PHYSICOCHEMICAL PROPERTIES AND PHYSIOLOGICAL FUNCTIONS OF FISH MYOSIN LIGHT CHAINS

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INTRODUCTION

1. Historical Background

Myosin is a contractile protein, being involved in many biological movements, such as muscle contraction, amoeba movement, cell division, etc. (Mannherz and Goody, 1976; Adelstein and Eisenberg, 1980). Muscle contraction is caused by a cycle attachment of the heads of myosin molecule which form 'cross-bridge' to actin as myosin thick filament slides over actin thin filament, using ATP as an energy source (Huxley, 1969; Huxley and Simmons, 1971). Myosin possesses ATP hydrolyzing ability (ATP-ase activity), and forms filamentous fiber under low ionic strength. Electron microscopy shows that myosin molecule consists of two globular heads (20 nm in length, 9 nm in width) and rod portion (130 nm in length). It is also established that the active site exists on the globular head (subfragment 1, S1), while rod portion is involved in filament formation (Lowey et al., 1969).

Myosin consists of two heavy chains (molecular weight $\sim$200,000) and four light chains (molecular weight $\sim$20,000) (Gershman et al., 1969; Gazith et al., 1970; Perrie and Perry, 1970; Weeds and Lowey, 1971; Yagi et al., 1975). Existence of light chains in myosin was first recognized by Tsao (1953). Light chains are non-covalently bound to heavy chain and are released from heavy chain by treatment with urea, guanidine hydrochloride, sodium dodecylsulfate (SDS) or heat (Tsao, 1953; Kominz et al., 1959; Gazith et al., 1970). Recently, immuno-

electron microscopy revealed that light chains are located in a region near the junction of the head with the rod (Flicker et al., 1983; Winkelmann et al., 1983). Light chains are synthesized independently of the heavy chains on different populations of polyribosomes (Sarkar and Cooke, 1970; Brivio and Florini, 1971).

It has been demonstrated that both quantitative and qualitative differences exist between light chains from different types of muscle : i. e., fast, slow and cardiac muscles (Seidel, 1967; Sarkar et al., 1971; Weeds et al., 1975; Weeds, 1969, 1976). Fast skeletal muscle myosins, including those from fish ordinary muscle, possess four light chains of three different molecular weights, designated LC1, LC2 and LC3 in the order of decreasing molecular weight (Weeds and Baker, 1968; Horvath and Gaetjens, 1972). LC2 can be exclusively released from myosin by treatment with a kind of sulfhydryl reagent, 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), without significant loss of ATPase activity of myosin (Weeds, 1969; Gazith et al., 1970). On the other hand, two other light chains, LC1 and LC3, are dissociated from myosin at alkaline pH (about pH 11), and thus called alkali light chains, A1 and A2, respectively (Gazith et al., 1970). Release of alkali light chains is accompanied by loss of ATPase activity of myosin (Stracher, 1969), and hence they are referred to as 'essential' light chains. The primary structures of alkali light chains from rabbit or chicken fast muscle myosins are very similar to each other, differing only in additional 41 residues at the N-terminal of A1, so-called 'difference peptide', which is rich in alanine, lysine and proline (Frank and Weeds,

1974; Matsuda et al., 1980). Alkali light chains are encoded by a single gene, differentially spliced to give the specific N-termini (Barton and Buckingham, 1985). A1 and A2 light chains exist in single fibers, thus being synthesized in the same muscle cell (Weeds et al., 1975; Pette and Schnez, 1977). In rabbit fast muscle myosin, A1 and A2 exist in a molar ratio of 2:1 (Frank and Weeds, 1974; Weeds et al., 1975), while the molar ratio is about 1:1 in chicken breast muscle myosin (Lowey and Risby, 1971). The non-stoichiometry of A1/A2 ratio is due to the existence of myosin isozymes (Hoh et al., 1976; Hoh, 1978) : a heterodimer containing both A1 and A2, and two homodimers containing two moles of either A1 or A2 per mole (Lowey and Risby, 1971; Weeds et al., 1975). Alkali light chains are Ca-binding proteins, which contain four homologous "EF-hand" structures of the type found in parvalbumin (Collins, 1974; Kretsinger and Barry, 1975), though the affinity for divalent ions is several times of magnitude lower than that of troponin C or calmodulin (Potter and Gergely, 1975; Crouch and Klee, 1980).

On the other hand, light chain composition has been found to change during development or differentiation (Pelloni-Müller et al., 1976; Syrový, 1979). In case of rabbit and chicken, embryonic 'fast' muscle myosin contains none or smaller amount of A2 (Sreter et al., 1972; Takahashi and Tonomura, 1975; Dabrowska et al., 1977) and lower ATPase activity (Trayer and Perry, 1966; Obinata, 1969) compared to adult myosin. However, during post-natal development, A2 content gradually increases, accompanied by the increase of myosin ATPase activity (Takahashi and Tonomura,

1975).

As described above, alkali light chains were considered to be essential for myosin ATPase activity. Actually, S1 isozymes associated with different alkali light chains, S1(A1) and S1(A2), give rise to different kinetics in actin-activated Mg-ATPase activity (Weeds and Taylor, 1975), although the kinetics of both isozymes are similar in the absence of actin. Rabbit S1(A1) shows higher affinity for actin and lower maximum velocity than S1(A2). Hybrid experiments of S1 heavy chain with foreign alkali light chains supported the view that alkali light chains are involved in actin-myosin interaction, and that such kinetic differences could solely be attributed to light chains (Wagner and Weeds, 1977). Kinetic differences are also maintained with regulated actin (actin-tropomyosin-troponin complex) (Wagner et al., 1979). Heavy meromyosin isozymes showed the same kinetic differences as those of S1 (Wagner, 1977). However, such differences in kinetics are recognized at low salt concentration under physiological conditions (Wagner et al., 1979). In this connection, the difference peptide of A1 has been demonstrated to bind directly to actin (Prince et al., 1981; Dalgarno et al., 1982). At higher salt concentration, the differential binding between these isozymes to regulated actin was maintained in the presence of Ca, but not in its absence (Trayer and Trayer, 1985). Recently, Sivaramakrishnan and Burke (1982) prepared S1 heavy chain free of alkali light chain and showed that heavy chain itself possessed full ATPase activity and actin-binding ability. However, free heavy chain was labile, and rapidly aggregated. Wagner and Stone (1983) reported that recombination of free heavy

chain with alkali light chains under dissociating conditions lessened the degree of denaturation, but once heavy chain was transferred to heavy chain-alkali light chain complex, recombination did not result in the stabilization of heavy chain. On the other hand, DTNB light chain, a kind of 'regulatory' light chain restores Ca-sensitivity of desensitized (EDTA light chain-free) scallop myosin, though myosin-linked regulation has never been detected in skeletal muscle (Kendrick-Jones et al., 1976). This light chain possesses divalent cation binding sites (Morimoto and Harrington, 1974), and is phosphorylated by the action of a highly specific myosin light chain kinase (Pires and Perry, 1977), but the significance of phosphorylation in situ is controversial. Incidentally, the analogous light chains in vertebrate smooth (Gorecka et al., 1976) and invertebrate striated muscle (Kendrick-Jones et al., 1976) are known to be involved in regulation of the actin-myosin interaction. As a whole, function(s) of both alkali and DTNB light chains in skeletal myosin is ambiguous at present.

Fish myosins are very similar to those from higher vertebrates in amino acid composition, sedimentation coefficient, intrinsic viscosity, and subunit composition (Connell and Howgate, 1959; Hamoir et al., 1960; Buttkus, 1966; Takashi, 1973; Watabe and Hashimoto, 1980). Fish ordinary muscle myosins so far reported give rise to three kinds of light chains, whose composition is similar to that of fast muscle myosins of higher vertebrates (Takashi et al., 1974; Watabe and Hashimoto, 1980; Watabe et al., 1983a). On the other hand, dark muscle myosin

has only two kinds of light chains (Watabe et al., 1983a), resembling those from slow skeletal or cardiac muscle myosins (Lowey and Risby, 1971, Sarkar et al., 1971; Weeds and Frank, 1972; Weeds, 1976). Fish myosins show species-specificity due to the differences in molecular weight (Focant et al., 1976; Seki, 1976). Several reports are available on fish myosin light chains. Namely, physicochemical properties such as amino acid profile have been examined on those from carp, trout, pike and mackerel (Focant and Huriaux, 1976; Perzanowska et al., 1978; Huriaux and Focant, 1977; Watabe et al., 1983a). Immunochemical cross-reactivity has also been examined on myosin light chains from mackerel (Watabe et al., 1983a; Dinh et al., 1985) and some freshwater fishes (Huriaux and Focant, 1985).

2. The Aim of the Present Study

Light chains from fast skeletal myosin are divided into two groups: one is DTNB light chain and the other, alkali light chain, which has two molecular species, A1 and A2. Several reports have already been made on species-specific patterns of light chains from fish myosin with reference to molecular weight (Focant et al., 1976; Seki; 1976; Perzanowska, 1977). However, they did not identify light chain species, and ended up with the comparison of electrophoretic patterns. So far, such identification has been performed only with a few species : trout, carp and mackerel (Huriaux and Focant, 1977; Perzanowska et al., 1978; Watabe et al., 1983a). In addition, non-stoichiometry of alkali

light chains in myosin has been noticed in higher vertebrates (Lowey and Holt, 1972) which indicated the exsistence of myosin isozymes with respect to alkali light chains. Corresponding data on fish myosin has been still lacking.

It has also been reported that light chain composition and ATPase activity of myosin change during animal development. That is, A2, if any, is detected in only a small amount compared to A1 in embryonic myosin, but through development, the amount of A2 increases with the increase of ATPase activity (Takahashi and Tonomura, 1975). As for fish, Perzanowska (1979) examined light chain composition in myosins from several developmental stages of trout, and claimed that the patterns were virtually similar throughout development. No further information is available.

As for fish myosin light chains, physicochemical properties such as amino acid profile have been reported on those from carp, trout, pike and mackerel (Focant and Huriaux, 1976; Huriaux and Focant, 1977; Perzanowska et al., 1978, Watabe et al., 1983a). They reveal that the properties of fish myosin light chains are similar to the counterparts of higher vertebrates, but variations are more or less recognized depending on fish species. However, such minor differences seem to be inconsistent with the species-specificity as described above. Immunological studies showed that A1 and A2 were homologous, though cross-reaction was also recognized between alkali and DTNB light chains (Silberstein and Lowey, 1977). It has also been reported that the reactivity among myosin light chains from ordinary, dark and cardiac muscles of mackerel is generally reminiscent of that of higher verte-

brates (Dinh *et al.*, 1985). However, cross-reactivity of light chains between different fish species is still unknown. Therefore, structural differences of light chains - both physicochemical and immunological - responsible for the said specificity, for the most part, remain to be elucidated.

On the other hand, the function(s) of light chains in skeletal muscle myosin is controversial. DTNB light chain seems to be nothing to do with the ATPase of skeletal myosin, while alkali light chains are still considered to be 'essential'. Weeds and Taylor (1975) separated S1 isozymes, S1(A1) and S1(A2), and compared their enzymatic properties. They claimed that both isozymes differed only in actin-activated Mg-ATPase activity. Namely, the maximum velocity (V_{max}) and the apparent dissociation constant for actin (K_m) were higher for S1(A2) than for S1(A1). However, such differences in kinetics disappeared as the salt concentration raised to physiological value (Wagner *et al.*, 1979). Burke and Sivaramakrishnan (1982) prepared S1 heavy chain free of alkali light chain, and found that this heavy chain showed full enzymatic activity and actin-binding ability, though it easily aggregated and denatured. In this connection, hybridization experiments disclosed that the kinetics in the absence of actin were the same whether the essential light chains came from fast, slow, cardiac or smooth muscle myosin (Wagner, 1981). Therefore, it is quite natural that alkali light chains could possess other functions than the modulation of actin-myosin interaction, for example, stabilization of myosin. Fast myosin S1 isozymes, S1(A1) and S1(A2), are considered to be good

materials for the study on the functions of alkali light chains, because they only differ in the difference peptide of A1, provided that their heavy chains are homogeneous. However, comparative studies on S1 isozymes are still poor and fragmentary at present.

Under these backgrounds, the author carried out the present study on myosin light chains from fish muscle. First of all, species-specificity of light chains with reference to molecular weight, isoelectric point and stoichiometry was examined. Developmental changes in light chain composition were also examined. Attempts were then made to isolate light chains from several fishes, and their physicochemical properties were compared. Immunological cross-reactivity between homologous and heterologous light chains from various fishes was also studied. In addition, attempts were made to assess the physiological role of alkali light chains in kinetics, using native S1, and hybrid S1 consisting of rabbit S1 heavy chain and fish alkali light chains. Finally, stabilities of S1 isozymes were compared, and the role of alkali light chains in stabilization of myosin was discussed.

3. Review of the Present Study

This thesis consists of five chapters. In Chapter I, identification and species-specificity of myosin light chains as well as their developmental changes are described. Isolation and characterization of light chains are mentioned in Chapter II. In Chapters III and IV, kinetic properties of myosin S1 isozymes and the role of alkali light chains in stabilization of myosin are described, followed by general discussion in Chapter V.

Chapter I deals with the identification of alkali and DTNB light chains in fish myosins as well as the developmental changes of light chain composition in fish myosin.

Myosins were purified from the ordinary muscle of 19 fish species, and alkali and DTNB light chains were selectively released from myosin by treatment with LiCl and DTNB, respectively. Identification was performed by simultaneous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analyses of myosin and each light chain fraction. Results obtained showed that fish myosins, like fast muscle myosins from higher vertebrates, gave rise to two alkali light chains (A1, A2) and DTNB light chain. The composition was approximately two moles each of alkali light chains and DTNB light chain per mole of myosin. Molecular weights were in a range of 25,000 - 30,000 for A1, 13,500 - 22,500 for A2, and 17,500 - 21,000 for DTNB light chain.

A1/A2 molar ratio also showed a remarkable species-specificity, ranging from 0.1 (grunt) - 2.6 (requiem shark). Most myosins showed light chain patterns typical to those of fast

muscle myosins : DTNB light chains had an intermediate molecular weights of A1 and A2. However, as for scombrid fishes, whose locomotory activity is very high, molecular weights of A2 were exclusively higher than those of DTNB light chain, whereas, in case of sardines and horse mackerel, the molecular weights of A2 and DTNB light chains were very similar to each other.

Furthermore, two-dimensional gel electrophoresis with combination of isoelectric focusing and SDS-PAGE revealed that the electropherogram was similar among related species. Light chains from requiem shark and rabbit myosins were clearly distinguished, though the molecular weights and molar ratios of corresponding light chains were very similar to each other. On the other hand, A1 and DTNB light chains were spotted in quite a narrow range, while A2 light chains were widely scattered, showing the most remarkable species-specificity of this light chain.

Developmental changes of light chain composition were examined for tilapia (_Tilapia nilotica_) and chum salmon myosins. The results obtained disclosed that the molecular weights and isoelectric points of light chains did not change throughout development. However, A1 content gradually increased with concomitant decrease of A2, while DTNB light chain remained almost constant, for both fish myosins. The compositional changes were unexpectedly opposite to those of higher vertebrates.

Chapter II deals with isolation of myosin light chains from the ordinary muscle of several fish species, and their physico-

chemical and immunological characterization are described.

Based on the molecular weights of A2 and DTNB light chain, fish myosins can be classified into the following three types: I, A2 > DTNB; II, A2 = DTNB; III, A2 < DTNB. Six species were selected as follows: skipjack, bonito and mackerel from type I, sardine and round herring from type II, and Alaska pollack from type III. Total light chain fraction was obtained by urea treatment, and separated into each component by DEAE-Toyopearl column chromatography or HPLC. Among these fishes, degradation of DTNB light chain during isolation was remarkable for a few fish species, probably due to proteolysis by endogenous proteases. As a result, A1 light chains from six species, A2 light chains from four species and DTNB light chains from three species were obtained in high homogeneity.

Amino acid compositions of these light chains were similar in that they were rich in aspartic and glutamic acids, alanine, glycine, etc., and poor in tyrosine and histidine. The contents of alanine, proline and lysine were generally higher in A1 than in A2, suggesting the presence of the difference peptide as in the case of higher vertebrates. On the other hand, amino acid profiles of DTNB light chain were similar among the fish species examined and to those of 'regulatory' light chains from other animals, suggesting that the structure of this class of light chains is very conservative during the molecular evolution.

Ultraviolet absorption spectra of alkali and DTNB light chains showed a maximum at around 278 nm and a minimum at around 250 nm, in addition to small peaks due to the high content of

phenylalanine at 253, 259, 265 and 269 nm. Extinction coefficients at 280 nm, along with the above spectra, showed less species-specificity compared to molecular weights and compositions of light chains.

In the next place, antibodies were raised in rabbits against isolated light chains, and immunological cross-reactivity was examined by double immunodiffusion test and immunoelectrophoresis. As a result, strong cross-reactivity was observed between A1 light chains from the above six fish species, although spurs, which indicate partial antigenic heterogeneity, appeared between phylogenetically distinct species, e.g., between skipjack and Alaska pollack. Myosins from 14 fish species, including above six fishes, were then analyzed by SDS-PAGE, and the cross-reactivity was examined by immunoblotting technique using antisera obtained as above. Strong cross-reactivity was observed irrespective of fish species and antiserum used, although the staining intensity was dependent on fish species. The results suggested that ubiquitous structures may exist throughout the three types of light chain from all the fishes.

Chapter III deals with isolation and kinetics of S1 isozymes associating different alkali light chains, S1(A1) and S1(A2).

Myosins were prepared from the ordinary muscle of tilapia, bigeye tuna, and fast skeletal muscle of rabbit. S1 fraction was obtained by α-chymotryptic digestion of each myosin in the presence of EDTA, and separated into two isozymes by DEAE-Toyopearl column chromatography. S1 isozymes thus obtained were free from DTNB light chain, associating one mole of either A1 or

A2 per mole of heavy chain. Ca- and EDTA-ATPase activity of these S1 isozymes showed similar pH optima, but was significantly different between the isozymes in particular pH ranges. When actin-activated Mg-ATPase was examined under various concentrations of KCl, the apparent affinity for actin ($1/K_m$) decreased with the increase of KCl concentration, while the maximum velocity (V_{max}) was not largely affected. The effect of KCl concentration was rather different between tilapia and rabbit: rabbit S1 showed lower values of K_m and V_{max} at low KCl concentration, and tilapia S1 isozymes did vice versa.

Attempts were then made to elucidate the structural differences of the heavy chain between these isozymes, because these kinetic differences could be due to heterogeneity of the heavy chain. S1(A1) and S1(A2) from tilapia and rabbit were digested by α-chymotrypsin, Staphylococcus aureus V8 protease and subtilisin, and the peptide maps were compared by SDS-PAGE. The fragmentation patterns of heavy chain were essentially the same between both isozymes. Furthermore, S1 isozymes were digested with trypsin, and its time courses were analyzed by SDS-PAGE. All S1's gave rise to three fragments of 50 K, 25 K and 20 K daltons in the absence of actin, and two fragments of 75 K and 20 K in its presence. The time courses of fragmentation were essentially the same between both isozymes. These results demonstrated both these S1 isozymes are homogeneous with respect to heavy chain. Incidentally, alkali light chains were different in susceptilibity to tryptic digestion:i.e., A1 was partially cleaved, while A2 was not under the above conditions.

In the next place, hybrid S1 was constituted from rabbit S1 heavy chain and alkali light chains of tilapia and bigeye tuna. Hybrid S1 isozymes were then separated with reference to associating light chains by anion-exchange chromatography, and their ATPase activities were compared under various conditions. The results obtained showed that Ca-ATPase activity was very similar to that of rabbit S1(A1), suggesting that this ATPase activity might be mostly related to the heavy chain. However, kinetic values for actin-activated Mg-ATPase shifted to those of native S1 isozymes associating the respective alkali light chains. These results suggest that alkali light chains directly interact with actin.

Then, hybridization was performed between rabbit S1(A1) and A2, or between S1(A2) and A1, and the exchange rate of alkali light chains was examined by pyrophosphate native gel electrophoresis. The affinity for the heavy chain was proved to be about two-fold higher for A2 than for A1.

Chapter IV deals with the stability of S1 isozymes, and with the role of alkali light chains in stabilization of S1.

First of all, stabilities of S1 isozymes from tilapia and rabbit were compared under various denaturing conditions. S1 isozymes in 25 mM Tris-maleate (pH 8.0) were incubated at a given temperature, and denaturation rate constants (K_D) were calculated from the remaining Ca-ATPase activity. K_D values at 30°C were 57 x 10^{-5} and 34 x $10^{-5}s^{-1}$ for tilapia S1(A1) and S1(A2), respectively, and 24 x 10^{-5} and 14 x $10^{-5}s^{-1}$ for rabbit S1(A1) and S1(A2), respectively.

Heat-induced turbidity changes at 340 nm were examined at 35 and 40°C for tilapia and rabbit S1 isozymes, respectively. The increasing rate and maximum value were higher for S1(A1), regardless of animal species. The turbidity values of 1% S1 solution after 30 min-incubation ($T_{30}^{1\%}$) were 17.8 and 11.8 for tilapia S1(A1) and S1(A2), 13.3 and 8.5 for bigeye tuna S1(A1) and S1(A2), and 11.7 and 4.3 for rabbit S1(A1) and S1(A2), respectively. Tryptic digestibility was enhanced by heat treatment with S1(A1), but remained almost unchanged with S1(A2). Furthermore, resistibilities against methanol and urea were clearly higher for S1(A2) than for S1(A1).

S1 solutions, after heat treatment at due time intervals, were subjected to ultracentrifugation, and the supernatants were analyzed by SDS-PAGE. As a result, the heavy chain rapidly decreased during heat treatment, while light chains, especially A1, partially released from the heavy chain, remaining in the supernatant. These results suggest that dissociation of light chain is closely related to the denaturation of heavy chain.

In the next place, tilapia and rabbit S1 isozymes were incubated in the presence of 0.2 - 5 fold molar excess of A1 or A2 against S1 isozymes, and their effects on the thermostability of isozymes were examined. Alkali light chains inhibited heat-induced turbidity increase, and the effect of A2 was clearly stronger. The higher the amount of light chain added, the smaller the turbidity increase. For example, when rabbit A1 was added to S1(A1) at 0.2, 0.5 and 1 fold molar excess, $T_{30}^{1\%}$ values were decreased to 4.4, 1.4, and 0.6, respectively. However,

rabbit light chains showed little inhibitory effect on the denaturation of tilapia S1, suggesting the specificity of stabilization effect. From these results, it was strongly suggested that alkali light chains play an important role in stabilization of the myosin head.

Finally, in order to prove the stabilizing effect of alkali light chains in situ, stabilities of S1 isozymes was compared in the presence of actin, ATP, KCl, and various ions. The data obtained showed that at higher salt concentration, the interaction between A1 and actin disappeared, although the affinity between heavy chain and actin persisted. It was also suggested that A1 and A2 light chains differ from each other in binding for ATP as well as sensitivity for KCl.

In Chapter V, the results described in the preceding chapters are summarized and discussed collectively.

All the results obtained in the present study revealed that the structures and properties of fish myosin light chains resemble each other irrespective of light chain types and fish species, although they are remarkably species-specific as far as their molecular weights and the ratios in myosin are concerned. It was also demonstrated that alkali light chains are directly involved in actin-myosin interaction, but more possibly, they could be involved in stabilization of myosin head.

Contents of this thesis have been published or are now in press as follows:

1. Identification of 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) and alkali light chains of piscine myosin. Nippon Suisan Gakkaishi, **48**, 827-832 (1982) (in collaboration with S. Watabe and K. Hashimoto).

2. Isolation and characterization of myosin light chains from skipjack ordinary muscle. Nippon Suisan Gakkaishi, **50**, 1729-1733 (1984) (in collaboration with S. Watabe and K. Hashimoto).

3. Physicochemical and immunological properties of myosin light chains from the ordinary muscle of marine teleost fishes. Comp. Biochem. Physiol., **90B**, 347-353 (1988) (in collaboration with S. Watabe and K. Hashimoto).

4. Comparison of myosin light chains from shark ordinary and rabbit fast muscles. Nippon Suisan Gakkaishi, **54**, 1069 (1988) (in collaboration with C. J. Chow, S. Watabe and K. Hashimoto).

5. Role of alkali light chains in stabilization of fast skeletal myosin subfragment 1. Muscle Energetics (ed. R. Paul), Alan R. Liss Inc. (New York) (in press) (in collaboration with A. Handa, T. Kobayashi, S. Watabe and K. Hashimoto).

6. Involvement of essential light chains in maintenance of myosin head structure. J. Muscle Res. Cell. Motil. (in press) (in collaboration with T. Kobayashi, K. Miki, S. Watabe and K. Hashimoto).

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ABBREVIATIONS

A	:	Actin
A1	:	Alkali light chain 1
A2	:	Alkali light chain 2
BSA	:	Bovine serum albumin
DTNB	:	5,5'-Dithio-bis-2-nitrobenzoic acid
DTT	:	Dithiothreitol
EDC	:	1-Ethyl-3-(3-dimethylamino)propyl carbodiimide
EGTA	:	Ethylene glycol bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid
HC	:	Myosin heavy chain
HMM	:	Heavy meromyosin
IEF	:	Isoelectric focusing
K_D	:	Inactivation rate constant
K_m	:	Apparent dissociation constant
LC	:	Light chain
PBS	:	Phosphate buffered saline
PMSF	:	Phenylmethylsulfonyl fluoride
S1	:	Myosin subfragment 1
S1(A1)	:	S1 associating with A1
S1(A2)	:	S1 associating with A2
SDS-PAGE	:	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
T_{340}	:	Turbidity at 340 nm
$T_t^{1\%}$	:	T_{340} of 1% S1 solution after t min incubation
TBS	:	Tris buffered saline
V_{max}	:	Maximum velocity

CHAPTER I

LIGHT CHAIN COMPOSITION OF FISH MYOSIN

Myosin is generally composed of four light chains of molecular weight of about 20,000, and two heavy chains of that of 200,000 (Gazith *et al.*, 1970; Lowey and Risby, 1971; Yagi *et al.*, 1975). The fast muscle myosins so far reported have three kinds of light chain (Weeds and Baker, 1968; Horvath and Gaetjens, 1972), in contrast to slow or cardiac myosins which have only two kinds of light chain clearly different from those of fast myosins (Sarkar *et al.*, 1971; Weeds and Pope, 1971; Sreter *et al.*, 1972; Weeds and Frank, 1972). Similar relationships are observed among ordinary, dark and cardiac myosins of fish, respectively (Dinh *et al.*, 1985). Fast myosin light chains are classified into two categories : alkali light chains (A1 and A2) and DTNB light chain (Kominz *et al.*, 1959; Gaetjens *et al.*, 1968; Weeds, 1969; Gazith *et al.*, 1970; Lowey and Risby, 1971; Weeds and Pope, 1971). Amino acid sequence analysis indicates that A1 and A2 are encoded by homologous genes (Frank and Weeds, 1974; Barton and Buckingham, 1985). The molecular weight of DTNB light chain has so far been reported to be intermediate between those of A1 and A2.

The stability of fish myosin is generally much lower than that of mammalian myosin when measured using ATPase activity or aggregation rate as a parameter (Connell, 1960, 1961; Buttkus, 1966; Chung *et al.*, 1967; Watabe and Hashimoto, 1980). In addition, the stability of fish myosin is remarkably species-speci-

fic: e.g., tilapia and carp myosins were about 30 and 10 times, respectively, more stable than mackerel myosin at 30°C, when compared by ATPase activity. In this connection, it has been demonstrated by SDS-PAGE that fish myosins show remarkable species-specificity with respect to light chains (Seki, 1976). Thus, it is quite probable that myosin light chains are closely related to the stability and physiological functions of fish myosins. Studies along those lines have been, however, quite poor and fragmentary.

On the other hand, the structural and enzymatic properties of myosins from higher vertebrates such as rabbit and chicken, are known to change during development (Pelloni-Müller et al., 1976). Actually, A2 is completely or almost completely absent in embryonic myosins (Sreter et al., 1972; Syrový, 1979). At higher developmental stage, the content of A2 increases, along with the increase of myosin ATPase activity (Takahashi and Tonomura, 1975). However, little is known about the developmental changes of light chain composition in fish myosin.

In this chapter, attempts were made to purify myosins from fish ordinary muscle, to identify three kinds of light chains, and to compare their stoichiometry in respective myosins. In addition, developmental changes in light chain composition were followed.

Section 1

Species-specificity of Light Chain Composition

Myosins from fish ordinary muscle contain three kinds of light chains: alkali light chains A1, A2 and DTNB light chain (Focant and Huriaux, 1976; Huriaux and Focant, 1977; Watabe _et al_., 1983a). These myosins are remarkably species-specific with reference to the molecular weights of light chains (Focant _et al_., 1976; Seki, 1976; Perzanowska, 1977). Such species-specificity has been applied to identification of fish species in raw and cooked fish (Seki _et al_., 1980). However, identification of molecular species of light chains has not fully been performed except those from carp, trout and mackerel (Focant and Huriaux, 1976; Watabe _et al_., 1983a). On the other hand, the molar ratio of alkali light chains is dependent on animal species, while DTNB light chains are generally present at two moles per mole of myosin. In case of rabbit, A1/A2 ratio is 2:1, while it is almost 1:1 in case of chicken and frog (Lowey and Holt, 1972; Focant and Huriaux, 1979). Such unequimolar ratio is an indirect evidence for the existence of myosin isozymes with reference to alkali light chains. As far as fish myosins are concerned, however, no information is available on such non-stoichiometry of alkali light chains at present.

In this section, identification of A1, A2 and DTNB light chain was attempted with myosins from 19 fish species, and their molecular weights and stoichiometry were examined. In addition, two-dimensional mapping of each light chain on the coordinates of

molecular weight and isoelectric point was performed.

Materials and Methods

Materials

Fish species examined were as follows : mackerel *Pneumatophorus japonicus japonicus*, skipjack *Katsuwonus pelamis*, bonito *Auxis tapeinosoma*, bluefin tuna *Thunnus thynnus*, yellowfin tuna *T. albacares*, bigeye tuna *T. obesus*, round herring *Etrumeus micropus*, sardine *Sardinops melanosticta*, horse mackerel *Trachurus japonicus*, bigeye scad *Thachurops crumenophthalmus*, yellowtail *Seriola quinqueradiata*, threadfish *Alectis cilialis*, sea bass *Lateolabrax japonicus*, cutlassfish *Trichiurus lepturus*, pike conger *Muraenesox cinereus*, greenling *Hexagrammos otakii*, pufferfish *Fugu pardale*, grunt *Parapristipoma trilineatum*, filefish *Navodon modestus*, chum salmon *Oncorhynchus keta*, tilapia *Tilapia nilotica*, Atka mackerel *Pleurogrammus azonus*, carp *Cyprinus carpio*, and requiem shark *Triakis scyllia*. These fish samples were frozen with dry ice immediately after capture and kept at -40°C until use. The ordinary muscle was carefully excised from the dorsal part of each species, avoiding contamination of dark muscle. In addition, rabbit fast muscle was used for comparison.

Preparation of myosin light chains

The following procedures were performed at 0 - 4°C unless otherwise stated. Myosins were prepared by the method of Stafford *et al*. (1979), except that the ammonium sulfate fractionation was carried out between 40 - 50% instead of 40 - 70%

saturation. The whole, DTNB and alkali light chains were prepared from each myosin as described below.

Whole light chains To a portion of myosin solution was added an equal volume of 50 mM Tris-HCl (pH 8.0) containing 8 M urea, 10 mM EDTA and 10 mM dithiothreitol (DTT) according to Lowey and Holt (1972). The mixture was stirred at room temperature for 30 min. After adding 10 volume of ice-cold water, the mixture was centrifuged at 5,000 x g for 15 min. The resulting supernatant was used as "whole light chain fraction".

DTNB light chain To another portion of myosin solution was added an equal volume of 10 mM Tris-HCl (pH 8.5) containing 0.5 M KCl, 20 mM DTNB and 20 mM EDTA according to Focant and Huriaux (1976). The mixture was allowed to stand at 0°C for 10 min, and to it was added 10 volume of ice-cold water, and the mixture centrifuged. The supernatant obtained was used as "DTNB light chain fraction".

Alkali light chains The precipitate obtained at the last step in the DTNB light chain separation was used for preparing alkali light chains by the method of Gaetjens *et al*. (1968). The precipitate was allowed to stand at 0°C for 10 min in 0.1 M glycine-KOH (pH 11.1) containing 2 M LiCl and 1 mM DTT. To the solution was added neutralized 2.5 M potassium citrate to a final concentration of 0.8 M. The mixture was allowed to stand for another 10 min, and centrifuged to obtain "alkali light chain fraction".

SDS-PAGE

SDS-PAGE was performed according to Weber and Osborn (1969) using 10% tube gels (6 mm I. D. x 100 mm) containing 0.1% SDS.

The electrode buffer was 0.1 M sodium phosphate (pH 7.0) containing 0.1% SDS. Electrophoresis was performed at a constant current of 8 mA per tube for 4 h at room temperature. After the run, gels were stained with Coomassie brilliant blue R-250. Molecular weights of light chains were measured using bovine serum albumin, ovalbumin, chymotrypsinogen A, and cytochrome c (Schwarz/Mann) as standard protein markers.

The myosins prepared as above were electrophoresed to estimate molar ratios of respective light chains. After the run, gels were subjected to staining, followed by extensive destaining, and were scanned at 550 nm with a Gelman ACD-18 densitometer. Molar ratios of respective light chains were calculated using their molecular weights, on the assumptions that their staining intensities per unit weight match with each other, and that each myosin contains four light chains.

Two-dimensional gel electrophoreis

Two-dimensional gel electrophoresis was performed essentially according to Mikawa et al. (1981). Briefly, myosin was dissolved in 9 M urea, 2% Nonidet P-40 (Sigma), 2% Ampholine (LKB, [pH 3.5 - 10]:[pH 4 - 6] = 1:4), 2% β-mercaptoethanol, and applied to the first dimensional isoelectric focusing using 3% polyacrylamide capillary gels (0.75 mm I. D. x 60 mm). These gels were subsequently subjected to the second dimensional SDS-PAGE according to Laemmli (1970) using 15% polyacrylamide slab gels. Isoelectric points (pI) were determined using standard pI markers (Oriental Yeast Co.). Protein staining was performed as described above.

Results and Discussion

Identification of light chains

The fish myosins used contained some actin, tropomyosin, and troponin, as seen in electropherograms of their whole light chain fractions (Fig. I-1-1). However, simultaneous analyses of the DTNB and alkali light chain fractions made it possible to identify respective light chains. The results obtained are schematically shown in Fig. I-1-2. Three light chains were observed in most fish myosins, but only two in those of round herring, sardine, bigeye scad and horse mackerel. In these four species, DTNB and A2 light chains were expected to possess completely or almost the same mobilities under the electrophoretic conditions adopted. When the whole light chain fraction of horse mackerel was electrophoresed in the presence of 8 M urea according to Perrie and Perry (1970), three bands appeared, one of which was DTNB light chain with an intermediate mobility (data not shown). This was also true for the three other fishes. It follows that A2 and DTNB light chains of these four species have the indistinguishable molecular weight, though both light chains differ from each other in net charge.

The molecular weights of the light chains are shown in Table I-1-1. The molecular weights of DTNB and A1 light chains fell in a fairly narrow range of 17,500 - 21,000 and 25,000 - 30,000, respectively. Another alkali light chain A2 elicited a widest range of 13,500 (requiem shark) - 22,500 (skipjack).

The molecular weight of A2 was larger than that of DTNB light chain in mackerel, skipjack, bonito and bigeye tuna,

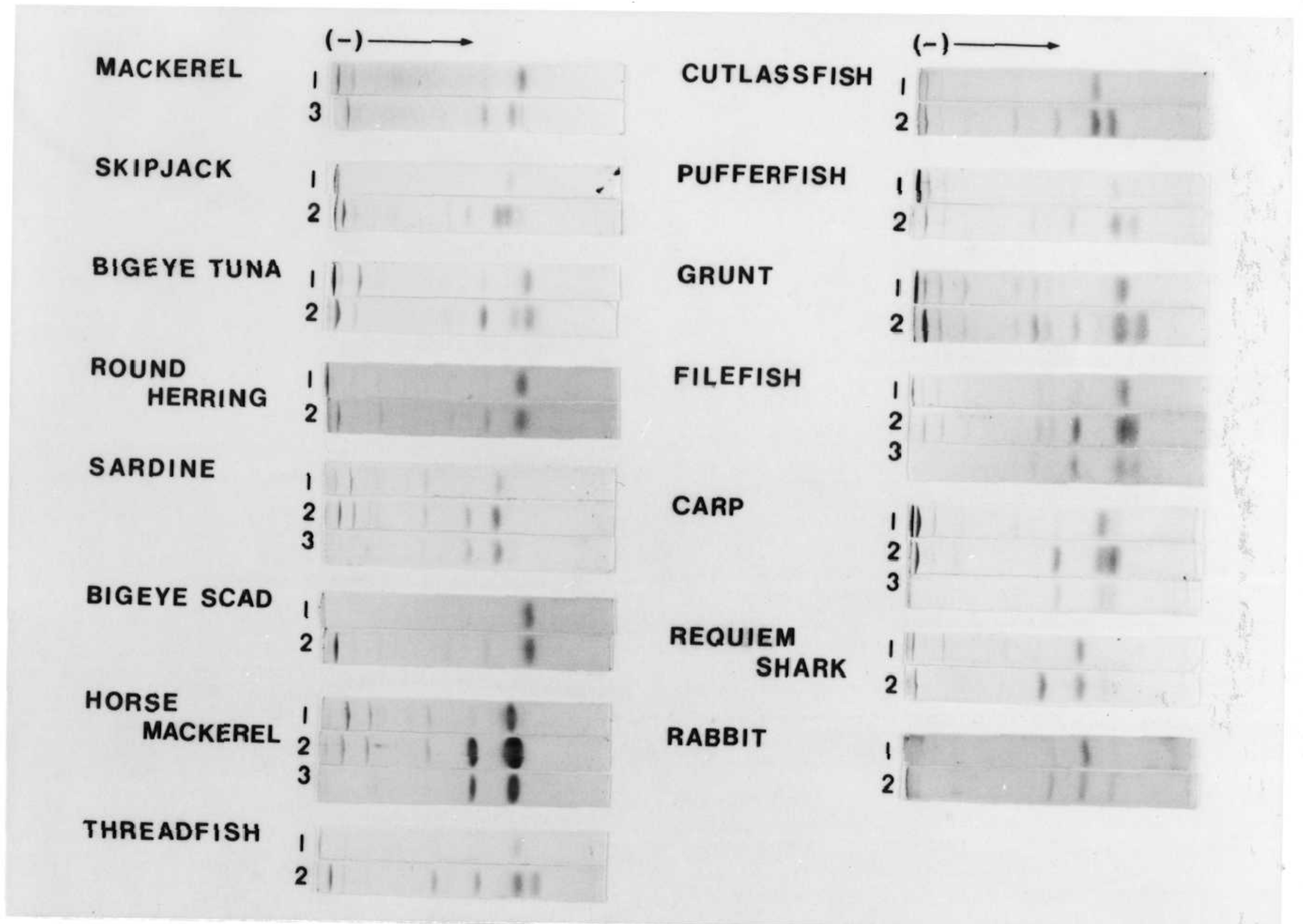


Fig. I-1-1. SDS-PAGE patterns of DTNB (1), whole (2), and alkali (3) light chain fractions from fish ordinary muscle myosins (10% gels).

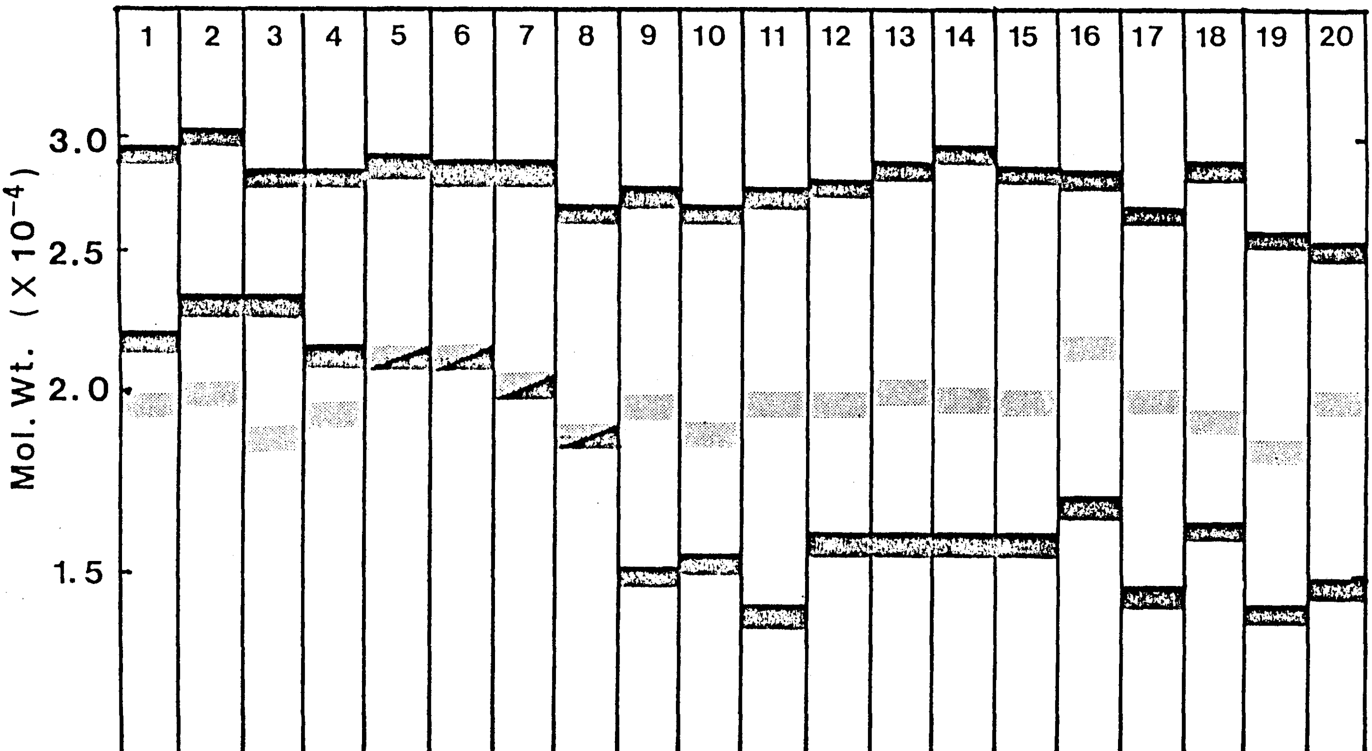


Fig. I-1-2. Schematic diagram of SDS-PAGE patterns of light chains from fish ordinary muscle myosins. Black and dotted bands represent alkali light chains (A1, A2) and DTNB light chains, respectively. 1, mackerel; 2, skipjack; 3, bonito; 4, bigeye tuna; 5, round herring; 6, sardine; 7, bigeye scad; 8, horse mackerel; 9, yellowtail; 10, threadfish; 11, sea bass; 12, cutlassfish; 13, pufferfish; 14, grunt; 15, filefish; 16, Alaska pollack; 17, Atka mackerel; 18, carp; 19, requiem shark; 20, rabbit.

Table I-1-1. Molecular weights of light chains from fish ordinary muscle myosins, as determined by SDS-PAGE

	Light chain	Mol. wt.		Light chain	Mol. wt.
Skipjack	A1	30,000	Sea bass	A1	27,000
	A2	22,500		DTNB	19,000
	DTNB	20,000		A2	13,500
Bonito	A1	27,500	Cutlassfish	A1	27,000
	A2	22,500		DTNB	19,000
	DTNB	18,000		A2	16,000
Bigeye tuna	A1	27,500	Pufferfish	A1	28,000
	A2	21,000		DTNB	19,000
	DTNB	19,000		A2	16,000
Mackerel	A1	29,000	Grunt	A1	28,500
	A2	21,500		DTNB	19,500
	DTNB	19,500		A2	16,000
Round herring	A1	28,000	Filefish	A1	28,000
	DTNB	21,000		DTNB	19,000
	A2	21,000		A2	16,500
Sardine	A1	28,000	Alaska pollack	A1	28,000
	DTNB	21,000		DTNB	19,000
	A2	21,000		A2	15,000
Bigeye scad	A1	28,000	Atka mackerel	A1	26,000
	DTNB	20,000		DTNB	19,000
	A2	20,000		A2	14,000
Horse mackerel	A1	26,000	Carp	A1	27,500
	DTNB	18,000		DTNB	18,000
	A2	18,000		A2	16,500
Yellowtail	A1	27,000	Requiem shark	A1	25,500
	DTNB	19,000		DTNB	17,500
	A2	15,000		A2	13,500
Threadfish	A1	26,500	Rabbit	A1	25,000
	DTNB	18,000		DTNB	18,500
	A2	15,000		A2	14,000

whereas all the known DTNB light chains fall between the two alkali light chains. All fishes possessing A2 which is larger than or equal to DTNB light chain in molecular weight, are migratory fishes, indicating that there may exist some relationship between the molecular weight of A2 and the locomotory activity of fish. Accordingly, fish myosins can be classified into the following three types based on the molecular weights of A2 and DTNB light chain : **I**, A2 > DTNB light chain; **II**, A2 = DTNB light chain; **III**, A2 < DTNB light chain.

Stoichiometry of light chains

Attempts were made to examine the light chain composition of these myosins. Fish myosins contained about 2 moles of DTNB light chain per mole as has already been reported for rabbit fast muscle myosin (Table I-1-2 and Fig. I-1-3). The remaining two moles were accounted for by A1 and A2. However, their relative ratios in myosin molecule fluctuated very widely among the fishes tested. The molar ratios of A1 to A2 were in a range from 0.1 (grunt) to 2.6 (requiem shark). It seems interesting from the viewpoint of comparative biochemistry that the light chains of requiem shark and rabbit myosins resembled each other not only in molecular weights but also in their molar ratios. Therefore, further analyses of light chains were performed by two-dimensional gel electrophoresis.

Mapping on two-dimensional coordinates

Electropherograms of myosin light chains on two-dimensional gel electrophoresis are shown in Fig. I-1-4a and b. The patterns were significantly species-specific. The spots of three

Table I-1-2. Light chain composition of fish ordinary muscle myosins

	Moles per mole of myosin			A1/A2
	DTNB-LC	A1	A2	
Skipjack	2.2	0.6	1.2	0.5
Bigeye tuna	2.4	0.9	0.7	1.3
Mackerel	2.4	0.5	1.1	0.4
Threadfish	2.5	0.6	1.5	0.4
Cutlassfish	1.9	0.6	1.5	0.4
Grunt	2.0	0.1	1.9	0.1
Pufferfish	2.2	0.6	1.2	0.5
Filefish	2.4	0.7	0.9	0.8
Carp	2.3	0.8	0.9	0.9
Requiem shark	2.2	1.3	0.5	2.6
Rabbit	2.0	1.4	0.6	2.3

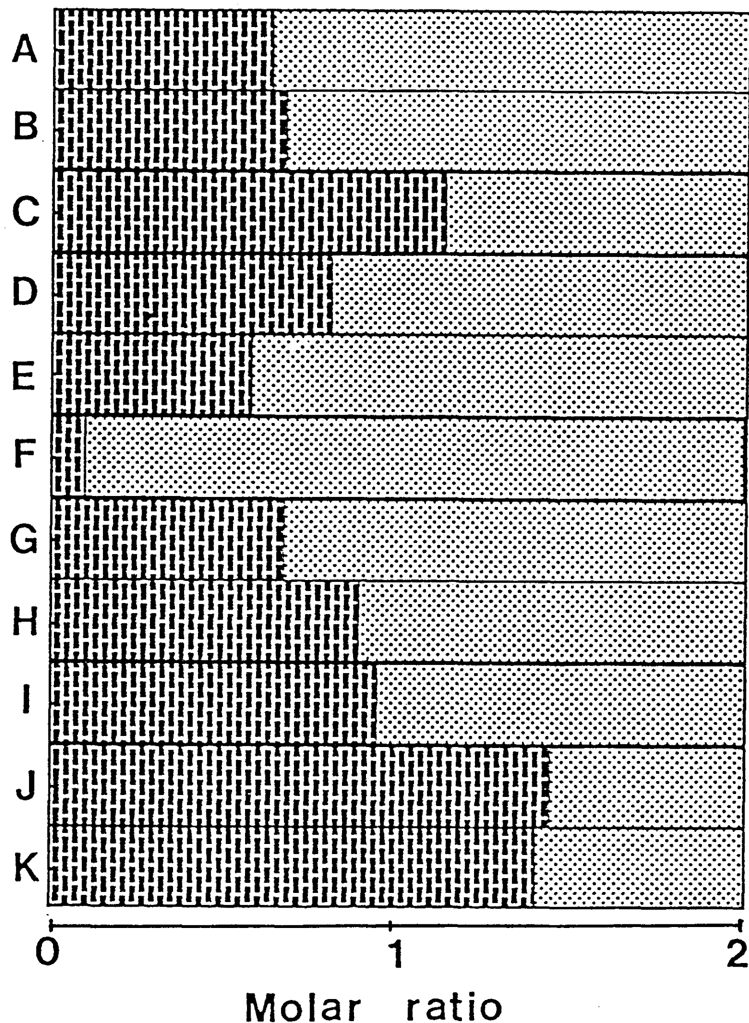


Fig. I-1-3. Alkali light chain composition of fish ordinary muscle myosins. Brick and dotted regions represent A1 and A2, respectively. Molar ratio was determined on the assumption that two moles of alkali light chains are contained per mole of myosin. A, mackerel; B, skipjack; C, bigeye tuna; D, threadfish; E, cutlassfish; F, grunt; G, pufferfish; H, filefish; I, carp; J, requiem shark; K, rabbit.

kinds of light chain from scombrid fishes, especially those from tunas, were overlayed with each other, while the isoelectric point of mackerel A1 to some extent shifted to the alkaline side (Fig. I-1-4b). Light chains from other fishes showed species-specific patterns, although the corresponding light chains (A1, A2 and DTNB light chain) showed similar molecular weights and isoelectric points.

As described above, myosin light chains from requiem shark and rabbit were similar in molecular weight and composition. When these myosins were subjected to two-dimensional gel electrophoresis, the corresponding light chains of both animals were separated clearly, though their isoelectric points were quite similar.

The isoelectric points and molecular weights as determined from the electropherograms were plotted on the same coordinate (Fig. I-1-5). A1 light chains of the fish myosins as well as rabbit myosin showed a molecular weight range of 21,000 - 22,900 and an isoelectric point range of 4.51 - 4.62. DTNB light chains were spotted in quite narrow ranges, 16,800 - 17,600 and 4.48 - 4.55, respectively. On the other hand, a remarkable species-specificity was recognized with A2 light chains, with a molecular weight and isoelectric point in the ranges of 14,000 - 19,500 and an isoelectric point range of 4.31 - 4.46. Sardine light chains were unique in that A1 and A2 showed the most alkaline and acidic isoelectric points, respectively, among the fish species examined. Such unique light chain composition might be related either to the higher locomotory activity of this

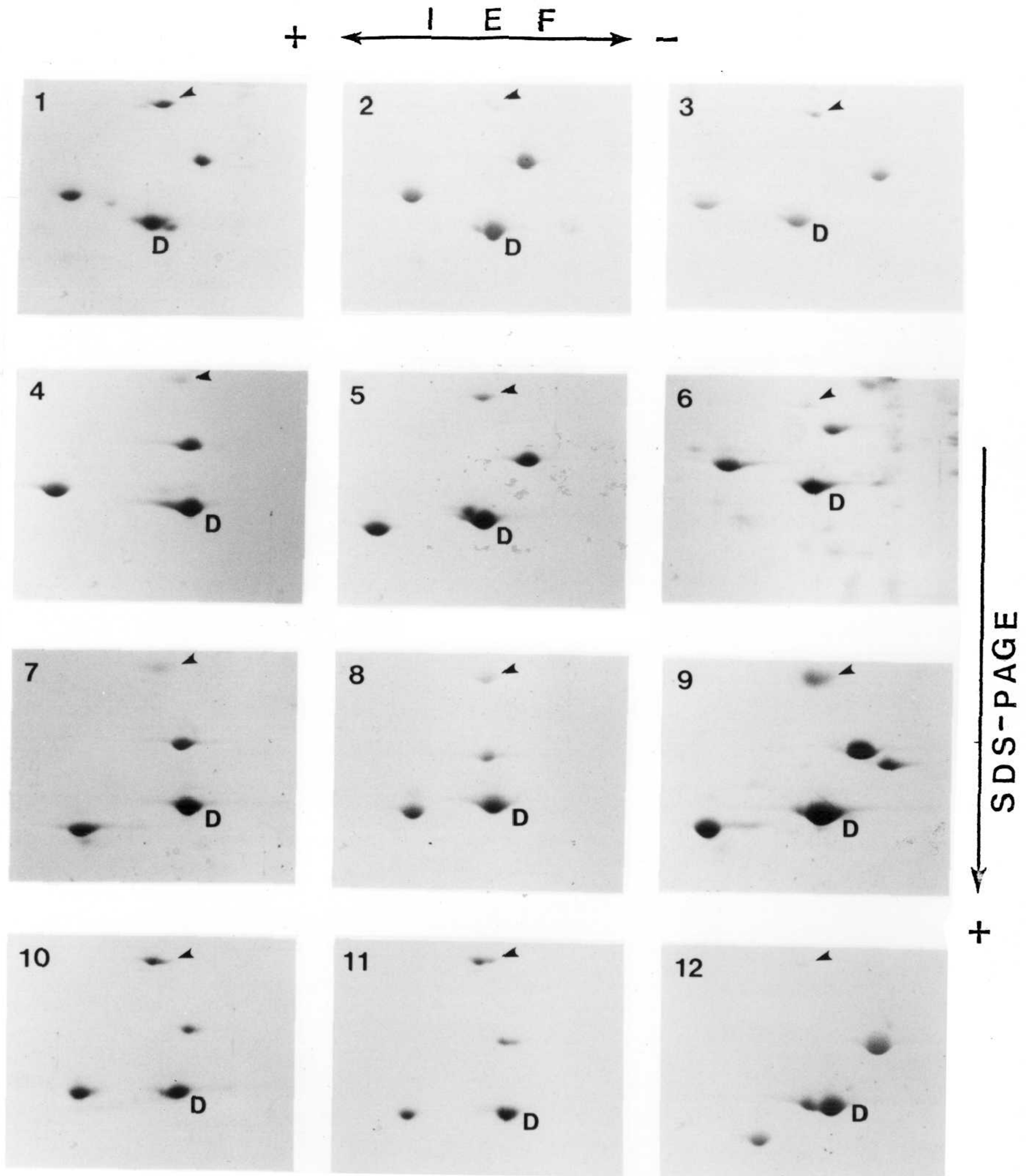


Fig. I-1-4a. Two-dimensional gel electrophoretic patterns of myosin light chains. D and IEF stand for DTNB light chain and isoelectric focusing, respectively. The arrowhead indicates actin. 1, skipjack; 2, bluefin tuna; 3, sardine; 4, horse mackerel; 5, yellowtail; 6, saury pike; 7, pike conger; 8, Atka mackerel; 9, chum salmon; 10, tilapia; 11, carp; 12, rabbit.

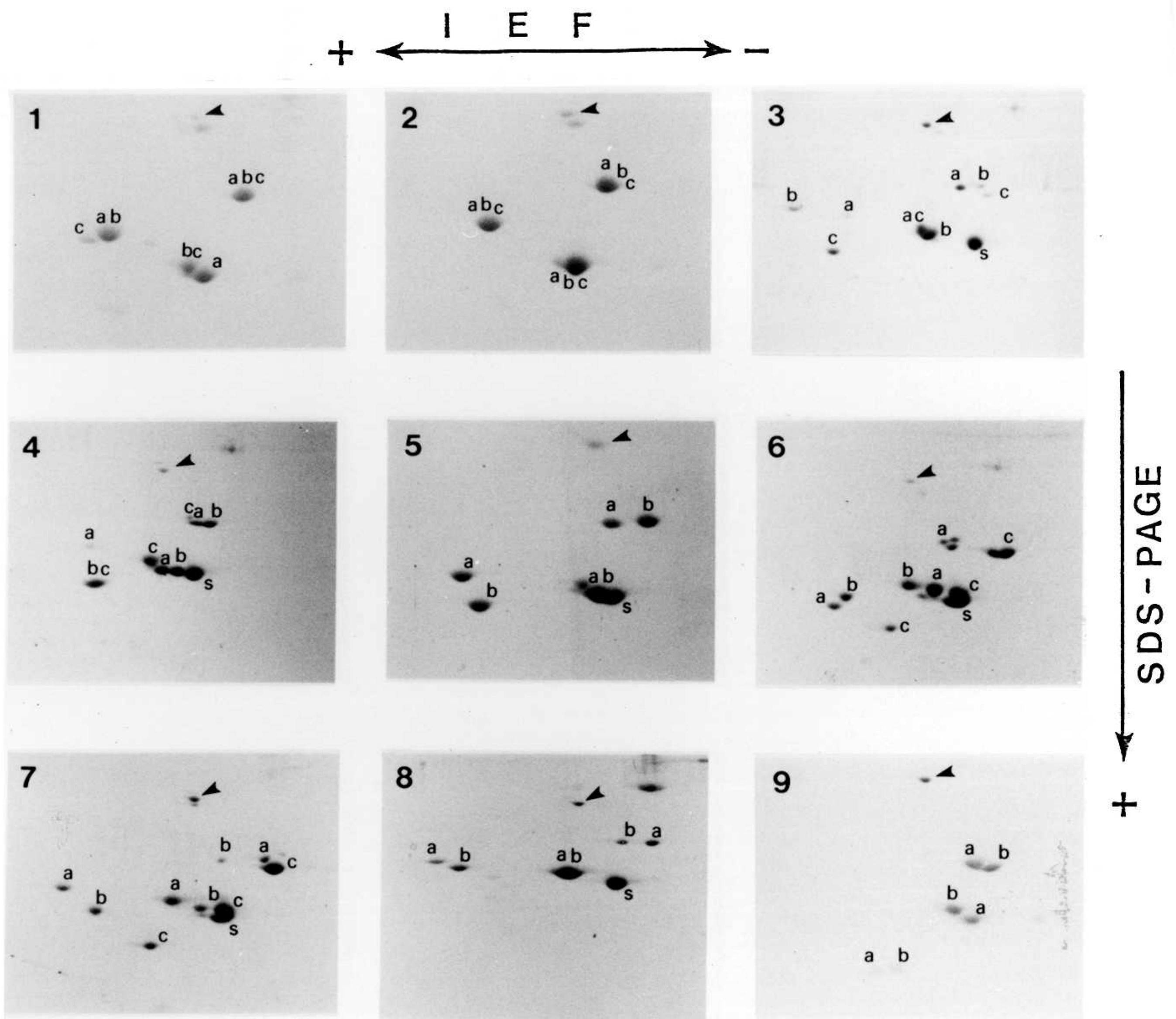


Fig. I-1-4b. Two-dimensional gel electrophoretic patterns of myosin light chains. IEF stands for isoelectric focusing. The arrowhead indicates actin. The letter "s" represents soybean trypsin inhibitor as a marker protein (mol. wt. 17,000, pI 4.55). 1, skipjack (a), bluefin tuna (b), and mackerel (c); 2, bluefin (a), yellowfin (b) and bigeye (c) tunas; 3, bluefin tuna (a), round herring (b), and chum salmon (c); 4, bluefin tuna (a), yellowtail (b), and pufferfish (c); 5, horse mackerel (a) and yellowtail (b); 6, pike conger (a), Atka mackerel (b), and rabbit (c); 7, sardine (a), carp (b), and rabbit (c); 8, sardine (a) and round herring (b); 9, requiem shark (a) and rabbit (b).

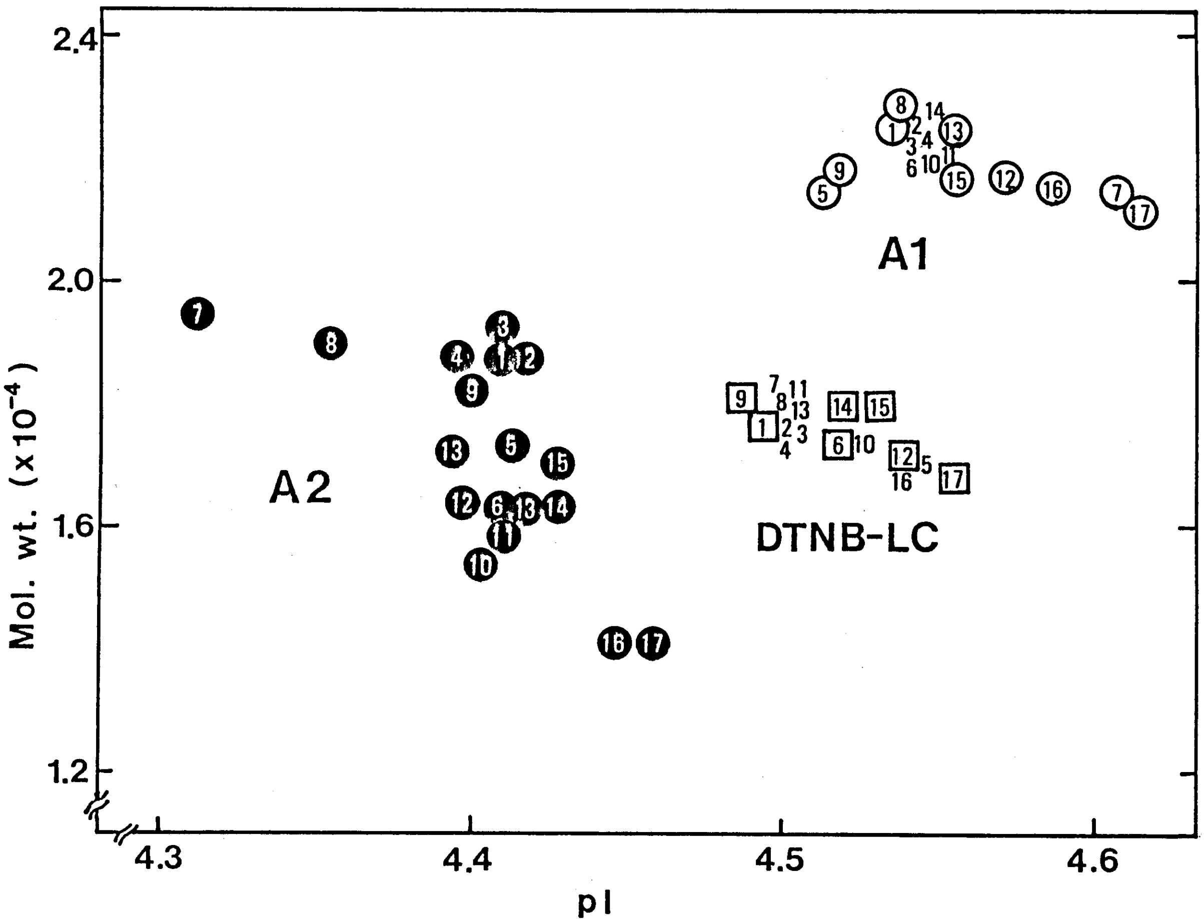


Fig. I-1-5. Two-dimensional mapping of myosin light chains on the coordinates of molecular weight and isoelectric point (pI). Open circle, square and closed circle represent A1, DTNB light chain, and A2, respectively. 1, bluefin tuna; 2, skipjack; 3, bonito; 4, mackerel; 5, horse mackerel; 6, yellowtail; 7, sardine; 8, round herring; 9, saury pike; 10, pike conger; 11, greenling; 12, pufferfish; 13, chum salmon; 14, trout; 15, tilapia; 16, requiem shark; 17, rabbit.

fish or to intensive phylogenetical differentiation of this fish. The molecular weights were slightly different from those determined by Weber-Osborn's method (Table I-1-1), probably because of differences in electrophoretic condition.

Frank and Weeds (1974) and Matsuda et al. (1980) elucidated the primary structures of alkali light chains from rabbit and chicken, and found that A1 and A2 differ only in "difference peptide" consisting of 41 residues at the N-terminal of A1. This peptide is rich in several amino acids such as lysine, proline and alanine. Especially, the higher content of lysine might be the reason for the most alkaline isoelectric point of A1 among the three light chains. It is quite strange that A1 light chains were spotted in quite a narrow range, although A2 light chains were dispersed on the coordinates. The difference peptide of A1 seems to be closely related to properties of this light chain in fish myosins, since the remarkable specificity of A2 is quenched by addition of this peptide. It is very probable that A1 and A2 possess different roles in muscle contraction.

Section 2

Developmental Changes in Light Chain Composition

As to myosins from higher vertebrates, profuse information is available on developmental and differential changes in their structural and enzymatic properties (Holland and Perry, 1969; Dow and Stracher, 1971; Sreter *et al*., 1975; Pelloni-Müller *et al*., 1976; Takano-Ohmuro *et al*., 1982). Actually, embryonic myosin contains smaller amounts of A2 (Sreter *et al*., 1975; Takahashi and Tonomura, 1975; Takahashi, 1976; Dabrowska *et al*., 1977) and shows lower ATPase activity than adult myosin (Trayer and Perry, 1966; Obinata, 1969). In contrast, slow muscle myosins remain virtually unchanged from birth to maturity in their light chain composition (Pelloni-Müller *et al*., 1976). In immunochemical properties (Obinata *et al*., 1976) and tryptic digestion pattern (Sreter *et al*., 1975), embryonic myosin resembles the slow skeletal myosin, though, at alkaline pH, the former myosin is as stable as fast muscle myosin.

Dow and Stracher (1971) found by SDS-PAGE that the whole muscle myosin from 16-day chick embryo showed two light chain bands, as did adult slow muscle myosin. The molecular weights of light chains from fetal fast muscle myosin were the same as those of adult fast myosin, but different from those of adult slow myosin (Takahashi and Tonomura, 1975). On the other hand, Sreter *et al*. (1972) reported that three light chains were detected in 16-day embryo myosin as in adult fast myosin, although A2 was always present in a smaller amount. The ATPase

activity of fetal fast myosin was about 50% that of adult myosin, though pH-activity curve of fetal myosin ATPase was similar to that of adult myosin (Takahashi and Tonomura, 1975).

Sreter et al. (1972) found that Ca-ATPase activity of myosin from cultivated myotubes was somewhat lower than that of myosin from 16-day embryonic breast or leg muscle. As development proceeded, A2 content in myosin increased with the concomitant increase of myosin ATPase activity (Takahashi and Tonomura, 1975).

In this connection, Buller et al. (1960) and Close (1972) showed that the contraction speed of mammalian fast muscle is slow at birth and gradually increases to the adult level during the early post-natal period. In addition, Drachman and Johnston (1973) reported that the change in contraction speed of fast muscle during the post-natal period was closely related to the increase in actomyosin ATPase activity. Incidentally, embryonic myosin does not contain 3-methylhistidine, while adult myosin does (Trayer et al., 1968).

As for fish myosin, Perzanowska (1979) examined myosins from the adult and embryo of trout Salmo trutta, and found that three kinds of light chains were present in embryonic myosins at different stages and that little electrophoretic changes occurred during development. However, no other information is available on developmental changes of fish myosin.

Under these circumstances, attempts were made to examine the subunit composition of myosins from embryonic (or fetal) and adult stages of chum salmon Oncorhynchus keta and tilapia Tilapia nilotica.

Materials and Methods

Materials

Chum salmon and tilapia at different stages were used as materials (Tables I-2-1 and I-2-2, and Figs. I-2-1 and I-2-2). Those specimes were stored at -80°C for not longer than one month.

Preparation of myosin

Myosin was prepared from the ordinary muscle or the decapitated specimens, by the method described in Section 1.

SDS-PAGE

SDS-PAGE was performed according to Laemmli (1970) using 10 - 20% polyacrylamide gradient gels of 0.5 mm thickness. Fifty and five microgram portions of myosin at each stage were applied to SDS-PAGE for protein staining and immunoblotting, respectively.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed as described in Section 1.

Immunoblotting

Anti-skipjack A1 antiserum was used. As for preparation of the antiserum, refer to **Materials and Methods** in Section 3 of Chapter II.

Immunoblotting was performed as shown in Scheme I-2-1. Briefly, an electrophoresed SDS-gel was overlayed on a Millipore GVHP membrane pre-treated with absolute methanol and blotting buffer (25 mM Tris-192 mM glycine, 0.1% SDS, 20% methanol). Transfer was carried out in this buffer at a constant current of

Table I-2-1. Specimens examined (chum salmon *Oncorhynchus keta*)

Stage No.	Length(cm)	Stage
1	1.8	(before hatching)
2	2.0	(〃)
3	3.0	(after hatching)
4	120	(adult)

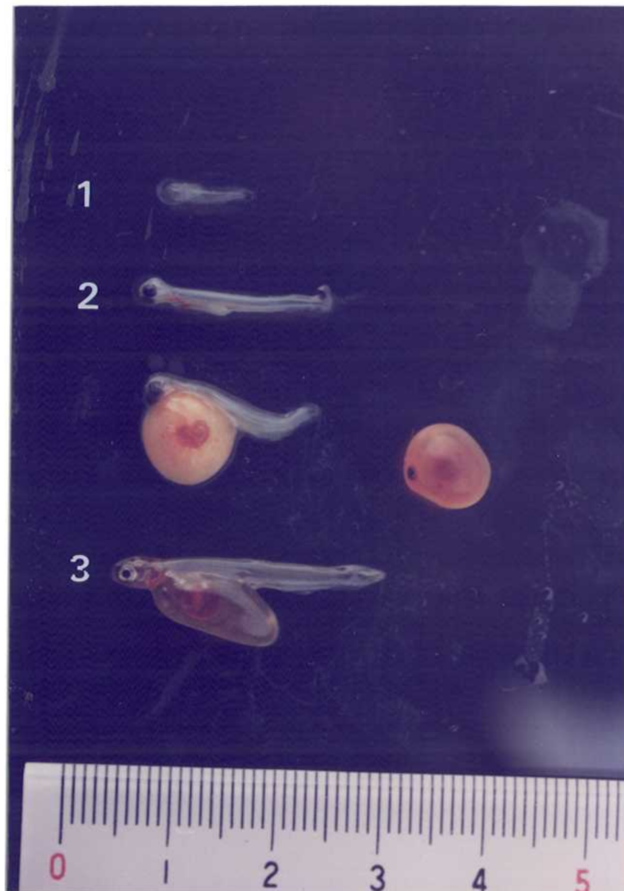


Fig. I-2-1. Chum salmon specimens at various stages. The numbers represent the stage Nos. in Table I-2-1.

Table I-2-2. Specimens examined (tilapia _Tilapia nilotica_)

Stage No.	Length(cm)	Weight(g)
1	1.7	0.1
2	2.5	0.3
3	3.0	0.5
4	5.0	3.5
5	9.0	11.0
6	15.0	60.0
7	35.0	300

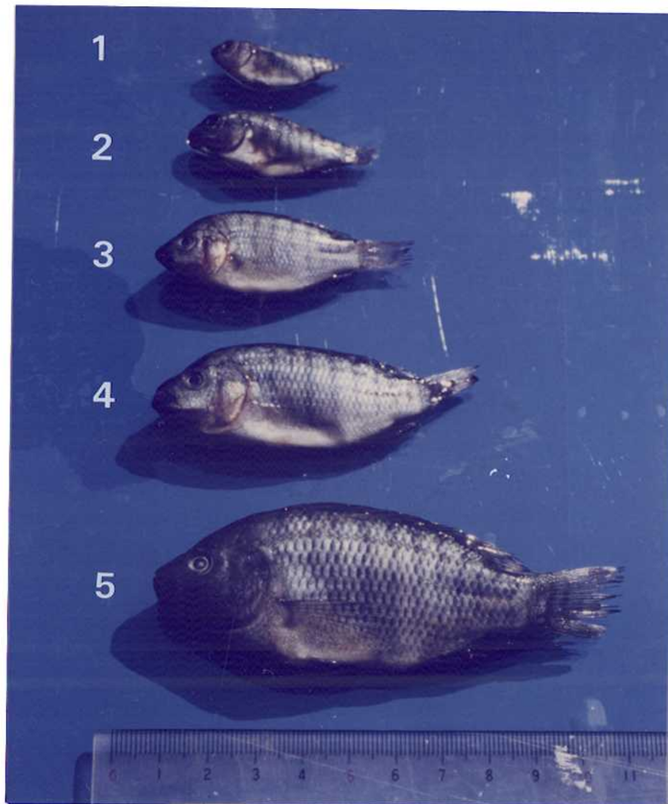


Fig. I-2-2. Tilapia specimens at various stages. The numt represent the stage Nos. in Table I-2-2.

Electrophoresed gel

- Overlayed on Millipore GVHP membrane and fixed firmly
- Transferred at a constant current of 0.2 A at 4°C for 1 h in 25 mM Tris-glycine (pH 8.3) containing 0.1% SDS and 20% methanol

(Transferred membrane)

- Treated with TBS (50 mM Tris-HCl, pH 8.0, containing 0.15 M NaCl) containing 2% BSA at room temperature for 1 h
- Washed with TBS containing 0.1% BSA at room temperature for 1 h
- Incubated in TBS containing 0.1% BSA and anti-skipjack A1 rabbit antiserum (500 - 1,000 fold diluted) at room temperature for 2 h
- Washed with ice-cold TBS for 5 min (repeated 3 times)
- Incubated in TBS containing 0.1% BSA and peroxidase-conjugated anti-rabbit IgG goat Fab (3,000 fold diluted) at room temperature for 1 h
- Washed with ice-cold TBS for 5 min (repeated 4-5 times)
- Color-developed in TBS containing 0.05% 3,3'-diaminobenzidine and 0.05% H_2O_2
- Washed with ice-cold TBS and then with distilled water

Immunoblotting pattern

Scheme I-2-1. Procedure of immunoblotting.

0.2 A for 1 h using a Trans-Blot Module of Mini-Protean II (Bio-Rad). After the transfer, the membrane was treated with Tris-buffered saline (TBS, 50 mM Tris-HCl, 0.15 M KCl, pH 8.0) containing 2% bovine serum albumin (BSA) at room temperature for 1 h, and then washed with TBS containing 0.1% BSA at room temperature for another 1 h. The washed membrane was then incubated in TBS containing 0.1% BSA and 500 - 1,000 fold diluted rabbit antiserum (first antibody) at room temperature for 2 h, and washed with ice-cold TBS three times. The membrane was incubated in TBS containing 0.1% BSA and horseradish peroxidase-conjugated anti-rabbit immunoglobulin G goat Fab (second antibody, 3,000 fold diluted) at room temperature for 1 h, and washed with ice-cold TBS 4 - 5 times.

Color development was performed in ice-cold TBS containing 0.05% 3,3'-diaminobenzidine (DAB) and 0.05% hydrogen peroxide, and was terminated by immersing the membrane in ice-cold TBS.

Results and Discussion

Developmental changes in SDS-PAGE pattern of chum salmon myosin are shown in Fig. I-2-3. Three kinds of light chains were detected irrespective of developmental stage. As shown in Table I-2-3, the molecular weights of A1, DTNB light chain and A2 were 20,500, 15,500 and 13,500, throughout all the stages. The molar ratio of alkali light chains (A1/A2) increased from 0.44 to 0.72, as a result of decrease of A2 light chain.

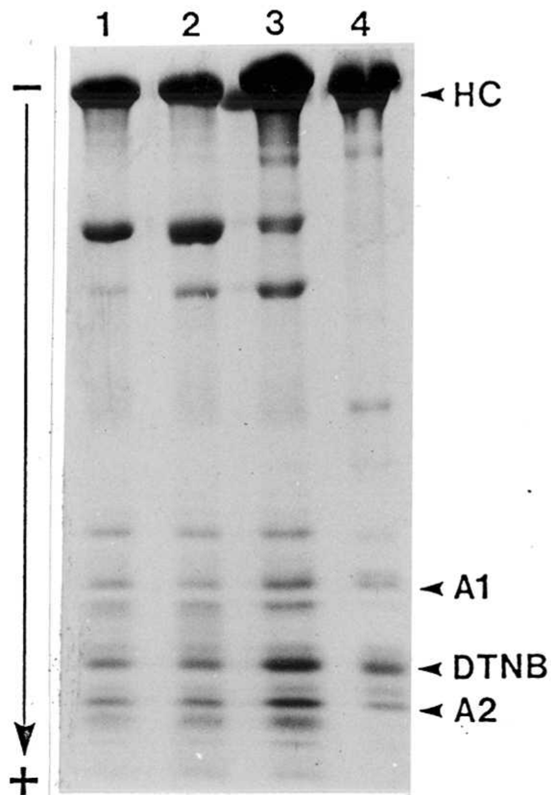


Fig. I-2-3. SDS-PAGE patterns of myosins from chum salmon at various stages. The numbers correspond to the stage Nos. in Table I-2-1.

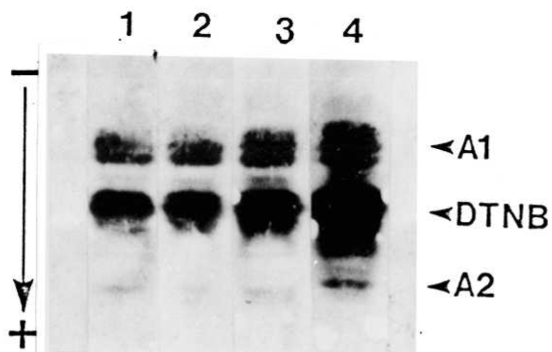


Fig. I-2-4. Immunoblotting patterns of myosin light chains from chum salmon at various stages. The numbers represent the stage Nos. in Table I-2-1.

Table I-2-3. Molecular weight and stoichiometry of myosin light chains released from chum salmon at various stages

Stage No.	LC	Mol. Wt.	Moles per myosin	A1/A2
1	A1	20,500	0.81	0.44
	DTNB	15,500	1.36	
	A2	13,500	1.83	
2	A1	20,500	0.86	0.61
	DTNB	15,500	1.73	
	A2	13,500	1.41	
3	A1	20,500	0.83	0.69
	DTNB	15,500	1.97	
	A2	13,500	1.20	
4	A1	20,000	0.63	0.72
	DTNB	15,500	2.49	
	A2	13,500	0.88	

In the next place, immunological cross-reactivity was examined by immunoblotting using anti-skipjack A1 rabbit antiserum, because fish myosin light chains showed a strong cross-reactivity irrespective of fish species, as described in Chapter II. As shown in Fig. I-2-4, the reactivity of A1 increased with the procession of developmental stage, suggesting that the molar ratio of this light chain increased during development. On the other hand, cross-reactivity was hardly recognized for A2 light chain. At earlier stages than stage No.1, myosin might be lacking A1. In case of chum salmon, however, it was impossible to prepare myosins from the earlier stages.

When chum salmon myosin was analyzed by two-dimensional gel electrophoresis, two spots appeared in the region of A1, and the ratio of higher molecular weight increased, along with the procession of development (Fig. I-2-5). This suggests the existence of both adult and embryonic forms of A1. However, changes in molecular weight and isoelectric point were hardly recognized for all the light chains.

Developmental changes in SDS-PAGE patterns of tilapia myosin are shown in Fig. I-2-6. Fetal myosins were different from adult counterparts in that A1 was not detected and that the molecular weight of DTNB light chain was smaller than that of adult form. The molecular weights of A2 and DTNB light chains were 19,500 and 17,500, respectively, and the molar ratios of both light chains were about 1:1 through stage Nos. 1 - 3 (data not shown).

Through the stage Nos. 4 - 7, the molecular weights of all light chains remained unchanged (Table I-2-4). The molar ratio

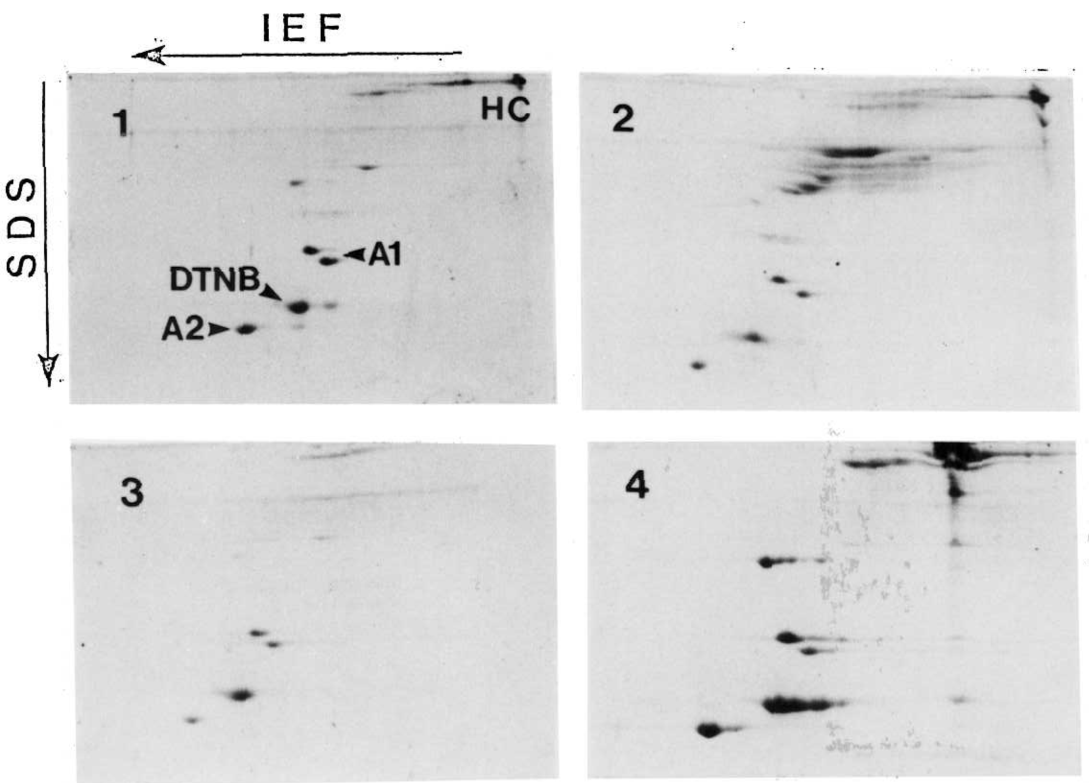


Fig. I-2-5. Two-dimensional gel electrophoretic patterns of myosins from chum salmon at various stages. The numbers correspond to the stage Nos. in Table I-2-1.

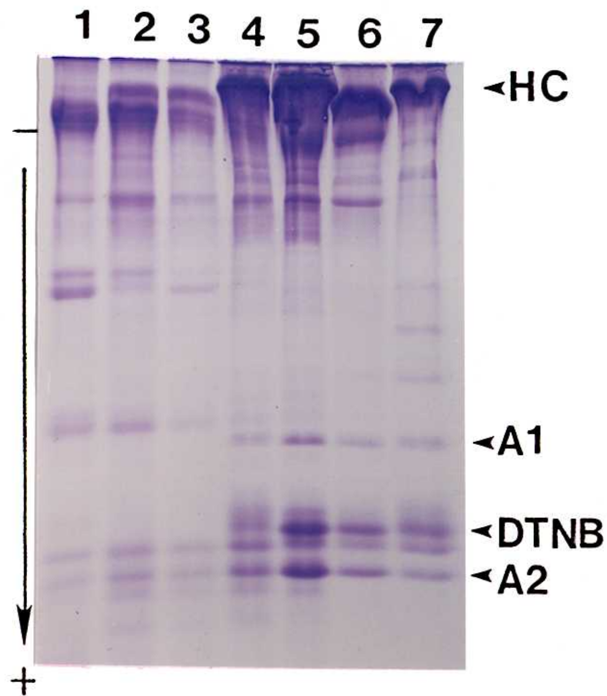


Fig. I-2-6. SDS-PAGE patterns of myosins from tilapia at various stages. The numbers represent the stage Nos. in Table I-2-2.

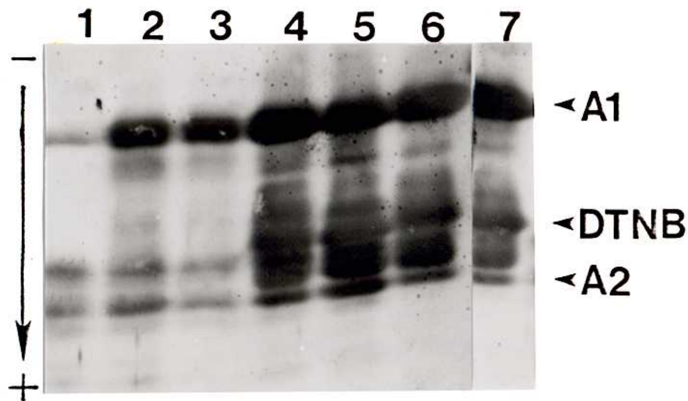


Fig. I-2-7. Immunoblotting patterns of myosin light chains from tilapia at various stages. The numbers represent the stage Nos. in Table I-2-2.

Table I-2-4. Molecular weight and stoichiometry of myosin light chains from tilapia at various stages

Stage No.	LC	Mol. Wt.	Moles per myosin	A1/A2
	A1	27,000	0.60	
4	DTNB	20,500	1.68	0.35
	A2	17,500	1.72	
	A1	27,000	0.67	
5	DTNB	20,500	1.78	0.43
	A2	17,500	1.55	
	A1	27,000	0.66	
6	DTNB	20,500	2.03	0.50
	A2	17,500	1.31	
	A1	27,000	0.68	
7	DTNB	20,500	2.04	0.53
	A2	17,500	1.28	

of alkali light chains (A1/A2) tended to increase during development. When immunoblotting patterns were compared, the reactivity of A1 clearly increased during development (Fig. I-2-7). A small amount of A1 was detected in fetal myosins at stage Nos. 1 - 3 by the immunoblotting technique. The isoelectric point of each light chain did not change during development, as demonstrated by two-dimensional gel electrophoresis (data not shown).

Syrový (1979) pointed out that, during development, the amount of A1 decreased in logissimus dorsi muscle of rat or rabbit, whereas the amount of A2 increased. In the present study, the total amount of A1 and A2 was almost constant throughout all the stages. Dabrowska et al. (1977) demonstrated in rabbit fast muscle that A2 is synthesized later than A1. However, this seems not to be the case with fish myosin. They also claimed that fetal myosin was heavily contaminated with histone which made it difficult to determine myosin light chains. No histone-like band was, however, detected in the present preparation of myosin, as revealed by two-dimensional gel electrophoresis. Takahashi (1976) reported that presence of a smaller amount of A2 in embryonic myosin was due to its inability to bind with embryonic heavy chain. This is an indirect evidence for the developmental changes in heavy chain structure. On the other hand, myosin ATPase activity is closely related with the increase of A2 content (Takahashi and Tonomura, 1975). In the present experiment, however, ATPase activity was not measured, because of scarcity of myosin available, especially at earlier developmental stages.

Summary

Myosins were purified from the ordinary muscle of various species of fish, and analyzed for light chain subunits by SDS-PAGE. Molecular weights were in a range of 17,500 - 21,000 for DTNB light chain, 25,000 - 30,000 for A1, and 13,500 - 22,500 for A2 light chain, respectively. In most myosins, DTNB light chain showed an intermediate molecular weight of A1 and A2. As for scombrid fishes examined, however, the molecular weight of A2 was clearly larger than that of DTNB light chain. As for sardines and horse mackerel, both light chains showed the same molecular weights. Quantitative analysis showed that each fish myosin contained approximately two moles each of DTNB light chain and alkali light chains per mole. However, the ratios of A1/A2 differed widely, from 0.1 to 2.6.

When these myosins were analyzed by two-dimensional gel electrophoresis, corresponding light chains were overlayed with each other between closely related species. However, A2 light chains were located in a wide region, while A1 and DTNB light chains in quite a narrow region.

In addition, developmental changes in light chain composition were examined on chum salmon and tilapia myosins. In both species, embryonic or fetal myosins possessed three kinds of light chains whose molecular weights and isoelectric points were similar to those of adult myosin. A1 content gradually increased, whereas A2 content decreased.

CHAPTER II

ISOLATION AND CHARACTERIZATION OF MYOSIN LIGHT CHAINS

Skeletal muscle myosin is composed of two heavy chains of a molecular weight of about 200,000, and four light chains of about 20,000 (Gaetjens *et al.*, 1968; Gazith *et al.*, 1970; Weeds and Lowey, 1971; Mannherz and Goody, 1976). Myosin light chains from fast skeletal muscle are classified into two groups :*i. e.*, alkali light chains, and DTNB light chain (Gaetjens *et al.*, 1968; Gazith *et al.*, 1970). The former consists of two heterologous components, A1 and A2, in the order of decreasing molecular weight (Gaetjens *et al.*, 1968). The primary structures of A1 and A2 from rabbit or chicken are highly homologous, differing only in "difference peptide" of N-terminal 41 residues in A1 (Frank and Weeds, 1974; Matsuda *et al.*, 1980). This observation indicated that these two protein isoforms are encoded by a single gene, differentially spliced to give the specific N-termini (Barton and Buckingham, 1985).

The myosins from fish ordinary muscle also possess four light chains of three different molecular weights (Watabe *et al.*, 1983a). As has been established in the previous chapter, fish myosin light chains consist of two alkali light chains and DTNB light chain, just like those of higher vertebrates, although a remarkable species-specificity in SDS-PAGE patterns was observed. As far as fish myosin light chains are concerned, several physicochemical properties such as amino acid profile have been reported on those from carp (Focant and Huriaux, 1976;

Huriaux and Focant, 1977), trout (Perzanowska *et al*., 1978), pike (Focant and Huriaux, 1976), and mackerel (Watabe *et al*., 1983a). Immunochemical cross-reactivity has also been examined on myosin light chains from mackerel ordinary, dark and cardiac muscles (Watabe *et al*., 1983a; Dinh *et al*., 1985) and from some fresh-water fishes (Huriaux and Focant, 1985). Based on the data so far reported, fish myosin light chains seem to have similar structures to the corresponding ones from higher vertebrates, although the primary structures of fish myosin light chains have not yet been determined. Therefore, the structural differences responsible for such species-specificity remain to be elucidated.

In the previous chapter, it was found that myosin light chains from fish ordinary muscle can be classified into three types, based on the molecular weights of A2 and DTNB light chains, with a possible relationship between the molecular weight of A2 light chain and the locomotory activity of fish. In this chapter, attempts were made to isolate myosin light chains from the ordinary muscle of several marine fishes, and to compare their physicochemical and immunological properties.

Section 1

Isolation of Light Chains

Myosin light chains have been isolated from higher vertebrates such as rabbit and chicken, and examined for various properties including primary structure and conformation. As far as fish myosin is concerned, several papers have so far been published: light chains were isolated from the ordinary muscle of carp, trout and mackerel (Focant and Huriaux, 1976; Perzanowska et al., 1978; Watabe et al., 1983a), and dark and cardiac muscles of mackerel (Watabe et al., 1983a; Dinh et al., 1985). However, properties of fish myosin light chains, for the most part, remain to be elucidated.

Isolation of light chains has been attempted by preparative gel electrophoresis (Yazaki et al., 1973), gel filtration (Perzanowska et al., 1978; Klotz et al., 1978), anion-exchange column chromatography (Lowey and Holt, 1972; Focant and Huriaux, 1976; Perzanowska et al., 1978; Watabe et al., 1984), isoelectric precipitation (Hiratsuka, 1980), isoelectric focusing (Vincent and Cummins, 1985), and high-performance liquid chromatography (Watabe et al., 1983b; Libera et al., 1984; Rushbrook, 1985). Among these methods, anion-exchange column chromatography seems to be the best way with respect to recovery and resolution.

In this section, attempts were made to isolate light chains from the ordinary muscle of six marine fishes, which were selected from the three types classified on the basis of molecular weights of A2 and DTNB light chains (Section 1 of Chapter I).

Materials and Methods

Materials

Fish specimens used are as follows : skipjack *Katsuwonus pelamis*, bonito *Auxis tapeinosoma*, and mackerel *Pneumatophorus japonicus japonicus* (type I), round herring *Etrumeus micropus*, sardine *Sardinops melanosticta* (type II), and Alaska pollack *Theragra chalcogramma* (type III). Refer to Section 1 of Chapter I for the classification of light chain type. These specimens were stored at -40°C until use. The following procedures were performed at 0 - 4°C unless otherwise stated.

Preparation of myosin

Myosins were prepared from the ordinary muscle of the above fish species as described in Section 1 of Chapter I.

Isolation of myosin light chains

Myosin was first treated with DTNB according to Focant and Huriaux (1976) in order to remove DTNB light chain as much as possible, because this light chain often interfered with separation of alkali light chains. Alkali light chain fraction was obtained from the DTNB-treated myosin by 4 M urea treatment according to Lowey and Holt (1972). Light chain fraction thus prepared was lyophilized, and extensively dialyzed against 50 mM potassium phosphate buffer (pH 6.5) containing 0.1 mM DTT. After centrifugation at 5,000 x g for 10 min, the supernatant was applied to a DEAE-cellulose or a DEAE-Toyopearl 650M column (2.5 x 40 cm) equilibrated with the same buffer. In case of DTNB light chain, a DEAE-cellulose column (2.5 x 40 cm) was exclusively used, because recovery was very low when a DEAE-Toyopearl

column was used. Light chains were eluted with a linear gradient from 0.05 M and 0.4 M potassium phosphate buffer (pH 6.5) containing 0.1 mM DTT at a flow rate of 40 ml/h.

High-speed gel filtration was performed using a TSK 3000SW column (7.5 mm x 60 cm, Tosoh Co.) equilibrated with 0.2 M potassium phosphate buffer (pH 6.0) containing 0.1 mM DTT and 2 M urea according to Watabe et al. (1983b).

Other analytical methods

SDS-PAGE was performed as described in Section 1 of Chapter I. Protein concentration was determined according to Lowry et al. (1951).

Results and Discussion

Elution profile of skipjack myosin light chains from a DEAE-cellulose column is shown in Fig. II-1-1. The order of elution was A1, A2 and DTNB light chain, in contrast to the order of A1, DTNB and A2 with myosin light chains so far reported. These differences in elution profile might stem from their differences in molecular size and net charge. In the present experiment, A1 and DTNB light chains were isolated relatively easily, but A2 was not even by repetition of column chromatography. Isolation of A2 was, however, achieved by high-speed gel filtration as shown in Fig. II-1-2. Incidentally, when skipjack light chains were subjected to a DEAE-cellulose column using a linear gradient from 0.05 - 1 M potassium phosphate (pH 6.0), peaks of light chains were hardly separated from each other (data not shown).

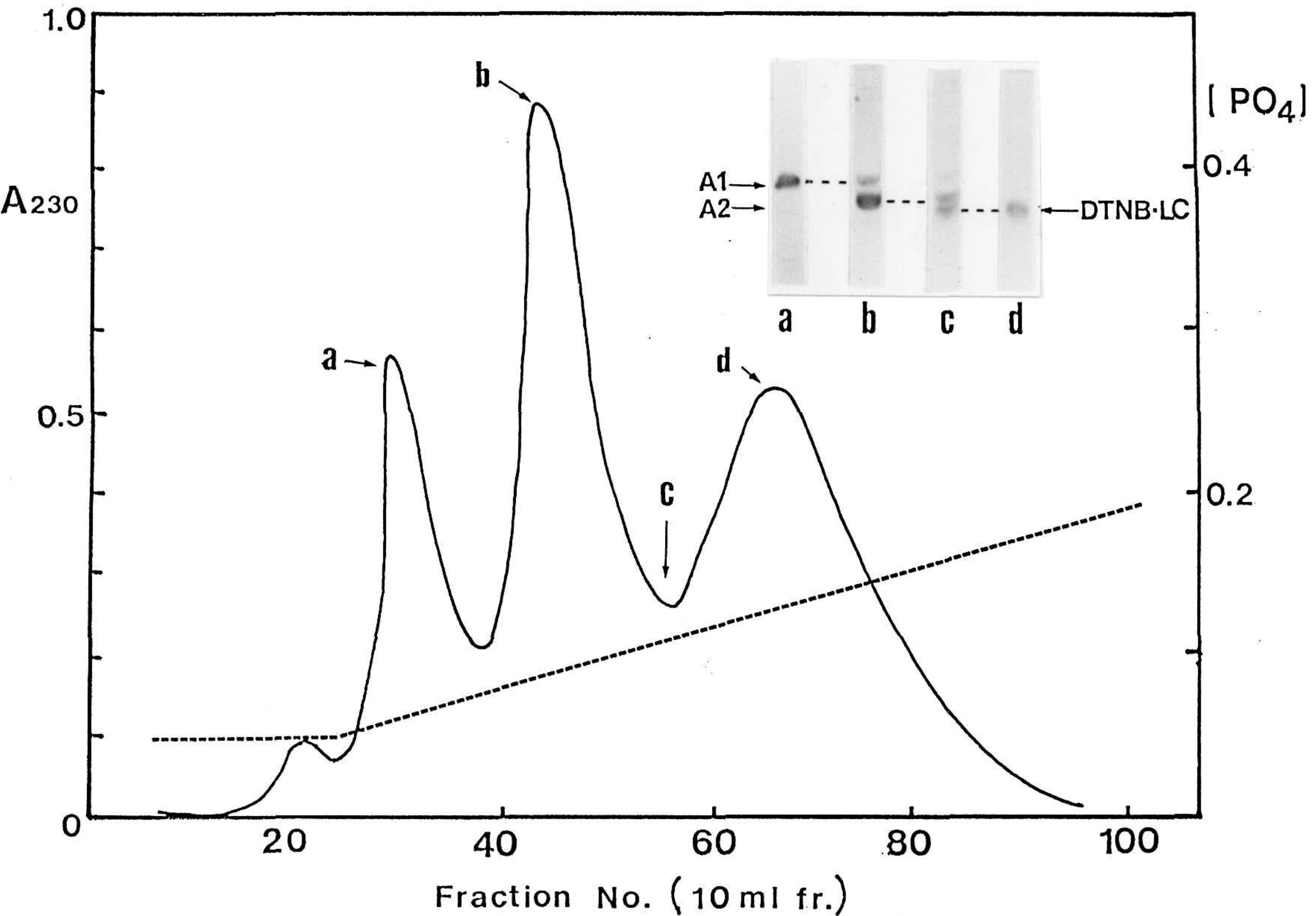


Fig. II-1-1. Elution profile of myosin light chains from skipjack ordinary muscle by DEAE-cellulose column chromatography. Inserted figures represent SDS-PAGE patterns of the fractions indicated by arrow. 10% gels. [PO_4] is given by a dotted line.

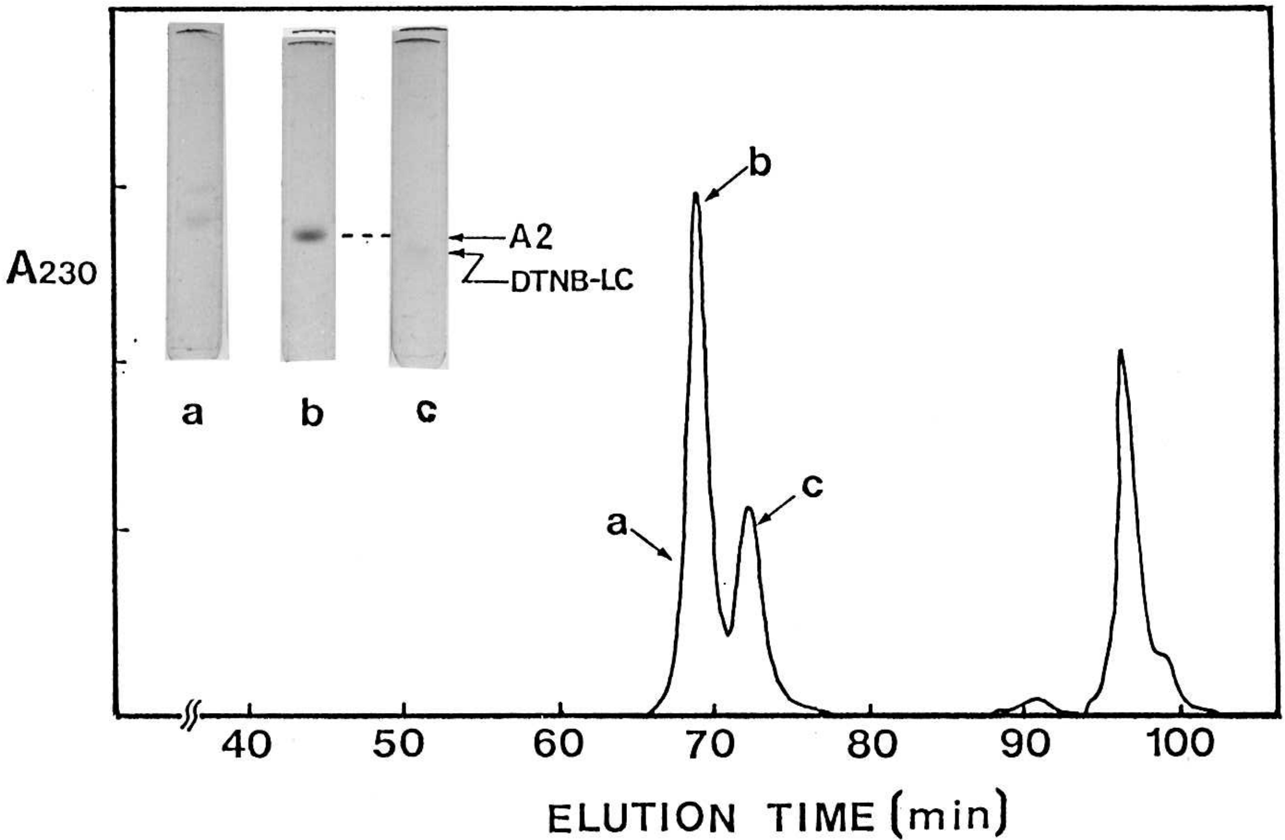


Fig. II-1-2. High-speed gel filtration of skipjack A2 light chain on a TSK 3000SW column. Inserted figures represent SDS-PAGE patterns of the fractions indicated by arrow. 10% gels.

DTNB light chain of skipjack myosin was rapidly decomposed during purification procedure, unlike the two alkali light chains. The decomposition was, however, partly inhibited by phenylmethylsulfonyl fluoride (PMSF). The decomposition might be associated with a high susceptibility of this light chain to endogenous proteases.

Elution profiles of myosin light chains from sardine and Alaska pollack ordinary muscle are shown in Figs. II-1-3 and II-1-4. Irrespective of fish species, A1 light chain was successfully separated from other light chains by the chromatographic technique as described above. However, major parts of A2 and DTNB light chains were eluted together, which necessitated repetition of the chromatography for further purification. In addition, DTNB light chain preparations from all the fishes were rapidly decomposed during purification processes, probably due to contaminating proteases. Such decomposition was especially remarkable in cases of bonito and sardine. Furthermore, this light chain was irreversibly adsorbed onto the DEAE-Toyopearl column, resulting in a poor yield. It was, therefore, necessary to apply DTNB light chain to a DEAE-cellulose column (2.5 x 40 cm), which made it possible to prepare this light chain from bonito (data not shown). Consequently, A1 light chain was isolated from all the species, A2 light chain from four species (skipjack, bonito, mackerel and round herring) and DTNB light chain from three species (skipjack, mackerel and bonito) (Fig. II-1-5).

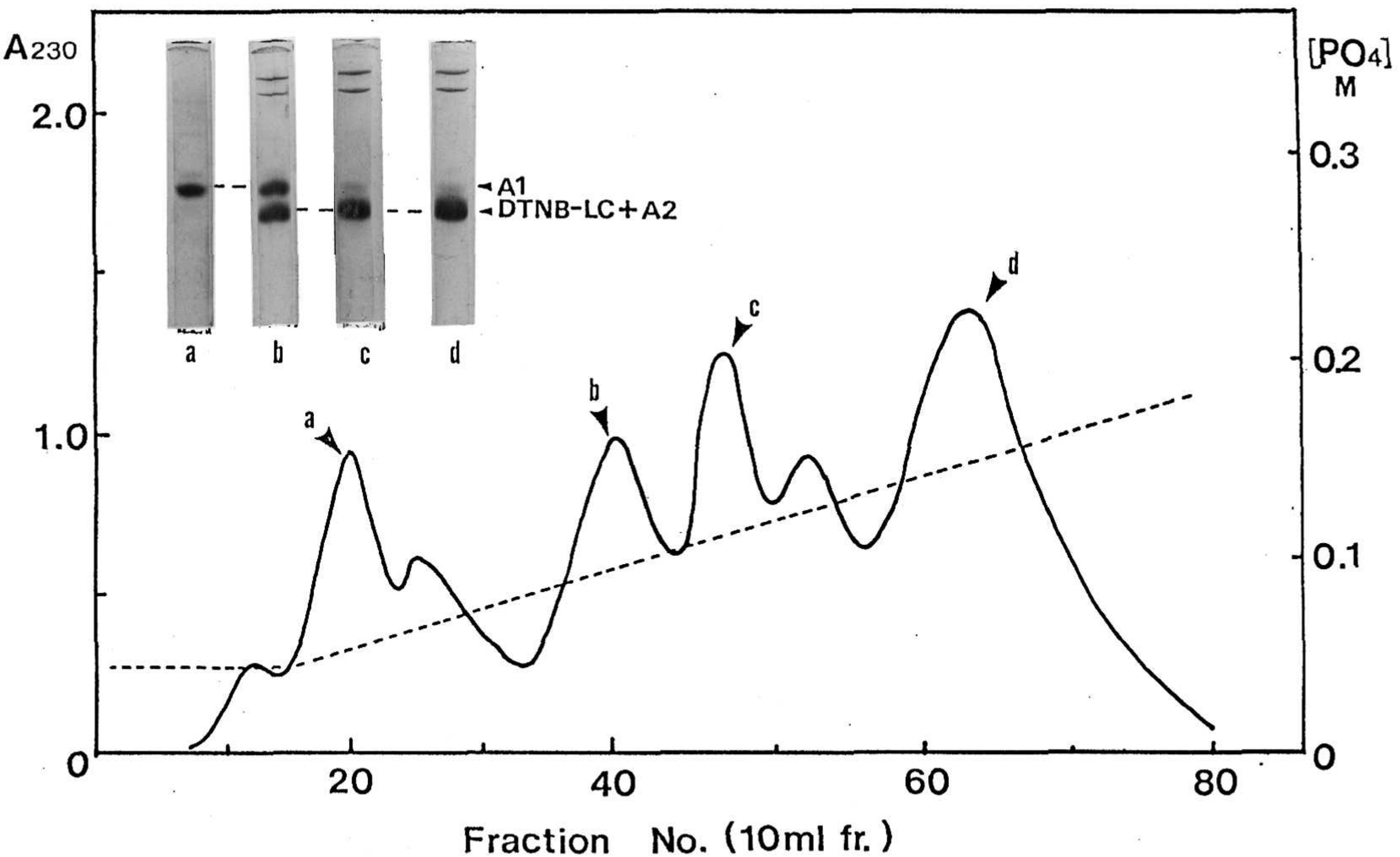


Fig. II-1-3. Elution profile of myosin light chains from sardine ordinary muscle on a DEAE-Toyopearl 650M column (2.5 x 40cm). Inserted figures represent SDS-PAGE patterns (15% gels) of the fractions indicated by arrow.

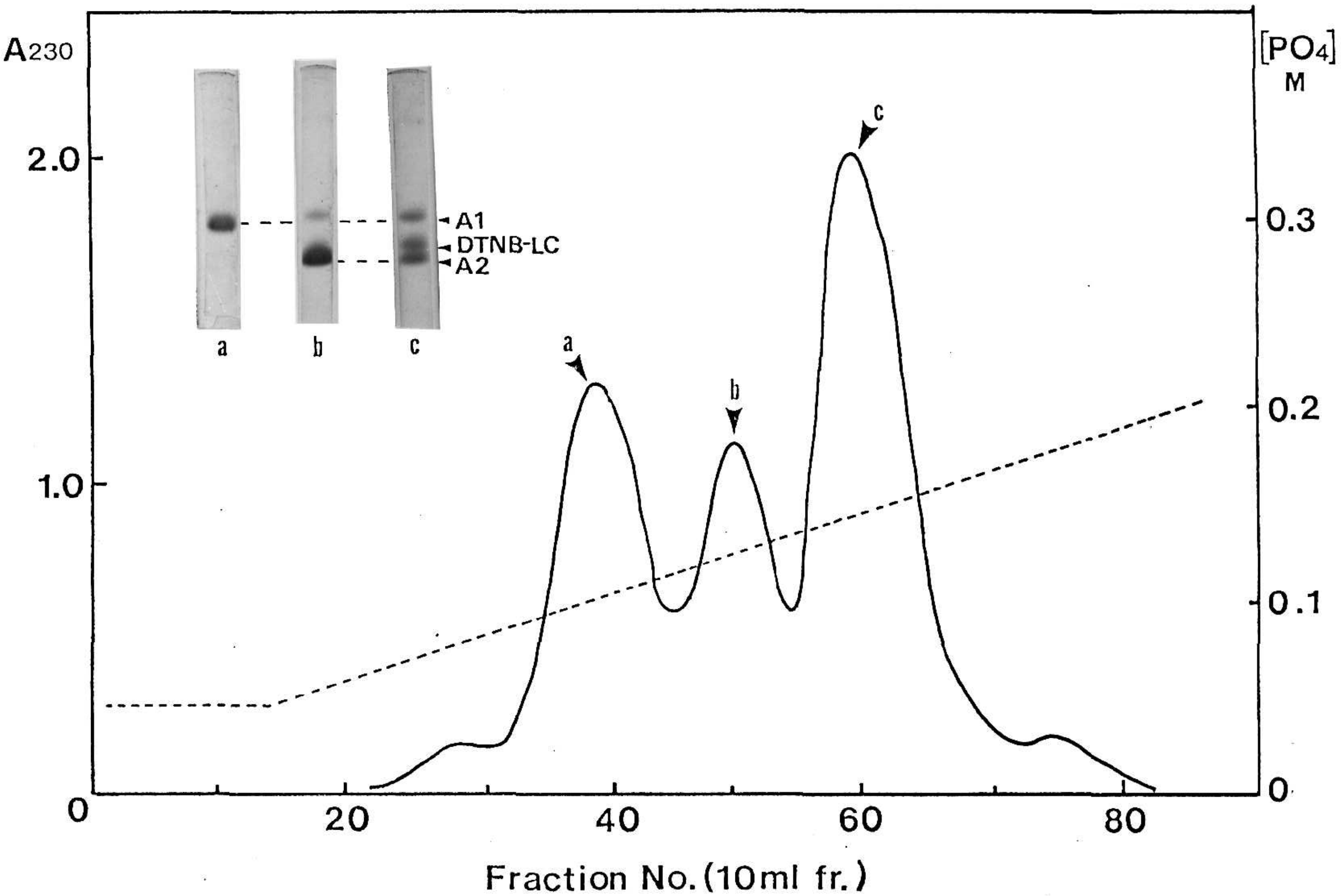


Fig. II-1-4. *Elution profile of myosin light chains from Alaska* pollack ordinary muscle on a DEAE-Toyopearl 650M column (2.5 x 40cm). Inserted figures represent SDS-PAGE patterns (15% gels) of the fractions indicated by arrow.

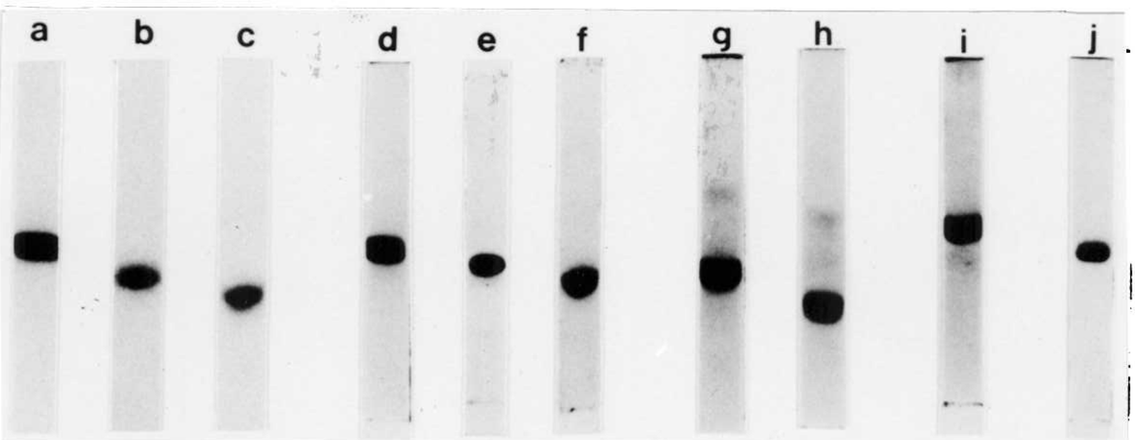


Fig. II-1-5. SDS-PAGE (10% gel) patterns of isolated myosin light chains. Skipjack A1 (a), A2 (b), DTNB light chain (c); bonito A1 (d), A2 (e), DTNB light chain (f); round herring A1(g), A2(h); sardine A1 (i); Alaska pollack A1 (j).

Section 2

Physicochemical Properties

As demonstrated in Chapter I, fish myosin light chains are quite similar to each other in molecular size and isoelectric point, though the molecular weights were generally larger than those of higher vertebrate counterparts. Remarkable species-specificity observed in SDS-PAGE patterns, however, suggested a wide diversity in their structures.

The physicochemical properties of myosin light chains from higher vertebrates such as rabbit and chicken have been examined in detail. According to the results obtained, A1 and A2 light chains are similar to each other in primary structure, except that A1 additionally possesses a peptide ("difference peptide") at the N-terminal. The difference peptide is featured by high contents of alanine, proline and lysine. Primary structures of fish myosin light chains have not been determined as yet. However, the contents of these amino acids are higher in A1 than in A2 for fish myosins, (Focant and Huriaux, 1976; Huriaux and Focant, 1977; Watabe et al., 1983a), suggesting that A1 differs from A2 by this kind of peptide in case of fish myosin as well.

In this section, myosin light chains isolated in the previous section were examined for physicochemical properties, such as amino acid composition and ultraviolet absorption spectrum.

Materials and Methods

Myosin light chains were isolated from various species of fish as described in the previous section and used.

SDS-PAGE was performed according to Laemmli (1970) using 15% polyacrylamide slab gels. Amino acid composition was analyzed on hydrolyzed light chain in 6 N HCl for 24 h, with a Hitachi 835 amino acid analyzer. Cysteine content was determined after conversion to cysteic acid according to Moore (1963). Ultraviolet absorption spectrum of each light chain was measured in 0.05 M potassium phosphate buffer (pH 6.8) at 1 mg/ml. Protein concentration was measured according to Lowry et al. (1951).

Results and Discussion

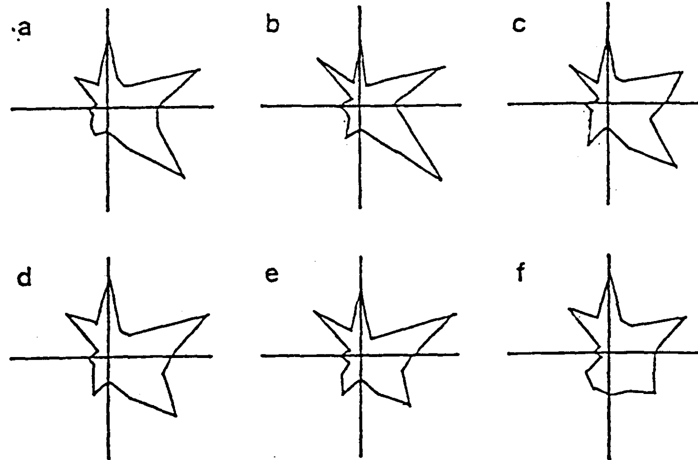
Apparent molecular weights of the isolated light chains are shown in Table II-2-1. The values ranged from 27,000 - 28,000 for A1, from 21,000 - 22,500 for A2, and from 18,000 - 21,000 for DTNB light chain.

Star diagrams for amino acid composition of these light chains are shown in Fig. II-2-1. Throughout the light chains examined, they were rich in aspartic and glutamic acids, alanine and lysine, while poor in histidine and tyrosine. As for A1 light chain, high similarity was recognized between skipjack and bonito, both of which are taxonomically related. These two A1 light chains were featured by a high content of alanine, which was comparable to that of mackerel A1 light chain (Watabe et al., 1983a). On the other hand, the diagrams of A1 light chains from

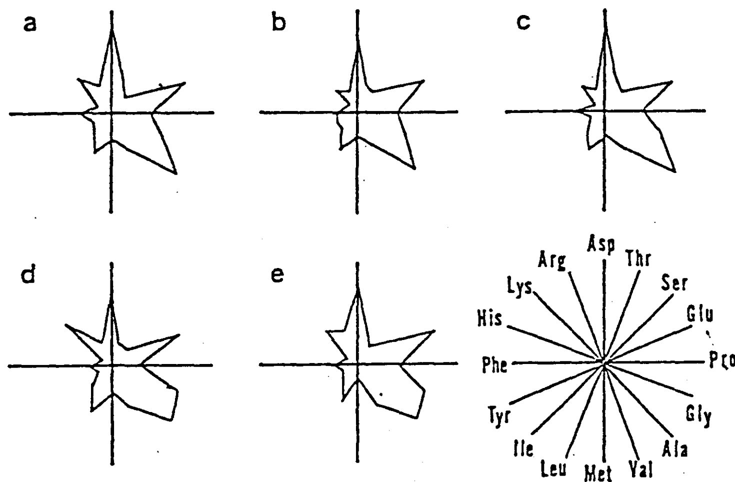
Table II-2-1. Molecular weights and extinction coefficients at 280 nm ($E^{1\%}_{1cm}$) of fish myosin light chains

Species	Light chain	Mol. wt.	$E^{1\%}_{1cm}$
Skipjack	A1	30,000	4.8
	A2	22,500	3.6
	DTNB-LC	20,000	4.6
Bonito	A1	27,500	5.1
	A2	22,500	5.5
	DTNB-LC	18,000	6.0
Mackerel	A1	27,000	5.8
	A2	22,500	5.5
	DTNB-LC	19,000	4.9
Round herring	A1	28,000	6.0
	A2	21,000	5.6
Sardine	A1	28,000	5.1
Alaska pollack	A1	28,000	5.1

A1



A2



DTNB-LC

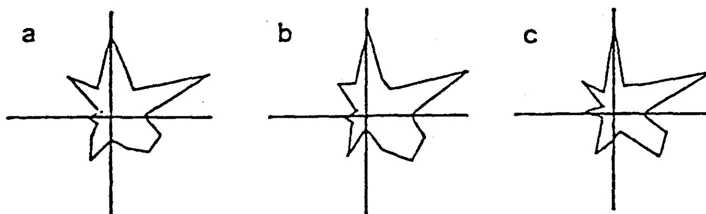


Fig. II-2-1. Star diagrams for amino acid composition of A1, A2 and DTNB light chains from the ordinary muscle of skipjack (a), bonito (b), mackerel (c), round herring (d), sardine (e) and Alaska pollack (f).

sardine and round herring resembled each other. These patterns were also comparable to those of A1 light chains from trout (Perzanowska _et_ _al_., 1978) and carp (Huriaux and Focant, 1977). A1 light chain from Alaska pollack was distinguished from those of other fish species by a high content of methionine.

The amino acid profiles of A2 light chain were also quite similar between closely related species. A2 light chains from sardine and round herring were featured by high contents of glycine and valine, as in the case of trout A2 light chain (Perzanowska _et_ _al_., 1978). In this connection, Focant and Huriaux (1976) reported that pike A2 light chain contained a large amount of leucine, as did the essential light chain of scallop (Kendrick-Jones _et_ _al_., 1976).

The contents of alanine, proline and lysine were higher in A1 than in A2 light chain. This tendency was more pronounced in skipjack and bonito. In the cases of rabbit and chicken, these amino acids are known to localize in the difference peptide (Frank and Weeds, 1974; Matsuda _et_ _al_., 1980). The present data supported the view that A1 light chain of fish myosin possesses a similar structure to those of higher vertebrates.

The amino acid profiles of DTNB light chain were very similar to each other, and also to those of rabbit DTNB light chain (Lowey and Holt, 1972) and scallop regulatory light chain (Kendrick-Jones _et_ _al_., 1976), while alkali light chains differed clearly between fish and higher vertebrates as described above. As described in Chapter I, the molecular weights and isoelectric

points of DTNB light chains fell in quite a narrow range regardless of fish species. Therefore, this light chain is considered to be a highly conservative protein from the viewpoint of molecular evolution.

Fig. II-2-2 shows the ultraviolet absorption spectra of the isolated light chains in 0.05 M potassium phosphate buffer (pH 6.8). All the light chains examined showed a maximum at around 278 nm and a minimum at around 250 nm, except DTNB light chain from bonito. In addition, they showed minor absorption maxima at 253, 259, 265 and 269 nm probably due to high contents of phenylalanine. These results, along with their amino acid compositions, suggested that the light chains of these species possess similar structures. The extinction coefficients at 280 nm ($E^{1\%}_{1cm}$) were calculated to be 5.1 - 6.0 for A1, 4.2 - 5.6 for A2, and 4.8 - 5.0 for DTNB light chain from fish ordinary muscle myosins (Table II-2-1), including those of skipjack and mackerel. The myosin light chains examined were not significantly species-specific in this respect.

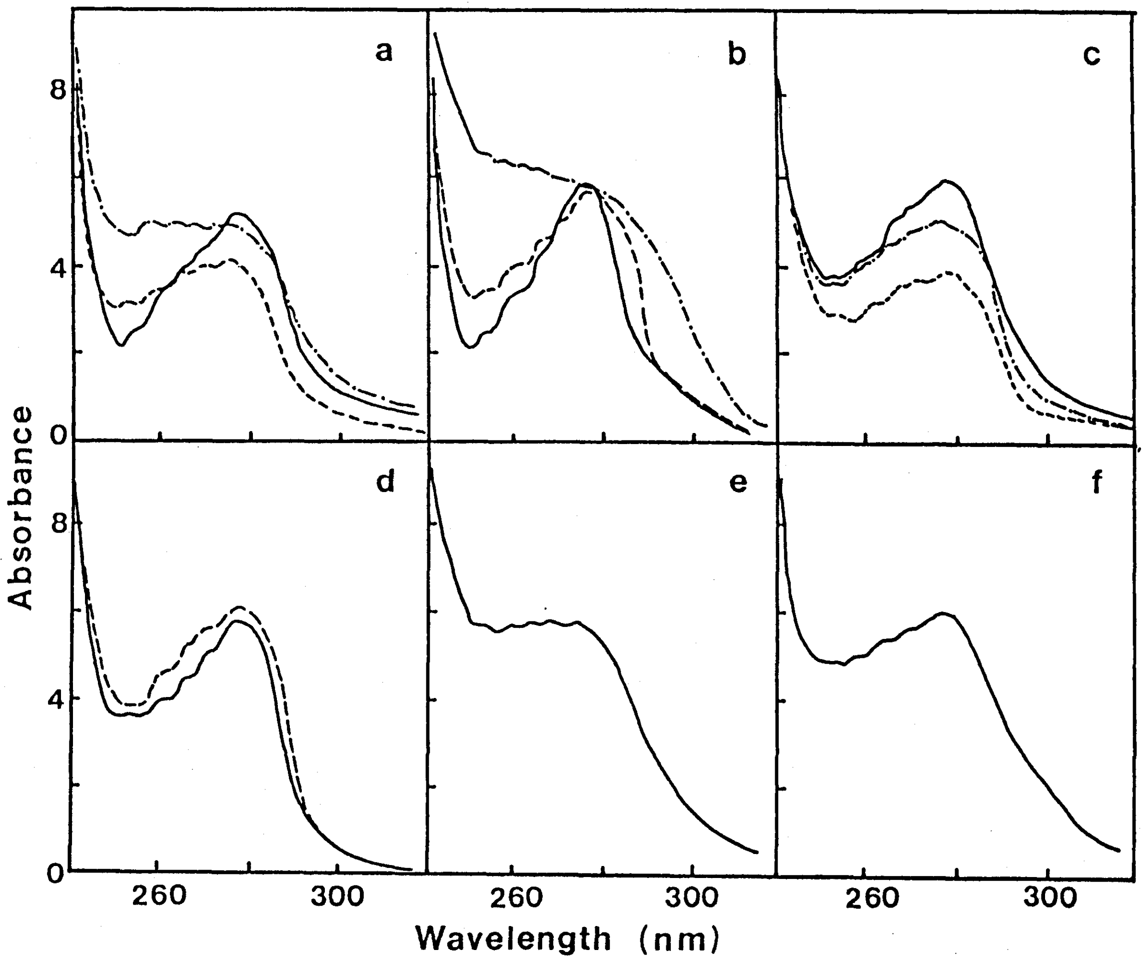


Fig. II-2-2. Ultraviolet absorption spectra of myosin light chains from skipjack (a), bonito (b), mackerel (c), round herring (d), sardine (e) and Alaska pollack (f) in 0.05 M potassium phosphate buffer (pH 6.8) at 1% concentration. Solid, broken, and chain lines represent A1, A2 and DTNB light chains, respectively.

Section 3

Immunological Properties

Fast skeletal myosins contain one pair of DTNB light chain and another pair of alkali light chains, A1 and A2 (Weeds and Lowey, 1971). A1, A2 and DTNB light chains from rabbit and chicken have some homology in their primary structures, supporting the view that they have evolved from a common anscestor (Frank and Weeds, 1974; Matsuda et al., 1980). Similarity of A1 and A2 light chains has also been demonstrated immunologically (Horvath and Gaetjens, 1972; Holt and Lowey, 1975a). Cross-reactivity was, however, hardly observed between either alkali light chains and DTNB light chain in immunodiffusion and immunoelectrophoreis (Silberstein and Lowey, 1977; Obinata et al., 1979), though a strong cross-reaction occurred between DTNB and alkali light chains in radioimmunoasssay (Silberstein and Lowey, 1977, 1981).

As for fish myosin light chains, immunological cross-reactivity of light chains from the ordinary and dark muscles of mackerel has been examined by immunodiffusion and enzyme-linked immunosorbent assay (Watabe et al., 1983a). However, no further information is available on fish myosin light chains.

In this section, antisera against light chains were prepared, and immunological cross-reactivity among three kinds of light chains and among fish species was examined by immunodiffusion, immunoelectrophoresis and immunoblotting.

Materials and Methods

Materials

Fish species examined were as follows : skipjack Katsuwonus pelamis, bonito Auxis tapeinosoma, bluefin tuna Thunnus thynnus, mackerel Pneumatophorus japonicus japonicus, sardine Sardinops melanosticta, round herring Etrumeus micropus, yellowtail Seriola quinqueradiata, horse mackerel Trachurus japonicus, pike conger Muraenesox cinereus, greenling Hexagrammos otakii, chum salmon Oncorhynchus keta, tilapia Tilapia nilotica, carp Cyprinus carpio, and requiem shark Triakis scyllia. These specimens were stored at -40°C until used. The ordinary muscle was exclusively used. Rabbit fast muscle was also used for comparison.

Preparation of myosin

Myosin was prepared as described in Section 1 of Chapter I.

Isolation of light chains

Myosin light chains were isolated as described in Section 1 of this chapter.

Preparation of antisera against fish myosin light chains

Antisera against myosin light chains were raised in adult female rabbits (about 3 kg). Each purified light chain (1 mg) was dissolved in 1 ml of 0.01 M sodium phosphate buffer (pH 7.0), and emulsified with an equal volume of complete Freund's adjuvand (Iatron Lab.). The emulsion thus prepared was subcutaneously injected into the back of a rabbit. This procedure was repeated 4 - 5 times at 2-week intervals. The immunized rabbits were then bled via the jugular vein. Antisera were prepared from the bloods and stored at -80°C until use.

As described in Section 1 in this chapter, A1 light chain of high purity was isolated quite easily, while it was difficult to separate A2 from DTNB light chain in all the fishes examined. Therefore, antisera were prepared mainly against A1 light chains from six fish species.

Immunological methods

Double immunodiffusion test was performed using 1% agarose plates (1mm thick) containing 0.1 M phosphate-buffered saline (PBS) and 0.1% sodium azide according to Ouchterlony (1953, 1958). About 1 μg of light chain and 5 μl of antiserum were poured into the peripheral and central wells, respectively, and the plate was allowed to stand overnight at room temperature. The precipitation lines were observed using an Immunoviewer (Kayagaki Co.).

Immunoelectrophoresis was carried out in 1.2% agarose gel plates of 1 mm thickness, using barbital buffer (pH 8.6, $\underline{I}$=0.05). About 1 μg of light chain was first pipetted into the wells, and run at 3 mA/cm at 4°C for 30 min. After the run, 30 μl of antiserum was poured into the troughs, and the plate was allowed to stand overnight at room temperature. After soaking in PBS overnight, the plate was dried, and stained with Coomassie brilliant blue 250-R.

Immunoblotting was performed for 14 fish species as described above according to the method in Section 2 of Chapter I.

Results and Discussion

The titer of the antisera against light chains depended on

preparation and fish species, although the antisera were made under similar conditions. It is uncertain at this stage whether the varying titers resulted from differences in antigenicity of light chain preparation used.

The cross-reactions among A1 light chains in immunodiffusion test are shown in Fig. II-3-1. Precipitation lines were formed among all the combinations of light chains and antisera, although the reactivity was different from each other. These results suggest that all these A1 light chains possess common antigenic sites. However, spurs appeared with some antigen-antiserum combinations, indicating the presence of partial antigenic heterogeneity among fish myosin light chains. For example, strong spurs were formed between skipjack and Alaska pollack A1 light chains against anti-skipjack A1 antiserum, as indicated by white arrows in Fig. II-3-1. These fishes are phylogenetically distinct species. On the other hand, precipitation lines were fused with each other between A1 light chains from related species, e. g., between skipjack and bonito, and between round herring and sardine. These immunological properties, along with physicochemical properties, suggest that there are little structural differences between these A1 light chains.

In immunoelectrophoresis, precipitation lines were observed at the positions where light chains were electrophoresed, with any combination of antigen and antiserum as in the case of Ouchterlony's double immunodiffusion test, though the mobilities of A1 light chains were somewhat different from each other. The representative patterns are given in Fig. II-3-2. Similar precipitation patterns were obtained irrespective of antisera

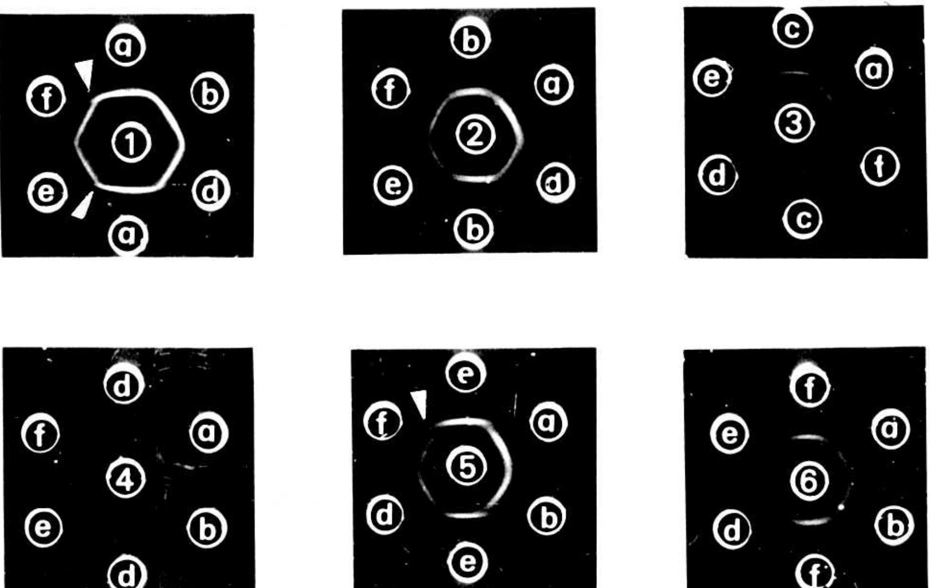


Fig. II-3-1. Ouchterlony's double immunodiffusion of A1 light chains with its homologous and heterologous antisera in 1% agarose gel plates. In the peripheral wells, 1 μg of A1 from skipjack (a), bonito (b), mackerel (c), round herring (d), sardine (e), and Alaska pollack (f) was placed. To the central wells, 5 μl of rabbit antisera against A1 from skipjack (1), bonito (2), mackerel (3), round herring (4), sardine (5), and Alaska pollack (6) was placed. The arrowhead indicates spurs of precipitation lines.

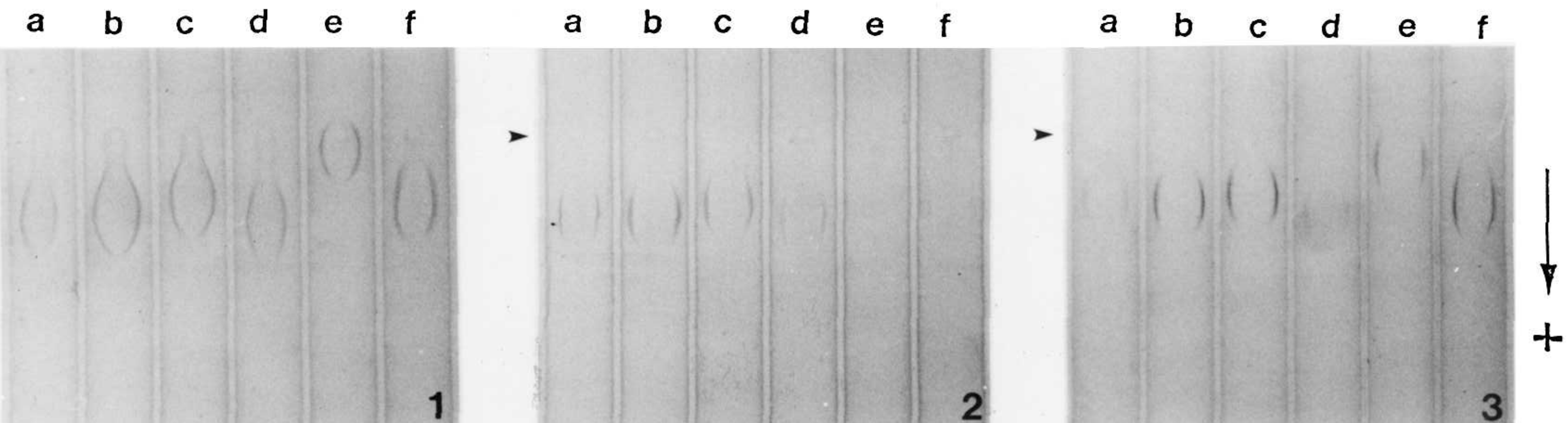


Fig. II-3-2. Immunoelectrophoresis of A1 light chain on 1.2% agarose gel plates containing 0.05 M barbital buffer (pH 8.6) and 0.1 mM sodium azide. One microgram of A1 light chains from skipjack (a), bonito (b), mackerel (c), round herring (d), sardine (e), and Alaska pollack (f) was electrophoresed, and subsequently, 30 µl of antisera against A1 light chains from skipjack (1), mackerel (2), and Alaska pollack (3) was poured into each trough, and allowed to stand overnight at room temperature. The arrowhead indicates the position where light chains were applied.

prepared, although the densities of precipitation lines were somewhat different according to the antiserum used, probably due to the heterologous antigenicity of A1.

Watabe et al. (1983a) reported previously that mackerel A1 and A2 light chains possessed similar structures as revealed by immunoelectrophoresis and enzyme-linked immunosorbent assay. These results suggest that alkali light chains from different animals possess, to some extent, common structures with each other, although myosin light chains of slow and cardiac muscles are shown to be immunologically different from those of fast muscle (Masaki, 1974; Obinata et al., 1979; Watabe et al., 1983a; Dinh et al., 1985).

In the next place, cross-reactivity was examined by immunoblotting technique among the three types of light chains (A1, A2 and DTNB light chain) from the ordinary muscle of 14 fish species. To begin with, cross-reactivity against anti-skipjack A1 antiserum was examined (Fig. II-3-3). A1 light chains from all the fishes as well as rabbit were strongly stained. Furthermore, DTNB light chains generally showed strong cross-reactivity, irrespective of the fish species. A2 light chains, including that of skipjack, were scarcely stained. The reactivity was generally higher for red-fleshed fishes, and relatively weaker for white-fleshed species.

When anti-skipjack A2 antiserum was used, A1 light chains of red-fleshed fishes were also stained, though less intensively. This tendency was in good agreement with the pattern obtained by using anti-skipjack A1 antiserum (Fig. II-3-3). However, A1

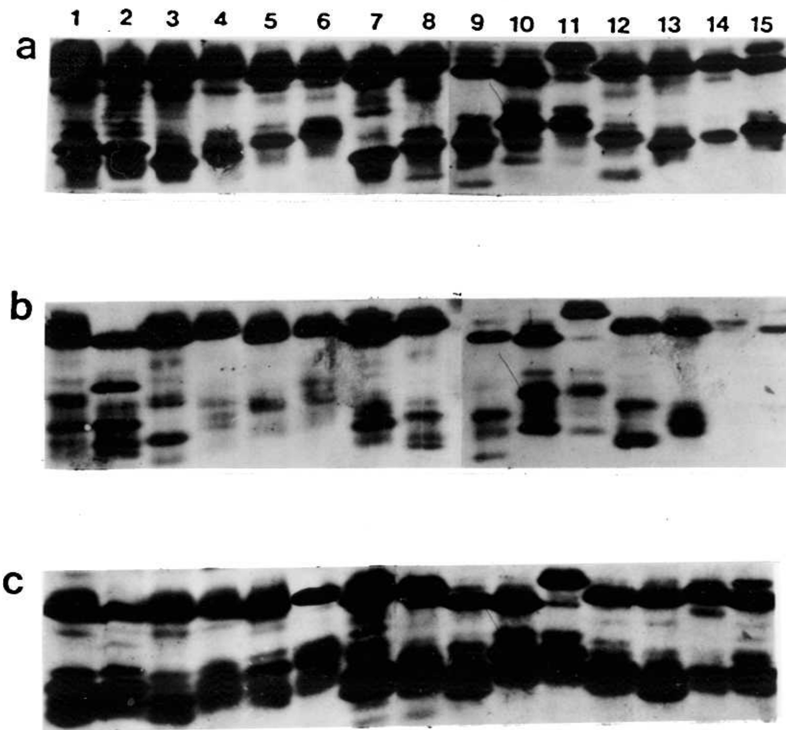


Fig. II-3-3. Cross-reactivity of myosin light chains from 14 fish species with antisera against skipjack A1 (a), A2 (b) and DTNB light chains (c). 1, skipjack; 2, bonito; 3, bluefin tuna; 4, mackerel; 5, sardine; 6, round herring; 7, yellowtail; 8, horse mackerel; 9, pike conger; 10, greenling; 11, chum salmon; 12, tilapia; 13, carp; 14, requiem shark; 15, rabbit.

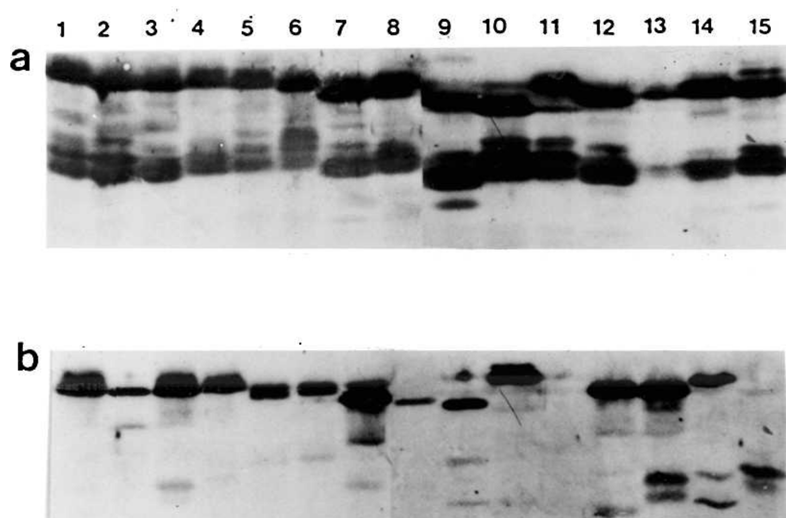


Fig. II-3-4. Cross-reactivity of myosin light chains from 14 fish species with antisera against round herring A1 (a) and Alaska pollack A1 (b). Refer to the legend of Fig. II-3-3 for fish species examined.

light chain of requiem shark and rabbit were not stained. Unexpectedly, little reaction was observed for A2 and DTNB light chain from most fish species. A2 light chain of greenling and tilapia were exceptionally stained, though the reason is not clear.

When anti-skipjack DTNB light chain antiserum was used, quite different patterns were obtained compared to those obtained by anti-A1 or A2 antiserum (Fig. II-3-3). Namely, A1 light chain was strongly stained for all the fish species as well as for rabbit. Furthermore, DTNB light chain and the band just below this light chain, which were not detected by Coomassie brilliant blue staining, were strongly stained. This band was supposed to be a decomposition product of DTNB light chain, because similar bands are often produced during preparation of fish myosin. A2 light chain was hardly stained, as was the case with anti-A1 or A2 antiserum. However, protein staining of the transferred membrane showed that A2 was steadily bound to the membrane. Such weak reactivity of A2 might have resulted from some structural damage of this protein during immunoblotting procedure.

In the next place, anti-round herring A1 antiserum was used (Fig. II-3-4a). This antiserum visualized A1 and DTNB light chains of white-fleshed fishes more strongly than those of red-fleshed ones. However, these light chains of carp were not stained. As described above, anti-skipjack A1 antiserum gave rise to the opposite results. It was suggested that the structures of myosin light chains from round herring might be closer to those of white-fleshed fishes.

Finally, anti-Alaska pollack antiserum was used (Fig. II-3-4b). This antiserum reacted well with A1 light chains except those of chum salmon and rabbit. The intensity was a little stronger for white-fleshed fishes than red-fleshed ones. A2 and DTNB light chains were hardly stained. This was reminiscent of the patterns obtained with anti-skipjack A2 antiserum.

As described above, the cross-reactivity was dependent on the antiserum used, though ubiquitous structures seem to exist throughout the fishes examined and the three types of light chain. In addition, strong cross-reaction was recognized with rabbit myosin light chains. However, intensity of cross-reactivity was generally stronger between taxonomically related species.

As described above, A1 differs from A2, only by its possessing the difference peptide of 41 residues at the N-terminal (Frank and Weeds, 1974). In good agreement with this sequence homology, the precipitation lines of chicken A1 and A2 fused with each other, when these light chains were diffused against anti-serum to total light chains, while the precipitation lines crossed that of DTNB light chain (Lowey and Holt, 1972). On the other hand, A1 and A2 were proved to be immunologically different from each other by a quantitative precipitation analysis (Lowey and Holt, 1972; Silberstein and Lowey, 1981). Such difference can largely be accounted for by the difference peptide. The antibody specific to the difference peptide can recognize S1 isozymes, heavy meromyosin and myosin associating different alkali light chains (Holt and Lowey, 1977; Silberstein and Lowey,

1981). However, anti-A2 antibodies which do not cross-react with A1 also exist (Silberstein and Lowey, 1981). Significant cross-reactivity was also reported between corresponding rabbit and chicken light chains (Holt and Lowey, 1975a, b). Watabe et al. (1983a) reported that anti-mackerel A1 (or A2) antiserum exhibited cross-reaction in both immunoelectrophoresis and enzyme-linked immunosorbent assay (ELISA), indicating an immunochemical similarity of these light chains. In addition, no precipitation line appeared when anti-A1 or A2 antiserum was diffused against DTNB light chain in immunoelectrophoresis. In ELISA, however, each pair showed cross-reactivity as high as 50 - 80%. These values are clearly higher than those obtained with heterologous alkali light chains (10 - 40%).

The cross-reactivity observed between the difference peptide of A1 and DTNB light chain demonstrated the difficulty inherent to the use of antibodies as specific markers. Sequence comparisons have led to the conclusion that both classes of myosin light chains are homologous, not only with each other, but also with Ca-binding proteins, troponin C and parvalbumin (Collins, 1974; Weeds and McLachlan, 1974). Actually, weak cross-reactivity was observed among myosin light chains and troponin C (Weeds and MaLachlan, 1974), consistent with the suggestion made on the basis of sequence homologies that these subunits contain similar structural domains (Silberstein and Lowey, 1981).

Summary

Myosin light chains (A1, A2, and DTNB light chain) were isolated from the ordinary muscle of six marine fishes, and the physicochemical and immunological properties were examined.

A1 was isolated from all fish species, A2 from four species, and DTNB light chain from three species. All the isolated light chains were rich in aspartic and glutamic acids, alanine, lysine, and poor in histidine and tyrosine. Between closely related species such as skipjack and bonito, the amino acid profiles of corresponding light chains were very similar to each other. A1 contained a larger amount of alanine, proline and lysine than A2 irrespective of fish species, suggesting the presence of difference peptide in fish A1 light chains. The isolated light chains showed absorption maximum at 278 nm, and a minimum at around 250 nm.

Structural similarities and differences of A1 light chains were further demonstrated immunologically using anti-A1 anti-serum. Precipitaion lines were fused with each other among closely related species, whereas strong spurs were observed among phylogenetically distinct species. By immunoblotting, it was demonstrated that fish myosin light chains possess, to some extent, ubiquitous structures, irrespective of the types of light chains.

CHAPTER III

PHYSIOLOGICAL FUNCTIONS OF MYOSIN LIGHT CHAINS

The interaction of actin with myosin head and the actin-activated Mg-ATPase of myosin are crucial events of the mechano-chemical transduction process in muscle and other motile systems. Myosin releases the head region on chymotrypsin or papain digestion, giving subfragment 1 (S1). It is well established that S1 inherits ATPase, actin-binding and light chain binding sites. Thus, S1 is thought to have a very important role in the force generation mechanism of muscle for such inherent properties.

S1 prepared by chymotryptic digestion in the presence of EDTA can be separated into two isozymes, S1(A1) and S1(A2), with respect to alkali light chains (Weeds and Taylor, 1975). In the absence of actin, no practical differences in ATPase profile have so far been recognized between both isozymes. In the presence of actin, however, both isozymes significantly differed from each other in Mg-ATPase at low salt concentration. In case of fast muscle myosin, S1(A1) showed a higher affinity for actin and lower maximum velocity than S1(A2) (Weeds and Taylor, 1975). Hybridization experiments demonstrated that these kinetic differences stemed from the associated alkali light chains, suggesting the involvement of the difference peptide of A1 in actin-myosin interaction (Wagner and Weeds, 1977). In addition, such kinetic differences are maintained in case of regulated actin (actin-tropomyosin-troponin complex) (Wagner *et al*., 1979). As the ionic strength increased, however, kinetic values of S1(A1)

increased up to those of S1(A2), while those of S1(A2) remained unchanged practically (Wagner et al., 1979; Reisler, 1980; Chalovich et al., 1984). Furthermore, free heavy chain showed an enzymatic activity similar to S1(A2) (Sivaramakrishnan and Burke, 1982). Anyway, physiological function of light chains has not been fully established at present.

As described in Chapter I, species-specificity was marked in alkali light chains. It follows that such specificity might reflect the stability of myosin or swimming ability of fish. S1 isozymes, associated with either A1 or A2 seem to be materials suitable to investigate the role of alkali light chains. Ikariya et al. (1981) prepared S1 from carp ordinary muscle, and found that it resembled rabbit fast myosin S1 in respect of physicochemical and enzymatic properties. However, no attempt has so far been made to isolate both isozymes from fish S1.

In this chapter, S1 isozymes were isolated from fish myosins and characterized. Alkali light chains in S1 was substituted by foreign alkali light chains, to examine its effect on S1 ATPase activity. Then, heavy chains in both isozymes were compared by peptide mapping and tryptic digestion methods. In addition, affinities of both alkali light chains for heavy chain and actin were examined.

Section 1

Isolation and Characterization of Myosin Subfragment 1

Fast myosin S1 can be separated into two isozymes with either of both alkali light chains, S1(A1) and S1(A2). S1 isozymes, differing only in alkali light chains, might give a clue to probe the role of alkali light chains. Separation of these isozymes has been achieved analytically by native gel electrophoresis (Hoh et al., 1976; Hozumi et al., 1979; Lowey et al., 1979). S1 isozymes were also prepared by ion-exchange chromatography (Yagi and Otani, 1974; Weeds and Taylor, 1975) and high-performance liquid chromatography (Toste, 1980). Affinity chromatography has also been applied, employing as the immobilized ligands, antibody against alkali light chains (Silberstein and Lowey, 1981), actin (Trayer et al., 1977) and nucleotide (Wagner, 1977; Winstanley et al., 1979). Although affinity chromatography succeeded in complete separation of both isozymes, application of this method has been limited for technical reasons. Alkali light chains, especially A1, are supposed to be involved in actin-myosin interaction, but their role in situ is still disputable (Sivaramakrishnan and Burke, 1982). In addition, ATPase activity in the absence of actin has not been sufficiently investigated. Furthermore, little information is available on fish S1.

In this section, S1 was prepared from ordinary muscle of tilapia and bigeye tuna as well as rabbit fast muscle, and each S1 separated into S1(A1) and S1(A2) by anion-exchange chromato-

graphy. Further attempts were made to elucidate their kinetics both in the absence and presence of actin.

Materials and Methods

Materials

Fresh specimens of tilapia and bigeye tuna were obtained, and stored at -80°C until used. Ordinary muscle was excised, and any trace of dark muscle was removed macroscopically.

The following procedures were carried out at 0 - 4°C unless otherwise stated.

Preparation of myosin

The sampled muscle was coarsely minced, washed with 5 volume of 6 mM sodium phosphate buffer (pH 7.0), and centrifuged at 5,000 x g for 10 min. This procedure was repeated two or three more times. The precipitate was extracted with 0.45 M KCl, 5 mM $MgCl_2$, 5 mM ATP, and 0.1 mM DTT (pH 6.4) for 15 - 30 min. The supernatant was then diluted 9 fold with ice-cold distilled water, and centrifuged at 5,000 x g for 10 min. The pellet was dissolved in 0.02 M Tris-maleate (pH 7.5) containing 0.5 M KCl and 0.1 mM DTT, and to it were added 10 mM ATP, 5 mM $MgCl_2$ and 0.5 mM EGTA (pH 7.5). The mixture was then subjected to ammonium sulfate fractionation and 40 - 60% saturated-fraction was obtained. Rabbit myosin was prepared from back (fast) muscle according to Kielly and Bradley (1956).

Preparation of S1

S1 was prepared with reference to Weeds and Taylor (1975) and Okamoto and Sekine (1985) using α-chymotrypsin (Sigma).

The above myosin fraction was extensively dialyzed against 20 mM phosphate buffer (pH 7.0) containing 0.12 M NaCl, 0.1 mM EDTA and 0.1 mM DTT, and the protein concentration was adjusted to about 10 mg/ml. Digestion was performed at α-chymotrypsin/myosin ratio of 1/130 (w/w) at 10°C for 30 min, and was terminated by addition of 0.2 mM PMSF. In case of rabbit myosin, digestion was performed at α-chymotrypsin/myosin ratio of 1/100 (w/w) at 25°C for 10 min. The digest was dialyzed overnight against 50 mM Tris-HCl (pH 8.0) containing 20 mM NaCl, and centrifuged at 10,000 x g for 30 min. Subsequently, the supernatant was applied to column chromatography. In case of bigeye tuna, protease inhibitors such as PMSF, Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK), antipain and pepstatin A (Sigma) were used, because proteolytic degradation was marked during purification procedure.

Isolation of S1 isozymes

S1 isozymes were isolated by anion-exchange chromatography essentially according to Weeds and Taylor (1975). Crude S1 fraction was applied to a DEAE-Toyopearl column (2.5 x 40 cm) equilibrated with 50 mM Tris-HCl (pH 8.0) containing 20 mM NaCl, and eluted with a linear gradient up to 0.15 M NaCl. Elution pattern was traced by absorbance at 280 nm. The fractions of S1 isozymes were separately pooled, concentrated by ultrafiltration (Amicon PM30 membrane), and used for the following experiments.

Preparation of actin

F-actin was prepared from acetone-dried powder of rabbit back (fast) muscle according to Spudich and Watt (1971).

Measurement of ATPase activity

Ca-ATPase activity was measured in a reaction mixture consisting of 25 mM Tris-maleate (pH 6 - 8.5) or glycine-KOH (pH 8.5 - 11), 5 mM $CaCl_2$, 50 mM KCl and 1 mM ATP at 25°C at S1 concentration of 0.03 - 0.05 mg/ml. In establishing the temperature-activity relationship, the activity was measured in 25 mM Tris-maleate (pH 7.5) at 20 - 45°C. EDTA-ATPase activity was measured as in case of Ca-ATPase activity, except that 5 mM EDTA was added instead of 5 mM $CaCl_2$, and KCl concentration was 0.5 M.

Actin-activated Mg-ATPase activity was measured at 25°C in a reaction mixture consisting of 25 mM Tris-maleate (pH 8.0), 1 mM $MgCl_2$, 1 mM ATP and 0 - 100 mM KCl, in the presence of 0.1 - 1 mg/ml of actin.

The liberated phosphorus was determined according to Fiske and SabbaRow (1925).

<u>Other analytical methods</u>

SDS-PAGE was carried out as described in Section 1 of Chapter I, using 15% slab gels. Protein concentration was determined according to Lowry <u>et al</u>. (1951) or by using the following extinction coefficients at 280 nm: 0.75 and 1.1 for S1 and actin, respectively.

Results and Discussion

<u>Isolation of S1 isozymes</u>

SDS-PAGE patterns of myosin and crude S1 from tilapia, bigeye tuna and rabbit are shown in Fig. III-1-1. DTNB light chain disappeared during chymotryptic digestion, completely from

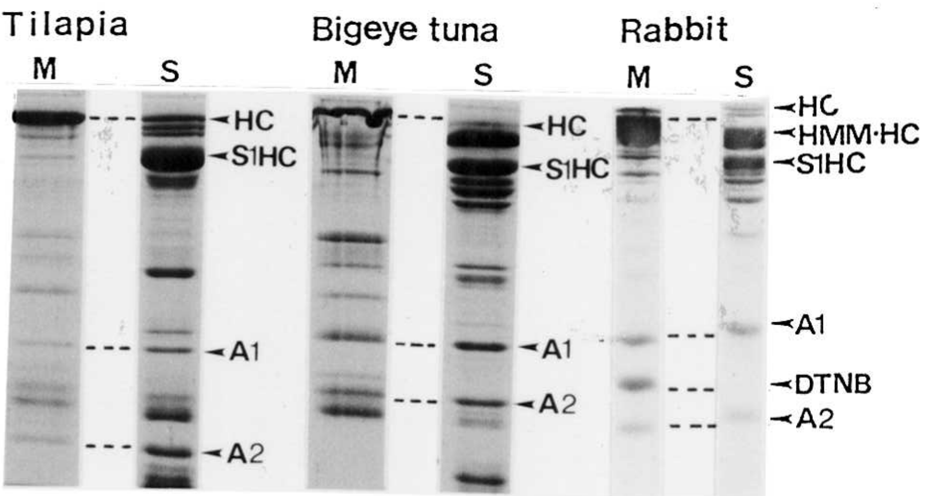


Fig. III-1-1. SDS-PAGE patterns of myosin (M) and crude S1 fraction (S) prepared from the ordinary muscle of tilapia, bigeye tuna and rabbit fast muscle. 15% gels. Abbreviations : HC = myosin heavy chain; HMM, heavy meromyosin; A, actin; A1 and A2, alkali light chains 1 and 2; DTNB, DTNB light chain.

rabbit myosin, and mostly from tilapia and bigeye tuna myosins. It is known that Ca or Mg protects cleavage at the S1/S2 junction by binding to DTNB light chain (Bagshaw, 1977; Balint _et al_., 1978). Thus, it is quite natural that DTNB light chain becomes susceptible to proteolysis in the absence of these cations. However, fish DTNB light chain showed some resistance against α-chymotrypsin.

The elution profile of tilapia S1 isozymes is shown in Fig. III-1-2A. The isozymes were eluted in the order of S1(A1) and S1(A2) as has been reported for rabbit S1 isozymes (Weeds and Taylor, 1975). Both isozymes were separated well from each other by single chromatography. Each S1 was devoid of any trace of DTNB light chain, and was associated with A1 or A2 stoichiometrically (1:1) (data not shown).

In case of bigeye tuna S1, degradation proceeded during chromatography, especially in the absence of protease inhibitors, and complicated elution pattern was obtained, and the recovery was very low. In spite of this, S1 isozymes of high purity were successfully obtained (data not shown).

As shown in Fig. III-1-2B, rabbit S1 isozymes were not separated under the chromatographic conditions adopted. However, S1(A1) obtained was proved to be of high purity, while S1(A2) was contaminated with S1(A1) up to 10%. This elution profile is in good accordance with the previous report (Weeds and Taylor, 1975; Sivaramakrishnan and Burke, 1981). The present chromatographic conditions distinguished the net charge of the difference peptide of A1, since light chain-based S1 isozymes seem to

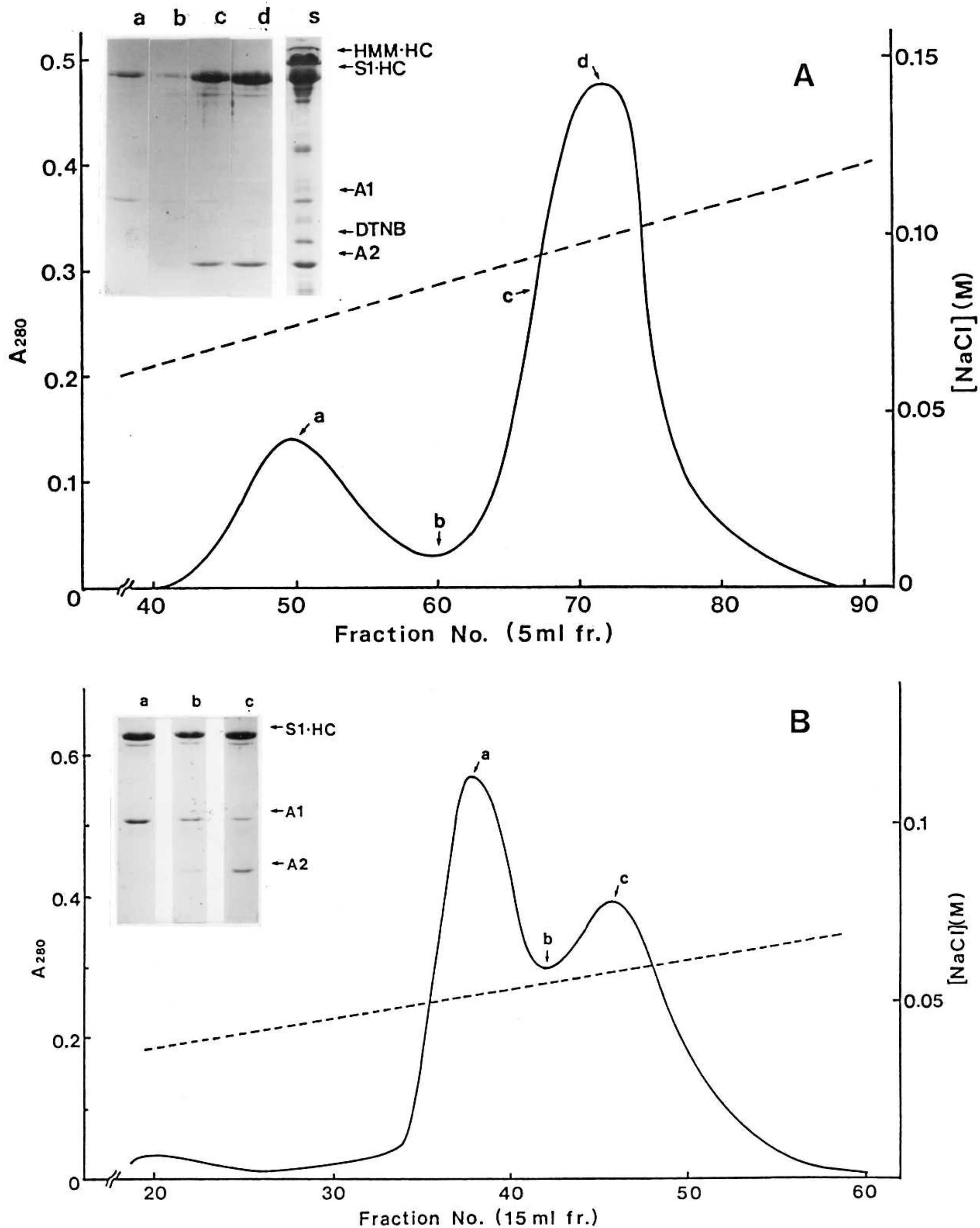


Fig. III-1-2. Elution profiles of S1 isozymes of tilapia (A) and rabbit (B) from a DEAE-Toyopearl column (2.5 x 40 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8.0) containing 20 mM NaCl. Elution was performed with a linear gradient up to 0.15 M NaCl at a flow rate of 40 ml/h. The inserted figures represent SDS-PAGE patterns of the fractions indicated by arrow. Abbreviations: DTNB, DTNB light chain; HC, heavy chain; HMM, heavy meromyosin. The letter 's' represents the applied sample.

differ only in this peptide. It has been reported that S1 isozymes can also be distinguishable in the interaction with immobilized nucleotide such as Sepharose-ADP only at very low ionic strength in the absence of Mg (Wagner, 1977; Winstanley et al., 1979; Burke et al., 1981). G-actin-Sepharose column has been used for separation of these isozymes (Winstanley et al., 1979). Lowey et al. (1979) isolated them by immunosorbents specific to the difference peptide of A1. These affinity chromatographic methods can well resolve S1 isozymes, but are troublesome and not suitable for preparation in a mass scale.

Effect of pH on Ca- and EDTA-ATPase activity

pH-Activity curves of Ca-ATPase of tilapia and rabbit S1 are shown in Fig. III-1-3. Minimum activity (ca. 0.8 μmol Pi/min·mg) was clearly observed at pH 7.0 - 7.5 for both tilapia S1 isozymes, whereas that of rabbit S1 was ambiguous. In case of tilapia S1 isozymes, the activity was higher for S1(A2), while vice versa for rabbit S1 isozymes. For both tilapia and rabbit S1, the activity at alkaline pH was very high, being above 4 μmol Pi/min·mg at pH 10. In case of bigeye tuna, the activity was generally higher with S1(A2) than with S1(A1) (data not shown). It seems likely that associated light chain might reflect pH-dependence of Ca-ATPase activity. On the other hand, Yagi et al. (1977) prepared two HMM isozymes and found no significant differences between their Ca-ATPase activities. Nakata et al. (1985) obtained two different S1's from each of rabbit fast skeletal and porcine cardiac myosins, and revealed that they were different in pH-dependence of Ca-ATPase and with reference to reactive lysine residue, though they were identical in heavy

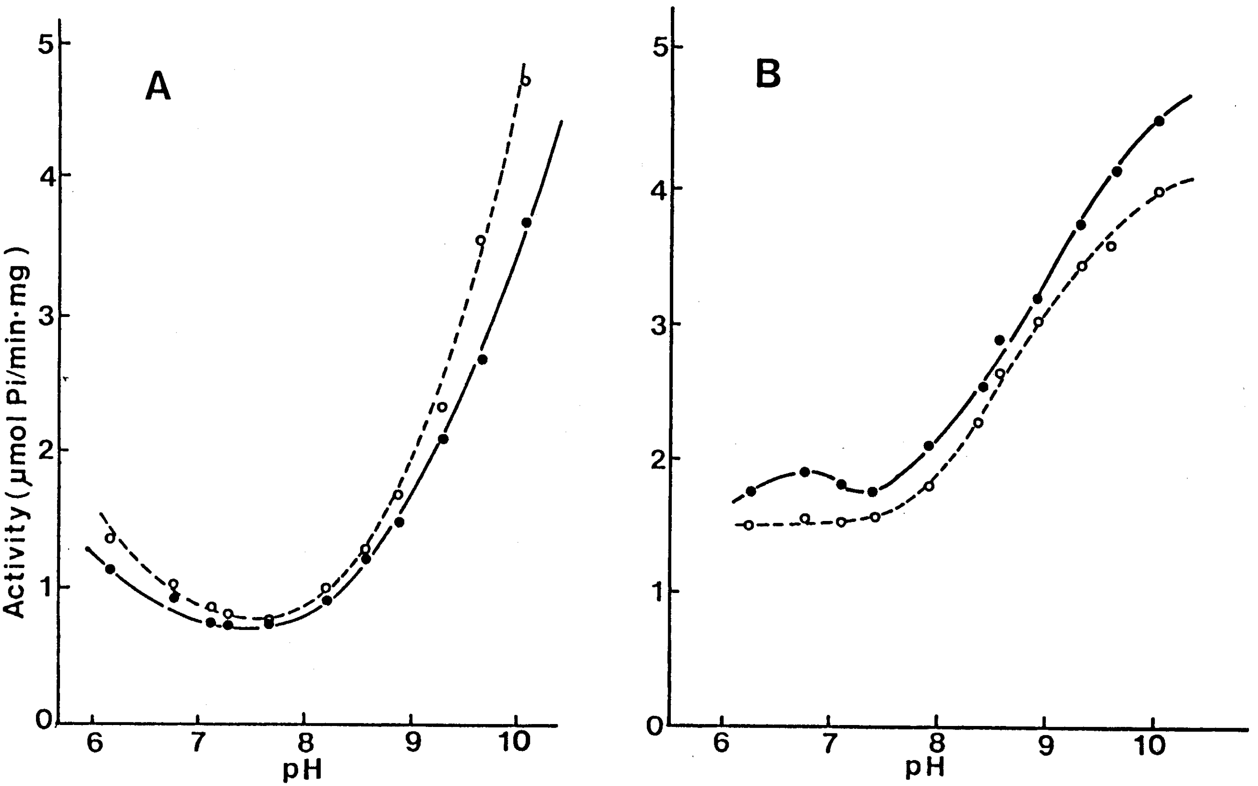


Fig. III-1-3. pH-Activity curves of Ca-ATPase activity of S1(A1) and S1(A2) of tilapia (A) and rabbit (B). Closed and open circles represent S1(A1) and S1(A2), respectively.

chain and light chain compositions.

pH-Activity curves of EDTA-ATPase of tilapia and rabbit S1 are shown in Fig. III-1-4. The maximum activity was observed at around pH 9, and the activity was rapidly decreased with the decrease of pH. Activity at neutral and acidic pH was very low throughout all the S1 isozymes. As was the case with Ca-ATPase activity, S1(A1) showed higher activity with tilapia, and lower activity with rabbit. In this connection, it was reported that EDTA-ATPase activity of rabbit S1(A2) was constantly higher by 5% than that of S1(A1) irrespective of KCl concentration (Mrakovcic-Zenic et al., 1981). The present results were unexpectedly contrary to their finding, but demonstrate that S1 isozymes showed different ATPase activities even in the absence of actin at particular pH range adopted.

Information on the pH-dependence of S1 ATPase activity is quite poor. The ATPase profiles of carp S1 were very similar to those of rabbit S1 in respect to KCl concentration and pH dependences, and actin-activated Mg-ATPase (Ikariya et al., 1981).

Temperature-activity curves of tilapia and rabbit S1 are shown in Fig. III-1-5. Both tilapia S1 isozymes showed optimum temperature at around 40°C, and the temperature-dependency was almost the same between both isozymes. In case of rabbit S1, the maximum activity was observed at around 45° C for both isozymes, and the activity was partially retained even at 50°C, indicating the higher thermostability of these S1's compared to tilapia counterparts. Rabbit S1(A1) generally showed higher activity than S1(A2). The activity was several times higher than

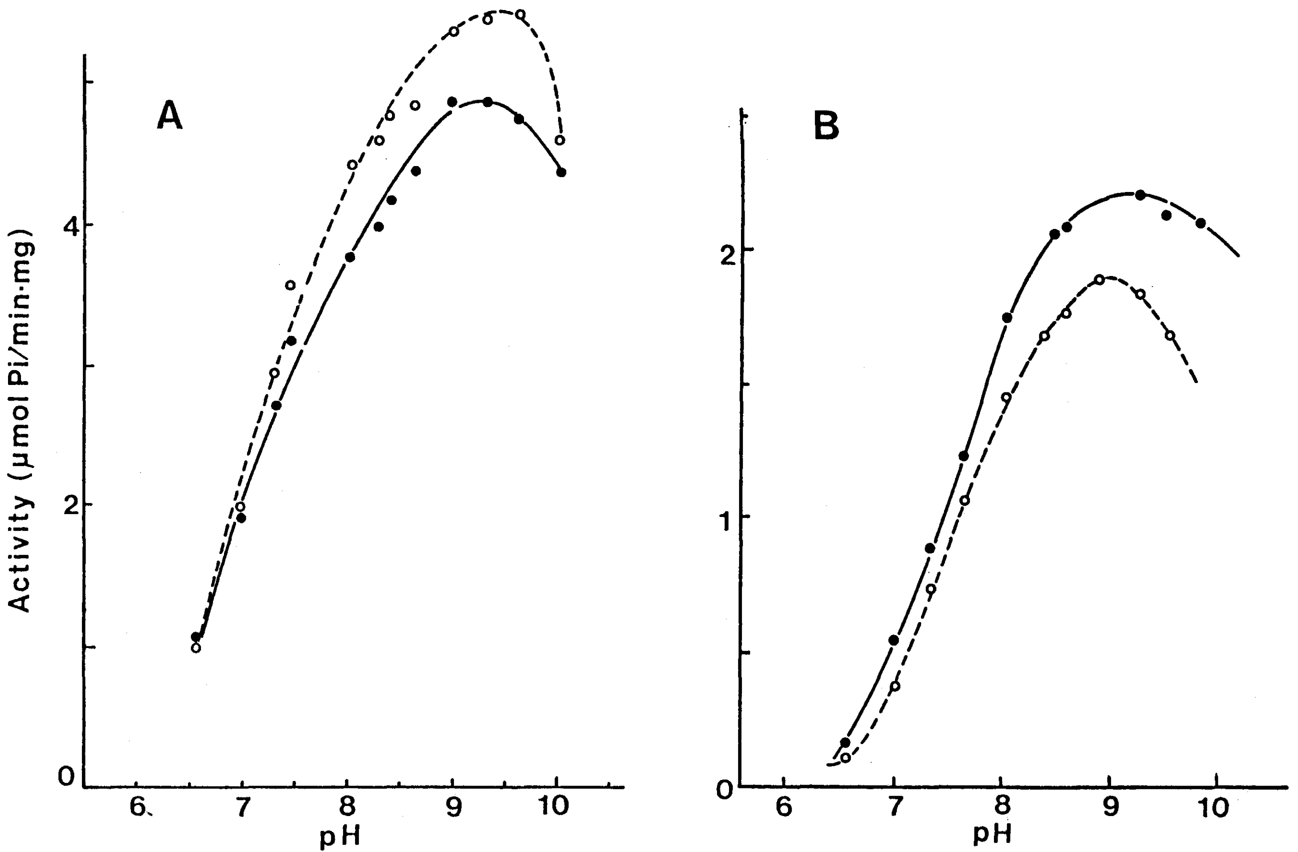


Fig. III-1-4. pH-Activity curves of EDTA-ATPase activity of S1(A1) and S1(A2) of tilapia (A) and rabbit (B). Closed and open circles represent S1(A1) and S1(A2), respectively.

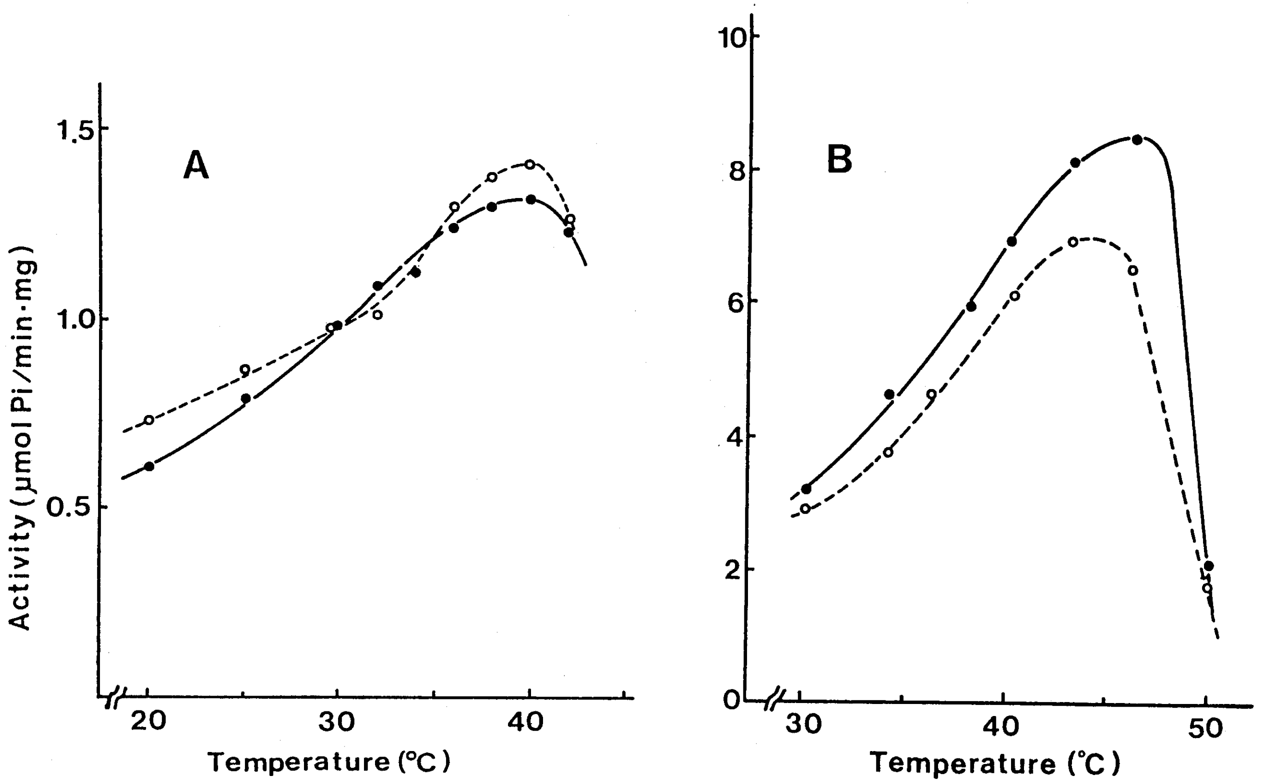


Fig. III-1-5. Temperature-activity curves of Ca-ATPase activity of S1(A1) and S1(A2) of tilapia (A) and rabbit (B). Closed and open circles represent S1(A1) and S1(A2), respectively.

tilapia S1. On the other hand, optimum temperature of bigeye tuna S1 was observed at around 25°C, suggesting the thermolability of this S1 (data not shown).

From the above results, significant differences in ATPase activity seem to exist between the isozymes under particular conditions, although the kinetics of S1 isozymes in the absence of actin have been regarded the same.

The effect of KCl concentration on the actin-activated Mg-ATPase activity of tilapia S1 isozymes is shown in Fig. III-1-6. Differences in actin-activation profiles were clearly observed between S1(A1) and S1(A2). Irrespective of KCl concentration, tilapia S1(A2) was much more activated by rabbit actin than S1(A1). However, as the KCl concentration was increased, the differences between both isozymes were decreased: the apparent affinity for actin ($1/K_m$), intercept of the abscissa, decreased and the maximum velocity (V_{max}), reciprocal of the intercept of the ordinate, tended to increase.

When the effect of KCl concentration was examined separately for both tilapia S1 isozymes (Fig. III-1-7), both kinetic values were critically affected with S1(A1), but in case of S1(A2), only the K_m was largely affected, but V_{max} remained almost constant. Thus, the effect of KCl concentration on actin-activation profile is clearly different between both isozymes.

In case of rabbit S1, KCl concentration also affected the actin-activation profile (Fig. III-1-8). As KCl concentration was increased, the differences between both isozymes were decreased. At low KCl concentration, S1(A1) was much more activated than S1(A2). In 0.1 M KCl, the activation was found to

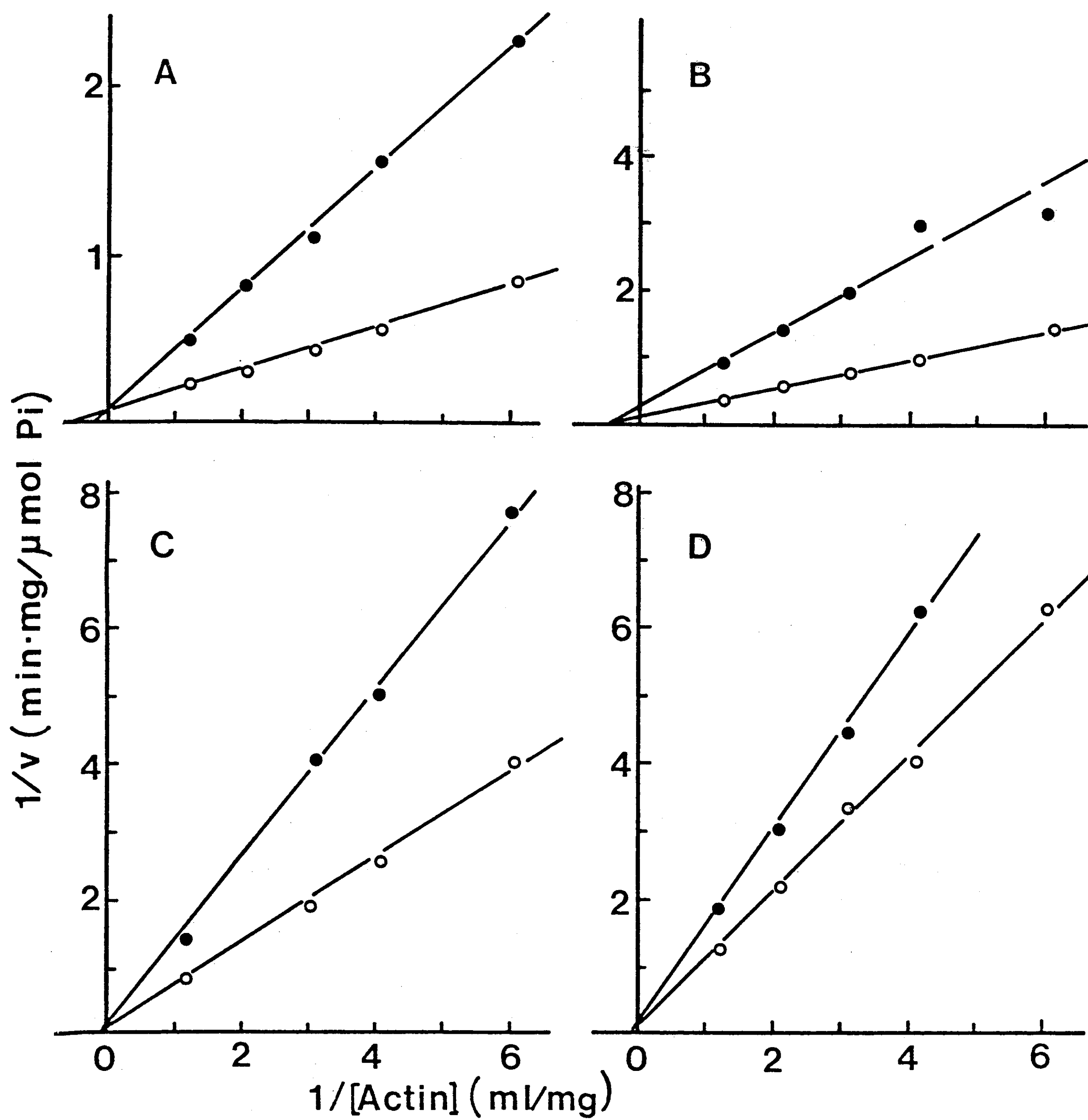


Fig. III-1-6. Lineweaver-Burk plots of actin-activated Mg-ATPase activity of tilapia S1(A1) and S1(A2) at various KCl concentrations. KCl concentration: A, 0 mM; B, 20 mM; C, 60 mM; D, 100 mM. Closed and open circles represent S1(A1) and S1(A2), respectively.

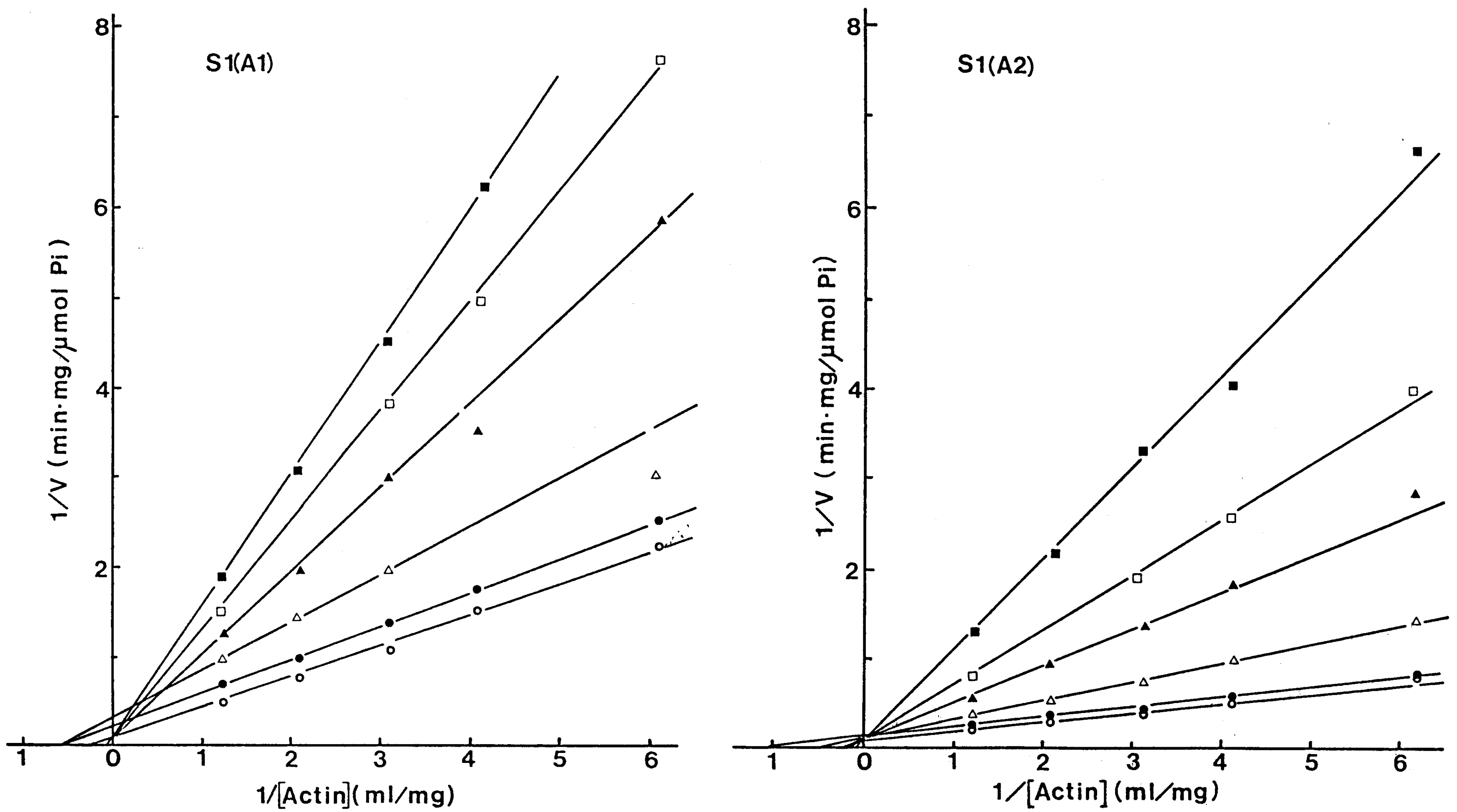


Fig. III-1-7. Effect of KCl concentration on actin-activated Mg-ATPase activity of tilapia S1(A1) and S1(A2). KCl concentration : ○, 0 mM; ●, 6 mM; △, 20 mM; ▲, 40 mM; □, 60 mM; ■, 100 mM.

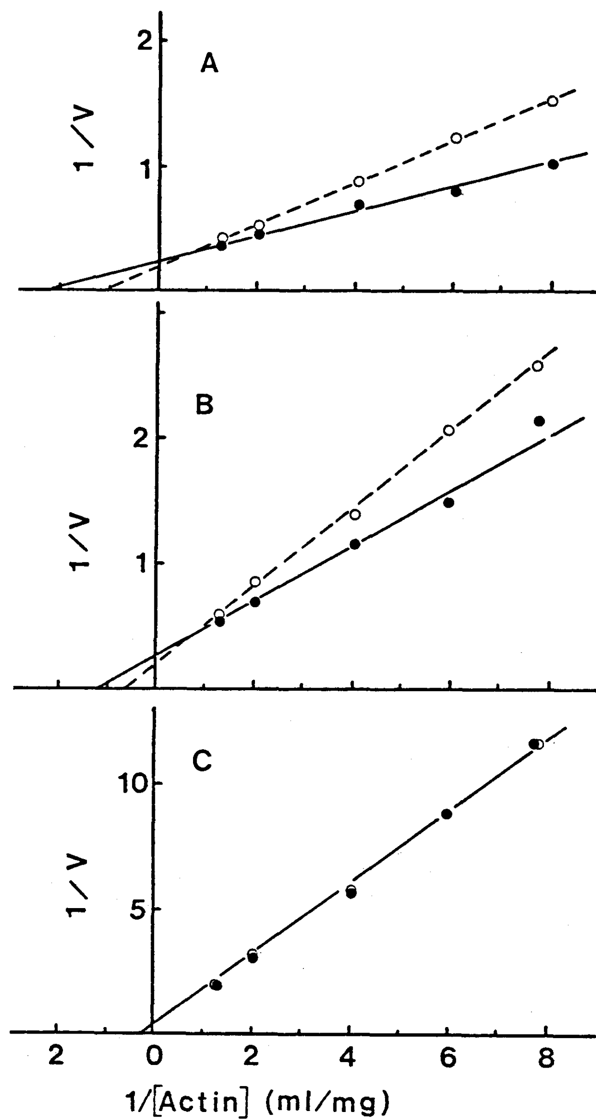


Fig. III-1-8. Lineweaver-Burk plots of actin-activated Mg-ATPase activity of rabbit S1(A1) and S1(A2) at various KCl concentrations. A, 20 mM KCl; B, 40 mM KCl; C, 100 mM KCl. Closed and open circles represent S1(A1) and S1(A2), respectively.

be the same. These activation profiles are quite different from those with tilapia S1 isozymes.

The kinetic constants of tilapia and rabbit S1 isozymes at each KCl concentration are shown in Tables III-1-1 and -2, respectively. In both S1, the affinity for actin generally decreased with the increase of KCl concentration. Tilapia S1(A1) and S1(A2) resembled rabbit S1(A2) and S1(A1), respectively, in respect of KCl effect. These results might be due to the intrinsic properties of both S1, or be ascribed to the differences in reaction profiles with rabbit actin. In this connection, Wagner *et al*. (1979) described that at high KCl concentration, the extrapolated V_{max} values are less reliable because of high K_m values.

In case of bigeye tuna S1 isozymes, actin-activation profiles were rather similar to those of rabbit S1 isozymes, whose S1(A1) was much more activated by actin than S1(A2) (Fig. III-1-9). In 20 mM KCl, K_m and V_{max} values were 40.5 μM and 1.0 μmol Pi/min·mg for S1(A1), and 28.6 μM and 1.3 μmol Pi/min·mg for S1(A2), respectively.

Wagner (1977) claimed that both V_{max} and K_m values were higher in HMM(A2) than in HMM(A1), indicating that the kinetic differences were intrinsic, and not secondarily caused by proteolysis at the head-tail junction. In this connection, Wagner (1981) described that the heavy chain could dominate actin-activated ATPase of myosin.

Wagner *et al.* (1979) claimed that the increase of ionic strength may change the rate-limiting process in the steady-state

Table III-1-1. Effect of KCl concentration on the kinetic constants of actin-activated Mg-ATPase activity of tilapia S1(A1) and S1(A2)

KCl concn.	S1(A1)		S1(A2)	
	K_m *1	V_{max} *2	K_m	V_{max}
0 mM	95.2	10.0	28.0	7.1
6 mM	47.6	4.5	25.0	7.1
20 mM	47.6	3.3	47.6	7.1
40 mM	298	10.0	95.2	8.3
60 mM	340	10.0	140	10.0
100 mM	396	10.0	397	12.5

*1 μM. *2 μmol Pi/min·mg.

Table III-1-2. Effect of KCl concentration on the kinetic constants of actin-activated Mg-ATPase activity of rabbit S1(A1) and S1(A2)

KCl concn.	S1(A1)		S1(A2)	
	K_m *1	V_{max} *2	K_m	V_{max}
0 mM	10.1	3.7	23.8	4.1
6 mM	12.9	3.1	17.0	2.6
20 mM	14.9	2.1	16.4	1.8
40 mM	34.0	2.3	34.0	1.8
60 mM	68.0	1.8	-	-
100 mM	68.0	1.8	68.0	1.8

*1 μM. *2 μmol Pi/min·mg.

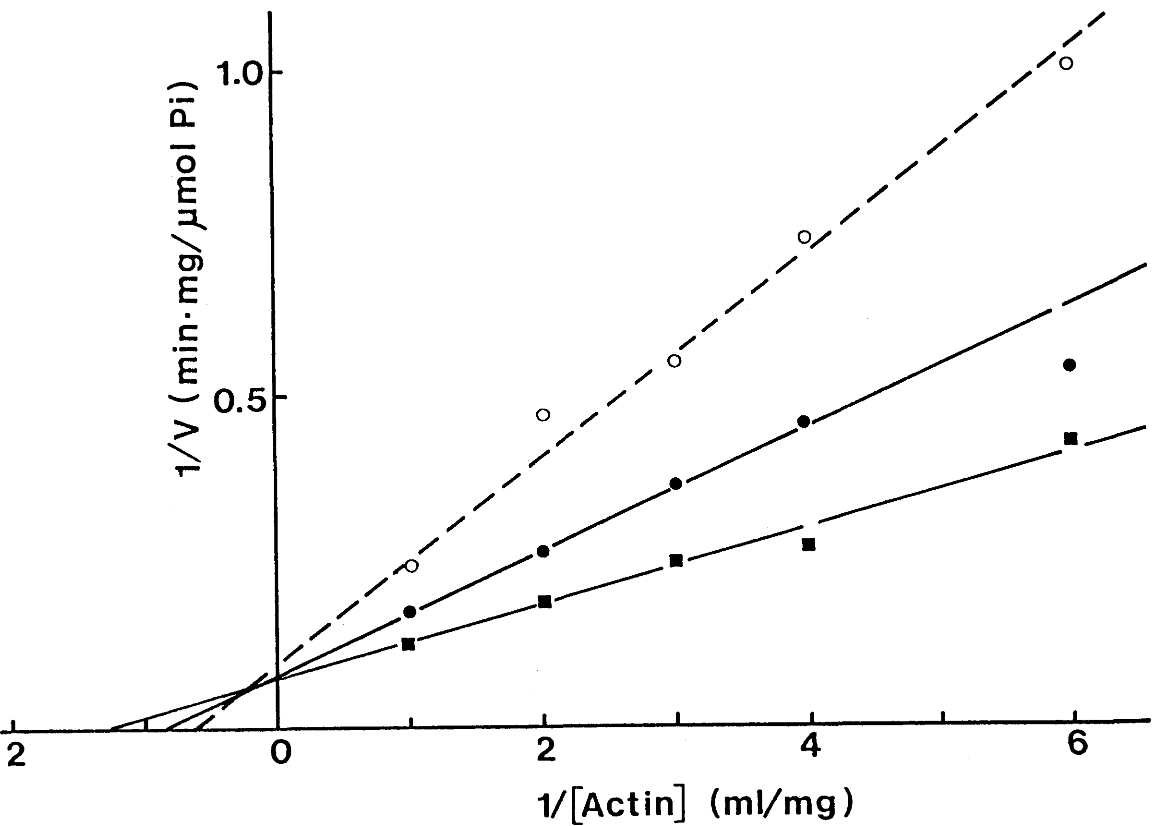


Fig. III-1-9. Lineweaver-Burk plots of actin-activated Mg-ATPase activity of bigeye tuna S1(A1) and S1(A2). Closed and open circles represent S1(A1) and S1(A2), respectively, in 20 mM KCl. Closed square represents S1(A1) in the absence of KCl.

hydrolysis of ATP by acto-S1(A1). Wagner (1981) also observed that A1 of S1(A1) was proteolytically cleaved into a small fragment during storage, resulting in an actin-activated ATPase similar to S1(A2). It follows that alkali light chains might also be involved in actin-myosin interaction.

At low ionic strength, in either the presence or absence of Ca, the binding of regulated actin was always stronger for S1(A1) than for S1(A2) (Trayer and Trayer, 1985). As the ionic strength was increased, the differential binding between these isozymes was still maintained in the presence of Ca, but not in its absence (Trayer and Trayer, 1985). Henry et al. (1985) prepared the difference peptide from A1 by thrombic digestion, and demonstrated by affinity column and NMR studies that this fragment directly interacted with actin. The terminal α-N-trimethyllysine residue was supposed to participate in the interaction (Henry et al., 1982). In this connection, the N-terminal regions of essential light chains from cardiac and slow muscle myosins are similar to that of A1 (Weeds, 1975, 1976). Slow muscle myosin S1 which contained the less anodic essential light chain (LC1a) also bound more tightly to immobilized actin (Winstanley et al., 1979). It follows that 'A1 type' light chains could regulate the actin-myosin interaction.

On the other hand, unlike S1 or HMM, Lineweaver-Burke or Eadie-Hofstee plots for myosin are biphasic, with two values for V_{max}, one corresponding to a low K_m and the other to a much higher K_m (Pope et al., 1981). They suggested that the presence of a particular alkali light chain does not influence the V_{max} of actomyosin under ionic conditions approximately physiological.

Sivaramakrishnan and Burke (1982) found that actin activated the Mg-ATPase activity of free heavy chain in the same manner as occurs with the native S1, claiming that alkali light chains are not essential for ATPase activity. Free heavy chain has also been isolated by immunoadsorption using NH_4Cl, and was shown to possess a similar ATPase to the native S1, although the activity was somewhat decreased (Wagner and Giniger, 1981).

Section 2

Preparation and Characterization of Hybrid Subfragment 1

In the preceding section, it was established that alkali light chains are involved in actin-myosin interaction, which was, however, markedly affected by salt concentration. On the other hand, the partial involvement of the heavy chain in kinetic differences between both S1 isozymes seemed to be also probable. Enzymatic properties of both S1's, which differ from each other only in light chain, might give more direct evidence for involvement of alkali light chains in kinetics of myosin.

Attempts have been made to obtain myosin hybrids (light chain exchanged) with intrinsic ATPase activity and actin-binding ability (Stracher, 1969; Dreizen and Richards, 1972; Okamoto and Yagi, 1977). Essential light chains of scallop myosin can be cross-hybridized with homologous light chains by mild heat treatment in the absence of regulatory light chain (Ashiba and Szent-Györgyi, 1985). In case of skeletal muscle myosin, however, light chains are associated to the heavy chain more tightly, which makes it difficult to carry out cross hybridization experiments. Wagner and Weeds (1977) succeeded in alkali light chain exchange at high salt concentration (4.7 M NH_4Cl). Ueno and Morita (1984) proposed to use 4.7 M ammonium buffer at pH 9.9 instead.

When bound to rabbit fast S1 heavy chain, essential light chains, whether from fast, slow or cardiac myosins, gave rise to comparable actin-activated ATPase activities (Wagner and Weeds,

1977). S1 consisting of fast muscle S1 heavy chain and cardiac essential light chain behaved like S1(A1) (Wagner, 1981). However, hybridization of essential light chains between taxonomically distinct animals has not yet been attempted.

In this section, hybrid S1's consisted of rabbit S1 heavy chain and fish alkali light chains were prepared, and their ATPase activities were compared to those of native S1 both in the presence and absence of actin.

Materials and Methods

Rabbit S1(A1) was prepared as described in Section 1 of this chapter. Light chains were isolated according to the method described in Section 1 of Chapter II.

Preparation and isolation of hybrid S1

Hybrid S1 was prepared essentially according to Wagner and Weeds (1977), and Ueno and Morita (1984).

Rabbit S1(A1) (50 μM) and alkali light chains (100 - 250 μM) were dissolved in 4.7 M ammonium buffer (pH 9.9) containing 2 mM DTT and 2 mM EDTA, and incubated at 4°C for 30 min. The mixture was dialyzed successively against 50 mM imidazole-HCl (pH 8.0) containing 2 M NH_4Cl, the same buffer containing 1 M NH_4Cl, and the same buffer without NH_4Cl for 4 h. The dialyzate was then applied to a DEAE-Toyopearl column equilibrated with the same buffer, and proteins were eluted with a linear gradient up to 0.15 M NaCl. The hybrid S1 fractions were pooled and concentrated by ultrafiltration, and used for the following experi-

ments.

Measurement of ATPase activity

Ca-ATPase and actin-activated Mg-ATPase activity were measured as described in Section 1 of this chapter.

Results and Discussion

Rabbit S1(A1) was first incubated with tilapia A1 and A2, and the hybrid S1's formed were subjected to DEAE-Toyopearl chromatography (Fig. III-2-1A). Unhybridized rabbit S1(A1) was first eluted, then S1 associating tilapia A1 [S1(tA1)], followed by S1 associating tilapia A2 [S1(tA2)].

Similar elution profiles were obtained when bigeye tuna A1 and A2 were hybridized to rabbit S1(A1), though rabbit S1(A1) and S1 associating bigeye tuna A1 [S1(bA1)] did not separate well (Fig. III-2-1B).

It was revealed for the first time that hybrid S1's were reconstituted with rabbit S1 heavy chain and fish alkali light chains. This is rather in accordance with the data in Chapter II that light chains from fish and rabbit have structures in common to some extent. The hybrid S1 thus prepared consisted of equimoles of heavy chain and light chain, as demonstrated by densitometric analysis of SDS-gel (data not shown).

Each hybrid S1 fraction was pooled, and ATPase activity was examined. The Ca-ATPase activity inherited that of rabbit S1(A1) used, irrespective of associated light chains (data not shown). However, the actin-activated Mg-ATPase activity was largely affected by associated light chain. As shown in Fig.

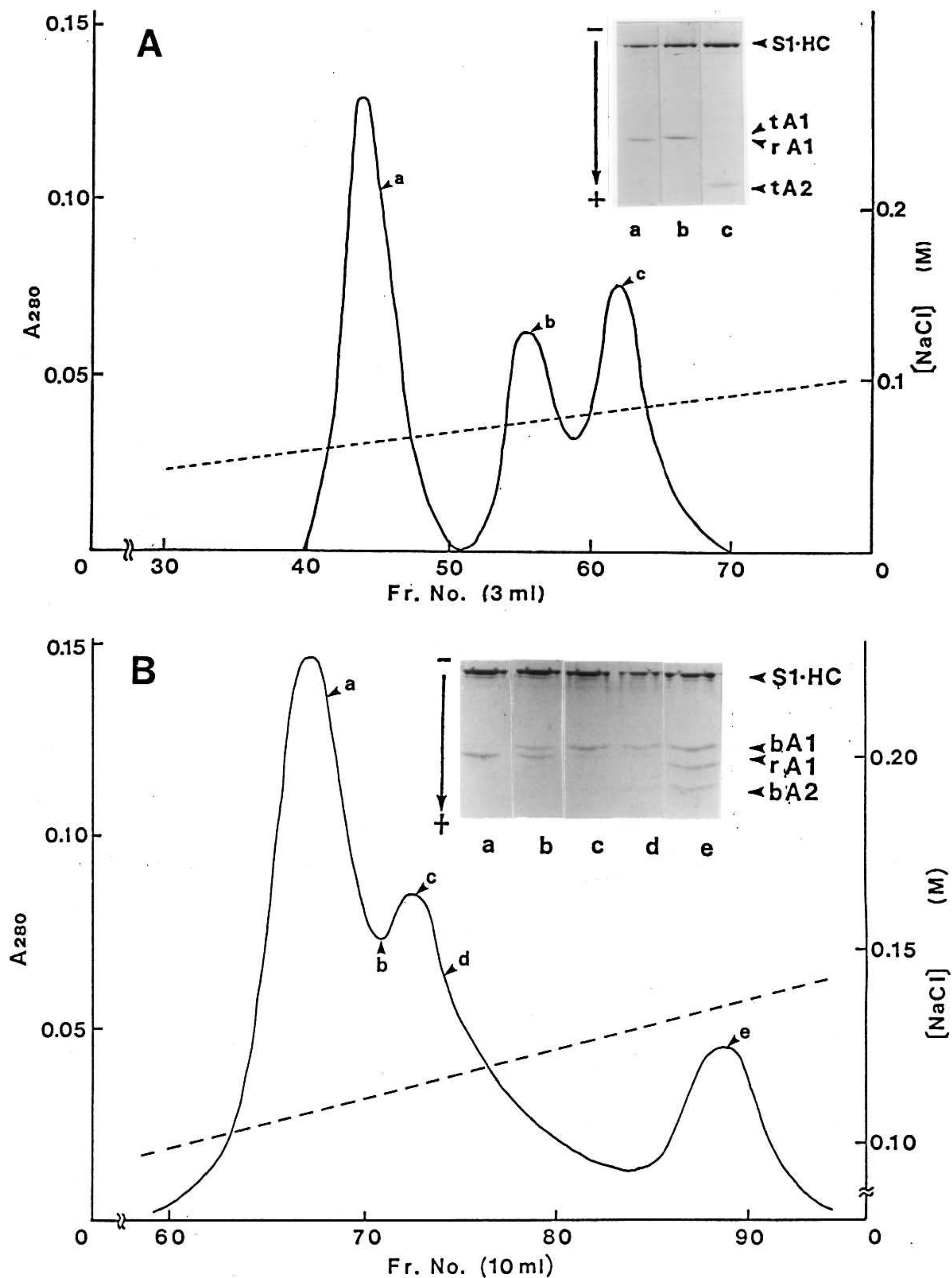


Fig. III-2-1. Elution profile of hybrid S1 consisting of rabbit S1 heavy chain and alkali light chains of tilapia (A) and bigeye tuna (B) from a DEAE-Toyopearl column (1 x 20 cm). Inserted figures represent the SDS-PAGE patterns of the indicated fractions. The letters t, b and r in inserted figures represent tilapia, bigeye tuna and rabbit, respectively.

III-2-2, the Lineweaver-Burk plots of this activity for hybrid S1's revealed similar kinetics observed with native S1 (Section 1 of this chapter). However, the difference between S1(tA1) and S1(tA2) was smaller than that between native ones. In case of S1(bA1) and S1(bA2), the activation profiles were very similar to native bigeye tuna S1(A1) and S1(A2), respectively (Fig. III-1-9). The kinetic constants of hybrid S1 in the presence of actin are shown in Table III-2-1. K_m values for S1(tA1) and S1(tA2) were very similar, 25.1 and 29.8 μM, respectively, in good agreement with native tilapia S1 isozymes. V_{max} of S1(tA2) was 4.1 μmol Pi/min·mg, being lower than that of native S1(A2), 7.2 μmol Pi/min·mg. On the other hand, the kinetic values of S1(bA1) and S1(bA2) were very similar to those of native bigeye tuna S1 isozymes: K_m for S1(bA1) and S1(bA2) were 23.0 and 47.4 μM, showing the higher affinity of S1(bA1) than S1(bA2). These data clearly indicate that alkali light chains are involved in the interaction of actin and myosin, in case of fish myosin as well.

Crasnier (1987) claimed that the hybrid S1 obtained under the same condition as adopted here was inactivated up to 60%. In the present experiment, ATPase activity of hybrid S1 was somewhat reduced probably due to high salt concentration. It seems likely that, during hybridization, heavy chain free of light chain suffered from an irreversible conformational change responsible for the inactivation.

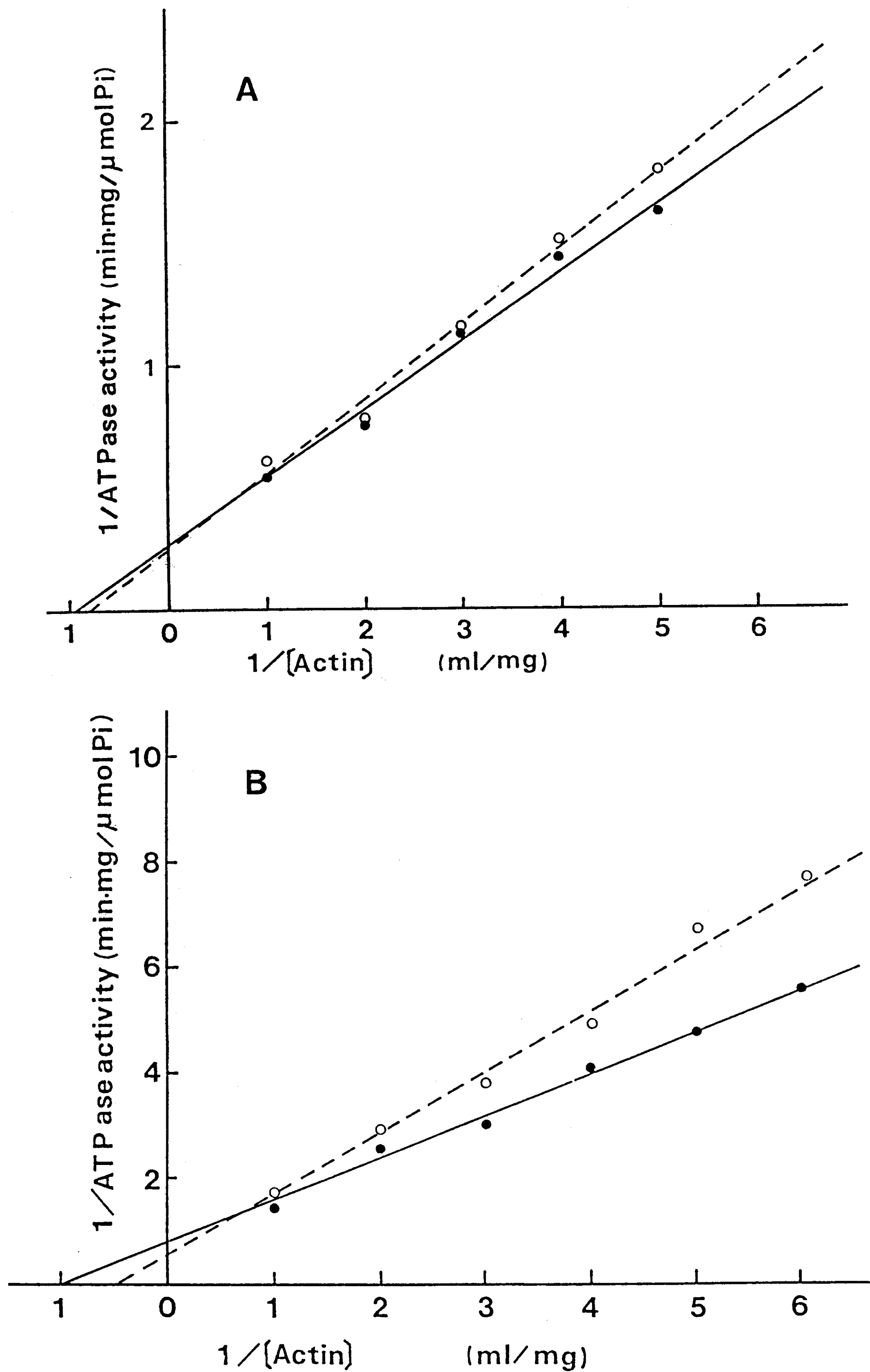


Fig. III-2-2. Lineweaver-Burk plots of actin-activated Mg-ATPase activity of hybrid S1 consisting of rabbit S1 heavy chain and alkali light chains of tilapia (A) and bigeye tuna (B). Closed and open circles represent S1(A1) and S1(A2), respectively.

Table III-2-1. Kinetic constants of actin-activated Mg-ATPase activity of hybrid S1

Associated light chain	Tilapia		Bigeye tuna		Rabbit
	A1	A2	A1	A2	A1
Km[*1]	25.1	29.8	23.0	47.4	14.8
Vmax[*2]	3.9	4.1	1.3	1.9	3.5

[*1] μM. [*2] μmol Pi/min·mg.

Section 3

Homogeneity of Heavy Chain in Subfragment 1 Isozymes

In the previous sections, it was demonstrated that kinetic differences really exist between S1 isozymes, especially in the presence of actin.

Actually, partial differences in primary structure have been reported for S1 heavy chains from rabbit (Starr and Offer, 1973; Pope et al., 1977; Tong and Elzinga, 1983) and chicken (Maita et al., 1987). However, Wagner and Weeds (1977) suggested that heavy chain heterogeneity almost equally distributed between S1 isozymes. Burke et al. (1986) isolated four species of S1 based on the differences of light and heavy chains, whose ATPase activities slightly differed from each other, and suggested that such differences could be due to different heavy chains. However, they regarded it unlikely that preferential binding exists between heavy and light chain isoforms. In this connection, Okamoto and Yount (1985) noticed a microheterogeneity in heavy chain sequence against the observation of Tong and Elzinga (1983), and ascribed this difference to a genetic variation in the rabbit used.

In this section, peptide maps and tryptic digestion patterns were compared between S1(A1) and S1(A2) in order to check the possibility of heterogeneity of heavy chain.

Materials and Methods

Peptide mapping

Peptide mapping of S1 isozymes was performed essentially according to Cleveland et al. (1977) and Whalen et al. (1979). Briefly, S1 was digested with α-chymotrypsin, *Staphylococcus aureus* V8 protease and subtilisin (Sigma) in the presence of SDS, and analyzed by SDS-PAGE using 12.5% polyacrylamide slab gels.

Tryptic digestion

Tryptic digestion of S1 isozymes was performed in 50 mM Tris-HCl (pH 7.5) containing 0.1 M KCl and 0.1 mM DTT, both in the absence and presence of rabbit F-actin. Protein concentration was in a range of 1.2 - 1.5 mg/ml. Digestion was performed at 10 and 25°C for tilapia and rabbit S1, respectively, with 1/100 (w/w) amount of trypsin for 5 - 60 min, and was terminated by addition of 5 fold (w/w) soybean trypsin inhibitor (Sigma, Type I). The digests were subjected to SDS-PAGE, and the fragmentation patterns stained with Coomassie brilliant blue were traced densitometrically.

Results and Discussion

Peptide maps of S1 isozymes from tilapia and rabbit are shown in III-3-1. Under the adopted conditions, A1 light chain was partially cleaved, and thus, the peptides possessing higher molecular weight than A1 have been derived from S1 heavy chain. The heavy chain-derived peptide maps were essentially the same between both S1 isozymes, irrespective of the protease used,

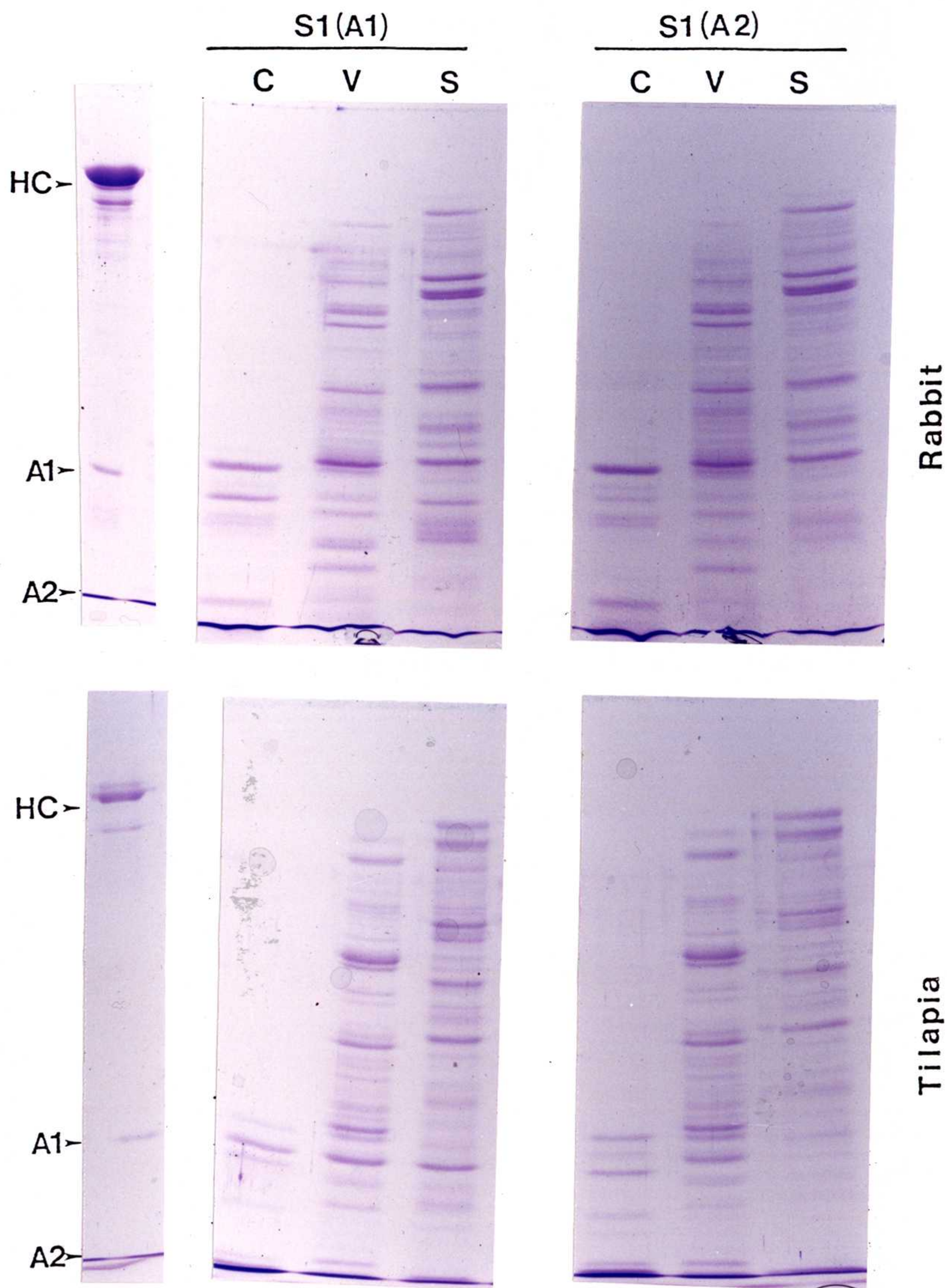


Fig. III-3-1. Peptide mapping of S1(A1) and S1(A2) of tilapia and rabbit in SDS gels. Abbreviations : C, α-chymotrypsin; V, Staphylococcus aureus V8 protease; S, subtilisin.

although the patterns were species-specific. These results indicate that structural differences are absent or negligible, if any, between the heavy chains of both S1 isozymes of each species.

Tilapia S1(A1) and S1(A2) gave rise to similar time courses of tryptic digestion (Fig. III-3-2). Both S1 isozymes gave three main fragments, 50 K, 25 K and 20 K. It is now established that S1 heavy chain is tryptically cleaved into three main fragments (Muhlrad and Hozumi, 1982; Mornet et al., 1984). However, in these experiments, mixture of isozymes were used. Therefore, this is the first finding that heavy chain of both S1 isozymes have essentially the same structures. On the other hand, A1 was more susceptible to tryptic digestion than A2. Essentially the same results were obtained with both rabbit S1's (data not shown). Tryptic digestion of S1(A1) released the difference peptide (Cardinaud, 1980). Proteolytic susceptibility of A1 has also been reported by Yamamoto and Sekine (1980). Incidentally, Kodama et al. (1987) reported that structures of squid mantle myosin and rabbit S1's are similar to each other, except that reactive lysil residue of the former resides on 50 K domain.

In the presence of actin, all S1 isozymes of both species gave rise to only two main fragments, 75 K and 25 K, suggesting that 20 K-50 K junction was protected by actin (data not shown). Mornet et al. (1979) found that actin protected S1 heavy chain from tryptic digestion at 20 K-50 K junction and from the resulting loss of acto-S1 Mg-ATPase activity. Tilapia S1 was more susceptible to trypsin than was rabbit S1:the former was cleaved into smaller fragments when digested, indicating that it was

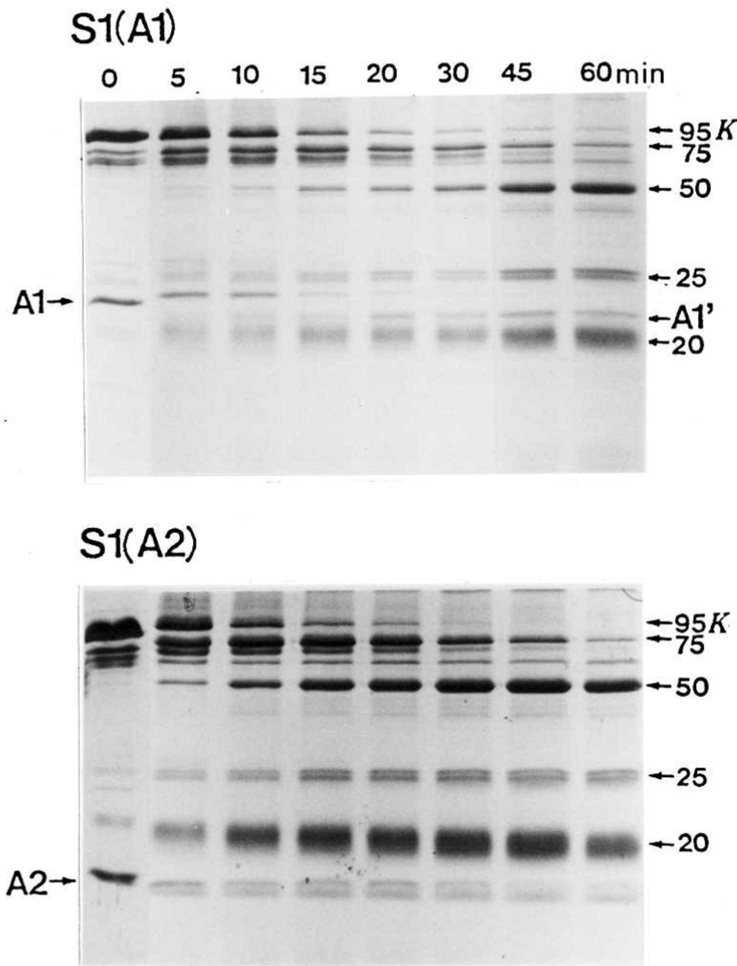


Fig. III-3-2. Time courses of tryptic digestion of tilapia S1(A1) and S1(A2). 15% SDS gels. The number at the top of each column represents digestion time.

split at many sites. So was the case with bigeye tuna S1 isozymes. S1 isozymes from both fishes seem to possess a fragile structure, as supported by the low optimum temperature of ATPase activity and high susceptibility to proteolysis (Section 1 of this chapter).

Tilapia S1(A1) and S1(A2) showed comparable time courses of the formation of major fragments during tryptic digestion (Fig. III-3-3). In this figure, only those of 95 K, 75 K and 50 K fragments are given. As digestion proceeded, 95 K fragment decreased rapidly at the initial stage, while 75 K fragment once increased a little, and then decreased gradually. 50 K fragment, on the other hand, increased along with the decrease of 95 K fragment. These fragmentation patterns are essentially the same between both isozymes. Time courses of the formation of 25 K and 20 K fragments were also similar between both isozymes (data not shown).

These tryptic digestion patterns, together with peptide mapping, showed that the heavy chains of both S1 isozymes could be completely or almost the same. Therefore, their kinetic differences might be ascribed to the differences between associated light chains.

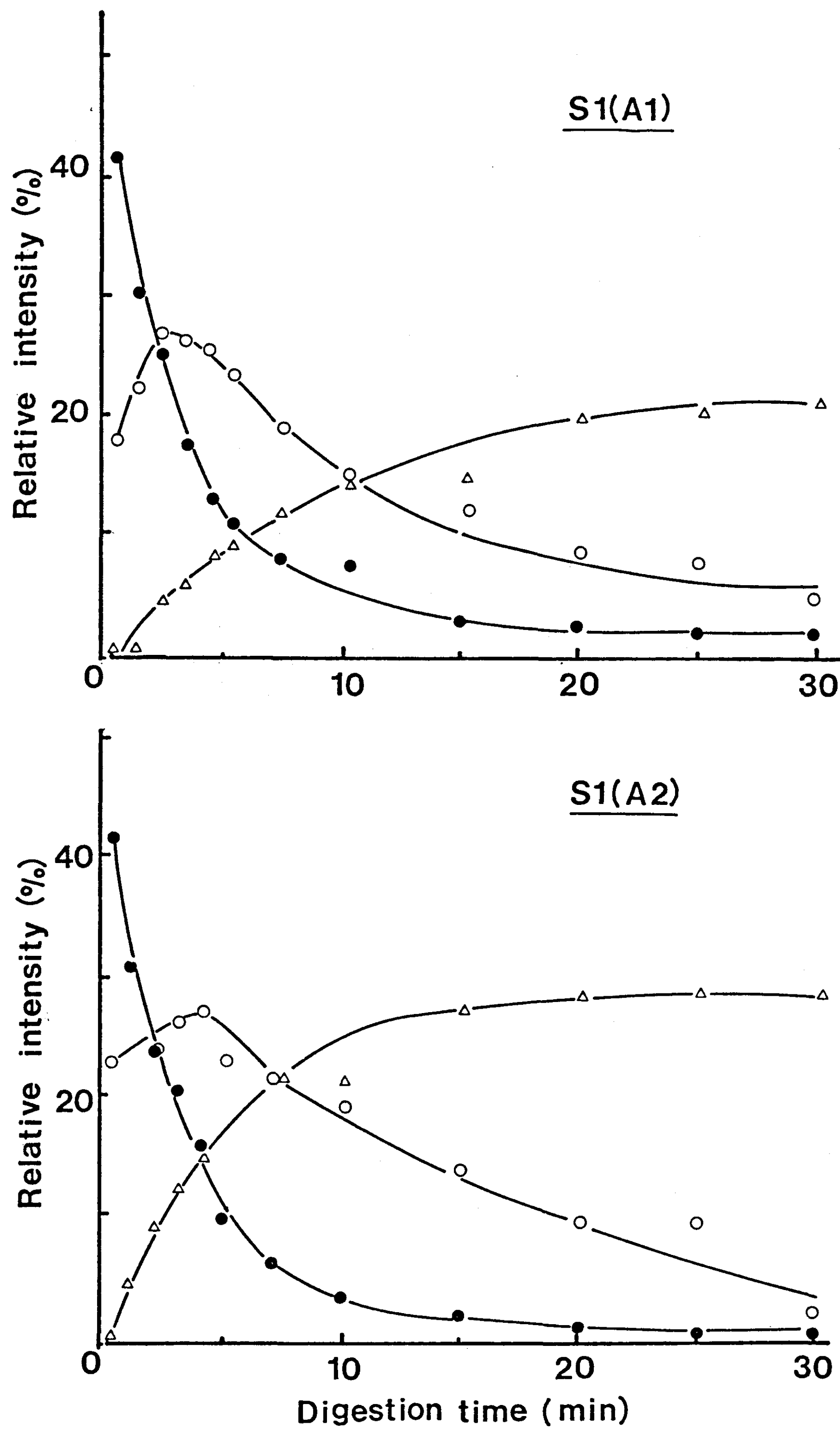


Fig. III-3-3. Time courses of the formation of major fragments during tryptic digestion of tilapia S1(A1) and S1(A2). ●, 95 K; ○, 75 K; △, 50 K fragments.

Section 4

Affinity of Alkali Light Chains for Heavy Chain and Actin

It is supposed that differences in net charge between A1 and A2 might affect the affinity of these light chains for the heavy chain, since the difference peptide of A1 contains many lysine residues and thus A1 is more positively charged than A2. On the other hand, the structure or the net charge of the heavy chain seems to be the same between the S1 isozymes as described in the preceding section. Therefore, such affinity differences might affect the physical or chemical properties of S1 isozymes. However, the affinity of alkali light chains for heavy chain has been controversial.

Dreizen and Richards (1972) observed that incubation of myosin at 37°C in the absence of ATP or at 0°C with concentrated NH_4Cl released A1 preferentially. On the other hand, Sarkar (1972) claimed that A2 was preferentially released when incubated in the presence of NH_4Cl. Leger and Marrotte (1975) and Wagner and Weeds (1977) have shown that both A1 and A2 were equally released from S1 by NH_4Cl treatment.

Burke and Sivaramakrishnan (1981a) found, by thermal exchange of alkali light chains in S1 or myosin, that A1 had higher affinity for the heavy chain. Ueno and Morita (1984) claimed that A2 was preferentially dissociated from the heavy chain when S1 was incubated in the presence of NH_4Cl at pH 9.9.

In all these experiments, light chains were hybridized with or dissociated from S1 or myosin, and then analyzed chromatogra-

phically. As a whole, recoveries of light chains were supposed to be low, which might have affected the apparent affinity. In this section, attempts were made to determine the affinities of light chains for heavy chain, directly by native gel electrophoresis.

Materials and Methods

Preparation of proteins

S1 isozymes were prepared from rabbit fast muscle as described in Section 1 of this chapter.

Alkali and DTNB light chains were isolated from rabbit fast muscle myosin as described in Section 1 of Chapter II.

Actin was prepared as described in Section 1 of this chapter.

Hybridization of alkali and DTNB light chains

Hybridization of light chains was performed in 4.7 M ammonium buffer (pH 9.9) as described in Section 2 of this chapter.

Pyrophosphate native gel electrophoresis

Native gel electrophoresis was performed in pyrophosphate gels essentially according to Hoh *et al*. (1976) using 3% polyacrylamide tube gels.

Measurement of affinity of S1 isozymes for F-actin

S1 and actin were incubated in 25 mM Tris-maleate (pH 8.0) in the presence of 0 - 100 mM KCl at room temperature for 2 h. The affinity was measured by turbidity at 350 nm.

Results and Discussion

Rabbit S1(A1) and S1(A2) were incubated in the excess amount of A2 and A1, respectively, and the ratio of hybrid formed was analyzed by native gel electrophoresis (Fig. III-4-1). The extent of hybrid formation was dependent on the amount of light chain added, but the rate showed a plateau at the light chain/S1 (LC/S1) molar ratio of about 3, suggesting the presence of maximum dissociation ratio.

In order to calculate this ratio, double reciprocal plot was performed between LC/S1 ratio and exchange rate (Fig. III-4-2). Each point was arranged linearly for both cases, and the maximum dissociation ratio was obtained by extrapolating LC/S1 to the infinite point. The values were 35.5% and 29.5% for S1(A1) and S1(A2), respectively. When the LC/S1 ratio required for the exchange of half of the maximum ratio, the values were 1.8 and 0.9 for A1 and A2, respectively, indicating about two fold higher affinity of A2 for heavy chain than A1 (Fig. III-4-3). Crasnier (1987) claimed that the exchange in 4.7 M NH_4Cl at pH 9.5 for recovery of S1(A1) from S1(A2) was attained by only a two-fold molar excess of A1 over S1(A2). Burke and Sivaramakrishnan (1981a,b) claimed that the subunit interaction was more thermostable in S1(A1) than in S1(A2), and that affinity of A2 for heavy chain was weaker than that of A1. Heavy chain-alkali light chain complex in S1 as well as myosin from rabbit fast muscle exists in a rapid equilibrium with the dissociated subunits at 37° C in the presence of MgATP (Burke and Sivaramakrishnan, 1981a,b; Sivaramakrishnan and Burke, 1981).

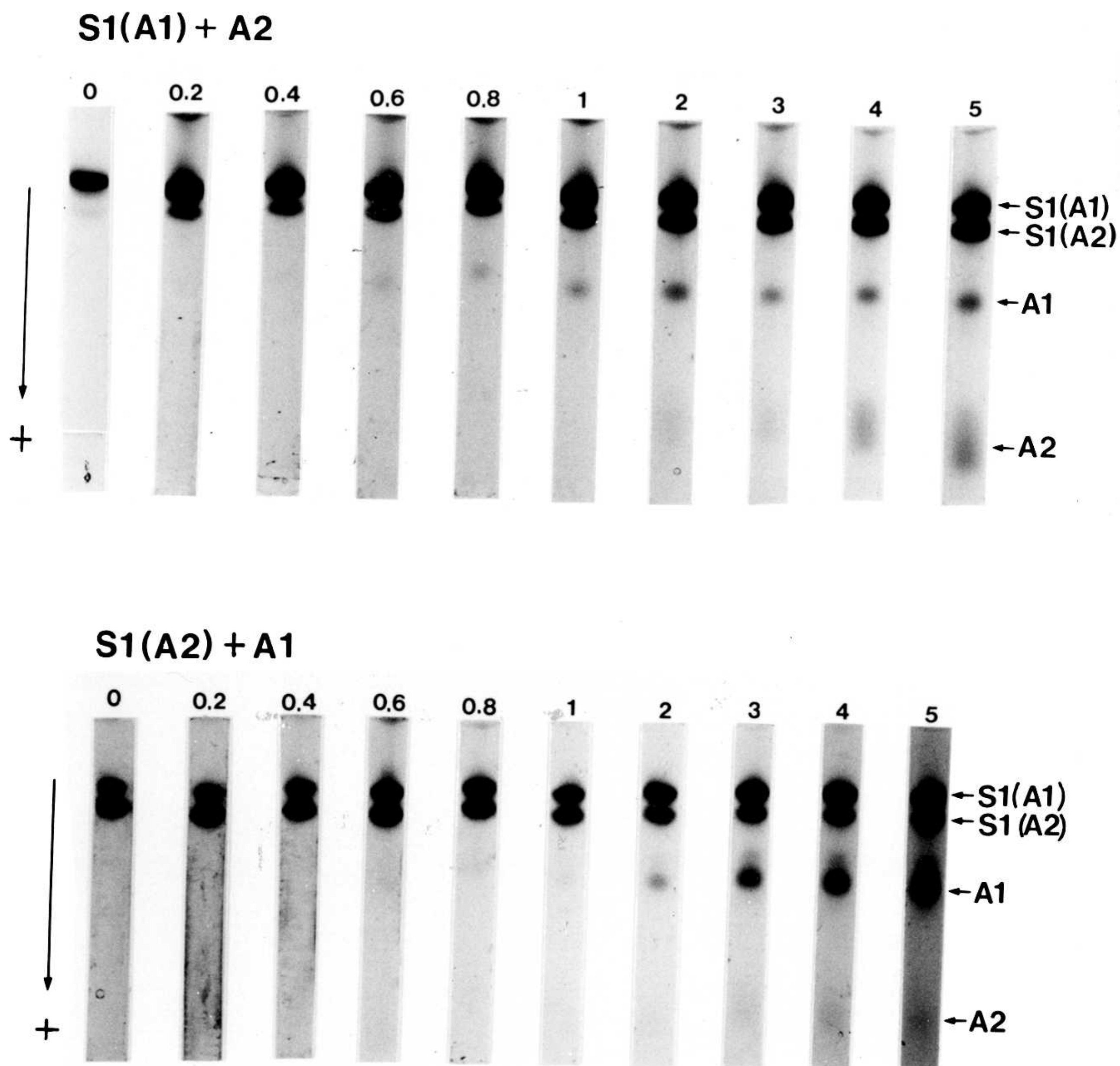


Fig. III-4-1. Native gel electrophoretic patterns of rabbit S1(A1) and S1(A2) formed in the presence of excess heterologous alkali light chains. The number at the top of each gel represents the molar ratio of light chain added.

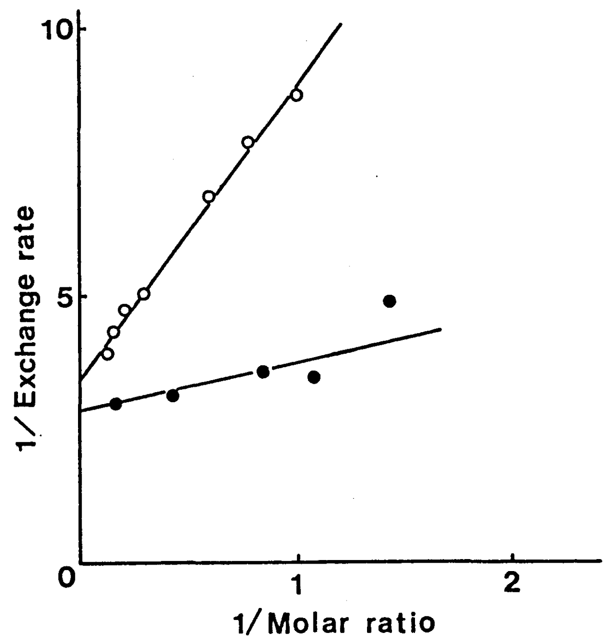


Fig. III-4-2. Double reciprocal plots of the molar ratio (A1 or A2/S1) vs the exchange rate of light chains. Open and closed circles represent A1 and A2, respectively.

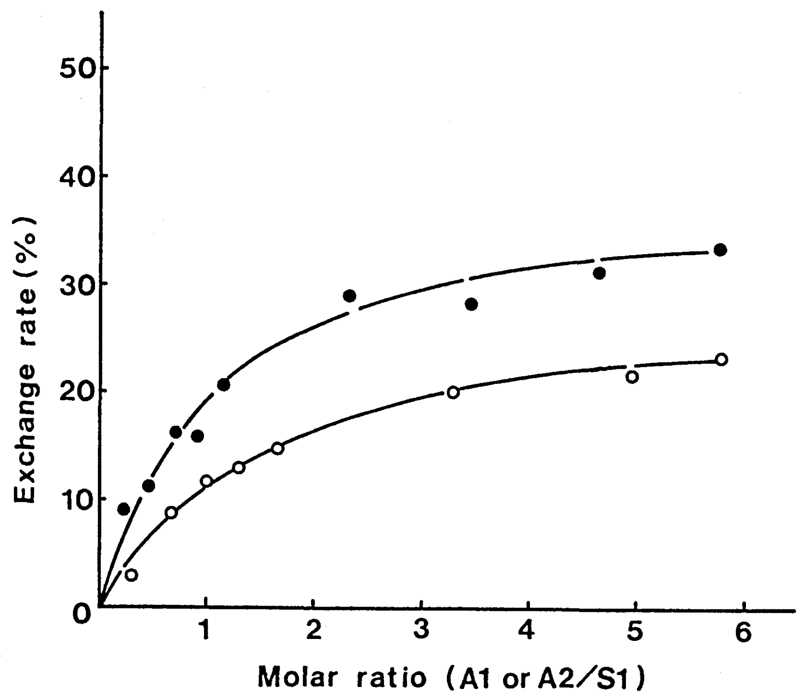


Fig. III-4-3. Effect of light chain/S1 molar ratio on the exchange rate of rabbit S1(A1) and S1(A2). Open and closed circles represent A1 and A2, respectively.

However, Waller and Lowey (1985) demonstrated that no significant exchange of alkali light chains occurred in myosin, due to the presence of DTNB light chain.

Incidentally, DTNB light chain was not bound to both S1 isozymes (data not shown). It seemed that both isozymes have lost the binding site for DTNB light chain. On the contrary, Hozumi et al. (1979) reported that DTNB light chain showed a strong affinity for S1(A1) than for S1(A2), suggesting that the loss of DTNB light chain during the DTNB treatment was mainly from S1(A2). This discrepancy might be due to differences in preparation procedure of S1 isozymes. Hozumi's S1 preparation might have partially retained the DTNB light chain binding site.

Ashiba and Szent-Györgyi (1985) found the blocking action of regulatory light chain on essential light chain exchange in scallop myosin, and suggested that parts of essential light chain may lie between regulatory light chain and heavy chain. In addition, they described that regulatory and essential light chains are chemically cross-linked, suggesting that both light chains are located in close proximity on the heavy chain. Furthermore, the exchange of alkali light chains in S1 was inhibited by DTNB light chain, while partial cleavage of the DTNB light chain of HMM increased the rate of alkali light chain exchange (Wagner and Stone, 1983).

Muhlrad and Morales (1984) isolated three tryptic fragments from S1 heavy chain, and demonstrated that 20K fragment has actin binding ability. Cross-linking studies showed that S1 formed a 1:1 complex with F-actin monomer by preferential binding of 20K fragment (Sutoh, 1983; Heaphy and Treager, 1984; Chen et al.,

1985). Turbidity increase was also observed as the result of interaction between actin and 20K fragment (Muhlrad and Morales, 1984).

In the next place, the affinities of S1 isozymes for actin were compared at various concentrations of KCl (Fig. III-4-4). As a result of formation of acto-S1 complex, the turbidity (absorbance at 350 nm) increased. The turbidity was dependent on the amount of S1 added at low S1 concentration, but reached a plateau at around 0.15 mg S1/ml. In the present experiment, acto-S1 complex was in rigor state, suggesting an extremely high affinity of both protein elements. Incidentally, the apparent dissociation constants of acto-S1 complex were estimated to be 5 x 10^{-7} in the rigor and 5 x 10^{-5}M in the active state (Wagner and Weeds, 1977).

The rates of acto-S1 complex formation were essentially the same between both isozymes, irrespective of KCl concentration. As described in Section 1 of this chapter, the affinity of S1 isozymes for actin, based on actin-activation profiles of Mg-ATPase activity, was markedly affected by KCl concentration. These differences seem to indicate that the turbidity was caused by some heavy chain-light chain association. The differences in affinity for actin between both S1 isozymes as observed in kinetic constants may be accounted for by associated alkali light chains. Alternatively, the turbidity increase might have stemed from limited region(s) of heavy chain, which is completely free from salt-induced conformational change.

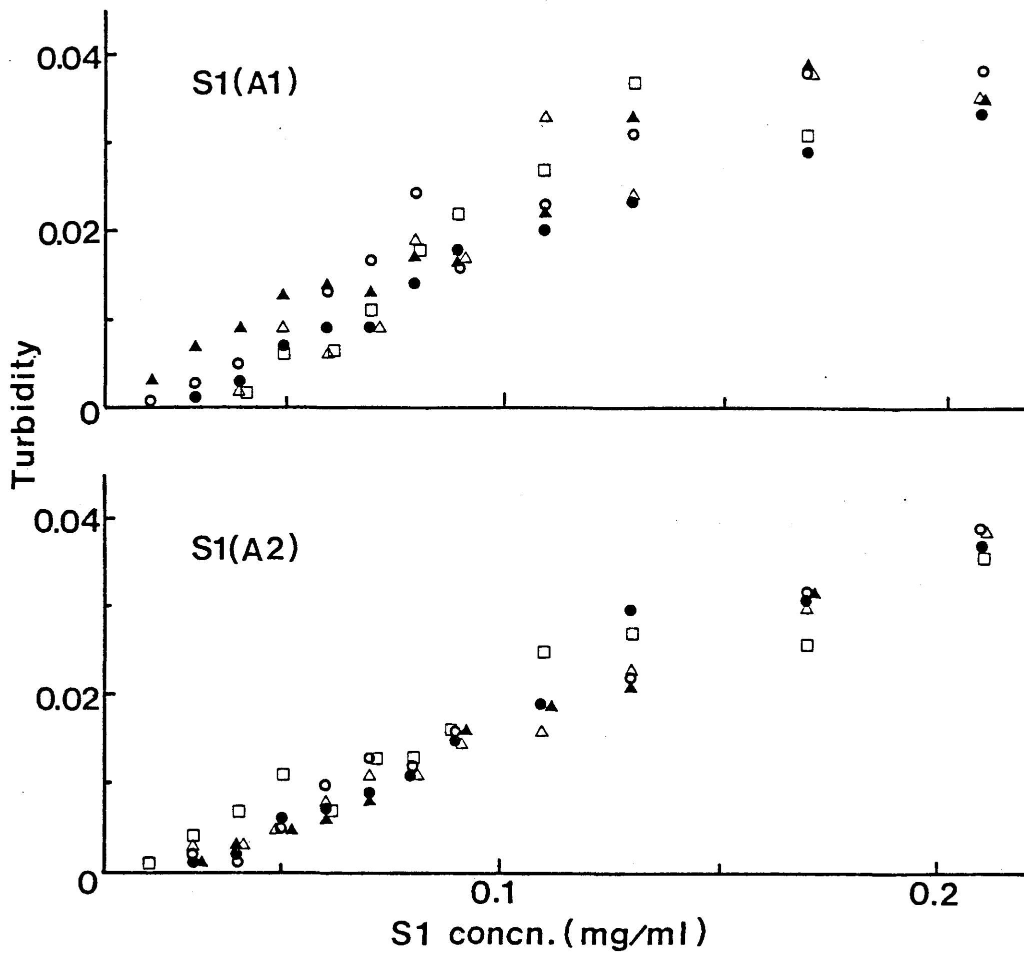


Fig. III-4-4. Effect of the concentration of rabbit S1 isozymes on the turbidity formed by interaction with actin in the presence of various concentrations of KCl. KCl concentration: ●, 0 mM; ○, 20 mM; ▲, 40 mM; △, 60 mM; □, 100 mM.

Summary

Both S1 isozymes associating different alkali light chains, S1(A1) and S1(A2), were isolated from the ordinary muscle of tilapia and bigeye tuna as well as from rabbit fast skeletal muscle.

In the absence of actin, both isozymes exhibited significant differences in ATPase activity over the pH range examined. They showed wide differences in kinetic constants (V_{max} and K_m) when measured for actin-activated Mg-ATPase activity at low salt concentrations. These differences tended to decrease or disappear as KCl concentration was increased. S1(A1) generally showed higher affinity for actin, suggesting that the difference peptide of A1 directly interacts with actin. However, tilapia S1 isozymes showed the opposite profiles in actin activation.

Hybrids consisting of rabbit S1 heavy chain and fish alkali light chains showed similar activation profiles to those of native S1, indicating that the kinetic differences are due to associated light chain, but not to heavy chain.

Both S1 isozymes disclosed essentially the same peptide maps and tryptic digestion patterns of heavy chain, further supporting the above view. On the other hand, A2 light chain was demonstrated to possess higher affinity for heavy chain than A1.