Properties of Actin and Stability of the Actomyosin Reconstituted from Milkfish (*Chanos chanos*) Actin and Myosin

Shann-Tzong Jiang,* Fu-Jin Wang, and Chin-San Chen

In order to investigate the effects of actin and myosin to actin ratio on the thermal stability of reconstituted actomyosin (R-AM), actin was extracted from the acetone powder of milkfish dorsal muscle and purified by reversible polymerization, gel filtration on Sephadex G-200, and DEAE-Sephadex A-50 chromatography. The intrinsic viscosities of G- and F-actins were 0.38 and 2.32 dL/g, respectively, while the total SHs of G-actin were 4.8–5.1 mol/43 000 g. The molecular weight was electrophoretically determined to be 43 000. The amino acid composition was similar to that of rabbit, but the methionine content was higher than for trout. R-AM was prepared by using the purified milkfish actin and myosin with 1:1, 1:0.5, and 1:0.25 weight ratios. The inactivation rate constant of R-AM Ca-ATPase (K_D) was significantly lower than that of myosin alone ($P < 0.01$) and increased with the decrease of actin content. All R-AM K_D values were significantly higher than for actomyosin except at 30 and 40 °C.

Freezing seafood can result in excellent or poor quality, depending upon how quickly the freezing is done after the catch, and upon storage temperatures (Arai et al., 1973; Arai, 1977; Suzuki et al., 1964, 1965; Suzuki, 1967; Hatano, 1968; Tokiwa and Matsumiya, 1969; Seki and Hasegawa, 1978; Fukuda et al., 1981; Fukuda, 1986; Jiang, 1977; Matsumoto, 1980). However, freeze-denaturation of fish protein frequently occurs during frozen storage, which consequently changes the functionality of fish protein, especially in that of myofibrillar proteins. The denaturation of actomyosin or myosin was caused by the progressive increase of intermolecular and intramolecular interactions due to the formation of hydrogen, ionic, hydrophobic, and disulfide bonds during freezing and subsequent storage (Hwang, 1987; Jiang et al., 1988a,b). Although solubility, viscosity, and ATPase activity of actomyosin and myosin decreased with duration of frozen storage, no significant changes in viscosity, biological activity, and solubility of actin were observed during frozen storage (Connell, 1960). According to previous studies (Hwang, 1987; Jiang et al., 1988a,b; Chen et al., 1988), the inactivation rate constant of actomyosin is lower than that of myosin. This study aimed to purify the actin and investigate the effects of actin on the thermostability of myosin.

MATERIALS AND METHODS

Extraction and Purification of Actin. Crude actin was extracted according to Spudich and Watt (1971). The fish meat was washed with 0.4 M sodium bicarbonate and then centrifuged at 3000*g* for 20 min. Residue obtained was homogenized with three volumes of precooled acetone. The homogenate was filtered through a Buchner funnel and washed three times with the precooled acetone and once with ether. The acetone powder was air-dried overnight at room temperature and stored at −20 °C. G-actin was then extracted with 200 mL of buffer A (2 mM Tris-HCl, 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM $CaCl_2$; pH 8.0) from 18 g of acetone powder at 0 °C for 30 min. The extraction at 0 °C was to minimize the contamination of troponins and tropomyosin (Drabikowski and Gergely, 1962). The resultant extracts were filtered through the Toyo filter paper (No. 5B). The residue was washed with 100 mL of buffer A and filtered again. Collected supernatants were cleared by centrifugation at 10000*g* for 1 h. KCl and $MgCl_2$ were added to final concentrations of 50 and 2 mM, respectively, and the G-actin was allowed to polymerize into F-actin for 2 h at 25 °C. After the concentration of KCl was adjusted to 0.6 M, the solution was stirred gently for 1.5 h and then centrifuged at 80000*g* for 3 h. The pellet was resuspended in 30 mL of buffer A and dialyzed with vigorous stirring for 3 days at 4 °C, changing to fresh buffer A every 24 h. The G-actin was cleared by centrifugation at 80000*g* for 3 h.

Gel Filtration on Sephadex G-200. The actin was further purified by Sephadex G-200 chromatography. The crude actin (45 mg) was passed through a Sephadex G-200 column (2.6 × 80) with a buffer solution containing 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM $CaCl_2$, and 2 mM Tris-HCl, at a flow rate of 10 mL/h. The absorbance at 280 nm was measured to evaluate the protein concentration. The purity of actin was judged by sodium dodecyl sulfate–polyacrylamide discontinuous gel electrophoresis (disc-SDS-PAGE).

DEAE–Sephadex A-50. The actin obtained by gel filtration on Sephadex G-200 was dialyzed against 2 mM Tris-HCl buffer (pH 8.0) overnight. A 15-mL portion of dialysate (0.8 mg/mL) was loaded on a DEAE–Sephadex A-50 column (2.6 × 40) and eluted with a gradient concentration of 0.0 M NaCl–2 mM Tris-HCl and 0.5 M NaCl–2 mM Tris-HCl buffers (pH 8.0) at a flow rate of 15 mL/h.

Determination of Sulfhydryls (SHs) of Actin. The total SHs of actin were determined according to Buttkus (1971) and calculated by the method of Ellman (1959). The reactive SHs were determined by incubating the actin at 5 °C for 1 h, in the absence of urea according to Buttkus (1971). The total and reactive SHs were expressed as moles of SH per 43 000 g of protein.

Determination of Molecular Weight (MW) of Actin. The MW of actin was determined by using disc-SDS-PAGE (Laemmli and Favre, 1973). Actin 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 containing 2% SDS, 5% 2-mercaptoethanol, and 62.5 mM Tris-HCl buffer (pH 6.8). On the top of the gel was pipetted 80 μL of 0.05% bromophenol blue–10% sucrose–solubilized protein sample. 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 0.1% Coomassie Brilliant blue–42% methanol–7% 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. The acrylamide concentration of resolving gel was 10%.

Determination of Amino Acid Composition. Actin was treated with performic acid according to Hirs (1956). After the performic acid was removed with freeze-drying, samples were subjected to acid hydrolysis and amino acid analysis (on an amino acid analyzer, Hitachi Model LKB 4150).

Determination of Viscosity. The intrinsic viscosity was measured at 10 °C on an Ostwald viscometer with total volume of 2 mL and outflow time of 30–40 s (130 s for water).

Determination of the Polymerization Ability of G-Actin. To the G-actin solution containing 0.2 mM ATP–0.2 mM Ca-

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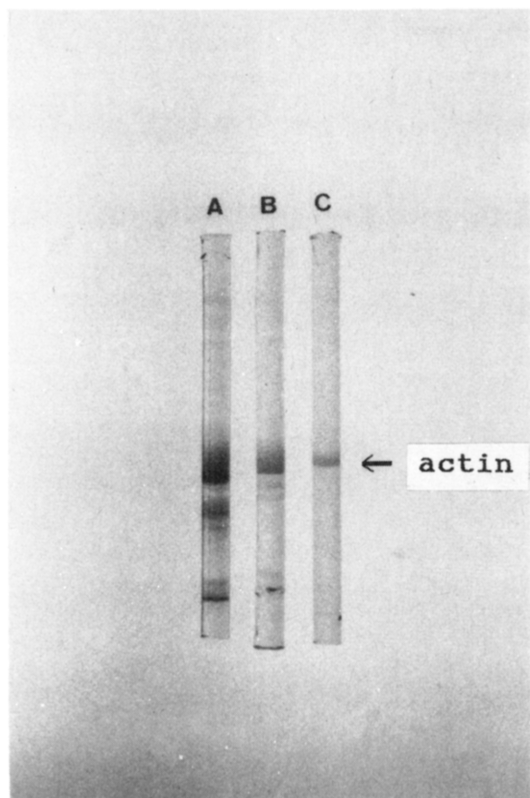

Figure 1. SDS–polyacrylamide gel electrophoretic patterns of actin: A, crude actin (26 μg) extracted from acetone powder with 0.5 mM ATP–0.2 mM $CaCl_2$–0.5 mM 2-mercaptoethanol–2 mM Tris-HCl (pH 8.0) for 20 min at 0 °C and purified by reversible polymerization; B, G-actin further purified gel filtration on Sephadex G-200 (30 μg); C, G-actin from B further purified by DEAE–Sephadex A-50 (44 μg).

Cl_2–0.5 mM 2-mercaptoethanol–2 mM Tris-HCl (pH 8.0) were added KCl and $MgCl_2$ to final concentrations of 0.1 M and 1.0 mM, respectively, separately. The changes in specific viscosity were measured on an Ostwald viscometer to evaluate the polymerization ability.

Preparation of Myosin. Myosin was prepared from milkfish dorsal muscle as in a previous study (Chen et al., 1988).

Preparation of Actomyosin. Actomyosin was extracted from milkfish dorsal muscle according to Noguchi and Matsumoto (1970).

Preparation of the Reconstituted Actomyosin (AM). KCl (to 0.1 M) and $MgCl_2$ (to 1 mM) were added to the G-actin solution and allowed to polymerize into F-actin for 1 h at 25 °C. To the F-actin solution was then added KCl to 0.6 M and the mixture stirred gently for 1.5 h.

The AM was reconstituted by mixing the purified myosin and F-actin with various weight ratios (1:1, 1:0.5, 1:0.25), dialyzing against a buffer consisting of 0.6 M KCl–5 mM Tris–maleate (pH 7.0) for 18 h, and centrifuging at 20000*g*, 4 °C, for 20 min. The resultant reconstituted AM was subjected to the following analysis.

Thermal Stability. Various reconstituted AMs (30-mL portions) were placed in plastic jars and incubated at 0, 10, 20, 30, and 40 °C. At definite time intervals, samples were removed and incubated in 25 °C water bath for 5 min to equilibrate the temperature of protein samples. The Ca-ATPase activity was determined according to Arai (1974) and defined as micromoles of inorganic phosphate liberated/milligram of protein within 1-min reaction at 25 °C. The inactivation rate constant (K_D) of reconstituted AM Ca-ATPase was calculated according to Arai et al. (1973); i.e., $K_D = (\ln C_0 - \ln C_t)/t$, where C_0 = Ca-ATPase activity before incubation, C_t = Ca-ATPase activity after incubation for time t, and t = incubation time (s).

RESULTS AND DISCUSSION

Purification of Actin. Milkfish actin extracted by the method of Spudich and Watt (1971) was still contaminated by tropomyosin and troponins (Figure 1A). For further purification, the actin solution was passed through Sephadex G-200 chromatography (Figure 2). The purity was judged by disc-SDS-PAGE. The actin purified by this step was still contaminated by tropomyosin and troponins (Figure 1B). Accordingly, actin was further purified by DEAE–Sephadex A-50 chromatography (Figure 3) and was shown to be electrophoretically homogeneous (Figure 1C).

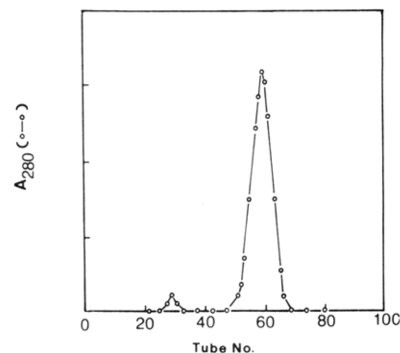

Figure 2. Purification of milkfish actin by gel filtration. 25 mg of G-actin was applied to Sephadex G-200 and eluted with 0.2 mM ATP–0.2 mM $CaCl_2$–0.5 mM 2-mercaptoethanol–2.0 mM Tris-HCl (pH 8.0) at 4 °C.

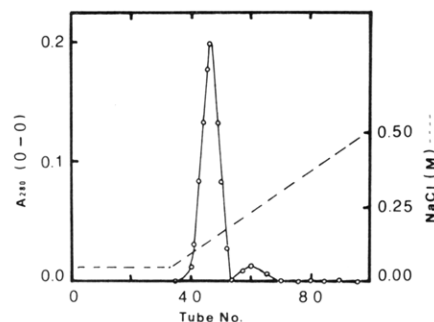

Figure 3. Chromatography of milkfish actin on DEAE–Sephadex A-50. 50 mg of actin in 2.0 mM Tris-HCl (pH 8.0) was applied and eluted by linear gradient of NaCl. The flow rate was 15 mL/h. Fractions of 5 mL were collected.

Table I. Amino Acid Composition of Actin

amino acid	milkfish, mol/43 000	trout,[a] mol/43 000	rabbit,[a] mol/43 000
Lys	21.0	23.5	20.1
His	8.1	9.6	7.7
Arg	19.2	20.9	19.8
Cys/2	4.8	5.0	5.1
Asp	36.8	36.5	36.9
Thr	30.2	29.1	26.7
Ser	23.0	25.1	22.3
Glu	45.2	42.1	42.5
Pro	19.4	21.7	20.0
Gly	31.3	29.0	30.1
Ala	30.2	31.1	30.9
Val	22.1	22.3	20.8
Met	15.1	9.8	16.1
Ile	27.2	26.0	27.3
Leu	30.4	30.5	27.9
Tyr	15.3	15.8	16.2
Phe	11.0	12.7	13.1
Try	–	3.1	4.1

[a] Data from the study of Bridgen (1971).

Properties of Milkfish Actin. According to the disc-SDS-PAGE, the molecular weight of actin calculated from its relative mobility was 43 000, which was approximately the same as that from tilapia and carp (Seki et al., 1973). The amino acid composition of milkfish actin was similar to that of trout and rabbit actin except for the

Table II. Content of SH in Milkfish Actin Compared with Tilapia and Rabbit Actin

source	total SH, mol/43 000	reactive SH, mol/43 000
milkfish	4.8–5.1	2.8
trout[a]	4.3–4.8	
rabbit[a]	4.7–4.9	

[a] Data from Tonomura (1973).

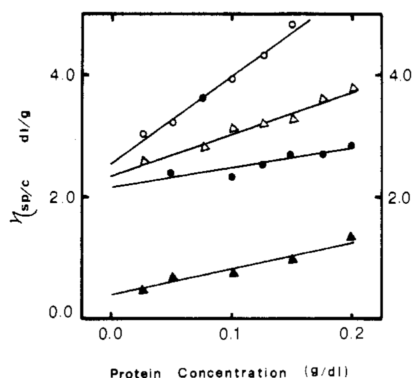

Figure 4. Concentration dependence of the decreased specific viscosity of F-actin, myosin, and reconstituted actomyosin: △, F-actin; ●, myosin; ○, reconstituted actomyosin; ▲, G-actin.

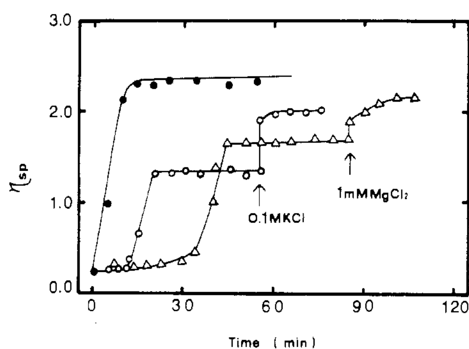

Figure 5. Effect of salts on the polymerization of purified G-actin from milkfish muscle: ●, KCl + $MgCl_2$; △, KCl added; ○, $MgCl_2$ added; ▲, either 0.1 M KCl or 1 mM $MgCl_2$ added at the arrow.

content of methionine (Table I). The methionine content was much higher than in trout, but almost the same in rabbit (Table I). The total and reactive SHs of G-actin were 4.8–5.1 and 2.8 mol/43 000 g of actin, respectively (Table II). It suggested that G-actin contained five SHs, and three appeared on the surface of molecule, similar to the G-actin of trout and rabbit (Bridgen, 1971, 1972).

The intrinsic viscosities of G-actin and F-actin were 0.38 and 2.32 dL/g, respectively (Figure 4). The intrinsic viscosity of F-actin was very close to that of tilapia and cod, 2.30 dL/g (Arai et al., 1974). The polymerization ability of G-actin was judged by changes in specific viscosity in the presence of KCl and $MgCl_2$, which was shown in Figure 5. The G-actin polymerized slower and had lower specific viscosity in the presence of either 0.1 M KCl or 1 mM $MgCl_2$ than of both salts. However, when the 0.1 M KCl added to the actin with 1 mM $MgCl_2$, and $MgCl_2$ to that with KCl, the specific viscosity increased rapidly to levels almost equal to that with both salts (Figure 5).

Stability of Reconstituted Actomyosin. The inactivation rate constant of reconstituted actomyosin (R-AM) Ca-ATPase increased with the decrease of actin content and with increase of incubation temperature (Table III). The inactivation rate constant of natural actomyosin (N-AM) is significantly lower than that of myosin ($P < 0.01$). Although actin per se has no enzymatic activity, it significantly modified the Ca-ATPase activity and stability of myosin in the actin–myosin complex (R-AM) (Table III). Myosin separated from actin had lower stability than N-AM and R-AM (Table III). The results suggested that actin plays a protective role on the stability of myosin. The Ca-ATPase activity was highest in N-AM, and then R-AM and myosin in this order (Table III). This is because actin and troponin–tropomyosin activated the Ca-ATPase of myosin (Spudich and Watt, 1971).

Table III. Inactivation Rate Constants[a] ($K_D \times 10^5$/s) of Various Reconstituted Actomyosin Ca-ATPase at Various Temperatures

temp, °C	AM[b]	myosin to actin ratio (w/w) 1:1	1:0.5	1:0.25	myosin
0	0.52 a[c]	1.1 b	1.26 b	1.75 c	1.82 c
10	0.84 a	1.27 b	1.57 c	2.38 d	2.74 e
20	1.23 a	1.50 b	2.48 c	2.70 d	3.86 e
30	7.08 a	7.29 a	8.06 b	8.95 c	9.67 d
40	31.41 b	29.21 a	32.36 b	35.91 c	39.34 d
Ca-ATPase act.[d]	0.63	0.44	0.41	0.35	0.32

[a] $K_D = (\ln C_0 - \ln C_t)/t$, where C_0 = Ca-ATPase activity before incubation, C_t = Ca-ATPase activity after t-s incubation, and t = incubation time (s). [b] Natural actomyosin (directly extracted from milkfish dorsal muscle). [c] Mean values bearing unlike characters in the same row differ significantly ($P < 0.01$). [d] Ca-ATPase activity was expressed as micromoles of inorganic phosphate per minute per milligram.

The role of SHs in actin is still not well understood. It has been postulated that they involve the polymerization of G-actin (Katz and Mommaerts, 1962; Drabikowski and Gergely, 1963), nucleotide binding (Strohman and Samorodin, 1962; Katz, 1963; Kuehl and Gergely, 1969), and myosin combination (Perry and Cotterill, 1964; Bailin and Barany, 1967). However, some studies indicated that modification of two or three cysteine residues of actin with (2-aminoethyl)isothiouronium (Seraydarian et al., 1968), with an azo dye (Lusty and Fasold, 1969), or with iodoacetate or iodoacetamide (Bridgen, 1972) showed no effect on the ability of G-actin to polymerize or bind myosin, ATP, and Ca. Our results seemed to suggest that, in spite of whether the SHs are involved in actin–myosin interaction or not, SHs appearing on the surface of F-actin might also compete with SHs on the myosin molecules for oxidation into disulfides during incubation, which consequently increased the stability of myosin in R-AM. The stability of N-AM was significantly higher than that of R-AM except those with 1:1 myosin to actin ratios at 30 and 40 °C. This is because the N-AM contains other proteins, such as troponins and tropomyosin, that may protect N-AM from thermal denaturation.

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