ミルクフィッシユのミオシンの精製とその性質

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Purification and Characterization of Milkfish (*Chanos chanos*) Myosin

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Milkfish myosin was isolated and purified by a method which consisted of the extraction with Guba-Straub solution, followed by the ultracentrifugation at 1000,000×g in the presence of $MgCl_2$ and ATP, the precipitation at low ionic strength, and DEAE-Sephadex A-50 chromatography. Milkfish myosin contained a 200 Kd heavy chain and three light chains with molecular weights of 22, 19, and 16 Kd. The amino acid composition was similar to those of cod and tilapia. The total SH group content of myosin, 34–38 mol/5×10^5 g, was higher than those of cod and tilapia, and close to that of rabbit myosin. According to the inactivation rate constant of myosin Ca-ATPase (Kd), the thermal stability of milkfish myosin was higher than that of tuna, but lower than that of carp. The intrinsic viscosity was 2.4 dl/g, close to that of carp and rabbit myosin.

Although denaturation of myofibrillar proteins of fish muscle during frozen storage have been studied by many workers, progress in studies on the denaturation of frozen fish myosin has been impeded due to the difficulty in isolating it in a highly purified state. During the extraction of fish myosin, actin is readily extractable and apt to bind myosin. Takashi *et al.*[1–3] isolated carp myosin, but it still showed some viscosity drop on the addition of ATP. However, Tsuchiya and Matsumoto[4] purified carp myosin to an ultracentrifugal homogeneity. This study aims to optimize the extraction and purification conditions, and characterize the purified myosin from milkfish *Chanos chanos*.

Materials and Methods

Extraction and Purification of the Myosin

Crude myosin was extracted according to Tsuchiya and Matsumoto.[4]

DEAE-Sephadex A-50 Chromatography

The crude myosin was dialyzed against 0.04 M Na-pyrophosphate buffer (pH 7.5) overnight. Fifteen ml dialyzed crude myosin solution (0.6 mg/ml) was loaded on a column (DEAE-Sephadex A-50; 2.6×70 cm), and eluted with a gradient concentration of 0.04 M Na-pyrophosphate buffer and 0.5 M KCl–0.04 M Na-pyrophosphate buffer (pH 6.2) at a flow rate of 15 ml/h. The absorbance of eluate at 280 nm was determined using an UV-detector (Single Path Monitor UV-1, Pharmacia Fine Chemicals, Co.). Fractions of 5 ml were collected with a fractional collector (Fractional Collector FRAC-100, Pharmacia Fine Chemical Co.). The purity of eluted myosin was determined by Disc-SDS polyacrylamide gel electrophoresis.

Determination of SH Group of Myosin

The total SH group of myosin was determined according to Buttkus,[5] and calculated by the method suggested by Ellman.[6] The total SH content was expressed as mol per 5×10^5 g of protein.

Determination of the Stability of Myosin

A myosin solution (1.0–3.0 mg/ml) was incubated at various temperatures (0, 10, 20, 30, and 40°C). At a definite time interval, a portion was removed and placed at 25°C for 5 min. The Ca-ATPase specific activity was determined according to Arai.[7] The rate constant for inactivation of myosin Ca-ATPase activity was calculated according to Arai *et al.*,[8] i.e. $K_D=(\ln Co-\ln Ct)/t$, where Co: Ca-ATPase activity before incubation; Ct: Ca-ATPase activity after t sec incubation; t: incubation time (s).

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Determination of Ca-ATPase Activity

To 1 m*l* of myosin solution (1–5 mg/m*l*), 0.5 m*l* of 0.5 M Tris-maleate buffer (pH 7.0), 0.5 m*l* of 0.1 M $CaCl_2$, 7.5 m*l* deionized water and finally 0.5 m*l* of 20 mM Na_2-ATP solution (pH 7.0) were added. The rate of release of inorganic phosphate at 25°C within 3 min reaction was determined after the addition of ATP. Five m*l* of 15% trichloroacetic acid was added to stop the reaction and the inorganic phosphate was determined according to the method of Arai.[8] The Ca-ATPase specific activity was shown as micromol of inorganic phosphate liberated per mg protein within 1 min for the reaction at 25°C.

Determination of Myosin Subunits and Their Molecular Weight

The myosin subunits and their molecular weight were determined by using Disc Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (Disc SDS-PAGE).[9] A myosin solution was dialyzed overnight against 62.5 mM Tris-HCl buffer (pH 6.8) at 5°C, and then incubated at 100°C for 3 min in a buffer consisting of 2% SDS, 5% 2-mercapto-ethanol and 62.5 mM tris-HCl buffer (pH 6.8). On the top of gel, 80 μl of 0.05% bromophenol blue —10% sucrose—solubilized protein sample was pipetted. After the electrophoretic run in a buffer consisting of 25 mM Tris, 0.192 M glycine and 0.1% SDS (pH 8.3), the gels were stained with a 0.1% Coomassie brilliant blue—42% methanol—17% acetic acid solution for 4–5 h. Destaining was done by immersion in a mixture of 30% methanol and 10% acetic acid for 8–10 h, as recommened by Seki.[10] The concentration of polyacryamide of resolving gel was 10%.

Distance scanning at the wave length of 585 nm, for which the staining solution has the maximum absorbance, was employed to analyze the band size of the gel on a UV-VIS microprocessor-controlled spectrophotometer system (2600, Gilford Instrument). The molecular weights of myosin subunits were determined by comparing the mobility of each subunit with standard proteins (High and Low Molecular Weight Proteins kits, Pharmacia Fine Chemical Co.).

Determination of Amino Acid Composition

Myosin was treated with performic acid according to Hirs.[11] After removing the performic acid with freeze-drying, sample was subjected to the acid hydrolysis and amino acid analysis (using an amino acid analyzer, LKB 4150).

Determination of Viscosity

The intrinsic viscosity of myosin was measured using an Ostwald Viscometer at 10°C.

Determination of ATP-sensitivity

The ATP-sensitivity was determined according to Arai.[7]

Results and Discussion

Purification of Myosin

The water soluble proteins involved in the muscle was almost removed by washing with 3 volumes of 0.05 M phosphate buffer 4 times (Fig. 1). It is well known that myofibril consists of two kinds of filaments (thick and thin filaments). Myosin is the protein forming the thick filament. Rabbit myosin is easily obtained from the skeletal muscle by a short-period extraction with a Guba-Straub solution. However, fish myosin is easily contaminated by actin during extraction, and forms actomyosin.[12] Many approaches have been employed to extract myosin from fish. Some investigators extracted myosin with 0.45–0.6 M KCl solution containing $MgCl_2$ and ATP or pyrophosphate to avoid the contamination of actin.[13–15] $MgCl_2$ prevents the decomposition of ATP, and ATP or pyrophosphate dissociates actomyosin into myosin and actin. For preparation of fish myosin, the extracting periods depend upon fish species, varying from 6–30 min. The recommanded extraction periods for cod, tuna, tilapia, and carp were 30, 15–30, 10, and 7 min, respectively.[2,15–17] The optimum extracting period after the addition of ATP-$MgCl_2$ for milkfish myosin was found to be 10 min (Fig. 2).

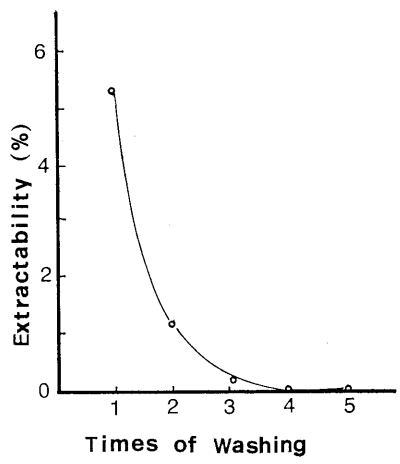

Fig. 1. Effect of the times of washing on the removal of water soluble proteins.

Table 1. ATP sensitivity of milkfish myosin before and after chromatography and actomyosin

	$\log \eta$	$\log \eta \cdot$ATP	ATP-sensitivity* (%)
Actomyosin	0.521	0.233	123.6
Myosin before chromatography	0.118	0.110	7.2
Myosin after chromatography	0.109	0.106	2.8

* ATP-sensitivity was caculated as follows:
$(\log \eta - \log \eta \cdot \text{ATP}) / \log \eta \cdot \text{ATP}$
where η and $\eta \cdot$ATP are the relative viscosity before and after the addition of ATP respectively.

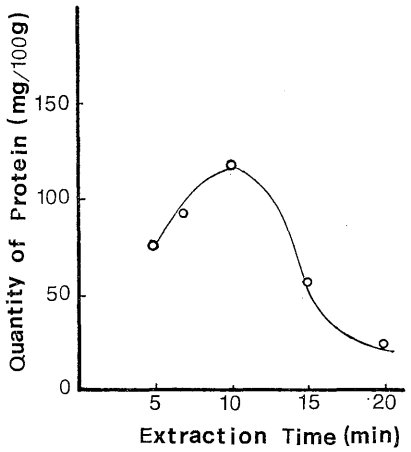

Fig. 2. Effect of the extracting time on the extractability of myosin.

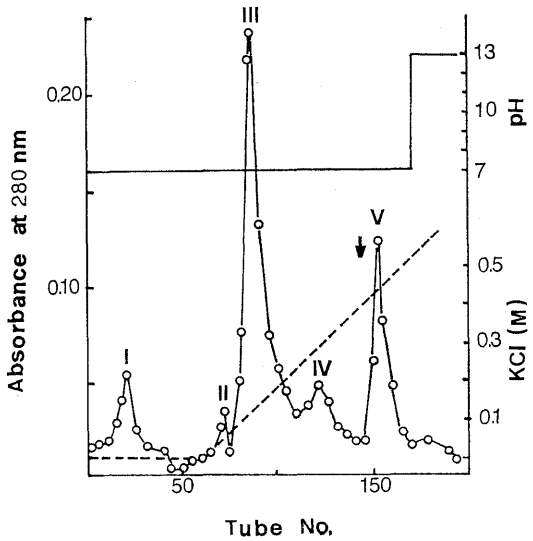

Fig. 3. Chromatography of milkfish myosin on DEAE-Sephadex A-50. (Myosin in 0.04 M Na-pyrophosphate, pH 7.5 was applied to a linear gradient of KCl. The flow rate was 15 ml/h. Fractions of 5 ml were collected. 0.5 M NaOH was applied at the place of arrow).

Although Tsuchiya and Matsumoto[4] reported that myosin could be purified from carp, the

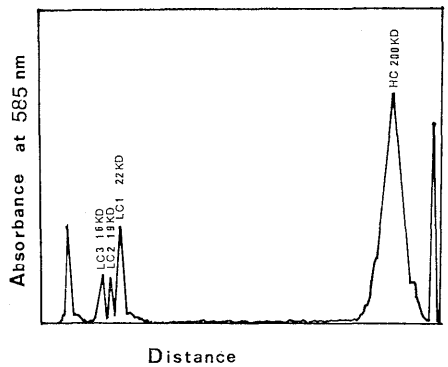

Fig. 4. SDS-Disc electrophoretic pattern of milkfish myosin.
The concentration of polyacrylamide gel was 10%.

myosin extracted from milkfish using the same procedures still showed high ATP-sensitivity (Table 1). For further purifying the myosin, the extracts from milkfish using the method of Tsuchiya and Matsumoto[4] was passed through DEAE-Sephadex A-50. Fractions from tube No. 80 to 100 were collected (Fig. 3) and analyzed with Disc-SDS PAGE (Fig. 4). As shown in Fig. 3, the first and second peaks from the left were considered to be actomyosin or aggregated myosin, and myosin dimer, respectively. The third peak was identified to be myosin (Fig. 4), where the 4th peak might be due to RNA having a maximum absorbance at 260 nm.[15] Collected eluate from tube No. 80 to 100 was diluted with 14 volumes chilled water and adjust to pH 6.4–6.6. After centrifugation at 5000×g, 0°C for 20 min, the preciptate was dissolved in 0.6 M KCl, pH 7.0 and characterized as follows.

Properties of Milkfish Myosin

The ATP-sensitivity of milkfish myosin after DEAE-Sephadex A-50 chromatography was 2.8%, which was lower than that before chromatography (myosin extracted by the method of Tsuchiya and Matsumoto[4]), and much lower than actomyosin (Table 1). According to the Disc-SDS PAGE

Table 2. Amino acid composition of milkfish and some other myosins

Amino acid	Milkfish	Cod*1	Tilapia*2	Rabbit*3
	(mol/10^5 g of myosin)			
Asp	82	85	90	85
Thr	42	36	40	44
Ser	32	45	39	39
Glu	112	145	159	157
Pro	28	25	21	22
Gly	48	44	43	40
Ala	70	71	79	78
Val	60	44	47	43
Met	19	20	25	23
Ile	43	35	39	42
Leu	73	77	88	81
Tyr	32	15	18	20
Phe	20	23	29	29
Lys	76	72	93	92
His	17	13	16	16
Arg	49	38	43	43
Try	—	—	—	—
Cys/2	7.6	7.2	7.5	8.8

*1 Data from ref. 29.
*2 Data from ref. 25.
*3 Data from ref. 30.

Table 3. Contents of SH groups in milkfish, and some other myosins

Species	SH groups per 5×10^5 g myosin
Milkfish	34–38
Cod*1	31–35
Tilapia*2	33–35
Rabbit*2	36.0–37.5

*1 Data from ref. 18.
*2 Data from ref. 24.

analysis, the milkfish myosin had a heavy chain with a molecular weight of 200 Kd and three light chains with molecular weights of 22, 19, and 16 Kd (Fig. 4).

The amino acid composition of milkfish myosin was similar to that of cod or tilapia[12] (Table 2). The total SH content calculated from the amino acid composition data and from DTNB method were 38 and 34 mol/5×10^5 g myosin, respectively (Table 3). The SH groups was close to that of other species and rabbit myosin.[18-26]

The Ca-ATPase activity of milkfish myosin was lower than that of rabbit or tilapia, but higher than carp. When the concentration of KCl in the reaction solution increased to 0.5 M, the Ca-ATPase activity of myosin from this species was higher than those of tilapia and carp, but lower

Table 4. Effect of KCl on the Ca-ATPase activity*1 of myosin from milkfish, tilapia, carp and rabbit

Myosin	0.06 M KCl	0.5 M KCl
Milkfish	0.551	0.315
Tilapia*2	0.643	0.142
Carp*2	0.286	0.157
Rabbit*2	1.080	0.464

*1 The Ca-ATPase activity was expressed as micromol of inorganic phosphate released within 1 min reactionat 25°C per mg of protein.
*2 Data from ref. 24.

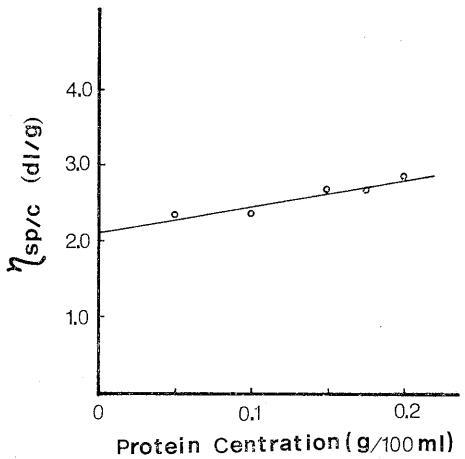

Fig. 5. Concentration dependence of the decreased specific viscosity of milkfish myosin.

Table 5. Rate constants for inactivation of milkfish myosin Ca-ATPase at various temperatures

Temperature (°C)	0	10	20	30	40
KD$\times10^5$ (s^{-1})	1.32	1.54	1.86	2.59	37.3

than that of rabbit[24] (Table 4). The intrinsic viscosity was 2.145 dl/g (Fig. 5), close to tilapia,[25] 2.15 dl/g, but higher than carp,[1] 1.75–1.90 dl/g and cod,[16] 2.00 dl/g.

The inactivation rate constant of actomyosin or myosin Ca-ATPase activity was frequently used for evaluating the thermal stability of fish muscle proteins.[17,27] The stability of myosin Ca-ATPase was considered to be highly related to the living environment temperature of fish.[17,18,27] As indicated in Table 5, the stability of milkfish myosin decreased with the increase of incubation temperature. The rate constant for inactivation of myosin Ca-ATPase (K_D) increased rapidly at 40°C (Table 5). As comparing the K_D values with other species, the stability of milkfish myosin was higher than yellowfin tuna, but lower than carp, tilapia, and rabbit[17,28] (Table 6).

Table 6. Rate constant for inactivation of milkfish myosin Ca-ATPase at 40°C, comparing with those of other myosins

Species	K_D ($\times 10^{-5}$/s)
Milkfish	37.3
Yellowfin tuna*1	38.8–49.2
Rabbit*1	4.1
Carp*2	25.4–26.7
Tilapia*2	13.1

*1 Data from ref. 17.
*2 Data from ref. 28.

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