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BIGHEAD CARP MYOSIN STABILITY TO HEAT AND FROZEN STORAGE

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Differential scanning calorimetry (DSC) was used to investigate thermal transitions of bighead carp muscle (Aristichthys nobilis Richardson). Three endothermic peaks were observed in DSC thermograms of fresh muscle. After addition of salt, transition temperatures shifted to lower temperatures. Preheating samples at 70°C caused virtual disappearance of all transition peaks. Low temperature storage (-18°C, for five weeks) caused changes in myosin transitions. The evidence suggests that changes infish protein during low temperature storage are different from those occurring during high temperature treatment.

Keywords: Bighead carp muscle, diffferential scanning calorimetry, protein stability, storage.

INTRODUCTION

The procedures commonly applied to fish processing include frozen storage and heat treatment. Both of these treatments are accompanied by chemical and microstructural changes that modify the quality of the final product (Dyer, 1951; Connell, 1968). It is believed that myosin and actin, the main contractile proteins, are largely responsible for the functional properties of flesh foods. The changes observed in texture as well as in the muscle water holding capacity have been related to myofibrillar protein denaturation (Wagner and Anon, 1985). The deterioration of fish muscle during processing and storage is mainly a result of denaturation of myosin which is thermally less stable than that of mammalian origin and this is reflected in the stability of the fish flesh to heat and frozen storage (Hoewll *et al.*, 1991). The denaturation of fish myosin initiates various other molecular processes e.g. protein-protein aggregations yielding a drier, tougher flesh (Dyer, 1951).

Carp *(Cyprinus carpio)* is in volume terms by far the most important aquaculture species in world fisheries (Jevtić, 1988; Frimodt, 1995). Bighead carp eat zooplankton and vegetation not consumed by other farmed carp species, so they can be grown successfully in conjunction with other species, adding to the total harvest of ponds without requiring additional feed. Aquaculture production of bighead carp has increased constantly in recent years (Jevtić, 1988; Frimodt, 1995). Bighead carp is similar in texture to common carp, with coarsely textured, greyish coloured meat of a somewhat distinct flavour compared to common carp

(Tumbas and Vujković, 1978). The objective of the present study was to determine the stability of myofibrillar proteins from bighead carp *(Aristichthys nobilis Richardson)* during both frozen storage and heat treatment. The stability of myofibrillar proteins from bighead carp muscle were determined by differential scanning calorimetry (DSC) of whole muscle samples (e.g. Howell *et al.*, 1991; Davies *et al.*, 1994) and the changes associated with myofibrillar protein denaturation were detected through observed alterations in protein extractability and SDS electrophoretic patterns (e.g. Huidobro and Tejada, 1995).

MATERIALS AND METHODS

Sample preparation

Fresh bighead carp (2-2.5 kg in body weights) were obtained from "Živača" fish-pond in Belgrade in the autumn (October, November). At this time the fish were beheaded, gutted, washed in ice and water and were brought to the laboratory by refrigerated transport, packed in ice. The fish were processed 24 after death to permit the resolution of rigor mortis. The skin, bones, red muscle and fat tissue were removed manually and the muscle was then minced. During handling, sample temperatures were kept at 4 °C.

Proximate analysis of muscle

Moisture was determined by drying for 16 h at 105°C, fat by Soxhlet extraction and protein by the Kjeldahl procedure (AOAC, 1995) as well as by the modified biuret method (Scopes, 1987) . The pH of each sample was determined in a homogenate of 5 g of muscle in 25 ml of distilled water. All analyses were performed in duplicate.

Calorimetry

Differential scanning calorimetry was performed on a Perkin-Elmer DSC II instrument. Samples were accurately weighed (10-20 mg) and sealed in aluminium sample pans. An equal weight of water was sealed in a separate pan and used as the reference. The scanning range was 20-90°C at a heating rate of 10°C/min. Triplicate samples were analyzed at an instrument sensitivity of 5 mcal/sec. In DSC, heat is supplied to both the reference and the sample pan so that no temperature difference exists between them. DSC is based on the principle that whenever a material undergoes a physical or chemical change heat is either liberated or absorbed. Thus, if a transition involving the evolution or absorption of heat occurs in the sample, more (absorption) or less (evolution) power is supplied to the sample to maintain the temperature of the two pans the same. This is registered as a peak or trough on the thermogram; in muscle, peaks seen as protein denaturation usually involve the absorption of heat (endotherm). DSC provides a means of measuring the thermal stabilities of proteins in whole systems, provided that the various proteins are denaturated in different temperature ranges, yielding distinguishable peaks (Stabursvik and Martens, 1980).

The parameters measured were the extrapolated onset temperature, To (i.e., that at which the denaturation reaction begins), peak maximum temperature, Tm and the specific enthalpy of the transition (denaturation enthalpy), ΔH per gram of wet matter (Davies *et al.*, 1994). After subtracting the base line data

(reference data), total denaturation enthalpies (ΔH) were estimated by measuring the area under the DSC transition curve. Partial areas of the separate "myosin" and "actin" components were estimated by splitting the thermogram into only two components (Davies *et al.*, 1994). Although these partial areas are expressed in units of specific enthalpy (Joules g-1) they are not strictly enthalpies of the individual components as the mass is that of the whole sample (Davies *et al.*, 1994). DSC were analyzed using software package, Origin, developed by MicroCal.

Effects of sodium chloride addition

To evaluate the effects of sodium chloride on heat stability of bighead carp muscle proteins, muscle was mixed with 1 - 5% (w/w) sodium chloride with a spatula before DSC measurements. Despite the small quantity of the sample used for DSC measurements, the results were highly reproducible indicating that the salt was adequately dissolved and uniformly distributed within the bulk sample.

Effects of frozen storage

After removing samples for analysis, fish samples (5 -10 g) were kept frozen at - 18 °C for 5 weeks. DSC analysis was performed after the period of storage.

Preheating effects

In order to analyze the effects of preheat treatments on the thermal transitions of proteins, muscle samples were put into separate test tubes and incubated in a water bath at 70°C for 5 and 30 minutes. After preheating, samples were cooled to 4°C and then sealed and scanned by DSC as described above.

Sodium Dodecyl Sulfate-Polyacryamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Gorg *et al.*, (1985) using an LKB, Pharmacia horizontal gel system. Molecular mass standards (Sigma) included β-galactosidase (116.000 Daltons), bovine serum albumin (66.000 Daltons), egg albumin (45.000 Daltons), carbonic anhydrase (29.000 Daltons) and myoglobin from equine skeletal muscle (17.800 Daltons). Protein bands were visualized by Coomassie brilliant blue R-250 staining.

Extractability of myofibrillar proteins

Myofibrillar proteins were extracted from ground muscle with three volumes (w/V) of buffer (0.3 M KCl, 0.10 M KH_2PO4 0.05 M K_2HPO_4, pH 6.52 ± 0.02) used for extraction of crude myosin (Starr and Offer, 1982). This was homogenized in an Ultra-turax (5 minutes at 8000 r/min) and gently stirred for 15 minutes at 4°C. The suspension was centrifuged at 10 000 r/min for 15 minutes and then the protein concentration was determined in the supernatant.

RESULTS

Proximate analysis

Isolated white muscle, from which the excess of fat had been previously removed was analysed. Moisture (81.8 ± 0.4 %), fat (0.4 ± 0.1 %) and crude protein content (16.2 ± 0.4 %) of bighead carp muscle and its pH (6.0-6.2) were in good agreement with values reported previously (Tumbas and Vujković, 1978).

Scanning calorimetry

The DSC thermograms of fresh bighead carp muscle and muscle containing different percentages of salt (NaCl) are shown in Figure 1. The thermograms comprised two distinctive peaks: the large myosin peak and the smaller actin peak (Howell *et al.*, 1991; Davies *et al.*, 1994). In order to describe the thermograms, the peak maximum temperatures Tm were used to represent the transition temperature. Values of Tm measured on different samples at the same heating rate were comparable. The coefficient of variation (CV) for these peak temperature measurements was less than 1%.

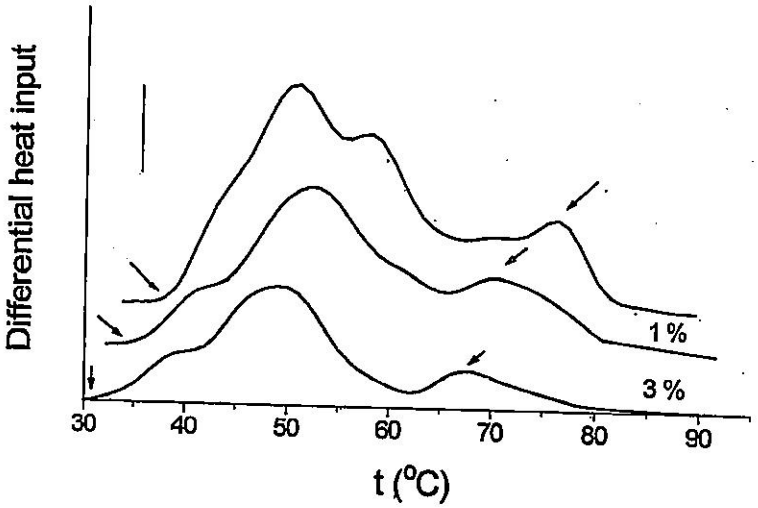

Figure 1. DSC thermograms of whole bighead carp muscle before and after salt addition. The upper thermogram is from the untreated sample and the lower thermograms are from the samples with 1% or 3% NaCl addition. Arrows at lower temperatures on each thermogram indicate To; arrows at higher temperatures indicate the actin peak (TmA). The vertical bar represents 0.334 $mJsec^{-1}$

The onset (To) of the first (myosin) transition in muscle containing no salt occurred at 38°C. The Tm of the transitions measured for myosin were 45°C (shoulder) 52°C and 59°C. The Tm of the transition measured for actin was 77°C (Figure 1). The total denaturation enthalpy (ΔH) estimated by measuring the area under the DSC transition curve was 17.57 Jg-1 and partial areas of the separate "myosin" and "actin" components were 9.84 Jg^{-1} and 2.52 Jg^{-1} respectively (Table 1).

In the presence of salt both myosin and actin from bighead carp muscle were destabilized to thermal denaturation. After the addition of salt to the muscle the To and Tm values of both myosin and actin transition shifted to lower temperatures as shown in Figure 1. At both 1% and 3% salt level, the To for myosin transition shifted from 38°C to 33°C, the Tm values for first two myosin transition shifted from 45C and 52°C to 39°C and 50C, respectively and the Tm for actin transitions shifted from 77°C to 69°C. The 59°C transition of myosin almost disappeared indicating that the protein had already passed the third transition prior to the DSC analysis. Denaturation enthalpies (Table 1) indicate that the extent of protein denaturation was salt concentration dependent.

Table 1. Analysis of DSC thermograms of bighead carp myofibrillar proteins from fresh muscle and muscle previously subjected to the selected experimental treatments

Selected experimental treatments	Onset temper-ature To (°C)	Peak maximum temperature Tm myosin			actin	Enthalpy ΔH		
		Tm1 (°C)	Tm2 (°C)	Tm3 (°C)	Tm (°C)	ΔH total (Jg^{-1})	ΔHmyosin (Jg^{-1})	ΔHactin (Jg^{-1})
Fresh muscle	38	45	52	59	77	17.57	9.84	2.52
Frozen muscle (-18°C 5 weeks)	33	37	47	58	77	10.28	4.61	2.41
Muscle with 1% NaCl	33	39	50	59	69	12.34	8.02	1.45
Muscle with 3% NaCl	30	39	49	58	68	8.63	6.95	0.74
Muscle heated at 70°C 5 min.	30	-	50	59	77	6.89	3.28	1.40
Muscle heated at 70°C 30 min.	-	-	-	-	-	3.01	-	-

Table 2. Soluble total proteins in buffer (0.3 M KCl 0.10 M KH_2PO_4 0.05 M K_2HPO_4, pH 6.52±0.02) for fresh and treated bighead carp muscle

Experimental treatments	Percent of soluble proteins
Fresh muscle	55.7 ± 1.7
Frozen muscle (-18°C 5 weeks)	27.4 ± 1.7
Muscle heated at 70°C 5 min.	43.4 ± 1.4
Muscle heated at 70°C 30 min.	26.0 ± 1.4

The effect of frozen storage (5 weeks at -18°C) on the DSC thermogram of bighead carp muscle is shown in Figure 2. After frozen storage the To of myosin transition shifted from 38°C to 33°C and the Tm values for the first two myosin transition shifted from 45°C and 52°C, to 37°C and 47°C respectively, whereas the Tm for actin transition (77°C) remained unaltered. The 59°C transition of myosin remained unaffected indicating that frozen storage did not affect the third transition prior to DSC analysis. The changes in total peak area (total denaturation enthalpy) reflects changes in the myosin peak area (Table 1). These results indicate that myosin is the protein affected by the freezing process, especially the region of the molecule that contribute to transitions at 45°C and 52°C.

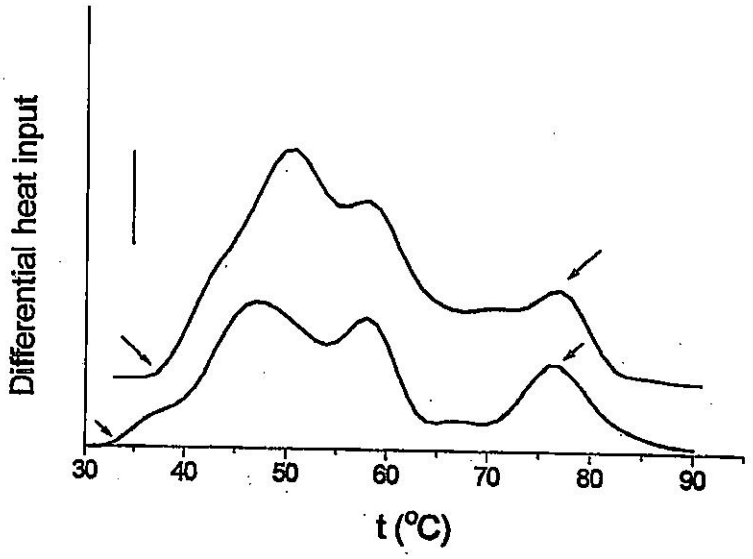

Figure 2. DSC thermograms of whole bighead carp muscle before and after - 18 °C for 5 weeks. The upper thermogram is from the unfrozen sample and the lower thermogram from the frozen one. Arrows at lower temperatures on each thermogram indicate To; arrows at higher temperatures indicate the actin peak (TmA). The vertical bar represents 0.334 mJsec-1

Figure 3. shows the effects of preheating treatments on the DSC thermograms of bighead carp muscle. After heating at 70oC for 5 min. the To of myosin transition shifted from 38°C to below 30°C. The 45°C and 52°C transitions of myosin were significantly decreased compared to those from untreated muscle indicating that the protein had already passed through the first two transitions prior to DSC analysis. The 59°C transition of myosin remained unaffected indicating that heating did not influence the third myosin transition prior to DSC analysis. The Tm (77°C) of the actin peak remained unchanged, but the area of the actin peak in the thermogram (Table 1) was reduced indicating that exposure to 70°C for 5 min. caused cooperative denaturation of actin.

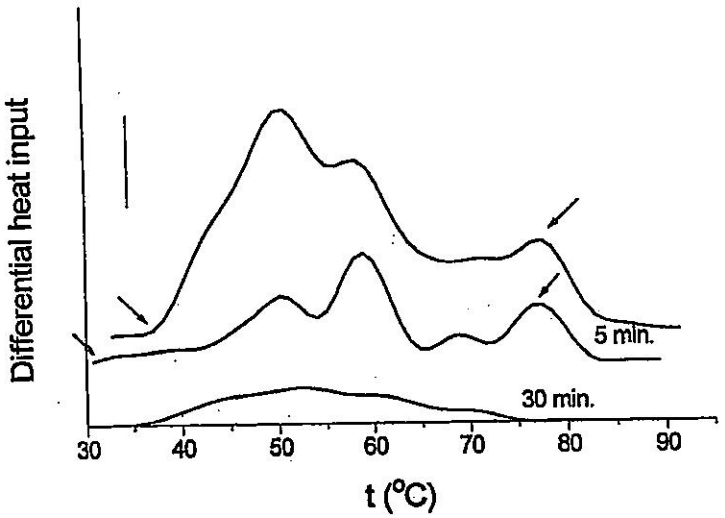

Figure 3. DSC thermograms of whole bighead carp muscle showing the effects of preheating. The upper thermogram is from an untreated sample and the lower thermograms from samples preheated at 70 °C for 5 or 30 minutes. Arrows at lower temperatures on each thermogram indicate To; arrows at higher temperatures indicate the actin peak (TmA). The vertical bar represents 0.334 mJsec-1

Progressive reduction in the enthalpy of denaturation occurred as the duration of heat treatment increased because heating at 70°C for 30 min. caused protein denaturation to the extent that no transition peaks could be distinctly observed (Figure 3).

Extractibility and SDS studies

The denaturation of myosin observed during frozen storage and heat pretreatment of fish muscle is a result of partial unfolding of the molecule with exposure of hydrophobic groups which leads to molecule aggregation with consequent decrease of protein solubility (Mackie, 1993). Indeed, extractability of myofibrillar proteins from bighead carp muscle after frozen storage and heat pretreatment decreased significantly compared to the untreated sample (Table 2).

The nature of extractable myofibrillar proteins reflects the changes that have taken place in the muscle during frozen storage and heat pretreatment and is demonstrated by the SDS-PAGE patterns (Figure 4). Figure 4 shows that, in SDS-PAGE of extractable proteins from muscle subjected to frozen storage or heat treatment, the bands characteristic for actin and myosin light chains (MLC) were present whereas the band for myosin heavy chain (MCH) had disappeared. The band for MHC is clearly seen in SDS-PAGE of insoluble residues.

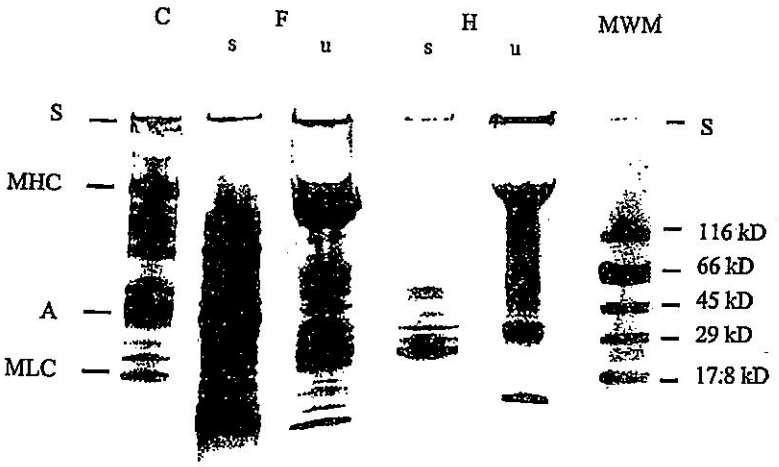

Figure 4. SDS-PAGE patterns of bighead carp proteins extracted from fresh muscle (C) and from muscle subjected to frozen storage at -18°C for 5 weeks (F) or to heat pretreatment at 70 °C for 5 min (H). Profiles for soluble (s) and insoluble (u) from extractable muscle proteins are shown. Start (S), myosin heavy chain (MHC), myosin light chain (MLC), actin (A).

DISCUSSION

In this study DSC combined with extractability and SDS-PAGE was applied in order to examine the stability of bighead carp muscle to heat and frozen storage. Myofibrillar proteins from bighead carp gave a characteristic DSC thermogram within the temperature range 20 - 90°C with two distinctive peaks: the large myosin and the smaller actin peak. The Tm of the transitions measured for myosinewere 45°C (shoulder), 52°C and 59°C. The Tm of the transition measured for actin was 77°C. The calorimetric enthalpy change (ΔH) for the endotherm estimated from the total area under the thermogram was 17.57 Jg^{-1} and partial enthalpy change for myosin was 9.84 Jg^{-1}. It is well established that myosin of fish muscle differs in stability depending on the habitat temperature of the species. In contrast to myosin, thermal stability of actin is not species dependent (Connell, 1961; Poulter *et al.*, 1985; Davies *et al.*, 1994; Howell *et al.*, 1991). Thermal stability of myofibrillar proteins from fresh bighead carp muscle corresponds to that reported in the literature for fish species with the habitat temperature of 20 - 27°C at which bighead carp lives (Davies *et al.*, 1994; Howell *et al.*, 1991). Thermal transitions of bighead carp muscle are comparable to those for carp reported in the literature. Both the To (38°C) and Tm (52°C) of the myosin peak from bighead carp were lower than the respective values reported in the literature for carp (To 46°C, Tm 56°C) under comparable conditions (Howell *et al.*, 1991). The Tm for bighead carp actin peak (77°C) is in good agreement with Tm (76.6°C) reported for carp (Howell *et al.*, 1991).

In agreement with previous studies on a range of fish muscles of different habitat temperature (Park and Lanier, 1989; Wu *et al.*, 1985; Beas *et al.*, 1990) our results show that the addition of salt has denaturing effects on both myosin and actin from bighead carp muscle whereas only myosin is predominantly affected

after frozen storage and heat pretreatment of bighead carp muscle. There appears to be a correlation between the extent of "freeze denaturation" of the muscle myosin and the habitat temperature of the fish (Davies *et al.*, 1994). The results of DSC analysis and protein extractability measurements demonstrate that denaturation of bighead carp myosin is considerably more pronounced during frozen storage than myosin from other fish species of similar habitat temperature (Davies *et al.*, 1994). The same applies for the addition of salt and heat pretreatment.

Fish skeletal myosins have complex multidomain structures containing two units of globular heads, S1, and a rodlike tail forming a coiled coil of α-helices similarly to mammalian myosins (Nakaya *et al.*, 1995). The myosin molecule itself is composed of noncovalently bound hexameric peptides: it contains two heavy chains with an approximate molecular mass of about 200 000 Da and four light chains with a molecular mass of about 20 000 Da. S1 has two noncovalently bound light chains and contains a site for ATP hydrolysis and a site for the interaction with actin.

DSC studies suggest that mechanisms of denaturation of myofibrillar proteins induced by the addition of salt and frozen storage are similar, whereas in muscle subjected to heat-pretreatment, different domains of the myosin molecule denaturate and aggregate in a different way. This may be explained by the finding that frozen storage causes a local increase of ionic strength as a consequence of freezing water migration from the myofibrillar space or dehydration of myofibrillar protein (Wagner & Anon, 1985; Mackie, 1993). At low ionic strenght myosin molecules aggregate to form filaments with a greater stability than the individual myosin molecule which exists at higher ionic strength. Thus the formation of filaments gives extra stability and this accounts for the higher temperatures required for transitions at low ionic strength. Salt decreases the heat stability of muscle proteins, causing them to denaturate at lower temperatures (Wu *et al.*, 1985; Howell *at al.*, 1991). Actin is also destabilized by increasing ionic strength and actin stability is known to be specific to salt type and concentration (Howell *at al.*, 1991). Increasing ionic strength reduces the stability of the myosin subunit, myosin molecules or myosin-myosin interaction within the myofibrillar proteins (Okada, 1981; Schut, 1976; Mackie, 1993). A possible explanation for these effects is that unfolding of myosin occurs in consecutive reactions with initial marked decrease in myosin-actin affinity due to denaturation of myosin heads, followed with dissociation and some denaturation of the myosin tail (Ogawa *et al.*, 1993; 1994). By this interpretation an increase of ionic strength caused by the addition of salt to bighead carp muscle would cause unfolding of the rod, whereas the rod domain would retain much of its structure throughout frozen storage. Then, more slowly, myosin continues to denaturate and aggregation of the denaturated proteins eventually occurs. This is accompanied by decreased solubility and viscosity (Davies *et al.*, 1994).

Bighead carp is similar in texture to common carp and is considered hardly distinguishable when prepared for market (Frimodt, 1995). Our results demonstrate that myosin and (to a lesser extent) actin from bighead carp muscle are more prone to denaturation upon addition of salt, frozen storage and heat-pretreatment, which are procedures commonly applied in fish processing, than was observed for commo· carp muscle and other fish species with habitat temperatures similar to that of bighead carp. This indicates that the deterioration

of bighead carp muscle during processing and frozen storage may be prominent thus affecting functional properties of bighead carp flesh.

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STABILNOST MIOZINA SIVOG TOLSTOLOBIKA PRI TERMIČKOJ OBRADI I SKLADIŠTENJU U ZAMRZNUTOM STANJU

RADIČEVIĆ TATJANA , RAIČEVIĆ SMILJANA i NIKETIĆ VESNA

SADRŽAJ

U ovom radu korišćena je diferencijalna skanirajuća kalorimetrija (DSC) da bi ispitali termalne prelaze mišićnog tkiva sivog tolstolobika *(Aristichthys nobilis Richardson).* Na DSC termogramu svežeg mišićnog tkiva uočljiva su tri endotermna pika. Nakon dodatka soli, temperature prelaza se pomeraju ka nizim vrednostima. Prethodno zagrevanje uzoraka na 70°C uzrokuje skoro potpuni nestanak pikova. Skladištenje na niskim temperaturama (-18°C, pet nedelja) izaziva promene, pre svega, u prelazima miozina. Rezultati ukazuju da se promene na proteinima mišićnog tkiva riba, nastale tokom skladištenja na niskim temperaturama, razlikuju od promena koje nastaju prilikom zagrevanja mišićnog tkiva.