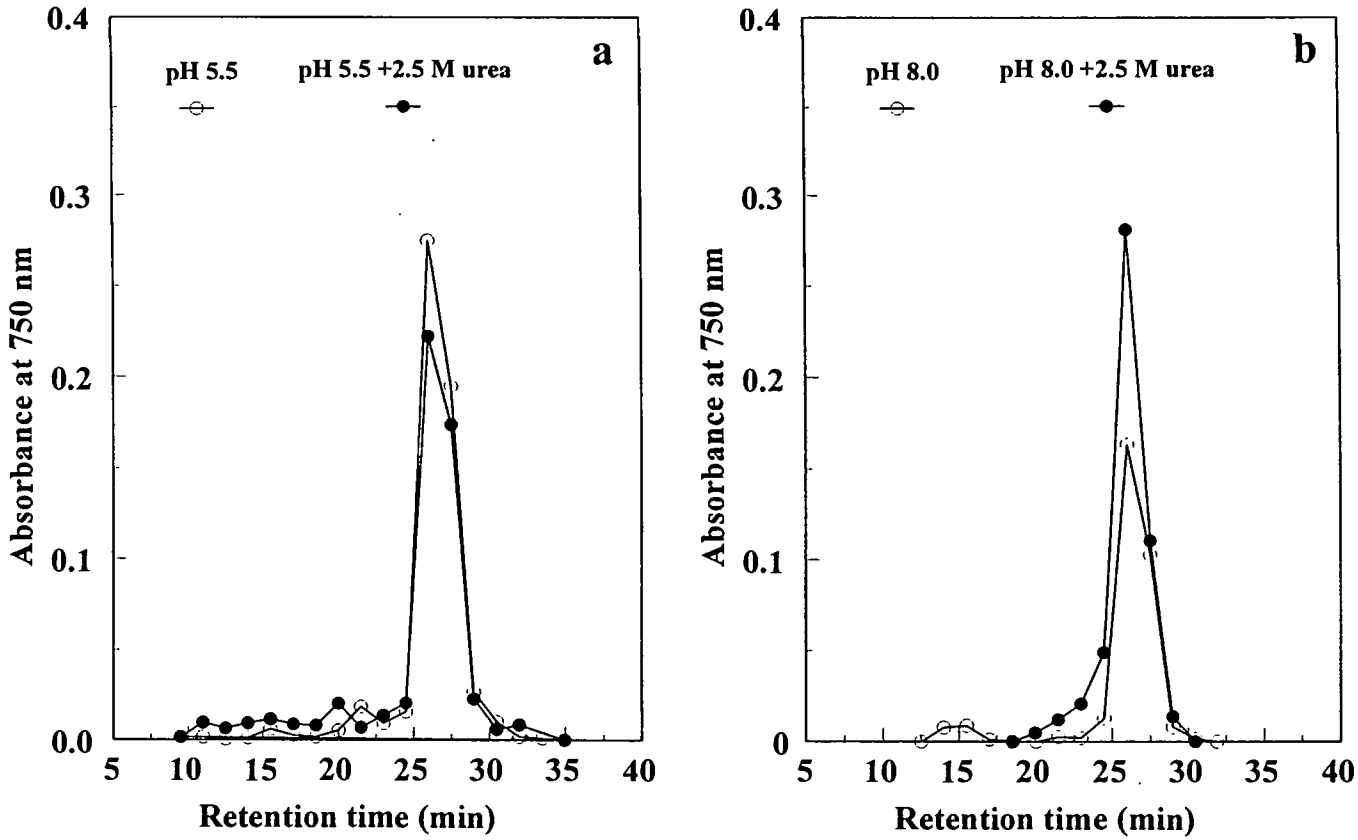

Fig. 2.6. Caseinolytic activity of Superose 12 HR 10/30 fractions of fish extract; (a) at pH 5.5, 55°C in the presence and absence of 2.5 M urea; (b) at pH 8.0, 55°C in the presence and absence of 2.5 M urea.

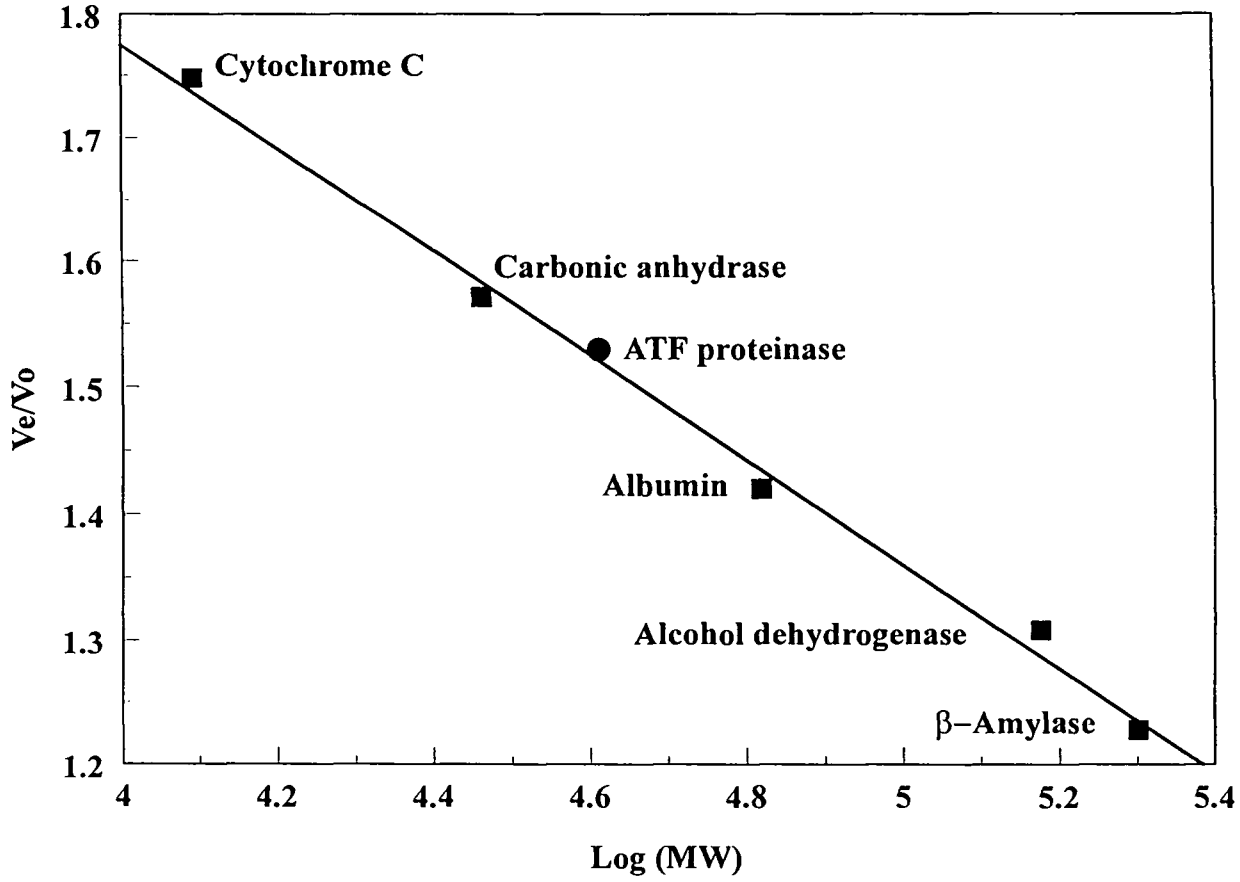

Fig. 2.7. Estimation of the native molecular weight of arrowtooth flounder proteinase based on elution volumn on Superose 12 HR 10/30 column. The molecular weight was estimated to be M_r 39,800.

parasite density and textural deterioration of the cooked fillets was observed in arrowtooth flounder (Greene and Babbitt, 1990). Cathepsin L was shown to be capable of hydrolyzing various types of protein substrates. In addition to myofibrillar proteins, cathepsin L was report to have very strong activity against various collagens and elastin (Kirschke and Barrett, 1987). Thus, it is presumed to cause partial disintegration of the original extracellular metrix structure which may play an important role in the tissue softening of fish.

Among the cathepsin L-like enzymes, the presence of cathepsin S was presumed by the retaining activity of fish extract after preincubation at pH 7.5. Cathepsin S is the only member of the lysosomal cathepsins that can retain proteolytic activity after prolonged exposure to neutral pH (Kirshke et al., 1986; Bromme et al., 1993). Cathepsin S is a lysosomally located, single-chain, cysteine proteinase with a high endopeptidase activity against proteins including elastin and collagen (Shi et al., 1992). Although its substrate specificity shows some similarities with cathepsin L, it was shown by biochemical and immunological methods and analysis of the amino acid sequences that cathepsin S and cathepsin L are distinct enzymes (Kirschke and Wiederanders, 1994). Therefore, autolytic activity or proteolytic activity on various substrates detected at pH 8.0 is apparently due to Z-Phe-Arg-NMec hydrolyzing activity mediated by cathepsin S.

Depending upon the pH, the distinctive response to urea may explain the presence of heat stable alkaline protease in fish extract, even though its contribution may be less than cathepsins. Since it exhibits little or no catalytic activity unless

assayed at a non-physiological, high temperature or is activated by protein denaturing agents like urea, the enzyme will contribute to the post-mortem degradation of muscle only when denaturing substances analogous to urea are produced during storage. The activation phenomenon was previously reported by Toyohara et al. (1987), and Kinoshita et al. (1990) as a unique characteristic of alkaline proteases that are known to be predominantly active at the physiological condition of marine flesh tissues. However, several studies have shown that cathepsins L and S are stable or activated in the presence of urea (Kirschke et al., 1989). Proteinase activity of cathepsin L against azocasein was increased in the presence of 3 M urea and was used as a unique characteristic to differentiate cathepsin L and B (Brömme et al., 1989). Recently the activity of cathepsin L recovered from Pacific whiting surimi wash water was found to be highly enhanced by the addition of 2.5 M urea against Z-Phe-Arg- NMec (Benjakul et al., 1998). Thus, cathepsin L or likes might exhibit hydrolytic activity on various protein substrates both at pH 5.5 and pH 8.0 and showed the identical responses to urea which were often used to characterize alkaline protease.

Identical activity bands resolved on the activity-stained substrate-gels at both pH 5.5 and 8.0 imply the involvement of several proteinases in the proteolytic activity However, only one activity peak was obtained on SEC-HPLC. This fraction was thought to be the most predominant proteinase found in ATF fish extract. This fraction also showed the previously observed effects of urea; the reduction of activity at pH 5.5 by the addition of urea but enhancement at pH 8.0.

Based on its estimated molecular weights of M_r 43,000 by activity-stained substrate-gel electrophoresis, or M_r 39,800 by SEC-HPLC, it is most likely to be cathepsin L or like. Cathepsin L and cathepsin S are relatively small when compared to the alkaline proteinase. The molecular weights of purified cathepsin L and cathepsin S are estimated to be 29 and 24 kDa, respectively (Kirschke and Barrett, 1987). However they are often found to be complexed with endogenous inhibitors, such as, cystatins and alpha-cysteine proteinase, which apparently increase the molecular weight of the proteinases by 11-14 kDa (An et al., 1995; Yamashita and Konagaya, 1992). The fact that cathepsin L has been found in a complex form with its natural inhibitor, dissociation of the cathepsin L-inhibitor complex may occur upon activation by urea.

Conclusion

Cathepsin L or cathepsin L-like enzyme was found to be a predominant heat-activated proteinase in arrowtooth muscle. The activity exhibited the maximum shift to lower temperatures by the addition of 2.5- 4.0 M urea with activity reduction at pH 5.5, but enhancement at pH 8.0. Although this phenomenon has been reported to be common to alkaline proteases, our study demonstrated that cathepsin L or cathepsin L like enzyme also showed the same response to urea as the alkaline proteases.

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Chapter 3

PHYSICOCHEMICAL CHANGES AND MECHANISM OF HEAT-INDUCED GELATION OF ARROWTOOTH FLOUNDER MYOSIN

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Abstract

Physicochemical changes of myosin during heating were investigated to elucidate the mechanism of heat-induced gelation of arrowtooth flounder (ATF) myosin at high ionic strength. Changes in dynamic properties indicated ATF myosin formed a gel in three different stages, as shown by the first increase in G' (storage modulus) at 28°C, followed by a decrease at 35°C and the second increase at 42°C. DSC thermogram showed the onset of myosin denaturation at 25°C, with two maximum transition temperatures at 30 and 36°C, respectively. The decrease in α-helical content indicated ATF myosin began to unfold at 10°C and the unfolding continued until it reached 65°C. Turbidity measurement showed myosin began to aggregate at 23°C and the aggregation was completed at 40°C. Surface hydrophobicity increased consistently in the temperature range studied, 20-65°C. Sulfhydryl contents decreased significantly at 20-30°C due to the formation of disulfide linkages, but remained constant at temperatures above 30°C. ATF myosin was shown to be extremely sensitive to heat, resulting in denaturation at a lower temperature than other fish myosins. Denaturation was initiated by the unfolding of the α-helical region in myosin followed by exposure of hydrophobic and sulfhydryl residues, which are subsequently involved in the aggregation and gelation processes.

Introduction

Heat-induced gelation of surimi, an intermediate product produced by repeated washing of minced fish flesh and mixing with cryoprotectants to extend its frozen shelf-life, is a fundamentally important step in manufacturing a variety of surimi seafoods such as kamaboko, fish meat gel, and crab and other shellfish analogs. The formation of a protein network in these final products contributes not only to the elastic texture but also to other functional properties, which are important to simulate the appearance, flavor, and texture of the natural meat counterparts (Park et al., 1997).

Heat-induced gelation of surimi is a complex physicochemical process involving structural and functional changes of myofibrillar proteins. The formation of a myofribrillar network can be represented by three stages, i.e., dissociation of myofibrillar structures, thermal denaturation, and aggregation reaction (Roussel and Cheftel, 1990). In the presence of salts, muscle fibers and proteins undergo major structural changes leading to the solubilization of myosin, actin, and a number of other myofibrillar constituents (Parsons and Knight, 1990). Partial unfolding of the protein structure is accelerated by an increase in temperature, resulting in the aggregation of unfolded regions between protein molecules to form a three dimensional network.

Formation of fish meat gel has been characterized, in respect to changes in three dimensional structure by three stages as heating proceeds: "suwari", "modori", and "kamaboko" (Suzuki, 1981). "Suwari" or the gel setting stage describes the

formation of a loose network when fish mince paste containing 2- 3% salt is heated to 40-50°C. It is mediated by transglutaminase and is the reaction wherein a three-dimensional network is formed which subsequently acts as the backbone of the final gel (Niwa et al., 1995). "Modori" describes the partial disruption of a loose network as the temperature is increased further over 50-60°C. Modori is associated with the action of endogenous heat-activated proteinases and/or the thermal behavior of myofibrillar proteins alone (Niwa, 1992). "Kamaboko" or gel enhancement refers to the formation of ordered, strong and elastic gels once the temperature reaches above 65-70°C.

The ability of surimi to form an elastic gel is largely derived from myosin (Sano et al., 1988), which is the major component of the myofibrillar proteins, comprising 55-60%. Myosin is a multidomain protein comprised of two large heavy chains and four light chains arranged into an asymmetrical molecule with two globular heads that are attached to a long α-helical rod-like tail (Privalov, 1982; Lopez-Lacomba et al. 1989). Myosin, in contrast to other protein components, has been shown to exclusively possess gelling ability. In surimi, myosin is present in a complex form with actin and other proteins, collectively called actomyosin, which can also form an elastic gel (Niwa, 1992). Since actin itself can not form a strong gel upon heating, it is thought that the gelling characteristics of actomyosin are derived from the myosin portions. However, binding with actin has been shown to modify the gelling characteristic of myosin

(Yasui et al., 1980). The changes in the rheological properties of myosin and actomyosin during heating at a similar condition indicated that myosin produces more elastic gels than actomyosin and the gelation reflected the three steps of texture formation previously described using fish mince (Sano et al., 1988).

According to the studies on physicochemical changes using myosin and its subfragments from several species of fish i.e., flying fish (Taguchi et al., 1987), carp (Sano et al., 1990a), croaker (Hamada, 1992), cod, and herring (Chan et al., 1992; Gill et al., 1992; Chan and Gill, 1994), it is agreed that heat-induced gelation of myosin at an high salt condition involves two processes, denaturation and aggregation (Stones and Stanley, 1992). Denaturation is a process where myosin undergoes conformational changes at or above denaturation temperature and aggregation is a process where denatured myosin molecules align themselves and interact with each other to form a three-dimensional network. However, the interactions involved in gelation have been shown to be dependent upon the species of fish employed (Taguchi et al., 1987; Sano et al., 1990b; Chan et al., 1993). Since myosin heavy chain is the main subunit involved in gelation (Samejima et al., 1984), differences in composition and structure of myosin heavy chain among various fish species may be responsible for the species difference in cross-linking ability and the gelation properties of fish muscle under the same conditions.

Arrowtooth flounder (ATF) is an abundant fish species caught off the North America/Pacific region. However, its utilization has been hampered due to the softening of muscle by heat stable proteases during heating (Greene and Babbitt,

1990; Wasson et al., 1992). In order to provide the basic information for future utilization of this fish species, we investigated physicochemical changes of arrowtooth flounder myosin during heating and its gelation mechanism at the high ionic strength condition normally used for surimi gel products.

Materials and methods

Reagents

Potassium chloride (KCl), sodium azide (NaN_3), Tris base, 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), 2-mercaptoethanol (βME), N,N,N',N'- tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), magnesium acetate, Tris maleate, adenosine triphosphate (ATP), sodium bicarbonate, magnesium chloride, sodium phosphate, potasium phosphate, glycerol, 8-anilino-1-naphthalene sulfonic acid (ANS), and 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) were purchased from Sigma Chemical Co. (St Louis, MO). Phenylmethylsulfonyl fluoride (PMSF) was purchased from Calbiochem Co. (La Jolla, CA). Sodium dodecyl sulfate (SDS) and urea were purchased from Bio-Rad Laboratories (Hercules, CA).

Sample

Arrowtooth flounder were obtained from the National Marine Fisheries Service, Utilization Research Laboratory, Kodiak, Alaska. Trawl-caught arrowtooth

flounder were obtained and manually filleted. The fillets were vacuum-packed in polyethelene bags, frozen at -20°C, and transported in dry ice to the OSU Seafood Laboratory. Frozen fillets were kept at -50°C until used.

Myosin preparation

Myosin was extracted by the method described by Martone et al. (1986) with slight modifications. All steps were performed at 0-4°C to minimize proteolysis and protein denaturation. Arrowtooth flounder fillets were finely chopped and added with 10 volumes of Buffer A (0.10 M KCl, 1 mM PMSF, 10 μM E-64, 0.02% NaN_3 and 20 mM Tris-HCl, pH 7.5). After incubation on ice for 10 min with occasional stirring, washed muscle was recovered by centrifugation at 1,000 *xg* for 10 min. The pellet was suspended in 5 volumes of Buffer B (0.45 M KCl, 5 mM ßME, 0.2 M $Mg(CH_3COO)_2$, 1 mM EGTA, and 20 mM Tris-maleate, pH 6.8) and then ATP was added to a final concentration of 10 mM. The mixture was kept on ice for 1 h with occasional stirring and centrifuged at 10,000 *xg* for 15 min. Supernatant was recovered and 25 volumes of 1 mM $NaHCO_3$ was slowly added and the mixture was incubated for 15 min on ice. Precipitated myosin was collected by centrifugation at 12,000 *xg* and resuspended gently with 5 volumes of Buffer C (0.50 M KCl, 5 mM βME and 20 mM Tris-HCl, pH 7.5), followed by the addition of 3 volumes of 1 mM $NaHCO_3$ and $MgCl_2$ to a final concentration of 10 mM. The mixture was incubated overnight and centrifuged at 22,000 *xg* for 15 min.

Myosin was recovered in the pellet and used immediately or stored in 50% glycerol at -20°C. The purity of extracted myosin was estimated by SDS-PAGE and densitometric analysis of the gels.

Dynamic rheological analysis

Rheological changes of myosin during thermal gelation were analyzed using a Bohlin CS-50 rheometer (Bohlin Instruments, Inc., Cranbury, N.J) which operated in the small-amphitude oscillatory mode according to the method described by Xiong (1993) with a slight modification. The rheometer was equipped with 20-mm parallel plate geometry with a gap of 1 mm. Samples were prepared as myosin suspension at 45 mg/mL in 0.6 M KCl, 20 mM phosphate buffer, pH 7.0. The sample was sheared at a fixed frequency of 0.1 Hz to minimize stress on the sample during network with a maximum strain amplitude of 0.015. These parameters have been previously determined to give linear response in the viscoelastic region (data not shown). Samples were heated from 25 to 80°C at 1°C/min using a Bohlin temperature control unit. To avoid sample evaporation during heating, a plastic cover was used. Data were collected every 60 s during shearing measurements. Network development was studied in real time by measuring changes in the storage modulus (G'), loss modulus (G") and loss tangent (tan δ) as a function of temperature.

Differential Scanning Calorimetry

Differential scanning calorimetry measures energy changes associated with the transition that occurs in protein molecules when subjected to heating (Davies et al., 1994). DSC studies were performed in a DuPont 910 differential scanning calorimeter (Du Pont Co., Wilmington, DE). To eliminate water condensation in and under the DSC cell, 40 mL/min of helium gas was purged through the purging port. The system was calibrated using DuPont calibration software with an indium thermogram. Myosin solution, prepared at 45 mg/mL in 0.6 M KCl, 20 mM potassium phosphate, pH 7.0 were sealed in hermatic DSC pans and accurately weighed to 14-20 mg wet weight on a semi-micro balance (Mettler Instrument Corp., Highstown, NJ). All samples were scanned at 10°C/min over the range of 2-100°C using a DuPont mechanical cooling accessory. An empty hermatic pan was used as a reference. Five determinations were made and the most representative thermograms with average onset and transition temperatures were reported.

Circular dichroism (CD)

Secondary structures of proteins were analyzed using a JASCO A-500 spectrophotometer (Japan Spectroscopic Co., Ltd., Tokyo, Japan) equipped with a temperature control unit and operated under constant nitrogen flush. Myosin pellet was dissolved in cold 0.6 M KCl, 20 mM potassium phosphate, pH 7.0 and centrifuged at 7,800 *xg* for 10 min. Supernatant was used for CD analysis after

dilution to 0.4 mg/mL in the same buffer. A circular quartz cell (path length of 0.1 cm) with a jacket for temperature control was used. Temperature inside the cell was monitored using a thermocouple while heating proceeded. Thermal scanning experiments were performed from 10 to 65°C at an average heating rate of 1.2°C/min. Scan speeds and time constant were chosen to allow sufficient response time and achieve a favorable signal to noise ratio. Molar ellipticities at 222 nm ($[\theta]_{222}$) were calculated using Equation (1) and a mean residue molecular weight of myosin of 115 (McCubbin and Kay, 1982). α-Helix content was estimated using Equation (2) on the basis of $[\theta]_{222}$ of poly-L-glutamic acid which possesses the complete helical structure (Holzwarth and Doty, 1965).

$$[\theta]_{222} = (\theta_{obs} \times 115)/(10 \times C'') \quad (1)$$

$$\alpha\text{-Helical content} = 100 \times \{[\theta]_{222}/ -40{,}000\} \quad (2)$$

Turbidity measurement

Myosin solution was prepared at 1 mg/mL in chilled 0.6 M KCl, 20 mM potassium phosphate, pH 7.0 and centrifuged at 7,800 *xg* for 10 min to remove insoluble debris. Myosin solution was placed in a cuvette (light path length, 1 cm), covered with Parafilms and heated linearly at an average rate of 1.3°C/min from 18 to 65°C. Turbidity was measured as the absorbance at 350 nm while heating proceeded using a spectrophotometer (Beckman Instrument, Inc., Redmond, WA)

equipped with a temperature controlled unit. Results were reported as absorbance per protein concentration (A_{350}/mg/mL).

Surface hydrophobicity

Surface hydrophobicity (S_0 ANS) was determined by the method of Li-Chan et al. (1985) with slight modifications. Myosin solution was diluted to 0.125, 0.25, 0.5 and 1 mg/mL in 0.6 M KCl, 20 mM phosphate, pH 7.0 and aliquoted into 2 mL in borosilicate tubes. Parafilm was placed on the tubes to prevent evaporation during heating. The tubes were placed in a circulating water bath at 20°C and allowed to equilibrate for 5 min prior to heating. A thermometer was used to monitor the temperature of protein solution in tubes that were placed in the water bath. Temperatures were recorded at 30 s intervals. The temperature rise in each run, from 20 to 80°C, was shown to be linear and the heating rate was estimated at 1.2-1.5°C/min. To 2.0 mL of diluted myosin solutions, 10 µL of 10 mM ANS dissolved in 50 mM phosphate, pH 7.0 were added. Fluorescence intensity was measured using a luminescence spectrophotometer (LS 50B, Perkin-Elmer Ltd., Beaconsfield, England) at excitation wavelength 374 nm and emission wavelength 485 nm. Fluorescence intensity was plotted against protein concentration, and the slope of the regression line was reported as S_0 ANS.

Determination of sulfhydryl (SH) content of myosin

Total and surface reactive SH were determined using 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) by the method of Ellman (1959). Aliquots (2 mL) of myosin solution (1 mg/mL) were linearly heated from 20 to 80°C in a temperature controlled waterbath as described previously in the section "Surface hydrophobicity". Samples were cooled down immediately, mixed and aliquoted into two portions to determine total and surface reactive SH. For total SH determination, 1 mL of heated sample was added to 3 mL of 0.2 M Tris-HCl, pH 6.8, containing 8 M urea, 2% SDS, and 10 mM EDTA, followed by 0.4 mL of 0.1% DTNB solution and incubated at 40°C for 25 min. Absorbance was measured at 412 nm and used to calculate total SH content using the molar extinction of 13,600 M^{-1} cm^{-1}. Surface reactive SH was determined by incubating 1 mL of the heated sample in 3 mL of 0.2 M Tris-HCl, pH 6.8 containing 2% SDS, and 10 mM EDTA at 5°C for 25 min. Surface reactive SH content was calculated from absorbance using the molar extinction of 14,150 M^{-1} cm^{-1} according to Riddles et al. (1983). A blank was prepared by replacing sample with 0.6 M KCl, 20 mM phosphate buffer, pH 7.0. Both total and surface reactive SH contents were expressed as mol/10^5 g protein.

Protein determination

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Myosin was first solubilized in 5% (w/v) SDS solution at 95 °C for 5 min and further incubated at 85°C for 30 min. Insoluble debris was removed by centrifugation at 7,800 *xg* for 10 min and supernatant was used for protein analysis.

Statistical analyses

Obtained data were analyzed statistically by analysis of variance (ANOVA). Mean difference was determined using the least significant difference (LSD) multiple range test (Statgraphics Version 6.0, Manugistics Inc., Rockville, MD). Significance of difference was established at $p \leq 0.05$.

Results

Myosin extract

The purity of extracted myosin was 85%, as estimated by the densitometric analysis of SDS-PAGE gel. The electrophoretic pattern showed that myosin of arrowtooth flounder was comprised of heavy chains (MHC) and at least two different types of light chains (Fig. 3.1). Their molecular weights were estimated at approximately 205, 23, and 21 kDa, respectively. Since no notable changes in the electrophoretic patterns of myosin was observed from incubation up to 30 min at

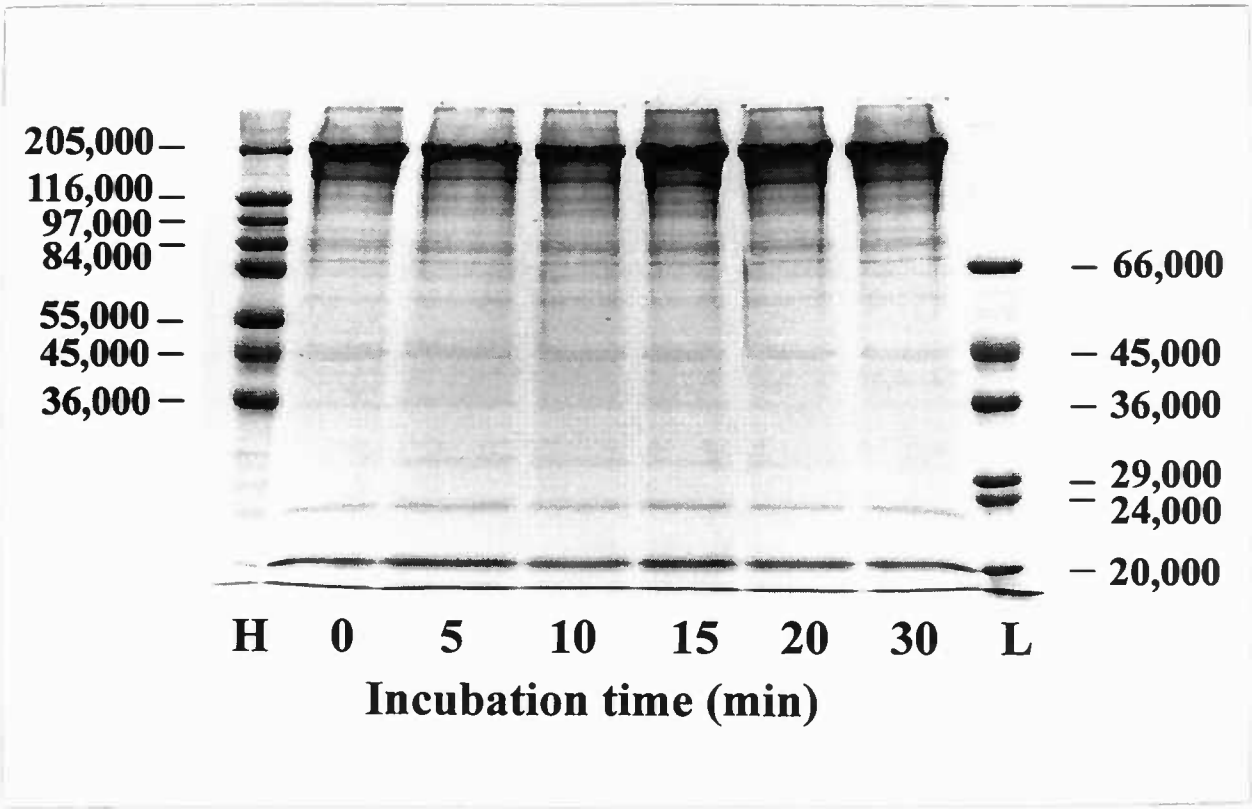

Fig. 3.1. SDS-PAGE pattern of ATF myosin before and after incubation at 55°C up to 30 min. H and L designates high and low molecular weight protein standards, respectively.

55°C, extracted myosin was postulated to be free of endogenous protease. The appearance of smear bands in the control group indicated the degradation products accumulated prior to myosin extraction. MHC was the main target of proteolytic activity in which its band disappeared completely by heat treatment for 20 min (Wasson et al., 1992). The results indicated the myosin preparation was free of endogenous enzymes, such as, proteases and transglutaminase, and suitable for further analyses.

Dynamic rheological properties

Typical changes in the rheological properties of ATF myosin during thermal scanning are shown in Figure 3.2. Of the rheological parameters assessed, storage modulus (G') and phase angle were used to evaluate gel formation. An increase in storage modulus (G'), a measure of energy recovered per cycle of sinussoidal shear deformation, indicated an increase in rigidity of the sample associated with the formation of an elastic gel structure (Egelandsdal et al., 1995). The first derivative plot showed that the apparent onset of heat-induced elastic myosin gel formation occurred at 28°C where G' began to rise and reached a maximum at 34°C. An increase in G' (approximately 3% of the maximun G') found in this temperature range was referred to as "gel setting" in which the loose gel structure was formed. However, the term should be distinguished from "suwari" observed with surimi mediated by endogenous fish transglutaminase. The slight decrease in G' during

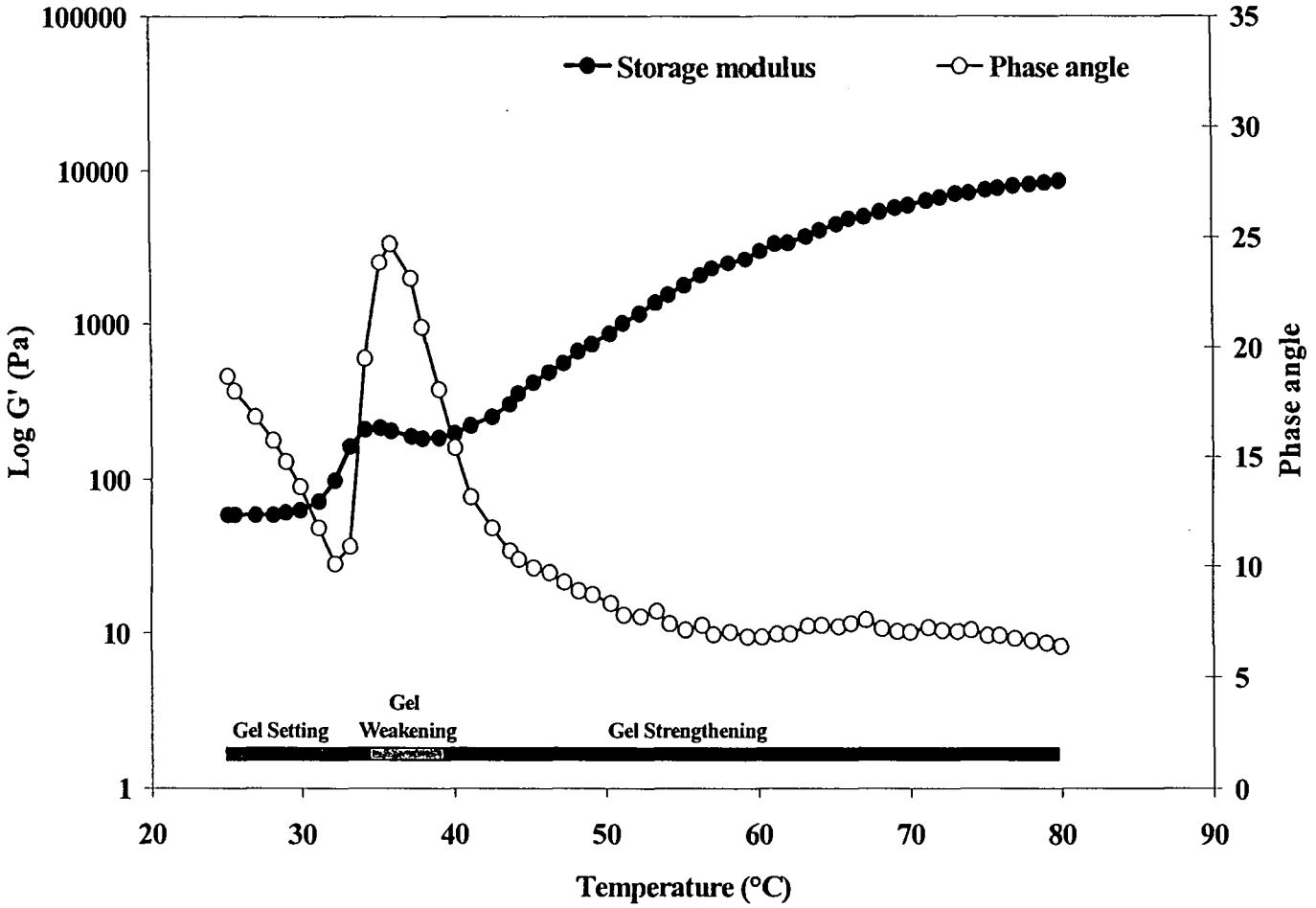

Fig. 3.2. Changes in rheological properties of ATF myosin suspension (45 mg/ mL) heated from 25 to 80 °C at a rate of 1 °C/min.

34-40°C was referred to as "gel weakening". Since ATF myosin used in this study was confirmed to be free of proteolytic activity, disruption of the initial gel structure was not due to the action of heat-activated proteases responsible for "modori" in surimi. Similar to the G' drop observed in chicken breast myosin (Xiong, 1997), it was postulated to be due to the helix-to-coil transformation of myosin, which leads to a large increase in fluidity of the semi-gel and may disrupt some of the protein network already formed (Sano et al., 1988). The onset of the second increase in G' was noticed at 42°C, where G' continuously increased thereafter. This final step was referred to as "gel strengthening", and it was ascribed to both an increase in the number of cross-links between protein aggregates and deposition of additional denatured proteins in the existing protein networks to strengthen the gel metrix (Xiong, 1997).

The phase angle decreased from 18° to 10° by heating from 25 to 32°C and increased rapidly to a maximum of 25° at 35°C. The phase angle continuously decreased thereafter to a minimum of 7° at 50°C and remained constant until the final stage of heating. The use of phase angle to evaluate network characteristics has the advantage of incorporating the contributions of both G' and G" into a single parameter as a way to evaluate the final network (Egelandsdal et al., 1995). Changes in the phase angle reflected a transition of the viscous myosin sol to the elastic myosin gel, which correlated to the changes of G' observed during heating.

DSC studies

Arrowtooth flounder myosin was found to undergo a multi-stage denaturation process as characterized by endothermic trough and peaks (Fig 3.3). The onset of denaturation was observed at 25°C, while the maximum transition temperature (T_{max}) was observed at 36°C with one transition peak detected at 30°C. The observed T_{max} was within the temperature range (25-46°C) observed among various fish species (Ogawa et al., 1993) and closely related to those of other cold-water fishes, i.e., cod and herring (Davies et al., 1994). However, it was lower than those of myosins extracted from chicken, turkey, beef, and pork, of which transition normally occurred at 45-55°C. The results suggested that ATF myosin is highly unstable to heat and its thermal susceptibility might be related to the habitat temperature of the living animal (Johnston et al., 1973).

CD measurements

An asymmetric configuration of myosin molecules with a relatively high content of α-helix allows application of CD to monitor changes in the secondary structure induced by heat. By heating linearly from 9 to 65 °C at 1.5°C/min, ATF myosin showed a decrease in helicity as temperatures increased (Fig. 3.4). The melting temperature (T_m), at which 50% of the initial helical structure is lost, was determined to be 34 °C. The first derivative plot shows the α-helical content started to decrease at 10°C and proceeded through at least three major stages before

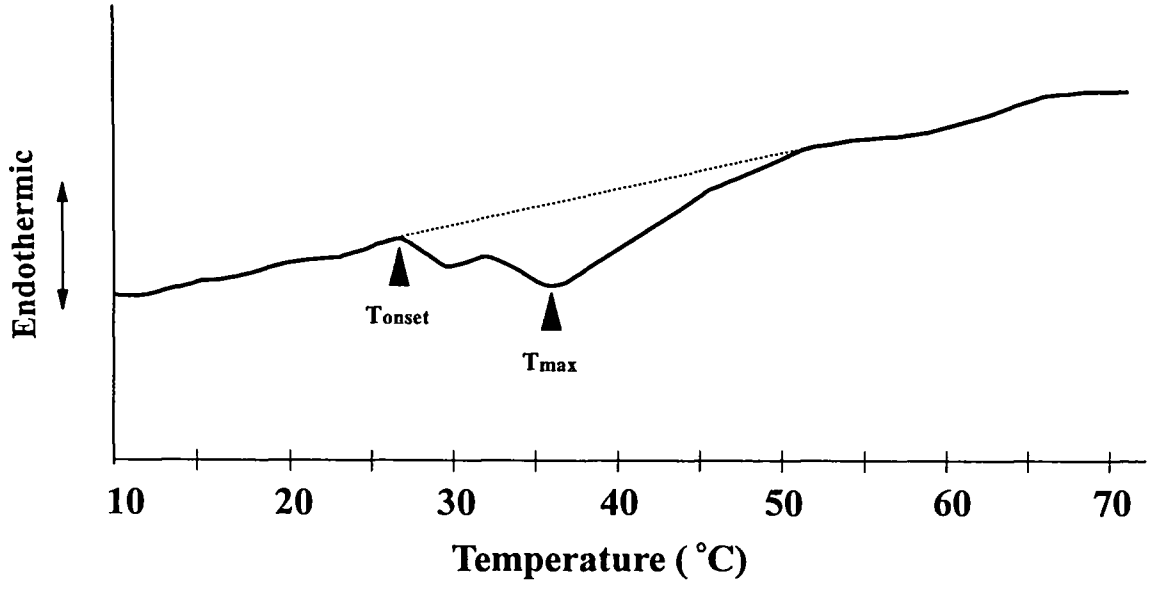

Fig. 3.3. Differential scanning calorimetric endotherm of ATF myosin in 0.6 M KCl, 20 mM Sodium phosphate buffer, pH 7.0, heated from 2 to 80 °C at a rate of 10°C/min.

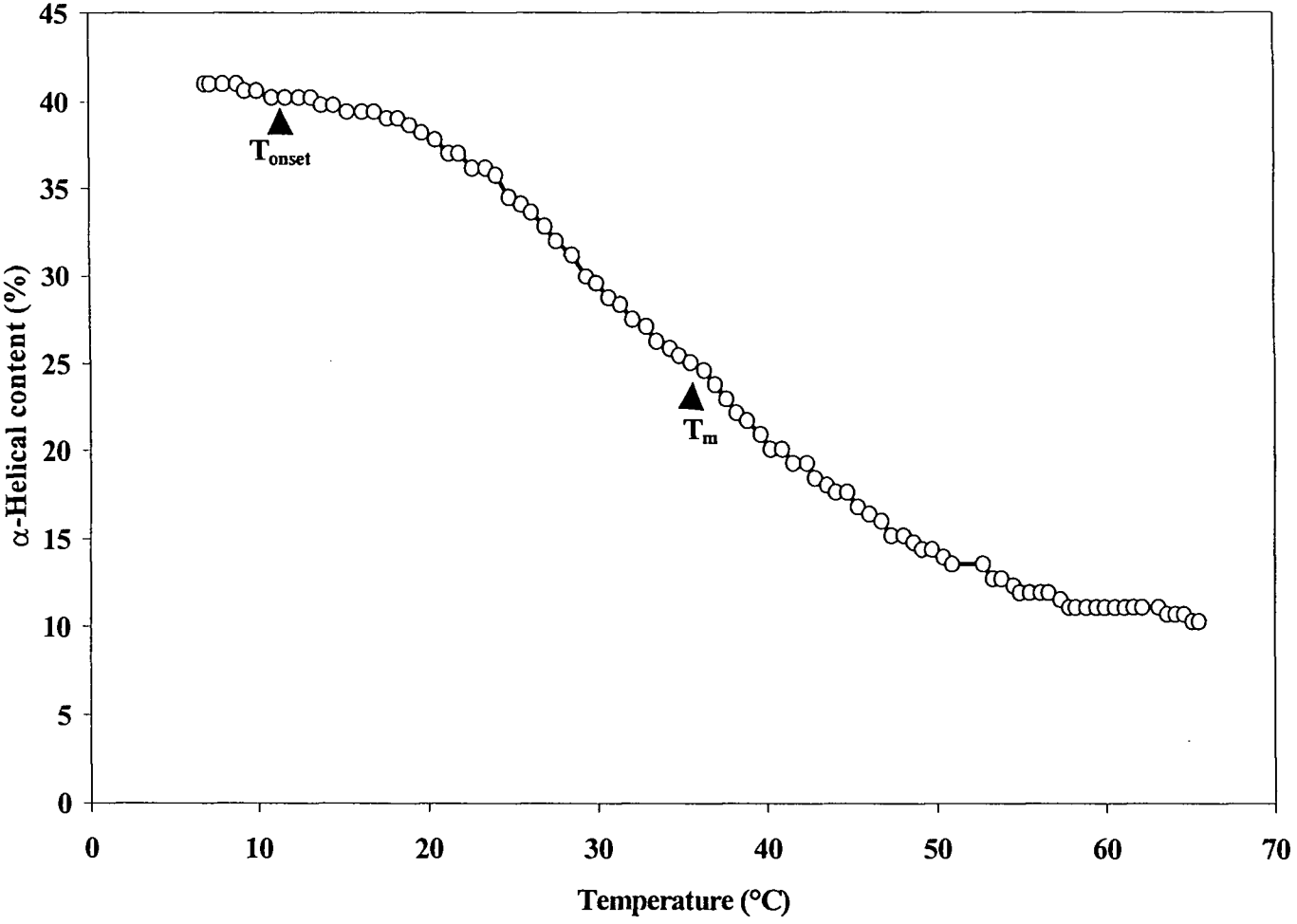

Fig. 3.4. α-Helical content as a function of temperature of ATF myosin in 0.6 M KCl, 20 mM Sodium phosphate buffer pH 7.0, heated from 2 to 80°C at a rate of 1.3°C/min. Each point is the mean of two determinations.

completely unfolding at 65 °C. According to Ogawa et al. (1993), the pattern of α-helical decrease in fish myosins by heating is classified by 3 types depending on the initial helicity and the number of transitions involved in the decrease. The pattern determined with ATF myosin was similar to walleye pollack. It exhibited slightly lower initial helicity prior to heating and decreased gradually during heating.

Thermal denaturation shown by the endothermic peaks of myosin (Fig. 3.3) was indicated due to the disruption of α-helical structure. However, the onset temperature of the unfolding process detected by CD was found to be at 10°C, 15°C lower than that detected by DSC. CD may be more sensitive to detect small changes than DSC, but differences in conditions used in the measurements, i.e., protein concentration and heating rate, may have contributed to the discrepancy. Increasing heating rate does affect the unfolding rate of the protein. An upward shift in transition temperature (Tmax) was commonly observed by increasing the heating rate (Park and Lanier, 1990). Change in myosin concentration, compared to heating rate, has little effect on transition temperatures (Goodno et al., 1975; Chan et al., 1992). Based on the same heating rate used for CD and dynamic test, it is concluded that denaturation of ATF myosin occurred before development of G' was noticeable.

Turbidity measurements

Turbidity development of ATF myosin was monitored from 20 to 65 °C (Fig.3.5). An increase in absorbance of heated fish myosin solution resulted from the formation of myosin aggregates (Gill et al., 1992), which were large enough to cause light scattering. The formation of myosin aggregates was enhanced sharply by an increase in temperature. The first derivative plot of the extent of turbidity-temperature curve showed the onset temperature of thermal aggregation was 23°C. A maximum was found at 40°C with two transition peaks at 25 and 30°C, respectively. Thermal aggregation of fish myosin is an ordered process in which the rate and extent of aggregation are specific to species and dependent on the concentration used (Chan and Gill, 1994). Species difference is attributed to the ability of myosin heavy chain to form cross-linking (Chan et al 1992). Compared with the results of the thermal-unfolding profile of heat-treated myosin molecule (Fig. 3.4), it is suggested that some conformational changes in myosin molecules seem to be the driving force for the acceleration of myosin-myosin interactions shown at the transition temperature (Ishioroshi et al., 1979). The temperature at which the maximum turbidity was noticed would have been higher if a higher concentration of myosin was used.

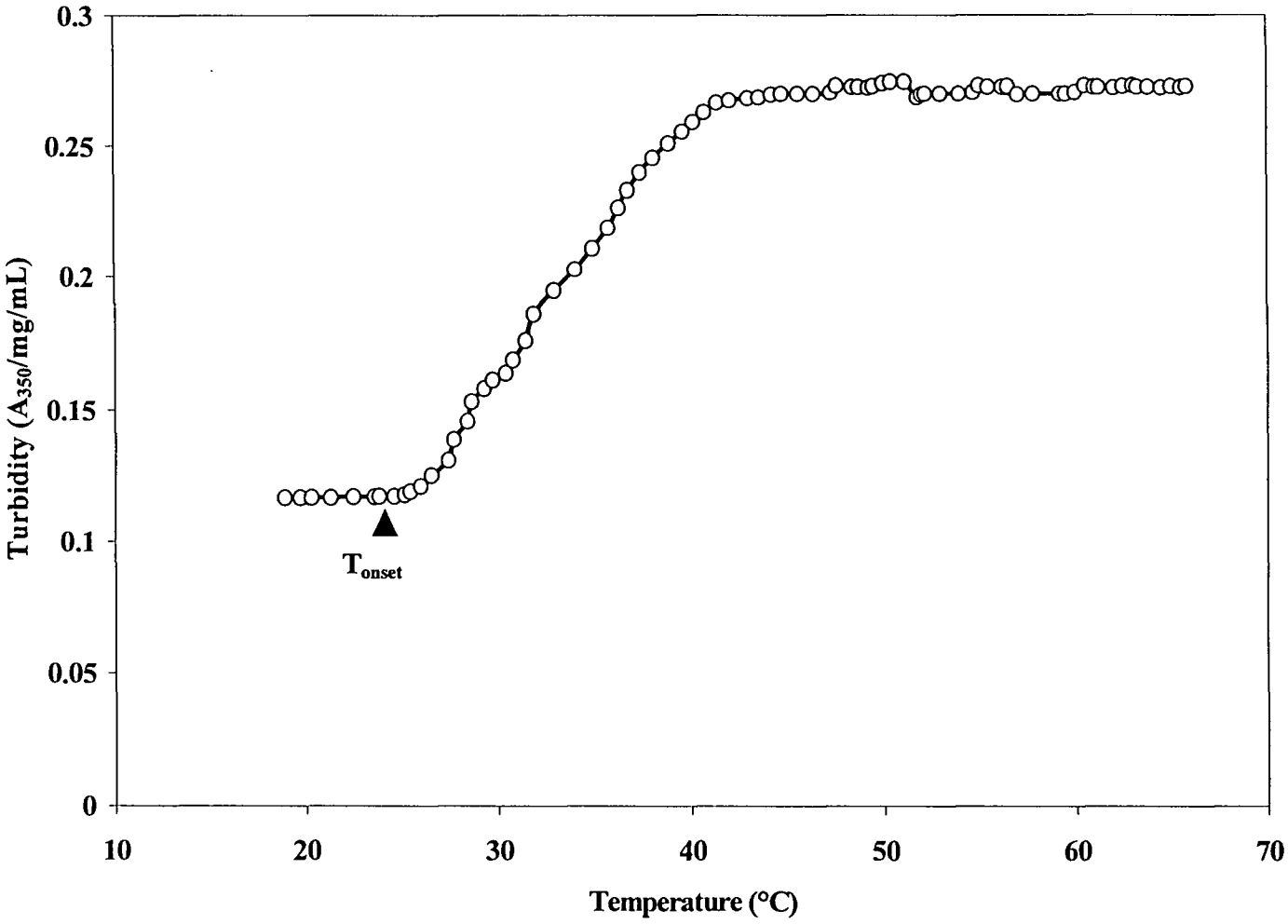

Fig. 3.5. Effect of temperature on turbidity of ATF myosin solution (1 mg/mL). The protein solutions were continuously heated from 20 to 65 °C at a rate of 1.3 °C/min.

Hydrophobicity

Surface hydrophobicity, determined by the ANS probe, increased nonlinearly with increase in temperature (Fig. 3.6). The increased hydrophobicity indicated the structural and conformational changes of ATF myosin during heating which causes the hydrophobic groups to become more exposed and bind with ANS. As heating proceeded, the slight decreases in surface hydrophobicity observed at 25, 45, 55 and 75°C were probably due to the aggregation of myosin, which competed for the same hydrophobic binding sites as ANS. The increased hydrophobicity during heating indicated the involvement of hydrophobic interactions in myosin gel formation at both the gel setting and strengthening stages.

Total and surface reactive sulfhydryl (SH) contents

The total SH content of ATF myosin was estimated at 6 moles/10^5 g protein, comparable to those of salmon (Lin and Park, 1998) and carp (Tsuchiya and Matsumoto, 1975) myosin. It was measured in the absence of urea; thus, about 15% of the total SH content was predicted to be the surface-reactive SH group. The total sulfhydryl content ofATF myosin decreased significantly by heating from 20 to 30°C, but remained relatively constant thereafter (Fig. 3.7). A decrease in SH content was reported to be due to the formation of disulfide bonds through the oxidation of SH groups or disulfide interchanges (Hayakawa and Nakai, 1985). Therefore, it implies that disulfide bonds are involved in the thermal aggregation of

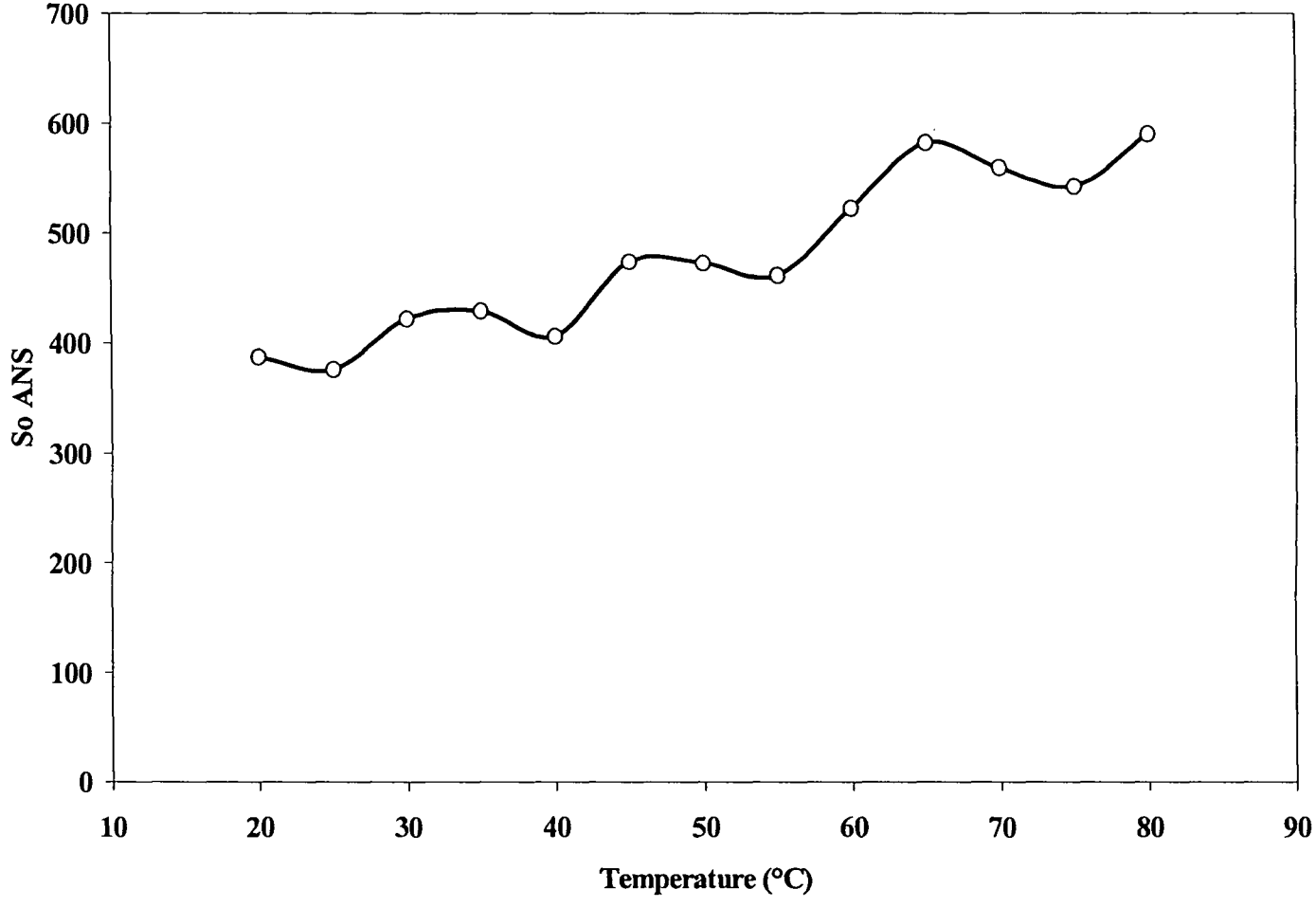

Fig. 3.6. Effect of temperature on surface hydrophobicity of ATF myosin solution (1mg/mL). The protein solutions were continuously heated from 20 to 65°C at a rate of 1.3°C/min.

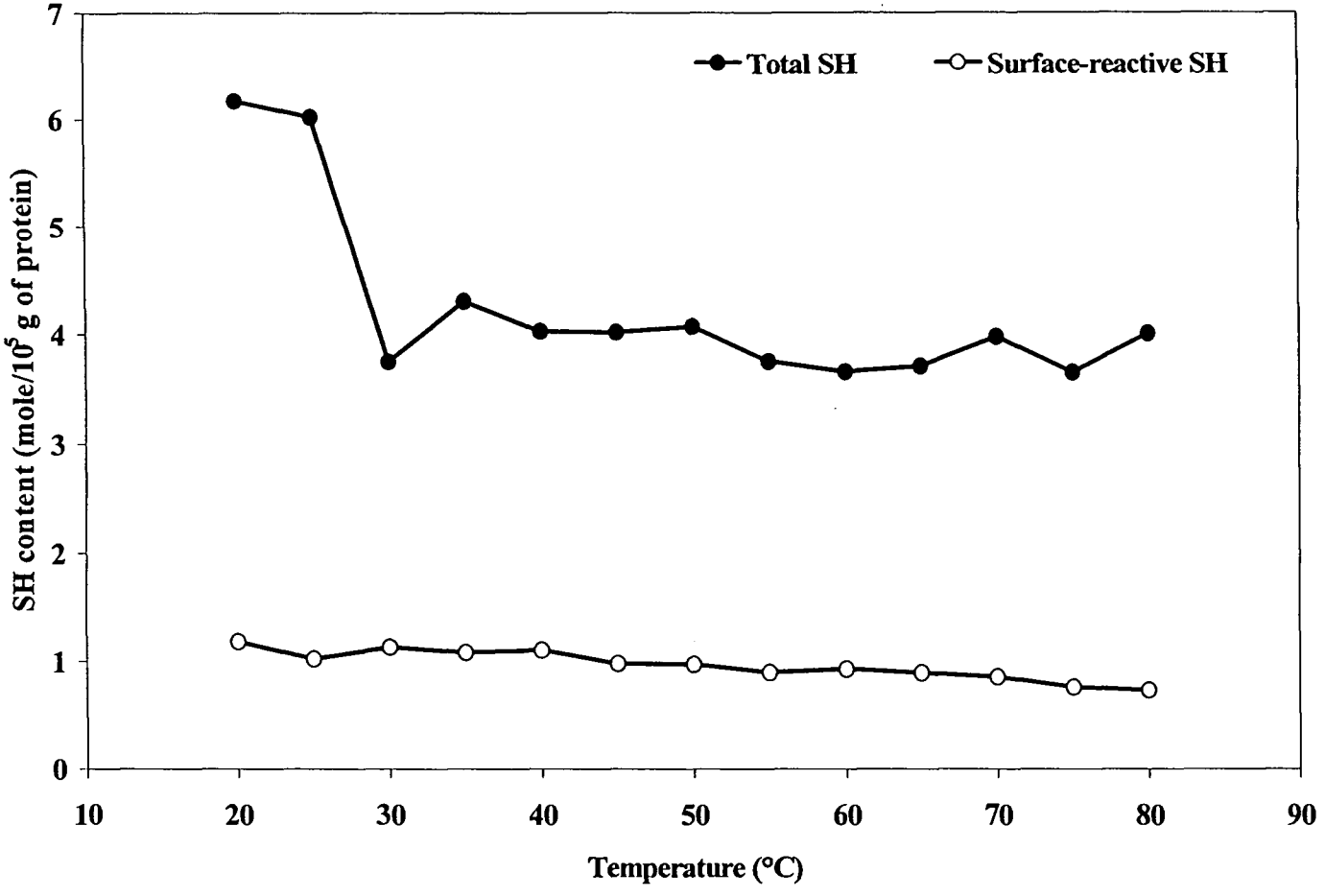

Fig. 3.7. Changes in total and surface SHs groups of ATF myosin, heated from 20 to 65°C at a rate of 1.3°C/min.

fish myosin. No significant changes were observed for surface-reactive SH content during heating.

Discussion

Heat-induced gelation of ATF myosin is a complex process comprised of several physicochemical changes. The observed denaturation and aggregation processes were similar to the mechanism proposed by Stone and Stanley (1992). ATF myosin was highly sensitive to heat, resulting in denaturation at a lower temperature compared with other reported fish myosin. Denaturation was initiated at 15 °C by unfolding of the α-helical region in myosin followed by exposure of the hydrophobic and sulfhydryl groups which are subsequently involved in the formation of intermolecular bonding during the aggregation process. Continuation of the aggregation of myosin at higher temperature resulted in the development of a three-dimensional structure, as shown by a significant increase in G' and a decrease in phase angle.

The ATF myosin molecule is thought to consist of a number of discrete, co-operative domains which could independently unfold during heating, similar to myosin from mammals (Privalov, 1982; Lopez-Lacomba et al. 1989), poultry (Wang and Smith, 1994) and fishes (Ogawa et al., 1993; Davies et al., 1994). The one large endothermic peak of ATF myosin observed in this study was thought to be a composite of several transitions deriving from each domain of the myosin

molecule, which has been found to unfold in different temperature ranges. Discrepancies in species and experimental conditions, such as, pH, ionic strength and heating rate (Park and Lanier, 1990), used in different studies make it difficult to determine melting order of the various, specific regions of ATF myosin by comparing the DSC thermogram with those of myosin from other fishes. It was postulated, however, that a hinge region near the center of the myosin rod may be the first domain to be unfolded because it has the lowest thermal stability and is susceptible to proteolysis (Wang and Smith, 1994).

Nonheated myosin molecules are known to be present in a monomeric form at a high salt concentration (ionic strength greater than 0.5) and at pH 6.0 or above (Morita et al., 1987). Therefore, an increase in absorbance with heated fish myosin solution resulted from the formation of myosin aggregates (Gill et al., 1992). ATF myosin began to aggregate at 23 °C prior to the completion of the denaturation process. Since myosin is a relatively large molecule, various proteolytic subfragments of myosin have been prepared and used to study their structure and function (Fig. 3.8). The heavy chain can be cleaved by papain near the globular end of the rod to produce a myosin rod and subfragment-1 (S-1) or by trypsin at the hinge region to produce light meromyosin (LMM) and heavy meromyosin, which can be further cleaved to yield S-1 and subfragment-2 (S-2) (Pearson and Young, 1989). The role of each discrete region of myosin in aggregation, however, has not been clearly determined. Gill and Conway (1989) concluded that the tail rather than the head portion of myosin was involved in thermal aggregation of cod myosin in

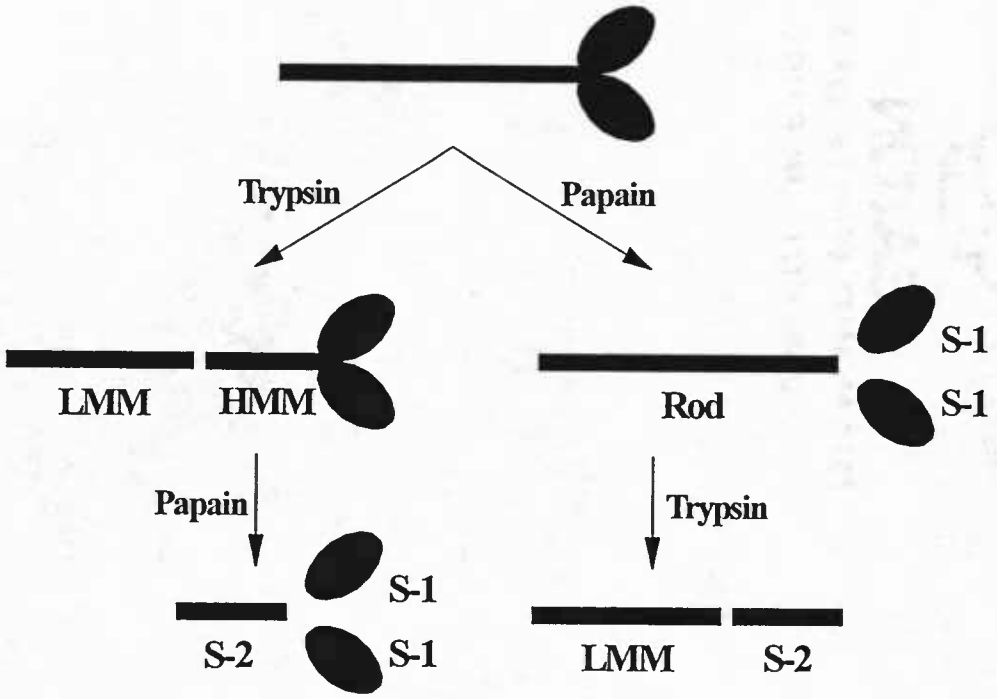

Fig. 3.8. Schematic diagram of a myosin and its subfragments (modified from Pearson and Young, 1989).

accordance to Sano et al. (1990a, b), who proposed that the initial stage of gel formation at 30-44°C of carp myosin was mainly attributed to the interaction between LMM. In contrast to those studies, Taguchi et al. (1987) proposed that aggregation of fish myosin was initiated by the inteaction between S-1 with the oxidation of SH groups followed by unfolding and interaction of LMM to form a gel network, similar to the mechanism proposed by Samejima et al. (1981) for rabbit myosin.

Since most of the sulfhydryl groups (~68%) in myosin are located in the head portion of myosin (Lowey et al., 1969), the significant decrease in SH groups observed at 20-30°C not only indicated the formation of disulfide bonds during gel formation but also implied the role of myosin heads at the initial aggregation, as reported by Taguchi et al. (1987) and Chan et al. (1993). Irreversible aggregation of S-1 from rabbit skeletal myosin (Samejima et al., 1981) and chicken breast muscle myosin (Smyth et al., 1998) at 45 °C was associated with the oxidation of SH groups. In addition to disulfide linkage, hydrophobic interaction may also be involved in the initial aggregation of myosin. Compared to the rod portion, which contains a large portion of charged amino acids, the head portion is relatively rich in hydrophobic amino acid residues (Maita et al., 1991). Dissociation of light chains from the head of myosin may lead to irregular refolding (Hamai and Konno, 1989) and create hydrophobic patches for intramolecular and intermolecular head association (Sharp and Offer, 1992) during the initial stage of heating.

It is evident that hydrophobic interaction was a major force involved in myosin aggregation at high temperature. The exposure of hydrohobic domains has been suggested as a prerequisite for the formation of large myosin aggregates (Wicker et al., 1986; Chan et al., 1992). The concomitant increase in surface hydrophobicity of myosin aggregate suggests the presence of hydrophobic interactions taking place in the thermal aggregation of fish myosins. Several studies have suggested that hydrophobic interactions are involved in the heat-induced gelation of fish myosin. Gill and Conway (1989) showed that the thermal aggregation of the cod MHC was the result of noncovalent forces and that the forces were weakened in the presence of hydrophobic, apolar substances. Hamada (1992) reported that hydrophobic bonds play a principal role in the gelation of myosin. Increase in protein hydrophobicity was shown to be responsible for the subsequent increase in the rigidity of myosin gels from fish, rabbit and chicken (Wicker et al. 1986) and other functional properties of muscle foods including emulsifying capacity (Li-Chan et al., 1985).

Conclusion

Heat-induced gelation of arrowtooth flounder myosin, prepared free of endogenous proteinases, involved denaturation and aggregation of myosin molecules. Denaturation was initiated by unfolding of the α-helical region in myosin followed by exposure of hydrophobic and sulfhydryl groups which are

subsequently involved in the formation of intermolecular bonding during the aggregation process and results in a three-dimensional structure. In the absence of endogenous proteinases, the thermal susceptibilty of ATF myosin provided the base to form high quality protein gels.

Acknowledgement

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Chapter 4

PROTEOLYSIS AFFECTS HEAT-INDUCED GELATION OF ARROWTOOTH FLOUNDER MYOSIN

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Abstract

A model system using arrowtooth flounder myosin and papain was developed to investigate how proteolysis affects heat-induced gelation of fish myosin. The addition of papain decreased the onset temperature and the rate at which G' developed during heating. Frequency sweep curves after the heating and cooling phase revealed that G' markedly decreased in proportion to the amount of papain that was added. However, use of E-64, a cysteine proteinase inhibitor, reversed the effects of papain and protected myosin heavy chain (MHC) from degradation, as observed on SDS-PAGE. DSC thermograms indicated papain significantly decreased the enthalpy required to induce myosin denaturation with no significant changes in the onset or maximum temperature. Thermal denaturation kinetics indicated a decrease in both the activation energy of the denaturation process and the denaturation rate of myosin. CD studies revealed a rapid decrease in the initial α-helical content, indicating the degradation of myosin molecules mostly occurred in the tail region. These results suggested that thermal denaturation of myosin was likely to be facilitated by proteolytic degradation. Although myosin gel could be formed, structural disruption caused by proteolysis, i.e., reduction in molecular size and loss in the structural domain, resulted in lowering the gelling ability and rigidity of the formed gel.

Introduction

Functional properties of a protein play an important role in determining the textural qualities and physical behavior of a food during preparation, processing, and storage. Since a particular functional property is governed by a specific conformation of a protein, any alterations of the physical, chemical, and conformational properties of the protein by extrinsic factors would influence the functionality displayed.

Ability to form a gel upon heating is an important functional property of myosin which is considered to be the primary component responsible for surimi gel formation (Sano et al., 1988). Myosin is a multidomain protein with two large heavy chains and four light chains arranged into an asymmetrical molecule with two globular heads attached to a long α-helical rod-like tail (Privalov, 1982; Lopez-Lacomba et al. 1989). Both head and tail portions of myosin have been shown to play an important role in gel formation (Taguchi et al., 1987; Chan et al., 1993). Structural changes that occur in the head and tail portions of myosin, as a result of thermal denaturation, enable myosin to form protein-protein interactions. The native conformation of myosin is of primary importance for proper gelation. In order to form highly viscoelastic gels, sequential unfolding of different protein structural domains and ordered formation of protein-protein interactions are essential (Sharp and Offer, 1992).

The gelling property of myosin is highly related to species and the conditions of myosin preparation. Species differences in gel forming ability of fish muscle are attributed to the differences in the cross-linking of myosin heavy chain (MHC) associated with the surface hydrophobicity displayed by the unfolded domains of the MHC and the temperature at which such domains unfold (Wicker et al., 1986; Chan et al., 1992). It is postulated that the cross-linking ability of the MHC may be localized at one or more discrete portions of the molecule and any structural/functional differences in these loci among different fish myosins might explain species differences in gel forming ability (Chan et al., 1993). In addition to the species, the ability to form myosin gel is also related to structure and has been shown to be highly dependent on ionic environment, pH, and heating (Xiong, 1997).

Proteolytic degradation of myofibrillar proteins, particularly myosin, is a major concern in surimi gel manufacture, especially those produced from the muscle of soft-texture fish species that contain high levels of endogenous proteinases, i.e., cathepsins and heat stable alkaline proteinase (An et al., 1996). Proteolysis can take place during postmortem storage and processing and results in undesirable flavor and texture alterations (Asghar and Bhatti, 1987). Gel-softening phenomenon or "modori" observed at 50-70°C was attributed to myosin hydrolysis by some heat-activated proteases (Lin and Lanier, 1980; Deng, 1981). Quality variations within a species during different seasons has been reported to be

associated with proteolysis (Beas et al., 1991). During and after spawning, before the feeding season, fish flesh loses its gel-forming ability as a result of increased proteolytic activity which leads to the proteolytic breakdown of muscle tissue. Such proteolysis accompanies a decrease in the myofibrillar protein level and an increased moisture retention in the tissue. Proteolysis has a detrimental effect on surimi quality by substantially lowering the gel strength (Morrissey et al., 1995). However, the mechanism that proteolysis interferes with during surimi gelation has not been clearly elucidated.

The objective of this study was to investigate how proteolysis affects the heat-induced gelation of fish myosin in the model system prepared with arrowtooth flounder (ATF) myosin and papain. Arrowtooth flounder is an abundant fish species caught off the North America/Pacific region, but suffers from softening of muscle by heat stable proteases during heating (Greene and Babbitt, 1990; Wasson et al., 1992). Papain is a proteolytic enzyme widely used in meat tenderization. Papain was used in the model because it was closely related to the cysteine proteinases found predominantly in fish muscle that play an important role in textural degradation.

Materials and methods

Reagents

Potassium chloride (KCl), sodium azide (NaN_3), Tris base, 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), 2-mercaptoethanol (βME), N,N,N',N'-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), magnesium acetate, Tris maleate, adenosine triphosphate (ATP), sodium bicarbonate, magnesium chloride, sodium phosphate, potasium phosphate, glycerol and papain (EC 3.4.22.4) were purchased from Sigma Chemical Co. (St Louis, MO). Phenylmethylsulfonyl fluoride (PMSF) was purchased from Calbiochem Co. (La Jolla, CA). Sodium dodecyl sulfate (SDS) and urea were purchased from Bio-Rad Laboratories (Hercules, CA).

Sample

Arrowtooth flounder were obtained from the National Marine Fisheries Service, Utilization Research Laboratory, Kodiak, Alaska. Trawl caught arrowtooth flounder were obtained fresh and hand-filleted. The fillets were vacuum-packed in polyethelene bags and frozen at -20° C before being immediately shipped in dry ice to the OSU-Seafood Laboratory. Frozen fillets were kept frozen at -50°C until used.

Myosin preparation

Myosin was extracted by the method described by Martone et al. (1986) with slight modifications. All steps were performed at 0-4°C to minimize proteolysis and protein denaturation. Arrowtooth flounder fillets were finely chopped and added with 10 volumes of Buffer A (0.10 M KCl, 1 mM PMSF, 10 μM E-64, 0.02% NaN_3 and 20 mM Tris-HCl, pH 7.5). After incubation on ice for 10 min with occasional stirring, washed muscle was recovered by centrifugation at 1,000*xg* for 10 min. The pellet was suspended in 5 volumes of Buffer B [0.45 M KCl, 5 mM ßME, 0.2 M $Mg(CH_3COO)_2$, 1 mM EGTA, and 20 mM Tris-maleate, pH 6.8], and ATP was added to a final concentration of 10 mM. The mixture was kept on ice for 1 h with occasional stirring and centrifuged at 10,000*xg* for 15 min. Supernatant was collected and added slowly with 25 volumes of 1 mM $NaHCO_3$ followed by incubation for 15 min on ice. Precipitated myosin was collected by centrifugation at 12,000*xg*, resuspended gently with 5 volumes of Buffer C (0.50 M KCl, 5 mM βME and 20 mM Tris-HCl, pH 7.5), and added with 3 volumes of 1 mM $NaHCO_3$ and $MgCl_2$ to a final concentration of 10 mM. The mixture was kept overnight on ice prior to centrifugation at 22,000*xg* for 15 min. Myosin was recovered as pellet and used immediately or stored at -20°C in 50% glycerol.

Electrophoretic analysis of ATF myosin

Extracted myosin was analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophroresis (SDS-PAGE) by the method of Laemmli (1970) to investigate purity and the extent of autolysis, if any, due to the presence of endogeneous proteases possibly associated with myosin. About 0.5 g of myosin were incubated in a 55°C waterbath for different time intervals. After incubation, 2.5 mL of prewarmed (80°C) 5 % (w/v) SDS was immediately added and the solution was then held in an 80 °C waterbath until the proteins were solubilized. Solubilized samples were mixed at 1:1 (v/v) ratio with the SDS-PAGE sample buffer containing 1.5 M βME and boiled for 3 min. The samples, 10 μg, were loaded on the gel made of 4% stacking and 10 % separating gels and subjected to electrophoresis at a constant voltage of 75 V using a Mini Protean II apparatus (BioRad Laboratories Inc., Richmond, CA). After electrophoresis, the gels were stained with 0.125% Coomassie brilliant blue R-250 in 25% ethanol and 10% acetic acid and destained with 25% ethanol and 10% acetic acid. Molecular weights of the proteins were estimated using high and low molecular weight standards (Sigma Chemical Co., St. Louis, MO). High molecular weight standards included rabbit muscle myosin (M_r 200,000), β-galactosidase (M_r 116,000), phosphorylase b (M_r 97,000), fructose-6-phosphate kinase (M_r 84,000), bovine serum albumin (M_r 66,000), glutamic dehydrogenase (M_r 55,000), ovalbumin (M_r 45,000), and glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000). Low molecular weight

standards included bovine serum albumin, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase (M_r 29,000), trypsinogen (M_r 24,000), trypsin inhibitor (M_r 20,000), α-lactalbumin (M_r 14,200), and aprotinin (M_r 6,500).

The intensity of the protein bands was quantitated by scanning gels with a HP Deskscan II (Hewlett-Packard Co., Mineapolis, MN) and analyzing the image with software (National Institutes of Health Image 1.54, Washington, DC.)

Papain activity assay

Papain activity was determined by using BANA as a substrate according to the modified method of Abe et al. (1994). Papain stock solution was prepared in 50 mM sodium acetate, pH 4.5 at a concentration of 3.2 mg/mL. To obtain the optimal absorbance range, papain solution was diluted 20 times in the same buffer, and 50 μL of the diluted solution was preincubated at 40 °C for 10 min with 450 μL of the assay buffer (25 mM sodium phosphate, pH 7.0, containing 20 mM βME). Reaction was started by adding 200 μL of 6.5 mM BANA and the mixture was incubated at 40 °C for 10 min. The reaction was stopped by adding 1 mL of 2% (v/v) HCl in ethanol and the color was developed by adding 1 mL of 0.06% (w/v) *p*-dimethylaminocinnamaldehyde in ethanol. Reaction products were measured spectrophotometerically at 540 nm. A blank was run in the same manner except that enzyme was added after the addition of the stopping reagent. One activity unit was defined as the changes in absorbance of 1 at 540 nm per minute.

Determination of degree of proteolysis of ATF mince

Extent of proteolysis of ATF mince by papain was monitored by the modified method of Greene and Babbitt (1990). Three grams of chopped muscle were heated linearly from 25 to 80 °C at the rate of 1.3 °C/min, with or without papain added at 2.5 and 5.0 mU/mg of protein, respectively. The reaction was stopped by adding 27 mL iced-cold 5% (w/v) trichloroacetic acid (TCA). The mixture was homogenized for 1 min using Polytron (Brinkmann Instruments, Westbury, NY) and kept on ice for 1 h followed by centrifugation at 5,000*xg* for 10 min to collect the supernatant. Oligopeptide content in the supernatant was measured by the method of Lowry et al. (1951). Proteolytic activity was expressed as micromole of tyrosine released per min.

Autolysis pattern and effect of papain on fish mince

Three grams of chopped muscle were heated linearly from 25 to 80 °C at the rate of 1.3 °C/min with or without papain added at 2.5 and 5.0 mU/mg of protein, respectively. The reaction was stopped by adding 27 mL 5% (w/v) SDS preheated to 95 °C. The mixture was homogenized for 1 min using Polytron (Brinkmann Instruments, Westbury, NY) and incubated at 85 °C for 30 min. Insoluble debris was removed by centrifugation at 7,800 *xg* for 10 min and supernatant was used for electrophoretic analysis as previously described in the section "Electrophoretic analysis of ATF myosin".

Dynamic rheological measurements

Rheological changes of myosin during thermal gelation were analyzed by the method of Visessanguan et al. (1999) using a Bohlin CS-50 rheometer (Bohlin Instruments, Inc., Cranbury, N.J), which operated in the small-amphitude oscillatory mode. Samples containing papain and/or E-64 were freshly prepared at 0°C before each run. The amount of enzyme added was equivalent to 2.5 and 5 mU/mg of protein, respectively, as determined by using BANA as a substrate. Samples were sheared at a fixed frequency of 0.1 Hz with a constant strain amplitude of 0.015 while heated over the range of 25-80°C at 1°C/min. Data were collected every 60 s during shearing measurements. Network development was studied in real time by measuring changes in the storage modulus (G'), loss modulus (G") and loss tangent (tan δ) as a function of increasing temperature. At the end of the heating cycle, samples were cooled and held at 25°C for 2 min. The resulting myosin gels were characterized by measuring dynamic properties as a function of oscillatory frequency using the same strain amplitude used in thermal scan.

Differential Scanning Calorimetry

DSC studies were performed in a DuPont 910 differential scanning calorimeter (Du Pont Co., Wilmington, DE) according to the method of Visessanguan et al. (1999). Samples containing papain at 2.5 mU/mg of protein

were freshly prepared at 0-4°C before each run. Samples with or without added papain were sealed in hermatic DSC pans and accurately weighed to 14 -20 mg wet weight on a semi-micro balance (Mettler Instrument Corp., Highstown, NJ). All samples were scanned at 10°C/min over the range of 2-100°C using a DuPont mechanical cooling accessory. An empty hermatic pan was used as a reference. At least three determinations were made and the most representative thermogram with average onset and transition temperatures was reported.

Thermal denaturation kinetic studies

Thermal denaturation kinetic constants were calculated by a dynamic method (Wagner and Añon, 1985). Samples, with or without papain (2.5 mU/mg protein), were scanned from 2-100°C at heating rates of 2, 5, 7, 10, 13, 16, and 20°C/min at least twice according to ANSI-ASTM E698/79 method (1979). Based on a plot of the logarithm of the program rate versus the peak temperature, corresponding activation energy (Ea), pre-exponential factor of the Arrhenius equation (Z), and rate constant (Kd) of myosin denaturation were calculated by the following equation (Ozawa, 1970):

$$-\ln (\beta / T^2_{max}) = \ln (ZR/Ea) - Ea/RT_{max}$$

where β represents the heating rate (K min^{-1}); T_{max}; peak temperature (K); and R; the gas constant (cal/mol/K).

α-Helical content

α-Helical content of myosin was analyzed using a JASCO A-500 spectrophotometer (Japan Spectroscopic Co., Ltd., Tokyo) equipped with a temperature control unit (Visessanguan et al., 1999). Myosin pellet was dissolved in cold 0.6 M KCl-20 mM potassium phosphate, pH 7.0 and centrifuged at 10,000 rpm for 10 min. The supernatant was used for circular dichroism (CD) analysis after being diluted to 0.4 mg/mL in the same buffer. Papain was added to the supernatant at the ratio of 2.5 mU/ mg protein at 0°C before each run. α-Helical content was estimated using Equation (1) on the basis of $[\theta]_{222}$ of poly-L-glutamic acid, which possesses the complete helical structure (Holzwarth and Doty, 1965):

$$\alpha\text{-Helical content} = (100 \times 115 \times [\theta]_{obs})/ (10 \times C'' \times -40{,}000)$$

where $[\theta]_{obs}$ and C" represented the observed ellipticities at 222 nm and protein concentration, respectively.

Protein determination

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Myosin was first solubilized for 5 min in 5% (w/v) SDS preheated to 95 °C and further incubated at 85 °C for 30 min. Insoluble debris was removed by centrifugation at 7,800 *xg* for 10 min and supernatant was used for protein analysis.

Statistical analyses

Data were analyzed by analysis of variance (ANOVA). Mean difference was determined using the least significant difference (LSD) multiple range test (Statgraphics Version 6.0, Manugistics Inc., Rockville, MD). Significance of difference was established at $p \leq 0.05$. The least square linear regression was applied and slopes were compared by student t-test (Ramsey and Schafer, 1996).

Results

Purity and autolytic activity associated with extracted myosin

The purity of extracted myosin was estimated to be 90% by the densitometric analysis of SDS-PAGE gel (Fig. 4.1). The appearance of smeared bands in the control group indicated degradation products accumulated prior to myosin extraction. Since no notable changes in the electrophoretic patterns of myosin was observed by incubating up to 30 min at 55°C, extracted myosin was

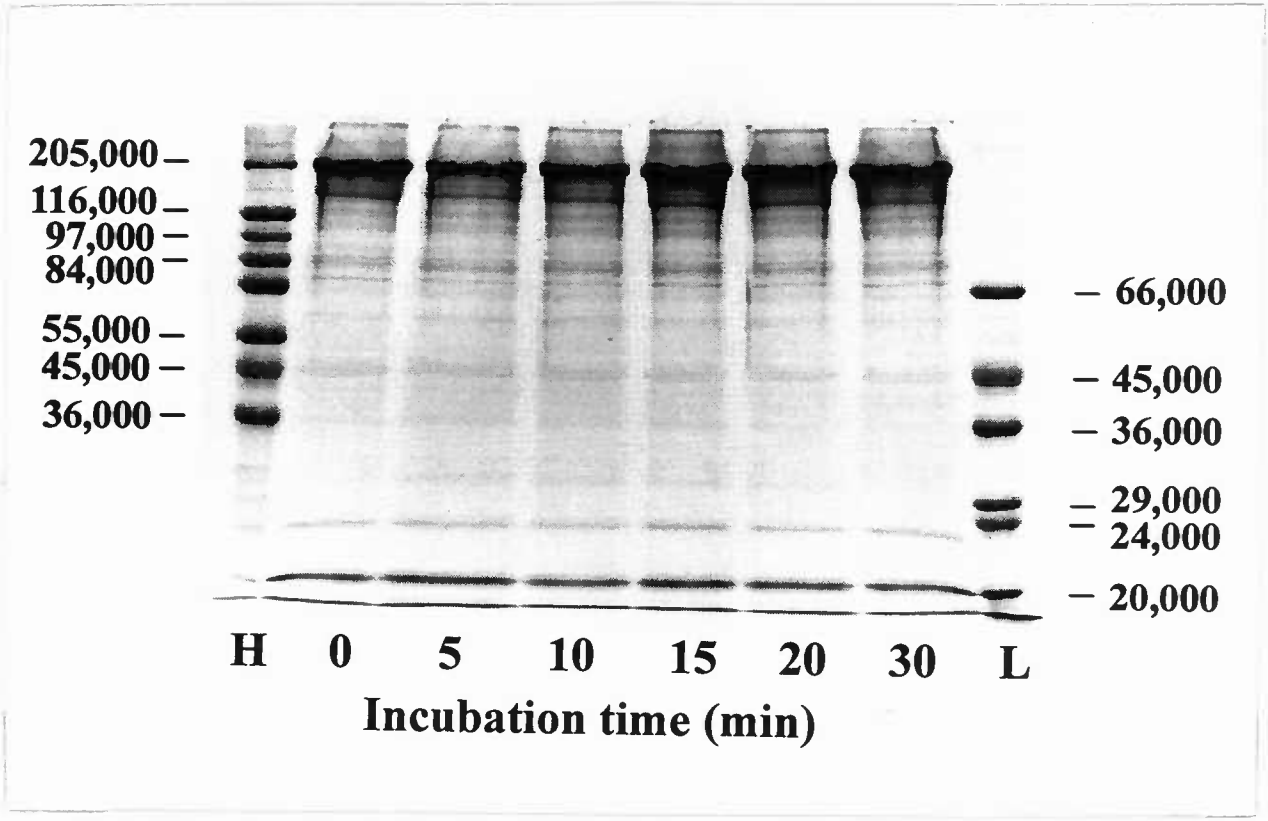

Fig. 4.1. SDS-PAGE pattern of ATF myosin before and after incubation at 55°C up to 30 min. H and L designates high and low molecular weight protein standards, respectively.

postulated to be free of contaminated proteinases and thus adequate for further analysis.

Autolysis and effect of papain on fish mince

Arrowtooth flounder mince was used as a substrate to compare the extent of proteolysis caused by papain and the extent of autolysis mediated by endogenous muscle proteinases. Autolytic activity of fish mince, estimated as an increase in oligopeptide content, was about 9 nmoles/min by linear heating from 25-80°C at the average of 1.3°C/min (Fig. 4.2). By adding papain at the concentrations used in the study, which were 2.5 and 5 mU/mg of protein, the extent of proteolysis caused by papain at the above ratios were determined to be about 1.6 and 1.9 times higher than that of autolysis of mince incubated under the same condition, respectively. Autolytic activity was substantially reduced by adding E-64, a cysteine protease inhibitor, while the remaining activity was assumed to be due to the activities of others proteinases.

Electrophoretic analyses of the degradation products indicated extensive proteolysis of myofibrillar proteins by the activities of endogenous proteinases present in the ATF mince or papain added (Fig. 4.3). Most bands of the major myofibrillar proteins, i.e., myosin heavy chain (MHC) (Mr~200,000), tropomyosin, troponin, and actin (Mr~45,000) disappeared after prolonged incubation with and without papain added. The disappearance of low molecular weight protein bands of

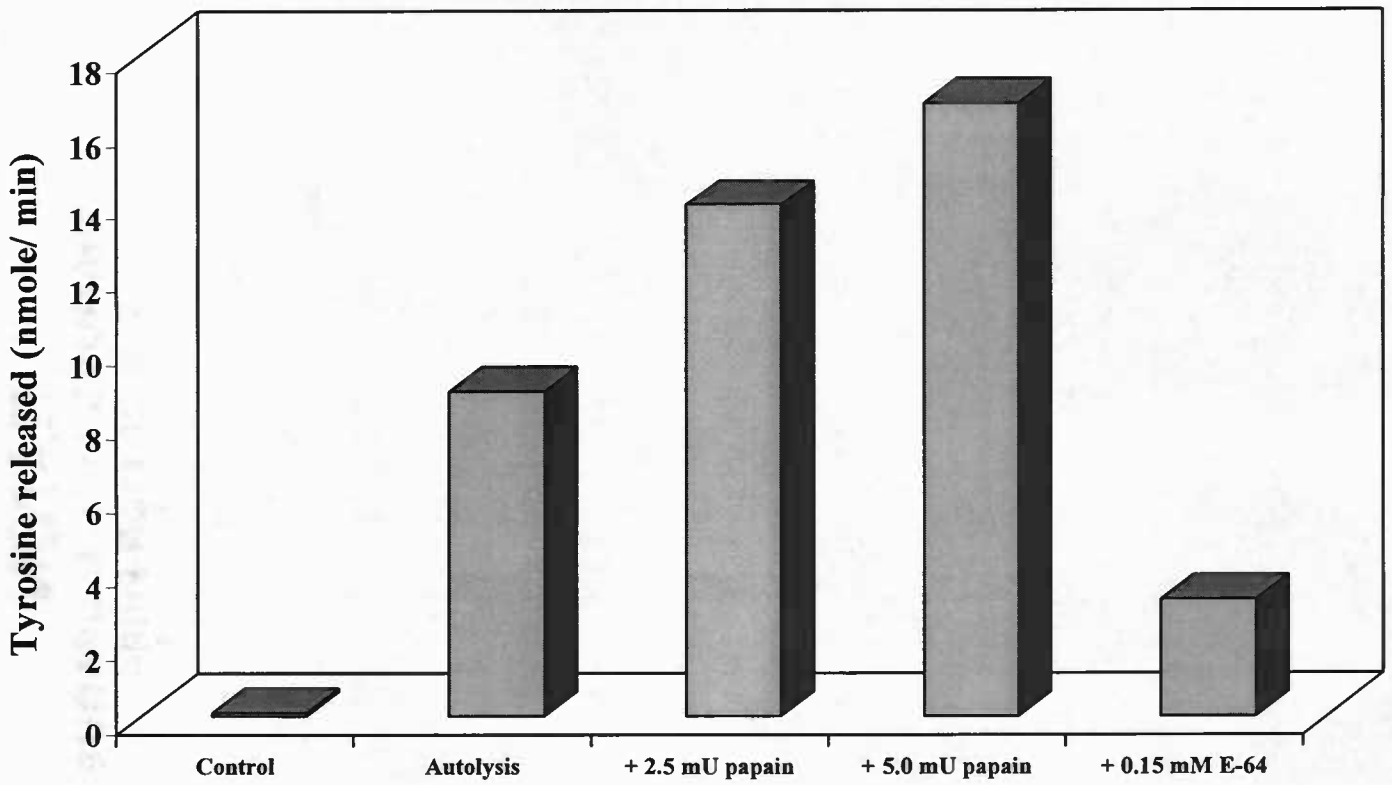

Fig. 4.2. Extent of proteolysis caused by the addition of papain and autolysis of ATF mince incubated by linear heating from 25-80°C at a rate of 1.3 °C/min

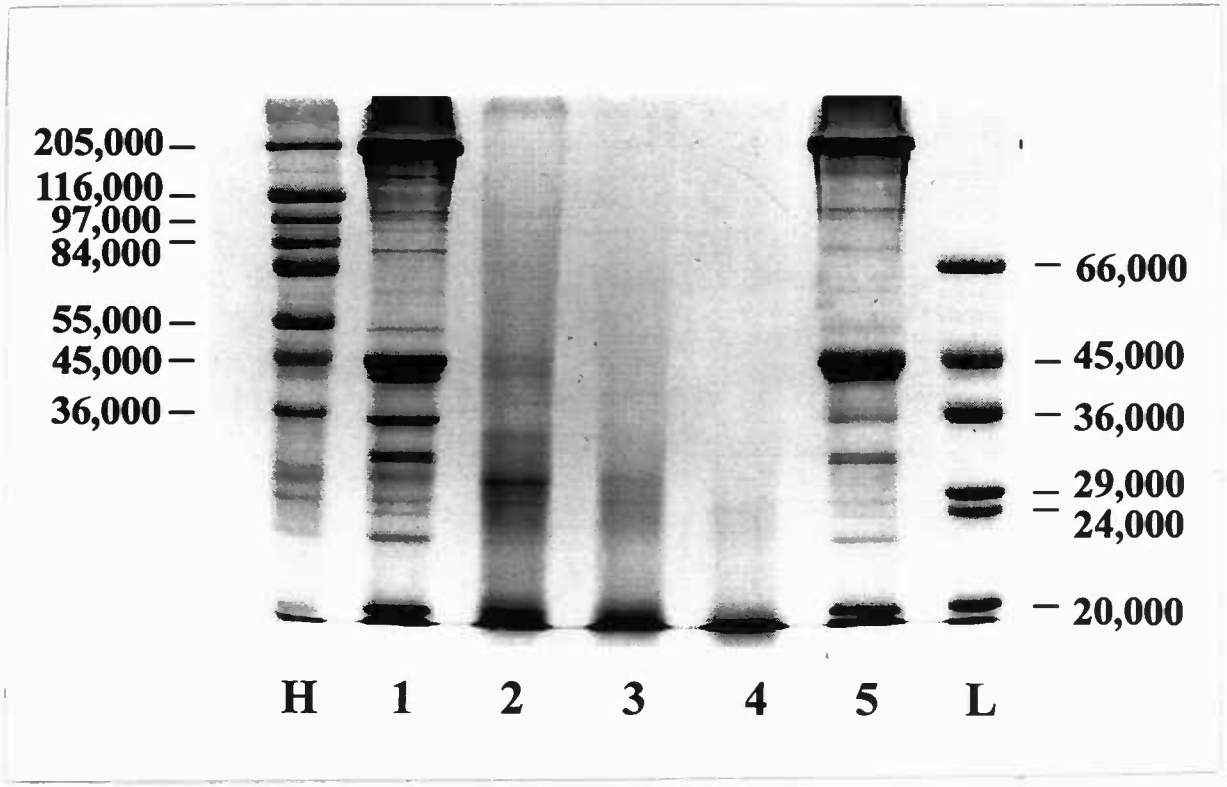

Fig. 4.3. SDS-PAGE pattern of ATF mince incubated under various conditions. H and L designated high and low molecular weight protein standards, respectively. 1: ATF mince incubated at 0°C (as a control); 2: mince incubated by linear heating from 25-80°C; 3: mince added with 2.5 mU papain and heated from 25-80°C; 4: mince added with 5.0 mU papain and heated from 25-80°; and 5: mince added with 0.15 mM E-64 and heated from 25-80°.

mince with papain added, compared to the mince incubated without papain, indicated the higher extent of proteolysis caused by papain. Those protein bands were presumed to undergo further proteolytic degradation until they were too small to be retained on the polyacrylamide gels. Only trace degradation of some proteins was observed in mince with added E-64, but no substantial degradation of MHC.

Effect of papain on heat-induced gelation of myosin

Changes in G' as a function of temperature indicated ATF myosin formed gel in 3 stages: (1) gel setting, (2) gel weakening, and (3) gel strengthening (Fig. 4.4). The similar pattern of gelation was also shown for with myosin with papain added. The two systems, however, showed the differences in apparent onset temperature. The first derivative plots showed that the apparent onset temperatures at which those stages were observed were shifted to significantly lower temperatures in the presence of papain ($P<0.05$). It was also evident that myosin, added with papain, exhibited a lower magnitude of G' for all stages of gel development. To confirm that the adverse effects were due to proteolytic degradation, E-64, a specific cysteine proteinase inhibitor, was added to the myosin system. Myosin added with E-64 both in the presence and absence of papain, exhibited 3 stages of gel development and higher G' values were obtained than myosin by itself. Besides the inhibitory action on proteinases, E-64 has an epoxy characteristic that enhances the gel formation of myosin. Since no differences were observed between the gelation characteristics of myosin alone and myosin added

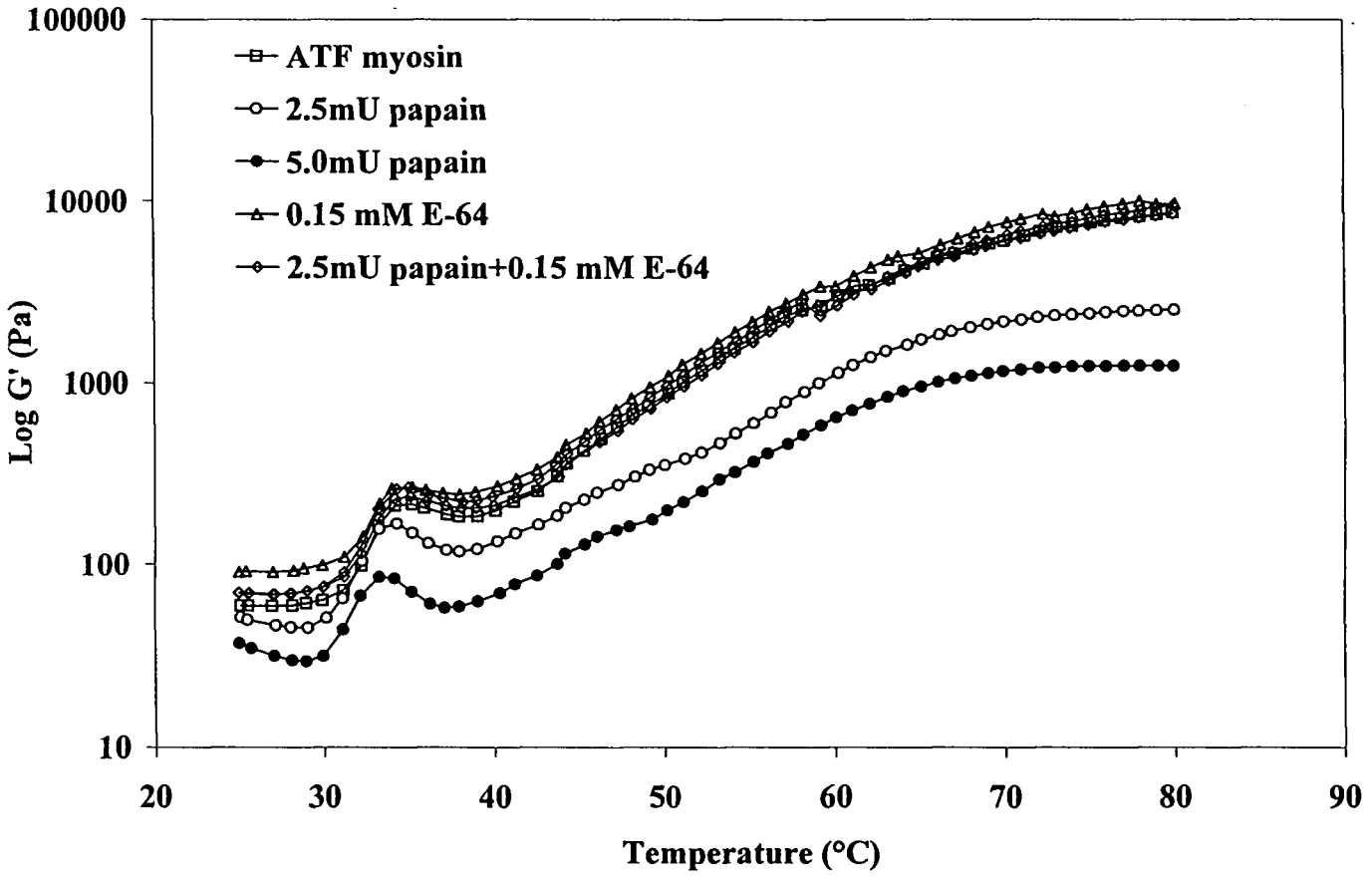

Fig. 4.4. Change in storage modulus (G') during thermal gelation of ATF myosin under various conditions.

with papain in the presence of E-64, the loss in the gel forming ability of myosin was concluded to be due to the proteolytic activity of papain.

More than 80% of G' were developed in the gel strengthening stage, where the rate of G' development was determined by linear regression analysis. In addition, the final G' values observed at the end of heating were compared to evaluate gel formation of myosin under various conditions (Figs. 4.5a, b). Among the various conditions tested, addition of papain resulted in the most severe reduction of both the rate of G' development and the final G' value. The values were reduced proportionally to the amount of papain added. When E-64 was added, myosin added with papain showed the same rate of G' development and final G' value as myosin gels without papain.

Effect of papain on phase angle

Addition of papain resulted in a higher magnitude of phase angle (tan δ) observed as heating proceeded (Fig 4.6). Myosin added with 5.0 mU papain showed the highest tan δ followed by myosin added with 2.5 mU papain and myosin without papain, respectively, while myosin added with E-64 showed the lowest tan δ. Corresponding to the changes in G' observed (Fig. 4.4), the decrease in the phase angle of myosin with and without added papain reflected a transition of viscous myosin sol to elastic myosin gel as heating proceeded. The phase angle is derived from both G' and G", which represent the elastic and viscous behavior,

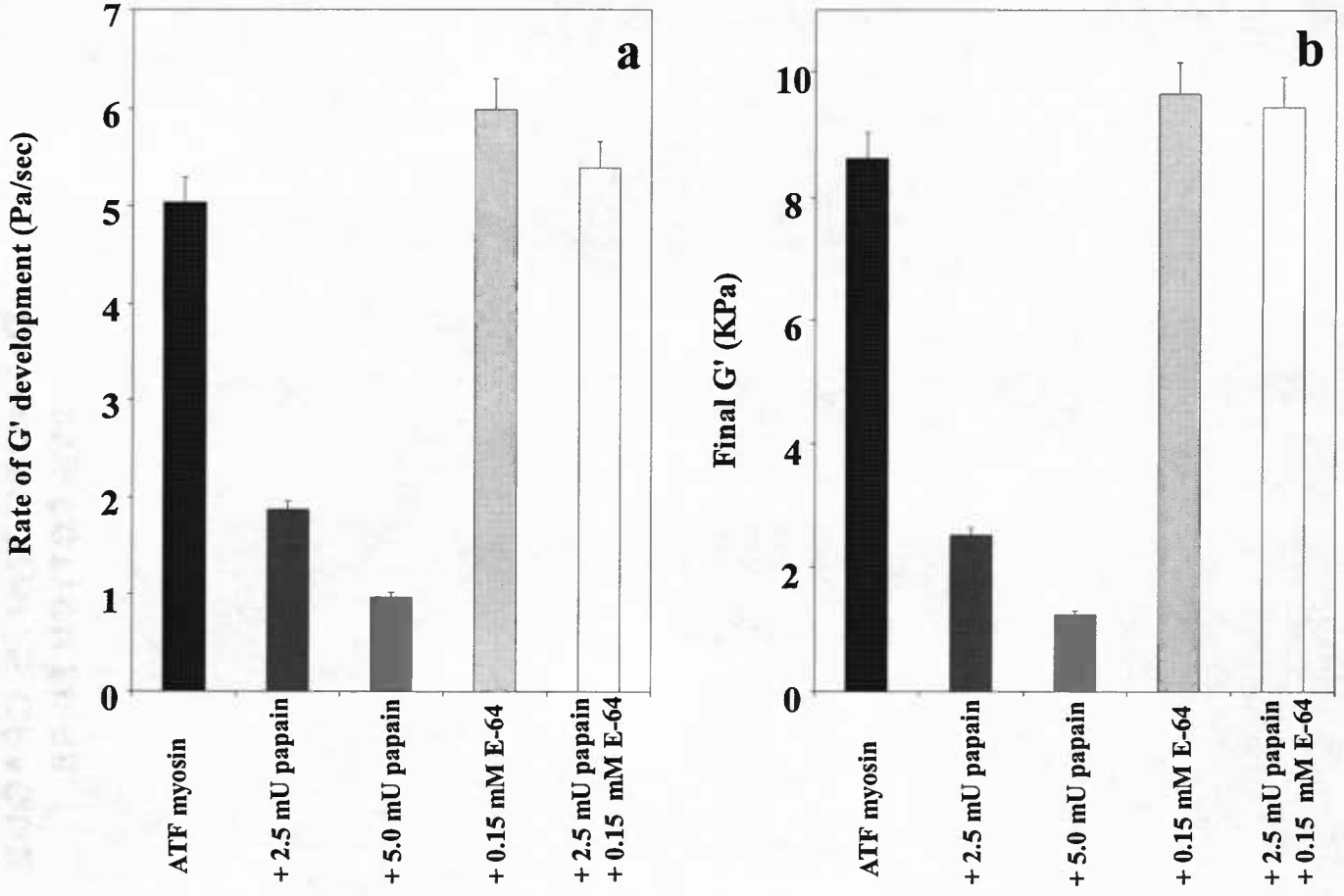

Fig. 4.5. Developing rate and final G' of ATF myosin gels formed under various conditions. Rate was estimated by linear regression of the obtained data within the temperature range that about 20 -80% of G' were developed and final G' was determined at 80°C.

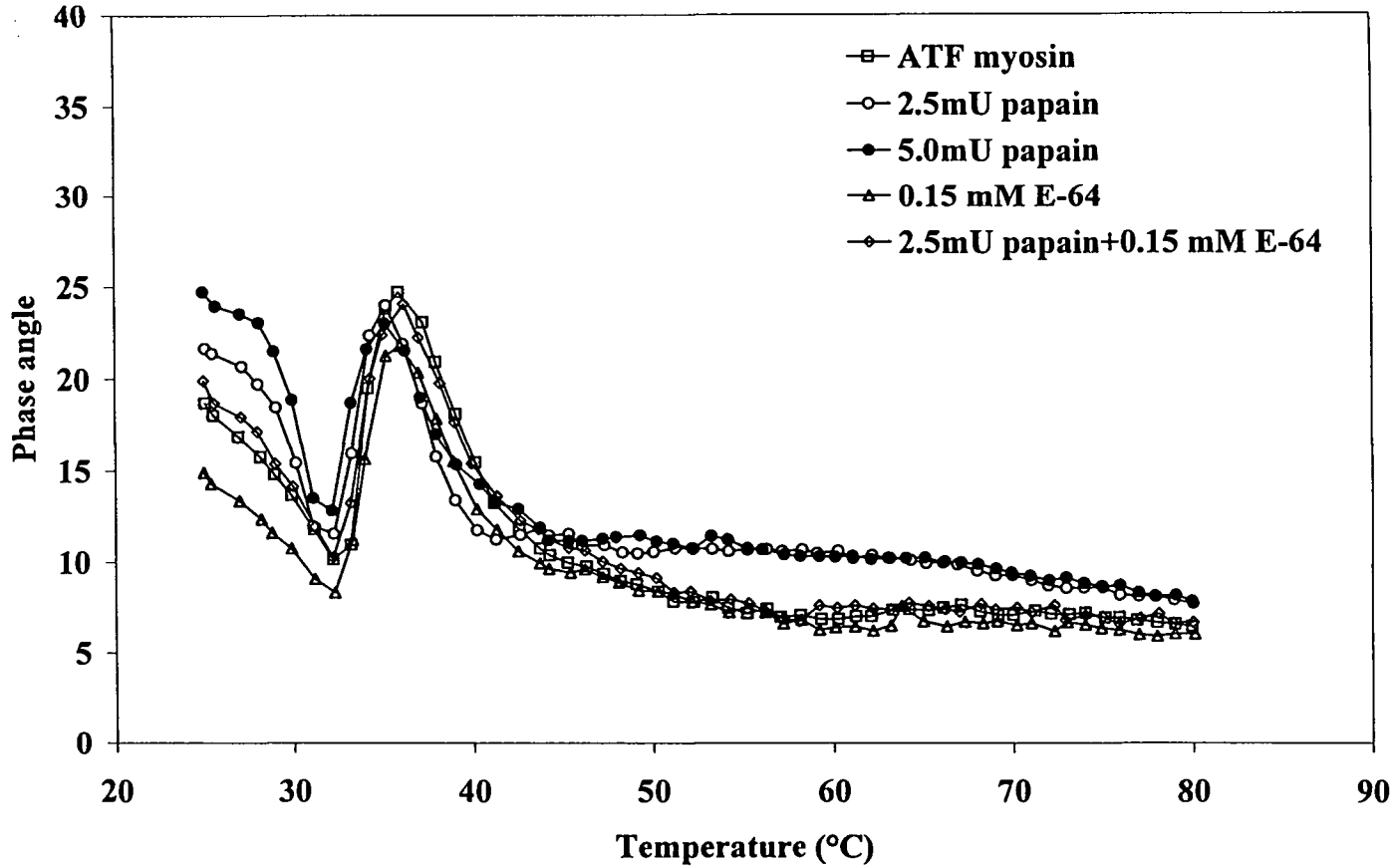

Fig. 4.6. Change in phase angle during thermal gelation of ATF myosin under various conditions.

respectively. For a completely elastic material, the phase angle is 0° and for a purely viscous fluid, the phase angle is 90° (Egelansdal et al., 1995). Therefore, myosin gels formed in the presence of papain were more viscous than myosin gels formed by itself and in the presence of E-64.

Effect of papain on frequency sweep curves

Frequency sweep curves show a linear relationship between log G' and the log of the frequency of myosin gels formed under various conditions (Fig. 4.7). Consistent with the results observed from thermal scanning experiments, myosin gel formed by itself showed the highest log G' in the frequency sweep scan, whereas myosin gel added with papain exhibited the lowest log G'. The G' was shown to be dependent on the amount of papain added, thus showing the lowest log G' with the highest concentration of papain added. Addition of E-64 resulted in an increase in log G' equivalent to the control ($P>0.05$). Generally, G' increased an the increase in frequency. For a totally elastic system, the G' values would be independent of frequency (Arntfield et al., 1989). Therefore, the slight dependence of frequency observed with the network reflected its viscoelastic nature. Since the protein gel network is generally developed at or above the denaturation temperature during the heating phase and continued during the cooling phase, frequency sweep of the gel can help evaluate the final gel properties, providing an overview of the rheological behavior as a function of frequency (Cai and Arntfield, 1997).

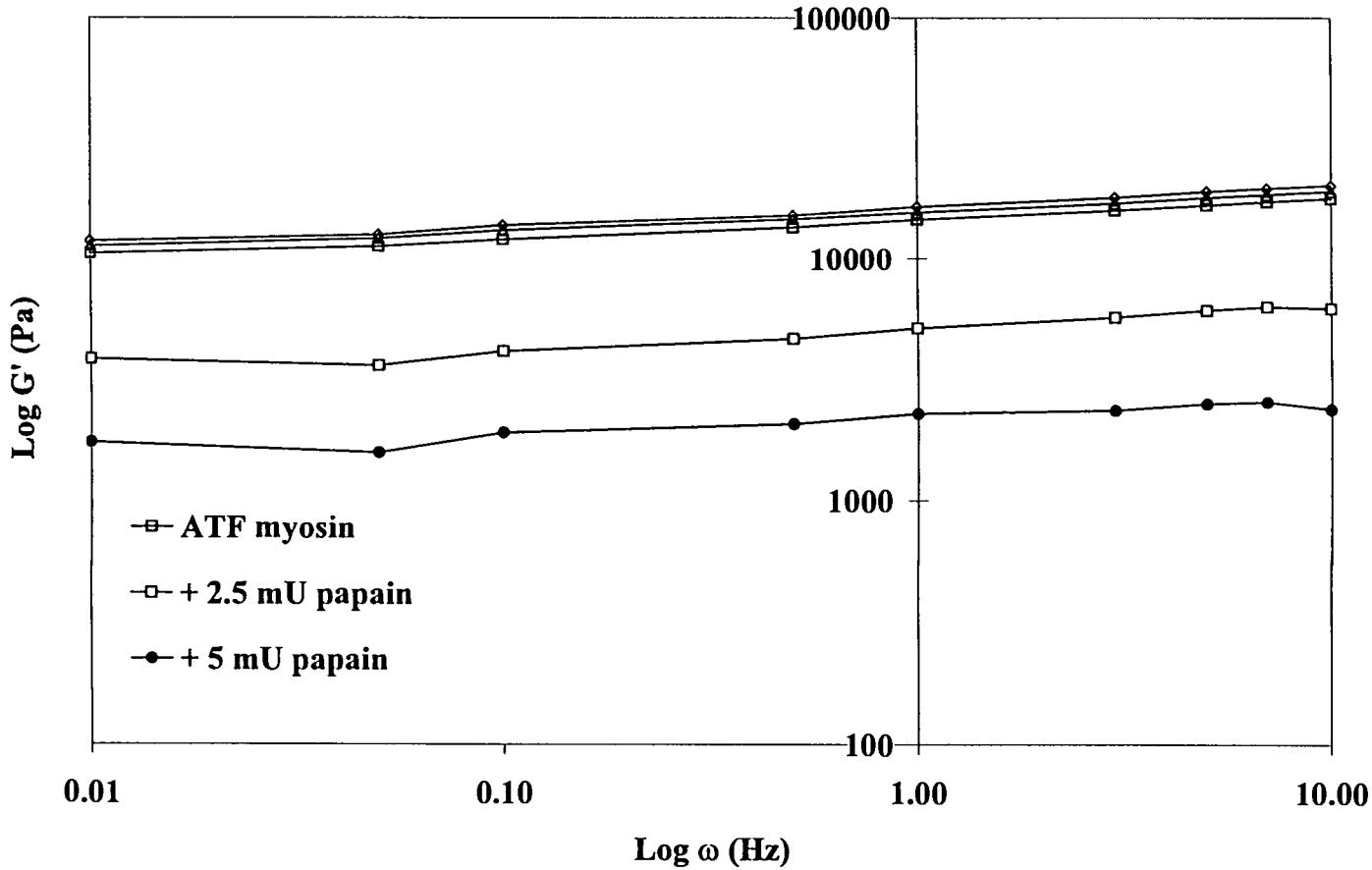

Fig. 4.7. Change in the storage modulus (G') as a function of oscillatory frequency (ω) of myosin gels formed under various conditions.

Electrophoretic study of myosin gels formed after and during dynamic testing

The electrophoretic analysis of myosin gels formed after dynamic testing is shown in Fig. 4.8. Myosin gel formed by itself showed the same pattern of protein bands as that of non-heated myosin suspension, indicating that no degradation of myosin occurred as a result of heat. However, extensive myosin degradation was observed in myosin gels formed in the presence of papain at both concentrations tested. Adding a cysteine proteinase inhibitor, E-64, reversed the effects of papain and protected myosin heavy chain (MHC) from degradation. The results indicated that proteolytic degradation of ATF myosin, particularly myosin heavy chain (MHC) was the main factor contributing to the decrease in the textural development of the gel.

To investigate the temperature range myosin was most susceptible at, myosin added with 2.5 mU papain was sampled during heating on a dynamic rheometer. The electrophoretic analysis of formed myosin gels is shown in Figure 4.9. Myosin suspension was linearly heated from 25°C to various temperatures in the range of 30-60°C under the same condition used in dynamic testing to determine the temperature range at which myosin was degraded. By heating up to 30°C, no significant loss of MHC band was observed. However, a substantial decrease in MHC band was observed when heated to 40°C or above. The results indicated myosin underwent substantial degradation between 30-40°C before being

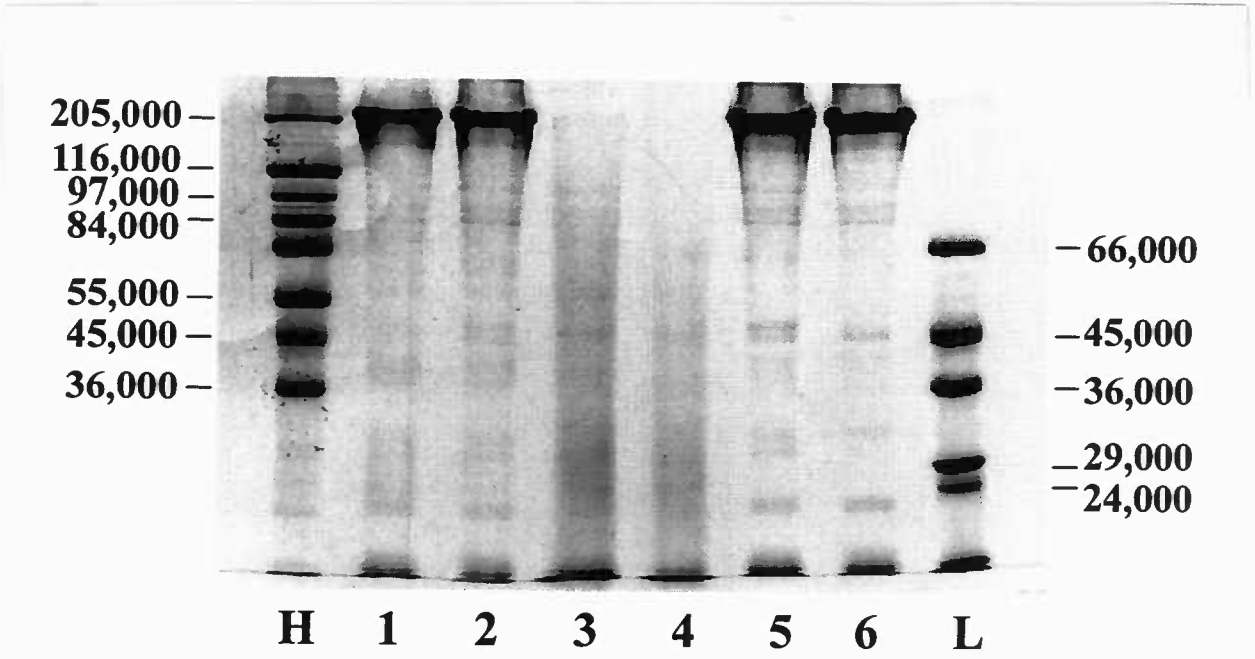

Fig. 4.8. SDS-PAGE pattern of ATF myosin gels formed under various conditions during frequency sweep scan. The gels were heated linearly to 80°C and cooled down to 25°C. H and L designated high and low molecular weight protein standards, respectively. 1: non-heated myosin suspension; 2: myosin (control); 3: myosin added with 2.5 mU papain; 4: myosin added with 5 mU papain; 5: myosin added with E-64 in the presence of 2.5 mU papain, and 6: myosin added only E-64.

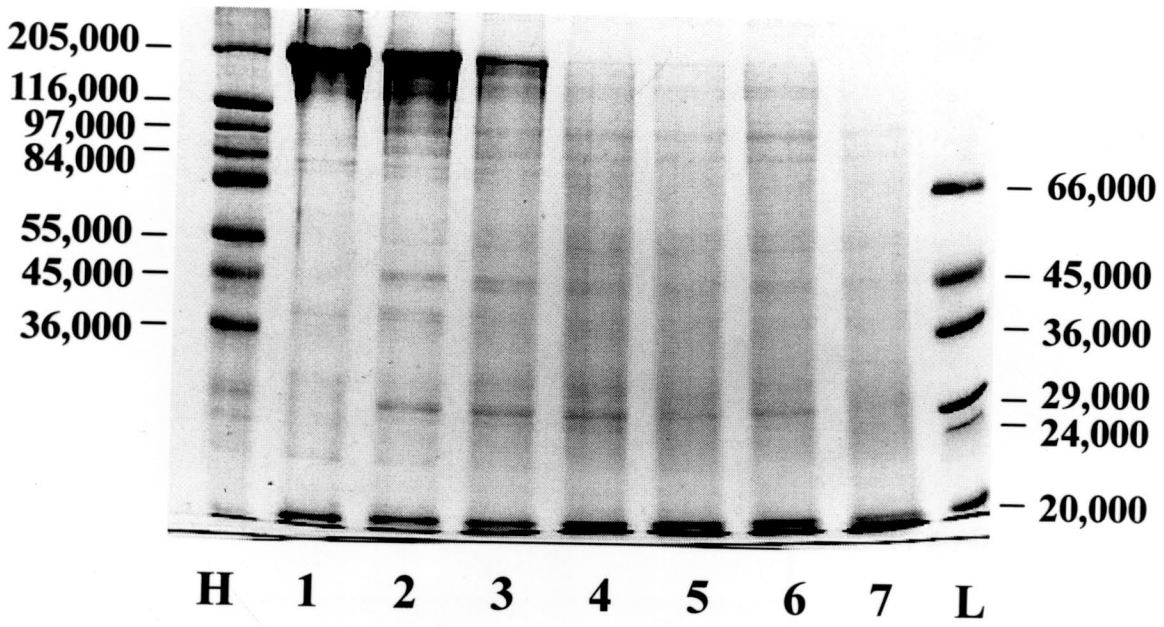

Fig. 4.9. SDS-PAGE pattern of ATF myosin added with 2.5 mU papain and linearly heated from 25°C to various temperatures 30, 40, 45, 50, 55, and 60°C (Lane 2 to 6). H and L designated high and low molecular weight protein standards, respectively. 1: non-heated myosin suspension.

totally degraded at higher temperatures. It is surprising, however, to see that even such severely degraded products were still actively involved in gel formation.

Effect of papain on thermal denaturation of myosin

Different patterns in endothermic peaks and troughs were observed with DSC thermograms of myosin and myosin added with papain (Fig. 4.10). Addition of papain significantly decreased the enthalpy required to induce myosin denaturation. Even though the onset (T_{onset}) and maximun transition temperatures (T_{max}) were shown not to be statistically different ($P>0.05$). The differences indicated changes in the thermal property of myosin in a way that made it more sensitive to heat since less energy was required for denaturation.

Kinetic studies were conducted based on the Arrhenius model to determine how proteolysis affects myosin denaturation. The kinetic parameters were calculated by the variable program rate method (Ozawa, 1970) based on the experimental fact that T_{max} is sensitive to heating rate (β) while the conversion at the reaction peak remains constant (Duswalt, 1974). A trend of upward shifts in T_{max} of myosin transition was observed as the heating rate increased (data not shown). Linear correlation of the plot between $-\ln(\beta/T^2_{max})$ versus $10^3/T_{max}$ was obtained with the coefficient >0.95 in which the slopes were found to be significantly different ($P<0.05$). The activation energy (Ea) and the pre-exponential factor of the Arrhenius equation (Z) for denaturation of myosin added with papain were

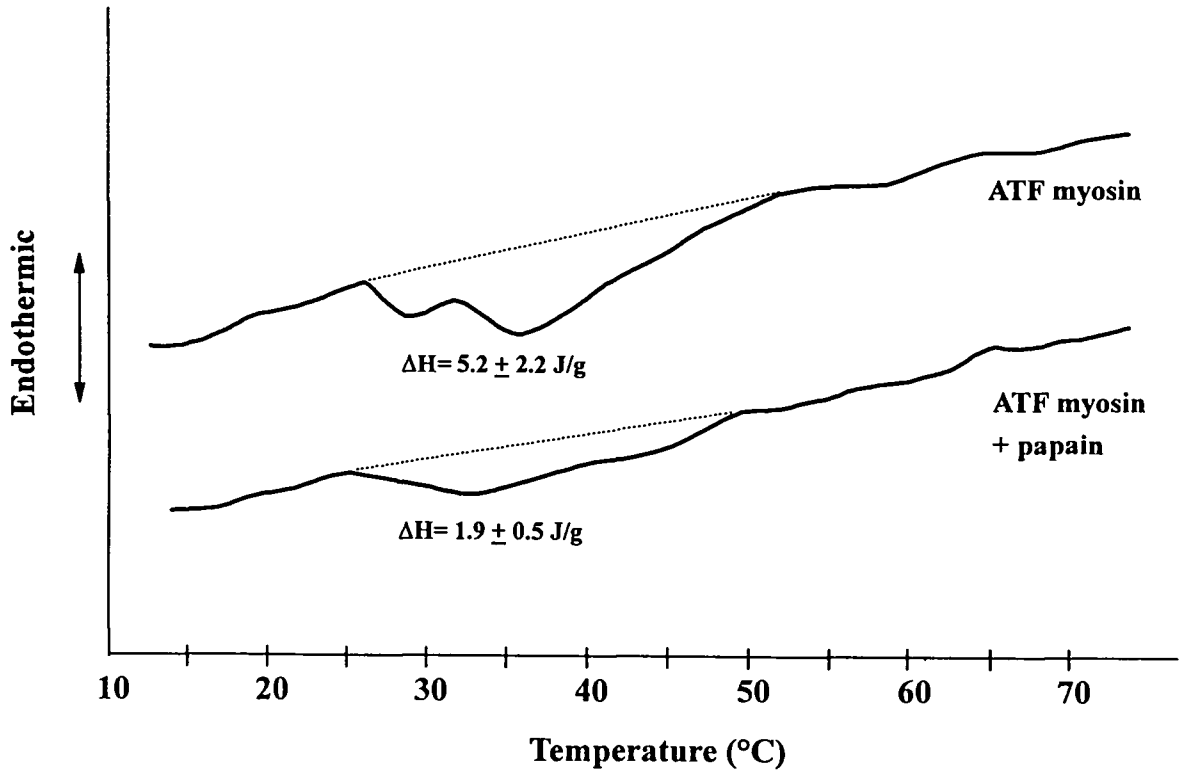

Fig. 4.10. Differential scanning calorimetric endotherm of ATF myosin and myosin added with 2.5 mU papain. Samples were prepared in 0.6 M KCl, 20 mM Sodium phosphate buffer, pH 7.0 and heated from 2 to 80 °C at a rate of 10°C/min.

significantly lower than those of myosin (Table 4.1). The results indicated myosin, in the presence of papain, has a tendency to undergo thermal denaturation more easily than myosin alone. However, with a higher rate constant (Kd) calculated at any selected temperatures, myosin was shown to denature more rapidly than myosin added with papain. Effects of temperature on the rates of myosin denaturation is shown in Fig. 4.11. With a rise in temperature, there was an increase in reaction rate. Q_{10} factor, defined as the ratio of rate for each 10°C rise in temperature, of myosin are higher than that of myosin addded with papain. The results indicated that papain decreased the thermal sensitivity of myosin to undergo the denaturation process. Even though the activation energy of the denaturation process was lowered by papain addition, myosin added with papain was found to denature at a slower rate than myosin alone.

Effect of papain on α-helical content of myosin

A rapid decrease in the initial α-helical content of ATF myosin was observed by the addition of papain prior to heating. Since the decrease was only observed after papain addition, it was mainly caused by papain rather than the thermal denaturation of myosin. By heating linearly from 9 to 65°C at 1.5°C/min, ATF myosin, both in the presence and absence of papain showed a decrease in helicity as temperatures increased (Fig. 4.12). The melting temperature (T_m), at which 50% of the initial helical structure is lost, of myosin added with papain was

Table 4.1. Denaturation kinetic constant of myosin heated in the presence and absence of papain

	Tmax (°C)	Ea (Kcal/mol)	Z (min^{-1})	Kd (min^{-1})			
				60°C	50°C	40°C	30°C
Control	36.3±1.2	12.0	1.76×10^{8}	2.17	1.24	6.79×10^{-1}	3.58×10^{-1}
+Papain	38.6± 5.3	10.6	1.19×10^{7}	1.44	8.78×10^{-1}	5.20×10^{-1}	2.97×10^{-1}

Tmax: Peak temperature at 10°C/min, Ea: Activation energy, Z: preexponential factor of Arrhenius equation, Kd: rate constant at selected temperatures. Values of Ea and Z were calculated from slope in (β/Tmax2) vs 10-3/ Tmax according to the dynamic method. Two or three samples of myosin were analyzed at 8 different rates of heating. Lines correlation coefficient were > 0.95. Slopes were significantly different ($P<0.05$).

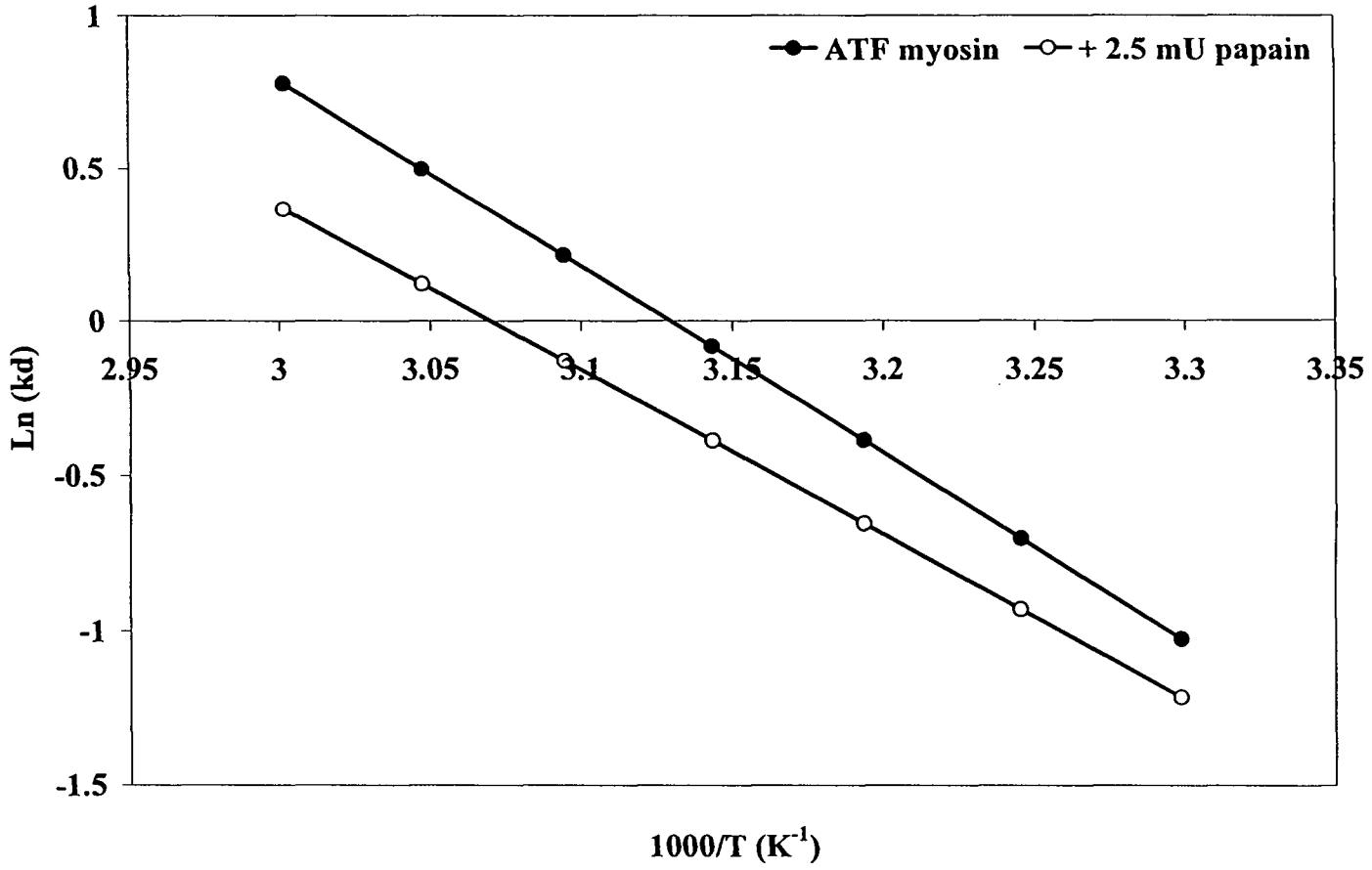

Fig. 4.11. Denaturation constants of myosin in the presence and absence of papain. Arrhenius plot (calculated from data presented in Table 1)

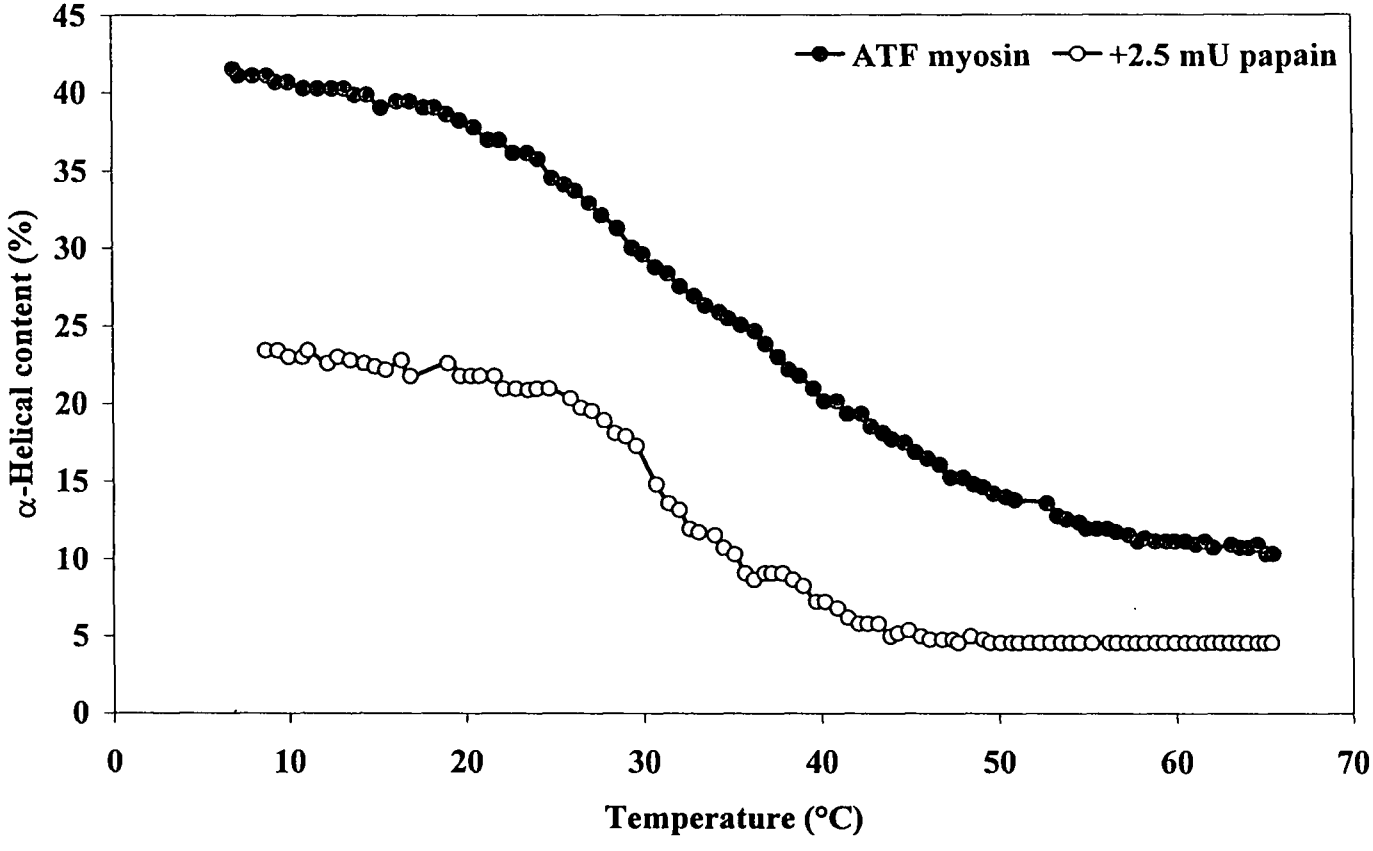

Fig. 4.12. α-Helical content as a function of temperature of ATF myosin and myosin added with 2.5 mU papain.

significantly lower than that of myosin alone (34°C). In addition, the first derivative plots also indicate that the α-helical content of myosin with papain added started to decrease at the same temperature (~10°C) as myosin alone but proceeded into fewer stages before completely unfolding at 65°C (Fig. 4.13).

Discussion

The myosin model used in this study indicated 3 major changes associated with proteolysis during heat-induced gelation. They included (1) an upward shift of onset temperature (T_{onset}) at which gel setting, gel weakening and gel strengthening were observed; (2) a significant decrease in the rate of gel development during gel strengthening; and (3) a significant decrease in rigidity of the formed gel. Heat-induced gelation of ATF myosin involves both denaturation and aggregation processes (Visessanguan et al., 1999). Since the onset temperature in dynamic test indicates the occurrence of a viscoelastic gel matrix, which normally occurs at or above the denaturation temperatures of protein, a temperature shift reflects the change in the thermal properties and stability of myosin during the process of denaturation. Furthermore, changes in the gel developing rate reflect the ability to form aggregates during the aggregation process, which eventually determines the properties of the final gels formed.

Thermal denaturation of myosin is as an endothermic process in which myosin changes its conformation to a more disordered structure without rupturing

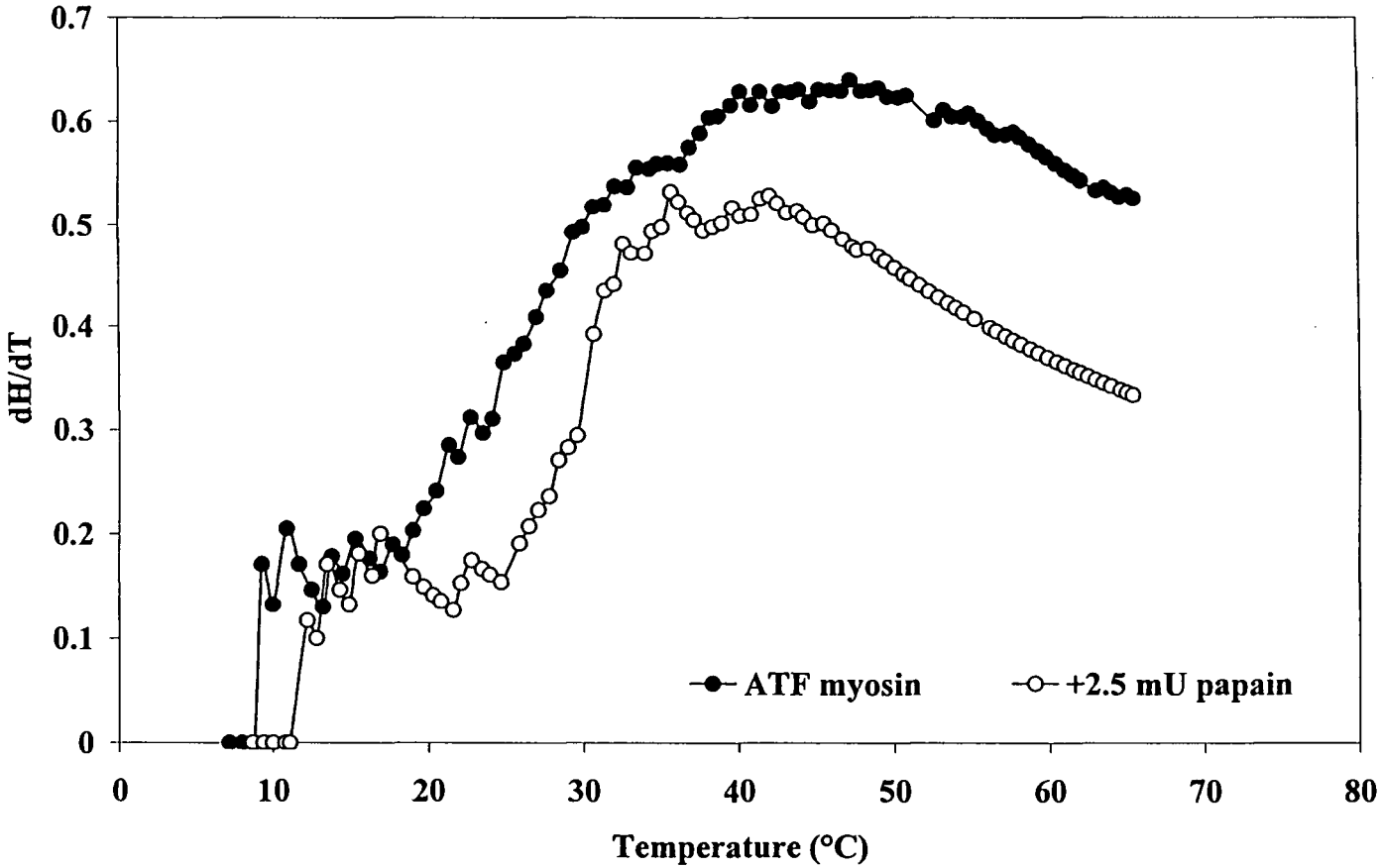

Fig. 4.13. First derivative plot of α-helical content as a function of temperature of ATF myosin and myosin added with 2.5 mU papain.

peptide bonds involved in the primary structure (Cheftel et al., 1985). However, in the presence of papain, denaturation of myosin was complicated by the rupture of the peptide bonds, producing degradation products that are heterogeneous in size and structure. Papain has a broad specificity of substrates, however, it has a marked preference for binding onto basic amino acid residues, such as, Arg and Lys (Cleveland et al., 1977) which are relatively rich in the rod portion of myosin. Under a condition for limited myosin was cleaved by papain into head and rod subfragments (Rattrie and Regenstein, 1977), which can be further hydrolyzed into smaller fragments or peptides unless enzymatic activity was stopped by adding a protease inhibitor.

Since degradation products are different in molecular composition and conformation, they are thought to be different in thermal properties as well. The differences in the shape of the heat flow curve and a significant decrease in enthalpy were observed as a result of papain addition. It is believed that the irregular shape of the thermogram was from composite of endothermic changes derived from each component of myosin that was partially degraded by papain and/or simultaneously denatured by heat. For intact myosin, each endothermic peak is derived from each domain structure which unfolds at a different temperature depending on its thermal stability (Ogawa et al., 1993). Fragmentation of myosin resulted in reduction of molecular size and the destruction of structural complexity, therefore a lesser amount of energy was required to unravel the residual molecular

structures. Unfolding of myosin has been shown to relate to the disruption of α-helical structure. As shown by CD studies, the significant decrease in enthalpy required for myosin denaturation in the presence of papain was probably due to an abrupt decrease in α-helical content. Since it occurred prior to heating, an abrupt decrease was thought to result from the partial hydrolysis of myosin molecules that probably unfolded a helical structure. Myosin, especially at a tail region, is highly susceptible for proteolysis (Privalov, 1982; Lopez-Lacomba et al. 1989).

Kinetic studies of myosin denaturation indicated papain probably facilitated the denaturation process by reducing the activation energy. However, the resulting myosin was less sensitive to heat and denatured at a slower rate than intact myosin. The intact structure of myosin allowed each structural domain unfolds step by step and reflected the cooperative nature of the transition from the native conformation to the least structured state. Thus, the results imply the importance of the intact structure of myosin in the denaturation process.

Since denaturation and aggregation are continuous processes, changes in denaturation characteristics may also affect subsequent aggregation. Denaturation of the least stable domain at low temperature enables myosin molecules to associate with one another via head to head interactions, possibly through disulfide bonds, so that interactions of uncoiled tail at a higher temperature can follow (Sharp and Offer, 1992). The decrease in rate of G' development implied that addition of papain also resulted in a decrease in the gelling ability of myosin. It was postulated

from the electrophoretic pattern of myosin gel formed in the presence of papain that the detrimental effects of proteolysis resulted from the degradation of MHC portions into smaller fragments. Even though the cross-links and deposition of small fragments may occur, the resulting gel structures are much weaker than those formed from intact myosin.

The gel property of myosin is highly correlated to the length of double stranded α-helical tail (Ishioroshi et al., 1982). Proteolysis resulted in the reduction in molecular size and a loss of structural domains, which are probably essential for molecular interaction and binding. The gel forming ability of myosin is associated with the surface hydrophobicity displayed by the unfolded domains of the MHC and the temperature at which such domains unfold. (Wicker et al. 1986). Unfolding of α-helical region in ATF myosin resulted in the exposure of hydrophobic and sulfhydryl groups which are subsequently involved in the formation of intermolecular bonding during the aggregation process (Visessanguan et al., 1999). Extent of aggregation of fish myosin seems to depend on the amount of hydrophobic surface exposed on the heated molecules (Chan et al., 1992; Wicker at al., 1986). An increase in surface hydrophobicity is found to be important in gelation and other functional properties of proteins. It is postulated that the cross-linking ability of the MHC may be localized at one or more discrete portions of the molecule (Wicker at al., 1986). The involvement of hydrophobic interaction is a prerequisite for the formation of large myosin aggregates and an elastic gel.

Consequently, anything that affects the surface hydrophobicity of myosin molecules may also affect the rheological properties of the muscle gel (Gill et al., 1992).

The lower gel modulus of myosin gels added with papain indicated the overall effect of proteolysis as a result of changes in myosin denaturation and aggregation. Similar to the detrimental effects on surimi gel strengths caused by endogenous muscle proteinases, this model system not only explains how proteolysis affects gelation but also emphasizes the importance of the structural integrity of myosin. The structural integrity of myosin facilitates the sequential unfolding and promotes the ordered formation of protein-protein interactions which is essential for the formation of highly elastic protein gels. Studies of the various fragments of myosin obtained by limited proteolysis have shown that gels prepared from myosin showed the highest rigidity over those prepared from rod, HMM, and S1 subfragments under identical pH, ionic conditions, and protein concentration (Ishioroshi et al., 1982). Maximal gel strength cannot be obtained from denatured myosin prior to the initiation of gelation (Niwa, 1992).

Conclusion

The model study indicates the effect of proteolysis on denaturation and gelation of myosin. Reduction in molecular size and structural integrity of myosin resulted in less sensitivity to heat denaturation and the ability to form a gel.

Although myosin gel could be formed, structural disruption caused by proteolysis resulted in gel weakening.

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Chapter 5

SUMMARY

Cathepsin L or likes were the most active proteinases that were primarily responsible for the autolysis of fish muscle at elevated temperatures. The proteinases exhibited the maximum activity at pH 5.5, 60°C and effectively hydrolyzed Z-Phe-Arg-NMec and all types of proteins substrates tested, i.e., casein, bovine serum albumin, hemoglobin, hide powder, and ATF myofibrils. The inhibition by E-64 indicated the proteinase belongs to the cysteine proteinase class. The proteinase in crude muscle extract was likely in a complex form with its natural inhibitor with an estimated M_r of 39,800, while the molecular weight of purified proteinase was estimated to be 27 kDa.

Heat-induced gelation of ATF myosin is a complex process comprised of several physicochemical changes. Denaturation was initiated at 15°C by unfolding of the α-helical region in myosin followed by the exposure of hydrophobic and sulfhydryl groups, which are subsequently involved in the formation of intermolecular bonding during the aggregation process. ATF myosin began to aggregate at 23°C prior to the completion of the denaturation process. Continuation of aggregation of myosin at higher temperature resulted in the development of a three-dimensional structure. Significant decrease in SH groups observed at 20-30°C indicated the formation of disulfide bonds during gel formation and implied the

role of myosin heads at the initial aggregation. In addition to disulfide linkage, hydrophobic interaction may also be involved in the initial aggregation of myosin.

The myosin model used in this study indicated the 3 major changes associated with proteolysis during heat-induced gelation. They included (1) an upward shift of the onset temperature (T_{onset}) at which gel setting, gel weakening and gel strengthening were observed; (2) a significant decrease in the rate of gel development during gel strengthening; and (3) a significant decrease in rigidity of the formed gel. Temperature shift reflects the change in thermal properties and stability of myosin during the process of denaturation. Kinetic studies of myosin denaturation indicated papain probably facilitated the denaturation process by reducing the activation energy. However, the resulting myosin was less sensitive to heat and denatured at a slower rate than the intact myosin. The decrease in rate of G' development implied that addition of papain resulted in the decrease in the gelling ability of myosin. Even though the cross-links and deposition of small fragments may occur, the resulting gel structures are much weaker than those formed from the intact myosin. Therefore, this model system not only explains how proteolysis affects gelation, but also emphasizes the importance of the structural integrity of myosin for the formation of highly elastic protein gels.

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APPENDIX

PURIFICATION OF CATHEPSIN L FROM ARROWTOOTH FLOUNDER MUSCLE

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Abstract

A protease exhibiting pH and temperature optima at 5.0-5.5 and 60°C was purified to electrophoretic homogeneity from muscle extract of arrowtooth flounder. The apparent molecular weight of the purified enzyme was determined to be 27 kDa by size exclusion chromatography and SDS-polyacrylamide gel electrophoresis. The protease had high activity and affinity toward Z-Phe-Arg-NMec with K_m and k_{cat} determined as 8.2 μM and 12.2 s^{-1}, respectively. The activity was inhibited by all sulhydryl reagents tested and activated by reducing agents. The characteristics of purified enzyme were consistent with those of cathepsin L from other species, suggesting cathepsin L as a major proteinase responsible for autolysis of fish flesh at elevated temperatures.

Introduction

Textural degradation of fish muscle during processing and cooking especially at elevated temperature is of the primary limitations for commercial utilization of Arrowtooth flounder, *Atheresthes stomias*. Arrowtooth flounder is found on the Pacific coast of the United States and Canada and is the most abundant species in the Gulf of Alaska along the Aleutian chain and the Bering Sea (Cullenberg, 1994). Similar to other underutilized fishery resources, this common problem is found to be due to the high proteinase activity in the muscle (Greene and Babbitt, 1990). Thermostable protease is of great concern for utilization of fish, in that it is stable at cooking temperatures and highly active at the postmortem/

physiological pH, resulting in damaged and/or softened fish textures and reduced gel strength in surimi.

The presence of a heatstable cysteine protease in arrowtooth flounder was first reported by Greene and Babbitt (1990) and later partially characterized by Wasson et al. (1992). The partially purified enzyme was shown to have an approximate molecular weight of 32,000 daltons and the optimal condition at pH 6.0-7.0 at 55°C against casein. Autolytic activity of arrowtooth flounder muscle analyzed at 60°C showed a major peak at pH 5.5 and was thought to be mediated by heat stable proteinases which are optimally active at slightly acidic pH. Based on its substrate specificity and some molecular properties or the partially purified fraction, cathepsin L or likes were presumed to be active proteinases in fish extract that primarily responsible for autolysis of arrowtooth muscle (Visessanguan et al., 1999).

Cathepsin L was found as a major proteinase to degrade the myofibrillar proteins in antemortem or postmortem muscle softening of chum salmon (Yamashita and Konagaya, 1990a), spotted mackerel (Lee et al., 1993), Pacific whiting (An et al., 1994) and Pacific hake (Masaki et al., 1993). Cathepsin L is a typical cysteine proteinase found in lysosomes and is considered to play an important role with cathepsins B and H in the degradation of both endogenous and exogenous protein taken up by lysozomes (See Aranishi et al., 1997). The majority of the basic enzymatic characteristics are common among the various samples of cathepsin L include the range of their molecular masses from 23,000 to 30,000,

consisting of two polypeptide chain linked by a disulfide bridge, a much higher endopeptidase activity than cathepsins B and H and a potent ability to act on the fluorescence substrate of Z-Phe-Arg-NMec and a variety of proteins specially collagens and elastin. The objective of this study was to isolate and characterize the endogenous proteinase responsible in muscle degradation of arrowtooth flounder.

Material and methods

Chemicals

N-carbobenzoxy-phenyalanine-arginine 7-amido-4-methylcoumarin (Z-Phe-Arg-NMec), N-carbobenzoxy-arginine-arginine 7-amido-4-methylcoumarin (Z-Arg-Arg-NMec), L-arginine 7-amido-4-methylcoumarin (L-Arg-NMec), 1-(L-trans-epoxysuccinyl-leucylamino)-4-guanidinobutane (E-64), pepstatin, leupeptin, phenanthrorine, ethylene diaminetetraacetic acid (EDTA), cytochrome C (horse heart), carbonic anhydrase, bovine serum albumin (BSA), hide powder azure, sweet potato-amylase, blue dextran, Tris/base, 2-mercapthoethanol (β-ME), p-chloromercuribenzoate, aprotinin, and dithiothreitol were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium caseinate was obtained from US Biochemical Corp. (Cleveland, OH). Iodoacetic acid and phenylmethylsulfonyl fluoride (PMSF) were purchased from Calbiochem Co. (La Jolla, CA).

Fish samples

Arrowtooth flounder were caught off the Alaska coast and filleted. The fillets were vacuum packed in polyethelene bags and frozen at –20°C before immediately shipped on dry ice to OSU Seafood Laboratory.

Purification of arrowtooth flounder proteinase

Arrowtooth flounder fillets were finely comminuted and centrifuged at 5,000 *xg* for 30 min to obtain sarcoplasmic fluid. The supernatant was then diluted with an equal volume of 20 mM Tris, pH 7.5, starting buffer (SB), and heated at 60°C for 3 min. After centrifugation at 10,000 RPM for 15 min, the supernatant was collected by filtering through cheese cloth. Prior to application of the sample to phenyl-Sepharose column (1.5x19 cm) at room temperature (~21°C), saturated ammonium sulfate (AS) was added to adjust the concentration to 1 M. After the column was washed with 1 M AS in SB until the absorbance at 280 nm (A_{280}) was less than 0.05, fractions (8 mL) with protease activity were eluted at 4°C with SB at a flow rate of 0.5 mL/min. Fractions were assayed for activity at pH 5.5 and for protein content. The fractions were pooled on the basis of activity and dialyzed against SB at 4°C for DEAE-Bio-Gel A chromatography. Samples were applied at 4°C to a DEAE-Bio-Gel A column (1.5x15 cm) previously equillibrated with SB. After washing overnight with SB, fractions were eluted with a linear gradient (300 mL) of 0-300 mM NaCl. The fractions (5 mL) were pooled and assayed for activity at pH 5.5 and protein content. Ammonium sulfate was added to 70% saturation to

precipitate proteins in pooled DEAE fraction. Precipitate was recovered by centrifugation and dissolved with a small volume of SB. Concentrated fractions was subjected to acidification by adding 2 volume of 0.05 M sodium citrate-0.05 M phosphate buffer pH 3.3 containing 1 mM EDTA and 1 mM β-ME. The mixture was incubated at 20°C for 10 min and concentrated by ultrafiltration using a Centricon 10 at 4°C for size exclusion chromatography. Size exclusion chromatography was carried out on a Superose 12 HR 10/30 column (Pharmacia LKB Biotechnology,Uppsala, Sweden) connected with a Bio-Rad HPLC pump (Model 2700, Bio-Rad Laboratories, Inc., Hercules, CA) and a UV detector (Bio-Rad Model 1706). Concentrated fraction, 100 μL, containing 5-10 mg protein were injected into the column. The proteins were eluted isocratically with 50 mM sodium phosphate buffer, pH 7.2 at a flow rate of 0.5 mL/min. Eluted proteins were monitored by absorbance at 280 nm and collected in 0.5 mL aliquots for further analyses. The proteinase activity of each fraction was determined at pH 5.5 using TCA-Azo.

Enzyme assay

Proteinase activity was assayed during the purification by the TCA-azo method (An et al., 1994) with McIlvaine's buffer, pH 5.5, containing 2 mM β-ME with azocasein as a substrate. All activity assays were carried out at 55°C. Blank was prepared in the same manner except that buffer was used., and activity was determined as the increase in A428 compared with that of blank.

Characterization of arrowtooth flounder proteinase

The proteinase separated on SEC-HPLC was estimated for its molecular weight by plotting relative elution volume (Ve/Vo) against the logarithm of M_r of the protein standards. The elution volume (Ve) was measured for each protein standard and the proteinase, and the void volume (Vo) was estimated by the elution volume of blue dextran (M_r 2,000,000). The standards used included horse heart cytochrome C (M_r 12,400), carbonic anhydrase (M_r 29,000), BSA (M_r 66,000), and sweet potato amylase (M_r 200,000).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970). PS fraction, DEAE fraction, and SEC-HPLC fraction about 20 μL were mixed at 1:1 (v/v) ratio with the SDS-PAGE sample buffer. The samples were loaded on the gel made of 4% stacking and 15% separating gels and subjected to electrophoresis at a constant voltage of 75 V using a Mini Protean II apparatus (BioRad Laboratories Inc., Richmond, CA). After electrophoresis, the gels were stained with 0.125% Coomassie brilliant blue R-250 in 25% ethanol and 10% acetic acid and destained with 25% ethanol and 10% acetic acid. Molecular weights of proteins were estimated using high and low molecular weight standards (Sigma Chemical Co., St. Louis, MO). Low molecular weight standards included bovine serum albumin (M_r 66,000), ovalbumin (M_r 45,000), glyceraldehyde-3-phosphate dehydrogenase (M_r 36,000), carbonic anhydrase (M_r 29,000), trypsinogen (M_r 24,000), trypsin inhibitor (M_r 20,000), α-lactalbumin (M_r 14,200), and aprotinin (M_r 6,500).

Substrate specificity

The hydrolytic activity on various synthetic substrates including Z-Phe-Arg-NMec, Z-Arg-Arg-NMec, and L-Arg-NMec were determined according to the method of Yamashita and Konagaya (1990a) with a slight modification. Fish extract, 20-25 μL, was diluted to 1000 μL with 0.1% Brij 35 and added with 500 μL of assay buffers (8 mM DTT McIlvaine's buffer, pH 5.5 or 8.0). To initiate the enzymatic reaction, 500 μL of 20 μM substrate solution was added to the mixture and then incubated at 55°C for 10 min. The reaction was stopped by adding 200 μL of 5 mM iodoacetic acid. The fluorescence intensity of aminomethylcoumarin was determined at excitation wavelength at 370 nm and emission wavelength at 460 nm using a luminescence spectrophotometer (LS 50B, Perkin-Elmer Ltd., Beaconsfield, England). A unit of activity was expressed as 1 nmole of methylcoumarin released/min.

Kinetic studies

Purified proteinase, 1.1 pmole as determined by E-64 titration, was diluted with 0.1% Brij to total volume of 1000 uL and incubated with 500 uL of the acetate buffer, pH 5.5 containing 8 mM DTT for 3 min. Then the activity was assayed with 500 μL of different final concentrations of Z-Phe-Arg-NMec ranging from 5 to 100 μM. Km and Vmax were determined. Values of kcat were calculated from the equation: Vmax/[E] = kcat, where [E] is the active enzyme concentration.

Active site titration

Active site concentration of the enzyme was determined by titration with E-64 by the method described by Barrett and Kirschke (1981). Fractions, 25 uL were preincubated with 25 uL of 6.25-500 nM E-64 and 500 uL of the assay buffer (acetate buffer pH 5.5, containing 8 mM DTT). The mixture was then analyzed for activity with Z-Phe-Arg-NMec for 10 min at 55°C as described under cathepsin L activity assay. Molarilty of the enzyme active sites was determined by linear plot of activity against E-64 molar concentrations, as described by Barrett and Kirschke (1981).

Effect of pH and temperature

Purified protease was assayed in buffer in the pH range 3.0-8.0, with 8 mM DTT at 55°C for 10 min. The temperature optima were determined by assaying purified protease for 10 min at various temperatures in buffer at pH 5.5. To determine heat stability, purified protease was incubated at 55°C in different time interval from 2-40 min and assayed for the residual activity at 55°C using Z-Phe-Arg-NMec as a substrate.

Inhibitor study

The purified proteinase was incubated with an equal volume of protease inhibitor stock solutions of E-64, sodium iodoacetate, leupeptin, sodium bisulfite, PMSF, soybean trypsin inhibitor, p-chloromercuribenzoate, aprotinin, pepstatin,

1,10-phenanthroline, dithiothreitol and EDTA at the concentrations listed in the text for 15 min at room temperature. The residual activity was analyzed by TCA-Lowry assay using casein as a substrate. Activity on casein was determined s tyrosine (Tyr) equivalents solubilized in TCA supernatants as measured by the Lowry assay (Lowry et al., 1951). One unit of activity was defined as that releasing 1nmol of Tyr/min.

Protein determination

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

Results and Discussion

Purification of arrowtooth flounder proteinases

The procedure employed for the purification of the most active endopeptidase at the elevated temperatures from arrowtooth flounder muscle was similar to the method used to purify cathepsin L from Pacific whiting (Seymour et al., 1994). Purification steps of proteinase are summarized in Table A.1. An increase in purification fold of 1.4 was obtained by heat treatment which was carried out to remove heat labile proteins and inactivate other proteolytic enzymes. Heat-treated fish extract was further purified by hydrophobic chromatography on phenyl-Sepharose which has been shown to be highly effective in concentrating cathepsin L activity. To avoid the difficulty in eluting due to the strong adsorption

Table A.1. Purification table of Arrowtooth flounder proteinase

Step	Total protein (mg)	Total activity* (U)	Specific activity (U/mg)	Purity (fold)	Yield (%)
Fish muscle extract	2,291	3,162	1.4	1	100
Heat treatment	1,479	2,869	1.9	1.4	64.6
Phenyl-Sepharose	234.6	2,081	8.9	6.4	10.2
DEAE-Sephadex	29.2	550.5	18.8	13.4	1.3
SEC-HPLC with acidification	2.9	224.5	76.9	54.9	0.1

Assay was done at 55°C for 10 min by using Z-Phe-Arg-NMec as a substrate.

of cathepsin L onto phenyl-Sepharose, the sample loading and washing were done at room temperature while sample elution was done at 4°C. A large amount of protein were removed with small loss in enzyme activity (Fig. A.1), leading to an increase in specific activity 6.4 folds. Hydrophobicity chromatography has proved effective in separating cathepsin B, H. and L from other proteins, but not for resolving the individual proteinases (Kirschke and Barrett, 1987; Mason et al., 1984).

A 2-fold purification was obtained by DEAE-Bio-Gel A (Fig. A.2). Ion exchange chromatography was used to remove contaminating cathepsin B activity during the purification of cathepsin L from rabbit liver (Mason et al., 1982). Ammonium sulfate precipitation and ultrafiltration through Microcon 10 (MWCO~ 10,000) were used to concentrate proteins in the pooled fraction for further acid treatment which was particularly important for the separation of enzymes from other contaminating proteins on size exclusion chromatography. Acidification was carried out to separate the enzymes from the enzyme-inhibitor complexes and Microcon 10 was used to separate low molecular weight protease inhibitor and concentrate the fraction for size exclusion chromatography. Working with an extract of the whole muscle encounters the difficulty that proteinases are partially complexed with endogenous inhibitors. This is especially a problem for cathepsin L, which bind very tightly to cystatins and alpha-cystein proeteinase inhibitors present in the tissue homogenate (Mason et al., 1985; Barrett et al., 1986; Kirschke and Barrett, 1987). Acid-treated fraction was separated on SEC-HPLC. Based on

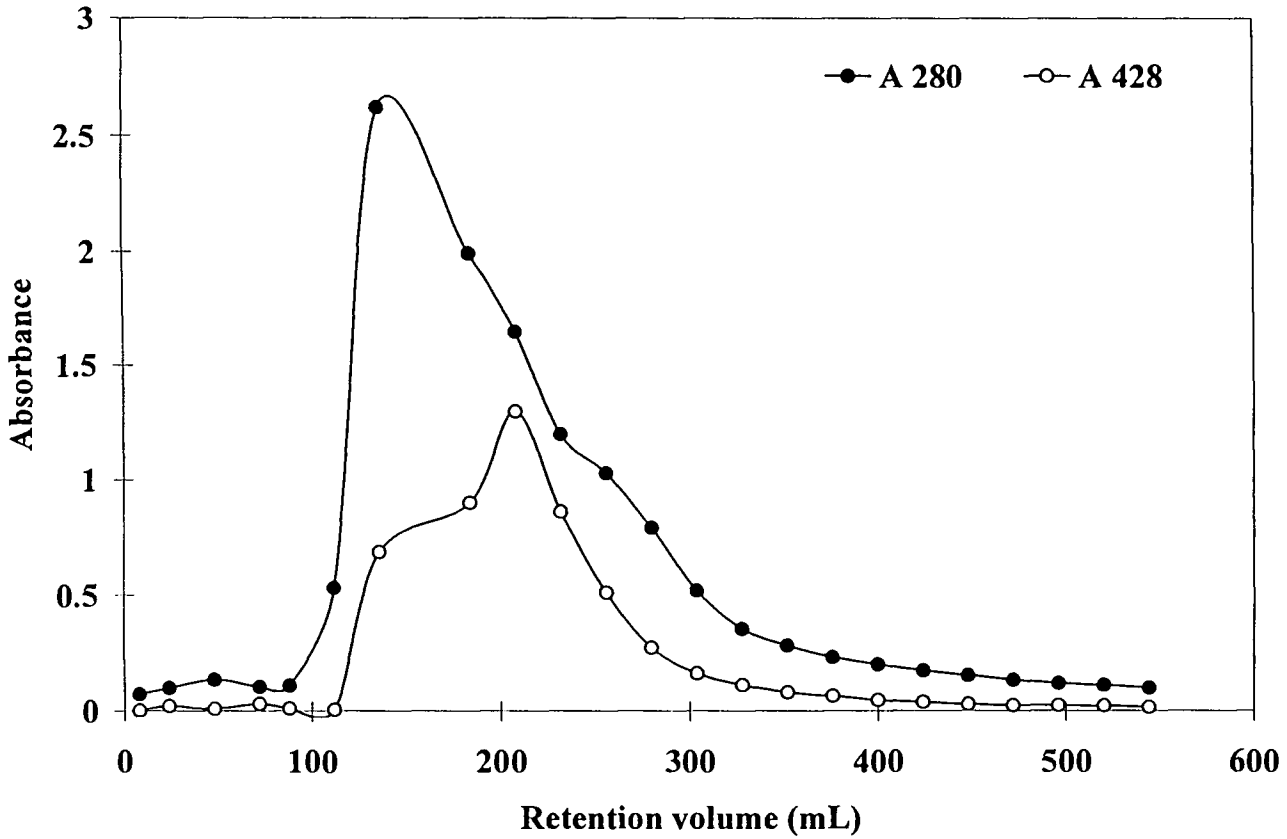

Fig. A.1. Elution profile of heat-treated arrowtooth flounder muscle extract on phenyl-Sepharose column. Enzyme solution was adjusted to 1 M AS in SB and applied to a phenyl-Sepharose column (x) at room temperature. After the column was washed with 1 M AS in SB, elution was carried out with SB. Fractions of 8 mL were collected at a flow rate 0.5 mL/ min. Activity as determined by TCA-azo assay is expressed as ΔA_{428}. Protein concentration was monitored by A_{280}.

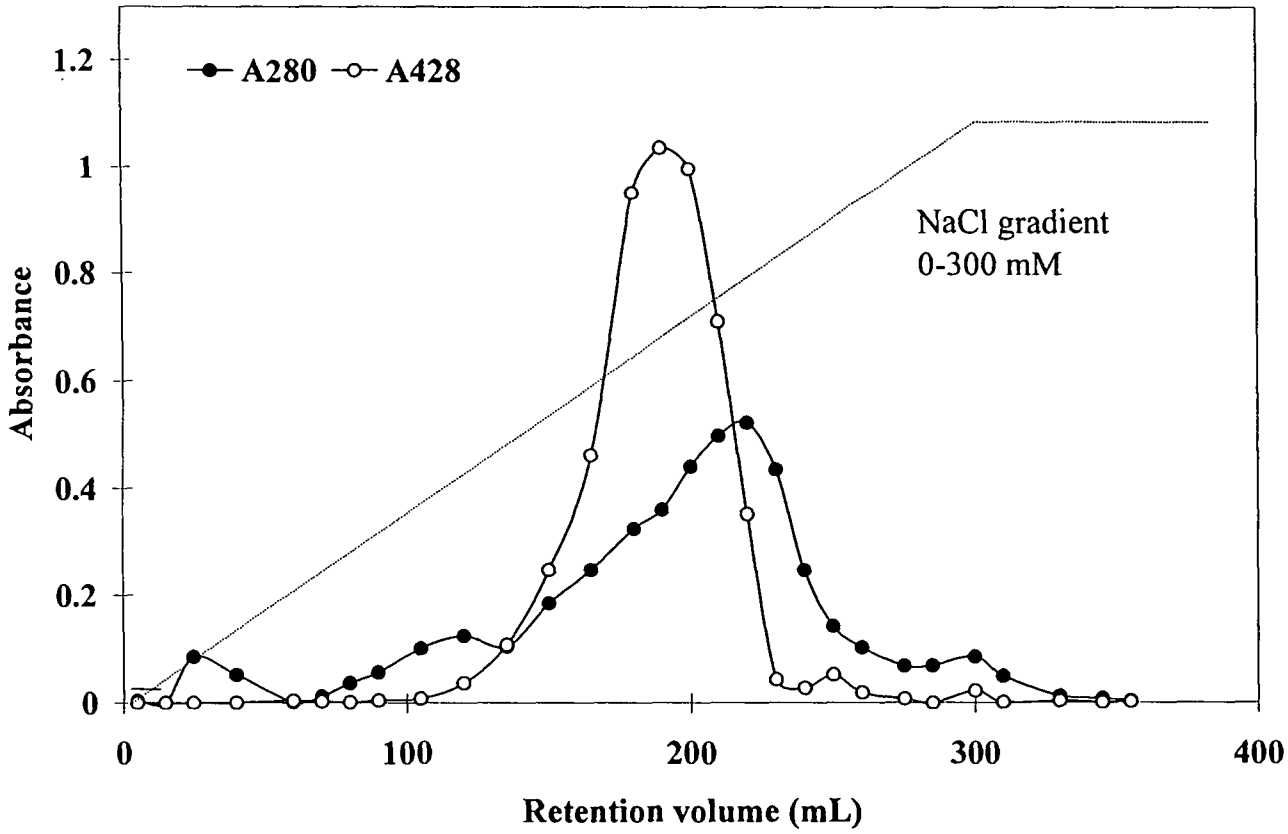

Fig. A.2. Elution profile of proteinase on DEAE-Bio-Gel A column. Pooled fractions from phenyl-Sepharose chromatography were dialyzed into SB and applied onto a DEAE-Bio-Gel A column (x) at 4°C. Fractions of 5 mL were eluted with a linear NaCl gradient from 0 to 300 mM. Activity as determined by TCA-azo assay is expressed as ΔA_{428}. Protein concentration was monitored by A_{280}.

molecular sieving, SEC-HPLC effectively fractionated cathepsin L from other high molecular weight proteins present in the pooled fraction. Only one proteolytic peak was observed with a peak eluted at the retention time of 23.9 min.(Fig. A.3). Analysis of fractions of this peak by SDS-PAGE revealed a single protein band (Fig. A.4).

Molecular properties the purified enzyme

A proteinase appeared to be a single polypeptide chain with the apparent molecular weight of 27 kDa by SDS-PAGE (Fig. A.4), corresponding to that estimated by size exclusion chromatography. Its molecular weight was slightly smaller than those of purified cathepsin L which have been estimated as 29 kDa for mammals (Kirschke and Barrett, 1987) and 30 kDa for salmon and mackerel muscles (Yamashita and Konagaya, 1990b; Lee et al., 1993). Cathepsin L generally consists of a heavy chain containing the active site and a low molecular weight light chain involving in stabilization of the active enzyme (Mason, 1986; Seymour et al., 1994). Since a light chain was removed by low pH treatment prior to size exclusion chromatography (Seymour et al., 1994), it was thought that a purified proteinase with M_r 27 kDa probably is a heavy chain of cathepsin L.

Compared to the estimated molecular weight of the active fraction (M_r 39,800) previously obtained by separating the muscle extract without acidification on the Superose 12 HR 10/30, the apparent decrease in the molecular weight of the purified proteinases by 12.8 kDa may be attributed to the presence of endogenous

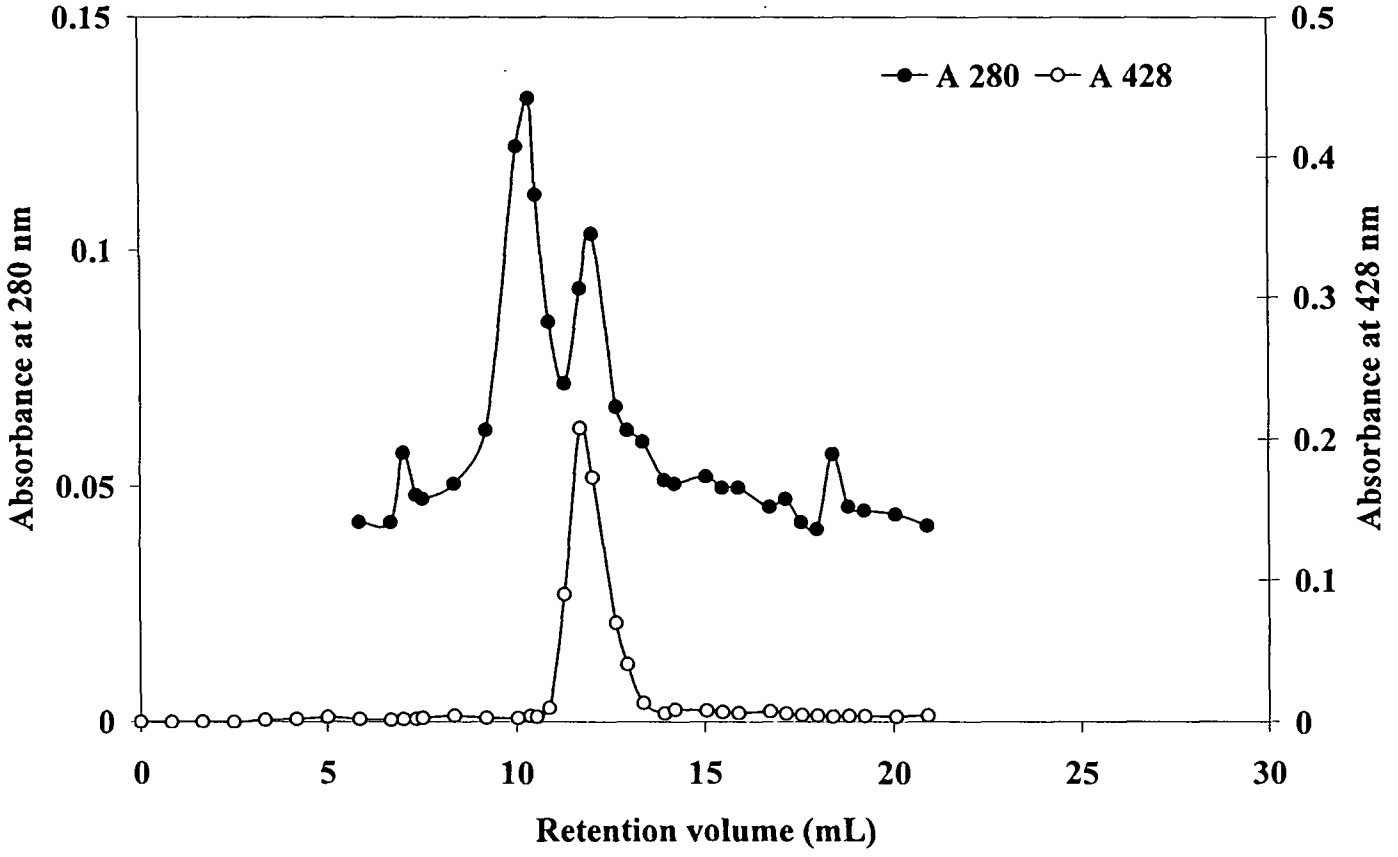

Fig. A.3. Superose 12 HPLC column elution profile of acid treated-DEAE pooled fraction. Samples were eluted with 50 mM sodium phosphate buffer, pH 7.2 at the flow rate of 0.5 mL/min. Activity as determined by TCA-azo assay is expressed as ΔA_{428}. Protein concentration was monitored by A_{280}.

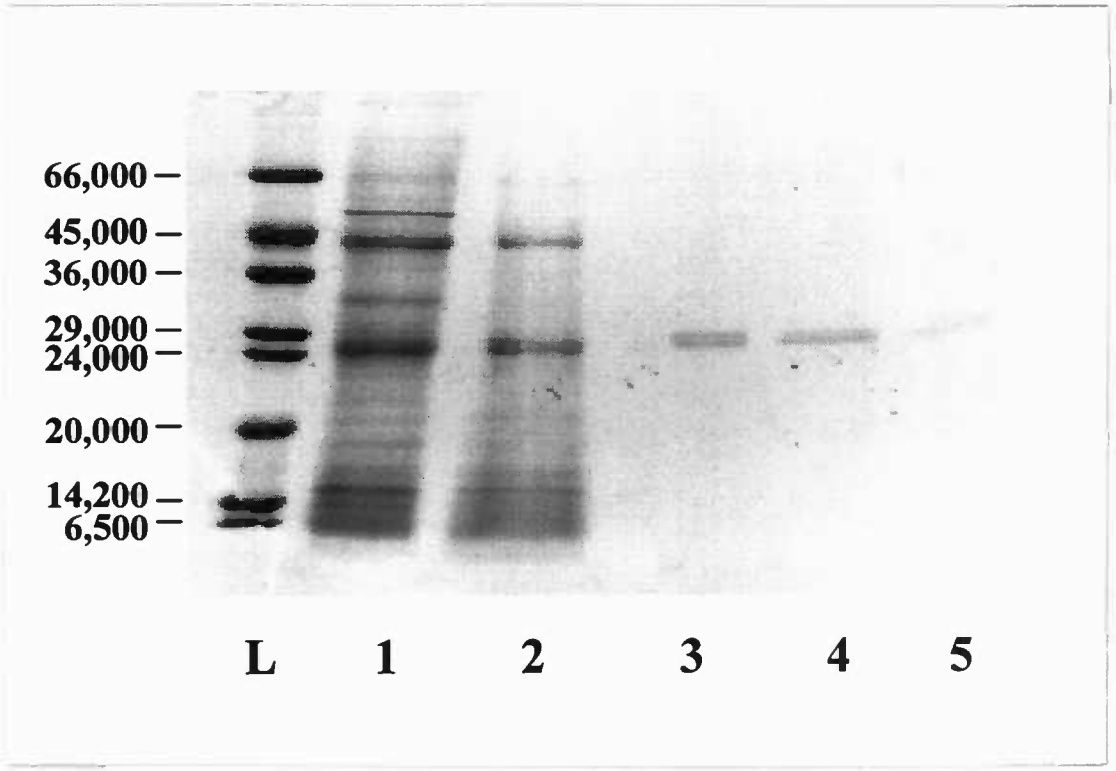

Fig. A.4. SDS-PAGE (15% T) of purified arrowtooth flounder proteinase. L, molecular weight standard; lane 1, PS fraction, 10 μg; lane 2, DEAE fraction, 10 μg; lane 3 to 5, SEC-HPLC fractions of activity peak from 3 different preparations, 1μg.

inhibitors. Cystatins and alpha-cysteine proteinase are often found to be complexed with proteinase (An et al., 1995; Yamashita and Konagaya, 1992). Yamashita and Konagaya (1992) reported the presence of a protease inhibitor in salmon of Mr 15,000 which copurified with cathepsin L.

Substrate specificity

Substrate specificity of the purified proteinase was examined using synthetic substrates specific for cathepsin B, H, and L. Among the substrates tested, the purified proteinase could hydrolyze only Z-Phe-Arg-NMec, a specific substrate commonly used to assay cathepsin L activity (Table A.2). Arginine is very efficient P1 residues for the hydrolysis of methylcoumarin substrates for cathepsins (Mason et al. 1984; 1985). Since the enzyme could not hydrolyze other tested substrates which has different amino acid residue at P2 position, hydrophobic amino acid at the position P2 is necessary for substrate specificity of arrowtooth flounder proteases. Cathepsin L shows a preference for rather bulky hydrophobic residues in the P2 position (Bohley and Seglan, 1992).

Kinetic studies

Kinetics of hydrolysis of Z-Phe-Arg-NMec were determined for ATF cathepsin L. The K_m and k_{cat} were determined as 8.2 μM and 12.2 (s^{-1}), respectively. Cathepsin L generally has high affinity for Z-Phe-Arg-NMec as shown by K_m and k_{cat} in the range of 1-5 μM and 8-30 s^{-1}, respectively (Mason,

Table A.2. Specificity and kinetic constants for the hydrolysis of peptide methylcoumarylamide substrates

Substrate	K_m (μM)	k_{cat}. (s^{-1})	k_{cat}/K_m ($mM^{-1}.s^{-1}$)
Z-Phe-Arg-NMec	8.2 ± 0.2	12.2 ± 1.8	1488
Z-Arg-Arg-NMec	ND	ND	ND
L-Arg-NMec	ND	ND	ND

Enzyme concentrations were determined by titration with E-64 and K_m and k_{cat} values determined at pH 5.5 and 30°C. Values represent means ± SD for two separate determinations per substrate concentrations. ND = Non-detectable

1986). ATF cathepsin L has slightly higher K_m than those from other species; however, it is lower than those of closely related enzymes such as cathepsins B and S which have relatively low affinity for Z-Phe-Arg-NMec (Brömme at al., 1989). K_m and k_{cat} values of cathepsin B of rabbit liver (Mason et al., 1989), human (Barrett and Kirschke, 1981), rat liver (Brömme at al., 1989), and salmon muscle (Yamashita and Konangaya, 1990b), have been found in the range of 75-223 μM and 238-1500 s^{-1}, respectively. Cathepsin S differs in catalytic properties in having lower catalytic efficiency and k_{cat}/K_m value against Z-Phe-Arg-NMec (Kirschke et al., 1984). The present study has shown that the purified enzyme has properties characteristic of cathepsin L.

Optimal pH

The effects of pH on the enzymic activity are shown in Fig. A.5. The optimum pH for hydrolysis of Z-Phe-Arg-NMec was found at at pH 5.0-5.5. The optimal pH value of the purified enzyme was similar to that of cathepsin L obtained from Pacific whiting (Seymour et al., 1994), salmon (Yamashita and Konagaya, 1990) and mackerel (Lee et al., 1993). The rapid decrease in enzymatic activity above the optimum pH values may indicate the susceptibility of the purified proteinase in an alkaline region.

Optimal temperature and its stability

Temperature also had a marked influence on the enzymatic activity. The maximum activity of the enzyme was observed at 60°C. (Fig.A.6). Arrowtooth flounder protease has slightly higher optimal temperatures compared to cathepsin L from other species which had optimal temperatures in the ranges of 45-50°C. However, the purified enzyme rapidly lost its activity after incubation at 55°C for 2.5 min (Fig. A.7). Dissociation from inhibitors makes enzyme more susceptible than in a complex. (Seymour et al., 1994). The heat dependent stability of cathepsin L purified from carp hepatopancreas gradually decreased at 40°C and largely lost the activity at temperature above 40°C (Aranishi et al., 1997). The optimal pH and temperature of the purified enzyme corresponded to those observed with crude extract and autolytic activity of arrowtooth flounder muscle (Visessanguan and An,

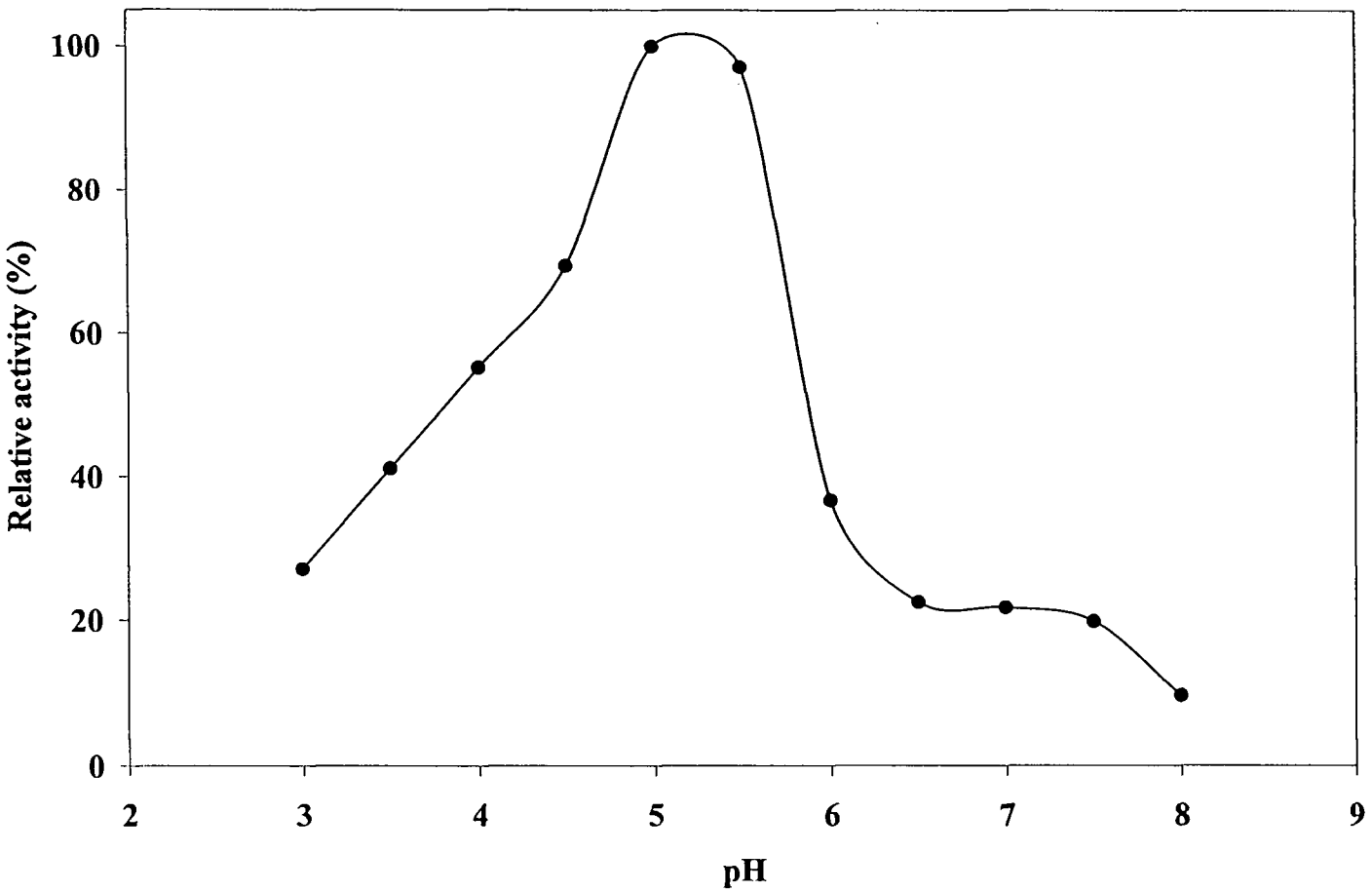

Fig. A.5. pH optima of purified arrowtooth flounder proteinase. Purified enzyme was assayed in the pH range 3.0-8.0 with 8 mM DTT at 55°C for 10 min. Activity was determined using Z-Phe-Arg-NMec as a substrate in duplicate at each pH.

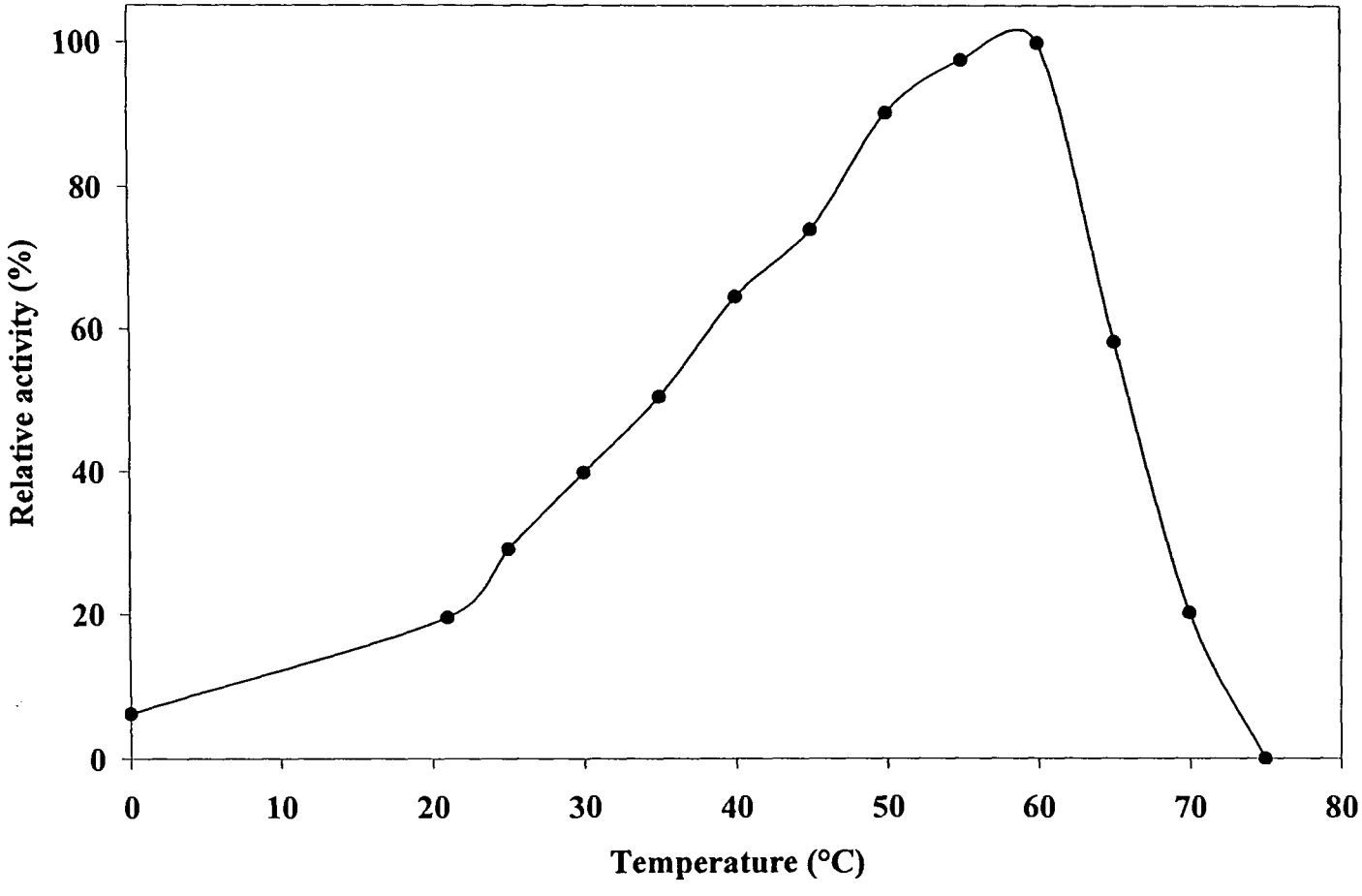

Fig. A.6. Temperature optima of purified arrowtooth flounder proteinase. Purified enzyme was assayed at 55°C for 10 min in acetate buffer pH 5.5 containing 8 mM DTT. Activity was determined using Z-Phe-Arg-NMec as a substrate in duplicate at each temperature.

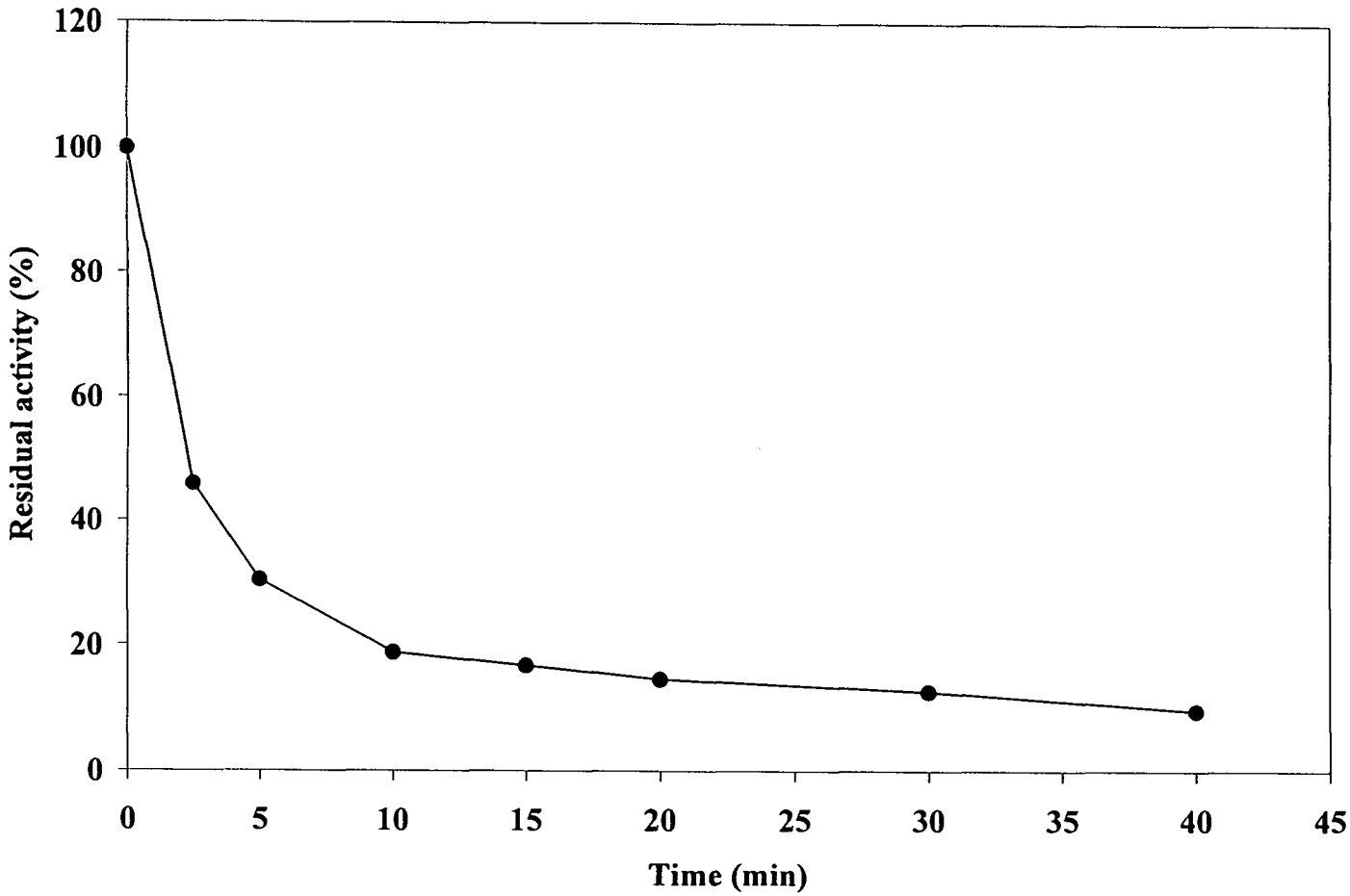

Fig. A.7. Residual activity of purified arrowtooth flounder proteinase after incubation at 55°C for various time. Activity was determined at 55°C for 10 min in acetate buffer pH 5.5 containing 8 mM DTT using Z-Phe-Arg-NMec as a substrate in duplicate at each temperature.

1999). Therefore, it evidently showed that cathepsin L is a predominant proteinase responsible for autolysis of arrowtooth flounder muscle.

Effect of inhibitors and activators

All four types of inhibitors, i.e., serine, cysteine, aspartic acid and metallo-protease, were tested with the purified proteinase to determine the class of active proteinase. Neither protease inhibitors known for serine nor metallo proteases showed a distinctive effect on the activity of the purified enzymes. The partial or complete inhibitions shown by thiol-blocking agents, e.g., HgCl, E-64 and iodoacetate indicate that the purified proteinase is a thiol proteinase (Table A.3). Enzymatic activity was enhanced by the thiol-activating agents, such as, DTT and EDTA which were used for specific activation of cysteine proteinase actvity.

Conclusion

A protease with a maximum activity at 60°C and pH 5.5, which has been implicated in texture degradation of arrowtooth flounder muscle, was purified and shown to be cathepsin L on the bases of molecular weight and enzymatic properties essentially consistent with those of cathepsin L from other species.

Table A.3. Effect of inhibitors and activators on purified ATF muscle proteinase

Inhibitors	Concentration	% Relative activity
Control	-	100
E-64	0.1 mM	0
Iodoacetate	1 mM	0
Sodium bisulfite	10 mM	25.4
PMSF	1 mM	82.3
1,10-phenanthroline	1 mM	59.1
Soybean trypsin inhibitor	0.1 g/L	88.0
PCMB	0.1 mM	67.2
$HgCl_2$	0.1 mM	62.8
Pepstatin A	1 mg/L	56.9
Carbon disulfide	10 mM	62.5
DTT	2 mM	314
EDTA	2 mM	196
DTT+EDTA	2 mM each	299

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