Structural stability of myosin rod from silver carp as affected by season

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ABSTRACT

Chymotrypsin digestion and Circular dichroism (CD) spectroscopy were used to study the structural stability of myosin rods prepared from silver carp in summer and winter. Differences in the thermal stability were clearly demonstrated by the chymotryptic digestion patterns and CD data obtained at pH 7.5 in 0.5 M KCl for myosin rods in both seasons, indicating the structural properties differed seasonally. In winter, the myosin rods were more susceptible to be cleaved into short (40 kDa) subfragments when digested at high temperature (30 ºC). The major peaks in unfolding profiles showed that transition temperatures for the rods were 35.0 ºC in winter, and 36.0 ºC, 41.0 ºC in summer. Rod subfragments (S-2 and LMM) were isolated and further analyzed by CD. In both winter and summer, the LMM fragments showed an unfolding transition at around 35.5 °C, but the S-2 fragments showed large seasonal difference; in winter, they showed a major unfolding peak around 35.0 °C and a minor peak at 40.5 °C, while in summer, they unfolded mostly at 44.5 °C, which was 8°C higher than that for the LMM fragments. Short (40 kDa) S-2 subfragments isolated from winter rods gave a single unfolding transition at 41.0 °C. These results suggest that differences in the thermal stability of myosin rods between winter and summer were due to differences in the stability of S-2 region.

Keywords: Silver carp, myosin, rod, chymotryptic digestion, unfolding, thermostability

Practical Application: The aim of this study was to detect the differences in the structure thermal stability of myosin rods between summer and winter, which is a very important portion for gel formation. The information will be very useful for producing surimi from freshwater fish species in different seasons.

Introduction

Myosin comprises two unique structures; a globular double-headed structure called subfragment-1 (S-1); and a long flexible coiled tail called a rod (Harrington and Rodgers 1984). The head contains ATPase and F-actin binding sites, and the tail region assembles to form filaments under physiological conditions of low salt. The structure of the rods is almost completely α-helical (Rodgers and others 1987). The amino terminal half of the rods, termed subfragment-2 (S-2), is water-soluble, and the rest of the rod (light meromyosin, LMM) is water-insoluble. Myosin in an isolated form or in actomyosin complex dissolved in high salt is cleaved at the heavy meromyosin (HMM)/LMM junction by chymotrypsin when the medium contains divalent cations to protect the cleavage at the S-1/rod junction (Weed and Pope 1977). This is commonly observed in skeletal muscle myosin, including fish myosin (Kato and Konno 1993). Internal structural changes in myosin can be easily detected using the chymotrypsin digestion technique (Takahashi and others 2005). The thermal stability of the rod portion is also species-specific similar to that of the S-1 portion of myosin, and the myosin rods from cold-water species are much more unstable than that those from warm-water species (Ogawa and others 1995). Rods play an important role in the formation of elastic gel (Samejima and others 1981). The thermal stability of rods is of great interest in connection with the gel-forming mechanism of myosin because the transition from a coiled-coil helix to random-coil or the unfolding of the rods is essential for gel formation (Rodgers and others 1987; Kato and Konno 1993; Ogawa and others 1995; Sasaki and others 2006).

Silver carp, *Hypophthalmichthys molitrix*, is one of the most abundant freshwater fish species in China. It is a temperate freshwater species, so it often experiences large seasonal fluctuations in body temperatures. There have been extensive studies concerning the effects of temperature acclimation on the ATPase activity (Johnston and others 1975), thermal stability (Heap and others 1985, Huang and others 1993, Nakaya and others 1995, 1997; Kakinuma and others 1998), myosin heavy-chain gene multiplicity (Watabe and others 1998) in goldfish and common carp. It is reasonable to consider that silver carp muscle contains multiple isoforms of myosin because of the seasonal temperature change. Recently, it has been reported fast skeletal muscle myosin heavy chains genes expressed in silver carp and grass carp as a result of temperature acclimation, and the deduced amino acid sequence of myosin isomers expressed differ from each other in the primary structure (Tao and others 2009; Fukushima and others 2009). We have also studied seasonal changes in myosin thermostablility and proposed that there are two types of myosin with different thermostability expressed in summer and winter by measuring the index of myosin ATPase inactivation (Yuan and others 2006). However, there was no further information about thermal stability of myosin rods.

In this paper, we investigated the difference in the thermal structural stability of myosin rods from silver carp between summer and winter using chymotrypsin digestion and CD spectroscopy measurement.

Materials and methods

Raw materials

Live silver carp (*Hypophthalmichthys molitrix*) were obtained from a Shanghai local market in summer (August and September) and winter (December and February) in 2006 and 2007. The fish were beheaded, and the dorsal white muscle was chopped finely. The minced meat was mixed with 10 % (w/w) sorbitol powder to prevent myosin denaturation, packaged into PET bags (12 x 14cm; 20±5 g each), and stored at -40 ºC until use. All samples were analyzed within four months. During the storage, it was found the myosin was not denatured according to the ATPase activity (unpublished data).

Preparation of myofibril

Myofibrils were prepared from the frozen muscle using the method described by Kato and Konno (1993), which involved repeated homogenization and washing in 0.1 M KCl 20 mM Tris-HCl (pH 7.5). The myofibril suspension was then filtered through two layers of cotton gauze, and the filtrate was used as myofibrils in the study.

Preparation of myosin rods and rod subfragments (S-2 and LMM)

Myosin in myofibrils was digested at 20 °C using 1/500 (w/w) of chymotrypsin to myofibrils in a medium of 0.05 M KCl, 20 mM Tris-maleate (pH 7.0) containing 1 mM ethylenediaminetetraacetic acid (EDTA). Proteolysis was terminated by the addition of 0.5

mM (final concentration) phenylmethlsulfonyl fluoride (PMSF). Rods were isolated from the digest using ammonium sulfate fractionation as described by Kato and Konno (1993). Myosin rods were then dissolved in 0.5 M KCl, 20 mM Tris-HCl (pH 7.5). The obtained myosin rods were further digested in the same medium containing 0.5 M KCl, 20 mM Tris-HCl (pH 7.5), and the digest was dialyzed against 0.05 M KCl, 20 mM Tris-maleate (pH 7.0) and 5 mM $MgCl_2$. The supernatant of the dialysate obtained by ultracentrifugation at 100,000 x g for 15 min was referred to as the water-soluble or S-2 fraction, and the pellet was referred to as the water-insoluble or LMM fraction. Purity of the protein samples was routinely checked by SDS polyacrylamide gel electrophoresis.

Chymotrptic digestion of myosin and myosin rod at various temperatures.

The internal structure of fish myosin in summer and winter was studied by digesting myofibrils in a medium containing 0.5 M KCl, 20 mM Tris-HCl (pH 7.5) and 1 mM $CaCl_2$, to cleave the myosin at HMM/LMM junction. Digestion was conducted by changing the temperature to study the temperature-dependent flexibility of myosin conformation using α-chymotrypsin to myofibril ratios of 1/40 (w/w) at 0 ºC, 1/100 (w/w) at 10 ºC, 1/500 (w/w) at 20 ºC, 1/3000 (w/w) at 30 ºC.

To study the temperature-dependent flexibility of rod conformation, chymotryptic digestion of the rods was conducted with monomeric rod in a medium containing 0.5 M KCl, 20 mM Tris-HCl (pH 7.5) using α-chymotrypsin to myofibril ratios of 1/40 (w/w) at 0 ºC, 1/100 (w/w) at 10 ºC, 1/500 (w/w) at 20 ºC, 1/3000 (w/w) at 30 ºC. The conditions are

suitable for cleaving rods into LMM and S-2 fragments (Kato and Konno 1993).

Polyacrylamide gel electrophoresis

SDS-PAGE electrophoresis was performed according to the method of Laemmli (1970), and Pozio and others (1977) using 7.5% polyacrylamide gels containing 0.1% SDS. Proteins of 5-10 μg /well were applied to the gel. Prestained protein marker (Biolabs inc., USA) including eight molecular weight markers (Ma) used: MBP-β galactosidase (175 kDa); MBP-paramyosin (83 kDa); glutamic dehydrogenase (62 kDa); aldolase (47.5 kDa); triosephosphate isomerase (32.5 kDa); β-Lactoglobulin A (25 kDa); lysozyme (16.5 kDa); and aprotinin (6.5 kDa). Gels were stained with 0.1 % Coomassie brilliant blue R-250 and destained with a solution containing 45% methanol and 9% acetic acid.

Circular dichroism measurement

Circular dichroism measurement was performed on a J 725 spectropolarimeter (JASCO, Tokyo). A constant N_2 flux and a jacketed cell of 0.1 cm optical path length were used, and the temperature was controlled by a peltier temperature control system (PTC-348 WI, JASCO). Measurements were made on the rod or rod fragments (LMM and S-2) solution at usually 0.5 mg/mL. Ellipticity at 222 nm was used as an index to detect the unfolding of the helix structure, and the negative value at 10 °C was taken as 100% for all the samples. Changes in the ellipticity at 222 nm upon heating of these samples from 10 to 70 ºC at a

rate of 1 °C/min were measured. Normalized change in ellipticity at 222 nm as an index of thermal unfolding of rods and their derivatives were plotted to detect the unfolding profile (Nakaya and Watabe 1997).

For the reversibility measurement of rod helix structure, the CD spectrum of rod solution was measured firstly at 10 °C, and then heated it from 10 to 70 °C at a rate of 1 °C/min, after cooling it to 10 °C and measured the CD spectrum again.

Determination of protein concentration

Protein concentrations were determined by the biuret method of Gonall and others (1949) using bovine serum albumin (BSA) as the standard. The protein concentration of chymotrypsin was determined by UV absorption measurement at 282 nm with an extinction coefficient ($E^{1\%}_{280nm}$) of 20.4.

All experiments were repeated at least three times to confirm the same conclusion from separate experiments with separate samples, and the mean values were presented instead of using statistic analysis of data obtained by a single experiment.

Results and discussion

Chymotryptic digestion of myofibrils at elevated temperature

There are several digestion sites within carp myosin rods, and chymotryptic digestion patterns of carp myofibrils change at various temperatures (Takahashi and others 2005), which suggests the temperature-dependent flexibility of myosin conformation. We wondered whether the difference in the internal structure between winter and summer myosin could be detected by chymotryptic digestion pattern when the temperature is raised from 0 to 30 °C. The digestion was performed in the medium of 0.5 M KCl, 20 mM Tris-HCl (pH 7.5) and 1 mM $CaCl_2$ to cleave the myosin at the HMM/LMM junction (Fig. 1).

At 0 °C, silver carp myosin from summer and winter fish was cleaved almost selectively at a single site producing only HMM (165 kDa) and LMM (60-70 kDa) fragments. The HMM and LMM were kept unchanged until complete cleavage of the myosin without further degradation. When the temperature was raised to 10 °C, LMM began to be further cleaved into shorter fragments, while HMM remained unchanged. The density of 165 kDa HMM from winter fish decreased remarkably at 20 °C suggesting further degradation into much shorter (135 kDa) fragments. The disappearance of 165 kDa HMM bands in the winter sample was more prominent at 30 °C, at which winter fish myosin produced a very small amount of 135 kDa HMM. The density of short HMM (135 kDa) did not explain the total conversion of ordinary HMM (165 kDa) to short HMM (135 kDa). Some of the 135 kDa HMM was probably degraded into very short fragments. Such degradation of HMM was not detected in summer fish at the same temperature. These results clearly indicated that winter silver carp myosin easily altered its conformation at relatively low temperature. As the cleavage site is within the myosin rod, we propose that the internal structure of

myosin rods between winter and summer fish differed. These results demonstrate that although winter and summer myosin rods contained similar cleavage sites at low temperature, overall conformation for myosin was less stable in winter than in summer.

Characterization of temperature-dependent chymotryptic digestion pattern of myosin rods in summer and winter

Chymotryptic digestion was used to compare the internal structure of myosin rods in summer and winter. Myosin rods were isolated from both winter and summer myofibrils of silver carp, and the purity of the rods was confirmed (Fig. 2). The characteristic digestion patterns of rods at 0 and 30 °C are shown in Fig. 3. As shown in Fig. 3, summer myosin rods generated fragments of mainly two sizes (65 and 70 kDa) without further degradation even digested at 30 °C, and the products were kept unchanged until a complete digestion of the rods. The winter myosin rods were similarly cleaved into 65 and 70 kDa fragments in an early phase of digestion (partial digestion of rod) at low temperatures (0 °C), while both fragments were further cleaved into shorter (about 60 kDa) fragments. When the digestion temperature was raised to 30 °C, the 65 kDa fragments were further degraded to shorter (e.g., 40 kDa) fragments. As a second cleavage was not observed in the summer myosin rods, these results from chymotryptic digestion indicated a more stable conformational structure in summer rods than in winter ones.

To determine if the obtained digested rod fragments were S-2 or LMM fragments, the digest was dialyzed against 0.05 M KCl, 20 mM Tris-maleate (pH 7.0) and 5 mM $MgCl_2$ to

separate the water-soluble (S-2) fraction from the water-insoluble (LMM) fraction. The SDS-PAGE patterns of digested rods and their separation fractions are shown in Fig. 4. According to the SDS-PAGE patterns of the separation fractions in the digests, the 70 kDa fragment in the digest of summer myosin rods were water-insoluble LMM, and the 65 kDa fragments were S-2. The size of LMM obtained in the digest of rods was the same as one directly prepared from myofibrils. Three fragments detected in the pattern for winter myosin rods were similarly separated into water soluble and insoluble fractions. The water-insoluble LMM fraction contained 70 and 65 kDa fragments, and the water soluble fraction contained 65, 60 and 40 kDa fragments. These results indicated that 70 kDa LMM and 65 kDa S-2 initially produced from winter myosin rods were further cleaved at the second sites on respective fragments due to the instable structure.

Comparison of the reversibility of helix structure in myosin rods of two seasons by circular dichroism spectra

We prepared myosin rods from fish collected in summer and winter, and their purity was confirmed (Fig. 2). Myosin rods are almost completely helical in structure, so unfolding of the structure of the rods upon heating could be used to study their thermal stability (Rodgers and others 1987). We firstly compared the CD spectra of myosin rods from winter and summer fish at 10 °C (Fig.5). Both samples showed large ellipticity (θ) peaks at 222 nm and 208 nm, characteristic of an α-helix structure. The magnitude of the ellipticity for both samples at 10 °C indicated their nearly 100% helical structure. The θ_{222}

nm/θ_{208} nm ellipticity ratio at 10 °C was 1.09, which was in agreement with a previous study of myosin rods in other species (King and Lehrer 1989).

Unfolded pollock myosin LMM has been reported to partially refold upon cooling (Ojima and others 1999). However, carp myosin rods show high reversibility of the helix structure (Sano and others (1990), Sasaki and others (2006)). We wondered whether the reversible refolding ability differed between the summer and winter myosin rods. The reversibility of two rod preparations was compared. CD spectra for the heated and cooled samples were compared as shown in Fig 5. Ellipticity for those samples was quite high in both seasons. We should mention that the characteristic spectrum for a helix structure disappeared when the two rods preparations reached 70 °C, and a flat spectrum corresponding to a random structure was obtained (data not shown). Thus, heating at high temperature almost completely unfolded the α-helix structure of rod. The recovered helix content calculated from the ellipticity upon cooling was about 92.6% and 80.3%, for the summer and winter rod preparations, respectively. Although rods lost their α-helix structure at 70 °C, more than 80% of the unfolded helix structure was refolded during the cooling process. The reversibility for winter myosin rods was a slightly lower than summer myosin rods, indicating the winter myosin rods were less stable.

Comparison of the unfolding profiles between winter and summer rod preparations

A decrease in the ellipticity upon heating is a useful index for detecting the unfolding process of rods (Samejima and others 1981; Nakaya and others 1997). To understand the

difference in the unfolding process between winter and summer rod preparations, we analyzed the unfolding profile as shown in Fig. 6. A gradual increase in temperature led to a gradual decrease in the negative magnitude of the ellipticity at 222 nm as a result of unfolding of the helix structure for all the samples. The winter rods began to unfold at a slightly lower temperature than summer rod. A marked decrease in α-helix content was observed at around 40 and 35 °C for the summer and winter rods, respectively. Temperature-dependent unfolding profiles between summer and winter rods were compared by calculating the derivative of the change of helix content. Summer rods generated two major helix unfolding transitions at 36.0 and 41.5 °C, but the winter rods mainly unfolded in a single phase with a transition temperature of 35.0 °C. A small transition peak (shoulder) at 40.5 °C was also detected in the winter sample. These two unfolding profiles were clearly distinguished from each other. It should be noted that both summer and winter rods unfolded at 35-36.0 °C, but the unfolding peak at 41.5°C characterized the summer rods.

Comparison of the unfolding profiles between winter and summer S-2 and LMM

To characterize the unfolding peaks, we further isolate S-2 and LMM fragments from the rods by chymotryptic digestion and ammonium sulfate separation. The unfolding profiles of S-2 and LMM from myosin rods in summer and winter were compared (Fig. 7). The SDS-PAGE patterns of isolated S-2 and LMM are shown in the figure 2. Winter LMM showed a broad unfolding transition at around 35 °C, which was the same as the one

detected in the winter rods. The winter S-2 showed two unfolding temperatures. A major unfolding occurred at around 35.0 °C which was the same temperature as a major unfolding temperature detected in the rods. S-2 contained a second unfolding temperature at 40.5 °C. The second peak explained the shoulder detected with the unfolding of the winter rods (Fig. 6). It was demonstrated that similar unfolding peaks of LMM and S-2 at around 35.0 °C generated an apparent single unfolding peak in the winter myosin rods. The summer LMM gave a sharp unfolding at around 36.5°C, which corresponded to the low unfolding peak detected in the summer rods. Thus, there was a small difference in the stability of LMM region for both summer and winter rods. The summer S-2 unfolded mostly at a single temperature (44.5 °C), which was 8°C higher than that for LMM. The unfolding peak corresponding to isolated S-2 was not detected in the parent rods. However, the summer rods contained a second unfolding peak at around 41°C. The peak might correspond to unfolding of the S-2 region. It was suggested that linkage of unstable LMM lowered the unfolding temperature of the S-2 region. Nevertheless, it was clear that the S-2 region of summer rods was much more stable than the winter S-2 region. We conclude that there was a small difference in the stability of LMM, while a large difference in the S-2 region between summer and winter myosin rods.

Fukushima and others (2009) recently reported that silver carp express different types of myosin heavy chains of fast skeletal muscle as a result of temperature acclimation, which differ from each other in the primary structure. The deduced amino acid sequences of myosin S-2 and LMM showed very high identities of 90-91% and 86-90% between low and high temperature accalimation, respectively. However, their loop-1 and loop-2 regions

in S-1 were highly variable. In this study, chymotryptic digestion patterns of the rod suggested marked differences in the S-2 part or hinge region between winter and summer myosin rods. Summer myosin rods produced 65 kDa S-2 and 70 kDa LMM, while winter myosin rods produced short (40 kDa) S-2 subfragments when digested at temperatures above 20 °C. Moreover, the transition temperature of summer myosin S-2 was about 4 °C higher than that of winter one upon unfolding. Therefore, it is difficult to explain the difference in the thermal structure stability judging from the identities of primary structure only.

Isolation of winter 40 kDa S-2 and its thermal unfolding profile

The unfolding profile for the winter 60 kDa S-2 gave two transient steps, so we wondered whether the short 40 kDa S-2 might give a single unfolding profile. We tried to isolate the 40 kDa S-2, which is the C-terminal region of 135 kDa HMM. As the SDS-pattern of chymotryptic digestion of winter myofibril showed 135 kDa HMM produced at 30 °C (Fig. 1), it was easy to isolate the 135 kDa HMM from the digest at 30 °C. The preparative procedures were practically the same as those used for S-1 preparation from the myofibril digest (Konno and others 1990). First, acto-HMM complex was collected as a pellet at 40% saturated ammonium sulfate in the absence of Mg-ATP. HMM in the complex was detached from F-actin upon addition of 2 mM Mg-ATP. F-actin was removed as a pellet at 40% saturated ammonium sulfate, leaving HMM in the supernatant. The HMM was collected as a pellet by raising the saturation to 55%. After

dialysis against 0.1 M KCl, 20 mM Tris-HCl (pH 7.5), and centrifugation at 20,000 g for 20 min, the soluble fraction was collected as HMM. As the 40 kDa S-2 is present as a coiled-coil structure in the 135 kDa HMM, it would be produced from the 135 kDa HMM by tearing off the subfragment-1 (S-1) region during digestion at high temperature, such as 30 °C. A further digestion of the 135 kDa HMM isolated in the presence of EDTA generated uniquely short (40 kDa) S-2 together with S-1 as described in Takahashi and others (2005), The 40 kDa S-2 was identified by N- and C-end sequencing, and demonstrated to locate the amino end of the rod portion. The separation of S-1 from the 40 kDa S-2 was simply achieved by applying the thermal treatment. The digest containing S-1 and 40 kDa S-2 was heated at 30 °C for 60 min. The heat-denatured S-1 almost completely resulted in aggregate formation (Takahashi and others 2005). In this study, we also confirmed that the 135 kDa HMM was gradually converted into 95 kDa and 40 kDa fragments (data not shown). We used the method to isolate 40 kDa S-2 from 135 kDa HMM by heating the digest at 30 °C for 60 min. The heat-denatured S-1 almost completely resulted in aggregation. The turbid solution was centrifuged at 20,000g for 20 min to remove aggregated materials. We found no 40 kDa S-2 sedimented by the procedure. SDS-PAGE analysis revealed that the supernatant contained the 40 kDa S-2 (Fig. 2). The thermal stability of the 40 kDa S-2 was studied by CD measurement. The unfolding profile of 40 kDa S-2 upon heating was shown in Fig. 8. There appears to be a single unfolding peak at 40.5 °C, which is very similar to the higher one for the 60 kDa S-2. As the 70 kDa LMM showed an unfolding peak at 35 °C, the unfolding peak for the 40 kDa S-2 was detected with the parent rod as the small shoulder peak at around 40.5 °C. However, a

high unfolding peak at around 44.5 °C was detected in summer myosin rod S-2, indicating that the unfolding peak of winter S-2 was at least 4°C lower than that of the summer one. This confirmed that winter S-2 is less stable than summer S-2.

The deduced amino acid sequences of myosin both in summer type and winter type were determined, so we searched the amino acid sequences in the National Center for Biotechnology Information (NCBI) database, and found some amino acid substitutions, for example, glycine was substituted by alanine in winter type myosin S-2 (Fukushima and others, 2009; Tao and others, 2009). It was reported that glycine is a predominant contributor to all the weak interactions and a cumulative effect of weak interactions seems to be important in thermal stability of proteins (Ibrahim and Pattabhi, 2004). However, it is difficult to understand the structure-stability relationship of proteins simply from the deduced amino acid sequences. In this study, the summer myosin rods showed a more stable conformation than that of winter one according to the results obtained from chymotryptic digestion and circular dichroism measurement, which was consistent with the results of S-1 obtained from ATPase index (Yuan and others 2005, 2006). To understand the structure-stability relationship of proteins, more intensive studies should be conducted. As many freshwater fish are temperate fish, which generally experience a wide range of seasonal variations in temperature and change fast skeletal myofibrils featured by a higher ATPase activity and lower thermal stability in cold water and by opposite properties in warm water (Johnston and others 1975; Heap and others 1985), this could be a good model for examining structure-stability relationships of proteins. Further study might be conducted on the thermal biochemical properties of myosin in other freshwater species

considering seasonal change.

Conclusion

The results of the present investigation show that there was a small difference in the stability of LMM, while a large difference in S-2 region between summer and winter myosin rods. This suggests that the characteristic thermal stability of silver carp myosin rods come from the difference in the stability of the S-2 region rather than the LMM region. Due to the unstable properties of winter myosin, it unfolded at lower transition temperature and was more liable to be digested to short fragments with increasing temperature than the summer myosin was. Gel-forming ability is an important property of myosin, so information on the properties of myosin function domains (S1, S2 and LMM) in different seasons might be very useful for their use. Future studies should be conducted to determine the most suitable season for surimi production.

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Figure captions:

Fig. 1. Temperature-dependent chymotryptic digestion patterns of myofibrils of summer and winter silver carp.

Myofibrils from summer (Left) and winter (Right) samples were digested in high ionic strength medium; 0.5 M KCl, 20 mM Tris-HCl (pH 7.5), 1 mM $CaCl_2$, using α-chymotrypsin of 1/40 (w/w) at 0 ºC (A), 1/100 (w/w) at 10 ºC (B), 1/500 (w/w) at 20 ºC (C), 1/3000 at 30 ºC (D).

Molecular weight markers (Ma) used are 1, MBP-β galactosidase (175 kDa); 2, MBP-paramyosin (83 kDa); 3, Glutamic dehydrogenase (62 kDa); 4, Aldolase (47.5 kDa); 5,Triosephosphate isomerase (32.5 kDa); 6, β -Lactoglobulin A (25 kDa); 7, Lysozyme (16.5 kDa); 8, Aprotinin (6.5 kDa)

Fig. 2. SDS-PAGE patterns of myosin rod and rod fragments prepared from myofibrils.

The purity of summer and winter myosin rod and its fragments (S-2 and LMM) prepared from myofibril was confirmed. The numerals of each lane represented as follows: 1. Summer myofibril; 2, summer myosin rod; 3, summer myosin LMM; 4, summer myosin S-2; 5, winter myosin rod; 6, winter myosin LMM; 7, winter myosin 65 kDa S-2; 8, winter myosin 40 kDa S-2. M is the abbreviation of molecular weight markers as Fig.1.

Fig. 3. Temperature-dependent chymotryptic digestion patterns of myosin rods of summer and winter silver carp.

Rods from summer (Left) and winter (Right) samples were digested in high ionic strength medium; 0.5 M KCl, 20 mM Tris-HCl (pH 7.5) , using α-chymotrypsin of 1/40 (w/w) at 0 ºC (A), 1/3000 at 30 ºC, (B). M is the abbreviation of molecular weight markers as Fig.1.

Fig. 4. Separation of the fragments in the digests of myosin rods of summer and winter silver carp at various temperatures.

Myosin Rods from summer (s) and winter (w) samples were digested for 30 min in high ionic strength medium; 0.5 M KCl, 20 mM Tris-HCl (pH 7.5), using α-chymotrypsin of 1/40 (w/w) at 0 ºC (A), 1/500 (w/w) at 20 ºC (B), and 1/3000 (w/w) at 30 ºC, 70, 60 and 40 kDa are sub-fragments of rod. Abbreviation: D, rod digest; Ws, water soluble fractions; Wi, water insoluble fractions. M is the abbreviation of molecular weight markers as Fig.1.

Fig. 5. Changes in the CD spectrum of myosin rods upon heating-cooling.

CD spectra of summer silver carp myosin rods (A) and of winter myosin rods (B) were measured before heating (solid line) and after heating and cooling (dotted line). CD spectra were measured at 10 ºC.

Fig.6. Comparison of the structural stability between summer and winter myosin rods

Rod solutions (0.5mg/mL) of summer and winter were heated from 10 to 70 ºC at a rate

of 1 ºC/min. 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.

Fig. 7. Comparison of the structural stability between myosin LMMs and S2 of summer and winter silver carp.

Rod subfragments, LMM, (A) and S-2, (B) solutions (0.5mg/mL) of summer and winter were heated from 10 to 60 ºC at a rate of 1 ºC/min. Derivatives of helix content at 222 nm (closed circles for summer and open circles for winter) were compared. SDS-PAGE patterns are of the subfragments produced from summer (s) and winter (w) rods. The molecular weight of these rod subfragments can be determined from Fig. 2 by comparing with protein markers.

Fig. 8. Unfolding profile of 40 kDa S-2 from winter rod.

40 kDa S-2 isolated from winter rod was heated from 20 to 60 ºC. Derivatives of helix content at 222 nm were calculated. The inset is SDS-PAGE pattern of 40 kDa S-2 isolated. The molecular weight of these rod subfragments can be referred from Fig. 2 by comparing with protein markers.

Fig. 1.

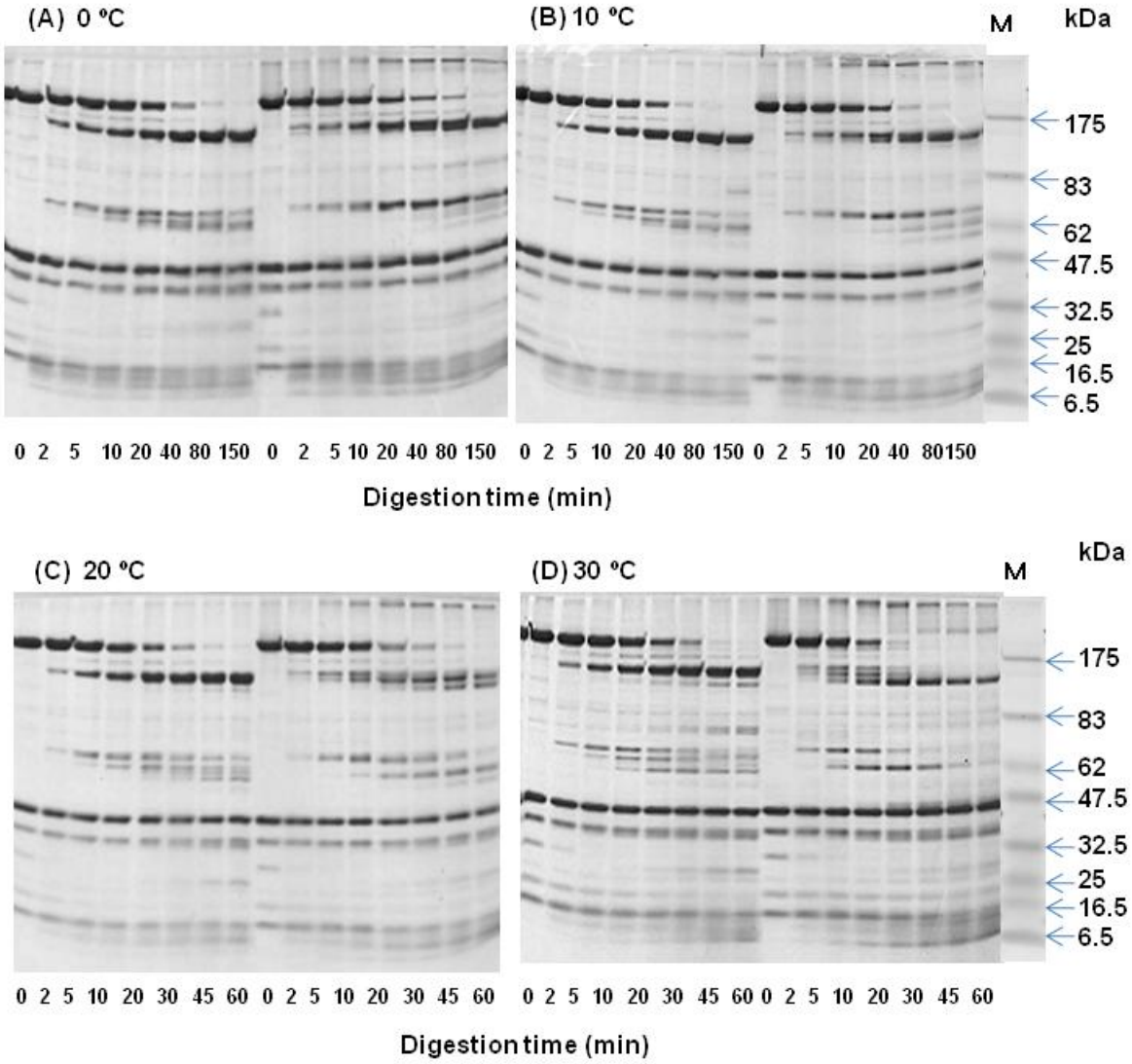
(A) 0 ºC
(B) 10 ºC
M
kDa
175
83
62
47.5
32.5
25
16.5
6.5
0 2 5 10 20 40 80 150 0 2 5 10 20 40 80 150
0 2 5 10 20 40 80 150 0 2 5 10 20 40 80150
Digestion time (min)
(C) 20 ºC
(D) 30 ºC
M
kDa
175
83
62
47.5
32.5
25
16.5
6.5
0 2 5 10 20 30 45 60 0 2 5 10 20 30 45 60
0 2 5 10 20 30 45 60 0 2 5 10 20 30 45 60
Digestion time (min)

Fig. 2.

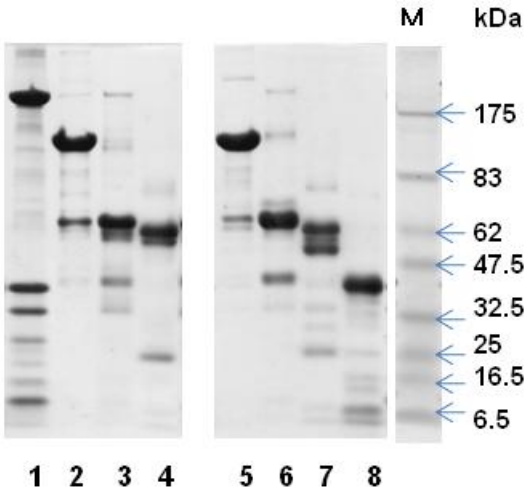
M
kDa
175
83
62
47.5
32.5
25
16.5
6.5
1 2 3 4 5 6 7 8

Fig. 3.

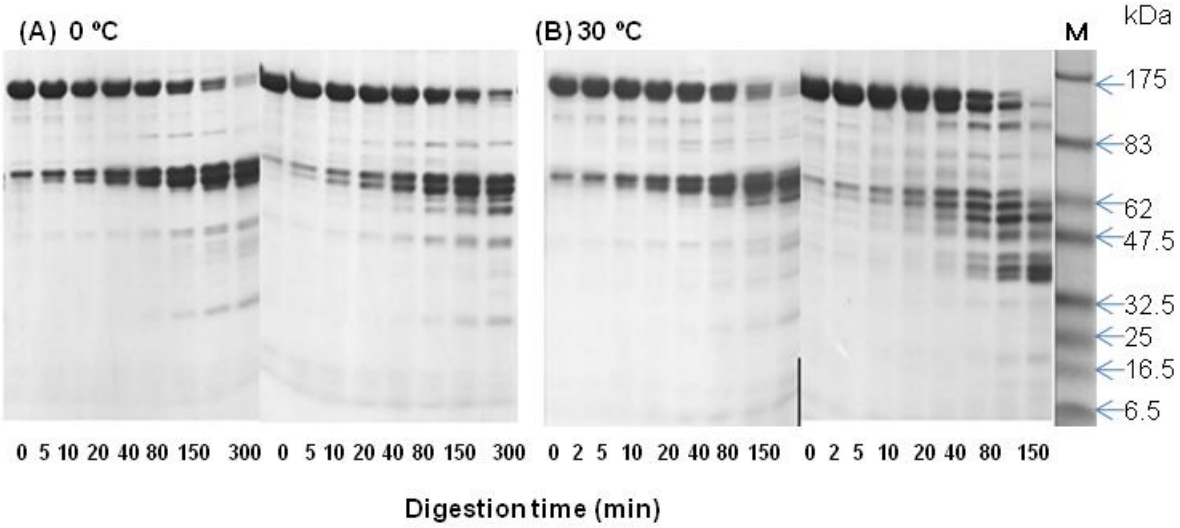
(A) 0 ºC
(B) 30 ºC
M
kDa
175
83
62
47.5
32.5
25
16.5
6.5
0 5 10 20 40 80 150 300 0 5 10 20 40 80 150 300
0 2 5 10 20 40 80 150 0 2 5 10 20 40 80 150
Digestion time (min)

Fig.4

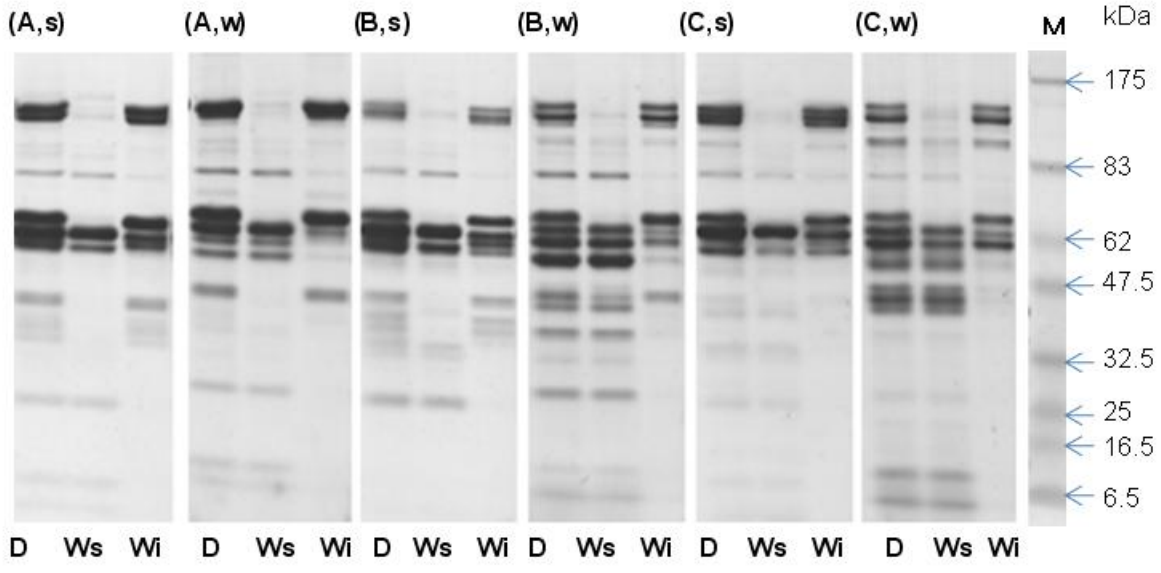
(A,s)
(A,w)
(B,s)
(B,w)
(C,s)
(C,w)
M
kDa
175
83
62
47.5
32.5
25
16.5
6.5
D Ws Wi D Ws Wi D Ws Wi D Ws Wi D Ws Wi D Ws Wi

Fig. 5

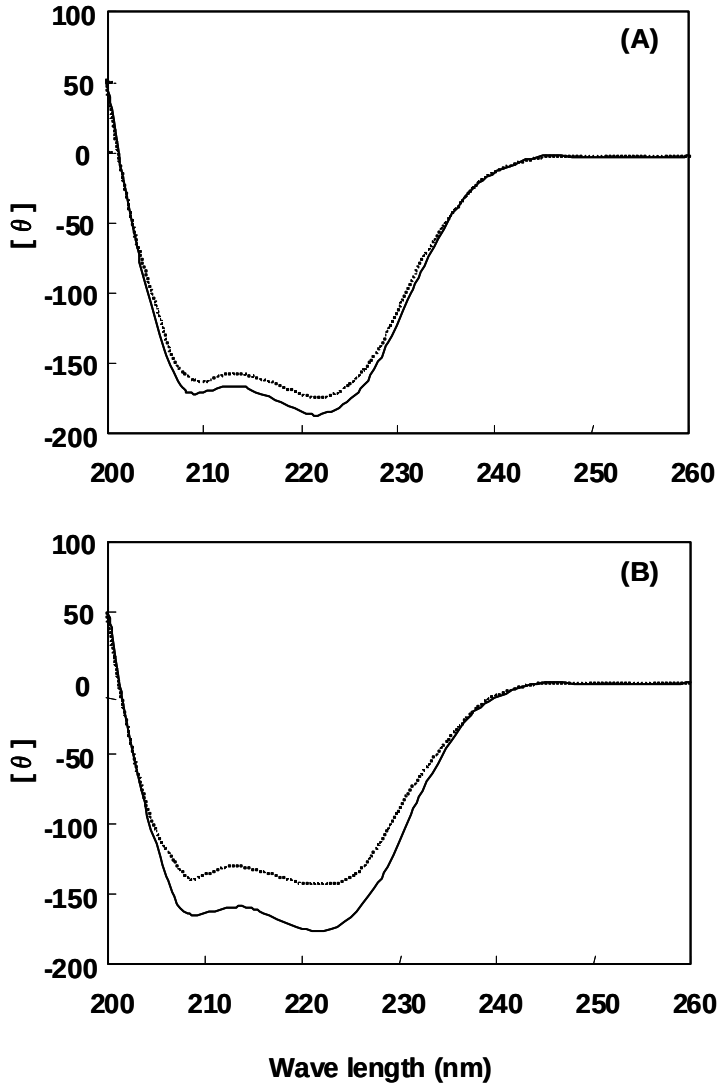
(A)
(B)
100
50
0
-50
-100
-150
-200
[θ]
200
210
220
230
240
250
260
Wave length (nm)

Fig. 6.

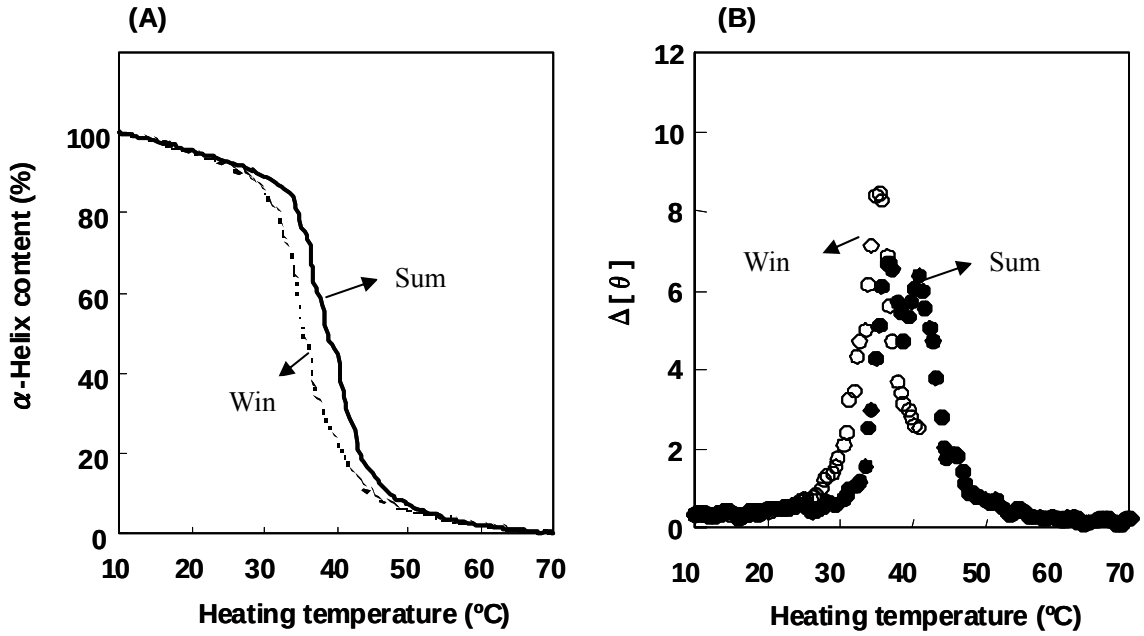
(A)
α-Helix content (%)
Sum
Win
Heating temperature (ºC)
(B)
Δ[θ]
Win
Sum
Heating temperature (ºC)

Fig. 7.

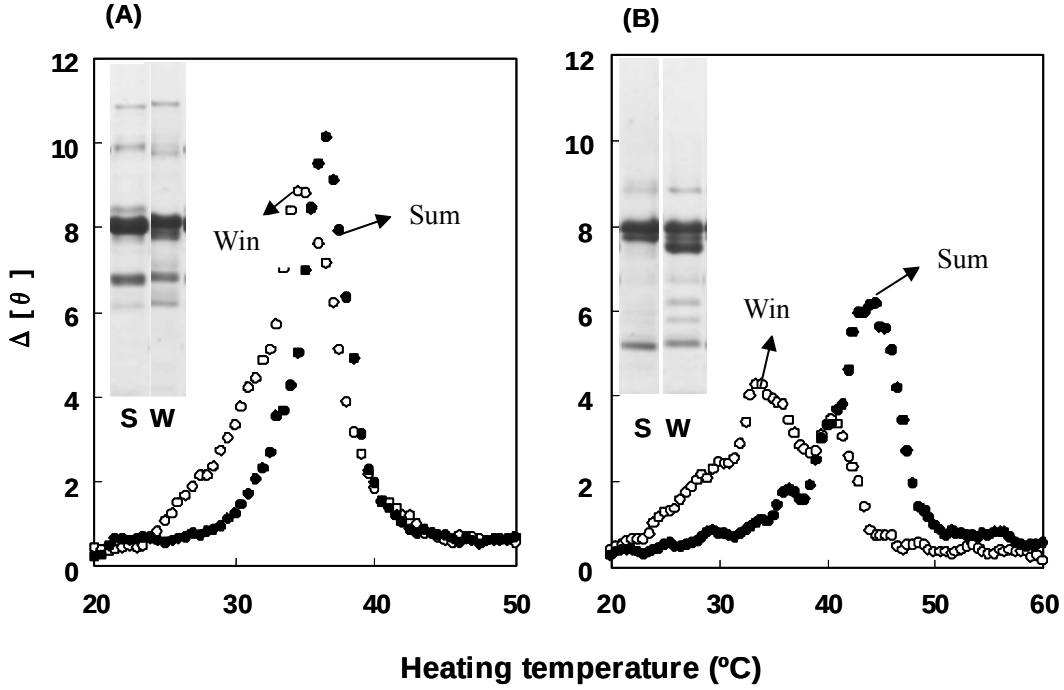
(A)
Δ [θ]
S W
Win
Sum
(B)
S W
Win
Sum
Heating temperature (ºC)

Fig. 8.