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**Seasonal Variation and Species-specificity of  
Several Chinese Freshwater Fish Myosin**

A dissertation

Presented to the Graduate School of Fisheries Sciences  
Hokkaido University

In Partial Fulfillment of  
the Requirements for the Degree of Doctor of Philosophy (Fisheries Science)

By

**Chunhong YUAN**

January 2006

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## List of Abbreviations

BTB: bromothymolblue

CD: circular dichroism

DTNB: 5, 5'-dithio bis (2-nitrobenzoate)

EDTA: ethylenediaminetetraacetic acid

HC: heavy chain component of myosin, S-1 and HMM

HMM: heavy meromyosin

kDa: kilodalton

LC: light chain component of myosin, S-1 and HMM

LMM; light meromyosin

PAGE: polyacrylamide gel electrophoresis

PMSF: phenylmethylsulfonyl fluoride

Rod: myosin rod

S-1: myosin subfragment-1

S-2: myosin subfragment-2

SDS: sodium dodecyl sulfate

Tris: Tris (hydroxymethyl) aminomethane

# General Introduction

## 1. Overview of China Fishery Industry

The Chinese fisheries production has been rapidly progressing in the last two decades. The production volume of aquatic products of China has ranked the first one among the world fisheries countries since 1990. It has reached 49.01million metric ton in 2004. Of the total production, approximately 38% is produced by inland aquaculture and 27% by marine aquaculture (CFYEC 2005). In volume terms, more than 30% of world seafood is produced and more than 70% of world aquaculture production is generated in China (FAO 2005).

Inland freshwater aquaculture is a major part of Chinese fishery industry. It is estimated that the harvesting of Chinese domestic aquatic product will keep on growing up, but most of the increments will come from fresh water aquaculture and a few marine aquaculture (CFYEC 2005). Fresh water aquaculture takes place in ponds, lakes, rivers, reservoirs and rice paddy fields, wide spread in almost whole China. In 2004, the total freshwater culture production was 18.92million metric ton, of which 17.21 million metric ton of freshwater fish takes 91%. The most common farmed species are grass carp, silver and bighead carp, common carp and crucian carp, which accounted for over 80% of the total freshwater fish production. Despite the fact that freshwater fish resources in China have expanded rapidly, their distribution sphere and storage period are very limited since most freshwater fish are transported as live fish without treatment, such as freezing and processing. Compared with marine resources, freshwater fisheries resources lack the diversity of consumption because of the backward transportation and processing technologies. A big gap existed between China and some developed countries in the world in the fish processing industry, particularly in fishery products finely processed. Only 30% of landed fish are processed compared to 70% in developed countries such as Japan (JFS 2005). Freshwater fish are almost always sold alive. Although the sector of freshwater fish processing has gained a great development, the volume of the processed aquatic product from freshwater fish only occupies 10% of the total according to

the statistics in 2004, even less with fine processing. As an animal protein resource, freshwater fish has not been fully utilized, compared with marine fish (Fukuda et al. 2001).

Due to backward post-harvesting technologies compared with fast increment of the production of freshwater fish, overproduction is the problem in harvesting season in some fish farming regions. The market value of freshwater fish drops because of the excess of supply over demand, which definitely would hinder a further development of fish farming. Furthermore, implementation of "Summer Fishing Moratorium" policy was started since 1996. The core idea of the policy is that environmental friendly and sustainable fishery development is crucially important. Under these situations, the development of processing industry is essential to reduce losses through poor handling of fish, to utilize over catch fish, and to raise the value of fish products. In addition, improving fish quality, flavor, healthy and safe fish products are another goals for Chinese government.

## **2. Development of Frozen Surimi from Freshwater Fish**

"Surimi", a raw material for thermal gel production, is an innovative material for Kamaboko and fish sausages. Industrialized surimi-making process was developed by Nishiya et al. (1960) of Hokkaido Fisheries Experiment Institute, who discovered the function of cryoprotectants in preserving protein of Alaska pollack functionality during frozen storage. Frozen surimi can be thought as stabilized myofibrillar proteins obtained from mechanically deboned fish flesh that is washed with water and blended with cryoprotectants to keep long frozen shelf-life. In the 1970s', surimi was developed as an intermediate foodstuff with high potential for free productivity of texturized products. It allows a manufacturer to imitate the texture and taste of a higher quality product, such as lobster tail, crab, abalone, shrimp and scallop using a relatively low cost material. Furthermore, surimi is also seen as an inexpensive source of protein.

The surimi industry mainly utilizes Alaska pollock because of its large fishery biomass, which covers 50-70 % of total surimi, but its proportion has been continuously reducing. Since 1991, efforts to use

other species have also been successful, through technical and marketing advances in Japan. Currently, a number of different species are being utilized in commercial surimi production. The most suitable species for surimi processing are those with white flesh and low fat content, including threadfin bream, whiting, hoki, and Mackerel (Guenneuguen and Morrissey 2005). In China, surimi industry has increased very quickly since 2001. The production of surimi and surimi based foods in 2004 was 329,808 metric ton compared to 8,350 metric ton in 2001. The material for surimi is almost from marine fish. In fact, most of freshwater fish are white meat with low fat (less than 5%). It is reasonable to think that freshwater fish might be a potential material for surimi production due to the large biomass, low market price and its characteristic with white flesh and low fat content. Some trials to develop frozen surimi from freshwater fish were carried out (Yamamoto et al. 1988; Tu et al. 1989; Fukuda et al. 2001). However, it seems very difficult to make the high utilization of the potential freshwater fish flesh in various ways including surimi processing without the information on the functional and biochemical properties of freshwater fish muscle proteins. Therefore, it is necessary to understand these properties of muscle proteins, especially for some major species of freshwater fish.

### **3. Functional Properties of Muscle Proteins**

Fish meat is an abundant protein resource that is widely used in food products. Seafood proteins, like those of all other muscle foods, may be classified as sarcoplasmic, myofibrillar and stroma-type. The functional properties of muscle proteins have a great influence on the quality of processed products. The major functional properties of the muscle proteins are solubility, water retention, gelling ability, and lipid emulsifying properties. Major myofibrillar proteins are myosin, actin, troponin, and tropomyosin, which account for 40-60 % of the total crude protein content of fish. Fish myofibrillar protein has excellent functional characteristics such as emulsifying properties, gel-forming ability, and water-holding capacity (Tanabe and Saeki 2001). Many kinds of seafood products, ready-to-fry fish portions, surimi-based products, and fish sausages and cakes take advantage of the functional properties of fish myofibrillar

protein.

Myosin comprises 50 to 60 % of the total myofibrillar proteins. It is confirmed that myosin plays the most important role in the gel formation, especially myosin rod portion (Samejima et al. 1981), while other muscle proteins seems not contribute, and possibly even inhibit the gel formation (Akahane et al. 1984; Konno 2003). As the heat-induced gelation results mainly from heat-polymerization of myosin, the cross-linking and aggregation of protein molecules into three-dimensional solid-like networks results in elastic gel with high water holding capacity (Sano et al. 1988).

One of the characteristic properties of fish muscle protein, especially myosin, is its unstable nature (Connell 1961). Its functional properties decreases easily upon protein denaturation, which is commonly defined as any non-covalent changes in the structure of a protein accompanying unfolding of helical structure without cleaving polypeptide chain (Lehninger 1982). Many efforts were devoted to elucidate the myosin denaturation process during the storage and processing of fish meat, and to develop effective additives to prevent myosin denaturation for long-term storage of fish meat (Konno 2005).

#### **4. Myosin Structure and Stability**

Myosin is a multifunctional hexamer consisting of two intertwined 220 kDa heavy chains (MHCs) and two pairs of different light chains (LCs), ranging from 17 to 22 kDa (Lowey and Risby 1971). Roughly half of MHCs at N-termini forms twin water-soluble globular heads (subfragment-1, S-1), while remaining C-termini portion makes up salt soluble coiled-coil  $\alpha$ -helical rod (Harrington and Rodgers 1984). The former, head portion of myosin molecule contains ATPase and F-actin binding sites, and the latter, tail region, assembles to form thick filaments under physiological conditions of low salt (Warrick and Spudich, 1987). A pair of light chains (LCs) non-covalently cross-links to C-terminal regions of S-1. There are two major cleavage sites by limited proteolysis with chymotrypsin under specified conditions. In high-salt medium, dissolved myosin was cleaved at the center of myosin rod into light meromyosin (LMM) and heavy meromyosin (HMM). In the presence of EDTA, the head/tail junction is selectively cleaved

producing myosin S-1 and rod (Weeds and Pope 1977).

It is generally accepted that fish myosins are much more unstable than mammalian counterparts (Connell 1961). This is mainly characterized by a quick loss of adenosine 5'-triphosphatase (ATPase) activity and aggregation. Furthermore, the thermal stability of the ATPase activity is related to the environmental temperature in which the species live (Johnston et al. 1973; Hashimoto et al. 1982). It has been reported that common carp and grass carp, inhabiting a wide range of environmental temperatures, express three types of myosin heavy chain isoforms with different thermal stabilities in relation to the adaptation to the seasonal change of environmental temperatures (Watabe et al. 1998; Tao et al. 2004). Thermal stability of fish myosin rod has also been studied. Rodgers et al. (1987) reported that myosin rod purified from Antarctic fish was less heat-stable than rabbit myosin rod. Other reports also demonstrated that carp myosin rod was much more unstable than rabbit one (Kato and Konno 1993b; Nakaya et al. 1995). Konno et al. (2000) also showed the denaturation of rod portion when heated as myofibrils by using chymotryptic digestion technique. As myosin rod or LMM is nearly 100 %  $\alpha$ -helical structure, circular dichroism spectroscopy technique was employed to show the unstable nature of tail portion by measuring the unfolding process of helix structure (King and Lehrer 1989). All results with myosin, S1, rod and LMM showed that the thermal stability of any regions of fish myosin is habitat temperature dependent. Unstable structure of fish myosin is rather favorable to survive at relatively low temperature, at which structural flexibility of myosin molecule is achieved by reducing the non-covalent bonds to form the structure (Watabe 2002). Protein stability is basically determined by a primary structure, namely, its amino acid sequence. At present, amino acid sequences of the myosin HC of the following species of fish by cloning walleye pollack (Ojima et al. 1998; Togashi et al. 2000), carp multiple isoforms (Kikuchi et al. 1999), white croaker fast skeletal muscle (Yoon et al. 2000) are determined. However, a complete explanation of the species-specific thermal stability of myosin by studying the primary structure is still hard.

## 5. Research Objectives

Species-specific thermal stability of fish myosin is important in two fields. The first interest comes from the concept, fish meat as food. Understanding unstable nature of fish myosin gave important information in the preservation, storage and processing of fish food. Another interest comes from the viewpoint of comparative biochemistry including the interest on the adaptation to the environment.

The objective of the present research is to characterize the myosin from various species of freshwater fish. There is little report on the structure properties of Chinese freshwater fish myosin at present. These are fundamental studies for the processing of fish meat of these species. The study was also carried out by considering the change in the properties of myosin due to the expressing of myosin isomers in different seasons. Thermal gelation of freshwater fish meat was one of the goals planned. Silver carp, was mainly used in the study because the species had a potential to be used as a raw material for processing due to its low prices and its large harvests. The study was constructed to establish the most favorable condition for thermal gel formation of silver carp meat. The gelling properties of meat from other species of fish were also compared. In all cases, the concepts include what is the species-specificity, and what is the consequence of adaptation to water temperature changing seasonally.

The summarized and specific objectives are as follows:

To understand seasonal variation of gelling properties of freshwater surimi from silver carp.

To investigate the seasonal change in the thermal stability of silver carp myosin.

To characterize the species-specific thermal stability among seven species of freshwater fish in summer and winter.

To understand the expression of myosin isomers in different seasons.

To understand the adaptation strategy for individual species of fish.



# Materials and Methods

## 1. Fish Species

Fish species used in the present study were listed as follows:

Five species belonging to carp (*Cyprinidae*) family

- 1) Silver carp (*Hypophthalmichthys molitrix*)
- 2) Bighead carp (*Aristichthys nobilis*)
- 3) Grass carp (*Ctenopharyogodon idella*)
- 4) Common carp (*Cyprinus carpio*)
- 5) Blunt snout bream (*Megalobrama amblycephala*)

Two species belonging to Perciformes order were also compared:

- 6) Largemouth bass (*Micropterus salmoides*) in sunfishes (*Centrarchidae*) family
- 7) Chinese Snakehead (*Ophiocephalus argus Cantor*) in snakeheads (*Channidae*) family

Photographs of the fish used are presented in Fig. 1.

## 2. Sampling of Muscle from Various Fish Species

The information on silver carp used for surimi production was shown in Table 1. The chemical composition of the surimi produced was also indicated in the Table. Live silver carp were purchased at local markets in Shanghai on September 14 and December 6 in 1999, March 21 and June 13 in 2000, respectively. Fish size used in this study ranged from 30 to 42 cm in length and 500 to 1200 g in weight.

Seven species of cultured freshwater fish were also purchased at local market in Shanghai from July 2003 to Jun 2004. The date for sampling and body weights of fish were indicated in Table 2. Natural silver carp was caught at Kasumigaura Lake (Ibaraki, Japan) in July, Sep 2003 and Mar 2004.

The dorsal white muscle was sampled from three specimens and minced together. The mince was added by 10 % sorbitol to prevent myosin denaturation during frozen storage (-40 °C). All the samples were used within 4 months.



Silver carp



Bighead carp



Blunt snout bream



Grass carp



Common carp



Largemouth bass



Snakehead

**Figure 1. Photographs giving a view of external morphology of different fish species used in the present study**

**Table 1. Body index parameters of silver carp used for preparing surimi and chemical composition of surimi.**

Season	Date M.D.Y.	Body index of material fish		Chemical composition of surimi		
		Body length(cm) Mean(min-max)	Body weight(g) Mean (min-max)	Moisture (%)	Proein (%)	Fat (%)
Autumn	9.14.1999	39.6 (37.5-42.5)	1150 (920-1265)	75.2	16.8	0.63
Winter	12.6.1999	34.8 (30.5-36.5)	760 (500-890)	74.8	16.7	0.49
Spring	3.20.2000	41.2 (38.6-42.2)	1280 (980-1340)	72.3	19.2	0.44
Summer	6.13.2000	40.8 (36.8-42.3)	1150 (950-1290)	74.2	17.3	0.45

**Table 2. Sampling date and body weight of fish samples used for the study**

Sampling date	Body weight (g) of seven fish species						
	Silver carp	Bighead carp	Grass carp	Blunt snout bream	Common carp	Largemouth bass	Snakehead
2003.7.16	920±50	720±40	2350±150	680±140	890±120	340±20	485±15
2003.8.14	970±90	1160±60	2390±130	890±160	1950±110	280±20	440±10
2003.9.17	1190±70	1350±90	2700±80	460±150	770±70	335±10	580±30
2003.10.15	860±40	830±45	2680±130	780±85	660±40	425±15	590±50
2003.11.17	810±60	970±80	2890±150	610±50	1770±180	420±20	680±30
2003.12.15	1210±120	1085±60	2450±160	510±110	880±170	405±10	525±25
2004.1.7	1150±95	1050±100	3460±200	550±30	1180±180	420±25	980±50
2004.2.17	730±60	1010±50	2180±150	340±30	ND	380±25	810±70
2004.3.15	1280±90	1100±50	2470±120	435±40	1980±190	365±20	630±40
2004.4.15	1080±105	745±40	4140±200	680±70	2140±220	360±10	800±10
2004.5.14	1980±150	1150±120	1660±180	550±40	1310±150	360±15	590±30
2004.6.14	1240±90	750±80	2280±160	650±60	655±60	340±15	760±80

Note: ND, no sample available

### 3. Chemicals

All chemicals used were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan)

### 4. Preparation of Frozen Surimi

Fish were killed, beheaded, degutted, skinned and washed with chilled water. Minced meat was separated immediately from fish fillet by using a belt drum type meat separator. The minced meat was washed thrice with chilled water, and finally with 0.3 % NaCl. Washing was performed by soaking mince for 15 min in 4 volumes of the solution (v/w). After washing the mince, residual black skin and fine bone

were removed from the meat by using a strainer. Final dewatering was carried out by centrifugation of the meat at 3,000 x g for 20 min. Sugar (4 g), sorbitol (4 g) and polyphosphates (0.3 g) were added per 100 g of the dewatered mince as cryoprotective compounds. The prepared surimi was immediately frozen at -40 °C for about 20 h and subsequently stored at -30 °C until use. The four surimi samples of silver carp prepared in different seasons were termed as autumn (Sept. 1999), winter (Dec. 1999), spring (Mar. 2000) and summer (June 2000) surimi, respectively.

### 5. Preparation of Thermal Gel

Frozen surim was tempered at room temperature for about 30 min before use. The surimi partially thawed was chopped in a vertical vacuum cutter (Model UMC 5E, Stephan machinery Co.,) connected with a circulating chiller. The protein concentration in the surimi were adjusted to 14.34 %, , by adding the solution containing 4 % sucrose, 4 % sorbitol, and 0.3 % polyphosphates. Consequently, the moisture contents were about 78.0 % . The above solution instead of water was used to maintain the sugar and polyphosphate content unchanged by decreasing protein content because these two are known to affect the gelation profile. Salt content coming from surimi itself was neglected in this calculation. pH of the surimi samples was about  $7.3 \pm 0.1$  for all seasons.

Surimi was chopped in the cutter with salt (3 % w/w) for 5min, and for 2 min under vacuum to remove air. The salt ground paste thus obtained was stuffed into polyvinylidene chloride casing tubes, 22 mm in diameter and 70 mm in length. Sealed tubes were incubated in water baths of 30, 40, 50, 60, 70 and 85 °C for 0-36 h. Incubation was stopped by cooling in ice-cold water. Rheological parameters of the gels, breaking force and breaking strain were measured after standing at room temperature ( $22 \pm 2$  °C) for 30min.

### 6. Characterization of Gel Properties

The breaking force and breaking strain of the thermal gel was measured on Rheometer (Type EZ-test, Shimadzu Co. Ltd.) using a spherical plunger of 5 mm in diameter with the penetration speed of the

plunger of 60 mm/min. For the measurement, cylindrical gel was cut from the heated gel tube with a size of 22 mm in diameter and 30 mm in height.

The textural properties of heated gel were characterized by plotting breaking strains against breaking forces, which is termed as gel texture map by Lanier (1986).

The gelation rate was defined as a reciprocal time (h) required for reaching the half-maximal breaking force (Kato et al. 1984).

### **7. Preparation of Myofibrils from Surimi or Frozen Meat**

Myofibrils were prepared from fish mince or surimi according to the method as described (Azuma and Konno 1998). Firstly, 5 grams of chopped surimi or frozen meat was washed twice with 6 volumes of 0.1 M KCl, 20 mM Tris-HCl (pH 7.5). Washed meat was then homogenized four times for 30 seconds each at 12,000 rpm with an interval of 30 s in the above medium. Centrifugation of the homogenate at 3 °C at 3,000 x g for 10 min, and re-suspension in the above solution was repeated for more than 4 times. The suspension finally obtained in the above medium was filtered through a layer of gauze to remove connective tissue. The filtrate was used as myofibril suspension in this study.

### **8. Preparation of Myosin**

Myosin was prepared from myofibrils according to the method described by Koseki et al. (1993). Myofibrils dissolved in 0.5 M KCl, 20 mM Tris-HCl (pH 7.5) were added by 2 mM  $Mg^{2+}$ -ATP to dissociate myosin from actin, immediately saturated ammonium sulfate solution was added to give 40 % saturation to remove actin as precipitate. Myosin in the supernatant was collected as pellet by raising the saturation to 55%. Myosin pellet was dissolved in and dialyzed against 0.5 M KCl, 20mM Tris-HCl (pH 7.5). The supernatant by the centrifugation at 20,000 x g for 20 min was used as myosin sample.

## 9. Chymotryptic Digestion of Myosin

Chymotryptic digestion of myosin or myofibril was conducted in the two media containing either 0.05 M KCl, 20 mM Tris-maleate (pH 7.0) with 1 mM EDTA, or 0.5 M KCl, 20 mM Tris-HCl (pH 7.5) with 1 mM  $\text{CaCl}_2$ . The former and the latter media were suitable for cleavage at Subfragment-1 (S-1)/rod and Heavy meromyosin (HMM)/Light meromyosin (LMM) junctions, respectively. The digestion temperature and the ratio of chymotrypsin to myosin (myofibrils) were changed optionally considering the objective. Chymotryptic digestion was quenched by adding 0.5 mM phenylmethylsulfonyl fluoride (PMSF).

## 10. Preparation of Rod

Rod was prepared from myofibrils according to the method by Kato and Konno (1993a, 1996). Myofibrils were finally suspended in 0.05 M KCl, 20mM Tris-maleate (pH 7.0), and 1 mM EDTA. Myosin in myofibrils was digested with chymotrypsin, which cleaved filamentous myosin into S-1 and rod. The conditions for digestion were listed in the Table 3. The digestion was quenched with 0.5 mM PMSF, and the digest was centrifuged. The pellet was dissolved in a solution of 0.5 M KCl containing 20 mM Tris-HCl (pH 7.5). Ammonium sulfate was added to the solution to give 40 % saturation, and the mixture was centrifuged. The pellet containing both the undigested myosin and S-1 produced bound to actin was discarded. Myosin rod remaining in the supernatant was allowed to precipitate by raising the saturation to 55 %. Rod in the pellet was dialyzed against 0.05 M KCl, 20 mM Tris-maleate (pH 7.0) or iced water. Filamentous rod was collected by centrifugation as a pellet, leaving tropomyosin and troponin in the supernatant. The pellet was dissolved in and dialyzed against 0.5 M KCl containing 20 mM Tris-HCl (pH 7.5). The obtained dialyzate was centrifuged at 20,000 x g for 15 min, and the supernatant was used as rod. Myosin rod was finally dissolved in 0.5 M KCl, 20 mM Tris-HCl (pH 7.5).

**Table 3. Conditions for digestion of myofibrils to prepare rod from various fish species**

	Silver carp, bighead carp, grass carp and blunt snout bream in winter	Silver carp, bighead carp, grass carp and blunt snout bream in summer	Common carp, large-mouth bass, snakehead in both winter and summer
Chymotrypsin(w/w)	1/100	1/500	1/500
Temperature (°C)	10	20	20
Digestion time(min)	30	30	30

### 11. Chymotryptic Digestion of Rod, and Its Fractionation

Rod dissolved in 0.5 M KCl and 20 mM Tris-HCl (pH 7.5) was digested with 1/200 (w/w) of chymotrypsin at 10 °C. The digest quenched with 1 mM PMSF was dialyzed against 0.05 M KCl, 20 mM Tris-maleate (pH 7.0) and 5 mM MgCl<sub>2</sub> to separate the components in the digest into water-soluble and water-insoluble fractions. The supernatant obtained by centrifugation at 100,000 × g for 15 min was referred to as the water-soluble or subfragment-2 (S-2) fraction and the pellet was referred to as the water-insoluble or LMM fraction.

### 12. Preparation of LMM (Light meromyosin)

LMMs were prepared from myofibrils by chymotryptic digestion. Myofibrils was digested in a medium of 0.5 M KCl, 20 mM Tris-HCl (pH 7.5), and 1 mM CaCl<sub>2</sub> at 10°C, using 1/100 (w/w) of chymotrypsin, i.e., the conditions are suitable for cleaving myosin into HMMs and LMMs. Isolation of LMM from the digest was exactly the same as used for isolation of rod from myofibril digest.

### 13. Preparation of S-2

S-2 was prepared from rod by digesting with chymotrypsin. The digestion of rods was conducted in a medium of 0.5 M KCl, 20 mM Tris-HCl (pH 7.5), 1 mM CaCl<sub>2</sub> at 10 °C, using 1/100 (w/w) of



chymotrypsin, i.e., the condition for cleaving rod into LMMs and S-2. The digest was dialyzed against 0.05 M KCl, 20 mM Tris-maleate (pH 7.0), 1 mM MgCl<sub>2</sub> to sediment LMM. The supernatant of the dialysate obtained by ultracentrifugation at 100,000 x g for 15 min was referred to as S-2.

Purity of the preparations was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

#### 14. Protein Concentration Determination

Protein concentration was measured using Biuret method (Gornall et al. 1949). Bovine serum albumin was used as a standard. The protein concentration of chymotrypsin was determined by the absorption at 282 nm assuming 2.04 for 1 mg/ml solution.

#### 15. Electrophoretic Analysis

SDS-PAGE was performed according to the method of Laemmli (1970) using 7.5 or 12.5 % polyacrylamide gels. Electrophoresis was conducted at a constant current of 15 mA/gel using a Compact-PAGE Apparatus (ATTO Corp., Tokyo, Japan). After electrophoresis, gel was stained with 0.125 % (w/v) Coomassie Brilliant Blue R-250 in 50 % (v/v) methanol and 10 % (v/v) acetic acid, and destained with 50 % (v/v) methanol and 10 % (v/v) acetic acid.

Myosin and subfragments content in the preparations was estimated by measuring the staining intensity of the corresponding bands on SDS-PAGE using Scion Image software (Frederick, Maryland, USA) as employed by Reidler (2000).

Ammonium sulfate in the sample was removed before preparation of SDS-PAGE sample. Protein solution was added by equal volume of 15 % TCA, and centrifuged at 1,000 x g for 30 min to sediment proteins. The protein pellet was suspended in acetone and centrifuged. The pellet was allowed to dry by standing overnight at room temperature. Finally, the pellet was dissolved in 20 mM Tris-HCl (pH7.5), 8 M urea, 2 % SDS, 2 % mercaptoethanol, 0.01 % bromothymolblue (BTB) by shaking overnight. The

solution was used as SDS-PAGE sample. The final protein content was adjusted to be about 1 mg/ml.

### **16. Heating of Myofibrils**

The myofibril suspension (2-6 mg/ml) in 0.1 M KCl, 20 mM Tris-HCl (pH 7.5) was incubated in water bath at various temperatures. Heating was stopped by cooling the sample in iced water. KCl concentration for the heating medium was changed from 0.1 M to 2 M. In the case of heating with high concentrations of KCl, heating was stopped by addition ice cold 20 mM Tris-HCl (pH7.5) buffer to reduce the KCl concentration less than 0.5 M. When the ATPase inactivation rate at 0 °C was measured, the equal volume of ice cold 2 M Na-glutamate solution was added to stop the ATPase inactivation.

### **17. Ca<sup>2+</sup>-ATPase Assay**

Ca<sup>2+</sup>-ATPase activity was measured at 25 °C in a medium of 0.5 M KCl, 5 mM CaCl<sub>2</sub>, 25 mM Tris-maleate (pH 7.0) and 1 mM ATP. Liberated Pi was determined to analyze ATP hydrolysis (Fisk and Subbarow 1925). The specific activity was expressed in μmol Pi/min/mg of myofibril. Thermal denaturation of myofibrils and myosin was studied by measuring the Ca<sup>2+</sup>-ATPase inactivation rate. The first order inactivation rate ( $k_D$ ) was estimated (Uchiyama et al. 1978).  $k_D = (\ln C_0 - \ln C_t) / t$ , where  $C_0$  and  $C_t$  are the relative remaining ATPase activity before and after heating for  $t$  second.

### **18. Thermodynamic Parameters of Thermal Inactivation**

Thermodynamic parameters (activation energy,  $E_a^\ddagger$ ) was calculated from the slope of the line obtained by plotting  $\log k_D$  against  $1/T$  (Arrhenius plot). The slope represents  $-E_a^\ddagger / 2.303 \cdot R$ . Inactivation rate was calculated from the mean value of triplicated experiments with separate samples of myofibrils. The linear regression lines were obtained from these experiments (R-squared value is higher than 0.99).

## 20. Thermal Inactivation Profile of Whole Myosin in the Myofibrils

For studying the thermal inactivation rates of myosin contained in the myofibrils, the following two methods were employed. The first one was to measure  $k_D$  of myofibrils at 2 M KCl, at which myosin completely loses the protection by F-actin and shows the rate corresponding to that of myosin itself (Wakameda and Arai 1985, 1986). Another method was  $k_D$  measurement with myosin preparation free from actin. In the latter case, the supernatant of the myofibrils at 40 % saturated ammonium sulfate in the presence of Mg-ATP (Koseki et al. 1993) was dialyzed against 0.5 M KCl and 20 mM Tris-HCl (pH 7.5) containing 1 M Na-glutamate. The dialysate was directly used as myosin preparation. Na-glutamate at 1 M was essential for the protection of myosin from denaturation during the dialysis and storage. The myosin sample contained practically no actin, although it contained regulatory proteins (tropomyosin and troponin). Recovery of myosin was estimated by SDS-PAGE analysis, and was proved to be nearly 100 %. As the sample always contained 1 M Na-glutamate, the direct comparison of  $k_D$  with others could not be done.

## 21. Estimation of Two Myosin Isomer Contents in Myofibrils

Myosin isomers seasonally expressed in fish muscle was studied by monitoring the thermal inactivation profile of myosin samples prepared as above. Inactivation rate was the index employed to detect myosin isomers. The principle was that respective isomers have their own specific inactivation rates, and that a mixture of two isomers produces the simple sum of the respective inactivation extents. Remaining activity for the mixture of two isomers could be calculated by using the following equation. Remaining activity  $= \alpha e^{-(k_1 t)} + (1-\alpha) e^{-(k_2 t)}$ , where  $k_1$  and  $k_2$  are inactivation rates ( $\text{sec}^{-1}$ ) of two isomers, and  $\alpha$  is the proportion of myosin with the inactivation rate of  $k_1$  in the mixture, and  $t$  is the incubation time (sec). Practically, the rates for summer and winter-type myosin with different inactivation rates were estimated experimentally.

When the summer and winter-type myosin showed a large difference in the inactivation rates,

coexistence of two isomers was easily detected by analyzing the inactivation profile representing a breaking point in the profile. Summer type myosin isomer content was estimated by extrapolating the line for the latter phase to incubation time zero.

## 22. Thermal Denaturation Mode of Myofibrils

Thermal denaturation of myofibrils when heated in the medium of 0.1 M KCl, 20 mM Tris-HCl (pH 7.5) was studied. Heating temperature was varied from species to species so as to make the thermal denaturation mode analysis easier. The indicators employed for detecting myosin denaturation upon heating of myofibrils were as follows:

(i)  $\text{Ca}^{2+}$ -ATPase inactivation.  $\text{Ca}^{2+}$ -ATPase activity was assayed in a medium of 0.5 M KCl, 20 mM Tris-maleat (pH 7.0), 5 mM  $\text{CaCl}_2$ , and 1 mM ATP at 25 °C.

(ii) Loss of salt-solubility. Heated myofibrils were dispersed in 0.5 M KCl and 20 mM Tris-HCl (pH 7.5), and left for 30 min in ice. Then the mixture was centrifuged at  $20,000 \times g$  for 15 min immediately after addition of 1 mM ATP and 1 mM  $\text{MgCl}_2$  ( $\text{Mg}^{2+}$ -ATP).  $\text{Mg}$ -ATP was added to remove the influence of F-actin, which decreases the solubility of myosin. Myosin content in the supernatant was estimated on SDS-PAGE.

(iii) Monomeric myosin content. This index was developed to distinguish monomeric myosin from salt-soluble myosin by Koseki et al. (1993). Saturated ammonium sulfate solution was added into dissolved myofibrils to give 40 % saturation in the presence of 1 mM  $\text{Mg}^{2+}$ -ATP., then centrifuged at  $20,000 \times g$  for 15 min. Myosin content in the supernatant was referred to as monomeric myosin. Myosin content in (ii) and (iii) was estimated by measuring the staining intensity of myosin HC band appearing on SDS-PAGE pattern.

(iv) Amount of chymotryptic fragments and their monomeric form. Myofibrils were digested with chymotrypsin at S-1/Rod junctions. The amount of chymotryptic fragments, S-1 and rod, were estimated by measuring their staining intensity on SDS-PAGE pattern.

### 23. Aggregate Formation of Rod upon Heating

Aggregate formation by rod upon heating was studied. Myosin rod in 0.5 M KCl, 20 mM Tris-HCl (pH 7.5) (usually at 2 mg/ml) was heated for 30 min at temperatures from 20 to 80 °C. The heated samples were cooled in ice-cold water. The aggregate formation by rod was studied by measuring the absorption at 350 nm of the heated-cooled solution, and by reading its light scattering intensity at 350 nm at 90° angle on spectrofluorometer (FVP-6200 Jasco, Tokyo, Japan). Separate samples for respective temperatures were prepared to obtain the temperature dependent aggregation profile.

### 24. Circular Dichroism

Circular dichroism (CD) spectrum of rod from various species in different seasons was measured in a cell of 1 mm light path length on spectropolarimeter (JASCO J-725, Tokyo, Japan). Unfolding of Rod, LMM, and S-2 (0.5 mg/ml in 20 mM Tris-HCl (pH 7.5), 0.5 M KCl ) was studied by monitoring the ellipticity change at 222 nm upon heating the samples linearly from 10 to 70 °C at a rate of 1°C /min (Ogawa et al. 1995). Temperature was controlled by JASCO PTC 348 WI temperature control unit.  $\alpha$ -Helix content was estimated from the ellipticity assuming that  $\alpha$ -helix content of rod was 100 % and 0 % at 10 °C and 70 °C respectively. The derivatives of the unfolding was calculated using the software supplied by JASCO to make unfolding profile upon heating.

# Results

## Chapter 1.

### Comparison of Gel-Forming Properties of Silver Carp Surimi Prepared in Four Seasons

Silver carp is eurythermal species, which can tolerate a large extent of temperature change throughout the year. For example, in Shanghai district, the water temperature of fishponds is higher than 30 °C in summer, while in winter it is as low as 4 °C. It is well accepted that the thermostability of myofibrillar protein in fish muscle differs from species to species and deeply depends on the habitat temperature (Johnston et al. 1973; Johnston and Goldspink 1975; Hashimoto et al. 1982). Some studies have also showed that the thermostability of myofibrillar ATPase in eurythermal fish changed by temperature acclimation (Huang et al. 1990; Guo and Watabe 1993). It was also reported that the thermostability of myofibrillar Ca-ATPase in silver carp fish muscle showed significant seasonal changes (Wang et al. 1997). Moreover, it has also been reported that the thermal stability of myofibrils determined the suitable gel forming temperatures (Kato et al. 1984). For the industrial use of silver carp surimi, it is necessary to know the gel forming properties of silver carp muscle proteins produced in different seasons. In this chapter, silver carp surimi in four seasons was prepared, and their gel forming abilities were investigated at various heating temperatures. Moreover, the thermal stability of myofibril proteins in four surimi was also investigated to understand the relationship between the gel forming temperature and thermal stability of myofibril proteins.

#### 1.1 Temperature-Dependent Gelation Patterns of Silver Carp

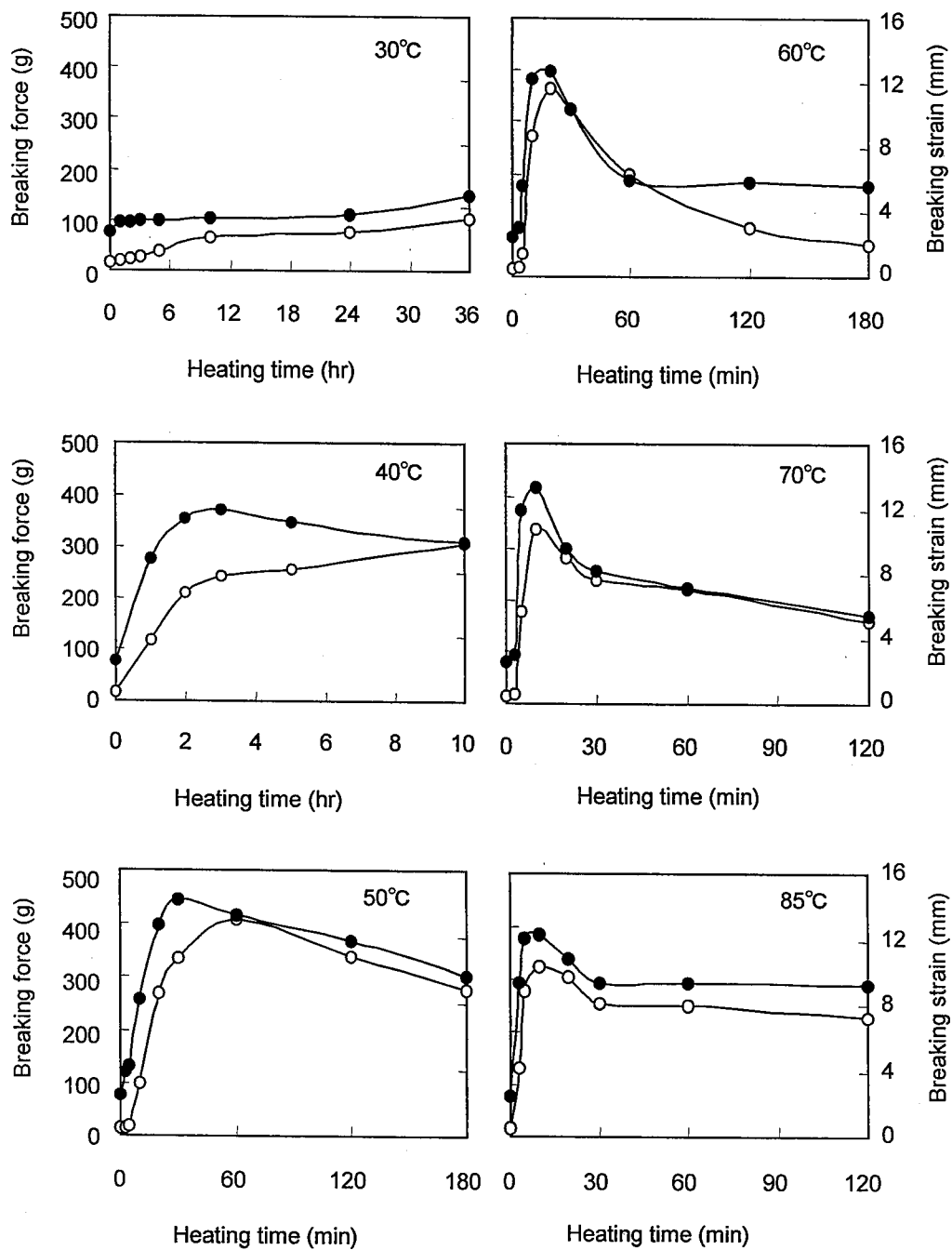
Gel forming properties of silver carp surimi in four different seasons were studied. Surimi was prepared from silver carp on the dates listed in Table 1. In the table, size of fish used and general component analysis of the surimi prepared are shown. The autumn surimi prepared on 14 September 1999 was used to study the temperature-dependent gelation. Salted surimi paste was heated at various temperatures from

30 to 85 °C for various time (0~36 h). The heating was stopped by cooling the sample in iced water. Their breaking force and strain were measured (Fig. 1-1). In general, breaking force and breaking strain proportionally changes at all temperatures examined. Moreover, there was a trend that the higher the incubation temperature is, the faster the gelation becomes. However, the autumn surimi failed to form hard gel at 30 °C even by the incubation for 36 hours probably because the temperature was too low to make gel for the autumn surimi. The breaking force achieved by 36 hours incubation was only about 100 g. At 40 °C, breaking force and breaking strain both increased continuously in 4 hours and kept high up to 10 hours incubation. A similar pattern was obtained at 50 °C, but the maximal breaking force and strain were obtained in much shorter incubation time of 30 minutes. Decrease in the both parameters in the later phase began to happen at 50 °C. The gelation profile at 60 °C was very characteristic, namely a quick increase in the breaking force and breaking strain in 15 to 20 minutes was followed by rapid and drastic decrease. This phenomenon is known as gel disintegration. That also was true for the gel formation at 70 °C, but the extent of the decrease was smaller than that at 60 °C. A slight decrease in the breaking force was also detectable at 85 °C upon prolonging the incubation time.

It is also noticed that the appearance of the formed gels was very temperatures dependent. For example, the gel formed at 40 °C was translucent and quite elastic, while the gel formed at higher temperatures such as 85 °C showed a marked opacity and great rigidity. However, the gel formed at 60 °C was liable to become soft and mushy showing typical properties of the disintegrated gel.

## **1.2 Comparison of Gelling Properties of Silver Carp Surimi Prepared in Four Seasons**

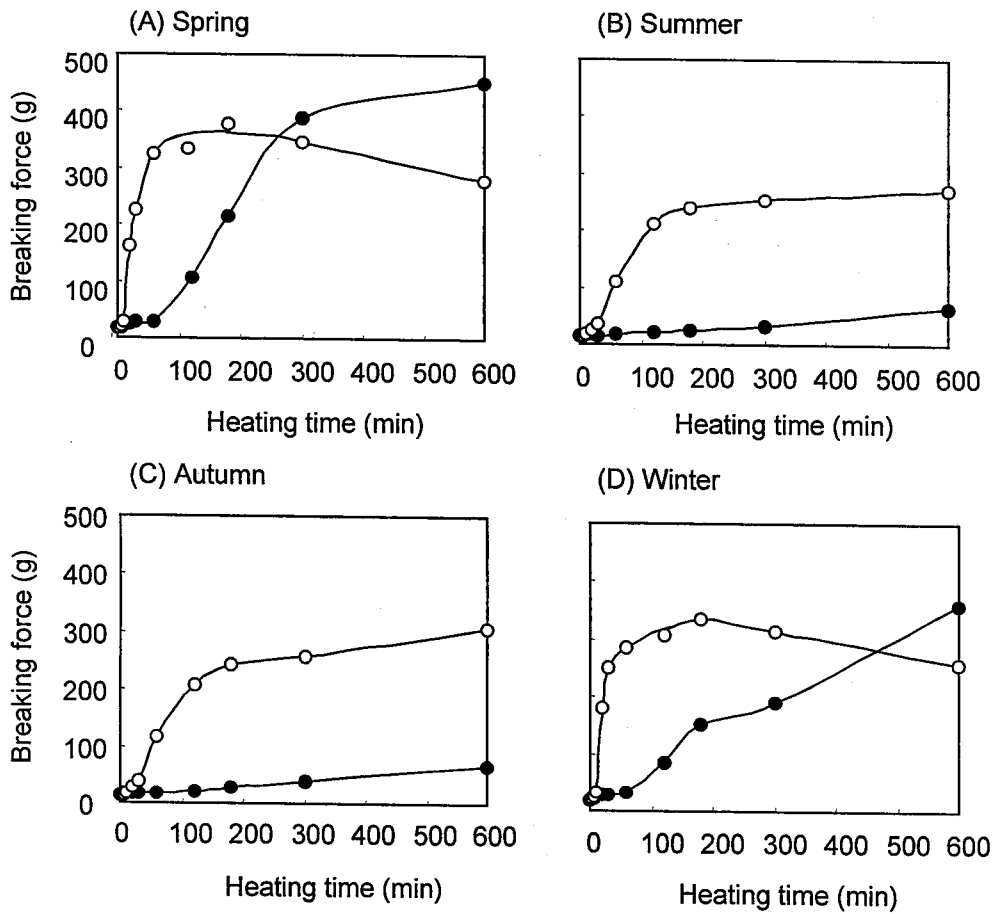
The gel forming properties of silver carp autumn surimi was quite temperature dependent. To compare the gel forming ability of surimi prepared in different seasons, the same set of experiments were carried out with surimi samples prepared in winter, spring and summer. Gelation profiles at 30 and 40 °C were compared to characterize surimi in different seasons. The gelation profiles at 30 and 40 °C are presented in Fig. 1-2. It was very clear that summer surimi as well as autumn surimi hardly formed gel at 30°C, but



**Figure 1-1. Temperature-dependent gel formation of autumn surimi.**

Changes in the breaking force (○) and breaking strain (●) at various temperatures were followed. Heating temperatures were indicated in the figures.





**Figure 1-2. Comparison of the gel formation at 30 °C and 40 °C among four surimi prepared in different seasons.**

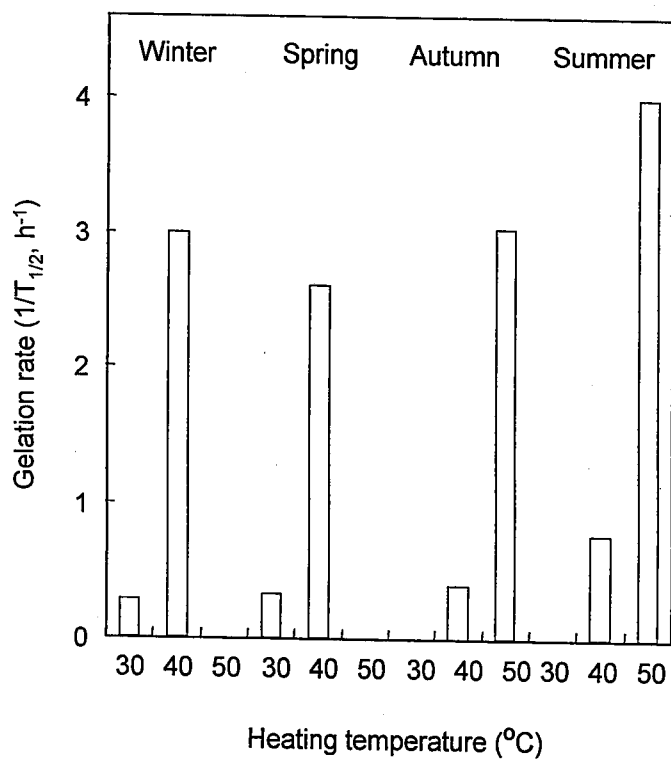
Breaking force increment upon heating at 30 °C (●) and 40 °C (○) was followed with four samples of spring, summer, autumn, and winter surimi.

formed gel at 40 °C. However, both spring and winter surimi gradually formed gel even at 30 °C, and showed faster gel forming process at 40 °C. Moreover, the breaking force for spring and winter surimi increased to the maximum and then decreased at 40 °C. The pattern was clearly distinguished from one with summer surimi. It was demonstrated that 30 °C was sufficiently high for spring and winter surimi to form gel, but not for summer and autumn surimi. It was concluded that gelation ability of silver carp was deeply dependent on the seasons for surimi preparation.

To compare the gelation forming ability of surimi in four seasons, the gelation rate defined as a reciprocal time to reach the half maximal breaking force at 30, 40 and 50 °C was estimated (Fig. 1-3). The rates for autumn and summer surimi at 30 °C were not calculated because no maximal breaking force was obtained under the conditions. As shown in Fig. 1-3, the gelation rates for winter and spring was similar at the same treatment temperature. That also was true for autumn and summer. Moreover, it required 10 °C higher to generate the same gelation rate for autumn and summer surimi compared with that for winter and spring surimi. It was concluded that surimi from silver carp could be clarified into two groups, summer- and winter- type from their gelation profile. Moreover, summer and autumn surimi required 10 °C higher temperature to produce similar gel than that for winter and spring surimi.

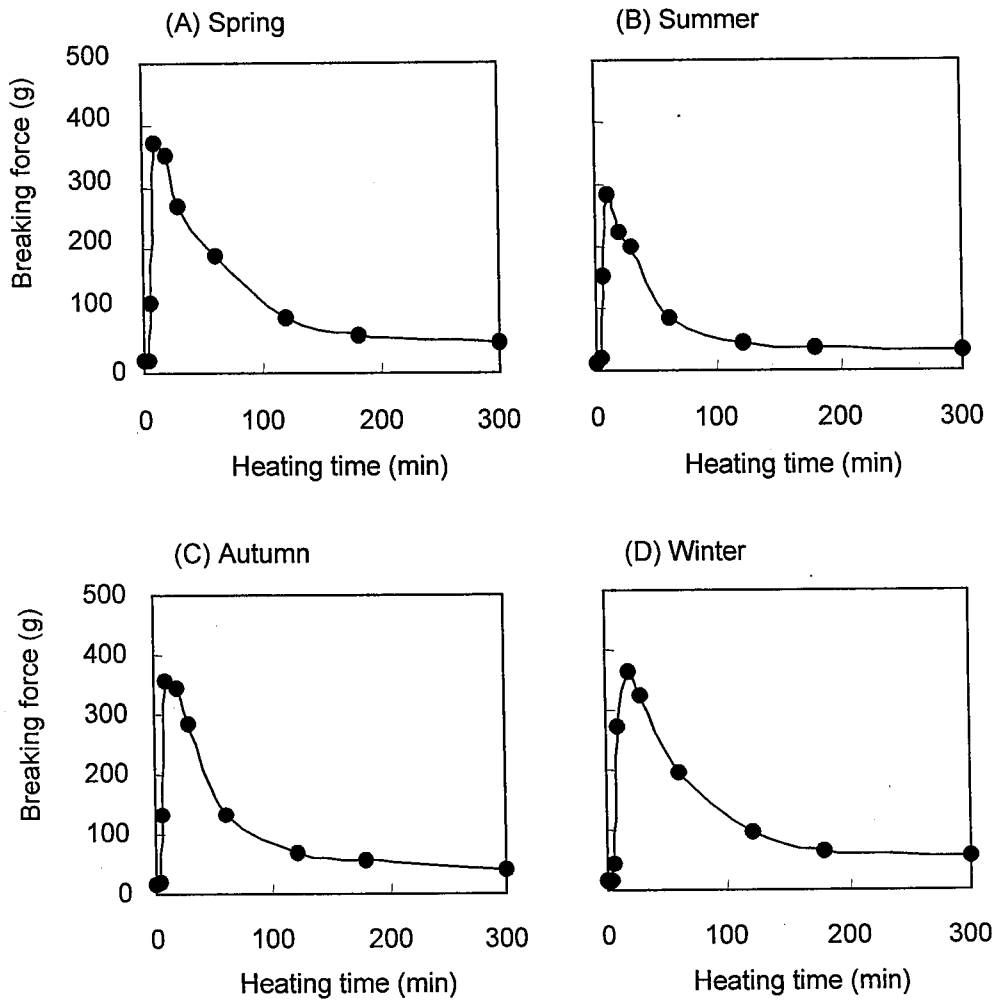
It is demonstrated in Fig. 1-1 that autumn surimi showed the disintegration at 60 °C. Similar disintegration was also observed with other three surimi samples from the results shown in Fig.1-4. Although the maximal breaking force achieved was a little different among samples, a general features for four surimi samples were very similar; breaking force reached the maximal level in very short period of 15 min, and a quick drop in breaking force was followed. The breaking force after incubation for 2 hours was about 1/5 that of the maximal value for all of samples. Therefore, it was concluded that the disintegration at around 60 °C was a common phenomenon for silver carp surimi prepared in all seasons.

When the temperature was raised to 85 °C, all of surimi gave similar changes in breaking force upon heating. The results are shown in Fig. 1-5. There is practically no difference in the breaking force achieved among four samples. The breaking force reached the maximal value in 10 min and its slight



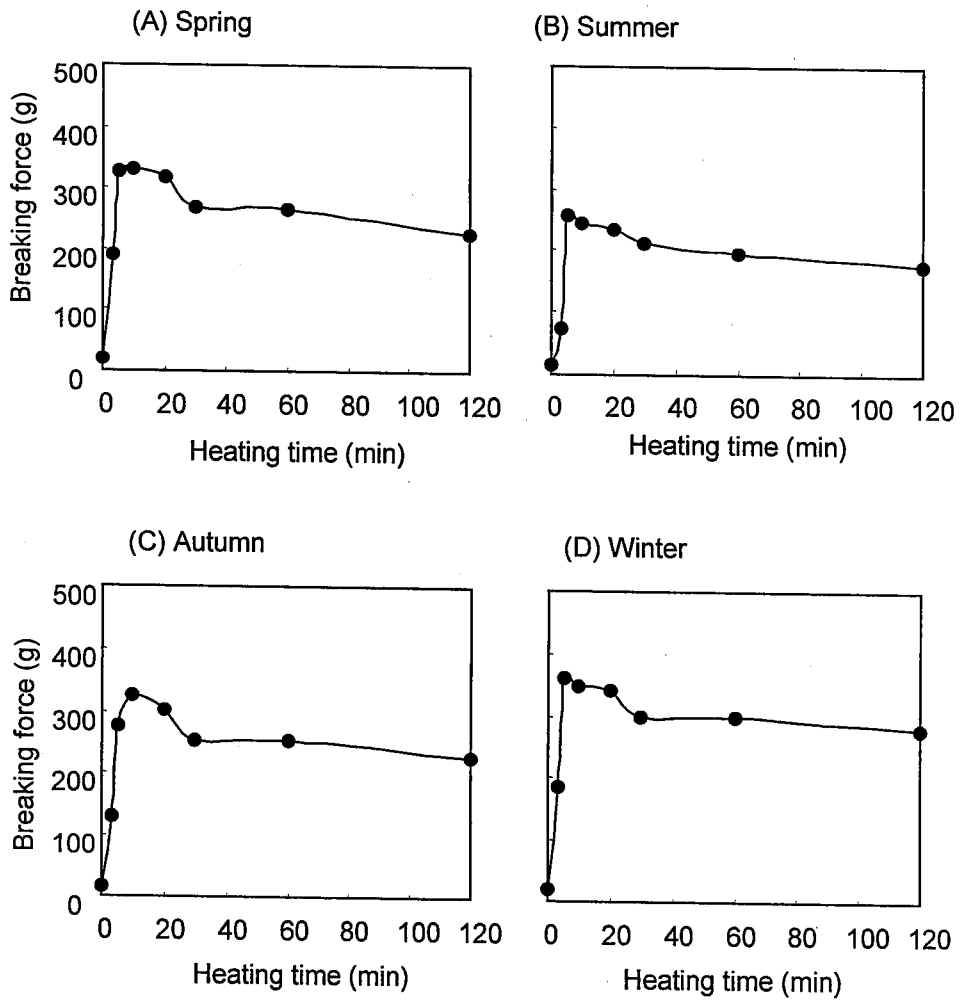
**Figure 1-3. Comparison of the gelation rates among four surimi samples.**

The gelation rates at 30, 40 and 50 °C were calculated from the gelation process as in Figs. 1-1 and 1-2 as a reciprocal time to reach the half maximal breaking force.



**Figure 1-4. Comparison of the disintegration profile among four surimi samples.**

Changes in the breaking force upon heating at 60 °C were followed with spring, summer, autumn, and winter surimi.

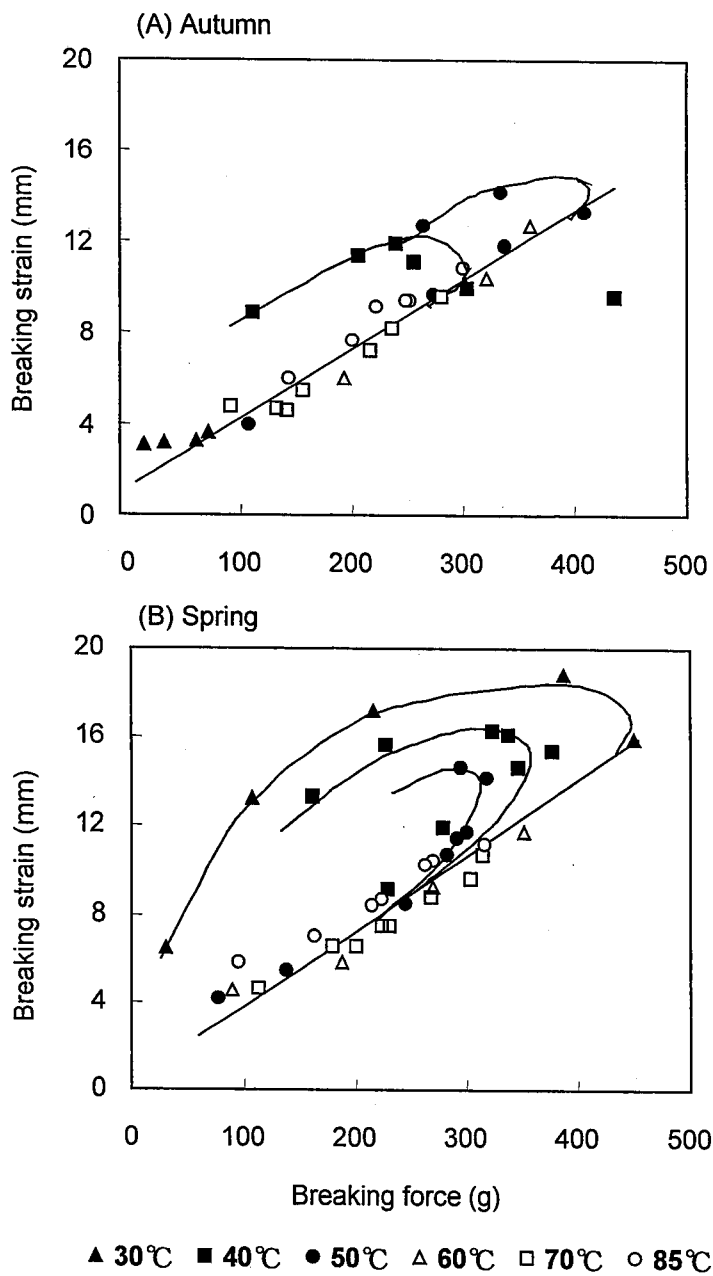


**Figure 1-5. Comparison of the changes in the breaking force upon heating at 85 °C among four samples of spring, summer, autumn, and winter surimi.**

decrease was also followed upon prolonging the incubation time.

### **1.3 Characterization of the Gel Forming Properties of Surimi by Texture Map**

There are many proposals to characterize the gel forming properties of surimi such as setting and disintegration index (Shimizu et al. 1981), spring constant (Abe 1994), contour map (Fukuda et al. 2001). To characterize the gel forming properties of silver carp surimi prepared in four seasons, the texture map proposed by Lanier (1986) was applied, which is an analysis by plotting breaking force against breaking strain. All of the breaking forces and breaking strains of the gel at various temperatures were used to yield the texture map. In this study, breaking strain against breaking force was plotted to emphasize the gel elasticity. As it was demonstrated that four surimi could be grouped into winter- and summer-type surimi by the above results, the two typical results with autumn and spring surimi were picked up to make texture map shown in Fig. 1-6A and B. First, it is interesting to find that almost all the data obtained above 60 °C ( $P < 0.001$ ) were on a single straight line in the texture map. The results indicated that the gel formed above were similar rheologically. With two surimi samples, the parameters for the gels produced above 60 °C were on a straight line. The slope for the line for both the spring surimi and the autumn surimi was identical (Fig. 1-6). The parameters for the gel obtained at 40 °C or 50 °C were not on the line, locating upper left region of the straight line. The results indicated that these gels rich in the breaking strain were much more elastic compared with the gel on the straight line. As autumn surimi did not form gel at 30 °C, plots were all located at the lower left corner of the plotting. Although the incubation time could not be seen in the figure, some of gelation profiles showed a gradual shift of the location from the upper left region of the line to on the line upon prolonging the incubation time. For example, when autumn surimi was incubated at 40 °C, the data in early phase were upper left of the straight line, while ones in latter phase were on the straight line. The results demonstrated that even though the heating temperature was the same, the gel properties changed with incubation time from elastic to rigid one. Therefore, heating temperature alone did not determine the gel properties, and heating time should also be



**Figure 1-6. Characterization of autumn and spring surimi by using texture map.**

To generate texture map, the breaking strain was plotted against the breaking force, which were taken from Fig. 1-1. The surimi analyzed (autumn and spring) and the temperatures were shown in the figures.

considered. The same tendencies were obtained with the surimi sample prepared in spring (Fig. 1-6B). Spring surimi formed gel at 30 °C, and was remarkably rich in breaking strain locating far from the line. The gel formed 40 °C was also elastic locating above the line in a very early phase, while the data were readily on the straight line soon. Shifting of the plot from upper region of the line to on the line was more obvious when heated at 50 °C, where only a couple of points in early phase were apart from the line. These results indicated that the gel formed at high temperatures and low temperatures were clearly distinguished in the texture map for both surimi samples. Comparing two texture maps, it was concluded that spring surimi seemed to require lower temperature by about 10 °C to produce a similar elastic gel. Moreover, the spring surimi required low temperature to produce the gel shift the points from the upper left of the line to on the line. As the gel properties for the gel prepared at high temperature were indistinguishable among different temperatures, the gel properties for the gel would be so-called “directly heated gel”. The gel rich in breaking strain locating upper region of the straight line would be referred to as so-called “set gel”. In other words, the linear relationship between breaking force and breaking strain might be the index for characterization of direct heated gels. The gel disintegration occurred at 60 °C. Nevertheless, all parameters for the gel at 60 °C were all on the same line indicating a parallel decrease of the breaking force and breaking strain upon heating. The difference in the gel formation between spring surimi and autumn surimi was well demonstrated in the texture map, so the mapping was found to be useful to characterize the gel properties.

#### **1.4 Comparison of Ca<sup>2+</sup>-ATPase Inactivation Rate of Myofibrils Prepared from the Four Surimi Samples at 40 °C**

The gel formation was always accompanied by the denaturation of muscle protein, especially myosin (Wu et al. 1985). As the temperature to induce gel formation differed from each other among surimi prepared in four seasons, it is reasonable to consider that the thermal stability of myosin in four surimi samples differ from each other. Myofibrils were prepared from 4 surimi samples, and the thermal stability

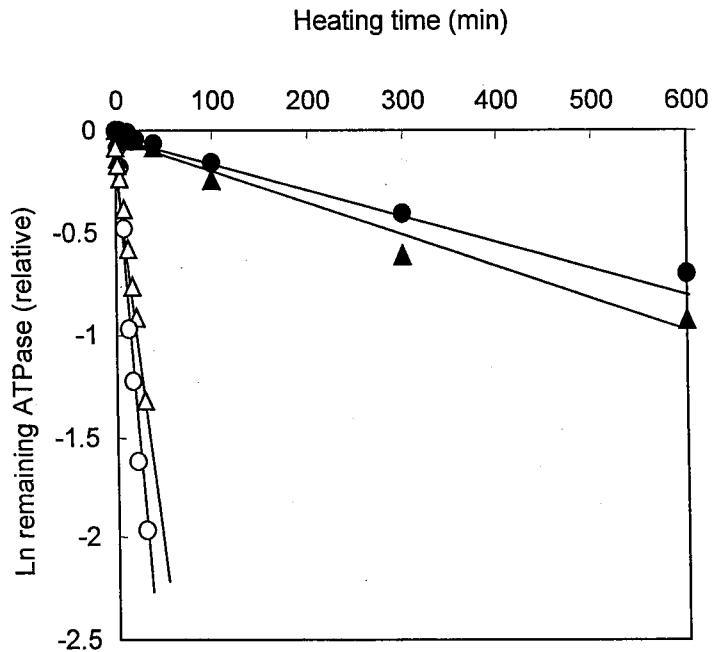


of myofibril was then compared by measuring the ATPase inactivation rate.

Myofibrils from surimi in four seasons were prepared routinely, and their thermal inactivation rates of Ca-ATPase at 40 °C were compared (Fig. 1-7). The rates were also classified into two groups, summer-type surimi (summer and autumn) with small inactivation rate and winter-type surimi (spring and winter) with large inactivation rate. The myofibrils from winter surimi showed the largest rate of  $129 \times 10^{-5} \text{ (sec}^{-1}\text{)}$ , and those from autumn surimi showed the smallest rate of  $2.36 \times 10^{-5} \text{ (sec}^{-1}\text{)}$ . Difference in the rates between these two myofibrils was about 60 times.

## 1.5 Discussion

Katoh et al. (1984) has reported that suitable heating temperature for gel formation is species-specific, and is deeply related to the thermal stability of myofibril. In this chapter, thermal gelation of silver carp surimi was studied by considering the preparation seasons. Silver carp surimi in any seasons formed elastic gels, suggesting a potential to be a raw material for thermal gel products. It is generally believed that freshwater fish meat such as common carp hard to form gel in Japan. However, it was firstly demonstrated that silver carp surimi had quite high gelation ability. Difference was observed in the temperatures for gel formation; summer-type surimi required 10 °C higher temperature to produce similar gel than that for winter-type surimi. The difference was well related to the different thermal stability of myofibril from surimi in four seasons. Myofibrils in summer-type surimi were very stable, while those in winter-type surimi were very unstable. The relationship between gelation temperature and myofibril thermal stability was consistent with the results by Katoh et al. (1984). Comparing the inactivation rates obtained with silver carp myofibril at 40 °C with those of other species of fish (Hashimoto et al. 1982), it was suggested that myofibrils in summer- and winter-type surimi were as stable as those of skipjack and sardine, respectively. Myofibrils in summer-type surimi required 10 °C higher heating temperature to provide the same inactivation rate with those of winter-type surimi. This would be reasonable because raising heating temperature by 10 °C would result in the acceleration of inactivation roughly by 40 to 100



**Figure 1-7. Comparison of the thermal inactivation of  $\text{Ca}^{2+}$  - ATPase of myofibrils prepared from four surimi at 40 °C.**

The myofibrils (2-3 mg/ml) prepared from four surimi were incubated at 40 °C, and remaining ATPase activity was analyzed by assuming the first order reaction. Myofibrils were from spring ( $\Delta$ ), summer ( $\blacktriangle$ ), autumn ( $\bullet$ ), and winter ( $\circ$ ) surimi.

times (Hashimoto et al. 1982). The difference in the stability of myosin in summer- and winter-type surimi seemed to be coincided with that in the gelling temperature by about 10 °C. It suggested that seasonal difference in the gel forming ability of silver carp would be derived from the seasonal difference in the thermal stability of myosin in surimi. Therefore, it is important to know the season for surimi production to find the suitable heating temperature for thermal gel formation.

Two-step heating is often introduced to improve thermal gel properties in Japan. Usually, the first heating (pre-heating) is performed at low temperatures such as 20~40 °C, then heated at high temperature for thermal gel production. Gel formed at such low temperatures is called “set gel”. Increased breaking force or strain by introducing pre-heating is referred to as setting effect. As set gel has the characteristic with high elasticity, the gel produced at low temperatures in this paper would be categorized into set gel due to its higher breaking strain (more elastic) than directly heated gel. Gel properties characteristic to set gel was clearly demonstrated by plotting the parameters in the texture map (Fig. 1-6). The temperature to form set gel changed seasonally. Thus, when the setting effect was expected, the pre-heating temperature should be carefully chosen considering the season of surimi production.

All of surimi samples prepared in different seasons showed a severe disintegration at 60°C similarly. The magnitude of disintegration was quite large. Thus, the phenomenon is another factor to be considered seriously in the thermal gel production from silver carp surimi.

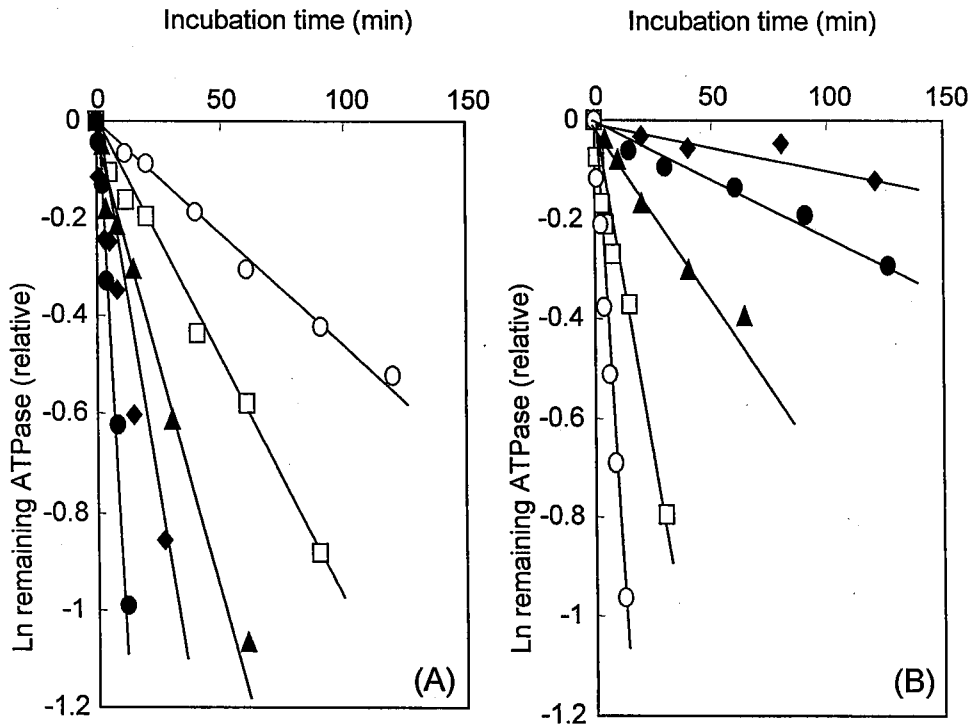
## **Chapter 2.**

### **Seasonal Expression of Two Types of Myosin Isomer with Different Thermal Stability**

Silver carp surimi prepared in different seasons exhibited different thermal gelation profiles, which was closely related to the different thermal stability of myosin in these surimi samples. Difference in the thermal stability of myosin between hot seasons and cold seasons was explained by the expression of myosin isomers with different thermal stability as the result of temperature acclimation studied with common carp (Hwang et al. 1990; Guo and Watabe 1993; Imai et al. 1997; Watabe et al. 1998). Thermal stability of myofibril in summer silver carp was much more stable than that in winter one by about 60 times. The difference in the thermal stability reported with carp was only about 4 times, while it was much less observed with silver carp. Although silver carp seems to use the same strategy expressing myosin isomers to overcome the water temperature change seasonally, the consequences were not the same as carp. Further studies are needed to understand the seasonal changes in the thermal stability of silver carp myosin adapting to the water temperature change. A large difference in the stability was obtained with myofibrils. A question may be raised whether the thermal stability of silver carp myosin itself as well as myofibrils in two seasons differed from each other. To answer the questions, myosin was isolated from myofibrils monthly, and its thermal stability was analyzed.

#### **2.1 Comparison of the Thermal Stability of Myofibril between Winter and Summer Seasons by Changing Heating Temperatures**

Thermal stabilities of myofibrils in Aug and Feb, representing summer- and winter-type myofibrils, were compared by varying the heating temperatures (32 °C to 47 °C). The results are shown in Fig. 2-1. Comparing the profile at the same temperature, it was clear that winter samples inactivated much quicker than summer ones at all temperatures tested. To give the same denaturation rate for winter-type as that of summer one, the incubation temperature should be lowered about by 7~10 °C. The data were analyzed by



**Figure 2-1. Temperature dependent  $\text{Ca}^{2+}$ -ATPase inactivation of myofibrils in Aug and Feb at 0.1 M KCl.**

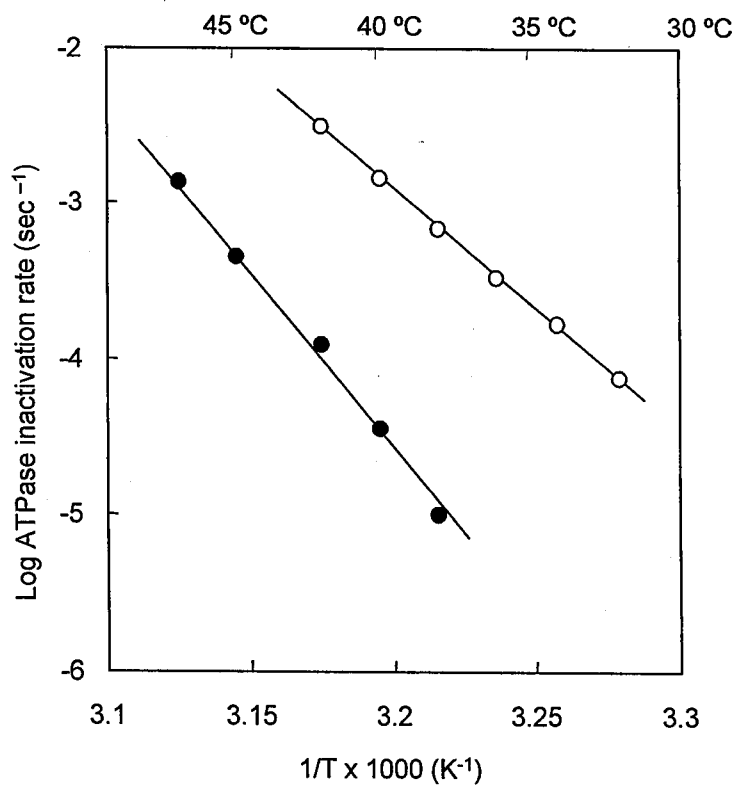
(A), Myofibrils in Aug. were heated at 40 - 47 °C. Heating temperatures were indicated as follows: 38°C (◆), 40 °C (●), 42 °C (▲), 45 °C(□), 47 °C (○)

(B), Myofibrils in Feb. were heated at 32 - 40 °C. Heating temperatures were indicated as follows: 32 °C (○), 34 °C(□), 36 °C (▲), 38°C (◆), 40 °C (●)

the Arrhenius plots (Fig.2-2). The plotting gave straight line for both myofibrils in different temperature ranges. The line for summer-type myofibrils located higher temperature range by about 10 °C. Temperature range for the lines with summer- and winter-type myofibrils were similar to those of skipjack tuna and sardine, respectively (Hashimoto et al. 1982). These results clearly demonstrated that seasonal thermal stability change of silver carp myosin was of such a large magnitude. Thermodynamic parameters from the lines were also calculated. The activation energy for summer and winter samples were 98.9 and 70.30 kcal/mol, respectively.

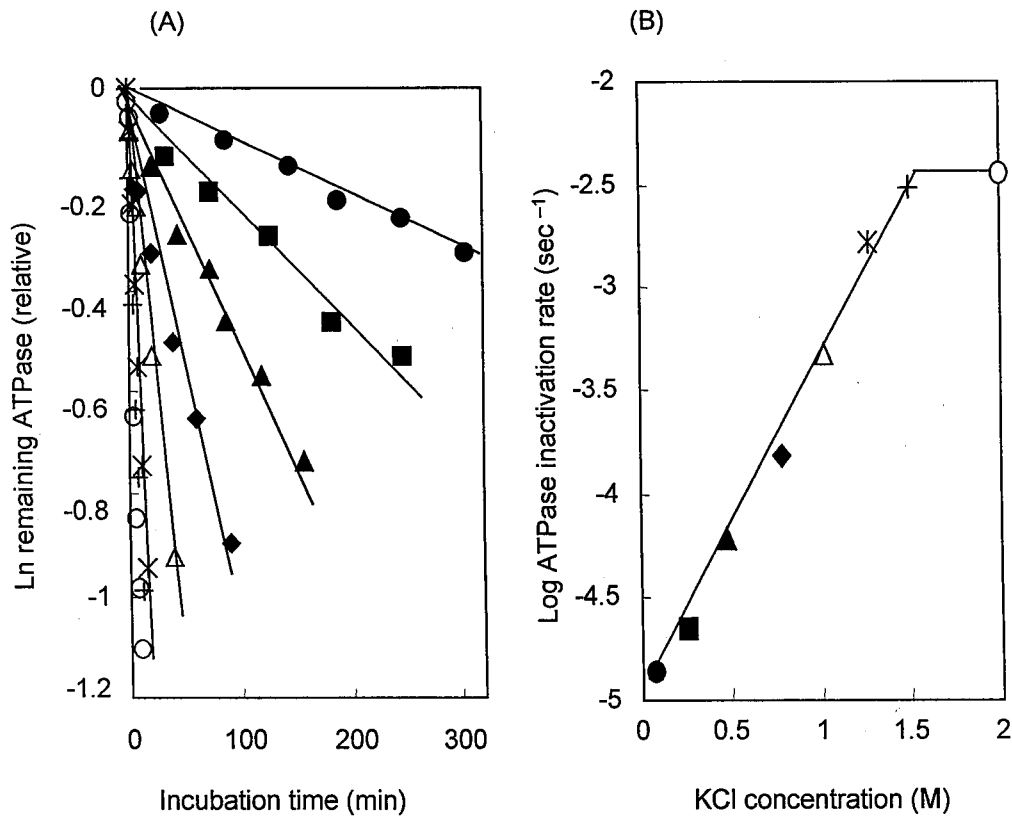
## **2.2 Comparison of Thermal Stability between Isolated Myosin and Myofibril at 2 M KCl**

It is essential to confirm that thermal stability of silver carp myosin itself differed from each other in two seasons. The simplest way is to isolate myosin from dorsal muscle in two seasons, and to analyze their stability. However, the method contains some problems; low recovery of myosin and myosin denaturation during the preparation process. These problems make it impossible to study thermal stability of whole myosin in myofibril. Therefore, it was not suitable to use for the objective in this study. It is reported that myosin is significantly stabilized by F-actin binding in low salt medium such as 0.1M KCl, and that increase in salt concentration gradually reduces the protection by F-actin, and protection can not be detected at very high concentrations such as 2 M KCl (Wakameda and Arai 1985, 1986; Takahashi et al. 2005b). Thermal stability of myofibrils at 2 M KCl can be used as an index for thermal stability of myosin itself. Loss of protection by F-actin was confirmed with myofibrils prepared from summer silver carp by treatment with high salt concentration (Fig. 2-3). The  $\text{Ca}^{2+}$ -ATPase inactivation rate of myofibrils gradually increased with increasing salt concentration. It reached the maximal level at around 1.5 M KCl (Fig. 2-3 A). The inactivation rates calculated in logarithmic values were plotted against KCl concentration in Fig. 2-3 B. The inactivation rate obtained at 2 M KCl with myofibril would be referred as the rate of myosin itself. To confirm that the inactivation rate of myofibrils at 2 M KCl can be used as the one for myosin, inactivation profile of myosin isolated from summer fish was compared with that of summer fish myofibrils at 2 M KCl at 35 °C (Fig. 2-4). Both samples exhibited similar inactivation



**Figure 2-2. Arrhenius plots of the inactivation rate of Ca<sup>2+</sup>-ATPase of summer and winter myofibrils.**

The inactivation rates in logarithmic values estimated from Fig. 2-1 were plotted against the reciprocal of heating temperatures (absolute temperatures). Myofibrils from Summer (Aug) (●) and winter (Feb) (○) were compared.



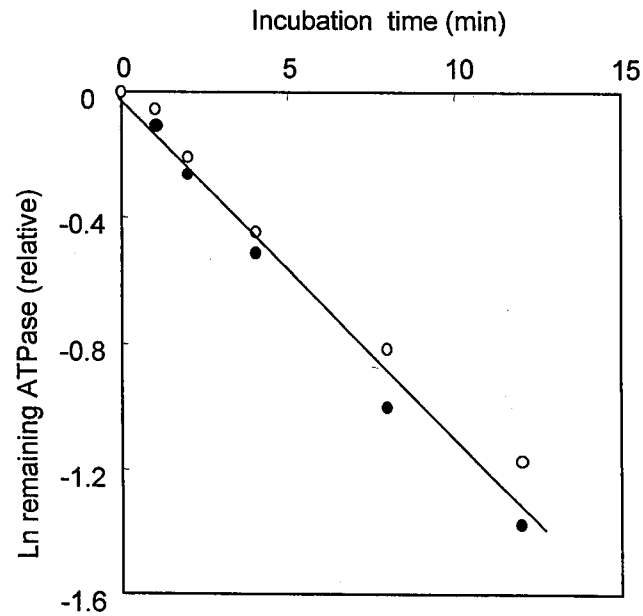
**Figure 2-3. KCl concentration dependent Ca<sup>2+</sup>-ATPase inactivation of myofibril in Aug.**

(A), Myofibrils were heated at 38 °C in the presence of various concentrations of KCl.

(B), ATPase inactivation rates in (A) were plotted against KCl concentrations.

KCl concentrations used are as follows: 0.1 M (●), 0.25 M (■), 0.5 M (▲) 0.75 M (◆), 1.0 M (△), 1.25M (⋈), 1.5 M (X), 2.0 M (○).





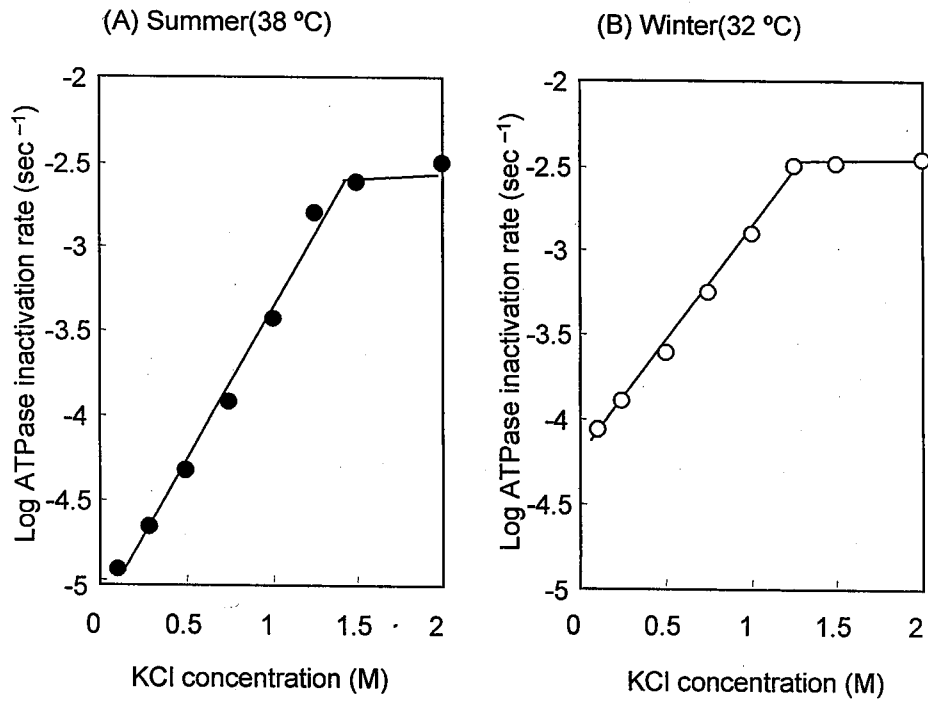
**Figure 2-4.  $\text{Ca}^{2+}$ -ATPase Inactivation profile for summer-type myosin and myofibril at 2 M KCl.**

Myofibrils were prepared from silver carp in Aug. Myosin was prepared from myofibrils using ammonium sulfate fractionation. ATPase inactivation profile of myofibrils (O) and myosin (●) were compared at 35 °C.

profiles. It is confirmed that the rate with myofibrils at 2 M KCl can be referred as an approximate rate of summer-type myosin itself. Comparison of the rate of myofibrils at 2 M KCl with that of isolated myosin from winter samples that could not be done because the latter one readily inactivated upon storage in ice, which will be described later. KCl concentration dependent inactivation rate with winter silver carp myofibrils were also studied (Fig. 2-5). As the inactivation rates above 1.5 M were almost constant for winter samples, the rate at 2 M KCl could be referred to as one for myosin itself. Assuming the inactivation rate at 0.1 M KCl is one with full stabilization by F-actin, the difference in the stability between myofibrils at 0.1 M KCl and at 2 M KCl was referred as the extent of stabilization by F-actin. Difference in the stabilization by F-actin between summer and winter myofibrils was observed. The extent of F-actin stabilization for winter and summer myosin were 30 and 200 times, respectively. The difference in the stabilization for summer myofibrils was more than 7 times than winter myofibrils. It was concluded that myosin in summer myofibrils was more strongly stabilized upon binding to F-actin than in winter ones.

### **2.3 Comparison of the Thermal Stability of Myosin between Winter and Summer Seasons**

Thermal inactivation rates of myosins in Aug and Feb, representing summer- and winter-type myosin, were compared by employing the above measuring system. Summer samples were heated from 30 to 38 °C, while winter samples were heated from 22 to 30 °C. The results are shown in Fig. 2-6. It was clear that winter samples inactivated much quicker than summer ones at the same temperature. For example, at 30 °C, the inactivation rate,  $k_D$  for Feb sample was  $220 \times 10^{-5} \text{ (s}^{-1}\text{)}$ , while the  $k_D$  for Aug sample was  $11.3 \times 10^{-5} \text{ (s}^{-1}\text{)}$ , showing a difference by about 20 times. To give the same denaturation rate for winter sample as summer one, the incubation temperature should be lowered about by 7~10 °C. The thermal stability of winter silver carp myosin was rather similar to that of Alaska pollack myosin (Takahashi et al. 2005b,c). The inactivation rates calculated in logarithmic values were plotted against the

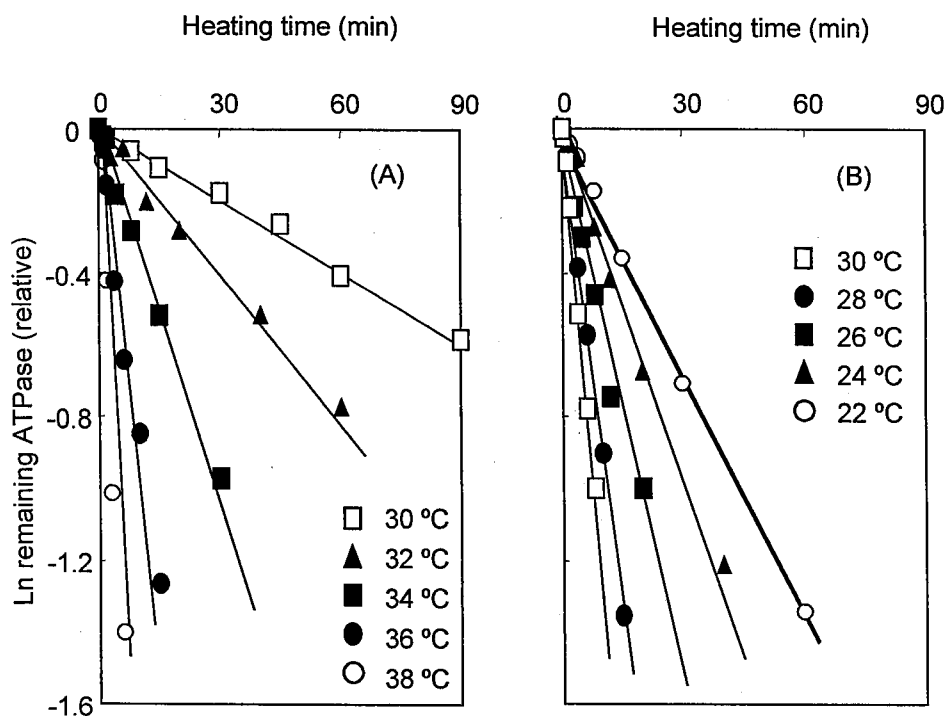


**Figure 2-5. Comparison of KCl concentration dependent  $\text{Ca}^{2+}$ -ATPase inactivation rate of summer and winter myofibrils**

Myofibrils from silver carp in Sept (A, Summer) (●) and Feb (B, Winter) (○) were compared.

(A), Summer myofibrils were heated at 38 °C (●)

(B), Winter myofibrils were heated at 32 °C (○).



**Figure 2-6. Temperature dependent of Ca<sup>2+</sup>- ATPase inactivation of myofibrils at 2 M KCl**

Myofibrils prepared from summer (A) and winter (B) were heated , at 2 M KCl and the inactivation profiles were compared. Heating temperatures were indicated in the figures.

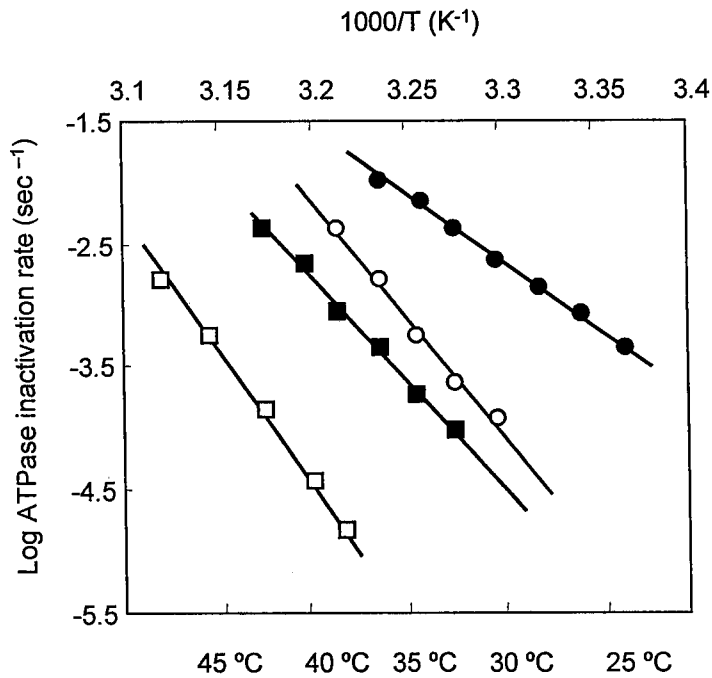
(A), Myofibrils in Jul were heated at the range of 30 - 38 ° C.

(B), Myofibrils in Feb were heated at the range of 22 - 30 ° C .

reciprocal of heating temperatures (absolute temperatures) in Fig. 2-7. The data with myofibril at 0.1 M KCl are also presented in Fig. 2-7 for comparison. It is clear to see the large difference in thermostability between summer and winter samples both in a form of myofibril (myosin with F-actin stabilization at 0.1 M KCl) and myosin (myosin without F-actin stabilization at 2 M KCl, which showed the difference in the F-actin stabilization. It was confirmed again that the extents of stabilization by F-actin for summer- and winter-type myosin were about 200 times and about 30 times, respectively. Consequently, summer silver carp myosin was kept very stable by using two strategies; high thermal stability of myosin itself, and large stabilization by F-actin.

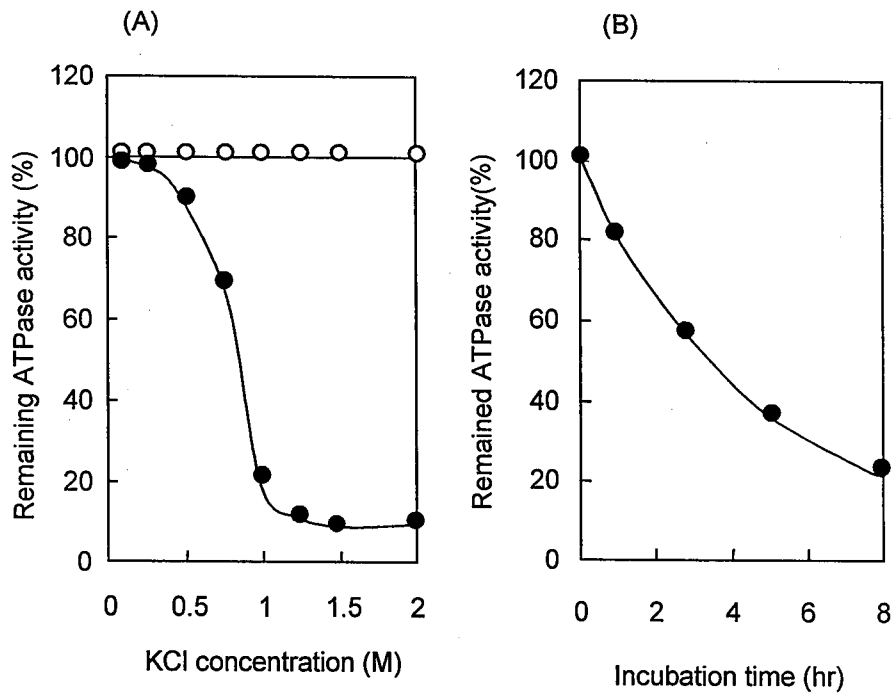
#### **2.4 The Instability of Winter Myosin**

A similar thermal inactivation rate of winter-type silver carp myosin to that of Alaska pollack myosin indicated that winter silver carp expresses very unstable myosin. To confirm that winter-type myosin is as unstable as pollack myosin, ATPase inactivation of myosin in ice was studied. Winter-type myofibrils were stored in ice overnight in the presence of various KCl concentrations from 0.1 to 2 M. For comparison, summer-type myofibrils were also examined. Remaining ATPase activity after overnight storage in ice was measured (Fig. 2-8). Practically no ATPase inactivation was detected at all KCl concentrations for summer samples, while winter samples lost the activity when the medium contained high concentrations of KCl. Inactivation was detected above 0.5 M KCl, and almost complete inactivation above 1.5 M (Fig. 2-8-A), which was similar as KCl-concentration dependent inactivation pattern (Fig. 2-5). As it was demonstrated that the inactivation rate of myofibrils at 2 M KCl could be referred as that of myosin itself, these results showed that winter-type myosin denatured even in ice. ATPase inactivation process was followed in ice. Myofibrils were incubated in ice in the presence of 2 M KCl, and the treatment was stopped by reducing the KCl concentration to 0.5 M KCl and adding 1 M Na-glutamate. Remaining activity decreased slowly with a half inactivation in 4 hours (Fig. 2-8-B). These results clearly demonstrated that winter silver carp myosin was quite unstable once it lost the protection by F-actin, and



**Figure 2-7. Arrhenius plots for inactivation rate constants ( $k_D$ ) of myofibrillar  $\text{Ca}^{2+}$ -ATPase of winter- and summer-type silver carp at 0.1 M and 2 M KCl.**

The inactivation rates in logarithmic values were plotted against the reciprocal of heating temperatures (absolute temperatures). The inactivation rates of myofibrils from Feb (■, ●), Aug (□, ○) were compared at 0.1 M (□, ■) and at 2 M KCl (○, ●).



**Figure 2-8. Ca<sup>2+</sup>-ATPase inactivation of myofibrils in ice.**

(A), Feb (●) and Aug (○) myofibrils were stored in ice overnight at various KCl concentrations. Remaining Ca<sup>2+</sup>-ATPase activity was measured.

(B), Ca<sup>2+</sup>-ATPase inactivation of Feb myofibril at 2 M KCl was followed in ice.

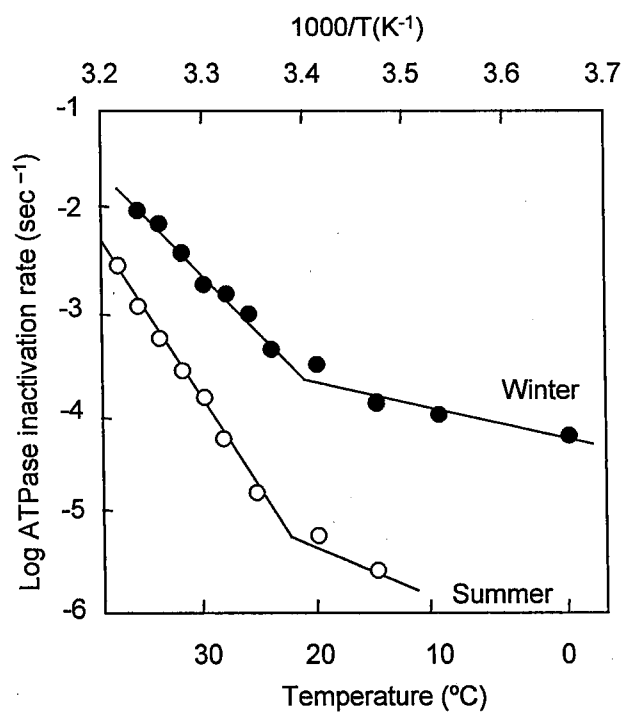
that conventional preparative method would lead myosin denaturation during the preparation including dialysis. As the information on the myosin denaturation at low temperature range seemed important for handling winter silver carp myosin, ATPase inactivation rates were measured at lower incubation temperature from 0 to 30°C. The rates were used to make Arrhenius plot (Fig. 2-9). Temperature dependent inactivation rates gave a breaking point at around 22°C for both samples. Lowering the temperature from 20 to 0 °C only reduced the rate by 4 times for winter-type. A similar tendency was obtained with summer-type although the rates itself were remarkably small. The estimated  $E_a$  values from the slopes of the two regions for winter myosin are 44.6 and 7.47 kcal/mol, respectively. The presence of the breaking point with low  $E_a$  value clearly showed that, lowering storage temperature below the breaking point (22°C) was less effective for suppressing the denaturation of winter-type myosin.

The above results were all obtained with silver carp cultured in China. It is still uncertain whether the above properties are commonly observable with silver carp. To answer the question, thermal stability of myofibrils of silver carp captured in Kasumigaura Lake (Ibaraki, Japan) in winter and summer seasons were investigated. The inactivation rates of myofibrils at 2 M KCl were compared with those of samples in China at various heating temperatures by Arrhenius plot (Fig. 2-10). A great difference in the inactivation rate of myosin between winter and summer Japanese fish was observed as well as Chinese fish, and the respective rates were the same as those obtained with Chinese silver carp in corresponding season. The above results indicated that silver carp in Japan was the same as in China judging from the myosin stability in summer and winter season fish.

## **2.5 Seasonal Expression of Myosin with Different Thermal Inactivation Rates in Silver Carp**

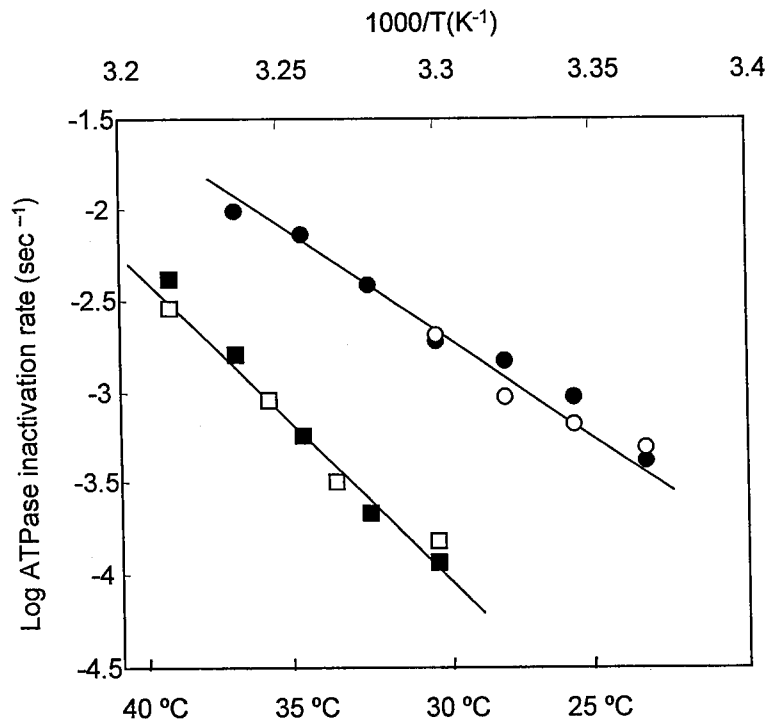
Two types of myosin with different thermal stability were proved to be expressed in winter and summer seasons. As the inactivation profiles for other seasons were not fully studied, it was still uncertain how many types of myosin with different thermal stability were expressed year round. There was no





**Figure 2-9. Ca<sup>2+</sup>-ATPase inactivation of winter- and summer-type silver carp myofibrils at 2 M KCl at low temperatures.**

The inactivation rates in logarithmic values were plotted against the reciprocal of heating temperatures (absolute temperatures). Myofibrils from Feb (●) and Aug (○) were compared.

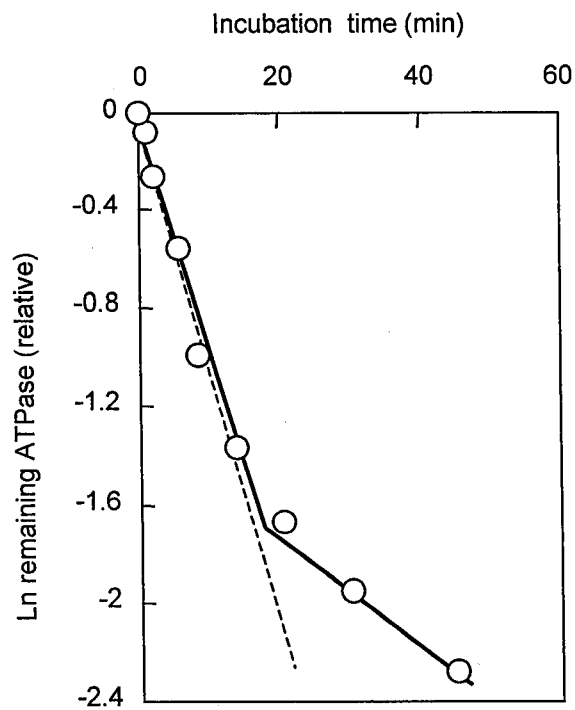


**Figure 2-10. Arrhenius plots of the inactivation rate of Ca<sup>2+</sup>-ATPase of silver carp sampled in China and Japan.**

The conditions were the same as in Fig. 2-6 except the temperatures. The inactivation rates in logarithmic values were plotted against the reciprocal of heating temperatures (absolute temperatures). Myofibrils from summer seasons (■, □) and winter seasons (●, ○) were compared. Closed symbols are sample in China (Shanghai) and open symbols are sample in Japan (Kasumigaura).

information when fish changes the expression of myosin isomers, either. To answer the question, an accurate analysis of the thermal inactivation profile should be done because difference in the inactivation rates was the index to distinguish the myosin isomers. In the previous section, thermal inactivation rate of myofibrils at 2 M KCl was referred to as the rate of myosin itself. However, the method contained a problem to employ for this purpose. As presented in Fig. 2-4, thermal inactivation profile of myofibrils at 2 M KCl was slightly different from isolated myosin in later phase of heating. ATPase inactivation of myofibrils was followed carefully until more than 90% inactivation was achieved (Fig. 2-11). The inactivation profile of myofibrils gave a breaking point at about 80 % inactivation. The inactivation profile in the early phase showed quick inactivation, which was similar to that of myosin obeying the first order reaction. However, the latter phase showed a slow inactivation. The profile with breaking point could be explained in two ways. The first explanation is that even summer type myofibril contains two types of myosin with different stabilities. Another explanation is an incomplete denaturation of actin leaving a small extent of protection in the latter phase. In the latter explanation, a quick decrease phase would come from the inactivation of free myosin detached from F-actin, and the latter slow phase would be produced by myosin bound to F-actin. The latter possibility was probable because Torigai and Konno reported with carp (1997) that salt treatment did not always denature actin completely. It also showed that isolated silver carp summer myosin did not show such a breaking point in the inactivation profile (Fig. 2-4).

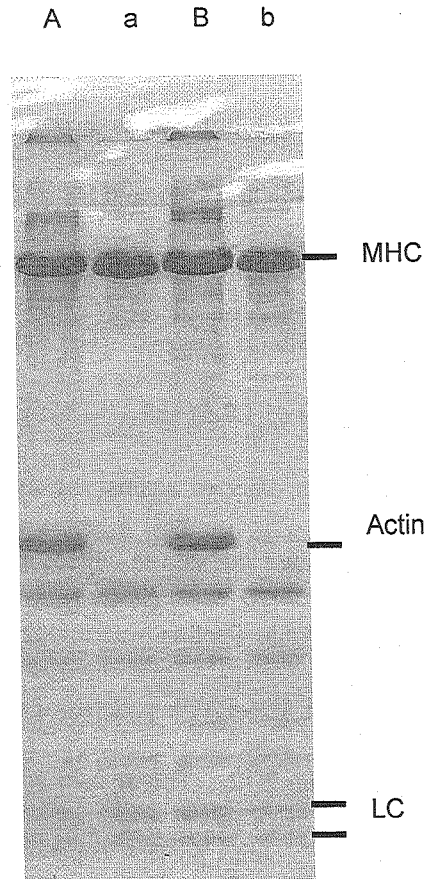
As the purpose of the section is to detect myosin isomers with different stability, the presence of slow phase due to incomplete removal of protection by actin was a serious problem for distinguishing stable myosin isomer and stabilized myosin by F-actin binding. In order to analyze the inactivation rate of myosin, a complete removal of protection by actin was required. As described before, the study with myosin conventionally isolated could not be used because the preparation contained problems of low yield of myosin and partial denaturation during the preparation due to low stability of winter-type myosin. Myosin preparation from myofibrils has been reported using ammonium sulfate fractionation in the presence of Mg-ATP (Koseki et al 1993). The method included a process to remove tropomyosin and



**Figure 2-11. Complete inactivation profile for summer-type silver carp myofibril at 2 M KCl.**

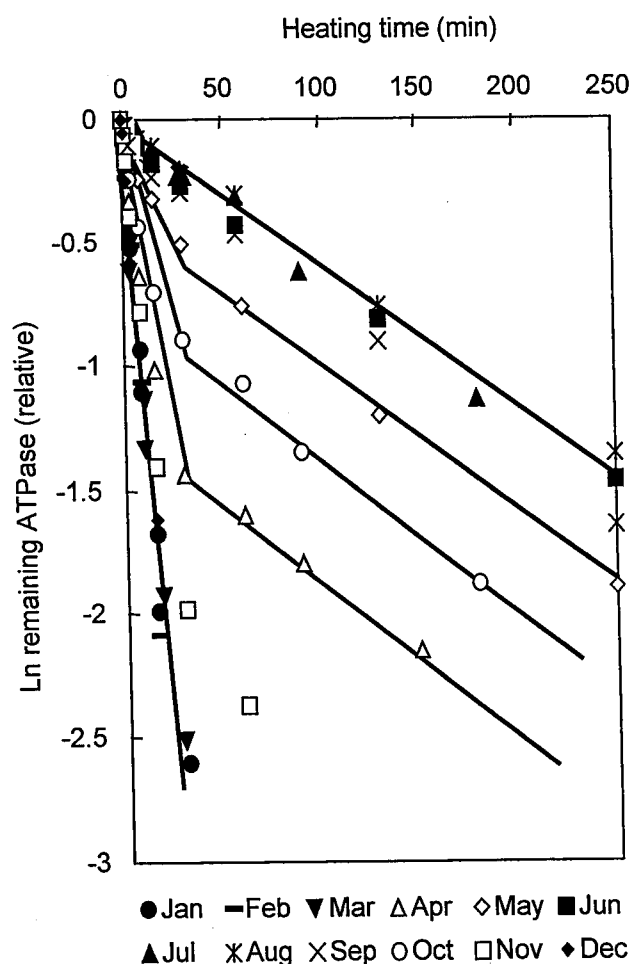
The inactivation profiles of myofibrils prepared in Aug at 35 °C was followed. The inactivation profile of summer-type myosin isolated from the myofibrils was also showed in the figure (---).

troponin from the myosin fraction in addition to the removal of actin. Low yield, and a partial denaturation could not be avoided during these procedures. The preparation method was modified for the purpose in this section. As described in Fig. 2-8, myosin in winter silver carp was too unstable to be dialyzed without loss of ATPase activity. Winter-type myosin in the fraction had to be stabilized by adding stabilizing compound for analysis of the stability. Several compounds were tested such as sorbitol (Ooizumi et al. 1981); Na-glutamate (Ooizumi et al. 1981); and Na-sulfate (Konno 1998). Among them, Na-glutamate at 1 M was chosen considering its high stabilizing effect, low viscosity, and high solubility at low temperature. The established method was as follows. The supernatant at 40 % saturation was dialyzed against 0.5 M KCl, 20 mM Tris-HCl (pH 7.5) and 1 M Na-glutamate for removing ammonium sulfate. Myosin in summer seasons was also dialyzed against the same solution for comparison. The dialysate was directly used as myosin. Because the step to collect myosin as pellet at 55 % saturation leaving troponin and tropomyosin in the soluble fraction was omitted, the myosin fraction contained almost all of tropomyosin and troponin. The SDS-PAGE patterns of myosin prepared from myofibrils in Aug and Feb by this method are shown in Fig. 2-12. The myosin preparation was completely free from actin, but contained almost all of regulatory proteins present in parent myofibrils. This was reasonable because regulatory proteins detach from F-actin in the presence of high concentration of salt (Spudich and Watt 1971). As myosin content in the supernatant at 40 % saturation was practically the same as in starting myofibrils, the yield of myosin by the method was almost 100 %. The SDS-PAGE provided other information about the protein components of myofibrils in two seasons. No difference was detected in the mobility for any bands migrating faster than actin band including components of either light chain or regulatory proteins between Aug and Feb samples (Fig. 2-12). To analyze the light chain composition, myosin preparations as above were dialyzed against low salt medium, and filamentous myosin was collected as pellet leaving water-soluble regulatory proteins in the supernatant. Winter myosin might have lost the activity by the procedures. There was no difference in the mobility for light chain components with summer and winter samples (data not shown).



**Figure 2-12. SDS-PAGE patterns for the myosin prepared from summer and winter myofibril by ammonium sulfate fractionation in the presence of  $Mg^{2+}$ -ATP.**

(A) and (B) are myofibrils in Aug and Feb used for myosin isolation, respectively. (a) and (b) are myosins isolated from (A), and (B), respectively. MHC, LC, and actin are myosin heavy chain, light chain components, and actin, respectively.



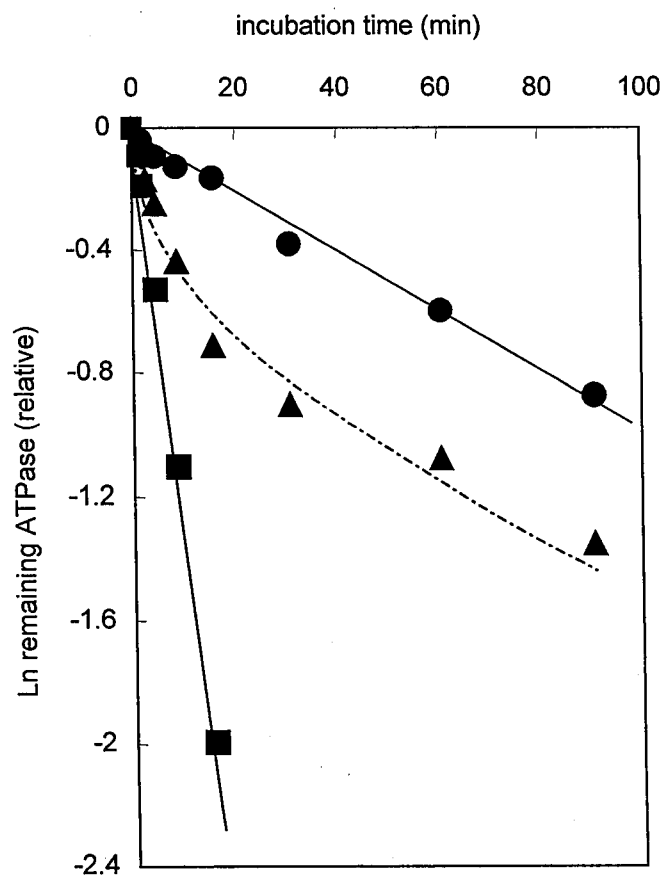
**Figure 2-13. Comparison of the inactivation profile of silver carp myosins in different seasons.**

Myosin was prepared from myofibrils sampled in different seasons by using ammonium sulfate fractionation at 40 % saturation. Myosin samples in 0.5 M KCl, 20 mM Tris-HCl (pH 7.5) and 1 M sodium glutamate were heated at 38 °C. The months sampled are presented in the figure.

As myosin contained 1 M Na-glutamate, heating temperature for inducing ATPase inactivation was raised to 38 °C. It should be noted that the inactivation rates obtained with the samples were the rates of myosin with a strong stabilization by Na-glutamate. A direct comparison of the inactivation rates obtained with those of other species of fish myosin could not be done. As presented in Fig. 2-12, the yield of myosin from winter myofibrils by the method was almost 100 %. This was true for all of myosin prepared year round. ATPase inactivation profiles of myosin in myofibrils prepared monthly are shown in Fig. 2-13.

The inactivation profile for the myosin in Dec, Jan, Feb and Mar was almost straight until almost complete inactivation (about 95 %). The results indicated that there was no detectable contamination of actin and only a single type of myosin existed in the samples. The myosin in Jun, July, Aug, and Sep also denatured according to the first order reaction, and the inactivation rate was much smaller than those for myosin in winter seasons. Difference in the rates between two seasons was about 20 times, which was the confirmation of the results obtained with myofibrils at 2 M KCl as shown in Fig. 2-7. Myosin prepared from the myofibrils in Nov, Apr, May, and Oct showed a breaking point in the inactivation process. The slope in the latter phase for these months was exact same as that one of summer-type myosin, while one for the early phase was a little similar to that of winter-type myosin. Thus, the existence of the breaking point in the profile was assumed a coexistence of summer- and winter-type myosin in the preparation. As the inactivation rates for summer and winter type myosin were estimated from the slopes in two seasons, apparent inactivation profile for the mixture of two types of myosin could be produced if the mixing ratio was assumed. Remaining activity for the mixture could be calculated by using the following equation. Remaining activity =  $\alpha * e^{k_1 t} + (1-\alpha) * e^{k_2 t}$ , where  $k_1$  and  $k_2$  are inactivation rate ( $\text{sec}^{-1}$ ) of summer- and winter-type myosin, respectively;  $\alpha$  is the proportion of summer type myosin in the mixture;  $t$  is the incubation time (sec). The inactivation profile of myosin in Oct was produced by assuming the mixing ratio of these two myosin to be 52:48 (summer-: winter-type myosin) (Fig. 2-14). The ratio of summer-type myosin in logarithmic value could also be easily estimated graphically from the inactivation profile by extrapolating the line of the latter phase to heating time zero. These results indicated that silver





**Figure 2-14. Simulation of the inactivation profile of silver carp myosin prepared from Oct myofibril consisted of summer- and winter-type myosin isomers.**

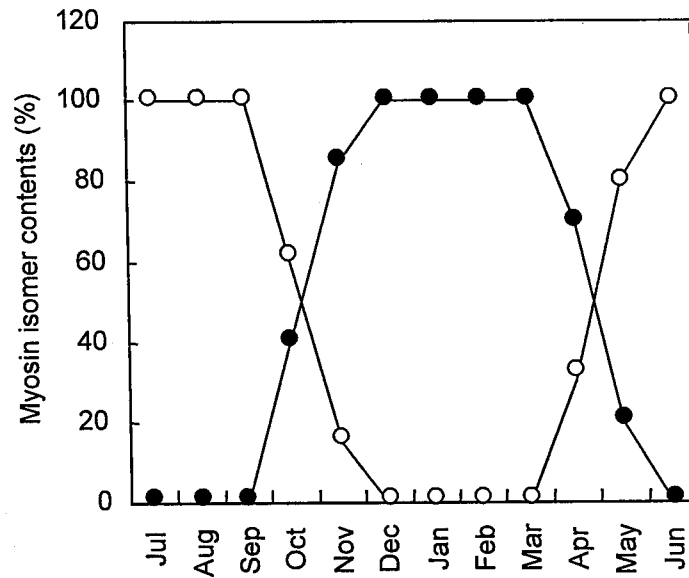
The measured inactivation profiles of myosin prepared from myofibril in Aug (●), Oct (▲), and Feb (■) were shown. Broken line was simulated profile for myosin in Oct myofibrils assuming the mixing ratio of summer- and winter-type myosin (52:48).

carp expresses only two types of isomers, summer- and winter-type when the thermal stability was the index to distinguish myosin isomers.

By extrapolating the latter phase of inactivation profiles to heating time zero, summer- and winter-type myosin contents in every sample were estimated. Their contents are presented in Fig. 2-15. Only summer-type myosin was detected in the samples from Jul to Sep. In Oct, amount of summer-type myosin decreased by a half, and winter-type myosin occupied the rest. Very small amount of summer-type myosin was detected in Nov. From Dec to Mar, only unstable winter-type myosin was detected. Summer-type myosin started to be expressed in Apr. The amount of summer-type increased in May, and no winter-type myosin was detected in June. It was concluded the period for the expression of both winter- and summer-type myosin isomers were 4 months, and two months were required for a full exchange of expression of two types of myosin.

## **2.6 Discussion**

It is well established that carp expresses several myosin isomers adapting to change in water temperature (Imai et al. 1997, Okamoto et al. 2002), and the system is the strategy for carp to survive year round. The system would be similarly working in silver carp because the fish expressed myosin isomers with different stability seasonally. In common carp dorsal muscle, at least three types of isomers were reported to be expressed by studying mRNA analysis (Imai et al. 1997). However, in the study, only two types of myosin were detected with silver carp judging only from the difference in the stability of the expressed myosin, winter- and summer-type myosin. Difference in the thermal stability between two types of silver carp myosin was much larger than that reported with carp (Hwang et al. 1990). The differences for the former and latter species of fish were more than 20 and only 4 times, respectively. These stabilities seemed intrinsic because silver carp caught in Kasumigaura Lake showed exactly the same stability as ones cultured in China. To emphasize the change in the thermal stability, the inactivation rates of summer- and winter-type myosin were compared with the rates for other fish species in the literatures (Murozuka et al



**Figure 2-15. Year-round changes in the contents of two types of myosin in silver carp.**

The amount of summer- (○) and winter-type (●) myosin were estimated from Fig. 2-13.

1976, Arai et al. 1976, Takahashi et al. 2005b). Summer-type silver carp myosin was proved to be as stable as tilapia myosin, and winter-type silver carp myosin was as unstable as Alaska pollack myosin. The myosin in the two fish species is known to be one of the most stable and unstable myosin. Such a large change in the stability with the myosin expressed in silver carp has not reported ever. Unstable winter-type silver carp myosin could not be stored in ice without denaturation. It was also indicated that lowering storage temperature was not successful in suppressing the denaturation; decrease in the temperature from 20 to 0 °C reduced the inactivation rate only by 4 times. The information seemed very useful in storing and processing of silver carp meat. Considering the water temperature of fishpond in Shanghai area in summer (above 30 °C) and winter seasons (near 0 °C), the change in the stability of summer- and winter-type myosin responding to the temperature change seemed reasonable. Moreover, two types of myosin had another system to overcome the temperature change. Summer-type myosin was much more strongly stabilized by F-actin than winter one. Consequently, summer-type myosin under physiological conditions was kept very stable due to the above reasons. Even though winter-type silver carp myosin was as unstable as Alaska pollack one, its stability in myofibril was more stable than Alaska pollack due to greater F-actin stabilization. It was reported that the magnitude of the F-actin stabilization was fish species-specific, and the magnitude was determined by myosin (Takahashi et al. 2005b). Therefore, it was concluded that summer myosin had two systems for surviving in summer seasons; stable myosin structure and high magnitude of stabilization by F-actin.

A careful analysis of myosin stability in the myofibrils preparations demonstrated that the period for the expression of both winter- and summer-type myosin isomers were 4 months, and the exchange of expression of two types of myosin took two months. Summer seasons were from Jun, to Sep, and winter seasons were from Dec. to Mar. In Chapter 1, the gel forming ability of surimi prepared in Jun and Sep were concluded to be identical, and that of surimi samples in Dec and Mar were also the same. The conclusion was reasonable because myosin in Jun. and Sep. are summer-type, and myosin in Dec. and Mar. were winter-type according to the stability of the expressed myosin isomer. Thus, the information

obtained in this chapter was practical information when considering surimi production from the fish species. Such a precaution was not necessary in utilization of marine fish species due to no seasonal changes in the thermal stability. As surimi usually contains polyphosphate to maintain its pH neutral, and polyphosphate is also well established to remove the protection of F-actin even in the presence of 0.5 M NaCl (Yagi and Arai 1986), a careful handling of winter silver carp surimi would be required after salting.

### **Chapter 3.**

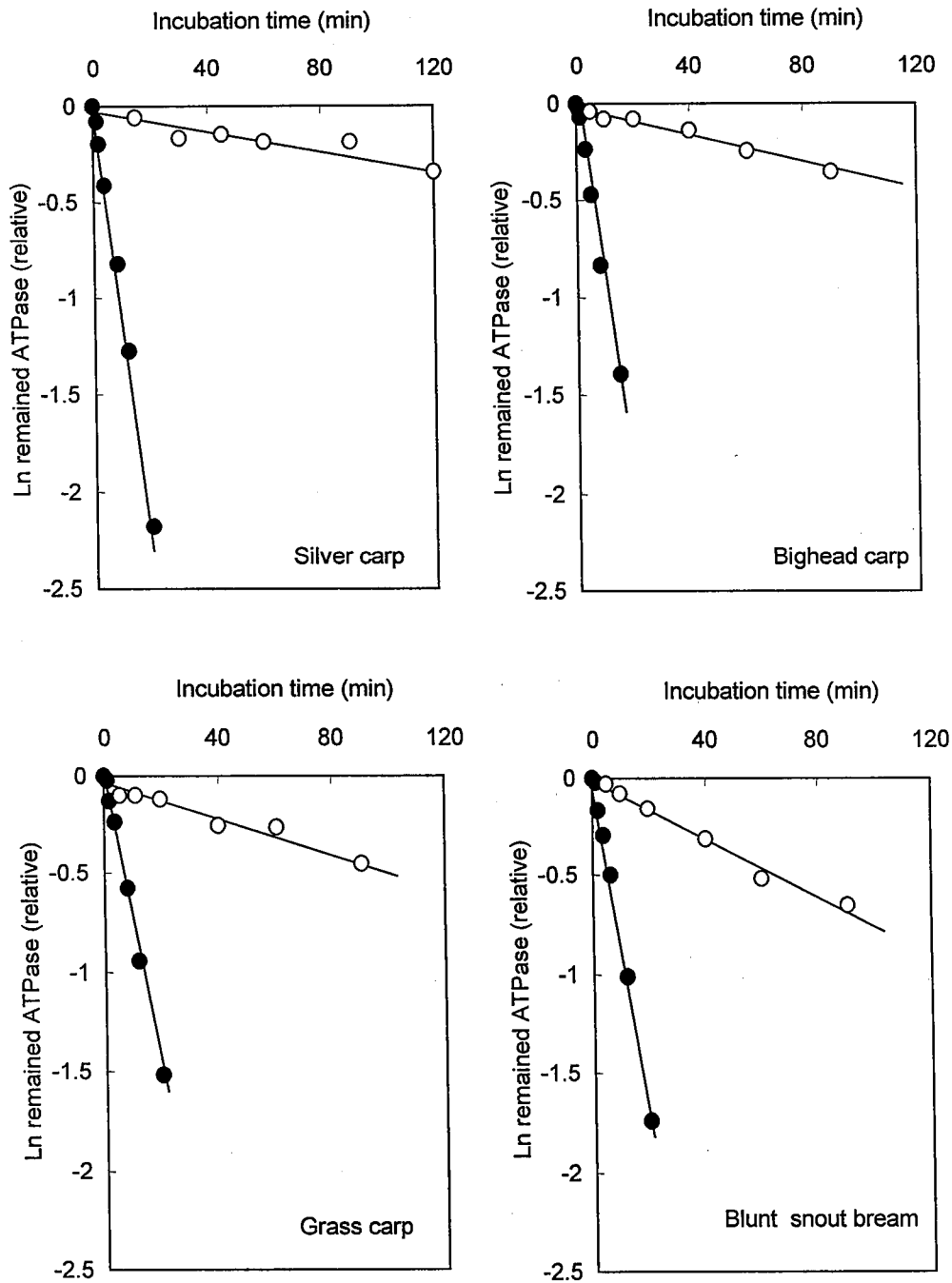
#### **Species-Specific Thermal Stability of Myosin and Its Seasonal Change**

In Chapter 2, the seasonal change in the thermal stability of myosin from silver carp was demonstrated, which was explained by its strategy to adapt to the environmental temperature change. Most of Chinese freshwater fish as well as silver carp are temperate fish, which can tolerate a large temperature change year roundly. It is still uncertain whether the conclusion drawn from the study with silver carp is applicable to other species of freshwater fish. To elucidate the seasonal expression of myosin isomers in other species of fish, same study as Chapter 2 was performed. Four fish species in carp family (bighead carp, grass carp, blunt snout bream, and common carp) and two species in perciformes order (largemouth bass and snakehead) were studied. These species of fish together with silver carp account for more than 80 % of the total harvest of freshwater fish in China. All fish species including silver carp sampled were cultured in the outskirts of Shanghai. It should also be pointed out that Chinese freshwater fish is usually polycultured, namely, various species of fish are cultured in the same pond. Usual combination is either silver carp or bighead carp, either grass carp or blunt snout bream, and common carp. The system is possible because of their different feeding; silver carp and bighead carp are plankton feeder, grass carp and blunt snout bream are herbivorous (weed eater) species, and common carp is omnivorous, feeding mainly on aquatic insects, crustaceans, annelids, mollusks, weed and tree seeds, wild rice, and aquatic plants. However, largemouth bass and snakehead are cultured separately due to their carnivoral feeding habit. Largemouth bass feeds minnows, carp, and any other available fish species including their own young fish. Adult snakehead prey on fish, frogs, aquatic birds and small mammals, while the juvenile snakehead fish prey on earthworms, water bugs, tadpoles, dragonfly larvae and other similar organisms. Snakehead is an air-breathing fish, which can survive out of water from 3 to 7 days. The fish can walk across land to find new sources of food in other lakes and streams by using its pectoral fins (Ge and Cai 2005). The suitable habitat temperature for all the fish species is around 25 °C, even though the optimal temperature is

somewhat species-specific. For all the fish species, they spawn in late spring or early summer. In winter season below 10 °C, they dwell on or near the bottom of fishponds or lake, and stop feeding and growth. The objective of this study is to detect the species-specific thermal stability and the seasonal change in the thermal stability of myosin from the seven fish species.

### **3.1 Comparison of the $\text{Ca}^{2+}$ -ATPase Inactivation Rate of Myofibril at 0.1 M KCl Prepared from Winter and Summer Fish of Seven Species**

Thermal stability of myosin from winter and summer fish for various species was compared. The ATPase inactivation profiles of myofibrils heated at 40 °C are shown in Fig. 3-1. The profiles for silver carp were also shown for comparison. Profiles differed from each other among fish species. Big head carp, grass carp, and blunt snout bream as well as silver carp showed a large difference (more than 20 times) in the ATPase inactivation rate of myofibril between winter and summer seasons. However, common carp showed a small difference (less than 4 times) in the inactivation rates, which was consistent with the report by Hwang et al. (1990). For snakehead and largemouth bass, there was no obvious change in the thermal stability of myofibrils between summer and winter seasons. The inactivation rates of summer and winter myofibrils are listed in Table 3-1. Thermal stabilities of myofibrils in summer season were rather similar to each other among fish species. The most stable and unstable species were silver carp and blunt snout bream with a difference of only 4.7 times. However, the inactivation rate of myofibrils in winter season showed much larger difference among species. Difference between the most stable and unstable fish species was about 27 times. Four species of carp family species except common carp showed a large seasonal change in the stability of myofibril. However, snakehead and largemouth bass showed a characteristic behavior. As snakehead and largemouth bass are cultured under similar water temperature as carp family, the water temperature seemed not determine the thermostability of myofibril for these species. Species-specific thermal stability of myofibrils in summer and winter seasons were also studied by changing heating temperatures, and the inactivation rates were used to make Arrhenius



**Figure 3-1-1. comparison of thermal inactivation profiles between summer and winter myofibrils from various fish species.**

The inactivation profiles of myofibril in Aug (○) and Feb (●) were compared at 40 °C. Fish species was presented in the figures.



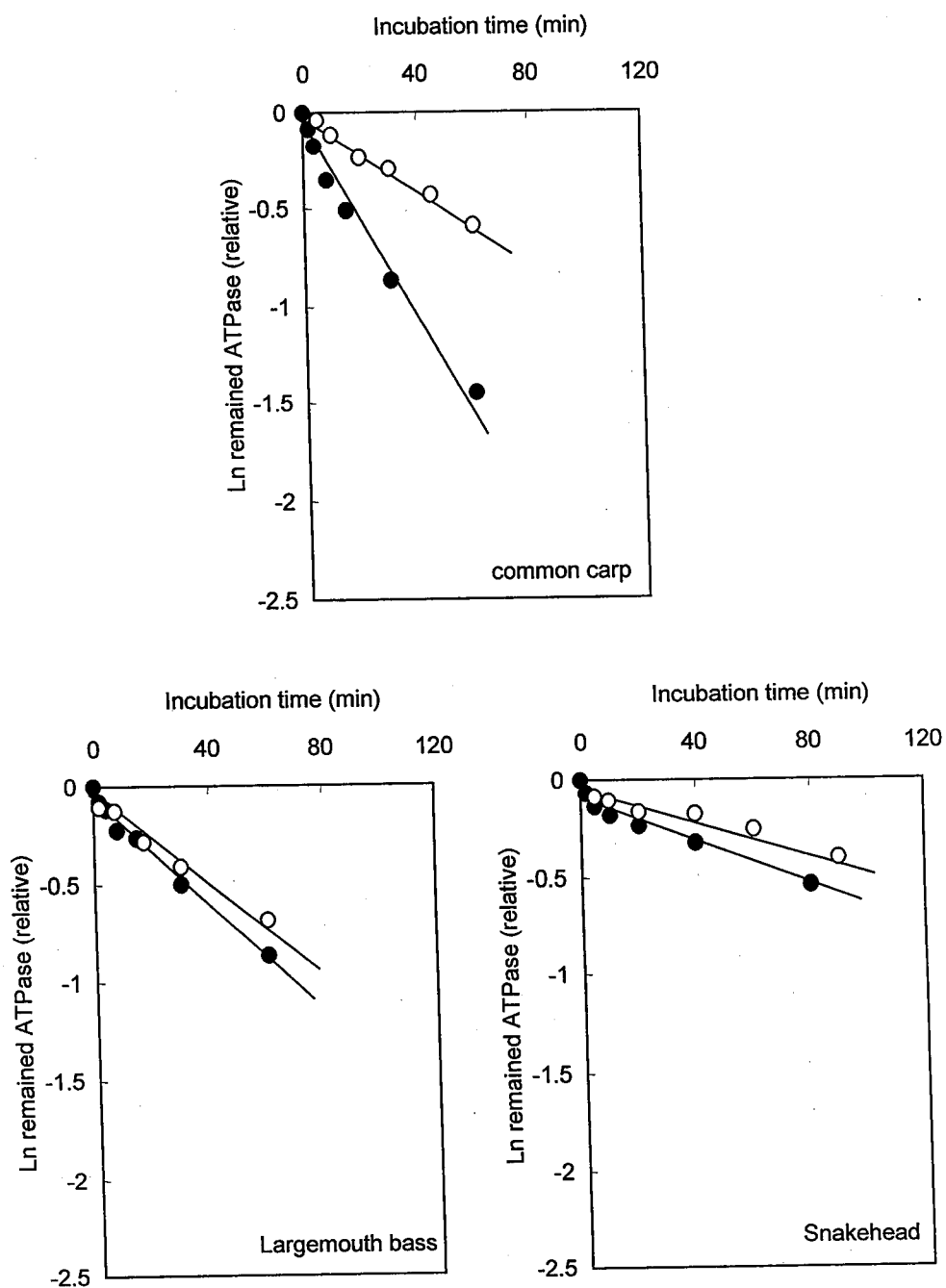


Figure 3-1-2. Comparison of thermal inactivation profiles between summer and winter myofibrils from various fish species.

The inactivation profiles of myofibril in Aug (○) and Feb (●) were compared at 40 °C. Fish species was presented in the figures.

**Table 3-1. Ca<sup>2+</sup>-ATPase inactivation rate of myofibrils of summer and winter season at 0.1 M and 2 M KCl.**

KCl	ATPase inactivation rate (X 10 <sup>5</sup> ) (sec <sup>-1</sup> )						
	0.1 M*1			2 M*2			
	Fish species	Summer	Winter	Ratio*3	Summer	Winter	Ratio*3
Silver carp	4.2	208.0	49.5	6.9	220.0	32.0	
Bighead carp	4.1	198.0	48.3	6.5	184.0	28.3	
Grass carp	7.9	198.0	25.1	6.7	225.0	33.6	
Blunt snout bream	15.2	165.0	10.8	16.2	183.0	11.3	
Common carp	17.6	45.8	2.6	17.1	40.5	2.4	
Largemouth bass	19.6	22.7	1.2	8.6	32.8	3.8	
Snakehead	6.3	7.6	1.2	8.7	55.6	6.4	

\*1. Myofibrils were heated at 40 °C.

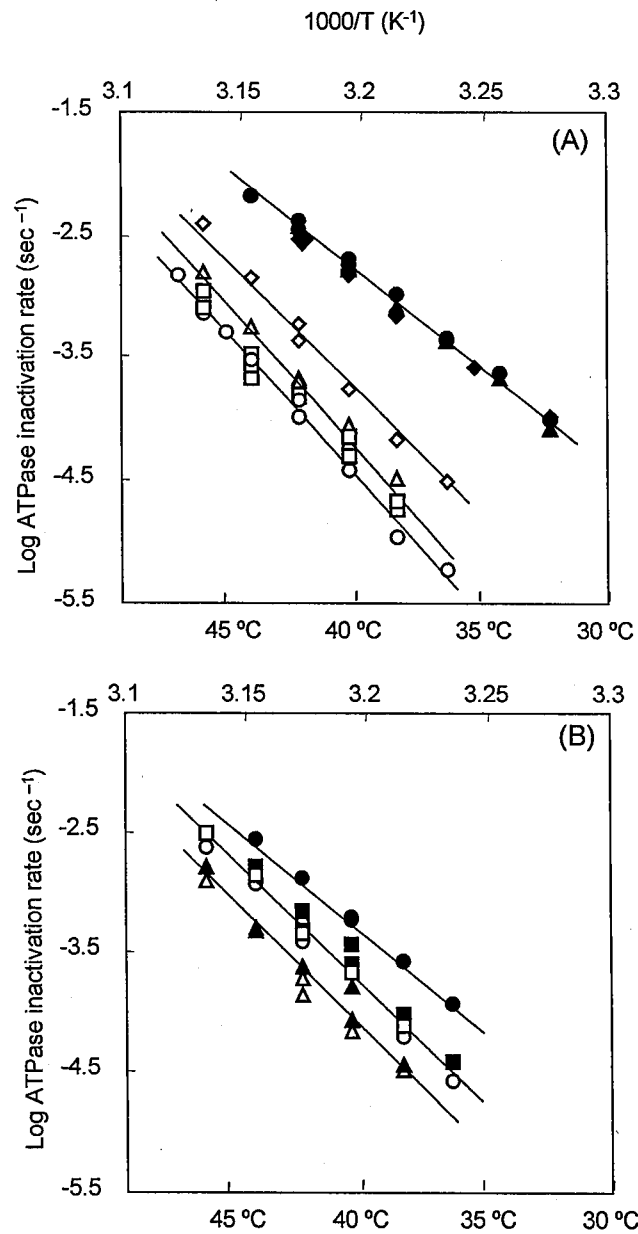
\*2. Myofibrils were heated at 30 °C.

\*3. The ratios were calculated as (Rate for winter/Rate for summer) .

plot (Fig. 3-2). Activation energies ( $E_a$ ) for the thermal denaturation with myofibril were estimated from the slope (Fig. 3-2).  $E_a$  of these fish species are listed in Table 3-2. Fish species were classified into three groups from the magnitude of the difference in the thermal stability between winter and summer season. The first group included four fish species (silver carp, bighead carp, grass carp, blunt snout bream), which was characterized by a large difference. The inactivation rates of myofibrils in winter season for these four species were identical, while there were some differences in summer season. The second one was common carp with a small difference in the inactivation rate between two seasons. The third one contained snakehead and largemouth bass. The group was very characteristic because no difference in the thermal stability of myofibril between two seasons. On the other hand, the activation energy ( $E_a$ ) for each group are similar. The mean values of  $E_a$  for each group are 93.0, 87.6, and 88.9 kcal/mol in summer, and 67.7, 85.8, and 91.5 kcal/mol in winter fish myofibrils. It is demonstrated that  $E_a$  in summer season were very similar among species, while there are large changes in winter. These results indicated that the first group fish decreased the activation energy in winter, while the other fish species still kept the activation energy unchanged.

### **3.2 Comparison the $Ca^{2+}$ -ATPase Inactivation Rate of Myofibril at 2 M KCl for Seven Species of Fish in Winter and Summer Seasons**

Thermal stability of myosin itself in summer and winter seasons for the above species was further compared. As described in Chapter 2, ATPase inactivation rate of myofibril at 2 M KCl was referred to as the thermal stability of myosin itself. These profiles are shown in Fig. 3-3. Heating temperature was set at 30 °C. Comparing the differences in the inactivation rates between summer and winter for the same species, the magnitudes at 2 M KCl were not always coincident with those at 0.1 M KCl. Four fish species belonging to the first group classified in the former section showed a large difference in the inactivation rates even with myosin (more than 10 times). Common carp showed a little difference in the inactivation rates (about 3 times) than that obtained with myofibrils at 0.1 M KCl. With snakehead

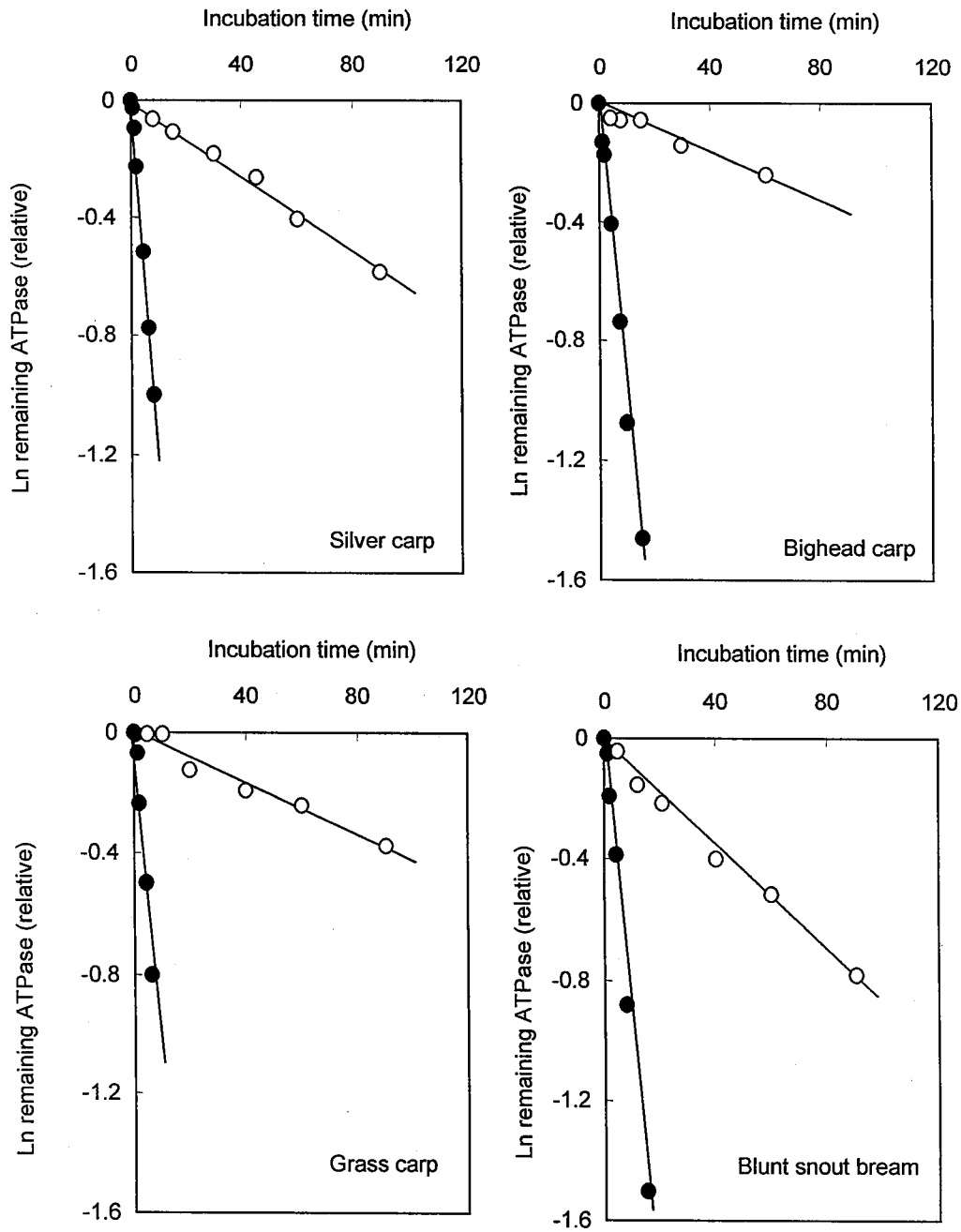


**Figure 3-2. Arrhenius plots of thermal inactivation rate of Ca<sup>2+</sup>-ATPase of summer and winter myofibrils from various species of fish.**

The inactivation rates in logarithmic values were plotted against the reciprocal of heating temperatures (absolute temperatures). Myofibrils from winter (closed symbols) and summer (open symbols) were compared. Heating was conducted at 0.1 M KCl, 20 mM Tris-HCl (pH 7.5). Fish species were indicated in the figure. Fish species in (A) are silver carp (●, ○), bighead carp (■, □), grass carp (▲, △), blunt snout bream (◆, ◇), and fish species in (B) are common carp (●, ○), largemouth bass (■, □), and snakehead (▲, △).

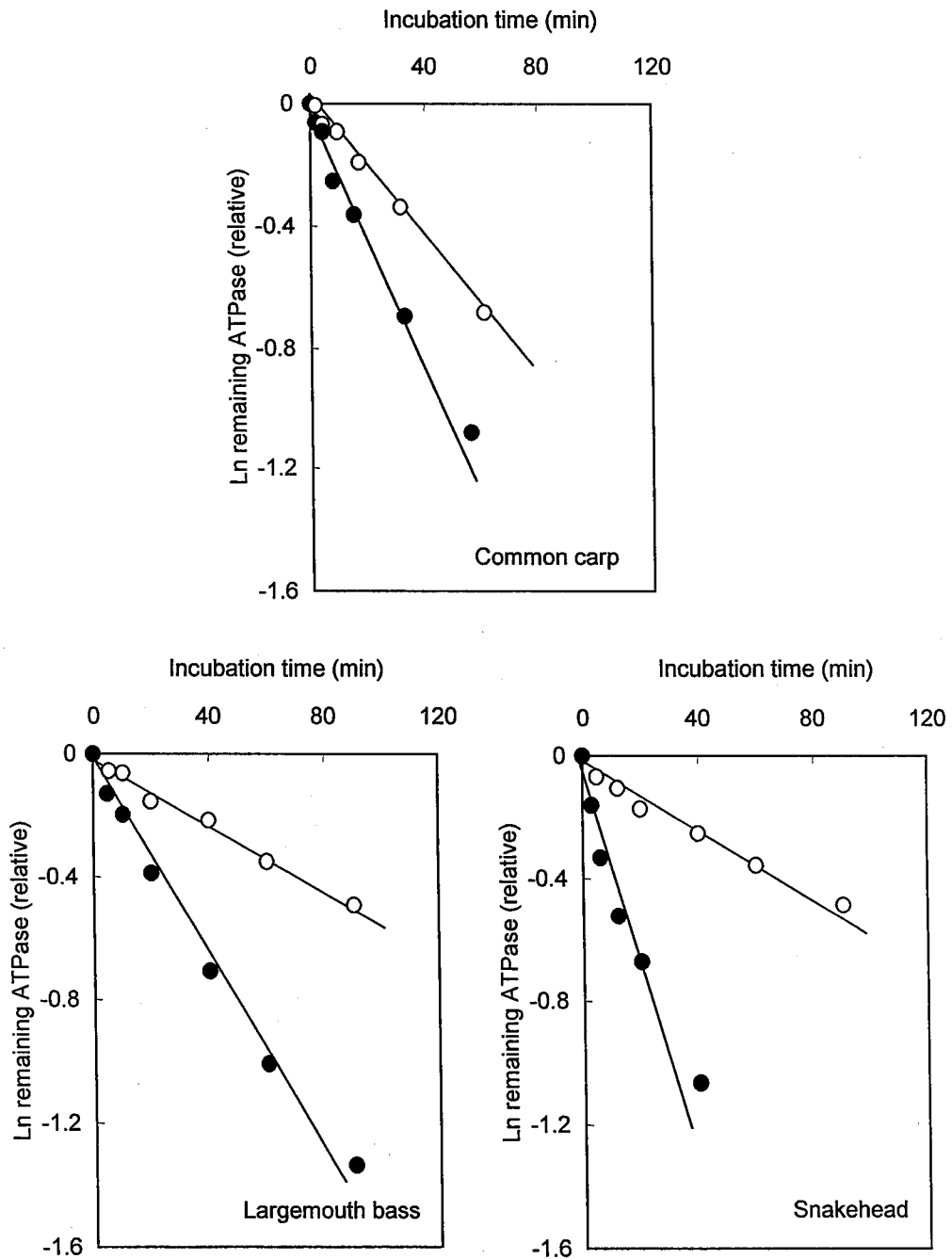
**Table 3-2. Activation energy for the thermal inactivation of summer and winter myofibril at 0.1 M and 2 M KCl.**

KCl concentration	Activation energy (kcal/mol)			
	2 M KCl		0.1 M KCl	
	Summer	Winter	Summer	Winter
Silver carp	88	45	93	68
Bighead carp	84	51	93	70
Grass carp	88	45	93	68
Blunt snout bream	87	42	93	66
Common carp	79	73	88	86
Largemouth bass	88	98	88	92
Snakehead carp	78	69	92	92



**Figure 3-3-1. Comparison of thermal inactivation profiles between summer and winter myosin from various fish species.**

The inactivation profiles of myofibril at 2 M KCl in Aug (○) and Feb (●) were compared at 30 °C . Fish species was indicated in the figures.



**Figure 3-3-2. Comparison of thermal inactivation profiles between summer and winter myosin from various fish species.**

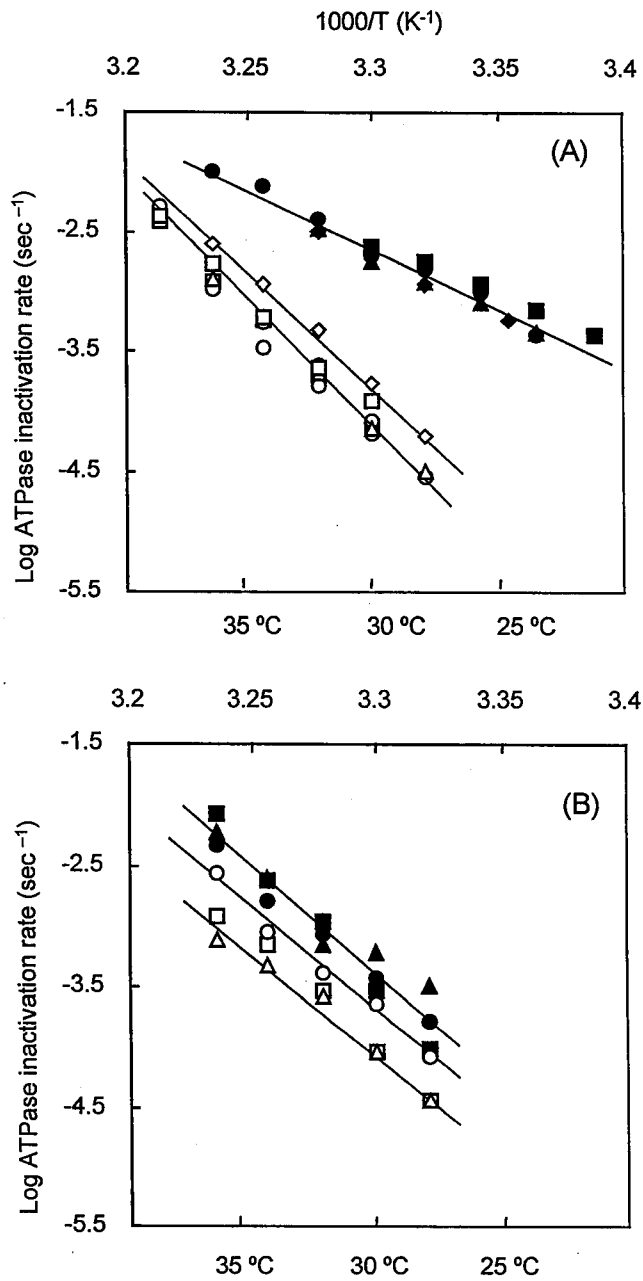
The inactivation profiles of myofibril at 2 M KCl in Aug (○) and Feb (●) were compared at 30 °C . Fish species was indicated in the figures.

and largemouth bass, a quite large difference was observed for myosin although the difference with myofibrils at 0.1 M KCl was negligible. The difference in the inactivation rates between two seasons was about 5~7 times for snakehead and largemouth bass. Only 3 times difference was obtained with common carp. The inactivation rates for myosin and myofibrils in two seasons with seven species were summarized in Table 3-1. Difference in the stabilities of myosin among fish species was also compared in summer and winter seasons. For summer myosin, the difference was only 2.6 times, while the difference was 6.9 times for winter myosin. The differences obtained with myosin were much smaller than those obtained with myofibrils. However, a similar tendency that a large difference in winter season and a small difference in summer season among species was again observed.

These differences in the thermal stabilities were confirmed by changing heating temperatures. The rates are presented as Arrhenius plot (Fig. 3-4). Activation energies ( $E_a$ ) for the thermal denaturation with myosin calculated from the slopes of Arrhenius plot are shown in Table 3-2. Similar result as with myofibril was obtained. Generally,  $E_a$  with myosin was a little lower than that with myofibril, and  $E_a$  with both myosin and myofibril in winter was much smaller than that in summer. However, largemouth bass was exceptional, of which  $E_a$  (98 kcal/mol) for winter myosin was oddly a little larger than summer one. All of myosin in summer fish showed large  $E_a$  around 80 kcal/mol. However, large difference was observed among fish species in winter. The first group (silver carp, bighead carp, grass carp, blunt snout bream) showed very small  $E_a$  (45.2 kcal/mol).  $E_a$  for both common carp and snakehead myosin showed a similar  $E_a$  of 70 kcal/mol (winter) and 78 kcal/mol (summer), respectively.

As presented in Table 3-1, snakehead and largemouth bass were unique because their thermal stability of myosin was different between summer and winter, while the stability at 0.1 M KCl was practically the same in two seasons. As described previously (Fig. 2-5), stabilization of myosin by F-actin (the difference in the inactivation rates between at 0.1 M KCl and 2 M KCl) for summer silver carp was greater than that for winter silver carp. To understand the difference in the extent of protection by F-actin between myosin in two seasons for various species of fish, KCl concentration dependent thermal inactivation rates were





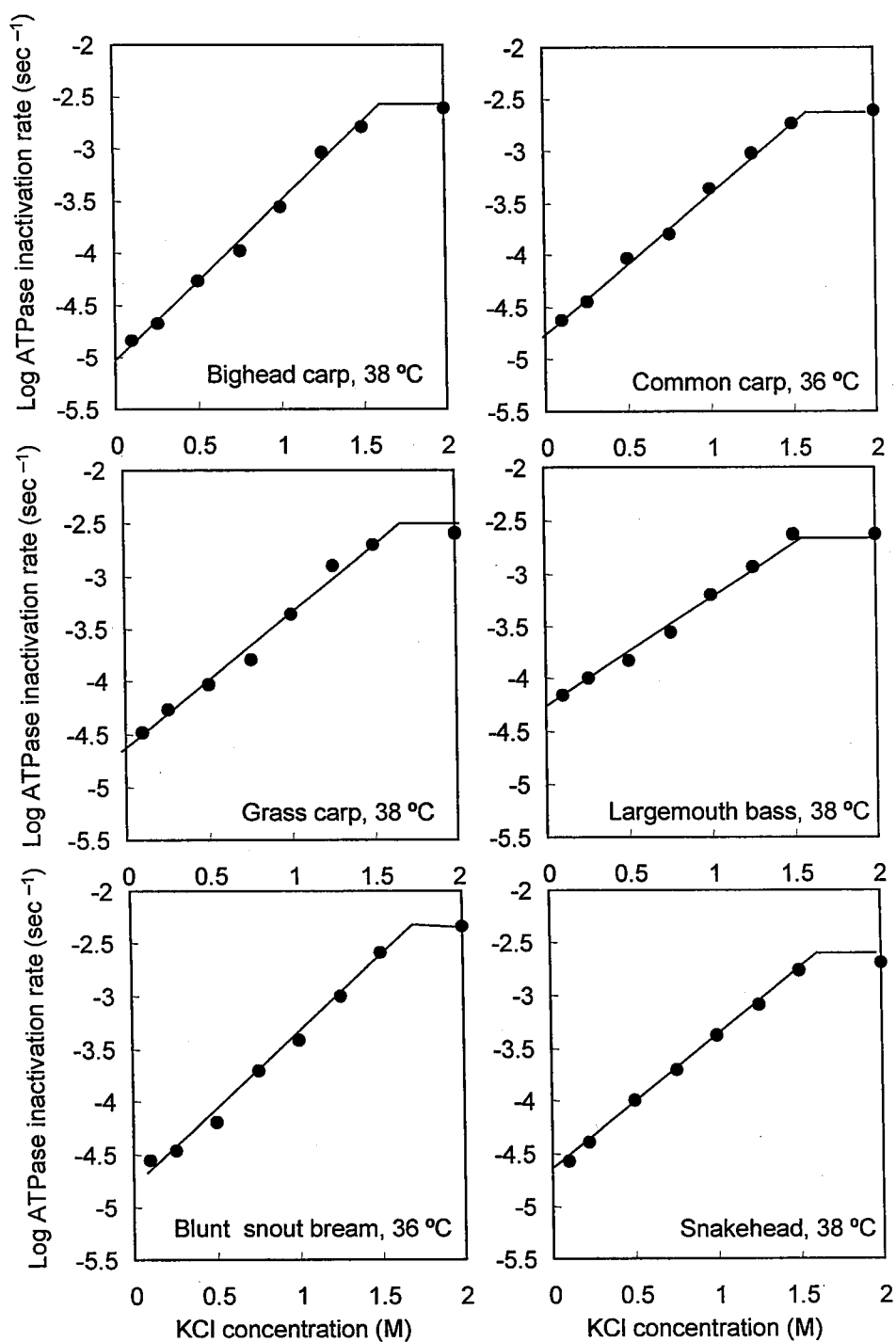
**Figure 3-4.** Arrhenius plots of thermal inactivation rates of  $\text{Ca}^{2+}$ -ATPase of summer and winter myosins from various species of fish.

Thermal inactivation rates of myofibrils at 2 M KCl from winter (closed symbols) and summer (open symbols) were compared. Symbols for indicating fish species were the same as in Fig. 3-2.

compared (Fig. 3-5). The extents of stabilization by F-actin estimated from Fig. 3-5 were listed in Table 3-3. It was common for the first group including silver carp, bighead carp, grass carp and blunt snout bream that myosin in summer season underwent much greater stabilization by F-actin, while less stabilization in winter season. There were some differences in the stabilization by F-actin with myosin in summer season, but no difference in the stabilization with one in winter season. In the case of common carp, little difference in the extent was observed between summer and winter seasons. In contrast, winter snakehead and largemouth bass underwent much greater stabilization by F-actin than summer ones. The reversed stabilization extent in two seasons made the same stability with myofibrils. Thermal stability of myosin under physiological conditions is important for surviving. Snakehead and large mouth bass took the strategy to keep the thermal stability unchanged year round. Therefore, another mechanism rather than temperature dependent thermal stability change of myosin is needed to understand the adaptation of the fish species to year round temperature change.

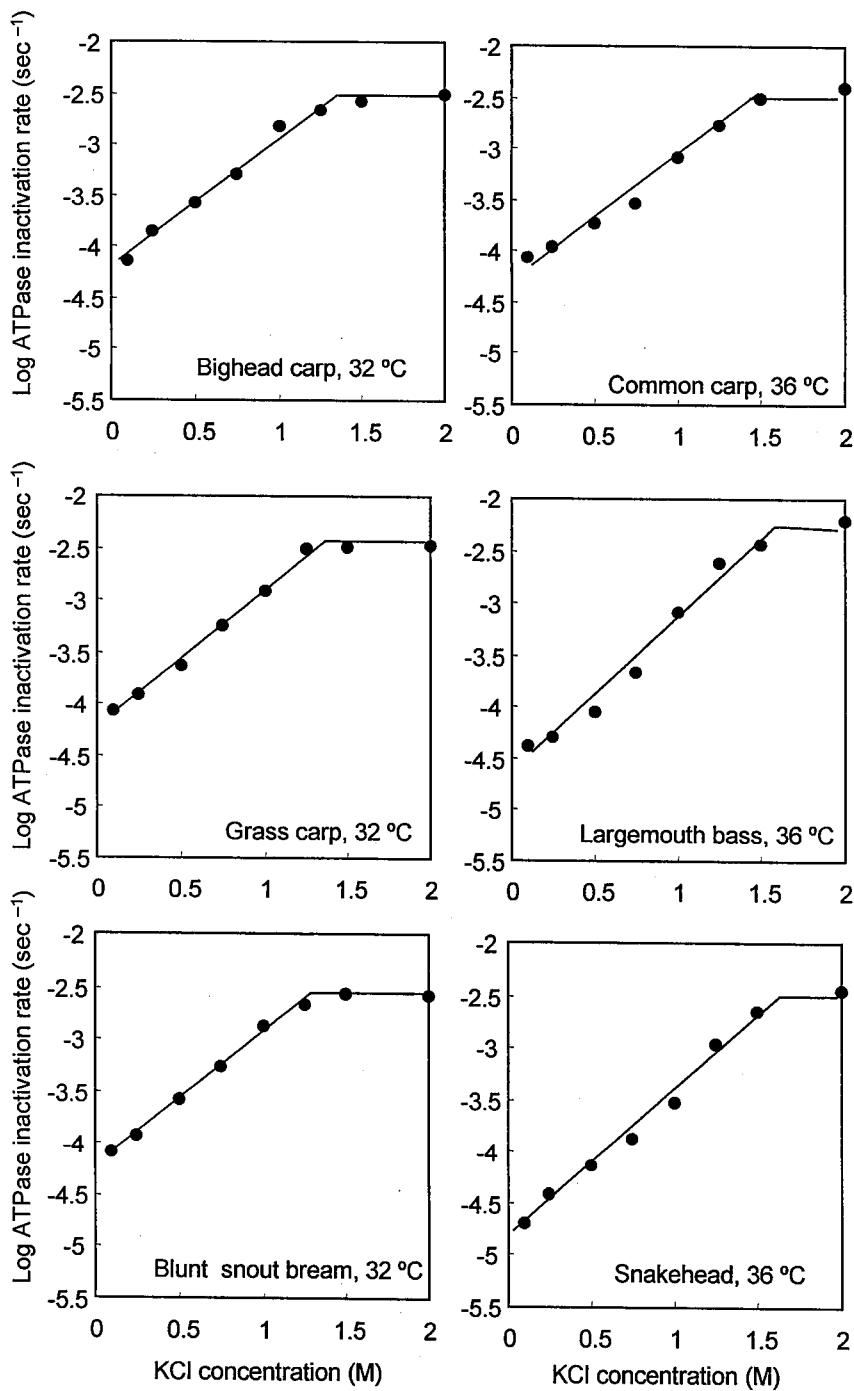
### **3.3 Seasonal Expression of Winter and Summer Type Myosin in Various Species of Fish**

In Chapter 2, it was demonstrated that silver carp expressed two types of myosin isomers with different thermal stabilities year round (Fig. 2-15). They were termed winter-type and summer-type myosin. As the species-specific different stabilities in winter and summer fish were confirmed with various species, it is important to know when myosin isomer expression took place year-roundly. Adaptation of common carp to temperature change was exclusively studied. Comparing the thermal denaturation rates between two seasons with myosin from various species of fish, seasonal expression of myosin isomers might be a general feature among species. To analyze the expression of myosin isomers, the same method of thermal inactivation profile analysis with myosin was employed as used for silver carp in the Chapter 2. Preparative method of myosin using ammonium sulfate fractionation in the presence of ATP-Mg was successfully applied to all of samples. Purity and recovery of myosin by the method for these fish species in all seasons were checked by SDS-PAGE. The recovery of myosin for all samples was about 100%.



**Figure 3-5-1. Comparison of KCl concentration dependent  $\text{Ca}^{2+}$ -ATPase inactivation rate among summer myofibrils from various species of fish.**

Fish species and heating temperatures are indicated in the figures.



**Figure 3-5-2. Comparison of KCl concentration dependent Ca<sup>2+</sup>-ATPase inactivation rate among summer myofibrils from various species of fish.**

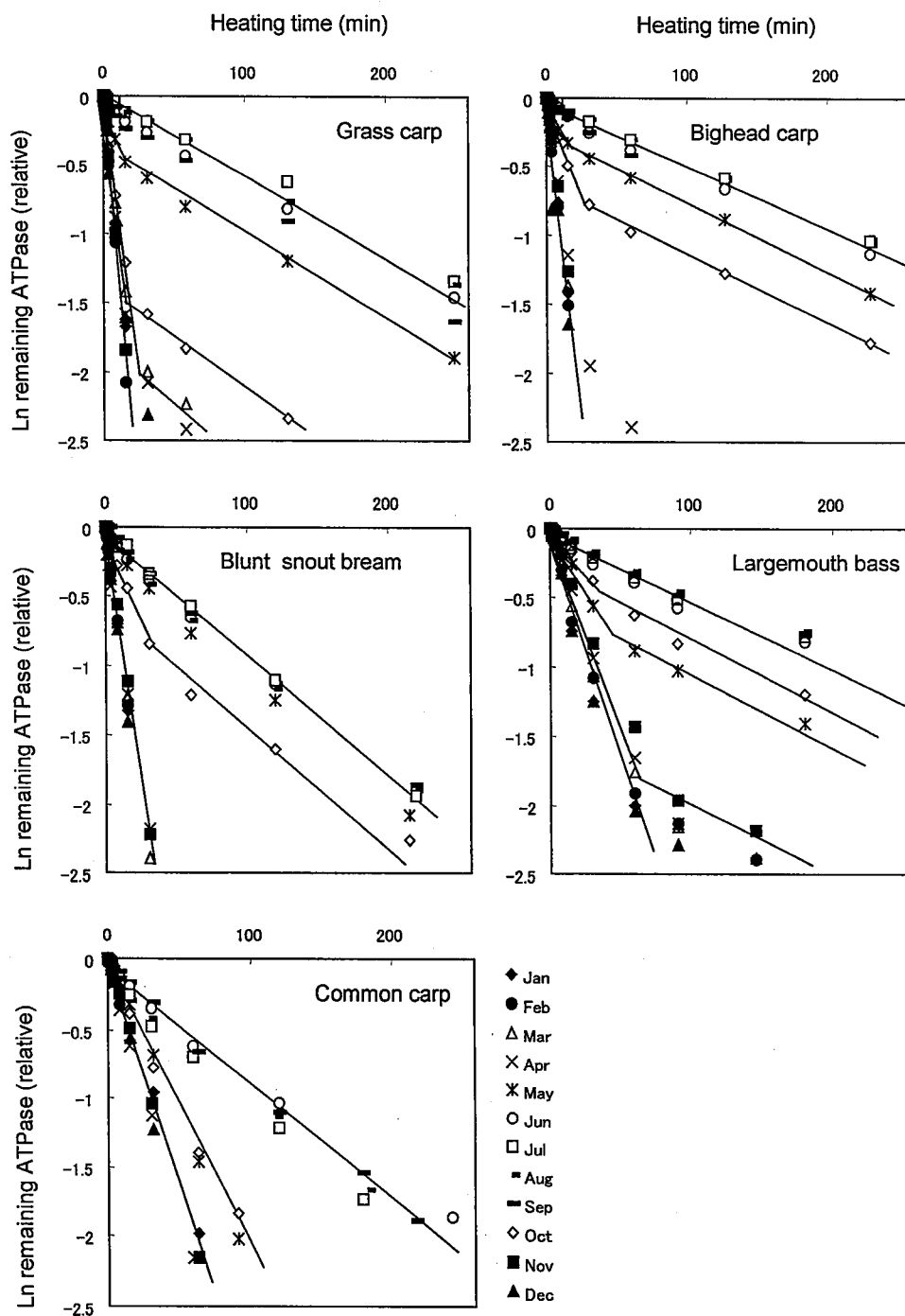
Fish species and heating temperatures are indicated in the figures.

**Table 3-3. The extent of stabilization by F-actin for various species of fish myosin.**

	Summer *	Winter*
Silver carp	200	30
Bighead carp	200	30
Grass carp	100	30
Blunt snout bream	100	30
Common carp	60	40
Largemouth bass	30	180
Snakehead	50	180

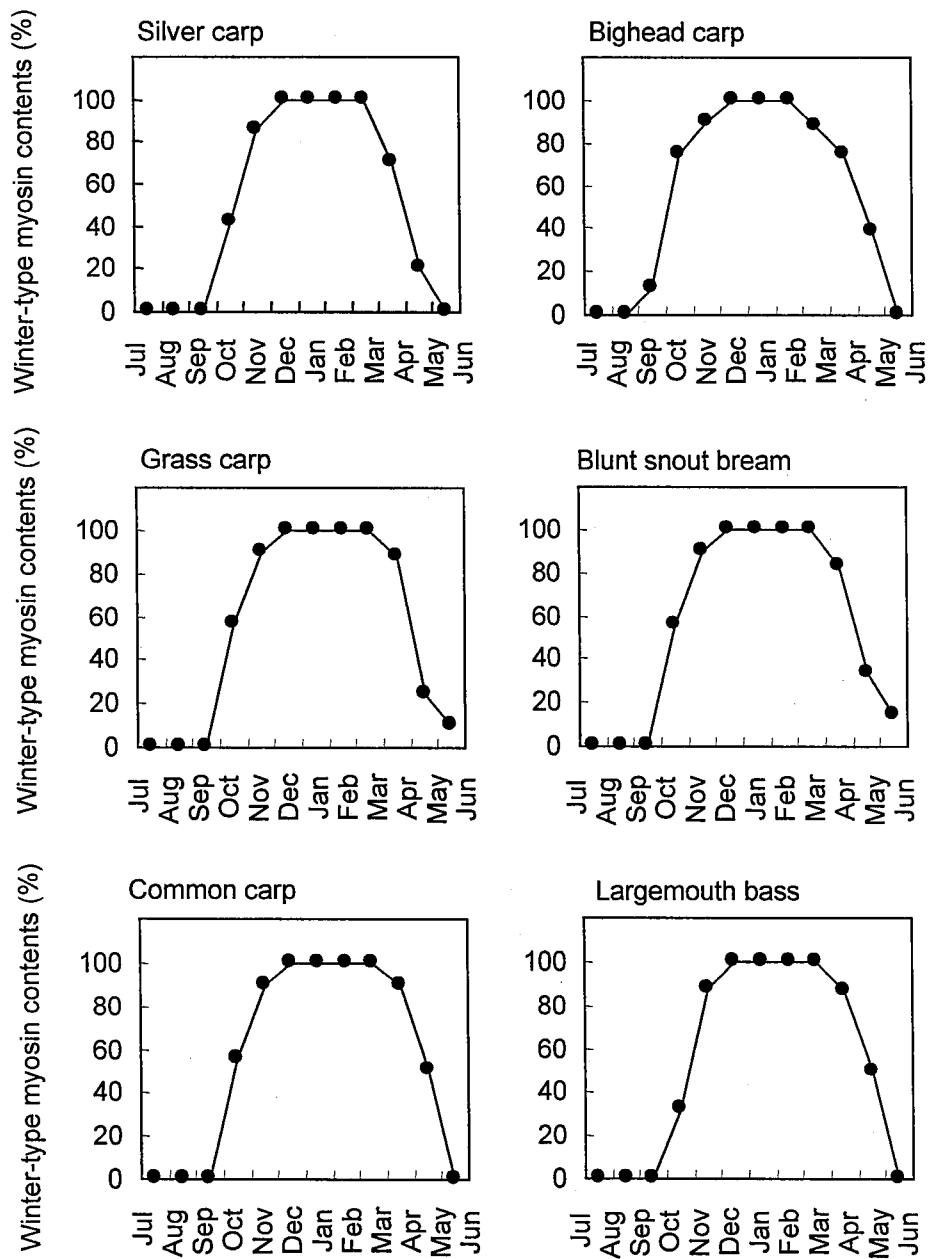
\*Stabilization at the temperatures was estimated from Fig. 2-5 and Fig. 3-5

Myosin preparation from myofibrils in two seasons showed no difference in the polypeptide compositions as studied by SDS-PAGE (data not shown). ATPase inactivation profiles of myosin prepared monthly are shown in Fig. 3-6. General profiles for bighead carp, grass carp, and largemouth bass were very similar to those of silver carp. Samples in winter and summer seasons gave a quick and slow inactivation profiles, respectively. All myosin prepared from the myofibrils in intermediate season (Nov, Apr, May, and Oct) showed a breaking point in the inactivation profiles, which was the same phenomenon observed with silver carp. The slopes in latter phases were similar to those of summer-type myosin. It was again concluded that only winter- and summer-type myosin were expressed for all of species judging from the thermal stability. Mixing ration of these two myosin isomers were simply estimated by extrapolating the line of latter phase to heating time zero as used with silver carp. However, the method was not well applied for common carp because the difference in the thermal stabilities between summer and winter were too small (only about 3 times) to analyze. The winter- and summer-type myosin contents for common carp were estimated by using inactivation profile analysis as described in Chapter 2. Inactivation profiles were simulated by changing the mixing ratios of these two isomers, and the ratio which gave best fit to the experimental data was obtained. Seasonal changes of winter-type myosin contents for six fish species are presented in Fig. 3-7. All of species expressed winter-type myosin in the same months, and the patters were exactly the same as that of silver carp. Summer-type myosin was detected from Jul to Sep (4 months) for all species studied. In Oct, expression of summer-type decreased by about half, and winter-type myosin occupied the rest. Summer-type myosin decreased in Nov. From Dec to Mar (4 months), only winter-type myosin was detected. Summer-type myosin started to be expressed again in Apr. The amount increased in May. All of myosin expressed in June was summer-type. Although there were several differences in the denaturation profiles in expressed myosin among fish species, expression pattern of myosin isomers were the same. Probably water temperature regulated myosin isomer expression for all species as reported with common carp. It should be noted that exchange in the expression of myosin isomer with different stabilities is the common strategy for freshwater fish species for



**Figure 3-6.  $\text{Ca}^{2+}$ -ATPase inactivation profiles of myosins prepared from various species of fish in different seasons.**

The method was same as Fig. 2-13. The myosin prepared from myofibrils by using ammonium sulfate fractionation was used. Fish species and sampling month are presented in the figures.



**Figure 3-7. Year-round changes in the contents of winter-type myosin in various species of fish.**

The amounts of winter-type myosin were estimated from Fig. 3-6. Fish species are presented in the figure.



surviving, although the thermal stability of myosin expressed was species-specific.

### **3.4 Discussion**

Year round expression of myosin isomers in dorsal muscle of seven freshwater fish species cultured near Shanghai was analyzed. All fish species studied tolerate a large temperature change from nearly 0 °C in winter season to above 30 °C in summer season. Freshwater fish, especially carp family are usually polycultured in the same fishpond. Accordingly, several species of fish in the pond underwent the same water temperature change year round. Freshwater fish grow faster in summer seasons, while they almost stop growing in winter seasons. A very low activity in winter season is a characteristic of freshwater fish, which is completely different from cold-water marine fish species that move actively for feeding. Therefore, the adaptation of fish to environmental temperature for freshwater fish should be considered differently from adaptation of marine fish.

Analysis of the thermal inactivation of myosin and myofibrils in winter and summer seasons for various species of fish revealed that all of freshwater fish expressed two types of myosin isomers seasonally. Myosin isomers with different stability were not distinguished by SDS-PAGE analysis because no different subunit compositions were detected for all of species between two seasons. Thermal stability was a sensitive index to distinguish two types of myosin in the preparation. Comparing the thermal stability of myosin in summer and winter for fish species, species were divided into three groups by the extent of difference in the thermal stability. The first group including carp family except common carp exhibited a large difference. The second group of common carp showed a small difference (about 4 times). The last group of snakehead and largemouth bass showed the identical stability for myofibrils from summer and winter fish. Similarly, stabilization extent of myosin by F-actin distinguished among fish species. Species belonging to the first group showed a larger extent of stabilization for summer myofibrils than that for winter ones. Summer myosin in the group was doubly stabilized; intrinsically stable myosin with greater stabilization by F-actin. This was not a common strategy for freshwater fish to survive. Myosin

in the second one showed the similar stabilization between two seasons. The third group were different from the above two. Winter myosin was stabilized by F-actin much greatly than summer myosin. The greater stabilization by F-actin in winter myosin compensated the less stability of myosin itself, resulted in that winter myofibrils were as stable as ones in summer. When the adaptation of fish to water temperature was discussed, thermal stability of myosin bound to F-actin at physiological conditions (stability of myofibrils) should be considered. Thus, the identical thermal stability of myofibrils in summer and winter was the consequence for the species for surviving. Compared with other species of fish, myosin in winter season for the third group was extraordinary stable. The fact is contradict with the idea that fish adapt to low temperature by altering myosin conformation more flexible, namely less stable structure (Johnston and Walesby 1977; Goldspink and Penney 1982). The story is well adapted to marine fish species that stays at the certain temperature year round. However, the story could not be adapted to freshwater fish species. It is certain that stable structure of myosin in winter season is unfavorable for an active movement at low temperature. The problem seems not serious because their activity in winter season was significantly suppressed and fish are dormancy staying on the bottom quietly without feeding. Stable structure of myosin for these species might not be problem for just staying quietly on the bottom of fishpond. However, fish such as silver carp, bighead carp, grass carp and blunt snout bream express unstable myosin in winter season comparable to that of Alaska pollack. However, the structure is not the essential factor for surviving because the fish are also inactive in winter. From the results, it was proposed that adaptation at high temperature in summer season is the event of water temperature adaptation for freshwater fish species.

It was proved that all fish species exchanged the expression of myosin isomers in the identical month. This fact was reasonable assuming that water temperature is the factor to regulate the expression. Expression of myosin isomers controlled by temperature is well established with common carp, in which gradual lowering or raising water temperature exchanges the expression of isomers (Imai et al. 1997). It has been reported that common carp expresses three types of myosin isomers (10 and 30 °C and

intermediate types) as detected by mRNA analysis (Imai et al. 1997). A further study is needed to answer the questions whether only two isomers were expressed actually in dorsal muscle of silver carp, the third type myosin isomer shows the same stability as either of two isomers, or the amount of third type myosin expressed is too small in quantity to detect by the less sensitive method of thermal stability analysis.

## Chapter 4.

### Thermal Denaturation Mode of Fish Myosin from Seven Species

In the former chapters, thermal stability of myosin from seven species of freshwater fish was compared by measuring their thermal inactivation of  $\text{Ca}^{2+}$ -ATPase. All of fish express two isomers with different stabilities seasonally. The month of expression exchange was identical probably because the expression was turned on by the water temperature change. Even though all fish underwent the same water temperature change, the stability of myosin expressed differed from species to species. The difference in the stability was remarkable for fish in winter season. Therefore, it is concluded that thermal stability of fish myosin was intrinsic, and habitat water temperature seems not the sole factor to determine the thermal stability of myosin itself. Although  $\text{Ca}^{2+}$ -ATPase inactivation provides information on S-1 denaturation, the index gives no information on the structural change of rod portion upon heating of myofibrils. Detection of rod denaturation seemed indispensable to elucidate thermal denaturation of myosin. Accordingly, myosin denaturation when heated as myofibrils in the medium of 0.1 M KCl, 20 mM Tris-HCl (pH 7.5) was carefully studied by employing several techniques in addition to ATPase inactivation. The indicators employed for detecting myosin denaturation upon heating of myofibrils were followings. (i)  $\text{Ca}^{2+}$ -ATPase inactivation. (ii) Loss of salt-solubility (Azuma and Konno 1998). (iii) Monomeric myosin content (Koseki et al. 1993). (iv) Amounts of chymotryptic fragments produced (S-1 and rod) and their monomeric form (Konno et al. 2000). It is proposed that thermal denaturation rates of S-1 and rod as studied by chymotryptic digestion characterized fish species (Takahashi et al. 2005c). For example, the preceded rod denaturation than S-1 characterized carp myofibrils, while the delayed rod denaturation characterized myofibrils of walleye pollack. The characteristic denaturation rate of S-1 and rod was well explained by the magnitude of S-1 stabilization by F-actin at low salt medium. Carp myosin was more strongly stabilized by F-actin than pollack myosin. It was also proved that S-1 was produced only from active myosin in the heated myofibrils by comparing the ATPase inactivation and S-1 production.

In the previous chapter, seasonal expression of myosin isomers with different stability was studied. In the present chapter, structural differences in winter-type and summer-type of myosin isomers were investigated in detail.

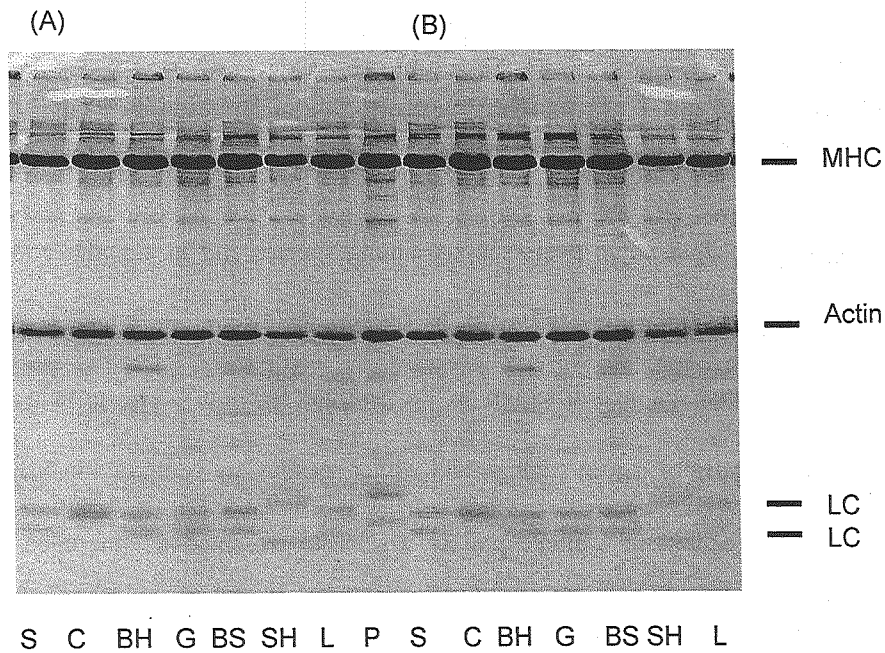
#### **4.1 Comparison of Myosin Compositions among Seven Species of Fish in Summer and Winter Season**

To compare the myosin light chain compositions among species and in summer and winter seasons, myosin was collected as actomyosin pellet at 38 % saturation of ammonium sulfate in the absence of Mg-ATP remaining tropomyosin and troponin in the soluble fraction. The protein compositions of myofibrils from seven species of fish in summer and winter seasons are compared in SDS-PAGE patterns (Fig. 4-1). SDS-PAGE pattern of walleye pollack was also presented as reference. As mentioned previously with silver carp, no difference was detected in the SDS-PAGE pattern of myofibrils from winter and summer fish for all of species examined. This is consistent with previous study by Tao et al. (2005), in which no difference was showed in the SDS-PAGE patterns of myosin from grass carp in different seasons.

The light chain composition for silver carp, bighead carp, grass carp and blunt snout bream were indistinguishable from each other. However, the patterns for common carp, largemouth bass, and snakehead were clearly distinguished from the above species in the mobility of light chain components. Although largemouth bass and snakehead could be distinguished from carp family fish, the identification of carp family species was practically impossible from the SDS-PAGE pattern.

#### **4.2 Comparison of Chymotryptic Digestion Patterns of Myofibrils among Seven Fish Species**

Firstly, chymotryptic digestion patterns for silver carp myofibrils from winter and summer fish were compared. Myofibrils were digested with chymotrypsin under two conditions, low-salt medium without

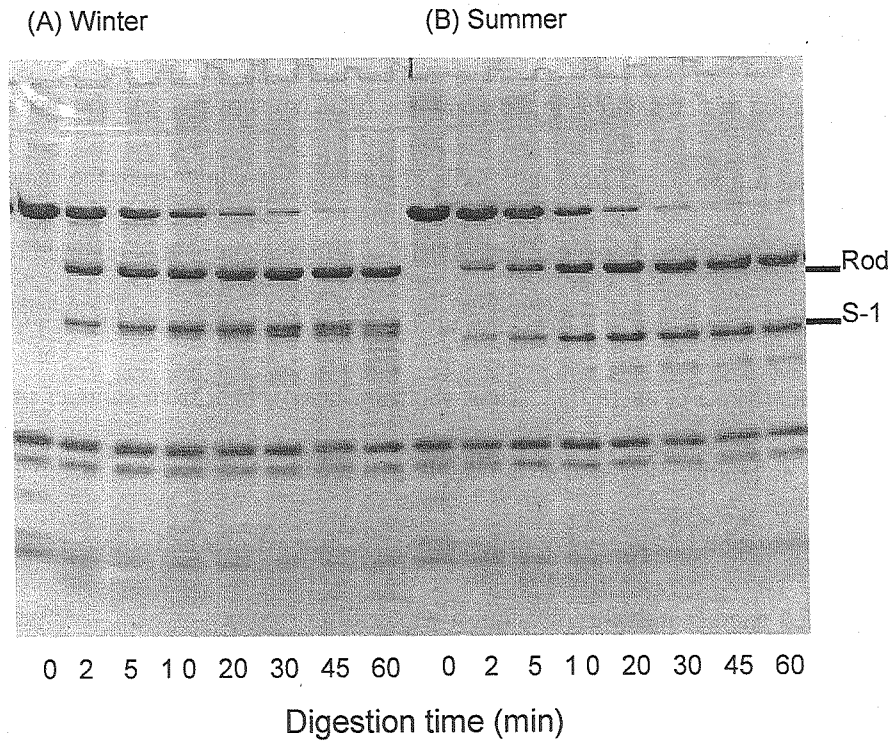


**Figure 4-1. Comparison of the protein composition between winter and summer myofibrils from various species of fish.**

(A) and (B) represent winter and summer samples, respectively. Silver carp (S), common carp (C), bighead carp (BH), grass carp (G), blunt snout bream (BS), snakehead (SH), largemouth bass (L), and walleye pollack (P) were compared. MHC, LC, and actin are myosin heavy chain, light chain components, and actin, respectively.

divalent cations (0.05 M KCl, 20 mM Tris-maleate (pH 7.0) with 1 mM EDTA), and high-salt medium with divalent cations (0.5 M KCl, 20 mM Tris-HCl (pH 7.5) with 1 mM CaCl<sub>2</sub>). Under the former conditions, myosin in both of winter and summer myofibrils was selectively cleaved into S-1 and rod (Fig. 4-2). However, there was a slight difference in the mobility of S-1 heavy chain (S-1 HC). S-1 HC produced from winter myofibrils was longer than that from summer ones. Different mobilities of two S-1 HCs were confirmed by co-migration of two samples in the same lane (data not shown). As the mobilities of parent myosin heavy chain and rod produced from myofibrils of summer and winter were the same, difference in the S-1 HC did not come from the cleavage of myosin HC at different site. It is reported that cleavage of rabbit skeletal myosin into rod and S-1 is always accompanied by tearing off of a short peptide from carboxyl end of S-1 HC (Maita et al. 1991). Thus, short S-1 heavy chain from summer myofibrils might be produced similarly as rabbit S-1. Winter S-1 might still retain the short piece at the carboxyl end of S-1. Although the production mechanism of different S-1 HC from winter and summer fish was uncertain, the sizes of S-1 seemed another index to distinguish winter from summer myosin. Actually, it was true that both S-1s with different size were produced with different ratio in intermediate season such as Oct. and May (Fig. 4-3). The same results were obtained from other species such as bighead carp, grass carp and blunt snout bream (data not shown). Such difference was not observed with common carp, largemouth bass and snakehead (data not shown).

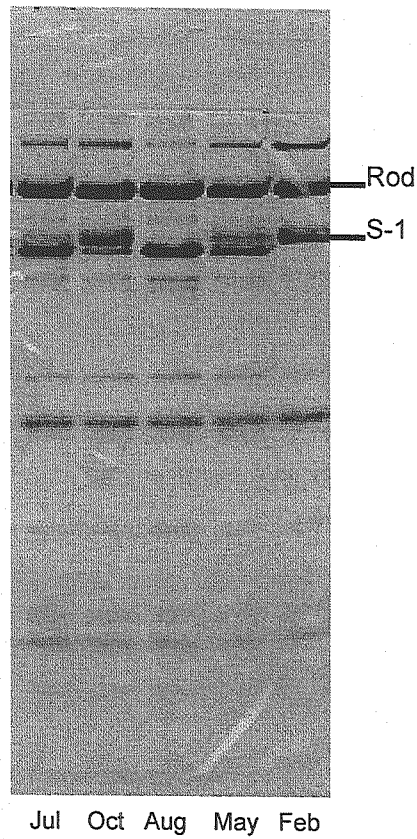
Internal structure of rod portion of summer and winter fish myosin was studied by digesting myofibrils in the medium containing 0.5 M KCl, 20 mM Tris-HCl (pH 7.5) and 1 mM CaCl<sub>2</sub>, in which myosin is expected to be cleaved at HMM/LMM junction. The digestion was conducted by changing the temperature (0, 10, 20, and 30 °C) to study the temperature dependent flexibility of myosin conformation. The chymotryptic digestion patterns of myofibrils are shown in Fig. 4-4. For summer silver carp, myosin was cleaved almost selectively at a single site producing HMM (165 kDa) and LMM (60-70 kDa), regardless of digestion temperature. HMM produced were kept unchanged until a complete cleavage of myosin HC without further degradation, while LMM seemed to be easily further cleaved into shorter LMM. However, in the case of



**Figure 4-2. Chymotryptic digestion patterns of winter- and summer-type myofibrils of silver carp.**

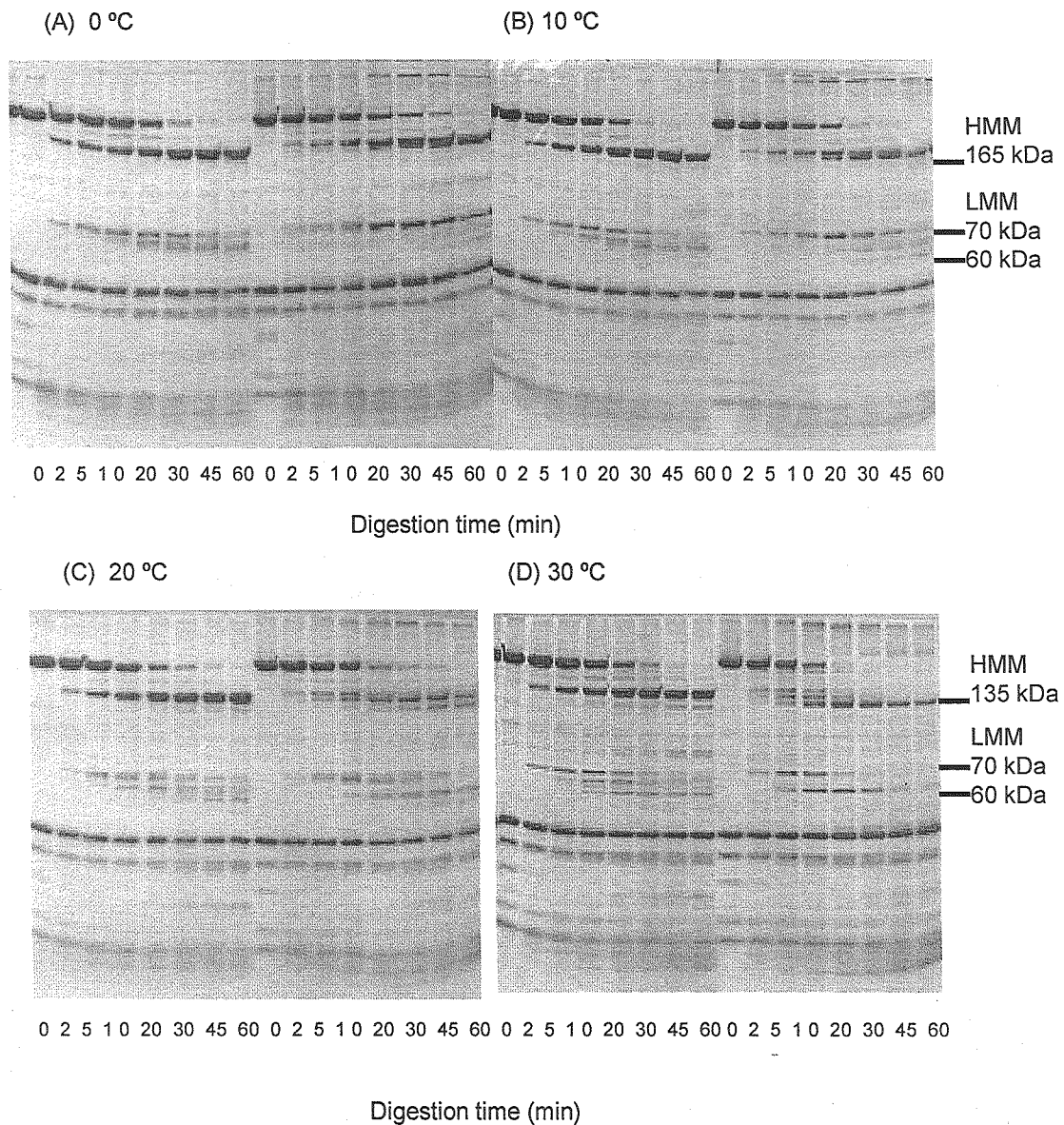
Myofibrils from winter (Winter, A) and summer (Summer, B) silver carp were digested in low ionic strength medium; 0.05M KCl, 20 mM Tris-maleat (pH 7.0), 1 mM EDTA at 10 °C. Using 1/100 (w/w) of  $\alpha$ -chymotrypsin.





**Figure 4-3. Comparison of the mobilities of rod and S-1 produced from summer and winter myofibrils by chymotryptic digestion.**

Myofibrils of silver carp fish in Jul, Oct, Aug, May, and Feb were digested in low ionic strength medium; 0.05M KCl, 20 mM Tris-maleate (pH 7.0), 1 mM EDTA for 30 min at 10 °C, using 1/100 (w/w) of  $\alpha$ -chymotrypsin.

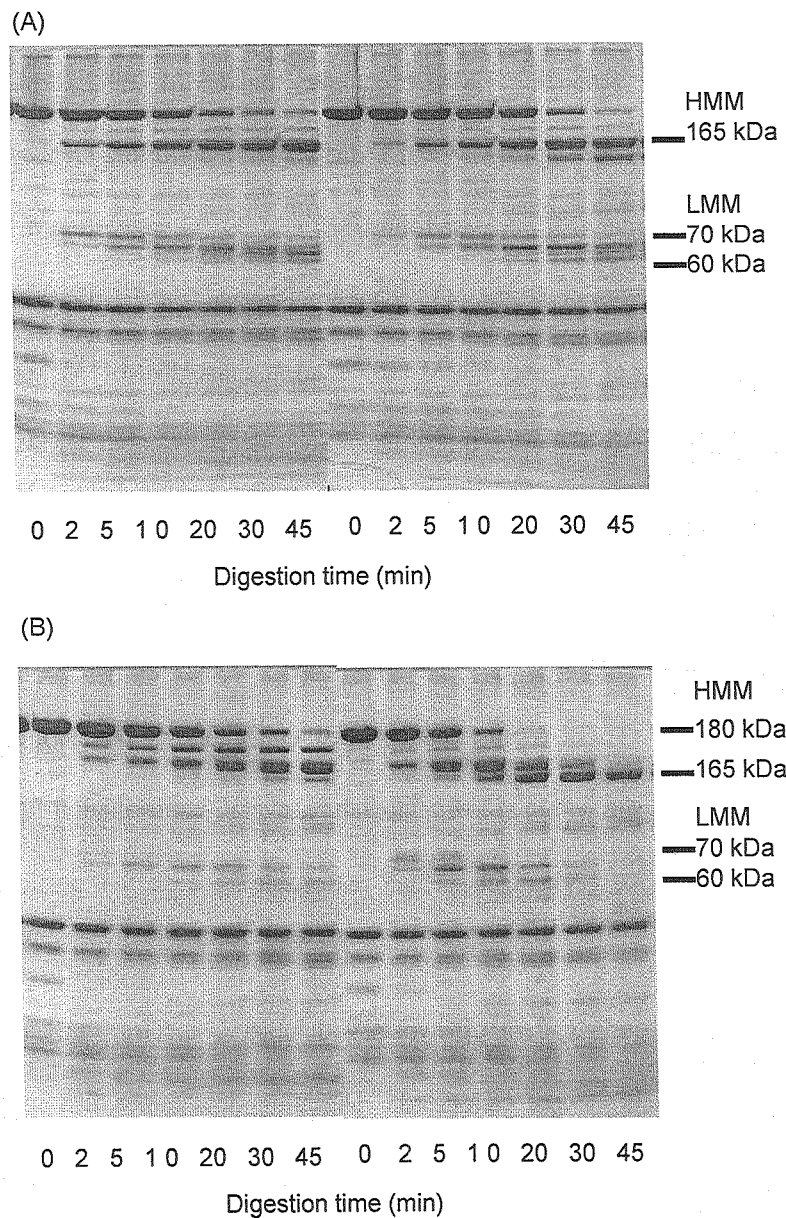


**Figure 4-4. Chymotryptic digestion patterns of summer and winter myofibrils of silver carp at various temperatures.**

Myofibrils from summer (Left) and winter (Right) samples were digested in high ionic strength medium; 0.5M KCl, 20 mM Tris-HCl (pH 7.5), 1 mM CaCl<sub>2</sub>, using  $\alpha$ -chymotrypsin of 1/40 (w/w) at 0 °C (A), 1/100 at 10 °C, (B), 1/400 at 20 °C, (C), and 1/3000 at 30 °C (D)

winter silver carp, it was indicated the temperature dependent digestion patterns. Myosin was cleaved selectively at a single site producing HMM (165 kDa) and LMM (60-70 kDa) at 0 °C. When the temperature was raised to 10 °C, both HMM and LMM began to be further cleaved into shorter fragments. Amount of HMM from winter fish decreased remarkably at 20 °C suggesting a further degradation into much shorter fragments. Shorter HMM (135 kDa) was detected in the digest, but the amount did not explain the total conversion of ordinary HMM (165 kDa) to short HMM (135 kDa). Probably, a part of HMM was degraded into much shorter fragments. Such degradation of HMM was not detected with summer one at the same temperature. These results clearly indicated that winter silver carp rod easily altered the conformation at relatively low temperature. The disappearance of HMM bands in winter sample was more prominent at 30 °C, at which winter fish myosin produced very small amount of HMM with a size of 135 kDa. These results demonstrated that although winter and summer myosin rods contained similar cleavage site within at low temperature, overall conformation for winter myosin rod was less stable than summer one. The fact seemed reasonable because S-1 portion of winter silver carp myosin was less stable.

To characterize the digestion pattern of myosin with other 6 species, the same sets of chymotryptic digestion in high-salt medium were conducted. Digestion of summer fish myofibrils for all species produced very similar HMM (165kDa) and LMM (60-70 kDa). However, some differences were found in the fragmentation of myosin among species of fish in winter season. Fish in carp family except common carp showed the same chymotryptic patterns producing single HMM and LMM as did silver carp (data not shown). Summer and winter common carp showed significantly different patterns. Summer common carp was cleaved selectively into HMM and LMM, which was similar to the cleavage pattern of silver carp. However, winter carp produced several HMM species with different mobilities. Production of much longer HMM (180 kDa) together with ordinary HMM (165 kDa) was characteristic. Production of large amount of 135 kDa HMM at 30 °C also characterized winter common carp (Fig. 4-5). The patterns for winter common carp were same as the one reported with Japanese common carp (Takahashi et al. 2005a).



**Figure 4-5. Comparison of Chymotryptic digestion patterns between summer and winter common carp myofibrils.**

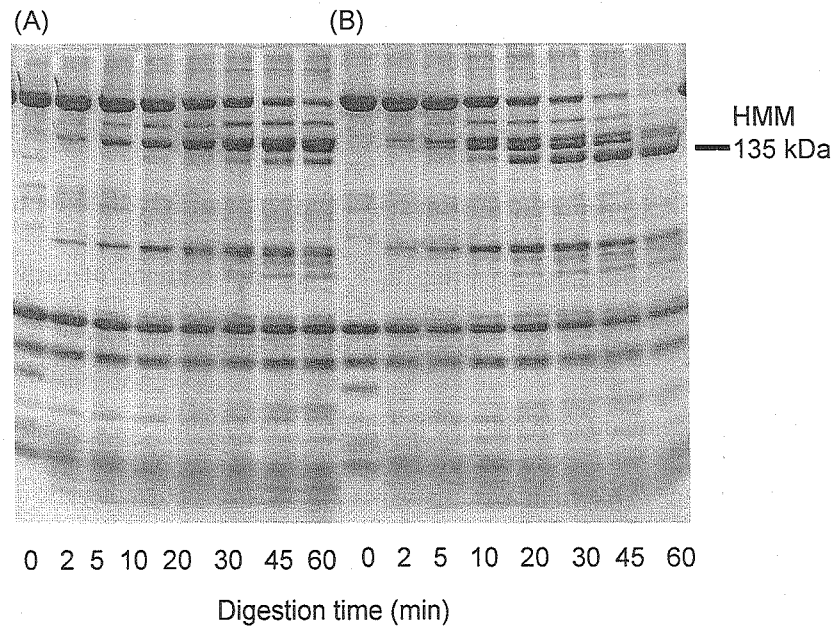
Myofibrils from summer (A) and winter (B) common carp were digested with  $\alpha$ -chymotrypsin at 20 °C (Left, 1/400, w/w) and 30 °C (Right, 1/3000) in high ionic strength medium; 0.5M KCl, 20 mM Tris-HCl (pH 7.5), 1 mM CaCl<sub>2</sub>. HMM and LMM produced are indicated on the side of the patterns.

Similar with common carp, winter largemouth bass produced large amount of HMM (135 kDa) when digested at 20 °C, while summer one mainly produced HMM (165 kDa) (Fig. 4-6). However, for snakehead, chymotryptic digestion patterns of both winter and summer myofibrils were identical each other (data not shown), similar to that for summer silver carp.

#### **4.3 Changes in the Chymotryptic Digestion Patterns of Myofibrils upon Heating**

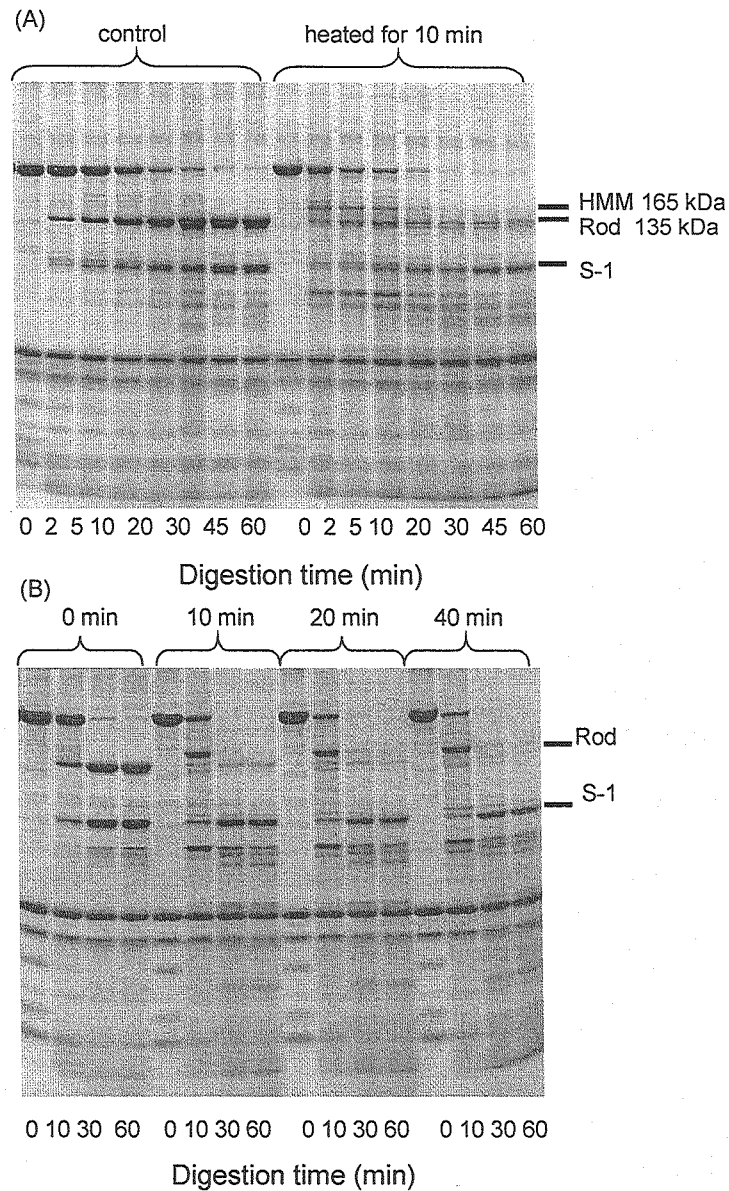
To detect the structural change occurring in S-1 and rod portions of carp myosin upon heating of myofibrils, chymotryptic digestion technique was successfully applied (Konno et al. 2000). The results were confirmed with common carp cultured in China. The chymotryptic digestion patterns of winter carp myofibrils heated at 40 °C for 10 min are shown in Fig. 4-7-A. Chymotryptic digestion was conducted in the medium so as to cleave myosin into S-1 and rod (Kato and Konno 1990). The digestion pattern for the heated myofibrils was more complicated and clearly distinguished from that for unheated myofibrils. Heated myofibrils produced two new bands migrating above rod and below S-1 HC in an early phase of digestion. It is noticed that the rod band (130 kDa) generated by the heated myofibrils was less dense than that in the control digest. It seemed that these new fragments came from the digestion of myosin at HMM/LMM junction exposed by heating. It was therefore confirmed that the fastest event, which occurred in the myosin molecule upon heating of myofibrils, was the irreversible exposure of the HMM/LMM junction (Konno et al 2000).

Structural changes of myosin for other species of fish upon heating were investigated by using the same method. The results obtained were very similar to those obtained with common carp. Summer silver carp myofibrils were heated at 44 °C for 0, 10, 20, 40mins. The chymotryptic digestion patterns for the heated samples are shown in Fig. 4-7-B. Control myofibrils produced reasonable amount of S-1 and rod. Heating myofibrils only for 10 min significantly altered the pattern; two new fragments above rod and below S-1 were produced. Amount of rod produced dropped remarkably (about 10 %), while a fairly large amount of S-1 was still produced from the heated myofibrils. The amount of S-1 produced



**Figure 4-6. Comparison of chymotryptic digestion patterns between summer and winter myofibrils of largemouth bass.**

Summer (A) and winter (B) myofibrils were digested in high ionic strength medium; 0.5M KCl, 20 mM Tris-HCl (pH 7.5), 1 mM CaCl<sub>2</sub> at 20 °C with  $\alpha$ -chymotrypsin (1/400, w/w).



**Figure 4-7. Changes in the chymotryptic digestion patterns of common carp and silver carp myofibrils upon heating.**

(A) Myofibrils prepared from common carp in Aug was heated at 40 °C for 0 (control) and 10 min (heated).

(B) Myofibrils prepared from silver carp in Aug was heated at 44 °C for 0, 10, 20, 40 min.

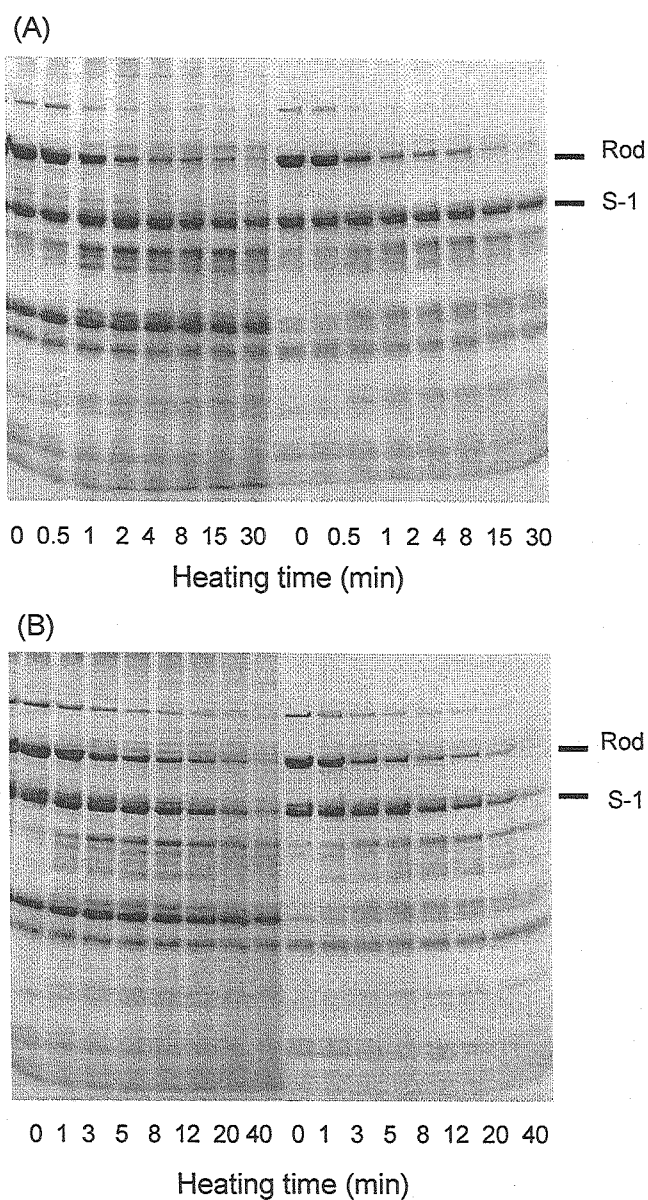
Sample were digested in low ionic strength medium; 0.05M KCl, 20 mM Tris-maleate (pH 7.0), 1 mM EDTA at 20 °C with chymotrypsin (1/500). Major products are indicated on the side of the patterns.

decreased gradually with incubation time. These results indicated that structural change occurring in rod portion was much faster than S-1 portion in summer silver carp. In other words, rod denaturation preceded S-1 denaturation remarkably in silver carp.

#### **4.4 Comparison of Thermal Denaturation Mode between Summer and Winter Silver Carp Myofibrils**

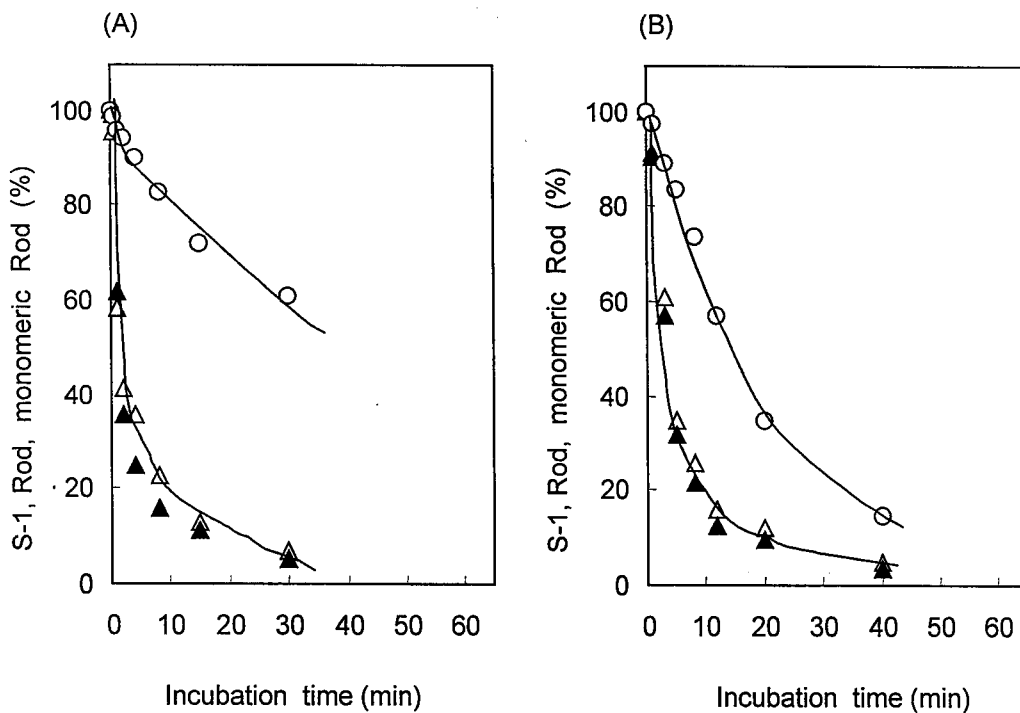
To elucidate the thermal denaturation profiles of silver carp myofibrils in winter and summer fish, structural changes of myosin upon heating was further studied. Considering the different thermal stability for summer and winter myofibrils as demonstrated in the former chapter, the heating temperatures were set at 44 °C and 38 °C for summer and winter myofibrils, respectively. Myofibrils were digested with chymotrypsin at 20°C for 45 min to achieve a complete conversion of myosin into S-1 and rod. Chymotryptic digests were further fractionated at 40 % saturated ammonium sulfate in the presence of Mg-ATP to separate aggregated fragments as precipitates. Principle of the method was the same as used in the estimation of monomeric myosin content in heated myofibrils. Fragments in the supernatant were referred to as monomeric products. Components in the supernatant were analyzed by SDS-PAGE. SDS-PAGE patterns of the digests and monomeric fractions are shown in Fig. 4-8. Both summer and winter myofibrils showed that much faster decrease in the amount of rod production than that of S-1. It was concluded that rod denaturation preceded S-1 denaturation in both myofibrils. Decreasing rates of S-1 and rod production were estimated by measuring the staining intensity of the corresponding bands in SDS-PAGE patterns. The results are shown in Fig. 4-9. Summer myofibrils heated for 30 min at 44 °C reduced the S-1 production to about 60 %, while rod production decreased to nearly zero. Heated winter myofibril for 40 min at 38 °C reduced the S-1 production to nearly 15%, while almost no monomeric rod was produced from the sample. Changes in the rod and S-1 production were analyzed by assuming the first order reaction. Decreasing rates of S-1 and rod in a monomeric form were compared (Fig. 4-10). The denaturation rates of rod and S-1 were  $29.7 \times 10^{-5} \text{ (s}^{-1}\text{)}$  and  $416 \times 10^{-5} \text{ (s}^{-1}\text{)}$  for summer myofibrils, and





**Figure 4-8. Changes in the amount of production of S-1, rod, and their monomeric form from silver carp myofibril upon heating.**

Myofibrils of silver carp heated at 44 °C (Aug, A) and at 38 °C (Feb. B) in 0.1 M KCl (pH 7.5) were digested for 30 min at 20 °C with chymotrypsin (1/500) as in Fig. 4-7. SDS-PAGE patterns of the digests (left) and their supernatant at 40% saturated  $(\text{NH}_4)_2\text{SO}_4$  with 1 mM ATP Mg as monomeric forms (right) are shown.



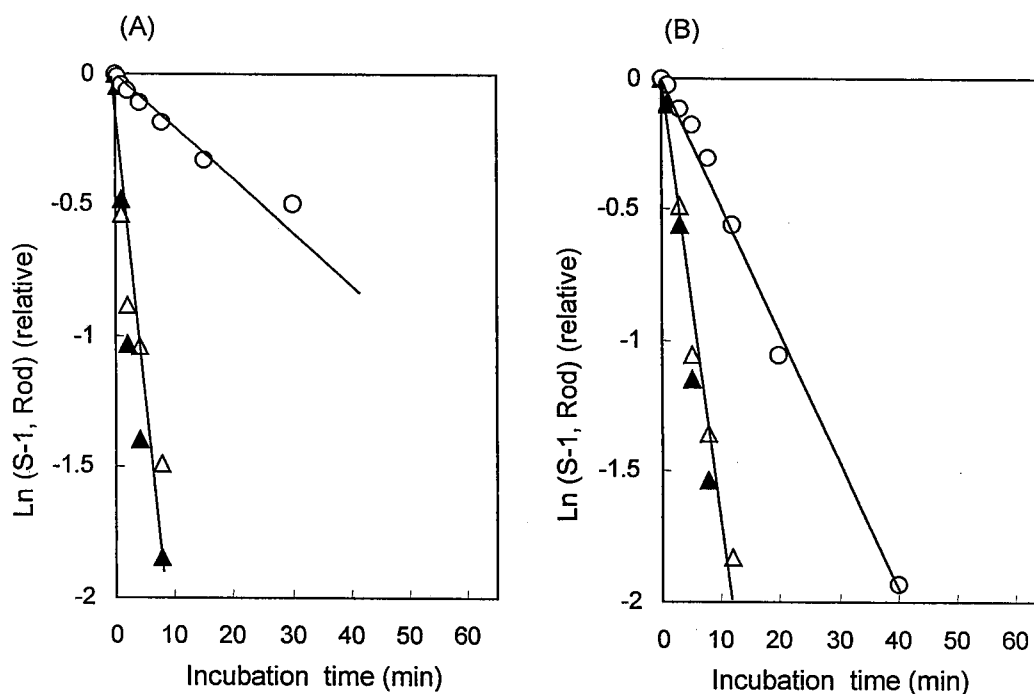
**Figure 4-9. Change in the amount of production of S-1 and rod from silver carp myofibril upon heating.**

Myofibrils were heated in 0.1 M KCl (pH 7.5) and were digested for 30 min at 20 °C with chymotrypsin (1/500) as in Fig. 4-7.

S-1( O ), rod produced ( Δ ), and monomeric rod ( ▲ ) were estimated from the SDS-PAGE patterns presented in Fig. 4-8.

(A) Myofibrils prepared from silver carp in Aug was heated at 44 °C.

(B) Myofibrils prepared from silver carp in Feb was heated at 38 °C.



**Figure 4-10. Quantitative analysis of the decrease in the S-1 and rod production and their monomeric form.**

Myofibrils suspended in 0.1 M KCl, 20 mM Tris-HCl (pH 7.5) were heated. S-1 (O), rod produced (Δ), and monomeric rod (▲) generated from the heated myofibrils were estimated from the SDS-PAGE patterns presented in Fig. 4-9. Their changes were analyzed by using the first order reaction mechanism.

(A), Myofibrils prepared from silver carp in Aug was heated at 44 °C.

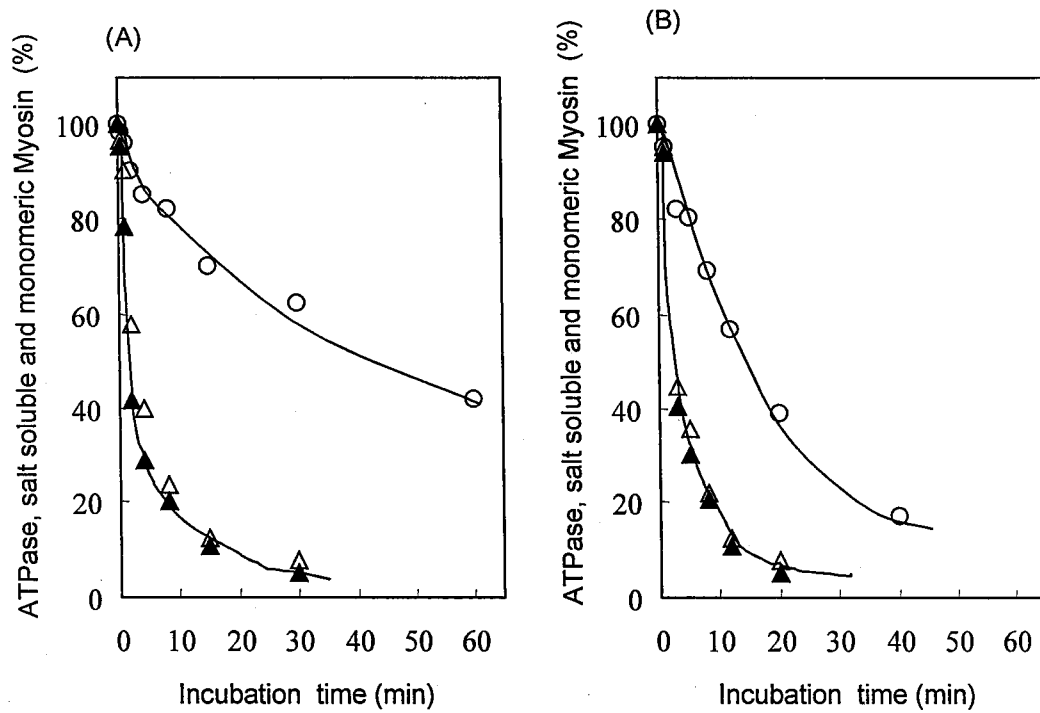
(B), Myofibrils prepared from silver carp in Feb was heated at 38 °C.

$77.2 \times 10^{-5} \text{ (s}^{-1}\text{)}$  and  $377 \times 10^{-5} \text{ (s}^{-1}\text{)}$  for winter myofibrils, respectively. These results indicated that the relative denaturation rate of rod compared to that of S-1 was about 15 times and 5 times for summer and winter myofibrils, respectively. The relative denaturation rate of rod in summer silver carp myofibrils was much greater than that reported with Japanese common carp (Takahashi et al. 2005c), while the relative denaturation rate of rod for winter one was very similar to that reported with carp.

Changes in the salt-solubility and monomeric myosin content were also measured. Decreasing profiles of these two are presented in Fig. 4-11. The amount of salt soluble myosin and monomeric myosin dropped very quickly upon heating with the identical rates. It was certain that soluble myosin were all monomeric, although the content decreased with duration. These decreases were faster than ATPase inactivation for both samples. It was also proved that rapid decrease of myosin solubility as well as monomeric myosin was also well consistent with the quick decrease of monomeric rod.

#### **4.5 Comparison of the Thermal Denaturation Modes of Myosin between Summer and Winter Fish with Other Six Species of Freshwater Fish.**

Difference in the denaturation profile of myofibrils upon heating between summer and winter fish was studied for other six species. S-1 and rod production from the heated myofibrils by chymotryptic digestion was the index to characterize the denaturation mode. Heating temperatures were varied considering the respective thermal denaturation rates to make the measurement easier. Decreases in the production of S-1 and rod and their monomeric form were compared between myofibrils in summer and winter fish (Fig. 4-12). Both of summer and winter myofibrils showed much faster decrease of rod than S-1 for all of species examined. There was a slight difference in the relative rod denaturation rate to S-1 among species. Although, a quantitative analysis was not done, decreasing profiles of S-1 production was similar to ATPase inactivation for all cases. Similarly, rapid decreases of myosin solubility upon heating seemed consistent with that of monomeric rod production. As ammonium sulfate fractionation did not sediment rod as well as S-1, all of S-1 and rod produced were monomeric as proved with silver carp.

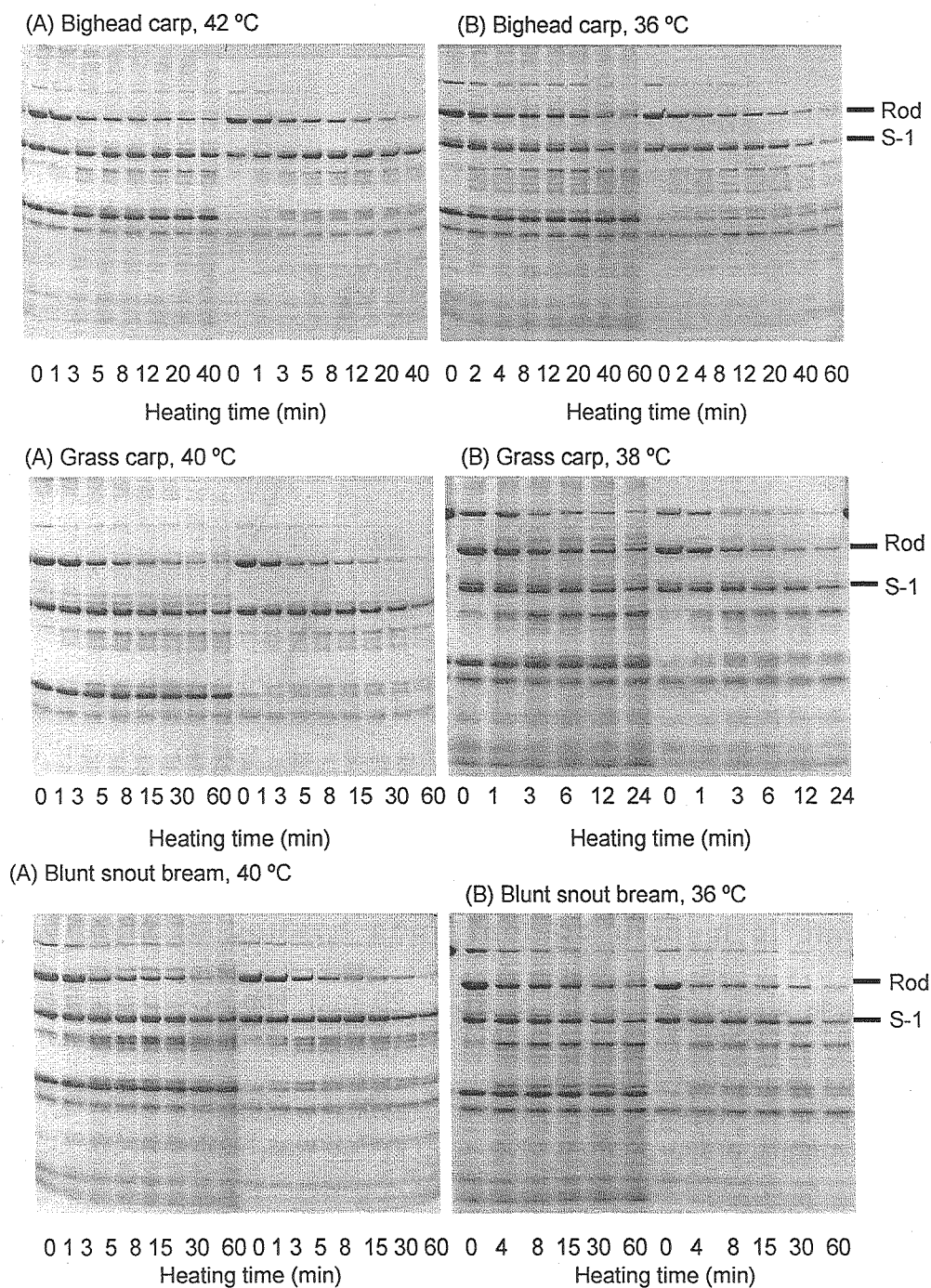


**Figure 4-11. Decrease in  $\text{Ca}^{2+}$ -ATPase activity, salt solubility, and monomeric myosin content upon heating of myofibrils.**

Myofibrils suspended in 0.1 M KCl , 20 mM Tris-HCl (pH 7.5) were heated. ATPase ( O ), myosin solubility( Δ ), and monomeric myosin ( ▲ ) were estimated.

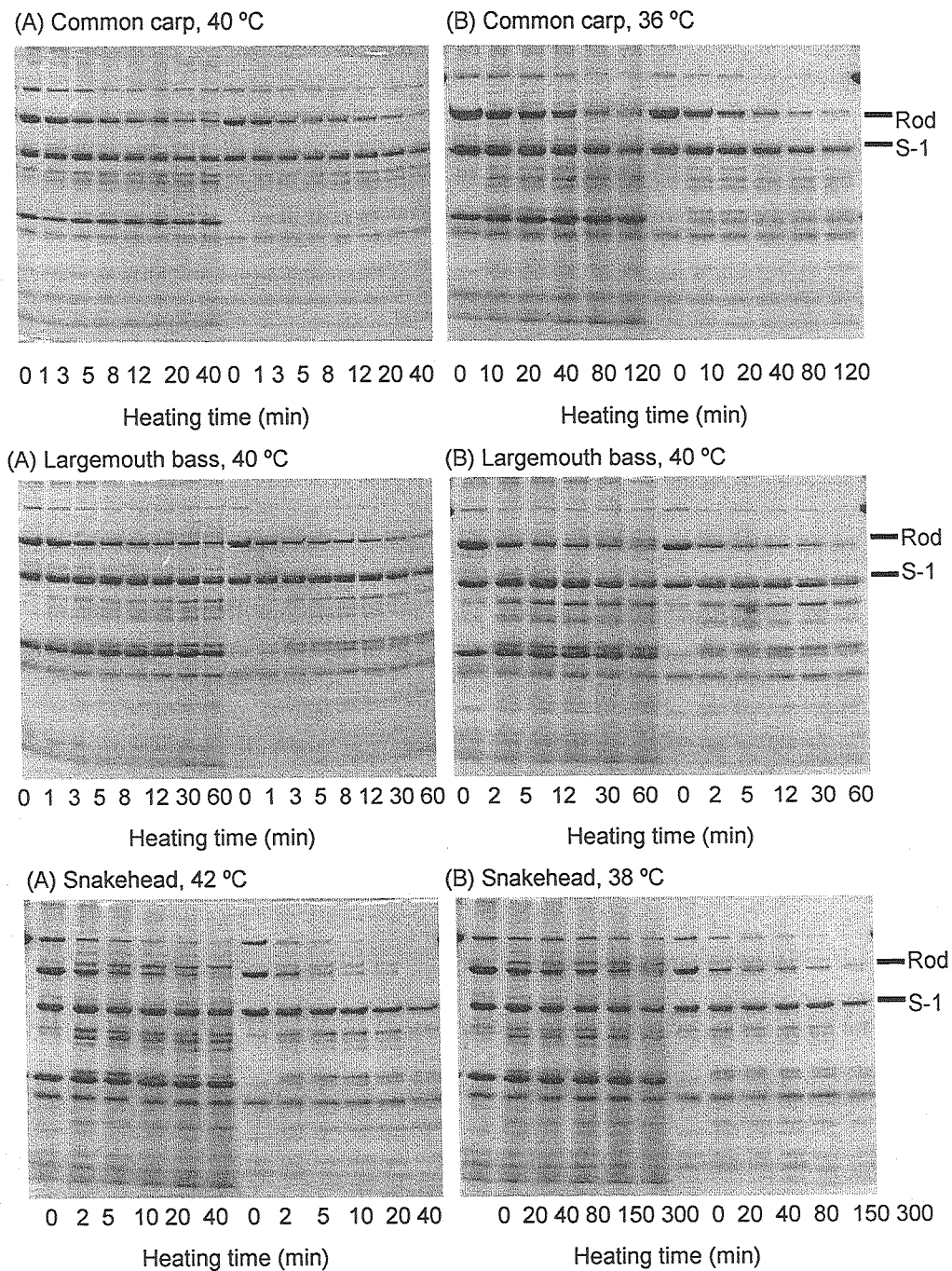
(A), Myofibrils prepared from silver carp in Aug was heated at 44 °C.

(B), Myofibrils prepared from silver carp in Feb was heated at 38 °C.



**Figure 4-12-1. Changes in the amounts of S-1 and rod produced from myofibrils of various species upon heating.**

Chymotryptic digestion was conducted as Fig. 4-8. (A) and (B) are myofibrils in summer and winter seasons. Species of fish and heating temperatures were indicated in the figures. Digests (left) and their monomeric forms (right) were estimated.



**Figure 4-12-2. Change in the amount of production of S-1 and rod from myofibrils of various species upon heating.**

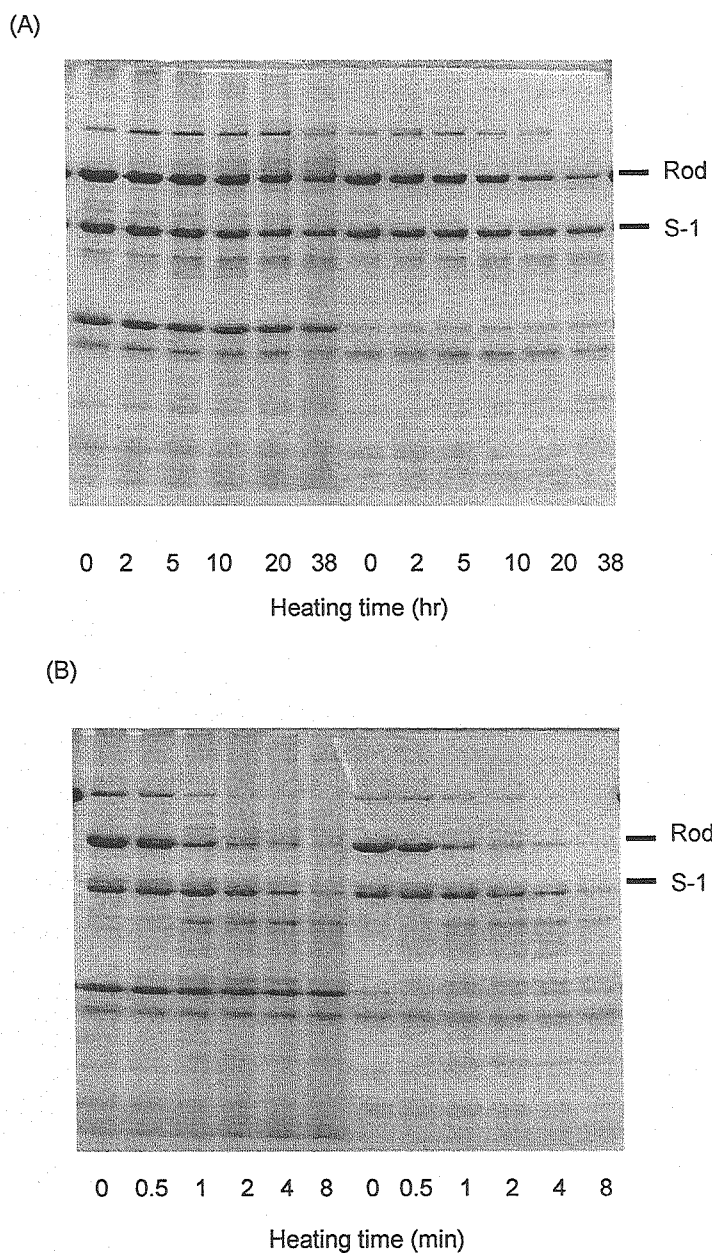
(A) and (B) are myofibrils in summer and winter seasons. Species of fish and heating temperatures were indicated in the figures. Digests (left) and their monomeric forms (right) were estimated. Chymotryptic digestion was conducted as Fig. 4-8.

#### **4.6 Temperature Dependent Thermal Denaturation Modes for Seven Species of Freshwater Fish in Summer and Winter Seasons.**

Thermal denaturation mode analysis of myofibrils for seven species of fish in summer and winter seasons revealed that rod denaturation preceded S-1 for all cases irrespective of fish species and seasons. However, the conclusion was led from the study at a fixed heating temperature, which was chosen to give a similar ATPase inactivation rate for respective myofibril samples. As myofibrils from winter fish was unstable, the heating temperatures were always lower than that for summer samples. To investigate the effect of heating temperature on the denaturation mode, silver carp myofibrils in summer season was heated at lower temperature of 36 °C. Oppositely, unstable myofibrils from winter fish were heated at elevated temperature of 42 °C. Consequently, the heating time for summer myofibrils was prolonged to 36 hr, while that for winter myofibrils were only 8 min. Decreasing profiles of S-1 and rod from the heated myofibrils are presented in Fig. 4-13. Different patterns from previous ones were obtained for both samples. Relative denaturation rate of rod to S-1 increased for winter sample by raising temperature. In contrast, relative rod denaturation was significantly suppressed by lowering heating temperature for summer myofibrils. These results indicated that apparent thermal denaturation mode was temperature dependent. The higher the temperature was, the faster the rod denaturation was, and vice versa.

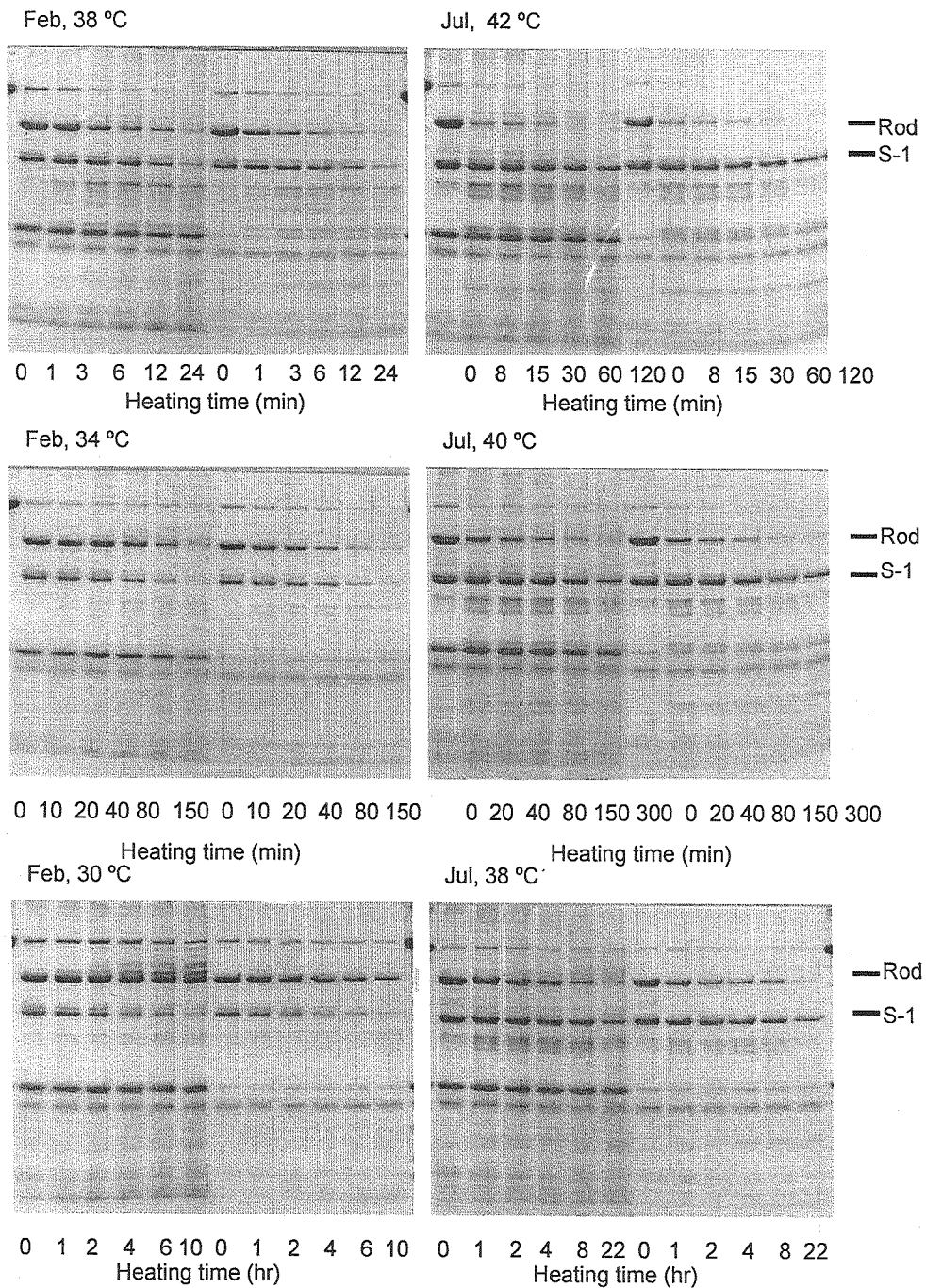
Temperatures dependent thermal denaturation rates of S-1 and rod were estimated with silver carp by changing heating temperatures from 30 °C to 44 °C. Changes in the production of S-1 and rod are presented in Fig. 4-14. The results clearly indicated that relative rod denaturation became slower with lowering the heating temperature. It should be pointed out that S-1 denaturation preceded rod denaturation when winter myofibrils were heated at 30 °C. As the decreasing rates could be calculated by assuming the first order reaction reaction, S-1 and rod denaturation rates at various temperatures were estimated to produce Arrhenius plot (Fig. 4-15). S-1 denaturation rates provided a straight line, while the denaturation rates of rod gave a breaking point in the line. The breaking point was around 36 °C and 40 °C for winter and summer myofibrils, respectively. Two lines for S-1 and rod denaturation crossed at





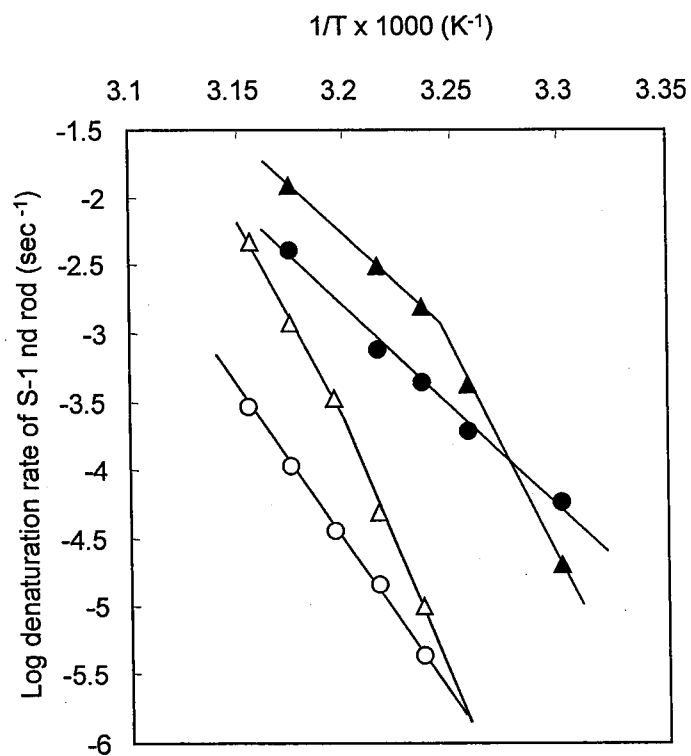
**Figure 4-13. Effect of heating temperature on the S-1 and rod denaturation of silver carp myofibril.**

Experiments were the same as in Fig. 4-8 except the heating temperatures. (A), Myofibrils from summer silver carp was heated at 36 °C, and (B), ones from winter silver carp was heated at 42 °C. Digests (left) and their monomeric forms (right) were estimated.



**Figure 4-14. Temperature dependent changes in the decreasing rate of S-1 and rod production, and their monomeric form upon heating myofibrils of silver carp in summer and winter seasons.**

Experiments were the same as in Fig. 4-8 except the heating temperatures. Month of sampling and heating temperatures were indicated in the figures. Digests (left) and their monomeric forms (right) were estimated.



**Figure 4-15.** Temperature dependent changes in the S-1 and rod denaturation rate for winter- and summer-type myofibrils of silver carp.

Decreasing rate of monomeric S-1 (○, ●) and rod (▲, △) production were measured by changing heating temperatures for winter- (closed) and summer-type (open) myofibrils.

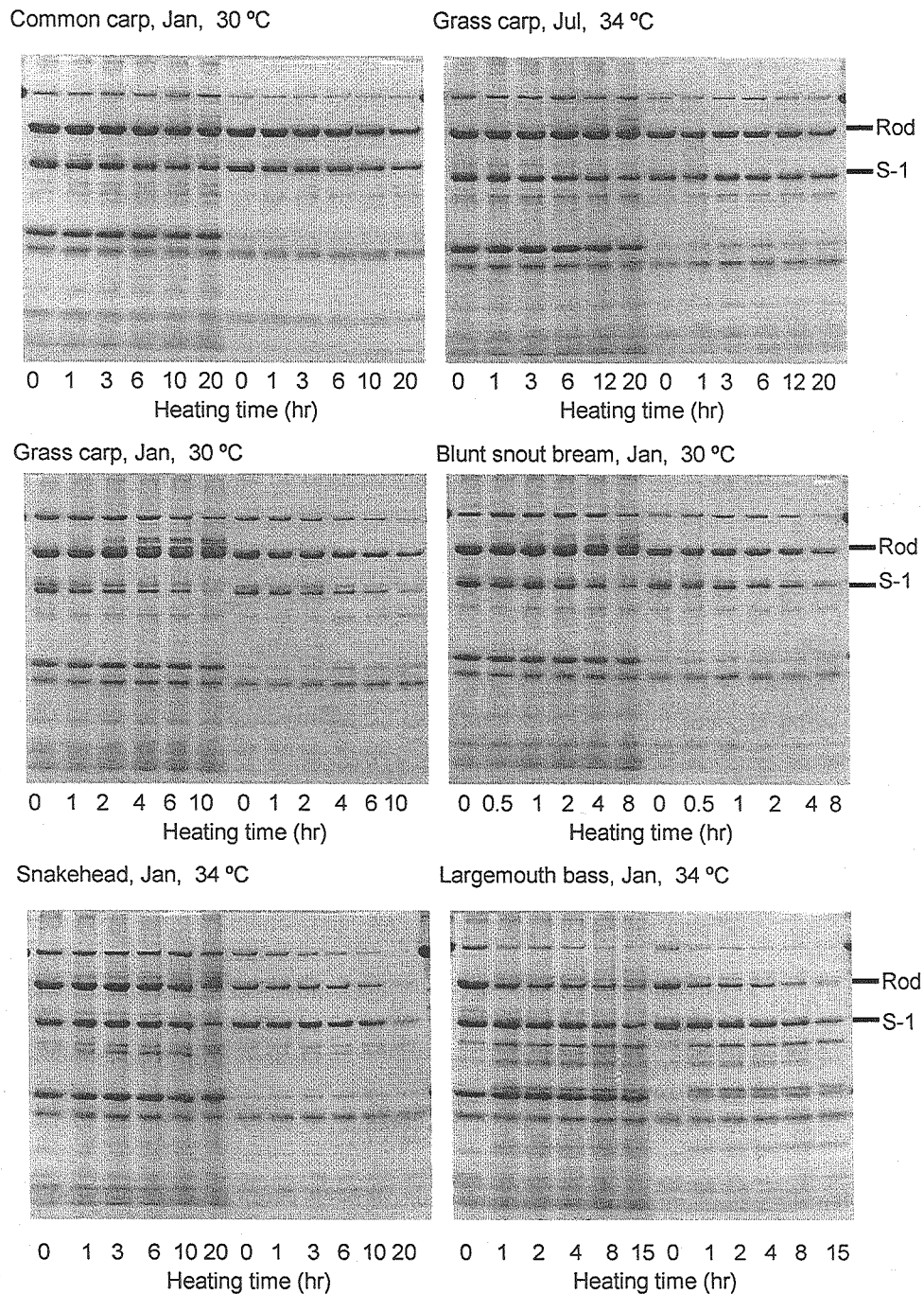
around 33 °C for winter and 35°C for summer myofibrils. Above the temperature, rod denatured faster than S-1.

Effect of heating temperature on the denaturation mode was always observed with other species of fish, namely decreased relative denaturation rate of rod at lower temperatures were equally observed for other six species (Fig. 4-16).

#### **4.7 Discussion**

Chymotryptic digestion of myofibrils from all freshwater fish species revealed that myosin in a filamentous was selectively cleaved at S-1/rod junction as reported with carp. However, the cleavage pattern within rod structure was species-specific as studied by the digestion at high-salt medium. Myosin from many fish species was selectively cleaved into HMM and LMM producing a single HMM and LMM. A typical pattern was obtained with silver carp. The pattern was rather similar to that of rabbit skeletal myosin (Kato and Konno 1993b). It should be noted that myosin from summer and winter fish showed practically the same cleavage pattern suggesting a similar structure in HMM/LMM cleavage region for both myosin molecules at 0 °C. Among the carp family fish, common carp was characteristic. Although the digestion pattern for summer myosin was almost identical to silver carp being cleaved into single HMM and LMM, winter myosin generated several species of HMM possessing longer (180k Da) and shorter (135 kDa) HC indicating that winter carp myosin contained at least three HMM/LMM cleavage sites. It has been reported that Japanese carp produces uniquely long HMM (Kato and Konno 1993b). The Japanese common carp used in the report would correspond to Chinese winter common carp. It was certain that common carp isomers contain characteristic amino acids sequences in respective myosin rods. As the digestion pattern for winter common carp was exceptional among carp family, it suggested that the structure of tail portion of winter common carp might be unique although the role of the structure is uncertain.

Thermal denaturation analysis of myofibrils from various species of fish in different seasons



**Figure 4-16. Effect of heating temperature on the S-1 and rod denaturation of various species of fish myofibrils.**

Experiments were the same as in Fig. 4-12 except the heating temperatures. Fish species, month of sampling, and heating temperature were in the figures. Digests (Left) and their monomeric forms (Right) were estimated.

demonstrated that denaturation mode characterized by the relative rod denaturation rate to S-1 were almost identical irrespective of seasons and species. In all cases, rod denaturation preceded S-1 denaturation. The pattern was consistent with the result reported with Japanese common carp. However, the relative rod denaturation rate over S-1 for summer silver carp was 15 times, which was remarkably greater than that for winter silver carp (5 times). The rate for winter fish was rather similar to that reported with Japanese common carp (Takahashi et al. 2005b). Takahashi et al. also reported that the relative rod denaturation rate was deeply correlated with the magnitude of the stabilization by F-actin. These might be reasonable because magnitude of stabilization by F-actin binding for summer silver carp myosin was much greater than winter one.

The same decrease of both  $\text{Ca}^{2+}$ -ATPase activity and production of S-1 indicated that S-1 was generated only from myosin that retained the  $\text{Ca}^{2+}$ -ATPase activity. In other words, S-1 denaturation could be studied by measuring the amount of S-1 produced from heated myofibrils. The conclusion was led from all of myofibrils studied. As the salt-solubility of myosin depends on its rod portion, the amount of rod was well correlated with the solubility and monomeric myosin content. These facts indicated that monomeric rod was generated only from myosin that retained solubility. As myosin denaturation was studied with heating myofibrils, the results indicated much more serious structure change in filament rod than that in S-1. The altered filament structure allowed chymotrypsin to penetrate to cleave myosin molecule at the center of rod portion (HMM/LMM junction).

Different from the denaturation patterns found with common carp, much slower rod denaturation than S-1 with pollack myofibril and the same denaturation rate of rod and S-1 with yellowtail myofibrils were reported (Takahashi et al. 2005b). All the myofibrils prepared from freshwater fish species showed quicker rod denaturation than S-1 regardless the season and species. Fish species examined in the present study belonged to the group termed as "carp group" by Takahashi et al. (2005b). It has also been reported that denaturation mode of carp changes dependent on the heating temperature (Yamamoto et al. 2002). The same conclusion was led with silver carp, in which rod denaturation was much slower than S-1 at low

temperatures. Thus, the denaturation pattern as defined by S-1, rod denaturation rate is temperature dependent. These results indicated that detection of S-1 denaturation measured by ATPase activity is the most sensitive indicator at low temperatures and salt solubility is the most sensitive and simple index for detecting myosin denaturation at high temperatures. Slow rod denaturation at low temperatures might include a high reversibility of rod structure upon heating and cooling due to its complete helix structure. Reversibility of the helical structure of rod portion became much greater compared with S-1 portion at lower temperature. The estimated activation energy for the rod denaturation at low temperature range was about 3 times greater than at high temperature range (Fig. 4-15). These findings provided information about the effect of temperature on the denaturation of head and tail portion, which would be important for storing and processing fish meat.

## Chapter 5.

### Species-Specific Structural Stability of Myosin Rod between Winter and Summer Season Fish

Myosin molecule consists of two distinct structures; two globular portions designated S-1, and an almost completely  $\alpha$ -helical coiled-coil tail, termed rod. Rod portion is composed of two portions, water-soluble S-2 and salt-soluble LMM. S-2 supports the projected head from the thick filaments, and LMM is a filament-forming domain of myosin molecule. In Chapters 2 and 3, structural stability of myosin expressed in winter and summer season fish were compared by using ATPase inactivation rate as the index. In Chapter 4, thermal denaturation mode of myosin heated as myofibrils in different seasons from various species of fish were investigated by employing chymotryptic digestion technique. For all of species of fish, rod denaturation precedes S-1 denaturation when heated as myofibrils.

There are many reports on the elucidation of the thermal gelation mechanism by employing model system of thermal aggregation of myosin or its subfragments in high-salt medium (Samejima et al., 1976, 1981; Chan et al. 1993; Higuchi et al. 2002). It was proved that rod portion of myosin molecule plays an important role to form an elastic gel (Samejima et al. 1976, 1981; Sano et al. 1990). Usually thermal gel is formed by heating at high temperature such as 90 °C, at which a complete unfolding of rod structure is expected. It is well recognized that thermal gelation is fish species-specific. However, a clear explanation for the different gelation properties among fish species has not been made. Therefore, it seems important to understand the thermal denaturation or unfolding of rod portion of myosin from various species of fish in two seasons at high temperatures because the thermal gel is one of the possible ways of utilization of freshwater fish meat. At present, there is no information on the thermal unfolding properties of myosin rod from freshwater fish except Japanese carp. Preliminary thermal gel formation by various freshwater fish meat has been reported (Fukuda et al. 2001). In the report, seasonal changes in the thermal stability of myosin was not considered. In Chapter 1, seasonal change in the gel forming ability of silver carp surimi was carefully investigated, and the difference in the temperature dependent gelation

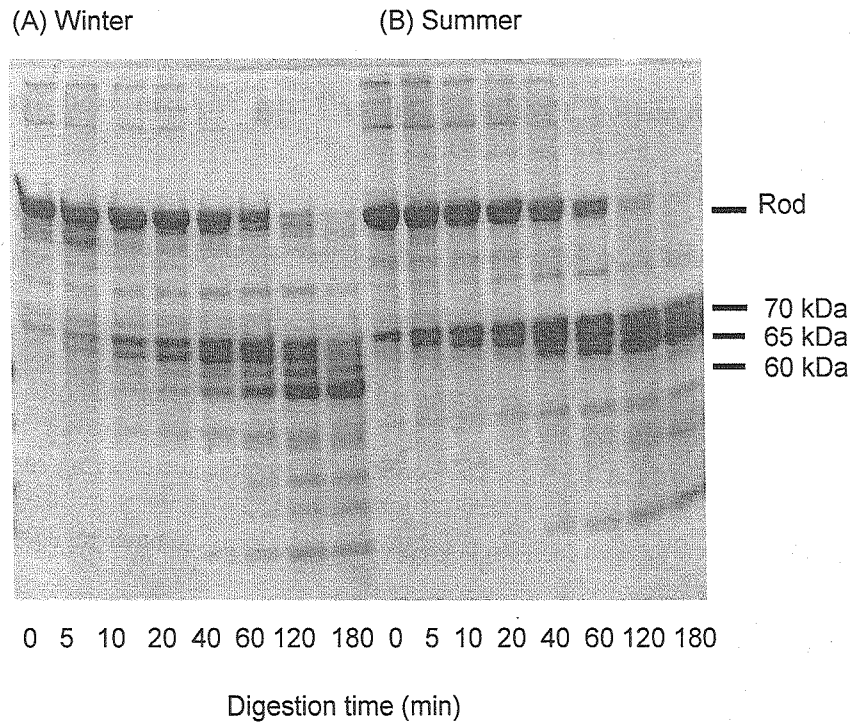


was explained by the different stability of myosin in different seasons.

In this chapter, the differences in the internal structure of myosin rod between summer and winter fish myosin and differences among fish species were studied. Thermal aggregation process of myosin rod was also studied as a model system of thermal aggregation of myosin molecule.

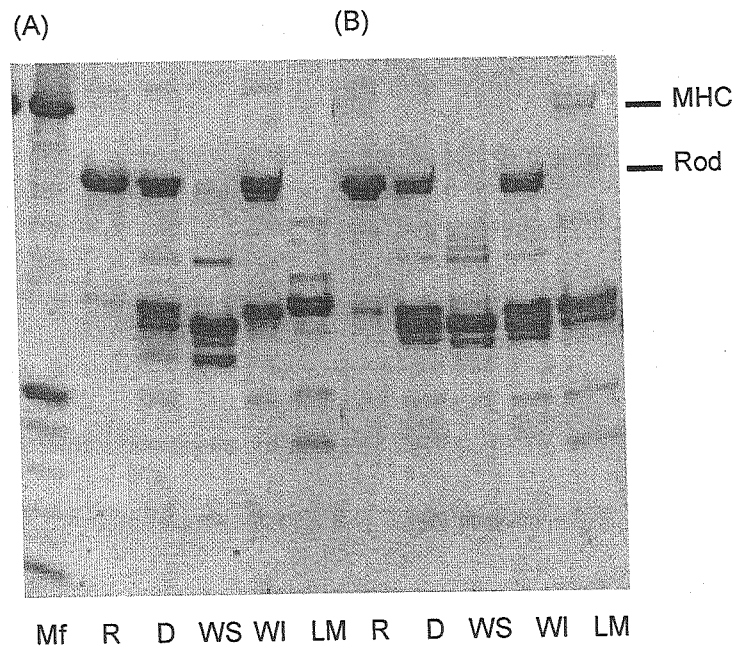
### **5.1 Chymotryptic Digestion Profile of Silver Carp Myosin Rod from Summer and Winter Fish**

Myosin rod was isolated from both winter and summer myofibrils of silver carp. To compare the internal structure of myosin rod in two seasons, firstly chymotryptic digestion pattern of rod was compared. Monomeric rod dissolved in a medium containing 0.5 M KCl, 20 mM Tris-HCl (pH 7.5) was digested with 1/200 (w/w) of chymotrypsin at 10 °C. The condition is suitable for cleaving rod into LMMs and S-2 (Kato et al. 1993). The digestion patterns are shown in Fig. 5-1. Summer myosin rod generated mainly two fragments with sizes of 65 and 70 kDa, and the products were kept unchanged until a complete digestion of rod. Winter myosin rod was similarly cleaved into 65 and 70 kDa in an early phase of digestion (30 min), while both fragments were further cleaved into much shorter fragment with a size of about 60 kDa. To identify these fragments whether S-2 or LMM, the digests were dialyzed against 0.05 M KCl, 20 mM Tris-maleate (pH 7.0) and 5 mM MgCl<sub>2</sub>, and centrifuged at 100,000 × g for 15 min. The supernatant of the dialysate obtained by ultracentrifugation was referred to as water-soluble or S-2 fraction, and the pellet was referred to as the water-insoluble or LMM fraction. The SDS-PAGE patterns of the fractions are shown in Fig. 5-2. For comparison, LMM was prepared by a direct digestion of myofibrils, and its pattern was also shown in the same figure. It was confirmed that the 70kDa fragment in the digest of summer myosin rod was water-insoluble LMM, and the 65 kDa fragment was S-2. The size of LMM obtained in the digest of rod was the same as one directly prepared from myofibrils. Three fragments detected in the pattern for winter myosin rod were similarly separated into water soluble and insoluble fractions. Water insoluble LMM fraction contained 70 and 65 kDa fragments, and 65 and 60 kDa were



**Figure 5-1. Chymotryptic digestion patterns of winter- and summer-type myosin rod of silver carp.**

Rods from winter (A) and summer (B) myofibrils were digested in high ionic strength medium; 0.5M KCl, 20 mM Tris-HCl (pH 7.5), 1 mM  $\text{CaCl}_2$  for 0-180 min at 10 °C by using 1/100 (w/w) of  $\alpha$ -chymotrypsin. Sizes of products were indicated on the side of the pattern.



**Figure 5-2. Separation of the fragments in the digests of silver carp myosin rod into water-soluble and water-insoluble fractions.**

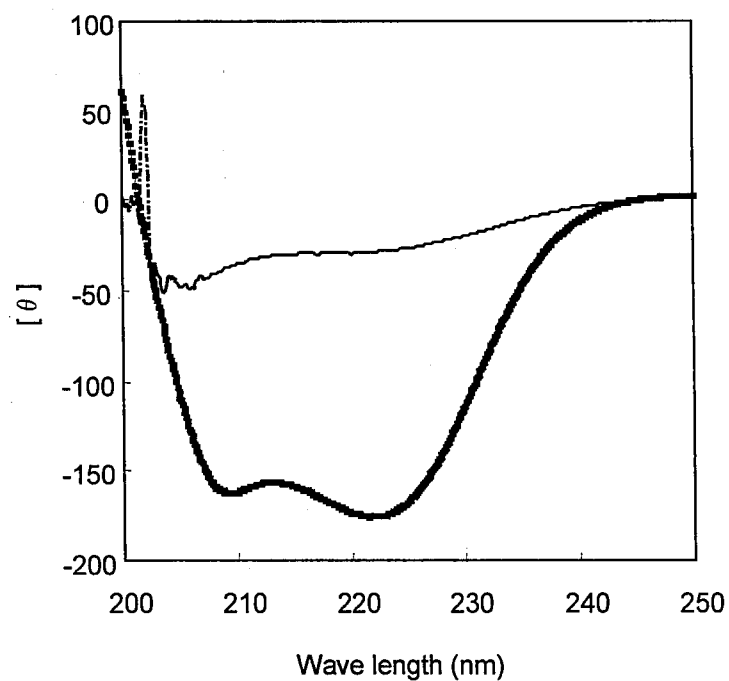
Myosin rods of winter (A) and summer (B) were digested as in Fig. 5-1 for 30 min at 10 °C. The digest was dialyzed against 0.05 M KCl, 20 mM Tris-maleate (pH 7.0), 5 mM MgCl<sub>2</sub>, and centrifuged at 100,000 x g for 20 min. Mf, R, D, WS, and WI are, myofibrils, rod, rod digest, water soluble fraction, and water insoluble fractions, respectively. LM is the LMM prepared from myofibrils by chymotryptoc digestion.

detected in the water soluble fraction. These results indicated that 70 kDa LMM and 65 kDa S-2 initially produced from winter myosin rod were further cleaved at the second sites on respective fragments. As the second cleavage was not observed with summer myosin rod, somewhat different structure of winter rod from summer one both in S-2 and LMM portions was indicated.

## 5.2 Characterization of Circular Dichroism Spectra of Silver Carp Myosin Rod

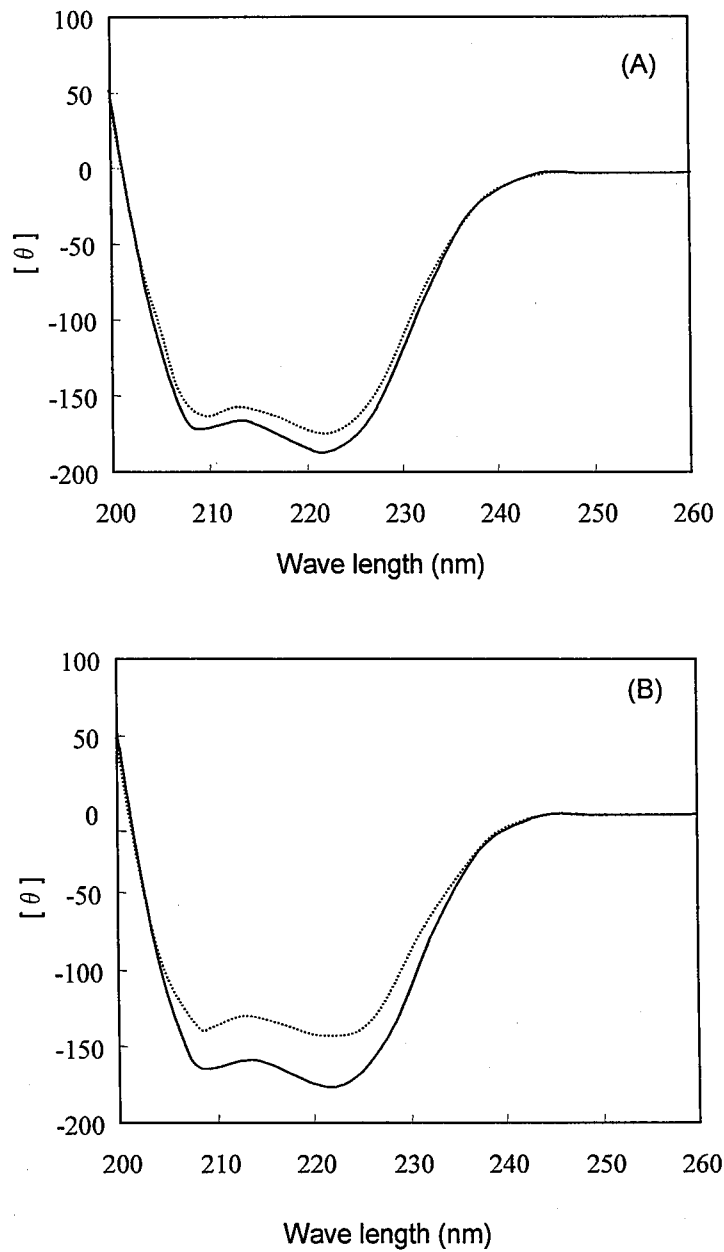
The circular dichroism (CD) spectra of myosin rod from both winter and summer silver carp at 10 °C were compared (Fig. 5-3). Both samples showed large negative ellipticity ( $\theta$ ) peaks at 222 nm and 208 nm, characteristic of  $\alpha$ -helix structure. The magnitude of the ellipticity for both samples at 10 °C indicated their nearly 100% helical structure. The ratio of  $\theta_{222\text{nm}}/\theta_{208\text{nm}}$  at 10 °C was 1.09, which was in agreement with previous study with other species of myosin rod (King and Lehrer 1989) (Fig. 5-3). The spectrum of summer myosin rod measured at 70 °C was also shown in Fig. 5-3. Characteristic spectrum for helix structure disappeared and flat spectrum corresponding to random structure was obtained. Thus, heating of rod at 70 °C almost completely unfolded the  $\alpha$ -helix structure of rod.

It is reported that unfolded myosin LMM partially refolded upon cooling (Ojima et al. 1999). Reversible refolding ability of these two rod samples was compared. Rod solution was heated from 10 to 70 °C, and then cooled to 10 °C. CD spectra for thus treated samples of summer and winter rod were compared. CD spectra of respective rod samples before heating are also presented as references in Fig. 5-4. Ellipticity for the heated and cooled was quite high for both myosin rods. Recovered helix contents calculated from the ellipticity upon cooling were about 92.6 and 80.3%, for summer and winter rods, respectively. It was clearly demonstrated that although rod lost the  $\alpha$ -helix structure at 70 °C, cooling process refolded significantly. As the reversibility for winter myosin rod was a slightly lower than summer myosin rod, less stable structure of winter myosin rod was suggested. High reversibility of helix structure of rod was coincidental with the results reported by Sano et al. (1994) studied with carp actomyosin.



**Figure 5-3. CD spectra of silver carp myosin rod at 10 and at 70 °C .**

CD spectra of silver carp myosin rod were measured at 10 °C (bold line), and at 70 °C (solid line) at 0.5 mg/ml by using 1 mm light pass length cell.

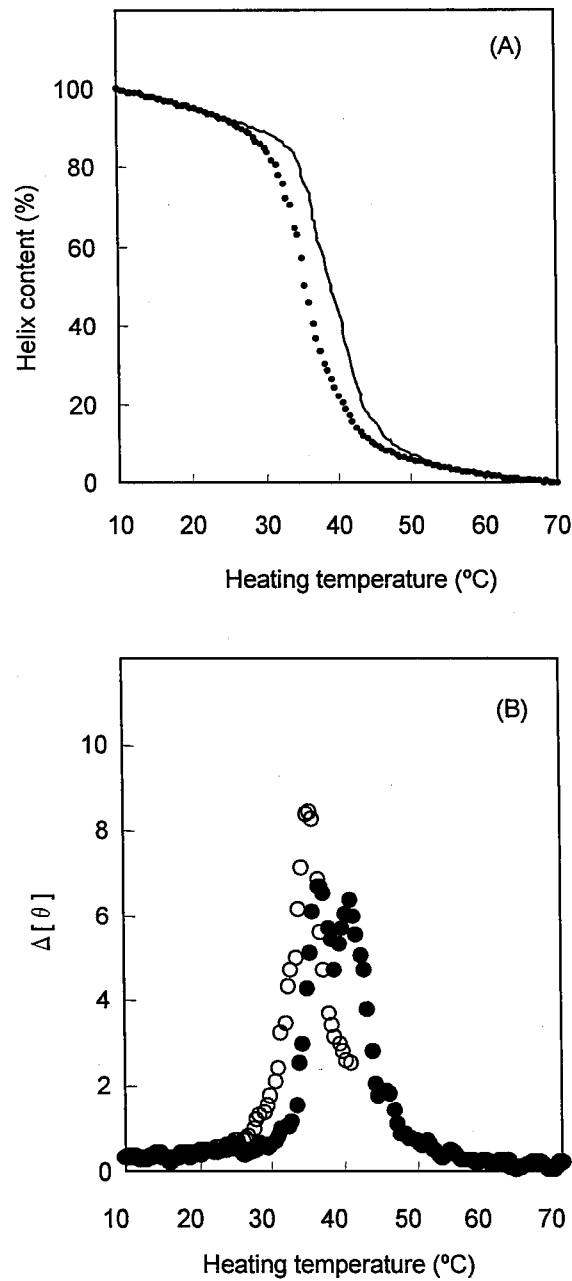


**Figure 5-4. Changes in the CD spectrum of myosin rod upon heating-cooling.**

CD spectra of summer silver carp myosin rod (A) and of winter myosin rod (B) was measured before heating (solid line) and after heating and cooling (dotted line). CD spectra was measured at 10 °C as in Fig. 5-3.

### 5.3 Comparison of the Unfolding Profiles between Winter and Summer Rods from Silver Carp

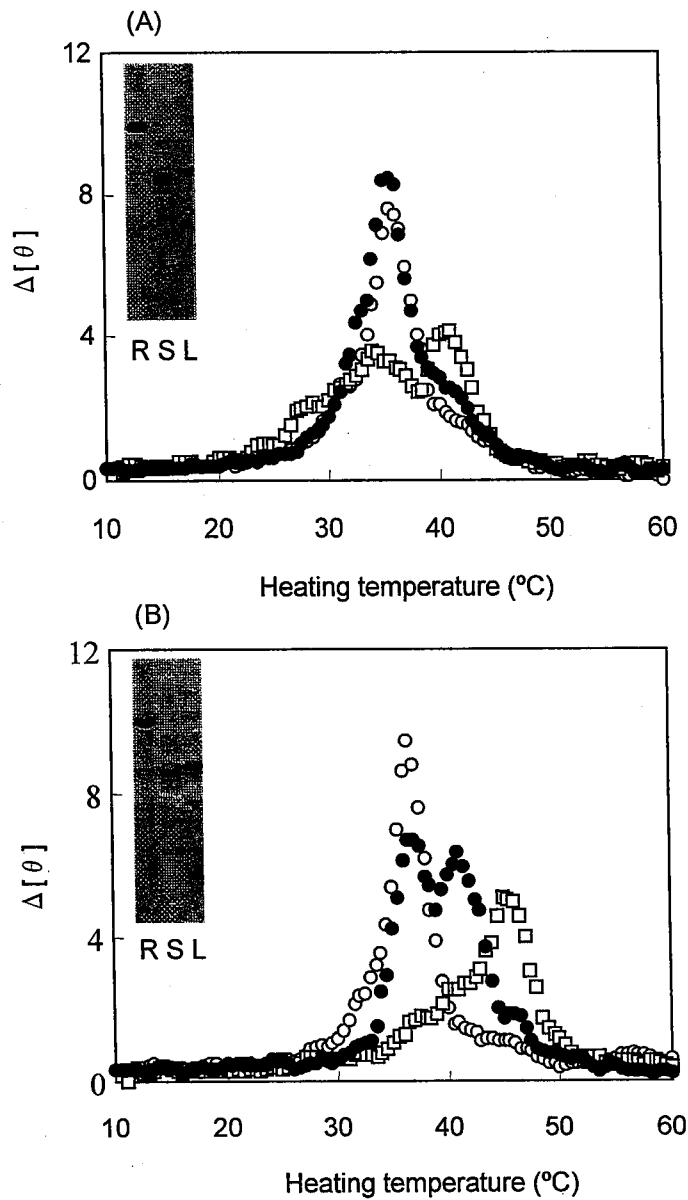
Unfolding process of helix structure of rod during a gradual heating from 10 to 70 °C at a rate of 1 °C/min was analyzed by reading the ellipticity ( $\theta$ ) at 222 nm (Takahashi et al. 2005a). Decrease in the ellipticity upon heating was a useful index for detecting the unfolding process of rod (Nakaya et al. 1997). Normalized change in  $\theta_{222\text{nm}}$  as an index of thermal unfolding of rod and their derivatives are shown in Fig. 5-5. It was demonstrated that winter rod began to unfold at a slightly lower temperature than summer rod by 5 °C. Remarkable decrease in  $\alpha$ -helix content was observed at around 40 and 35 °C for summer and winter rod, respectively. Temperature dependent unfolding profile between summer and winter myosin rod was carefully studied by taking the derivative of the change. Summer myosin rod generated two major unfolding transitions at 36.5 and 41.5 °C. However, winter rods roughly unfolded in a single phase with a transition temperature of 35.5 °C. A small transition peak at 40.5 °C was also detected with winter sample. These two unfolding profiles were clearly distinguished from each other. It should be noted that the transition temperature for winter rod was similar to that lower one detected with summer rod. In other words, the presence of unfolding peak at higher temperature (41.5 °C) characterized summer myosin rod. To characterize the unfolding peaks detected with two myosin rod samples, contribution of S-2 and LMM portions in the patterns was further studied. S-2 and LMM were isolated from rod by chymotryptic digestion. SDS-PAGE patterns for LMM and S-2 used are inserted in Fig. 5-6. Unfolding profiles for these fragments were compared with those of myosin rod using their derivative of ellipticity (Fig. 5-6). Winter LMM showed single unfolding transition at 36 °C, which was the same as one detected with winter rod. Winter S-2 showed two unfolding peaks with a major unfolding at around 36 °C and a minor unfolding at higher range of 40.5 °C, which explained the shoulder detected with the unfolding of winter rod (Fig. 5-5). It was also demonstrated that similar unfolding peaks of winter LMM and S-2 generated an apparent single unfolding peak of winter myosin rod. Summer LMM gave a sharp unfolding at around 36°C, which corresponded to low unfolding peak detected with summer rod. The unfolding temperature



**Figure 5-5. Comparison of the structural stability between summer and winter myosin rod of silver carp.**

Rod solutions of summer and winter were heated from 10 to 70 °C. Decrease in ellipticity at 222 nm (A, bold line for summer, and dotted line for winter) and its derivatives (B, closed circles for summer and open circles for winter) were compared.





**Figure 5-6. Comparison of unfolding profiles of myosin rod, subfragment-2 and light meromyosin fractions from silver carp in both winter and summer seasons.**

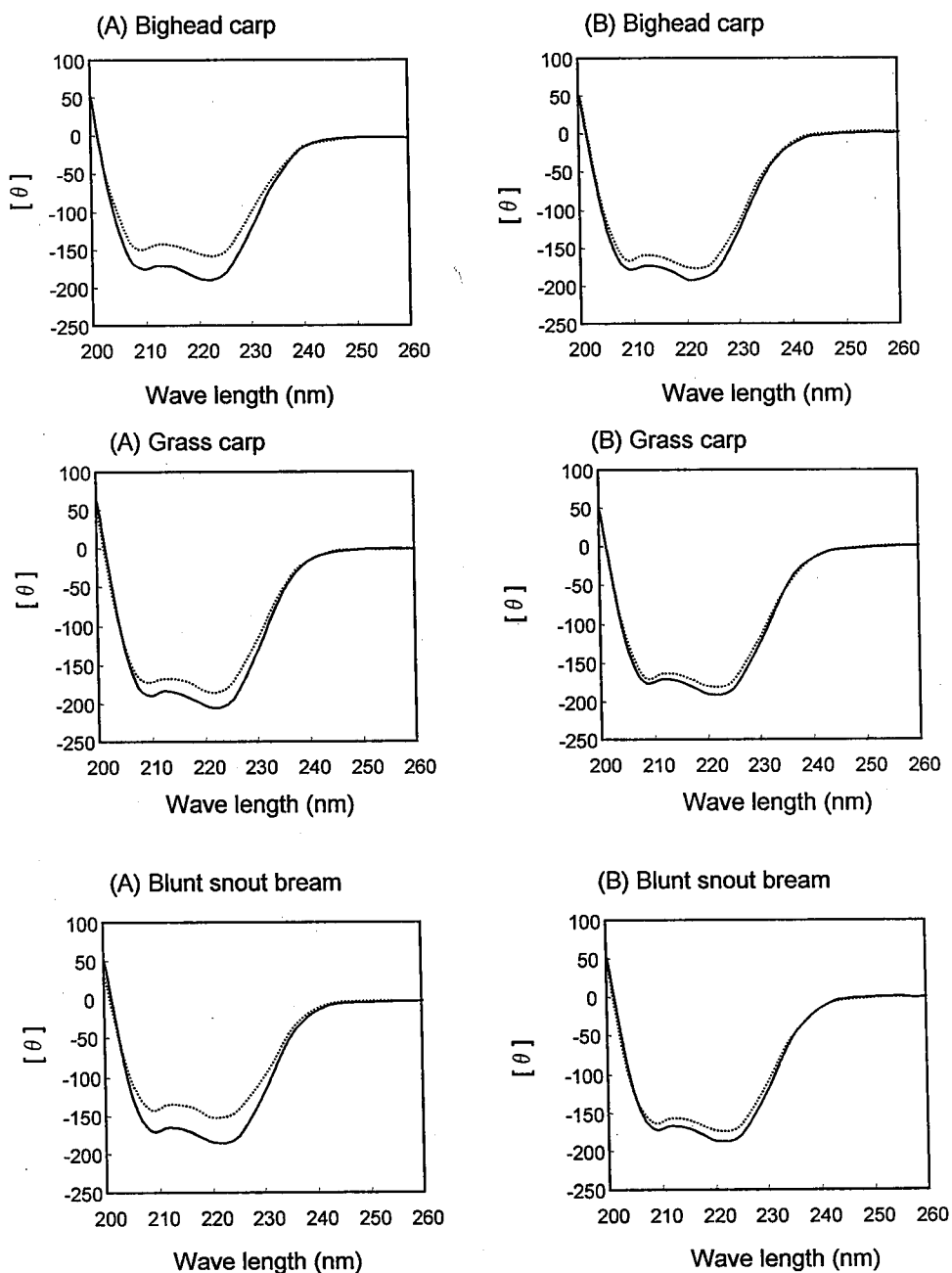
(A) and (B) are samples in winter and summer seasons, respectively. Derivatives of unfolding profiles of myosin rod (●), S-2 (□), and LMM (○) are compared. Inserted SDS-PAGE patterns are rod (R), S-2 (S), and LMM (L), respectively.

for summer LMM was rather similar to winter LMM, it was indicated that there was practically no difference in the stability of LMM region for both of summer and winter rod. Summer S-2 unfolded almost at a single temperature (46 °C), which was 10°C higher than that for LMM. The identical unfolding peak corresponding to isolated S-2 was not detected with parent rod. The second unfolding peak at around 40.5°C detected with rod might correspond to unfolding of S-2 region. Unfolding temperature of S-2 as a portion in rod was a little lower than that of S-2 as isolated form, which suggested that connection with unstable LMM seemed to lower the unfolding temperature of neighboring S-2. It was concluded that there was a small differences in the stability of LMM, while a large difference in S-2 region between summer and winter myosin rod. In other words, characteristic thermal stability of silver carp myosin rod came from the difference in the stability of S-2 region rather than LMM region.

#### **5.4 Species-Specific Structural Stability of Myosin Rod**

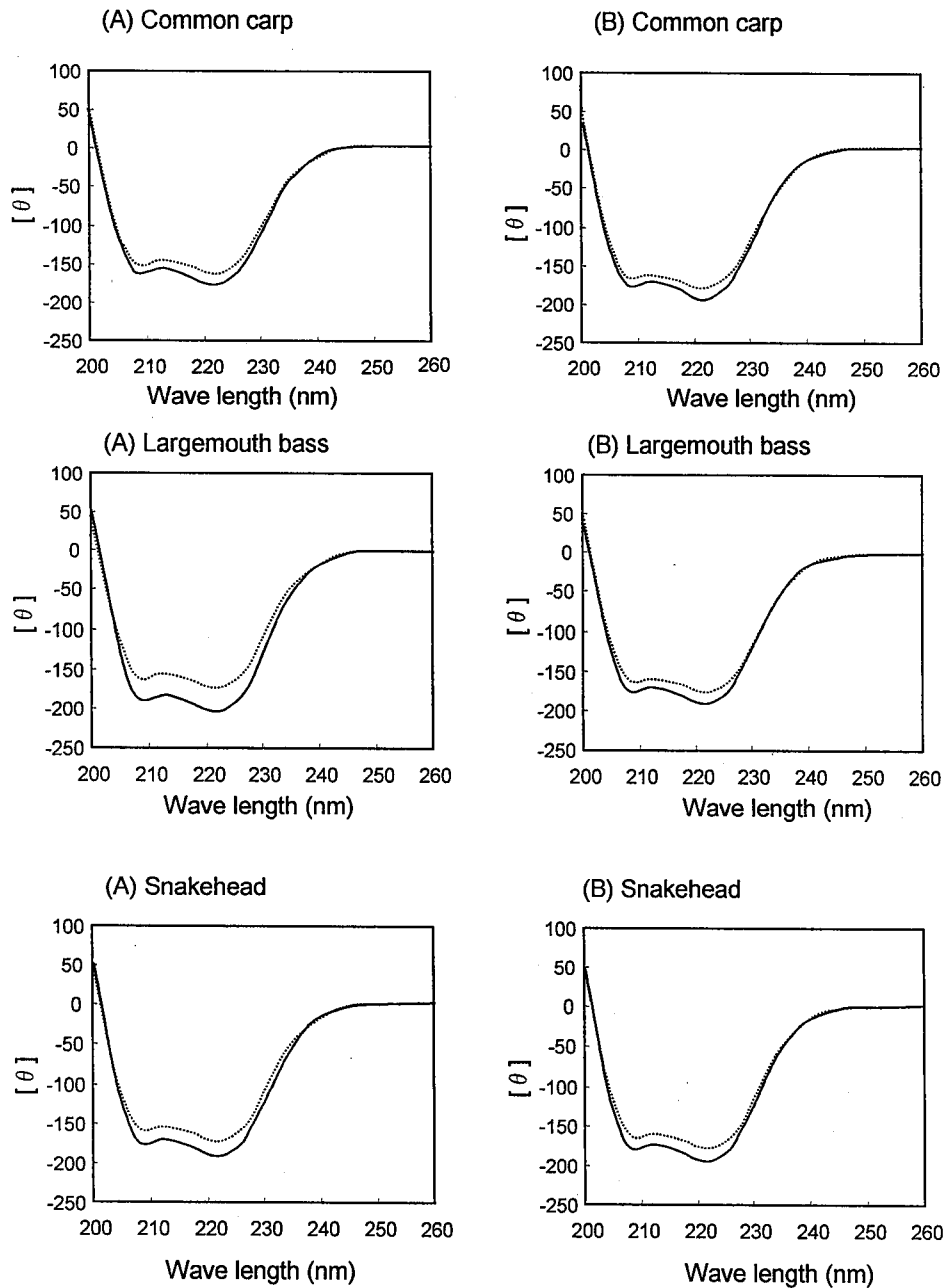
To clarify the difference in the thermal stability of myosin rod among seven species of freshwater fish, unfolding profiles of myosin rod from summer and winter fish were compared upon heating. First, CD spectra of rod from winter and summer fish among fish species were compared. Reversible refolding ability of rod upon heating to 70 °C and subsequently cooled was also compared among fish species. The CD spectra are shown in Fig. 5-7. There was no difference in the ellipticity for all of rod isolated from any species of fish and in any seasons. It was confirmed that all of myosin rod were almost completely helix structure at 10°C. Moreover, recovery in the helical structure was quite high for all of samples. Recovery extents for all cases are listed in Table 5-1. Generally, rod in summer season had higher refolding extent than that in winter season. However, the refolding ability of rod from common carp and snakehead exceptionally gave no difference between two seasons.

Unfolding profiles of rod from six species of fish upon heating from 10 to 70 °C were compared (Fig. 5-8). Winter myosin rods of bighead carp, grass carp, blunt snout bream and largemouth bass unfolded at lower temperature than summer rods by about 5 °C, which was similar trend as observed with silver carp



**Figure 5-7-1. Reversibility of the helix structure upon heating and cooling for various species of myosin rod.**

Experimental design was the same as studied with silver carp myosin rod in Fig. 5-4. (A) and (B) are samples in winter and summer seasons, respectively. Species of fish of samples were indicated in the figures. Spectra before (solid) and after heating and cooling (dashed line) were similarly measured as in Fig. 5-4.



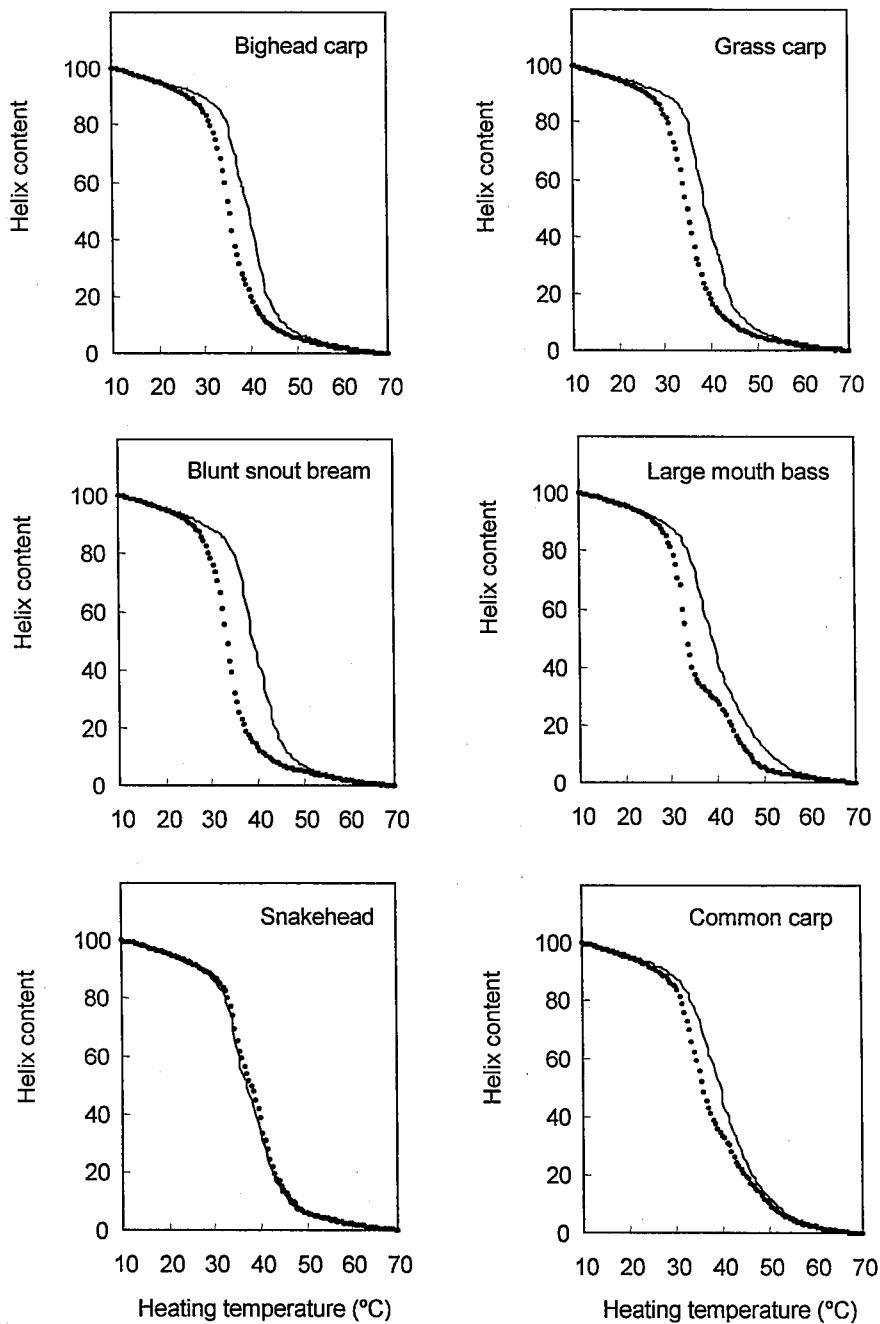
**Figure 5-7-2. Reversibility of the helix structure upon heating and cooling for various species of myosin rod.**

Experimental design was the same as studied with silver carp myosin rod in Fig. 5-4. (A) and (B) are samples in winter and summer seasons, respectively. Species of fish of samples were indicated in the figures. Spectra before (solid) and after heating and cooling (dashed line) were similarly measured as in Fig. 5-4.

**Table 5-1. Refolding extent of rod upon heating and cooling of rod.**

Seasons	Reversibility(%)*	
	Summer	Winter
Silver carp	93	80
Bighead carp	93	82
Grass carp	95	89
Blunt bream	94	81
Common carp	94	94
Largemouth bass	94	85
Snakehead	91	90

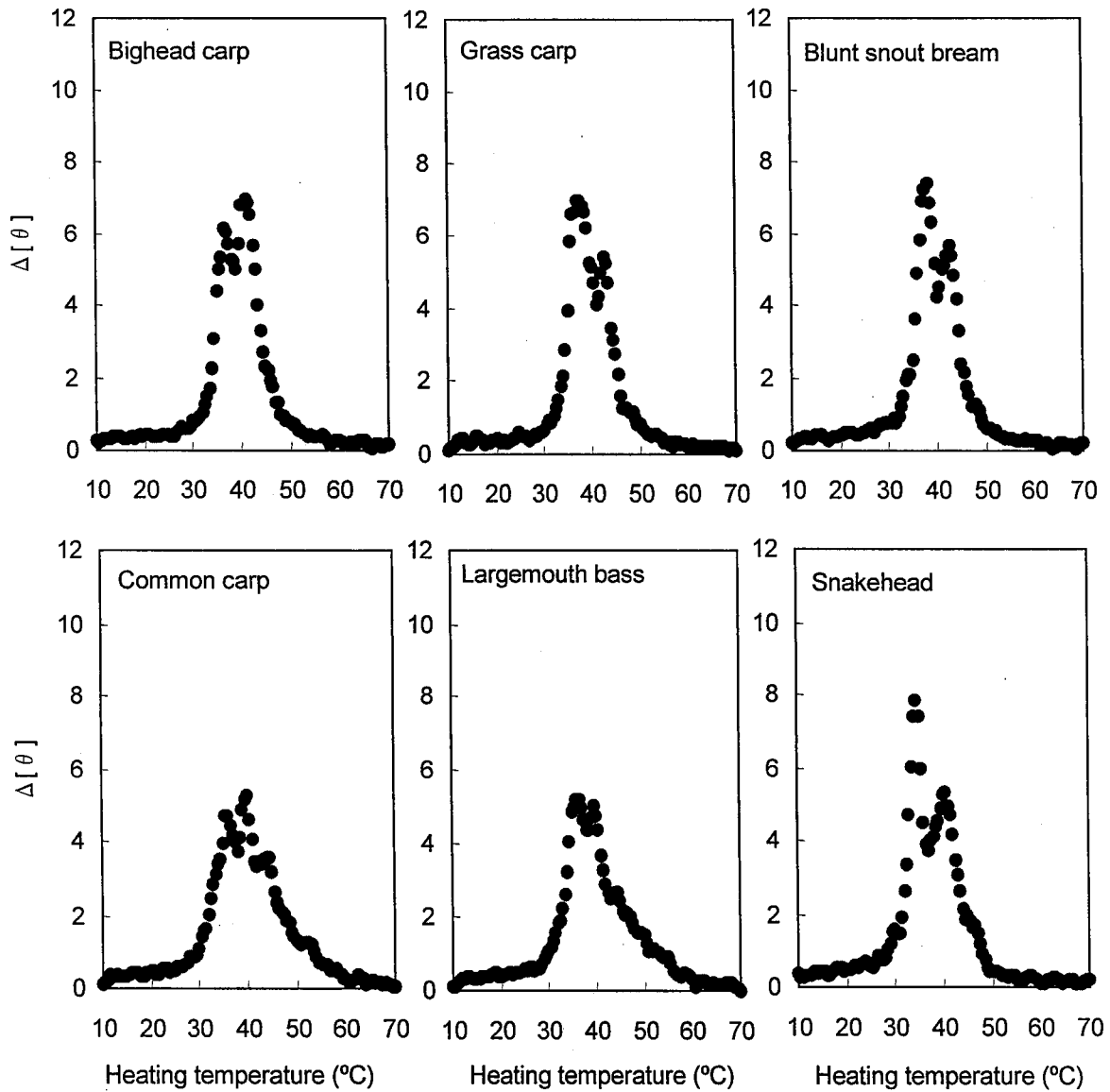
\*Rod was heated from 10 to 70 °C, and then cooled to 10 °C. Reversibility was estimated from the ellipticity at 222 nm



**Figure 5-8 Unfolding profiles of myosin rod from six species of fish in both winter and summer seasons.**

Decrease in helix content was monitored by reading the value at 222 nm. The relative helical values were estimated by assuming a helix content at 10 and at 70°C to be 100 and 0 %, respectively. Rod in summer (solid) and winter (dotted line) were compared. Species were in the figures.

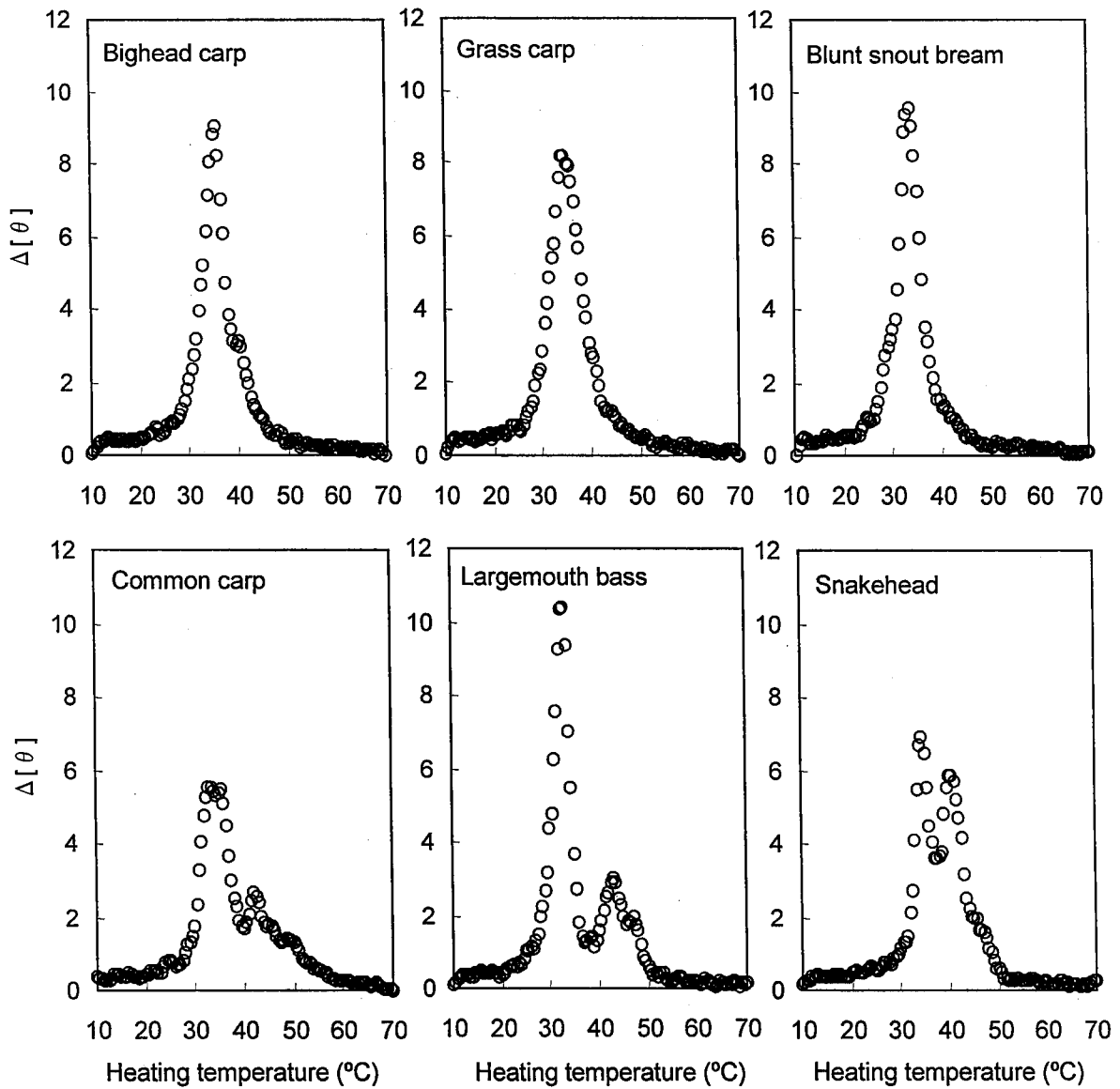
myosin rod (Fig. 5-5). Difference in the unfolding temperature for common carp rod between two seasons was only about 2.5 °C. Surprisingly, snakehead myosin rod in two seasons showed exactly the same unfolding profiles. The derivatives of the unfolding were obtained to compare the unfolding more carefully. The derivatives are presented in Figs. 5-9-1 and 2. All of summer myosin rod generated two unfolding peaks although relative peak heights were different. The transition temperatures for these two peaks are presented in Table 5-2. It was demonstrated that respective unfolding temperatures for rod were rather similar among fish species in summer season. The results strongly showed that thermal stability of rod in summer fish was similar among species, which was the same conclusion derived from the thermal inactivation rate of ATPase in Chapter 2 on the thermal stability of S-1 portion. On the other hands, unfolding profiles for winter myosin rod differed from species to species. Bighead carp, grass carp and blunt snout bream showed practically a monophasic unfolding which was exactly the same as silver carp myosin rod. The unfolding temperatures for these rods were also the same as winter silver carp rod. Common carp, bigmouth bass, and snakehead exhibited two unfolding peaks for winter rod similarly to summer one. Lower unfolding peak for these rods was similar to one detected with rod from above three species of carp family fish. Unfolding profiles for winter and summer myosin rod were compared for respective species of fish (Fig. 5-9-3). A single unfolding for winter bighead carp, blunt snout bream, and grass carp rod occurred at a slightly lower temperature range than that of lower unfolding temperature for summer myosin rod. With common carp both summer and winter rods exhibiting two unfolding peaks, unfolding peak at lower temperature was also lower than that for summer myosin rod. However, the higher unfolding temperature of both summer and winter rods was similar. For snakehead, unfolding profiles for winter and summer rods were identical. In the case of largemouth bass, unfolding of winter rod showed a rather higher transition temperature (47 °C) than that found in summer one. Although the region in rod for producing unfolding at such a high temperature was not identified, the result suggested that winter myosin rod of largemouth bass contained less stable LMM and more stable S-2 region than summer one.



**Figure 5-9-1. Derivatives of unfolding profiles of myosin rod from six species of fish in summer season.**

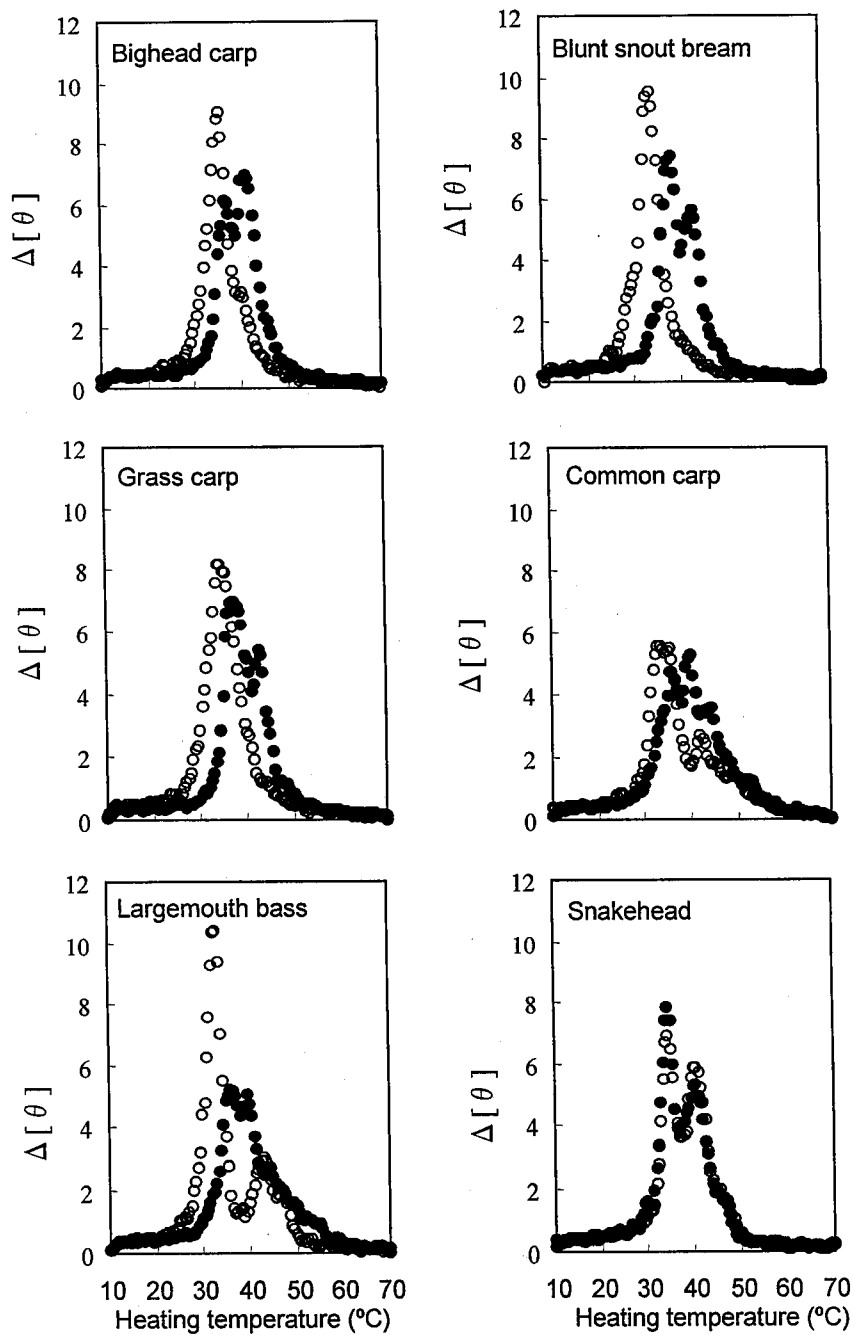
The derivatives of the decrease in helix content as in Fig. 5-8 were calculated. Species were in the figures.





**Figure 5-9-2. Derivatives of unfolding profiles of myosin rod from six species of fish in winter season.**

The derivatives of the decrease in helix content as in Fig. 5-8 were calculated. Species were in the figures.



**Figure 5-9-3. Comparison of unfolding profiles of myosin rod from six species of fish between summer and winter seasons.**

The derivatives of the decrease in helix content as in Fig. 5-8 were calculated. Species were in the figures. Summer (closed ) and winter (open) symbols were compared.

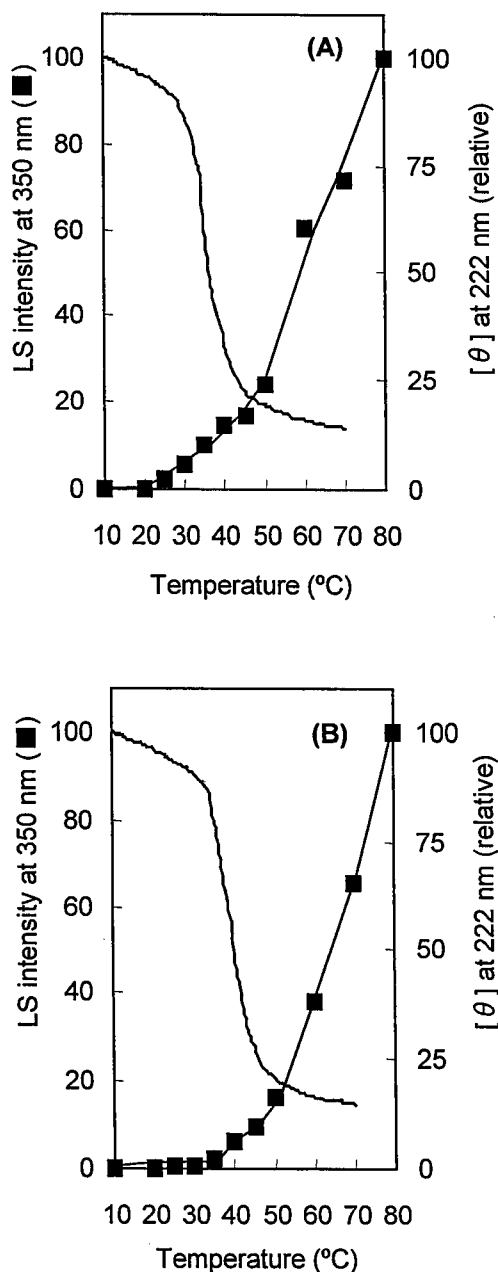
**Table 5-2. Transition temperatures in the unfolding profiles for seven species of rod.**

Seasons	Transition temperature (°C) *	
	Summer	Winter
Silver carp	36.5, 41.0	35.5, (40.5)
Bighead carp	36.5, 41.0	35.5, (40.5)
Grass carp	37.5, 43.0	35.0
Blunt snout bream	38.0, 42.5	33.5
Common carp	36.0, 40.0, 44.5, 52.5	33.5, 35.5, 42.5, 50.0
Largemouth bass	36.0, 39.5	33.0, 43.0, 47.0
Snakehead	34.5, 40.5	34.5, 40.5

\* Transition temperature was the peak temperature in the derivatives of the change in ellipticity at 222 nm upon heating. Temperatures in parenthesis denote the shoulder.

## **5.5 Relationship between Unfolding Profile of Rod and Aggregation**

It is well established that myosin rod is directly involved in thermal gelation by myosin. Thermal gelation mechanism contains heat-induced unfolding of myosin and its subsequent irregular association to form network aggregates (Yasui et al. 1979; Samejima et al. 1981; Ojima et al. 1999). In order to obtain basic information on thermal aggregation of myosin, temperature dependent aggregate formation by rod from various species of fish in summer and winter was studied considering their characteristic unfolding profiles. First, the unfolding and aggregation properties of summer and winter silver carp rods were compared. For the measurement of rod aggregation upon heating, rod solution in 0.5 M KCl, 20 mM Tris-HCl (pH 7.5) (usually 2 mg/ml) was heated for 30 min at various temperatures from 20 to 80 °C, and cooled in ice-cold water. The heated rod solutions at all temperatures were clear, and no turbidity was detected by naked eyes. Although the data were not presented, the turbidity could not be detected by measuring absorption at 350 nm even for 80 °C heated samples. Moreover, centrifugation of the heated rod solution at  $100,000 \times g$  for 15 min practically did not sediment aggregates. Much more sensitive light scattering intensity measurement at 350 nm detected aggregate formation by rod. As presented in Fig. 5-10, the light scattering intensity increased with raising the heating temperature for the heated rod solution as a result of aggregation. General features for winter and summer myosin rod were similar to each other. However, there was a slight difference in the temperature to begin aggregation between two samples. No increase was detected when heated below 25 and 35 °C for winter and summer rod, respectively. Relative helix contents for summer and winter rod at these temperatures were roughly 90 % and 80%, respectively. Above respective temperatures, both winter and summer rod started to form aggregates. It was commonly observed that aggregation was remarkable after a complete unfolding of rod above 45 and 50°C for winter and summer silver carp myosin rod. Unfolding of whole rod structure seemed required for aggregate formation. Thus, high unfolding peak at around 40 °C detected in summer myosin rod would explain higher temperature for aggregation because unfolding temperatures for LMM region for both samples were the same. In other words, low unfolding of S-2 region in winter myosin rod seemed reduce the



**Figure 5-10. Aggregation and unfolding of silver carp myosin rod in winter and summer seasons upon heating.**

Rod solution of winter (A) and summer (B) seasons were heated for 30 min at respective temperatures, and its light scattering intensity at 350 nm was measured (■). Unfolding profiles of rod (solid line) were taken from Fig. 5-5.

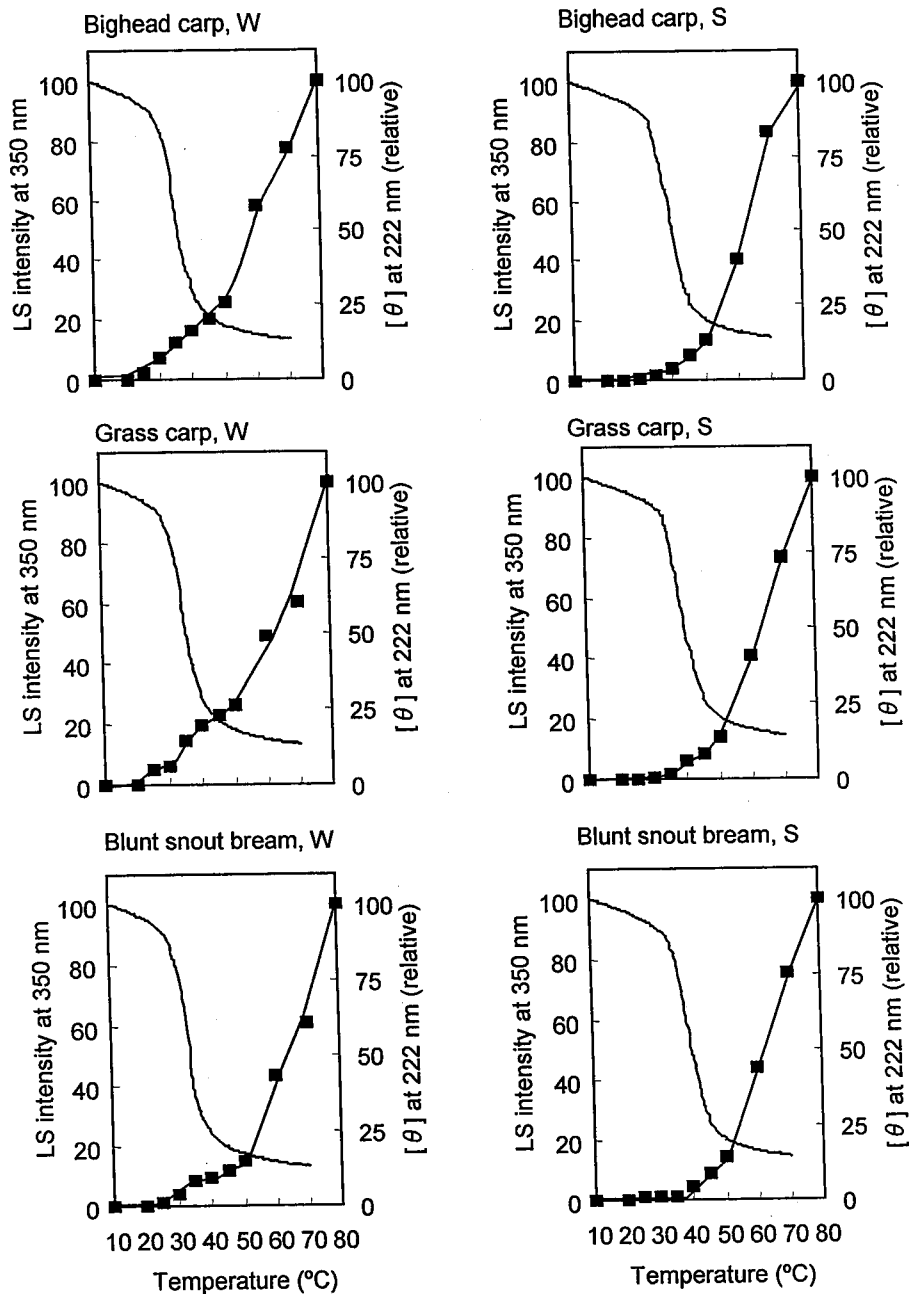
aggregation temperature.

### **5.6 Thermal Aggregation and Unfolding of Rod from Various Species**

To study the relationship between aggregate formation of rod and unfolding with other species, thermal aggregation and unfolding patterns of rod from six species of fish in winter and summer are compared. Comparison was made in Fig. 5-11-1, 2, and 3 and the summary of unfolding and aggregate formation was listed in Table. 5-3. Species were classified into three groups from the difference in the aggregation pattern between winter and summer rods. The first group included bighead carp, grass carp and blunt snout bream, in which winter rod formed aggregate at lower temperature than summer one, similar with silver carp.. For these species, aggregation readily started at temperature where a quite low extent of unfolding was achieved. The second group included common carp and snakehead, in which both summer and winter rods started the aggregation at similar temperature. Unfolding extent at the temperature where aggregate started was much larger than the first group. An exceptional specie was largemouth bass, in which winter rod began to aggregate at higher temperature than summer rod (Fig. 5-11-2). The unique aggregation profile of largemouth bass was explained by the presence of exceptionally stable S-2 region in winter rod (Fig. 5-9). In other words, the stable S-2 prevented aggregates formation by rod. The finding would support the conclusion that a complete unfolding of whole rod structure was essential for aggregation.

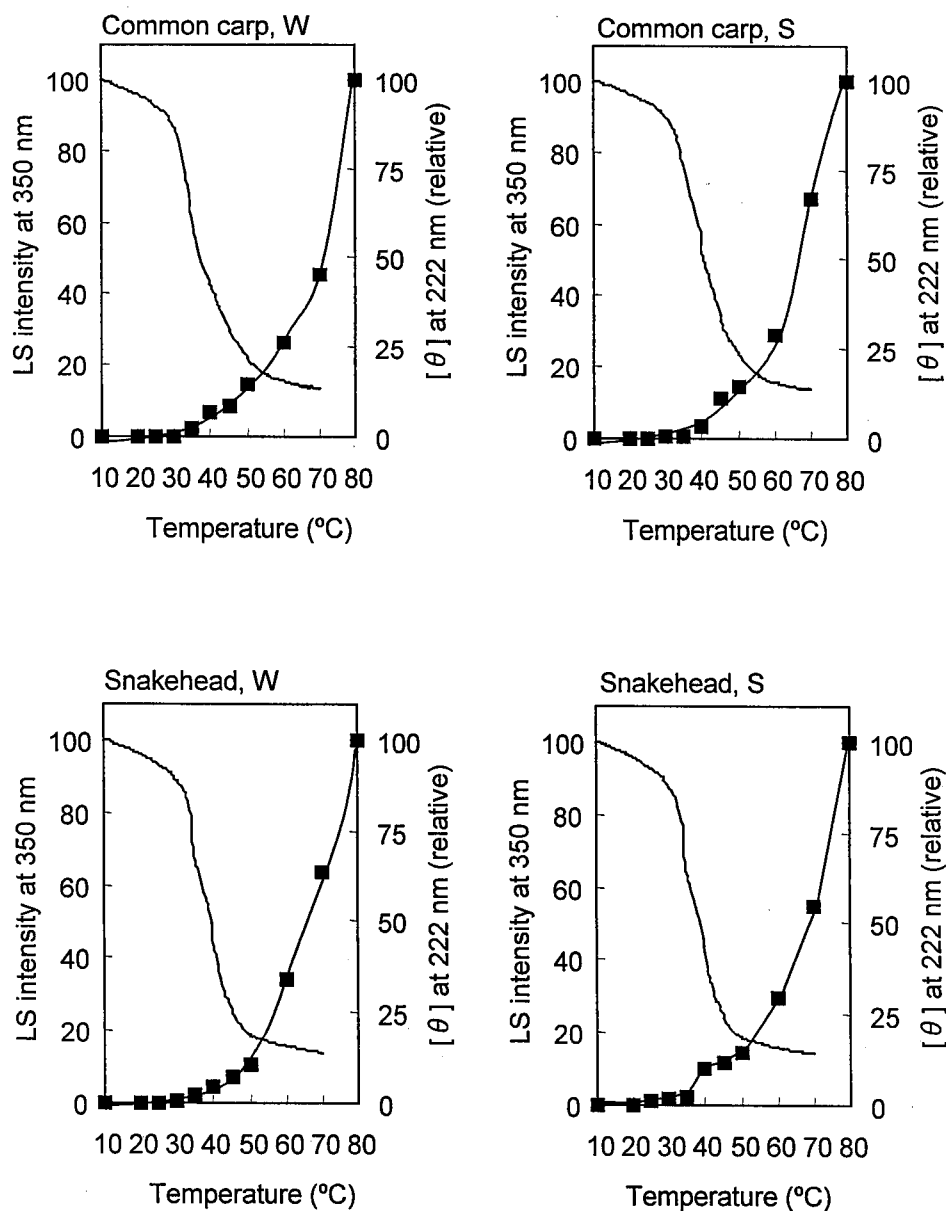
### **5.7 Discussion**

Thermal gelation is a process converting surimi sol to gel upon heating. The process is considered to be a reaction to form three-dimensional network structure by aggregated myosin molecules. These networks might be formed non-covalently by the thermally denatured myosin molecules. Binding forces such as hydrophobic interaction, hydrogen bonding, and electrostatic interaction are involved. In addition to the non-covalent bondings, it is reported that covalent bonds catalyzed by endogenous transglutaminase is



**Figure 5-11-1. Aggregation and unfolding of myosin rod of various species of fish in winter and summer seasons upon heating.**

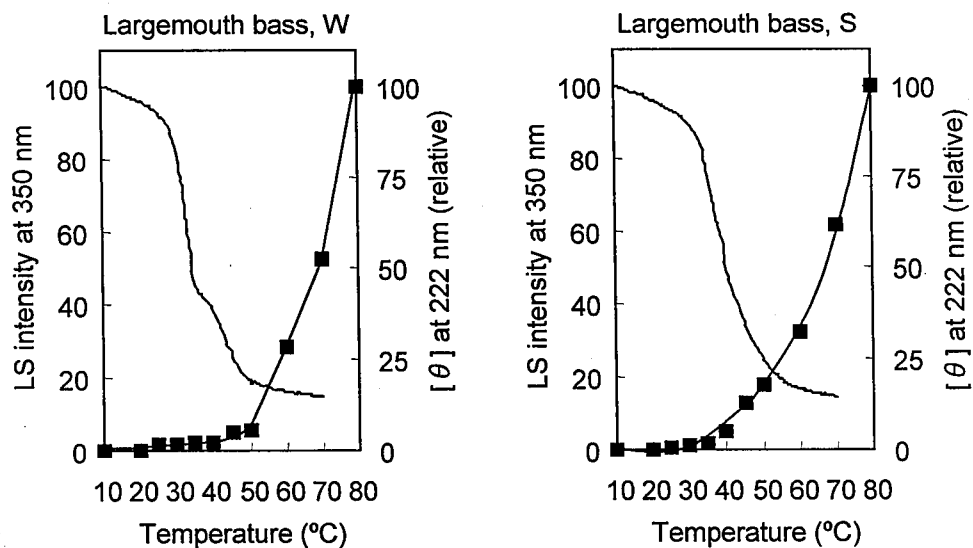
Rod solution of winter (A) and summer (B) seasons were heated for 30 min at respective temperatures, and its light scattering intensity at 350 nm was measured (■). Unfolding profiles of rod (solid line) were taken from Fig. 5-8. Species and seasons (winter, w and summer, S) were in the figures.



**Figure 5-11-2** Aggregation and unfolding of myosin rod of various species of fish in winter and summer seasons upon heating.

Rod solution of winter (W) and summer (S) seasons were heated for 30 min at respective temperatures, and its light scattering intensity at 350 nm was measured (■). Unfolding profiles of rod (solid line) were taken from Fig. 5-8. Species and seasons (winter, w and summer, S) were in the figures.





**Figure 5-11-3. Aggregation and unfolding of myosin rod of large mouth bass in winter and summer seasons upon heating.**

Rod solution of winter (W) and summer (S) seasons were heated for 30 min at respective temperatures, and its light scattering intensity at 350 nm was measured (■). Unfolding profiles of rod (solid line) were taken from Fig. 5-8.

**Table 5-3. The summary of unfolding and aggregate formation by seven species of myosin rod upon heating.**

Seasons	Fish species	Unfolding *1 (peak)	T <sub>1/2</sub> (°C) *2	T <sub>agg 1/2</sub> (°C) *3	H <sub>agg</sub> (%) *4
Summer	Silver carp	2	39.5	40.0	48.0
	Bighead carp	2	39.5	40.0	48.0
	Grass carp	2	39.5	40.0	45.0
	Blunt snout bream	2	40.0	40.0	50.0
	Common carp	3	39.5	45.0	20.0
	Largemouth bass	3	39.5	40.0	34.0
	Snakehead	2	36.5	44.0	17.0
Winter	Silver carp	1	35.5	30.0	82.0
	Bighead carp	1	34.5	30.0	85.0
	Grass carp	1	34.5	30.0	83.0
	Blunt snout bream	1	34.5	30.0	83.0
	Common carp	2	35.0	45.0	20.0
	Largemouth bass	2	34.0	47.0	9.0
	Snakehead	2	36.5	44.0	17.0

\*1. Number of unfolding peak in the decreasing profile of ellipticity at 222 nm.

\*2. The temperature to give 50% helix content.

\*3. The temperature to start the aggregate formation.

\*4. Helix content at the temperature when the aggregate formation begins.

involved in the network formation (Wan and Seki 1992; Niwa et al. 1993; Araki and Seki 1993). The latter factor is not the determinant one in the thermal gel formation by fish meat because a direct cooking of salted meat forms elastic thermal gel without covalent cross-linking of myosin. The fact suggested that myosin aggregation through denaturation process is the most important event in the thermal gelation. It is also well recognized that thermal gelling characteristics are species-specific. Since myosin is the responsible protein for thermal gelation, the difference in the gelation properties should come from the different properties of myosin molecule.

The different thermal gelation properties between summer and winter surimi demonstrated in Chapter 1 was deeply dependent on different thermal stability as measured by ATPase inactivation. Under the circumstances, it seemed important to provide the information on the structural characteristics of myosin rod between summer and winter season fish. Structural properties of rod were characterized by analyzing their unfolding profiles upon heating. CD measurement of myosin rod from all fish species revealed that rod was almost completely helix structure irrespective of the seasons and species. Rod lost the helix structure upon heating up to 70 °C. However, species-specific and myosin isomer specific unfolding process of helix structure were demonstrated. Silver carp myosin rod showed a typical pattern usually detected with carp family fish such as grass carp, bighead carp, and blunt snout bream, namely single unfolding peak with winter rod and two unfolding peaks with summer rod. The unfolding profile of rod was well explained by unfolding profiles of respective S-2 and LMM. Two unfolding peaks for summer fish myosin rod was explained by the unfolding of S-2 at high temperature and that of LMM at low temperature. A single unfolding for winter rod was also explained by similar unfolding temperatures of S-2 and LMM. As the LMM for winter and summer myosin had the same stability, different stability of S-2 region distinguished these two rods. These two rods showed different aggregation profiles; winter rod tended to form aggregates at lower temperature than summer one. Unfolding of rod structure and its aggregation had a close relationship. High temperature for aggregation for summer myosin rod seemed to come from the stable S-2 region. The results seemed consistent with the results of surimi, in which winter

surimi formed gel at 30 °C, while summer surimi formed gel at 40 °C.

In all cases, aggregation was significant above the temperature for a complete unfolding of whole rod structure. Thus, stable S-2 in rod structure seemed to suppress the aggregation by rod. Fish species were grouped by aggregate formation. A typical profile for the first group was the case of silver carp, in which aggregate started at low level of unfolding. Other group included common carp and largemouth bass that contained more stable S-2 region. The rod did not form aggregates until most stable S-2 region unfolded at higher temperature. Different from common carp, myosin rod in Alaska pollack, which is a typical fish that forms excellent elastic thermal gel, unfolds monophasically and aggregation starts even at a high content of helix structure.. Winter rod of the first group such as silver carp classified in this chapter was rather similar to pollack. Common carp, largemouth bass, and snake head were similar to carp.

## General Discussion

For the effective utilization of freshwater fish as protein resources, it seems essential to understand the properties of muscle protein of these species of fish. Especially, the information on the stability or denaturation of the protein is important because biochemical properties of fish myosin determines the quality of final products. It is well known that thermal stability of myosin is deeply dependent on the environmental temperature at which fish live (Johnston et al. 1973; Hashimoto et al. 1982). There are accumulated studies on the thermostability of myofibrillar ATPase in eurythermal fish from the viewpoint of temperature acclimation (Huang et al. 1990; Guo et al. 1994; Tao et al 2005). However, there is little report on the difference in thermal stability for those fish living at the same habitat temperature. For a full understanding of seasonal change in the thermal stability of myosin, myosin expressed in winter and in summer seasons with seven species of fish was investigated in detail. The objectives of the study are as follows; difference in the thermal stability of myosin in summer and winter seasons, characterization of myosin of seven species of freshwater fish in summer and winter seasons, types of myosin expressed year-roundly, strategy of adaptation to the change in water temperature, and seasonal change in gelling properties of freshwater surimi.

In Chapter 2 and Chapter 3, thermal stability of myosin from seven species of freshwater fish in winter and summer seasons was investigated by measuring  $\text{Ca}^{2+}$ -ATPase inactivation rate upon heating. Myofibrils from seven species of fish in two seasons gave different results. Although thermal stability was species-specific, fish species was roughly classified into three groups by the difference in the thermal stability of myofibril between winter and summer seasons. The first group including silver carp, bighead carp, grass carp, and blunt snout bream showed a quite large change in thermal stability. The second one was common carp that showed a small difference in the stability. The last one included largemouth bass and snakehead, which showed the same stability in two seasons. Thermal stability of myosin in the first group in summer season was as stable as tilapia, one of the most stable fish, and myosin in winter season

was as unstable as walleye pollack one, one of the most unstable species. The difference seemed reasonable because the water temperature in summer reaches 30 °C, and that in winter season became lower than 5 °C. Both are suitable temperatures for tilapia and pollack, respectively. Consequently, unstable winter silver carp myosin could not be stored in ice without denaturation. The information seems very important for the utilization of fish meat in winter because nobody has pointed out such an unstable nature of winter silver carp myosin. Such a large seasonal change in myosin stability observed with silver carp has not been reported ever. However, the story is not always applicable to other freshwater species because change in the stability for common carp was only 3 times. Moreover, myosin of winter largemouth bass and snakehead showed a similar stability to that of summer fish. Comparing the thermal stability among species in winter and in summer, it was demonstrated that thermal stability of myofibril in summer season was quite similar among species, while the thermal stability in winter season was rather species-specific. Adaptation is usually accompanied by altering myosin conformation more flexible at the habitat temperature, namely less stable structure for cold-water fish species (Johnston and Walesby 1977, Goldspink and Penney 1982). Supposing pollack myosin has the ideal conformation for keeping active movement at low habitat temperature of 5-10 °C, myosin of largemouth bass and snakehead in winter season seems too stable to be functional. Although the same story was proposed with freshwater fish (Heap et al. 1985; Hwang et al. 1990), it could not be applied to freshwater fish species because very stable winter myosin was found in largemouth bass and snakehead in the present study. It is certain that stable structure of myosin in winter season was unfavorable for an active movement. Considering actual behavior of fish in winter season, stable structure seems not unfavorable because the fish do not swim actively but stay on the bottom quietly. The stable structure might have no problem for the species for surviving in winter season. As similar thermal stability was observed with above seven species of fish myosin in summer season, the myosin structure for all of species was rigid enough for keeping their conformation functional above 30 °C. High stability for summer myosin seemed essential for surviving at high water temperature, and the adaptation to high temperature in summer season seemed the strategy for

surviving.

As winter and summer myosin for all fish species studied showed different thermal stability, the myosin expressed in winter and in summer was thought as different type of myosin isomer. The system to express several myosin isomers adapting to change in water temperature is considered as the strategy for carp to survive year round (Imai et al. 1997). Nucleotide sequence analysis on cDNA clones showed three myosin heavy chain isoforms in fast skeletal muscle of carp in an acclimation-temperature -dependent manner. As the objective of this study is to utilize muscle protein as food, the thermal stability was used as the index to distinguish the expressed myosin isomers. The technique can not distinguish myosin isomers with the same stability if present. For estimation of myosin isomers present in myofibrils, thermal stability of whole myosin was analyzed by measuring  $\text{Ca}^{2+}$ -ATPase inactivation of samples monthly prepared. It was demonstrated that only two types of myosin were detected, namely, unstable winter type and stable summer type. Myosin in certain periods showed the identical inactivation rate indicating an expression of the same type of myosin isomer in the season. Fish in the intermediate season expressed both types of myosin with different ratios. Co-existing of two types of isomer in the preparation was easily observed by the existing of breaking point in the thermal inactivation profile. The mixing ratio of these two myosin isomers was simply estimated from the inactivation profile graphically. Twelve months were divided into summer seasons of 4 months from June to September, winter seasons of 4 months from December to March, and two transition seasons of 2 months between summer and winter seasons. As year round myosin isomer expression pattern was similar among species although the stability among species differed from each other, water temperature change would be the trigger to start the expression of another isomer.

For better understandings myosin denaturation, the thermal denaturation of myosin in myofibrils was investigated by employing various indexes such as salt solubility, monomeric myosin content, and chymotryptic digestion as well as ATPase inactivation in Chapter 4. Combined indexes gave information on the conformational change of myosin upon heating. Salt solubility and monomeric myosin content

decreased much faster than ATPase inactivation. The event was similarly observed with myofibrils in two seasons for all of species of fish irrespective of the stability. The pattern was similar to one reported with Japanese common carp by Takahashi et al. (2005b). Therefore, quick loss of salt solubility was a common characteristic denaturation pattern for Chinese freshwater fish myosin. It was also demonstrated that ATPase inactivation was well correlated with the loss of production of S-1 from heated myofibrils for all the species studied. Thus, the statement that S-1 was generated only from myosin retaining ATPase activity was proved in the study. In other words, S-1 denaturation could be studied by measuring either ATPase inactivation or the decrease in the amount of S-1 produced from heated myofibrils. Similarly, salt-solubility and monomeric myosin content was well correlated with the amount of rod produced for all of species. These facts indicated that monomeric rod was generated only from myosin that retained solubility. The pattern obtained was not unchangeable because denaturation mode became opposite by lowering heating temperature. All myofibrils examined in the study showed a quicker rod denaturation than S-1 when heated at high temperatures ( $>33\text{ }^{\circ}\text{C}$ ) regardless of the season and species. However, S-1 and rod denaturation turned into opposite when heated at low temperatures ( $<33\text{ }^{\circ}\text{C}$ ). Temperature dependent S-1 and rod denaturation with silver carp myofibrils clearly showed that S-1 denaturation rates gave a straight line, while rod denaturation rates gave a breaking point in the line with the transition temperature of  $33\text{ }^{\circ}\text{C}$ . The activation energy for the denaturation of rod at low temperature range was about 3 times greater than that at high temperature range. S-1 and rod denaturation detected was irreversible structural changes. Probably, reversibility of the conformational change occurring at rod portion was much greater than that at S-1 portion at low temperature range. The fact is important considering the storage of dorsal muscle of the fish species at low temperature.

To characterize internal structure of myosin rod between two seasons and among fish species, myofibrils were digested with chymotrypsin in a dissolved form at  $0.5\text{ M KCl}$  expecting cleavages at HMM/LMM junction in rod. Digestion temperatures were varied from  $0$  to  $30\text{ }^{\circ}\text{C}$  to study the temperature dependent flexibility of rod portion. At low temperature ( $10\text{ }^{\circ}\text{C}$ ), it was proved that myosin in all species of fish in



summer season showed a very similar digestion pattern with a selective cleavage practically at a single site producing single HMM (about 165 kDa). However, myosin in winter fish showed rather species-specific digestion pattern. Cyprinid family except common carp and largemouth bass and snakehead myosin was similarly cleaved as summer myosin producing a single HMM (165 kDa), while common carp produced uniquely long HMM (180 kDa) indicating an additional cleavage at the site located near C-terminal region. Digestion of myosin at elevated temperature provided the information on the flexibility of the rod portion of myosin in winter and summer fish. Effect of temperature was clearly demonstrated with winter silver carp myofibrils. HMM produced at low temperature tended to be cleaved further resulted in a decrease in the HMM production at high temperature. Production of characteristically short 135 kDa HMM from largemouth bass and common carp in winter season was unique especially at 30 °C. Such short HMM was reported with Japanese common carp (Kato and Konno 1993b; Takahashi et al. 2005a). Therefore, Japanese common carp used in the reference was winter type. As the pattern for common carp differed from in two seasons, a small conflict in the reports on the substructure of myosin rod would be explained by the myosin used for analysis.

The structural stability of rod portion of myosin for various species of fish in different seasons was further studied in Chapter 5. As the rod portion of myosin molecule is almost helical structure, unfolding of helical structure is the index to study the structural differences. As presented in Chapter 3, rod was easily prepared from myofibrils by digestion with chymotrypsin although the conditions were a little different. Unfolding profiles of rod in winter and summer upon heating from 10 to 70 °C were compared. For summer samples, all unfolding profiles generated two unfolding peaks although transition temperatures and the peak heights for these two peaks were a little different. The results strongly indicated that thermal stability of myosin rod in summer fish was similar among species, which was the same conclusion derived from the thermal inactivation rate of ATPase in Chapter 3 and the chymotryptic digestion pattern of myosin in Chapter 4. On the other hand, unfolding profiles for winter myosin rod differed from species to species. Unfolding profiles were classified into two groups. Winter myosin rods of silver carp, bighead carp, grass

carp, and blunt snout bream showed a single large unfolding peak at around 35 °C. The rest of fish species (common carp, bigmouth bass, and snakehead) showed two unfolding peaks. Unfolding temperature for lower one at around 35 °C for the latter group was very similar among species, and was also similar to that of the single peak obtained with the first group. Comparing the unfolding temperatures between summer and winter myosin rod, the low unfolding temperature around 36 °C detected in summer rod was a little higher than the single unfolding temperature for winter one in the first group. Additional unfolding peak at high temperature at around 45 °C characterized the summer rod. Surprisingly, snakehead myosin rod in two seasons showed exactly the same unfolding profiles. Contribution of S-2 and LMM regions in the characteristic unfolding profiles of winter and summer silver carp myosin rod was studied by preparing these two fractions from rod of two seasons. LMM from summer and winter rod showed a single transition peak at around 36 °C indicating that thermal stability of LMM region in two seasons was the same. A large difference was observed in the unfolding of S-2. Although both S-2 fractions showed two unfolding peaks, temperature to generate major peak was different. S-2 from summer rod showed unfolding at high temperature (47 °C), which corresponded to the second unfolding peak detected with summer myosin rod. On the other hand, transition temperature (36 °C) for winter S-2 was rather similar to that of LMM, which is the explanation of a single unfolding of winter myosin rod. It was concluded that structural stability in rod portion between two seasons was determined by the S-2 region rather than LMM region.

One of the possible ways of utilization of fish muscle protein is surimi production. It is well recognized that thermal gelling characteristics are fish species-specific. In Chapter 5, thermal aggregation process of myosin rod was studied as a model system of thermal aggregation. Relationship between thermal aggregation and unfolding were investigated considering different unfolding properties of rod in different seasons. In all cases, aggregation was significant above the temperature where a complete unfolding of whole rod structure was achieved. Consequently, the rod that contained stable S-2 was proved to be hard to form aggregates. The temperature to induce a complete unfolding was determined by the most stable

region of rod, namely S-2 region. Species were classified into three groups from the difference in the aggregation pattern between winter and summer rods. The first group included silver carp, bighead carp, grass carp and together with blunt snout bream, in which winter rod formed aggregate at lower temperature than summer one. For these species in winter, aggregation readily started with a quite low extent of unfolding. The second group included common carp and snakehead, in which both summer and winter rods started the aggregation at similar temperature. Unfolding extent at the temperature where aggregate started was much larger than the first group. An exceptional species was largemouth bass, in which winter rod began to aggregate at higher temperature than summer rod. The unique aggregation profile was explained by the presence of exceptionally stable S-2 region in winter rod. Generally, the stability of S-2 region, the most stable region in rod, seemed determine the aggregation temperature for rod.

For the practical application of the study, thermal gelation properties of surimi prepared from silver carp in four seasons were compared in Chapter 1. Overall conclusion was that gelation temperature was determined by the different stability of myosin. Summer season surimi judging from the thermal stability of myofibrils (Jun and Sep) required 10 °C higher temperature to produce similar gel than that for winter (Dec and Mar) surimi. The relationship between gelation temperature and thermal stability was consistent with the results by Katoh et al. (1984). Two-step heating is often introduced to improve thermal gel properties in Japan. Usually, the first heating (pre-heating) is performed at low temperatures such as 20~40 °C, then heated at high temperature for thermal gel production. Gel formed at such low temperatures is called "set gel". Increased breaking force or strain by introducing pre-heating is referred to as setting effect. The effect of two-step heating was also investigated preliminarily for both winter and summer surimi. Applying the two-step heating technique in the thermal gel production increased the breaking force for the final product. Preheating of winter surimi at 30 °C for 4-6 hours increased the breaking force from 350 to 700 g, by twice increment. Preheating of summer surimi at 35 °C, about 5 °C higher than that for winter surimi, gave a similar effect. Different suitable temperature for pre-heating is also explained by different stability of myosin in these two surimi samples. It is reported that setting

effect is attributed to myosin cross-linking catalyzed by transglutaminase. It is also reported that common carp generates practically no cross-linking products during pre-heating step, although the muscle contains a quite high transglutaminase activity. Preliminary analysis of myosin cross-linking reaction during preheating was conducted to understand the setting effect observed with silver carp surimi. Incubation of salted winter surimi at 30 °C gradually generated myosin cross-linked products revealed on 3 % polyacrylamide/0.5 % agarose gel. Summer surimi also formed cross-linked products at a little higher temperature. A general gelation profiles for silver carp surimi was rather similar to those of Alaska pollack although a suitable heating temperature was a little high than that of pollack. These results open the possibility of silver carp muscle as a material for surimi production. The lowest price of silver carp among freshwater fish species is also favorable for surimi production.

Generally, gel produced at relatively low temperature is called as 'set gel', one formed at high temperature is called as 'heated gel' or 'directly heated gel', and one formed by two-step heating is termed as 'two-step heated gel'. The definition seems reasonable, however, there is no clear identification for 'set gel' and 'heated gel'. Texture map in which breaking strain was plotted against breaking force well distinguished these two gels. Irrespective of gel properties, the parameters for the directly heated gel were on a straight line. This also was true for the disintegrated gel producing at around 60 °C. However, the parameters for some gels produced at low temperature were not on the line. They located above the line indicating more elastic properties of the gel. The temperature to produce heated gel for summer was higher than winter surimi by about 5-10 °C. Surprisingly, gel properties changed from 'set gel' to 'heated gel' in the heating process. The gels produced in an early phase of heating had properties characteristic of 'set gel', but the gel produced in later phase at the same temperature showed the properties of heated gel. The fact clearly demonstrated that the properties were not only determined by heating temperature and also by heating time. The transition temperature from 'set gel' to 'heated gel' was different from each other with summer and winter surimi.

As a result of fast economic growth of China in two decades, people's life style has changed. The style

includes the changes in the consumption style of fish. Distribution of live fish is becoming limited in modernized city, and the proportion of processed food increases. The information on the properties of muscle protein of various species of fish in different seasons is very useful for their utilization. One of the promising examples was the utilization of silver carp in surimi production. However, it should be further studied when is the most suitable season for surimi production. The season should be very carefully determined considering the physiology of fish, reproduction system of fish culture, water temperature used for processing, and gel forming ability of surimi produced in different seasons.

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## Abstract in Japanese

中国の淡水魚生産量は年々増えており、現在全世界生産量の65%を占める。しかし、昔ながらの活魚流通が主流で、加工製品としての消費は少ない。今後、近代化に伴う生活に合わせた魚肉の利用形態の多様化が望まれる。魚肉を食料として捉えた場合、筋肉たんぱく質の基礎的な知見なしでは開発も難しいが、コイを除き淡水魚に関する知見はないに等しい。中国の淡水魚養殖には食性の異なる複数の魚種を同じ池で生産させるという大きな特徴がある。その結果、これら数種の魚は夏(30℃)、冬(0℃)の大きな水温の周年変化を同じように受けていることになる。コイを用いた温度馴化の研究から、コイは水温変化に合わせて熱安定性の異なるミオシンを発現して温度変化に対応しているといわれている。この結論が、魚種が変わっても同じであるのかについての明確な結論はない。本研究では中国で広く生産されているハクレン、コクレン、タントウホウ、ソウギョ、コイ、ライギョおよびオオクチバスについて、季節による熱安定性の異なる筋肉たんぱく質、ミオシンの発現および同時期に発現されたミオシンの種間の違いの二面から検討した。

夏、冬の魚の筋原繊維(Mf)の熱安定性をATPase失活から調べた。ハクレン、コクレン、タントウホウ、ソウギョでは夏と冬のMfの熱安定性には20倍の差があったが、コイでは4倍、ライギョ、オオクチバスでは両季節で同じであった。夏のハクレンは最も安定なティラピア程度、冬のハクレンは不安定の魚種として知られているスケトウダラ程度の安定性で、その差は極めて大きかった。これらの魚が同じような水温で生活していることを考えると、Mfの熱安定性は水温のみによって決定されないことを示している。夏のMfでは魚種間の差はほとんどなかったが、冬のMfでは大きな差があり、夏、冬の熱安定性の差が小さい魚種では冬のMfが非常に安定であることが特徴であった。低温でたんぱく質が機能するためには、そのエネルギー条件にあった柔らかい構造が必要になるが、ライギョ、オオクチバスの冬のミオシンは強固過ぎる。この矛盾は魚の活動から説明された。寒帯産の海産魚は低温でも活発に動き回る必要があるのに対し、淡水魚は冬の間、餌も採らず、池の底で静かにしている。このような安定な構造を有しても、生きてい

くための障害とはならないと考えられる。すなわち、いずれの淡水魚も夏の高水温に適応したミオシンを発現することに共通性があり、冬のみオシンの熱安定性はそれほど重要でないことが示唆された。

熱安定性の異なるミオシンがどの季節に発現されるかを知るため、全ての魚種から毎月筋肉を採取し、そのミオシンの熱安定性を解析した。夏の4ヶ月(6-9月)、冬の4ヶ月(12-3月)はそれぞれ同一の安定性を示したので、安定性の異なる2種のミオシンのみが発現されていると推定した。2ヶ月の移行期には、2種のミオシンの比が少しずつ変化し、2種のミオシンが交換されていた。この夏冬2種のミオシンの発現の時期は魚種によらず同じであり、夏冬の熱安定性の違いとは無関係であったので、発現そのものは水温で制御されていることが推定された。

Mfを加熱したときのミオシンの変性を塩溶解性、単量体量、およびキモトリプシン消化による頭部(S-1)と尾部(Rod)の生成量の変化から追跡した。いずれの魚種のいずれの季節のMfでもATPaseの失活に先立ち、塩溶解性、単量体量が平行して減少した。そして、ATPaseの失活とS-1の変性が、塩溶解性の低下とRodの変性が対応した。それゆえ、検討した全ての魚種のMfの加熱で、RodがS-1より先に変性するという共通した様式があることが分かった。

魚種ごとのミオシンRodの内部構造の違いをキモトリプシン消化が比較した。夏のミオシンの低温(10°C)での消化ではいずれも単一のHMM(165 kDa)とLMMに選択的に切断された。しかし、冬のみオシンの切断は魚種によって異なった。コイ以外は夏の切断とあまり差がなかった。コイでは特徴的に長いHMM(180 kDa)が生成され、別の切断箇所が存在することが分かった。これは、日本で用いられているコイの切断パターンとよく似ていた。温度を上昇させた消化から、夏に比べ冬のハクレンのRodの内部構造はかなり脆弱であることが分かった。

Rodの構造を比較するため、単離したRodの加熱によるUnfoldingを比較した。いずれの魚種の夏のMfから調製したRodはよく似た2つのUnfoldingピークを示した。この結果はS-1の部分だけでなくRodの部分の安定性は調べた全ての魚種でよく似ていることが結論された。しかし、冬のRodは魚種ごとに大きな違いを示した。ハクレン、コクレン、タントウホウ、ソウギョでは夏の低い温度域のUnfoldingと似た温度帯(35°C)で一つのUnfoldingを示した。他の魚種ではや



はり2つのピークを示す Unfolding であった。このとき得られた低温でのピークはハクレンなどで得られたピークとよく似ていた。それゆえ、熱安定性の高い部分を持つことがこれらの魚種の特徴であった。これらの Unfolding の由来を知るため、夏冬のハクレン Rod を LMM と S-2 に切断してさらに検討した。すると、LMM には夏冬の差がなく、35°C 付近で Unfolding が起きた。しかし、S-2 については、両者で異なった。夏の S-2 は高温で Unfolding が起こったが、冬の S-2 は LMM と類似した低温で Unfolding が起きた。このことが Rod の Unfolding の違いの原因であると結論した。ライギョの Rod では夏冬の差が認められず、頭部 S-1 部分のみならず、Rod 部分の構造もあまり変化していないことが確かめられた。Rod の熱凝集は安定な S-2 を有するものほど起こりにくいことが認められた。そして、Rod 全体の Unfolding が起きた後に凝集が著しく進行することが分かった。すなわち、安定な S-2 を有しない冬のハクレン Rod の熱凝集は夏のそれより 10°C 程度低い温度で起きた。

魚肉の利用の一つの形態はすり身製造である。これまで、淡水魚のすり身のゲル形成能はいずれも劣っており、スケトウダラのすり身のようなゲルが形成されないと報告されている。そこで、生産量が多く、価格の低いハクレンを用いて、ゲル化特性を検討した。これまでの結果に基づき、夏および冬の期間に調製したすり身を用いて検討した。すると、夏（6, 9月）、冬（12, 3月）のミオシンを持つすり身はそれぞれ同じようゲル形成をした。夏のすり身は冬より 10°C 高温でゲルを形成した。この温度差はミオシンの有する熱安定性の差で説明できた。この結果は、熱安定性の異なる魚種では最適加熱温度が異なるという結果を支持するものであった。同じ魚種でありながら、漁獲時期が異なると、それらから調製したすり身のゲル形成が大きく変化するという事実はこれらの魚種をすり身材料として利用する場合に非常に重要なことである。これらは、海産魚では考慮する必要のないことである。

以上の結果は、これから中国産の各種淡水魚の貯蔵、利用を考えるときに貴重な情報となると考えられる。また、これまで、淡水魚のゲル形成能は劣り、ゲル物性に優れたものは製造できないといわれていたが、少なくともハクレンは十分なゲル形成能を有し、さらにスケトウダラに匹敵する坐り効果が得られたため、すり身製造に適した魚種であることを明らかにした。