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EPITOPE MAPPING OF ANTI-MYOSIN MONOCLONAL ANTIBODIES

A Thesis

Presented to

The Faculty of the Department of Chemistry

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Susie Grant

May 1995

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ABSTRACT

EPITOPE MAPPING OF ANTI-MYOSIN MONOCLONAL ANTIBODIES

by Susie Grant

The focus of this research project is to characterize six monoclonal antibodies to skeletal muscle myosin by Western blotting techniques. These antibodies define three previously unknown subsets of slow muscle myosin, and two subsets of adult fast myosin present in human, mouse, and rat skeletal muscles. Rat skeletal myosin was cleaved by enzymatic digestion into head and tail subfragments, and the location of each of the six monoclonal epitopes was assigned using gel electrophoresis and Western blotting techniques. Previous studies were done using rabbit or chicken muscle material and cloned gene products. Due to the extensive sequence homology seen amongst muscle myosin, it was expected that susceptible digestion sites would be comparable in rats. Through Western blot analysis of enzymatic digests of rat muscle myosin, it was found that all six monoclonal antibodies react with the myosin head S-1 subfragment.

To my mom and dad for all their love, support and encouragement. And to my brother David who is always 'proud of his little sister', I'm proud of you.

TABLE OF CONTENTS

Introduction . . . . page 1

Project Goals . . . . page 17

Materials and Methods

Buffers . . . . page 19

Reagents . . . . page 21

Theory . . . . page 22

Results . . . . page 35

Discussion . . . . page 55

Conclusion . . . . page 61

Future Projects . . . . page 62

References . . . . page 63

LIST OF FIGURES

Figure 1: Phase-contrast light micrograph of a skeletal muscle fiber.

Figure 2: Electron micrograph of a longitudinal section of a skeletal muscle fiber.

Figure 3: Electron micrograph of a single sarcomere.

Figure 4: High magnification of an electron micrograph of a single sarcomere.

Figure 5: Electron micrograph showing cross-bridges between thick and thin filaments.

Figure 6: The sliding filament model.

Figure 7: Schematic diagram of a myosin molecule.

Figure 8: Enzymatic cleavage of myosin by trypsin and papain.

Figure 9: Illustration of the two hinges regions in a myosin molecule.

Figure 10: Representation of the self-aggregation of myosin.

Figure 11: A representation of the reaction of MHC.F36 with S-1 and S-1 subfragments.

Figure 12: A representation of the reaction of mAb25 with tryptic digests.

Figure 13: Schematic of myosin monoclonal antibody epitopes.

Figure 14: Absorbance spectra of nucleic acids.

Figure 15: Absorbance spectra of the aromatic amino acid residues.

Figure 16: Reactive group and covalent intermedite of papain.

Figure 17: Mechanism of iodoacetic acid oxidation.

Figure 18: Structure of sodium dodecyl sulfate.

Figure 19: Log-linear relationship of molecular weight and relative mobility.

Figure 20: Log-linear correlation of molecular weight and relative mobility.

Figure 21: Representation of a 'gel sandwich'.

Figure 22: Amplification of the antibody epitope signal with AP-ABC®

Figure 23: BCIP/NBT reaction with alkaline phosphatase for band development on Western blots.

Figure 24: SDS-PAGE analysis comparing the ATP and PP_i preps.

Figure 25: Spectral comparison of the ATP and PP_i preps.

Figure 26: SDS-PAGE analysis comparing homogenization vs. grinding only preps (Prep S8).

Figure 27: Post-dialysis spectral analysis of Prep S8, 'G' sample.

Figure 28: SDS-PAGE analysis of Digestion #5.

Figure 29: SDS-PAGE analysis of Digestion #6.

Figure 30: Preliminary Western blots for mAbs MHC.25 and F36.

Figure 31: Western blots of mAbs S84, S95, F74, and FS26 showing high background and low reactivity.

Figure 32: Western blot reactivities of mAbs F36 and S95 with the corresponding stained gels.

Figure 33: Western blot reactivities of mAbs F36 and S84 with the corresponding stained gels.

Figure 34: Western blot reactivities of mAbs F36 and FS26 with the corresponding stained gels.

Figure 35: Western blot reactivities of mAbs F36 and F74 with the corresponding stained gels.

Figure 36: Semi-log plots of the stained gels corresponding to Western blots in Figures 32-35.

LIST OF TABLES

Table 1: Reactions of expressed gene products with myosin monoclonal antibodies.

Table 2: Summary of myosin purifications.

Table 3: Comparison of ATP and PP_i extraction buffers.

Table 4: Summary of papain digestions.

Table 5: Summary of the reactivities of the six monoclonal antibodies with the myosin heavy chain.

Table 6: Molecular weights of S-1 and rod fragments calculated from the corresponding semi-log plots of the stained gels.

Table 7: Major species reacting in the Western blot S-1 samples.

LIST OF ABBREVIATIONS

A_{260}:	absorbance at 260 namometers
$A_{280/260}$	ratio of absorbances at 280 namometers and 260 namometers
A_{280}:	absorbance at 280 namometers
A_{340}:	absorbance at 340 namometers
aa:	amino acid
Abs:	absorbance
AP-ABC®:	alkaline phosphatase-avidin biotin complex
ATP:	adenosine triphosphate
BCIP-NBT:	5-bromo-4-chloroindoxyl phosphate-NitroBlue tetrazolium
C-terminal:	carboxyl-terminal
C:	Celcius
CAPS:	3-[cyclohexylamino]-1-propane sulfonic acid
cDNA:	copy deoxyribonucleic acid
Cys:	cysteine
DTT:	dithiothreitol
EDTA:	ethylenediaminetetraacetic acid
ELISA:	enzyme-linked immunosorbent assay
HMM:	heavy meromyosin
IAA:	iodoacetic acid
Ig:	immunoglobulin
K+:	potassium ion
kDa:	kilodalton
LC:	myosin light chain
LMM:	light meromyosin

M:	molar
mAb:	monoclonal antibody
Mg^{2+}:	magnesium ion
mg:	milligram
MHC:	myosin heavy chain
mL:	milliliter
mM:	millimolar
MW:	molecular weight
N-terminal:	amino-terminal
nm:	nanometer
PP_i:	pyrophosphate
Prep:	preparation
SDS-PAGE:	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SDS:	sodium dodecyl sulfate
ßME:	ß-mercaptoethanol
Supn:	supernatant
µL:	microliter
WWB:	Western Wash Buffer

INTRODUCTION

Muscle Structure. Skeletal muscle, as well as striated muscle, consists of myosin and actin filaments running across the length of the muscle fibers (Figure 1). Running lengthwise within each fiber are myofibrils (Stryer, 1988) (Figure 2). The functional unit is known as the sarcomere. The myosin and actin filaments are known as the thick and thin filaments, respectively.

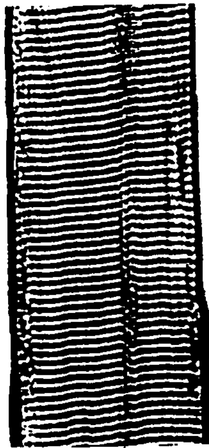


Figure 1: Phase-contrast light micrograph of a skeletal muscle fiber. The A bands are dark and the I bands are light. (Copied with permission from Stryer, 1988. Courtesy of H.E. Huxley.)

Figure 2: Electron micrograph of a longitudinal section of a skeletal muscle fiber. The myofibrils run diagonally from the upper left to the lower right. Again, the A bands appear dark and the I bands appear light. (Copied with permission from Stryer, 1988. Courtesy of H.E. Huxley.)

Figure 3 illustrates an electron micrograph of a single sarcomere in skeletal muscle. The actin filaments are anchored at the edges of the sarcomere at the Z lines, and

make up the two I bands on the outer edges of the sarcomere (Stryer, 1988) (Figures 3 and 4). The myosin filaments are anchored at the M line in the center of the sarcomere. The dark regions clearly show the overlap of the thick and thin filaments known as the A band (Stryer, 1988). Striated muscle derives its name from its striated appearance resulting from the banded structure of the repeating sarcomeres in the myofibrils (Voet and Voet, 1990)

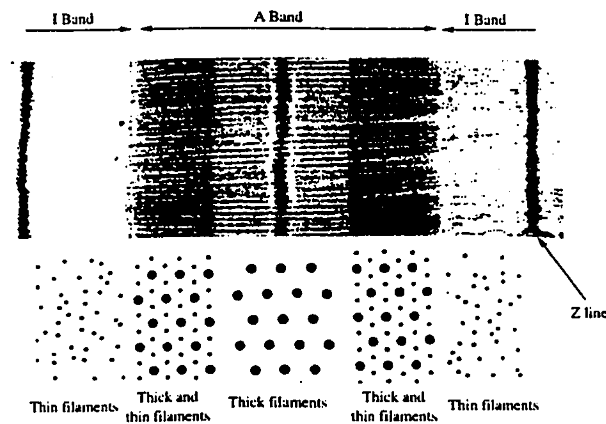


Figure 3: Electron micrograph of a single sarcomere (Upper half of figure) and diagram of a cross-section through each band (Lower half of figure). (Copied with permission from Stryer, 1988. Courtesy of H.E. Huxley.)

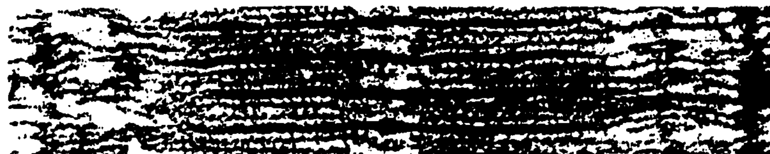


Figure 4: High magnification of an electron micrograph of a single sarcomere. Note the shortened (contracted) appearance of this sarcomere. (Copied with permission from Stryer, 1988. Courtesy of H.E. Huxley.)

The thick and thin filaments are connected through cross-bridges by the interaction of myosin with the actin filaments (Figure 5). These cross-bridges are formed specifically by the enzymatic head of the myosin molecule which is responsible for adenosine triphosphate (ATP) hydrolysis and muscle contraction. Recent X-ray crystallographic analysis of the globular myosin head has identified the actin contact zone of the cross-bridges (Rayment et al., 1993).

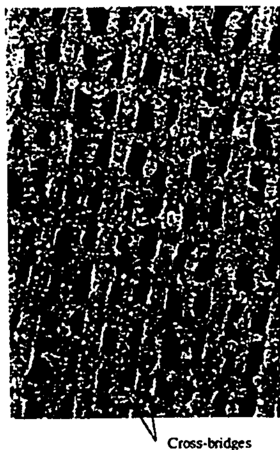


Figure 5: Electron micrograph showing cross-bridges between the thick and thin filaments. (Copied with permission from Stryer, 1988. Courtesy of H.E. Huxley.)

Muscle Contraction and the Sliding Filament Model. Muscle contraction is an ATP-driven process which involves the sliding of myosin and actin filaments. Muscle contraction occurs by the sliding of the actin and myosin filaments past one another as described by the sliding filament model (Huxley, 1958; see Stryer, 1988 for a recent discussion). The thick and thin filaments do not shorten, but the entire sarcomere shortens as the filaments slide past one another. This action forms an increased overlap of the thick and thin filaments (Figure 6). In addition, ATP hydrolysis occurs simultaneously with the binding and release of actin by myosin (Voet and Voet, 1990).

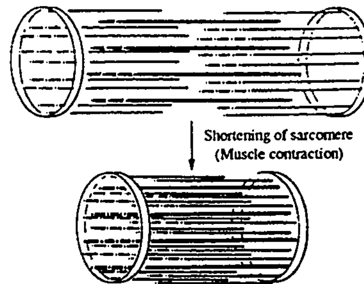


Figure 6: The sliding filament model.
(Copied with permission from Stryer, 1988. Courtesy of H.E. Huxley.)

Myosin Function. As was already discussed, myosin is responsible for both ATPase activity as well as actin binding. In addition, myosin assembles into filaments at physiological pH and ionic strength (Stryer, 1988). Both its enzymatic activity and actin binding capability are necessary for muscle contraction. The structure of myosin itself is partially responsible for these functions.

Myosin Structure and Proteolytic Subfragments. Myosin, which comprises the thick filament in myofibrils, is a 520 kilodalton (kDa) molecule consisting of two heavy chains and four light chains. Each heavy chain is approximately 220 kDa (Bandman, 1985) while each light chain ranges from 16 kDa to 25 kDa (Weeds and Lowey, 1971; Lowey, 1986; Lowey and Risby, 1971; Rayment et al., 1993). The myosin heavy chains are often abbreviated as MHC. At the N-terminus, the molecule has two globular heads connected to a long tail (Lowey, 1986). The two heavy chains form the two globular heads with two light chains (LC1 and LC2) associated with each head. The tail of the myosin molecules is a coiled-coil alpha-helix of the two heavy chains' C-terminal ends. Figure 7 clearly illustrates the coiled-coil nature of the C-terminus and the globular heads located at the N-terminus.

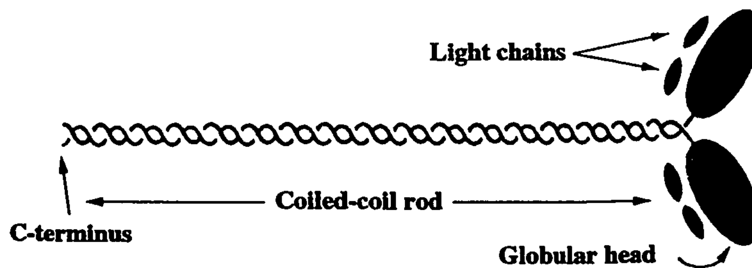


Figure 7: Schematic diagram of a myosin molecule.
(Adapted from Stryer, 1988.)

The globular head of myosin is often referred to as the S-1 fragment and contains both the ATPase site and the actin-binding site. The tail or rod fragment, on the other hand, is responsible for self-aggregation of myosin at low ionic strength and assembly into thick filaments (Lowey, 1986). Papain digestion of rabbit or chicken striated muscle myosin, in the presence of Mg^{2+}, creates primarily two fragments, the S-1 fragment (~95 kDa) and the rod fragment (~125 kDa) (Margossian et al.. 1975).

Chymotrypsin or trypsin digests of myosin give rise to different subfragments known as heavy meromyosin (HMM) and light meromyosin (LMM) (Mihalyi and Szent-Gyorgyi, 1953). HMM contains the globular head and a portion of the tail giving rise to a 150-kDa fragment (Voet and Voet, 1990). LMM, on the other hand. contains only a portion of the tail and is approximately 70 kDa (Voet and Voet, 1990). The cleavage of myosin by chymotrypsin or trypsin occurs at a second hinge region between the HMM and LMM subfragments. In addition, the HMM subfragment can be further cleaved at its hinge region giving rise to the S-1 and S-2 subfragments (Figure 8). Thus, myosin contains two hinge regions which are susceptible to enzymatic digestion (Figure 9).

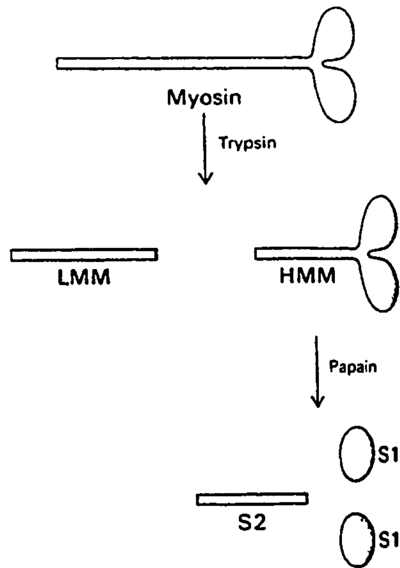


Figure 8: Enzymatic clevage of myosin by trypsin and papain.
(Copied with permission from Stryer, 1988.)

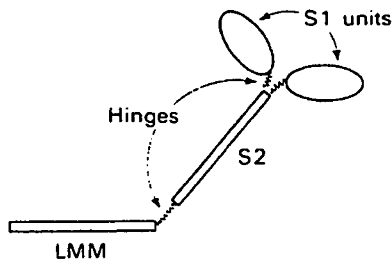


Figure 9: Illustration of the two hinge regions in a myosin molecule.
(Copied with permission from Stryer, 1988.)

The importance of the hinge regions is clearly manifested when investigating the mechanism of muscle contraction. As was mentioned earlier, myosin can self-aggregate at physiological ionic strength. When this occurs, the myosin molecules are oriented so that the globular heads are sticking out along the thick filament (Figure 10).

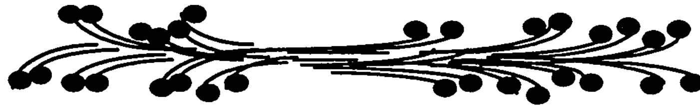


Figure 10: Representation of the Self-Aggregation of Myosin
(Adapted from Voet and Voet, 1990, Figure 34-46)

This orientation with the S-1 heads lined up allows for actin binding. The flexibility of the second hinge region, located between the LMM and HMM subfragments (Stryer, 1988), allows myosin to bend up towards the actin filaments. The second hinge region, located between the S-1 and S-2 subfragments, allows for the "power stroke" which occurs in muscle contraction as the actin and myosin filaments slide past one another (Stryer, 1988).

Since crystallization of S-1 has now been achieved (Rayment et al., 1993), and since myosin has been the subject of extensive biochemical investigation over the last 50 years, much is known about its structure and function. The S-1 fragment itself is composed of three subdomains. Beginning with the N-terminus, the S-1 fragment contains 27-kDa, 50-kDa, and 20-kDa fragments (Dan-Goor et al., 1990). These fragments arise from tryptic digestion of the S-1 fragment (Dan-Goor et al., 1990). The subdomains of S-1 are important with regard to identification of epitopes for anti-myosin monoclonal antibodies.

Anti-Myosin Monoclonal Antibodies: Immunoblots. Two anti-myosin monoclonal antibodies (mAbs), MHC.25 and MHC.F36, have been characterized by Western blot analysis with rabbit myosin (Dan-Goor et al., 1990). The epitopes of both have been shown to reside in the S-1 region of the myosin heavy chain. [These reagents were earlier referred to as 'mAb.25' and 'N3.36', respectively (Silberstein, personal communication).]

Furthermore, the specific subdomains identified by these antibodies have been located and will be discussed in detail below.

The antibodies were raised against human skeletal myosin and the studies were carried out with myosin isolated from back and leg muscles of rabbit (Dan-Goor et al., 1990). These regions are generally chosen because of the high yield of material obtained. Isolation of S-1 was accomplished through digestion of myosin filaments with chymotrypsin. The S-1 itself was digested into sub-subfragments with trypsin. In all cases, the digests were analyzed by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and either stained with Coomassie Blue or transferred to nitrocellulose for immunoblotting.

Western Results. In order to localize the epitope of mAb MHC.F36, tryptic digests were prepared for S-1 heavy chain fragments. The digestion was carried out in both the presence and absence of ATP. Trypsin cleaves the S-1 (95 kDa) heavy chain into 27-kDa, 50-kDa, and 20-kDa fragments with an intermediate 75-kDa fragment appearing during digestion (Dan-Goor et al., 1990); these fragments are in order beginning with the N-terminus. In the presence of ATP, an additional cleavage is made creating a 22-kDa fragment instead of the 27-kDa fragment. This particular fragment lacks the N-terminus (Dan-Goor et al., 1990). Western blot analysis indicates that MHC.F36 reacts with the 95-kDa, 75-kDa, and 27-kDa fragments (Figure 11). Therefore, the epitope must reside in the N-terminal region of the S-1 heavy chain. This was verified by additional digests and an 'anti-N-terminus' antibody (Dan-Goor et al., 1990).

Tryptic digests of S-1 heavy chain were also reacted with mAb MHC.25. Since the 27-kDa, 50-kDa, and the 20-kDa fragments are the domains of S-1, these results indicate that the epitope is located on the 50-kDa domain of S-1 (Dan-Goor et al., 1990). These results are summarized in Figure 12.

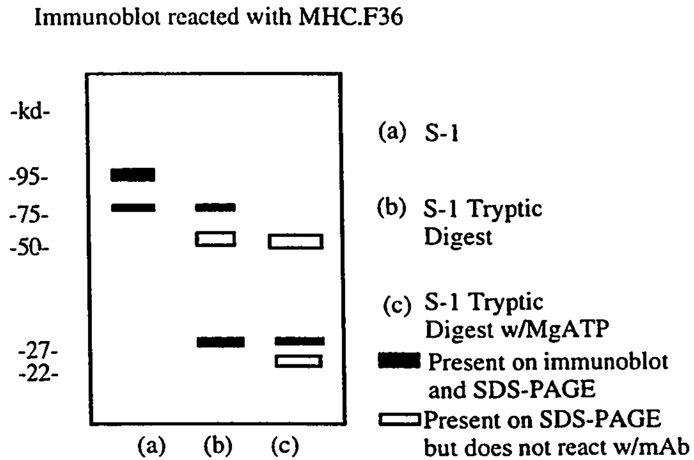


Figure 11: A Representation of the Reaction of MHC.F36 with S-1 and S-1 Subfragments (Adapted from Dan-Goor et al., 1990, Figure 1.)

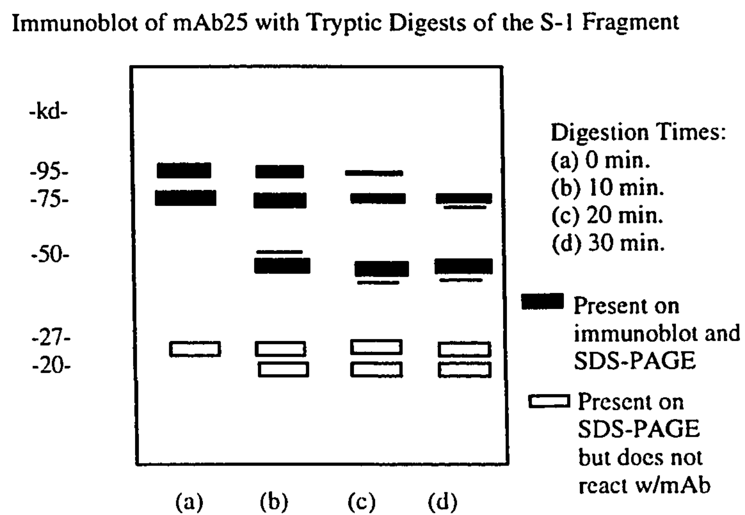


Figure 12: A Representation of the Reaction of mAb25 with Tryptic Digests (Adapted from Dan-Goor et al., 1990, Figure 3.)

An even more accurate localization of the mAb MHC.25 epitope was made by careful analysis of further digests. The 75-kDa fragment was further fragmented and incubated with both mAb MHC.25 and the 'anti-N-terminus' antibodies. The smallest fragment reacting with both antibodies was 30 kDa. Because of the positive reaction with the 'anti-N-terminus antibody, this 30-kDa fragment is an N-terminal fragment. Since the 27-kDa fragment resides at the N-terminus, the 30-kDa piece must contain the 27-kDa domain and a 3-kDa portion of the N-terminus of the 50-kDa domain. Since mAb MHC.25 does not react with the 27-kDa fragment, the epitope must reside on the 3-kDa N-terminal region of the 50-kDa domain (Dan-Goor et al., 1990). These results have been confirmed by cleavage with two additional methods (Dan-Goor et al., 1990).

Inhibition Studies. Dan-Goor et al. have shown that the epitopes for both mAbs MHC.25 and MHC.F36 lie in the S-1 portion of myosin. According to their unpublished results, both antibodies react with human, rabbit, and chicken skeletal muscle myosin (Silberstein, personal communication). This indicates some sort of homology between species and will be discussed later in more detail. Furthermore, Dan-Goor et al. have carried out inhibition studies with ATP and actin to determine their effects on antibody binding. In the presence of mAb MHC.25, both K^+ and actin-activated ATPase activities are inhibited. This would indicate that either this 3-kDa piece of the 50-kDa domain of S-1 has a direct role in the ATPase activity or that antibody-binding in some way affects the ATP binding site (Dan-Goor et al., 1990). In any case, the sites for ATPase activity and antibody binding are interrelated in some as yet unknown way.

In addition, higher levels of mAb MHC.25 are required for positive reaction with the actin-myosin complex than for isolated myosin. This result may indicate that the epitope is near the actin binding site of myosin (Dan-Goor et al., 1990). However, the decrease in affinity for antibody in the presence of actin could also be explained by

allosteric effects such as an actin-induced conformational change which may inhibit MHC.25 binding. Both of these hypotheses are supported by other data (Dan-Goor et al., 1990).

In conclusion, the most important aspect of this work is the localization of the epitopes of both mAbs MHC.25 and MHC.F36 on the S-1 region of myosin. The current work is concerned, in part, with an attempt to verify these findings with rat myosin. The rationale for using rat myosin is that sequence homology between species indicates findings with rat myosin will be consistent with previous studies (See *Project Goals*). In addition, a variety of rat MHC cloned genes are now available, which will allow future correlation of histochemistry with nucleic acid analyses.

Myosin Isozymes. It has been shown by various studies that different forms of myosin exist during different developmental stages. In addition, two major categories of myosin in the adult have been identified as 'fast' and 'slow' myosin based on ATPase histochemical fiber typing and, to some extent, speed of ATP hydrolysis and fiber contraction (Bandman, 1985: Barany, 1967; and Pette, 1980). The differences in myosin isozymes which appear throughout development in embryonic, neonatal, and adult myosin and between fast and slow myosins have also shown differences in antibody reactivity (Bandman, 1985 and Hughes et al., 1993).

Current work has identified at least three different fast myosin isozymes in chicken (Bandman, 1985), and three different slow isozymes in humans and rats (Hughes et al.. 1993) that first appear at different stages in development. The differences between fast and slow myosin may be related to their expected function and can be identified by their association with epitopes unique to fast or slow myosins. Therefore. it is likely that epitopes which distinguish between fast versus slow myosins would be localized near the ATPase function site, i.e., the S-1 head. Furthermore, if fast isozymes differ subtly in their

rate of ATP hydrolysis, epitope location would be predicted to be in the enzymatic S-1 head. The same rationale applies to the differences between fast and slow isozymes. On the other hand, since functions of the various fast and slow isozymes are unknown, differences in function may involve sarcomere assembly. Thus, these epitopes might be localized in the myosin rod.

Anti-Myosin Monoclonal Antibodies: Gene Cloning. It is known that different muscle fibers have characteristic rates of contraction giving rise to what is known as slow and fast muscle fibers. These rates of contraction are dictated by the rate of ATP hydrolysis (Barany, 1967). In addition, different muscle fibers contain different isozymes of myosin (Hughes et al., 1993). Some of these different isozymes are expressed at different stages of development. Hughes et al. have postulated the existence of various isozymes of myosin heavy chain and used monoclonal antibodies to examine their diversity. This has been accomplished in conjunction with gene cloning.

The myosin heavy chain from a variety of different sources was analyzed to study the various isozymes. Both human and rat myosin were used for analysis of neonatal, embryonic, and adult tissue. Human ß-cardiac slow MHC was cloned into an expression vector with subsequent introduction into *Dictyostelium* for production (Hughes et al., 1993). All other rat and human MHC's were isolated and cloned into bacterial or mammalian expression vectors and expressed in *E. coli* or cos cells (monkey kidney cells), respectively. The processing for all samples was similar.

The isoforms were then analyzed through the use of monoclonal antibodies. Different antibodies were used to distinguish between different isozymes and give proof of their existence. In order to insure that the antibodies were in fact reacting with epitopes on the MHC, Hughes et al. took several precautions. Firstly, each antibody was tested with the purified myosin immunogen on Enzyme-Linked Immunosorbent Assays (ELISA).

Secondly, Western blots with whole cell lysates of the muscle cells showed reaction of the antibody with only a 220-kDa protein. This, in fact, corresponds to the MHC. Finally, Western blot results using purified myosin showed reactions with either the MHC bands or its fragments.

For the purposes of this discussion, only the localization of epitopes of the antibodies for the MHC will be analyzed although the bulk of Hughes et al.'s work resides in the identification of three slow myosin isoforms. The four antibodies used were MHC.S84, MHC.S95, MHC.FS26, and MHC.F74.[1] The antibodies were raised against MHC antigens from human skeletal muscle (Hughes et al., 1993). Table 1 is a partial list of the results obtained by Hughes et al. from reactions of various MHC isozymes with these four monoclonal antibodies.

The antibodies were raised against neonatal and adult myosins. The mAb FS26 resulted from immunization with neonatal myosin and does not react with embryonic myosin. The mAbs F74, S84, and S95 resulted from immunization with adult myosin and do not react with either embryonic or neonatal myosins. Thus, antibody specificity differs with regard to adult versus embryonic and fast versus slow. The mAb F74 is specific for a subset of fast myosin (adult), whereas, mAbs S84 and S95 are specific for slow myosins, possibly of the ß-cardiac type.

Table 1 shows the mAb reactions with cloned gene products, namely, S-1, S-2, LMM, MHC lacking the N-terminus, and a 130-kDa N-terminal fragment containing S-1 and a portion of the rod fragment.

---

[1] These reagents were earlier referred to as A4.840, A4.951, N2.261, and A4.74, respectively (Silberstein, personal communication). The current nomenclature refers to their myosin heavy chain reactivity, 'MHC', and to their fast or slow myosin specificities, eg. S84 and S95 react only with slow fibers, F74 reacts only with a subset of fast fibers, and FS26 reacts with a subset of fast and slow fibers.

Cloned Gene Product			mAb Reactions in Westerns			
Cloned Gene Product	**Isoform**	**Source of MHC**	**MHC.S84**	**MHC.S95**	**MHC.FS26**	**MHC.F74**
MHC Lacking the N-terminus	ß-cardiac/ slow	rat cDNA in bacteria	+	–	–	–
130 kDa N-term fragment	ß-cardiac/ slow	human cDNA in dictyostelium	–	+	+	–
LMM Fragment	fast	human cDNA in bacteria	–	–	–	–
LMM Fragment	embryonic	human cDNA in bacteria	–	–	–	–
S-1 Fragment (head)	neonatal	human cDNA in cos cells	–	–	–	–
S-2 and LMM Fragment (rod)	neonatal	human cDNA in bacteria	–	–	–	–

Table 1: Reactions of expressed gene products with myosin monoclonal antibodies. (Adapted from Table 1, Hughes et al., 1993)

The source of the cDNAs[2] used, either rat or human, or embryonic, neonatal, or adult cardiac, is also shown together with the expression system. Hughes et al. were unable to get any positive reactions with embryonic or neonatal MHC, as expected from previous immunohistochemical work with the slow mAbs (S84, S95, and FS26). The lack of reaction of the LMM with mAbs F74 and FS26 may be due to the epitopes being in the

[2] Copy DNA encoding the fragment of interest.

deleted HMM region. Alternatively, this particular fast MHC gene may code for a different isozyme than is recognized by either mAb F74 or mAb FS26.

The mAb MHC.S84 gave a positive reaction for the rat slow myosin-derived fragment lacking the N-terminus. Thus, the epitope resides on some other portion of the MHC. Furthermore, the lack of mAb S84's reaction with the 130-kDa N-terminus suggests that the epitope resides somewhere in the rod, namely, in the 90-kDa C-terminal end of the rod. However, this could also mean that the human cardiac clone used may code for a different slow MHC isozyme than is recognized by mAb S84. Since mAbs S84, S95, and FS26 recognize three distinct slow skeletal muscle myosin isozymes (Hughes et al., 1993), it is expected that these three myosins are encoded by three different genes. Suprisingly, mAbs S95 and FS26 both react with the human 130-kDa N-terminal cardiac cDNA expression product. This may mean that the ß-cardiac MHC isozyme, unlike slow skeletal muscle myosins, contains both epitopes on the same molecule. Since both mAbs MHC.S95 and MHC.FS26 reacted with the 130-kDa N-terminal fragment, these may both be anti-S-1 monoclonal antibodies even though a significant portion of the rod sequence (beyond the first hinge region) is present in this expression vector (Figure 13). The mAb MHC.F74 did not react with any of the fragments tested and thus its epitope remains unknown.

An attempt will be made to verify and extend these findings with rat myosin by localization of the epitopes to either the S-1 head or the rod tail of myosin. The following figure represents a schematic of the location of the six monoclonal antibodies to date combining both Western blot results from Dan-Goor et al. and gene cloning results from Hughes et al. (Figure 13).

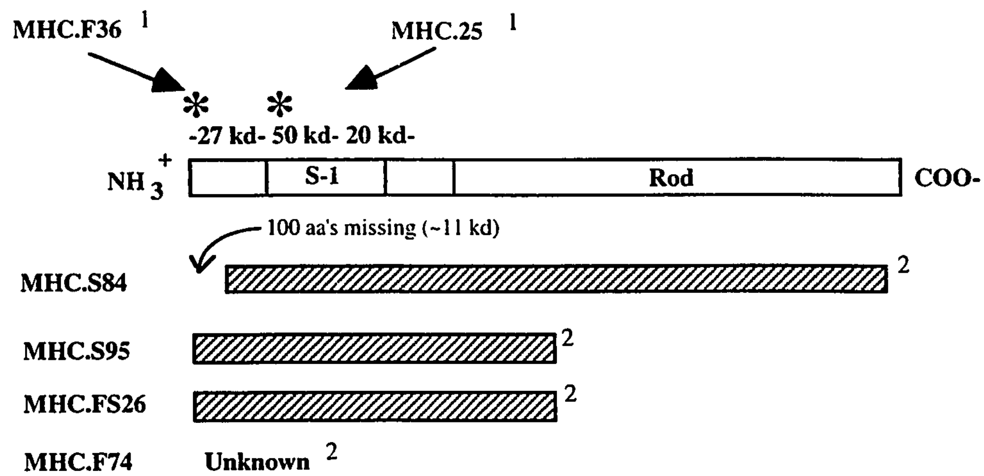


Figure 13: Schematic of myosin monoclonal antibody epitopes. The open bar represents the linear amino acid sequence of the myosin heavy chain. The asterisks and the shaded bars indicate that the epitope resides somewhere in the depicted region. ([1]Dan-Goor et al., 1990; [2]Hughes et al., 1993)

PROJECT GOALS

The focus of this project is the characterization of six monoclonal antibodies with respect to binding of S-1 or rod fragments from papain digests of purified rat myosin. The six mAbs to be studied are MHC.25, MHC.F36, MHC.S95, MHC.FS26, MHC.S84, and MHC.F74. Two of the antibodies, MHC.25 and MHC.F36, have been identified as anti-S-1 as previously discussed. The mAbs MHC.S95 and MHC.FS26 react with a 130-kDa N-terminal fragment containing the S-1 fragment and a portion of the rod fragment. In addition, the mAb MHC.S84 epitope is not within the 11-kDa fragment of the N-terminus. Finally, the location of the epitope for mAb MHC.F74 is not known beyond the fact that it binds to the myosin heavy chain (Webster et al., 1988).

Previously, these enzymatic digestions and localization of epitopes have been performed with rabbit or chicken muscle material. In the case of enzymatic digestions, it is expected that the susceptible digestion sites of rat myosin will correspond to the two susceptible hinge regions in rabbit myosin. Furthermore, the assignment of epitopes of the six monoclonal antibodies for previous work done with gene cloning of human myosin and proteolytic digestion of chicken myosin will be verfied with rat myosin.

It is expected that verification will be successful because of the DNA sequence homology of myosin genes existing between species. Myosin from different species such as *Dictyostelium*, *Caenorhabditis elegans*, chicken, and rabbit share many other features (Stryer, 1988). Besides the presence of a globular head and long tail and having similar sizes, all known myosins contain the amino acid sequence Gly-Glu-Ser-Gly-Ala-Gly-Lys-Thr (Stryer, 1988) within the molecule. Furthermore, the lysine in this sequence is responsible for binding the phosphate of ATP (Stryer, 1988).When comparing percent amino acid similarity in the S-1, S-2, and LMM regions of sequenced myosins, rabbit vs chicken, rabbit vs. rat, and rat vs. chicken myosins all show similarities in the 80-91%

range (Warrick and Spudich, 1987) Additional patterns of homology will be discussed below. It is also important to note that the epitope specificities of these mAbs: fast vs. slow and adult, neonatal, and fetal myosin types, are conserved between rats, mice, and humans (Silberstein, personal communication).

Although myosins from widely divergent organisms show evolution in terms of amino acid sequence, parallel myosins show a pattern of homology(op. cit.). It has been observed that muscle myosins show more homology than non-muscle myosins, and more conservation exists in the head region than the tail region. In the case of rat skeletal myosin versus myosin from the slime mold *Dictyostelium*, conserved sequences in the head appear at the same relative positions. Furthermore, the tail portions have repeating groups of charged and hydrophobic amino acids (ibid). This holds true for other comparisons of different myosins as presented by Warrick and Spudich. Although myosins between species are not entirely conserved, the overall chemical properties are maintained with respect to charge, hydrophobicity, and size. In addition, although homologies are not uniform throughout the tail region, the repeating patterns themselves contribute to the formation of a coiled-coil structure with a hydrophobic core (ibid). More importantly, the differences occuring between myosins are thought to contribute to species specific properties and do not affect the overall myosin structure and function.

All six antibodies recognize adult myosins, and either fast or slow as indicated by their notation as 'F', 'S', or 'FS'. The significance of localizing the epitopes of these six antibodies in head versus tail lies in the possibility of insight into myosin function. The methods used to verify and extend previous findings with regard to the epitopes include myosin purification, papain digestions, SDS-PAGE analysis, and Western blotting. A detailed description of the process theories and purposes will be given in the following sections.

MATERIALS AND METHODS

Buffers

Myosin Purification:

1. ATP Extraction Buffer: 0.3 M Potassium Chloride, 0.15 M Potassium Phosphate, 5 mM Magnesium Chloride, 0.02 M EDTA, 5 mM ATP, pH 6.7 (Ionic Strength = 0.77 M)
2. Pyrophosphate Extraction Buffer: 0.1 M Sodium Pyrophsphate, 5 mM EDTA, 0.1 mg/mL Cysteine, pH 8.3 (Ionic Strength = 0.62 M)
3. Myosin Buffer: 0.60 M Sodium Chloride, 10 mM Sodium Phosphate, pH 7.0 (Ionic Strength = 0.60 M)
4. Pellet Dispersion Buffer #1: 1 M Potassium Chloride, 50 mM Potassium Phosphate, 20 mM EDTA, pH 6.7 (Ionic Strength = 1.2 M)
5. Pellet Dispersion Buffer #2: 3 M Potassium Chloride, 50 mM Potassium Phosphate, pH 6.7 (Ionic Strength = 3.1 M)

Papain Digestions:

6. Ammonium Acetate Buffer: 0.2 M Ammonium Acetate, 2 mM Magnesium Chloride, pH 7.0
7. 2 mg/mL Papain Stock Solution in Ammoniun Acetate Buffer
8. 10 mM Iodoacetic Acid Stock Solution in Ammoniun Acetate Buffer
9. 10 mM Dithiothreitol Stock Solution in Ammoniun Acetate Buffer

SDS-PAGE Analysis:

10. 2XSDS Sample Buffer: 10% (v/v) SDS, 0.02% (v/v) Bromphenol Blue, 0.05% (v/v) Pyronin Y, 20% (v/v) Glycerol, 125 mM Tris pH 6.8, 4 mM DTT
11. 1X Gel Running Buffer: 0.1% (v/v) SDS, 0.384 M Glycine, 0.05M Tris-Base
12. Gel Fix: 10% (v/v) Acetic Acid

13. Gel Stain: 0.025% (m/v) Coomassie Brillant Blue, 10% (v/v) Methanol, 10% (v/v) Acetic Acid

14. Gel Destain: 25% (v/v) Methanol, 10% (v/v) Acetic Acid

Western Blotting:

15. Transfer Buffer: 10 mM CAPS (3-[cyclohexylamino]-1-propane sulfonic acid) pH 10.5, 5% (v/v) Methanol

16. Western Wash Buffer: 0.5 M Sodium Chloride, 20 mM Borate, 0.05% (v/v) Tween 20, pH 8.2

17. 10X Borate Buffer: 200 mM Borate, pH 8.2

18. BCIP/NBT Buffer: 100 mM Tris-HCl, pH 9.5

19. Molecular Weight Marker Developing Buffer: Phosphate Buffered Saline pH 7.2, 0.3% (v/v) Tween-20, 2 μL/mL Pelikan Fount India Ink.

20. Cell Culture Supernatants: Hybridoma clones are cultured in growth medium (5% (v/v]) Fetal Calf Serum and 5% (v/v) Hyclone Supplement in Dulbecco's Modified Eagle's Medium) for 24 to 48 hours. The cells are removed by centrifugation at 1000 g for ten minutes. The supernatant is harvested using sterile techniques and stored at 4 °C.Prior to incubation with Western blots, sodium azide is added to 0.02% (m/v).

Reagents

Unless otherwise indicated, all chemicals used are reagent grade. All water used in buffer preparation and/or dilution is Millipore Milli-Q® water. Below are the descriptions of specific reagents and their vendors.

Myosin Purification:

All reagents used in purification of myosin are reagent grade. The only specific requirement is high quality water such as Millipore Milli-Q® water.

Papain Digestions:

1. Papain: 2X Crystallized Suspension in 0.05 M Sodium Acetate. pH 4.5, 0.01% (v/v) Thymol. Obtained from Sigma Chemical Company. Catalog # P-3125.

SDS-PAGE Analysis:

2. Denaturing Jule Gels: Non-gradient, 7.5% Acrylamide in 0.375 M Tris-HCl, pH 8.8, 0.1% (v/v) SDS, 0.2% (m/v) Sodium Azide. Gel Thickness: 0.75 mm. Obtained from Jule, Inc., Catalog # 7.5D.75BMC10.
3. High Molecular Weight Standard Mixture. Obtained from Sigma Chemical Comopany, Catalog # SDS-6H.

Western Blotting:

4. Vectastain Alkaline Phosphatase Mouse IgG Kit: Alkaline Phosphatase Mouse IgG. The kit contains the following reagents: Blocking Serum (Normal Horse Serum), Biotinylated Anti-IgG, Reagent A (Avidin DH), and Reagent B (Biotinylated Alkaline Phosphatase H). Obtained from Vector Laboratories, Inc., Catalog # AK-5002.
5. Alkaline Phosphatase Substrate Kit (BCIP/NBT): Obtained from Vector Laboratories, Inc., Catlaog # SK-5400.

Theory

Myosin Purification. Myosin purification from rat muscle is carried out in a five day process. Unless otherwise indicated, all procedures are carried out at 2-6 °C . In addition, gel samples are collected at every stage for process analysis. On Day 1, the rats are sacrificed and leg muscle is isolated at room temperature and packed on ice for 15-90 minutes, depending on the number of animals used. Homogenization with a meat grinder serves to expose the sarcoplasm for extraction with either ATP or pyrophosphate extraction buffer. At least three passes through the meat grinder are required. In addition, some preparations included a blender homogenization after the meat grinder but prior to extraction. Extraction is based on the solubility of myosin and the ionic strength must be greater than 0.3 M for myosin to be soluble. The ionic strength of the extraction buffers are 0.62 M for the pyrophosphate buffer and 0.77 M for the ATP buffer. The minced muscle and extraction slurry are diluted two-fold with cold distilled water and then filtered over cheesecloth with the mince being discarded after preparation of a gel sample. The remaining supernatant is then slowly diluted an additional six-fold with cold water to final ionic strengths of 0.05 M and 0.06 M, respectively. The extract is then set in Florence flasks on ice and left overnight to allow the myosin precipitate to settle out.

Day 2 involves collection of the myosin precipitate and dialysis against Myosin Buffer (0.6 M ionic strength) to resolubilize myosin. The precipitate is collected from the Florence flasks by aspiration of the supernatant and subsequent collection of the remaining precipitate slurry. The collected material is centrifuged at 8000 rpm for 30 minutes. A minimum amount of Pellet Dispersion Buffer #1 is overlayed onto the pellet over ice for 30 minutes. The viscous solution is then dialyzed against the myosin buffer.

On Day 3, the dialyzed crude myosin solution is diluted two-fold dropwise with cold water using slow stirring with a glass rod to precipitate the actomyosin.

Actomyosin is a complex of actin filaments with bound myosin and is a major contaminant. Furthermore, at 0.3 M , actomyosin is not soluble, whereas myosin is marginally soluble. The solution is then centrifuged at 8000 rpm for 30 minutes to separate the large molecular weight insoluble actomyosin complexes from the lower molecular weight soluble myosin species. The pellet is discarded and the resulting supernatant is then further centrifuged at 20,000 rpm for one hour to further remove aggregates. The pellet, if any, is discarded. The myosin is then reprecipitated by an eight-fold dilution with cold water to an ionic strength of 0.04 M to concentrate the product and to further improve purification, and then left overnight to settle out.

Day 4 requires collection of the myosin precipitate following the same procedure as that carried out in Day 2. The pellet is spun at 8000 rpm for 30 minutes followed by addition of a minimum amount of pellet dispersion buffer for collection of pellet. Overlaying is done with the high ionic strength Pellet Dispersion Buffer #2. The dispersed pellet is then dialyzed against 0.6 M ionic strength myosin buffer overnight.

On Day 5, the myosin solution is clarified by ultracentrifugation at 20,000 rpm for two hours. This step serves to remove fat, and to isolate monodisperse monomers of myosin by sedimenting any denatured material. If any fat is present at the top of the supernatant following ultracentrifugation, it is aspirated off and discarded. Protein determination and gel electrophoresis are then carried out on the final material. If desired, the myosin is glycerinated with an equal volume of glycerol by dropwise addition on a stirplate or by gentle rocking of the tube and stored at -20 °C .

Spectral Analysis. Spectral analysis is performed using an HP diode array UV/Visible spectrophotometer. Spectra are obtained for the range of 200 nm through 400 nm. Absorbance is recorded at 260 nm, 280 nm, and 340 nm. At 260 nm, the absorbance is primarily due to nucleic acids, both single and double stranded (Figure 14).

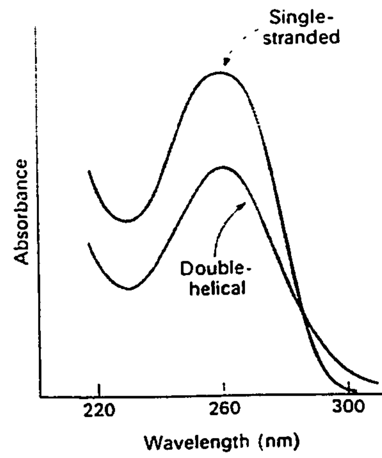


Figure 14: Absorbance spectra of nucleic acids.
(Copied with permission from Stryer, 1988.)

At 280 nm, the actual protein content is responsible for the majority of the absorbance. More specifically, at this wavelength, the amino acid residues tyrosine, tryptophan, and phenyalanine have absorbance maxima with the absorption due to tyrosin and tryptophan dominating the protein's absorption spectra (Figure 15).

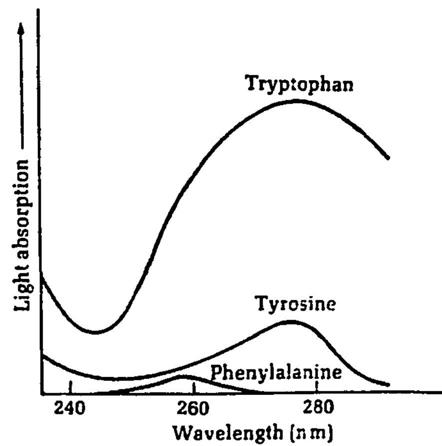


Figure 15: Absorbance spectra of the aromatic amino acid residues.
(Copied with permission from Lehninger, 1970.)

Finally, at 340 nm, there is no absorbance due to protein or nucleic acid content, such that any decrease in observed light intensity is caused by particles, such as denatured myosin aggregates. If the amount of absorbance at 340 nm exceeds 10% of the absorbance at 280 nm, large errors are introduced by the extrapolation of the baseline performed to subtract the light scattering contribution to apparent A_{280} values. An example of this will be discussed in detail in the Results section. Protein concentration is determined according to Beer's Law: A = Ecl, using E_{280} for a 1% myosin solution = 5.0 (Silberstein, 1979). The pathlength, or 'l', is one centimeter, and the extinction coefficient, 'E', for myosin at 280 nm, is 5.0 for a 1% solution (i.e., 10 mg/mL = 1 gm/100 cc), and the units of 'c' are in gm/dl.

Papain Digestions. Proteolytic digestion of myosin is accomplished by using papain. As was earlier discussed, papain preferentially cleaves myosin at the first hinge region giving rise to the S-1 and rod subfragments. Papain is classified as a *thiol protease* containing an active-site cysteine that acts much like serine 195 in chymotrypsin (Stryer, 1988). It contains 212 residues with its essential sulfydryl group being Cys25 (Kamphius et al., 1984). Papain cleaves peptide bonds at the interior of the polypeptide chain (Garret and Grisham, 1995), with arginine and lysine residues following phenylalanine being the preferred cleavage site (Schechter and Berger, 1968). Its reactive group and covalent intermediate are shown in Figure 16.

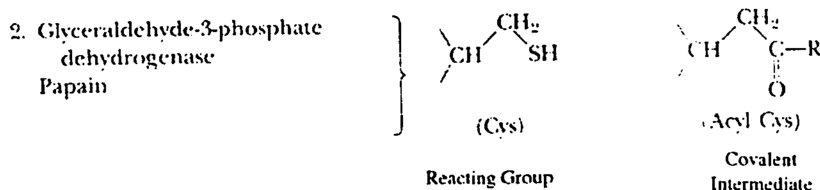


Figure 16: Reactive group and covalent intermediate of papain.
(Copied with permission from Garret and Grisham, 1995.)

Prior to digestion with papain, the myosin is precipitated in an ammonium acetate digestion buffer at 0.2 M ionic strength to increase concentration. In addition, precipitation in the presence of Mg^{2+} allows the myosin to self-aggregate and hide the second hinge region from papain (Margossian et al., 1975). After centrifugation, the supernatant is discarded and the precipitated myosin is dispersed in a minimal amount of digestion buffer. Digestion is performed at 16° C for varying lengths of time. The reaction mixture is stirred every two minutes with a chilled miniature glass stirring rod. Digestion at the lowered temperature is necessary to discourage irreversible aggregation of myosin interfering with results. The length of time, concentration of myosin, and amount of papain were varied to determine optimal conditions for preparation of S-1 and rod subfragments. In some experiments, dithiothreitol (to final concentration of 0.5 mM) is added to ensure that papain is in the reduced state. Iodoacetic acid (IAA) is added to terminate the reaction (to final concentration of 5 mM). IAA oxidizes sulfhydryls (Figure 17), thus, addition of IAA destroys papain's active group.

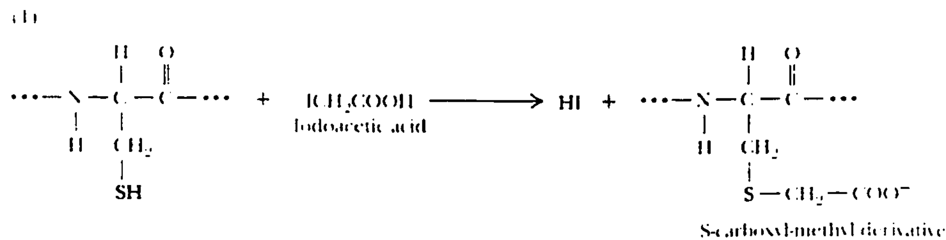


Figure 17: Structure of iodoacetic acid.
(Copied with permission from Garret and Grisham, 1995.)

A gel sample is made of the reaction mixture by removing an aliquot. The remainder of the digestion mixture is then microfuged for removal of the supernatant presumably containing the soluble S-1 fragment and another gel sample is made. The remaining pellet is then washed with a low ionic strength solvent and microfuged a total of four times to remove

any soluble material. The remaining pellet contains any insoluble material including undigested myosin, the rod subfragment, and any fragments still bound.

Gel samples are prepared for SDS-PAGE analysis by addition of an equal volume of SDS sample buffer containing both a denaturant and a reducing agent for proper sample mobility, followed by immediate boiling for three minutes. The following gel samples are prepared for each digestion performed: undigested myosin, digestion mixture, soluble S-1 head, and insoluble material. SDS-PAGE analysis is then performed.

SDS-PAGE Gel Electrophoresis. Analysis of myosin purifications and papain digestions is performed using SDS-PAGE gel electrophoresis. SDS-PAGE analysis utilizes the detergent sodium dodecyl sulfate (SDS). SDS is an amphipathic molecule that serves as a strong denaturant (Voet and Voet, 1990). SDS has a long nonpolar tail and a polar sulfate group at the head which associates with the sodium atom (Figure 18).

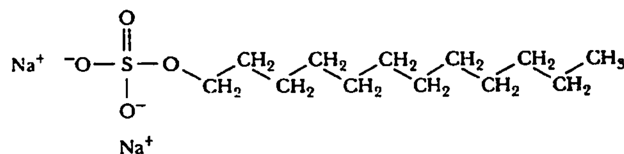


Figure 18: Structure of sodium dodecyl sulfate.
(Copied with permission from Garret and Grisham, 1995.)

By competing with hydrophobic binding sites on the interior of the protein, SDS causes denaturation. In addition, because of the negatively charged sulfate group, any hydrophobic R-groups gain a net negative charge and any postively charged R-groups get neutralized. The net result is a negatively charged protein. Therefore, SDS masks the native charge of the protein and the resulting net charge of the molecule is based on overall size. Thus, electrophoresis separates the molecules based on molecular weight, the larger molecules being retarded longer by the gel.

Migration in an SDS-PAGE gel follows a log-linear relationship (Stryer, 1988). In other words, the relative mobility of the proteins is linearly proportional to the logarithm of their molecular weight (Figure 19). The power of SDS-PAGE analysis lies in the ability to identify proteins based on their mobility with respect to known standards (Sigma, Catalog # SDS-6H). With each run of proteins, eg. papain digested material, known standards are run and the fragments identified based on mobility.

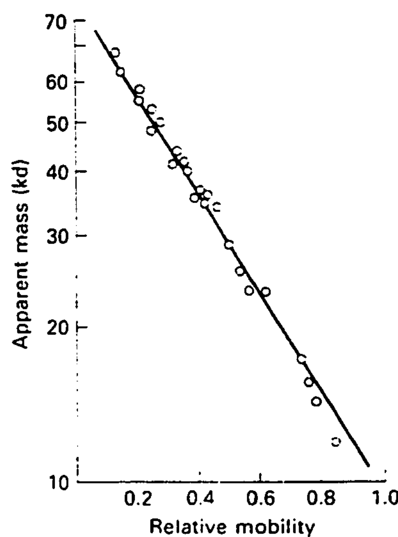


Figure 19: Log-linear relationship of molecular weight and relative mobility. (Copied with permission from Stryer, 1988.)

Sample preparation is required for SDS-PAGE analysis to insure proper mobility. First of all, addition of SDS sample buffer precedes analysis. This sample buffer contains both a denaturant (SDS) and a reducing agent (dithiothreitol). Dithiothreitol (DTT) reduces disulfide bonds (Stryer, 1988). In addition, prior to application of the samples onto the gel, samples are boiled for three to five minutes to inactivate any remaining proteases and increase the myosin denaturation rate. Samples are stored frozen at -20 °C to

discourage any further proteolytic activity. Additional reducing agent may be added upon thawing the samples (5 µL 2-ßME per 100 µL sample, see *Discussion*).

Electrophoresis is carried out at room temperature at 25 mA in 1X Running Buffer for approximately one hour to two hours, using a mini slab gel format (8 x 10 mm, 0.75 mm gel thickness, 10 lanes; obtained from Jule, Inc. Part #7.5D.75BMC10), or until the dye front is 0.2 to 2.0 cm from the bottom of the gel. Prior to staining, the gel is fixed for ten minutes to immobilize the bands and remove SDS. Unless otherwise noted, gels contained 7.5% acrylamide. Gels are stained overnight at room temperature and destained until background is minimized. Gels are then photographed and dried for storage.

Identification of myosin fragments is achieved through analysis of their migration based on molecular weights. Myosin (220 kDa), S-1 (95 kDa), rod, (125 kDa), HMM (150 kDa), and LMM (70 kDa) can all be identified by this procedure. Actin (42 kDa) is also of interest as it is a major contaminant of the purification process. The molecular weights of the standards and their respective relative mobilities are plotted in an log-linear fashion followed by calculation of myosin fragment molecular weights by plotting their relative mobilities on the standard curve. An example of this is shown in Figure 20.

Western Blotting. Western blotting allows for the identification of a particular protein through staining with a specific antibody (Stryer. 1988). After electrophoresis on the SDS-PAGE gel, the bands are transferred from the gel to a nitrocellulose sheet. The transfer process calls for the formation of a "gel sandwich" where the gel is placed next to the nitrocellulose sheet (Bio-Rad Trans-Blot Transfer Medium, 0.45 micron, Part #162-0113) and surrounded by Whatman paper (Figure 21). The entire sandwich is then placed in the transfer box (Bio-Rad Mini Trans-Blot. Catalog #153BR; or Hoefer TE Series Transphor Electrophoresis Unit, Model #: TE Series) with Transfer Buffer at 4 °C and transferred

overnight on a stirplate at 30 or 60 volts (depending on the geometry of the transfer box) which corresponds to approximately 250 mA (Hockfield et al, 1993). A power supply which regulates at constant current is preferred to regulation at constant voltage to prevent overheating.

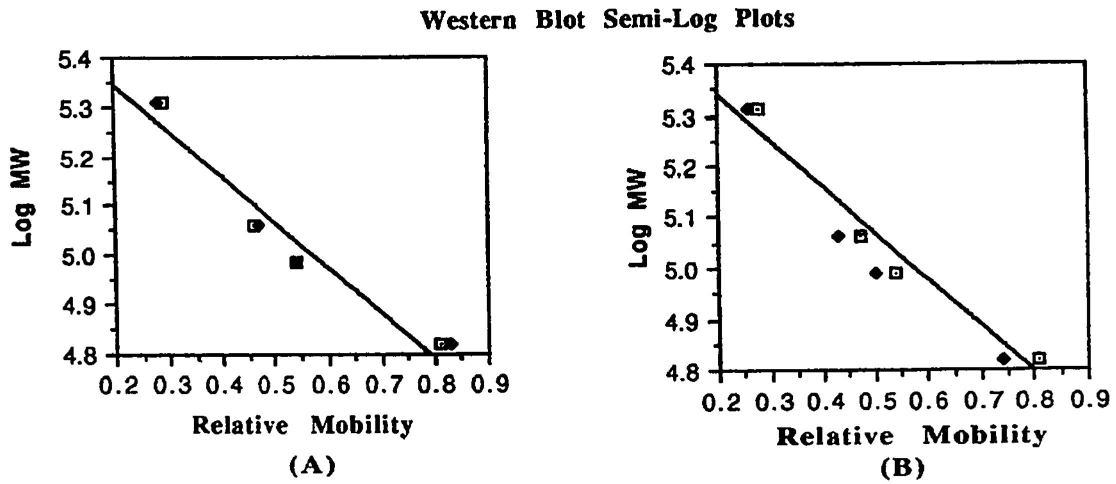


Figure 20: Log-linear correlation of molecular weight and relative mobility. (A) Semi-log plot corresponding to Western Blots A1 (squares) and B1 (diamonds) run on 3/27/95. The solid line represents the linear regression curve. The respective equations and correlation coefficients are: $y = 5.5 - 0.92x$, $R^2 = 0.94$ and $y = 5.5 - 0.87x$, $R^2 = 0.94$. 'y' represents the log MW and 'x' represents the relative mobility. (B) Semi-log plots corresponding to Western Blots A2 and B2 run on 3/30/95, same format as described in (A). The respective equations and correlation coefficients are: $y = 5.5 - 0.91x$, $R^2 = 0.95$ and $y = 5.5 - 1.00x$, $R^2 = 0.96$.

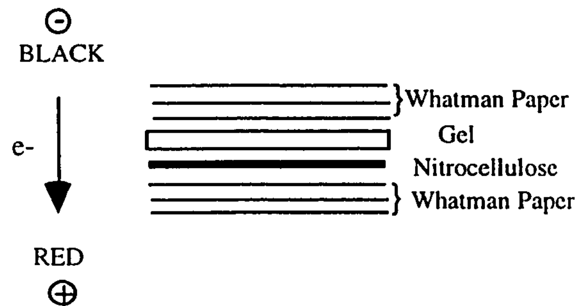


Figure 21: Representation of a 'gel sandwich'.

After transfer of the protein bands, the molecular weight marker lane is cut away from the nitrocellulose paper and dried for later staining. The molecular weight standard lane can be developed by various methods including visualization with India Ink as described by Hockfield et al. The following step-by-step process represents the exact materials and procedure required per blot after transfer onto the nitrocellulose paper. Prior to incubation with antibody, the rest of the nitrocellulose sheet is blocked in Western Wash Buffer for 15 minutes to block any non-specific binding sites.

Western Blot Protocol. The following summarizes the Western blotting procedure.

1. Block blot for 15 minutes in Western Wash Buffer.

2. Transfer blot carefully to a "Seal-a-meal" baggie.

3. Incubate blot with desired monoclonal antibody overnight on a rocking platform at 4 °C according to the following:

   1.5 mL 10X Borate Buffer
   12 mL Primary Antibody
   1.5 mL H_2O

4. Wash blot four times with Western Wash Buffer (4 X 15 mL) over a period of at least ten minutes.

5. Add biotinylated reagent to blot as follows and incubate overnight on a rocking platform at 4 °C :

   2.0 mL 10X Borate
   2 drops Normal Horse Serum (Vectastain Alkaline Phosphatase Mouse IgG Kit, Catalog # AK-5002)
   2 drops Biotinylated Horse Anti-Mouse IgG (Vectastain Alkaline Phosphatase Mouse IgG Kit, Catalog # AK-5002)
   18 mL H_2O

6. Wash blot four times with Western Wash Buffer as above.

7. Prepare Vectastain ABC-AP® complex and allow to stand at least 20 minutes prior to use:

   1.5 mL 10X Borate Buffer

3 drops Reagent A (Vectastain Alkaline Phosphatase Mouse IgG Kit, Catalog # AK-5002, Avidin DH)
3 drops Reagent B (Vectastain Alkaline Phosphatase Mouse IgG Kit, Catalog # AK-5002, Biotinylated alkaline phosphatase)
13.5 mL H_2O

Incubate blot with Vectastain ABC-AP® complex for at least one hour on a rocking platform at 4° C .

8. Rinse blot with 2 X 15 mL of each of the following: Western Wash Buffer, H_2O, and 100 mM Tris-HCl, pH 9.5. All washes should take a total of five to ten minutes.

9. Prepare the BCIP/NBT Substrate in 40 mL 100 mM Tris-HCl, pH 9.5:

16 drops Reagent 1, mix. (Alkaline Phosphatase Substrate Kit, Catalog # SK-5400, BCIP)
16 drops Reagent 2, mix. (Alkaline Phosphatase Substrate Kit, Catalog # SK-5400, NBT)
16 drops Reagent 3, mix. (Alkaline Phosphatase Substrate Kit, Catalog # SK-5400, $MgCl_2$)

Development can be carried out in a petri dish.

Vectastain Alkaline Phosphatase Mouse IgG Kit. As described above, after incubation with primary antibody and subsequent washing in Western Wash Buffer (WWB), the blot is then incubated with a biotinylated reagent (Vectastain Kit, Biotinylated Horse Anti-Mouse IgG) that is specific for immunoglobin G, both heavy and light chains. Thus, the biotinylated reagent recognizes the immobilized antibody. After incubation and subsequent washing, the blot is then subjected to the ABC-AP® complex. The ABC-AP® complex is a biotinylated alkaline phosphatase-avidin complex which is prepared by mixing biotinylated alkaline phosphatase and avidin DH (Vectastain Reagents A and B, respectively) thirty minutes prior to blot incubation according to the manufacturer's instructions (Step 7 in the above protocol). This pre-mixed ABC-AP® complex has unoccupied avidin binding sites available for reaction with the biotinylated anti-mouse IgG bound to the blot. Since each biotinylated reagent (both the horse IgG and alkaline phosphatase) has been derivatized with several biotin molecules, and each molecule of

avidin has four binding sites for biotin, extensive amplification of the signal is achieved with a high degree of specificity (Figure 22).

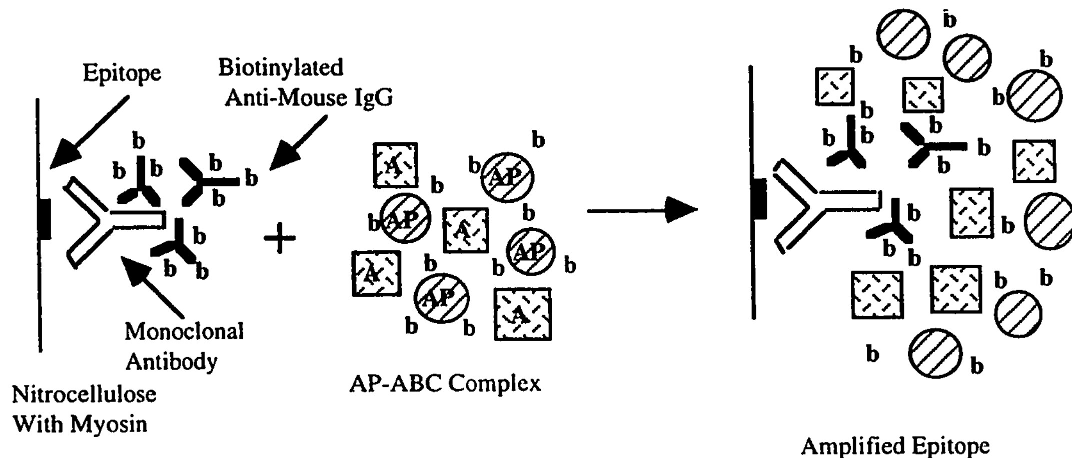


Figure 22: Amplification of the antibody epitope signal with AP-ABC.

<u>Vectastain Alkaline Phoshatase Substrate Kit.</u> The final step involves development with 5-bromo-4-chloro-indoxyl phosphate-NitroBlue Tetrazolium (BCIP-NBT) at room temperature. The BCIP/NBT reagents are added to a solution containing Tris-HCl at pH 9.5. BCIP, upon reaction with alkaline phosphatase, forms an indoxol compound which can in turn react with NBT (Figure 23). The final product formed by this reaction is formazan which is a colored compound and is insoluble in situ. Thus, the epitope of the monoclonal antibody is localized and visualized through this procedure. In order to further localize the epitope on myosin, all myosin fragments are subjected to Western blotting with each of the six monoclonal antibodies.

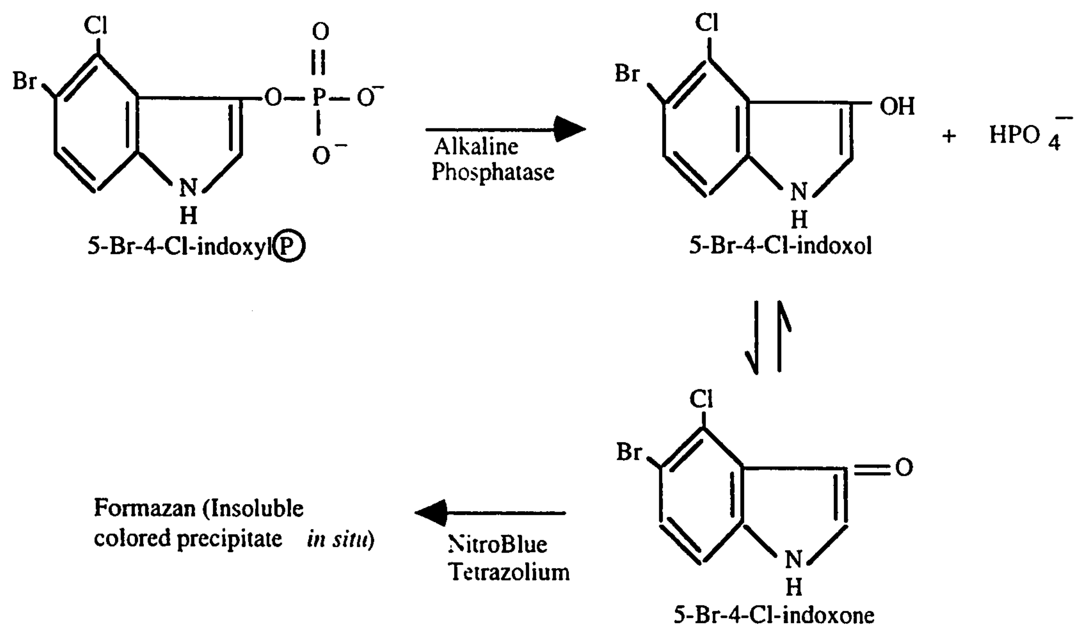


Figure 23: BCIP/NBT Reaction with Alkaline Phosphatase for band development on Western Blots

RESULTS

Myosin Purification. Several myosin preparations have been undertaken with varying degrees of success. Each of the preparations were run in-parallel with regard to extraction buffers and means for exposing myofibrils. The comparisons were made in an attempt to optimize the purification process in terms of overall yield and quality of product. An optimal purification process would give high quality, monodisperse myosin while at the same time having a high yield. In addition, repetitions served as a good method to trouble-shoot most aspects of the process itself. Table 2 summarizes the results of these purifications.

Date/Code	Starting Material (Rat Leg Muscle)	Yield (Based on A_{280})	Comments
8-93 / (S3)	48 g	400 mg*	Material used for gels, digestions, and Westerns
8-94 / (S4)	200 g	0 mg	Contaminated Water
9-94 / (S5)	247 g	0 mg	Unsuccessful exposure of myofibrils (Two passes through meat grinder)
10-94 / (S6)	51 g/ 51 g	62 mg/ 88 mg*	ATP vs. PP_i Extraction Buffer
1-95 / (S7)	80 g/ 82 g	0 mg	Temperature during prep rose above maximum limit (Grinding vs. Homogenization)
2-95 / (S8)	106 g/ 103 g	28 mg/ 0 mg	Grinding Only vs. Homogenization

Table 2: Summary of myosin purifications.
(*High A_{340}% of A_{280} introduces large error in yield calculation)

Prep S3 was successful in producing sufficient material for the use in preliminary papain digestions, SDS-PAGE analysis, and Western blots. The next two purifications, Prep S4 and Prep S5, had unforeseen technical problems.

First of all, no useable material was obtained from Prep S4 because of a problem with the in-house distilled water system. As the use of water is a critical component of myosin precipitation at three steps during the process, any inconsistencies can ruin the preparation. It is postulated that because of the problems with the in-house distilled water at the time of the preparation, the water was contaminated. Contamination could have caused the water to be at a high ionic strength; therefore, the myosin did not precipitate as expected. It was at this point that the decision to use a higher quality water was made. In all subsequent purifications, Millipore Milli-Q® water was used to insure a high degree and consistency of water purity.

Prep S5 was discontinued after the first precipitation step as no myosin was present. This was attributed to incomplete exposure of myofibrils during the grinding step. If this occurs, an insufficient amount of myosin is exposed to the extraction buffer and thus remains insoluble. It became clear that all subsequent purifications must involve at least three passes of the muscle through the grinder. In addition, a blade with a smaller frit size was chosen.

Prep S6 involved the comparison of ATP extraction buffer and pyrophosphate (PP_i) extraction buffer. This was undertaken to determine the relative efficiencies of the two buffers to extract myosin. Both extraction buffers gave rise to high quality myosin, however, upon close inspection of the gels and spectral analysis, it is clear that the ATP extraction buffer produces higher quality material. SDS-PAGE analysis clearly shows the step-by-step purification process with quantitatively equal loads from both the ATP and the

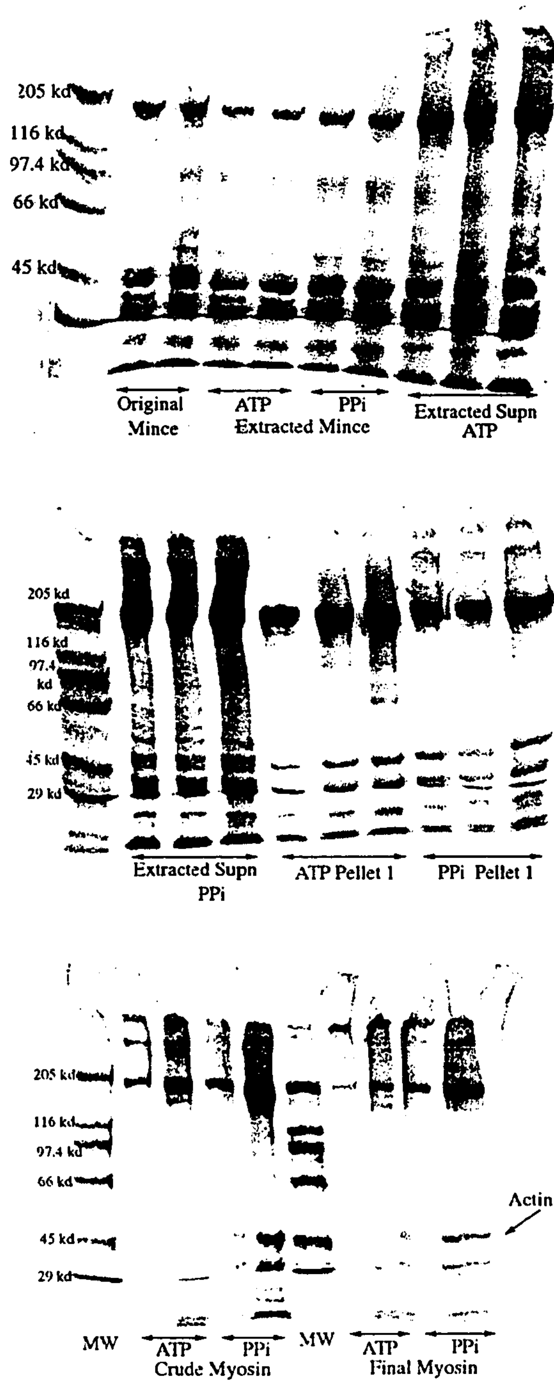


Figure 24: SDS-PAGE analysis of myosin Prep S6 comparing the ATP and PP_i extraction buffers. Samples are indicated at the bottom of each lane and and show the sequential purification process. In this figure, 'kd' represents kilodaltons.

PP_i preparations (Figure 24). As indicated, actin contamination is sequentially decreased throughout the purification process. Furthermore, the ATP prep, although lower in concentration, has a smaller percentage of actin contamination. In addition, the ATP prep also has less contamination due to nucleic acids as seen in a comparison of the spectra (Figure 25).

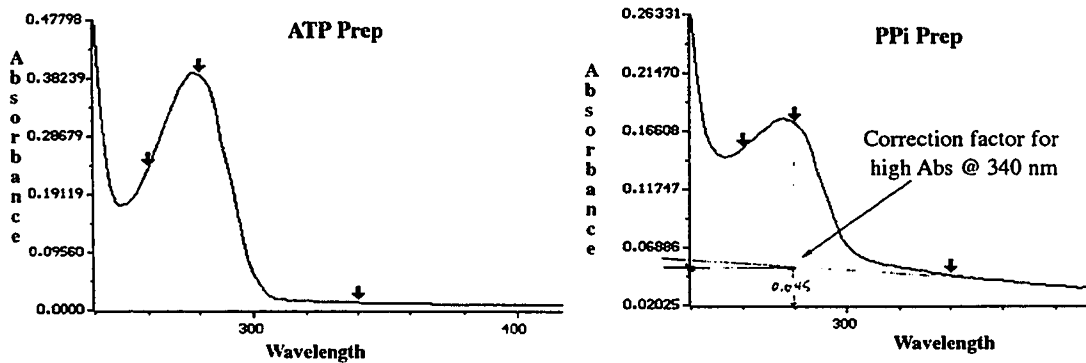


Figure 25: Spectral comparison of the ATP and PP_i preps. The ATP sample is a 10-fold dilution, whereas the PP_i prep is a 40-fold dilution. The extrapolation to correct for a high A_{340} is shown for the PP_i prep.

The relative absorption at 260 nm (an absorbance maximum for nucleic acids), as compared to the absorption at 280 nm, is clearly lower for the ATP prep. More importantly, absorbance due to light scattering (observed at 340 nm) is much lower for the ATP prep indicating that less aggregated myosin is present. The calculated concentration is corrected for high absorption at 340 nm by extrapolation of the baseline from long wavelengths. The corresponding value on this line at 280 nm is subtracted from the orginal 280 nm value. The extrapolation and subsequent subtraction from the A_{280} reading is

shown directly on the spectrum for the PP_i prep. The overall comparison of the two preparations is presented in Table 3.

Property	ATP Extraction Buffer	PP_i Extraction Buffer
Starting Material	51 grams	51 grams
Final Concentration	7.79 mg/mL	11 mg/mL
Final Yield (Based on A_{280})	62 mg	88 mg*
$A_{280/260}$	1.66	1.13
A_{340}% of A_{280}	3%	28%

Table 3: Comparison of ATP and PP_i Extraction Buffers.
(*High A_{340}% of A_{280} ratio introduces large error in yield calculation)

An attempt to compare grinding only versus grinding plus homogenization with the blender was undertaken in Prep S7. The ground mince was dispersed in a low ionic strength buffer, and homogenized for three fifteen second bursts in a chilled Virtis blender cup. The addition of homogenizing the ground mince with a blender was expected to further expose the myofibrils resulting in a higher yield of myosin. However, at the last precipitation step, very little material was present in both preparations and the preparation was discontinued. Upon careful analysis of the working conditions, it was discovered that the cold-room temperature was 12 °C . Previous work has proven that, above 8 °C , any attempt to isolate myosin is unsuccessful. Thus, careful monitoring of the temperature of the coldroom has become necessary.

A second attempt was made to compare the two methods of exposing myofibrils in Prep S8. Both methods showed high yield at the earlier stages of the preparation. However, very little material was present after the final precipitation in the homogenization prep. Upon analysis of the gels, it is evident that a substantial amount of myosin was present prior to this step. However, in earlier steps there was a large amount of actin contamination (Figure 26).

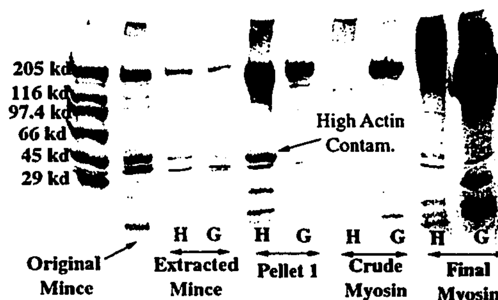
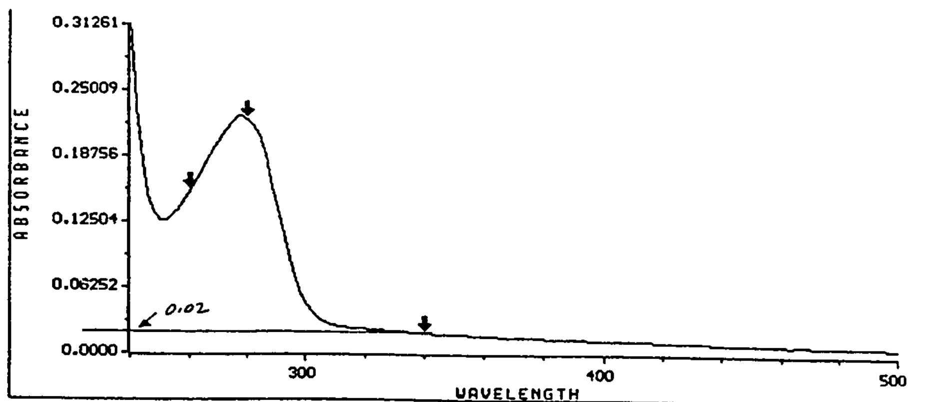


Figure 26: SDS-PAGE analysis comparing homogeniztion vs. grinding only preps. Homogenization samples are designated 'H', and grinding only samples are designated 'G'. Note the presence of high actin contamination in the 'H' Pellet 1. In this figure, 'kd' represents kilodaltons.

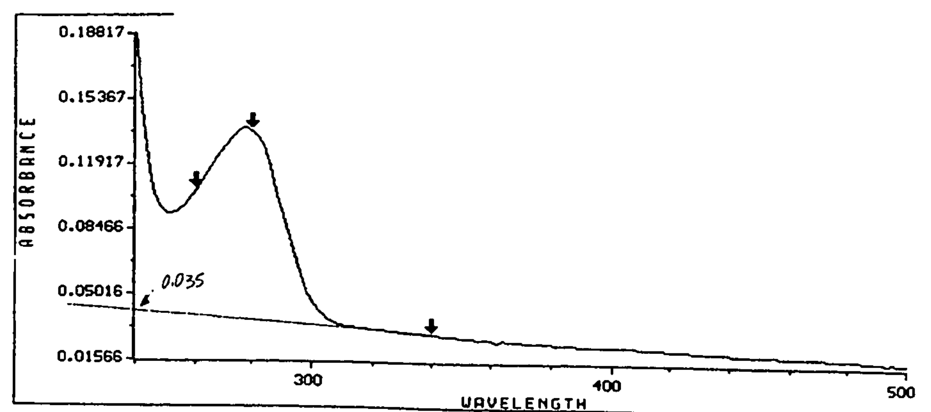
It is postulated that the majority of myosin precipitated out in the earlier stages of the process as actomyosin, as indicated by the large amount of material collected at the actomyosin precipitation stage. The higher molecular weight material in the 'H' final myosin sample is not pure myosin. Evidence of this is the lack of myosin light chains in the 16-25 kDa range that are clearly visible in the 'G' sample. On the other hand, the grinding only portion of the preparation resulted in superior material of the highest quality and, in fact, was so concentrated and viscous, spectral analysis was extremely difficult. Because of the viscosity of the solution, attempts at accurate pipetting were unsuccessful giving rise to inconsistent and unreliable calculated concentrations. The spectra below represent a post-glycerination and post-dialysis analysis of myosin concentration (Figure 27). Hand-calculated concentration values are shown directly on the figure, as well as corrections for high absorbance and 340 nm.



Annotated Wavelengths:

	Wavelength		Result
1 :	Wavelength =	340	Result = 0.019226
2 :	Wavelength =	280	Result = 0.223679
3 :	Wavelength =	260	Result = 0.154160

Concentration: 8.95 mg/mL
$A_{280/260}$ 1.45
Corrected A_{280}= 0.223679 - 0.02 = 0.203679
Corrected concentration = 8.15 mg/mL
Corrected $A_{280/260}$ 1.31



Annotated Wavelengths:

	Wavelength		Result
1 :	Wavelength =	260	Result = 0.105103
2 :	Wavelength =	280	Result = 0.136139
3 :	Wavelength =	340	Result = 0.029556

Corrected A_{280}= .1361139 – .035 = 0.1011
Corrected concentration = 8.09 mg/mL
Corrected $A_{280/260}$= 0.96

Figure 27: Post-dialysis spectral analysis of Prep S8, 'G' sample. The top spectrum represents a 20-fold dilution, and the bottom spectrum represents a 40-fold dilution. Both show corrections for high A_{340}.

Papain Digestions. Several papain digestions of myosin were performed in an attempt to produce S-1 and rod fragments. Preliminary digestions showed inadequate digestion of the myosin and subsequent improvements were made with each digestion. Table 4 summarizes some of these digestion protocols.

Digestion # / Date	Material Used/ Concentration	Amount of Papain	Amount of DTT	Amount of IAA	Notes
#4 10-94	Prep S6 (ATP,PP_i) 15 mg/mL	0.005 mg/ mg Myosin	0 mM	1 mM	Comparison of Digestion Times (5, 10, 20 Minutes)
#5 1-95	Prep S6 (ATP) 15 mg/mL	0.005 mg/ mg Myosin	0.0 - 0.5 mM	5 mM	Comparison of Amount of Reducing Agent
#6 3-95	Prep S8 30 mg/mL	0.005- 0.015 mg/ mg Myosin	0.5 mM	5 mM	Comparison of Amounts of Papain

Table 4: Summary of Papain Digestions

Papain Digestion #4 was a comparison of the ATP versus PP_i extracted material. The digestions were done in parallel with quantitatively equal amounts of material. In both cases, it appeared that auto-degradation (due to contaminating endogenous proteases) was more active than the papain itself. Furthermore, the ATP extracted material showed a small amount of S-1 fragment present while that found in the PP_i material was insignificant. Therefore, it was concluded that the papain was not functioning properly which could be a result of it not being in the reduced state. If papain is not in the reduced state, it is unable to maintain catalytic activity. Thus, a reducing agent (DTT) was added to the mixture prior to digestion in subsequent digestions to ensure that papain is in the proper state. In addition, SDS-PAGE analysis showed evidence of non-reduced material not entering the gel and being retarded at the top of the gel giving a pronounced smearing effect. To facilitate subsequent SDS-PAGE analyses, ß-mercaptoethanol was added to all

subsequent gel samples upon boiling to ensure that the material is fully reduced in order to properly electrophorese.

A comparison of the effect of using different amounts of reducing agent was examined in Digestion #5. The amounts of DTT added were 0.00 mM (control), 0.25 mM, and 0.50 mM. All digestions were carried out for a total of 20 minutes. In addition, an increased amount of IAA was added to terminate the reaction in order to compensate for the additional reducing agent added at the beginning of the reaction. A final concentration of 5 mM IAA was used , as opposed to the 1 mM IAA used previously. A digestion time of 20 minutes was chosen to allow for adequate digestion of the myosin. SDS-PAGE analysis shows a successful digestion of the myosin into soluble fragments migrating at a molecular weight of 95 kDa, presumably the S-1 head. (Figure 28). It also shows that addition of DTT does indeed improve the efficiency of papain and that 0.25 mM is sufficient for cleavage into S-1 type fragments. What appears to be the rod fragment (125 kDa) may be present in the digestion mixture and the apparent S-1 fragment is present in the S-1 samples as indicated. It should be noted that some auto-degradation of myosin has occured as indicated by the satellite bands in the undigested myosin sample. Furthermore, further digestion of the S-1 head into the 27-kDa, 50-kDa, and 20-kDa sub-subfragments has not been accomplished.

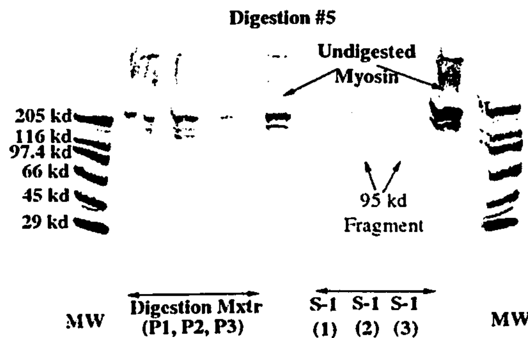


Figure 28: SDS-PAGE analysis of Digestion #5. Samples P1, P2, and P3 [4 uL each] and S-1 (1), (2), and (3) [10 uL each] correspond to 0.00 mM DTT, 0.25 mM DTT, and 0,50 mM DTT, respectively. The ATP extracted material from Prep S6 was used as the starting material.. The date of the gel run was 1/27/95. In this figure, 'kd' represents kilodaltons.

In an attempt to produce S-1 sub-subfragments (i.e., the 75-, 50-, 27-, and 20-kDa fragments), increasing amounts of papain were used in Digestion #6. The amounts used were 0.005, 0.010, and 0.015 mg papain per mg myosin. The pre-digested myosin itself was at a higher concentration (30 mg/mL) than in previous digestions. Reducing agent (DTT) was used at 0.5 mM and the reaction was again terminated with 5 mM IAA after 20 minutes. Figure 29 clearly shows increased amounts of S-1 and rod fragments with increasing papain concentrations. The rod samples appeared smeared which was due to an insufficient amount of ß-mercaptoethanol in the gel sample. The S-1 sub-subfragments are still not evident at these concentrations of papain and digestion times. The improvement in digestion is probably due to both the increased amount of papain and the higher concentration of pre-digested myosin. Major problems still present include MHC contamination in the S-1 fraction and degradation of the MHC in the rod sample (See *Discussion*). Note that the 'rod' sample is actually the washed pellet and contains all the insoluble material present after completion of the digestion.

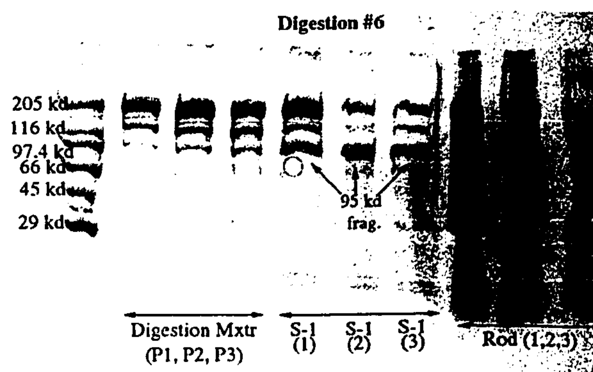


Figure 29: SDS-PAGE analysis of Digestion #6. Samples P1, P2, and P3, S-1 (1), (2), and (3), and Rod (1, 2, and 3) correspond to 0.005, 0.010, and 0.015 mg papain per milligram myosin. respectively. The corresponding loads are 4 ul for the digestion mixture, 10 uL for the S-1 sample and 3 uL for the rod sample. The date of the gel run was 3/3/95 and material was used from myosin Prep S8. In this figure, 'kd' represents kilodaltons.

Western Blotting. The Western blot results indicate that all six monoclonal antibodies react with the S-1 portion of the myosin heavy chain of rat myosin. Table 5 summarizes these results with rat myosin as compared to previous results with other myosins.

Monoclonal Antibody	Previous Finding / Myosin Used	Current Finding with Rat Myosin
MHC.25	Anti-S-1 / Rabbit Myosin[1]	Anti-S-1
MHC.F36	Anti-S-1 / Rabbit Myosin[1]	Anti-S-1
MHC.S95	Anti-130 kDa N-term fragment / Cloned Gene Product[2]	Anti-S-1
MHC.FS26	Anti-130 kDa N-term fragment / Cloned Gene Product[2]	Anti-S-1
MHC.S84	Not at the N-terminus / Cloned gene product[2]	Anti-S-1
MHC.F74	Unknown[2]	Anti-S-1

Table 5: Summary of the reactivities of Six Monoclonal Antibodies with the Myosin Heavy Chain. ([1]Dan-Goor et al., 1990; [2]Hughes et al., 1993)

Western blot analysis was carried out with several different myosin preparations and papain digested material. It was not possible to draw clear conclusions from preliminary results due to some technical problems which will be discussed in the following section. While mAb25 and mAb F36 consistently showed anti-S-1 reactivity (Figure 30), preliminary results obtained for the other four antibodies were unclear as they

had high background and low reactivity (Figure 31). However, it was expected that mAb25 and mAb F36 would give these results, as they are high affinity antibodies. Since it was verified that both mAb25 and mAb F36 are in fact anti-S-1, F36 was used as a control in later Western blots as its intensity is lower and is more interpretable than mAb25.

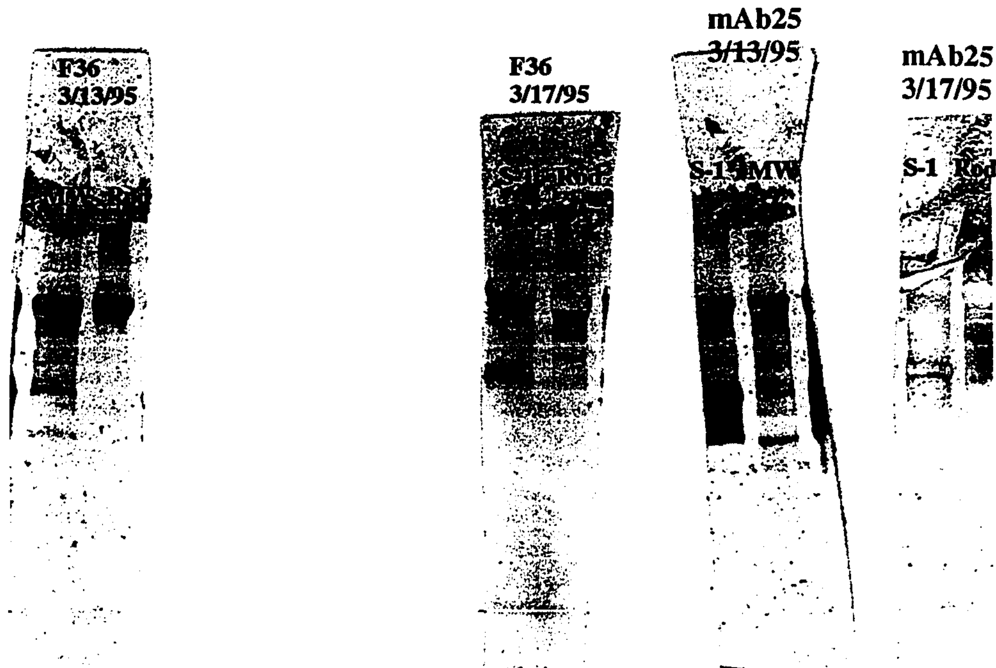


Figure 30: Reactivities of mAbs MHC.25 and F36. Note that the relative intensity of the reaction is much greater for mAb MHC.25 than for mAb F36. However, the 95-kDa S-1 fragments react strongly in both cases, as well as S-1 degradation products. Extremely intense reactions appear as a white-out of excluded product. Note the reaction of the MHC and its degradation products in the MW lanes (MHC is used as the 205 kDa high molecular weight standard). Other contaminating MHC degradation products appear in the rod samples (See *Discussion*). Material from Digestion #6 was used for these blots.

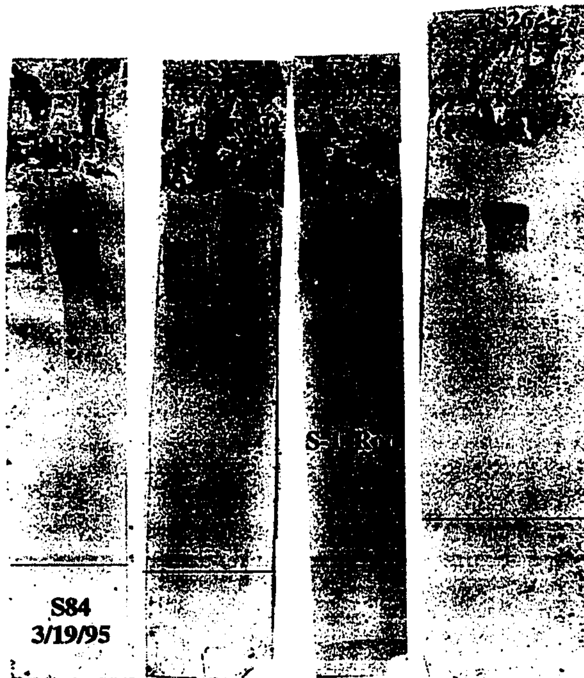


Figure 31: Preliminary Western blots of mAbs S84, S95, F74, and FS26 showing high background and low reactivity. The date of the blot and samples loaded are shown directly on the blot. Material was used from Digestion #6.

Technically speaking, when analyzing the best possible data, mAbs S84, S95, FS26, and F74 all indicated anti-S-1 reactivity (Figures 32 -35). These sets of data were superior for several reasons. Firstly, these transfers were carefully controlled for proper temperature and voltage. Overheating of earlier transfers may have preferentially removed lower molecular weight samples by causing electrophoresis completely through the nitrocellulose paper, thus explaining the lack of any bands below 150 kDa in the preliminary blots shown above (See *Discussion*). Secondly, these blots had a much improved signal-to-noise ratio as compared to the preliminary blots. This was primarily due to the use of fresh substrate for each set of blots and simultaneous development. Also, the ABC-AP® and BCIP-NBT reagents were received two days prior to use, and were not subject to erratic freeze/thaw cycles in the laboratory refrigerator. In addition, as indicated

by the corresponding SDS-PAGE gels (Figures 32-35), these samples had the least amount of myosin heavy chain contamination in the S-1 sample. Finally, these gels and Western blots were run for a longer period of time to increase band separation of the high molecular weight myosin S-1 and rod species.

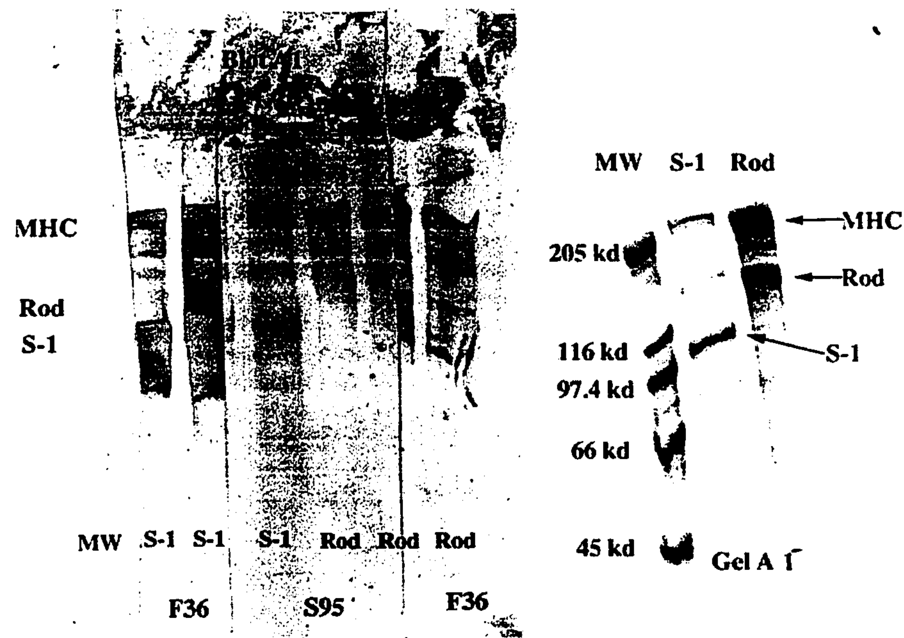


Figure 32: Western blot reactivities of mAbs F36 and S95 shown along with the corresponding stained gel.The gel samples and mAbs used are indicated directly on the blot and gel. Material was used from Digestion #6. In this figure. 'kd' represents kilodaltons.

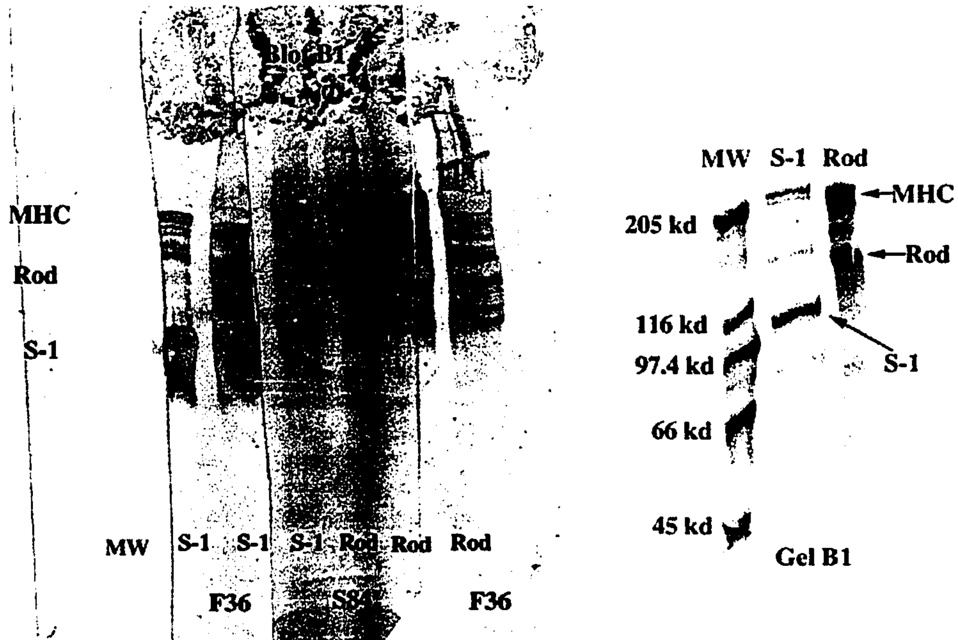


Figure 33: Western blot reactivities of mAbs F36 and S84 shown along with the corresponding stained gel.The gel samples and mAbs used are indicated directly on the blot and gel. Material was used from Digestion #6. In this figure, 'kd' represents kilodaltons.

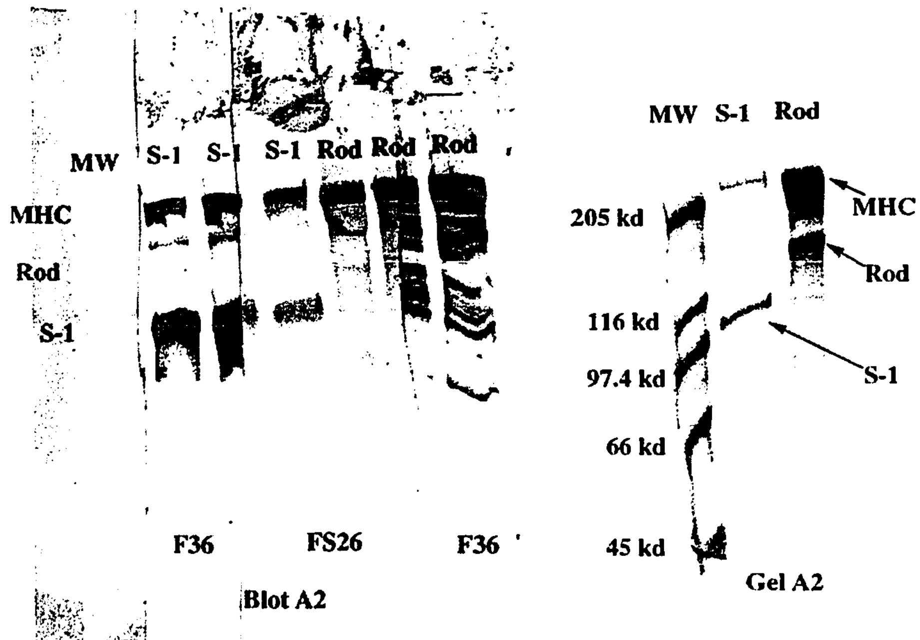


Figure 34: Western blot reactivities of mAbs F36 and FS26 shown along with the corresponding stained gel.The gel samples and mAbs used are indicated directly on the blot and gel. Material was used from Digestion #6.In this figure, 'kd' represents kilodaltons.

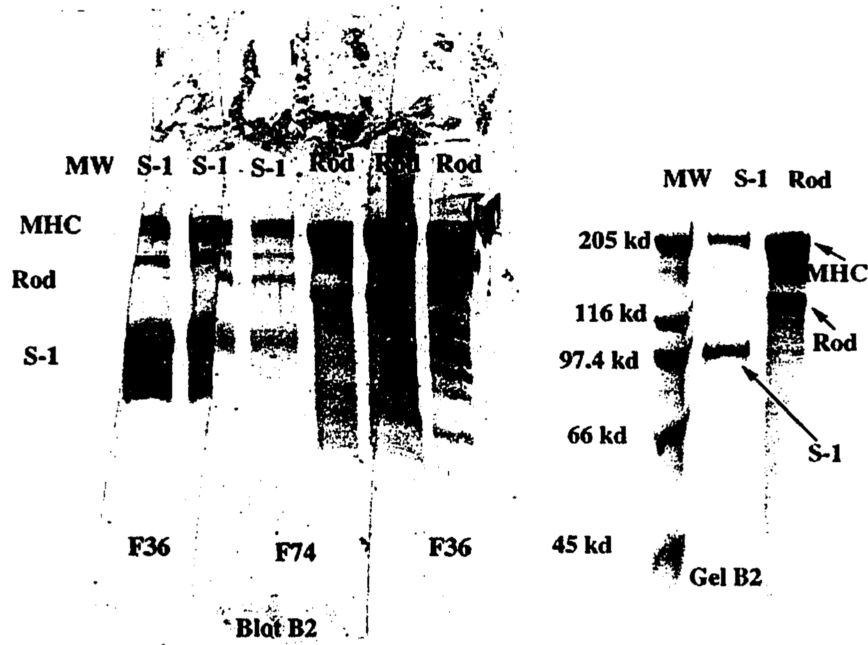


Figure 35: Western blot reactivities of mAbs F36 and F74 shown along with the corresponding stained gel.The gel samples and mAbs used are indicated directly on the blot and gel. Material was used from Digestion #6. In this figure, 'kd' represents kilodaltons.

The reactive bands in the rod samples are apparently due to post-SDS-PAGE proteolytic activity of papain on previoulsy undigested myosin (See *Discussion*). This is a direct result of the rod sample containing the insoluble papain. Note that many of these bands react strongly with mAb F36, indicating that the S-1 epitope is present in these samples. However, the major rod species present in the sample (refer to stained gels) does not appear to react with mAb F.36. This holds true for the other antibodies as well.

In addition to the 95-kDa fragment, the S-1 gel samples show the presence of two minor bands. One of these has a slightly slower mobility than the rod sample and may be the 150-kDa HMM fragment. This fragment does react with the six monoclonal antibodies as shown by the Western blots. The second minor fragment migrates between the 97-kDa and 66-kDa standards and may be the 70-kDa LMM fragment. This fragment does not appear to react on the Western blots.

The semi-log plots corresponding to these gels are shown in Figure 36. Table 6 contains the average molecular weights for the major species in the S-1 and rod samples in each of the corresponding gels.

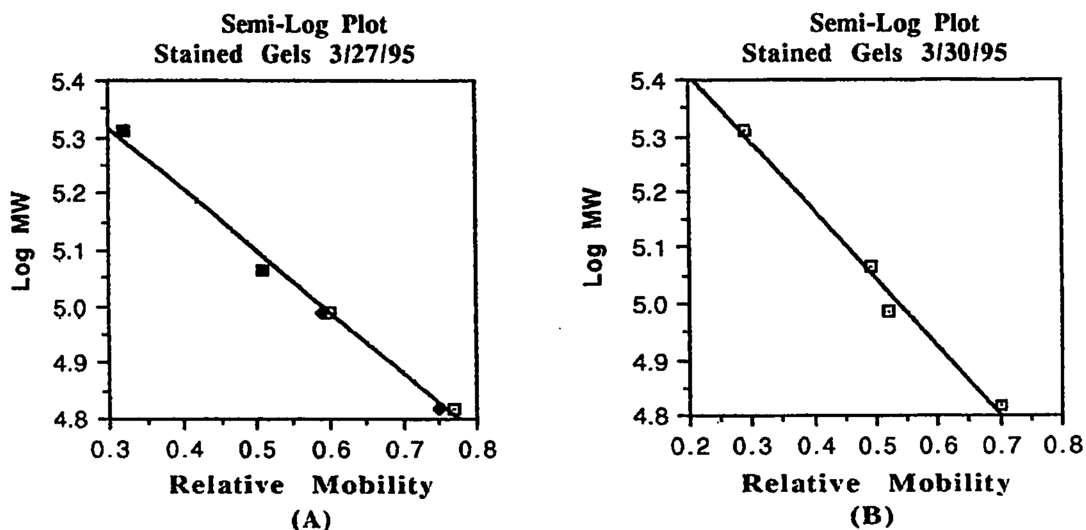


Figure 36: Semi-log plots of the stained gels corresponding to Figures 32-35. (A) Linear regression of Gels A1 (squares) and B1 (diamonds). The solid line represents the linear regression curve. 'y' represents log MW and 'x' represents relative mobility. The respective equations and correlation coefficients are: (A) $y = 5.6 - 1.09x$, $R^2 = 0.99$ and $y = 5.7 - 1.14x$, $R^2 = 0.99$. (B) $y = 5.7 - 1.21x$, $R^2 = .99$ and $y = 5.6 - 1.09x$, $R^2 = 0.99$.

Date of Gel Run	Gel Code	S-1 MW (kDa)	Rod MW (kDa)
3/27/95	A1	106	139
3/27/95	B1	103	135
3/30/95	A2	94	139
3/30/95	B2	99	131
Average MW	n/a	**101 + 5**	**136 $\pm$ 4**

Table 6: Molecular weights of S-1 and Rod fragments calculated from the corresponding semi-log plots of the stained gels.

The semi-log plots for each of the Western blots corresponding to the stained gels were shown in *Materials and Methods* (Figure 20). These standard curves were generated from the molecular weight standard lane of the blots which were stained with India ink. Table 7 lists the calculated molecular weights of the major species reacting in the blots in the S-1 samples.The major species reacting in the rod samples are not calculated due to the following: (1) the presence of contaminating post-SDS-PAGE fragments containing the S-1 epitope, and (2) post SDS-PAGE digestion sites are not expected to be restricted to the flexible hinge regions due to the denaturation of myosin in SDS. Thus, the assignment of molecular weight values to these fragments would have no known correlation to S-1, rod, HMM, or LMM fragments.

Monoclonal Antibody	Date	Blot Code	MW of S-1 (kDa)
F36	3/27/95	A1	110
S95	3/27/95	A1	115
F36	3/27/95	B1	111
S84	3/27/95	B1	111
F36	3/30/95	A2	117
FS26	3/30/95	A2	117
F36	3/30/95	B2	109
F74	3/30/95	B2	109

Table 7: Major species reacting in the Western blot S-1 samples.

As shown by Blot A1 (3/27/95) in Figure 32, mAb S95 is relatively low affininty as compared to mAb F36. Although the major species reacting are the same for both mAbs, the minor banding patterns are different. This is clearly evident in the banding patterns of the rod samples. The mAb F36 shows strong satellite bands under the MHC which are not reacting with mAb S95. This would indicate that the eptitopes reside in different areas of the S-1 fragment. Blot B1 (Figure 33) compares the reactions of mAb F36 and mAb S84, and also show strong anti-S-1 reactivity. Again, the rod samples show different banding patterns. When comparing the satellite bands below the MHC, the mAb S84 blot shows a positive reaction with a third band. The mAb F36 blot, on the other hand, does not react with this third satellite band as indicated by its absence. Furthermore,

no reaction occurs with the lower molecular weight bands in mAb S84 as in mAb F36. Therefore, these epitopes as well are probably different. Based on the reactivities of Blots A2 and B2 (Figures 34 and 35), both mAbs FS26 and F74 are anti-S-1. But once again, the patterns of the lower molecular weight bands differ from those of mAb F36. Although mAbs F36 and FS26 look very similar, the relative intensities of the first two satellite bands in the mAb F36 S-1 sample are very different from the first three satellite bands in the mAb FS26 blot, indicating differing epitopes. Thus, all six monolonal antibodies appear to have different epitope maps based on the reactions of the contaminating proteolytic bands. Finally, as indicated by immunoblot reactivity and molecular weight calculations, although preliminary results are questionable, analysis of the later data, using more refined techniques, indicates that all six antibodies are anti-S-1.

DISCUSSION

Myosin Purification. It has been shown that rat myosin can be successfully purified from rat muscle tissue. In addition, some aspects of the procedure itself have been identified as critical. If these factors are taken into consideration, a successful myosin purification with high yield can become routine. The most difficult factor involved when working with myosin is its susceptibility to denaturation and degradation when exposed to high temperature (>12 °C). First of all, the temperature of the working conditions throughout the entire preparation must be between 2-8 °C . Also, the muscle tissue, precipitation flasks, etc. must be kept on ice. The lowered temperature is necessary to prevent aggregation and denaturation of myosin as well as to inhibit any endogenous contaminating protease activity. The gel samples must also be maintained on ice prior to preparation and stored at –20 °C . Upon use, they must go directly from the freezer into the boiling water bath and be kept on ice prior to loading on the gel. This is due to the relative stability of proteases in SDS, as compared to myosin. Many proteases are resistant to denaturation and may in fact survive in the denaturing solution longer than the protein (i.e. myosin) itself (Harris and Angal, 1989). This may cause proteolytic artifacts that were not present in the original sample necessitating the use of protease inhibitors in the sample buffers (ibid). Thus, an irreversible papain inhibitor such as E-64 (L-trans-epoxysuccinyl leucylamido[4-guanidino]butane) or chloracetyl-OH-Leu-Ala-Gly-NH_2 (ibid), should be included in the rod samples (which contain insoluble papain) in future work.

Another important factor in the purification of myosin is ionic strength. At many stages of the preparation, myosin is precipitated and solubilized based on its solubility as a function of ionc strength. As discussed earlier, myosin is soluble at or above an ionic strength of 0.3 M. Actin, on the other hand, is not soluble at this ionic strength , requiring that the buffers used at the different steps must have the ionic strength required

for either precipitation and/or solubilization of myosin and/or actin depending on what result is desired. If the buffers are of incorrect ionic strength, the preparation will not be successful (as was the case when the house-distilled water had excessive ion contamination).

Related to ionic strength, actin contamination is another important factor to consider for a successful purification. On Day 3 of the myosin purification protocol a dropwise dilution of the crude myosin to 0.3 M ionic strength serves to precipitate actin. If the ionic strength is any lower, the myosin will also be insoluble and will be discarded along with the actin. Another outcome may be that the actin does not precipitate and remains with the preparation increasing the risk of myosin subsequently precipitating out as actomyosin and being discarded. Thus, it is important to remove most of the actin in the earlier stages of the preparation and keeping it at a minimum throughout the final stages.

Efficient exposure of myofibrils is another critical step to insure adequate extraction of myosin from the muscle tissue. It has been observed that if the myofibrils are not sufficiently exposed, myosin is not successfully extracted. Adequate exposure can be as simple as an additional pass through the meat grinder during Day 1 of the procedure. Simple though it may be, if this is not done properly, the purification will not be successful. Furthermore, overexposure (obtained by blender homogenization post-grinding, eg. Prep S8) results in too much actin contamination and ultimately, low myosin yield. Thus, it has been shown that purification of myosin is an intricate process requiring great attention to details.

Papain Digestion. Through SDS-PAGE analysis and Western blotting, it has been shown that papain digestion of rat myosin gives rise to S-1 and rod fragments. Furthermore, several additions to the initial digestion procedure have been made for optimization. First of all, the use of non-degraded, monodisperse myosin proves to give superior results (i.e.

Digestion #6 with material from Prep S8). In addition, the concentration of the myosin in the digestion mixture has an effect on the extent of digestion. As discussed earlier, myosin resuspended to 30 mg/mL gives the largest amount of digestion fragments, as compared to the 15 mg/mL material used earlier.

The papain itself must be in the proper form. As was discussed earlier, for papain to cleave myosin, it must be in the reduced state. Activation can be achieved by adding a reducing agent prior to the start of digestion. The reducing agent of choice was DTT and worked sufficiently at a final concentration of 0.25 mM.

Not only does the activation of papain affect the digestion, but the amount of papain also contributes to a successful digestion. It was clearly shown in Digestion #6 that increasing amounts of papain directly relate to increasing extents of digestion. At 0.015 mg papain per milligram myosin, the S-1 and rod fragments are clearly present in satisfactory amounts. Over-digestion at these concentrations is not a factor as digestion into the 27-kDa, 50-kDa, and 20-kDa S-1 sub-subfragments does not occur. If desired, this may possibly be achieved by further increasing papain concentration, or by using other proteases (Dan-Goor et al., 1990).

In order to properly analyze the gel samples from the papain digests (as well as all other SDS-PAGE analysis), it has been shown that addition of a reducing agent prior to loading the gels decreases the amount of smearing at the top of the gel. The smearing results from non-aggregated and/or non-reduced material being retarded at the top of the lane. The addition of ß-mercaptoethanol (approximately 2 µL/ 50 µL sample) was sufficient to eliminate evidence of smearing as it ensures that full reduction of the material within the gel sample is accomplished. It is postulated that upon storage, the ßME present in the original sample is either degraded or evaporated resulting in insufficient activity. ß-

mercaptoethanol addition was performed after re-boiling of the frozen samples, prior to loading. This greatly facilitated analysis of all gels performed using this procedure.

Western Blotting. Western blotting has shown that all six monoclonal antibodies have linear epitopes recognizable after SDS denaturation of the antigen. In the process of localizing the epitopes, several aspects of Western blotting procedure proved to be important. First and foremost, when working with nitrocellulose, one must be aware of its fragility. In addition, all the reagents must be used according to manufacturer's specifications. This includes proper concentrations and corresponding incubation times. Early Western results indicate that if excessively dilute concentrations are used, band development is not visualized.

Superior results were attained when using non-degraded, high quality myosin. As shown by the Western blots, the different antibodies showed marked differences in the extent of band development. This observation may be attributed to a number of different phenomena. First of all, the concentrations of the antibody supernatants were not adjusted identically and the most concentrated cell culture supernatants available were used. Although an effort was made to use the same lot of each specific antibody for consistency, the differences between antibodies was not controlled. However, ELISA analysis indicated that all mAb solutions were in the 2-20 ug/mL range (data not shown). The concentration of each specific antibody was not determined prior to incubation resulting in possible differences between antibodies. Thus, more concentrated antibodies may allow for greater amplification and band development. Secondly, differences occur in the affinity of the antibodies for their specific epitopes. The extent of antibody binding is dependent on the affinity of the mAbs for the epitope and the concentration of reactants. The affinity of a particular mAb may also be more or less affected by the denatured conformation of the protein. Thus, the antibody affinity of a mAb which recognizes a strictly linear protein

determinant will be less affected than one which recognizes a conformationally-dependent determinant. Because of differences in antibody specificity and affinity, different loads on the gels may be necessary to increase the epitope concentration. This can usually be determined by trying several different loads and exposing them to the same antibody solution.

Some of the blots appeared to have high backgrounds in comparison to the bands themselves. This is due to the freshness of the development buffer. Blots developed in fresh buffer have access to fresh substrate. As the substrate becomes exhausted, the amount available to bind to the sites decreases dramatically. Therefore, in order to achieve the best possible results, fresh BCIP/NBT solution is required for each blot. The maximum number of blots for each BCIP/NBT solution is two. Any blots developed after this show significant background artifacts.

Significant technical problems were encountered in preliminary Western blots. It seemed as though all S-1 samples had a significant amount of myosin heavy chain contamination. The most likely explanation would be that some of the pellet may have been taken up during pipetting of the S-1 supernatant during gel sample preparation. In order to alleviate this problem, the S-1 supernatant should be re-spun for an additional five to ten minutes after being removed from the insoluble material. It is unlikely that contamination occurs during gel loading, as the S-1 sample is loaded first and any spillover into other wells would show contamination of the rod sample from the S-1 lanes. This does not occur. Furthermore, the loading syringe is rinsed five times with running buffer between loading of different samples. This was done consistently for the last gel series. Furthermore, the rod sample shows more degradation than the S-1 sample, suggesting that the insoluble papain is the culprit, as discussed earlier. The reactivities of the rod samples

with mAb F36 confirm that S-1 containing degradation products exist (Figures 30 and 31). The use of an irreversible inhibitor in the rod sample should alleviate this problem.

Another problem affecting Western blot analyis is degradation of the samples during storage. This leads to the appearance of satellite bands which are due to auto-degradation and/or contaminating proteases, and are not present in the original papain digested samples. This makes identification of the bands and localization of the epitopes more difficult. The method used to retard this auto-degradation is to keep the samples on ice after re-boiling and prior to loading onto the gel and to consider addition of an irreversible oxidant as discussed earlier.

Preliminary blots did not show anti-S-1 reactivity as indicated by the lack of the 95-kDa band reactivities with most mAbs. This could be attributed to another problem encountered in the early stages of Western blot analysis. Prior to transfer, the transfer buffer must be equilibrated to 4-8 °C . If the buffer starts out at room temperature, the transfer runs at a higher temperature which causes increased current for a given voltage setting and faster migration. Further electrophoresis continues to increase temperature even more. When this occurs, the lower molecular weight fragments are electrophoresed faster and may in fact be transferred through the paper faster than the higher molecular weight fragments. This phenomenon may explain the fact that the 95-kDa bands are not present in earlier blots for mAbs S84, S95, FS26, and F74. These antibodies are lower affinity antibodies and if elimination of the 95-kDa fragment on the nitrocellulose paper occurred to any appreciable extent, it would not be recognized. On the other hand, even if such a significant amount of the 95-kDa band was eliminated, mAb25 and mAb F36 would still shown strong reactivity. Thus, the most reliable data indicates that all six monoclonal antibodies tested are anti-S-1, as discussed previously in the *Results*.

CONCLUSION

Myosin has been successfully purified from rat muscle tissue. Isolation of myosin was verified by both spectral analysis and SDS-PAGE. In addition, when subjected to papain digetion, rat myosin is cleaved into the S-1 and rod fragments. These fragments were again identified through SDS-PAGE and assignment of molecular weights according to migration as compared to standards. This is consistent with previous findings with rabbit and chicken myosin.

Furthermore, localization of the epitopes for the following six antibodies has been accomplished through Western blot analysis of the digestion fragments: mAbs MHC.25, MHC.F36, MHC.S95, MHC.FS26, MHC.S84, and MHC.F74. All appear to have epitopes on the S-1 portion of myosin. Previous findings in rabbit and chicken myosin show both mAb MHC.25 and mAb MHC.F36 having epitopes in the S-1 portions (Dan-Goor et al., 1990). The mAbs MHC.S95 and MHC.FS26 react with a 130 kDa N-terminal fragment as shown with cloned gene products (Hughes et al., 1993). Previously, the only information known about mAb MHC.S84 was that its epitope was not at the N-terminus (Hughes et al., 1993). Previously. nothing was known about the location of the epitope for mAb MHC.F74 other than it reacted with the myosin heavy chain (Hughes et al., 1993). Thus, the Western results are consistent with the known previous findings.

FUTURE PROJECTS

Although the epitopes of the six monoclonal antibodies have been determined to reside on the S-1 portion of myosin, further localization can be achieved. In order to assign the epitopes to the 27-kDa, 50-kDa, or 20-kDa sub-subfragments. these subfragments must first be isolated. This can be achieved by a variety of methods. First of all, the S-1 fragment itself can be subjected to digestion by either papain or trypsin for further digestion. Also, myosin can be digested according to the protocol followed here with some minor changes. Increasing the papain concentration to 0.03 and 0.06 mg papain per milligram myosin may provide sufficient digestion to isolate these fragments. In order to determine the presence of the S-1 sub-subfragments, SDS-PAGE analysis should be carried out on 12% acrylamide gels for sufficient separation of these lower molecular weight fragments.

Another significant project would be to validate the findings with rat myosin by Western blot analysis with an anti-rod antibody. This would help to further verify that the epitopes are all, in fact, on the S-1 fragment. Thus, four additional projects would significantly enhance the results of this project: (1) digestion with greater amounts of papain, (2) digestion of the S-1 fragment, (3) analysis on 12% acryamide gels. and (4) incubation with an anti-rod antibody.

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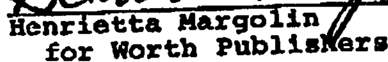 3/14/95

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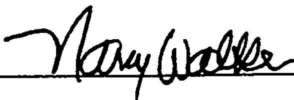

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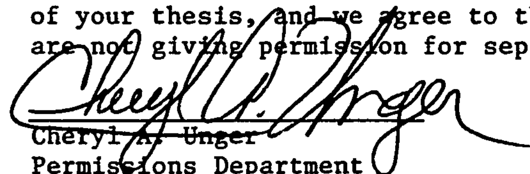

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