MYOSIN-LIKE PROTEINS IN PLANTS

BY

QIAO LIN

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STATEMENT

All research reported in this thesis
is original and my own,
except where acknowledgement is given,
and has not been submitted
for any other degree.

Q. Lin

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ABSTRACT

Immunoblotting with anti-pan myosin antibody strongly and reliably identified a 165 kDa polypeptide from mung bean extracts made with either extraction buffer or TCA solution. It was considered as a putative myosin heavy chain. A 155 kDa mung bean polypeptide was also identified by immunoblotting with this antibody although the detection was not so consistent as that of the 165 kDa polypeptide. The 155 kDa polypeptide was unlikely to be a proteolytic fragment of the 165 kDa polypeptide since it was detected in extracts made with TCA which minimizes proteolysis (Wu & Wang 1984). A mung bean 110 kDa polypeptide was considered to be a proteolytic fragment of the 165 or 155 kDa polypeptide because it was more easily detected with anti-pan myosin in the absence than in the presence of proteolytic inhibitors and was never detected in extracts make directly with TCA. Only one single polypeptide of 165/160 kDa was identified in pea, wheat and *Arabidopsis* with anti-pan myosin.

Four monoclonal antibodies were raised to the mung bean 165 kDa polypeptide, and together with four commercial anti-myosin antibodies, were used in immunoblotting to detect epitopes on proteins from mung bean, pea, wheat, *Arabidopsis* and *Chara*. At least six epitopes were recognized by the eight antibodies, all of which were on the heavy chain of rabbit skeletal muscle myosin. Five of these epitopes were on the 165/160 kDa polypeptide of the four higher plants. Four polypeptides (200, 175, 124 and 110 kDa) of *Chara*, however, each shared ≤ 3 epitopes with the muscle myosin heavy chain. The impressive similarity of epitopes the mung bean 165 kDa polypeptide shared with the muscle myosin heavy chain strongly supports the case that it is a myosin heavy chain.

The mung bean 155 kDa polypeptide shared only one epitope with the muscle myosin heavy chain, supporting the view that it is not a myosin heavy chain. The mung bean 165 kDa but not the 155 kDa polypeptide was specifically immunoprecipitated by anti-pan myosin, which again strengths the view that the

155 kDa polypeptide is not a proteolytic fragment of the 165 kDa polypeptide. The mung bean 165 kDa polypeptide was immunofluorescently colocalized with actin in the phragmoplast of mung bean root tip cells and in cytoplasmic filaments that probably contain actin. This strengthens the case that it is a myosin heavy chain which together with actin, may play role in cytokinesis.

The mung bean 165 kDa polypeptide, but not the 155 and 110 kDa ones, bound to an ADP-agarose column, demonstrating that only the 165 kDa polypeptide can be an ADP/ATP-utilizing enzyme. The binding and elution of the 165 kDa polypeptide in the presence of EDTA suggests that it is an K^+-EDTA-ATPase rather than a kinase. This greatly strengthens the case that it is a myosin heavy chain.

Fractionation of mung bean crude extracts with $(NH_4)_2SO_4$ showed that the 165 kDa polypeptide fraction accounts for only a small fraction of the total K^+-EDTA-ATPase activity of the crude extract. The other K^+-EDTA-ATPase activity may be accounted for by non-myosin(s) and/or other myosin isoform(s) that can not be detected by anti-pan myosin. Since K^+-EDTA-ATPase activity alone is not a specific and reliable indicator of the mung bean 165 kDa polypeptide, immunoblotting which is more specific and reliable was used to monitor the partial purification of this polypeptide.

The mung bean 165 kDa polypeptide was partially purified, with its fraction having a total protein yield of 0.02-0.05% (or 0.041-1.0 mg/100 g tissue). Analysis of ATPase activity during the partial purification showed that the polypeptide at most accounts for only a very small fraction of the total ATPase activity of the crude extract. Both the yield and specific activity of the K^+-EDTA-ATPase activity of the 165 kDa polypeptide fraction decreased particularly at the early steps of the purification. The K^+-EDTA-ATPase specific activity of the partially purified 165 kDa polypeptide fraction was 0.016 μmol/min/mg which was increased 7.3-fold by ADP-agarose affinity chromatography. The partially purified mung bean 165 kDa polypeptide fraction did not exhibit actin-activated Mg^{2+}-ATPase activity.

Preliminary attempts were made to rapidly purify the 165 kDa polypeptide by immunoaffinity chromatography to see if any ATPase activity could be preserved and to see whether it could be adsorbed to antibody-coated beads and catalyze their actin-dependent movement. Neither approach was successful.

Therefore, the important enzymatic evidence that the 165 kDa polypeptide is a myosin heavy chain is still lacking although the immunological evidence strongly suggests that it is.

LIST OF ABBREVIATIONS

A	absorbance
abstra	abstract
ACPS	ATP-containing perfusion solution
AFPS	ATP-free perfusion solution
Anon	anonymous
BSA	bovine serum albumin
CM	carboxymethyl
C-terminus	carboxyl-terminus
CDPK	calcium-dependent protein kinase
Con-A	concanavallin A
CNBr	cyanogen bromide
d	day
DABCO	1,4-diazabicyclo [2,2,2]octane
DAPI	4,6-diamidino-2-phenylindole
DEAE	diethylaminoethyl
dH_2O	distilled H_2O
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid
ELISA	enzyme-linked immunosorbant assay
F-actin	fibrous actin
Fig	figure
FITC	fluorescein isothiocyanate
G-actin	globular actin
HMW	high molecular weight
HMM	heavy meromyosin
h	hour
Ig	immunoglobulin
kDa	kilo dalton
LMM	light meromyosin
LMW	low molecular weight
MB165	antibody against the mung bean 165 kDa polypeptide
Mes	2-[N-morpholino]ethanesulfonic acid
min	minute
Mops	3-[N-morpholino]propanesulfonic acid

M_r	relative molecular mass
MRLC-C	myosin regulatory light chain-protein C
n	nucleus
nl	neutral line
N-terminus	amino-terminus
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PE	phosphate-EGTA buffer
PEG	polyethylene glycol
Pi	inorganic phosphate
Pipes	piperazine-N,N'-bis[2-ethanesulfonic acid]
PMSF	phenylmethylsulfonyl fluoride
R_f	relative migration value
rpm	revolutions per minute
S-1	subfragment-1
S-2	subfragment-2
SDS	sodium dodecyl sulfate
TBS	Tris buffered saline
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TPCK	N-tosyl-1-phenylalanine chloromethyl ketone
Tris	tris (hydroxymethyl)aminomethane
TWEEN-20	polyoxyethylenesorbitan monolaurate
vol	volume
UV	ultra violet
V	voltage

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1.1 INTRODUCTION

Cellular motility can be divided into three basic forms: migration of cells across a surface, morphogenetic movements or changes in cell shape, and movement of organelles (LeBlanc & Leinwand 1991). The last form of motility can take place in the absence of the first two forms (Warrick & Spudich 1987). The macromolecules which drive these movements are referred to as molecular motors which are mechanochemical enzymes expressing ATPase activities. These include microtubule-based molecular motors such as kinesin and dynein (Vale 1987; Porter & Johnson 1989; Vallee & Shpetner 1990; Schroer & Sheetz 1991) and actin-based myosin.

Myosin is defined as any protein which binds to F-actin (fibrous actin) and has its ATPase activity activated. Myosins express various levels of Ca^{2+}-, K^{+}-EDTA- and Mg^{2+}-ATPase activities, but only the Mg^{2+}-ATPase activity is expressed under physiologically relevant conditions and is activated by F-actin (Korn & Hammer 1988).

Myosin was first noticed and named in nematode by Kuhne (1864), but its ATPase activity was not discovered until 75 years later (Engelhardt & Ljubimowa 1939). Actin was discovered subsequently by Albert Szent-Gyorgyi and his colleagues (Szent-Gyorgyi 1942). Since then, extensive work has been done on the biochemistry, biophysics and molecular genetics of muscle myosins (Taylor 1979; Eisenberg & Greene 1980; Adelstein & Eisenberg 1980; Morales *et al.* 1982; Huxley & Faruqi 1983; Harrington & Rodgers 1984; Amos 1985; Eisenberg & Hill 1985; Hibberd & Trentham 1986; Emerson & Berstein 1987; Wade & Kedes 1989; Fyrberg & Goldstein 1990).

Myosin is a ubiquitous protein that probably, like actin, exist in all eukaryotic cells (LeBlanc & Leinwand 1991). They can be classified into two distinct

isoforms: I and II. Myosin II is the two-headed, filamentous myosin capable of self-aggregation to form large filaments familiar from muscles; myosin I is the newly found, single-headed, nonfilamentous myosin. They will be described separately below.

1.2 STRUCTURAL PROPERTIES OF MYOSIN II

1.2.1 Basic structure

Myosin II refers to a family of myosins that share similarities with those myosins found in muscle. Most nonmuscle myosins closely resemble muscle myosin in general structure. Myosins II are large, highly asymmetric proteins composed of two heavy chains, two essential light chains and two regulatory light chains. Each heavy chain (M_r 160-240 kDa) has an amino-terminus (N-terminus) domain (about 90 kDa) that folds into a globular head with which one of each pair of light chains (12-27 kDa) are non-covalently associated. The remainder of the heavy chain dimerizes to form a long, α-helical coiled-coil rod known as the tail (Schliwa 1985; Kiehart 1990).

Proteolytic enzymes cleave myosin into a number of functional domains. Limited digestion with trypsin yields heavy meromyosin (HMM) comprising the heads and part of the tail, and light meromyosin (LMM) comprising the distal two thirds of the tail. Further limited proteolysis of HMM with papain or chymotrypsin yields two identical HMM subfragment-1 (S-1) comprising the two heads and one HMM subfragment-2 (S-2) (Lowey *et al.* 1969). The cleavage site between HMM and LMM has less ordered secondary and tertiary structure than the rest of the rod and forms a flexible "hinge" joining LMM in the myosin backbone to HMM which is free to interact with adjacent F-actin. Another flexible site seems to be located in the junction between S-1 and S-2. Both sites are considered to play important roles in the power stroke of myosin (Huxley 1963; Kendrick-Jones *et al.* 1971; Harvey & Cheung 1982; 1.5). Both HMM and S-1 are soluble and bind to F-actin in the absence of ATP in an arrowhead pattern, a property used to detect F-actin and

determine its polarity in muscle (Huxley 1963) and nonmuscle cells (Schliwa 1985). LMM is rather insoluble and self-aggregates under physiological conditions, indicating that it contains the region responsible for myosin filament formation (Huxley 1963; Kendrick-Jones *et al.* 1971).

The primary structure of a number of muscle and nonmuscle myosins has been determined by sequencing proteins or, more commonly, myosin genes. The first complete primary structure of a myosin heavy chain protein was deduced from the sequence of the *unc-5* gene of the nematode *Caenorhabditis elegans* that encodes the myosin heavy chain B of body wall muscle (Karn *et al.* 1983). DNA sequencing has been the most practical way to deduce the primary structures of nonmuscle myosins which form only about 1% of total cell protein (Warrick & Spudich 1987; Emerson & Berstein 1987; Korn & Hammer 1988).

Comparison of the sequences of various myosins from widely divergent organisms show something of the evolution of the myosin structure. The nucleic acid sequences of myosin show limited conservation, but the predicted amino acid sequences indicate that nonmuscle myosins show more divergence from the muscle myosins than occurs within the muscle myosin family (Warrick & Spudich 1987). Conservation in the head exceeds that in the tail, but is not as great as the conservation found in other cytoskeletal proteins, particularly actin (Vandekerckhove & Weber 1978). Areas of sequence conservation may indicate regions that play important roles in myosin function. In the myosin head, these regions include the ATP binding site, the actin binding site, the site of ATP hydrolysis and a highly conserved fourth site of unknown function.

1.2.2 Myosin head

Mapping of the substructure of the globular head S-1 has been pursued mainly by proteolytic fragmentation, cross-linking and specific labelling of various reactive groups (Harrington & Rodgers 1984). In rabbit skeletal muscle myosin, S-1 can be further cleaved into subfragments of 25, 50 and 20 kDa, which occur in that order from the N-terminus to the carboxyl-terminus (C-terminus). The tail (LMM-S-2)

can be cleaved into fragments of 55 and 70 kDa, in the same sequential order. Myosins from other species do not show exactly the same digestion pattern. However, this cleavage pattern is better conserved among muscle myosins than among nonmuscle myosins (Warrick & Spudich 1987).

The S-1 fragment interacts with actin through a strong binding site in the 20 kDa subfragment and a much weaker one in the 50 kDa subfragment (Yamamoto & Sekine 1979; Mornet *et al.* 1981a, b; Greene 1984; Chen *et al.* 1985a, b, 1987; Katoh *et al.* 1985; Sutoh 1982b, 1983). Cross-linking two highly reactive thiols in the 20 kDa subfragment (Gallager & Elzinga 1980; Karn *et al.* 1982) inhibits ATPase activity and traps Mg^{2+}-ATP (Wells & Yount 1979, 1980, 1982; Wells *et al.* 1980a, b). Both the ATP-binding site (Szilagyi *et al.* 1979; Sutoh *et al.* 1986) and a reactive lysine residue essential for myosin ATPase activity (Mornet *et al.* 1980; Hozumi & Muhlrad 1981; Miyanishi & Tonomura 1981) lie in the 25 kDa subfragment.

The regulatory light chain has been localized by electron microscopy on S-1 near the head-tail junction, that is the "neck" region (Flicker *et al.* 1981, 1983; Yamamoto *et al.* 1985). The essential light chain extends from the neck region to the actin-binding site (Sutoh 1982a; Flicker *et al.* 1981; Burke *et al.* 1983; Okamoto *et al.* 1986; Mitchell *et al.* 1986). The regulatory and essential light chain may be arranged in closed proximity, and some interplay might occur between these chains (Wallimann & Szent-Gyorgyi 1981a, b; Wallimann *et al.* 1982; Hardwicke *et al.* 1982; Harvey & Cheung 1982).

A recently discovered putative calmodulin/light chain-binding domain, referred to as the IQ motif, is present as one or more tandem repeats in the neck region of the head-tail junction of all myosins (Cheney & Mooseker 1992). One tandem repeat is an extremely basic unit of about 23 amino acids.

1.2.3 Myosin tail

Numerous studies have demonstrated that the domains that drive filament assembly lie within the LMM portion of the myosin tail. S-2 is soluble under

physiological conditions and may be loosely bound to the thick filament surface (Harrington & Rodgers 1984). In every filament-forming myosin tail, there is a basic 28-residue repeat unit in which the smallest repeat contains seven amino acids (the heptad repeat). Small, generally hydrophobic amino acids are found in the first and fourth positions of this repeat. The heptad repeat forms two turns of an α-helix and the hydrophobic residues form the inner surfaces of the coiled-coil. The fifth and seventh residues of the heptad repeat are frequently occupied by acidic and basic residues respectively and form the outer surface of the coiled-coil. Proper positioning of the hydrophobic residues is thought to be important for the interaction of the two heavy chains in the formation of dimers, and the alignment of the charged resides in the promotion of thick filament assembly (McLachlan & Karn 1983; Karn *et al.* 1983; McLachlan 1984; Harrington & Rodgers 1984; Warrick & Spudich 1987; Korn & Hammer 1988). The vertebrate muscle myosin tail is α-helical to its C-terminus, but heavy chains of nematode myosin, *Drosophila* muscle myosin and *Acanthamoeba* myosin II end in nonhelical regions (Karn *et al.* 1983; Strehler *et al.* 1986; Rozek & Davidson 1986; Cote *et al.* 1984; Hammer *et al.* 1986, 1987). A nonhelical tailpiece is not predicted from the amino acid sequence of *Dictyostelium* myosin II (Korn & Hammer 1988).

1.2.4 Myosin light chains

Both myosin regulatory and essential light chains belong to a group of divalent cation-binding proteins including troponin C and calmodulin which are believed to have evolved by gene duplication and reduplication from a common ancestor. They share distinct similarities in amino acid sequence and possess common structural features including similar Ca^{2+}-binding sites (Collins *et al.* 1973; Collins 1974, 1976b, 1991; Kretsinger 1980; Baba *et al.* 1984). Evolutionary changes in the amino acid sequences of many contemporary proteins have resulted in loss of Ca^{2+}-binding ability, while maintaining many features of their three dimensional structures (Collins 1974, 1976a, b). The light chain composition of nonmuscle myosin is similar to that of smooth muscle myosin (Clarke & Spudich 1977).

The regulatory light chains can be phosphorylated (Weeds *et al.* 1977) and are believed to play a role in the regulation of contraction by Ca^{2+} and phosphorylation, which is well established in smooth muscle and nonmuscle cells (Szent-Gyorgyi 1980; Kendrick-Jones & Scholey 1981; Kendrick-Jones *et al.* 1982; Kuznicki 1986; Kuznicki & Barylko 1988; Korn & Hammer 1988). The essential light chains are not phosphorylatable; they can be removed from myosin without loss of ATPase activity, and S-1 can hydrolyze ATP in the absence of light chains (Wagner & Giniger 1981; Sivaramakrishnan & Burk 1982). It therefore seems unlikely that this class of light chain is truly essential to the myosin ATPase activity. Their role in the contractile mechanism is somewhat more ambiguous than that of the regulatory light chains.

Recent work has just started to shed light on the function of essential light chains in contractile mechanisms. The actin-activated Mg^{2+}-ATPase activity of molluscan myosin is regulated by direct binding of Ca^{2+} to myosin. The essential light chains of scallop myosin are required for the specific Ca^{2+} binding to myosin, most likely to provide the specific Ca^{2+}-binding site (Kwon *et al.* 1990). Comparison of the gene sequence of *Dictyostelium* myosin essential light chain with those of other essential light chains from a variety of sources has identified a highly conserved domain which might play an important role in light chain function (Pollenz & Chisholm 1991).

1.3 STRUCTURAL PROPERTIES OF MYOSIN I

Myosin I is a more recently identified diverse collection of single-headed, nonfilamentous myosins. It was first found in *Acanthamoeba* (Pollard 1971; Pollard & Korn 1972, 1973a, b) and subsequently in *Dictyostelium,* bovine and chicken intestinal brush borders, *Drosophila* (Korn & Hammer 1988; Pollard *et al.* 1991; Cheney & Mooseker 1992), rat kidney brush border (Coluccio 1991), bovine adrenal medulla, adrenal cortex and brain (Barylko *et al.* 1992). The available evidence strongly suggests that myosin I is present in all eukaryotic species.

Myosin I comprises one heavy chain (M_r 100-140 kDa) (Pollard 1984; Cheney & Mooseker 1992) and one to four light chains. The light chains of intestinal brush border myosin I are up to four calmodulins (Howe *et al.* 1980; Coluccio & Bretscher 1987; Swanljung-Collins & Collins 1991). Myosin I consists of two major structural domains: a globular head and a short, slightly asymmetric tail.

Myosin I heads contain nearly all of the sequences that are conserved in the heads of muscle and nonmuscle myosins II (1.2.1 & 1.2.2). A well conserved sequence of myosin head which distinguishes most known myosins I from myosins II lies C-terminus to the phosphorylation site of *Acanthamoeba* myosins I (Brzeska *et al.* 1989). In the heavy chain of *Acanthamoeba* myosins I, a single threonine (myosin IA) or serine (IB, IC) residue must be phosphorylated to permit actin-activation of the Mg^{2+}-ATPase (Brzeska *et al.* 1989). The phosphorylation sites are located between the ATP- and actin-binding sites. Myosin I tails are highly variable and shows no significant sequence similarity to those of myosins II, but have some common features related to the function (Korn & Hammer 1988; Pollard *et al.* 1991). All tails lack heptad repeats so that no myosin I polymerizes to form filaments like myosin II.

The heads of all myosins (I and II) have a similar ATP-sensitive actin-binding site. Binding occurs when actin activates the Mg^{2+}-ATPase of myosin (1.5). *Acanthamoeba* myosins IA, IB and ID, and *Dictyostelium* myosins IB and ID have a second F-actin-binding site which is located in the C-terminus 30 kDa of the heavy chain tail (Lynch *et al.* 1986; Doberstein & Pollard 1992; Hammer 1991; Cheney & Mooseker 1992). It is ATP-independent, has no effect on ATP hydrolysis and is located in a glycine-proline-alanine-rich region that is unique to myosin I. This, like the filament forming tail of myosin II, provides a mechanism by which myosin can cross-link actin filaments and move one filament relative to the other through the cross-bridge cycle at the actin-binding site that is coupled to ATP hydrolysis (Korn & Hammer 1988; Pollard *et al.* 1991). The chicken and bovine intestinal brush border myosins I have three putative calmodulin-binding

sites just C-terminus to the head (Hoshimaru *et al.* 1989; Hayden *et al.* 1990). The myosins I of *Acanthamoeba,* which do not bind calmodulin, lack these sites.

Morphological and biochemical studies of *Acanthamoeba, Dictyostelium* and vertebrate epithelial cell show that myosin I associates with the plasma membrane, but questions remain about the association of myosin I with the membranes of cytoplasmic organelles (Pollard *et al.* 1991). *In vitro* experiments suggest that myosins I bind to negatively charged surfaces provided by anionic phospholipids (Adams & Pollard 1989). However, the high affinity protein-lipid electrostatic interaction alone cannot account for the emerging evidence that myosin I isoforms are bound to specific membranes, such as the microvillar plasma membrane of the brush border. It is suggested that the specific localization is accomplished by a combination of nonspecific concentration of myosin I on the surface of membrane via lipid interactions and relatively weak associations with membrane proteins (Pollard *et al.* 1991). The membrane-binding site lies in the basic, N-terminus part of the tail of both *Acanthamoeba* and brush border myosins I. In *Acanthamoeba,* the binding site lies between the head and C-terminus ATP-insensitive actin-binding domains (Adams & Pollard 1989; Doberstein & Pollard 1989, 1992; Hayden *et al.* 1990).

Novel myosin heavy chains have also been described. An *Acanthamoeba* gene potentially encodes a 177 kDa polypeptide, which is close in size to a myosin II heavy chain (Horowitz & Hammer 1990). Its tail shows essentially no similarity to either myosin I or II except for a C-terminus region (about 50 amino acids) homologous to that of myosin I. Sequence analysis predicts that it will not form a myosin II-like coiled-coil structure, implying it is single-headed and nonfilamentous. Therefore, this new protein has been tentatively classified as a high molecular weight form of myosin I.

Two novel heavy chains of 174 kDa and 132 kDa are encoded by the *Drosophila ninaC* gene which expresses two overlapping mRNAs (Montell & Rubin 1988). Each polypeptide consists of a central myosin I-like head domain

linked to a C-terminus domain that, uniquely among all characterized myosins, is a kinase domain that may catalyze autophosphorylation (Pollard *et al.* 1991). The *ninaC* gene products could be members of a diverse family of molecules which include a myosin head as part of their domain structure (Mooseker 1989).

Recently, novel myosin heavy chains have been identified that share characteristics of both myosins I and II (Cheney & Mooseker 1992). They are the 215 kDa gene product of the *dilute* coat-colour locus of mouse (Mercer *et al.* 1991), the 180 kDa product of the yeast *MYO2* gene (Johnston *et al.* 1991), and the 190 kDa calmodulin-binding protein (p190) from vertebrate brains (Larson *et al.* 1990; Espindola *et al.* 1992). Chicken brain p190 and mouse *dilute* protein share 91% deduced amino acid identity (Espindola *et al.* 1992; Cheney & Mooseker 1992). p190 is biochemically and immunologically distinct from the heavy chains of brain myosin II and of brush border myosin I (Espindola *et al.* 1992).

All three novel myosin heavy chains contain a neck region with six tandem repeats of a putative binding unit for myosin light chain or calmodulin (Cheney & Mooseker 1992). After the tandem repeats, there is a tail domain with the heptad repeat of an α-helical coiled-coil, followed by a globular domain bearing no obvious sequence similarity with the tails of other myosins. The *dilute* gene product has numerous proline residues in its tail which would preclude α-helix formation and assembly into thick filaments (Mercer *et al.* 1991). p190 molecules are dimers with two rather large heads, a central rod-like segment and two C-terminal globular domains, but p190 does not appear to form filaments (Cheney & Mooseker 1992).

It is hypothesized (Pollard *et al.* 1991) that all contemporary myosins evolved from a primitive myosin consisting of a small myosin I-like head and a short tail capable of binding membranes. This original myosin acquired extra domains by duplication of the sequence encoding its head and its fusion with a gene encoding a tropomyosin-like molecule. This eventually led to myosin II molecules with two heads and a long α-helical coiled-coil tail. In some multicellular metazoan

organisms, however, a specialized form of myosin II evolved that is capable of forming sarcomeres.

1.4 FILAMENT FORMATION BY MYOSIN

The assembly of myosin into filaments is a property common to all myosins II. The thick filaments in vertebrate striated muscle are bipolar structures in which myosin molecules are arranged with their tails in the backbone and their heads along the surface. Packing is anti-parallel near the center of the filament (the bare central zone) and parallel throughout the remainder of the structure (Huxley 1963).

All myosins II self-associate *in vitro* at low ionic strength to form filamentous structures that resemble those isolated directly from cells. The process of assembly and the final structure of the filaments depend on the source of myosin. The dimensions of myosin filaments formed *in vitro* are variable and depend on a variety of conditions, such as ionic strength, pH, divalent cation composition and the presence or absence of copolymerizing proteins (Schliwa 1985; Sinard *et al.* 1990).

In both vertebrate muscle (Suzuki *et al.* 1978; Onishi *et al.* 1978; Kendrick-Jones *et al.* 1983) and nonmuscle (Scholey *et al.* 1980; Craig *et al.* 1983; Broschat *et al.* 1983) myosins, phosphorylation of regulatory light chains controls both filament formation and actin-activated Mg^{2+}-ATPase activity (1.7.3). Heavy chain phosphorylation occurs in a number of nonmuscle myosins, including *Physarum, Dictyostelium, Acanthamoeba,* leukemic myeloblasts, macrophages, lymphocytes and brain cells (Kuznicki 1986; Kuznicki & Barylko 1988; Korn & Hammer 1988).

Phosphorylation of *Physarum* myosin II is required for both thick filament formation and actin activation of Mg^{2+}-ATPase activity (Ogihara *et al.* 1983; Takahashi *et al.* 1983; 1.7.4). For *Acanthamoeba* and *Dictyostelium* myosins II, heavy chain phosphorylation inhibits both filament assembly and actin activation of Mg^{2+}-ATPase activity (Kuczmarski & Spudich 1980; Peltz *et al.* 1981; Pagh & Gerisch 1986; Korn *et al.* 1988; Sinard & Pollard 1989; Ravid & Spudich 1989).

For *Acanthamoeba* myosin II, the sites of phosphorylation are located on three serine residues within the nonhelical tailpiece of each heavy chain (Collins & Korn 1980, 1981; Collins *et al.* 1982a; McClure & Korn 1983; Cote *et al.* 1984). Proteolytic removal of serines from the tailpiece suggest that the two serines closest to the C-terminus are not required for either actin-activated Mg^{2+}-ATPase activity or filament formation (Sathyamoorthy *et al.* 1990; Ganguly *et al.* 1990). In the tail of *Dictyostelium* myosin II heavy chain, two domains required for assembly and phosphorylation, respectively, have been identified (O'Halloran *et al.* 1990). Three threonine residues in the C-terminus 34 kDa domain are phosphorylated (Pagh *et al.* 1984; Vaillancourt *et al.* 1988; Luck-Vielmetter *et al.* 1990). This 34 kDa domain is not necessary for *in vivo* contractile activity, but is critical for regulating the extent of myosin assembly *in vivo* and for proper control of cortical localization (Egelhoff *et al.* 1991).

The basic model of myosin assembly has been established by studies on purified myosin from vertebrate skeletal muscle, which show that assembly is initiated by anti-parallel packing of myosin rods and that the filament then elongates by parallel packing in each end of the bipolar filament (Huxley 1963). An important feature of myosin filament structure is that an intermediate level of organization occurs between dimer and full-sized filament: the subfilament (Davis 1985). Some models for filament assembly suggest that, at least in striated muscles, myosin assembles via a subfilament intermediate (Wray 1979; Squire 1981).

Acanthamoeba myosin II minifilaments are proposed to assemble from monomers through antiparallel dimers and tetramers by three successive dimerization steps, resulting in small bipolar assemblies containing from 8 to 16 myosin molecules each (Sinard *et al.* 1989, 1990; Sinard & Pollard 1989). Functional regions required for individual steps in the assembly have been identified on the tail of myosin II (Sinard *et al.* 1990; Rimm *et al.* 1990). Myosins from skeletal muscle (Reisler *et al.* 1980), smooth muscle (Trybus & Lowey 1987), vertebrate nonmuscle cells (Niederman & Pollard 1975), *Dictyostelium*

(Kuczmarski *et al.* 1988), and probably all other myosins as well can also form small bipolar minifilaments.

Whether nonmuscle myosins exist as filaments, oligomers or even monomers in cells, is not very clear. Generally, but not always, the filaments of the enzymatically more active state of nonmuscle myosin are larger and more stable *in vitro* than those of the enzymatically less active state (Kuznicki *et al.* 1983; Korn & Hammer 1988). This suggests that nonmuscle myosins functions *in vivo* in the form of bipolar aggregates (Clarke & Spudich 1977). Such an idea has recently been supported by the localization of bipolar filaments of *Dictyostelium* myosin II *in vivo* by immunofluorescence (Yumura & Fukui 1985) and immunogold electron microscopy (Yumura & Kitanishi-Yumura 1990a). Myosin II in the cytoplasm of *Acanthamoeba* is organized into rodlike filaments that may be octameric minifilaments (Baines & Korn 1990) and larger filaments formed by the aggregation of minifilaments (Yonemura & Pollard 1992). The finding of bipolar filaments implies that myosin and actin in nonmuscle cells produce the motive force for some forms of cell movement when they are both organized as filaments, by a mechanism analogous to the sliding-filament model of actomyosin in muscle cells (Clarke & Baron 1987; Yumura & Kitanishi-Yumura 1990a).

Nonmuscle myosin II is considered to be distributed between disassembled and assembled filament pools (Herman & Pollard 1981) in response to changes in divalent cation concentration (Condeelis *et al.* 1976), osmotic shock (Behnke *et al.* 1971), chemotactic stimulation (Yumura & Fukui 1985; Fukui & Yumura 1986; Fukui *et al.* 1991; Yumura & Kitanishi-Yumura 1992) or mitosis (Fukui & Inoue 1991).

1.5 MODELS FOR THE MECHANISM OF FORCE PRODUCTION

Models of the mechanism of force production have been deduced exclusively from studies on striated muscle. Although equivalent evidence is not available from other cells, the similarities in the properties of actin and myosin and their

arrangement in other cells, are consistent with the view that striated muscle exemplifies a common principle (Sheterline 1983).

Contraction of striated muscle is caused by an active sliding of actin filaments relative to myosin filaments, with the length of each set of filaments remaining constant. Although the molecular mechanism of the sliding process in striated muscle remains controversial, the most widely held view is that the elements responsible for the generation of contractile force reside in the cross-bridges (the HMM region of myosin) which extend from the myosin filament and interact cyclically with the F-actin (A. F. Huxley 1957, 1974; H. E. Huxley 1969; Eisenberg & Hill 1978; Harrington & Rodgers 1984).

In H. E. Huxley's model of the cross-bridge (Huxley 1969, 1971; Huxley & Kress 1985), the myosin head, together with S-2, swings out from the myosin filament to bind to the F-actin. Attachment of the myosin head to F-actin releases ADP and inorganic phosphate (Pi) from the hydrolysed ATP. Meanwhile, the myosin head changes its conformation so that the angle it makes with the actin filament alters. This results in relative displacement of the actin and myosin filaments. As soon as ADP and Pi are lost from the myosin head, a new MgATP-myosin head complex is formed which lets the myosin head detach from the actin filament. The cyclic attachment and detachment of the myosin head to F-actin move the latter relative to the myosin filament. It is proposed that the most likely seat of the force-developing mechanism is the globular part of HMM and its attachment to actin filaments. The force originates in a tendency for the myosin head to rotate relative to the actin filament, and is transmitted to the myosin filament by the S-2 portion of the myosin molecule action as an inextensible link. Flexible points at each end of S-2 permit S-1 to rotate, and allow for variations in the separation between filaments (Huxley 1969).

The rotating cross-bridge model, however, is not universally accepted since the conformational change of the myosin head during the power stroke is not observed by some highly sensitive spectroscopic techniques (Alberts & Miake-Lye 1992),

such as fluorescence anisotropy (Yanagida 1985) and electron spin resonance (Thomas 1987).

Harrington proposes that a conformational change within the S-2 region rather than head rotation generates force (Harrington 1971, 1975, 1979; Harrington & Rodgers 1984; Ueno & Harrington 1986a, b). It is proposed that the S-2 link is helical in the resting state where it is bound to the thick filament surface but shortens to a coil after the myosin head attaches to an actin filament in a cross-bridge cycle and the S-2 link swings away from the filament surface. The melting in the force-generating element causes a small angular rotation of the head about its point of attachment on the actin filament. At the end of the power stroke, the released cross-bridge returns to the stable resting state position where S-2 is bound to the thick filament surface. Rotation of the head acts like a spring to take up tension during force generation. The head returns to its initial orientation when tension on the cross-bridge decreases to zero.

The ability of isolated heads attached to a solid support to move actin filaments (Sheetz & Spudich 1983; Toyoshima *et al.* 1987; Hynes *et al.* 1987) strongly argues that force is generated within the myosin head itself (Redowicz *et al.* 1990; Huxley 1990). One way to reconcile the Huxley and Harrington theories is to consider that S-1 alone is sufficient to move actin under no-load conditions, but that the contribution of the rod may be needed to generate force in an actively working muscle (Harrington *et al.* 1990).

Even though it is considered that in both muscle and nonmuscle cells, force is generated by the sliding of actin filaments past myosin, there are fundamental differences between the two systems in terms of the subcellular and supramolecular organization of the contractile proteins. Both skeletal and smooth muscle cells are extremely specialized for generation a force in one dimension only, shortening of the sarcomere in the former, and of the entire cell in the latter. Nonmuscle cells also generate linear forces, but in addition, the contractile proteins are sometimes arranged in a more complex three-dimensional lattice so that the force generated

within the lattice will result in complicated three-dimensional deformations. Furthermore, the contractile machinery of nonmuscle cells can be remodeled rapidly both in space and time, whereas the paracrystalline array of skeletal muscle remains virtually unchanged. Thus a fundamental difference between skeletal and nonmuscle systems lies in the degree of plasticity of the contractile apparatus (Schliwa 1985).

1.6 FUNCTIONS OF MYOSIN IN CELL MOTILITY

1.6.1 Myosin II

Myosins II generate muscle contraction, and may play roles in nonmuscle cell motility, including ameboid movement (Fukui & Yumura 1986), chemotaxis (Fukui & Yumura 1986; Fukui *et al.* 1990), capping of surface receptors (Carboni & Condeelis 1985; Pasternak *et al.* 1989; Fukui *et al.* 1990), cytokinesis (Fujiwara & Pollard 1976; Mabuchi & Okuno 1977; De Lozanne & Spudich 1987; Schroeder 1987; Egelhoff *et al.* 1990) and nuclear segregation (Watts *et al.* 1987). A nonmuscle myosin heavy chain-like polypeptide has been localized *in situ* in nuclear pore complexes in *Drosophila* (Berrios & Fisher 1986; Newmeyer & Forbes 1988; Berrios *et al.* 1991). It is proposed that annular subunits of the nuclear pore complex are formed by the heads of myosin molecules, the cylindrical wall of the pore lumen is formed by myosin tails. Myosin may play a role in hydrolysis of ATP required for transport through the pore.

In *Drosophila*, a mitotic mutation, sqh^1, disrupts cytokinesis, resulting in the accumulation of enormous numbers of chromosomes in the normally diploid cells of larvae. The *sqh* gene has been cloned and mapped on the X chromosome. Independently, the regulatory light chain of nonmuscle myosin (MRLC-C) has been biochemically purified, and the gene that encodes it cloned, sequenced and mapped to the same site on the X chromosome as the *sqh* gene (Karess *et al.* 1991). It has been found out that the *sqh* gene encodes the MRLC-C protein. These studies provide genetic proof that the myosin regulatory light chain is required for

cytokinesis, suggesting a role for the protein in regulating contractile ring function, and establishing a genetic system to evaluate its function.

Dictyostelium discoideum and *Saccharomyces cerevisiae* can be used to study the function of myosins by genetic manipulations. *Dictyostelium* contains a single-copy gene (*mhcA*) encoding the heavy chain of a myosin II (De Lozanne *et al.* 1985; Warrick *et al.* 1986). A myosin-defective mutant (*hmm*) has been created by using gene targeting to disrupt the *mhcA* gene (De Lozanne & Spudich 1987). The *hmm* cells which express a C-terminally truncated myosin unable to assemble into thick filaments show the same phenotype as when myosin II is depleted by expression of antisense RNA which is complementary to *mhcA* mRNA (Knecht & Loomis 1987). They survive and display many forms of cell movement including moving at reduced velocity, phagocytosis and chemotaxis (Wessels *et al.* 1988; Fukui *et al.* 1990). Studies of the cytoskeletal organization and physiological responses of this mutant demonstrate that myosin II is significantly involved in organizing the cortical cytoskeleton and participates in the cortical motile activities of *Dictyostelium* cells, including rounding up, constriction of cleavage furrows, capping surface receptors and establishing cell polarity.

Dictyostelium myosin null mutants, *mhcA*$^-$ cells, have been generated by gene targeting (Manstein *et al.* 1989). These mutants provide genetic proof that the myosin II gene is required for growth in suspension, normal cell division and sporogenesis, rapid cellular translocation, cellular polarity, rapid particle movement and cAMP response (Manstein *et al.* 1989; Soll *et al.* 1990). Cloned *mhcA* gene has been reintroduced into the *mhcA*$^-$ null mutants by the use of a direct functional selection (Egelhoff *et al.* 1990). The rescued cells are normal for cytokinesis and are fully competent for sporogenesis. The complementation of both the cytokinesis defect and the developmental defect of the null cells rules out the possibility that secondary mutations play a role in these phenotypes. The demonstration that a direct functional selection can be used to introduce cloned genes into *Dictyostelium* offers a new versatile method for studying cloned genes and for confirming

phenotypes of mutant cell lines. The feasibility of introducing altered myosin genes into myosin null cells makes it possible to identify domains of the protein that are critical for the *in vivo* activities in which myosin is involved (Egelhoff *et al.* 1991).

In yeast, a myosin heavy chain mutant has been created by disrupting the *MYO1* gene that encodes a myosin II protein (Watts *et al.* 1987). The mutant phenotype shows that an intact myosin heavy chain gene is essential for cytokinesis and nuclear migration, and is required to maintain the cell type specific budding pattern and the correct localization and deposition of chitin and cell wall components during cell growth and division (Rodriguez & Paterson 1990).

1.6.2 Myosin I

The association of myosin I with membranes, identified by biochemical studies, and cellular localization, suggests that myosin I may play a key role in motile events involving plasma membrane and membranous organelles, such as amoeboid locomotion, phagocytosis, chemotaxis and organelle movement (Pollard *et al.* 1991). Several types of potential cellular movements have been suggested to exist which can be powered by myosin I due to its association with membrane lipids (Adams & Pollard 1989). Not all of them have been identified in cells so far.

Acanthamoeba myosin II occurs in the cytoplasm and appears to be concentrated in the cellular cortex, but myosins I are preferentially distributed near the plasma membrane (Gadasi & Korn 1980; Hagen *et al.* 1986; Baines & Korn 1989). *Acanthamoeba* myosin II has also been localized to a range of particles in the cytoplasm which are considered to be myosin filaments (Yonemura & Pollard 1992). The smallest and most numerous cytoplasmic particles are distributed throughout the endoplasm. The largest particles, however, are concentrated in the cleavage furrow of dividing cells and in the tail of locomoting cells.

Acanthamoeba myosin IB and/or IA are localized to spots that do not correspond clearly to any of the phase-dense particles in the cytoplasm (Yonemura & Pollard 1992). They may correspond to small vesicles of intermediate density and low sedimentation coefficient that bind substantial amounts of myosin I in

cellular homogenates and that may participate in endocytotic membrane traffic. Myosins IA and IB are also concentrated at the leading edge of many locomoting cells, and are localized to the membrane of a single cytoplasmic vacuole of variable size that is presumed to be the contractile vacuole. Furthermore, myosin IA is localized to novel intercellular contacts and myosin IB to the plasma membrane, especially the tips of filopodia. The functions of the intercellular contacts are unknown but it is suggested that myosin IA may accumulate some membranes or membrane molecules at the sites of these contacts. *Acanthamoeba* myosin IC has been localized to the plasma membrane and the contractile vacuole membrane (Baines & Korn 1990).

Beads coated with myosins IA and IB move unidirectionally on actin cables of *Nitella* (Albanesi *et al.* 1985a). Organelles isolated from *Acanthamoeba* also move along *Nitella* actin cables, and the movement is inhibited by monoclonal antibodies to myosin I rather than those to myosin II (Adams & Pollard 1986). These results indicate that myosin I may be the motor for some vesicle movements.

The second actin-binding site on the tail of *Acanthamoeba* myosin I heavy chain gives myosin I the potential to crosslink actin filament and generate force. *In vitro* studies have shown that myosin I can crosslink actin filaments and cause superprecipitation in the presence of ATP (Fujisaki *et al.* 1985; Albanesi *et al.* 1985b; Lynch *et al.* 1986). This establishes the potential for myosin I to participate in a wide range of contractile processes generally thought to be powered by myosin II. Such movements would employ a sliding filament mechanism like bipolar filaments of myosin II. So far, however, no cellular movement is known to be powered in this way by myosin I (Pollard *et al.* 1991).

That much motility continues in the *Dictyostelium mhcA*$^-$ null mutant provides definite proof that the *mhcA* gene is neither an essential gene nor is needed in many forms of cell motility (Manstein *et al.* 1989). Myosin I might function in some forms of cell motility in the absence of myosin II.

Dictyostelium myosin I is localized at the leading edges of the lamellipodial projection of migrating amoebae, in regions which are devoid of myosin II, whereas myosin II is concentrated in the posterior of the cells (Fukui *et al.* 1989). It is suggested that forces generated by myosin I may cause extension at the leading edge of a motile cell, while myosin II may be involved in the contraction of the posterior of the advancing cell. In addition, myosin I may be important in phagocytosis, since it is also concentrated beneath the plasma membrane at sites of particle ingestion. Proof of the roles of myosin I requires disruption of its genes.

In a mutant deficient in myosin IB heavy chain, chemotactic streaming and aggregation are delayed, and the rate of phagocytosis is reduced (Jung & Hammer 1990; Wessels *et al.* 1991). Mutant cells form lateral pseudopodia and turn more often, and exhibit depressed average instantaneous cell velocity. They also exhibit a decrease in the average instantaneous velocity of intracellular particle movement and an increase in the degree of randomness in particle direction. It is suggested therefore that myosin IB plays a role in these processes. It has been deduced that at least five genes encode myosin I heavy chains in *Dictyostelium* (Jung & Hammer 1990). If there is considerable overlap of function between these isoforms, multiple gene disruptions within a single cell may be necessary to generate a more striking myosin I-deficient phenotype.

Intestinal brush border myosin I, the 110 kDa-calmodulin complex, tethers microvillar actin filaments to the plasma membrane in the brush border of intestinal epithelial cells (Mooseker 1985). Brush border myosin I can move plastic beads and membrane fragments unidirectionally along actin cables of *Nitella* (Mooseker & Coleman 1989; Mooseker *et al.* 1989), and fluorescent actin filaments when it is immobilized on nitrocellulose-coated coverslips (Collins *et al.* 1990). It may contribute to microvillar motility and vitamin D-regulated calcium transport across the brush border (Bikle *et al.* 1982, 1984, 1991; Bikle & Munson 1984, 1985). Myosin I is also localized on small vesicles in the terminal web of intestinal epithelial cells (Drenckhahn & Dermietzel 1988). It may transport these vesicles

along the microvillar rootlets to the site where they fuse with the plasma membrane and then anchor the newly expanded plasma membrane to the actin bundle (Fath *et al.* 1990).

The products of the *Drosophila ninaC* gene, the 174 kDa and 132 kDa myosin heavy chains, are localized in the eye to the rhabdomeral microvilli and the cytoplasm adjacent to the rhabdomeres respectively (Matsumoto *et al.* 1987; Montell & Rubin 1988; Hicks & Williams 1992; Porter *et al.* 1992). The microvilli have only two axial actin filaments (Arikawa *et al.* 1990), and are connected to the surrounding plasma membrane by myosin I radial links. The *ninaC* mutation which affects myosin I disrupts *Drosophila* visual photo reception. One hypothetical function of myosin I is to translocate newly synthesized membranes and/or photo-transduction cascade components distally into rhabdomeres along the axial actin cables (Fyrberg & Goldstein 1990; Hicks & Williams 1992). It is suggested that the tail of the 174 kDa myosin causes association with the rhabdomeres and that the kinase domain phosphorylates a rhabdomeric protein important in phototransduction (Porter *et al.* 1992). The 174 kDa protein may function as a component of the microvillar cytoskeleton (perhaps analogous to the 110 kDa brush border myosin I), and the 132 kDa protein may play a role in the adjacent cytoplasm by effecting some aspect of photoreceptor membrane turnover (Hicks & Williams 1992).

Mutation of the *MYO2* gene of yeast, which encodes a novel myosin heavy chain, causes marked disorganization of the actin cytoskeleton and delocalized distribution of actin cortical patches (Johnston *et al.* 1991). Secretory vesicles accumulate and the mutant is defective in localization of cell growth. The novel myosin may transport secretory vesicles along actin cables to the site of bud development.

A 150 kDa calmodulin-binding polypeptide in *Dictyostelium* may be the *Dictyostelium* homologue of the *dilute*/p190/*MYO2* class of novel myosin heavy chains (Zhu & Clarke 1992). The association of this 150 kDa polypeptide with

contractile vacuole membranes indicates that it may play a role in movement of the vacuole to the plasma membrane.

1.7 REGULATION OF ACTIN-MYOSIN INTERACTION

Regulation of actin-myosin interaction can be viewed as either actin-linked or myosin-linked according to which protein is the focus of the regulatory processes. The actin-linked regulatory systems include the actin-associated proteins troponin-tropomyosin, caldesmon and calponin. Myosin-linked regulatory systems include the direct binding of Ca^{2+} to myosin, the phosphorylation of myosin light chains and/or heavy chains. The dominant regulatory process in vertebrate striated (skeletal and cardiac) muscle is actin-linked, whereas myosin-linked systems provide the major regulatory mechanisms in molluscan muscle and vertebrate smooth muscle, as well as in nonmuscle cells. These several systems will be discussed separately later. Many cells contain dual and perhaps multiple regulatory systems (Adelstein & Eisenberg 1980; Kendrick-Jones & Scholey 1981; Leavis & Gergely 1984; Zot & Potter 1987; Korn & Hammer 1988; Trybus 1991; Sobue & Sellers 1991). Sections 1.7.1 to 1.7.4 refer to myosin II, and myosin I is discussed in 1.7.5.

1.7.1 Vertebrate striated muscle: troponin-tropomyosin regulation

In vertebrate striated muscle, the actin-activated Mg^{2+}-ATPase activity is regulated by the binding of Ca^{2+} to the regulatory complex (tropomyosin, troponin I, T and C) bound to actin filaments. At low Ca^{2+} concentration (10^{-7} M or lower), the regulatory complex inhibits the interaction between myosin and actin. When the concentration of Ca^{2+} reaches about 10^{-5} M or greater, the binding of Ca^{2+} to troponin C fully relieves the inhibition and fully activates the myosin Mg^{2+}-ATPase activity (Leavis & Gergely 1984; Zot & Potter 1987).

1.7.2 Molluscan muscle: direct binding of Ca^{2+} to myosin light chain

In molluscan muscles, contraction is regulated by the direct binding of Ca^{2+} to myosin (Szent-Gyorgyi *et al.* 1973; Kendrick-Jones & Scholey 1981). The binding

of Ca^{2+} activates the ATPase of myosin. Regulatory light chains are essential for the Ca^{2+} sensitivity of the ATPase, and for specific Ca^{2+} binding to myosin (Szent-Gyorgyi *et al.* 1973; Chantler & Szent-Gyorgyi 1980), but the specific Ca^{2+}-binding site probably lies in the essential light chain (Collins *et al.* 1986; Kwon *et al.* 1990).

1.7.3 Vertebrate smooth muscle and nonmuscle: phosphorylation of myosin; caldesmon and calponin regulation

In vertebrate smooth muscle myosin, the actin-activated ATPase activity is regulated by the phosphorylation of myosin regulatory light chains. Light chain phosphorylation is regulated by two enzymes, a Ca^{2+}/calmodulin-dependent kinase and a Ca^{2+}-insensitive phosphatase. At high Ca^{2+} concentrations, phosphorylation of myosin light chain increases the actin-activated Mg^{2+}-ATPase activity (Kendrick-Jones & Scholey 1981; Trybus 1991).

In vertebrate nonmuscle cells, actomyosin activity is regulated by the phosphorylation of myosin. Myosins can be classified into two groups with respect to the location of phosphorylation sites. In one group, both the light chains and heavy chains are phosphorylated, while in the other group, only light chains are phosphorylated (Kuznicki 1986; Kuznicki & Barylko 1988; Korn & Hammer 1988). Like vertebrate smooth muscle, phosphorylation of regulatory light chains of vertebrate nonmuscle myosin by the Ca^{2+}/calmodulin-dependent light chain kinase increases the actin-activated Mg^{2+}-ATPase activities.

Caldesmon is a major actin- and calmodulin-binding protein found in smooth muscle and many nonmuscle cells (Sobue *et al.* 1988; Mabuchi & Wang 1991; Bryan & Lee 1991). It inhibits the actin-activated Mg^{2+}-ATPase, and the inhibition can be reversed by calmodulin in a Ca^{2+}-dependent manner (Sobue *et al.* 1985; Pritchard & Moody 1986; Okagaki *et al.* 1991; Sobue & Seller 1991). Calponin is an actin-, calmodulin- and tropomyosin-binding protein present in many vertebrate smooth muscles (Takahashi *et al.* 1986, 1987, 1988; Abe *et al.* 1990). It inhibits the

actin-activated myosin Mg^{2+}-ATPase activity, and the inhibition can be reversed by calmodulin only in the presence of Ca^{2+}.

1.7.4 Lower eukaryotes: phosphorylation of myosin and direct Ca^{2+} binding to myosin light chain

In *Acanthamoeba,* phosphorylation of the myosin II heavy chain by heavy chain-specific kinase activates actin-activated Mg^{2+}-ATPase activity (Collins & Korn 1980; Collins *et al.* 1982b; Pollard 1982c; Kuznicki *et al.* 1983). The specific activity of each myosin II molecule within a filament is independent of its own state of phosphorylation but is regulated by a phosphorylation-dependent conformational change in the myosin filament as a whole (Atkinson *et al.* 1989; Ganguly *et al.* 1990).

In *Dictyostelium,* the ATPase activity of myosin II is regulated by both heavy chain and light chain phosphorylation. Heavy chain phosphorylation has no effect in the absence of light chain phosphorylation. Phosphorylation of the heavy chain by heavy chain kinase inhibits the actin-activated Mg^{2+}-ATPase activity (Kuczmarski & Spudich 1980; Cote & McCrea 1987; Ravid & Spudich 1989).

In *Physarum,* enzymatic activity of myosin is regulated by both the direct binding of Ca^{2+} to myosin light chain and phosphorylation of the heavy chain. Direct Ca^{2+} binding to Ca^{2+}-binding light chains inhibits the actin-activated Mg^{2+}-ATPase activity (Kohama & Kendrick-Jones 1982, 1986; Kohama 1987, 1988; Kohama *et al.* 1991b). Phosphorylation of the heavy chain by the heavy chain-specific kinase activates the actin-activated Mg^{2+}-ATPase activity (Ogihara *et al.* 1983; Takahashi *et al.* 1983), and the Ca-inhibition is only observed with phosphorylated myosin (Kohama 1990). These studies have been carried out at a conventional actin concentration, which is comparable with that of myosin by weight. However, when the concentration of actin is increased by 10 times, the Ca-inhibition is observed with dephosphorylated myosin. Since actin is quite abundant in plasmodia of *Physarum* (Ogihara *et al.* 1983; Kohama 1987), it is suggested that

myosin phosphorylation plays virtually no role in regulating actin-myosin-ATP interaction *in vivo* (Kohama *et al.* 1991a).

1.7.5 Myosin I: phosphorylation of myosin heavy chain and direct Ca^{2+} binding to light chain

Light chain phosphorylation has not been observed in myosin I (Korn & Hammer 1988; Pollard *et al.* 1991). Phosphorylation of the heavy chain by heavy chain kinase is required for actin-activated Mg^{2+}-ATPase activities of both *Acanthamoeba* and *Dictyostelium* myosins I (Maruta & Korn 1977b; Maruta *et al.* 1978; Hammer *et al.* 1983; Cote *et al.* 1985; Brzeska *et al.* 1990). The activity of *Acanthamoeba* heavy chain kinase is highly activated by autophosphorylation which is inhibited by Ca^{2+}-calmodulin (Maruta & Korn 1977b; Brzeska *et al.* 1990, 1991; Kulesza-Lipka *et al.* 1991). Chicken brush border myosin I has up to four calmodulins as light chains. Physiological Ca^{2+} concentrations stimulate actin-activated Mg^{2+}-ATPase activity with three or four calmodulin light chains bound to myosin heavy chain, but inhibit with less than two bound (Swanljung-Collins & Collins 1991). The association of the myosin I with the membrane might also affect the Ca^{2+} regulation of its ATPase activity.

1.8 PLANT MYOSINS AND THEIR ROLES IN CELL MOTILITY

Plant myosins are much less characterized than those of other eukaryotes due to difficulties of protein purification from plants and lack of successful cloning of their genes. Biochemical and/or immunological methods have mainly been used to identify and characterize plant myosins, and explore their possible roles in cell motility.

1.8.1 Purification and biochemical characterization of plant myosins and putative myosins

So far, plant myosin has been purified from the alga *Nitella flexilis* (Kato & Tonomura 1977), from parenchyma cells of the fruit of tomato (*Lycopersicon esculentum*) (Vahey & Scordilis 1980; Vahey *et al.* 1982) and from conducting

tissues of the petiole of *Heracleum sosnowskyi* (Sokolov *et al.* 1985; Turkina *et al.* 1987). Purified *Nitella* myosin exhibits ATPase activities characteristic of myosin (Kato & Tonomura 1977). At high ionic strength, its ATPase activity is activated maximally by EDTA, partially by Ca^{2+}, and least by Mg^{2+}. At low ionic strength, its Mg^{2+}-ATPase activity is activated by rabbit skeletal muscle F-actin. The M_r of *Nitella* myosin heavy chain is slightly higher than that of rabbit skeletal muscle myosin, estimated by SDS-PAGE. Ultracentrifuge studies of mixtures of *Nitella* myosin and muscle F-actin at low ionic strength indicate that *Nitella* myosin binds to muscle F-actin in the absence of ATP. At low ionic strength, *Nitella* myosin aggregates to form bipolar filaments with a central bare zone and globular projections at the ends.

Vahey & Scordilis (1980) briefly presented studies of isolating myosin from parenchymal cells of tomato fruit. A putative 220 kDa myosin heavy chain was estimated by SDS-PAGE of crude tomato actomyosin. At high ionic strength, the ATPase activity of the isolated myosin is said to be maximal in the presence of K^+-EDTA, and lowest in the presence of Mg^{2+}, but no original data are presented in the paper. At low ionic strength, the Mg^{2+}-ATPase activity can be activated by either muscle skeletal F-actin or tomato F-actin. Ultracentrifuge studies are said to show that the myosin interacts with muscle F-actin to form an actomyosin complex that can be dissociated by ATP; however, no data are presented. Tomato myosins in crude extract aggregate to form bipolar filaments.

Later, the same group reported thoroughly the purification of a 130 kDa tomato myosin that contains a 100 kDa heavy chain and light chains of 16 kDa and 14 kDa (Vahey *et al.* 1982). Its ATPase activity is activated maximally by K^+-EDTA, partially by Ca^{2+}, and least by Mg^{2+} at high ionic strength. At low ionic strength, its Mg^{2+}-ATPase activity can be activated by muscle F-actin or tomato F-actin. The myosin binds to muscle F-actin in the absence of ATP. A Coomassie blue-stained gel of the purified myosin demonstrates weak bands of heavy and light chains and there seem to be several other faint bands on the gel.

Since the major difficulty in the purification of tomato myosin is considered to be the sensitivity of the enzyme to proteolytic degradation (Vahey & Scordilis 1980), potent proteolytic inhibitors (EDTA, PMSF) were extensively used in purifying the 130 kDa protein. The relationship between the HMW myosin reported before and the 130 kDa protein was not discussed by the authors. Although no indication of proteolysis was recorded during purification, the possibility that the 100 kDa polypeptide is a proteolytic fragment of a HMW myosin heavy chain cannot be excluded. Nevertheless, the 130 kDa ATPase from tomato exhibits all of the properties of a myosin enzyme.

Purified myosin of *Heracleum sosnowskyi* has Mg^{2+}-ATPase activity that can be activated by muscle F-actin at low ionic strength (Turkina *et al.* 1987). It binds to muscle F-actin as demonstrated by both ultracentrifuge and electron microscopic studies. The myosin forms bipolar filaments *in vitro* and these aggregate into thick bundles.

Myosin has also been partially purified from leaves of a flowing plant *Egeria densa* (Ohsuka & Inoue 1979). The partially purified myosin fraction is still contaminated with several very prominent proteins as shown by SDS-PAGE. The M_r of its heavy chain is 180 kDa. Its ATPase activities differ from those of other plant myosins identified so far. At high ionic strength, its ATPase activity is activated most by Ca^{2+}, partially by Mg^{2+}, and is lowest in K^+-EDTA. A similar case is the myosin isolated from *Amoeba proteus* whose ATPase activity is activated by Ca^{2+}, inhibited by K^+-EDTA and Mg^{2+}, resulting in lowest K^+-EDTA-ATPase activity at high ionic strength (Condeelis 1977). Furthermore, the Mg^{2+}-ATPase activity of the partially purified *Egeria* myosin is not affected by skeletal muscle F-actin at low ionic strength. Nevertheless, ultracentrifuge and electron microscopic studies show that the myosin binds to muscle F-actin and forms bipolar filaments *in vitro*.

A putative myosin has been reported to be partially purified from bulbs of onion (*Allium cepa*) by tracking myosin-like enzymatic activity during the

purification (Pesacreta *et al.* 1991; abstract only). Its ATPase activity at higher ionic strength is activated by K^+-EDTA and is lowest in the presence of Mg^{2+}. Its Mg^{2+}-ATPase activity can be activated by muscle F-actin. A 100 kDa polypeptide identified by immunoblotting with anti-pan myosin antibody is considered to be responsible for the K^+-EDTA-ATPase activity.

Tendrils of pea (*Pisum sativum* L.) that exhibit coiling movement have been used to prepare a fraction enriched for putative myosin by step elution of a crude extract on DEAE-cellulose (Ma & Yen 1989). The fraction's ATPase activity is activated most by K^+-EDTA, partially by Ca^{2+}, and least by Mg^{2+} at high ionic strength. Its Mg^{2+}-ATPase activity is activated by skeletal muscle F-actin at low ionic strength. Native polyacrylamide gel electrophoresis shows that the putative pea myosin which can be identified on the gel by its ATPase reaction has a M_r of 440 kDa. SDS-PAGE of the putative myosin extracted from the native gel shows a 165 kDa heavy chain and light chains of 17 kDa and 15 kDa.

A putative myosin has been partially isolated from pollen of snake gourd (*Luffa cylindrica*) (Ma & Yen 1988). Its ATPase activity at high ionic strength is activated maximally by K^+-EDTA, partially by Ca^{2+}, and is lowest in the presence Mg^{2+}. No data regarding actin-activated Mg^{2+}-ATPase activity are presented. The heavy chain is suggested to be a 165 kDa polypeptide that is enriched in the ATPase fraction. Another putative myosin of pollen of Chinese cabbage (*Brassica pekinensis*) has been identified by its ATPase reaction after native polyacrylamide gel electrophoresis of pollen crude extract, and SDS-PAGE of this protein extracted from the native gel shows a 220 kDa myosin heavy chain (Yen *et al.* 1986).

Purification data, in regard to protein yields and ATPase activities, in most reports of plant myosins or putative myosins are incomplete except in the case of the 130 kDa tomato myosin purification. Usually, data for only the final fraction were presented while full results of each purification step are absent. Most purified or partially purified plant myosins or putative myosins exhibit actin-activated ATPase activities. Since no controls for ATPase activities associated with

exogenous actin fractions added are presented except in the case of the 130 kDa tomato myosin, any ATPase activities associated with exogenous actin are not discounted in calculating the actin-activated ATPase activities of the plant myosin/putative myosin fractions.

The characterization of plant myosins has been hindered by the difficulties of purifying this plant enzyme. Only a few plant myosins have been purified so far. Characterization of these has shown subunit composition, ATPase activities, ability to form filaments and to bind F-actin. However, no immunochemical and immunocytochemical characterization have been studied on the purified or partially purified plant myosins or putative myosins except for the 100 kDa polypeptide which cross-reacts with anti-pan myosin antibody (Pesacreta *et al.* 1991). Molecular details of plant myosin, such as its primary structure are still not available. Only recently is there a preliminary report of isolating a complementary DNA encoding an unconventional myosin from *Arabidopsis thaliana,* but sequence data is not presented (Kinkema & Schiefelbein 1992; abstract only).

Most of the purified/partially purified plant myosins/putative myosins contain HMW heavy chains. Some are myosins II that form filaments *in vitro* and the others are either myosin II or novel myosins containing HMW heavy chains. The existence of myosin I in plant has not been confirmed. The 130 kDa myosin purified from tomato and the 100 kDa putative myosin heavy chain of onion suggest such proteins, but the possibility of their being proteolytic products of HMW myosins cannot be excluded.

1.8.2 Immunochemical identification of putative plant myosins

Putative plant myosin heavy chains can be identified in plant extracts by immunoblotting without protein purification. Monoclonal anti-pan myosin antibody has identified a putative myosin heavy chain of 200 kDa in onion root tip cells (Parke *et al.* 1986), two putative heavy chains of 200 kDa and 110 kDa in *Chara* internodal cells (Grolig *et al.* 1988), and a 100 kDa polypeptide in the partially purified myosin-like protein fraction of onion (Pesacreta *et al.* 1991). A putative

myosin heavy chain of 175 kDa from *Nicotiana* pollen tubes reacts with both monoclonal anti-pan myosin and anti-fast myosin antibodies (Tang *et al.* 1989a). Two putative myosin heavy chains of 220-230 kDa and 85 kDa have been identified in *Ernodesmis verticillata* with an affinity-purified polyclonal antiserum against *Dictyostelium* myosin heavy chain (La Claire 1991).

The 110 kDa polypeptide of *Chara* and the 85 kDa one of *Ernodesmis* are unlikely to be the proteolytic fragments because of their consistent presence in TCA extracts which minimizes proteolysis (Wu & Wang 1984). However, further study is needed to confirm whether the LMW putative myosin heavy chains are myosin I heavy chains.

Each of the polypeptides reacting with the monoclonal anti-pan myosin antibody has been shown to share only one epitope present in authentic myosins so that it is sensible to describe them as putative myosin heavy chains. A stronger case is the putative myosin heavy chain of *Nicotiana* pollen tube which has two epitopes recognized by two monoclonal anti-myosin antibodies.

1.8.3 Immunocytological characterization and possible functions of putative plant myosins

Immunocytochemical studies, especially immunofluorescence, have been used to localize putative plant myosins, and therefore reveal possible roles myosin may play in plants. Plant myosin, by interacting with actin, is considered to play important roles in motility, such as cytoplasmic streaming, membrane and organelle movement and cytokinesis.

The internodal cells of characean algae are the most favourable model system to study the cytoplasmic streaming of plants due to their giant size and simple organization (Allen & Allen 1978; Kamiya 1981; Tazawa & Shimmen 1987; Williamson 1991). The cytoplasmic streaming is inhibited by Ca^{2+}. It is now widely accepted that the motive force driving the cytoplasmic streaming is produced by the interaction between subcortical actin bundles and motile

endoplasmic myosin, but the coupling between myosin and endoplasm and the mechanism of force generation remain to be elucidated.

Three hydrodynamic models of viscous coupling between motile myosin and endoplasm have been proposed (Nothnagel & Webb 1982). Only one of the models, in which myosin is incorporated into a fibrous or membranous network or gel extending into the endoplasm, is considered to be able to generate the observed cytoplasmic streaming easily. Filaments required for cytoplasmic streaming of *Chara* have been found by electron microscopy to associate with the endoplasmic reticulum (Williamson 1979), and portions of a continuous network of endoplasmic reticulum which pervades the streaming cytoplasm contact the actin bundles, according to both electron and video microscopy (Kachar & Reese 1988). It is suggested that the endoplasmic reticulum contains myosin and that the continuous network of endoplasmic reticulum provides a means of exerting motive forces on cytoplasm deep inside the cell, distant from the subcortical actin cables where the motive force is generated.

Two putative myosin heavy chains have been identified in *Chara* internodal cells (1.8.2). The putative myosins are immunofluorescently located to subcortical actin bundles, small organelles and endoplasmic strands which may be osmotically damaged endoplasmic reticulum (Grolig *et al.* 1988; Williamson 1991, 1993). Organelles and the bulk of endoplasm have been observed to move differently in characean cells (Williamson 1975; Mustacich & Ware 1977; Kachar 1985; Kamitsubo 1986). Therefore two separate force-generating mechanisms have been proposed to operate in characean cells: individual organelles reacting directly with the actin bundles, and organelles trapped within and moved by tangled, myosin-containing endoplasmic strands (Grolig *et al.* 1988).

Pollen tubes exhibit vigorous cytoplasmic streaming using mechanisms identical or at least closely comparable to those involved in intracellular movements in other plant cells (Heslop-Harrison & Heslop-Harrison 1989b). Actin filaments occur in bundles, mainly longitudinally oriented, throughout the vegetative cells.

The actin bundles associate with membranes of the endoplasmic reticulum, surfaces of organelles and vegetative nuclei (Staiger & Schliwa 1987; Lancelle & Hepler 1989; Tang *et al.* 1989b; Heslop-Harrison & Heslop-Harrison 1988, 1989c).

Putative myosin has been identified in pollen of Chinese cabbage (Yen *et al.* 1986) and snake gourd (Ma & Yen 1988) and in *Nicotiana* pollen tubes (1.8.2). The 175 kDa putative myosin is immunofluorescently localized to the surface of organelles, vegetative nuclei and generative cells of angiosperm pollen grains and tubes (Tang *et al.* 1989a; Heslop-Harrison & Heslop-Harrison 1989a). Organelles from *Lilium* pollen tubes slide along actin bundles in characean cell models, and the movement is regulated by Ca^{2+} (Kohno & Shimmen 1988a, b). All these results indicate that cytoplasmic streaming in pollen tubes is driven by interaction between the myosin-bearing membranes of different classes of organelles and actin filaments. It has been suggested that the motility system of pollen tube is analogous to that of characean algae and that the two force-generation mechanisms proposed by Grolig *et al.* (1988) may also be present in pollen tubes to power the movement of individual organelles, and large vegetative nuclei and generative cells separately (Heslop-Harrison & Heslop-Harrison 1989b).

Two putative myosin heavy chains have been identified in *Ernodesmis verticillata* (1.8.2). In intact cells of *Ernodesmis* which lack cytoplasmic streaming, putative myosin has been localized immunofluorescently on chloroplast surfaces, in nuclei, in cytoplasmic strands between plastids, and in association with pyrenoids primarily in apical chloroplasts (La Claire 1991). During wound-induced cytoplasmic contraction, putative myosin is colocalized with actin near the plasma membrane in longitudinal bundles and in a reticulum in regions of cytoplasmic contraction. These results indicate that myosin and actin are associated with contractility during the wound-healing of *Ernodesmis*.

Immunolocalization of putative myosins on the surfaces of amyloplasts of *Alopecurus pratensis* (Heslop-Harrison & Heslop-Harrison 1989a), chloroplasts of *Ernodesmis* (La Claire 1991) and vegetative nuclei of angiosperm pollen grains and

tubes (Tang *et al.* 1989a; Heslop-Harrison & Heslop-Harrison 1989a), suggests the involvement of myosin in the organelle movement of chloroplasts and nuclei (Williamson 1993).

In *Euglena gracilis,* putative myosin has been immunofluorescently colocalized with actin in pellicle strips beneath the plasma membrane; however, no immunochemical identification of this protein has been presented (Lonergan 1985). The coincidence of the putative myosin and actin fluorescence patterns with the pellicle ridges suggests that an actomyosin contractile system could operate to alter pellicle strip positioning.

A putative myosin heavy chain has been identified in onion root tip cells (1.8.2). The putative myosin is colocalized with actin by immunofluorescent staining to the cytokinetic phragmoplast (Parke *et al.* 1986). This suggests that myosin and actin may play an important role in plant cytokinesis, possibly transporting vesicles which contain cell plate precursors to the growing cell plate.

1.8.4 Regulation of plant actin-myosin interaction

Direct evidence of any regulation mechanism is not yet available in any plant. The regulatory systems of other nonmuscle actin-myosin interaction are mainly myosin-linked systems including the direct binding of Ca^{2+} to myosin and the phosphorylation of myosin light and/or heavy chains (1.7). These regulatory systems may also exist in plants. Plant actomyosin may also be regulated by a variety of mechanism that differ from those of other nonmuscle systems. Physiological, biochemical and immunological studies have demonstrated the possible mode of Ca^{2+}- or Ca^{2+}/calmodulin-mediated regulation of plant actin-myosin interaction (Williamson 1991).

Ca^{2+} regulates numerous physiological processes in plant cells (Hepler & Wayne 1985; Allan & Hepler 1989). Cytoplasmic streaming in the characean algae, unlike the Ca^{2+}-activated motility in muscle and many nonmuscle cells (1.7), is inhibited by high concentration of Ca^{2+} (Tazawa & Shimmen 1987) as probably is cytoplasmic streaming in other plants (Hepler & Wayne 1985; Williamson 1993).

Cytoplasmic streaming in characean cells is inhibited by high concentrations of Ca^{2+} ($\geq 10^{-6}$ M) and the Ca^{2+}-sensitive site lies on myosin not actin (Tazawa & Shimmen 1987; Williamson 1991, 1993). In plasma membrane-permeabilized or gently perfused cells, Ca^{2+} inhibition of cytoplasmic streaming is reversible when Ca^{2+} concentrations are lowered (Tominaga *et al.* 1983, 1987). However, such reversibility in response to Ca^{2+} is lost in rapidly vacuole-perfused cells (Williamson 1975). Physiological studies of characean cell models show that inhibitors of protein phosphatases inhibit streaming even in the absence of Ca^{2+} and that ATP-γ-S strongly inhibits the recovery of streaming that has been inhibited by 10 μM Ca^{2+} (Tominaga *et al.* 1987). Added protein phosphatase, however, restores streaming even in the presence of Ca^{2+}. Inhibitors of calmodulin do not affect the Ca^{2+}-induced cessation of streaming but inhibit the recovery of the streaming when Ca^{2+} concentration is lowered (Tominaga *et al.* 1983). Therefore, it is suggested that streaming is inhibited when a component is phosphorylated by a Ca^{2+}-activated, calmodulin-independent protein kinase and reactivated when the component is dephosphorylated by a Ca^{2+}/calmodulin-activated protein phosphatase (Tominaga *et al.* 1987). Since the Ca^{2+}-sensitive site lies on myosin not actin, myosin is considered the most probable candidate for the substrate of phosphorylation-dephosphorylation although it is possible that the streaming is controlled indirectly by phosphorylation of a regulatory protein.

A Ca^{2+}-dependent but calmodulin-independent protein kinase (CDPK), which can be activated by micromolar Ca^{2+}, has been purified from soybean and characterized (Harmon *et al.* 1987; Putnam-Evans *et al.* 1990). A monoclonal antibody against this protein localizes CDPK with F-actin bundles in interphase onion and soybean root tip cells and in *Tradescantia* pollen tubes, and to the phragmoplast/cell plate during cytokinesis of onion root tip cells (Putnam-Evans *et al.* 1989). *In vitro* studies show that soybean CDPK does not interact directly with actin; therefore it may be associated with an actin-binding protein. It was suggested that CDPK regulates the assembly of actin, or the interaction of actin with myosin.

A putative CDPK identified in *Chara* by immunoblotting with antibodies against soybean CDPK exhibits Ca^{2+}-dependent kinase activity after SDS-PAGE (McCurdy & Harmon 1992a). It localizes to subcortical actin bundles, and to the surface of small organelles and other membrane components of the streaming endoplasm. Endoplasmic CDPK is extracted from cells by perfusion with ATP or high Ca^{2+}. In both localization and selective extraction from *Chara* cells, the putative CDPK closely resembles putative *Chara* myosins (Grolig *et al.* 1988). CDPK may mediate the Ca^{2+}-induced inhibition of cytoplasmic streaming in characean cells.

Since soybean CDPK efficiently phosphorylates myosin light chains of chicken gizzard (Putnam-Evans *et al.* 1990) and light chains of intact myosin molecules, one role for CDPK in plants may be the phosphorylation of a myosin light chain (McCurdy & Harmon 1992a). A 16-18 kDa polypeptide recognized by an antibody to gizzard myosin light chains has been identified in *Chara* as a putative myosin light chain (McCurdy & Harmon 1992b). Soybean CDPK phosphorylates this polypeptide *in vivo* at high Ca^{2+} concentrations (10^{-4}), which supports the view that phosphorylation by CDPK of a myosin light chain is involved in the Ca^{2+}-induced inhibition of cytoplasmic streaming in *Chara*.

A putative *Chara* calmodulin identified by immunoblotting in *Chara* cells is widely distributed in the endoplasm, where it seems to associate with organelles aligned along actin bundles, but not with the actin bundles themselves (Jablonsky *et al.* 1990). It is undetectable, however, in rapidly perfused cells where the inhibition of high Ca^{2+} is irreversible. This is consistent with the model proposed by Tominaga *et al.* (1987) in which calmodulin is required to restore the activity of myosin, but not to inhibit it.

Cytoplasmic streaming of pollen tubes is regulated by Ca^{2+}, like that of characean cells. It is inhibited by Ca^{2+} higher than 10^{-5} M in lily pollen tubes (Kohno & Shimmen 1988b). Lily pollen tube organelles move along characean actin bundles; the movement is inhibited by Ca^{2+} at 10^{-5} M levels and the

inhibition is reversible (Kohno & Shimmen 1988a, b). The organelle motility is lost after N-ethylmaleimide or heat treatment of organelles, suggesting that myosin-linked regulation underlies the Ca^{2+} inhibition (Kohno & Shimmen 1988a). However, the *in situ* actin filaments of pollen tubes are fragmented at high Ca^{2+} concentrations and the fragmentation is irreversible (Kohno & Shimmen 1988b). Therefore, the Ca^{2+} inhibition of cytoplasmic streaming can be attributed to both inactivation of myosin and fragmentation of actin, and the irreversibility of the inhibition *in situ* is attributed to the irreversible fragmentation of actin filaments (Kohno & Shimmen 1988b).

Calmodulin has been immunofluorescently colocalized to actin and putative myosin in several cases in plants, which may indicate its role in regulating actomyosin activity. In the alga *Ernodesmis*, calmodulin and putative myosin colocalize with actin to extensive, longitudinal bundles and a reticulum in the cortical cytoplasm of regions showing wound-induced cytoplasmic streaming (Goddard & La Claire 1991; La Claire 1989, 1991). Calmodulin antagonists inhibit cytoplasmic motility and actin bundles do not assemble or are poorly formed. Calmodulin may regulate the formation of functional actin bundles during wound-induced cytoplasmic contraction in *Ernodesmis*. It may also regulate actomyosin interaction during the contraction. Calmodulin, together with actin, putative myosin and tubulin, has also been localized to phragmoplasts of onion and pea root tip cells during cytokinesis (Gunning & Wick 1985; Wick *et al.* 1985; Wick 1988). It is suggested that calmodulin may be involved in microtubule dynamics and possible actomyosin activities such as vesicle fusion in the phragmoplast.

1.8.5 Conclusions

The evidence suggests that plant myosins are involved in diverse forms of cell motility, like myosins of other eukaryotes, but information regarding plant myosin is very limited. Only a few plant myosins have been purified and further studies related to their cellular distribution and function have not been reported. Investigations of the detailed molecular structure and enzymatic regulation of these

myosins, which required considerable amounts of highly purified proteins, have not been possible. On the other hand, putative plant myosins have been identified immunochemically and their cellular localization determined immunocytochemically, without purifying these enzymes. These studies widen the knowledge of plant myosin and of the possible roles it plays in plant.

My research work on plant myosin-like proteins starts with the identification of a putative myosin heavy chain by immunoblotting with anti-pan myosin antibody. This antibody cross-reacts with many authentic myosins from animal cells and with putative myosin heavy chains of some plants (1.8.2). A 165 kDa polypeptide of mung beans was strongly and reliably detected by anti-pan myosin on immunoblots, and was considered as a putative myosin heavy chain. To further investigate the 165 kDa polypeptide and confirm its identity as a myosin, purification was extensively studied but proved to be very difficult. The partially purified fraction containing the 165 kDa polypeptide had low K^+-EDTA-ATPase activity but did not exhibit actin-activated Mg^{2+}-ATPase activity.

The impurity, low protein yield and ATPase activity of the partially purified 165 kDa polypeptide fraction made it impractical to further characterize this polypeptide using the partially purified fraction. Therefore, monoclonal antibodies were raised against the 165 kDa polypeptide. The value of anti-myosin antibodies has been shown before (1.8.2; 1.8.3), and they permit studies of putative plant myosins without the hindrance of tough protein purification. Epitopes of mung bean proteins and rabbit skeletal muscle myosin were detected by immunoblotting with antibodies to the 165 kDa polypeptide and with commercial anti-myosin antibodies. Eight antibodies recognized at least six epitopes, all of which were on muscle myosin heavy chain. The mung bean 165 kDa polypeptide shared five of these epitopes with the muscle myosin heavy chain. This impressive degree of epitope similarity strengthens the status of the 165 kDa polypeptide as a myosin heavy chain.

Furthermore, mung bean root tip cells and *Chara* internodal cells were labelled immunofluorescently with antibodies to the 165 kDa polypeptide, with anti-myosin and with anti-actin. Colocalization of the 165 kDa polypeptide with actin in phragmoplasts and perhaps actin filaments of mung bean root tip cells strengthens the case for the 165 kDa polypeptide having some association with actin and is consistent with its being a myosin heavy chain.

CHAPTER 2

MATERIALS AND GENERAL METHODS

CHAPTER 2

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2.1 PLANT MATERIALS

The higher plants mung bean (*Phaseolus mungo*), pea (*Pisum sativum* cv Greenfeast), wheat (*Triticum aestivum*), *Arabidopsis thaliana* var Columbia, and the alga *Chara corallina* were used.

Seeds of mung bean, pea, and wheat were grown in the dark at room temperature, either in vermiculite moistened with tap water in plastic trays or in commercially available plastic "bean-sprouters" moistened with tap water. Plants (roots and shoots unless otherwise stated) were harvested after 4-5 days, washed with tap water, and finally rinsed several times with distilled water (dH_2O). *Arabidopsis* was grown in the light on vertical agar plates at room temperature (Baskin *et al.* 1992). The seedlings were harvested after 7 days and rinsed with dH_2O before protein extraction. Soil-cultured *Chara* was grown in the glass-house as previously described (Williamson & Hurley 1986). Apical cuttings of soil-cultured *Chara* were temporarily cultured in a plastic container filled with tap water, and cells were rinsed with fresh tap water before use.

2.2 CHEMICALS, IMMUNOCHEMICALS AND BUFFERS

All chemicals were at least analytical-grade purity. Basic laboratory chemicals were supplied by Ajax Chemicals, Clyde Industries Ltd., Auburn, Australia. Unless otherwise stated, all the other chemicals and immunochemicals were supplied by Sigma Chemical Co., St. Louis, MO., USA.

All buffers used for protein purification or in other experiments carried out at 0-4°C were adjusted to the desired pH when cooled to 0-4°C. Buffers which contained the reducing agent dithiothreitol (DTT, Cleland's reagent) were freshly

made up and used within 24 h because DTT is easily oxidized by oxygen and so loses its ability to protect active sulfhydryls of enzymes.

ATP was from equine muscle, in the form of disodium salt crystalline. It could be added directly into solutions when making up buffers or, more often, was made up as concentrated stock solution in dH_2O and adjusted to pH 7.0 with KOH. The ATP solution was immediately distributed into small aliquots and stored at -20°C.

2.3 PREPARATION OF CHROMATOGRAPHY MEDIA

The chromatography media are listed below.

1. Gel filtration:

   Sephadex G-25, Medium (Pharmacia Fine Chemicals AB, Uppsala, Sweden).

   Sephacryl S-400, Superfine (Pharmacia).

2. Ion exchange chromatography:

   Cation exchange chromatography:

   diethylaminoethyl (DEAE)-cellulose: DE52 (Whatman Biosystems Ltd., Kent, England, UK).

   DEAE-Sephacel (Pharmacia).

   DEAE-Sepharose CL-4B (Pharmacia).

   DEAE-Sephadex A-50 (Pharmacia).

   Anion exchange chromatography:

   carboxymethyl (CM)-cellulose: CM52 (Whatman).

   phosphocellulose*.

3. Hydrophobic interaction chromatography:

   phenyl Sepharose CL-4B (Pharmacia).

4. Hydroxylapatite (Bio-Rad Laboratories, Richmond, CA., USA).

5. Affinity chromatography:

   Novobiocin-Sepharose 6B* [prepared by the method of Staudenbauer & Orr

(1981)].

ADP-agarose (ADP attached through ribose hydroxyls via six-carbon spacers to 4% beaded agarose; Sigma).

F-actin Sepharose 4B* [prepared by the method of Winstanley *et al.*(1977)].

* Kindly prepared by Dr. Peter. P. Jablonsky of this Group.

All chromatography media were supplied as liquid suspensions and ready for use except Sephadex G-25, hydroxylapatite, DE52 and CM52 which were pretreated according to the manufacturers' instructions. All the prepared chromatography media were stored at 0-4°C with 0.02% (w/v) NaN_3.

2.4 COLUMN CHROMATOGRAPHY

Columns [1.6 x (8, 13 cm), 2.6 x (13, 53 cm), 4.9 x 60 cm; Bio-Rad] were packed and run by gravity-induced flow. A slightly higher flow rate than that to be used in the experiment was used for packing. Columns were equilibrated with more than 3 column volumes of starting buffers. Small samples were applied by Pasteur pipettes, large sample volume by gravity-induced flow. For gel filtration, sample volume represented 1-5% of the column bed volume. Eluate was collected with a LKB UltraRac fraction collector (LKB-Produkter AB, Bromma, Sweden). All procedures were carried out at 0-4°C. Protein content of the eluate was monitored by A_{280} determinations (Shimadzu UV-265 spectrophotometer; Shimadzu Corporation, Kyoto, Japan), or sometimes by protein assay reagent (Spector 1978).

Linear concentration (ionic strength) gradients used in gradient elution were formed in a Gradient Mixer GM-1 (Pharmacia).

2.5 EXTRACTION OF PROTEINS FROM HIGHER PLANTS

2.5.1 Extraction with buffer

The extraction buffer was 0.34 M sucrose, 10 mM imidazole, pH 7.0, 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM N-tosyl-L-phenylalanine chloromethyl ketone (TPCK), 1 mM ethylenediaminetetraacetic acid (EDTA), 40 mM sodium metabisulfite, 1 mM DTT. TPCK was prepared as 50 mM stock in 95% ethanol, and stored at -20°C. PMSF was freshly dissolved in a small volume of dimethyl sulphoxide (DMSO) and added directly into plant tissues before homogenization rather than mixed with the extraction buffer prior to homogenization since PMSF hydrolyses quite rapidly (Scopes 1987). For extraction of protein from less than 15 grams (g) of plant tissue, both PMSF and DTT could be prepared as 200 mM stocks, and stored at -20°C before use. All procedures of protein extraction and fractionation were carried out at 0-4°C.

(i) Small-scale extraction

1-3 g of plant tissue were ground in prechilled mortar and pestle with extraction buffer (1 ml/g of tissue). The extract was spun at 13,000 rpm for 15 minutes (min) in a microcentrifuge (Biofuge A; Foss Electric Pty. Ltd., Australia) to remove cell debris. After spinning at 10,000 rpm for 5 min to clarify, the supernatant was ready for electrophoresis sample preparation (2.8) or other experiments.

(ii) Medium to large scale extraction

20 g to 1 kg of plant tissues were homogenized in a waring blender with an equal volume of extraction buffer. Cell debris was removed by centrifuging at 14,500 rpm for 20 min in rotor JA-20, or 12,500 rpm for 24 min in JA-14, or 9,500 rpm for 26 min in JA-10 (Beckman Instruments Inc., Palo Alto. CA., USA). The supernatant was twice filtered through six layers of cheese cloth to remove floating lipid.

2.5.2 Extraction with trichloroacetic acid (TCA)

Protein proteolysis can be minimized by extraction of protein directly with TCA (Wu & Wang 1984). 1-3 g of plant tissue were ground in a prechilled mortar

and pestle directly with ice-cold 20% (w/v) TCA (1 ml/g of tissue). After immediate filtering through one layer of cheese cloth to remove cell debris, the extract was kept on ice for 20-30 min and protein precipitate collected by centrifugation at 7,000 rpm for 3 min in a Microfuge 11 (Beckman). The protein precipitate was washed three times with acetone and collected at 7,000 rpm for 2 min. The protein precipitate was air-dried or dried in a desiccator using a vacuum pump. The dried precipitate was ready for electrophoresis sample preparation (2.8) or could be stored at -20°C.

2.6 $(NH_4)_2SO_4$ FRACTIONATION

Solid $(NH_4)_2SO_4$ (Merck, Darmstadt, Germany) was slowly added into a protein solution, with constant gentle stirring. The solution was kept stirring for 15 min after all salt had dissolved to allow complete equilibration between dissolved and aggregated proteins. Protein precipitates were collected by centrifugation at 10,500 rpm for 10 min in JA-20, or at 9,000 rpm for 12 min in JA-14 rotor. The supernatant was used for further $(NH_4)_2SO_4$ fractionation if needed. The $(NH_4)_2SO_4$ pellet was dissolved in a suitable buffer for desalting, or stored at -20°C.

2.7 DESALTING AND CONCENTRATING PROTEIN SAMPLES

$(NH_4)_2SO_4$ pellets were resuspended in buffer used for the equilibration of various chromatographic media, then desalted by chromatography on a Sephadex G-25 column. Desalting efficiency was monitored by adding a drop of saturated $BaCl_2$ to 100 µl of fraction samples. The peak protein fractions were pooled. Alternatively, samples were desalted by dialysis against a large volume of buffer with constant stirring. Dialyzed samples were clarified by centrifugation at 10,500 rpm for 10 min in JA-20, and the supernatant used for chromatography. Unless otherwise stated, $(NH_4)_2SO_4$ fractions and the other protein samples were desalted by Sephadex G-25 chromatography. Small samples ($\leq$ 1 ml) used in ATPase assays

(2.14) were desalted on disposable columns (PD-10; Pharmacia) prepacked with 9.1 ml of Sephadex G-25 medium.

Protein samples were concentrated by dialysing against Aquacide II (Calbiochem, La Jolla, CA., USA). Samples could also be concentrated and/or desalted by ultrafiltration through membrane YM 10 (M_r cut-off 10 kDa; Amicon Danvers, MA., USA) in an Amicon ultrafilter.

2.8 PREPARATION OF PLANT PROTEIN SAMPLES FOR SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Generally, three volumes of the protein supernatant were mixed with one volume of 4x stock of SDS-PAGE sample buffer, boiled for 3 min in a water bath, and clarified at 7,000 rpm for 2 min in the Microfuge 11 to remove any insoluble residues. The 1x SDS-PAGE sample buffer was 0.0625 M Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) ß-mercaptoethanol, 0.002% (w/v) bromophenol blue (Laemmli 1970).

High concentrations of ions such as NH_4^+, SO_4^{2-}, K^+, Na^+ precipitate SDS. Such samples were desalted by chromatography or by dialysis against buffer (K^+< 0.1 M, Na^+< 0.5 M). Dried protein pellets were resuspended with several volumes of SDS-PAGE sample buffer, boiled for 3 min and clarified at 7,000 rpm for 2 min.

2.9 SDS-PAGE

SDS-PAGE was by the method of Laemmli (1970). High molecular weight (HMW) proteins (210-66 kDa) were resolved by 7% SDS-PAGE, low molecular weight (LMW) proteins (66-14 kDa) by 12-14% SDS-PAGE. Small slab gels (Mini-Protean II; Bio-Rad) were loaded with ≤ 40 µl of sample in each of 10 wells, or with ≤ 400 µl in a large "streak" well. SDS-6H and SDS-7B (prestained) kits (Sigma) were used as HMW standards and SDS-7 as LMW standards. Gels were normally run at 180 V constant voltage for 35-50 min until the dye front was 5-10

mm from the bottom. Gels were stained with silver (Wray *et al.* 1981) or with 0.1% (w/v) Coomassie brilliant blue R in 40% (v/v) methanol, 10% (v/v) acetic acid for 0.5 h or overnight, and destained in 40% methanol, 10% acetic acid.

2.10 IMMUNOBLOTTING

Proteins in acrylamide gels were electrophoretically transferred (Towbin *et al.* 1979) to nitrocellulose membrane (Hybond-C, 0.45 μm; Amersham International plc., UK) using a Mini-Protean II transfer unit (Bio-Rad) at 40 V constant voltage for 12 h, or occasionally at 70-75 V for 1-2 h. HMW proteins were routinely transferred overnight.

Immunoblotting was essentially as described before (Grolig *et al.* 1988). All primary antibodies (2.11) were diluted with 1% (w/v) bovine serum albumin (BSA) in TBS-Tween [Tris buffered saline plus 0.05 (v/v) Tween-20] and incubated with the blot for 1 h. The second and third antibodies varied with the blot developing systems which were either alkaline phosphatase (Grolig *et al.* 1988) or peroxidase. With peroxidase detection, the second antibody was sheep anti-mouse Ig, biotinylated whole antibody (1:300 dilution for 1 h; Amersham) which was detected with streptavidin-biotinylated peroxidase complex (1: 400 dilution for 0.5 h; Amersham). The peroxidase substrate 4-chloro-1-naphthol was prepared as 3 mg/ml stock in methanol that was stored at 4°C in dark for up to one month. The developing solution was made up freshly by diluting one volume of the chloronaphthol stock with 5 volumes of TBS buffer and adding H_2O_2 to 0.03% (v/v). In controls, the first antibody was replaced with 1% (w/v) BSA in TBS-Tween buffer. Unless otherwise stated, all blots were developed with the alkaline phosphatase system.

M_r of polypeptides were determined on immunoblots or blots stained with Ponceau using a SDS-6H standard curve of relative migration value (R_f) vs. M_r.

2.11 MONOCLONAL ANTIBODIES USED IN IMMUNOBLOTTING AND IMMUNOFLUORESCENT STAINING

Antibody	Ig class	Product form	Host	code
anti-pan myosin[a]	IgM	concentrated cell culture supernatant	mouse	RPN. 1169
anti-fast myosin[b]	IgM	concentrated cell culture supernatant	mouse	RPN. 1167
anti-skeletal myosin (fast)*	IgG_1	ascites fluid	mouse	M-4276
anti-myosin (skeletal and smooth)*	-	delipidized, whole antiserum	rabbit	M-7648
anti-myosin (light chain)*	IgM	ascites fluid	mouse	M-4401
anti-actin C4[c]	IgG_1	ascites fluid	mouse	

a. Amersham; it is the J14 antibody of Parke *et al.* (1986).

b. Amersham.

c. Gift from Dr. J. Lessard, Children's Hospital Research Foundation, Cincinnati, OH., USA (Otey *et al.* 1986).

* Sigma.

2.12 PHOTOGRAPHY OF GELS AND BLOTS

All photographs were taken on Kodak Technical Pan Film (Eastman Kodak Co., Rochester, NY., USA) at 100 ASA, using a Pentax SP-500 camera (Asahi Opt Co., Japan). A green filter was used when photographing Ponceau-stained blots.

2.13 ASSAY OF PROTEIN CONCENTRATION

Protein was determined by the method of Spector (1978) using BSA, fraction V, as standard.

2.14 ATPase ASSAY

ATPase activities were measured by determination of Pi released according to the method of Ames (1966). The x/y (abscissa/ordinate) ratio of the standard curve (Fig. 2.1) was 38.5 so that Pi (nmol) released in an ATPase reaction was 38.5 x A_{820}.

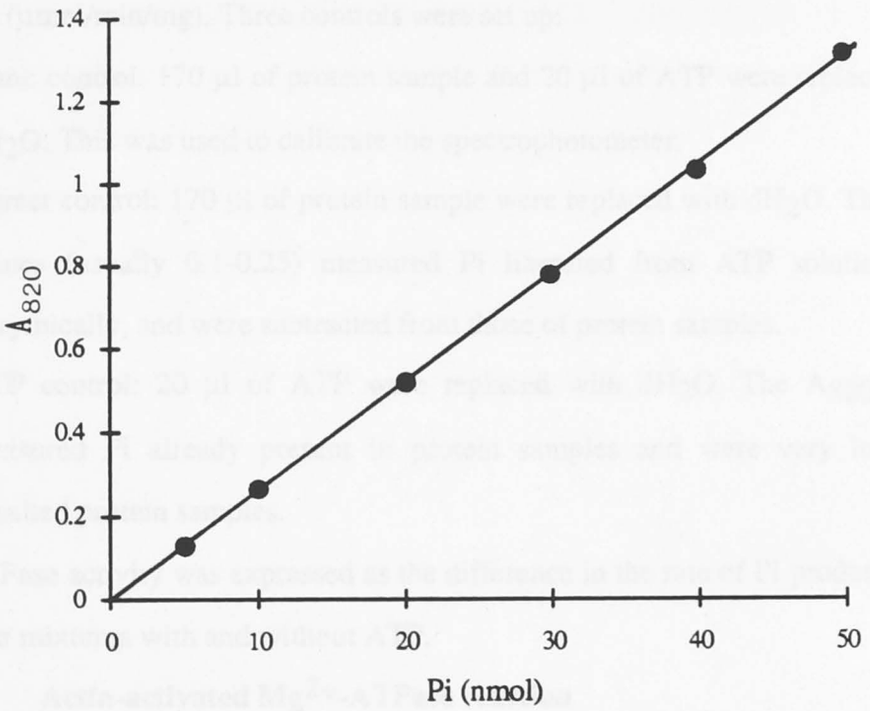

Fig. 2.1. Standard curve of Pi assay. The x/y (abscissa/ordinate) ratio of the standard curve was 38.5.

2.14.1 K^+-EDTA- /Ca^{2+}- /Mg^{2+}-ATPase reactions

Protein samples were desalted on a Sephadex G-25 column equilibrated with 0.34 M sucrose, 10 mM imidazole, pH 7.0, 1 mM DTT. ATPase reactions followed the method of Pollard & Korn (Pollard 1982b). Reaction buffer (210 µl) in 400 µl reaction mixture was 0.5 M KCl, 10 mM imidazole, pH 7.0, and 1 mM EDTA or 5 mM Ca_2Cl or 5 mM $MgCl_2$. About 40 µg of protein in 170 µl were used in each reaction. When testing elution fractions of chromatography, 60 µl of eluate were

used in each reaction (Fig. 4.6-4.9). The reaction mixtures were preincubated at 36°C for 5 min, and ATPase reactions were initiated by adding 1 mM ATP from a 20 mM stock. The reaction (at 36°C for usually 15 min) was stopped by adding 100 µl of 55% (w/v) TCA solution and the mixture was clarified by spinning at 10,000 rpm for 3 min in the Microfuge. The supernatant was used for Pi assay. The specific activity of the ATPase was expressed as µmol Pi liberated per min per mg protein (µmol/min/mg). Three controls were set up:

1. Blank control: 170 µl of protein sample and 20 µl of ATP were replaced with dH_2O. This was used to calibrate the spectrophotometer.
2. Extract control: 170 µl of protein sample were replaced with dH_2O. The A_{820} values (usually 0.1-0.25) measured Pi liberated from ATP solution non-enzymically, and were subtracted from those of protein samples.
3. ATP control: 20 µl of ATP were replaced with dH_2O. The A_{820} values measured Pi already present in protein samples and were very low with desalted protein samples.

ATPase activity was expressed as the difference in the rate of Pi production for reaction mixtures with and without ATP.

2.14.2 Actin-activated Mg^{2+}-ATPase reaction

Actin-activated Mg^{2+}-ATPase reaction was according to the method of Pollard (1982b). Rabbit skeletal muscle actin was purified up to the stage of ion exchange chromatography according to the method of Pardee & Spudich (1982). Reaction conditions were the same as for K^+-EDTA- /Ca^{2+}- /Mg^{2+}-ATPase reactions except that the reaction buffer was 20 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10 mM imidazole, pH 7.0, and 0.5 mg/ml rabbit muscle actin. Five controls were set up:

1. Blank control: protein sample, ATP and F-actin were replaced with dH_2O.
2. and 3. Extract control (- actin ATPase reaction) and ATP control (- actin ATPase reaction) were the same as those of 2.14. 1.

4. Extract control (+ actin reaction): protein sample was replaced with dH_2O. The A_{820} values measured Pi liberated from ATP nonenzymically and from any ATPase activity associated with the F-actin preparation.
5. ATP control (+ actin reaction): ATP and F-actin were replaced with dH_2O.

2.15 PREPARATION OF RABBIT SKELETAL MUSCLE MYOSIN

Rabbit skeletal muscle myosin was prepared up to the stage of ion exchange chromatography according to the method of Margossian & Lowey (1982). Purified myosin was stored in buffer (0.3 M KCl, 0.25 M K-phosphate, pH 6.5) containing 50% glycerol at -20°C.

CHAPTER 3

IDENTIFICATION AND PRELIMINARY INVESTIGATIONS OF THE CHROMATOGRAPHIC BEHAVIOUR OF A 165 kDa PUTATIVE MYOSIN HEAVY CHAIN FROM MUNG BEAN

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3.1 INTRODUCTION

Myosin has been isolated from a large number of nonmuscle cells of vertebrates, invertebrates and lower eukaryotes (Taylor & Condeelis 1979; Korn 1978, 1982; Korn & Hammer 1988; Pollard 1982a; Pollard *et al.* 1991), and from a few plants cells (1.8.1). Although myosin represents 55% of total cell protein in rabbit skeletal muscle, it constitutes less than 2% of total protein in nonmuscle cells (Taylor & Condeelis 1979; Schliwa 1985). Compared with myosin, actin is much more abundant in nonmuscle cells than in muscle cells. The ratio of actin/myosin varies from 6 in rabbit skeletal muscle to 110 in human platelets and 157 in *Dictyostelium* (Schliwa 1985).

Based on the assumption that the properties of nonmuscle myosins will be very similar to those of skeletal muscle myosin, a traditional procedure for extracting myosin from muscle and nonmuscle cells is to extract cells in 0.6 M KCl, precipitate actomyosin at low ionic strength, separate myosin from actin by $(NH_4)_2SO_4$ precipitation, and subject the material solubilized in 0.5 M KCl to gel filtration on an agarose column (Korn 1978). In practice, the properties of nonmuscle myosin from different sources are quite variable, so that, no single procedure purifies all nonmuscle myosins but a suitable combination of different procedures is considered likely to be able to purify any type of myosin (Pollard 1982a, b).

Generally, three types of extracting solutions can be used to solubilize myosin on lysis of cells: high ionic strength, pyrophosphate and sucrose buffers. Both the high ionic strength and pyrophosphate buffers solubilize myosin by dissociating myosin filaments into monomers. Whether all myosin in sucrose buffers is

monomeric is unknown, but, a number of nonmuscle myosins are soluble in sucrose. There are no absolute reasons making one buffer type better than the others. Sucrose buffers have two advantages: they minimize rupture of lysosomes and hence problems of proteolysis and their low ionic strength allows extracted proteins to be fractionated by ion exchange chromatography (Pollard 1982a). After protein extraction, actomyosin precipitation, $(NH_4)_2SO_4$ fractionation, gel filtration, ion exchange chromatography, hydroxylapatite chromatography and affinity chromatography have all been used in different combinations to purify different nonmuscle myosins.

In this chapter, putative myosin heavy chains have been identified by immunoblotting in mung bean as well as wheat and pea. The behaviour of the mung bean putative myosin heavy chain was investigated on a diverse range of chromatography media in order to devise a scheme for its purification and the distribution of K^+-EDTA-ATPase studied in $(NH_4)_2SO_4$ fractions of mung bean crude extracts.

3.2 MATERIALS AND METHODS

All procedures of protein fractionation and purification were carried out at 0-4°C. Unless otherwise stated, mung bean crude extract was prepared from seedlings (roots and shoots) as described in 2.5.1.

3.2.1 Extraction of putative myosin from different tissues of higher plants

Proteins of mung bean, pea and wheat were extracted from plant roots and/or shoots with either extraction buffer or TCA solution using mini-scale quantities (2.5) and immunoblotted with anti-pan myosin antibody. M_r of polypeptide bands of different plants were measured on both the same and different blots, and the mean M_r of each polypeptide from at least three experiments was taken.

3.2.2 Comparison of different extraction buffers

Several types of buffers were tested to extract putative myosin from mung bean:

1. High ionic strength: 0.6 M NaCl, 10 mM imidazole, pH 7.0, 1 mM EDTA, 2 mM PMSF, 1 mM TPCK, 40 mM sodium metabisulfite, 1 mM DTT.
2. Pyrophosphate: 50 mM sodium pyrophosphate, 10 mM imidazole, pH 7.0, 1 mM EDTA, 2 mM PMSF, 1 mM TPCK, 40 mM sodium metabisulfite, 1 mM DTT.
3. Sucrose: 0.34 M sucrose, 10 mM imidazole, pH 7.0, 1 mM EDTA, 2 mM PMSF, 1 mM TPCK, 40 mM sodium metabisulfite, 1 mM DTT.

3.2.3 K^+-EDTA-ATPase investigation and $(NH_4)_2SO_4$ fractionation of mung bean crude extract

Mung bean crude extract was fractionated with $(NH_4)_2SO_4$ of 0-30%, 30-50%, 50-70%, 70-90% saturation, and in a subsequent experiment with 0-30%, 30-40%, 40-50%. The desalted crude extract and $(NH_4)_2SO_4$ fractions were assayed for K^+-EDTA-ATPase (2.14.1) and immunoblotted.

3.2.4 Effects of proteases on a putative myosin heavy chain from mung bean

Both mung bean crude extract and the desalted 0-45% $(NH_4)_2SO_4$ fraction of the crude extract were used to test the effects of proteases on putative myosin heavy chain.

(i) Crude extract

Mung bean proteins were extracted separately with the extraction buffer, and with buffer containing 0.34 M sucrose, 10 mM imidazole, pH 7.0, 1 mM DTT (i.e. the extraction buffer without 1 mM EDTA, 2 mM PMSF, 1 mM TPCK, 40 mM sodium metabisulfite). Aliquots of both crude extracts were kept at 0-4°C for 0, 9, 19, 24, 32, 42, 55, 65 and 72 h. All protein samples were examined by immunoblotting.

(ii) 0-45% $(NH_4)_2SO_4$ fraction

The 0-45% fraction of mung bean crude extract was desalted in 0.34 M sucrose, 10 mM imidazole, pH 7.0, 1 mM DTT. The desalted fractions with or without protease inhibitors were kept at 0-4°C for 0, 18, 30, 77 and 138 h, and examined by immunoblotting.

3.2.5 Testing different forms of chromatography for the purification of mung bean putative myosin heavy chain

A 0-45% $(NH_4)_2SO_4$ fraction of mung bean crude extract was used in all pilot experiments with different chromatography media unless otherwise stated. The $(NH_4)_2SO_4$ pellet was desalted in the desired starting buffer for the subsequent column chromatography or batch test. Immunoblotting was used to monitor the chromatography of putative myosin heavy chain. Pilot experiments were as follows.

(i) Ion exchange chromatography

(a) DEAE-cellulose

Four groups of experiments are described below:

1. 88 ml (640 mg of protein) of the desalted 0-45% $(NH_4)_2SO_4$ fraction were applied to a column (2.6 x 6.3 cm) of DE52 equilibrated with starting buffer containing 0.34 M sucrose, 20 mM Tris-HCl, pH 8.0, 1 mM DTT. The column was eluted with 0.2 M, 0.6 M and 1 M NaCl steps in the starting buffer.

2. Three types of buffer systems were tested on DE52 columns with one-step elution. About 4 ml (7.3-20 mg of protein) of the desalted 0-45% fraction were applied to a column (1.6 x 1.6 cm) of DE52. Three starting buffers were:

10 mM NaCl, 20 mM Tris-HCl, pH 8.0, 1 mM DTT.

50 mM sodium pyrophosphate, 20 mM Tris-HCl, pH 8.0, 1 mM DTT.

0.34 M sucrose, 10 mM imidazole, pH 7.0, 1 mM DTT.

Elution was with 1 M NaCl in the starting buffers.

3. 400 g of mung bean were extracted with the extraction buffer. The crude extract was applied to a column (4.9 x 10.9 cm) of DE52 equilibrated with starting buffer (0.34 M sucrose, 10 mM imidazole, pH 7.0, 1 mM DTT), and the column was eluted with 0.1 M, 0.4 M and 0.6 M NaCl steps in the starting buffer (NaCl was replaced by KCl in some experiments).

4. Experiments were the same as described in 1 and 3 except that extraction buffer contained 10 mM NaF and that buffers subsequently used were 0.34 M sucrose, 10 mM imidazole, pH 7.0, 1 mM DTT, 10 mM NaF.

(b) DEAE-Sephacel

3-5 ml of the desalted 0-45% $(NH_4)_2SO_4$ fraction were applied to a column (1.6 x 1.6 cm) of DEAE-Sephacel equilibrated with starting buffer containing 0.34 M sucrose, 20 mM Tris-HCl, pH 8.0, 1 mM DTT. The column was eluted with 1 M and 2 M NaCl steps in the starting buffer. A batch method was used to test the following buffers of different pH values:

0.34 M sucrose, 10 mM imidazole, pH 7.0 /7.5, 1 mM DTT.

0.34 M sucrose, 20 mM Tris-HCl, pH 8.0 /8.5, 1 mM DTT.

Elution was with 1 M NaCl in the starting buffers.

For the batch method, 1.5 ml (gel volume) of DEAE-Sephacel were added into each of four 15 ml Pyrex glass centrifuge tubes. Gel was equilibrated with ten, 6 ml aliquots of the appropriate concentrated starting buffer (0.5 M imidazole /Tris-HCl instead of 10 mM imidazole /20 mM Tris-HCl in the starting buffers), and then with ten, 6 ml aliquots of the starting buffer. Each time, gel was collected by centrifuging at 2,500 rpm for 1 min in a Clements 2000 centrifuge. 2-3 ml (5 mg of protein) of the desalted 0-45% fraction were added to each tube and mixed for 10-15 min. Unbound proteins were removed by centrifugation, and the gel was washed with 1.5 ml of the starting buffer. 1.5 ml of the elution buffer were mixed with the gel for 10-15 min. Supernatants of the gel after incubating with protein sample, with washing buffer and with elution buffer were examined by immunoblotting.

(c) DEAE-Sepharose CL-4B and DEAE-Sephadex A-50

For DEAE-Sepharose CL-4B, batch tests were carried out as described in (b). For DEAE-Sephadex A-50, the same batch test was used except that only the pH 8.0 buffer was tested.

(d) CM-cellulose

4.4 ml (7.2 mg of protein) of the desalted 0-45% $(NH_4)_2SO_4$ fraction were applied to a column (1.6 x 1.4 cm) of CM-cellulose. The starting buffer was 0.34 M sucrose, 20 mM Mes, pH 6.5, 1 mM DTT. Elution buffers were 1 M and 2 M NaCl in the starting buffer.

Starting buffers of 6 different pH values were batch-tested as described in (b):

0.34 M sucrose, 20 mM sodium acetate, pH 5.0 /5.5, 1 mM DTT.

0.34 M sucrose, 20 mM Mes, pH 6.0 /6.5, 1 mM DTT.

0.34 M sucrose, 10 mM imidazole, pH 7.0 /7.5, 1 mM DTT.

Elution buffers were 1 M NaCl in the starting buffers.

(ii) Hydrophobic chromatography

Six different buffer systems were tested on columns [1.6 x (1.5-1.8) cm] of Phenyl-Sepharose CL-4B. The buffers classified into two groups according to starting buffers:

1. Starting buffers:

   1 M /3 M /4 M NaCl /1M $(NH_4)_2SO_4$, 20 mM Tris-HCl, pH 8.0.

   Each column was eluted with steps of 20 mM Tris-HCl, pH 8.0 and 10 mM glycine-NaOH, pH 9.8.

2. Starting buffer:

   4 M NaCl, 10 mM NaH_2PO_4-Na_2HPO_4, pH 6.8.

   The column was eluted with steps of 10 mM NaH_2PO_4-Na_2HPO_4, pH 6.8 and 10 mM glycine-NaOH, pH 9.8.

All 0-45% $(NH_4)_2SO_4$ pellets were desalted with the starting buffers without NaCl or $(NH_4)_2SO_4$, and solid NaCl or $(NH_4)_2SO_4$ was added to the desalted fraction to make the final concentration described in the starting buffer. 4.5-8 ml (11-19 mg of protein) of the desalted samples were applied to the columns.

(iii) Hydroxylapatite

5-9 ml (10-20 mg of protein) of the desalted 0-45% $(NH_4)_2SO_4$ fraction were applied to a column [1.6 x (1.2-1.6) cm] of hydroxylapatite equilibrated with 0.34 M sucrose, 1 mM DTT buffered at pH 8.0 (10 mM /20 mM Tris-HCl) or pH 7.0 (10 mM imidazole). The column was eluted with KH_2PO_4 steps in the range of 0.01 M to 0.5 M in the starting buffer.

(iv) Affinity chromatography

Mung bean crude extract was applied to a DE52 column as described in 3.2.5 (i) (a) 3, and the column was eluted with 0.4 M NaCl in the starting buffer. The 0-45% $(NH_4)_2SO_4$ fraction of the 0.4 M NaCl eluate from DE52 was dialysed against the starting buffer of the appropriate affinity column in order to keep the sample in a small volume. For both novobiocin-Sepharose 6B and ADP-agarose column, gels were regenerated by washing with 3 column volumes of 2 M KCl, 6 M urea solution, then with >10 column volumes of dH_2O, and finally with >4 column volumes of starting buffer.

(a) Novobiocin-Sepharose 6B

3 ml of the desalted 0-45% fraction were applied to a column (1.6 x 2 cm) of novobiocin-Sepharose 6B (2.3). Two starting buffers were tested:

50 mM KCl, 10 mM imidazole, pH 7.0, 1 mM EDTA, 1 mM DTT.

0.34 M sucrose, 10 mM imidazole, pH 7.0, 1 mM EDTA, 1 mM DTT.

Each column was eluted with 1 M KCl and 5 M urea steps in the starting buffer.

(b) ADP-agarose

2.5 ml of the desalted 0-45% fraction were applied to a column (1.6 x 2.1 cm) of ADP-agarose at a flow rate of < 10 ml/h. After sample application, the chromatography was stopped for 2 h before washing and elution to facilitate the equilibrium between ligands of the gel and proteins of the sample. Two buffer systems were tested:

1. Starting buffer: 0.6 M ammonium acetate, 2 mM sodium pyrophosphate, pH 6.5, 5 mM EDTA, 0.25 mM DTT.

   Elution buffer: 0.6 M NH_4Cl, 2 mM sodium pyrophosphate, pH 6.5, 5 mM EDTA, 0.25 mM DTT.

2. Starting buffer: 0.34 M sucrose, 10 mM imidazole, pH 7.0, 1 mM DTT, (± 2 mM EDTA).

   Elution buffers: 1 M KCl and 5 M urea steps in the starting buffer; or 50 mM ATP in the starting buffer.

(c) F-actin Sepharose 4B

Mung bean crude extract was applied to a DE52 column as described in 3.2.5 (i) (a) 3, and the column was eluted with 0.4 M KCl in the starting buffer. 81 ml (77 mg of protein) of the 0.4 M KCl eluate were applied at a flow rate of 30 ml/h to a column (1.6 x 6.3 cm) of glutaraldehyde cross-linked F-actin Sepharose (2.3) equilibrated with 0.5 M KCl, 20 mM Tris-HCl, pH 7.6, 1 mM Mg_2SO_4, 1 mM DTT. The chromatography was stopped for 2 h before washing and elution with steps of:

0.5 M KCl, 20 mM Tris-HCl, pH 7.6, 3 mM ATP, 3 mM Mg_2SO_4, 1 mM DTT.

0.5 M KCl, 20 mM Tris-HCl, pH 7.6, 2 mM sodium pyrophosphate, 1 mM $MgSO_4$, 1 mM DTT.

1.0 M KCl, 20 mM Tris-HCl, pH 7.6, 1 mM $MgSO_4$, 1 mM DTT.

The column was regenerated with the third elution buffer.

(v) Gel filtration

(a) Calibration

Two known M_r proteins, rabbit muscle myosin (470 kDa) and thyroglobulin (669 kDa; Pharmacia) were used to calibrate the gel filtration column, and hemocyanin was used to determine the void volume. Rabbit muscle myosin (2.15) was further purified by chromatography on DE52 (Margossian & Lowey 1982). 5 mg of thyroglobulin and 6 mg of hemocyanin were dissolved in 4.4 ml (6.8 mg of protein) of rabbit muscle myosin solution, and the mixture was clarified at 10,000 rpm for 10 min. The supernatant was applied to a column (2.6 x 46 cm) of Sephacryl S-400 equilibrated with 0.04 M sodium pyrophosphate, pH 7.5, 0.001 mM DTT, at a flow rate of 60 ml/h.

(b) Chromatography of the 165 kDa polypeptide fraction of mung bean

Mung bean (580 g) was extracted with the extraction buffer [2.5.2 (ii)], the crude extract (812 ml, 1835 mg of protein) fractionated with 0-45% $(NH)_2SO_4$, the 0-45% fraction desalted (176 ml, 725 mg of protein) and applied to a DE52 column (2.6 x 17.5 cm) equilibrated with starting buffer (0.34 M sucrose, 10 mM imidazole, pH 7.0, 1 mM DTT). The flow-through fraction (131 ml, 148 mg of protein) was fractionated with 0-45% $(NH_4)_2SO_4$, desalted (53 ml, 90.6 mg of protein), applied at a flow rate of 13 ml/h to a ADP-agarose column (2.6 x 3.3 cm) equilibrated with the starting buffer, and the column left overnight. Proteins were eluted with 100 ml of a 0-0.8 M KCl gradient in the starting buffer, and the peak fractions (24 ml) pooled and concentrated to 5 ml by Aquacide II. 5 ml (1.1 mg of protein) of the partially purified 165 kDa polypeptide fraction were applied to the

previously calibrated Sephacryl S-400 column and eluted as before. Eluate fractions were monitored by 7% SDS-PAGE and gels were stained with silver.

3.3 RESULTS

3.3.1 Immunochemical identification of putative myosin heavy chains in higher plants

Monoclonal anti-pan myosin antibody against the myosin heavy chain of mouse 3T3 cells cross-reacts immunochemically and immunocytochemically with putative myosin heavy chains from *Allium* (200 kDa; Parke *et al.* 1986), *Nicotiana* (175 kDa; Tang *et al.* 1989a) and *Chara* (200 and 110 kDa; Grolig *et al.* 1988). It was used to identify putative myosin heavy chains in crude extracts of mung bean, wheat and pea by immunoblotting. Polypeptides of 165 kDa and 155 kDa were detected in mung bean (Fig. 3.1, lanes 2, 4 and 5), of 165 kDa in wheat (Fig. 3.1, lane 6), and of 160 kDa in pea (Fig. 3.1, lane 7). Protein extracts from either shoots or roots showed the same bands as those from seedlings (data not shown), irrespective of whether the extracts were made with extraction buffer or TCA.

For mung bean, three bands other than the 165 kDa and 155 kDa ones were also detected on blots using the streptavidin-biotinylated peroxidase complex (Fig. 3.1, lane 2). They were not myosin heavy chain bands because they also stained in the control incubated without anti-pan myosin (Fig. 3.1, lane 1). The heavy chain of rabbit skeletal muscle myosin was clearly detected by anti-pan myosin antibody (Fig. 3.1, lane 8). Generally, both the 165 kDa and 155 kDa polypeptides of mung bean could be detected by either alkaline phosphatase or peroxidase, and quite often, the 165 kDa band was stronger than the 155 kDa one on blots. Sometimes, only the 165 kDa band was detected. This varied with different experiments.

The 155 kDa polypeptide of mung bean did not seem to be a proteolytic product of the 165 kDa one. Even when proteins were extracted from mung bean directly with TCA, which minimizes proteolysis (Wu & Wang 1984), the 155 kDa and 165 kDa bands still coexisted (Fig. 3.1, lane 4). Since the 165 kDa polypeptide was the one most constantly detected by immunoblotting, together with other evidence which will be described later, it was considered to be a putative myosin heavy chain of mung bean and subsequent experiments were focused on this polypeptide.

3.3.2 Extraction of putative mung bean myosin with different buffers

The high ionic strength, pyrophosphate and sucrose buffers used to extract various nonmuscle myosins were tested for their ability to extract putative myosins from mung bean. When aliquots of crude extracts containing the same quantities of protein were resolved by SDS-PAGE and immunoblotted, the 165 kDa band in extracts made with sucrose buffer seemed to be the strongest when 20 μg of protein were loaded (Fig. 3.2, lane 3; compare lanes 1 and 5). Because the low ionic strength of the sucrose buffer facilitates ion exchange chromatography, it was chosen to extract myosin from mung bean as it was for work on *Nitella* (Kato & Tonomura 1977), *Egeria densa* (Ohsuka & Inoue 1979) and *Pisum* (Ma & Yen 1989).

3.3.3 K^+-EDTA-ATPase investigation and $(NH_4)_2SO_4$ fractionation of mung bean crude extract

Mung bean crude extracts catalysed ATP-dependent production of Pi in the presence of K^+-EDTA. The rate of reaction was constant for 25 min then decreased (Fig. 3.3), and K^+-EDTA-ATPase activity was proportional to protein concentration up to 140 μg /ml of reaction mixture (Fig. 3.4). The mean rate of K^+-EDTA-ATPase activity at pH 7.0 was 0.116 ± 0.023 μmol/min/mg (10 determinations).

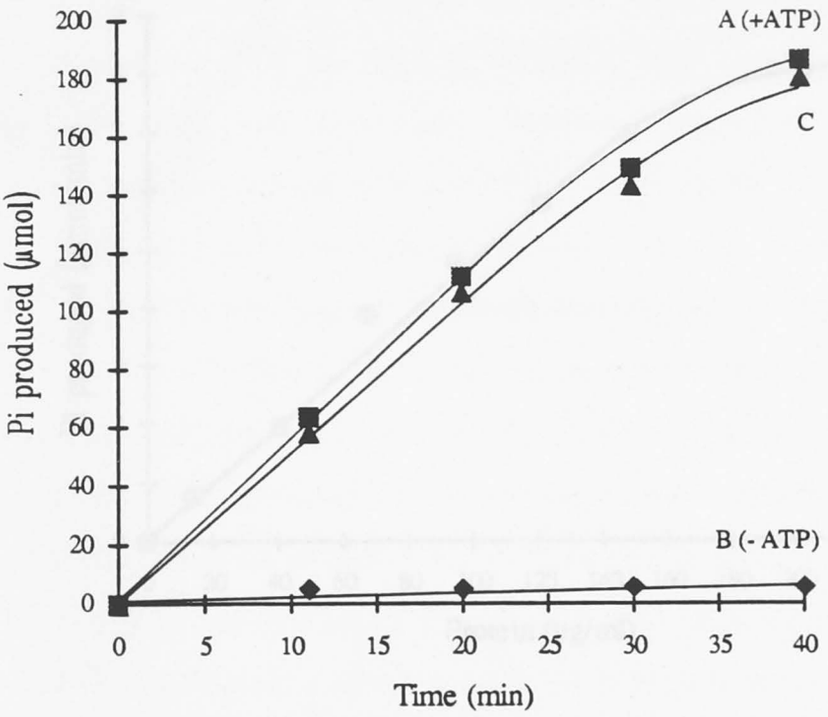


Fig. 3.3. Time course of Pi production catalysed by mung bean crude extract. Extract-dependent Pi production (C) was calculated from the expression A-B when A was with ATP and B without. Reactions A and B contained 96.25 µg protein/ml of reaction mixture.

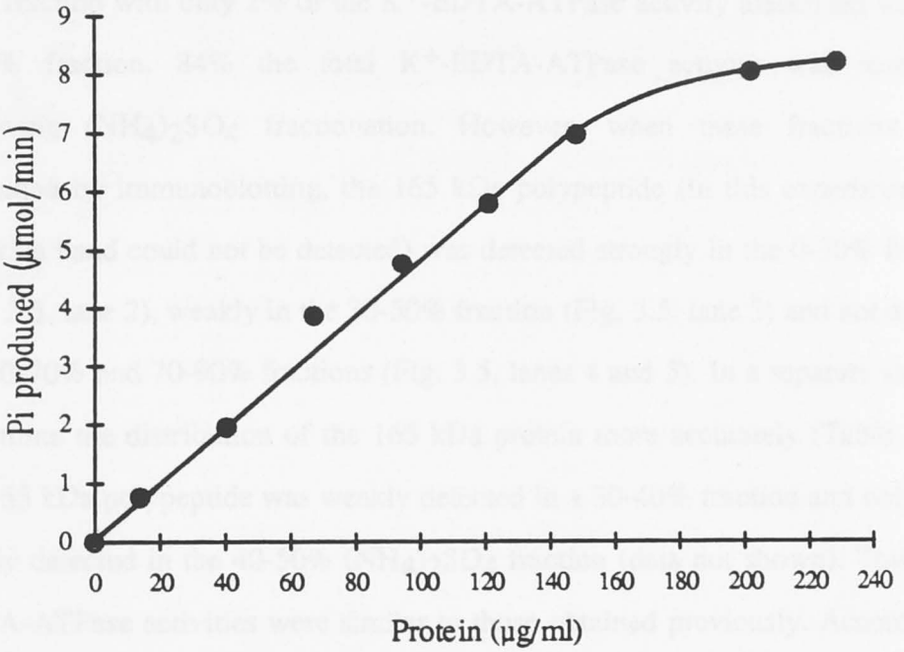


Fig. 3.4 The effect of protein concentration on K^+-EDTA-ATPase activity associated with mung bean crude extract. Reaction mixtures were as described for K^+-EDTA-ATPase assays with the concentration of protein in the reaction mixture as specified.

During fractionation of mung bean crude extract, K^+-EDTA-ATPase activity was usually higher than Ca^{2+}-ATPase, and Mg^{2+}-ATPase activity was the lowest (4.3.2). Therefore, K^+-EDTA-ATPase activity was mainly assayed during the fractionation. When the crude extract was fractionated with $(NH_4)_2SO_4$ (Table 3.1A), 48.7% of the total K^+-EDTA-ATPase activity was associated with the 50-70% fraction with only 2% of the K^+-EDTA-ATPase activity associated with the 0-30% fraction. 84% the total K^+-EDTA-ATPase activity was recovered following $(NH_4)_2SO_4$ fractionation. However, when these fractions were examined by immunoblotting, the 165 kDa polypeptide (in this experiment, the 155 kDa band could not be detected) was detected strongly in the 0-30% fraction (Fig. 3.5, lane 2), weakly in the 30-50% fraction (Fig. 3.5, lane 3) and not at all in the 50-70% and 70-90% fractions (Fig. 3.5, lanes 4 and 5). In a separate study to determine the distribution of the 165 kDa protein more accurately (Table 3.1B), the 165 kDa polypeptide was weakly detected in a 30-40% fraction and only very faintly detected in the 40-50% $(NH_4)_2SO_4$ fraction (data not shown). Total K^+-EDTA-ATPase activities were similar to those obtained previously. Accordingly, 0-45% $(NH_4)_2SO_4$ was used to fractionate the 165 kDa polypeptide in subsequent studies (Fig. 3.5, lanes 6 and 7; the 155 kDa band was detected in this experiment).

Table 3.1 $(NH_4)_2SO_4$ fractionation of mung bean crude extract K^+-EDTA-ATPase activity.

A.

$(NH_4)_2SO_4$ fraction	Total protein mg	K^+-EDTA-ATPase activity		% of total crude extract activity
		Specific µmol/min/mg	Total µmol/min/mg	
Crude	122.6	0.057	7.0	100
0-30%	12.4	0.011	0.14	2.0
30-50%	35.5	0.05	1.78	25.4
50-70%	13.1	0.26	3.41	48.7
70-90%	4.2	0.135	0.57	8.1

B.

$(NH_4)_2SO_4$ fraction	Total protein mg	K^+-EDTA-ATPase activity	
		Specific µmol/min/mg	Total µmol/min/mg
Crude	110.0	0.107	11.8
0-30%	7.6	0.024	0.18
30-40%	28.0	0.018	0.5
40-50%	21.8	0.069	1.5

3.3.4 Effects of proteases on mung bean 165 kDa polypeptide

Several experiments were carried out to test how stable the 165 kDa polypeptide was during extraction and fractionation. The 165 kDa band was clearly detected in mung bean crude extracts while the 155 kDa band was weakly detected in this experiment (Fig. 3.6). The 165 kDa band of the crude extract made with extraction buffer (Fig. 3.6A) was detected longer than that of the crude extract made with a similar buffer lacking protease inhibitors (Fig. 3.6B). A 110 kDa band that reacted with the anti-pan myosin antibody, was detected after 32 h in the crude extract with protease inhibitors (Fig. 3.6A, lane 5) and after 9 h in the crude extract without inhibitors (Fig. 3.6B, lane 2), and became stronger as the 165 kDa band became weaker and finally disappeared. Sometimes, the 110 kDa band was present in crude extracts freshly made with extraction buffer, especially during large-scale extraction, but its detection was not constant. When proteins were directly extracted with TCA, the 110 kDa band was never detected by immunoblotting (Fig. 3.1, lane 4). It is therefore suggested that the 110 kDa polypeptide is a proteolytic product of the 165 or 155 kDa polypeptides.

The 165 kDa polypeptide was more stable in a 0-45% $(NH_4)_2SO_4$ fraction than in the crude extract: the strength of the 165 kDa band was constant for up to 77 h (Fig. 3.7, lanes 1-4), but the band was only faintly detected after 138 h (Fig. 3.7, lane 5). Protease inhibitors had little effect on this time course (data not shown). It was therefore concluded that it was important to include PMSF, EDTA and TPCK in the extraction buffer and to apply the crude extract to the next step of purification immediately since the 165 kDa polypeptide was more liable to proteolysis in crude extracts than in partially purified fractions.

3.3.5 Tests of chromatographic properties of mung bean 165 kDa polypeptide

Various chromatography media were examined to build up a picture of the chromatographic properties of the 165 kDa polypeptide in order to devise a procedure for its purification.

(i) Ion exchange chromatography

DEAE-cellulose has been used to purify myosin from *Physarum* (Adelman & Taylor 1969), *Acanthamoeba* (Pollard & Korn 1973a, b; Maruta & Korn 1977a; Pollard *et al.* 1978; Maruta *et al.* 1979; Lynch *et al.* 1989), *Dictyostelium* (Cote *et al.* 1985), *Drosophila* (Kiehart & Feghali 1986) and Ehrlich ascites tumour cells (Kuznicki & Filipek 1988), and to fractionate putative myosin from pea (Ma & Yen 1989). DEAE-Sephadex has been used to purify myosin from *Physarum* (Adelman & Taylor 1969) and from *Egeria densa* (Ohsuka & Inoue 1979). Cation-exchange chromatography has been used in the purification of onion putative myosin (Pesacreta *et al.* 1991). So, several ion exchanges were tested first to isolate the 165 kDa polypeptide from mung bean.

When the desalted 0-45% $(NH_4)_2SO_4$ fraction of mung bean crude extract was applied to a DE52 column equilibrated with 0.34 M sucrose, 20 mM Tris-HCl, pH 8.0, 1 mM DTT, large quantities of protein bound to the column although the bulk flowed through. Most bound proteins were eluted by 0.2 M and 0.6 M NaCl, and little further was eluted further by 1 M NaCl. The 165 and 155 kDa polypeptides mainly existed in the flow-through fractions and were at most very weakly detected in the 0.2 M NaCl or 0.6 M NaCl fractions (data not shown). When testing NaCl, sodium pyrophosphate and sucrose buffers, the 165 kDa polypeptide of the 0-45% fraction did not bind to DE52 even though large amounts of protein bound and were eluted with 1 M NaCl (data not shown). Similarly, the 165 kDa polypeptide of the 0-45% $(NH_4)_2SO_4$ fraction did not bind to other anion exchange media (DEAE-Sephacel, DEAE-Sepharose CL-4B and DEAE-Sephadex A-50) in column and/or batch format using several buffers.

After testing these various anion exchangers, the cation exchanger CM-cellulose was tested. Less protein bound to CM52 than to DE52, and was eluted by 1 M NaCl. The 165 kDa polypeptide of the 0-45% $(NH_4)_2SO_4$ fraction again flowed through the column. If the desalted 0-45% fraction was applied to a CM52 column first, the flow-through fraction adjusted to pH 7.0 and applied to a column of DE52 equilibrated with 0.34 M sucrose, 20 mM Tris-HCl, pH 8.0, 1 mM DTT, the 165 kDa polypeptide flowed through both columns (data not shown). When buffers of pH ranging from 5 to 7.5 were used in batch tests of CM52, the 165 kDa polypeptide was always found in unbound fractions.

From the results discussed above, the 165 kDa polypeptide in the 0-45% fraction of crude extracts was not able to bind to a diverse range of ion exchangers. Mung bean crude extract was therefore applied directly to DE52. Most proteins bound to the column. From immunoblotting, all of the 165 kDa polypeptide, together with the 155 kDa polypeptide, bound to the column, and could be eluted by 0.4 M NaCl (Fig. 3.8, lane 3). When the 0.4 M NaCl eluate was fractionated with $(NH_4)_2SO_4$ (0-45% saturation), desalted and reapplied to a DE52 column, all the 165 kDa polypeptide (together with the 155 kDa one) again bound and could be subsequently eluted with 0.4 M NaCl (data not shown, see 4.3.1).

The chromatographic behaviour of the 165 kDa polypeptide (together with the 155 kDa one) on DE52 was the same when the extraction buffer contained NaF (data not shown) or not.

(ii) Hydrophobic chromatography

Hydrophobic chromatography relies on the hydrophobic interaction between aliphatic chains on the chromatography medium and hydrophobic regions on the surface of the proteins (Scopes 1987). Hydrophobic interactions strengthen with increasing salt concentration. Proteins that are strongly adsorbed to hydrophobic media even at low salt concentration are generally those with a low water solubility, including those (like the mung bean 165 kDa polypeptide) that precipitate in the low range of $(NH_4)_2SO_4$ saturation (20-40%).

Fragments and subunits (HMM, native S-1, denatured S-1 and light chains) of rabbit skeletal muscle myosin bind to a column of phenyl Sepharose CL-4B and elute with different conditions (Borejdo *et al.* 1984). It is suggested that phenyl Sepharose CL-4B may be useful to obtain purified S-1 with a high ATPase activity. So far, hydrophobic chromatography has not been used in the purification of nonmuscle myosins. Since mung bean 165 kDa polypeptide was precipitated by $(NH_4)_2SO_4$ of 0-45% saturation, the 0-45% fraction was tested on phenyl Sepharose CL-4B.

When the 0-45% fraction was applied to a column of phenyl Sepharose CL-4B equilibrated with the starting buffer 3 M NaCl, 10 mM Tris-HCl, pH 8.0, a small amount of protein bound to the column and was mainly eluted by 20 mM Tris-HCl, pH 8.0 (Fig. 3.9). Most 165 kDa polypeptide flowed through the column (data not shown). All of the 165 kDa polypeptide flowed through the column when using other buffer systems.

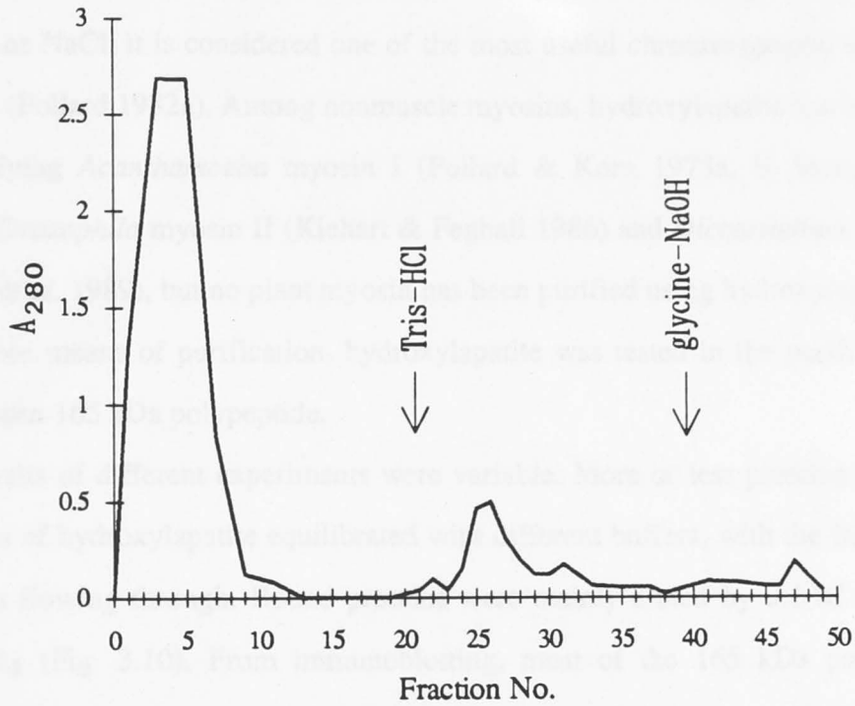


Fig. 3.9 Phenyl-Sepharose CL-4B chromatography of the 0-45% $(NH_4)_2SO_4$ fraction of mung bean crude extract. 4.5 ml (11 mg of protein) of the sample were applied to a column (1.6 x 1.5 cm) of phenyl-Sepharose CL-4B equilibrated with the starting buffer (3 M NaCl, 20 mM Tris-HCl, pH 8.0). The column was eluted with steps of 20 mM Tris-HCl, pH 8.0 (fractions 21-39) and 10 mM glycine-NaOH, pH 9.8 (fractions 40-49). Fraction volume was 1.3 ml.

(iii) Hydroxylapatite

Hydroxylapatite is an insoluble, crystalline form of calcium phosphate. Unlike ion exchangers or affinity adsorbents, such inorganic materials do not have a readily explainable mode of action (Scopes 1987). Adsorbed at low K-phosphate concentrations, all protein can be eluted by increasing phosphate concentrations, with basic proteins tending to require stronger buffers than acidic ones. KCl, NaCl, $CaCl_2$ and $MgCl_2$ do not elute acidic proteins, whereas basic proteins are eluted by

KCl and NaCl, and even more readily by $CaCl_2$ and $MgCl_2$ (Bernardi *et al.* 1972; Gorbunoff 1985).

Since most myosins tested bind to hydroxylapatite even in high concentrations of KCl or NaCl, it is considered one of the most useful chromatography media for myosin (Pollard 1982a). Among nonmuscle myosins, hydroxylapatite has been used in purifying *Acanthamoeba* myosin I (Pollard & Korn 1973a, b; Maruta *et al.* 1979), *Drosophila* myosin II (Kiehart & Feghali 1986) and *Dictyostelium* myosin I (Fukui *et al.* 1989), but no plant myosin has been purified using hydroxylapatite. As a possible means of purification, hydroxylapatite was tested in the purification of mung bean 165 kDa polypeptide.

Results of different experiments were variable. More or less proteins bound to columns of hydroxylapatite equilibrated with different buffers, with the bulk of the proteins flowing through. Bound proteins were mainly eluted by 0.1 M or 0.2 M KH_2PO_4 (Fig. 3.10). From immunoblotting, most of the 165 kDa polypeptide flowed through columns whatever the starting buffer. Sometimes, a little 165 kDa polypeptide could be detected in fractions eluted with 0.06 M, 0.1 M or 0.2 M KH_2PO_4 (data not shown).

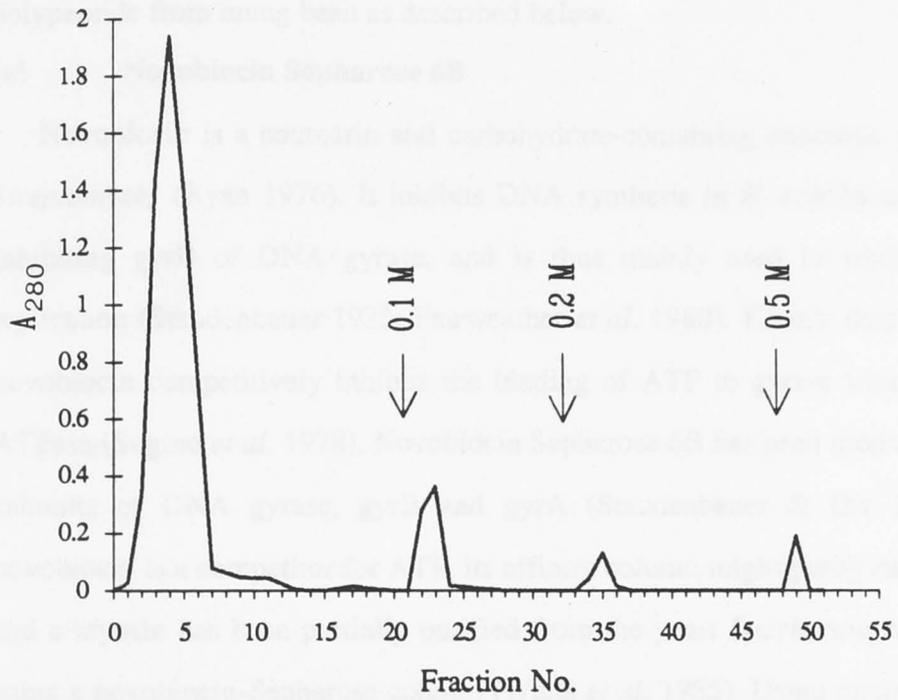


Fig. 3.10 Hydroxylapatite chromatography of the 0-45% $(NH_4)_2SO_4$ fraction of mung bean crude extract. 9 ml (18 mg of protein) of the sample were applied to a column (1.6 x 1.6 cm) of hydroxylapatite equilibrated with the starting buffer (0.34 M sucrose, 20 mM Tris-HCl, pH 8.0, 1 mM DTT). The column was eluted with steps of 0.1 M (fractions 21-32), 0.2 M (fractions 33-47), and 0.5 M (fractions 48-51) KH_2PO_4 in the starting buffer. Fraction volume was 1.3 ml.

(iv) Affinity chromatography

Compared with other forms of chromatography, affinity chromatography is a more recently introduced method for the purification of myosin. Affinity chromatography can separate myosin isoforms by different elution conditions, and separate active from inactive myosin (Pollard 1982a). Its specificity and rapidity make affinity chromatography an effective method to purify myosin, and affinity

chromatography is increasingly used to purify nonmuscle myosins when other chromatography fails. However, no plant myosin has been purified using affinity chromatography. Three affinity columns were tested to isolate the 165 kDa polypeptide from mung bean as described below.

(a) **Novobiocin Sepharose 6B**

Novobiocin is a coumarin and carbohydrate-containing antibiotic produced by *Streptomyces* (Ryan 1976). It inhibits DNA synthesis in *E. coli* by competitively inhibiting gyrB of DNA gyrase, and is thus mainly used in studies of DNA replication (Staudenbauer 1975; Fairweather *et al.* 1980). Kinetic data suggest that novobiocin competitively inhibits the binding of ATP to gyrase which is also an ATPase (Sugino *et al.* 1978). Novobiocin Sepharose 6B has been used to purify two subunits of DNA gyrase, gyrB and gyrA (Staudenbauer & Orr 1981). Since novobiocin is a competitor for ATP, its affinity column might purify other ATPases and a myosin has been partially purified from the yeast *Saccharomyces cerevisiae* using a novobiocin-Sepharose column (Watts *et al.* 1985). Using mung bean, when the desalted 0-45% $(NH_4)_2SO_4$ fraction of the 0.4 M NaCl eluate from DE52 was applied to the column of novobiocin Sepharose 6B, the bulk of the 165 kDa polypeptide flowed through although some of the 165 kDa polypeptide was bound when proteins were eluted from the column with 1 M KCl (data not shown).

(b) **ADP-agarose**

During force production, myosin interacts reversibly with both actin and adenine nucleotides, which makes ADP/ATP affinity columns a useful method to separate myosin from other cellular proteins (Trayer & Trayer 1975). Since ATP immobilized to gel can be hydrolyzed by myosin during chromatography and thus change the property of the column, ADP columns are mainly used (Pollard 1982a). ADP columns have been used to separate isoforms of muscle myosin and its proteolytic fragments (HMM, S-1). For nonmuscle myosins, an ADP column has been used to purify myosin II from pig platelet (Trayer & Trayer 1975), myosin I

(Maruta *et al.* 1979; Lynch *et al.* 1989) and myosin II (Maruta & Korn 1977a) from *Acanthamoeba,* and myosin I from *Dictyostelium* (Cote *et al.* 1985).

Using mung bean when the 0-45% $(NH_4)_2SO_4$ fraction of the 0.4 M NaCl eluate from DE52 was applied to the column of ADP-agarose, all the 165 kDa polypeptide bound to the column irrespective of whether the column was equilibrated in sucrose-imidazole (containing EDTA or not) or ammonium acetate buffer (Fig. 3.11 A, lane 3). In contrast, all of the detectable 155 kDa and 110 kDa polypeptides were present in the flow-through fraction (Fig. 3.11 A, lane 2). Bound 165 kDa polypeptide could be eluted by 1 M KCl or 50 mM ATP using sucrose-imidazole buffer, or by 0.6 M NH_4Cl using ammonium acetate buffer. The proteins from these fractions, resolved on SDS-PAGE, were similar (data not shown). Centrifuging the resuspended and dialyzed 0-45% pellet against the ammonium acetate buffer rather than the sucrose-imidazole buffer resulted in a small pellet. The 165 kDa polypeptide was detected by immunoblotting in the pellet (data not shown) perhaps because of its low solubility in the 0.6 M ammonium buffer of pH 6.5. Therefore, sucrose buffer was routinely used in ADP-agarose chromatography. ADP-agarose columns were discarded following three cycles of regeneration since their binding capacity decreased substantially by regeneration in 2 M KCl and 6 M urea (data not shown).

(c) F-actin Sepharose 4B

Actin affinity chromatography has been used to purify muscle myosin and its proteolytic fragments, HMM and S-1 (Pollard 1982a). Nonmuscle myosin has not been reported to be purified by such affinity chromatography. Basically, there are two methods to couple actin to agarose gel. In one method, G-actin (globular actin) (Bottomley & Trayer 1975), F-actin or F-actin plus tropomyosin, both stabilized by glutaraldehyde crosslinking (Winstanley *et al.* 1977), or F-actin stabilized by phalloidin (Winstanley *et al.* 1979) can be directly coupled to CNBr-activated agarose gel. Alternatively, G-actin incubated with the gel in the presence of phalloidin polymerizes into stabilized F-actin that is trapped within the agarose

beads (Grandmont-Leblanc & Gruda 1977). The latter type of affinity column binds muscle myosin in high salt (0.5 M KCl) but it has low capacity.

A glutaraldehyde-crosslinked F-actin column was examined to isolate mung bean 165 kDa polypeptide. When the 0.4 M KCl eluate from DE52 was applied to the F-actin column, very few proteins bound to the column and the 165 kDa polypeptide flowed through (data not shown). The efficiency of the column was later examined by applying 5 mg (2 ml) of purified rabbit skeletal muscle myosin (2.15) to the column. Substantial amount of muscle myosin bound to the column, and could largely be eluted by ATP buffer (data not shown).

(v) Gel filtration

Gel filtration has been successfully and widely used in the purification of nonmuscle myosins since few other cellular components have the large Stokes radius of myosin II (Pollard 1982a). Bio-Gel A-15 m (200-400) has been used to purify myosin from *Acanthamoeba* (Maruta & Korn 1977a; Pollard *et al.* 1978; Maruta *et al.* 1979; Collin & Korn 1980), *Dictyostelium* (Clark & Spudich 1974), human platelet (Pollard *et al.* 1974), and *Drosophila* (Kiehart & Feghali 1986). Another widely used form of agarose beads is Sepharose 4B. It has been used to purify myosin from Ehrlich ascites tumour cells (Kuznicki & Filipek 1988), and the higher plants *Egeria densa* (Ohsuka & Inoue 1979) and *Heracleum sosnowskyi* (Turkina *et al* 1987). Sepharose CL-4B has been used to purify myosin from tomato (Vahey *et al.* 1982).

Elution profiles of the partially purified 165 kDa polypeptide fraction and the standard M_r proteins from Sephacryl S-400 are shown in Fig. 3.12. The void volume was 91.1 ml determined by hemocyanin. Elution volumes of rabbit skeletal muscle myosin, thyroglobulin and the partially purified mung bean 165 kDa polypeptide were: 104.6 ml, 144.6 ml and 92.1 ml, respectively. The 165 kDa polypeptide was eluted in a protein peak with some other plant proteins at the very beginning of the elution profile (Fig. 3.12, lanes 4 and 5), just after the void volume.

3.4 DISCUSSION

3.4.1 Immunoblotting

Immunocytochemical and biochemical studies show that anti-pan myosin antibody recognizes an epitope present on the heavy chain of muscle myosin from skeletal, smooth, cardiac muscle, and various non-muscle myosins [Parke *et al.* 1986; Anonymous (Anon.) 1987]. Putative myosin heavy chains have been identified immunochemically and immunocytochemically with anti-pan myosin antibody in onion (Parke *et al.* 1986), *Chara* (Grolig *et al.* 1988) and *Nicotiana* pollen tubes (Tang *et al.* 1989a). A 100 kDa polypeptide has also been identified by immunoblotting with anti-pan myosin in a partially purified fraction of onion exhibiting ATPase activities consistent with a myosin (Pesacreta *et al.* 1991).

In mung bean extracts made with either extraction buffer or TCA, two polypeptides (165 and 155 kDa) were immunochemically detected with anti-pan myosin. There was no tissue-specific distribution of the two polypeptides between roots and shoots. The 165 kDa polypeptide was constantly detected. However, the detection of the 155 kDa polypeptide in different mung bean extracts was variable which might, in part, be due to the efficiency of electrophoretic transfer of this polypeptide. In wheat and pea, only one polypeptide of 165/160 kDa was constantly detected with anti-pan myosin. Therefore, the 165/160 kDa polypeptides of mung bean, wheat and pea were considered as putative myosin heavy chains.

An additional mung bean polypeptide of 110 kDa was detected in the absence of proteolytic inhibitors but not when extracts were extracted with TCA, conditions which inhibit proteolytic degradation of plant proteins (Wu & Wang 1984). These properties suggested that the 110 kDa polypeptide was a proteolytic fragment of either the 165 kDa or 155 kDa polypeptide.

3.4.2 ATPase activity

The most fundamental criterion for identifying a putative myosin is its ability to interact physically and enzymatically with actin (Clark & Spudich 1974). That is,

myosin is able to physically interact with, and has its ATPase activity modified by, F-actin. In most cases, myosins *in vitro* under conditions of high ionic strength (K^+) express high levels of K^+-EDTA and Ca^{2+}-ATPase activities but very low levels of Mg^{2+}-ATPase activity. Only the Mg^{2+}-ATPase activity is expressed under physiological conditions and is activated by F-actin. The high ionic strength prevents any actin in crude protein fractions from activating the Mg^{2+}-ATPase activity. In contrast to myosin, most other ATPases are more active in Mg^{2+} than Ca^{2+} and are inactive in EDTA (Pollard 1982b). Therefore, assay of K^+-EDTA-ATPase activity (or Ca^{2+}-ATPase activity in some cases) has usually been used to monitor myosin-like activities in isolating myosins or putative myosins from nonmuscle cells including plants (Kato & Tonomura 1977; Ohsuka & Inoue 1979; Vahey *et al.* 1982; Ma & Yen 1988, 1989; Pesacreta *et al.* 1991). Most plant myosin ATPases are also more active in K^+-EDTA than Ca^{2+} at high ionic strength (1.8.1).

When mung bean crude extracts were fractionated with $(NH_4)_2SO_4$, the bulk of the K^+-EDTA-ATPase activity did not fractionate with the 165 kDa polypeptide determined by immunoblotting, suggesting that the 165 kDa polypeptide contained at most a very small fraction of the total K^+-EDTA-ATPase activity of the crude extract. There are several possible explanations. First, myosin isoforms are common in muscle and nonmuscle cells (Emerson & Bernstein 1987; Wade & Kedes 1989; Cheney & Mooseker 1992) and may also exist in plant cells. Accordingly, two or more isoforms of myosin may be present in mung bean but without being identified by immunoblotting with the anti-pan myosin. Unidentified myosin isoform(s) may account for some or all of the K^+-EDTA-ATPase activity in fractions not containing the 165 kDa polypeptide.

Alternatively, the bulk of the K^+-EDTA-ATPase activity unassociated with the 165 kDa polypeptide may also be contributed partially or wholly by non-myosin ATPase(s) in mung bean extract. Similar results have been obtained by Vahey *et al.* (1982) in which three K^+-EDTA-ATPase peaks were eluted from Sepharose CL-

4B. Two of the peaks with lower specific activity are not tomato myosin because they do not have actin-activated Mg^{2+}-ATPase activity and do not bind to rabbit F-actin in the absence of Mg^{2+}ATP. Similarly, when fractionating pea tendril crude extract on DEAE-cellulose monitored by Ca^{2+}-ATPase activity, several ATPase peaks appear (Ma & Yen 1989). Only a very small ATPase peak fraction is considered containing putative myosin; however, Ma & Yen (1989) do not state how exactly the putative myosin fraction is determined. So, K^+-EDTA- or Ca^{2+}-ATPase activities other than those of myosin do exist in some plants. Accordingly, plant proteins catalysing K^+-EDTA- /Ca^{2+}-ATPase activity cannot be designated myosins without further evidence. Actin-activated ATPase activity and even F-actin binding assays are required in addition to the K^+-EDTA- or Ca^{2+}-ATPase activity to identify plant myosin.

Likewise during purification of *Acanthamoeba* myosin II, a peak of Ca^{2+}-ATPase activity is eluted together with vesicular material in the void volume ahead of the Ca^{2+}-ATPase peak of myosin II on Bio-Gel A-15 m (Maruta & Korn 1977a). Since the Mg^{2+}-ATPase activity of this void fraction is not activated by F-actin and the enzyme does not bind to a F-actin-DNase-agarose affinity column, this fraction is not related to myosin and may be a membrane-associate ATPase. So, multiple fractions of K^+-EDTA- or Ca^{2+}-ATPase activity are possibly present when purifying some nonmuscle myosins, and again other assays are needed to identify the myosin fraction when encountering such problems.

Therefore in mung bean crude extract, the K^+-EDTA-ATPase activity in $(NH_4)_2SO_4$ fractions other than that of 165 kDa polypeptide may be contributed by other myosin isoform(s) or by other non-myosin ATPase(s), or by some combination of these enzymes. K^+-EDTA-ATPase activity alone is therefore not a specific and reliable indicator of the 165 kDa putative myosin heavy chain in mung bean extracts and cannot be used to detect the 165 kDa polypeptide during its purification. Accordingly, immunoblots, which provide greater reliability and

specificity, were used to monitor the 165 kDa polypeptide during the development of a protocol for its partial isolation from mung bean extract.

3.4.3 ADP-agarose chromatography

ADP and ATP affinity chromatography has been used to isolate some ATP-utilizing enzymes such as kinases and ATPases including myosin (Trayer 1974; Trayer *et al.* 1974; Trayer & Trayer 1975; Scouten 1981). Three types of ADP-agarose are mainly used for isolating myosins and their subfragments: ADP is linked to agarose via a spacer by N^6 or C8 of the adenine, or by the ribose (Pollard 1982b), the third one being used in the present study of mung bean 165 kDa polypeptide. Myosin binds to ADP columns in the presence of Mg^{2+}, Ca^{2+}, or EDTA, representing three conditions under which its ATPase activity can be expressed *in vitro* (Trayer 1974; Trayer & Trayer 1975). Bound myosin can be eluted by salt (KCl, NH_4Cl), ATP, ADP or pyrophosphate.

When purifying rabbit muscle myosin from a crude extract by ADP affinity chromatography, EDTA (5 mM) is included in the buffer since most other ATP/ADP-utilizing enzymes require a divalent cation to express activity (Trayer & Trayer 1975). Using the same buffer, pig platelet myosin is purified from a crude extract on an ADP column (Trayer & Trayer 1975). Similar results are obtained by replacing EDTA with Mg^{2+} (5 mM). When purifying kinases on ADP columns, no EDTA is added in buffers (Trayer *et al.* 1974; Trayer & Trayer 1974). ADP columns are also used to purify *Acanthamoeba* myosins I (Maruta *et al.* 1979; Lynch *et al.* 1989) and II (Maruta & Korn 1977a) with EDTA (1 or 2 mM) in elution buffers. Nevertheless, *Dictyostelium* myosin I has been purified on an ADP column using buffers that do not contain either EDTA or Mg^{2+} (Cote *et al.* 1985).

Another type of ADP-agarose in which ADP is linked to agarose from its phosphate via a spacer is used for the affinity chromatography of nicotinamide nucleotide-dependent dehydrogenases such as lactate dehydrogenase (Trayer *et al.* 1974; Trayer 1974). The dehydrogenase is eluted by low concentrations of a

specific displacing ion, NAD^+, but not by KCl. Lactate dehydrogenase also binds to N^6-ADP-Sepharose and can be eluted by AMP but not by ATP (Trayer & Trayer 1974). However, AMP-agarose is mainly used in isolating these dehydrogenases (Scouten 1981). ATP-analogue-agarose affinity chromatography has been used in purifying Na^+, K^+-ATPase from bovine brain tissue (Anderton *et al.* 1974).

Chromatography of mung bean protein fraction on ADP-agarose columns separated the 165 kDa polypeptide from the 155 kDa and 110 kDa polypeptides recognized by anti-pan myosin. The 165 kDa polypeptide bound to the column while the other two polypeptides were obtained in the unbound fraction, demonstrating that only the 165 kDa polypeptide could be an ADP/ATP-utilizing enzyme. The binding and elution of the 165 kDa polypeptide in the presence of EDTA was consistent with a K^+-EDTA-ATPase activity rather than a kinase, most of which require Mg^{2+} and/or Ca^{2+} for activity (Trayer 1974; Trayer *et al.* 1974; Polya & Davies 1983; Polya *et al.* 1983; Davies & Polya 1983).

The 155 kDa polypeptide contained the sequence of amino acids which is recognized by anti-pan myosin antibody. The 110 kDa polypeptide that was thought to be a proteolytic fragment of the 165 or 155 kDa polypeptide did not bind to the ADP-agarose column indicating that it did not contain the required parts of the S-1 of the myosin heavy chain. The epitope of anti-pan myosin lies on the tail of muscle myosin heavy chain (Tang *et al.* 1989a). The 110 kDa polypeptide could be the tail part of the 165 kDa polypeptide since this would not bind ADP. However, the 110 kDa polypeptide could be a proteolytic fragment of the 155 kDa polypeptide that itself does not bind ADP-agarose.

3.4.4 Other types of chromatography

Gel filtration can differentiate myosin II and I due to their substantially different M_r. The M_r of muscle and nonmuscle myosin II is about 400-550 kDa (Taylor & Condeelis 1979; Fukui *et al.* 1989) while that of the single-headed, globular myosin I has remarkably lower M_r than myosin II. Three isoforms of

Acanthamoeba myosin I have the native M_r of about 180 kDa, or 162 kDa (IC) (Maruta *et al.* 1979; Lynch *et al.* 1989).

In gel filtration, muscle myosin eluted ahead of thyroglobulin even though its M_r (470 kDa) is less than that of thyroglobulin (669 kDa). This is because myosin II is a two-headed, highly asymmetric protein with a long coiled-coil tail (1.2), giving it a higher Stokes radius than a globular protein of the same M_r. The 165 kDa polypeptide eluted ahead of the rabbit muscle myosin, consistent with it too being oligomeric and/or highly asymmetric. Such properties are characteristic of myosins II and probably some unconventional myosins such as brain p190 that form dimers (Cheney & Mooseker 1992; 1.3). They are not characteristic of myosins I (1.3). Such properties are not, however, diagnostic for myosin.

Several differences were observed between the chromatographic behaviour of the putative myosin heavy chain from mung bean and the behaviour of myosin heavy chains of muscle/nonmuscle myosins (Pollard 1982b). These include:

1. The failure of the 165 kDa polypeptide to bind the anion exchanger DE52 following desalting of the $(NH_4)_2SO_4$ fraction although the 165 kDa polypeptide in crude extracts bound DE52. When the DE52-bound 165 kDa polypeptide was eluted and subsequently fractionated with $(NH_4)_2SO_4$, it again bound DE52. This suggested that an extract-dependent modification of the putative myosin occurred in the 0-45% $(NH_4)_2SO_4$ fraction by a factor which was separated from the 165 kDa polypeptide when DE52 was added directly to crude extracts and the polypeptide eluted with salt. The modification could change the net charge on the molecule of the 165 kDa polypeptide. One possible explanation for such change may be due to different degrees of phosphorylation of the polypeptide. Since phosphorylation of myosin heavy or light chain widely regulates the actin-activated Mg^{2+}-ATPase activities of nonmuscle myosins (1.7) and is also implicated in regulating the ATPase activities of plant myosins (1.8.4), NaF, a phosphatase inhibitor which has been used in several cases of nonmuscle myosin extraction and purification (Kato & Tonomura 1977; Turkina *et al.* 1987), was tested in fractionating the mung bean

165 kDa polypeptide. No matter whether the extraction buffer contained NaF or not, the 165 kDa polypeptide in a crude extract bound to DE52. When it was subsequently eluted and fractionated with $(NH_4)_2SO_4$, it bound again to DE52. However, the 165 kDa polypeptide in the 0-45% $(NH_4)_2SO_4$ fraction of a crude extract did not bind to DE52 (data not shown). Therefore, NaF had no effect on the binding of the 165 kDa polypeptide to DE52, and the nature of the modification remains unknown.

2. The majority of the 165 kDa polypeptide did not bind novobiocin-Sepharose 6B. Since novobiocin-Sepharose has only been used in purifying myosin from yeast (Watts *et al.* 1985), it may not be a versatile affinity medium to isolate myosin.

3. The 165 kDa polypeptide did not bind to glutaraldehyde cross-linked F-actin coupled to Sepharose although rabbit muscle myosin was bound and eluted from the column with 3 mM ATP. F-actin columns prepared by Winstanley *et al.* (1977, 1979) were originally used to purify muscle myosin fragments (but not myosins II) and the columns were equilibrated with low salt, 5 mM triethanolamine-HCl buffer (pH 7.5) in which myosin fragments (but not intact myosins II) are still soluble. Therefore, the 0.5 M KCl in the starting buffer which allowed some muscle myosin to bind in my experiment might be too high for the much smaller quantities of mung bean putative myosin to bind to the column in detectable amounts. It is also possible that any actin-binding site of the 165 kDa polypeptide was altered during extraction or was blocked by another protein(s).

3.4.5 Conclusions

The mung bean 165 kDa polypeptide was identified as a putative myosin heavy chain by immunoblotting. The distribution of the 165 kDa polypeptide and K^+-EDTA-ATPase activity between $(NH_4)_2SO_4$ fractions indicated that the polypeptide at most accounted for a small fraction of total K^+-EDTA-ATPase activity of the crude extract. The retention of the 165 kDa polypeptide on ADP-agarose in the presence of EDTA indicated that it was probably an ATPase. After

testing a range of chromatography media many of which have been successfully used to purify nonmuscle myosins from different species, only DE52 and ADP-agarose columns could reliably bind mung bean 165 kDa polypeptide. Only chromatography on ADP-agarose completely resolved the 165 kDa polypeptide from the 155 kDa and 110 kDa ones, the latter two being obtained in the unbound fraction. Therefore, DE52 and ADP-agarose were used in subsequent studies reported in Chapter 4 to isolate this polypeptide from mung bean. Gel filtration of the 165 kDa polypeptide excludes the possibility that it is a myosin I and is consistent with it being the heavy chain of an highly asymmetric myosin (II or unconventional).

CHAPTER 3

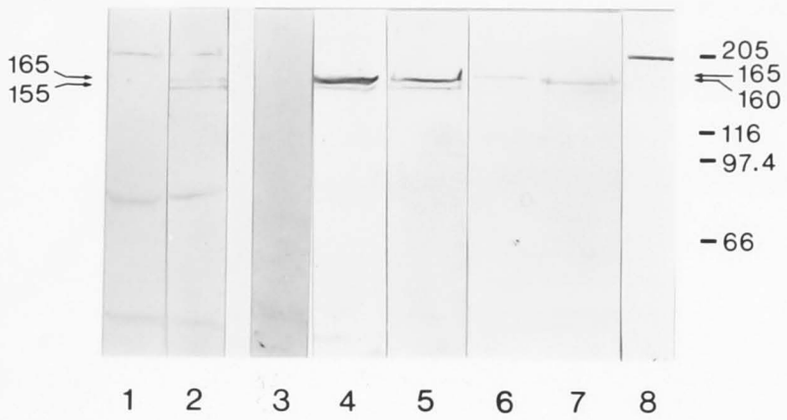
FIGURES

Fig. 3.1 **Identification of putative myosin heavy chains of higher plants by immunoblotting with anti-pan myosin antibody.** Putative myosin was extracted from seedlings of mung bean, wheat and pea with the extraction buffer (1-3, 5-8), or from those of mung bean with TCA solution (4). Proteins of crude extracts were resolved by 7% SDS-PAGE, and immunoblotted. Blots (1) and (2) were developed with peroxidase and the rest with alkaline phosphatase. (1) and (3), controls of mung bean extracts in which anti-pan myosin was replaced by 1% BSA (w/v) in TBS-Tween buffer; (2), (4) and (5), mung bean, two polypeptide bands were detected: 165 kDa and 155 kDa; (6), wheat, 165 kDa band; (7), pea, 160 kDa band; (8), rabbit skeletal muscle myosin, 205 kDa heavy chain band.

Fig. 3.2 **Extraction of putative myosin from mung bean with different buffers.** (1) and (2), high ionic strength buffer; (3) and (4), sucrose buffer; (5) and (6), pyrophosphate buffer. Protein contents of crude extract loaded on the gel were: (1), (3), and (5), 20 μg; (2), (4), and (6), 60 μg. Proteins were immunoblotted with anti-pan myosin antibody.

Fig. 3.5 **$(NH_4)_2SO_4$ fractionation of mung bean crude extract.** Mung bean crude extract was fractionated with $(NH_4)_2SO_4$ of 0-30%, 30-50%, 50-70% and 70-90% saturation: (1)-(5), or of 0-45% saturation: (6) and (7). Desalted $(NH_4)_2SO_4$ fractions and the crude extract were resolved by SDS-PAGE and immunoblotted. (1)-(5), 30 μg of proteins were loaded, showing a 165 kDa band; (6) and (7), showing both 165 and 155 kDa bands. (1) and (6), crude extract; (2), 0-30% $(NH_4)_2SO_4$ fraction; (3), 30-50%; (4), 50-70%; (5), 70-90%; (7), 0-45%.

1


165
155
205
165
160
116
97.4
66
1 2 3 4 5 6 7 8

2


165
1 2 3 4 5 6

5

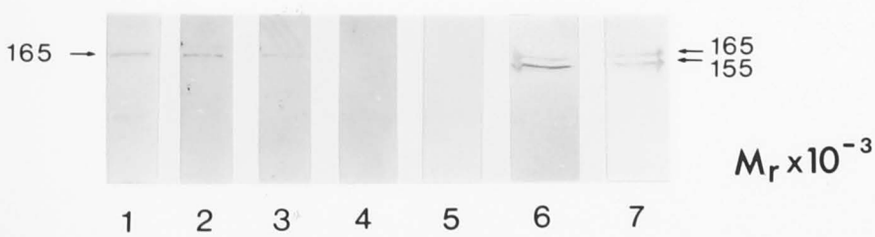
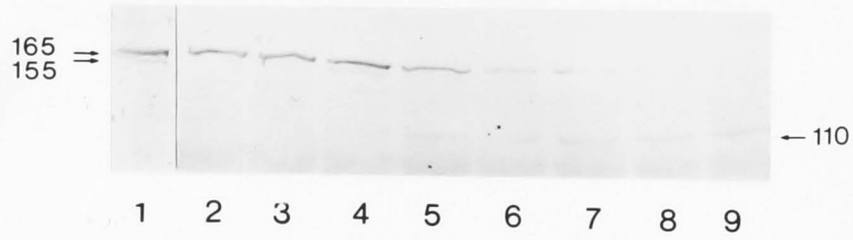

165
165
155
$M_r \times 10^{-3}$
1 2 3 4 5 6 7

Fig. 3.6 **Effects of proteases on putative myosin heavy chain in mung bean crude extract.** Mung bean putative myosin was extracted with the extraction buffer (A), or with buffer containing sucrose, 10 mM imidazole, pH 7.0, 1 mM DTT (B). Both crude extracts were kept at 0-4°C for 0 h (A1, B1), 9 h (A2, B2), 19 h (A3, B3), 24 h (A4, B4), 32 h (A5, B5), 42 h (A6, B6), 55 h (A7, B7), 65 h (A8, B8), and 72 h (A9, B9). All proteins were resolved by SDS-PAGE, and immunoblotted.

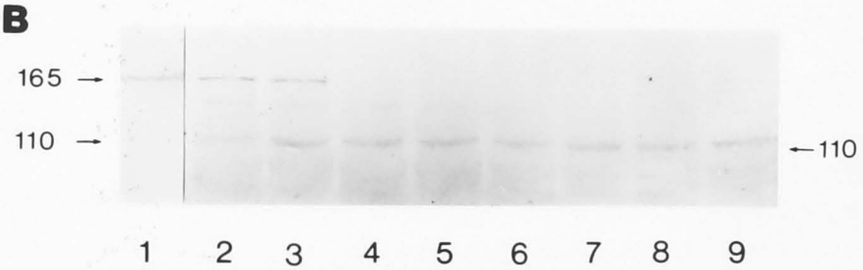
Fig. 3.7 **Effects of proteases on putative myosin heavy chain in desalted 0-45% $(NH_4)_2SO_4$ fraction.** The 0-45% $(NH_4)_2SO_4$ fraction of mung bean crude extract was desalted in 0.34 M sucrose, 10 mM imidazole, pH 7.0, 1 mM DTT. The desalted fraction was kept at 0-4°C for a series of time length: 0 h (1), 18 h (2), 30 h (3), 77 h (4), and 138 h (5). Protein samples were resolved by SDS-PAGE and immunoblotted.

Fig. 3.8 **Chromatography of mung bean crude extract on DE52.** Mung bean crude extract was applied to a DE52 column, and proteins were eluted stepwise with 0.1, 0.4 and 0.6 NaCl. Eluates were examined by immunoblotting. (1), crude extract; (2), 0.1 M NaCl fraction; (3), 0.4 M fraction; (4), 0.6 M NaCl fraction.

6A


165
155
110
1 2 3 4 5 6 7 8 9

6B


165
110
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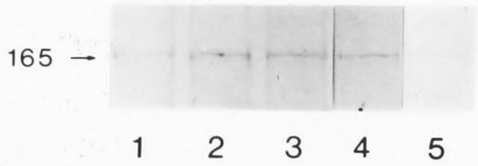
7


165
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8

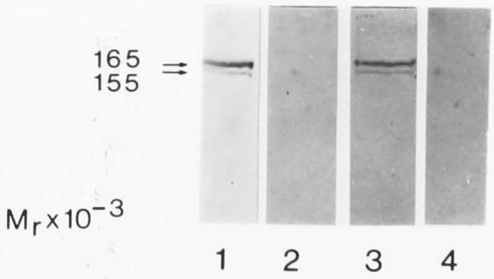
165
155
Mr x 10-3
1 2 3 4

Fig. 3.11 ADP-agarose chromatography of mung bean myosin fraction. 3 ml of the desalted 0-45% $(NH_4)_2SO_4$ fraction of the 0.4 M NaCl eluate from DE52 were applied to a column (1.6 x 2.1 cm) of ADP-agarose equilibrated with the starting buffer (0.6 M ammonium acetate, 2 mM sodium pyrophosphate, pH 6.5, 5 mM EDTA, 0.25 mM DTT). The column was eluted with 0.6 M NH_4Cl, 2 mM sodium pyrophosphate, pH 6.5, 5 mM EDTA, 0.25 mM DTT (fractions 20-28). Immunoblots of selected fractions are shown at the top (A). (1), 0-45% $(NH_4)_2SO_4$ fraction applied; (2), flow-through fraction; (3), bound fraction of the column.

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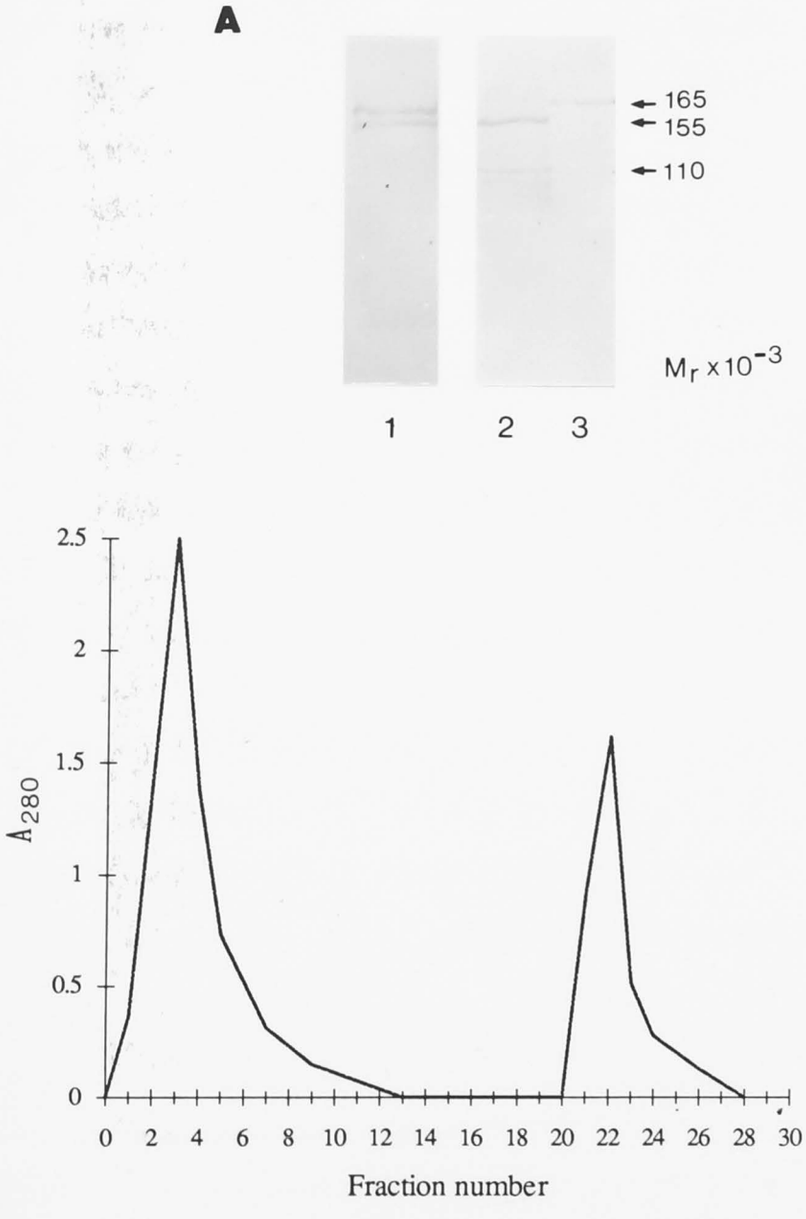
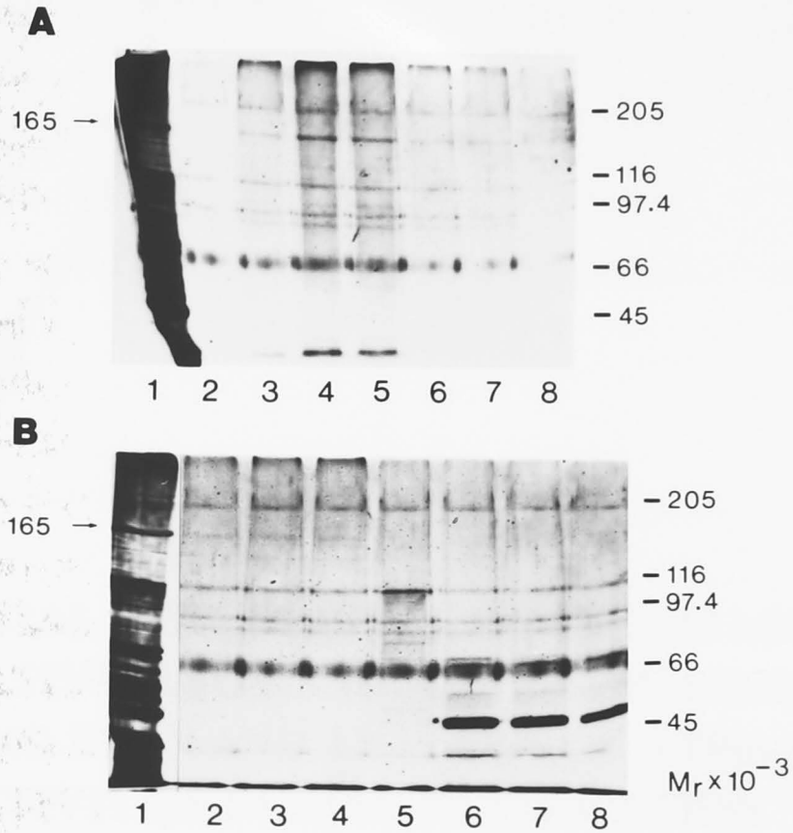


Fig. 3.12 Gel filtration of partially purified mung bean 165 kDa polypeptide on Sephacryl S-400. The column (2.6 x 46 cm) of Sephacryl S-400 was equilibrated with 0.04 M sodium pyrophosphate, pH 7.5, 0.001 mM DTT. It was calibrated with rabbit skeletal muscle myosin (470 kDa) and thyroglobulin (669 kDa), and the void volume determined by hemocyanin. 5 ml (1.1 mg of protein) of the partially purified mung bean 165 kDa polypeptide fraction were applied to the calibrated column. The void volume was 91.1 ml. The elution volumes (ml) of muscle myosin, thyroglobulin and mung bean 165 kDa polypeptide were: 104.6, 144.6 and 92.1, respectively. Selected eluate fractions resolved by SDS-PAGE and stained with silver are shown at the top (A) and (B). (1), the partially purified 165 kDa polypeptide fraction applied to the Sephacryl S-400 column. Corresponding elution volumes (ml) of the other fractions were: A (2), 26.1; A (3), 88.7; A (4), 92.1; A (5), 95.5; A (6), 119.7; A (7), 123.1; A (8), 130; B (2), 133.4; B (3), 136.9; B (4), 138.6; B (5), 171.3; B (6), 208.7; B (7), 212.1; and B (8), 215.5. Note the prominent 165 kDa band in lanes (4) and (5).


A
165
205
116
97.4
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B
165
205
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97.4
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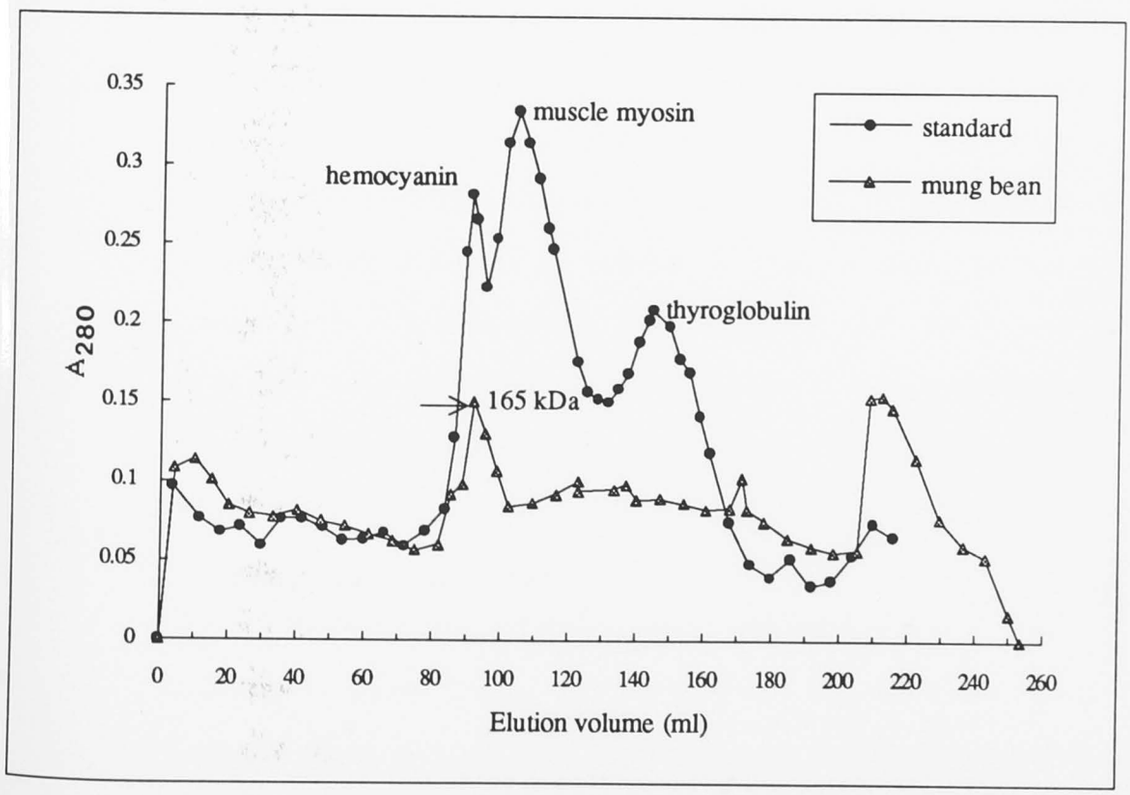
45
$M_r \times 10^{-3}$
1 2 3 4 5 6 7 8


0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
A_{280}
muscle myosin
hemocyanin
thyroglobulin
165 kDa
standard
mung bean
0 20 40 60 80 100 120 140 160 180 200 220 240 260
Elution volume (ml)

CHAPTER 4

PARTIAL PURIFICATION OF THE 165 kDa PUTATIVE MYOSIN HEAVY CHAIN FROM MUNG BEAN AND ANALYSIS OF ATPase ACTIVITIES DURING PURIFICATION

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4.1 INTRODUCTION

There are substantial differences in ATPase activities of various nonmuscle myosins according to published results (Korn 1978; Taylor & Condeelis 1979). In muscle and nonmuscle cells, myosins I express maximal ATPase activities with K^+-EDTA, while activities of myosins II are usually maximal in K^+-EDTA when compared to Ca^{2+} but the activities of some myosins II are the reverse (Clark & Spudich 1974; Korn 1982; Cote *et al.* 1985; Kohama *et al.* 1983, 1986). Most Mg^{2+}-ATPase activities are activated by F-actin, but some are not. It seems likely that the ability to interact enzymatically with actin is common to all myosins, but may not be preserved in purification procedures developed by monitoring some other more stable property of the molecule (Clark & Spudich 1974).

K^+-EDTA- or Ca^{2+}-ATPase activity is usually used to monitor the purification of plant myosins or putative myosin. For plant myosins (1.8.1) purified from *Nitella* (Kato & Tonomura 1977) and tomato (Vahey & Scordilis 1980; Vahey *et al.* 1982), and putative myosins partially purified from onion (Pesacreta *et al.* 1991) and preliminarily fractionated from pea (Ma & Yen 1989), ATPase activities are activated maximally by EDTA, partially by Ca^{2+}, and are lowest in the presence of Mg^{2+}, under high ionic strength conditions. The Mg^{2+}-ATPase activities are activated by rabbit skeletal muscle F-actin at low ionic strength. Mg^{2+}-ATPase of myosin from *Heracleum sosnowskyi* (Turkina *et al.* 1987) is activated by F-actin at low ionic strength.

The partially purified *Egeria* myosin, however, exhibits higher Ca^{2+}-ATPase activity than Mg^{2+}-ATPase activity, and its K^+-EDTA-ATPase activity is the lowest (Ohsuka & Inoue 1979). There is no inhibition of myosin ATPase activity

by Mg^{2+} at high ionic strength. At low ionic strength, *Egeria* myosin binds to skeletal muscle F-actin but its Mg^{2+}-ATPase activity is not affected by F-actin, which demonstrates that the actin-activated ATPase activity of some plant myosin may not be detected during purification.

After examining the chromatographic behaviour of the mung bean 165 kDa putative myosin heavy chain in Chapter 3, a scheme for partially purifying the 165 kDa polypeptide myosin heavy chain was developed and monitored by immunoblotting with anti-pan myosin in this chapter. ATPase activities were also examined during the partial purification, and the 165 kDa polypeptide was shown at most to account for a very small fraction of the total K^+-EDTA-ATPase activity of mung bean extract. The partially purified 165 kDa polypeptide fraction had K^+-EDTA-ATPase activity but did not exhibit F-actin-activated Mg^{2+}-ATPase activity.

4.2 MATERIALS AND METHODS

4.2.1 Partial purification of the mung bean 165 kDa polypeptide monitored by immunoblotting

Mung bean seedlings (900 g) were extracted with extraction buffer [2.5.1 (ii)]. The crude extract was mixed (batch processing) with DE52 (370-400 ml) equilibrated with starting buffer (0.34 M sucrose, 10 mM imidazole, pH 7.0, 1 mM DTT) and left for about 25 min with gentle stirring with a glass stirring bar. The slurry was filtered on a Buchner funnel and washed with 8 liters of the starting buffer containing 0.1 M NaCl until the A_{280} of the filtrate declined to 0.12. The cellulose cake was resuspended in a small volume of 0.1 M NaCl buffer, and poured into a glass column (4.9 x 36 cm).

The DE52 column was eluted with 0.4 M NaCl in the starting buffer. The peak protein fractions (A_{280}: 0.2-0.476) were pooled and fractionated with 0-45% $(NH_4)_2SO_4$. Following centrifugation, the 0-45% pellet was desalted on a column (4.9 x 32.8 cm) of Sephadex G-25 equilibrated with the starting buffer (0.34 M sucrose, 10 mM imidazole, pH 7.0, 1 mM DTT), and protein fractions were pooled and where necessary concentrated to $\leq$ 200 ml by dialyzing against Aquacide II powder for 1.5 h. Desalted protein was applied to a second DE52 column (2.6 x 19 cm) equilibrated with the starting buffer (0.34 M sucrose, 10 mM imidazole, pH 7.0, 1 mM DTT). The column was eluted with 500 ml of a 0-0.4 M NaCl gradient in the starting buffer, at a flow rate of 55 ml/h. The 165 kDa polypeptide monitored by immunoblotting, was eluted by 0.138-0.158 M NaCl. These fractions (26 ml) were pooled and concentrated to 16 ml by dialyzing against Aquacide II for 2 h. The concentrated fraction was desalted by dialyzing overnight against 2.5 liter of the starting buffer. The dialyzed material was applied at a flow rate of 10 ml/h to a column (1.6 x 2.5 cm) of ADP-agarose equilibrated with the starting buffer (0.34 M sucrose, 10 mM imidazole, pH 7.0, 1 mM DTT). Chromatography was stopped for 2 h before washing. Protein was eluted with 100 ml of a 0-0.8 M KCl gradient, and then 1 M KCl in the starting buffer, at a flow rate of 50 ml/h. The 165 kDa

polypeptide was eluted in a peak by 0.29-0.43 M KCl, and the peak fractions were pooled. The pooled fractions (16.9 ml) were concentrated to 3.2 ml by dialyzing against Aquacide II, and then desalted by dialyzing against 3 liter of the starting buffer. A typical procedure for the partial purification of the 165 kDa polypeptide is summarized in Fig. 4.1.

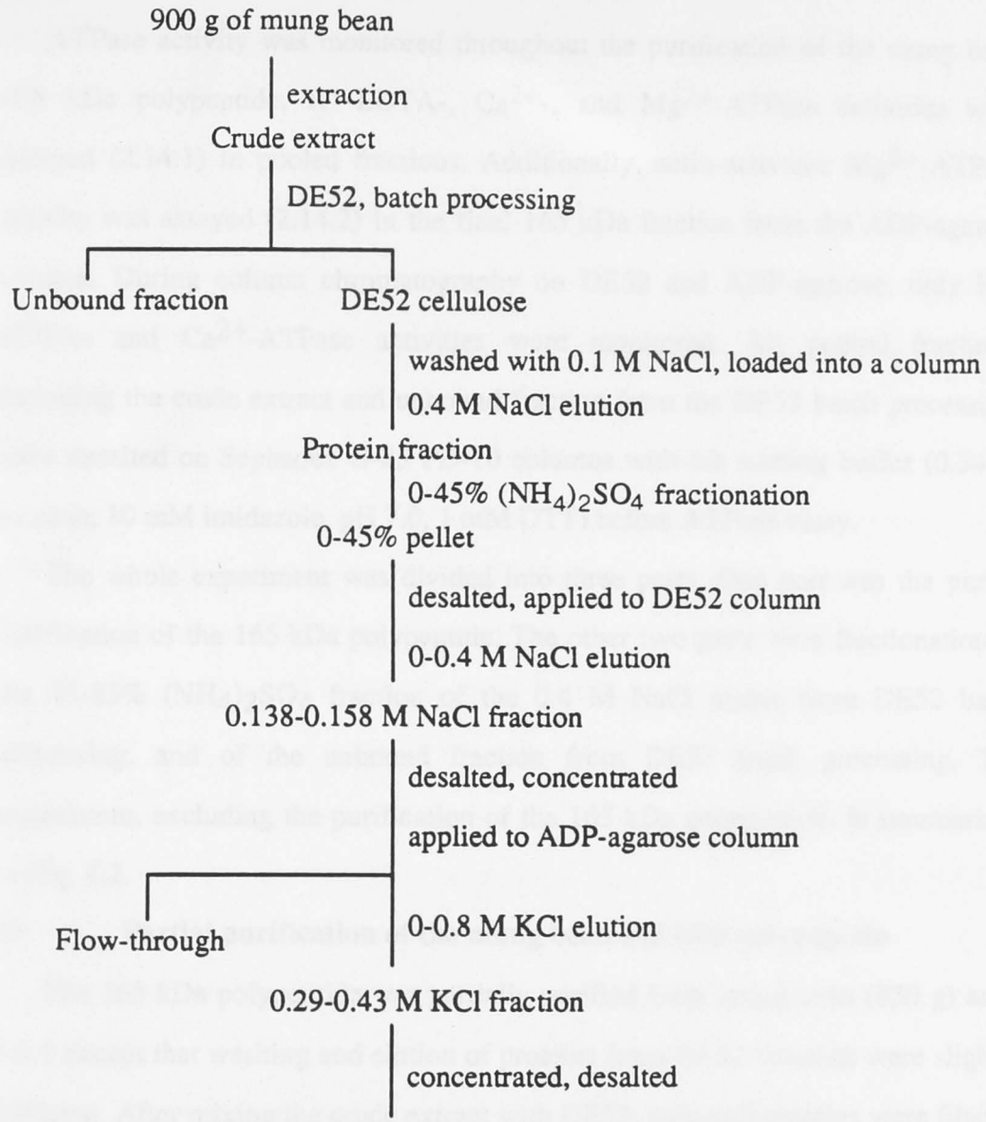


Fig. 4.1 Flow diagram for the partial purification of the mung bean 165 kDa polypeptide

4.2.2 Analysis of ATPase activities during purification

ATPase activity was monitored throughout the purification of the mung bean 165 kDa polypeptide. K^+-EDTA-, Ca^{2+}-, and Mg^{2+}-ATPase activities were assayed (2.14.1) in pooled fractions. Additionally, actin-activated Mg^{2+}-ATPase activity was assayed (2.14.2) in the final 165 kDa fraction from the ADP-agarose column. During column chromatography on DE52 and ADP-agarose, only K^+-EDTA- and Ca^{2+}-ATPase activities were monitored. All pooled fractions, including the crude extract and unbound fraction from the DE52 batch processing, were desalted on Sephadex G-25 PD-10 columns with the starting buffer (0.34 M sucrose, 10 mM imidazole, pH 7.0, 1 mM DTT) before ATPase assay.

The whole experiment was divided into three parts. One part was the partial purification of the 165 kDa polypeptide. The other two parts were fractionation of the 45-85% $(NH_4)_2SO_4$ fraction of the 0.4 M NaCl eluate from DE52 batch processing, and of the unbound fraction from DE52 batch processing. The experiment, excluding the purification of the 165 kDa polypeptide, is summarized in Fig. 4.2.

(i) Partial purification of the mung bean 165 kDa polypeptide

The 165 kDa polypeptide was partially purified from mung bean (850 g) as in 4.2.1 except that washing and elution of proteins from DE52 columns were slightly different. After mixing the crude extract with DE52, unbound proteins were filtered through and the cellulose cake was washed with 1 liter of the starting buffer (0.34 M sucrose, 10 mM imidazole, pH 7.0, 1mM DTT). After loading the cellulose into a column, protein was eluted with 1 liter steps of 0.05 M and 0.4 M NaCl in the starting buffer. Peak fractions of ATPase activities of the 0.4 M NaCl eluate were pooled. The pooled fraction (156 ml) was fractionated with $(NH_4)_2SO_4$ of 0-45% and 45-85% saturation. The desalted 0-45% fraction (163 ml) was applied to a column (2.6 x 18 cm) of DE52 and protein eluted with a 0-0.3 M NaCl gradient in the starting buffer, at a flow rate of 58 ml/h. Peak fractions of ATPase activities, eluted by 0.095-0.139 M NaCl, were pooled. The pooled fraction (71 ml) was then

precipitated with 0-45% $(NH_4)_2SO_4$, desalted (26.3 ml) and applied to an ADP-agarose column (1.6 x 2.5 cm). Protein was eluted with a 0-0.8 M KCl gradient in the starting buffer. The 165 kDa polypeptide was eluted in a peak between 0.076 M and 0.13 M KCl, and the peak fractions were pooled.

(ii) Fractionation of the 45-85% $(NH_4)_2SO_4$ fraction of the 0.4 M NaCl eluate

The desalted 45-85% $(NH_4)_2SO_4$ fraction (29.5 ml) was applied to a column (2.6 x 7.8 cm) of DE52 equilibrated with the starting buffer (0.34 M sucrose, 10 mM imidazole, pH 7.0, 1mM DTT). The column was eluted with 250 ml of 0-0.4 M NaCl gradient, then 0.6 M and 1 M NaCl in the starting buffer. Peak fractions of ATPase activities eluted by 0.098-0.167M NaCl were pooled (46.8 ml).

(iii) Fractionation of the unbound fraction of the DE52 batch processing

The unbound fraction (1300 ml) of the DE52 batch processing was fractionated with 0-85% $(NH_4)_2SO_4$. The 0-85% pellet was desalted on a column (4.9 x 56 cm) of Sephadex G-25 equilibrated with the starting buffer (0.34 M sucrose, 10 mM imidazole, pH 7.0, 1mM DTT). The desalted fraction (345 ml) was applied to a column (2.6 x 7.9 cm) of phosphocellulose equilibrated with the starting buffer. Flow-through and bound fractions of the column were fractionated separately as described below.

(a) Flow-through fraction

The flow-through fraction (345 ml) was applied to a column (2.6 x 6.4 cm) of hydroxylapatite equilibrated with the starting buffer. The flow-through fractions were collected.

(b) Bound fraction

The phosphocellulose column was eluted with 250 ml of a 0-0.5 M NaCl gradient and then 1 M NaCl in the starting buffer, at a flow rate of 90 ml/h. Peak fractions of ATPase activities were eluted by 0.095-0.19 M NaCl. The peak fractions (51 ml) were pooled and concentrated to 6.4 ml with the salt concentration diluted by half through ultrafiltration. The sample (6.4 ml) was applied to a column

(1.6 x 2.5 cm) of ADP-agarose equilibrated with the starting buffer (0.34 M sucrose, 10 mM imidazole, pH 7.0, 1mM DTT). The column was eluted with 100 ml of 0-0.8 M KCl gradient in the starting buffer. Proteins were eluted in a peak by 0.17-0.25 M KCl.

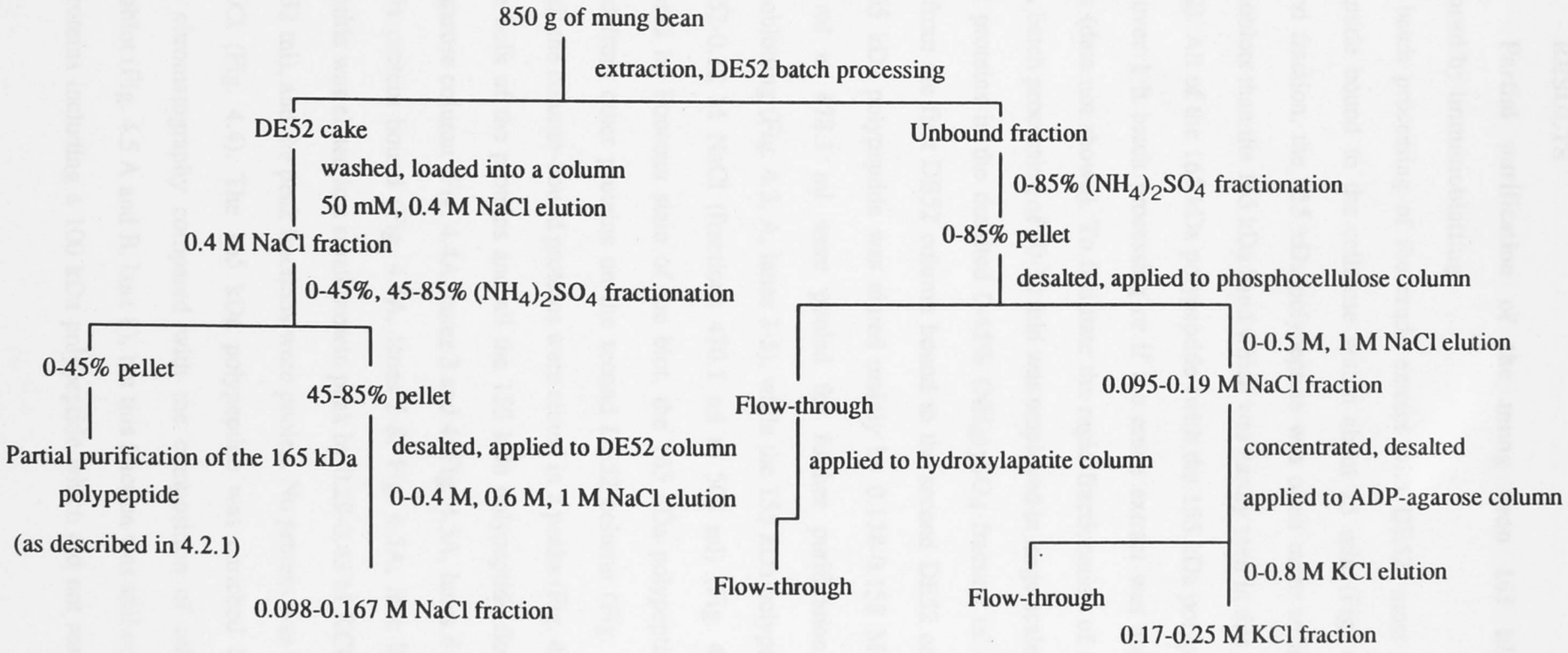


Fig. 4.2 Flow diagram for fractionation of two fractions unused in the partial purification of the mung bean 165 kDa polypeptide

4.3 RESULTS

4.3.1 Partial purification of the mung bean 165 kDa polypeptide monitored by immunoblotting

In batch processing of the crude extract with DE52, most of the 165 kDa polypeptide bound to the cellulose within about 25 min (Fig. 4.5A, 3). In the unbound fraction, the 155 kDa polypeptide was often more strongly detected on immunoblots than the 165 kDa band which was hardly seen in this experiment (Fig. 4.5A, 2). All of the 165 kDa polypeptide (with the 155 kDa polypeptide) bound to DE52 over 1 h batch processing or if the crude extract was applied to a DE52 column (data not shown). To facilitate the rapid fractionation of the crude extract (3.3.4), batch processing of 20-30 min was employed in large-scale purification.

All proteins in the desalted 0-45% $(NH_4)_2SO_4$ fraction of the 0.4 M NaCl eluate from the first DE52 column bound to the second DE52 column (Fig. 4.3). The 165 kDa polypeptide was eluted mainly by 0.138-0.158 M NaCl (fractions 452.1 ml to 478.1 ml were pooled for further purification) as shown by immunoblotting (Fig. 4.3, A, lanes 2-5), while the 155 kDa polypeptide was eluted by 0.152-0.18 M NaCl (fractions 470.1 ml to 505 ml) (Fig. 4.3A, lanes 4-8). Monitored by Ponceau stain of the blot, the 165 kDa polypeptide was not well resolved from other proteins on the second DE52 column (Fig. 4.5B, lane 5) in which all the column-bound proteins were eluted in 2 peaks (Fig. 4.3).

The bulk of the proteins and all the 155 kDa polypeptide flowed through the ADP-agarose column (Fig. 4.4A, lanes 3 and 4; Fig. 4.5A, lanes 6 and 7) while the 165 kDa protein bound (Fig. 4.4A, lanes 5-8; Fig. 4.5A, lane 8). The 165 kDa polypeptide was eluted in a small protein peak by 0.29-0.43 M KCl (fractions 116.4 ml to 132 ml), and the peak fractions were pooled. No protein was further eluted by 1 M KCl (Fig. 4.4). The 165 kDa polypeptide was enriched by ADP-agarose affinity chromatography compared with the composition of other fractions on immunoblot (Fig. 4.5 A and B, lane 8), but this fraction was still contaminated with other proteins including a 100 kDa polypeptide which did not react with anti-pan

myosin and did not always appeared as prominent as in this experiment. However, a 0-0.8 M KCl gradient could not separate the 165 kDa polypeptide from these proteins, and all proteins eluted in one peak (Fig. 4.4). Step elution by 50 mM ATP in the sucrose starting buffer was tried. ATP eluted the 165 kDa polypeptide, but did not resolve the 165 kDa polypeptide from other proteins (data not shown).

The total protein in the final ADP-agarose fraction containing the 165 kDa polypeptide fraction represented 0.05% of the total protein in the initial extract (or 1.0 mg protein/100 g tissue) (Table 4.1, 7). Most proteins were removed by the DE52 batch processing and 0.4 M NaCl elution which had a yield of 24.9% (Table 4.1, 2). In the fractions retained from the second DE52 column and from the ADP-agarose, 6.9% and 5.7% of the proteins applied were recovered (Table 4.1, 5 and 7), the lowest protein recoveries along the whole purification procedure.

Table 4.1 Partial purification of the mung bean 165 kDa polypeptide

Fraction	Protein			
	Vol (ml)	Total (mg)	Yield (%)[a]	recovery from previous step (%)[b]
1. Crude extract	1330	1862	-	-
2. 1st DE52, 0.4 M NaCl	1030	463.5	24.9	24.9
3. 0-45% $(NH_4)_2SO_4$, desalted	268	318.9	17.1	68.8
4. 0-45% $(NH_4)_2SO_4$, desalted, concentrated	168	295.7	15.9	92.7
5. 2nd DE52, 0.138-0.158 M NaCl	26	20.5	1.1	6.9
6. 2nd DE52, 0.138-0.158 M NaCl, desalted, concentrated	16	16.2	0.9	79
7. ADP-agarose, 0.29-0.43 M KCl	3.2	0.92	0.05	5.7

a. Protein yield was the percentage of total protein amount of the crude extract in a protein fraction.

b. Recovery from previous step was the percentage of protein in the previous fraction that was recovered in the current fraction.

4.3.2 Analysis of ATPase activities during purification of the 165 kDa polypeptide

The procedure for the partial purification of the 165 kDa polypeptide described in 4.3.1 was developed by immunoblotting. However, one of the most fundamental criteria for identifying a myosin is its actin-activated Mg^{2+}-ATPase activity at low ionic strength. As myosin *in vitro* usually expresses high level of K^+-EDTA- or Ca^{2+}-ATPase activity at high ionic strength, such ATPase assays are normally used to monitor the purification of myosin. In order to see whether the partially purified 165 kDa polypeptide monitored by immunoblotting retained the ATPase activities characteristic of myosin, ATPase activities were monitored together with immunoblotting during its purification.

After the DE52 batch processing of mung bean crude extract, most of the 165 kDa polypeptide bound to the cellulose, but some remained in the unbound fraction (data not shown). The cellulose cake was transferred to a column after being washed with 1 liter of the starting buffer to remove unbound proteins, and the DE52 column was eluted with 50 mM NaCl. Proteins were eluted in a low and broad peak by 50 mM NaCl. There were several low specific ATPase activities in the eluate fractions (Fig. 4.6), and no 165 kDa polypeptide could be detected by immunoblotting (data not shown). A high protein peak was eluted by 0.4 M NaCl, and peaks of both K^+-EDTA- and Ca^{2+}-ATPase activities were eluted just in front of the first protein peak (Fig. 4.7). The peak of K^+-EDTA-ATPase activity was slightly higher than that of Ca^{2+}-ATPase activity. Instead of pooling all the protein-rich fractions as described in 4.2.1, only fractions containing the peak of ATPase activities (from fractions 46 to 66) were pooled. By immunoblotting, the 165 kDa polypeptide was mainly eluted in fractions from 46 to 61 (data not shown). The pooled fraction was fractionated with 0-45% $(NH_4)_2SO_4$, desalted, and applied to the second DE52 column.

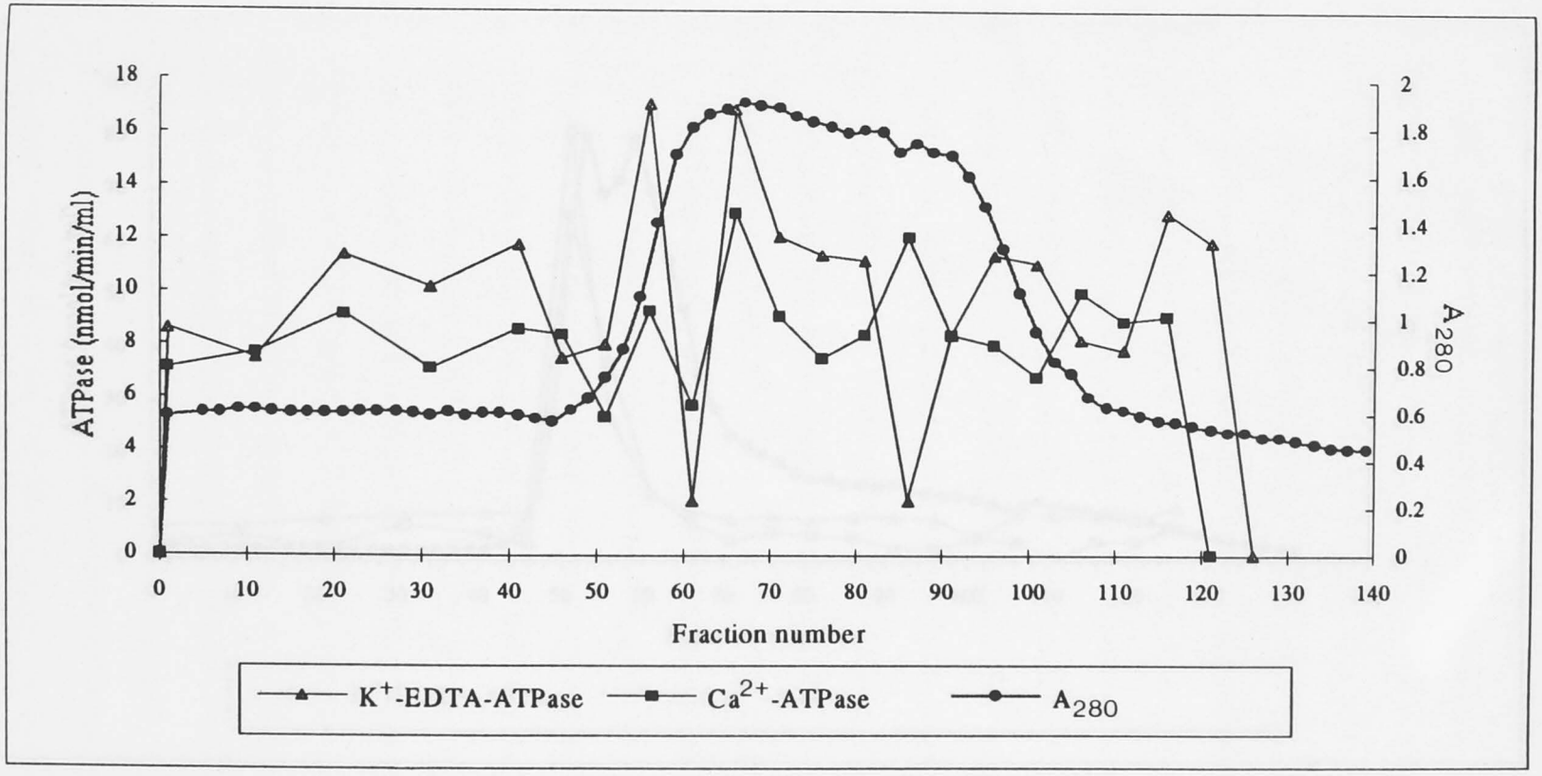


Fig. 4.6 DEAE-cellulose chromatography of mung bean crude extract after batch processing. The column (4.9 x 22 cm) of DE52 was washed with 1 liter of 50 mM in the starting buffer. K^+-EDTA- and Ca^{2+}-ATPase activities were monitored in selected fractions.

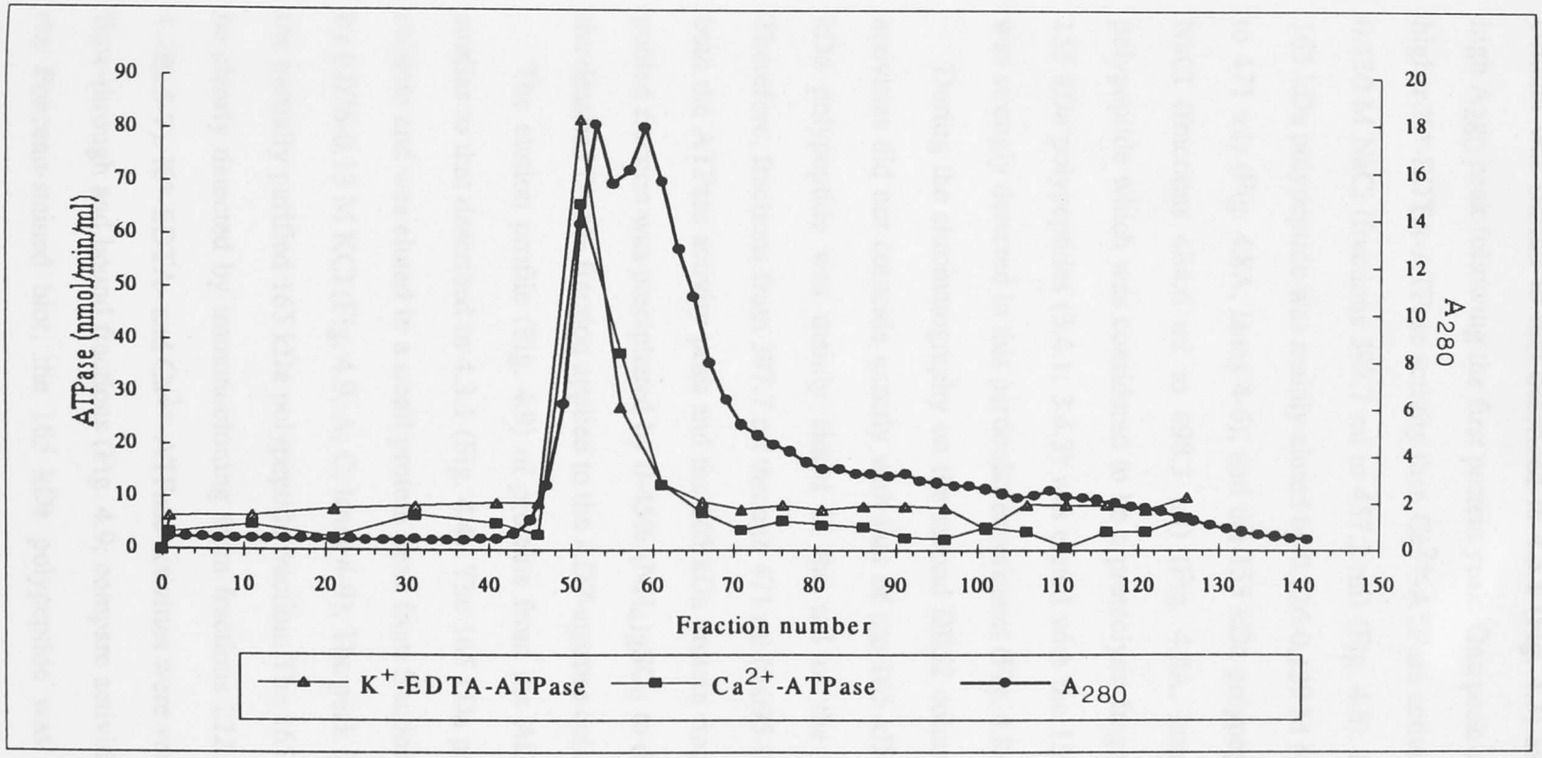


Fig. 4.7 DEAE-cellulose chromatography of mung bean crude extract after batch processing. After elution with 50 mM NaCl as shown in Fig. 4.6, protein was eluted from the DE52 column with 1 liter of 0.4 M NaCl in the starting buffer. K^+-EDTA- and Ca^{2+}-ATPase activities were monitored in selected eluate fractions. The 165 kDa polypeptide was eluted in a peak containing ATPase activities by 0.4 M NaCl, and peak fractions from 44 to 66 were pooled for further purification

The 0-0.3 M NaCl elution profile (Fig. 4.8) of proteins from the second DE52 column was similar to that described in 4.3.1 (Fig. 4.3) except that there was no high A_{280} peak following the first protein peak. One peak of ATPase activity, with higher K^+-EDTA-ATPase activity than Ca^{2+}-ATPase activity was eluted by 0.095-0.130 M NaCl (fractions 397.7 ml to 457.2 ml) (Fig. 4.8). By immunoblotting, the 165 kDa polypeptide was mainly eluted by 0.126-0.139 M NaCl (fractions 450.2 ml to 471 ml) (Fig. 4.8A, lanes 4-6), and the 155 kDa polypeptide by 0.147-0.155 M NaCl (fractions 484.6 ml to 498.3 ml) (Fig. 4.8A, lanes 8-9). The 110 kDa polypeptide which was considered to be a proteolytic fragment of the 165 kDa or 155 kDa polypeptides (3.4.1; 3.4.3) was eluted with the 155 kDa polypeptide, and was strongly detected in this particular experiment (Fig. 4.8A, lanes 7-9).

During the chromatography on the second DE52 column, the peak of ATPase activities did not coincide exactly with that of the 165 kDa polypeptide. The 165 kDa polypeptide was mainly eluted in the tail of the ATPase activity peak. Therefore, fractions from 397.7 ml through 471 ml (0.095-0.139 M NaCl) covering both the ATPase activity peak and the 165 kDa protein fractions were pooled. The pooled fraction was precipitated by 0-45% $(NH_4)_2SO_4$ to decrease the volume and the desalted 0-45% fraction applied to the ADP-agarose column.

The elution profile (Fig. 4.9) of proteins from the ADP-agarose column was similar to that described in 4.3.1 (Fig. 4.4). The 165 kDa polypeptide bound to the column and was eluted in a small protein peak from fractions 221.2 ml to 227.9 ml by 0.076-0.13 M KCl (Fig. 4.9, A; C, lanes 4-9). The peak fractions were pooled as the partially purified 165 kDa polypeptide fraction. The 165 kDa polypeptide could be clearly detected by immunoblotting from fractions 222.5 ml to 225.2 ml (Fig. 4.9B, 5-7). K^+-EDTA- and Ca^{2+}-ATPase activities were very low and scattered the flow-through and bound fractions (Fig. 4.9; compare activities with Fig. 4.8). From the Ponceau-stained blot, the 165 kDa polypeptide was enriched in the ADP-agarose bound fraction, but was still contaminated with many other proteins including the 100 kDa polypeptide (Fig. 4.9C, 5-8). The total protein yield of the

ADP-agarose purified 165 kDa polypeptide fraction in this experiment was 0.02% (or 0.041 mg/100 g tissues) (Table 4.2, 6).

From Table 4.2, it can be seen that the specific activities of K^+-EDTA-, Ca^{2+}- and Mg^{2+}-ATPases were decreasing along the purification. Usually, the specific activity of K^+-EDTA-ATPase was the highest, as shown in Table 4.2. Therefore, K^+-EDTA-ATPase activity was mainly discussed in this experiment.

The specific activity of K^+-EDTA-ATPase of the crude extract, 0.175 μmol/min/mg, was the highest among all fractions. It decreased greatly, about 10-fold, after the DE52 batch processing and 0.4 M NaCl elution (0.016 μmol/min/mg), and the yield of the K^+-EDTA-ATPase activity at this step was only 2.5% (Table 4.2, 2). At later purification steps, the specific activity of K^+-EDTA-ATPase declined further (Table 4.2, 3-5) but increased at the final ADP-agarose step (Table 4.2, 6). The specific activity of K^+-EDTA-ATPase of the ADP-agarose purified 165 kDa polypeptide fraction was 0.016 μmol/min/mg which was higher than those of three previous steps, and was about 7 times that of the $(NH_4)_2SO_4$ fraction applied to ADP-agarose (Table 4.2, 6). However, only 0.58% of the protein applied to the ADP column was recovered in the bound fraction (Table 4.2, 6) and part of the K^+-EDTA-ATPase activity flowed through the column (Fig. 4.9). Therefore, only 6% of the K^+-EDTA-ATPase activity applied was recovered in the ADP-agarose fraction (Table 4.2, 6). The high specific activity of Mg^{2+}-ATPase of the ADP-agarose bound fraction in this experiment (0.064 μmol/min/mg) was unusual since there was no Mg^{2+}-ATPase activity in the preceding two fractions and usually Mg^{2+}-ATPase activity was lower than K^+-EDTA-ATPase activity. However, no actin activation of the Mg^{2+}-ATPase activity at low ionic strength could be detected in the final ADP-agarose fraction (Table 4.2, 6).

The yield of K^+-EDTA-ATPase activity in the partially purified 165 kDa polypeptide fraction was very low, only 0.0014% of the initial activity (Table 4.2, 6). Both the total and specific activities of K^+-EDTA-ATPase decreased

dramatically after the DE52 batch processing and 0.4 M NaCl elution (Table 4.2, 2).

Table 4.2 ATPase activities during the partial purification the mung bean 165 kDa polypeptide.

Fraction	Protein			ATPase activity									
				K^+-EDTA-ATPase			Ca^{2+}-ATPase		Mg^{2+}-ATPase		Actin-activated Mg^{2+}-ATPase		
	Vol (ml)	Total (mg)	Yield (%)	Yield (%)	Total μmol/min	Specific μmol/min/mg	Total μmol/min	Specific μmol/min/mg	Total μmol/min	Specific μmol/min/mg	- actin μmol/min/mg	+ actin μmol/min/mg	activation
1. Crude extract	1338	2369.6	100	100	414.7	0.175	225.1	0.095	170.6	0.072	-	-	-
2. 1st DE52, 0.4 M NaCl	156	668	28.2	2.5	10.7	0.016	9.4	0.014	7.3	0.011	-	-	-
3. 0-45% $(NH_4)_2SO_4$, desalted	163	414	17.5	0.6	2.4	0.0059	2.0	0.0049	1.9	0.0047	-	-	-
4. 2nd DE52, 0.095-139M NaCl	72	186.7	7.9	0.2	1.0	0.0052	0.9	0.0047	0	0	-	-	-
5. 0-45% $(NH_4)_2SO_4$, desalted	26.3	59.9	2.5	0.02	0.1	0.0022	0.2	0.0029	0	0	-	-	-
6. ADP-agarose, 0.076-0.13 M NaCl	8.1	0.35	0.02	0.0014	0.006	0.016	0	0	0.02	0.064	0.030	0.026	0

Protein yield was the percentage of total protein amount of the crude extract in a protein fraction.

Activity yield was the percentage of ATPase activity of the crude extract in a protein fraction.

Table 4.3 ATPase activities of unused fractions from the partial purification of the mung bean 165 kDa polypeptide.

Fraction	Protein			ATPase activity						
				K^+-EDTA-ATPase			Ca^{2+}-ATPase		Mg^{2+}-ATPase	
A.	Vol (ml)	Total (mg)	Yield (%)	Yield (%)	Total µmol/min	Specific µmol/min/mg	Total µmol/min	Specific µmol/min/mg	Total µmol/min	Specific µmol/min/mg
1. Crude extract	1338	2369.6	100	100	414.7	0.175	225.1	0.095	170.6	0.072
2. 45-85% $(NH_4)_2SO_4$, desalted	29.5	81	3.4	0.5	2.2	0.027	1.6	0.02	2.0	0.024
3. 2nd DE52, 0.098-0.167 M NaCl	46.8	52.2	2.2	0.4	1.8	0.034	1.6	0.031	1.4	0.027
B.										
2. 1st DE52, unbound fraction	1300	934.7	39.4	62.9	260.8	0.279	145.8	0.156	108.4	0.116
3. 0-85% $(NH_4)_2SO_4$, desalted	345	659	29.3	55.1	228.7	0.347	187.8	0.285	160.1	0.243
4. Phosphocellulose, flow-through	345	532	22.5	40.8	169.2	0.318	127.1	0.239	119.2	0.224
5. Hydroxylapatite, flow-through	337	466.1	19.7	42	174.3	0.374	111.9	0.24	105.3	0.226
6. Phosphocellulose, 0.095-0.19 M NaCl	51	12.1	0.5	0.6	2.6	0.218	1.5	0.127	1.2	0.096

Definitions of yields of protein and ATPase activity were the same as described in Table 4.1.

A. Fractionation of the supernatant of 0.4 M NaCl fraction after 0-45% $(NH_4)_2SO_4$ precipitation.

B. Fractionation of the unbound fraction of DE52 batch processing.

4.3.3 ATPase activities in other fractions and attempted purification of a K^+-EDTA-ATPase

During the purification of the mung bean 165 kDa polypeptide using monitoring by immunoblotting, most of the ATPase activities were lost, especially after the DE52 batch processing and 0.4 M NaCl elution (4.3.2). To trace the lost ATPase activity, other fractions unused in the purification were fractionated and monitored for ATPase to see whether any protein with the appropriate ATPase activity for a myosin could be identified.

When the 0.4 M NaCl fraction from the first DE52 column was fractionated with 0-45% and 0-85% $(NH_4)_2SO_4$, slightly more K^+-EDTA-ATPase activity was recovered in the 0-45% fraction (yield: 0.6%; Table 4.2, 3) than the 45-85% fraction (yield: 0.5%; Table 4.3A, 2). However, the 45-85% fraction had a K^+-EDTA-ATPase specific activity of 0.027 μmol/min/mg (Table 4.3A, 2) which was about 4.6 times that of the 0-45% fraction containing most of the 165 kDa polypeptide (Table 4.2, 3). Proteins of the desalted 45-85% fraction all bound to the second DE52 column. Peaks of both K^+-EDTA-ATPase activity (peak top: 74.1 nmol/min/ml) and Ca^{2+}-ATPase activity (peak top: 78.8 nmol/min/ml) which were slightly in front of the protein peak (A_{280} of peak top: 1.71) were eluted by 0.098-0.167 M NaCl. No protein was further eluted by 0.6 M and 1 M NaCl (data not shown). The pooled peak fractions of ATPase activities had a K^+-EDTA-ATPase activity of 0.034 μmol/min/mg (Table 4.3A, 3). However, no proteins reacting with anti-pan myosin antibody could be detected by immunoblotting in either the 45-85% $(NH_4)_2SO_4$ fraction or the pooled DE52 fraction. The fact that ATPase activities in the 45-85% fraction were higher than those in the 0-45% fraction has been discussed before (3.3.3; 3.4.2)

During the purification of the mung bean 165 kDa polypeptide, a small amount of the 165 kDa polypeptide did not bind to DE52 after the batch processing, and 62.9% of the initial K^+-EDTA-ATPase activity in the crude extract was recovered in the unbound fraction (Table 4.3B, 2). The specific activities of the K^+-EDTA-,

Ca^{2+}-, and Mg^{2+}-ATPases of the unbound fraction were higher than those of the crude extract. Most ATPase activities in the unbound fraction were recovered in its 0-85% $(NH_4)_2SO_4$ fraction (Table 4.3B, 3). The majority of proteins, together with most ATPase activities of the desalted 0-85% fraction flowed through a phosphocellulose column. 40.8% of the K^+-EDTA-ATPase activity was recovered in the flow-through fraction (Table 4.3B, 4). Only small amounts of proteins bound to the column and were eluted in a small peak (A_{280} of peak top: 0.57) by 0.095-0.19 M NaCl, and no proteins were eluted further by 1 M NaCl (data not shown). The eluate fractions also contained a peak of K^+-EDTA-ATPase activity (peak top: 112.6 nmol/min/ml) and Ca^{2+}-ATPase activity (peak top: 85.6 nmol/min/ml). But only 0.6% of the K^+-EDTA-ATPase activity was recovered in the bound fraction from the phosphocellulose (Table 4.3B, 6). By immunoblotting, the 165 kDa polypeptide that remained in the unbound fraction of DE52 batch processing could be detected only weakly in the flow-through fraction of phosphocellulose (data not shown).

The flow-through fraction from the phosphocellulose column was applied to a column of hydroxylapatite, and most proteins with high ATPase activities flowed through (Table 4.3B, 5). The 165 kDa band was weakly detected in the flow-through fraction. Since the fraction bound to phosphocellulose still had high K^+-EDTA-ATPase activity (0.218 μmol/min/mg) (Table 4.3B, 6), it was applied to an ADP-agarose column.

Slightly more than half of the proteins bound to the ADP-agarose column and were eluted in a peak (A_{280} of peak top: 0.728), together with a small peak of K^+-EDTA-ATPase activity (peak top: 3.0 nmol/min/mg), by 0.17-0.25 M KCl. No Ca^{2+}-ATPase activity was present in the bound fraction. A peak of K^+-EDTA-ATPase activity (peak top: 136.2 nmol/min/ml) and Ca^{2+}-ATPase activity (peak top: 100.7 nmol/min/ml) was present in the flow-through fractions. No 165 kDa polypeptide was detected by immunoblotting in either the flow-through or bound fractions. No HMW proteins could be detected on either a Ponceau-stained blot or a

Coomassie blue-stained gel. The flow-through fractions contained proteins of < 97 kDa, and the bound fractions of < 80 kDa (data not shown). No particular protein seemed to be enriched in the bound fractions.

4.4 DISCUSSION

4.4.1 Partial purification of the 165 kDa polypeptide

The 165 kDa polypeptide was partially purified from mung bean, and was completely separated from the 155 and 110 kDa polypeptides by ADP-agarose affinity chromatography. The 165 kDa polypeptide was still contaminated with other proteins (Fig. 4.5B, 8; Fig. 4.9C, 4-9) so that its purification was far from complete. Mung bean protein sample was chromatographed on ADP-agarose in the absence of EDTA. As discussed in 3.4.3, EDTA inhibits most muscle ATP/ADP-binding enzymes which require a divalent cation to express activity and is used in purifying some nonmuscle myosins on ADP column. The binding of the 165 kDa polypeptide to ADP-agarose was unaffected by the presence or absence of EDTA, consistent with it having EDTA and other ATPase activity [3.3.5 (iv) (b)]. However, numerous other proteins were retained on the column in both experiments.

The total protein yield of this partially purified 165 kDa polypeptide fraction was low, 0.02-0.05% (Table 4.2, 6; Table 4.1, 7) or 0.041-1.0 mg/100 g tissues. Since the partial purified fraction was far from homogenous, the protein yield of the 165 kDa polypeptide itself would be still lower. The protein yields of some nonmuscle myosins purified so far are: 0.2% from tomato (Vahey *et al.* 1982), 0.28% from human platelet (Pollard *et al.* 1974), 5-10% from *Drosophila* (Kiehart & Feghali 1986), 0.5% for *Acanthamoeba* myosin II (Pollard *et al.* 1978), 0.005% for *Dictyostelium* myosin I (Cote *et al.* 1985), and 0.02% /0.028% /0.076% for *Acanthamoeba* myosins IA /IB /IC (Maruta *et al.* 1979) respectively. Therefore, purified plant myosin and some myosins I of lower eukaryotic cells generally have low protein yields, probably due to their low abundance in cells, whereas some nonmuscle cells give much high protein yields of purified myosins.

If protein yield is in terms of mg/100 g tissues, purified *Nitella* myosin has a yield of 0.1 mg/100 g tissues (Kato & Tonomura 1977), while the partially purified

myosin fraction of *Egeria densa* has a total protein yield of only 0.014-0.071 mg/100 g tissues (Ohsuka & Inoue 1979).

4.4.2 ATPase activities during the partial purification of the 165 kDa polypeptide

During the partial purification of the mung bean 165 kDa polypeptide, fractions containing most of the 165 kDa polypeptide had only a very small fraction of the total K^+-EDTA-ATPase activity of the crude extract. Only 2.5% of the initial K^+-EDTA-ATPase activity remained in the 165 kDa polypeptide fraction eluted by 0.4 M NaCl from DE52 after batch processing (Table 4.2, 2) while 62.9% of the K^+-EDTA-ATPase activity went to the unbound fraction that was depleted in the 165 kDa polypeptide (Table 4.3B, 2). This was the step of the purification scheme where both the yield and specific activity of the K^+-EDTA-ATPase decreased most drastically from those of the crude extract. When the 0.4 M NaCl eluate was fractionated with $(NH_4)_2SO_4$, the 0-45% fraction containing the 165 kDa polypeptide had a K^+-EDTA-ATPase activity yield of 0.6%, slightly higher than that of the 45-85% fraction, but its specific K^+-EDTA-ATPase activity was much lower than that of the 45-85% fraction (Table 4.2, 3; Table 4.3A, 2). This accorded with the results of $(NH_4)_2SO_4$ fractionation of crude extracts discussed before (3.3.3; 3.4.2). Therefore, the 165 kDa polypeptide fraction retained only a small part of the total K^+-EDTA-ATPase activity and had a low specific ATPase activity.

When the 0-45% $(NH_4)_2SO_4$ fraction was resolved on another DE52 column using gradient elution, neither the 165 nor 155 kDa polypeptide coincides with the peak ATPase activity. The 165 kDa polypeptide was eluted in the tail of the peak of K^+-EDTA-ATPase activity and the 155 kDa polypeptide even later (Fig. 4.8). The 165 kDa polypeptide at most accounts for only a small part of the K^+-EDTA-ATPase activity in the 0-45% $(NH_4)_2SO_4$ fraction and therefore was at most a minor K^+-EDTA-ATPase in mung bean crude extract. Nevertheless, it remains

possible that the ATPase peak came from a small fraction of the active 165 kDa polypeptide eluting rather earlier and at the detection limit for immunoblotting.

The specific activity of the K^+-EDTA-ATPase in the 165 kDa polypeptide fractions decreased during purification to 0.0022 μmol/min/mg (Table 4.2, 5) prior to ADP-agarose affinity chromatography which increased it 7.3-fold to 0.016 μmol/min/mg (Table 4.2, 6). The binding of the 165 kDa polypeptide to ADP-agarose in the presence of EDTA [3.3.5 (iv) (b)] and the increase of the K^+-EDTA-ATPase specific activity of the ADP-agarose purified 165 kDa polypeptide fraction were consistent with the 165 kDa polypeptide having K^+-EDTA-ATPase activity. Since the 165 kDa polypeptide at most accounted for a minor part of the K^+-EDTA-ATPase activity of mung bean extract, any increase in its specific ATPase activity during earlier purification steps could be masked by the removal of other ATPase(s) exhibiting high K^+-EDTA-ATPase activity. Therefore, an increase in the specific K^+-EDTA-ATPase activity of the 165 kDa polypeptide need not be expected at all steps of purification.

However, the failure to observe a continuous increase in the K^+-EDTA-ATPase specific activity during the purification of the 165 kDa polypeptide and the impurity of the ADP-agarose purified fraction make it uncertain that the 165 kDa polypeptide actually had K^+-EDTA-ATPase activity although its retention on ADP-agarose in EDTA suggests that it did. Most or all (if the 165 kDa polypeptide showed no ATPase activity) K^+-EDTA-ATPase activity of mung bean extract was in any event accounted for by non-myosin ATPase(s) and/or by myosin isoform(s) which was/were not identified by anti-pan myosin antibody (3.4.2).

In other plants, where myosin purification was usually monitored by ATPase assays, the specific activity of K^+-EDTA- or Ca^{2+}-ATPase increases in purified or partially purified fractions. However, full record of the ATPase activities at each purification step is only available in the case of the 130 kDa tomato myosin, which shows that the K^+-EDTA-ATPase specific activity increases during purification. The K^+-EDTA-ATPase specific activity (μmol/min/mg) is 0.15 (Kato & Tonomura

1977) and 0.126 (Vahey *et al.* 1982) in the purified myosins of *Nitella* and tomato, respectively; and is 0.0736 in the partially enriched putative pea myosin fraction (Ma & Yen 1989). These are higher than the 0.016 μmol/min/mg of the partially purified 165 kDa polypeptide fraction of mung bean.

Furthermore, Mg^{2+}-ATPase activities of these purified or partially purified myosins or putative myosins are activated by actin filament at low ionic strength as is the Mg^{2+}-ATPase activity of the purified myosin from *Heracleum* (Turkina *et al.* 1987). In addition to ATPase activities characteristic of myosin, other criteria have been used to identify these purified proteins as myosins: purified *Nitella* myosin forms bipolar filaments *in vitro* (Kato & Tonomura 1977); the 130 kDa tomato myosin binds to muscle F-actin (Vahey *et al.* 1982) and the *Heracleum* myosin forms bipolar filament and binds to muscle F-actin (Turkina *et al.* 1987).

The partially purified mung bean 165 kDa polypeptide fraction did not exhibit any actin activation of its Mg^{2+}-ATPase activity (Table 4.2, 6), and thus this important piece of enzymatic evidence that the 165 kDa polypeptide is a myosin heavy chain is still lacking. Nevertheless, there are substantial differences in ATPase activities of various nonmuscle myosins and not all myosins exhibit actin-activated Mg^{2+}-ATPase activity (4.1). The ATPase activity of the partially purified *Egeria* myosin at high ionic strength is maximal in Ca^{2+} (0.006 μmol/min/mg), lower in Mg^{2+} and lowest in K^{+}-EDTA (Ohsuka & Inoue 1979). Its Mg^{2+}-ATPase activity is not affected by muscle F-actin at low ionic strength. However, because it forms bipolar filament *in vitro,* it is considered a myosin. The differences in the actin-activated Mg^{2+}-ATPase activities may reflect differences in assay conditions, presence of contaminants, or basic enzymatic properties of the myosin (Taylor & Condeelis 1979). It is not known whether the partially purified 165 kDa polypeptide shared the same property of exhibiting no actin-activated Mg^{2+}-ATPase as the partially purified *Egeria* myosin but several potential explanations can be foreseen.

It is known that purification of an enzyme from plant is difficult because of the low protein content, highly active proteases and inactivation by oxidative enzymes and phenolics (Anderson 1968; Loomis 1974; Wu & Wang 1984; Cremer & Van de Walle 1985). Proteases destroyed the mung bean 165 kDa polypeptide in the absence of protease inhibitors, which could be inhibited to some degree by protease inhibitors included in the extraction buffer (3.3.4). Since the 165 kDa polypeptide in the 0-45% $(NH_4)_2SO_4$ fraction of the crude extract was stable for up to 77 h in the absence of protease inhibitors (3.3.4), protease inhibitors were not included in buffers at the later steps after tissue extraction. Enzyme protective compounds including sodium metabisulfite, EDTA and DTT were added in the extraction buffer, and DTT was included in all buffers. The purification was a long process, however, and part of the 165 kDa putative myosin heavy chain may be degraded by proteolysis and/or inactivated by other detrimental compounds of mung bean. Therefore, it is possible that the low yield of K^+-EDTA-ATPase activity and the absence of actin-activated Mg^{2+}-ATPase activity was due to the degradation and/or denaturation of this protein during purification, and including protease inhibitors at later steps of the purification may preserve the activity of putative myosin to some degree.

For nonmuscle myosins, the actin-activated Mg^{2+}-ATPase activity is regulated by different systems (1.7), and thus many myosins require activating enzymes to express their actin-activated ATPase activities, such as the heavy chain kinase of *Acanthamoeba* myosin I (Pollard & Korn 1973a; Maruta & Korn 1977b) or light-chain kinases of other nonmuscle and smooth muscle myosins (Adelstein & Conti 1975; Dabrowska *et al.* 1977; Kuznicki 1986; Kuznicki & Barylko 1988; Trybus 1991). When the activating enzyme is separated from myosin during purification, the myosin loses its actin-activated ATPase activity.

Enzyme-regulated actin-myosin interaction has been implicated in some plants, such as the Ca^{2+}-activated protein kinase/phosphatase model in *Chara* (1.8.4). Such enzymes may also be present in other plants and essential for myosins to

express their actin-activated Mg^{2+}-ATPase activities. There was 1 mM EDTA in the extraction buffer but it might not keep Ca^{2+} sufficiently low to inactivate possible mung bean CDPK. The absence of the actin-activated Mg^{2+}-ATPase activity in the partially purified 165 kDa polypeptide fraction may be caused by the separation of some activating enzyme or other cofactor from the 165 kDa polypeptide during the purification. Adding back fractions to the 165 kDa polypeptide fraction could be tested to find any activating enzymes. However, to date no such activating enzymes or other cofactors have been reported to be essential for expressing the actin-activated Mg^{2+}-ATPase activities of the purified or partially purified plant myosin or putative myosin.

4.4.3 Attempted purification of a K^+-EDTA-ATPase from fractions unused in the partial purification of the 165 kDa polypeptide

As discussed in 4.4.2, most K^+-EDTA-ATPase activity of the mung bean crude extract with high specific activity was in the unbound fraction from the DE52 batch processing, and may be contributed by non-myosin ATPase(s) and/or myosin isoform(s) which was/were not identified by anti-pan myosin antibody. When fractionating the unbound fraction, most K^+-EDTA-ATPase activity flowed through the phosphocellulose column and through the subsequent hydroxylapatite column, as did the bulk of the protein (Table 4.3B, 4 and 5). All these unbound and flow-through fractions maintained a high K^+-EDTA-ATPase specific activity, which made it impossible to isolate any particular ATPase fraction. When the 0.095-0.19 M NaCl fraction from the phosphocellulose was applied to the ADP-agarose column, most K^+-EDTA-ATPase activity flowed through. Since the salt concentration of the 0.095-0.019 M NaCl phosphocellulose fraction was desalted half by ultrafiltration rather than the usual dialysis, the salt concentration in the sample may be a bit high for some proteins to bind to the ADP column.

When purifying *Acanthamoeba* myosin I, three isoforms of myosin I can be eluted as three discrete peaks from a phosphocellulose column by a linear 0-0.6 M

KCl gradient (Lynch *et al.* 1989). Cation exchange chromatography has also been used in the purification of putative myosin from onion (Pesacreta *et al.* 1991). However, no particular mung bean protein seemed to be resolved and enriched by phosphocellulose.

LMW proteins were obtained from both the flow-through (< 97 kDa) and bound (< 80 kDa) fractions of ADP-agarose. Myosin I heavy chains are usually 100-140 kDa (1.3). Several putative plant LMW myosins have been identified but not confirmed yet (1.8.1; 1.8.2). A 130 kDa myosin with a heavy chain of 100 kDa has been purified from tomato (Vahey *et al.* 1982), but the possibility of its being a proteolytic product of a HMW myosin has not been excluded. Another two LMW putative myosin heavy chains identified only by immunoblotting of plant TCA extracts are the 110 kDa polypeptide of *Chara* (Grolig *et al.* 1988) and the 85 kDa one of *Ernodesmis* (La Claire 1991). The ADP-bound proteins of < 80 kDa were well below the M_r range of authentic myosin I and below that of the smallest putative myosin I identified so far. It was therefore considered doubtful whether the bound proteins contained any possible myosin isoform.

When fractionating the 45-85% $(NH_4)_2SO_4$ fraction of the 0.4 M NaCl eluate from DE52 on another DE52 column, all K^+-EDTA-ATPase activity bound and the proteins eluted in one peak. As a result, isolating any protein with the appropriate ATPase activity of myosin by monitoring the K^+-EDTA-ATPase activity from the unused protein fractions of partially purifying the 165 kDa polypeptide was not successful. The widely spread K^+-EDTA-ATPase activity and poor resolution of proteins on chromatography during the fractionation make it impossible to isolate one particular protein exhibiting high K^+-EDTA-ATPase activity. Although other K^+-EDTA-ATPases in unused fractions of purifying the 165 kDa polypeptide suggested the presence of myosin isoform(s), no other polypeptides reacting with anti-pan myosin were found. The potential polypeptide(s) could have been below the detection limit in unpurified fractions or was/were not recognized by anti-pan

myosin. Examination of actin-activation of all these other ATPases may tell whether any were myosins.

In previous studies of protein purification of plant myosins and putative myosins (1.8.1), K^+-EDTA- or Ca^{2+}-ATPase were used to monitor the purification except in the case of onion putative myosin where an anti-myosin was also used (Pesacreta *et al.* 1991). Multiple peaks of K^+-EDTA- or Ca^{2+}-ATPase activity exist in tomato (Vahey *et al.* 1982) and pea (Ma & Yen 1989). Tomato myosin fractions were identified by additional assays of actin-activated Mg^{2+}-ATPase activity and actin binding (Vahey *et al.* 1982), while Ma & Yen (1989) do not show exactly how they conclude that a small fraction of very low Ca^{2+}-ATPase activity contains putative myosin. The purified or partially purified myosins or putative myosin fractions show highest K^+-EDTA-ATPase activity at high ionic strength and most also exhibit actin-activated ATPase activity at low ionic strength except the partially purified *Egeria* myosin (Ohsuka & Inoue 1979). When working on mung bean putative myosin, the lack of detectable actin-activated ATPase activity and the high K^+-EDTA-ATPase activities, probably unrelated to myosins, made definite biochemical identification impossible.

4.4.4 Conclusions

The 165 kDa polypeptide was partially purified from mung bean. The partially purified fraction had low K^+-EDTA-ATPase activity that increased with ADP-agarose chromatography and no detectable actin-activated Mg^{2+}-ATPase activity. The 165 kDa polypeptide at most accounted for only a very small fraction of the total K^+-EDTA-ATPase activity of mung bean extract. Its retention on ADP-agarose in the presence of EDTA was consistent with a K^+-EDTA-ATPase activity but until further purification is achieved this cannot be taken as proven. The K^+-EDTA-ATPase activity unassociated with the 165 kDa polypeptide may be contributed by non-myosin ATPase(s) and/or myosin isoform(s) which was/were

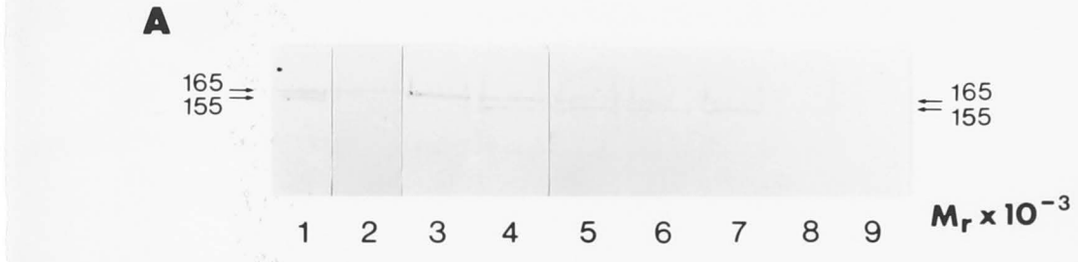
not identified by anti-pan myosin antibody. The lack of enzymatic evidence leave the identity of the 165 kDa polypeptide as a myosin heavy chain unconfirmed yet.

The quantity and purity of the fractions containing the 165 kDa polypeptide greatly limited its further biochemical characterization. Nevertheless, they provided a chance to raise monoclonal antibodies against this polypeptide. No antibody against a plant myosin has been reported so far and any antibodies against the mung bean 165 kDa polypeptide will show how extensive are its homologies with authentic myosins. The antibodies to the 165 kDa polypeptide were shown to cross-react with muscle myosin, and together with several commercial anti-myosin antibodies, were used to identify by immunoblotting putative myosin heavy chains in mung bean and several other plants. Cellular distributions of putative myosins were also characterized by immunofluorescence in mung bean root tip cells and *Chara* internodal cells. The raising of antibodies and immunochemical and immunocytochemical characterization of putative plant myosins will be discussed in Chapter 5.

CHAPTER 4

FIGURES

Fig. 4.3 DEAE-cellulose chromatography of the 165 kDa polypeptide fraction. 168 ml of the desalted 0-45% $(NH_4)_2SO_4$ fraction of the 0.4 M NaCl eluate (from the first DE52) were applied to a column (2.6 x 19 cm) of DE52 equilibrated with the starting buffer (0.34 M sucrose, 10 mM imidazole, pH 7.0, 1 mM DTT). The column was eluted with a gradient of 0-0.4 M NaCl in the starting buffer. The 165 kDa polypeptide was eluted between fractions 452.1 ml and 478.1 ml by 0.138-0.158 M NaCl, and these fractions were pooled for further purification on an ADP-agarose column. Immunoblots of selected fractions are shown at the top (A). (1), crude extract. Corresponding elution volumes (ml) of the other fractions were: (2), 454.1 ml; (3), 462 ml; (4), 470.1 ml; (5), 478.1 ml; (6), 482.8; (7), 491.7; (8), 505 ml; and (9), 598.8 ml.


A
165
155
165
155
1 2 3 4 5 6 7 8 9
$M_r \times 10^{-3}$

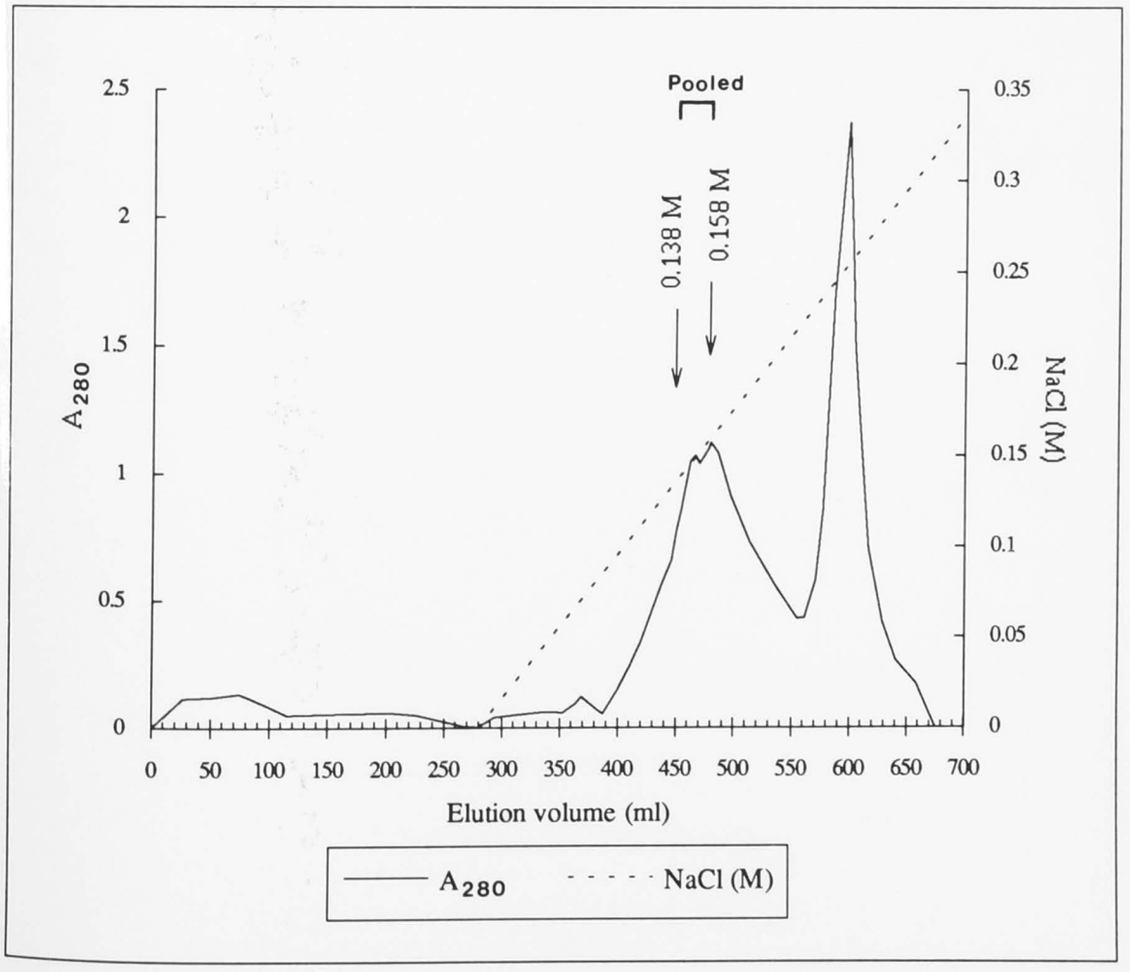
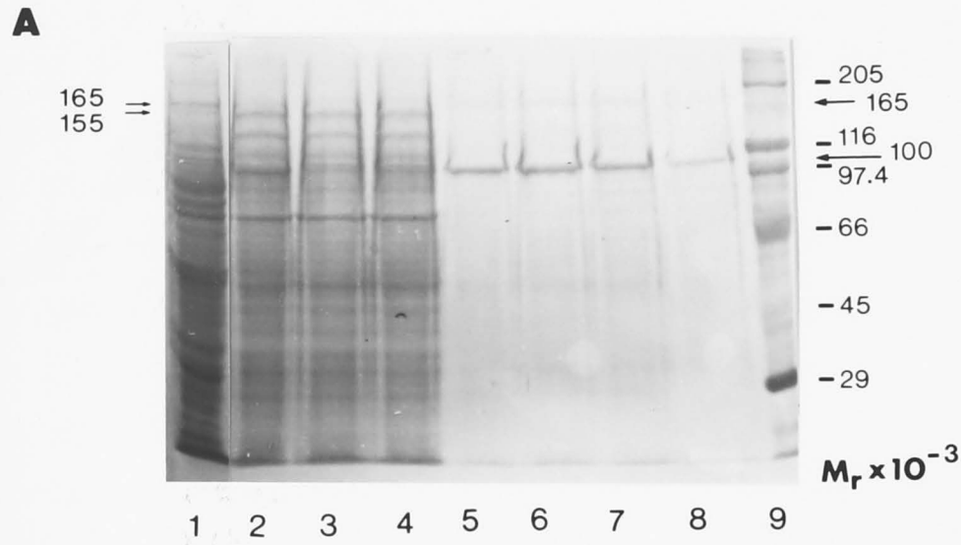

Pooled
0.138 M
0.158 M
A_{280}
NaCl (M)
Elution volume (ml)
A_{280}
NaCl (M)

Fig. 4.4 **ADP-agarose chromatography of the 165 kDa polypeptide fraction from the DE52 column.** 16 ml of the 0.138-0.158 M NaCl fraction from DE52 were applied to a column (1.6 x 2.5 cm) of ADP-agarose equilibrated with the starting buffer (0.34 M sucrose, 10 mM imidazole, pH 7.0, 1 mM DTT). The column was eluted with a gradient of 0-0.8 M KCl, and then 1 M KCl in the starting buffer. The 165 kDa polypeptide was eluted in a small protein peak, and fractions from 116.4 ml to 132 ml (0.29-0.43 M NaCl) were pooled. Selected fractions resolved by SDS-PAGE and the blots stained with Ponceau were shown at the top (A). (1), crude extract; (2), 0.138-0.158 M NaCl fraction applied to the ADP-agarose column; (9), SDS-6H. Corresponding elution volumes (ml) of the other fractions were: (3), 19.6; (4), 22; (5), 119; (6), 120.2; (7), 121.6; and (8), 122.9.


A
165
155
205
165
116
100
97.4
66
45
29
$M_r \times 10^{-3}$
1 2 3 4 5 6 7 8 9

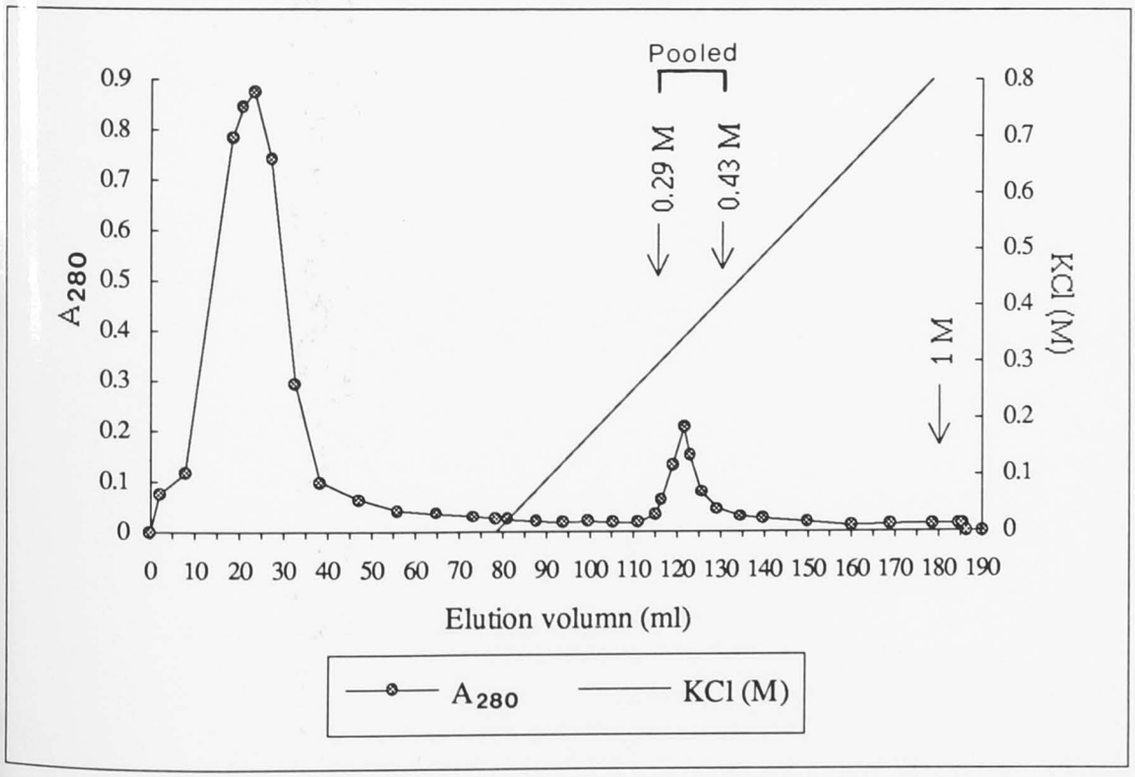

Pooled
0.29 M
0.43 M
1 M
A_{280}
KCl (M)
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170 180 190
Elution volumn (ml)
A_{280}
KCl (M)

Fig. 4.5 **Immunoblot of the 165 kDa polypeptide in fractions obtained during the partial purification of this polypeptide.** A, immunoblot; B, the same blot stained with Ponceau. (1), crude extract; (2), unbound fraction of DE52 batch processing; (3), 0.4 M NaCl eluate from the first DE52; (4), 0-45% $(NH_4)_2SO_4$ fraction of the 0.4 M NaCl eluate, desalted and concentrated; (5), 0.138-0.158 M NaCl fraction of the second DE52, concentrated and desalted; (6), flow-through (fraction 22 ml) of ADP-agarose; (7), pooled flow-through fraction of ADP-agarose; (8), 0.29-0.43 M KCl fraction of ADP-agarose, concentrated and desalted; and (9), SDS-6H.

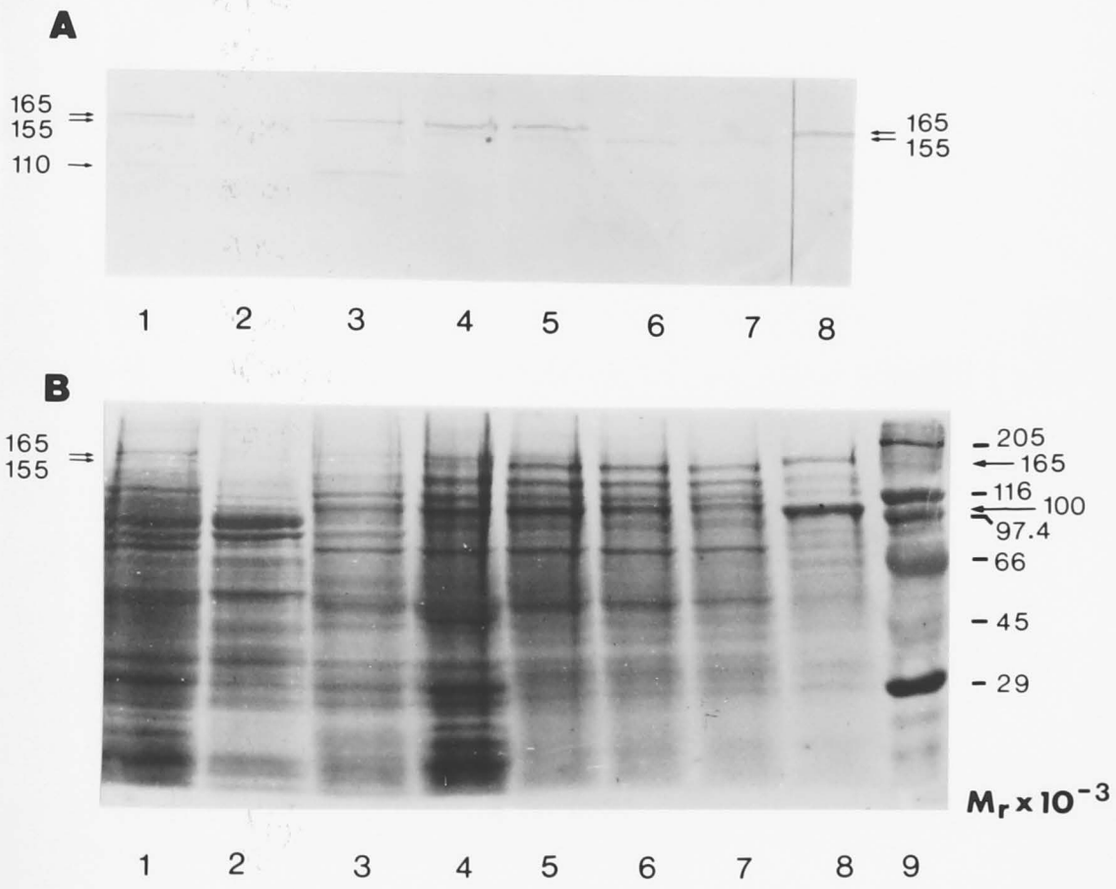
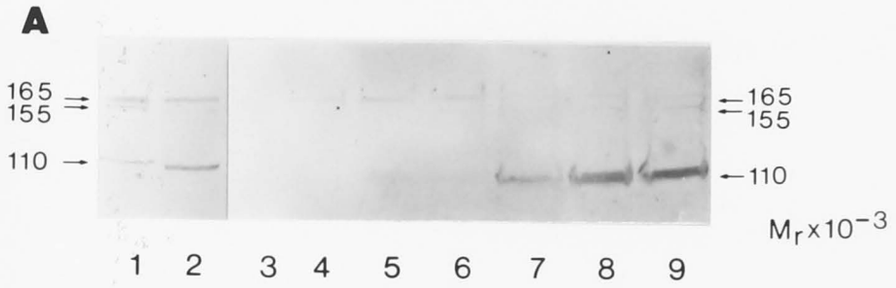

A
165
155
110
165
155
1 2 3 4 5 6 7 8
B
165
155
205
165
116
100
97.4
66
45
29
$M_r \times 10^{-3}$
1 2 3 4 5 6 7 8 9

Fig. 4.8 **DEAE-cellulose chromatography of the 165 kDa polypeptide fraction.** 163 ml of the desalted 0-45% $(NH_4)_2SO_4$ fraction of the 0.4 M NaCl eluate (from the first DE52) were applied to a column (2.6 x 18 cm) of DE52 equilibrated with the starting buffer (0.34 M sucrose, 10 mM imidazole, pH 7.0, 1 mM DTT). The column was eluted with a gradient of 0-0.3 M NaCl in the starting buffer (from fraction 243.6 ml). One peak of ATPase activities from fractions 397.7 ml to 457.2 ml was eluted by 0.095-0.13 M NaCl. By immunoblotting, the 165 kDa polypeptide was mainly eluted from fractions 450.2 ml to 471 ml by 0.126-0.139 M NaCl. Fractions from 397.7 ml (0.095 M NaCl) to 471 ml (0.139 M NaCl) were pooled for further purification on ADP-agarose column. Immunoblots of selected fractions are shown at the top (A). (1), crude extract; (2), 0-45% $(NH_4)_2SO_4$ fractions of the 0.4 M NaCl eluate applied to the second DE52 column. Corresponding elution volumes (ml) of the other fractions were: (3), 443.2; (4), 450.2; (5), 457.2; (6), 471; (7), 477.8; (8), 484.6; and (9), 498.3.


A
165
155
110
165
155
110
Mr x10⁻³
1 2 3 4 5 6 7 8 9

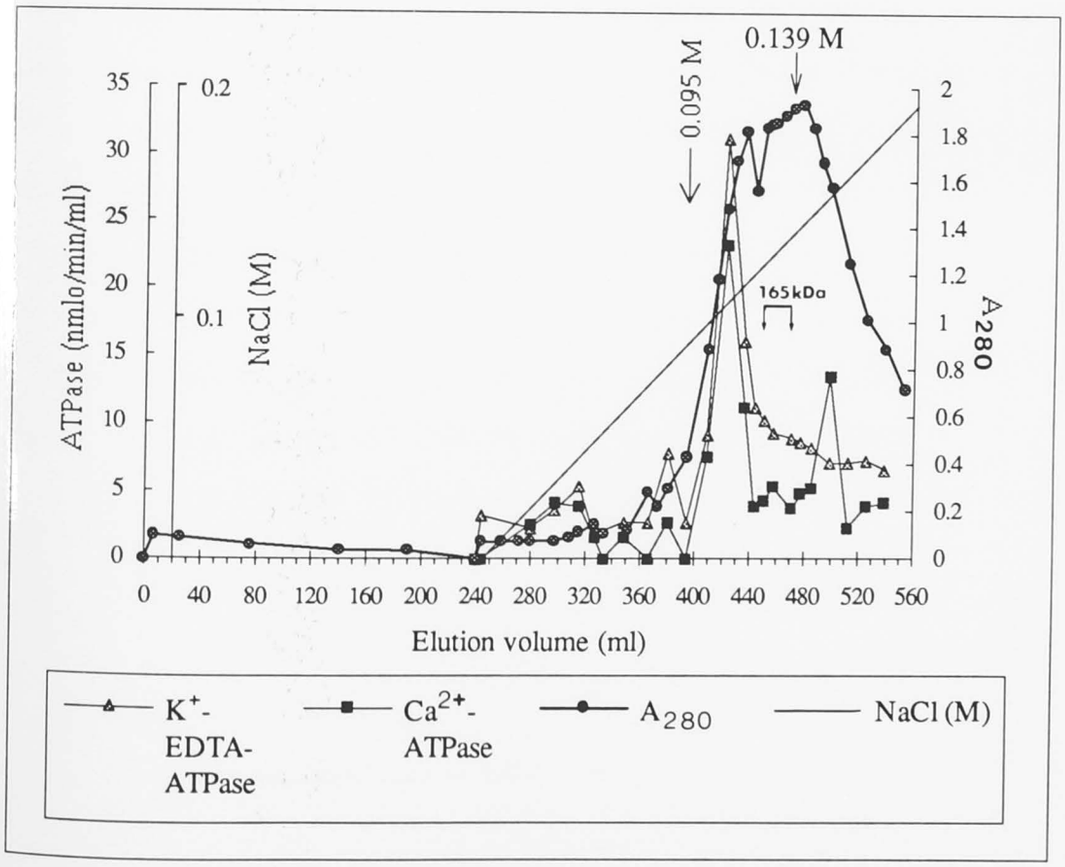

0.139 M
0.095 M
165kDa
ATPase (nmlo/min/ml)
NaCl (M)
A280
Elution volume (ml)
K+-EDTA-ATPase
Ca2+-ATPase
A280
NaCl (M)

Fig. 4.9 ADP-agarose chromatography of the 165 kDa polypeptide fractions from a DE52 column. 26.3 ml of the 0-45% $(NH_4)_2SO_4$ fraction of the 0.095-0.139 M NaCl fraction from the second DE52 column were applied to a column (1.6 x 2.5 cm) of ADP-agarose equilibrated with the starting buffer (0.34 M sucrose, 10 mM imidazole, pH 7.0, 1 mM DTT). The column was eluted with a gradient of 0-0.8 M KCl in the starting buffer (from fraction 213.1 ml). The 165 kDa polypeptide was eluted in a small protein peak, and fractions from 221.2 ml to 227.9 ml (0.076-0.13 M KCl) were pooled. Part of the whole elution profile, from fractions 211.7 ml to 261.7 ml, is shown separately in (A). Immunoblots (B) and Ponceau-stained blots (C) of selected fractions are shown below (A). (1), crude extract; (2), 0-45% $(NH_4)_2SO_4$ fraction applied to the ADP-agarose column; (10), SDS-6H. Corresponding elution volumes (ml) of the other fractions were: (3), 27; (4), 221.2; (5), 222.5; (6), 223.9; (7), 225.2; (8), 226.6; and (9), 227.9.

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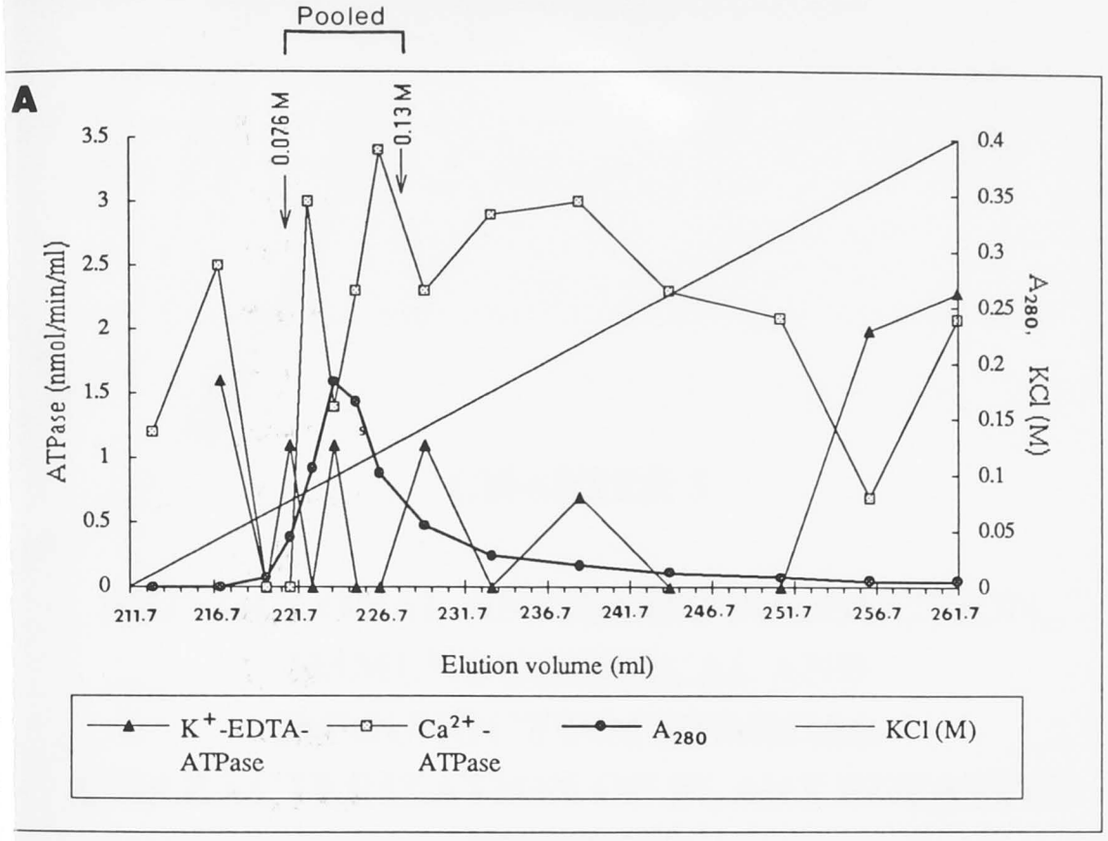
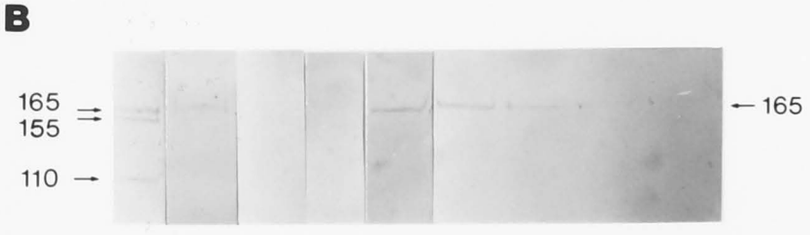
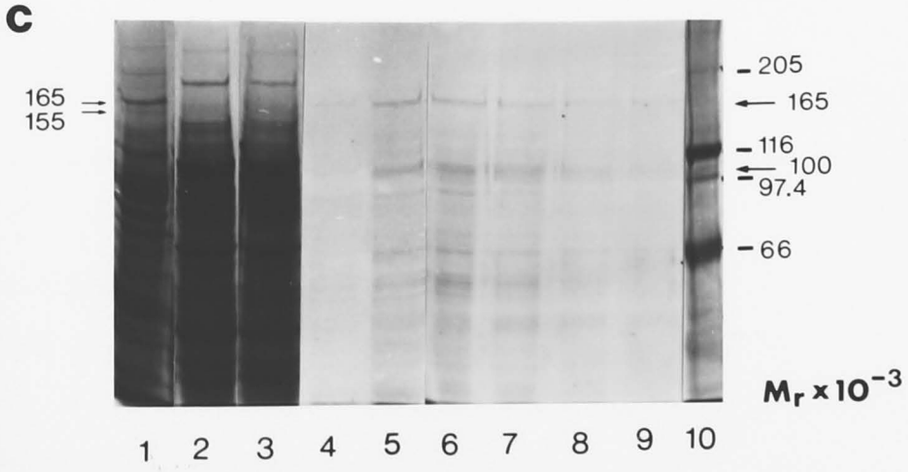

Pooled
A
0.076 M
0.13 M
ATPase (nmol/min/ml)
A_{280}, KCl (M)
Elution volume (ml)
211.7
216.7
221.7
226.7
231.7
236.7
241.7
246.7
251.7
256.7
261.7
K^+-EDTA-ATPase
Ca^{2+}-ATPase
A_{280}
KCl (M)


B
165
155
110
165
1 2 3 4 5 6 7 8 9


C
165
155
205
165
116
100
97.4
66
$M_r \times 10^{-3}$
1 2 3 4 5 6 7 8 9 10

CHAPTER 5

MONOCLONAL ANTIBODY PRODUCTION, IMMUNOCHEMICAL AND IMMUNOCYTOCHEMICAL CHARACTERIZATION OF PLANT PUTATIVE MYOSINS

CHAPTER 5

MONOCLONAL ANTIBODY PRODUCTION, IMMUNOCHEMICAL AND IMMUNOCYTOCHEMICAL CHARACTERIZATION OF PLANT PUTATIVE MYOSINS

5.1 INTRODUCTION

Monoclonal antibody production allows experimenters to use impure antigens to produce specific antibodies that recognize particular sites on antigens known as epitopes (Harlow & Lane 1988). Epitopes on protein antigens are local surface structures that can be formed by contiguous or noncontiguous amino acid sequences. A monoclonal antibody recognizes only one specific epitope and thus can be used to detect that epitope. Because monoclonal antibodies recognize relatively small regions of antigens, occasionally they can find similar epitopes on other molecules, which forms the molecular basis for cross-reaction. Therefore, monoclonal antibodies can be used to detect similar epitopes on related molecules, like those of proteins such as myosins which contain highly conserved regions.

Different proteins sharing a functional relationship may have similar epitopes. Nine monoclonal antibodies against *Acanthamoeba* myosins I (IA and IB) also cross-react with a 34 kDa nuclear actin-binding protein of *Acanthamoeba* (Kiehart *et al.* 1984; Hagen *et al.* 1986; Rimm & Pollard 1989). Since the 30 kDa C-terminus region of *Acanthamoeba* myosin I heavy chain contains the unique ATP-independent actin-binding site (1.3), it is suggested that these antibodies may recognize some features of the actin-binding site alone and result in the cross reactivity between myosin I and the nuclear actin-binding protein. However, the presence of similar epitopes does not necessarily imply a functional relationship. A monoclonal antibody against α-tubulin also cross-reacts with performic acid-oxidized actin and *E. coli* rec A protein that is the catalytic subunit of the cyclic AMP-dependent muscle protein kinase (Wehland *et al.* 1984).

Myosin antibodies have been one of the most useful tools in studying the structure and function of nonmuscle myosin. Myosin antibodies microinjected into cells cause changes in cellular activity that have been used to explore myosin's function in cell motility (Mabuchi & Okuno 1977; Kiehart *et al.* 1982; Sato & Grasser 1990). Antibodies directed against specific fragments of myosin have helped in dissecting the functional domains of myosin molecule and the mechanism of myosin-actin interaction (Dan-Goor *et al.* 1990; Dan-Goor & Muhlrad 1990, 1991). Immunoblotting identifies the presence and polypeptide composition of putative myosins from unpurified protein fraction. Immunofluorescence is widely used to determine the intracellular distribution of putative nonmuscle myosin and actin.

Immunofluorescent localization of plant actin has been well characterized recently (Clayton & Lloyd 1985; Parthasarathy *et al.* 1985; Seagull *et al.* 1987; Traas *et al.* 1987; Kakimoto & Shibaoka 1987a, b; Schmit & Lambert 1987; Palevitz 1987a, b, 1988; McCurdy *et al.* 1988; Tang *et al.* 1989a; McCurdy & Gunning 1990; Lloyd 1988; Heslop-Harrison & Heslop-Harrison 1989b, c, 1991). Immunofluorescent localization of plant putative myosin, however, is much less characterized than those of plant actin and other nonmuscle myosins. It is believed that plant myosin and actin interact in a similar way to that involved in the sliding filament mechanism of muscle actomyosin. The colocalization of myosin and actin implies that actomyosin may be involved in many cellular functions, as seen in the many forms of cell motility shown by nonmuscle cells (1.6).

Putative myosin has been immunofluorescently localized in *Euglena* (Lonergan 1985), *Ernodesmis* (La Clair 1991), *Chara* internodal cells (Grolig *et al.* 1988), onion root tip cells (Parke *et al.* 1986) and *Nicotiana* pollen tubes (Tang *et al.* 1989a; Heslop-Harrison & Heslop-Harrison 1989a). Putative myosin and actin are colocalized to pellicle strips of *Euglena* (Lonergan 1985), *Chara* subcortical actin bundles (Grolig *et al.* 1988), longitudinal bundles and a reticulum during wound-induced cytoplasmic contractions of *Ernodesmis* (La Clair 1991), and

phragmoplasts of mung bean root tip cells (Parke *et al.* 1986). The immunofluorescent localization of putative myosin, especially the colocalization of putative myosin and actin, helps to elucidate functions of plant myosin and mechanisms of cytoplasmic streaming in plants (1.8.3).

The 165 kDa polypeptide was identified as a putative myosin heavy chain of mung bean myosin in previous chapters. Since anti-pan myosin recognized both the 165 and 155 kDa polypeptides of mung bean, further monoclonal antibodies against the 165 kDa polypeptide were raised in this chapter. Antibodies to the mung bean 165 kDa polypeptide and several commercial anti-myosin antibodies were used in immunoblotting to detect epitopes on proteins of mung bean, pea, wheat, *Arabidopsis* and *Chara,* and to see how many epitopes various plant proteins share with the heavy chain of rabbit skeletal muscle myosin. The antibodies to the 165 kDa polypeptide, anti-myosin and anti-actin antibodies were also examined in immunofluorescent labelling of *Chara* and mung bean root tip cells in order to localize putative myosin and actin structures and thus reveal the roles in which plant myosin and actin may be involved.

5.2 MATERIALS AND METHODS

5.2.1 Production of monoclonal antibodies (MB165) against the mung bean 165 kDa polypeptide

Animal handling and hybridoma culturing was performed by Ms. Janet Elliott of the Plant Cell Biology Group.

(i) Antigen

Partially purified mung bean 165 kDa polypeptide (the desalted and concentrated 0.29-0.43 M KCl ADP-agarose fraction in 4.3.1), and mung bean crude extract were resolved by 7% SDS-PAGE, transferred to nitrocellulose, and stained with Ponceau. The clear 165 kDa band of the partially purified fraction (Fig. 4.5B, lane 8) or the crude extract (Fig. 4.5B, lane 1) was cut out cautiously to avoid contamination, and destained completely with several washes of dH_2O. The air-dried nitrocellulose could also be stored at -20°C for a few weeks before use. Ten nitrocellulose strips of the 165 kDa band were ground into small pieces in a mortar and pestle (with or without liquid nitrogen), suspended with phosphate-buffered saline (PBS: 2.68 mM KCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4, 0.137 M NaCl; 700-800 µl for bands from partially purified fraction, 500 µl for crude extract) and used as antigen.

(ii) Antibody production

All immunization and boosts were done intraperitoneally. 400 µl of antigen (from 43.2 µg of the partially purified fraction) were injected into each of two 8-week female BALB/c mice. Each mouse was boosted at week 2 (350 µl of the same antigen from 45.9 µg of the partially purified 165 kDa polypeptide fraction) and at weeks 13, 16, 19 and 21 (500 µl of the 165 kDa polypeptide antigen from 530 µg of mung bean crude extract each time). 20-30 µl of blood were collected from the orbital sinus and immunoblotted to test for antibody production.

Three days after the final boost, splenocytes from the mouse with positive immune response were fused with Sp_2O mouse myeloma cells by polyethylene glycol (PEG) 1500. Hybridoma lines were screened by immunoblotting 11 days

after the fusion. From 22 positive lines, 4 were selected, cloned by limiting dilution and injected into 2,6,10,14-tetramethylpentadecane primed BALB/c mice to produce ascites fluids. Ascites fluids were stored with 0.05% NaN_3 at 0-4°C, or at -80°C for later use. Four ascites fluids, containing antibodies to the mung bean 165 kDa polypeptide antibody (MB165) were named: MB165/1, MB165/2, MB165/3 and Mb165/4 (clone numbers: E3.20 2D10/44, E3.20 3G5/64, E3.20 4G3/23 and E3.20 3E9/29, respectively). All were IgM (J. Elliott, personal communication).

5.2.2 Immunoassays used in monoclonal antibody production

Immunoblotting was used to test sera after immunization and for routine screening during hybridoma production. Enzyme-linked immunosorbant assay (ELISA) was used to confirm positive clones screened by immunoblotting.

(i) Immunoblotting

Blots of mung bean crude extract [2.5.1 (i)] were used. These could be air-dried blots after Ponceau staining and stored at -20°C before use. A Miniblot apparatus (Bio-Rad) allowed 24 samples to be processed using, at various times, both alkaline phosphatase and peroxidase detection. Pre- and post-immunization sera were tested at dilutions of 1: 50 and 1: 100 while culture supernatants were used neat. Anti-pan myosin was used as a positive control, hybridoma culture medium as a negative control.

(ii) ELISA

ELISA was carried out in a 96-well polyvinylchloride (PVC) microtiter plate at room temperature using 50 µl aliquots of each solution. Additions were: mung bean crude extract, diluted 1: 1 with PBS to give 45-60 µg of protein/50 µl (1 h); hybridoma culture supernatant (1 h); sheep anti-mouse Ig, biotinylated whole antibody (1: 1000 dilution; Amersham) (1 h); streptavidin-biotinylated peroxidase complex (1: 500 dilution; Amersham) (0.5 h). Antibodies were diluted with PBS-Tween (0.05% v/v). Plates were washed 3 times with PBS-Tween between each step. The plate was incubated with 0.2 M 2,2'-azino-bis(3-ethylbenzthiazoline-6-

sulphonic acid), 2.6 mM citric acid, 2.4 mM sodium citrate, pH 4.5 for 20 min, color development stopped with 30 mM NaF solution and A_{405} values determined in a plate reader (Titertek Multiskan Plus MK II; Flow Laboratories Australasia Pty. Ltd., North Ryde, NSW., Australia). The mean A_{405} of controls (tissue culture supernatant with PBS-Tween) was subtracted from each reading.

5.2.3 Preparation of protein samples for immunoblotting

(i) Immunoblotting with anti-myosin and MB165

(a) Higher plants

For mung bean, pea and wheat, proteins were extracted as described in 2.5.1 (i). *Arabidopsis* seedlings were extracted similarly [2.5.1 (i)] but concentrated by mixing 900 µl of crude extract with an equal volume of ice-cold 20% (w/v) TCA. The protein precipitates were collected after 1 h on ice, washed as described in 2.5.2 and the dried pellet resuspended with 300 µl of 1x SDS-PAGE sample buffer.

(b) *Chara*

Protein extracts of *Chara* were prepared as described before (Grolig *et al.* 1988).

(c) Rabbit skeletal muscle myosin

Purified rabbit skeletal muscle myosin (2.15) was diluted 1: 1 with dH_2O, and mixed 1: 1 with 2x SDS-PAGE sample buffer.

(ii) Immunoblotting with anti-actin

(a) Chicken gizzard actin

Acetone powder of chicken gizzard was prepared according to Ebashi (1985). Actin was extracted from a small amount of acetone powder with 1 mM $NaHCO_3$, clarified at 7,000 rpm for 2.5 min, and the supernatant mixed 1: 1 with 2x SDS-PAGE sample buffer.

(b) *Chara*

Chara internodal cells were perfused with ATP-containing perfusion solution (ACPS; Grolig *et al.* 1988) for 13 min to remove the tonoplast and the bulk of

streaming endoplasm. Alternatively, *Chara* actin was selectively removed by perfusing cells with ACPS for 3 min and then with low salt solution (Williamson *et al.* 1985). After perfusion, the contents of 7 cells of each perfusion type were squeezed into 90 µl of 20% (w/v) TCA solution [5.2.3 (i) (b)], and the pellet resuspended with 90 µl of 2x SDS-PAGE sample buffer.

All samples were prepared as described in 2.8 for SDS-PAGE.

5.2.4 Immunoblotting

(i) With anti-myosin and MB165

In 7% SDS-PAGE, usually 30-35 µl of mung bean, 20 µl of *Arabidopsis*, 18-20 µl of pea, wheat or *Chara* sample, or 4 µl of rabbit skeletal muscle myosin were loaded into a single sample well. Antibodies (2.11) in the following table were tested.

Antibody	Mung bean	Pea	Wheat	*Arabidopsis*	*Chara*	Muscle
anti-skeletal myosin (fast)	neat	neat	neat	neat	neat	1: 800
anti-myosin (smooth and skeletal	neat	neat	neat	neat	neat	1: 800
anti-fast myosin	neat	neat	neat	neat	neat	1: 800
anti-pan myosin	1: 100	1: 100	1: 100	1: 100	1: (1-40)	1: 300
MB165/1	1: 100	1: 100	1: 100	1: 100	1: 80	1: 200
MB165/2	1: 100	1: 100	1: 100	1: 100	1: 80	1:200
MB165/3	1: 200	1: 200	1: 200	1: 200	1: 100	1: 200
MB165/4	1: 150	1: 150	1: 150	1: 150	1: 100	1: 200

(ii) With anti-actin

4 µl of chicken gizzard actin and 30 µl of *Chara* proteins were resolved by 12% SDS-PAGE and immunoblotted with C4 anti-actin (1: 400).

5.2.5 Immunofluorescent staining

Antibodies used are listed below.

Antibody	*Chara*	Mung bean
anti-pan myosin	neat	1: 50
anti-fast myosin	1: 2	-
MB165/1	1: 200	1: 400
MB165/2	1: 50	1: 100
MB165/3	1: 200	1: 400
MB165/4	1: 50	1: 100
C4 anti-actin	1: 400	1: 400

(i) *Chara*

A *Chara* internodal cell was perfused with ATP-free perfusion solution (AFPS; Grolig *et al.* 1988) for 1-2 min to remove the tonoplast and the bulk of streaming endoplasm. All later steps were performed by perfusion (personal communication from Dr. Geoffrey O. Wasteneys). The cell was fixed with 1% (v/v) glutaraldehyde in AFPS for 20 min. The cell was washed for 5 min with AFPS, then for 2 x 5 min with PBS containing 0.02% (w/v) NaN_3 (PBS/NaN_3; PBS was 0.131 M NaCl, 5.11 mM Na_2HPO_4, 1.56 mM KH_2PO_4, pH 7.4). The cell was treated for 2 x 5 min with 1 mg/ml sodium borohydride freshly made up with PBS/NaN_3, then washed with PBS/NaN_3 to remove any bubbles caused by sodium borohydride. Sodium borohydride treatment was used to reduce glutaraldehyde-induced fluorescence, but was eliminated when using anti-pan myosin, MB165/2 and MB165/4. Then, the cell

was blocked three times with 0.8% BSA (w/v) in PBS/NaN_3 (BSA/PBS/NaN_3). Antibodies were diluted with BSA/PBS/NaN_3. All primary antibodies were detected with FITC-conjugated sheep anti-mouse Ig (affinity purified; Silenus Laboratories, Dandenong, Australia) at 1: 40 dilution. Each antibody was incubated for 30 min, followed by 3 x 5 min washes with BSA/PBS/NaN_3. The cell was finally washed for 2 x 3 min with PBS/NaN_3. The cell was bisected, and the cell piece was mounted on a coverslip with its cytoplasmic side facing down. In controls, the primary antibodies were replaced with BSA/PBS/NaN_3.

(ii) Mung bean

(a) Single labelling

Mung bean (2.1) root tips about 1.5 mm long were fixed for 1 h with fresh 4% (w/v) paraformaldehyde in phosphate-EGTA buffer (PE: 50 mM KH_2PO_4-K_2HPO_4, pH 6.8, 5 mM EGTA) and then washed for 3 x 10 min with PE. The root tips were digested for 25 min with 1% (w/v) cellulysin (Calbiochem Corp., La Jolla, CA., USA) in 0.4 M mannitol, and washed 3 x 5 min with PE. All steps used constant gentle shaking.

Coverslips cleaned in acetone and 70% ethanol were coated with 1 mg/ml poly-L-lysine in PBS. The root tips were squashed gently between pretreated coverslips, large lumps removed and the coverslips air-dried. Cells were extracted for 10 min with 1% (v/v) Triton X-100 in PE, washed for 3 x 5 min with PE, extracted for 10 min with methanol at -20°C and rinsed briefly with PE.

Antibodies (see Table) diluted with 1% (w/v) BSA in PBS were detected as for *Chara*. Each antibody incubation was carried out for 1 h in a wet chamber with cell sides of the coverslips facing down onto the antibody solutions on Parafilm, followed by 3 x 5 min washes with PE. Nuclei were stained for 30 seconds with 4, 6-diamidino-2-phenylindole (DAPI, 0.1 mg/ml in PBS) and rinsed twice with PE for 2 min each. In the control, the primary antibody was replaced with BSA/PBS.

(b) Double labelling

Anti-pan myosin antibody (IgM) and MB165/1 (IgM) were used in double labelling with C4 (IgG_1). IgM antibody was visualized by FITC-conjugated sheep anti-mouse IgM (Serotec, Oxford, UK) and IgG_1 by rhodamine-conjugated anti-mouse IgG_1 (given by Dr. David McCurdy of this Group), both used at 1: 50 dilution. Anti-pan myosin antibody or MB165/1 labelling was carried out first followed by C4 labelling. Each antibody was incubated for 1 h. Other steps were the same as those used in the single labelling.

In controls, incubation with anti-pan myosin, MB165/1 or C4 was replaced with BSA/PBS. Controls also included single labelling with one IgM or IgG_1 primary antibody followed by the inappropriate class- or sub-class-specific second antibody to detect any cross reactivity.

(iii) Microscopy

Coverslips were mounted in anti-fade mounting medium Moviol 4-88 (Hoechst, Frankfurt, FRG), containing 10% (w/v) of 1,4-diazabicyclo [2,2,2]octane (DABCO). Specimens were kept in the dark for at least 1 h before examination. Cells were viewed with a 100x oil-immersion objective (NA: 1.3) on an Axioplan Universal incident-light fluorescence microscope (Zeiss, FRG). A red-suppressing filter was used to exclude autofluorescence from *Chara* chloroplasts. Photographs were taken on Kodak T-Max 400 film at 400 ASA (exposure adjustment: -2, auto exposure time) or 1600 ASA (exposure adjustment: -1, auto exposure time).

5.3 RESULTS

5.3.1 Production of monoclonal antibodies against the 165 kDa polypeptide

Antibodies were successfully elicited with PBS-solubilized antigen whereas nitrocellulose dissolved with DMSO (Harlow & Lane 1988) was fatal and subcutaneous implants of nitrocellulose (Harlow & Lane 1988) failed to generate useful hybridomas. Both alkaline phosphatase and the less sensitive peroxidase detection proved useful in immunoblot screening with peroxidase detecting only the stronger of the positive hybridoma cells detectable with phosphatase (data not shown). Positive clones were confirmed by ELISA (data not shown) and four monoclonal anti-165 kDa polypeptide antibodies (MB165/1, 165/2, 165/3 and 165/4) obtained in the form of ascites fluids were used in all subsequent work.

5.3.2 Identification of putative myosin heavy chains of plants by immunoblotting

The overall results of immunoblotting are summarized in Table 5.1. Of the two mung bean polypeptides (165 and 155 kDa) recognized by anti-pan myosin antibody (Fig. 5.1, lane 5), only the 165 kDa polypeptide was detected by all four MB165 (Fig. 5.1, lanes 6-9), anti-myosin (smooth and skeletal) (Fig. 5.1, lane 3) and anti-fast myosin (Fig. 5.1, lane 4). Anti-skeletal myosin did not cross-react with any bands of mung bean (Fig. 5.1, lane 2), pea (Fig. 5.2, lane 2), wheat (Fig 5.3, lane 2), *Arabidopsis* (Fig. 5.4, lane 2) or *Chara* (Fig. 5.5, lane 2). All antibodies except anti-skeletal myosin also detected polypeptides of 160 kDa in pea (Fig. 5.2), 165 kDa in wheat (Fig. 5.3) and 165 kDa in *Arabidopsis* (Fig. 5.4). All antibodies strongly reacted with the 205 kDa heavy chain of rabbit skeletal myosin (Fig. 5.6).

In *Chara,* four polypeptides (200, 175, 124 and 110 kDa) were identified by immunoblotting, varying with different antibodies (Table 5.1). Neither anti-skeletal myosin nor anti-myosin (smooth and skeletal) detected any polypeptide (Fig. 5.5, lanes 2 and 3). Both anti-fast and anti-pan myosin antibodies detected a 124 kDa

polypeptide (Fig. 5.5, lanes 4, 5 and 7). Additionally, anti-pan myosin antibody recognized a 200 kDa polypeptide (Fig. 5.5, lanes 6 and 7) and the 175 kDa (Fig. 5.5, lane 8) one. Of four MB165, MB165/3 and MB165/4 identified the 175 kDa polypeptide constantly (Fig. 5.5, lanes 11-13). MB165/3 also identified the 110 kDa polypeptide sometimes (Fig. 5.5, lane 12). MB165/1 and MB165/2 usually had smear of high background (Fig. 5.5, lanes 9 and 10), and sometimes seems to detect a faint and fuzzy band of 175 kDa, but this was not convincing due to the high background (data not shown). When *Chara* proteins were precipitated by acetone, the same pattern of polypeptides could be detected as was detected following TCA precipitation (data not shown).

In immunoblotting, *Chara* proteins were much more difficult to detect than those of higher plants. The 200, 175 and 124 kDa polypeptides of *Chara* were not all recognized by anti-pan myosin at the same time (Fig. 5.5, lanes 5-8), and the 175 kDa polypeptide was the most difficult to detect. Such inconsistency of polypeptide detection may be due to the different content of each protein in different sample preparations.

Table 5.1 Identification of putative myosin heavy chains by immunoblotting (+: positive; -: negative; ±: dubious).

Tissue	M_r x 10^{-3}	anti-skeletal myosin	anti-myosin (smooth and skeletal)	anti-fast myosin	anti-pan myosin	MB 165/1	MB 165/2	MB 165/3	MB 165/4
Mung bean	165	-	+	+	+	+	+	+	+
	155	-	-	-	+	-	-	-	-
Pea	160	-	+	+	+	+	+	+	+
Wheat	165	-	+	+	+	+	+	+	+
Arabidopsis	165	-	+	+	+	+	+	+	+
Chara	200	-	-	-	+	-	-	-	-
	175	-	-	-	+	±	±	+	+
	124	-	-	+	+	-	-	-	-
	110	-	-	-	-	-	-	+	-
Rabbit muscle myosin	205	+	+	+	+	+	+	+	+

5.3.3 Immunofluorescent labelling of *Chara* internodal cells with anti-actin, anti-myosin and MB165

In immunofluorescence, the labelling patterns with the four MB165 were nearly identical to those with anti-pan myosin and anti-fast myosin (Fig. 5.7-5.11), and similar to the results for perfused *Chara* cells presented by Grolig *et al.* (1988). MB165/2 and MB165/4 gave much weaker immunofluorescent staining than MB165/1 and MB165/3. The same structures of *Chara* cell were labelled with MB165/2 and MB165/4 as with MB165/1 and MB165/3, but they were less distinct. In the control without incubation with the first antibody, nothing was labelled except autofluorescent vesicles lying around chloroplasts between the focal plane of subcortical actin bundles and the cell wall (Fig. 5.7, e). These vesicles caused some out-of-focus fluorescence visible in photographs.

On one side of the neutral line (nl), antibodies labelled subcortical actin bundles (asterisks), small organelles associated with the actin bundles and endoplasmic strands, tangled (arrows heads) or not (arrows) (anti-pan myosin, Fig. 5.7, a and b; anti-fast myosin, Fig. 5.8, a-c; MB165/1, Fig. 5.9, a-c; MB165/3, Fig. 5.10, a and b; MB165/2, Fig. 5.11, a and b; MB165/4, Fig. 5.11, c and d). Patches of compact fluorescence were considered by Grolig *et al.* (1988) to be tangled endoplasmic strands. Endoplasmic strands, single or branched, often connected two adjacent actin bundles and usually showed a beaded pattern of fluorescence (arrows, Fig. 5.7-5.11). Sometimes, nuclei (n) were also labelled (Fig. 5.9, c).

On the other side of the neutral line, antibodies labelled the actin bundles and small organelles associated with them but very few small patches of tangled endoplasmic strands (anti-pan myosin, Fig. 5.7, c; anti-fast myosin, Fig. 5.8, d; MB165/1, Fig. 5.9, d; MB165/3, Fig. 5.10, c). Usually, fluorescent staining of this side of neutral line was weaker than the opposite side with its many tangled endoplasmic strands, as clearly seen at the neutral line (Fig. 5.8, c). The staining of actin by C4, however, was confined to the actin bundles (Fig. 5.7, d) and was of equal intensity on both sides of the neutral line (data not shown). On immunoblots,

C4 recognized the single band of both chicken gizzard actin (42 kDa) and *Chara* actin (43 kDa, Williamson *et al.* 1985) from cells perfused with ACPS, while no polypeptide was detected after actin was removed from *Chara* with low salt (data not shown).

Sodium borohydride was used to reduce the glutaraldehyde-induced autofluorescence. However, no labelling or sometimes very faint fluorescent actin bundles could be observed with anti-pan myosin after sodium borohydride treatment (data not shown). Moreover, some structures remained weakly labelled and many areas unlabelled with MB165/2 and MB165/4 after such treatment (data not shown). Therefore, sodium borohydride treatment may block the labelling of cellular structures with some, perhaps weak, antibodies.

5.3.4 Immunofluorescent labelling of mung bean root tip cells with anti-actin, anti-pan myosin and MB165

Since all four MB165 antibodies recognized only the 165 kDa polypeptide of mung bean, they were used to localize immunofluorescently this putative myosin heavy chain in mung bean root tip cells. Anti-pan myosin has been used in immunofluorescent localization of a 200 kDa putative myosin heavy chain of onion root tip cells (Parke *et al.* 1986), and therefore was used as a positive control here. Actin was also labeled for comparison with putative myosin. In controls without the incubation with primary antibodies, no cellular structure was labelled (Fig. 5.12, n).

(i) C4 anti-actin

(a) Interphase

Mung bean root tip cells in interphase contained extensive arrays of actin filaments that were more prominent in elongated cells than in isodiametric ones (Fig. 5.12, a-m). In elongated cells, thick longitudinal actin bundles were present in the endoplasm and were usually close to nuclei (Fig. 5.12, c-e). Fine actin filaments, longitudinal or in random arrays, were present in the cell cortex (Fig. 5.12, b, f and h).

The actin filaments in small, isodiametric cells (Fig. 5.12, e and f, i-k) were less prominent than those in elongated cells. A fine, random network of actin filaments was present in the cortex of some cells (Fig. 5.12, i) but more often, short or rod-like actin filaments in random arrays existed in both endoplasm and cell cortex (Fig. 5.12, e, f, j and k). Sometimes, slightly elongated cells also showed short actin filaments (Fig. 5.12, l). Perinuclear actin filaments were present in a few isodiametric or slightly elongated cells, with fine filaments extending from them into the cytoplasm (Fig. 5.12, a and m).

(b) Preprophase/prophase

Preprophase/prophase cells were recognized by their condensed chromatin. The extensive arrays of long actin filaments seen in interphase disappeared and only short actin filaments were present in both endoplasm and cortex and were especially prominent in elongated cells (Fig. 5.13, a-f). The cortex usually had more actin filaments than the endoplasm except in elongated cells. In some cells, only diffuse cytoplasmic staining was observed, with a few short actin filaments sometimes (Fig. 5.13, g and h). No transverse cortical actin filaments coinciding with the preprophase band of microtubules (Palevitz 1987a; Kakimoto & Shibaoka 1987a; McCurdy *et al.* 1988; McCurdy & Gunning 1990) were observed.

(c) Metaphase

In metaphase, random short actin filaments and punctate staining were present in endoplasm and cell cortex, but mostly excluded from the spindle (Fig. 5.14, a-h). The staining of short filaments was particularly distinct in elongated cells (Fig. 5.14, g and h). The cortex usually displayed more filaments than the endoplasm except in elongated cells. In some cells, only diffuse or punctate staining with a few rod-like actin filaments were observed (Fig. 5.14, i and j).

(d) Anaphase

Anaphase cells were recognized by the separation of daughter chromosomes and the absence of a cell plate. Both endoplasm and cell cortex exhibited random arrays of short actin filaments and punctate staining, mostly excluded from the

spindle (Fig. 5.15, a-j). Usually, more actin filaments were present in the cortex than the endoplasm. Additionally, long actin filaments (Fig. 5.15, b and i) and a mesh of actin filaments (Fig. 5.15, f and g) could be observed in the cortex sometimes. In some cells, the cytoplasm only displayed diffuse and punctate staining (Fig. 5.15, m and n). Occasionally, a prominent accumulation of actin was present in the midplane at late anaphase (Fig. 5.15, k and l), as in onion root tip cells (Palevitz 1987b). Within the actin staining, some short rod-like structures could be recognized.

(e) Cytokinesis

Actin was localized in the phragmoplast of mung bean root tip cells (Fig. 5.16, a-f), usually with amorphous (Fig. 5.16, a and b) but sometimes with punctate and occasionally short rod-like staining in it (Fig. 5.16, c-f). In some cells, only diffuse actin staining was found in the phragmoplast (Fig. 5.16, g-n). The cytoplasm usually had diffuse or punctate staining (Fig. 5.16, m and n), but a few short and occasionally some long actin filaments were present in endoplasm and cortex of some cells (Fig. 5.16, g-l).

(ii) Anti-pan myosin and MB165

Immunofluorescent labelling of mung bean root tip cells with anti-pan myosin or MB165 gave the same result whether the cells were extracted with prechilled methanol or not. However, the cytoplasm of cells extracted with methanol was not preserved as well as the cytoplasm in unextracted cells. Since actin could only be stained well in cells treated with methanol, cells labelled with anti-pan myosin or MB165 were also extracted with methanol in order to compare the localization of putative myosin and actin under the same conditions.

The labelling of mung bean root tip cells with anti-pan myosin (Fig. 5.17) was very similar to that with MB165/1 (Fig. 5.18 and 5.19) and MB165/3 (Fig. 5.20). In interphase, cells displayed diffuse or punctate cytoplasmic staining (anti-pan myosin, Fig. 5.17, a; MB165/1, Fig. 5.18, a; MB165/3, Fig. 5.20, a). One or occasionally two brightly stained filaments occurred in the endoplasm of some cells

(anti-pan myosin, Fig. 5.17, b-h; MB165/1, Fig. 5.18, b-e; MB165/3, Fig. 5.20, b-d). Short filaments were in random directions but often close to the periphery of nucleus. Long filaments running longitudinally in close proximity to nuclei were also observed in the endoplasm of a few elongated cells (anti-pan myosin, Fig. 5.17, e-h). These long filaments were very similar to the actin filaments in the endoplasm described before [5.3.4 (i) (a)]. Occasionally, a long filament was present in the cell cortex (anti-pan myosin, Fig. 5.17, i and j; MB165/1, Fig. 5.18, f and g).

During mitosis, one or two filaments were present in some cells. In prophase (anti-pan myosin, Fig. 5.17, k and l; MB165/1, Fig. 5.18, h and i; MB165/3, Fig. 5.20, e and f), metaphase (MB165/1, Fig. 5.18, j and k; MB165/3, Fig. 5.20, g and h) and anaphase (anti-pan myosin, Fig. 5.17, q and r; MB165/1, Fig. 5.19, a and b; MB165/3, Fig. 5.20, i and j), cells exhibited weak, diffuse or punctate cytoplasmic staining. Sometimes, one or occasionally two filaments were present in the endoplasm of a metaphase (anti-pan myosin, Fig. 5.17, m and n; MB165/1, Fig. 5.18, l and m) or anaphase cell (anti-pan myosin, Fig. 5.17, o and p; MB165/1, Fig. 5.19, c and d). Filaments were not observed in prophase cells, but a larger number of cells would need to be examined before it is clear that they are completely absent.

During cytokinesis, the phragmoplast showed amorphous labelling (anti-pan myosin, Fig. 5.17, s and t; MB165/1, Fig. 5.19, e-h; MB165/3, Fig. 5.20, k-n). Occasionally, a filament was labelled elsewhere in the cytoplasm (MB165/1, Fig. 5.19, e and f; MB165/3, Fig. 5.20, m and n). In some cases, the phragmoplast was not labelled more intensely than the cytoplasm which showed diffuse or punctate staining (anti-pan myosin, Fig. 5.17, u and v; MB165/1, Fig. 5.19, i-o). One or two short filaments could be detect in the endoplasm (MB165/1, Fig. 5.19, m) and/or cortex of some cells in cytokinesis (MB165/1, Fig. 5.19, j, k and n).

MB165/2 and MB165/4 gave generally similar but much weaker staining than MB165/1 and MB165/3, and no distinct filaments were detected. Interphase cells

exhibited diffuse or punctate staining of the cytoplasm (Fig. 5.21, a and b) while in prophase (Fig. 5.21, c and d), metaphase (Fig. 5.21, e and f) and anaphase (Fig. 5.21, g and h) cells, cytoplasm was weakly stained. During cytokinesis, the cell plate (Fig. 5.21, i-l) rather than the phragmoplast (Fig. 5.21, m and n) was often labelled.

(iii) Double labelling

Double labelling with anti-actin and anti-pan myosin or MB165/1 was attempted to determine whether the filaments labelled with anti-pan myosin and MB165 also contained actin. Double labelling, however, was not successful: actin filaments were detected but neither anti-pan myosin nor MB165/1 labelled filaments although both showed diffuse or punctate cytoplasmic staining (data not shown). When anti-pan myosin and MB165/1 were tested in single labelling using the FITC-conjugated anti-mouse IgM employed in the double labelling experiment, filaments were detected but very much less frequently than in the experiments using FITC-conjugated anti-mouse Ig [5.3.4 (ii)]. The reasons for this effect are not understood.

5.4 DISCUSSION

5.4.1 Epitopes detected by anti-myosin and MB165 antibodies and the identification of plant putative myosin heavy chains by immunoblotting

Myosin heavy chains are large molecules (1.2.1) and thus have many potential epitopes. If a plant polypeptide shares several epitopes with the heavy chain of rabbit skeletal muscle myosin, it is more likely to be a genuine myosin heavy chain than if it shares only one. When using immunoblotting to decide how many different epitopes were recognized by anti-myosin and the MB165 antibodies, two antibodies were considered to recognize different epitopes if a polypeptide reacted with only one of them. Of eight monoclonal antibodies tested, both MB165/1 and MB165/2 recognized the same polypeptides from each of six tissues, and could therefore be recognizing the same epitope (Table 5.1). It cannot be concluded from this reasoning that they do recognize the same epitope since blotting further species could reveal a polypeptide recognized by only one of them. The other six antibodies recognized six different epitopes. Since the detection of the *Chara* 175 kDa polypeptide with MB165/1 and MB165/2 was equivocal, it was not conclusive whether the two antibodies recognized the same epitope as anti-myosin (smooth and skeletal) or MB165/4. Therefore, the eight antibodies detected at least six different epitopes all of which existed in the rabbit myosin heavy chain.

The four MB165 cross-reacted with rabbit skeletal muscle myosin heavy chain as did the four anti-myosin antibodies. Although the MB165 antibodies were raised against a mung bean protein, they worked at higher dilution with rabbit muscle myosin heavy chain than with plant proteins [5.2.4 (i)]. This is probably because each protein to which the antibodies bound in plant crude extract was present in far lower amounts than purified rabbit muscle myosin in SDS-PAGE. All antibodies except anti-skeletal myosin detected the 160 kDa polypeptide of pea, and the 165 kDa one of mung bean, wheat and *Arabidopsis*. Anti-skeletal myosin is specific for the myosin heavy chain of either human or animal skeletal muscle extract in

immunoblotting, and does not stain human or animal cardiac or smooth muscle myosin or nonmuscle myosins of tissue culture cells (Anon. 1988).

All the recognized six epitopes were on the heavy chain of rabbit muscle myosin; and five were also on the polypeptides of 165 kDa from mung bean, wheat and *Arabidopsis*, and on the 160 kDa polypeptide from pea. The impressive degree of similarity of epitopes between the four polypeptides of higher plants and rabbit muscle myosin heavy chain greatly strengthens the case that these plant polypeptides are myosin heavy chains. No plant putative myosin heavy chain has been identified which shares so many similar epitopes with rabbit muscle myosin heavy chain (1.8.2). The mung bean 155 polypeptide detected by anti-pan myosin antibody does not contain the epitopes recognized by any of the other seven antibodies. This further supported the view that it is not a putative myosin heavy chain (see 3.4.1; 3.4.3).

In *Chara*, none of the four polypeptides detected by immunoblotting shared so many epitopes with muscle myosin heavy chain as did the 165/160 kDa polypeptides of higher plants (Table 5.1). The number of epitopes of *Chara* polypeptides that were shared with muscle myosin heavy chain and the 165/160 kDa polypeptides of four higher plants were: $\geq$ three on 175 kDa, two on the 124 kDa and one on the 200 and 110 kDa polypeptides. The 124 and 110 kDa polypeptide were unlikely to be the proteolytic fragments of the 200 or 175 kDa polypeptide because *Chara* proteins were precipitated immediately with TCA (Wu & Wang 1984) after being squeezed out of cells. Therefore, these four polypeptides were considered to be putative myosin heavy chains of *Chara*. It is possible that multiple isoforms of myosin heavy chains exist in *Chara*. In another alga *Ernodesmis*, putative myosin heavy chains of 220-230 and 85 kDa are also identified by immunoblotting protein extracts made with either buffer or TCA extraction (La Claire 1991).

A 110 kDa polypeptide identified with anti-pan myosin by Grolig. *et al.* (1988) was not detected with the same antibody in my experiments which detected a 124

kDa polypeptide. Only two M_r standards of 205 and 94 kDa were used next to the 110 kDa polypeptide in SDS-PAGE by Grolig *et al.* (1988) while three standards of 205, 116 and 97.4 kDa were used in my experiment. These will provide more accurate M_r determination than the two used by Grolig *et al.* The 110 and 124 kDa recognized with anti-pan myosin by Grolig *et al.* may be the same polypeptide whose M_r was determined differently in two experiments.

The epitopes of anti-pan myosin and anti-fast myosin have been shown by immunoblotting to lie on LMM and S-1 of rabbit skeletal muscle myosin heavy chain, respectively (Tang *et al.* 1989a). Anti-pan myosin identified both 165/160 and 124/110 polypeptides (Table 5.1; Grolig *et al.* 1988), indicating that these HMW and LMW putative myosin heavy chains probably shared an epitope on their tail regions. The conservation of amino acids in the head of myosin is significantly greater than that in its tail (1.2; 1.3). However, for myosin II, even quite distantly related organisms have a >25% amino acid homology in the tail (Warrick & Spudich 1987). Compared with myosin II, myosins I have variable and very dissimilar tails, and several known primary sequences of myosin I tails show no significantly similarity to those of muscle myosin (Korn & Hammer 1988; Pollard *et al.* 1991).

Nevertheless, nine monoclonal antibodies to heavy chains of *Acanthamoeba* myosins I (IA and IB) bind to the heavy chain of myosin IA, eight of them bind to that of myosin IB and also cross-react with that of *Acanthamoeba* myosin II (Kiehart *et al.* 1984; Hagen *et al.* 1986). Epitopes of these eight antibodies are localized to the tail region distal to the head-tail junction of myosin II heavy chain and to the corresponding 30 kDa C-terminus tail region of myosin I heavy chain. These indicate the possible homology in these tails of *Acanthamoeba* myosin I and II, and that myosin I is more closely related to myosin II than was originally apparent from amino acid sequence data. It is suggested that there are at least common epitopes in the tail region of myosin I and II, but close similarity is improbable because of the distinct structural differences between myosin I and II

(Hagen *et al.* 1986). Common epitopes might arise from folding of the polypeptide chain to bring together small clusters of amino acids from different parts of the sequence. These shared amino acids occurring in small, scattered groups would not be picked by simple coalignment of large sequences.

In deducing which of the epitopes of anti-myosin and MB165 antibodies exist on plant proteins, it would be preferable to confirm the failure of certain antibodies to react with specific polypeptides using assays other than immunoblotting such as immunoprecipitation and ELISA. However, a single antibody may not work well in different immunoassays (Harlow & Lane 1988) and since the antibodies were selected because they worked on blots, epitope detection by immunoblotting is less of a problem than it might be. Therefore, the assignment of different epitopes by immunoblotting is preliminary but it provides much more reliable information about plant myosin than relying on reaction with one or at most two antibodies as previous work has done.

5.4.2 Immunofluorescent labelling of *Chara* internodal cells

The same structures of perfused *Chara* internodal cells were labelled with anti-pan myosin, anti-fast myosin and with the four MB165 antibodies. The labelling was similar to that with anti-pan myosin reported before (Grolig *et al.* 1988) except that nuclei considered as autofluorescent by Grolig *et al.* were antibody-labelled and that beaded endoplasmic strands and nuclei recorded only in intact cells by them were observed in perfused cells. Perfusion of 1-2 min rather than 5 min (Grolig *et al.* 1988) before fixation could prevent some endoplasmic strands and big organelles like nuclei from being washed away. The beading of endoplasmic strands may result from their association with small organelles (Grolig *et al.* 1988) or they may be osmotically damaged endoplasmic reticulum (Williamson 1991, 1993). The different staining with anti-myosin or MB165 antibodies on two sides of the neutral line having oppositely directed streaming *in vivo* may reflect a situation where endoplasm is more readily removed from cells if it is streaming in the same

direction as the initial flow of perfusion solution (Grolig *et al.* 1988; McCurdy & Harmon 1992a)

Anti-fast myosin and MB165/4 reacted only with the 124 and 175 kDa polypeptides respectively. These proteins therefore lie on the actin bundles and small organelles attached to them, and the endoplasmic strands (tangled or not). Since the same structures were also labelled with anti-pan myosin localizing three polypeptides (200, 175 and 124 kDa) and MB165/3 localizing two polypeptides (175 and 110 kDa), the 200 and 110 kDa may be colocalized to some or all of these labelled structures. MB165/1, MB165/2 and MB165/4 each displayed different relative strengths in immunoblotting and immunofluorescence. The strength of the reaction in immunoblotting was: MB165/3 > MB165/4 > MB165/1 = MB165/2 (5.3.2), while that in immunofluorescence was: MB165/1 = MB165/3 > MB165/2 = MB165/4.

A 175 kDa polypeptide of *Nicotiana* pollen tubes is identified by immunoblotting with both anti-pan myosin and anti-fast myosin antibodies and is immunolocalized to small vesicles and/or organelles in the pollen tubes (Tang *et al.* 1989a). It is also localized to the generative cell and vegetative nuclear envelopes by anti-fast myosin but not by anti-pan myosin. The different staining patterns of the nuclei seen with two antibodies recognizing the same polypeptide may be caused by the organization and/or anchorage state of the putative myosin molecules on the nuclear surface differing from those on the vesicles and/or organelles. For example, the rod portion of the putative myosin heavy chain may be deeply embedded in the nuclear envelopes so that the epitope is inaccessible to anti-pan myosin, while the polypeptide may be associated with the membranes of vesicles and organelles by a different mechanism in which epitopes of both anti-pan and anti-fast myosin are exposed (Tang *et al.* 1989a). In the present study, MB165/4 and MB165/3 worked well in immunoblotting, but MB165/4 was much weaker than MB165/3 in immunofluorescence. It is possible that in cellular structures, the epitope recognized by MB165/4 on the 175 kDa polypeptide was less accessible

than that recognized by MB165/3 on the 110 kDa polypeptide, due to the different molecular organization of two polypeptides.

MB165/1 and MB165/2 did not clearly detect any *Chara* polypeptide on immunoblots, but sometimes seemed to recognize the 175 kDa polypeptide albeit with a high background. However, MB165/1 was strong in immunofluorescence and MB165/2 was weak but still labelled the same structures as the other MB165. If the epitopes of MB165/1 and MB165/2 on *Chara* polypeptides were formed by noncontiguous amino acid sequences, they could be destroyed by polypeptide denaturation after SDS-PAGE making them unrecognizable or only partially recognizable to MB165/1 and MB165/2 although they were accessible to antibodies in cellular structures.

The subcortical actin bundles were the only place that was labelled by both C4 anti-actin and anti-myosin/MB165. It is well established that the motive force driving the cytoplasmic streaming is produced at the interface between the actin bundles and the endoplasm (1.8.3). The colocalization of polypeptides recognized by anti-myosin or MB165 with actin increases the chances that the polypeptides are putative myosins involved in force generation for cytoplasmic streaming.

In perfused *Chara* cells, the presence of small organelles attached to actin bundles and tangled endoplasmic strands is ATP-sensitive (Grolig *et al.* 1988). Perfusion of ACPS containing a higher concentration of Ca^{2+} inhibits cytoplasmic streaming, tangled endoplasmic strands disappear and only actin bundles and attached small organelles labell with anti-pan myosin. Therefore, the small organelles and endoplasmic strands are believed to be myosin-associated structures responsible for cytoplasmic streaming, and dual mechanisms of force generation have been put forward: individual organelles reacting directly with and moving along the actin bundles; organelles trapped within and moved by tangled, myosin-containing endoplasmic strands.

5.4.3 Immunofluorescent labelling of mung bean root tip cells with anti-actin antibody

In mung bean root tip cells, actin staining was observed in interphase and at all stages of mitosis. Several similarities are evident when comparing the actin staining patterns of mung bean root tip cells during the cell cycle with those seen in root tip cells of other species fixed with 4% formaldehyde: extensive arrays of actin filaments are present in interphase cells; actin staining appears at late anaphase in the midplane region when initial, elongated microtubule elements of the phragmoplast are evident between daughter chromosomes; and actin is localized to phragmoplast but not mitotic spindle (onion: Clayton & Lloyd 1985; Palevitz 1987b; wheat: McCurdy *et al.* 1988; McCurdy & Gunning 1990). Other staining properties vary with different work.

In preprophase, the transverse cortical actin filaments colocalized with microtubules in the preprophase band of onion root cells (Palevitz 1987a, 1988) were not detected in mung bean cells. Transverse actin filament occupying the central region of cell cortex at early preprophase or the entire cortical surface at late preprophase have also been observed in wheat root tip cells (McCurdy *et al.* 1988; McCurdy & Gunning 1990), but not in mung bean cells.

In wheat root tip cells, only short fragments of actin filaments are present in the cell cortex at prophase (some prophase cells have transverse cortical actin filaments), metaphase and anaphase (McCurdy & Gunning 1990). The short filaments detected in mung bean cells from prophase through cytokinesis were longer than the short fragments of actin filaments observed in wheat. Furthermore, long and distinct or meshwork-like actin filaments were present in the cortex of some cells in anaphase. Long actin filaments were also present in the endoplasm and cortex of some mung bean cells in cytokinesis, which may be an early sign of interphase filaments. Therefore, even though the extensive arrays of actin filaments in interphase disappear at the onset of mitosis, short or sometimes long actin filaments were present throughout mitosis in mung bean root tip cells. The

detection of transverse preprophase actin filaments or actin filaments in mitosis may be a question of proper preservation of actin filament during fixation.

In plant root tip cells fixed with 4% formaldehyde, the commonly observed cortical actin filaments and thick subcortical actin bundles at interphase, and the intensive actin staining in phragmoplasts may represent the most stable actin structures in cell division. In many cases, the network of actin filaments in plant cells is partially sensitive to fixation by conventional aldehydes, and therefore methods of mild treatment have been explored (Lloyd 1988). These methods include fixation with paraformaldehyde at low concentration (0.5-2%) for short time (15 min or less) (Parthasarathy *et al.* 1985; Seagull *et al.* 1987; Schmit & Lambert 1987), formaldehyde fixation after lysine or tropomyosin treatment which stabilizes actin filaments (Kakimoto & Shibaoka 1987a, b), mild extraction with detergent and/or DMSO to avoid aldehyde fixation (Traas *et al.* 1987; Lloyd & Traas 1988; Heslop-Harrison & Heslop-Harrison 1991), and electroporation which bypasses both aldehyde fixation and detergent extraction (Traas *et al.* 1987; Lloyd & Traas 1988).

The sensitivity of plant actin filaments to conventional aldehyde fixation, however, has been conclusively demonstrated only in higher plant cells with large vacuoles (McCurdy & Gunning 1990). Hydrolytic enzymes, phenolic compounds, and especially, calcium ions stored in vacuoles may rapidly disrupt filaments if the vacuoles are ruptured during specimen processing. Therefore, it is argued that the ability to preserve fine networks of transverse cortical actin filaments in preprophase of wheat root tip cells indicates that aldehyde fixation may not be a limitation to obtaining a complete picture of distribution of actin filament during mitosis of densely cytoplasmic meristematic cells (McCurdy *et al.* 1988; McCurdy & Gunning 1990).

Besides the preprophase band and phragmoplast that are commonly labelled with actin, several studies using the new methods of mild treatment reveal more and complicated actin structures during cell cycle than those using conventional

aldehyde fixation. Several classes of interconnected arrays of actin filaments are present at interphase and the network of actin filaments persist throughout mitosis although it is organized differently during division (Schmit & Lambert 1987; Seagull *et al.* 1987; Traas *et al.* 1987; Lloyd & Traas 1988). Actin filaments associated with the mitotic spindle are observed in a few cases (Seagull *et al.* 1987; Lloyd & Traas 1988), but probably do not provide the motive force for chromosome transport because treatment with cytochalasin B or D which disrupt actin filaments does not affect the transport (Lloyd 1988).

Phragmosomal actin filaments radiating from nucleus to cortex at preprophase are present in dividing carrot suspension culture cells (Traas *et al.* 1987; Lloyd & Traas 1988), and are suggested to guide the outgrowing phragmoplast to the opposing cortex previously occupied by the preprophase band of microtubules. Therefore, actin filament may be involved in the spatial control of cell division (Lloyd & Traas 1988). Actin in the phragmoplast suggests that an actin-based contractile system may be involved in the transport of vesicles containing cell plate precursors to the midline and/or in vesicle fusion and organization of the cell plate (Clayton & Lloyd 1988; Schmit & Lambert 1987; Palevitz 1987b). However, the vesicle movements in the phragmoplast are never directly observed and it remains uncertain whether actin- or microtubule-based motors or both support such movements (Williamson 1993)

The short or sometimes long actin filaments observed in mitotic mung bean root tip cells support the idea that actin filaments are present throughout cell division. The short filaments were very similar to those present during interphase in isodiametric cells. However, long cortical actin filaments in random arrays were observed in some isodiametric cells and meshwork-like actin filaments were also occasionally present in the cortex of anaphase cells. The short actin filaments may be produced from long filaments that were sensitive to aldehyde fixation, or both short and long filaments may be present *in vivo* with the long ones being more sensitive to the aldehyde fixation. The actin filaments present at mitosis in mung

bean cells may be involved in some functions observed in other higher plants discussed before, such as maintaining the integrity of the cell cortex and exerting spatial control over cell division; however, clarifying these functions in mung bean requires further extensive studies of actin filaments.

5.4.4 Immunofluorescent labelling of mung bean root tip cells with anti-pan myosin and MB165 antibodies

The staining patterns of mung bean root tip cells labelled with anti-pan myosin were very similar to those with MB165/1 or MB165/3, and share some similarities with those of onion root tip cells labelled with anti-pan myosin (Parke *et al.* 1986). Interphase cells of onion root tips display diffuse cytoplasmic staining within which spherical dots are labelled to various degrees and these dots disappear at the onset of mitosis (Parke *et al.* 1986). The small dots look like the punctate cytoplasmic staining of mung bean root tip cells, but the big ones were not detected in mung bean cells. The structural nature of these various dots is not known but they may represent different organelles in the cytoplasm. Neither the preprophase band nor the mitotic spindle of either mung bean or onion (Parke *et al.* 1986) root tip cells were labelled with anti-pan myosin or with MB165 antibodies.

Actin and putative myosin heavy chain have been colocalized to the phragmoplast of onion root tip cells, and it is suggested that plant myosin and actin may be involved in cytokinesis (Parke *et al.* 1986). As mentioned in 5.4.3, actomyosin in the phragmoplast may transport vesicles containing cell plate precursors to the growing cell plate. The phragmoplast of mung bean root tip cells was labelled with anti-actin, anti-pan myosin, MB165/1 and MB165/3. The colocalization of the mung bean 165 kDa polypeptide with actin in the phragmoplast further supports the view that it was a myosin heavy chain. The mung bean 155 kDa polypeptide recognized by anti-pan myosin may also be localized in the phragmoplast if the epitope was accessible to the antibody since no extra sites were stained only by anti-pan myosin. MB165/2 and MB165/4 were much weaker

than MB165/1 and MB165/3, and surprisingly, they labelled the cell plate rather than the phragmoplast. The explanation for such labelling is not evident at the moment.

The one or two filaments labelled with anti-pan myosin, MB165/1 and MB165/3 in interphase and sometimes in metaphase and anaphase cells of mung bean root tips have not been reported in onion root tip cells (Parke *et al.* 1986) even though similar fixation (4% vs 3.7% paraformaldehyde) was used. It is unknown whether the filaments labelled with anti-pan myosin, MB165/1 and MB165/3 also contained actin since double labelling was not successful. Since the filaments in interphase cells did not form a network like the actin filaments, it was difficult to judge whether putative myosin and actin were colocalized. Nevertheless, the long filaments (one of which labelled with anti-pan myosin nearly extended the whole length of the cell, see Fig. 5.17, e and f), lying in close proximity to the nuclei of interphase cells, looked very much like the longitudinal subcortical actin filaments of interphase mung bean cells.

It is unknown why the number of the putative myosin-reactive filaments is much less than that of the actin filaments if the putative myosin is colocalizing with the actin filaments. It may be a genuine result that not all actin filaments are associated with the putative myosin, but there is no obvious biological reason why only some actin filaments should have associated myosin. It may also be an artefact of preparation which, for some reason, does not preserve the putative myosin on many actin filaments.

Putative myosin has been colocalized with the subcortical actin bundles of *Chara* and both may be involved in force generation of the cytoplasmic streaming (Grolig *et al.* 1988; 5.4.2). In *Ernodesmis,* putative myosin has been localized in the longitudinal actin bundles formed after wounding, and it is believed that actin and myosin are directly associated with the wound-induced cytoplasmic contraction in such alga cells (La Claire 1991). Therefore, the filaments labelled with anti-pan myosin and MB165 in mung bean root tip interphase cells may represent the

putative myosin localized on actin filaments, where both may be involved in the cytoplasmic streaming.

5.4.5 Conclusions

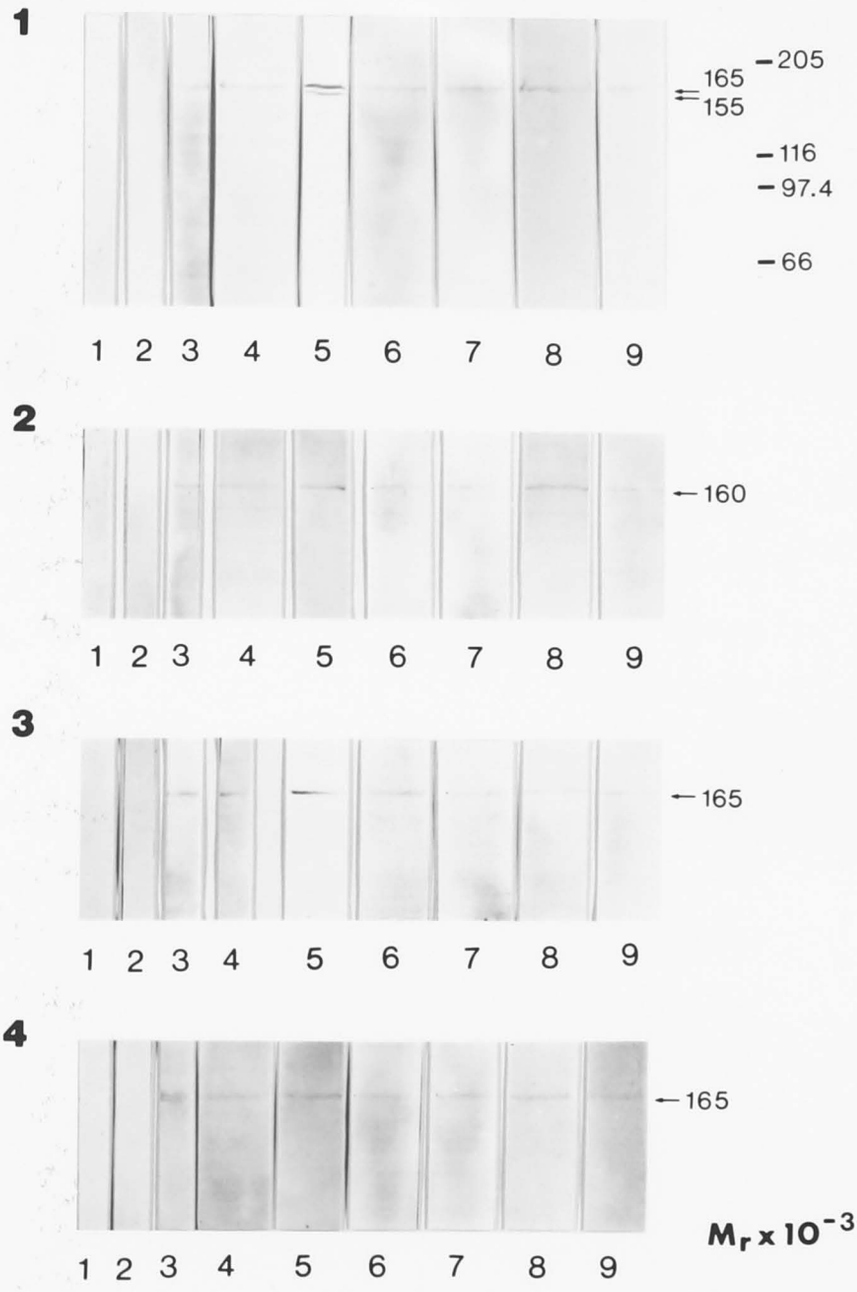
Six epitopes were recognized by four anti-myosin antibodies and four MB165 antibodies. All epitopes were present on the heavy chain of rabbit skeletal muscle myosin. Each of the 165 or 160 kDa polypeptide of mung bean, pea, wheat and *Arabidopsis* shared five epitopes with rabbit muscle myosin heavy chain. This makes the case that these polypeptides are myosin heavy chains much stronger than when they were identified with only one antibody. In contrast, the 155 kDa mung bean polypeptide only had the epitope recognized by anti-pan myosin, supporting that it was not a myosin heavy chain. Four putative myosin heavy chains of *Chara* (200, 175, 124 and 110 kDa) shared 1-3 epitopes with muscle myosin heavy chain and with the 165/160 kDa polypeptides of higher plants; not as many as those higher plant proteins shared with muscle myosin heavy chain.

Immunofluorescently, the mung bean 165 kDa polypeptide was colocalized with actin in phragmoplasts of mung bean root tip cells by anti-pan myosin, MB165/1 and MB165/3. This strengthens the case that the polypeptide is a myosin heavy chain and that, together with actin, it may play a role in cytokinesis. In *Chara,* the 175 and 125 kDa polypeptide that can be localized individually by MB165/4 and anti-fast myosin localize to the actin bundles, suggesting that they may be involved in force generation for cytoplasmic streaming. Neither the 200 nor the 110 kDa polypeptide is recognized individually so that one, both, or neither of them may be localized to the actin bundles.

CHAPTER 5

FIGURES

Fig. 5.1-Fig. 5.4 Identification of putative myosin heavy chains of plants by immunoblotting. Fig. 5.1, mung bean; Fig. 5.2, pea; Fig. 5.3, wheat; Fig. 5.4, *Arabidopsis*. (1) was the control without incubation of the first antibody. Each sample was immunoblotted with 8 antibodies: (2), anti-skeletal myosin; (3), anti-myosin (smooth and skeletal); (4), anti-fast myosin; (5), anti-pan myosin; (6), MB165/1; (7), MB165/2; (8), MB165/3; and (9), MB165/4.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165
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$M_r \times 10^{-3}$

Fig. 5.5 **Identification of putative myosin heavy chains of *Chara* by immunoblotting.** (1) was the control without incubation of the first antibody. Eight antibodies were tested: (2), anti-skeletal myosin; (3), anti-myosin (smooth and skeletal); (4), anti-fast myosin; (5)-(8), anti-pan myosin; (9), MB165/1; (10), MB165/2; (11) and (12), MB165/3; and (13), MB165/4.

Fig. 5.6 **Identification of the rabbit skeletal muscle myosin heavy chain by immunoblotting.** (1) was the control without incubation of the first antibody. Eight antibodies were tested: (2), anti-skeletal myosin; (3), anti-myosin (smooth and skeletal); (4), anti-fast myosin; (5), anti-pan myosin; (6), MB165/1; (7), MB165/2; (8), MB165/3; and (9), MB165/4.

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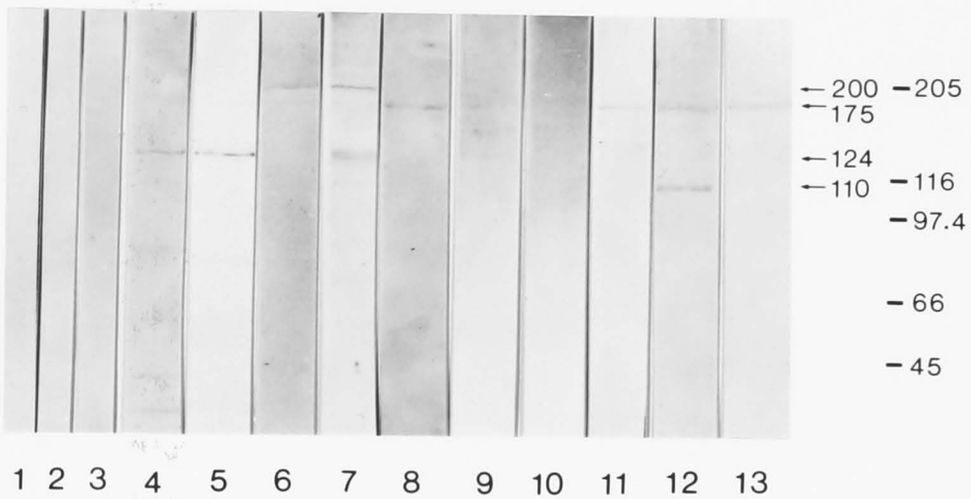
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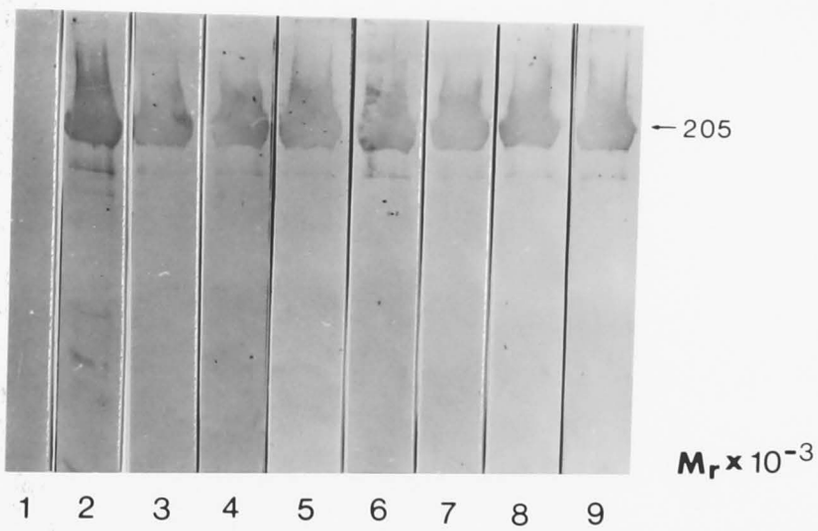
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Fig. 5.7. Immunofluorescent labelling of *Chara* internodal cells with anti-pan myosin (a-c) and C4 anti-actin. (a) and (b), On one side of the neutral line, anti-pan myosin labelled actin bundles (asterisks) and small organelles attached to them, tangled endoplasmic strands (arrow heads) and endoplasmic strands (arrows) connecting two adjacent actin bundles. (c), On the other side of the neutral line, anti-pan myosin labelled actin bundles and small organelles attached to them. (d), Actin was solely localized to actin bundles. Fuzzy fluorescent dots were contributed by autofluorescent vesicles on the chloroplast layer beneath the actin bundles. (e), Control in which incubation with the first antibody was eliminated. Some cells had bright autofluorescent vesicle around chloroplasts. (magnification: x 816)

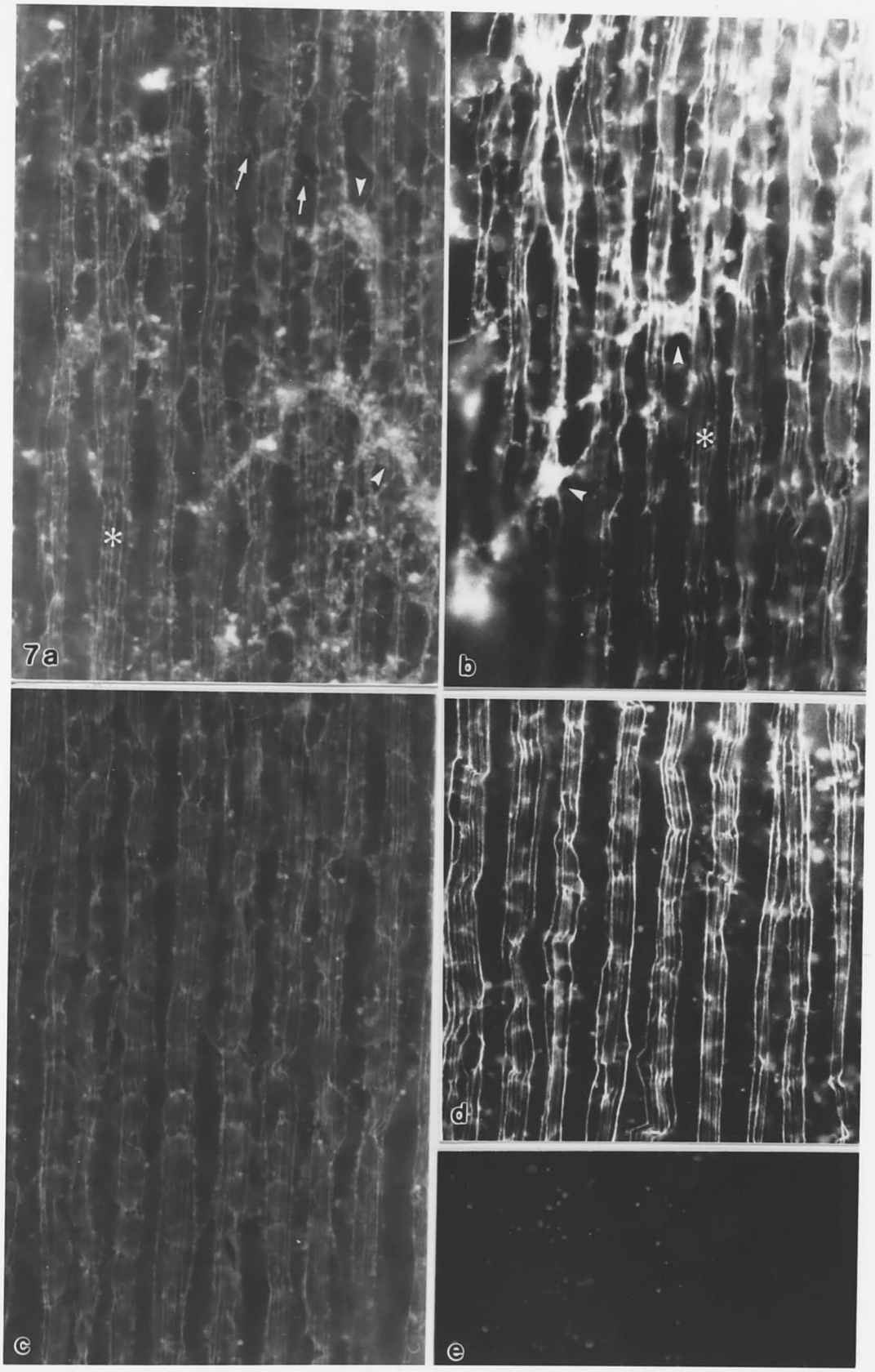
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Fig. 5.8 Immunofluorescent labelling of *Chara* cells with anti-fast myosin. (a), (b) and left hand part of (c), On one side of the neutral line (nl), The antibody labelled actin bundles (asterisks), small organelles attached to them, tangled endoplasmic stands (arrow heads) and endoplasmic strands (arrows) connecting two adjacent actin bundles. Right hand side of (c) and whole of (d), On the other side of the neutral line, the antibody labelled actin bundles and small organelles on them, with very few small tangled endoplasmic strands sometimes. The fluorescent staining was weaker than the opposite side of the neutral line, as clearly seen in (c). (magnification: x 816)

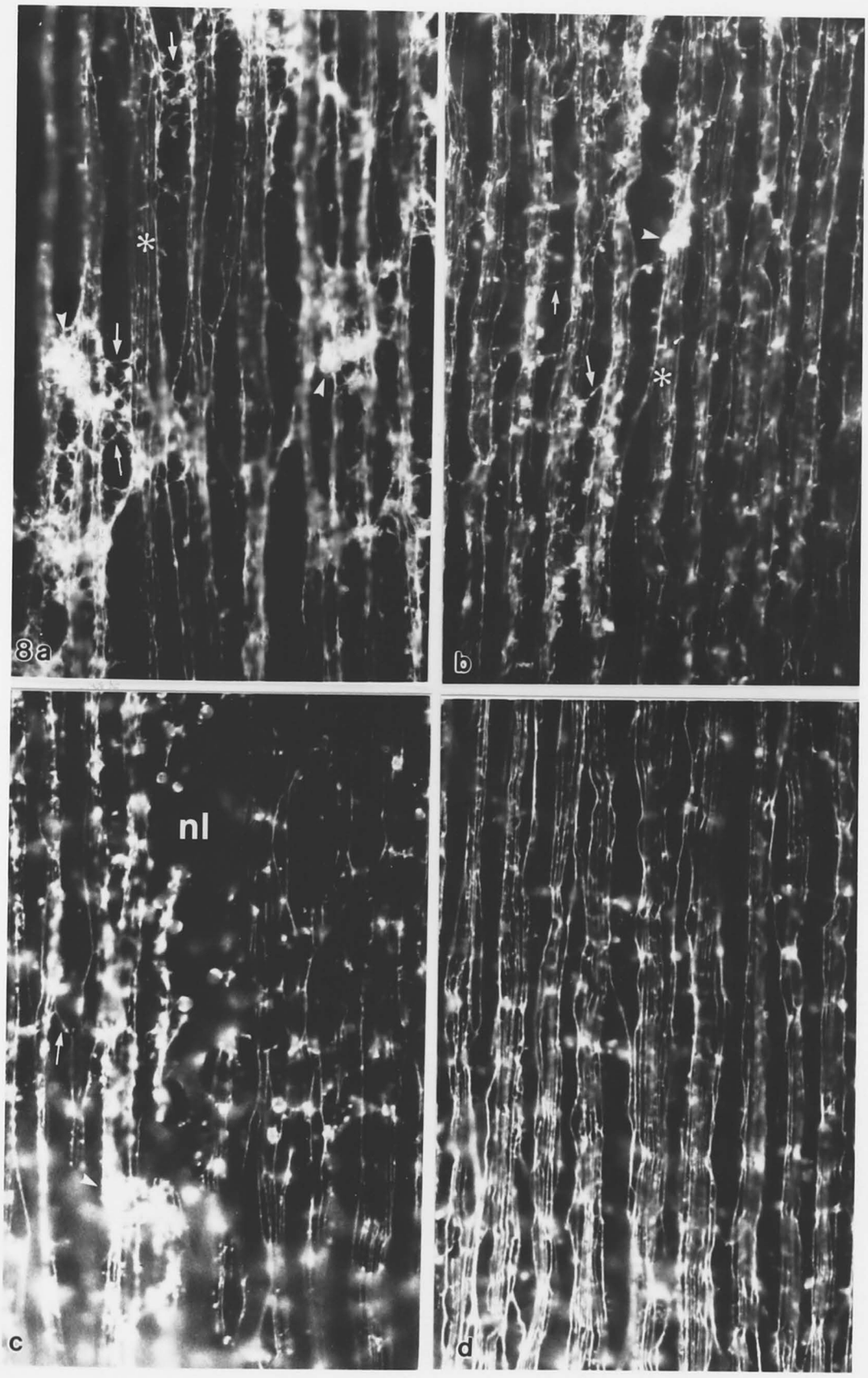
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Fig. 5.9 Immunofluorescent labelling of *Chara* cells with MB165/1. (a)-(c), Actin bundles with attached small organelles (asterisks), tangled endoplasmic strands (arrow heads), and endoplasmic strands, branched or not (arrows). Sometimes, nuclei (n) could be observed as in (c). (d), On the other side of the neutral line, actin bundles and small organelles associated with them were immunofluorescently labelled. (magnification: x 816)

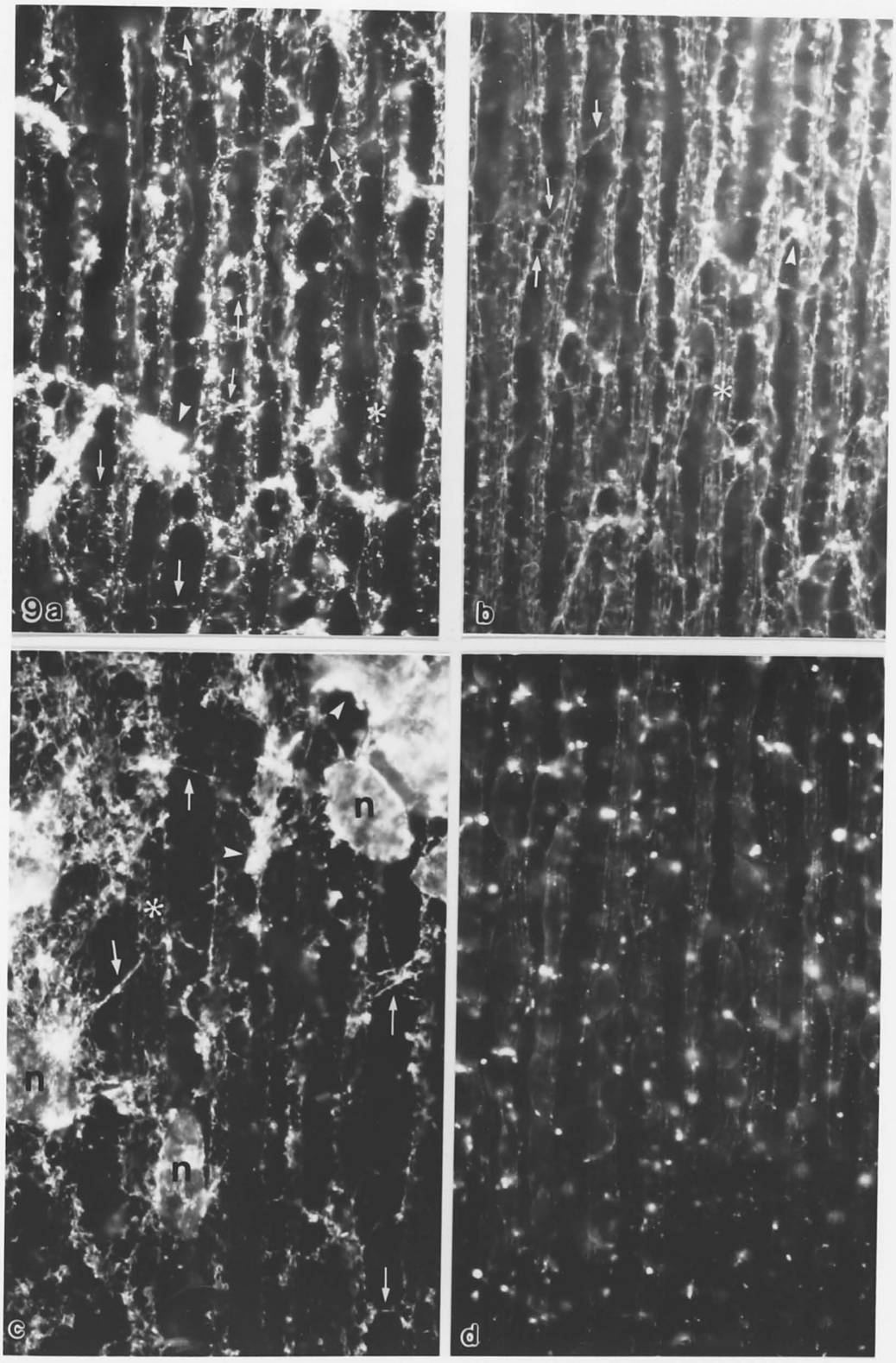
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Fig. 5.10 Immunofluorescent labelling of *Chara* cells with MB165/3. (a) and (b), Actin bundles with attached small organelles (asterisk), tangled endoplasmic strands (arrow heads), and endoplasmic strands, branched or not, connecting adjacent actin bundles (arrows). (c), On the other side of neutral line, actin bundles and attached small organelles were more weakly labelled than those of the opposite side in (a) and (b). Very few tangled endoplasmic strands were detected. (magnification: x 816)

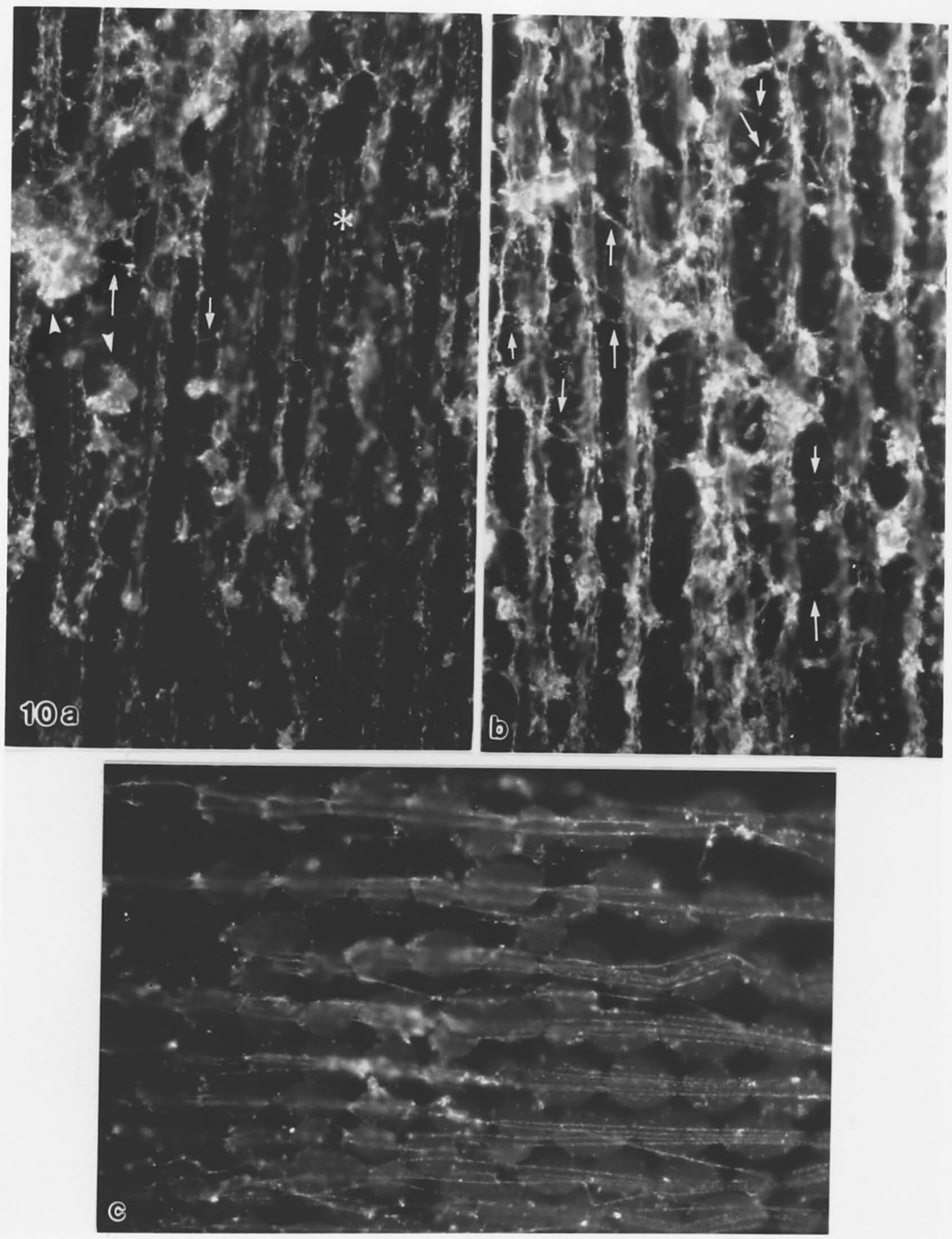
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Fig. 5.11 Immunofluorescent labelling of *Chara* cells with MB165/2 (a and b) and MB165/4 (c and d). Actin bundles and small organelles associated with them (asterisks), tangled endoplasmic strands (arrow heads), endoplasmic strands connecting two adjacent actin bundles (arrows) were immunofluorescently labelled. The fluorescence was indistinct due to the weakness of the staining achieved with MB165/2 and MB165/4. (magnification: x 816)

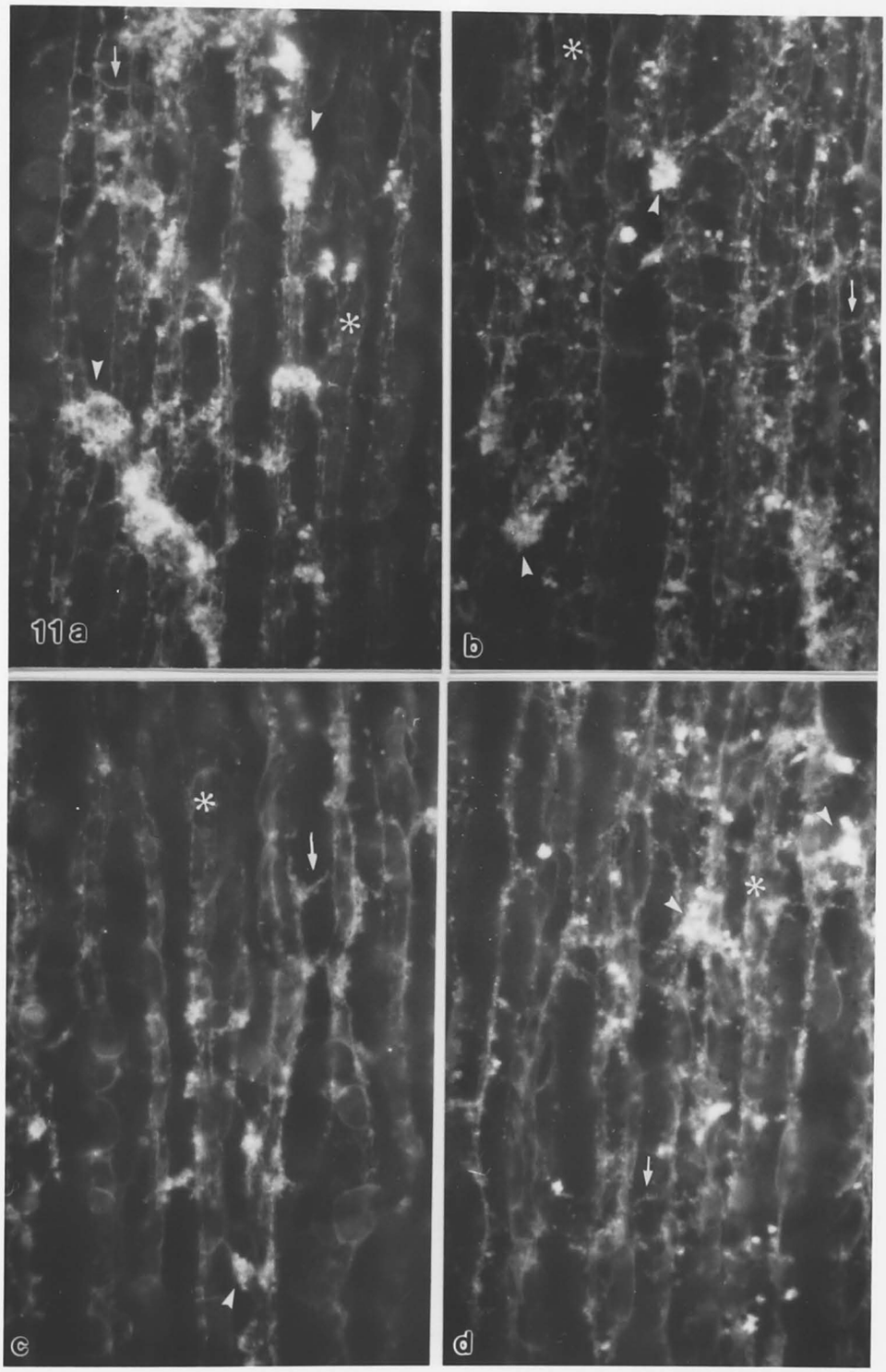
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Fig. 5.12 Mung bean root tip cells in interphase labelled with C4 anti-actin (a-m). (a), Internal view (bracket), showing perinuclear actin filaments. (b), Surface view of the same cell, showing a network of cortical actin filaments, longitudinal or in more random arrays. (c) and (d), Internal view of elongated cells, showing thick subcortical longitudinal actin filaments close to nuclei. (e), Internal view, showing longitudinal actin filaments in an elongated cell and short, random actin filaments in an isodiametric cell. (f), Surface view of the same cells, showing fine cortical actin filaments, random in the isodiametric cell and more longitudinal in the elongated cell. (g) Internal view of an elongated cell. (h), Surface view of the same cell, showing fine longitudinal actin filaments in cortex. (i), Surface view of an isodiametric cell (bracket), showing random fine actin filaments in cortex. (j), Internal view of three isodiametric cells, showing random short, rod-like actin filaments in endoplasm. (k), Surface view of the same cells, showing random short cortical actin filaments. (l), Internal view of an elongated cell, showing random short cortical actin filament with one long subcortical actin filament. (m), Internal view, showing perinuclear actin filaments with fine filaments radiating into the cytoplasm. (n), Control in which incubation with the first antibody was replaced with BSA/PBS; nothing was stained. (magnification: x 954)

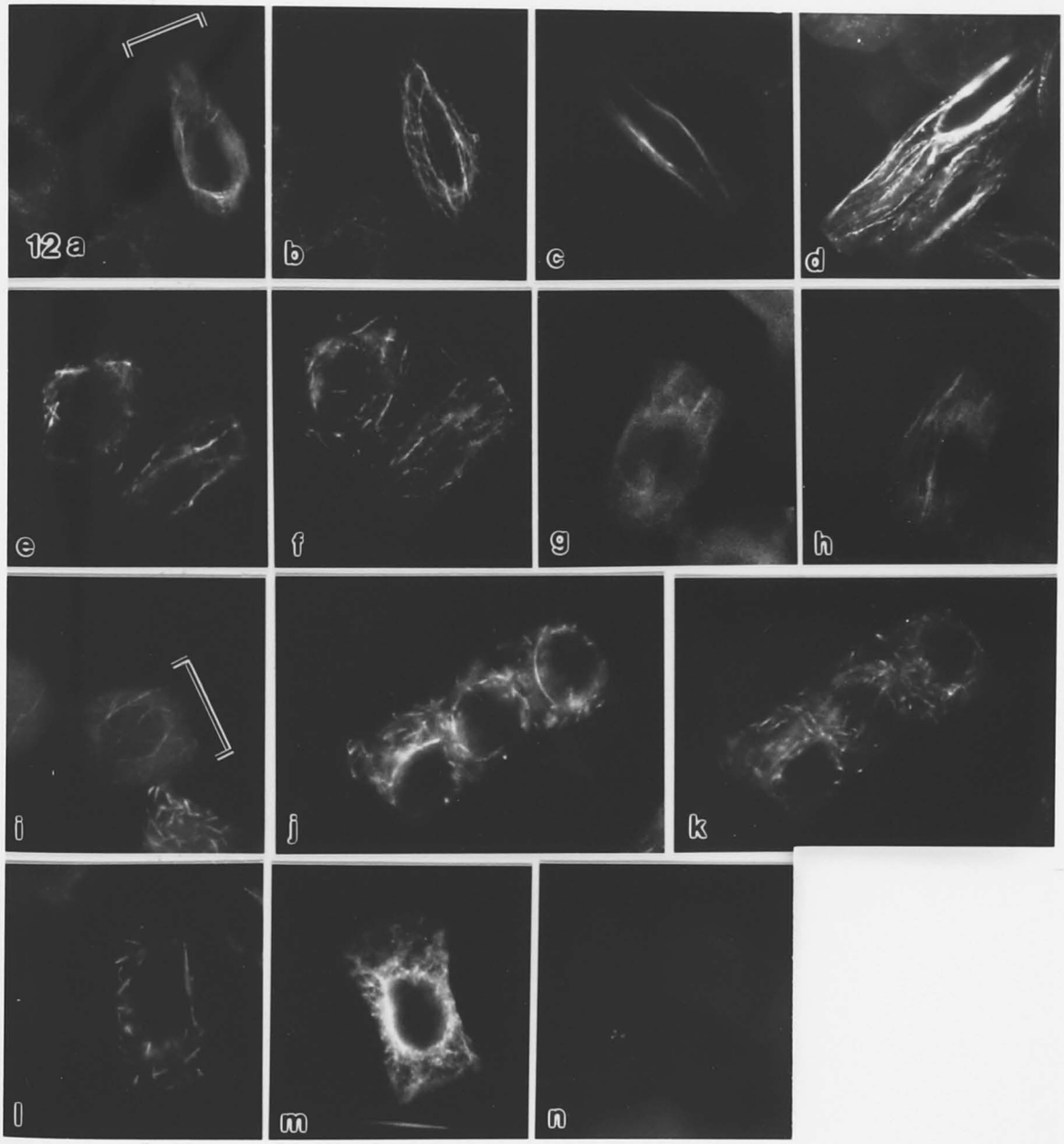
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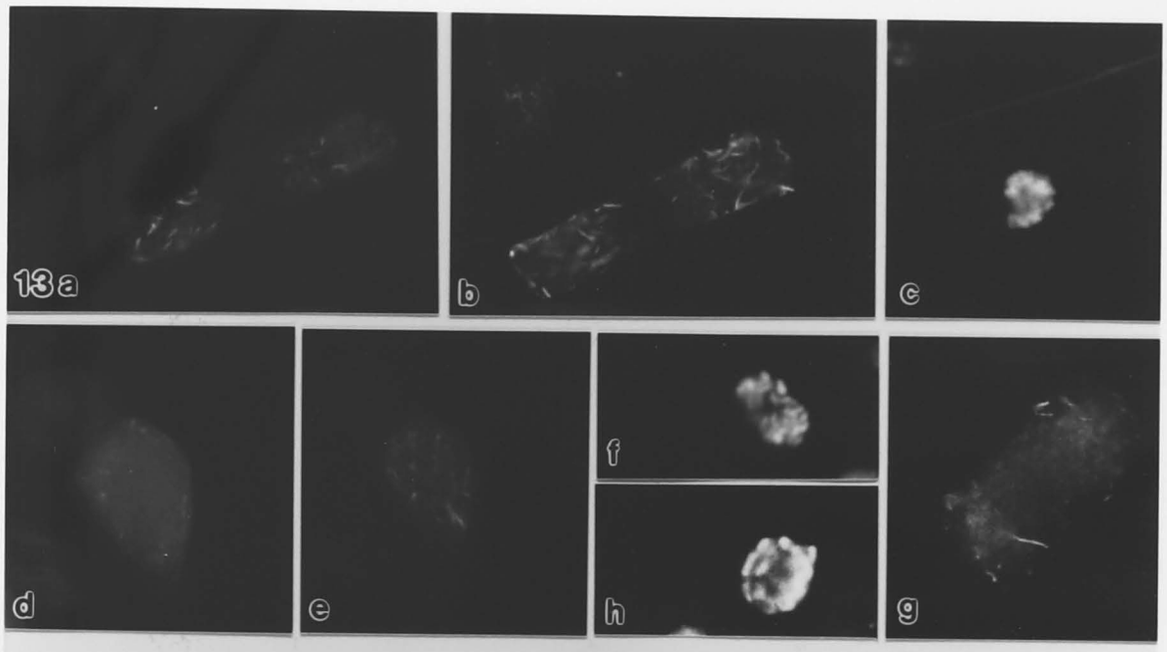
Fig. 5.13 Mung bean root tip cells in prophase stained with C4. (a), Internal view, showing random short actin filaments in endoplasm. (b), Surface view of the same cell, showing random short actin filament in cortex. (c), DAPI of the same cell, showing condensed chromatin. (d), Internal view, weak, diffuse and punctate staining only. (e), Surface view of the same cell, showing random short actin filaments in cortex. (f), DAPI of the same cell. (g), Internal view, showing diffuse staining with a few short actin filaments. (h), DAPI of the same cell. (magnification: x 954)

Fig. 5.14 Mung bean root tip cells in metaphase stained with C4. (a), Internal view, showing random short actin filaments in endoplasm but excluded from the mitotic spindle. (b), Surface view of the same cell, showing random short actin filaments in cortex. (c), DAPI of the same cell, showing chromosomes aligned at metaphase plate halfway between the poles. (d), Internal view, showing diffuse and punctate staining. (e), Surface view of the same cell, showing short actin filament in cortex. (f), DAPI of the same cell. (g), Internal view, showing random short actin filaments. (h), DAPI of the same cell. (i), Internal view, showing diffuse, punctate staining with a few short rod-like actin filaments. (j), DAPI of the same cell. (magnification: x 954)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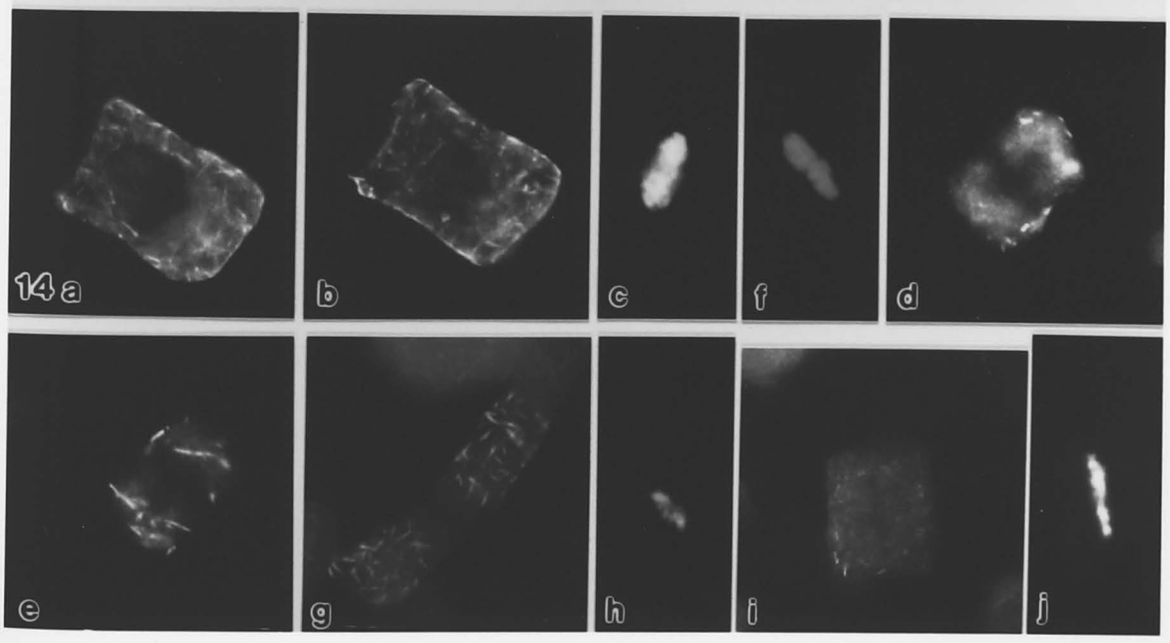
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Fig. 5.15 Mung bean root tip cells in anaphase labelled with C4. (a), Internal view (bracket), showing punctate staining with a few short actin filaments in endoplasm. (b), Cortical view of the same cell, showing several long actin filaments in cortex. (c), Adjusted cortical view of the same cell, showing random short actin filaments in cortex. (d), DAPI of the same cell. (e), Internal view (bracket), showing random short actin filament and punctate staining in endoplasm. (f), Cortical view of the same cell, showing random meshwork-like actin filaments in cortex. (g), DAPI of the same cell. (h), Internal view, showing punctate staining and some short actin filaments. (i), Cortical view of the same cell, showing one long actin filament in cortex. (j), DAPI of the same cell. (k), late anaphase, showing intense fibrous staining in midplane region between two daughter sets of chromosomes. (l), DAPI of the same cell. (m), Diffuse and punctate staining throughout the cytoplasm. (n), DAPI of the same cell. (magnification: x 954)

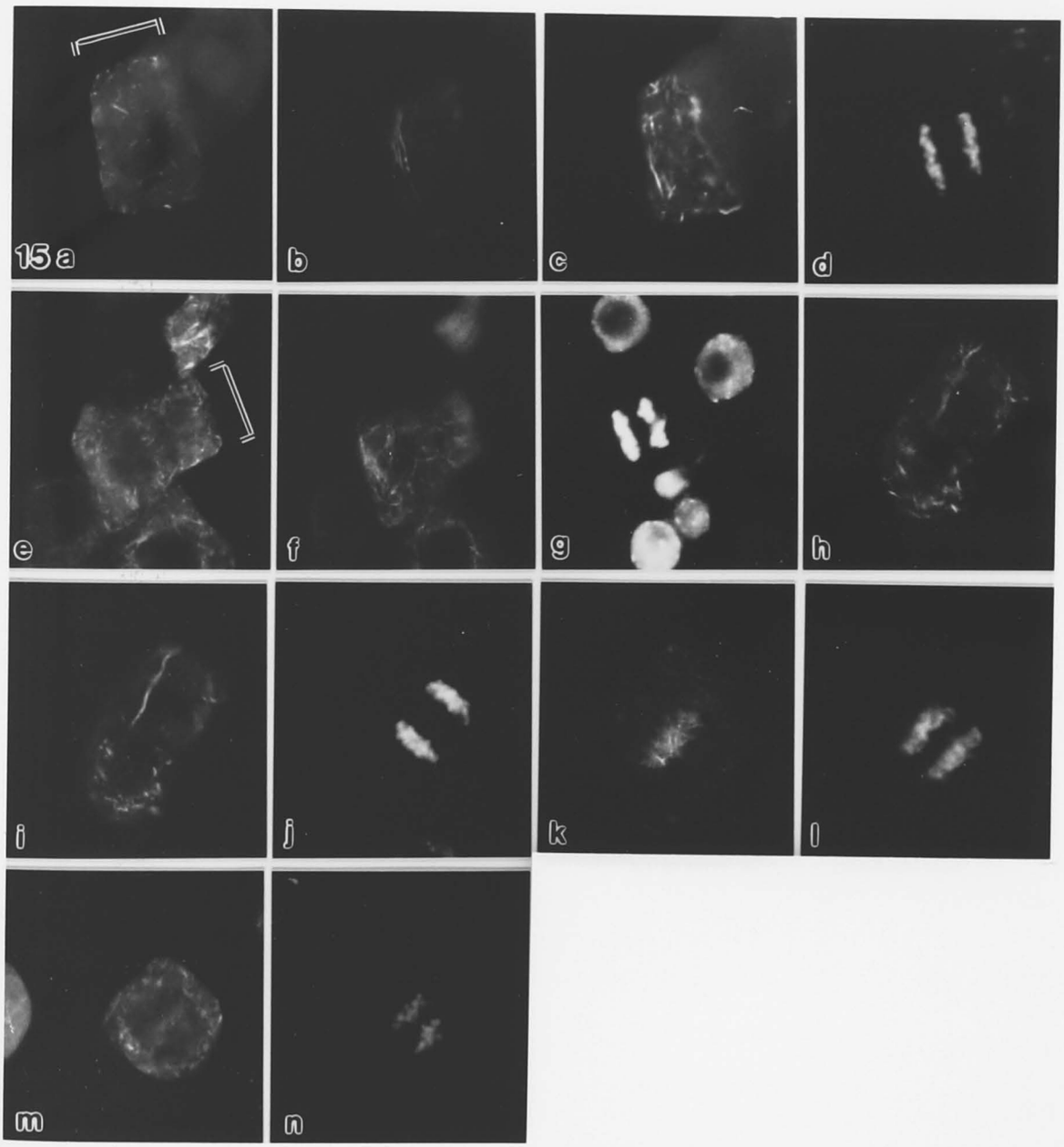
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Fig. 5.16 Mung bean root tip cells in cytokinesis labelled with C4. (a), Amorphous staining of a phragmoplast. (b), DAPI of the same cell. (c), Stained phragmoplast containing very short rod-like elements. (d), DAPI of the same cell. (e), A cell (bracket) showed stained phragmoplast with punctate staining in it. (f), DAPI of the same cell. (g)-(n), Phragmoplasts were not labelled with C4 and display weak diffuse staining. (g), Internal view, showing short rod-like and punctate staining of the endoplasm. (h), Surface view of the same cell, showing punctate staining and short actin filaments in cortex. (i), DAPI of the same cell. (j), Internal view, showing punctate staining of cytoplasm, with one long actin filament in endoplasm (arrow). (k), Surface view of the same cell, showing one long actin filament in cortex (arrow), probably a continuation of the filament observed in (j). (l), DAPI of the same cell. (m), General, punctate staining of cytoplasm. (n), DAPI of the same cell. (magnification: x 954)

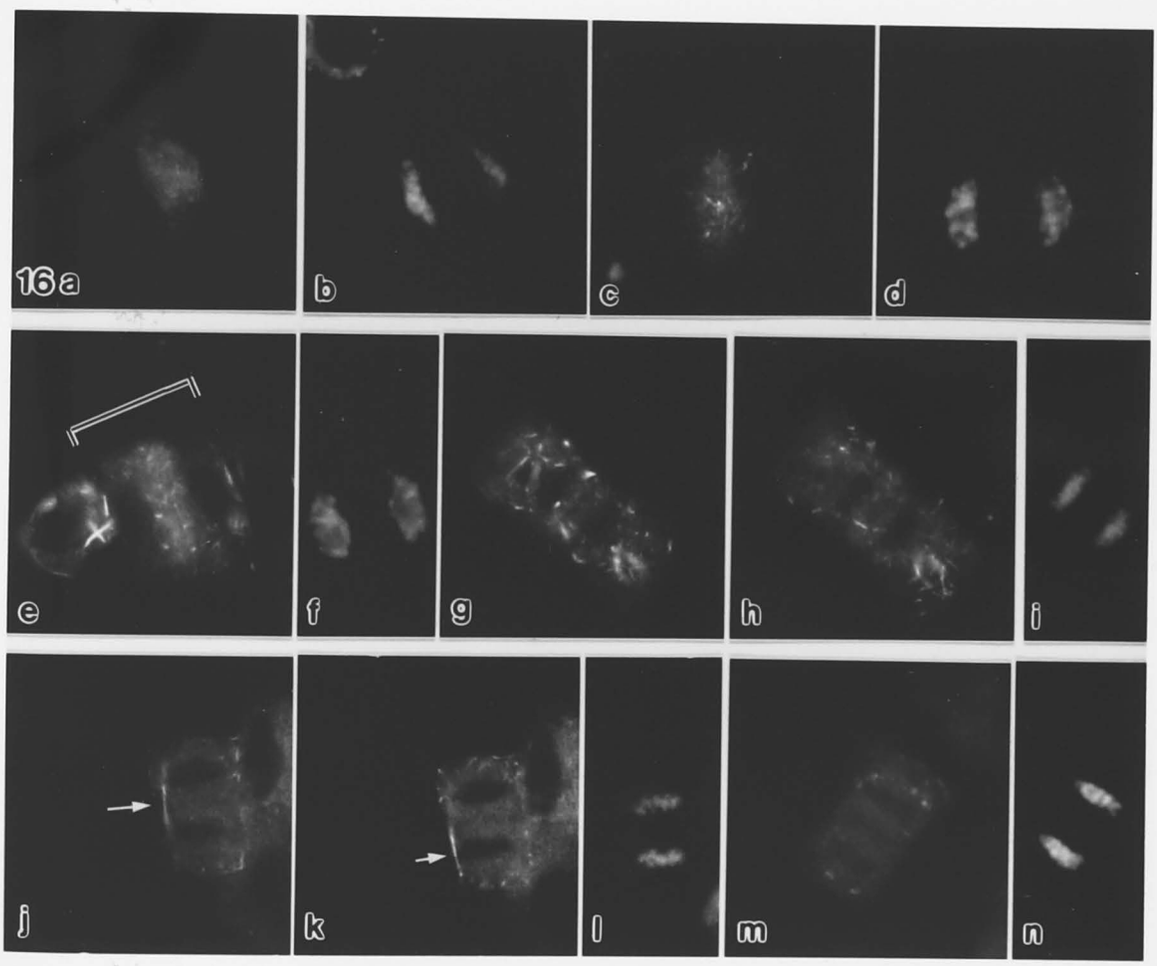
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Fig. 5.17 Mung bean root tip cells labelled with anti-pan myosin. (a)-(j), Interphase. (a), Diffuse and punctate staining of cytoplasm. (b)-(d), One filament in endoplasm. (e), One longitudinal subcortical filament. (f), Adjusted view of the same cell, showing part of the same filament in close proximity to nucleus. (g), Two subcortical filaments (arrows). (h), Adjusted view of the same cell, showing the other part of the same subcortical filaments on two sides of the nucleus (arrows). (i), Internal view, showing one filament (arrow). (j), Cortical view of the same cell, showing the other part of the same filament in cortex (arrow). (k), Prophase, showing weak, diffuse staining of cytoplasm. (l), DAPI of the same cell. (m), Metaphase, showing one filament in endoplasm. (n), DAPI of the same cell. (o)-(r), Anaphase. (o), One filament in endoplasm. (p), DAPI of the same cell. (q), weak, diffuse staining of the cytoplasm. (r), DAPI of the same cell. (s)-(v), Cytokinesis. (s), Intense, amorphous staining of a phragmoplast on two sides of the cell plate (arrow). (t), DAPI of the same cell, at different focal plane from that of (s). (u), No special staining in a phragmoplast, diffuse staining of the cytoplasm. (v), DAPI of the same cell. (magnification: x 954)

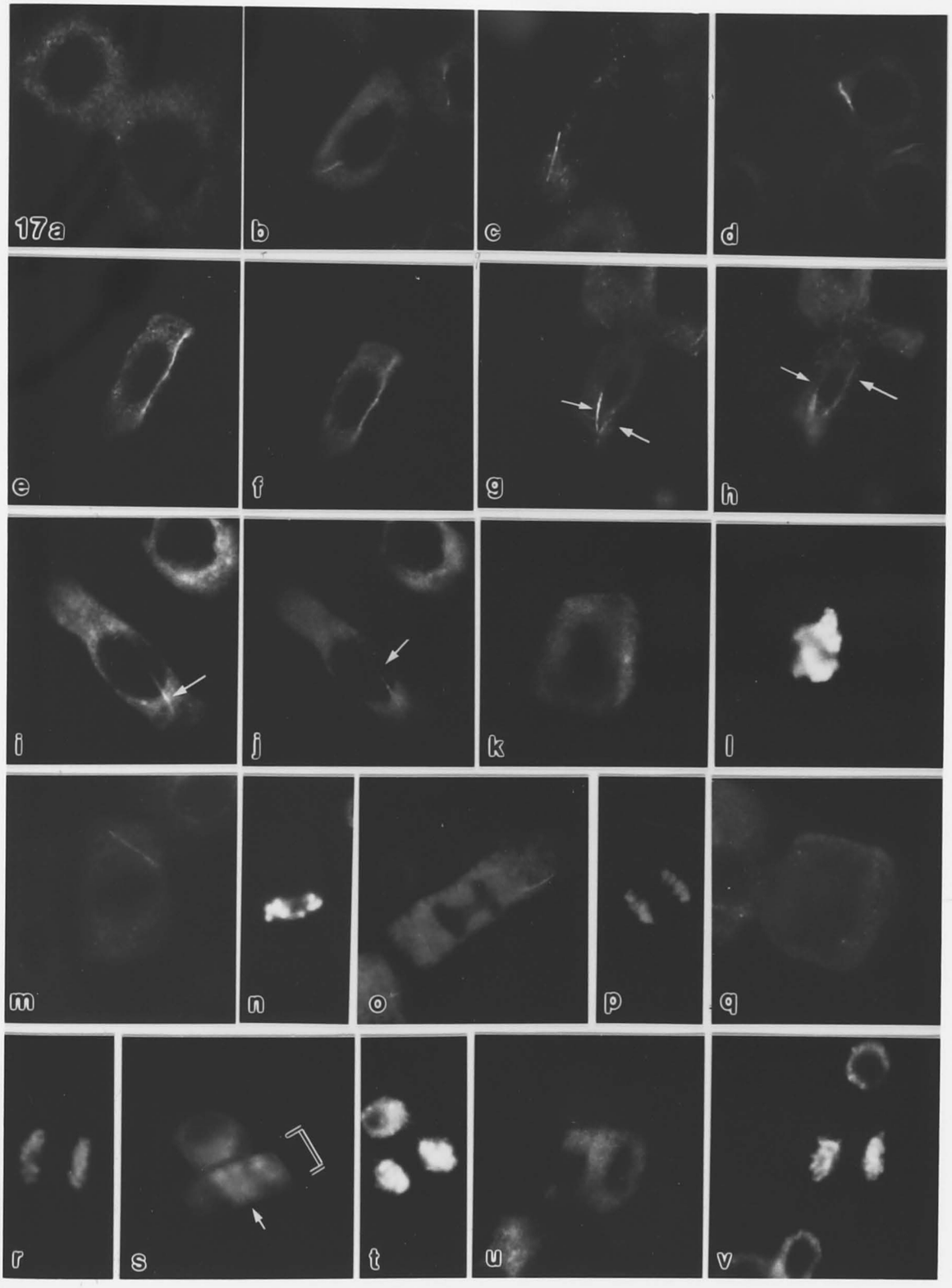
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Fig. 5.18 Mung bean root tip cells in interphase, prophase and metaphase labelled with MB165/1. (a)-(g), Interphase. (a), Diffuse or punctate staining of the cytoplasm. (b)-(e), One or occasionally two filaments in the endoplasm. (f), Internal view, showing a short length of filament. (g), Surface view of the same cell, showing the continuation of the same filament in cortex. (h), Prophase, showing weak, diffuse staining of cytoplasm. (i), DAPI of the same cell. (j)-(m), Metaphase. (j), Weak diffuse staining of cytoplasm. (k), DAPI of the same cell. (l), Two filaments in endoplasm (arrows). (m), DAPI of the same cell. (magnification: x 954)

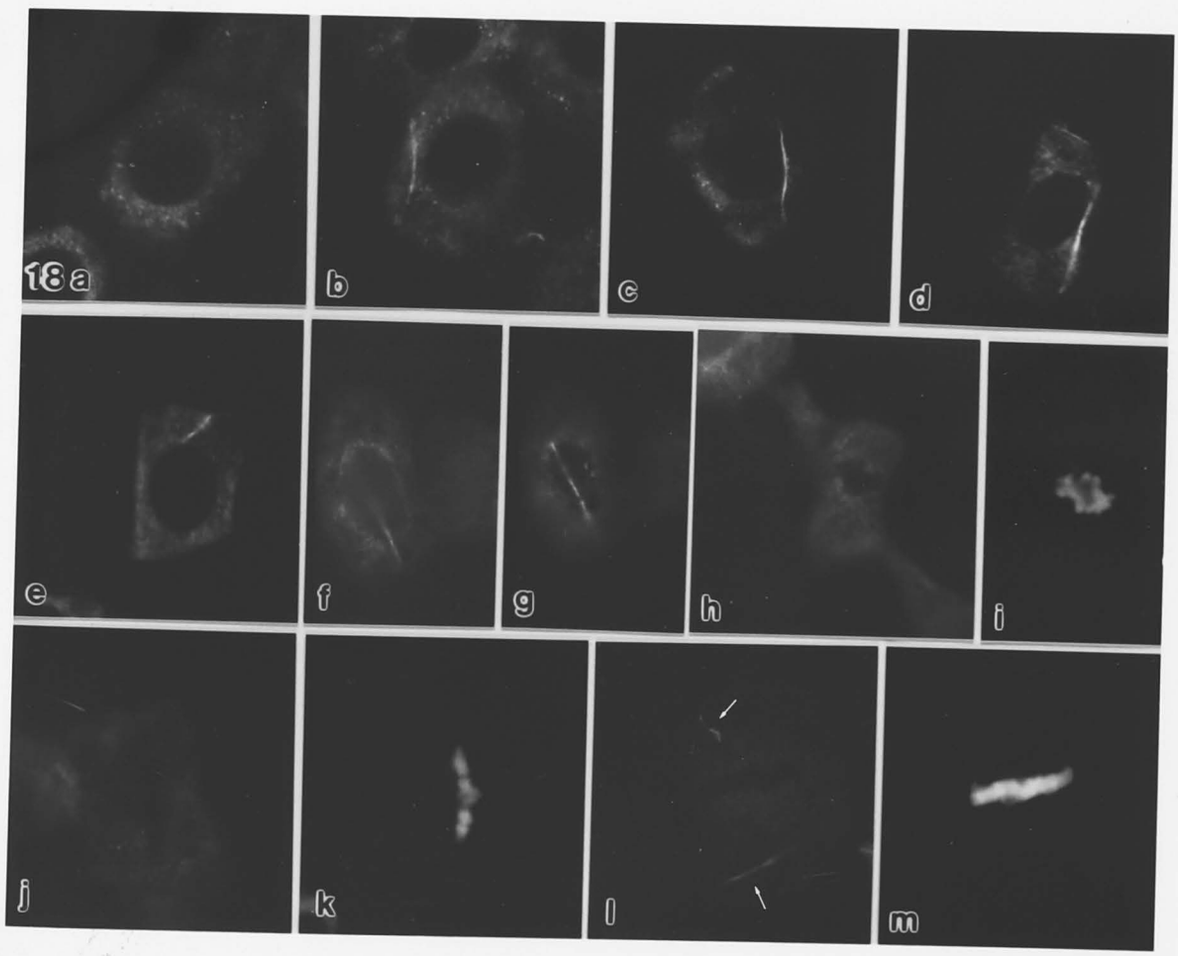
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Fig. 5.19 Mung bean root tip cells in anaphase and cytokinesis labelled with MB165/1. (a)-(d), Anaphase. (a), Weak cytoplasmic staining particularly in the region of the future phragmoplast. (b), DAPI of the same cell. (c), One filament in endoplasm (arrow). (d), DAPI of the same cell. (e)-(o), Cytokinesis. (e), Intense staining of a phragmoplast, with one filament in endoplasm (arrow). (f), DAPI of the same cell. (g), Phragmoplast staining on two sides of the cell plate. (h), DAPI of the same cell. (i), Internal view, showing weak, diffuse staining of a phragmoplast with diffuse and punctate cytoplasmic staining. (j), Surface view of the same cell, showing one short filament in cortex (arrow). (k), Adjusted view of the same cell, showing another short filament in the cortex (arrow). (l), DAPI of the same cell. (m), Internal view, showing one short filament in punctate stained cytoplasm (arrow), with no specific staining in the phragmoplast. (n), Surface view of the same cell, showing the other part of the same filament in cortex (arrow). (o), DAPI of the same cell. (magnification: x 954)

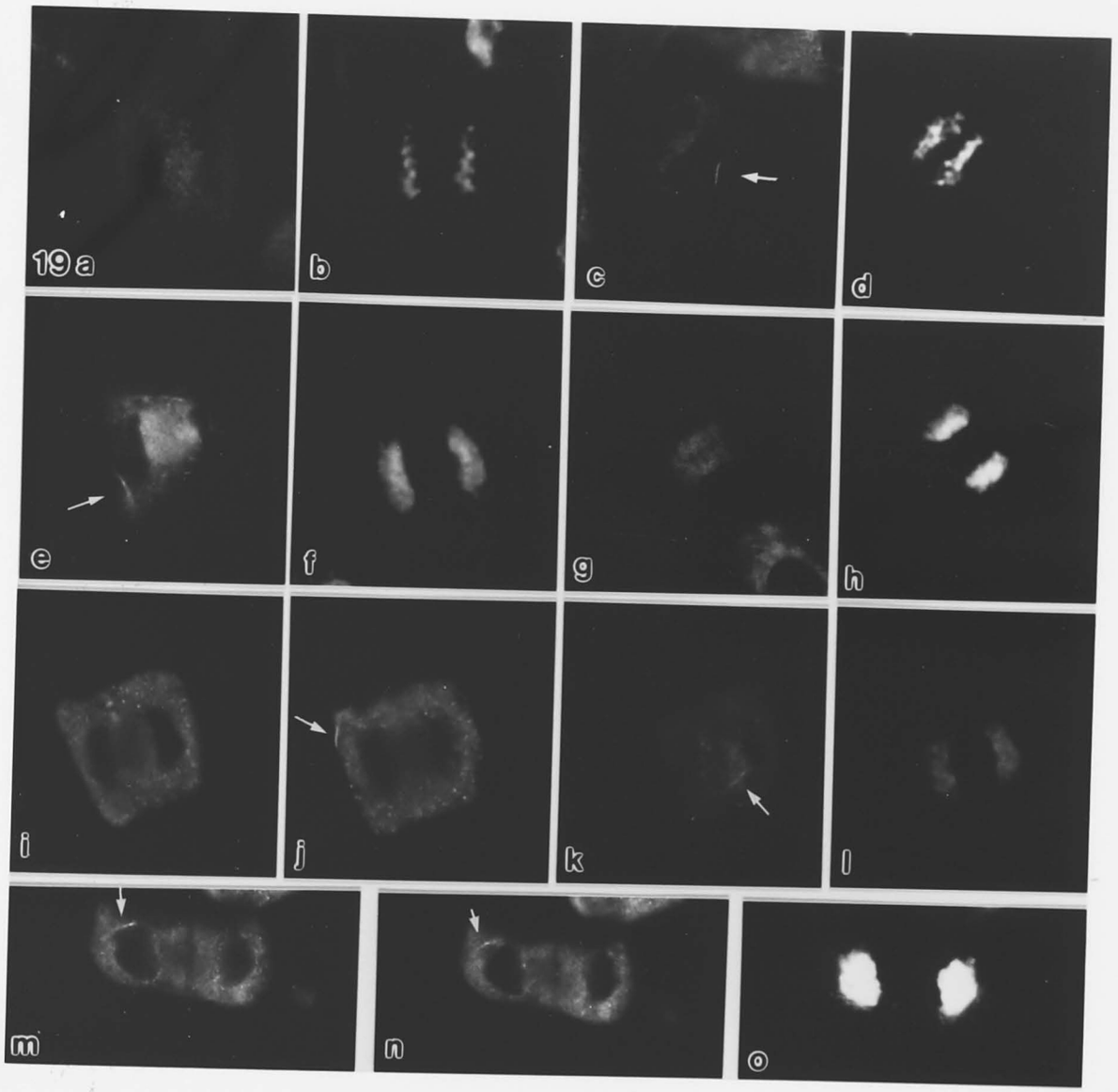
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Fig. 5.20 Mung bean root tip cells labelled with MB165/3. (a)-(d), Interphase. (a), General punctate staining of cytoplasm. (b)-(d), Filaments in endoplasm. (e), Prophase, weak diffuse staining of cytoplasm. (f), DAPI of the same cell. (g), Metaphase, showing weak diffuse and punctate staining of cytoplasm. (h), DAPI of the same cell. (i), Anaphase, showing weak general cytoplasmic staining. (j), DAPI of the same cell. (k)-(n), Cytokinesis. (k), Amorphous phragmoplast staining on two sides of the cell plate (arrow). (l), DAPI of the same cell. (m), One filament in the endoplasm (arrow). The phragmoplast was more strongly stained than the cytoplasm. (n), DAPI of the same cell. (magnification: x 954)

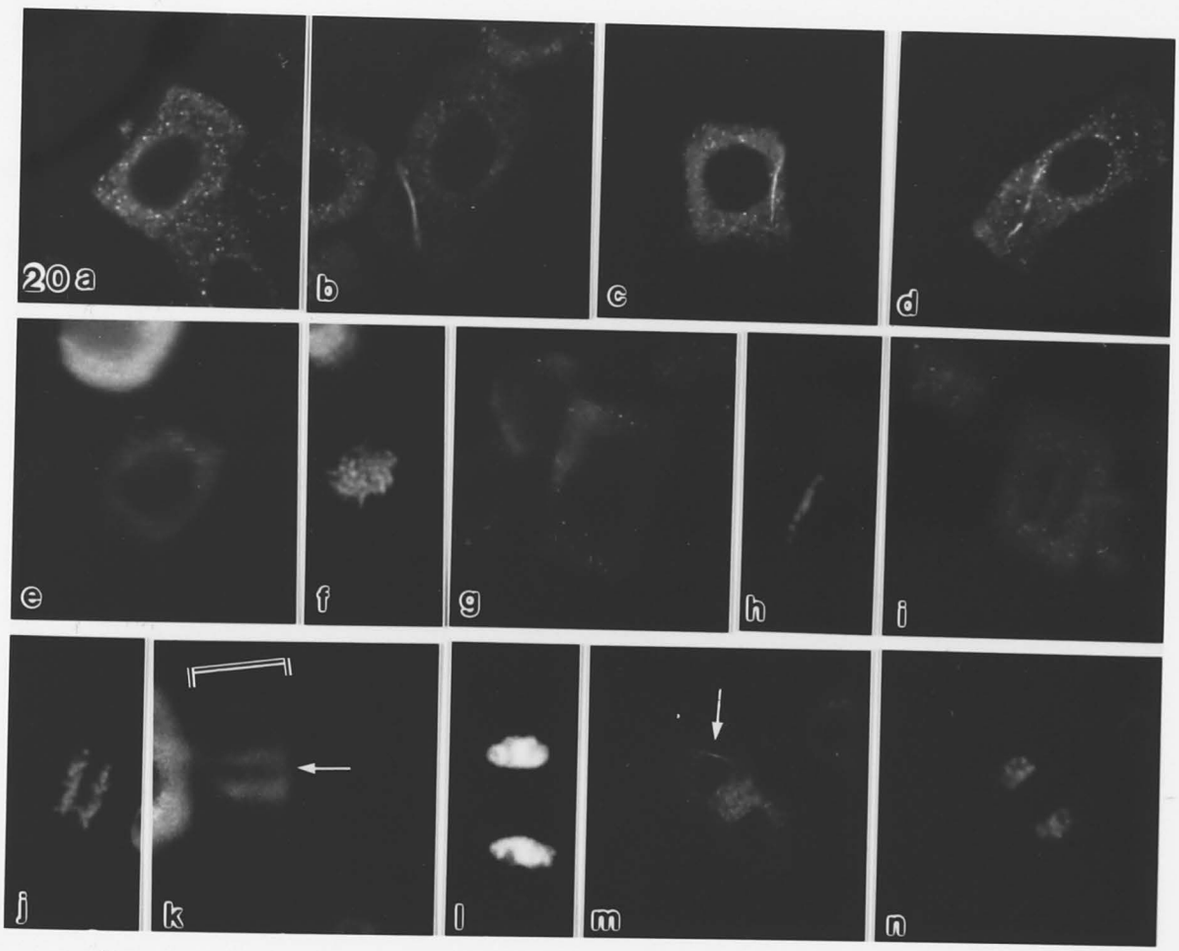
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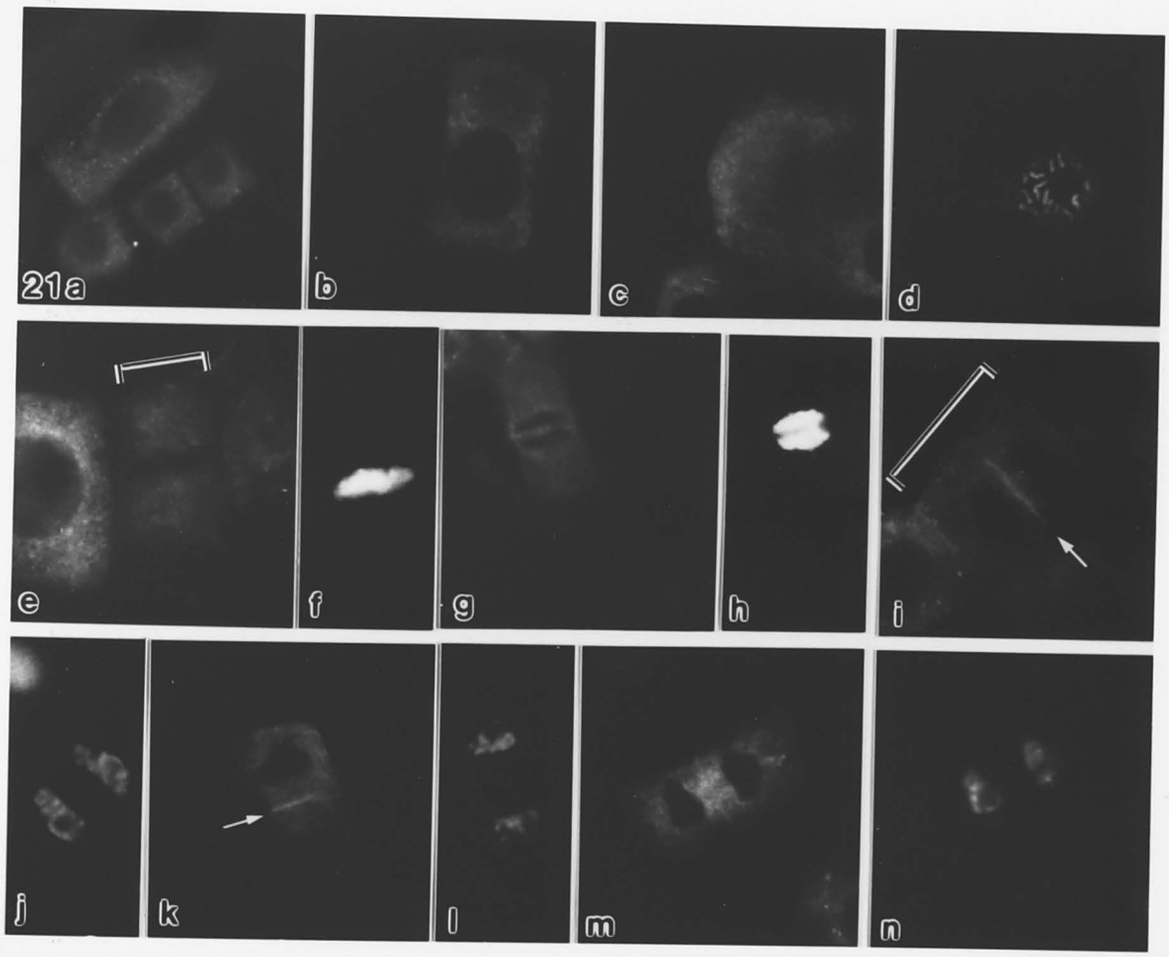
Fig. 5.21 Mung bean root tip cells labelling with MB165/2 and MB165/4. (a), (i) and (j) were labelled with MB165/2, and the rest with MB165/4. (a) and (b), Interphase, showing diffuse or punctate cytoplasmic staining. (c), Prophase, showing weak cytoplasmic staining. (d), DAPI of the same cell. (e), Metaphase (bracket), showing weak cytoplasmic staining. (f), DAPI of the same cell. (g), Anaphase, showing weak cytoplasmic staining. (h), DAPI of the same cell. (i)-(n), Cytokinesis. (i) and (k), Intense staining in cell plate between two daughter cells, resulting in a bright line (arrows). (j) and (l), DAPI of (i) and (k), respectively. (m), Phragmoplast was slightly more strongly stained than cytoplasm. (n), DAPI of the cell. (magnification: x 954)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CHAPTER 6

IMMUNOAFFINITY CHROMATOGRAPHY AND CHARACTERIZATION OF THE MUNG BEAN 165 kDa POLYPEPTIDE FRACTION

CHAPTER 6

IMMUNOAFFINITY CHROMATOGRAPHY AND CHARACTERIZATION OF THE MUNG BEAN 165 kDa POLYPEPTIDE FRACTION

6.1 INTRODUCTION

Immunoblotting with anti-myosin and MB165 antibodies demonstrates that the mung bean 165 kDa polypeptide shares at least five epitopes with the heavy chain of rabbit skeletal muscle myosin while the 155 kDa polypeptide only shares one. This strongly supports the view that the 165 kDa polypeptide is a myosin heavy chain. The immunofluorescent colocalization of the 165 kDa polypeptide with actin in the phragmoplast of mung bean root tip cells is also consistent with this view. The binding of the 165 kDa but not the 155 kDa polypeptide to ADP-agarose showed that only the 165 kDa can be an ATPase. Together, these observations greatly strengthen the view that the 165 kDa polypeptide is a myosin heavy chain and that the 155 kDa one is not.

The small quantity, impurity and the lack of detectable actin-activated Mg^{2+}-ATPase activity of the partially purified 165 kDa polypeptide fraction made the further purification and biochemical characterization of this polypeptide impractical. For this reason, a number of preliminary studies were made to see whether the MB165 antibodies could achieve a rapid immunoaffinity purification of the 165 kDa mung bean protein or could selectively precipitate it with a solid phase so its association with other polypeptides in native conditions could be assessed and its ability to support movement of antibody-coated beads investigated.

Immunoaffinity chromatography has rarely been used in purifying nonmuscle myosins. *Acanthamoeba* myosin I or II can be purified with a column coupled with antibodies specific for *Acanthamoeba* myosin I or II (Pollard 1982b), but no plant myosins or putative myosins (1.8.1) have been purified using antibody affinity columns. Because recognition of native antigens involved, immunoaffinity

chromatography like immunoprecipitation can be used to identify any light chains that might associate with the putative myosin heavy chain from mung bean.

In vitro motility assays directly demonstrate force generation by actomyosin. Myosin-coated polystyrene beads (Sheetz & Spudich 1983; Shimmen & Yano 1984) or organelles (Adams & Pollard 1986) can move unidirectionally over actin cables of characean cells like the *in vivo* cytoplasmic streaming. Alternatively, fluorescently labelled actin filaments can move along myosin-coated surface (Kron & Spudich 1986; Toyoshima *et al.* 1987; Kron *et al.* 1992). The assays can be made quantitative to study myosin and the regulation of myosin-actin interaction (Shimmen & Yano 1985, 1986; Vale *et al.* 1984; Okagaki *et al.* 1989; Kohama & Shimmen 1985; Kohama *et al.* 1991a). The velocity of movement is principally determined by the type of myosin rather than of actin.

In plants, endoplasmic organelles isolated from *Chara* (Shimmen & Tazawa 1982) and pollen tube organelles (Kohno & Shimmen 1988a, b; Kohno *et al.* 1990) move along actin bundles in characean cells and muscle actin filaments move on a surface coated with a crude extract of pollen tubes (Kohno *et al.* 1991). However, none of the purified and partially purified plant myosins (1.8.1) have been studied using such assays. If beads coated with mung bean 165 kDa polypeptide fractions move unidirectionally along *Chara* actin bundles, it would confirm that the polypeptide is an authentic myosin heavy chain. This Chapter records the results of preliminary experiments using these three immunological techniques.

6.2 MATERIALS AND METHODS

6.2.1 Purification of antibodies from ascites fluids

3.7 ml of MB165/1, 10.3 ml of MB165/2, 9.2 ml of MB165/3 and 2.5 ml of MB165/4, were pooled and clarified by centrifuging at 10,500 rpm for 10 min in JA-20. 12 ml of the pooled ascites supernatant were applied to a column (2.6 x 7.6 cm) of CM Affi-Gel blue (Bio-Rad) equilibrated with 250 ml of starting buffer (0.01 mM K_2HPO_4, pH 7.25, 0.15 M NaCl), washed with 160 ml of the buffer, and flow-through fractions pooled. The column was regenerated with 150 ml of 2 M NaCl in the starting buffer, equilibrated with 500 ml of the starting buffer and used to process the other 12 ml of pooled supernatant. The column was eluted and regenerated with 350 ml of 2 M NaCl in the starting buffer. Eluate fractions were monitored by ELISA [5.2.2 (ii)] and 13.5% SDS-PAGE.

The 0-50% $(NH_4)_2SO_4$ pellet from the combined flow-through fraction was desalted in the starting buffer and repurified on the CM Affi-Gel Blue column as before. 23 ml of the pooled flow-through fraction were applied to a DEAE-Sephacel column (1.7 x 9.8 cm) equilibrated with 0.01 M K_2HPO_4, pH 7.25, 0.15 M NaCl. The column was eluted with 160 ml of a 0-0.5 M NaCl gradient, and then with 0.8 M NaCl in the starting buffer. Antibodies were eluted in one protein peak by 0.074-0.12 M NaCl, and the peak was pooled.

6.2.2 Immunoaffinity chromatography of mung bean 165 kDa polypeptide fraction

(i) Preparing the antibody column

16 ml of the purified MB165 (6.2.1) were concentrated against Aquacide II overnight, and dialyzed against 3 liters of coupling buffer (0.1 M $NaHCO_3$, pH 8.5, 0.5 M NaCl) for 6 h. 1 ml (6.5 mg of protein) of MB165 was made up to 5 ml with coupling buffer.

1 g of freeze-dried cyanogen bromide (CNBr)-activated Sepharose 4B (Pharmacia) was washed and reswollen on a sintered glass funnel with 500 ml of 1

mM HCl and with 100 ml of coupling buffer. The equilibrated gel was mixed with 5 ml of MB165 and incubated for 4.5 h with rotation and several gentle shakes by hand. The buffer was removed by filtering and active groups blocked by with 100 ml of 0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl. Protein concentrations of MB165 solution (5 ml) added to the gel and the supernatant after 4.5 h incubation were measured to monitor the efficiency of protein coupling. The gel was resuspended in 20 ml of the Tris buffer, and incubated with rotation for 6 h with several gentle shakes. Filtered gel was washed 3 times with alternating 125 ml of acetate buffer (0.1 M sodium acetate, pH 4.0, 0.5 M NaCl) and 133 ml of the Tris buffer before a final wash with 125 ml of the acetate buffer and then with 190 ml of imidazole buffer (0.34 M sucrose, 10 mM imidazole, pH 7.0). The MB165-Sepharose gel was resuspended with an equal volume of the imidazole buffer.

(ii) Immunoaffinity chromatography

The crude extract from 100 g of mung bean was fractionated with DE52 batch processing (4.2.1) and 67.5 ml (83.6 mg of protein) of the 0.4 M NaCl eluate were fractionated with 0-45% $(NH_4)_2SO_4$. 17 ml of the desalted 0-45% fraction were applied to a column (1.6 x 0.8 cm) of MB165-Sepharose equilibrated with 0.34 M sucrose, 10 mM imidazole, pH 7.0 (DTT was avoided as it can break the disulfide linkages between the heavy and light chains of antibody). The pooled flow-through fraction was reapplied to the column. The second pooled flow-through fraction was applied to the column at a flow rate of 40 ml/h, using a peristaltic pump (Varioperpex; LKB). Chromatography was stopped overnight and the column eluted sequentially with 1 M NaCl; 3 M $MgCl_2$; 10 mM Tris-HCl, pH 8.0; and 0.1 M glycine, pH 2.5. Eluate fractions were monitored by immunoblotting with anti-pan myosin antibody.

6.2.3 Immunoprecipitation of mung bean proteins

(i) Coupling antibodies to anti-mouse IgM-agarose

500-600 µl of goat anti-mouse IgM (µ-chain specific)-agarose were loaded into a small column, and washed with 10 ml of TBS. 250 µl of anti-pan myosin or anti-fast myosin antibody were applied to the column cyclically for 1.5 h at room temperature and left overnight at 0-4°C. The column was washed with 15 ml of TBS, and the antibody-agarose resuspended in an equal volume of TBS.

(ii) Immunoprecipitation

Mung bean crude extract was prepared as described in 2.5.1 (i) except that DTT was omitted. 1 ml of crude extract was mixed with 200 µl of antibody-agarose in a 1.5 ml eppendorf tube, rotated for 1 h at room temperature, and the gel was pelleted at 10,000 rpm for 3 min. The gel was washed with 3 x 1 ml of 0.1 M Tris-HCl, pH 7.5, 0.6 M NaCl, with 4 x 1 ml of 0.1 M Tris-HCl, pH 7.5, and collected by centrifugation. The gel was resuspended with an equal volume 2x SDS-PAGE sample buffer, then with 2 gel volumes of 1x SDS-PAGE sample buffer. The resuspended gel was boiled for 3 min in a water bath, cooled, and clarified at 7,000 rpm for 2 min. The supernatant was used in immunoblotting. Three controls were:

1. 1 ml of crude extract was replaced with extraction buffer.
2. 1 ml of crude extract was mixed with anti-mouse IgM-agarose.
3. 1 ml of extraction buffer was mixed with anti-mouse IgM-agarose.

6.2.4 *In vitro* motility assay of mung bean 165 kDa polypeptide fraction

(i) Coating Covaspheres with antibodies

The whole procedure was carried out at 0-4°C. 30 µl of green fluorescent Covaspheres MX suspension (0.75 µm diameter; Duke Scientific Corporation, Palo Alto, CA., USA) were mixed with 30 µl of anti-pan myosin antibody and 30 µl of dH_2O. The mixture was incubated with rotation for 75 min. Covaspheres were pelleted at 10,000 rpm for 10 min, resuspended in 300 µl of 1% (w/v) BSA in TBS, and incubated for 10 min with rotation to block unreacted sites. Pelleted

Covaspheres were washed twice with 300 µl of imidazole buffer (0.34 M sucrose, 10 mM imidazole, pH 7.0) and resuspended in 30 µl of same buffer.

For MB165 labelling, 30 µl of Covaspheres were mixed with 60 µl of MB165/1, MB165/2, MB165/3, or MB165/4 for 75 min. In the blocking step, the Covaspheres were resuspended with 60 µl of each MB165 in 240 µl TBS in order to increase the concentration of antibody on the bead surface. The other steps were the same as those for anti-pan myosin antibody labelling.

(ii) Reacting antibody-Covaspheres with mung bean proteins

The whole procedure was carried out at 0-4°C. 200 µl of mung bean crude extract [2.5.1 (i)] were incubated for 1 h with 10 µl of antibody-labelled Covaspheres diluted 10-fold with buffer. Pelleted Covaspheres were washed with 2 x 300 µl of ACPS and resuspended with 10 µl ACPS. In controls, mung bean crude extract was replaced with extraction buffer.

To check whether the 165 kDa polypeptide bound to antibody-labelled Covaspheres, 10 µl of Covaspheres suspension labelled with either anti-pan myosin antibody or with each of the four MB165 were mixed with 1 ml of mung bean crude extract, and incubated for 1 h with rotation. Pelleted Covaspheres were washed for 10 min with rotation in 1 ml of TBS. The Covaspheres were collected, resuspended with 15 µl of 1x SDS-PAGE sample buffer, and boiled for 3 min. Pelleted Covaspheres were resuspended with 25 µl of 1x SDS-PAGE sample buffer, boiled for 3 min, the two supernatants combined and resolved by 7% SDS-PAGE.

(iii) Coating Covaspheres directly with protein samples

Proteins of five fractions (Table 4.1, 1-3, 5 and 6) containing the 165 kDa polypeptide during the partial purification of this polypeptide were coupled as described in (ii) to Covaspheres prewashed twice with the imidazole buffer (i) except that Covaspheres unlabelled with antibodies and diluted 1: 100 were used for protein coating.

In positive controls, rabbit skeletal muscle myosin (2.15) (50-100 μg) was coupled directly to Covaspheres prewashed with buffer (0.3 M KCl, 0.15 M K-phosphate, pH 6.5) as described in (ii) except that Covaspheres unlabelled with antibodies and diluted 1: 100 were used for protein coating.

(iv) ***In vitro* motility assay**

A *Chara* internodal cell was perfused with ACPS as described by Williamson (1975) for 5 min to remove the endoplasm of the cell. About 5 μl of the Covaspheres suspension were introduced by perfusion. Movement of beads was observed with a 40 x objective using an Axiovert 35 M inverted microscope equipped for incident-light fluorescence (Zeiss, Oberkochen, FGR).

6.3 RESULTS

6.3.1 Purification of antibodies from ascites fluids

Common problems encountered during immunoaffinity purification using monoclonal antibodies are low-affinity reactions or cross-reactions due to the single epitope. Pooled monoclonal antibodies may overcome these problem (Harlow & Lane 1988). Therefore, four MB165 were pooled to make a single antibody affinity column. CM Affi-Gel Blue is an affinity chromatography medium made by coupling Cibacron Blue F3GA to CM Bio-Gel A crosslinked agarose beads (Anon. 1984). It is conveniently used in purifying antibodies by selectively adsorbing both albumin and protease from serum, and thus was tried in purifying MB165.

The first elution profile of ascites fluid (12 ml) from the CM Affi-Gel Blue column is shown in Fig. 6.1. From ELISA tests, most antibodies flowed through the column, some bound to the column and could be eluted by 2 M NaCl (data not shown). The heavy chains of IgM antibodies migrate with a M_r of approximately 70-80 kDa in SDS-PAGE (Harlow & Lane 1988) as shown in Fig. 6.1A. Besides antibodies, some other proteins, especially the 66 kDa protein, also flowed through the column (Fig. 6.1A, lanes 1-4). In the 2 M NaCl fraction, the 66 kDa protein was the major protein (Fig. 6.1A, lanes 5-7). The elution profile of the other 12 ml of ascites from the CM Affi-Gel Blue column was similar to Fig. 6.1.

Since flow-through fractions were still contaminated with the 66 kDa protein, the combined flow-through fractions (Fig. 6.1A, lane 8) were fractionated with 0-50% $(NH_4)_2SO_4$ (Fig. 6.1A, lane 9) and reapplied to the regenerated CM Affi-Gel Blue column. The third profile of antibodies from the CM Affi-Gel Blue column was similar to Fig. 6.1. Some IgM bound to the column (data not shown), and many IgM antibodies were lost after several steps of fractionation (Fig. 6.1A, lane 10). All proteins bound to the DEAE-Sephacel column (Fig. 6.2), but antibodies eluted with other proteins and no good resolution was achieved (data not shown). Peak fractions of 0.074-0.12 M NaCl (fractions 75.9 ml to 91.9 ml) were pooled, concentrated, and used to prepare the antibody column.

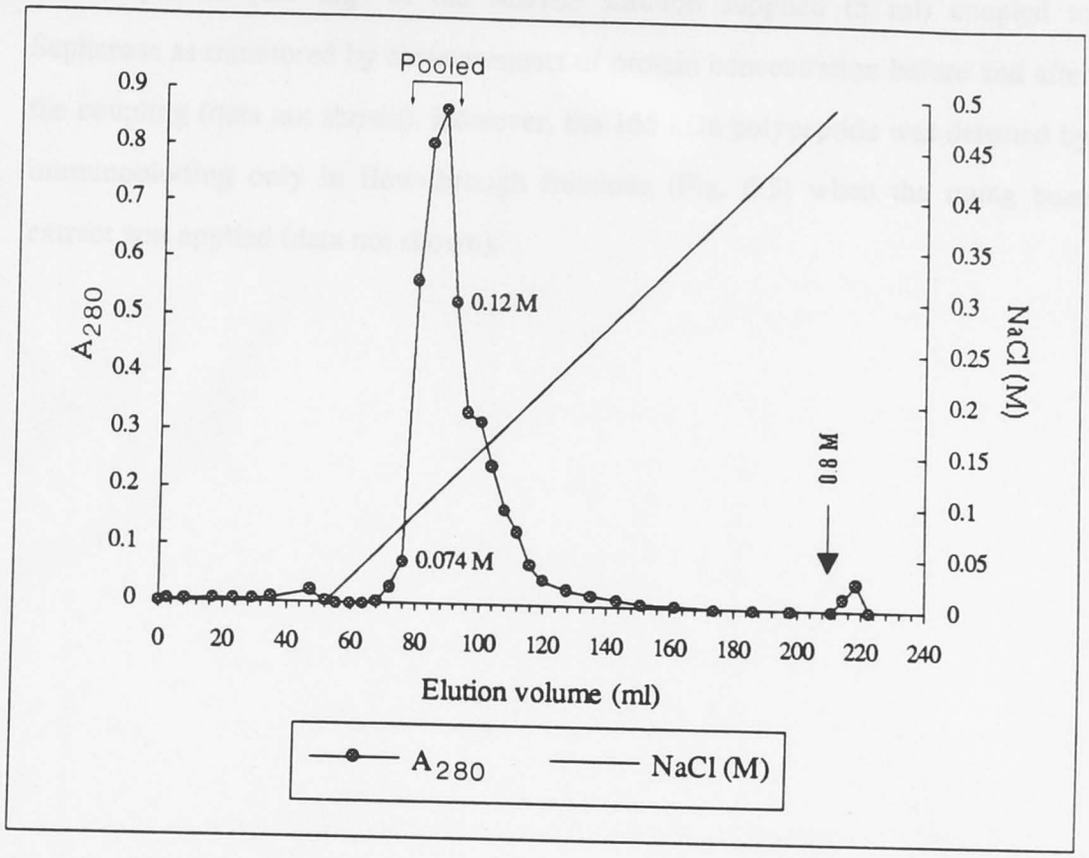


Fig. 6.2 DEAE-Sephacel chromatography of antibody fraction. 17.7 ml of the 0-50% $(NH_4)_2SO_4$ fraction of the flow-through from the CM Affi-Gel Blue column were applied to a column (1.7 x 9.8 cm) of DEAE-Sephacel equilibrated with starting buffer (0.01 M K_2HPO_4, pH 7.25, 0.15 M NaCl). The column was eluted with a gradient of 0-0.5 M NaCl (fractions 53.9 ml to 210.5 ml), and then 0.8 M NaCl (from fraction 212.5 ml) in the starting buffer. Antibodies were eluted in a peak by 0.074-0.12 M NaCl, and peak fractions were pooled.

6.3.2 Immunoaffinity purification of mung bean 165 kDa polypeptide

All protein (6.5 mg) of the MB165 solution supplied (5 ml) coupled to Sepharose as monitored by measurements of protein concentration before and after the coupling (data not shown). However, the 165 kDa polypeptide was detected by immunoblotting only in flow-through fractions (Fig. 6.3) when the mung bean extract was applied (data not shown).

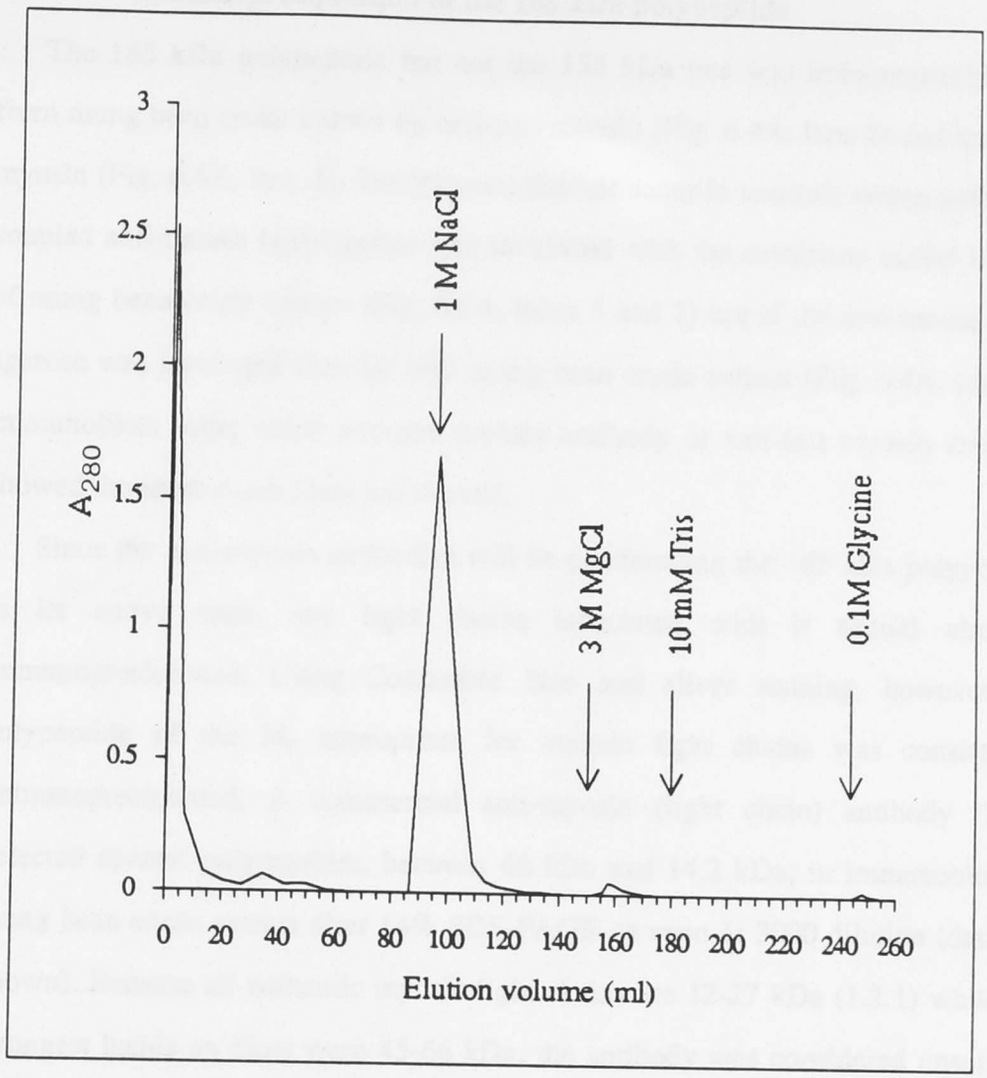


Fig. 6.3 MB165-Sepharose chromatography of mung bean 165 kDa polypeptide. 17 ml of 0-45% $(NH_4)_2SO_4$ fraction of the 0.4 M NaCl eluate from DE52 were applied to a column (1.6 x 0.8 cm) of MB165-Sepharose equilibrated starting buffer (0.34 M sucrose, 10 mM imidazole, pH 7.0). The column was eluted with steps of 1 M NaCl (fractions 95.6 ml to 149.6 ml), 3 M $MgCl_2$ (fractions 151.9 ml to 177.8 ml), 10 mM Tris-HCl, pH 8.0 (fractions 179.5 ml to 242.3 ml), and 0.1 M glycine, pH 2.5 (fractions 244.3 ml to 254.7 ml).

6.3.3 Immunoprecipitation of the 165 kDa polypeptide

The 165 kDa polypeptide but not the 155 kDa one was immunoprecipitated from mung bean crude extract by anti-pan myosin (Fig. 6.4A, lane 2) and anti-fast myosin (Fig. 6.4A, lane 6). Precipitation did not occur in controls where antibody-coupled anti-mouse IgM-agarose was incubated with the extraction buffer instead of mung bean crude extract (Fig. 6.4A, lanes 3 and 7) nor if the anti-mouse IgM-agarose was incubated directly with mung bean crude extract (Fig. 6.4A, lane 4). Immunoblots using either anti-pan myosin antibody or anti-fast myosin antibody showed the same result (data not shown).

Since the anti-myosin antibodies will be precipitating the 165 kDa polypeptide in its native state, any light chains associated with it should also be immunoprecipitated. Using Coomassie blue and silver staining, however, no polypeptide of the M_r appropriate for myosin light chains was consistently immunoprecipitated. A commercial anti-myosin (light chain) antibody (2.11) detected several polypeptides, between 66 kDa and 14.2 kDa, in immunoblots of mung bean crude extract after 14% SDS-PAGE, at even 1: 3000 dilution (data not shown). Because all authentic myosin light chains are 12-27 kDa (1.2.1) while the strongest bands on blots were 45-66 kDa, the antibody was considered unsuitable for detecting light chains of mung bean myosin.

6.3.4 *In vitro* motility assays of mung bean 165 kDa polypeptide

Beads coated directly with rabbit skeletal muscle myosin moved unidirectionally along *Chara* actin bundles and in opposite directions on both sides of the neutral line (personal communication from Dr. Richard E. Williamson). However, no movement of beads coated directly with mung bean 165 kDa polypeptide fractions was observed. To attempt to increase selectively the concentration of the 165 kDa polypeptide on the bead surface, anti-pan myosin antibody and four MB165 were coupled to Covaspheres. However, no bead movement was observed.

To confirm that the 165 kDa polypeptide was coupled to the antibody labelled beads, proteins eluted from beads were monitored by 7% SDS-PAGE. From the Ponceau-stained blot, the 165 kDa polypeptide together with some other proteins were captured by beads labelled with either anti-pan myosin or MB165 (Fig. 6.5).

6.4 DISCUSSION

6.4.1 Immunoaffinity chromatography

The purification of IgM from ascites was inefficient. The CM Affi-Gel Blue mainly absorbed the 66 kDa protein while the flow-through antibody fractions were still contaminated with many other proteins. IgM was easily lost after several cycles of the affinity chromatography. A bigger column to avoid recycling the ascites fluid through the column twice may prevent some loss of IgM antibodies. CM Affi-Gel Blue gel efficiently removes protease and albumin from serum, and over 80% of serum IgG will pass through a CM Affi-Gel Blue column without adsorption or retardation (Anon. 1984). However, the gel may not be optimal for purifying IgM from ascites.

Mung bean 165 kDa polypeptide did not bind to the immunoaffinity column. This may result from the low concentration of MB165 on the column since IgM antibodies were not well purified from ascites. Alternatively, if the affinity of MB165 for the 165 kDa polypeptide is low, the antibody affinity column may likewise not work well. Improved antibody purification and further attempts to optimize conditions may improve the efficiency of the antibody affinity column.

6.4.2 Immunoprecipitation

The 165 kDa polypeptide of mung bean, but not the 155 kDa polypeptide was specifically immunoprecipitated by both anti-pan myosin and anti-fast myosin antibodies. Although anti-pan myosin recognizes both the 165 and 155 kDa polypeptides on immunoblots (3.3.1), its recognition of only the 165 kDa polypeptide under the native conditions of immunoprecipitation shows that the epitope it recognizes in the two polypeptides cannot be identical. This further strengthens the argument that the 155 kDa polypeptide is not a proteolytic fragment of the 165 kDa polypeptide.

6.4.3 *In vitro* motility assays

The 165 kDa polypeptide, with other plant proteins, bound to Covaspheres labelled with anti-pan myosin or MB165. This confirms the ability of MB165 to recognize the native 165 kDa polypeptide and suggests that further efforts with immunoaffinity columns might provide successful large scale purification. However, no unidirectional bead movements along the actin cables of *Chara*, could be discerned. Two reasons why an authentic myosin might not catalyze movement can be foreseen: the concentration of the 165 kDa polypeptide on the Covaspheres might not be high enough or the antibodies may not capture the myosin in an orientation that permits reaction with actin. The force generated by actin and myosin may then not be strong enough to move the bead along the actin cables.

In the case of *in vitro* motility assay of rabbit skeletal muscle myosin, the velocity of bead movement is independent of myosin concentration on the bead surface for concentrations above a critical value which is about 20 μg myosin/2.5 x 10^9 beads of 1 μm in diameter (Sheetz *et al.* 1984). Therefore, it may be better to use a more purified 165 kDa polypeptide fraction in motility assays. However, if the actin-activated Mg^{2+}-ATPase activity was lost during purification, the 165 kDa polypeptide would not be able to drive the bead movement.

The velocity (μm/s) of bead movement is 2-6 with rabbit skeletal muscle myosin, 0.1-0.6 with gizzard smooth muscle, 0.5-1.5 with *Dictyostelium* myosin (Sheetz *et al.* 1984) and 1-1.8 with *Physarum* myosin (Kohama & Shimmen 1985). The organelles of pollen tube move along actin cables at 26 μm/s (Kohno & Shimmen 1988a). For the organelles from *Acanthamoeba*, the organelle movement driven by *Acanthamoeba* myosin I were too slow (0.23 μm/s) to be discerned by eye but became obvious when speeded up by time-lapse video microscopy (Adams & Pollard 1986). In the case of mung bean, the beads might move too slowly to be distinguished by eye, and observation could be helped by using time-lapse video microscopy.

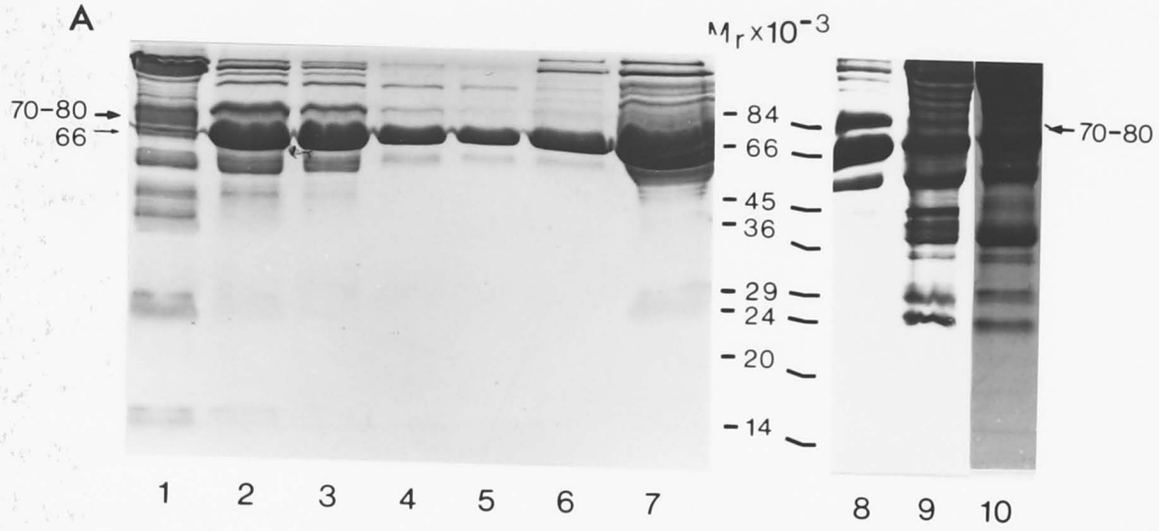
6.4.4 Conclusions

Immunoaffinity purification of the 165 kDa polypeptide from mung bean was not successful and neither was the *in vitro* motility assay. This leaves it undecided whether the 165 kDa polypeptide is an authentic myosin. The 165 but not the 155 kDa polypeptide was specifically immunoprecipitated with anti-pan myosin antibody, which further supports the view that the 155 kDa polypeptide is unlikely to be a proteolytic fragment of the 165 kDa polypeptide. Because the MB165 antibodies do recognize native 165 kDa polypeptide, more thorough studies of this type using the MB165 antibodies are desirable in the hope of recovering the 165 kDa polypeptide in an enzymically active form.

CHAPTER 6

FIGURES

Fig. 6.1 CM Affi-Gel Blue chromatography of ascites fluid. 12 ml of the pooled ascites fluid of the four MB165 antibodies were applied to a column (2.6 x 7.6 cm) of CM Affi-Gel Blue equilibrated with starting buffer (0.01 M K_2HPO_4, pH 7.25, 0.15 M NaCl). Flow-through fractions up to fraction 138.9 ml were pooled. The column was eluted and regenerated (from fraction 159.5 ml) with 2 M NaCl in the starting buffer. Selected eluate fractions resolved by 13.5% SDS-PAGE and stained with Coomassie R are shown at the top (A). Corresponding elution volumes (ml) of the fractions were: (1), 19.9; (2), 58.4; (3), 72.4; (4), 114.4; (5), 159.5; (6), 181.2; and (7), 192. (8), combined flow-through fractions of two chromatography runs of the CM Affi-Gel Blue column; (9), 0-50% $(NH_4)_2SO_4$ fraction of the combined flow-through fraction; (10), pooled flow-through fraction of the 0-50% $(NH_4)_2SO_4$ fraction on the CM Affi-Gel Blue column. The M_r of IgM heavy chain is approximately 70-80 kDa.


A
$M_r \times 10^{-3}$
70-80
66
84
66
45
36
29
24
20
14
70-80
1 2 3 4 5 6 7
8 9 10

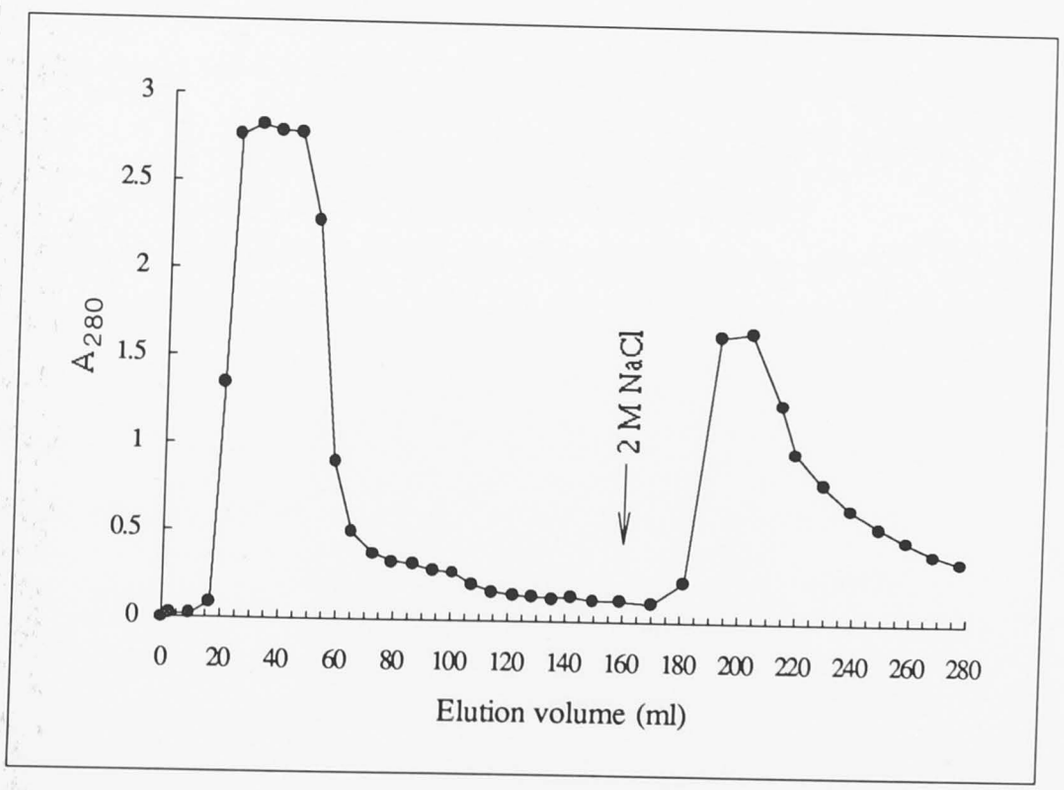
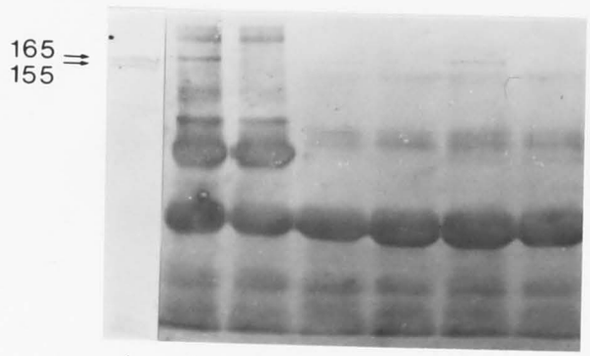

A_{280}
3
2.5
2
1.5
1
0.5
0
2 M NaCl
0 20 40 60 80 100 120 140 160 180 200 220 240 260 280
Elution volume (ml)

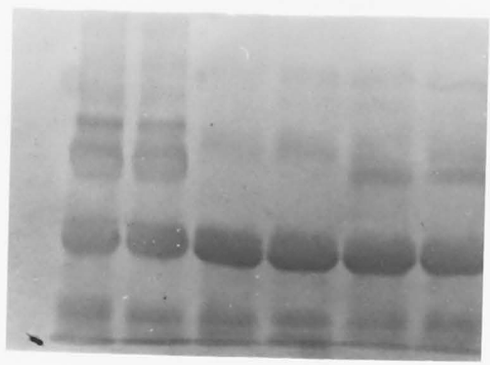
Fig. 6.4 **Immunoprecipitation of mung bean 165 kDa polypeptide.** Mung bean 165 kDa polypeptide was immunoprecipitated with anti-mouse IgM-agarose coupled with anti-pan myosin antibody (2) or anti-fast myosin antibody (6). Blot A was immunoblotted with anti-pan myosin antibody, and blot B was a control without incubation of the blot with the primary antibody. (1), mung bean crude extract; (2) and (6), immunoprecipitated mung bean 165 kDa polypeptide. Controls were: (3), anti-pan myosin coupled anti-mouse IgM-agarose was incubated with extraction buffer instead of mung bean extract; (4), anti-mouse IgM-agarose was incubated with mung bean crude extract; (5), anti-mouse IgM-agarose was incubated with the extraction buffer. (7), anti-fast myosin coupled anti-mouse IgM-agarose was incubated with extraction buffer instead of mung bean extract.

Fig. 6.5 **Coating of anti-myosin antibody labelled Covaspheres with mung bean 165 kDa polypeptide.** Ponceau-stained blots of: (1), mung bean crude extract. Covaspheres were labelled with five antibodies respectively and incubated with mung bean crude extracts: (2), MB165/1; (3), MB165/2; (4), MB165/3; (5), MB165/4; and (6), anti-pan myosin antibody.

4A



4B



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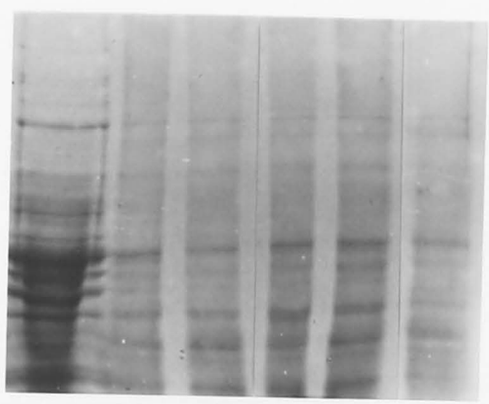

$M_r \times 10^{-3}$

CHAPTER 7

GENERAL DISCUSSION

CHAPTER 7

GENERAL DISCUSSION

In mung bean, polypeptides of 165 and 155 kDa are detected by immunoblotting with anti-pan myosin antibody in protein extracts made with either extraction buffer or with TCA solution that minimizes proteolysis (Wu & Wang 1984). This indicates that the 155 kDa polypeptide is not a proteolytic fragment of the 165 kDa one. The 165 kDa polypeptide is the one most constantly detected and was therefore considered as a putative heavy chain of mung bean myosin. An additional 110 kDa polypeptide is much more easily detected in the absence than in the presence of proteolytic inhibitors but is never detected in extracts directly made with TCA. Therefore, it is considered to be a proteolytic fragment of either the 165 or 155 kDa polypeptide. In other higher plants (pea, wheat and *Arabidopsis*), only one single polypeptide of 165/160 kDa is detected by anti-pan myosin.

Epitopes of proteins from mung bean, pea, wheat, *Arabidopsis* and *Chara* are detected by immunoblotting with four monoclonal antibodies raised to the mung bean 165 kDa polypeptide (MB165) and four commercial monoclonal anti-myosin antibodies [anti-skeletal myosin (fast), anti-myosin (smooth and skeletal), anti-fast myosin and anti-pan myosin]. At least six different epitopes are recognized by these eight antibodies, all of which are on the heavy chain of rabbit muscle myosin. The 165/160 kDa polypeptides of all four higher plants share five of these six epitopes with the heavy chain of rabbit muscle myosin. However, none of the four immunoreactive *Chara* polypeptides (200, 175, 124 and 110 kDa) share so many epitopes (≤ 3) with the heavy chain of rabbit muscle myosin.

The high degree of similarity of epitopes between the mung bean 165 kDa polypeptide and the heavy chain of rabbit muscle myosin greatly strengthens the case that it is a myosin heavy chain. No putative plant myosin heavy chain has been shown previously to share so many epitopes with the heavy chain of muscle myosin (1.8.2). The mung bean 155 kDa polypeptide, however, has only one epitope

recognized by anti-pan myosin, supporting the earlier conclusion that it is not a myosin heavy chain.

The mung bean 165 kDa, but not the 155 kDa, polypeptide is specifically immunoprecipitated by either anti-pan myosin or anti-fast myosin. Since antibodies recognize native antigens in immunoprecipitation, the results show that the epitopes anti-pan myosin recognizes are not identical on the 165 and 155 kDa polypeptides. This again strengthens the argument that the 155 kDa polypeptide is not a proteolytic fragment of the 165 kDa one.

The 165 kDa polypeptide is immunofluorescently colocalized with actin in the phragmoplast of mung bean root tip cells and perhaps in cytoplasmic filaments by MB165/1, MB165/3 or anti-pan myosin antibody. The 200 kDa putative onion myosin heavy chain has also been colocalized with actin in the phragmoplast of onion root tip cells by anti-pan myosin (Parke *et al.* 1986). This colocalization strengthens the case that the 165 kDa polypeptide is a myosin heavy chain which together with actin, may play a role in cytokinesis.

ADP affinity chromatography has been used to isolate some ATP/ADP-utilizing enzymes such as kinases and ATPases including myosin (Trayer 1974; Trayer *et al.* 1974; Trayer & Trayer 1975; Scouten 1981; Pollard 1982b). When purifying rabbit muscle myosin and most nonmuscle myosins using ADP-agarose columns, EDTA (1-5 mM) is included in the buffer since this provides conditions in which the K^+-EDTA-ATPase activity of myosin is expressed *in vitro* while most other ADP/ATP-utilizing enzymes require a divalent cation to express activity (Trayer & Trayer 1975; Maruta & Korn 1977a; Maruta *et al.* 1979; Lynch *et al.* 1989). Most kinases require Mg^{2+} and/or Ca^{2+} for activity (Trayer 1974; Trayer *et al.* 1974; Polya & Davies 1983; Polya *et al.* 1983; Davies & Polya 1983).

The mung bean 165 kDa polypeptide, but not the 155 and 110 kDa ones, binds to an ADP-agarose column, showing that only the 165 kDa can be an ADP/ATP-utilizing enzyme. The binding and elution of the 165 kDa polypeptide to the ADP column in the presence of EDTA suggests that it is an K^+-EDTA-ATPase rather

than a kinase. This strongly supports the view that the 165 kDa polypeptide is a myosin heavy chain.

Fractionation of mung bean crude extracts with $(NH_4)_2SO_4$ demonstrates that the 165 kDa polypeptide fraction accounts for only a small fraction of the total K^+-EDTA-ATPase activity of the crude extract. The other K^+-EDTA-ATPase activity may be contributed by non-myosin ATPase(s) and/or other myosin isoform(s) that can not be detected by anti-pan myosin. Nevertheless, if myosin isoforms exist they are also not discernible by immunoblotting with the other three anti-myosin and four MB165 antibodies which also detect only the 165 kDa polypeptide. Because K^+-EDTA-ATPase activity alone is not a specific and reliable indicator of the 165 kDa polypeptide, immunoblotting - which provides greater specificity and reliability - was used to monitor the partial purification of the 165 kDa polypeptide from mung bean.

Multiple peaks of K^+-EDTA- and Ca^{2+}-ATPase activities have been reported in purifying the 130 kDa tomato myosin (Vahey *et al.* 1982) and in fractionating the putative pea myosin (Ma & Yen 1989) respectively, and other assays in addition to K^+-EDTA- /Ca^{2+}-ATPase activity including actin-activated Mg^{2+}-ATPase activity and even F-actin binding assays are required to identify plant myosin. When preparation of this thesis was nearly complete, Kohno *et al.* (1992) reported partial purification of a myosin from lily pollen tubes which has a heavy chain of 120 kDa. A crude extracts of pollen tubes has high ATPase activity that is considered to be due to other proteins. Therefore, it was concluded that ATPase activity cannot be used as marker of myosin of lily pollen tubes during purification, which is consistent with my findings in mung bean. Kohno *et al.* used *in vitro* motility assays to monitor their purification.

Analysis of ATPase activity during the partial purification of the mung bean 165 kDa polypeptide shows, as already discussed, that the polypeptide at most accounts for only a very small fraction of the total ATPase activity of the crude extract. Both the yield and specific activity of the K^+-EDTA-ATPase of the 165

kDa polypeptide fraction decreased particularly after DE52 batch processing and after elution by 0.4 M NaCl from the DE52 column (Table 4.2). The K^+-EDTA-ATPase specific activity of the partially purified 165 kDa polypeptide fraction was low (0.016 μmol/min/mg) although it was increased 7.3-fold by ADP-agarose affinity chromatography. The failure to observe a continuous increase in the K^+-EDTA-ATPase specific activity during the purification of the 165 kDa polypeptide and the impurity of the partially purified fraction from ADP-agarose leave it uncertain whether the 165 kDa polypeptide actually has K^+-EDTA-ATPase activity. Chromatography, however, on ADP-agarose suggests that it does.

The partially purified 165 kDa fraction from ADP-agarose does not exhibit detectable actin-activated Mg^{2+}-ATPase activity. Preliminary studies of *in vitro* motility assays, attempting to detect the actin-activated Mg^{2+}-ATPase activity of the 165 kDa polypeptide fraction, were unsuccessful. Therefore, the important enzymatic evidence that the 165 kDa polypeptide is a myosin heavy chain is still lacking although there is strong immunological evidence that it is. Even though the actin-activated Mg^{2+}-ATPase activity is one of the most fundamental criteria for identifying a putative myosin, it may not be detected in all purified nonmuscle myosins (Korn 1978; Taylor & Condeelis 1979). One such example of plant myosin is the partially purified *Egeria* myosin fraction that does not exhibit actin-activated Mg^{2+}-ATPase activity (Ohsuka & Inoue 1979).

Enzymes are difficult to purify from plants due to the low protein content, highly active proteases and the frequent inactivation caused by oxidative enzymes and phenolics that mix after the rupture of vacuoles (Anderson 1968; Loomis 1974; Wu & Wang 1984; Cremer Van de Walle 1985). During the partial purification of myosin from lily pollen tubes, the velocity at which actin filaments move in the *in vitro* motility assays increases with purification from the crude extract to DE52 fractionation. However, the velocity decreases with further purification, which is possibly due to denaturation of myosin (Kohno *et al.* 1992). These are thus several similarities with the study described in this thesis. One clear advantage of the

immunological strategy adopted in my study is in the detection of proteolysis. No studies are presented by Kohno *et al.* (1992) to confirm that the LMW lily myosin is not a proteolytic product of a HMW myosin.

There is considerable scope for further work with the mung bean 165 kDa polypeptide. The absence of actin-activated Mg^{2+}-ATPase activity from and the low yield of K^{+}-EDTA-ATPase activity in the partially purified mung bean 165 kDa polypeptide fraction may be due to degradation and/or denaturation of this protein. Therefore, more effective means of protecting myosin from degradation and denaturation should be developed. The separation of any possible activating enzyme or cofactor necessary for expressing the actin-activated Mg^{2+}-ATPase activity of myosin during purification may also lead to the loss of such ATPase activity. Such phenomenon has never been reported in plant myosin purification but could be tested by adding back earlier discarded fractions and testing ATPase activity.

Preliminary attempts to purify the mung bean 165 kDa polypeptide using an MB165-immunoaffinity column were unsuccessful. Since the MB165 coupled Covaspheres recognize the native 165 kDa polypeptide, further efforts with immunoaffinity columns using MB165 antibodies might rapidly purify the 165 kDa polypeptide in an enzymically active form and so remove the final doubts about its status as a myosin heavy chain.

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